Original Paper

FAM3A Protects HT22 Cells Against Hydrogen Peroxide-Induced Oxidative Stress Through Activation of PI3K/Akt but not MEK/ERK Pathway

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Key Words

FAM3A • Oxidative stress • Mitochondria • Akt • ERK

Abstract

Background/Aims: Oxidative stress-induced cell damage is involved in many neurological diseases. FAM3A is the first member of family with sequence similarity 3 (FAM3) gene family and its biological function remains largely unknown. **Methods:** This study aimed to determine its role in hydrogen peroxide (H_2O_2) induced injury in neuronal HT22 cells. The protective effects were measured by cell viability, lactate dehydrogenase (LDH) release and apoptosis, and oxidative stress was assayed by reactive oxygen species (ROS) generation, ATP synthesis and lipid peroxidation. By using selective inhibitors, the involvement of PI3K/Akt and MEK/ERK pathways were also investigated. **Results:** The results of fluorescence staining revealed that H_2O_2 significantly decreased the expression of FAM3A protein, which was shown to be subcellularly located in mitochondria. Up-regulation of FAM3A by lentivirus transfection markedly increased cell viability and decreased LDH release after H_2O_2 treatment. The anti-apoptotic activity of FAM3A was demonstrated by the reduced mitochondrial cytochrome c release, decreased activation of caspase-3 and the results of flow cytometry. Overexpression of FAM3A attenuated intracellular ROS generation and loss of ATP production induced by H_2O_2 , and subsequently inhibited lipid peroxidation. In addition, overexpression of FAM3A significantly increased the activation of Akt and ERK in H_2O_2 injured HT22 cells. By using Akt and ERK specific inhibitors, we found that inhibition of PI3K/Akt, but not MEK/ERK pathway, partially prevented FAM3A-induced protection against H_2O_2 . **Conclusion:** These results suggest that FAM3A has protective effects against H_2O_2 -induced oxidative stress by reducing ROS accumulation and apoptosis, and these protective effects are dependent on the activation of PI3K/Akt pathway.

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Introduction

Reactive oxygen species (ROS), the chemically reactive molecules formed by incomplete one-electron reduction of oxygen, play important roles in cell signaling and homeostasis [1].

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However, increased ROS production under aberrant metabolic processes leads to oxidative stress, which has been implicated in the pathophysiology of various neurological disorders [2-4]. It is well known that the mammalian brain has high amount of oxygen but low levels of antioxidant enzymes, making it particularly sensitive to oxidative damage [5, 6]. The ROS-induced oxidative stress in neuronal cells triggers a mechanism that, through the release of cytochrome c and activation of caspase-3, leads to intrinsic apoptosis. Hydrogen peroxide (H₂O₂), a highly reactive ROS, is thought to be the radical most responsible for oxidative neuronal damage. It has been widely used to mimic *in vitro* oxidative stress in many different cell types, including HT22 hippocampal neuronal cells [7, 8].

The family with sequence similarity 3 (FAM3) gene family is a cytokine-like gene family identified in 2002 with four members: FAM3A, FAM3B, FAM3C and FAM3D [9]. It is shown that FAM3B is highly expressed in pancreas, FAM3D in placenta, and FAM3A and FAM3C in almost all tissues. Increasing evidence suggests that these FAM3 family members are involved in the development and progression of many human diseases, such as diabetes and cancer [10]. Most of the studies of FAM3 family proteins are focused on FAM3B, which is also known as pancreatic-derived factor (PANDER) due to its high abundance in pancreatic islets [11]. Previous studies showed that dysregulated FAM3B could promote the pathogenesis of type 2 diabetes by decreasing pancreatic β cell function and hepatic insulin sensitivity [12]. However, the physiological role of FAM3A remains largely unknown. FAM3A mRNA is ubiquitously expressed in almost all tissues of human and rodent, and FAM3A protein is composed of 230 amino acid residues [9]. Recent studies reported that FAM3A is a target gene of peroxisome proliferator-activated receptor γ (PPAR γ) [13], and overexpression of FAM3A significantly attenuates hyperglycemia, insulin resistance and fatty liver via Ca²⁺-mediated activation of PI3K/Akt signaling [14].

In the present study, we reported that FAM3A is expressed in HT22 hippocampal neuronal cells, where its expression was significantly decreased by H₂O₂ treatment. Overexpression of FAM3A markedly attenuated H₂O₂-induced cytotoxicity and apoptosis with suppressed oxidative stress in HT22 cells. These effects of FAM3A were found to be mediated by PI3K/Akt, but not MEK/ERK signaling.

Materials and Methods

Cell culture

The neuronal HT22 cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum (FBS) and incubated at 37°C with 5% CO₂, and 95% humidity.

Cell viability assay

Cell viability assay was performed using the cell proliferation reagent WST-1 following the manufacture's protocol (Roche, Basel, Switzerland). Briefly, cells were cultured at a concentration of 3×10^5 in microplates in a final volume of 100 μ l/well culture medium. After various treatments, 10 μ l WST-1 was added into each well and incubated for 4 h at 37°C. Then, 100 μ l/well culture medium and 10 μ l WST-1 was added into one well in the absence of cells, and its absorbance was used as a blank position for the enzyme-linked immunosorbent assay (ELISA) reader. The cells were shaken thoroughly for 1 min on a shaker and the absorbance of the samples was measured using a microplate reader.

Lactate dehydrogenase (LDH) release assay

Cytotoxicity was determined by the release of LDH using a diagnostic kit according to the manufacturer's instructions. Briefly, 50 μ l of the supernatant from each well was incubated with the reduced form of nicotinamide-adenine dinucleotide (NADH) and pyruvate for 15 min at 37°C and the reaction was stopped by adding 0.4 M NaOH. The activity of LDH was calculated from the absorbance at 440 nm, and the background absorbance from the culture medium that was not used for any cell cultures was subtracted from all of the absorbance measurements.

Immunocytochemistry (ICC)

HT22 cells were fixed for 30 min with 4% paraformaldehyde, rinsed twice with PBS and subsequently incubated with blocking solution (PBS containing 1% bovine serum albumin, 0.4% Triton X-100 and 4% normal goat serum) for 20 min. Next, cells were incubated with primary antibody (1:500; anti-FAM3A) at 4°C overnight. The cells were then rinsed twice and incubated with fluorescein isothiocyanate (FITC) labeled secondary antibody (1:600; goat anti-rabbit) for 1 h at room temperature. Mito tracker (Red) and DAPI were used to stain mitochondria and nucleus, respectively.

Lentivirus construction and transfection

The coding sequence of FAM3A was amplified by RT-PCR with sequences: forward, 5'-TCA TGA GCA GCG TCA AAGAC-3'; reverse, 5'-AGG GTA CCT TCA TGC AGTGG-3'. The PCR fragments and the pGC-FU plasmid were digested with Age I and then ligated with T4 DNA ligase to produce pGC-FU-FAM3A. To generate the recombinant Lentivirus LV-FAM3A, 293T cells were co-transfected with of the pGC-FU plasmid (20 µg) with a cDNA encoding FAM3A, pHelper1.0 plasmid (15 µg) and pHelper 2.0 plasmid (10 µg) by using Lipofectamine 2000 (100 µl). After 48 hours, supernatant was harvested and the viral titer was calculated by transducing 293T cells. As a control, we also generated a control lentiviral vector that expresses GFP alone (LV-Control).

Flow cytometry

HT22 cells were harvested 24 h after exposure to H₂O₂, washed with ice-cold Ca²⁺ free PBS, and re-suspended in binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 1 mM MgCl₂, 5 mM KCl, and 2.5 mM CaCl₂). Cell suspension was then incubated in 0.5 µg/ml FITC-conjugated annexin V (AV) at room temperature in darkness for 15 min. Cellular DNA was stained by addition of 5 µl of PI (50 µg/ml) at room temperature for 5 min, and the cells were analyzed by FACScan analysis with Cellquest software.

Quantification of cytochrome c release

Cytochrome c release was assessed after subcellular fraction preparation. HT22 cells were lysed and centrifuged for 10 min at 750 g at 4°C, and the pellets containing the nuclei and unbroken cells were discarded. The supernatant was then centrifuged at 15 000 g for 15 min. The resulting supernatant was removed and used as the cytosolic fraction. The pellet fraction containing mitochondria was further incubated with PBS containing 0.5% Triton X-100 for 10 min at 4°C. After centrifugation at 16 000 g for 10 min, the supernatant was collected as mitochondrial fraction. The levels of cytochrome c in cytosolic and mitochondrial fractions were measured using the Quantikine M Cytochrome C Immunoassay kit obtained from R&D Systems (Minneapolis, MN, USA). Data were expressed as ng/mg protein.

Measurement of ROS generation

Intracellular ROS were evaluated by the probe 2', 7'-dichlorofluorescein diacetate (DCFH-DA, Sigma). HT22 cells were incubated with 50 µg/ml DCFH-DA for 1 h at 37°C in the dark and then re-suspended in PBS. Fluorescence was read using an excitation wavelength of 480 nm and an emission wavelength of 530 nm with a fluorescence plate reader.

Measurement of ATP synthesis

Isolated mitochondria were utilized to measure ATP synthesis with a luciferase/ luciferin-based system as described previously [15]. 30 µg of mitochondria enriched pellets were resuspended in 100 µl of buffer A (150 mM KCl, 25 mM Tris-HCl, 2 mM potassium phosphate, 0.1 mM MgCl₂, pH 7.4) with 0.1% BSA, 1 mM malate, 1 mM glutamate and buffer B (containing 0.8 mM luciferin and 20 mg/ml luciferase in 0.5 M Tris-acetate, pH 7.75). The reaction was initiated by addition of 0.1 mM ADP and monitored for 5 min using a microplate reader.

Measurement of lipid peroxidation

Malonyldialdehyde (MDA) and 4-hydroxynonenal (4-HNE), two index of lipid peroxidation, were determined by using assay kits from Cell Biolabs and strictly following the manufacturer's instruction. The absorbance of the samples was measured by a microplate (ELISA) reader.

Western blot analysis

Equivalent amounts of total protein (40 µg per lane) were loaded and separated by 10 % SDS-PAGE gels, and transferred to polyvinylidene difluoride (PVDF) membranes. Immunoblot was performed and the membrane was developed with ECL. The amount of protein was determined using software Image J with normalization to the control value.

Statistical analysis

Statistical analysis was performed using SPSS 16.0, a statistical software package. Statistical evaluation of the data was performed by *t*-test or one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons.

Results

Expression of FAM3A following H₂O₂-induced injury in HT22 cells

HT22 cells were incubated in the presence of H₂O₂ at different concentrations (10, 50, 100, 500 µM or 1 mM) for 24 h, and the results of cell viability (Fig. 1A) and LDH release (Fig. 1B)

Fig. 1. Expression of FAM3A following H₂O₂-induced injury in HT22 cells. HT22 cells were treated with H₂O₂ at different concentrations for 24 h, and cell viability (A) and LDH release (B) were assayed. (C) HT22 cells were treated with 500 µM H₂O₂, and the distribution of FAM3A was shown as immunofluorescence staining for FAM3A (green), mitochondria (red) and DAPI (nuclei). Scale bar: 10 µm. The expression of FAM3A in mitochondrial and cytosolic fractions was detected by western blot (D). The expression of FAM3A at different time points after H₂O₂ treatment was detected by western blot (E). Data are shown as mean ± SEM (n=5). **p* < 0.05 vs. Control.

Fig. 2. Overexpression of FAM3A attenuates H_2O_2 -induced toxicity. HT22 cells were transfected with lentivirus expressed FAM3A (LV-FAM3A) or control lentivirus (LV-Control) for 72 hours, and the expression of FAM3A was detected by western blot (A). After lentivirus transfection, HT22 cells were treated with 500 μM H_2O_2 for 24 h. The cell viability (B) and LDH release (C) were assayed, and the apoptotic cell death was detected by flow cytometry (D). The expression of cleaved caspase-3 (C-Cas-3) was detected by western blot (E). The cytochrome c content in mitochondrial (F) and cytosolic fractions (G) was measured. Data are shown as mean $\pm$ SEM (n=5). * p < 0.05 vs. Control. # p < 0.05 vs. LV-Control. n.s., not statistically significant.

showed that H_2O_2 induced dose-dependent cytotoxicity in HT22 cells. Because 500 μM H_2O_2 caused nearly half of the cells to die, this concentration was used in the following experiments. After treatment with 500 μM H_2O_2 for 24 h, the results of fluorescence staining showed that the expression of FAM3A in H_2O_2 treated cells was significantly reduced as compared with control group, but the distribution of FAM3A was unaffected (Fig. 1C). To confirm the expression of FAM3A in the mitochondria, we also detected the expression of FAM3A in mitochondrial and cytosolic fractions (Fig. 1D). The results showed that the expression of FAM3A in mitochondrial and total cell homogenates was decreased after H_2O_2 exposure,

Fig. 3. Overexpression of FAM3A reduces H₂O₂-induced oxidative stress. HT22 cells were transfected with LV-FAM3A or LV-Control for 72 h, and treated with 500 μ M H₂O₂. Intracellular ROS generation was shown as immunofluorescence staining (A) for DCF (green) and DAPI (nuclei), and relative ROS levels were calculated (B). Scale bar: 100 μ m. The ATP content (C) and lipid peroxidation (D, E) were assayed. Data are shown as mean $\pm$ SEM (n=5). **p* < 0.05 vs. Control. #*p* < 0.05 vs. LV-Control.

whereas no expression of FAM3A was detected in cytosolic fractions. As shown in Fig. 1E, H₂O₂ treatment induced a decrease in FAM3A expression in a time-dependent manner in HT22 cells.

Overexpression of FAM3A attenuates H₂O₂-induced toxicity

To investigate the effect of FAM3A on H₂O₂-induced cytotoxicity, HT22 cells were transfected with lentivirus expressed FAM3A (LV-FAM3A) and control lentivirus (LV-Control). Immunoblot analysis indicated that exogenous FAM3A was expressed in HT22 cells by transfection with LV-FAM3A, whereas LV-Control had no

such effect (Fig. 2A). Overexpression of FAM3A significantly increased the cell viability, but decreased LDH release after H₂O₂ exposure (Fig. 2B and 2C). In addition, LV-FAM3A transfection reduced the number of AV⁺/PI⁺ and AV⁺/PI⁻ cells as measured by flow cytometry (Fig. 2D), and also decreased the cleavage of caspase-3 in H₂O₂ treated cells (Fig. 2E), suggesting the anti-apoptotic activity of FAM3A. As shown in Fig. 2F, mitochondrial cytochrome c release induced by H₂O₂, as evidenced by a decrease in mitochondrial cytochrome c content, was

Fig. 4. Involvement of PI3K/Akt pathway in FAM3A-induced protection. HT22 cells were transfected with LV-FAM3A or LV-Control for 72 h, and treated with 500 μM H_2O_2 . The expression of p-Akt, t-Akt, p-ERK and t-ERK were detected by western blot (A) and calculated (B). After transfection with LV-FAM3A and LV-Control, HT22 cells were treated with wortmannin (W, 1 μM) or LY294002 (LY, 10 μM), and PD98059 (PD, 20 μM) or U0126 (U, 2 μM), and the expression of p-Akt, t-Akt, p-ERK and t-ERK were detected by western blot (C) and calculated (D). After letivirus transfection and inhibitors treatment, HT22 cells were treated with 500 μM H_2O_2 . The cell viability (E), LDH release (F), ROS generation (G) and ATP content (H) were measured. Data are shown as mean $\pm$ SEM (n=5). * p < 0.05 vs. Control. # p < 0.05 vs. LV-Control. & p < 0.05 vs. LV-FAM3A.

partially prevented by FAM3A overexpression. In consistent, the H_2O_2 -induced increase in cytosolic cytochrome c content was decreased by FAM3A overexpression (Fig. 2G).

Overexpression of FAM3A reduces H_2O_2 -induced oxidative stress

To determine whether FAM3A affects the intracellular ROS production, HT22 cells were transfected with LV-FAM3A or LV-Control and exposure to H_2O_2 . As shown in Fig. 3A, the intracellular ROS generation was measured by DCFDA-based fluorescence staining analysis. ROS production induced by H_2O_2 was reduced after transfection with LV-FAM3A, but was not decreased by transfection with LV-Control (Fig. 3B). FAM3A overexpression also preserved ATP generation after H_2O_2 treatment (Fig. 3C). Moreover, we also detected lipid peroxidation by measuring MDA and 4-HNE content. The results showed that H_2O_2 treatment significantly

increased the production of MDA and 4-HNE, which were both partially reversed by FAM3A overexpression (Fig. 3D and 3E).

Involvement of PI3K/Akt pathway in FAM3A-induced protection

To investigate the potential molecular mechanism underlying FAM3A induced protection, western blot was performed to detect the activation of Akt and ERK in HT22 cells (Fig. 4A). The results showed that FAM3A overexpression significantly increased the expression of p-Akt and p-ERK, but had no effect on t-Akt and t-ERK expression (Fig. 4B). To further determine the involvement of Akt and ERK pathways in FAM3A induced protection, as shown in Fig. 4C, HT22 cells were treated with wortmannin (W, 1 μ M) or LY294002 (LY, 10 μ M), and PD98059 (PD, 20 μ M) or U0126 (U, 2 μ M). Treatment with W and LY selectively inhibited Akt activity, and ERK phosphorylation was suppressed by PD and U (Fig. 4D). Intriguingly, increased expression of p-ERK induced by FAM3A in HT22 cells was also markedly decreased by W and LY, indicating that the increased activation of MEK/ERK induced by FAM3A might be the results indirectly from its effect on PI3K/Akt pathway. Furthermore, the results of cell viability (Fig. 4E), LDH release (Fig. 4F), intracellular ROS generation (Fig. 4G) and ATP measurement (Fig. 4H) in the presence of inhibitors showed that FAM3A induced protection against H₂O₂ treatment was partially prevented by inhibition of PI3K/Akt pathway, but not by inhibition of MEK/ERK pathway.

Discussion

Among four members of FAM3 family proteins, the role of FAM3B in glucose and lipid metabolism has been intensively investigated [9, 16]. However, the biological function of FAM3A, especially in central nervous system, remains poorly understood. In the present study, we found that FAM3A expression was reduced in HT22 hippocampal neuronal cells after H₂O₂ exposure. For the first time, we demonstrated that neuronal overexpression of FAM3A exerted protective effects against ROS related oxidative stress. These findings indicate that deregulated FAM3A expression might be an ideal therapeutic target for the treatment of neurological disorders where neuronal oxidative stress contributes to the pathology.

Regarding possible mechanisms underlying the protective effects observed here, we found that FAM3A overexpression significantly reduced apoptosis as measured by flow cytometry. H₂O₂ and other ROS have been found to induce many types of DNA damage, such as DNA breaks, base and sugar modifications, DNA-protein crosslinks, depurination and depyrimidination [17]. They also disrupt the membrane integrity of mitochondria and increase the formation of mitochondrial transition pores, which in turn promotes the release of pro-apoptotic factors and aggravates DNA damage [18]. The roles of FAM3 family proteins in cell survival and apoptosis are not fully understood. Previous studies showed that recombinant FAM3B treatment or adenovirus mediated FAM3B overexpression induced betaTC3 cell and mouse islet apoptosis [16, 19]. However, a recent report indicated that FAM3B was essential for cell survival and knockdown of FAM3B triggered apoptosis through p53 dependent pathway [20]. These opposite results might be explained by the different tissues and *in vitro* models used. Our results of caspase-3 activation and cytochrome c release measurement indicated that FAM3A exerts anti-apoptotic activity in neuronal cells. Therefore, the FAM3 family proteins might play different roles in cell survival and apoptosis in various tissue and cell types, which warrant further investigations.

Previous studies using northern analysis indicated that the FAM3A mRNA was ubiquitously expressed in almost all tissues of humans and rodents [9]. Our results confirmed the expression of FAM3A protein in neuronal HT22 cells. In addition, we observed that FAM3A is subcellularly located mainly in the mitochondria, which was consistent with previous findings in liver and vascular smooth muscle cells [14, 21]. Thus, we speculated that the role of FAM3A under oxidative stress conditions might be related to the regulation of mitochondrial energy metabolism and ROS generation. The results of DCF fluorescence

staining and measurement of MDA and 4-HNE indicated that overexpression of FAM3A markedly attenuated ROS generation and lipid peroxidation, suggesting that suppressed oxidative stress products may be responsible for metabolic improvement after FAM3A overexpression. ROS are mainly produced by mitochondria, and oxidative stress-induced ROS production contributes to neuronal death by oxidation of many important lipids, eventually leading to aggravated mitochondrial dysfunction [22, 23]. We found that overexpression of FAM3A also preserved the intracellular ATP generation after H₂O₂ exposure, which further confirmed the role of FAM3A in mitochondrial function regulation under oxidative stress conditions.

In response to oxidative stress, various signal transduction pathways are triggered, among which PI3K/Akt and MEK/ERK are two important cascades. The serine and threonine protein kinase, which is a critical downstream target of PI3K, plays a key role in regulating cellular functions and results from extracellular stimulus [24]. Equally, as one member of mitogen-activated protein kinases (MAPKs) family, ERK can transduce a large number of extracellular information into intracellular responses [25]. Some authors have suggested that FAM3A elicit signaling such as Akt and ERK phosphorylation in liver and vascular smooth muscle cells [14, 21]. Consistently, our results showed that both p-Akt and p-ERK expression were up-regulated by FAM3A overexpression, suggesting the possible involvement of these cascades in FAM3A induced protection. Akt has been known as an endogenous protective factor against cell death in many kinds of insults, and it can inhibit apoptosis in many ways, both upstream and downstream of mitochondrial perturbation [26]. In contrast, the role of ERK in cell death was disputable. Despite the volume of evidence supporting the p-ERK elevation as detrimental effects that were essential for oxidative stress and inflammation-related cell death, numerous studies demonstrated that ERK activation contributed to protective effects of many neuroprotectants [27, 28]. In our study, overexpression of FAM3A-induced protection against cell death and oxidative stress was partially prevented by both wortmannin and LY294002, but not by PD98059 and U0126. Therefore, it is reasonable to assume that the activation of PI3K/Akt, but not MEK/ERK, mediates FAM3A-induced protection against H₂O₂-induced cytotoxicity.

It is well known that signaling pathways can crosstalk with each other wherein one pathway can signal to either enhance or suppress signaling by another [29-31]. Whether cross-inhibition or cross-activation occurs between these pathways is dependent on different conditions. The interaction of PI3K/Akt with MEK/ERK has shown crosstalk on multiple levels. Akt was shown to positively regulate Raf phosphorylation and ERK activation in prostate cancer cells in response to androgen depletion [32]. A previous study indicated that Gi-coupled receptor- and G β γ -stimulated ERK activation is attenuated by the PI3K inhibitors wortmannin and LY294002 or by overexpression of a dominant negative mutant of the p85 subunit of PI3K [33]. In our study, both wortmannin and LY294002 were demonstrated to inhibit not only the activation of Akt, but also ERK, which means that the activation of ERK in our *in vitro* conditions is dependent on the activity of PI3K/Akt signaling pathway at the level of PI3K. However, PD98059 and U0126 inhibited the activation of ERK, with no effect on Akt activity, suggesting that FAM3A induced activation of Akt was not dependent on MEK/ERK pathway. In addition, treatment with PD98059 and U0126 had no effects on FAM3A induced protection against H₂O₂ exposure in HT22 cells. Intriguingly, our results showed that ERK1/2 inhibition in H₂O₂ + LYFAM3A + PD or U treated cells increases the phosphorylation levels of Akt to levels that are equal to that obtained with LV-FAM3A + H₂O₂ treated cells. It might be ruled out that this increase in Akt with ERK inhibition contributes to the protection against H₂O₂ toxicity in that the levels of cell viability, ROS and ATP with ERK inhibition are equal to that of the LV-FAM3A control. However, significant inhibition on FAM3A-induced protection was detected after treatment Akt inhibitors. Thus, whether ERK inhibition-induced regulation on Akt activity was involved in FAM3A-induced protection needs to be further determined. From our results, we think that the involvement FAM3A in moderating the PI3K/Akt pathway could occur at the level upstream of MEK/ERK. The exact mechanisms need further investigation in other neuronal cells and also *in vivo* conditions.

Thus, these observations provided a new element to the crosstalk between PI3K/Akt and MEK/ERK pathways under oxidative stress conditions.

Conclusions

In summary, FAM3A is expressed in HT22 cells, where it affects ROS generation and mitochondrial ATP production, and inhibits apoptotic cell death via a PI3K/Akt mediated, but MEK/ERK independent, signaling pathway. Overexpression of FAM3A is beneficial for preventing oxidative stress induced by H₂O₂ in neuronal cells.

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Disclosure Statement

The authors report no conflicts of interest.

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