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# Effects of metformin and phenformin on apoptosis and epithelial-mesenchymal transition in chemoresistant rectal cancer

Ji-Hye Park[1] | Young-heon Kim[1] | Eun Hyeh Park[1] | Sun-Joo Lee[2] | Hyewon Kim[2] | Areumnuri Kim[1] | Seung Bum Lee[1] | Sehwan Shim[1] | Hyosun Jang[1] | Jae Kyung Myung[1,2] | Sunhoo Park[1,2] | Su-Jae Lee[3] | Min Jung Kim[1]

[1]Laboratory of Radiation Exposure & Therapeutics, National Radiation Emergency Medical Center, Korea Institute of Radiological & Medical Science, Seoul, Korea

[2]Laboratory of Experimental Pathology, Department of Pathology, Korea Institute of Radiological & Medical Science, Seoul, Korea

[3]Department of Life Science, Research Institute for Natural Sciences, Hanyang University, Seoul, Korea

**Correspondence**
Su-Jae Lee, Laboratory of Molecular Biochemistry, Department of Life Science, Research Institute for Natural Sciences, Hanyang University, 222, Wangsimni-ro, Seongdong-gu, Seoul 04763, Korea.
Email: sj0420@hanyang.ac.kr
Min-Jung Kim, Laboratory of Radiation Exposure & Therapeutics, National Radiation Emergency Medical Center, Korea Institute of Radiological & Medical Science, 75 Nowon-ro, Nowon-gu, Seoul 01812, Korea.
Email: kimmj74@kirams.re.kr

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## Abstract

Recurrence and chemoresistance in colorectal cancer remain important issues for patients treated with conventional therapeutics. Metformin and phenformin, previously used in the treatment of diabetes, have been shown to have anticancer effects in various cancers, including breast, lung and prostate cancers. However, their molecular mechanisms are still unclear. In this study, we examined the effects of these drugs in chemoresistant rectal cancer cell lines. We found that SW837 and SW1463 rectal cancer cells were more resistant to ionizing radiation and 5-fluorouracil than HCT116 and LS513 colon cancer cells. In addition, metformin and phenformin increased the sensitivity of these cell lines by inhibiting cell proliferation, suppressing clonogenic ability and increasing apoptotic cell death in rectal cancer cells. Signal transducer and activator of transcription 3 and transforming growth factor-β/Smad signaling pathways were more activated in rectal cancer cells, and inhibition of signal transducer and activator of transcription 3 expression using an inhibitor or siRNA sensitized rectal cancer cells to chemoresistant by inhibition of the expression of antiapoptotic proteins, such as X-linked inhibitor of apoptosis, survivin and cellular inhibitor of apoptosis protein 1. Moreover, metformin and phenformin inhibited cell migration and invasion by suppression of transforming growth factor β receptor 2-mediated Snail and Twist expression in rectal cancer cells. Therefore, metformin and phenformin may represent a novel strategy for the treatment of chemoresistant rectal cancer by targeting signal transducer and activator of transcription 3 and transforming growth factor-β/Smad signaling.

**KEYWORDS**
chemoresistant rectal cancer, metformin, phenformin, phosphorylated-signal transducer and activator of transcription 3 (Ser-727), transforming growth factor β receptor type 2

Ji-Hye Park and Young-heon Kim contributed equally to this work.

## 1 | INTRODUCTION

Colorectal cancer (CRC) is the third most common malignant cancer and the 4th most frequent cause of cancer-related death worldwide.[1] Most patients with CRC are treated with surgery; however, adjuvant treatments, such as radiotherapy and chemotherapy, are also required. CRC is a complex disease with a variable clinical presentation and molecular characteristics in response to treatment, even in tumors with similar histopathological characteristics.[2] Therefore, because of a lack of treatment strategies specific for rectal cancer, adjuvant chemotherapy regimens for patients with CRC are often applied in the treatment of rectal cancer. Although combinations of many advanced therapies for CRC have accelerated early detection and improved survival, treatment of CRC, particularly rectal cancer, remains challenging and requires molecular targeted therapy.[3]

Many studies have demonstrated that there is an association between type 2 diabetes (T2DM) and CRC. Metformin, a standard clinical drug used to treat T2DM, decreases the incidence of tumor development and increases the survival rates of patients with T2DM and various types of cancer, including breast, colon, lung and prostate cancers.[4] Another T2M drug, phenformin, has also been shown to have anticancer effects in several cancers, including lung cancer, lymphoma and breast cancer.[5] Although these drugs have been used for many years to treat T2DM, the mechanisms of action of these drugs in cancer are still unclear. Both of these drugs are known to function as AMP-dependent protein kinase (AMPK) agonists and inhibit complex1in the mitochondrial electron transport chain.[6] Moreover, metformin and phenformin inhibit cell proliferation, angiogenesis, the epithelial-mesenchymal transition (EMT) and tumor growth in various cancers, including breast cancer, prostate cancer, lung cancer, melanoma, ovarian cancer and colon cancer.[7-12] In colon cancer, metformin and phenformin inhibit the proliferation of CRC cells by blocking glycolysis, activating AMPK and stimulating reactive oxygen species production.[13-15] Indeed, in vivo studies have demonstrated the selective suppression of tumor growth in p53-deficient tumor cell xenografts.[16] However, other mechanisms of metformin and phenformin in rectal cancer have not been elucidated.

Transforming growth factor (TGF) β receptor type 2 (TGFBR2), a member of the TGF-β/Smad pathway, is involved in tumorigenesis. The expression of TGFBR2 is altered in various malignancies, including metastatic breast cancer,[17] CRC[18] and prostate cancer.[19] TGF-β signaling is an important pathway contributing to cancer development and has various functions in the regulation of cell growth, differentiation, apoptosis, EMT, angiogenesis and metastasis in a cellular context-dependent manner.[20,21] Accumulating evidence has demonstrated that the EMT is a highly conserved developmental process in which the properties of epithelial cells are lost, and cells acquire the properties of mesenchymal cells, exhibiting an invasive phenotype.[22] Therefore, the EMT can regulate the invasion, metastasis, and therapeutic resistance of cancer cells, making it a promising target for cancer therapy.

In this study, we evaluated the effects of metformin and phenformin on apoptosis and the EMT in rectal cancer cells. Our findings suggested that metformin and phenformin represent effective therapeutic agents for the treatment of chemoresistant rectal cancer.

## 2 | MATERIALS AND METHODS

### 2.1 | Cell culture and chemical reagents

The rectal cancer cell lines SW837 and SW1463 and the colon cancer cell lines HCT116 and LS513 were obtained from the ATCC. Rectal and colon cancer cell lines were cultured in Leibovitz's 15 (Welgene) at 37°C in an atmosphere containing 1% $CO_2$ or in RPMI 1640 (Lonza) at 37°C in an atmosphere containing 5% $CO_2$ containing 10% FBS (Corning) and gentamycin (50 μg/mL; Lonza), respectively. 5-Fluorouracil (5-FU), TGFBR inhibitor (SB525334), metformin and phenformin were obtained from Sigma-Aldrich. The signal transducer and activator of transcription 3 (STAT3) inhibitor STATTIC was purchased from Calbiochem. For assessing apoptosis, apoptotic protein assay (ARY009) was purchased and was performed according to the instructions provided using the R&D system.

### 2.2 | Irradiation

Colon and rectal cancer cells were irradiated with a 137Cs laboratory γ-irradiator (Gammacell 3000 Elan; MDS Nordion) at a dose rate of 3.25 Gy/min for the time required to apply a prescribed dose at room temperature.

### 2.3 | Apoptosis assay by flow cytometry (FACS) and immunofluorescence

Apoptosis analysis was performed using an FITC Annexin V Apoptosis Detection Kit I (BD Pharmigen), according to the manufacturer's instructions. Briefly, chemical-induced or radiation-induced apoptotic cells were collected (approximately $5 \times 10^5$ cells) at the indicated time points and resuspended in 1× diluted binding buffer. For staining, annexin V-FITC and propidium iodide (PI) were added to each sample, and the mixture was incubated for 5 minutes at room temperature in the dark. The cells were analyzed immediately using a BD FACS CANTO II flow cytometer.

Immunofluorescence was used for the microscopic experiments. Briefly, cells were rinsed with PBS, and then treated with the recommended volume of annexin V and PI in binding buffer. After incubation in the dark, at room temperature for 20 minutes, cells were visualized under the microscope.

### 2.4 | Western blot analysis

Cells were lysed using lysis buffer (40 mmol/L Tris-HCl [pH 8.0], 120 mmol/L NaCl, 0.1% Nonidet-P40) supplemented with protease and phosphatase inhibitors. For western blot analysis, the cell lysates were separated by sodium dodecyl sulfate polyacrylamide

gel electrophoresis and transferred to a nitrocellulose membrane (Amersham, UK). The membranes were blocked with 5% skim milk, incubated with primary antibodies overnight at 4°C, and subsequently reacted with the appropriate HRP-conjugated secondary antibodies (anti–mouse IgG-HRP and anti–rabbit IgG-HRP; Cell Signaling Technology). Bands were detected with ECL Plus Western Blotting Detection Reagents from GE Healthcare.

Antibodies specific for the following factors were used for western blotting: AKT, AMPKα, phospho-AMPKα, cleaved-poly (ADP-ribose) polymerase (PARP), cleaved caspase-3, c-Myc, mammalian target of rapamycin (mTOR), protein kinase A (PKA), phospho-PKA, phospho-Smad2, phospho-STAT3 (Ser-727), Smad2, STAT3, survivin, X-linked inhibitor of apoptosis protein (XIAP), PUMA, β-catenin (Cell Signaling Technology); phospho-AKT, Bak, Bax, cellular inhibitor of apoptosis protein-1 (cIAP1), epidermal growth factor receptor, myeloid leukemia cell differentiation protein, TGF-β1, TGFBR2, slug, Bad, β-actin (Santa Cruz Biotechnology; phospho-mTOR, snail, twist, vimentin (Abcam); N-cadherin (BD Bioscience); and Zeb1 (Sigma).

### 2.5 | Cell survival assays

MTT assays were conducted using HCT116, LSD513, SW837 and SW1463 cells. Cells were treated with different concentrations of metformin (0.5, 1, 5, 10 or 50 mmol/L) and phenformin (2.5, 5, 10, 50 or 100 μmol/L) for 72 hours. After 72 hours, cells were incubated with 0.5 mg/mL MTT at 37°C. After 3 hours, 10% sodium dodecyl sulfate was added to solubilize the formazan crystals, and the absorbance was measured at 595 nm using a microplate reader (Thermo Fisher Scientific).

### 2.6 | Colony forming assay

To test the effects of ionizing radiation (IR), 5-FU, metformin and phenformin on the survival of HCT116, SW837, LS513 and SW1463 cells, the cells were seeded at different densities ($1 \times 10^4$ to $3 \times 10^4$ cells) on 60-mm or 35-mm dishes and then cultured for 7-14 days. The colonies were fixed with methanol, stained with crystal violet to determine the survival efficiency, and counted to analyze the treatment effects.

### 2.7 | Transforming growth factor-β receptor type 2 knockdown and overexpression

For transient silencing of the *STAT3* and *TGFBR2* genes, cells were transfected with nontargeting siRNA and siRNA targeting *STAT3* and *TGFBR2* (*STAT3* siRNA duplexes, CAGCCUCUCUGCAGAAUUCAAUU, UUGAAUUCUGCAGAGAGGCUGUU [Genolution Pharmaceuticals]; TGRBR2 [Santa Cruz Biotechnology]) for 48 hours using Lipofectamine 2000 (Invitrogen), according to the manufacturer's recommendations. To re-overexpress TGFBR2, we purchased a pCMV5B-TGFBR2 wt (#11766) from Addgene, deposited by Jeff Wrana (University of Toronto, Ontario, Canada), transfected into the siRNA-mediated TGFBR2 knocked-down cell.

### 2.8 | Transwell assays

For migration assays, cells were seeded in the upper chambers of Transwells (Corning) and incubated for 72 hours in the presence of inhibitors or siRNA. To observe the cells that migrated into the lower chamber, the Transwell membranes were fixed with 4% paraformaldehyde and stained with 0.05% crystal violet (Sigma-Aldrich). Cells on the undersurface of the membrane were counted under a light microscope. For invasion assays, cells were plated in the upper compartments of the Matrigel (BD Bioscience). The invading cells in the lower chamber were fixed, stained and counted under a light microscope.

### 2.9 | Human tissue microarray with immunohistochemical staining

Human colon cancer tissue microarray slides were obtained from AccuMax ISU ABXIS and contained 32 colon cancer specimens. After baking and deparaffinization, the slides were boiled in a pressure cooker filled with 10 mmol/L sodium citrate (pH 6.0) and then immunostained with antibodies targeting phospho-STAT3 (Ser-727; 1:25; Cell Signaling Technology) and TGFBR2 (1:50; Santa Cruz Biotechnology). Spots were evaluated by estimating the intensity of tumor cells. Samples were considered positive if 30% or more of the tumor cells were immunostained.

### 2.10 | Xenograft mouse studies

All animal experiments were approved and performed in accordance with the Korea Institute of Radiological and Medical Science (KIRAMS) Animal Care and Use Committee (Seoul, Korea). For xenografts experiments, $5 \times 10^6$ SW837 cells were injected subcutaneously into the right flank of 6 to 8-week-old male athymic nude mice that were purchased from the Orient Bio. Mice were randomized to 3 treatment groups (n = 6 per group) once the meat tumor volume reached approximately 65 $mm^3$. Metformin and phenformin were diluted with PBS and administered at 100 mg/kg/d and 14 mg/kg/d, respectively, via i.p. injection. Tumors were measured twice weekly using calipers, and volume was calculated as 1/2 × long diameter × short $diameter^2$.

### 2.11 | Statistical analysis

Statistical significance of the differences between mean values was calculated with unpaired Student's *t* tests using SPSS (version 12.0; SPSS Inc.) or Excel (Microsoft) software packages. Results with *P*-value of <0.05 were considered statistically significant.

## 3 | RESULTS

### 3.1 | Rectal cancer cells were resistant to ionizing radiation and 5-fluorouracil treatment

Because IR and 5-FU are the first-line treatments for patients with CRC, we investigated the apoptotic response to IR and 5-FU in

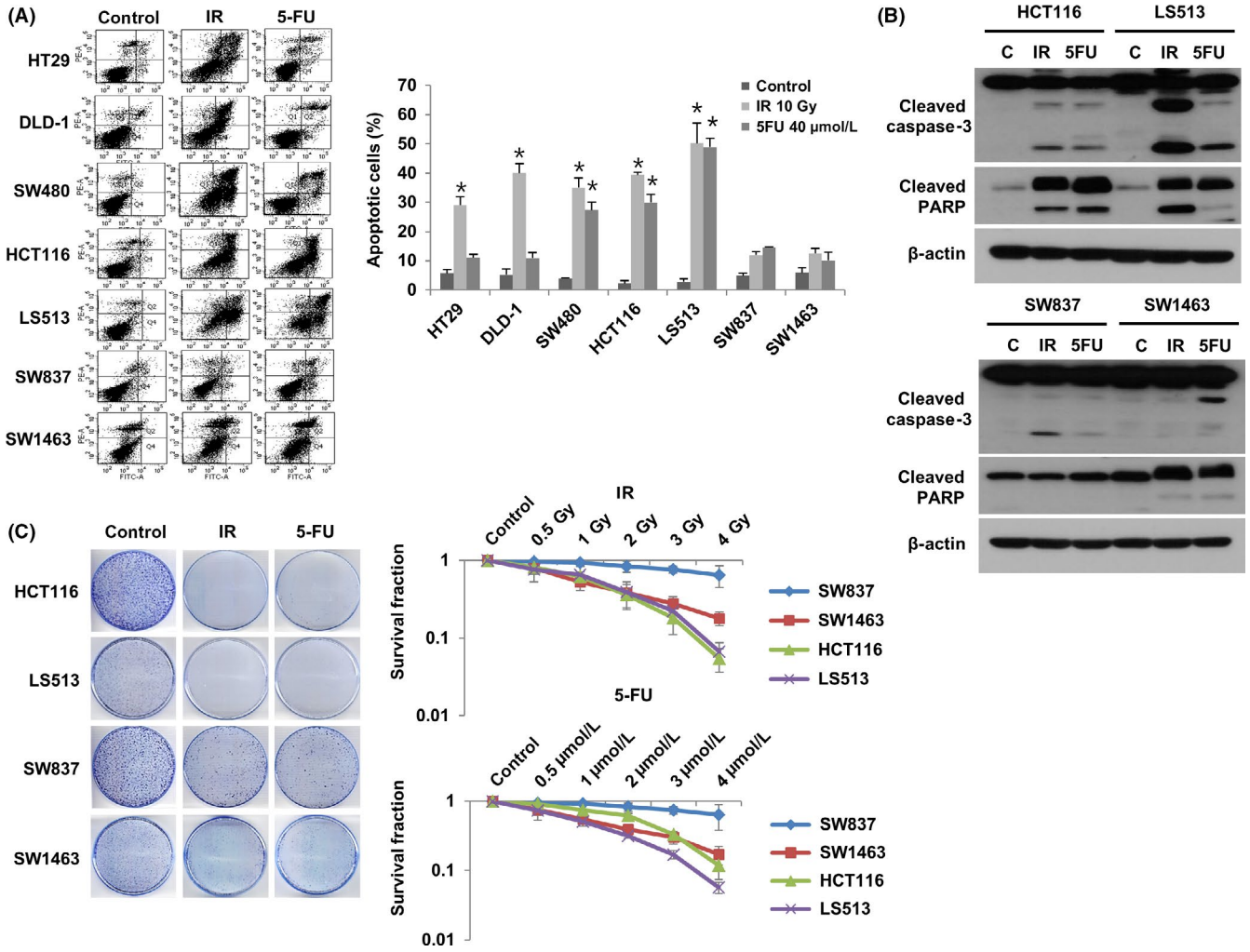



**FIGURE 1** Rectal cancer cells were resistant to ionizing radiation (IR) and 5-fluorouracil (5-FU) treatment. A, The cell apoptosis was measured by annexin V staining and flow cytometry analysis in indicated cells treated with 10 Gy and 40 μmol/L 5-FU. *$P < 0.05$ compared with control (Student's *t* test). B, Indicated cell lines were treated with 10 Gy IR and 40 μmol/L 5-FU for 48 h and these cell lysates were subjected to western blot analysis for the detection of cleaved caspase-3 and cleaved-PARP expression. β-actin expression was used for normalization. C, Colony formation assay was performed with indicated cells treated with 3 Gy and 3 μmol/L 5-FU (left panel). Graph showing quantification of relative colony numbers in the different doses of IR or 5-FU (right panel)

CRC cell lines by annexin V/PI staining. Among the various cancer cell lines, the colon cancer cell lines HCT116 and LS513 were particularly sensitive to IR and 5-FU. In contrast, rectal cancer cells SW837 and SW1463 were more resistant to apoptosis than colon cancer cells (Figure 1A). To further confirm the resistance of rectal cancer cells to IR and 5-FU, we conducted colony formation assays. Colonies of both colon and rectal cells were inhibited by IR and 5-FU in a dose-dependent manner, whereas colonies of colon cancer cells were significantly reduced compared with the rectal cancer cells, indicating that rectal cancer cells showed higher radioresistance and chemoresistant to IR and 5-FU, respectively (Figure 1B). In addition, the proliferation and colony formation rates of both cell types were inhibited by IR and 5-FU. Next, we carried out western blotting to investigate changes in apoptotic markers in response to IR and 5-FU in colon and rectal cancer cells. Cleaved caspase-3 and cleaved PARP were strongly increased in IR-treated and 5-FU-treated HCT116 and LS513 colon cancer cells (Figure 1C). Collectively, these results indicated that SW837 and SW1463 rectal cancer cells were more resistant to IR- and 5-FU-induced apoptosis.

## 3.2 | Metformin and phenformin increased apoptotic cell death in rectal cancer cells

Because metformin and phenformin have been found to have potential applications as anticancer drugs in various cancer cell lines[7-12] and metformin has been shown to have positive clinical outcomes in patients with T2DM and CRC,[4] we next examined whether metformin and phenformin exhibited antiproliferative effects in rectal cancer cells. By screening colon and rectal cancer cells following treatment with metformin and phenformin, we found that rectal cancer cells showed significantly decreased proliferation compared with colon cancer cells (Figure 2A, left). In addition, to investigate

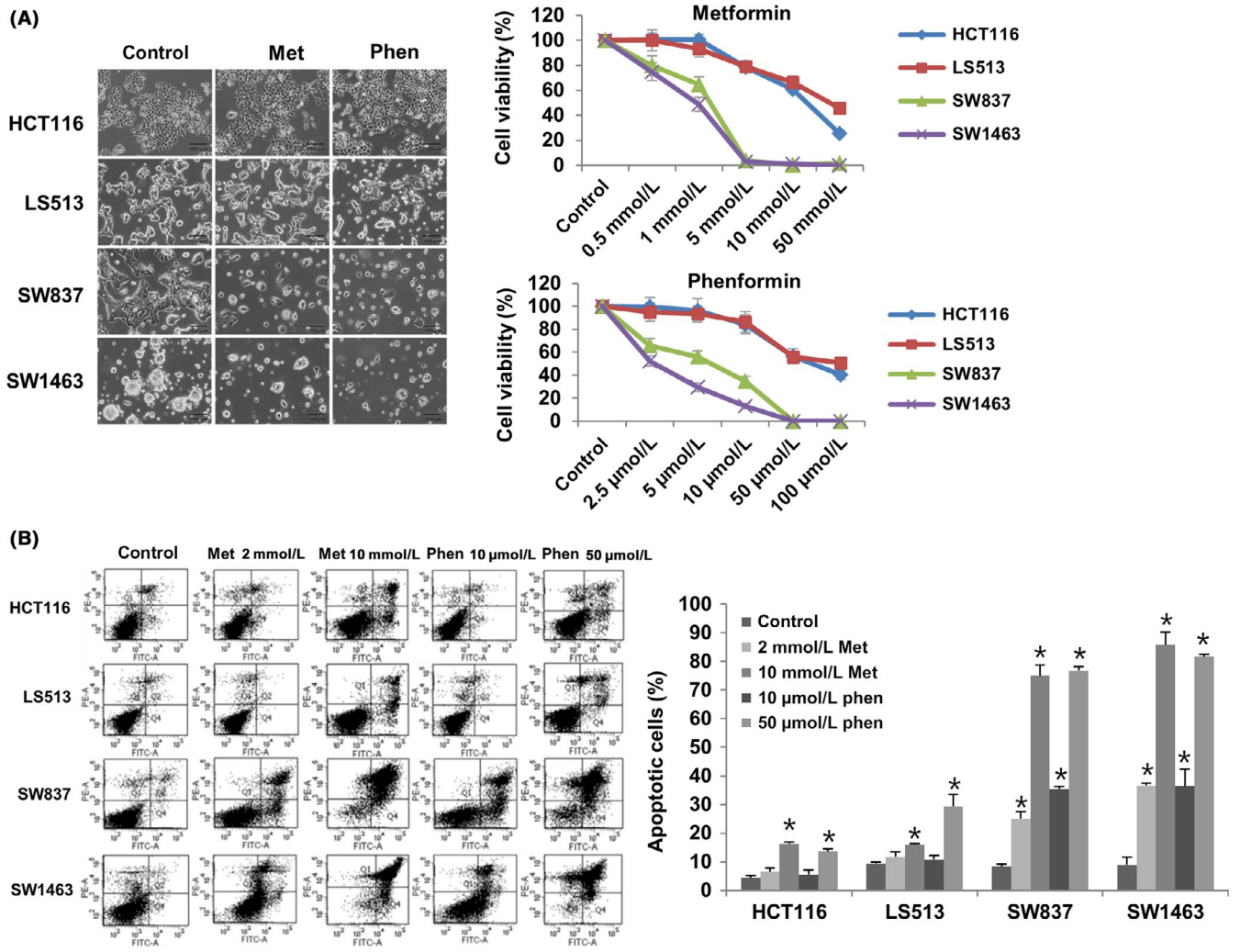



**FIGURE 2** Metformin and phenformin increased apoptotic cell death in rectal cancer cells. A, Bright-field imaged of the indicated cell lines treated with 2 mmol/L metformin (Met) and 10 μmol/L phenformin (Phen) (left panel). Cytotoxicity of Met (0.5, 1, 5, 10 or 50 mmol/L) and Phen (2.5, 5, 10, 50, 100 μmol/L) for 72 h in indicated cell lines was analyzed using the MTT assay (right panel). B, Cell lines were treated with indicated dose of Met and Phen for 48 h and apoptosis was measured by annexin V staining and flow cytometry analysis. $^*P < 0.05$ compared with control (Student's *t* test). C, Colony formation assay was performed with indicated cells treated with 1 mmol/L Met and 5 μmol/L Phen and the graph shows the quantification of relative colony numbers. $^*P < 0.05$ compared with control (Student's *t* test). D, Indicated cells were stained with FITC-annexin V and PI post–treatment and viewed under fluorescent microscope (scale bars in 100 μm). E, Cell lysates of indicated cell lines treated with Met and Phen were assayed by western blot for expression of indicated proteins. F, Met and Phen inhibited the growth (left panel) and weight (middle panel) of SW837 xenograft tumors. $^*P < 0.05$ compared with control. The right panel shows the xenograft tumor of mice treated with Met, Phen or control

the sensitivity of metformin and phenformin in rectal cancer cells, MTT assays were performed, and half-maximal inhibitory concentration ($IC_{50}$) values were determined. The $IC_{50}$ values of metformin and phenformin were: 34.4 mmol/L and 93.75 μmol/L, respectively, for HCT116 cells, 40 mmol/L and 100 μmol/L, respectively, for LS513 cells; 1.02 mmol/L and 2.4 μmol/L, respectively, for SW837 cells; and 8.75 mmol/L and 8.75 μmol/L, respectively, for SW1463 cells (Figure 2A, right). Notably, the $IC_{50}$ values for SW837 and SW1463 cells were significantly reduced compared with those of HCT116 and LS513 cells, indicating that rectal cancer cells were sensitize to metformin and phenformin. Thus, we confirmed that metformin and phenformin induced apoptosis in rectal cancer cells. To assess apoptosis, SW837 and SW1463 cells were treated with metformin (2 and 10 mmol/L) and phenformin (10 and 50 μmol/L) for 48 hours and analyzed by flow cytometry with annexin V-FITC/PI staining (Figure 2B, left). The percentages of apoptotic cells induced by metformin and phenformin were 25.0% and 35.4%, respectively, for SW837 cells and 36.6% and 36.4%, respectively, for SW1463 cells (Figure 2B, right). Induction of the apoptotic cell population by metformin and phenformin was significantly increased in rectal cancer cells compared with control cells. To determine whether metformin and phenformin affected rectal cancer cell proliferation, we performed colony forming assays. Notably, treatment with 1 mmol/L metformin and 5 μmol/L phenformin significantly inhibited colony forming activity in SW837 and SW1463 cells (Figure 2C). To further confirm the induction of apoptosis by metformin and phenformin, we investigated

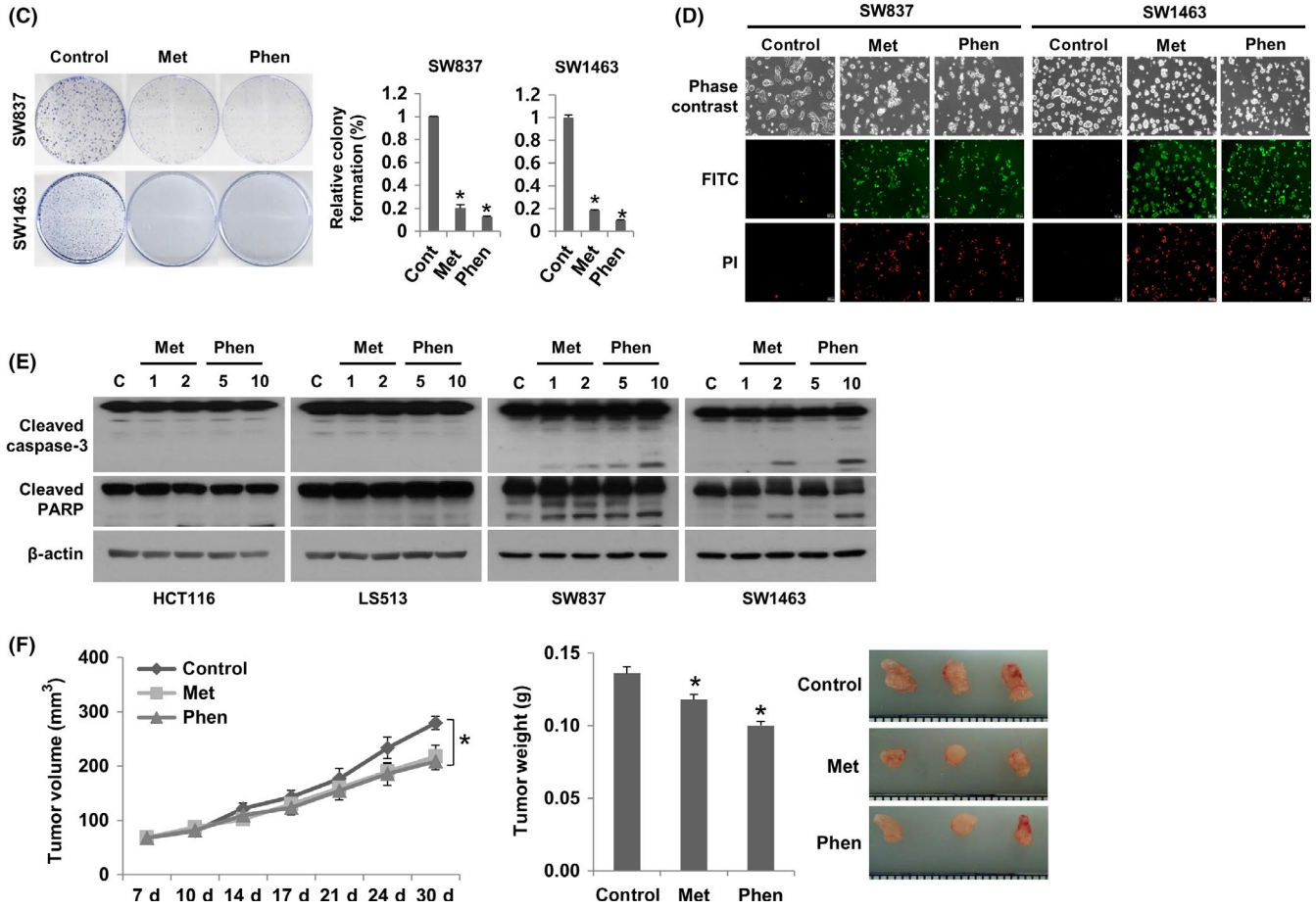



**FIGURE 2** Continued

fluorescence staining by FITC-Annexin-V and PI staining, and the expression of cleaved caspase-3 and cleaved-PARP by western blotting. As shown in Figure 2D, FITC and PI staining was detected only in cells treated with metformin and phenformin (Figure 2D). Consistent with our above results, cleaved caspase-3 and cleaved-PARP levels were significantly increased in metformin-treated and phenformin-treated SW837 and SW1463 rectal cancer cells (Figure 2E). Furthermore, mice bearing SW837 tumor xenografts treated with metformin and phenformin displayed decreased tumor volume of 22% and 25%, respectively, compared to approximately 280 $mm^3$ for control mice (Figure 2F). Taken together, these data suggested that metformin and phenformin induced antiproliferative/antisurvival activity via a caspase-dependent apoptotic pathway.

### 3.3 | Metformin and phenformin attenuated the expression of antiapoptotic proteins in rectal cancer cell lines by suppressing transforming growth factor-β receptor type 2/Smad2 and STAT3 signaling

Because TGF-β/Smad and STAT3 signaling pathways play important roles in CRC progression by reducing apoptosis and increasing cellular proliferation and survival,[23,24] we next examined whether these signaling pathways were activated by metformin and phenformin in CRC cells. Among the 4 cell lines, protein levels of TGF-β1, TGFBR2, phospho-Smad2 and phospho-STAT3 (Ser-727) were highly expressed in the 2 rectal cancer cells compared with the 2 colon cancer cells (Figure 3A). We next explored whether the expression levels of these proteins were involved in mediating the growth inhibitory effects of metformin and phenformin. Metformin and phenformin decreased the levels of TGFBR2, phospho-Smad2 and phospho-STAT3 (Ser-727) in SW837 and SW1463 cells (Figure 3B, left). To elucidate how the apoptotic pathway was affected by metformin and phenformin, we evaluated the expression of apoptosis-related proteins using western blotting. Metformin and phenformin inhibited the antiapoptotic proteins XIAP, cIAP1 and survivin (Figure 3B, middle). However, the expression levels of the pro–apoptotic proteins Bak, Bax, Bad and PUMA were not significantly altered (Figure 3B, right).

Next, we examined whether STAT3 and TGFBR2 were directly involved in mediating apoptosis in rectal cancer cells. STAT3 inhibition by 5 μmol/L STATIC, which selectively inhibits the activation, dimerization and nuclear translocation of STAT3, significantly suppressed the growth of SW837 rectal cancer cells. Moreover, STATTIC combined with IR or 5-FU slightly increased apoptosis in rectal cancer cells to 29.1% (Figure 3C, left). Consistent with this, siRNA-mediated STAT3 knockdown increased the apoptotic effects of IR or 5-FU to 37.5% (Figure 3C, right). We further confirmed the apoptotic effect of STAT3 inhibition on rectal cancer cells through annexin V and PI fluorescence staining assay (Figure 3D), indicating that STAT3 was

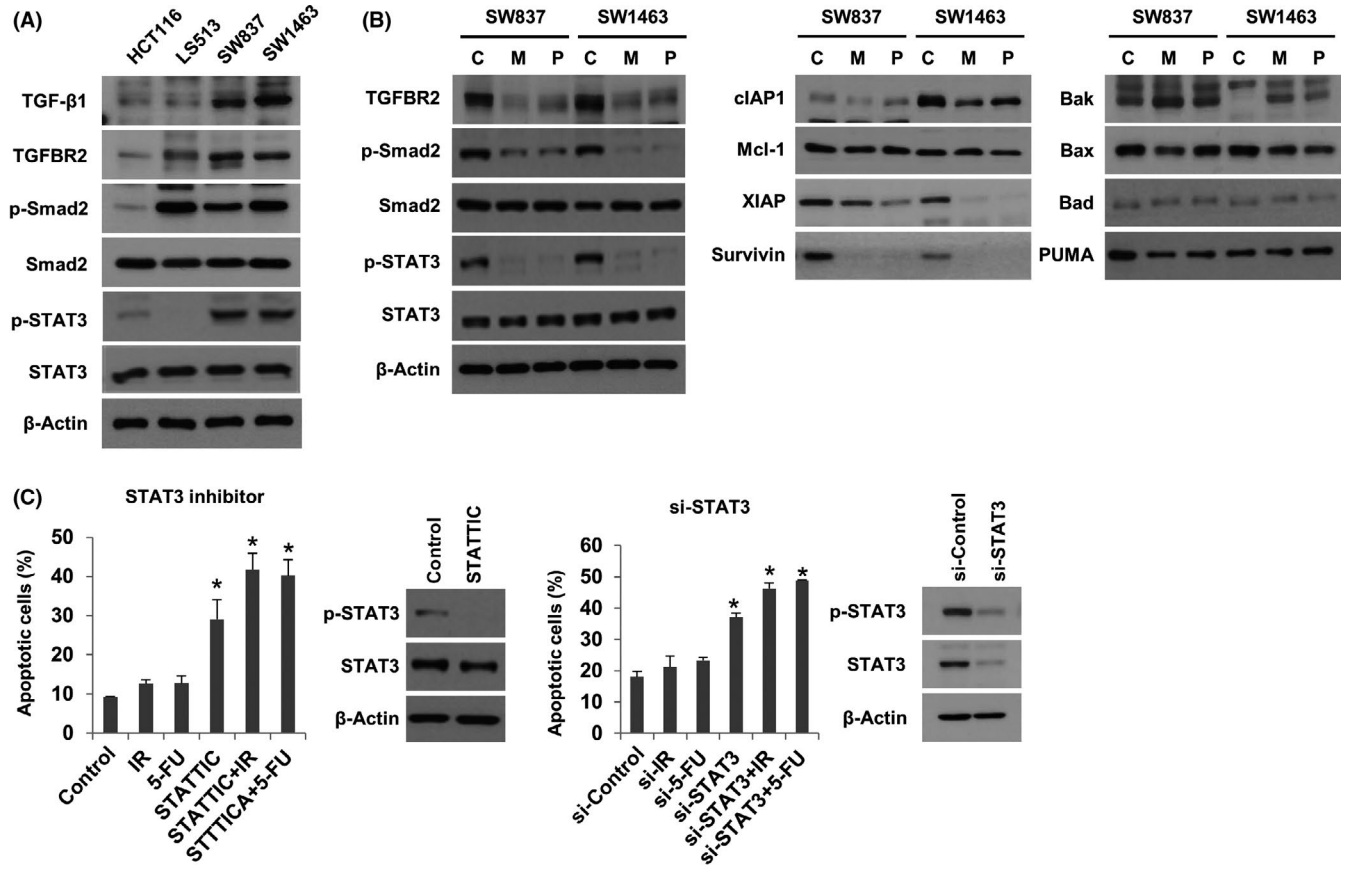



**FIGURE 3** Metformin and phenformin attenuated the expression of antiapoptotic proteins in rectal cancer cell lines by suppressing transforming growth factor β receptor type 2/Smad2 (TGFBR2) and STAT3 signaling. A, The protein expression of transforming growth factor β (TGF-β)/Smad and STAT3 signaling pathways in the indicated cells was measured using western blotting. B, Lysates of SW837 and SW1463 cells treated with 2 mmol/L Met and 10 μmol/L Phen for 48 h were assayed by western blotting for expression of indicated proteins. C, SW837 cells were pre–treated with 5 μmol/L STAT3 inhibitor (STATTIC) or transfected with 50 nmol/L STAT3 siRNA, and additionally cultured with 10 Gy and 40 μmol/L 5-FU. After 60 h, cells were stained with annexin V and then the apoptosis was measured by flow cytometry. The bar graph on the left shows quantification of the FACS results. *$P < 0.05$ compared with control (Student's *t* test). The indicated proteins were detected by western blotting. D, Indicated cells were stained with FITC-annexin V and PI post–treatment and viewed under fluorescent microscope. E and F, To investigate the effect of STAT3 on apoptosis-related protein expression, cell lysates prepared as in (C) were assayed by western blotting and human apoptosis array for expression of indicated proteins. G and H, To examine the involvement of TGFBR2 in cell apoptosis regulation, SW837 cells were pre–treated with 10 μmol/L TGFBR inhibitor (SB525334) or transfected with 20 nmol/L TGFBR2 siRNA and additionally cultured with the 10 Gy and 40 μmol/L 5-FU. The FACS results measuring apoptosis were quantified and shown as a bar graph (E), and the expression of apoptosis-related proteins was confirmed by western blot (F)

involved in the regulation of apoptosis in rectal cancer cells. Because the antiapoptotic proteins XIAP, cIAP1 and survivin were regulated by metformin and phenformin (Figure 3B, middle), we explored whether inhibition of STAT3 affected the expression of 35 apoptosis-related proteins with human apoptosis array. SW837 cells were inhibited by STAT3, 5 μmol/L STATTIC or 50 nmol/L STAT3 siRNA, and total cell extracts were isolated and then subjected to western blot and apoptosis array analysis (Figure 3E,F). Consistent with Figure 3B, we found that expression of antiapoptotic proteins, XIAP and cIAP1 were suppressed by STATTIC or STAT3 siRNA, whereas expression of pro–apoptotic proteins Bax and Bad were not changed (Figure 3E,F). We next investigated the roles of TGF-β signaling in rectal cancer cells. Treatment with the TGFBR inhibitor SB525334 or TGFBR2 siRNA did not significantly affect apoptosis in rectal cancer cells compared with that in IR-treated and 5-FU-treated cells (Figure 3G). Moreover, inhibition of TGF-β/Smad signaling by SB525334 or siRNA had no effect on the expression of antiapoptotic proteins (Figure 3H). Collectively, our data suggested that STAT3 signaling, but not TGF-β/Smad signaling, contributed to apoptosis in rectal cancer.

### 3.4 | Metformin and phenformin inhibited the epithelial-mesenchymal transition by blocking of transforming growth factor-β receptor type 2 expression in rectal cancer cells

To evaluate the inhibitory effects of metformin and phenformin on malignant progression of rectal cancer, we first performed Transwell migration and invasion assays in SW837 cells treated with 1 mmol/L metformin and 5 μmol/L phenformin. Metformin and phenformin significantly decreased the migration and invasion abilities of

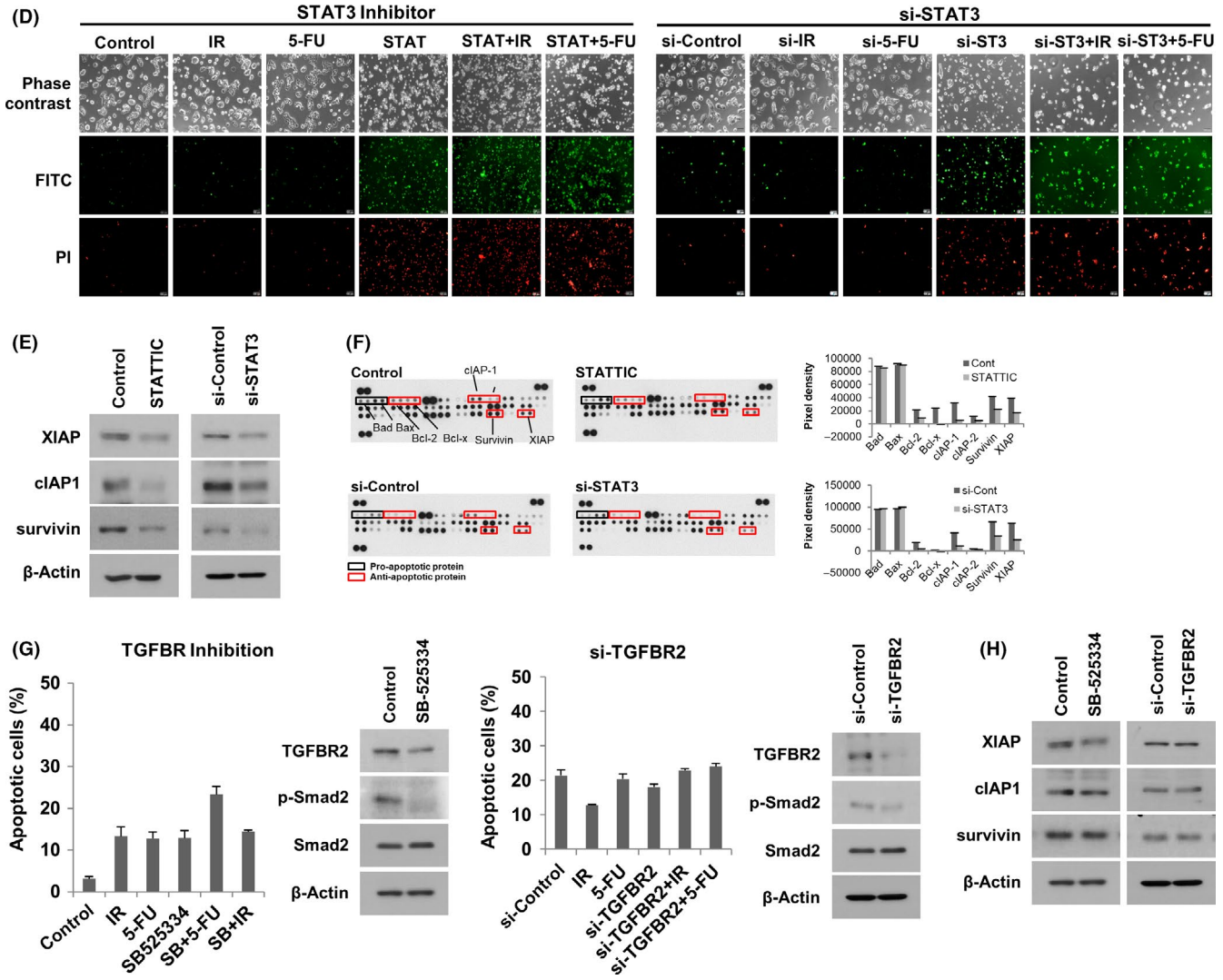



**FIGURE 3** Continued

SW837 cells (Figure 4A). To determine the roles of metformin and phenformin in the EMT phenotype in rectal cancer cells, we evaluated the expression of mesenchymal markers, such as N-cadherin, vimentin, Snail, Twist, Slug and Zeb1. The expression levels of these markers were downregulated in rectal cancer cells treated with metformin and phenformin (Figure 4B). Because TGF-β signaling plays crucial roles in cell growth and the EMT,[25] we also explored whether TGFBR2 expression was involved in inhibition of the metformin- and phenformin-mediated EMT. To block the activity or expression of TGFBR2, SW837 rectal cancer cells were treated with a 10 μmol/L TGFBR inhibitor or 20 nmol/L TGFBR2-targeting siRNA. As shown in Figure 3G, inhibition of TGF-β activity and TGFBR2 expression did not affect the cell apoptosis rate (Figure 4C) but decreased migration and invasion in SW837 rectal cancer cells (Figure 4D,F). We also observed reduced expression of mesenchymal markers, such as N-cadherin and vimentin, and EMT regulators, such as Snail, Twist, Slug and Zeb1, in SW837 cells treated with metformin and phenformin (Figure 4E,G). Moreover, treatment with TGFBR2 siRNA reversed the changes in the ability of invasion and migration, and EMT marker expression in the TGFBR2-overexpressing SW837 cells (Figure 4F,G). Together, these findings suggested that metformin and phenformin significantly inhibited the EMT phenotype, including the expression of EMT-related molecules and the migration and invasion abilities of the cells, by targeting TGF-β signaling.

### 3.5 | Phospho-STAT3 (Ser-727) and transforming growth factor-β receptor type 2 levels in human colon and rectal cancer tissues

In previous studies, phospho-STAT3 (Ser-727) and TGFBR2 have been shown to act as oncogenes in various cancers, including breast, lung, colorectal and prostate cancers.[19,26-28] However, their roles in rectal cancer are unclear. We therefore determined by western analysis that the expression of STAT3 (Ser-727) and TGFBR2 are decreased in mouse tumor tissues treated with metformin and phenformin, as shown in Figure 2F (Figure S1). In addition, we first investigated differences in the expression levels of phospho-STAT3 (Ser-727) and TGFBR2 in CRC, including 32 colon cancer cases and 73 rectal cancer cases. The levels of phospho-STAT3 (Ser-727) and TGFBR2 were increased in CRC (Figure 5A,B). Collectively, these results supported that

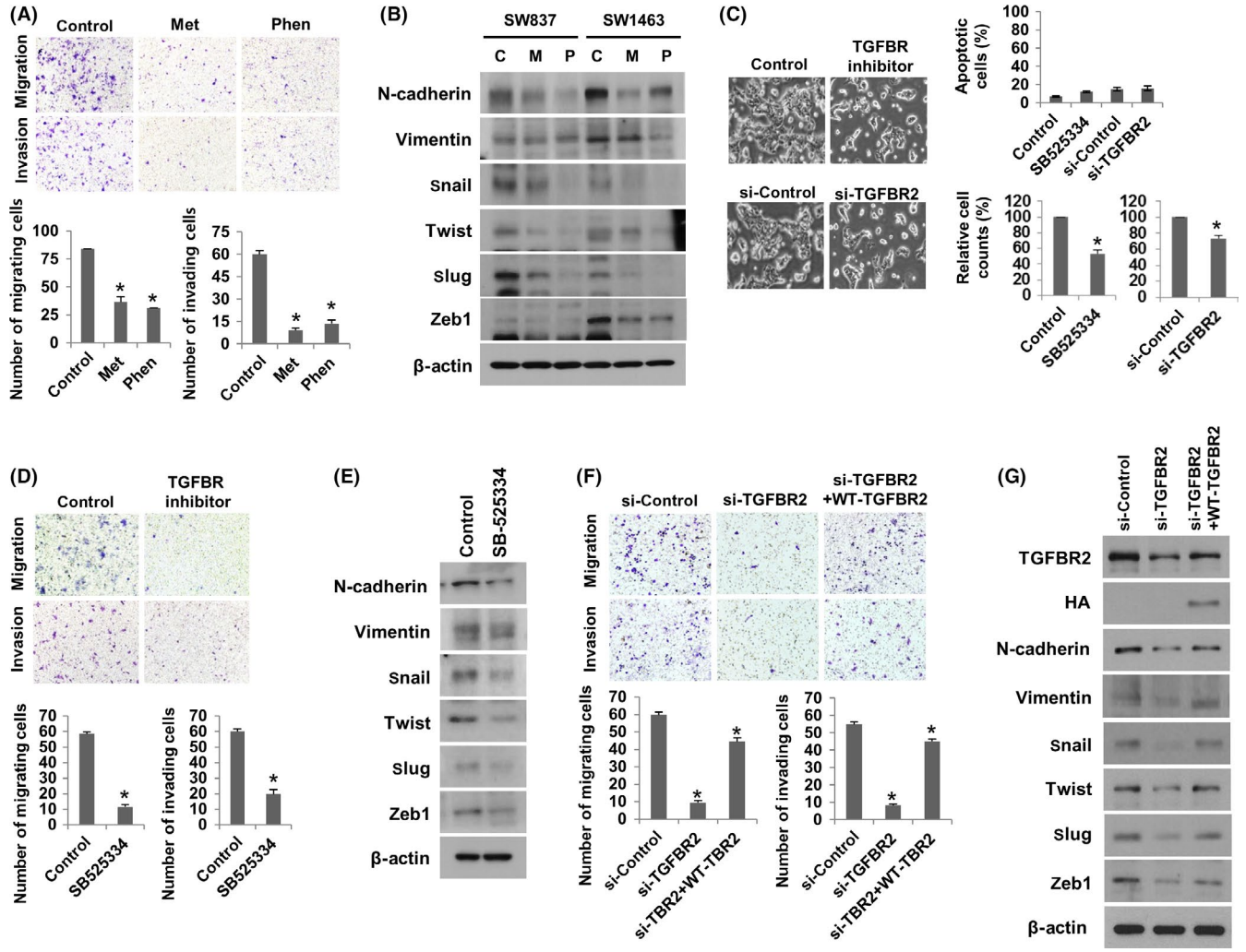


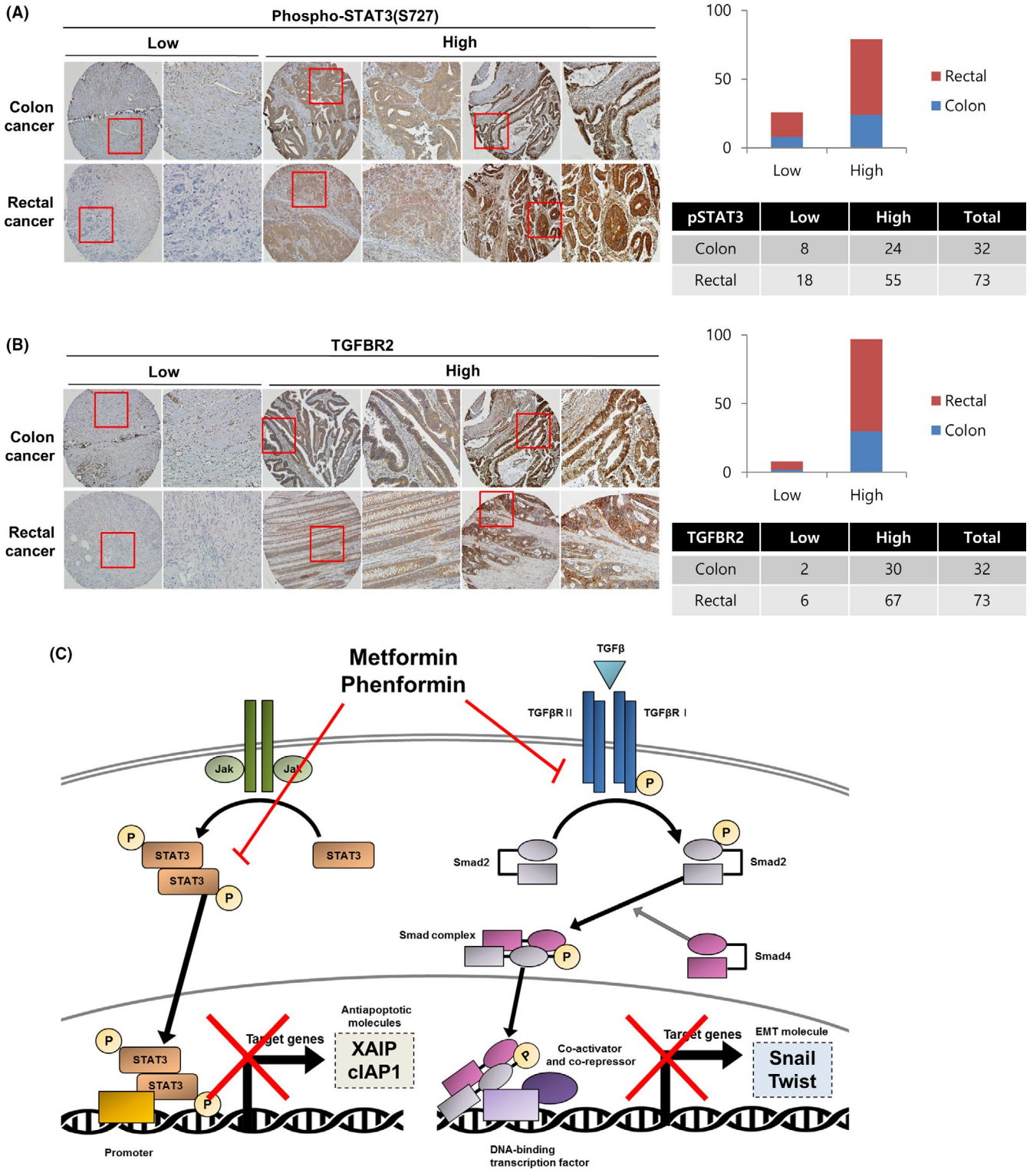

**FIGURE 4** Metformin and phenformin inhibited the EMT by blocking of TGFBR2 expression in rectal cancer cells. A, The migration (upper panel) and invasion (lower panel) by the SW837 cells treated with Met and Phen were analyzed and quantified. B, The expression levels of epithelial and mesenchymal (EMT) markers in the indicated cells were analyzed using western blot. C, Cells were treated with 10 μmol/L TGFBR inhibitor or transfected with 20 nmol/L siRNA against TGFBR2. Cell morphology was examined under a light microscope. The graph shows the relative viability of cells. D-G, To investigate the effect of TGFBR2 in EMT phenomenon, TGFBR2 inhibition or siRNA-mediated TGFBR2 knockdown cells (D and E), and rescued TGFBR2 WT (F and G) cells were subjected to chamber transwell assays of cellular migration or invasion (D and F) and to western blotting for analysis of indicated EMT marker expression (E and G). Bars in A, D and F indicate measurements of migration and invasion abilities. *$P < 0.05$ compared with control (Student's *t* test)

phospho-STAT3 (Ser-727) and TGFBR2 acted as oncogenes, suggesting that these molecules may be new therapeutic targets in early and metastatic rectal cancer, respectively.

## 4 | DISCUSSION

In this study, we showed that metformin and phenformin decreased the expression of pro–apoptotic proteins by inhibiting STAT3 phosphorylation at Ser-727 and suppressed invasion and migration by inhibiting TGFBR2-mediated signaling. These data demonstrated, for the first time, that rectal cancer progression, including apoptosis, invasion and migration, was controlled by metformin- and phenformin-mediated phospho-STAT3 (Ser-727) and TGFBR2 signaling. A proposed working model for metformin and phenformin in rectal cancer progression is presented in Figure 5C.

Recent epidemiological studies have demonstrated that metformin and phenformin play important roles in cancer progression[5,29]; however, the molecular mechanisms underlying this effect are unclear. AMPK-dependent signaling has been shown to mediate the effects of metformin and phenformin on cell growth. In bladder cancer, especially, metformin or phenformin and gefinitib, a well-known EGFR tyrosine kinase inhibitor, cooperated to inhibit cell growth via AMPK and EGFR pathways.[30,31] However, this mechanism was not active in our system; indeed, metformin and phenformin did not alter the expression of AMPK-related molecules (data not shown). Several reports have demonstrated that an AMPK-independent pathway also plays an important role in cell growth inhibition




| pSTAT3 | Low | High | Total |
|---|---|---|---|
| Colon | 8 | 24 | 32 |
| Rectal | 18 | 55 | 73 |

| TGFBR2 | Low | High | Total |
|---|---|---|---|
| Colon | 2 | 30 | 32 |
| Rectal | 6 | 67 | 73 |

**FIGURE 5** Phospho-STAT3 (Ser-727) and TGFBR2 levels in human colon and rectal cancer tissues. A and B, Representative immunohistochemistry images and bar graphs showing the expression of phosphor-STAT3 (Ser-727) (A) and TGFBR2 (B) in 32 colon and 73 rectal cancer tissues. C, Proposed model for metformin and phenformin in rectal cancer progression

and the TGF-β-induced EMT. Growing evidence has emerged that metformin combined with targeted drugs overcomes resistance and enhances therapeutic efficacy.[32] For example, metformin increases the sensitivity of tyrosine kinase inhibitor-resistant lung cancer cells through inhibition of interleukin-6/STAT3 signaling and EMT reversal.[33] According to a recent study, constitutively active STAT3 or silencing of STAT3 regulates metformin-induced growth inhibition and apoptosis in triple-negative breast cancer cells.[34] Our data also

demonstrated that metformin inhibited phospho-STAT3 (Ser-727) and decreased the expression of STAT3-mediated antiapoptotic proteins in rectal cancer cells. Several studies have illustrated the combined effects of metformin and 5-FU in tumor growth in various cancers, including esophageal cancer, colon cancer and oral squamous cell carcinoma.[35-37] Although our immunohistochemical staining results showed that phospho-STAT3 (Ser-727) was upregulated in CRC, colon cancer was sensitive to IR and 5-FU, whereas rectal cancer showed resistance to these therapeutic modalities. Furthermore, we found that a STAT3-specific inhibitor or siRNA against STAT3 not only significantly increased the number of apoptotic cells in rectal cancer but also had combined effects with IR and 5-FU. Thus, we suggest that phospho-STAT3 (Ser-727) may be an important downstream mediator of metformin- and phenformin-induced apoptosis.

In this study, we found that the metformin- and phenformin-mediated EMT occurred via TGF-β rather than STAT3 activation. Metformin has been shown to inhibit the TGF-β1-induced EMT, which plays a key role in cancer progression.[38] Xiao et al[39] demonstrated that metformin directly interacts with TGF-β1 and inhibits TGF-β1-induced TGFBR2 dimerization. In our study, we found that both TGF-β1 and TGFBR2 were more highly expressed in rectal cancer cells than in colon cancer cells. Growing evidence has shown that metformin modulates the stability of various proteins, such as c-Myc, mTOR, KLE5 and sirtiun 1, in cancer cells.[40-43] Therefore, we examined whether TGFBR2 was a target of metformin. Expression of TGFBR2 was decreased by metformin and phenformin in rectal cancer cells. However, metformin and phenformin also altered the expression of *TGFBR2* mRNA (data not shown). Therefore, other modulators may regulate *TGFBR2* at the mRNA level. For example, the H3K27 methyltransferase enzyme EZH2 contributes to TGFBR2 silencing by promoter hypermethylation in prostate cancer.[44] Moreover, *miR-93* and *miR-204*, which are aberrantly expressed in various malignant tumors, including prostate and breast cancers, respectively, regulate cancer progression by targeting TGFBR2.[41,45] Taken together, our findings suggested that metformin and phenformin may regulate *TGFBR2* mRNA expression through other regulators. Further studies are needed to clarify the exact mechanisms of *TGFBR2* mRNA regulation by metformin and phenformin.

The use of metformin in rectal cancer has recently been reported to significantly improve pathologic complete response rates and survival rates, suggesting the need for prospective studies.[43] Previous studies have shown that low expression of TGFBR2 is related to a poor prognosis in some cancers, including oral,[46] cervical[47] and breast cancers.[28] In contrast, another study suggested that high expression of TGFBR2 may be a poor prognostic indicator for overall survival in estrogen receptor α-negative breast cancer.[48] Li et al[49] also showed that zinc finger protein 32, an important transcription factor associated with cancer, binds to the *TGFBR2* promoter and elevates its expression at the mRNA and protein levels, thereby increasing drug resistance in lung cancer. Consistent with this, our results showed that TGFBR2 was highly expressed in rectal cancer cells and tissues. Taken together, our findings supported the oncogenic roles of phospho-STAT3 (Ser-727) and TGFBR2 in rectal cancer progression. These results could have important implications regarding the critical roles of phospho-STAT3 (Ser-727) and TGFBR2 in tumor development through metformin- and phenformin-induced inhibition of apoptosis and the EMT.

In this study, we demonstrated that metformin and phenformin regulated chemoresistance in rectal cancer cells by controlling cell growth and the EMT via phospho-STAT3 (Ser-727) and TGFBR2. These findings provide insight into the oncogenic activities of phospho-STAT3 (Ser-727) and TGFBR2 and support that metformin and phenformin may have therapeutic roles in rectal cancer treatment. Moreover, TGFBR2 may represent a promising molecular target for controlling the resistance of rectal cancer to chemotherapy.

## DISCLOSURE

The authors declare that they have no conflicts of interest.

## ORCID

*Ji-Hye Park* https://orcid.org/0000-0003-0674-5110
*Young-heon Kim* https://orcid.org/0000-0002-4766-3181
*Jae Kyung Myung* https://orcid.org/0000-0001-9612-5323

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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