Title: Aquaporin 3 promotes human extravillous trophoblast migration and invasion

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Abstract

Problem: Does aquaporin 3 (AQP3) affect the migration and invasion of human extravillous trophoblast (HTR8/Svneo) cells?

Method of Study: A lentivirus infection system was used to construct stable cell lines with either AQP3 knockdown or overexpression. RT-PCR and western blotting were used to verify the efficiencies of AQP3 knockdown or overexpression in HTR8/Svneo cells at mRNA and protein levels, respectively. Cell Counting Kit-8 and flow cytometry assays were used to detect the influence of AQP3 knockdown or overexpression on proliferation and apoptosis of HTR8/Svneo cells. In addition, wound healing and Transwell invasion assays were used to detect the effects of AQP3 knockdown or overexpression on migration and invasion capabilities of HTR8/Svneo cells. An Agilent gene chip was used to screen for significant differentially expressed genes after AQP3 knockdown. Finally, mechanisms by which AQP3 influences the migration and invasion of HTR8/Svneo cells were explored using bioinformatic analysis.

Results: Compared with controls, migration and invasion capabilities of HTR8/Svneo cells were significantly reduced after AQP3 knockdown, and significantly increased after AQP3 overexpression. Subsequent bioinformatic analysis of gene chip

expression profiles indicated downregulation of genes related to adhesion such as PDGF-B, as well as signaling pathways (such as PIK3/AKT, NF-κB, and TNF) after AQP3 knockdown.

Conclusions: AQP3 could significantly promote migration and invasion capabilities of human extravillous trophoblasts, it may mediate embryo invasion and adhesion to endometrium by regulating PDGF-B、PIK3/AKT signaling pathways, although this requires further verification.

Keywords: aquaporin 3 (AQP3), embryo implantation, human extravillous trophoblast, migration, invasion

Introduction

Recurrent implantation failure (RIF) is one of the bottlenecks of *in vitro* fertilization-embryo transfer and its derivative techniques. Currently, there is no uniform definition on RIF. However, widely accepted standards include an age less than 40 years and failure to achieve a clinical pregnancy after transfer of at least four good-quality embryos in a minimum of three fresh or frozen cycles [1]. Incidence of RIF is up to 10%–15% [2], but its pathogenesis is still unclear. Many studies have reported that two-thirds of RIF cases can be attributed to endometrial receptivity, while the other third is caused by inherent factors within embryos [3]. Currently, most investigations on embryo implantation focus on this interaction from the aspect of endometrial receptivity, whereas few investigate the embryo's capacity for implantation. Our previous study found high expression of AQP3 in the cell membrane of trophoblasts in blastocysts of Kunming mice [4]. AQP3 could significantly promote both adhesion and expansion capabilities of blastocysts[5], suggesting that AQP3 participates in the process of trophoblasts invading the endometrium. However, it is unknown if AQP3 is expressed in human extravillous trophoblasts (EVT), or whether it participates in human embryo implantation. If so, the mechanism by which AQP3 regulates embryo implantation is unknown. All of these issues were addressed in the current study.

Methods and materials

Methods

Cell culture

The human trophoblast cell line HTR8/SVneo has been widely applied as an early invasion and migration model of extravillous cytotrophoblasts [6]. HTR8/SVneo, which was provided by American Type Culture Collection (USA), was cultured with Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS; Gibco, USA) at 37°C and 5% CO_2.

Construction of stable cell lines

Plasmids containing AQP3 knockdown, AQP3 overexpression, or their respective negative control plasmids were purchased from Weijiang Biotechnology (China). Lentiviral packaging was performed in 293T cells according to the manufacturer's instructions (Suzhou GenePharma, China). Lentivirus in the supernatant was collected to transfect cells. Strict phenotype selection was performed on stably infected HTR8/SVneo cells with 0–10 μg/mL puromycin (MPbio, USA) to use resistance as a screening index. Furthermore, cells were stably cloned in 0.5 μg/mL puromycin.

Total RNA extraction and RT-PCR

Total RNA was extracted according to the instructions of a TRIzol kit (Taraka Biotechnology, China), and measured with a spectrophotometer (Nanodrop 2000; Thermo Scientific, USA). cDNA was synthesized from total RNA (1 μg) using a fluorescent quantitative reverse transcription kit (Takara, Japan) and RT-PCR was performed by fluorescent quantitative PCR kit (Takara). PCR primer sequences were as follows: GAPDH forward 5'-GAAGCTCATTTCCTGGTATGACA-3' and reverse 5'-GGGAGATTCAGTGTGGTGGG-3'; AQP3 forward 5'-ACCATCAACCTGGCCTTTGG-3' and reverse 5'-GGGGACGGGGTTGTTGTAG-3'. The $2^{-\Delta\Delta CT}$ method was used to quantify relative expression of AQP3 mRNA.

Western blotting

Cells were collected and lysed with RIPA lysis buffer and phenylmethylsulfonyl fluoride (Beyotime Biotechnology, China) on ice for 30 min, quantified by bicinchoninic acid assay, and loaded for SDS-PAGE electrophoresis. After transfer to a polyvinylidene fluoride membrane, the membrane was blocked in 5% skim milk, sealed for 1 h at room temperature with shaking, and incubated with an AQP3 primary antibody (Abcam, UK; 1:1000) at 4°C. Finally, the membrane was incubated with horseradish peroxidase-labeled secondary antibody (1:20000) at room temperature for 1 h, and developed by enhanced chemiluminescence. Image Pro-Plus 6.0 software (Media Cybernetics, USA) was used to analyze gray values.

Cell proliferation/CCK-8 assay

HTR8/Svneo cells (100 μL; 1 × 10^5 cells/mL) in vector control, knockdown, and overexpression groups were added into 96-well plates in triplicate, and cultured at 37°C overnight. CCK-8 kit reagent (10 μL; Dojindo, Japan) was added into each well and incubated for 2 h, 3 h, and 4 h. Optical density at 450 nm of each well was detected each time point with a multifunctional microplate reader.

Flow cytometry assay

Annexin V-APC/7-AAD double staining was performed on HTR8/Svneo cells according to the instructions of an Annexin V-APC/7AAD Apoptosis Detection Kit [Multisciences (Lianke) Biotech, China]. Live, early apoptotic, and late apoptotic or necrotic cells were classified using flow cytometry (AccuriC6, Becton Dickinson, USA) and Flow Jo 7.6.1 software (FlowJo, USA).

Wound healing assay

HTR8/Svneo cells (1 × 10^6 cells/mL) were seeded in a six-well plate and routinely cultured in an incubator. When cells grew into a monolayer, they were treated with mitomycin for 1 h to inhibit cell division. Next, a sterile 10 μL-pipette tip was used to

scrape cell culture plates. Scraped cells were washed twice with phosphate-buffered saline, cultured in serum-free medium, incubated in an incubator, and photographed at 0, 6, 24 and 48 h after scratching. Image Pro-Plus 6.0 was used to measure scratch depth at any five sites at the same time point to calculate migration rates, thus reflecting cell mobility and migratory capabilities.

Transwell invasion assay

A Transwell invasion system (8-μm, 24-well; Corning, UK) coated with Matrigel (40 μL; Becton Dickinson) was used. Briefly, 1×10^5 cells were suspended in DMEM without serum, and seeded in the upper chamber. DMEM containing 10% FBS was then added to the lower chamber and the plate was incubated at 37°C and 5% CO_2. After 24 h, cells were fixed with methanol and stained with 0.1% crystal violet. The quantity of colored cells in five random visual fields was counted using an inverted microscope (Nikon, Japan).

Whole genome expression profile

Gene expression profiles of AQP3-shRNA and CON-shRNA were analyzed by two-color gene expression microarray (Agilent Technologies, USA) according to the instructions of a Low Input Quick Amp Labeling Kit Two-Color (Agilent). Total RNA obtained in the extraction phase was used as a template, and the first strand of cDNA was reverse transcribed using T7 RNA polymerase. The second strand of cDNA was used as the synthesis template to perform *in vitro* transcription and promote generation of cRNA. An Agilent cRNA labeling kit was used to incorporate cRNA with Cy-3, which allowed purification and qualification of cRNA (Nanodrop 2000). After hybridization, washing, and chip scanning, data were extracted to perform bioinformatic analysis using Agilent Feature Extraction Software. Doing q-PCR verfication for FDGF-B,FOS and Snail1, which showed significantly decrease in the results of the gene expression profile experiment.

Statistical analysis

Data were analyzed by SPSS23.0 software (IBM, USA). Each experiment was performed in triplicate, and data were expressed as mean ± standard deviation (SD). Migration rate and invasion index were analyzed using a two independent-samples t-test. Proliferation and apoptosis rates were analyzed by analysis of variance. $P < 0.05$ was considered statistically significant.

Results

Verification of AQP3 knockdown and overexpression efficiency

After construction of stable cell lines, AQP3 knockdown and overexpression efficiencies at mRNA and protein levels in HTR8/Svneo cells were detected using RT-PCR and western blotting, respectively. The results indicated that the AQP3-shRNA group had significantly downregulated AQP3 mRNA (Fig. 1a) and protein (Fig. 1c) levels compared with the CON-shRNA group, suggesting effective AQP3 knockdown in HTR8/SVneo. Compared with the CON-OE group, AQP3 expression was significantly upregulated at mRNA (Fig. 1b) and protein (Fig. 1d) levels in AQP3-overexpressing cells, suggesting effective AQP3 overexpression in HTR8/SVneo.

AQP3 knockdown expression affected the apoptosis of HTR 8/SVneo cells but had no significant effect on their cell proliferation

Influence of AQP3 knockdown and overexpression on HTR8/Svneo apoptosis as detected by flow cytometry, cell proliferation as detected by CCK-8. Apoptosis rates of AQP3-shRNA, vector control, and AQP3-OE groups were 7.157% ± 0.4391%, 5.36% ± 0.2594%, and 4.727% ± 0.1984%, respectively. Compared with the control group, apoptosis in the AQP3-shRNA group was significantly upregulated, but the AQP3-OE group exhibited no statistical significance. Proliferation rates in AQP3-shRNA, vector control, and AQP3-OE groups after 2 h, 3 h, and 4 h of CCK-8 addition were detected. The results indicated no significant differences (Fig. 2).

Correlation of migratory and invasion capability of HTR8/Svneo with AQP3 expression

After shRNA interference with HTR8/Svneo cells (AQP3-shRNA), wound healing assay results indicated that cell migration rates at 6 h, 24 h, and 48 h were significantly reduced compared with the CON-shRNA group. In contrast, after AQP3 overexpression (AQP3-OE), cell migration rates at 6 h, 24 h, and 48 h after scratching were significantly upregulated (Fig. 3a). We further used transwell assay to detecte the influence of AQP3 on invasion capability of trophoblasts. The results demonstrated that after AQP3 knockdown, numbers of invading cells in the AQP3-shRNA group were downregulated by 50.25% compared with the CON-shRNA group, and numbers of invading cells in the AQP3-OE group were upregulated by 34.38% compared with the CON-OE group after overexpression，the difference were statistically significant (Fig. 3b).

Whole genome expression profile

To further study signaling pathways regulated by AQP3 during embryo implantation, an Agilent gene expression microarray was used to examine AQP3-shRNA and CON-shRNA groups. Genes with differential expression fold-changes ≥ 2 and $q < 0.05$ as screened with Significance Analysis of Microarrays software were taken as significant differentially expressed genes. The results indicated that after AQP3 gene downregulation, there were 311 significant differentially expressed genes (150 upregulated and 161 downregulated) (Fig. 4). The result of gene ontology (GO) analysis of genes with differential expression (fold-change>2) between AQP3 knockdown and its control group indicated enrichment for genes involved in angiogenesis, cell migration, inflammatory response, cell adhesion, and extracellular matrix recombination. Eleven differentially expressed genes related to cell migration (GO: 0030335) were statistically enriched ($P = 0.000119$); notably, all were critical factors (e.g. PDFGF-B, FOS and SNAIL1) that were significantly downregulated. Fourteen differentially expressed genes related to cell adhesion (GO: 0007155), such

as ICAM-1, COL18A, and JUP, were statistically enriched (P=000345) and significantly downregulated (Fig5a,5b; Fig6). Screening of differentially expressed genes by Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis revealed participation primarily in tumor cell metastasis, adhesion, and apoptosis, as well as MAPK, PIK3-AKT, cell adhesion-related, tumor necrosis factor (TNF), and NF-κB signaling pathways. Among them, the majority of genes involved in cell migration, adhesion, PIK3 and NF-κB signaling pathway were down-regulated, and AQP3-shRNA was significantly down-regulated compared with CON-shRNA, with statistically significant differences (Fig. 5c, Table1). Of all these pathways, changes in cell migration and adhesion-related signaling were the most significant.

To verify the expression of some differentially expressed genes selected from the results of whole genome expression profile

The results of q-PCR verfication for FDGF-B,FOS and Snail1 showed that, the mRNA expression level of FDGF-B in AQP3-shRNA group was significantly lower than that in CON-shRNA group（$P<0.05$）, while FOS、Snail1 mRNA expression were lower than that of CON-shRNA group, but there were no significant difference (P>0.05) (Fig. 7a). The protein level of FDGF-B was further verified, Western blot showed that the FDGF-B protein level in AQP3-shRNA group was significantly lower than that in CON-shRNA group (P<0.0001) (Fig. 7b).

Discussion

Assisted reproductive techniques bring hope for infertility patients. With continuous improvement of this technology, success rates have also increased. However, many patients still do not achieve pregnancy after multiple cycles of embryo transplantation, a condition referred to as recurrent implantation failure (RIF). Current clinical regimens for RIF include removal of maternal endometrial lesions, improvement of thin endometrium, increasing endometrial receptivity, anticoagulation therapy, immunotherapy, blastocyst transfer, and assisted hatching[7]. However, the effectiveness is unsatisfactory, especially if no clear reason

for RIF is found (e.g. detection of uterine cavity lesions by hysteroscope). For idiopathic RIF, the newest strategy is to perform preimplantation genetic screening, that is, detection of preimplantation chromosome number and structural abnormalities to exclude embryos carrying genetic defects. Approximately 30%–40% of screened blastocysts have the possibility of implantation failure. Therefore, deep investigation of critical links and molecular mechanisms of embryo implantation into the endometrium is important to identify RIF therapeutic targets. Embryo implantation is an important step of mammalian reproduction, and is critical for determining pregnancy. Two factors that determine embryo implantation are the implantation capability of the embryo and receiving status of the endometrium. The process of embryo implantation includes localization, adhesion, and invasion of the maternal endometrium until embedment into the matrix. This behavior of the embryo invading the endometrium at a specific time and space is the primitive motive for embryo implantation. The invasion process is completed when EVT differentiate from cytotrophoblasts [8]. Decidualization happens in the endometrium during the implantation window after blastocyst adhesion. Endometrial interstitial cells gradually transform into decidual stromal cells, which receive the invasion of EVT [9]. It has been shown that invasion of trophoblasts into the endometrium is similar to the metastasis of malignant tumors[10], as both involve cell invasion. Interestingly, an *in vitro* co-culture study reported that trophoblasts in mouse embryos exhibited stronger invasion than malignant tumor cells[11, 12]. Directional migration of trophoblasts, which involves a series of cell signaling events, is a central step of invasion behavior. At present, widely accepted cell migration mechanisms include: (1) actin depolymerization, transport of ions into cells, and increased osmotic pressure in the front of cells; (2) penetration of water through the cell membrane to increase local hydrostatic pressure, whereby the cell membrane forms local crowning including ruga, pseudopod, and vesicles; and (3) actin re-polymerization. Thus, migration speed can be controlled by osmotic pressure in extracellular medium. This means that high osmotic pressure accelerates migration, while low pressure slows down migration[13]. During this process, AQPs play a critical

role for compliance with the acceleration of intracellular and extracellular osmotic pressure changes, as well as rapid changes of cellular morphology [14, 15].

AQPs, a type of channel protein, can regulate levels of water and small molecular substances (such as glycerin, urea, and nitrogen), which is very important for the maintenance of body fluid equilibrium[16]. Multiple AQPs have been detected in embryos before implantation[17-19]. Expression of AQP3 lasts from zygophase to the blastula stage[20], and is the most abundant aquaporin expressed in villi during early pregnancy [21]. This suggests that AQP3 may have significance in the early growth and implantation of embryos. In addition, AQP3 is expressed in multiple malignant tumor cells [22]. Studies performed by Chikuma *et al.*[23, 24] demonstrated that AQP3 participates in both tissue oncogenicity and tumor cell migration. A breast cancer study[25] indicated that estradiol directly upregulated AQP3 expression via the ERE promoter in AQP3, and further enhanced the activity and invasion of tumor cells by upregulating epithelial-mesenchymal transition (EMT) and molecules related to actin cytoskeletal rearrangements. Chen[26] *et al.* found that AQP3 increased expression and secretion of matrix metalloproteinase 3 by inducing activation of prostate tumor cells through ERK1/2, and further promoted cell migration and invasion. Hou[27] *et al*. found that downregulation of AQP3 expression in non-small cell lung cancer (NSCLC) cells under hypoxia, resulted in significant downregulation in the activity of relevant molecules in HIF-1α/VEGF and Raf/MEK/ERK signaling pathways. Moreover, proliferation, migration and invasion capabilities of NSCLC cells were significantly downregulated. Thus, they believed that AQP3 delayed growth of NSCLC cells via these two signaling pathways and promoted both the migration and invasion of tumor cells. However, mechanisms by which AQP3 influences human reproduction are not as clear as those by which malignant tumor metastasis is promoted. A recent study on endometrial receptivity reported that estradiol and progesterone directly upregulated expression of AQP3 via its ERE and PRE promoters, and induced EMT via AQP3 to promote the migration and invasion of RL95-2 (an endometrium cell line) with high receptivity[28]. The invasion process of trophoblasts into the maternal endometrium and metastasis of malignant tumors both rely on cell invasion

behaviors. Our previous studies reported expression of AQP3 in four-celled, eight-celled, morula, and blastula stages of mouse embryos, with highest expression on the membrane of blastula-stage trophoblasts[4, 5]. Upon co-culture of blastocysts and endometrial somatic cells, heparin-binding epidermal growth factor-like growth factor (HB-EGF) induced AQP3 upregulation in blastocyst trophoblasts, which exhibited gradually enhanced adhesion and expansion capability with increasing AQP3 upregulation. Furthermore, EGF receptor inhibitors and ERK inhibitors inhibited AQP3 expression in mouse embryos in a concentration-dependent manner. As AQP3 expression decreases, adhesion and expansion of mouse embryos to the endometrium is gradually reduced, suggesting the HB-EGF/EGFR/ERK/AQP3 signal transduction pathway participates in regulation of embryo implantation. Reca Alejandra *et al.*[29] found that silencing AQP3 expression via siRNA or inhibition of AQP3 by $CuSO_4$ could significantly reduce the migratory capability of EVT, although the mechanism was unclear. Indeed, it was unknown whether AQP3 participates in blastocyte invasion and, if so, which signal transduction pathways regulate embryonic trophoblasts to make them appropriately invade endometrium.

In our study, the human HTR8/Svneo cell line was used as a model of early embryonic EVT invasion and migration to construct AQP3 knockdown and overexpression cells. Our results indicate that overexpression of AQP3 significantly promoted the migration and invasion of cells, were consistent with the results of Huang *et. al.*[25]. After AQP3 knockdown, the migratory and invasion capabilities of cells were significantly reduced, and apoptosis was significantly upregulated, which correspond with the results of Xiong *et al*.[30] .Moreover, this study showed that there was no significant change in the proliferation of HTR8/SVneo cells after inhibition of AQP3 expression, while xiong observed that siRNA-mediated downregulation of AQP3 could significantly inhibit the proliferation of NSCLC cells. As the XWLC-05 cell line was applied to their study, but HTR8/Svneo was used in ours, one possible reason for the discrepancies is that differences in cell lines lead to different influences of AQP3 knockdown on cell proliferation.

On the other hand, bioinformatics analysis of gene chip expression profiling data showed that key genes related to migration, such as platelet-derived growth factor PDGF-B、Snai1、FOS were significantly down-regulated after AQP3 knockdown expression. Further q-PCR validation of these two key genes revealed that PDGF-B mRNA expression was significantly downregulated after knockdown expression ($P<0.05$), while Snai1、FOS mRNA expression was downregulated, but the difference were not statistically significant ($P=0.61$). We continued western blot validation of PDGF-B genes, found out that expression of PDGFB protein levels were also significantly downregulated ($P<0.0001$). Schwenke[31] et.al found that PDGF-B trigger undirected motility in endometrial stromal cells, while pathway inhibitor studies have shown that ERK1/2,PI3K/Akt and p38 signaling are associated with chemotactic motility, whereas chemokines (PDGF-B) are mainly dependent on PI3 kinase/ Akt activation. Jing[32] et al. found that PDGFB and PI3K/AKT signaling pathways have co-expression networks together with the false detection rate is very low, and PDGFB promote the metastasis of oral squamous cell carcinoma through the PI3K/AKT signaling pathway. Therefore, we speculate that there are co-expression networks AQP3, PI3K/AKT as well as PDGFB regulate PDGFB expression through PI3K/AKT signaling pathways and mediate the migration and invasion of extravillous trophoblastic cells, thereby mediating embryo implantation.

Conclusions

Collectively, these results reveal that AQP3 is an important positive regulatory factor for fetal-maternal crosstalk during the first trimester of pregnancy, whereby it actively promote migration and invasion ability of trophoblast cells, and may mediate the extension of pseudopodia and migration of blastocyst trophoblast cells through the regulation of AQP3/PI3K/AKT/PDGFB signaling pathway, thus achieving embryo implantation, although the underlying mechanism requires a further investigation.

Abbreviations

AQP3：aquaporin 3； RIF: Recurrent implantation failure; EVT: Extravillous trophoblasts; EMT: Epithelial-mesenchymal transition; HB-EGF: Heparin-binding epidermal growth factor-like growth factor.

Ethical Approval and Consent to participate

This study was approved by the Ethics Committee of Guangdong Women and Children Hospital and has been performed in accordance with the principles of Declaration of Helsinki.

Consent for publication

Not applicable.

Availability of supporting data

Not applicable.

Competing interests

The authors have no conflict of interest to declare.

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Authors' contributions

Nong YQ designed the study and contributed to article revision. Li SF performed the research and contributed to writing the article. They share first authorship. Liu WJ, Fan L, Chen Y and Huang QW were contributed to experiment design and performed. Zhang XQ and Zhang QY were responsible for analysis of data. Liu FH designed the work, provided technical guidance and final approved of manuscript. All authors read and approved the final manuscript.

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Figure legends

Fig 1. Verification of AQP3 knockdown and overexpression efficiency

Compared with the CON-shRNA group, AQP3 knockdown resulted in (a) downregulation of AQP3 mRNA expression by 82% ($P < 0.0001$), and (c) downregulation of AQP3 protein levels by 53% ($P < 0.01$). Compared with the CON-OE group, AQP3 overexpression resulted in (b) upregulation of AQP3 mRNA expression by 1128-fold ($P < 0.0001$), and (d) upregulation of AQP3 protein levels by 9-fold ($P < 0.01$) (t-test). **$P < 0.01$; ****$P < 0.0001$.

Fig 2. Influence of AQP3 knockdown and overexpression on HTR8/Svneo cell apoptosis and proliferation

Q2 (APC+/7AAD+) indicates late apoptotic/necrotic cells, Q3 (APC+/7AAD–) indicates early apoptotic cells, Q4 (APC–/7AAD–) indicates living cells. Apoptosis rates of AQP3-shRNA, Control, and AQP3-OE groups were 7.157% ± 4.391%, 5.36% ± 2.594%, and 4.727% ± 1.984%, respectively. Compared with the control, apoptosis in the

AQP3-shRNA group was significantly upregulated by 33.5% ($P < 0.05$). However, the rate of apoptosis in HTR8/Svneo cells in the AQP3-OE group was downregulated by 11.8% ($P > 0.05$) (one-way ANOVA). Proliferation rates of HTR8/SVneo cells as detected by CCK-8 assay while apoptosis rates as analyzed by flow cytometry. Proliferation rates in AQP3-shRNA, vector control, and AQP3-OE groups at 2 h after CCK-8 addition were 79.2% ± 1.935%, 79.63% ± 1.335%, and 83.4% ± 2.022%, respectively, which were not significantly different ($P > 0.05$). After 3 h, rates were 94.17% ± 2.198%, 95% ± 1.552%, and 98.27% ± 1.924%, and were not significantly different ($P > 0.05$). After 4 h rates were 100.32% ± 1.732%, 100.38% ± 1.027%, and 100.70% ± 1.387%, which were not significantly different ($P > 0.05$) (two-way ANOVA). #, compared with vector control group, * $P< 0.05$, NS:$P > 0.05$.

Fig 3. Migration rates(4×) of AQP3 and number of invading cells(10×) after AQP3 knockdown and overexpression.

At 6 h after scratch, average migration rates of AQP3-shRNA and CON-shRNA groups were 20.96% ± 0.4032% and 35.22% ± 1.099%, respectively. Cell migration in the AQP3-shRNA group was downregulated by 40.50% compared with the CON-shRNA group ($P < 0.0001$). Migration rates at 24 h were 43.29% ± 0.5446% and 57.79% ± 1.531%, respectively. AQP3-shRNA was downregulated by 25.09% compared with CON-shRNA ($P < 0.0001$). Migration rates at 48 h were 57.32% ± 0.5719% and 86.57% ± 0.8777%, respectively. AQP3-shRNA was downregulated by 33.79% compared with CON-shRNA ($P< 0.0001$). At 6 h after scratch, average migration rates of AQP3-OE and CON-OE groups were 40.35% ± 2.074% and 34.95 ± 0.7426, respectively. AQP3-OE was upregulated by 15.44% compared with the CON-OE group ($P < 0.05$). At 24 h, migration rates were 80.31% ± 1.425% and 60.59% ± 1.425%, respectively; thus, the AQP3-OE group was upregulated by 32.56% compared with the CON-OE group ($P < 0.0001$). Migration rates at 48 h were 99.61% ± 0.2702% and 87.34% ± 1.09%, respectively; thus, AQP3-OE was upregulated by 14.05% compared with the CON-OE group ($P < 0.0001$) (two-way ANOVA), (×4 amplification) (Fig. 3a). After AQP3 knockdown, numbers of invading cells in AQP3-shRNA and CON-shRNA groups were 59.4 ± 1.29and 119.4 ± 2.952. numbers in the AQP3-shRNA group were

downregulated by 50.25% compared with the CON-shRNA group ($P < 0.0001$). After AQP3 overexpression, numbers of invading cells were 159.7 ± 3.046 and 118.9 ± 2.914, respectively, numbers in the AQP3-OE group were upregulated by 34.38% compared with the CON-OE group ($P<0.0001$) (t-test), (×10 amplification) (Fig.3b). All data are expressed as mean ± SD, * $P< 0.05$, **** $P < 0.0001$.

Fig 4. Clustering gene expression patterns: after AQP3 gene downregulation, there were 311 significant differentially expressed genes (150 upregulated and 161 downregulated). J: AQP3-shRNA, K: CON-shRNA. Red represents gene upregulation, while green represents gene downregulation.

Fig 5. GO enrichment analysis of differentially expressed genes after AQP3 downregulation.

red represents result of biological enrichment, green represents result of cell component enrichment and blue represents result of molecular function enrichment,(Fig.5a,b). Some entries of the Pathway on KEGG enrichment analysis of differentially expressed genes after down-regulation of AQP3 genes (Fig.5c).

Fig 6. KEGG enrichment analysis of cellular pathways associated with differentially expressed genes after AQP3 downregulation.

Expression abundance of genes involved in cell migration, adhesion, PIK3, and NF-κB signaling pathway J: AQP3-shRNA, K: CON-shRNA. The size of each dot represents expression abundance, while difference is shown by color: blue, down-regulated significantly ($P < 0.05$); red, up-regulated significantly ($P < 0.05$).

Fig 7. PDGF-B, FOX, Snail1 expression in HTR8/Svneo cells.

(a) Compared to the CON-shRNA group, the mRNA relative expression of FDGF-B in AQP3-shRNA group decreased significantly ($P=0.0012$), The mRNA relative expression of FOS and Snail1 were down-regulated, but the differences were not statistically significant (P values were 0.063,0.233, respectively). (b) The PDGF-B protein levels in the AQP3-shRNA group were significantly lower to the CON-shRNA group ($P<0.0001$). **$P<0.005$, **** $P<0.0001$, NS: $P > 0.05$.

Table 1. Functional categories of selected genes differentially expressed in of HTR8/Svneo cells AQP3-shRNA and CON-shRNA.

A list of differentially expressed genes discussed in the text with fold changes and statistical significances is shown.