

12-Lipoxygenase promotes epithelial–mesenchymal transition via the Wnt/ β -catenin signaling pathway in gastric cancer cells

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Background: 12-Lipoxygenase (12-LOX) plays a major role in the progression and metastasis of various types of cancer. In gastric cancer (GC), the expression level of 12-LOX is significantly up-regulated; however, its function, and underlying mechanism of action remain unclear.

Methods: The mRNA and protein expression levels of 12-LOX were assessed using quantitative reverse transcription polymerase chain reaction (qRT-PCR) and Western blot analyses, respectively, in GC cell lines. 12-LOX expression was stably up-regulated using lentiviral vector in BGC823 and MGC803 cells, and cell-counting kit-8 (CCK8), colony formation, and invasion assays were performed to verify the function of 12-LOX in proliferation and metastasis. In addition, the expression levels of epithelial-mesenchymal transition (EMT) differentiation markers and downstream targets of the Wnt/ β -catenin signaling pathway were examined by Western blotting. A nude mouse model of tumor growth and metastasis was established to investigate the role of 12-LOX in vivo.

Results: Our findings demonstrate that 12-LOX mRNA and protein were highly expressed in GC cell lines. 12-LOX overexpression promoted GC cell proliferation, migration, and invasion both in vitro and in vivo. In addition, up-regulation of 12-LOX promoted the EMT in GC cells, as reflected by a decrease in E-cadherin expression and an increase in N-cadherin and Snail expression. 12-LOX overexpression in GC cells also increased the expression of multiple downstream targets of the Wnt/ β -catenin signaling pathway.

Conclusion: These findings revealed that 12-LOX functions as an oncogene in promoting GC cell proliferation and metastasis in vitro and in vivo. In addition, 12-LOX might regulate the EMT via the Wnt/ β -catenin signaling pathway, indicating a potential role for 12-LOX as a target in GC treatment.

Keywords: 12-lipoxygenase, EMT, Wnt/ β -catenin signaling pathway, gastric cancer

Introduction

Gastric cancer (GC) is the fifth most commonly diagnosed cancer worldwide. Over one million new GC diagnoses were made in 2018.¹ Despite rapid advancement in surgical techniques and chemotherapy, GC is still the third highest cause of cancer-related deaths, with 1 in every 12 deaths globally, especially in Eastern Asia and Eastern Europe.^{2,3} Among diverse factors influencing GC-related death, invasion and metastasis is the major factor. The median survival for advanced metastatic patients is approximately 1 year.⁴ Therefore, elucidating the underlying mechanisms of GC development and metastasis is an urgent need in the field of oncology.

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Previous studies have revealed that epithelial–mesenchymal transition (EMT) is a multiple-step process in which epithelial cells lose their polarity, exhibit junction break down and then eventually acquire a mesenchymal phenotype.⁵ EMT is a dynamic and fundamental process that plays a pivotal role in embryogenesis, organ fibrosis, and tumor progression and metastasis.⁶ Down-regulation and up-regulation of E-cadherin and N-cadherin expression, respectively, are crucial features in EMT. Many signaling pathways and molecules including TGF- β , tumor necrosis factor- α , Wnt, Notch and sonic hedgehog (Shh) are implicated in the induction of EMT.⁷ Extensive studies indicate that the Wnt/ β -catenin signaling pathway plays a critical role in regulating the EMT during tumor development and metastasis.^{8,9} Upon canonical Wnt/ β -catenin signaling pathway activation, β -catenin enters the nucleus and activates LEF/TCF-mediated transcription of Wnt target genes including Cyclin D1, c-Myc, and TCF.^{10,11} EMT has previously been observed in multiple GC cell lines including MKN45, BGC823, MGC803 and SGC7901.^{8,12}

12-Lipoxygenase (12-LOX) is a key member of the lipoxygenase gene family, which plays a vital role in many physiological and pathophysiological processes including vascularization, inflammation, arteriosclerosis and cancer.^{13–15} It was reported that 12-LOX is up-regulated in a variety of cancers, such as prostate, lung, gastric, colorectal and breast cancer.^{16,17} Increasing evidence suggests that 12-LOX is an important factor in the genesis and metastasis of cancer. Dilly et al reported that in advanced-stage and high-grade prostate cancer, 12-LOX is overexpressed. Further studies found that 12-LOX promoted cancer progression by modulating cell proliferation, angiogenesis and metastasis.^{18–20} Our previous research explored the relationship between 12-LOX expression and clinicopathological features of GC by immunohistochemistry (IHC). We found that 12-LOX was positively correlated with the aggressive phenotype of GC.²¹ However, the mechanism of action of 12-LOX is poorly understood.

The aim of the present study was to investigate the function of 12-LOX in GC cells and its involvement in the mechanism of action of EMT. We analyzed 12-LOX levels in GC cell lines and confirmed that 12-LOX was overexpressed. Our results showed that 12-LOX overexpression promoted the proliferation, migration and invasion of GC cell lines (BGC823 and MGC803) *in vivo* and *in vitro*. Furthermore, we found that 12-LOX altered the

expression of EMT markers and promoted EMT via the Wnt/ β -catenin signaling pathway, thus providing a potential therapeutic target for cancer therapy.

Materials and methods

Cell culture

The human GC cell lines (BGC823, MGC803, AGS and HGC27) and normal gastric epithelial GES-1 cells were obtained from the Chinese Academy of Sciences (Shanghai, China). MGC803 cells were cultured in DMEM (HyClone, GE Healthcare Life Sciences, Logan, UT, USA). The other cell lines were cultured in RPMI-1640 (HyClone). The culture medium was supplemented with 10% FBS. All cells were cultured in a humidified atmosphere of 37°C at 5% CO₂.

Transfection and generation of stable cell lines

To investigate further the function of 12-LOX, a lentivirus construct expressing 12-LOX (LV-12-LOX-Puromycin) and a negative control virus were obtained from Shanghai Genechem Co.,Ltd.(Shanghai, China). BGC823 (2.0×10^5) and MGC803 (1.0×10^5) cells were seeded in six-well plates and allowed to adhere for 24 hrs. The cells were then transfected following the manufacturer's instructions with a multiplicity of infection of 50 (BGC823) and 100 (MGC803) using polybrene (10 μ g/mL) for 8 hrs. Matching empty lentiviral vectors were transfected (named as NC) using the same method. After 48 hrs, the cells were treated with puromycin (Merck, Kenilworth, NJ, USA) for 2 weeks to select stable cells. Stable overexpression of 12-LOX in the selected BGC823 and MGC803 cells was confirmed using quantitative reverse transcription polymerase chain reaction (qRT-PCR) and Western blotting.

Real-time qPCR assays

Total RNA was extracted from cells or mouse tumor tissues using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The cDNA was synthesized using a cDNA synthesis kit (Thermo Scientific, Waltham, MA, USA). qRT-PCR was conducted with SYBR Green (Roche, Basel, Switzerland). The transcript levels were normalized to that of β -actin, which was used as the endogenous control. Each experiment was performed at least in triplicate, and the relative gene expression was calculated using the method of $2^{-\Delta\Delta C_t}$ values. The following primers were used,

12-LOX forward 5'-CCTTCCAGTCCCAGAGTCA-3'; 12-LOX reverse 5'-GCCATCGTCACATCTTCCTT-3'; E-cadherin forward 5'-ATTTTCCCTCGACACCCGAT-3'; E-cadherin reverse 5'-TCCCAGGCGTAGACCAAGA-3'; β -actin forward 5'-CCTGGCACCCAGCACAAT-3'; β -actin reverse 5'-GGGCCGGACTCGTCATAC-3'.

Western blot analysis

Cells lysates were extracted using radioimmunoprecipitation assay buffer (Beyotime, Shanghai, China) and protein concentrations were quantified using the BCA protein assay kit. Equal amounts of protein were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% milk solution at room temperature for 1 hr and incubated with the relevant primary antibodies at 4°C overnight. On the following day, the membranes were washed and then incubated with secondary antibodies at room temperature for 1 hr. Labeled bands were visualized by the enhanced chemiluminescence (ECL) reagent (Beyotime, Shanghai, China) using X-ray film and an ECL system (Bio-Rad, Hercules, CA, USA). The gray values of the bands were evaluated using the Image J software (National Institutes of Health, Bethesda, MD, USA).

All the primary antibodies used in this study were obtained from Cell Signaling Technology (Danvers, MA, USA): monoclonal rabbit primary antibodies against E-cadherin (cat. no. 3195T), N-cadherin (cat. no. 13116T), Snail (cat. no. 3879S), β -catenin (cat. no. 8480T), c-Myc (cat. no. 13987S), CyclinD1 (cat. no. 2978S) and TCF1/TCF (cat. no. 2203S). The monoclonal rabbit primary antibody against 12-lipoxygenase (cat. no. ab168384) was purchased from Abcam (Cambridge, MA, USA). β -Actin mouse monoclonal antibody (cat. no. K10601) was purchased from TransGen Biotech (Beijing, China).

Cell migration and invasion assays

Cell migration and invasion assays were performed using Transwell (24-well format) with 8- μ m polycarbonate membranes (BD Biosciences, San Jose, CA, USA) with or without Matrigel. Briefly, 1×10^5 cells were plated in the upper chamber filled with serum-free medium. RPMI 1640 containing 10% FBS was added to the lower chamber. For the invasion assay, the bottoms of the Transwell chambers were coated with extracellular Matrigel (BD Biosciences). After incubation at 37°C for 24 hrs, the cells remaining in the upper chamber were removed with a wet cotton swab. Subsequently, the migratory and invading cells on the

lower surface of the membrane were stained with 0.1% crystal violet and counted under a light microscope. The number of cells on the bottom was calculated in five random fields (200 \times). Each experiment was independently repeated three times.

Cell proliferation assay

Cell proliferation was evaluated using a cell counting kit-8 (CCK8) assay (Dojindo, Japan). BGC823 and MGC803 cells (2×10^3) were plated onto 96-well plates in triplicate. Each well contained 100 μ L of medium supplemented with 10 μ L of CCK8 solution. After incubating the cells at 37°C for 1 hr, the absorbance at 450 nm was measured to assess the degree of cell proliferation.

Colony formation assay

Stably transfected and non-transfected BGC823 and MGC803 cells were plated onto 6-well plates at a density of 500 cells/well and cultured in medium with 10% FBS at 37°C exposed to 5% CO₂. The medium was changed every 2 days. After 14 days, the cells were fixed in 10% methanol and stained with 0.1% crystal violet. The number of colonies (>50 cells) were determined using Photoshop software.

Xenograft model

The effects of 12-LOX on tumorigenesis and metastasis were investigated in vivo using a mouse xenograft assay. BGC823 cell (1×10^6 in phosphate-buffered saline) stably transfected with LV-12-LOX-puromycin or the negative control vector were inoculated subcutaneously in the flank regions of 6-week-old BALB/C nude mice (n=6 for each group). Tumor volumes were recorded every 3 days until 21 days postinoculation. We measured tumor volume using the following formula: volume=length \times width²/2. At 21 days postinoculation, all mice were sacrificed, the tumors were excised and their weights recorded. To evaluate tumor metastasis, 2×10^6 BGC823 cells stably overexpressing 12-LOX were inoculated into nude mice in the OE group via the tail vein, and the normal BGC823 cells and cells transfected with the negative control vector were inoculated by the same method. After 60 days, all mice were sacrificed, and the lungs were isolated and examined microscopically using H&E staining. This study was approved by the animal use committee of Fujian Medical University Union Hospital. All the experiments involving animals followed the National Institutes of Health guidelines.

IHC

The mouse tumor tissues were fixed with 4% paraformaldehyde, embedded in paraffin and cut into 4- μ m sections. The sections were baked at 60°C for 1 hr and then deparaffinized and rehydrated. Antigen retrieval was performed by heating the sections in a microwave oven in 0.01 M citrate buffer (pH=6.0) for 20 mins. The sections were soaked in 3% hydrogen peroxide for 15 mins to remove endogenous peroxidase. After washing with PBS, the sections were blocked with normal goat serum and incubated at 4°C overnight with Ki-67 primary antibody (cat. no. ab156956, Abcam, Cambridge, MA, USA). On the following day, the sections were incubated with secondary antibodies at room temperature for 30 mins. After washing, the sections were stained with diaminobenzidine and counterstained with hematoxylin. Finally, all the sections were dehydrated and sealed.

Statistical analysis

Results were obtained from three independent experiments and values are presented as the means \pm standard error of the mean. All statistical tests were analyzed using a one-way analysis of variance and a $*P < 0.05$ was considered statistically significant.

Results

12-LOX is up-regulated in GC cell lines

In order to explore the function of 12-LOX in gastric carcinoma, we performed real-time qPCR assay and Western blot analysis to investigate 12-LOX expression levels in GC cells. As shown in Figure 1A, compared with normal gastric epithelial cells (GES-1), the mRNA and protein levels of 12-LOX were up-regulated in diverse GC cells including BGC823, MGC803, AGS and HGC27. Additionally, BGC823 and MGC803 cells exhibited lower expression of 12-LOX than the other cancer cell lines. This result is consistent with our previous study, which found that a high expression level of 12-LOX was associated with malignant progression in GC patients.²¹ To demonstrate the role of 12-LOX in the progression of GC, a lentiviral vector specifically targeting 12-LOX was constructed and transfected into BGC823 and MGC803 cells to elevate 12-LOX expression. The qRT-PCR and Western blot analyses performed to confirm the transfection efficiency (Figure 1B and C) indicated that 12-LOX was obviously overexpressed compared with the level of the control BGC823 and MGC803 cells.

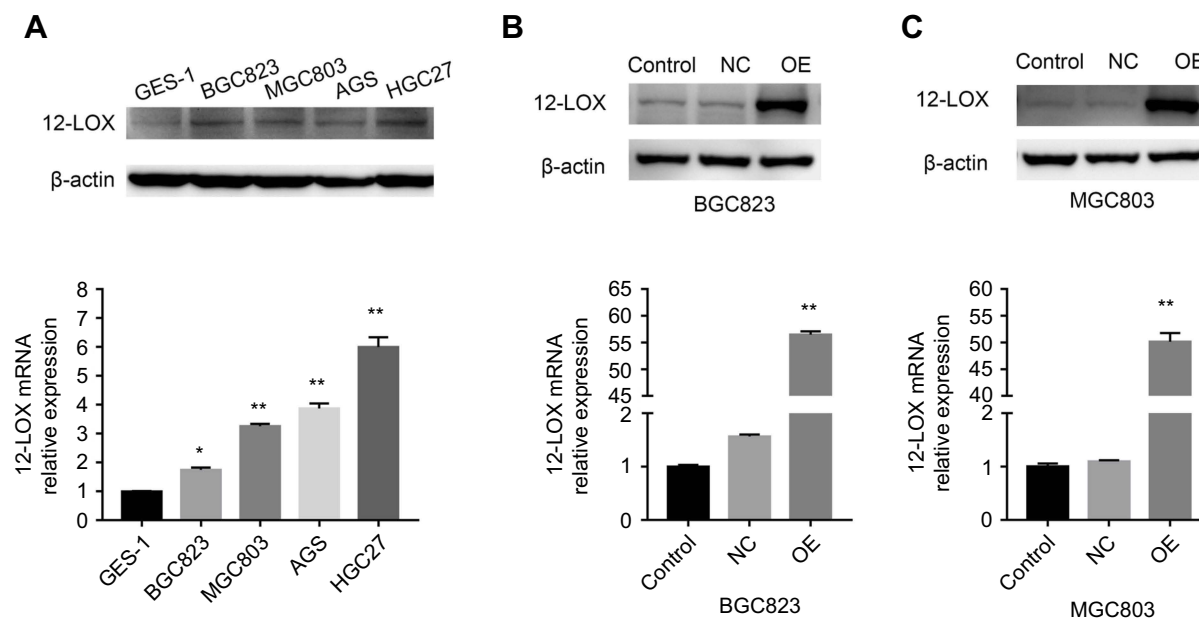


Figure 1 Analysis of 12-LOX expression in human GC cell lines and generation of 12-LOX overexpression cell lines. **(A)** Real-time PCR and Western blot analysis of 12-LOX expression in BGC823, MGC803, AGS, and HGC27 human GC cells and the immortalized human gastric epithelial line GES-1. **(B)** Efficiency of LV-12-LOX transfection was evaluated by real-time PCR and Western blotting. 12-LOX was overexpressed significantly in BGC823-OE cells compared with control and NC cells. **(C)** Real-time PCR and Western blotting confirmed 12-LOX overexpression in MGC803 cells. $*P < 0.05$, $**P < 0.01$ versus the corresponding control group.

Abbreviations: 12-LOX, 12-lipoxygenase; GC, gastric cancer; NC, negative control group; OE, 12-LOX overexpression group.

12-LOX overexpression promotes GC cell proliferation, migration and invasion in vitro

Previous studies revealed that 12-LOX is amplified and has an important role in the process of tumorigenesis in various tumors.¹⁸ Therefore, we investigated whether 12-LOX plays a significant role in regulating cell proliferation and metastasis in GC cells. CCK8 assays were used to evaluate the cell proliferation ability of BGC823 and MGC803 cells. The proliferation rate of BGC823 and MGC803 cells transfected with the 12-LOX overexpression vector was markedly improved compared with the control (Figure 2A). In order to evaluate the 12-LOX function in malignant transformation, a colony formation assay was performed. 12-LOX up-regulation markedly elevated the colony forming capacity of both BGC823 and MGC803 cells ($P < 0.01$ vs controls, Figure 2B). Furthermore, Transwell migration assays were utilized to evaluate the effects of 12-LOX on the migration and mobility of GC cells. Our results indicated that enhancing the level of 12-LOX markedly increased migration and invasion of both BGC823 and MGC803 cells (Figure 2C). In summary, these findings suggest that up-regulation of 12-LOX promoted GC cell growth, migration and invasion in vitro.

12-LOX up-regulation promotes EMT in GC cells

It has been demonstrated that EMT is closely associated with tumorigenesis and metastasis. As shown in Figure 3A, overexpression of 12-LOX resulted in a morphological change in GC cells; the cobblestone-like epithelial cells were converted to spindle-shaped mesenchymal cells. We performed Western blot analysis to quantitate the expression levels of EMT markers. The results showed that up-regulation of 12-LOX in BGC823 and MGC803 cells reduced E-cadherin expression significantly. In contrast, the expression of N-cadherin and Snail was significantly increased ($P < 0.05$ vs controls, Figure 3B). In general, these results showed that up-regulation of 12-LOX promoted EMT in GC cells.

12-LOX regulates the Wnt/ β -catenin signaling pathway in GC cells

Extensive research indicates that the Wnt/ β -catenin signaling pathway plays a critical role in the development and regulation of EMT and tumor metastasis. To further explore the underlying mechanism of how 12-LOX promotes EMT

in GC cells, the expression of the downstream targets of the Wnt/ β -catenin signaling pathway was analyzed using Western blot analysis (Figure 3C). We found that cells transfected with the 12-LOX vector showed significantly increased expression of β -catenin, TCF-1, CyclinD1 and c-Myc, which are all involved in inducing EMT. These results indicated that 12-LOX might promote GC cell EMT by activating the Wnt/ β -catenin signaling pathway.

12-LOX increases growth and metastasis of GC cells in vivo

Furthermore, we evaluated the influence of 12-LOX overexpression on tumor growth and metastasis in vivo in a BGC823 xenograft model. BGC823 cells stably transfected with either LV-12-LOX-puromycin or a negative control vector were inoculated subcutaneously into the flank regions of nude mice. The subcutaneous tumor size was measured every 3 days. The results revealed that compared with the control and NC group, 12-LOX overexpression markedly increased the growth of subcutaneous tumors of the transfected group ($P < 0.05$, Figure 4A and B). The tumor weight of the BGC823-OE group was also substantially increased compared with that of the other two groups ($P < 0.05$, Figure 4C). We also investigated the E-cadherin levels of the xenograft tumors using qRT-PCR. The results revealed that overexpression of 12-LOX also reduced the expression of E-cadherin in vivo ($P < 0.01$, Figure 4D). Additionally, H&E staining was performed on xenograft tumors from the control (CON), negative control (NC) and overexpression (OE) groups. As shown in Figure 4E, the control and negative control groups showed more normal cells, while the group overexpressing 12-LOX had a higher number of malignant cells. The expression level of Ki-67 in tumors was evaluated using IHC (Figure 4F). To investigate the function of 12-LOX in tumor metastasis, a nude mouse lung metastasis model was established by tail vein injection. As shown in Figure 5, 12-LOX overexpression markedly increased the number and size of the lung metastatic foci. Taken together, these observations indicate that 12-LOX overexpression played a significant role in the progression and metastasis of GC.

Discussion

12-LOX is a metabolizing enzyme of the eicosanoid pathway, which metabolizes arachidonic acid to 12-hydroxyeicosate-traenoic acid (12-HETE). 12-LOX has been identified as a crucial player in diabetes, skin diseases, thrombosis and

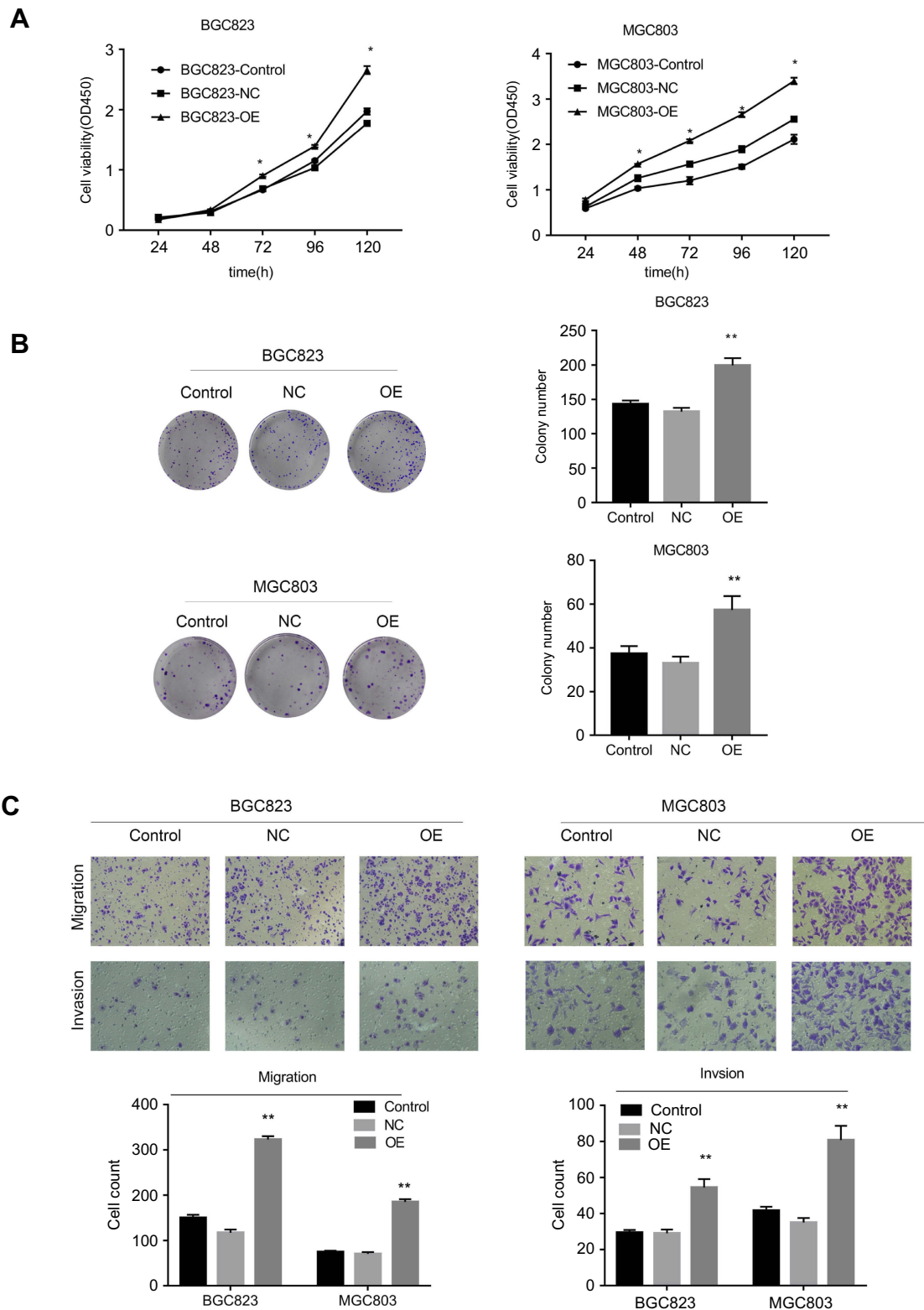
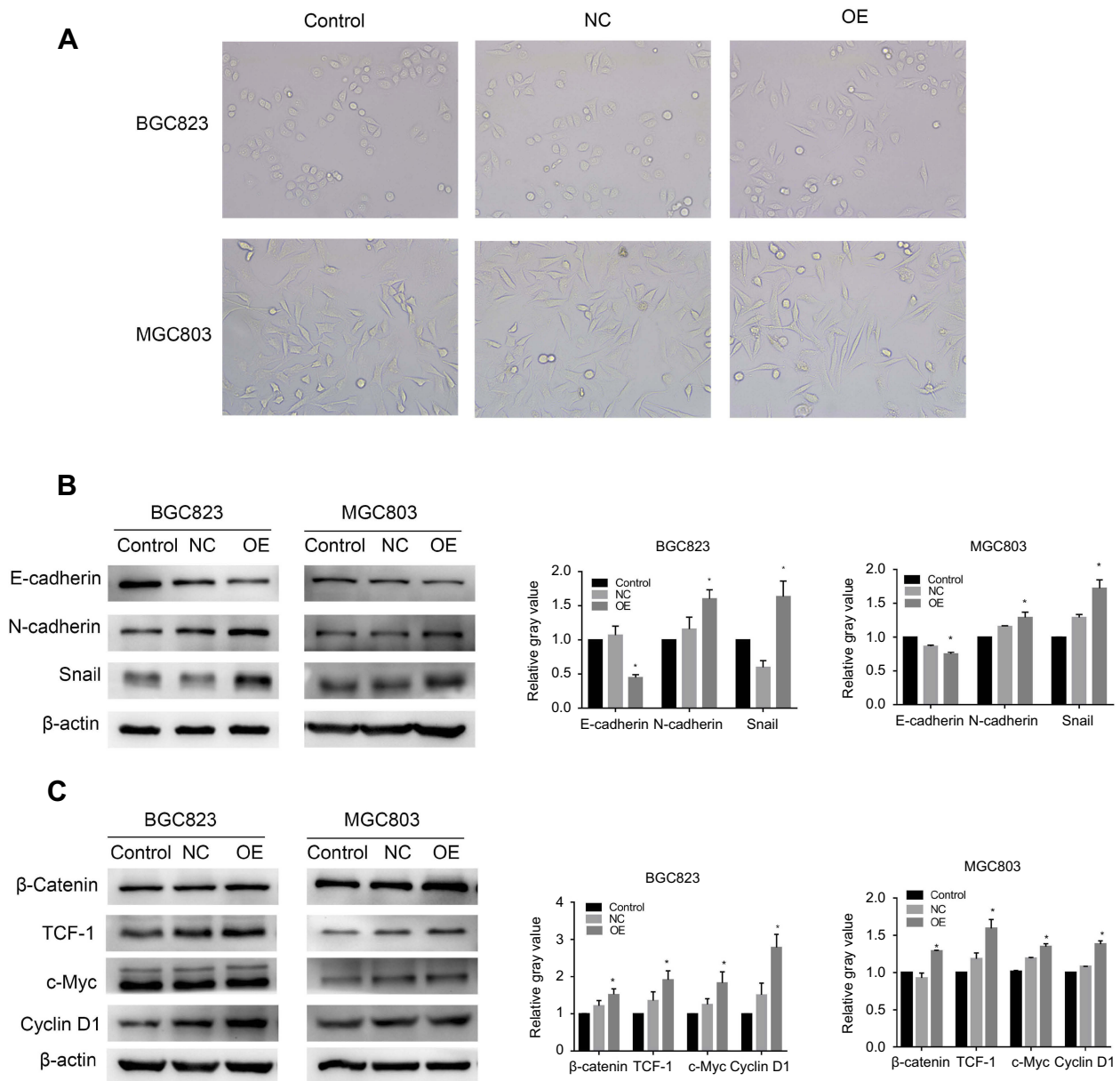


Figure 2 12-LOX overexpression promoted GC cell proliferation, migration and invasion in vitro. **(A)** Cell proliferation rates were increased in BGC823-OE and MGC803-OE cells compared with control and NC cells based on the CCK8 assay. **(B)** Overexpression of 12-LOX enhanced the colony formation ability in BGC823 and MGC803 cells. **(C)** Transwell assay revealed that up-regulating 12-LOX expression promoted BGC823 and MGC803 migration and invasion. Original magnification $\times 200$. * $P < 0.05$, ** $P < 0.01$ versus the corresponding control group.

Abbreviations: CCK8, cell-counting kit-8; 12-LOX, 12-lipoxygenase; GC, gastric cancer; NC, negative control group; OE, 12-LOX overexpression group.



cancers.^{15,22–27} There is increasing evidence that 12-LOX and 12-HETE promote tumor angiogenesis, growth and metastasis.²⁸ Genetic studies suggest that mutations of 12-LOX are closely associated with tumorigenesis.²⁹ Prasad et al reported a connection between the 12-LOX Arg261Gln polymorphism and the susceptibility to breast carcinoma.³⁰ In the present study, we investigated the function of 12-LOX in GC cells and its underlying mechanism of action in EMT for the first time to the best of our knowledge. First, we measured the mRNA and protein expression of 12-LOX in

several GC cell lines, and compared with normal gastric epithelial (GES-1) cells, the mRNA and protein levels of 12-LOX were up-regulated. This result is consistent with our previous study, which showed that high expression of 12-LOX might be a useful biomarker for predicting a worse prognosis in GC patients.²¹ Next, our experiments showed that both in vitro and in vivo, overexpression of 12-LOX elevated the growth rate of GC cells, suggesting that 12-LOX enhanced the proliferation of GC cells. These findings are consistent with earlier findings by Guo et al showing that

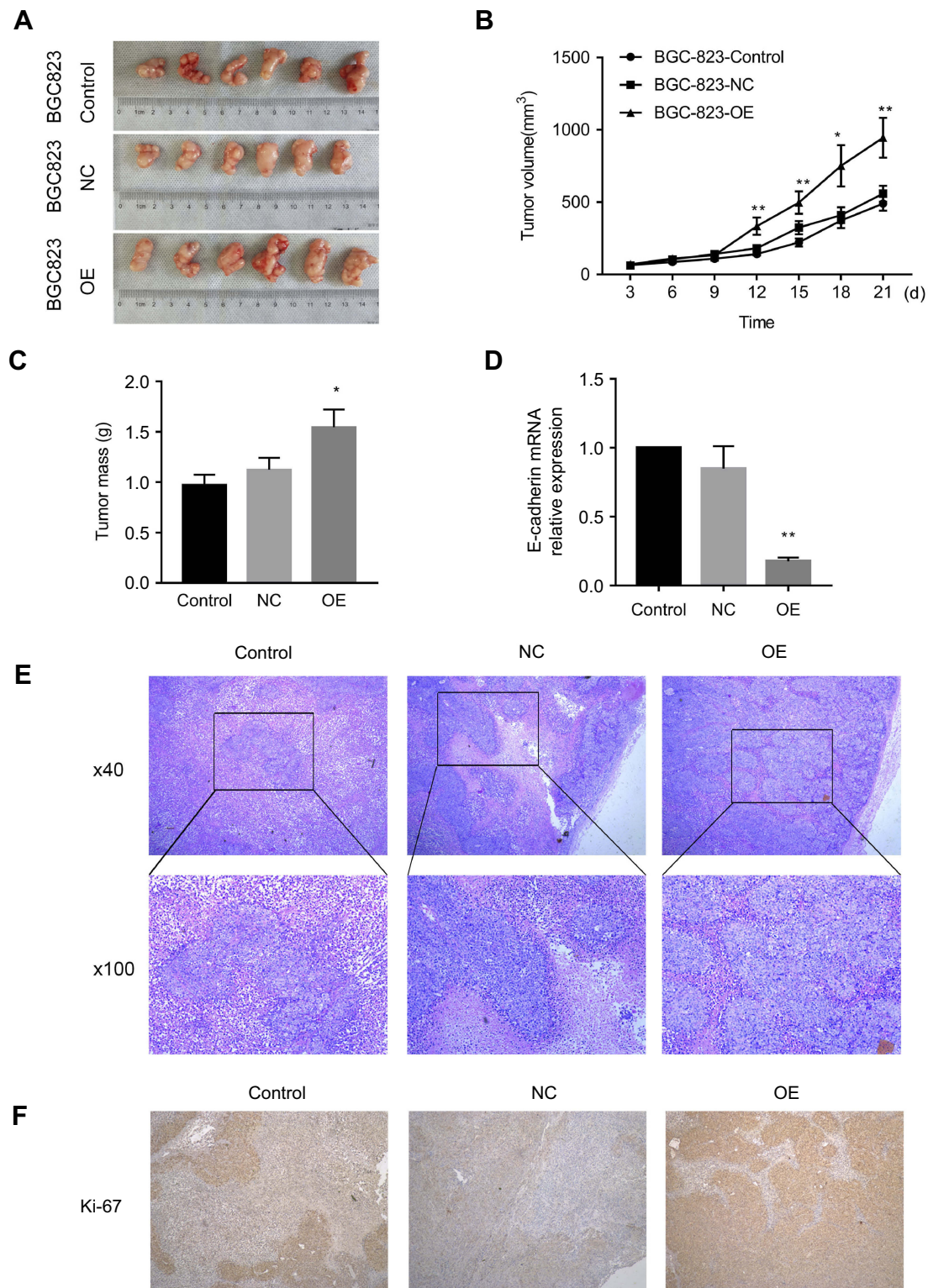


Figure 4 12-LOX promoted GC cell growth in the xenograft model. **(A)** Image of tumor samples acquired 21 days after subcutaneous injection. **(B)** Tumor volume curves were measured every 3 days in all three groups. **(C)** Weights of subcutaneous tumors in individual mice were recorded. **(D)** E-cadherin mRNA levels of tumors in all three groups were determined by qRT-PCR. **(E)** H&E staining was performed to evaluate the xenograft tumors of the three groups. **(F)** Expression of Ki-67 in xenograft tumor was detected using IHC. Original magnification $\times 100$. * $P < 0.05$, ** $P < 0.01$.

Abbreviations: IHC, immunohistochemistry; 12-LOX, 12-lipoxygenase; GC, gastric cancer; NC, negative control group; OE, 12-LOX overexpression group; qRT-PCR, quantitative real-time polymerase chain reaction.

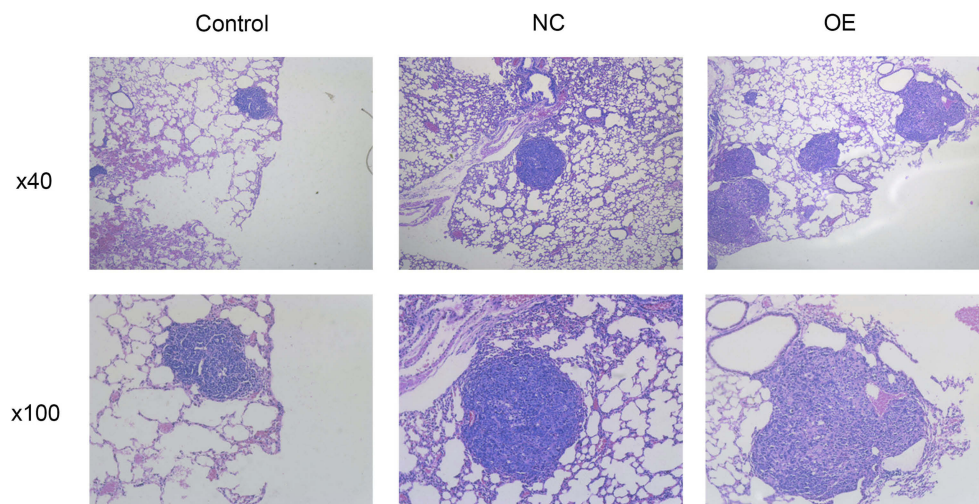


Figure 5 12-LOX promoted GC cell metastasis in vivo.

Notes: Representative H&E staining of a lung metastatic tumor of BGC823 cells transfected with the respective 12-LOX vector.

Abbreviations: 12-LOX, 12-lipoxygenase; GC, gastric cancer; NC, negative control group; OE, 12-LOX overexpression group.

12-LOX and 12-HETE increased the growth and survival of ovarian cancer.¹⁶ Moreover, Xu et al found that inhibition of 12-LOX decreased cell proliferation and induced hepatocellular cell apoptosis, suggesting that 12-LOX functions as a lipid mediator in its carcinogenic impact.³¹

EMT is a physiological process where epithelial cells are converted to mesenchymal cells by losing cellular polarity and cell–cell adhesion and acquiring invasive and migratory abilities. Recent studies indicate that EMT is a fundamental process in tumor progression and metastasis.³² E-cadherin characterizes epithelial cells and plays a vital role in the maintenance of intracellular binding structures.³³ The loss of E-cadherin is considered a critical event in the process of EMT.³⁴ Snail is a transcription factor involved in repressing the transcription of the E-cadherin gene by binding to the E-box of the E-cadherin promoter.³⁵ Dysregulation of Wnt/ β -catenin signaling genes has been reported in a variety of tumors including gastric, colorectal, breast and lung cancers.^{8,9,36–39} Klampfl et al reported that in colorectal cancer, elevating the level of 12-LOX promoted Caco2 and SW480 cell migration by reducing the expression of E-cadherin and integrin- β 1.⁴⁰ Additionally, 12-LOX activity modulated by integrin β 4 is strongly correlated with the stage/grade of prostate cancer.⁴¹ 12-LOX and 12-HETE promote prostate cancer cell migration and invasion by enhancing matrix metalloproteinase 9 expression.¹⁸ Therefore, we investigated whether the same molecular mechanism is operating in GC. In the present study, we found that overexpression of 12-LOX in GC cells resulted in a morphological

change, where cobblestone-like epithelial cells convert to spindle-shaped mesenchymal cells. Furthermore, enhancing the levels of 12-LOX resulted in a change of differentiation markers, which reduced the expression of the epithelial marker E-cadherin and concomitantly increased the expression of the mesenchymal marker N-cadherin. Investigating the relationship between 12-LOX and the Wnt/ β -catenin signaling pathway revealed that 12-LOX might promote the EMT by increasing the levels of TCF-1, Cyclin D1 and c-Myc, which are major downstream targets of the Wnt/ β -catenin signaling pathway. Overexpression of 12-LOX enhanced the migration and invasion abilities of GC cells. Finally, a subcutaneous xenograft model further confirmed that 12-LOX promoted GC growth and metastasis in vivo. Therefore, it is reasonable to assume that 12-LOX promoted EMT via the Wnt/ β -catenin signaling pathway in GC cells. A limitation of the present study is that we only investigated the downstream regulation of 12-LOX. The upstream regulatory mechanism of 12-LOX is not understood and deserves future in-depth investigations.

Conclusion

In summary, our results revealed that 12-LOX functions as an oncogene in inducing EMT by activating the Wnt/ β -catenin signaling pathway and plays an important role in the development and metastasis of GC. Therefore, 12-LOX is a potential therapeutic target for GC.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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