



# Molecular hydrogen alleviates asthma through inhibiting IL-33/ILC2 axis

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## Abstract

**Background** Asthma is one of the most common noninfectious chronic diseases characterized by type II inflammation. This study aimed to investigate the effects of molecular hydrogen on the pathogenesis of asthma.

**Methods** OVA sensitized asthma mouse model and house dust mite treated 16HBE cellular model were established and hydrogen/oxygen mixture was used to treat asthmatic mice and 16HBE cells. Serum and BALF cytokines were measured with specific ELISA assays. E-cadherin and ZO-1 were detected by immunohistochemical staining and expression of caspase 3 and 9, NF- $\kappa$ B, IL-33 and ST2 was assessed by quantitative real-time PCR, western blot and/or immunofluorescence. IL-33 promoter activity was analyzed by dual-luciferase assay. ILC2 population was assayed by flow cytometry and differentially expressed miRNAs were detected using miRNA array.

**Results** Serum and BALF levels of IL-33 and other alarmin and type II cytokines were greatly increased by OVA and inhibited by H<sub>2</sub> in asthmatic mice. The expression of NF- $\kappa$ B (p65) and ST2 was upregulated by OVA and suppressed by H<sub>2</sub>. ILC2 population was markedly increased in OVA-induced asthmatic mice, and such increase was inhibited by H<sub>2</sub>. E-cadherin and ZO-1 levels in airway tissues of asthmatic mice were significantly lower than that of control mice, and the reduction was recovered by H<sub>2</sub> treatment. H<sub>2</sub> alleviated HDM induced apoptosis of 16HBE cells, upregulation of IL-33 and ST2, and elevation of IL-33 promoter activity. A group of miRNAs differentially expressed in HDM and HDM + H<sub>2</sub> treated 16HBE cells were identified.

**Conclusions** These data demonstrated that H<sub>2</sub> is efficient in suppressing allergen-induced asthma and could be developed as a therapeutics for asthma and other conditions of type II inflammation.

**Keywords** Hydrogen · Asthma · ILC2 · IL-33 · Type II cytokine

## Background

Asthma is one of the most common noninfectious chronic diseases, affecting more than 300 million people worldwide [1]. Asthma is characterized by various immunological mechanisms [2] and resulted from complex gene-environment interactions with heterogeneous clinical presentation [3]. The central theme of asthma is type 2-mediated allergic inflammatory responses that promote barrier defenses at mucosal surfaces [4]. This type 2 immune response

perpetuates the production of type 2 cytokines including IL-4, IL-5, IL-9, and IL-13 [4, 5] and antibody switching to IgE [6]. Innate type 2 response does require T4<sup>+</sup> cells [7, 8] but relies on group 2 innate lymphoid cells (ILC2s) [9, 10].

Group 2 innate lymphoid cells were first identified as IL-25 dependent non-T non-B immune cells which were not originated from spleen and bone marrow [11]. ILC2s were found in mesenteric lymph node (MLN), spleen, lung, peritoneum, liver [10, 12], and they were essential for clearing parasite infections [9, 10, 12]. ILC2s influence Th2 cell-mediated lung inflammation through regulating the production of Th2 cytokines. IL-33 plus IL-2, IL-7, IL-25, and thymic stromal lymphopoietin (TSLP) activated lung ILC2s responded to helminth infection [12] and allergen-induced tissue damage [13, 14]. Lung ILC2s produced large quantity of IL-5 and IL-13 but not IL-4 [13]. ILC2 activation was mostly mediated by IL-33 which was a damage-induced

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cytokine released from necrotic epithelial cells in active form [15] and its expression is induced in type 2 pneumocytes upon exposure to allergens [8].

Since Ohsawa et al. demonstrated that hydrogen gas attenuated focal ischemia and reperfusion caused oxidative stress and brain injury through selectively eliminating the hydroxyl radical [16], increasing evidence showed the potential of hydrogen gas in preventing and relieving different diseases and conditions [17]. Molecular hydrogen is believed to reduce oxidative stress in alleviating various conditions [18–20]. Meanwhile, oxidative stress is heavily implicated in the pathogenesis of asthma [21, 22]. This study aims to explore the effects of hydrogen gas on asthma and the underlying molecular mechanisms.

## Materials and methods

### Asthma model

Female ICR mice (5–6 weeks old) were purchased from Changzhou Cavens Laboratory Animal Ltd. (Changzhou, China). The mice were maintained at  $25 \pm 2^\circ\text{C}$  with a 12 h light/dark cycle and fed with a standard mouse chow and water ad libitum. All experiments were approved by the Ethical Committee for Animal Experiments of Naval Medical University, and strictly carried out in accordance with the approved guidelines.

Mice were randomly divided into four groups ( $n=6$  each): (1) control group, (2) 60%  $\text{H}_2$  group, (3) asthma group, and (4) asthma + 60%  $\text{H}_2$  group. The mice were sensitized with ovalbumin (OVA) +  $\text{Al}(\text{OH})_3$  (both from Sigma, St Louis, MO) (100  $\mu\text{g}$  OVA and 3 mg  $\text{Al}(\text{OH})_3$  in 300  $\mu\text{l}$  PBS) or PBS (300  $\mu\text{l}$  PBS) by subcutaneous injection on day 1, 8 and 15. This was then followed by a nasal drip of 20  $\mu\text{l}$  OVA (5  $\mu\text{g}$  OVA in 20  $\mu\text{l}$  PBS) or PBS (20  $\mu\text{l}$  PBS) on day 22 and 29. To assess the effects of hydrogen gas on asthma, the mice of 60%  $\text{H}_2$  group and asthma + 60%  $\text{H}_2$  group were housed in a specific airtight device (Wuxi Puhe BioMedical Tech, Jiangying, China) producing an air mixture containing 60%  $\text{H}_2$  and 21%  $\text{O}_2$  for two weeks (2 h per day) beginning on day 30.

### Cellular model

16HBE cells were obtained from Puhe Biotechnology Company (WuXi, China), and maintained in DMEM high glucose medium containing 10% foetal bovine serum (Thermo Fisher Scientific Inc., Waltham, MA). The cellular model of asthma was established by treating 16HBE cells with 10  $\mu\text{g}/\text{m}$  house dust mite extract (HDM, *Dermatophagoides pteronyssinus*, STALLERGENES GREER, Lenoir, NC) for

12 h, and treated with hydrogen in the same way as animal experiments.

### Hydrogen gas production

The mixed gas consisting of  $\text{H}_2$  and  $\text{O}_2$  (66.7%: 33.3% v/v) was produced by the AMS-H-01 hydrogen nebulizer (Asclepius, Shanghai, China) used in animal experiments, which was designed to produce hydrogen by electrolyzing water, whereas, the hydrogen incubator (PH-1-A) used in the cell experiments and adjustable three gas chamber (PH-2-A) used for animal experiments were from Puhe Biotechnology Company (Wuxi, China). Thermal trace GC ultra-gas chromatography (Thermo Fisher, MA, USA) was used to monitor the concentration of hydrogen gas in the box.

### BALF collection

Using a 1 ml syringe, 0.5 ml aliquots of 0.9% NaCl solution was injected into the trachea and lungs through the tracheal cannula, the chests of mice were gently massaged and rinsed for approximately 1 min, and then the liquid was withdrawn with the same syringe. The process was repeated three times, and the total quantity of BALF was 1.2 ml for each sample. The cellular fraction from BALF was collected by centrifugation at 1500 rpm for 10 min at  $4^\circ\text{C}$  and resuspended in 1 ml saline.

### Immunohistochemical staining

The airway tissue were fixed with 4% paraformaldehyde and embedded in paraffin. For immunohistochemistry, deparaffinized sections were incubated with 3%  $\text{H}_2\text{O}_2$  for 5 min to block endogenous peroxidase activity. After blocking with 10% foetal bovine serum, sections were stained with anti-ZO-1 (ab190085) or anti-E-cadherin (CST3195S) antibodies over night at  $4^\circ\text{C}$  followed by secondary Abs. Sections were washed three times with PBS buffer. The colour was developed using DAB substrate-chromogen solution (Biocare Medical), and images were obtained under a microscope with 40 magnification (OLYMPUS-IX71). The protein levels (staining intensity) were analyzed using Image J (NIH, Bethesda, MD).

### Flow cytometry

Cells in BALF were collected and prepared to cell suspension ( $3 \times 10^6$  cells/ml). The Fc receptor blocker (Cat # 130-092-575, Miltenyi Biotec, Shanghai, China) was incubated at room temperature for 10 min, then the intraprep permeabilization reagent (Cat # A07802, Beckman Coulter Life Sciences, Indianapolis, IN) was incubated at  $4^\circ\text{C}$  for 20 min. After PBS washing, the corresponding antibodies

were incubated at room temperature and away from light for 40 min. Flow cytometry was performed and results were analyzed with CELL Quest software.

## ELISA

Serum, BALF supernatant of mice or cell culture medium were collected for ELISA assay of IL-4, IFN- $\gamma$ , IL-33, IL-25, TSLP, MCP-1, IL-5, IL-9, IL-13 (Elabscience, Houston, TX) and ST2 (ab213871, Abcam) according to the manufacturers' recommendation. The data were expressed as cytokine (pg/ml) for each sample.

## Immunofluorescence staining

Cells were fixed with 4% PFA for 15 min and washed with 0.2% Triton X-100 in PBS for 10 min. Then blocked with 1% BSA in PBS, and incubated with monoclonal anti-IL-33 antibody (ab187060, abcam) and anti-ST2 (60112-1-Ig, Proteintech) antibody, followed by DyLight488 goat anti-rabbit IgG secondary antibodies (GAR4882, Multi Sciences) and anti-mouse CY3 secondary antibodies (SA00009, Proteintech) away from light for 1 h at room temperature before being counterstained with Hoechst and mounted.

## CCK-8 assay

5000 16HBE cells/well in 100  $\mu$ l of complete medium were seeded in 96-well plates and culture at 37 °C for 24 h in a humidified incubator with 5% CO<sub>2</sub>. Then the cells were treated with 10  $\mu$ g/ml of HDM and or H<sub>2</sub> for 24 h before adding 10  $\mu$ l of CCK-8 solution to each well and incubating the plate for 3 h in the incubator. The absorbance at 450 nm was measured using a microplate reader (Molecular Devices, San Jose, CA).

## Western blot

Protein quantification was performed. Protein lysate was introduced to SDS-PAGE and subsequently electrotransferred to a polyvinylidene fluoride membrane. Western blotting was carried out as previously reported. The primary antibodies used were listed as follows: anti-IL33 (abcam, ab187060, 1:1000), anti-caspase 3 (abcam, ab90437, 1:1000), anti-cleaved caspase 3 (abcam, ab49822, 1:500), anti-caspase 9 (abcam, ab52298, 1:500), anti-cleaved caspase 9 (abcam, ab2324, 1:1000), anti-p65 (Active Motif, 39159, 1:1000), anti-p-p65 (abcam, ab76307, 1:5000), anti-ST2 (Proteintech, 60112-1-Ig, 1:2000), anti-GAPDH (abcam, ab181602, 1:10000). Secondary antibodies included goat anti-rabbit IgG-HRP (Abcam, Cambridge, MA, USA).

## Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted from airway tissues using RNeasy mini kits (Qiagen, Venlo, Netherlands). Reverse transcription was performed with the SuperScript® III First-Strand Synthesis System (Life Tech, Shanghai, China) according to the supplier's instructions using 1 g total RNA. Quantitative real-time PCR was performed using the SYBR® Green PCR Master Mix (Life Tech) on a ABI 7300 (Applied Biosystems, Foster City, CA) with the following program: 95°C for 3 min followed by 40 cycles of 95°C for 30 s, 58°C for 15 s, and 68°C for 30 s. Primers (Table S1) were synthesized by Shanghai Sangon Biotech Co., Ltd (Shanghai, China). The relative transcription levels were calculated with the 2<sup>- $\Delta\Delta C_t$</sup>  method using gapdh as the internal control.

## Luciferase reporter gene assays

The effect of HDM on IL-33 promoter luciferase reporter assays was determined. The IL-33 promoter region was constructed to the expression vector of pGL3-basic. 16HBE were seeded in 24-well plates (3  $\times$  10<sup>4</sup> cells/well) and maintained for 24 h. Cells were then serum-starved (0.1% FBS/DMEM) for 24 h, transfected with the pGL3-basic and pRL-TK reporter vector using LipofectAMINE reagent (0.2  $\mu$ g DNA/well; 1  $\mu$ l of LipofectAMINE/well in a total volume of 140  $\mu$ l/well) in serum-free media for 4 h and subsequently treatment with 10  $\mu$ g/ml HDM or/and 60% H<sub>2</sub> for an additional 12 h/24 h. Cells were then treated with test agents at the indicated concentrations for 6 h and luciferase activity measured using a luciferase assay kit and a microplate luminometer (Luminoskan Ascent; LabSystems, Helsinki, Finland). Luciferase activity was normalized to Renilla luciferase.

## MiRNA microarray

RNeasy Micro Kit (Qiagen) was used to extract RNA from 16HBE cells following manufacturer's instructions. The Whole Human miRNA Microarray was a broad view that represents all known miRNAs in the human transcriptome. Sequences were compiled from a broad source survey, and then verified and optimized by alignment to the assembled human transcriptome.

Sample labeling and array hybridization were performed according to the Agilent miRNA Microarray System with miRNA Complete Labeling and Hyb Kit protocol (Agilent Technology). Briefly, total miRNA from each sample was labeled with Cyanine 3-pCp under the action of T4 RNA ligase. The labeled cRNA over the procession of inspissation and desiccation and then redissolved with water. 1  $\mu$ g of each labeled cRNA was fragmented by

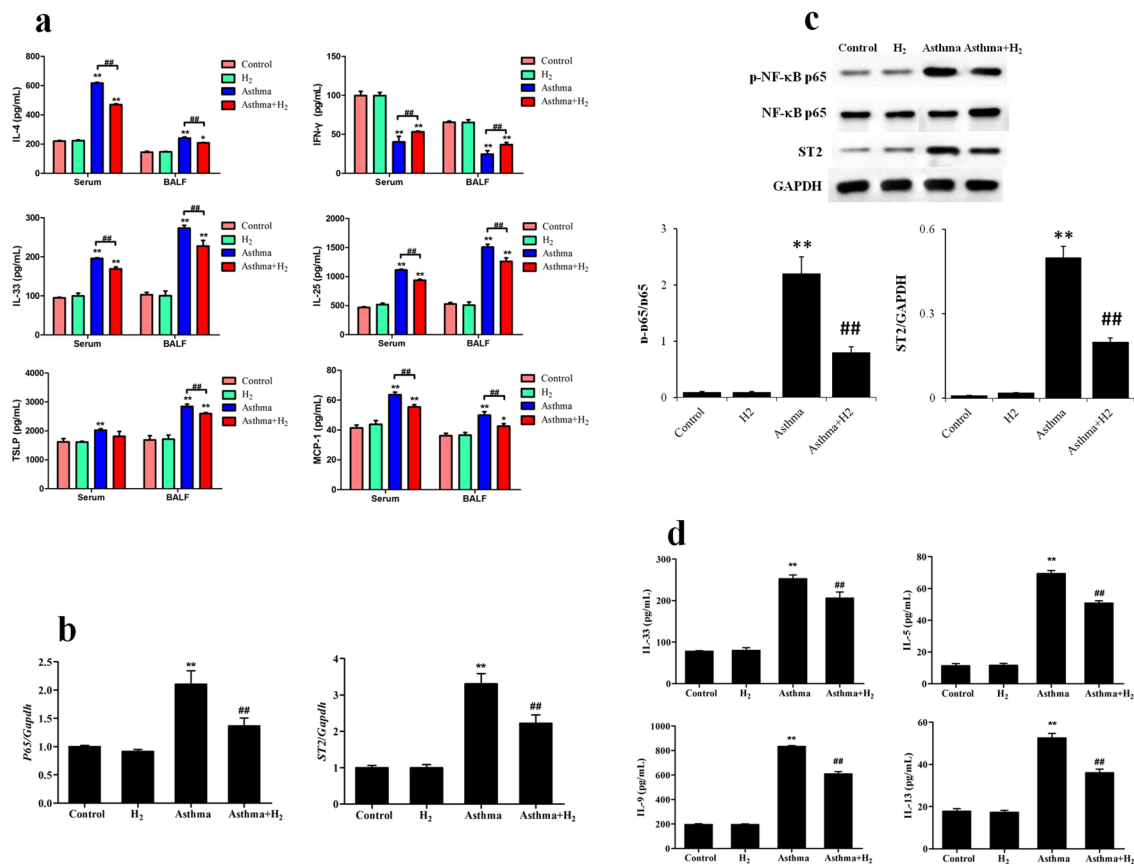


**Fig. 1** H<sub>2</sub> inhibited OVA induced overexpression of type II inflammation cytokines. Mice were given OVA according to specified regimen (Refer to experimental methods) and / or H<sub>2</sub> inhalation. **a** Serum and BALF levels of IL-4, IL-25, IL-33, TSLP, INF- $\gamma$ , and MCP-1 were measured by ELISA assays. **b** Total RNA was extracted from mouse airway tissues and mRNA levels of NF- $\kappa$ B p65 and ST2 were analyzed by quantitative real-time PCR. **c** Protein samples were prepared

from mouse airway tissues and NF- $\kappa$ B p65 (total protein and phosphorylated form), and ST2 were detected by western blot. **d** BALF cells from either control or asthmatic mice were collected and cultured under normal conditions or with H<sub>2</sub> treatment for 12 h. Medium IL-5, IL-9, IL-13, and IL-33 levels were assessed by ELISA.  $n=6$ . \* $p<0.05$  and \*\* $p<0.01$  compared to control; ## $p<0.01$  compared to asthma group

adding 11  $\mu$ l 10 $\times$  Blocking Agent and 2.2  $\mu$ l of 25 $\times$  Fragmentation Buffer, then heated at 60  $^{\circ}$ C for 30 min, and finally 55  $\mu$ l 2 $\times$ GE Hybridization buffer was added to dilute the labeled cRNA. 100  $\mu$ l of hybridization solution was dispensed into the gasket slide and assembled to the gene expression microarray slide. The slides were incubated for 17 h at 65  $^{\circ}$ C in an Agilent Hybridization Oven. The hybridized arrays were washed, fixed and scanned with using the Agilent Microarray Scanner (part number G2505C).

The chip is washed and scanned using the Agilent Microarray Scanner. Agilent Feature Extraction software is used to collect the chip probe signal value. Chip standardization was carried out with Agilent GeneSpring GX v14.9 software.

## Statistics

All quantitative data are expressed as mean  $\pm$  SD. The software SPSS Statistics 22.0 (SPSS, Chicago, IL) was used to perform the statistical analysis. One-way ANOVA (analysis of variance) was used to verify the differences between groups.  $p<0.05$  was regarded statistically significant.

## Results

### Molecular hydrogen inhibited type 2 cytokine production in OVA-induced asthma mice

Ovalbumin strongly induced the production of danger signal mediators and type II cytokines including interleukin (IL) 4, IL-25, IL-33, and thymic stromal lymphopoietin

(TSLP) while inhibited Th1 mediator interferon gamma (INF- $\gamma$ ) (Fig. 1a). The serum and bronchoalveolar lavage fluid (BALF) levels of monocyte chemoattractant protein 1 (MCP-1) were also significantly increased by OVA (Fig. 1a). Inhalation of hydrogen did not show any effect on cytokine production of control non-asthma mice but markedly reduced serum and BALF levels of IL-4, IL-25, IL-33, TSLP, and MCP-1 in OVA-induced asthmatic mice (Fig. 1a).

Moreover, the expression levels of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) and IL-33 receptor ST2 (tumorigenicity 2 receptor) in airway tissues of OVA-induced asthmatic mice were significantly higher than those of control mice, and hydrogen inhalation suppressed the upregulation of NF- $\kappa$ B and ST2 in asthmatic mice (Fig. 1b, c).

When cultured in vitro, BALF cells from asthmatic mice excreted more than 2.5 fold of IL-5, IL-9, IL-13, and IL-33 compared to BALF cells from control mice, which was substantially inhibited by treatment with hydrogen gas (Fig. 1d).

### Hydrogen protected airway epithelial barrier from asthma associated damages

OVA induction of asthma caused obvious damages to airway epithelium and reduced levels of cell adhesion protein E-cadherin and tight junction protein ZO-1 (Fig. 2a, b). Inhalation of hydrogen gas attenuated OVA-induced damages to airway epithelial barrier and partially but significantly increased E-cadherin and ZO-1 protein levels (Fig. 2a, b).

The markers of apoptosis caspase 3 and caspase 9 were overexpressed and the cleaved active form of Caspase 3 and Caspase 9 proteins were drastically increased in asthmatic mouse airway tissue, which was markedly inhibited by hydrogen inhalation (Fig. 2c, d).

### The increase of group 2 innate lymphoid cell population in asthmatic mice was inhibited by hydrogen inhalation

As ILC2s play a critical role in asthma [8, 13], the population of lineage<sup>−</sup>ICOS<sup>+</sup>ST2<sup>+</sup> ILC2 was assessed by flow cytometry. In OVA-induced asthmatic mouse BALF, ICOS<sup>+</sup>ST2<sup>+</sup> cells were about 61.2% of lineage<sup>−</sup> population, which was significantly higher than 42.4% and 40.4% from control mouse with or without inhaling hydrogen gas. Inhalation of hydrogen gas reduced ICOS<sup>+</sup>ST2<sup>+</sup> cells to about 50% of lineage<sup>−</sup> population in asthmatic mouse BALF (Fig. 3).

### Hydrogen protected human bronchial epithelial cells from HDM induced apoptosis

Treating human bronchial epidermal cells (16HBE) with 10  $\mu$ g/ml HDM significantly reduced their viability while exposure to 60% hydrogen alone did not change the viability of 16HBE cells (Fig. 4). However, hydrogen gas relieved HDM caused reduction of viability of 16HBE cells (Fig. 4).

### Molecular hydrogen suppressed the upregulation of IL-33 and ST2 in 16HBE cells

HDM treatment increased mRNA levels of both IL-33 and ST2 of 16HBE cells by more than 5 times (Fig. 5a). Meanwhile, the levels of medium IL-33 (Fig. 5b) and cellular IL-33 protein (Fig. 5c, d) were also significantly elevated by HDM. The augmentation of IL-33 at mRNA and protein levels by HDM was markedly inhibited by hydrogen exposure (Fig. 5a–d). The luciferase report assay confirmed that HDM activated the transcriptional activity of IL-33 promoter and hydrogen gas inhibited such activation for more than 50% (Fig. 5e). The protein level of ST2 in HDM treated cells was significantly higher than control or hydrogen only cells while hydrogen plus HDM treated cells had lower ST2 protein level than HDM only cells but higher than that of control cells (Fig. 5d).

### Identification of an asthmatic microRNA signature of human airway epithelial cells

As microRNAs were strongly implicated in different aspects of asthma [23, 24], a microRNA array was employed to identify the changes of microRNA expression associated to HDM and or hydrogen treatment of 16HBE cells. Using fold change of 1.5 of log2 value and  $p < 0.05$  as the cut-off, 21 miRNAs were upregulated and 19 miRNAs downregulated by HDM (Table S2). Compared to HDM treated 16HBE cells, H<sub>2</sub> caused overexpression of 48 miRNAs and downregulation of 21 miRNAs (Table S2). A miRNA signature composed of 22 miRNAs was identified (Fig. 6). Of them, levels of 17 miRNAs were significantly reduced and 5 increased by HDM while H<sub>2</sub> treatment essentially reversed the effects of HDM (Fig. 6).

## Discussion

Asthma has many clinical presentations with different types of excessive immunological responses [2, 3] which involve many immune cells and fibroblasts and epithelial cells [25]. Activation of airway epithelial cells and release of cytokines including IL-25, IL-33, and TSLP play a major role in onset of asthma [26]. IL-25 and IL-33 from activated airway

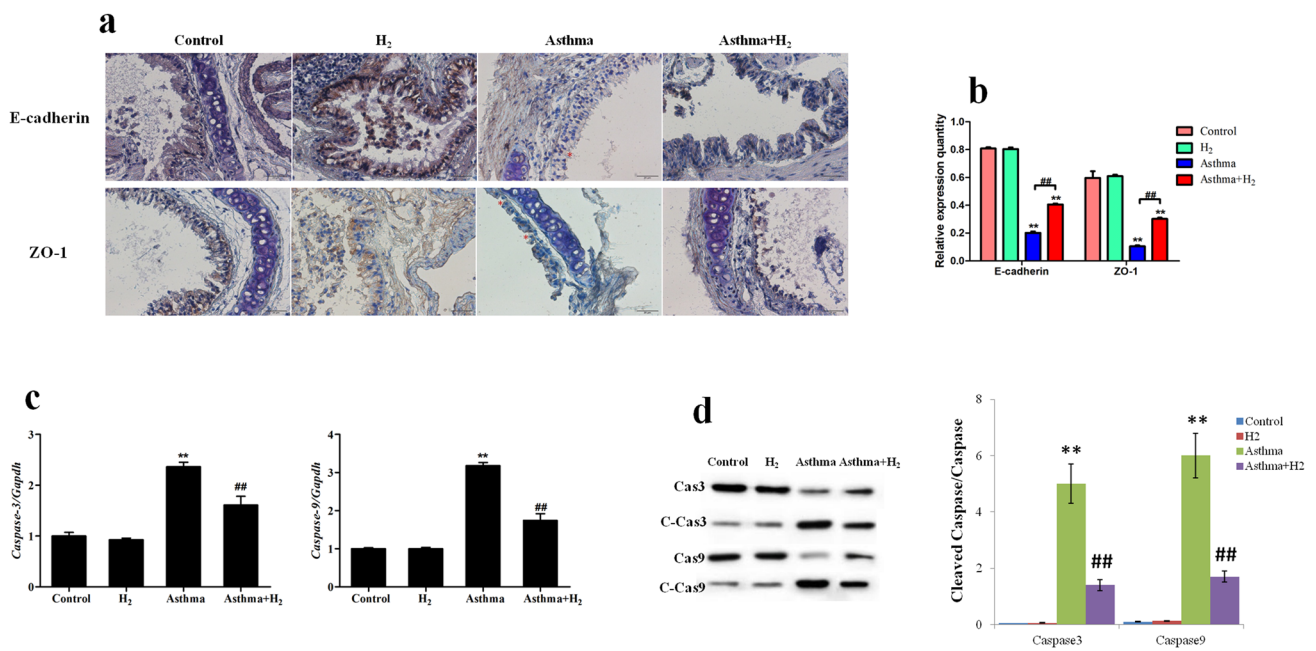


**Fig. 2** H<sub>2</sub> ameliorated OVA induced disruption of airway epithelial barrier. **a** Mouse airway E-cadherin and ZO-1 proteins were detected by immunohistochemical staining with specific antibodies. **b** Total RNA was extracted from mouse airway tissues and mRNA levels of caspase 3 and caspase 9 were analyzed by quantitative real-time

PCR. **c** Protein samples were prepared from mouse airway tissues and Caspase 3 and 9 (unprocessed and cleaved forms) were detected by western blot. \*\* $p < 0.01$  compared to control; ## $p < 0.01$  compared to asthma group

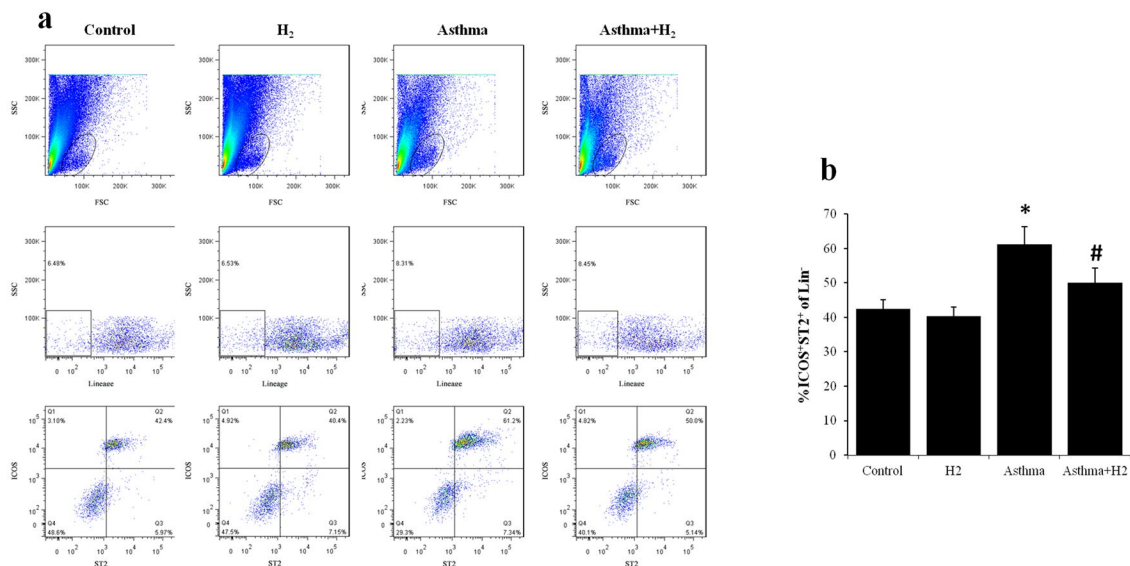


**Fig. 3** H<sub>2</sub> inhibited OVA induced activation of ILC2. **a** Cells collected from BALF were stained with antibody against lineage, ICOS, and ST2 and serially gated for lineage<sup>+</sup>ICOS<sup>+</sup>ST2<sup>+</sup> cells. **b** Quantita-

tive analysis of lin-ICOS<sup>+</sup>ST2<sup>+</sup> population in each group. \* $p < 0.05$  compared to control; # $p < 0.05$  compared to asthma group

epithelial cells and other sources induce the expansion and activation of ILC2s which produce type II inflammatory cytokines [8, 11, 25, 26]. The current study demonstrated

that molecular hydrogen inhibited IL-33 expression in HDM activated human bronchial epithelial cells (16HBE) at transcriptional level and might also at post-transcription level.

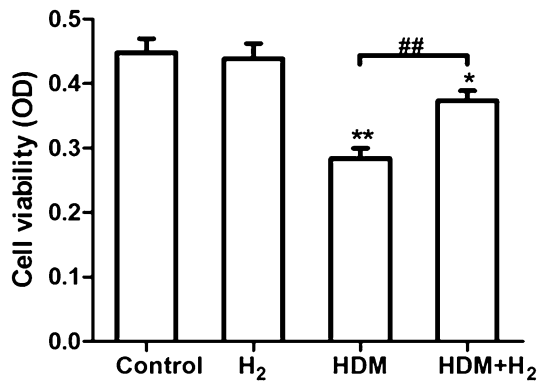


**Fig. 4** H<sub>2</sub> alleviated HDM induced damage to 16HBE cells. 16HBE cells were treated with or without 10 µg/ml HDM under normal condition or exposed to H<sub>2</sub> for 12 h. Cell viability was assessed by CCK-8 assay. \* $p < 0.05$  and \*\* $p < 0.01$  compared to control; ## $p < 0.01$  compared to HDM

IL-33 mRNA and protein levels of 16HBE cells were greatly increased by HDM, which was inhibited by hydrogen gas. These changes were at least partially due to the alteration of transcription activity as IL-33 promoter activity was strongly enhanced by HDM. Hydrogen gas alone did not change IL-33 promoter activity, and mRNA and protein levels of IL-33 in 16HBE cells but significantly suppressed HDM induced overexpression of IL-33, indicating that molecular hydrogen not only acted as an antioxidant [16, 19] but also modulated IL-33 expression through regulating its transcription in HDM activated 16HBE cells. Meanwhile, IL-33 receptor ST2 exhibited a similar pattern to that of IL-33 in response to HDM and hydrogen treatments, implying that molecular hydrogen blocked the auto-amplification of IL-33/ST2 pathway [27].

MicroRNAs (miRNAs) are a class of highly conserved small non-coding RNA molecules with 18–22 nucleotides long and many microRNAs are implicated in asthma [28, 29]. Serum levels of many miRNAs including miR-21, miR-125b, miR-126, miR-145, miR-148a, miR-221, miR-338, and miR-485-3p were increased in asthmatic patients [30–32]. In nasal biopsy samples, miR-18a, miR-126, let-7e, miR-155, miR-224 were downregulated and miR-498, miR-187, miR-874, miR-143, miR-886-3p were upregulated in asthmatic patients [33]. We identified that 21 miRNAs were upregulated and 19 miRNAs were downregulated by HDM in human bronchial epithelial cell line 16HBE. Among them, miR-1246 was the most upregulated miRNA by HDM treatment for more than 3.2 fold, which was in line with the finding in airway brushing samples from steroid naïve patients [34]. MiR-21-3p was upregulated more than 1.4 fold in 16HBE cells by HDM and this upregulation was completely abolished by hydrogen gas (-1.53 fold for HDM + H<sub>2</sub> vs HDM; -0.21 fold for HDM + H<sub>2</sub> vs Control).

MicroRNA-21 was found to drive severe, steroid-insensitive allergic airway disease through targeting phosphatase and tensin homolog (PTEN) to regulate PI3K pathway [35]. We speculate that these miRNAs may play a role in different aspects of asthma pathogenesis. However, it requires further investigations to elucidate the role of different miRNA in asthma and how they are regulated by allergens and molecular hydrogen (Fig. 7).

IL-33 has been shown as the major player for activation of ILC2s [8, 25–27], the increased level of IL-33 was found in both serum and bronchoalveolar lavage fluid in OVA induced asthmatic mice, which would activate lung ILC2 population. Lineage<sup>+</sup>ICOS<sup>+</sup>ST2<sup>+</sup> ILC2 population was significantly increased in OVA-induced asthmatic mice. Hydrogen gas reduced serum and BALF IL-33, IL-4, IL-25, and TSLP levels as well as lineage<sup>+</sup>ICOS<sup>+</sup>ST2<sup>+</sup> ILC2 population of asthmatic mice, indicating molecular hydrogen was capable of mitigating OVA induced type II inflammation through targeting IL-33/ST2 pathway (Fig. 7) [27].

Airway epithelial barrier integrity plays a critical role in the pathogenesis of asthma as it is not only the first line of defense against allergens and other environmental stimuli but also a major source of IL-33, IL-25, and TSLP [26, 36]. The current data showed that the airway epithelial integrity was compromised in asthmatic mice where the levels of cell adhesion molecule E-cadherin and tight junction protein ZO-1 were markedly lower than that of control mice. It has been shown that IL-33 activated ILC2s disrupted airway epithelial barrier through IL-13 mediated inhibition of expression of tight junction proteins [37]. Therefore, it would be possible to ameliorate asthma by improving airway epithelial integrity through inhibiting airway epithelial ER stress and apoptosis [38]. Molecular hydrogen indeed suppressed the activation of caspase 3 and 9 and restored the levels of E-cadherin and ZO-1 in asthmatic mouse airway tissue. Moreover, house dust mite induced loss of viability of 16HBE cells was also abrogated by hydrogen, indicating the protective effect of hydrogen on airway epithelial cells upon exposure to allergens (Fig. 7).

In summary, molecular hydrogen suppressed OVA induced upregulation of mRNA and protein levels of IL-33 and ST2 in mouse airway, elevation of serum and BALF IL-33 and other alarmin and type II cytokine levels, activation of ILC2, and disruption of airway epithelial barrier integrity. Hydrogen gas inhibited HDM induced apoptosis of 16HEB cells and overexpression of IL-33 and ST2 in 16HEB cells. HDM promoted IL-33 promoter activity was inhibited by molecular hydrogen. Moreover, a miRNA signature associated with asthmatic response and hydrogen inhibition of such responses were identified. Although the exact mechanism governing IL-33 upregulation in airway epithelial cells upon allergen exposure [39], the current data presented appealing evidences to show that mechanisms

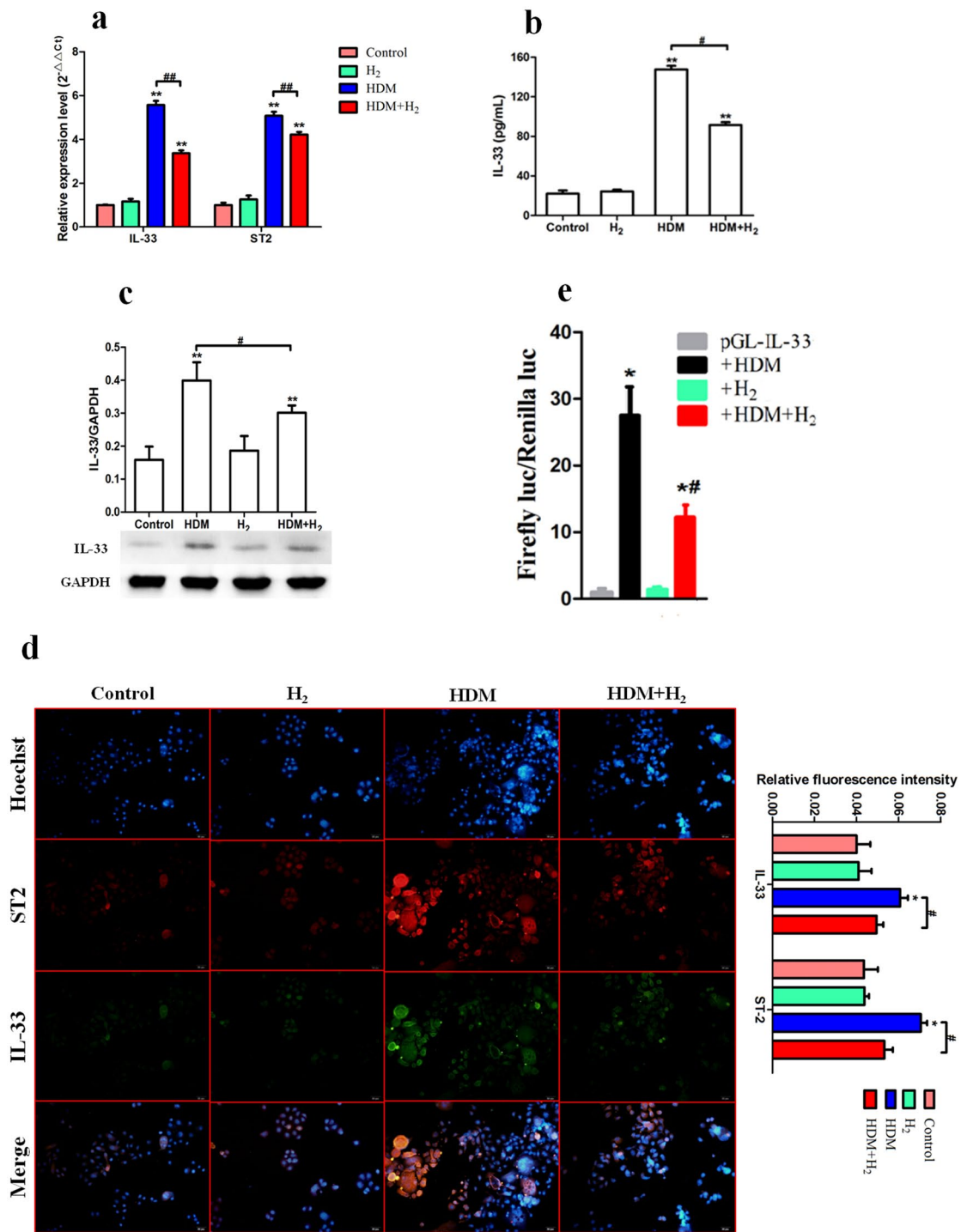


**Fig. 5** H<sub>2</sub> inhibited HDM induced overexpression of IL-33 and ST2. 16HBE cells were treated with or without 10  $\mu$ g/ml HDM under normal condition or exposed to H<sub>2</sub> for 12 h. **a** IL-33 and ST2 mRNA levels were assayed by quantitative real-time PCR. **b** Medium IL-33 level was measured by ELISA. **c** Cellular IL-33 protein was detected

by western blot. **d** Cellular IL-33 and ST2 proteins were detected by immunofluorescence. **e** IL-33 promoter activity was assessed by dual-luciferase assay. \* $p$  < 0.05 and \*\* $p$  < 0.01 compared to control; ## $p$  < 0.01 compared to HDM

**Fig. 6** H<sub>2</sub> reversed the changes of miRNA expression caused by HDM. 16HBE cells were treated with or without 10 µg / ml HDM under normal condition or exposed to H<sub>2</sub> for 12 h. Total RNA was isolated and subjected to miRNA array assay. A heatmap showing a group of miRNAs that up- or downregulated by HDM and reversed by H<sub>2</sub>

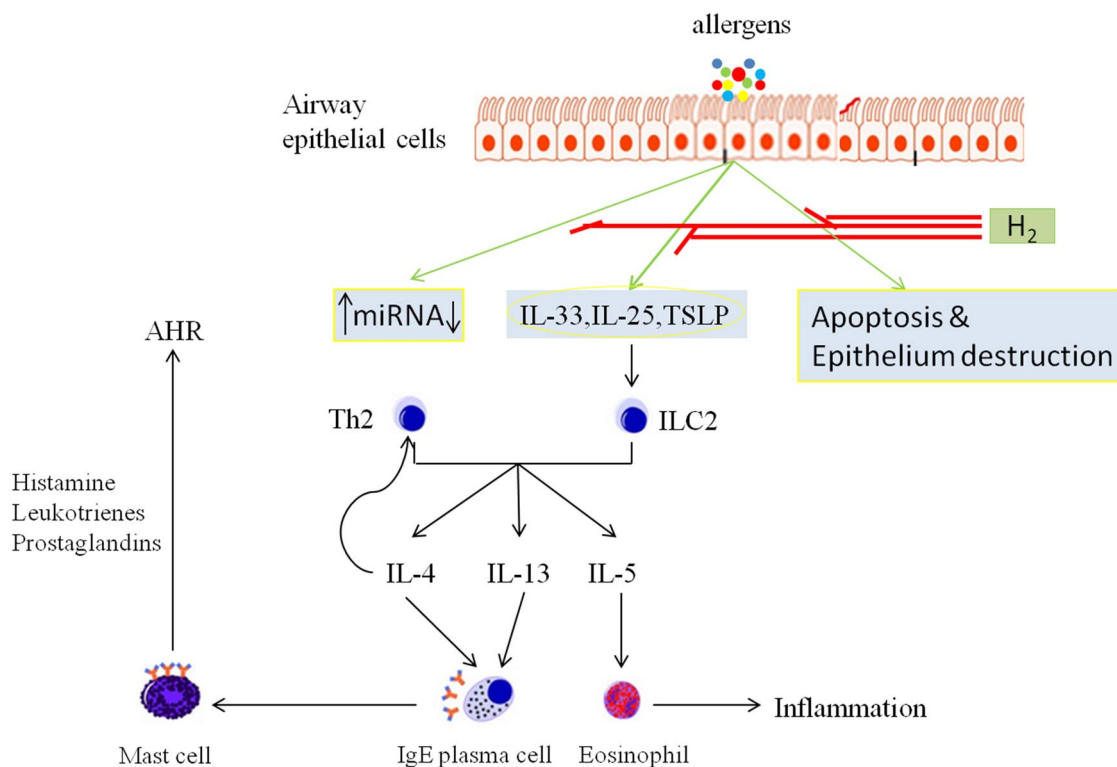
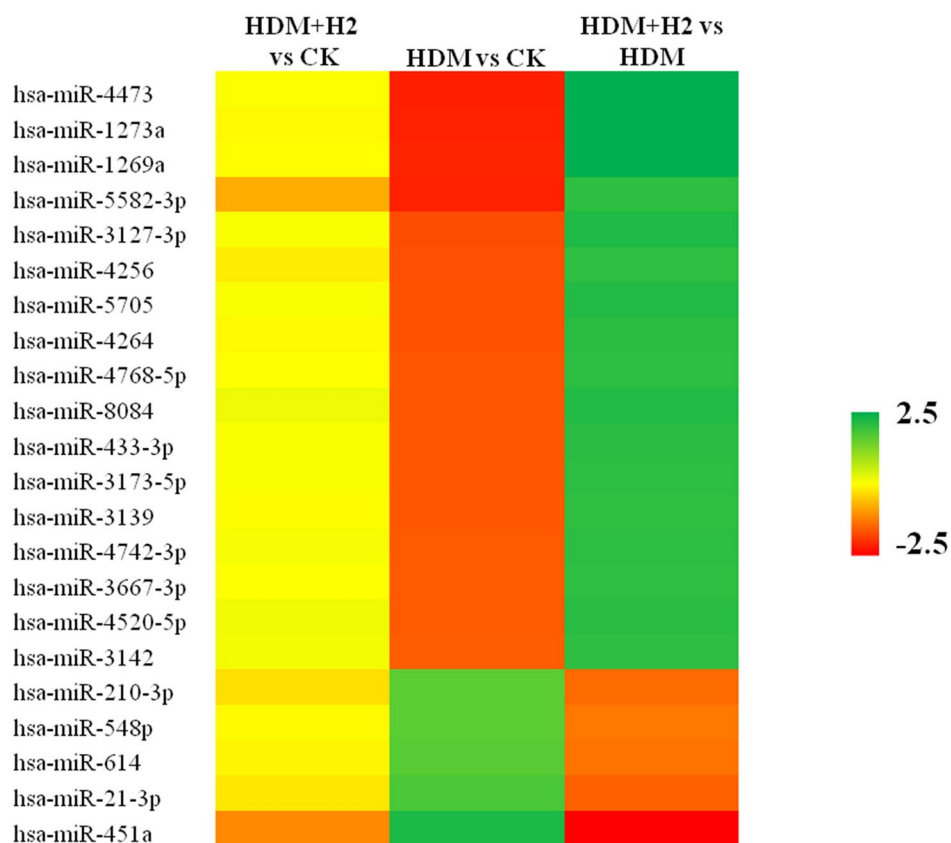


**Fig. 7** A working model for how molecular hydrogen protect against asthma. Please refer to text for details

involving transcriptional and post-transcriptional regulation were implicated in both asthma induction and the amelioration of asthma by molecular hydrogen.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s00011-021-01459-w>.

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**Table S1.** Primers used for RT-PCR

| Gene            |                       | Sequence              |
|-----------------|-----------------------|-----------------------|
| p65(Mouse)      | CAGCCGGAGCCACCAACAA   | CAGGGACGACGCCATAGA    |
| Caspase9(Mouse) | CGGTGGACATTGGTTCTG    | CACATTGTTGATGATGAGGC  |
| Caspase3(Mouse) | TGACTGGAAAGCCGAAAC    | CTGGATGAACCACGACCC    |
| ST2(Mouse)      | CTGGGATTTATGCTTGTG    | CTGATCCACGTACTGTCG    |
| GAPDH(Mouse)    | GGTGAAGGTCGGTGTGAACG  | CTCGCTCCTGGAAGATGGTG  |
| ST2(human)      | AACCTAACTTGCTCTGCTTGT | CTTTGATTTTGCCCTTCCTCT |
| IL33(human)     | TACTTTTGCTTTGGAGGATG  | CTGGTCTGGCAGTGGTTTTT  |
| GAPDH(human)    | AGAAGGCTGGGGCTCATTG   | AGGGGCCATCCACAGTCTTC  |



|                 |       |
|-----------------|-------|
| 3142            |       |
| hsa-miR-6840-5p | 1.643 |
| hsa-miR-323a-3p | 1.645 |
| hsa-miR-6806-3p | 1.647 |
| hsa-miR-4802-5p | 1.650 |
| hsa-miR-5582-3p | 1.651 |
| hsa-miR-4743-3p | 1.658 |
| hsa-miR-6755-5p | 1.659 |
| hsa-miR-7151-5p | 1.663 |
| hsa-miR-6807-3p | 1.666 |
| hsa-miR-4742-3p | 1.667 |
| hsa-miR-4768-5p | 1.670 |
| hsa-miR-3173-5p | 1.672 |
| hsa-miR-4264    | 1.678 |
| hsa-miR-433-3p  | 1.680 |
| hsa-miR-4520-5p | 1.686 |
| hsa-miR-8084    | 1.736 |
| hsa-miR-3929    | 1.737 |
| hsa-miR-5705    | 1.738 |
| hsa-miR-3127-3p | 1.775 |
| hsa-miR-1273a   | 2.149 |
| hsa-miR-1269a   | 2.152 |
| hsa-miR-4263    | 2.158 |
| hsa-miR-4446-5p | 2.168 |
| hsa-miR-3692-3p | 2.187 |
| hsa-miR-        | 2.231 |

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