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.

1   **lncRNA H19 mediates TGF-β1-induced epithelial to mesenchymal transition in bovine**
2   **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

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

92   including bladder cancer(Luo et al. 2013) and   Gastric (Song et al. 2013; Yang et al. 2012),

93   and the exact mechanism is still elusive(Jiang et al. 2016). Otherwise, H19 RNA was related

94   to the development of inflammatory diseases, such as ulcerative colitis(Chen et al. 2016)

95   and osteoarthritis(Steck et al. 2012) and organ fibrosis including liver fibrosis(Song et al.

96   2017), renal fibrosis(Xie et al. 2016) and pulmonary fibrosis (Tang et al. 2016). A recent

97   research linked H19 upregulation to the TGFβ-induced EMT process(Matouk et al. 2013).

98   In this study, we compared the expression of H19 in inflammatory tissue and mammary

99   epithelial cells to their normal counterparts. Using immortalized mammary epithelial cells line

100   of MAC-T cells, we then investigated the roles of H19 in the TGF-β1-induced EMT in

101   MAC-T cells, suggesting that H19 mediate the mastitis-caused mammary fibrosis. And this

102   finding will lead to a better understanding of the pathological mechanism of bovine mammary

103   gland fibrosis caused by mastitis.

104

105   **MATERIALS AND METHODS**

106   Cell culture and treatment

107   The mammary alveolar cell-T (MAC-T) cell line was a gift from Prof. Mark D. Hanigan

108   (Virginia Polytechnic Institute and State University, Blacksburg, VA). MAC-T cells were

109   cultured in complete DMEM/F12 medium (GIBCO BRL, Life Technologies, Burlington, ON)

110   supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 μg/ml

111   streptomycin (Gibco BRL) at 37 °C in an incubator with 5% CO_2. Confluence cells were

112   subcultured by digestion with 0.15% trypsin and 0.02% EDTA.

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

114   dishes were respectively treated by LPS (Sigma-Aldrich, St. Louis, MO) at 10 ng/μl for 3 h,

115   LTA (Inviogen, San Diego, CA) at 20 ng/μl for 12 h, or TGF-β1 (Creative BioMart, Shirley,

116   NY) at 10 ng/ml for 36 h according to recommended concentrations of our previous

117   publication(Zhang et al. 2016).

118

119   RNA extraction and real-time PCR

120   Total mRNA was extracted from mammary tissue and cells by using TriZol solution

121   (TransGene, Shanghai, China) and the RNA Easy Kit (TransGene) according to the

122   manufacturer's instructions. Total RNA concentration was measured by the spectrophotometer

123   (ND 2.0; Nano Drop Technologies, Wilmington, DE), and 3μg of total mRNA was

124   reverse-transcribed into cDNA using TransScript II First-Stran cDNA Synthesis SuperMix

125   (TransGene). Quantitative primers were designed based on the sequences in the National

126   Center of Biotechnology Information Database and synthesized by Sangon Biotech (Shanghai,

127   China). The primers were provide in Additional file 1. Quantitative real time was performed

128   with an iQ5 light cycler (Bio-Rad, Hemel Hempstead, UK) in 20 μl reactions. GAPDH was

129   used as the reference gene.

130

131   Western blotting

132   Cells were lysed in PRO-PREP Protein Extraction Solution (iNtRON Biotechnology, Inc,

133   Gyeonggi-do, Korea) to prepare protein lysates according to manufacturer's instructions. Cell

134   lysates were centrifuged at 12000 rpm for 10 min at 4°C. Protein concentration was measured

135   using Bradford Easy Protein Quantitative Kit (TransGene). Equal protein extraction was

136　separated in 10% polyacrylamide gels (Sigma-Aldrich).After that, proteins were transferred to

137　the PVDF membranes. Then the membranes were blocked with 10% non-fat milk and

138　incubated with anti-α-SMA (Abcam, Cambridge, UK), anti-collagen I (Abcam),

139　anti-E-cadherin (Abcam), anti-N-cadherin (Bioss, Beijing, China), albumin (Bioss), MMP9

140　(Bioss) and anti-GAPDH (TransGene) antibodies at 4 °C overnight. After washing

141　membranes with TBST for three times for 5 min each time, membranes were incubated with

142　secondary antibodies. Finally, immunoreactive proteins were detected using an enhanced

143　chemiluminescence detection kit (Beyotime, shanghai, china).

144

145　ELISA

146　　　MAC-T cells were cultured for 24 h in fresh serum-free medium after treatment with

147　LPS or LTA. Then, the collected medium was centrifuged at 12000 rpm for 5min to remove

148　cell debris. TNF-α secreted from MAC-T in the medium was detected according to the

149　manufacturer's instructions of ELISA kits (Huzhen Biological Technology Co, LTD, Shanghai,

150　China).

151

152　Establishment of stable MAC-T cell clone with overexpressed H19

153　　　The full length H19 gene sequence from NCBI was synthesized by Sangon Biotech,

154　which includes BamHI and NotI enzyme restriction sites. The product synthesized was

155　digested with the BamHI and NotI enzymes and ligated into the BamHI and NotI sites of the

156　CD513B-1 basic vector(System Biosciences, Mountain View, CA, USA), yielding the

157　CD513B-1-H19 construct. One day before transfection, *MAC-T* cells were seeded into 60-mm

158 culture dishes at about 80% confluence. Cells were transfected with CD513B-1-H19 *or*

159 CD513B-1 control plasmids using electroporation with transfer buffer in a 4 mm gap cuvette

160 at 510 V for one pulse. After 8 h of transfection, cells were subcultured to 50% confluence in

161 medium containing 0.6 μg/ml puromycin (Sigma-Aldrich, St. Louis, MO, USA). When all

162 MAC-T cells in the nontransfected control culture were killed, puromycin-resistant clones

163 were picked and passaged in medium containing half the concentration of puromycin. H19

164 targeted cell clones were screened by real time PCR and flag fluorescence.

165

166 Luciferase reporter assays

167   Series of upstream fragments of H19 gene sequence were amplified by PCR from

168 genomic DNA using primers containing enzyme restriction sites, and the primers were listed

169 in Additional file 1. These fragments of H19 gene sequence were respectively cloned into the

170 PGL4.10 plasmids (promega, Madison, WI, USA). MAC-T cells were transfected with a

171 mixture of pRL-TK-renilla-luciferase plasmid and PGL4.10-reporter plasmids. The PGL4.10

172 plasmid was used as control vector. After transfection for 8 h, MAC-T cells transfected with

173 PGL4.10-reporter plasmids and control vectors were treated with TGF-β1 as aforementioned.

174 Finally, the luciferase activities were detected by Dual-Luciferase Reporter analytical

175 instrument (promega).

176

177 Statistical analysis

178   The results are expressed as means ± standard deviation (SD). All data from at least three

179 independent for each parameter were analyzed with ANOVA (SPSS11.5 software). A p-value

180   of < 0.05 was considered statistically significant.

181

182   **RESULTS**

183   **Expression of H19 in mammary tissue and epithelial cells of bovine**

184   To investigate if H19 mediates the process of bovine mastitis, we first explored the

185   expression of H19 in normal and inflammatory mammary tissue of bovine. Results from

186   qPCR analysis showed that H19 was significantly up-regulated in inflammatory mammary

187   tissue compare with normal tissue (Figure.1). Subsequently, we further detected the H19

188   expression in inflammatory MAC-T cells in an epithelial cell model of mastitis by treating

189   MAC-T cells with LPS or LTA according to the recommended concentration of previous

190   publication(Zhang et al. 2016). Both LPS and LTA could induce an obvious inflammatory

191   response in MAC-T cells as indicated by elevated TNF-α mRNA expression (Figure.2A) and

192   protein secretion (Figure. 2B). Consistent with the expression of H19 in tissue level, we found

193   that the H19 expression was also up-regulated in inflammatory epithelial cells induced by

194   LPS and LTA in vitro (Figure. 2C).

195

196   **TGF-β1 was highly expressed in inflammatory epithelial cells and induced EMT and**

197   **enhanced ECM protein expression**

198   TGF-β1 has been shown to play an essential role in the suppression of inflammation,

199   yet recent studies have revealed the positive roles of TGF-β1 in inflammatory

200   response(Yoshimura et al. 2010). Here we found that TGF-β1 had an increased expression in

201   both LPS- and LTA-induced inflammatory MAC-T cells (Figure.3A). TGF-β1 could induce

202   EMT phenotypes in epithelial cells in vitro and has been associated with EMT in vivo

203   (Moustakas & Heldin 2016; Risolino et al. 2014). To investigate whether if increased TGF-β1

204   in epithelial cells under inflammatory condition caused mammary fibrosis via EMT, we

205   incubated MAC-T cells with exogenous TGF-β1 to examine the occurrence of EMT. The

206   western blot results showed that the expressions of α-SMA and N-cadherin, two well-known

207   EMT markers, were significantly upregulated in TGF-β1-treated MAC-T cells compared to

208   untreated cells, while E-cadherin expression was down-regulated in MAC-T cells after

209   treatment with TGF-β1 compared to untreated cells (Figure.3B), suggesting TGF-β1 could

210   induce EMT in epithelial cells of MAC-T. In addition, TGF-β1 treatment enhanced the protein

211   expression levels of several extracellular matrix (ECM) proteins, including albumin, collagen

212   1, and MMP9, in MAC-T cells (Figure.3B). Interestingly, we found that TGF-β1 treatment

213   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

215   To investigate whether if H19 mediated TGF-β1-induced EMT in MAC-T cells, we

216   established H19 stably overexpressed MAC-T cell clones by introducing of the lentiviral

217   vector of CD513B-1 containing the H19 cDNA (Figure.4A). High-level GFP-expression was

218   detected by direct viewing with a fluorescent microscope in MAC-T cells transfected by both

219   CD513B-1 and CD513B-1-H19 vectors after selection with puromycin (Figure.4B). The

220   overexpression of H19 in MAC-T cells was further confirmed by RT-qPCR (Figure. 4C).

221   Western blot results showed that overexpression of H19 significantly promoted

222   TGF-β1-induced EMT in MAC-T cells compared to CD513B-1 control vector-transfected

223   cells, as indicated by increased α-SMA and N-cadherin expression and decrease E-cadherin

224   expression (Figure. 4D). In addition, overexpression of H19 also promoted TGF-β1-induced

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

226

227   **TGF-β1-stimulated H19 requires the AKT activation**

228   A previously publication linked H19 expression to PI3K/AKT pathway in response to

229   TGF-β1 treatment(Matouk et al. 2014). Here, we found that TGF-β1 treatment could activate

230   AKT protein in MAC-T cells, and this activation was effectively inhibited by LY 294002, a

231   specific chemical inhibitor of the PI3K/AKT pathway (Figure. 5A). Furthermore, the

232   inhibition of activation of PI3K/AKT pathway by LY294002 was able to abolished

233   TGF-β1-induced up-regulation of H19 in MAC-T cells (Figure. 5B). These data suggest that

234   TGF-β1-induced EMT in MAC-T cells is mediated by the TGF-β1/AKT/H19 axis.

235

236   **Identification of H19 promoter region**

237   To identify the promoter region of H19 gene responded to TGF-β1 treatment, luciferase

238   reporter plasmids were constructed by inserting each truncation of a 4679 bp upstream

239   segment of H19. All constructs were confirmed by DNA sequencing. The luciferase activities

240   were tested by luciferase assay in MAC-T cell line. We did not find the H19 promoter region

241   within the upstream 4976bp sequences of H19responsible for TGF-β1-induced EMT in

242   MAC-T cells. As showed in Figure. 6, transcriptional activity was not different among all

243   examined segments.

244

245   **DISCUSSION**

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

247   carcinogenesis and embryogenesis, and induction of EMT by different approaches (e.g.

248   hypoxia, TGFβ) in cancer cells is accompanied by H19 upregulation(Oviedo-Boyso et al.

249   2007). EMT has been taken as the potential sources of myofibroblasts in tissue fibrosis(Zou et

250   al. 2017), and bovine mastitic usually can cause varying degrees of fibrosis process of

251   mammary glands. We speculated that H19 upregulation might be involved in the mammary

252   fibrosis induced by mastitic.

253   Here we found that exposure of MAC-T cells to TGF-β1 stimulation could cause them

254   undergo EMT mediated by H19 upregulation, representing another source of fibroblasts

255   involved in mammary gland fibrosis of bovine.

256   We first observed that the expression level of H19 was upregulated in both mastitic

257   mammary tissue compared to normal tissue, indicating that H19 was involved in the immune

258   response of mammary glands during the process of bovine mastitic. During bacterial invasion,

259   mammary epithelial cells play an important role in inducing a relevant innate immune

260   response in mammary glands by immunological factor release(Zhang et al. 2016). H19

261   upregulation was also observed in inflammatory MAC-T cells induced by the gramnegative

262   and gram-positive bacterial cell wall components LPS and LTA, suggesting H19 is involved

263   in the immune response process of epithelial cells triggered by pathogens.

264   Following inflammation, some types of cells produce TGF-β1, and excessive TGF-β1

265   contributes to a pathologic excess of tissue fibrosis(Branton & Kopp 1999). EMT is

266   recognized as playing an important role in repair and scar formation following epithelial

267   injury. The evidence that TGF-β1 induces EMT in alveolar epithelial cells in vitro and in vivo

268   suggests that alveolar epithelial cells may serve as a source of myofibroblasts in lung

269 fibrosis(Willis & Borok 2007). In this study, we found TGF-β1 had an upregulated expression

270 in MAC-T cells under LPS or LTA stimulation. To investigate whether the excessive TGF-β1

271 has an effect on MAC-T cells through an autocrine manner, thus leads to an EMT process, we

272 treated MAC-T cells with exogenous TGF-β1. TGFβ1-induced protein expression changes of

273 several well-known EMT makers, such as downregulation of E-cadherin and upregulation of

274 α-SMA and N-cadherin, were observed in MAC-T cells, indicating TGFβ1-induced EMT

275 occurred. Myofibroblasts release a variety of excessive extracellular matrix proteins

276 contributing to organ fibrosis(Hinz et al. 2007). We found that TGFβ1 stimulates increased

277 expression of extracellular matrix proteins including Collagen type 1, MMP9, and albumin in

278 MAC-T cells.

279   An interesting finding is that TGFβ1 stimulates the expression of H19 in MAC-T cells.

280 We next established an epithelial cell line with stable overexpressed H19 level to assess the

281 relation between H19 and TGF-β1-induced EMT in MAC-T cells. We found that H19

282 overexpression could enhance the TGF-β1-induced EMT in MAC-T cells, indicating a high

283 level of H19 expression was associated with EMT, which is consistent with the previous

284 observation that TGF-β1 is associated with EMT and inflammation (Franco et al. 2010; Gal et

285 al. 2008; Salgado et al. 2017).

286   Accumulating studies have evidenced that AKT was an important regulator of EMT in

287 the majority of cell types (Larue & Bellacosa 2005; Lee & Han 2010; Maseki et al. 2012). In

288 our study, we found TGFβ1 activated PI3K/AKT signal pathway in MAC-T cells, and the

289 specific inhibitor of the PI3K/AKT pathway was able to inhibit the activation of AKT. What's

290 more, this inhibitor also abated TGF-β1-induced upregulation of H19 in MAC-T cells,

291 suggesting that H19 expression increase in MAC-T cells was induced by TGF-β1 through

292 PI3K/AKT pathway. This finding is consistent with the results from a previously publication

293 that linked H19 expression to PI3K/AKT pathway in response to TGF-β1 treatment(Matouk

294 et al. 2014). Finally, we tried to identify the promoter region responsive to TGF-β1 treatment

295 by using luciferase reporter assay to detect the upstream 4.679kb sequence of H19 gene, but

296 no promoter responsive to TGF-β1 was found within the examined sequence regions. Further

297 researches should be done in the future to demonstrate the mechanism in which TGF-β1

298 regulate the expression of H19 in MAC-T cells.

299 **CONCLUSIONS**

300 In summary, we show evidence that H19 had increased expressions in both bovine

301 mastitic tissue and inflammatory bovine epithelial cells, and lncRNA H19 mediates

302 TGF-β1-induced EMT and ECM protein synthesis in bovine epithelial cells through

303 PI3K/AKT Signaling Pathway. Additional studies are clearly needed to both demonstrate the

304 molecular mechanism of H19 responsive to TGF-β1 and address whether EMT occurs in vivo

305 during bovine mastitis. Based on our results we suggest that mammary epithelial cells might

306 be one source for myofibroblastsin vivo in the mammary glands under an inflammatory

307 condition, thereby contributing to mammary gland fibrosis.

311 **ADDITIONAL INFORMATION AND DECLARATIONS**

313 The study was supported by grants from National Natural Science Foundation of China (No.

314 31402165).

Competing Interests

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316 No competing interests declared

317 **Author Contributions**

318 All authors contributed significantly to this research and preparation of the manuscript. Y.Z.

319 and G.MQ conceived and designed the experiments. W.Y. L.XZ Q.SP L.XU K.Z and Q.SZ

320 performed the experiments and analyzed the data. W.Y. and G.MQ wrote the manuscript. All

321 authors have been involved in the drafting, critical revision and final approval of the

322 manuscript for publication.

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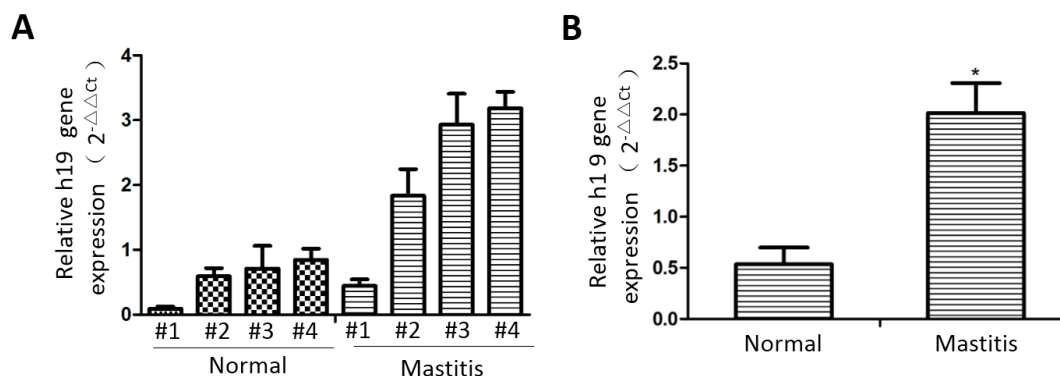
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433   **FIGURE LEGENDS**

434

435   **Figure. 1 The expression of H19 in normal and mastitic tissue of bovine mammary**

436   **glands.** (**A**) The expression of H19 in normal (n=4) and mastitic (n=4) tissue of mammary

437   glands assessed by RT-qPCR. GAPDH was used as an internal control. (**B**) Statistic analysis

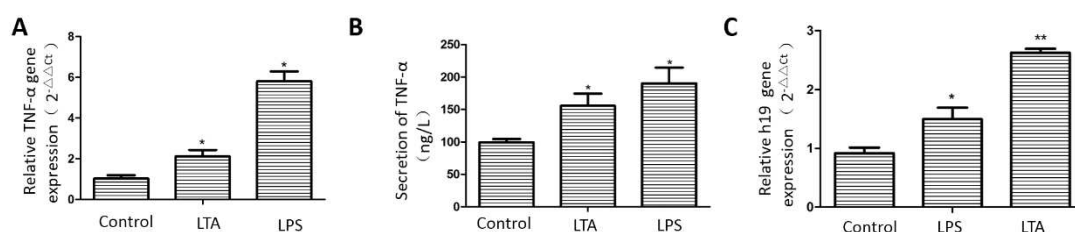438   of the expression of H19 between normal and mastitis group. *p<0.01 vs Normal.

439

440   **Figure. 2 The expression of H19 in MAC-T cells with or without LPS or LTA treatment.**

441   (**A** and **B**) An in vitro inflammatory epithelial cell model induced by LPS or LTA stimulation

442   was established. TNF-α was used as an indicator of MAC-T cell inflammatory responses to

443   stimulus. TNF-α mRNA expression in treated cells was analyzed by RT-qPCR (A), and

444   TNF-α protein secretion in the medium was measured with an ELISA kit (B). (C) The

445   expression of H19 in MAC-T cells analyzed by RT-qPCR. Untreated MAC-T cells were used

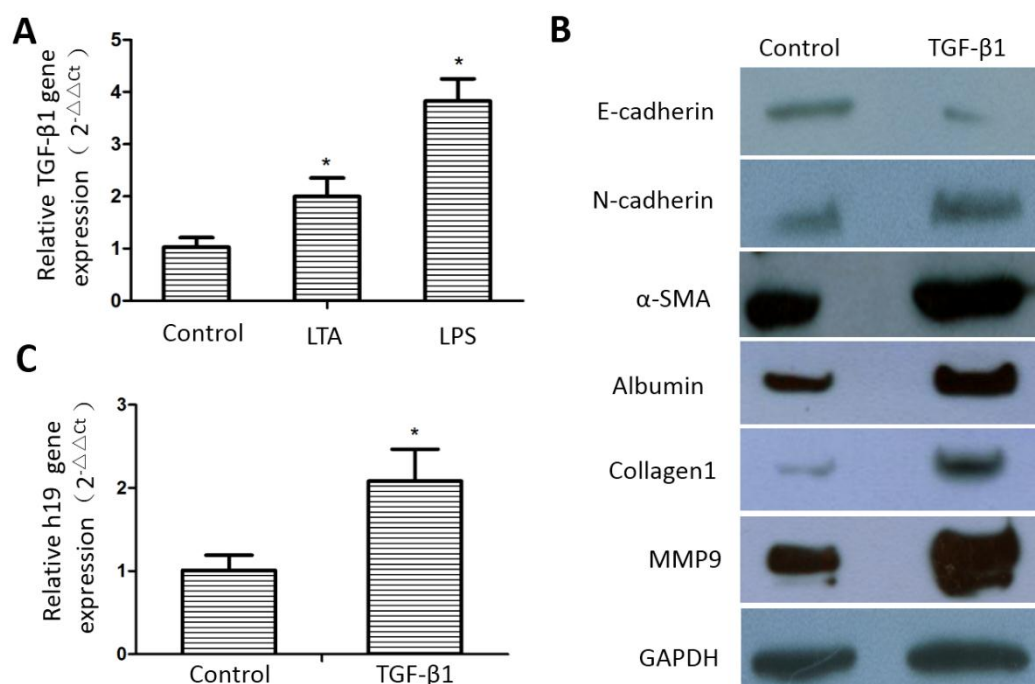446   as control. GAPDH was used as internal control. *p<0.05, **p<0.01 vs Control.

447

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.

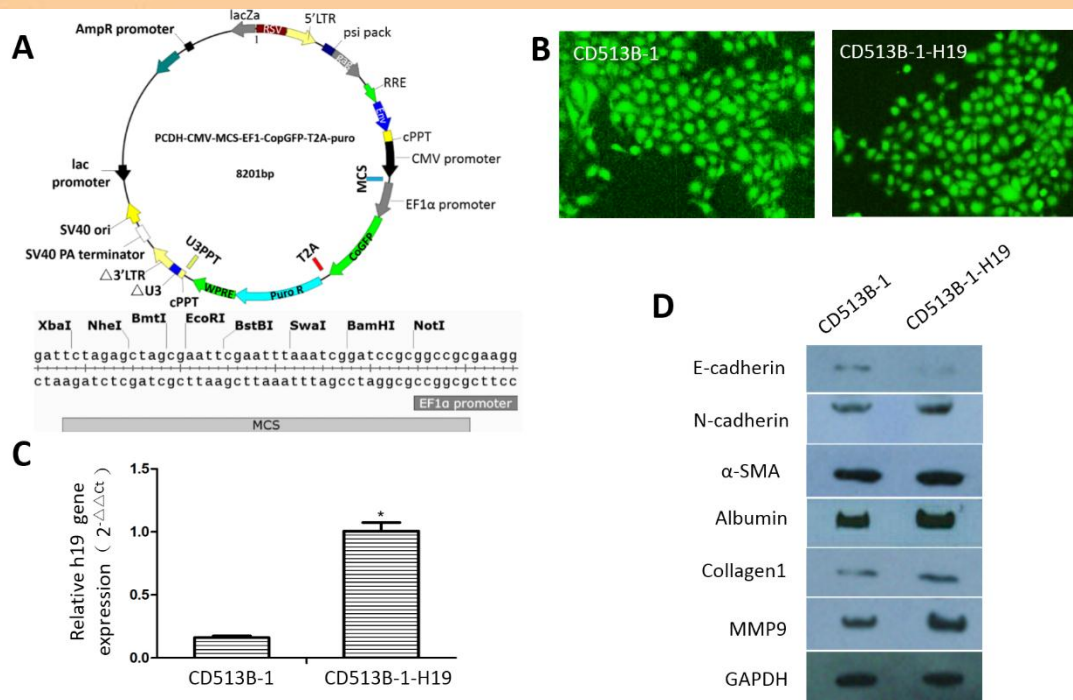GAPDH was used as an internal control. *p<0.01 vs control.

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.
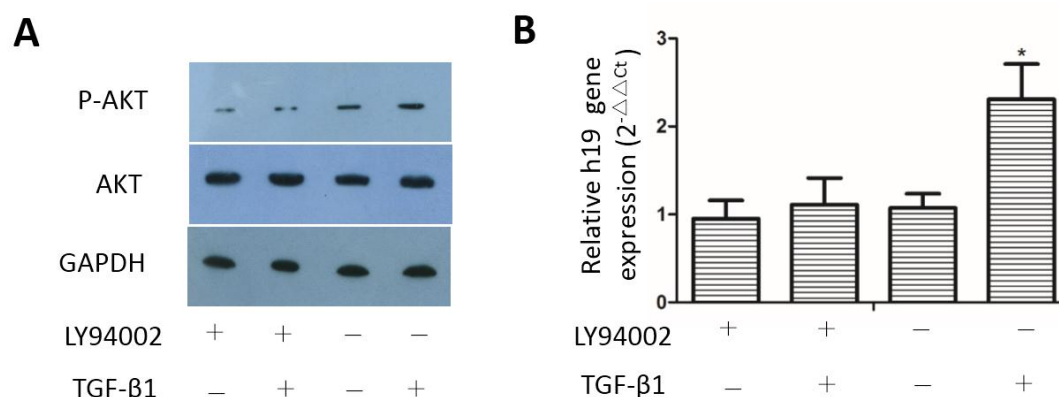
A

P-AKT

AKT

GAPDH

| LY94002 | + | + | − | − |
| TGF-β1 | − | + | − | + |

B

467

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.
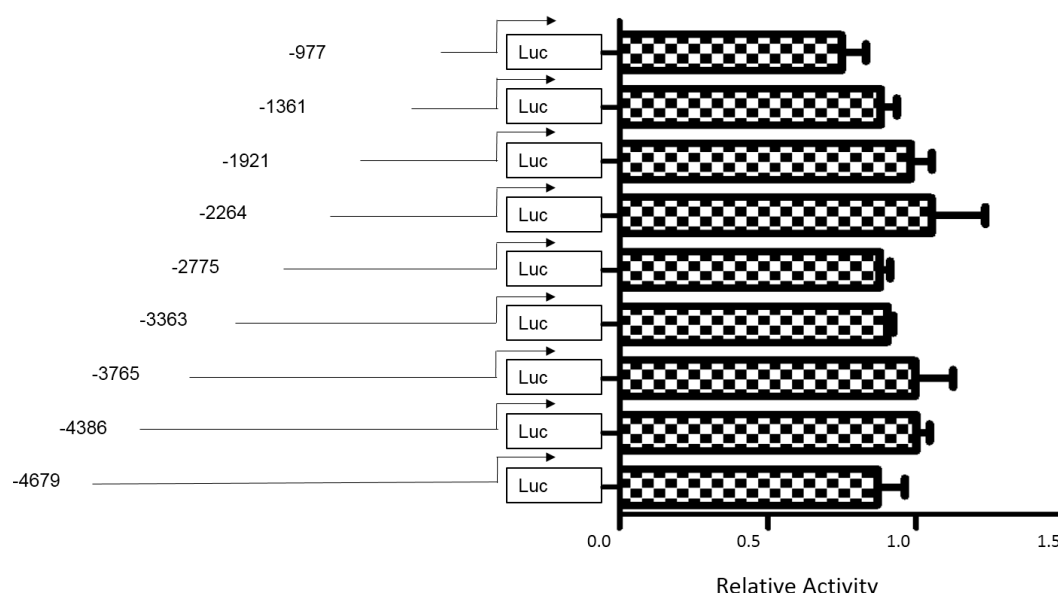

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Figure. 6 Functional analysis of different lengths of putative promoter sequences of H19 in MAC-T cells. The different 5' truncated fragments of upstream of H19 gene sequences

478   were generated and inserted into the Luc-vector. The length of fragments was indicated

479   relative to the transcription start site, and the activity of the fragments constructs relative to a

480   promoter-less construct was given. p>0.05 among all groups.