Article

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Upregulation of MFAP5 Enhances COL1A1 Expression, Promoting Epithelial-Mesenchymal Transition in Gastric Cancer Cells

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Background: Gastric cancer (GC) is one of the most common types of cancer. Earlier research has suggested an association of microfibril-associated protein 5 (MFAP5) and collagen type I alpha 1 (COL1A1) with the progression of various tumors. However, the specific roles and mechanisms of action of MFAP5 and COL1A1 in the context of GC are yet to be fully elucidated. Thus, the objective of this study is to investigate the functions of MFAP5 and COL1A1 in the epithelial-mesenchymal transition (EMT) of GC and to unravel the associated molecular mechanisms.

Methods: We examined the MFAP5 expression level in GC through real-time polymerase chain reaction (RT-PCR), western blotting, and immunohistochemistry. Subsequently, shRNA interference was employed to knockdown the expression of *MFAP5* or *COL1A1* in GC cells. Cell viability assay, Transwell assay, RT-PCR, and western blotting were then used to explore the impact of MFAP5 and COL1A1 on GC progression and metastasis, along with GC cell proliferation, migration, and EMT.

Results: Increased MFAP5 levels were observed in both GC tissues and cells ($p < 0.05$), with decreased MFAP5 levels significantly impeding GC cell activity and GC progression and metastasis ($p < 0.05$). Additionally, the pronounced reduction in the COL1A1 expression level effectively alleviated the migration and EMT processes induced by MFAP5 overexpression in GC cells ($p < 0.05$).

Conclusions: These results indicate that MFAP5 plays a role in initiating the process of EMT in GC cells through the upregulation of COL1A1 expression.

Keywords: gastric cancer; epithelial-mesenchymal transition; MFAP5; COL1A1

Introduction

Gastric cancer (GC), a formidable malignancy, is a prominent contributor to global cancer incidence [1]. Despite advances in the treatment of GC, its persistent invasive and metastatic characteristics continue to pose significant challenges [2]. Epithelial-mesenchymal transition (EMT) is a pivotal event in tumor progression, transforming tumor cells from polarized epithelial cells to ones exhibiting mesenchymal characteristics, imparting increased migratory and invasive capabilities [3–5]. Therefore, a comprehensive understanding of the EMT process of GC cells holds substantial clinical implications for impeding tumor progression.

In recent years, microfibril-associated protein 5 (MFAP5) has attracted widespread attention as a protein abundant in the extracellular matrix [6,7]. While MFAP5 has been found to be overexpressed in different cancers, its precise role and underlying mechanisms in GC remain unclear [8,9]. Our research team is dedicated to unraveling the specific functions of MFAP5 in the development and progression of GC, along with elucidating its regulatory role in EMT.

A key molecule of interest in this context is collagen type I alpha 1 (COL1A1), a crucial constituent of the extracellular matrix intricately involved in EMT [10,11]. The potential association between increased MFAP5 expression and elevated COL1A1 levels in GC remains uncertain. We hypothesize that MFAP5 may promote EMT in GC cells by upregulating COL1A1 expression, thereby contributing to tumor development and progression.

In this study, we conducted in-depth analyses of the expression patterns of MFAP5 and COL1A1 in GC, further validating their interrelationship through cell experiments. Additionally, we uncovered the potential molecular mechanism linking MFAP5 to COL1A1, aiming to reveal how they collaboratively drive EMT. By comprehensively understanding the roles of MFAP5 and COL1A1 in GC, we aimed to provide robust support for the development of new therapeutic strategies and precision medicine approaches to effectively halt GC development and progression, including metastasis. This research establishes a crucial foundation for related therapeutic investigations.

Materials and Methods

Cell Culture

Human gastric cancer cell line SNU-16 (iCell-h430) and human gastric mucosal epithelial cell line GES-1 (iCell-h062) were procured from iCell Bioscience, Inc. (Shanghai, China) and cultured in DMEM (iCell-138-0001, iCell Bioscience, Inc., Shanghai, China) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin in an incubator at 37 °C with 5% CO_2. The culture medium was replaced every 2–3 days. Upon reaching an 80% fusion rate, the cells were passaged. All cell lines were validated by short tandem repeat and tested free of mycoplasma contamination.

Animals and Establishment of GC Model

Female BALB/c mice were acquired from the Experimental Animal Resources Platform of the Chinese Academy of Sciences (Shanghai, China) and N-methyl-N-nitrosourea (MNU, N3385, Sigma Chemical, St. Louis, MO, USA) was dissolved in distilled water at a concentration of 240 ppm and was freshly prepared twice a week for administration in drinking water in light-shielded bottles. The mice drank water containing 240 ppm MNU and were exposed for a total of five weeks. Finally, the neck of the mice was dislocated and the stomach tissues were collected. This study has been approved by the Experimental Animal Ethics Committee of Changchun University of Chinese Medicine (Approval no: 2024013).

RNA Interference

Recombinant lentiviruses encoding an shRNA against either human *MFAP5* or human *COL1A1*, along with *pLNCX2-MFAP5* and *pLNCX2*-vector, were designed and prepared by GeneChem (Shanghai, China). The target sequence for shRNA-*MFAP5* was: 5′-CCGGCGGGATGAGAAGTTTGCTTGTCTCGAGACAAGCAAACTTCTCATCCCGTTTTTTG-3′. The target sequence for *COL1A1*-shRNA was: 5′-AAAACACCAGTTTACGACGTATGTATTCGTACATACGTCGTAAACTGGTGC-3′. A negative control, scrambled (scr)-shRNA, with a target sequence 5′-TTCTCCGAACGTGTCACGTTT-3′ was used, along with *pLNCX2-MFAP5*-F: 5′-CACCGCTTGTCTGTAAGGAACACGAACGAATTCGTGTTCCTTACAGACAAG-3′; *pLNCX2-MFAP5*-R: 5′-AAAACTTGTCTGTAAGGAACACGA-3′, *pLNCX2*-vector-F: 5′-TGGCAGTACATCTACGTATTAGTCATCGC-3′, and *pLNCX2*-vector-R: 5′-GTGGATAGCGGTTTGACTCACGGGGATTT-3′. Lentivirus was introduced into cells according to the manufacturer's instructions. The efficiency of *MFAP5* and *COL1A1* knockdown was quantified using RT-PCR and western blotting.

Table 1. Primer sequences were used in this study.

Primer name	Primer sequence (5′–3′)
Human-*MFAP5*-F	GCATCGGCCGGTTAAACAAT
Human-*MFAP5*-R	TCACAGGGAGGAAGTCGGAA
Mouse-*MFAP5*-F	CAGTCCTGCTTCACCAGTTTAC
Mouse-*MFAP5*-R	AAGTCGGAAGTAGTTGGAGCG
E-cadherin (E-ca)-F	CGAGAGCTACACGTTCACGG
E-cadherin-R	GGGTGTCGAGGGAAAAATAGG
N-cadherin (N-ca)-F	TTTGATGGAGGTCTCCTAACACC
N-cadherin-R	ACGTTTAACACGTTGGAAATGTG
Vimentin-F	GACGCCATCAACACCGAGTT
Vimentin-R	CTTTGTCGTTGGTTAGCTGGT
Actin-F	ACACTGTGCCCATCTACG
Actin-R	TGTCACGCACGATTTCC
COL1A1-F	GCCTCTGCTCTCCGACCTCTC
COL1A1-R	CTGCTTTGTGCTTTGGGAAGTTGTC

MFAP5, microfibril-associated protein 5; *COL1A1*, collagen type I alpha 1; F, Forward; R, Reverse.

RNA Extraction and Real-Time Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from cells using total RNA extraction kit (OSR-M610, Tiangen, Beijing, China), and its quantity and quality were assessed using a spectrophotometer. RNA was transcribed into cDNA using reverse transcriptase (MR101-01/02, Nuoweizan, Nanjing, China). Subsequently, primer pairs were designed, and RT-PCR, including cDNA template, primers, DNA polymerase, reaction buffer, and fluorescent probe, were set up. During the reaction, we monitored the fluorescent signal in real time, and the cycle number (Ct value) at which the signal reached its threshold was recorded. Finally, the relative expression level was calculated using the $2^{-\Delta\Delta Ct}$ method, which compared the Ct values of the experimental group with the Ct values of the control group. The primer sequences are provided in Table 1.

Western Blotting

Total protein was extracted from tissues or cells, and its concentration was measured using a bicinchoninic acid assay to ensure equal loading amounts. Subsequently, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used to separate the proteins based on their size, followed by transfer to a polyvinylidene fluoride or nitrocellulose membrane. To prevent nonspecific binding, the membrane was blocked in 5% non-fat milk, followed by incubation with anti-MFAP5 (1:1000; cat no. PA5-52706; Thermo Fisher Scientific, Waltham, MA, USA), anti-COL1A1 (1:1000; cat no. PA5-29569; Thermo Fisher Scientific, Waltham, MA, USA), anti-E-cadherin (1:1000; cat. no. A20798; ABclonal, Inc., Wuhan, China), anti-N-cadherin (1:1000; cat. no. A19083; ABclonal, Inc., Wuhan, China), or anti-vimentin (1:1000; cat. no. A19607; ABclonal, Inc., Wuhan, China) antibodies,

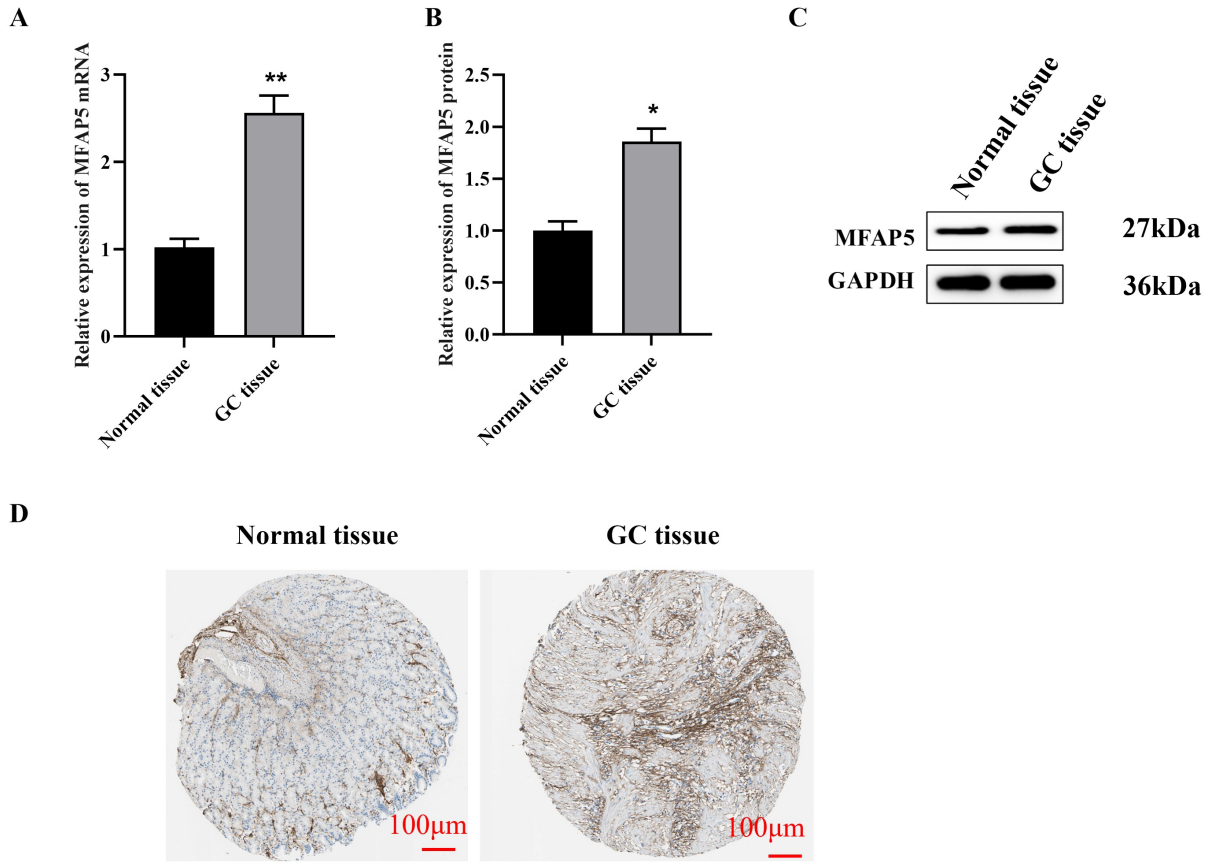

Fig. 1. High expression of microfibril-associated protein 5 (MFAP5) in gastric cancer. (A) Expression of MFAP5 in normal gastric tissues and gastric cancer (GC) tissues in mice as determined by RT-PCR. (B,C) Expression of MFAP5 in normal gastric tissues and gastric cancer tissues in mice as determined by western blotting. (D) Immunohistochemical assessment of the staining intensity of MFAP5 in normal gastric tissues and GC tissues in mice (n = 6). (*$p < 0.05$, **$p < 0.01$). RT-PCR, real-time polymerase chain reaction.

GAPDH (1:2000; cat no. USAPA1-987; Thermo Fisher Scientific, Waltham, MA, USA). After washing, the membrane was incubated with the respective enzyme- or fluorescent probe-conjugated secondary antibodies (cat no. ZB-2305 and ZB-2301, ZSGB-BIO, Beijing, China). After washing, the detection of the target protein bands was accomplished through chemiluminescent or fluorescent methods. Images were captured, and the grayscale values of target protein bands were analyzed using ImageJ software (version 1.5f, National Institutes of Health, Bethesda, MD, USA).

Immunohistochemical Staining

The tissues were fixed in 4% paraformaldehyde at room temperature overnight, dehydrated, embedded, and then sectioned. The tissues were sliced into 5 μm slices and incubated with the primary antibody MFAP5 (ab203828, Abcam, Cambridge, MA, USA). It tested positive with the DAB detection kit (ab64260, Abcam, Cambridge, MA, USA) and was analyzed using an Olympus microscope (CX23, Olympus, Tokyo, Japan).

Cell Counting Kit-8 (CCK-8) Assay

SNU-16 cells were cultured as indicated and divided into treatment and control groups. The treatment group underwent transfection, while the control group underwent mock transfection. Subsequently, the CCK-8 assay (CA1210, Solarbio, Beijing, China) was performed according to the manufacturer's instructions, and cell viability was assessed by measuring the absorbance at a wavelength of 450 nm in a microplate reader (CMax Plus, Molecular Devices Corporation, Silicon Valley, CA, USA). Finally, the differences between the groups were compared.

Transwell Migration and Invasion Assays

For the migration assay, cells were suspended in a medium and allowed to migrate through the pores toward the lower chamber, which contained the chemoattractant. After a specified incubation period, cells were removed from the upper surface of the insert, and those that migrated to the lower surface were fixed, stained, and quantified.

For the invasion assay, Matrigel, which mimics the basement membrane, was applied to the Transwell cham-

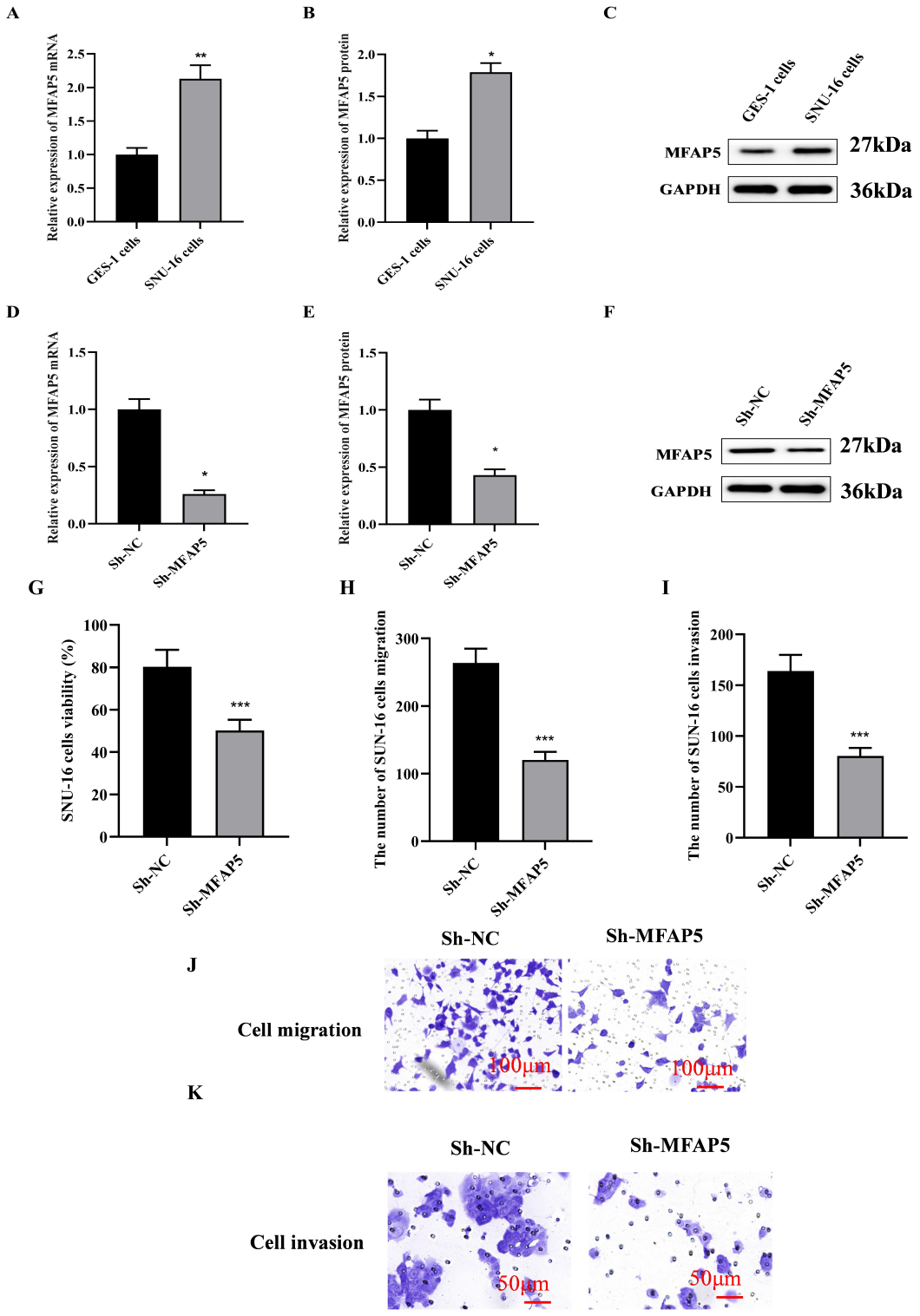

Fig. 2. Impact of MFAP5 on cell proliferation and migration *in vitro*. (A) mRNA levels of *MFAP5* in gastric cancer cells (SNU-16) and normal gastric mucosal epithelial cells (GES-1). (B,C) Protein levels of MFAP5 in gastric cancer cells and normal gastric mucosal cells. (D) mRNA levels of *MFAP5* in SNU-16 cells after transfection with sh-*MFAP5*. (E,F) Protein levels of MFAP5 in SNU-16 cells after transfection with sh-*MFAP5*. (G) Proliferative capacity of SNU-16 cells. (H–K) Migration and Invasion capabilities of SNU-16 cells (n = 6). (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

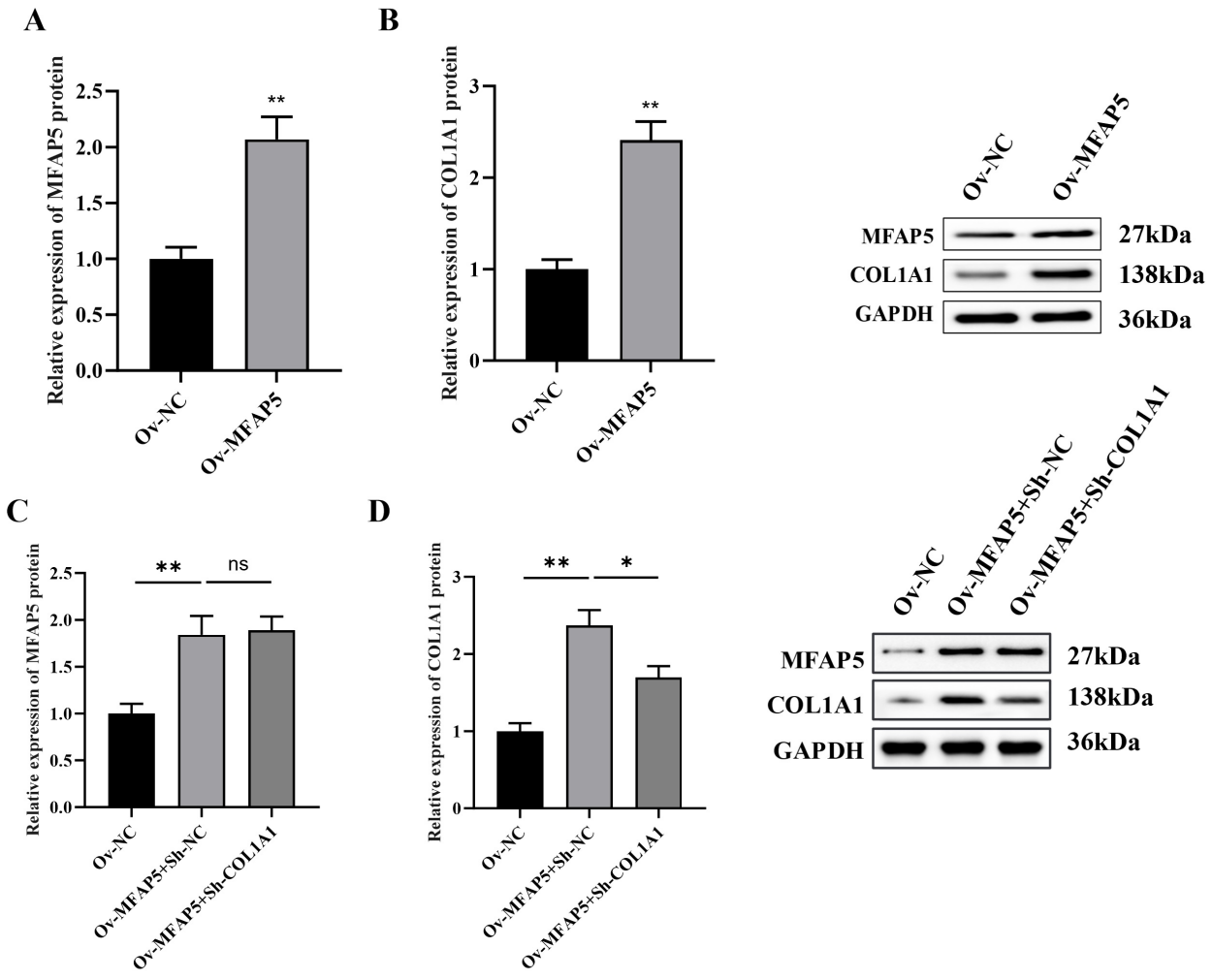

Fig. 3. Upregulation of COL1A1 expression by MFAP5. (A–D) Quantitative analysis of MFAP5 and COL1A1 protein expression (n = 6). (*$p < 0.05$, **$p < 0.01$, ns, no significant difference).

ber to create a barrier that cells must penetrate. Cells were plated in the upper chamber, and after incubation, those that invaded the Matrigel and reached the lower chamber were evaluated by a light microscope (CX23, Olympus, Tokyo, Japan).

Statistical Analysis

Statistical analysis was carried out using GraphPad software (version 8.0, GraphPad software Inc., San Diego, CA, USA). The comparison of two datasets was performed using a *t*-test, while the comparison of more than two datasets was performed using one-way analysis of variance. Subsequent post hoc analyses were carried out using Tukey's method. Results were expressed as mean ± standard deviation and statistical significance was determined at a threshold of $p < 0.05$.

Results

MFAP5 Expression is Upregulated in GC

As depicted in Fig. 1A–C, the mRNA and protein concentrations of MFAP5 significantly increased in GC tissues compared to normal tissues ($p < 0.05$). Subsequently, we used immunohistochemistry to examine the localization of MFAP5 in GC tissues. The results revealed predominant MFAP5 staining in the cytoplasm of GC tissues (Fig. 1D). Taken collectively, these findings underscore the elevated presence of MFAP5 in GC tissues at both mRNA and protein levels, as well as its localization within the cytoplasm.

MFAP5 Enhances Gastric Cancer Cell Proliferation and Migration

In the initial phase of our investigation, we examined the MFAP5 expression levels in SNU-16 and GES-1 cells and observed an upregulation in both mRNA and protein levels ($p < 0.05$) (Fig. 2A–C). Next, we used shRNA to

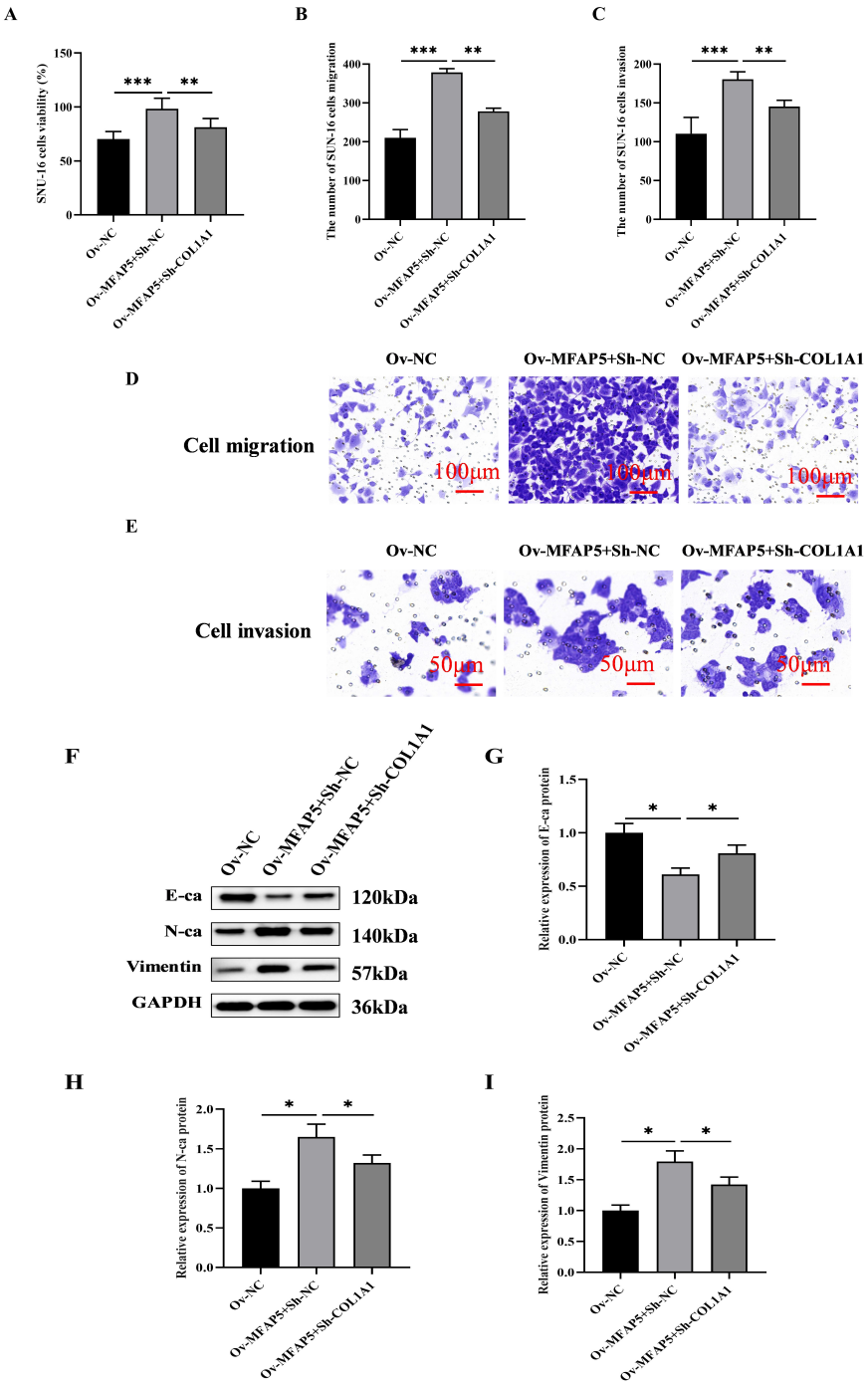

Fig. 4. MFAP5 regulates GC cell proliferation and mesenchymal transition through the upregulation of COL1A1. (A) SUN-16 cell proliferation, as determined by Cell Counting Kit-8 (CCK-8) assay. (B–E) SUN-16 cell migration and invasion, as determined by the Transwell assay. (F–I) Protein levels of E-cadherin (E-ca), N-cadherin (N-ca), and vimentin in SUN-16 cells, as determined by western blotting (n = 6). (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

knockdown MFAP5 in SNU-16 cells. The MFAP5 mRNA and protein levels in the silenced cells were 74% and 54% lower in the silenced cells than those in the control cells, respectively ($p < 0.05$) (Fig. 2D–F).

Transfection with shRNA-*MFAP5* significantly decreased cell viability subsequent to MFAP5 downregulation ($p < 0.001$) (Fig. 2G). Furthermore, migration and invasion in MFAP5-silenced SNU-16 cells were significantly reduced compared to the control cells ($p < 0.001$) (Fig. 2H–K).

Upregulation of MFAP5 Enhances the Expression of COL1A1 in Gastric Cancer

To further investigate the regulatory mechanism between MFAP5 and COL1A1 in GC, we employed two approaches: overexpression of MFAP5 through transfection of the MFAP5-overexpressing plasmid into GC cells and knockdown of COL1A1 through transfection of the shRNA-*COL1A1*. We observed a significant increase in both MFAP5 and COL1A1 protein levels after MFAP5 overexpression (Ov) ($p < 0.01$) (Fig. 3A,B). Compared to the Ov-MFAP5+sh-NC group, the COL1A1 protein levels were significantly downregulated in the Ov-MFAP5+sh-*COL1A1* group ($p < 0.05$) (Fig. 3C,D).

MFAP5 Influenced the Viability and EMT Process of GC Cells by Modulating the Expression of COL1A1

We silenced COL1A1 in SUN-16 cells overexpressing MFAP5. The results from the CCK-8 assay showed a significant increase in SUN-16 cell proliferation in the Ov-MFAP5+sh-NC group compared to the Ov-NC group ($p < 0.001$). In contrast, the proliferation of SUN-16 cells in the Ov-MFAP5+sh-*COL1A1* group showed a significant decrease compared to the Ov-MFAP5+sh-NC group ($p < 0.01$) (Fig. 4A). The Transwell assay results indicated that, while the overexpression of MFAP5 significantly facilitated the migration of GC cells ($p < 0.001$), co-transfection of Ov-MFAP5+sh-*COL1A1* markedly suppressed migration compared to the Ov-MFAP5+sh-NC group ($p < 0.01$) (Fig. 4B–E).

Subsequent assessments examined the impact of MFAP5 and COL1A1 on proteins involved in EMT. In the Ov-MFAP5+sh-NC group, the E-cadherin (E-ca) level was found to be lower than that in the Ov-NC group ($p < 0.05$), whereas in the Ov-MFAP5+sh-*COL1A1* group, the E-ca level was significantly higher than that in the Ov-MFAP5+sh-NC group ($p < 0.05$) (Fig. 4F,G). Additionally, in the Ov-MFAP5+sh-NC group, the levels of N-cadherin (N-ca) and vimentin were higher in the Ov-NC group ($p < 0.05$). However, in the Ov-MFAP5+sh-*COL1A1* group, the levels of N-ca and vimentin were significantly lower than those in the Ov-MFAP5+sh-NC group ($p < 0.05$) (Fig. 4F,H,I). These findings suggest that MFAP5 may modulate GC cell proliferation and migration through its intricate interplay with COL1A1, thereby influencing key proteins involved in EMT.

Discussion

To the best of our knowledge, this study is the first to investigate the role and the mechanism of action of MFAP5 in GC. Our findings reveal that MFAP5 is upregulated in GC, and its high expression correlates positively with this aggressive malignancy.

Existing research indicates that MFAP5 serves as a potential tumor-promoting factor in different types of cancer, including pancreatic cancer, bladder cancer, and breast cancer [12–14]. Another investigation revealed that MFAP5 enhances the migration and invasion capabilities of head and neck squamous cell carcinoma through the protein kinase B (AKT) signaling pathway [15]. This finding aligns with the findings of our study that showed an association between increased MFAP5 expression and enhanced GC cell migration. These observations indicate that MFAP5 exerts diverse effects in different types of cancer, serving as a biomarker and providing new insights into potential therapeutic strategies for GC.

Additionally, COL1A1, which belongs to the collagen family, participates in EMT, a process intricately linked to the progression of malignant tumors. Several studies have suggested a correlation between elevated COL1A1 expression and tumor infiltration and metastasis [10,16]. These observations have led us to speculate that MFAP5 promotes GC progression by regulating COL1A1 expression. After identifying a correlation between MFAP5 and COL1A1 expression, we performed transfection experiments to validate the role of MFAP5 in modulating COL1A1 expression in GC cells. The knockdown of MFAP5 expression led to a significant reduction in GC cell migration and invasion. Earlier studies have indicated that MFAP5 can serve as an independent prognostic factor for adverse patient outcomes [12,17], as it influences endothelial cell behavior and promotes cell survival. Xu *et al.* [15] reported that MFAP5 facilitates migration and invasion of head and neck squamous cell carcinoma via the AKT pathway, whereas Li *et al.* [18] demonstrated that MFAP5 could serve as a diagnostic and prognostic biomarker for intrahepatic cholangiocarcinoma and that the upregulation of MFAP5 significantly enhances the invasive capability of intrahepatic cholangiocarcinoma tumors. These findings align with our results. Our findings suggest that increased MFAP5 expression promotes GC proliferation and migration, whereas reduced MFAP5 expression has the opposite effect. Additionally, RT-PCR assays and western blot analyses demonstrated that the elevated *MFAP5* level led to increased expression of N-ca and vimentin, along with the decreased expression of E-ca, whereas knockdown of *COL1A1* significantly reversed EMT induced by *MFAP5* overexpression.

EMT refers to the phenomenon where epithelial cells transform into mesenchymal cells, resulting in altered cell morphology and heightened migratory abilities [19]. This process plays a crucial role in the early stages of tumor progression and metastasis [20]. Thus, identifying and understanding the signaling mechanisms that promote EMT may offer new therapeutic approaches for strategies aimed at inhibiting this cellular transition [21]. EMT involves various signaling pathways, including Transforming Growth Factor beta (TGF-β), Nuclear Factor kappa B (NF-κB), Wingless/Integrated (Wnt), and others [22–24], and an earlier study has demonstrated a close association between COL1A1 and EMT initiation and regulation [10]. In GC,

COL1A1 interacts with the PI3K/AKT signaling pathway to facilitate the acquisition of a more invasive phenotype by tumor cells [25]. In our study, the upregulation of COL1A1 is associated with MFAP5 in GC cells, thereby promoting EMT in GC cells.

Clearly, our study indicates that the overexpression of MFAP5 represents a tumorigenic phenomenon, leading to the aggressive behavior of GC cells. Therefore, we hypothesize that MFAP5 may promote EMT in GC by upregulating COL1A1. Nevertheless, additional studies are required to uncover the novel mechanisms by which MFAP5 induces the increased expression of COL1A1.

Strengths and Weaknesses of the Study

This study provides preliminary evidence of the roles of MFAP5 and COL1A1 in GC, but the experimental data is relatively limited. Lack of clinical validation: Laboratory results have not been clinically validated. Validating the research findings in actual patients is a crucial step for further investigation to assess the feasibility and applicability of the study results. Focus limitation: The study primarily focuses on MFAP5 and COL1A1, without delving into other potential molecular participants in GC. This focus may lead to an incomplete understanding of the overall mechanism.

Comprehensive assessment of MFAP5 and COL1A1 expression levels in GC using various experimental techniques. Utilization of shRNA interference technique to study the functions of MFAP5 and COL1A1 in GC cells, aiding in validating their roles in GC development and metastasis. Despite limitations, this study provides important clues for further research into the molecular mechanisms of GC.

Limitations of the Study

Although the research provides preliminary evidence on the roles of MFAP5 and COL1A1 in GC, the experimental data is limited.

The Focus of the Study

The study primarily focuses on MFAP5 and COL1A1, without delving into other potential molecular players in GC. This focus may lead to an incomplete understanding of the overall mechanisms.

Lack of Clinical Validation

Laboratory results have not been clinically validated. Validating the research findings in actual patients is a crucial step for further investigation to assess the feasibility and applicability of the study results. Overall, future research can overcome these limitations by adopting more comprehensive and multi-faceted research strategies to advance the understanding of the molecular mechanisms underlying GC.

Conclusions

In summary, our findings elucidate the pivotal roles played by MFAP5 and COL1A1 in the transition of GC cells from the epithelial phenotype to the mesenchymal phenotype. It is likely that MFAP5 influences EMT by upregulating COL1A1 expression.

Availability of Data and Materials

Data to support the findings of this study are available on reasonable request from the corresponding author.

Author Contributions

YS, JE, and FW performed the research. XW and JE provided help and advice on the experiments. FW, YS and XW contributed to the analysis and interpretation of the data. All authors were involved in the drafting and critical revision of the manuscript. All authors have read and approved the final manuscript. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

This study has been approved by the Experimental Animal Ethics Committee of Changchun University of Chinese Medicine (Approval no: 2024013).

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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