Metformin Accelerates the Growth of BRAF$^{V600E}$-Driven Melanoma by Upregulating VEGF-A

Matthew J. Martin¹, Robert Hayward¹, Amaya Viros¹, and Richard Marais¹,²
The antidiabetic drug metformin has antitumor activity in a variety of cancers because it blocks cell growth by inhibiting TORC1. Here, we show that melanoma cells that are driven by oncogenic BRAF are resistant to the growth-inhibitory effects of metformin because RSK sustains TORC1 activity even when AMP-activated protein kinase (AMPK) is activated. We further show that AMPK targets the dual-specificity protein phosphatase DUSP6 for degradation and this increases ERK activity, which then upregulates the VEGF-A protein. Critically, this drives angiogenesis and accelerates the growth of BRAF-driven tumors in mice. Unexpectedly, however, when VEGF signaling is inhibited, instead of accelerating tumor growth, metformin inhibits tumor growth. Thus, we show that BRAF-driven melanoma cells are resistant to the antigrowth effects of AMPK and that AMPK mediates cell-autonomous and cell-nonautonomous effects that accelerate the growth of these cells in vivo.

**SIGNIFICANCE:** Metformin inhibits the growth of most tumor cells, but BRAF-mutant melanoma cells are resistant to metformin in vitro, and metformin accelerates their growth in vivo. Unexpectedly, VEGF inhibitors and metformin synergize to suppress the growth of BRAF-mutant tumors, revealing a combination of drugs that may be effective in these patients. Cancer Discov; 2(4):344–55. ©2012 AACR.
standard tissue culture plastic, metformin and AICAR only modestly affected the growth of these cells (Supplementary Fig. S1A); however, when the cells were grown in soft agar, whereas NRAS-mutant cell growth was inhibited by metformin and AICAR by 68% to 100%, BRAF-mutant cell growth was insensitive to these agents (Fig. 1A and Supplementary Fig. S1B and S1C). Thus, NRAS-mutant melanoma cells were sensitive to the antigrowth effects of metformin and AICAR, whereas BRAF-mutant cells were resistant.

To test whether this difference arose because AMPK was not activated in the BRAF-mutant cells, we Western blotted cell extracts for AMPKα phosphorylation on threonine-172 and ACC phosphorylation on S79. AICAR induced slow phosphorylation of AMPKα and ACC in BRAF-mutant SK-Mel28 and A375 cells, with no increase apparent for the first 2 hours but robust phosphorylation at 6 hours and beyond (Fig. 1B and C). AICAR also activated AMPK in NRAS-mutant D04 cells, with clear increases in AMPKα and ACC phosphorylation apparent within an hour of AICAR treatment (Fig. 1D). We also found that metformin and glucose starvation activated AMPKα in A375 cells (Supplementary Fig. S1D and S1E) and that phosphorylation of ACC was increased in response to 12-hour metformin and AICAR treatment in 5 additional BRAF-mutant melanoma lines (Supplementary Fig. S1F). Thus, AMPK was activated in BRAF-mutant melanoma cells, albeit with slow kinetics, but it did not inhibit their growth.

**RSK Mediates BRAF-Mutant Melanoma Cell Resistance to AMPK**

To investigate why BRAF-mutant cells are resistant to AMPK, we examined TORC1 signaling because this pathway is a key AMPK target in cells (19). We show that 4E-BP1 and rpS6 phosphorylation (Supplementary Fig. S2A) was suppressed in metformin-treated NRAS-mutant (D04, MM415, and WM1366), but not BRAF-mutant (A375, Mel-HO, and SK-Mel28) cells (Fig. 2A). Thus TORC1 signaling was insensitive to AMPK in BRAF-mutant cells. TORC1 can be activated by 2 pathways (5). In the canonical pathway downstream of RAS, phosphatidylinositol 3-kinase activates Akt, which inhibits TSC1/2, allowing Rheb to activate TORC1 (Supplementary Fig. S2A). In the noncanonical pathway downstream of RAS, ERK activates the protein kinase RSK, which then inhibits TSC1/2 and directly activates TORC1 (Supplementary Fig. S2A) and accordingly, both BRAF and MEK inhibitors block RSK phosphorylation in A375 cells (Supplementary Fig. S2B).

We measured RSK activity by Western blotting for phosphorylation of S380/S386 on RSK1/RSK2. We show that RSK activity was generally greater in BRAF-mutant than NRAS-mutant cells (Fig. 2B) and that metformin and AICAR activated RSK in A375 cells (Fig. 2C and Supplementary Fig. S2C). Critically, metformin did not block 4E-BP1 or rpS6 phosphorylation in A375 cells, whereas the RSK inhibitor BI-D1870 suppressed phosphorylation of both (Fig. 2D). Note also that metformin and BI-D1870 cooperated both to inhibit rpS6 phosphorylation and to activate AMPK in A375 cells (Fig. 2D). Critically, whereas neither metformin nor BI-D1870 inhibited the growth of A375, SK-Mel28, or WM-266.4 cells, together these agents strongly inhibited the growth of these cells (Fig. 2E).

We observed similar results with RNA interference (RNAi). Thus, whereas downregulation of RSK1 and RSK2 did not affect the growth of A375 cells, it did make these cells more sensitive to the growth-inhibitory effects of metformin (Fig. 2F). Note that the loss of either protein alone did not sensitize A375 cells to metformin (Fig. 2F), demonstrating that these proteins are functionally redundant. Finally, constitutively active myristoylated RSK1 (20) increased RSK and rpS6 phosphorylation in NRAS-mutant D04 cells (Fig. 2G) and caused them to become resistant to the growth-inhibitory effects...
of metformin and AICAR (Fig. 2H). We conclude that RSK mediates the resistance of BRAF-mutant melanoma cells to metformin.

**Metformin Accelerates the Growth of BRAF Tumors and Induces Expression of VEGF-A**

Next, BRAF-mutant cells were grown as tumor xenografts in metformin-treated nude mice. Metformin did not affect the weight of the mice (Supplementary Fig. S3A) or increase their blood lactate levels (Supplementary Fig. S3B), showing that it was well tolerated. Surprisingly, metformin increased the size of A375 (BRAF-mutant) xenografts by 3.2-fold at 50 days (Fig. 3A; \( P < 0.0001 \)) and Mel-HO (BRAF-mutant) xenografts by 2.3-fold (Fig. 3B; \( P < 0.034 \)), but decreased D04 (NRAS-mutant) xenografts in size by 42% (Supplementary Fig. S3C; \( P < 0.0033 \)). Thus, despite having little effect on
BRAF-mutant cell growth in vitro, metformin accelerated their growth in vivo.

Histologic examination of the tumors revealed a dramatic increase in the size and number of CD31-positive vessels in A375 xenografts from metformin-treated mice (Fig. 3C and Supplementary Fig. S3D), and we confirmed that these structures were blood vessels by staining for endoglin/CD105 (Fig. 3D). We used a human-specific antibody to show that metformin increased VEGF-A protein production by A375 melanoma cells in vivo (Fig. 3E) and that metformin, phenformin, AICAR, and A-769662 all increased VEGF-A protein production in BRAF-mutant, but not NRAS-mutant melanoma cells (Fig. 3F and Supplementary Fig. S3E).

Metformin Cooperates with Anti-VEGF Therapies to Suppress the Growth of BRAFV600E-Mutant Tumors

To investigate the importance of VEGF-A to the response of BRAF-mutant tumors to metformin in vivo, nude mice bearing A375 xenografts were treated with the VEGF receptor inhibitor axitinib. Metformin accelerated the growth of the tumors (2.2-fold increase in tumor size at day 50), whereas axitinib had no effect (Fig. 4A). When these agents were combined, they suppressed A375 tumor growth by 45% (Fig. 4A). Note that no such cooperation was seen in vitro (Fig. 4B), and axitinib and metformin did not cooperate to inhibit the growth of NRAS-mutant cells either in vitro or in vivo (Supplementary Fig. S4A and S4B).

These effects on A375 xenografts were reproduced with the use of the VEGF-neutralizing antibody bevacizumab. Metformin increased A375 tumor growth by 2.0-fold, and bevacizumab reduced tumor growth by 34%, but together they reduced tumor growth by 64% (Fig. 4C). Finally, these observations were also reproduced with the use of short-hairpin RNA (shRNA). MDA-MB-435 cells were engineered to express 2 independent VEGF-A shRNA probes. These constructs blocked metformin and AICAR-induced VEGF-A protein production by MDA-MB-435 cells (Fig. 4D) but did not affect their growth in vitro (Fig. 4E). Critically, whereas metformin accelerated the growth of MDA-MB-435 tumors expressing control shRNA, it induced regression in tumors expressing VEGF-A shRNA (Fig. 4F).

Taken together, these data show that VEGF-A drives the accelerated growth of BRAF-mutant melanoma in...
Metformin-treated mice, but when VEGF-A signaling was inhibited, metformin unexpectedly switched from a growth promoter to a growth inhibitor.

**AMPK Increases VEGF-A Protein Production in BRAF-Mutant Cells**

Next we investigated how metformin upregulated VEGF-A in BRAF-mutant cells. We show that the AMPK inhibitor compound C blunted VEGF-A upregulation by AICAR in A375 cells (Fig. 5A) and that AMPKα1 depletion by RNAi blocked VEGF-A upregulation in metformin and AICAR-treated A375, Mel-HO, and MDA-MB-435 cells (Fig. 5B and Supplementary Fig. S5A and S5B). AMPKα2 depletion did not affect VEGF-A protein production in A375 cells (Supplementary Fig. S5C).

Next, we examined VEGF-A upregulation in SK-Mel5 melanoma cells because although these cells express BRAF*V600E*, they lack LKB1 and thus cannot activate AMPK when treated with AICAR (21, 22). We confirmed that LKB1 was not expressed in SK-Mel5 cells and that AICAR did not activate AMPK in them (Fig. 5C). Critically, AICAR did not upregulate VEGF-A in SK-Mel5 cells (Fig. 5C). As an important control, we show that the calcium ionophore A23187 activated AMPKα and upregulated VEGF-A in SK-Mel5 cells and that the CAMKK inhibitor STO-609 blunted both responses (Fig. 5C and D). This finding suggests that AMPK is still activated by CAMKKII in these cells and shows that the VEGF-A gene still responded to AMPK in SK-Mel5 cells, providing further evidence that AMPK upregulated VEGF-A in BRAF-mutant cells.

**AMPK Stimulates VEGF-A Protein Production Through ERK**

To determine how AMPK upregulated VEGF-A, we show that AICAR increased VEGF-A mRNA levels in A375 cells (Fig. 5E). Because VEGF-A is a hypoxia-regulated gene (23), we investigated whether the hypoxia-inducible transcription factors (HIF) regulated these responses. However, our experiments were performed in 20% oxygen, and HIF-1α was not expressed (Supplementary Fig. S6A). Furthermore, metformin and AICAR did not induce HIF-1α expression in these cells (Supplementary Fig. S6B), and HIF1α siRNA did not block VEGF-A upregulation by metformin or AICAR (Supplementary Fig. S6C and S6D). In addition, we were unable to detect HIF-2α by Western blot in these cells, and HIF-2α siRNA did not block metformin or AICAR-mediated VEGF-A upregulation (Supplementary Fig. S6C and S6D). Finally, we also show that although
hypoxia upregulated the basal level of VEGF-A in A375 cells, metformin and AICAR further upregulated VEGF-A in the hypoxic cells (Supplementary Fig. S6E). We conclude that metformin and AICAR upregulate VEGF-A independently of hypoxia.

In previous studies investigators have shown that VEGF-A expression is also regulated by ERK signaling in some cells (24), so we examined whether ERK regulated VEGF-A in metformin-treated BRAF-mutant melanoma cells. We found that the MEK inhibitor PD184352 downregulated
the basal levels of VEGF-A mRNA in A375 cells (Fig. 5E). We also discovered that PD184352 and the BRAF inhibitors PLX4720 and 885-A (25) blocked VEGF-A upregulation in AICAR-treated cells (Fig. 5F). Furthermore, BRAF depletion by siRNA also blocked VEGF-A upregulation by metformin and AICAR (Fig. 5G). Note that BRAF depletion did not block AICAR or metformin-driven ACC phosphorylation (Fig. 5G), showing that BRAF depletion did not inhibit AMPK through an unknown cryptic mechanism. Furthermore, while conducting this experiment, we noted that metformin and AICAR activated ERK in the scrambled siRNA control samples (Fig. 5G, lanes 1–3), but not in the cells in which BRAF was depleted (Fig. 5G lanes 4–9). As a follow-up to this observation, we found that ERK activation by AICAR occurred with slow kinetics and in the absence of increased MEK phosphorylation (Fig. 5H) or BRAF activation (Supplementary Fig. S6F).

**AICAR Induces Degradation of DUSP6 Protein in BRAF-Mutant Melanoma Cells**

Because metformin and AICAR activated ERK without activating upstream signaling, we examined whether AMPK disrupted ERK pathway negative feedback loops. The dual specificity phosphatase DUSP6 is an ERK-negative regulator and a transcription target of BRAFMAPK/ERK signaling in melanoma cells (26–28). Consensuise with this, we show that PD184352 strongly suppressed (>99% inhibition) DUSP6 mRNA in A375 cells (Fig. 6A), and this suppression was accompanied by loss of the DUSP6 protein (Fig. 6B, lanes 1, 2). Conversely, we found that ERK activation by AICAR was accompanied by an increase in DUSP6 mRNA (~4.2-fold increase; see Fig. 6A), but unexpectedly this was accompanied by a reduction rather than increase in DUSP6 protein (Fig. 6B, lanes 1, 3, 4).

These data suggest that AMPK targets DUSP6 for degradation, and, accordingly, we found that DUSP6 protein was downregulated in A375 xenografts from metformin-treated mice (Fig. 6C and Supplementary Fig. S7A), correlating with increased ERK activation (Supplementary Fig. S7B). We also show that the proteasome inhibitor MG132 prevented DUSP6 protein loss in PD184352-treated cells (Fig. 6D) but increased DUSP6 protein in AICAR-treated cells (Fig. 6D). Finally, we show that DUSP6 depletion by 2 distinct siRNAs activated ERK (Fig. 6E) and upregulated VEGF-A (Fig. 6F) in A375 cells. We conclude that by targeting DUSP6 for degradation, AMPK activated ERK and upregulated VEGF-A.

**DISCUSSION**

The antidiabetic drug metformin blocks cancer cell growth in vitro (11, 12), delays the onset of tumors in mice (15), and decreases the lifetime risk of cancer in humans (9, 10). Thus, metformin has antitumor activity in a variety of cancers, but we show here that BRAF-mutant melanoma cells are resistant to this drug because RSK activity is elevated. Support for this
conclusion comes from our observation that NRAS-mutant cells had low RSK activity and were sensitive to metformin but could be made resistant to metformin by the expression of constitutively active RSK. Conversely, BRAF-mutant melanoma cells had high RSK activity, were resistant to metformin, and could be made sensitive to metformin by inhibition or depletion of RSK. Critically, metformin blocked TORC1 signaling in NRAS-mutant cells, whereas RSK inhibitors blocked TORC1 signaling in BRAF-mutant cells. Thus, unlike most other cancer cells so far tested, in our study BRAF-mutant melanoma cells were resistant to AMPK, and we show that this was mediated by RSK sustaining TORC1 signaling.

Our results were unexpected because it has been reported that AICAR does not activate AMPK in BRAF-mutant melanoma cells (16, 17), but we clarify this apparent contradiction by showing that AICAR activated AMPK with slow kinetics in these cells, explaining why its activation was missed in the earlier studies. We further show that metformin and glucose starvation activated AMPK in BRAF-mutant cells. These data confirm that AMPK could be activated in BRAF-mutant melanoma cells, albeit with uncharacteristically slow kinetics. A plausible explanation for the delay in AMPK activation is that RSK activity is elevated. The role of RSK in AMPK regulation is controversial because although in some studies authors report that RSK inhibits cell growth through LKB1 (29), others report that RSK inhibits LKB1-mediated AMPK activation (16, 17), and in other studies RSK has been reported not to play a role in AMPK regulation (30, 31). We note that expression of an LKB1 isoform lacking the RSK phosphorylation sites results in constitutive AMPK activation in SK-Mel28 melanoma cells and also inhibits their growth (16, 17). However, the relative contribution of AMPK compared with other LKB1 substrates, of which there are at least 13 (32), to this growth suppression is unknown. We show that RSK inhibition increased basal and metformin-stimulated AMPK activation in A375 cells (Fig. 2D) and posit that RSK antagonizes AMPK to delay but not block its activation in these cells. Our results may explain some of the apparently discordant previously published results.

A second reason that our results were unexpected is that it has been reported that the growth of SK-Mel28 cells in soft agar was inhibited by metformin and AICAR (18). However, we were unable to replicate that result, and in our study the number and size of colonies formed by SK-Mel28 cells in soft agar were unaffected by metformin or AICAR (Supplementary Fig. S1B). Furthermore, we have confirmed that 1 mouse and 8 human BRAF-mutant cell lines were resistant to metformin in soft agar (Fig. 1A and Supplementary Fig. S1C), suggesting that the SM-Mel28 clone used in the previous study is not representative of the majority of other BRAF-mutant cells. One possibility is that the cells acquired sensitivity to AMPK, something that would occur if, for example, they downregulated RSK.

While we were conducting this study, it was reported that metformin inhibits the growth of A375 cells xenografts in mice (33). The basis of this difference is unclear, but we obtained consistent acceleration of BRAF-mutant melanoma cell growth in vivo with 3 different BRAF-mutant cell lines (Figs. 3A, 3B, 4F). Notably, our results are consistent with those of Phoenix and colleagues (34), who also observed accelerated growth of MDA-MB-435 cells in metformin-treated mice. A notable difference between the studies is that whereas we initiated drug treatment at the same time as implanting the cells and delivered the drug through the oral route (as did Phoenix and colleagues [ref. 34]), Tomic and colleagues (33) initiated drug treatment 5 days after inoculating the cells and delivered the drug by intraperitoneal injection. These differences could plausibly account for the discrepancies in the results.

Another unexpected finding of our study was that metformin accelerated BRAF-mutant tumor growth by upregulating VEGF-A. When VEGF was inhibited using genetics (shRNA), antibodies (bevacizumab), or small molecules (axitinib), instead of accelerating tumor growth, metformin suppressed tumor growth. Thus, in addition to driving the growth of BRAF-mutant cells in vivo, VEGF was critical for their survival. Thus, we have identified an unexpected “synthetic lethality,” whereby metformin and VEGF inhibitors cooperate to suppress BRAF-mutant tumor growth. We note that in previous studies investigators have shown that integrin inhibitors also upregulate VEGF-A and then cooperate with anti-VEGF therapies to suppress tumor growth (35), showing intriguing parallels with our findings.

Figure 7. Model for signaling networks controlled by AMPK in BRAF-mutant melanoma cells. The activity of each protein is represented according to the color code bar below. The relative level of interaction between the components is indicated by the thickness of the lines/arrows between them. A, under basal conditions oncogenic BRAF activates ERK, which then drives DUSP6 expression to modulate ERK signaling. ERK also activates RSK, which activates TORC1 to drive protein translation. ERK also induces expression of low levels of VEGF-A. B, in metformin-treated cells, AMPK is activated and targets the DUSP6 protein for degradation. This results in increased ERK activity and although this increases DUSP6 mRNA levels the protein does not accumulate. ERK also activates RSK, which maintains TORC1 activity despite AMPK activation, and it upregulates VEGF.
Metformin has been reported to upregulate VEGF-A in MDA-MB-435 cells (34), but the origin of these cells is controversial, as they express markers consistent with a melanoma line rather than triple-negative breast cancer cells (25, 36–39). Thus, although we confirm that metformin upregulates VEGF-A in MDA-MB-435 cells, our findings clarify that this is a response of BRAF-mutant melanoma cells rather than triple-negative breast cancer cells. Furthermore, we have elucidated the mechanisms underlying this response and based on our observations, we propose the following model to explain how this network controls BRAF-mutant melanoma cell growth in vitro and in vivo. We posit that under normal conditions (Fig. 7A) ERK is activated downstream of oncogenic BRAF and induces DUSP6, which feeds back to fine-tune ERK activity. ERK also activates RSK, which is largely responsible for maintaining TORC1 signaling, and it induces low levels of VEGF-A expression. These events presumably exist in equilibrium. When the cells are treated with metformin (Fig. 7B), although RSK delays AMPK activation, once it is activated it targets DUSP6 for degradation and disrupts feedback equilibrium to increase ERK activity. Although this increases DUSP6 mRNA levels, the protein does not accumulate because it is persistently degraded by AMPK. ERK then further activates RSK, sustaining TORC1 activity despite AMPK activation. We posit that these cell-autonomous effects explain why BRAF-mutant melanoma cells are resistant to AMPK, and the consequent upregulation of VEGF-A drives cell-nonautonomous events in vivo that increase vascular density and accelerate tumor growth.

Our results have 2 apparently contradictory clinical implications. First, they suggest that metformin should not be prescribed to diabetic patients with BRAF-mutant melanoma because it may accelerate the growth of their tumors. Conversely, they suggest that metformin and anti-VEGF agents could be combined to treat these same tumors. BRAF drugs such as vemurafenib mediate impressive responses in BRAF-mutant melanoma patients, but responses are limited because most patients develop resistance and approximately 20% of patients have primary resistance to these agents (3, 4). Thus, alternative treatments are needed even for BRAF-mutant tumors and the metformin/anti-VEGF combination we describe may have clinical utility.

In summary, we show that AMPK drives cell-autonomous and cell-nonautonomous events in BRAF-mutant melanoma cells. RSK mediates the cell-autonomous events and allows the cells to escape the antigrowth effects of AMPK. The cell-nonautonomous effects are mediated by VEGF-A and drive tumor growth. Intriguingly, we have also identified a cooperative response between VEGF signaling antagonists and metformin that slows tumor growth. Our findings therefore have clear implications for diabetic and melanoma patients, but may also provide new melanoma treatment strategies that bear further exploration.

METHODS

Reagents

Antibodies for phospho-ACC, phospho-AMPKα, total AMPKα, phospho-MEK, phospho-S6, phospho-S6K1, phospho-4EBP1, phospho-RSK, RSK2, HIF-1α, and LKB1 were from Cell Signaling Technology (Cambridge-Biosciences). Antibodies for BRAF, ERK2, and AMPKα1 were from Santa Cruz Biotechnology, Inc. The DUSP6 antibody was from Abcam. Phospho-ERK1/2 and tubulin antibodies were from Sigma-Aldrich. Anti-RSK1 was from Millipore. A-77652 and BI-D1870 were from the Medical Research Council Protein Phosphorylation Unit, University of Dundee. Metformin, phenformin, AICAR, A23177, STO-609, and rapamycin were from Sigma-Aldrich. Axitinib was purchased from Selleck Chemicals. 885-A was synthesized on contract by Evotec AG. PD184352 and PLX4720 were synthesized in house.

Preparation of Cell Lysates and Western Blotting

The details of preparation are described in the Supplementary Materials.

Cell-Culture Techniques

Details regarding cell lines, their growth conditions, mutation status, and source are found in Supplementary Table S1. Cell viability was by SRB assay (40) and growth in soft agar as described (41), with macroscopic (>0.1-mm) colonies scored (10 fields per sample; triplicate determinations) after 2 weeks. The number of colonies formed/total number of cells plated (%) was calculated and is expressed relative to appropriate controls. Statistical analysis was by Student t test. VEGF-A protein levels were measured by human-specific sandwich ELISA (R&D Systems). Gene expression measurements by quantitative real-time PCR- and siRNA-mediated gene depletion were as described previously (25). Cells transfected with shRNA vectors (SA Biosciences) were selected in puromycin (1 μg/mL). Further details can be found in the Supplementary Materials.

Xenograft Studies

All animal procedures were approved by the Animal Ethics Committee of the Institute of Cancer Research in accordance with National Home Office regulations under the Animals (Scientific Procedures) Act 1986 and according to the guidelines of the Committee of the National Cancer Research Institute. A total of 2.5 × 10^6 A375, 2.5 × 10^6 Mel-HO, 5 × 10^5 D-04, 4 × 10^6 MDA-MB-435/NS, or 4 × 10^6 MDA-MB-435/shiV.3 cells were injected into the flanks of female (5-8 mice per group) nude mice (Charles River) as described previously (42). Metformin (300 mg/kg) was administered in drinking water 1 day before cells were injected, assuming an average water consumption of 5 ml per day per mouse. Axitinib was dissolved in a vehicle of 0.5% w/v carboxymethyl cellulose and administered daily by oral gavage at a dose of 10 mg/kg, beginning 14 days after injection of cells. Bevacizumab was injected intraperitoneally twice weekly. Tumor length and width were measured using calipers and tumor volume calculated using the following formula: Volume = 0.5236 × length × width^2.

Immunohistochemistry

Tumor vessel density was analyzed in ~600 mm^2 formalin fixed tumors. Then, 3-μm sections were stained for endothelin (CD105, dilution 1:60, Novoceastra) detection with VECTOR M.O.M. Immunodetection Basic Kit (Vector Laboratories). The number of endothelial positive vessels in 5 randomly selected high-powered fields for control tumors (n = 6) or metformin (n = 9)–treated mice was determined. The average vessel area was calculated as the sum of the vessel area (SA) in 5 high-powered fields per tumor with area expressed as maximum length × maximum width (A = a × b), with the average taken for all water treated and metformin treated animals.

Statistical Analysis

The Student t test was performed for mRNA expression, tumor xenografts, soft agar, and VEGF-A ELISA assays, the Mann–Whitney ranks test was performed for the blood vessel number and area.
Disclosure of Potential Conflicts of Interest

All Institute of Cancer Research authors are part of a “Rewards to Inventors Scheme,” which could provide financial benefit to any authors that contribute to programs that are subsequently commercialized.

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