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Cancer Prevention Research

Research Article

See Perspective on p. 500

Metformin Prevents Liver Tumorigenesis by Inhibiting Pathways Driving Hepatic Lipogenesis

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Abstract

A number of factors have been identified that increase the risk of hepatocellular carcinoma (HCC). Recently it has become appreciated that type II diabetes increases the risk of developing HCC. This represents a patient population that can be identified and targeted for cancer prevention. The biguanide metformin is a first-line therapy for the treatment of type II diabetes in which it exerts its effects primarily on the liver. A role of metformin in HCC is suggested by studies linking metformin intake for control of diabetes with a reduced risk of HCC. Although a number of preclinical studies show the anticancer properties of metformin in a number of tissues, no studies have directly examined the effect of metformin on preventing carcinogenesis in the liver, one of its main sites of action. We show in these studies that metformin protected mice against chemically induced liver tumors. Interestingly, metformin did not increase AMPK activation, often shown to be a metformin target. Rather metformin decreased the expression of several lipogenic enzymes and lipogenesis. In addition, restoring lipogenic gene expression by ectopic expression of the lipogenic transcription factor SREBP1c rescues metformin-mediated growth inhibition. This mechanism of action suggests that metformin may also be useful for patients with other disorders associated with HCC in which increased lipid synthesis is observed. As a whole these studies show that metformin prevents HCC and that metformin should be evaluated as a preventive agent for HCC in readily identifiable at-risk patients. *Cancer Prev Res; 5(4); 544–52. ©2012 AACR.*

Introduction

Primary liver cancer is the fifth most common cancer and has the third highest rate of mortality worldwide. Hepatocellular carcinoma (HCC) represents approximately 85% of all primary liver cancer. Although the incidence of some cancers is declining, incidences of HCC are increasing worldwide (1, 2). Even in the United States it is the fastest growing cause of cancer-related deaths in men (1, 3). Overall 5-year survival is less than 15%, and for patients with advanced stage disease, 5-year survival is less than 2% (4). Therefore identification and chemopreventive intervention of patients at risk for HCC represents one of the best strategies for reducing morbidity and mortality from this cancer.

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A number of risk factors have been identified that increase the risk of HCC. Hepatitis B virus (HBV) and hepatitis C virus (HCV) represent the most significant risk factors for HCC (5). However, epidemiologic evidence shows an increasing role for diabetes in the development of HCC. Patients with type II diabetes have a 2- to 3-fold increased relative risk of HCC (3, 6, 7). Furthermore, the comorbidities of obesity and diabetes are reported to account for 37% of HCC cases in patients without HBC or HCV infection (8). Several studies have shown an increase in HCC in rodent models of diabetes, illustrating a more direct link between diabetes and HCC (9–11). Therefore diabetics represent a patient population at risk for HCC that can readily be identified.

Metformin is a biguanide that has been used for the treatment of type II diabetes and nonalcoholic fatty liver disease (NAFLD; refs. 12, 13). Metformin has an excellent therapeutic index with few side affects being associated with long-term treatment. Although studies show that diabetes increases the relative risk of HCC, several studies now show that treatment of diabetics with metformin was associated with a reduced risk of HCC (14, 15). Despite this association between metformin and reduced HCC, an anticancer role for metformin has not been examined in the main organ site of action. Therefore we set out to determine the role of metformin as a chemopreventive agent in HCC.

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Materials and Methods

Cell culture and growth assays

H4IIE and McA-RH7777 rat hepatoma, HepG2 human hepatoblastoma cell lines were obtained from American Type Culture Collection (ATCC). ATCC characterizes cell lines by short tandem repeat profiling. Experiments were conducted with cells within 6 months of receipt or resuscitation. Huh7 human hepatocarcinoma were also used but authentication was not done. All cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and pen/strep. Experiments were conducted with cells at less than 25 passages after receipt. A total of 5,000 cells per well were seeded in a 96-well plate and allowed to attach for 24 hours. Cells were then treated with 250 μmol/L to 10 mmol/L metformin. Cells were treated for 48 hours and cell growth determined using WST-1 (Roche) as per manufacturer's instructions. For lipogenic rescue experiments H4IIE and McA-RH7777 rat hepatoma cell lines were transfected with a vector control or constitutively active SREBP1c (CA-SREBP1c) construct (Addgene). This construct encodes the cleaved, nuclear form of SREBP1c, which is constitutively active. Cells were selected in G418 and induction of Srebp1c and lipogenic gene expression confirmed by reverse transcriptase PCR (RT-PCR). Cells were seeded into 60-cm^2 dishes and treated with metformin for 72 hours and cell growth assessed using a hemocytometer.

In vivo experiments

C57BL/6J mice were obtained from Jackson Labs and maintained by Animal Care facility at the University of Maryland, Baltimore. All mice procedures were done in accordance with University of Maryland IACUC care guidelines. Forty, 2-week-old male mice (10 mice per group) were injected intraperitoneally with 25 mg/kg body weight diethylnitrosamine (DEN). Several pups died shortly after DEN treatment reducing the number of mice per group to 7. Mice were weaned at 4 weeks of age and fed control chow or chow containing metformin (Base diet was AIN-76-A; Bioserv) at a dose of 250 mg/kg body weight for 24 weeks or 36 weeks. Before euthanasia, mice were weighed and fed and fasting glucose levels obtained (Aviva Accuchek). At the termination of experiment mice were euthanized by CO_2 asphyxiation followed by cervical dislocation. Livers were harvested and fixed in formalin or snap frozen for subsequent RNA or protein analysis. Visible tumors were also counted in 36-week group. For short-term metformin experiments, mice were given control or metformin chow for 2 weeks and then liver and muscle removed for analysis.

Hepatic histology

Livers were embedding in paraffin and 5 μm sections cut and stained with hematoxylin and eosin (H&E) by the University of Maryland Pathology Core and Mass Histology Services (Worcester, MA). Pathologic analysis including tumors number and size was determined blinded by a pathologist (W.T).

RNA isolation and reverse transcription

RNA was isolated from liver tissue or cells using TRIzol reagent (Invitrogen) and cDNA synthesized as previously described (16). Real-time PCR was done with SYBR Green reagent (Applied Biosystems) using the primers shown in Supplementary Table S1. Actin or 18S were used as endogenous controls.

Western blotting

Livers were homogenized, protein extracted, separated by SDS-PAGE, and transferred to nitrocellulose as previously described (16). Immunoblotting was done for AMPK, p-AMPK, p-TSC2, ACLY, p-ACLY, ACC, p-ACC, and FASN (Cell Signaling) followed by horseradish peroxidase secondary antibodies (Jackson Immunological). Actin was used as a control and proteins visualized using chemiluminescence (Pierce).

Triglyceride analysis

Triglyceride levels in liver tissue were determined by a colorimetric method using a triglyceride quantification kit (BioVision). Briefly, 50 mg of liver tissue was homogenized in 1 mL solution containing 5% NP-40 in water and absorbance was read at 570 nm as per manufacturer's recommendations.

Cell growth assay

H4IIE and McA-RH7777 rat hepatoma, Huh7 human hepatocarcinoma, HepG2 human hepatoblastoma cell lines were obtained from ATCC and grown in DMEM supplemented with 10% FBS and pen/strep. Experiments were conducted with cells at less than 25 passages after receipt. A total of 5,000 cells per well were seeded in a 96-well plate and allowed to attach for 24 hours. Cells were then treated with 250 μmol/L to 10 mmol/L metformin. Cells were treated for 48 hours and cell growth determined using WST-1 (Roche) as per manufacturer's instructions. To access viability, cells were plated in 60-cm^2 dishes, treated with metformin as described. Trypan blue exclusion was used to access viability using the Countess Cell Counter. For lipogenic rescue experiments H4IIE and McA-RH7777 rat hepatoma cell lines were transfected with a vector control or constitutively active SREBP1c (CA-SREBP1c) construct (Addgene). This construct encodes the cleaved, nuclear form of SREBP1c, which is constitutively active. Cells were selected in G418 and induction of Srebp1c and lipogenic gene expression confirmed by RT-PCR. Cells were seeded into 60-cm^2 dishes and treated with metformin for 72 hours and cell growth assessed using a hemocytometer.

Statistical analysis

Data are presented as mean ± SD or SE with indicated number of samples shown in figure legends. Statistical significance of differences between groups was analyzed by Student t test, and at least $P < 0.05$ was considered to be statistically significant. Level of significance is indicated in figure legends. To determine the number of mice for these

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studies, a Wilcoxon (Mann–Whitney) rank sum test was used with a 0.05 two-sided significance level.

Results

Metformin prevents DEN-induced tumorigenesis

Metformin has been investigated for cancer in a number of different organ sites. One of the primary indications for metformin is inhibition of hepatic glucose output by the liver. However, previous studies have not examined the effect of metformin in liver cancer. Therefore we treated several different HCC cell lines with increasing doses of metformin. Metformin decreased the growth of the cell lines in a dose-dependent manner after 48 hours (Fig. 1A–D). Viability did not seem to be affected by metformin at the doses used (Supplementary Fig. S1A–C). It should be pointed out that viability was determined using trypan blue exclusion on cells remaining attached to the plate. Therefore any nonviable cells that had detached would be missed. However, we did not observe significantly fewer cells in the metformin-treated samples after 48 hours compared with the start of the experiment. Nevertheless, at much higher doses of metformin we do observe a decrease in viability (data not shown). Although these data showed that metformin inhibits established liver cancer cell growth, it raises the question as to whether metformin can reduce liver carcinogenesis.

We investigated the effect of metformin on HCC using the liver-specific carcinogen DEN. Following treatment and weaning, mice were fed metformin-containing chow and mice euthanized after 24 or 36 weeks. Body weight of mice did not differ between control- and metformin-treated mice (Supplementary Fig. S2A). In addition, the ratio of liver weight to body weight was also not different (Supplementary Fig. S2B). No visible surface tumors were present on the livers of either control- or metformin-treated mice after 24 weeks. H&E examination of livers showed that metformin-treated mice developed 57% fewer tumors compared with control chow-fed mice (Fig. 2A). The size of the liver tumors from metformin-treated mice was also reduced approximately 37% compared with control mice (Fig. 2B). Despite the decrease in tumor formation, there was no histopathologic difference between tumors of control- or metformin-treated mice (Fig. 2C). Often an increase in liver to body weight ratio shows an increase in liver tumor burden. The lack of a difference in our studies may be due to both groups having tumors present, albeit there were fewer tumors in

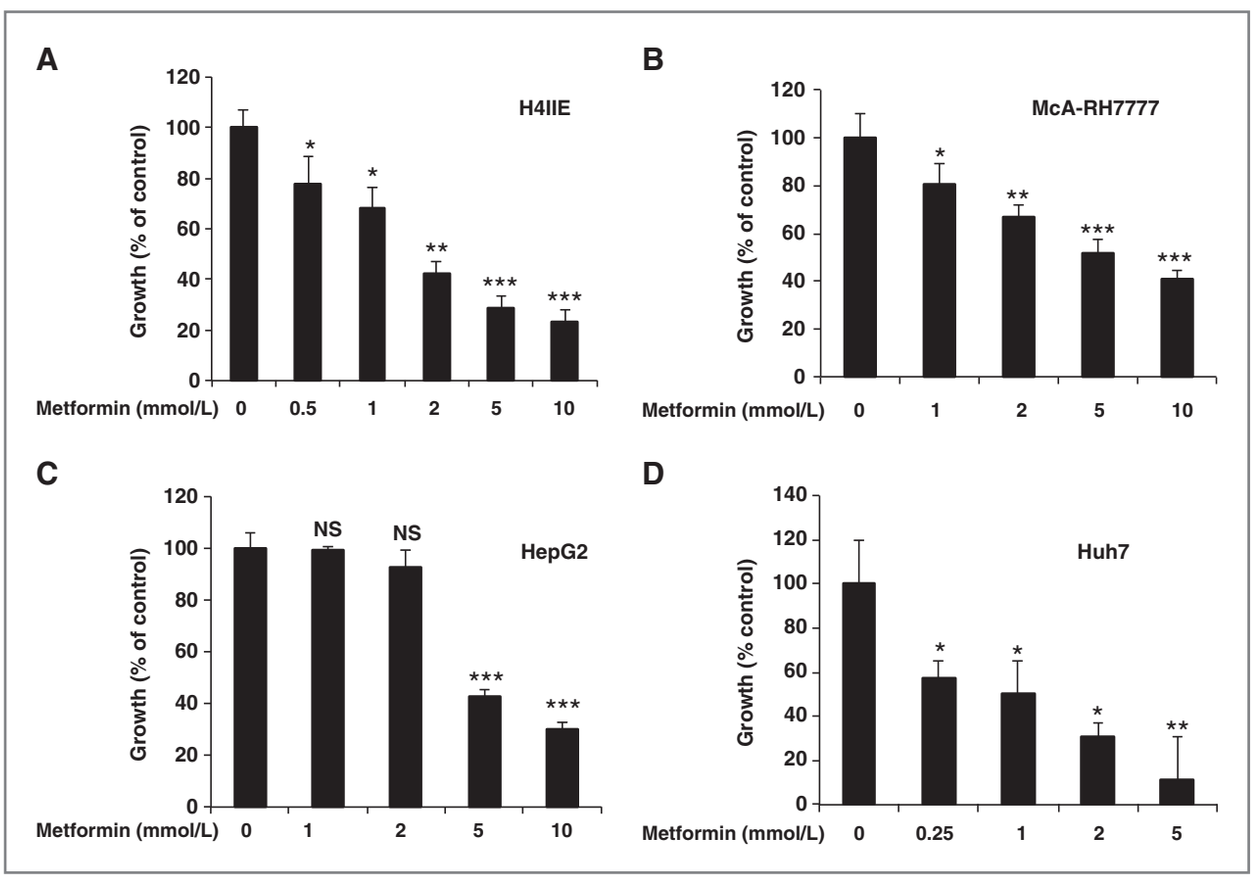

Figure 1. Metformin decreases the growth of HCC cell lines. Dose-dependent decrease in cell growth in (A) H4IIE (B) McA-RH7777, (C) HepG2, and (D) Huh7 cell lines treated with the indicated doses of metformin as described in Materials and Methods. Cell growth was measured using Wst-1 colorimetric assay. $N = 6 \pm$ SE. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$.

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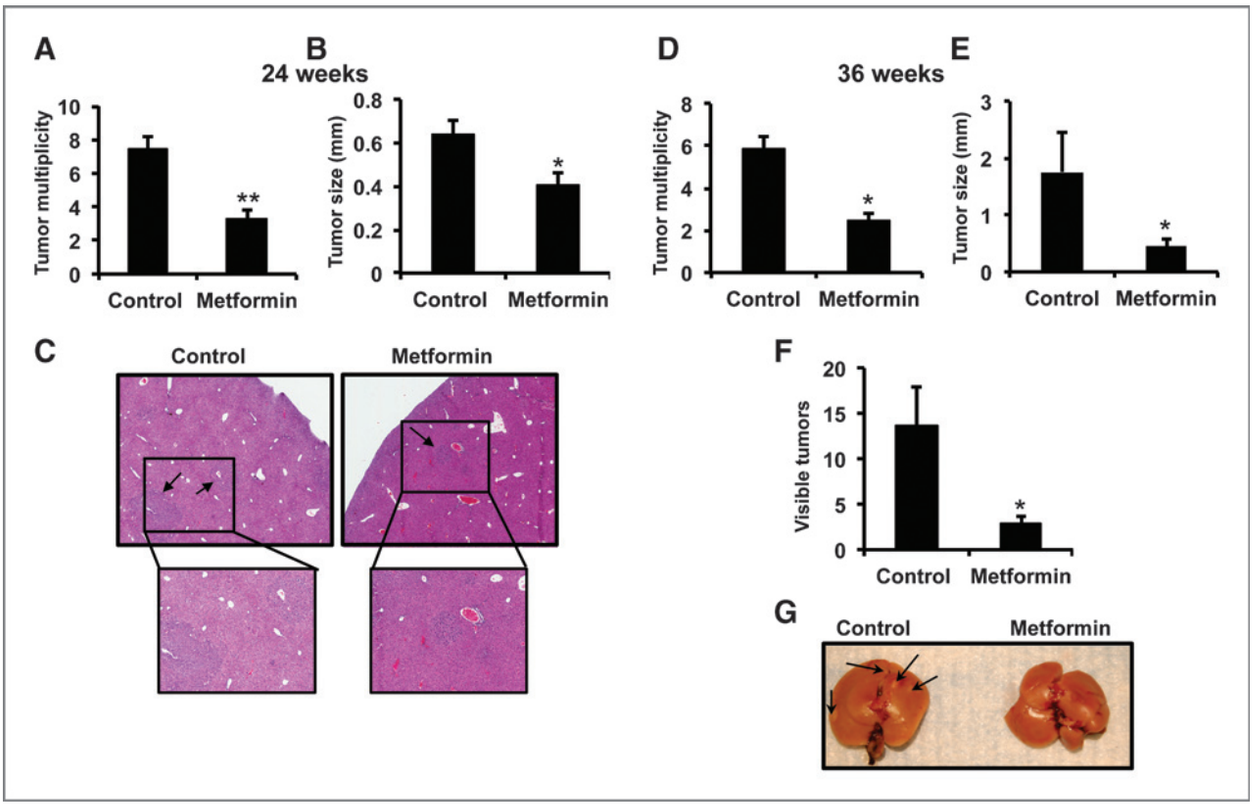

Figure 2. Metformin inhibits DEN-induced hepatocellular carcinogenesis. A, metformin reduces liver tumor multiplicity compared control treated mice at 24 weeks. B, reduced liver tumor size in mice treated with metformin at 24 weeks. C, representative H&E staining of paraffin-embedded liver sections. Sections also indicate no change in pathology of tumors. D, tumor number from livers of mice treated with control or metformin diet for 36 weeks. E, reduced liver tumor size in mice treated with metformin at 36 weeks. F, metformin reduces visible surface tumors after 36 weeks following DEN treatment. G, representative liver from control or metformin-treated mice after 36 weeks following DEN treatment. Mice were injected with DEN at 2 weeks of age and then placed on control or metformin chow diet after weaning. Mice were euthanized 24 or 36 weeks after DEN treatment. $N = 7$ mice per group $\pm$ SE, *, $P < 0.05$; **, $P < 0.001$.

metformin-treated mice. In addition, the increased ratio is usually associated with abundant tumor burden, which was not observed in our studies. In the 36-week group, metformin treatment reduced the number of tumor number, as determined by histopathology, to almost 60% (Fig. 2D). The size of tumors was also reduced significantly (Fig. 2E). In addition, visible tumors on the surface of the liver were reduced nearly 80% (Fig. 2F and G, white spots are a reflection of light).

Metformin suppresses liver tumorigenesis independent of AMPK

Previous studies have shown that metformin exerts its effect via activation of AMP kinase (17–20). The total protein levels of AMPK in the livers of metformin-treated mice did not change compared with control-fed mice. Interestingly, the active form of AMPK, phosphorylated AMPK (pAMPK), did not change following treatment with metformin for 24 weeks (Fig. 3A and Supplementary Fig. S3A). We also confirmed that AMPK was not active by examining phosphorylation of the AMPK target, TSC2 which was unaltered following treatment with metformin (Supplementary Fig. S3A).

The most well-known role of metformin is glycemic control and not necessarily AMPK activation. Metformin mediates its antidiabetic effects in part by inhibiting the expression of PEPCK and G6Pase, key mediators of hepatic gluconeogenesis (12, 17, 21). Metformin treatment suppressed the expression of PEPCK and G6Pase mRNA and protein (although the effect on G6pase protein was less pronounced) (Fig. 3B and C and Supplementary Fig. S3B). We also examined whether metformin had a physiologic effect on treated mice. Control mice had normal fed glucose levels (~165 mg/dL), whereas metformin decreased fed glucose levels (143 mg/dL), although it was still in the normal fed glucose range (Fig. 3D). Fasting glucose in control mice was reduced to 96 mg/dL and 76 mg/dL in metformin-treated mice (Fig. 3E). This confirmed that metformin reduces circulating glucose levels in DEN-treated mice without inducing clinical hypoglycemia (glucose levels <55 mg/dL). These data showed that metformin alters glucose homeostasis without increasing AMPK activity in the liver. This was in agreement with recent studies showing that metformin treatment reduces glucose production independent of AMPK activation (22).

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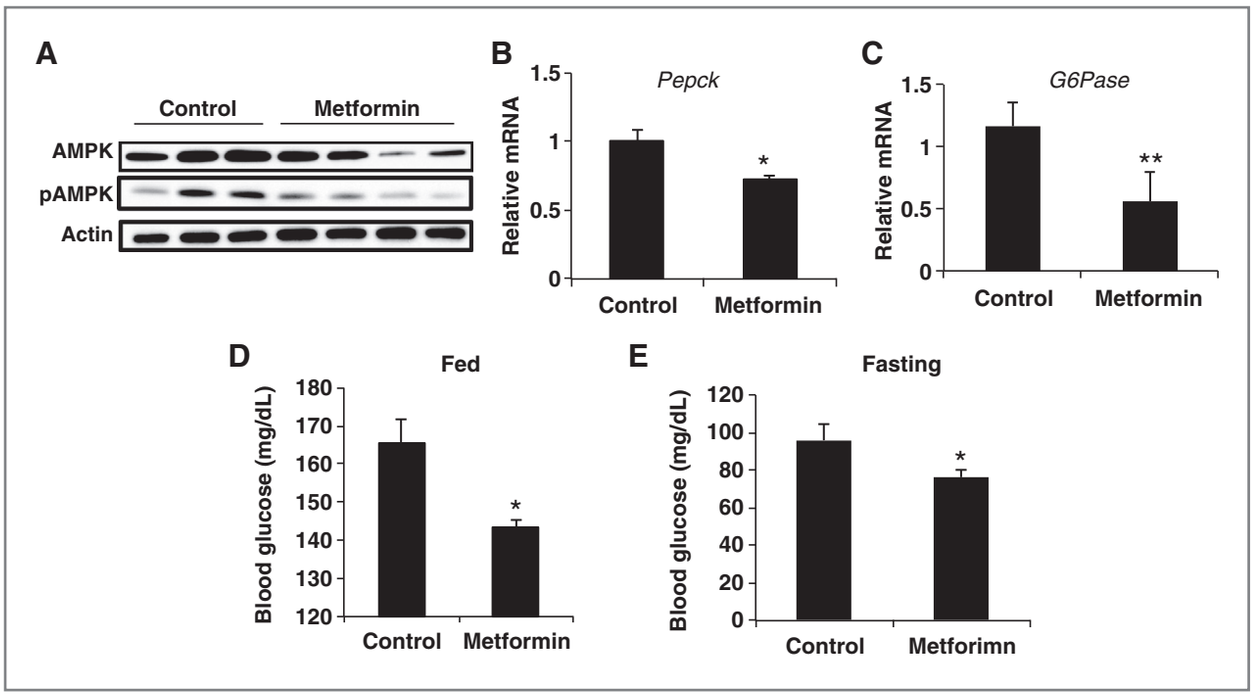

Figure 3. Metformin does not increase AMPK activation but does promote glucose homoeostasis. A, metformin does not increase AMPK expression or phosphorylation. Protein extracts from livers of control and metformin-treated mice were separated by SDS-PAGE and immunoblotted for AMPK, phospho-AMPK, and actin as a control. Metformin decreases (B) *Pepck* and (C) *G6Pase* mRNA expression in livers of treated mice. RNA was isolated from livers of control and metformin-treated mice and RT-PCR conducted for *Pepck* and *G6pase*. Metformin reduces (D) fed and (E) fasting glucose levels. Mice were fed or fasted overnight, tail vein blood obtained and glucose levels determined. $N = 4–6 \pm$ SD. *, $P < 0.05$; **, $P < 0.005$.

The lack of metformin-induced AMPK activation was surprising given the evidence in the literature. However, the first report of AMPK activation *in vivo* by metformin was shown in muscle and not liver. To confirm this tissue-specific effect on AMPK, we treated mice with metformin for 2 weeks and examined pAMPK in liver and muscle. Treatment with metformin increased pAMPK in muscle from metformin-treated mice (Supplementary Fig. S4A). In contrast, there was no change to liver pAMPK (Supplementary Fig. S4B). This showed that oral administration of metformin activates AMPK in muscle but not in the liver. The effects of metformin on blood glucose and gluconeogenic gene expression confirmed the physiologic efficacy of metformin in our experiments, despite the lack of AMPK activation in the liver. These data strongly suggested that metformin inhibited liver tumorigenesis in an AMPK-independent manner.

Metformin inhibits tumor growth by reducing lipogenesis

De novo lipogenesis from glucose has become recognized as an important pathway in cancer (23–25). Increased lipogenesis has been shown in liver tumors compared with normal adjacent tissue (26). The tumors showed increased expression of key enzymes regulating fatty acid synthesis and lipogenesis. Therefore we examined the effect of metformin on lipogenic gene expression during DEN-induced carcinogenesis. Acetyl-CoA carboxylase (ACC) is the rate-limiting enzyme in the synthesis of fatty acids, which converts acetyl CoA to malonyl CoA. ACC mRNA and protein levels were reduced in metformin-treated mice (Fig. 4A and D and Supplementary Fig. S5). AMPK decreases lipogenesis in part by phosphorylating ACC, which reduces ACC activity. Surprisingly, phosphorylation of ACC was actually reduced in livers from metformin-treated mice (Fig. 4D and Supplementary Fig. S5). It is unclear why pACC is reduced (more active), but might reflect compensation by the liver as a result of reduced lipid synthesis. Regardless, the lack of an increase in ACC phosphorylation further shows that metformin does not activate AMPK in these studies and that the mechanism of metformin-mediated growth inhibition is AMPK independent. We then examined the expression of fatty acid synthase (FASN), a large multifunctional enzyme that synthesizes palmitate from acetyl CoA and malonyl CoA. FASN mRNA and protein levels were significantly decreased from livers of mice treated with metformin (Fig 4B and D and Supplementary Fig. S5). In order for glucose to be used for *de novo* fatty acid synthesis, the enzyme ATP citrate lyase (ACLY) is required to make acetyl CoA available in the cytoplasm for fatty acid synthesis. The expression of ACLY RNA levels was reduced in the livers from metformin-treated mice (Fig. 4C). In addition, the protein expression of ACLY was also reduced (Fig. 4D and Supplementary Fig. S5). We also examined the expression of phosphorylated active ACLY. pACLY levels were also reduced from the livers of metformin-treated mice (Fig.

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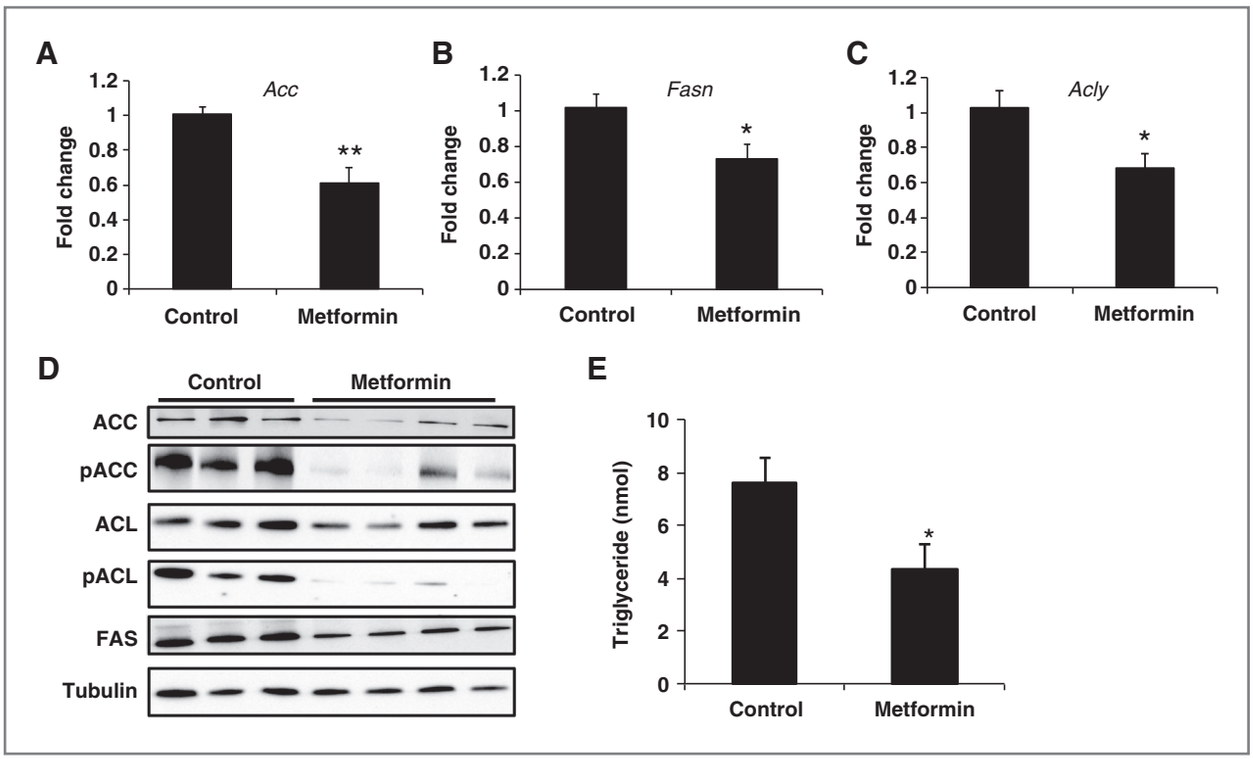

Figure 4. Metformin decreases the expression of enzymes driving hepatic lipogenesis. Treatment of mice with metformin decreases the expression of fatty acid synthesis genes, (A) *Acc* and (B) *Fasn*. C, metformin decreases the expression of the citrate cleavage enzyme, *Acly*. D, metformin decreases the protein expression of ACC, FASN, and ACLY, and active ACLY (phosphorylated). Metformin treatment does not increase AMPK activation as evidence by lack of an increase in ACC phosphorylation. Total cell lysates were prepared from liver tissues extracted from control and metformin-treated mice. Immunoblotting was done for ACC, p-ACC, ACLY, p-ACLY, and FAS. Tubulin was used as an endogenous control. E, metformin decreases liver triglycerides. For RT-PCR and triglycerides levels, $N = 4–6 \pm$ SD. *, $P < 0.05$; **, $P < 0.005$.

4D and Supplementary Fig. S5). Lipogenesis in the liver is a function of increased fatty acid synthesis and fatty acid esterification into triglycerides. Therefore to confirm the effect of metformin on hepatic lipogenesis, we examined triglyceride concentrations in the livers of control- and metformin-treated mice. As expected based on previous studies, metformin treatment significantly reduced liver triglyceride levels (Fig. 4E). Together, these data showed that metformin coordinates the downregulation of pathways driving *de novo* lipogenesis from glucose.

Induction of lipogenic gene expression rescues the growth inhibiting effects of metformin

Next we examined the effect of metformin on the expression of lipogenic genes in the HCC cell lines described above. Similar to what we observed *in vivo*, metformin reduced the expression of ACLY, ACC, and FASN in the HCC cell lines (Fig. 5A–D). These data strongly suggested an association between a reduction in lipogenic gene expression and inhibition of growth by metformin. Therefore, we wanted to determine whether the reduction in lipogenic gene expression is mediating the growth effects of metformin. Rather than overexpress a single lipogenic gene, we wanted to restore the expression of all 3 lipogenic genes. SREBP1c is a key transcription factor driving lipogenic gene expression in the liver (27). In addition, SREBP1c is associated with the lipogenic phenotype observed in cancer (28, 29). We used a cleaved nuclear form of SREBP1c, which is constitutively active (CA-SREPB1c). Ectopic expression of CA-SREBP1c in H4IIE and MCA-RH7777 hepatoma cell lines induced the expression of Srepb1c and all 3 lipogenic genes (Supplementary Fig. S6A–D). Cells were then treated with metformin and effects on cell growth determined. Metformin treatment of the vector control cells with the indicated doses reduced growth approximately 40% in both cell line (Fig. 5E and F). However, in the CA-SREBP1c expressing cells, growth inhibition was significantly reduced, with growth being restored to untreated levels. This showed that the anticancer effects of metformin are in part mediated by reducing lipogenic gene expression.

Discussion

HCC is typically associated with a poor prognosis. Most patients are diagnosed with advanced disease which has a 5-year survival of approximately 2%. Therefore prevention of HCC represents the best strategy to reduce mortality and morbidity. This requires the identification of patients at risk for HCC and the development of safe chemopreventive agents. Type II diabetics have significant increased risk for developing HCC (3, 6, 7). The increased risk represents a

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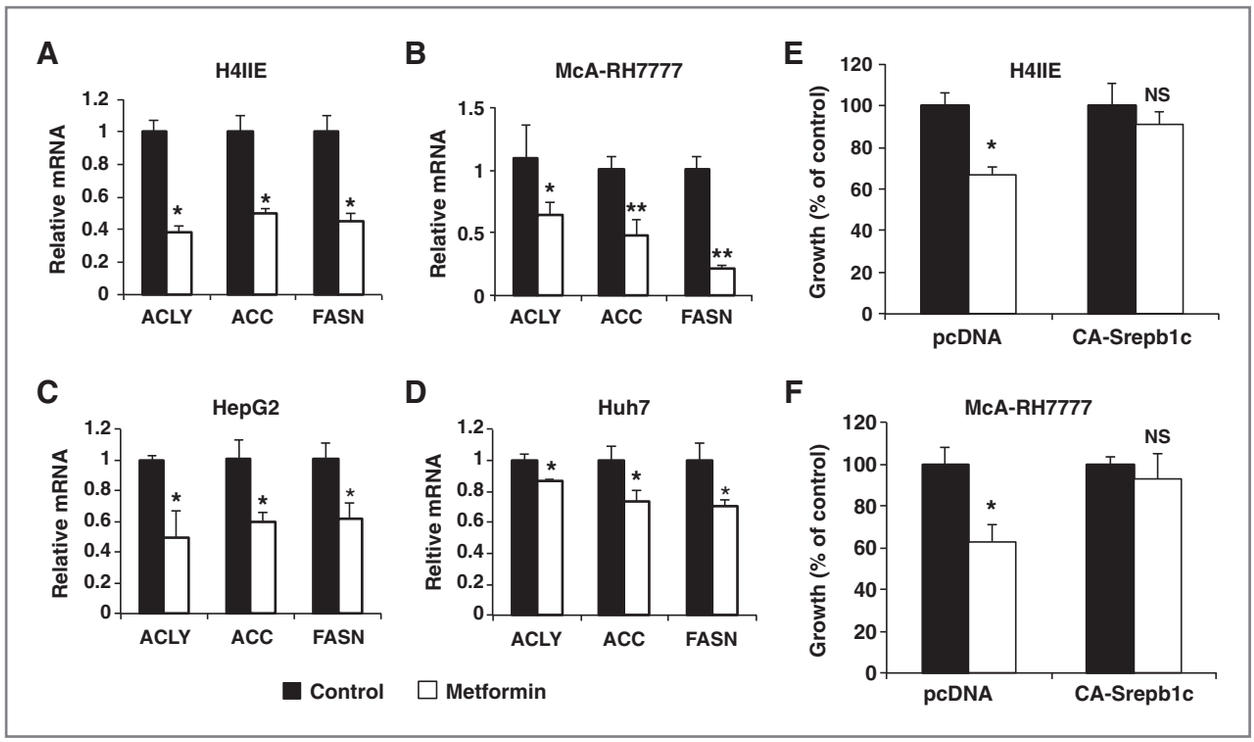

Figure 5. Inhibition of lipogenic gene expression is responsible for the growth effects of metformin. Treatment with metformin decreases lipogenic gene expression in (A) H4IIE, (B) MCA-RH7777, (C) HepG2, and (D) Huh7 HCC cell lines. Expression was normalized to β-actin and expressed relative to control. $N = 3 \pm$ SD, *, $P < 0.05$; **, $P < 0.005$ compared with control. Induction of lipogenic gene expression by ectopic expression of SREBP1c rescues metformin-mediated growth inhibition in (E) H4IIE and (F) MCA-RH7777 HCC cell lines. Stable pcDNA vector control and CA-SREBP1c expressing cell lines were established. Cells were treated with metformin for 72 hours and cell growth determined using a hemocytometer. $N = 3 \pm$ SD, *, $P < 0.01$.

growing health concern because diabetes rates are increasing, due in part to the obesity epidemic. Metformin is a first-line drug of choice for the treatment of type II diabetes. In addition to its antidiabetic effects, preclinical studies show that metformin has anticancer properties *in vitro* and *in vivo* (6, 19, 30–34). Epidemiologic evidence shows a significant reduction in HCC in diabetic patients taking metformin (14, 15). Surprisingly, there have been no preclinical studies on the ability of metformin to inhibit HCC despite the liver being the main metformin responsive tissue. The studies we describe here show that metformin significantly protects against HCC formation and tumor growth. In addition, our data shows this is in part via downregulation of multiple steps in *de novo* lipogenesis.

Several potential mechanisms have been proposed for inhibitory action of metformin on tumor growth (19, 35–37). Early reports suggested that metformin exerts its effect via activation of the energy sensor AMPK (17–20). However, in our studies we did not observe an increase in phosphorylated AMPK in the livers of treated mice, although fed and fasting glucose levels as well as gluconeogenic targets were reduced. This was further confirmed by the lack of phosphorylation of AMPK downstream targets, ACC and TSC2. This is in line with several recent studies highlighting AMPK-independent effects of metformin on glucose homeostasis and tumor growth *in vitro* and in *vivo* (22, 34, 38–40). Although administration of metformin to mice did not alter AMPK activation in liver, we did observe activation in muscle. Indeed, the original manuscript describing metformin-mediated activation of AMPK *in vivo* was shown in muscle (18).

These studies and others still contradict several studies showing AMPK activation in the liver by metformin. One likely explanation may be that mice were treated for an extended period of time in our experiments whereas the other studies used short-term treatment (17). In addition, it was recently shown by Memmott and colleagues that AMPK activation by metformin in the liver may be route dependent (34). They showed that intraperitoneal, but not oral, metformin treatment increased the phosphorylation of AMPK in liver. It is believed that intraperitoneal administration leads to a higher systemic concentration compared with oral administration (34). It is important to note that metformin is currently approved for oral administration, and therefore intraperitoneally administered metformin is not clinically appropriate.

This prompted us to investigate other potential mechanisms responsible for the chemopreventive effects of metformin. *De novo* lipogenesis represents a common feature of many types of cancers and in particular HCC. The expression and activity of the 2 main fatty acid synthesis enzymes, ACC and FASN, are elevated in several different cancer types including HCC. ACC is the rate-limiting step of *de novo* fatty acid synthesis, which converts acetyl CoA to malonyl CoA.

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FASN generates palmitate from acetyl CoA and malonyl CoA. Similarly our studies show that metformin reduces FASN expression in the livers of treated mice. In addition, we observe a decrease in ACC expression as well. In support of the potential of metformin to inhibit cancer growth via fatty acid synthesis, recently Algire and colleagues showed that metformin treatment of tumor-bearing mice led to a reduction in FASN expression in tumors (37). Although metformin is reported to reduce fatty acid synthesis by activating AMPK, as we did not observe an increase in AMPK activation, an AMPK-independent mechanism is most likely responsible. Regardless, these studies are the first to show the effect of metformin on lipogenic pathways in an autochthonous cancer model.

ACC and FASN use acetyl CoA for *de novo* lipogenesis in the cytoplasm. However acetyl CoA derived from glucose is generated in the mitochondria. In order for acetyl CoA to participate in fatty acid synthesis, it must be made available in the cytoplasm. Cells accomplish this by converting acetyl CoA and oxaloacetate (OAA) in the TCA cycle into citrate, which can then be exported to the cytoplasm. In the cytoplasm ATP-citrate lyase (ACLY) converts citrate back to OAA and acetyl CoA. We show that metformin also reduces ACLY expression. This would further reduce the ability of cells to carry out *de novo* lipogenesis from glucose. The importance of ACLY in cancer is highlighted by studies showing that genetic or chemical inhibition of ACLY has anticancer effects (41, 42). The ability of metformin to reduce cell growth by inhibiting the gene expression of several genes driving fatty acid synthesis was confirmed using a genetic rescue approach. SREBP1c is a master regulator of lipogenesis in the liver. In addition, ACLY, ACC, and FASN are direct transcriptional targets of SREBP1c. Ectopic expression of SREBP1c induced lipogenic gene expression and blocked the growth inhibitory effects of metformin in HCC cell lines. This shows that the ability of metformin to reduce cell growth is in part mediated via inhibition of expression of multiple lipogenic genes. Therefore unlike inhibitors of fatty acid synthesis that target only one step, our data shows that metformin regulates multiple pathways involved in fatty acid synthesis.

The studies we describe here were done in nondiabetic mice. However, metformin is most likely as effective in diabetic or obese conditions. Indeed, recent studies show that metformin is more effective at reducing tumor growth in mice receiving a diet promoting obesity and diabetes (31, 37). In addition, the mechanism of action suggests it may be more effective in diabetic models. Diabetes is associated with increased fatty acid synthesis and hepatic steatosis (25, 43) both of which promote HCC (25, 44). Future studies will determine the ability of metformin to protect against HCC in diet and genetic rodent models of diabetes and obesity.

Our data also has relevance for cancer prevention with regard to other risk factors associated with HCC. NAFLD and obesity are independent risk factor for HCC. Both of these conditions are associated with increased lipogenesis and hepatic steatosis. However, it should be noted that NAFLD, obesity, and diabetes are often comorbidities and found in most cases of HCC. These studies also have bearing for patients with HBV and HCV. Many patients with HBV and HCV have hepatic steatosis and increased fatty acid synthesis (45–48). The significance of this is underscored by a 100-fold increased risk of HCC in patients the comorbidities of diabetes and obesity with hepatitis (49). Therefore this represents a particularly important candidate group for consideration for metformin as a chemoprevention approach.

One of the advantages of metformin is its relatively safe toxicity profile. In addition, metformin is already approved for diabetes and therefore its introduction into the clinic streamlined. There are currently more than 20 clinical trials investigating the role of metformin as an anticancer agent (50). However, none of these are investigating the ability of metformin to reduce cancer in the liver, its main target organ of action. Before these trials it would be valuable to determine at what time point during liver carcinogenesis metformin is effective. In conclusion our data shows that metformin inhibits HCC in part by inhibition of hepatic lipogenesis. This provides a rationale for clinical trials into the efficacy of metformin in HCC in patients that can readily be identified such as diabetics and other pathologies associated with hepatic lipogenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Cancer Prevention Research

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