Muc5ac Production Inhibited by Decreased IncRNA H19 via PI3K/Akt/NF-κB in Asthma

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Introduction: LncRNAs play important roles in multiple diseases including asthma, while there are a few reports on the role of IncRNA H19 about asthma. This study aimed to investigate the roles and mechanisms of IncRNA H19 in asthma.

Methods: We detected IncRNA H19 and Muc5ac mRNA by establishing a murine asthma model and an in vitro inflammation model. Regulatory roles of IncRNA H19 in asthma were explored by IncRNA H19 overexpression or knockdown in vitro. To study its mechanisms, we detect p-NF-κB and p-Akt expression, and treated 16-HBE cells with inhibitors of PI3K.

Results: Firstly, we established a murine asthma model and an in vitro inflammation model. We found that IncRNA H19 expression was decreased, while Muc5ac mRNA was increased in lung tissues of murine asthma model and in the in vitro inflammation model. IncRNA H19 overexpression increased Muc5ac mRNA expression and IncRNA H19 knockdown decreased Muc5ac mRNA expression in 16-HBE cells. Moreover, IncRNA H19 overexpression further increased Muc5ac expression in TNFα-induced in vitro inflammation model. IncRNA H19 knockdown decreased p-Akt and p-NF-κB expression. Inhibitors of PI3K abolished Muc5ac induced by IncRNA H19 overexpression. Although miR-675-3p was increased by IncRNA H19 overexpression, it had no regulatory effects on Muc5ac expression.

Discussion: These results demonstrated that IncRNA H19 positively regulates Muc5ac expression through PI3K/Akt/NF-κB pathway in the in vitro inflammation model. Therefore, this study indicated that decreased IncRNA H19 in asthma might play a protective role relieving mucus overproduction, and IncRNA H19 might be a potential target for asthma treatment.

Keywords: asthma, IncRNA H19, Muc5ac, NF-κB, Akt

Background

Asthma is a chronic allergic disease affecting almost more than 300 million people around the world, which impairs life qualities of asthmatics and even threatens their lives.1 Typical asthmatic symptoms, including wheezing, dyspnea, chest tightness, and cough, are caused by airflow obstruction which is thought to result from inflammation-triggered AHR and mucus overproduction.2 Therefore, elucidating the regulatory mechanism of mucus overproduction might provide new insights into asthma treatment.

The glycoprotein components of airway mucus is primarily composed of secreted polymeric mucins, Muc5ac and Muc5b. In the airways, Muc5ac is specifically expressed in goblet cells, whereas Muc5b is expressed in submucosal...
miR-675-3p and functions in numerous diseases. Via diverse mechanisms, LncRNA H19 exon 1 encodes mechanisms of Muc5ac production are unclear. In asthma and regulate the production of Muc5ac. In this study, we therefore established a murine asthma model and an in vitro inflammation model to explore the roles and mechanisms of lncRNA H19 on Muc5ac expression in asthma. We found that lncRNA H19 expression was decreased, while Muc5ac mRNA was increased in lung tissues of murine asthma model and in the in vitro inflammation model. Moreover, we found that lncRNA H19 positively regulated Muc5ac mRNA expression through PI3K/Akt/NF-κB pathway in the in vitro inflammation model.

Materials and Methods
Mice
BALB/c female mice aged 6–8 weeks under specific pathogen-free conditions were purchased from Chongqing Medical University, where the animal experiments took place. The Institutional Animal Care and Use Committee of Chongqing Medical University approved all animal experiments. The approval number is SYXK (Chongqing) 2012–0001. Animals were treated in accordance with Guide for the Care and Use of Laboratory Animals (8th edition, National Academies Press).

Establishment of Asthma Model
BALB/c female mice were divided randomly into two groups, 6–8 mice per group. Mice in asthmatic group were intraperitoneally sensitized with 100 μg OVA (Sigma-Aldrich, St. Louis, MO, USA) suspended to 1 mg of aluminum hydroxide (Thermo Scientific, Waltham, MA, USA) in 200 μL of sterile phosphate-buffered saline (PBS). After sensitization on days 1 and 8, mice were challenged by aerosolization with 5% OVA for 30 minutes each day from day 14 to day 21. The control group was sensitized and challenged with PBS in the same way. On day 22, mice were anesthetized with 1.5% sodium pentobarbital by intraperitoneal injection and sacrificed for analysis. The experiments were performed for three times.

Analysis of Bronchoalveolar Lavage Fluid (BALF)
Tracheal intubation was performed using an indwelling needle after mice were anesthetized. BALF was obtained by lavaging 5 times with 1 mL of PBS. The cell free supernatant was used for detection of cytokines by ELISA kits after centrifugation at 800 g for 5 min at 4°C. After resuspension in 1 mL of PBS, the total number of inflammatory cells were counted by modified Neubauer
Counter under a microscope. After cytopsin and Wright’s staining, neutrophils, eosinophils, lymphocytes, and macrophages were differentially counted under a microscope.\textsuperscript{27}

**Cell Culture**

Human bronchial epithelial cell line 16-HBE was purchased from American Type Culture Collection (ATCC) and cultured in 1640 medium (Hyclone) supplemented with 10% of fetal bovine serum (FBS) and 1% of penicillin and streptomycin at 37 °C with 5% of CO\(_2\). After confluence, the cells were plated in 6-well plates at a density of 2.5\(\times\)10\(^5\) per well for siRNAs transfection, or 5\(\times\)10\(^5\) per well for plasmids transfection. Almost after 24 hours, the cells were transfected with siRNAs (Tsingke, China) targeting IncRNA H19 (si-NC as controls) or phblv-H19 (GenScript, Nanjing) plasmids (phblv-vc as controls) using lipofectamine (lipo) 2000 (Invitrogen, USA). The sequences of siRNAs (from 5’ to 3’), miR-675-3p mimics and inhibitors are listed in Table 1. After overexpression of IncRNA H19 for 24 hours, 16-HBE cells were pre-treated with inhibitors of PI3K or DMSO for 40 minutes and then treated with TNF\(\alpha\) for 24 hours.

**Reverse Transcription-Quantitative Polymerase Chain Reaction**

Total RNA from cells and murine lung tissues were harvested and prepared using isoplus (Invitrogen) according to the manufacturer’s instructions. Then the cDNA products were produced using a PrimeScript\textsuperscript{TM}RT reagent Kit (Takara, Shiga, Japan) according to the manufacturer’s protocol. Q-PCR Primers (Sangon, Shanghai, China) specific for IncRNA H19, Muc5ac and GAPDH were listed in Table 2. All RNA expressions were normalized to GAPDH gene expression. Q-PCR Primers (Sangon, Shanghai, China) specific for miR-675-3p mimics and inhibitors were listed in Table 2. All microRNA expressions were normalized to U6 gene expression.

**Western Blotting**

The cells were lysed by RIPA lysis buffer (Beyotime, China) supplemented with 1% of PMSF. Subsequently, the total protein content was measured using a BCA protein assay kit (Beyotime Inc., China). After separation of total protein by 10% of SDS-PAGE, protein and prestained protein ladder (Thermo Scientific, Canada) were electro-transferred onto PVDF membranes (Millipore, United States). Then the PVDF membranes were blocked with 5% of nonfat-dried milk for 2 hours at 37°C and incubated respectively with primary antibodies to p-NF-κB, p-Akt and GAPDH (CST, United States) at 4°C overnight. After washing for 15 minutes per time and for 3 times, the membranes were incubated with HRP-conjugated secondary antibodies for 1 hour at 37°C. Finally the membranes were visualized using Image Lab and analyzed using Image J.

**ELISA**

IFN-\(\gamma\), IL-5, IgE levels were detected by commercial ELISA kits (Biolegend, San Diego, CA, USA) according to the manufacturer’s instructions.

**Histopathological Analysis**

After fixing in 4% of paraformaldehyde, the lung tissues were embedded in paraffin and finally made into sections (5\(\mu\)m). The sections were stained with Hematoxylin and eosin (HE) to evaluate lung inflammation, or Periodic

### Table 1 siRNAs, miRNA-675-3p Mimics and Inhibitors Used in This Study

| Sequences (5’-3’) | siRNA3-F | siRNA3-R | siRNA1-F | siRNA1-R | siRNA415-F | siRNA415-R | siRNA940-F | siRNA940-R | siRNA NC-F | siRNA NC-R | miRNA-675-3p mimics sense | miRNA-675-3p mimics antisense | mimics control | miRNA-675-3p inhibitors | Inhibitors control |
|-------------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|-----------------|-----------------|---------------|------------------------|----------------|
|                   | CCAACAUCAAAGACACCAUATT | AUGCUGUCUUGAUGUUGGGC | CUCUUUGUUUCUGAGCUUUCTT | GAAAGCUCAAGAAACAGAGAC | GCGGGUCUGUUUCUUUACUUTT | AGUAAAGAAACAGACCGGCUU | CAUUUGCAUCGGUGAGUGUUGTT | CAACUCCAAACUGUGCAAAAGAC | UUCUCCGAACGUGUCAGGUUTT | AGUGACACGGUGGGAAGAATT | CUGUAUGCCUCACCGCUCA | AGCGGUGAGGGCAUACAGUU | UGUACUACACAAAAUGACUG | UGACGGUGAGGGCAUACAG | CAGUACUUUUGUGAGUACAA |

Abbreviation: siRNA, small interfering RNA.
Acid-Schiff (PAS) to examine mucus secretion in airways under the light microscope.

**Statistical Analysis**

The data was shown as mean ± SD from repeated experiments for at least three times. Statistical evaluation was performed using Student’s t-test or Wilcoxon-Mann–Whitney test. P values less than 0.05 were considered statistically significant.

**Results**

**Murine Asthma Model Was Successfully Established**

To study the role of lncRNA H19 in asthma, murine asthma model was established (Figure 1A). The total number of nucleated cells in BALF was significantly higher in asthmatic group than control group, as were the number of eosinophils, neutrophils, lymphocytes and monocytes, respectively (Figure 1B). IgE and Type 2 cytokine IL-5 in BALF were increased in asthmatic group compared with control group, while Type 1 cytokine IFN-γ was decreased (Figure 1C). Moreover, HE staining showed increased inflammation in asthmatic group than in control group. PAS staining showed that mucus secretion was much more in asthmatic group than in control group (Figure 1D). These results suggested successful establishment of murine asthma model.

**LncRNA H19 Was Decreased, While Muc5ac Was Increased in Murine Asthma Model and in 16-HBE Cells Treated with TNFα**

It was reported that increased mucins in airways of asthmatic were primarily composed of Muc5ac, not Muc5b, and that Muc5ac played a pathologic role.3 We found that Muc5ac was significantly increased in lung tissues of

| Primers          | Sequences (5′-3′)                      |
|------------------|----------------------------------------|
| homo-H19-F       | GACATGACATGGTCCGGTGT                   |
| homo-H19-R       | ATGTTGGTGTTTCTGGGAGC                   |
| homo-Muc5ac-F    | CCCAAACCTGCTTCGCAAC                    |
| homo-Muc5ac-R    | TCATCACACAGCTTGGC                    |
| homo-GAPDH-F     | GCAGTGAGCCGAGTACGTG                   |
| homo-GAPDH-R     | TTGAGACACCGATCAGCT                    |
| mmu-H19-F        | AATGACTCAATCGGTGCCTTC                |
| mmu-H19-R        | CAGGGATACGTGCTTCACG                   |
| mmu-Muc5ac-F     | CCCGTGAGGCCGACAG                      |
| mmu-Muc5ac-R     | ATGTTGCGTGCTGGATCTGA                 |
| mmu-GAPDH-F      | TGAAAGTCGCCAGGACACCT                 |
| mmu-GAPDH-R      | CGCCTGATGATGCCCTAC                   |
| miR-675-3p-F     | AGTGCAGGGAGTCCAG                   |
| miR-675-3p-R     | GTCGTATCGAATGCGGGTTTCACG             |
| miR-675-3p-RT    | U6-F                                  |
|                  | CTCGCTTGCAGCACA                      |
|                  | U6-R                                  |
|                  | ACGCCTACGAATTTGCGT                   |

Table 2 q-PCR Primers Used in This Study
asthmatic mice compared with control group, and Muc5ac was much higher than Muc5b (Figure 2A). We hypothesized that lncRNA H19 might be involved in asthma and regulate expression of Muc5ac. Firstly, we detected lncRNA H19 expression in murine asthma model. The results showed that lncRNA H19 in lung tissues was significantly decreased in asthma group compared with control group (Figure 2B). TNFα is an important inflammatory mediator which contributes to Muc5ac overproduction in asthma. Therefore, it was used to treat 16-HBE cells to construct in vitro inflammation model of asthma. Subsequently, we detected expression of lncRNA H19 and Muc5ac in the in vitro inflammation model. Consistent with in vivo study, obviously increased Muc5ac mRNA expression (Figure 2C) and decreased lncRNA H19 expression were detected (Figure 2D) when 16-HBE cells were stimulated with TNFα. These results indicated that lncRNA H19 expression was negatively correlated with Muc5ac expression both in in vivo and in vitro model, and lncRNA H19 might be involved in the regulation of Muc5ac in asthma.

**LncRNA H19 Positively Regulated Muc5ac Expression**

Since lncRNA H19 was decreased in asthma, we deduced that lncRNA H19 plays an important role in asthma. In order to investigate the role of lncRNA H19 in pathogenesis of asthma, plasmid vectors overexpressing lncRNA H19 were constructed and siRNAs targeting lncRNA H19 were synthesized. Plasmids phblv-vc and phblv-H19 were

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*Figure 1* Successful establishment of murine asthma model. (A) Protocol of murine asthma model by using OVA. (B) The number of leukocytes, neutrophils, eosinophils, lymphocytes and monocytes in BALF of murine asthma group and control group. (C) The levels of IgE, IFNγ and IL-5 in BALF were determined by Elisa analysis. (D) HE (haematoxylin and eosin) and PAS (periodic acid-Schiff stain) analysis of lung tissues in murine asthma group and control group, and histopathology score (PA) and periodic acid-Schiff stain score (PAS). The study was repeated for three times. **P<0.01; ***P<0.001, ****P<0.0001.
successfully transfected into 16-HBE cells (Figure 3A), and lncRNA H19 was obviously increased in phblv-H19 treated group compared with phblv-vc treated group (Figure 3B). siRNA-415 was the most effective in knocking down lncRNA H19 expression among the four siRNAs (Figure 3C), which was used in subsequent experiments as si-H19. Muc5ac mRNA expression was increased in phblv-H19 group compared with phblv-vc group, whereas it was decreased in si-H19 group compared with si-NC group (Figure 3D). These results suggested that lncRNA H19 positively regulated Muc5ac mRNA expression.

TNFα treatment of 16-HBE transfected with plasmids phblv-vc or siRNAs si-NC showed a decrease in lncRNA H19 (Figure 3E and G) and an increase in Muc5ac expression (Figure 3F and H), which is consistent with the in vivo and in vitro results described earlier. lncRNA H19 was significantly increased in plasmids phblv-H19 transfected TNFα treatment group compared with plasmids phblv-vc transfected TNFα treatment group (Figure 3E), and Muc5ac expression was significantly higher in the former group than the latter group (Figure 3F). Similarly, lncRNA H19 was significantly decreased in si-H19 transfected TNFα treatment group compared with si-NC transfected TNFα treatment group (Figure 3G), and Muc5ac expression was significantly lower in the former group than the latter group (Figure 3H). These results further demonstrated that lncRNA H19 positively regulated TNFα-induced Muc5ac mRNA expression (Figure 3F and H). Therefore, we speculated that decreased lncRNA H19 expression in asthma might be a protective mechanism partly relieving mucin overproduction in asthma.

Figure 2 Decreased lncRNA H19 and increased Muc5ac in murine asthma model and 16-HBE cells treated with TNFα. q-PCR analysis of Muc5ac, Muc5b (A) and lncRNA H19 (B), in lung tissues of murine asthma group and control group. q-PCR analysis of LncRNA Muc5ac (C) and lncRNA H19 (D) in 16-HBE cells treated with TNFα for 24 hours. The study was repeated for three times. *P<0.05; **P<0.01; ***P<0.001.

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LncRNA H19 Regulated Muc5ac Expression via PI3K/Akt/NF-κB Pathway in 16-HBE Cells

NF-κB was widely demonstrated as a downstream transcription factor of PI3K/Akt, binding to promoter region of Muc5ac gene. To further explore mechanisms which mediated effects of lncRNA H19 on Muc5ac expression, lncRNA H19 was knocked down in 16-HBE cells by transfection with si-H19. p-Akt and p-p65 were reduced in knockdown group compared with control group (Figure 4A). TNFα treatment rapidly activated Akt and NF-κB in 16-HBE cells (Figure 4B). Moreover, PI3K inhibitor blocked effects of lncRNA H19 on Muc5ac expression, no matter with (Figure 4D) or without TNFα treatment (Figure 4C). These results indicated that lncRNA H19 regulated Muc5ac expression via PI3K/Akt/NF-κB pathway in vitro inflammation model.

LncRNA H19 Regulated miR-675-3p Production, Which Had No Effects on Muc5ac Expression

LncRNA H19 encodes miR-675-3p from its exon 1 (Figure 5A), and plays important roles in numerous diseases via regulation of mir-675 production. We found that lncRNA H19 overexpression definitely increased miR-675-3p production by transfection with plasmids phblv-H19 in 16-HBE cells (Figure 5B). To explore if miR-675-3p regulates Muc5ac expression, miR-675-3p was overexpressed or knocked down in 16-HBE cells by transfection with miR-675-3p mimics or inhibitors, respectively (Figure 5C). However, Muc5ac mRNA expression was not significantly changed (Figure 5D). These results indicated that lncRNA H19 regulated Muc5ac expression not through regulation of miR-675-3p production.

Discussion

Currently, reports on lncRNAs are increasing day by day. LncRNA H19 is a maternally expressed and conserved long noncoding RNA, widely expressed in many organs, and its roles have been largely reported in many diseases except asthma. In our study, we found that lncRNA H19 positively regulated Muc5ac mRNA expression via PI3K/Akt/NF-κB pathway in the in vitro inflammation model. Moreover, decreased lncRNA H19 levels were detected in the in vitro inflammation model and the murine asthma model. These results indicated that decreased lncRNA H19 in asthma might be a protective mechanism partly by reducing mucin overproduction in asthmatic airways.

TNFα is one of cardinal inflammatory factors in asthma, mediating persistent activation of NF-κB, which is proved to bind to the promoter region of Muc5ac gene. TNFα was elevated in asthmatic airways and induced EGFR expression in airway epithelium. Subsequently, EGFR-ligands increased Muc5ac production at both gene and protein levels. Furthermore, TNFα was reported to activate RhoA expression and thus leading to AHR. Anti-TNFα therapy were effective and could alleviate...
inflammatory cell infiltration, inflammation damages in airways and AHR. Because of crucial roles of TNFα in asthmatic airways and Muc5ac expression, we treated bronchial epithelial cells 16-HBE with TNFα and thereby induced Muc5ac production. In the in vitro inflammation model, Muc5ac was increased, while lncRNA H19 was decreased. Moreover, increased Muc5ac and decreased lncRNA H19 in lung tissues were also detected in murine asthmatic group compared with control group. Our results in the in vitro and in vivo models were consistent. These results were also consistent with asthmatics.

In our study, lncRNA H19 was demonstrated to positively regulate Muc5ac mRNA expression by transfection with plasmids pHBLV-vc and pHBLV-H19 in 16-HBE cells. lncRNA H19 overexpression increased TNFα-induced Muc5ac expression, while lncRNA H19 knockdown reduced TNFα-induced Muc5ac expression. Interestingly, lncRNA H19 was decreased while Muc5ac was increased both in murine asthma model and in the in vitro inflammation model. These results indicate the points as follows: 1) LncRNA H19 positively regulates Muc5ac expression, and overexpression of lncRNA H19 promotes the development of asthma. 2) Decreases of lncRNA H19 are probably a protective mechanism in murine asthma model. Otherwise the expression of Muc5ac was higher. 3) It deserves further study about the mechanisms of decreases of lncRNA H19 in asthma.

Positive regulation of Muc5ac by lncRNA H19 and their opposite changes in asthma model implied that Muc5ac mRNA expression might be increased by other mechanisms such as EGFR. As reported, TNFα was elevated in asthmatic airways and induced EGFR expression in airway epithelium. EGFR-ligands binding to EGFR increased Muc5ac production at both gene and protein levels. H19 was reported to activate EGFR. Consequently, lncRNA H19 probably promotes Muc5ac production through EGFR. And thus Muc5ac expression was partly inhibited by downregulation of lncRNA H19 through some mechanisms in murine asthma model.

LncRNAs functions in numerous diseases through various mechanisms, including regulation of transcription factors, microRNAs, regulatory proteins, chromatin-
modifying complexes and mRNA targets.\textsuperscript{39} To further explore the mechanism of Muc5ac regulation by lncRNA H19, lncRNA H19 knockdown were achieved by transfection with si-H19 in 16-HBE cells, resulting in decreased p-Akt, p-NF-κB and Muc5ac. NF-κB is demonstrated as a transcription factor for Muc5ac in numerous studies, containing a binding site at the promoter region of Muc5ac gene.\textsuperscript{28} Moreover, NF-κB was reported as a downstream transcription factor of PI3K/Akt.\textsuperscript{30–42} In the in vitro inflammation model, TNFα rapidly activated Akt and NF-κB in 16-HBE cells. In our study, transcriptional levels of Muc5ac gene were increased by lncRNA H19 overexpression when treated with or without TNFα. However, inhibitors of PI3K abolished the promotive effect of lncRNA H19 on Muc5ac expression through abrogation of phosphorylation of Akt and subsequent activation of NF-κB. Therefore, our study indicated that lncRNA H19 regulated Muc5ac expression through PI3K/AKT/NF-κB pathway.

\textbf{miR-675-3p is a microRNA encoded in exon 1 of lncRNA H19 and can be processed from it. lncRNA H19 was reported to regulate intestinal epithelial barrier function via miR-675.}\textsuperscript{28} Therefore, we wondered whether lncRNA H19 regulated Muc5ac expression through miR-675-3p in asthma. In our study, overexpression of lncRNA H19 increased production of miR-675-3p in 16-HBE cells.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{miR-675 processed from lncRNA H19 had no regulatory effects on Muc5ac mRNA expression in 16-HBE cells. (A) miRNA-675 encoded by exon 1 of lncRNA H19 gene. (B) q-PCR analysis of miR-675 in 16-HBE cells after overexpression of lncRNA H19 for 24 hours. After overexpression (left) and knockdown (right) of miR-675-3p in 16-HBE cells for 24 hours, miR-675-3p (C) and Muc5ac expressions (D) were assayed by q-PCR. The study was repeated for three times. ***P<0.01; ****P<0.001; **P<0.0001, ns, not significant.}
\end{figure}
However, miR-675-3p had no effects on Muc5ac expression.

To sum up, our study demonstrated that lncRNA H19 positively regulated Muc5ac expression through PI3K/AKT/NF-κB pathway. However, there were some limitations of our study as are follows: The effect of lncRNA H19 overexpression on Muc5ac expression in murine asthma model had not been studied. Furthermore, it needs to prove that whether H19 promotes Muc5ac production through EGFR.

Many asthmatics are suffering from airway obstruction and airflow limit. Currently, the common therapy is combination treatments of various bronchodilators and corticosteroids. However, the therapeutic effects are limited and temporary. Besides, corticosteroids have side effects such as osteoporosis and pneumonia. Thus, new treatments for asthma are urgently needed. Given that Muc5ac is a dominant component of gels formed in airways of asthmatics, it has become a major drug target for asthma treatment. Therefore, our study about the regulative role of lncRNA H19 in Muc5ac expression might provide a new insight into asthma treatment from the perspective of long noncoding RNA.

Data Sharing Statement
The data generated and analysed during this study are available from the corresponding author on reasonable request.

Author Contributions
All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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