

Research Article

Metformin-Mediated Bambi Expression in Hepatic Stellate Cells Induces Prosurvival Wnt/ β -Catenin SignalingNanthakumar Subramaniam¹, Mara H. Sherman², Renuka Rao¹, Caroline Wilson¹, Sally Coulter¹, Annette R. Atkins², Ronald M. Evans^{2,3}, Christopher Liddle¹, and Michael Downes²**Abstract**

AMP-activated protein kinase (AMPK) regulates lipid, cholesterol, and glucose metabolism in specialized metabolic tissues, such as muscle, liver, and adipose tissue. Agents that activate AMPK, such as metformin and 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside (AICAR), have beneficial effects on liver glucose and lipid metabolism. In addition, AMPK activation in proliferating hepatic stellate cells (HSC) induces growth arrest and inhibits hepatic fibrosis. As metformin and AICAR act in different ways to achieve their effects, our aim was to examine the effects of AMPK activation in quiescent HSCs with these two agents on HSC function. We found that phospho-AMPK levels were markedly upregulated by both AICAR and metformin in quiescent HSCs. However, although AICAR treatment induced cell death, cells treated with metformin did not differ from untreated controls. AICAR-mediated HSC cell death was paralleled by loss of expression of the TGF- β decoy receptor Bambi, whereas metformin increased Bambi expression. Transfection of siRNA-Bambi into HSCs also induced cell death, mimicking the effects of AICAR, whereas overexpression of Bambi partially rescued AICAR-treated cells. As Bambi has previously been shown to promote cell survival through Wnt/ β -catenin signaling, a reporter incorporating binding sites for a downstream target of this pathway was transfected into HSCs and was induced. We conclude that although AICAR and metformin both activate AMPK in quiescent HSCs, AICAR rapidly induced cell death, whereas metformin-treated cells remained viable. The finding that metformin increases Bambi expression and activates Wnt/ β -catenin signaling provides a possible mechanistic explanation for this observation. These results suggest that AICAR and metformin may confer disease-specific therapeutic benefits. *Cancer Prev Res*; 5(4); 553–61. ©2012 AACR.

Introduction

Hepatic fibrosis underlies most forms of chronic liver disease (1, 2), the twelfth most common cause of death in the United States (3). The main causes of hepatic fibrosis in industrialized countries include chronic hepatitis C virus (HCV) infection, alcohol abuse, and nonalcoholic fatty liver disease (NAFLD; ref. 4). NAFLD has emerged as a considerable public health concern, as its major risk factors, obesity and insulin resistance, have reached epidemic proportions worldwide. Furthermore, conditions that induce hepatic fibrosis, such as HCV and hepatitis B infection,

alcoholism, and NAFLD, are the most common risk factors for the development of hepatocellular carcinoma (HCC; ref. 5), suggesting that therapeutic intervention for liver fibrosis may hold great value in the prevention of HCC.

Independent of etiology, chronic liver disease follows a common progression from inflammation to fibrosis, and finally, to cirrhosis. Hepatocyte damage often serves as a triggering event in the pathogenesis of liver fibrosis, which initiates a complex cross-talk between hepatocytes and non-parenchymal liver cells. Injured or apoptotic hepatocytes can directly activate hepatic stellate cells (HSC) (reviewed in ref. 6) and also release reactive oxygen species (ROS) and cytokines, leading to recruitment of inflammatory immune cells and to activation of resident macrophages (Kupffer cells; ref. 7). Activated Kupffer cells and injured hepatocytes also secrete profibrogenic cytokines, including TNF- α , TGF- β 160₁, and fibroblast growth factor, which stimulate the activation of HSCs (8). HSCs reside in the space of Disse; in normal liver, HSCs are quiescent and are characterized in part by the presence of cytoplasmic lipid droplets, which serve as the major site of retinol storage. In response to hepatic injury, HSCs undergo rapid activation and transdifferentiate into myofibroblast-like cells with a multitude of acquired morphologic and functional properties. Activated

Authors' Affiliations: ¹Storr Liver Unit, Westmead Millennium Institute and Sydney Medical School, University of Sydney, Westmead, New South Wales, Australia; and ²Gene Expression Laboratory, ³Howard Hughes Medical Institute, The Salk Institute for Biological Studies, La Jolla, California

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

Corresponding Author: Michael Downes, Gene Expression Laboratory, The Salk Institute for Biological Studies, La Jolla, CA. E-mail: downes@salk.edu

doi: 10.1158/1940-6207.CAPR-12-0053

©2012 American Association for Cancer Research.

HSCs lose lipid droplets, obtain proliferative and contractile capabilities, express myogenic markers, such as α -smooth muscle actin and myocyte enhancer factor-2, and secrete pathologic extracellular matrix (ECM) proteins such as collagen type I (4). In addition to matrix protein production, HSCs contribute to tissue repair in the injured liver by secreting proinflammatory cytokines (such as TGF- β , RANTES, and MCP-1) and adhesion molecules (including integrins, VCAM-1, and ICAM-1) that promote immune cell recruitment and activation (9). Whereas transient HSC activation promotes wound healing and tissue repair, chronic HSC activation results in excessive ECM accumulation, destruction of liver cytoarchitecture, and eventually, to cirrhosis and liver failure (4). The extent and duration of HSC activation are naturally limited by senescence; in mice lacking key senescence mediators, HSCs undergo aberrant proliferation in response to hepatic damage, leading to excessive liver fibrosis (10). Pharmacologic means by which to limit the activation state of HSCs, by inhibiting proliferation or promoting senescence or apoptosis, therefore, represents an appealing therapeutic avenue in chronic liver disease. Furthermore, given the established connection between HSC activation and hepatic fibrosis (6) and the implications of hepatic fibrosis in the development of HCC as described above, strategies to limit HSC activation may also be of value for cancer prevention.

Although insulin resistance has long been implicated in the progression of hepatic fibrosis, the molecular mechanisms underlying this relationship remain unclear. Adiponectin, a hormone secreted by adipose tissue, is markedly decreased in plasma under conditions of obesity and type 2 diabetes, and adiponectin levels are directly correlated with insulin sensitivity and inversely with the presence of metabolic syndrome (11, 12). Administration of adiponectin ameliorates liver damage during experimental steatohepatitis and reduces the development of fibrosis (13, 14). In agreement with these findings, adiponectin has been shown to decrease proliferation, migration, and fibrogenic gene expression and to induce apoptosis in activated HSCs (14, 15). Subsequent studies determined that adiponectin principally inhibits profibrogenic actions of HSCs via AMP-activated protein kinase (AMPK; refs. 16, 17), a known downstream effector through which adiponectin induces glucose usage and fatty acid oxidation (18). AMPK is an energy sensor that maintains cellular energy homeostasis in response to various stimuli including stress conditions, nutrient deprivation, exercise, and adipocyte-derived hormones (19, 20). AMPK can also be activated pharmacologically by 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR) and by metformin, a widely used antidiabetic drug (21, 22). Drugs that activate AMPK have well-established beneficial effects on liver glucose and lipid metabolism, which may augment their antifibrogenic properties. In HSCs, AMPK is thought to oppose activation by inhibiting the mTOR pathway, a known AMPK target (23), and perhaps, by inhibiting NF- κ B (16). AICAR and metformin also inhibit platelet-derived growth factor-induced proliferation and migration in activated HSCs (17).

Although activation of AMPK was shown to negatively regulate the activated HSC phenotype, the effect of AMPK activating compounds on quiescent HSCs remains unknown. Although quiescent HSCs serve a physiologic function in the liver and excess apoptosis and cytokine release can exacerbate hepatic fibrosis, maintenance of quiescent HSC viability is a desirable outcome of therapeutic strategies for chronic liver disease.

This study aimed to evaluate the role of AMPK in quiescent primary rat HSCs *in vitro*. Although AICAR and metformin both activated AMPK in quiescent HSCs to the same extent, these agents elicited distinct effects on HSC function. AICAR rapidly induced cell death, whereas HSCs treated with metformin remained viable. We found that activation of BMP and activin membrane-bound inhibitor (Bambi), a TGF- β pseudoreceptor, by metformin promotes the survival of quiescent HSCs. Use of siRNA against Bambi in quiescent primary rat HSCs resulted in a dramatic decrease in viable cells, and we further showed that metformin-induced Bambi expression activates a prosurvival Wnt/ β -catenin signaling pathway unique to quiescent HSCs (24).

Materials and Methods

HSC isolation, cell culture, and transfection

HSCs were isolated from male Sprague-Dawley rats by *in situ* pronase, collagenase perfusion, and single-step Histodenz gradient as previously reported (25). Isolated HSCs were cultured in Dulbecco's modified Eagle's medium (GIBCO) containing 20% fetal calf serum (FCS; JRH) on plastic for 40 hours. This medium was removed and fresh medium without FCS but in the presence or absence of aminoimidazole carboxamide ribonucleotide (AICAR) or metformin at concentrations as indicated in the figure legend, $1\alpha, 25(\text{OH})_2$ vitamin D₃ (calcitriol; 10 nmol/L), LPS (15 ng/mL), cycloheximide (1 mg/mL), and TGF- β_1 (2 ng/mL) were added to the cells for various time points. For transfection, HSCs were grown to 50% confluence in 6-well plates and a human Bambi expression vector transfected using FuGENE-HD (Roche), as recommended by the manufacturer. For siRNA transfection (siRNA-Bambi or siRNA-AMPK), Lipofectamine RNAiMAX (Invitrogen) was used as recommended by the manufacturer. Six hours later, different treatments were given to cells as indicated in figure legends. Each experimental condition was measured in triplicate, and the values given represent the mean \pm SD from 2 to 3 experiments. Student unpaired *t* test was done to assess the significance of treatments versus controls. Statistically significant differences ($P < 0.05$) are indicated by asterisk.

RNA isolation and quantitative real-time PCR

Total RNA from cultured HSCs was isolated using RNeasy (Qiagen) according to the manufacturer's instructions. A260/280 ratios were determined by a NanoDrop ND-1000 spectrophotometer and the integrity of the total RNA was verified using a Bioanalyzer (Agilent Technologies). Five hundred nanogram of deoxyribonuclease treated total

RNA was reverse transcribed into cDNA using SuperScript III reverse transcriptase (Invitrogen). Quantification of mRNA expression was done by quantitative real-time PCR (qPCR) using a StepOnePlus qPCR platform (Applied Biosystems). The following primer pairs were used for the amplification of genes of interest:

rat Bambi forward 5'-tcacatctgctgcagttgg-3'
 rat Bambi reverse 5'-catcacagtagcatctgatctcg-3'
 ratIL-1 β forward 5'-tacatgtctctcccgaggag-3'
 ratIL-1 β reverse 5'-atcatccacagtagcacagagg-3'
 ratTGF β 1 forward 5'-cctggaaaggctcaacac-3'
 ratTGF β 1 reverse 5'-cagttctctctgtggagctga-3'
 rat TRAIL forward 5'-ccaaatcgactagcttgc-3'
 rat TRAIL reverse 5'-tcaaaggttctcaaagtcacctc-3'
 human BAMBI forward 5'-tgcacagatgtctctctct-3'
 human BAMBI reverse 5'-gaagtcagctctgcacctt-3'
 rat MCP-1 forward 5'-agcatccagctgctgtctc-3'
 rat MCP-1 reverse 5'-gatcatctgcccagtgaaatgag-3'
 rat β -catenin forward 5'-cgaggactcaataccattcc-3'
 rat β -catenin reverse 5'-agcgtttctgtagctctg-3'
 rat Bcl-2 forward 5'-gtacctgaaccggcatctg-3'
 rat Bcl-2 reverse 5'-ggggccatatagttccacaa-3'
 Luciferase forward 5'-gcctgaagtctctgattaagt-3'
 Luciferase reverse 5'-acactcgcgtcgaagt-3'

For standardization, rat Sp1 was amplified using the following primers:

rat Sp1 forward 5'-gctatagcaaacaccccaggt-3'
 rat Sp1 reverse 5'-gatcagggtgttctctctt-3'

Western immunoblot analysis

HSCs treated with or without AICAR (500 μ mol/L), metformin (1 mg/mL), calcitriol (10 nmol/L), LPS (15 ng/mL), or TGF- β ₁ (2 ng/mL) for 2 hours were dissolved in whole-cell extraction buffer [25 mmol/L Tris-Cl, pH 8.0, 10% (w/v) glycerol, 2 mmol/L EDTA, 0.2 mmol/L dithiothreitol (DTT), 1% Triton X-100, 1.5 mmol/L MgCl₂ and 200 mmol/L NaCl] and lysed on ice for 1 hour, then centrifuged at 14,000 rpm for 15 minutes, 4°C. Twenty microgram of solubilized HSC extracts were analyzed on 10% SDS-PAGE gels. Gels were electroblotted onto Hybond-P Extra nitrocellulose membrane (Amersham Biosciences) and blocked for 4 hours, 22°C with PBS containing 5% skim milk powder. To check for equal protein loading/transfer, the membrane was stained with Ponceau S solution (Sigma). After removing the stain by washing in water, the membrane was probed with monoclonal antibodies (phospho-AMPK or phosphor-S6RP at 1/250; Chemicon) in PBS overnight at 4°C, followed by anti-rabbit peroxidase conjugate (1/10,000; Sigma) for 1 hour at 22°C. Immunoreactive bands were detected by chemiluminescence (Lumi-Light^{PLUS}; Roche Diagnostics). In addition to Ponceau S staining, the membranes were also probed with β -actin or CTGF antibodies for loading control.

Apoptosis assay

To measure apoptosis, Caspase-3/7 Assay Kit from AnaSpec (Campus Dr, Fremont, CA) was used according to the manufacturer's instructions. Briefly, rat HSCs were treated with AICAR (500 μ mol/L) or metformin (1 mg/mL) for the time points indicated in the figure and lysed in the buffer provided. HSCs transfected with siRNA-Bambi were lysed 18 hours after transfection. Caspase activity was measured after the incubation of 125 μ L of cell extract with 70 μ L of caspase-3/7 substrate (60 minutes at room temperature) by fluorescence spectroscopy at Ex/Em = 490 nm/520 nm.

Results

AICAR and metformin have distinct effects on quiescent hepatic stellate cell survival but identical impact on AMPK activation and inhibition of mTOR pathway

Previously published studies have focused on the effect of AMPK activation in activated HSCs. The results from these studies were promising in that AMPK-activating agents AICAR and metformin inhibited proliferation and profibrogenic activity while promoting apoptosis of activated HSCs (16, 17). The effect of AMPK activation in quiescent HSCs was the focus of this study. To determine the effect of AMPK activation on quiescent HSC survival, primary rat HSCs maintained in culture for 48 hours were treated with AICAR or metformin, and cell viability was assessed. HSCs treated with AICAR for 24 hours showed dramatic induction of cell death even at the lowest concentration used (100 μ mol/L), whereas metformin-treated HSCs remained viable with a normal appearance, even at the highest concentration employed (1,000 μ g/mL; Fig. 1A, Supplementary Fig. S1). We next examined the kinetics of AICAR-induced HSC cell death. For this purpose, HSC viability was assessed over a 24-hour time course following application of 500 μ mol/L AICAR. Cell density began to decrease as early as 3 hours after AICAR administration, with progressive loss of viable cells until near complete cell death occurred by 24 hours (Fig. 1B). This loss of cell viability occurred coincident with an increase in caspase-3/7 activity, consistent with an induction of cell apoptosis (Fig. 1C). These results suggested that AICAR and metformin have distinct impacts on quiescent HSC survival.

Previous studies have reported that treatment with AMPK activators AICAR and metformin results in a dose-dependent increase in Thr-172 phosphorylation of α -AMPK in activated HSCs (17). A difference in AMPK phosphorylation could potentially account for the distinct responses to AICAR and metformin in quiescent HSCs. We therefore compared the time course of α -AMPK Thr-172 phosphorylation in quiescent HSCs treated with AICAR or metformin. Western immunoblot analysis shows maximal induction of Thr-172 phosphorylation of α -AMPK in quiescent HSCs treated with either of these drugs after 2 hours (Fig. 1D). This result implied that the extent of AMPK activation does not explain the different outcomes of these treatments. Therefore, we reasoned that metformin may induce a

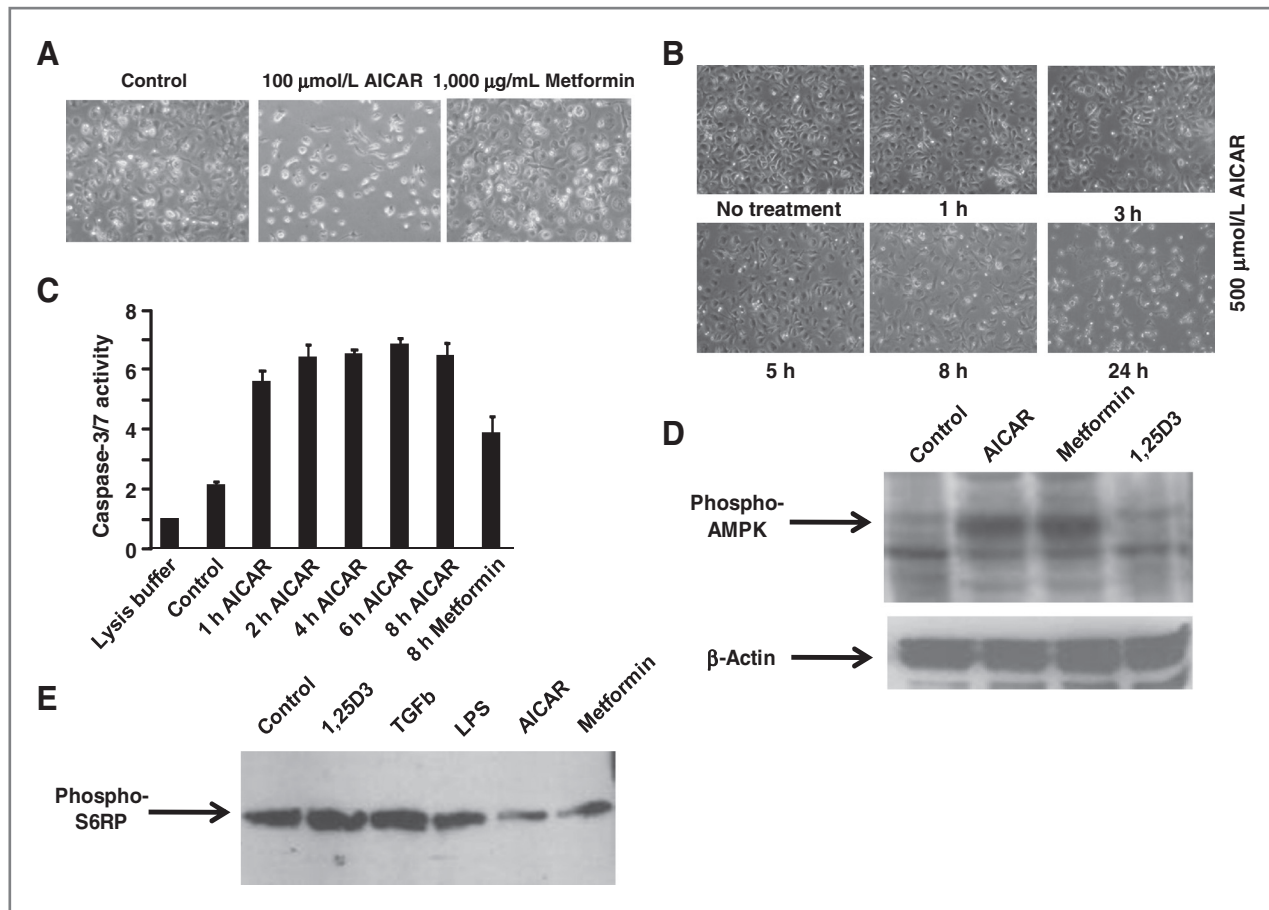


Figure 1. Effect of AICAR and metformin on quiescent HSCs. **A**, light microscopy images of quiescent HSCs treated with 100 $\mu\text{mol/L}$ AICAR or 1 mg/mL metformin for 24 hours. **B**, light microscopy images of HSCs treated with 500 $\mu\text{mol/L}$ AICAR for indicated times. Representative images are shown ($n = 3$). **C**, caspase activity in HSCs treated with AICAR (500 $\mu\text{mol/L}$) or metformin (1 mg/mL). **D**, Western immunoblot analysis of phosphorylated AMPK with β -actin as a loading control and **E**, phosphorylated S6 ribosomal protein (S6RP) in HSCs after 2-hour treatment with indicated agents.

downstream or AMPK-independent response that protects quiescent HSCs from cell death or alternatively, AICAR may activate mechanisms that impede HSC survival.

AMPK regulates cellular metabolism, in part, by suppressing the mTORC1 via direct phosphorylation of TSC2 (23) and mTORC1 subunit Raptor (26), consequently decreasing mTORC1-directed cell growth. AMPK activation results in rapid dephosphorylation of mTORC1 substrates 4EBP1 and S6K (23), leading to decreased phosphorylation of S6K substrate S6 ribosomal protein (S6RP). We postulated that differential mTORC1 inhibition by AICAR and metformin may underlie the varying responses to these drugs in quiescent HSCs. To assess the degree of mTORC1 inhibition by AMPK-activating agents in quiescent HSCs, phosphorylated S6RP was measured after 2 hours of treatment with these compounds (Fig. 1E). Although HSC activation through AMPK-independent pathways [vitamin D signaling (27), TGF- β 1 signaling (28), and TLR4/LPS signaling (29)] did not significantly affect S6RP phosphorylation, AICAR and metformin treatment induced similar reductions in S6RP phosphorylation. Given this similarity in AICAR- and met-

formin-induced inhibition of mTOR signaling, it is unlikely that differential mTORC1 inhibition was responsible for the observed AICAR-induced cell death.

AICAR suppresses inflammatory gene expression in primary hepatic stellate cells

During liver injury various inflammatory mediators induce cell damage and apoptosis (29, 30). We next explored the possibility that AICAR-induced cell death in HSCs was due to induction of proinflammatory chemokines/cytokines such as MCP-1 and interleukin (IL)-1 β . Contrary to our expectation, MCP-1 and IL-1 β gene expression progressively decreased in HSCs upon AICAR treatment (Fig. 2A). Expression levels of the metabolic genes *PEPCK* and *LPL* were found to increase with AICAR treatment, consistent with the known actions of AMPK activators (Supplementary Fig. S2). In addition, AICAR treatment completely abrogated lipopolysaccharide (LPS)-induced expression of MCP-1 and IL-1 β , suggesting that proinflammatory factors do not play a role in this event (Fig. 2B).

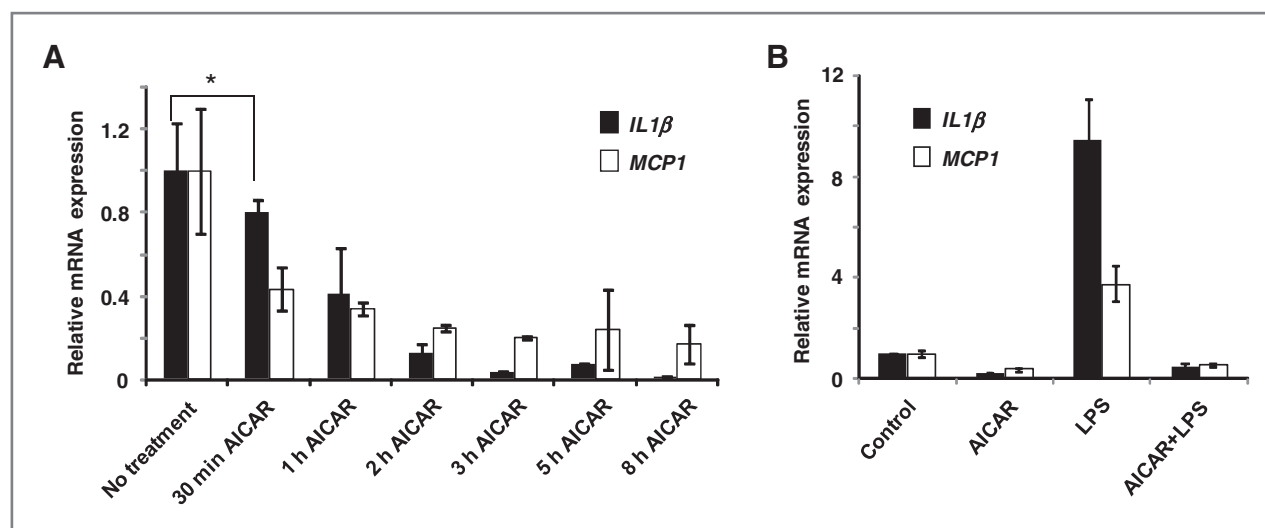


Figure 2. Inflammatory gene modulation by AMPK activation in HSCs. A, relative *IL1 β* and *MCP1* mRNA expression in quiescent HSCs treated with 500 μ mol/L AICAR. B, relative *IL1 β* and *MCP1* expression in HSCs treated with AICAR (500 μ mol/L) and/or LPS for 10 hours.

AICAR-induced HSC cell death parallels loss of Bambi and silencing of AMPK restores Bambi expression and partially rescues HSCs from death

The TGF- β pseudoreceptor Bambi functions as a negative regulator of TGF- β signaling, a potent cytokine that is a prime mediator of hepatic fibrogenesis (31). Downregulation of Bambi and consequent sensitization to TGF- β signaling is an integral component of HSC activation (29). As Bambi may function not only to promote the quiescent HSC phenotype but also to promote quiescent HSC survival, we wished to determine whether AICAR and metformin could elicit differential effects on Bambi expression, thereby altering HSC survival. Interestingly, expression levels of Bambi progressively declined over time in AICAR-treated cells (Fig. 3A). In contrast, metformin treatment induced an increase in Bambi expression for up to 10 hours, followed by a return to baseline after 24 to 32 hours (Fig. 3B). These results suggested that differential expression of Bambi may account for the differences in HSC survival. To further examine the role of AMPK in Bambi expression and HSC survival, the effects of AICAR and metformin treatment were examined after prior siRNA knockdown of AMPK expression. AICAR-treated control cells (500 μ mol/L AICAR for 15 hours after transfected with a scrambled siRNA) seemed poised to undergo apoptosis/necrosis and expressed markedly reduced levels of Bambi, consistent with our earlier findings (Fig. 3C and D). However, although HSCs lacking AMPK resembled control cells, these cells were partially rescued from AICAR-induced cell death and maintained normal levels of Bambi expression (Fig. 3C and D). In contrast, siRNA knockdown of AMPK had little effect on the response of HSCs to metformin. A similar induction of Bambi was seen in control (transfected with scrambled siRNA) and AMPK knockdown cells, and no effect on cell viability was observed (Fig. 3E and data not shown). Together these results suggested that AICAR-mediated HSC cell death is due, in part, to AMPK-dependent inhibition of

Bambi expression, and that metformin-mediated HSC cell survival is attributable to an AMPK-independent increase in Bambi.

Ectopic expression of Bambi improves HSCs survival upon treatment with AICAR

During the resolution phase of hepatic fibrosis, a crucial mechanism that operates to reduce injury is an increase in the apoptosis of activated HSCs (32). Given our finding that AICAR induces cell death/apoptosis in quiescent HSCs, we next asked whether this effect was mediated by changes in Bambi expression. HSCs transfected with siRNA targeting Bambi showed reduced viability, similar to that seen with AICAR treatment (Fig. 4A). Furthermore, HSCs transfected with human BAMB1 displayed increased resistance to AICAR-induced cell death (Fig. 4A). As activated HSCs have been shown to be sensitive to apoptosis induction by TNF-related apoptosis-inducing ligand (TRAIL) and subsequent activation of a protease cascade (32), we investigated whether a similar signaling pathway occurs in quiescent cells. The apoptotic response in quiescent HSCs after AMPK activation, with or without manipulation of Bambi expression, was measured by the expression of the proapoptotic gene *TRAIL* and antiapoptotic gene *Bcl-2*. Results showed that *TRAIL* levels were increased by 3- to 4-fold upon AICAR treatment, whereas antiapoptotic *Bcl-2* levels fell 3-fold, consistent with induction of apoptosis (Fig. 4B). Ectopic overexpression of Bambi increased *Bcl-2* expression 2-fold without significantly altering *TRAIL* expression. After AICAR treatment, ectopic Bambi expression partially increased HSC survival, and *TRAIL* expression was reduced by 50% compared with control (Fig. 4A and B), though *Bcl-2* expression was diminished by AICAR independent of ectopic Bambi expression. In addition, siRNA-mediated reduction of Bambi had minor effects on *TRAIL*, although significantly reducing the expression of antiapoptotic *Bcl-2* gene (Fig. 4B). In HSCs treated with metformin, *TRAIL*

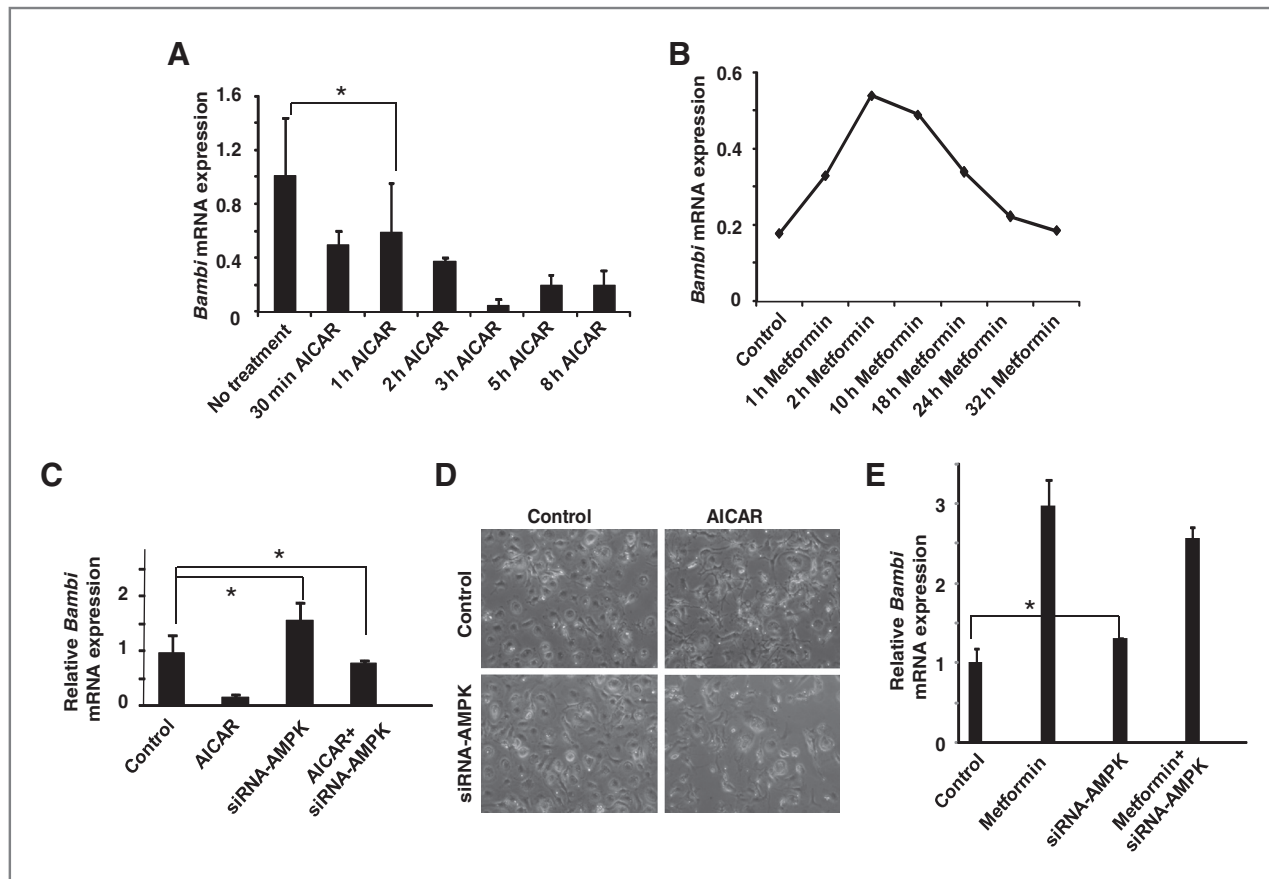


Figure 3. AICAR and metformin conversely modulate Bambi. A, induction of *Bambi* mRNA in HSCs treated with 500 $\mu\text{mol/L}$ AICAR and B, 1 mg/mL metformin. C, *Bambi* mRNA expression in HSCs after siRNA knockdown of AMPK in the presence or absence of AICAR and D, light microscopy images of HSCs after siRNA knockdown of AMPK and treated with AICAR. E, *Bambi* mRNA expression after siRNA knockdown of AMPK in the presence or absence of metformin.

levels gradually decreased with time (Fig 4C), suggesting that metformin functions differently to AICAR and is protective against TRAIL-dependent cell death. Furthermore, consistent with a prosurvival role for Bambi, siRNA knockdown of Bambi in HSCs resulted in a marked increase in caspase activity (Fig. 4D).

Ectopic expression of Bambi induces the Wnt/ β -catenin pathway in HSCs

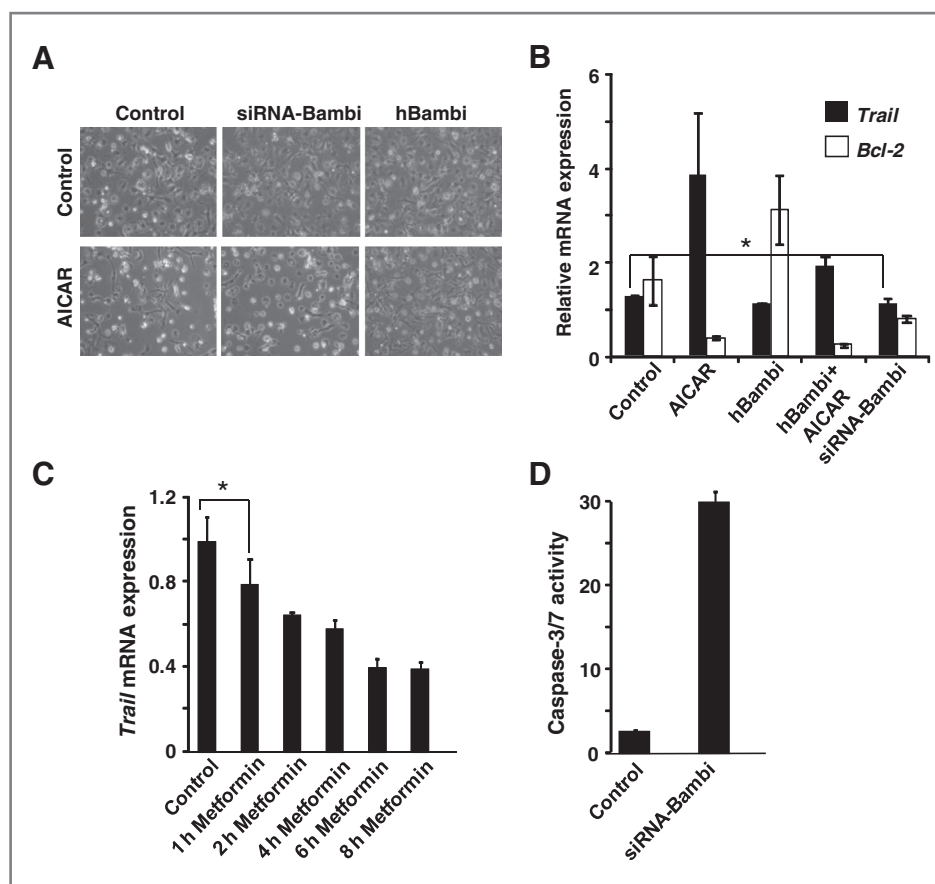
Bambi has been shown to induce Wnt/ β -catenin signaling in other cellular systems leading to cell proliferation and survival (33). This prompted us to investigate whether Bambi-dependent partial rescue of AICAR-induced cell death was, in part, due to induction of the Wnt/ β -catenin signaling pathway. Supporting this hypothesis, transfection of HSCs with increasing amounts of Bambi significantly increased β -catenin mRNA and protein (Fig. 5A and B). To confirm that Bambi augments the functional Wnt/ β -catenin signaling pathway, quiescent HSCs were transfected with a β -catenin-dependent TCF-4/ELF-binding luciferase reporter from the fibronectin promoter/enhancer. This reporter was induced several-fold by Bambi, showing that a true activation of Wnt/ β -catenin signaling operates in quiescent HSCs in

response to heightened Bambi expression (Fig. 5A). In addition, a reduced β -catenin expression was observed on Bambi knockdown (Fig. 5B).

Bambi mRNA expression is stabilized by addition of cycloheximide in quiescent HSCs

To determine whether metformin acts directly to upregulate Bambi, quiescent HSCs were treated with metformin with and without cycloheximide, a *de novo* protein synthesis inhibitor. Inhibition of protein synthesis markedly enhanced the induction of Bambi by metformin (Fig. 6A), consistent with reported results (34). Interestingly, though the extent of stabilization of Bambi mRNA by cycloheximide in AICAR-treated cells was also significant, it was less robust, suggesting that different protein synthesis-independent regulatory mechanisms were operating. In addition, metformin-mediated Bambi induction was not found in activated HSCs (Fig. 6B), in which metformin treatment reduced Bambi expression. Taken together, these data implied that in quiescent HSCs, metformin partially stabilizes Bambi mRNA, protecting cells from death, whereas AICAR fails to elicit Bambi expression and promotes apoptosis.

Figure 4. Ectopic expression of Bambi partially rescues AICAR-induced cell death. **A**, light microscopy images of HSCs transfected with human Bambi or siRNA targeting Bambi treated with or without AICAR. Representative images from $n = 3$. **B**, *Trail* and *Bcl-2* expression in cells from (A). **C**, *Trail* expression in HSCs treated with metformin (1 mg/mL). **D**, caspase activity in HSCs after Bambi knockdown by siRNA.



Discussion

Sustained AMPK activation by AICAR, metformin, and adiponectin in activated HSCs has been linked to apoptosis (16, 35). Similarly, AMPK-mediated cell death has been reported in other cell types treated with these AMPK activators (36). However, AICAR can induce apoptosis inde-

pendent of AMPK through activation of the mitochondrial apoptotic pathway (37). This study revealed contrasting effects of the AMPK activators AICAR and metformin in quiescent HSCs. Although both drugs activated AMPK, AICAR selectively induced cell death through an AMPK-dependent mechanism and reduced Bambi expression.

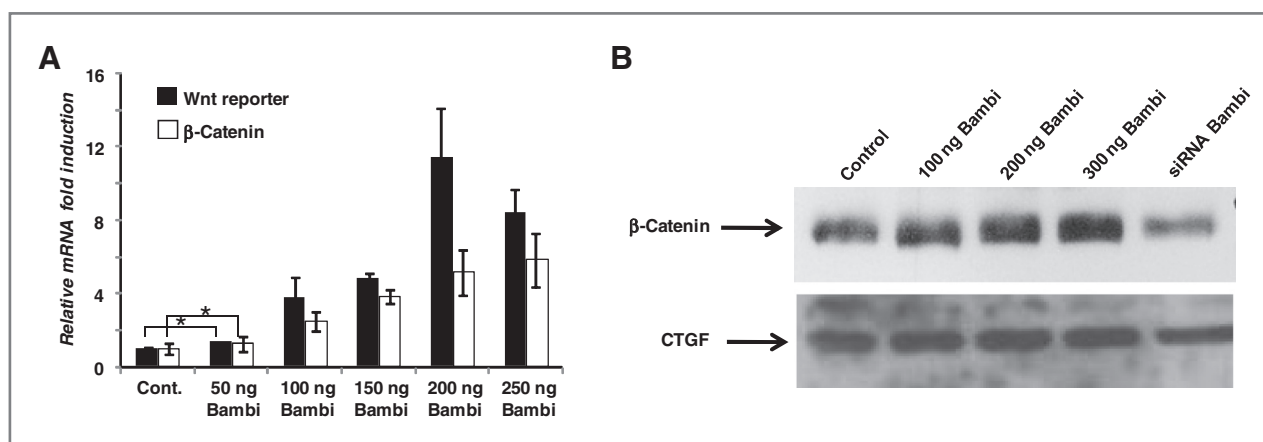


Figure 5. Bambi induce Wnt/ β -catenin signaling in HSC. HSCs were transfected either with human Bambi expression plasmid or with siRNA against Bambi and after 24 hours RNA and protein extracted from the cells. **A**, quantification of β -catenin and fire-fly *luciferase* mRNA was done by reverse transcriptase PCR (RT-PCR). **B**, protein extracted from the cells were analyzed by Western immunoblot analysis for β -catenin expression. CTGF (connective tissue growth factor) was used as a loading control.

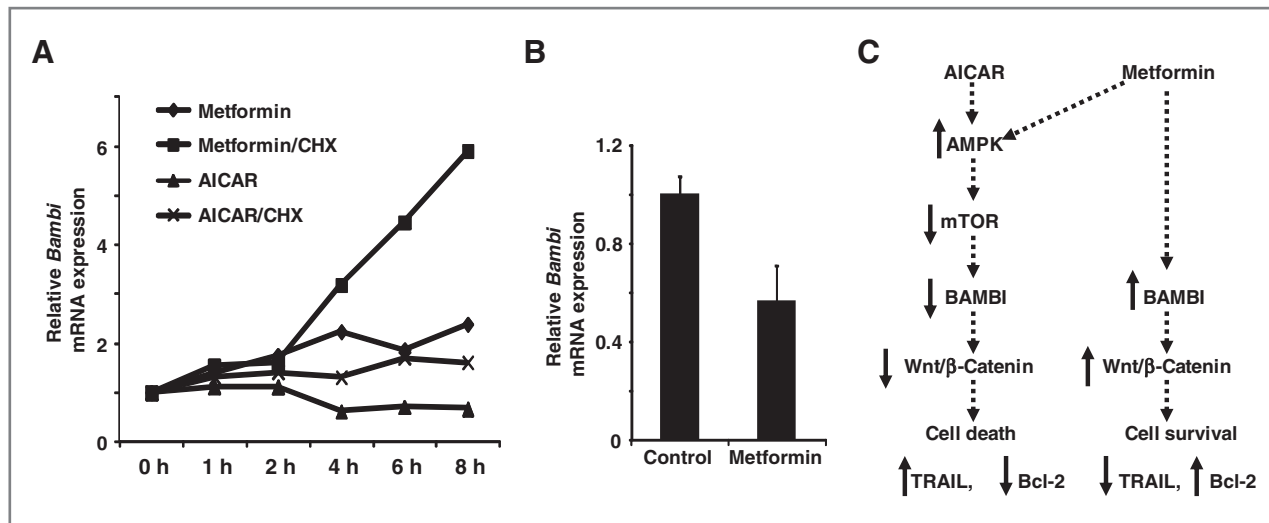


Figure 6. Posttranscriptional regulation of Bambi. A, effects of cycloheximide on Bambi mRNA levels in quiescent HSCs. Cells were treated with metformin (1 mg/mL) or AICAR (200 μ mol/L) in the presence or absence of cycloheximide (CHX, 1 mg/mL). B, HSCs were activated in culture (7 days growth) before overnight treatment with metformin (1 mg/mL). Bambi mRNA was quantified by RT-PCR. C, proposed signaling events in quiescent HSCs upon AICAR and metformin treatment.

AMPK knockdown partially rescued AICAR-induced HSC death and restored Bambi expression, consistent with an AMPK-dependent Bambi modulation. In contrast, metformin treatment induced Bambi expression independent of AMPK. These studies revealed a correlation between Bambi expression levels and quiescent HSC survival that was substantiated by studies in HSCs with targeted abrogation or ectopic expression of Bambi. Mechanistically, increased Bambi expression was linked to increased expression of antiapoptotic factor Bcl-2, whereas AICAR induced expression of the proapoptotic factor TRAIL as well as enhanced caspase activity. Furthermore, we report that Bambi induces the Wnt/ β -catenin signal transduction pathway, implicating this pathway in metformin-induced cell survival. Thus, the apoptotic response of quiescent HSCs depends on the balance of pro- and antiapoptotic signals (32).

This study revealed that the AMPK activators AICAR and metformin operate differently in quiescent HSCs such that AICAR induces cell death in an AMPK-dependent manner whereas metformin, though inducing prosurvival factors, did not significantly effect overall cell survival. These findings may have implications on the therapeutic use of these AMPK activators as treatments for chronic liver disease and as preventative therapies for HCC (38, 39, 40).

Disclosure of Potential Conflicts of Interest

R.M. Evans is an investigator of the Howard Hughes Medical Institute and March of Dimes Chair in Molecular and Developmental Biology at the Salk Institute.

References

- Brenner DA. Molecular pathogenesis of liver fibrosis. *Trans Am Clin Climatol Assoc* 2009;120:361–8.
- Friedman SL. Liver fibrosis—from bench to bedside. *J Hepatol* 2003;38 Suppl 1:S38–53.
- Heron M, Hoyert DL, Murphy SL, Xu J, Kochanek KD, Tejada-Vera B. Deaths: final data for 2006. *Natl Vital Stat Rep* 2009;57:1–134.
- Battaller R, Brenner DA. Liver fibrosis. *J Clin Invest* 2005;115:209–18.

Authors' Contributions

Conception and design: N. Subramaniam, R.M. Evans, C. Liddle, and M. Downes.

Development of methodology: N. Subramaniam, R. Rao, C. Wilson, C. Liddle.

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Liddle.

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): N. Subramaniam, R. Rao, C. Wilson, S. Coulter, A.R. Atkins, C. Liddle, and M. Downes.

Writing, review, and/or revision of the manuscript: N. Subramaniam, M. H. Sherman, A.R. Atkins, R.M. Evans, C. Liddle, and M. Downes.

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Wilson, S. Coulter, R.M. Evans, C. Liddle.

Study supervision: N. Subramaniam, C. Liddle, and M. Downes.

Acknowledgments

The authors thank E. Ong and S. Ganley for administrative assistance, and Ruth Yu for useful discussion.

Grant Support

This work was supported, in part, by grants from Ipsen/Biomeasure, The Helmsley Charitable Trust, Howard Hughes Medical Institute, NIH (DK062434, DK090962), and National Health and Medical Research Council of Australia Project grant 512354 and 632886. R.M. Evans is supported in part by a Stand Up to Cancer Dream Team Translational Cancer Research Grant, a Program of the Entertainment Industry Foundation (SU2C-AACR-DT0509). M.H. Sherman is supported by a NIH T32 training grant CA009370.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received January 28, 2012; revised February 27, 2012; accepted February 28, 2012; published OnlineFirst March 9, 2012.

5. Yang JD, Harmsen WS, Slettedahl SW, Chaiteerakij R, Enders FT, Therneau TM, et al. Factors that affect risk for hepatocellular carcinoma and effects of surveillance. *Clin Gastroenterol Hepatol* 2011;9:617–23 e1.
6. Rombouts K, Marra F. Molecular mechanisms of hepatic fibrosis in non-alcoholic steatohepatitis. *Dig Dis* 2010;28:229–35.
7. Kisseleva T, Brenner DA. Role of hepatic stellate cells in fibrogenesis and the reversal of fibrosis. *J Gastroenterol Hepatol* 2007;22 Suppl 1: S73–8.
8. Bataller R, Brenner DA. Hepatic stellate cells as a target for the treatment of liver fibrosis. *Semin Liver Dis* 2001;21:437–51.
9. Knittel T, Dinter C, Kobold D, Neubauer K, Mehde M, Eichhorst S, et al. Expression and regulation of cell adhesion molecules by hepatic stellate cells (HSC) of rat liver: involvement of HSC in recruitment of inflammatory cells during hepatic tissue repair. *Am J Pathol* 1999;154: 153–67.
10. Krizhanovsky V, Yon M, Dickins RA, Hearn S, Simon J, Miething C, et al. Senescence of activated stellate cells limits liver fibrosis. *Cell* 2008;134:657–67.
11. Tilg H, Hotamisligil GS. Nonalcoholic fatty liver disease: Cytokine-adipokine interplay and regulation of insulin resistance. *Gastroenterology* 2006;131:934–45.
12. Matsuzawa Y. Therapy insight: adipocytokines in metabolic syndrome and related cardiovascular disease. *Nat Clin Pract Cardiovasc Med* 2006;3:35–42.
13. Xu A, Wang Y, Keshaw H, Xu LY, Lam KS, Cooper GJ. The fat-derived hormone adiponectin alleviates alcoholic and nonalcoholic fatty liver diseases in mice. *J Clin Invest* 2003;112:91–100.
14. Kamada Y, Tamura S, Kiso S, Matsumoto H, Saji Y, Yoshida Y, et al. Enhanced carbon tetrachloride-induced liver fibrosis in mice lacking adiponectin. *Gastroenterology* 2003;125:1796–807.
15. Ding X, Saxena NK, Lin S, Xu A, Srinivasan S, Anania FA. The roles of leptin and adiponectin: a novel paradigm in adipocytokine regulation of liver fibrosis and stellate cell biology. *Am J Pathol* 2005;166:1655–69.
16. Caligiuri A, Bertolani C, Guerra CT, Aleffi S, Galastri S, Trappoliere M, et al. Adenosine monophosphate-activated protein kinase modulates the activated phenotype of hepatic stellate cells. *Hepatology* 2008;47:668–76.
17. Adachi M, Brenner DA. High molecular weight adiponectin inhibits proliferation of hepatic stellate cells via activation of adenosine monophosphate-activated protein kinase. *Hepatology* 2008;47:677–85.
18. Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, et al. Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat Med* 2002;8:1288–95.
19. Hardie DG. AMP-activated protein kinase: an energy sensor that regulates all aspects of cell function. *Genes Dev* 2011;25:1895–908.
20. Mihaylova MM, Shaw RJ. The AMPK signalling pathway coordinates cell growth, autophagy and metabolism. *Nat Cell Biol* 2011;13: 1016–23.
21. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, et al. Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest* 2001;108:1167–74.
22. Hardie DG. AMP-activated protein kinase as a drug target. *Annu Rev Pharmacol Toxicol* 2007;47:185–210.
23. Inoki K, Zhu T, Guan KL. TSC2 mediates cellular energy response to control cell growth and survival. *Cell* 2003;115:577–90.
24. Zhu NL, Wang J, Tsukamoto H. The Ncoad-Wnt pathway causes epigenetic peroxisome proliferator-activated receptor gamma repression in hepatic stellate cells. *J Biol Chem* 2010;285:30463–71.
25. Weiskirchen R, Gressner AM. Isolation and culture of hepatic stellate cells. *Methods Mol Med* 2005;117:99–113.
26. Gwinn DM, Shackelford DB, Egan DF, Mihaylova MM, Mery A, Vasquez DS, et al. AMPK phosphorylation of raptor mediates a metabolic checkpoint. *Mol Cell* 2008;30:214–26.
27. Abramovitch S, Dahan-Bachar L, Sharvit E, Weisman Y, Ben Tov A, Brazowski E, et al. Vitamin D inhibits proliferation and profibrotic marker expression in hepatic stellate cells and decreases thioacetamide-induced liver fibrosis in rats. *Gut* 2011;60:1728–37.
28. Hellerbrand C, Stefanovic B, Giordano F, Burchardt ER, Brenner DA. The role of TGFbeta1 in initiating hepatic stellate cell activation *in vivo*. *J Hepatol* 1999;30:77–87.
29. Seki E, De Minicis S, Osterreicher CH, Kluwe J, Osawa Y, Brenner DA, et al. TLR4 enhances TGF-beta signaling and hepatic fibrosis. *Nat Med* 2007;13:1324–32.
30. Pradere JP, Troeger JS, Dapito DH, Mencin AA, Schwabe RF. Toll-like receptor 4 and hepatic fibrogenesis. *Semin Liver Dis* 2010;30: 232–44.
31. Wanninger J, Neumeier M, Bauer S, Weiss TS, Eisinger K, Walter R, et al. Adiponectin induces the transforming growth factor decoy receptor BAMBI in human hepatocytes. *FEBS Lett* 2011;585:1338–44.
32. Shen H, Fan J, Minuk G, Gong Y. Apoptotic and survival signals in hepatic stellate cells. *Zhong Nan Da Xue Xue Bao Yi Xue Ban* 2007;32: 726–34.
33. Shin HW, Park SY, Lee KB, Jang JJ. Down-regulation of Wnt signaling during apoptosis of human hepatic stellate cells. *Hepatogastroenterology* 2009;56:208–12.
34. Xavier S, Gilbert V, Rastaldi MP, Krick S, Kollins D, Reddy A, et al. BAMBI is expressed in endothelial cells and is regulated by lysosomal/autolysosomal degradation. *PLoS One* 2010;5:e12995.
35. Canto C, Auwerx J. AMP-activated protein kinase and its downstream transcriptional pathways. *Cell Mol Life Sci* 2010;67:3407–23.
36. Mihaylova MM, Shaw RJ, Carling D, Mayer FV, Sanders MJ, Gamblin SJ. The AMPK signalling pathway coordinates cell growth, autophagy and metabolism AMP-activated protein kinase: nature's energy sensor. *Nat Cell Biol* 2011;13:1016–23.
37. Garcia-Garcia C, Fumarola C, Navarathnam N, Carling D, Lopez-Rivas A. AMPK-independent down-regulation of cFLIP and sensitization to TRAIL-induced apoptosis by AMPK activators. *Biochem Pharmacol* 2010;79:853–63.
38. Friedman SL. A deer in the headlights: BAMBI meets liver fibrosis. *Nat Med* 2007;13:1281–2.
39. Behari J. The Wnt/beta-catenin signaling pathway in liver biology and disease. *Expert Rev Gastroenterol Hepatol* 2010;4:745–56.
40. Sekiya T, Adachi S, Kohu K, Yamada T, Higuchi O, Furukawa Y, et al. Identification of BMP and activin membrane-bound inhibitor (BAMBI), an inhibitor of transforming growth factor-beta signaling, as a target of the beta-catenin pathway in colorectal tumor cells. *J Biol Chem* 2004;279:6840–6.

Cancer Prevention Research

Metformin-Mediated Bambi Expression in Hepatic Stellate Cells Induces Prosurvival Wnt/ β -Catenin Signaling

Nanthakumar Subramaniam, Mara H. Sherman, Renuka Rao, et al.

Cancer Prev Res 2012;5:553-561. Published OnlineFirst March 9, 2012.

Updated version Access the most recent version of this article at:
doi:[10.1158/1940-6207.CAPR-12-0053](https://doi.org/10.1158/1940-6207.CAPR-12-0053)

Supplementary Material Access the most recent supplemental material at:
<http://cancerpreventionresearch.aacrjournals.org/content/suppl/2012/03/08/1940-6207.CAPR-12-0053.DC1>

Cited articles This article cites 40 articles, 4 of which you can access for free at:
<http://cancerpreventionresearch.aacrjournals.org/content/5/4/553.full#ref-list-1>

Citing articles This article has been cited by 3 HighWire-hosted articles. Access the articles at:
<http://cancerpreventionresearch.aacrjournals.org/content/5/4/553.full#related-urls>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

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

Permissions To request permission to re-use all or part of this article, use this link
<http://cancerpreventionresearch.aacrjournals.org/content/5/4/553>.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.