Metformin Blocks Melanoma Invasion and Metastasis Development in AMPK/p53-Dependent Manner

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Abstract

Metformin was reported to inhibit the proliferation of many cancer cells, including melanoma cells. In this report, we investigated the effect of metformin on melanoma invasion and metastasis development. Using different in vitro approaches, we found that metformin inhibits cell invasion without affecting cell migration and independently of antiproliferation action. This inhibition is correlated with modulation of expression of proteins involved in epithelial-mesenchymal transition such as Slug, Snail, SPARC, fibronectin, and N-cadherin and with inhibition of MMP-2 and MMP-9 activation. Furthermore, our data indicate that this process is dependent on activation of AMPK and tumor suppressor protein p53. Finally, we showed that metformin inhibits melanoma metastasis development in mice using extravasation and metastasis models. The presented data reinforce the fact that metformin might be a good candidate for clinical trial in melanoma treatment.

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Introduction

Metastatic melanoma is one of the most aggressive and highly proliferative human malignancies with a median survival of only 6 to 9 months once distant sites become seeded from skin (1). Typically, primary lesions progress to malignant tumors through a multistep process including dysplasia, radial growth phase (RGP), invasive vertical growth phase (VGP), and metastasis.

Melanoma is a neoplasm of neuroectodermal origin; this melanoma cells may not undergo classic epithelial-mesenchymal transition (EMT)-like changes. However, their ability to invade into the dermis is associated with an EMT-like phenotype characterized by changes in expression of cell–cell adhesion molecules as the cadherin family. During malignant transformation, there is loss of expression of E-cadherin in favor of the N-cadherin (2, 3). These changes allow the melanocytes to escape keratinocytes control, and after crossing the stratum basale, to interact with new cell types such as fibroblasts or vascular endothelial cells which promote tumor progression and metastasis. Melanocytes cross the basal lamina by secreting matrix metalloproteinases (MMP) such as collagenases MMP-2 and MMP-9, which will degrade a major constituent of the basal lamina, collagen IV. Several transcription factors that belong to the Snail superfamily of zinc finger transcription factors including Snail/SNAI1 and Slug/SNAI2 are involved in this mechanism. For example, these 2 proteins are central regulators of EMT during neural crest cell migration and cancer (4, 5). In addition, it was shown in melanoma that Slug functions as a melanocyte-specific factor required for the strong metastatic propensity of this tumor (6). More interesting, Slug is a p53 target that antagonizes p53-mediated apoptosis (7) and invasion (8). Elevated mortality caused by melanoma is attributed to its strong propensity to form distal metastases in organs, such as lung, liver, brain, and bones, and its notorious resistance to all current therapies (9). The new important challenge is thus to discover new therapeutic drugs that inhibit melanoma cell proliferation but also exhibit antimetastatic properties.

The oral antidiabetic drug metformin belongs to the family of biguanides and is the most widely used antidiabetic drug in the world. This drug has been shown to inhibit the energy-sensitive AMPK/mTOR signaling pathway that leads to reduced protein synthesis and cell proliferation. Recent studies indicate that metformin can potentially be used as an efficient anticancer drug in various neoplasms such as prostate carcinoma, breast, lung, and pancreas cancers (10, 11). These results were confirmed by retrospective epidemiologic studies that reported a decrease in cancer risk in patients with diabetes treated with metformin (12). In addition, metformin was
reported by several groups, including ours, to inhibit melanoma cell proliferation (13–16). In our previous study, we showed that metformin dramatically impairs the growth of melanoma tumors in vitro and in vivo by inducing autophagic cell death leading to massive apoptosis (13). Interestingly, in the present study, we show also that metformin, independently of its effect on melanoma cell survival, could display anti-invasive and antimetastatic properties.

Materials and Methods

Reagents and antibodies

Metformin was purchased from Sigma-Aldrich. Dulbecco’s Modified Eagle Medium (DMEM), penicillin/streptomycin, and trypsin were from Invitrogen and fetal calf serum (FCS) from Hyclone. Slug, Snail, p53, HSP90, AMPKα1, and AMPKα2 antibodies were purchased from Santa Cruz Biotechnology (TEBU). Anti-AMPKα and phospho-acetyl coA carboxylase (Ser79) antibodies were from Cell Signaling. Antibodies against fibronectin were from BD Biosciences. Antibody to human SPARC was purchased from Hematologic Technologies. Antibody to human N-Cadherin was purchased from Invitrogen. Antibody to human S100 was purchased from Abcam.

Cell lines

All melanoma cell lines were purchased from American Tissue Culture Collection (ATCC) and used within 6 months between resuscitation and experimentation. The ATCC authentication protocols include testing for mycoplasma, bacteria, fungi contamination, confirmation of species identity, and detection of cellular contamination or misidentification using COI for interspecies identification and DNA profiling as well as cytogenetic analysis, flow cytometry, and immunocytochemistry with consistent refinement of cell growth conditions as well as documentation systems, ensuring traceability. Cells were grown in RPMI-1640 (A375, WM9, and SKMel28) or in DMEM (1205Lu, Mel501, and Mewo) supplemented with 10% FCS and penicillin/streptomycin (100 U/mL/50 μg/mL) at 37°C and 5% CO₂. Patient melanoma cells were prepared as described (13) after obtaining informed consent from this patient. Briefly, biopsy was dissected and digested for 1 to 2 hours with collagenase A (0.33 U/mL), dispase (0.85 U/mL), and DNase I (144 U/mL) with rapid shaking at 37°C. Large debris were removed by filtration through a 70-mm cell strainer. Viable cells were obtained by Ficoll gradient centrifugation.

siRNA transfection

Transfection of duplex siRNAs (50 nmol/L) was carried out using Lipofectamine RNAiMAX (Invitrogen) in Opti-MEM (Invitrogen) as described (17). The day after transfection, metformin was added to the medium and proteins were extracted 24 hours after the addition of metformin. Stealth siRNA targeting AMPKα1, AMPKα2, and p53 were purchased from Invitrogen, whereas AMPK siRNA were from Dharmacon. As nonspecific control, scramble sequence siRNAs were used.

Infection with adenovirus

Adenoviruses encoding a dominant-negative form (Ad AMPK-DN) of subunits α1 and α2 of AMPK were a generous gift of Dr. Foufelle (INSERM, UMR-S 872, Paris, France). An adenovirus of which the expression cassette contains the major late promoter with no exogenous gene was used as control (Ad control). Adenoviruses were propagated in human embryonic kidney 293 cells and stored at −80°C. 1205Lu cells were infected for 24 hours with the Ad AMPK-DN before the metformin treatment.

Luciferase assays

Melanoma cells were seeded in a 24-well plate, and transient transfections were conducted the following day using 2 μL Lipofectamine (Gibco-BRL) and 0.3 μg of PG13-Luc, a p53-dependent firefly luciferase reporter gene in a 200-μL final volume. pCMVβGal plasmid was cotransfected to control the variability of transfection efficiency in the reporter assays. The day after the transfection, metformin was added to the medium. At 24 hours after stimulation, cells were harvested in 50 μL of lysis buffer and soluble extracts assayed for luciferase and β-galactosidase activities. All transfections were repeated several times using different plasmid preparations. Luciferase assays were conducted exactly as described (18).

Western blot assays

Protein were extracted in buffer containing 50 mmol/L Tris HCl, pH 7.5, 15 mmol/L NaCl, 1% Triton X-100, and 1× protease and phosphatase inhibitors. Briefly, cell lysates (30 μg) were separated by SDS-PAGE, transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore), and then exposed to the appropriate antibodies. Proteins were visualized with the ECL System from Amersham. The Western blot analyses shown are representative of at least 3 independent experiments. Quantification of Western blot analyses was conducted using ImageJ software.

Coimmunoprecipitation assays

For the co-immunoprecipitation experiments, 1205Lu melanoma cells were treated for 24 hours with metformin 10 mmol/L and lysed in Fischer buffer. Fifty microliters of protein G agarose (0.33 U/mL) were added to the lysates (30 μg) were separated by SDS-PAGE, transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore), and then exposed to the appropriate antibodies. Proteins were visualized with the ECL System from Amersham. The Western blot analyses shown are representative of at least 3 independent experiments. Quantification of Western blot analyses was conducted using ImageJ software.

Invasion assays

Boyden chambers (8.0-μm pores, Transwell; Corning, Inc.) were coated with 1 mg/mL Matrigel (BD Biosciences) and were placed into 24-well chambers containing medium supplemented with 10% FCS. The cells were
resuspended in FCS-starved medium, loaded into the top chamber. Five hours later, adherent cells to the underside of the filters were fixed with 4% paraformaldehyde (PFA) and stained with 0.4% crystal violet, and 5 random fields at × 20 magnification were counted. Results represent the average of triplicate samples from 3 independent experiments.

Three-dimensional spheroid growth
Melanoma spheroids were prepared using the liquid overlay method. Briefly, 500 μL of melanoma cells (20,000/mL) were added to a 24-well plate coated with 1.5% agar (Difco). Plates were left to incubate for 72 hours; by this time, cells had organized into 3-dimensional (3D) spheroids. Spheroids were then harvested using a P1000 pipette. The medium was removed and the spheroids were implanted into a gel of bovine collagen I containing MEM (Invitrogen). Normal melanoma medium was overlaid on top of the solidified collagen. After different times, pictures of the invading spheroids were taken using a Zeiss microscope.

MMP activity measurement
The culture media from stimulated cells were harvested and incubated in a 96-well plate with 0.2 mmol/L of NH2-RA-Dpa-LGLP-AMC as a substrate for various times at 37°C. MMP activity was measured in quadruplicate by quantifying the emission at 460 nm (excitation at 390 nm) in the presence or absence of 10 μmol/L CP471474. The enzyme activities were expressed in arbitrary units per mg of protein.

Substrate zymography
The culture media from 1205Lu melanoma cells were concentrated in centrifugal filter unit and loaded on 10% SDS-PAGE containing 1 mg/mL type I collagen (BD Biosciences). To estimate the protein concentration, SDS-PAGE containing 1 mg/mL type I collagen (BD Biosciences) was run in parallel. Western Blot analysis was performed using 10% SDS-PAGE containing 1 mg/mL type I collagen (BD Biosciences) at 37°C under agitation for 30 minutes. Lysates were clarified by brief spinning, and protein concentration was evaluated by bicinchoninic acid technique (BCA protein assay kit, Pierce).

Following electrophoresis, proteins were renatured by incubating gels in 2.5% Triton X-100 for 2 hours at 37 °C. Gels were then washed 3 times in distilled water and incubated in substrate buffer (50 mmol/L Tris, pH 7.4, and 1 mmol/L CaCl2) at 37°C for 24 hours with gentle shaking. Gels were stained with 0.1% Coomassie Blue R-250 (Sigma) and destained in 7% acetic acid. Enzymatic activities appear as cleared bands in a dark background.

In vivo studies
1205Lu cells stably transfected with a vector encoding luciferase come from Dr. Tartare-Deckert team. A total of 1 × 10⁶/150 μL PBS 1205Lu-Luc cells were injected via the tail vein of nude mice (Harlan Laboratories). The mice were treated with or without intraperitoneal injection of 60 mg/kg metformin every day. Melanoma cells were visualized in the animal after intraperitoneal injection of 50 mg/kg luciferin (Caliper Life Sciences) by bioluminescence imaging using a Photon Imager (Biospace Lab). Mice were sacrificed and the lungs were excised, fixed, and serially sectioned. S100 (1:100) and Slug (1:100) immunostaining was conducted.

To conduct pulmonary extravasation analysis, 1.5 × 10⁶ 1205Lu cells were labeled for 1 hour with CellTracker Green (Invitrogen) and injected via the tail vein of nude mice. After 24 hours, mice were sacrificed, and the lungs were harvested for analysis with a Zeiss Inverted scope.

Statistical analysis
Results are presented as mean ± SE with experiment numbers indicated in the figure legends. Statistical significance was assessed using the Student t test. P ≤ 0.05 was accepted as statistically significant.

Results
Metformin inhibits cell invasion but not cell migration
We previously showed that the antidiabetic drug metformin (Fig. 1A) induced melanoma cell death after long-term treatment of 96 hours (13). We now wanted to determine whether metformin was able to inhibit migration and invasion properties of melanoma cells at early times. As presented in cell migration assay using Boyden chambers (Supplementary Fig. S1A and S1B), metformin did not inhibit migration of 1205Lu and A375 melanoma cell lines after 24 hours. Results were confirmed using wound healing assay (Supplementary Fig. S1C). We next determined the metformin capacity to inhibit cell invasion using Boyden chambers coated with Matrigel (Fig. 1B). Metformin decreases cell invasion in a dose-dependent manner in melanoma cell lines 1205Lu, A375, and WM9. At concentration of 10 mmol/L, metformin inhibits by 95%, 90%, and 60% cell invasion in 1205Lu, A375, and WM9 cells, respectively. Similar results were obtained with cells freshly isolated from patients (Fig. 1B).

To determine whether these anti-invasive effects of metformin are apoptosis mediated, we carried out an experiment in which we analyzed apoptosis in our conditions. As presented in Supplementary Fig. S2, we observed in all tested cell lines, absence of PARP cleavage and decrease of procaspase-3 expression in response to metformin. As expected, apoptosis control staurosporine induces cleavage of PARP and decrease of procaspase-3 expression. These results indicated that the anti-invasive effect of metformin is not mediated by apoptosis.

Tumor invasion was then analyzed in a more physiologic context; WM9 melanoma cells were grown as spheroids embedded in collagen. Metformin significantly reduced cell invasion into collagen (Fig. 1C). To confirm that invasion inhibition is not due to apoptosis induced by metformin, we performed the same experiment in...
presence of apoptosis inhibitor, QVD. As expected, contrary to QVD alone, association of QVD with metformin blocks invasion, indicating that apoptosis does not account for the inhibitory effects on cell invasion mediated by metformin.

**Metformin decreases expression of proteins involved in EMT**

To determine proteins involved in the inhibition of invasion mediated by metformin, we checked by Western blot analysis expression of proteins involved in EMT.

Figure 2A indicates that metformin inhibited in a dose-dependent manner expression of key proteins involved in this process such as fibronectin, N-cadherin, or SPARC in 1205Lu melanoma cells (Fig. 2A). In contrast, vimentin expression was not modified by metformin. Levels of both transcription factors Slug and Snail that initiate EMT were also decreased.

Similar results were found in A375 melanoma cells and in patient-isolated melanoma cells (Fig. 2B and C, respectively).

**Metformin inhibits activation of matrix MMPs in melanoma cells**

We next examined MMP activities in melanoma cells treated with metformin. Total MMP activity level was assessed using a broad-spectrum fluorogenic MMP substrate on 1205Lu melanoma cells treated by metformin (Fig. 3A). Metformin (10 mmol/L) induced a slight but significant decrease of approximately 30% of total MMP activities. In addition, cell-associated metalloproteinase activities were assessed by type I collagen substrate gel...
Metformin Inhibits Melanoma Invasion

AMPK is involved in inhibition of invasion mediated by metformin

To determine whether AMPK activation plays a role in inhibition of invasion by metformin, we abrogated AMPK activation by metformin using infection of dominant-negative forms of AMPK adenoviruses (AdAMPK DN) in 1205Lu melanoma cells (Fig. 4). As expected, infection of AdAMPK DN α1 and α2 increases the expression of AMPK α1 and α2 indicating that dominant-negative forms of AMPK are expressed in the cells. Furthermore, basal and metformin-stimulated phosphorylation of direct AMPK substrate, acetyl coA carboxylase is abolished in cells infected by AdAMPK DN α1 and α2 showing that AMPK activation is inhibited.

In parallel, we observed that Slug and SPARC are inhibited in response to metformin in cells infected with Ad control. In contrast, expression of AMPK DN constructs abrogates these inhibitory effects.

Finally, we tested metformin capacity to inhibit invasion using Boyden chambers in presence (Ad control) or absence (AdAMPK DN α1 and α2) of active AMPK. Interestingly, metformin-induced inhibition of invasion was abolished in presence of dominant-negative forms of AMPK. Taken together, these results suggest an implication of AMPK in the inhibitory effects of metformin in invasion.

Transcription factor p53 is involved in inhibition of invasion mediated by metformin

As AMPK is involved in p53 activation, we wondered whether this transcription factor could play a role in inhibition of invasion mediated by metformin. First, we verified that in our system, metformin is able to activate p53. For this, reporter assay using a promoter–luciferase construct that contains p53-binding sites revealed that treatment with 5 and 10 mmol/L metformin led to approximately 10-fold and 20-fold induction of p53 promoter activity, respectively (Fig. 5A). As expected, actinomycin D (ActD) was used as positive control of p53 activation leads to increase of p53 promoter activity comparable with 10 mmol/L metformin.

We next wanted to determine whether upon metformin stimulation of melanoma cells, AMPKα could associate with p53 to induce p53 activation. We immunoprecipitated p53 from 1205Lu cells stimulated or not with metformin (10 mmol/L) for 24 hours. Proteins were then blotted with antibodies to either p53 or AMPKα (Fig. 5B). In unstimulated conditions, p53 is poorly associated...
with AMPKα, but after metformin treatment, a large increased amount of p53 is co-immunoprecipitated with AMPKα. As control, total blots were presented and showed no major modification of AMPKα level and a decrease in Slug, N-cadherin, and fibronectin expressions in response to metformin. We conclude that in intact 1205Lu melanoma cells, p53 associates with AMPKα in a metformin-dependent fashion.

Furthermore, we asked whether decreasing levels of p53 could prevent inhibition of invasion induced by metformin (Fig. 5C). We observed that siRNA-mediated downregulation of p53 prevents downregulation of Slug (bottom) and inhibition of invasion (top) induced by metformin. Similar results were obtained in another melanoma cell line, Mel501 stably transfected with shp53 RNA (Supplementary Fig. S3).

Regarding a functional role for p53 in mediating anti-invasion properties of metformin, melanoma cells harboring a mutated TP53 gene (Mewo, SKmel28, and HMV2 cells) exhibited resistance to metformin-mediated inhibition of invasion using Western blot analyses and Boyden chamber assays (Fig. 5D and Supplementary Fig. S4A and S4B). Interestingly, re-expression of wild-type (WT) p53 expression by adenoviruses infection in Mewo cells re-sensitizes cells to metformin (Fig. 5D) and restores the inhibition of invasion and the decrease in Slug and N-cadherin expressions in response to metformin.

Our results show that inhibition of invasion induced by metformin occurs through an AMPKα/p53-dependent mechanism.

**Metformin inhibits melanoma metastasis development in mice using extravasation and metastasis models**

Finally, to assess a potential antimetastatic effect of metformin in vivo, extravasation and lung metastasis models were conducted in immunodeficient nude mice. Green-labeled human melanoma cells 1205Lu treated or not 24 hours by metformin were injected in the caudal vein of 6-week-old female athymic nude mice, and their ability to extravasate through the pulmonary parenchyma was evaluated (Fig. 6A). As shown in figure, the control 1205Lu cells treated with PBS give much more micrometastases in the lungs than 1205Lu cells treated by metformin. Quantification of experiments by counting extravasated cells using inverted microscope confirmed this result. In addition, similar experiment carried out with Mewo cells harboring p53 mutation shows the incapacity of metformin to inhibit extravasation in lungs (Supplementary Fig. S5A), confirming the implication of p53 in this process in vivo.

In another experiment, 1205Lu melanoma cells (1.5 x 10⁶) stably expressing luciferase were injected in caudal vein of 6-week-old female athymic nude mice and were then treated daily with an intraperitoneal injection of vehicle or metformin (2 mg/mouse/d) over a period of 39 days (Fig. 6B). Seven days after cell injection, bioluminescence was detected in the lungs of all mice. Importantly, a 3-fold increase in bioluminescence intensity was observed in the lungs of mice treated with vehicle compared with lungs of mice treated by metformin. This result...
reflects the decreased capacity of cells in mice treated by metformin to metastase in lung in vivo. After 39 days, bioluminescence intensity was very weak in lungs of mice treated by metformin in comparison of lungs of control mice. This inhibition was not found when we used Mewo cells with inactive p53 (Supplementary Fig. S5B).

To confirm the molecular mechanisms involved in the antimetastatic effects of metformin in vivo, Slug expression was studied by immunofluorescent staining on tumor sections from mice treated with vehicle or metformin (Fig. 6C). S100 staining was used to detect melanoma cells in lungs. Sections of lung tumors from mice treated with metformin showed a significant decrease in Slug staining compared with sections of tumors from control mice injected with vehicle. Quantification of ratio Slug:S100 confirms this observation. Thus, the reduction of metastases observed in metformin-treated mice seems to be, at least in part, related to the inhibition of the expression of Slug protein.

Discussion

The discovery of new therapeutic compounds is a very important challenge to treat advanced melanomas that are resistant to existing therapies. For this purpose, using in vitro and in vivo approaches, we and others previously showed the potent effects of the antidiabetic drug metformin on reduction of melanoma cells growth and xenograft development (13–16). One crucial step of melanoma development appears to be promotion of an EMT-like transition, a process having central role during RGP and VGP progression, melanoma invasion, and metastatic dissemination of melanoma cells (19, 20). In this study, we show that metformin inhibits melanoma invasion by regulating the EMT-like regulatory factors and show the critical role of the AMPK/p53 axis in this process.

The mode of action and the biologic consequences of the antidiabetic drug metformin in cancer cell migration and invasion are poorly understood. Indeed, only few studies done in fibrosarcoma, carcinoma, and ovarian carcinoma cells evoke the role of metformin in this process (21–23). The current study was designed to evaluate the anti-invasive potential of metformin in melanoma and to analyze the molecular mechanisms involved in this process.

Using different in vitro approaches, we found that metformin inhibits cell invasion without affecting cell migration. The fact that migration was not affected by metformin indicates that cells are functional and shows that inhibition of invasion mediated by metformin observed after 24 hours of treatment is not due to induction of apoptosis observed in cells treated by drug after a longer period of time (96 hours; ref. 13).
More interesting, we found the same results using more physiologic tumor invasiveness in 3D collagen matrix, an assay that mimics 3D invasion by melanoma cells. To definitively confirm that invasion inhibition is not due to apoptosis induced by metformin, we carried out the same experiment in presence of an apoptosis inhibitor and we observed that metformin inhibited invasion in the same manner in presence or not of inhibitor.

To elucidate the mechanism by which metformin inhibited melanoma cell invasion, we focused our interest on proteins involved in EMT transition. In melanoma, the transcriptional factors of the Snail family Snail and Slug are required for the control of the expression of genes involved in EMT such as N-cadherin, SPARC, or fibronectin and for the development of local tumor invasion and promotion of distant metastasis (24, 25). Interestingly, we found that metformin inhibited in a dose-dependent manner the expression of transcription factors Slug and Snail in association with inhibition of EMT proteins N-cadherin, SPARC, and fibronectin. Furthermore, it is well-established that melanoma cell invasion depends on MMP expression and activity especially MMP-2 and MMP-9 (26). Both MMP are highly expressed in melanoma cells, and a direct relationship between melanoma progression and MMP expression and activity has been well-established in many studies (26). Moreover, inhibition of MMP activity has been previously investigated as a new therapeutic strategy to control metastatic spreading. As presented, metformin decreases global MMP activity and, more specifically, expression and activity of MMP-2 and MMP-9. Taken together these results indicate that decrease in EMT protein
were treated or not with metformin (60 mg/kg) for 39 days. The bioluminescence resulting from the presence of lung metastasis was quantified in mice receiving a short-term treatment of melanoma cells (24 hours) with metformin or PBS and labeled with Green Cell Tracker and then injected via the tail vein in nude mice. After 24 hours, the lungs of the mice were imaged (left) and the number of micrometastasis were counted (right). B and C, after injection of $1 \times 10^6$ 1205Lu melanoma cells expressing luciferase into the tail vein, the nude mice were treated or not with metformin (60 mg/kg) for 39 days. The bioluminescence resulting from the presence of lung metastasis was quantified with a Photon Imager. The results after 7 days were quantified and presented in B. A characteristic image of 3 mice per group and quantification after 39 days were presented in C. The bars indicate the mean ± SD of triplicate samples. *, $P < 0.05$; **, $P < 0.001$. D, immunofluorescence on sections of lungs was conducted with Slug and S100 antibodies. Hoechst was used to labeled nucleus (left). Quantification of the ratio Slug/S100 was evaluated with ImageJ (right).

Figure 6. Effects of metformin on melanoma invasion in vivo. 1205Lu melanoma cells were treated for 24 hours with 10 mmol/L of metformin or PBS and labeled with Green Cell Tracker and then injected via the tail vein in nude mice. After 24 hours, the lungs of the mice were imaged (left) and the number of micrometastasis were counted (right). B and C, after injection of $1 \times 10^6$ 1205Lu melanoma cells expressing luciferase into the tail vein, the nude mice were treated or not with metformin (60 mg/kg) for 39 days. The bioluminescence resulting from the presence of lung metastasis was quantified with a Photon Imager. The results after 7 days were quantified and presented in B. A characteristic image of 3 mice per group and quantification after 39 days were presented in C. The bars indicate the mean ± SD of triplicate samples. *, $P < 0.05$; **, $P < 0.001$. D, immunofluorescence on sections of lungs was conducted with Slug and S100 antibodies. Hoechst was used to labeled nucleus (left). Quantification of the ratio Slug/S100 was evaluated with ImageJ (right).

expressions and MMP inhibitions are, at least in part, mechanisms by which metformin negatively regulates melanoma invasiveness.

Metformin action is mainly mediated by AMPK activation; however, several recent reports indicate that some effects of metformin in cancer cell lines could be mediated by an AMPK-independent pathway (13, 27–29). In our model, AMPK inactivation by AMPK dominant-negative construct (30) or inhibition of AMPK expression by siRNA (data not shown) inhibits the effect of metformin on melanoma invasion, suggesting that the effects of this drug are mediated through an AMPK-dependent mechanism.

Several recent studies indicate that AMPK activation is involved in this mechanism (data not shown) as described in human melanoma (8). We therefore examined the regulation of Slug and invasion by p53 in p53-knockdown cells and found that Slug and invasion regulation by metformin is dependent of both expression and mutational status of p53. Accordingly, we observed that reversion of the EMT process, including modification of N-cadherin, SPARC, and fibronectin expression requires activation of p53. However, the mechanism by which p53 induces Slug degradation remains unclear and needs further investigations. Indeed, our experiments show that MDM2 is not involved in this mechanism (data not shown) as described in lung carcinoma cells (8).

Finally, we have evaluated the antimalanoma activity of metformin in mouse models of extravasation and melanoma metastasis of lungs.

One of the key events in cancer metastasis is extravasation. Extravasation of cancer cells is a complicated process which involves adhesive interactions, chemotaxis, and invasion. Our in vivo experiments indicate that short-term treatment of melanoma cells (24 hours) with metformin inhibited the capacity of tumor cells to extravasate in lungs to create micrometastasis. Similar results
were observed when mice were pretreated with metformin instead of the injected cells (data not shown).

Importantly, in models of lung metastasis, the doses of metformin administered to the mice (2 mg/25 g/d) are in the same range to those administered to patients with diabetes (3 g/75 kg/d). We found that short-term administration of metformin (7 days) reduces markedly the development of melanoma metastasis in lungs mice and almost abolished metastasis after 39 days. The dramatic effect of the metformin after 39 days on the inhibition of metastases in lungs is probably due to a combined effect of the drug on the induction of the cell death and an inhibition of the invasion. Experiments are ongoing in our laboratory to confirm this hypothesis.

To determine whether the molecular events observed in cell lines were also found in mice, we conducted immunohistologic staining of mouse tumor sections. Experiments showed a decrease of Slug expression in the tumors of metformin-treated mice. Furthermore, we observed that metformin was inactive for inhibition of extravasation and metastasis when melanoma harbors p53 mutation. These results suggest that in vivo, p53 activation induced by metformin is required for Slug inhibition and the decrease in melanoma metastasis dissemination in lung.

More recently, it was reported that metformin could increase melanoma growth in xenograft mice models of melanoma A375 cells harboring B Raf V600E mutation through increase of angiogenesis (36). In our experiments, we did not observe such effects in all the tested melanoma cell lines (possessing or not B Raf V600E mutation). This discrepancy could be due to high level of metformin used by Martin and colleagues and could result in off-target effects due to lactic acidosis described when concentrations of drug are elevated in serum (37, 38).

In summary, we show for the first time that metformin inhibits melanoma invasion and metastasis development through AMPK/p53 axis activation. This finding brings new clues to the understanding of metformin action in melanoma development. Finally, taking into account the drastic effects of metformin on melanoma cell growth, survival, invasion, and metastasis development in mice, it might be worth evaluating metformin treatment in patients suffering from metastatic melanoma. However, our data indicate that mutational status of p53 must be considered before providing a patient with the metformin therapy.

Disclosure of Potential Conflicts of Interest
R. Ballotti has honoraria from speakers bureau from Roche. No potential conflicts of interest were disclosed by the other authors.

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Conception and design: M. Cerezo, S. Tartare-Deckert, S. Rocchi
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Study supervision: S. Rocchi

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