Diet and tumor LKB1 expression interact to determine sensitivity to anti-neoplastic effects of metformin *in vivo*

C Algire[1], L Amrein[2], M Bazile[2], S David[2], M Zakikhani[2] and M Pollak[1]

[1]*E423 Segal Cancer Centre of the Jewish General Hospital, Departments of Experimental Medicine and Oncology, McGill University, Montréal, Québec, Canada and* [2]*Lady Davis Institute for Medical Research, Jewish General Hospital, Montréal, Québec, Canada*

Hypothesis-generating epidemiological research has suggested that cancer burden is reduced in diabetics treated with metformin and experimental work has raised questions regarding the role of direct adenosine monophosphate-activated protein kinase (AMPK)-mediated anti-neoplastic effects of metformin as compared with indirect effects attributable to reductions in circulating insulin levels in the host. We treated both tumor LKB1 expression and host diet as variables, and observed that metformin inhibited tumor growth and reduced insulin receptor activation in tumors of mice with diet-induced hyperinsulinemia, independent of tumor LKB1 expression. In the absence of hyperinsulinemia, metformin inhibited only the growth of tumors transfected with short hairpin RNA against LKB1, a finding attributable neither to an effect on host insulin level nor to activation of AMPK within the tumor. Further investigation *in vitro* showed that cells with reduced LKB1 expression are more sensitive to metformin-induced adenosine triphosphate depletion owing to impaired ability to activate LKB1-AMPK-dependent energy-conservation mechanisms. Thus, loss of function of LKB1 can accelerate proliferation in contexts where it functions as a tumor suppressor, but can also sensitize cells to metformin. These findings predict that any clinical utility of metformin or similar compounds in oncology will be restricted to subpopulations defined by host insulin levels and/or loss of function of LKB1.

Keywords: metformin; insulin; cancer; LKB1

Introduction

Metformin, a biguanide commonly prescribed for treatment of type II diabetes, is currently being investigated with respect to its anti-neoplastic activity. This research is motivated in part by hypothesis-generating epidemiological studies, which suggest that diabetic patients receiving metformin have substantially (~40%) reduced cancer burden compared with diabetic patients receiving other therapies (Evans *et al*., 2005; Currie *et al*., 2009; Libby *et al*., 2009; Bodmer *et al*., 2010; Landman *et al*., 2010).

Metformin and other biguanides are mild inhibitors of complex I of the respiratory chain, and are known to reduce adenosine triphosphate (ATP) levels and increase the ratio of adenosine monophosphate to ATP in the cell (El Mir *et al*., 2000; Owen *et al*., 2000; Hardie, 2006; Dykens *et al*., 2008). The tumor suppressor LKB1 (Shackelford and Shaw, 2009) phosphorylates and activates adenosine monophosphate-activated protein kinase (AMPK) at Thr^{172} when the ratio of intracellular adenosine monophosphate/ATP increases (Shaw *et al*., 2004; Jones *et al*., 2005). Metformin-induced energy stress can be overcome by activating the LKB1-AMPK signaling pathway, which inhibits downstream targets, such as mammalian target of rapamycin complex 1, to downregulate processes that consume energy and activate processes that generate ATP, in keeping with the physiological role of AMPK as a key regulator of cellular energy homeostasis (Shaw *et al*., 2004; Jones *et al*., 2005; Hardie, 2007; Shackelford and Shaw, 2009). In addition, an AMPK-independent, rag GTPase-dependent pathway by which metformin inhibits mammalian target of rapamycin complex 1 has recently been described (Kalender *et al*., 2010). By decreasing mitochondrial ATP production, metformin can indirectly activate LKB1-AMPK signaling *in vitro* in transformed cells, with consequences including inhibition of protein synthesis (Dowling *et al*., 2007), proliferation (Zakikhani *et al*., 2006, 2008) and expression of fatty acid synthase (Algire *et al*., 2010).

Multiple *in vivo* models have provided further evidence for the anti-neoplastic activity of metformin. For example, it has been shown that metformin suppresses polyp formation in ApcMin/+ mice (Tomimoto *et al*., 2008), inhibits *in vivo* growth of p53 null cancers (Buzzai *et al*., 2007), attenuates tumorigenesis in phosphatase and tensin homolog-deficient mice (Huang *et al*., 2008) and slows proliferation of triple-negative breast cancer cells (Liu *et al*., 2009); however, these studies did not investigate the anti-neoplastic effects of metformin in the context of variation of dietary energy intake or insulin levels. This issue is critical as all epidemiological evidence for an anti-neoplastic action of

Correspondence: Dr M Pollak, E423 Segal Cancer Centre of the Jewish General Hospital, Departments of Experimental Medicine and Oncology, McGill University, 3755 Côte Ste. Catherine, Montréal, Québec, Canada H3T 1E2.
E-mail: michael.pollak@mcgill.ca

metformin is derived from type II diabetic patients and metformin has important endocrine effects at the whole organism level in the setting of type II diabetes, apart from any direct effects on neoplastic cells. These endocrine effects are a consequence of actions of the drug on hepatocytes, where administration of metformin leads to the inhibition of gluconeogenesis (Shaw *et al*., 2005), which causes a decline in glucose output and therapeutically significant reductions in the elevated glucose and insulin levels seen in type II diabetics (DeFronzo and Goodman, 1995). Shaw *et al*. (2005) showed that the effects of metformin on gluconeogenesis require the LKB1-dependent phosphorylation of AMPK; however, a recent report (Foretz *et al*., 2010) suggests that metformin-induced inhibition of gluconeogenesis is dependent on the energy state within hepatocytes and is independent of AMPK phosphorylation.

As hyperinsulinemia can stimulate *in vivo* growth of certain neoplasms (Venkateswaran *et al*., 2007; Pollak, 2008; Novosyadlyy *et al*., 2010), the insulin-lowering action of metformin may contribute to its anti-neoplastic activity. Previous results (Algire *et al*., 2008) showed that metformin attenuated tumor growth in a murine model of type II diabetes, while having no effect on tumor growth in mice on a control diet, thus raising questions regarding the 'direct' AMPK-mediated anti-neoplastic effects of metformin vs the 'indirect' insulin-lowering actions of metformin as the mechanism by which metformin attenuates tumor growth in *in vivo* models.

In the present study, we engineered LKB1-deficient cell lines using short hairpin RNA (shRNA) against LKB1 and studied the anti-neoplastic activity of metformin, treating both host diet and expression of LKB1 by neoplastic cells as variables in order to better understand the 'direct' vs 'indirect' effects of metformin.

Results

LKB1 knockdown leads to resistance to the direct in vitro *AMPK-dependent growth inhibitory effects of metformin*

Figure 1a shows that transfection of MC38 colon carcinoma or Lewis lung carcinoma (LLC1) cells with shRNA against LKB1 reduced the expression of LKB1. For LKB1 shRNA, two shRNAs were used and the results shown are from one selected colony from each shRNA ('F' or 'G'). The phosphorylation and activation of AMPK requires the presence of active LKB1 (Shaw *et al*., 2004) and previous reports (Zakikhani *et al*., 2006, 2008) have shown that, when LKB1 is functional, exposure of neoplastic cells to 5 mM metformin indirectly leads to the activation of AMPK, resulting in decreased cell proliferation. Following 48 h of metformin exposure, we observed (Figure 1b) that both MC38 and LLC1 cells transfected with control shRNA were growth inhibited by metformin, whereas those cells transfected with shRNA against LKB1 (+F or +G) were resistant to growth inhibition by the drug. Figure 1c shows that those cells transfected with shRNA against LKB1 (referred to as MC38-LKB1- and LLC1-LKB1-) are resistant to AMPK activation and downstream phosphorylation of acetyl CoA carboxylase compared to cells transfected with control shRNA. These observations, with the proliferation results (Figure 1b), indicate that LKB1 deficiency renders the

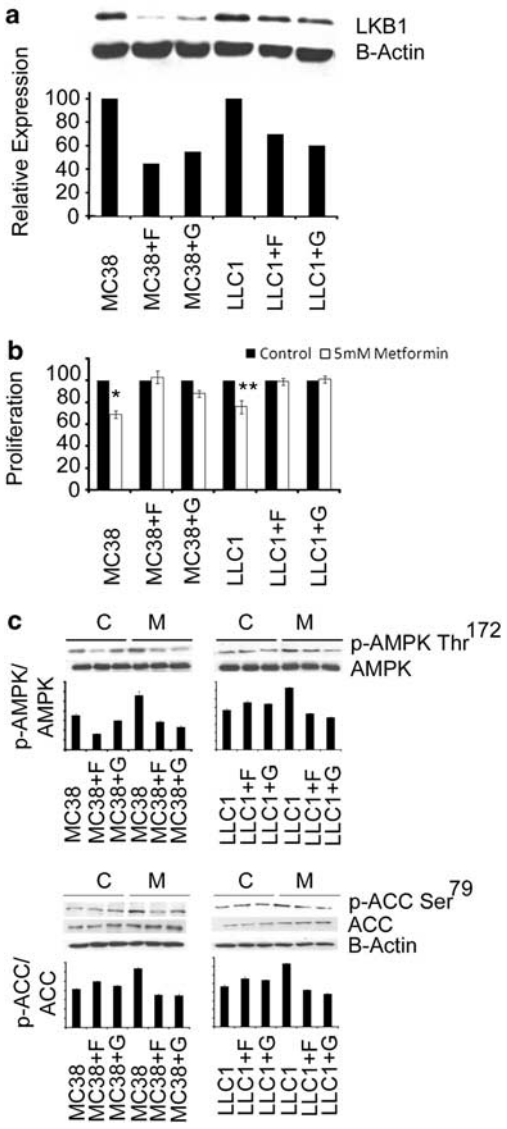

Figure 1 Effects of LKB1 knockdown and metformin on proliferation and p-AMPK Thr^{172} in LLC1 and MC38 cells. (**a**) Western blot demonstrating knockdown of LKB1 following transfection of MC38 and LLC1 cells with 'F' or 'G' shRNAs against LKB1. Lanes labeled MC38 and LLC1 show results for the control cells following transfection with empty vector. (**b**) Cells were treated with or without 5 mM metformin for 48 h and proliferation was assessed by MTT assay. MC38 and LLC1 were significantly growth inhibited by metformin (MC38: 30% growth inhibition; $*P<0.003$, LLC1: 25% growth inhibition; $**P<0.002$) whereas the proliferation of cells transfected with shRNA against LKB1 was not significantly altered by metformin. (**c**) p-AMPK Thr^{172} and p-ACC Ser^{79} were assessed by western blot after cells were treated with or without 5 mM metformin for 48 h. For both MC38 and LLC1, p-AMPK Thr^{172} increased following metformin exposure, but this was attenuated in cells transfected with shRNA against LKB1. C, control; M, metformin.

MC38 and LLC1 cells insensitive to the direct growth-inhibitory effects of metformin under standard tissue culture conditions.

Tumor LKB1 expression is not essential for the in vivo *anti-neoplastic activity of metformin*

A total of 60 mice were randomly assigned to either a control or a high-fat diet at 5–6 weeks of age. Following 12 weeks on the respective diets, the mice on the high-fat diet displayed significantly greater weight gain (Figure 2a) and hyperinsulinemia (Figure 2b) compared with mice on the control diet. After mice had received the assigned diets for 12 weeks, 2×10^5 MC38 colon carcinoma cells were injected sub-cutaneously on the left flank, 2×10^5 MC38-LKB1- cells were injected sub-cutaneously on the right flank, and tumor growth was observed for 17 days. Starting 24 h following tumor injection, mice were given daily i.p. injections of either 50 mg/kg metformin or vehicle control. Metformin significantly reduced the hyperinsulinemia observed in the mice on the high-fat diet (high-fat diet vs high-fat diet + metformin: 4.92 ± 1.30 vs 1.90 ± 0.15 ng/ml; $P<0.05$), while having no significant effect on insulin levels in the control mice (Figure 2c).

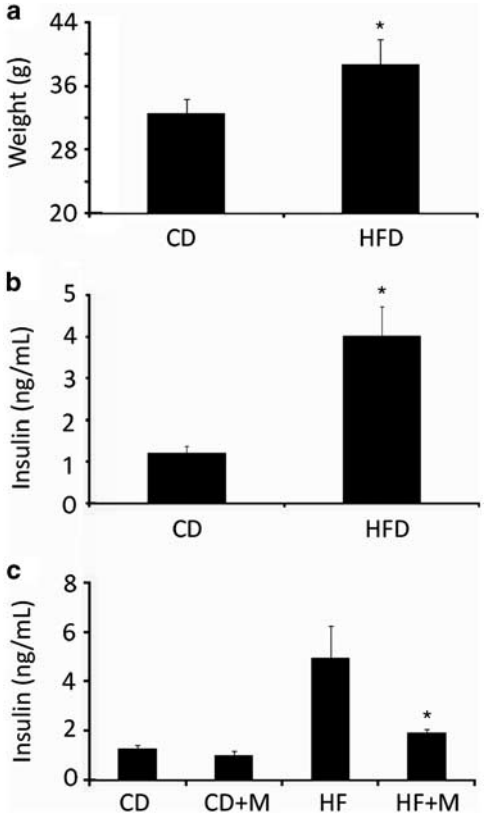

Figure 2 Metformin attenuates diet-induced hypersinulinemia, but has no effect in mice on a control diet. (**a**) Body weight of mice given the high-fat diet for 12 weeks was increased relative to that of mice on the control diet (*$P<0.0001$, $n=30$). (**b**) Blood insulin level of mice following 12 weeks on the high-fat diet was significantly higher than that of mice on the control diet (*$P<0.006$, $n=15$). (**c**) Following 12 weeks on the respective diets, mice received daily i.p. injections of 50 mg/kg metformin or vehicle for 16 days. Metformin had no effect on insulin levels of mice on the control diet, but significantly reduced the hypersinsulinemia observed in mice on the high-fat diet (*$P<0.05$, $n=15$). CD, control diet; HFD, high-fat diet; met, metformin.

As shown in Figures 3a and b, the high-fat diet accelerated tumor growth compared with the control diet, regardless of LKB1 expression (tumor size on day 17: high fat diet $= 1172 \pm 84\,mm^3$ vs control diet $605 \pm 76\,mm^3$, $P<0.015$). LKB1 expression did not affect the tumor volume of mice on either diet (tumor size on day 17: high-fat diet: MC38 $1201 \pm 128\,mm^3$ vs MC38-LKB1- $1143 \pm 127\,mm^3$; $P=0.15$; control diet: MC38 $638 \pm 81\,mm^3$ vs MC38-LKB1- $572 \pm 136\,mm^3$; $P=0.55$).

Metformin completely attenuated the stimulatory effect of the high-fat diet on MC38 tumor growth (tumor size on day 17: high-fat diet: $1201 \pm 128\,mm^3$ vs high-fat diet + metformin: $658 \pm 78\,mm^3$; $P<0.02$, $n=15$) while having no significant effect on the growth of MC38 tumors in mice on the control diet (Figure 3a). Importantly, metformin attenuated MC38-LKB1- tumor growth in mice on both the control diet and high-fat diets (Figure 3b) (high-fat diet: $1143 \pm 127\,mm^3$ vs high-fat diet + metformin: $544 \pm 99\,mm^3$; $P<0.001$, control diet: $572 \pm 136\,mm^3$ vs control diet + metformin: $326 \pm 57\,mm^3$; $P<0.003$), thus demonstrating that LKB1 expression by the tumor is not critical for metformin

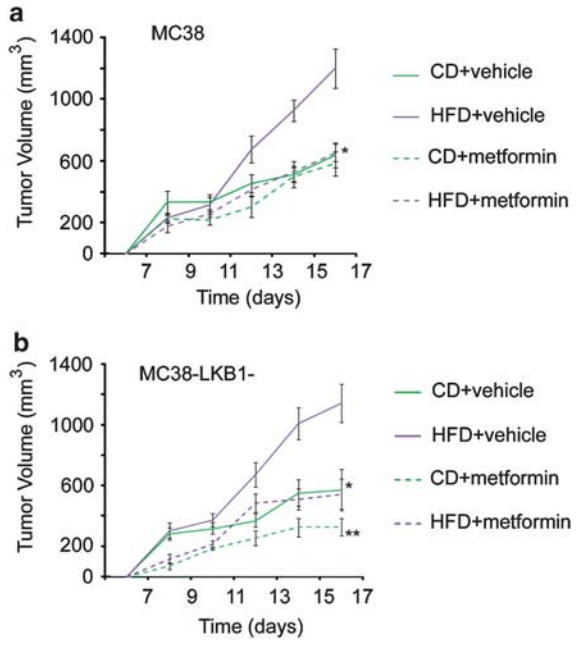

Figure 3 Effects of diet and LKB1 knockdown on the growth of MC38 colon cancer cells *in vivo*. (**a**) *In vivo* tumor growth of MC38 cells transfected with control shRNA. The high-fat diet led to significantly increased tumor growth relative to the control diet. For mice on the high-fat diet, metformin significantly reduced tumor growth (*$P<0.02$; $n=15$); however, metformin had no effect on growth of tumors of mice on the control diet. (**b**) *In vivo* tumor growth of MC38-LKB1- cells. We observed the same stimulatory effect of the high-fat diet on growth of MC38-LKB1- cells. Metformin abolished the stimulatory effect of the high-fat diet on tumor growth of MC38-LKB1- cells (*$P<0.001$, $n=15$). Unlike MC38, metformin significantly attenuated growth of MC38-LKB1- cells in mice on the control diet (**$P<0.003$).

action *in vivo*. The observation that metformin inhibited growth of MC38-LKB1- tumors in mice on the control diet was unexpected, as this action could be attributed to neither a reduction in insulin levels nor direct activation of the LKB1-AMPK pathway in neoplastic cells.

Intracellular signaling analyses in vivo

The administration of metformin leads to the phosphorylation of AMPK at Thr^{172}, an event that is dependent on the presence of active LKB1 (Shaw *et al*., 2004). We measured AMPK phosphorylation in the tumor tissue to determine whether the dose of metformin used was sufficient to activate AMPK in MC38 cells, and to confirm that LKB1 knockdown by shRNA was sufficient to prevent this action. We found elevated p-AMPK (Figure 4a), independent of diet, in the tumors that expressed LKB1, taken from mice that were administered metformin.

We detected increased insulin receptor tyrosine phosphorylation at Tyr^{972} in tumors taken from mice on the high-fat diet relative to those from mice on the control diet (Figure 4b), regardless of LKB1 expression, consistent with the insulin levels measured in the blood. This observation is consistent with previous reports (Novosyadlyy *et al*., 2010 reviewed in Pollak, 2008) that neoplasms can be growth-stimulated by insulin, and by our observation that MC38 cells are mitogenically responsive to physiological insulin concentrations *in vitro*. The increased insulin receptor activation associated with the high-fat diet was attenuated by metformin, consistent with the insulin-lowering effects of the drug. There was no significant effect of metformin on insulin levels or phosphorylation of the insulin receptors of tumors in mice on the control diet.

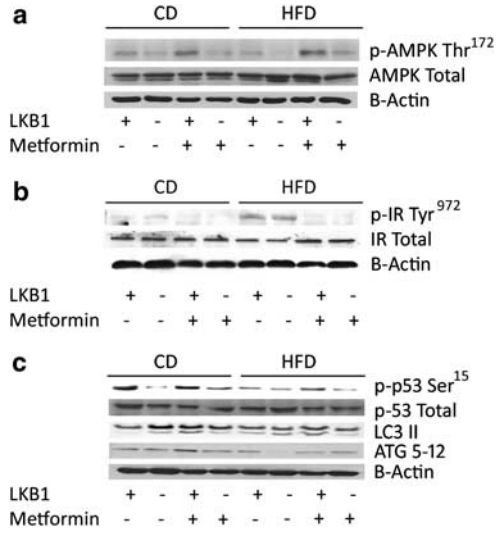

Figure 4 Effects of diet and LKB1 knockdown on AMPK activation, insulin receptor activation, and autophagy. (**a**) Phosphorylated AMPK at Thr^{172} in tumors arising from MC38 cells was detected following administration of daily 50 mg/kg i.p. metformin, regardless of diet, but was not observed in tumors arising from MC38-LKB1- cells. (**b**) Insulin receptor activation (Tyr^{972}) is increased in tumors of mice on the high-fat diet, independent of LKB1 expression. Metformin abolished the effect of the high-fat diet on tumor insulin receptor activation. (**c**) Metformin administration was associated with increased phosphorylation of p53, in an LKB1-dependent manner, and also with LKB1-dependent autophagy, as assessed by LC3 cleavage and Atg12-Atg5 binding. CD, control diet; HFD, high-fat diet.

A previous report (Buzzai *et al*., 2007) demonstrated that metformin-induced energy stress causes neoplastic cells to undergo autophagy in order to conserve energy, an action that is dependent on the presence of functional p53 and AMPK. To investigate the possibility that AMPK-dependent autophagy may reduce energy stress and alleviate the anti-neoplastic action of metformin *in vivo* for mice on the control diet, we examined phosphorylation of p53 at Ser^{15}, and the autophagy markers cleaved LC3 and Atg12 bound to Atg5. We observed higher levels of phosphorylated p53 in tumors from mice that were administered metformin compared with those treated with vehicle (Figure 4c). We also noted that metformin increased autophagy only in tumors where LKB1 was expressed. This suggests that LKB1/AMPK-dependent autophagy may protect tumors from the energy stress induced by metformin, and that LKB1 deficiency may sensitize cells to metformin-induced energy stress in mice on a control diet. Each tumor was also analyzed for expression of LKB1, and we observed that following 17 days of tumor growth *in vivo*, the MC38 cells continued to express the shRNA and LKB1 was stably knocked down for the duration of the *in vivo* experiment (data not shown).

LKB1- cells are sensitive to metformin under conditions of low glucose in vitro

The observation that metformin can inhibit *in vivo* growth of MC38-LKB1- tumors in mice on the control diet suggests that a mechanism of action other than reduction of host insulin levels or activation of the LKB1-AMPK pathway must exist. We carried out *in vitro* cell growth assays to examine possible interactions between metformin, glucose concentration and LKB1 expression in determining cell proliferation and cell death. As shown in Figure 1, MC38 and LLC1 cells transfected with shRNA against LKB1 are resistant to the growth-inhibitory effects of metformin when grown in regular DMEM medium (~25 mM glucose) with 5 mM metformin for 48 h. Figure 5a shows the effects of varying glucose concentration and exposure to metformin on cell number. Inhibition of MC38 and LLC1 cell proliferation by metformin was observed, and did not change significantly with change in glucose concentration; however, LKB1- cells were more sensitive than the control cells to metformin-induced growth inhibition at low glucose concentration (2.5 mM) and were resistant to metformin at high glucose concentration (15 mM) (Figures 5a and b). These results are compatible with previous *in vitro* evidence for an LKB1-dependent growth-inhibitory action of metformin at high glucose concentrations (Zakikhani *et al*., 2006, 2008) and with the perspective that the LKB1-AMPK signaling pathway, by inhibiting energy-consuming processes, protects cells during periods of sub-optimal energy supply (Shaw *et al*., 2004; Shackelford and Shaw, 2009).

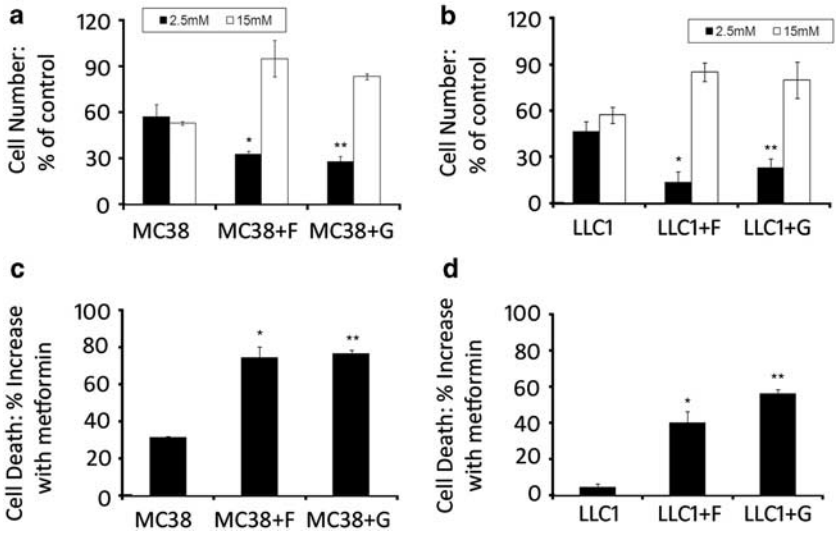

Figure 5 LKB1 knockdown leads to sensitivity to metformin *in vitro* under conditions of low glucose. Bars represent cell number in the presence of metformin as a percentage of cell number in the absence of metformin. (**a**) MC38 cells were growth inhibited by metformin at both glucose concentrations. Both MC38-LKB1- cell lines were more sensitive than MC38 cells to metformin at low glucose concentrations (MC38 vs MC38 + F: $*P<0.03$; MC38 vs MC38 + G: $**P<0.05$). The MC38-LKB1- cells were resistant to metformin at high glucose concentrations. (**b**) LLC1 cells were also growth inhibited by metformin at both glucose concentrations; however, LLC1 + F and LLC1 + G were more sensitive to metformin under conditions of low glucose (LLC1 vs LLC1 + F: $*P<0.02$; LLC1 vs LLC1 + G: $**P<0.02$) and were resistant to metformin in high glucose. (**c**, **d**) MC38 cells displayed a modest (30%) increase in cell death when treated with metformin, whereas MC38-LKB1- cells displayed nearly 75% increase in cell death (MC38 vs MC38 + F; $*P<0.002$; MC38 vs MC38 + G: $**P<0.001$). Metformin increased cell death by 5% in LLC1, and by 35% and 49% for LLC1 + F and LLC1 + G, respectively (LLC1 vs LLC1 + F: $*P<0.02$; LLC1 vs LLC1 + G: $**P<0.001$). At high glucose, metformin had no effect on cell death.

Following the observation that LKB1- cells are sensitive to metformin under low glucose conditions, we investigated the effects of glucose and metformin on cell death *in vitro*. Cells were treated with 5 mM metformin under conditions of high or low glucose, and analyzed by flow cytometry for Annexin IV and 7-amino actinomycin as markers of apoptosis and necrosis, respectively. We observed an effect of metformin on cell death primarily through necrosis, and this effect was dependent on LKB1 expression. Figure 5c shows that treatment with metformin led to a 30% increase in necrotic MC38 cells, whereas MC38-LKB1- showed a 75% increase in necrosis when treated with metformin for 72 h. Figure 5d shows the same trend in LLC1 cells. LLC1 cells treated with metformin displayed an $\sim$5% increase in necrosis, whereas LLC1-LKB1- cells displayed 45 and 54% increase in cell death for cells transfected with shRNA F and G, respectively. These results were observed at low glucose concentrations; we did not observe an effect of metformin on cell death when cells were cultured in high glucose, regardless of LKB1 expression. Additional flow cytometry data are available in Supplementary Figures 1a and 1b.

Metformin increases glucose consumption and lactate production in MC38 and LLC1 cells

It has been shown that cells treated with metformin increase glucose consumption, an action that is dependent on AMPK (Buzzai *et al.*, 2007). In order to determine whether LKB1 expression had an effect on increased glucose consumption, we treated cells with 5 mM metformin in either low glucose (2.5 mM) or high glucose (15 mM) concentrations for 48 h. As shown in Figure 6a, when MC38 cells were treated with metformin, we observed a significant increase (greater than twofold) in glucose consumption at both glucose concentrations. In contrast, in MC38-LKB1- cells, we observed a minimal increase in glucose consumption at both glucose concentrations. This was true for both MC38 + F and MC38 + G cell lines. We observed the same results in both the LLC1 cells and the LLC1-LKB1- cells (Figure 6b). In LLC1 cells, we observed a greater effect of glucose concentration on basal glucose uptake than was observed with MC38; however, the fold increase in glucose consumption with metformin treatment was the same for both cell lines. Data shown in Figures 6c and d reveal increased lactate production by both MC38 and LLC1 cells when exposed to metformin, suggesting increased glycolysis when taken together with the results of the glucose consumption assays shown in Figures 6a and b. This would be expected to compensate in part for the decreased ATP production due to the effect of metformin on oxidative phosphorylation; however, LKB1- cells show a small increase in lactate production, indicating less effective upregulation of glycolysis in an attempt to compensate for metformin-induced energy stress.

LKB1 expression influences cellular ATP levels following metformin exposure

Consistent with previous data demonstrating that biguanides reduce mitochondrial ATP production

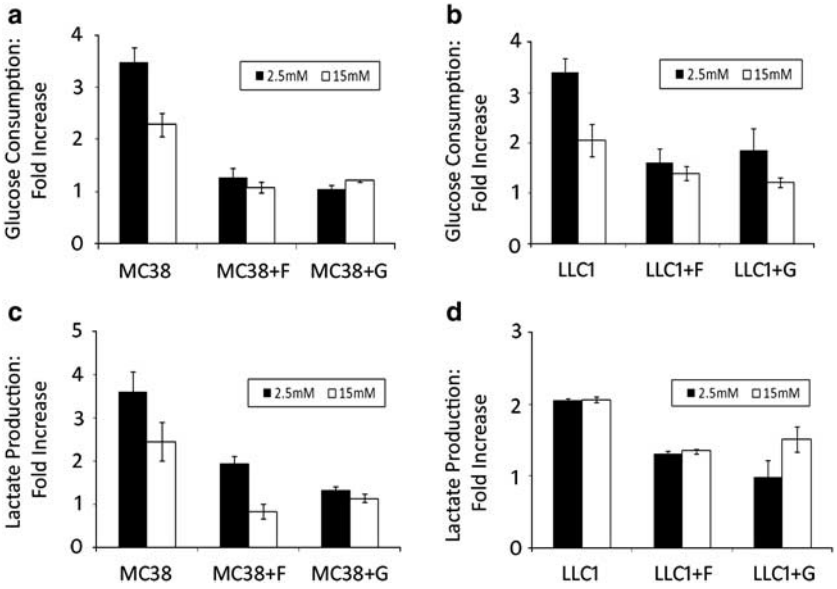

Figure 6 LKB1 expression is required for metformin induced increase in glucose consumption and lactate production. (**a**, **b**) MC38 and LLC1 cells were treated in 2% FBS DMEM with either 2.5 or 15 mM glucose, with or without 5 mM metformin for 48 h. Data are expressed as the fold change in glucose consumption when cells were treated with metformin compared with vehicle. For both MC38 and LLC1 cells, we observed a substantial metformin-induced increase in glucose consumption at both high and low glucose concentrations. We observed very little change in glucose consumption in MC38-LKB1- and LLC1-LKB1- cells. (**c**, **d**) MC38 and LLC1 cells were treated in 2% FBS DMEM with either 2.5 or 15 mM glucose, with or without 5 mM metformin for 48 h. We observed ~2.5-fold increased lactate production in both MC38 and LLC1 cells treated with metformin compared with vehicle at both glucose concentrations; however, we observed only a 1.3 fold increase in lactate production for MC38-LKB1- and LLC1-LKB1- cells.

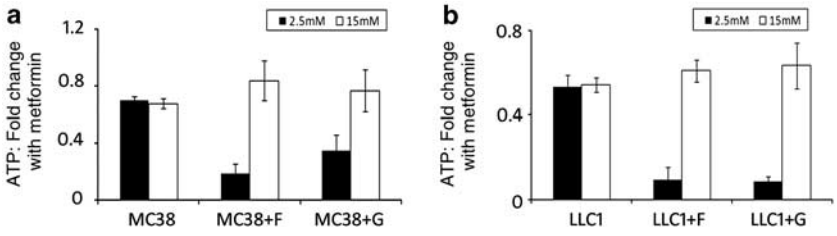

Figure 7 LKB1 expression influences cellular ATP levels following metformin exposure. MC38 (**a**) and LLC1 (**b**) cells were treated in 2.5 or 15 mM glucose for 48 h with or without 5 mM metformin. Data are expressed as fold change in cellular ATP concentration in the presence of metformin compared with vehicle. We observed that metformin decreased ATP levels in MC38 cells at both high and low glucose concentrations. In MC38-LKB1-, metformin depleted ATP to a greater effect (to 20 and 34% of control conditions in MC38 + F and MC38 + G, respectively), while having minimal effect on ATP depletion when these cells were treated at high glucose concentrations. LLC1 cells also showed greater ATP depletion (to 10% of control) in the absence as compared with the presence of LKB1 expression, similar to MC38.

(Dykens *et al*., 2008), we observed decreased cellular ATP concentration with metformin exposure; however, we observed an important influence of LKB1 expression on this effect. Following 48-h exposure to 5 mM metformin, both MC38 and LLC1 cells showed a significant decrease in ATP of 40–50% under both low and high glucose conditions (Figure 7). These results are consistent with the action of metformin as a mild inhibitor of complex I of the respiratory chain and with our observations that metformin treatment leads to AMPK phosphorylation in these cells (Figure 1c). The magnitude of the decrease in ATP observed was not dependent on glucose concentration in either cell line. In LKB1- cells, we observed a substantial decrease in ATP when cells were cultured at low glucose (~80% reduction in MC38-LKB1- and 90% in LLC1-LKB1-). Under conditions of 15 mM glucose, we did not observe a decrease of the same magnitude as in conditions of low glucose. In MC38-LKB1- cells (for both MC38 + F and MC38 + G) we observed only a 15% decrease in ATP, whereas in LLC1-LKB1- cells we observed a 40–50% decrease. These results suggest that for cells that do not express LKB1, the ATP-depleting effects of metformin are exaggerated when cells are cultured in low glucose and may lead to increased cell death by necrosis.

Discussion

Previous laboratory models have demonstrated the anti-proliferative activity of metformin, but have not comprehensively addressed the role of indirect actions of the drug on the host as distinct from direct effects on

transformed cells. This is an important issue, as the epidemiologic studies that suggest anti-neoplastic activity of the drug were confined to patients with type II diabetes, where metformin lowers insulin levels. It is unclear if the effects of the drug on cancer end points in diabetic individuals, which have an increased cancer burden relative to non-diabetics (Larsson *et al*., 2005, 2007), are relevant to the general population.

By using an experimental model that allowed us to treat diet and LKB1 expression by the neoplasm as variables, we characterized an interaction between host nutritional status and the anti-neoplastic activity of metformin. In mice with diet-induced hyperinsulinemia, insulin receptor activation in neoplastic tissue and growth of an insulin-responsive tumor were increased relative to that seen in mice on the control diet, in keeping with epidemiological evidence for an adverse effect of type II diabetes on neoplastic disease (Vigneri *et al*., 2009). Metformin abolished the excess tumor growth associated with the high-fat diet and hyperinsulinemia, regardless of LKB1 expression by the tumor. This anti-neoplastic activity was associated with a decline in circulating insulin levels and with reduced insulin receptor activation in neoplastic tissue, suggesting similarities to the mechanisms underlying tumor growth inhibition by dietary restriction (Kalaany and Sabatini, 2009; Pollak, 2009). Dietary restriction involves reduction of insulin and IGF-I levels from normal to sub-normal ranges, although metformin reduces insulin levels significantly only in the setting of baseline hyperinsulinemia. This is consistent with our observations that the indirect actions of metformin involving reduced activation of insulin receptors in neoplastic tissues were confined to animals with diet-induced hyperinsulinemia.

In mice without diet-induced hyerinsulinemia, the anti-neoplastic activity of metformin was not related to any change in insulin levels, but was instead confined to LKB1-deficient cancer. Although we and others have observed direct, LKB1-AMPK-dependent growth inhibition of cancer cells *in vitro* (Zakikhani *et al*., 2006, 2008), we did not observe such an effect *in vivo*, even though we documented that the metformin administration protocol we employed activated the LKB1-AMPK pathway in neoplastic cells when it was functional. Rather, LKB1-deficient tumors showed greater growth inhibition by metformin *in vivo*, together with *in vitro* observations of increased necrosis and reduced ATP levels in response to metformin exposure combined with low glucose concentrations.

Previous work (Russell III *et al*., 2004; Sakamoto *et al*., 2005) indicated that loss of function of LKB1 or AMPK in muscle can adversely affect the energy balance in that tissue. Our results suggest that there are conditions in the context of neoplasia where LKB1-dependent AMPK signaling acts in a manner consistent with its ancient evolutionary role to favor cell survival under conditions of energy stress, and that, under certain conditions, loss of function of the tumor suppressor LKB1 therefore leads to sensitivity to metformin. The fact that sensitivity is conferred by loss of a tumor suppressor predicts a favorable therapeutic index, and indeed metformin is well tolerated systemically at doses that inhibit growth in the LKB1-deficient tumor model. Somatic cell mutations of LKB1 are common in human lung, cervical and squamous cancers (Sanchez-Cespedes *et al*., 2002; Ji *et al*., 2007; Wingo *et al*., 2009) and a variety of LKB1 null neoplasms arise in patients with Peutz–Jeghers syndrome (van Lier *et al*., 2010). Although LKB1 functions upstream of 12 AMPK-related kinases (Shackelford and Shaw, 2009) and shRNA against LKB1 may also affect these related kinases, we attributed our results to impaired activation of AMPK and downstream targets, as our results are consistent with those observed in AMPK null mouse embryonic fibroblasts under similar experimental conditions as shown in Buzzai *et al*. (2007). In addition, we did not observe differences in tumor volume between MC38 and MC38-LKB1 tumors in either dietary group, thus implying that, in our model, knockdown LKB1 does not affect tumor volume.

Our results justify translational research regarding the activity of biguanides in these settings, as a situation where loss of a tumor suppressor confers sensitivity to a therapeutic strategy represents an attractive therapeutic opportunity.

There is enthusiasm for investigation of novel therapies that target tumor cell metabolism (Kroemer and Pouyssegur, 2008; Tennant *et al*., 2010), and the large number of ongoing and planned clinical trials of biguanides represent important examples of this research direction. Our results suggest that any clinical anti-neoplastic activity of this agent will vary according to the metabolic characteristics of patients and the molecular pathology of tumors. Therefore, rigorous evaluation of the clinical activity of biguanides in cancer patients will require the use of relevant predictive biomarkers, as well as the selection of a compound and dose capable of achieving sufficient drug levels in tumors as well as liver. Recently reported experimental data (Engelman and Cantley, 2010; Hosono *et al*., 2010; Memmott *et al*., 2010; Pollak, 2010) raise the possibility that the reduction in tumor burden associated with metformin use may be attributable to a prevention effect targeting at-risk epithelial cells rather than a treatment effect on transformed cells. The mechanisms we describe, including indirect growth inhibition related to reduction in insulin levels, direct AMPK dependent growth inhibition, and cell death associated with metformin-induced ATP deficiency in LKB1 deficient cells, are likely relevant to both treatment and prevention, and deserve investigation in the context of both Peutz-Jeghers syndrome and many common epithelial tumors.

Materials and methods

Animals

The 60 male C57BL/6 mice were purchased from Charles River (Saint-Constant, Québec, Canada) at 5–6 weeks of age and were put on either a high fat or a control diet *ad libitum* for 12 weeks. Diets were purchased from Harlan Teklad (Madison, WI, USA).

The high-fat diet consisted of 18.8% protein, 39.8% fat (lard) and 41.4% carbohydrate, and the diet provided 4.3 kcal per gram consumed. The control diet consisted of 16% protein, 3.5% fat and 60% carbohydrate, and provided 3.3 kcal per gram consumed. All protocols were approved by the McGill University Animal Care and Handling Committee.

Metformin
Following 12 weeks on the respective diets, 15 mice in each dietary group were subdivided into groups that were administered metformin (Sigma Aldrich, St-Louis, MO, USA). Mice were given daily i.p. injections of metformin at a dose of 50 mg/kg dissolved in 0.2 ml phosphate-buffered saline. Vehicle-treated mice were given daily i.p. injections of phosphate-buffered saline of equal volume.

Cells
In vivo and *in vitro* experiments were done with MC38 colon carcinoma, a mouse tumor cell line derived from a C57BL/6 mouse (generously donated by Dr Pnina Brodt) and LLC1 purchased from the ATTC (Manassas, VA, USA). Cells were transfected with either a control shRNA or shRNA against the expression of LKB1 using two shRNAs. All reagents and shRNA constructs were purchased from Open Biosystems (Huntsville, AL, USA). Cells were maintained in DMEM with 4 μg/ml puromycin to maintain the growth of transfected cells.

Insulin ELISA
Insulin ELISA was performed on blood taken from mice following 12 weeks on the respective diets and again at the time of sacrifice. Insulin ELISA kits were purchased from Millipore (Billerica, MA, USA).

Immunoblotting
Lysates were made from tissue taken at the time of sacrifice. Antibodies against LKB1, p-p53, p53 total, p-AMPK, AMPK total, ATG, LC3, p-acetyl CoA carboxylase, ACC Total and B-Actin were purchased from Cell Signaling Technologies (Danvers, MA, USA). Antibodies against p-insulin receptor and insulin receptor total were purchased from Millipore. Secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Bands were quantified with Scion Image (Frederick, MD, USA).

Cell death analysis
Cell death was measured using the Annexin V: PE Apoptosis Detection Kit I (BD Biosciences, San Jose, CA, USA). Briefly, cells were harvested at 36 (LLC1) or 72 h (MC38), collected, and resuspended in Annexin V buffer with Annexin V and 7-amino actinomycin for 20 min. Cells were resuspended in phosphate-buffered saline and analyzed by flow cytometry using a FACS Calibur (Beckton Dickenson, Franklin Lakes, NJ, USA) equipped with CellQuest software (BD Biosciences).

ATP measurement
Cellular ATP levels were measured using the Invitrogen ATP Determination Assay (Carlsbad, CA, USA) (Fantin *et al*., 2006). Cells were treated in 2% fetal bovine serum DMEM supplemented with either 2.5 or 15 mM glucose, with or without 5 mM metformin, for 48 h. The kit was used as per the manufacturer's instructions, with 2×10^5 cells per well.

Glucose consumption
Supernatants from treated cells were used for glucose consumption as described in Blake and McLean (1989). Results were indexed to cell-free media and to the number of cells.

Lactate production assay
Lactate production was measured with supernatants collected from treated cells and results were indexed to the number of cells. Lactate was quantified using BioVision Lactate Assay Kit purchased from BioVision (Mountain View, CA, USA).

Statistical analyses
For tumor data, before statistical analysis, data were square-root transformed to satisfy the assumptions of analysis. Statistical significance was evaluated using the GLM Procedure. A one-way analysis of variance was used to determine pairwise comparisons of means and least-squares means multiple unpairwise comparisons of means (LSMEANS statement with Bonferroni correction) were applied. All statistical analyses were performed using Statistical Analysis System software, version 9.1.3 (SAS Institute, Cary, NC, USA), with the P values <0.05 considered significant. P values are given for analysis of data over the 17-day tumor growth period.

For direct comparison between two groups (such as comparing dietary effect on insulin levels) the student's t-test was used in the Microsoft Excel Program (Seattle, WA, USA).

Conflict of interest

The authors declare no conflict of interest.

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