Small Interfering RNA Targeting α-Fodrin Suppressing the Immune Response of Sjögren's Syndrome Mice

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To the Editor: Sjögren's syndrome (SS) is a multisystem chronic autoimmune disease, and mainly affects the lacrimal and salivary glands, leading to ocular and oral dryness. SS is characterized by a large quantity of autoantibodies and involves multiple systems including the lymphatic system, blood, lungs, kidneys, liver, muscles, and joints. Late complications include interstitial pulmonary fibrosis, peripheral neuropathy, tracheobronchial sicca, blindness, arthralgia, oral candidiasis, and lymphoma. The main histopathological feature of SS is lymphocytic infiltration of the exocrine glands, resulting in acinar cell death, probably through apoptosis.[1] The exact etiology and pathogenesis of SS are still not clear.

Nonobese diabetic (NOD) mice are the most common animal model used to investigate the disease properties of SS. In 1997, the α-fodrin antibodies were found in the salivary glands of a mouse model of SS. This indicated that anti-α-fodrin antibodies are potential biomarkers for SS diagnosis. A specific autoantibody against 120,000 α-fodrin was found in the early onset of autoimmune sialadenitis of NOD mice, and its production correlated closely with autoimmune sialadenitis. It indicated that the 120,000 α-fodrin molecule may be an important autoantigen in the pathogenesis of SS. Multiple subsets of $CD4^+$ Th cells regulate the process of adaptive immunity. Th cells are currently divided into three major subsets: Th1 cells, Th2 cells, and Th17 cells.[2] Interleukin (IL)-4 and IL-6 were secreted by Th2 cells. IL-17 was secreted by Th17 cells. Some reports have shown that the proportion of proinflammatory Th2 and Th17 cells, and the levels of cytokines, such as IL-4, IL-6, and IL-17, were abnormal in both animal models and patient samples of SS.[3] This study was to silence α-fodrin using gene-specific siRNA to investigate the role of α-fodrin in SS mice. In this study, a siRNA eukaryotic expression vector targeting the α-fodrin gene was constructed. Then, the siRNA vector was inoculated into the SS mice to investigate the therapeutic effect of silencing the α-fodrin gene in NOD mice.

This study was approved by the Ethics Committee of Baotou Medical College. All animal experiments complied with ARRIVE guidelines and were carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978). The siRNA targeting α-fodrin (location at positions 238–256 and 405–423) was designed according to complementary DNA (cDNA) sequence (SEQ ID NM001076554.2) using BLOCK-iT™ RNAi Designer (http://rnaidesigner.lifetechnologies.com/rnaiexpress/): siRNA1, CCCTTAGGCGTCAGAAGCT; siRNA2, GCTGAAGTGCAGGCCAACT. The siRNA was chemically synthesized and annealed to two double-stranded DNA, then digested with BamHI and Hind III. The digested double-stranded oligos were inserted into the downstream of U6 promoter in linearized pGFP-V-RS vector (Origene, USA). Recombination was confirmed using restrictive enzyme digestion and sequencing (Invitrogen, USA).

The NOD mice (28-week-old) were randomly divided into four groups to undergo tail-vein injection (6 in each group): the negative control (phosphate buffered saline) group, the siRNA control (pGFP-V-RS vector) group, α-fodrin siRNA1 group, and α-fodrin siRNA2 group. The 5 mg/kg vector was injected into NOD mice once a week for 2 weeks. Peripheral blood was collected by tail-vein after injection (on 1, 3, and 5 days). NOD mice were sacrificed after 2 weeks. The silencing efficiency was determined using real-time quantitative polymerase chain reaction (RT-qPCR). Subsequent experiments focused on siRNA targeting α-fodrin, which was identified as the most effective methods for inhibiting α-fodrin expression as previously described.

Total RNA was extracted from the peripheral blood using TRIzol® reagent (Takara Biomedical Technology Co., Ltd., Japan) in accordance with the manufacturer's instructions. The cDNA was synthesized through the reverse transcription reaction by RT reaction mix (Takara Biomedical Technology Co., Ltd.) and used for RT-qPCR. The primers of α-fodrin and β-actin were as follows: α-fodrin, sense, 5'-GACCGAATCCGTGGAGTTAT-3' and antisense, 5'-ACTCGCTCAGTTTCGCTCTT -3'; β-actin,

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sense, 5'-CGCGAGAAGATGACCCAGAT-3', and antisense, 5'-GCACTGTGTTGGCGTACAGG-3'. Specific primers for α-fodrin were designed using the Primer 5 software program (Palo Alto, CA, USA) and synthesized by Shanghai Sangon Biological Engineering Technology and Services Co. Ltd. The ELISA kits (R and D Systems Europe Co., Ltd. Dundee, UK) were obtained to analyze the levels of IL-4, IL-6, and IL-17 in the tail-vein blood of mice. The experiment was performed in accordance with the manufacturer's protocols. All experiments were performed in triplicate.

After euthanasia, whole lacrimal glands and lungs were surgically removed from each mouse and placed in 10% formalin for 24 h for hematoxylin and eosin staining according to standard methods. Stained sections were observed for structure and leukocyte infiltration of glands and lungs under a microscope. Paraffin-embedded sections were deparaffinized and then were incubated overnight at 4°C with anti-α-fodrin antibody. Goat anti-RABBIT IgG was used as the secondary antibody (Abcam PLC, Cambridge, UK). The intensity of the red fluorescence was observed by fluorescence microscope (Olympus, Japan). The results were analyzed by Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Maryland, US) and expressed by integral optical density/area.

The data were expressed as mean ± standard error (SE). Statistical evaluation was determined using repeated measures analysis of variance and pairwise comparison. The statistical analyses were performed using SPSS version 13.0 software (IMB Inc., Chicago, IL, US). A $P < 0.05$ was considered as statistically significant.

Under RT-qPCR, the expression of α-fodrin mRNA was found to be significantly lower in the α-fodrin siRNA1 group (0.48 ± 0.01) than those in the negative control (1.00 ± 0.00, $P < 0.010$) and siRNA control groups (0.97 ± 0.06, $P < 0.010$) in lung tissues of NOD mice; and the same trend could be found in the α-fodrin siRNA2 group (0.41 ± 0.06; all $P < 0.010$). The levels of α-fodrin mRNA were not significantly different between the negative control and siRNA control groups ($P = 0.980$) and between the α-fodrin siRNA1 and α-fodrin siRNA2 groups ($P = 0.891$; Figure 1a).

Under immunohistochemistry, the expression of α-fodrin protein was found to be significantly lower in the α-fodrin siRNA1 group (0.12 ± 0.03) than those in the negative control (1.40 ± 0.05, $P < 0.010$) and siRNA control groups (1.32 ± 0.05, $P < 0.010$) in lung tissues of NOD mice; and this trend was also found in the α-fodrin siRNA2 group (0.08 ± 0.06, all $P < 0.010$). The levels of α-fodrin protein were not significantly different between the negative control and siRNA control groups ($P = 0.980$) and between the α-fodrin siRNA1 and α-fodrin siRNA2 groups ($P = 0.940$; Figure 1b). In the negative control and siRNA control groups, the structure of alveolar was damaged and fused into bulla structure. A large number of lymphocytes also infiltrated the pulmonary interstitium. The level

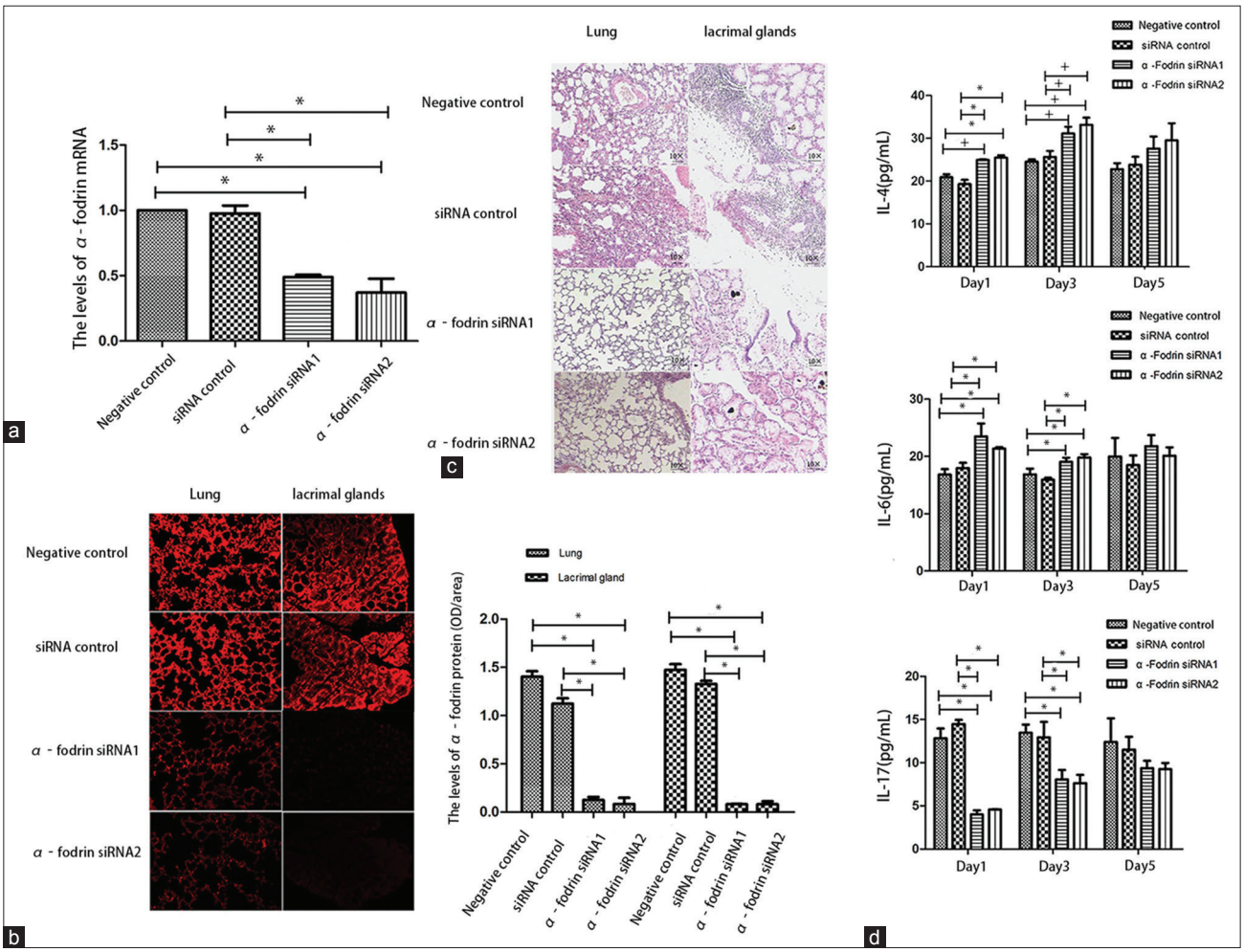

Figure 1: (a) Levels of α-fodrin mRNA in lung tissues of nonobese diabetic mice by real-time quantitative polymerase chain reaction. (b) Under immunohistochemistry, levels of α-fodrin protein in lung and lacrimal glands of nonobese diabetic mice. (c) Histological appearance of lung and lacrimal gland (HE staining, ×10). (d) Levels of inflammatory factors in nonobese diabetic mice. *$P < 0.01$, †$P < 0.05$.

of pathologic changes in siRNA control group was higher that of the negative control group. Pulmonary interstitium was lower in α-fodrin siRNA1 and α-fodrin siRNA2 groups, compared with the negative control and siRNA control groups. Pathologic changes of lacrimal gland in the negative control and siRNA control groups accorded with SS including cell arrangement, alveoli fusion, and lymphocyte infiltration. Lymphocyte infiltrations were significantly less pronounced in the lacrimal glands of α-fodrin siRNA1 and α-fodrin siRNA2 groups than those in the negative control and siRNA control groups [Figure 1c].

The levels of IL-4 were significantly higher after 1 and 3 days in α-fodrin siRNA1 and siRNA2 groups than in the negative control (day 1: 24.95 ± 0.13 vs. 20.93 ± 0.70, P = 0.013, and 25.49 ± 0.55 vs. 20.93 ± 0.70, P = 0.006; day 3: 31.10 ± 1.56 vs. 24.57 ± 0.50, P = 0.039, and 33.15 ± 1.68 vs. 24.57 ± 0.50, P = 0.010) and siRNA control groups (day 1: 24.95 ± 0.13 vs. 19.30 ± 1.0, P = 0.002 and 25.49 ± 0.55 vs. 19.30 ± 1.0, P = 0.001; day 3: 31.10 ± 1.56 vs. 24.64 ± 1.41, P = 0.040, and 33.15 ± 1.68 vs. 24.64 ± 1.41, P = 0.010); however, the differences were not significant after 5 days (P > 0.05; Figure 1d). The level of IL-6 was significantly higher after 1 and 3 days in α-fodrin siRNA1 and siRNA2 groups than in the negative control (day 1: 26.81 ± 0.90 vs. 16.80 ± 0.97, P < 0.010, and 27.67 ± 0.18 vs. 16.80 ± 0.97, P < 0.010; day 3: 19.67 ± 0.12 vs. 15.50 ± 0.21, P < 0.010, and 20.42 ± 0.11 vs. 15.50 ± 0.21, P < 0.010) and siRNA control groups (day 1: 26.81 ± 0.90 vs. 17.93 ± 0.92, P < 0.010, and 27.67 ± 0.18 vs. 17.93 ± 0.92, P = 0.00 < 0.010; day 3: 19.67 ± 0.12 vs. 15.90 ± 0.35, P < 0.010, and 20.42 ± 0.11 vs. 15.90 ± 0.35, P < 0.010); however, the differences were not significant after 5 days (P > 0.05; Figure 1d). The level of IL-17 was significantly lower after 1 and 3 days in α-fodrin siRNA1 and siRNA2 groups than in the negative control group (day 1: 4.00 ± 0.49 vs. 12.83 ± 1.12, P < 0.010, and 4.56 ± 0.03 vs. 12.83 ± 1.12, P < 0.010; day 3: 9.40 ± 0.26 vs. 15.80 ± 0.40, P < 0.010, and 8.63 ± 0.27 vs. 15.80 ± 0.40, P < 0.010) and siRNA control group (day 1: 4.00 ± 0.49 vs. 14.47 ± 0.92, P < 0.010, and 4.56 ± 0.03 vs. 14.47 ± 0.92, P < 0.010; day 3: 9.40 ± 0.26 vs. 16.27 ± 0.15, P < 0.010, and 8.63 ± 0.27 vs. 16.27 ± 0.15, P < 0.010); however, the differences were not significant after 5 days (P > 0.050; Figure 1d).

This study sought to determine whether α-fodrin might be involved in controlling the secretion of inflammatory factors in SS mice. By injecting α-fodrin siRNA into the tail vein of mice, it was possible to investigate whether there was any temporal relationship between the expression of α-fodrin and the levels of IL-4, IL-6, and IL-17 *in vivo*. Initial results showed that the levels of α-fodrin mRNA and protein were significantly reduced by siRNA targeting of *α-fodrin* in NOD mice, indicating that α-fodrin siRNA could inhibit the expression of α-fodrin and be used in the following experiments.

Reports showed that T cells predominated in the lesions. SS is characterized by T and B lymphocytes, with infiltration of exocrine glands. $CD4^+$ T cells seem to respond to autoantigens, such as α-fodrin, on apoptotic cells. Cytokines are crucial mediators of T cells, including Th2 and Th17 cells, which contribute to the pathology of SS. In this study, the levels of IL-4 and IL-6 secreted by Th2 cells were higher; however, the levels of IL-17 secreted by Th17 cells were lower in the α-fodrin siRNA1 and siRNA2 groups than in the negative control and the siRNA control groups. This indicated that the proportion of Th2 and Th17 was rendered abnormal by the control of α-fodrin. Th2 cells promote B cells to secrete IgG and IgE and cause immediate-type hypersensitivity reactions, jointly called humoral immunity. Th17 cells and Treg cells exist simultaneously in many tissues, and the dynamic balance between them may be closely related to the occurrence of an appropriate level of immune response in the body. Th17 cells cannot only induce Th17 cells to produce by secreting TGF-β. In the absence of exogenous TGF-β and the presence of IL-6, Treg cells themselves can also differentiate into Th17 cells.[4] In this study, results suggested that the levels of IL-4 and IL-6 were suppressed, and the level of IL-17 was promoted by α-fodrin in SS mice. Immune elimination could be the reason why the difference among all the groups was not significant 5 days after injection.

Currently, SS is present in 0.3–5.0% of the population. The clinical diagnosis of the SS is critical because there is an almost 44-fold risk of lymphoma related to this disease. Some reports showed that the diagnostic specificity and sensitivity of anti-α-fodrin antibodies for SS were 0.80–1.00 and 0.60–0.90, respectively. In October 2016, the American College of Rheumatology (ACR)/European League Against Rheumatism combined with ACR and AECG standard formulated the classification standard of primary SS. This standard is based on the weighted sum of 5 items, such as pathological lymphoid foci of labial glands, anti SSA/Ro antibody, corneal staining, Schirmer test, and natural saliva flow rate. In this study, the levels of inflammatory cell infiltration in the lung and the lacrimal gland of the NOD mice were lower after injection of α-fodrin siRNA1 and siRNA2. This indicated that α-fodrin could promote inflammatory cell infiltration in SS mice model. Anti-α-fodrin IgG and IgA antibodies were critical to diagnosing pSS.[5]

The results of this study underscored the complexity of the immune regulation mechanism by α-fodrin in the SS mice. Our ultimate goal was to understand the role of α-fodrin in regulating the levels of inflammatory factors in pathogeny of SS mice. Further studies are needed to confirm the mechanism, which may help us better understand the pathogenesis of SS.

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Conflicts of interest

There are no conflicts of interest.

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