

Altered FOXO1 Transcript Levels in Peripheral Blood Mononuclear Cells of Systemic Lupus Erythematosus and Rheumatoid Arthritis Patients

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FOXO forkhead transcription factors play an important role in controlling lymphocyte activation and proliferation. To evaluate the possibility that FOXO transcriptional expression is dysregulated in systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) patients, we determined the quantities of FOXO1, FOXO3a, and FOXO4 transcripts in peripheral blood mononuclear cells (PBMCs) from normal controls as well as from SLE and RA patients. Results showed that FOXO1 and FOXO3a are dominant FOXO factors at the transcript level in PBMCs. Statistical analysis showed that the FOXO1 transcript levels in RA patients and in SLE patients with active disease activity were significantly lower than those in normal controls, and the FOXO1 transcript levels were inversely correlated with lupus disease activity. In contrast, the differences in FOXO3a and FOXO4 transcript levels between normal controls and patients were not significant. These data suggest that the transcriptional dysregulation in FOXO1 is possibly linked to the pathogenesis of SLE and RA.

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INTRODUCTION

The forkhead transcription factors, denoted as Forkhead-box (Fox) factors, belong to a group of nuclear proteins that contain conserved winged helix/forkhead DNA binding domain (1). More than 100 forkhead transcription factors have been identified in different animal species and are further classified into subfamilies. Expressed in a variety of tissues, forkhead transcription factors have been shown to mediate various cellular functions, especially in controlling embryonic developmental processes, metabolism, and cell growth (1-3). In addition, certain forkhead transcription factors participate in the regulation of immune functions, as exempli-

fied by the function of Foxp3 in programming the development of naturally occurring CD4⁺ CD25⁺ regulatory T cells and by the role of Foxn1 in thymic development (4,5).

The members of the forkhead box class O (FOXO) subfamily of forkhead transcription factors, FOXO1, FOXO3a, and FOXO4, have been shown to mediate various cellular functions, including cell proliferation, tumor suppression, metabolism, and oxidative stress responses (6,7). The activities of these FOXO factors are tightly regulated at the protein level via posttranslational modification, including phosphorylation by kinases, such as protein kinase B (also known as Akt, a downstream mediator of phos-

phatidylinositol 3-kinase signaling) and inhibitor of nuclear factor- κ B kinase (8-12). Transcriptional activity of FOXO factors can be inactivated through phosphorylation. FOXO factors can be transported from the nucleus to the cytoplasm and subjected to proteasomal degradation (10). Recently, accumulated evidence has suggested the important role of FOXOs in controlling lymphocyte activation and immune functions, as demonstrated in recent studies on human or mouse primary lymphocytes and lymphocyte cell lines, which showed that the inactivation of FOXO activity via phosphatidylinositol 3-kinase/Akt signaling activation leads to cell proliferation and activation, cell survival, and B-cell class-switch recombination (13-20). More evidence for the important role of FOXOs in regulating lymphocyte homeostasis comes from the findings in FOXO gene-knockout mice, in which Foxo1-deficiency results in embryonic lethality. Nevertheless, the expression of a dominant-negative mutant Foxo1 transgene in

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T cells has been shown to affect thymocyte survival in mice (21–23). Foxo3a-deficient mice developed mild autoimmune diseases during old age, with spontaneous T-cell activation, lymphoproliferation, and increased NF- κ B activation (24). Foxo4-deficient mice were found to have a normal phenotype, a finding that, together with the mild autoimmune phenotype in Foxo3a-deficient mice, can be explained by the joint action of FOXO1, FOXO3a, and FOXO4 to guard lymphocyte activation (12,21,25,26).

Due to the critical role of FOXO factors in the control of lymphocyte activation demonstrated in mice and in human lymphocyte *in vitro*, we hypothesized that the dysregulation in FOXO gene expression may be connected to the development of systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA), two human autoimmune diseases with tissue damages mediated by the overactivation of autoreactive lymphocytes. In this study, we examined our hypothesis by comparing the transcript levels of FOXO1, FOXO3a, and FOXO4 in human peripheral blood mononuclear cells (PBMCs) from healthy individuals with those in PBMCs from SLE and RA patients. We then performed statistical analysis to evaluate the possible dysregulation of the FOXO gene transcript levels in SLE and RA patients, and we also designed experiments to profile the relative transcript abundance of FOXO genes in human PBMCs.

MATERIALS AND METHODS

Study Populations

We recruited 16 healthy individuals, 30 SLE patients, and 16 RA patients into this study from Cathay General Hospital, Taipei, Taiwan. The diagnoses of RA and SLE were based on the classification criteria of American College of Rheumatology (27,28). The disease activities of SLE patients were evaluated with the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) (29). Based on the SLEDAI scores, SLE patients were as-

Table 1. Sequences of Gene-Specific Primer Sets Used in PCR

<i>Genes</i>	<i>Primer sequences</i>
FOXO1	Forward: 5'-GCAGATCTACGAGTGGATGGTC-3' Reverse: 5'-AAACTGTGATCCAGGGCTGTC-3'
FOXO3a	Forward: 5'-CTCAAGGATAAGGGCGACAG-3' Reverse: 5'-TCGTCCTGGACTTCATCCAAC-3'
FOXO4	Forward: 5'-AGCGACTGACACTTGCCAGAT-3' Reverse: 5'-AGGGTTCAGCATCCACCAAGAG-3'
GAPDH	Forward: 5'-ATGACCCCTTCATTGACCTCA-3' Reverse: 5'-GAGATGATGACCCCTTTGGCT-3'

signed to one of two study groups, the active SLE group (14 patients with active disease and SLEDAI score >3) and the inactive SLE group (16 patients with inactive disease and SLEDAI score \leq 3). Based on the presence or absence of treatment with etanercept (a TNF- α -blocking agent), RA patients were assigned to one of two study groups, the RA without etanercept group (11 patients) and the RA with etanercept group (five patients with the effective control of joint inflammation by the etanercept treatment). This study was approved by the institutional review board for research ethics at Cathay General Hospital, Taiwan. Informed consent was provided from all blood donors.

Preparation of PBMCs and Total RNA Isolation

Whole blood samples in heparin-containing tubes were collected from healthy individuals, SLE patients, and RA patients, and PBMCs were isolated immediately by Ficoll-Hypaque density centrifugation. Total RNA was then extracted from PBMCs by using the RNeasy mini-kit (Qiagen, Valencia, CA, USA), according to the manufacturer's protocol.

Semiquantitative Reverse Transcription-Polymerase Chain Reaction

Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed to determine the transcript levels of FOXO1, FOXO3a, and FOXO4 in human PBMCs, and the amplification of GAPDH transcripts was used as the control to normalize the transcript levels of these FOXOs. RT was performed by using the oligo-dT primer (Invitrogen,

Carlsbad, CA, USA), and 0.5 μ g total RNA in a 25- μ L reaction mixture, containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM DTT, 5 mM MgCl₂, 2.5 mL dNTP (10 mM), 10 U RNasin, and 200 U MMLV reverse transcriptase (Invitrogen). The RT reaction was carried out at 39 °C for one hour to synthesize cDNAs. Then, PCR was performed to amplify cDNAs in a 25- μ L reaction mixture containing 50 nmol of each gene-specific primer, 3 mL RT product, 2.5 mM dNTP, 1X PCR buffer (5 mM Tris-HCl pH 8.3, 42.5 mM KCl, 0.1% Triton X-100), 0.5 mL Taq polymerase (Promega, Madison, WI, USA), and 2 mM MgCl₂. The sequences of gene-specific primers used in PCR are presented in Table 1. The cDNAs of FOXO1, FOXO3a, and FOXO4 transcripts were all amplified for 19 cycles (1 min at 94 °C, 1 min at 59 °C, and 1 min at 72 °C), and the cDNAs of GAPDH transcripts were amplified for 17 cycles (1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C). The PCR cycling numbers had been optimized to avoid the amplification saturation. Five microliters of the RT-PCR product was separated on 2% agarose gels, which were subsequently stained with ethidium bromide. DNA fragments in gels were then visualized and quantified by using the ChemiGenus2 imaging system (Syngene, Frederick, MD, USA), based on the known concentrations of DNA markers. The specificity of RT-PCR DNA fragments was determined by restriction fragment length polymorphism analysis and by direct sequencing of some RT-PCR DNA fragments purified from agarose gels with the use of a GeneClean kit (Qiagen).

Western Blot Analysis

Whole cell lysates obtained from PBMCs were fractionated on a 10% reducing sodium dodecyl sulfate-polyacrylamide gel and then transferred to an Immobilon-P membrane (Millipore, Bedford, MA, USA) according to the manufacturer's instructions. The membrane was incubated overnight with a mouse anti-FOXO1 monoclonal antibody (Sigma, St Louis, MO, USA) in the TBST buffer (20 mM Tris-HCl pH 7.6, 137 mM NaCl, and 0.5% Tween 20) with 5% skim milk. After being washed with the TBS-T buffer, the membrane was incubated with HRP-conjugated anti-mouse IgG antibody for five hours. Immunoreactive protein bands on the membrane were then visualized by using an ECL Western-blotting detection reagent (Pierce, Rockford, IL, USA) and an image processing system (ChemiGenius XE2). The actin protein in cell protein extracts was used as the internal control for FOXO1 in the Western blot analysis.

Statistical Analysis

We used the nonparametric Mann-Whitney rank-sum test to test the significance of the difference in the transcript levels of FOXO1, FOXO3a, and FOXO4 between the control group and each of the disease groups (RA without etanercept, RA with etanercept, active SLE, and inactive SLE groups). Simple linear regression was used to test the correlation between the FOXO1 transcript levels and the SLEDAI scores. *P*-values were calculated and considered significant if less than 0.05.

RESULTS

Profiling FOXO Gene Transcripts in PBMCs

To gain a better insight into the relative transcript abundance of FOXO1, FOXO3a, and FOXO4 genes in human PBMCs, the transcript levels of these FOXOs in PBMCs of healthy individuals were evaluated by RT-PCR experiments with the same PCR-cycling conditions for amplifying each FOXO gene tran-

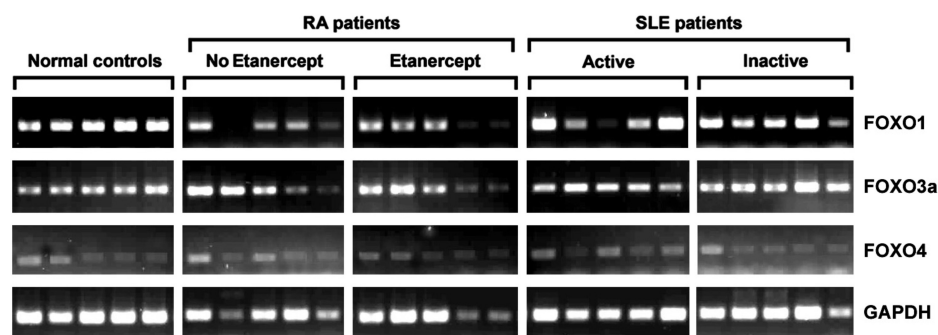


Figure 1. RT-PCR experiments were performed to amplify FOXO1, FOXO3a, FOXO4, and GAPDH transcripts in PBMCs isolated from normal controls, from RA patients with or without the etanercept treatment, and from SLE patients with active or inactive disease (19 PCR amplification cycles for FOXO1, FOXO3a, and FOXO4 transcripts; 17 cycles for GAPDH transcripts). Shown are some representative results of RT-PCR DNA products visualized in gels.

scripts. Some representative RT-PCR results are shown in Figure 1, and it appears that all FOXO1, FOXO3a, and FOXO4 transcripts can be detected in PBMCs of healthy individuals with slight interindividual variation. The transcript levels of FOXO genes in PBMCs were determined by quantifying the amounts of DNA products derived from the RT-PCR amplification of FOXO transcripts and were normalized according to GAPDH transcript levels, and FOXO1 and FOXO3a transcripts appeared to be more abundant than FOXO4 transcripts in normal PBMCs (Table 2). When we used the same experimental methods to assay the relative transcript abundance of FOXO1, FOXO3a, and FOXO4 genes in PBMCs from SLE and RA patients, we obtained results similar to those for PBMCs from healthy individuals, except that the interindividual variation in the FOXO transcript levels was more obvious in SLE and RA patients, as interpreted by the wide distribution of the amounts of

gene-specific RT-PCR DNA products (Figure 2 and Table 2). These data suggest that in human PBMCs FOXO1 and FOXO3a are the dominant FOXO members at the mRNA level.

Decreased FOXO1 Expression in SLE and RA Patients

To evaluate the possibility that the transcriptional expression of FOXO genes is dysregulated in SLE and/or RA patients, the quantities of FOXO1, FOXO3a, and FOXO4 transcripts in PBMCs isolated from controls and from SLE and RA patients were determined by quantifying DNA products produced by the RT-PCR amplification of FOXO transcripts. Statistical analysis was then conducted to determine the significance of the differences in the quantities of FOXO1, FOXO3a, and FOXO4 transcripts between controls and patients. Meanwhile, to evaluate the influence of lupus disease activity and treatment with a TNF- α -blocking agent on the FOXO tran-

Table 2. Means and Standard Errors of the Quantities of DNA Products Produced by the RT-PCR Amplification of FOXO Transcripts for Different Study Groups.

	Normal controls	RA without etanercept	RA with etanercept	Active SLE	Inactive SLE
FOXO1	42.7 \pm 3.5	29.7 \pm 4.3	25.8 \pm 4.8	29.4 \pm 5.6	33.2 \pm 5.1
FOXO3a	31.1 \pm 3.4	34.7 \pm 6.9	29.0 \pm 4.9	33.1 \pm 3.1	33.1 \pm 3.14
FOXO4	16.2 \pm 2.7	20.4 \pm 5.7	10.7 \pm 3.2	21.8 \pm 3.7	20.9 \pm 4.3

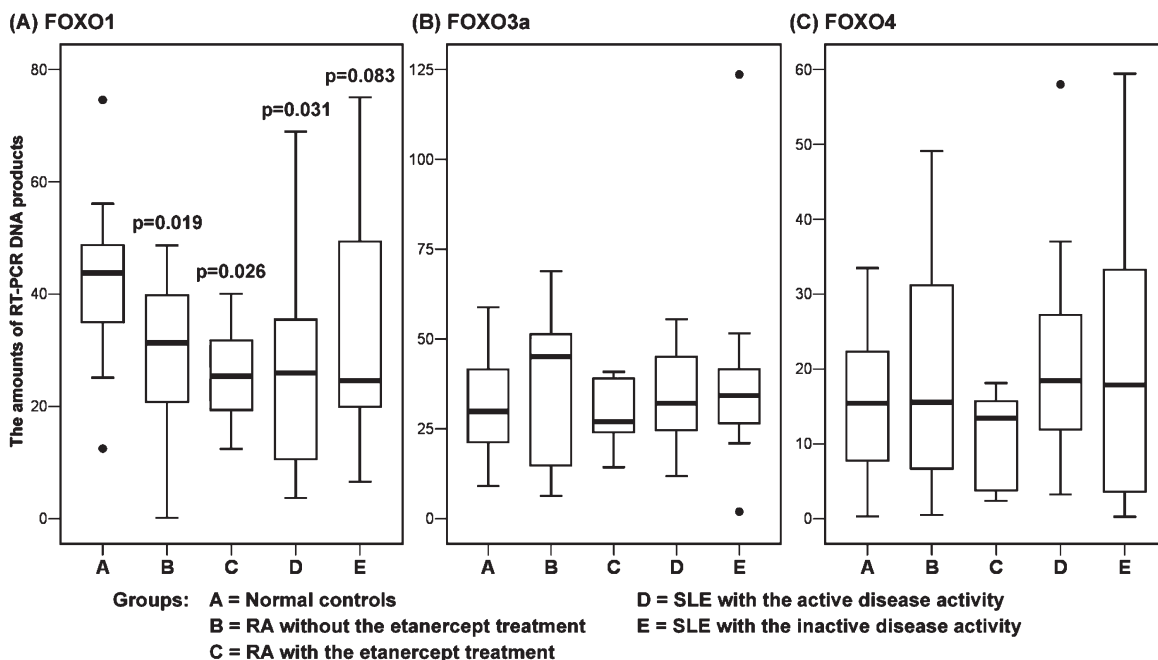


Figure 2. The quantities of FOXO1, FOXO3a, and FOXO4 transcripts in PBMCs of normal controls and SLE and RA patients were determined by RT-PCR and the subsequent quantification of gene-specific RT-PCR DNA products. The FOXO transcript levels were normalized according to the GAPDH transcript levels. The distributions (boxplots with medians and quartiles) of the quantities of FOXO1, FOXO3a, and FOXO4 transcripts for various study groups are presented. *P* values of the statistical comparisons for the FOXO1 transcript levels are indicated. *P* values of the statistical comparisons for the FOXO3a and FOXO4 transcript levels are all more than 0.5 (data not shown).

script levels, SLE patients were divided into active SLE and inactive SLE groups and RA patients into RA with etanercept and RA without etanercept groups in the statistical comparisons. From the statistical comparison for the FOXO1 transcript levels, we found that RA patients with or without etanercept treatment and SLE patients with the active disease activity were significantly different from controls (all *P* values less than 0.05), that the significance for the difference between normal controls and SLE patients with the inactive disease activity was marginal (*P*=0.083), and that there was no significant difference between different disease groups. It appears that RA patients and SLE patients with active disease have decreased FOXO1 transcript levels in PBMCs, and even SLE patients with the inactive disease also tend to have this phenomenon (Table 2, Figure 2). On the other hand, when different pairs of study groups were statistically analyzed for the difference in the FOXO3a and FOXO4

transcript levels, no significant differences were found. These data indicate that FOXO1 transcripts in PBMCs are down-regulated in SLE and RA patients, and that treatment with the TNF- α -blocking agent appears to have no affect

on the FOXO transcript levels in RA patients. In addition to the transcript levels, the protein levels of FOXO1 in PBMCs of SLE and RA patients were also found to be down-regulated, as demonstrated by Western blot analysis (Figure 3).

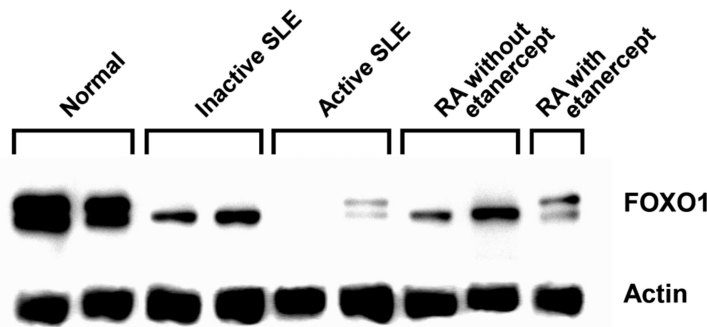


Figure 3. FOXO1 protein expression in PBMCs of normal individuals, RA patients, and SLE patients. Western blot analysis was performed by using whole cell lysates of PBMCs obtained from 12 normal individuals, 15 RA patients (10 patients without etanercept, 5 patients with etanercept), and 20 SLE patients (10 active and 10 inactive patients). The actin protein levels were also evaluated and used as internal controls for cell protein extracts. The representative of the results from similar Western blot experiments is shown.

Inverse Correlation of FOXO1 Transcript Levels with SLE Disease Activity in PBMCs

We then determined whether the FOXO1 transcript levels in PBMCs are correlated with the lupus disease activity in SLE patients with active disease. Therefore, the quantities of FOXO1 transcripts in PBMCs determined by quantifying DNA products from the RT-PCR amplification of FOXO1 transcripts for SLE patients with active disease were used in the simple linear regression analysis to test their correlation with SLEDAI scores. As shown in Figure 4, quantities of FOXO1 transcripts were found to be inversely correlated with SLEDAI scores, suggesting a negative correlation of FOXO1 transcript levels in with lupus disease activity in PBMCs from SLE patients. We also found that the FOXO3a and FOXO4 transcript levels were not significantly correlated with SLEDAI scores (data not shown).

DISCUSSION

The FOXO forkhead factors appear to critically regulate lymphocyte activation. In this study, we evaluated the transcript profiles of FOXO1, FOXO3a, and FOXO4 genes in PBMCs from healthy individuals as well as in PBMCs from SLE and RA patients. FOXO1 and FOXO3a seem to be the dominant FOXO members expressed at the mRNA level in PBMCs of healthy individuals, and FOXO1 transcripts are slightly more abundant than FOXO3a transcripts. Similar transcript patterns were also found in PBMCs of SLE and RA patients, albeit with increased interindividual variation. These results are somewhat discordant with the finding in mice that FOXO3a is the dominant FOXO member expressed in spleen (30,31), and the discrepancy may be due to the difference in tissues used to assay FOXO transcripts. On the other hand, the finding that FOXO4 is less abundant than FOXO1 and FOXO3a seems to parallel the finding in mice, suggesting that FOXO4 plays a relatively minor role in mediating FOXO activity in human immune cells, as extrapolated by the observation of normal

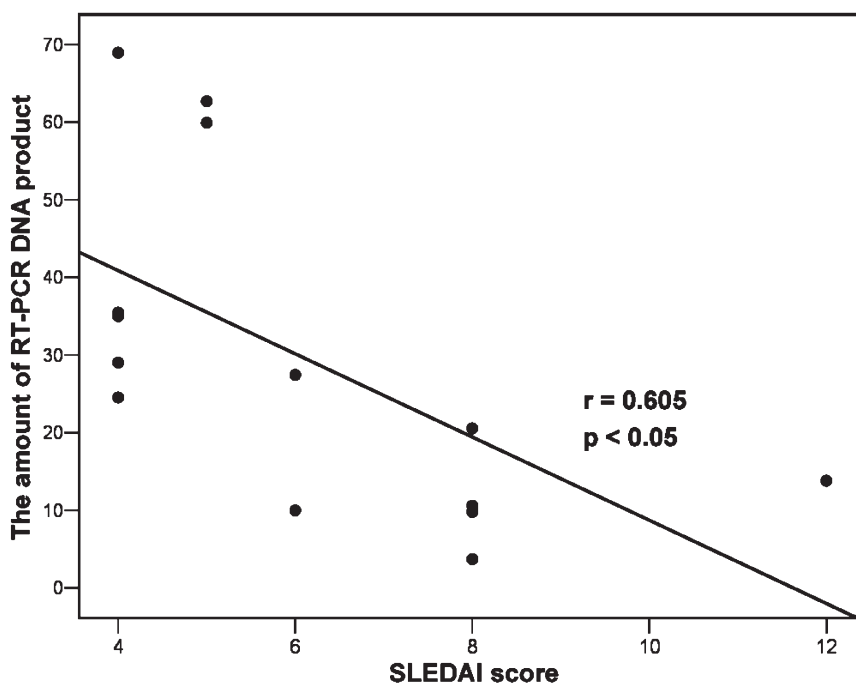


Figure 4. The correlation between the SLEDAI scores and the quantities of FOXO1 transcripts in PBMCs of SLE patients with active disease. The amounts of RT-PCR DNA products amplified from FOXO1 transcripts in PBMCs of SLE patients with active disease were quantified and simple linear regression analysis was used to test their correlation with the SLEDAI scores.

phenotype in FOXO4-deficient mice (21). However, further study on the transcript and protein levels of each FOXO member in different immune cell subpopulations may be needed to elucidate the relative roles of each FOXO protein in controlling human immune functions, although it has been reported that FOXO1 is the dominant FOXO member, with very low levels of FOXO3 and FOXO4 occurring in human peripheral blood T cells (16).

The important role of FOXOs in controlling lymphocyte activation has prompted us to hypothesize that the FOXO gene expression is dysregulated in SLE and RA patients. In this study, we examined this hypothesis by comparing FOXO1, FOXO3a, and FOXO4 transcript levels in PBMCs from healthy individuals with those in PBMCs from SLE and RA patients, and the results showed that the transcript levels of FOXO1, but not FOXO3a or FOXO4, in SLE and RA patients are significantly different from those in healthy individuals. The FOXO1

transcript levels in PBMCs appear to be down-regulated both in SLE patients and in RA patients. In SLE patients, the FOXO1 transcript levels in PBMCs are even negatively correlated with the lupus disease activity in SLE patients. Our data suggest that although FOXO activity appears to be mainly controlled at the protein level by the nuclear export and degradation of FOXO factors, FOXO1 activity can be regulated at the transcript level to control cell activation. The down-regulation of FOXO1 protein levels in PBMCs is also evidenced in SLE and RA patients, and therefore these data suggest the possibility that the down-regulation of FOXO1 expression may be involved in the reduction of the lymphocyte activation threshold and contribute to the development of SLE and RA autoimmune diseases with lymphocyte over-activation. However, our results do not exclude the possibility that FOXO factors influence the development of SLE and RA at the protein level. In addition, we

simply analyzed the FOXOs transcript levels in PBMCs in this study, and therefore it remains to be seen whether the expression of FOXOs in different immune cell populations is dysregulated in SLE and RA patients, as shown in lupus-prone mice, in which FOXO activity is down-regulated in helper T cells (24).

The mechanism that mediates the down-regulation of FOXO1 transcript levels in PBMCs of SLE and RA patients remains to be clarified. However, because it has recently been shown in mouse B cells that BCR signaling down-regulates FOXO1 transcriptional expression (32), it is possible that FOXO1 transcripts are down-regulated in lymphocytes after activation and therefore are found to be decreased in PBMCs of RA and SLE patients with enhanced lymphocyte activation. In addition, although TNF- α has been reported to activate the FOXO1 activity in human fibroblasts (33), our results suggest that the transcript levels of FOXO1 as well as FOXO3a and FOXO4 in PBMCs are not influenced by TNF- α in RA patients. The lack of the reversal of FOXO1 transcript down-regulation in RA patients receiving anti-TNF- α therapy also suggests that the down-regulation of the FOXO1 transcript levels is not likely to be related to joint inflammation.

Taken together, our results provide the first evidence that FOXO1 gene expression in PBMCs is down-regulated in SLE and RA patients, suggesting that the regulation of FOXO1 at the transcript level may be linked to the pathogenesis of SLE and RA. Thus FOXO1 transcript levels in PBMCs may serve as a biomarker of lupus disease activity in SLE patients. Further investigation into the expression patterns of FOXO factors in different immune cell subpopulations may improve our insight into the role of different FOXO members in the pathogenesis of SLE and RA.

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