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Actin cytoskeleton aggregation involves the water channel protein aquaporin 1-mediated human chondrocyte degeneration

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ABSTRACT

Water metabolism and actin cytoskeleton remoulding act as essential characters in the process of osteoarthritis (OA). However, the relation between water channel protein aquaporin 1 (AQP1) and actin filament during chondrocytes (CHs) degeneration is not evident. Therefore, the present study aimed to evaluate the role of actin remoulding in the AQP1 mediated CHs degeneration. Primary CHs were collected from human hip cartilage and were degenerated from long-time monolayer culture or IL-1β stimulation. Besides, the CHs were transfected with AQP1-specific siRNA or vectors to mediate the AQP1 gene expression. The potent inhibitor of actin polymerization Cytochalasin D was also supplemented during culture. RT-PCR was performed to determine the relative gene expression. AQP1 and F-actin fluorescence staining were performed to determine the AQP1 and F-actin organization. Moreover, the cell area and viability were also analyzed. AQP1 and F-actin organization were both increased during seven days' CHs culture or three days' IL-1β stimulation. Silencing of AQP1 prevented the cell area spreading and degenerated phenotype of CHs with suppression of F-actin aggregation in both natural or IL-1β-caused inflammatory-related degeneration. Besides, upregulating the AQP1 in the CHs via gene editing promoted the cell area spreading, and F-actin accumulation, and accelerated the CHs degeneration, which can be alleviated by Cytochalasin D treatment. These findings suggested that AQP1-mediated human CHs degeneration is related to F-actin aggregation.

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Introduction

Osteoarthritis (OA) generally refers to a class of degradative diseases in the joints and surrounding tissues of the human body, which has many types and complicated etiologies (1,2). It is usually considered to be related to various factors such as inflammation-promoting factors, autoimmune reactions, pathogenic microbial infections, metabolic disorders, trauma, and systemic degenerative diseases (3). In recent years, studies have mostly focused on the pathological process of cartilage degeneration. Articular cartilage has no internal vascular system or lymphatic system support, so it relies on adjacent tissues (subchondral bone and synovium) to provide nutrients, which are essential for maintaining the survival of the chondrocytes (CHs) (4). Water accounts for 65% to 80% of cartilage capacity. Among them, 1/3 of the water content is in CHs, and the rest of the water is combined with the extracellular matrix (ECM) (5).

The ECM is a highly variable ion and osmotic pressure environment. Changes in the ion and osmotic environment in cartilage can affect the osmotic pressure and volume of CHs and adjust the synthesis and degradation speed of ECM (6). In the process of cell volume adjustment, water is one of the essential factors. In normal conditions, the osmotic pressure of the articular cavity is estimated to be around 404 mOsm, which decreases to 301 mOsm with increased water content in the early OA (7). Aquaporins (AQPs), a specific pore protein family on the cell membrane, are the primary molecular basis for the transmembrane transport of water molecules and can mediate the passive transmembrane transport of free water to maintain the balance of osmotic pressure inside and outside the cell (8,9). It has been found that the subtypes of AQPs expressed in CHs are AQP1 and AQP3, and aquaporin 1 (AQP1) is highly expressed during the occurrence and development of OA, which causes CHs to absorb water and swell and aggravate the process of cartilage degeneration (10).

The enhanced activity of AQP1 causes CHs to absorb water and swell, eventually rupture and die (11). However, the underlying molecular mechanism of AQP1 in CHs degeneration is not fully elucidated. The cytoskeleton plays a vital role in maintaining cell morphology and keeping the internal structure of cells in order, which is also closely related to cell movement, energy conversion, information transmission, and cell differentiation (12,13). The swelling of CHs is bound to be accompanied by an increase in cell volume and the cytoskeleton's remodelling. The microfilament skeleton, also known as actin filament (AF), is one of the main components of the cytoskeleton and is closely related to OA (14). Sliogeryte et al. (15) reported CH dedifferentiation is associated with increased F-actin organization. Hua et al. (16) found F-actin cytoskeleton aggregation participates in the long-term high glucose-caused CH apoptosis. Whereas, whether the actin cytoskeleton

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plays a role in the AQP1-mediated CHs degeneration is not apparent.

This study used primary human CHs to explore how AQP1 affected the actin cytoskeleton during monolayer culture naturally degeneration and IL-1β caused degeneration. Thus, a better understanding of the AQP1 properties could identify targets for preventing the pathological changes of the CHs degeneration in OA.

Materials and Methods

CHs isolation

This study protocol was approved by the Ethics Committee of the People's Hospital of Ningxia Hui Autonomous Region. We recruited 12 patients undergoing femoral neck fracture in our hospital to collect the hip joint. The CHs were isolated from the articular cartilage as in the previous description (16). Briefly, the cartilage was removed from the cartilage and cut into small pieces. The fragments were sequentially digested in the mixture of 0.15% trypsin and 0.25% type I collagenase (Sigma, St. Louis, MO, USA) for six hours. The cell pellets were collected after centrifuged and filtered with the sieve. We re-suspended the CHs with DMEM/F12 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Gibco, Rockville, MD, USA) and cultured in 75 mm flask at 37°C and 5% CO_2 for the following treatments.

CHs treatments

The primary CHs initiatively undergo dedifferentiation in the monolayer culture in vitro, during which they lose the chondrogenic phenotype (17). After one culture passage, CHs were cultured in a monolayer for seven days to observe the natural degeneration. Additionally, we also treated the CHs with IL-1β (10 ng/ml, R&D Systems, Minneapolis, MN, USA) for three days to cause inflammatory degeneration as previously reported (18). Besides, we transfected the CHs with vectors carrying the AQP1 gene to overexpress the AQP1 and transfected AQP1 siRNA to downregulate AQP1 gene expression. To disrupt the F-actin polymerization, we used the Cytochalasin D (CD, 1 μM, Sigma-Aldrich, St. Louis, MO, USA) to treat CHs while culturing (19,20).

Transfection of AQP1 siRNA and vector

CHs were plated on 6-well plates in serum-free medium and transiently transfected with 4.8 siRNAs (CAT# 146917, Thermo Fisher Scientific, Waltham, MA, USA) mixed transfection reagent or 1.0 μg of the full-length human AQP1 vector (pCMV6-Entry, CAT# RC205304, OriGene, USA) using Lipofectamine 2000 (11668027, Invitrogen, Carlsbad, CA, USA) as per manufacturers' protocol.

Immunofluorescence (IF) of F-actin and AQP1

The polymerization of F-actin and AQP1 protein expression was determined by measuring the indicated density. After treatment, CHs were treated with 4% paraformaldehyde for fixation, 0.1 % Triton-X for permeabilization, and 5% bovine serum albumin (BSA) for blocking. Then, cells were incubated with anti-AQP1 (ab219055, Abcam, Cambridge, MA, USA) overnight at 4°C. The next day, the cells were subsequently incubated with Alexa Fluor488 conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA). The F-actin filament was stained with Phalloidin-iFluor 594 Reagent (ab176757, red, Abcam, Cambridge, MA, USA), and the nuclei were stained with DAPI in the dark at room temperature. The staining intensity was analyzed using IMAGE J (Version 5.1, Media Cybernetics, Inc., Silver Springs, MD, USA).

Real-time PCR analysis

RNA was isolated from CHs after treatments using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as per manufacturers' protocol. 25 ng of RNA of each sample were reverse-transcribed into cDNA with a reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Relative gene expression was measured by normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using one-step SYBR Green Master (TOYOBO, Japan) according to the method of $2^{-\Delta\Delta Ct}$. The primers used for real-time PCR are listed in Table 1.

Cell area measurement

CHs were stained with an F-actin filament to visualize the outline. The cell area was calculated using IMAGE J (Version 5.1, Media Cybernetics, Inc., Silver Springs, MD, USA). software as previously mentioned (21). For one group, at least 100 cells were analyzed at least three experiments.

Cell viability assay

According to the manufacturer's instructions, the cell viability was analyzed by the CCK8 assay (ab228554, Abcam, Cambridge, MA, USA). After treatments, the spectrophotometrical intensity of the CCK8 product was measured at 570 nm using a microplate reader (Labsystems Multiskan, Finland). Cell viability was shown as a percentage relative to the non-treated value in triplicate experiments.

Statistical Analysis

Data were analyzed from at least three experiments using the Statistic Package for Social Science (SPSS) 20.0

Table 1. Primer sequences for PCR.

Gene name	Forward (5'>3')	Reverse (5'>3')
Collagen II	CCTGGCAAAGATGGTGAGACAG	CCTGGTTTTCCACCTTCACCTG
Collagen I	CCTGGTGCTAAAGGAGAAAGAGG	ATCACCACGACTTCCAGCAGGA
MMP-3	CACTCACAGACCTGACTCGGTT	AAGCAGGATCACAGTTGGCTGG
ADAMTS-4	AGGCACTGGGCTACTAC	GGGATAGTGACCACATTGTT
IL-6	AGACAGCCACTCACCTCTTCAG	TTCTGCCAGTGCCTCTTTGCTG
IL-8	GAGAGTGATTGAGAGTGGACCAC	CACAACCCTCTGCACCCAGTTT
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

software (IBM, Armonk, NY, USA). The results were expressed as mean ± standard deviation (SD). Unpaired one-way analysis of variance (ANOVA) was used to compare the differences between the two groups. Statistical significance was determined by P-value < 0.05.

Results

AQP1 and F-actin filament were increased while CHs degeneration

Since the CHs naturally dedifferentiate during the monolayer culture, we tested the morphology, AQP1 and F-actin levels, and the chondrogenic phenotype of CHs after 1 day, 3 days, and 7 days' culture. Within 7 days of culture, the cell viability was not significantly changed (Figure 1A). However, the cell area was gradually increased from day 1 to day 7 (Figure 1B). Compared to 1 day's culture, the collagen II gene expression was significantly decreased 7 days later, and the collagen I level was massively increased from day 3 to day 7 (Figure 1C). Additionally, the AQP1 protein expression was also gradually increased from day 1 to day 7, and the F-actin density was also increased 7 days later after monolayer culture (Figure 1D). Therefore, CHs were obviously observed to degenerate after 7 days' culture with a bigger volume, higher AQP1 expression, and more F-actin filament.

Suppressing AQP1 expression prevents F-actin aggregation and CHs degeneration

To clear the effects of AQP1 during CHs degeneration, we compared the AQP1 gene-silenced CHs with the normal CHs after 7 days' vitro monolayer culture. The potent inhibitor of actin polymerization was also used during culture to eliminate the effect of actin aggregation. After 7 days' culture, silencing of AQP1 significantly delayed the increase of the cell area compared to the normal CHs, which was also achieved when the actin aggregation was abolished by CD treatment (Figure 2A). As shown in Figures 2B and 2C, the CD was sufficient to suppress the F-actin filament aggregation, and silencing the AQP1 gene also prevented the increase of the F-actin filament. However, CD could not affect the AQP1 levels. Moreover, both AQP1 silencing and CD treatments played a role in protecting collagen II content and inhibiting collagen I expression compared to the non-treated CHs on day 7 (Figure 2D). Apart from this, we also tested the MMP-3 and ADAMTS expression, which would degrade the ECM. Fortunately, AQP1 deficiency and suppressing F-actin aggregation prevented the increase of MMP-3 and ADAMTS within 7 days' culture (Figure 2E). Furthermore, the inflammatory response was also inhibited, resulting from the suppression of AQP1 expression or F-actin aggregation (Figure 2F). Thus, direct suppressing the F-actin aggregation by CD could prevent the CHs degeneration, and silencing AQP1 might delay the CHs degeneration via preventing the F-actin aggregation.

Suppressing AQP1 expression prevents F-actin aggregation in IL-1β treatment

In addition to the natural degeneration process, we tested the relation between AQP1 and F-actin aggregation in the inflammatory condition. The normal CHs or AQP1-silenced CHs were cultured in IL-1β for three days, and the CD was also used to inhibit the actin poly-

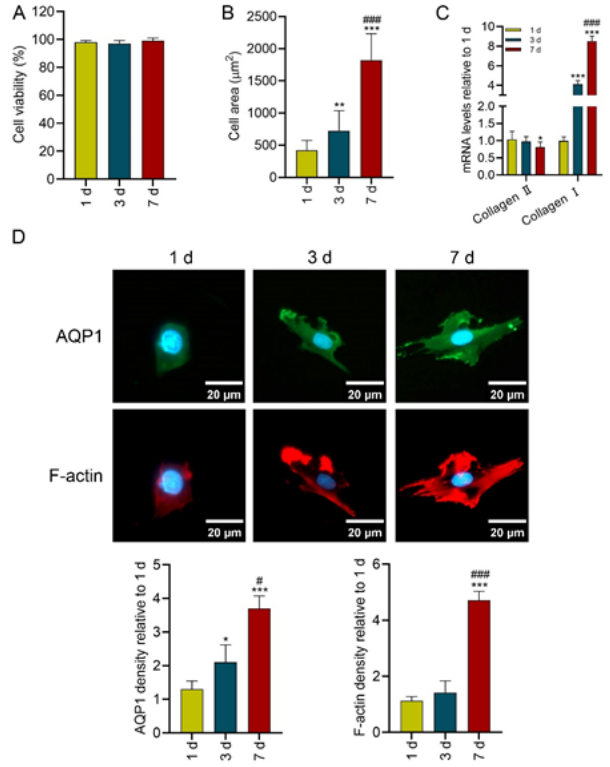

Figure 1. AQP1 and F-actin filament were increased while CHs degeneration. CHs were cultured for 1/3/7 days. (A) Cell viability was determined by CCK8 assay. (B) The cell area measurement by ImageJ. (C) RT-PCR analysis for collagen II and collagen I by normalization to GAPDH. (D) Immunofluorescence staining of the AQP1 and F-actin and quantitative analysis of the fluorescence intensity. Results are expressed as mean ± SD. (*P < 0.05, **P < 0.01, ***P < 0.001 compared to 1 d; #P < 0.05, ###P < 0.01 compared to 3 d).

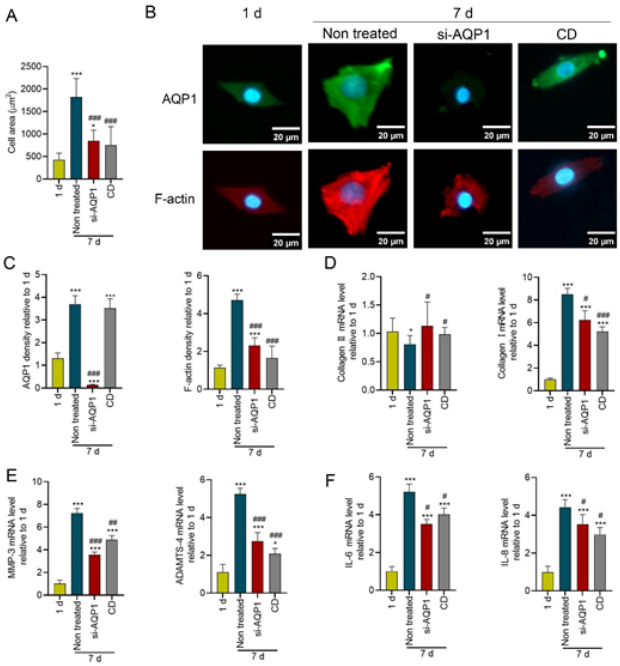

Figure 2. Suppressing AQP1 expression prevents F-actin aggregation and CHs degeneration. (A) The cell area measurement by ImageJ. (B) Immunofluorescence staining of the AQP1 and F-actin, and (C) quantitative analysis of the fluorescence intensity. (D-F) The RT-PCR analysis for collagen II, collagen I, MMP-3, ADAMTS-4, IL-6, and IL-8 by normalization to GAPDH. Results are expressed as mean ± SD. (*P < 0.05, ***P < 0.001 compared to 1 d; #P < 0.05, ###P < 0.01 compared to non-treated group).

merization in the IL-1β treated normal CHs. In contrast to the control group with non-treatment, IL-1β stimulated the cell's spreading with a bigger area, which could be resisted by AQP1 silencing or CD treatment (Figure 3A). The influence on the cell viability of IL-1β was also alleviated by AQP1 silencing or CD treatment (Figure 3B). Besides, AQP1 silencing protected the collagen II level, and the increased collagen I expression could be inhibited by AQP1 silencing or CD treatment (Figure 3C). Moreover, the IL-1β also promoted the increase of AQP1 and F-actin expression. Like CD treatment, the suppressing of the AQP1 gene effectively mitigated the IL-1β caused F-actin aggregation (Figure 3D). As we all know, IL-1β amplified the MMP-3, ADAMTS, IL-6, and IL-8 expression, which could be interrupted via AQP1 silencing and F-actin suppressing (Figure 3E, F). Therefore, in the pathological inflammation state, silencing of AQP1 also alleviated the F-actin aggregation, which delayed CHs degeneration.

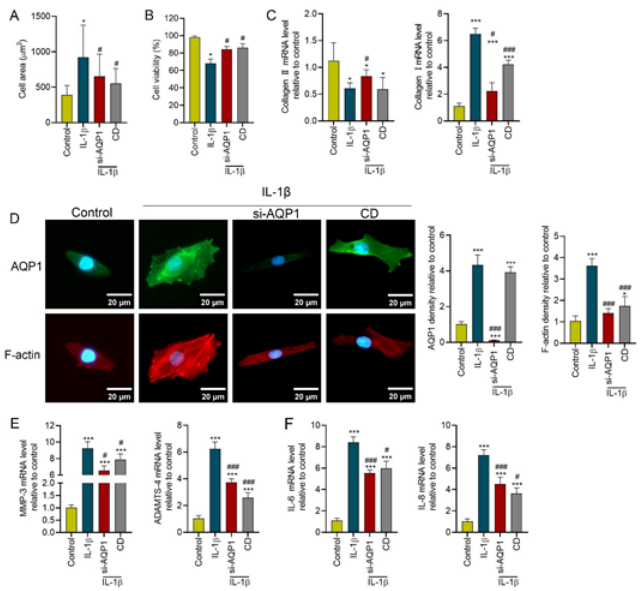

Figure 3. Suppressing AQP1 expression prevent F-actin aggregation in IL-1β treatment. (A) The cell area measurement by ImageJ. (B) Cell viability was determined by CCK8 assay. (C) The RT-PCR analysis for collagen II and collagen I by normalization to GAPDH. (D) Immunofluorescence staining of the AQP1 and F-actin, and quantitative analysis of the fluorescence intensity. (E-F) The RT-PCR analysis for MMP-3, ADAMTS-4, IL-6, and IL-8 by normalization to GAPDH. Results are expressed as mean ± SD. (*P < 0.05, ***P < 0.001 compared to control group; #P < 0.05, ###P < 0.01 compared to IL-1β group).

Disrupting F-actin aggregation alleviated the AQP1-mediated CHs degeneration

Due to the AQP1 and F-actin expression being synchronously upregulated during CHs degeneration, and suppression of AQP1 resulted in the interrupting of F-actin aggregation. We speculated that the upregulated AQP1 might play a role in F-actin's aggregation and caused CHs degeneration. Thus, we also overexpressed the AQP1 in CHs via vector transfection, and the F-actin content and phenotype were tested. The normal CHs cultured for 3 days without any treatments were grouped as controls. Meanwhile, the AQP1-overexpressed CHs were also monolayer cultured for 3 days with or without CD treatments. As shown in Figure 4A, the overexpression of APQ1 led to the polymerization of F-actin, which can be abolished by CD treatment. However the CD did not affect the AQP1 level. From the above data, the cell viability was stable within 3 days' culture, but it was significantly reduced when the AQP1 expression was increased (Figure 4B). Besides, AQP1 overexpression also accelerated the spreading of cells, which could be abolished by CD treatment (Figure 4C). During 3 days' culture, the overexpression of AQP1 did not affect the collagen II content but promoted the collagen I expression, and the prevention of F-actin aggregation could resist the collagen I production (Figure 4D). For the analysis of MMP-3, ADAMTS-4, IL-6, and IL-8, AQP1 gene overexpression was only significantly aggravated the MMP-3 and ADAMTS-4 expression, and the CD treatment could suppress the MMP-3 synthesis (Figure 4E, F). Thus, AQP1 upregulation contributed to F-actin's aggregation and accelerated the CHs degeneration, which can be alleviated by suppressing F-actin content.

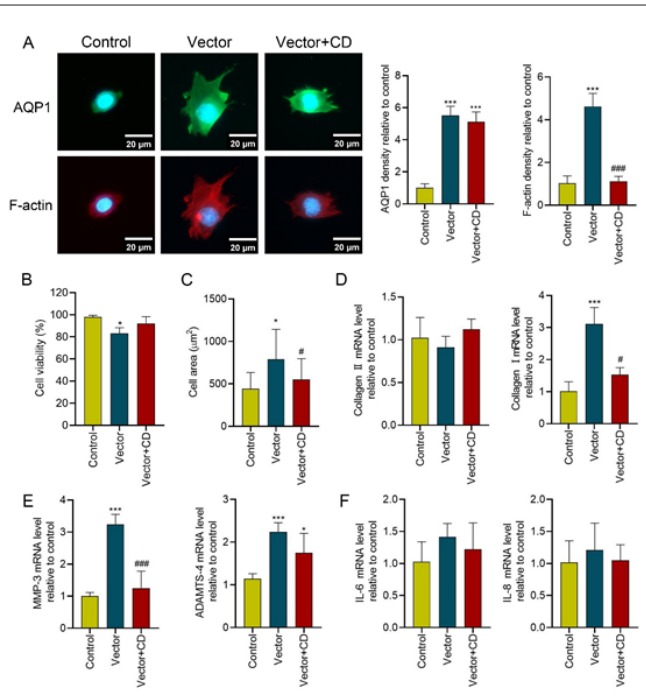

Figure 4. Disrupting F-actin aggregation alleviated the AQP1-mediated CHs degeneration. (A) Immunofluorescence staining of the AQP1 and F-actin, and quantitative analysis of the fluorescence intensity. (B) Cell viability was determined by CCK8 assay. (C) The cell area measurement by ImageJ. (D-F) The RT-PCR analysis for collagen II, collagen I, MMP-3, ADAMTS-4, IL-6, and IL-8 by normalization to GAPDH. Results are expressed as mean ± SD. (*P < 0.05, ***P < 0.001 compared to control group; #P < 0.05, ###P < 0.01 compared to vector group).

Discussion

For the first time, our study uncovered the role of upregulated AQP1 in CHs degeneration, involving the aggregation of F-actin microfilaments. The cytoskeleton has the function of maintaining its original morphology, and the reorganization of the actin cytoskeleton can lead to changes in chondrogenic phenotype (14). The actin cytoskeleton is present in two forms: the monomeric G-actin, and the polymer filaments F-actin (polymerized from G-actin) (22). The F-actin is an essential part of the cytoske-

leton and constitutes lots of high-order intracellular structures (23,24). Studies have shown that CHs undergo dedifferentiation after monolayer culture or suffer from harmful treatments changing from spherical to flat, leading to a fibroblast-like phenotype (25). The type II collagen and proteoglycan synthesized by CHs also decrease, while the synthesis of type I collagen increases, and the actin structure is elongated and denser (17). However, after CD's addition, the dedifferentiated CHs became spherical again and re-expressed type II collagen and proteoglycan. CD is a chemical substance that can destroy the microfilament structure of CHs and add caps to the ends of the microfilaments to shorten and lyse the microfilament skeleton (26).

In recent years, AQP1 has been recognized to play an essential role in the occurrence and development of OA. Geyer et al. (27) listed AQP1 as a new candidate gene for OA pathophysiology, revealing cartilage damage. The synovial fluid in the joint cavity plays a role in joint movement and weight-bearing and reduces the friction between cartilage. Musumeci et al. (28) confirmed that AQP1 is strongly expressed in fibro-chondrocytes, which may be used to regulate tissue degeneration in OA joints. Our study found the silencing of the AQP1 gene could prevent F-actin's polymerization, thereby alleviating the CHs dedifferentiation. The inactivation of AQP1 resulted in inhibiting cell swelling caused by abnormal water transport and maintained the original morphology of CHs, which also contributed to the F-actin stability. On the contrary, overexpressing the AQP1 gene in CHs promoted abnormal water transport and enlarged the cell volume, partly resulting from the polymerization of F-actin. Thus, after inhibiting the F-actin polymerization by CD treatment, the AQP1 upregulation caused cell area to increase, and the degenerated phenotype was alleviated. Similar results were verified from the study from Monzani et al. (29). They found silencing of AQP1 gene expression led to the deficiency of F-actin organization in human endothelial and melanoma cell lines. Therefore, our data provided a possible mechanism that AQP1 mediated the CHs degeneration involving the F-actin polymerization.

In addition to the natural dedifferentiation in the monolayer culture, inflammation is another fundamental factor that causes CHs degeneration. IL-1β and TNF-α substantially increase the cellular stiffness and F-actin level (30). Li et al. (31) and Pritchard et al. (32) have stated that IL-1β stimulation increased the F-actin of CHs via the RhoA pathway. There are other reports (33-39) in this regards.

Besides, Haneda et al. (40) reported that IL-1β treatment significantly promoted the AQP1 expression in hip explant cartilage, and the downregulating of AQP1 could decrease the catabolic process in OA CHs. Our results pointed out a new understanding of the AQP1-regulated of F-actin structure under inflammatory conditions. The suppression of AQP1 could inhibit the organization of F-actin and attenuate the degenerated phenotype of CHs.

In conclusion, we demonstrated that the aggregation of F-actin in the degenerated CHs is positively related to the overexpressed AQP1. Further, the suppression of AQP1 inhibits the F-actin organization and protects CHs degeneration, and the disruption of F-actin aggregation also alleviates the AQP1-mediated CHs degeneration. Our study elucidated a relationship between AQP1 and F-actin polymerization in CH homeostasis; thus, providing a new understanding of AQP1-mediated OA progression.

Conflict of interests

The authors declared no conflict of interest.

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