MicroRNA-219c-5p May Participate in Bladder Fibrosis in Multiple Sclerosis Mice by Regulating Fibronectin-1

Bowen L[1,2], Yafei D[1], Peng L[1], Wang T[1], Siyuan H[1], Zhankui J[1,2], Chaohui G[1,2] and Jinjian Y[1,2]*

[1]Department of Urology, Zhengzhou University First Affiliated Hospital, China

[2]Zhengzhou Institute of Urology, Zhengzhou University First Affiliated Hospital, China

Abstract

Background: We found that the bladder of the multiple sclerosis mice was significantly fibrosis. This study aimed to investigate the relationship between Fibronectin-1 (*FN1*) and bladder fibrosis, as well as microRNAs involved in the regulation of *FN1*.

Methods: The degree of bladder smooth muscle fibrosis was observed by immunohistochemistry. And we used RT-qPCR (quantitative real-time polymerase chain reaction) and Western blot to identify the expression of *FN1* in different grades of fibrosis bladder. It was found by bio information website analysis that miR-1a-3p, miR-219c-5p and miR-3572-3p may prevent inhibition of *FN1* synthesis. Thus, miR-1a-3p, miR-219c-5p and miR-3572-3p were overexpressed or knocked down in bladder smooth muscle cells (BMSCs), and the respective transfection efficiency and *FN1* knockdown efficiency were detected by RT-qPCR. We discovered that only overexpression and knockdown of miR-219c-5p met the expected results. The dual luciferase reporter assay was used to determine the targeting relationship between miR-219c-5p and *FN1*. Flow cytometry and Cell Counting Kit 8 (CCK8) experiments confirmed that miR-219c-5p reduced *FN1* and affected the biological activity of smooth muscle cells.

Results: As bladder fibrosis worsens, the expression of *FN1* is raised, while the expression levels of miR-1a-3p, miR-219c-5p, and miR-3572-3P are decreased. The results of RT-qPCR after transfection showed that only miR-219c-5p was the best to regulate *FN1*. The results of the dual luciferase reporter gene indicated that miR-219c-5p is targeted to bind to *FN1*. CCK8 assay and cell cycle assay showed that overexpression of miR-219c-5p inhibited the proliferation of BMSCs, while knockdown of miR-219c-5p promoted the proliferation of BMSCs. Apoptosis assay showed that overexpression of miR-219c-5p promoted apoptosis, while knockdown of miR-219c-5p inhibited apoptosis of BMSCs.

Conclusion: Our findings indicate that up-regulation of *FN1* and down-regulation of miR-219c-5p play an important role in the development of bladder fibrosis. And miR-219c-5p may be involved in bladder fibrosis by targeting *FN1* expression.

Keywords: Multiple sclerosis; Experimental autoimmune encephalomyelitis; Bladder fibrosis; Fibronectin-1; microRNA-219c-5p

Abbreviations

MS: Multiple Sclerosis; EAE: Experimental Autoimmune Encephalomyelitis; BSMCs : Bladder Smooth Muscle Cells; ECM: Extracellular Matrix; FBS: Fetal Bovine Serum; *FN1*: Fibronectin-1; qPCR: Quantitative real-time PCR; α-SMA: α-Smooth Muscle Actin; CS: Clinical Score

Introduction

Multiple Sclerosis (MS) is a diffuse inflammatory autoimmune disease marked by a multiplicity of spatial and multiple symptoms of symptoms and signs [1,2]. It is currently believed that MS patients are mainly myelin-specific T cell-mediated autoimmune demyelination diseases. Demyelination of the urinary reflex nerve leads to inconsistency in the movement of the bladder detrusor and sphincter. Therefore, MS patients often have frequent urination, urgency and urinary retention [3-5]. So far, although many reports have investigated the possible mechanisms of the development of lower urinary tract symptoms in MS, the mechanism of bladder fibrosis in the ultimate outcome of bladder dysfunction has not been clearly elucidated [6,7]. The EAE model is currently the most commonly used animal model of MS. Myelin autoantigen specifically activates brain-assisted T cells,

causing inflammatory infiltration of the central nervous system and loss of myelin. Biochemical, immunological and pathological features are very close to MS, so they are widely used to assess the etiology of multiple sclerosis and to find different treatment options [8-10]. Bladder fibrosis is the ultimate outcome of bladder dysfunction [11,12]. Fibrosis is described as the "wrong wound healing process" and there may be many mechanisms involved in its development, but they are all based on the activation of bladder smooth muscle cells and the resulting Extracellular Matrix (ECM) components [13]. Wang et al. [14] first reports that microRNA-101b is induced by hypoxia and represses fibrosis of bladder smooth muscle cells by inhibiting the expression of TGFβR1 through TGF-β signaling pathway, and it may be an anti-fibrotic miRNA for therapy. Altuntas CZ et al. [7] first reports that bladder fibrosis and bladder remodeling corresponding to the severity of EAE may be due to increased expression of CTGF and increased connective tissue growth. Antifibrotic therapy may be an effective way to control bladder dysfunction, but it has not been well studied [15-17]. Fibronectin-1 is an extracellular macromolecular membrane protein present on the surface of animal cells and is the major non-collagen glycoprotein in the ECM and basement membrane. It is also very abundant in the ECM of various tumors including osteosarcoma, leiomyosarcoma, and gastric cancer [18]. Fibronectin-1, which belongs to the FN family, is involved in a variety of cellular biology processes and plays a role in diseases such as fibrosis [19-21]. We found that the more severe the bladder fibrosis, the more *FN1*. Then, if the synthesis of *FN1* can be inhibited, whether the progression of fibrosis can be alleviated or even retarded.

Numerous studies have shown that miRNAs are involved in many cellular biological processes such as cell invasion, cell migration, cell proliferation, apoptosis, and cell cycle. Therefore, miRNAs may be critical for the diagnosis and treatment of certain diseases [22]. Many miRNAs have been found in the brain spinal cord tissue of EAE mice as a biomarker for MS. Bruinsma et al. [23] report that miR-219 may be a candidate biomarker for MS diagnosis by detecting the loss of miR-219 in the patient's cerebrospinal fluid. MicroRNAs (miRNAs) are short-chain non-coding RNAs of approximately 22 nucleotides in length, whose primary function is tantamount to participate in biological metabolism by inducing mRNA degradation. Many miRNAs turned out to be involved in the development of fibrosis, including miR-26b-5p and miR-200c [24,25]. Notably, regulation of these miRNA expressions can prevent fibrogenesis *in vitro* and *in vivo*, suggesting the importance of miRNAs as potential targets for the treatment of fibrosis diseases [26]. Used Target Scan (http://www.targetscan.org), Miranda (http://www.mirdb.org) and miRBase (http://www.mirbase.org) for prediction and *FN1* Interacting microRNAs. After comparative analysis, it was found that miR-1a-3p, miR-219c-5p and miR-3572-3p all suggested a target for interaction with *FN1*.

The bladder wall is mainly composed of a mucosal layer, a submucosa, a muscle layer and an outer membrane. Among them, bladder fibrosis is mainly caused by the pathological proliferation of BMSCs in the muscular layer of the bladder wall. A considerably amount of matrix deposition in the cytoplasm of BMSCs affects bladder detrusor compliance and leads to bladder remodeling and fibrosis. Therefore, BSMCs were used as experimental models for our study. To investigate whether microRNAs can target the inhibition of *FN1* synthesis and block the progression of bladder fibrosis, we overexpressed and knocked down microRNAs in primary bladder smooth muscle cells (BMSCs) *in vitro*. The results of RT-qPCR after

transfection showed that only miR-219c-5p was the best to regulate *FN1*. In this study, we established an EAE model to simulate bladder fibrosis in MS patients and found that bladder fibrosis is directly proportional to *FN1*. Through *in vitro* experiments on bladder smooth muscle primary cells, we found that miR-219c-5p can inhibit the progression of bladder smooth muscle fibrosis by targeting *FN1*. This study aimed to investigate the significance of miR-219c-5p expression in bladder fibrosis *in vitro* and *in vivo*. In addition, this study determined that miR-219c-5p may directly target the *FN1* gene, and the miR-219 c-5p/*FN1* signaling pathway may contribute to the diagnosis and treatment of bladder fibrosis.

Materials and Methods

Animals

Forty female C57BL/6J mice (4 weeks to 9 weeks old) were purchased from The Charles River. All mice were kept in the SPF animal room of the Zhengzhou University Animal Experimental Center and exposed to a 12:12 hour light: dark cycle (light from 6 am to 6 pm) in the absence of specific pathogens. The experimental protocol was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University.

Experimental autoimmune encephalomyelitis

To induce chronic EAE, animals were injected subcutaneously with an emulsion of MOG 35-55 in complete Freund's adjuvant (CFA) (Sigma, USA). Then the pertussis toxin (List Biological Laboratories, Campbell, CA; 0.2 µg per animal) in PBS on days 0, 3, and 7 after immunization [7,27]. The mice were scored daily according to the following signs of nerve damage: 0: no disease; 1: tail weakness and/or moderately awkward gait and/or poor righting ability; 2: tail weakness and/or moderate awkward Gait and; 3: hind limb paralysis or mild forelimb weakness (or both); 4: limb paralysis; 5: quadriplegia with urine detained or dying. Another group of animals was injected subcutaneously with CFA emulsion and analyzed as a normal control group.

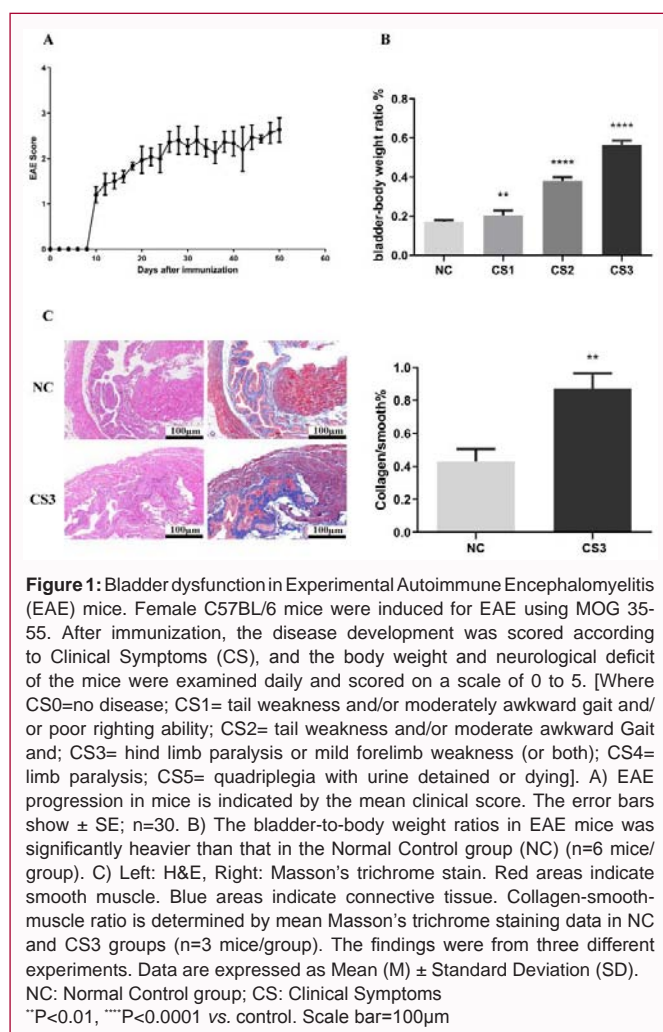### Prediction of potentially targeted miRNAs that inhibit *FN1*

Used Target Scan (http://www.targetscan.org), Miranda (http://www.mirdb.org) and miRBase (http://www.mirbase.org) for prediction and *FN1* Interacting microRNAs. After comparing Context++score, Context ++score percentile, Weighted context ++score, Conserved branch length, it was found that miR-1a-3p, miR-219c-5p and miR-3572-3p may interact with *FN1*.

Mice bladder smooth muscle cells (BSMCs) primary culture

The BMSCs were purchased from Otwo Biotech (HTX3129; Shenzhen, China) and verified by a-SMA immunofluorescence. The BMSCs were cultured in Dulbecco's Modified Eagle Medium - High Glucose (DMEM-H; Gibco, Thermo Fisher Scientific, Waltham, MA) containing 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, Waltham, MA) 100 U/ml cyan-streptomycin. Used α-SMA (MA1-06110; Thermo Fisher Scientific, Waltham, MA; 1:200) and Goat Anti-Rabbit IgG H&L (Alexa Fluor˚ 488) (ab150077; Abcam, USA; 1:1000) immunofluorescence staining to identify BSMCs. Cells with passage numbers 3 to 10 were used only for all experiments.

Overexpression or knockdown of miRNAs in BMSCs

One day before the transfection, the cells were starved, that is, the cells were seeded in a medium supplemented with 2% FBS at a density of 5×10^4 cells/ml. The day of transfection was changed to 10% FBS

Figure 1: Bladder dysfunction in Experimental Autoimmune Encephalomyelitis (EAE) mice. Female C57BL/6 mice were induced for EAE using MOG 35-55. After immunization, the disease development was scored according to Clinical Symptoms (CS), and the body weight and neurological deficit of the mice were examined daily and scored on a scale of 0 to 5. [Where CS0=no disease; CS1= tail weakness and/or moderately awkward gait and/or poor righting ability; CS2= tail weakness and/or moderate awkward Gait and; CS3= hind limb paralysis or mild forelimb weakness (or both); CS4= limb paralysis; CS5= quadriplegia with urine detained or dying]. A) EAE progression in mice is indicated by the mean clinical score. The error bars show ± SE; n=30. B) The bladder-to-body weight ratios in EAE mice was significantly heavier than that in the Normal Control group (NC) (n=6 mice/group). C) Left: H&E, Right: Masson's trichrome stain. Red areas indicate smooth muscle. Blue areas indicate connective tissue. Collagen-smooth-muscle ratio is determined by mean Masson's trichrome staining data in NC and CS3 groups (n=3 mice/group). The findings were from three different experiments. Data are expressed as Mean (M) ± Standard Deviation (SD). NC: Normal Control group; CS: Clinical Symptoms
**P<0.01, ****P<0.0001 *vs.* control. Scale bar=100µm

Table 1: List of primers.

Gene name	Primer sequences
β-actin	F-AAATGTGGCTGAGGACTTTGTAC
	R-GGACTTCCTGTAACCACTTATTTCA
U6	F-GCTCGCTTCGGCAGCACA
	R-GAGGTATTCGCACCAGAGGA
FN1	F-ACCCGTTTTCATCCAACAAGAG
	R-CGGTATCCAGACACCACACTATCA
miR-1a-3p	UAUGUAUGAAGAAAUGUAAGGU
miR-219c-5p	GCUCUCAACGCACCUGCAGG
miR-3572-3p	GACCCCUUUCUUCCUGUUCACAU

FN1: Fibronectin-1; F: Forward; R: Reverse

was subjected to reverse transcription reaction using ReverTra Ace qPCR RT Master Mix and gDNA Remover (FSQ-301; TOYOBO, Japan). Primers used are listed in Table 1. As described above, the cDNA obtained by reverse transcription was used for real-time PCR. qPCR was performed using a SYBR-Green mix kit (Applied Biosystems; Roche, Inc.) and a 20 µl reaction system at 95°C for 2 seconds, 60°C for 20 sec, and 70°C for 10 sec (40 cycles). Relative miRNA expression levels and other indicators were calculated in triplicates using the $2^{-\Delta\Delta CT}$ method. Expression levels were normalized to β-Actin or U6. The primer sequences are shown in Table 1.

Hematoxylin and Eosin (H&E) and Masson trichrome staining

Mouse bladders were soaked overnight with 4% paraformaldehyde. A 5 µm paraffin sections were made for analysis of bladder fibrosis. According to the protocol, paraffin sections were stained with Hematoxylin and Eosin (H&E) and Masson. The amount of collagen, the amount of smooth muscle cells, and the ratio of collagen to smooth muscle cells was calculated by using an image analysis system (Image J 1.80). The ratio of collagen to smooth muscle cells was used to quantify the degree of fibrosis in bladder tissue.

Immunohistochemistry

Immunohistochemical (IHC) staining of Streptavidin-Peroxidase (SP) was used to detect the expression of *FN1* in bladder tissue. After sectioning, conventional dewaxing, gradient ethanol hydration, and antigen retrieval were performed in sequence. Subsequently, it was incubated with 3% hydrogen peroxide for 20 min, serum blocked for 10 min, and primary antibody anti-rabbit anti-mouse *FN1* antibody (ab45688; Abcam, USA; 1:200) was incubated overnight at 4°C. They were then incubated with horseradish peroxidase-labeled goat anti-rabbit secondary antibody (ab205718; Abcam, USA; 1:2000) for 30 min and finally stained with DAB (Sigma, USA). After counterstaining with hematoxylin, all slides were hydrated, clarified, fixed and observed, and the cell positive index was counted.

Cell proliferation assay

Cell proliferation was tested using Cell Counting Kit 8 (CCK8) (CK04; DOJINDO, Japan; 10 µl). The BMSCs to be transfected were inoculated into 96-well plates. Each well was inoculated with about 1000 cells, and 6 wells were repeated for each group. After 24 h, 48 h, 72 h and 96 h of transfection, 10 µl of CCK8 was added to each well and incubated for 1 h before being tested on the machine. Sample absorbance was read at 450 nm on a microplate reader (SpectraMax-M5, Sunnyvale, CA).

medium and transfected with miRNA mimic, mimic NC, miRNA inhibitor and inhibitor NC (RIBOBIO, Guangzhou, China), using riboFECT™cP Transfection Kit (RIBOBIO, Guangzhou, China) according to the manufacturer's instructions.

miR-219c-5p target prediction and luciferase reporter assay

Potential miR-219c-5p binding sites were predicted using online database programs with different algorithms, including Target Scan (http://www.targetscan.org), Miranda (http://www.mirdb.org) and miRBase (http://www.mirbase.org). For the luciferase assay, BSMCs (3×10^3 cells/well) were first seeded in 96-well plates. Then, 1 day later, luciferase reporter plasmid [pmiR-*FN1*-Wild Type (WT) or pmiR-*FN1*-Mutant (Mut) (RIBOBIO, Guangzhou, China) was co-transfected with mir-219c-5p mimic or mimic NC to BMSCs. Luciferase activity was determined 48 h after transfection used the GLOMAX 96 spectrophotometer (Promega Corporation) depending on the manufacturer's protocol. Firefly luciferase activity was normalized *via* Renilla luciferase activity.

Quantitative real time polymerase chain reaction

Total RNA was isolated from bladder tissue or BSMCs using Trizol (Leagene Biotechnology, Beijing, China) according to the manufacturer's protocol. For RT-qPCR of miRNA, reverse transcription reaction was performed using ReverTra Ace qPCR RT Master Mix (FSQ-101; Japan TOYOBO). However, RT-PCR of *FN1*

Table 2: Bladder weight, *FN1* and miR-219c-5p expression in mice with different EAE scores.

Variable	EAE Score				F Value	P Value
	0	1	2	3		
Body weight	27.99 ± 0.56	27.91 ± 0.78	25.81 ± 1.05	25.68 ± 0.87	0.5882	0.646
Bladder weight to total body weight, %	0.17 ± 0.01	0.20 ± 0.02	0.38 ± 0.02	0.56 ± 0.02	1002	<0.0001
FN1	1.00 ± 0.10	1.23 ± 0.70	1.59 ± 0.14	3.01 ± 0.10	342.7	<0.0001
miR-1a-3p	3.40 ± 0.53	2.73 ± 0.25	1.93 ± 0.40	1.03 ± 0.15	23.6	<0.0001
miR-219c-5p	3.10 ± 0.10	1.73 ± 0.08	1.23 ± 0.06	1.00 ± 0.10	376.4	<0.0001
miR-3572-3p	4.50 ± 0.50	3.53 ± 0.55	2.03 ± 0.25	1.03 ± 0.15	44.6	<0.0002

Values are means ± SE; n=6 mice/group. P<0.05 by ANOVA among four groups; Expression levels were normalized to β-Actin or U6

Flow cytometry

After 48 h of transfection, cells were treated with 0.25% trypsin and cells were harvested. Approximately 1×10^5 cells were counted, fixed with 75% cold ethanol (Solarbio Life Sciences, Shanghai, China) for 4 h, and then treated with 0.5% Triton-X100 for 10 min. Triton-X100 was removed by centrifugation and stained with 200 μl Propidium Iodide (PI) (BD, USA) at room temperature for 30 min in the dark. A flow cytometer (ACCURI C6 PLUS, BD, USA) was used to analyze the cell cycle and the experiments were repeated three times.

Similarly, cells were treated with 0.25% trypsin without Ethylenediaminetetraacetic acid. About 1×10^5 cells were counted, and 10 μl of each of Annexin V-Fluorescein Isothiocyanate (FITC) (BD, USA) and PI (BD, USA) was added and incubated at room temperature for 15 min in the dark. The rate of apoptosis (percentage of apoptosis cells relative to the total number of cells) was estimated by flow cytometry. Representative results for three independent experiments are shown.

Western blot assay

An equal amount of each protein sample (20 μg) was separated using a 12% Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) at a voltage of 120V. The membrane was transferred to a Polypropylene Difluoride (PVDF) membrane by a constant flow of 400 mA for 5 h under ice bath conditions. The PVDF membrane was immersed for 30 min at room temperature using 5% skim milk powder for blocking non-specific bands. The PVDF membrane was incubated with a primary antibody, rabbit anti-mouse-*FN1* (ab45688; Abcam, USA; 1:10000), and β-Actin (AC026; ABclonal, Wuhan, China; 1:10000) at 4°C overnight. After washing once with TBS and 3 times with TBST, the PVDF membrane was incubated with goat anti-rabbit anti-IgG secondary antibody (AS003; ABclonal, Wuhan, China; 1:10000) conjugated to specific horseradish peroxidase for 1 hour at room temperature. The PVDF membrane was washed 5 times with TBST and then detected using an Enhanced Chemiluminescence (ECL) system. Grayscale analysis of protein bands was performed using Image-J software (National Institutes of Health, Bethesda, MA, USA) and normalized to the gray value of β-Actin. This experiment was replicated three times.

Statistical analysis

All data were from three independent replicates and are shown as Mean (M) ± Standard Deviation (SD). A comparison between the two sets of samples was performed using Student's t test. One-way ANOVA or two-way ANOVA was used for comparison between single factors or two factor multiple groups. P<0.05 was deemed to represent a statistically significant difference. Statistical analysis was performed using SPSS software (SPSS for Windows 21.0; SPSS Inc., Chicago, IL, USA).

Results

Bladder dysfunction in EAE mice

On the 10th day after immunization, EAE mice were observed to show signs of neurological deficit, while the normal control mice (immunized with CFA alone) were normal. And we found that the symptoms of EAE mice were not persistently aggravated, but rather a cycle of sustained remission-relapse. However, the mice in the remission phase were not completely normal and still had symptoms of weakness in the tail. Figure 1A shows the mean Clinical Symptom score (CS) from day 0 to day 50. The Normal Control mice (NC) showed no damage throughout the time period. At 50 days post-immunization, the bladder-body weight ratio of the EAE model group (CS 1/2/3 group) was significantly higher than that of the NC group (Figure 1B), whereas the mean body weight was not significantly different in NC mice and EAE mice (Table 2). HE and Masson staining showed that the CS3 group was significantly more fibrous than the NC group (Figure 1C). Therefore, the higher the CS score of EAE mice, the more severe the bladder fibrosis.
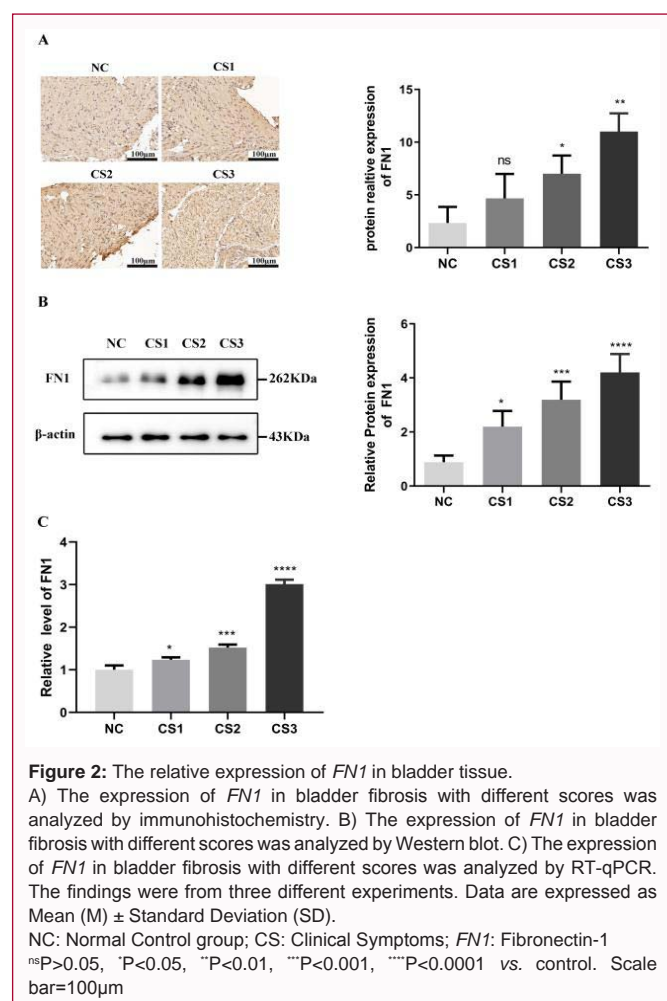
Expression of *FN1* in bladder tissues

We found a potential correlation between bladder fibrosis and *FN1* by detecting protein and mRNA expression levels in varying degrees of fibrosis bladder. As the clinical symptoms of EAE model mice deteriorated, the expression level of *FN1* increased significantly. We detected an increase in *FN1* expression by immunohistochemistry, Western blot analysis and RT-qPCR as bladder fibrosis was aggravated (Figure 2).

Predict miRNAs acting on *FN1* and detect miRNA expression in bladders with different degrees of fibrosis

Used Target Scan (http://www.targetscan.org), Miranda (http://www.mirdb.org) and miRBase (http://www.mirbase.org) for prediction and *FN1* Interacting microRNAs. After comparing Context++score, Context++score percentile, Weighted context++score, Conserved branch length, it was found that miR-1a-3p, miR-219c-5p and miR-3572-3p may interact with *FN1* (Figure 3A). The expression levels of miRNAs in the bladder of different scores were detected by RT-qPCR (Figure 3B-3D). We found that the expression levels of miR-1a-3p, miR-219c-5p and miR-3572-3p decreased with the increase of bladder fibrosis. That is, the opposite of the *FN1* trend.

Identification of bladder smooth muscle cells and miRNA transfection

Bladder Smooth Muscle Cells (BSMCs) were identified by

Figure 2: The relative expression of *FN1* in bladder tissue.
A) The expression of *FN1* in bladder fibrosis with different scores was analyzed by immunohistochemistry. B) The expression of *FN1* in bladder fibrosis with different scores was analyzed by Western blot. C) The expression of *FN1* in bladder fibrosis with different scores was analyzed by RT-qPCR. The findings were from three different experiments. Data are expressed as Mean (M) ± Standard Deviation (SD).
NC: Normal Control group; CS: Clinical Symptoms; *FN1*: Fibronectin-1
ⁿˢP>0.05, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 *vs.* control. Scale bar=100μm

Figure 3: Predict miRNAs acting on FN1 and detect miRNA expression in bladders with different degrees of fibrosis. A) This sites predicts the targeting of miR-1a-3p, miR-219c-5p, miR-3572-3p targeting *FN1*. B-D) The expression levels of miR-1a-3p, miR-219c-5p, miR-3572-3p in different fibrotic bladders were detected by RT-qPCR. The findings were from three different experiments. Data are expressed as Mean (M) ± Standard Deviation (SD).
NC: Normal Control group; CS: Clinical Symptoms; *FN1*: Fibronectin-1
ⁿˢP>0.05, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 *vs.* control

immunofluorescence using α-SMA (Figure 4A). The transfection efficiency of miR-1a-3p, miR-219c-5p and miR-3572-3p was detected by RT-qPCR (Figure 4B). The effect of miR-1a-3p, miR-219c-5p and miR-3572-3p on *FN1* was detected by RT-qPCR (Figure 4C). We found that only miR-219c-5p achieved the expected results in the case of no difference in transfection efficiency between miR-1a-3p, miR-219c-5p and miR-3572-3p. That is, *FN1* was decreased when overexpressing miR-219c-5p and *FN1* was increased when miR-219c-5p was knocked down. In summary, miR-219c-5p is most likely one of the miRNAs acting on *FN1*.

miR-219c-5p binding to *FN1*

The bio information prediction websites such as, Target Scan (http://www.targetscan.org), Miranda (http://www.mirdb.org) and miRBase (http://www.mirbase.org) were used to infer the potential targeting relationship between miR-219c-5p and *FN1*. The sequence of the 3'-UTR regions where miR-219c-5p bind to *FN1* mRNA is shown in Figure 5A. The results of the dual luciferase reporter assay showed that miR-219c-5p reduced *FN1*-WT luciferase activity by 49% (P<0.01) and had no effect on *FN1*-MUT luciferase activity (Figure 5A). That is, miR-219c-5p can target *FN1* and affect the synthesis of *FN1*.

miR-219c-5p targeted FN1 for suppressing BSMC proliferation

The effect of miR-219c-5p on cell proliferation in BSMCs is shown in Figure 5B. There was no significant difference in the survival
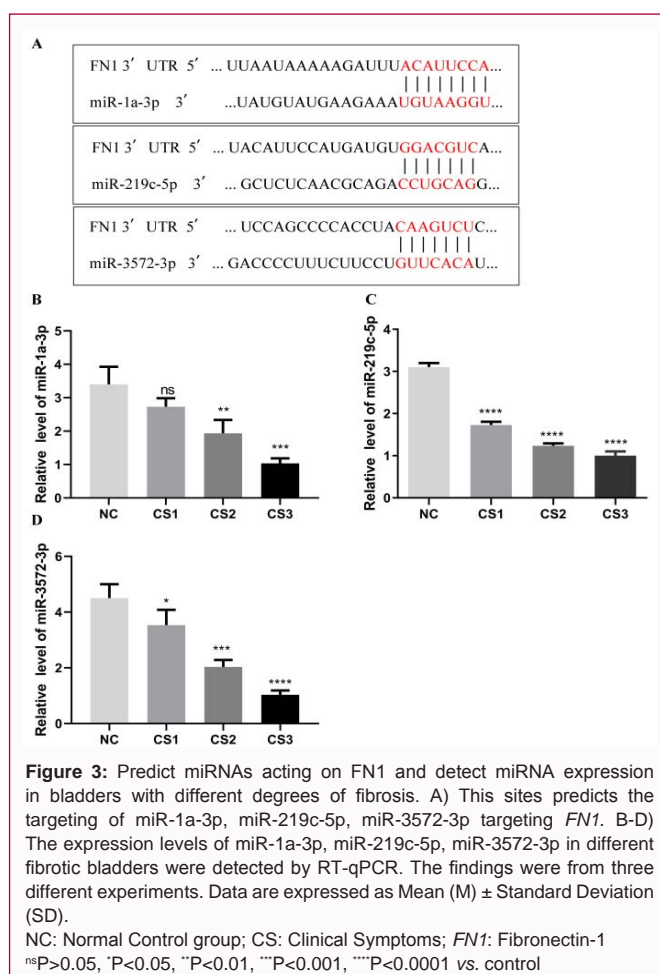
rate of BSMCs cells between the blank and NC groups (all P>0.05). Compared with the blank and NC groups, the viability of the BSMCs in the miR-219c-5p group decreased (P<0.05), whereas cell viability in the miR-219c-5p inhibitor group increased (P<0.05).

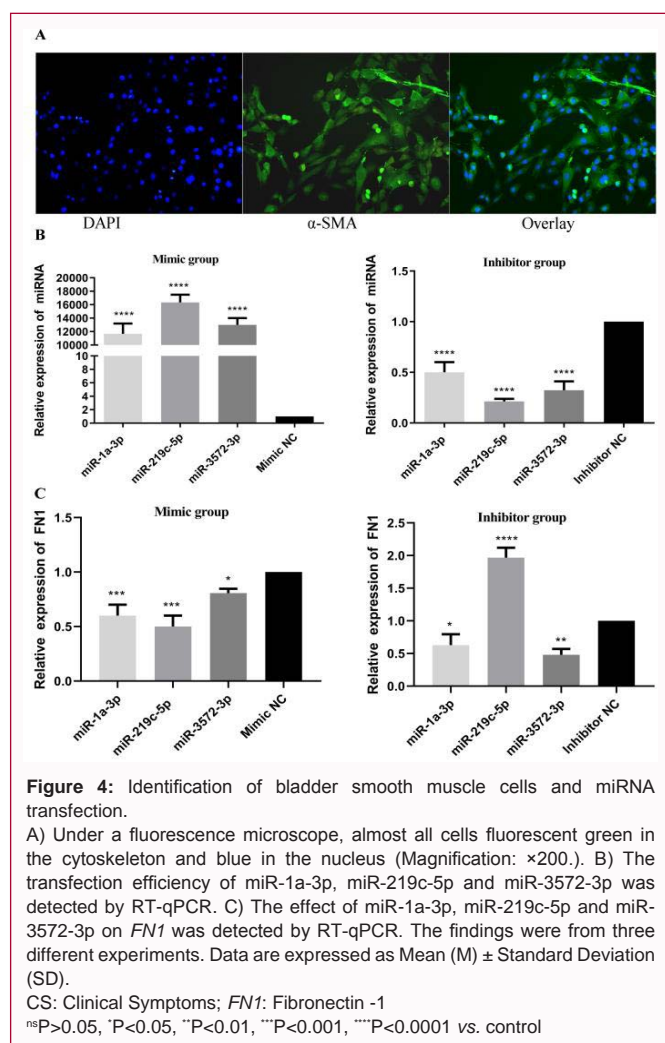### Effect of miR-219c-5p on the BSMC cycle

Compared with the blank and NC groups, the number of BMSCs in the G1 phase of the miR-219c-5p group increased, but decreased in the S/G2 phase, while the number of cells in the G1 phase of the miR-219c-5p inhibition group decreased, but increased in the S/G2 phase (P<0.05 in all cases) (Figure 6A). These data indicate that miR-219c-5p can inhibit the cell cycle by regulating the expression of the *FN1* gene and thereby arresting BMSCs in the G1 phase.

### Effect of miR-219c-5p on cell apoptosis in BSMCs

The apoptosis rate of the miR-219c-5p mock group increased significantly compared with the blank and NC groups (P<0.05). However, the apoptosis rate of the miR-219c-5p-inhibited group reduced significantly compared with the blank and NC groups (P<0.05) (Figure 6B). These results indicate that miR-219c-5p can promote BSMC apoptosis.

Discussion

In this study, we found that bladder fibrosis in EAE mice was aggravated with clinical symptoms. Moreover, we found that bladder fibrosis aggravation positively correlates with *FN1*. Therefore, we speculated whether the synthesis of *FN1* can be inhibited by certain miRNA targeting, thereby blocking the progression of bladder

Figure 4: Identification of bladder smooth muscle cells and miRNA transfection.
A) Under a fluorescence microscope, almost all cells fluorescent green in the cytoskeleton and blue in the nucleus (Magnification: ×200.). B) The transfection efficiency of miR-1a-3p, miR-219c-5p and miR-3572-3p was detected by RT-qPCR. C) The effect of miR-1a-3p, miR-219c-5p and miR-3572-3p on *FN1* was detected by RT-qPCR. The findings were from three different experiments. Data are expressed as Mean (M) ± Standard Deviation (SD).
CS: Clinical Symptoms; *FN1*: Fibronectin -1
nsP>0.05, *P<0.05, ¨P<0.01, ¨¨P<0.001, ¨¨¨P<0.0001 *vs.* control

Figure 5: miR-219c-5p binding to FN1 and miR-219c-5p targets FN1BSMC proliferation.
A) Binding of miR-219c-5p and *FN1* 3'-UTR and detection of the luciferase activity of BMSCs (n=3). B) The OD$_{450}$ values of cells were measured *via the* CCK8 assay. The findings were from three different experiments.
Data are expressed as Mean (M) ± Standard Deviation (SD). WT: Wild Type; MUT: Mutant; *FN1*: Fibronectin-1; 3 'UTR: Three Prime Untranslated Regions
nsP>0.05, *P<0.05, ¨P<0.01, ¨¨P<0.001, ¨¨¨P<0.0001 *vs.* Mimic NC or Inhibitor NC

fibrosis. Three sites, such as Target Scan (http://www.targetscan. org), were used to predict microRNAs that interact with *FN1*. After comparative analysis, it was found that miR-1a-3p, miR-219c-5p and miR-3572-3p all suggested a target for interaction with *FN1*. By overexpressing or knocking down miR-1a-3p, miR-219c-5p and miR-3572-3p in bladder smooth muscle *in vitro*, we found that only miR-219c-5p targeted *FN1* was better. The luciferase reporter assay confirmed that miR-219c-5p can target the inhibition of *FN1* synthesis. Moreover, CCK8 and flow functional assays also confirmed that miR-219c-5p can regulate the function of BMSCs by targeting the regulation of *FN1* synthesis. Urinary dysfunction such as frequent urination, urgency, urinary retention is common problems in patients with MS. The treatment of MS patients with bladder dysfunction remains a clinically difficult problem because there is at present no effective treatment. Altuntas et al. [7] first suggested that the EAE model induced by PEP139-151 is similar to the urinary dysfunction in patients with neurogenic bladder. Previous studies have shown that the neurological damage symptoms of EAE mice are associated with PGE2 EP3/EP4 (Prostaglandin E2 E-prostaglandin receptor 3/4) receptors, providing a new target for neurogenic bladder target therapy [28]. Wang et al. [29] report that the proliferation of human bladder smooth muscle cells is mainly regulated by the extracellular signal-regulated kinase 1/2 activity by the exchange factor directly activated by cAMP pathway. These findings not only propose a new idea for the treatment of neurogenic bladder, but also support our
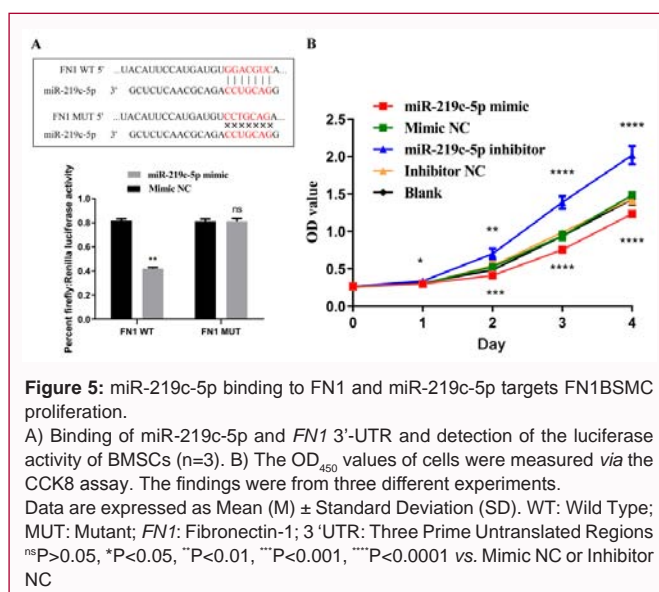
findings from the side.

Fibrosis is described as "wrong body repair" and its development may involve many mechanisms, but activation of myofibroblasts and subsequent production of excess extracellular groups are essential for fibrosis. Similarly, bladder fibrosis is thought to be involved in the activation of bladder smooth muscle cells and their accumulation of excess extracellular matrix. Some studies have shown that miRNAs are involved in smooth muscle cell function, including cell proliferation, cell cycle, and apoptosis [24,30,31]. There is growing evidence that many miRNAs play key regulatory roles in bladder fibrosis. Duan et al. [32] revealed a novel miR-133 regulatory factor that targets the TGF-β-Smad3 signaling pathway to modulate TGF-β1-induced changes in a BSMC phenotype. A novel anti-fibrotic function of miR-133 is proposed, which represents a potential target for diagnosis and treatment strategies of bladder fibrosis.

FN1 is a high molecular weight glycoprotein in the extracellular matrix with complex biological functions as a core component. Fibroblasts, vascular endothelial cells, hepatocytes, vascular smooth muscle cells, and other cell types secrete these functional proteins, which regulate cell adhesion, proliferation, and differentiation, maintenance of cell morphology, promotion of cell migration, ion exchange, signal transduction, and other functions. Numerous studies have reported the regulation of *FN1*-mediated fibrosis by miRNAs. Gunadi et al. [33] reported that miRNA-206 targeting *FN1* is abnormally expressed in multifactorial Hirschsprung's disease and can be used as a potential targeting molecule.

Numerous studies have shown that miRNAs are involved in the tissue fibrosis process. Jing et al. [34] reported that miR-27a can target AMP-activated protein kinase alpha 2 catalytic subunit and activate TGF-β1 and Smad3 signaling pathways, thereby reducing detrusor fibrosis in streptozotocin-induced diabetic rats. Moon et al. [35] reported that *FN1* is an important functional protein in the development of skin fibers. Therefore, to explore whether *FN1* is involved in the development of bladder fibrosis, and whether miRNA can target *FN1* to regulate the progression of bladder fibrosis. Used Target Scan (http://www.targetscan.org), Miranda (http://www. mirdb.org) and miRBase (http://www.mirbase.org) prediction found miR-1a-3p, miR-219c-5p and miR-3572-3p may interact with *FN1*.
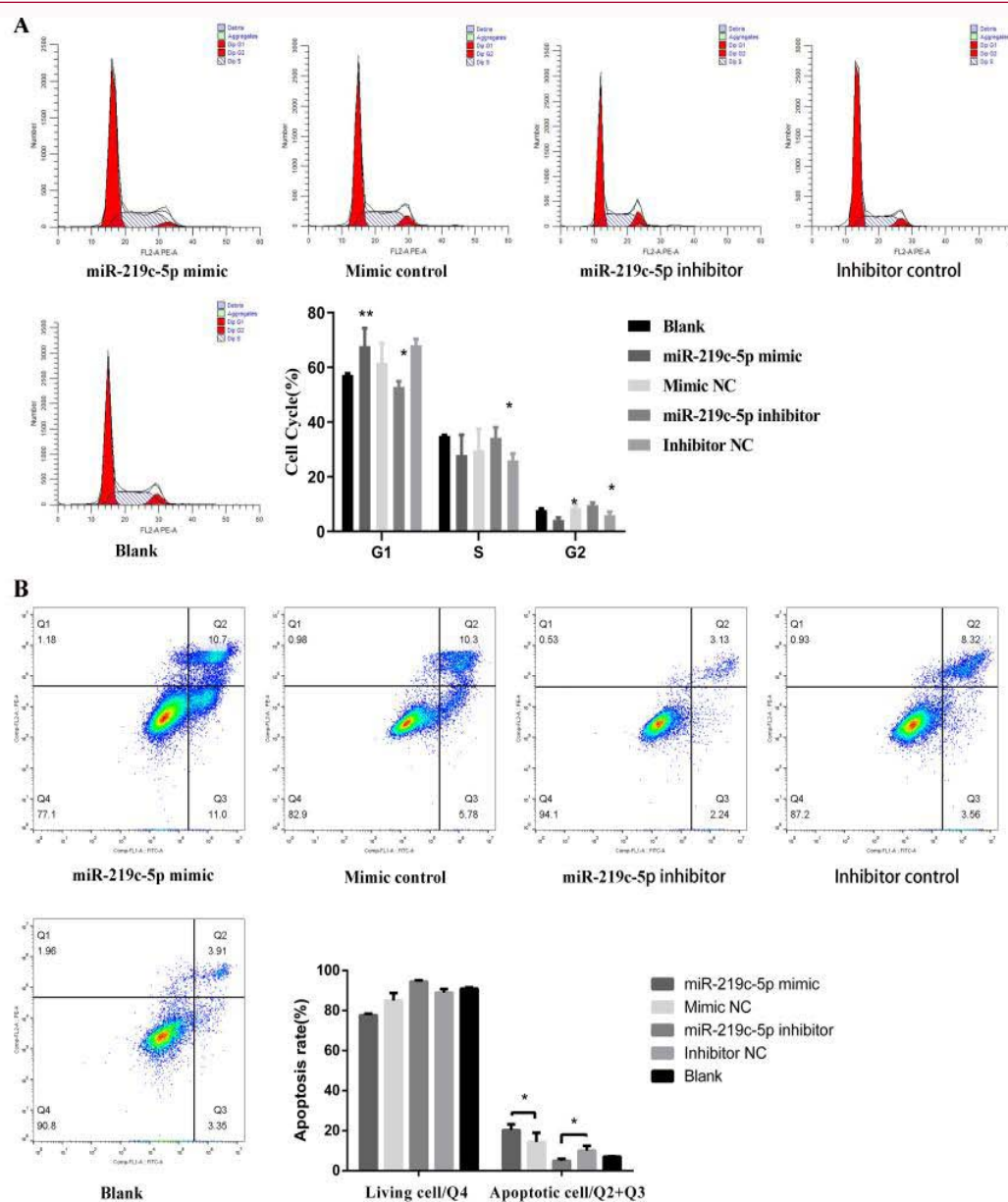
Figure 6: Effect of miR-219c-5p on cell cycle and apoptosis of BMSCs.
A) Compared with the blank and NC groups, the number of BMSCs in the G1 phase of the miR-219c-5p group increased, but decreased in the S/G2 phase, while the number of cells in the G1 phase of the miR-219c-5p inhibition group decreased, but increased in the S/G2 phase (P<0.05 in all cases). B) The apoptosis rate of the miR-219c-5p mock group increased significantly compared with the blank and NC groups (P<0.05). However, the apoptosis rate of the miR-219c-5p-inhibited group reduced significantly compared with the blank and NC groups (P<0.05). The findings were from three independent experiments. Data are expressed as Mean (M) ± Standard Deviation (SD). P<0.05
**P<0.01 *vs*. Mimic NC or Inhibitor NC

Taken together, this study reported that miR-219c-5p is better targeted to regulate *FN1* than miR-1a-3p and miR-3572-3p. This study demonstrated that miR-219c-5p blocks fibrosis of bladder smooth muscle cells by targeting inhibition of *FN1* expression. However, the experimental deficiency is that the effect of miR-219c-5p regulating *FN1* on bladder fibrosis has not been demonstrated in animals.

Conclusion

In conclusion, this study confirmed that *FN1* and miR-219c-5p are associated with bladder fibrosis in MS mice. It also shows that miR-219c-5p can regulate the proliferation and apoptosis of bladder smooth muscle cells by targeting *FN1*. Thus, miR-219c-5p and *FN1*

are potentially promising biomarkers for the diagnosis and treatment of bladder fibrosis.

Declarations

Ethical approval and consent to participate

The experimental protocol was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University (2019-KY-202).

Availability of supporting data

The datasets generated and analysed during the current study are available the Target Scan (http://www.targetscan.org), Miranda (http://www.mirdb.org), miRBase (http://www.mirbase.org).

Funding

Authors' Contribution

JY and ZJ have made significant contributions to the concept and design. CG and TW collected data. The data was analyzed and interpreted by YD and PL. BL participated in the drafting of the manuscript. JY and SH participated in critical revisions of important knowledge content. All authors have finally approved the version to be released and agree to be responsible for all aspects of the work.

Acknowledgement

References

1. Hafler DA, Slavik JM, Anderson DE, O'Connor KC, De Jager P, Baecher-Allan C. Multiple sclerosis. Immunol Rev. 2005;204:208-31.

2. Steinman L. Multiple sclerosis: a two-stage disease. Nat Immunol. 2001;2(9):762-4.

3. Fernandez O. Mechanisms and current treatments of urogenital dysfunction in multiple sclerosis. J Neurol. 2002;249(1):1-8.

4. Kalsi V, Fowler CJ. Therapy insight: bladder dysfunction associated with multiple sclerosis. Nat Clin Pract Urol. 2005;2(10):492-501.

5. Litwiller SE, Frohman EM, Zimmern PE. Multiple sclerosis and the urologist. J Urol. 1999;161(3):743-57.

6. Al-Izki S, Pryce G, Giovannoni G, Baker D. Evaluating potential therapies for bladder dysfunction in a mouse model of multiple sclerosis with high-resolution ultrasonography. Mult Scler. 2009;15(7):795-801.

7. Altuntas CZ, Daneshgari F, Izgi K, Bicer F, Ozer A, Sakalar C, et al. Connective tissue and its growth factor CTGF distinguish the morphometric and molecular remodeling of the bladder in a model of neurogenic bladder. Am J Physiol Renal Physiol. 2012;303(9):F1363-9.

8. Steinman L, Zamvil SS. Virtues and pitfalls of EAE for the development of therapies for multiple sclerosis. Trends Immunol. 2005;26(11):565-71.

9. Petry KG, Boullerne AI, Pousset F, Brochet B, Caille JM, Dousset V. Experimental allergic encephalomyelitis animal models for analyzing features of multiple sclerosis. Pathol Biol (Paris). 2000;48(1):47-53.

10. Altuntas CZ, Daneshgari F, Liu G, Fabiyi A, Kavran M, Johnson JM, et al. Bladder dysfunction in mice with experimental autoimmune encephalomyelitis. J Neuroimmunol. 2008;203(1):58-63.

11. Anumanthan G, Tanaka ST, Adams CM, Thomas JC, Wills ML, Adams MC, et al. Bladder stromal loss of transforming growth factor receptor II decreases fibrosis after bladder obstruction. J Urol. 2009;182(4 Suppl):1775-80.

12. Taylor JA, Zhu Q, Irwin B, Maghaydah Y, Tsimikas J, Pilbeam C, et al. Null mutation in macrophage migration inhibitory factor prevents muscle cell loss and fibrosis in partial bladder outlet obstruction. Am J Physiol Renal Physiol. 2006;291(6):F1343-53.

13. Slama A, Khouni H, Sriha B, Brini K, Ben Sorba N, Taher Mosbah A: Bladder fibrosis caused by eosinophilic cystitis. Ann Urol (Paris). 2003;37(5):272-4.

14. Wang N, Duan L, Ding J, Cao Q, Qian S, Shen H, et al. MicroRNA-101 protects bladder of BOO from hypoxia-induced fibrosis by attenuating TGF-beta-smad2/3 signaling. IUBMB Life. 2019;71(2):235-43.

15. Siddiqui NY, Helfand BT, Andreev VP, Kowalski JT, Bradley MS, Lai HH, et al. Biomarkers implicated in lower urinary tract symptoms: systematic review and pathway analyses. J Urol. 2019;202(5):880-9.

16. Andersson KE, Fry C, Panicker J, Rademakers K. Which molecular targets do we need to focus on to improve lower urinary tract dysfunction? ICI-RS 2017. Neurourol Urodyn. 2018;37(S4):S117-26.

17. Peyronnet B, Mironska E, Chapple C, Cardozo L, Oelke M, Dmochowski R, et al. A comprehensive review of overactive bladder pathophysiology: on the way to tailored treatment. Eur Urol. 2019;75(6):988-1000.

18. Sobolewski P, Murthy NS, Kohn J, El Fray M. Adsorption of fibrinogen and fibronectin on elastomeric poly(butylene succinate) copolyesters. Langmuir. 2019;35(26):8850-9.

19. Liao YX, Zhang ZP, Zhao J, Liu JP. Effects of fibronectin 1 on cell proliferation, senescence and apoptosis of human glioma cells through the PI3K/AKT signaling pathway. Cell Physiol Biochem. 2018;48(3):1382-96.

20. Gao R, Feng Q, Tan G. microRNA-613 exerts anti-angiogenic effect on nasopharyngeal carcinoma cells through inactivating the AKT signaling pathway by down-regulating FN1. Biosci Rep. 2019;39(7).

21. Matalliotaki C, Matalliotakis M, Rahmioglu N, Mavromatidis G, Matalliotakis I, Koumantakis G, et al. Role of FN1 and GREB1 gene polymorphisms in endometriosis. Mol Med Rep. 2019;20(1):111-6.

22. Rupaimoole R, Slack FJ. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. Nat Rev Drug Discov. 2017;16(3):203-22.

23. Bruinsma IB, van Dijk M, Bridel C, van de Lisdonk T, Haverkort SQ, Runia TF, et al. Regulator of oligodendrocyte maturation, miR-219, a potential biomarker for MS. J Neuroinflammation. 2017;14(1):235.

24. Yang L, Dong C, Yang J, Yang L, Chang N, Qi C, et al. MicroRNA-26b-5p inhibits mouse liver fibrogenesis and angiogenesis by targeting PDGF receptor-beta. Mol Ther Nucleic Acids. 2019;16:206-17.

25. Zhang H, Sun Z, Li Y, Fan D, Jiang H. MicroRNA-200c binding to FN1 suppresses the proliferation, migration and invasion of gastric cancer cells. Biomed Pharmacother. 2017;88:285-92.

26. Si M, Wang Q, Li Y, Lin H, Luo D, Zhao W, et al. Inhibition of hyperglycolysis in mesothelial cells prevents peritoneal fibrosis. Sci Transl Med. 2019;11(495).

27. Falcao AM, van Bruggen D, Marques S, Meijer M, Jakel S, Agirre E, et al. Disease-specific oligodendrocyte lineage cells arise in multiple sclerosis. Nat Med. 2018;24(12):1837-44.

28. Xue R, Jia Z, Kong X, Pi G, Ma S, Yang J. Effects of PGE2 EP3/EP4 receptors on bladder dysfunction in mice with experimental autoimmune encephalomyelitis. Am J Physiol Renal Physiol. 2013;305(12):F1656-62.

29. Lai J, Ai J, Luo D, Jin T, Liao B, Wang K, et al. β-Adrenoceptor signaling regulates proliferation and contraction of human bladder smooth muscle cells under pathological hydrostatic pressure. J Cell Biochem. 2019;120(10):17872-86.

30. Guo JH, Su C, Jiang SY, Wang F, Feng X, Wang JT. MicroRNA-1 regulates fibronectin expression in human trabecular meshwork cells under oxidative stress. Zhonghua Yan Ke Za Zhi. 2019;55(5):355-60.

31. Ma L, Ma J, Ou HL. MicroRNA219 overexpression serves a protective role during liver fibrosis by targeting tumor growth factor beta receptor 2. Mol Med Rep. 2019;19(3):1543-50.

32. Duan LJ, Qi J, Kong XJ, Huang T, Qian XQ, Xu D, et al. MiR-133 modulates TGF-β1-induced bladder smooth muscle cell hypertrophic and fibrotic response: implication for a role of microRNA in bladder wall remodeling caused by bladder outlet obstruction. Cell Signal. 2015;27(2):215-27.

33. Gunadi, Budi NYP, Kalim AS, Santiko W, Musthofa FD, Iskandar K, et al. Aberrant expressions of miRNA-206 target, FN1, in multifactorial Hirschsprung disease. Orphanet J Rare Dis. 2019;14(1):5.

34. Wu J, Liu S, Yuan ZW, Liu JH, Li JM, Chen T, et al. MicroRNA-27a suppresses detrusor fibrosis in streptozotocin-induced diabetic rats by targeting PRKAA2 through the TGF-BETA1/Smad3 signaling pathway. Cell Physiol Biochem. 2018;45(4):1333-49.

35. Moon SJ, Bae JM, Park KS, Tagkopoulos I, Kim KJ. Compendium of skin molecular signatures identifies key pathological features associated with fibrosis in systemic sclerosis. Ann Rheum Dis. 2019;78(6):817-25.