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Oxygen-glucose deprivation regulates BACE1 expression through induction of autophagy in Neuro-2a/APP695 cells

Rong-fu Chen[1], Ting Zhang[1], Yin-yi Sun[1], Ya-meng Sun[1], Wen-qi Chen[1], Nan Shi[2], Fang Shen[1], Yan Zhang[2], Kang-yong Liu[1, 2], Xiao-jiang Sun[1, *]

1 Department of Neurology, Sixth People's Hospital Affiliated to Shanghai Jiao Tong University, Shanghai, China
2 Zhoupu Hospital, Pudong New District, Shanghai, China

Correspondence to:
Xiao-jiang Sun, Ph.D.,
sunxj155@sohu.com.

orcid:
0000-0003-2061-7262 (Xiao-jiang Sun)

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Abstract

Our previous findings have demonstrated that autophagy regulation can alleviate the decline of learning and memory by eliminating deposition of extracellular beta-amyloid peptide (Aβ) in the brain after stroke, but the exact mechanism is unclear. It is presumed that the regulation of beta-site APP-cleaving enzyme 1 (BACE1), the rate-limiting enzyme in metabolism of Aβ, would be a key site. Neuro-2a/amyloid precursor protein 695 (APP695) cell models of cerebral ischemia were established by oxygen-glucose deprivation to investigate the effects of Rapamycin (an autophagy inducer) or 3-methyladenine (an autophagy inhibitor) on the expression of BACE1. Either oxygen-glucose deprivation or Rapamycin down-regulated the expression of BACE1 while 3-methyladenine up-regulated BACE1 expression. These results confirm that oxygen-glucose deprivation down-regulates BACE1 expression in Neuro-2a/APP695 cells through the introduction of autophagy.

Key Words: *nerve regeneration; brain Injury; oxygen-glucose deprivation; cerebral ischemia; stroke; autophagy; beta-site APP-cleaving enzyme 1 (BACE1); beta-amyloid peptide; 3-methyladenine (3-MA); Rapamycin; neural regeneration*

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Introduction

With increasing human life expectancy, the prevalence of ischemic stroke and associated disability has increased; ischemic stroke accounts for 80% of all occurrences of stroke and about two-thirds of stroke patients have cognitive impairment to different degrees. Many studies have suggested that cognitive decline is closely related to the deposition of extracellular β-amyloid peptide (Aβ) in the brain (Henon et al., 2001; Pendlebury and Rothwell, 2009; Landau et al., 2012; Pimentel-Coelho and Rivest, 2012). Therefore, preventing Aβ production and/or promoting Aβ degradation can improve cognitive decline in patients or mice (Hachinski and Munoz, 1997; Zhang et al., 2007; McAllister et al., 2010; Chetelat et al., 2012; Kawahara et al., 2012). Aβ is a polypeptide of 37–43 amino acids and is derived from β-amyloid precursor protein (APP) by β- and α-secretase cleavage (Nunan and Small, 2000). APP is a type I transmembrane glycoprotein that can be cleaved by α-, β-, and γ-secretases, and Vassar (2005) identified the β-secretase as the β-site APP-cleaving enzyme 1 (BACE1), the key rate-limiting enzyme in Aβ metabolism (Vassar, 2001). BACE1 triggers Aβ generation and acts as a key regulator of Aβ metabolism. In addition, up-regulation of BACE1 expression has been shown to increase Aβ production (Sun et al., 2006), and regulation of BACE1 activity can affect Aβ generation (Gravenfors et al., 2012; Obregon et al., 2012; Zhu et al., 2012; Yun et al., 2013). Moreover, application of a BACE1 inhibitor is expected to decrease Aβ expression in patients with Alzheimer's disease (Kacker et al., 2012). These data suggest that reduction of Aβ generation through down-regulation of BACE1 expression might be practicable and beneficial.

Autophagy, or cellular self-digestion, is a cellular catabolic pathway in which misfolded or aggregated proteins and damaged organelles are transported to lysosomes for degradation (Levine and Kroemer, 2008). Studies have shown that the β-site APP-cleaving enzyme can be degraded *via* the lysosomal pathway (Koh et al., 2005). Thus, reduction of Aβ generation might indirectly result from inhibition of BACE1 metabolism. Yu et al. (2005) discovered that autophagy regulation could modulate Aβ generation in the brain of mice, thus improving learning and memory. A previous study has shown that autophagy regulation affected Aβ generation (Zhang et al., 2011). The present study focused on the relationship between autophagy and BACE1.

In ischemic stroke, regional cerebral blood flow is inadequate or blocked. Neurons in the affected areas are short of oxygen and glucose, subsequently leading to neuronal loss in the central necrosis area and cellular stress injury in the penumbral region of brain infarction (Dirnagl et al., 1999). Therefore, a oxygen-glucose deprivation (OGD) model might mimic the pathological changes of cerebral ischemia and mouse neocortical cell cultures has become an *in vitro* model in its research (Goldberg and Choi, 1993). A cell model of cerebral ischemia was established as previously described (Wang et al., 2010). This was applied in the following experiments to evaluate BACE1 expression with autophagy regulators and to explore pathways involved in BACE1 metabolism. The results might provide some useful information for clinical treatment of post-stroke cognitive decline.

Materials and Methods

Creation of a cell model of cerebral ischemia *in vitro*

Neuro-2a/amyloid precursor protein 695 (Neuro-2a/APP695) cell lines, stably over-expressing wild-type human APP695 protein, were provided by Professor Chun-jiu Zhong from the Department of Neurology of Zhongshan Hospital of Fudan University, China. Neuro-2a/APP695 cells over-expressed APP695 protein, so the intervention effect of β-site APP-cleaving enzyme by drugs or treatment could be induced easily. They were cultured in mixed media [44.5% Dulbecco's modified Eagle's medium (DMEM), 44.5% Opti-MEM®, 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 U/mL streptomycin, 200 μg/mL G418; Thermo Fisher Scientific, Waltham, MA, USA) in an incubator with 100% saturated humidity and 5% CO_2 (Heraeus, Hanau, Germany) at 37°C. A cell model of OGD *in vitro* was established according to a previous report (Wang et al., 2010). Cells were passaged at around 80% confluence, and cultured as usual. When cells were in the logarithmic growth phase, the cell culture medium was replaced with OGD culture medium. The study was approved by the Local Institutional Ethical Committee of the Sixth People's Hospital of Shanghai, China.

Preparation of autophagy-inducing or -inhibiting drugs

Rapamycin (2.5 mg/mL), a liquid reagent itself, dissolves easily in cell culture medium (working concentration 200 ng/mL) while 3-methyladenine (3-MA), a poorly water-soluble drug, should be dissolved with sterilized phosphate buffered saline (PBS) solution beforehand. Before each trial, a stock solution of 100 mM 3-MA in PBS was heated in a water bath at 50°C, and then diluted into working concentration (5 mM) with culture medium, avoiding white flock formation.

Evaluation of proliferation rate of Neuro-2a/APP695 cells with OGD treatment

The cell proliferation rate was detected by a cell proliferation-cytotoxicity assay kit (CCK-8, CK04; Dojindo, Kumamoto, Japan). Grouping was designed as the following: control group, OGD group (OGD for 1 hour), OGD + Rapamycin group (intervention with 200 ng/mL Rapamycin, Sigma-Aldrich, St. Louis, MO, USA), and OGD + 3-MA group (intervention with 5 mM 3-MA, Sigma-Aldrich). Before OGD treatment, cells were pre-incubated with Rapamycin (200 ng/mL) or 3-MA (5 mM) for 1 hour, and then followed by OGD treatment along with a drug intervention (200 ng/mL Rapamycin or 5 mM 3-MA) for 1 hour respectively. At the same time, the cell culture medium was replaced with OGD culture medium (sugar-free DMEM, 1.0 mM NaCN (Sigma-Aldrich), penicillin, streptomycin, and 2% FBS). Cells in the logarithmic growth phase were collected and planted at a density of 1×10^4 cells per well in a 96-well plate. After incubation for 24 hours, cells were treated with OGD or interventional drugs, and 10 μL CCK-8 solution (10 mg/mL) was added to the cell medium of all groups, and incubation was continued for 2 hours. Finally, the optical density value at 450 nm was measured with a multifunctional microplate reader (BioTek Instruments, Inc., VT, USA) using a reference wavelength of 630 nm to detect a yellow-colored product (formazan), which is soluble in the tissue culture medium. The amount of the formazan dye generated by the activity of dehydrogenases in cells is directly proportional to the number of living cells. Relative cell viability was compared with the control group by percentage optical density.

Evaluation of apoptosis ratio of Neuro-2a/APP695 cells with OGD treatment

When a cell culture reached 80% confluence, cells were trypsinized, resuspended, and then planted in 6-well plates at an appropriate density. After 24 hour incubation, cells were treated with OGD or an intervention drug (200 ng/mL Rapamycin or 5 mM 3-MA) for 1 hour. Next, cells were rinsed with ice-cold 1 × binding buffer once and digested for 20 minutes with ethylenediamine tetra acetic acid-free trypsin. Cell lysates were collected in flow cytometry tubes and centrifuged at 1,000 r/min at 4°C for 5 minutes. After centrifugation, 1 × binding buffer was added to each tube at 1×10^6 cells /mL, and 100 μL of the cell re-suspension solution was transferred into a new flow cytometry tube. Then the cell suspensions were mixed with 5 μL of Annexin-V-fluorescein isothiocyanate (20 μg/mL, V13241; Invitrogen, Carlsbad, CA, USA), shaken gently, incubated for 15 minutes at room temperature in the dark, and then re-suspended with 400 μL 1 × binding buffer, mixed with 1 μL propidium iodide (PI; 100 μg/mL, V13241; Invitrogen) for 5 minutes before detection by flow cytometry. A tube without propidium iodide was used as a negative control. After staining, apoptotic cells, dead cells and live cells exhibited different fluorescence. These populations can easily be distinguished using a flow cytometer (FACSAria, Becton Dickinson Company, Franklin Lakes, NJ, USA). The relative cell apoptosis ratio was evaluated by percentage of the control group. The total number of cells (all events) was 30,000.

Western blot assay

Target proteins in the cell lysates of all groups were detected by western blot assay. First, cells were collected after their

respective treatment and fully lysed in radioimmunoprecipitation assay lysis buffer [1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1% NP-40, 50 mM Tris-HCl (pH 7.5), 250 mM sodium chloride] containing phenylmethanesulfonyl fluoride at 0°C for 20 minutes. Following centrifugation at 12,000 × *g* for 30 minutes at 4°C in a supercentrifuge (CP80 MX, Hitachi, Tokyo, Japan), supernatants were collected, and stored at −80°C. After determining the protein concentration by a BCA protein assay kit (Thermos Fisher Scientific, Waltham, MA, USA), equal amounts of proteins were separated using SDS-polyacrylamide gel electrophoresis at 80 V constant voltage for 20 minutes, followed by 120 V constant voltage for 90 minutes. The proteins were then wet-transferred to polyvinylidene difluoride membranes (Merck Millipore: Calbiochem, Darmstadt, Germany) at 250 mA constant current for 90 minutes. Membranes were then blocked with 5% skim milk in Tris-buffered saline and Tween 20 (TBS-T) at room temperature for 1 hour, followed by incubation at 4°C overnight with corresponding primary antibodies: (i) monoclonal anti-β-actin antibody produced in mouse (1:5,000; A5441; Sigma-Aldrich); (ii) rabbit anti-LC3-B monoclonal antibody, (1:1,000; L7543; Sigma-Aldrich). This antibody can detect protein of molecular weight major at 18 kDa and minor at the 16-kDa band. Immunoblotting of microtubule-associated protein 1-light chain 3 (LC3) usually gives two bands: LC3-I (18 kDa) and LC3-II (16 kDa). The amount of LC3-II or the LC3-II/LC3-I ratio correlates with the number of autophagosomes; (iii) rabbit anti-Beclin1 polyclonal antibody (1:1,000; sc-11427; Santa Cruz Biotechnology, Santa Cruz, CA, USA); and (iv) rabbit anti-BACE1 polyclonal antibody (1:500; 195111; Merck Millipore, Calbiochem, Darmstadt, Germany). Membranes were then rinsed three times for 10 minutes each using 1 × TBS-T solution, and then incubated with appropriate secondary antibodies (horse-radish peroxidase-labeled goat anti-rabbit or anti-mouse IgG, 1:10,000; Cell Signaling Technology, Danvers, MA, USA) at room temperature for 2 hours. Membranes were then rinsed three times for 10 minutes each using 1 × TBS-T solution. Finally, protein bands were detected using an electrochemiluminescence kit (Merck Millipore, Darmstadt, Germany) in the darkroom. Image J software (NIH, Bethesda, MA, USA) was used to compare the density of bands on a western blot image after flatbed scanning. The relative optical density of the target protein of each group could be calculated compared to the optical density of β-actin protein. An ultrastructural observation of Neuro-2a/APP695 cells with OGD treatment was performed by transmission electron microscopy.

Cells in the logarithmic growth phase were seeded in plates. After 24 hour incubation, adherent cells were pretreated with intervention drugs (200 ng/mL Rapamycin or 5 mM 3-MA) for 1 hour, and followed by with OGD for 1 hour in the presence of Rapamycin (200 ng/mL) or 3-MA (5 mM) according to group. Cells were then digested with trypsin, collected in 1.5-mL Eppendorf tubes, centrifuged for 5 minutes at 2,000 r/min, and fixed in 2.5% glutaraldehyde at 4°C overnight. Next day, the samples were rinsed three times with 0.1 M PBS for 15 minutes each, and then fixed with osmic acid for 2–3 hours. Afterwards, samples were rinsed three times with 0.1 M PBS for 15 minutes each. Finally, samples were dehydrated in 50–90% ethanol and 90% acetone and fixed. The cells were embedded and the blocks were solidified with 2.5% glutaraldehyde, sectioned with an ultramicrotome (70 nm thickness), and double stained with 3% uranyl acetate followed by lead citrate. Sections from different groups were finally observed by transmission electron microscopy (TEM) (JEM-1230; Jeol, Tokyo, Japan) and photographed. Nuclear membrane, mitochondrial morphology, and autophagosome or autolysosome were observed in particular.

Statistical analysis

Data were expressed as the mean ± SD. SPSS 17.0 software (SPSS, Chicago, IL, USA) was used for statistical analysis. Differences among the groups were determined using one-way analysis of variance, and the least significance difference *post hoc* tests were used for comparisons between groups. Results were statistically significant at the $\alpha = 0.05$ level.

Results

Significant decrease of cell proliferation rates of Neuro-2a/APP695 cells with OGD

Compared with the control group, cell proliferation rate of OGD group decreased significantly by approximately 25% ($P < 0.01$; **Figure 1**). Cells in OGD + Rapamycin group were pre-incubated with Rapamycin (200 ng/mL) for 1 hour, followed by OGD for 1 hour in the presence of Rapamycin (200 ng/mL). The same procedure was followed with cells of OGD + 3-MA group, with 3-MA (5 mM). Cell proliferation rates of both OGD + Rapamycin and OGD + 3-MA groups showed a decline compared with the control group, respectively ($P < 0.05$; **Figure 1**), but no statistically significant difference was found between the two groups ($P > 0.05$).

Effects of autophagy regulation and OGD on apoptosis ratios of Neuro-2a/APP695 cells

Cell apoptosis was measured by Annexin V-propidium iodide (PI) double staining (**Figure 2**). Cell apoptosis ratio of the control group (5.67 ± 0.91%) was significantly lower than that of the other three groups ($P < 0.05$). Moreover, there was no significant difference in the apoptosis ratio between the other three groups ($P > 0.05$). The highest apoptosis ratio occurred in the OGD group (11.20 ± 0.72%), less than 11.92%, which indicated that cells with OGD treatment in our experiments showed a predominating phenomenon of autophagy, not apoptosis.

Effects of autophagy regulation and OGD on LC3 expression in Neuro-2a/APP695 cells

As mentioned before, cells were pre-incubated with Rapa (200 ng/mL) or 3-MA (5 mM) for 1 hour, followed by OGD for 1 hour in the presence of Rapamycin (200 ng/mL) or 3-MA (5 mM) in its respective group. Cells were then collected and subjected to immunoblotting. Compared with the control group, LC3 expression increased in the cells of the

three other groups, There were significant differences between the control and OGD and OGD + Rapamycin groups (each at $P < 0.01$) but it was not significant between the control and OGD + 3-MA groups ($P > 0.05$; **Figure 3**). Rapa increased LC3 expression in cells between the OGD and OGD + Rapamycin groups, but no significant statistical difference existed between them ($P > 0.05$), however, 3-MA did significantly inhibit LC3 expression (OGD group versus OGD + 3-MA group, $P < 0.01$). In addition, Rapamycin increased LC3 expression while 3-MA suppressed it (OGD + Rapamycin group *versus* OGD + 3-MA group, $P < 0.01$).

Effects of autophagy regulators and oxygen-glucose deprivation on Beclin1 expression in Neuro-2a/APP695 cells

Compared with the control group, Beclin1 expression increased in cells of both OGD and OGD + Rapa groups ($P < 0.05$), but decreased in OGD + 3-MA group ($P < 0.05$; **Figure 4**). The level of Beclin1 was reduced remarkably in cells of OGD + 3-MA group compared with OGD or OGD + Rapamycin group ($P < 0.05$, $P < 0.01$, respectively).

Effects of autophagy regulation and OGD on BACE1 expression in Neuro-2a/APP695 cells

BACE1 expression decreased in cells of OGD and OGD + Rapamycin groups, and increased only in the OGD + 3-MA group. There was no statistical difference in BACE1 expression between the control and each of the other three groups ($P > 0.05$; **Figure 5**). However, compared with the OGD group, BACE1 expression increased in cells of the OGD + 3-MA group ($P < 0.05$; **Figure 5**). Moreover, BACE1 expression reduced in cells of OGD + Rapamycin group compared with OGD + 3-MA group ($P < 0.05$; **Figure 5**).

Effects of autophagy regulation and OGD on the morphology and ultrastructure of Neuro-2a/APP695 cells

Under transmission electron microscope, cells in the control group displayed intact plasma and nuclear membranes, normal mitochondrial morphology, no autophagosome or autolysosome, and several small vacuoles (**Figure 6**). In the OGD group, cells were observed with approximately normal morphology, but autolysosomes were observed with a double-membrane structure (red arrows in **Figure 6**) and the phenomenon of nuclear margination in cells after OGD treatment. Cells of OGD + Rapamycin group presented with an intact morphology, no karyopyknosis, and no vacuolization in mitochondria. The formation of autophagosome was observed along with a double-membrane structure (not yet enclosed) in a cell (red arrows in **Figure 6**). In contrast, cells of OGD + 3-MA group displayed intact cell membranes, no obvious autophagosome or autolysosome, abundant vacuolization, and irregular mitochondrial morphology.

Discussion

Ischemic stroke is one of the leading causes of cognitive impairment; however, the mechanisms by which cognitive decline occurs after stroke are still not fully understood (Pluta et al., 2011). Cerebral ischemia has been reported to lead to intracellular deposition of Aβ in the brain (Bulbarelli et al., 2012), which eventually causes cognitive decline. However, related research on the mechanism of post-stroke cognitive decline is still in its infancy and needs urgent attention. Earlier experimental results showed that Aβ expression increased after cerebral ischemia (Zhang et al., 2011) and many studies have reported that BACE1 activity may affect Aβ generation (Sun et al., 2006; Cole and Vassar, 2007; Gravenfors et al., 2012; Obregon et al., 2012; Zhu et al., 2012; Yun et al., 2013). A prior study had reported that BACE1 was degraded through the lysosomal pathway (Koh et al., 2005). Therefore, BACE1 might be degraded through an autophagy-lysosomal pathway. There are three types of autophagy in mammalian cells: macroautophagy (usually termed autophagy), micro-autophagy, and chaperone-mediated autophagy (CMA). Macroautophagy (hereinafter referred to as autophagy) predominantly serves as a cell survival mechanism and occurs at low basal levels in all cells. CMA involves selective translocation of the cytosolic proteins. The mechanism of micro-autophagy is still unclear. Pharmacological agents (*e.g.*, Rapamycin) or stress (*e.g.*, starvation or various pathologies) can stimulate autophagy (Mizushima et al., 2008), but the target of Rapamycin in mammals (mTOR) negatively regulates autophagy. Under condition of nutrient deprivation or starvation, mTOR is inhibited, which activates the Atg13-ULK1/2-FIP200 complex to initiate autophagy. Rapamycin, selectively inhibits the mTOR complex 1 (mTORC1), inducing autophagy. The phosphoinositide-3-kinase (PI3K) pathway is the major downstream signaling pathway regulated by mTORC1, and up-regulation of PI3P (metabolite of the class III PI3K) can stimulate autophagy, while inhibition of PI3K by 3-MA can suppress autophagy (Levine and Kroemer, 2008; Ravikumar et al., 2010).

It was reported that moderate up-regulation of autophagy could decrease Aβ generation, thus alleviating cognitive impairment (Spilman et al., 2010; Majumder et al., 2011). Previous results showed that OGD also induced autophagy and the expression of LC3 and Beclin1 (an essential autophagy protein) increased (Tassa et al., 2003), which was consistent with previous reports (Klionsky et al., 2008; Mizushima et al., 2010). Beclin 1, a Bcl2 interacting protein, is the mammalian ortholog of yeast Atg6/Vps30. It is an essential autophagy protein that has been linked to multiple processes including tumor suppression, protection against some cardiac and neurological degenerative diseases. Oxygen-glucose deprivation and some drugs such as Rapamycin can introduce autophagy, and raise protein Beclin1expr ession. In the experiment, Beclin1 expression increased in cells of both OGD and OGD + Rapamycin groups. Meanwhile, LC3 is a soluble protein with a molecular mass of approximately 17 kDa that is distributed ubiquitously in mammalian tissues and cultured cells. During autophagy, autophagosomes engulf cytoplasmic components. Concomitantly, a cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine to form LC3-phosphatidylethanolamine conjugate (LC3-II), which is recruited to autophagosomal membranes. Autophagosomes fuse with lysosomes to form autolysosomes,

and components in the autophagosome are degraded by lysosomal hydrolases. At the same time, LC3-II in the autolysosomal lumen is degraded. So, lysosomal turnover of the autophagosomal marker LC3-II reflects autophagic activity, and detecting LC3 by immunoblotting has become a reliable method for monitoring autophagy (Klionsky et al., 2008; Mizushima et al., 2010). In addition, autophagy inhibitor 3-MA could suppress the expression of autophagy-specific proteins markedly.

BACE1 might be degraded through the autophagy-lysosomal pathway, thereby impairing the fusion of autophagosome-lysosome or the lysosomal proteolytic function. This might lead to a reduction of BACE1 clearance and subsequently increase Aβ generation (Boland et al., 2008; Lai and McLaurin, 2012). Thus, BACE1 becomes one of the effective targets for reducing Aβ generation (Ohno et al., 2004, 2007; Laird et al., 2005; McConlogue et al., 2007). In our experiments, BACE1 decreased in cells of both OGD and OGD + Rapa groups, while it increased in OGD + 3-MA group, because autophagy was induced by OGD or Rapa but inhibited by 3-MA. Obregon et al. (2012) discovered that reduction of BACE1 activity could decrease Aβ production (Obregon et al., 2012), and in this present experiment we showed that up-regulation of autophagy could decrease BACE1 expression. Therefore, it will be necessary to check whether up-regulation of autophagy decreases Aβ expression by detection of the Aβ peptide in future experiments.

Hypoxia-inducible factor 1, a core transcription factor responsible for maintaining oxygen homeostasis, facilitates moderate responses of cells to hypoxia under physiological or pathological conditions. Hypoxia-induced autophagy is dependent on the HIF-1α/AMP-activated protein kinase (AMPK) signaling pathway (Mazure and Pouyssegur, 2010; Hu et al., 2012). However, during OGD, AMPK is activated, which can induce autophagy through the inhibition of mTOR. Moreover, Rapamycin (an inhibitor of FRAP, an inducer of autophagy) can suppress HIF-1 expression through the PI3K/PTEN/AKT/FRAP pathway and inhibit mTOR-dependent HIF-1α mRNA translation (Zhong et al., 2000; Semenza, 2010). A recent study reported that CMA or nutrient-deficient conditions could lead to a reduced HIF-1α expression. Further studies have indicated that CMA decreased HIF-1α expression through the interaction between HIF-1α and HSC70 or LAMP2A, two major target proteins of CMA (Hubbi et al., 2013). It was thought that activation of autophagy could down-regulate HIF-1 expression. In addition, previous studies have shown that up-regulation of HIF-1α could increase BACE1 expression (Sun et al., 2006; Zhang et al., 2007). Our results show that BACE1 expression decreased in cells of OGD + Rapamycin group, but increased in OGD + 3-MA group, thus it was hypothesized that up-regulation of autophagy decreased BACE1 expression through the interaction between HIF-1 and CMA. Further research should be done to check these correlations and elucidate the mechanisms involved. Although CMA differs from autophagy, there might be a cross-talk between CMA and autophagy that has a synergistic effect to regulate BACE1 metabolism. This could be another area for further investigations.

The integrity of structure and function of lysosome is also crucial to autophagy. More interventions such as chloroquine or leupeptin should be made to check lysosomal function in these cells. BACE1 is a rate-limiting enzyme in the production of Aβ, thus the amount of Aβ should also be measured, the level of BACE1 does not fully represent Aβ production. Thus the quantity of Aβ metabolism should also be measured as well as its key rate-limiting enzyme BACE1.

The results of this study show that autophagy regulation could affect the expression of BACE1, and that regulation of BACE1 expression through OGD might be *via* an autophagy-dependent pathway in Neuro-2a/APP695 cells.

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Author contributions: *RFC, KYL and XJS designed the study. RFC, TZ, YYS, YMS and WQC performed the experiments. KYL conducted statistical analysis. NS, FS, and YZ were responsible for cell culture and western blot analysis. XJS conceived the study, coordinated the entire procedure, and helped to write the paper. All authors approved the final version of the paper.*
Conflicts of interest: *None declared.*

References

Boland B, Kumar A, Lee S, Platt FM, Wegiel J, Yu WH, Nixon RA (2008) Autophagy induction and autophagosome clearance in neurons: relationship to autophagic pathology in Alzheimer's disease. J Neurosci 28:6926-6937.

Bulbarelli A, Lonati E, Brambilla A, Orlando A, Cazzaniga E, Piazza F, Ferrarese C, Masserini M, Sancini G (2012) Abeta42 production in brain capillary endothelial cells after oxygen and glucose deprivation. Mol Cell Neurosci 49:415-422.

Chetelat G, Villemagne VL, Villain N, Jones G, Ellis KA, Ames D, Martins RN, Masters CL, Rowe CC, Group AR (2012) Accelerated cortical atrophy in cognitively normal elderly with high beta-amyloid deposition. Neurology 78:477-484.

Cole SL, Vassar R (2007) The Alzheimer's disease beta-secretase enzyme, BACE1. Mol Neurodegener 2:22.

Dirnagl U, Iadecola C, Moskowitz MA (1999) Pathobiology of ischaemic stroke: an integrated view. Trends Neurosci 22:391-397.

Goldberg MP, Choi DW (1993) Combined oxygen and glucose deprivation in cortical cell culture: calcium-dependent and calcium-independent mechanisms of neuronal injury. J Neurosci 13:3510-3524.

Gravenfors Y, Viklund J, Blid J, Ginman T, Karlstrom S, Kihlstrom J, Kolmodin K, Lindstrom J, von Berg S, von Kieseritzky F, Bogar K, Slivo C, Swahn BM, Olsson LL, Johansson P, Eketjall S, Falting J, Jeppsson F, Stromberg K, Janson J, Rahm F (2012) New aminoimidazoles as beta-secretase (BACE-1) inhibitors showing amyloid-beta (Abeta) lowering in brain. J Med Chem 55:9297-9311.

Hachinski V, Munoz DG (1997) Cerebrovascular pathology in Alzheimer's disease: cause, effect or epiphenomenon? Ann N Y Acad Sci 826:1-6.

Henon H, Durieu I, Guerouaou D, Lebert F, Pasquier F, Leys D (2001) Poststroke dementia: incidence and relationship to prestroke cognitive decline. Neurology 57:1216-1222.

Hu YL, DeLay M, Jahangiri A, Molinaro AM, Rose SD, Carbonell WS, Aghi MK (2012) Hypoxia-induced autophagy promotes tumor cell survival and adaptation to antiangiogenic treatment in glioblastoma. Cancer Res 72:1773-1783.

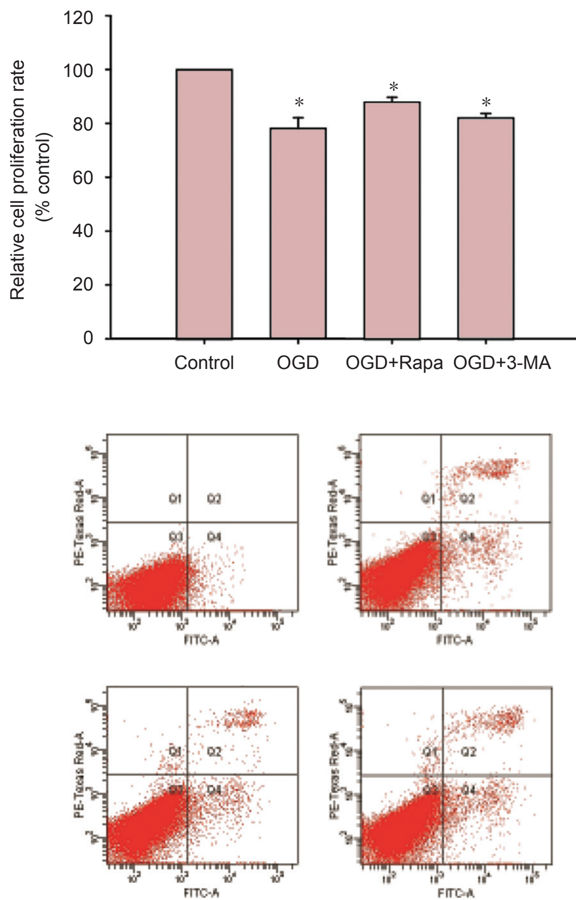

Figure 1 Effects of autophagy regulation and oxygen-glucose deprivation (OGD) on proliferation rate in Neuro-2a/ amyloid precursor protein 695 cells (cell counting kit-8 assay).
Control group: Untreated; OGD group: OGD for 1 hour; OGD + Rapa group: pretreated with Rapamycin (200 ng/mL) for 1 hour followed by OGD for 1 hour in the presence of Rapamycin (200 ng/mL); OGD + 3-MA group: pretreated with 3-methyladenine (5 mM) for 1 hour followed by OGD in the presence of 3-methyladenine (5 mM). *$P < 0.05$, *vs.* control group (mean ± SD, one-way analysis of variance followed by the least significance difference *post hoc* test). The experiment was replicated three times, and there were at least triplicate wells for each trial.

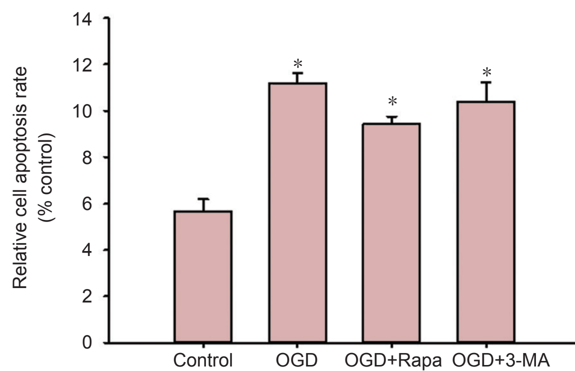

Figure 2 Effects of autophagy regulation and oxygen-glucose deprivation (OGD) on apoptosis ratio in Neuro-2a/amyloid precursor protein 695 cells (flow cytometry).
Control group: Untreated; OGD group: OGD for 1 hour; OGD + Rapa group: pretreated with Rapamycin (200 ng/mL) for 1 hour followed by OGD for 1 hour in the presence of Rapamycin (200 ng/mL); OGD + 3-MA group: pretreated with 3-methyladenine (5 mM) for 1 hour followed by OGD in the presence of 3-methyladenine (5 mM). In cell clustering of flow cytometer, Q1 represents live cells, Q2 represents necrotic or late apoptotic cells, Q3 represents cells with mechanical injury, and Q4 represents early apoptotic cells. *$P < 0.05$, *vs.* control group (mean ± SD, one-way analysis of variance followed by the least significance difference *post hoc* test). The experiment was replicated three times.

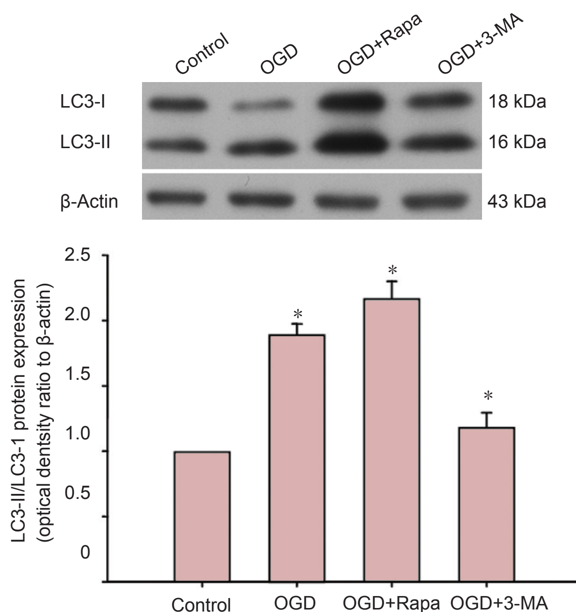

Figure 3 Effects of autophagy regulation and oxygen-glucose deprivation (OGD) on microtubule-associated protein 1 light chain 3 (LC3) expression in Neuro-2a/amyloid precursor protein 695 cells (western blot assay).
Control group: Untreated; OGD group: OGD for 1 hour; OGD + Rapa group: pretreated with Rapamycin (200 ng/mL) for 1 hour followed by OGD for 1 hour in the presence of Rapamycin (200 ng/mL); OGD + 3-MA group: pretreated with 3-methyladenine (5 mM) for 1 hour followed by OGD in the presence of 3-MA (5 mM). *$P < 0.05$, *vs.* control group (mean ± SD, one-way analysis of variance followed by the least significance difference *post hoc* test). The experiment was replicated three times.

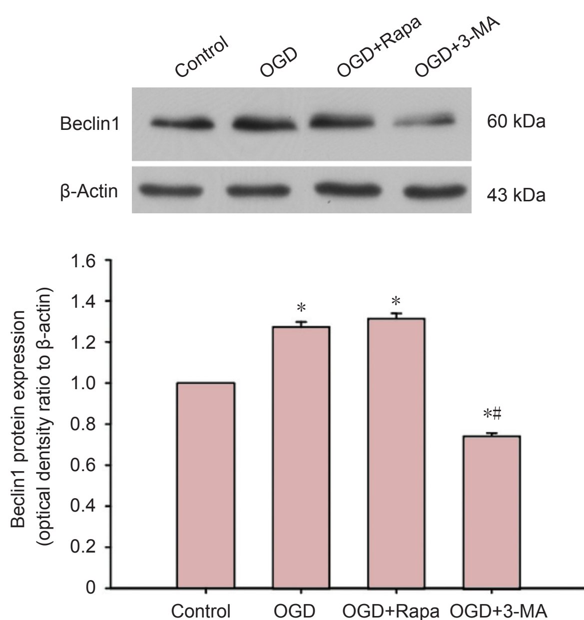

Figure 4 Effects of autophagy regulation and oxygen-glucose deprivation (OGD) on Beclin1 expression in Neuro-2a/amyloid precursor protein 695 cells (western blot assay).

Control group: Untreated; OGD group: OGD for 1 hour; OGD + Rapa group: pretreated with Rapamycin (200 ng/mL) for 1 hour followed by OGD for 1 hour in the presence of Rapamycin (200 ng/mL); OGD + 3-MA group: pretreated with 3-methyladenine (5 mM) for 1 hour followed by OGD in the presence of 3-methyladenine (5 mM). *$P < 0.05$, *vs.* control group; #$P < 0.05$, *vs.* OGD + Rapa group (mean ± SD, one-way analysis of variance followed by the least significance difference *post hoc* test). The experiment was replicated three times.

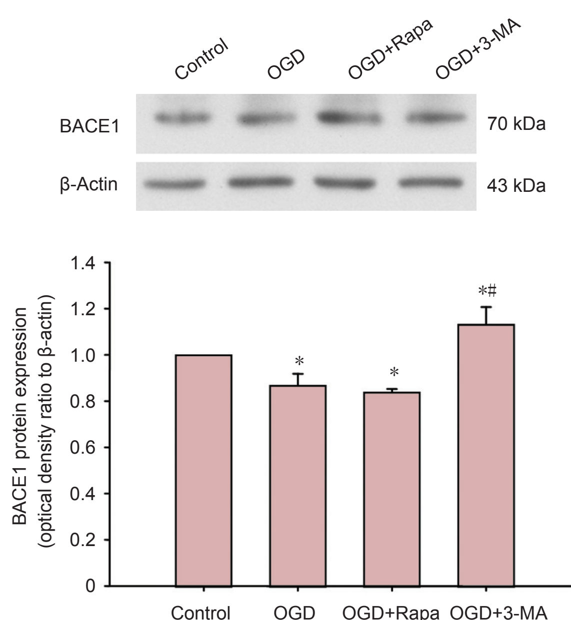

Figure 5 Effects of autophagy regulation and oxygen-glucose deprivation (OGD) on BACE1 expression in Neuro-2a/amyloid precursor protein 695 cells (western blot assay).

Control group: Untreated; OGD group: OGD for 1 hour; OGD + Rapa group: pretreated with Rapamycin (200 ng/mL) for 1 hour followed by OGD for 1 hour in the presence of Rapamycin (200 ng/mL); OGD + 3-MA group: pretreated with 3-methyladenine (5 mM) for 1 hour followed by OGD in the presence of 3-methyladenine (5 mM). *$P < 0.05$, *vs.* OGD group; #$P < 0.05$, *vs.* OGD + Rapa group (mean ± SD, one-way analysis of variance followed by the least significance difference *post hoc* test). The experiment was replicated three times. BACE1: Beta-site APP-cleaving enzyme 1 .

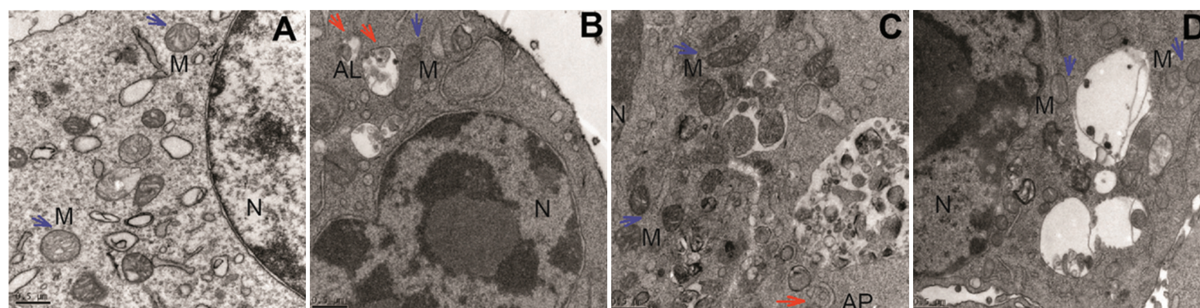

Figure 6 Effects of autophagy regulation and oxygen-glucose deprivation (OGD) on the morphology and ultrastructure of Neuro-2a/amyloid precursor protein 695 cells (transmission electron microscopy).

Control group: Untreated; (B) OGD group: OGD for 1 hour; (C) OGD + Rapa group: pretreated with Rapa (200 ng/mL) for 1 hour followed by OGD for 1 hour in the presence of Rapamycin (200 ng/mL); (D) OGD + 3-MA group: pretreated with 3-methyladenine (5 mM) for 1 hour followed by OGD in presence of 3-methyladenine (5 mM). In B, red arrows indicate autolyosomes. In C, red arrows indicate autophagosomes that are in the process of formation. Blue arrows in every plot mark mitochondria. Scale bars: 500 nm. N: Nucleus; M: mitochondria; AL: autolyosome; AP: autophagosome; 3-MA: 3-methyladenine.

Hubbi ME, Hu H, Kshitiz, Ahmed I, Levchenko A, Semenza GL (2013) Chaperone-mediated autophagy targets hypoxia-inducible factor-1alpha (HIF-1alpha) for lysosomal degradation. J Biol Chem 288:10703-10714.

Kacker P, Bottegoni G, Cavalli A (2012) Computational methods in the discovery and design of BACE-1 inhibitors. Curr Med Chem 19:6095-6111.

Kawahara K, Suenobu M, Yoshida A, Koga K, Hyodo A, Ohtsuka H, Kuniyasu A, Tamamaki N, Sugimoto Y, Nakayama H (2012) Intracerebral microinjection of interleukin-4/interleukin-13 reduces beta-amyloid accumulation in the ipsilateral side and improves cognitive deficits in young amyloid precursor protein 23 mice. Neuroscience 207:243-260.

Klionsky DJ, Abeliovich H, Agostinis P (2008) Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. Autophagy 4:151-175.

Koh YH, von Arnim CA, Hyman BT, Tanzi RE, Tesco G (2005) BACE is degraded via the lysosomal pathway. J Biol Chem 280:32499-32504.

Lai AY, McLaurin J (2012) Inhibition of amyloid-beta peptide aggregation rescues the autophagic deficits in the TgCRND8 mouse model of Alzheimer disease. Biochim Biophys Acta 1822:1629-1637.

Laird FM, Cai H, Savonenko AV, Farah MH, He K, Melnikova T, Wen H, Chiang HC, Xu G, Koliatsos VE, Borchelt DR, Price DL, Lee HK, Wong PC (2005) BACE1, a major determinant of selective vulnerability of the brain to amyloid-beta amyloidogenesis, is essential for cognitive, emotional, and synaptic functions. J Neurosci 25:11693-11709.

Landau SM, Mintun MA, Joshi AD, Koeppe RA, Petersen RC, Aisen PS, Weiner MW, Jagust WJ; Alzheimer's Disease Neuroimaging Initiative (2012) Amyloid deposition, hypometabolism, and longitudinal cognitive decline. Ann Neurol 72:578-586.

Levine B, Kroemer G (2008) Autophagy in the pathogenesis of disease. Cell 132:27-42.

Majumder S, Richardson A, Strong R, Oddo S (2011) Inducing autophagy by rapamycin before, but not after, the formation of plaques and tangles ameliorates cognitive deficits. PLoS One 6:e25416.

Mazure NM, Pouyssegur J (2010) Hypoxia-induced autophagy: cell death or cell survival? Curr Opin Cell Biol 22:177-180.

McAllister C, Long J, Bowers A, Walker A, Cao P, Honda S, Harada N, Staufenbiel M, Shen Y, Li R (2010) Genetic targeting aromatase in male amyloid precursor protein transgenic mice down-regulates beta-secretase (BACE1) and prevents Alzheimer-like pathology and cognitive impairment. J Neurosci 30:7326-7334.

McConlogue L, Buttini M, Anderson JP, Brigham EF, Chen KS, Freedman SB, Games D, Johnson-Wood K, Lee M, Zeller M, Liu W, Motter R, Sinha S (2007) Partial reduction of BACE1 has dramatic effects on Alzheimer plaque and synaptic pathology in APP Transgenic Mice. J Biol Chem 282:26326-26334.

Mizushima N, Yoshimori T, Levine B (2010) Methods in mammalian autophagy research. Cell 140:313-326.

Mizushima N, Levine B, Cuervo AM, Klionsky DJ (2008) Autophagy fights disease through cellular self-digestion. Nature 451:1069-1075.

Nunan J, Small DH (2000) Regulation of APP cleavage by alpha-, beta- and gamma-secretases. FEBS Lett 483:6-10.

Obregon D, Hou H, Deng J, Giunta B, Tian J, Darlington D, Shahaduzzaman M, Zhu Y, Mori T, Mattson MP, Tan J (2012) Soluble amyloid precursor protein-alpha modulates beta-secretase activity and amyloid-beta generation. Nat Commun 3:777.

Ohno M, Cole SL, Yasvoina M, Zhao J, Citron M, Berry R, Disterhoft JF, Vassar R (2007) BACE1 gene deletion prevents neuron loss and memory deficits in 5XFAD APP/PS1 transgenic mice. Neurobiol Dis 26:134-145.

Ohno M, Sametsky EA, Younkin LH, Oakley H, Younkin SG, Citron M, Vassar R, Disterhoft JF (2004) BACE1 deficiency rescues memory deficits and cholinergic dysfunction in a mouse model of Alzheimer's disease. Neuron 41:27-33.

Pendlebury ST, Rothwell PM (2009) Prevalence, incidence, and factors associated with pre-stroke and post-stroke dementia: a systematic review and meta-analysis. Lancet Neurol 8:1006-1018.

Pimentel-Coelho PM, Rivest S (2012) The early contribution of cerebrovascular factors to the pathogenesis of Alzheimer's disease. Eur J Neurosci 35:1917-1937.

Pluta R, Jolkkonen J, Cuzzocrea S, Pedata F, Cechetto D, Popa-Wagner A (2011) Cognitive impairment with vascular impairment and degeneration. Curr Neurovasc Res 8:342-350.

Ravikumar B, Sarkar S, Davies JE, Futter M, Garcia-Arencibia M, Green-Thompson ZW, Jimenez-Sanchez M, Korolchuk VI, Lichtenberg M, Luo S, Massey DC, Menzies FM, Moreau K, Narayanan U, Renna M, Siddiqi FH, Underwood BR, Winslow AR, Rubinsztein DC (2010) Regulation of mammalian autophagy in physiology and pathophysiology. Physiol Rev 90:1383-1435.

Semenza GL (2010) Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. Oncogene 29:625-634.

Spilman P, Podlutskaya N, Hart MJ, Debnath J, Gorostiza O, Bredesen D, Richardson A, Strong R, Galvan V (2010) Inhibition of mTOR by rapamycin abolishes cognitive deficits and reduces amyloid-beta levels in a mouse model of Alzheimer's disease. PLoS One 5:e9979.

Sun X, He G, Qing H, Zhou W, Dobie F, Cai F, Staufenbiel M, Huang LE, Song W (2006) Hypoxia facilitates Alzheimer's disease pathogenesis by up-regulating BACE1 gene expression. Proc Natl Acad Sci U S A 103:18727-18732.

Tassa A, Roux MP, Attaix D, Bechet DM (2003) Class III phosphoinositide 3-kinase-Beclin1 complex mediates the amino acid-dependent regulation of autophagy in C2C12 myotubes. Biochem J 376:577-586.

Vassar R (2001) The beta-secretase, BACE: a prime drug target for Alzheimer's disease. J Mol Neurosci 17:157-170.

Vassar R (2005) beta-Secretase, APP and Abeta in Alzheimer's disease. Subcell Biochem 38:79-103.

Wang Y, Li Y, Dalle Lucca SL, Simovic M, Tsokos GC, Dalle Lucca JJ (2010) Decay accelerating factor (CD55) protects neuronal cells from chemical hypoxia-induced injury. J Neuroinflammation 7:24.

Yu WH, Cuervo AM, Kumar A, Peterhoff CM, Schmidt SD, Lee JH, Mohan PS, Mercken M, Farmery MR, Tjernberg LO, Jiang Y, Duff K, Uchiyama Y, Naslund J, Mathews PM, Cataldo AM, Nixon RA (2005) Macroautophagy-a novel Beta-amyloid peptide-generating pathway activated in Alzheimer's disease. J Cell Biol 171:87-98.

Yun SM, Cho SJ, Song JC, Song SY, Jo SA, Jo C, Yoon K, Tanzi RE, Choi EJ, Koh YH (2013) SUMO1 modulates Abeta generation via BACE1 accumulation. Neurobiol Aging 34:650-662.

Zhang T, Yan W, Li Q, Fu J, Liu K, Jia W, Sun X, Liu X (2011) 3-n-Butylphthalide (NBP) attenuated neuronal autophagy and amyloid-beta expression in diabetic mice subjected to brain ischemia. Neurol Res 33:396-404.

Zhang X, Zhou K, Wang R, Cui J, Lipton SA, Liao FF, Xu H, Zhang YW (2007) Hypoxia-inducible factor 1alpha (HIF-1alpha)-mediated hypoxia increases BACE1 expression and beta-amyloid generation. J Biol Chem 282:10873-10880.

Zhong H, Chiles K, Feldser D, Laughner E, Hanrahan C, Georgescu MM, Simons JW, Semenza GL (2000) Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. Cancer Res 60:1541-1545.

Zhu L, Su M, Lucast L, Liu L, Netzer WJ, Gandy SE, Cai D (2012) Dynamin 1 regulates amyloid generation through modulation of BACE-1. PLoS One 7:e45033.

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