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## Metformin prevents tobacco carcinogen-induced lung tumorigenesis

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### Abstract

Activation of the mTOR pathway is an important and early event in tobacco carcinogen-induced lung tumorigenesis, and therapies that target mTOR could be effective in the prevention or treatment of lung cancer. The biguanide metformin, which is widely prescribed for the treatment of type II diabetes, might be a good candidate for lung cancer chemoprevention because it activates AMPK, which can inhibit the mTOR pathway. To test this, A/J mice were treated with oral metformin after exposure to the tobacco carcinogen NNK. Metformin reduced lung tumor burden by up to 53% at steady-state plasma concentrations that are achievable in humans. mTOR was inhibited in lung tumors but only modestly. To test whether intraperitoneal administration of metformin might improve mTOR inhibition, we injected mice and assessed biomarkers in liver and lung tissues. Plasma levels of metformin were significantly higher after injection than oral administration. In liver tissue, metformin activated AMPK and inhibited mTOR. In lung tissue, metformin did not activate AMPK but inhibited phosphorylation of IGF-1R/IR, Akt, ERK, and mTOR. This suggested that metformin indirectly inhibited mTOR in lung tissue by decreasing activation of IGF-1R/IR and Akt upstream of mTOR. Based on these data, we repeated the NNK-induced lung tumorigenesis study using intraperitoneal administration of metformin. Metformin decreased tumor burden by 72%, which correlated with decreased cellular proliferation and marked inhibition of mTOR in tumors. These studies show that metformin prevents tobacco carcinogen-induced lung tumorigenesis, and support clinical testing of metformin as a chemopreventive agent.

### Keywords

metformin; AMPK; mTOR; chemoprevention; lung cancer

### Introduction

Lung cancer is the leading cause of cancer-related death in both men and women in the United States, and new targets are needed for the prevention and treatment of lung cancer. The serine-threonine kinase mTOR could be a good target because it is an important regulator of processes critical to tumorigenesis, such as cellular metabolism, growth, and proliferation. Many cancers, including lung cancer, are characterized by aberrant activation

of mTOR (reviewed in (1)). Although activating mutations in mTOR itself have not been identified, deregulation of upstream components that regulate mTOR are prevalent in lung cancer. The prototypic mechanism of mTOR regulation in cells is through activation of the PI3K/Akt pathway, but mTOR receives input from multiple signaling pathways, including the LKB1/AMPK pathway (reviewed in (2)).

Exposure of A/J mice to the tobacco-specific carcinogen NNK induces K-Ras-mediated lung tumorigenesis (3). Lung tumors that develop in these mice are characterized by increased activation of the mTOR pathway, and treatment with the mTOR inhibitor rapamycin prevents lung tumorigenesis in this model by 90% (4). Prevention of NNK-induced lung tumorigenesis by rapamycin is associated with inhibition of the mTOR pathway in tumor cells and with decreased lung- and tumor-associated regulatory T cells (5), which can suppress lung immunity and create a permissive environment for tumor development (reviewed in (6)). This suggests that rapamycin could be an effective chemopreventive agent in patients at high risk to develop lung cancer. However, the development and application of rapamycin as a chemopreventive agent may be limited because of toxicities. The identification of other drugs that inhibit mTOR that are well tolerated could increase opportunities for lung cancer chemoprevention.

One candidate is the biguanide metformin, which is the most widely prescribed drug for the treatment of type II diabetes. Long-term treatment is associated with few adverse effects (7). Metformin is an effective anti-diabetic agent because it activates AMPK, a master regulator of cell metabolism (8–10). AMPK activation may also play a role in preventing the formation and progression of cancer because AMPK is activated by the tumor suppressor LKB1 (11). The LKB1/AMPK pathway is linked to inhibition of tumor growth through inhibition of the mTOR pathway (12). Interestingly, metformin use in humans is associated with a decrease in cancer incidence (13, 14), but whether this is related to activation of AMPK is unclear. Because AMPK inhibits the mTOR pathway, drugs that activate AMPK could be tested in the NNK-induced mouse model of lung tumorigenesis.

We investigated if two different routes of administration of metformin were effective in preventing NNK-induced lung tumorigenesis. Administration of oral metformin significantly prevented lung tumorigenesis by 40–50%, but only modestly inhibited the mTOR pathway in tumors. Intraperitoneal administration of metformin produced higher plasma concentrations of metformin, which increased inhibition of the mTOR pathway in tumors and reduced lung tumorigenesis by ~75%. Inhibition of mTOR in lung tissue was surprisingly independent of AMPK, and correlated with decreased circulating levels of IGF-I and insulin, suggesting that chemoprevention of lung tumors by metformin was indirect. This mechanism of chemoprevention is similar to that observed in diabetics and provides an underpinning for the decreased cancer incidence associated with metformin use in diabetic patients.

## Methods

### Materials

The following antibodies were obtained from Cell Signaling Technologies (Beverly, MA): p-AMPK (T172), pan-AMPK $\alpha$ , p-ACC (S79), p-S6 (S235/236), 4E-BP1, p-IGF-1R/IR (Y1135/1146), p-Akt (T308), and p-ERK (T202/Y204). Anti-mouse/rat Foxp3 (clone FJK-15s) and CD3 antibodies were obtained from eBioscience (San Diego, CA) and Dako (Carpinteria, CA), respectively. Ki-67 antibody was purchased from Vision Biosystems (Norwell, MA). NNK was obtained from EaglePicher Pharmaceuticals (Lenexa, KS). Metformin, RIPA buffer, protease inhibitor cocktail, and phosphatase inhibitors were purchased from Sigma Chemicals (St. Louis, MO). Recombinant mouse IGF-1 and

HUMULIN R were obtained from R&D Systems (Minneapolis, MN) and Eli Lilly (Indianapolis, IN), respectively.

### **Tumorigenesis and biomarker studies in A/J mice**

A/J mice were obtained from Jackson Laboratories (Bar Harbor, ME), and housed according to the guidelines of the Animal Care and Use Committee of the National Institutes of Health (NIH). For the tumorigenesis studies, 6-week old female A/J mice were fed the AIN-93G diet (15, 16) and given three weekly injections of 100 mg/kg NNK, as previously described (4). One week following the last dose of NNK, mice were switched to the AIN-93M diet and randomized into a control group, or groups that received 1 mg/mL or 5 mg/mL metformin dissolved in drinking water (n=15 mice/group). Mice were treated with this dosing schedule with metformin for thirteen weeks, and their water was changed weekly. For the tumorigenesis study in which metformin was administered by intraperitoneal injection, mice were treated daily with saline or 250 mg/kg metformin beginning one week after the last dose of NNK (n=15 mice/group). This dosing schedule with metformin was continued for 13 weeks. At the end of the tumorigenesis studies, mice were anesthetized using isoflurane, and blood was collected by cardiac puncture using BD vacutainer vials containing EDTA (Franklin Lakes, NJ). Plasma was isolated by centrifugation of the blood samples and sent to the Laboratory of Proteomics and Analytical Technologies (Frederick, MD) for metformin pharmacokinetic analysis. Lung and liver tissues were also harvested, fixed in formalin overnight, and processed by Histoserv (Germantown, MD) for immunohistochemical analysis, or stained with H&E or Oil Red O. To determine tumor multiplicity, peripheral lung adenomas were counted using a dissecting microscope. Tumor volume was calculated by multiplying three dimensions of each tumor. Two dimensions were measured using a micrometer, and the third dimension was estimated by averaging these dimensions. Tumor burden was calculated as the sum of individual lung tumor volumes in each mouse.

For the biomarker studies, 8-week old female A/J mice were fed the tumorigenic diet AIN-93M and were injected with saline or 250 mg/kg metformin QDX3 (n=3 mice/group). Mice were anesthetized with isoflurane 2 h after the last injection of metformin, and liver or lung tissues were harvested and flash-frozen in liquid nitrogen. Because AMPK is exquisitely sensitive to the metabolic state of the animal, tissues were harvested early in the morning when the mice were in a “fed” state. Biomarker studies were performed at least three times.

### **Immunoblotting analysis**

Frozen tissues were pulverized on dry ice, and lysates were prepared using RIPA buffer supplemented with protease and phosphatase inhibitors. Immunoblotting analysis of tissue lysates was performed as described previously (17). For immunoblotting analysis of p-AMPK or p-IGF-1R/IR, antibodies were used at a dilution of 1:500. All other antibodies were used at a dilution of 1:1000. Densitometry was performed using NIH Image software. To determine differences in the phosphorylation of 4E-BP1 using an antibody that recognizes total 4E-BP1, changes in the ratio of the top band (hyperphosphorylated 4E-BP1) to the sum of the bottom two bands (hypophosphorylated 4E-BP1) were quantified.

### **Immunohistochemistry**

Formalin-fixed lung tissues were paraffin-embedded, sectioned, and placed on slides (Histoserv Inc., Gaithersburg, MD). Immunohistochemical analysis was performed on lung tissues from five mice per treatment group. Antigen retrieval was performed using target retrieval solution (pH 6.0) and a decloaking chamber (Dako, Carpinteria, CA). Tissues were incubated in p-AMPK (1:50), p-S6 (1:100), Ki-67 (1:2000), Foxp3 (1:25), or CD3 (1:600) antibodies per the manufacturers' recommendations. To verify staining specificity, tissues

were also incubated in the absence of primary antibody. Detection was performed using VECTASTAIN Elite ABC kits (Vector Laboratories, Burlingame, CA).

Immunohistochemical analysis for p-AMPK or p-S6 was quantified by assigning a score of absent (0), minimal (1), moderate (2), or high (3) staining to each tumor cell. All tumors in a single, randomly selected section of lung tissue containing all lobes were scored. The staining index was calculated for each tumor by multiplying the staining intensity by its distribution. The staining indices were then averaged for all tumors in mice from each treatment group. Ki-67 staining was quantified by counting the number of Ki-67+ cells per tumor, and these values were averaged for all tumors in mice from each treatment group. Lung-associated %Foxp3+/CD3+ cells were determined by averaging the number of Foxp3+ and CD3+ cells in ten 40X magnification fields containing normal lung parenchyma. Tumor-associated %Foxp3+/CD3+ cells were determined by counting the number of Foxp3+ or CD3+ cells per tumor. Tumor-associated %Foxp3+/CD3+ cells were then averaged for all tumors from mice in each treatment group. The investigator was blinded to the identities of the samples.

### **Pharmacokinetic analysis of metformin plasma levels**

Sample preparation and analysis were performed as previously described (18). Analysis was performed on plasma collected from 9 mice per treatment group at the end of the tumorigenesis study using oral administration of metformin, and from 6 mice injected with 250 mg/kg metformin intraperitoneal qd x 7, 2 hours after the last injection.

### **Quantitative RT-PCR for OCT1**

Lung and liver tissues were harvested from 10-week old A/J mice (n=3) and stored in RNAlater (Ambion, Austin, TX). RNA was isolated from tissues using an electric homogenizer and the RNeasy kit (Qiagen, Valencia, CA). Quantitative RT-PCR for OCT1 was performed using a 2-step process. cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit. Quantitative PCR was subsequently performed using SYBR Green PCR Master Mix and run on an ABI PRISM 7500 (Applied Biosystems, Foster City, CA). Expression of OCT1 mRNA was normalized to GAPDH to control for differences in total mRNA between samples. Primer sequences for mouse OCT1 and GAPDH were used as previously published (19).

### **Blood glucose and plasma IGF-1 and insulin analysis**

Blood glucose levels were assessed in mice fed the tumorigenic diet AIN-93G/M or the cereal diet NIH-07 for 16 weeks. Mice were fasted for 4 h prior to blood collection from the saphenous vein, and glucose levels were measured using a One Touch Ultra2 glucometer (n=5 mice/diet). Plasma levels of IGF-1 and insulin were measured using the Mouse/Rat IGF-1 ELISA (Diagnostic Systems Laboratories, Webster, TX) and the Rat/Mouse Insulin ELISA kit (Millipore, Billerica, MA), respectively. These analyses were performed on plasma obtained from 5 mice/treatment group at the end of the tumorigenesis studies.

### **Statistical Analyses**

All analyses were performed using a two-tailed Student's t-Test.

## Results

### Oral metformin significantly prevents NNK-induced lung tumorigenesis in A/J mice, but only modestly inhibits the mTOR pathway in lung tumors

To investigate if metformin prevents NNK-induced lung tumorigenesis, A/J mice were treated with 1 or 5 mg/mL metformin dissolved in drinking water for 13 weeks, beginning one week after administration of NNK (Figure 1a). These doses of metformin were used because they have been previously shown to activate AMPK in multiple tissues in mice (20, 21). Metformin was well tolerated and did not significantly affect the weight of mice throughout the course of treatment (data not shown). To determine if oral metformin produced levels that could be achieved in humans, plasma concentrations were assessed at the end of 13 weeks. Mice that received metformin at 1 mg/ml achieved 0.45  $\mu\text{g/mL}$ , and mice at 5 mg/ml achieved 1.70  $\mu\text{g/mL}$ , which is consistent with steady-state values that have been reported in patients (0.5–2  $\mu\text{g/mL}$ ) (Figure 1b). Although metformin did not affect tumor incidence or pathology, lung tumor multiplicity was decreased by 28% (1 mg/ml) and 32% (5 mg/ml). Treatment with 5 mg/mL metformin also decreased average tumor volume by 34% (Figure 1c, left and center panels). Overall tumor burden was decreased by 39% (1 mg/mL) and 53% (5 mg/mL). (Figure 1c, right panel). To determine if oral metformin inhibited the mTOR pathway in lung tumors, immunohistochemical analysis was performed using antibodies specific for the phosphorylated form of S6, a component downstream of mTOR. Oral metformin inhibited phospho-S6 by 20% in lung tumors (Figure 1d). Taken together, these results show that oral metformin significantly prevented tumor growth, but to a lesser extent than rapamycin, which previously decreased lung tumorigenesis by 90% in this model system (4).

### Intraperitoneal administration of metformin produces higher plasma levels of metformin that cause tissue-specific modulation of the AMPK and mTOR pathways

Because oral metformin only modestly affected the mTOR pathway in lung tissue, we investigated if another route of administration with metformin might be more effective in modulating this pathway in mice. A short-term biomarker study was done using intraperitoneal injections of metformin, because we hypothesized that peak plasma concentrations would be higher than that achieved with oral administration. Pharmacokinetic analysis demonstrated that plasma levels of metformin 2 h after the last injection were higher (4.0  $\mu\text{g/mL}$ ) than with either oral dosing schedule. Immunoblotting analysis of lysates of liver tissue harvested from mice treated with intraperitoneal metformin showed that it activated hepatic AMPK and increased the phosphorylation of a substrate of AMPK, acetyl Co A carboxylase (ACC) (Figure 2a). Additionally, this dosing schedule with metformin inhibited the mTOR pathway in liver tissue, as demonstrated by a trend toward decreased phosphorylation of both S6 and 4E-BP1, although some variability in these markers between mice was observed. Conversely, short-term oral administration of 5mg/mL metformin did not activate AMPK nor inhibit the mTOR pathway in liver tissue (Figure 2b). This suggests that higher plasma metformin concentrations are better able to activate AMPK and inhibit the mTOR pathway.

Although intraperitoneal administration of metformin activated hepatic AMPK, it did not activate AMPK in lung tissue (Figure 2c). Nonetheless, metformin still inhibited the mTOR pathway in lung tissue. Akt and ERK phosphorylation were also inhibited, suggesting that an upstream receptor tyrosine kinase might be inhibited by metformin. Clinical studies have shown that metformin treatment is associated with decreases in plasma levels of insulin and IGF-1 in both diabetic and non-diabetic patients (22, 23). Immunoblotting analysis using an antibody specific for the phosphorylated, or active forms, of the IGF-1 and insulin receptors, showed that metformin decreased their activation in lung tissue. These results could suggest



that intraperitoneal administration of metformin inhibits the mTOR pathway in lung tissue independently of AMPK by decreasing the response to insulin or IGF-1.

The fact that metformin activates AMPK in liver but not lung tissue indicates a tissue specific response to metformin, which could be related to differences in the level of expression of the organic cation transporter 1 (OCT1). This receptor is required for tissue uptake of metformin in mice, and polymorphisms in the gene that encodes OCT1 are associated with decreased response to metformin in diabetic patients (19). Quantitative RT-PCR analysis of liver and lung tissues harvested from A/J mice demonstrated that OCT1 expression was 17-fold higher in liver tissue (Figure 2d), suggesting that the inability of metformin to activate AMPK in lung tissue was due to diminished uptake.

### **Intraperitoneal administration of metformin is more effective than oral administration in preventing NNK-induced lung tumorigenesis in A/J mice**

Because intraperitoneal administration of metformin significantly inhibited the mTOR pathway in lung tissue, we investigated if this dosing schedule with metformin was more effective in preventing NNK-induced lung tumorigenesis in A/J mice. Mice were fed the tumorigenic AIN-93G/M diets and treated with daily intraperitoneal injections of saline or metformin, beginning one week after NNK administration (Figure 3a). This dosing schedule with metformin was surprisingly well tolerated and did not significantly affect the weight of mice throughout the course of the study (data not shown). Metformin was highly effective. Tumor multiplicity decreased by 66%, average tumor volume decreased by 50%, and overall tumor burden decreased by 72% (Figure 3b–d). Phosphorylation of S6 decreased by 40% (Figure 4a). Thus, intraperitoneal metformin was more effective in preventing NNK-induced lung tumorigenesis and inhibiting mTOR than oral metformin. However, consistent with the results of our short-term biomarker studies, this occurred in the absence of AMPK activation in lung tumors (Supplementary Figure 1).

Previous studies have demonstrated that metformin inhibits cancer cell proliferation *in vitro* by a mechanism that is dependent upon inhibition of mTOR-induced protein translation (24). To investigate if metformin inhibits tumor cell proliferation *in vivo*, immunohistochemical analysis was performed on lung tissues using an antibody specific for Ki-67, a marker of cellular proliferation (Figure 4b). We observed that inhibition of NNK-induced lung tumorigenesis by metformin was associated with a 67% decrease in tumor cell proliferation. TUNEL analysis demonstrated that metformin did not induce apoptosis in tumor cells (data not shown). Collectively, these data demonstrate that prevention of NNK-induced lung tumorigenesis by metformin is associated with decreased tumor cell proliferation but not increased apoptosis.

The ability of metformin to prevent NNK-induced lung tumorigenesis could also be due to effects on the tumor microenvironment. We previously demonstrated that NNK increases lung- and tumor-associated Foxp3+ regulatory T cells (Treg) in A/J mice, which are required for K-ras mediated lung tumorigenesis, and that rapamycin prevented the induction of lung associated Foxp3+ cells by NNK (5). Because metformin also inhibits the mTOR pathway in NNK-induced lung tumors in A/J mice, we investigated if treatment with metformin was associated with decreases in lung- and tumor-associated Treg. Immunohistochemical analysis using antibodies specific for Foxp3, a marker of Treg, and CD3, a pan-T cell marker, showed that metformin treatment decreased lung-associated Treg by 65% and decreased tumor-associated Treg by 50% (Figure 4c). Because we have previously demonstrated that Foxp3+ regulatory T cells are required for K-ras mediated lung tumorigenesis (5), decreases in lung- and tumor-associated Treg might contribute to the ability of metformin to prevent NNK-induced lung tumorigenesis in A/J mice.

## Metformin is a well-tolerated drug that prevents fatty liver disease in A/J mice fed the tumorigenic diet AIN-93G/M

Because there were no published studies that demonstrated the safety of daily intraperitoneal injections of metformin over many weeks, we harvested livers from mice at the end of the tumorigenesis study to assess hepatotoxicity. Examination of H&E stained sections showed that livers from the control group contained a large number of vacuoles that stained with Oil Red O (Figure 5a). These lipid-laden vacuoles were substantially decreased both in size and number in livers from mice treated with metformin (Figure 5b). The presence of vacuoles was most likely due to diet and not NNK, because we did not observe liver vacuolization in mice that were exposed to NNK but were fed the cereal diet NIH-07 (Supplementary Figure 2). Interestingly, the AIN-93G and AIN-93M diets are reformulations of the AIN-76A diet, which has been previously reported to cause fatty liver disease in rats (25). These results suggest that the tumorigenic AIN-93G and AIN-93M diets induce fatty liver disease in A/J mice, which is prevented by treatment with metformin. To confirm that diabetes was not induced by the AIN-93G/M diet (26), glucose and insulin levels were compared in mice fed either AIN-93G/M or NIH-07 diets. No differences in blood glucose (Figure 5c) or insulin levels (data not shown) were observed. This demonstrates that metformin prevented tobacco carcinogen-induced tumor growth in a non-diabetic mouse model of lung tumorigenesis.

## Inhibition of the mTOR pathway in tumors is associated with decreases in levels of circulating IGF-1 and insulin

Because we showed that metformin decreased phosphorylation of IGF-1 and/or insulin receptors in lung tissue, and metformin is known to decrease circulating levels of these hormones in patients, plasma levels of insulin and IGF-I were assessed at the end of the tumorigenesis studies. Oral and intraperitoneal administration of metformin decreased insulin by ~20% and 35%, respectively (Figure 6a). Additionally, oral metformin at either 1 or 5 mg/ml decreased levels of circulating IGF-1 by ~20% (Figure 6b left panel). Intraperitoneal injection of metformin did not decrease IGF-I levels, but the levels of IGF-1 in mice injected with saline were significantly lower than in mice that were not injected (Figure 6b middle and right panels), suggesting that stress associated with daily injections could have obscured an inhibitory effect of intraperitoneal metformin on circulating levels of IGF-1. These results support the hypothesis that metformin inhibits the mTOR pathway in normal lung epithelium and in lung tumors by decreasing circulating levels of IGF-1 or insulin, which contributes to its ability to prevent NNK-induced lung tumorigenesis.

To investigate the responsiveness of lung and liver tissues to exogenously administered IGF-I or insulin, IGF-1 or insulin were injected into the peritoneal cavity and tissues were processed for immunoblotting analysis. IGF-1 rapidly activated the IGF-1R/Akt/mTOR pathway in lung tissue (Figure 6c). It also increased phosphorylation of IGF-1R and Akt in liver tissue, but did not affect phosphorylation of S6. The tissue specific response to insulin was opposite that of IGF-I, in that insulin had no effect on the mTOR pathway in lung tissue, but increased phosphorylation of the insulin receptor, Akt and S6 in liver tissue. These data suggest that lung tissue is more responsive to IGF-I and that liver tissue is more responsive to insulin. Given that metformin decreased levels of IGF-I and insulin, prevention of lung tumorigenesis by metformin might be related to multiple mechanisms in different organs.

## Discussion

A majority of lung cancer cases are associated with tobacco use (85–90%), and over 90 million current or former smokers in the US are at permanent increased risk to develop lung cancer (27). Thus, there is great need for the development of new chemotherapeutic

strategies for the prevention of this disease. Our studies demonstrate that metformin is effective in preventing tumor growth in a mouse model of tobacco carcinogen-induced lung tumorigenesis. Oral administration of 1 mg/mL or 5 mg/mL metformin decreased lung tumor burden in mice by 38% and 53%, respectively. The steady state levels of metformin in mice given 5 mg/mL are similar to those in diabetic patients using metformin, suggesting the possibility that clinical prevention of lung cancer could be achieved with standard oral dosing. Despite this efficacy of oral metformin, intraperitoneal administration of metformin was more effective than oral administration and decreased tumor burden by 72%. Because plasma levels of metformin were higher using this route of administration, these results could support the development of more potent analogues of metformin such as phenformin. Phenformin is more effective at decreasing hyperglycemia in diabetic patients, and preclinical studies demonstrate that phenformin is a more potent activator of AMPK and activates AMPK in a broader range of tissues than metformin (21). However, phenformin treatment is associated with lactic acidosis in patients, which could limit its clinical potential as a chemopreventive agent (28).

Inhibition of tumorigenesis by metformin could be related to mTOR inhibition within tumors that is an indirect effect of diminished circulating levels of growth factors such as insulin and IGF-1. Inhibition of mTOR correlated with decreased tumor cell proliferation in A/J mice, which is consistent with *in vitro* studies that demonstrate that metformin is a cytostatic agent that decreases cancer cell proliferation by inhibiting mTOR-dependent protein translation (24). Metformin inhibited mTOR in lung tissue independently of AMPK activation, which suggests that lung tissue does not respond directly to metformin due to insufficient uptake. Consistent with this, lung tissue from A/J mice expresses 17-fold less OCT1 than liver tissue, which did show AMPK activation after metformin administration. Inhibition of the mTOR pathway by metformin was associated with decreased phosphorylation of IGF-1/insulin receptors and decreased levels of circulating IGF-1 and insulin, suggesting that they might contribute to tumorigenesis in this model. In agreement with this, a recent study using mice genetically engineered to overexpress IGF-1 in lung tissues demonstrated that IGF-1 enhances NNK-induced lung tumorigenesis in FVB mice (29). These data suggest that the prevention of lung tumorigenesis by metformin could be due to effects on other tissues that decrease circulating levels of growth factors.

This is the first study to demonstrate the safety and efficacy of metformin in a tobacco carcinogen-driven mouse model of lung tumorigenesis, but other studies have investigated the ability of metformin to prevent tumor growth *in vivo*. For example, studies performed using multiple xenograft models have shown that treatment with metformin modestly inhibited tumor growth (30–32). The modest effect of metformin observed in some of these xenograft models could be due to the use of immunocompromised mice or the fact that the subcutaneous compartment where tumors are placed is not analogous to the microenvironment within organs. A role for the microenvironment in the response to metformin is supported by the fact that metformin decreased lung- and tumor-associated Treg, which is a requirement for K-ras induced lung tumorigenesis originally described in studies using rapamycin. In a syngeneic, orthotopic model of lung cancer, LLC1, investigators showed that metformin effectively inhibited tumor growth, but only in mice that were fed a high calorie diet (33). However, the role of the mTOR pathway in promoting tumor growth in this model is not clear, and inhibition of the mTOR pathway in tumors was not assessed. Metformin was also tested in a Pten<sup>+/-</sup>Lkb1<sup>fl/+</sup> mouse model (21). Pten and LKB1 are tumor suppressors that negatively regulate mTOR, and tissues from these mice were characterized by activation of the mTOR pathway. However, administration of metformin to Pten<sup>+/-</sup>Lkb1<sup>fl/+</sup> mice only increased tumor latency by one month, and did not affect tumor incidence or morphology. Although this study used a similar oral dosing schedule, they did not observe decreases in serum levels of insulin. This could be related to



loss of LKB1 and/or Pten in different tissues such as liver. Collectively, these studies suggest that indirect effects of metformin on the tumor microenvironment and on circulating levels of growth factors could be important to the prevention of tumorigenesis.

Metformin is a promising candidate for lung cancer chemoprevention. Clinical studies have shown that long-term treatment with metformin is associated with few adverse effects in diabetic, as well as non-diabetic, patient populations (7, 34). Similarly, the dosing schedules with metformin used in our studies appeared to be well tolerated and did not cause weight loss or hepatotoxicity. Metformin also prevented diet-induced fatty liver disease in A/J mice. This effect of metformin has been previously demonstrated in mouse models of hepatosteatosis (35), as well as in patients with non-alcoholic fatty liver disease (NAFLD) (reviewed in (36)). Even though the tumorigenic diets used in our studies caused lipid accumulation in the liver, they did not induce hyperglycemia or hyperinsulinemia. This demonstrates that metformin was effective in preventing tobacco carcinogen-induced tumor growth in a non-diabetic mouse model of lung tumorigenesis. An additional advantage of metformin as a chemopreventive agent for lung cancer is that it inhibits tumor formation independently of AMPK activation in lung tissue. Metformin activates AMPK by a mechanism that is dependent upon its upstream kinase, the tumor suppressor LKB1 (37). Somatic mutations in LKB1 are an early and frequent event in lung cancer, and are associated with smoking (38–40). Because the effects of metformin are independent of AMPK in lung tissue, the presence of LKB1 mutations would not preclude response to metformin. Taken together, the safety and efficacy of metformin provide strong rationale for a clinical prevention trial with metformin in heavy smokers that are at high risk to develop lung cancer.

## Supplementary Material

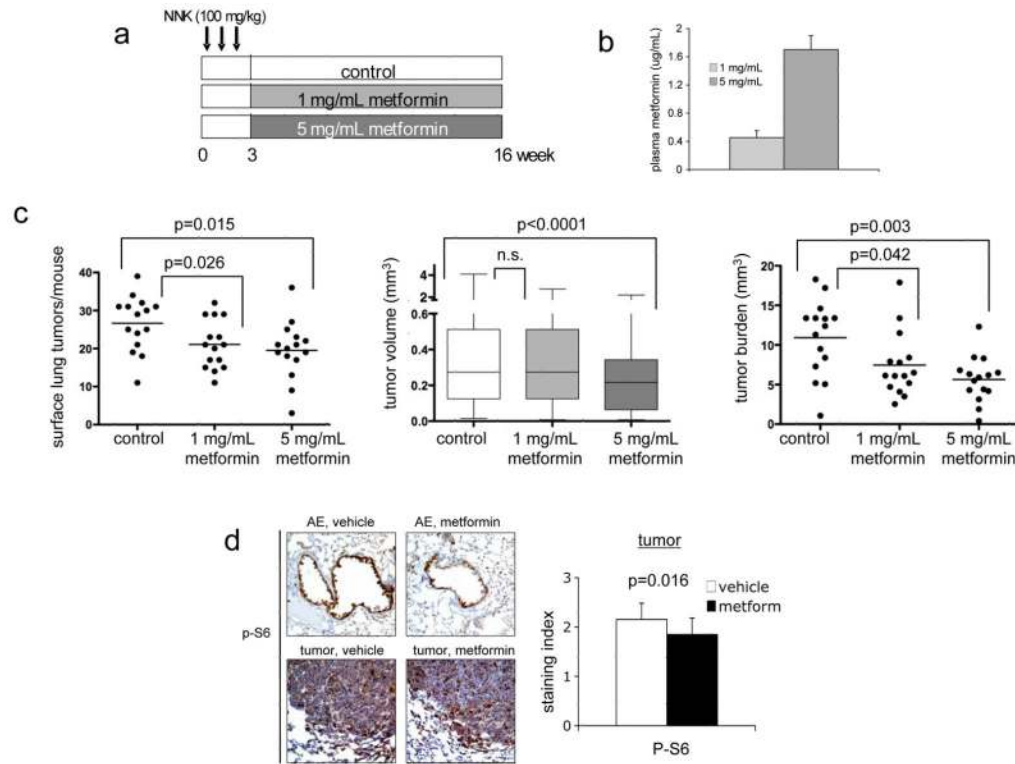
Refer to Web version on PubMed Central for supplementary material.

## References

1. Chiang GG, Abraham RT. Targeting the mTOR signaling network in cancer. *Trends Mol Med.* 2007; 13:433–42. [PubMed: 17905659]
2. Memmott RM, Dennis PA. Akt-dependent and -independent mechanisms of mTOR regulation in cancer. *Cell Signal.* 2009; 21:656–64. [PubMed: 19166931]
3. Belinsky SA, Devereux TR, Maronpot RR, Stoner GD, Anderson MW. Relationship between the formation of promutagenic adducts and the activation of the K-ras protooncogene in lung tumors from A/J mice treated with nitrosamines. *Cancer Res.* 1989; 49:5305–11. [PubMed: 2670201]
4. Granville CA, Warfel N, Tsurutani J, et al. Identification of a Highly Effective Rapamycin Schedule that Markedly Reduces the Size, Multiplicity, and Phenotypic Progression of Tobacco Carcinogen-Induced Murine Lung Tumors. *Clin Cancer Res.* 2007; 13:2281–9. [PubMed: 17404113]
5. Granville CA, Memmott RM, Balogh A, et al. A central role for Foxp3+ regulatory T cells in K-Ras-driven lung tumorigenesis. *PLoS One.* 2009; 4:e5061. [PubMed: 19330036]
6. Zou W. Regulatory T cells, tumour immunity and immunotherapy. *Nat Rev Immunol.* 2006; 6:295–307. [PubMed: 16557261]
7. Bolen S, Feldman L, Vassy J, et al. Systematic review: comparative effectiveness and safety of oral medications for type 2 diabetes mellitus. *Ann Intern Med.* 2007; 147:386–99. [PubMed: 17638715]
8. Hundal HS, Ramlal T, Reyes R, Leiter LA, Klip A. Cellular mechanism of metformin action involves glucose transporter translocation from an intracellular pool to the plasma membrane in L6 muscle cells. *Endocrinology.* 1992; 131:1165–73. [PubMed: 1505458]
9. Stumvoll M, Nurjhan N, Perriello G, Dailey G, Gerich JE. Metabolic effects of metformin in non-insulin-dependent diabetes mellitus. *N Engl J Med.* 1995; 333:550–4. [PubMed: 7623903]

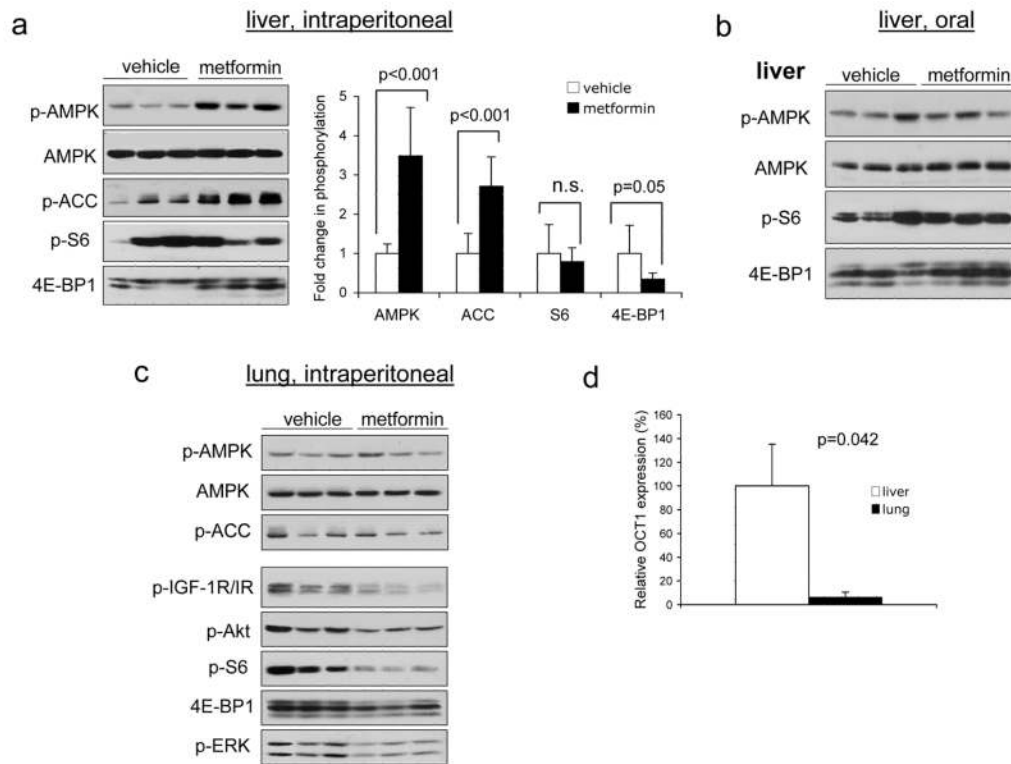
10. Zhou G, Myers R, Li Y, et al. Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest*. 2001; 108:1167–74. [PubMed: 11602624]
11. Shaw RJ, Kosmatka M, Bardeesy N, et al. The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. *Proc Natl Acad Sci U S A*. 2004; 101:3329–35. [PubMed: 14985505]
12. Shaw RJ, Bardeesy N, Manning BD, et al. The LKB1 tumor suppressor negatively regulates mTOR signaling. *Cancer Cell*. 2004; 6:91–9. [PubMed: 15261145]
13. Libby G, Donnelly LA, Donnan PT, Alessi DR, Morris AD, Evans JM. New users of metformin are at low risk of incident cancer: a cohort study among people with type 2 diabetes. *Diabetes Care*. 2009; 32:1620–5. [PubMed: 19564453]
14. Evans JM, Donnelly LA, Emslie-Smith AM, Alessi DR, Morris AD. Metformin and reduced risk of cancer in diabetic patients. *Bmj*. 2005; 330:1304–5. [PubMed: 15849206]
15. Hecht SS, Morse MA, Amin S, et al. Rapid single-dose model for lung tumor induction in A/J mice by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and the effect of diet. *Carcinogenesis*. 1989; 10:1901–4. [PubMed: 2791206]
16. Reeves PG, Nielsen FH, Fahey GC Jr. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J Nutr*. 1993; 123:1939–51. [PubMed: 8229312]
17. Memmott RM, Gills JJ, Hollingshead M, et al. Phosphatidylinositol ether lipid analogues induce AMP-activated protein kinase-dependent death in LKB1-mutant non small cell lung cancer cells. *Cancer Res*. 2008; 68:580–8. [PubMed: 18199555]
18. Wang Y, Tang Y, Gu J, Fawcett JP, Bai X. Rapid and sensitive liquid chromatography-tandem mass spectrometric method for the quantitation of metformin in human plasma. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2004; 808:215–9.
19. Shu Y, Sheardown SA, Brown C, et al. Effect of genetic variation in the organic cation transporter 1 (OCT1) on metformin action. *J Clin Invest*. 2007; 117:1422–31. [PubMed: 17476361]
20. Ma TC, Buescher JL, Oatis B, et al. Metformin therapy in a transgenic mouse model of Huntington's disease. *Neurosci Lett*. 2007; 411:98–103. [PubMed: 17110029]
21. Huang X, Wullschleger S, Shpiro N, et al. Important role of the LKB1-AMPK pathway in suppressing tumorigenesis in PTEN-deficient mice. *Biochem J*. 2008; 412:211–21. [PubMed: 18387000]
22. Berker B, Emral R, Demirel C, Corapcioglu D, Unlu C, Kose K. Increased insulin-like growth factor-I levels in women with polycystic ovary syndrome, and beneficial effects of metformin therapy. *Gynecol Endocrinol*. 2004; 19:125–33. [PubMed: 15697073]
23. Goodwin PJ, Pritchard KI, Ennis M, Clemons M, Graham M, Fantus IG. Insulin-lowering effects of metformin in women with early breast cancer. *Clin Breast Cancer*. 2008; 8:501–5. [PubMed: 19073504]
24. Dowling RJ, Zakikhani M, Fantus IG, Pollak M, Sonenberg N. Metformin inhibits mammalian target of rapamycin-dependent translation initiation in breast cancer cells. *Cancer Res*. 2007; 67:10804–12. [PubMed: 18006825]
25. Medinsky MA, Popp JA, Hamm TE, Dent JG. Development of hepatic lesions in male Fischer-344 rats fed AIN-76A purified diet. *Toxicol Appl Pharmacol*. 1982; 62:111–20. [PubMed: 7064147]
26. Surwit RS, Feinglos MN, Rodin J, et al. Differential effects of fat and sucrose on the development of obesity and diabetes in C57BL/6J and A/J mice. *Metabolism*. 1995; 44:645–51. [PubMed: 7752914]
27. IARC. IARC Monographs on the Evaluation of the Carcinogenic Risks of Chemicals to Humans. Lyon, France: International Agency for Research on Cancer; 2004. Tobacco smoking and tobacco smoke.
28. McGuinness ME, Talbert RL. Phenformin-induced lactic acidosis: a forgotten adverse drug reaction. *Ann Pharmacother*. 1993; 27:1183–7. [PubMed: 8251683]
29. Kim WY, Jin Q, Oh SH, et al. Elevated epithelial insulin-like growth factor expression is a risk factor for lung cancer development. *Cancer Res*. 2009; 69:7439–48. [PubMed: 19738076]

30. Buzzai M, Jones RG, Amaravadi RK, et al. Systemic treatment with the antidiabetic drug metformin selectively impairs p53-deficient tumor cell growth. *Cancer research*. 2007; 67:6745–52. [PubMed: 17638885]
31. Ben Sahra I, Laurent K, Loubat A, et al. The antidiabetic drug metformin exerts an antitumoral effect in vitro and in vivo through a decrease of cyclin D1 level. *Oncogene*. 2008; 27:3576–86. [PubMed: 18212742]
32. Phoenix KN, Vumbaca F, Claffey KP. Therapeutic metformin/AMPK activation promotes the angiogenic phenotype in the ERalpha negative MDA-MB-435 breast cancer model. *Breast Cancer Res Treat*. 2009; 113:101–11. [PubMed: 18256928]
33. Algire C, Zakikhani M, Blouin MJ, Shuai JH, Pollak M. Metformin attenuates the stimulatory effect of a high-energy diet on in vivo LLC1 carcinoma growth. *Endocr Relat Cancer*. 2008; 15:833–9. [PubMed: 18469156]
34. Tang T, Lord JM, Norman RJ, Yasmin E, Balen AH. Insulin-sensitising drugs (metformin, rosiglitazone, pioglitazone, D-chiro-inositol) for women with polycystic ovary syndrome, oligo amenorrhoea and subfertility. *Cochrane Database Syst Rev*. 2009:CD003053.
35. Lin HZ, Yang SQ, Chuckaree C, Kuhajda F, Ronnet G, Diehl AM. Metformin reverses fatty liver disease in obese, leptin-deficient mice. *Nat Med*. 2000; 6:998–1003. [PubMed: 10973319]
36. Stein LL, Dong MH, Loomba R. Insulin sensitizers in nonalcoholic fatty liver disease and steatohepatitis: Current status. *Adv Ther*. 2009; 26:893–907. [PubMed: 19921118]
37. Shaw RJ, Lamia KA, Vasquez D, et al. The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin. *Science*. 2005; 310:1642–6. [PubMed: 16308421]
38. Ghaffar H, Sahin F, Sanchez-Cepedes M, et al. LKB1 protein expression in the evolution of glandular neoplasia of the lung. *Clin Cancer Res*. 2003; 9:2998–3003. [PubMed: 12912948]
39. Matsumoto S, Iwakawa R, Takahashi K, et al. Prevalence and specificity of LKB1 genetic alterations in lung cancers. *Oncogene*. 2007; 26:5911–8. [PubMed: 17384680]
40. Ji H, Ramsey MR, Hayes DN, et al. LKB1 modulates lung cancer differentiation and metastasis. *Nature*. 2007; 448:807–10. [PubMed: 17676035]



**Figure 1. Oral administration of metformin prevents NNK-induced lung tumorigenesis in A/J mice and is associated with modest inhibition of the mTOR pathway**

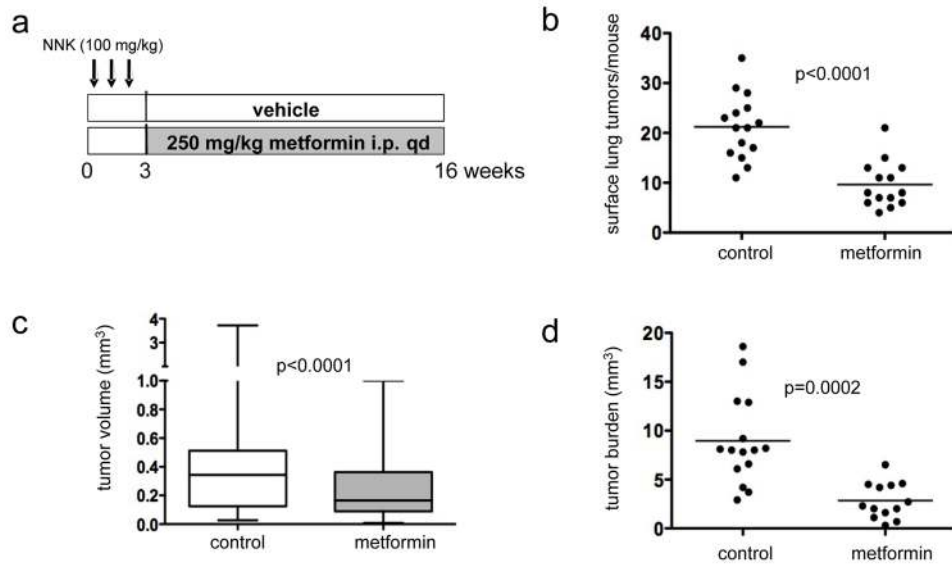
a) Schema for the prevention study with oral metformin in a tobacco carcinogen-driven mouse model of lung tumorigenesis. b) Mean plasma levels of metformin in A/J mice treated with 1 or 5 mg/mL metformin orally were determined using LC-MS/MS. c) Tumor multiplicity (left panel), tumor volume (middle panel) and tumor burden (right panel) were assessed after 13 weeks of oral administration of 1 mg/mL or 5 mg/mL metformin in A/J mice. For the dot plots, each point represents a mouse and the lines represent the median values. For the box and whisker graph, boxes indicate interquartile range, lines indicate median, and whiskers indicate minimal and maximal values. ‘n.s.’ is not significant. d) Representative images of IHC analysis for P-S6 in airway epithelium (AE) and lung tumors from control mice and from mice that received oral metformin (left panel), and quantification of IHC analysis (right panel). Graph shows mean staining indices and error bars represent SD. IHC analysis was performed on 5 mice/treatment group.



**Figure 2. Intraperitoneal administration of metformin causes tissue-specific modulation of the AMPK and mTOR pathways in A/J mice**

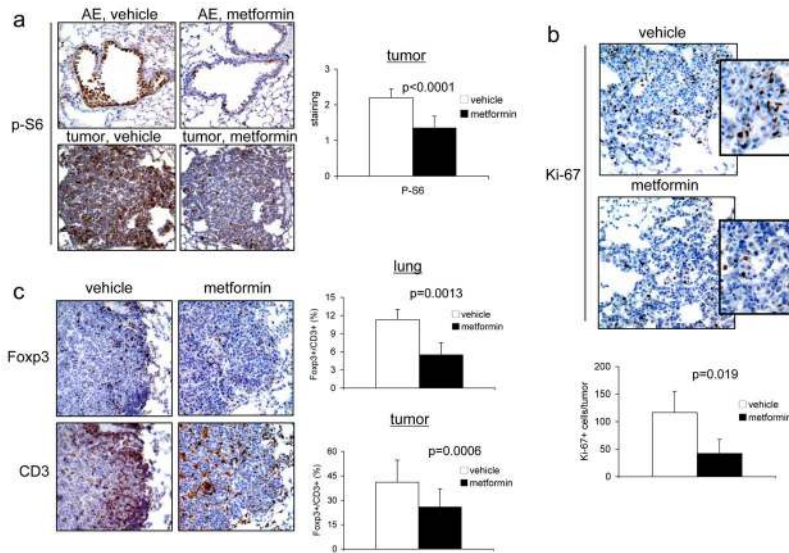
a–c) Immunoblotting analysis of components of the AMPK pathway and pathways downstream of IGF-1R/IR that converge on mTOR in liver (a and b) and lung tissues (c). Tissues were harvested from mice treated short-term with intraperitoneal injections of 250 mg/kg metformin (qd x 3) in two independent studies, and representative blots from these studies are shown. Densitometry was performed on immunoblots of liver lysates from both studies, which is presented in the right panel of (a). b) Liver tissue was also harvested from mice treated with 5 mg/mL metformin orally for 7 d, for comparison. d) OCT1 expression in liver and lung tissues from A/J mice was assessed by RT-PCR.





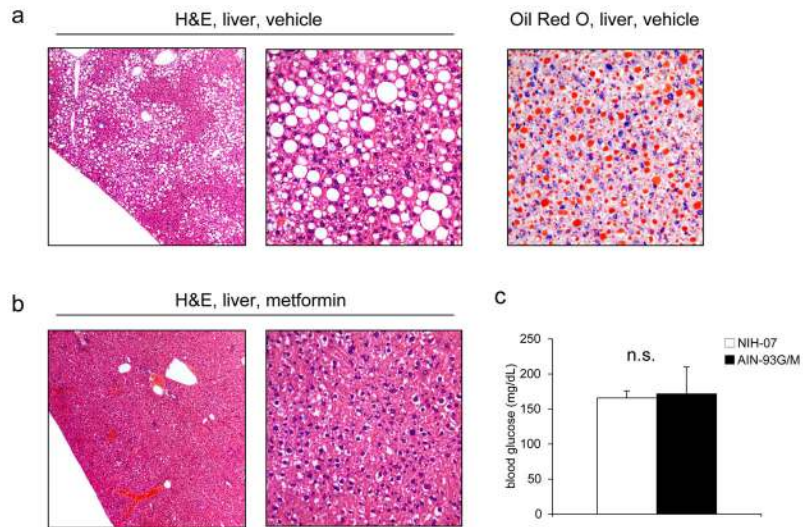
**Figure 3. Administration of metformin by intraperitoneal injection prevents NNK-induced lung tumorigenesis**

a) Schema for the prevention study with metformin administered by intraperitoneal injection in a tobacco carcinogen-driven mouse model of lung tumorigenesis. Tumor multiplicity (b), tumor volume (c) and tumor burden (d) were assessed after 13 weeks of administration of 250 mg/kg metformin qd by intraperitoneal injection in A/J mice. For the dot plots in (b) and (d), each point represents a mouse and the lines represent the median values. For the box and whisker graph shown in (c), boxes indicate interquartile range, lines indicate median, and whiskers indicate minimal and maximal values.



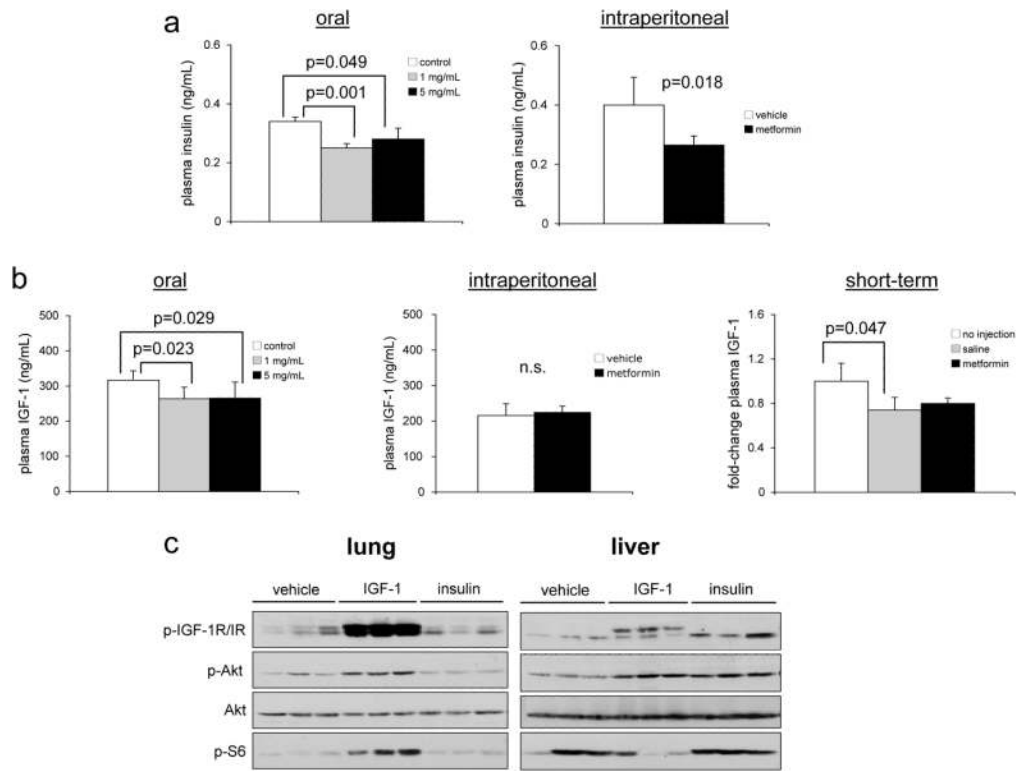
**Figure 4. Intraperitoneal administration of metformin inhibits the mTOR pathway in lung tissue, decreases in tumor cell proliferation, and decreases lung- and tumor-associated Fxp3+ Treg**

a) Representative images of IHC analysis for p-S6 in airway epithelium (AE) and lung tumors from vehicle-treated mice and from mice that received intraperitoneal metformin (left panel), and quantification of IHC analysis (right panel). Graph shows mean staining indices and error bars represent SD. b) Representative images of IHC analysis for Ki-67, a marker of cellular proliferation, in lung tumors from vehicle and metformin treated mice (upper panels), and quantification of IHC analysis (bottom panel). Graph shows mean number of Ki-67+ cells/tumor and error bars represent SD. c) Representative images of IHC for tumor-associated Fxp3+ and CD3+ cells in A/J mice treated with vehicle or intraperitoneal metformin (left panel), and quantification of IHC analysis for lung- and tumor-associated percent Fxp3+/CD3+ cells (upper and lower right panels, respectively). For (a), (b), and (c), IHC analysis was performed on 5 mice/treatment group.



**Figure 5. The tumorigenic diet AIN-93G/M causes fatty liver disease in A/J mice, which is prevented by metformin, but does not cause hyperglycemia**

a) H&E (right panel) or Oil Red O staining (left panel) of liver sections from A/J mice fed AIN-93G/M and treated with vehicle. b) H&E staining of liver sections from A/J mice fed AIN-93G/M and treated with 250 mg/kg metformin QD i.p. for 13 weeks. For (a) and (b), images were taken at 10× and 40× magnification. c) Blood glucose levels in A/J mice that were fed the cereal diet NIH-07 or the tumorigenic diet AIN-93G/M (n=5 mice/group). Graph shows mean values, and error bars represent SD.



**Figure 6. Metformin-induced inhibition of the mTOR pathway in lung tissue is associated with decreases in circulating levels of IGF-1 and insulin**  
a–b) Plasma levels of insulin (a) or IGF-1 (b) were assessed by ELISA in A/J mice treated with oral or intraperitoneal administration of metformin at the end of the tumorigenesis studies. Graphs show mean values and error bars represent SD. This analysis was performed on 5 mice/treatment group. Plasma IGF-1 levels were also assessed in mice given intraperitoneal injections of saline or 250 mg/kg metformin qd x 3 (b, far right panel). (c) Immunoblotting analysis of IGF-1R/IR/Akt/mTOR pathway in liver and lung tissues harvested from A/J mice 0.5 h after administration of 0.5 mg/kg IGF-1 or 0.75 U of insulin.