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ORIGINAL RESEARCH

Overexpression of Aquaporin 1 in Synovium Aggravates Rat Collagen-Induced Arthritis Through Regulating β-Catenin Signaling: An in vivo and in vitro Study

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Introduction: Previous studies have confirmed that aquaporin 1 (AQP1) is up-regulated in synovium of rheumatoid arthritis (RA), but its exact pathogenic mechanisms in RA are unclear. This study revealed the pathogenic role of AQP1 in rat collagen-induced arthritis (CIA) and the underlying mechanisms related to β-catenin signaling.

Materials and Methods: Secondary paw swelling and pathological changes of ankle joints were used to evaluate the severity of rat CIA. Synovial AQP1 and β-catenin expression were measured by immunohistochemistry (IHC) and Western blot assay. AQP1 siRNA was applied to knockdown AQP1 in cultured CIA fibroblast-like synoviocyte (FLS). Assays of MTT, PCNA immunofluorescence and transwell were performed to detect cell proliferation, migration and invasion. The protein levels of β-catenin pathway members and ratio of TOP/FOP luciferase activity were also measured.

Results: In vivo, we revealed that synovial AQP1 and β-catenin expressions in CIA rats were higher than normal rats, and synovial AQP1 expression of CIA rats increased in parallel with secondary paw swelling and total pathological score on joint damage. Correlation analysis of IHC results indicated that synovial AQP1 expression positively correlated with β-catenin expression in CIA rat. In vitro, AQP1 siRNA apparently reduced the proliferation, migration and invasion of CIA FLS by inhibiting β-catenin signaling pathway. As an activator of β-catenin signaling, lithium chloride (an inhibitor of GSK-3β) reversed the inhibitory effects of AQP1 siRNA on the cultured CIA FLS.

Conclusion: We concluded that the overexpression of synovial AQP1 aggravated rat CIA by promoting the activation of FLS through β-catenin signaling pathway.

Keywords: aquaporin 1, collagen-induced arthritis, fibroblast-like synoviocyte, rheumatoid arthritis, β-catenin signaling

Introduction

Rheumatoid arthritis (RA) is an inflammatory autoimmune disease, featuring joint swelling, proliferative synovitis and synovial inflammation, leading to inevitable joint damage and functional disability.[1] The etiology and pathophysiology of RA are not fully clear, but improved knowledge of the various factors in RA pathogenesis has caused apparent advances in recent decades.[2] Although disease-modifying anti-rheumatic drugs and biologic therapies are recommended for treating RA patients, at least 30% of patients are resistant to the available therapies and long-term medication

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usually causes serious side effects.[3] Therefore, the development of novel treatments aiming at specific factors would contribute to understanding the complicated mechanisms in RA, with improved outcomes.

Aquaporin 1 (AQP1), belonging to the aquaporin proteins family, is expressed in various tissues including synovium and is selectively permeated by water driven by osmotic gradient.[4,5] Trujillo et al first reported that AQP1 could be expressed in synoviocytes and synovial micro-vessels, and AQP1 expression in synovial tissues was up-regulated in people with RA compared with that in normal and those with osteoarthritis (OA).[4] Mobasheri et al further verified that synovial AQP1 overexpression in RA might be related to the formation of hydrarthrosis and synovitis.[5] However, the exact pathologic role of AQP1 in RA and the underlying mechanisms are unknown. The activated fibroblast-like synoviocyte (FLS) shows a lot of tumor-like aggressive phenotypes and plays an important role in the pathogenesis of RA.[6] Interestingly, except for regulating influx and outflow of water, AQP1 can promote the proliferation, migration and angiogenesis of tumor cells.[7–9] Whether AQP1 can enhance the abnormal behaviors of RA FLS as it does in tumor cells remains to be further clarified. Previous studies have revealed that β-catenin signaling is involved in the activation of RA FLS and inhibition of β-catenin signaling has great potential as a therapeutic target of RA.[10] Recently, the interaction of AQP1 and β-catenin signaling has been verified in other diseases,[11–14] we therefore speculated that the overexpression of synovial AQP1 enhanced the abnormal behaviors of RA FLS by activating β-catenin signaling, which may be involved in RA pathogenesis.

Collagen-induced arthritis (CIA) is a widely-used experimental model to explore RA pathogenesis and screening new drugs for treatment of RA.[15–17] We revealed that the synovial AQP1 overexpression aggravated rat CIA, and β-catenin signaling might be implicated in the pathogenesis of AQP1 in CIA. In vitro, we observed the inhibitory effects of AQP1 siRNA on cell proliferation, migration, invasion and β-catenin signaling pathway in CIA FLS. We also found that lithium chloride (LiCl, an activator of β-catenin signaling) could reverse the inhibitory effects of AQP1 siRNA on CIA FLS. This study aims to provide new experimental evidence that AQP1 might be involved in the pathogenesis of RA by regulating β-catenin signaling.

Materials and Methods

Reagents

Chicken type II collagen (CCII) and incomplete Freund's adjuvant (IFA) were obtained from Chondrex (Redmond, WA, USA). 4′,6-diamidino-2-phenylindole (DAPI), LiCl and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were bought from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), trypsin and Dulbecco's modified Eagle's medium (DMEM) were obtained from Gibco (Carlsbad, CA, USA). Antibodies of AQP1 (ab9566), β-catenin (ab16051), c-myc (ab32072), matrix metalloproteinase 9 (MMP9) (ab76003) and proliferating cell nuclear antigen (PCNA) (ab92552) were bought from Abcam (Cambridge, UK). Cyclin D1 (#55,506) and phosphor (p)-GSK-3β (Ser9) (#9323) were purchased from Cell Signaling Technology (Beverly, MA, USA).

Induction and Evaluation of Rat CIA

Male Sprague-Dawley rats (120–130 g) were bought from the Laboratory Animals Center of Anhui Medical University and housed under conventional laboratory conditions. After 7-day acclimatization, rats were randomly separated into normal group and CIA group (30 rats per group). The experimental protocols were approved by the Ethical Committee on Animal Research at School of Pharmacy of Anhui Medical University, in accordance with National Institutes of Health guide for the care and use of Laboratory animals (No. 8023, revised 1978). Rat CIA model was induced according to the previous references.[15] Briefly, CCII was dissolved in 0.01 M acetic acid at 4 mg/mL and emulsified thoroughly with an equal volume of IFA. 0.2 mL of emulsion was intradermally injected into the left hind paw. The first injection day was defined as day 0. Seven days later, rats were given a booster injection of an equal amount of CCII emulsion into the base of the tail or back at multiple sites. The volume of non-injected hind paw was measured by a toe volume meter every four days from day 12 to 36 after CIA induction. The secondary swelling defined as the change in paw volume on each time point (ΔmL) was used to evaluate the secondary arthritis of CIA rats.

Pathological Examination and Assessment of Ankle Joint Damage

Rats were euthanized on day 36 after CIA induction. The ankle joint tissues were collected, decalcified by 10% EDTA for 2 weeks, embedded in paraffin and sectioned

at 5 μm thickness for hematoxylin and eosin (HE) staining. The ankle joint sections with HE staining were histologically evaluated by a trained researcher blinded to the specimens. The assessments of ankle joint damage were performed on the basis of synovial hyperplasia, inflammatory cells infiltrate, cartilage damage and pannus formation, with a score from 0 to 4: 0 = no changes; 1 = mild; 2 = moderate; 3= severe; 4 = very severe.[18] Total pathological score of ankle joint damage in CIA rats was the sum of individual pathological index score, with a score from 0 to 16.

Immunohistochemistry Assay for AQP1 and β-Catenin

The immunohistochemistry (IHC) assay was applied with classic protocols.[19,20] The tissue sections were deparaffinized, hydrated and microwave-treated in citrate buffer for antigen retrieval. Endogenous peroxidase activity was quenched by 3% hydrogen peroxide. Nonspecific staining was blocked by 5% goat serum. Sections were "reacted" with anti-AQP1 or β-catenin at 4°C overnight, and then incubated with the biotinylated-secondary antibody and avidin-biotin horseradish peroxidase complex (Zhongshan Goldenbridge Biotechnology, Beijing, People's Republic of China) at 37°C for 30 min. Sections were visualized by diaminobenzidine, lightly counterstained with hematoxylin and observed by Nikon 80i light microscope. The numbers of immune-positive stained cells and total cells were counted at five fields per slide. The percentage of immune-positive cells in synovial tissues (number of positive cells/total cells×100%) was calculated by a trained pathologist.

Rat FLS Preparation and Experimental Grouping

Rat FLS were cultured from synovial tissues by tissue explant cultivation.[21,22] Briefly, fresh synovial tissues obtained from knee joint were cut into small pieces, rinsed thoroughly, incubated in flat-bottomed bottles and further cultured in DMEM adding 20% FBS at 37°C in 5% CO_2 for 7 days. Tissue pieces were removed and adherent cells were cultured for another 3 days. At 70%–80% confluence, cells were digested with 0.25% trypsin, split at a 1:2 ratio and cultured continually. Most cells after 2 passages kept a homogeneous population of FLS. The cells were identified to be FLS by their morphology and expression of vascular cell adhesion molecule-1 (VCAM-1) by immunofluorescence cytochemistry. Primary synovial cells between passages 2 and 4 were used for subsequent experiments. Cells were divided into five groups, including normal FLS group (normal FLS without treatment), CIA FLS group (CIA FLS without treatment), negative control (NC) siRNA group (CIA FLS transfected with NC siRNA), AQP1 siRNA group (CIA FLS transfected with AQP1 siRNA) and LiCl group (CIA FLS transfected with AQP1 siRNA + LiCl).

Small Interfering RNA (siRNA) and Transfection

Rat AQP1 siRNA (5′-GCUGUACUCAUCUACGACUTT −3′) and NC siRNA (5′-ACGUGACACGUUCGGAGAATT -3′) were purchased from GenePharma Company (Shanghai, People's Republic of China). Cells were seeded in 6-well plates at a density of 1×10^6 cells/well and incubated at 50%–60% confluence before transfection. Then, cells were transfected with AQP1 siRNA or NC siRNA using Lipofectamine 2000 (Thermo Fisher Scientific, PA, USA) according to the manufacturer's instructions. The silencing effects of AQP1 siRNA were confirmed by Western blot analysis.

Cell Viability

Cell viability was measured by MTT assay. After transfection of AQP1 siRNA or NC siRNA, cells were seeded in 96-well plates at 5×10^3 cells/well and incubated in DMEM adding 10% FBS at 37°C, 5% CO_2 for adherence. In LiCl group, CIA FLS with AQP1 siRNA transfection were treated with LiCl at concentration of 10 mmol/L. Cells in different groups were cultured for 48 h. 4 h before the end of 48 h-cultivation, 20 μL of MTT (5 mg/mL) was added into each well and continually incubated. The supernatants were carefully removed after centrifugation. The formazan crystals were dissolved in 120 μL of dimethyl sulfoxide and oscillated for 1 min. The absorbance at 490 nm was measured using a microplate reader and cell viability value was defined as a ratio versus normal FLS group.

PCNA Immunofluorescence Assay

After appropriate treatment according to experimental groupings, cells were adhered on coverslips placed in 6-well plates at 1×10^5 cells/well. Then, cells were fixed with 4% paraformaldehyde, permeabilized by 0.1% Triton X-100 and blocked with goat serum. Cells were incubated with rabbit anti-PCNA at 4°C overnight, followed by FITC-conjugated goat anti-rabbit IgG (diluted 1:100) in the dark for 1 h at room temperature. Cell nuclei were

counterstained with DAPI (1 μg/mL) in the dark for 5 min. The fluorescence was examined using a fluorescent microscope (Olympus, Tokyo, Japan) and typical photos were taken. The number of PCNA-positive cells and total cells were counted in five different fields of each section, and the percentage of PCNA-positive cells was calculated.

Transwell Migration and Invasion Assay

Cell migration and invasion were determined using Transwell chambers with 8 μm pores (Millipore, MA, USA). For the cellular migration assay, cells in serum-free DMEM were added into the upper chamber at a density of 5×10^4 cells/well. DMEM containing 10% FBS was added into the lower chamber. After 24 h of incubation at 37°C, cells remaining on the upper surface of the insert were scraped off with cotton swabs, and cells migrating through the membrane to the lower surface of the insert were stained with 0.1% crystal violet for 20 min. The cellular invasion assay was performed as described in the migration assay, but with matrigel pre-coated inserts. The numbers of migrating or invading cells were counted in five randomly selected fields under a microscope and typical images were photographed.

Western Blot Analysis

Synovial tissues or cultured FLS were treated with RIPA lysis buffer containing protease inhibitors. The supernatant was collected and the protein level of the lysate was determined by Bradford assay. Equal amounts of proteins (20 μg) were run on by 10% SDS-PAGE electrophoresis and transferred onto PVDF membranes, which were then blocked with 5% skim milk in Tris-buffered saline. Membranes were probed with the primary antibodies at 4°C overnight, and incubated at 37°C with HRP-conjugated secondary antibodies (Cell Signaling Technology, MA, USA) for 2 h. The immunoreactive proteins were visualized by Super Signal West Femto Trial Kit (Thermo Fisher Scientific, PA, USA). The protein bands were scanned and quantified by densitometry using Image J. The relative levels of target proteins were normalized by the internal control β-actin.

TOP/FOP Flash Luciferase Assay

TOP flash plasmid (#21-170) and FOP flash plasmid (#21-169) were purchased from Merck Company (Hesse, Germany). After appropriate treatment according to groupings, cells were seeded in 24-well plates at a density of 1×10^5 cells/well. Then, cells were transfected with TOP flash or FOP flash firefly luciferase plasmid, and internal control pRL-TK Renilla luciferase vector (Promega Corp, # E6921) using Lipofectamine 2000. After 48 h of cultivation, the firefly fluorescence and the Renilla fluorescence were measured using a dual-luciferase assay kit (Promega Corp, #E1910). The luciferase activity of each sample was normalized with the respective Renilla luciferase activity. The ratio of TOP and FOP flash was recognised as the activation level of transcription factors in Wnt/β-catenin signaling pathway.[23]

Statistical Analysis

Statistical analyses were performed by SPSS 16.0 software. The experimental data were analyzed by Independent-samples *t*-test or One-way ANOVA followed by LSD post hoc test. Pearson's correlation test was used to examine the correlation between synovial AQP1 expression and pathological parameters of rat CIA or synovial β-catenin expression. Data were all shown as mean ± standard error of the mean (SEM) and $P < 0.05$ was considered to be statistically significant.

Results

Evaluation of CIA in Rats

CIA rats showed an obvious swelling of non-injected hind paws (i.e., secondary paw swelling) on day 36 after CIA induction, caused by a systemic autoimmune response (Figure S1A). The secondary paw swelling, measured every 4 days from day 12 to 36, was used to evaluate the development of rat CIA (Figure S1B). The secondary paw swelling of CIA rats was significantly increased compared with that of normal rats on every time point. HE staining was used to reveal the pathological changes of ankle joints: no cartilage damage or inflammation was found in normal ankle joint sections, whereas CIA ankle joint sections showed many pathological characteristics resembling RA, such as synovial hyperplasia, inflammatory cells infiltration, cartilage damage and pannus formation (Figure S1C). The pathological scores of the previously mentioned parameters of ankle joint damage were exhibited in Figure S1D.

AQP1 and β-Catenin Were Overexpressed in Synovial Tissues of CIA Rat

IHC assay was used to observe in situ expression of AQP1 and β-catenin in rat synovial tissues: AQP1 was mainly localized at cell membrane/cytoplasm (Figure 1A) and β-catenin was mostly localized at cell cytoplasm/nucleus (Figure 1B). The semi-quantified results of IHC assay revealed that the positive

staining percentages of AQP1 and β-catenin in synovium of CIA rats were dramatically elevated compared with normal rats (Figure 1C). In addition, Western blot results indicated that the levels of AQP1 and β-catenin protein in synovial tissues of CIA rats were much higher than normal rats (Figure 2), consistent with IHC results. These results demonstrated that there was overexpression of AQP1 and β-catenin in synovim of CIA rats.

AQP1 Overexpression in Synovium Aggravated Rat CIA: Involvement of β-Catenin Signaling Activation

Total pathological score of joint damage was the sum of individual pathological index score, with a score from 0 to 16. Herein, the secondary hind paw swelling on day 36 and total pathological score were applied to represent the severity of rat CIA. Correlation analysis indicated that the percentage of AQP1 immune-positive cells in synovial tissues of CIA rats was correlated positively with the secondary hind paw swelling on day 36 (Figure 3A, $r = 0.849$, $P < 0.01$) and total pathological score on ankle joint damage (Figure 3B, $r = 0.878$, $P < 0.01$), indicating that synovial AQP1 expression level may have been associated with the severity of rat CIA. In addition, correlation analysis of IHC results revealed that there was a significant positive correlation between AQP1 positive staining percentage and β-catenin positive staining percentage in synovial tissues of CIA rats (Figure 3C, $r = 0.901$, $P < 0.01$). Taken together, AQP1 overexpression in synovial tissues aggravated rat

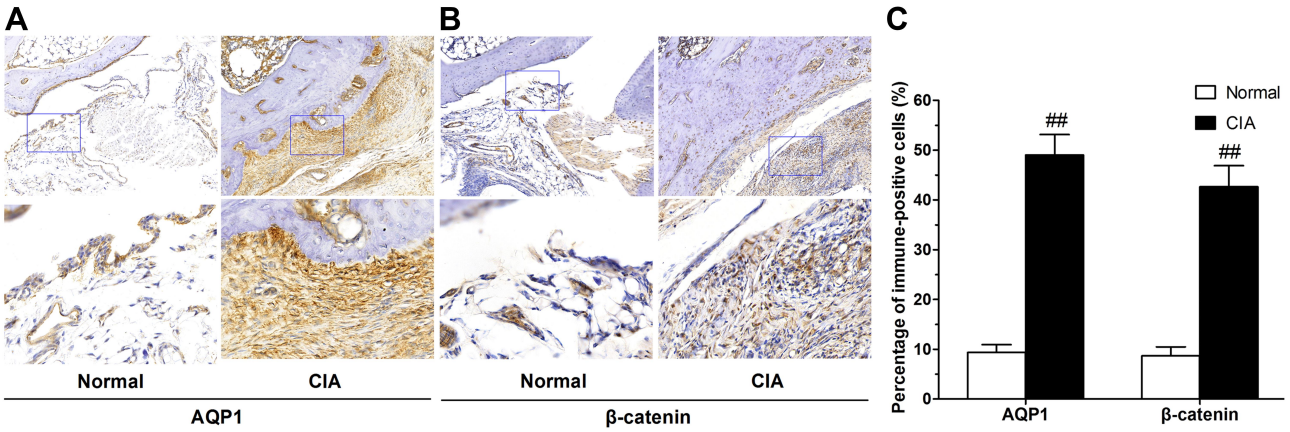

Figure 1 Immunohistochemistry assay for AQP1 and β-catenin expression in synovim of ankle joints from normal and CIA rats. Typical images of AQP1 (**A**) and β-catenin (**B**) expression in synovial tissues. High-power images (×400) show amplifications of regions boxed in blue in low-power images (×100). AQP1 and β-catenin were expressed at low levels in synovim from normal rats, whereas the relatively strong staining of AQP1 and β-catenin could be observed in CIA rats. (**C**) Percentages of AQP1 and β-catenin immune-positive cells in synovial tissues of normal and CIA rat. Data are mean ± SEM (n = 30). $^{\#\#}P < 0.01$ compared with normal rat group.
Abbreviations: AQP1, aquaporin 1; CIA, collagen-induced arthritis.

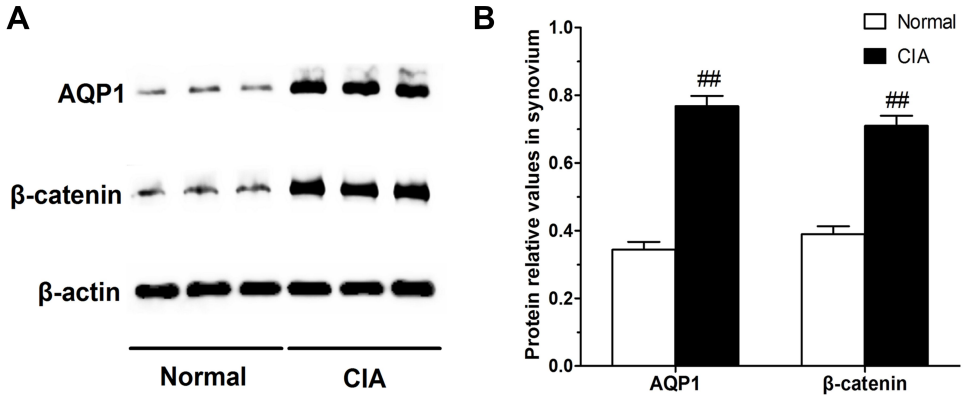

Figure 2 Western blot analysis for AQP1 and β-catenin protein in synovial tissues. (**A**) Typical examples of AQP1 and β-catenin protein expression in synovial tissues from normal and CIA rats. (**B**) The quantitative analysis of AQP1 and β-catenin protein relative values, β-actin serves as the house-keeping protein. Data are mean ± SEM (n = 6). $^{\#\#}P < 0.01$ compared with normal rat group.
Abbreviations: AQP1, aquaporin 1; CIA, collagen-induced arthritis.

CIA, and β-catenin signaling might be implicated in the pathogenesis of AQP1 in rat CIA.

AQP1 siRNA Transfection Reduced AQP1 Protein Level in CIA FLS

The cultured cells had a spindle shape, accordant with FLS morphological feature. The positive expression of VCAM-1 by immunofluorescence staining confirmed that the cultured cells were consistent with the intimal subpopulation of FLS (Figure 4A). AQP1 protein level in CIA FLS was significantly increased compared with that in normal FLS (Figure 4B), as is consistent with our in vivo results. AQP1 siRNA was used to knockdown AQP1 expression in CIA FLS and the knockdown efficiency was confirmed with Western blot analysis (Figure 4C). AQP1 siRNA transfection in CIA FLS caused a significant reduction of AQP1 protein level compared with non-treated CIA FLS group, whereas NC siRNA exhibited no effect on AQP1 expression. AQP1 protein level in AQP1 siRNA group was 15% of that in NC siRNA group.

Inhibition of CIA FLS Proliferation by AQP1 siRNA Was Abolished by LiCl

In Figure 5A, the viability of CIA FLS was obviously higher than that of normal FLS. AQP1 siRNA transfection in CIA

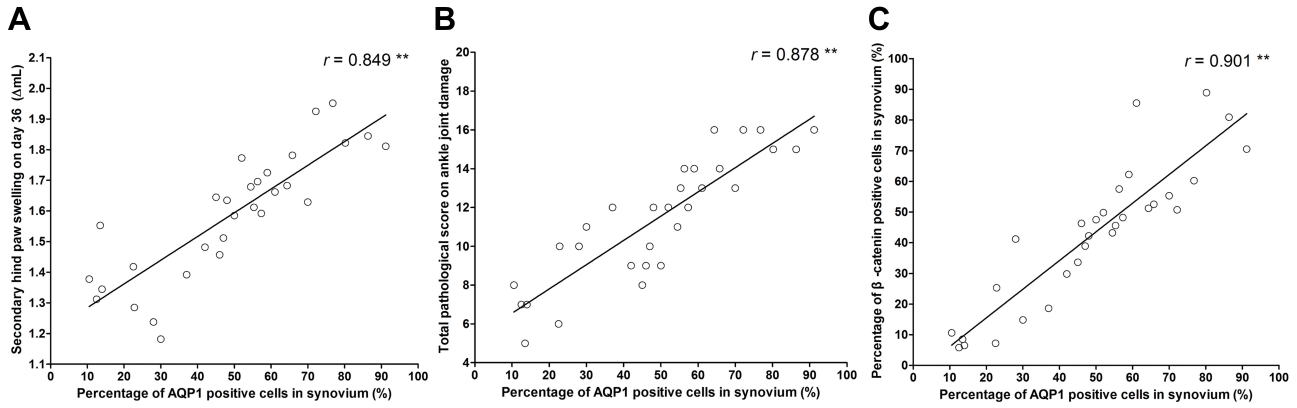

Figure 3 Correlations between synovial AQP1 positive cell percentage of CIA rats and secondary hind paw swelling (**A**) or total pathological score on joint damage (**B**) or synovial β-catenin positive cell percentage (**C**). The correlation analysis is performed by Pearson's correlation test (n = 30) and asterisks (*) show the statistical significance. $^{**}P$ <0.01.
Abbreviations: AQP1, aquaporin 1; mL, milliliter; *r*, correlation coefficient.

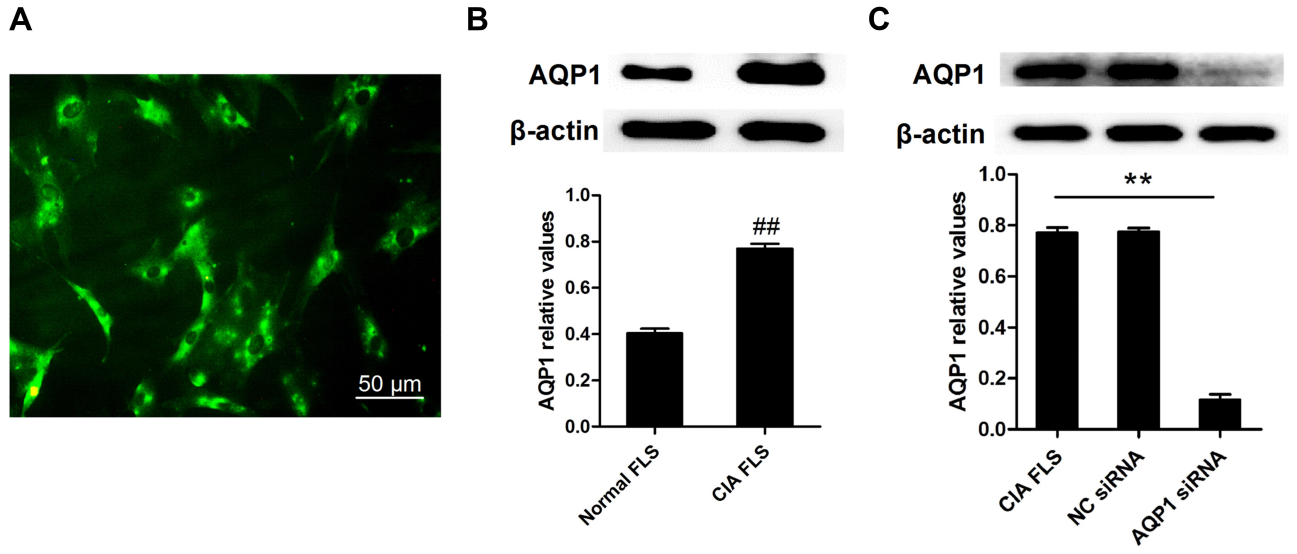

Figure 4 AQP1 siRNA effectively reduced AQP1 protein level in cultured CIA FLS. (**A**) FLS identification by VCAM-1 expression using immunofluorescence staining (×200). (**B**) Protein bands and quantitative analysis of AQP1 in normal and CIA FLS. (**C**) Protein bands and quantitative analysis of AQP1 from non-treated CIA FLS, NC siRNA-transfected CIA FLS and AQP1 siRNA-transfected CIA FLS. β-actin serves as the house-keeping protein. $^{\#\#}P$ < 0.01 compared with normal FLS group. $^{**}P$ <0.01 compared with CIA FLS group. The data are mean ± SEM of three independent experiments performed in triplicate.
Abbreviations: AQP1, aquaporin 1; CIA, collagen-induced arthritis; FLS, fibroblast-like synoviocyte; NC, negative control; siRNA, small interfering RNA.

FLS remarkably reduced the cell viability compared with non-treated CIA FLS group, while NC siRNA showed no obvious effect on CIA FLS viability. We further assayed the percentage of PCNA-positive cells by immunofluorescence assay (Figure 5B). The percentage of PCNA-positive cells in CIA FLS group was much higher than normal FLS group (Figure 5C). AQP1 siRNA significantly reduced the percentage of PCNA-positive cells in contrast to CIA FLS group. Compared to AQP1 siRNA group, LiCl as an activator of β-catenin signaling canceled the inhibitory effect of AQP1 siRNA on CIA FLS proliferation and increased the percentage of PCNA-positive cells, indicating that β-catenin signaling might be involved in AQP1 siRNA-induced inhibition of CIA FLS proliferation.

Suppression of CIA FLS Migration and Invasion by AQP1 siRNA Was Canceled by LiCl

In the transwell assay, FLS were seeded into the upper chambers without or with pre-coated matrigel for measuring migration or invasion (Figure 6). The numbers of

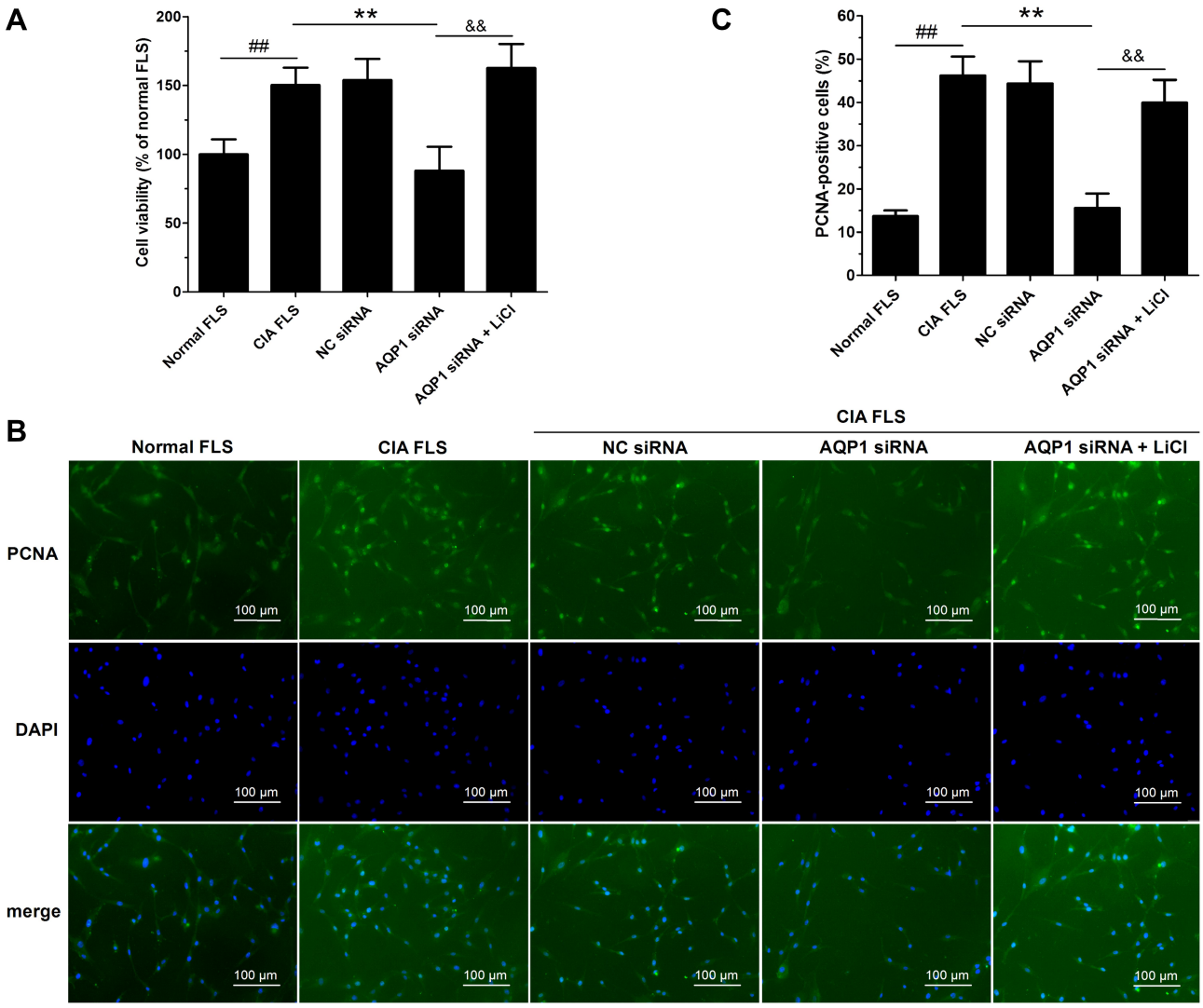

Figure 5 Inhibition of CIA FLS proliferation by AQP1 siRNA was abolished by LiCl. (**A**) The cell viability of different FLS groups, detected by MTT assay. (**B**) Typical photos of PCNA immunofluorescence staining from different FLS groups (×100). (**C**) Quantitative statistical results of PCNA-positive cells (%). The data are mean ± SEM of three to five independent experiments performed in triplicate. $^{\#\#}P < 0.01$ compared with normal FLS group. $^{**}P < 0.01$ compared with CIA FLS group. $^{\&\&}P < 0.01$ compared with AQP1 siRNA group.

Abbreviations: AQP1, aquaporin 1; CIA, collagen-induced arthritis; DAPI, 4′,6-diamidino-2-phenylindole; FLS, fibroblast-like synoviocyte; LiCl, lithium chloride; NC, negative control; PCNA, proliferating cell nuclear antigen; siRNA, small interfering RNA.

migrated and invasive cells in CIA FLS group were much higher than those in normal FLS group. AQP1 siRNA significantly reduced the numbers of migrated and invasive cells in contrast to CIA FLS group, while NC siRNA exhibited no effect on CIA FLS migration and invasion. Interestingly, LiCl reversed the inhibitory effect of AQP1 siRNA on CIA FLS migration and invasion, implying that β-catenin signaling might be involved in AQP1 siRNA-induced inhibition of CIA FLS migration and invasion.

AQP1 siRNA Inhibited the Activation of β-Catenin Signaling Pathway in CIA FLS

We observed whether AQP1 siRNA could regulate β-catenin signaling in CIA FLS using Western blot analysis and TOP/FOP flash luciferase assay (Figure 7). We found that the protein levels of β-catenin signaling pathway members in CIA FLS, including β-catenin, p-GSK-3β (Ser9), c-myc, cyclin D1 and MMP9, were much higher than those in normal FLS (Figure 7A and B). Compared with CIA FLS group, AQP1 siRNA could significantly reduce the protein

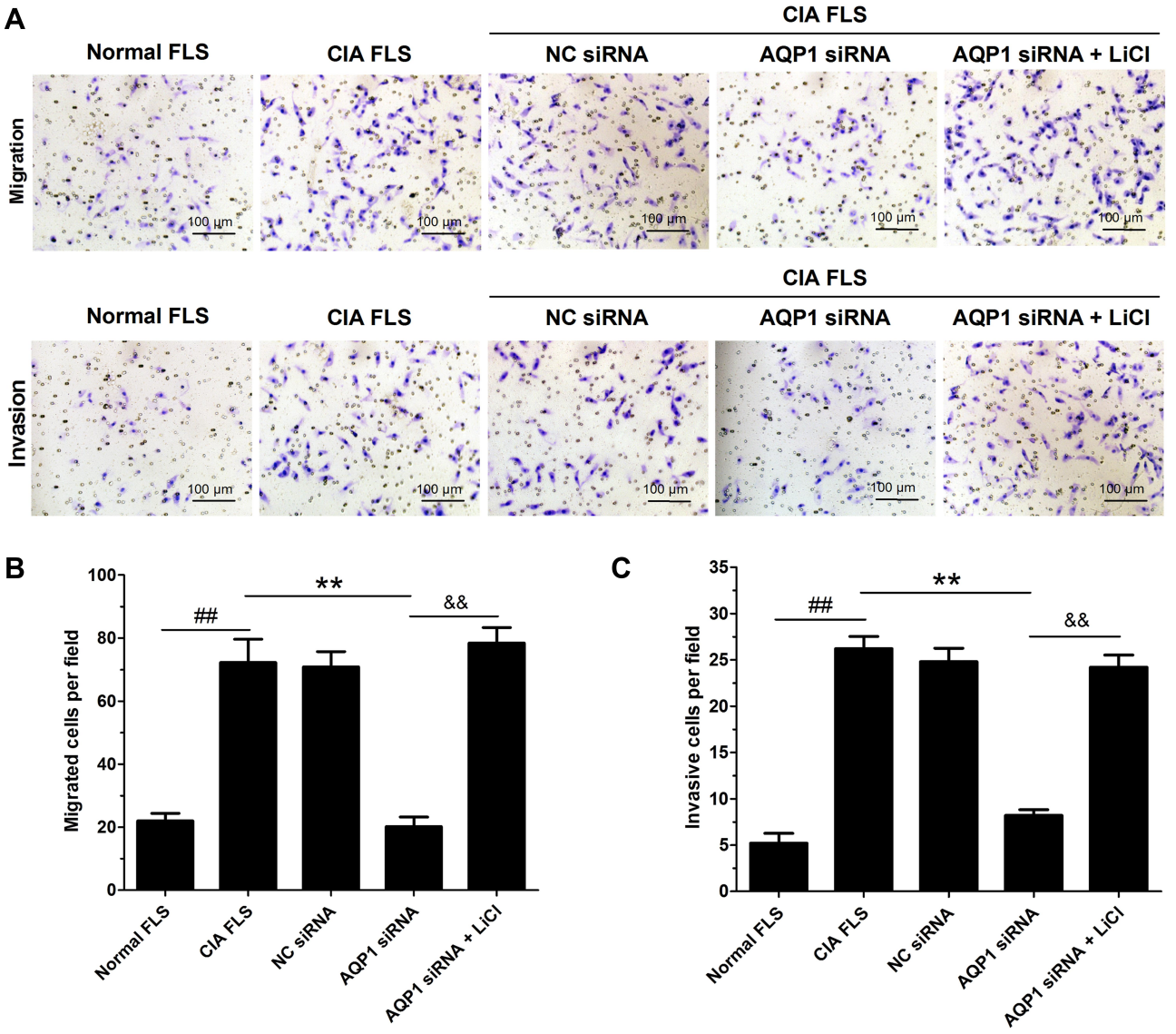

Figure 6 Suppression of CIA FLS migration and invasion by AQP1 siRNA was canceled by LiCl. (**A**) Typical photos of cells that crossed the membrane coated without or with matrigel, measured by transwell assay for detecting migration or invasion (×100). (**B**) Histogram shows numbers of migrated cells per microscopic field. (**C**) Histogram shows numbers of invasive cells per microscopic field. The data are mean ± SEM of three independent experiments performed in triplicate. $^{\#\#}P < 0.01$ compared with normal FLS group. $^{**}P < 0.01$ compared with CIA FLS group. $^{\&\&}P < 0.01$ compared with AQP1 siRNA group.

Abbreviations: AQP1, aquaporin 1; CIA, collagen-induced arthritis; FLS, fibroblast-like synoviocyte; LiCl, lithium chloride; NC, negative control; siRNA, small interfering RNA.

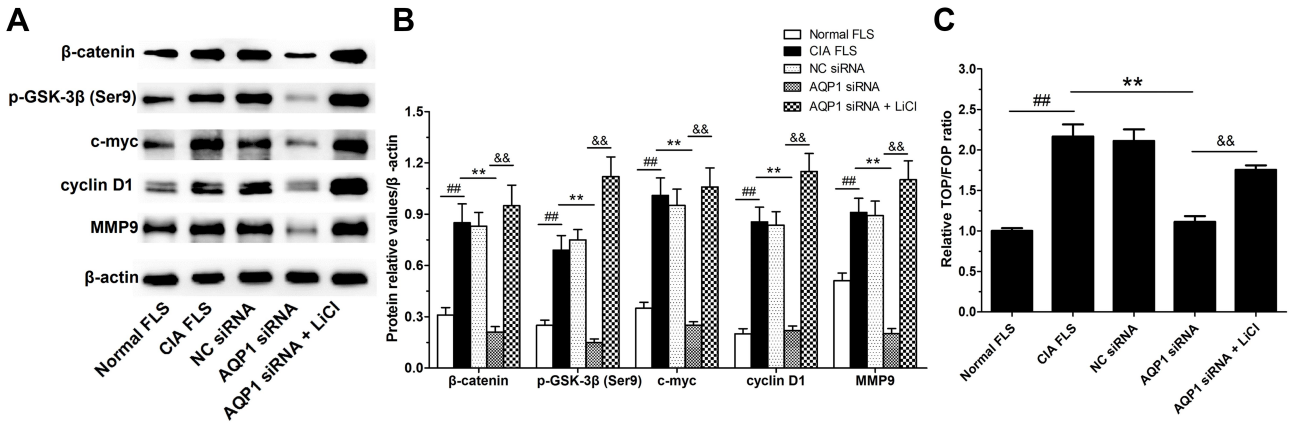

Figure 7 AQP1 siRNA inhibited the activation of β-catenin signaling in CIA FLS. (**A**) The protein expressions of β-catenin signaling pathway members such as β-catenin, p-GSK-3β (Ser9), c-myc, cyclin D1 and MMP9, detected by Western blot analysis. (**B**) The quantitative analysis of protein relative values, β-actin serves as house-keeping protein. (**C**) The ratio of TOP/FOP, an indicator of the activity of TCF/LEF transcription factors. The data are mean ± SEM of three independent experiments performed in triplicate. $^{\#\#}P < 0.01$ compared with normal FLS group. $^{**}P < 0.01$ compared with CIA FLS group. $^{\&\&}P < 0.01$ compared with AQP1 siRNA group.

Abbreviations: AQP1, aquaporin 1; CIA, collagen-induced arthritis; FLS, fibroblast-like synoviocyte; FOP, T cell factor (TCF) reporter plasmid containing mutated TCF binding site; LiCl, lithium chloride; MMP9, matrix metalloproteinase 9; NC, negative control; p-GSK-3β, phosphor-GSK-3β; siRNA, small interfering RNA; TOP, T cell factor (TCF) reporter plasmid containing TCF binding site.

levels of β-catenin signaling pathway members. As shown in Figure 7C, the ratio of TOP/FOP in CIA FLS was significantly increased when compared with that in normal FLS, further indicating the activation of β-catenin signaling in CIA FLS, whereas AQP1 siRNA apparently inhibited the ratio of TOP/FOP in contrast to CIA FLS group. As we expected, LiCl canceled the inhibitory effect of AQP1 siRNA on β-catenin signaling in CIA FLS.

Discussion

Rat CIA model shares many pathological changes of RA, such as joint damage, extremities swelling and synovial inflammation, highlighting the value of this animal model for studying RA pathology and therapeutic drugs.[15–17] In this study, rat CIA model was successfully set up, evidenced by increased secondary hind paw swelling and the pathological changes of ankle joints. We revealed that there existed the overexpression of AQP1 in synovial tissues from CIA rats, consistent with previous findings that synovial AQP1 expression was up-regulated in RA joints compared to normal and OA joints.[4,5] Particularly, we verified that synovial AQP1 expression of CIA rats increased in parallel with the secondary paw swelling and total pathological score on ankle joint damage, indicating that the synovial AQP1 overexpression was associated with the severity of rat CIA.

Increasing evidence has revealed that Wnt/β-catenin signaling is involved in RA pathogenesis, and its inhibition may be an effective treatment for RA.[10] β-catenin is an important regulator in Wnt/β-catenin pathway and β-catenin as a transcriptional co-activator can bind with T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factor family,[24] causing target gene transcriptions (e.g., c-myc, cyclin D1 and MMPs) and regulating cell proliferation, migration and invasion.[25] Interestingly, the cross-talk between AQPs and β-catenin signaling has been verified in many types of normal and tumor cells.[11,12,20] AQP1 can co-immunoprecipitate with β-catenin and FAK or lin7 to form stable complex. Then, AQP1 improves β-catenin protein level through reducing the ubiquitination and degradation of β-catenin and finally activates β-catenin signaling.[11,12] GSK-3β can promote the phosphorylation of β-catenin and the phosphorylated β-catenin can be degraded by the ubiquitin/proteasome pathway. A recent study has indicated that AQP3 overexpression can increase p-GSK-3β (Ser9) protein levels and inactivate GSK-3β, causing β-catenin accumulation in nucleus and the activation of β-catenin signaling.[20] Given the verified interaction of AQPs and β-catenin signaling in other diseases, we hypothesized that β-catenin signaling would play an important role in the pathogenesis of AQP1 in RA. In this study, β-catenin was overexpressed in both CIA rat synovial tissues and cultured CIA FLS, similar to previous findings that β-catenin is highly-expressed in synovium and cultured RA FLS.[26,27] Importantly, correlation analysis of IHC results indicated that the synovial AQP1 expression was positively correlated with synovial β-catenin expression in CIA rats, implying that β-catenin signaling might be involved in the pathogenic role of AQP1 in rat CIA.

FLS, a cell type composed of synovial lining layer, is the crucial effector cell in inflammatory arthritic diseases including RA.[6] The activated FLS enhances the productions of inflammatory mediators and MMPs, eventually causing synovial inflammation and joint damage in RA patients.[28] RA FLS displays multiple tumor-like phenotypes, such as aggressive proliferation, apoptosis resistance, enhanced migration and invasion, all of which are critical factors of synovial hyperplasia and cartilage erosion caused by RA FLS.[6,28] The imbalance between proliferation and apoptosis of RA FLS leads to synovial hyperplasia.[29] RA FLS migration is partly responsible for spreading arthritis destruction to distant joints.[30] RA FLS invasion is considered to be as aggressive as tumor cells and RA FLS can invade articular cartilage even in absence of immune cells.[31] Therefore, inhibition of FLS activation highlights the therapeutic potential for RA. Previous studies have indicated that AQP1 can enhance cell proliferation, migration and invasion of certain classes of cancer cells.[7–9] Herein, coupled with an increased level of AQP1 protein, the cultured CIA FLS exhibited excessive proliferation, promoted migration and invasion, suggesting that AQP1 overexpression might enhance these abnormal behaviors of CIA FLS. On the contrary, we found that AQP1 knockdown by siRNA suppressed cell proliferation, migration and invasion of the cultured CIA FLS, accordant to previous reports describing a role of AQP1 siRNA or AQP1 inhibitor reducing the proliferation and migration of tumor cells.[32–34] Our findings provided new evidence of the potential association between AQP1 and the aggressive nature of RA FLS.

β-catenin signaling has been confirmed to be involved in the activation of RA FLS by regulating cell proliferation, migration and invasion.[10,35,36] To clarify the inhibitory effect of AQP1 siRNA on CIA FLS was mediated by β-catenin signaling, we observed whether AQP1 siRNA could suppress β-catenin signaling in CIA FLS. As expected, AQP1 siRNA significantly reduced the protein levels of β-catenin signaling pathway members, including β-catenin, p-GSK-3β (Ser9), c-myc, cyclin D1 and MMP9. The key step of β-catenin signaling activation is that β-catenin accumulated in nucleus regulating gene expression together with TCF/LEF transcription factor.[37] TOP/FOP luciferase assay was used to detect the transcriptional activity of TCF/LEF. TOP flash plasmid with TCF binding sequences in upstream of luciferase promoter regulates luciferase expression according to β-catenin activity, whereas FOP flash plasmid with mutated TCF binding sequences shows no dependence on β-catenin activity. The ratio of TOP/FOP was regarded as an activation indicator of β-catenin signaling.[38] In this study, AQP1 siRNA remarkably reduced the ratio of TOP/FOP in CIA FLS, further indicating its inhibitory effect on β-catenin signaling. LiCl as an inhibitor of GSK-3β can promote the phosphorylation of GSK-3β (Ser9) and increase the protein level of β-catenin, thus this compound is used as a classic activator of β-catenin signaling.[39] In the present study, LiCl abolished the inhibitory effect of AQP1 siRNA on CIA FLS proliferation, migration and invasion, associated with the recovery of the activity of β-catenin signaling. Our results suggested that AQP1 siRNA exhibited inhibitory effects on the activation of CIA FLS by suppressing β-catenin signaling.

In summary, we concluded that the synovial AQP1 overexpression aggravated rat CIA by promoting FLS activation through β-catenin signaling pathway. This study presents certain experimental evidence that AQP1 could be a therapeutic target for RA and AQP1 inhibitors might be considered as a kind of potential anti-rheumatic agent for RA therapy. However, in view of the fact that AQP1 can be expressed in various tissues including synovial tissue, inhibition of AQP1 might lead to a variety of systemic side effects. Further studies are needed to improve the targeting selectivity of AQP1 inhibitors to the inflamed synovium and reduce the possible adverse reactions.

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Disclosure

The authors declare that they have no conflicts of interest for this work.

References

1. Walsh DA, McWilliams DF. Mechanisms, impact and management of pain in rheumatoid arthritis. *Nat Rev Rheumatol*. 2014;10(10):581–592.
2. Keith MP, Edison JD, Gilliland WR. Progress toward personalized treatment of rheumatoid arthritis. *Clin Pharmacol Ther*. 2012;92(4):440–442. doi:10.1038/clpt.2012.124
3. Lampropoulos CE, Orfanos P, Bournia VK, et al. Adverse events and infections in patients with rheumatoid arthritis treated with conventional drugs or biologic agents: a real world study. *Clin Exp Rheumatol*. 2015;33(2):216–224.
4. Trujillo E, Gonzalez T, Marin R, et al. Human articular chondrocytes, synoviocytes and synovial microvessels express aquaporin water channels; upregulation of AQP1 in rheumatoid arthritis. *Histol Histopathol*. 2004;19(2):435–444.

5. Mobasheri A, Moskaluk CA, Marples D, et al. Expression of aquaporin 1 (AQP1) in human synovitis. *Ann Anat*. 2010;192(2):116–121. doi:10.1016/j.aanat.2010.01.001
6. Bartok B, Firestein GS. Fibroblast-like synoviocytes: key effector cells in rheumatoid arthritis. *Immunol Rev*. 2010;233(1):233–255.
7. Hoque MO, Soria JC, Woo J, et al. Aquaporin 1 is overexpressed in lung cancer and stimulates NIH-3T3 cell proliferation and anchorage-independent growth. *Am J Pathol*. 2006;168 (4):1345–1353. doi:10.2353/ajpath.2006.050596
8. Wei X, Dong J. Aquaporin 1 promotes the proliferation and migration of lung cancer cell in vitro. *Oncol Rep*. 2015;34(3):1440–1448. doi:10.3892/or.2015.4107
9. Chow PH, Kourghi M, Pei JV, et al. 5-hydroxymethyl-furfural and structurally related compounds block the ion conductance in human aquaporin-1 channels and slow cancer cell migration and invasion. *Mol Pharmacol*. 2020;98(1):38–48. doi:10.1124/mol.119.119172
10. Miao CG, Yang YY, He X, et al. Wnt signaling pathway in rheumatoid arthritis, with special emphasis on the different roles in synovial inflammation and bone remodeling. *Cell Signal*. 2013;25 (10):2069–2078. doi:10.1016/j.cellsig.2013.04.002
11. Monzani E, Bazzotti R, Perego C, et al. AQP1 is not only a water channel: it contributes to cell migration through Lin7/beta-catenin. *PLoS One*. 2009;4(7):e6167. doi:10.1371/journal.pone.0006167
12. Meng F, Rui Y, Xu L, et al. Aqp1 enhances migration of bone marrow mesenchymal stem cells through regulation of FAK and β-catenin. *Stem Cells Dev*. 2014;23(1):66–75. doi:10.1089/scd.2013.0185
13. Wang W, Li F, Sun Y, et al. Aquaporin-1 retards renal cyst development in polycystic kidney disease by inhibition of Wnt signaling. *FASEB J*. 2015;29(4):1551–1563. doi:10.1096/fj.14-260828
14. Yun X, Jiang H, Lai N, et al. Aquaporin 1-mediated changes in pulmonary arterial smooth muscle cell migration and proliferation involve beta-catenin. *Am J Physiol Lung Cell Mol Physiol*. 2017;313 (5):L889–L898. doi:10.1152/ajplung.00247.2016
15. Wang Q, Ye C, Sun S, et al. Curcumin attenuates collagen-induced rat arthritis via anti-inflammatory and apoptotic effects. *Int Immunopharmacol*. 2019;72:292–300. doi:10.1016/j.intimp.2019.04.027
16. Ahmad Khan M, Sarwar A, Rahat R, et al. Stigmasterol protects rats from collagen induced arthritis by inhibiting proinflammatory cytokines. *Int Immunopharmacol*. 2020;85:106642. doi:10.1016/j.intimp.2020.106642
17. Korani M, Jamshidi M. The effect of aqueous extract of trachyspermum ammi seeds and ibuprofen on inflammatory gene expression in the cartilage tissue of rats with collagen-induced arthritis. *J Inflamm Res*. 2020;13:133–139. doi:10.2147/JIR.S236242
18. Wang QT, Wu YJ, Huang B, et al. Etanercept attenuates collagen-induced arthritis by modulating the association between BAFFR expression and the production of splenic memory B cells. *Pharmacol Res*. 2013;68(1):38–45. doi:10.1016/j.phrs.2012.11.003
19. Jia W, Wu W, Yang D, et al. Histone demethylase JMJD3 regulates fibroblast-like synoviocyte-mediated proliferation and joint destruction in rheumatoid arthritis. *FASEB J*. 2018;32(7):4031–4042. doi:10.1096/fj.201701483R
20. Zhou Y, Wang Y, Wen J, et al. Aquaporin 3 promotes the stem-like properties of gastric cancer cells via Wnt/GSK-3beta/beta-catenin pathway. *Oncotarget*. 2016;7(13):16529–16541. doi:10.18632/oncotarget.7664
21. Huang XY, Zhang XM, Chen FH, et al. Anti-proliferative effect of recombinant human endostatin on synovial fibroblasts in rats with adjuvant arthritis. *Eur J Pharmacol*. 2014;723:7–14. doi:10.1016/j.ejphar.2013.10.068
22. Cai L, Li C-M, Chen W-N, et al. Penta-acetyl geniposide induces apoptosis of fibroblast-like synoviocytes from adjuvant-induced arthritis rats in vitro, associated with inhibition of NF-kappaB activation. *Pharmacol Rep*. 2019;71(6):1006–1013. doi:10.1016/j.pharep.2019.05.014
23. Takemaru K, Yamaguchi S, Lee YS, et al. Chibby, a nuclear β-catenin -associated antagonist of the Wnt/Wingless pathway. *Nature*. 2003;422(6934):905–909. doi:10.1038/nature01570
24. Clevers H, Nusse R. Wnt/beta-catenin signaling and disease. *Cell*. 2012;149(6):1192–1205. doi:10.1016/j.cell.2012.05.012
25. MacDonald BT, Tamai K, He X. Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Dev Cell*. 2009;17(1):9–26. doi:10.1016/j.devcel.2009.06.016
26. Sen M, Reifert J, Lauterbach K, et al. Regulation of fibronectin and metalloproteinase expression by Wnt signaling in rheumatoid arthritis synoviocytes. *Arthritis Rheum*. 2002;46(11):2867–2877. doi:10.1002/art.10593
27. Xiao CY, Pan YF, Guo XH, et al. Expression of beta-catenin in rheumatoid arthritis fibroblast-like synoviocytes. *Scand J Rheumatol*. 2011;40(1):26–33. doi:10.3109/03009742.2010.486767
28. Mor A, Abramson SB, Pillinger MH. The fibroblast-like synovial cell in rheumatoid arthritis: a key player in inflammation and joint destruction. *Clin Immunol*. 2005;115(2):118–128. doi:10.1016/j.clim.2004.12.009
29. Smith MD, Weedon H, Papangelis V, et al. Apoptosis in the rheumatoid arthritis synovial membrane: modulation by disease-modifying anti-rheumatic drug treatment. *Rheumatology (Oxford)*. 2010;49 (5):862–875. doi:10.1093/rheumatology/kep467
30. Lefevre S, Knedla A, Tennie C, et al. Synovial fibroblasts spread rheumatoid arthritis to unaffected joints. *Nat Med*. 2009;15 (12):1414–1420.
31. Karouzakis E, Gay RE, Gay S, et al. Epigenetic control in rheumatoid arthritis synovial fibroblasts. *Nat Rev Rheumatol*. 2009;5(5):266–272. doi:10.1038/nrrheum.2009.55
32. Klebe S, Griggs K, Cheng Y, et al. Blockade of aquaporin 1 inhibits proliferation, motility, and metastatic potential of mesothelioma in vitro but not in an in vivo model. *Dis Markers*. 2015;2015:286719. doi:10.1155/2015/286719
33. Wu Z, Li S, Liu J, et al. RNAi-mediated silencing of AQP1 expression inhibited the proliferation, invasion and tumorigenesis of osteosarcoma cells. *Cancer Biol Ther*. 2015;16(9):1332–1340. doi:10.1080/15384047.2015.1070983
34. Dorward HS, Du A, Bruhn MA, et al. Pharmacological blockade of aquaporin-1 water channel by AqB013 restricts migration and invasiveness of colon cancer cells and prevents endothelial tube formation in vitro. *J Exp Clin Cancer Res*. 2016;35:36. doi:10.1186/s13046-016-0310-6
35. Sen M, Carson DA. Wnt signaling in rheumatoid synoviocyte activation. *Mod Rheumatol*. 2020;Volume 13(1):5–9. doi:10.3109/s101650200001
36. Sun J, Yan P, Chen Y, et al. MicroRNA-26b inhibits cell proliferation and cytokine secretion in human RASF cells via the Wnt/GSK-3beta/beta-catenin pathway. *Diagn Pathol*. 2015;10:72. doi:10.1186/s13000-015-0309-x
37. Luo W, Yan D, Song Z, et al. miR-126-3p sensitizes glioblastoma cells to temozolomide by inactivating Wnt/beta-catenin signaling via targeting SOX2. *Life Sci*. 2019;226:98–106. doi:10.1016/j.lfs.2019.04.023
38. Yang HC, Wang JY, Bu XY, et al. Resveratrol restores sensitivity of glioma cells to temozolamide through inhibiting the activation of Wnt signaling pathway. *J Cell Physiol*. 2019;234(5):6783–6800. doi:10.1002/jcp.27409
39. Liu J, Ju P, Zhou Y, et al. Six2 is a coordinator of LiCl-induced cell proliferation and apoptosis. *Int J Mol Sci*. 2016;17(9):1504. doi:10.3390/ijms17091504