

## Paracrine Hedgehog Signaling Drives Metabolic Changes in Hepatocellular Carcinoma

Isaac S. Chan<sup>1,4</sup>, Cynthia D. Guy<sup>2</sup>, Yuping Chen<sup>1</sup>, Jiuyi Lu<sup>1</sup>, Marzena Swiderska-Syn<sup>1</sup>, Gregory A. Michelotti<sup>1</sup>, Gamze Karaca<sup>1</sup>, Guanhua Xie<sup>1</sup>, Leandi Krüger<sup>1</sup>, Wing-Kin Syn<sup>1,5</sup>, Blair R. Anderson<sup>3</sup>, Thiago A. Pereira<sup>1</sup>, Steve S. Choi<sup>1</sup>, Albert S. Baldwin<sup>4</sup>, and Anna Mae Diehl<sup>1</sup>

### Abstract

Hepatocellular carcinoma (HCC) typically develops in cirrhosis, a condition characterized by Hedgehog (Hh) pathway activation and accumulation of Hh-responsive myofibroblasts. Although Hh signaling generally regulates stromal–epithelial interactions that support epithelial viability, the role of Hh-dependent myofibroblasts in hepatocarcinogenesis is unknown. Here, we used human HCC samples, a mouse HCC model, and hepatoma cell/myofibroblast cocultures to examine the hypothesis that Hh signaling modulates myofibroblasts' metabolism to generate fuels for neighboring malignant hepatocytes. The results identify a novel paracrine mechanism whereby malignant hepatocytes produce Hh ligands to stimulate glycolysis in neighboring myofibroblasts, resulting in release of myofibroblast-derived lactate that the malignant hepatocytes use as an energy source. This discovery reveals new diagnostic and therapeutic targets that might be exploited to improve the outcomes of cirrhotic patients with HCCs. *Cancer Res*; 72(24); 6344–50. ©2012 AACR.

### Introduction

Hepatocellular carcinoma (HCC) is one of the most common deadly forms of cancer worldwide (1). HCCs typically develop in cirrhotic livers (1). The latter compromises recovery from extensive liver resection and restricts chemotherapy options and efficacy. Therefore, survival depends mainly upon detection of tumors that are small enough to be safely ablated. Better screening and preventative strategies are needed, however, because the number of HCCs that are already advanced at diagnosis is increasing, and the population at risk for HCC is growing due to the increasing incidence of cirrhosis (1). Improved understanding of the early events in hepatocarcinogenesis would help to optimize prevention, early diagnosis, and treatment of HCCs.

Evidence that HCCs occur in 1% to 5% of cirrhotic patients annually suggests that the cirrhotic microenvironment promotes the outgrowth of malignant hepatocytes (2). However, the mechanisms involved remain obscure. One possibility is

that that stromal–epithelial interactions fuel HCC growth because deregulated, and excessively fibrogenic, repair of liver injury causes cirrhosis itself (3). The major producers of fibrous matrix during liver injury are myofibroblasts, and cirrhotic livers harbor large numbers of these cells. The role of myofibroblasts in HCC pathogenesis/progression is unclear, however, despite evidence that myofibroblast-derived factors mediate key aspects of the wound-healing response including matrix turnover, recruitment of inflammatory cells, vascular remodeling, and outgrowth of liver epithelial progenitors (4). The pivotal importance of myofibroblasts in cirrhosis pathogenesis justifies evaluating their role in hepatocarcinogenesis.

A key regulator of myofibroblasts is Hedgehog (Hh), a developmental morphogenic signaling pathway (5). Hh pathway activity is barely detectable in healthy livers but becomes robust during all types of liver injury. Injured liver epithelial cells are important drivers of this process because injury stimulates the wounded epithelia to produce and release Sonic hedgehog (Shh) and Indian Hedgehog (Ihh) ligands, as well as other soluble factors, that promote Hh signaling in neighboring Hh-responsive stromal cells (6, 7). Like other Hh-responsive stromal cells, myofibroblasts express the Hh ligand transmembrane receptor, Patched (Ptc; ref. 8). Interaction of epithelia-derived Hh ligands with Ptc results in the activation of Smoothened, the Hh signaling-competent coreceptor. This leads to accumulation and nuclear localization of glioma family proteins (Gli1, Gli2, Gli3), which regulate the transcription of Hh-responsive genes that control proliferation, viability, and differentiation of the stromal cells. Exchange of paracrine signals between Hh-producing epithelia and Hh-responsive stroma orchestrates organogenesis during development.

**Authors' Affiliations:** <sup>1</sup>Division of Gastroenterology, Department of Medicine, <sup>2</sup>Department of Pathology, <sup>3</sup>Center for Human Genetics, Duke University Medical Center, Durham; <sup>4</sup>Department of Genetics, The University of North Carolina School of Medicine, Chapel Hill, North Carolina; and <sup>5</sup>Liver Regeneration and Repair Group, The Institute of Hepatology, Foundation for Liver Research, London, United Kingdom

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**Corresponding Author:** Anna Mae Diehl, Division of Gastroenterology, Duke University Medical Center, 595 LaSalle Street, Snyderman Building, Suite 1073, Durham, NC 27710. Phone: 919-684-4160; Fax: 919-684-4183; E-mail: diehl004@mc.duke.edu

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Similar mechanisms are presumed to modulate some types of carcinogenesis based on findings in mouse models of pancreatic and prostate cancer (9, 10). Although increased Hh signaling has been documented in human HCCs (11), and liver myofibroblasts are known to be an Hh-responsive cell type, the possibility that HCC growth might be regulated by paracrine Hh signaling between myofibroblasts and malignant hepatocytes has not, to our knowledge, been examined.

Recently, we showed that treating a mouse model of fibrosis-associated HCCs with an Hh signaling inhibitor caused advanced HCCs to regress (12). Tumor involution was accompanied by myofibroblast loss and fibrosis improvement. This suggests that the anti-cancer actions of the Smoothed antagonist may have resulted from deletion of Hh-responsive myofibroblasts and justifies further work to identify how myofibroblasts might support the growth of malignant hepatocytes. Here, we evaluate the hypothesis that Hh signaling modulates myofibroblasts' metabolism to generate fuels for neighboring malignant hepatocytes. Given that HCCs, like many other epithelial cancers, exhibit enhanced glycolysis (i.e., the Warburg effect; ref. 13), we asked whether HCC glycolytic activity was influenced by Hh signaling in tumor-associated myofibroblasts.

## Materials and Methods

### Human subjects

The Duke Department of Pathology computer database was searched for cases of HCC arising in nonalcoholic fatty liver disease (NAFLD) patients from 2007 through 2011. Five cases were identified from resections, hepatectomies, and explants. Random tissue blocks containing both tumor and adjacent nontumor were used.

### Mice and cell culture

Ten *Mdr2*<sup>-/-</sup> mice (age, 51–59 weeks) with advanced liver fibrosis and HCCs were treated with either vehicle [dimethyl sulfoxide (DMSO), *n* = 5] or 40 mg/kg GDC-0449 (*n* = 5) for 9 days as described previously (12).

Liver myofibroblasts (8B cells, M. Rojkind, George Washington University, Washington, DC; ref. 14) were cultured alone (monoculture) or in a Transwell coculture system with HepG2 cells (American Type Culture Collection), Huh7.5 cells (C. Rice, Rockefeller University, New York, NY), or Panc 10.05 cells (Duke Cell Culture Facility, Durham, NC). Myofibroblast monocultures were also treated with PBS control or 1000 ng/mL recombinant Shh ligand with or without DMSO vehicle or 3 μmol/L GDC-0449, or conditioned medium from the other cells with or without IgG control or 10 μg/mL 5E1 antibody for 24 hours. HepG2 and Huh7.5 cells were grown alone or treated with lactate with or without FX11. See Supplementary Methods for details.

### Statistical analyses

Mean data were compared using the Student *t* test. Differences were considered significant when *P* < 0.05.

## Results and Discussion

### Malignant epithelia produce Hh ligands and stroma is enriched with Hh-responsive, glycolytic myofibroblasts in human HCC

To investigate Hh ligand expression and localization in HCCs, we conducted immunohistochemistry for SHH ligand in archived paraffin-embedded tissues from 5 patients with HCCs and cirrhosis caused by NAFLD. All of the HCCs showed increased expression of SHH relative to their capsules and adjacent nontumorous liver tissue (Fig. 1A). Within tumor nodules, malignant hepatocytes were a major source of SHH ligand. In contrast, nuclear staining for the Hh-regulated transcription factor, GLI2, was confined to the tumor-associated stroma. Compared with adjacent nontumor liver, HCCs were also significantly enriched with GLI2(+) stromal cells (Fig. 1B). These findings suggest that malignant hepatocytes produce Hh ligands that promote Hh signaling in adjacent stromal cells.

To assess glycolytic activity in these HCCs, we stained sections for pyruvate kinase M2 (PKM2), a rate-limiting glycolytic enzyme and well-validated marker of glycolysis (15). Unexpectedly, we found that PKM2 staining localized to HCC stroma, rather than to the malignant hepatocytes themselves (Fig. 1C). Dual staining for GLI2 and PKM2 confirmed that the Hh-responsive stromal cells were glycolytic and showed that HCC stroma harbored greater numbers of glycolytic cells than adjacent nontumorous liver (Fig. 1B and C). To further characterize these glycolytic stromal cells, we costained other sections for PKM2 and α-smooth muscle actin (α-SMA), a myofibroblast marker (4). Expression of PKM2 colocalized with α-SMA, showing that the glycolytic tumor-associated stromal cells were Hh-responsive myofibroblasts (Fig. 1D).

### Hh inhibitor depletes glycolytic myofibroblasts from tumor stroma in murine HCC model

Progressive liver injury and fibrosis occur in *Mdr2*<sup>-/-</sup> mice due to deficient transport of phosphatidyl choline into bile. Primary HCCs emerge spontaneously between 50 and 60 weeks of age, modeling the natural evolution of HCCs during fibrogenic repair of various types of chronic liver injury (16). Therefore, we used immunohistochemistry to characterize the tumor-associated stroma in HCCs that were microdissected from these mice. As noted in our human HCC cohort (Fig. 1B–D), tumor-associated stroma in the mouse model was enriched with cells that coexpressed α-SMA and PKM2 (Fig. 2A). Therefore, glycolytic myofibroblasts localize within the HCC-associated stroma in both species, and this seems to occur irrespective of the etiology of the underlying liver disease. In human HCCs, the glycolytic myofibroblasts costained for GLI2 (Fig. 1B) and, thus, were presumed to be Hh-responsive. To examine the role of Hh signaling in regulating glycolytic activity in the murine tumor stromal cells, we compared expression of *Glut1*, a glucose transporter, and several key glycolytic enzymes in mRNA isolated from HCCs of *Mdr2*-deficient mice that had been treated with either vehicle or the Smoothed antagonist, GDC-0449, for 9 days before sacrifice. Treatment with the Hh inhibitor reduced expression of mRNAs encoding *Gli2*, *Glut1*,

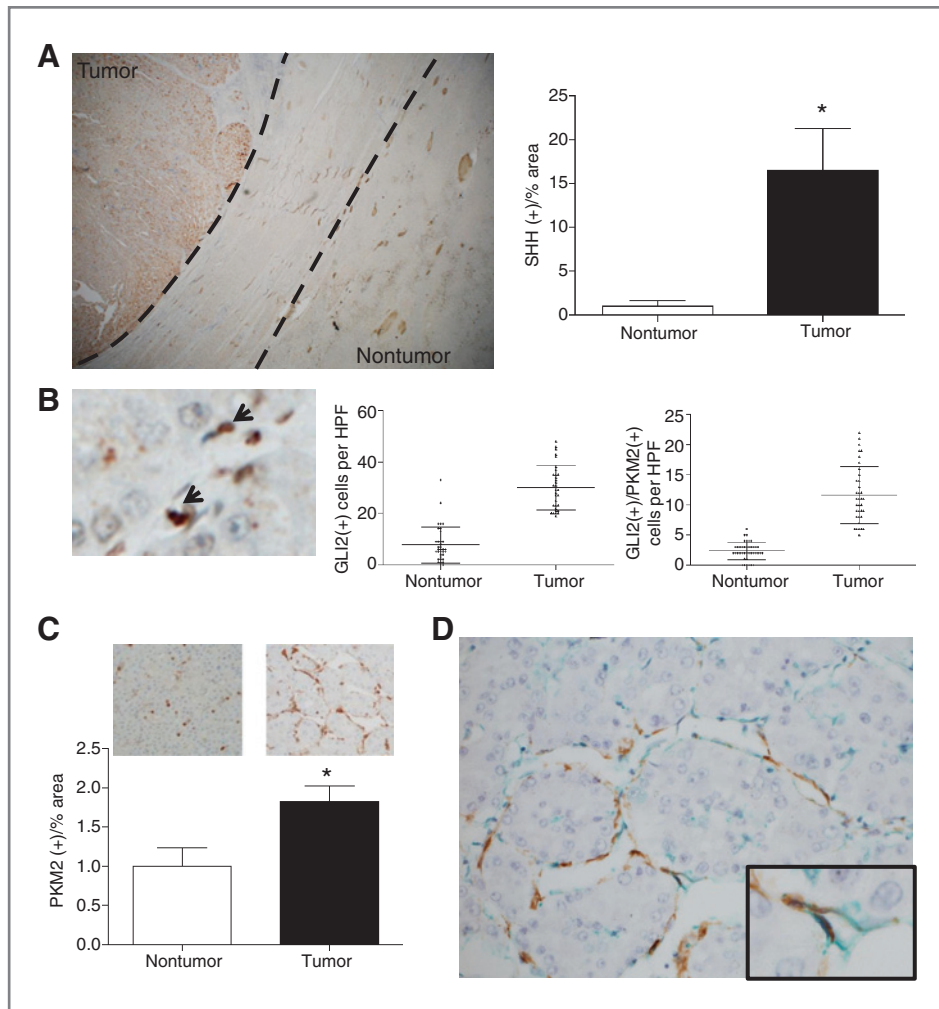
glycolytic enzymes, and  $\alpha$ -SMA (Fig. 2B). Immunohistochemistry confirmed that reduced expression of myofibroblast- and glycolysis-associated mRNAs was paralleled by depletion of tumor stromal cells that expressed  $\alpha$ -SMA, PKM2, and monocarboxylate transporter 4 (MCT4), a facilitator of lactate export (ref. 17; Fig. 2C).

**Paracrine Hh signaling between hepatoma cells and myofibroblasts stimulates glycolysis in myofibroblasts**

To determine whether hepatoma cells generate soluble Hh ligands that might stimulate glycolytic activity in Hh-responsive myofibroblasts, we compared Gli-luciferase reporter activity in Shh-LightII cells that were exposed to conditioned medium from HepG2 cells, cocultured with HepG2 cells in a Transwell system, or treated with control medium without or with recombinant SHH (Fig. 3A). Like recombinant SHH, exposure to HepG2 cell-derived soluble factors significantly increased Hh signaling in the Shh-LightII cells. The stimulatory effect of HepG2 conditioned medium was abrogated by adding 5E1, an Hh-neutralizing antibody that blocks HH ligand-Ptc interaction (ref. 18; Fig. 3A). Moreover, when HepG2 cells were

replaced with cells that do not generate Hh ligands (Panc 10.05 cells; ref. 19) and experiments were repeated, no change in Shh-LightII cell luciferase activity was observed (Supplementary Fig. S1A). The aggregate data, therefore, indicate that HepG2 cells generate soluble, biologically active Hh ligands.

To determine whether these HepG2-derived Hh ligands functioned as inducers of myofibroblast glycolysis, we cultured a well-characterized rat liver myofibroblasts line (8B cells; ref. 14) alone (monoculture) or in the Transwell system with HepG2 cells and assessed myofibroblasts glycolytic activity. Coculturing myofibroblasts with HepG2 cells induced myofibroblasts expression of mRNAs that encode key glycolytic enzymes (Fig. 3B) and increased their lactate:pyruvate ratio, a measure of glycolytic activity (Fig. 3C). Treating myofibroblasts with HepG2 cell conditioned media had similar effects (Fig. 3D). The stimulatory effects of HepG2 conditioned medium on myofibroblasts glycolysis were attenuated by adding 5E1 to block Hh ligand-Ptc interactions (Fig. 3E), suggesting that Hh ligands are the factors that malignant hepatocytes release to induce glycolytic activity in neighboring myofibroblasts. To verify that activating Hh signaling in myofibroblasts

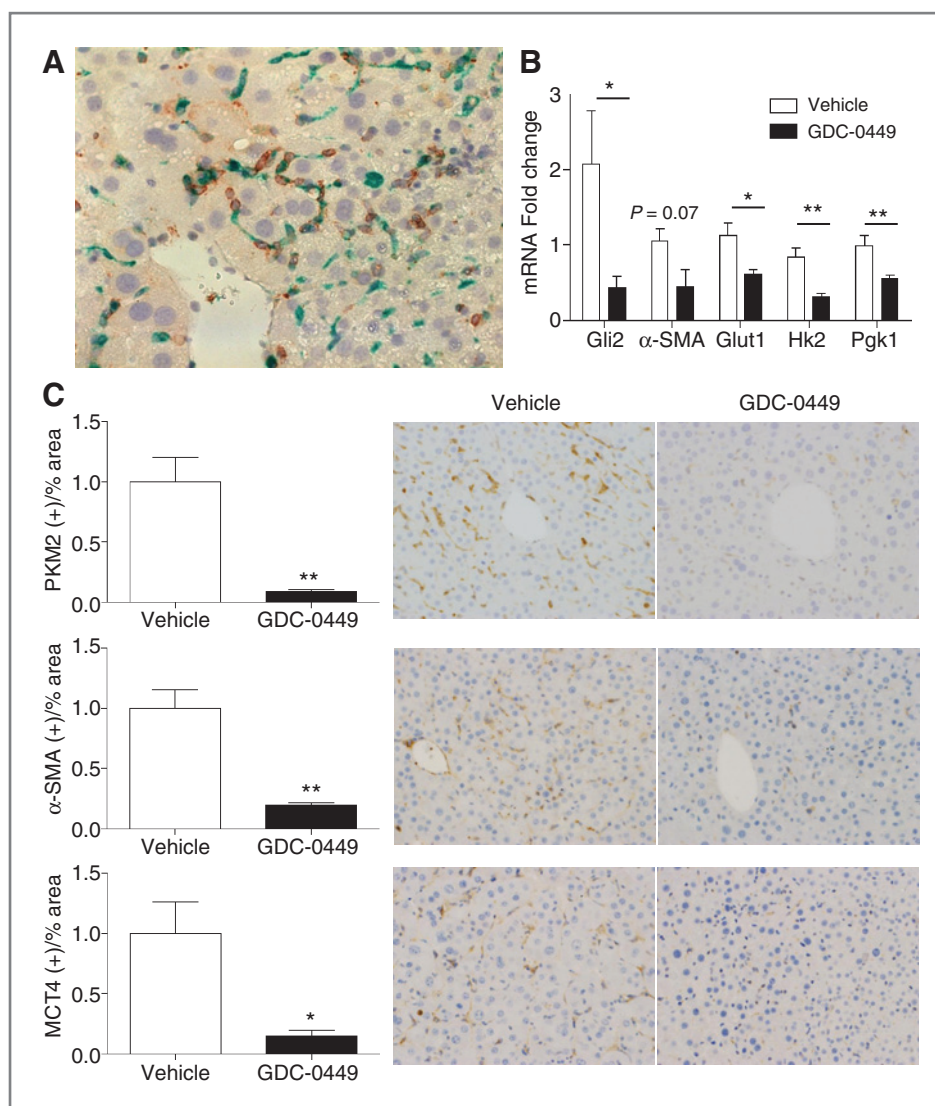


**Figure 1.** Evidence for paracrine Hh signaling between malignant epithelia and tumor stroma in human HCCs. Tumor and adjacent nontumorous liver from 5 patients with HCCs were evaluated by immunohistochemistry and quantitative morphometry. Representative sections are shown and morphometric (mean  $\pm$  SEM) or cell count (mean  $\pm$  SD) data are graphed. \*,  $P < 0.05$  for tumor versus nontumorous tissue. A, SHH ligand (brown,  $\times 50$ ). Dotted lines enclose fibrotic capsule. B, GLI2 (arrows,  $\times 400$ ). C, PKM2 (brown) in nontumor and tumor nodules ( $\times 400$ ). D, colocalization of PKM2 (green) with  $\alpha$ -SMA (brown) in tumor nodule ( $\times 400$ ,  $\times 1,000$  insert).

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**Figure 2.** Murine HCC stroma is enriched with Hh-dependent glycolytic myfibroblasts. Mdr2-deficient mice with HCCs were treated with the Hh inhibitor GDC-0449 or vehicle ( $n = 5$  mice per group); effects on stroma of microdissected tumor nodules were evaluated by morphometry of immunostained sections and quantitative reverse-transcription (qRT-PCR). Representative sections and mean  $\pm$  SEM data are displayed (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$  GDC-0449-treated vs. vehicle-treated groups). A, colocalization of PKM2 (green) with  $\alpha$ -SMA (brown) in tumor from vehicle-treated mouse ( $\times 400$ ). B, qRT-PCR for Gli2,  $\alpha$ -SMA, Glut1, Hk2, and Pgk1 in liver tumor mRNA. C, tumor sections from vehicle- and GDC-0449-treated mice show (top) PKM2, (middle)  $\alpha$ -SMA, and (bottom) MCT4 (brown,  $\times 200$ ).

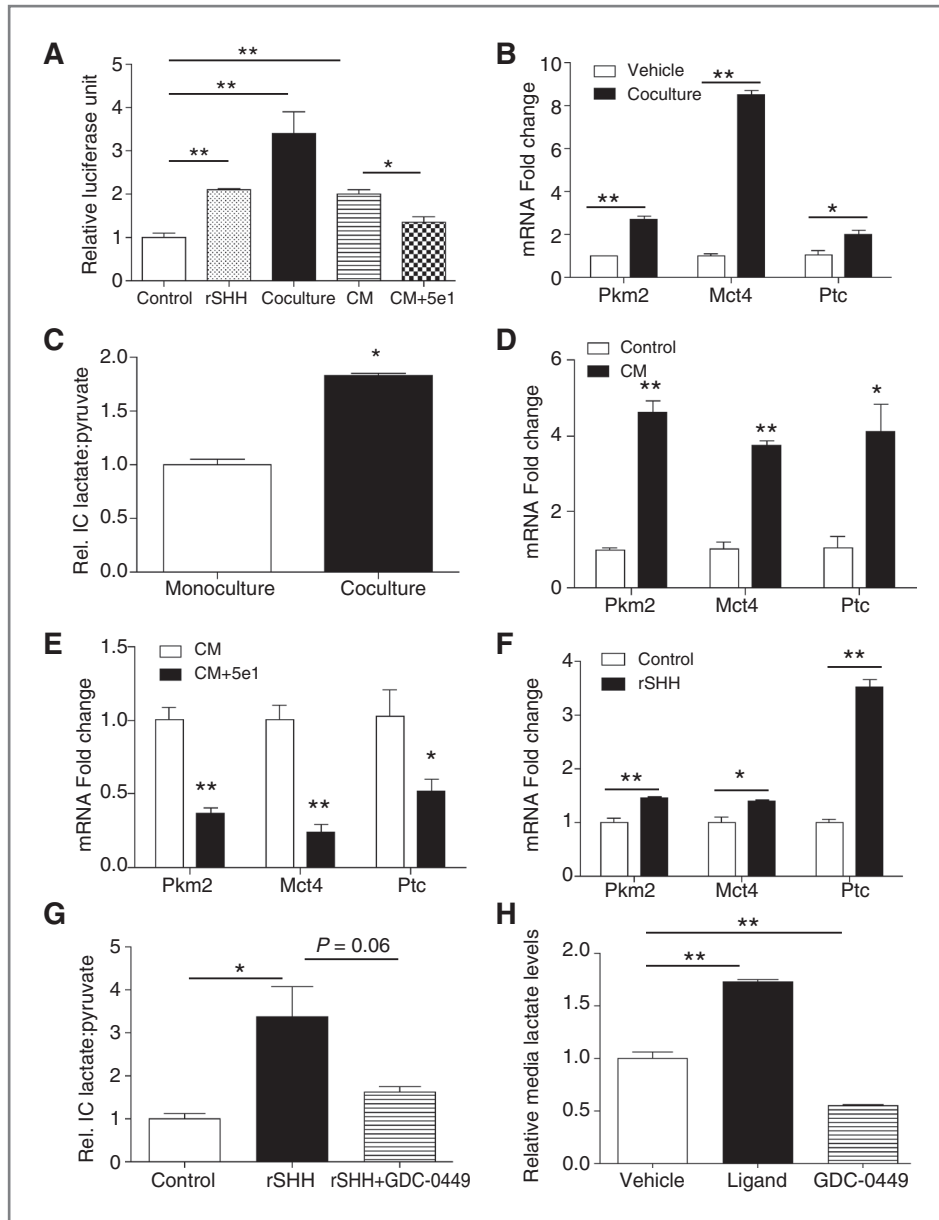


promotes glycolysis, we treated monocultures of liver myfibroblasts with recombinant SHH (rSHH) ligand. Compared with vehicle-treated myfibroblasts, myfibroblasts treated with rSHH ligand had increased expression of genes encoding glycolytic enzymes (Fig. 3F) and higher lactate:pyruvate ratios (Fig. 3G). GDC-0449, a direct antagonist of the Hh signaling intermediate, Smoothed, reversed the effects of rSHH ligand, confirming that myfibroblast glycolysis is regulated by canonical Hh signaling (Fig. 3G). Treating myfibroblasts with rSHH also significantly increased their secretion of lactate into the media, whereas adding GDC-0449 reduced media lactate below basal levels, showing that Hh signaling also regulates myfibroblast secretion of lactate (Fig. 3H). Finally, to determine whether cancer cells that do not generate Hh ligands can induce myfibroblast glycolysis, we compared expression of *pkm2* and *mct4* mRNAs in myfibroblasts that were exposed to soluble factors derived from Panc 10.05 cells (Supplementary Fig. S1C and S1D). As predicted by the data shown in Supplementary Fig. S1A, Panc 10.05 cells were unable to induce

myfibroblasts expression of *Ptc*, a known Hh target gene (Supplementary Fig. S1B). However, they did increase myfibroblasts expression of both glycolysis markers, albeit at significantly lower levels than were induced by HepG2 cells. Thus, while malignant hepatocytes release Hh ligands to activate glycolysis in neighboring stromal cells, pancreatic cancer cells are able to achieve this via other mechanisms that do not require Hh-Ptc paracrine interactions.

#### Lactate generated by glycolytic myfibroblasts fuels lipogenesis in HepG2 cells

Nevertheless, our aforementioned findings identified a novel Hh-dependent mechanism whereby malignant hepatocytes modulate the metabolic activity of tumor-associated myfibroblasts. Because tumor stroma is generally believed to support the growth of malignant epithelial cells, we used the Transwell coculture system to evaluate the related hypothesis that myfibroblast-derived glycolytic end-products (such as lactate) enhance net energy homeostasis of malignant

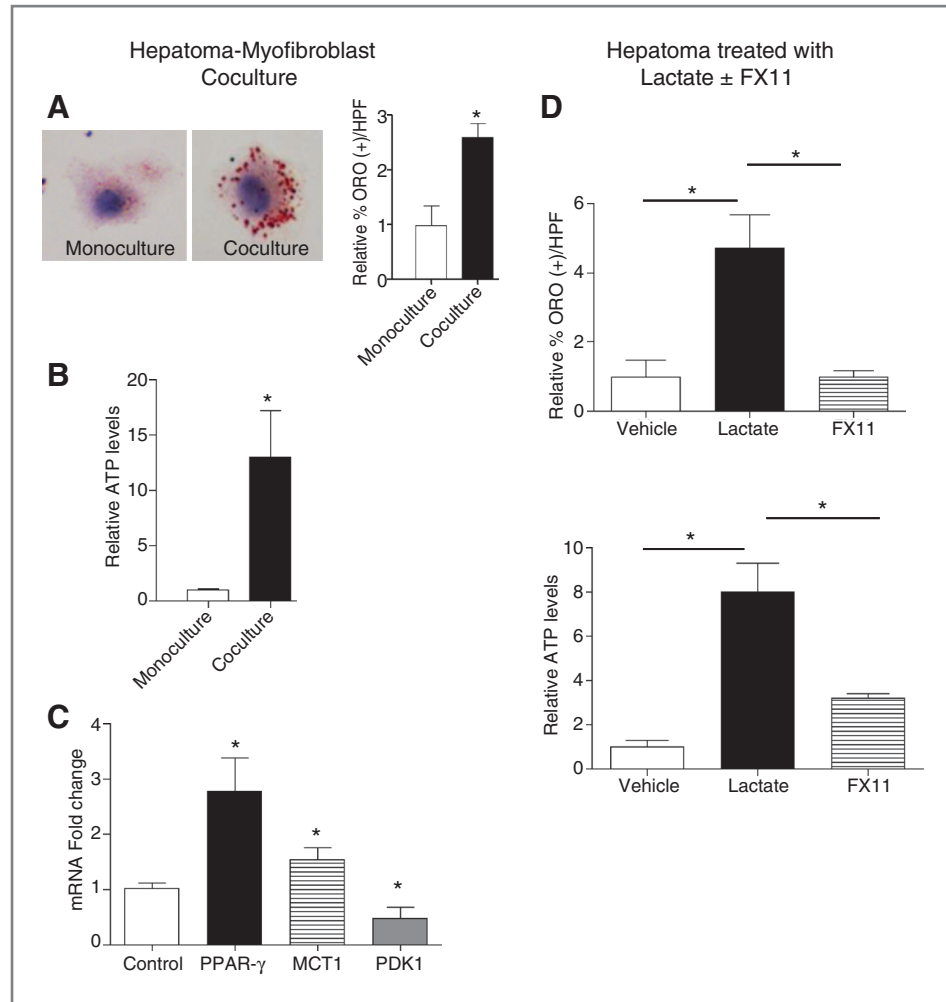


**Figure 3.** Paracrine Hh signaling stimulates myofibroblast glycolysis. A, Gli-luciferase reporter activity in Shh-LightII cells incubated with control medium, 1,000 ng/mL recombinant SHH ligand, cocultured with HepG2 in a Transwell system, incubated with HepG2 conditioned media (CM) with or without 5E1 neutralizing antibody (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ). B, Pkm2 and Mct4 mRNA levels in myofibroblasts grown in monoculture or cocultured with HepG2s in Transwells. C, intracellular (IC) lactate:pyruvate ratio in myofibroblasts monocultured or cocultured with HepG2 cells. D, Pkm2, Mct4, and Patched mRNA levels in myofibroblasts grown in control media or with HepG2 conditioned media. E, Pkm2, Mct4, and Patched mRNA levels in myofibroblasts grown in HepG2 conditioned media with or without 5E1 neutralizing antibody. F, Pkm2 and Mct4 mRNA levels in myofibroblasts treated with vehicle or recombinant SHH ligand (rSHH-L). G, intracellular lactate:pyruvate ratio in myofibroblasts after treatment with rSHH-L. H, lactate exported into media by myofibroblasts treated with control media, rSHH-L, or rSHH-L + GDC-0449. Mean  $\pm$  SEM data from triplicate experiments are graphed. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  versus respective controls.

hepatocytes. Compared with monocultured HepG2 cells, HepG2 cells that were cocultured with liver myofibroblasts showed significant accumulation of Oil Red O-stained lipid droplets (Fig. 4A). Because lipid accumulation occurs during energy excess, we compared the ATP content of mono- and cocultured HepG2 cells and found significantly higher ATP content in the cocultured HepG2 cells (Fig. 4B). Consistent with increased lipogenesis during coculture, cocultured HepG2 cells expressed higher mRNA levels of the lipogenic transcription factor *Ppar $\gamma$*  than monocultured HepG2 cells (Fig. 4C). Coculture also enhanced HepG2 expression of monocarboxylate transporter 1 (*Mct1*), which encodes a lactate transporter (17), but suppressed expression of pyruvate dehydrogenase kinase (*Pdk1*), which encodes an enzyme that gates entry of pyruvate into the tricarboxylic acid (TCA) cycle (Supplemen-

tary Fig. S3). These findings suggest that malignant hepatocytes import myofibroblast-derived lactate and convert it into pyruvate to fuel ATP and lipid biosynthesis. To assess this issue more directly, we treated monocultured HepG2 cells with lactate in the absence or presence of FX11. FX11 inhibits the activity of lactate dehydrogenase (LDH), thereby blocking the interconversion of lactate and pyruvate (20). Treating HepG2 cells with lactate significantly increased lipid accumulation and ATP content. Both responses were prevented when cells were pretreated with FX11 to inhibit intracellular conversion of lactate into pyruvate (Fig. 4D). Similar results were obtained when another hepatoma cell line, Huh7.5, was cocultured with liver myofibroblasts or treated directly with lactate (Supplementary Fig. S4), providing reassurance that the findings were not restricted to a single liver cancer cell line.

**Figure 4.** Lactate generated by glycolytic myofibroblasts fuels lipogenesis in HepG2 cells. **A**, Oil Red O (ORO) staining of HepG2 cells grown alone or cocultured in Transwells with myofibroblasts and quantified by morphometry. Intracellular ATP (**B**) and MCT1, PPAR $\gamma$ , and PDK1 (**C**) mRNA levels in HepG2 cells cultured alone or in Transwells with myofibroblasts. **D**, change in Oil Red O staining and ATP in HepG2s after treatment with 40 mmol/L lactate or FX11. Mean  $\pm$  SEM data from triplicate experiments are graphed. \*,  $P < 0.05$  versus respective control.



The aggregate data, therefore, support a model whereby malignant hepatocytes generate Hh ligands to orchestrate the construction of an Hh-responsive stroma that nurtures further growth of the malignant epithelia. The cancer-associated process resembles epithelial–stromal interactions that are triggered by injury to nonmalignant liver epithelial cells. When damaged, such cells begin to produce Hh ligands that also act in a paracrine fashion to promote accumulation of Hh-dependent myofibroblasts (5). In nontumorous cirrhotic livers, myofibroblasts are a major source of fibrous matrix but also produce various factors that promote the survival of residual liver epithelial cells (4). Here, we identify end products of Hh-dependent changes in myofibroblasts' metabolism as novel trophic factors for malignant hepatocytes by showing that Hh signaling in myofibroblasts stimulates glycolysis and that malignant hepatocytes use myofibroblast-derived lactate to generate ATP and fuel lipogenesis (Supplementary Fig. S5). Evidence that the lactate-induced responses are blocked by treating malignant hepatocytes with an inhibitor of LDH suggests that the improved epithelial energy balance occurs because malignant hepatocytes convert the assimilated lactate into pyru-

vate, which is then shunted into the TCA cycle to increase mitochondrial ATP production. In HCCs, therefore, increased aerobic glycolytic activity (i.e., the Warburg effect) is an end result of collaborations between malignant hepatocytes and glycolytic myofibroblasts in the tumor-associated stroma. Via this process, the malignant hepatocytes reap the benefits of the excess lactate generated by glycolysis without becoming glycolytic themselves, thereby fully retaining the capacity for oxidative phosphorylation and efficient ATP synthesis. Although this concept is contrary to conventional dogma, which localizes the Warburg effect to the malignant cells themselves (15), it is consistent with other recent reports of lactate production by stroma in breast cancer (21) and raises the intriguing possibility that Hh-mediated switches in stromal cell metabolism also occur in cancers other than HCCs. In any case, evidence for increased glycolytic activity in tumor-associated myofibroblasts has important diagnostic and therapeutic implications. It suggests that positron emission tomographic (PET) scans might be deployed to identify HCCs that are particularly enriched with glycolytic stroma. The latter information might facilitate HCC detection and could also have

prognostic significance because highly glycolytic tumors tend to have more aggressive biology (22). Knowing which HCCs are most enriched with glycolytic stroma would also justify, and help to refine, novel treatment approaches for HCCs, supporting consideration of Hh inhibitors, LDH antagonists, and glycolysis inhibitors, as potential therapies for some patients with this life-threatening disease.

#### Disclosure of Potential Conflicts of Interest

S.S. Choi is a consultant/advisory board member of Onyx Pharmaceutical. No potential conflicts of interest were disclosed by the other authors.

#### Authors' Contributions

**Conception and design:** I.S. Chan, C.D. Guy, Y. Chen, B.R. Anderson, S.S. Choi, A. M. Diehl

**Development of methodology:** I.S. Chan, C.D. Guy, Y. Chen, M. Swiderska, G. Karaca, G. Xie, B.R. Anderson, S.S. Choi, A.M. Diehl

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** I.S. Chan, C.D. Guy, J. Lu, G.A. Michelotti, L. Krüger, W.-K. Syn, T.A. Pereira, A.M. Diehl

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** I.S. Chan, C.D. Guy, Y. Chen, W.-K. Syn, T.A. Pereira, S. S. Choi, A.M. Diehl

**Writing, review, and/or revision of the manuscript:** I.S. Chan, G.A. Michelotti, B.R. Anderson, S.S. Choi, A.S. Baldwin, A.M. Diehl

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** I.S. Chan, L. Krüger, S.S. Choi, A.M. Diehl

**Study supervision:** I.S. Chan, S.S. Choi, A.S. Baldwin, A.M. Diehl

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