# Expression of *S100A8* is induced by interleukin-1α in TR146 epithelial cells through a mechanism involving CCAAT/enhancer binding protein β

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Abstract. Calprotectin in mucosal epidermal keratinocytes has an important role in fighting microbial infections. S100A8 belongs to the S100 protein family and is a subunit of calprotectin (heterodimer complex of \$100A8/A9). Interleukin-1a (IL-1 $\alpha$ ) is one of the cytokines produced by oral keratinocytes. The primary aims of the present study were to investigate the effect of IL-1a on the expression of S100A8 and its underlying molecular mechanism in oral epithelial cells. Determining the molecular mechanism of the induced expression of S100A8 by IL-1 $\alpha$  aims to improve current understanding of the roles of calprotectin during the infection of mucosal epithelial cells. The expression analysis indicated that IL-1 $\alpha$  significantly induced the expression of S100A8 in human TR146 epithelial cells at the mRNA and protein levels, respectively. The reporter assay demonstrated that the upregulatory effect of S100A8 induced by IL-1a was dependent on the S100A8 promoter specific region (-165/-111). The results of electrophoresis mobility shift assay and chromatin immunoprecipitation assay also demonstrated that the CCAAT/enhancer binding protein  $\beta$  (C/EBP $\beta$ ) binding site (-113/-109) in the S100A8 promoter region was involved into the upregulatory effect on the expression of S100A8 induced by IL-1a. Taken together, these results suggested that the activation of the expression of S100A8 induced by IL-1 $\alpha$  in TR146 epithelial cells involves a mechanism by which the binding activity of C/EBP $\beta$  to the specific site (-113/-109) of the S100A8 promoter is increased.

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#### Introduction

Mucosal epidermal keratinocytes release proinflammatory cytokines during the fast innate immune response to microbial infection (1-3). Interleukin-1 $\alpha$  (IL-1 $\alpha$ ) is one of the cytokines produced by oral keratinocytes (3-8). The binding of IL-1 $\alpha$  to its cell surface IL-1 receptor induces the activation of nuclear factor-kB, c-Jun N-terminal kinase and p38-mitogen activated protein kinase (MAPK) target gene transcription (9,10). Functionally, pro-IL-1 $\alpha$  has a nuclear localization signal at its N-terminus and binds HS-1-associated protein X-1 to allow for translocation into the nucleus; it then binds transcription activators to directly affect target gene transcription (9,10). In response to IL-1 $\alpha$ , the expression levels of antimicrobial proteins/peptides, including calprotectin (a heterodimer complex of S100A8/A9), defensin and adrenomedullin, are significantly upregulated in epidermal keratinocytes (1-3,5-7). Calprotectin has a variety of antimicrobial activities in keratinocytes and is important in mucosal innate immunity (1-8). In addition, tumor-suppressive roles of calprotectin in head and neck squamous cell carcinoma (HNSCC) have been reported (11,12). Mechanistically, calprotectin negatively regulates G<sub>2</sub>/M cell cycle progression and growth in a protein phosphatase  $2\alpha$ -dependent manner in HNSCC (11,12).

The human *S100A8* promoter has been well characterized (13). Transcription factors that can regulate the expression of human *S100A8* include CAAT enhancer binding protein  $\alpha$  (C/EBP $\alpha$ ) (14), C/EBP $\beta$  (15) and hypoxia inducible factor-1 (16). However, several copies of Ets transcription factor, E-Box and C/EBP $\beta$  consensus sequences have been observed in the murine *S100A8* promoter region (17).

Although IL-1 $\alpha$  has been reported to increase the expression of *S100A8* in the HaCaT human keratinocyte cell line (5,6), the mechanism underlying the effect of IL-1 $\alpha$  on human *S100A8* in keratinocytes remains to be fully elucidated. Determining the molecular mechanism underlying the expression of *S100A8* induced by IL-1 $\alpha$  may provide a better understanding of the roles of calprotectin during the infection of mucosal epithelial cells. Therefore, to the best of our knowledge, the results of the present study are the first to provide a conceivable mechanism underlying the effects of human *S100A8* induced by IL-1 $\alpha$  in epidermal keratinocytes.

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## Materials and methods

Cell culture and IL-1 $\alpha$  treatment. The human TR146 epithelial cancer cells (ATCC, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% FBS (Gemini Bio-Products, Sacramento, CA, USA). The TR146 cells were incubated at 37°C in an incubator with 5% CO<sub>2</sub>. Recombinant IL-1 $\alpha$  (Sino Biological, Inc., Beijing, China) was dissolved and cell treatment was then performed as described previously (1). Briefly, cells were seeded 10<sup>5</sup> per well into 24-well plates. Following overnight incubation at 37°C, cells were washed with Dulbecco's phosphate-buffered saline (DPBS), different concentrations of IL-1 $\alpha$  were added or bovine serum albumin (BSA; vehicle, 50 µg/ml BSA in DPBS).

Reverse transcription-quantitative polymerase chain reaction analysis (RT-qPCR) analysis. Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, Inc.). Reverse transcription was performed with a FastQuant RT kit and gDNase (Tiangen Biotech Co., Ltd., Beijing, P. R. China). RT-qPCR with SYBR Green I was then conducted. Primers for the qPCR amplification of human S100A8 [primer pair S100A8-1 forward (F)/S100A8-1 reverse (R)] (1), and  $\beta$ -actin (ACTB; primer pair ACTB-1F/ACTB-1R) (18) were used (Table I). Each reaction was performed in a 20  $\mu$ l volume containing 1XSYBR qPCR MasterMix (Fermentas; Thermo Scientific, Inc.), 50 nM of each primer and 1 µl cDNA. The cycling conditions were: 2 min at 95°C, followed by 40 cycles at 95°C for 5 sec and 60°C for 30 sec. The mRNA expression levels of human S100A8 were standardized to the mRNA expression of  $\beta$ -actin. The RT-qPCR results were quantified using the 2<sup>- $\Delta\Delta Cq$ </sup> method (19).

Plasmid construction. A PCR fragment (primer pair PA8-1/PA8-2) was amplified from human blood genomic DNA (Promega Corporation, Madison, WI, USA) using Herculase® II Fusion DNA Polymerase (Agilent Technologies, Inc., Santa Clara, CA, USA), which was then ligated into pGL3-basic (Promega Corporation) via the NheI and BglII restriction sites, to generate the pGL3(-3096/+246) construct. The cycling conditions were as follows: 2 min at 94°C, followed by 36 cycles at 94°C for 20 sec, 63°C for 30 sec and 72°C for 90 sec, followed by a final extension at 72°C for 3 min. A 7,906-bp fragment (PA8-3/pGL3-S) was amplified from pGL3 (-3096/+246) and this fragment was then self-ligated to generate the pGL3(-3096/-1) construct. To generate S100A8 promoter 5'deletion mutants, fragments of 6,697 bp (DPA8-1/pGL3-AS), 5,739 bp (DPA8-2/pGL3-AS), 5,488 bp (DPA8-3/pGL3-AS), 5,229 bp (DPA8-4/pGL3-AS), 5,053 bp (DPA8-5/pGL3-AS) and 4,882 bp (DPA8-6/pGL3-AS) were amplified from pGL3 (-3096/-1), respectively (Table I). The products were purified and self-ligated to generate pGL3(-1887/-1), pGL3(-929/-1), pGL3(-678/-1), pGL3(-419/-1), pGL3(-243/-1) and pGL3 (-72/-1), respectively. Similarly, fragments of 5,067 bp (DDA8-1/pGL3-AS), 5,042 bp (DDA8-2/pGL3-AS), 5,003 bp (DDA8-3/pGL3-AS), 4,975 bp (DDA8-4/pGL3-AS) and 4,921 bp (DDA8-5/pGL3-AS) were amplified from the pGL3 (-419/-1) construct (Table I), and these the fragments were purified and self-ligated to generate pGL3(-257/-1), pGL3(-232/-1), pGL3(-193/-1), pGL3(-165/-1) and pGL3(-111/-1), respectively. The cycling conditions for amplification of self-ligated fragments were: 2 min at 94°C, followed by 36 cycles at 94°C for 20 sec, 63°C for 30 sec and 72°C for 4 min, followed by a final extension at 72°C for 5 min. Primer pairs (M3-1/M3-2) and pGL3(-257/-1) were used for amplification to generate the pGL3(-257/-1)-M3 constructs using the QuikChange XL site-directed mutagenesis kit (Stratagene; Agilent Technologies, Inc.; Table I). All plasmid constructs were confirmed by automated sequencing (Sangon Biotech Co., Ltd., Shanghai, China).

*RNA interference*. The C/EBP $\beta$  small interfering (si)-RNA sequence (5'-UUGGCCACUUCCAUGGGUCUAAAGG-3'), as described previously (20), was synthesized by Sangon Biotech Co., Ltd. C/EBP $\beta$  was silenced by transfecting the cells with 25 nM C/EBP $\beta$  siRNA using HiperFect transfection reagent (Qiagen, Inc., Valencia, CA, USA), following which the cells were collected for protein expression analysis. Non-specific siRNA (Sangon Biotech Co., Ltd.) was used as the negative control.

Transfection and dual luciferase assay. The TR146 cells were grown to 60-80% confluency, following which the cells were transfected with a firefly luciferase construct and a *Renilla* luciferase construct, pRL-TK (20:1 ratio), using lipofectamine  $3000^{TM}$  (Thermo Fisher Scientific, Inc.). The luciferase activities were measured after 40 h (Promega Corporation). The luciferase activity was normalized to *Renilla* luciferase activity.

Electrophoretic mobility shift assays (EMSA). The EMSA was conducted as previously described (21). Briefly, 5'-biotin-labeled single-strand probes were synthesized by Sangon Biotech Co., Ltd. Double-stranded oligonucleotide probes were prepared by diluting equimolar quantities of complementary oligonucleotides in 1X STE buffer (100 mM NaCl, 50 mM Tris-HCl and 1 mM EDTA, pH 8.0), incubated at 95°C for 3 min, and then slowly cooled to room temperature. Nuclear extracts from the TR146 cells were extracted using a nuclear extraction kit (Biyuntian, Shanghai, China). The EMSA reaction mixtures were incubated on ice for 30 min with or without unlabeled competitor, prior to adding end-labeled oligonucleotides for 20 min on ice. For the competitive assays, a 200-fold molar excess of cold oligonucleotides was added to the binding reaction prior to the addition of the hot-labeled oligonucleotides. The binding reactions were analyzed by transferring the reactants to positively charged nylon membranes (cat. no. 11209299001, Roche Diagnostics, Indianapolis, IN, USA). For the supershift assay, the nuclear extracts containing 5  $\mu$ g protein were incubated with 500 ng C/EBPa (D-5, cat. no. sc-365318; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) or C/EBPß antibodies (cat. no. 23431-1-AP; Wuhan Sanying Biotechnology, Wuhan, China) on ice for 30 min prior to adding end-labeled oligonucleotides for 20 min on ice. The samples were electrophoresed on a 5% non-denaturing polyacrylamide gel in 0.5X Tris-borate-EDTA buffer. Detection was conducted using Lightshift electrophoretic mobility shift reagent (Pierce; Thermo Fisher Scientific, Inc.).

Table I. Oligonucleotides used in the present study.

RT-qPCR analysis       GGGCATCATGTTGACCGAGC         \$100A8-1F       GGGCATCATGTTGACCGAGC         \$100A8-1R       GTAACTCAGCTACCTATGTGGGCTT         ACTB-1F       GACGACATGGGAGAAATCTG         ACTB-1R       ATGATCTGGGTCATCTTCTC         Plasmid construction       PA8-1         PA8-2       GAAGATCTGTCCAGCCTAGGAGACAATGTGCC         pGL3-S       AGATCTGCGATCTAAGAAGCTTGCCAGC         pPA8-1       CCCGGACATGGGAAAAGCTCAG         pPA8-2       GGTGGGGGAGGAGGATTTGTTCCTCC         pPA8-3       CCCGGACATGGGAAAGCTCAGG         pPA8-4       CCCGGACATGGGAAAGCTCAG         pPA8-5       AGGCTGCCAGGGCAGGCC         pPA8-6       CCTGATGCCTGAAGCCTGTGGG         pPA8-5       AAGCAAGTGGAACCCGGTAAC         pPA8-6       TCTGATGCCTGAAGCCTGTGGG         pDA8-7       CCCGGCACACCCGGAAAAAGGCC         pDA8-8       GGCTACCACCCGGTAAGAGCTGTGGGA         pDA8-9       CTGCATCTCTTTCCGCTTCCC         pDA8-1       CCCGGCCAAAATGGAAACGAGCC         pDA8-2       CTACCTGCTTTTCCCCTTTCCGCTCC         pDA8-3       TGCCTCTCTTCTCCCTTCCCC         pDA8-4       TCCCCACCCAAATTTTCATCTCGC         pDA8-5       CAACTCTGCTCTTCCCCTTCCCC         pDA8-6       TCCCCACCCCAAATTTTCATCTCGC         pD	Primer name	Oligonucleotide sequence (5'-3') <sup>a</sup>
S100A8-IFGGGCATCATGTTGACCGAGCS100A8-IRGTAACTCAGCTACTCTTGTGGCTTACTB-IFGACGACATGGGACAAATCTGACTB-IRATGATCTGGGGACAATCTGCPA8-1CTAGCTAGCAGGGACTGAGCCCTTCCTGTAAACATGPA8-2GAAGATCTGTCCAGCCTAGGAGCAATGTGCCpA8-3GCAGGGCTGAGAGGCAGCTCCpGL3-SAGATCTGCGAGCACTGAGCAGCTGCDPA8-1CCCGGACATGGGAAAAGCTCAGDPA8-2GGTGGGGAGGGAGGATTTGTTCCTCCDPA8-3CCCGGACATGGGAAAAGCTCAGDPA8-4TCCCGTCTTGGACCCTTGAAACDPA8-5AGCTAGGCAGGCAGGCDPA8-5AGCCAGGCCAGAGGCDPA8-6TCTGATGGCCTGAGAGCTGGGGDPA8-6TCTGATGGCCTGAAGCTGGGGDPA8-7CCCGCCCGAAAAAGGCCGGTACDDA8-8CCCCCACCCAAAATGTTCCTCCDDA8-1CCCGCACCCAGAAAAAGGCCDDA8-1CCCCCACCCAAAATTTCATCTGCDDA8-3TCCCCACCCAAAATTTCATCTGCDDA8-4TCCCCACCCAAAATTTCATCTGCDDA8-5CAACTCTGGCAGGAAACCAGGGGAEPIsTCCCCACCCAAAATTTCATCTGCEPIsCTGCCACCCACAATTTCCACCCEP2sCTGCCACCTGGTTGCCACTGCAGAEP3asGCTGAATGGCCAATCCACTGCAGGEP3asGTCTCTCTTTCCGCTTCCh-1TCCCCCACCACAGCGTCAGAGAGCCh-2CAGCTGCCCACAGCTTCAGGAGAGCCh-3GTACTGATGTGGGAAGGAGCh-4ACCTAGTGTGGGAAGGAGGAGCh-4ACCTAGTGTGGGAAGGAGGAGCTGTAGCAACCCMutagenesis of C/EBPB binding sites*M3-2M3-2GAGTTGCTACAGTCTCGAAGGCTGCAACCAGGTGAATGTGGCAACCCAGGTGAAGCTGTAGCAACCCAGGTGAATGTGCAACCAGGTGAAATGTGGCAACCAGGTGAATGCC	RT-qPCR analysis	
SI00A8-IRGTAACTCAGCTACTCTTGTGGCTTACTB-IFGACGACATGGAGAAAATCTGACTB-IRATGATCTGGGTCATCTCPlasmid constructionPA8-1CTAGCTAGCAGGACAGCCCTCCTGTAAACATGPA8-3GCAGGGCTGAGAGGCACTCCpGL3-SAGATCTGCGATCTAAGTAAGCTTGGCATTCDPA8-1CCCGGACATGGGAAAAGCTCAGDPA8-2GGTGGGGAGAGGATTGTTGCTCCCDPA8-3CTCCATCTCCCAGGGCATGGTCDPA8-4TGCGGTCTTTGGACCTTGAAACDPA8-5AAGCAAGTGGATGCCAGCAGCDPA8-6TCTGATGGCCTGAAGCTGTGGGpGL3-ASGGCCTAGCACGCGAAGCCDPA8-6TCTGATGGCCTGAAGCTGTGGGDPA8-6CTCGATGGCCTGAGAGCCDDA8-6CTCGATGCCCGGAACCCGGTACDDA8-1CCCAGCACCCGGAAAAAGGCCDDA8-2CTACCTGCTTTTCCCTCTGGGCACDDA8-3TGCCTTCCTCTTTCCGGTCCDDA8-3TGCCTTCCTGGCAGGAGAGCTGTCEMSA assaysTCCCCACCAAAATTTTCATTCTGCEP1sCCCCGCACAAATTTTCGGTGGGGAEP2asCTGCACCTGGTTGGGAAGCACGGGAEP3aCATCACCTGGTTGGGAAGCAGAGCEP3asCATCACCTGGTTGGCACAGGGAACEP3asGTCCTGCTTTCCACCAGGGAAGCEP3asCATCACCTGGTTGCCACAGGGAACEP3asCATCACCTGGTTGCCACAGGGAGACEP3asCATCACCTGGTTGCCACAGGGAGACEP3asGTCCTGCTTTCCCCTTTCCGCTTCCh-1TGCCTCCTTTCCGCTTCCh-2CAGCTGCTCACAGCTTCAGGAGGAGACCh-3GTACTGGTGGGACATTACMutagenesis of C/EBPβ binding sites"Ma3-1M3-2GCCACATTCACCTGGTTGGAAGCCTGTAGCACGTGCGCACCTCGAAGCACGTGCGCACCTCCGAGGCTGAA	S100A8-1F	GGGCATCATGTTGACCGAGC
ACTB-1FGACGACATGGAGAAAATCTGACTB-1RATGATCTGGGTCATCTTCTPlasmid constructionPlas-1CTAGCTAGCAGGGACTGAGCCCTTTCCTGTAAACATGPA8-2GAAGATCTGTCCAGCCTAGGAGACAATGTGCCpGL3-SGCAGGGCTGAGAGGCAGCTCpA8-3CCCGGACATAGTAAGCTTGGCATTCDPA8-1CCCGGACATGGGAAAGCTCAGDPA8-2GGTGGGGAGAGGCCTTCTCCDPA8-3CTCCATCTCCCCAGGCATGGTCDPA8-4TGCGGTCTTGGACCCTTGAAACDPA8-5AAGCAAGTGGATGCCAGCAGCDPA8-6TCTGATGGCCTGAAGCGGGGApGL3-ASGGCTAGCACCAGGAAAAGAGCCDDA8-1CCCAGCACCCGAAAAAGAGCCDDA8-2CTACCTGCTTTTCCTCTGGGCACDDA8-3TGCCTCCTCTTTCGGCCACDDA8-4TCCCCACCCAAAATTTCATCTGCDDA8-5CAACTCTGGCAGGAGAAGCTGTCEMSA assaysTEP1sTCCCCACCCAAAATTTTCATTCTGCEP2sCTGCACAGTGATGCAACAGGAGACAGGAAGCEP3sGCTGGCCACACGGTAACCAGGGAAGCEP3sGCTGCTCCTCTTCCGCTTCCh1TCCCTCCTCTTCCGCTTCCh2CAGCTGCCACACGGTAAGAGGAGAGCEP3sGTCCTGGTTGCAACCAGGGAAGGAGEP3sGTCCTCTCTTCCGCTTCCh2CAGCTGCCCACAGCTTCAGGCh3GTACTGTGTGGGAAGGAGGAGAGCH3-1GCCCACTTCACCTGGTTGAGCACTGAAGCACCAGGTGAATGTGCGCACCACGGTAACCAGGTGAATGTGCGCACCCACGGTAACCAGGTGAATGTGCGCACCACGGTGAATGTGCGCACCACGGTGAATGTGCGCACCCACGGTGAAGCACGGTGAATGTGCGCACCCACGGTGAAGCACGAGGACCh3-2GCTGCTCCCTCTCTCGAGGCCTCAACCAGGTGAAGCACCTGTAGCACACCGGTAACCAGGTGAAGCACCACGGTGAAGCACCACGGTAACCAGGTGAAGCACCACGGTGAAGCACCACGGTAACCAGGTGAACCAGGGACACTCTAGCCCCCCCAACCCTGGTAGCAACC	S100A8-1R	GTAACTCAGCTACTCTTTGTGGCTT
ACTB-1RATGATCTGGGTCATCTTCCPlasmid constructionPA8-1CTAGCTAGCAGGGACTGAGCCCTTTCCTGTAAACATGPA8-2GAAGATCTGTCCAGCCTAGGAGACAATGTGCCPA8-3GCAGGGCGAGAGGCAGCTCCpGL3-SAGATCTGCGATCTAAGTAAGCTTGGCATTCDPA8-1CCCGGACATGGGAAAAGCTCAGDPA8-2GGTGGGGAGAGGAGTTGTTCCTCCDPA8-3CTCCATCTCCCAGGCATGGCAACCDPA8-4TGCGGTCTTTGAAACAGCGGGDPA8-5AAGCAAGTGGAGCCCTTTGAAACDPA8-6TCTGATGGCCTGAAGCCTGGGGACDDA8-7GGCTAGCACGCGTAAAGAGCCCDDA8-1CCAGCAGCCCAGAAAAGAGCCDDA8-3TGCCTTCTTTCCTTCTGGGCACDDA8-3TGCCTCCCTTTTCCTTCGGGCACDDA8-3TGCCTCCCCCACAAATTTCATTCTGCDDA8-4TCCCCACCCAAAATTTCATCTGCEPIsTCCCCACCCAAAATTTTCATCTGCEPIsCCGCACGGTAGAACCAGGGAAGCEP2sCTGCACAGTGATGCCACATTCACCEP2sCTGCACAGTGATGCCACATTCACCEP3sGTCTCTGGTTTCCCACAGAGACEP3sGTCCTCGTTTCCCAGAGACCAGGAAACEP3sGTCCTCGTTTCCCACACAGAGACEP3sGTCCTCGTTTCCACCAGGGAAGCEP3sGTCTCTGGTTGCAGAGACCAGAGACEP3sGTCCTCGTTTCCACCAGGGAAGCCh1TGCCTCCTTTCCGCTTCCh2CAGCTGCCACAGCTTCAGCh3GTACTGGTGGGACATTACMuteneesis of C/EBPB binding sites"Ma32M3-1GCCACTTCACCTGGTTGAGCACTGTAGCACACGGTGAACCAGG	ACTB-1F	GACGACATGGAGAAAATCTG
Plasmid construction           PA8-1         CTAGCTAGCAGGACTGAGCCCTTCCTGTAAACATG           PA8-2         GAAGATCTGTCCAGCCTAGGAGACAATGTGCC           PA8-3         GCCAGGCTGAGAGAGCAGCTCC           pGL3-S         AGATCTGCGATCTAAGTAAGCTTGGCATTC           DPA8-1         CCCGGACATGGGAAAAGCTCGGC           DPA8-2         GGTGGGAGAGAGGATTGTTCCCC           DPA8-3         CTCCATCTCCCAGGCATGGTC           DPA8-4         TGCGGTCTTGGACCCTTTGAAAC           DPA8-5         AAGCAAGTGGATGCCAGCAGC           DPA8-6         TCTGATGCACGCGAAAAGCTCGGGAC           DPA8-6         CCCAGCAGCCCAGAAAAAGAGCC           DDA8-1         CCAGCAGCCCAGAAAAAGAGCC           DDA8-5         CAGCTGCTCTTTTCCTCTGTGGGAC           DDA8-1         CCCCACCCAAAAATTTTCATTCTGC           DDA8-3         GCCTGCTCCTTTTCCCTCC           DDA8-4         TCCCCACCCAAAATTTTCATTCTGC           DDA8-5         CAACTCTGGCAGGAAGAGCTGTC           EMSA         GCCGCACCACAAAATTTTCATTCTGC           EP1as         CCCCACCCACAAAATTTTCATTCTGC           EP2as         GGTGGGAATGGCAATCACGAGAC           EP2as         GTGCTCTGTTTCCACTGGCAGG           EP3as         GTCTCTGGTTTCTCAACCAGAGACCAGAGAC           EP3as         GTCTCTGGTTTCTCAACCAGAGAC           Ch-	ACTB-1R	ATGATCTGGGTCATCTTCTC
PA8-1CTAGCTAGCAGGGACTGAGCCTTTCCTGTAAACATGPA8-2GAAGATCTGTCCAGCCTAGGAGACCAATGTGCCPA8-3GCAGGCTGCAGCAGCCCpGL3-SAGATCTGCGATCTCAGTAAGCTTGGCATTCDPA8-1CCCCGGACATGGGAAAAGCTCAGDPA8-2GGTGGGGAGAGGATTTGTTCCTCCDPA8-3CTCCATCTCCCAGGGCATGGTCDPA8-4TGCGGTCTTGGACCTTTGAAACDPA8-5AAGCAAGTGGATGCCCGGAGCDPA8-6TCTGATGGCCTGAAGCTGGTGGDPA8-1CCCAGCCGGAAAAGGCCDDA8-1CCCAGCCCGAAAAAGAGCCDDA8-1CCCGCACGCCGAAAAAGAGCCDDA8-2CTACCTGCTTTTCCTTCTGGGGACDDA8-3TGCCTTCTCTTTCCGCTTCCCDDA8-4TCCCCACCCAAAATTTTCATTCTGCDDA8-5CAACTCTGGCAGGAGAGAGCTGTCEMSA assaysEEP1sCCCCCACCCAAAATTTTCATTCTGCEP1asCCTGCACTGGTTGAGAAACAGGGAGAEP3asCATTCACCTGGTTGAGAAACAGAGACEP3asCATTCACCTGGTTGAGAAACAGAGACEP3asCATTCACCTGGTTGAGAAACAGAGACCh-1TGCCTTCTCTTCCGCTTCCh-3GTACATGATGTGGGAAGGAGCh-3GTACATGATGTGGGAAGGAGCh-3GTACATGATGTGGGAAGGAGCh-4ACCTAGTGTGGGAAGAGGAGCh-3GTACATGATGTGGGAAGGAGCh-4CCCTGCTTCCGGTTGAGGACGACTGTAGCAACCCMutagenesis of C/EBPβ binding sites*M3-2M3-2GAGTTGCTACAGTCTCGGACCTCAACCAGGTGAAGCACTGTAGCACCC	Plasmid construction	
PA8-2GAAGATCTGTCCAGCCTAGGAGACAATGTGCCPA8-3GCAGGGCTGAGAGGCGCCpGL3-SAGATCTGCGATCTAAGTAAGCTCGCATTCDPA8-1CCCGGACATGGGAAAAGCTCAGDPA8-2GGTGGGGAGAGGATTTGTTCCTCCDPA8-3CTCCATCTCCCAGGCCATGGTCDPA8-4TGCGGTCTTGGACCCTTTGAAACDPA8-5AAGCAAGTGGATGCCAGCAGCDPA8-6TCTGATGGCCTGAAGCTGGGGpGL3-ASGGCTACCACGCCAGAAAAGCTCCGGTACDDA8-1CCAGCAGCCAGAAAAGAGCCDDA8-2CTACCTGCTTTTCCTTCTGGGCACDDA8-3TGCCTTCCTCTTTCCTTCTGGCACDDA8-4TCCCCACCCAAAATTTTCCATTCTGCDDA8-5CAACTCTGGCAGGAGAGCTGTCEMSA assaysEEP1sTCCCCACCCAAAATTTTCATTCTGCEP1asGCAGAATGGAAAATTTGGCGAGGAGACEP3asGGTGAATGGCCAATCACTGTGCAGEP3asGTCCTGGTTTCCCAACCAGGGAAACEP3asGTCCTCGGTTTCCCAACCAGGAGAACEP3asGTCCTCGCTTTCCGCTTCCh-1TGCCTTCCTCTTCCGCTTCCh-2CAACTGGTGGGAAGGAGGCh-3GTACATGATGTGGGAAGGAGCh-4ACCTAGTGTGGGAAGGAGCh-3GTACATGATGTGGGAAGGAGCh-4ACCTAGTGTGGGAAGGAGMutagenesis of C/EBPβ binding sites"M3-1Mutagenesis of C/EBPβ binding sites"M3-2MaterM3-2GCCACATTCACCTGGTTGGACATTAC	PA8-1	CTAGCTAGCAGGGACTGAGCCCTTTCCTGTAAACATG
PA8-3GCAGGGCTGAGAGGCAGCTCCpGL3-SAGATCTGCGATCTAAGTAAGCTTGGCATTCDPA8-1CCCGGACATGGGAAAAGCTCAGDPA8-2GGTGGGAGAGGATTGTTCCTCCDPA8-3CTCCATCTCCCAGGCATGGTCDPA8-4TGCGGTCTTTGAACCDPA8-5AAGCAAGTGGATGCAGCAGCDPA8-6TCTGATGGCCTGAAGCAGCGGTACDDA8-7GGCTAGCACGCCTAAGAGCTCGGTACDDA8-7CCCAGCAGCCCAGAAAAAGAGCCDDA8-7CCAGCAGCCCAGAAAAAGAGCCDDA8-1CCAGCAGCCCAGAAAAAGAGCCDDA8-2CTACCTGCTTTCCTGTGGCACDDA8-3TCCCCACCCAAAATTTCATCTGCDDA8-4TCCCCACCCAAAATTTCATCTGCDDA8-5CAACTCTGGCAGGGAGAAGCTGTCEMSA assaysFFP1sCCCCACCCAAAATTTTCATCTGCEP1asGCGAATGGAAATTTTCATCTGCEP2asGGTGAATGTGGCAATCACTGTGCAGEP3sCTCCTCTGTTCCGCTTCCh-1TGCCTTCCTCTTTCCGCTTCCh-2CAGCTGCCACAGCTCAGGCh-3GTACATGATGTGGGAAGGAGCh-3GTACATGATGTGGGAAGGAGCh-3GCCACATTCACCTGGTTGAGACATCACMutagenesis of C/EBPβ binding sites"M3-2	PA8-2	GAAGATCTGTCCAGCCTAGGAGACAATGTGCC
pGL3-SAGATCTGCGATCTAAGTAAGCTTGGCATTCDPA8-1CCCGGACATGGGAAAGCTCAGDPA8-1GCTGGGCATGGGAAAGCTCAGDPA8-2GGTGGGGAGAGGATTTGTTCCTCCDPA8-3CTCCATCTCCCAGGCATGGTCDPA8-4TGCGGTCTTTGGACCCTTTGAAACDPA8-5AAGCAAGTGGCTGAAGCTCGGGAGDPA8-6TCTGATGGCCTGAAGCTCGGTACDDA8-1CCAGCAGCCAGAAAAAGAGCCDDA8-2CTACCTGCTTTTCCTCTGGGCACDDA8-3TGCCTTCCTCTTTCCGCTTCCDDA8-3TGCCTTCCTCTTTCCGCTTCCDDA8-4CCCCCACCCAAAATTTTCATTCTGCDDA8-5CAACTCTGGCAGGAGAAGCTGTCEMSA assaysTEP1sTCCCCACCCAAAATTTTCATTCTGCEP2sCTGCACAGTGATTGCCACATTCACCEP2asGGTGAATGTGGCAATCACTGTGCAGEP3asCATTCACCTGGTTGAGAAACCAGAGACEP3asGCTCTCGTTTCCCACCAGGTGAATGCh-1TGCCTTCTCTTTCCGCTTCCh-1TGCCTTCTCTTTCCGCTTCCh-1CCCCACCCACAGCTTCAGCh-1CCCCACCCACAGCTTCAGCh-1TGCCTTCTCTTTCCGCTTCCh-1TGCCTTCTCTTTCCGCTTCCh-1TGCCTTCTCTTTCCGCTTCCh-1CCCTGCTCTCTCAGCh-1CCCTACTGTGTGGAAGGAGCh-1TGCCTTCTCTTTCCGCTTCCh-2CAGCTGCCCACAGCTTCAGCh-3CTCTGGTTGGGAAGGAGCh-4ACCTAGTGTGGAAGGAGMutagenesis of C/EBP\B binding sites*TM3-2GAGTTGCTACCTGGTTCCAGGGGAACTGTAGCAACCCCM3-2GAGTTGCTACAGTCTCTGAAGGCCCAAGCTGTAGCAACCCC	PA8-3	GCAGGGCTGAGAGGCAGCTCC
DPA8-1CCCGGACATGGGAAAAGCTCAGDPA8-2GGTGGGAGAGAGATTTGTTCCTCCDPA8-3CTCCATCTCCCAGGGCATGGTCDPA8-4TGCGGTCTTTGGACCCTTTGAAACDPA8-5AAGCAAGTGGATGCCAGCAGCDPA8-6TCTGATGGCCTGAAGCTGTGGGpCL3-ASGGCTAGCACGCGTAAGAGACCCGGTACDDA8-1CCAGCAGCCCAGAAAAAGAGCCDDA8-2CTACCTGCTTTTCCTTCTGGGCACDDA8-3TGCCTTCCTCTTTCCGCTTCCDDA8-5CAACTCTGGCAGGGAGAAGCTGTCEMSATCCCCACCCAAAATTTTCATTCTGCEMSATCCCCACCCAAAATTTTCATTCTGCEP1sTCCCCACCCAAAATTTTCGTTGCGGGGAEP2sCTGCACAGTGATTGCCACATTCACCEP2asGGTGAATGTGGCAATCACTGTGCAGEP3asCATTCACCTGGTTGCAGAGACCEP3asCATCCTGGTTCCCCTTCCh-1TGCCTTCTCTTTCCGCTTCCh-2CAGCTGCCCACAGCTTCAGCh-3GTACATGATGTGGGAAGGAGCh-3GTACATGATGTGGGAAGGAGCh-3GTACATGATGTGGGAAGGAGCh-3GTACATGATGTGGGAAGGAGCh-3GTACATGATGTGGGAAGGAGCh-3GTACATGATGTGGGAAGGAGCh-3GTACATGATGTGGGAAGGAGCh-3GTACATGATGTGGGAAGGAGCh-3GTACATGATGTGGGAAGGAGCh-3GTACATGATGTGGGAAGGAGCh-3GTACATGATGTGGGAAGGAGCh-3GTACATGATGTGGGAAGGAGCh-4ACCTAGATGTGGGAAGGAGMutagenesis of C/EBPβ binding sites*JACATGATCACCTGGTTGAGCACTCAGGGAACTGTAGCAACCCCM3-2GAGTTGCTACAGTCTCGAGGGCTCAACCAGGTGAAGGGGG	pGL3-S	AGATCTGCGATCTAAGTAAGCTTGGCATTC
DPA8-2GGTGGGGAGAGGATTTGTTCCTCCDPA8-3CTCCATCTCCCAGGGCATGGTCDPA8-3TGCGGTCTTGGACCCTTTGAAACDPA8-4TGCGGTCTTGGACCCTTGAAACDPA8-5AAGCAAGTGGAGCCAGCAGCDPA8-6TCTGATGGCCTGAAGCTGTGGGpGL3-ASGGCTAGCACGCGTAAGAGCTCGGTACDDA8-1CCAGCAGCCCAGAAAAAGAGCCDDA8-2CTACCTGCTTTTCCGCTCTCCDDA8-3TGCCTTCCTCTTCCGGCACDDA8-4TCCCCACCCAAAATTTCATTCTGCDDA8-5CAACTCTGGCAGGAGAAGCTGTCEMSA assaysEEP1sTCCCCACCCAAAATTTTCATTCTGCEP2sCTGCACAGTGATGAAAACCAGAGAGCEP2sCTGCACAGTGATGACACCAGTGCAGEP3asGGTGAATGTGGCAATCACCAGTGCAGEP3asGTCTCTGGTTTCCACCAGGAGAGCEP3asGTCTCTGGTTTCCCACTTCCCh-1TGCCTTCCTCTTTCCGCTTCCh-2CAGCTGCCCACAGCTCAGCh-3GTACATGATGTGGGAAGGAGCh-4ACCTAGTGTGGGAAGAGAGMutagenesis of C/EBPβ binding sites*TM3-2GAGTTGCTACAGTCTGGAAACCAGAGGCAATGGCC	DPA8-1	CCCGGACATGGGAAAAGCTCAG
DPA8-3CTCCATCTCCCAGGGCATGGTCDPA8-4TGCGGTCTTTGGACCCTTTGAAACDPA8-5AAGCAAGTGGATGCCAGCAGCDPA8-6TCTGATGGCCTGAAGCTGTGGGpGL3-ASGGCTAGCACGCGTAAGAGCTCGGTACDDA8-1CCAGCAGCCCAGAAAAAGAGCCDDA8-2CTACCTGCTTTTCCTTCTGGGCACDDA8-3TGCCTTCCTCTTCCGGTACDDA8-4TCCCCACCCAAAATTTTCATTCTGCDDA8-5CAACTCTGGCAGGAGAAGCTGTCEMSA assaysTEP1sTCCCCACCCAAAATTTTCATTCTGCEP2sCTGCAACGAGTACACCAGGGAAGCEP3sGCTAGCTGGTTGGCAATCACGGGAGAEP3sCATTCACCTGGTTGGGAATCACAGGACAEP3sCATCCACCTGGTTGGAAACCAGAGACEP3sCATCCACTGGTTGGAAACCAGAGACEP3sGTCCTCGGTTTCCAACCAGGGAATGACh-1TGCCTTCCTCTTTCCGCTTCCh-2CAGCTGCCCACAGCTTCAGCh-3GTACATGATGTGGGAAGGAGCh-4ACTAGATGTGGGAAGGAGAGAMutagenesis of C/EBPβ binding sites"M3-1M3-2GAGTTGCTACAGTCTTGGAAGCCTCTGAACCAGGTGAATGGCGC	DPA8-2	GGTGGGGAGAGGATTTGTTCCTCC
DPA8-4TGCGGTCTTTGGACCCTTTGAAACDPA8-5AAGCAAGTGGATGCCAGCAGCDPA8-6TCTGATGGCCTGAAGCTGTGGGpGL3-ASGGCTAGCACGCGTAAGAGCTCGGTACDDA8-1CCAGCAGCCCAGAAAAAGAGCCDDA8-2CTACCTGCTTTTCCTTCTGGGCACDDA8-3TGCCTTCCTCTTTCGGTCACDDA8-4TCCCCACCCAAAATTTTCATTCTGCDDA8-5CAACTCTGGCAGGAGAAGCTGTCEP1sTCCCCACCCAAAATTTTCATTCTGCEP1sCCGCACCCAAAATTTTCGGGGGAEP2sCTGCACAGTGATGGCACATTCACCEP3sGCTGCACAGTGATGGCAGAGAGCEP3sGTCCTGGTTTCTCAACCAGGTGAAGCEP3sGTCCTGGTTTCCCAATTCACCEP3sGTCCTGGTTTCCCAAGGAGAACAGAGACCh-1TGCCTTCCTTTCCGCTTCCh-2CAGCTGCCCACAGCTCAGCh-3GTACATGATGGGAAGGAGCh-4ACTAGATGTGGGAAGGAGMutagenesis of C/EBPβ binding sites"MagacattagGCTCAACCAGGTCAAGCCAGGTGAATGGCCM3-1GCCACATTCACCTGGTTGAGGCCTCAACCAGGTGAATGTGCCM3-2GAGTTGCTACAGTCTCTGAGCCAACCAGGTGAATGTGCC	DPA8-3	CTCCATCTCCCAGGGCATGGTC
DPA8-5AAGCAAGTGGATGCCAGCAGCDPA8-6TCTGATGGCCTGAAGCTGTGGGpGL3-ASGGCTAGCACGCCTGAAGCTCGGTACDDA8-1CCAGCAGCCCAGAAAAAGAGCCDDA8-2CTACCTGCTTTTCCTTCTGGGCACDDA8-3TGCCTTCCTCTTTCCGCTTCTCCDDA8-4TCCCCACCCAAAATTTTCATTCTGCDDA8-5CAACTCTGGCAGGAGAAGCTGTCEMSA assaysTEP1sTCCCCACCCAAAATTTTCATTCTGCEP2sCTGCACAGTGATTGCCACATTCACCEP3sCATTCACTGGTTGAGCAATCACGAGGAEP3sCATTCACCTGGTTGAGAAACCAGAGACEP3sGTCTCTGGTTTCCAACCAGGTGAATGCh-1TGCCTTCCTTTCCGCTTCCh-2CAGCTGCCCACAGCTTCAGCCh-3GTACATGTGGGAAGGAGCh-4ACCTAGTGTGGGAAGGAGMutagenesis of C/EBPß binding sites"Magana GCCCTCTGTGAGGCCTCCAGAGCTGTAGCAACTCCM3-1GCCACATTCACCTGGTTGAGGAGCTGAAGCACTCCM3-2GAGTTGCTACAGTCTCTGAAGGCTCCAACCAGGTGAATGTGCGCA	DPA8-4	TGCGGTCTTTGGACCCTTTGAAAC
DPA8-6TCTGATGGCCTGAAGCTGTGGGpGL3-ASGGCTAGCACGCGTAAGAGCTCGGTACDDA8-1CCAGCAGCCCAGAAAAAGAGCCDDA8-2CTACCTGCTTTTCCTTTGGGCACDDA8-3TGCCTTCCTCTTTCCGCTTCTCCDDA8-4TCCCCACCCAAAATTTTCATTCTGCDDA8-5CAACTCTGGCAGGGAGAAGCTGTCEMSA assaysTCCCCACCCAAAATTTTCGTCTGCEP1sTCCCCACCCAAAATTTTGGGTGGGGGAEP2sCTGCACAGTGATTGCCACATTCACCEP2asGGTGAATGTGGCAATCACTGTGCAGEP3sCATTCACCTGGTTGAGAAACCAGAGACEP3asGTCCTCGTTTCCGCTTCCh-1TGCCTTCCTCTTTCCGCTTCCh-2CAGCTGCCCACAGCTTCAGCh-3GTACATGATGTGGGAAGGAGCh-4ACCTAGTGGTGAGCACTTCACMutagenesis of C/EBPβ binding sites"TM3-1GCCACATTCACCTGGTTGAGAGCCTTCAGAGACTGTAGCAACTCCM3-2GAGTTGCTACAGTCTCTGAAGCGCTCAACCAGGTGAATGTGCGCACTCCACAGGTGAATGTGGCAATGTGCGCAACCAGGTGAATGTGGCAATGTGCGCAACCAGGTGAATGTGCCCACAGCTCCACAGGTGAATGTGCCCACAGCTCCACAGGTGAATGTGCGCAACTCC	DPA8-5	AAGCAAGTGGATGCCAGCAGC
pGL3-ASGGCTAGCACGCGTAAGAGCTCGGTACDDA8-1CCAGCAGCCCAGAAAAAGAGCCDDA8-2CTACCTGCTTTTCCTTTGGGCACDDA8-3TGCCTTCCTCTTTCCGGCTCCCDDA8-4TCCCCACCCAAAATTTTCATTCTGCDDA8-5CAACTCTGGCAGGGAGAAGCTGTCEMSA assaysTCCCCACCCAAAATTTTCATTCTGCEP1sTCCCCACCCAAAATTTTGGTGGGGAEP2sCTGCACAGTGATTGCCACATTCACCEP2asGGTGAATGTGGCAATCACTGTGGCAGEP3sCATTCACCTGGTTGAGAAACAAGAGCEP3sGTCTCTGTTTCCACCAGGTGAATGCh-1TGCCTTCCTCTTCCACGGTGAATGCh-2CAGCTGCCCACAGCTTCAGCh-3GTACATGATGTGGGAAGGAGCh-4ACCTAGTGATGTGGGAAGGAGMutagenesis of C/EBPβ binding sites <sup>a</sup> M3-1GCCACATTCACCTGGTTGAGAGCCTTCAGAGACTGTAGCAACTCTM3-2GAGTTGCTACAGTCTCTGAAGGCTCAACCAGGTGAATGTGGCAATGTCGC	DPA8-6	TCTGATGGCCTGAAGCTGTGGG
DDA8-1CCAGCAGCCAGAAAAAGAGCCDDA8-2CTACCTGCTTTTCCTTCTGGGCACDDA8-3TGCCTTCCTCTTTCCGCTTCTCCDDA8-4TCCCCACCCAAAATTTTCATTCTGCDDA8-5CAACTCTGGCAGGGAGAAGCTGTCEMSA assaysEEP1sTCCCCACCCAAAATTTTCATTCTGCEP1asGCAGAATGAAAATTTTGGCTGGGGAEP2sCTGCACAGTGATTGCCACATTCACCEP2asGGTGAATGTGGGCAATCACTGTGCAGEP3sCATTCACCTGGTTGAGAAACAGAGACEP3sGTCTCCTGTTTCTCAACCAGGTGAATGChIP assayCATCCTCGTTTCCGCTTCCh-1TGCCTTCCTCTTCCGCTTCCh-2CAGCTGCCCACAGCTCAGCh-3GTACATGATGTGGGAAGGAGCh-4ACCTAGTGATGTGGAACATTACMutagenesis of C/EBPβ binding sites"M3-1M3-1GCCACATTCACCTGGTTGAGAGCCTCAGGAGACTGTAGCAACTCCM3-2GAGTTGCTACAGTCTCTGAAGGCTCAACCAGGTGAATGTGGCAATGTCGCCACAGCTCCAGGTGAAGCACTCC	pGL3-AS	GGCTAGCACGCGTAAGAGCTCGGTAC
DDA8-2CTACCTGCTTTTTCCTTCTGGGCACDDA8-3TGCCTTCCTCTTTCCGCTTCTCCDDA8-4TCCCCACCCAAAATTTTCATTCTGCDDA8-5CAACTCTGGCAGGAGAAGCTGTCEMSA assaysEEP1sTCCCCACCCAAAATTTTCATTCTGCEP1asGCAGAATGAAAATTTTGGGTGGGGGAEP2sCTGCACAGTGATTGCCACATTCACCEP3asGGTGAATGTGGCAATCACTGTGCAGEP3asGTCTCTGGTTTCTCAACCAGGTGAATGCh-1TGCCTTCCTCTTTCCGCTTCCh-2CAGCTGCCCACAGCTTCAGCh-3GTACATGATGTGGGAAGGAGCh-4ACCTAGTGATGTGGAAGGAGMutagenesis of C/EBPβ binding sites"GCCACATTCACCTGGTTGAGAGGCTTCAGCAGCTGTAGCAACTCM3-1GCCACATTCACCTGGTTGAGGGCTCAACCAGGTGAATGTGGC	DDA8-1	CCAGCAGCCCAGAAAAAGAGCC
DDA8-3TGCCTTCCTCTTTCCGCTTCTCCDDA8-4TCCCCACCAAAATTTTCATTCTGCDDA8-5CAACTCTGGCAGGGAGAAGCTGTCEMSA assaysTCCCCACCCAAAATTTTCATTCTGCEP1sTCCCCACCCAAAATTTTCGGTGGGGAEP2sCTGCACAGTGATTGCCACATTCACCEP2asGGTGAATGTGGCAATCACTGTGCAGEP3asCATTCACCTGGTTGAGAAACCAGAGACCh-1TGCCTTCTCTCTTCCGCTTCCh-2CAGCTGCCACAGCTTCAGCh-3GTACATGATGTGGGAAGGAGCh-4ACCTAGTGTGGGAAGGAGMutagenesis of C/EBPβ binding sites*GCCACATTCACCTGGTTGAGAGCCTTCAGAGACTGTAGCAACTCM3-1GCCACATTCACCTGGTTGAGAGCTCAACCAGGTGAATGTGGGCAATGTGGGAATGTGGGAATGTGGGCAATGTGGGAAGCTGTAGCAACTCM3-1GCCACATTCACCTGGTTGAGCCTCAACCAGGTGAAGAATGTGGGCM3-2GCACATTCACGTCTCGAAGGCTCAACCAGGTGAAGAATGTGGC	DDA8-2	CTACCTGCTTTTTCCTTCTGGGCAC
DDA8-4TCCCCACCAAAATTTTCATTCTGCDDA8-5CAACTCTGGCAGGGAGAAGCTGTCEMSA assaysTCCCCACCCAAAATTTTCATTCTGCEP1sTCCCCACCCAAAATTTTGGGTGGGGGAEP2sGCAGAATGAAAATTTTGGGTGGGGGAEP2sCTGCACAGTGATGCCACATTCACCEP2asGGTGAATGTGGCAATCACTGTGCAGEP3sCATTCACCTGGTTGAGAAACCAGAGACEP3asGTCTCTGGTTTCTCAACCAGGTGAATGChIP assayTCCCTTCTCTTTCCGCTTCCh-1TGCCTTCCTCTTTCCGCTTCCh-2CAGCTGCCCACAGCTTCAGCh-3GTACATGATGTGGGAAGGAGCh-4ACCTAGTGTGGGAAGGAGMutagenesis of C/EBPβ binding sites"GCCACATTCACCTGGTTGAGAGCCTTCAGAGACTGTAGCAACTCM3-1GCCACATTCACCTGGTTGAAGGCTCAACCAGGTGAATGTGGGCAM3-2GAGTTGCTACAGTCTCTGAAGGCTCAACCAGGTGAATGTGGGCA	DDA8-3	TGCCTTCCTCTTTCCGCTTCTCC
DDA8-5CAACTCTGGCAGGGAGAAGCTGTCEMSA assaysTCCCCACCCAAAATTTTCATTCTGCEP1sTCCCCACCCAAAATTTTCGGTGGGGAEP1asGCAGAATGAAAATTTTGGGTGGGGAEP2sCTGCACAGTGATTGCCACATTCACCEP2asGGTGAATGTGGCAATCACTGTGCAGEP3sCATTCACCTGGTTGAGAAACCAGAGACEP3asGTCTCTGGTTTCTCAACCAGGTGAATGCh-1TGCCTTCCTCTTTCCGCTTCCh-2CAGCTGCCCACAGCTTCAGCh-3GTACATGATGTGGGAAGGAGCh-4ACCTAGTGTGGGAAGGAGMutagenesis of C/EBPβ binding sites"M3-1GCCACATTCACCTGGTTGAGGCCTTCAGAGACTGTAGCAACTCM3-2GAGTTGCTACAGTCTCTGAAGGCTCAACCAGGTGAATGTGGGCAACTCT	DDA8-4	TCCCCACCCAAAATTTTCATTCTGC
EMSA assaysEP1sTCCCCACCAAAATTTTCATTCTGCEP1asGCAGAATGAAAATTTTGGGTGGGGAEP2sCTGCACAGTGATTGCCACATTCACCEP2asGGTGAATGTGGCAATCACTGTGCAGEP3sCATTCACCTGGTTGAGAAACCAGAGACEP3asGTCTCTGGTTTCTCAACCAGGTGAATGChIP assayTGCCTTCCTCTTTCCGCTTCCh-1TGCCTTCCTCTTTCCGCTTCCh-2CAGCTGCCCACAGCTTCAGCh-3GTACATGATGTGGGAAGGAGCh-4ACCTAGTGTGGGAAGGAGMutagenesis of C/EBPβ binding sites <sup>a</sup> GCCACATTCACCTGGTTGAGCCTTCAGAGACTGTAGCAACTCM3-1GCCACATTCACCTGGTTGAGCCTTCAGAGACTGTAGCAACTCCM3-2GAGTTGCTACAGTCTCTGAAGGCTCAACCAGGTGAATGTGGCA	DDA8-5	CAACTCTGGCAGGGAGAAGCTGTC
EP1sTCCCCACCAAAATTTTCATTCTGCEP1asGCAGAATGAAAATTTTGGGTGGGGAEP2sCTGCACAGTGATTGCCACATTCACCEP2asGGTGAATGTGGCAATCACTGTGCAGEP3sCATTCACCTGGTTGAGAAACCAGAGACEP3asGTCTCTGGTTTCTCAACCAGGTGAATGChIP assayTCCCTCCTCTTTCCGCTTCCh-1TGCCTTCCTCTTTCCGCTTCCh-2CAGCTGCCCACAGCTTCAGCh-3GTACATGATGTGGGAAGGAGCh-4ACCTAGTGATGTGGGAAGGAGMutagenesis of C/EBPβ binding sites <sup>a</sup> GCCACATTCACCTGGTTGAGCCTTCAGAGACTGTAGCAACTCM3-1GCCACATTCACCTGGTTGAGCCTTCAGAGACTGTAGCAACTCCM3-2GAGTTGCTACAGTCTCTGAAGGCTCAACCAGGTGAATGTGGCA	EMSA assays	
EP1asGCAGAATGAAAATTTTGGGTGGGGAEP2sCTGCACAGTGATTGCCACATTCACCEP2asGGTGAATGTGGCAATCACTGTGCAGEP3sCATTCACCTGGTTGAGAAACCAGAGACEP3asGTCTCTGGTTTCTCAACCAGGTGAATGChIP assayTGCCTTCCTCTTTCCGCTTCCh-1TGCCTTCCTCTTTCCGCTTCCh-2CAGCTGCCCACAGCTTCAGCh-3GTACATGATGTGGGAAGGAGCh-4ACCTAGTGATGTGGGAAGGAGMutagenesis of C/EBPβ binding sites <sup>a</sup> GCCACATTCACCTGGTTGAGCCTTCAGAGACTGTAGCAACTCM3-1GCCACATTCACCTGGTTGAGCCTTCAGAGACTGTAGCAACTCM3-2GAGTTGCTACAGTCTCTGAAGGCTCAACCAGGTGAATGTGGGCCTCAACCAGGTGAATGTGGGCCTCAACCAGGTGAATGTGGGCCTCAACCAGGTGAATGTGGCCCTCAACCAGGTGAATGTGGCCCTCAACCAGGTGAATGTGGCCCTCAACCAGGTGAATGTGGCCCCCACAGGTGAATGTGGCCCCCACAGGTGAATGTGGCCCCCACAGGTGAATGTGGCCCCCACAGGTGAATGTGGCCCCCACAGGTGAATGTGGCCCCCACAGGTGAATGTGGCCCCCACAGGTGAATGTGGCCCCACAGGTGAATGTGGCCCCCACAGGTGAATGTGGCCCCCACAGGTGAATGTGGCCCCCACAGGTGAATGTGGCCCCCACAGGTGAATGTGGCCCCCACAGGTGAATGTGGCCCCCACAGGTGAATGTGGCCCCACCAGGTGAATGTGGCCCCCACCAGGTGAATGTGGCCCCCACCAGGTGAATGTGGCCCCCCACCAGGTGAATGTGGCCCCCACCAGGTGAATGTGGCCCCCACCAGGTGAATGTGGCCCCCCACCAGGTGAATGTGGCCCCCACCAGGTGAATGTGGCCCCCCACCAGGTGAATGTGGCCCCCCACCAGGTGAATGTGGCCCCCCCC	EP1s	TCCCCACCCAAAATTTTCATTCTGC
EP2sCTGCACAGTGATTGCCACATTCACCEP2asGGTGAATGTGGCAATCACTGTGCAGEP3sCATTCACCTGGTTGAGAAACCAGAGACEP3asGTCTCTGGTTTCTCAACCAGGTGAATGChIP assayTGCCTTCCTCTTTCCGCTTCCh-1TGCCTTCCTCTTTCCGCTTCCh-2CAGCTGCCCACAGCTTCAGCh-3GTACATGATGTGGGAAGGAGCh-4ACCTAGTGATGTGGGAAGGAGMutagenesis of C/EBPβ binding sites <sup>a</sup> GCCACATTCACCTGGTTGAGCCTTCAGAGACTGTAGCAACTCM3-1GCCACATTCACCTGGTTGAGCCTTCAGAGACTGTAGCAACTCM3-2GAGTTGCTACAGTCTCTGAAGGCTCAACCAGGTGAATGTGGCA	EP1as	GCAGAATGAAAATTTTGGGTGGGGA
EP2asGGTGAATGTGGCAATCACTGTGCAGEP3sCATTCACCTGGTTGAGAAACCAGAGACEP3asGTCTCTGGTTTCTCAACCAGGTGAATGChIP assayTGCCTTCCTCTTTCCGCTTCCh-1TGCCTTCCTCTTTCCGCTTCCh-2CAGCTGCCACAGCTTCAGCh-3GTACATGATGTGGGAAGGAGCh-4ACCTAGTGATGTGGACATTACMutagenesis of C/EBPβ binding sites <sup>a</sup> GCCACATTCACCTGGTTGAGCCTTCAGAGACTGTAGCAACTCM3-1GCCACATTCACCTGGTTGAGCCTTCAGAGACTGTAGCAACTCM3-2GAGTTGCTACAGTCTCTGAAGGCTCAACCAGGTGAATGTGGC	EP2s	CTGCACAGTGATTGCCACATTCACC
EP3sCATTCACCTGGTTGAGAAACCAGAGACEP3asGTCTCTGGTTTCTCAACCAGGTGAATGChIP assayTGCCTTCCTCTTTCCGCTTCCh-1TGCCTTCCTCTTTCCGCTTCCh-2CAGCTGCCCACAGCTTCAGCh-3GTACATGATGTGGGAAGGAGCh-4ACCTAGTGATGTGGACATTACMutagenesis of C/EBPβ binding sites <sup>a</sup> GCCACATTCACCTGGTTGAGCCTTCAGAGACTGTAGCAACTCM3-1GCCACATTCACCTGGTTGAGCCTTCAGAGACTGTAGCAACTCM3-2GAGTTGCTACAGTCTCTGAAGGCTCAACCAGGTGAATGTGGC	EP2as	GGTGAATGTGGCAATCACTGTGCAG
EP3asGTCTCTGGTTTCTCAACCAGGTGAATGChIP assayTGCCTTCTTTCCGCTTCCh-1TGCCTTCCTCTTTCCGCTTCCh-2CAGCTGCCACAGCTTCAGCh-3GTACATGATGTGGGAAGGAGCh-4ACCTAGTGATGTGGACATTACMutagenesis of C/EBPβ binding sites <sup>a</sup> GCCACATTCACCTGGTTGAGCCTTCAGAGACTGTAGCAACTCM3-1GCCACATTCACCTGGTTGAGCCTTCAGAGACTGTAGCAACTCM3-2GAGTTGCTACAGTCTCTGAAGGCTCAACCAGGTGAATGTGGC	EP3s	CATTCACCTGGTTGAGAAACCAGAGAC
ChIP assayTGCCTTCCTCTTTCCGCTTCCh-1TGCCTTCCTCTTTCCGCTTCCh-2CAGCTGCCACAGCTTCAGCh-3GTACATGATGTGGGAAGGAGCh-4ACCTAGTGATGTGGACATTACMutagenesis of C/EBPβ binding sites <sup>a</sup> GCCACATTCACCTGGTTGAGCCTTCAGAGACTGTAGCAACTCM3-1GCCACATTCACCTGGTTGAGCCTTCAGAGACTGTAGCAACTCM3-2GAGTTGCTACAGTCTCTGAAGGCTCAACCAGGTGAATGTGGGC	EP3as	GTCTCTGGTTTCTCAACCAGGTGAATG
Ch-1TGCCTTCCTCTTTCCGCTTCCh-2CAGCTGCCACAGCTTCAGCh-3GTACATGATGTGGGAAGGAGCh-4ACCTAGTGATGTGGACATTACMutagenesis of C/EBPβ binding sitesaGCCACATTCACCTGGTTGAGCCTTCAGAGACTGTAGCAACTCM3-1GCCACATTCACCTGGTTGAGCCTTCAGAGACTGTAGCAACTCM3-2GAGTTGCTACAGTCTCTGAAGGCTCAACCAGGTGAATGTGGC	ChIP assay	
Ch-2CAGCTGCCCACAGCTTCAGCh-3GTACATGATGTGGGAAGGAGCh-4ACCTAGTGATGTGGACATTACMutagenesis of C/EBPβ binding sitesaGCCACATTCACCTGGTTGAGCCTTCAGAGACTGTAGCAACTCM3-1GCCACATTCACCTGGTTGAGCCTTCAGAGACTGTAGCAACTCM3-2GAGTTGCTACAGTCTCTGAAGGCTCAACCAGGTGAATGTGGC	Ch-1	TGCCTTCCTCTTTCCGCTTC
Ch-3GTACATGATGTGGGAAGGAGCh-4ACCTAGTGATGTGGACATTACMutagenesis of C/EBPβ binding sitesaGCCACATTCACCTGGTTGAGCCTTCAGAGACTGTAGCAACTCM3-1GCCACATTCACCTGGTTGAGCCTCAGAGACTGTAGCAACTCM3-2GAGTTGCTACAGTCTCTGAAGGCTCAACCAGGTGAATGTGGC	Ch-2	CAGCTGCCCACAGCTTCAG
Ch-4ACCTAGTGATGTGGACATTACMutagenesis of C/EBPβ binding sitesaGCCACATTCACCTGGTTGAGCCTTCAGAGACTGTAGCAACTCM3-1GCCACATTCACCTGGTTGAGCCTTCAGAGACTGTAGCAACTCM3-2GAGTTGCTACAGTCTCTGAAGGCTCAACCAGGTGAATGTGGC	Ch-3	GTACATGATGTGGGAAGGAG
Mutagenesis of C/EBPβ binding sites <sup>a</sup> GCCACATTCACCTGGTTGAGCCTTCAGAGACTGTAGCAACTC         M3-1       GCCACATTCACCTGGTTGAGCCTTCAGAGACTGTAGCAACTC         M3-2       GAGTTGCTACAGTCTCTGAAGGCTCAACCAGGTGAATGTGGC	Ch-4	ACCTAGTGATGTGGACATTAC
M3-1GCCACATTCACCTGGTTGAGCCTTCAGAGACTGTAGCAACTCM3-2GAGTTGCTACAGTCTCTGAAGGCTCAACCAGGTGAATGTGGC	Mutagenesis of C/EBPβ binding sites <sup>a</sup>	
M3-2 GAGTTGCTACAGTCTCTGAAGGCTCAACCAGGTGAATGTGGC	M3-1	GCCACATTCACCTGGTTGAGCCTTCAGAGACTGTAGCAACTC
	M3-2	GAGTTGCTACAGTCTCTGAAGGCTCAACCAGGTGAATGTGGC

<sup>a</sup>Mutated nucleotides are shown in bold. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; F, forward; R, reverse; ACTB,  $\beta$ -actin; EMSA, electrophoretic mobility shift assays; ChIP, chromatin immunoprecipitation; C/EBP $\beta$ , CCAAT/enhancer binding protein  $\beta$ .

Chromatin immunoprecipitation (ChIP) assays. ChIP assays were performed using the Magna ChIP<sup>TM</sup> A/G kit (EMD Millipore, Billerica, MA, USA) according to the manufacturer's protocol. Antibodies against C/EBP $\beta$  (cat. no. 23431-1-AP; Wuhan Sanying Biotechnology) were combined with protein A/G magnetic beads and were then incubated for 4 h at 4°C and rotated. Normal mouse IgG (Santa Cruz Biotechnology, Inc.) was used as the negative control. Elution of the protein/DNA complexes and reverse cross-links of the protein/DNA complexes to free DNA

were then performed. For amplification of the *S100A8* promoter (-193/-45), the Ch-1/Ch-2 primer pairs (Table I) were used. Serving as the control for the absence of C/EBP $\beta$  binding sites, the Ch-3/Ch-4 primer pairs (Table I) were used to amplify the upstream fragment of human *S100A8* (-2861/-2723). The thermocycling conditions were as follows: 2 min at 94°C, followed by 32 cycles at 94°C for 20 sec, 59°C for 30 sec, and 72°C for 30 sec, and then a final extension at 72°C for 3 min. The PCR products were detected by 1.5% agarose gel electrophoresis.

Western blot analysis. The cells were washed with Dulbecco's phosphate-buffered saline (Gibco; Thermo Fisher Scientific, Inc.) and were then extracted using mammalian cell lysate buffer (Biyuntian). The cell extracts were centrifuged at 12,000 x g for 5 min at 4°C and the supernatants were collected. The protein concentrations were determined using a bicinchoninic acid protein concentration detection kit (Biyuntian). The cell extracts (20 µg protein) were separated by 12% DS-PAGE, transferred onto 0.22-µM nitrocellulose membranes, and incubated overnight at 4°C with rabbit anti-myeloid-related protein-8 (an alias of \$100A8; cat. no. ab196689, Abcam, Cambridge, MA, USA) or mouse anti-\beta-actin (cat. no. TA-09, OriGene Technologies, Inc., Beijing, China) at 1:2,000 dilution. The membranes were washed and then incubated 2 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit antibodies or goat anti-mouse antibodies (cat. nos. EM35111-01 and EM35110-01, EMAR Biotechnology, Beijing, China) at 1:3,000 dilution. The immunoreactions were visualized using Clarity<sup>TM</sup> Western ECL substrate (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and exposed to Amersham Hyperfilm ECL film (Amersham; GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). The protein bands were evaluated using Quantity One software (version 4.6.5, Bio-Rad Laboratories, Inc.).

*Bioinformatics analysis and statistical analysis.* TRANSFAC 7 (http://gene-regulation.com/pub/databases.html) online prediction software was used to analyze transcription factor binding for *S100A8* promoter region. Between three and six independent experiments were conducted in the present study. Statistical analysis was performed with SPSS version 19.0 (IBM Corp., Armonk, NY, USA). Comparisons between two groups were performed with Student's t-test, and multiple comparisons were conducted with one-way analysis of variance followed by Bonferroni's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

# Results

IL-1 $\alpha$  activates the expression of S100A8 in TR146 epithelial cells. The effect of IL-1 $\alpha$  on the expression of S100A8 in human TR146 epithelial cells was investigated by RT-qPCR and western blot analyses. The results revealed that treatment with various concentrations (5-100 ng/ml) of IL-1 $\alpha$ significantly upregulated the expression levels of S100A8 at the mRNA level (Fig. 1A). The maximal induction effects were observed with 10-50 ng/ml IL-1 $\alpha$ . The results also indicated that the inductive effects on the expression of S100A8 induced by various concentrations (5-100 ng/ml) of IL-1 $\alpha$  were detected at the protein level (Fig. 1B). Taken together, these results suggested that IL-1 $\alpha$  significantly induced the expression of S100A8 in human TR146 epithelial cells through a mechanism associated with transcriptional regulation.

Promoter region of -165/-111 is responsible for the upregulation of S100A8 by IL-1a treatment. To localize the promoter region that is responsible for the upregulation of S100A8 induced by IL-1a treatment, a series of promoter fragments of S100A8 were cloned into a luciferase reporter gene vector, pGL3-basic, to generate several deletion mutants. Following



Figure 1. Induction of the expression of *S100A8* by IL-1 $\alpha$  in TR146 epithelial cells. (A) RT-qPCR analysis of the mRNA expression of *S100A8*;  $\beta$ -actin was used as the internal control. (B) Western blot analysis was conducted to analyze the protein expression of S100A8. TR146 cells were treated with vehicle (50  $\mu$ g/ml bovine serum albumin in Dulbecco's phosphate-buffered saline) or various concentrations of IL-1 $\alpha$  in fresh medium and incubated for 24 h, following which the cells were collected for RT-qPCR or western blot analyses. Bars represent the mean  $\pm$  standard error of the mean from three to six determinations. \*P<0.05 vs. control. IL, interleukin; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

the transfection of these constructs into TR146 cells, the cells were treated with IL-1 $\alpha$ , and were then collected for luciferase activity assays. The results revealed that IL-1 $\alpha$ treatment significantly enhanced promoter activity following transfection with the pGL3 (-1887/-1), pGL3 (-929/-1), pGL3 (-678/-1), pGL3 (-419/-1) and pGL3 (-243/-1) constructs, but not with the pGL3 (-72/-1) construct (Fig. 2A). These results indicated that the promoter region potentially responsible for the induced gene expression of S100A8 by IL-1a may be located in the-243/-72 promoter region. In order to further locate the associated promoter region for the induced gene expression of S100A8 by IL-1 $\alpha$ , a series of deletion mutants were also constructed. The luciferase assays showed that IL-1a treatment significantly induced promoter activity for the pGL3 (-257/-1), pGL3 (-232/-1), pGL3 (-193/-1) and pGL3 (-165/-1) reporter gene constructs, but not for the pGL3 (-111/-1) reporter gene construct (Fig. 2B). Taken together, these results suggested that the -165/-111 promoter region of S100A8 may be responsible for the inductive effects of IL-1 $\alpha$ .

IL-1 $\alpha$  treatment induces the binding of C/EBP $\beta$  to a specific site in the promoter region. As IL-1 $\alpha$  can affect gene transcription via the transcription factor C/EBP $\beta$  (22,23), the present study analyzed the -165/-111 promoter region of human S100A8 using online prediction software for transcription factor binding, TRANSFAC 7 (http://gene-regulation.com/pub/databases.html) (24). The results revealed that there are three potential transcription factor C/EBP $\beta$  binding sites in this region. Subsequently, three pairs of EMSA primers (-166/-142 for E1; -145/-121 for E2 and -128/-102 for E3) were designed to detect whether IL-1 $\alpha$  treatment affects the combination of



Figure 2. Localization of the promoter region responsible for the upregulation of *S100A8* induced by IL-1 $\alpha$  in TR146 cells. (A) Deletion analysis for the promoter -1887/-1 region. (B) Deletion analysis for the promoter -257/-1 region. Constructs were transfected into TR146 cells for 10 h, followed by the addition of 20 ng/ml IL-1 $\alpha$ . After 12 h, the cells were collected for the luciferase activity assay. Bars represent the mean ± standard error of the mean from three to six determinations. \*P<0.05 vs. control. NS, not significant; IL, interleukin.

transcription factors to these primers (Fig. 3A). No significant differences were observed when comparing C/EBP $\beta$  binding and alterations in IL-1 $\alpha$  treatment with E1 primers (Fig. 3B). In addition, the EMSA results revealed no significant difference in C/EBP $\beta$  binding following IL-1 $\alpha$  treatment with the E2 primer (Fig. 3C). Notably, the results demonstrated that the binding activity between the C/EBP $\beta$  binding site (-113/-109) in E3 primer pairs and the transcription factor C/EBP $\beta$  were significantly enhanced following IL-1 $\alpha$  treatment (Fig. 3D). In addition, the ChIP assay verified the binding activity between C/EBP $\beta$  and the-193/-45 promoter region *in vivo* (Fig. 3E). These results suggested that the C/EBP $\beta$  binding site (-113/-109) of the *S100A8* gene promoter may be associated with the upregulatory effect on the expression of *S100A8* induced by IL-1 $\alpha$ .

C/EBP $\beta$  is critical in the process of S100A8 activation induced by IL-1 $\alpha$ . Finally, to further elucidate the role of the transcription factor C/EBP $\beta$  binding site (-113/-109) in the S100A8 gene promoter, the pGL3 (-257/-1) reporter gene vector was used as a template to generate the mutated C/EBP $\beta$ binding site construct pGL3 (-257/-1)-M3 (mutation at the -113/-109 site) (Fig. 4A). Following transfection with these constructs, the cells were collected subsequent to IL-1 $\alpha$  treatment, and the effect of IL-1 $\alpha$  treatment on S100A8 promoter activity was analyzed. The results demonstrated that the inductive effect of IL-1 $\alpha$  treatment was attenuated following pGL3(-257/-1)-M3 transfection, whereas the inductive effect remained following of pGL3(-257/-1) transfection (Fig. 4B). By contrast, silencing C/EBP $\beta$  significantly decreased *S100A8* promoter activity following pGL3(-419/-1) construct transfection (Fig. 4C and D). Taken together, these results suggested that activation of the expression of *S100A8* induced by IL-1 $\alpha$  in TR146 epithelial cells may involve a mechanism associated with the increased binding activity of C/EBP $\beta$  to a specific site (-113/-109) of the *S100A8* promoter.

#### Discussion

Due to the important roles of human S100A8 in infectious diseases and tumors (1-8,11,12), a number of studies have investigated the mechanism underlying the transcriptional regulation of human S100A8 (15-17,25-27). The upregulation of S100A8 by fibroblast growth factor-2 and IL-1 $\beta$ , and its downregulation by transforming growth factor- $\beta$  in murine fibroblasts has been previously observed (25). Through activation of the protein kinase A signaling pathway and subsequent stimulation of C/EBPβ binding to the S100A8 promoter, prostaglandin E2 has been reported to upregulate the expression of human S100A8 (15). Mechanistically, the process of the induced expression of S100A8 by glucocorticoids was positively regulated by protein kinase A and negatively regulated by protein kinase C (26). Glucocorticoids increase the transcription and mRNA half-life of human S100A8; the upregulation process requires new protein synthesis, IL-10, products of the cyclooxygenase-2 pathway, and both the extracellular signal-regulated kinase (ERK)-1/2 and p38 MAPK signaling pathways (26). Furthermore, the expression of human S100A8 is induced by polyinosinic:polycytidylic acid, a



Figure 3. Induction of C/EBP $\beta$  binding to the specific site in the *S100A8* promoter region by IL-1 $\alpha$ . (A) Oligonucleotide sequences used for EMSA analysis. Bold letters indicate the potential binding sites for transcription factor C/EBP $\beta$ . (B) Promoter region (-166/-142) sequence used for EMSA analysis. (C) Promoter region (-145/-121) sequence used for EMSA analysis. (D) Promoter region (-128/-102) sequence used for EMSA analysis. (E) C/EBP $\beta$  binding to the *S100A8* promoter *in vivo*. Chromatin immunoprecipitation assays were performed using DNA from TR146 cells and specific antibodies. Compared with the competitive assay (left-hand panels of B-D), a longer exposure time was performed for the supershift assay (right-hand panels of B-D). Lanes: 1, no IL-1 $\alpha$ ; 2, IL-1 $\alpha$ ; 3, 200-fold cold oligo; 4, no antibody (negative control); 5, C/EBP $\beta$  antibody; 6, C/EBP $\alpha$  antibody. Square brackets indicate the position of the DNA/protein complex involving C/EBP $\beta$ . \*Positions of supershift bands. At least three experiments were performed. NE, nuclear extracts; Input, sheared DNA prior to immunoprecipitation was used for amplification; C/EBP $\beta$ , CCAAT/enhancer binding protein  $\beta$ ; EMSA, electrophoretic mobility shift assays.



Figure 4. Role of C/EBP $\beta$  in the activation of *S100A8* expression induced by IL-1 $\alpha$ . (A) Schematic illustration of the C/EBP $\beta$  binding site and its mutation in the *S100A8* promoter region, the C/EBP $\beta$  binding site (-113/-109) is underlined. The mutated oligos in the binding site are shown in bold. (B) Mutational analysis of the C/EBP $\beta$  binding site (-113/-109) in the *S100A8* promoter region and its effect on *S100A8* promoter activity. (C) siRNA silencing of C/EBP $\beta$  downregulated the expression of the protein. (D) siRNA silencing of C/EBP $\beta$  attenuated *S100A8* promoter activity. pGL3 (-419/-1) was used. Constructs were transfected into TR146 cells for 10 h, followed by the addition of 20 ng/ml IL-1 $\alpha$ . After 12 h, the cells were collected for luciferase activity assays. Bars represent the mean  $\pm$  standard error of the mean from three to six determinations. \*P<0.05 vs. control. NS, not significant; C/EBP $\beta$ , CCAAT/enhancer binding protein  $\beta$ ; IL-, interleukin; siRNA, small interfering RNA.

double strand RNA mimetic, and its induction is dependent on the p38, ERK MAPK and protein kinase R-dependent signaling pathways (27). Notably, the p38 MAPK signaling pathway is critical in the process of tumor necrosis factor- $\alpha$ - and IL-17A-induced expression of S100A8 in human keratinocytes (28).

It has been demonstrated that IL-1 $\alpha$  can affect gene transcription by increasing C/EBPβ-dependent transcriptional activity (22,23). A previous report also revealed that IL-1 $\alpha$  promotes the expression of stromal-derived factor-1 in vascular smooth muscle cells by upregulating C/EBPB in an inhibitor of NF- $\kappa$ B kinase  $\beta$  signaling-dependent manner (29). An IL-1 $\alpha$ -induced increase in the binding of C/EBP $\beta$  to the 11β-hydroxysteroid dehydrogenase type 1 P2 promoter in human A549 epithelial cells has also been reported (30). In the present study, the results revealed that IL-1 $\alpha$  treatment induced the expression of S100A8 in TR146 epithelial cells, and the inductive effect occurred at the transcriptional level. In addition, the activated expression of S100A8 induced by IL-1 $\alpha$ in TR146 epithelial cells may involve a mechanism associated with increasing the binding activity of C/EBP $\beta$  to a specific site (-113/-109) of the S100A8 promoter. Overall, the results of the present study are consistent with the findings reported in previous studies (22,23,29,30). Taken together, these similar findings support the hypothesis that IL-1 $\alpha$  increases the binding activity of the transcription factor C/EBPB to the promoter of specific genes and this may be a common regulatory mechanism that affects target gene expression.

In conclusion, the present study provided novel mechanistic insights into the transcriptional regulation of human S100A8 in TR146 epithelial cells. However, the detailed molecular mechanism requires further clarification.

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#### Availability of data and materials

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

## **Authors' contributions**

MQ, YZ, KZ and XZ made substantial contributions to the conception and design of the study. YG and XZ analyzed the results. MQ, YG and XZ drafted the manuscript. All the authors read and approved the final manuscript.

### Ethics approval and consent to participate

Not applicable.

#### Patient consent for publication

Not applicable.

# **Competing interests**

The authors confirm that they have no competing interests.

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