Metformin inhibits pancreatic cancer cell and tumor growth and downregulates Sp transcription factors

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Metformin is a widely used antidiabetic drug, and epidemiology studies for pancreatic and other cancers indicate that metformin exhibits both chemopreventive and chemotherapeutic activities. Several metformin-induced responses and genes are similar to those observed after knockdown of specificity protein (Sp) transcription factors Sp1, Sp3 and Sp4 by RNA interference, and we hypothesized that the mechanism of action of metformin in pancreatic cancer cells was due, in part, to downregulation of Sp transcription factors. Treatment of Panc1, L3.6pL and Panc28 pancreatic cancer cells with metformin downregulated Sp1, Sp3 and Sp4 proteins and several pro-oncogenic Sp-regulated genes including bcl-2, survivin, cyclin D1, vascular endothelial growth factor and its receptor, and fatty acid synthase. Metformin induced proteasome-dependent degradation of Sp1 in L3.6pL and Panc28 cells, whereas in Panc1 cells metformin decreased microRNA-27a and induced the Sp repressor, ZBTB10, and disruption of miR-27a:ZBTB10 by metformin was phosphatase dependent. Metformin also inhibited pancreatic tumor growth and downregulated Sp1, Sp3 and Sp4 in tumors in an orthotopic model where L3.6pL cells were injected directly into the pancreas. The results demonstrate for the first time that the anticancer activities of metformin are also due, in part, to downregulation of Sp transcription factors and Sp-regulated genes.

Introduction

Metformin or N,N′-dimethyl biguanide is an oral hypoglycemic drug with a remarkable record of safety that has been prescribed worldwide for the treatment of Type II diabetes, and metformin also protects against many other diseases (1–3). Metformin directly inhibits mitochondrial oxidative phosphorylation and decreases hepatic ATP pools required for gluconeogenesis (4,5). Metformin also increases adenosine monophosphate-activated protein kinase pathway that is important for maintaining cellular energy homeostasis under various stress conditions (6,7). There is also evidence that metformin-induced mitochondrial effects and activation of the tumor-suppressor gene liver kinase B1 may also play a role in the antidiabetic effects of metformin (8,9).

Abbreviations: CDDO-Me, methyl 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid; FAS, fatty acid synthase; MKP, mitogen-activated protein kinase phosphatase; miRNA, messenger RNA; mTOR, mammalian target of rapamycin; PARP, poly ADP-ribose polymerase; RNAi, RNA interference; ROS, reactive oxygen species; SOV, sodium orthovanadate; Sp, specificity protein; VEGF, vascular endothelial growth factor.

The potential role of metformin as a cancer chemopreventive and chemotherapeutic agent became apparent in studies showing that diabetes using metformin exhibited decreased cancer rates compared with diabetics not using this drug (9–18). Studies with cancer cells and in vivo models have confirmed the anticancer activity of metformin (19–21) and some of these reports have demonstrated inhibition of mammalian target of rapamycin (mTOR) signaling. For example, treatment of ovarian cancer cells with metformin induced a time- and dose-dependent increase in phosphorylation of AMPK and this was accompanied by decreased phosphorylation of the mTOR downstream kinases p70S6K and S6K (22).

Although inhibition of mTOR reportedly contributes to the anticancer activity of metformin, several reports show that metformin also affects responses/genes that may be independent of mTOR signaling. For example, metformin decreased cyclin D1 and E2F1 and induced p27 in LNCaP prostate cancer cells, decreased bcl-2 protein expression in ovarian cancer cells and enhanced poly ADP-ribose polymerase (PARP) cleavage in breast cancer cells (19–21). Metformin also inhibited nuclear factor-kappaB signaling and downregulated p65 (nuclear factor-kappaB) in endometrial and breast cancer cell lines (23,24). Knockdown of specificity protein (Sp) transcription factors Sp1, Sp3 and Sp4 in pancreatic and other cancer cell lines by RNA interference (RNAi) decreased expression of many of the same genes downregulated by metformin including bcl-2, cyclin D1 and p65 (nuclear factor-kappaB) (25–36). Moreover, Sp silencing also inhibited cancer cell growth and induced apoptosis and cleaved PARP, and similar results were observed for other anticancer agents such as curcumin and methyl 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO-Me), which also downregulated Sp, Sp3 and Sp4 in pancreatic cancer cells (30–34). Based on these data, we hypothesized that the anticancer activity of metformin may also be due, in part, to downregulation of Sp transcription factors that are overexpressed in pancreatic and other cancer cell lines (25–36). Results of this study show that metformin induced downregulation of Sp1, Sp3 and Sp4 through proteasome-dependent and proteasome-independent pathways in pancreatic cancer cells and tumors, and we also observed downregulation of several Sp-regulated genes. Thus, the anticancer activity of metformin in pancreatic cancers is also due, in part, to downregulation of Sp transcription factors.

Materials and methods

Cell lines, antibodies, plasmids and reagents

Human pancreatic cancer cell lines Panc1 cells were purchased from American Type Culture Collection (Manassas, VA). Cells were purchased >6 months ago and were not further tested or authenticated by the authors. Panc28 and L3.6pL pancreatic cancer cell lines were provided by The University of Texas M.D. Anderson Cancer Center and maintained as described (25,32,33). Sp1 antibody was purchased from Millipore (Temecula, CA); Sp3, Sp4, vascular endothelial growth factor (VEGF), survivin, bcl2, cyclin D1 and ubiquitin antibody (P4D1) were purchased from Santa Cruz Biotech (Santa Cruz, CA); Fatty acid synthase (FAS) and ZBTB10 antibody were purchased from Cell Signalling (Duveres, MA) and Bethyl (Montgomery, TX), respectively. MirVanaTM miRNA extraction kit, the reverse transcription and real-time PCR amplification kits were purchased from Applied Biosciences. ZBTB10 expression vector and empty vector (pCMV6-XL4) were purchased from Origene (Rockville, MD). Metformin was purchased from Calbiochem (Darmstadt, Germany). The mitogen-activated protein kinase phosphatase-5 (MKP-5) and MKP-1 expression plasmids were kindly provided by Dr Donna Peelh (Stanford University, Stanford, CA) and Dr Stephen M.Keye (University of Dundee, Dundee, Scotland), respectively.

Cell proliferation assay and annexin V staining

Panc28, Panc1 and L3.6pL pancreatic cancer cells (7.5 × 104 per well) were plated in 12 well plates and allowed to attach for 24 h and growth inhibition (cell counting) by metformin was determined as described (32,33). Apoptosis was analyzed by apoptotic and necrotic assay kit, which contained fluorescein isothiocyanate–annexin-V, ethidium homodimer III and Hoechst 3342. All

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three pancreatic cancer cell lines \((1 \times 10^5)\) were seeded in two-chambered cover glass slides and left to attach overnight. The cells were treated with metformin for 18–24 h. Apoptosis, necrotic and healthy cell detection kit was used according to manufacturer’s protocol.

**Western blot analyses and immunoprecipitation**

All three pancreatic cancer cells \((3 \times 10^5)\) per well were seeded in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium in six well plates. After 24 h, cells were treated with different concentrations of metformin and/or glutathione, glutoxin for 36 h. Cell lysates were obtained as described (32,33) and quantitated with Bradford reagent. For immunoprecipitation of ubiquitinated Sp proteins, Panc28 pancreatic cancer cells were treated with metformin with or without glutoxin. Cells for each treatment group were lysed using 2x STT lysis buffer \([1 M \text{ Tris (pH 7.5)}, 5 M \text{ NaCl}, 0.5\% \text{ Triton}\] with the addition of protease inhibitor cocktail (1:1000). Immunoprecipitation was carried out as reported previously (36). Western blot analysis was determined as described (32,33), and Immunoblot western chemiluminescence substrates \((\text{Millipore, Billerica, MA})\) were used to develop images captured on a Kodak 4000 MM Pro image station.

**Small interfering RNA interference assay**

Panc28, Panc1 and L3.6pL pancreatic cancer cells were treated \((1 \times 10^5\) per well) in six well plates in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium supplemented with 2.5% charcoal or metformin plus glutoxin without antibiotic and left to attach for 24 h. Knockdown of Sp1, Sp3 and Sp4 along with iLamin as control was carried out using Lipofectamine 2000 reagent according to the manufacturer’s instructions. Small inhibitory RNAs were prepared by Sigma–Aldrich \((\text{St Louis, MO})\).

**Quantitative real-time PCR and luciferase assay**

Total RNA was isolated from Panc1 cells using the RNeasy Protect Mini kit \((\text{Qiagen, Valencia, CA})\) according to the manufacturer’s protocol. RNA was eluted with 40 μl of RNase-free water and stored at −80°C. RNA was reverse transcribed using Superscript II reverse transcriptase \((\text{Invitrogen, Carlsbad, CA})\) according to the manufacturer’s protocol. Complementary DNA was prepared using a combination of oligodeoxythymidylic acid \((\text{Applied Biosystems, Foster City, CA})\), deoxynucleoside triphosphate mix and Superscript II reverse transcriptase \((\text{Invitrogen})\). Each PCR was carried out in triplicate in a 20 μl volume using SYBR Green Master mix \((\text{Invitrogen})\) for 15 min at 95°C for initial denaturing, followed by 40 cycles of 95°C for 30 s and 60°C for 1 min in the 7500 fast real-time PCR system \((\text{Applied Biosystems})\). Values for each gene were normalized to expression levels of TATA-binding protein \((\text{TPB})\). Primers were purchased from Integrated DNA Technologies. The following primers were used:

1. TBP \((\text{F}): 5’-\text{TGCACAGGAGGCCAAGATGGA-3’}\)
2. TBP \((\text{R}): 5’-\text{CATCAGACGCTCCCAACCA-3’}\)
3. ZBTB10 \((\text{F}): 5’-\text{GCTGAGTATTAGTATTGTCG-3’}\)
4. ZBTB10 \((\text{R}): 5’-\text{CTGAGTGGTTGATGGAACA-3’}\)

MirVana™ miRNA extraction kit was used for the extraction of miRNA according to manufacturer’s protocol. Quantification of miRNA \((\text{RNU6B and miRNA-27a})\) was determined using a Taqman miRNA kit \((\text{Applied Biosystems})\) according to the manufacturer’s protocol with real-time PCR. U6 small-nuclear RNA was used as a control to determine relative miRNA expression. For luciferase assays, cells were transfected with various amounts of plasmids \((\text{i.e. miR-27a (400 ng) and β-gal (40 ng)})\) and luciferase activity \((\text{normalized to β-gal})\) was determined as described (32,33).

**Orthotopic nude mice study and immunohistochemical staining**

Male athymic nude mice \((\text{NCI-nu})\) were housed and maintained under specific pathogen-free conditions in approved facilities. L3.6pL cells were harvested from subconfluent cultures by a brief exposure to 0.25% trypsin and 0.02% ethylenediaminetetraacetic acid and used to produce tumors. Trypsinization was stopped with medium containing 10% fetal bovine serum, and the cells were washed once in serum-free medium and resuspended in Hank’s balanced salt solution. Only suspensions consisting of single cells with >90% viability were used for the injections. Injection of cells into the pancreas was done as described previously (33). The remaining mice were divided into two groups \((\text{at least four animals per group})\) and treated (orally) with vehicle \((\text{control})\) or 250 mg/kg metformin daily. Mice were killed when moribund (4–5 weeks after injection), and body weights were recorded. Primary tumors in the pancreas were excised, measured and weighed. For immunohistochemistry, tumor tissue specimens were fixed in 10% formaldehyde, embedded in paraffin and sectioned into 3–5 mm thick slices, and immunostaining was carried out essentially as described (25,33).

**Results**

Treatment of Panc28, Panc1 and L3.6pL cells with 5–20 mM metformin for 48 or 72 h significantly inhibited growth and IC\(_{50}\) values were 26, 30 and 18 mM (48 h) and 19, 24 and 14 mM (72 h), respectively, in the three cell lines \((\text{Figure 1A})\). Significant growth inhibition was observed at ≤5 mM metformin and this was comparable with concentrations used in previous studies \((19–21)\). However, in subsequent studies, we used higher concentrations at shorter time points to determine the primary pathways affected by metformin in pancreatic cancer cells. Moreover, these experiments were carried out at higher cell densities, and Supplementary Figure 1, available at Carcinogenesis Online, shows that 20 mM metformin inhibited cell growth by only 20–25% and minimal toxicity was observed. The effects of metformin on induction of apoptosis was investigated using annexin V staining as an endpoint, and treatment with 20 mM metformin for 18–24 h significantly enhanced annexin V staining in Panc28, Panc1 and L3.6pL cells \((\text{Figure 1B–D})\), demonstrating that metformin inhibits growth and induces apoptosis in pancreatic cancer cells. Previous studies show that metformin decreased bcl-2 and induced PARP cleavage in cancer cells \((20,21)\), and Figure 2A confirms that treatment of Panc28, Panc1 and L3.6pL not only decreased bcl-2 but induced PARP cleavage but also decreased expression of the antiapoptotic survivin protein.

Knockdown of Sp transcription factors by RNAi or agents that downregulate Sp1, Sp3 and Sp4 in pancreatic cancer cells induced apoptosis and decreased cell growth and migration and expression of Sp-regulated genes such as bcl-2, survivin, cyclin D1, VEGF, VEGFR1 and FAS \((25,32,33,35,37)\). Figure 2B–D show that metformin also decreased expression of Sp1, Sp3 and Sp4 and Sp-regulated cyclin D1, VEGF1, VEGFR1 and FAS proteins in Panc28, Panc1 and L3.6pL cells; cyclin D1 was also downregulated in Panc1 and L3.6pL but not in Panc28 cells where regulation of cyclin D1 was Sp independent. The effects of metformin on expression of Sp1, Sp3, Sp4 and Sp-regulated gene products were not confined to pancreatic cancer cells because comparable effects were observed in prostate and colon cancer cells \((\text{Supplementary Figure 2, available at Carcinogenesis Online})\). The effects of metformin on cell growth inhibition, induction of apoptosis and downregulation of Sp proteins and Sp-regulated genes are comparable with responses observed after Sp knockdown by RNAi in these cell lines and demonstrate that this pathway plays a role in the anticancer activity of metformin \((31–35)\).

The mechanisms of drug-induced downregulation of Sp1, Sp3 and Sp4 in pancreatic cancer cells is drug-dependent and include activation of proteasomes and induction of reactive oxygen species \((\text{ROS})\) \((25,32–34)\). Treatment of Panc28, L3.6pL and Panc1 cells with 15 mM metformin for 36 h decreased expression of Sp1, Sp3 and Sp4 proteins and cotreatment with the antioxidants glutathione and/or DTT did not attenuate the metformin-induced effects in these cell lines \((\text{Figure 3A})\). In contrast, metformin-induced downregulation of Sp1, Sp3 and Sp4 in Panc28 and L3.6pL cells was attenuated after cotreatment with the proteasome inhibitor glitoxin \((\text{Figure 3B})\), whereas glitoxin did not block Sp degradation in Panc1 cells. Using Panc28 cells as a model, we observed that after treatment with metformin alone, proteasome inhibitors glitoxin for 24 h, cell lysates immunoprecipitated with antibodies against Sp1, Sp3 or Sp4 and exhibited increased formation of multiple bands after staining with ubiquitin antibodies \((\text{Figure 3C})\). These results were similar to those previously observed for the non-steroidal anti-inflammatory drug tolfenamic acid in pancreatic cancer cells \((25)\). A recent report showed sumoylation of Sp1 increased proteasome-dependent degradation by inducing nuclear to cytosolic export of Sp1 \((\text{38})\) and therefore, we investigated the effects of metformin on degradation of Sp1, Sp3 and Sp4 in the absence or presence of leptomycin B, an inhibitor of nuclear export \((\text{Figure 3D})\). In untreated cells, Sp1, Sp3 and Sp4 were exclusively found in nuclear extracts, and treatment with metformin alone or in combination with leptomycin B resulted in downregulation of Sp1, Sp3 and Sp4, demonstrating that the
degradation process was nuclear and not dependent on export of Sp1, Sp3 and Sp4 to the cytosol.

Like metformin, the triterpenoid anticancer agent CDDO-Me also decreased Sp1, Sp3, Sp4 and Sp-regulated genes in Panc1 cells due to downregulation of miR-27a and induction of the transcriptional repressor ZBTB10, which is regulated by miR-27a (33). Metformin also significantly decreased miR-27a levels in Panc1 cells (Figure 4A) and decreased luciferase activity in cells transfected with a construct containing the +36 to −603 region of the miR-27a promoter (Figure 4B). Metformin-mediated downregulation of miR-27a was accompanied by increased expression of ZBTB10 messenger RNA (mRNA) levels (Figure 4C), and there was a
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Fig. 2. Metformin decreases expression of antiapoptotic and Sp proteins. (A) Panc28, Panc1 and L3.6pL cells were treated with vehicle, 10 or 20 mM metformin for 36 h, and whole cell lysates were analyzed by western blots as outlined in the Materials and methods. Panc28 (B), Panc1 (C) and L3.6pL (D) cells were treated with vehicle, 10, 15 or 20 mM metformin for 36 h, and whole cell lysates were analyzed by western blots as outlined in the Materials and methods.
time-dependent induction of ZBTB10 protein 6–24 h after treatment followed by decreased expression after 24–36 h. Similar time-dependent induction of ZBTB10 has also been observed for other compounds (data not shown). Metformin also increased luciferase activity in Panc1 cells transfected with a 3′-UTR (ZBTB10)-luc construct containing a miR-27a binding site, whereas induction was not observed using the 3′-UTR (ZBTB10)-luc construct with mutations in the miR-27a binding sequence (Figure 4D). These results were consistent with those previously observed with CDDO-Me in Panc 1 cells (33); however, in contrast to CDDO-Me, metformin-induced repression of Sp transcription factors was ROS independent (Figure 4A).

Drugs such as curcumin and thiazolidinediones that downregulate Sp proteins (32,39) also induce the dual specificity phosphatases MKP-5 and MKP-1 (40,41), and recent studies show that MKP-1 overexpression decreases Sp1 in breast cancer cells (42). Therefore,
we initially investigated the role of metformin-induced phosphatase activity on Sp downregulation using the phosphatase inhibitor sodium orthovanadate (SOV). Results in Figure 5A show that 20 μM SOV significantly inhibited metformin-induced downregulation of Sp1, Sp3 and Sp4 and also blocked downregulation of miR-27a and induction of ZBTB10 in Panc1 cells cotreated with metformin plus SOV. Interestingly, SOV alone increased miR-27a levels but did not affect ZBTB10 expression. We also showed that metformin
Fig. 5. Role of phosphatases in metformin-induced repression of Sp proteins. (A) SOV inhibits metformin-induced responses. Panc1 cells were treated with 15 mM metformin alone or in combination with 20 μM SOV. Sp proteins and RNA levels were determined by western blots and real-time PCR, respectively, as outlined in the Materials and methods. (B) Metformin induces MKP-1 and MKP-5. Panc1 cells were treated with 15 mM metformin, and mRNA and protein levels were determined as outlined in (A). (C) MKP-1 and MKP-5 disrupt miR-27a:ZBTB10. Panc1 cells were transfected with MKP-1 or MKP-5 expression plasmids or treated with metformin in the presence or absence of transfected siCtl or siMKP-1 or siMKP-5, and RNA or protein levels were determined as outlined in (A). (D) MKP-1 and MKP-5 expression downregulates Sp proteins. MKP-1 or MKP-5 was overexpressed in Panc1 cells, and whole cell lysates were analyzed by western blots as outlined in the Materials and methods. Significant ($P < 0.05$) induction (A, B) or inhibition (A, C) is indicated (*).
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induced MKP-1 and MKP-5 mRNA and protein expression in Panc1 cells (Figure 5B). Moreover, overexpression of MKP-1 or MKP-5 decreased miR-27a and increased ZBTB10 protein expression and knockdown of MKP-1 (siMKP-1) or MKP-5 (siMKP-5) attenuated induction of ZBTB10 by metformin (Figure 5C). Overexpression of MKP-1 or MKP-5 decreased expression of Sp1, Sp3 and Sp4 proteins (Figure 5D), thus confirming a critical role for these metformin-induced phosphatases in downregulating Sp proteins through modulation of miR-27a:ZBTB10. We also examined the possible role of LKB-AMPKα in mediating activation of MKP-1/MKP-5; however, metformin induced both phosphatases in the presence or absence of the AMPKα inhibitor compound C (Supplementary Figure 3, available at Carcinogenesis Online).

The in vivo anticancer activity of metformin was investigated in an orthotopic model of pancreatic cancer in which L3.6pL cells are injected directly into the pancreas of athymic nude mice (25). Previous in vivo xenograft studies with Panc1 cells reported that 250 mg/kg/day inhibited tumor growth (43) and therefore, we used this dose in this study with the highly aggressive L3.6pL cells. Metformin (250 mg/kg) was administered daily for 28 days after injection of the cancer

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Fig. 6. Metformin inhibits pancreatic (L3.6pL) tumor growth and downregulates Sp proteins in vivo. (A) Mice bearing L3.6pL cells in the pancreas were treated with corn oil (control) or 250 mg/kg/d metformin. At the end of the treatment (28 days), pancreatic tumor volumes and weights were determined as outlined in the Materials and methods. (B) Lysates from a portion of each tumor were analyzed by western blots and quantitated (relative to β-actin; control values set at 100%) as outlined in the Materials and methods. Significant (*P < 0.05) decreases in protein in tumors from metformin-treated mice compared with controls are indicated (*). (C) Immunostaining of tumors from control and metformin-treated mice for FAS expression was carried out as outlined in the Materials and methods. Hematoxylin and eosin staining of tumors from control and treated mice did not exhibit any striking morphological differences.

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cells, and treatment with metformin significantly decreased tumor volume and weight compared with control animals (Figure 6A) and this was not accompanied by changes in body weight or evidence for toxicity (data not shown). Analysis of tumor lysates from control and metformin-treated mice showed some variability in expression of Sp1, Sp3 and Sp4 proteins; however, there was a significant decrease in protein levels in tumors from mice treated with metformin (Figure 6B). Moreover, immunohistochemical analysis (Figure 6C) confirmed decreased staining of FAS in tumors from the metformin-treated animals. Results of the in vivo and in vitro studies demonstrate that metformin-induced downregulation of Sp transcription factors also contributes to the anticancer activity of this antidiabetic drug; however, this does not exclude other pathways including mTOR inhibition or decreased mitogen expression.

Discussion

Pancreatic cancer is the fourth major cause of cancer deaths and it is estimated that in the USA >343,920 new cases of pancreatic cancer will be diagnosed and there will be 37,390 deaths from this disease in 2012 (44). Risk factors for pancreatic cancer include obesity and diabetes and both of these conditions are interrelated because obesity is also a risk factor for Type II diabetes. It was reported that diabetic patients using metformin had a significantly lower risk for pancreatic cancer than patients using other antidiabetic drugs (14). Results reported for metformin in this study were comparable with those observed for the non-steroidal anti-inflammatory drug tolfenamic acid and the experimental anticancer agent CDDO-Me in pancreatic cancer cell lines (25,33). Both of these compounds downregulate Sp1, Sp3 and Sp4 transcription factors that are overexpressed in pancreatic cancer cells and also downregulated several Sp-regulated genes associated with cell proliferation (cyclin D1), metabolism (FAS), apoptosis (bcl-2 and survivin) and angiogenesis (VEGF and VEGFR1) (25,33,35). The growth inhibitory and apoptotic responses induced by tolfenamic acid and CDDO-Me in pancreatic cancer cells are also observed after knockdown of Sp1, Sp3 and Sp4 (individually and combined) by RNAi (30–34), confirming that drug-induced downregulation of Sp transcription factors contributes to their anticancer activity. Results summarized in Figures 1 and 2 demonstrate that metformin inhibited pancreatic cancer cell growth, induced apoptosis and downregulated Sp transcription factors and Sp-regulated genes. Similar results were also observed in vivo (Figure 6), suggesting that the anticancer activity of metformin was also due, in part, to downregulation of Sp transcription factors.

Tolfenamic acid induces proteasome-dependent degradation of Sp1, Sp3 and Sp4 in Panc1 cells, whereas the effects of CDDO-Me are due to induction of ROS and ROS-dependent induction of the Sp repressor ZBTB10 through downregulation of miR-27a (25,33). Metformin activated the proteasome pathway in Panc28 and L3.6plp, cells (Figure 3) and these effects were comparable with those observed for tolfenamic acid in pancreatic cancer cells (25). Moreover, tolfenamic acid and metformin inhibited pancreatic tumor growth and downregulated Sp1, Sp3 and Sp4 protein expression in an orthotopic model for pancreatic cancer in which L3.6plp cells were injected directly into the pancreas (Figure 6). Metformin-mediated activation of the proteasome pathway in Panc28 cells was accompanied by enhanced ubiquitination of Sp1, Sp3 and to a lesser extent Sp4 (Figure 3B) and based on results of cotreatment with leptomycin B (Figure 3C), the action of the proteasomes was nuclear, whereas in HeLa cells sumoylation of nuclear Sp1 and subsequent nuclear export are key factors in proteasome (cytosolic)-dependent degradation of this transcription factor (38). The mechanisms of activation of nuclear proteasomes by metformin are currently being investigated.

Previous studies in pancreatic and other cancer cell lines (33) have identified a transcriptional pathway for Sp downregulation that involves drug-induced ROS and ROS-dependent downregulation of miR-27a and induction of ZBTB10, which is a transcriptional repressor that competitively binds GC-rich sequences to inactivate gene expression (29,33). Although both CDDO-Me and metformin decreased miR-27a, induced ZBTB10 and decreased expression of Sp transcription factors, the effects of metformin in Panc1 cells were not reversed after cotreatment with antioxidants, indicating a proteasome and ROS-independent mode of action for Sp downregulation by metformin in this cell line.

Previous reports showed that compounds, such as curcumin and rosiglitazone that induced MKP-5 and MKP-1, respectively, in prostate and glioma cells (40,41) also downregulated Sp1 and other Sp proteins in cancer cell lines (31,32,39,41). Moreover, a recent study also showed that overexpression of MKP-1 in breast cancer cells decreased Sp1 protein (42). Based on these reports, we showed that metformin induced MKP-1 and MKP-5 expression in Panc1 cells (Figure 4B), and the role of induced phosphatases in mediating metformin-induced downregulation of Sp proteins was confirmed by showing the inhibitory effects of SOV (Figure 5A) and by demonstrating that MKP-1 and MKP-5 overexpression also downregulates Sp1, Sp3 and Sp4 (Figure 5D). Induction of phosphatases also plays a critical role in metformin-mediated disruption of miR-27aZBTB10 (Figure 5A) and the subsequent downregulation of Sp proteins, and the mechanism of MKP-1- and MKP-5 induction by metformin and the role of phosphatases in downregulation of miR-27a are currently being investigated.

Results of this study demonstrate that metformin downregulates Sp transcription factors and pro-oncogenic Sp-regulated genes including FAS in pancreatic cancer cells and tumors and in other cancer cell lines (Supplementary Figure 1, available at Carcinogenesis Online) and this is consistent with previous studies showing that metformin downregulates several Sp-regulated genes (19–21,23). Although mTOR and Sp transcription factors regulate some common genes, mTOR activation did not affect Sp1, Sp3 or Sp4 expression and silencing of Sp transcription factors did not alter mTOR expression (data not shown); however, other interactions between mTOR and Sp proteins are currently being investigated in pancreatic cancer cells. Because silencing of Sp1, Sp3 and Sp4 in pancreatic cancer cells results in growth inhibition and induction of apoptosis (32–35), the anticancer activity of metformin, which also downregulates Sp proteins, is due, in part, to downregulation of these transcription factors. Results of this study will facilitate development of clinical applications of metformin alone or in combined therapies because several Sp-regulated genes (FAS, survivin) are associated with radiation and drug resistance.

Supplementary material

Supplementary Figures 1–3 can be found at http://carcin.oxfordjournals.org.

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References

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