Metformin suppresses gastric cancer progression through calmodulin-like protein 3 secreted from tumor-associated fibroblasts

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Abstract. Gastric cancer is one of the most common malignant tumor types worldwide, with a high morbidity and associated mortality. The interaction between gastric cancer cells and their microenvironment has a significant role in their maintenance and progression. Gastric tumor-associated fibroblasts (TAFs) are among the major regulators of the gastric cancer microenvironment. Metformin, a classical anti-diabetic drug, is known to prevent cancer progression. However, the effect of metformin on gastric TAFs and TAF-associated cancer progression has remained to be elucidated. In the present study, TAFs were isolated from gastric cancer patients, pre-treated with metformin and then co-cultured with gastric cancer cell lines. It was demonstrated that pre-treatment with 200 μM metformin reduced the stimulatory effect of TAFs on the proliferation of gastric cancer cells in co-culture, suggesting that metformin impairs the tumor-promoting role of TAFs. Using tandem mass tags-based quantitative proteomic analysis, it was identified that metformin significantly affected the secretion of 32 proteins (14 upregulated and 18 downregulated) in the culture medium of gastric TAFs. Among these proteins, calmodulin-like protein 3 (Calml3) was 2.88-fold upregulated in the culture medium of gastric TAFs after metformin treatment and a further experiment using recombinant Calml3 indicated its suppressive effect on the clonogenicity of gastric cancer cells. It was concluded that metformin suppresses gastric cancer through stimulating Calml3 secretion from TAFs, which represents a novel anticancer mechanism of metformin.

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Introduction

Gastric cancer is one of the most common types of malignancy worldwide and the second biggest health burden in China (1-3). *Helicobacter pylori* infection is the most important cause of proximal gastric cancer (4). Altered cell apoptosis, proliferation and certain modifications to tumor suppressor genes, which may lead to inflammation, also contribute to gastric oncogenesis (5). Surgery remains the only curative therapy for gastric cancer. Peri-operative and adjuvant chemotherapy as well as chemoradiation therapy may improve the outcome for patients with resectable gastric cancer (6). Cisplatin and fluoropyrimidine-based chemotherapy with the addition of trastuzumab is widely used in human epidermal growth factor receptor 2-positive patients that are fit for chemotherapy (7).

Tumors that comprise a mass of malignant epithelial cells are surrounded by multiple non-cancerous cell populations, including fibroblasts, vascular endothelial cells, adipocytes and immune regulatory cells (8-10). Fibroblasts were first described as non-vascular, non-epithelial and non-inflammatory cells

of the connective tissue, which are embedded in the fibrillar matrix of the connective tissue (11). Fibroblasts are responsible for the deposition of the extracellular matrix (ECM), regulation of epithelial differentiation and inflammation, as well as wound healing (12). Activated fibroblasts in the tumor microenvironment are known as tumor-associated fibroblasts (TAFs) (13,14). TAFs have recently been identified to have important roles in the proliferation, metastasis and radio-/chemoresistance of cancer cells (10,15,16). Gastric cancer-associated TAFs are major regulators of the tumor microenvironment in this malignancy (17,18).

1-(Diaminomethylidene)-3,3-dimethylguanidine (metformin) is an oral anti-diabetic drug, which decreases the risk of cancer in patients with type 2 diabetes. Metformin exerts antitumor effects on multiple cancer cell lines, including ovarian (19), prostate (20), breast (21) and lung cancer cells (22). However, the mechanisms underlying the antitumor effect of metformin are complex, which include adenosine monophosphate-activated protein kinase, the phosphatidylinositol-3-kinase/Akt pathway, mammalian target of rapamycin, lipogenesis and apoptosis (23-25). A previous study by our group reported that metformin suppresses the progression of gastric cancer through the hypoxia-inducible factor-1α/pyruvate kinase isoenzyme M2 pathway (26). However, the effect of metformin on TAFs and the tumor microenvironment has remained elusive.

The present study focused on the antitumor effects of metformin on TAFs regarding their subsequent interaction with cancer cells. Using a proteomics analysis, calmodulin-like protein 3 (Calml3) was identified as one of the most upregulated factors in the culture medium of gastric TAFs after treatment with metformin. Although Calml3 overexpression and knockdown models have previously demonstrated that Calml3 is able to inhibit the genesis and initiation of metastasis in hepatocellular carcinoma (HCC) (27), it has remained elusive whether Calml3 secreted from TAFs exerts any antitumor effect on gastric cancer cells. The present study was designated to reveal the antitumor effect of metformin by regulating the tumor microenvironment and provide a better understanding of the roles of Calml3.

Materials and methods

Reagents. Metformin was purchased from Sangon Biotech (Shanghai, China). Bovine serum albumin (BSA) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). A Cell Counting Kit (CCK)-8 and trypsin were purchased from Beyotime Institute of Biotechnology (Haimen, China). Recombinant human Calml3 protein was purchased from Sino Biological (Beijing, China).

Isolation of TAFs from gastric cancer tissues. TAFs were isolated from surgically resected gastric cancer specimens from six patients treated at the First People's Hospital of Xuzhou (Suzhou, China) from January to September 2015. The six patients were male with and age of 55-60 years (median age, 57 years). Normal gastric tissues were obtained from 6 age- and gender-matched trauma patients. All patients provided written informed consent for their tissues to be used for scientific research. Ethical approval of the study was obtained from the First People's Hospital of Xuzhou (Suzhou, China). TAFs were isolated as reported previously (28) and were pooled together. Gastric cancer samples were placed in RPMI-1640 medium (HyClone; GE Healthcare, Little Chalfont, UK) containing 4% meropenem (Haibin Pharmaceutical Co., Ltd, Shenzhen, China). Tissues were cut into pieces of ~3 mm^3 in volume with a scalpel, placed in 25-cm^2 culture flasks and covered with 1 ml fetal bovine serum (FBS; HyClone; GE Healthcare). Next, the flasks were inverted for 48 h. After 48 h, RPMI-1640 medium supplemented with 20% FBS (HyClone; GE Healthcare) was added to the flasks, and the flasks were inverted again. Culture was performed in an incubator with humidified air containing 5% CO_2 at 37˚C. Following tissue attachment (2-3 days), the culture medium [Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% FBS] was changed twice per week for the next 2-3 weeks. Under these conditions, fibroblasts were explanted from tissue fragments, whereas other types of cell were mostly retained within the tissue. The fibroblasts formed multiple dense colonies that spread out on the culture dish. After 10-14 days, the cultured cells were briefly trypsinized in an incubator for 5 min and re-seeded into new flasks (passage 1). After reaching confluence, which occurred every 3-4 days, the cultured cells were split at a ratio of 1:2. All fibroblasts used in the experiments of the present study were between passages 4 and 9.

Cell culture. The SGC-7901, BGC823, GES-1 and MGT-803 (29) human gastric cancer cell lines and the human gastric TAFs were cultured in DMEM supplemented with 10% FBS, 1% penicillin-streptomycin and 1 mM sodium pyruvate. These cells were grown at 37˚C in a humidified atmosphere with 5% CO_2.

Cell viability assay. After centrifugation and re-suspension, cells were seeded in 96-well plates at 4,000 cells/well. The cells were co-cultured in a Transwell chamber with TAFs pre-treated with or without different concentrations of metformin. After 48 h, CCK-8 solution (Beyotime Institute of Biotechnology) was added and the plates were cultured for an additional 2 h, according to the manufacturer's instructions. The optical density was determined at 450 nm using a microplate reader (Synergy™ Neio; Biotek, Winooski, VT, USA). The cell viability of each group was determined in triplicate.

Immunofluorescence assay. Cells were washed with PBS, fixed with 4% formaldehyde and blocked with 1% BSA in PBS for 1 h at room temperature. Cells were then incubated overnight at 4˚C with a primary antibody against α-smooth muscle actin (1:1,000 dilution; cat. no. ab5694; Abcam, Cambridge, UK) and subsequently with cyanine 3-conjugated secondary antibodies (1:2,000 dilution; cat. no. A0507; Beyotime Institute of Biotechnology, Haimen, China) for 1 h at room temperature. Nuclei were stained using DAPI and images were captured using an FV1200 confocal microscope (Olympus, Tokyo, Japan).

Protein extraction and tryptic digest. Gastric TAF cells were treated with 0.2 mM metformin or PBS as a control for 12 h. Equal quantities of cells from triplicate wells were mixed to generate one sample for each group. Culture medium

of TAFs treated with or without metformin was collected individually. The protein solution was reduced with 10 mM DTT for 1 h at 37˚C and alkylated with 20 mM iodoacetic acid (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 45 min at room temperature in the dark. The protein sample was diluted with 100 mM TEAB buffer to reach a final urea concentration of <2 M. Finally, trypsin was added at a 1:50 trypsin-to-protein ratio for the first digestion overnight at 37˚C, and 1:100 trypsin-to-protein ratio for a second 4-h digestion at 37˚C. From each sample, ~100 μg protein was digested with trypsin for subsequent analysis.

Tandem mass tag (TMT) labeling. After digestion, tryptic peptides were desalted using a Strata X C18 SPE column (Phenomenex, Torrance, CA, USA) and vacuum-dried. Peptides were reconstituted in 0.5 M TEAB and processed with a 6-plex TMT kit according to the manufacturer's protocol. One unit of each TMT reagent (defined as the amount of reagent required to label 100 μg of protein) was thawed, reconstituted in 24 μl acetonitrile (ACN), and added to each sample. Next, the peptide mixtures were incubated for 2 h at room temperature. The mixtures were pooled, desalted and dried by vacuum centrifugation.

High-performance liquid chromatography (HPLC) fractionation. The labeled peptide sample was then fractionated by high-pH reverse-phase HPLC using an Agilent 300 Extend C18 column (5-μm particles, 4.6 mm inner diameter and 250 mm length; Agilent Technologies, Inc., Santa Clara, CA, USA). Peptides were separated first, with a gradient of 2-60% ACN in 10 mM ammonium bicarbonate in water (pH 10) over 80 min, and the eluate was collected in 80 fractions. Next, the peptides were combined into 18 fractions and dried by vacuum centrifugation.

Quantitative proteomic analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS). Peptides were dissolved in 0.1% formic acid (FA) and directly loaded onto a reverse-phase pre-column (Acclaim PepMap 100; Thermo Fisher Scientific, Inc.). Next, these peptides were separated using a reverse-phase analytical column (cat. no. 164568; Thermo Fisher Scientific, Inc.). The gradient began with an increase from 8 to 22% in solvent B (0.1% FA in ACN, HPLC grade) over 26 min, followed by a further increase to 35% over 6 min, rising up to 80% over 4 min and this concentration remaining constant for the last 4 min, all at a steady flow rate of 300 nl/min on an EASY-nLC 1000 ultra (U)PLC system (Thermo Fisher Scientific, Inc.). Spectra were obtained using a Q Exactive™ plus hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Inc.).

The peptides were subjected to nano spray ionization source ionization (LTQ Oribtrap; Thermo-Fisher Scientific, Inc.) followed by MS/MS in a Q Exactive™ plus that was coupled online to the UPLC. Intact peptides were detected at a resolution of 70,000. Peptides were fragmented for MS/MS using a nomalized collision energy of 33%, and the resulting ion fragments were detected at a resolution of 17,500. A top-20 data-dependent method was applied for the top 20 precursor ions above a threshold ion count of $2x10^4$ in the MS survey scan, with 30.0-sec dynamic exclusion. The electrospray voltage applied was 2.0 kV. The automatic gain control setting of $5x10^4$ ions was used to prevent overfilling of the ion trap. For MS scans, the m/z scan range was 350-1,800. A fixed first mass was set at and m/z ratio of 100.

Database search. The resulting spectra were processed using Mascot search engine (v.2.3.0; http://data-pc/mascot/home.html). Tandem mass spectra were searched against the UniProt *Homo sapiens* database (20,274 sequences; https://www.uniprot.org/). Trypsin/proline was specified as a cleavage enzyme allowing up to two missing cleavages. The mass error was set to 10 ppm for precursor ions and 0.02 Da for fragment ions. Carbamidomethyl on Cys, TMT-6plex (N-term) and TMT-6plex (K) were specified as fixed modifications, and oxidation on Met was specified as a variable modification. As cut-off criteria, a false discovery rate of <1% and a peptide ion score of >20 were used.

First, the mass error of all the identified peptides was checked. The distribution of the mass error was near zero, and usually <0.02 Da. Most of the identified peptides were between 8 and 16 amino acids in length, which agrees with the expected length of tryptic peptides and indicates that tryptic digestion was efficient.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation. GO terms may be assigned to three domains: i) Cellular component, ii) Molecular function and iii) Biological process. GO annotation of the proteome was derived from the UniProt-GOA database (www.http://www.ebi.ac.uk/GOA/). The identified protein IDs were first converted to UniProt IDs and mapped to GO IDs in the UniProt database. Proteins lacking a UniProt-GOA annotation were analyzed using InterProScan (http://www.ebi.ac.uk/interpro/interproscan.html) to annotate the protein's functional sites based on protein sequence alignment. The proteins were then classified by GO and KEGG annotation.

Cell clonogenic assay. To perform a standard clonogenic assay, cells were seeded in 6-well plates at 1,000 cells/well. After treatment with medium containing different concentrations of recombinant Caml3 for 48 h, cells were grown for 7-10 days to allow for colony formation, subsequently fixed with pure methanol for 15 min at 37˚C, and then stained with crystal violet (0.1%) for 15 min at 37˚C. Clusters consisting of ≥50 cells were considered as colonies.

Statistical analysis. Values are expressed as the mean ± standard error of the mean of at least three independent experiments. The results were evaluated by one-way analysis of variance followed by the Student-Neuman-Keuls post hoc test, or Student's t-test to determine statistical significance. The statistical analyses were performed using GraphPad Prism 6 software (GraphPad, Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Co-culture with gastric TAFs increases the viability of gastric cancer cells. TAFs were isolated from gastric cancer tissue (Fig. 1A). A TAF-specific marker, α-SMA, was detected

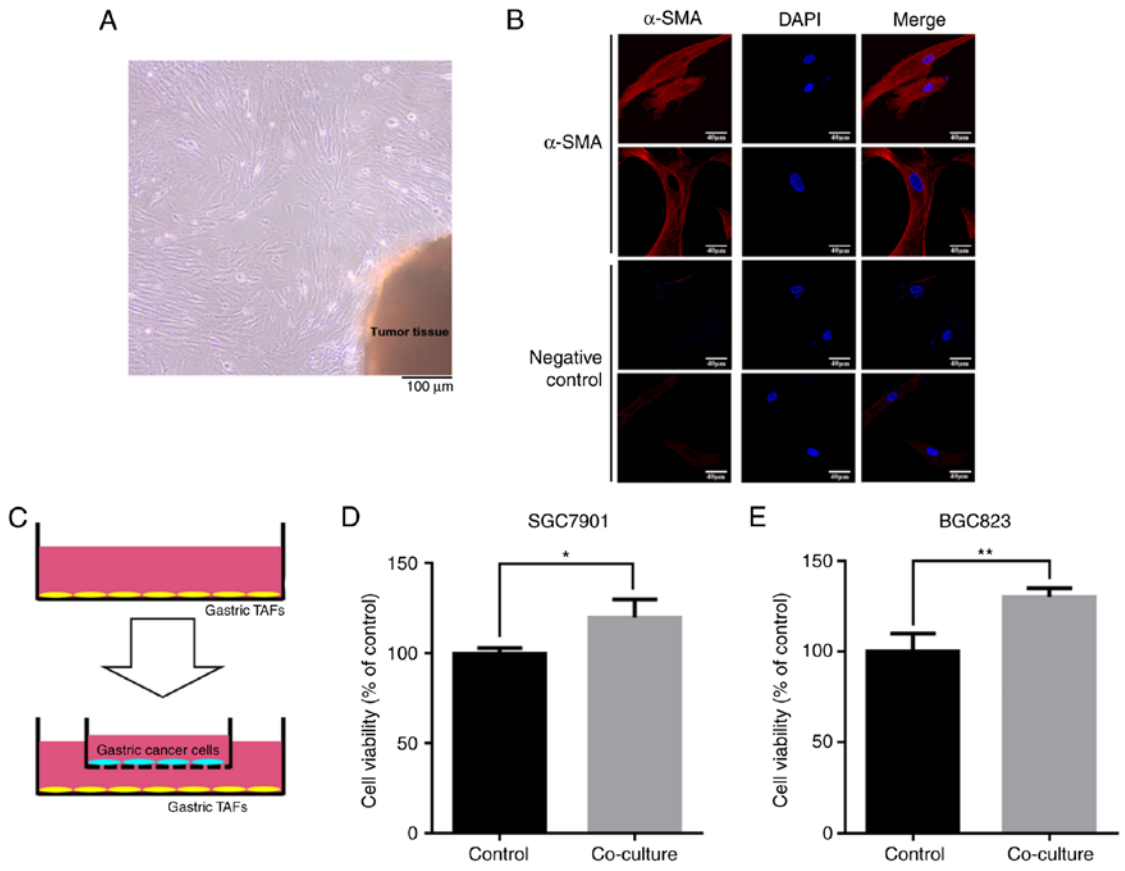


Figure 1. Co-culture with gastric TAFs enhances the proliferation of gastric cancer cells. (A) TAFs were isolated from fresh gastric cancer specimens (scale bar, 100 μm). (B) Immunofluorescence images demonstrating α-SMA distribution (red) in TAFs. Cell nuclei were counterstained with DAPI (blue). Negative control: Primary antibody was omitted (scale bar, 40 μm). (C) Co-culture diagram of gastric cancer cells and gastric TAFs. (D and E) Co-culture of gastric cancer cells and gastric TAFs increased the proliferation of gastric cancer cells. *P<0.05, **P<0.01 compared with the control group. TAFs, tumor-associated fibroblasts; SMA, smooth muscle actin.

by immunocytochemistry to confirm the identity of the TAFs (Fig. 1B). The fibroblasts from normal gastric tissues did not express α-SMA at detectable levels (data not shown). Gastric cancer cells were seeded into a Transwell chamber and co-cultured with the isolated gastric TAFs (Fig. 1C). The cell viability assay demonstrated that gastric cancer cells grew faster when co-cultured with gastric TAFs than normally cultured gastric cancer cells (Fig. 1D and E). This suggests that gastric cancer TAFs promote the proliferation of gastric cancer cells, probably through the modulation of the cancer microenvironment.

Metformin decreases the effect of TAFs on the proliferation of co-cultured gastric cancer cells. Next, it was investigated whether metformin affected the tumor-promoting role of TAFs isolated from gastric cancer. Gastric TAFs were treated with different concentrations of metformin for 24 or 48 h. At concentrations of up to 1 mM, metformin only slightly influenced the viability of the TAFs (Fig. 2A and B). Next, gastric cancer cells were co-cultured with gastric TAFs in a Transwell chamber (Fig. 2C). Co-culture with TAFs that had been pre-treated with 0.2 mM metformin for 48 h, significantly decreased the proliferation of gastric cancer SGC-7901 and MGT-803 cells, compared with gastric cancer cells co-cultured with metformin-untreated TAFs (Fig. 2D and E). Although the proliferation of GES-1 was decreased when co-cultured with TAFs pre-treated with metformin, the difference was not significant (Fig. 2F). It was observed that metformin reduced the stimulatory effect of TAFs on the proliferation of gastric cancer cells, probably by altering the extracellular proteins secreted by gastric TAFs into the culture medium.

Proteomic analysis of proteins secreted from TAFs after metformin treatment. To investigate the proteomic changes in secreted proteins from gastric TAFs following metformin treatment, the proteomic profile of TAF-secreted proteins was investigated by TMT-based protein quantification (Fig. 3A). In metformin-treated TAFs, >360 differentially expressed proteins compared with those in untreated TAFs were successfully identified, including 14 significantly upregulated and 18 significantly downregulated proteins (Fig. 3B). The upregulated proteins included calmodulin-like protein 3 (Calml3), tropomyosin α-3 chain (TPM3), tissue-type plasminogen activator (PLAT) and nucleoside diphosphate kinase A (NME1).

To further determine the function and features of the identified proteins, GO annotation was performed, including protein domain, pathway and subcellular localization enrichment analysis. The differentially expressed proteins were summarized for each GO category. The proportion of differentially expressed proteins representing each subcellular location is summarized in Fig. 4A. Among the upregulated proteins, 37% were extracellular proteins, 29% were cytosolic proteins

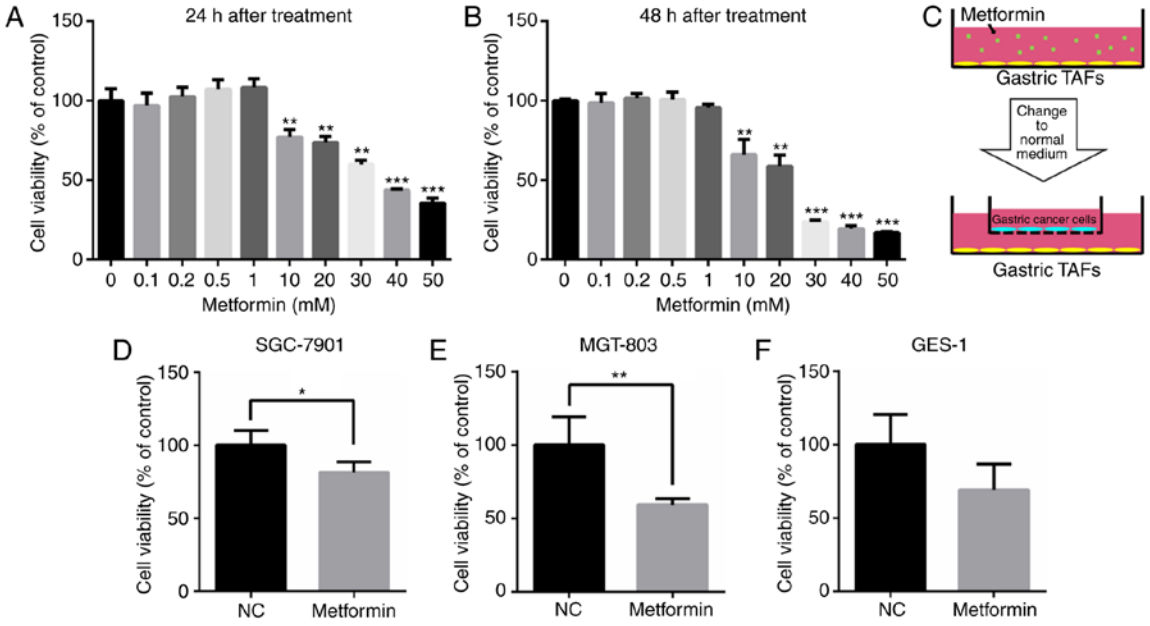
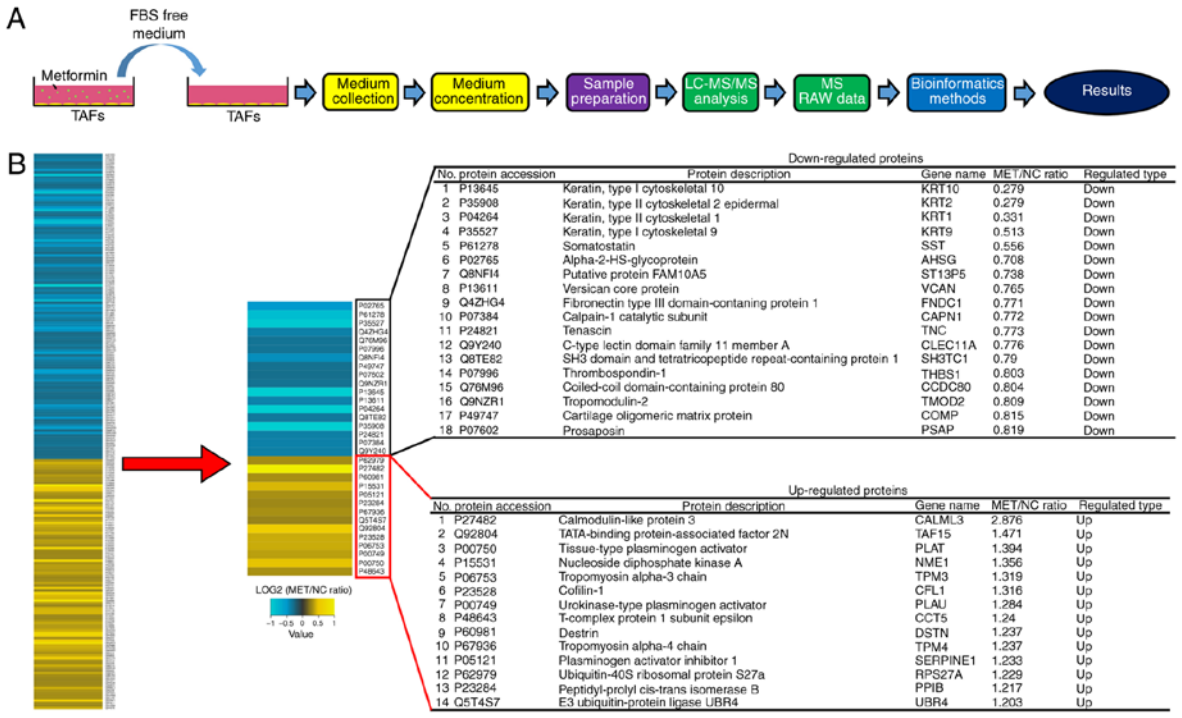


Figure 2. Metformin-treated TAFs exert an antiproliferative effect on gastric cancer cells. (A and B) TAFs were treated with the indicated concentrations of metformin for (A) 24 or (B) 48 h and the viability of gastric cancer cells in subsequent co-culture was measured using a Cell Counting Kit-8-based assay. (C) Co-culture diagram of metformin-treated gastric TAFs and gastric cancer cells. (D-F) Co-culture with metformin-treated TAFs inhibited the proliferation of gastric cancer cells. *P<0.05, **P<0.01, ***P<0.001 compared with the control group. TAFs, tumor-associated fibroblasts; NC, negative control.



Down-regulated proteins

No.	protein accession	Protein description	Gene name	MET/NC ratio	Regulated type
1	P13645	Keratin, type I cytoskeletal 10	KRT10	0.279	Down
2	P35908	Keratin, type II cytoskeletal 2 epidermal	KRT2	0.279	Down
3	P04264	Keratin, type II cytoskeletal 1	KRT1	0.331	Down
4	P35527	Keratin, type I cytoskeletal 9	KRT9	0.513	Down
5	P61278	Somatostatin	SST	0.556	Down
6	P02765	Alpha-2-HS-glycoprotein	AHSG	0.708	Down
7	Q8NFI4	Putative protein FAM10A5	ST13P5	0.738	Down
8	P13611	Versican core protein	VCAN	0.765	Down
9	Q4ZHG4	Fibronectin type III domain-containing protein 1	FNDC1	0.771	Down
10	P07384	Calpain-1 catalytic subunit	CAPN1	0.772	Down
11	P24821	Tenascin	TNC	0.773	Down
12	Q9Y240	C-type lectin domain family 11 member A	CLEC11A	0.776	Down
13	Q8TE82	SH3 domain and tetratricopeptide repeat-containing protein 1	SH3TC1	0.79	Down
14	P07996	Thrombospondin-1	THBS1	0.803	Down
15	Q76M96	Coiled-coil domain-containing protein 80	CCDC80	0.804	Down
16	Q9NZR1	Tropomodulin-2	TMOD2	0.809	Down
17	P49747	Cartilage oligomeric matrix protein	COMP	0.815	Down
18	P07602	Prosaposin	PSAP	0.819	Down

Up-regulated proteins

No.	protein accession	Protein description	Gene name	MET/NC ratio	Regulated type
1	P27482	Calmodulin-like protein 3	CALML3	2.876	Up
2	Q92804	TATA-binding protein-associated factor 2N	TAF15	1.471	Up
3	P00750	Tissue-type plasminogen activator	PLAT	1.394	Up
4	P15531	Nucleoside diphosphate kinase A	NME1	1.356	Up
5	P06753	Tropomyosin alpha-3 chain	TPM3	1.319	Up
6	P23528	Cofilin-1	CFL1	1.316	Up
7	P00749	Urokinase-type plasminogen activator	PLAU	1.284	Up
8	P48643	T-complex protein 1 subunit epsilon	CCT5	1.24	Up
9	P60981	Destrin	DSTN	1.237	Up
10	P67936	Tropomyosin alpha-4 chain	TPM4	1.237	Up
11	P05121	Plasminogen activator inhibitor 1	SERPINE1	1.233	Up
12	P62979	Ubiquitin-40S ribosomal protein S27a	RPS27A	1.229	Up
13	P23284	Peptidyl-prolyl cis-trans isomerase B	PPIB	1.217	Up
14	Q5T4S7	E3 ubiquitin-protein ligase UBR4	UBR4	1.203	Up

Figure 3. Proteomic analysis of secreted proteins from gastric TAFs treated with metformin. (A) Experimental design of the proteomic analysis. (B) A tandem mass tags quantitative proteomic analysis was performed to identify proteins that were dysregulated in the culture medium of metformin-treated TAF. Compared with the negative control cells, 14 upregulated and 18 downregulated proteins were identified in metformin-treated TAFs. TAFs, tumor-associated fibroblasts; LC-MS/MS, liquid chromatography tandem mass spectrometry; FBS, fetal bovine serum; MET, metformin; NC, negative control.

and 14% were nuclear proteins. Among the downregulated proteins, 33% were extracellular proteins, 28% were nuclear proteins and 17% were mitochondrial proteins. The results of the protein domain enrichment analysis and KEGG pathway enrichment analysis suggested that transcriptional dysregulation was significantly enhanced in cancer, whereas the extracellular matrix-receptor interaction pathway was the most significantly downregulated pathway (Fig. 4B and C). Finally, GO enrichment analysis of upregulation and downregulated proteins was performed (Figs. 5 and 6). Proteins involved in the positive regulation of protein and actin filament depolymerization were enriched among the dysregulated proteins.

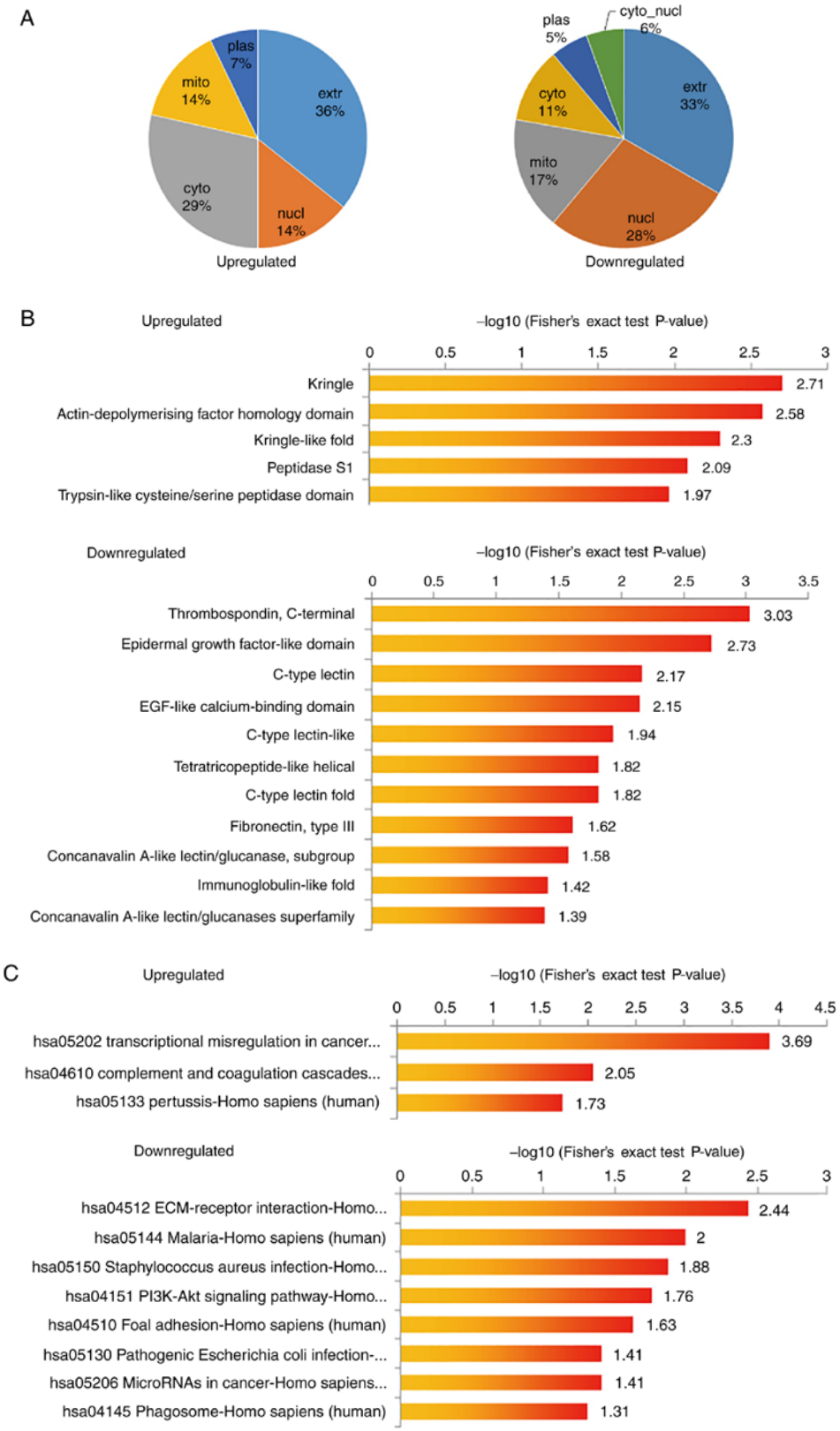


Figure 4. Subcellular location, protein domain enrichment and KEGG pathway enrichment analysis of upregulation and downregulated proteins. (A) Subcellular location of upregulation and down-regulated proteins (metformin-treated cells vs. control group). (B) Protein domain enrichment analysis of upregulation and downregulated proteins. (C) KEGG pathway enrichment analysis of upregulation and downregulated proteins. KEGG, Kyoto Encyclopedia of Genes and Genomes; extr, extracellular; plas, plasma; cyto, cytosol; mito, mitochondria; nucl, nucleus; hsa, *Homo sapiens*; ECM, extracellular matrix; PI3K, phosphoinositide-3 kinase; EGF, epidermal growth factor.

Calml3 exerts a tumor-suppressive role in gastric cancer cells. The suppressive effect of metformin on the stimulatory role of TAFs on gastric cancer cells was likely to be exerted through the upregulation and downregulation of certain proteins released

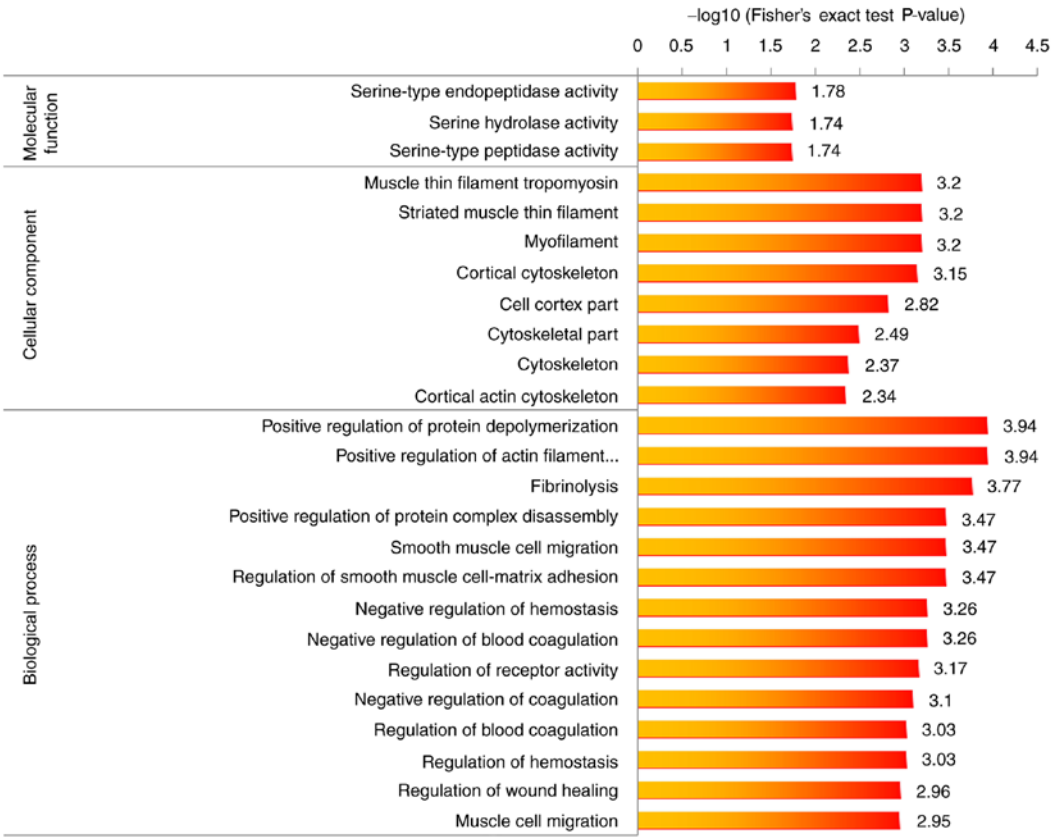


Figure 5. Gene Ontology enrichment analysis of upregulated proteins.

into the culture medium by TAFs. The further investigations were focused on the upregulated proteins, which may have potential antitumor activity. Among the dysregulated proteins identified in TAFs subjected to metformin treatment, Calml3 was most significantly upregulated, with a 2.88-fold increase in metformin-treated TAFs compared with untreated TAFs. To explore whether Calml3 secreted from gastric TAFs mediated the tumor-suppressive role of metformin, gastric cancer cells were incubated with culture medium containing different concentrations of recombinant Calml3 protein. A clonogenic assay revealed that Calml3 not only decreased the surviving fraction of gastric cancer cells, but also decreased the size of cell clones in two gastric cancer cell lines (Fig. 7A and B). These results demonstrate that Calml3 secreted from TAFs exerts a tumor-suppressive role in gastric cancer cells. Calml3 may account for the antitumor effect of metformin on the tumor microenvironment.

Discussion

Gastric cancer is one of the leading causes of cancer-associated mortality worldwide (1-3). It is clear that tumor-associated fibroblasts have an important role in the initiation and progression of gastric cancer. In previous co-culture experiments, TAFs increased the proliferation and progression of cancer cells and promoted angiogenesis (30,31). Injection of immortalized prostate epithelial cells together with TAFs, but not with normal fibroblasts, led to tumor formation in mice (30). The tumor growth promoting function of TAFs may be due to the secretion of cytokines that have a positive regulatory role in cancer (32). Orimo *et al* (31) reported that stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated stromal cell-derived factor-1α secretion. Vermeulen *et al* (33) reported that TAFs secrete hepatocyte growth factor and activate β-catenin-dependent transcription, and subsequently the clonogenicity of cancer stem cells. Exosomes derived from fibroblasts have also emerged as positive mediators of cancer progression (34-36). Thus, targeting TAFs or inhibiting the tumor-promoting role of TAFs has emerged as a novel strategy in cancer treatment (8,9).

Metformin is an oral anti-diabetes drug that has been widely studied due to its antitumor activity (19-22). Most previous studies have focused on its direct antitumor effect on cancer cells. However, it has remained elusive whether metformin affects the tumor microenvironment or TAFs. In the present study, gastric cancer cells were co-cultured in a Transwell system with TAFs that had been pre-treated with metformin. The results indicated that the proliferation and clonogenicity were reduced in gastric cancer cells co-cultured with metformin-treated TAFs, which indicates that metformin inhibited the tumor-promoting role of TAFs that were isolated from gastric cancer cells. The culture medium from TAFs was concentrated and analyzed by LC-MS/MS to quantify the proteomic differences in metformin-treated and untreated TAFs. Proteomic analysis revealed that metformin affected the secretion of 32 proteins (14 upregulated and 18 downregulated) in the culture medium of gastric TAFs. Although the upregulated as well as the downregulated proteins are likely to be responsible for the antitumor effect of metformin, the

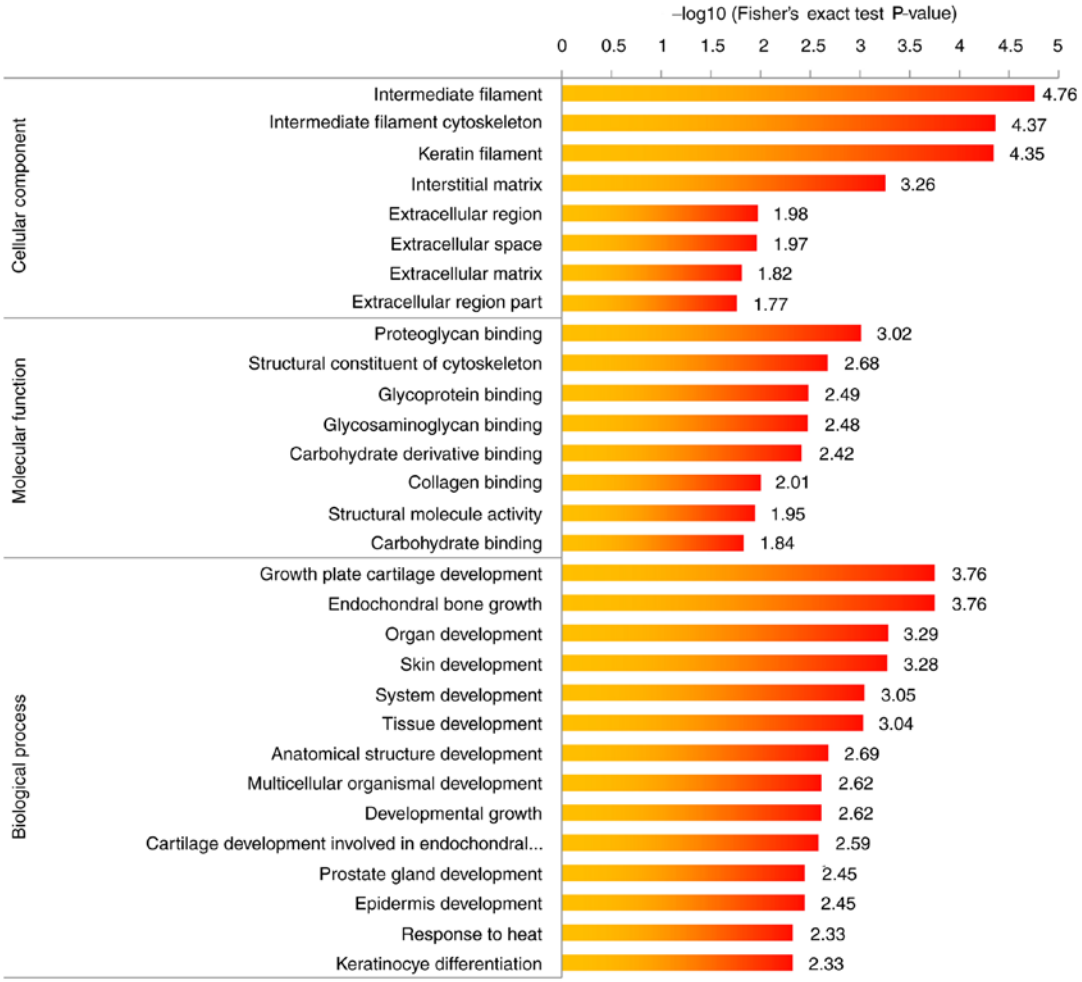


Figure 6. Gene Ontology enrichment analysis of downregulated proteins.

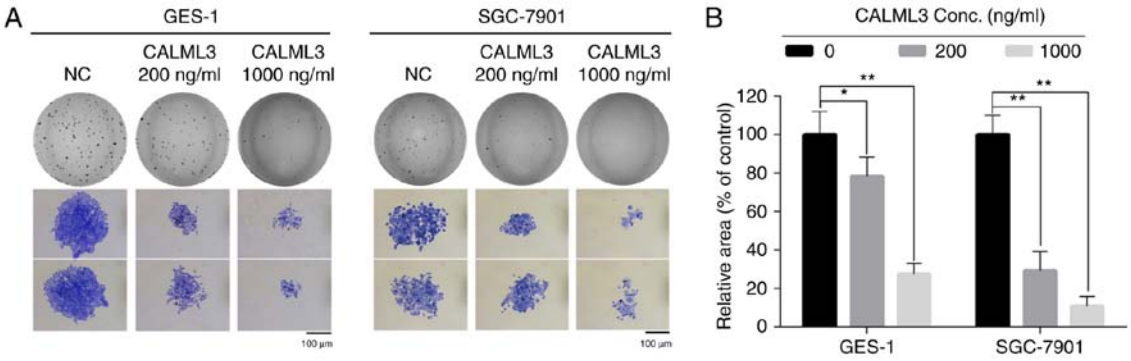


Figure 7. Calml3 has a tumor-suppressive role in gastric cancer cells. (A) Clonogenic ability of GES-1 and SGC-7901 gastric cancer cells after treatment with recombinant Calml3 (magnification, 100 μm). (B) Relative colony size in the groups of GES-1 and SGC-7901 cells after Calml3 treatment. *P<0.05, **P<0.01 compared with the control group. Calml3, calmodulin-like protein 3; Conc., concentration; NC, negative control.

present study we only focused on upregulated proteins, which may have potential tumor suppressor activity. Among these, Calml3 was 2.88-fold upregulated in the culture medium of gastric TAFs after treatment with 0.2 mM metformin. Further studies on the proteomic profiles of TAF secretion with other concentrations of metformin are needed.

Calml3 is a 148-amino acid calcium sensor protein (37). Rogers *et al* (38) reported that Calml3 is downregulated in invasive ductal carcinoma and lobular carcinoma compared with that in normal breast epithelium. Furthermore, Calml3 is expressed in the normal oral mucosa, and is downregulated during its malignant transformation (39), suggesting a potential tumor-suppressive role. A recent study by Yang *et al* (27) using Calml3 gain- and loss-of-function experiments suggested that Calml3 inhibits the genesis and metastasis of HCC. However, their study focused on Calml3 expressed in HCC cells, and they did not assess the potential antitumor effects of Calml3 secreted from TAFs. In the present study,

recombinant Calml3 protein was added into the culture medium of gastric cancer cells to imitate the culture medium of TAFs treated with metformin. It was revealed that Calml3 exerted a tumor-suppressive effect on these gastric cancer cells by inhibiting their clonogenicity and proliferation. However, the physiological protein levels of Calml3 and whether the concentration of Calml3 used in the clonogenic assay of the present study (200 ng/ml or 1,000 ng/ml, which were determined in preliminary experiments) is physiologically achievable remains elusive. In addition, the mechanism underlying the tumor-suppressive role of Calml2 warrants further investigation.

In conclusion, the present study indicated that metformin alters the proteins secreted from TAFs to reduce their proliferative effect on gastric cancer cells. In particular, the levels of Calml3 were increased, which exerted a tumor-suppressive effect on gastric cancer cells. The present study provides novel evidence for the antitumor effects of metformin. The present study may also provide a novel antitumor strategy using Calml3, although further investigation is required in the future.

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Availability of data and materials

The data and materials are available from the corresponding authors on request.

Authors' contributions

SZ and CY conceived and designed the study. GC, CY, ZT, JZ and QW performed the molecular biology experiments. GC, FA, JZ and SL drafted the manuscript and prepared the figures. FA, JC, SL and QW isolated the TAFs. JC, ZT and QZ performed the statistical analysis. QZ and SZ modified the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All patients provided written informed consent for their tissues to be used for scientific research. Ethical approval of the study was obtained from the First People's Hospital of Xuzhou (Suzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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