Journal of Inflammation Research

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ORIGINAL RESEARCH

Lentivirus-Mediated Overexpression or Silencing of Aquaporin 1 Affects the Proliferation, Migration and Invasion of TNF-α-Stimulated Rheumatoid Arthritis Fibroblast-Like Synoviocytes by Wnt/β-Catenin Signaling Pathway

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Introduction: Previous studies have confirmed the pathologic role of synovial aquaporin 1 (AQP1) in rheumatoid arthritis (RA), but its associations with the abnormal biologic behaviors of fibroblast-like synoviocytes (FLS) remain unclear. Herein, we examined the roles of AQP1 in the proliferation, migration and invasion of TNF-α-stimulated RA FLS (MH7A cells) and explored the underlying mechanisms.

Materials and Methods: Lentivirus-mediated AQP1 overexpression or silencing MH7A cells was constructed. Assays of MTT, flow cytometry (PI staining and Annexin V-PE/7-AAD staining), TMRM staining, wound-healing, transwell and phalloidin staining were performed to detect cell proliferation, cycle distribution, apoptosis, migration and invasion. The involvement of Wnt/β-catenin pathway was revealed by Western blot and β-catenin immunofluorescence staining.

Results: AQP1 overexpression promoted cell proliferation of TNF-α-stimulated MH7A by facilitating transformation from G0/G1 to S phase and inhibiting cell apoptosis (ie, reduced apoptosis rates, raised mitochondrial membrane potential, increased Bcl-2 protein level and decreased levels of Bax and cleaved caspase 3 protein). Also, AQP1 overexpression increased the migration index as well as the numbers of migrated and invasive cells. Furthermore, AQP1 overexpression promoted the activation of Wnt/β-catenin pathway, and XAV939, an inhibitor of Wnt/β-catenin, canceled the above effects of AQP1 overexpression on MH7A cells. As expected, AQP1 silencing exhibited the opposite effects on TNF-α-stimulated MH7A cells, which could be reversed by LiCl, an activator of Wnt/β-catenin.

Conclusion: AQP1 can affect the proliferation, migration and invasion of MH7A cells by Wnt/β-catenin signaling pathway, and AQP1 can be as a crucial determiner that can regulate RA FLS biologic behaviors.

Keywords: aquaporin 1, rheumatoid arthritis, fibroblast-like synoviocytes, Wnt/β-catenin signaling pathway, biologic behaviors

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Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by synovial hyperplasia, joint inflammation, cartilage damage and bone destruction.[1,2] About 1% of adults worldwide suffer from RA, causing huge

Received: 25 March 2021
Accepted: 23 April 2021
Published: 14 May 2021

economic losses and heavy burdens to patients.[3] Although the pathogenic mechanism of RA is not fully understood, increasing evidence has revealed that fibroblast-like synoviocytes (FLS) plays a critical role in RA pathogenesis.[4] RA FLS as the main cell type of synovial lining layer has a lot of tumor-like biologic behaviors, such as aggressive proliferation, apoptosis resistance, enhanced abilities of migration and invasion.[5] The imbalance between proliferation and apoptosis of RA FLS contributes to the massive synovial hyperplasia.[6] The elevated RA FLS migration to cartilage or bone and the following invasion of extracellular matrix are the key events in RA joint destruction.[7] Therefore, inhibition of the proliferation, migration and invasion of RA FLS may be a promising strategy for RA treatment. However, the molecular mechanisms involved in RA FLS activation remain unclear. Searching novel factors or mechanisms that can regulate the biologic behaviors of RA FLS is urgently needed for RA.

Aquaporins (AQPs), a family of membrane transport proteins, can participate in regulating the influx and outflow of water or small molecules.[8] AQP1 as an important member of mammalian AQPs can be expressed in many organs and tissues including RA synovial tissues.[9] In addition to controlling water influx and outflow, AQP1 can play important roles in regulating various biologic processes, such as cell proliferation, migration, invasion and inflammatory response.[10–12] Increasing evidence reveals that AQP1 overexpression in RA synovium contributes to hydrarthrosis formation, joint swelling and synovial inflammation.[13] Recently, we reported that the elevation of synovial AQP1 worsened the severity of rat collagen-induced arthritis (CIA) by facilitating the aggressive phenotype of CIA FLS, and AQP1 siRNA could inhibit the proliferation, migration and invasion of cultured CIA FLS, as implying the possible relationships between AQP1 and the biologic behaviors of RA FLS.[14] However, there is absence of direct evidence whether AQP1 can affect RA FLS biologic processes and further studies are still needed. It is well known that Wnt/β-catenin signaling pathway is activated in synovium of RA patients and experimental RA animals as well as in cultured RA FLS,[15,16] and this pathway is closely involved in regulating the biologic behaviors of RA FLS.[17] The interactions of AQP1 and Wnt/β-catenin pathway in many diseases have been revealed in studies from ours and other groups.[12,14,18] However, it is still unknown whether Wnt/β-catenin pathway is implicated in the regulation of AQP1 on the biologic behaviors of RA FLS.

In this study, we revealed the effects of lentivirus-mediated AQP1 overexpression or silencing on proliferation, apoptosis, migration and invasion of TNF-α-stimulated RA FLS (MH7A) and explored the mechanisms related to Wnt/β-catenin pathway. We intended to provide direct evidence that AQP1 can regulate the biologic behaviors of RA FLS by Wnt/β-catenin pathway, and the inhibition of AQP1 may be a prospective strategy for the treatment of RA.

Materials and Methods

Reagents

TNF-α was purchased from Peprotech (Rocky Hill, NJ, USA). MTT and lithium chloride (LiCl) was bought from Sigma-Aldrich (St. Louis, MO, USA). XAV939 was got from MedChem Express (Newark, NJ, USA). Tetramethyl rhodamine methyl ester (TMRM) was bought from BestBio Company (Shanghai, China). Propidium iodide (PI) and fluorescent phalloidin were purchased from Beyotime Biotechnology (Haimen, Jiangsu, China). Transwell filter and Annexin V-PE/7-ADD kit were bought from BD Biosciences (USA). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco (Carlsbad, CA, USA). Antibodies of AQP1 (ab9566), Bax (ab32503), Bcl-2 (ab182858), cleaved caspase 3 (ab2302), c-myc (ab32072), MMP-2 (ab92536), MMP-9 (ab76003), Wnt1 (ab15251) and β-catenin (ab32572) were bought from Abcam (Cambridge, UK). Antibodies of cyclin D1 (#55506), GSK-3β (#12456) and p-GSK-3β (Ser9) (#9323) were purchased from Cell Signaling Technology (Beverly, MA, USA).

Cell Culture, Lentivirus Transduction and Generation of Stable Cell Lines

RA FLS (MH7A cells) were obtained from Jennio Biotech Co., Ltd. (Guangzhou, China) and cultured in DMEM at 37°C and 5% CO_2. After 70–80% confluence, the adherent cells were trypsinized, split at a 1:2 ratio and cultured. Human AQP1 (NM_198098.3) lentivirus (LV-AQP1), negative control (LV-AQP1 NC), shRNA lentivirus targeting AQP1 (LV-shAQP1) and scrambled shRNA lentivirus (LV-shRNA NC) were purchased from Hanheng Biotechnology (Shanghai, China). All lentiviruses were labeled with green fluorescent protein (GFP). The target sequence of AQP1 was 5′-CCAGCGAGTTCAAGAA GAAGCTCTT-3′ for shAQP1, and 5′-TTCTCCGAA CGTGTCACGTAA-3′ for shRNA-NC. Lentivirus

transduction and stable cell line selection were performed as previously described.[19] Briefly, MH7A cells were seeded in a 6-well plate (1×10^5 cells/well) for adherence. Then, the cells were transfected with lentivirus vectors at a multiplicity of infection (MOI) of 30 in the presence of 5 μg/mL polybrene. After 72 h of transduction, puromycin (8 μg/mL) was added and stable cell lines were screened for 3 weeks. The infection efficiency was determined by the fluorescence intensity of GFP and Western blot assay.

Cell Groupings and Treatment

The cells were divided into five groups, including control group (non-transfected MH7A), LV-AQP1 NC group (LV-AQP1 NC transfected MH7A), LV-AQP1 group (LV-AQP1 transfected MH7A), LV-shRNA NC group (LV-shRNA NC transfected MH7A) and LV-shAQP1 group (LV-shAQP1 transfected MH7A). MH7A cells in all groups were stimulated with TNF-α (10 ng/mL) before the specific assays. In those experiments of intervening Wnt/β-catenin pathway, the cells were divided into three groups, including LV-AQP1 NC group, LV-AQP1 group, XAV939 (40 μM) group (LV-AQP1 transfected MH7A + XAV939) or LV-shRNA NC group, LV-shAQP1 group, LiCl (10 mmol/L) group (LV-shAQP1 transfected MH7A + LiCl).

Cell Proliferation Assay

MTT assay was performed to evaluate cell proliferation. MH7A cells were seeded in a 96-well plate (5×10^3 cells/well) for adherence. Then, MH7A cells were cultured for 24 h or 48 h with proper treatments according to the groupings in the presence of TNF-α. 4 h before the end of cultivation, 20 μL of MTT (5 mg/mL) was added into each well for further incubation. After the removal of the supernatants, DMSO (150 μL/well) was added to fully dissolve the formazan crystals. A microplate reader was applied to measure the absorbance at 490 nm. The cell viability values were defined as the ratios of the corresponding control group.

Cell Cycle Analysis

Cell cycle distributions were detected by flow cytometer using PI staining method. MH7A cells were seeded in 6-well plate (1×10^5 cells/well) for adherence. After 24 h of synchronization in serum-free medium, MH7A cells were cultured for another 48 h in the presence of TNF-α. MH7A cells were harvested, suspended in PBS, centrifuged and fixed with 75% ethanol and stored at 4°C for 12 h. The cells were stained with PI solution adding RNase A for 30 min in dark at 37°C. The stained cells were analyzed by flow cytometer (Beckman Coulter, Brea, CA, USA), and the percentages of cells in G0/G1, S and G2/M phases were analyzed by MODFIT LT software programs.

Annexin V-PE/7-AAD Double Staining Analysis

The apoptosis assay was detected by flow cytometer using Annexin V-PE/7-AAD staining method. Briefly, MH7A cells were seeded in 6-well plate (1×10^5 cells/well) for adherence, and cultured for 48 h with proper treatments according to groupings in the presence of TNF-α. MH7A cells were harvested, washed by PBS, centrifuged and suspended in binding buffer. Then, 5 μL of Annexin V-PE and 5 μL of 7-AAD were added to 400 μL of cell suspension, and the mixed solution was incubated for 15 min at a room temperature in dark. The samples were analyzed by flow cytometer and the percentages of various cell subpopulations were analyzed using FlowJo 7.6 software.

Mitochondrial Membrane Potential Analysis

The mitochondrial membrane potential was detected by the staining of TMRM, a cell-permeant cationic lipophilic red fluorescent dye. MH7A cells were seeded in 6-well plate (1×10^5 cells/well) for 48 h-cultivation in the presence of TNF-α. Then, the cells were incubated with TMRM staining solution (200 nM) at 37°C for 30 min. The red fluorescence was examined by a fluorescent microscope and typical photos were taken. Moreover, cells with TMRM staining were detected by flow cytometry and the experimental data were analyzed using FlowJo software.

Wound-Healing Assay

Wound-healing assay was performed to detect the cellular migration ability. MH7A were seeded in a 6-well plate (1×10^5 cells/well) and cultured with proper treatments according to groupings in presence of TNF-α. When reaching to 90–100% confluence, a line within cells was softly scraped by sterile micropipette tips in each well. After the removal of cellular debris, the cells were cultured in serum-free medium adding TNF-α for another 24 h. Typical images were taken at 0 h (the initial) and 24 h (the end). Relative migration index was defined as

follows: [(the scratch width at 0 h)-(the scratch width at 24 h)]/(the scratch width at 0 h).

Transwell Cell Migration and Invasion Assay

Transwell chambers (8 μm) were used to measure cellular migration and invasion. MH7A suspended in serum-free medium were added into the upper chamber (5×10^4 cells/well). The medium containing 10% FBS as an inducer were added to the lower chamber. After 24 h of cultivation with proper treatments according to the groupings in the presence of TNF-α, the residual cells on the upper chamber were softly wiped off using cotton swabs, and the migrated cells on the lower side of the membrane were fixed by 4% paraformaldehyde and stained by 0.1% crystal violet. The migrated cell numbers were counted at five random regions per well under a microscope and typical photos were taken. In the case of cell invasion assay, similar experiments were performed by the transwell chambers, but pre-coated with the matrigel matrix.

Immunofluorescence Assay for β-Catenin and Phalloidin Staining for F-Actin

MH7A cells were seeded onto a cover glass placed in a 6-well plate. In the process of β-catenin immunofluorescence assay, the cells were fixed by paraformaldehyde, permeated by TritonX-100 and blocked by goat serum. Then, the cells were incubated with rabbit anti-β-catenin overnight at 4°C, incubated with TRITC-conjugated goat anti-rabbit IgG (1:100) for 1 h at room temperature, and counterstained using DAPI (1 μg/mL) for 5 min in dark. In the case of phalloidin staining, MH7A were incubated by fluorescent phalloidin and counterstained with DAPI. The cover glass was put on slide and mounted by the antifade mounting medium. The fluorescence was detected by a fluorescent microscope and typical photos were taken.

Western Blot Assay

MH7A cells were treated with RIPA lysis buffer adding protease inhibitors and the proteins were isolated with an extraction kit. Bradford assay was performed to detect the protein levels in supernatants. The proteins were separated by 10% SDS-PAGE electrophoresis and then transferred to polyvinylidene fluoride (PVDF) membrane. The PVDF membranes were blocked with 5% skim milk at room temperature for 2 h and incubated with the primary antibodies overnight at 4°C. After thorough washing with TBST buffer, the PVDF membranes were incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies at 37°C for 2 h. The protein bands were visualized by chemiluminescence with the enhanced chemiluminescent detection kit (Thermo Scientific, PA, USA) and quantified by ImageJ software.

Statistical Analysis

Statistical analysis was performed by SPSS 19.0 software and graphs were generated using GraphPad Prism 8.0 software. The experimental data were shown as mean ± standard error of the mean (SEM). Statistical analysis between groups was carried out by ANOVA, followed by Tukey's HSD post hoc test, and $P < 0.01$ was considered to be statistically significant.

Results

Efficiency of Lentivirus-Mediated AQP1 Overexpression or Silencing in MH7A

As shown in Figure 1A, most MH7A cells showed GFP-positive signals at 72 h after transfection, indicating that the lentiviruses infected MH7A with high efficiency. After 3 rounds of puromycin addition, AQP1 protein level was detected by Western blot to further confirm the efficiency of AQP1 overexpression or RNAi lentivirus in MH7A cells. AQP1 protein level in LV-AQP1 group was increased by 71% compared with that in LV-AQP1 NC group (Figure 1B, $P < 0.01$), and AQP1 protein level in LV-shAQP1 group was decreased by 85% compared with that in LV-shRNA NC group (Figure 1C, $P < 0.01$). LV-AQP1 NC or LV-shRNA NC transfection showed no effect on AQP1 expression compared with the non-transfected control group.

Effects of AQP1 Overexpression or Silencing on Proliferation and Cell Cycle of TNF-α-Stimulated MH7A Cells

MTT results showed that AQP1 overexpression led to the significant elevations of cell proliferation of TNF-α-stimulated MH7A at 24 h and 48 h when compared with LV-AQP1 NC group, while AQP1 silencing caused the remarkable reductions of cell proliferation of TNF-α-stimulated MH7A at 24 h and 48 h as contrasted to LV-shRNA NC group (Figure 2A and B). In Figure 2C and D, results of flow cytometry using PI staining method indicated that AQP1 overexpression reduced G0/G1 phase population and increased S phase population of TNF-α-stimulated MH7A

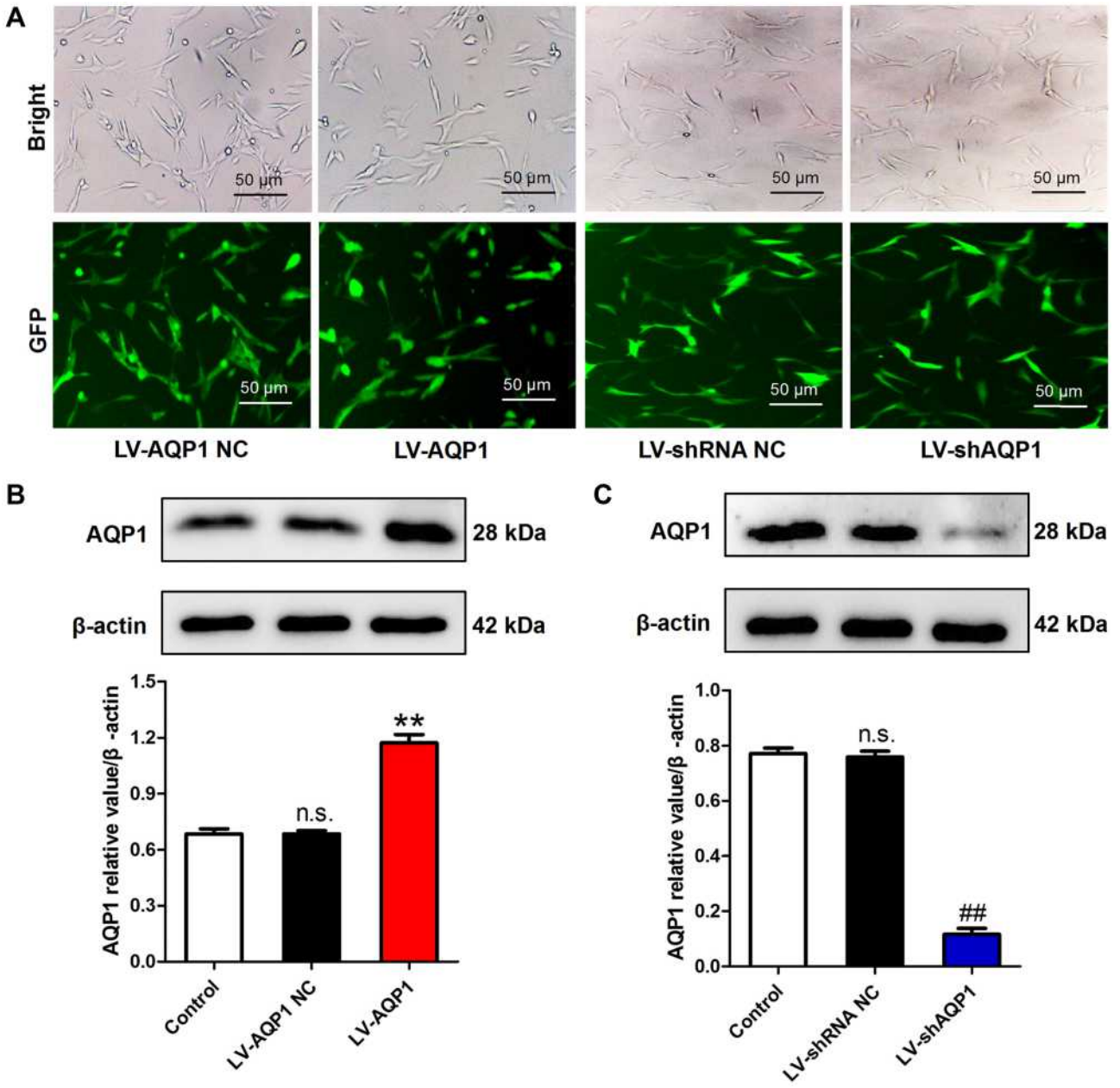

Figure 1 Efficiency of lentivirus-mediated AQP1 overexpression or silencing in MH7A cells. (**A**) GFP expression was observed in the infected MH7A cells by a fluorescence microscopy at 72 h after infection (×100). (**B**) Results of Western blot revealed the increased AQP1 expression in LV-AQP1 group. (**C**) Results of Western blot revealed the reduced AQP1 expression in LV-shAQP1 group. The data are mean ± SEM of three independent experiments performed in triplicate. n.s. compared with non-transfected control group. $^{**}P < 0.01$ compared with LV-AQP1 NC group. $^{\#\#}P < 0.01$ compared with LV-shRNA NC group.
Abbreviation: n.s., no significant difference.

cells, facilitating transformation from G0/G1 phase to S phase. Contrarily, AQP1 silencing increased G0/G1 phase population and reduced S phase population, causing G0/G1 phase arrest. There were no differences in the cell population in G2/M phase among various groups. LV-AQP1 NC or LV-shRNA NC transfection showed no effect on cell proliferation and cell cycle distribution in TNF-α-stimulated MH7A cells.

Effects of AQP1 Overexpression or Silencing on Apoptosis of TNF-α-Stimulated MH7A Cells

The results of flow cytometry assay using Annexin V-PE/7-AAD staining method showed that the apoptosis rate of TNF-α-stimulated MH7A cells in LV-AQP1 group was

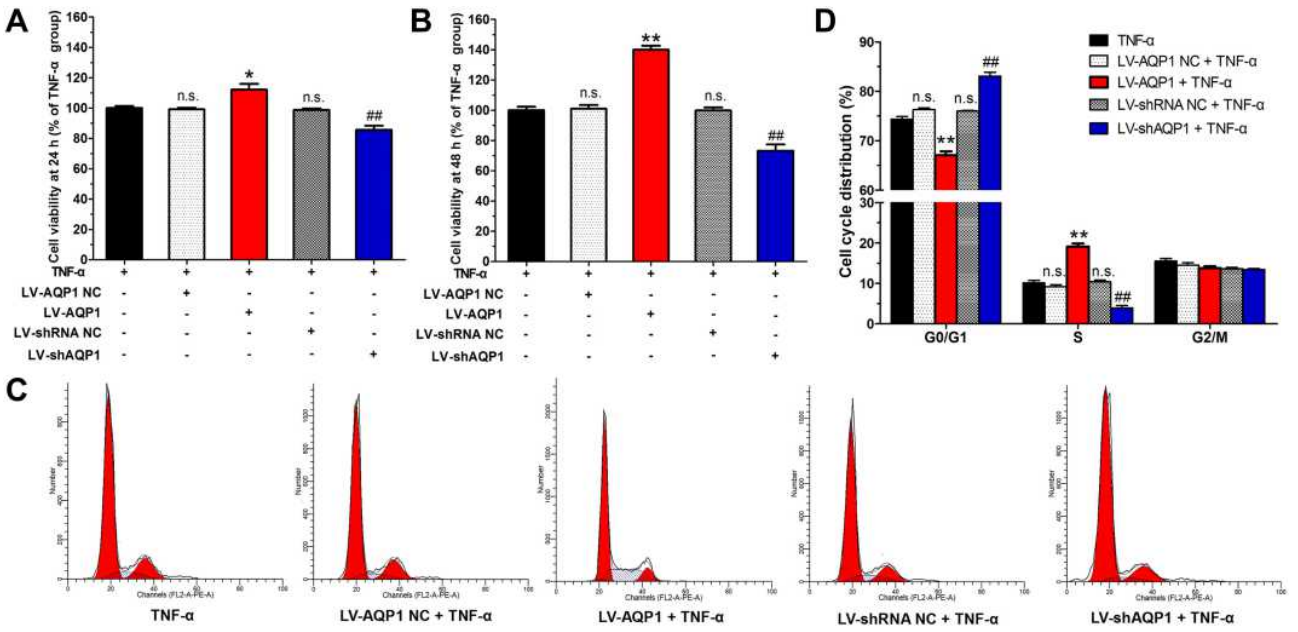

Figure 2 Effects of AQP1 overexpression or silencing on proliferation and cell cycle of TNF-α-stimulated MH7A cells. (**A**) Cell viability at 24 h (MTT assay). (**B**) Cell viability at 48 h (MTT assay). (**C**) Typical graphs of cell cycle distribution in various groups, detected by flow cytometer using PI staining method. (**D**) Cell population in each cell cycle stage (%). The data are mean ± SEM of three to five independent experiments performed in triplicate. n.s. compared with non-transfected control group. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with LV-AQP1 NC group. $^{\#\#}P < 0.01$ compared with LV-shRNA NC group.
Abbreviation: n.s., no significant difference.

markedly decreased compared with that in LV-AQP1 NC group, while the apoptosis rate in LV-shAQP1 group was apparently increased contrasted to that in LV-shRNA NC group (Figure 3A and B). The red fluorescence intensity of TMRM staining was detected to represent mitochondrial membrane potential, an important index to reflect the mitochondrial function. As shown in Figure 3C, AQP1 overexpression increased the mitochondrial dye aggregation featured by the strong fluorescence intensity, while AQP1 silencing reduced the mitochondrial dye aggregation featured by the weak fluorescence intensity. In Figure 3D, the quantitative results revealed that AQP1 overexpression significantly increased the mitochondrial membrane potential contrasted to LV-AQP1 NC group, but AQP1 silencing apparently reduced the mitochondrial membrane potential compared with LV-shRNA NC group, as indicating that AQP1 can affect the stabilization of mitochondrial membrane potential and the mitochondria function in MH7A cells. In Figure 3E and F, AQP1 overexpression raised the level of anti-apoptotic protein Bcl-2, but reduced the levels of pro-apoptotic proteins Bax and cleaved caspase 3 in TNF-α-stimulated MH7A cells. As we expected, AQP1 silencing showed the opposite effects on these apoptosis-related proteins. Furthermore, the ratio of Bcl-2/Bax protein in LV-AQP1 group was much higher than that in LV-AQP1 NC group, while the ratio of Bcl-2/Bax protein in LV-shAQP1 group was much lower than that in LV-shRNA NC group (Figure 3G). LV-AQP1 NC or LV-shRNA NC transfection had no effects on cell apoptosis and apoptosis-related protein expressions in TNF-α-stimulated MH7A.

Effects of AQP1 Overexpression or Silencing on Migration and Invasion of TNF-α-Stimulated MH7A Cells

The results of wound-healing assay revealed that AQP1 overexpression markedly increased the migration index of TNF-α-stimulated MH7A cells when compared with LV-AQP1 NC group, but AQP1 silencing significantly reduced the migration index as contrasted to LV-shRNA NC group (Figure 4A and B). The results of transwell assay indicated that the numbers of migrated and invasive cells in LV-AQP1 group were larger than those in LV-AQP1 NC group, while the numbers of migrated and invasive cells in LV-shAQP1 group were smaller than those in LV-shRNA NC group (Figure 4C–F). The fluorescent phalloidin staining was carried out to observe the dynamic reorganization of F-actin, which is beneficial to cell migration and invasion. In Figure 4G, AQP1 overexpression caused the elevation of F-actin expression, the abnormality of

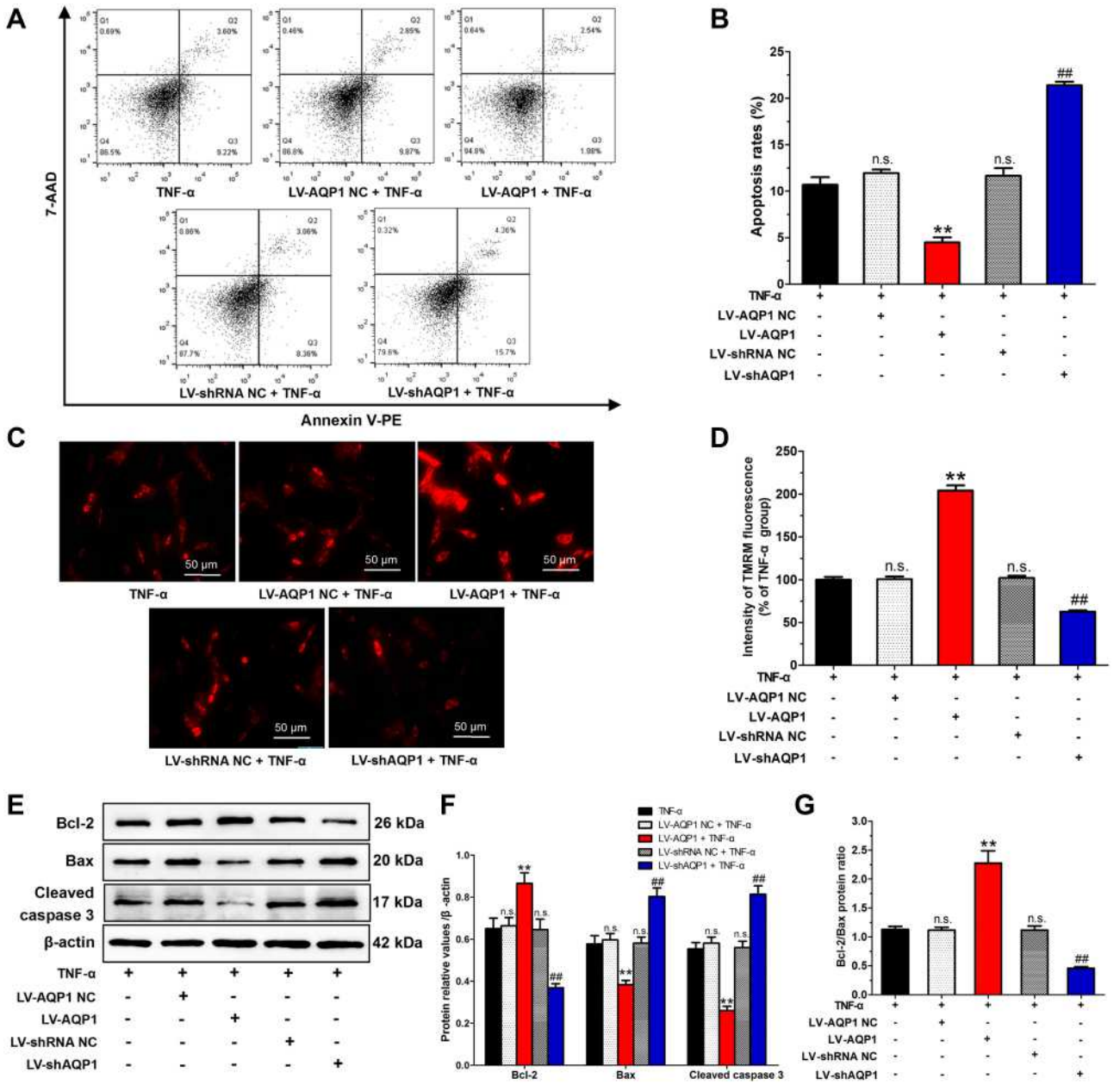

Figure 3 Effects of AQP1 overexpression or silencing on the apoptosis of TNF-α-stimulated MH7A cells. (**A**) Typical examples of cell apoptosis in various groups, detected by flow cytometer using Annexin V-PE/7-AAD staining method. (**B**) Histogram of apoptosis rates (%). (**C**) Typical images of TMRM staining (red) from various groups (×100). (**D**) Histogram of intracellular TMRM fluorescence intensity. (**E**) Typical examples of Bcl-2, Bax, cleaved caspase 3 protein expressions from various groups (Western blot). (**F**) Quantitative results of protein values, β-actin serves as housekeeping protein. (**G**) Histogram of the ratios of Bcl-2 to Bax protein. The data are mean ± SEM of three independent experiments performed in triplicate. n.s. compared with non-transfected control group. $^{**}P < 0.01$ compared with LV-AQP1 NC group. $^{\#\#}P < 0.01$ compared with LV-shRNA NC group.

Abbreviation: n.s., no significant difference.

fiber morphology and arrangement as well as the formation of protuberance and pseudopodia in TNF-α-stimulated MH7A cells. However, the F-actin expression was low in LV-shAQP1 group, with almost no formation of protuberance or pseudopodia. LV-AQP1 NC or LV-shRNA NC transfection had no effects on migration, invasion and F-actin cytoskeletal reorganization of MH7A cells.

Effects of AQP1 Overexpression or Silencing on Wnt/β-Catenin Signaling Pathway in TNF-α-Stimulated MH7A Cells

Western blot results indicated that AQP1 overexpression remarkably increased the protein levels of Wnt/β-catenin

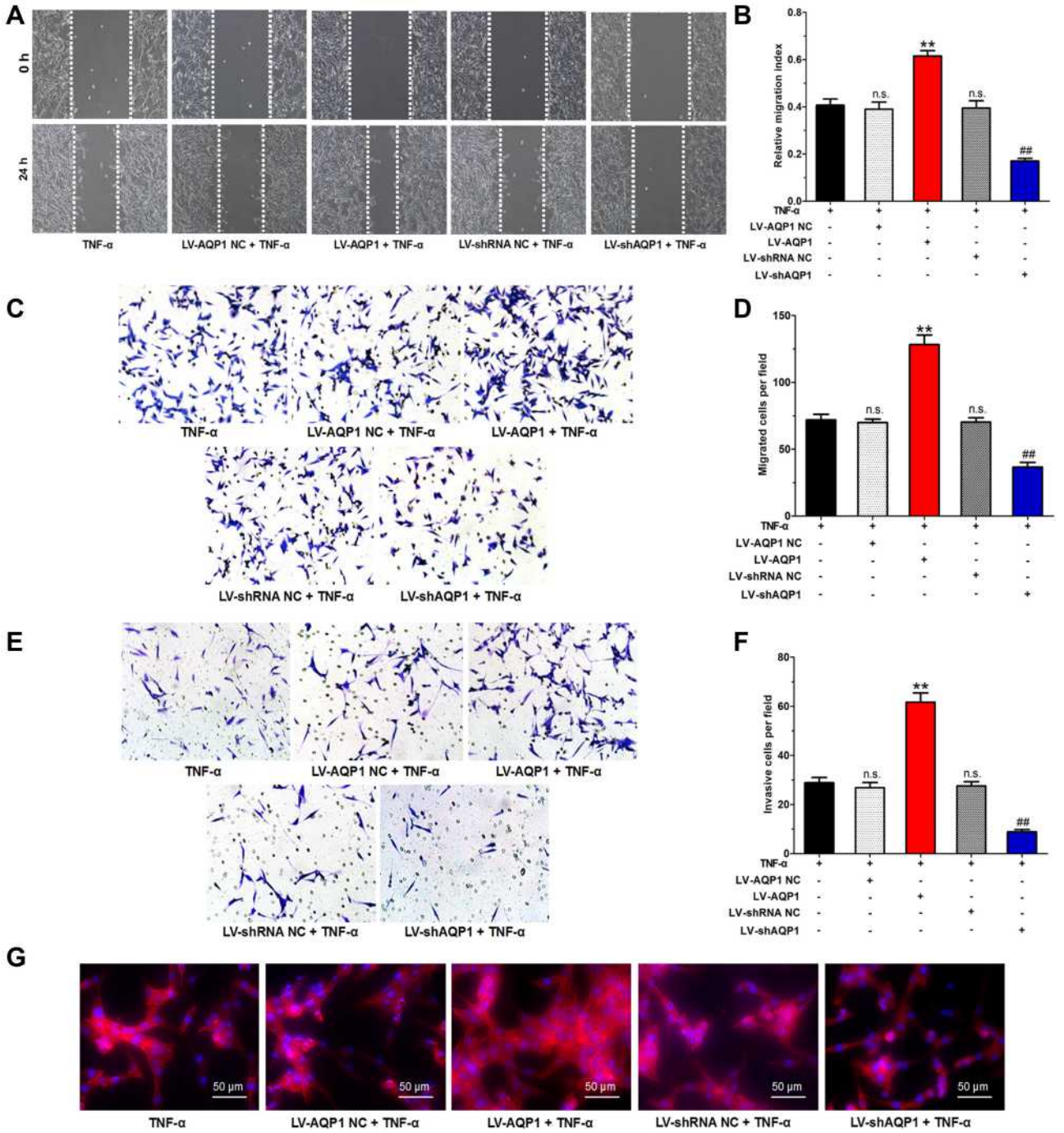

Figure 4 Effects of AQP1 overexpression or silencing on migration and invasion of TNF-α-stimulated MH7A cells. (**A**) Typical photos indicated the initial (0 h) and final (24 h) positions of cells after scraping from various groups (wound-healing assay, ×100). (**B**) Histogram of the relative migration indexes. (**C**) Typical photos of MH7A cells crossing through the membrane coated without matrigel from various groups (transwell assay, ×100). (**D**) Histogram of the migrated cell numbers per microscopic field. (**E**) Typical photos of MH7A cells crossing through the membrane coated with matrigel from various groups (transwell assay, ×100). (**F**) Histogram of the invasive cell numbers per microscopic field. (**G**) Typical photos of fluorescent phalloidin staining from various groups (×100). Fluorescent phalloidin was used to stain cells to make F-actin (red) visible and DAPI was used to stain the cell nuclei (blue). The data are mean ± SEM of three independent experiments performed in triplicate. n.s. compared with non-transfected control group. $^{**}P < 0.01$ compared with LV-AQP1 NC group. $^{\#\#}P < 0.01$ compared with LV-shRNA NC group.
Abbreviation: n.s., no significant difference.

signaling pathway key members (Wnt1, β-catenin and p-GSK-3β (Ser9)) and the target genes (like c-myc, cyclin D1, MMP-2 and MMP-9) in TNF-α-stimulated MH7A compared with LV-AQP1 NC group (Figure 5A and B). As we expected, AQP1 silencing exhibited the opposite effects on the expressions of the above proteins in TNF-α-stimulated

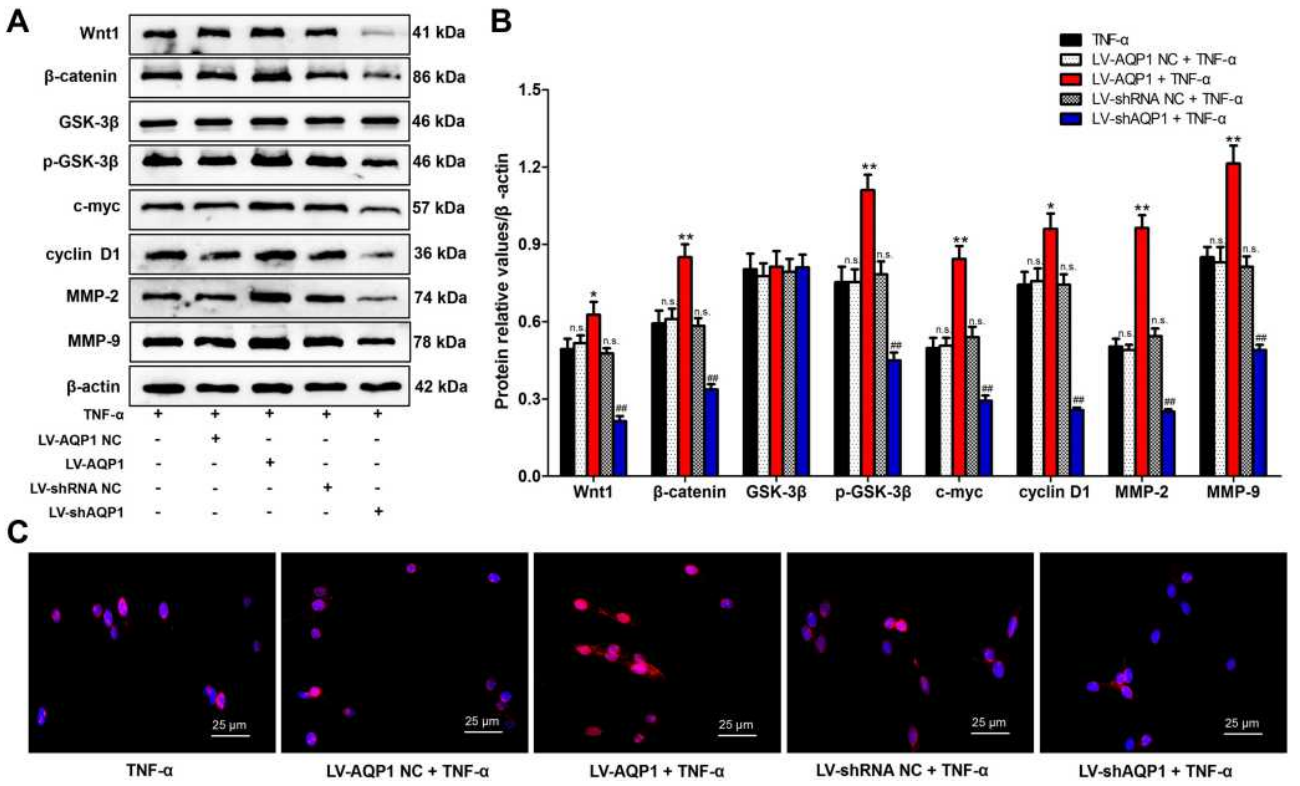

Figure 5 Effects of AQP1 overexpression or silencing on Wnt/β-catenin signaling pathway in TNF-α-stimulated MH7A cells. (**A**) Typical examples of protein expressions of Wnt/β-catenin pathway key members and the target genes from various groups, detected by Western blot. (**B**) Quantitative statistical results of protein relative values, β-actin served as the housekeeping protein. (**C**) Typical photos of β-catenin nuclear translocation from various groups (×200). Immunofluorescence staining was carried out to make β-catenin (red) visible and DAPI was used to stain the cell nuclei (blue). The data are mean ± SEM of three independent experiments performed in triplicate. n.s. compared with non-transfected control group. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with LV-AQP1 NC group. $^{\#\#}P < 0.01$ compared with LV-shRNA NC group.
Abbreviation: n.s., no significant difference.

MH7A cells. To be mentioned, AQP1 overexpression or silencing had no effects on the expression of GSK-3β protein. In Figure 5C, the results of β-catenin immunofluorescence staining revealed that AQP1 overexpression enhanced β-catenin staining in nucleus, while AQP1 silencing reduced the nuclear translocation of β-catenin. LV-AQP1 NC or LV-shRNA NC transfection did not affect this signaling pathway in MH7A cells.

Intervention of Wnt/β-Catenin Reversed the Effects of AQP1 Overexpression or Silencing on the Biologic Behaviors of TNF-α-Stimulated MH7A Cells

To further verify the involvement of Wnt/β-catenin pathway in the regulatory effects of AQP1 on the biologic behaviors of MH7A cells, XAV939 (an inhibitor of Wnt/β-catenin) or LiCl (an activator of Wnt/β-catenin) were applied to treat AQP1 overexpression or silencing MH7A cells. XAV939 (40 μM) treatment inhibited the AQP1 overexpression-induced activation of Wnt/β-catenin pathway in MH7A cells (Figure 6A and B). Moreover, XAV939 canceled the effects of AQP1 overexpression on the proliferation, apoptosis, migration and invasion of MH7A cells (Figure 6C–G). As we expected, LiCl (10 mmol/L) treatment rescued the AQP1 silencing-induced inhibition of Wnt/β-catenin pathway in MH7A cells (Figure 7A and B). Furthermore, LiCl reversed the effects of AQP1 silencing on the proliferation, apoptosis, migration and invasion of MH7A cells (Figure 7C–G). These above results further revealed that the effects of AQP1 on the biologic behaviors of MH7A cells were mediated by the regulation of Wnt/β-catenin signaling pathway.

Discussion

As we know, the synovium is the primary site of RA inflammatory process, and RA FLS as an important component of the synovium plays a crucial role in the pathological process of RA. RA FLS is the main effector cell that is responsible for initiating and driving the inflammatory response and for the invasive nature of rheumatoid synovium.[20] RA FLS activated in the chronic

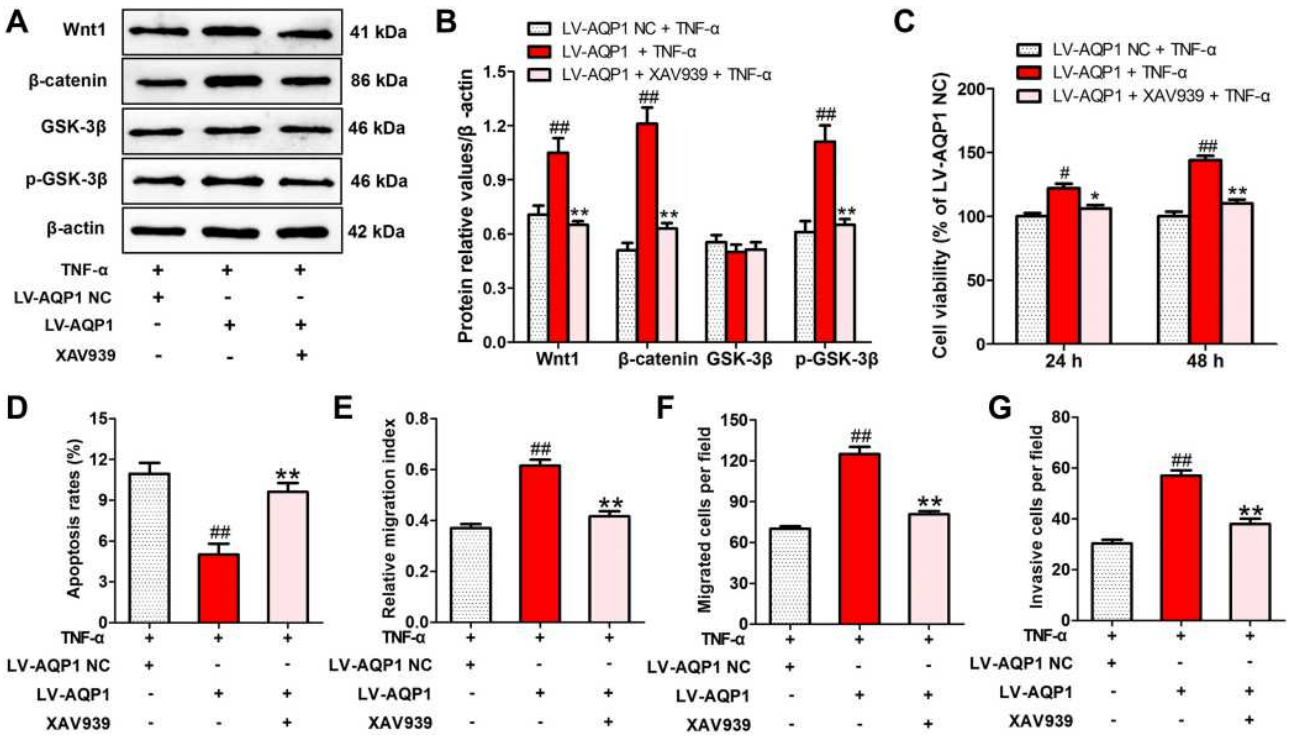

Figure 6 Inhibition of Wnt/β-catenin pathway by XAV939 canceled the effects of AQP1 overexpression on the biologic behaviors of TNF-α-stimulated MH7A cells. (**A**) Typical examples of protein expressions of Wnt/β-catenin pathway key members. (**B**) Quantitative statistical results of protein relative values. (**C**) Cell viability at 24 h and 48 h. (**D**) Histogram of apoptosis rate (%). (**E**) Histogram of the relative migration indexes. (**F**) Histogram of the migrated cell numbers per microscopic field. (**G**) Histogram of the invasive cell numbers per microscopic field. The data are mean ± SEM of three to five independent experiments performed in triplicate. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with LV-AQP1 NC group. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with LV-AQP1 group.

inflammatory environment can escape the growth limits of contact inhibition, enhance migration and acquire invasive ability.[21] The search of novel factors or mechanisms that regulate the biologic behaviors of RA FLS and therefore inhibition of the activity of RA FLS is necessary for the treatment of RA. TNF-α is a crucial cytokine in the inflammatory cascade and can stimulate RA FLS to produce various inflammatory mediators. RA FLS with TNF-α stimulation was widely served as an in vitro model to explore RA pathogenesis and screen potential therapeutic drugs.[22,23] In this study, we evaluated the effects of lentivirus-mediated AQP1 overexpression or silencing on the proliferation, apoptosis, migration and invasion of TNF-α-stimulated MH7A cells, and revealed the underlying mechanisms related to Wnt/β-catenin signaling pathway.

Aggressive proliferation of RA FLS has been suggested to contribute to synovial hyperplasia, the basic pathological feature of RA.[24] Cell cycle transformations among different phases are tightly regulated to prevent uncontrolled cell proliferation. Previous studies have revealed that the increase of cell population in S phase lead to the abnormal proliferative ability of RA FLS, and inhibition of cell transformation from the G0/G1 phase to S phase can exhibit anti-proliferative effect on RA FLS.[25] Interestingly, it has been reported that AQP1 can influence cell cycle progression and contribute to maintain the balance between proliferation and apoptosis.[26] Particularly, AQP1 knockdown in osteosarcoma cells inhibited cell proliferation, increased cell population in G0/G1 phase and reduced cell population in S phase.[27] In this study, we found that AQP1 overexpression time-dependently promoted MH7A proliferation by facilitating transformation from G0/G1 phase to S phase. Contrarily, AQP1 silencing inhibited MH7A proliferation in a time-dependent manner by inducing G0/G1 phase arrest. These findings indicate that the AQP1 can affect the proliferation of RA FLS by regulating cell cycle distribution.

Cell proliferation is a dynamic process, including cell regeneration and cell death, and the ratio of the two aspects determines the overall number of cells.[28] Apoptosis, also known as programmed cell death, is considered to be an important mechanism that modifies tissue composition and homeostasis.[29] The disorderly or inadequate apoptosis of RA FLS is a main cause of synovial

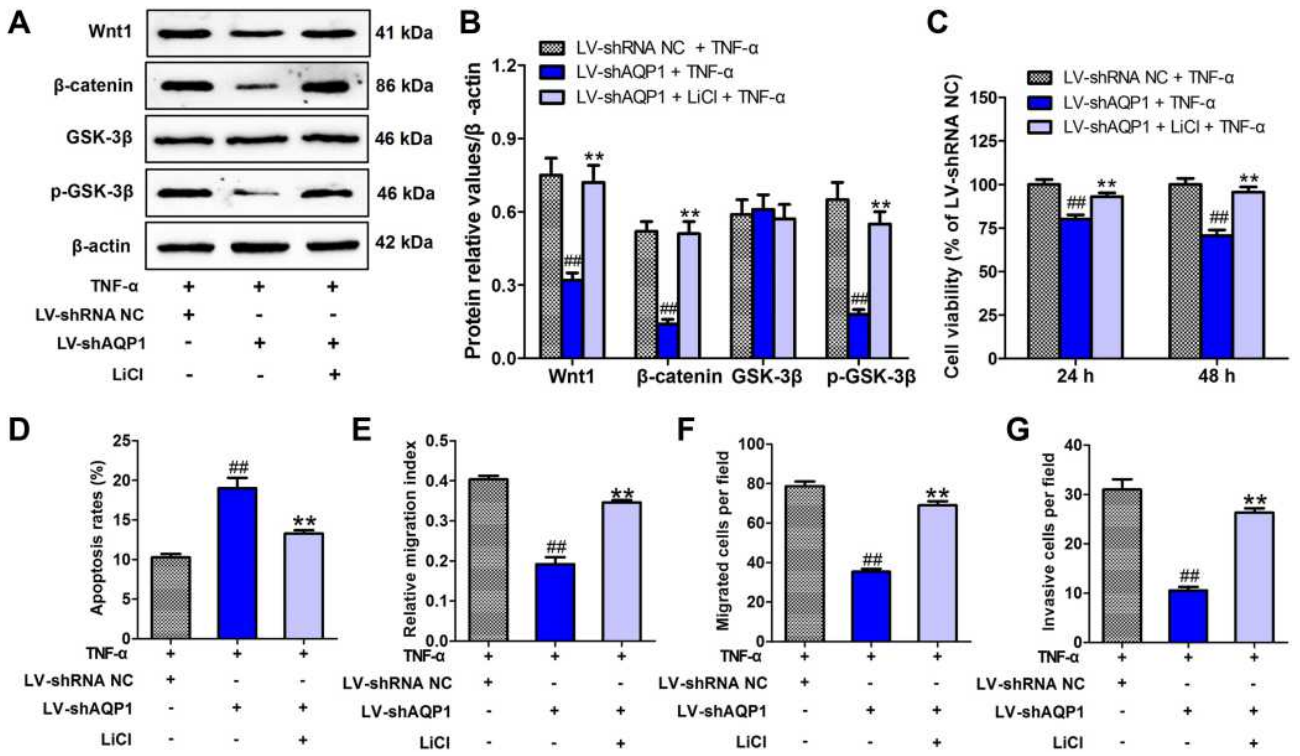

Figure 7 Activation of Wnt/β-catenin pathway by LiCl reversed the effects of AQP1 silencing on the biologic behaviors of TNF-α-stimulated MH7A cells. (**A**) Typical examples of protein expressions of Wnt/β-catenin pathway key members. (**B**) Quantitative statistical results of the protein relative values. (**C**) Cell viability at 24 h and 48 h. (**D**) Histogram of apoptosis rate (%). (**E**) Histogram of the relative migration indexes. (**F**) Histogram of the migrated cell numbers per microscopic field. (**G**) Histogram of the invasive cell numbers per microscopic field. The data are mean ± SEM of three to five independent experiments performed in triplicate. $^{\#\#}P < 0.01$ compared with LV-shRNA NC group. $^{**}P < 0.01$ compared with LV-shAQP1 group.

membrane continuous thickness.[30] Interventions to induce RA FLS apoptosis and thus keep the equilibrium of RA FLS proliferation and apoptosis may be a meaningful strategy for RA treatment. In this study, we found that AQP1 overexpression reduced the apoptosis of TNF-α-stimulated MH7A cells, while AQP1 silencing increased the apoptosis of MH7A cells. The effects of AQP1 on the apoptosis of MH7A cells might be related to regulating the stabilization of mitochondrial membrane potential and the function of mitochondria. The mitochondrial pathway plays a critical role in the apoptosis of RA FLS, with Bax, Bcl-2, and caspase-3 proteins being implicated.[31] Bax exerts the pro-apoptotic effects, while Bcl-2 exerts a wide range of anti-apoptotic effects. The damaged mitochondria cause the activation of caspase 3, which is an important executor of cell apoptosis and the common downstream effector of many apoptotic pathways.[32] In this study, AQP1 overexpression increased Bcl-2 protein expression in TNF-α-stimulated MH7A cells, while reduced Bax and cleaved caspase 3 protein expressions, resulting in an elevated Bcl-2/Bax protein ratio. As expected, AQP1 silencing exhibited the opposite effects on the expressions of apoptosis-related proteins and the ratio of Bcl-2/Bax protein. These findings suggest that AQP1 is closely involved in the apoptosis of RA FLS.

The migration and invasion of RA FLS into cartilage and bone is well associated with RA joint destruction.[33] RA FLS can invade into extracellular matrix and destroy cartilage and bone by releasing matrix degrading enzymes and chemokines.[7] The activated RA FLS facilitates the productions of inflammatory mediators that further promote the migration and invasion of RA FLS, finally causing synovial inflammation and joint damage in RA.[34] Previous studies have revealed that AQP1 can affect the migration and invasion of many tumor cells.[35] Notably, we reported that AQP1 siRNA could inhibit the abilities of migration and invasion of cultured CIA FLS, as implying the potential involvement of AQP1 in RA FLS migration and invasion.[14] In this study, AQP1 overexpression promoted the migration and invasion of TNF-α-stimulated MH7A cells, while AQP1 silencing showed the inhibitory effects on MH7A migration and invasion. The dynamic reorganization of actin cytoskeleton is an iconic event in the process of cell migration and invasion. Phalloidin

staining results indicated that AQP1 overexpression increased F-actin expression and F-actin stress fiber intensity, and promoted the cytoskeletal reorganization of TNF-α-stimulated MH7A cells, while AQP1 silencing inhibited the cytoskeletal reorganization in MH7A. Our findings provide direct evidence that AQP1 can affect the migration and invasion of RA FLS by regulating the cytoskeletal reorganization.

Accumulating evidence has revealed that the activation of Wnt/β-catenin pathway is involved in RA pathogenesis, associated with regulating the proliferation, migration and invasion of RA FLS,[17,36] and the inhibition of this pathway may be an effective strategy for RA.[37] Notably, the intimate interaction of AQP1 and Wnt/β-catenin pathway has been disclosed in many diseases.[12,38] AQP1 can co-immunoprecipitate with β-catenin and other proteins to form the stable complex and increase β-catenin protein level by reducing β-catenin degradation, then the elevated β-catenin nuclear translocation causes the activation of Wnt/β-catenin pathway.[18,39] Recently, we found that the elevated expression of synovial AQP1 aggravated the severity of rat CIA by Wnt/β-catenin pathway.[14] In this study, lentivirus-mediated AQP1 overexpression promoted the activation of Wnt/β-catenin pathway in TNF-α-stimulated MH7A cells, as evidenced by the increased protein levels of pathway key members and target genes as well as the elevation of β-catenin nuclear translocation. Contrarily, AQP1 silencing produced the inhibitory effect on Wnt/β-catenin pathway in MH7A cells. XAV939, an inhibitor of Wnt/β-catenin, can promote β-catenin degradation and prevent the entry of β-catenin into nucleus.[40] LiCl, an activator of Wnt/β-catenin, can promote the phosphorylation of GSK-3β (Ser9) and increase β-catenin accumulation in nucleus.[41] As we expected, the effects of AQP1 overexpression or silencing on the proliferation, migration and invasion of MH7A cells could be respectively reversed by the treatment of XAV939 or LiCl, further indicating that Wnt/β-catenin pathway is involved in the effects of AQP1 on the biologic behaviors of MH7A cells.

In short, we conclude that AQP1 can affect the proliferation, apoptosis, migration and invasion of TNF-α-stimulated MH7A cells by Wnt/β-catenin signaling pathway. This study presents direct evidence that AQP1 is a crucial determiner that can regulate the biologic behaviors of RA FLS and the inhibition of AQP1 may be a prospective strategy for the treatment of RA.

Acknowledgments

This work was supported by National Natural Science Foundation of China (81102273, 81972040), Program for Outstanding Young Talents of Higher Education Institution of Anhui Province (gxyqZD2016045), Program of Inflammation and Immune Mediated Diseases Laboratory of Anhui Province (IMMDL202001) and Research Fund of Anhui Medical University (2018xkj022).

Disclosure

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

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