Metformin inhibits epithelial-mesenchymal transition of oral squamous cell carcinoma via the mTOR/HIF-1α/PKM2/STAT3 pathway

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Abstract. Epithelial-mesenchymal transition (EMT) serves an important role in the formation and development of various types of cancer, including oral squamous cell carcinoma (OSCC). Metformin, used for treating type 2 diabetes, has been revealed to exert an anticancer effect in various types of cancer, including liver, breast and colorectal cancer. However, its role in the EMT of OSCC has been rarely reported. Therefore, the present study aimed to investigate the effects of metformin on EMT and to identify its underlying mechanism in OSCC. Firstly, EMT was induced in CAL-27 cells using $CoCl_2$. Subsequently, the effects of metformin on cell viability, migration and xenograft growth were evaluated *in vitro* and *in vivo*. Reverse transcription-quantitative PCR was performed to detect the expression levels of E-cadherin, vimentin, snail family transcriptional repressor 1, mTOR, hypoxia inducible factor 1α, pyruvate kinase M2 and STAT3. The results demonstrated that metformin abolished $CoCl_2$-induced cell proliferation, migration, invasion and EMT. Moreover, metformin reversed EMT in OSCC by inhibiting the mTOR-associated HIF-1α/PKM2/STAT3 signaling pathway. Overall, the present findings characterized a novel mechanism via which metformin modulated EMT in OSCC.

Introduction

Oral squamous cell carcinoma (OSCC) is the sixth most common type of cancer in the world (1), and is the most common primary oral cancer type in the oral maxillofacial region, with a 5-year survival rate of 50-60% (2). Smoking and alcohol are major risk factors for oral cancer, both exerting synergistic effects (3). Epithelial-mesenchymal transition (EMT) serves an important role in tumorigenesis and tumor development. EMT is a process that involves loss of cell polarity and cell-cell adhesion conferring tumor cells the ability to migrate and metastasize (4).

mTORs, functioning as mechanistic targets, are regulators of cell proliferation and metabolism (5). mTOR principally controls cell metabolism by regulating the translation and transcription of metabolic genes (6). It has been revealed that mTOR can be activated in kidney cancer and accelerates cancer progression (7). Previous studies have reported that suppressing the mTOR-associated signaling pathway can inhibit EMT (8-10).

Hypoxia inducible factor 1α (HIF-1α) leads to insufficient blood supply and hypoxia in the tumor microenvironment and affects tumor metabolism (11). Moreover, the upregulation of HIF-1α serves a crucial role in tumorigenesis, tumor angiogenesis, glycolysis and chemoresistance (12). The downstream factor of HIF-1α, pyruvate kinase M2 (PKM2), can interact with HIF-1α to regulate cancer metabolism (13). In addition, STAT3 can promote EMT progression (14), and HIF-1α, PKM2 and STAT3 are all modulated by mTOR (15,16).

Metformin, used for treatment of type 2 diabetes, has been associated with decreasing cancer incidence and mortality (17). A previous study revealed that metformin decreased the risk of liver, breast and colorectal cancer (2). Other studies have indicated that metformin inhibits EMT in prostate cancer, cervical cancer and rectal cancer (18-20). However, to the best of our knowledge, no reports have studied EMT in OSCC. Moreover, the potential mechanism via which metformin inhibits tumor growth is yet to be fully elucidated. Therefore, the aims of the present study were to investigate the role of metformin on inhibiting $CoCl_2$-induced EMT in OSCC cells and to examine whether EMT could be suppressed via the mTOR/HIF-1α/PKM2/STAT3 signaling pathway.

Materials and methods

Cell lines and culture. The human OSCC CAL27 cell line was acquired from the Department of Oral and Maxillofacial

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Surgery, Tooth Development and Maxillary Reconstruction and Regeneration Laboratory at Jilin University (Changchun, China). Cells were cultured in Dulbecco's modified Eagle's (DMEM) medium supplemented with 10% FBS and 100 mg/ml penicillin/streptomycin (all purchased from Invitrogen; Thermo Fisher Scientific, Inc.). Cells were cultured at 37˚C in a humid incubator with 5% CO_2.

Metformin (MedChemExpress) and $CoCl_2$ (Sigma-Aldrich; Merck KGaA) were dissolved in PBS (Invitrogen; Thermo Fisher Scientific, Inc.) at a stock concentration of 160 mM and 500 μM, respectively. Both were stored at -80˚C.

Cell proliferation assay. Cells were seeded at a density of $1x10^4$ cells/well in 96-well plates and cultured overnight at 37˚C. After treatment by indicated concentrations of $CoCl_2$ (0, 50, 100, 200, 300, 400 and 500 μM) and metformin (0, 2.5, 5, 10, 20, 40, 80 and 160 mM) for 24 h at 37˚C, 10 μl the Cell Counting Kit-8 (CCK-8) reagent (Invigentech) was added to each well according to the manufacturer's protocol. After culturing for 2 h, the absorbance was measured at 490 nm using a microplate reader (BioTek Instruments, Inc.). After the screening process, the medium of optimal concentrations (10 mM metformin with or without 300 μM $CoCl_2$) was used to culture cells for 24 h at 37˚C. Then, the above process were repeated. All results were measured three times.

Cell migration assay. A wound-healing assay was performed to assess cell migration, and 10^6 cells/well were seeded onto 6-well plates for 24 h at 37˚C. A wound was scraped using a 1,000-μl pipette tip, and plates were washed three times with PBS. The cells were cultured in fresh serum-free medium containing 300 μM $CoCl_2$, with or without 10 mM metformin, for 24 h at 37˚C. Images were captured at the time points of 0 and 24 h after wounding. The migration rate was quantified using the following equation: (0-h scratching distance - 24-h scratching distance)/0-h scratching distance. Representative images were obtained at x40 magnification using an Olympus light microscope (Olympus Corporation). All experiments were repeated at least three times.

Cell invasion assay. Transwell assay was performed using 24-well Transwell units (Corning, Inc.) with an 8-μm pore size polycarbonate membrane which has been precoated with Matrigel (Becton Dickinson) for 1 h at 37˚C. Cells ($1x10^5$), 300 μM $CoCl_2$, with or without 10 mM metformin, were suspended in 100 μl DMEM without FBS were seeded into the upper unit, while 600 μl DMEM with 10% FBS, was added to the lower units. After incubation for 24 h at 37˚C, cells on the upper side of the membrane were removed using PBS-soaked cotton swabs. The membrane was fixed in paraformaldehyde for 30 min at 37˚C and then stained with 0.1% crystal violet for 30 min at room temperature. Cell numbers under the membrane were counted using an Olympus light microscope (magnification, x400; Olympus Corporation).

RNA isolation and reverse-transcription-quantitative (RT-q) PCR. Cells were cultured in DMEM with 10% FBS containing 300 μM $CoCl_2$, with or without 10 mM metformin (metformin and $CoCl_2$ were added at the same time), for 48 h at 37˚C. Total RNA was extracted using TRIzol® (TRIeasyTM Total RNA Extraction reagent; Shanghai Yeasen Biotechnology Co., Ltd.) from the specified treated cells and maintained at -20˚C for 12 h. Total RNA was reverse transcribed using Hifair™ II 1st Strand cDNA Synthesis SuperMix (TRIeasy™ Total RNA Extraction reagent; Shanghai Yeasen Biotechnology Co., Ltd.) for qPCR under the recommended conditions: 25˚C for 5 min, 42˚C for 30 min, 85˚C for 5 min and holding at 4˚C (GeneAmp PCR System 9700; Thermo Fisher Scientific, Inc.). cDNA corresponding to 25 ng RNA was used for qPCR using a HieffTM qPCR SYBR® Green Master mix (Shanghai Yeasen Biotechnology Co., Ltd.). The following thermocycling conditions were used: Initial denaturation at 95˚C for 5 min and 40 cycles at 95˚C for 10 sec and 60˚C for 30 sec (ProFlex PCR System; Thermo Fisher Scientific, Inc.). The expression levels of human β-actin, E-cadherin, vimentin, snail family transcriptional repressor 1 (Snail1), mTOR, HIF-1α, PKM2 and STAT3 were detected. Gene expression normalized to β-actin was calculated using the $2^{-\Delta\Delta Cq}$ method (21). The RT-qPCR primers were as follows: β-actin forward, 5'-CTCCATCCT GGCCTCGCTGT-3' and reverse, 5'-GCTGTCACCTTCACC GTTCC-3'; E-cadherin forward, 5'-GCCTCCTGAAAAGAG AGTGGAAG-3' and reverse, 5'-TGGCAGTGTCTCTCC AAA TCCG-3'; vimentin forward, 5'-AGGCAAAGCAGG AGTCCACTGA-3' and reverse, 5'-ATCTGGCGTTCCAGG GACTCAT-3'; Snail1 forward, 5'-ATCTGCGGCAAGGCG TTTTCCA-3' and reverse, 5'-GAGCCCTCAGATTTGACC TGTC-3'; mTOR forward, 5'-AGCATCGGATGCTTAGGA GTGG-3' and reverse, 5'-CAGCCAGTCATCTTTGGA GACC-3'; HIF-1α forward, 5'-TAGCCGAGGAAGAAC TATGAAC-3' and reverse, 5'-CTGAGGTTGGTTACTGTT GGTA-3'; PKM2 forward, 5'-ATGGCTGACACATTCCTG GAGC-3' and reverse, 5'-CCTTCAACGTCTCCACTG ATCG-3'; and STAT3 forward, 5'-CTTTGAGACCGAGGT GTATCACC-3' and reverse, 5'-GGTCAGCATGTTGTACCA CAGG-3'.

Xenograft mouse studies. To investigate whether metformin inhibited $CoCl_2$-induced EMT *in vivo*, the subcutaneous xenografted growth of OSCC cells was monitored. For the experiment, cells were cultured in DMEM with 20% FBS containing 300 μM $CoCl_2$, with or without 10 mM metformin, for 48 h at 37˚C. There were 28 male BALB/C nude mice (Shanghai Vital River Laboratory Animal Technology Co., Ltd.; age, 4-6 weeks; weight, 15-20 g) were housed under specific pathogen-free conditions, with food and water provided *ad libitum*. After 1 week of acclimation, the mice were randomly divided into four groups (seven mice per group) and injected with $5x10^6$ indicated OSCC cells subcutaneously which was resuspended by PBS into the right flank. Xenograft tumor volume and weight were measured every other day. After 24 days, nude mice were euthanized by cervical dislocation and tumors were collected. Tumor volume (mm3) was calculated as follows: 1/2 x long diameter (mm) x short diameter $(mm)^2$. The present study was approved by the Animal Research Ethics Committee of Jilin University. All animal treatments were performed in accordance with the Regulations of the Administration of Affairs Concerning Experimental Animals.

Statistical analysis. Statistical analysis was performed using SPSS v21 (IBM Corp.) and GraphPad Prism 8 (GraphPad

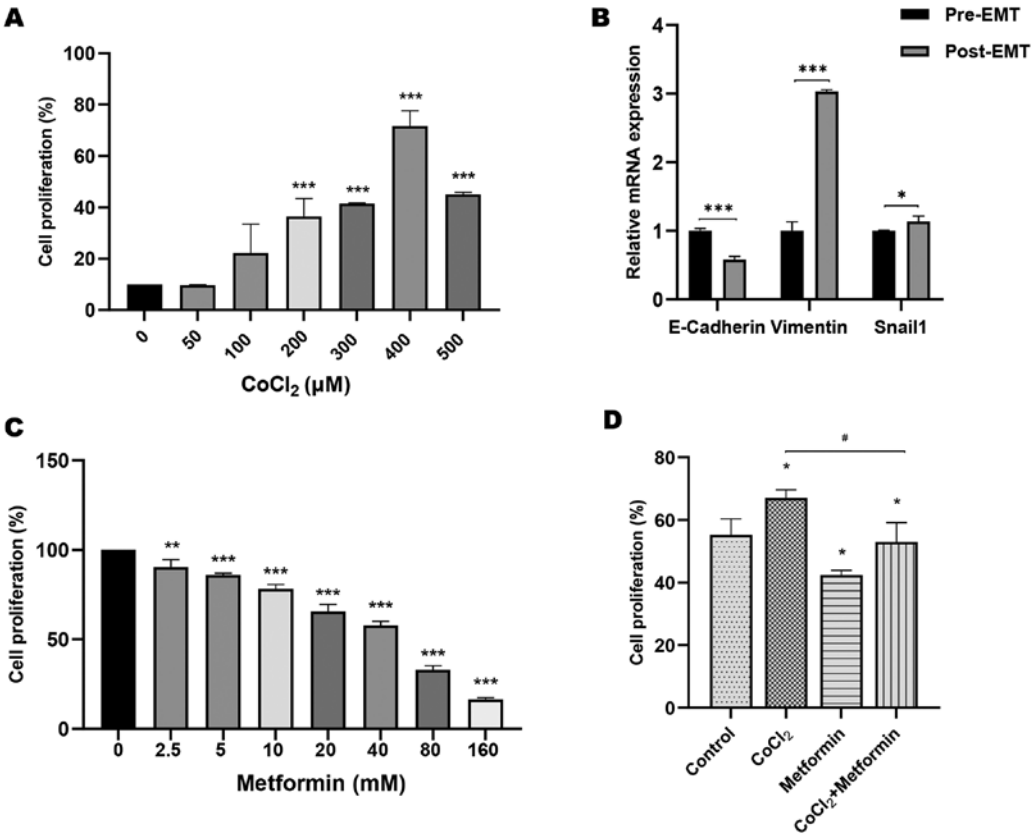

Figure 1. $CoCl_2$ promotes the proliferation and induces EMT of OSCC cells. (A) CAL-27 cells were treated with $CoCl_2$ (0-500 μM) for 24 h and cell proliferation was evaluated using a CCK-8 assay. ***P<0.001 vs. 0 μM. (B) Reverse transcription-quantitative PCR was used to detect the expression levels of E-cadherin, vimentin and Snail1. *P<0.05; ***P<0.001. (C) Metformin inhibited the proliferation of OSCC cells, evaluated via CCK-8 assay. **P<0.01; ***P<0.001 vs. 0 mM. (D) CAL-27 cells were treated with metformin (10 mM) with or without $CoCl_2$ (300 μM). CCK-8 assay was used to compare the four groups, revealing that metformin inhibited $CoCl_2$-induced proliferation. *P<0.05; #P<0.05 vs. control. CCK-8, Cell Counting Kit-8; OSCC oral squamous cell carcinoma; EMT, epithelial-mesenchymal transition; Snail1, snail family transcriptional repressor 1.

Software, Inc.). Data are presented as the mean ± SD of three independent experiments. One-way ANOVA was used for comparisons among multiple groups (with Tukey's post-hoc test), and unpaired t-test was used for comparisons between two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

$CoCl_2$ promotes proliferation and induces EMT of OSCC cells. Cells were treated with 0, 50, 100, 200, 300, 400 and 500 μM $CoCl_2$ to evaluate the potential effect of $CoCl_2$ in OSCC cells. $CoCl_2$ at concentrations ≥100 μM could induce cell proliferation (Fig. 1A). The concentration of 300 μM $CoCl_2$ with the lowest error bar was chosen for subsequent experiments. Moreover, the expression levels of E-cadherin were significantly upregulated in the absence of $CoCl_2$ (pre-EMT). After cells were stimulated with $CoCl_2$, E-cadherin expression was significantly decreased (post-EMT). Compared with the pre-EMT state, vimentin and Snail1 expression was significantly increased post-EMT by $CoCl_2$ treatment (Fig. 1B). These data indicated that $CoCl_2$ promoted cell proliferation and induced EMT when OSCC cells were stimulated with $CoCl_2$.

Metformin prevents proliferation, migration, invasion and EMT of OSCC cells induced by $CoCl_2$. Cells were treated with 0, 2.5, 5, 10, 20, 40, 80 and 160 mM metformin to investigate the underlying anti-proliferative effect of metformin in OSCC. Proliferation of cells treated with metformin decreased significantly in a dose-dependent manner compared with that of untreated cells (Fig. 1C). A concentration of 10 mM metformin was used for CCK-8, wound-healing, Transwell, RT-qPCR and nude mice xenograft assays. Moreover, compared with the group treated with $CoCl_2$, cell proliferation in the $CoCl_2$ + metformin group was significantly attenuated by the addition of metformin (Fig. 1D). Additionally, cells were treated with 300 μM $CoCl_2$, with or without 10 mM metformin and the result revealed that $CoCl_2$ significantly increased the migration of cells compared with the control group. This phenomenon could be abolished by the addition of metformin (Fig. 2A and B).

The markers of EMT were detected using RT-qPCR. In the $CoCl_2$ group, E-cadherin expression was decreased, while vimentin and Snial1 expression was increased, which all could be reversed by metformin (Fig. 2C-E). *In vivo* compared with the control group, the volume and weight of xenografts in the metformin group were reduced. Using $CoCl_2$ alone promoted tumor growth, which could be inhibited by the addition of metformin (Fig. 3A and B). These findings suggested that metformin inhibited the cell proliferative, migratory and invasive abilities, as well as reversed $CoCl_2$-induced EMT.

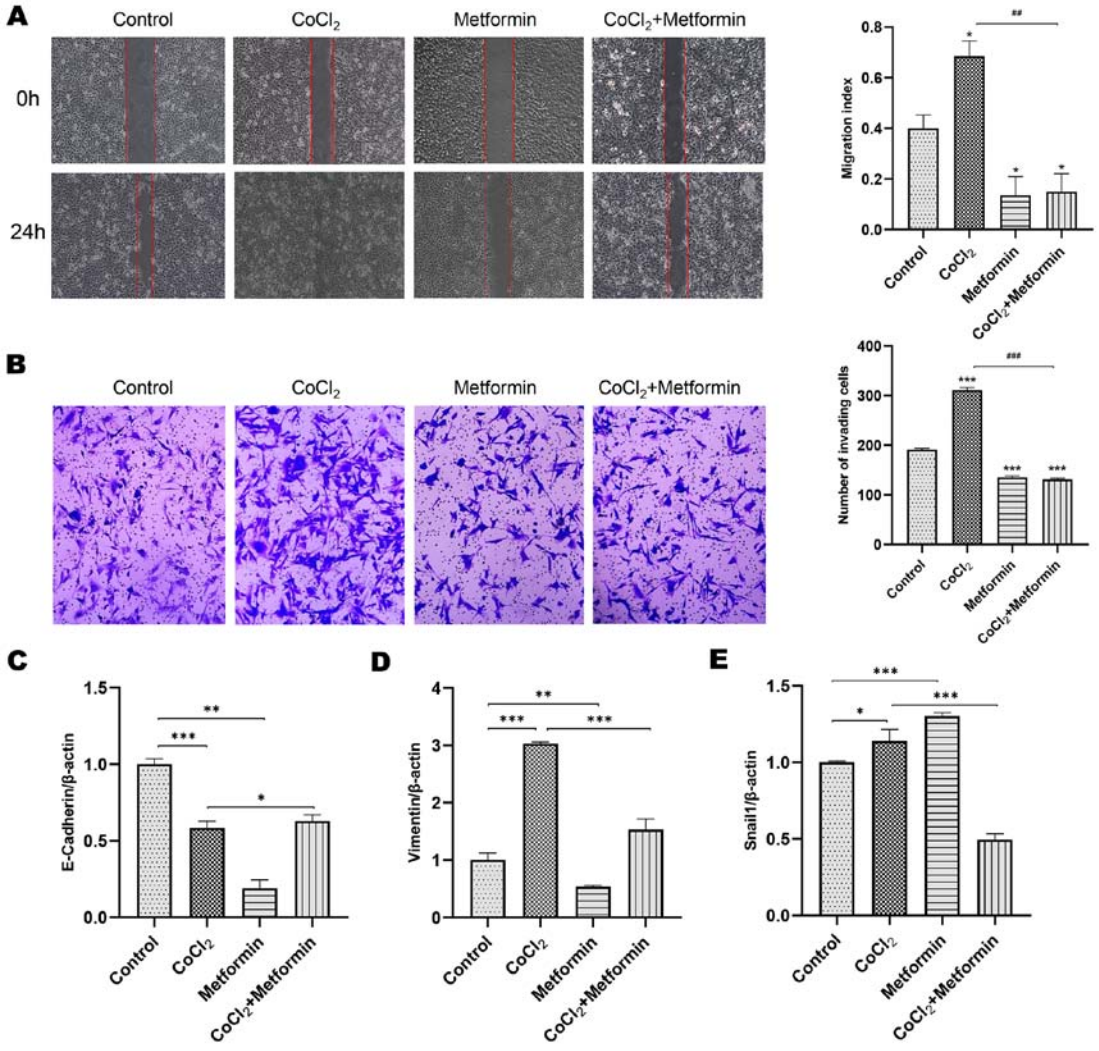

Figure 2. Metformin inhibits the migration and invasion of oral squamous cell carcinoma cells. (A) Wound-healing assay was used to analyze the relative cell migration distance after 24 h (magnification, x40) (B) Transwell assay was used to evaluate cell invasion (magnification, x200). Metformin inhibited $CoCl_2$-induced EMT. *P<0.05; ***P<0.001; $^{\#\#}$P<0.01; $^{\#\#\#}$P<0.001 vs. control. (C-E) Expression levels of EMT markers were detected via reverse transcription-quantitative PCR. (C) E-cadherin, (D) vimentin and (E) Snail1. Data are presented as the mean value of cells in five fields based on three independent experiments. *P<0.05; **P<0.01; ***P<0.001. EMT, epithelial-mesenchymal transition; Snail1, snail family transcriptional repressor 1.

Metformin prevents EMT of OSCC induced by $CoCl_2$ via the mTOR/HIF-1α/PKM2/STAT3 signaling pathway. The expression levels of mTOR, HIF-1α, PKM2 and STAT3 in the EMT process were detected via RT-qPCR. High expression levels of mTOR and PKM2 in $CoCl_2$-induced EMT were identified, which were inhibited by metformin (Fig. 4A and C). Moreover, HIF-1α was upregulated in the $CoCl_2$ group compared with the control group, but its expression was highest in the metformin group. In the $CoCl_2$ + metformin group, HIF-1α's expression was decreased compared with the $CoCl_2$ group (Fig. 4B). Thus, it was suggested that metformin suppressed $CoCl_2$-induced EMT, but using metformin independently did not exert this effect. STAT3 expression was significantly increased in the $CoCl_2$ group, but was significantly decreased by the addition of metformin (Fig. 4D).

Discussion

EMT is a major process in tumor metastasis, as well as a vital factor involved in mortality in patients with OSCC (22). Previous studies have reported that $CoCl_2$ at an appropriate concentration promotes cell proliferation and induces EMT in liver and mammary gland cancer (23,24). The present study used $CoCl_2$ to induce EMT and then investigated the underlying mechanism of metformin inhibition on $CoCl_2$-induced EMT in OSCC.

Metformin, as an antidiabetic drug, has been revealed to exert effects to decrease cancer incidence and mortality rates in various types of human cancer (17). The use of metformin in diabetic patients is associated with a decreased incidence in cancer types, including pancreatic, liver and colon cancer, and can decrease cancer-associated mortality (25). Moreover, several studies have reported that metformin inhibits tumor growth via multiple mechanisms, including by suppressing tumor cell proliferation (26) and EMT, affecting tumor autophagy and metabolism (12,27), and inducing apoptosis of cancer stem cells (28). Although no studies on the effects of metformin on EMT in OSCC, previous studies have demonstrated that metformin suppresses EMT in other types of cancer, including cervical and breast carcinoma (4,18).

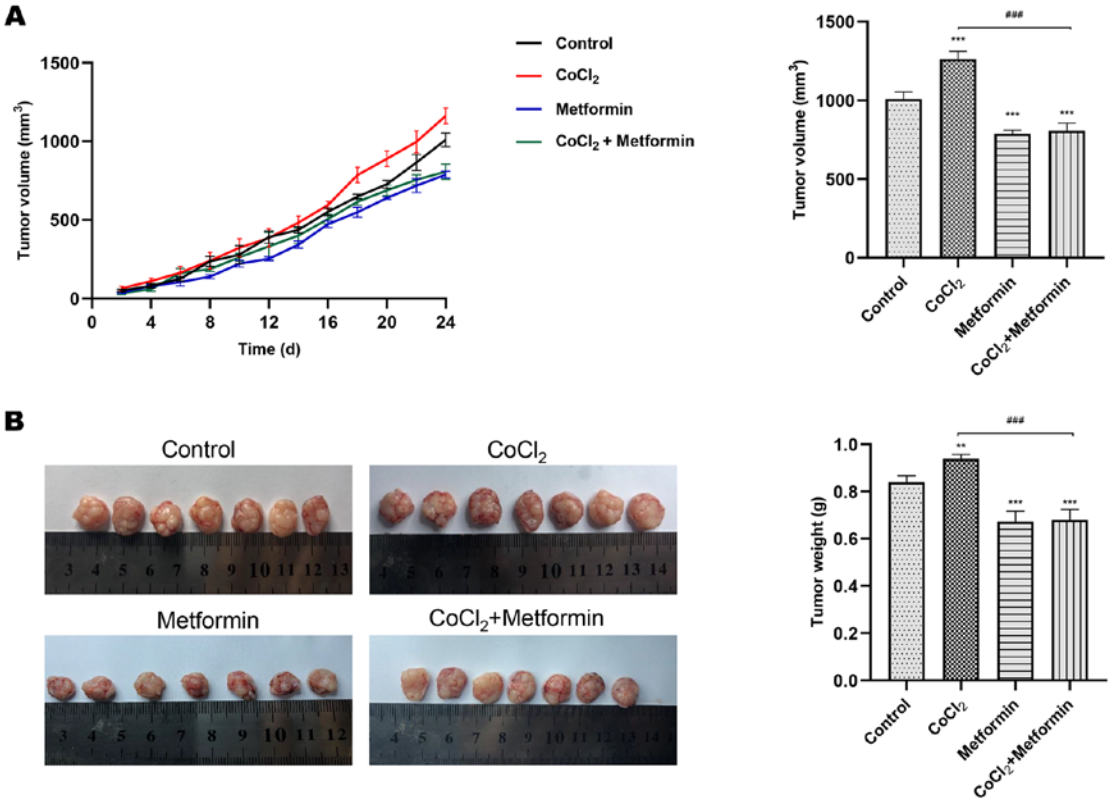

Figure 3. Metformin inhibits $CoCl_2$-induced EMT *in vivo*. (A) Tumor volume and (B) tumor weight were measured. **P<0.01; ***P<0.001; $^{###}$P<0.001 vs. control.

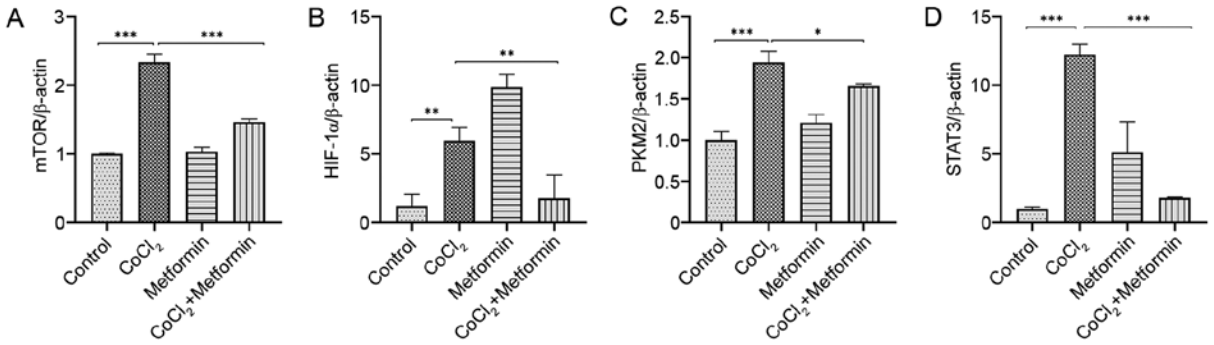

Figure 4. Metformin inhibits $CoCl_2$-induced EMT via the mTOR-associated HIF-1α/PKM2/STAT3 signaling pathway. CAL-27 cells were treated with metformin (10 mM) with or without $CoCl_2$ (300 μM) and the expression levels of (A) mTOR, (B) HIF-1α, (C) PKM2 and (D) STAT3 were detected via reverse transcription-quantitative PCR. *P<0.05; **P<0.01; ***P<0.001. EMT, epithelial-mesenchymal transition; HIF-1α, hypoxia inducible factor 1α; PKM2, pyruvate kinase M2.

The present study demonstrated that metformin inhibited $CoCl_2$-induced proliferation, migration, invasion and EMT in OSCC.

The inhibitory effect of metformin on EMT in cervical carcinoma via the mTOR-p70s6k relative signaling pathway has been observed (19). mTOR can accelerate cell proliferation and adjust cellular energy homeostasis (29). In EMT, overactivation of mTOR is closely associated with tumor progression (30). Thus, targeting the mTOR-associated pathway is the key to research.

mTOR regulates HIF-1α, and overexpression of HIF-1α facilitates tumor metastasis and EMT in colorectal cancer (15). Moreover, PKM2 is an important downstream executor of HIF-1α; the expression and function of PKM2 is associated with HIF-1α in feedforward loops, and HIF-1 is also a target gene of the PKM2/STAT3 signaling pathway (16). Metformin inhibits the expression levels of HIF-1α and PKM2 in gastric cancer (12), as wel as preventing the activation of STAT3 in breast cancer (31) and colorectal cancer (20). The aforementioned studies suggest that the mechanism of metformin suppressing $CoCl_2$-induced EMT in OSCC may also occur by inhibiting the mTOR-regulated HIF-1α/PKM2/STAT3 signaling pathway. The present results confirmed that metformin significantly inhibited $CoCl_2$-induced EMT via the mTOR/HIF-1α/PKM2 signaling pathway in OSCC cells. However, there are certain limitations to the current study, and additional cell lines are required to further validate the present results. In addition, the expression levels of markers

including mTOR/HIF-1α/PKM2/STAT3 should be further analyzed using western blotting or immunohistochemical staining. Additionally, rapamycin, an mTOR inhibitor, should be used to confirm that metformin inhibits EMT via the mTOR-associated HIF-1α/PKM2/STAT3 signaling pathway.

In conclusion, the present study demonstrated that EMT served an important role in the proliferation, migration and invasion of OSCC cells. Metformin was capable of reversing $CoCl_2$-induced EMT. Furthermore, it was identified that metformin exerted its effects by suppressing mTOR, HIF-1α, PKM2 and STAT3 activation. The current results were obtained using OSCC cell models *in vitro* and xenograft nude-mice models *in vivo*, and indicated that metformin may offer a novel strategy for the treatment of patients with OSCC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WY and ZY conceived the experiments. YL and BS designed the experiments. WY, YL, XL, XM, BS and ZY performed the experiments. WY and YL analyzed the data and wrote the manuscript. ZY and BS reviewed and edited the manuscript, and acquired the funds. All authors read and approved the final manuscript, and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was approved by the Animal Research Ethics Committee of Jilin University (Changchun, China). All animal treatments were performed in accordance with the Regulations of the Administration of Affairs Concerning Experimental Animals.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J and Jemal A: Global cancer statistics, 2012. CA Cancer J Clin 65: 87-108, 2015.
2. Wang Z, Lai ST, Xie L, Zhao JD, Ma NY, Zhu J, Ren ZG and Jiang GL: Metformin is associated with reduced risk of pancreatic cancer in patients with type 2 diabetes mellitus: A systematic review and meta-analysis. Diabetes Res Clin Pract 106: 19-26, 2014.
3. Galeone C, Edefonti V, Parpinel M, Leoncini E, Matsuo K, Talamini R, Olshan AF, Zevallos JP, Winn DM, Jayaprakash V, *et al*: Folate intake and the risk of oral cavity and pharyngeal cancer: A pooled analysis within the International Head and Neck Cancer Epidemiology Consortium. Int J Cancer 136: 904-914, 2015.
4. Zhang R, Zhang P, Wang H, Hou D, Li W, Xiao G and Li C: Inhibitory effects of metformin at low concentration on epithelial-mesenchymal transition of CD44(+)CD117(+) ovarian cancer stem cells. Stem Cell Res Ther 6: 262, 2015.
5. Hua H, Kong Q, Zhang H, Wang J, Luo T and Jiang Y: Targeting mTOR for cancer therapy. J Hematol Oncol 12: 71, 2019.
6. de la Cruz López KG, Toledo Guzmán ME, Sánchez EO and García Carrancá A: mTORC1 as a Regulator of Mitochondrial Functions and a Therapeutic Target in Cancer. Front Oncol 9: 1373, 2019.
7. Doan H, Parsons A, Devkumar S, Selvarajah J, Miralles F and Carroll VA: HIF-mediated Suppression of DEPTOR Confers Resistance to mTOR Kinase Inhibition in Renal Cancer. iScience 21: 509-520, 2019.
8. Baek SH, Ko JH, Lee JH, Kim C, Lee H, Nam D, Lee J, Lee SG, Yang WM, Um JY, *et al*: Ginkgolic acid inhibits invasion and migration and TGF-β-induced EMT of lung cancer cells through PI3K/Akt/mTOR inactivation. J Cell Physiol 232: 346-354, 2017.
9. Deng J, Bai X, Feng X, Ni J, Beretov J, Graham P and Li Y: Inhibition of PI3K/Akt/mTOR signaling pathway alleviates ovarian cancer chemoresistance through reversing epithelial-mesenchymal transition and decreasing cancer stem cell marker expression. BMC Cancer 19: 618, 2019.
10. Choi D, Kim CL, Kim JE, Mo JS and Jeong HS: Hesperetin inhibit EMT in TGF-β treated podocyte by regulation of mTOR pathway. Biochem Biophys Res Commun 528: 154-159, 2020.
11. Guo Y, Xiao Z, Yang L, Gao Y, Zhu Q, Hu L, Huang D and Xu Q: Hypoxia-inducible factors in hepatocellular carcinoma (Review). Oncol Rep 43: 3-15, 2020.
12. Chen G, Feng W, Zhang S, Bian K, Yang Y, Fang C, Chen M, Yang J and Zou X: Metformin inhibits gastric cancer via the inhibition of HIF1α/PKM2 signaling. Am J Cancer Res 5: 1423-1434, 2015.
13. Hasan D, Gamen E, Abu Tarboush N, Ismail Y, Pak O and Azab B: PKM2 and HIF-1α regulation in prostate cancer cell lines. PLoS One 13: e0203745, 2018.
14. Lin C, Ren Z, Yang X, Yang R, Chen Y, Liu Z, Dai Z, Zhang Y, He Y, Zhang C, *et al*: Nerve growth factor (NGF)-TrkA axis in head and neck squamous cell carcinoma triggers EMT and confers resistance to the EGFR inhibitor erlotinib. Cancer Lett 472: 81-96, 2020.
15. Lai HH, Li JN, Wang MY, Huang HY, Croce CM, Sun HL, Lyu YJ, Kang JW, Chiu CF, Hung MC, *et al*: HIF-1α promotes autophagic proteolysis of Dicer and enhances tumor metastasis. J Clin Invest 128: 625-643, 2018.
16. Li YH, Li XF, Liu JT, Wang H, Fan LL, Li J and Sun GP: PKM2, a potential target for regulating cancer. Gene 668: 48-53, 2018.
17. Heckman-Stoddard BM, DeCensi A, Sahasrabuddhe VV and Ford LG: Repurposing metformin for the prevention of cancer and cancer recurrence. Diabetologia 60: 1639-1647, 2017.
18. Liu Q, Tong D, Liu G, Xu J, Do K, Geary K, Zhang D, Zhang J, Zhang Y, Li Y, *et al*: Metformin reverses prostate cancer resistance to enzalutamide by targeting TGF-β1/STAT3 axis-regulated EMT. Cell Death Dis 8: e3007, 2017.
19. Cheng K and Hao M: Metformin inhibits TGF-β1-induced epithelial-to-mesenchymal transition via PKM2 relative-mTOR/p70s6k signaling pathway in cervical carcinoma cells. Int J Mol Sci 17: E2000, 2016.
20. Park JH, Kim YH, Park EH, Lee SJ, Kim H, Kim A, Lee SB, Shim S, Jang H, Myung JK, *et al*: Effects of metformin and phenformin on apoptosis and epithelial-mesenchymal transition in chemoresistant rectal cancer. Cancer Sci 110: 2834-2845, 2019.
21. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408, 2001.

22. Joseph JP, Harishankar MK, Pillai AA and Devi A: Hypoxia induced EMT: A review on the mechanism of tumor progression and metastasis in OSCC. Oral Oncol 80: 23-32, 2018.
23. Kong D, Zhang F, Shao J, Wu L, Zhang X, Chen L, Lu Y and Zheng S: Curcumin inhibits cobalt chloride-induced epithelial-to-mesenchymal transition associated with interference with TGF-β/Smad signaling in hepatocytes. Lab Invest 95: 1234-1245, 2015.
24. Li S, Zhang J, Yang H, Wu C, Dang X and Liu Y: Copper depletion inhibits CoCl2-induced aggressive phenotype of MCF-7 cells via downregulation of HIF-1 and inhibition of Snail/Twist-mediated epithelial-mesenchymal transition. Sci Rep 5: 12410, 2015.
25. De Santi M, Baldelli G, Diotallevi A, Galluzzi L, Schiavano GF and Brandi G: Metformin prevents cell tumorigenesis through autophagy-related cell death. Sci Rep 9: 66, 2019.
26. Abdel-Wahab AF, Mahmoud W and Al-Harizy RM: Targeting glucose metabolism to suppress cancer progression: Prospective of anti-glycolytic cancer therapy. Pharmacol Res 150: 104511, 2019.
27. Chen H, Lin C, Lu C, Wang Y, Han R, Li L, Hao S and He Y: Metformin-sensitized NSCLC cells to osimertinib via AMPK-dependent autophagy inhibition. Clin Respir J 13: 781-790, 2019.
28. Lei Y, Yi Y, Liu Y, Liu X, Keller ET, Qian CN, Zhang J and Lu Y: Metformin targets multiple signaling pathways in cancer. Chin J Cancer 36: 17, 2017.
29. Polivka J Jr and Janku F: Molecular targets for cancer therapy in the PI3K/AKT/mTOR pathway. Pharmacol Ther 142: 164-175, 2014.
30. Quan C, Sun J, Lin Z, Jin T, Dong B, Meng Z and Piao J: Ezrin promotes pancreatic cancer cell proliferation and invasion through activating the Akt/mTOR pathway and inducing YAP translocation. Cancer Manag Res 11: 6553-6566, 2019.
31. Esparza-López J, Alvarado-Muñoz JF, Escobar-Arriaga E, Ulloa-Aguirre A and de Jesús Ibarra-Sánchez M: Metformin reverses mesenchymal phenotype of primary breast cancer cells through STAT3/NF-κB pathways. BMC Cancer 19: 728, 2019.