Stat3-Mediated Activation of MicroRNA-23a Suppresses Gluconeogenesis in Hepatocellular Carcinoma by Down-Regulating Glucose-6-Phosphatase and Peroxisome Proliferator-Activated Receptor gamma, Coactivator 1 Alpha

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Considerable effort has been made in elucidating the mechanism and functional significance of high levels of aerobic glycolysis in cancer cells, commonly referred to as the Warburg effect. Here we investigated whether the gluconeogenic pathway is significantly modulated in hepatocarcinogenesis, resulting in altered levels of glucose homeostasis. To test this possibility, we used a mouse model (mice fed a choline-deficient diet) that develops nonalcoholic steatohepatitis (NASH), preneoplastic nodules, and hepatocellular carcinoma (HCC), along with human primary HCCs and HCC cells. This study demonstrated marked reduction in the expressions of G6pc, Pepck, and Fbp1 encoding the key gluconeogenic enzymes glucose-6-phosphatase, phosphoenolpyruvate carboxykinase, fructose-1,6-phosphatase, respectively, and the transcription factor Pgc-1α in HCCs developed in the mouse model that correlated with reduction in serum glucose in tumor-bearing mice. The messenger RNA (mRNA) levels of these genes were also reduced by ≈80% in the majority of primary human HCCs compared with matching peritumoral livers. The expression of microRNA (miR)-23a, a candidate miR targeting PGC-1α and G6PC, was up-regulated in the mouse liver tumors as well as in primary human HCC. We confirmed PGC-1α and G6PC as direct targets of miR-23a and their expressions negatively correlated with miR-23a expression in human HCCs. G6PC expression also correlated with tumor grade in human primary HCCs. Finally, this study showed that the activation of interleukin (IL)-6-Stat3 signaling caused the up-regulation of miR-23a expression in HCC.

Conclusion: Based on these data, we conclude that gluconeogenesis is severely compromised in HCC by IL6-Stat3-mediated activation of miR-23a, which directly targets PGC-1α and G6PC, leading to decreased glucose production.

U nit recently, little effort has been made to understand the significance of Otto Warburg’s pioneering demonstration more than eight decades ago that proliferating cancer cells metabolize 8 to 10-fold more glucose to lactic acid than corresponding normal tissues under aerobic conditions.1 The relatively heavy dependence of the tumors on glycolysis even in the presence of abundant oxygen occurs with a concomitant reduction in oxidative phosphorylation. The predominance of aerobic glycolysis characteristic of most cancer cells facilitates conversion of pyruvate produced during glycolysis to lactate, which is secreted into the blood instead of oxidation to completion.2-4 Increased glucose uptake and metabolism correlates with poor prognosis of many tumor types,2,5,6 supporting the notion that metabolic alterations may contribute to the malignant phenotype.7

As articulated by Levine and Puzio-Kuter,3 several compelling issues contributed to the lack of interest in the altered carbohydrate metabolism of cancer cells. First, whereas the conversion of pyruvate to lactate...
results in the recovery of oxidized nicotinamide adenine dinucleotide (NAD+) required for the maintenance of glycolysis and for continued cell proliferation in vivo, this process is energetically inefficient, producing just two out of 36 adenosine triphosphate (ATP) molecules for each mole of glucose expended. Second, it is not known how such a dramatic shift in energy metabolism occurs and how this altered metabolism results in cancer phenotype. Third, the relationship between metabolism deregulation and oncogene activation/tumor suppressor gene inactivation has not been established. After more than eight decades since Warburg proposed his hypothesis for the elicitation of cancer phenotype, the long-abandoned idea for the dependence of cancer cells on increased utilization of glycolysis has been resurrected, and has also provided an impetus for the development of novel anticancer drugs.

Although considerable effort has been made in understanding the functional significance of higher rates of glycolysis and production of lactate in malignant cells, the possibility of altered gluconeogenesis that could potentially facilitate the glycolytic pathway has not been explored. A mouse model for hepatocellular carcinoma (HCC) is an ideal system to determine potential modifications in the gluconeogenesis pathway for two reasons. First, only the liver contains a full complement of all the enzymes essential for gluconeogenesis, particularly glucose-6-phosphatase, catalytic subunit (G6PC), which converts glucose-6-phosphate (G6P) to glucose. Glucose is then released to the circulation and subsequently utilized by other tissues, including muscle. Second, a suitable mouse model for HCC is now available, which can induce liver tumors by feeding a choline-deficient and amino acid-defined (CDAA) diet in the absence of any exogenous chemicals or virus. Further, tumorigenesis in this model involves steatosis, inflammation, fibrosis, and insulin resistance that are the hallmarks of human HCC. We have used this model to study alterations in gluconeogenesis during the process of liver tumorigenesis. Surprisingly, we found dramatic inhibition of key gluconeogenic enzymes and of a transcription factor involved in this metabolic pathway in the liver tumors. We also explored the molecular mechanisms underlying the suppression of these enzymes and probable functional significance of this observation.

Materials and Methods

Mice and Diet. The animal model and the dietary regimen to induce HCC have been described. All animal experiments were carried out under protocols approved by the Ohio State University Institutional Laboratory Animal Care and Use Committee.

Human HCC Specimens. Primary human hepatocellular tumor and adjacent peritumoral tissue specimens were obtained from the Cooperative Human Tissue Network at the Ohio State University James Cancer Hospital. Tissue specimens were procured in accordance with the Ohio State University Cancer Institutional Review Board guidelines. Detailed information of patients is listed in Supporting Table 1.

Statistical Analysis. Statistical analysis of differences between groups was performed by unpaired Student's t test and P ≤ 0.05 was considered statistically significant. A paired Student's t test was used to analyze differences in expression of miR and messenger RNA (mRNA) levels among tumors and paired peritumoral tissues in real-time reverse-transcription polymerase chain reaction (qPCR) analysis. The correlation between miR-23a level and G6PC, PEPCK, and PGC-1α mRNA levels was analyzed by two-tailed Pearson Correlation Test. Correlation between G6PC expression and clinicopathological characteristics in human HCCs was analyzed by Wilcoxon Mann-Whitney test or one-way analysis of variance (ANOVA) test.

All other materials and methods are described in the Supporting Information.

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Results

**Development of HCC in Mice Fed CDAA Diet for 71 to 84 Weeks.** Previously, we have shown that C57BL/6 mice fed a CDAA diet progressively developed nonalcoholic steatohepatitis (NASH) (∼32 week) and preneoplastic nodules (∼65 week). In this study, mice were fed with CDAA and a control CSAA diet for a longer time (71 to 84 weeks) and examined for the development of HCC. As previously reported, mice fed the CDAA diet showed significant weight increase up to the preneoplastic stage (∼65 week).
Fig. 2. The expression of gluconeogenic genes is reduced in CDAA diet-induced mouse HCC model. (A) G6pc, Pepck, Fbp1, Pc, and Pgc-1α mRNA level decreased during hepatocarcinogenesis. The mRNA levels were measured by qPCR in the liver of mice fed a CSAA and CDAA diet for 32, 65, and 84 weeks (n = 4-5 per group with the exception of no tumor in 32 weeks and 1 tumor in 65 weeks). Data are represented as mean ± standard deviation (SD). (B) Western blot analysis of G6PC and PGC-1α protein level in liver extracts from mice fed a CSAA and CDAA diet as in (A). (C) G6PC phosphatase activity in liver tissues was measured by colorimetric methods using G6P as substrate (n = 4-5 mice per group). Data are represented as mean ± SD.
Although body weight started to decrease after 65 weeks due to tumor development, all mice still gained weight when comparing the final to initial body weight (Fig. 1A). After 71 week, almost all mice fed a CDAA diet (20 out of 21) developed NASH, and the majority of these mice (16 out of 20) developed hepatic tumors (Fig. 1B). In contrast, although 15 out of 21 mice fed the CSAA diet developed NASH, because of higher calorie and fat content in the CSAA diet compared with a standard chow diet, only six of them developed tumors. Moreover, the liver-to-body-weight ratio, tumor number, and largest tumor weight were much higher in mice fed the CDAA than those fed a CSAA diet (Fig. 1C). Histopathological analysis confirmed that most of these tumors were grade II HCC (Fig. 1B,D). Hematoxylin and eosin (H&E) staining showed massive inflammation in the livers of mice fed the CDAA diet compared with those fed a CSAA diet (Fig. 1E). Masson’s trichrome staining revealed severe fibrosis in CDAA diet livers, but not in CSAA diet livers (Fig. 1E). A two-fold increase in hydroxyproline level, which reflects the amount of collagen, corroborated severe fibrosis in CDAA diet livers compared with CSAA diet livers (Fig. 1E). Ki-67 staining indicated that cells in CDAA diet livers were highly proliferative (Fig. 1F). Furthermore, an increased alanine aminotransferase (ALT) level in sera suggested liver damage in tumor-bearing mice fed the CDAA diet compared with a CSAA diet (Supporting Table 2). We also observed a significant decrease in serum glucose level in tumor-bearing mice after overnight fasting (167.55 ± 14.42 versus 256.36 ± 21.89 mg/dL, P = 0.0029) (Supporting Table 2).

![Fig. 3. G6PC, PEPCK, FBP1, and PGC-1α expression are significantly inhibited in primary human HCC. (A) G6PC, PEPCK, FBP1, PC, and PGC-1α mRNA levels were measured by qPCR in HCC and pair-matched peritumoral liver tissues. Each data point represents one patient and horizontal bars denote the mean ± standard error of the mean (SEM). (B) Western blot analysis of G6PC protein level in six pairs of HCC and matching livers. The signal was quantified using Kodak Imaging software and the data were normalized to Ku-70. T: tumor, N: nontumor matching liver. (C) G6PC phosphatase activity in six pairs of HCC and matching livers.](image-url)

| Characteristics                          | T/N  | G6PC Expression (T/N) | P Value |
|------------------------------------------|------|----------------------|---------|
| Age                                      |      | Mean ± SEM Median    |         |
| >60                                      | 14   | 0.60±0.21 0.29       | P=0.62* |
| ≤60                                      | 15   | 0.53±0.23 0.23       |         |
| Gender                                   |      |                      |         |
| Male                                     | 17   | 0.60±0.21 0.27       | P=0.73* |
| Female                                   | 12   | 0.48±0.19 0.23       |         |
| Tumor grade (histological differentiation)|      |                      |         |
| Well                                     | 7    | 1.24±0.38 0.95       | P=0.0066†|
| Moderate                                 | 16   | 0.39±0.18 0.15       |         |
| Poor                                     | 6    | 0.23±0.10 0.17       |         |
| Tumor size                               |      |                      |         |
| >5cm                                     | 23   | 0.49±0.15 0.27       | P=0.89* |
| ≤5cm                                     | 6    | 0.84±0.49 0.14       |         |
| Vascular invasion                        |      |                      |         |
| Yes                                      | 14   | 0.49±0.23 0.23       | P=0.35* |
| No                                       | 15   | 0.63±0.21 0.31       |         |
| Tumor stage                              |      |                      |         |
| Stage I                                  | 14   | 0.67±0.22 0.35       | P=0.49† |
| Stage II                                 | 10   | 0.62±0.32 0.19       |         |
| Stage III                                | 5    | 0.16±0.05 0.23       |         |

T and N denote tumor and peritumoral liver tissues, respectively.

*Wilcoxon Mann-Whitney test. †One-way ANOVA test.
Genes Encoding G6PC, PEPCK, Fructose-1,6-Bisphosphatase (FBP1), Pyruvate Carboxylase (PC), and Peroxisome Proliferator-Activated Receptor gamma, Coactivator 1 alpha (PGC-1α) Are Down-Regulated in the CDAA Diet-Induced Mouse HCC Model. We postulated that higher glycolysis in HCC is accompanied by suppression of gluconeogenesis preventing conversion of G6P to glucose and its release into blood as well as other tissues. To test this possibility, we used the CDAA diet-induced mouse HCC model described above. To determine the gluconeogenesis level in CDAA diet-induced mouse HCC, we first examined the expression of genes involved in gluconeogenesis at different stages of tumorigenesis. qPCR analysis showed that the levels of mRNAs corresponding to G6pc, Pepck, Fbp1, and Pc, key enzymes involved in gluconeogenesis, dramatically decreased in the liver tumors developed in mice at week 84. The tumor formed in one mouse at 65 weeks also exhibited markedly reduced levels of these enzymes (Fig. 2A). Similarly, the level of PPARGC1A (Pgc-1α), a transcription factor that regulates G6pc and Pepck expression, also decreased in tumors (Fig. 2A). Western blot analysis showed that G6PC and PGC-1α protein levels were significantly diminished in tumors (Fig. 2B). The G6pc phosphatase activity in liver tissues also declined gradually during tumorigenesis (Fig. 2C).

G6PC, PEPCK, FBP1, and PGC-1α Expression Are Significantly Reduced in Primary Human HCC. Next, we assessed the expression of these gluconeogenic enzymes and PGC-1α expression in human primary HCC. qPCR analysis showed that the expression of most of these genes was significantly reduced in human primary HCCs compared with matching livers (Fig. 3A). The mRNA levels of G6PC, PEPCK, and PGC-1α decreased by 73%-80% in 24, 26, and 24 pairs, respectively, out of 30 pairs of HCC examined. The mRNA level of FB1 was not significantly altered in human HCC. Western blot analysis showed a dramatic reduction (50%) in G6PC protein level in primary HCC (Fig. 3B). The curtailed G6PC enzyme activity correlated with its low level of expression in the human HCC specimens (Fig. 3C).

G6PC Expression Correlates with Tumor Grade in Human Primary HCCs. To address the clinical relevance of gluconeogenesis inhibition, we analyzed the correlation between G6PC mRNA level and clinical features in 29 human primary HCC patients by...
comparing the mRNA level of \( G6PC \) in different subgroups of patients based on clinical parameters. One-way ANOVA revealed significant correlation between \( G6PC \) expression and tumor grade \((P = 0.0066)\) (Table 1). The mRNA level of \( G6PC \) was \( \approx 32\% \) and \( \approx 19\% \), respectively, in moderately and poorly differentiated tumors compared with that in well-differentiated tumors. However, there was no significant association between \( G6PC \) expression and tumor invasion status and tumor stage. No significant difference was observed in \( G6PC \) expression among HCC subgroups with regard to age, gender, and tumor size.

**Expression of MiR-23a, a Candidate MicroRNA Targeting PGC-1α and G6PC, Is Elevated in CDAAD Diet-Induced Liver Tumors and in Human Primary HCC.** Next, we sought to identify possible mechanisms underlying the down-regulation of genes encoding the key proteins involved in gluconeogenesis in

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**Fig. 5.** PGC-1α and G6PC are direct targets of miR-23a. (A) Luciferase assay. H293FT cells were cotransfected with the reporters and miR-23a or NC RNA (50 nM). After 48 hours, RLU2/RLU1 activity was measured. (B,C) Overexpression of miR-23a reduces PGC-1α and G6PC expression in Hep3B cells and mouse hepatocytes. Hep3B cells and mouse hepatocytes were transfected with miR-23a or NC RNA (50 nM) followed by measurements of PGC-1α and G6PC mRNA, protein levels, and G6PC phosphatase activity. (D,E) Depletion of miR-23a increases PGC-1α and G6PC expression in Hep3B cells and mouse hepatocytes. Hep3B cells and mouse hepatocytes were transfected with anti-miR-23a or anti-NC RNA (100 nM) followed by measurement of PGC-1α and G6PC mRNA, protein levels, and G6PC phosphatase activity.
diet-induced HCC model and primary HCC. MicroRNAs (miRs) regulate various biological processes including metabolism by inhibiting target gene expression. We explored the potential involvement of specific miRs in regulating gluconeogenesis. Analysis of the 3' untranslated region (UTR) of PGC-1α mRNA revealed two conserved miR-23a sites predicted by three different databases (TargetScan, Pictar, and miRanda) (Fig. 4A). Interestingly, G6PC 3'UTR also harbors one miR-23a site predicted by TargetScan. Previously, miR-23a has been shown to regulate glutamine metabolism by targeting glutaminase in lymphoma and prostate cancer cells. Another study demonstrated its up-regulation in HCC. To determine whether miR-23a can regulate PGC-1α and G6PC expression in liver tumors, we first measured its expression in a diet-induced HCC model by qPCR. The results displayed a gradual increase in miR-23a expression during tumorigenesis in the livers of mice fed a CDAA diet compared with those fed CSAA (control) (Fig. 4B). Its expression increased from 1.65-fold \((P = 0.08)\) in the livers from mice fed a CDAA diet at week 65 to 2.5-fold \((P = 0.019)\) in the tumors from mice fed a CDAA diet at 84 weeks compared with those fed a CSAA diet. It should be noted that a 2.5-fold difference in miR levels is considered highly significant, as this level of increase in a single miR can decrease expression of multiple genes.

To address the functional relevance of enhanced miR-23a levels in human HCC, miR-23a expression was analyzed in human primary HCCs and paired peritumoral liver tissues. Among the 30 HCC samples analyzed, miR-23a expression was significantly elevated \((\approx 2\text{-fold})\) in 21 HCC samples \((P = 0.0046)\) (Fig. 4C). Comparison of miR-23a level and mRNA levels corresponding to PGC-1α, G6PC, and PEPCK in HCCs exhibited inverse correlation between miR-23a and PGC-1α \((r = -0.37, P = 0.047)\), G6PC \((r = -0.55, P = 0.002)\), and PEPCK \((r = -0.35, P = 0.059)\) (Fig. 4D). In contrast, there was no correlation between miR-23a and FBPI or PC levels (data not shown).

**PGC-1α and G6PC Are Direct Targets of MiR-23a.** To determine whether PGC-1α and G6PC are direct targets of miR-23a, wildtype and mutant 3'UTRs lacking miR-23a binding sites (3'UTRAΔ) were cloned into psiCHECK2 luciferase reporter vector downstream of the Renilla luciferase coding region. The constructs were then cotransfected with miR-23a precursor or negative control (NC) RNA into H293FT cells. The overexpression of miR-23a \((\approx 15\text{-fold})\) was confirmed by real-time PCR (Supporting Fig. 1). It should be noted that ectopic expression of specific miR in cell lines after transfecting miR mimics results in higher expression of the miR than the in vitro level. The relative luciferase activity was reduced by 40% \((P = 0.0001)\) and 25% \((P = 0.002)\) in PGC-1α and G6PC wildtype 3'UTR, respectively, but not in respective mutant 3'UTRs or psiCHECK2 vector alone (Fig. 5A), suggesting that miR-23a cognate sites are essential for negative regulation of luciferase expression driven by PGC-1α-3'UTR and G6PC-3'UTR.

To confirm that miR-23a can indeed suppress expression of endogenous PGC-1α and G6PC, Hep3B cells and mouse hepatocytes were transfected with miR-23a or NC RNA, followed by measurements of their mRNA and protein levels. PGC-1α and G6PC mRNA levels decreased by \(\approx 40\%-50\%\) \((P = 0.01\) and 0.02, respectively) in both cells overexpressing miR-23a (Fig. 5B,C). A more than 30% reduction in PGC-1α protein level was observed in both Hep3B and hepatocytes (Fig. 5B). The G6PC protein level was reduced by 28% upon expression of miR-23a in hepatocytes compared with NC RNA (Fig. 5C). Next, we measured G6PC enzyme activity because it was not detectable by western blot analysis in Hep3B cells. The glucose-6-phosphatase activity in Hep3B cells transfected with miR-23a precursor indeed decreased by \(\approx 50\%\) compared with cells transfected with control RNA (Fig. 5C).

We also performed the reverse experiment by transfecting anti-miR-23a in Hep3B cells and mouse
hepatocytes, which resulted in \( \approx 90\% \) depletion of miR-23a (data not shown). PGC-1\( \alpha \) (Fig. 5D) and G6PC (Fig. 5E) mRNA levels increased by \( 20\% \sim 70\% \) in both cells depleted of miR-23a. The respective protein levels and glucose-6-phosphatase activity also increased by \( 30\% \sim 49\% \) in these cells (Fig. 5D,E). These results indicate that miR-23a interferes with PGC-1\( \alpha \) and G6PC expression at both the mRNA and protein levels. Taken together, these data demonstrate that PGC-1\( \alpha \) and G6PC are direct targets of miR-23a.

This is a functionally significant observation, as nearly \( 90\% \) of glucose-6 phosphatase is present in the liver. Consequently, suppression of G6PC will block the last critical reaction essential for the production of glucose and its release to blood for uptake by glucose-requiring tissues.

**MiR-23a Inhibits Glucose Production in Hepatocytes.** As indicated earlier, we observed significant reduction in the glucose levels in the sera of tumor-bearing mice fed a CDAA diet (Supporting Table 2) consistent with inhibition of gluconeogenesis in HCC. Because PGC-1\( \alpha \) and G6PC are involved in gluconeogenesis in liver, we investigated the potential regulation of hepatic gluconeogenesis by miR-23a, resulting in decreased glucose production. For this purpose, the miR-23a precursor or anti-miR inhibitor was transfected into hepatocytes isolated from mouse liver followed by replacement of the culture medium with...
glucose production medium 48 hours later. After 6 hours, glucose synthesis was analyzed by measuring the glucose concentration in the culture supernatant. The amount of glucose released from hepatocytes revealed a 40% reduction in glucose production after ectopic expression of miR-23a (Fig. 6A), whereas suppressing miR-23a expression increased its formation by 30%. Overexpression or depletion of miR-23a was confirmed by real-time PCR analysis (Fig. 6B). Taken together, these data indicate that miR-23a negatively regulates glucose production.

**IL-6 and Stat3 Signaling Pathway Modulate the Expression of MiR-23a.** Next, we sought to delineate the potential mechanisms underlying up-regulation of hepatic miR-23a during hepatocarcinogenesis in mice fed a CDAA diet. Analysis of miR-23a promoter region using rVISTA program identified a potential Stat3 binding site that is conserved in human and mouse (Supporting Fig. 2). Stat3 has been shown to inhibit gluconeogenesis by suppressing PGC-1α, G6PC, and PEPCK expression. This observation coupled with the present demonstration of miR-23a-mediated inhibition of gluconeogenesis prompted us to hypothesize that Stat3 may be involved in the regulation of miR-23a expression in liver. To test this hypothesis, we first measured Stat3 levels in the extracts of livers from a CDAA diet-fed mice by western blot analysis. The phospho-Stat3 level indeed was higher in the tumor tissues compared with the matching liver tissues (Supporting Fig. 3). To assess further the potential regulation of miR-23a expression by Stat3, Hep3B cells were treated with cryptotanshinone, a specific inhibitor of Stat3 phosphorylation, for 12 hours followed by measurements of the miR-23a level. Inhibition of Stat3 mitigated miR-23a expression by 80% compared with the vehicle (dimethyl sulfoxide, DMSO)-treated cells (Fig. 7A). Similarly, knocking down Stat3 expression in Hepa cells by small interfering RNA (siRNA) reduced miR-23a expression by more than 50% (Fig. 7A), suggesting that Stat3 positively regulates miR-23a expression.

It is well known that IL-6 activates Stat3 to suppress gluconeogenesis. The serum IL-6 level was indeed 2-fold higher in tumor-bearing mice fed a CDAA diet compared with control mice fed a CSAA diet (Fig. 7B). This observation suggested the possibility that IL-6 may regulate miR-23a expression. To confirm this notion, Hep3B and Hepa cells were treated with IL-6 for 12 hours and miR-23a levels were analyzed by real-time PCR, which showed an 2-fold increased expression of miR-23a in both cell lines (Fig. 7C). To further test if the Stat3 signaling pathway regulates miR-23a expression by activating its promoter, we cloned the mouse miR-23a promoter region into psiCHECK2 vector upstream of the Renilla luciferase coding region and performed luciferase assay after knocking down Stat3 or with or without IL-6 treatment. Knockdown of Stat3 expression in Hepa cells with siRNA reduced the luciferase activity by 50% compared with control siRNA-transfected cells (Fig. 7D). In contrast, IL-6 treatment increased luciferase activity by 30% compared with untreated cells. As expected, luciferase activity driven by psiCHECK2 alone did not respond to siRNA or IL-6 treatment (Fig. 7D).

To determine if Stat3 directly binds to the miR-23a promoter region, chromatin immunoprecipitation (ChiP) analysis was performed in Hepa cells transfected with Stat3 siRNA (siStat3) or control siRNA (siNC), and Hepa cells treated with or without IL-6. The results showed an 25-fold enrichment of Stat3 at the miR-23a promoter region compared with no antibody control samples (Fig. 7E). Similarly, we observed an 2.6-fold increase in Stat3 enrichment at the miR-23a promoter in Hepa cells treated with IL-6 compared with untreated cells. In contrast, recruitment of Stat3 to the same region was reduced by 80% in Stat3-depleted cells (Fig. 7E). These data, taken together, indicate that Stat3 can directly bind to the promoter region of miR-23a and increase its expression.

**Discussion**

**Overall Significance of the Study.** Although there has been considerable progress in understanding the functional significance and probable mechanisms of the Warburg effect, the potential role of gluconeogenesis during transformation of normal cells to cancer cells, particularly in liver cancer, has not been explored. We took advantage of a mouse model system that mimics many of the biochemical and pathological changes characteristic of NASH and produces HCC. It should be noted that this model has now been successfully utilized by many laboratories to address important genetic, epigenetic, and signaling mechanisms involved in hepatocarcinogenesis. We have now shown that this model can be effectively used to study alterations in gluconeogenesis at different stages of hepatocarcinogenesis and the potential mechanisms. A key observation in the present study was the almost complete suppression of glucose-6-phosphatase gene expression and its enzymatic activity in HCC. Because it is the last enzyme in the gluconeogenesis pathway, as well as one of the four irreversible enzymes in this pathway, and the majority of this enzyme (>80%) is
present in the liver, its inhibition will have major functional consequences. Accordingly, the glucose production by way of gluconeogenesis will be severely blocked, resulting in markedly reduced release of glucose into the circulation and consequently curtailing the supply of glucose for energy requirements in other tissues such as exercising muscles. Suppression of the genes encoding the rate-limiting enzyme FBP1, and PEPCK, an enzyme acting upstream of FBP1 as well as a key transcription factor involved in the expression of G6PC and PEPCK, further leads to drastic reduction of gluconeogenesis.

**Role of MiR-23a in Suppression of Gluconeogenesis.** To our knowledge, this is the first report that demonstrates the role of a specific miR in regulating gluconeogenesis in HCC. A key observation is that enhanced phospho-Stat3 levels (Supporting Fig. 3) caused a marked increase in miR-23a expression in HCC produced in the mouse model (Fig. 4B), causing suppression of two key miR-23a targeted genes involved in gluconeogenesis. The difference in Stat3 phosphorylation between the tumors and peritumoral liver tissues in the 84 week CDAA group was not as dramatic as the 65 week group, because the peritumoral regions were full of microscopic tumors at week 84. It was technically difficult to dissect the tumor-free matching liver tissues by microdissection at this stage of tumor development. The direct role of Stat3 in miR-23a expression was confirmed by (1) significant reduction in miR-23a expression following treatment of HCC cells with Stat3 specific siRNA; (2) enhanced miR-23a expression upon treatment of the two HCC cell lines Hep3B and Hepa cells with the cytokine IL-6, which is known to cause inflammation and activation of the oncogenic transcription factor Stat3 in the liver; and (3) demonstration of direct binding of Stat3 to miR-23a promoter and its enrichment at the miR promoter following treatment of cells with IL-6. IL-6-Stat3 signaling pathway has been shown to inhibit gluconeogenesis by suppressing G6pc and Pepck in Stat3 null mice. Previously, Stat3 has been shown to suppress G6PC and PEPCK expression by directly binding to the promoter region. Our study has revealed another layer of complexity in the regulation of these enzymes involving miR-23a. The present study has shown that the IL-6-Stat3 signaling pathway inhibits gluconeogenesis, in part by up-regulating miR-23a and compromising glucose production by directly targeting G6PC and PGC-1α (see Supporting Fig. 4 for a proposed model).

Recently, SIRT1 has been shown to regulate acetylation and phosphorylation of Stat3 and thus suppresses its inhibitory effect on gluconeogenesis. It will be of interest to determine if SIRT1 regulates miR-23a expression. miR-23a does not appear to be involved in the regulation of FBP1 expression, as there is no miR-23a binding site in its 3’UTR.

**How Does Reduced Gluconeogenesis Facilitate Tumorigenesis?** The drastic reduction in gluconeogenesis is reflected in the significant decrease in the level of glucose released from hepatocytes following increased expression of miR-23a, or in augmented glucose release in response to miR-23a suppression (Fig. 6A). Further, the glucose level in sera from the tumor-bearing mice was markedly diminished compared with normal sera (Supporting Table 2). Although the level of upstream enzyme PEPCK was also curtailed, drastic reduction in G6PC is likely to result in accumulation of G6P. Because G6P is utilized in the hexose monophosphate (HMP) shunt pathway for glucose metabolism, the tumors could metabolize excess G6P by way of this pathway and produce ribose-5-phosphate used in nucleotide synthesis. The enhanced production of this key building block of nucleic acids is probably an important means of meeting the basic requirement for rapid cell division and growth of tumors. The increased utilization of glycolytic and HMP shunt pathways in HCC, depending on the extent of accumulation of G6P due to block in gluconeogenesis, may contribute to the survival of the tumor cells under hypoxic conditions.

**Role of Glutaminase in HCC Proliferation and Survival.** The role of other factors relevant to cellular energy metabolism in cancer merits discussion. One such factor is glutamine, which has been recently rediscovered as an important growth-stimulating amino acid after nearly four decades since the initial observations of its potential role in energy metabolism. Recently study has shown that glutaminase 1 (GLS), which converts glutamine to glutamate, is up-regulated in lymphoma and prostate cancer cell lines by Myc oncogene through its repression of miR-23a/b, two miRs targeting GLS. Interestingly, we observed marked stimulation of GLS expression in the diet-induced mouse HCC (data not shown) irrespective of the increased expression of miR-23a, suggesting that GLS is probably regulated by some other mechanisms in liver cancer. The increased expression of GLS results in elevated levels of reduced glutathione (GSH) that scavenges reactive oxygen species. It is conceivable that NADPH required for GSH synthesis could be efficiently generated by augmented HMP shunt pathway resulting from inhibition of G6PC and accumulation of G6P in HCC. Further study is required to address this issue, which is beyond the scope of this work.
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