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Therapeutic Metformin/AMPK Activation Promotes the Angiogenic Phenotype in the ERα Negative MDA-MB-435 Breast Cancer Model

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Abstract

Metformin, a first line treatment for type 2 diabetes, has been implicated as a potential anti-neoplastic agent for breast cancers as well as other cancers. Metformin is known to work in part through the activation of AMP-dependent kinase (AMPK). AMPK is a key regulator of cellular energy homeostasis, especially under stress conditions where biosynthetic pathways are blocked by the phosphorylation of downstream AMPK substrates. Stimulation of AMPK by metformin resulted in a significant repression of cell proliferation and active MAPK1/2 in both estrogen receptor α (ERα) negative (MDA-MB-231, MDA-MB-435) and positive (MCF-7, T47D) human breast cancer cell lines. However, when ERα negative MDA-MB-435 cells were treated with metformin, they demonstrated increased expression of vascular endothelial growth factor (VEGF) in an AMPK dependent manner; while the ERα positive MCF-7 cells did not. Systemic therapy with metformin was tested for efficacy in an orthotopic model of ERα negative breast cancer performed in athymic nude mice. Surprisingly, metformin therapy significantly improved tumorigenic progression as compared to untreated controls. The metformin-treated group showed increased VEGF expression, intratumoral microvascular density and reduced necrosis. Metformin treatment was sufficient, however, to reduce systemic IGF-1 and the proliferation rate of tumor cells in vascularized regions. The data presented here suggests that, although metformin significantly represses breast cancer cell growth *in vitro*, the efficacy with respect to its therapeutic application for ERα negative breast cancer lesions *in vivo* may result in promotion of the angiogenic phenotype and increased tumorigenic progression.

Keywords

AMPK; Angiogenesis; Breast Carcinoma; MAPK; Metformin; VEGF

Introduction

The biguanide metformin is a first line treatment for patients with type 2 diabetes [1]. Metformin is known to be an insulin sensitization agent and it promotes reduced circulating insulin and glucose levels in hyper-glyceamic and hyper-insulineamic patients [2]. Interestingly, metformin has many other beneficial effects including the lack of additional weight gain or moderate weight loss when compared to other sulfonylureas. Metformin also has beneficial cardiovascular effects and is useful as a treatment for polycystic ovarian

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syndrome, see Kirpichnikov et al. for a review [1]. Some of these beneficial effects of metformin have been shown to work through the activation of the AMP-dependent kinase (AMPK). Treatment with metformin results in the activation of AMPK both *in vitro* and *in vivo* [3-6]. The activation of AMPK is well known to inhibit the expression of gluconeogenic genes and promote the expression of enzymes required for fatty acid oxidation [4].

Recent clinical studies have revealed that metformin treatment has been associated with reduced cancer risk. In a study of more than 10,000 diabetic patients being treated with metformin or other sulfonylureas, those that were treated with sulfonylureas had an increased risk of cancer-related mortality when compared to those patients on metformin [7]. In a second study using a smaller cohort, it was observed that patients treated with metformin had a lower incidence of cancer when compared to patients on other treatments [8]. Interestingly, this effect appeared to improve with higher doses of metformin. These two independent studies have led to the investigation of the use of metformin as an anti-neoplastic agent. Recently, one such study demonstrated that metformin was able to activate AMPK in a human breast carcinoma cell line, which resulted in decreased proliferation and a general decrease in protein synthesis *in vitro* [9]. Metformin has also been shown to inhibit glioma cell growth in low density cultures while promoting apoptosis in higher density cultures [10]. AMPK activation by 5-aminoimidazole-4-carboxamide riboside (AICAR) has also been shown to repress the growth of multiple myeloma cells, keratinocytes and MDA-MB-231 breast carcinoma cells *in vitro* [11-13]. A study by Rattan et al. demonstrated that a number of different cancer cell lines, including MCF-7 cells, experienced growth inhibition *in vitro* when treated with AICAR, and an *in vivo* glioma tumor model was inhibited with AICAR treatment [14]. Thus, AMPK activation has been demonstrated to have a potent anti-proliferative effect even in transformed tumor cells. However, there has been little work evaluating the potential utility of metformin with *in vivo* models of cancer. One recent study demonstrated that treatment with metformin selectively inhibited p53-null colon tumor growth, but not in wild-type p53 tumor cells [15]. Spontaneous breast tumor development was delayed in mice chronically treated with metformin in the Her-2/neu transgenic model where the tumors that did arise were smaller in size compared to untreated controls [16]. While metformin has been implicated in inhibiting cancer initiation and/or proliferation, its activation of AMPK may have undesired effects, especially when applied to phenotypically different breast or other cancer cell subtypes.

AMPK is a heterotrimeric protein composed of a catalytic alpha subunit, and two regulatory subunits, beta and gamma. The alpha subunit contains a serine/threonine protein kinase catalytic domain [17]. AMPK has been identified as a primary sensor of cellular energy change by responding to increases in AMP:ATP ratios, including hypoxia, nutrient deprivation, heat shock, metabolic poisoning and exercise in muscle tissues [18,19]. In addition, AMPK activation has been shown to significantly promote vascular endothelial growth factor (VEGF) expression [20-22] and angiogenesis [23,24]. However, activation of AMPK by metformin, its effect on VEGF expression, and the effect on tumor growth is not understood.

This study examined the effect of metformin on cell proliferation and cell survival in human breast carcinoma cell lines *in vitro* and in tumor progression *in vivo*. Specifically, this study analyzed the effect of metformin on repressing human breast cancer cell proliferation through AMPK activation and down regulation of the MAPK pathway. In addition, AMPK-dependent induction of survival genes such as VEGF and glucose transporter-1 (Glut-1) was examined in estrogen receptor alpha (ERα) positive and negative cells. The results of this study suggest that, while metformin reduces breast carcinoma cell proliferation both *in vitro* and *in vivo*, the activation of AMPK leads to significant VEGF production, angiogenesis and tumor progression. Metformin may be an effective anti-neoplastic agent as a preventative approach for some cancer cell types; however, the effect upon angiogenesis must be considered when metformin is employed as a therapeutic regime.

Materials and Methods

Cell Line and Culture Conditions, Antibodies and Chemical Effectors

Cell lines were purchased from the American Type Culture Collection (Manassas, VA). Human Breast Carcinoma cell lines, MDA-MB-435S and MDA-MB-231, were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, and penicillin (100 units/ml)-streptomycin (100 μg/ml). Human Breast Carcinoma cell lines, MCF-7 and T47-D, were maintained with DMEM or RPMI supplemented with 10% FBS, penicillin (100 units/ml)-streptomycin (100 μg/ml), and 0.01 mg/ml bovine insulin or 0.2 units/ml bovine insulin, respectively. Cells were cultured at 37°C in 5% CO_2. Antibodies used for immunoblots for AMPKα1 and AMPKα2 were from US Biological (Swampscott, MA), and antibodies for p-AMPK(Thr172), phospho-p44/42 MAPK(Thr202,Tyr204), and p44/42 MAPK were from Cell Signaling Technology (Beverly, MA), anti-phospho-Acetyl CoA carboxylase (Ser79) antibody was from Upstate (Lake Placid, NY) and the antibody for β-actin was from Abcam (Cambridge, MA). Metformin was purchased from Sigma-Aldrich (St. Louis, MO) and Compound C was from Calbiochem/EMD Biosciences (San Diego, CA).

Cell Growth Assays

Cell growth was determined using Methylthiazolyldiphenyl-tetrazolium bromide (MTT) (Sigma) following the manufacturer's protocol. Experiments were performed at least three times where n > 6 per experiment. Briefly, cell lines were plated at approximately 50% confluence in a 96 well plate and cultured with or without metformin (5 mM). MTT was added to the cell culture media at $1/10^{th}$ media volume, incubated for 2-4 hours at 37°C, solubilized with isopropanol and spectrophotometric absorbance measured at 570 nm and background at 690 nm. Results are expressed as mean ± SD.

SDS-PAGE and Immunoblots

Cytoplasmic extracts were obtained using Triton-X-100 lysis buffer (50mM Hepes, pH 7.5, 10mM sodium pyrophosphate, 150mM NaCl, 100mM NaF, 0.2mM $NaOVa_4$, 1mM EGTA, 1.5mM $MgCl_2$, 1% Triton X-100 and 10% glycerol, protease and phosphatase inhibitors added before use) and total cell extracts were obtained using RIPA lysis buffer (1X PBS, 1% NP40, 0.1% SDS, and 1.0% deoxycholate, protease and phosphatase inhibitors added before use). After indicated treatments cells were washed twice with cold PBS and lysed with appropriate lysis buffer. Protein concentrations were determined by Biorad DC Assay Kit according to manufacturers protocol (BioRad, Inc). Protein extracts were separated on 10% SDS-PAGE gels and transferred to nitrocellulose membranes. Immunoblots were performed using various primary antibodies and horseradish peroxidase-conjugated species appropriate IgG secondary antibodies diluted in blocking buffer according to manufacturer's protocols. Blots were developed using ECL reagents (KPL) on a Kodak Multimodel Imager (2000MM). Quantification of immunoblots was completed using the Kodak MI software.

Orthotopic Xenograft Breast Cancer Tumor Model

The tumor model was performed as previously described [25]. Briefly, 1×10^6 MDA-MB-435 cells expressing green fluorescent protein (GFP) were injected subcutaneously in the mammary fat pad of female athymic (nu/nu) mice and tumor growth determined throughout 12 weeks by external caliper measurements of each tumor and the estimated volume was calculated as $width^2 \times length \times 0.52$ to approximate ellipsoid volume. Animals were treated by oral delivery through autoclaved drinking water containing no drug or 5 mg/ml of metformin. The estimated dose of metformin per mouse was 750 mg/kg/day. Amber glass water bottles were replaced every three days. Ten mice were analyzed per treatment group. Animal weight and blood glucose was monitored weekly. Blood was drawn from the saphenous vein of each mouse,

alternate sides were used each week, and blood glucose levels were determined using the One Touch glucometer (Life Scan, Milpitas, CA).

Insulin-like Growth Factor (IGF-1) ELISA Assay

IGF-1 levels were determined by the Quantikine Mouse IGF-1 Immunoassy from R&D Systems, Inc. (Minneapolis, MN). Blood was obtained by cardiac blood draw at harvest. Blood samples were allowed to clot for two hours at room temperature and centrifuged for 20 minutes at 1000 × g. Serum was removed and stored at -80°C until assayed. Sample or control IGF-1 was assayed in duplicate.

Immunohistochemistry

Paraffin sections and cryosections and postfixed slides were used for immunohistochemical analyses with hemotoxylin and eosin stain and with antibodies to platelet endothelial cell adhesion molecule (PECAM-1 or CD31) (Pharmingen, San Diego, CA), VEGF (R&D Systems), proliferation with Ki67 (Dako North America, Inc., Carpinteria, CA) and apoptosis with ApopTag TUNEL kit (Intergen, Purchase, NY). Secondary detection was performed with appropriate biotinylated secondary antibodies and VectaStain Elite kit (Vector, Inc., Burlingame, CA) with diaminobenzidine substrate. Counterstain was performed with 1% methyl green. Negative control slides were obtained by omitting the primary antibody. The images were quantified by positive stain density using image analysis and recognition software, Image Pro Plus (Media Cybernetics, Silver Spring, MD), and averaged for three high-power fields/section/animal for 5 animals/group for each treatment group.

VEGF ELISA Assay

VEGF capture ELISA was performed as described previously [26]. Culture supernatants in triplicate were collected from cell culture wells and cleared by centrifugation. The samples were buffered with Tris-HCl pH 7.5 to a final concentration of 1 mM prior to analysis.

Statistical Analysis

Data from individual experiments were represented as mean ± standard error unless otherwise stated. Statistical comparison of groups was performed using 2-tailed student t-test or ANOVA test with appropriate tests for equal variances. Statistical significance was defined and indicated as $p \leq 0.05$ (*) or $p \leq 0.01$ (**).

Results

Metformin promotes AMPK activation in MDA-MB-435 breast carcinoma cells

Metformin is a known AMPK activator. To confirm AMPK activation in the MDA-MB-435 breast carcinoma cells by metformin, cells were treated with increasing doses of metformin for 24 hours, Figure 1A. Since AMPK phosphorylation at Thr172 has been associated with activation [27], the phosphorylation of AMPK at Thr172 was assessed by immunoblot to determine AMPK activation. Metformin treatment resulted in a dose dependent increase in AMPK activity. Expression levels of p-AMPK were quantified and normalized to total AMPK alpha 1 and beta-actin protein expression, Figure 1B.

A commonly used downstream target of AMPK, phosphorylated acetyl-CoA carboxylase (p-ACC) was also evaluated in the same set of extracts. Metformin treatment resulted in increased phosphorylation of ACC over the dose curve and was quantified and normalized to beta-actin protein expression, Figure 1C.

Metformin inhibits growth of human breast carcinoma cell lines *in vitro*

Metformin has been shown to affect the proliferation of several cancer cell lines *in vitro*, including the human breast cancer line, MCF-7 [9,10]. To assess the effect of metformin on the growth of breast cancer cell lines with various phenotypes, MDA-MB-435, MDA-MB-231, MCF-7 and T47-D cell lines were treated with 5 mM metformin and assessed for proliferation over time using the MTT cell viability assay. Interestingly, all cell lines were significantly affected by metformin treatment, Figure 2A-D. The growth of MCF-7 and T47-D cells was repressed by 82% and 96% respectively, when compared to untreated controls. However, the growth of the highly proliferative and invasive MDA-MB-435 and MDA-MB-231 cells was only affected by 40% and 29%, respectively. The level of growth inhibition and effect on cell viability by metformin on MDA-MB-435 cells was confirmed using direct cell counts and flow cytometery with propidium iodide labeling (data not shown). Cell viability remained greater than 94% across multiple experiments with 5 mM metformin treatment.

Metformin promotes repression of MAPK signaling in human breast carcinoma cell lines *in vitro*

Metformin treatment resulted in a large increase in phosphorylated AMPK in both MDA-MB-435 and MCF-7 cells when treated with metformin for 24 hours compared to untreated controls, Figure 3A. Coincident with the metformin-induced AMPK phosphorylation there was a decrease in phosphorylated MAPK1/2 (p-MAPK1/2) in both the MDA-MB-435 and the MCF-7 cells, although the MCF-7 cells were repressed to a greater degree, 63% vs. 80% respectively, Figure 3A. There was no change in the total MAPK protein with metformin treatment when signals were normalized to β-actin controls.

The expression level of AMPK catalytic subunits in MDA-MB-435 and MCF-7 cell lines were also evaluated by immunoblot, Figure 3B. The AMPKα1 isoform was predominant in both cell lines, Figure 3B. Interestingly, metformin treatment moderately reduced AMPKα1 in both cells with no discernable change in AMPKα2. Metformin was similarly effective at increasing the phosphorylation level of AMPK and repressing MAPK in the ERα negative MDA-MB-231 and the ERα positive T47-D cells (data not shown).

Systemic metformin treatment affects the growth of ERα negative MDA-MB-435 breast tumors *in vivo*

To determine if the metformin effect on cell proliferation observed *in vitro* would repress tumor growth *in vivo*, an orthotopic xenograft model of human breast cancer was implemented. Athymic nude mice were injected with MDA-MB-435 cells into the mammary fat pad and were treated with control drinking water or water containing metformin for a predicted dose of 750 mg/kg/day continuously beginning the same day as implantation. The metformin treatment group surprisingly demonstrated significantly increased tumor growth after 40 days of treatment, Figure 4A. At harvest, metformin-treated mice had an average tumor size four times that of untreated control mice. To assure that metformin treatment was effective in the mice, IGF-1 was evaluated in serum samples since IGF-1 has been shown to be repressed in both mice [16] and humans [28] by metformin therapy. The average serum IGF-1 was 33% lower in the metformin treated mice compared to the control group and was statistically significant, Figure 4B. Average blood glucose was not significantly different between the groups at any point during the treatment, Table 1.

Metformin treatment leads to increased viability and angiogenesis in MDA-MB-435 xenograft tumors

To investigate the effect of metformin treatment on overall tumor viability and angiogenesis, paraffin sections of tumors from each group were assessed by hematoxylin and eosin (H&E)

staining, Figure 5A. Tumor sections from control and metformin-treated groups were analyzed by quantitative digital analysis for the necrotic area and total area of each tumor and the average percent necrosis calculated for each group, Figure 5A. Tumors treated with metformin showed a significant reduction in necrotic area per section, which is surprising since necrosis is typically associated with increased tumor size. Angiogenesis was assessed by immunostaining tumor sections with an antibody to platelet endothelial cellular adhesion molecule 1 (PECAM-1/CD31) to label endothelial cells and the vasculature, Figure 5B. Analysis of three high power fields in each tumor section where viable tumor exists was quantified for each group, Figure 5B. Those tumors treated with metformin demonstrated a two-fold increase in the number of vessels compared to control tumors. The affect of metformin on tumor cell proliferation rates were also assessed *in situ* using an immunostain for the proliferation marker, Ki67, Figure 5C. Three high power fields per section were quantified in those areas where the most angiogenesis was determined by CD31 staining. The tumor cell proliferation in the highly vascularized areas was significantly decreased, by 47%, in those tumors treated with metformin when compared to control, Figure 5C.

Metformin treatment promotes VEGF expression in MDA-MB-435 cells but not MCF-7 cells in an AMPK dependent manner

To elucidate why metformin-treated breast tumors had significantly more vascular development than the control tumors, the expression of VEGF, a potent angiogenic growth factor, was assessed *in vitro.* MBA-MB-435 and MCF-7 cells not treated and treated with various doses of metformin were analyzed for VEGF mRNA expression using quantitative RT-PCR, Figure 6A. Only MDA-MB-435 cells demonstrated a significant increase in VEGF mRNA expression with metformin treatment for 24 hours with maximal induction of 2.5-fold with 1 mM and 10 mM metformin. To demonstrate the dependence of AMPK activity on the increased VEGF, the AMPK inhibitor compound C was used, Figure 6B. Cells treated with 10 mM metformin demonstrated a clear increase in VEGF mRNA expression when compared to controls. However, when compound C was combined with the maximum dose of metformin treatment, the increase of VEGF mRNA expression was inhibited. To determine whether the increased mRNA levels translated into synthesis and secretion of VEGF, the cell culture media was assessed by ELSIA for VEGF protein. Only the MDA-MB-435 cells produced an increase in secreted VEGF protein when treated with 5 mM metformin, whereas MCF-7 cells showed no increase and low VEGF levels overall, Figure 6C. In addition, MCF-7 cells had significantly less VEGF protein production at baseline when compared to the MDA-MB-435 cells. Interestingly, when another stress-induced gene was examined, glucose transporter-1(Glut-1), metformin treatment led to a significant increase in Glut-1 in both cell lines when compared to control, Figure 6D. These data suggest that metformin activation of AMPK may selectively promote VEGF expression in the ERα negative MDA-MB-435 cell line.

Metformin promotes VEGF expression in vascularized areas of breast tumor xenografts

Since metformin was able to induce VEGF expression in ERα negative MDA-MB-435 cells *in vitro*, it was critical to reassess the human breast xenograft tumors for their potential to express VEGF with metformin treatment. To evaluate localized VEGF production *in situ* within different areas of the tumor, the tumors were immunostained with an anti-VEGF antibody, Figure 7A. Small pockets of positively stained areas were observed in the untreated tumors, however the metformin treated tumors demonstrated multiple clusters of positive stained areas. Those areas that demonstrating the most VEGF staining were quantified using pixel-by-pixel analysis in three fields per section which showed a dramatic increase in VEGF levels in the metformin-treated tumors, Figure 7B. Since the areas of VEGF staining did not correspond to necrotic areas of the metformin-treated tumors, it was expected that areas of vascularization and perfusion would correspond to the VEGF expression observed in the metformin treated animals. To evaluate this possibility, tumor sections were double-stained

for tumor vessels using CD31 as well as VEGF, Figure 7C. VEGF was almost non-existent in areas with good vascularization in the control non-treated tumors whereas the metformin-treated tumors had significant clusters of VEGF staining directly adjacent to tumor microvessels. Immunostaining for carbonic anhydrase IX (CAIX), a hypoxia induced protein [29], was performed on each section to determine whether these microdomains might be areas of hypoxia; however there was no appreciable CAIX staining in either the control or the metformin-treated groups in the same areas of viable tumor, suggesting that this induction of VEGF was not due to lack of vascular flow within the tumor (data not shown).

Discussion

A recent report demonstrated that metformin treatment of MCF-7 breast carcinoma cells reduced cell proliferation *in vitro* and suggested that metformin could be used as an anti-neoplastic agent in breast cancer [9]. This study aimed to investigate the effect of metformin on several breast cancer lines *in vitro* as well as whether systemic metformin may be sufficient to repress to ERα negative breast tumor growth *in vivo*. It has been shown that a well known AMPK activator, AICAR, can repress cell growth, including MCF-7 cells [12,14]. AICAR has also been shown to repress the proliferation of multiple myeloma cells while activating AMPK and repressing MAPK *in vitro* [11]. However, in early studies AICAR was not effective in significantly promoting and sustaining AMPK activation in the MDA-MB-435 cell line (data not shown/unpublished findings).

The therapeutic benefits of metformin have been shown to require the AMPK upstream kinase LKB1 [30]. To assure that LKB1 is present in our cell lines we evaluated the expression of LKB1 by qRT-PCR and direct immunoblot which showed expression in each of the four human breast carcinoma cell lines, Supplemental Figure 1. However, this is in direct contrast to findings by Shen et al. that demonstrated both MDA-MB-435 and MDA-MB-231 cells lack LKB1 [31]. Possible epigenetic repression mechanisms may be at work in tumor cell lines maintained for extended periods in research laboratories, something that may affect AMPK activation studies, but have been controlled for in this study.

Screening the effectiveness of metformin on breast cancer phenotypes *in vitro* showed that all four human breast cancer cell lines demonstrated metformin activation of AMPK, repression of MAPK signaling and reduce cell proliferation. The two estrogen receptor positive (ERα positive) cell lines, MCF-7 and T47-D, demonstrated nearly a complete repression of cell growth under optimal growth conditions, while the estrogen receptor negative (Erα negative) cell lines, MDA-MB-435 and MDA-MB-231 demonstrated only a partial inhibition (45-63% of control). These levels of proliferation repression observed with metformin treatment correlated well with reduced phosphorylated MAPK1/2, suggesting that one primary effect of metformin was to limit MAPK signaling. These data suggest that although both ERα positive and ERα negative breast cancer cells demonstrate reduced cell growth with metformin treatment, the ERα negative cells are not as sensitive to metformin treatment with respect to limiting cell proliferation *in vitro*. To further examine metformin as a potential treatment for breast cancer through systemic intervention, a well-characterized orthotopic breast cancer tumor model was implemented [25,32]. The metformin treated group demonstrated a clear decrease in serum IGF-1 as expected confirming metformin-dependent effects *in vivo*. The tumors in the metformin treated group were larger, as determined by external caliper measurements, and were found to have reduced intra-tumoral necrosis. This dramatic effect of systemic metformin, was in sharp contrast to the observed repression of proliferation observed for the tumor cells *in vitro*. Careful histological analysis of the tumors demonstrated that metformin treatment did reduce tumor cell proliferation in highly vascularized areas, indicating that within close proximity of perfused vessels, metformin may be sufficient to block the

proliferation signal *in vivo* as well. However, metformin appeared to significantly promote a strong angiogenic response in these tumors as determined by microvascular density analysis.

Since metformin treatment promoted tumor growth *in vivo*, especially after showing significant repression of tumor cell growth *in vitro*, and considering that there was a significant increase in angiogenesis in metformin treated tumors, we hypothesized that the activation of AMPK by metformin may have resulted in excess VEGF production in these tumors. It has previously been demonstrated that AMPK activation can contribute to increased VEGF expression and angiogenesis [21-24]. Direct assessment of this possibility revealed the surprising discovery that the Erα negative cell line, MDA-MB-435, showed a significant increase in VEGF mRNA and VEGF protein secretion with metformin treatment *in vitro*. This increase in VEGF mRNA expression was shown to be dependent on AMPK activity. The use of the AMPK chemical inhibitor, compound C, blocked the metformin induced increase of VEGF mRNA. In contrast, the ERα positive MCF-7 cell line did not respond with increased VEGF expression at all, even though another survival gene, Glut-1, was positively induced with metformin treatment in both cell lines. When tumor samples were analyzed to determine whether this might explain the increased angiogenesis observed in the metformin treated animals, there was a clear increase in VEGF staining in the metformin treated tumors when compared to control tumors. Interestingly, the VEGF staining was associated, and in fact adjacent to, CD31 positive vessels suggesting that the increased VEGF expression was occurring in well vascularized areas. These areas did not demonstrate CAIX expression indicating that they were not stressed by hypoxia or possible areas of aberrant vascular flow. These data indicate that metformin can contribute to significant VEGF expression in the ERα negative MDA-MB-435 breast tumors, which may override its anti-proliferation effect. This increase in VEGF mediated by metformin and AMPK activation, particularly in non-stressed tumor cells apparently results in an effective pro-angiogenic response and effective tumor progression. Since AMPK did induce survival gene expression, such as Glut-1, but not VEGF expression in the ERα positive MCF-7 cells, determining whether metformin would promote or repress tumors in this model is something that has to be tested directly.

The potential utility of AMPK activating agents, such as metformin, in repressing tumor proliferation, could be a promising approach to limiting tumor progression or recurrence in certain breast cancer subtypes. However, there should be a level of caution with respect to global application of this therapeutic approach, especially for breast cancer. The data presented here indicate that phenotypic differences in tumor cells could dictate alternative responses to AMPK activation with metformin. One adverse response of excess VEGF expression could result in a strong angiogenic response that may supercede the anti-proliferative effect seen with most tumor cells treated with metformin. It is likely that Erα positive breast cancer phenotypes may be selectively responsive to therapeutic intervention by metformin since the activation of VEGF production is significantly muted in these cells as compared to ERα negative cell lines and the anti-proliferative effect of metformin is substantial. It is still not clear whether specific transformation pathways could augment the induction of VEGF through AMPK activation or the effect of metformin on the proliferation rates. However, it is interesting that the Her-2/neu model of endogenous initiation and breast cancer progression appears to be delayed with metformin treatment [16], suggesting that metformin may still be effective during early tumor initiation events.

Activation of AMPK by various stimuli, including metformin, has been shown to lead to inhibition of fatty acid metabolism and specifically inhibition of fatty acid synthase (FAS) [5,33-36]. However, a study by Menendez et al. found that the inhibition of FAS lead to a significant and drastic increase in VEGF expression [37]. Similar to our findings, this study indicates that treatments that would inhibit fatty acid metabolism and FAS should be thoroughly investigated before being used as a cancer therapy.

Since metformin is a first line therapeutic for type-2 diabetes and may be applied to a large number of women for treatment of metabolic disorders, the possible effect that metformin may have on the promotion of breast cancer lesions may be possible, but likely would be restricted to pre-menopausal women who are more likely to have ERα negative lesions. The widespread therapy with metformin in the elderly could produce a chemopreventative effect on tumor progression in post-menopausal women since the majority of primary breast lesions in this cohort are more likely to be phenotypically ERα positive [38,39]. However, given a particular case where a primary lesion was ERα negative, it may be prudent to consider alternative metabolic therapeutics that would not increase the risk of recurrence amplification of the tumorigenic and angiogenic pathways by AMPK signaling and increased VEGF production.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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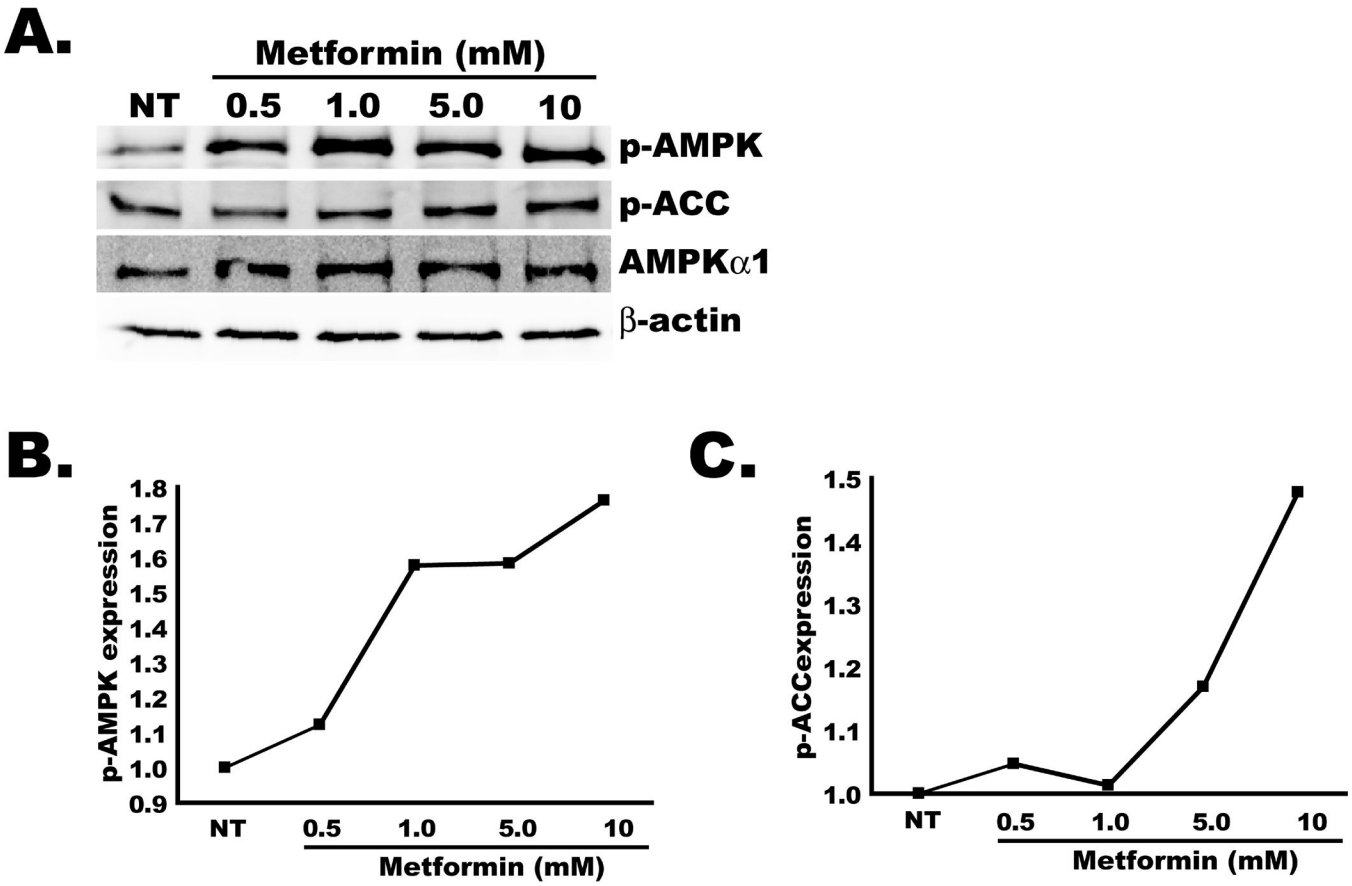

Figure 1. Metformin treatment promotes AMPK activity in MDA-MB-435 breast cancer cells

A. Human breast carcinoma cells, MDA-MB-435, were treated with increasing doses of metformin. Immunoblots were performed on cytoplasmic extracts and analyzed for **p-AMPK** (Thr172), **p-ACC** (Ser79), **AMPKα1**, and **β-actin** proteins. **B.** Expression levels of p-AMPK and p-ACC were quantified and normalized to total protein levels.

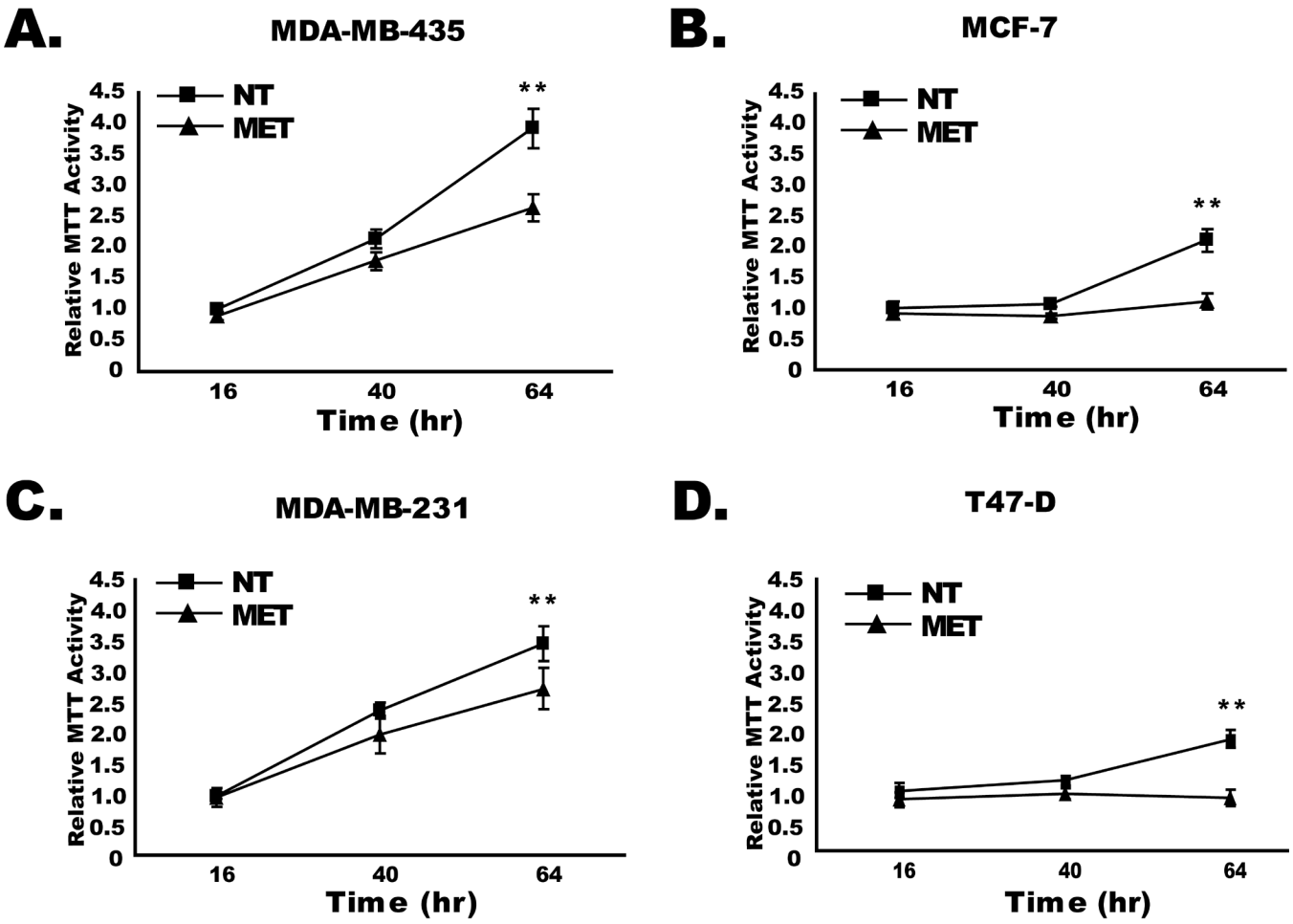

Figure 2. Metformin represses breast carcinoma cell line growth *in vitro*

Human breast carcinoma cell lines were treated with (▲) and without (■) metformin and cell growth was determined via MTT assay over time (hrs). **A.** MDA-MB-435 cell line. **B.** MCF-7 cell line. **C.** MDA-MB-231cell line. **D.** T47-D cell line. Data is represented as average relative MTT activity ± standard deviation. (** = $p \leq 0.01$)

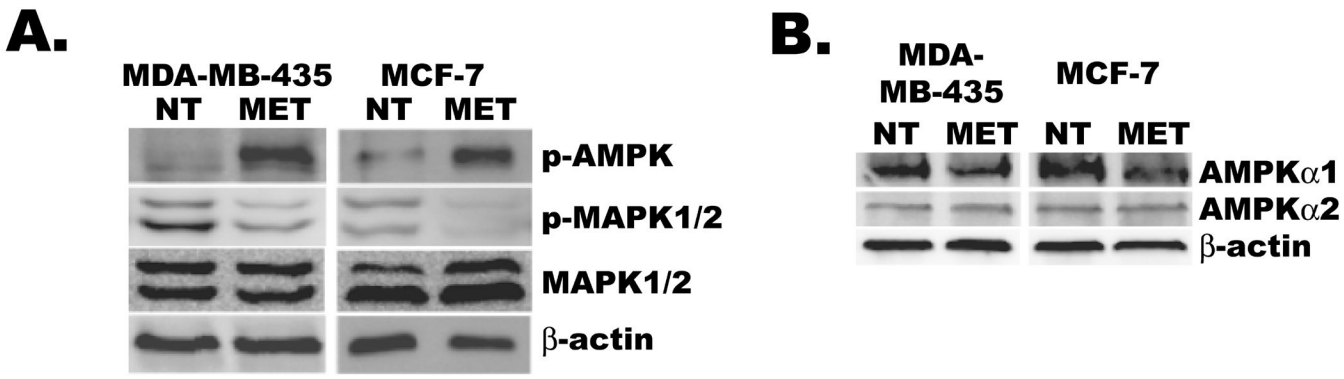

Figure 3. Metformin activates AMPK and represses phosphorylation of MAPK *in vitro*

MDA-MB-435 and MCF-7 cells were treated for 24 hours with or without **(NT)** metformin **(MET)**. **A.** Cytoplasmic extracts were analyzed for **p-AMPK** (Thr172), **p-MAPK1/2** (Thr202, Tyr204), total **MAPK1/2**, and **β-actin**. **B.** Total cell extracts were analyzed for AMPK alpha isoform expression (**AMPKα1, AMPKα2**) and **β-actin**. Metformin inhibition of p-MAPK1/2 expression was quantified by normalizing p-MAPK1/2 levels to total MAPK1/2 protein expression then to β-actin loading control. Repression of p-MAPK1/2 in MDA-MB-435 was 63% and in MCF-7 was 80% when compared to control samples.

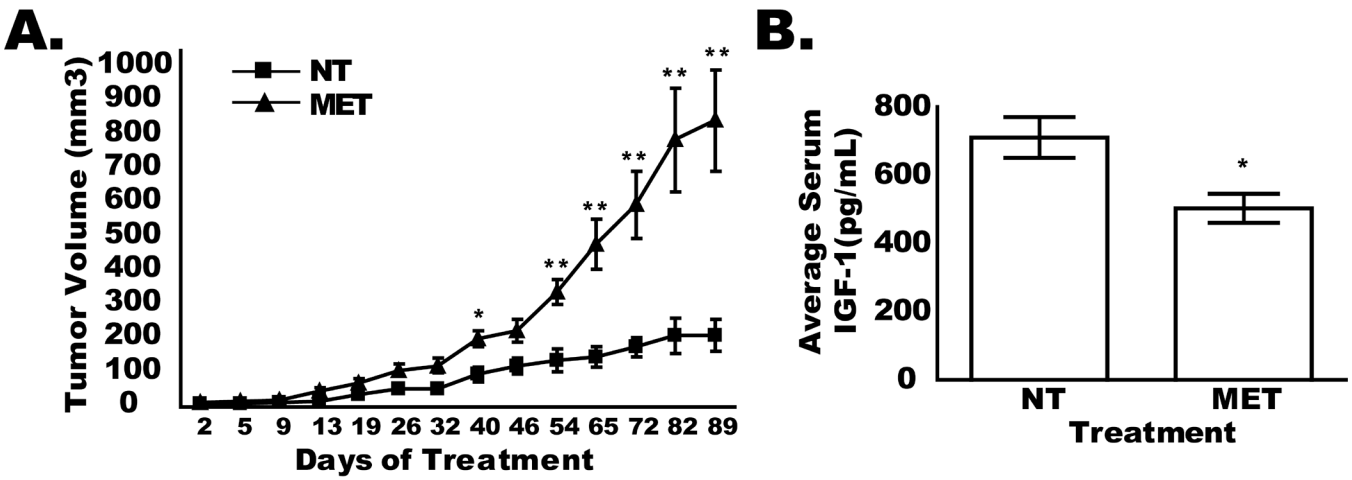

Figure 4. Effect of Systemic Metformin Treatment on Xenograft Breast Tumor Growth

Athymic female mice with orthotopic xenograft MDA-MB-435 breast tumors were treated with control (■)or metformin (▲) drinking water. **A.** Tumor growth was measured over time (days) using external measurements to estimate tumor volume (mm^3). **B.** Average serum IGF-1 was determined for each group – control (**NT**) or metformin (**MET**) at harvest. (* = $p \leq 0.05$, ** = $p \leq 0.01$)

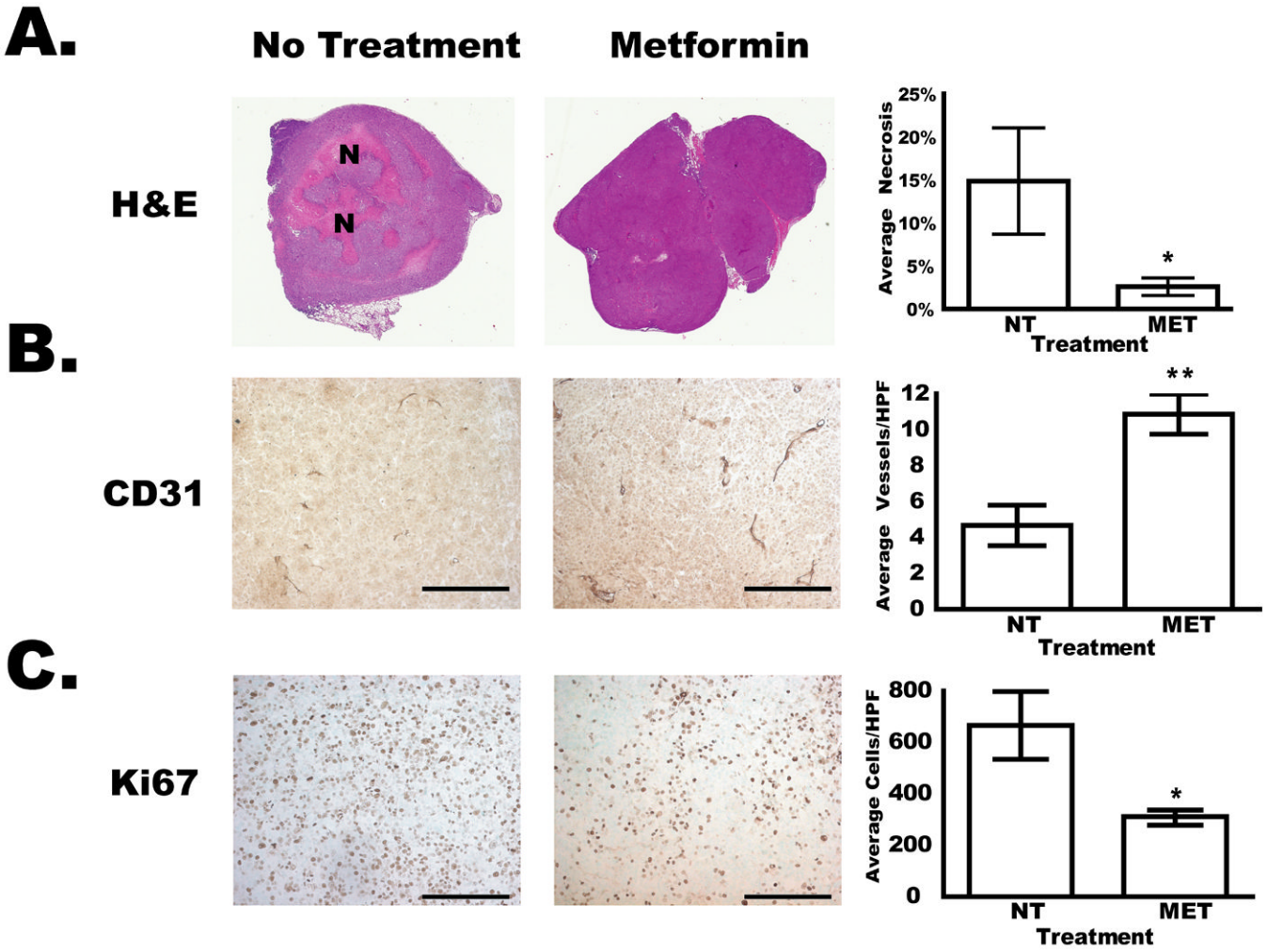

Figure 5. Effect of Metformin Treatment on Tumor Viability, Angiogenesis, and Proliferation

Immunohistochemical analysis of human breast xenograft tumors and quantification of three 40X images/animal for each of the stains. **A.** Scan of representative **H&E** stained tumor sections and quantification of tumor viability in control/**no treatment** and **metformin** treated tumors. Necrotic (**N**) areas are indicated. **B**. Representative images and quantification of **CD31** immunostains from no treatment or metformin treated tumors. **C.** Representative images and quantification of **Ki67** immunostains from no treatment or metformin treated tumors. (bar = 100 microns) **CD31** and **Ki67** images were quantified by measuring the density of the positive stained area in three 40X images/animal and normalized to average vessel size or average positive cell size respectively. (* = $p \leq 0.05$)

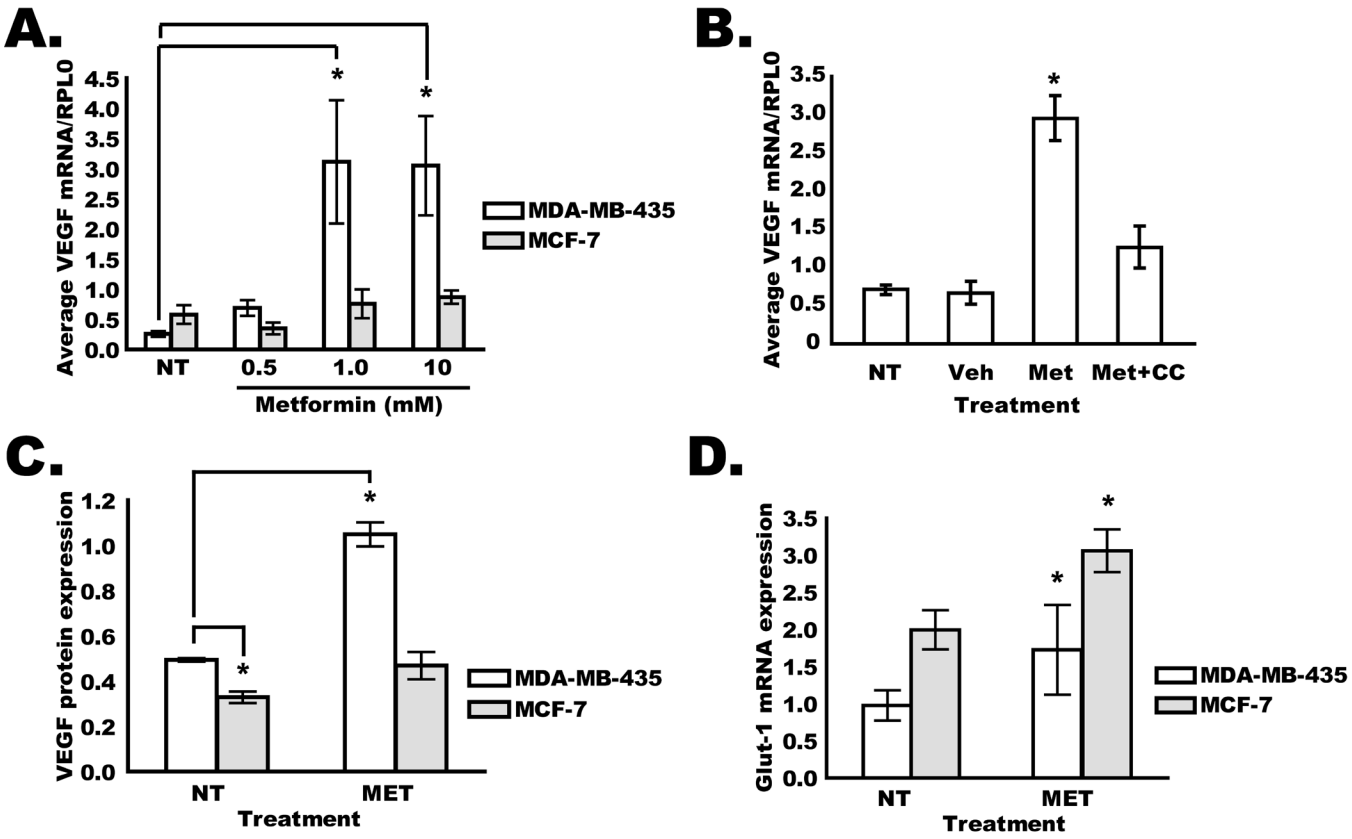

Figure 6. Effect of Metformin on VEGF production in breast carcinoma cell lines

A. Human breast carcinoma cells were treated with and without indicated doses of metformin for 24 hours. **A.** Quantitative RT-PCR for VEGF mRNA expression normalized to control gene in MDA-MB-435 cells (□) and MCF-7 cells (■)**. B.** MDA-MB-435 cells were treated with controls (**NT** or **Veh**), metformin (**Met**), or metformin and the AMPK inhibitor, compound C (**CC**), in combination and VEGF mRNA expression was determined by qRT-PCR. **C.** Conditioned media from MDA-MB-435 (□) and MCF-7 (■) cell culture samples treated with and without metformin were assessed for the presence of VEGF protein by ELISA and normalized to total protein. **D.** Quantitative RT-PCR for Glut-1 mRNA expression normalized to control gene in MDA-MB-435 cells (□) and MCF-7 cells (■). (* = $p \leq 0.05$)

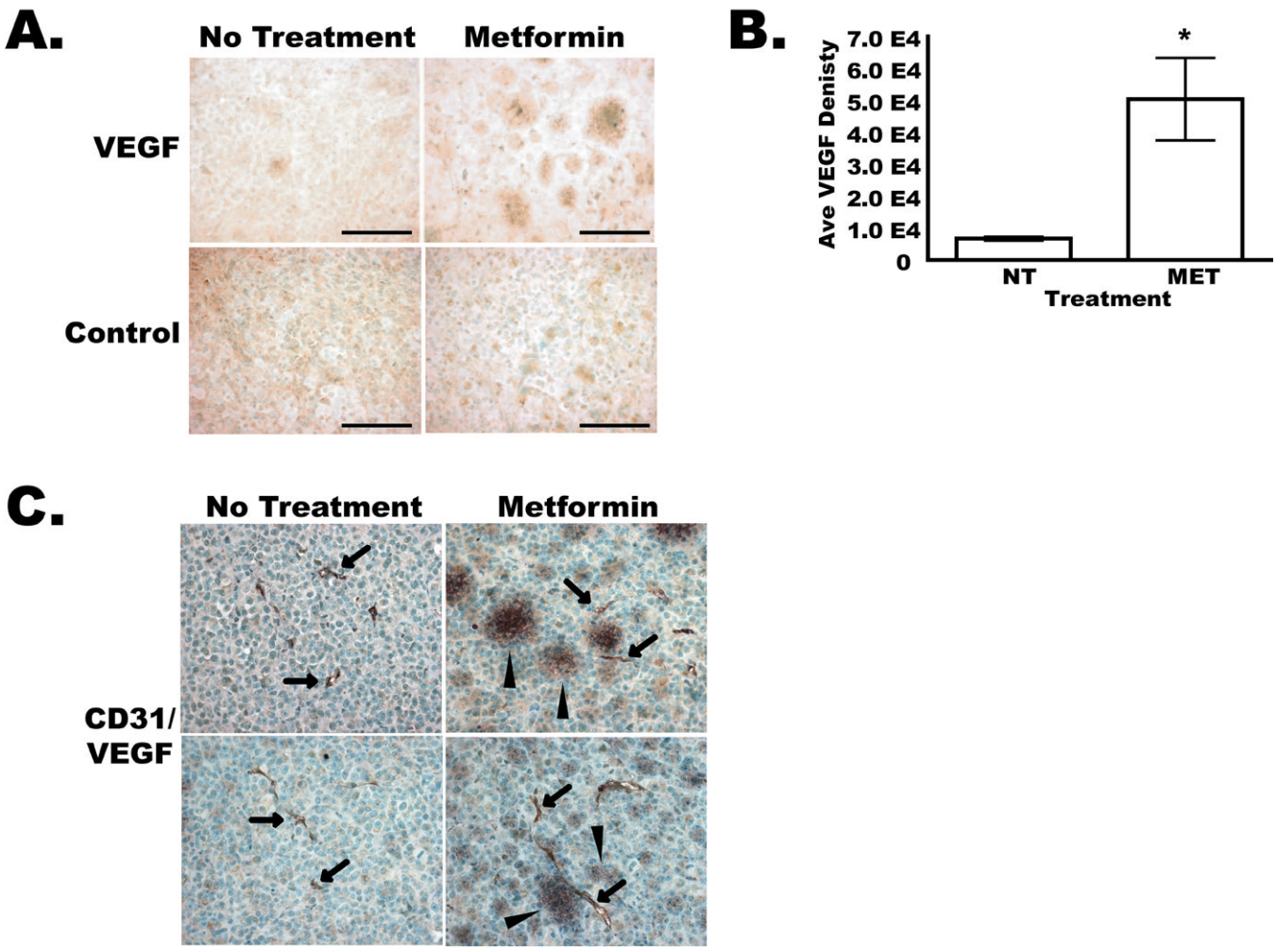

Figure 7. Effect of Metformin on VEGF expression *in vivo*

A. Tumor sections from the human breast tumor xenografts were assessed by immunostain for **VEGF**. Secondary alone **control** slides are also shown. (bar = 100 microns) **B.** Quantification of three 40X images/animal for VEGF expression normalized to cell density. **C.** Immunostained tumor sections for both VEGF (purple) and CD31 (brown). Vessels are indicated black arrows and pockets of VEGF are indicated by white arrow heads. (* = $p \leq 0.05$)

Table 1

Effect of Metformin on Blood Glucose Levels

Representative average blood glucose levels for no treatment control mice and mice treated with metformin during breast tumor model (average ± SEM). Asterisks indicate that animals were fasted for 4 hours before testing.

Time	Group	
Treatment Week	No Treatment (mg/dL)	Control (mg/dL)
Week 1	120.75±7.75	139.00±13.15
Week 5*	90.40±10.07	92.00±10.89
Week 8*	83.00±9.37	75.00±12.03