PI3K/AKT/mTOR Signaling Pathway Regulates MMP9 Gene Activation via Transcription Factor NF-κB in Mammary Epithelial Cells of Dairy Cows

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Research

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Abstract

Background

Matrix metalloproteinase 9 (MMP9) plays a pivotal role in mammary ductal morphogenesis, angiogenesis, and glandular tissue architecture remodeling. However, the molecular mechanism of MMP9 expression in mammary epithelial cells of dairy cows remains unclear. The current study aimed to explore the underlying mechanism of MMP9 expression.

Results

In this study, to determine whether the PI3K/AKT/mTOR/NF-κB signaling pathway participates in the regulation of MMP9 expression, we treated mammary epithelial cells with specific pharmacological inhibitors of PI3K (LY294002), mTOR (Rapamycin), or NF-κB (Celastrol), respectively. Western blotting results indicated that LY294002, Rapamycin, and Celastrol markedly decreased MMP9 expression and P65 nuclear translocation. Furthermore, we found that NF-κB (P65) overexpression resulted in elevated expression of MMP9 protein and activation of MMP9 promoter. In addition, we observed that Celastrol markedly decreases P65-overexpression-induced MMP9 promoter activity. Moreover, the results of the promoter assay indicated that the core regulation sequence for MMP9 promoter activity may be located -80bp to -420bp downstream from the transcription start site.

Conclusions

These observations indicated that the PI3K/AKT/mTOR signaling pathway is involved in MMP9 expression by regulating MMP9 promoter activity via NF-κB in the mammary epithelial cells of dairy cows.

Introduction

Matrix metalloproteinases (MMPs) are a multigene family of zinc-dependent endopeptidases enzymes that degrade the extracellular matrix (ECM) [1, 2]. Until now, 26 different MMPs have been confirmed in humans. Based on substrate specificity and domain homologies, MMPs were divided into six groups: collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and other non-classified MMPs [3]. Typical MMP consists of several conserved domains including pre-domain, propeptide, catalytic domain, and hemopexin domain. Among these domains, the catalytic domain consists of 170 amino acids (AAs) and contains a conserved three histidine sequence which is required for zinc chelation and plays an important role in MMP activity [3].

Given their role in the degradation of the extracellular matrix, MMPs, and the plasminogen activator (PA) system may play also a critical role in the extensive remodeling that occurs in the bovine mammary gland during development, lactation, and involution [4]. The mammary gland undergoes many changes in structure and function, including cyclic expansions corresponding to the hormonal changes induced by the estrous/menstrual cycle, as well as the dramatic changes that occur during pregnancy, lactation, and involution during the lifetime of the female [5]. MMPs play a pivotal role in mammary ductal morphogenesis, angiogenesis, and glandular tissue architecture remodeling [6]. For example, remodeling of the mammary tissue is also linked with the destruction of the lobular-alveolar structure of the gland and the extracellular matrix during mammary involution [7]. At the same time, the metalloproteinase (MMP) system is activated and induces the proteolysis of extracellular matrix components [8].

Among MMPs, MMP9 has been shown to regulate many cellular processes, such as tissue repair, angiogenesis, apoptosis, cell migration, and wound healing [9]. In the mammary gland, MMP9 preferentially degrades ECM during angiogenesis and tissue remodeling. The previous study indicated that MMP9 activity is regulated at three levels: gene transcription and mRNA stability, enzymatic form activation, and inhibition by endogenous inhibitors such as tissue inhibitors of metalloproteinases (TIMPs) [10]. The MMP9 gene can be induced by a variety of oncogene products, cytokines, mitogens, and phorbol ester [11]. These stimulators can upregulate the expression of MMP9 by modulating the activation of transcription factors such as NF-κB and AP-1 through the PI3K/AKT and MAPK signaling pathways. It is well known that human MMP9 promoter contains cis-acting regulatory elements for transcription factors including an NF-κB site (located at -600bp), an SP-1 site (located at -558bp), and two AP-1 sites (located at -79bp and -533bp) [12].

Phosphatidylinositol-3-kinase is an intracellular protein with catalytic activity, which can regulate the phosphorylation of downstream Akt and constitutes the PI3K/Akt signaling transduction pathway [13]. The PI3K/AKT signaling pathway plays an important role in the regulation of cell survival, growth, proliferation, angiogenesis, and metabolism. In MDA-MB-231 cells, CoQ0 suppressed MMP-9 protein expression by inhibiting the PI3K/AKT/NF-κB pathway [14]. PA effectively suppressed human breast cancer MDA-MB-231 cell migration, invasion, and cell motility by abating the expression of NF-κB and AP-1 to reduce the expression of MMP-9 through ERK/PI3K/Akt/mTOR signaling pathway [15]. MMP9 secretion is mediated by 12-LOX in PC-3 cells via the activation of the PI3K/AKT/NF-κB signaling pathway [16].

However, the molecular mechanism governing MMP9 expression in dairy cow mammary epithelial cells has been poorly understood. In the current study, we aimed to investigate the mechanism by which PI3K/AKT/mTOR pathway is involved in NF-κB activation and MMP9 expression. We hypothesized that the PI3K/AKT/mTOR signaling pathway regulates MMP9 expression through NF-κB (P65) in mammary epithelial cells of dairy cows. To test our hypothesis, we evaluated the effects of LY294002, Rapamycin, and Celastrol on NF-κB (P65) nuclear translocation and MMP9 expression in mammary epithelial cells. We then investigated the crucial role of NF-κB (P65) in the regulation of the MMP9 promoter activity.

Materials And Methods
Cell culture

Mammary epithelial cells were isolated from mammary parenchymal tissues of lactating Holstein dairy cows as described previously [17]. All animal experimental procedures were approved by the Northeast Agricultural University Animal Care and Use Committee (Harbin, China). Mammary epithelial cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified 5% CO₂ incubator. Pure mammary epithelial cells were obtained after three passages and identified by immunofluorescence for Cytokeratin-18 and β-casein [18]. All experiments were conducted using cells within seven passages.

Western blotting

Mammary epithelial cells were seeded in 6-well plates. Then cells were pre-treated with LY294002 (PI3K inhibitor), Rapamycin (mTOR inhibitor), or Celastrol (NF-κB inhibitor) for 24 h, respectively. DMSO was used as a negative control. Whole-cell lysates were prepared from mammary epithelial cells using RIPA buffer. The total protein concentration was determined using a BCA protein assay kit (Beyotime Shanghai, Chain). Protein was separated by 10% SDS-PAGE and then transferred to nitrocellulose (NC) membranes. Immunoblot analysis was performed using antibodies against MMP9 (1:4000 dilution; Proteintech), P-mTOR (1:1000 dilution; Cell Signaling Technology, Shanghai China), mTOR (1:1000 dilution; Abcam, Shanghai China), P-AKT (1:1000 dilution; Cell Signaling Technology, Shanghai China), AKT (1:1000 dilution; Cell Signaling Technology, Shanghai China), P-P65 (1:1000 dilution; Bioss, Beijing, China), P65 (1:1000 dilution; Bioss, Beijing, China), Histone3 (1:1000 dilution; Bioss, Beijing, China), and β-actin (1:4000 dilution; Proteintech) overnight at 4°C. Horseradish peroxidase-conjugated IgG was used as a secondary antibody (1:10000 dilution; ZSGB-BIO, Beijing, China). The protein expression levels were detected using ECL chemiluminescence (Biosharp) and analyzed using Image J.

Preparation of nuclear extract

Mammary epithelial cells were seeded in 6-well plates. Cells were grown to 90% confluence and treated with LY294002, Rapamycin, or Celastrol, respectively. Cells were collected after 4 h of treatment. Cytoplasmic and nuclear protein was prepared using Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Shanghai, Chain). The nuclear extracts and cytoplasmic extracts were stored at −80°C.

Construction of dairy cow MMP9 promoters

A series of fragments from the 5′-flank of the corresponding MMP9 promoter were purchased from Sangon Biotech (Shanghai, Chain). All MMP9 promoters are ≥ 3,500 bp long (KpnI and BglII sites were included in DNA fragments so that these DNA fragments could be digested with KpnI and BglII, and then subcloned into the PGL3-Basic KpnI/BglII site. The MMP9 promoter sequence was confirmed by DNA sequencing, and the resultant reporter plasmids were named MMP9-640-Luc, MMP9-420-Luc, MMP9-380-Luc, and MMP9-80-Luc respectively.

Transient Transfection, and Dual-luciferase Reporter Assay

Mammary epithelial cells were seeded in 6-well plates. Cells were grown to 80% confluence and co-transfected with 2 µg of various MMP9 promoter-luciferase reporter constructs and NF-κB (P65) expression vector (purchased from Sangon Biotech) using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s protocol. After 48 h of treatment, luciferase activities were determined by the Dual-Luciferase Reporter Assay System (Promega, USA) according to the manufacturer’s protocol. Luciferase activity was normalized in each cell lysate to Renilla activity co-transfected as an internal control. In addition, when mammary epithelial cells were co-transfected with MMP9 promoter-luciferase reporter constructs and NF-κB(P65) expression vector for 6 h, cells were treated with Celastrol for 48 h, and 0.1% DMSO was used as a negative control. All transient transfections were repeated in three independent experiments.

Statistical analysis

All experiments were performed in triplicate and the results are expressed as means ± SEM. T-test was used to compare means between two groups. One-way ANOVA followed by Tukey post hoc test was used to compare means between three groups. The minimum significance level was set at a P-value of less than 0.05 for all analyses.

Results

LY294002 attenuates MMP9 expression and P65 nuclear translocation in mammary epithelial cells

To determine whether PI3K/AKT signaling pathway is involved in MMP9 protein expression, mammary epithelial cells were treated with LY294002 (a PI3K inhibitor) for 24 h. Compared with control, LY294002 markedly decreased the phosphorylation level of mTOR and AKT, and the expression level of MMP9 protein (P < 0.01; Fig. 1A, B). However, there was no noticeable change in the level of AKT and mTOR protein expression under the same treatment conditions. Subsequently, we investigated whether LY294002 inhibited the translocation of NF-κB (p65) into the nucleus. Mammary epithelial cells were treated with
LY294002 for 4 h and compared with the control group, LY294002 significantly reduced the level of P65 protein in the nucleus by Western blot analysis ($P < 0.01$; Fig. 1C, D), and markedly increased the expression of P65 protein in the cytosolic extracts ($P < 0.01$; Fig. 1C, D).

**Rapamycin decreased MMP9 expression and P65 nuclear translocation in mammary epithelial cells**

Mammary epithelial cells were treated with Rapamycin, an mTOR inhibitor, for 24 h. Compared with the control, treatment of mammary epithelial cells with Rapamycin tremendously decreased the expression of MMP9 and the p-mTOR protein level ($P < 0.01$; Fig. 2A, B). However, the expression level of mTOR protein was not significantly changed. To investigate whether Rapamycin can affect NF-κB(p65) nuclear translocation, mammary epithelial cells were treated with Rapamycin for 4 h. Western blot analysis showed that treatment with Rapamycin significantly increased the protein levels of P65 in the cytoplasm, and diminished nuclear P65 protein compared with the control ($P < 0.01$; Fig. 2C, D).

**Celastrol suppressed MMP9 expression and P65 nuclear translocation in mammary epithelial cells**

To test whether MMP9 protein expression can be regulated by the transcriptional factor NF-κB in the mammary epithelial cells of dairy cows, mammary epithelial cells were treated with Celastrol (an NF-κB inhibitor) for 24 h. We observed that Celastrol significantly decreased the expression of MMP9 compared with control ($P < 0.01$; Fig. 3A, B). Next, we found that Celastrol treatment caused a marked increase in the expression of P65 in the cytosol, whereas the expression of P65 in the nucleus was significantly decreased compared with the control group ($P < 0.01$; Fig. 3C, D).

**Transcription factor NF-κB(P65) overexpression enhanced MMP9 expression and MMP9 gene promoter activity in mammary epithelial cells**

To determine whether MMP9 expression was directly regulated by NF-κB, mammary epithelial cells were transfected with NF-κB (P65) expression vector. After transfection for 48h, we observed a significant increase in P65, MMP9, and p-P65 expression in the P65 overexpression group compared with the empty vector group ($P < 0.05$; Fig. 4A, B). Then, the effect of transcription factor NF-κB(P65) on the transcriptional regulation of MMP9 was examined using an MMP9 promoter-luciferase construct, which contains 640bp, 420 bp, 380bp, and 80bp of proximal MMP9 promoter sequence upstream of a luciferase reporter. Mammary epithelial cells were transiently co-transfected with a reporter plasmid containing MMP9 promoter sequence and NF-κB(P65) expression vector for 48h. Our results demonstrated that the luciferase reporter gene activity of plasmid linked to the 640bp, 420bp, and 380bp fragment of MMP9 promoter is significantly increased in the P65 overexpression treated group compared with the untreated group ($P < 0.01$; Fig. 4C). Under the same conditions, there was no noticeable change in the M80-Luc group. Moreover, the luciferase activity of the M420-Luc group was highest in cells treated with P65 overexpression ($P < 0.01$; Fig. 4C).

Next, to further determine the inhibitory effect of Celastrol on P65 overexpression-induced MMP9 gene transcription factor activity, we performed a promoter assay using transiently co-transfected mammary epithelial cells with a luciferase reporter gene linked to a 420bp fragment from the MMP9 promoter region and an NF-κB(P65) expression vector. After transfection for 6h, cells were treated with Celastrol, and DMSO was used as a negative control. Compared with the control group, treatment with Celastrol resulted in a significant decrease in the promoter activity of the MMP9 gene ($P < 0.01$; Fig. 4D).

**Discussion**

The mammary gland is a unique glandular organ, which distinguishes mammals from all other animals and functions to produce and secrete milk to nourish offspring. The mammary gland undergoes many changes in structure and function during pregnancy, lactation, and involution. Because of the significant amount of ECM remodeling that must take place during these different stages, MMPs would be expected to play a pivotal role in mammary morphogenesis. Previous studies have shown that PI3K/AKT signaling pathway is involved in regulating MMP9 expression [19, 20]. In the current study, we demonstrated that LY294002, a PI3K/AKT pharmacological inhibitor reported to be the first synthetic reversible PI3K inhibitor and can inhibit the catalytic activity of PI3K [21], reduced the phosphorylation of AKT and the expression of MMP9 protein. In addition, LY294002 significantly suppressed the nuclear translocation of NF-κB (P65). Similarly, LY294002 significantly decreased MMP9 protein level and AKT phosphorylation in human HCC cells [19], and NF-κB activation in MDA-MB-231 human breast cancer cells[14]. Previous studies demonstrated that the PI3K/AKT pathway has a close connection with the NF-κB signaling pathway, in which the phosphorylation of Akt can activate NF-κB [22]. Inhibition of the PI3K/AKT pathway can block phosphorylation of AKT and subsequently suppress the activation of NF-κB [23]. Based our findings, we confirmed that the expression of MMP9 was regulated by PI3K/AKT signaling pathway.

The mTOR pathway is involved in many intracellular processes, such as translation and protein synthesis through its substrates [24], cell growth, and apoptosis. Moreover, PI3K and/or AKT can regulate mTOR activation [25]. In this study, we demonstrated that Rapamycin, a classic mTOR inhibitor that blocks mTOR activity through a mechanism involving an interaction with FKBP12 and a subsequent interaction with TORC1 to inhibit mTOR kinase activity [26], and suppressed expression of MMP9 in mammary epithelial cells. This agrees with the results of Park, who showed that MDA-MB-231 cells pretreated with mTOR siRNA or Rapamycin strongly inhibited MMP9 expression [15]. A more recent study demonstrated that Rapamycin decreased the expression of MMP9 and inhibited the colony formation of tumor cells in the human colorectal cancer cell line HCT116 [27]. These findings support the importance of the mTOR pathway in regulating MMP9 expression. We also showed that Rapamycin has a bigger influence on P65 nuclear translocation, and similar findings were reported by Han, who demonstrated that EPA could inhibit NF-κB (P65) translocation from the cytoplasm into the nucleus by blocking the
phosphorylation of mTOR in SKOV-3 cells [28]. Previous studies also showed that AKT-dependent regulation of NF-κB is controlled by mTOR through interaction with IKK in PTEN-null/inactive prostate cancer cells [29]. It is well established that AKT controls NF-κB activation via mTOR. These results provide good evidence that NF-κB is an important downstream effector of mTOR, which is involved in MMP9 expression in mammary epithelial cells.

NF-κB is a dimeric transcription factor that regulates a target gene's expression through binding to its promoter. NF-κB is initially located in the cytoplasm in an inactive form complexed with IκB, an inhibitory factor, which binds NF-κB and masks its nuclear localization signal, thereby preventing NF-κB translocation into the nucleus [30, 31]. Activation of NF-κB is initiated through phosphorylation of IκB by a macromolecular cytoplasmic IκB kinase (IKK). Exposure of cells to a variety of extracellular stimuli leads to the rapid phosphorylation, ubiquitination, and ultimately proteolytic degradation of IκB, which frees NF-κB to translocate to the nucleus where it regulates gene transcription including MMP9 [32].

Transcriptional regulation of MMP9 is believed to be the most important component for MMP9 expression. Previous studies have demonstrated that the MMP9 promoter has several transcription factor binding motifs, including NF-κB [33]. In human MCF-7 cells, NF-κB directly regulates the transcription of MMP9 [34]. Puerarin treatment effectively negated the expression of MMP9 by inhibition of the NF-κB pathway in LPS-activated MCF-7 and MDA-MB-231 cells [35]. Another study indicated that KA inhibits the PMA-induced activation of MMP-9 by suppressing NF-κB activation in HT-1080 cells [36]. In the current study, we showed that P65 overexpression significantly enhanced the phosphorylation level of P65 and increased MMP9 protein expression and gene promoter activity. Celastrol, an NF-κB inhibitor, remarkably reduced MMP9 protein expression and NF-κB(P65) nuclear translocation in mammary epithelial cells. Moreover, Celastrol also suppressed P65 overexpression-induced MMP9 gene promoter activity. The results of the promoter assay indicated that dairy cows' MMP9 gene promoter may contain cis-acting regulatory elements for transcription factors such as NF-κB and may be located at -80 ~ -420bp. It is well established that NF-κB plays a significant role in the transcriptional regulation of MMP9.

Here, we examined PI3K/AKT/mTOR signaling to the MMP9 promoter in a defined system. However, the MMP9 promoter could be regulated by a network of signaling cascades because the promoter contains cis-regulatory regions that are targets of multiple signaling pathways. It remains to be seen whether PI3K/AKT/mTOR signaling can synergize or co-operate with other signaling components to further enhance MMP9 promoter activity. Therefore, further experiments will be necessary to investigate the molecular mechanism of PI3K/AKT/mTOR signaling on the transcriptional regulation of MMP9.

Conclusion

In conclusion, we show that MMP9 protein expression was mediated by transcription factor NF-κB through PI3K/AKT/mTOR signaling pathway in mammary epithelial cells of dairy cows. In addition, our study demonstrated that the core regulation sequence of transcription factor NF-κB for MMP9 promoter activation may be located in -80bp to -420bp.

Abbreviations

| Abbreviation | Description                                |
|--------------|--------------------------------------------|
| MMP9         | Matrix metalloproteinase 9                 |
| mTOR         | mammalian target of rapamycin              |
| DMSO         | Dimethyl Sulfoxide                         |

Declarations

Acknowledgments

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Authors' contributions

Chen Su performed experiments and carried out the statistical analysis. Yongjin and Huilin drafted the manuscript and revised the manuscript. Feng Zhao, Bo Qu, Bing Zhao, and Yingjun designed the experiments. Yingjun supervised the study and helped the revision of this manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding authors upon reasonable request.

Ethics approval and consent to participate

All operations in this research strictly abide by the ordinances of the Administration of Laboratory Animals (College of Life Science, Northeast Agricultural University, Harbin). All animal experimental procedures were authorized by the Northeast Agricultural University Animal Care and Use Committee.
Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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**Figures**

**A**

| LY294002 (25μM) | − | + |
|------------------|---|---|
| p-AKT           |   |   |
| AKT             |   |   |
| p-mTOR          |   |   |
| mTOR            |   |   |
| MMP9            |   |   |
| β-actin         |   |   |

**B**

![Control](#) ![LY294002](#)

![Relative Protein Level](#)

**C**

| LY294002 (25μM) | − | + |
|------------------|---|---|
| P65 (Nuclear)    |   |   |
| Histone H3       |   |   |
| P65 (Cytoplasmic)|   |   |
| β-actin          |   |   |

**D**

![Control](#) ![LY294002](#)

![Relative Protein Level](#)

**Figure 1**

LY294002 attenuates MMP9 expression and P65 nuclear translocation in mammary epithelial cells. (A) Mammary epithelial cells were pretreated with 25μM LY294002 (a selective antagonist of PI3K/AKT) for 24h. DMSO was used as a negative control. The expression of P-AKT, AKT, P-mTOR, mTOR MMP9 was measured by Western blot analysis. β-actin expression was evaluated as an internal control. (B) Quantification of P-AKT, AKT, P-mTOR, mTOR, and MMP9 expression from the Western blots in panel A. (C) Mammary epithelial cells were treated with 25μM LY294002 for 4h. Nuclear and cytoplasmic extracts were...
prepared, and Western blot analysis was performed. Histone H3 and β-actin were used as an internal control for nuclear and cytoplasmic extracts respectively. (D) Quantification of nuclear and cytoplasmic P65 expression from the Western blots in panel C. One representative image of three different experiments for each of the analyses performed is shown. Data are expressed as mean±SEM of three independent experiments. *P < 0.05 and **P < 0.01 compared with the control.

Figure 2

Rapamycin decreased MMP9 expression and P65 nuclear translocation in mammary epithelial cells. (A) Mammary epithelial cells were pretreated with 0.1 μM Rapamycin (an mTOR inhibitor) for 24h. The expression of P-mTOR, mTOR, and MMP9 was measured by Western blot analysis. β-actin expression was evaluated as an internal control. (B) Quantification of P-mTOR, mTOR, and MMP9 expression from the Western blots in panel A. (C) Mammary epithelial cells were treated with 0.1 μM Rapamycin for 4h. Nuclear and cytoplasmic extracts were prepared, and Western blot analysis was performed. Histone H3 and β-actin were used as an internal control for nuclear and cytoplasmic extracts, respectively. (D) Quantification of nuclear and cytoplasmic P65 expression from the Western blots in panel C. One representative image of three different experiments for each of the analyses performed is shown. Data are expressed as mean±SEM of three independent experiments. *P < 0.05 and **P < 0.01 compared with the control.
Figure 3

Celastrol suppressed MMP9 expression and P65 nuclear translocation in mammary epithelial cells. (A) Mammary epithelial cells were pretreated with 0.3 μM Celastrol (an NF-κB inhibitor) for 24h. The expression of MMP9 was measured by Western blot analysis. β-actin expression was evaluated as an internal control. (B) Quantification of MMP9 expression from the Western blots in panel A. (C) Mammary epithelial cells were treated with 0.3 μM Celastrol for 4h. Nuclear and cytoplasmic extracts were prepared and Western blot analysis was performed. Histone H3 and β-actin were used as an internal control for nuclear and cytoplasmic extracts respectively. (D) Quantification of nuclear and cytoplasmic P65 expression from the Western blots in panel C. One representative image of three different experiments, for each of the analyses performed, is shown. Data are expressed as mean±SEM of three independent experiments. *P < 0.05 and **P < 0.01 compared with the control.
Effect of transcription factor NF-κB (P65) on MMP9 promoter activity in mammary epithelial cells. (A) Mammary epithelial cells were transfected with NF-κB (P65) expression vector for 48h. The levels of P65, P-P65, and MMP9 were determined by Western blotting. Protein expression levels of β-actin in cell lysates were used as a control. (B) Quantification of P65, P-P65, and MMP9 expression from the Western blots in panel A. (C) Mammary epithelial cells were co-transfected with a series of MMP9-PGL3 plasmid including MMP9-640-Luc (M640-Luc), MMP9-420-Luc (M420-Luc), MMP9-380-Luc (M380-Luc), MMP9-80-Luc (M80-Luc), which contains a deletion of the MMP9 gene promoter 5’-flanking region, and NF-κB (P65) overexpression vector for 48h. Luciferase activity was normalized to the Renilla activity in each cell lysate. (D) Mammary epithelial cells were co-transfected with M420-Luc plasmid and NF-κB(P65) overexpression vector for 6h. Then cells were treated with Celastrol, and DMSO was used as a negative control. Luciferase activity was normalized with the Renilla activity in each cell. Data are expressed as mean ± SEM of three independent experiments. For B, means without a common letter (a-b) differ at P < 0.05. For C and D, *P < 0.05; **P < 0.01.