Metformin Promotes AMP-activated Protein Kinase-independent Suppression of ΔNp63α Protein Expression and Inhibits Cancer Cell Viability*

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The blood glucose modifier metformin is used to treat type II diabetes and has also been shown to possess anticancer activities. Recent studies indicate that glucose deprivation can greatly enhance metformin-mediated inhibition of cell viability, but the molecular mechanism involved in this inhibition is unclear. In this study, we report that, under glucose deprivation, metformin inhibited expression of ΔNp63α, a p53 family member involved in cell adhesion pathways, resulting in disruption of cell matrix adhesion and subsequent apoptosis in human squamous carcinoma cells. We further show that metformin promoted ΔNp63α protein instability independent of AMP-activated protein kinase and that WWP1, an E3 ligase of ΔNp63α, was involved in metformin-mediated down-regulation of ΔNp63α levels. In addition, we demonstrate that a combination of metformin and the glycosylation inhibitor 2-deoxy-D-glucose significantly inhibited ΔNp63α expression and also suppressed xenographic tumor growth in vivo. In summary, this study reveals a new mechanism for metformin-mediated anticancer activity and suggests a new strategy for treating human squamous cell carcinoma.

Metformin is widely used to treat type II diabetes. Metformin can decrease blood glucose via enhancing cell sensitivity to insulin (1), inhibition of the mitochondrial respiratory chain (complex I), activation of AMP-activated protein kinase (AMPK), and inhibition of glucagon-induced elevation of cAMP. Metformin can also suppress hepatic glucose production and enhances peripheral glucose uptake (2–4).

In recent years, accumulating clinical evidence indicates that metformin may have anticancer activities. It has been reported that tumor incidence is reduced in cancer patients with diabetes treated with metformin (5). Metformin can inhibit tumor cell growth and survival, induce cellular senescence (6, 7), and enhance cancer cell chemosensitivity (8). At the molecular level, metformin has been shown to inhibit mammalian target of rapamycin (mTOR) signaling (9) and suppress expression of cyclin D1 and ErbB2 (Her2) (10–12).

AMPK is a critical energy sensor in maintaining cell metabolism homeostasis. AMPK is a heterotrimer consisting of three subunits (α, β, and γ). Metabolic stresses, including glucose deprivation and hypoxia, can active AMPK via phosphorylation of Thr-172 mediated by upstream kinases, including LKB1 and calcium/calmodulin-dependent protein kinase kinase (CaMKK) (13–15). AMPK has been shown to suppress acetyl-CoA carboxylase and mTORC1 kinase activity (16, 17) to stimulate p53-dependent apoptosis (18) and modulate Sirt 1 activity (19). It has been reported that metformin can activate AMPK via Thr-172 phosphorylation to induce cell cycle arrest (11) and apoptosis (20).

p63 is a p53 family member involved in multiple facets of biological processes, including embryonic development, cell proliferation, differentiation, survival, apoptosis, senescence, and aging (21). The p63 gene encodes multiple protein isoforms that are derived from two different promoters at the N terminus and alternative splicing at the C terminus. ΔNp63α is the predominant p63 protein isoform expressed in epithelial cells and is essential for epithelial development (22). ΔNp63α is frequently overexpressed in squamous cell carcinoma and has been reported to promote cancer cell proliferation and survival (23). On the other hand, it has been shown that ΔNp63α is a master transcriptional regulator of cell adhesion program through regulation of several adhesion molecules, including integrin β4, integrin α6, fibronectin1, and E-cadherin (24).

2-Deoxy-D-glucose (2-DG) is a glucose analog in which the 2-hydroxyl group is replaced by hydrogen. It suppresses glycolysis by competitively inhibiting the production of glucose-6-P from glucose (25). Because 2-DG hampers cell growth, it was developed as a potential tumor-therapeutic drug, and it is currently in clinical trials (26). However, it is not completely clear how 2-DG inhibits cell growth.

In this study, we show that metformin inhibited ΔNp63α expression, resulting in disruption of cell matrix adhesion and subsequent apoptosis in human squamous carcinoma FaDu cells. Glucose deprivation dramatically facilitated the action of...
metformin. Combination of metformin and 2-DG significantly inhibited ΔNp63α expression and xenograft tumor growth in vivo. Together, this study reveals a new mechanism for metformin-mediated anticancer activity and suggests a new strategy for treating human squamous cell carcinoma.

**Results**

**Metformin Reduces Cancer Cell Viability under Glucose Deprivation**—It has been reported that metformin possesses anticancer activities, but the molecular mechanism(s) is/are not entirely clear. To examine the effects of metformin on human squamous cancer cells, we first treated human head and neck squamous cell carcinoma FaDu cells in the absence or presence of metformin. As shown in Fig. 1, A and B, Metformin treatment of FaDu cells (grown under 4.5 mg/ml glucose) for 36 h had little effect on viability. Treatment with metformin for a longer time (48 h) led to significantly reduced cell viability, examined by MTS and trypan blue exclusion assays. By contrast, cell viability was comparable in the absence of metformin. Because culturing cells consumed glucose, we speculated that glucose concentration may be an important factor affecting metformin efficacy. We therefore examined the influence of glucose deprivation (1.0 mg/ml) on the effects of metformin. As shown in Fig. 1, C and D, treatment of metformin for 24 h dramatically reduced cell viability. In addition, data from Western blotting analyses for cleaved PARP1, a marker of apoptosis, and data from FACS analyses indicated a significant increase in apoptosis.
2-DG is an inhibitor of Glycolysis. Therefore, we examined whether 2-DG can affect the effect of metformin on cancer cell viability. As shown in Fig. 1, G–J, treatment with metformin or 2-DG, alone had little effect on the viability of FaDu cells under 4.5 mg/ml glucose. By sharp contrast, combined treatment of metformin and 2-DG significantly reduced FaDu cell viability, as evidenced by MTS and trypan blue exclusion assays, Western blotting for cleaved PARP1, and FACS analyses. Furthermore, similar to FaDu cells, metformin significantly reduced the cell viability of human lung adenosquamous carcinoma H596 cells and human mucoepidermoid pulmonary carcinoma H292 cells under glucose deprivation or treatment with 2-DG (Fig. 1, K and L).

Metformin Reduces Cell Matrix Adhesion Concomitant with Reduced ΔNp63α Expression—The abovementioned results indicated that metformin can dramatically reduce cancer cell viability under glucose deprivation. Notably, metformin treatment resulted in dramatic cell detachment under either low glucose (1.0 mg/ml) or with 2-DG (Fig. 2, A and B). We therefore examined the effects of metformin on the expression of adhesion molecules involved in cell matrix interaction, including integrin β1, integrin β4, integrin α6, and fibronectin 1. As shown in Fig. 2C, metformin significantly inhibits these adhesion molecules, except for integrin β1. Because ΔNp63α is known to regulate the cell adhesion program in epithelial cells (24), we investigated whether metformin can impact the expression of ΔNp63α. Treatment of metformin for 24 h did not affect ΔNp63α expression at 4.5 mg/ml glucose whereas it led to a dramatic decrease in ΔNp63α expression at 1.0 mg/ml glucose (Fig. 2D) in a dose- and time-dependent manner (Fig. 2, E and F). In addition, 2-DG alone had little effect on ΔNp63α expression. However, 2-DG significantly enhanced metformin-induced down-regulation of ΔNp63α under 4.5 mg/ml glucose (Fig. 2G). Moreover, metformin also dramatically decreased
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ΔNp63α expression in H596 and H292 cells under 1.0 mg/ml glucose (Fig. 2H). Together, these findings indicate that metformin can significantly down-regulate ΔNp63α expression under glucose deprivation concomitant with cell matrix disruption.

Metformin Induces ΔNp63α protein Instability Independent of AMPK—We next investigated the molecular mechanism with which metformin down-regulates ΔNp63α protein expression. We examined the effects of metformin on ΔNp63α protein half-life by cycloheximide (CHX) treatment. As shown in Fig. 3A, metformin dramatically reduced ΔNp63α protein half-life. In addition, metformin-induced down-regulation of ΔNp63α protein was significantly, but not completely, blocked by MG132, suggesting that the proteasome is largely involved in metformin-mediated ΔNp63α protein instability (Fig. 3B).

Because we have shown previously that WWP1, an ubiquitin E3 ligase, regulates ΔNp63α protein stability (27), we examined the effect of metformin on WWP1 expression. As shown in Fig. 3C, metformin evidently increased WWP1 protein levels but not ITCH, which is documented as another ΔNp63α E3 ligase (28). Silencing of WWP1 led to a clear increase in ΔNp63α protein levels and restored, in part, expression of ΔNp63α upon metformin treatment (Fig. 3D). These data indicate that WWP1 is important in metformin-mediated destabilization of ΔNp63α.

It has been reported that metformin can inhibit mTOR signaling to impact cell proliferation and survival (29). We therefore examined the effect of mTOR on the expression of ΔNp63α. Our data showed that metformin significantly inhibited phosphorylation of both mTOR and S6K concomitant with down-regulation of ΔNp63α. Knockdown of mTOR1 or rapamycin treatment led to a marked reduction in ΔNp63α protein.
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Levels (Fig. 3, E–G). These data suggest that mTOR signaling is involved in metformin-mediated down-regulation of $\Delta$Np63$\alpha$. Furthermore, Q-PCR analyses showed that metformin significantly decreased steady-state $\Delta$Np63$\alpha$ mRNA levels (Fig. 3H).

It is well documented that metformin can activate AMPK (30, 31) and inhibit mTOR signaling (9). When examined, metformin reduced phosphorylation of mTOR and S6K, as expected. However, metformin had little effect on AMPK phosphorylation in FaDu cells (Figs. 2 and 3F), suggesting that metformin failed to activate AMPK in this experimental setting. To further examine the role of AMPK in metformin-mediated regulation of $\Delta$Np63$\alpha$, we performed shRNA experiments. As shown in Fig. 3I, silencing of AMPK$\alpha_1$ had no significant effect on $\Delta$Np63$\alpha$ expression. Furthermore, metformin could still dramatically decrease $\Delta$Np63$\alpha$ expression upon silencing of AMPK$\alpha_1$ (Fig. 3K). Together, these data indicate that metformin inhibits $\Delta$Np63$\alpha$ expression independently of AMPK.

$\Delta$Np63$\alpha$ is critical in metformin-induced cell matrix adhesion and cell death—Because metformin disrupts cell matrix adhesion and induces subsequent apoptosis concomitant with down-regulation of $\Delta$Np63$\alpha$ expression, we first demonstrated that suspension of FaDu cells led to a significant decrease in cell viability (Fig. 4A). We also found that silencing of p63 led to significant down-regulation of several adhesion molecules, including fibronectin and integrin $\beta_4$ (Fig. 4B). We therefore investigated whether reduced $\Delta$Np63$\alpha$ expression accounts for metformin-mediated cell detachment and apoptosis. As shown in Fig. 4, C–E, ectopic expression of $\Delta$Np63$\alpha$ failed to reduce cell detachment and/or apoptosis, suggesting that other factors, in addition to $\Delta$Np63$\alpha$, are critical to overcome the effects of metformin. Ectopic expression of $\Delta$Np63$\alpha$ increased expres-
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Combination of Metformin and 2-DG Inhibits ΔNp63α Expression and Suppresses Tumor Growth in Vivo—Because our aforementioned results indicate that glucose deprivation or 2-DG significantly enhances metformin-induced cell apoptosis, we examined the effects of metformin and 2-DG on tumor cell growth in vivo. Results from tumor xenograph experiments showed that metformin and 2-DG together significantly inhibited tumor growth, as evidenced by measurement of tumor volume (reduced about 50%) (Fig. 5A), weight (reduced about 40%) (Fig. 5B), and tumor size (Fig. 5C). Notably, metformin and 2-DG together dramatically down-regulated p63 expression, as evidenced by immunohistochemical staining of p63 in tumor sections (Fig. 5D). These results indicate that inhibition of glycolysis is critical in metformin-mediated suppression of tumor growth in vivo.

Discussion

Metformin is frequently used to treat type II diabetes. Emerging evidence indicates that metformin also has antitumor activities. In this study, we show that metformin induces cell death upon glucose deprivation or inhibition of glycolysis. We further demonstrate that metformin promotes ΔNp63α instability independent of AMPK, leading to disrupted cell matrix adhesion and subsequent cell apoptosis. In addition, we show that WWP1 is critical in metformin-mediated inhibition of ΔNp63α stability. Moreover, we demonstrate that combination of metformin and 2-DG significantly inhibits xenographic tumor growth in vivo.

It has been well documented that metformin can decrease blood glucose levels via several means, including enhancing cell sensitivity to insulin (1), suppressing hepatic glucose production, and enhancing peripheral glucose uptake (2–4). Notably, it has been reported that glucose deprivation significantly impacts metformin anticancer activity (32, 33), and most recently, Sabatini and co-workers (34) showed that glucose limitation is important for cancer sensitivity toward phenformin, a member of the biguanide class like metformin; however, the mechanisms remain unclear. Our data clearly demonstrate that glucose deprivation or inhibition of glycolysis by 2-DG is essential for metformin effects on cell viability, and down-regulation of ΔNp63α is critically important in this process. Notably, glucose deprivation has a more profound effect on metformin-induced apoptosis than that of 2-DG, suggesting that use of 2-DG (20 mM) under normal glucose (4.5 mg/ml) cannot mimic glucose deprivation (1.0 mg/ml) with regard to down-regulation of ΔNp63α.
Our results show that metformin effectively induces cell detachment from the matrix and subsequent apoptosis under glucose deprivation. Work by us and other groups has demonstrated that AΔNp63α functions as a master transcription regulator for a set of genes involved in cell adhesion, including integrins and fibronectin (24) as well as CD82 (35). Our results show that metformin inhibits AΔNp63α expression upon glucose deprivation, resulting in decreased expression of fibronectin, integrin α6, and integrin β4, which leads to cell detachment from the matrix and subsequent apoptosis, a process termed anoikis (36). Notably, ectopic expression of AΔNp63α can completely reverse metformin-induced cell detachment and apoptosis in the presence of exogenous fibronectin. In our experimental system, exogenous fibronectin is required likely because of inefficient restoration of fibronectin expression in the presence of metformin.

How does metformin inhibit AΔNp63α expression? We show that metformin promotes proteasome-dependent AΔNp63α protein degradation. ITCH and WWP1 are two major ubiquitin E3 ligases of AΔNp63α (27, 28). Notably, treatment of metformin leads to up-regulation of WWP1 expression. Silencing of WWP1 leads to partial recovery of AΔNp63α expression in the presence of metformin, suggesting that WWP1 and other factors as well play important roles in metformin-mediated inhibition of AΔNp63α. In addition, it has been reported that metformin can inhibit mTOR signaling. In this study, we also found that metformin inhibits mTOR signaling and that silencing of mTOR or inhibition of mTOR by rapamycin can also decrease AΔNp63α expression. Furthermore, metformin can also suppress AΔNp63α mRNA expression. Together, our data indicate that metformin impacts AΔNp63α expression at multiple levels.

AMPK plays a critical role in maintaining cell energy homeostasis and is important in the regulation of various biological processes, including cell proliferation, survival, senescence, and autophagy (16, 17, 37). It has been reported that metformin inhibits cancer cell growth via activation of AMPK, leading to activation of p53 (38) and inhibition of the mTOR pathway (29). In this study, we show that metformin inhibits of AΔNp63α expression in an AMPK-independent manner. First, metformin fails to activate AMPK, whereas it inhibits AΔNp63α expression in FaDu cells. Second, knockdown of PRKAA1 (AMPKα1) does not affect AΔNp63α expression. Most importantly, silencing of AMPKα1 does not block metformin-mediated down-regulation of AΔNp63α expression. Notably, metformin effects on cancer cell survival independent of AMPK have been reported previously (39, 40).

This study reveals a new mechanism by which metformin induces anoikis under glucose deprivation (Fig. 6). It is of interest that combination of metformin and 2-DG significantly inhibits AΔNp63α expression and xenographic tumor growth in a mouse model, suggesting a new strategy for treating squamous cell carcinoma.

**Experimental Procedures**

**Cell Culture and Drug Treatments—**Human head and neck squamous cell carcinoma (HNNSCC) FaDu and HEK 293T cells were cultured in DMEM supplemented with 10% FBS (Hyclone). Human lung adenosquamous carcinoma H596 cells were cultured in DMEM supplemented with 10% FBS and 1% FBS. Notably, ectopic expression of AΔNp63α can completely reverse metformin-induced cell detachment and apoptosis in the presence of exogenous fibronectin. In our experimental system, exogenous fibronectin is required likely because of inefficient restoration of fibronectin expression in the presence of metformin.

**Glucose Deprivation—**Cells at 80% confluence were transfected using Lipofectamine 2000. Cells grown to 75–85% confluency were treated with chemical compound(s), as indicated. Metformin (PHR1084), 2-DG (D8375), cycloheximide (R750107), and MG132 (M8699) were purchased from Sigma.

**Plasmid Transfection, Lentiviral Infection, and RNA Interference—**Cells at 80% confluence were transfected using Lipofectamine 2000 transfection reagent (Invitrogen). Lentiviruses were amplified by transfected HEK 293T cells with psPAX2 and pMD2.G packaging plasmids and lentivirus expression plasmid using Lipofectamine 2000. Viruses were collected at 60 h after transfection. Cells at 50% confluence in the presence of 10 µg/ml Polybrene were infected with a recombinant lentivirus or an empty vector, followed by 12-h incubation at 37 °C with 5% CO2. Lentivirus-based shRNAs specific for GFP (GAAGCAGCACGACTTCTTC), WWP1-1 (TCTGTAACTAAAGGTGGTCCA), WWP1-2 (CCTCTCTAAATGGTAATTT), and PRKAA1 (GTATGATGTCAGATGGTGAATT) were constructed using procedures described previously (41).

**Western Blotting Analyses and Immunohistochemistry Analyses—**Cells were washed with cold PBS, and resuspended in EBC (250 mM NaCl, 50 mM Tris (pH 8.0), 0.5% Nonidet P-40, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 2 µg/ml aprotinin, and 2 µg/ml leupeptin). Equal amounts of total protein were loaded separated by SDS-PAGE, transferred to PVDF membranes (Millipore), and hybridized to an appropriate primary antibody and HRP-conjugated secondary antibody for subsequent detection by enhanced chemiluminescence. Specific antibodies for p63 (sc-8431, 1:200) or actin (sc-1615, 1:200) were purchased from Santa Cruz Biotechnology. Antibodies for PARP1 (9532, 1:1000), phospho-AMPK (Thr-172, 2535, 1:1000), AMPKα1 (2532, 1:1000), phospho-mTOR (2971, 1:1000), mTOR (2972, 1:1000), phospho-S6 kinase protein (Thr-389, 9206, 1:1000), Integrin β4 (4707, 1:1000), or Integrin α6 (3750, 1:1000) were purchased from Cell Signaling Technology (Danvers, MA). Antibodies for WWP1 (ab104440, and human mucoepidermoid pulmonary carcinoma H292 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. All Cells were grown in media supplemented with 100 units/ml penicillin and 100 µg/ml streptomycin. Cells were maintained in a humidified 37 °C incubator under a 5% CO2 atmosphere. Cells grown to 75–85% confluence in high-glucose DMEM (4.5 mg/ml glucose) or low-glucose DMEM (1.0 mg/ml glucose) were treated with chemical compound(s), as indicated. Metformin (PHR1084), 2-DG (D8375), cycloheximide (R750107), and MG132 (M8699) were purchased from Sigma.

![FIGURE6. Schematic of metformin-mediated anti-cancer activity](image-url)
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1:10,000), Integrin β1 (ab52971, 1:1000), ITCH (ab109018, 1:1000), or fibronectin (ab6328, 1:2000) were purchased from Abcam (Cambridge, MA). Immunohistochemistry was performed according to procedures described previously (42).

FACS, Cell Viability, and Protein Stability Assays—For the Annexin V and PI double staining assay (C1062, Beyotime), both floating and adherent cells were collected and subjected to FACS analyses according to the instructions of the manufacturer. The cell viability assay (MTS) was performed using the CellTiter 96 kit (Promega) as described in the instructions of the manufacturer. The trypan blue exclusion assay (C0011, Beyotime) was performed according to the instructions of the manufacturer. Owing to the protein half-life of ΔNp63α, FaDu cells were grown in DMEM containing 1.0 mg/ml glucose in the presence or absence of 10 mm metformin for 12 h, followed by addition of CHX (50 μg/ml). Cells were then collected at the indicated time intervals (0, 3, 6, 9, and 12 h). Cell lysates were subjected to Western blotting analyses.

Quantitative PCR—Total RNA was extracted from cells using the RNeasy Plus mini kit (74134, Qiagen) and reverse-transcribed. Q-PCR was carried out for ΔNp63α (F, GAAAA-CAATGCCAGAATA; R, GCGGGCGTGGTCTGTGTTA), Fibronectin1 (F, AGTTGATAAGAGGAATTTG; R, TAAAGCTATCTCCATTAGTGAAG), Integrin β1 (F, CACCG-CTTGCTAAGACG; R, TGGTGTGAGTGTGTTA), E-cadherin (F, GGATGTGCTGGATGTGAATG; R, CACCATGAGCGAAGCAACAT; R, TGGTGTGAGTGTGTTA), and GAPDH (F, GGAGCGCAAAAAGGTCATCATC; R, GAGGGGCAATCCACAGTCT). The Q-PCR reactions were performed in the CFX-960 real-time PCR system (Bio-Rad) using the Bio-Rad SsoFast EvaGreen Supermix kit according to the instructions of the manufacturer. Relative quantitation values were calculated using the ∆∆Ct method.

In Vivo Tumor Formation Assay—All animal care and animal experiments in this study were carried out according to the principles outlined in the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. Female BALB/c nude mice at an age of 5 weeks were used to establish xenografts. Cells (2 × 10⁶/ml) were subcutaneously injected into the right scruff of each nude mouse, 8 mice/group. On day 16 (when tumor volumes reached ~250 mm³), nude mice were intraperitoneally injected with metformin (400 mg/kg) and 2-DG (400 mg/kg) every other day. The volumes of xenograft tumors were measured 16, 20, 24, 28, and 32 days after inoculation. The tumor volumes were calculated according to the following formula: volume = length × width² / 2. The xenografts were dissected, and weights were measured 32 days after inoculation.

Statistical Analysis—Quantitative data were analyzed statistically using Student’s t test to assess significance. Data are presented as means ± S.E.

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