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miR-223-3p promotes autoreactive T_h17 cell responses in experimental autoimmune uveitis (EAU) by inhibiting transcription factor FOXO3 expression

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ABSTRACT: Pathogenic T helper (T_h)17 cells are key mediators of autoimmune diseases such as uveitis and its animal model, experimental autoimmune uveitis (EAU). However, the contribution of microRNAs (miRs) to the intrinsic control of pathogenic T_h17 cells in EAU remains largely unknown. Here, we have reported that miR-223-3p was significantly up-regulated in interphotoreceptor retinoid-binding protein–specific T_h17 cells, and its expression was enhanced by IL-23–signal transducer and activator of transcription 3 signaling. Knockdown of miR-223-3p decreased the pathogenicity of T_h17 cells in a T-cell transfer model of EAU. Mechanistic studies showed that miR-223-3p directly repressed the expression of forkhead box O3 (FOXO3), and FOXO3 negatively regulated pathogenic T_h17 cell responses partially *via* suppression of IL-23 receptor expression. Thus, our results reveal an important role for miR-223-3p in autoreactive T_h17 cell responses and suggest a potential therapeutic avenue for uveitis.—Wei, Y., Chen, S., Sun, D., Li, X., Wei, R., Li, X., Nian, H. miR-223-3p promotes autoreactive T_h17 cell responses in experimental autoimmune uveitis (EAU) by inhibiting transcription factor FOXO3 expression. FASEB J. 33, 13951–13965 (2019). www.fasebj.org

KEY WORDS: IL-23 · pathogenic Th17 cells · IL-23R · GM-CSF · IL-22

T helper (T_h)17 cells are critically involved in the pathogenesis of various autoimmune disorders, including uveitis and its animal model, experimental autoimmune uveitis (EAU) (1–3). T_h17 cells require IL-6 and TGF-β for their development and IL-23 for their effector functions (4–6). Increasing data demonstrate that T_h17 cells induced by IL-23 are more pathogenic than those induced by IL-6 and TGF-β (7). IL-23 stabilizes pathogenic T_h17 cell phenotype by increasing expression of IL-23 receptor (IL-23R) and endows T_h17 cells with pathogenic effector functions (8, 9). IL-23 also drives the expression of granulocyte-macrophage colony-stimulating factor (GM-CSF) (10, 11), which is crucial for the pathogenicity of T_h17 cells. Although much is known about the key factors that drive T_h17 cell differentiation and pathogenicity, very little is known about the cell-intrinsic mechanisms that regulate pathogenic function of T_h17 cells.

MicroRNAs (miRs) are noncoding single-stranded RNAs of about 22 nt that regulate gene expression at the post-transcriptional level *via* translational repression or mRNA degradation (12). miRs have been revealed to control dynamic aspects of autoimmunity (13). Moreover, published works have characterized a series of miRs involved in uveitis pathogenesis (14–16). Regarding the role of miRs in T_h17 cells in uveitis, miR-155 has been shown to promote T_h17 responses by targeting E26 transformation specific-1 (Ets-1) in $CD4^+$ T cells from patients with active Behçet's disease (BD) (17). Furthermore, Chang *et al.* (18) reported that miR-20a-5p suppressed T_h17 cell responses by targeting oncostatin M and C-C motif chemokine ligand 1 in $CD4^+$ T cells in patients with active Vogt-Koyanagi-Harada disease. However, the contributions of miRs to

ABBREVIATIONS: APC, antigen-presenting cell; B6, C57BL/6; BD, Behçet's disease; CFA, complete Freund's adjuvant; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; EAU, experimental autoimmune uveitis; FOXO, forkhead box O; Foxp3, forkhead box protein 3; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-1R1, IL-1 receptor 1; IL-23R, IL-23 receptor; IRBP, interphotoreceptor retinoid-binding protein; miR, microRNA; Mut, mutant; PBMC, peripheral blood mononuclear cell; qRT-PCR, quantitative RT-PCR; ROR-γt, retinoic acid receptor–related orphan receptor-γt; siRNA, small interfering RNA; STAT3, signal transducer and activator of transcription 3; T_h, T helper; T_{reg}, regulatory T; WT, wild type

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pathogenic T_h17 cells induced by IL-23 in uveitis are still largely unknown.

The forkhead box O (FOXO) family (FOXO1, 3, 4, and 6) of transcription factors plays important roles in innate and adaptive immune responses (19). For example, FOXO3 has been shown to promote the expression of forkhead box protein 3 (Foxp3) in regulatory T (T_{reg}) cells (20). Moreover, Caroline *et al.* reported that FOXO3 enhanced pathogenic T_h1 cell differentiation by inducing eomeseodermin expression in experimental autoimmune encephalomyelitis (EAE) (21). In $FOXO1^{-/-}$ $FOXO3^{-/-}$ T cells, genes associated with T_h17 cell differentiation were up-regulated and IL-17 production was increased (22), suggesting FOXO3 may be involved in regulating the production of IL-17. However, whether and how FOXO3 is involved in induction of pathogenic T_h17 cells remains unknown.

In this study, we showed that miR-223-3p was significantly up-regulated in interphotoreceptor retinoid-binding protein (IRBP)–specific T_h17 cells in EAU. miR-223-3p positively regulated pathogenic T_h17 cell responses by directly targeting FOXO3, and FOXO3 inhibited IL-17 production partially *via* suppression of IL-23R expression. Furthermore, knockdown of miR-223-3p in T cells dramatically reduced the severity of EAU and decreased autoreactive T_h17 cell percentages. Our study reveals a previously unknown T-cell–intrinsic miR pathway that promotes autoreactive T_h17 cells and identifies the miR-223-3p–FOXO3–IL-23R axis as a potential therapeutic target in uveitis.

MATERIALS AND METHODS

Mice

Pathogen-free female C57BL/6 (B6) (10-wk-old) mice were purchased from Vital River Laboratory Animal Technology (Beijing, China). All animal experiments conformed to the *Statement for the Use of Animals in Ophthalmic and Vision Research* (Association for Research in Vision and Opthalmology, Rockville, MD, USA), and the protocol was approved by the Institutional Animal Care and Use Committee of Tianjin Medical University.

Antibodies, cytokines, and peptides

FITC-conjugated or phycoerythrin-conjugated antibodies against mouse IL-17, IFN-γ, CD4, CD11c, and Foxp3 were purchased from BioLegend (San Diego, CA, USA). Antibody to FOXO3 was purchased from Cell Signaling Technology (Danvers, MA, USA), and antibody to β-actin was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Recombinant murine IL-12, IL-23, IL-4, and GM-CSF were purchased from R&D Systems (Minneapolis, MN, USA). A truncated form of IRBP $peptide_{1–20}$ (aa 1–20 of human IRBP) was synthesized and purified by Sangon Biotech (Shanghai, China). *Mycobacterium tuberculosis* strain H37RA was obtained from Difco Laboratories (Detroit, MI, USA). Pertussis toxin was purchased from MilliporeSigma (Burlington, MA, USA). WP1066 (Calbiochem, San Diego, CA, USA) was dissolved in DMSO for storage and use.

T-cell preparation and differentiation

$CD4^+$ T cells were purified from the spleen or draining lymph nodes of $IRBP_{1–20}$–immunized B6 mice or naive mice by positive selection using a combination of FITC-conjugated anti-mouse CD4 antibodies and anti-FITC antibody-coated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), followed by separation using an autoMACS Separator Column (Miltenyi Biotec) according to the manufacturer's protocol. The purity of the isolated cells was determined by flow cytometric analysis using phycoerythrin-conjugated anti-CD4 antibodies.

$CD4^+$ T cells were isolated from immunized B6 mice and cocultured with irradiated antigen-presenting cells (APCs) and immunized antigen (10 μg/ml) alone (T_h0 cells) or in the presence of IL-12 (10 ng/ml) for T_h1 cell polarization or IL-23 (20 ng/ml) for T_h17 cell polarization. Forty-eight hours later, cells were collected for further analysis.

Generation of bone marrow–derived dendritic cells

Dendritic cells (DCs) were generated from B6 mice as previously described in refs. 23 and 24. Briefly, bone marrow cells from B6 mice were isolated and cultured for 6 d in complete Roswell Park Memorial Institute (RPMI) medium 1640 containing 10 ng/ml GM-CSF (R&D Systems) and 10 ng/ml IL-4 (R&D Systems). DCs were enriched using magnetically activated cell sorting separation and then treated with miR-223-3p mimics or control mimics.

EAU induction and evaluation

For active induction of EAU, B6 mice were injected subcutaneously over 6 spots on the tail base and flank with the emulsion containing equal volumes of 200 μg of $IRBP_{1–20}$ in PBS and complete Freund's adjuvant (CFA) and intraperitoneally with 350 ng of pertussis toxin (MilliporeSigma). For adoptive transfer, $CD4^+$ T cells transfected with miR-223-3p inhibitor or control inhibitor were stimulated with 10 μg/ml of $IRBP_{1–20}$ for 48 h in the presence of APCs under T_h17 cell polarizing conditions. Then, activated T-cell blasts were separated by density gradient centrifugation (Ficoll; GE Healthcare, Waukesha, WI, USA) and injected into naive B6 mice.

To examine mice for clinical signs of EAU by indirect fundoscopy, the pupils were dilated using 1.25% phenylephrine hydrochloride ophthalmic solution and 0.5% tropicamide. Fundoscopic grading of disease was performed using the scoring system previously reported in Thurau *et al*. (25). Histopathological evaluation was performed on eye sections at the end of the experiment, and disease was graded on the basis of cellular infiltration and structural changes as previously reported in Zhang *et al*. (26).

Transfection

miR-223-3p mimics and control mimics, as well as miR-223-3p inhibitor and control inhibitor, were designed and synthesized by Gene Pharma (Suzhou, China). DCs and autoreactive $CD4^+$ T cells were transfected with these RNAs at the final concentration of 180 and 300 nM, respectively, using Lipofectamine 2000 Reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The FOXO3-specific small interfering RNAs (siRNAs) were obtained from Gene Pharma, and the following sequences were used: for FOXO3 siRNA, 5′-UGAUGAUCCACCAAGAGCUCUUGCC-3′(sense) and 5′-GGCAAGAGCUCUUGGUGGAUCAUCA-3′ (antisense); for control siRNA, 5′-UUCUCCGAACGUGUCA-CGUTT-3′ (sense) and 5′-ACGUGACACGUUCGGAGAATT-3′ (antisense). FOXO3 siRNAs were transfected into autoreactive $CD4^+$ T cells or mouse T-cell lymphoma cell line EL4 (27) at the final concentration of 200 nM. The overexpression lentiviral

plasmid pReceiver-Lv201–IL-23R was purchased from GeneCopoeia (Rockville, MD, USA). Four micrograms of the pReceiver-Lv201-IL–23R or the control plasmid were used for each transfection.

Lentivirus infection

The overexpression lentiviral plasmid pReceiver-Lv201-FOXO3 was purchased from GeneCopoeia. Lentivirus was produced according to the manufacturer's instructions. $CD4^+$ T cells isolated from EAU mice were infected with the concentrated lentivirus by spinning at 1000 *g* for 90 min. Subsequently cells were cultured at 37°C for 24 h, followed by T_h17 cell polarizing induction. After 48 h, culture supernatants were collected for ELISA detection and cells were lysed for real-time quantitative RT-PCR (qRT-PCR) analysis.

Luciferase reporter assay

Five algorithms (TargetScan, *www.targetscan.org*; PicTar, *http://pictar.mdc-berlin.de/*; Tarbase, *www.microrna.gr/tarbase*; StarBase, *http://starbase.sysu.edu.cn/index.php*; and miRTarBase, *http://miRTarBase.mbc.nctu.edu.tw/*) were performed to predict the targets of miR-223-3p. Luciferase reporter assay was performed to test the targeted relationship between miR-223-3p and FOXO3. Firstly, the wild-type (WT) mouse FOXO3 3′UTR fragment containing the putative miR-223-3p binding site or mutant (Mut) FOXO3 3′UTR fragment in the miR-223-3p binding site was amplified from mouse spleen cDNA and cloned into the pMIR-Report luciferase miR expression reporter vector (Promega, Madison, WI, USA). The following sequences of primers were used: FOXO3 3′UTR-WT forward, 5′-GGACTAGTATCATCCGTAGTGAACTCATGGATG-3′; FOXO3 3′UTR-WT reverse, 5′-CCCAAGCTTTAGAGTTCTGTTCCAC-GGGTAAGGG-3′; FOXO3 3′UTR-Mut forward, 5′-GGACTAGTAGCAGACCCTCACGTAACGAGAAGACCTACAGAGAAAACC-3′; FOXO3 3′UTR-Mut reverse, 5′-CCCAAGCTTTCAAAGGAAACAAACACAAGACGAC-3′. Human embryonic kidney 293T cells (2.5×10^4 cells/well) were cultured in the 96-well plates. Twenty-four hours later, 75 ng recombinant pMIR-Report vector containing WT or Mut FOXO3 3′UTR and 3.75 ng internal control plasmid pRL-TK vector (Promega) were cotransfected with miR-223-3p mimics (100 nM) or control mimics by Lipofectamine 2000 reagent (Thermo Fisher Scientific). Luciferase activity was measured 48 h later using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol.

miR microarray, gene expression and real-time qRT-PCR analysis

Total RNA was extracted from naive or autoreactive $CD4^+$ T cells using Trizol reagent (Thermo Fisher Scientific) according to the manufacturer's protocol. Purified RNA was prepared, according to the manufacturer's instructions (CapitalBio, Beijing, China) for miR array analysis with an 8×60 K miR Microarray Kit (Agilent Technologies, Santa Clara, CA, USA). Microarray data were analyzed by the GeneSpring GX software (Agilent Technologies). The Gene Expression Omnibus (GEO) database (*http://www.ncbi.nlm.nih.gov/geo/*) was sought to obtain gene expression profiles in uveitis patients and healthy volunteers, and GSE70403, GSE17114, GSE61399 and GSE10080 were selected for exploring the correlation of FOXO3 and the pathogenic T_h17 pathway. Real-time qRT-PCR was performed using Sybr Green Master Mix and an ABI 7900 HT Sequence Detection System (both from Thermo Fisher Scientific). For miR analysis, reverse transcription and real-time qRT-PCR primers for miRs and U6 small nuclear RNA are listed in **Table 1**. Relative miR expression was calculated using the $2^{-\Delta\Delta Ct}$ cycle threshold method with the expression of U6 as an internal control. For mRNA analysis, the gene-specific primers for real-time qRT-PCR are listed in **Table 2**. The relative expression level of mRNA was normalized to that of endogenous gene glyceraldehyde-3-phosphate dehydrogenase using the method previously described.

Flow cytometric analysis and ELISA

For intracellular cytokine staining, aliquots of T cells were stimulated with 50 ng/ml of phorbol myristic acetate, 1 μg/ml of ionomycin, and 1 μg/ml of brefeldin A (MilliporeSigma) for 4 h; then they were fixed, permeabilized overnight, and intracellularly stained with antibodies. Stained cells were analyzed on a flow cytometer (FACS Calibur; BD Biosciences, San Jose, CA, USA). FlowJo software (TreeStar, Ashland, OR, USA) was used to analyze the acquired data.

The cytokines in the culture supernatants were detected with commercially available ELISA kits (R&D Systems).

Western blot analysis

EL4 T cells were lysed with RIPA buffer (Solarbio, Beijing, China) containing PMSF (Solarbio) and phosphatase-protease inhibitor cocktail (Cell Signaling Technology). The protein concentration was measured with a Bicinchoninic Acid Assay Kit (Solarbio). Equal amounts of protein (20 μg) were loaded and resolved on SDS-polyacrylamide gel and transferred onto PVDF membrane. The membranes were blotted with antibodies specific for FOXO3 (Cell Signaling Technology) or β-actin (Santa Cruz Biotechnology) and a horseradish peroxidase–linked secondary antibody. The bands were obtained and analyzed as we previously described in Xiao *et al*. (24).

Statistical analysis

All the experiments were repeated 3 times or more. The results are presented as means ± SD, or means ± SEM. Statistical analysis was performed by an unpaired, 2-tailed Student's *t* test, 1- or 2-way ANOVA. A value of $P < 0.05$ was considered statistically significant.

RESULTS

miR-223-3p is up-regulated in IRBP-specific T_h17 cells

Given that autoreactive $CD4^+$ T cells play a major role in autoimmune uveitis, we performed microarray analysis detecting the miR expression profiles between naive and autoreactive $CD4^+$ T cells. As shown in **Fig. 1*A***, the results highlighted up-regulated expression of 2 distinct miRs (miR-223-3p and miR-210-3p) in autoreactive $CD4^+$ T cells, which was further confirmed by real-time qRT-PCR analysis (Fig. 1*B*). In addition, the data also revealed significantly up-regulated expression of miR-223-3p and miR-210-3p in $CD4^+$ T cells from the CFA^+ IRBP–immunized group compared with that in CFA-immunized

TABLE 1. *Sequences of primers for stem-loop real-time RT-PCR*

Name	Primer sequence, 5′–3′		
	Reverse transcription	Forward	Reverse
miR-223-3p	GTCGTATCCAGTGCAGGGTC CGAGGTATTCGCACTGGAT ACGACTGGGGT	GCGCGTGTCAGTTTGTCAAAT	
miR-210-3p	GTCGTATCCAGTGCAGGGTC CGAGGTATTCGCACTGGA TACGACTCAGCC	CGCTGTGCGTGTGACAGC	
miR universal			AGTGCAGGGTCCGAGGTATT
U6		CGCTTCGGCAGCACATATAC	TTCACGAATTTGCGTGTCATC

mice (Fig. 1*B*), suggesting possible roles of miR-223-3p and miR-210-3p in IRBP-specific T-cell responses in EAU. Kinetic studies showed that the expression of miR-223-3p and miR-210-3p was dramatically up-regulated in IRBP T_h17 cells at d 7 or 13 after immunization, respectively (Fig. 1*C–E*). The expression of these 2 miRs reached a maximum around d 21 after immunization, followed by a decline. The kinetics expression changes of miR-223-3p, but not miR-210-3p, correlated well with EAU disease severity. In addition, we detected an up-regulation of miR-223-3p and miR-210-3p expression in uveitogenic T cells in response to increasing doses of $IRBP_{1–20}$ (Fig. 1*F, G*). To investigate the expression of miR-223-3p and miR-210-3p in T_h cell subsets, we cultured $CD4^+$ T cells from EAU mice with APC and antigen under different T_h cell polarizing conditions *in vitro* and found significantly increased miR-223-3p and miR-210-3p expression in T_h17 cells compared with T_h0 cells (Fig. 1*H, I*). Additionally, we found that IL-23 induced the expression of miR-223-3p and miR-210-3p in a dose-dependent manner. (Fig. 1*J, K*). Signal transducer and activator of transcription 3 (STAT3) is one of the major downstream molecules of IL-23 signaling (28). IL-23 activates STAT3 in T_h17 cells from patients with BD (29). To further determine whether STAT3 is involved in the regulation of miR-223-3p expression, autoreactive T cells from EAU mice were pretreated with different doses of WP1066, a STAT3 inhibitor, and then cocultured with APC in the presence of IL-23. As shown in Fig. 1*L*, WP1066 reversed IL-23–induced up-regulation of miR-223-3p in a dose-dependent manner (Fig. 1*L*), indicating a correlation between IL-23–STAT3 signaling and miR-223-3p.

miR-223-3p positively regulates pathogenic T_h17 cell responses

We next determined whether miR-223-3p and miR-210-3p directly regulated T_h17 cell responses *in vitro*. T cells isolated from immunized B6 mice were transfected with miR-223-3p, miR-210-3p mimics, or control mimics and then cocultured with APC in the presence of immunized antigen under T_h17 cell polarizing conditions. As shown in **Fig. 2*A, B***, ELISA assay revealed that miR-223-3p overexpression significantly promoted IL-17 secretion, whereas miR-210-3p overexpression had no significant effect on this cytokine. Because of the stronger effect of miR-223-3p on IL-17 production, we next focused on the role of miR-223-3p in the regulation of autoreactive T_h17 cells. Further analysis revealed that miR-223-3p overexpression significantly increased the percentages of

TABLE 2. *Sequences of primers in this study for real-time qRT-PCR*

Gene	Primer sequence, 5′–3′	
	Forward	Reverse
GAPDH	CATGGCCTTCCGTGTTCCTA	GCGGCACGTCAGATCCA
IL-23p19	CATAGCTGCCCGGGTCTTT	GGCACTAAGGGCTCAGTCAGA
IL-12p35	GCGTGGGAGTGGGATGTG	GCAAAACGATGGCAAACCA
IL-1β	AGTTGACGGACCCCAAAAGA	GGACAGCCCAGGTCAAAGG
IL-6	CCACGGCCTTCCCTACTTC	TTGGGAGTGGTATCCTCTGTGA
ROR-γt	CCTCAGCGCCCTGTGTTTT	GCATGCAGCTTTTGCCTGTT
IRF4	TCCTCTGGATGGCTCCAGATGG	CACCAAAGCACAGAGTCACCTG
IL-17	CCTGGCGGCTACAGTGAAG	TTTGGACACGCTGAGCTTTG
GM-CSF	CACCCGCTCACCCATCAC	TTCTTTGATGGCCTCTACATGCT
IL-23R	CAGAGGACATCCTGCTTCAGGTA	GATGGCCAAGAAGACCATTCC
IL-1R1	CCTCGGAATGAGACGATCGA	CGTGACGTTGCAGATCAGTTG
IL-22	CTTTCCTGACCAAACTCAGCAA	TGGTCGTCACCGCTGATG
Foxp3	CCCTGCCCTTGACCTCAA	GCCTCAGTCTCATGGTTTTGG
FOXO3	GGCAAAGCAGACCCTCAAACT	TGAGAGCAGATTTGGCAAAGG

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IRF4, IFN regulatory factor 4.

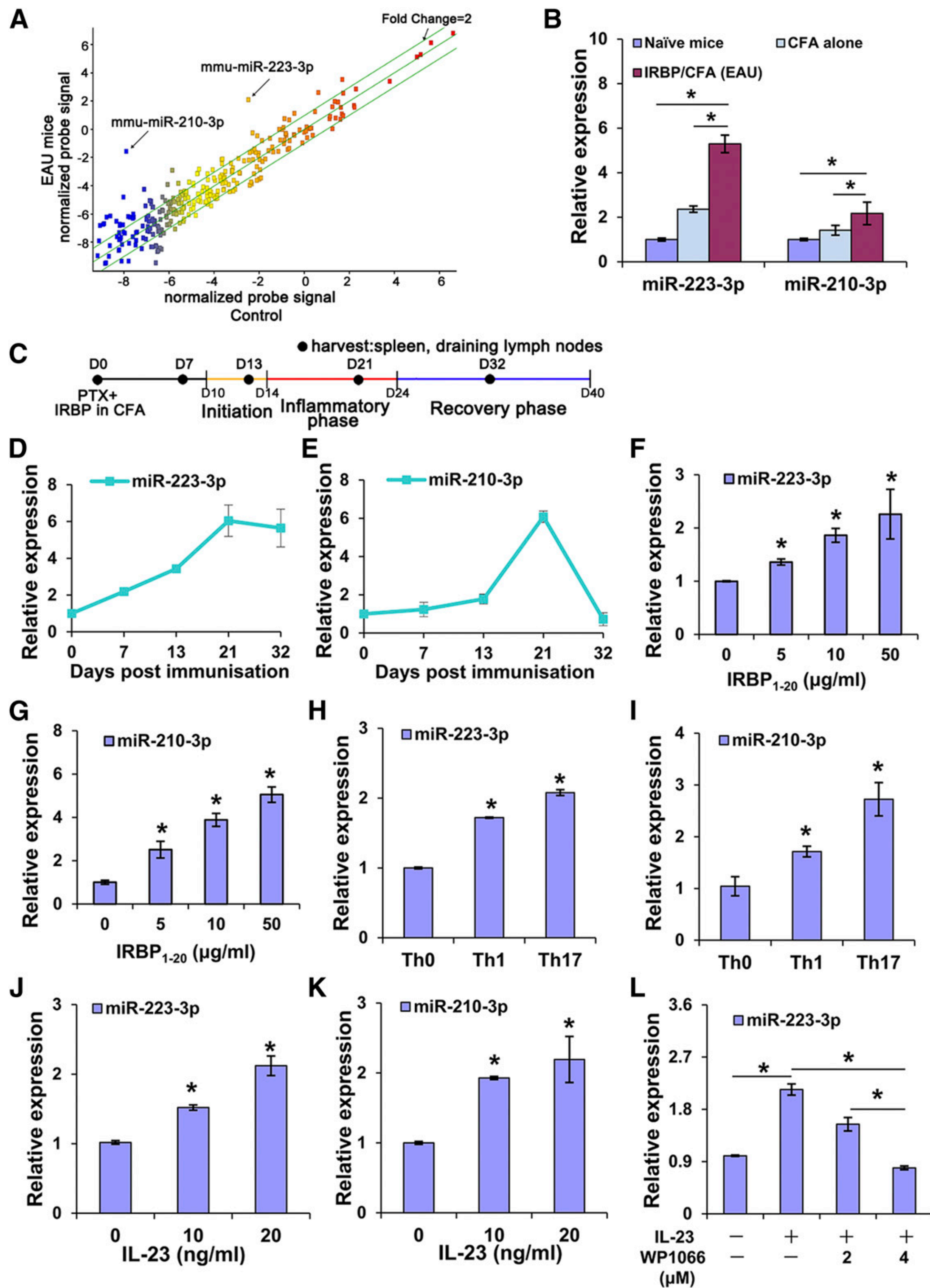

Figure 1. miR-223-3p is highly up-regulated in IRBP-specific T_h17 cells. *A*, *B*, *D*, *E*) $CD4^+$ T cells were isolated from EAU mice and stimulated with $IRBP_{1–20}$ in the presence of APCs for 48 h *in vitro*. *A*) Microarray assay of miR expression profiles was performed comparing $CD4^+$ T cells from naive mice with those from EAU mice. The scatter plot shows the miR normalized probe signal, applying a cutoff of 2, and highlights up-regulated expression of 2 distinct miRs [*Mus musculus* (mmu)-miR-223-3p and mmu-miR-210-3p]. *B*) The expression of miR-223-3p and miR-210-3p, detected by real-time qRT-PCR, in $CD4^+$ T cells isolated from naive mice, CFA-immunized mice, and IRBP and CFA–immunized EAU mice. *C*) Schematic representation of the onset and progression of actively induced EAU. *D*, *E*) Changes in expression levels, detected by real-time qRT-PCR, of miR-223-3p (*D*) and miR-210-3p (*E*) at different days following immunization, normalized to d 0 response. *F*–*K*) $CD4^+$ T cells were isolated from immunized mice at d 13 postimmunization and cultured under indicated conditions. The relative expression of miR-223-3p and miR-210-3p, detected by real-time qRT-PCR, is shown. *L*) miR-223-3p expression, detected by real-time qRT-PCR, in autoreactive T_h17 cells treated with different doses of WP1066. Data are from 3 independent experiments. The data are presented as means ± SD. $*P < 0.05$.

IL-17–producing T cells compared with that in the control group (26.3 *vs.* 17.6%, Fig. 2*C*). In addition, miR-223-3p overexpression significantly increased the expression of some pathogenic T_h17 cell–related genes—IL-17, retinoic acid receptor–related orphan receptor-γt (ROR-γt), IL-22, GM-CSF, and IFN regulatory factor 4—at 48 h after stimulation with $IRBP_{1–20}$. There was a very small and statistically insignificant increase in IL-23R expression at 48 h after stimulation (Fig. 2*D, E*). However, the kinetic data revealed significantly increased expression of IL-23R at 72 h after stimulation (Fig. 2*F*).

To further confirm the role of miR-223-3p in pathogenic T_h17 cells, $CD4^+$ T cells isolated from EAU mice were transfected with miR-223-3p inhibitor or control inhibitor. As shown in Fig. 2*G*, miR-223-3p was indeed silenced by miR-223-3p inhibitor, which was accompanied by a dramatically decreased IL-17 production (Fig. 2*H*) and T_h17 cell percentages (19.1 *vs.* 27.9%, Fig. 2*I*). In addition, miR-223-3p knockdown significantly decreased the expression of IL-17, ROR-γt, IL-22, IL-1 receptor 1 (IL-1R1), IL-23R, and GM-CSF (Fig. 2*J*). Collectively, these findings suggest that miR-223-3p is an important regulator that positively modulates the pathogenicity of T_h17 cells *in vitro*.

miR-223-3p promotes DC-driven T_h17 responses

Previous studies have shown that miR-$223^{-/-}$ DCs exhibited decreased ability to drive T_h17 cell responses in EAE (30). To determine whether miR-223-3p in DCs affects IRBP-specific T_h17 cell responses in EAU, we cocultured miR-223-3p mimic– or control mimic–transfected DCs with autoreactive T cells from immunized B6 mice under T_h17 cell polarizing conditions in the presence of immunized antigen. As shown in Fig. 2*K–M*, the percentages of antigen-specific IL-17–producing T cells and the production of IL-17 were significantly increased in autoreactive T cells cocultured with miR-223-3p mimic–transfected DCs compared with those in T cells cocultured with control mimic–transfected DCs. These data indicate that DC-intrinsic miR-223-3p contributes to pathogenic T_h17 cell responses in EAU.

IL-1β, IL-6, and IL-23 produced by DCs are critically involved in pathogenic T_h17 cell development (31, 32). Given the effect of DC-intrinsic miR-223-3p on T_h17 cell responses, we next examined the role of miR-223-3p in regulation of T_h17 cell polarizing cytokine expression in DCs. DCs pretreated with miR-223-3p mimics or control mimics were stimulated with LPS for 24 h and then were subjected to real-time qRT-PCR analysis. As indicated in Fig. 2*N*, overexpression of miR-223-3p resulted in significantly enhanced IL-1β and IL-23 expression, suggesting miR-223-3p contributes to the creation of a favorable environment for pathogenic T_h17 cell development.

Silencing miR-223-3p decreases pathogenicity of T_h17 cells

To address the $CD4^+$ T-cell–intrinsic function of miR-223-3p in EAU, we used an IRBP-specific T-cell–induced EAU model (**Fig. 3*A***), in which activated T cells cause EAU when transferred into naive B6 mice (24). For this, T cells isolated from immunized B6 mice were transfected with control or miR-223-3p inhibitor, then *in vitro* activated by coculturing with APC and immunized antigen. Two days later, the T cells were collected and transferred into syngeneic WT B6 mice, and the recipient mice were monitored for the development of EAU. As shown in Fig. 3*B*, mice that received T_h17 cells transfected with miR-223-3p inhibitor developed less severe EAU, as compared with mice that received T_h17 cells transfected with control inhibitor. Consistent with the clinical scores, massive infiltration of inflammatory cells and prominent retinal folds were observed in eyes of mice that received T_h17 cells transfected with control inhibitor relative to that in mice that received T_h17 cells with miR-223-3p inhibitor (Fig. 3*C*). Compared with the mean histopathological scores of 1.68 ± 0.29 in control mice, the eyes of mice given transfer of T_h17 cells with miR-223-3p inhibitor exhibited less retinal inflammation, with a mean score of 0.95 ± 0.36 ($P < 0.05$; Fig. 3*C*). Further analysis revealed that T cells isolated from mice given transfer of T cells with miR-223-3p inhibitor had dramatically lower percentages of IRBP-specific T_h17 and T_h1 cells compared with the control group (6.9 *vs.* 13.3% and 13.5 *vs.* 19%, respectively; Fig. 3*D*). Correlating with the FACS data, the production of IL-17 and IFN-γ was significantly decreased in the T cells from mice receiving T_h17 cells transfected with miR-223-3p inhibitor compared with that in the control group (Fig. 3*E*). In addition, we found that there was a higher percentage of $Foxp3^+$ T_{reg} cells in splenic cells from mice given transfer of T_h17 cells transfected with miR-223-3p inhibitor than those from control mice given T_h17 cells transfected with control inhibitor (Fig. 3*F*). Collectively, these results indicate that silencing miR-223-3p decreases pathogenic function of T_h17 cells and thereby ameliorates the development of EAU.

FOXO3 is a functional target of miR-223-3p

To define the mechanisms by which miR-223-3p modulates T_h17 cell responses, we used a combination of 5 different computational programs—TargetScan, PicTar, Tarbase, StarBase, and miRTarBase—to identify the putative targets of miR-223-3p (**Fig. 4*A***). Among the predicted targets, FOXO3, which plays an important role in regulating T-cell function, was selected for further study. As shown in Fig. 4*B*, 3′-UTR of FOXO3 contained 1 highly conserved putative binding element for the miR-223-3p.

To determine whether FOXO3 represents direct targets of miR-223-3p, we constructed reporter plasmids by cloning the mouse WT and Mut 3′-UTR of FOXO3 genes into the pMIR-Report luciferase vectors. By cotransfection of the reporter plasmids and internal control pRL-TK-*Renilla* luciferase plasmids with miR-223-3p mimics or control mimics, we observed that miR-223-3p suppressed the luciferase activity of the reporter containing the WT, but not the Mut 3′-UTR of FOXO3 (Fig. 4*C*), suggesting that miR-223-3p specifically targets FOXO3 and regulates its expression.

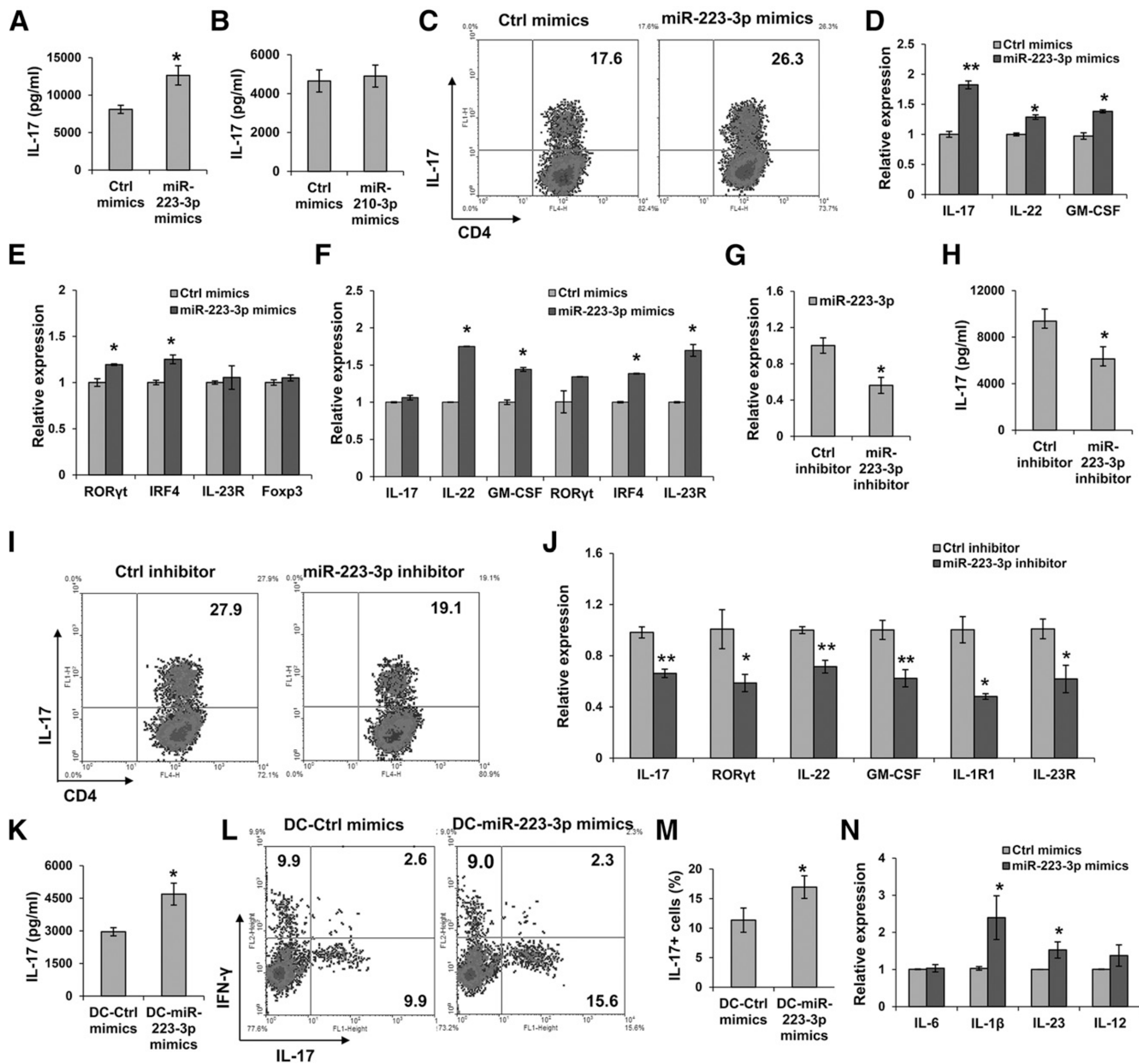

Figure 2. miR-223-3p promotes T_h17 cell responses *in vitro*. *A–J*) $CD4^+$ T cells isolated from immunized mice were transfected with miR-223-3p or miR-210-3p and control mimics (300 nM) (*A–F*) or inhibitor (300 nM) (*G–J*) and cocultured with irradiated APCs in the presence of $IRBP_{1–20}$ under T_h17 cell polarizing conditions. *A, B, H*) ELISA analysis of IL-17 production in the culture supernatants. Data are from 3 to 5 independent experiments. *C, I*) Representative flow cytometry showing $IL\text{-}17^+$ T cells. *D, E, G*) Real-time qRT-PCR analysis of relative expression of miR-223-3p and T_h17 cell–related cytokines, transcription factors, and surface receptors at 48 h after stimulation with $IRBP_{1–20}$. *F, J*) Real-time qRT-PCR analysis of T_h17 cell–related gene expression at 72 h after stimulation. *K, L*) DCs transfected with miR-223-3p or control mimics were cocultured with $CD4^+$ T cells from EAU mice under T_h17 cell polarizing conditions. ELISA analysis of IL-17 secretion in the supernatants (*K*). Representative flow cytometry showing $IL\text{-}17^+$ T cells (*L*). *M*) Summary data showing the percentage of $IL\text{-}17^+$ T cells. *N*) DCs pretreated with miR-223-3p mimics or control mimics were stimulated with 100 ng/ml LPS for 24 h. Real-time qRT-PCR analysis of T_h17 cell polarizing cytokine expression. Data are representative of 3 independent experiments. Ctrl, control. The data are shown as means ± SD. $^*P < 0.05$, $^{**}P < 0.01$.

We next sought to examine whether miR-223-3p influenced endogenous FOXO3 expression. EL4 T cells were transfected with miR-223-3p inhibitor or control inhibitor; the expression of FOXO3 was examined by real-time qRT-PCR or Western blot. As shown in Fig. 4*D, E*, the mRNA and protein levels of FOXO3 were significantly increased in EL4 T cells after transfection with miR-223-3p inhibitor compared with their control, indicating that FOXO3 expression could be regulated by miR-223-3p *via* translational inhibition and mRNA degradation. Given the increased levels of miR-223-3p in mice with EAU and the regulation of FOXO3 expression by miR-223-3p, we investigated the expression levels of FOXO3 in T_h17 cells in EAU. The kinetic studies showed that the expression of FOXO3 dramatically decreased during EAU development (Fig. 4*F*). Correlation analysis (33) revealed a negative correlation between miR-223-3p and FOXO3 expression in T_h17 cells in EAU (Fig. 4*G*). These findings together suggest that FOXO3 is a functional target of miR-223-3p.

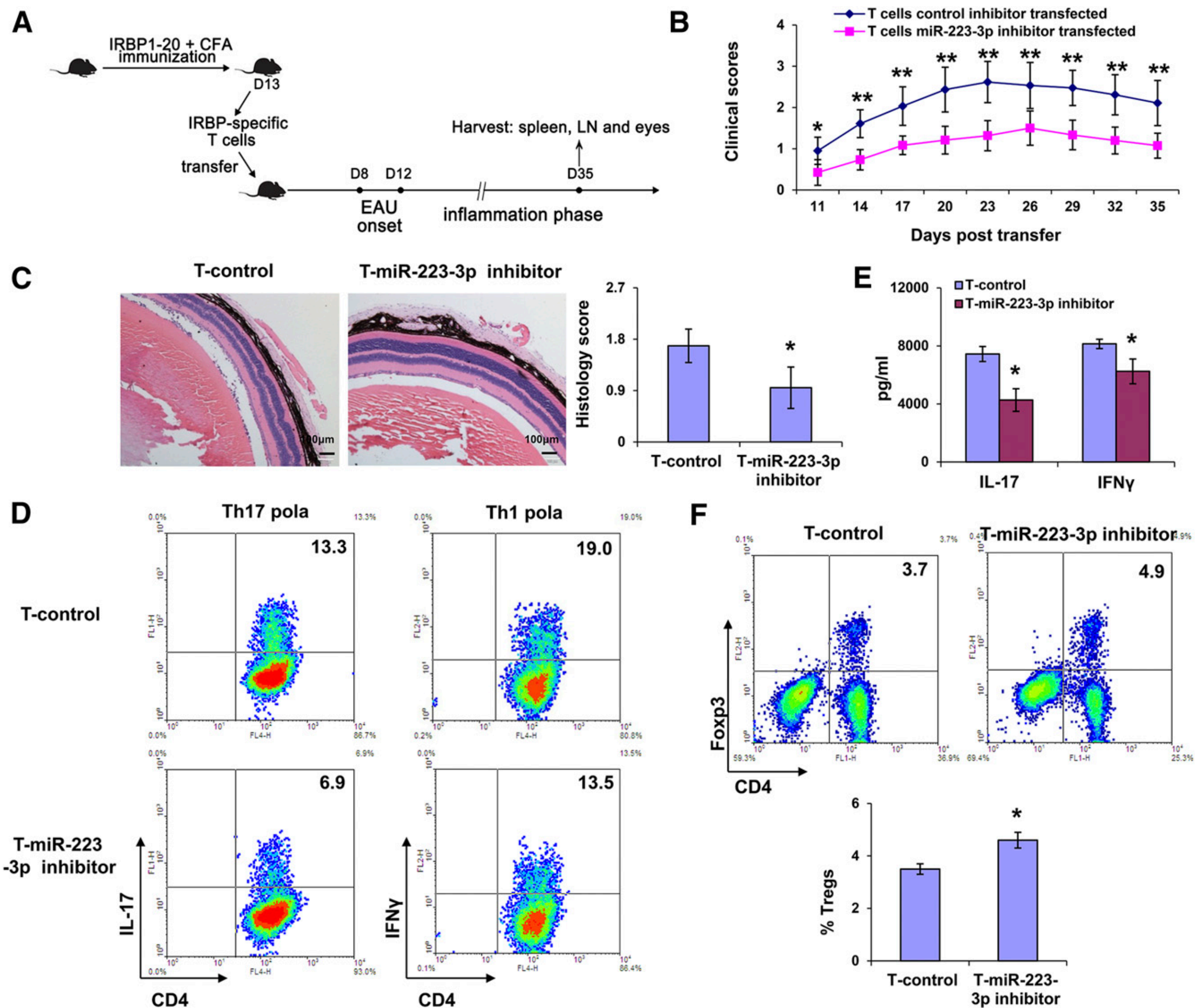

Figure 3. Silencing miR-223-3p in CD4$^+$ T cells ameliorates EAU. *A*) Schematic diagram of EAU induced by adoptive transfer. LN, lymph node. *B*) Disease score of adoptive EAU in naive B6 mice ($n = 6$/group) injected with autoreactive CD4$^+$ T cells transfected with miR-223-3p (T-miR-223-3p) or control (T-control) inhibitor. *C*) Hematoxylin and eosin staining and histopathological score of eyes from the transferred EAU mice ($n = 6$). Scale bars, 100 μm. *D*, *E*) CD4$^+$ T cells were isolated from adoptive EAU mice ($n = 3$) and stimulated with IRBP$_{1–20}$ in the presence of APCs under T$_h$17 cell polarization (T$_h$17 pola) or T$_h$1 cell polarization (T$_h$1 pola). *D*) Intracellular cytokine staining for IL-17$^+$ and IFN-γ$^+$ T cells. *E*) ELISA analysis of IL-17 and IFN-γ secretion in the culture supernatants. *F*) Flow cytometric analysis of the percentages of CD4$^+$Foxp3$^+$ cells among T cells isolated from the transferred EAU mice ($n = 3$). Data are representative of 3 independent experiments. The data are shown as means ± SD. $^*P < 0.05$, $^{**}P < 0.01$.

miR-223-3p promotes pathogenic T$_h$17 cell responses via suppression of FOXO3

FOXO3 has been demonstrated to control the development and function of T$_{reg}$ cells (34, 35). To determine the role of FOXO3 in T$_h$17 cells, a gain- and loss-of-function assay was performed using FOXO3 siRNA or lentivirus-mediated FOXO3 overexpression in EL4 or autoreactive T cells. As shown in **Fig. 5*A*, *B***, knockdown of FOXO3 by FOXO3 siRNA significantly increased IL-17 production in autoreactive T cells. Further analysis revealed that the expression of pathogenic T$_h$17 cell signature genes (IL-17, ROR-γt, IL-22, GM-CSF, and IL-1R1) were significantly increased when FOXO3 was knocked down in both autoreactive T cells (Fig. 5*C*) and EL4 T cells (Fig. 5*D*). In addition, increased FOXO3 expression mediated by lentivirus (Fig. 5*E*) greatly inhibited the expression of IL-17, ROR-γt, IL-22, GM-CSF, and IL-1R1 in autoreactive T$_h$17 cells (Fig. 5*F*), and the secretion of IL-17 was also significantly reduced after FOXO3 overexpression (Fig. 5*G*). These data indicate that FOXO3 negatively controls T$_h$17 cell responses, acting as a suppressor of T$_h$17 cell development.

We next examined whether the knockdown of FOXO3 counteracts decreased T$_h$17 cell response induced by miR-223-3p inhibitor. Cotransfection with FOXO3 siRNA, but not control siRNA, significantly rescued the decrease in mRNA and protein expression of IL-17 induced by miR-223-3p inhibitor in both EL4 T cells and autoreactive

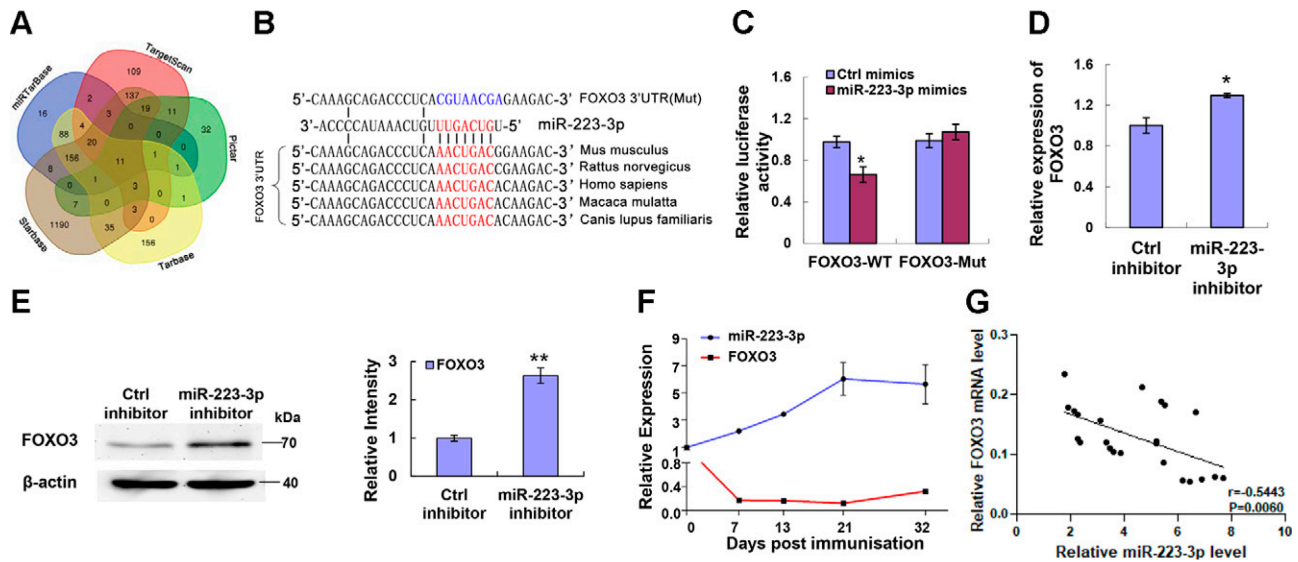

Figure 4. FOXO3 is a functional target of miR-223-3p. *A*) Venn diagram analysis of target genes of miR-223-3p as predicted by TargetScan, miRTarBase, PicTar, Tarbase, and Starbase. *B*) Conserved miR-223-3p target sites (in red letters) in the 3′UTR of FOXO3 mRNA among multiple species. Mutation of the miR-223-3p binding sites (in blue letters) is shown above. *C*) Luciferase activity was measured in 293T cells cotransfected with the reporter containing the WT or Mut 3′-UTR of FOXO3 and miR-223-3p mimics or control mimics. *D, E*) Real-time qRT-PCR and Western blot analysis of FOXO3 expression in EL4 T cells transfected with miR-223-3p or control inhibitor. *F*) Dynamic expression of miR-223-3p and FOXO3 at indicated times after disease induction was detected by real-time qRT-PCR, normalized to d 0 responses. *G*) Correlation analysis was performed between miR-223-3p and FOXO3 expression in T_h17 cells during EAU development. *P* values were calculated by the Pearson correlation test. Ctrl, control. Data are from 3 to 6 independent experiments. The data are presented as means ± SD. $^*P < 0.05$, $^{**}P < 0.01$.

T_h17 cells (Fig. 5*H, I*), suggesting miR-223-3p regulates IL-17 expression through repressing its target FOXO3. These findings indicate that miR-223-3p regulates T_h17 cell function through down-regulation of FOXO3.

IL-23R regulation by miR-223-3p–FOXO3 is involved in pathogenic function of autoreactive T_h17 cells

IL-23R is critical for T_h17 cell–mediated autoimmunity *in vivo* (36). To understand the molecular mechanisms by which FOXO3 suppresses pathogenic T_h17 cell responses, we investigated the involvement of FOXO3 in IL-23R expression. We observed that knockdown of FOXO3 in autoreactive T cells resulted in a marked enhancement of IL-23R expression (**Fig. 6*A***), whereas increased expression of FOXO3 had an inhibitory effect on the IL-23R expression (Fig. 6*B*). These results suggest that FOXO3 is a repressor of IL-23R expression in T_h17 cells.

As shown in Fig. 2*J*, IL-23R expression was significantly reduced in miR-223-3p inhibitor–transfected T_h17 cells. In contrast, the time course analysis showed that T_h17 cells transfected with miR-223-3p mimics displayed enhanced IL-23R expression in comparison with control cells. Furthermore, the knockdown of FOXO3 rescued decreased IL-23R expression induced by miR-223-3p inhibitor in T_h17 cells (Fig. 6C). Collectively, these data indicate that the inhibition of FOXO3 by miR-223-3p is one of the mechanisms for modulating IL-23R expression in T_h17 cells.

We next examined whether overexpression of IL-23R could rectify the defective IL-17 production induced by miR-223-3p inhibitor in T_h17 cells. Autoreactive T cells were transfected with plasmid control or IL-23R plasmid or miR-223-3p inhibitor. As shown in Fig. 6*D*, increased expression of IL-23R drove more IL-17 production than the control transfection group, and overexpression of IL-23R significantly reversed the decreased IL-17 production induced by miR-223-3p inhibitor. Consistent with ELISA analysis, real-time qRT-PCR analysis showed that the expression of IL-17, IL-22, and GM-CSF in miR-223-3p inhibitor–transfected T_h17 cells was partially reversed after overexpression of IL-23R (Fig. 6*E–G*). These results indicate that the reduction of IL-23R induced by miR-223-3p inhibitor is involved in the decreased pathogenic cytokine expression in T_h17 cells.

FOXO3 negatively correlated with T_h17 cell pathway in human uveitis

To extrapolate our results in mice to human subjects, we searched the Gene Expression Omnibus database for raw data from gene expression profiles in patients with uveitis and healthy volunteers, focusing on correlation of FOXO3 and the pathogenic T_h17 cell pathway. As expected, the expression of FOXO3 was down-regulated in peripheral blood mononuclear cells (PBMCs) from patients with uveitis in a clinical data set (GSE70403) (37), whereas the expression of pathogenic T_h17 cell signature genes (including IL-17A, IL-17F, IL-1R1, IL-23R, and IL-22) was up-regulated (**Fig. 7*A***). We further performed the correlation analysis between FOXO3 and T_h17 cell signature genes and found that IL-23R, IL-17A, GM-CSF, and

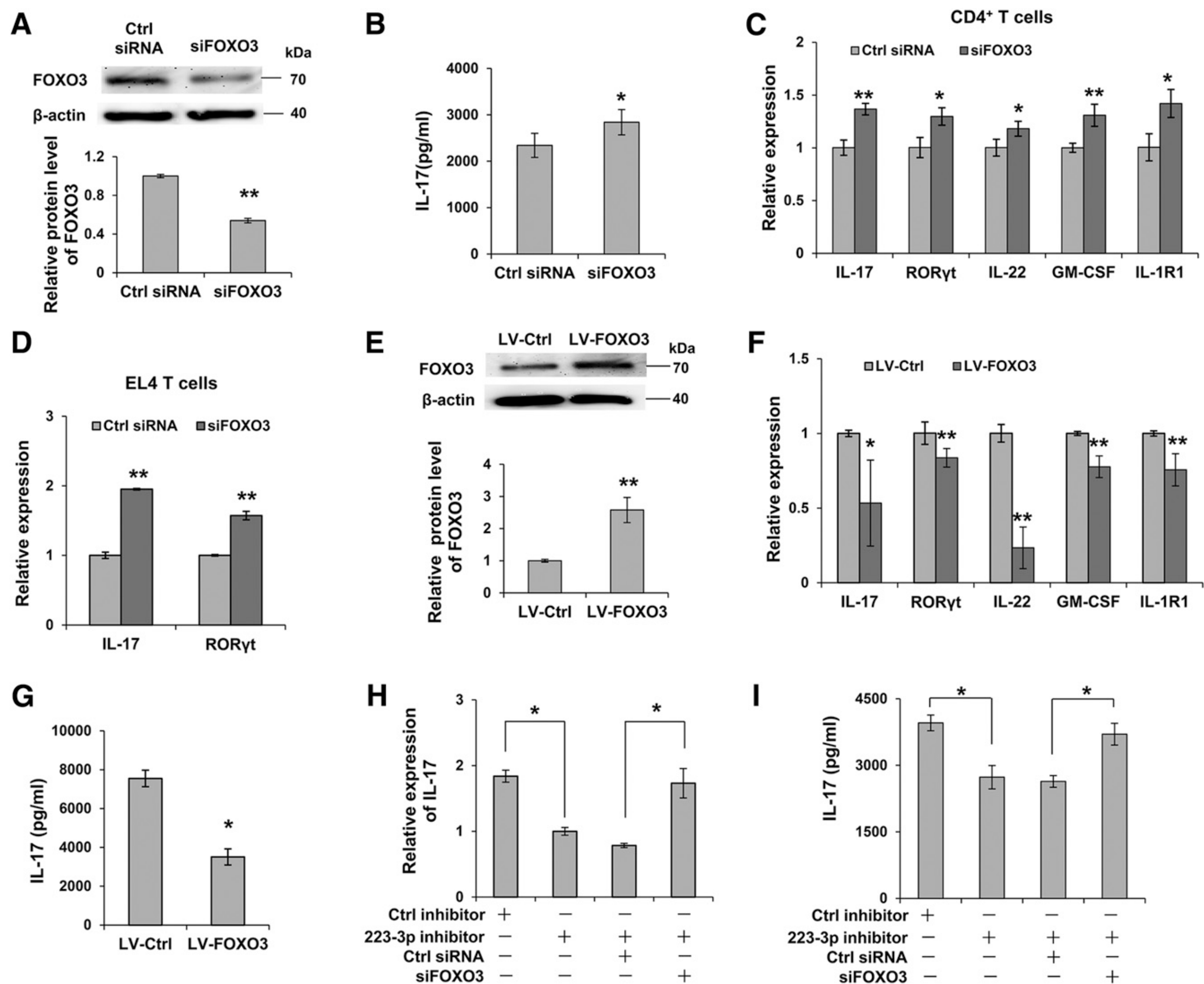

Figure 5. miR-223-3p promotes the pathogenic function of T_h17 cells *via* repression of FOXO3 expression. *A–G*) EL4 T cells or autoreactive $CD4^+$ T cells were transfected with FOXO3 siRNA and control siRNA (200 nM) or infected with FOXO3 lentivirus (LV-FOXO3) or control lentivirus (LV-control). *A, E*) FOXO3 protein level was analyzed by Western blot. *B, G*) IL-17 secretion was determined by ELISA. *C, D, F*) Relative expression levels of T_h17 cell signature genes in EL4 T cells and autoreactive T_h17 cells were measured by real-time qRT-PCR. *H, I*) EL4 T cells or autoreactive $CD4^+$ T cells were transfected with indicated oligonucleotide. *H*) Real-time qRT-PCR analysis of IL-17 expression in EL4 T cells. *I*) ELISA analysis of IL-17 production in autoreactive T_h17 cells. Ctrl, control; siFOXO3, FOXO3 siRNA. Data are representative of at least 3 independent experiments. The data are shown as means ± SD. *$P < 0.05$, **$P < 0.01$.

ROR-γt correlated negatively with FOXO3 in the data sets GSE17114 (38), GSE61399 (29), and GSE10080 (Fig. 7*B–E*). In addition, in the PBMCs of patients with active BD, human miR-223 was found to be up-regulated compared with quiescent BD (39) (Fig. 7*F*). These results support that there is a potential crosstalk between the miR-223-3p–FOXO3–IL-23R axis and pathogenic T_h17 cell responses in human uveitis.

DISCUSSION

Pathogenic T_h17 cells are the critical drivers of autoimmune diseases, and functional regulators of pathogenic T_h17 cells represent potential targets for clinical diagnosis or treatment of autoimmune disorders (40). Here, we showed that miR-223-3p positively regulated autoreactive T_h17 cell responses. Using a transferred EAU model for uveitis, we demonstrated that silencing miR-223-3p in autoreactive T cells substantially ameliorated EAU pathogenesis, consistent with the positive correlation between miR-223-3p expression and disease in patients with uveitis. Mechanistically, miR-223-3p targeted FOXO3 in autoreactive T_h17 cells. Moreover, FOXO3 negatively regulated pathogenic T_h17 cell responses partially *via* suppressing IL-23R expression.

miR-223-3p has been studied in many fields, particularly as a participant in cancers (41–43), ischemic heart disease (44, 45), pulmonary arterial hypertension (46), rheumatoid arthritis (47), and EAE (30). In this study, we focus on the role of miR-223-3p in pathogenic T_h17 cell responses in EAU, which has not yet been clearly illustrated. Our findings that miR-223-3p is up-regulated in autoreactive $CD4^+$ T cells are consistent with previous results obtained in other autoimmune diseases (48–52). The association among miR-223-3p,

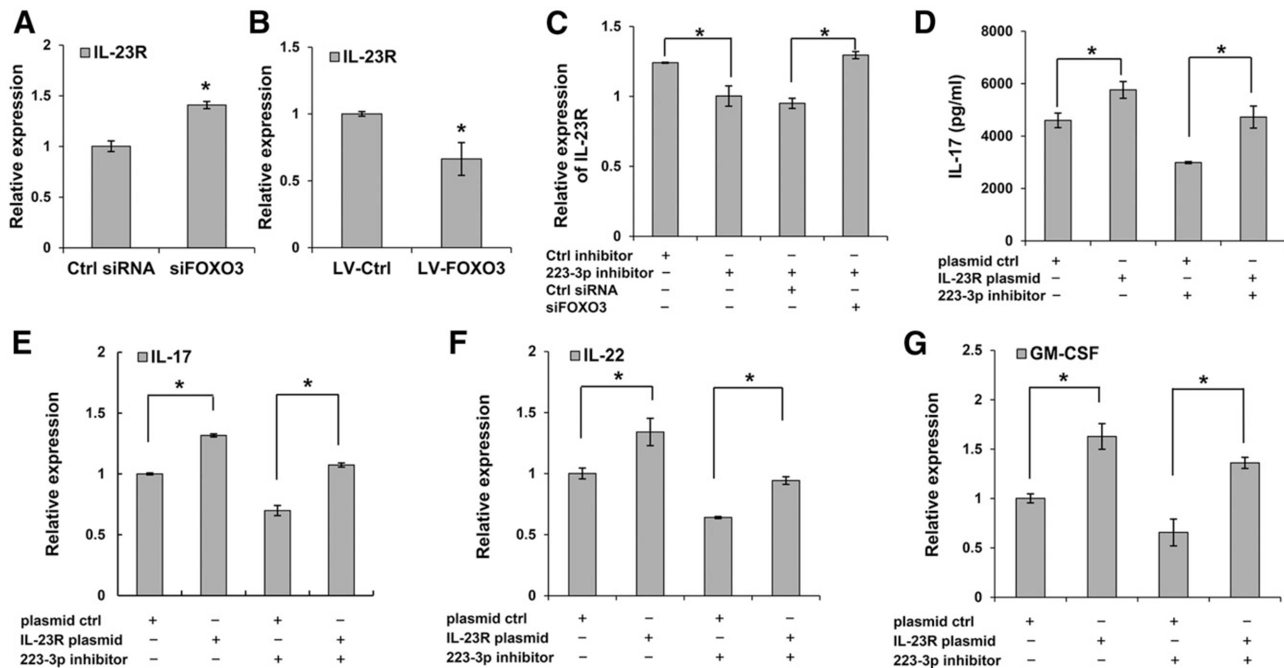

Figure 6. IL-23R is involved in pathogenic T_h17 cell responses. *A–C*) $CD4^+$ T cells from immunized B6 mice were transfected with indicated oligonucleotide (*A*, *C*) or infected with FOXO3 lentivirus (LV-FOXO3) or control lentivirus (LV-control) (*B*) and stimulated with the $IRBP_{1-20}$ and APCs under T_h17 cell polarizing conditions. Relative IL-23R mRNA expression was determined by real-time qRT-PCR. *D–G*) $CD4^+$ T cells isolated from EAU mice were transfected with indicated plasmid alone or in combination with miR-223-3p inhibitor and cocultured with irradiated APCs in the presence of antigen for 72 h. *D*) IL-17 production was detected by ELISA. *E–G*) The relative expression of IL-17, IL-22, and GM-CSF was analyzed by real-time qRT-PCR. Ctrl, control; siFOXO3, FOXO3 siRNA. Data represent means ± SEM from 3 independent experiments. $^*P < 0.05$.

uveitis, and EAU has also been observed. miR-223 has been shown to be significantly elevated in the retina, and its levels correspond with the clinical score of the EAU in rats (14). In the serum of patients with noninfectious uveitis, miR-223-3p was found to be significantly up-regulated compared with healthy controls (16). In PBMCs of active BD, miR-223-3p was found to be significantly up-regulated compared with quiescent BD (39). However, in PBMCs of patients with BD, miR-223-3p was found to be significantly down-regulated as compared with healthy controls (15). Indeed, our present findings of miR-223-3p dysregulation are partially in line with these previous studies. It should be noted that miR-223-3p levels changed dynamically at different stages during EAU development. The expression of miR-223-3p was up-regulated at the onset and reached the maximum at the peak stage but was decreased when the disease remitted. The difference in tissue and cells types, different type of uveitis, and disease stage may account for the discrepancy in these studies.

Development of T_h17 cells is programmed by the orphan nuclear receptor ROR-γt. Our data showed that miR-223-3p overexpression in uveitogenic T cells significantly increased ROR-γt mRNA expression and IL-17 production, suggesting that miR-223-3p concurrently affects autoreactive T_h17 cell lineage commitment and function. In addition, miR-223-3p also significantly up-regulated expression of GM-CSF and IL-22, which are critical for the pathogenicity of T_h17 cells in autoimmune disease (11). IFN-γ–producing T_h1 cells also play a role in the pathogenesis of EAU (53, 54). Here, we showed that miR-223-3p was also up-regulated in IRBP-specific T_h1 cells, and down-regulation of miR-223-3p significantly repressed IRBP-specific T_h1 cell responses *in vivo*. The precise mechanisms underlying the action of miR-223-3p on T_h1 cell responses in EAU are still to be identified. Given that both T_h1 and T_h17 pathogenic T cells contribute to uveitis pathogenesis, manipulation of miR-223-3p and its signal pathways may offer a new potential therapeutic strategy for uveitis.

Pathogenic T_h17 cell responses are critically regulated by DC-derived cytokines IL-1β and IL-23 (55). IL-1β is critical for expansion of pathogenic T_h17 cells (56), and IL-23 stabilizes pathogenic T_h17 cell phenotype (7). In this study, we observed that overexpression of miR-223-3p significantly increased the expression of IL-1β and IL-23 by DCs, suggesting miR-223-3p may create a favorable cytokine milieu that supports the generation of pathogenic T_h17 cells. Indeed, we found that miR-223-3p–overexpressing DCs possessed an enhanced ability to promote the expansion of IRBP T_h17 cells and IL-17 production, indicating miR-223-3p also regulates DC-driven autoreactive T_h17 cell responses. Our results here are further supported by a previous report (ref. 30) showing that miR-223 enhanced myeloid DC-induced activation of pathogenic T_h17 cells in EAE.

In a subsequent study, we found that miR-223-3p and FOXO3 displayed an inverse pattern of expression in EAU, and we identified FOXO3 as a target gene of miR-223-3p in autoreactive $CD4^+$ T cells. In agreement with our findings,

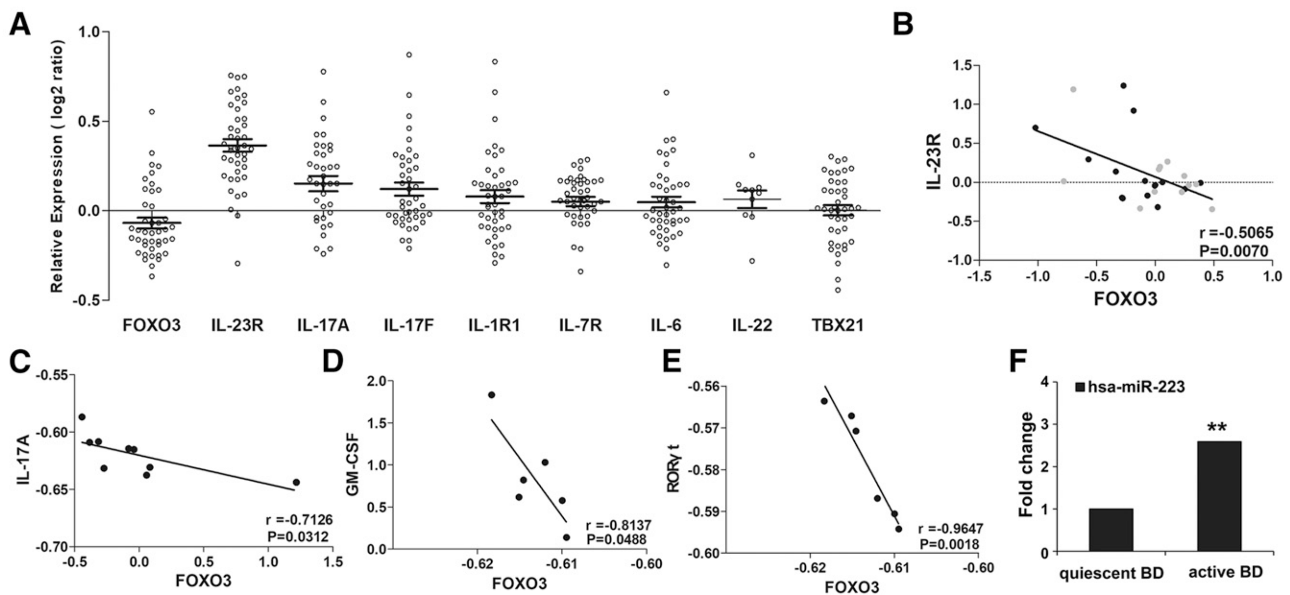

Figure 7. FOXO3 negatively correlates with T_h17 cell pathway in human uveitis. *A*) Expression profiles of FOXO3 and T_h17 cell signature genes in PBMCs from BD ($n = 41$) *vs.* those of healthy control by analyzing the clinical dataset GSE70403. *B*, *C*) Correlation of IL-23R and IL-17A gene expression *vs.* that of FOXO3. Gray and black dots represent healthy volunteers and BD, respectively. *D*, *E*) Correlation of GM-CSF and RORγt gene expression *vs.* that of FOXO3 in human PBMCs stimulated with anti-CD3 and -CD28 beads and IL-23. The *z* score transformed values of published dataset GSE17114 (*B*), GSE61399 (*C*), and GSE10080 (*D*, *E*) were used. *P* values were calculated by the Pearson correlation test (*B–E*). *F*) Analysis of the published clinical data (39) showed up-regulation of Homo sapiens (has)-miR-223 in patients with active BD. $^{**}P < 0.01$.

miR-223-3p was inversely correlated with FOXO3 in prostate cancer (57). To further decipher the effect of FOXO3 on T_h17 cell development, a gain- and loss-of-function assay was conducted using FOXO3 siRNA or lentivirus-mediated FOXO3 overexpression, and we demonstrated that FOXO3 negatively regulated the expression of pathogenic T_h17 cell signature genes, indicating that FOXO3 may act as a repressor of autoreactive T_h17 cell generation and function. Our findings that silence of FOXO3 compromised the activity of miR-223-3p inhibitor in T_h17 cells indicate the existence of a regulatory pathway in which autoreactive T cells enhance expression of miR-223-3p, which inhibits FOXO3 expression to promote T_h17 cell responses.

FOXO1 and FOXO3 have been demonstrated to promote the expression of Foxp3 and T_{reg} cell generation (34). High expression of miR-223 has been shown to be associated with lower numbers of T_{reg} cells in tobacco smokers (58). In this study, we observed that spleen cells from mice receiving T cells transfected with miR-223-3p inhibitor displayed a higher percentage of T_{reg} cells, suggesting decreased miR-223-3p may favor the generation of T_{reg} cells. Although T_h17 and T_{reg} cells play opposite roles in the regulation of autoimmunity, these 2 subsets can interconvert under specific conditions (59–61). Park *et al.* (62) demonstrated that FOXO3 ablation promotes T_{reg}-to-T_h17-like cell conversion, suggesting FOXO3 may function as an intrinsic modulator in T cells that decides between the T_h17 and T_{reg} cell choices. Our studies here suggest that this lineage choice is also influenced by miR-223-3p: miR-223-3p biases T cells toward T_h17 cell lineage but inhibits T_{reg} cell differentiation by suppressing FOXO3 expression. Therefore, we proposed that by modulating FOXO3 expression, miR-223-3p might be a post-transcriptional regulator controlling T_h17-to-T_{reg} cell plasticity in EAU. FOXO3 also drives pathogenic T_h1 cell differentiation *via* inducing eomesodermin expression in EAE, and deletion of FOXO3 ameliorates EAE (21). Further studies are needed to investigate the impact of FOXO3 on IRBP T_h1 cell responses and examine its role in modulation of EAU disease severity.

IL-23R is a key molecule of pathogenic T_h17 cell signatures (63). In the present study, we focused on IL-23R as key target of FOXO3 to explain its regulation on T_h17 cell pathogenic function. We found that overexpression of FOXO3 in T_h17 cells led to decreased IL-23R expression, indicating that FOXO3 negatively regulated the expression of IL-23R in T_h17 cells. ROR-γt induces the expression of IL-23R by binding directly to the Il-23R promoter in T_h17 cells (64). We found 1 putative FOXO3 binding element close to the ROR-γt binding region on the mouse Il-23R locus (Supplemental Fig. S1*A*, *B*), indicating that FOXO3 might inhibit IL-23R expression *via* interference of ROR-γt activity in uveitogenic T_h17 cells and therefore control the pathogenicity of T_h17 cells. Of note, miR-223-3p directly targeted FOXO3 to promote IL-17 production, silencing FOXO3 enhanced IL-23R expression, and IL-23 treatment significantly enhanced the expression of miR-223-3p and IL-23R (36). Therefore, we proposed that miR-223-3p, together with IL-23, FOXO3, and IL-23R may form a positive feedback loop to stabilize the pathogenic T_h17 cell phenotype and contribute to constant IL-17 production in EAU (**Fig. 8**).

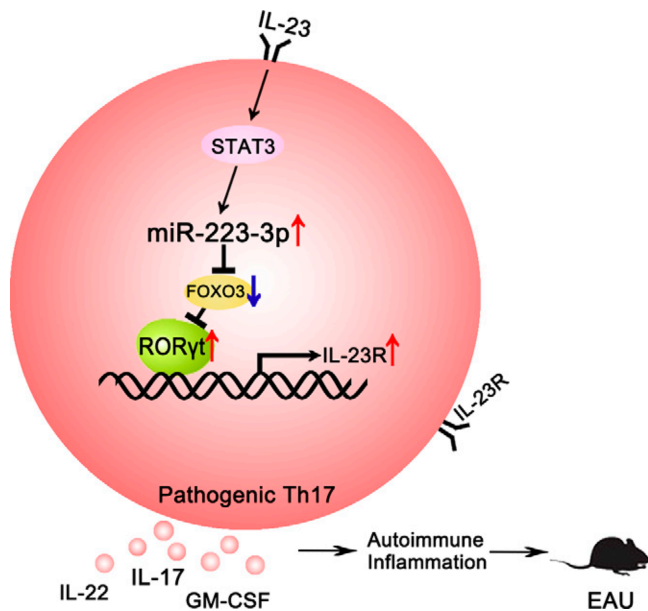

Figure 8. Schematic showing the molecular mechanisms underlying miR-223-3p to regulate pathogenic T_h17 cell responses in EAU. IL-23 activates STAT3 in T_h17 cells, which increases the expression of miR-223-3p. Through down-regulation of the transcription factor FOXO3, miR-223-3p enhances ROR-γt transcription activity and further induces the expression of IL-23R, resulting in an enhancement of pathogenic T_h17 cell responses. IL-23, miR-223-3p, FOXO3, and IL-23R form a positive feedback loop, which stabilizes the pathogenic T_h17 cell phenotype and contributes to constant IL-17 production in the EAU model.

miRs are essential for pathogenic function of T_h17 cells (65). Various studies have been undertaken to reveal the regulatory network of miRs in the regulation of pathogenic T_h17 cells in autoimmunity (65–69). Our study here adds another wrinkle to the relationship between miRs and pathogenic T_h17 cells. Specifically, we have demonstrated that the suppression of FOXO3 *via* miR-223-3p is an important mechanism by which T_h17 cells acquire pathogenic function in EAU. Thus, the miR-223-3p–FOXO3 axis may be an important therapeutic target for uveitis. FJ

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AUTHOR CONTRIBUTIONS

Y. Wei, S. Chen, and X. Li performed the experiments; Y. Wei contributed to writing the manuscript; Y. Wei and S. Chen analyzed the data; D. Sun, R. Wei, and X. Li contributed to editing the manuscript and designing the project; and H. Nian conceived the study, designed and supervised the overall project, and wrote the manuscript.

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