

Bone Marrow Mesenchymal Stem Cells Derived miRNA-130b Enhances Epithelial Sodium Channel by Targeting PTEN

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Research

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Bone marrow mesenchymal stem cells derived miRNA-130b enhances epithelial sodium channel by targeting PTEN

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Abstract

Aims: Acute lung injury (ALI) is a clinical syndrome with high morbidity and mortality, and severe pulmonary edema is one of the characteristics of ALI. Epithelial sodium channel (ENaC) located on the apical side of alveolar type 2 epithelial (AT2) cells is the primary rate limiting segments in alveolar fluid clearance. Many preclinical studies have revealed that mesenchymal stem cells (MSCs) based therapy has great therapeutic potential for ALI, while the role of ENaC in this process is rarely known.

Methods: We studied the effects of bone marrow-derived MSCs (BMSCs) on the protein/mRNA expression and activity of ENaC in primary mouse AT2 and H441 cells by co-culture with them, respectively. Moreover, the changes of miRNA-130b in AT2 cells were detected by qRT-PCR, and we studied the involvement of phosphatase and tensin homolog deleted on chromosome ten (PTEN) in miRNA-130b regulated ENaC.

Results: Our results demonstrated that BMSCs could increase the expression of miRNA-130b, which showed adverse effects on the protein expression of α/γ -ENaC and PTEN in AT2 cells. SiRNA-mediated downregulation of PTEN could increase the protein expression of α/γ -ENaC in AT2 cells, supporting PTEN as a negative regulator of ENaC.

Conclusion: In summary, miRNA-130b in BMSCs can enhance ENaC at least partially by targeting PTEN, which may provide a promising new direction for therapeutic strategy in ALI.

Keywords: epithelial sodium channels, bone marrow mesenchymal stem cells, miRNA-130b, acute lung injury

Introduction

Acute lung injury (ALI) is a common clinical syndrome with high morbidity and mortality caused by sepsis, pneumonia, trauma, *etc* [1, 2]. ALI and acute respiratory distress syndrome (ARDS) are characterized by an inflammatory response, alveolar edema, and hypoxemia. Approximately 40% of the ALI/ARDS patients are linked with viral and bacterial pneumonia [3, 4]. Lipopolysaccharide (LPS) is widely used to induce ALI models, since LPS can attack pulmonary microvascular endothelial cells, resulting in leakage of protein-rich edema fluid related with pulmonary endothelial cell injury, barrier dysfunction and inflammation [5]. Enhanced alveolar fluid clearance (AFC) could accelerate the clearance of edematous fluid accumulated in the alveoli [6]. In the lungs, amiloride-sensitive epithelial sodium channel (ENaC) is the primary determinant of AFC, a driving force to remove edema fluid from alveolar spaces on the ion transport-dependent mechanism [7, 8]. During the recovery of ALI, edematous fluid can be reabsorbed to the interstitium either by paracellular pathways or by diffusion driven by an osmotic gradient that is established by active apical Na^+ uptake, in part by the ENaC and Na^+ transport through the Na^+/K^+ -ATPase pumps [9]. There are mainly 3 subunits (α , β , and γ) to make up ENaC which transports Na^+ from apical to basolateral side of alveolar epithelial cells and regulates the transport of water [10, 11]. The α -subunit is required for Na^+ conductance, while β - and γ -subunits are needed to enhance the channel activity [12].

Mesenchymal stem cells (MSCs) are pluripotent stem cells, which are characterized by their ability to differentiate into multiple cell lines and exert antiproliferative, immunomodulatory and anti-inflammatory effects [13]. MSCs have the ability to regulate the immune response to tissue damage and promote repair *in vivo*, and have been suggested to act on a variety of lung diseases (including ALI) [14]. In addition, MSCs can release a variety of cytokines and growth factors, signal lipids, exosomes and microRNAs (miRNAs, miRs), many of which may be involved in a variety of lung diseases [15-17]. By performing microarray hybridization, Chen TS tested the RNAs for the presence of miRNAs in MSC, which expressed at least 151

miRNAs including some passenger miRNA sequences [18]. We have previously testified several miRNAs, including miR-124-5p, miR-130b, and so on. In our recently published paper, we investigated miR-124-5p, which existed in MSCs-conditioned medium, and found that the miR-124-5p was involved in the regulation of MSCs-conditioned medium in LPS-induced ALI by targeting α -ENaC [19]. Related studies have shown that miR-130b in MSCs can regulate the inflammatory response, cell function and gene expression of various diseases [20-22], whereas its role in ALI and the relative mechanisms are seldom studied. Therefore, we chose miR-130b as our main research object and co-cultured with AT2 cells to explore its role in the regulation of MSCs in LPS-induced ALI, aiming to provide a novel direction for therapeutic strategy in ALI.

Several bioinformatic websites such as TargetScan (<http://www.targetscan.org/>), PicTar (<http://pictar.mdc-berlin.de/>), and miRBase (<http://www.mirbase.org/>) were used to predict the targets of miR-130b, and a few targets were therefore found, such as phosphatase and tensin homolog deleted on chromosome ten (PTEN), IGF-1, PPAR- γ and CSF-1, etc. Moreover, PTEN was reported to be the target of miR-130b and a negative regulator in the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway that can up-regulate ENaC [23], which is the key step for the edematous fluid accumulation in ALI [24]. Thus we chose PTEN as the interested gene of miR-130b to study its role in pulmonary fluid transport accordingly. We speculate that BMSCs may affect ENaC through miR-130b targeting PTEN, and thus have a certain therapeutic effect on ALI.

Materials and methods

BMSC culture

All experimental protocols relating to C57 mice were performed according to the guidelines and regulations of Animal Care and Use Ethics Committee, and were approved by China Medical University (No.CMU2019088). The isolation and culture method of BMSCs has been described previously [25]. In

brief, the femora were removed from C57 male mice and the medullary cavity of femora was washed with DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS, Gibco, New York, NY, USA), 10 ng/mL recombinant mouse basic fibroblast growth factor (PeproTech, Rocky Hill, NJ, USA), 100 IU penicillin, and 100 µg/mL streptomycin, then the bone marrow was collected. After the cell suspension was mixed, the cells were cultured in 5% CO₂-95% air at 37°C for 24 h, and then the medium was changed to remove the non-adherent tissues and cells.

Alveolar type 2 epithelial cell culture

Alveolar type 2 epithelial (AT2) cells were isolated and cultured as previously described [19]. Isolated lungs from newborn mice (within 24 h) were separated by lobes in cold PBS. The lung tissue was digested with trypsin and collagenase (Sigma, Saint Louis, MO, USA) for 30 min, respectively. Cells were filtrated and cultured in 5% CO₂, 37°C atmosphere in DMEM/F12 medium (containing 10% FBS, 100 IU penicillin, and 100 µg/ml streptomycin) for 45 min. Unattached cells were collected and the above culture process was repeated 4 times to remove lung fibroblast cells. Then, the cell suspension was transferred to the culture dish coated IgG and incubated for 30 min to remove lymphocytes, macrophages, and neutrophils. Finally, unattached cells were adjusted to $2-3 \times 10^6$ /ml and the medium was changed after 72 h for the first time and then changed every other day.

Co-culture of BMSCs and AT2 cells

BMSCs were passaged after 80% confluence and the cells of the 2nd and 3rd passages were used to culture in 24-mm diameter Transwell inserts. After 24 h, the BMSC inserts were transferred to 6-well plates with co-cultured AT2 cells at the bottom, both the inserts and lower culture wells were washed three times with PBS, and then the medium was switched to DMEM/F12 without FBS for 24 h.

Ussing chamber assay

H441 cells obtained from the American Type Culture Collection were seeded onto 6.5-mm diameter mouse

tail collagen I pre-coated Transwell inserts ($\sim 6 \times 10^6$ cells/cm²), and cultured with RPMI-1640 medium containing 10% FBS, 100 IU penicillin, 100 μ g/ml streptomycin, 10 μ g/ml insulin, and 50 nM dexamethasone in 5% CO₂-95% air at 37°C. After 24 h, the medium and nonadherent cells in the apical compartment were removed to adapt the cells to air-liquid interface culture, and the medium in the basolateral compartment was replaced. Trans-epithelial electrical resistance was monitored by an epithelial volt-ohm-meter (WPI, Sarasota, FL, USA), and cell-growing inserts with a resistance $> 400 \Omega \text{ cm}^2$ were used.

Measurements of trans-epithelial electrical resistance and short-circuit current (*I_{sc}*) were performed as previously described [26]. Briefly, H441 monolayers were mounted in ussing chambers (Physiologic Instruments, San Diego, CA, USA) and bathed on both sides with solutions containing (in mM) 120 NaCl, 25 NaHCO₃, 3.3 KH₂PO₄, 0.83 K₂HPO₄, 1.2 CaCl₂, 1.2 MgCl₂, 10 HEPES, and 10 mannitol (apical compartment)/10 glucose (basolateral compartment). The trans-epithelial *I_{sc}* level was measured with 3M KCl, 4% agarose salt bridges placed 3 mm on either side of the membrane, which were connected on either side to Ag-AgCl electrodes. Both sides with the previously mentioned bath solution (pH 7.4) as designed were bubbled continuously with 95% O₂-5% CO₂ gas mixture and the temperature was 37°C. H441 monolayers were short circuited to 0 mV, and *I_{sc}* level was measured with an epithelial voltage clamp. A 10 mV pulse of 1 s duration was imposed every 10 s to monitor trans-epithelial electrical resistance. When the *I_{sc}* was stable, 100 μ M amiloride was pipetted into the apical side. Data were collected using the Acquire and Analyse program version 2.3.

Cell transfection

PTEN-siRNA (siPTEN), miR-130b mimic (Mimic), miR-130b inhibitor (Inhibitor), negative control (NC, the negative control of miR-130b mimic or PTEN-siRNA), inhibitor NC (the negative control of miR-130b inhibitor), and siRNA-mate were purchased from GenePharma (Shanghai, China). The final concentration of

miR-130b mimics, miR-130b inhibitors, and PTEN-siRNA were 30 nM, 60 nM, and 200 nM, respectively.

All transfection reagents were removed after 6 h and cells were used 72 h after transfection.

Western blot assays

The cell lysates were separated by SDS-PAGE (10% polyacrylamide gels) and transferred onto PVDF membrane. Membrane blockade was blocked with 5% BSA for 1 h at room temperature, and then incubated with diluted primary antibodies overnight: α -ENaC (1:2000, PA1-920A, Thermo Fisher, Waltham, MA, USA), γ -ENaC (1:2000, ab3468, Abcam, Cambridge, MA, USA), PTEN (1:1000, 9552S, Abcam, Cambridge, MA, USA) and β -actin (1:1000, sc-47778, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were washed three times and incubated with HRP conjugated goat-anti-rabbit or goat-anti-mouse secondary antibody (1:5000, ZSGB-BIO, Beijing, China) at room temperature for 1 h. The protein bands were visualized using ECL kit on a Tanon-5200 chemiluminescence detection system (Tanon, Shanghai, China), and the intensity of each specific band was quantified with Image J program.

Quantitative real-time PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and quantified by NanoDrop 2000C spectrophotometer (Thermo, Wilmington, DE, USA). In brief, total RNA and miRNA were synthesized into cDNA using PrimeScript RT reagent kit with gDNA Eraser and Mir-X miRNA First-Strand Synthesis Kit (TaKaRa, Kusatsu, Shiga, Japan). Quantitative real-time PCR (qRT-PCR) was then applied using SYBR Premix Ex Taq II (TaKaRa, Kusatsu, Shiga, Japan) in the ABI 7500 qRT-PCR System with the following primers: α -ENaC forward (5'-AAC AAA TCG GACTGC TTC TAC-3') and reverse (5'-AGC CAC CAT CAT CCA TAA A-3'), β -ENaC forward (5'-GGG ACC AAA GCA CCA AT-3') and reverse (5'-CAG ACG CAG GGA GTC ATAG-3'), γ -ENaC forward (5'-GCACCG TTC GCC ACC TTC TA-3') and reverse (5'-AGG TCA CCA GCA GCT CCT CA-3'), and GAPDH forward (5'-AGA AGG CTG GGG CTC ATT TG-3') and reverse (5'-AGG GGC CAT CCA CAG TCT TC-3'). Relative expression of mRNA/miRNA

was calculated using the $2^{-\Delta(\Delta CT)}$ method, and GAPDH/U6 was used as a reference.

Statistical analysis

Data were expressed as the mean \pm SE. We evaluated the power of sample size first to meet $P < 0.05$. Normality and homoscedasticity test was done by Levene and Shapiro-Wilk test before applying parametric tests. For comparison of two groups, we used Student's two-tailed t-test; for comparison of multiple groups, we performed one-way analysis of variance (ANOVA) followed by Bonferroni's test for all the groups of the experiment. When the data did not pass the normality or homoscedasticity test, we used a non-parametric t-test (Mann-Whitney U test). Statistical analysis was performed with Origin 8.0.

Results

BMSCs increase α/γ -ENaC protein and mRNA expression

ENaC is mainly composed of α , β and γ subunits, that are all indispensable for efficient AFC [27]. To investigate the effect of BMSCs on the expression of ENaC in AT2 cells, we applied Western blot and qRT-PCR assays in normal or LPS-treated AT2 cells co-cultured with BMSCs. The data of the other groups in the same experiment were compared with that of the Control group (divided by the Control value). Firstly, BMSCs could increase the expression of α/γ -ENaC protein in both normal and LPS-treated AT2 cells (Fig. 1A-C), indicating the beneficial effects of BMSCs in ALI. Due to the lack of suitable antibody for the Western blot assay, we didn't detect the β -ENaC expression. Furthermore, the results of qRT-PCR verified that BMSCs enhanced protein expression of ENaC was due to the higher transcription level in AT2 cells after LPS administration (Fig. 1D-F). BMSCs can enhance both the protein and mRNA expression of ENaC, supporting our hypothesis that ENaC is involved in the protective effects of BMSCs in ALI.

BMSCs enhance amiloride-sensitive Isc in H441 monolayers

Human bronchoalveolar epithelial-derived Clara (H441) cells have been extensively applied in studying the function of ENaC in the lung, and ENaC properties of H441 are similar to those of primary AT2 cells [28], which could hardly grow into monolayers. To further confirm the regulation of BMSCs on ENaC activity, we measured *I_{sc}* in confluent H441 monolayers. As shown in Fig. 2A-B, amiloride-sensitive *I_{sc}* (ASI) was defined as the difference between the total current and the amiloride-resistant current, and BMSCs significantly rescued the ASI reduction induced by LPS for 12 h. The above data further indicate that BMSCs can promote the ion transport of lung epithelium and possible edema fluid absorption, through increasing ENaC activity both under normally physiological and LPS-induced pathological conditions.

MiR-130b in BMSCs increase α/γ -ENaC protein expression

Growing evidence has indicated that miRNAs secreted by MSCs played a role in the treatment of lung diseases [29]. Compared with Control group that expressed miR-130b in normal AT2 cells, exposure to LPS caused a significant decrease of miR-130b (Fig. 3A). Conversely, BMSCs rescued the miR-130b reduction in LPS-treated cells. This indicates that miR-130b is likely to be a key factor for BMSCs to enhance ENaC.

In order to verify whether miR-130b affects ENaC, AT2 cells were transfected with miR-130b mimics (Mimic) or inhibitor (Inhibitor), respectively. The effect of miR-130b on ENaC was examined by Western blot analysis. As expected, miR-130b inhibitor abrogated the promotion effect of miR-130b on α/γ -ENaC protein level (Fig. 3B-D), supporting that miR-130b may be involved in the BMSCs enhanced ENaC expression.

MiR-130b and BMSCs reduce PTEN protein expression

Potential miR-130b targets were predicted using in silico approaches, and according to the bioinformatic website prediction. Supplementary Fig. 1 showed one of the screening results from the websites, which showed that PTEN could be a potent target for miR-130b according to the values shown on the left of this screenshot. Besides, the related study reveals that miR-130b in BMSCs can target PTEN [30]. To test this

hypothesis, AT2 cells were transfected with miR-130b mimics or inhibitors, respectively. Transfection of miR-130b mimics resulted in a significant decrease of PTEN expression compared with the miR-130b mimics negative control (NC) group, while the inhibition of miR-130b showed an augment compared with the miR-130b inhibitor negative control (Inhibitor NC) group (Fig. 4A-B).

Based on the higher expression of miR-130b in AT2 cells after co-culture with BMSCs and the above adverse effects of miR-130b and PTEN, we hypothesized that BMSCs may downregulate PTEN in LPS-treated AT2 cells. To confirm this hypothesis, we applied Western blot to examine the effect of BMSCs on PTEN protein expression level. As expectedly, BMSCs suppressed the increase of PTEN in AT2 cells after LPS administration (Fig. 4C-D), supporting that PTEN is at least one of the targets in BMSCs regulating ENaC expression.

BMSCs reduce PTEN and increase α/γ -ENaC protein expression *via* miR-130b

Based on the above results, we speculate that miR-130b may exert the intermediate effect between BMSCs and PTEN/ENaC. To confirm this assumption, miR-130b mimic or miR-130b inhibitor was transfected into BMSCs, respectively, which were then co-cultured with the AT2 cells. After co-culture with BMSCs overexpressing miR-130b, PTEN protein expression in AT2 cells decreased significantly, while BMSCs transfected with miR-130b inhibitor increased PTEN protein expression (Fig. 5A-B). Furthermore, miR-130b overexpression in BMSCs increased α/γ -ENaC protein expression in AT2 cells accordingly (Fig. 5C-E), indicating that BMSCs play a vital role in ENaC regulation through miR-130b, which may be achieved by targeting PTEN.

PTEN gene knockdown enhanced the protein expression of α/γ -ENaC in AT2 cells

In order to further verify whether miR-130b exerts a protective effect on LPS-reduced ENaC by targeting PTEN, we knocked down the PTEN gene by siRNA to find out the role of PTEN in miR-130b regulation of ENaC. The siRNA transfection efficiency was verified by the Western blot analysis (Supplementary Fig. 2).

The protein expression of PTEN in the LPS-treated AT2 cells was reduced through miR-130b transfection, whereas inhibition of miR-130b displayed a contrasting effect (Fig. 6A-B). Meanwhile, both upregulating miR-130b and knocking down PTEN resulted in the increase of α/γ -ENaC protein level. In addition, when PTEN-siRNA and miR-130b mimic were co-transfected into LPS-treated AT2 cells, the expression of α/γ -ENaC protein were higher than that of PTEN-siRNA transfected alone, showing the synergistic effects (Fig. 6C-E). These results indicate that miR-130b can enhance the expression of α/γ -ENaC protein in LPS-treated AT2 cells, and PTEN participates in the regulation process of miR-130b on ENaC, which may be involved in BMSC treatment of ALI.

Discussion

ALI is a serious lung disease characterized by sustained edema and lung tissue injury [31], which leads to respiratory failure, with high morbidity and mortality [32]. The discovery of novel and effective therapeutic targets for ALI is of great significance for improving the life quality of patients, and MSCs may be a promising alternative for ALI treatment, as a cell-based therapy [33]. Research data have shown that MSCs reversed ALI-induced decrease of AFC, foremostly contributed by ENaC-mediated fluid transport [34, 35]. In this study, we first found that the expression of ENaC protein and mRNA in AT2 cells increased after the administration of BMSCs, contrary to the effects of LPS alone. To test the effects of BMSCs on ion transport function, *Isc* was measured by using chamber assay and we revealed that BMSCs promoted ASI associated with ENaC activity in intact H441 monolayers.

MiRNAs secreted by MSCs are involved in many lung diseases, including ALI, which are especially important in lung homeostasis and development [36]. Of note, miR-130b has been reported to exert key roles in various inflammatory diseases, which suppress IL-6 and TNF- α in mitigating LPS-induced vascular inflammation [20, 21], whereas its role in ALI is seldom studied. We found that miR-130b is lowly

expressed in LPS-treated AT2 cells, and BMSCs can increase the expression of miR-130b. Besides, transfection of miR-130b in either AT2 cells directly or BMSCs indirectly could increase α/γ -ENaC protein expression, indicating miR-130b a key factor in BMSC upregulation of ENaC.

PTEN as a potential miR-130b target was predicted according to the relative websites, which exerted protective effects in animal models of ALI [37]. The degradation of PTEN by miR-130b may activate PI3K/Akt, which inhibits ENaC degradation from plasma membrane through Nedd4-2 [10, 38]. PTEN/PI3K/Akt has been approved to be the underlying molecular mechanism of miR-130b-mediated cellular protection in several labs [23, 39, 40]. An innovative identification in our study revealed that the expression of PTEN in AT2 cells decreased after the administration of BMSCs, further studies showed that overexpressed miR-130b could reduce PTEN protein expression and *vice versa*, revealing that PTEN might be a target of miR-130b modulating ENaC. Powerful evidence for the targeting effect of PTEN mediating miR-130b regulated ENaC was explored by PTEN siRNA, and we found that LPS-induced α/γ -ENaC reduction were abrogated by knocking down PTEN, while transfection with PTEN-siRNA and miR-130b exerted coincident effects on the expression of α/γ -ENaC in AT2 cells after LPS administration.

Conclusions

MiR-130b may enhance ENaC and involve in the BMSCs-based therapy of ALI by targeting PTEN in LPS-treated AT2 cells, which represents a promising direction for therapeutic strategy of ALI.

Abbreviations

ALI: Acute lung injury; ENaC: Epithelial sodium channel; AT2: Alveolar type 2 epithelial; MSCs: Mesenchymal stem cells; BMSCs: Bone marrow-derived MSCs; PTEN: Phosphatase and tensin homolog deleted on chromosome ten; ARDS: Acute respiratory distress syndrome; LPS: Lipopolysaccharide; AFC: Alveolar fluid

clearance; MiRs: MicroRNAs; Isc: Short-circuit current.

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Author contributions

H.N conceived and designed the study. H.Z, Y.D, Y.H, and Z.Z performed the study. H.Z, Y.L, Z.Z, and Y.H analyzed the data. H.Z and H.N drafted the manuscript. Y.H revised the draft of manuscript. All authors corrected and approved the final version of the manuscript.

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Availability of data and materials

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

Ethics approval and consent to participate

This study followed the national guidelines and protocols of the National Institutes of Health and was approved by the Local Ethics Committee for the Care and Use of Laboratory Animals of China Medical University.

Competing interests

All authors declare that they have no competing interests.

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Figure Legends

Fig. 1 BMSCs increase protein and transcription level of ENaC in AT2 cells. **(A)** Representative Western blot measurement of α - and γ -ENaC protein expression in AT2 cells treated with LPS for 12 h and/or co-cultured with BMSCs for 24 h. Blots for β -actin were used as internal controls. **(B-C)** Graphical representation of data obtained from Western blot and quantified through gray analysis (α - or γ -ENaC/ β -actin). **(D-F)** qRT-PCR results for ENaC mRNAs. Relative level of ENaC mRNA were calculated as α -, β - or γ -ENaC/GAPDH ratios. * P < 0.05, ** P < 0.01, compared with control; & P < 0.05, compared with LPS, n = 5-6.

Fig. 2 I_{sc} level in H441 monolayers is enhanced by BMSCs. **(A)** Representative I_{sc} traces after H441 monolayers were treated with LPS for 12 h and/or co-cultured with BMSCs for 24 h, then amiloride (100 μ M) was applied. **(B)** Statistic ASI in H441 monolayers. ASI is defined as the total current value minus the plateau current value after adding amiloride, and the initial ASI is set to 100%. * P < 0.05, compared with control; & P < 0.05, compared with LPS, n = 4.

Fig. 3 MiR-130b increases protein level α / γ -ENaC in AT2 cells. **(A)** The result of real-time PCR assays shows miR-130b level in AT2 cells treated with LPS for 12 h and/or co-cultured with BMSCs for 24 h. The relative level of miR-130b were calculated as miR-130b/U6 ratio. **(B)** Representative Western blot measurement of α - and γ -ENaC protein expression in AT2 cells transfected with miR-130b mimic/inhibitor for 72 h. Blots for β -actin were used as internal controls. **(C, D)** Graphical representation of data obtained from Western blot assays for α - and γ -ENaC subunits. Bands were quantified using gray analysis (α -ENaC/ β -actin and γ -ENaC/ β -actin). ** P < 0.01, compared with control; & P < 0.05, compared with LPS; # P < 0.05, compared with miR-130b mimic negative control

(NC); $\$P < 0.05$, compared with miR-130b inhibitor negative control (Inhibitor NC), n = 4-6.

Fig. 4 MiR-130b and BMSCs decrease protein level PTEN in AT2 cells. **(A, C)** Representative Western blot measurement of PTEN protein expression in AT2 cells transfected with miR-130b mimic/inhibitor or co-cultured with BMSCs, respectively. **(B, D)** Graphical representation of data obtained from Western blot assays for PTEN. Bands were quantified using gray analysis (PTEN/ β -actin). $*P < 0.05$, compared with miR-130b mimic negative control (NC); $\&P < 0.05$, compared with miR-130b inhibitor negative control (Inhibitor NC); $\#P < 0.05$, compared with control; $\$P < 0.05$, compared with LPS, n = 4-5.

Fig. 5 Effects of BMSCs transfected with miR-130b mimic/miR-130b inhibitor on protein expressions of PTEN and α/γ -ENaC in AT2 cells. **(A, C)** Representative Western blot measurement of PTEN and α/γ -ENaC protein expression in AT2 cells co-cultured with BMSCs that were transfected with miR-130b mimic/inhibitor. **(B, D and E)** Graphical representation of data obtained from Western blot assays for PTEN and α/γ -ENaC. Bands were quantified using gray analysis (PTEN/ β -actin, α -ENaC/ β -actin and γ -ENaC/ β -actin). $*P < 0.05$, $**P < 0.01$, compared with miR-130b mimic negative control (NC); $\&P < 0.05$, compared with miR-130b inhibitor negative control (Inhibitor NC), n = 4.

Fig. 6 *PTEN* gene knockdown enhanced the protein expression of α/γ -ENaC in AT2 cells. **(A, C)** Representative Western blot measurement of PTEN and α/γ -ENaC protein expression in LPS-treated AT2 cells transfected with miR-130b mimic/inhibitor or siPTEN. **(B, D and E)** Graphical representation of data obtained from Western blot assays for PTEN and α/γ -ENaC. Bands were

quantified using gray analysis (PTEN/ β -actin, α -ENaC/ β -actin and γ -ENaC/ β -actin). ** $P < 0.01$, compared with control; & $P < 0.05$, compared with LPS + negative control (NC); # $P < 0.05$, compared with LPS + inhibitor negative control (Inhibitor NC); \$ $P < 0.05$, compared with LPS + siPTEN, n = 4-6.

Figure 1

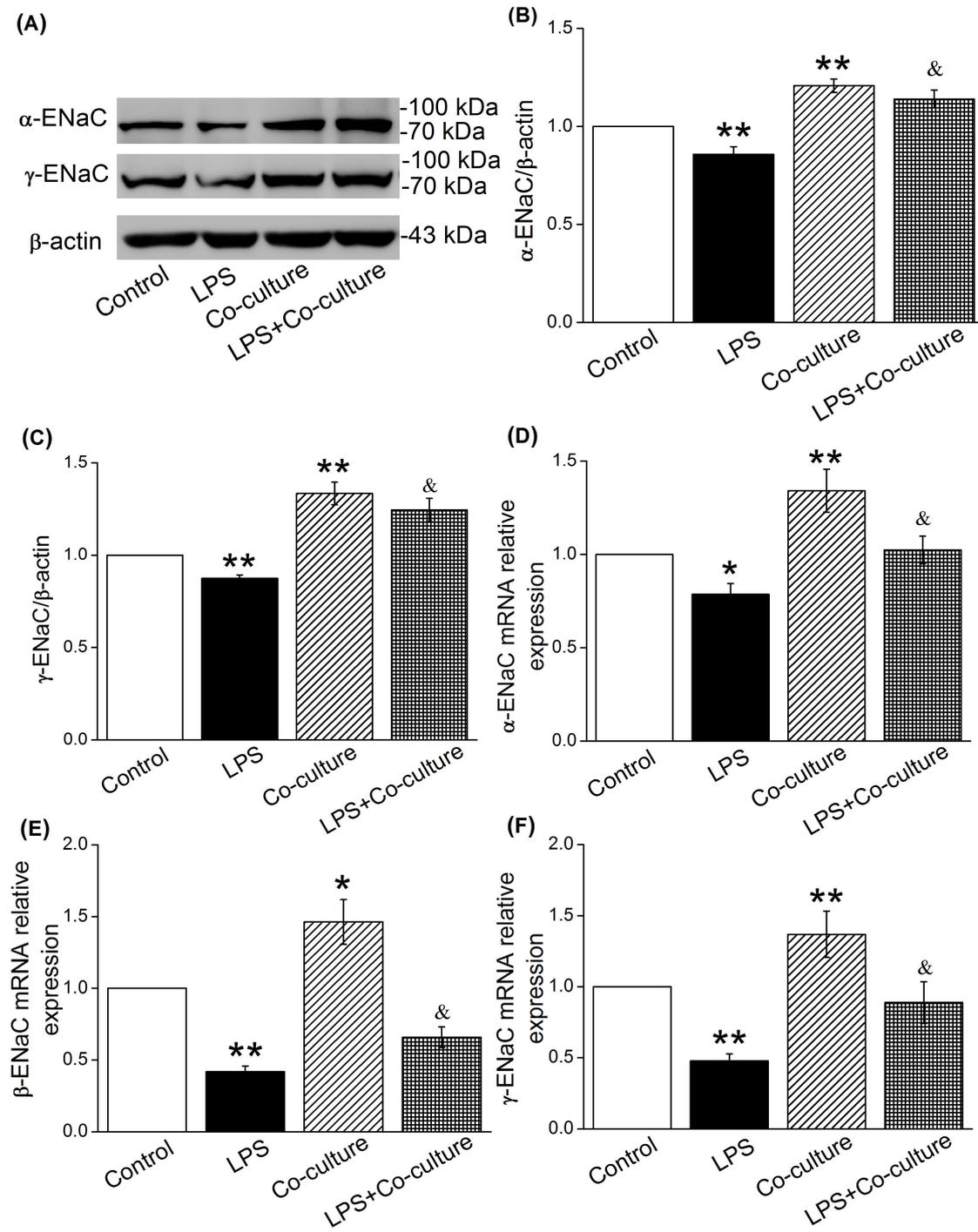


Figure 2

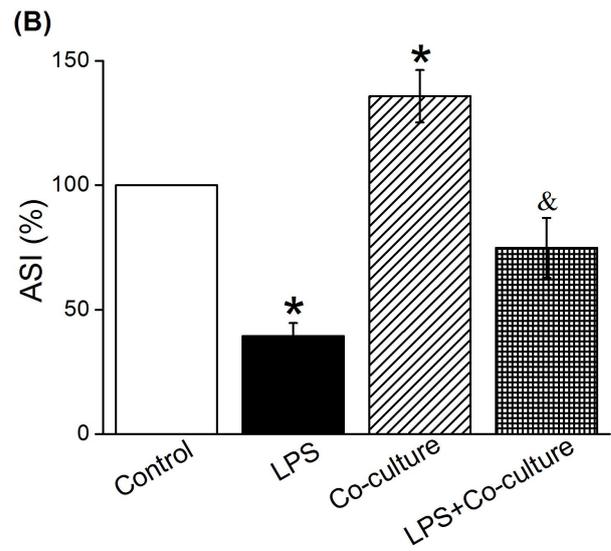
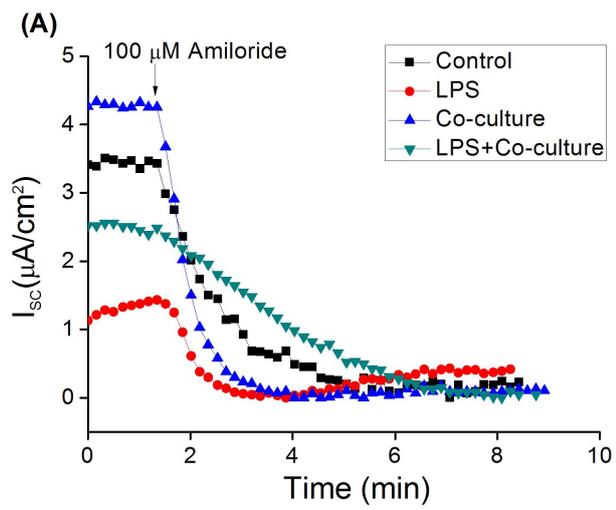


Figure 3

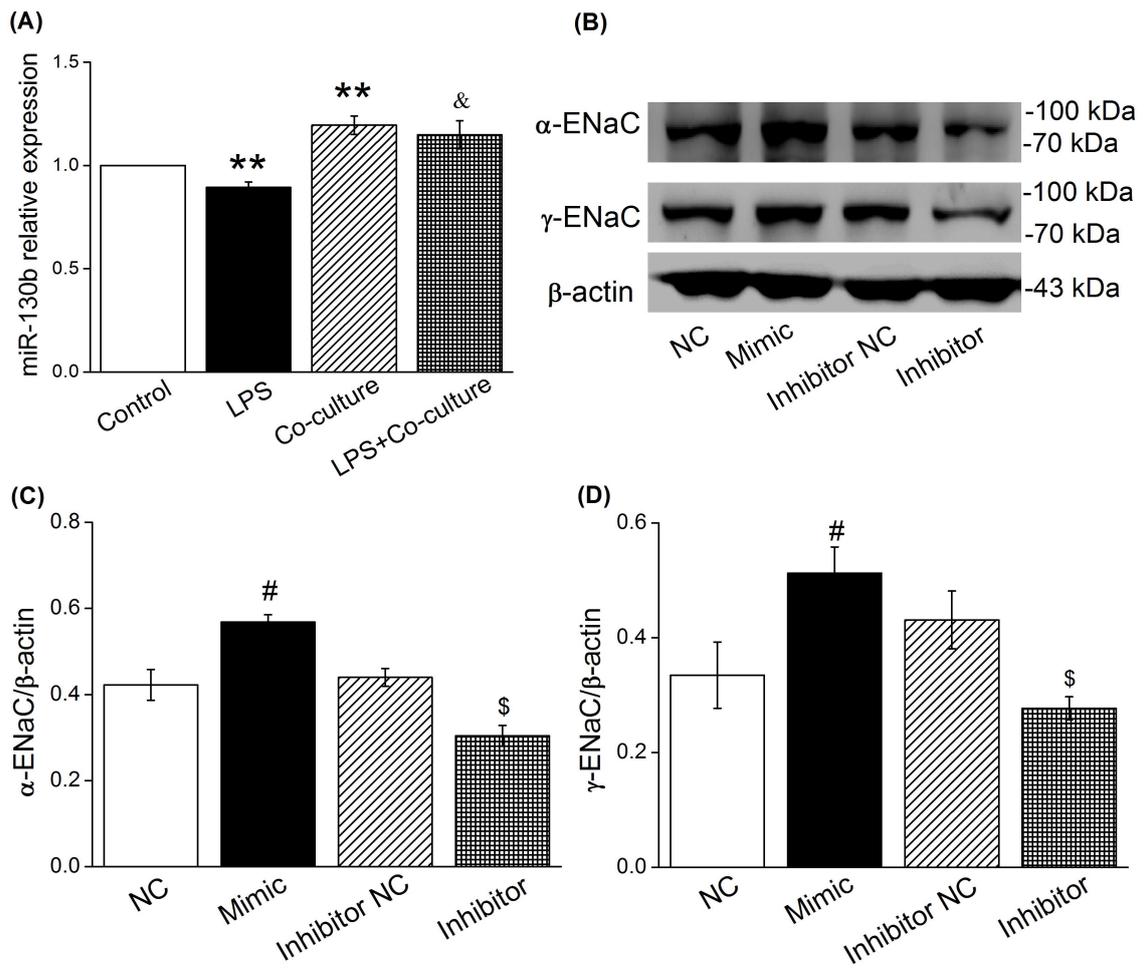


Figure 4

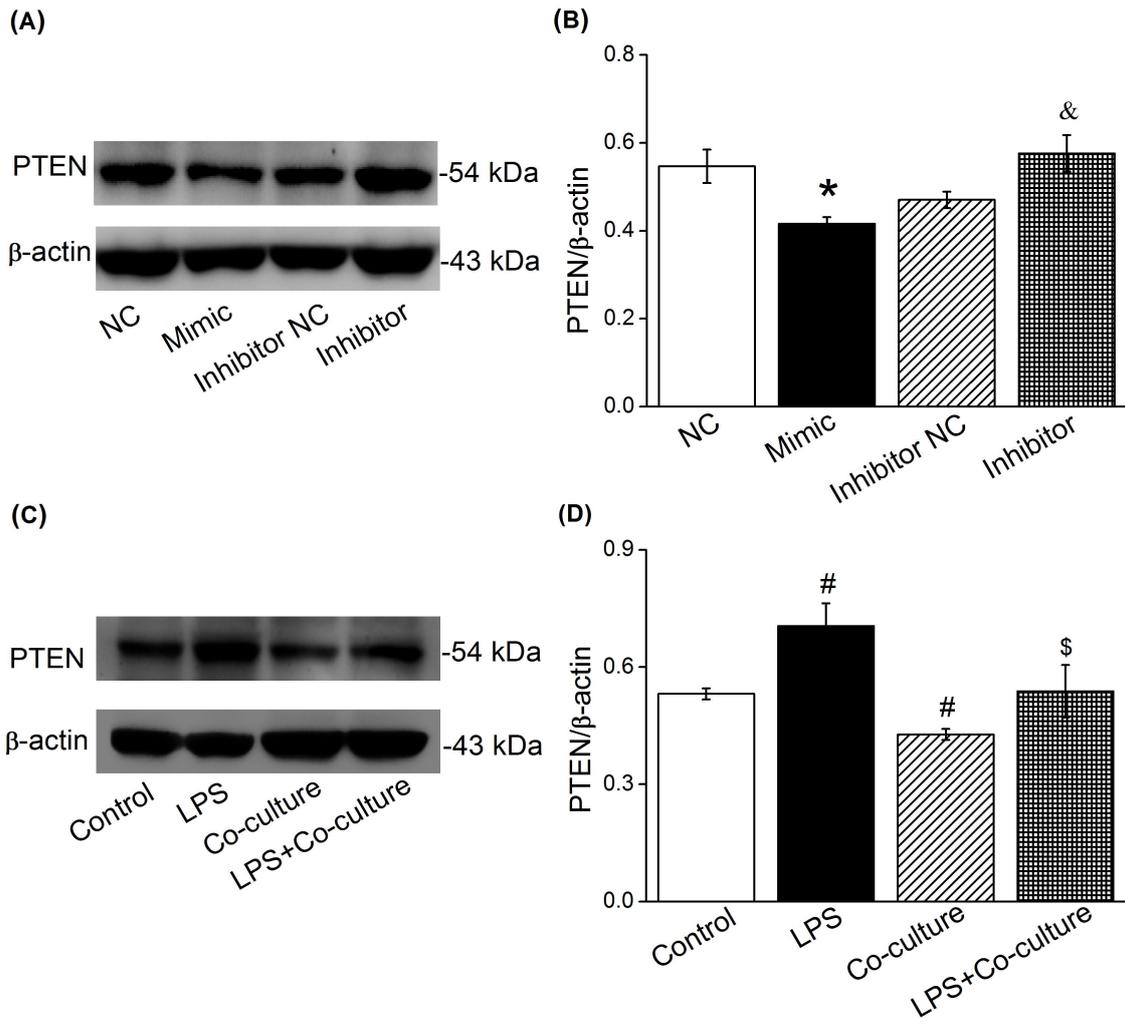


Figure 5

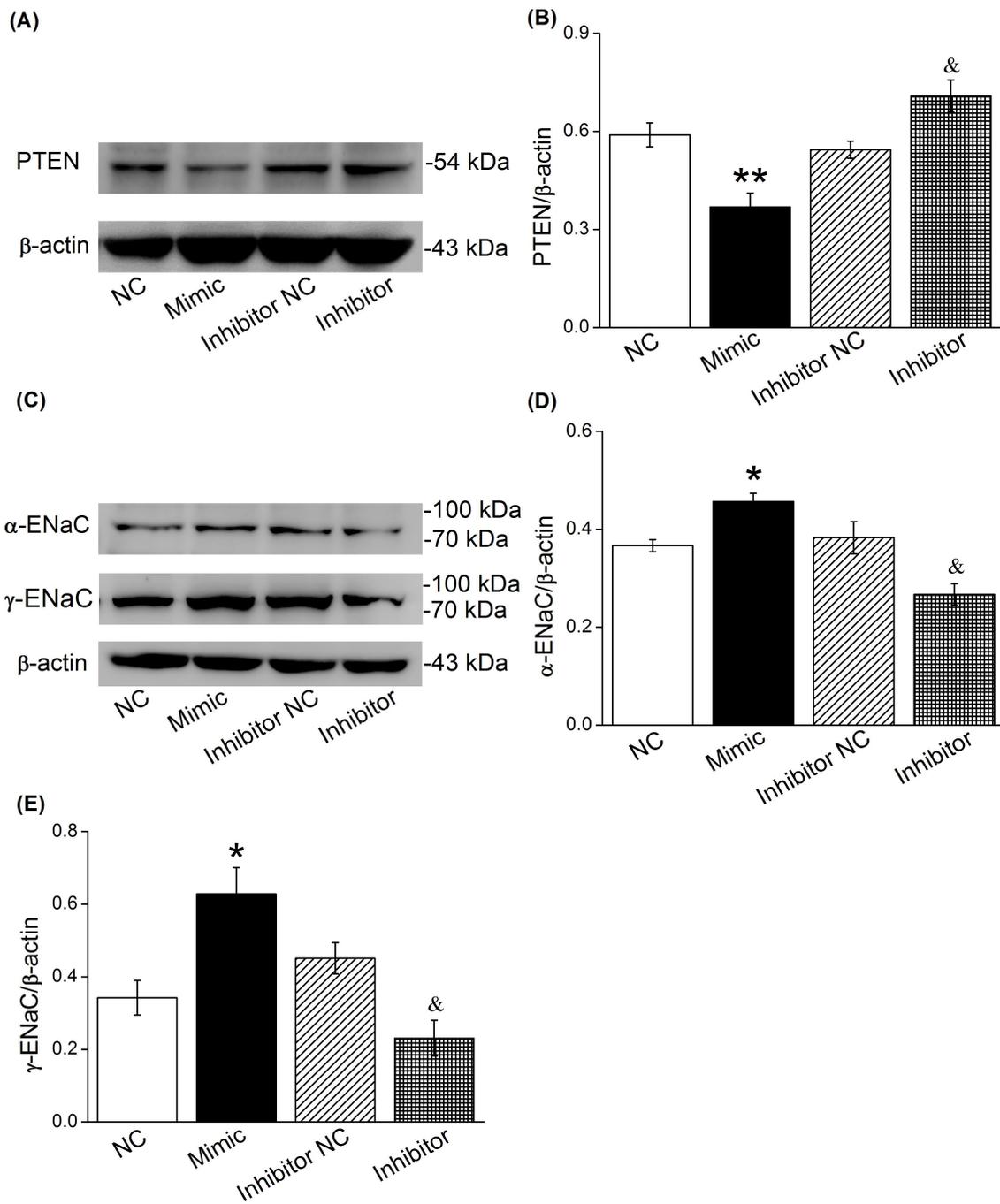
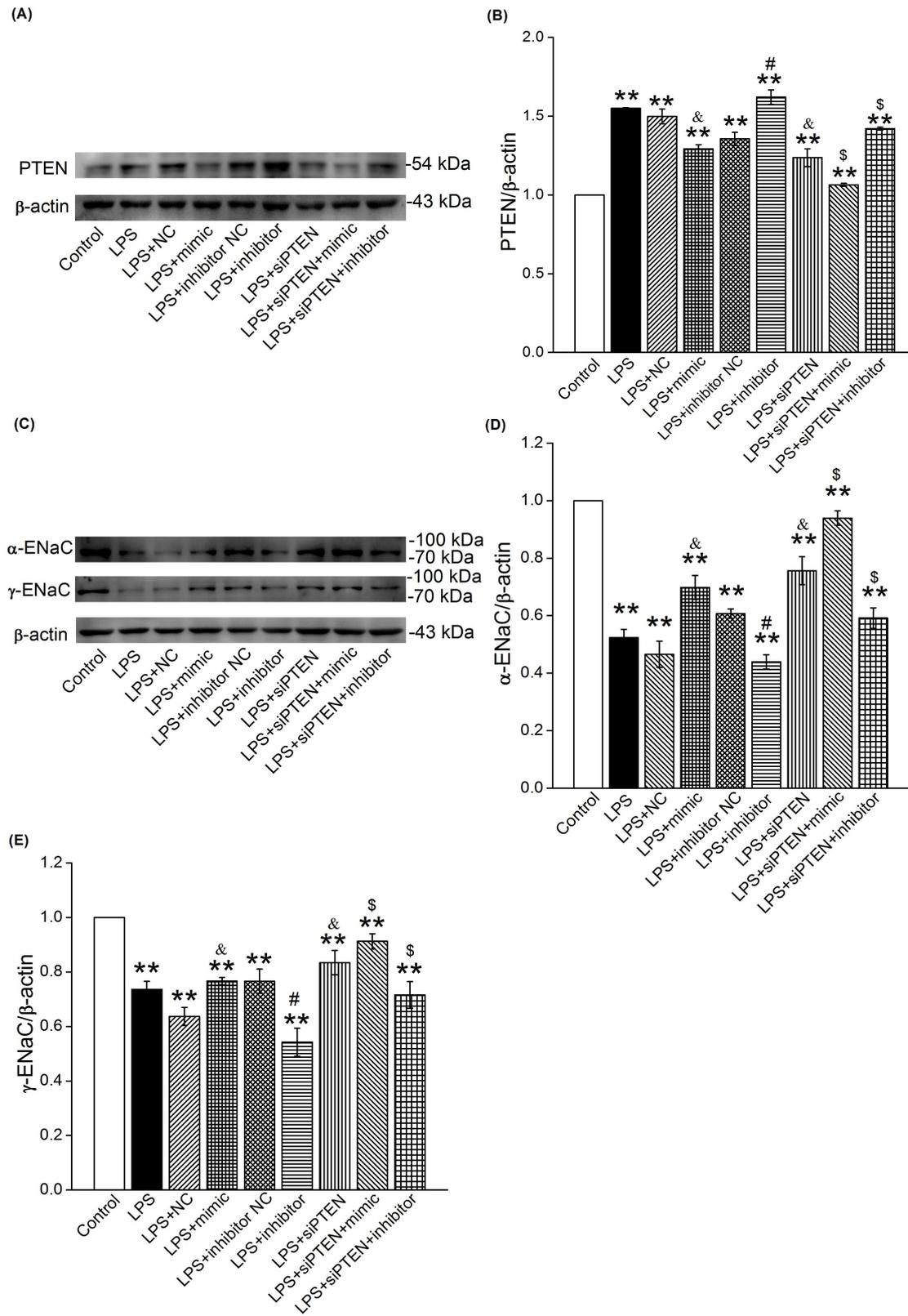


Figure 6



Supplementary information

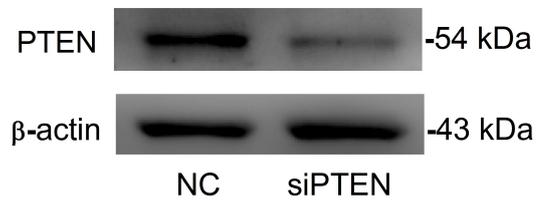
Supplementary Fig. 1 The screening results from the websites. The left of this screenshot showed the corresponding scores of miR-130b binding sites, and the right were the two typical miR-130b/PTEN alignments.

Supplementary Fig. 2 Knockdown identification of *PTEN* gene. **(A)** Representative Western blot measurement of PTEN transfected with PTEN-siRNA (siPTEN). **(B)** Graphical representation of data obtained from Western blot assays. Bands were quantified using gray analysis (PTEN/ β -actin).

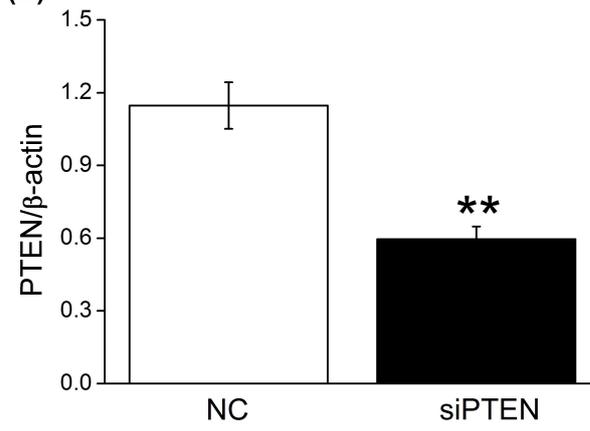
** $P < 0.01$, compared with negative control (NC), $n = 4$.

Supplementary Figure 2

(A)



(B)



Figures

Figure 1

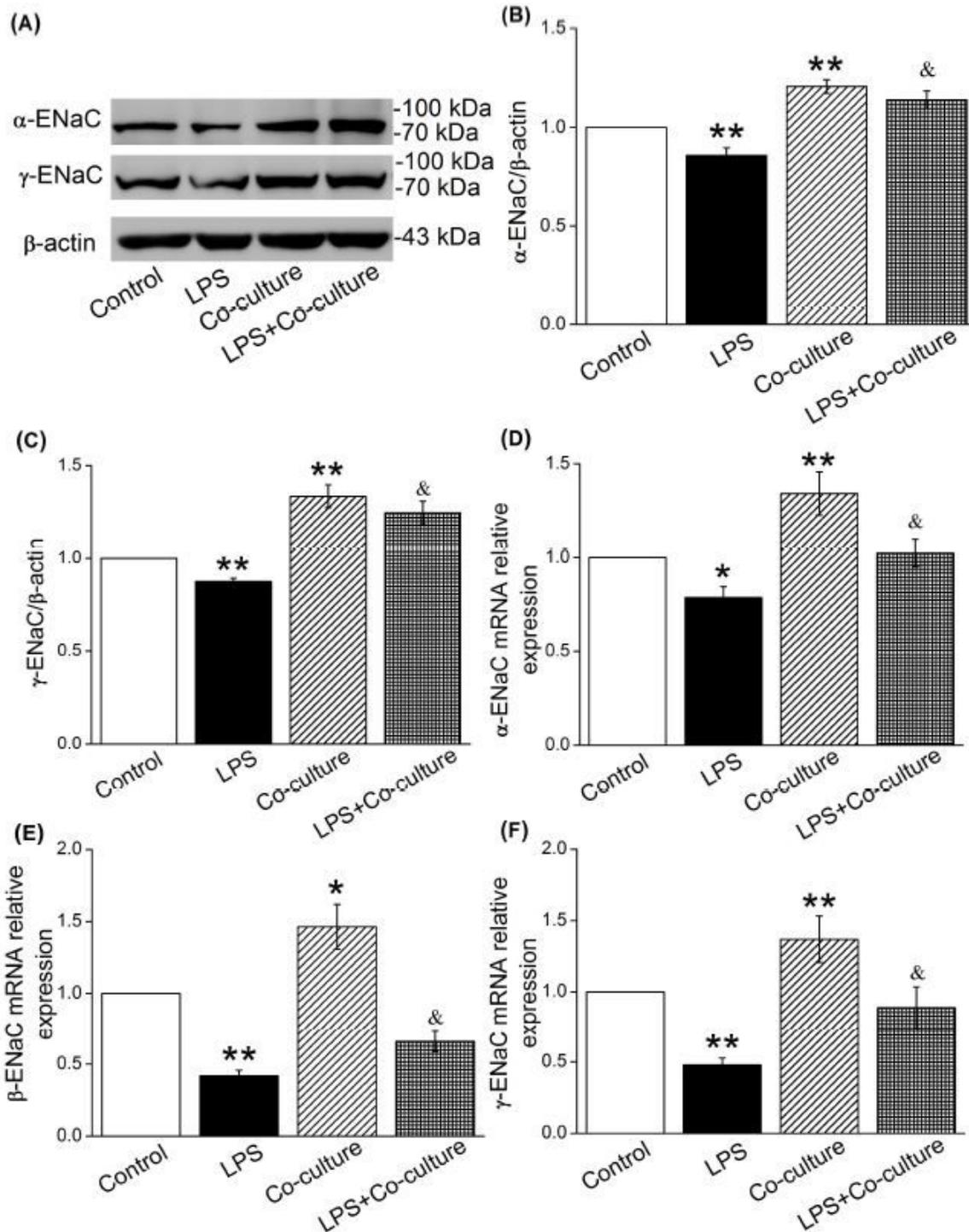


Figure 1

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Figure 2

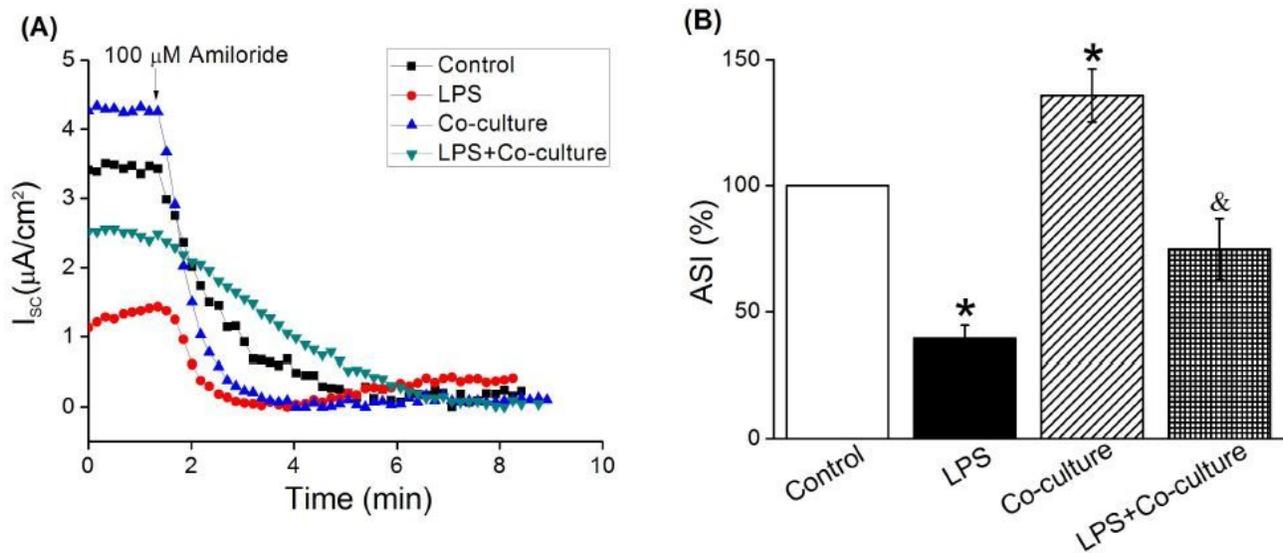


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Figure 3

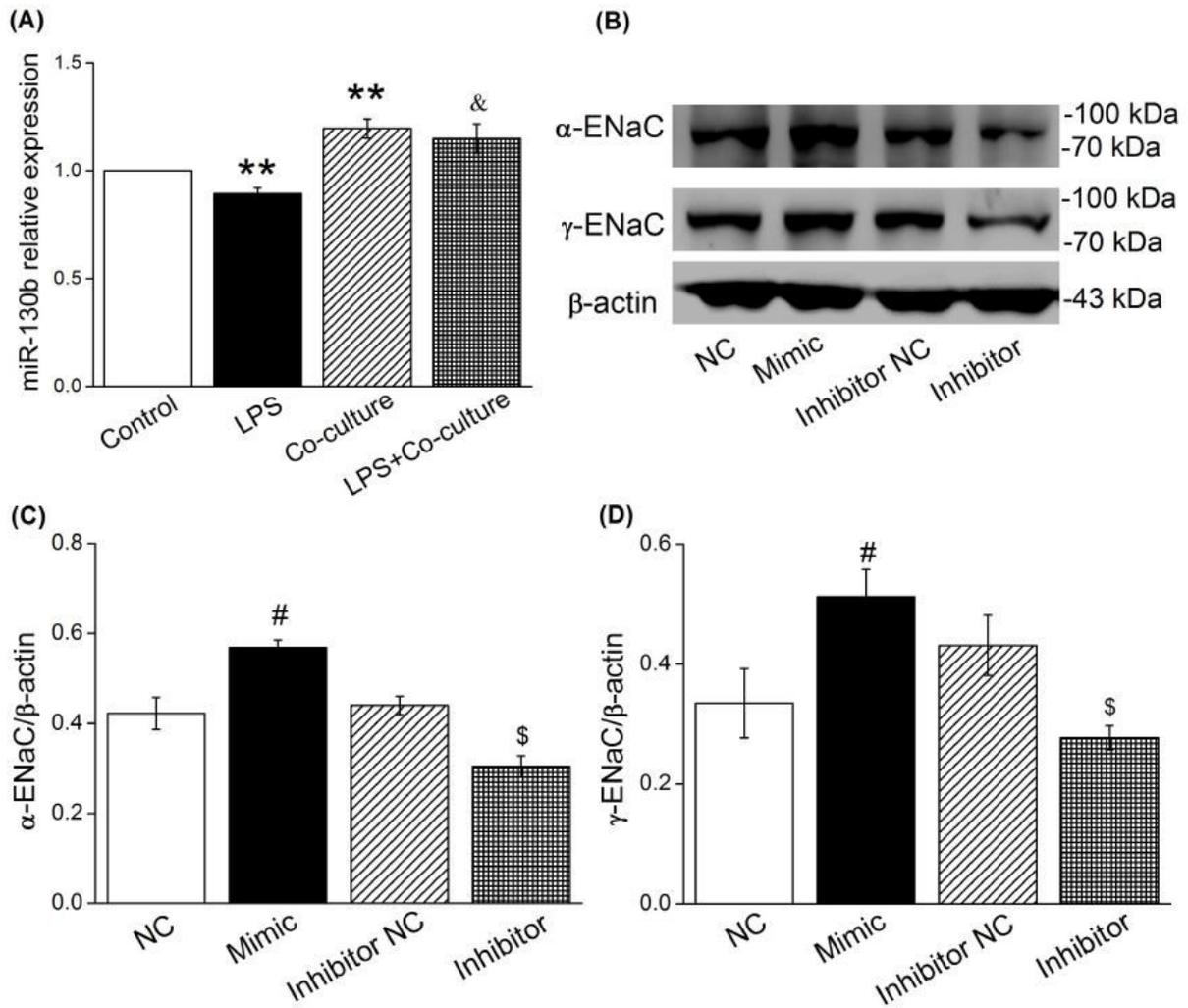


Figure 3

MiR-130b increases protein level α/γ-ENaC in AT2 cells. (A) The result of real-time PCR assays shows miR-130b level in AT2 cells treated with LPS for 12 h and/or co-cultured with BMSCs for 24 h. The relative level of miR-130b were calculated as miR-130b/U6 ratio. (B) Representative Western blot measurement of α- and γ-ENaC protein expression in AT2 cells transfected with miR-130b mimic/inhibitor for 72 h. Blots for β-actin were used as internal controls. (C, D) Graphical representation of data obtained from Western blot assays for α- and γ-ENaC subunits. Bands were quantified using gray analysis (α-ENaC/β-actin and γ-ENaC/β-actin). **P < 0.01, compared with control; &P < 0.05, compared with LPS; #P < 0.05, compared with miR-130b mimic negative control (NC); §P < 0.05, compared with miR-130b inhibitor negative control (Inhibitor NC), n = 4-6.

Figure 4

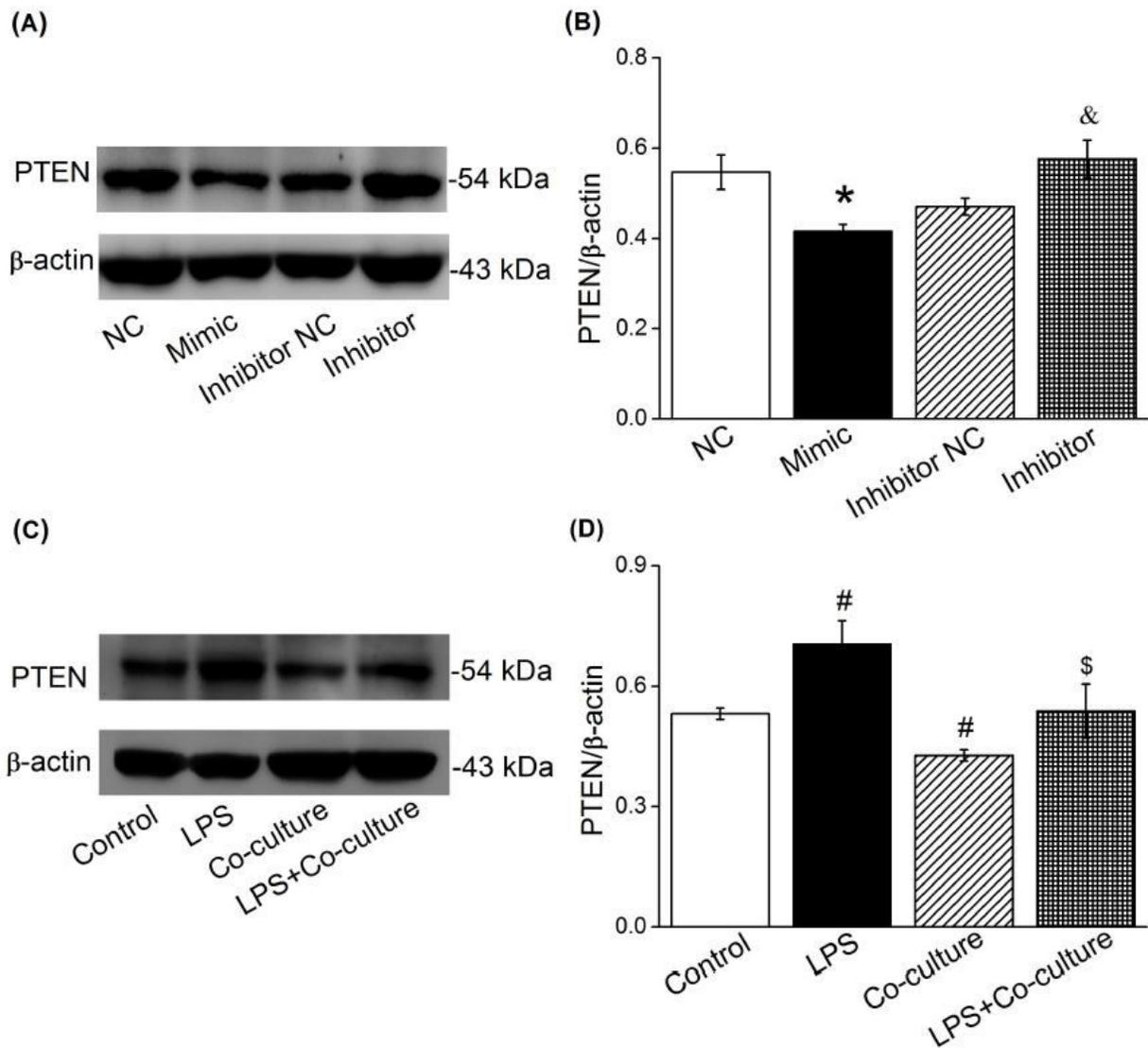


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Figure 5

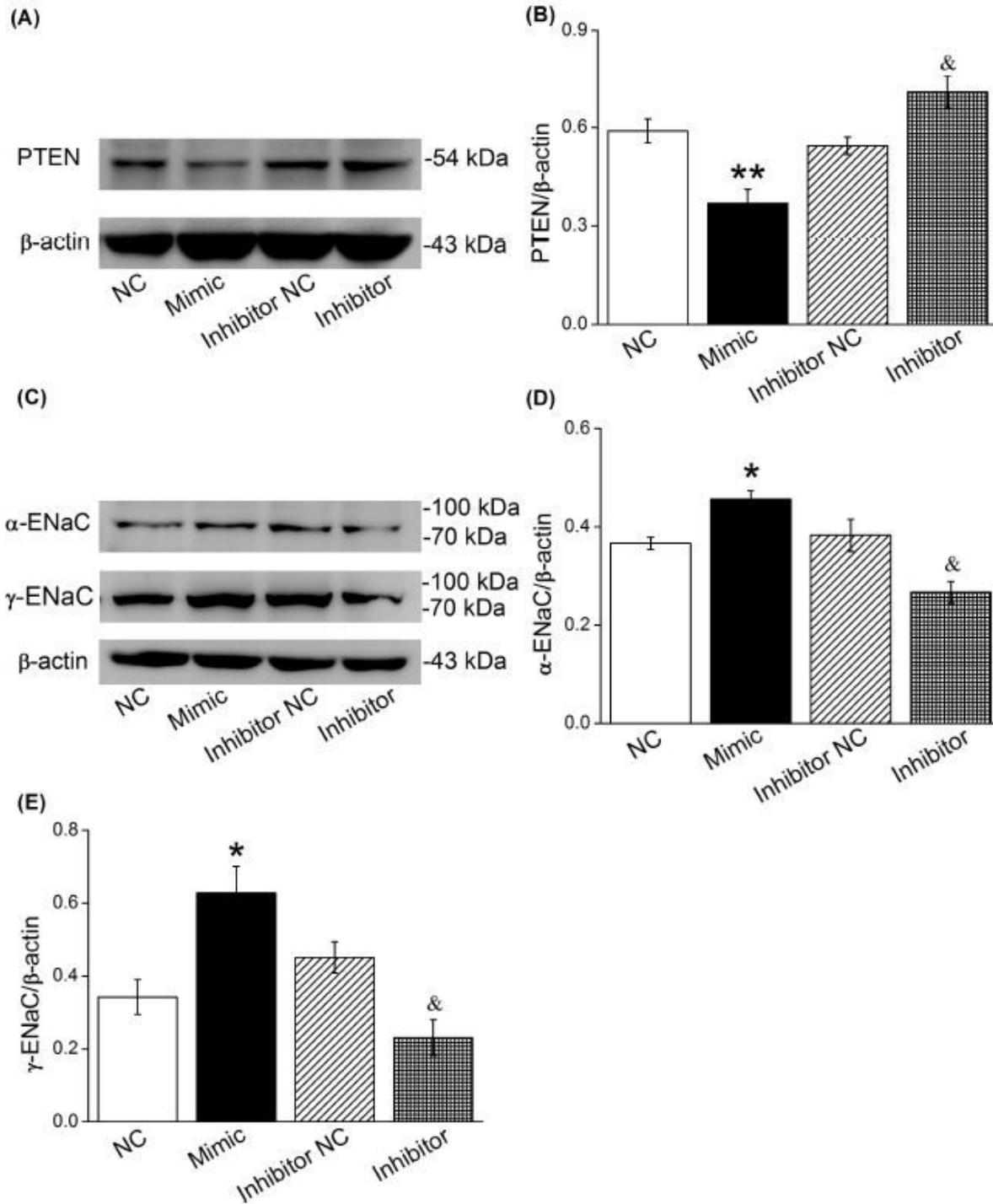


Figure 5

Effects of BMSCs transfected with miR-130b mimic/miR-130b inhibitor on protein expressions of PTEN and α/γ-ENaC in AT2 cells. (A, C) Representative Western blot measurement of PTEN and α/γ-ENaC protein expression in AT2 cells co-cultured with BMSCs that were transfected with miR-130b mimic/inhibitor. (B, D and E) Graphical representation of data obtained from Western blot assays for PTEN and α/γ-ENaC. Bands were quantified using gray analysis (PTEN/β-actin, α-ENaC/β-actin and γ-

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Figure 6

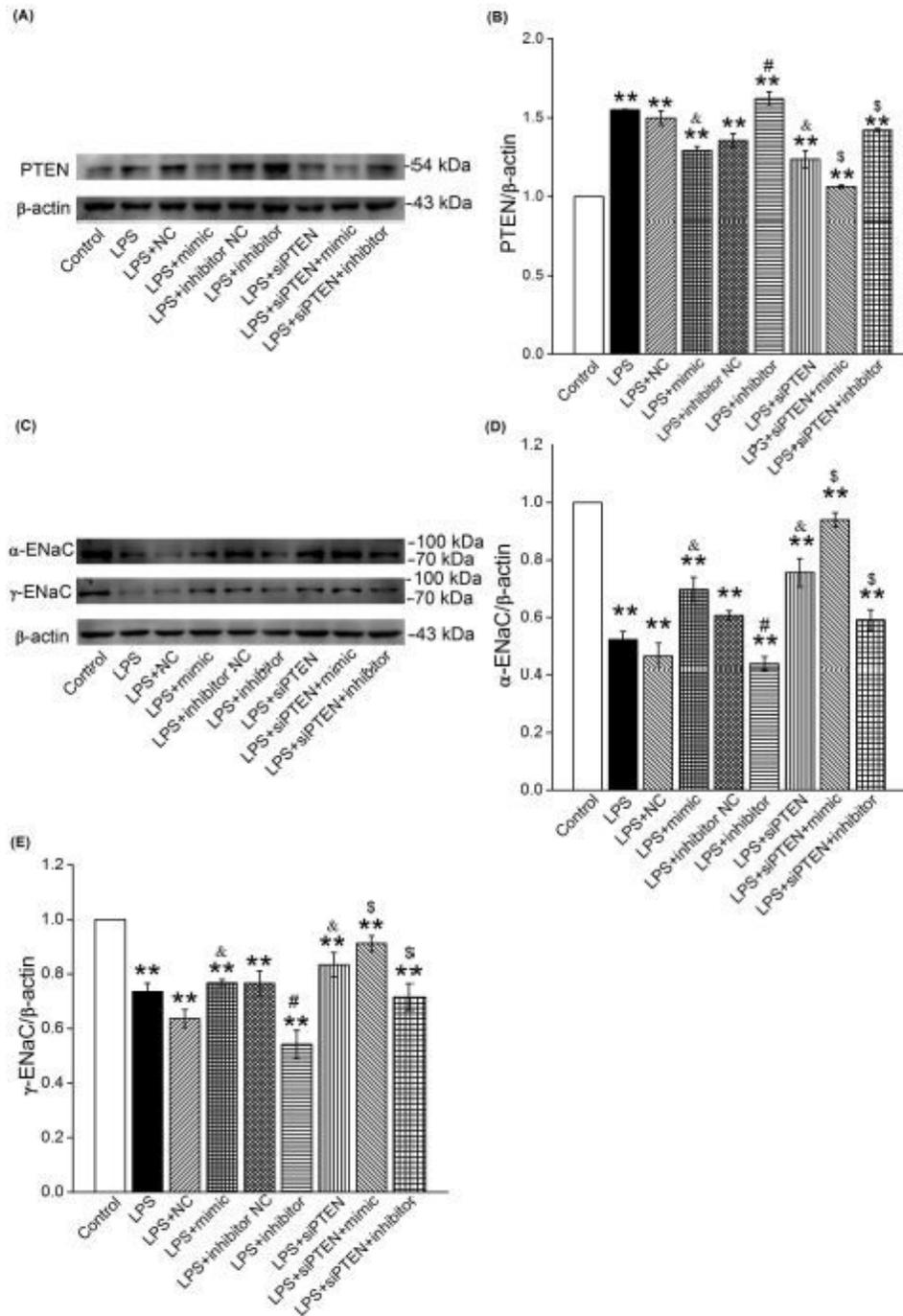


Figure 6

PTEN gene knockdown enhanced the protein expression of α/γ -ENaC in AT2 cells. (A, C) Representative Western blot measurement of PTEN and α/γ -ENaC protein expression in LPS-treated AT2 cells transfected with miR-130b mimic/inhibitor or siPTEN. (B, D and E) Graphical representation of data obtained from

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Supplementary Files

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