

Adipose-derived stem cells postpone the progression of Sjögren's syndrome by upregulating the Hippo signaling pathway

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Abstract. The aim of the present study was to explore the effect and mechanism of action of adipose-derived stem cells (ADSCs) on Sjögren syndrome (SS) to develop novel and more effective methods for SS treatment. ADSCs, dexamethasone or normal saline was injected into the submandibular gland (SMG) of three 12-week-old non-obese diabetic (NOD) mice. The degree of lymphocyte infiltration was considered as a criterion for judging disease progression, hematoxylin and eosin staining was performed to observe the pathological state, and the expression levels of TAZ, E-cadherin and α -catenin were assessed by western blotting. ADSC transplantation triggered an inhibitory effect on the progression of SS, which was slightly stronger compared with that of dexamethasone treatment. This was found to be related to the Hippo signaling pathway. In addition, TAZ protein expression levels decreased gradually with the progression of the disease; immunofluorescence staining showed that the expression of E-cadherin and TAZ followed similar trends. Notably, the expression of TAZ, p-TAZ, E-cadherin and α -catenin in NOD mice were lower compared with that in Control mice. Similarly, the ratio of p-TAZ/TAZ also decreased, which means that the activation level of Hippo signal pathway decreased. The results suggest that ADSCs may exert a therapeutic effect against SS and may postpone its progression by upregulating the Hippo signaling pathway.

Introduction

Sjögren's syndrome (SS) is a complex chronic inflammatory autoimmune disease. Its etiology is known to be related to the production of autoantibodies, but the specific details remain unclear (1). Previous studies have shown that the pathogenesis of SS is centered on abnormal accumulation of B lymphocytes (2,3). However, some studies have shown that T follicular helper cells, T helper (Th)17 and Th22 cells also serve an important role in the pathogenesis of SS (3). SS mainly affects the salivary and lacrimal glands, leading to dry mouth and eyes, as well as other exocrine glands and extraglandular organs, resulting in multisystem symptoms that ultimately lead to a declining quality of life for patients (4). The degree of lymphocyte infiltration of submandibular glands (SMGs) is often considered as an index to judge the progress of SS (5). Previous studies have shown that SS is also associated with an increased incidence of atherosclerosis, interstitial lung disease and even lymphoma (5-7). Currently, there is no effective treatment for SS. The most common clinical treatment methods are local substitution therapy and systemic immunotherapy (7). Nevertheless, glucocorticoids such as dexamethasone (DEX) have been considered effective therapeutic agents (8). At present, targeted therapy is thought to delay the progression of SS (9). For example, Gandolfo *et al* (10) suggest that targeting B-lymphocyte activating factor can delay the progression of SS. Similarly, de Vita *et al* (11) suggest that sequential treatment with belimumab to inhibit the proliferation of B lymphocytes may also be an effective treatment. However, at present, no drug is considered effective against SS in the long term. Therefore, it is important to develop novel therapies for SS treatment.

Adipose-derived stem cells (ADSCs) are stem cells capable of multidirectional differentiation and are produced by adipose tissue. They have self-renewal ability and can secrete a variety of cytokines. Therefore, ADSCs are thought to serve specific roles in the onset and progression of some diseases, such as the occurrence and development of breast cancer, cervical cancer and other types of tumors (12,13), as well as of a variety of autoimmune diseases. At present, the transplantation of ADSCs for the treatment of autoimmune diseases is an important topic in academic circles. For example, Zhang *et al* (14) observe that transplantation of ADSCs can improve the balance between

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Th17 and regulatory T cells to support the treatment of lupus nephritis. Another study reported that ADSCs can induce the downregulation of IL-17 expression, thereby delaying the progression of systemic lupus erythematosus (SLE) (15).

The Hippo signaling pathway is a core pathway that regulates organ size, cell proliferation and differentiation. This signaling pathway is thought to be also related to the occurrence and development of a number of autoimmune diseases (16). In fact, a previous study suggests that tRNA-derived small RNA-21109 can inhibit the Hippo signaling pathway and thus polarization of M1 macrophages, thereby delaying SLE progression (17). Another study shows that the inhibition of the Hippo signaling pathway can inhibit TGF- β accumulation and thus delay the progression of rheumatoid arthritis (18). In addition, Enger *et al.* (19) note that the Hippo signaling pathway is necessary for the development of the SMG, and its imbalance is related to the occurrence of Sjögren's syndrome. Transcriptional coactivator with PDZ-binding motif (TAZ), Yes-associated protein (YAP) and α -catenin are three proteins involved in the Hippo signaling pathway (16). Another study reported that altered expression of these proteins may be related to the progression and severity of SS (17). E-cadherin can affect the changes of cell adhesion; E-cadherin-mediated cell adhesion has been shown to influence the activity of the Hippo pathway (18,19). However, the possible role of ADSCs in delaying the progression of SS and their mechanism of action have not yet been examined. Therefore, the present study investigated the effect and mechanism of action of ADSCs on SS in order to assess whether ADSC transplantation can help delay the progression of SS, with the final aim to contribute to the development of novel methods for SS treatment.

Materials and methods

Ethics approval and consent to participate. Experiments were performed under a project license (approval no. 20201002) granted by the Institutional Ethics Board of Stomatological Hospital of Shandong University (Shandong, China), in compliance with Chinese national or institutional guidelines for the care and use of animals.

Mouse model of SS. All animal experiments were performed according to the guidelines provided by the National Institutes of Health Guide for the Care and Use of Laboratory Animals (15) and the study was approved by the Animal Ethics Committee of Shandong University. A total of 75 female non-obese diabetic (NOD) mice (10-week-old) and 15 female Control mice (10-week-old) used in the study were purchased from Huafukang Biotechnology Co., Ltd. Control mice are normal BALB/c mice. The mice were housed in accordance with animal welfare regulations, under specific-pathogen-free conditions at 25°C, humidity of 50% and a 12-h light/dark cycle. All NOD mice had free access to food and water. The mice were sacrificed by cervical dislocation. Inflammatory cells could be detected in the exocrine glands of NOD mice of ~12 weeks of age and obvious exocrine dysfunction appeared at ~16 weeks; therefore, these mice were used as the main model to study SS progression, as previously described (20). NOD mice were randomly divided into three groups (n=15 NOD mice/group): i) ADSC group; ii) DEX group and iii) untreated NOD Control

group. A total of 1×10^5 ADSCs (Guangzhou Saliat Stem Cell Science and Technology Co., Ltd.) suspended at 200 μ l PBS and injected into the SMG of NOD mice in the ADSC group. According to a previous study, ADSCs can influence immune responses and hence are key cell sources for tissue repair and regeneration (21). The DEX group NOD mice were injected with 200 μ l 0.1% DEX (MilliporeSigma) (8). Each Control group NOD mouse was injected with 200 μ l PBS solution.

Collection of tissue. In preliminary experiments, lymphocyte infiltration in the SMGs of NOD mice appeared at ~13 weeks of age. Therefore, when the mice reached 12 weeks of age, ADSCs (5×10^5 cells/ml; 0.2 ml/mouse), 0.1% DEX (4.125-8.25 mg/kg) and PBS were injected into the SMGs of ADSC, DEX and NOD Control group mice, respectively. A total of three mice in each group were sacrificed every week between 13 and 17 weeks of age. All animal experiments were repeated three times (8). After sacrificing the mice, SMGs were collected and SMG samples were fixed in 4% formalin at 37°C for 72 h. The fixed tissues were then dehydrated in a series of graded ethanol solutions, immersed in xylene and embedded in paraffin. Sections were cut at a thickness of 3-4 μ m using a sliding microtome and then kept at 37°C overnight.

Hematoxylin and eosin (H&E) staining. The sections were stained with hematoxylin at room temperature for 5 min, 1% HCl-alcohol differentiation for 5-30 sec and then with eosin staining solution for 5 min at room temperature. The sections were dehydrated with 95% alcohol for 5 min at room temperature, then cleared with xylene for 5 min and finally sealed with neutral balsam. The tissue sections were subsequently visualized under a light microscope at a magnification of x20 and x200 (Leica Microsystems GmbH). All parameters were assessed by a pathologist (Mr. Guimiao Xing; School and Hospital of Stomatology, Cheeloo College of Medicine; Shandong, China). Lymphocyte aggregates (LAs) were defined as sharp-edged dense groups of at least 50 lymphocytes; the size of these LAs was irrelevant. Each section was prepared as a 1x1 mm square to ensure the consistency of the total observation area and the number of LAs was detected on this basis. The amount of LA represents the degree of lymphocyte infiltration (8,19,20).

Antibodies and vectors. Anti-E-cadherin (cat. no. 564186; BD Transduction Laboratories; BD Biosciences), anti- α -catenin (cat. no. 610193; BD Transduction Laboratories; BD Biosciences), anti-TAZ (cat. no. ab224239; Abcam), anti-p-TAZ (cat. no. 59971; CST) and anti-GAPDH (cat. no. ab8245; Abcam) antibodies, as well as DAPI (cat. no. ab285390; Abcam) were used in western blotting and immunofluorescence staining assays.

The lentiviral vector (V0009) containing short hairpin (sh)RNAs targeting human TAZ (sh-TAZ) or a non-targeting oligonucleotide was bought from Wuhan Miaolingbio Co., Ltd. The target sequence for sh-TAZ was 5'-AGGTAATCTCAATCACA-3' and the target sequence for the negative control shRNA (sh-NC) was 5'-GAGAACTATCTCATAACC A-3'. Briefly, the lentiviral constructs for sh-TAZ and sh-NC were constructed into pLV3-U6-TAZ(human)-shRNA1-EF1a-turboRFP-Puro (cat. no. P37411; Wuhan Miaolingbio Co., Ltd.).

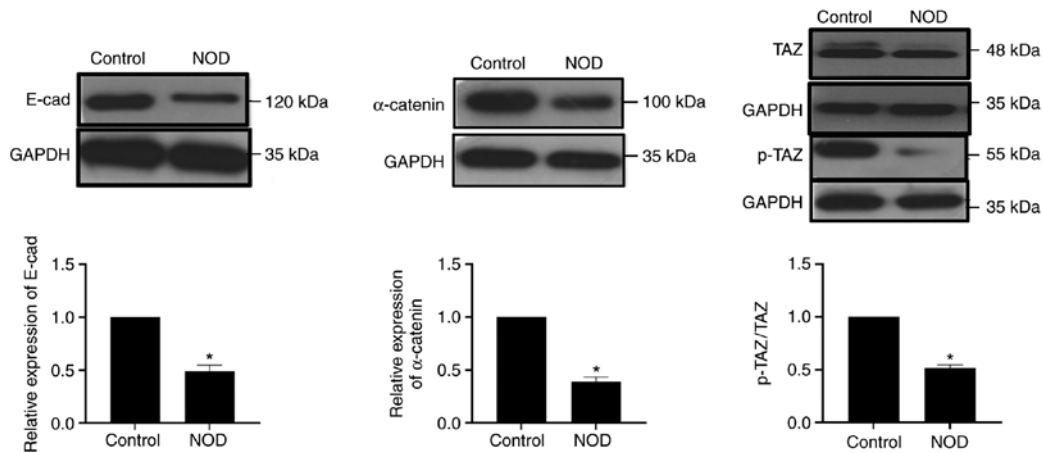


Figure 1. Sjögren's syndrome occurrence is accompanied by a downregulation of the Hippo signaling pathway. Western blotting analysis showed that the protein expression levels of E-cadherin, α -catenin, p-TAZ and TAZ were higher than those of SMGs in 17-week-old NOD mice. * $P < 0.05$ vs. Control. E-cad, E-cadherin; NOD, non-obese diabetic; p-, phosphorylated; TAZ, transcriptional coactivator with PDZ-binding motif.

All the plasmids were co-transfected with pHelper 1.0 (20 μ g) and pHelper 2.0 (10 μ g) into 293T cells (National Collection of Authenticated Cell Cultures). The knockdown efficiency was confirmed by western blotting analysis. A total of 1 μ g (50 pmol) shRNA was combined with serum-free diluent to a final volume of 25 μ l. shRNA plasmids (4 μ g) were co-transfected with the packaging vectors using TransLipid Transfection Reagent (Beijing TransGen Biotech Co., Ltd.) in accordance with the manufacturer's recommendations. Cells were cultured at 37°C in a 5% CO₂ incubator for 12 h, after which, the culture medium containing infection mixture was removed and replaced with complete culture medium. After 48 h incubation, the cell supernatant rich in lentiviral particles was collected and centrifuged for 10 min at 4°C and 3,000 \times g; then the supernatant was filtered with 0.45 μ m PVDF membrane and stored separately at -80°C. At 15 weeks, the sh-TAZ lentiviral particles (0.2 ml/mouse; effective titer, 5 \times 10⁹ TU/ml) were subsequently injected into the tail vein of NOD mice (n=15) to construct a TAZ-knockdown NOD mouse model; the sh-NC viral particles (0.2 ml/mouse; effective titer, 5 \times 10⁹ TU/ml) were injected into the tail vein of NOD mice (n=15); 0.2 ml/mouse PBS was injected into the Control group (n=15 NOD mice). ADSCs were injected into SMGs of the shRNA-treated mice at 13 weeks; ADSCs were not injected into Control group mice, which received PBS. At 17 weeks, the mice were killed and SMG tissues were collected.

Immunofluorescence staining. For immunofluorescence analysis, 3 \times 10⁵ SMG acinar cells were seeded on 6-well glass slides for 24–48 h, fixed with 4% paraformaldehyde for 15 min at 25°C and then washed with PBS. After blocking with 10% normal goat serum (WGAR1009-5 Wuhan Servicebio Technology Co., Ltd.) 37°C for 48 h, the cells were incubated overnight with the primary antibodies against TAZ and E-cadherin (each 1:1,000) at 4°C. Then, the slides were washed with PBS and incubated with FITC-conjugated goat anti-rabbit IgG (1:1,000; cat. no. ab6717; Abcam) and FITC-conjugated goat anti-mouse IgG (1:1,000; cat. no. ab6785; Abcam) for 1 h at room temperature. Thereafter, the slides were washed with PBS, stained with DAPI and examined under a fluorescence microscope (Olympus Corporation). ImageJ software

v1.8.0.112 (National Institutes of Health) was used to analysis the average intensity of expression.

Western blotting. The SMG lysates of were extracted using RIPA lysis buffer. The homogenates were centrifuged at 12,000 \times g for 15 min at 4°C, and the protein concentrations were determined using a BCA kit (Thermo Fisher Scientific, Inc.). Proteins (30 μ g) were separated by 10% SDS-PAGE, transferred to PVDF membranes (MilliporeSigma) and blocked in 5% non-fat milk at 25°C for 1.5 h. The membranes subsequently were incubated at 4°C overnight with rabbit E-cadherin, p-TAZ, TAZ, α -catenin and GAPDH (1:1,000 each). Following three washes with TBS +0.1% Tween-20, the membranes were incubated with HRP-conjugated goat anti-rabbit and goat anti-mouse secondary antibodies (1:20,000; cat. nos. G1213-100UL and G1214-100UL, respectively; Wuhan Servicebio Technology Co., Ltd.) for 2 h at room temperature and developed with ECL Reagent (MilliporeSigma). Densitometric analysis was conducted using ImageJ software (version 1.44p; National Institutes of Health).

Statistical analysis. All statistical analyses were performed using GraphPad Prism 8.0 software (GraphPad Software, Inc.). Two-tailed unpaired t-tests were used to analyze two groups. One-way analysis of variance followed by Tukey's test was used to analyze multiple groups. All results are presented as mean \pm SEM. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

SS occurrence is accompanied by downregulation of the Hippo signaling pathway. To explore the relationship between the Hippo signaling pathway and SS occurrence, SMG tissues were collected from NOD mice and normal BALB/c Control mice of the same age (17 weeks) and proteins extracted for quantification. As expected, the expression of TAZ, p-TAZ, E-cadherin and α -catenin in NOD mice was lower compared with that in Control mice. Similarly, the ratio of p-TAZ/TAZ also decreased (Fig. 1). The above experiments demonstrated

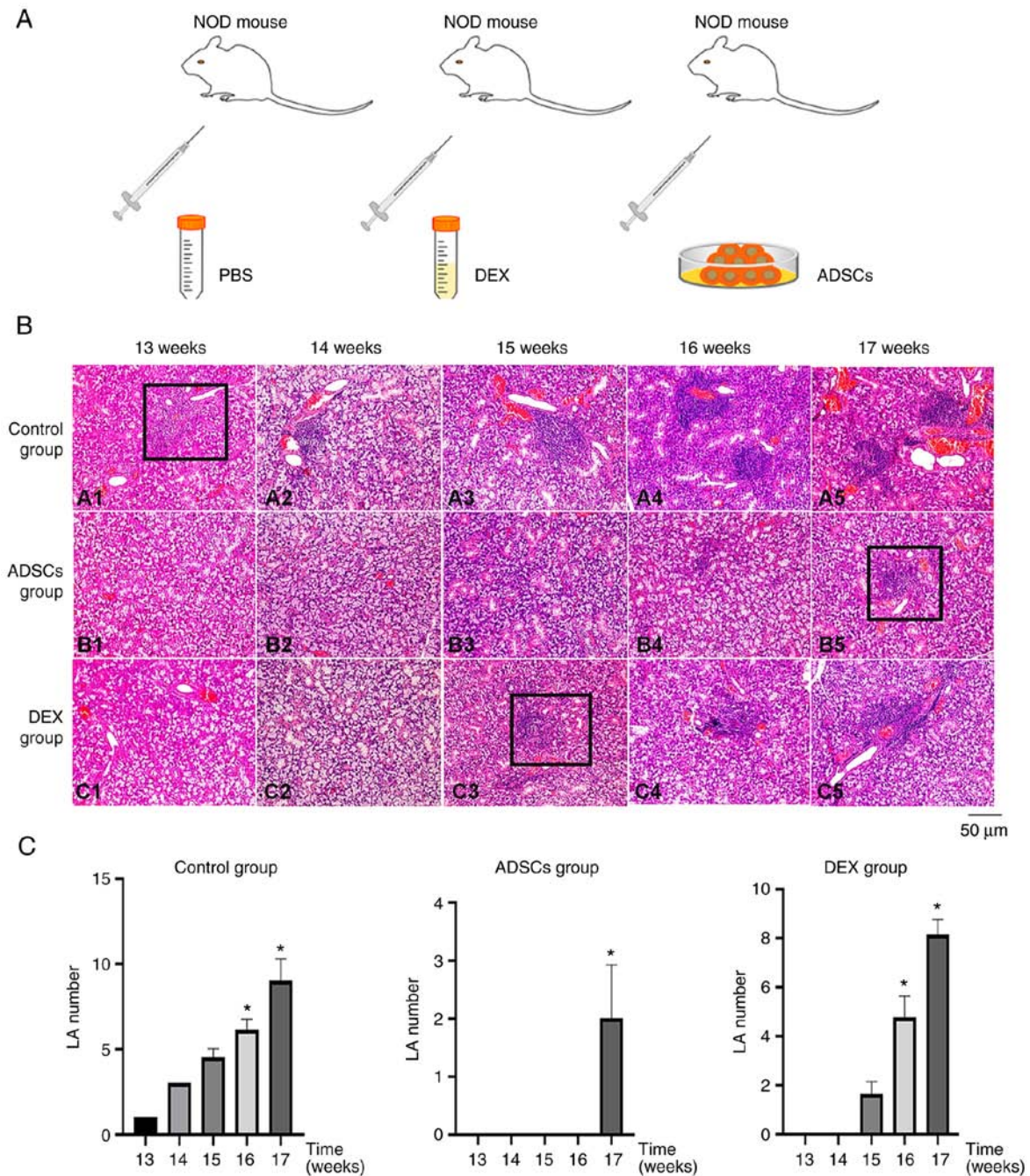


Figure 2. ADSCs may delay the infiltration of lymphocytes and thus Sjögren's syndrome progression. (A) NOD mice were randomly divided into Control (PBS-), DEX- and ADSC-treatment groups (n=15 mice/group). (B) Hematoxylin and eosin staining showed that, between 13 and 17 weeks, the degree of lymphocyte infiltration in SMGs of NOD mice in the ADSC group (B1-B5) was significantly lower compared with the Control group (A1-A5), whereas the degree of lymphocyte infiltration in SMGs of NOD mice in the DEX group (C1-C5) was between the two. (C) At the same time, the number of LAs in the different groups was detected (black rectangles in B) and counted. *P<0.05 vs. 13 weeks. ADSCs, adipose-derived stem cells; DEX, dexamethasone; LA, lymphocyte aggregate; NOD, non-obese diabetic; SMG, submandibular gland.

that, along SS occurrence, the Hippo signaling pathway was inhibited and the expression of its key proteins decreased.

ADSCs postpone the infiltration of lymphocytes and thus SS progression. To verify whether ADSCs exert a therapeutic effect on SS, NOD mice were randomly divided into three groups (Fig. 2A). H&E staining revealed that the SMGs of the NOD Control (PBS-), DEX- and ADSC-treated groups showed obvious lymphocyte infiltration at 13, 14, 15, 16 and 17 weeks of age (Fig. 2B). To clarify the results, the number of LAs were

detected and counted (Fig. 2C). These results suggested that ADSC injection may postpone the infiltration of lymphocytes and the progression of SS, with a stronger effect compared with that of DEX treatment.

ADSCs postpone the progression of SS by upregulating the Hippo signaling pathway. In previous experiments, it was found that ADSCs could postpone the progression of SS (21); however, their molecular mechanism of action is not clear. Therefore, the present study explored the mechanism by which

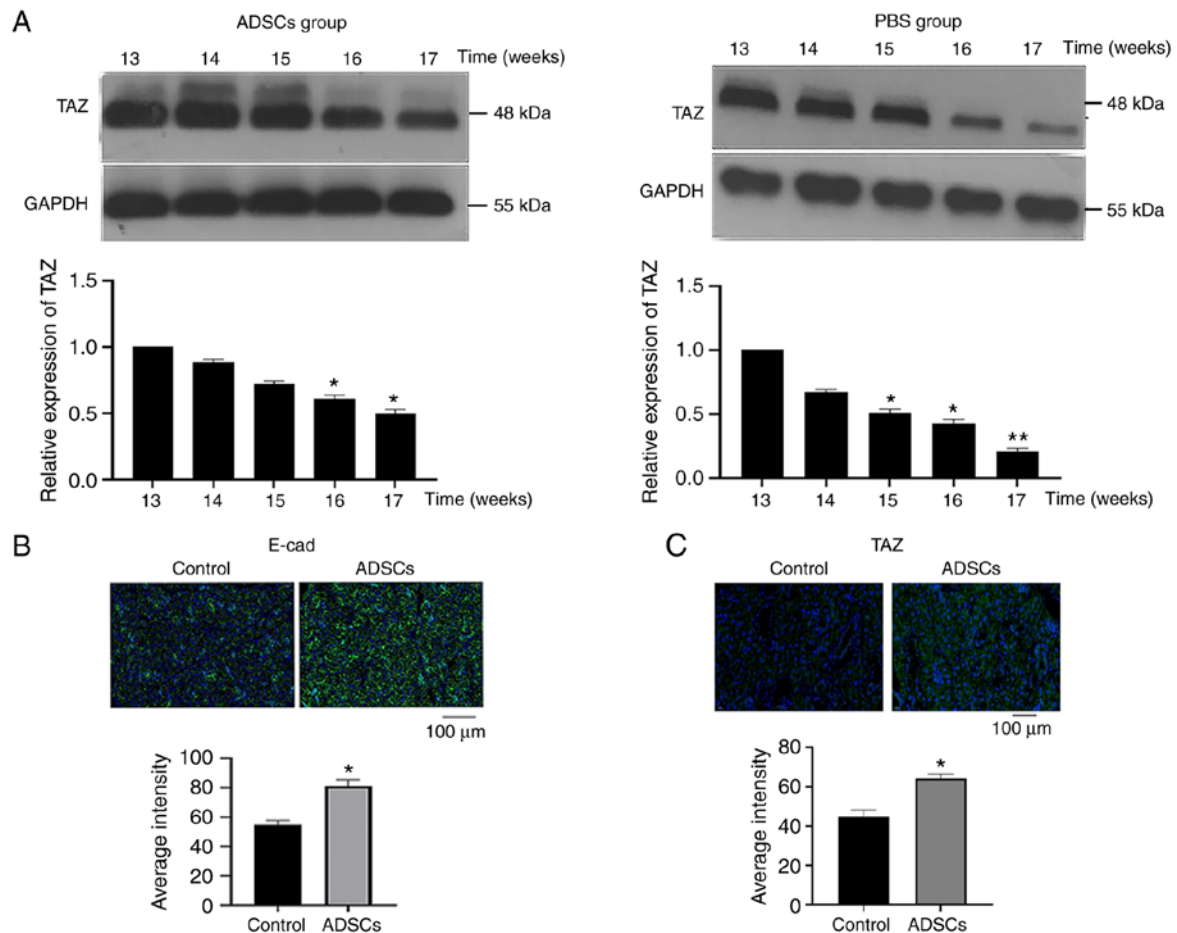


Figure 3. ADSCs delay the progression of Sjögren's syndrome by upregulating the Hippo signaling pathway. (A) Western blotting analysis showed that the protein expression levels of TAZ in the ADSC group and Control group decreased gradually between 13 and 17 weeks, and that the downward trend of TAZ in the ADSC group was slower compared with the Control group. (B) Immunofluorescence staining showed that the expression of E-cad (green) in SMGs from mice in the ADSC group was significantly higher compared with that in the Control group. DAPI (blue) was used to stain the nucleus. (C) Immunofluorescence showed that the expression of TAZ in SMGs of ADSC group was significantly higher than that of the Control group. DAPI (blue) was used to stain the nucleus. * $P < 0.05$, ** $P < 0.01$. ADSCs, adipose-derived stem cells; E-cad, E-cadherin; SMG, submandibular gland; TAZ, transcriptional coactivator with PDZ-binding motif.

ADSCs may trigger such postponement using western blotting and immunofluorescence. First, altered TAZ protein expression was observed in the SMGs of mice following transplantation of ADSCs. In fact, compared with the results shown in the NOD Control group, the decline of TAZ expression in SMGs was postponed following ADSC treatment (Fig. 3A). Next, the expression of E-cadherin and TAZ was assessed in the SMGs of NOD mice treated with ADSCs and of the NOD Control group by immunofluorescence. Moreover, at 17 weeks, ADSC treatment was found to increase the expression of E-cadherin and TAZ compared with the Control group (Fig. 3B and C).

Knocking down the expression of TAZ may reduce the postponing effect of ADSCs on SS. As aforementioned, the expression of TAZ and E-cadherin decreased during the pathogenesis of SS, whereas ADSC treatment seemed to postpone the progression of SS by increasing the expression of TAZ and E-cad. To further support these results, a gene knockdown experiment was performed by injecting shRNA lentiviral particles targeting TAZ into the caudal vein of NOD mice (with ADSCs treated) at week 15 (Fig. 4A). After the mouse SMG tissue was removed at week 17, the protein expression levels were detected; the western blotting results verified that

the expression of TAZ was knocked down (Fig. 4B). Then, the SMGs of NOD mice were stained to determine the progress of SS by estimating the degree of lymphocyte infiltration by counting LAs (Fig. 4C). The results suggested that the effect of ADSCs was weakened after knocking down TAZ, but it was still stronger compared with that of the sh-NC group. TAZ is an important protein in Hippo signaling pathway; the above results suggested that ADSCs may delay the progression of SS by upregulating the activity of Hippo signaling pathway.

Discussion

SS is a complex autoimmune disease that mainly causes dry mouth and dry eyes. At present, its etiology has not been fully elucidated and there is no effective treatment for this condition (22). Therefore, exploring SS pathogenesis is required to develop new methods for SS treatment.

The Hippo signaling pathway is a core pathway that regulates organ size, cell proliferation and differentiation (23). In addition, the effector protein TAZ of the catenin-related Hippo signaling pathway becomes increasingly phosphorylated, consistent with the activation of the Hippo signaling pathway (24). Another previous study reported that

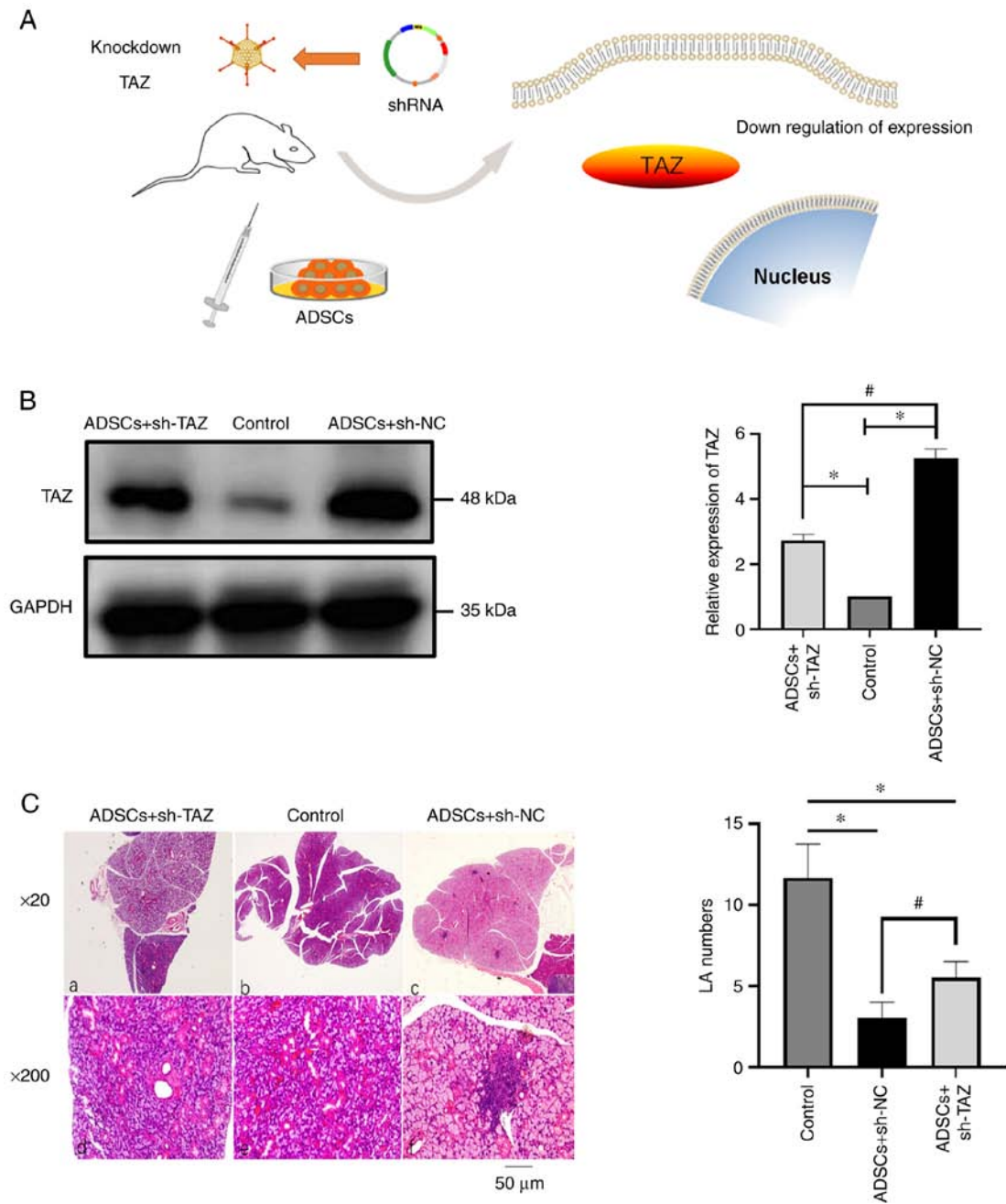


Figure 4. Knocking down the expression of TAZ reduces the postponing effect of ADSCs on SS. (A) shRNA lentivirus vector targeting TAZ was injected into tail vein of NOD mice (with ADSCs treated). (B) The expression of TAZ decreased after lentivirus transfection in SMGs in mice. * $P < 0.05$, # $P < 0.05$ vs. sh-NC. (C) Knocking down TAZ reduced the delaying effect of ADSCs on SS, but this effect is still stronger than the PBS-treated Control group. * $P < 0.05$; # $P < 0.05$. ADSCs, adipose-derived stem cells; NOD, non-obese diabetic; sh, short hairpin RNA; SMG, submandibular gland; SS, Sjögren's syndrome; TAZ, transcriptional coactivator with PDZ-binding motif.

E-cadherin-mediated cell adhesion can affect the activity of the Hippo signaling pathway (25). The present study results demonstrated that the progression of SS was accompanied by a decrease in the expression of TAZ and E-cadherin. Conversely, transplantation of ADSCs upregulated the Hippo signaling pathway and increased the expression of Hippo signaling-related proteins. The TAZ knockdown experiment suggested that TAZ may serve an important role in the process of ADSCs delaying the progression of SS. In addition, it has been previously noted that the expression of E-cadherin will change with the progression of autoimmune diseases (26,27). The reason for these phenomena may be the

different pathogenesis of the different autoimmune diseases. It was hypothesized that in addition to E-cad, there remained a number of proteins that served a role in this process, which will be the focus of future studies.

ADSCs have been considered good candidates for the treatment of various autoimmune diseases, such as multiple sclerosis, osteoarthritis, Crohn's disease and type 1 diabetes, against which they have shown a good therapeutic effect (28-32). A previous study suggested that transplantation of ADSCs can effectively alleviate dry eye symptoms caused by autoimmune factors (33). The present study found that ADSCs can reduce the infiltration of lymphocytes in the SMG,

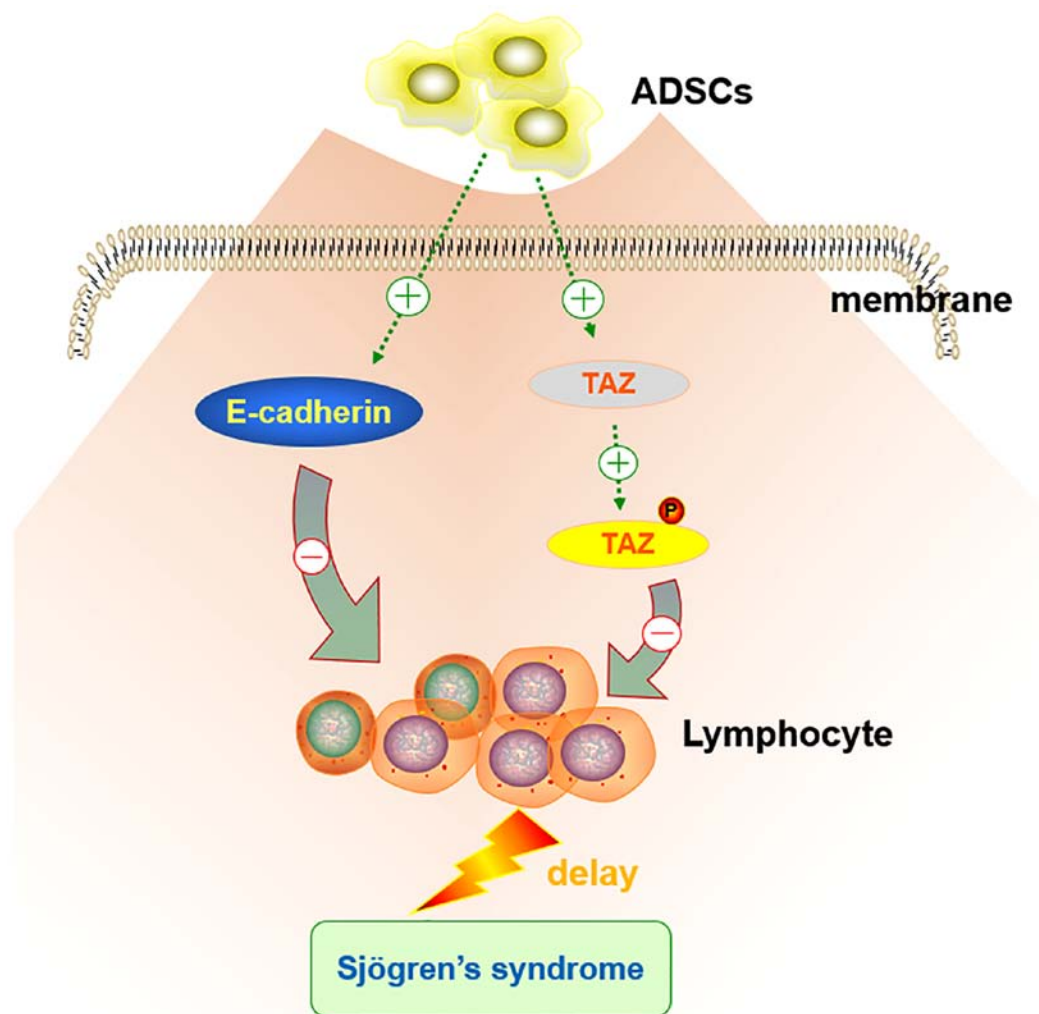


Figure 5. A brief schematic diagram of this study. NOD, non-obese diabetic; ADSCs, adipose-derived stem cells; E-cad, E-cadherin; TAZ, transcriptional coactivator with PDZ-binding motif.

thus ADSCs were considered to delay the progression of SS. The mechanism of this effect of ADSCs may be to increase the expression of E-cad and TAZ. It has been proposed that the expression of E-cadherin is negatively correlated with the activation level of Hippo signaling pathway (34). This is contrary to the results of the present study. The changes in protein expression as the disease progresses may be a potential explanation; but the reason for this difference will be one of the focuses of future research. In fact, whether SS or another autoimmune disease, treatment strategies are still the focus of modern research. ADSCs have been used in the treatment of a number of diseases, such as rheumatoid arthritis (35), but its detailed mechanism still needs to be explored. In different diseases, the mechanism of action of ADSCs is also different. Exploring these mechanisms is not only conducive to the development of new treatments, but also conducive to improving our better understanding of the pathogenesis of these diseases. Overall, the present study suggested that ADSCs may delay the progress of SS, which is accompanied by the upregulation of Hippo signaling pathway activation. Therefore, transplantation of ADSCs may be a potential method for the treatment of SS.

The present study had some limitations. For example, it did not explore how ADSCs upregulated the Hippo signaling pathway. In addition, it did not explore the adverse effects of its treatment. These limitations will be the focus of future research. Nevertheless, the present study provided new strategies for the treatment of SS. Future studies will continue to pursue the search for effective treatment methods for SS and will also explore other mechanisms by which ADSCs may delay SS progression.

In conclusion, ADSCs may delay the progression of SS, with a stronger effect compared with that of DEX; this therapeutic effect is mainly achieved through the upregulation of the Hippo signaling pathway (Fig. 5). However, our understanding of the role of ADSCs in SS remains limited. The study of cell-cell interactions between ADSCs and SS tissues may result in important developments of novel treatment strategies for SS, which should be the focus of future research.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZL and GL guided the project, analyzed the data and wrote the manuscript. XF and XX conceived the technical details and designed the experiments. ZL, QZ and GX performed the experiments. XF, GL and ZL confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Experiments were performed under a project license (approval no. 20201002) granted by Institutional Ethics Board of Stomatological Hospital of Shandong University (Shandong, China), in compliance with Chinese national or institutional guidelines for the care and use of animals. Informed consent was obtained from each patient upon their recruitment to the present study, which was approved by the Institutional Research Ethics Committee of School of Stomatology, Shandong University (approval no. 20201002).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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