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## **Alteration of glucose metabolism in cultured astrocytes after AQP9-small interference RNA application**

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**Abstract :**

Aquaglyceroporin-9 (AQP9) facilitates diffusion of water and energy substrates such as glycerol and monocarboxylates. AQP9 is present in plasma membrane and mitochondria of astrocytes and catecholaminergic neurons, suggesting that it plays a role in the energetic status of these cells. Using specific small interference RNA directed against AQP9 in astrocyte cultures, we showed that glycerol uptake is decreased which is associated with an increase in glucose uptake and oxidative metabolism. Our results not only confirm the presence of AQP9 in astrocytes but also suggest that changes in AQP9 expression alter glial energy metabolism.

## **Introduction:**

Aquaporins (AQPs) form a family of proteins with 13 members ubiquitously expressed in mammalian tissues. AQPs were first proposed to be involved in water diffusion and water homeostasis. A subgroup of AQPs are permeable to water, glycerol as well as other small solutes (e.g. monocarboxylates, copper, arsenite) and are named *aquaglyceroporins* (e.g. AQP9) (Badaut, 2010). In the brain, AQP9 was found in tanycytes of the mediobasal hypothalamus, in astrocytes and in catecholaminergic neurons (Arcienega et al., 2010; Badaut, 2010). At the subcellular level, AQP9 was found in the plasma membrane and in the internal mitochondrial membrane (Amiry-Moghaddam et al., 2005). These immunohistochemical results are now debated because Rojek and collaborators reported the presence of AQP9 immunostaining in AQP9<sup>-/-</sup> mouse brain. The authors explained this result by the fact that this immunostaining is likely due to non-specific labeling with commercial antibodies (Rojek et al., 2007) and they questioned the presence of AQP9 in the brain. However, this result contrasts with several other studies using RT-PCR and in situ hybridization indicating the presence of AQP9 mRNA, and revealing the presence of the AQP9 protein by western blot in rat and mouse brains (Arcienega et al., 2010; Mylonakou et al., 2009).

AQP9 was also observed in the liver (Badaut et al., 2008) and its expression has been shown to be regulated by the physiological feeding state mediated by the insulin response element (IRE) present in the promoter of the gene (Kuriyama et al., 2002). AQP9 expression in liver is increased in rodent models of diabetes (Badaut et al., 2008), (Kuriyama et al., 2002). All these results support the concept that AQP9 facilitates glycerol use for neoglucogenesis during fasting periods (Badaut, 2010). The brain is known to be also involved in energy balance, and we have previously shown that the brain AQP9 expression is negatively regulated by insulin application, similarly to the observation in liver (Badaut et al., 2008). Furthermore, glycerol is already known to be used by brain tissue as a metabolite (Davis et al., 1981; McKenna et al., 1986; Seiler et al., 1969; Tildon and Roeder, 1980).

Based on these observations, we hypothesized that AQP9 could facilitate glycerol uptake in astrocytes and decreased AQP9 expression would affect astrocyte energy metabolism.

## **Results:**

### **1/ Reduced AQP9 expression after siRNA application.**

The application of the siAQP9 caused a significant decrease of AQP9 expression of  $67\% \pm 2\%$  compared to CTL astrocyte cultures, treated with a non-targeted siRNA (siGLO,  $p < 0.05$ ) (Fig.1A, B). Astrocytes treated with siAQP4 exhibited a small but non-significant decrease of  $33\% \pm 8\%$  compared to CTL astrocyte cultures (Fig.1B). The hematoxylin staining (Fig.2A,B) and cell counting (Fig.2C) did not reveal any differences in cell density between CTL, siAQP4 and siAQP9-treated conditions, suggesting that the decrease in AQP9 protein level is not a consequence of cell death.

### **2/ Functional consequences of AQP9 knockdown on glycerol uptake and astrocyte energy metabolism.**

$^{14}\text{C}$ -glycerol uptake was significantly decreased by  $31\% \pm 6\%$  ( $p < 0.05$ ) in siAQP9 treated cultures compared to CTL cultures (Fig.3A). Application of siAQP4 did not change glycerol uptake compared to the CTL group (Fig.3A). As glycerol is a putative energy metabolite for the central nervous system and potentially for astrocytes (McKenna et al., 1986; McKenna et al., 2006), we then tested whether a decrease in AQP9 expression induced changes in glucose metabolism and in lactate release by astrocytes. Application of the siAQP9 induced a significant increase in  $^3\text{H}$ -2-deoxyglucose uptake that reached  $161\% \pm 20\%$  ( $P < 0.05$ ) compared to CTL cultures (Fig.3B). The application of siAQP4 did not change the uptake of  $^3\text{H}$ -2-deoxyglucose (Fig.3B). Although glucose uptake was increased, the release of lactate was not changed under the same conditions (Fig.3C). Next, the possible changes in oxidative metabolism were studied using the MTT-staining, a marker of the oxidative pathway that is also used for cell number counting. Since we did not observe any changes in the number of cells, we supposed then that changes in MTT staining must be related to oxidative metabolism. Our experiments revealed that MTT-staining was reduced by  $41\% \pm 8\%$  ( $p < 0.05$ ) in the siAQP4 condition and increased by  $17\% \pm 9\%$  ( $p < 0.05$ ) in the siAQP9 treated cultures compared to the control (Fig.3D).

Finally, the metabolic response of astrocytes induced by glutamate was then evaluated. Values for <sup>3</sup>H-2-deoxyglucose uptake by astrocytes after glutamate application were normalized to the basal value for each experimental group to assess the response to glutamate on glucose metabolism for each condition (Fig.4A, B). Glutamate application induced a significant increase of <sup>3</sup>H-2-deoxyglucose uptake (Fig.4A), which is paralleled by an increase in the release of lactate into the extracellular space in the 3 groups (Fig.4B). The metabolic response to glutamate application in astrocytes does not appear to be altered by the application of siAQP4 or siAQP9.



## **Discussion:**

In the brain, AQP9 expression was described in astrocytes and in catecholaminergic neurons by several groups, although recent results obtained with AQP9-KO mice questioned the presence of this channel protein in the brain. Using siRNAs against AQP9 on astrocyte cultures, we showed that a reduction in AQP9 expression induced a decrease in glycerol uptake and changes in astrocyte energy metabolism. For the first time, AQP9 was shown to contribute in astrocytes to glycerol diffusion and indirectly to energy metabolism. In fact, glycerol is known to be an energy substrate for brain tissue and also for astrocytes (McKenna et al., 1986). Glycerol is an important intermediate that can enter in the glycerol phosphate shuttle, a pathway that has been suggested to take place in astrocytes (McKenna et al., 1986). An interesting observation reported in this study is that a decrease in AQP9 expression (and glycerol uptake) in astrocytes increased their glucose uptake (Fig.2B) and potentially their oxidative metabolism, measured with MTT staining. The increase in oxidative metabolism might help to restore NAD<sup>+</sup>/NADH and FAD/FADH<sub>2</sub> balance, possibly perturbed by the decrease of glycerol uptake in siAQP9 treated cultures. A decrease of glycerol uptake may affect the glycerol phosphate shuttle, which is essential for maintaining a favorable NAD<sup>+</sup>/NADH ratio in the cell. Therefore oxidative metabolism is increased to maintain this ratio stable. In addition, glycerol might be used by the astrocyte as an energy substrate and the decrease of glycerol availability after siAQP9 application is compensated by an increase in glucose uptake and oxidative metabolism. On the basis of the *in vitro* work reported here, it can be proposed that AQP9 *in vivo* might play a role in energy metabolism in astrocytes.

Interestingly, despite such a possible implication in oxidative metabolism, AQP9 expression does not appear to alter the glycolytic response of astrocytes to an external signal such as glutamate. Such an observation emphasizes the point that glycolytic and oxidative metabolism in astrocytes can be compartmentalized and are likely regulated differently (Bouzier et al., 1998; Zwingmann et al., 2001).

AQP4 knockdown does not change the level of glycerol and glucose uptake. However, we observed a significant decrease in MTT staining (Fig.2) without significant cell death

(Fig.1), suggesting that oxidative metabolism is impaired when AQP4 is decreased. This unexpected result may be related to new observations that AQP4 facilitates CO<sub>2</sub> diffusion through the cell membrane (Musa-Aziz et al., 2009). A decrease of AQP4 expression may decrease the diffusion of CO<sub>2</sub> and affect the astrocyte intracellular pH. This may have direct consequences on mitochondrial physiology. Alternatively, AQP4 might facilitate O<sub>2</sub> diffusion and decreased AQP4 expression might limit the access of O<sub>2</sub> to the mitochondrial respiratory chain.

In summary, our data show that AQP9 is present in astrocytes and involved in energy metabolism, facilitating glycerol movements through the membrane. We previously described increase of AQP9 expression in astrocytes after stroke (Badaut et al., 2001; de Castro Ribeiro et al., 2006), suggesting that the extracellular increase of glycerol after brain injury (Bertrand et al., 1992; Frykholm et al., 2001) is possibly cleared by the astrocytes and used as energetic substrate. Interestingly, AQP4 is also linked with oxidative metabolism, since when its expression is decreased oxidative metabolism is changed. In other words, under pathological conditions, an increase in AQP4 expression might also help in O<sub>2</sub> diffusion and oxidative pathway, in addition to water diffusion.

## **Material and Methods**

### **Astrocyte cultures and siRNA tests**

Astrocyte cultures were prepared from mouse neurospheres (Brunet et al., 2004). After dissociation of the neurospheres, cells were plated into 12-well tissue culture plates (Costar 3524, Corning, NY) for differentiation into astrocytes using Fetal Calf Serum (10%) that was added to the culture 4 days prior to siRNA experiments.

AQP silencing protocol was adapted from (Badaut et al., 2011). Briefly, SMART-pool®-siRNA (20nM, Dharmacon-Research) against AQP4 (siAQP4), AQP9 (siAQP9), non-targeted siRNA (siGLO-RISC-free, control condition) were mixed with Dharmafect® (Dharmacon-Research), diluted 1:50 in Opti-MEM (Invitrogen) 20 minutes before to be added to the cell medium 4 days prior to perform the experiments. In the three groups (CTL, siAQP4, siAQP9) the level of AQP9 expression was confirmed by Western blot analysis.

### **Western blot analysis**

Astrocyte cultures were harvested in cells lysis buffer (RIPA buffer, Cell signaling) and sonicated for 30 sec. Ten µg of protein were subjected to SDS-polyacrylamide gel electrophoresis on a 10% gel (Nupage, Invitrogen, Carlsbad, CA). Proteins were then transferred to a polyvinylidene-fluoride membrane (PerkinElmer, Germany). The blot was incubated with a polyclonal antibody against AQP9 (Chemicon, California, 1:1,000) and a monoclonal antibody against actin (Sigma, Switzerland, 1:25,000) in Odyssey-blocking buffer (LI-COR-Bioscience, Germany) overnight at 4°C. After washing, the membrane was incubated with two fluorescence-coupled secondary antibodies (1:10,000; anti-rabbit-Alexa-Fluor-680nm, Molecular-Probes, Oregon and anti-mouse IR-Dye-800-nm, Roche, Germany) for 2 hours at room temperature. Fluorescence was measured using an infra-red scanner (Odyssey-LI-COR, Germany) (Badaut et al., 2011) and analyzed on 3 separate cultures for each group.

### **Histology and image analysis**

Hematoxylin stained cells were automatically counted in 3 separate cultures for each group with 5-ROIs, each one containing 65 fields (422 $\mu$ m x 338 $\mu$ m) after setting up threshold and morphology parameters using Mercator software (Explora-Nova, France).

### **<sup>14</sup>C-glycerol uptake measurement**

Glycerol uptake was studied by incubating cultured cells with <sup>14</sup>C-glycerol (1  $\mu$ Ci) and unlabeled glycerol (final concentration 2 mM, specific activity 0.5  $\mu$ Ci/ $\mu$ mol) in the presence or absence of HgCl<sub>2</sub> (1 mM). Incubation took place at 37°C for 10 min in DMEM (D5030) at pH 7.4 and determination of non transporter-mediated uptake was performed at 4°C. Then, the cultures were washed with the same buffer at 4°C containing HgCl<sub>2</sub> (1 mM) to block AQP9. Cells were harvested in 500  $\mu$ l of PBS buffer containing NaOH (0.1M) and triton X100 (1%) and radioactivity was measured by scintillation counting. Data presented are an average of 4 separate cultures for each group.

### **<sup>3</sup>H-2-Deoxyglucose uptake measurement**

Glucose utilization was determined by measuring <sup>3</sup>H-2-Deoxyglucose (2DG) uptake (Pellerin and Magistretti, 1994). The procedure was similar to the one described above for glycerol measurement except that <sup>3</sup>H-2-DG (American-Radiolabeled-Chemicals) at 1  $\mu$ Ci/ml (specific activity 60 Ci/mmol) was used as a tracer. The incubation time was 20 min and glutamate (200 $\mu$ M) was added together with the medium containing the tracer. To determine non-transporter-mediated uptake, incubation was performed at 4°C. Results, which represent glucose transporter-mediated uptake, were calculated by subtracting from total counts the portion which was not inhibited at 4°C. Data presented are an average of 5 separate cultures for each group.

### **Lactate release measurement**

Supernatants from 2DG uptake experiments were collected to determine lactate production. Two 100 $\mu$ l aliquots for each supernatant were placed in a 96-well plate. To each well was added 100  $\mu$ l of a solution containing lactate dehydrogenase 70U/ml (L-

2500 Sigma, Buchs, Switzerland) and NAD 15mM (53-84-9, Acros organics, Geel, Belgium) in glycine-semicarbazide buffer 0.33M, pH 10. After 1 h at 37°C, plates were read at 340 nm to detect production of NADH. A standard curve with known concentrations of lactate was used to determine lactate concentrations, corrected for the amount of protein in the same culture well. Data presented are an average of 5 separate cultures for each group.

### **MTT measurement**

Thiazolyl Blue Tetrazolium Bromide (MTT) staining was used to evaluate mitochondrial oxidative activity. MTT was prepared at 5mg/ml in PBS and added to the cell medium at a final concentration of 5 µg/ml. Cells were collected in DMSO and formazan was measured at 595 nm (TECAN SUNRISE, Software Magellan). The intensity of staining is linearly related to oxidative activity. Data presented are an average of 4 separate cultures for each group.

### **Statistical analysis**

All data are presented as mean ± SEM and statistical analysis was carried out using GraphPad InStat version 3.05 (GraphPad Software, San Diego CA) and (Sigmastat, SPSS Inc.). A Kolmogorov and Smirnov-test was first performed to assess the Gaussian distribution of the data. Data which passed the test were analyzed with an ANOVA followed by Tukey-Kramer multiple comparison tests.

## **Figure legends**

### Figure 1:

- A)** Western blot for AQP9 exhibiting a specific band (red) around 30 kDa in CTL, siAQP4 and siAQP9 conditions. The specific band for actin (green) located at 42 kDa, was used as a loading control for the 3 conditions. AQP9 intensity is significantly reduced in the siAQP9 condition compared to siAQP4 and CTL. In cultures treated with siAQP4, a decrease of AQP9 expression was observed, but did not reach significance when assessed quantitatively (see B)
- B)** Western blot quantification showing that AQP9 intensity is significantly decreased in the siAQP9 condition compared to siAQP4 and CTL (\*,  $p < 0.05$ ) in three independent experiments.

### Figure 2:

- A and B)** Hematoxylin staining of CTL and siAQP9 treated cultures did not show changes in cell density. Bar = 50  $\mu$ m
- C)** Quantification of cell density confirms the visual impression that siAQP4 and siAQP9 did not change cell numbers.

### Figure 3

- A)** Glycerol uptake after application of non-targeted siRNA (CTL), siAQP4 and siAQP9. Glycerol uptake is significantly decreased in siAQP9 treated cultures compared to siAQP4 and CTL (\*,  $p < 0.05$ )
- B)** Glucose utilization as assessed by  $^3\text{H}$ -deoxyglucose uptake after application of non-targeted siRNA (CTL), siAQP4 and siAQP9.  $^3\text{H}$ -deoxyglucose uptake was significantly increased in siAQP9 treated cultures compared to siAQP4 and CTL

**C)** No significant difference in lactate release was observed after application of non-targeted siRNA (CTL), siAQP4 and siAQP9.

**D)** MTT staining showed a significant decrease after application of siAQP4 compared to non-targeted siRNA (CTL) (#,  $p < 0.05$ ). In contrast, application of siAQP9 showed a significant increase of the MTT staining compared to non-targeted siRNA (CTL) (\*,  $p < 0.05$ ).

#### Figure 4

**A)** Glutamate application induced an increase of  $^3\text{H}$ -2-deoxyglucose uptake in the 3 groups that reached  $154\% \pm 9\%$  in CTL (non-targeted siRNA),  $162\% \pm 9\%$  in siAQP4 and  $139\% \pm 12\%$  in siAQP9 groups (\*,  $p < 0.05$ ).

**B)** Glutamate application induced enhancement of lactate release into the extracellular space reached  $157\% \pm 15\%$  in CTL (non-targeted siRNA),  $138\% \pm 24$  in siAQP4 and  $155\% \pm 27\%$  in siAQP9 groups (\*,  $p < 0.05$ ).

## Disclosure/Conflict of Interest

No conflict of interest



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Figure 1

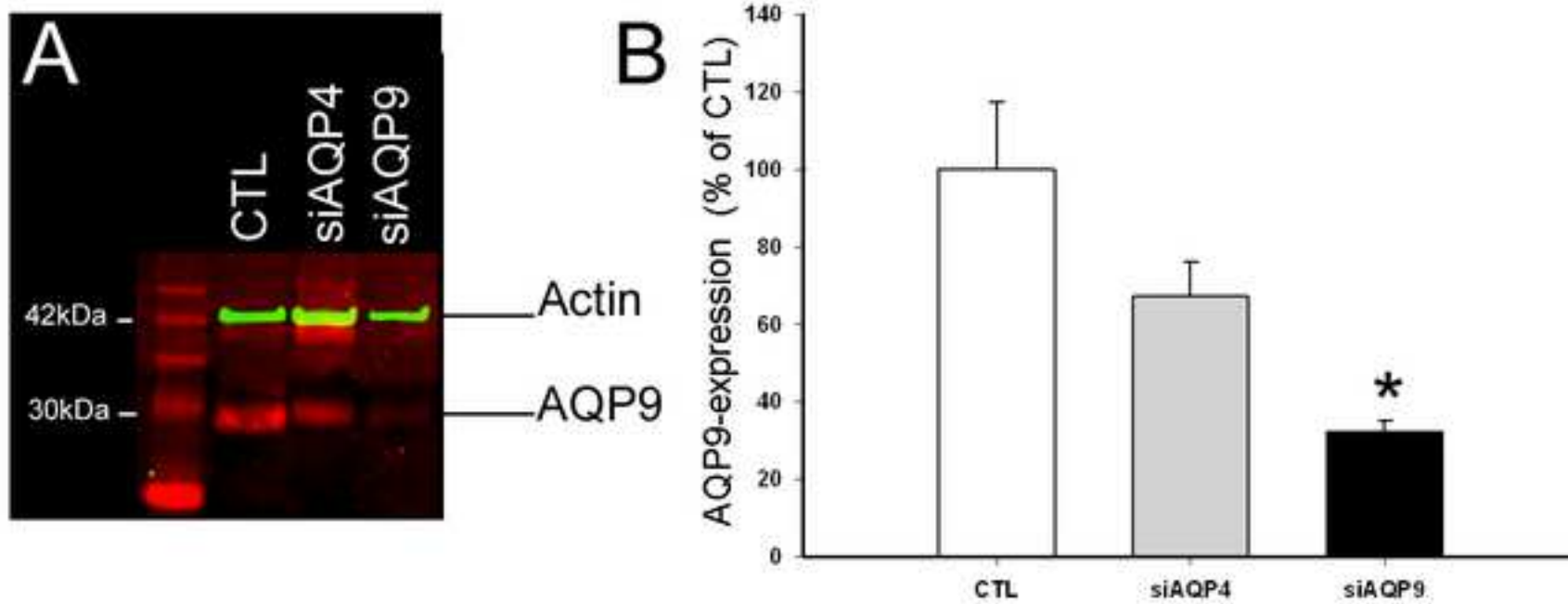


Figure 2

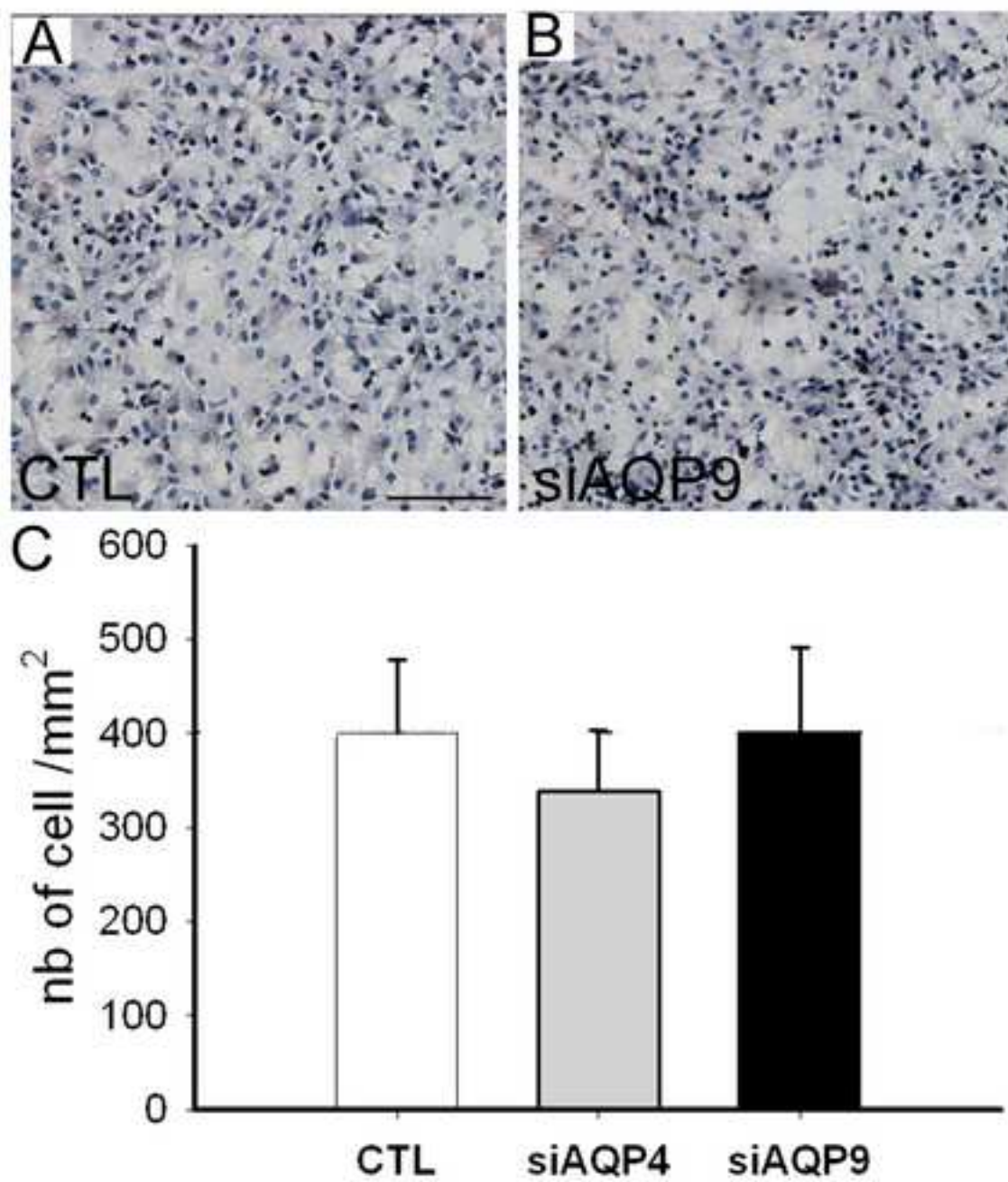


Figure 3

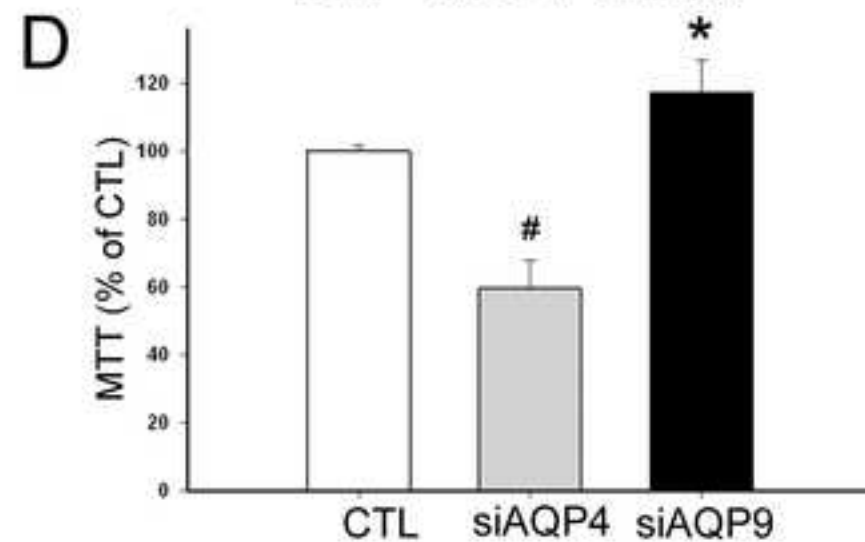
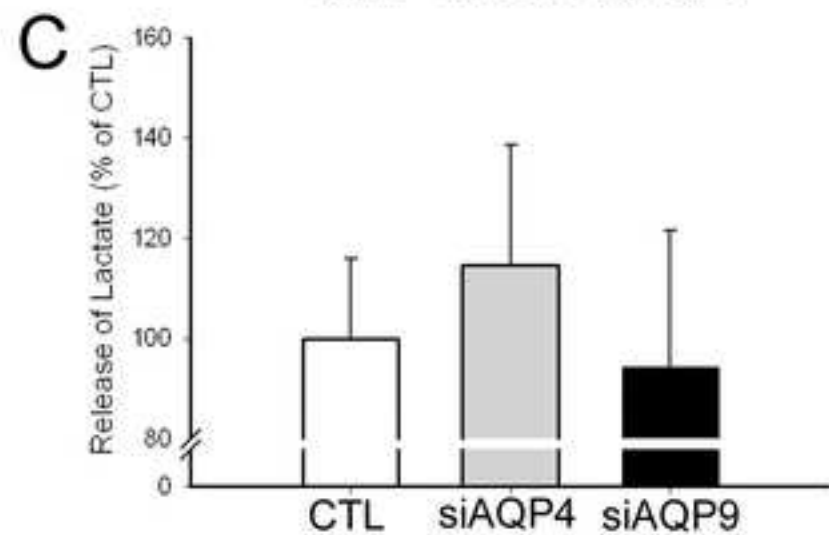
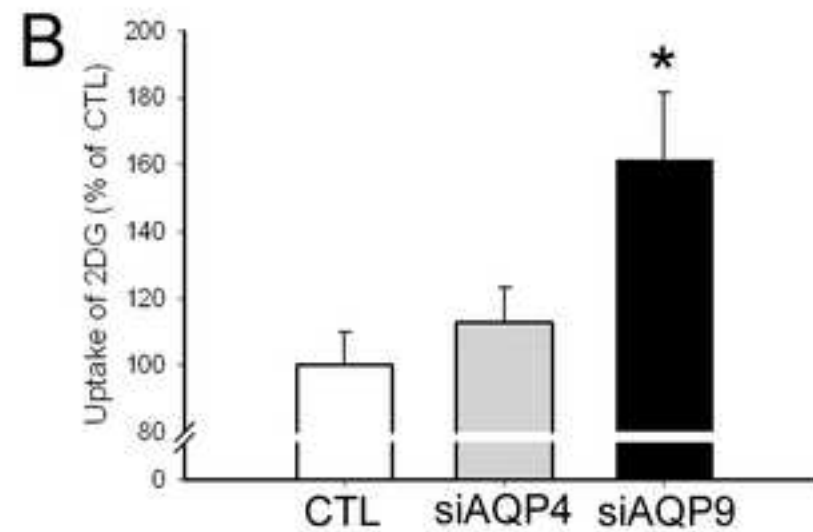
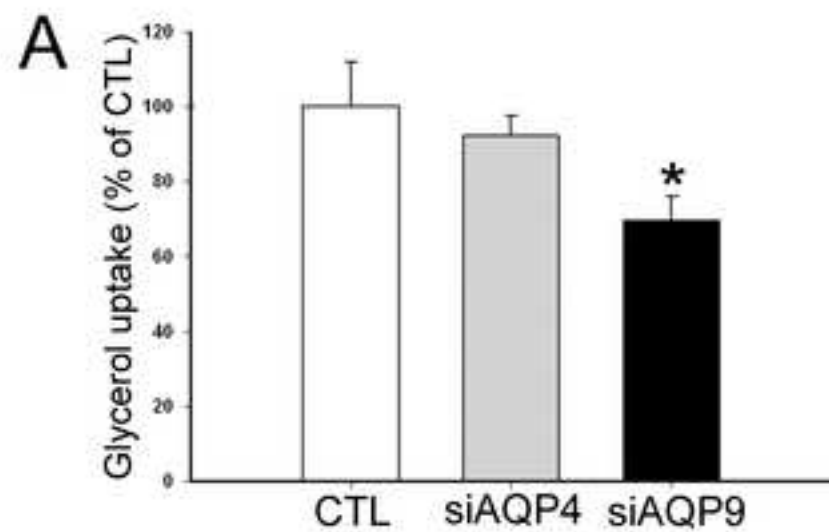


Figure 4

