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The blocking of aquaporin-3 (AQP3) impairs extravillous trophoblast cell migration

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ABSTRACT

Several aquaporins (AQPs) are expressed in extravillous (EVT) and villous trophoblast cells. Among them, AQP3 is the most abundant AQP expressed in chorionic villi samples from first trimester, followed by AQP1 and AQP9. Although AQP3 expression persists in term placentas, it is significantly decreased in placentas from preeclamptic pregnancies. AQP3 is involved in the migration of different cell types, however its role in human placenta is still unknown.

Here, we evaluated the role of AQP3 in the migration of EVT cells during early gestation.

Our results showed that Swan 71 cells expressed AQP1, AQP3 and AQP9 but only the blocking of AQP3 by $CuSO_4$ or the silencing of its expression by siRNA significantly attenuates EVT cell migration.

Our work provides evidence that AQP3 is required for EVT cell migration and suggests that an altered expression of placental AQP3 may produce failures in placentation such as in preeclampsia.

1. Introduction

Development of the human placenta and its different epithelial trophoblasts is critical to ensure the normal fetal growth and the maintenance of a healthy pregnancy. In this process, trophoblast cells differentiate along the villous or the extravillous trophoblast (EVT) pathways [1,2]. EVT cells proliferate, migrate and invade into the maternal decidua and the inner myometrium, remodeling the maternal spiral arteries. On the other hand, the villous trophoblasts form the outermost layer of the chorionic villi and play a central role in the regulation of feto-maternal exchange.

Failures of these events may lead to placental hypoperfusion, tissue injury, and trophoblast hypoxia and their consequences, including fetal death, growth restriction, and preeclampsia [2–6]. Preeclampsia is a gestational disorder associated with alterations in placental development and the differentiation of trophoblast cells, however, the underlying molecular mechanisms of this syndrome are still unknown [6].

Aquaporins (AQPs) are water channel proteins that allow the rapid movement of water across the plasma membrane in response to osmotic/hydrostatic pressure gradients [7,8]. The expression of several AQPs was found from blastocyst stages to term placenta and fetal membranes [9]. Escobar and coworkers have recently described that AQP3 was the most abundant AQP expressed in chorionic villi sample from first trimester, followed by AQP1 and AQP9 [10]. Thus, these proteins might be important in the normal fetal growth and homeostasis.

AQP3 is a member of the aquaporin family that can permeate water, urea and glycerol. It is expressed in trophoblast cells throughout gestation, but its physiological role is still unknown [10–12]. Apart from the classical functions in transcellular water transport, recent studies have revealed that AQPs may cooperate in different cellular processes, such as apoptosis, cell migration and proliferation [13–15].

Regarding the role of AQP3 in human placentas, we recently explored the participation of AQPs in placental programmed cell death and observed that the inhibition of AQP3 abrogates the trophoblast apoptosis [16]. Although the apoptosis of the trophoblast cells is a normal event that increases throughout gestation, it is exacerbated in placentas from pregnancies complicated by preeclampsia [2,3].

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In this context, we assumed that an increase in AQP3 might correlate with the increase in trophoblast apoptosis observed in preeclampsia, but in subsequent experiments, we found a reduced expression of AQP3 in these placentas [17].

Taken into account that preeclampsia is associated with defects in placental development during the early stages of pregnancy and the abnormal expression of AQP3 was found in the third trimester when the maternal syndrome is established, and the placenta is completely formed, we speculated that a normal AQP3 expression and function may be required for normal placentation.

Along with this idea, increasing evidence from both *in vitro* and *in vivo* experiments suggested that AQP3 could facilitate tumor cell migration [18]. Since EVT cells display a phenotype strikingly similar to that of cancer cells [19,20] we hypothesized that alterations in AQP3 since early stages of placenta development could be associated with pregnancy disorders. However, up to now, the role of AQP3 during early gestation was unexplored.

In the present study, we investigated the participation of AQP3 in EVT cell migration during placentation.

2. Methods

2.1. Cell culture

Swan 71 cell line (derived by telomerase-mediated transformation of a 7 week cytotrophoblast isolate) was kindly provided by Dr. Gil Mor [21]. Cells were cultured in DMEM-F12 (Life Technologies, Inc. BLR, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Laboratorio Natocor, Cordoba, Argentina), 5 mM L-Glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B in humidified air at 37 °C with 5% CO_2.

For the general blocking of AQPs, 50 μM $HgCl_2$ (Sigma–Aldrich, St. Louis, MO, USA), a nonselective inhibitor of AQPs, was added to the cultured medium [22,23]. Hg^{2+} is a nonselective inhibitor of AQPs by binding to the cysteine residue in the transmembrane domain of AQPs. For specific blocking of AQP3, 100 μM $CuSO_4$ (Sigma–Aldrich, St. Louis, MO, USA), was added to the culture medium [22,24]. $CuSO_4$ is a potent inhibitor of AQP3 which binds to three extracellular amino acid residues of AQP3 (Trp128, Ser152, and His241). Other AQPs were also blocked using 100 μM tetraethylammonium chloride (TEA; Sigma–Aldrich, St. Louis, MO, USA) and 100 μM phloretin (TEA; Sigma–Aldrich, St. Louis, MO, USA). TEA inhibits AQP1 by binging to the Tyr186 located at the extracellular part of the protein [22,25] while phloretin blocks AQP9 by electrostatic interactions with the Asg216, the Asp69 and the His151 of the protein [22,26].

2.2. Transfection with AQP3 siRNA

Cells were also transfected with human specific siRNA pools of 2–5 target specific 19–25 nt pools (AQP3-1 sc-sc-29713; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) with siRNA Transfection

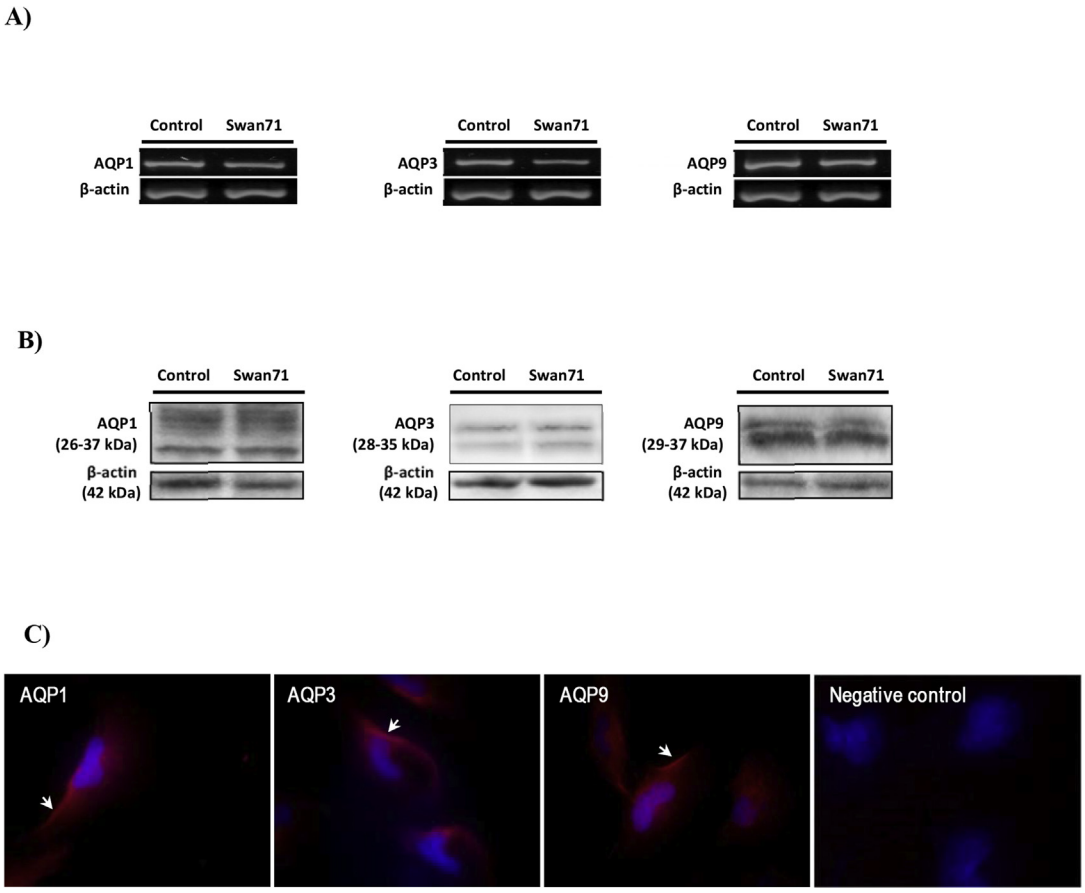

Fig. 1. Expression of AQP3 in Swan 71 cells.
A) Detection of AQP1, AQP3 and AQP9 mRNA in Swan 71 cells. Control: Term placenta.
B) Western blot of AQP1, AQP3 and AQP9 in Swan 71 cells. In all cases, two bands corresponding to the glycosylated and the non-glycosylated forms of AQPs were observed in Swan 71 cells. Control: Term placenta.
C) Immunofluorescence showing AQP1, AQP3 and AQP9 expression (white arrows) in plasma membrane of Swan 71 cells. *Magnification x1000.*

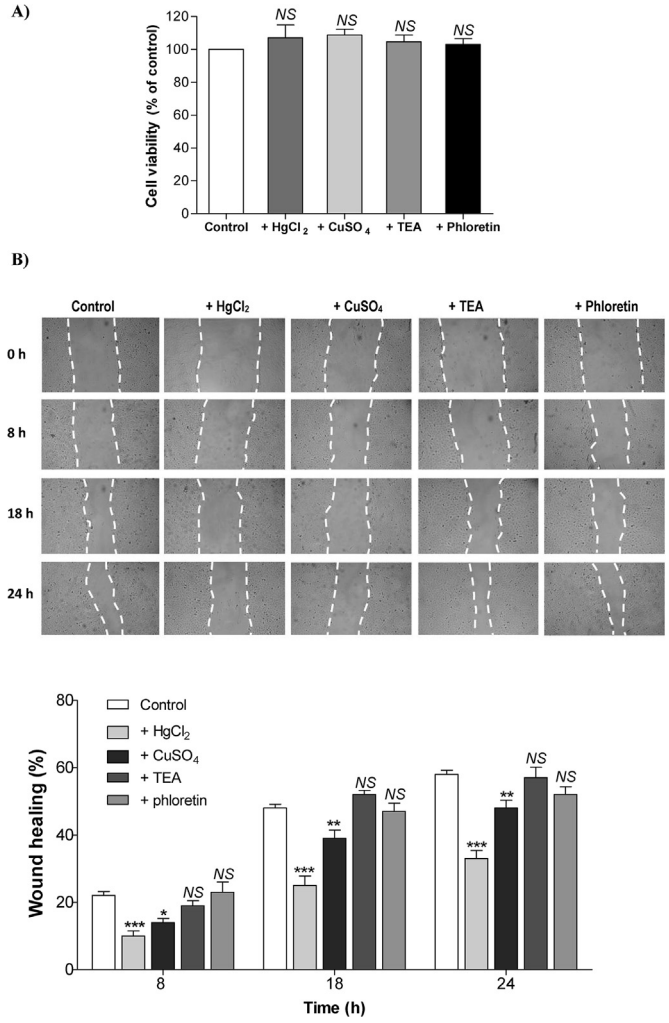

Fig. 2. Effect of blocking AQP1, AQP3 and AQP9 on the repair of wounds in Swan 71 cell monolayers.
A) The effect of $HgCl_2$ (50 μM), $CuSO_4$ (100 μM), TEA (100 μM) and phloretin (100 μM) on cell viability was examined using MTT *($p>0.05$, $n = 6$).* B) Cells were incubated in the absence or presence of $HgCl_2$, $CuSO_4$, TEA and phloretin. Wound was performed. The results were expressed as reinvasion percentage of the wound compared to time 0 and values are presented as means ± SEM of four wells from three different experiments *($^{*}p < 0.05$, $^{**}p<0.01$, $^{***}p<0.001$).*

Reagent® (sc-29528, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), according to the manufacturer's instructions, to silence AQP3 expression. Experiments were conducted 72 h post transfection. Western blotting was used to demonstrate AQP3 suppression. Scrambled siRNA-A (sc-37007) and PBS were used as controls.

2.3. Cell viability

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich Corp., San Luis, MO, USA) assay.

7×10^3 cells per well were loaded in a 96-well plate and cultured in DMEM-F12 with 10% FBS for 24 h. After treatments the medium was removed and 100 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT 0.5 mg/mL; Sigma-Aldrich Corp., San Luis, MO, USA) was added in the absence of light; formazan crystals were produced over a 2 h incubation period. To dissolve crystals, 100 μL of isopropanol was added to each well and the optical density at 540 nm was measured on ELISA Reader.

2.4. RT-PCR assay

Total RNA from trophoblast cells was isolated by using an SV Total RNA isolation system (Promega Co., USA). Reverse transcription was performed for 60 min on 5 μg of total RNA by using moloney murine leukemia virus reverse transcriptase, oligo (dT)15 primer and 400 μM of each deoxyribonucleotide triphosphate (dNTP) at 42 °C. PCR (30 cycles) at 94 °C for 60 s, at 58 °C for 60 s and at 72 °C for 60 s, followed by a final extension of 10 min at 72 °C, was carried out by using 5 μM of a specific oligonucleotide primers for human AQP3, using a specific pair of primers (sense 5′-CCTGAACCCTGCGGTGACC-3′ and antisense 5′- GGCATAGCCGG AGTTGAAGC-3′) [11,17]. We also assessed the expression of AQP1 (sense 5′-AGATCAGCATCTTCCGTG-3′ and antisense 5′-AGTTGTG TGTGATCACCG-3′) and AQP9 (sense 5′- CATCAACCCAGCTGTGTCT-3′ and antisense 5′-CAGCCACTGTTCAGTCCCA-3′) [11,27].

Control experiments were performed without the addition of reverse transcriptase to evaluate the absence of genomic DNA amplification.

2.5. Western blot

Cell lysates were obtained in Lysis buffer (0.3 M NaCl, 25 mM HEPES, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1% Triton X-100, pH 7.4) containing 0.2 mM phenylmethanesulfonyl fluoride (PMSF, Sigma-Aldrich Corp., San Luis, MO, USA) and 0.01x Protease Inhibitor Cocktail Set III (Calbiochem®, EMD Millipore Corporation, Darmstadt, Germany). The total protein concentration was measured using a BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific Inc. Waltham, MA, USA). Total protein lysates (50 μg) were resolved by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes (Hybond ECL, Amersham Pharmacia Biotech Ltd, Pittsburgh, PA USA), and blocked for 1 h with 5% nonfat dry milk in Phosphate-buffered saline (PBS, pH 7.5) with 0.1% Tween 20. After blocking, membranes were incubated overnight with the primary antibodies: anti-AQP1 antibody (Alpha Diagnostic International Inc., San Antonio, TX, USA; 1:1000), anti-AQP3 antibody (Alpha Diagnostic International Inc., San Antonio, TX, USA; 1:1000) or anti-AQP9 antibody (Alpha Diagnostic International Inc., San Antonio, TX, USA; 1:1000) and then with a goat anti-rabbit immunoglobulin G ([IgG] Jackson ImmunoResearch Laboratories, Inc. West Grove, PA, USA; 1:10,000) conjugated to peroxidase. Immunoreactivity was detected using the Enhanced Chemiluminescence (ECL) Western Blotting Analysis System (ECL plus, Amersham Pharmacia Biotech Ltd, Pittsburgh, PA USA) according to the manufacturer's instructions.

2.6. Immunofluorescence assay

Treated and untreated cells grown on coverslips were washed, fixed with 4% paraformaldehyde and blocked with goat serum. Expression of AQP1, AQP3 and AQP9 was detected with the antibodies described previously. Cells were incubated with Texas Red® Goat Anti-Rabbit IgG Antibodies (Vector Laboratories, Burlingame, CA, USA). After washing, cells were mounted with Fluoroshield mounting medium with DAPI (Abcam, Cambridge, MA, USA) to label nuclei and analyzed with an epifluorescent microscope (Nikon, Eclipse E:200).

2.7. Wound healing assay

The participation of AQPs in cell migration, was evaluated by wound healing assays. 5×10^4 cells were inoculated in 24-well plates, cultured until confluent, and then starved for 24 h in DMEM-F12 without FBS. After each treatment, the confluent cell

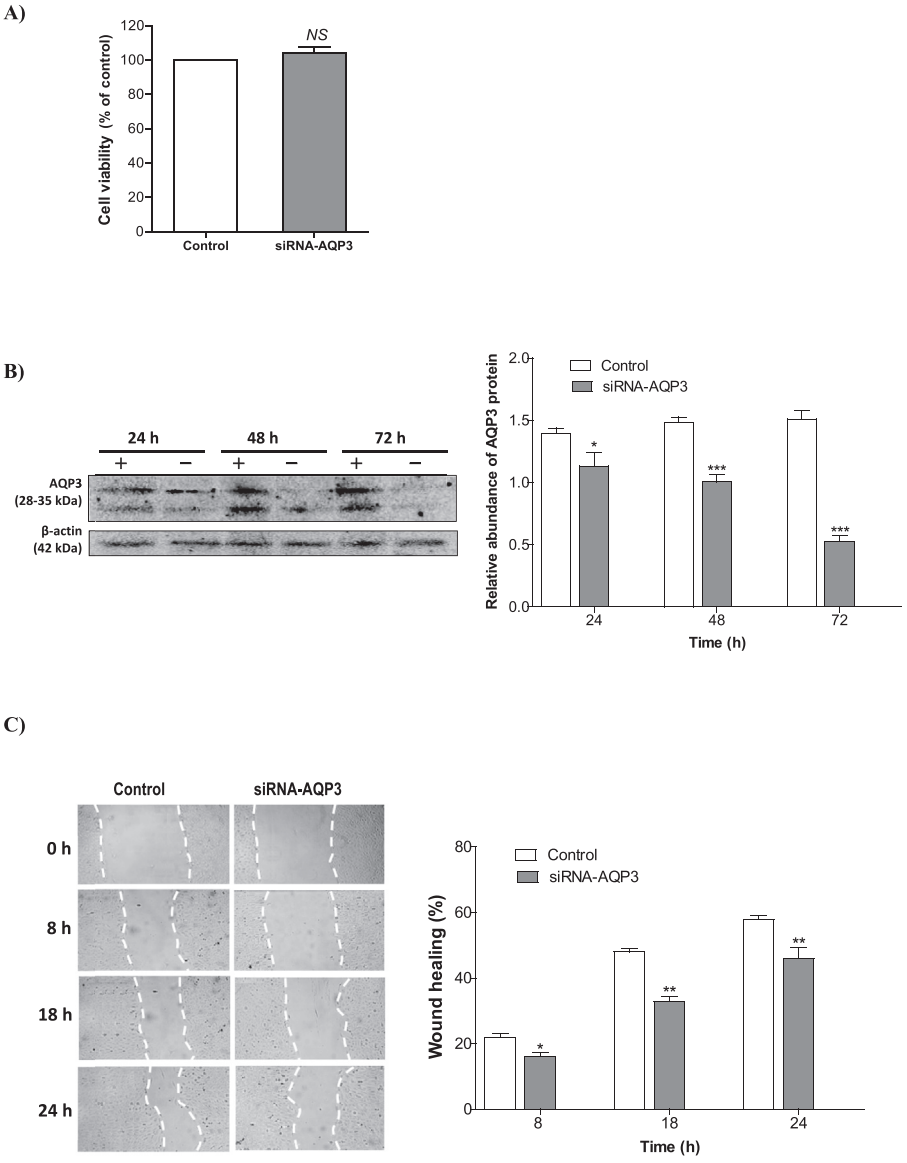

Fig. 3. Effect of silencing of AQP3 expression on the repair of wounds in Swan 71 cell monolayers.
A) Cell viability was examined by MTT *(p>0.05, n = 3).*
B) Densitometry analysis showed AQP3 suppression after 24 h, 48 h and 72 h of siRNA transfection *(n = 3, *p < 0.05; ***p < 0.001).*
C) After 72 h of siRNA transfection, wounds were performed. AQP3 silencing significantly attenuates Swan 71 cell migration. Results were expressed as reinvasion percentage of the wound compared to time 0 and values are presented as means ± SEM of four wells from three different experiments *(*p < 0.05, **p < 0.01).*

monolayer was scraped in a straight line with a 200 μL pipette tip to create a wound. Wounded monolayers were then washed twice with PBS to remove cell debris, and incubated in DMEM-F12 supplemented with 0.5% FBS for 24 h. Representative images were taken after 8, 18 and 24 h. TScratch® software was used to quantify the data and scratch distance at 0 h was considered as 0% of gap closure [28].

2.8. Statistical analysis

Statistical analysis of data was performed by GraphPad Prism v5 software (GraphPad Software, Inc. La Jolla, CA, USA). Results represent mean ± SEM. Comparisons were performed using one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc tests where appropriate. Student's t-test was used when the values of two groups were analyzed. Values were considered significantly different when $p < 0.05$.

3. Results

First, we investigated the expression of AQP1, AQP3 and AQP9 at the transcriptional and protein levels in Swan 71 cells. Total RNA was extracted and RT-PCR analysis was performed. An expected band of ~400-bp similar to the band obtained with total RNA from term placenta used as positive control was observed (Fig. 1A).

Regarding protein expression, immunoblotting showed two bands corresponding to the glycosylated and the non-glycosylated forms of AQPs in all cases (Fig. 1B). No bands were observed when the primary antibody was omitted or when the antibody was preadsorbed with the specific peptide (data not shown).

In addition, immunofluorescence showed the expression of AQP1, AQP3 and AQP9 in the plasma membrane of Swan 71 cells (Fig. 1C).

After establishing that Swan 71 cells expressed AQP1, AQP3 and AQP9 we tested the contribution of these proteins in cell migration

of EVT cells.

To investigate the possible involvement of AQPs in the mechanism of EVT cell migration during wound healing, we examined the effect of $HgCl_2$ on the recovery of wounds artificially produced in Swan 71 cell monolayers. We observed that the reinvasion of the wound was significantly reduced after the addition of 50 μM $HgCl_2$ to the medium. Then, we inhibited AQP3 with $CuSO_4$, a specific blocker of AQP3 water and glycerol permeability, and we also observed a reduced cell migration. We also used TEA to block AQP1 and phloretin to block AQP9. In both cases, cell migration was not affected (Fig. 2 B). In all the conditions, cell viability was not modified (Fig. 2A) and the expressions of the AQPs were not affected (data not shown).

To confirm the contribution of AQP3 in EVT cell migration during wound healing, AQP3 expression was silencing by siRNA.

We assessed the efficiency of the transfection by Western blot at 24, 48 and 72 h. AQP3 protein expression in the knockdown cells was decreased to less than 30% compared to the control cells at 72 h post transfection (Fig. 3A). These results confirmed the effectiveness of the knockdown in Swan 71 cells. We have not observed any difference in cell viability between the control cells and knockdown cells (Fig. 3B). After 72 h post transfection, we found that cell migration was significantly reduced (Fig. 3C).

4. Discussion

During placentation, EVT cells migrate to promote the remodeling of the maternal vessels and to establish an adequate blood supply between mother and fetus. EVT cells use the same molecular machinery as those of malignant tumors for growth, migration and invasion [19,20]. However, the development of the placenta is a complex and tightly regulated process and failures in this process result in gestational disorders such as preeclampsia [2–6]. Previously, we reported that AQP3 is expressed in human term placentas, and it significantly decreased in placentas complicated by preeclampsia [11,17]. However, its role in human placenta remains uncertain.

Since AQP3 expression persists from zygote to blastocyst stages [29] and is the most AQP abundant expressed in chorionic villi from first trimester [10], we believe that this protein may be essential for early placenta and fetal development.

Here we demonstrated that Swan 71 cells expressed AQP1, AQP3 and AQP9.

In addition, we also found that the general blocking of AQPs with $HgCl_2$ and the specific blocking of AQP3 with $CuSO_4$ significantly reduced EVT cell migration. Because both $HgCl_2$ and $CuSO_4$ at the concentrations used did not affect the viability of Swan 71 cells after 24 h of treatment (the end point of the wound healing assay), the impairing effect of $HgCl_2$ and $CuSO_4$ observed does not seem to be attributable to the cytotoxicity of these compounds. These results strongly suggest that the inhibition of AQP3 results in a delay of cytotrophoblast cells wound healing. Consistent with these findings, we also showed that the specific silencing of AQP3 expression also impaired EVT cell migration.

Therefore, our results provide evidence that AQP3 expressed in EVT cells may be required for cell migration during early gestation.

We also speculated that the abnormal expression of AQP3 found in placentas from preeclamptic women [17], might be also present in the trophoblast stem cell and persists after its differentiation in EVT and villous trophoblast cells.

Along with this idea, impairs in placentation at the very beginning of pregnancy may affect villous trophoblast as well as EVT development, resulting in the occurrence of pregnancy disorders such as preeclampsia and growth restriction [2,3].

In conclusion, we showed for the first time that AQP3 participates in EVT cell migration suggesting that the dysregulation of this protein may be involved in the pathophysiology of preeclampsia.

Declaration of interest

The author declares that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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