

Systemic Treatment with the Antidiabetic Drug Metformin Selectively Impairs p53-Deficient Tumor Cell Growth

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Abstract

The effect of the antidiabetic drug metformin on tumor growth was investigated using the paired isogenic colon cancer cell lines HCT116 p53^{+/+} and HCT116 p53^{-/-}. Treatment with metformin selectively suppressed the tumor growth of HCT116 p53^{-/-} xenografts. Following treatment with metformin, we detected increased apoptosis in p53^{-/-} tumor sections and an enhanced susceptibility of p53^{-/-} cells to undergo apoptosis *in vitro* when subject to nutrient deprivation. Metformin is proposed to function in diabetes treatment as an indirect activator of AMP-activated protein kinase (AMPK). Treatment with AICAR, another AMPK activator, also showed a selective ability to inhibit p53^{-/-} tumor growth *in vivo*. In the presence of either of the two drugs, HCT116 p53^{+/+} cells, but not HCT116 p53^{-/-} cells, activated autophagy. A similar p53-dependent induction of autophagy was observed when nontransformed mouse embryo fibroblasts were treated. Treatment with either metformin or AICAR also led to enhanced fatty acid β -oxidation in p53^{+/+} MEFs, but not in p53^{-/-} MEFs. However, the magnitude of induction was significantly lower in metformin-treated cells, as metformin treatment also suppressed mitochondrial electron transport. Metformin-treated cells compensated for this suppression of oxidative phosphorylation by increasing their rate of glycolysis in a p53-dependent manner. Together, these data suggest that metformin treatment forces a metabolic conversion that p53^{-/-} cells are unable to execute. Thus, metformin is selectively toxic to p53-deficient cells and provides a potential mechanism for the reduced incidence of tumors observed in patients being treated with metformin. [Cancer Res 2007;67(14):6745–52]

Introduction

The biguanide metformin is the most widely used drug for treatment of type 2 diabetes (1, 2). The primary systemic effect of metformin is the lowering of blood glucose levels. The effects of metformin on blood glucose has been explained through reduced hepatic gluconeogenesis and increased glucose uptake in skeletal muscles (3, 4). Metformin treatment leads to activation of AMP-

activated protein kinase (AMPK) *in vitro* and *in vivo* (5–7). The molecular mechanisms by which metformin leads to AMPK activation are poorly understood. Nevertheless, through activation of AMPK, metformin treatment decreases the expression of gluconeogenic genes and increases fatty acid oxidation in hepatocytes (7–9). In addition, studies in mice lacking expression of LKB1 in the liver show that the glucose-lowering effect of metformin is dependent on the LKB1/AMPK pathway (10).

Beyond the reported effects of metformin on insulin-responsive tissues, little is known about the effects of metformin on other cell types. However, AMPK is a intracellular fuel sensor that regulates cellular homeostasis in response to energy stress in all eukaryotic cells (11). Physiologically, AMPK is activated upon increase in the AMP/ATP ratio within the cell and induces a cellular response characterized by an up-regulation of catabolic pathways that generate ATP and by a down-regulation of ATP-consuming processes. The ability of cells to maintain a proper supply of ATP and intracellular metabolites is critical for survival. It has been suggested that AMPK activation promotes the survival of bioenergetically stressed stromal cells in part through p53 activation (12). Recent reports have linked p53 to metabolic regulation. Matoba et al. identified p53 as a regulator of SCO2 (synthesis of cytochrome *c* oxidase 2). Loss of p53 decreases SCO2 activity and reduces the coupling efficiency of mitochondrial oxidative phosphorylation (13–15). In addition, TIGAR (TP53-induced glycolysis and apoptosis regulator) has been identified as a target of p53. By lowering the level of fructose-2,6-bisphosphate, TIGAR redirects glucose into the pentose phosphate pathway, thus enhancing NADPH production and increasing the capacity of cells to handle redox stress (13). p53 induction leads to activation of the autophagy pathway through DRAM (damage-regulated autophagy modulator), a lysosomal protein required for fusion of autophagosomes (16). In turn, autophagy has been shown to be required for cells to survive growth factors withdrawal and/or nutrient deprivation (17). The ability of p53 to induce autophagy has been reported to depend on AMPK (18).

Recently, two reports link the treatment of metformin with a lower risk of cancer in patients. Evans et al. (19) did a pilot observational study reporting that among patients with type 2 diabetes, those treated with metformin have a lower incidence of cancer compared with those untreated. In an independent study, using administrative databases of a population-based cohort, Bowker et al. (20) reported that type 2 diabetes patients who use metformin have a lower cancer-related mortality compared with those that use sulfonylureas or insulin. Despite the wide use of metformin in patients and the link between cellular metabolism and cancer, there is no clear understanding of the role of metformin on tumor growth. This observation, together with the increasing evidence

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

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doi:10.1158/0008-5472.CAN-06-4447

of the role of p53 in regulation of cellular metabolism, prompt us to investigate the effects of metformin in correlation with the tumor-suppressor p53 on both cellular metabolism and tumor growth.

Here, we report that treatment with either AMPK activators metformin or AICAR selectively inhibits tumor growth of p53-deficient colon cancer cells *in vivo*. We show that cells treated with metformin or AICAR activate a p53-dependent metabolic response that contributes to continued *in vivo* and *in vitro* cell survival. These findings highlight p53 as a required component of an adaptive metabolic response that promotes cell survival after systemic treatment with metformin or AICAR.

Materials and Methods

Cell lines and cell culture. The colon cancer HCT116 p53^{+/+} and p53^{-/-} cell lines have been described previously. HCT116 cell lines were maintained in McCoy's 5A supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 µg/mL streptomycin (Invitrogen), and 2 mmol/L L-glutamine. MEF AMPKα^{+/+} and AMPKα^{-/-} cells were described previously (21). MEF AMPKα^{+/+} and AMPKα^{-/-} cells were grown in DMEM supplemented with 10% FBS, 100 units/mL penicillin, 100 µg/mL streptomycin, and 2 mmol/L L-glutamine. Cell concentration was determined with a Coulter Z2 particle analyzer. Cell viability was determined by the exclusion of 2 µg/mL propidium iodide (Invitrogen) by a LSR flow cytometer (BD Biosciences).

Reagents. AICAR was obtained from Toronto Research Chemicals, Inc. Metformin, fatty acid-free bovine serum albumin (BSA), [9,10-³H]palmitate, etomoxir, and Dowex 1 × 8-200 ion exchange resin were obtained from Sigma. Rapamycin was purchased from Calbiochem.

Mice xenografts. Early-passage HCT116 cells were harvested, washed twice in PBS, and resuspended in 200 µL PBS before being inoculated s.c. at both flanks (p53^{+/+} cells on the left flank and p53^{-/-} cells on the right flanks) of nude mice (6- to 8-week-old females; Charles River). Metformin (250 mg × kg body weight) or AICAR (500 mg × kg body weight) was dissolved in 200 µL PBS and administered daily with i.p. injections. The control group received vehicle only (200 µL PBS). Tumor volume (mm³) was measured twice a week and estimated from caliper measurements using the formula $\pi/6 \times A \times B^2$ (*A* is larger diameter, *B* is smaller diameter). For the experiment in Figs. 1 and 2, samples are as follows: for metformin-treated mice, *n* = 6; for AICAR-treated mice, *n* = 5; and for PBS treated mice, *n* = 4. The experiment was repeated four times with a total of *n* = 29 for metformin-treated mice, *n* = 24 for AICAR-treated mice, and *n* = 28 for PBS-treated mice. Statistical analysis was done using paired two-tailed Student's *t* test. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Infections. The pEGFP-LC3 construct (kindly provided by Tamotsu Toshimori, National Institute of Genetics, Mashima-Shizuoka, Japan) was previously described. The p53^{+/+}GFP-LC3 and p53^{-/-}GFP-LC3 mouse embryonic fibroblast cell lines were created by retroviral transduction of parental cell lines with MIGRI-GFP-LC3 and selected for green fluorescent protein (GFP)-positive clones by MoFlow.

Western blot analysis. Cells were lysed in radioimmunoprecipitation assay buffer containing protease inhibitors (Complete; Roche Applied Science) and phosphatase inhibitor cocktails I and II (Sigma). Protein concentrations were determined using the BCA Protein Assay kit (Pierce Biotechnology). Equal amounts of total protein (15 µg) were resolved on 4% to 12% NuPage Bis-Tris polyacrylamide gels (Invitrogen). Nitrocellulose membranes were blocked in PBS containing 5% nonfat dry milk and 0.1% Tween 20 with the following antibodies: anti-phospho-Ser⁷⁹ Acetyl CoA Carboxylase (Upstate), anti-phospho-Ser¹⁵ p53 (Cell Signaling Technology), or anti-actin (Santa Cruz Biotechnology). Anti-LC3 antibody was raised in rabbits against the peptide MPSEKTFKRRTEFQRVC (Quality Control Biochemicals). Bands were detected using horseradish peroxidase-labeled secondary antibodies and enhanced chemiluminescence detection kit (Amersham).

Fatty acid oxidation assay. To measure fatty acid oxidation, we modified an assay in which oxidation of [9,10-³H]palmitate results in formation of ³H₂O. Briefly, cells were seeded in six-well plates at 3 × 10⁵ per well in triplicate and incubated overnight in 1 mL medium containing 3 µCi/mL [9,10-³H]palmitate (70 nmol/L), bound to 10 µmol/L BSA, in the presence or absence of 2 mmol/L metformin or 1 mmol/L AICAR. Tritiated water was recovered by ion exchange treatment on Dowex 1 × 8-200 columns. Supernatants were applied to 3 mL Dowex 1 × 8-200 columns and eluted with 2 mL water. ³H₂O was quantified by liquid scintillation counting. Fatty acid oxidation rates are shown as CPT-1-dependent oxidation. Counts due to nonspecific oxidation, as shown by inability to be inhibited by 60 µmol/L etomoxir, were subtracted from total counts. Data are normalized to cell number (22).

Rate of oxygen consumption in intact cells. Measurements of oxygen consumption of whole cells were done as described previously (23). Briefly, HCT116 p53^{+/+} and p53^{-/-} cells were incubated in medium with or without 2 mmol/L metformin for 20 h. Following incubation, 4 × 10⁶ cells were resuspended in 200 µL medium with or without metformin and analyzed by using a water-jacketed, airtight 3-mL chamber with a polarographic oxygen electrode. Measurements were made at 37°C with constant stirring.

Immunofluorescence. Cells were cultured on glass coverslips in six-well plates, washed once with PBS, and fixed in 4% paraformaldehyde for 30 min. Coverslips were mounted using Immu-Mount (Thermo Shandon) and images were captured using a Zeiss 510 confocal microscope. For quantification of autophagic cells, cells with >10 GFP-LC3 punctuate dots were considered positive. Data obtained from counting triplicates of 100 cells was average (mean ± SD).

Electron microscopy. Tumors were resected from mice on day 14 after initiation of treatment. Tumors were fixed with 2.5% glutaraldehyde/2% formaldehyde with 0.1 mol/L sodium cacodylate and stored at 4°C until embedding. Tumor samples were postfixed with 2% osmium tetroxide followed by an increasing gradient dehydration step using ethanol and propylene oxide. Tumors were then embedded in LX-112 medium (Ladd) and sections were cut ultrathin (90 nm), placed on uncoated copper grids, and stained with 0.2% lead citrate and 1% uranyl acetate. Images were examined with JEOL-1010 electron microscope (JEOL) at 80 kV. For quantification of autophagic cells, positive cells were counted from a minimum of 30 independent cells.

Glucose and lactate assays. p53^{+/+} and p53^{-/-} cell lines were cultured in complete medium 10% FCS in the absence or presence of different concentration of metformin. After 48 h, media from samples were collected and analyzed for glucose and lactate concentration using colorimetric kits according to manufacturer's instructions (CMA/Microdialysis).

Terminal deoxyribonucleotide transferase-mediated nick-end labeling assay. Tumors were excised at day 6 of treatments, formalin fixed, and embedded in paraffin. Sections were cut at 5 µm and deparaffinized and dehydrated before treatment. The assay was done using the terminal deoxyribonucleotide transferase (TdT)-mediated nick-end labeling (TUNEL) method (Biotin-16-dUTP, Boehringer-Mannheim; TdT, Amersham).

Results

Metformin and AICAR treatment inhibits growth of HCT116 p53^{-/-} xenografts in nude mice. To investigate whether the antidiabetic drug metformin affects tumor growth, we used the paired isogenic human colon carcinoma cell lines, HCT116 p53^{+/+} and p53^{-/-}. Nude mice were injected in the lateral side of the left flank with HCT116 p53^{+/+} and of the right flank with p53^{-/-} cells. Four days after injection, animals were treated with daily i.p. injections of either metformin (250 mg × kg body weight) or an equal volume of saline solution. Tumor progression was followed by measurements of tumor volume over time. As previously reported, loss of wild-type p53 in a p53^{-/-} derivative of HCT116 accelerates tumorigenesis in untreated animals (Fig. 1A). However, in mice treated with metformin, we measured a significant reduction of growth of tumors from HCT116 p53^{-/-} cells when

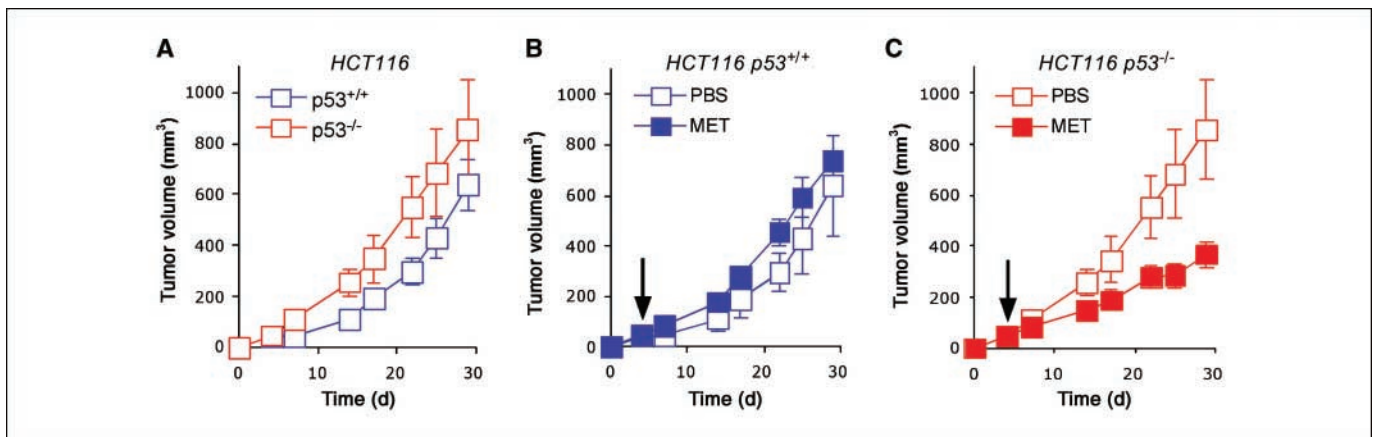
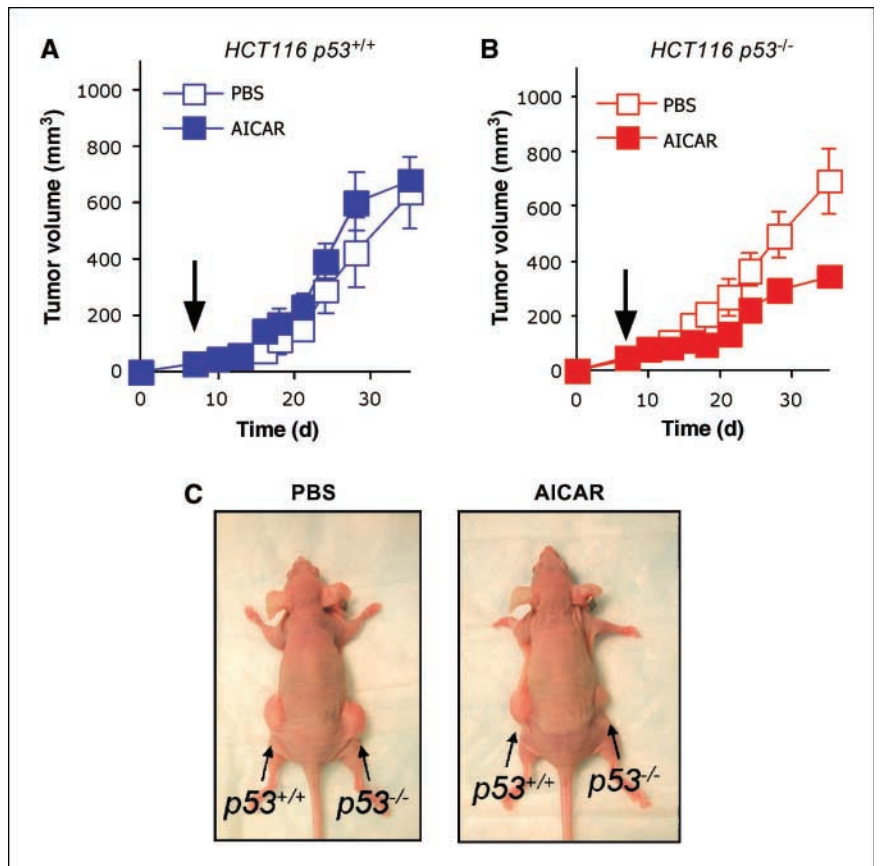


Figure 1. Systemic treatment with the antidiabetic drug metformin selectively inhibits growth of human colon HCT116 p53^{-/-} tumor xenografts in nude mice. Each nude mouse was s.c. injected with 3×10^6 HCT116 p53^{+/+} cells (on the left flank) and with 3×10^6 HCT116 p53^{-/-} cells (on the right flank). Mice bearing tumors were treated daily with i.p. injections of metformin (250 mg \times kg body weight; *MET*). The control group received equal volume (200 μ L) of vehicle only (*PBS*). Treatment started 4 d after implantation (arrow). Tumor volume was measured, at indicated time points, from xenografts derived from (A) p53^{-/-} or p53^{+/+} cells of vehicle-treated mice, (B) p53^{+/+} cells, and (C) p53^{-/-} cells of mice treated with metformin or PBS. Tumor volumes were measured as indicated in Materials and Methods. Points, mean; bars, SE. Data are representative of three independent experiments.

compared with the growth of the tumors from p53^{+/+} cells in the opposite flank. After 1 month of treatment with metformin, the average tumor volume of p53^{-/-} xenografts was >50% smaller than p53^{-/-} xenografts from mice treated with vehicle control (Fig. 1C; $P < 0.01$). Although a systemic effect of metformin on cell growth was observed in p53^{-/-} xenografts, no significant difference in tumor volumes were observed between metformin and control-treated mice bearing p53^{+/+} tumors (Fig. 1B).

Because the antidiabetic effects of metformin are AMPK dependent (7), we next tested whether AICAR, a widely used AMPK activator, could recapitulate these results. Similar results were obtained when mice were injected daily with 500 mg \times kg of AICAR. Mice bearing HCT116 tumor xenografts were treated with AICAR or PBS and tumor volume was measured over a period of 35 days. Following AICAR treatment, average tumor volume was reduced by 50% compared with vehicle-treated tumors in p53^{-/-}

Figure 2. Treatment with the AMPK activator AICAR selectively inhibits growth of HCT116 p53^{-/-} tumor cells *in vivo*. Mice were ectopically implanted with 4×10^6 HCT116 p53^{+/+} cells on the left flank and with 4×10^6 HCT116 p53^{-/-} on the right flank. Seven days after injection, mice were treated with daily i.p. injections of 500 mg \times kg dose of AICAR or equal volume (200 μ L) of vehicle (*PBS*). Arrow, 1st day of treatment. Tumor volume (mm³) was measured at the indicated time points and the mean tumor volumes were calculated for xenografts derived from p53^{+/+} cells (A) or p53^{-/-} cells (B) of mice treated with AICAR or saline solution (*PBS*). C, digital photographs of mice treated with AICAR (right) or PBS (left) on the last day of the experiment. Data are representative of three independent cohorts of mice.



xenografts (Fig. 2B). AICAR treatment resulted in no inhibition of tumor growth in p53^{+/+} xenografts compared with vehicle treatment (Fig. 2A). Although daily AICAR treatment had significantly reduced tumor volume selectively in p53-deficient tumors, no observable systemic toxicity was noted in p53^{+/+} tumors of mice treated for 30 to 35 days (Fig. 2C).

Metformin induces cell death in p53-deficient HCT116 cells under nutrient limitation. Tissue sections taken from the xenografts at day 6 of treatment were next subjected to histologic analysis. Apoptotic cells were observed in clusters in the p53^{-/-} tumors treated with metformin, but were not observed in either the PBS-treated p53^{-/-} tumors or in the p53^{+/+} tumors from either PBS- or metformin-treated animals. TUNEL assay of the tumor sections of xenografted regions of HCT116 p53^{-/-} cells showed that these regions were composed of apoptotic cells and infiltrating macrophages containing apoptotic nuclei. Only scattered TUNEL-positive cells were seen in the PBS-treated tumors (Fig. 3A).

The fact that the cell death observed in the tumors was regional suggested that this cell death might result from areas, which were underperfused or at the border between vascularized zones. Tumor cells in such regions might be either hypoxic and/or nutrient deficient. To determine if the cell death correlated with such a bioenergetic compromise, the ability of HCT116 p53^{+/+} and p53^{-/-} cells to survive under nutrient deprivation was examined *in vitro*. HCT116 cells were incubated in glucose-deficient medium or in medium containing full glucose (25 mmol/L) and cell viability was measured after 24 h of metformin treatment. The viability of p53^{-/-} cells was significantly decreased compared with that of p53^{+/+} cells when treated with metformin in the absence of glucose (Fig. 3B). There was no significant difference in the viability of p53^{+/+} and p53^{-/-} cells treated with metformin when grown in the presence of glucose.

Previously, we reported that cancer cells can maintain their viability by switching from glucose catabolism to alternative energy sources such as oxidation of fatty acids in response to glucose limitation (24). We then tested whether during glucose deprivation and metformin treatment, p53^{+/+} cells were relying on fatty acid

oxidation as their main fuel source for survival. We measured viability of HCT116 p53^{+/+} and p53^{-/-} cells treated with metformin during glucose deprivation when fatty acid oxidation was inhibited by etomoxir, an inhibitor of CPT-1 (25). The addition of etomoxir only slightly decreased the cell survival of both p53^{+/+} and p53^{-/-} in cells treated with metformin in the absence of glucose, suggesting that the catabolism of additional substrates such as amino acids contributes to promoting cell survival in the absence of glucose. There was no toxicity induced by etomoxir treatment in cells grown in the presence of glucose (Fig. 3B).

Analysis of autophagic markers in p53^{+/+} and p53^{-/-} cells after treatment with metformin or AICAR. In the absence of growth factors or nutrient availability, autophagy is an essential pathway for cell survival (26–28). To determine the level of autophagy in the HCT116 xenografts, ultrathin sections of the tumors resected from nude mice after 14 days of metformin treatment were analyzed by electron microscopy. Photomicrographs show the presence of increased number of autophagosomes in p53^{+/+} xenografts from metformin-treated animals compared with p53^{+/+} tumors of control-treated mice (Fig. 4A). A significant increase in the percentage of autophagosome-positive cells was observed in p53^{+/+} tumors from mice treated with metformin (Fig. 4B). In contrast, the percentage of autophagosome-positive cells in p53-deficient xenografts from metformin-treated mice did not differ from the percentage of autophagosome-positive cells observed in control mice.

During autophagy, microtubule-associated protein light chain 3 (LC3) is specifically processed from a cytosolic full-length form (LC3-I) to a cleaved membrane-bound form (LC3-II; ref. 29). As an independent method to detect activation of autophagy, we analyzed the appearance of LC3-II form in HCT116 p53^{+/+} and p53^{-/-} cells by Western blot analysis. The conversion from the LC3-I to the LC3-II form was markedly increased in p53^{+/+} cells when compared with p53^{-/-} cells after treatment either with metformin or AICAR (Fig. 4C), recapitulating the results observed *in vivo*. It was recently reported that p53 is an AMPK target and that activation of p53 induces autophagy in an AMPK-dependent

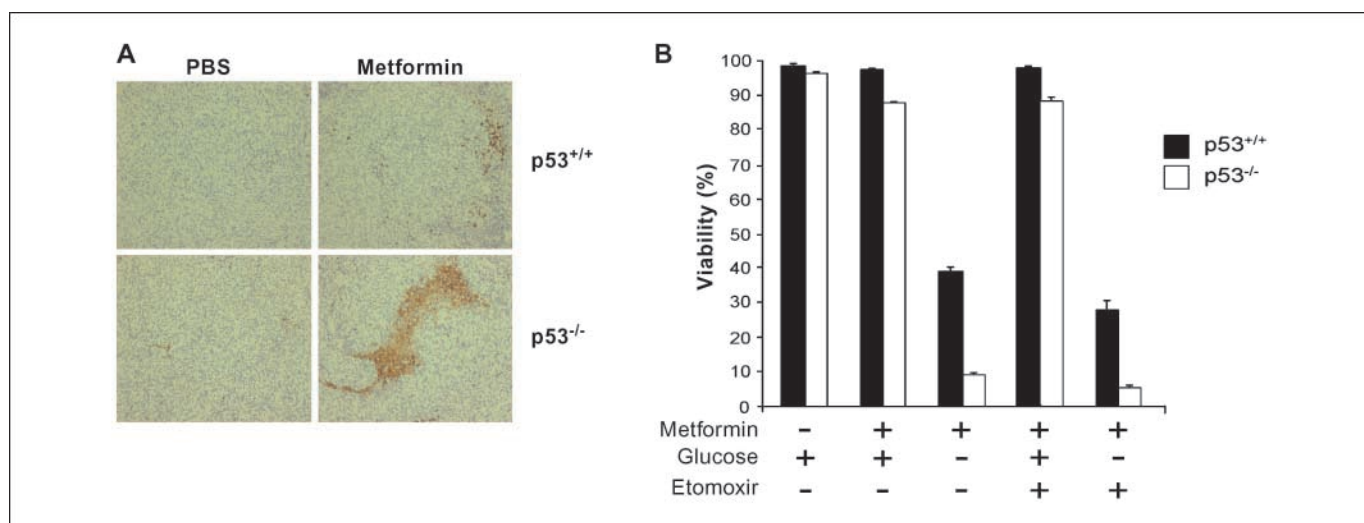
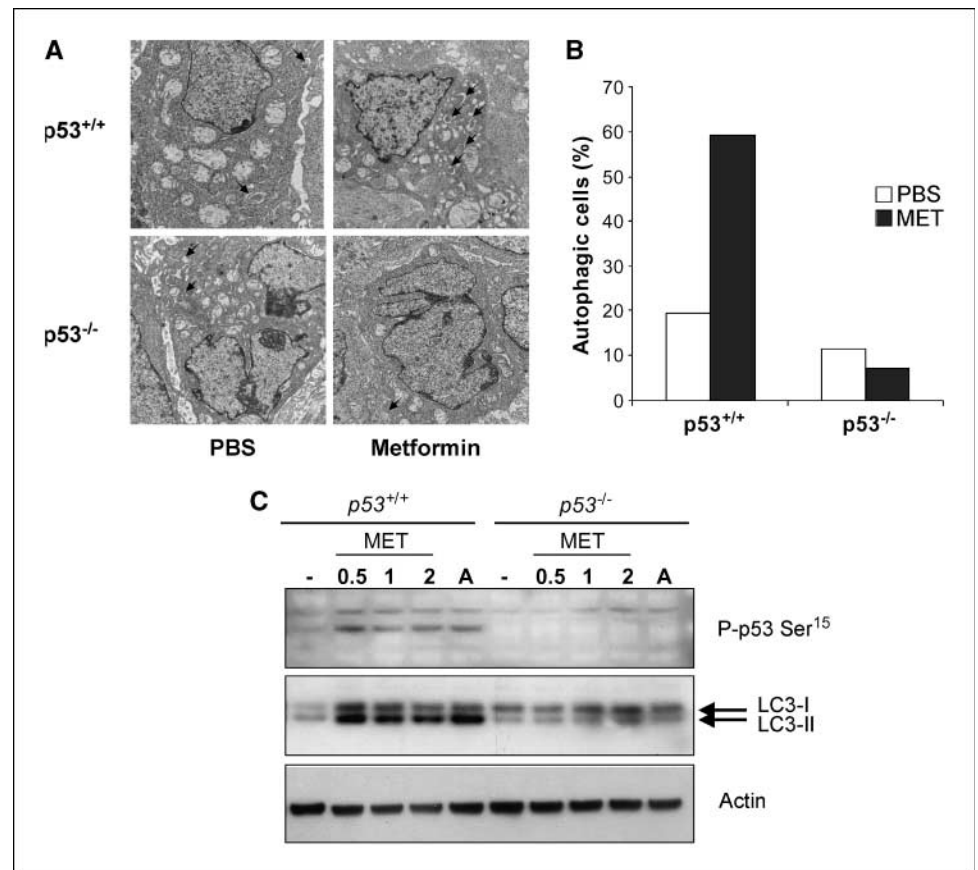


Figure 3. Metformin treatment induces apoptosis in cells lacking p53 under nutrient deprivation. A, TUNEL assay of cross-sectioned tumors from xenografted HCT116 p53^{+/+} and p53^{-/-} cells of mice treated with metformin (right) or PBS (left) for 6 d. B, percent viability was measured by propidium iodide exclusion for HCT116 p53^{+/+} and p53^{-/-} cells. Cells were grown in complete medium containing full glucose (25 mmol/L) or no glucose, with or without 2 mmol/L metformin or 100 μ mol/L etomoxir. Cell viability was measured 24 h after glucose withdrawal and drug treatments. In all conditions, cells were cultured in the presence of 10% serum. Columns, mean of duplicate samples; bars, SD. Representative of three independent experiments.

Figure 4. HCT116 p53^{+/+} cells induce autophagy *in vivo* and *in vitro* after treatment with metformin or AICAR. **A**, images of HCT116 p53^{+/+} or p53^{-/-} cells obtained by electron microscopy from ultrathin sections prepared from tumor xenografts of mice treated for 14 d with metformin or PBS. *Arrows*, typical double-membrane autophagic vesicles. Results are representative of two independent experiments. **B**, quantification analysis of double-membrane, autophagosome-positive cells from photomicrographs of experiments in (A), expressed on a percentage basis. **C**, whole-cell lysates were prepared from HCT116 p53^{+/+} and p53^{-/-} cells after 1 h treatment with increasing amount of metformin or 1 mmol/L AICAR (A). Western blot analysis was done to determine the level of phosphorylated p53 at Ser¹⁵ and the level of LC3-I/LC3-II with an antibody that recognizes both forms. Samples were probed for actin as a loading control. Results are representative of three independent experiments.



manner (18, 30). Therefore, we examined whether increased LC3-II accumulation was associated with p53 activation. Coincident with the appearance of the LC3-II form, metformin or AICAR treatment induced p53 phosphorylation at Ser¹⁵ in p53^{+/+} cells (Fig. 4C).

Similar results were obtained in untransformed mouse embryo fibroblasts (MEF). During autophagy, LC3 redistributes from the cytoplasm to autophagic vesicles (27). The intracellular localization of LC3 in p53^{+/+} and p53^{-/-} MEFs transduced with a GFP-LC3 construct was examined by fluorescence microscopy. Following treatment with metformin or AICAR, LC3 redistributed from a diffuse cytoplasmic staining to punctate structures only in p53^{+/+} cells, whereas in p53^{-/-} cells GFP-LC3 fluorescence remained diffusely cytoplasmic (Fig. 5A and B). The accumulation of punctate GFP-LC3 fluorescence in p53^{+/+} cells after metformin or AICAR treatment was comparable with LC3 relocalization observed after rapamycin treatment, a known inducer of autophagy (Fig. 5C). Although GFP-LC3 fluorescence remained diffusely cytoplasmic in p53^{-/-} cells after treatments with metformin or AICAR, punctate GFP-LC3 relocalization was observed after rapamycin treatment (Fig. 5B and C).

To investigate whether AMPK is necessary for metformin-induced autophagy, MEF AMPK^{+/+} and AMPK^{-/-} cells were transiently transfected with the GFP-LC3 construct and analyzed for autophagic structures by fluorescence microscopy. Metformin treatment induced relocalization of LC3 in AMPK^{+/+} cells, whereas there was no significant change in the AMPK^{-/-} cells (Fig. 5D).

Metformin and AICAR activate fatty acid β -oxidation in a p53-dependent manner. The induction of fatty acid β -oxidation has been reported as a marker of metabolic reprogramming in

response to glucose deprivation (24). We therefore tested whether either MEF p53^{+/+} or p53^{-/-} cells activate β -oxidation after treatment with metformin or AICAR. AICAR markedly increased the rate of fatty acid β -oxidation in p53^{+/+} cells, but not in p53^{-/-} cells. Compared with AICAR, metformin modestly stimulated β -oxidation, but the effects of metformin on β -oxidation were still p53 dependent (Fig. 6A). Because treatment with metformin has been reported to inhibit respiratory chain complex I in hepatocytes (31, 32), the rate of oxygen consumption in intact cells after treatment with metformin was measured. As shown in Fig. 6B, metformin blocked mitochondrial respiratory activity by >70% in all cell lines. Similar effects of AICAR or metformin on fatty acid oxidation and oxygen consumption were also observed in HCT116 p53^{+/+} and p53^{-/-} cells (Supplementary Fig. S1 and S2A).

Metformin stimulates glycolysis in a p53-dependent manner. If mitochondrial respiration is impaired, cells can compensate through an enhanced rate of glycolysis to improve bioenergetics. Glucose consumption and lactate production were measured in the MEF p53^{+/+} and p53^{-/-} cell lines after treatment with metformin. Metformin induced glucose consumption as well as lactate production in a dose-response manner in p53^{+/+} cells (Fig. 6C). No significant stimulation of glucose consumption or lactate production was detected in cells lacking p53. We observed the same p53 dependency on the effect of metformin on glucose consumption and lactate production in HCT116 cells (Supplementary Fig. S2B and S2C). To address whether the effects of metformin on glucose metabolism were dependent on AMPK, MEF AMPK^{+/+} and AMPK^{-/-} cells were analyzed. As shown in Fig. 6D, the rate of glucose consumption and lactate production were highly induced

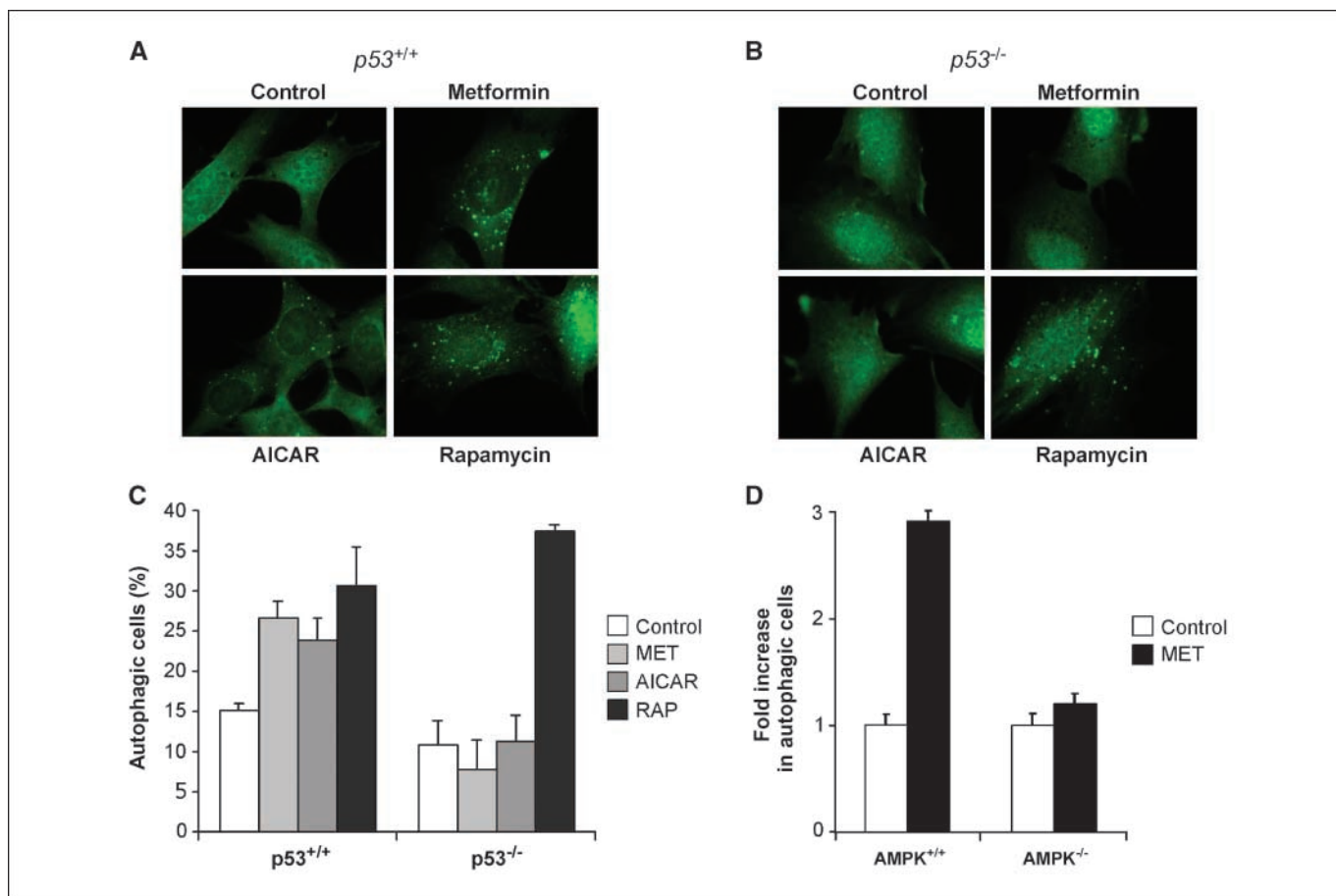


Figure 5. Activation of autophagy by metformin or AICAR is p53 dependent. *A*, MEF p53^{+/+} and *B*) p53^{-/-} cells stably expressing GFP-LC3 were incubated in the absence (*Control*) or presence of 2 mmol/L metformin, 1 mmol/L AICAR, or 25 nmol/L rapamycin (*RAP*) and analyzed by fluorescence microscopy. *C*, quantification of autophagic cells from the experiment shown in (*A*) and (*B*). *D*, metformin-induced activation of autophagy is AMPK dependent. MEF AMPK $\alpha^{+/+}$ and AMPK $\alpha^{-/-}$ cells were transiently transfected with GFP-LC3 and treated with (*MET*) or without (*Control*) 2 mmol/L metformin. Cell viability was measured 24 h after metformin treatment by propidium iodide exclusion. Data are representative of three independent experiments.

by metformin in the AMPK^{+/+}, whereas AMPK^{-/-} cells did not show any significant change, indicating that AMPK is required for metformin-induced glycolysis.

Discussion

Recent population-based studies of type 2 diabetes patients have reported that metformin treatment is associated with a reduced cancer incidence and mortality (19, 20). Here, we investigated whether metformin has a direct effect on growth of HCT116 colon cancer cells *in vivo*. We report that treatment with either metformin or AICAR, two known activators of AMPK, reduces tumor growth in xenografts of HCT116 cells deficient for p53. In contrast, metformin and AICAR have little to no effect on the growth of xenografts of HCT116 p53^{+/+} cells. This selectivity correlated with the ability of HCT116 and untransformed MEFs to engage in a p53-dependent metabolic response that promoted cell survival.

p53 is a tumor suppressor that is often mutated in cancer. In response to genotoxic stresses, p53 induces a transcriptional response that can result in cell cycle arrest, senescence, or apoptosis. However, a recent study revealed a prosurvival role for p53 in cells metabolically impaired by glucose limitation (12). Activation of p53 allows cells to respond to glucose deprivation by

arresting their proliferation until glucose is restored. The ability of glucose deprivation to induce p53 was found to be AMPK dependent. AMPK activation promotes cell survival in response to glucose deprivation by activating the catabolism of fatty acids through β -oxidation and by enhancing the efficiency of the ability to take up and capture remaining extracellular glucose. The data presented here suggest that the ability of cells to engage in a metabolic response to pharmacologic AMPK activators is p53 dependent. p53 is required to redirect the cell available nutrients into catabolic pathways that can supply ATP production and cell survival.

Metabolic adaptations are critical to maintain survival during conditions of energetic stress. Autophagy is a survival pathway that allows cells to degrade and metabolize part of the cytoplasm when access to extracellular nutrients is limited (17). Autophagy has been reported to be especially important for the survival of tumor cells in the center of tumors (33). The data presented here support a role for p53 in the induction of autophagy in response to metformin or AICAR. The fact that p53-deficient cells induce autophagy by rapamycin treatment suggest that p53 is not necessary for autophagy in general. However, p53 seems to be required for autophagy induced after treatment with metformin or AICAR. Fang et al. (34) have reported that p53 activates autophagy in response to etoposide treatment through inhibition of mammalian

target of rapamycin activity and showed that AMPK is involved in this pathway. The present work shows that p53 is also required for the induction of autophagy by pharmacologic activators of AMPK and we genetically determined that the mechanism of this metformin-mediated p53-dependent autophagy is dependent on AMPK.

AMPK signaling pathway is an evolutionary conserved mechanism that allows eukaryotic cells to coordinate the way that nutrients are used to generate ATP during conditions of bioenergetic impairment. In a previous work, the AMPK-dependent activation of fatty acid oxidation was shown to enhance the ability of glioblastoma cancer cells to survive under conditions of glucose deprivation (24). Here, we show that the cellular AMPK activators metformin and AICAR stimulate fatty acid oxidation in both HCT116 and MEF cells and that p53 is required for this effect. Under conditions of glucose limitation, p53-deficient cells showed an impaired ability to survive when treated with metformin compared with p53^{+/+} cells. However, pharmacologic inhibition of fatty acid oxidation only slightly abrogated the p53-dependent cell survival of these cells, suggesting that catabolism of other nutrients such as amino acids may contribute to ATP production upon glucose deprivation. A recent work highlights the prosurvival role of autophagy in cancer cells (35). Amaravadi et al. showed that inhibition of autophagy enhances p53-induced apoptosis, providing evidence that autophagy can be an adaptive mechanism of survival in tumor recurrence after p53 activation of Myc/p53ER^{TAM} lymphomas. AMPK-dependent induction of autophagy may provide an alternative strategy for p53-dependent tumor survival under conditions of metabolic stress.

The mechanisms by which metformin activates AMPK is indirect and poorly understood. Metformin has been previously described as a complex I inhibitor in hepatocytes. Therefore, metformin could activate AMPK by compromising oxidative phosphorylation. Consistent with this, treatment with metformin strongly blocked oxygen consumption in MEFs. Complex I inhibition also resulted in suppression of β -oxidation of fatty acids due to the inhibition of NADH oxidation. One rate-limiting step in mitochondrial β -oxidation is catalyzed by L-3-hydroxyacyl-CoA dehydrogenase, which requires NAD⁺ as a cofactor. NADH generated by this step is then reoxidized by complex I of the respiratory chain. Severe impairment of mitochondrial oxidative phosphorylation increases mitochondrial NADH/NAD⁺ ratio, which may explain the limited activation of β -oxidation observed in metformin-treated cells. To compensate for the metformin-dependent inhibition of oxidative phosphorylation, we found that p53^{+/+} cells but not p53^{-/-} cells enhance their rate of glycolysis. In addition, we showed that AMPK is required for this metabolic switch because the effects of metformin on glycolysis were completely abrogated in AMPK^{-/-} fibroblasts. These data suggest that p53 is a regulator of multiple adaptive metabolic responses that compensate for bioenergetic compromise and not simply an activation of mitochondrial oxidative phosphorylation as previously suggested (14).

Metabolic alterations are hallmarks in the pathogenesis of cancer. Cancer cells are often under a variety of stress stimuli, such as hypoxia and lack of nutrients. To successfully overcome these stress stimuli and meet their high metabolic demand, it is crucial that cancer cells engage proper adaptive responses to provide sufficient ATP supply and support survival. Although loss of p53 confers a

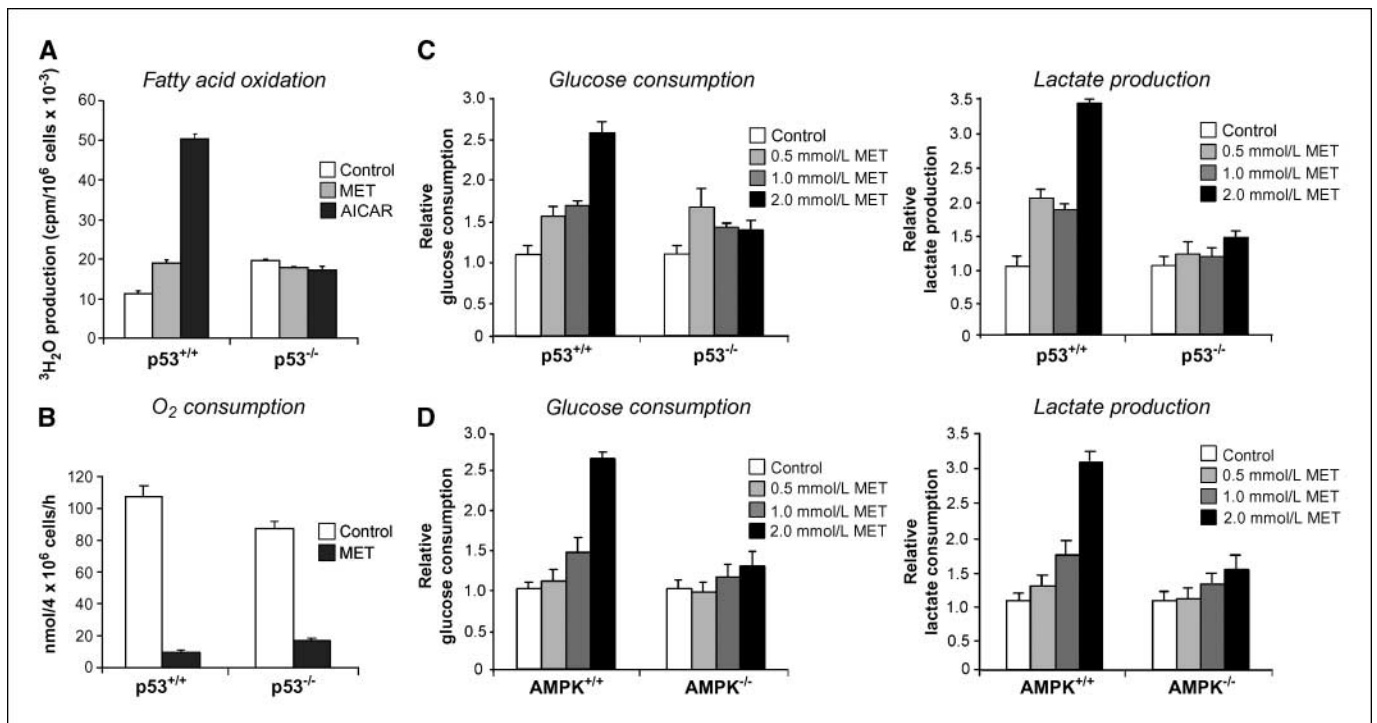


Figure 6. Metformin or AICAR treatment activates a p53-dependent metabolic response. *A*, fatty acid oxidation was determined by measuring ³H₂O produced by MEF cells after incubation with [9,10-³H]palmitate. MEF p53^{+/+} and p53^{-/-} cells were incubated in medium containing 2 mmol/L metformin, 1 mmol/L AICAR, or without any drug (Control). *B*, mitochondrial respiratory activity of MEF p53^{+/+} and p53^{-/-} cells was measured by an oxygen consumption assay. Cells were incubated in medium in the presence or absence of 2 mmol/L metformin for 20 h. Cells (4 × 10⁶) were resuspended in 200 μ L conditioned medium with or without 2 mmol/L metformin and analyzed. *C*, glucose consumption and lactate production were measured after incubation of MEF p53^{+/+} and p53^{-/-} cells or (*D*) MEF AMPK^{+/+} and AMPK^{-/-} cells with or without indicated amounts of metformin for 48 h. Columns, mean of triplicate samples; bars, SD. Representative of three independent experiments.

selective growth advantage to cancer cells, loss of p53 impairs the ability of cancer cells to respond to metabolic changes induced by metformin or AICAR and to survive under conditions of nutrient deprivation. In fact, we observed increased apoptosis *in vivo* and an impaired ability to survive under conditions of glucose limitation *in vitro* when p53-deficient tumor cells were treated with metformin. These findings suggest a potential benefit of metformin use in patients harboring p53-deficient tumors, which are often resistant to existing forms of chemotherapy or radiotherapy.

Acknowledgments

Received 12/4/2006; revised 4/27/2007; accepted 5/16/2007.

Grant support: NIH and National Cancer Institute.

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We thank the members of the Thompson laboratory for helpful discussion and critical reading of the manuscript; Daniel Martinez of the Pathology Core laboratory (Children's Hospital of Philadelphia, PA); and Neelima Shah and Q.C. Yu at the University of Pennsylvania Biomedical Imaging Core for the excellent technical assistance.

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Cancer Res 2007;67:6745-6752.

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