

# siRNA-mediated knockdown of T-bet and ROR $\gamma$ t contributes to decreased inflammation in pre-eclampsia

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**Abstract.** Abnormal immune response resulting from disordered T helper (Th)1/Th2 and Th17/regulatory T cells (Treg) cytokine expression has been demonstrated to serve an important role in the pathogenesis of preeclampsia (PE). However, the role of transcription factors regulating Th cell differentiation contributing to PE remain unclear. To determine whether a decrease in the expression of the T cell lineage transcription factor T-bet can restore immune balance and alleviate the systemic inflammatory response present in PE, 30 patients diagnosed with PE were assessed and compared with healthy pregnant controls. The expression of the transcription factors T-bet and retinoic acid receptor-related orphan receptor (ROR) $\gamma$ t were increased in the peripheral blood mononuclear cells of PE patients compared with controls, consistent with the presence of abnormally high T-bet:GATA3 and ROR $\gamma$ t:forkhead box (FOX) P3 ratios. The present study additionally identified a high-efficiency, specific small interfering (si)RNA that can downregulate ROR $\gamma$ t and T-bet mRNA levels and inhibit protein expression. This effective siRNA was transfected into activated CD4<sup>+</sup> T cells derived from patients with PE to observe the changes to transcription factor expression and attempt to elucidate the regulatory mechanism of T cell subsets. It was identified that knockdown of ROR $\gamma$ t induced increased expression of FOXP3 and that the ratios of ROR $\gamma$ t:FOXP3 and interleukin (IL)-17A:IL-10 were subsequently decreased. The results suggested that siRNA-mediated knockdown of T-bet regulated the immune balance of Th17/Tregs via changes to ROR $\gamma$ t and FOXP3. When siRNA against ROR $\gamma$ t and T-bet were used in combination, a stronger ability to regulate immune balance was observed. These results imply that Th1- and Th17-type immunity is dominant in PE and that the siRNA-mediated

knockdown of certain Th1 and Th17 cell transcription factors may be an effective therapeutic target for promoting immune balance in CD4<sup>+</sup> T cell subgroups and ameliorating local and generalized inflammation in PE.

## Introduction

Successful pregnancy relies on the subtle regulation of the maternal immune system to allow for the tolerance of the semi-allogenic fetus at the maternal-fetal interface. The maternal-fetal interface is thought to be the origin of preeclampsia (PE), a complex illness involving multiple organ dysfunction that occurs during human gestation, affecting 4-5% of all pregnancies (1). PE is characterized by serious hypertensive disorder and proteinuria that can result in restriction of fetal growth, premature birth, and increase the risk of fetal and maternal morbidity and mortality. Previous studies have demonstrated that proper immuno-regulation is crucial during pregnancy and that maternal-fetal tolerance is required for successful pregnancy (2,3).

Naive CD4<sup>+</sup> T cells possess a high level of plasticity and differentiate into T helper (Th)1 and Th2 and T regulatory (Treg) cells to execute their immunological functions (4,5). Specific master transcription factors T-bet and GATA binding protein 3 (GATA3) control the Th1 and Th2 differentiation, respectively. Predominant Th1 type immunity has been reported to be correlated with PE pregnancies; increased Th1-type cytokines, including tumor necrosis factor (TNF)- $\alpha$  and interferon (IFN)- $\gamma$ , are detected in PE plasma (6). Th2 cells, a novel CD4<sup>+</sup> lymphocyte subpopulation, produce anti-inflammatory cytokines against pro-inflammatory cytokines released from Th1 cells to maintain the immune tolerance state and accommodate the semiallogenic fetus at the maternal-fetal interface (7). Previous studies identified a new subset of CD4<sup>+</sup> helper cells, the Th17 lineage, with the receptor-related orphan receptor (ROR) $\gamma$ t as the master transcription factor and expressing pro-inflammatory cytokines (8), including interleukin (IL)-17 and IL-22. In PE, the increased level of IL-17 in serum is correlated with high blood pressure and immune disorders (9). Treg cells are forkhead box (FOX) P3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> cells, along with other transcription factors. FOXP3 is the master regulator responsible for the development, maintenance and suppressive function of Treg cells. Regarded as one of the most important subsets of suppressor CD4<sup>+</sup> T cells, Treg cells are crucial for

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anti-inflammatory responses and confer immune-tolerance during pregnancy (10).

T-bet, GATA3, ROR $\gamma$ t, FOXP3 are the major T-cell transcription factors that regulate the differentiation of T lymphocyte subsets. Their coordinated regulation is crucial for maintaining immune homeostasis during pregnancy (11). Previous studies have demonstrated that over-expression of Th1 cells and an imbalance of Th1/Th2 cells are predominant factors in the development of PE (12,13). However, more recent findings have implied a less significant role for Treg cells in the activation of inflammatory responses in PE. Instead, it is hypothesized that Th1 and Th17 immunity can act through the increased expression of IL-23 from dendritic cells (14-16). To further define the precise roles of Th1, Th2, Th17 and Treg, the present study used specific small interfering (si)RNAs (17) to inhibit ROR $\gamma$ t and T-bet in PE CD4<sup>+</sup> T lymphocytes and thus investigate whether the siRNA-mediated knockdown of these transcription factors may be able to correct the immune imbalance and alleviate inflammation responses present in PE.

## Materials and methods

**Study participants.** 30 PE patients were recruited from the Department of Obstetrics at the Nantong Women and Children Health Care Hospital between October 2015 and January 2016 (mean age 27.8±2.5 years; mean gestational age 34.1±2.3 weeks). Then 30 healthy pregnant women (mean age 27.7±3.1 years; mean gestational age 33.7±1.1 weeks) were recruited simultaneously as a control group. There were no significant differences between the two groups in terms of maternal and gestational ages. PE diagnosis was defined as severe gestational hypertension (systolic blood pressure of at least 140 mmHg and/or diastolic blood pressure of at least 90 mmHg on 2 occasions at least 4 h apart) and proteinuria (>30 mg/dl in at least 2 random urine specimens) after 20 weeks' gestation. Patients had no other obstetric or medical complications or histories of autoimmune disorders. All experimental procedures using human samples were performed with the approval of the Nantong Women and Children Health Care Hospital Ethics Committee and all the participants provided informed consent.

**Cell preparation.** Blood samples (10 ml) were collected from each participant, peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation at 2,000 x g for 20 min at 4°C (GE Healthcare Life Sciences, Little Chalfont, UK) and CD4<sup>+</sup> T cells were sorted by immune-magnetic beads coated with anti-human CD4 antibodies (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). CD4<sup>+</sup> T cells were washed twice and resuspended in RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), placed in 24-well plates in the presence of anti-CD3 (cat. no. 14-0037-82) monoclonal antibodies (mAbs) 20  $\mu$ l (diluted 1:10; eBioscience; Thermo Fisher Scientific, Inc.) and anti-CD28 mAbs (cat. no. 16-0288-81) 10  $\mu$ l (diluted 1:2; eBioscience; Thermo Fisher Scientific, Inc.), and cultured at 37°C in 5% CO<sub>2</sub> to induce cell differentiation. On day 3, 500,000 U/l IL-2 (Changsheng Gene Pharmaceutical Co., Ltd., Changchun, China), was added to maintain cell growth and

half of the medium was renewed. On day 6, suspended cells were collected.

**siRNA design.** siRNAs were designed using Primer 3 and the specificity was tested using the Primer-BLAST program (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). Then, 3 pairs of ROR $\gamma$ t or T-bet specific double-stranded siRNAs were designed to target distinct sets of RNA sequences and the siRNAs were chemically synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). Details are presented in Table I.

**Transfection of cells with siRNA.** To establish a stable transfection and evaluate transfection efficiency, 6-FAM-siRNA (Shanghai GenePharma Co., Ltd.) was transfected into cells simultaneously to measure the efficiency of transfection. In all, 3 conditions were tested on 2.5x10<sup>4</sup> cells in each well of a 24-well plate with 30 pmol siRNA and 1, 2 or 3  $\mu$ l DMRIE-C (Life Technologies; Thermo Fisher Scientific, Inc.); following 6 h transfection, efficiency was ascertained by fluorescence microscopy.

The medium was removed and cells were washed with OptiMem<sup>®</sup> I (Gibco; Thermo Fisher Scientific, Inc.) 1 day prior to transfection. Cells were then suspended at a concentration of 5x10<sup>4</sup>/ml and 2.5x10<sup>4</sup> cells were seeded in a 24-well plate and incubated at 37°C in 5% CO<sub>2</sub> in a humidified incubator. After 24 h and at 80% confluence the cells were transfected with siRNA using DMRIE-C (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. There were 5 groups: Blank control (BC) transfected with blank liposome, negative control (NC) transfected with scrambled-siRNA, group 1 transfected with T-bet-siRNA, group 2 transfected with ROR $\gamma$ t-siRNA and group 3 transfected with T-bet-siRNA and ROR $\gamma$ t-siRNA. DMRIE-C reagent was diluted in OptiMem<sup>®</sup> I medium in a 0.5 ml Eppendorf tube, mixed at room temperature for 10 min and then diluted siRNA was added to the mixture and incubated for 30 min to form the siRNA/transfection reagent complex. The complex was then added to the cells and cultured in serum-free medium. Following transfection for 4-5 h at 37°C in 5% CO<sub>2</sub>, the medium in each well was replaced with complete RPMI 1640 without antibiotics and incubated for 48 h at 37°C prior to subsequent experiments.

**SYBR Green reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Inc.) and complementary DNA (cDNA) was synthesized from 1  $\mu$ g RNA using the RevertAid<sup>™</sup> First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.). RT-qPCR was conducted with the aid of the Rotor-gene Q Realtime PCR Platform and SYBR Green PCR Master Mix (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. Oligonucleotide primer pairs used for the analysis were as follows: T-bet, sense AATGTGACCCAGATGATTGTGC and anti-sense ATGCTGGTGTCAACAGATGTGTAC; GATA3, sense ACCGGCTTCGGATGCAA and anti-sense TGCTCTCCTGGCTGCAGAC; ROR $\gamma$ t, sense TAACCAAAAATG GATGGGATG and anti-sense, AAGCTGTGGCCTCAA GGAT; FOXP3, sense GAGAAGGAGAAGCTGAGTGCCAT and anti-sense AGCAGGAGCCCTTGTCGGAT; GAPDH,

Table I. siRNA sequences designed for T-bet and ROR $\gamma$ t with scrambled siRNA as the negative control.

siRNA	Sense	Antisense
siT-bet-918/940	UUAGUUUCCCAAUGAAACUU	GUUUCAUUUGGGAAACUAAAG
siT-bet-1124/1146	UGUAAUCUCGGCAUUCUGGUA	CCAGAAUGCCGAGAUUACUCA
siT-bet-1837/1859	AAUAAACACUGUUUCUGUCCU	GAACAGAAACAGUGUUAUUAG
siROR $\gamma$ t-368/390	CCCGAGAUGCUGUCAAGUUTT	AACUUGACAGCAUCUCGGGTT
siROR $\gamma$ t-629/651	CCUCAUAUCCAACAACUUTT	AAGUUGUUGGAAUAUGAGGTT
siROR $\gamma$ t-713/735	GGCAGAGAGAGCUUCUAUATT	UAUAGAAGCUCUCUCUGCCTT
Scrambled siRNA	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT

si, small interfering; ROR $\gamma$ t, retinoic acid receptor-related orphan receptor  $\gamma$ t.

sense ACGGCAAATTCAACGGGACAGTCA and anti-sense TGGGGGCATCGGCAGAAGG. Primers were synthesized by Shanghai GenePharma Co., Ltd. The amplifications were conducted in a 36-well plate in a 20  $\mu$ l reaction volume containing 1X Real-time PCR Master Mix, 0.2  $\mu$ M primer, 1 U rTaq polymerase, 2  $\mu$ l cDNA samples and RNase-free water. The samples were run in triplicate for the target and for house-keeping genes using the following conditions: Initial denaturation for 3 min at 95°C, followed by a total of 40 cycles (denaturation 12 sec at 95°C and annealing 40 sec at 62°C) and extension for 30 sec at 72°C. Statistical analysis of the mean value as the representative of each investigated gene and the  $2^{-\Delta\Delta C_q}$  method were used to analyze relative changes in gene expression (18).

**Western blot analysis.** Cells were homogenized in lysate buffer containing 10 mM Tris-HCl (pH 7.4), 1% Triton X-100, 1% sodium deoxycholate, 5 mM EDTA, 1 mM PMSF, 10 mg/l aprotinin and 50 mg/l leupeptin. The homogenate was then centrifuged at 12,000 x g for 10 min at 4°C to collect the supernatant. Protein concentration was determined using a BCA protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The supernatant was diluted in 5X SDS loading buffer and was boiled. Then 20  $\mu$ g of protein were subjected to SDS polyacrylamide gel electrophoresis (5-10% gradient gels) and transferred to polyvinylidene difluoride filter membranes. The membrane was then blocked with 5% nonfat milk for 2 h at room temperature, and probed with the following monoclonal antibodies: Anti-T-bet mAbs, cat. no. sc21749; anti-GATA3-mAbs, cat. no. 268x; anti-ROR $\gamma$ t mAbs, cat. no. Sc6062; anti-FOXP3 mAbs, cat. no. sc-166212, at a dilution of 1:100. Membranes were washed in Tris-buffered saline containing 0.5% Tween-20 and incubated with 5% nonfat dry milk overnight at 4°C. Following incubation with the appropriate horseradish peroxidase conjugated anti-human IgG monoclonal anti-body (diluted 1:100, cat. no. sc-2453), All monoclonal antibodies were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). Proteins were detected with an enhanced chemiluminescent system (Pierce, Thermo Fisher Scientific, Inc.).

**Flow cytometry.** For intracellular cytokine staining, lymphocytes were collected and cultured in 96-well plates (200  $\mu$ l and  $2 \times 10^9$  cells per well). 500 ng/ml Ionomycin (Invitrogen;

Thermo Fisher Scientific, Inc.) and 10 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) were added and cultured at 37°C in 5% CO<sub>2</sub> for 3 h, then treated with 2.5  $\mu$ mol monensin for 2 h. Cells were collected and the supernatant was removed by centrifugation at 1,500 x g for 5 min at room temperature; surface staining was performed by incubation with the corresponding fluorescently labelled antibodies on ice for 15 min in the dark. Mouse monoclonal antibodies (mAb) were used at a dilution of 1:20 and provided by eBioscience (Thermo Fisher Scientific, Inc.). The antibodies were as follows: fluorescein isothiocyanate (FITC)-labelled anti-human IL-17A (IgG1, cat. no. 11-7179), anti-human interferon- $\gamma$  (IgG1, cat. no. 53-7319), anti-human FOXP3 (IgG2a, cat. no. 11-4776-41), phycoerythrin (PE)-labelled IL-10 (IgG1, cat. no. 53-7108-41), perCy5.5-labeled anti-human CD8 (IgG1, cat. no. 45-0088-41). Cells were washed twice with staining buffer and fixed in 500  $\mu$ l of 40 g/l paraformaldehyde (PFA) at 4°C for 30 min. Cells were washed twice using staining buffer, then intracellular staining was performed with the Cytofix-Cytoperm buffer kit (BD Biosciences, Franklin Lakes, NJ, USA). Following cell membrane permeation for 30 min at 4°C, the supernatant was removed and incubated with fluorescently labeled antibodies (BD Biosciences) against intracellular cytokines. Following a final wash, 300  $\mu$ l of 40 g/l PFA was added and flow cytometric measures were performed on a FACSCalibur flow cytometer (and analyzed using FlowJo software version 7.6.1; FlowJo, LLC., Ashland, OR, USA).

**Statistical analysis.** Statistical analysis was performed using GraphPad Prism v.5.0a (GraphPad Software, Inc. La Jolla, CA, USA). All data were expressed as the mean  $\pm$  standard error. Comparison between two groups were performed by paired or unpaired Student's t-test, as appropriate. One-way analysis of variance followed by Dunnett's test were used for comparisons among multiple treatment groups. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Expression of T-bet and ROR $\gamma$ t mRNA is increased in PE.** The expression levels of T-bet and ROR $\gamma$ t mRNA in patients with PE were significantly higher compared with normal pregnancies (P<0.05; Table II), while no difference was observed in GATA3

Table II. mRNA expression of transcription factors in peripheral blood mononuclear cells from normal pregnancy and PE.

Groups	n	T-bet	GATA3	T-bet/GATA3	ROR $\gamma$ t	FOXP3	ROR $\gamma$ t/FOXP3
Normal	30	0.029 $\pm$ 0.007	0.052 $\pm$ 0.012	0.556 $\pm$ 0.095	0.018 $\pm$ 0.004	0.036 $\pm$ 0.017	0.535 $\pm$ 0.124
PE	30	0.057 $\pm$ 0.011 <sup>a</sup>	0.037 $\pm$ 0.010	1.695 $\pm$ 0.746 <sup>a</sup>	0.026 $\pm$ 0.003 <sup>a</sup>	0.031 $\pm$ 0.007	0.896 $\pm$ 0.203 <sup>a</sup>

<sup>a</sup>P<0.05 vs. normal pregnancy. GATA binding protein 3; ROR $\gamma$ t, retinoic acid receptor-related orphan receptor  $\gamma$ t; FOX, forkhead box; PE, preeclampsia.

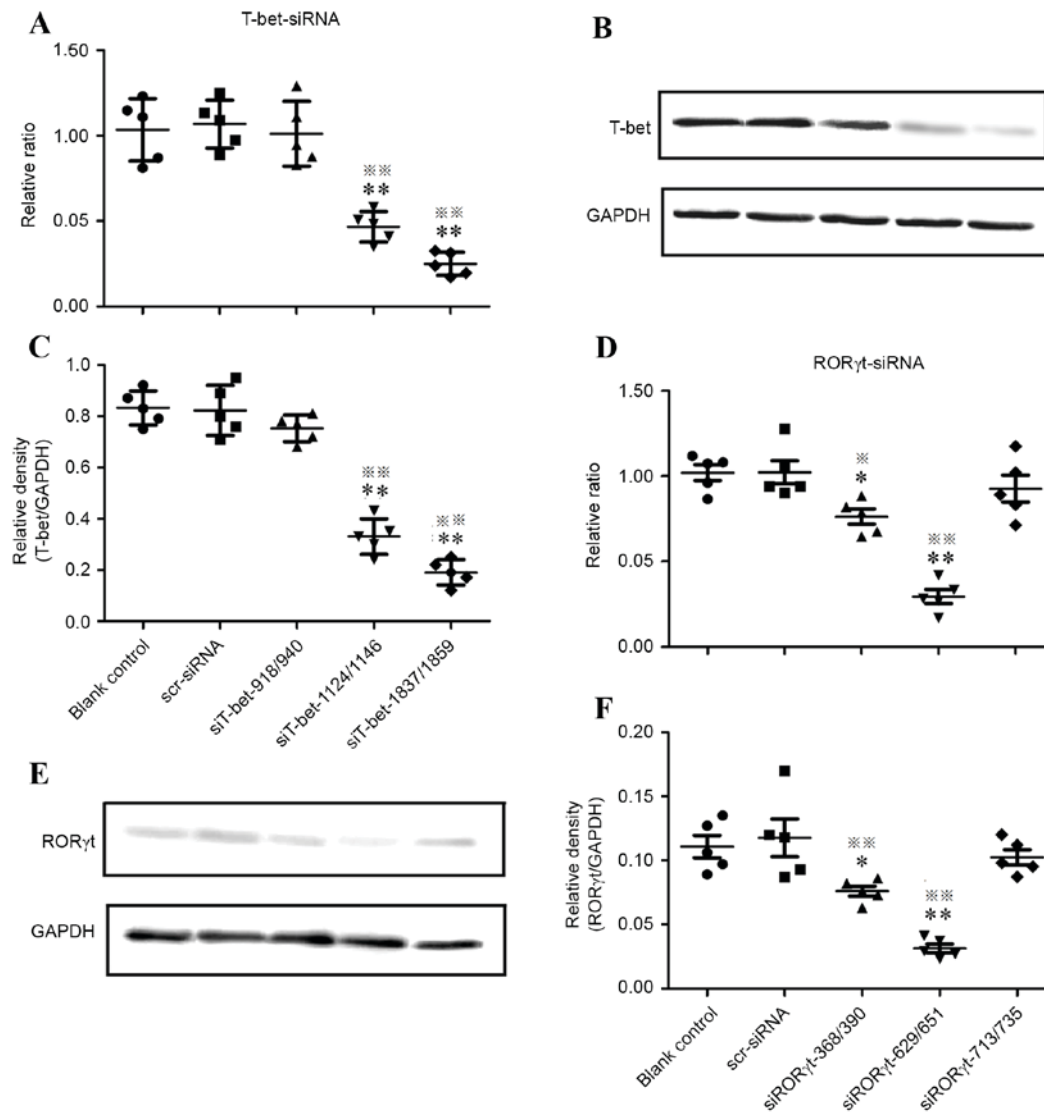


Figure 1. Determination of gene and protein expression levels in activated CD4<sup>+</sup> T cells treated by 3 potential siRNAs and scr-siRNA at 48 h by RT-qPCR and western blot analysis. The mRNA levels and protein abundance were normalized to GAPDH and errors bars are standard error of the mean of  $\geq 5$  independent measurements. (A) Scatter diagram analysis for mRNA levels of T-bet, siT-bet-1124/1146 and 1837/1859 resulted in the reduction of T-bet levels in CD4<sup>+</sup> T cells. (B) Western blot analysis confirmed the results of RT-qPCR. GAPDH served as the internal control. (C) Scatter diagram analysis for optical density by western blotting. T-bet protein levels were downregulated in siT-bet-1124/1146 and 1837/1859 group. (D) mRNA levels of ROR $\gamma$ t; si-ROR $\gamma$ t-368/390 and 629/651 resulted in the reduction of ROR $\gamma$ t levels in CD4<sup>+</sup> T cells. (E) Western blot analysis for ROR $\gamma$ t protein. (F) Scatter diagram analysis for optical density by western blotting. ROR $\gamma$ t protein levels were down-regulated in si-ROR $\gamma$ t-368/390 and 629/651 group. \*P<0.05, vs. blank control; \*\*P<0.01, vs. blank control; \*P<0.05, vs. scr-siRNA; \*\*P<0.01, vs. scr-siRNA. si, small interfering; scr, scrambled; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; ROR $\gamma$ t, retinoic acid receptor-related orphan receptor  $\gamma$ t.

and FOXP3 mRNA levels among the groups. The ratio of T-bet:GATA3 and ROR $\gamma$ t:FOXP3 mRNA in PBMC of patients with PE were significantly higher compared with normal pregnancy patients (P<0.05), indicating a Th1 and Th17-shift.

*Transfection efficiency.* Activated CD4<sup>+</sup> T cells transfected with 6-FAM-siRNA were observed using fluorescence microscopy. Cells emitting a green fluorescent signal were considered to be transfected successfully. The optimum transfection conditions

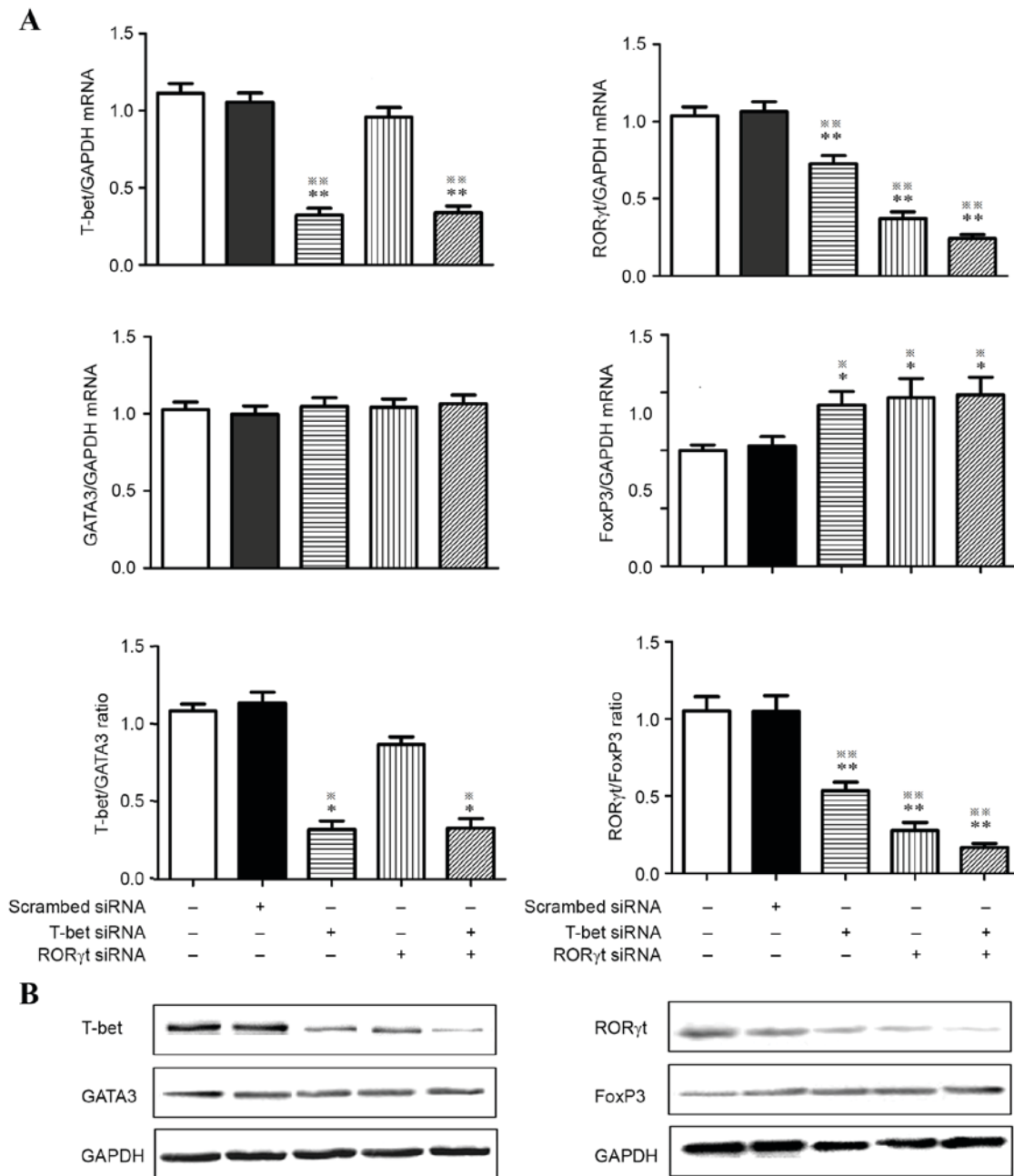


Figure 2. RT-qPCR and western blot analysis were performed to detect the expression of T-bet, GATA3, RORγt and FOXP3 in activated CD4<sup>+</sup> T cells treated with T-bet-specific siRNA and RORγt-specific siRNA alone or in combination. (A) Gene expression of T-bet, GATA3, RORγt, FOXP3, T-bet:GATA3 ratio and RORγt:FOXP3 ratio from activated CD4<sup>+</sup> T cells of preeclampsia. (B) Western blot analysis confirmed the results of RT-qPCR. GAPDH served as internal control. \*P<0.05, vs. blank control; \*\*P<0.01, vs. blank control; \*P<0.05, vs. scr-siRNA; \*\*\*P<0.01, vs. scr-siRNA. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; GATA3, GATA binding protein 3; RORγt, retinoic acid receptor-related orphan receptor γt; FOX, forkhead box; si, small interfering.

involved mixing 30 pmol siRNA with 2 μl DMRIE-C, giving a transfection efficiency of ~73.6%.

**siRNA efficiency.** RT-qPCR and western blot analysis were performed to screen for the most effective siRNAs. Gene and protein expression were normalized to GAPDH. As demonstrated in Fig. 1, in all the T-bet-specific siRNA treated groups, no statistically significant differences were observed in the levels of T-bet mRNA between the blank control and nonspecific siRNA control (P>0.05). In the siT-bet-1837/1859 treated group, T-bet mRNA knockdown

efficiency decreased by 75% and the relative ratio was 0.250±0.068; western blot analysis demonstrated that the relative density of T-bet protein was 0.250±0.068, significantly lower compared with the blank and nonspecific siRNA controls (P<0.01; Fig. 1A-C).

Of all RORγt-specific siRNAs treated groups, the siRORγt-629/651 group demonstrated over 70% knockdown efficiency and the relative ratio was 0.293±0.091; western blot analysis demonstrated that the level of RORγt protein expression was 0.049±0.013, significantly lower compared with the blank and nonspecific siRNA controls (P<0.01; Fig. 1D-F),

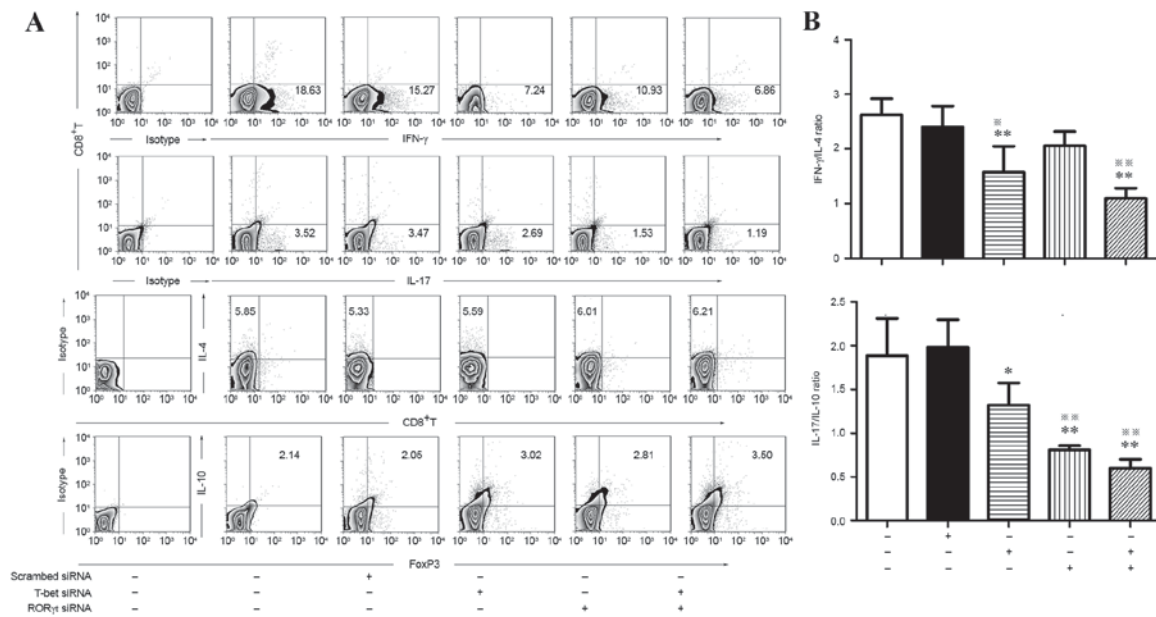


Figure 3. Flow cytometric analysis of IFN- $\gamma^+$  (Th1), IL-4 $^+$  (Th2), IL-17 $^+$  (Th17) and FOXP3 $^+$  IL-10 $^+$  (Treg) subsets in activated CD4 $^+$  T cells treated by T-bet-specific siRNA and ROR $\gamma$ t-specific siRNA alone or in combination. (A) Typical results for IFN- $\gamma^+$  Th1, IL-4 $^+$  Th2, IL-17 $^+$  Th17 and IL-10 $^+$  Treg subsets detected by flow cytometry. (B) Comparison of IFN- $\gamma$ /IL-4 and IL-17/IL-10 between different groups. \* $P < 0.05$ , vs. blank control; \*\* $P < 0.01$ , vs. blank control; \* $P < 0.05$ , vs. scr-siRNA; \*\* $P < 0.01$ , vs. scr-siRNA. IFN, interferon; IL, interleukin; FOX, forkhead box; si, small interfering; ROR $\gamma$ t, retinoic acid receptor-related orphan receptor  $\gamma$ t; scr, scrambled.

while no significant difference was observed between the blank and nonspecific siRNA controls.

These data suggested that siROR $\gamma$ t-629/651 and siT-bet-1837/1859 were the most efficient and specific siRNAs to downregulate mRNA levels, silence genes and inhibit protein expression.

*siROR $\gamma$ t-629/651 and siT-bet-1837/1859 affect the expression of T lymphocyte transcription factors.* It was next investigated whether siROR $\gamma$ t-629/651 or siT-bet-1837/1859 alone or in combination affected T lymphocyte subsets. As demonstrated in Fig. 2, T-bet mRNA levels were decreased by 70% and western blot analysis demonstrated a clear decrease in protein expression (25-40%) in the T-bet knockdown and T-bet/ROR $\gamma$ t double knockdown groups compared with the BC and NC groups. Comparison of mRNA and protein levels of the transcription factor GATA3 demonstrated no significant differences among the 5 groups. A decreased ratio of T-bet: GATA3 mRNA was observed in the T-bet and T-bet/ROR $\gamma$ t double knockdown groups compared with the BC and NC groups. Knockdown of T-bet or ROR $\gamma$ t alone led to a significant decrease (30 and 60%, respectively) in ROR $\gamma$ t gene expression along with a significant increase (40%) in FOXP3 gene expression under the same experimental conditions. The T-bet/ROR $\gamma$ t double knockdown demonstrated a marked decrease (75%) in ROR $\gamma$ t gene expression and a marked increase (50%) in FOXP3 gene expression compared with the BC and NC groups. The ratio of ROR $\gamma$ t: FOXP3 mRNA notably increased in each siRNA group. To confirm these results, T lymphocyte cell expression of transcription factors was analyzed by western blot analysis and it was determined that T-bet knockdown assays led to a decrease in ROR $\gamma$ t and an increase in FOXP3 protein expression. This change was more evident in the T-bet/ROR $\gamma$ t double

knockdown group. It was also confirmed that treatment with siRNA did not alter the expression of GATA3.

*RNAi knockdown of ROR $\gamma$ t and T-bet affects T lymphocyte cytokine expression.* To investigate whether siROR $\gamma$ t-629/651 and siT-bet-1837/1859 would influence the expression of cytokines by T lymphocytes, flow cytometry was employed to identify IFN- $\gamma^+$  (Th1), IL-4 $^+$  (Th2), IL-17 $^+$  (Th17) and FOXP3 $^+$  IL-10 $^+$  (Treg); all of which are cytokines secreted by subsets of CD4 $^+$  T cells. Multi-cytokine analysis demonstrated that compared with the control group and scrambled-siRNA, knockdown of T-bet alone demonstrated a marked decrease in IFN- $\gamma$  and IL-17 levels and an increase in IL-10 levels. Knockdown of ROR $\gamma$ t alone led to a marked decrease in IL-17 and an increase in IL-10. In the T-bet/ROR $\gamma$ t double knockdown group, a marked decrease of IFN- $\gamma$  and IL-17 and a significant increase in IL-10 was observed. Notably, no significant differences in IL-4 were detected among the 5 groups (Fig. 3).

The ratio of IFN- $\gamma$ :IL-4 and IL-17:IL-10 was analyzed in each group and it was identified that the ratio of IFN- $\gamma$ :IL-4 was significantly decreased in the T-bet knockdown and T-bet/ROR $\gamma$ t double knockdown groups compared with the control and scramble-siRNA groups. The ratio of IL-17:IL-10 was significantly lower in the siROR $\gamma$ t, siT-bet and combination groups compared with the control and scramble-siRNA groups (Fig. 3).

## Discussion

PE has been reported to result from an excessive maternal response toward existing inflammation originating at the maternal-fetal interface. A longitudinal study of the changes in cytokine expression during pregnancy identified a general

trend toward enhanced expression of counter-regulatory cytokines and a dampening of inflammatory cytokine expression (19), implying that changes in cytokine balance may underlie the development of PE. Previous studies also have demonstrated that increased expression of Th1 and Th17-specific cytokines may be responsible for activating the inflammatory response characteristic of this disorder (20,21).

Certain transcription factors are known to act as 'master regulators' in controlling the differentiation of T lymphocyte subpopulations. The present study compared Th1, Th2, Th17 and Treg cell transcription factor mRNA expression in normal pregnancy and PE and identified that the expression of a Th1-specific transcription factor, T-bet, and a Th17-specific transcription factor, ROR $\gamma$ t, were increased in PE; consistent with previous studies suggesting a pro-inflammatory state (14,22).

Little is known about the function of transcription factors in regulating T lymphocyte subsets. The present study hypothesized that downregulation of transcription factor expression in CD4<sup>+</sup> T cells would affect the expression and balance of T lymphocyte subsets. High-efficiency siRNA was used to inhibit T-bet or ROR $\gamma$ t gene expression and it was identified that ROR $\gamma$ t levels decreased not only following ROR $\gamma$ t siRNA treatment, however also in the T-bet siRNA treatment group and in the combination treatment group, accompanied by marked increases of FOXP3 expression. A number of studies have demonstrated that Th17 cells contribute to disease predominantly via changes to Th1 cells and that suppression of T-bet ameliorates the severity of disease by limiting the differentiation of Th17 cells via regulation of IL-23R (16,23). These results imply that IL-12-driven Th1 cells and IL-23-driven Th17 cells may be associated and may arise from the same T-bet-expressing precursor. T-bet not only serves an important role in regulating IL-12-driven Th1 cells, however can additionally influence the differentiation of IL-23-driven Th17 cells (24,25). Functional associations between Treg and Th17 cells have been reported and they are suggested to arise from common precursors in a reciprocal manner and conduct diametrically opposing functions (26). FOXP3 has been demonstrated to bind to ROR $\alpha$  and ROR $\gamma$ t and inhibit their biological activity; this can be blocked by exposure to TGF- $\beta$  and lead to rapid induction of ROR $\gamma$ t (27,28). The results of the present study support a reciprocal pathway between the Th1/Th17 and Th17/Treg subsets, indicating a mechanism in support of the increased expression of FOXP3 and decreased expression of ROR $\gamma$ t observed in the siT-bet group.

Cytokines are important mediators of immune responses and their expression profiles are used to categorize the functional status of the immune system. The present study has demonstrated that Th1 and Th17-type immunity is present in PE. The increased production of pro-inflammatory cytokines promotes systemic inflammation and pro-inflammatory cytokines, such as TNF- $\alpha$ , IL-6, IFN- $\gamma$  and IL-17, which are produced by activated Th1 and Th17 cells, have been identified to contribute to widespread vascular endothelial malfunction and vasospasm in PE. In the vasculature, increased cytokines promote the expression of the potent vasoconstrictor ET-1 and stimulate B-cell secrete agonistic autoantibody to the Ang II to further increase the blood pressure (29). In addition, pro-inflammatory cytokines also can promote the expression

of adhesion molecules in the vasculature and lead to increased vascular permeability that aggravates the symptoms of this disorder (30). In the placenta, overexpression of TNF- $\alpha$  and IL-6 leads to a significant reduction in the activities of caspases in trophoblasts, suggesting that excess and/or aberrant trophoblast death can be induced by pro-inflammatory cytokines (31). In contrast, anti-inflammatory cytokines produced by Th2 and Treg cells, including IL-4, IL-5 and IL-10, can have protective effects in pregnancy; their decreased expression may cause abnormal trophoblast differentiation and invasion, immune maladaptation, and exaggerated systemic inflammatory response, all of which have been proposed to be responsible for the development of PE (32). In the present study, knockdown of ROR $\gamma$ t was identified to inhibit IL-17A expression, which was followed by an increase in IL-10 expression. Knockdown of T-bet may reverse the ratio of IL-17A:IL-10 and IFN- $\gamma$ :IL-4 by depressing IFN- $\gamma$  and IL-17A expression and promoting IL-10 expression. Knockdown of T-bet in combination with ROR $\gamma$ t significantly increased the expression of IL-10 and inhibited IL-17A. Based on the propensity of Tregs to convert to a Th17 phenotype following exposure to inflammatory cytokines, it has been proposed that overexpression of pro-inflammatory cytokines may suppress the tolerance system and thus shift the T lymphocyte balance from pro-inflammatory to anti-inflammatory and ameliorate the imbalance observed in PE (33).

In summary, the present study identified increased expression of pro-inflammatory cytokines accompanied by enhanced expression of the transcription factors T-bet and ROR $\gamma$ t in patients with PE compared with normal pregnant controls. In addition, diminished anti-inflammatory cytokine expression was observed, possibly accentuating the systemic inflammation observed in PE. These results, along with previous observations led to the hypothesis that restoration of an impaired immune response in PE may be possible by regulating transcription factor expression. The results of the present study demonstrated that siROR $\gamma$ t-629/650 and siT-bet-1837/1859 were effective in silencing ROR $\gamma$ t and T-bet gene expression, respectively, suggesting that siRNA-mediated knockdown of T-bet in combination with ROR $\gamma$ t may be an effective therapeutic treatment for regulating immune imbalance in PE. Further studies will be required to elucidate the mechanism of CD4<sup>+</sup> T cell transcription factors in PE with the aim of developing interventions to ameliorate the adverse outcomes of the disease.

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