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lncRNA H19 mediates TGF-β1-induced epithelial to mesenchymal transition in bovine epithelial cells through PI3K/AKT Signaling Pathway

Increased levels of long noncoding RNA H19 (H19) have been observed in many inflammatory and organ fibrosis diseases including ulcerative colitis, osteoarthritis, liver fibrosis, renal fibrosis and pulmonary fibrosis. However, the role of H19 in bovine mastitis and mastitis-caused fibrosis is still unclear. In our study, H19 was characterized as a novel regulator of EMT induced by transforming growth factor-β1 (TGF-β1) in bovine mammary alveolar cell-T (MAC-T) cell line. We found that H19 was highly expressed in bovine mastitis tissues and inflammatory MAC-T cells induced by virulence factors of pathogens. TGF-β1 was also highly expressed in inflammatory MAC-T cells, and exogenous TGF-β1 could induce EMT, enhance extracellular matrix protein expression, and upregulate H19 expression in epithelial cells. Stable expression of H19 significantly promotes EMT progression and expression of ECM protein induced by TGF-β1 in MAC-T cells. Furthermore, by using a specific inhibitor of the PI3K/AKT pathway, we demonstrated that TGF-β1 upregulated H19 expression through PI3K/AKT pathway. All these observations imply that the lncRNA H19 modulated TGF-β1-induced epithelial to mesenchymal transition in bovine epithelial cells through PI3K/AKT signaling pathway, which suggests that mammary epithelial cells might be one source for myofibroblasts in vivo in the mammary glands under an inflammatory condition, thereby contributing to mammary gland fibrosis.

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lncRNA H19 mediates TGF-β1-induced epithelial to mesenchymal transition in bovine epithelial cells through PI3K/AKT Signaling Pathway

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ABSTRACT

Increased levels of long noncoding RNA H19 (H19) have been observed in many inflammatory and organ fibrosis diseases including ulcerative colitis, osteoarthritis, liver fibrosis, renal fibrosis and pulmonary fibrosis. However, the role of H19 in bovine mastitis and mastitis-caused fibrosis is still unclear. In our study, H19 was characterized as a novel regulator of EMT induced by transforming growth factor-β1 (TGF-β1) in bovine mammary alveolar cell-T (MAC-T) cell line. We found that H19 was highly expressed in bovine mastitis tissues and inflammatory MAC-T cells induced by virulence factors of pathogens. TGF-β1 was also highly expressed in inflammatory MAC-T cells, and exogenous TGF-β1 could induce EMT, enhance extracellular matrix protein expression, and upregulate H19 expression in epithelial cells. Stable expression of H19 significantly promotes EMT progression and expression of ECM protein induced by TGF-β1 in MAC-T cells. Furthermore, by using a specific inhibitor of the PI3K/AKT pathway, we demonstrated that TGF-β1 upregulated H19 expression through PI3K/AKT pathway. All these observations imply that the lncRNA H19 modulated TGF-β1-induced epithelial to mesenchymal transition in bovine epithelial cells through PI3K/AKT signaling pathway, which suggests that mammary epithelial cells might be one source for myofibroblasts in vivo in the mammary glands under an inflammatory condition, thereby contributing to mammary gland fibrosis.

Keywords: Mastitis, Epithelial cells, MAC-T, H19, TGF-β1, Bovine

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INTRODUCTION

Bovine mastitis is an ordinary disease of dairy herds caused by changes in metabolism, physiological trauma or contagious or environmental pathogenic microorganisms(Oviedo-Boyso et al. 2007). Mammary tissue was damaged by products from bacterial pathogen and enzymes released from stroma cells and secretory cells in the immune response process if infection persists(Zhao & Lacasse 2008). The repair process in the damaged mammary tissue is usually accomplished by fibrosis, which can start during an inflammatory response(Benites et al. 2002). Both mastitis and mastitis-caused fibrosis affect dairy industry due to the reduction of milk production and increased costs of treatments.

Epithelial mesenchymal transition (EMT) is a process that epithelial cells gradually acquire certain characteristics of the mesenchymal cells to produce fibroblasts and myofibroblasts(He et al. 2017). It is characterized by the morphological change of epithelial cells from cobblestone-shape to spindle-shape and expression changes of some EMT markers, such as E-cadherin decrease and the elevation of vimentin and α-SMA(Thiery 2002). Accumulating research evidenced that EMT plays a role in the genesis of fibroblasts during organ fibrosis(Becker-Carus 1972). What's more, Transforming Growth Factor-β1 (TGF-β1) can induce EMT during organ fibrosis, and it is regarded as a master regulator of EMT progression (Moustakas & Heldin 2016; Risolino et al. 2014).

The long non-coding RNA (lncRNA) H19 gene is an imprinted maternally expressed gene located on chromosome 29 in bovine, and it plays a vital role on mammalian development (Keniry et al. 2012). Increasing evidence showed that H19 may have either oncogenic or tumor suppressor properties based on its opposite expression changes in various cancers

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including bladder cancer(Luo et al. 2013) and Gastric (Song et al. 2013; Yang et al. 2012), and the exact mechanism is still elusive(Jiang et al. 2016). Otherwise, H19 RNA was related to the development of inflammatory diseases, such as ulcerative colitis(Chen et al. 2016) and osteoarthritis(Steck et al. 2012) and organ fibrosis including liver fibrosis(Song et al. 2017), renal fibrosis(Xie et al. 2016) and pulmonary fibrosis (Tang et al. 2016). A recent research linked H19 upregulation to the TGFβ-induced EMT process(Matouk et al. 2013).

In this study, we compared the expression of H19 in inflammatory tissue and mammary epithelial cells to their normal counterparts. Using immortalized mammary epithelial cells line of MAC-T cells, we then investigated the roles of H19 in the TGF-β1-induced EMT in MAC-T cells, suggesting that H19 mediate the mastitis-caused mammary fibrosis. And this finding will lead to a better understanding of the pathological mechanism of bovine mammary gland fibrosis caused by mastitis.

MATERIALS AND METHODS

Cell culture and treatment

The mammary alveolar cell-T (MAC-T) cell line was a gift from Prof. Mark D. Hanigan (Virginia Polytechnic Institute and State University, Blacksburg, VA). MAC-T cells were cultured in complete DMEM/F12 medium (GIBCO BRL, Life Technologies, Burlington, ON) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL) at 37 °C in an incubator with 5% CO_2. Confluence cells were subcultured by digestion with 0.15% trypsin and 0.02% EDTA.

For cell treatments with reagents, cells grown to around 80% confluence in culture

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dishes were respectively treated by LPS (Sigma-Aldrich, St. Louis, MO) at 10 ng/μl for 3 h, LTA (Inviogen, San Diego, CA) at 20 ng/μl for 12 h, or TGF-β1 (Creative BioMart, Shirley, NY) at 10 ng/ml for 36 h according to recommended concentrations of our previous publication(Zhang et al. 2016).

RNA extraction and real-time PCR

Total mRNA was extracted from mammary tissue and cells by using TriZol solution (TransGene, Shanghai, China) and the RNA Easy Kit (TransGene) according to the manufacturer's instructions. Total RNA concentration was measured by the spectrophotometer (ND 2.0; Nano Drop Technologies, Wilmington, DE), and 3μg of total mRNA was reverse-transcribed into cDNA using TransScript II First-Stran cDNA Synthesis SuperMix (TransGene). Quantitative primers were designed based on the sequences in the National Center of Biotechnology Information Database and synthesized by Sangon Biotech (Shanghai, China). The primers were provide in Additional file 1. Quantitative real time was performed with an iQ5 light cycler (Bio-Rad, Hemel Hempstead, UK) in 20 μl reactions. GAPDH was used as the reference gene.

Western blotting

Cells were lysed in PRO-PREP Protein Extraction Solution (iNtRON Biotechnology, Inc, Gyeonggi-do, Korea) to prepare protein lysates according to manufacturer's instructions. Cell lysates were centrifuged at 12000 rpm for 10 min at 4°C. Protein concentration was measured using Bradford Easy Protein Quantitative Kit (TransGene). Equal protein extraction was

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separated in 10% polyacrylamide gels (Sigma-Aldrich).After that, proteins were transferred to the PVDF membranes. Then the membranes were blocked with 10% non-fat milk and incubated with anti-α-SMA (Abcam, Cambridge, UK), anti-collagen I (Abcam), anti-E-cadherin (Abcam), anti-N-cadherin (Bioss, Beijing, China), albumin (Bioss), MMP9 (Bioss) and anti-GAPDH (TransGene) antibodies at 4 °C overnight. After washing membranes with TBST for three times for 5 min each time, membranes were incubated with secondary antibodies. Finally, immunoreactive proteins were detected using an enhanced chemiluminescence detection kit (Beyotime, shanghai, china).

ELISA

MAC-T cells were cultured for 24 h in fresh serum-free medium after treatment with LPS or LTA. Then, the collected medium was centrifuged at 12000 rpm for 5min to remove cell debris. TNF-α secreted from MAC-T in the medium was detected according to the manufacturer's instructions of ELISA kits (Huzhen Biological Technology Co, LTD, Shanghai, China).

Establishment of stable MAC-T cell clone with overexpressed H19

The full length H19 gene sequence from NCBI was synthesized by Sangon Biotech, which includes BamHI and NotI enzyme restriction sites. The product synthesized was digested with the BamHI and NotI enzymes and ligated into the BamHI and NotI sites of the CD513B-1 basic vector(System Biosciences, Mountain View, CA, USA), yielding the CD513B-1-H19 construct. One day before transfection, *MAC-T* cells were seeded into 60-mm

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culture dishes at about 80% confluence. Cells were transfected with CD513B-1-H19 *or* CD513B-1 control plasmids using electroporation with transfer buffer in a 4 mm gap cuvette at 510 V for one pulse. After 8 h of transfection, cells were subcultured to 50% confluence in medium containing 0.6 μg/ml puromycin (Sigma-Aldrich, St. Louis, MO, USA). When all MAC-T cells in the nontransfected control culture were killed, puromycin-resistant clones were picked and passaged in medium containing half the concentration of puromycin. H19 targeted cell clones were screened by real time PCR and flag fluorescence.

Luciferase reporter assays

Series of upstream fragments of H19 gene sequence were amplified by PCR from genomic DNA using primers containing enzyme restriction sites, and the primers were listed in Additional file 1. These fragments of H19 gene sequence were respectively cloned into the PGL4.10 plasmids (promega, Madison, WI, USA). MAC-T cells were transfected with a mixture of pRL-TK-renilla-luciferase plasmid and PGL4.10-reporter plasmids. The PGL4.10 plasmid was used as control vector. After transfection for 8 h, MAC-T cells transfected with PGL4.10-reporter plasmids and control vectors were treated with TGF-β1 as aforementioned. Finally, the luciferase activities were detected by Dual-Luciferase Reporter analytical instrument (promega).

Statistical analysis

The results are expressed as means ± standard deviation (SD). All data from at least three independent for each parameter were analyzed with ANOVA (SPSS11.5 software). A p-value

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of < 0.05 was considered statistically significant.

RESULTS

Expression of H19 in mammary tissue and epithelial cells of bovine

To investigate if H19 mediates the process of bovine mastitis, we first explored the expression of H19 in normal and inflammatory mammary tissue of bovine. Results from qPCR analysis showed that H19 was significantly up-regulated in inflammatory mammary tissue compare with normal tissue (Figure.1). Subsequently, we further detected the H19 expression in inflammatory MAC-T cells in an epithelial cell model of mastitis by treating MAC-T cells with LPS or LTA according to the recommended concentration of previous publication(Zhang et al. 2016). Both LPS and LTA could induce an obvious inflammatory response in MAC-T cells as indicated by elevated TNF-α mRNA expression (Figure.2A) and protein secretion (Figure. 2B). Consistent with the expression of H19 in tissue level, we found that the H19 expression was also up-regulated in inflammatory epithelial cells induced by LPS and LTA in vitro (Figure. 2C).

TGF-β1 was highly expressed in inflammatory epithelial cells and induced EMT and enhanced ECM protein expression

TGF-β1 has been shown to play an essential role in the suppression of inflammation, yet recent studies have revealed the positive roles of TGF-β1 in inflammatory response(Yoshimura et al. 2010). Here we found that TGF-β1 had an increased expression in both LPS- and LTA-induced inflammatory MAC-T cells (Figure.3A). TGF-β1 could induce EMT phenotypes in epithelial cells in vitro and has been associated with EMT in vivo

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(Moustakas & Heldin 2016; Risolino et al. 2014). To investigate whether if increased TGF-β1 in epithelial cells under inflammatory condition caused mammary fibrosis via EMT, we incubated MAC-T cells with exogenous TGF-β1 to examine the occurrence of EMT. The western blot results showed that the expressions of α-SMA and N-cadherin, two well-known EMT markers, were significantly upregulated in TGF-β1-treated MAC-T cells compared to untreated cells, while E-cadherin expression was down-regulated in MAC-T cells after treatment with TGF-β1 compared to untreated cells (Figure.3B), suggesting TGF-β1 could induce EMT in epithelial cells of MAC-T. In addition, TGF-β1 treatment enhanced the protein expression levels of several extracellular matrix (ECM) proteins, including albumin, collagen 1, and MMP9, in MAC-T cells (Figure.3B). Interestingly, we found that TGF-β1 treatment was able to up-regulate H19 expression in MAC-T cells (Figure.3C).

H19 mediated TGF-β1-induced EMT and ECM protein expression in MAC-T cells

To investigate whether if H19 mediated TGF-β1-induced EMT in MAC-T cells, we established H19 stably overexpressed MAC-T cell clones by introducing of the lentiviral vector of CD513B-1 containing the H19 cDNA (Figure.4A). High-level GFP-expression was detected by direct viewing with a fluorescent microscope in MAC-T cells transfected by both CD513B-1 and CD513B-1-H19 vectors after selection with puromycin (Figure.4B). The overexpression of H19 in MAC-T cells was further confirmed by RT-qPCR (Figure. 4C). Western blot results showed that overexpression of H19 significantly promoted TGF-β1-induced EMT in MAC-T cells compared to CD513B-1 control vector-transfected cells, as indicated by increased α-SMA and N-cadherin expression and decrease E-cadherin expression (Figure. 4D). In addition, overexpression of H19 also promoted TGF-β1-induced

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expression increase of Albumin, Collagen 1, and MMP9 (Figure. 4D).

TGF-β1-stimulated H19 requires the AKT activation

A previously publication linked H19 expression to PI3K/AKT pathway in response to TGF-β1 treatment(Matouk et al. 2014). Here, we found that TGF-β1 treatment could activate AKT protein in MAC-T cells, and this activation was effectively inhibited by LY 294002, a specific chemical inhibitor of the PI3K/AKT pathway (Figure. 5A). Furthermore, the inhibition of activation of PI3K/AKT pathway by LY294002 was able to abolished TGF-β1-induced up-regulation of H19 in MAC-T cells (Figure. 5B). These data suggest that TGF-β1-induced EMT in MAC-T cells is mediated by the TGF-β1/AKT/H19 axis.

Identification of H19 promoter region

To identify the promoter region of H19 gene responded to TGF-β1 treatment, luciferase reporter plasmids were constructed by inserting each truncation of a 4679 bp upstream segment of H19. All constructs were confirmed by DNA sequencing. The luciferase activities were tested by luciferase assay in MAC-T cell line. We did not find the H19 promoter region within the upstream 4976bp sequences of H19responsible for TGF-β1-induced EMT in MAC-T cells. As showed in Figure. 6, transcriptional activity was not different among all examined segments.

DISCUSSION

It has been reported that H19 upregulation is linked to the process of EMT in

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carcinogenesis and embryogenesis, and induction of EMT by different approaches (e.g. hypoxia, TGFβ) in cancer cells is accompanied by H19 upregulation(Oviedo-Boyso et al. 2007). EMT has been taken as the potential sources of myofibroblasts in tissue fibrosis(Zou et al. 2017), and bovine mastitic usually can cause varying degrees of fibrosis process of mammary glands. We speculated that H19 upregulation might be involved in the mammary fibrosis induced by mastitic.

Here we found that exposure of MAC-T cells to TGF-β1 stimulation could cause them undergo EMT mediated by H19 upregulation, representing another source of fibroblasts involved in mammary gland fibrosis of bovine.

We first observed that the expression level of H19 was upregulated in both mastitic mammary tissue compared to normal tissue, indicating that H19 was involved in the immune response of mammary glands during the process of bovine mastitic. During bacterial invasion, mammary epithelial cells play an important role in inducing a relevant innate immune response in mammary glands by immunological factor release(Zhang et al. 2016). H19 upregulation was also observed in inflammatory MAC-T cells induced by the gramnegative and gram-positive bacterial cell wall components LPS and LTA, suggesting H19 is involved in the immune response process of epithelial cells triggered by pathogens.

Following inflammation, some types of cells produce TGF-β1, and excessive TGF-β1 contributes to a pathologic excess of tissue fibrosis(Branton & Kopp 1999). EMT is recognized as playing an important role in repair and scar formation following epithelial injury. The evidence that TGF-β1 induces EMT in alveolar epithelial cells in vitro and in vivo suggests that alveolar epithelial cells may serve as a source of myofibroblasts in lung

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fibrosis(Willis & Borok 2007). In this study, we found TGF-β1 had an upregulated expression in MAC-T cells under LPS or LTA stimulation. To investigate whether the excessive TGF-β1 has an effect on MAC-T cells through an autocrine manner, thus leads to an EMT process, we treated MAC-T cells with exogenous TGF-β1. TGFβ1-induced protein expression changes of several well-known EMT makers, such as downregulation of E-cadherin and upregulation of α-SMA and N-cadherin, were observed in MAC-T cells, indicating TGFβ1-induced EMT occurred. Myofibroblasts release a variety of excessive extracellular matrix proteins contributing to organ fibrosis(Hinz et al. 2007). We found that TGFβ1 stimulates increased expression of extracellular matrix proteins including Collagen type 1, MMP9, and albumin in MAC-T cells.

An interesting finding is that TGFβ1 stimulates the expression of H19 in MAC-T cells. We next established an epithelial cell line with stable overexpressed H19 level to assess the relation between H19 and TGF-β1-induced EMT in MAC-T cells. We found that H19 overexpression could enhance the TGF-β1-induced EMT in MAC-T cells, indicating a high level of H19 expression was associated with EMT, which is consistent with the previous observation that TGF-β1 is associated with EMT and inflammation (Franco et al. 2010; Gal et al. 2008; Salgado et al. 2017).

Accumulating studies have evidenced that AKT was an important regulator of EMT in the majority of cell types (Larue & Bellacosa 2005; Lee & Han 2010; Maseki et al. 2012). In our study, we found TGFβ1 activated PI3K/AKT signal pathway in MAC-T cells, and the specific inhibitor of the PI3K/AKT pathway was able to inhibit the activation of AKT. What's more, this inhibitor also abated TGF-β1-induced upregulation of H19 in MAC-T cells,

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suggesting that H19 expression increase in MAC-T cells was induced by TGF-β1 through PI3K/AKT pathway. This finding is consistent with the results from a previously publication that linked H19 expression to PI3K/AKT pathway in response to TGF-β1 treatment(Matouk et al. 2014). Finally, we tried to identify the promoter region responsive to TGF-β1 treatment by using luciferase reporter assay to detect the upstream 4.679kb sequence of H19 gene, but no promoter responsive to TGF-β1 was found within the examined sequence regions. Further researches should be done in the future to demonstrate the mechanism in which TGF-β1 regulate the expression of H19 in MAC-T cells.

CONCLUSIONS

In summary, we show evidence that H19 had increased expressions in both bovine mastitic tissue and inflammatory bovine epithelial cells, and lncRNA H19 mediates TGF-β1-induced EMT and ECM protein synthesis in bovine epithelial cells through PI3K/AKT Signaling Pathway. Additional studies are clearly needed to both demonstrate the molecular mechanism of H19 responsive to TGF-β1 and address whether EMT occurs in vivo during bovine mastitis. Based on our results we suggest that mammary epithelial cells might be one source for myofibroblastsin vivo in the mammary glands under an inflammatory condition, thereby contributing to mammary gland fibrosis.

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ADDITIONAL INFORMATION AND DECLARATIONS

Funding

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Competing Interests

No competing interests declared

Author Contributions

All authors contributed significantly to this research and preparation of the manuscript. Y.Z. and G.MQ conceived and designed the experiments. W.Y. L.XZ Q.SP L.XU K.Z and Q.SZ performed the experiments and analyzed the data. W.Y. and G.MQ wrote the manuscript. All authors have been involved in the drafting, critical revision and final approval of the manuscript for publication.

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FIGURE LEGENDS

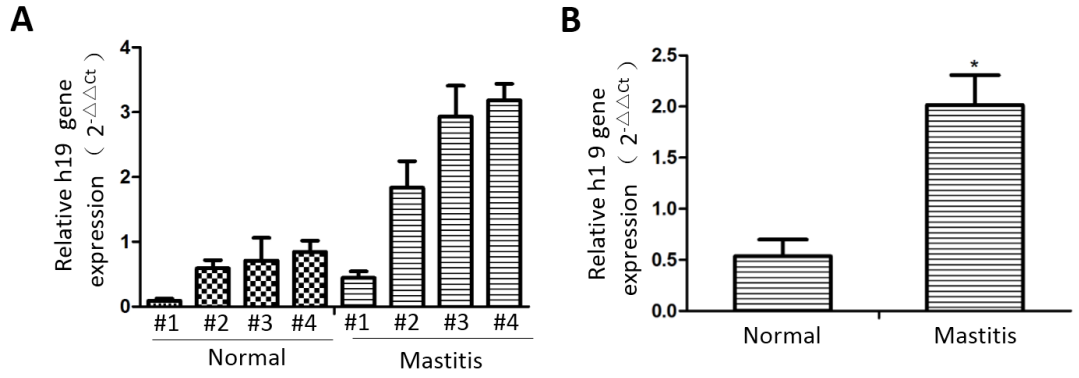

Figure. 1 The expression of H19 in normal and mastitic tissue of bovine mammary glands. (**A**) The expression of H19 in normal (n=4) and mastitic (n=4) tissue of mammary glands assessed by RT-qPCR. GAPDH was used as an internal control. (**B**) Statistic analysis of the expression of H19 between normal and mastitis group. *p<0.01 vs Normal.

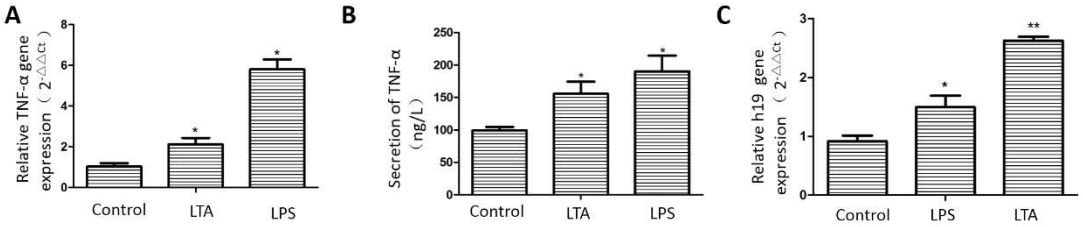

Figure. 2 The expression of H19 in MAC-T cells with or without LPS or LTA treatment. (**A** and **B**) An in vitro inflammatory epithelial cell model induced by LPS or LTA stimulation was established. TNF-α was used as an indicator of MAC-T cell inflammatory responses to stimulus. TNF-α mRNA expression in treated cells was analyzed by RT-qPCR (A), and TNF-α protein secretion in the medium was measured with an ELISA kit (B). (C) The expression of H19 in MAC-T cells analyzed by RT-qPCR. Untreated MAC-T cells were used as control. GAPDH was used as internal control. *p<0.05, **p<0.01 vs Control.

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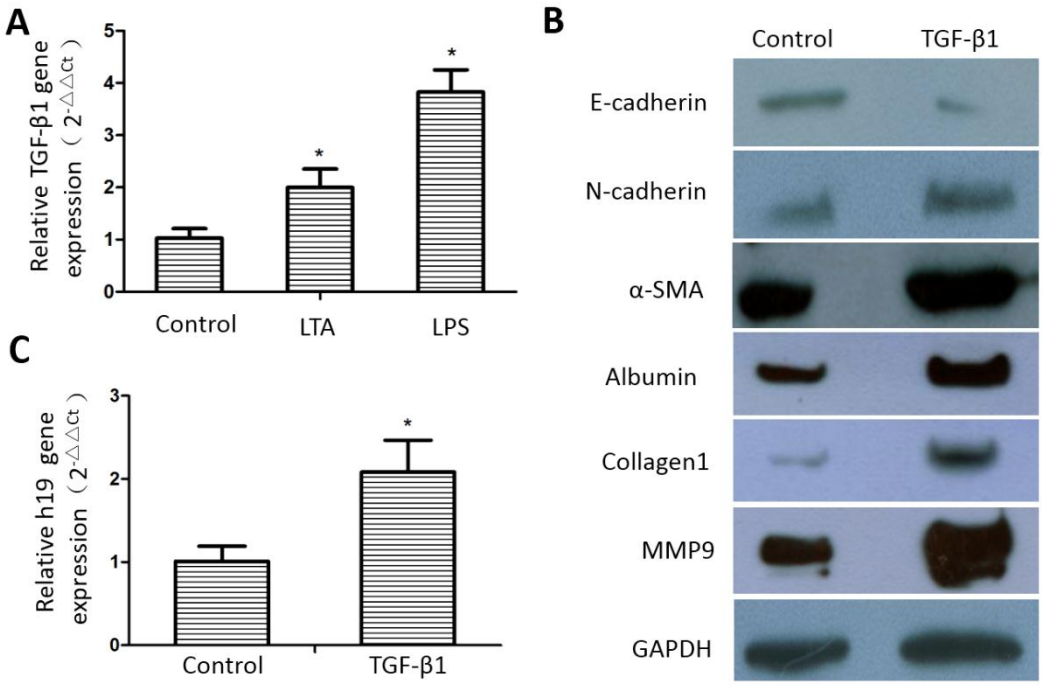

Figure. 3 The expression, EMT induction, and promotion of ECM protein expression of TGF-β1 in MAC-T cells. (**A**) The Expression of TGF-β1 in inflammatory MAC-T cells analyzed by RT-qPCR. (**B**)Expression changes of EMT markers (E-cadherin, N-cadherin and α-SMA) and ECM protein (Albumin, MMP9, Collagen type I) in MAC-T cells upon TGF-β1 treatments analyzed by western blot. (**C**) The expression of H19 in MAC-T cells upon TGF-β1 treatments analyzed by RT-qPCR. Untreated MAC-T cells were used as control. GAPDH was used as an internal control. *p<0.01 vs control.

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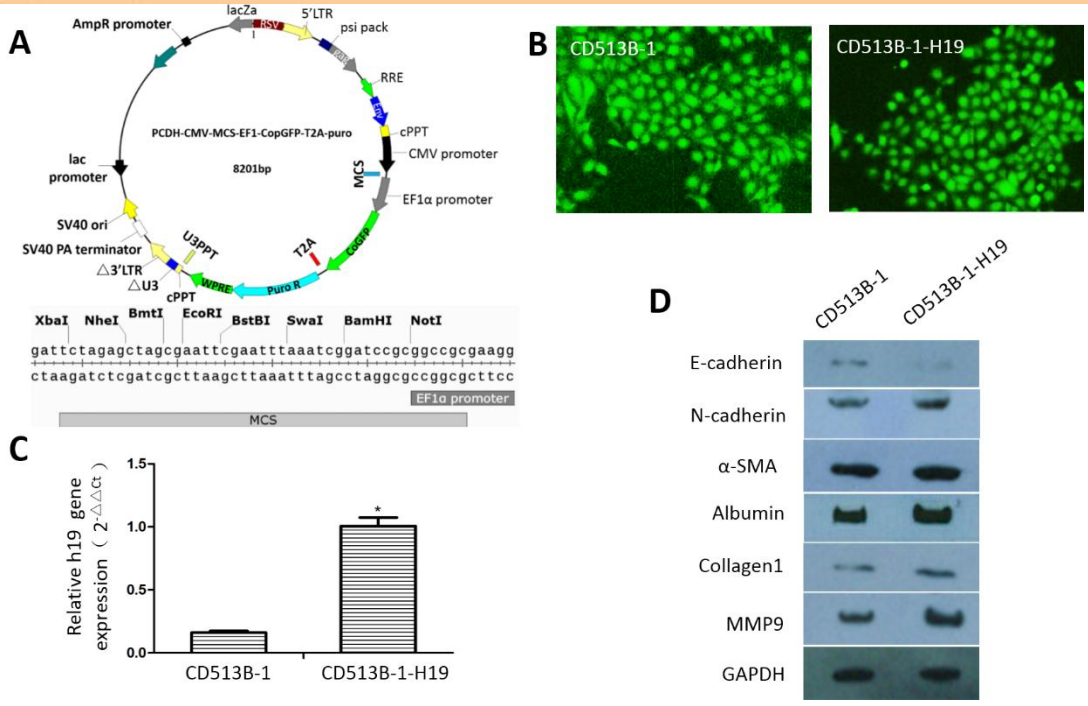

Figure. 4 H19 promotes EMT progression and ECM protein expression induced by TGF-β1 in MAC-T cells. (**A**) The information of lentviral vector of CD513B-1. BamHI and NotI sites from the CD513B-1 were chosen to construct the expression plasmid. (**B**) Fluorescence microscopy examination of MAC-T cells transduced withlentviral vectors. GFP expression was observed under fluorescence microscopy. Left, MAC-T cells infected with CD513B-1 as control; right, MAC-T cells infected with CD513B-1-H19. (**C**) Analysis of H19 expression in MAC-T cells with empty vector or CD513B-1-H19 by RT-qPCR. (**D**) The expression of EMT marker proteins E-cadherin, N-cadherin, and α-SMA, and ECM proteins Albumin, Collagen 1 and MMP9 were evaluated in H19-overexressing MAC-T cells by western blot. Control vector-transduced cells were used as control. GAPDH was used as an internal control. * $p<0.05$ vs control.

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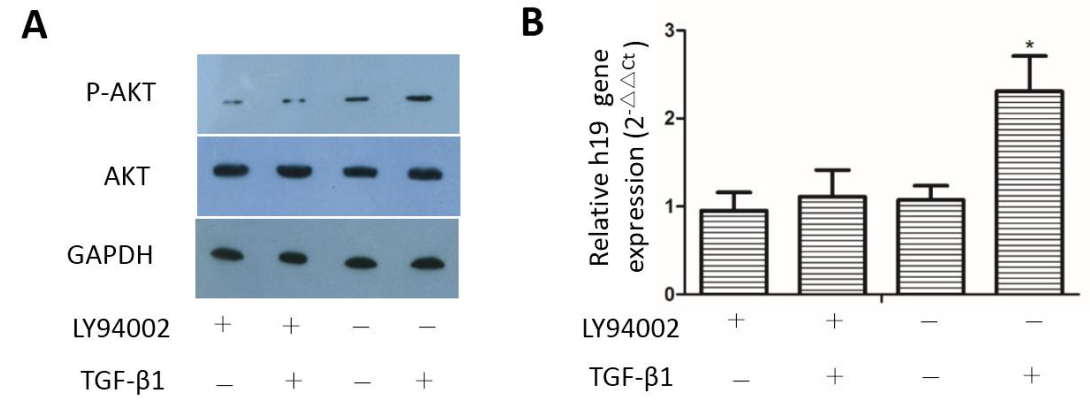

Figure. 5 Inhibition of the PI3K/AKT pathway suppressed the activition of TGF-β1-induced upregulation of H19 expression. Confluent MAC-T cells were serum-starved for 24 h and pretreated with the inhibitor at 20 μmol/l in serum-free media for 30 min, then the cells were treated with TGF-β1 at 2 ng/ml for 36 h. (**A**) Phosphorylated Akt and total Akt expressions in cells were analyzed by Western blot. (**B**) The expression of H19 in cells was analyzed by RT-qPCR. GAPDH was used as an internal control. * p <0.05 vs control.

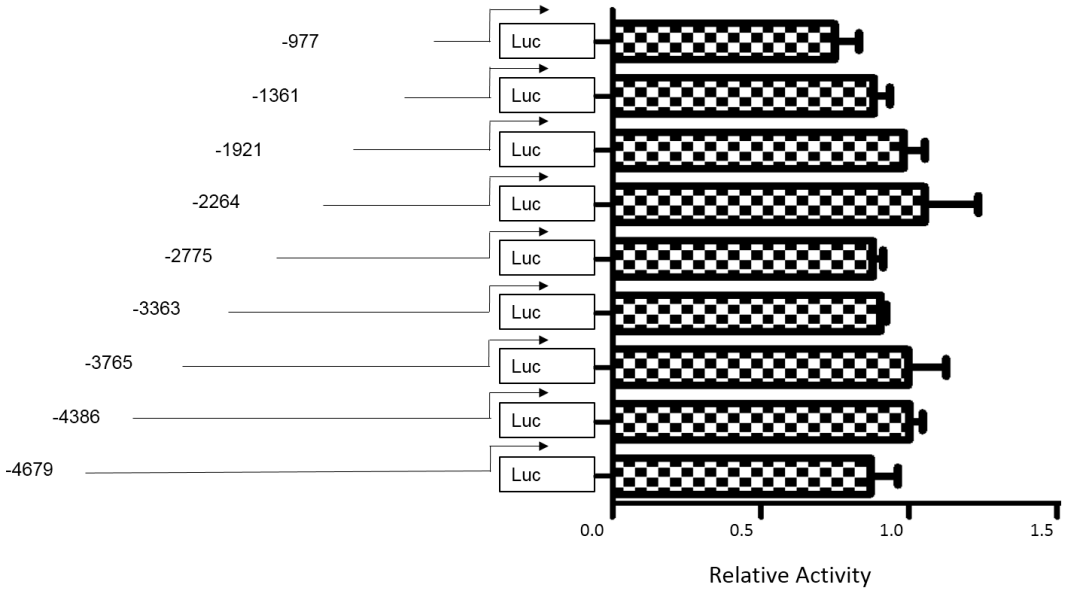

Figure. 6 Functional analysis of different lengths of putative promoter sequences of H19 in MAC-T cells. The different 5' truncated fragments of upstream of H19 gene sequences

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were generated and inserted into the Luc-vector. The length of fragments was indicated relative to the transcription start site, and the activity of the fragments constructs relative to a promoter-less construct was given. $p>0.05$ among all groups.

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