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Basic Study

Taurine alleviates activated hepatic stellate cells through inhibiting autophagy and inducing ferroptosis

Li Sen *et al*. Effect of taurine on autophagy and ferroptosis in hepatic stellate cells

Abstract

BACKGROUND

Liver fibrosis is a compensatory response during the tissue repair process in chronic liver injury, and finally leads to liver cirrhosis or even hepatocellular carcinoma. The pathogenesis of hepatic fibrosis is associated with the progressive accumulation of activated hepatic stellate cells (aHSCs) differentiate into fibroblasts and produced an excessive of extracellular matrix (ECM). Myofibroblasts are the main source of the excessive ECM responsible for the hepatic fibrosis. Therefore, targeting on aHSCs, the principal ECM producing cells in the injured liver, is a promising therapeutic target for the treatment of hepatic fibrosis.

AIM

To explore the effect of taurine on activated HSCs proliferation and the mechanisms implicated.

METHODS

Human HSCs (*LX-2*) were randomly divided into five groups, including normal group, PDGF-BB (20 ng/mL) treated group, and low, medium, and high dosage of taurine(10 mmol/L, 50 mmol/L, 100 mmol/L) with PDGF-BB (20 ng/mL) treated group. CCK8

method was performed to evaluate the effect of taurine on the activated HSCs viability. ELISA kits were taken to estimate the effect of taurine on the levels of reactive oxygen species (ROS), MDA, GSH, and iron concentration. Transmission electron microscope(TEM) was applied to observe the effect of taurine on the autophagosomes and ferroptosis features in activated HSCs. Quantitative real time-PCR and Western blot analysis were performed to detect the effect of taurine on the expression of α-SMA, Collagen I, Fibronectin1, LC3B, ATG3, ATG5, Beclin1, ATG7, ATG9, ATG12, ATG14, PTGS2, SLC11A2 and P62.

RESULTS

Results showed that taurine promoted the death of activated HSCs and reduced the deposition of extracellular matrix. Treatment with taurine could alleviate autophagy in HSCs to inhibit activation of HSCs, by decreasing of autophagosomes formation, downregulating of the LC3-II and Beclin1 protein expression, and upregulating p62 protein expression. Meanwhile, treatment with taurine triggered ferroptosis and ferritinophagy to eliminate activated HSCs characterized by iron overload, lipid ROS accumulation, glutathione depletion, and lipid peroxidation. Furthermore, bioinformatics analysis demonstrated that taurine had a direct targeting effect on NCOA4, exhibiting the best average binding affinity of -20.99 kcal/mol.

CONCLUSION

Treatment of taurine on liver fibrosis by mechanisms that involved inhibition of autophagy and trigger of ferroptosis and ferritinophagy in HSCs to eliminate activated HSCs.

Key Words: hepatic stellate cells; autophagy; ferroptosis; molecular docking; taurine

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Core Tip: We report our first results based on the previous studies that treatment of taurine could alleviate liver fibrosis by inhibiting hepatic stellate cells activation and inhibiting activated hepatic stellate cells proliferation. Considering the important role of autophagy and ferroptosis in the process of liver fibrosis pathology, we used molecular biology tests and bioinformatic methods to identify the effect of taurine on autophagy and ferroptosis in hepatic stellate cells in vitro. This study demonstrated for the first time that experimentally provoked taurine could inhibit autophagy in HSCs to inhibit activation of HSCs, while trigger ferroptosis and ferritinophagy to eliminate activated HSCs. The mechanism of ferritinophagy is associated with taurine have a direct targeting effect on NCOA4 by exhibiting the best average binding affinity of -23.95 kcal/mol.

INTRODUCTION

Liver fibrosis is a compensatory response during the tissue repair process in chronic liver injury, and finally lead to liver cirrhosis or even hepatocellular carcinoma. It is reported that liver fibrosis has a high incidence rate and mortality in the world[1,2]. Liver fibrosis is a common pathological occurrence and is initiated as a result of chronic liver injury due to alcohol, viral hepatitis, drugs, toxins, nonalcoholic steatohepatitis (NASH), autoimmune liver disease, and so on. The pathogenesis of hepatic fibrosis is associated with the progressive accumulation of activated hepatic stellate cells (aHSCs), which would lead to differentiate into fibroblasts and produce an excessive of extracellular matrix (ECM)[3]. In normal liver, HSCs remain quiescent that contain retinoid (vitamin A) and numerous lipid droplets. however, in response to liver injury, HSCs transform into the highly activated, proliferative, motile, and contractile myofibroblast phenotype by receiving either autocrine or paracrine signaling from

injured hepatocytes and immune cells. Myofibroblasts are the main source of the excessive ECM responsible for the hepatic fibrosis[4]. Since aHSCs are the principal ECM producing cells in the injured liver, targeting on aHSCs is a promising therapeutic target for the treatment of hepatic fibrosis.

Autophagy is a conservative way of cell self degradation, which involves the process of lysosomes engulfing their own cytoplasm or organelles to achieve intracellular nutrition and energy reuse. Autophagy has been implicated in major liver pathologies, such as HCV infection and hepatocarcinoma. Several studies have shown that autophagy dysbiosis can exacerbate liver diseases[5]. For example, a decreased autophagosome number is found in liver tissue of alcoholic liver disease model rats, while an increased autophagosome number is observed in hepatitis C-infected patients[6]. Besides, in a disease of α-1 antitrypsin (a1AT) deficiency, which results in protein aggregates and chronic liver injury, and autophagy stimulation reduces the hepatic load of aggregated protein and reverses fibrosis. Furthermore, studies also showed that autophagy is an important process during HSC activation. Treatment of HSCs with the inhibitors bafilomycin A1, hydroxychloroquine or 3-methyladenine hamper several characteristic features of the activated phenotype, such as proliferation and expression of ACTA2, PDGFR-β and PROCOL1a1, in both mouse and human-derived HSCs[7,8]. Bafilomycin A1-treated HSCs present a higher number of large lipid droplets when compared with control cells, further suggesting the important role of autophagy in HSC lipid droplet metabolism. The above mentioned findings also indicated that autophagy maybe therapeutic target for anti-fibrosis.

Taurine, a sulfur-containing amino acid, had a wide range of protective activity towards cytotoxicity and oxidative stress produced in hepatocytes or other tissues, especially antioxidation, anti-inflammatory, as well as anti-apoptotic activities[9,10]. In liver, taurine is an endproduct of sulfur amino acid catabolism and its biosynthetic ability is reduced in the case of liver diseases. Exogenous supplemented with taurine can prevent liver injury caused by different harmful substances and also can inhibit extracellular matrix (ECM) deposition in the damaged liver to prevent liver fibrosis[11].

Miyazaki *et al*[12]reported that the anti-fibrogenesis effect of taurine in rats is associated with inhibiting the proliferation of aHSCs. Our previous studies have also demonstrated that taurine can inhibit HSCs proliferation and promote cell apoptosis significantly, and its molecular mechanism mainly involved in p38 MAPK-JNK-Caspase9/8/3 pathway[13]. Overall, these results show that taurine serves as an effective anti-inflammatory to prevent liver disease.

To determine the mechanism by which treatment with taurine protects against hepatic fibrosis, the present study was performed to observed the effect of taurine on autophagy and ferroptosis for providing more data to taurine therapy.

MATERIALS AND METHODS

Materials

Human HSCs (*LX-2*) were purchased from XiangYa Central Experiment Laboratory, Central South University, Changsha, Hunan Province, China. taurine was provided by Yuanlong Pearl Co. Ltd., Beihai, Guangxi Zhuang Autonomous Region, China. Dulbecco's minimum essential medium (DMEM) was obtained from Hyclone (Logan, UT, USA). Fetal bovine serum (FBS) was purchased from Biochrom AG (Berlin, German). Streptomycin sulfate and penicillin were supplied by North China Pharmaceutical, China. CCK8 kits was purchased from Beyotime Biotechnology (Shanghai, China). Taurine was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). PDGF-BB was provided by PeproTech (NO. L1019).

Culture and treatment of HSCs

The HSCs were cultured in DMEM supplemented with 10% FBS, 100U/mL penicillin and 100U/mL streptomycin in an incubator at 37 °C and 5% CO_2. The fresh culture media were replaced every other day. When the cell density achieved approximately 80% confluence, cells were trypsinized and resuspended in DMEM at a concentration of 1×10^5/mL. For taurine-treated cells, the supernatant was discarded after centrifugation, and the cells were incubated for 48 h in DMEM containing 10, 50,

and 100 mmol/L taurine and 20ng/mL PDGF-BB, while the control group were incubated in DMEM containing the same concentration of HSCs were randomly received treatment with or without taurine.

Cell viability assay

Cell viability was evaluated with a CCK8 Cell Counting Kit (Beyotime Institute of Biotechnology, C0037) according to the manufacturer's instructions. Briefly, HSC cells were plated in a 96-well plates (Sigma, CLS9898) and exposed to various concentrations of the cytotoxic compounds for the indicated times. The 10 μl CCK8 reagents were added to each well and incubated at 37°C in 5% CO_2 for 4 h, and then the plates were measured at 450 nm using the Thermo MK3 Molecular Device (Morrisville, NC, USA).

Estimation of Reactive Oxygen Species (ROS) level by ELISA kits

Intracellular ROS level was measured using an oxidationsensitive fluorescent probe DCFH-DA (Solarbio, CA1410) according to the manufacturer's instructions.

Estimation of MDA and GSH levels by ELISA kits

The relative MDA and GSH concentration in cell lysates was assessed using a ELISA Kit (Nanjing Jiancheng Bioengineering institute, A003-1 and A006-2-1) according to the manufacturer's instructions.

Estimation of iron concentrations by ELISA kits

The relative iron concentration in cell lysates was assessed using an Iron Assay Kit (Nanjing Jiancheng Bioengineering institute, A039) according to the manufacturer's instructions.

Observation of autophagosomes and ferroptosis features by transmission electron microscope TEM

HSC-LX2 cells were seeded onto 4-well Chambered Coverglassat a density of 2×10^4 cells/mL (14,000 cells/well). Images were acquired using the HIATACHI HT7700 transmission electron microscope.

RNA isolation and real-time PCR

Total RNA was isolated and qPCR performed using the QuantiTect SYBR Green PCR Kit (Thermo, F-415XL) in accordance with the manufacturer's instructions. ACTB levels were taken for normalization and fold change was calculated using 2−ddCt. Primer Sequence was showed in Table 1.

Western blot

HSC cells were lysed using a mammalian lysis buffer (Beyotime, P0013B) and immunoblotting was performed according to the manufacturer's guidelines (Bio/Rad, Hercules, CA, USA). Densitometry analysis was performed using the ImageJ software.

Statistics

All the data were expressed as the mean ± standard deviation (SD) and were analyzed by GraphPad Prism (GraphPad Software, San Diego, CA, USA). To compare the data of two groups, unpaired Student's t-test was used and one-way ANOVA with Bonferroni test post hoc was used for multiple group comparisons. P values were all two sided and $P < 0.05$ was considered as statistically significant.

RESULTS

Taurine suppressed PDGF-BB-induced HSC proliferation

The effect of taurine on aHSCs viability was assessed by the CCK8 assay. There is no significant difference among these groups at 0 and 24 h. The proliferation of HSC (LX-2) was promoted by PDGF-BB (20 ng/mL) at 48 and 72 h ($P < 0.01$ Figure 1A). The aHSCs viability was significantly decreased in cells cultured with different concentration of taurine for 48h compared to that cultured without taurine but activated by PDGF-BB (Results showed in Figure 1A). Therefore, we selected 48 h as the working time and 50 mmol/L of the taurine as the treated concentration in the subsequent experiments.

Taurine inhibited the expression of α-SMA, CollagenI and Fibronectin1

The expression of α-SMA, CollagenI and Fibronectin1 at the protein level in PDGF-BB-induced groups were significantly higher than that in the control group. The upregulated expression of α-SMA, CollagenI and Fibronectin1 induced by PDGF-BB were significantly inhibited by taurine ($P < 0.01$, Figure 1B and C).

Taurine induced autophagy in hepatic stellate cells

The expression of P62, LC3B, ATG5, and Beclin1 showed by Western blotting experiments that the expression of P62 was significantly inhibited by taurine, while the expression of LC3B, ATG5, and Beclin1 was increased, as shown in Figure 2A. Typical ferroptosis was observed after taurine treatment, such as appearance of vacuoles in cells, edge depression of nuclear membrane. Meanwhile, we found that the number of autophagosomes was significantly increased, as shown in Figure 2B.

Taurine induced ferroptosis in hepatic stellate cells

Results of western blotting experiment showed that the expression of GPX4 proteins was significantly downregulated by taurine treatment, while the expression of PTGS2 and SLC11A2 proteins were upregulated, as shown in Figure 3A. Meanwhile, the kit test showed that the level of MDA and ROS was upregulated after taurine treatment, while the level of GSH was downregulated, as showed in Figure 3B and 3C for details. It is suggested that the lipid peroxidation was enhanced and ferroptosis occurs after taurine treatment. Moreover, ferroptotic cell morphological changes obviously were observed by transmission electron microscopy, such as the number of mitochondria were reduced, the length of mitochondrial was shorten, shrinkage, disappearance of mitochondrial cristae, as shown in Figure 3D. Furthermore, iron kit test showed that the deposition of iron ions was increased in hepatic stellate cells after taurine treatment, as shown in Figure 3E. All of the above results suggested that taurine can induce ferroptosis in hepatic stellate cells.

Effect of taurine on the ferritinophagy of hepatic stellate cells

The results of bioinformactics showed that there is a direct interaction between NCOA4 and FTH1, and taurine has a good docking effect with NCOA4 and FTH1. It is predicting that taurine and NCOA4 may have a direct targeting effect, as shown in Figure 4 for details.

DISCUSSION

It is well known that nearly half of the disease deaths in the developed countries are closely related to chronic fibroproliferative diseases, especially hepatic fibrosis [14-16]. HSCs activation is associated with the development of hepatic fibrosis and inhibiting on the activated HSCs proliferation has been identified as an important way for prevention and treatment of hepatic fibrosis[17]. A series of studies have implicated the important role of ferroptosis in the event of HSC activation over the past decade [18,19]. Although taurine could protect against hepatic fibrosis in rats by inhibiting activated HSCs proliferation[20,21], there are no in-depth reports focusing on the effect of taurine on HSCs ferroptosis in hepatic fibrosis. Elucidation of the mechanisms governing the ferroptosis of activated HSCs may provide a therapeutic approach for taurine to control liver fibrosis. In the current study, we initially demonstrated that taurine inhibited the proliferation of activated HSCs *in vitro*, as manifested by the significant reduction of cellular viability and the extracellular matrix expression, respectively. Thus, it is conceivable that the anti-fibrosis effect of taurine was associated with inhibiting PDGF-BB-induced activation of HSCs and promoting their death at the same time.

In recent years, many studies have found that drugs can improve the pathological damage of liver fibrosis by regulating ferroptosis in HSCs. Recently, Yang Zheng et.al indicated that curcumol induced ferroptosis in HSCs by promoting autophagy and mediating the degradation of NCOA4 and FTH1 complexes to release iron ions[2]. Moreover, Kuo Chan-Yen et.al reported that chrysophanol can impair HBx-induced activation of HSCs *via* endoplasmic reticulum stress, ferroptosis-dependent or

GPX4-independent pathways[23]. Furthermore, Zili Zhang et.al indicated that dihydroartemisinin could trigger ferroptosis to eliminate activated HSCs by regulating iron overload, lipid ROS accumulation, glutathione depletion, and lipid peroxidation[22]. In our study, we showed that taurine could inhibit activated HSC proliferation *in vitro*. The induction of ferroptosis is required for taurine to inhibit HSC proliferation. Our findings together with previous reports indicated that taurine triggered ferroptotic events including iron overload, lipid ROS generation, GSH depletion, and lipid peroxidation product MDA accumulation. Furthermore, results also showed that taurine can upregulate the expression of PTGS2 and SLC11A2 protein and downregulate the expression of GPX4 protein. Our above results are consistent with previous studies[2,15,22,24]. According to our results, the expression of PTGS2 in PDGF-BB-induced activated HSCs was significantly upregulated. Prostaglandin endoperoxide synthase 2 (PTGS2), also known as cyclooxygenase-2 (Cox-2), is the key enzyme in prostaglandin biosynthesis, and acts both as a peroxidase and as a dioxygenase[25,26]. It is reported that PTGS2 is associated with ferroptosis for the upregulation of it's expression after treatment with Rsl3 and erastin in mice[15,27]. As a matter of fact, PTGS2 was found to be significantly elevated in cells undergoing ferroptosis[28]. In spite of the the exact role of PTGS2 in the ferroptotic cell death cascade is still unclear, targeting on PTGS2 was an effective way to promote cell death. Additionally, several studies have also showed that the activation or upregulation of SLC11A2 promoted ferroptosis in hypoxia/reoxygenation treated myocardial cells[59], and the ferroptosis was reduced while SLC11A2 was knockdown in rats after subarachnoid hemorrhage[28]. Otherwise, glutathione peroxidase 4 (GPX4) is equipped with GSH to prevent cells from ferroptosis by reducing oxidized phospholipids, ROS production, and iron uptake. Here, we found that the expression of SLC11A2 was significantly upregulated in PDGF-BB-induced aHSCs after taurine treatment, while the expression of GPX4 was significantly downregulated. Altogether, these data indicated that taurine stimulated ferroptosis in aHSCs by increasing PTGS2 and SLC11A2 expression, and decreasing GPX4 expression to promote cell death. Although much

more research is needed to uncover the molecular mechanism of ferroptosis in activated HSCs, taurine treatment by inducing ferroptosis had become a potential strategy for eliminating activated HSCs.

As we known, the ways of autophagy regulates the development of liver injury and fibrosis included regulation of different cytokines secreted in the liver. As we known, the formation of essential autophagy consists of four molecular subunits, which included *ATG6/Beclin1, LC3, ATG9/VMP1*, and *ULK1* complex[29]. It is reported that *Beclin1* is a typical gene of autophagy for its up-regulation stimulated the occurrence of autophagy[30,31], while P62 is a specific autophagy protein for its expression is negatively correlated with autophagy level. Furthermore, P62 is an important bridge between LC3 and ubiquitin substrate to be degraded by autophagosome when autophagy is activated. In the process of degradating, P62 would bind to autophagy membrane protein LC3, thus forming the ubiquitin substrate and transporting to autophagosome[32]. Interestingly, we observed the numbers of autophagosome increased in PDGF-BB-induced aHSCs, but it decreased after treated by taurine. Meanwhile, our data showed that the levels of LC3-II and Beclin1 protein expression was downregulated by taurine, while the level of p62 protein was upregulated. In our study, it is suggested that taurine can inhibit HSC activation effectively by inhibiting autophagy. Lien F.R. Thoen reported that increased autophagic flux was observed during HSC activation and autophagy can induce HSC activation[33]. In previous studies, several pieces of research have demonstrated that autophagy promotes digestion of lipid droplets in quiescent HSC, thereby facilitating HSC activation and promoting liver fibrosis. Besides, autophagy is regarded as a cytoprotective and anti-fibrotic mechanism in most liver cell types and is crucial for metabolic homeostasis of hepatocytes[34].

A series of evidence indicated a vital relationship between ferroptosis and autophagy[27,35]. Autophagy is identified as an upstream mechanism in the induction of ferroptosis by regulating cellular iron homeostasis and cellular ROS generation[36]. Based on the relationship between autophagy and ferroptosis, Mancias JD *et al*

proposed a new conception named ferritinophagy in 2014[37]. Ferritinophagy was regarded as a cell-selective autophagy mediated by nuclear receptor coactivator 4 (NCOA4), and was involved in iron metabolism related-pathophysiologic process[16,37]. Due to the limitation of funds, we didn't carry out the experimental verification of NCOA4 gene knockout and overexpression. Otherwise, bioinformatics found that taurine has a good docking effect with NCOA4, demonstrating that taurine have a direct targeting effect on NCOA4. NCOA4 is a key target for regulating the process of ferritin phagocytosis. Studies have showed that NCOA4 depletion inhibits the delivery of ferritin to the lysosome, and NCOA4-mediated ferritinophagy modulates susceptibility to ferroptosis[38]. Besides, Cao Yu-meng reported that inhibiting of autophagy would upregulate the expression of NCOA4 and promote degradation of FTH1, and finally promote ferroptosis in hepatocyte[39]. It is further revealed that NCOA4 is a promising target for anti-hepatic fibrosis. Therefore, many investigators are looking at ways to mediate the expression of NCOA4 or to regulate HSCs' ferritinophagy to alleviate liver fibrosis. For example, Zheng Yang *et al* reported that curcumol inhibited the activation of HSCs by increasing the expression of NCOA4 to mediate the migration of FTH1 for degradation in autophagolysosomes [2]. Ma Mingyue *et al* found that Schisandrin B could ameliorate hepatic fibrosis by inducing NCOA4-mediated ferritinophagy to promote activated HSCs senescence[16]. Xiu Zhiru *et al* showed that caryophyllene oxide regulated NCOA4, LC3 II, and FTH1 to promote ferritinophagy[29]. As a result, our data provided further evidence for the notion that taurine inhibited HSCs activation by regulating the expression of NCOA4 and mediating ferritinophagy.

Altogether, our data demonstrated that the mechanism of taurine on anti-fibrosis in HSCs through three ways, which included inhibition of autophagy to inhibit HSCs activation, induction of ferritinophagy and ferroptosis to promote activated HSCs death (Details was showed in Figure 5).

CONCLUSION

Taurine inhibited autophagy of hepatic stellate cells, promoted ferroptosis and ferritinophagy of hepatic stellate cells, thus inhibiting the activation of hepatic stellate cells to alleviate hepatic fibrosis.

ARTICLE HIGHLIGHTS

Research background

HSCs activation is associated with the progression of hepatic fibrosis. Inhibiting on the proliferation of activated HSCs has been identified as an important way for prevention and treatment for hepatic fibrosis. Although taurine could protect against hepatic fibrosis in rats by inhibiting activated HSCs proliferation, there is no in-depth reports focusing on the effect of taurine on ferroptosis in HSCs. Elucidation of the mechanisms that regulating the ferroptosis in activated HSCs may provide a more effective therapy for taurine to target on liver fibrosis.

Research motivation

Our previous studies found that treatment of taurine could alleviate liver fibrosis by inhibiting hepatic stellate cells activation and inhibiting activated hepatic stellate cells proliferation. Considering the important role of autophagy and ferroptosis in the process of liver fibrosis pathology, we used molecular biology tests and bioinformatic methods in the present study to identify the effect of taurine on autophagy and ferroptosis in hepatic stellate cells *in vitro*.

Research objectives

To explore the effect of taurine on activated HSCs proliferation and the mechanisms implicated.

Research methods

Human HSCs (*LX-2*) were randomly divided into five groups, including normal group, PDGF-BB (20 ng/mL) treated group, and Low, Medium, and High dosage of taurine(10

mmol/L, 50 mmol/L, 100 mmol/L) with PDGF-BB (20 ng/mL) treated group. CCK8 method was performed to evaluate the effect of taurine on the activated HSCs viability. ELISA kits were taken to estimate the effect of taurine on the levels of reactive oxygen species (ROS), MDA, GSH, and iron concentrations. Transmission electron microscope(TEM) was applied to observe the effect of taurine on the autophagosomes and ferroptosis features in activated HSCs. Quantitative real time-PCR and Western blot analysis were performed to detect the effect of taurine on the expression of α-SMA, Collagen I, Fibronectin1, LC3B, ATG3, ATG5, Beclin1, ATG7, ATG9, ATG12, ATG14, PTGS2, SLC11A2 and P62.

Research results

Results showed that taurine promotes the death of activated HSCs and reduces the deposition of extracellular matrix. Treatment with taurine could alleviate autophagy in HSCs to inhibit activation of HSCs with decreasing of autophagosomes formation, downregulating the expression of the LC3-II and Beclin1 protein, and upregulating the expression of p62 protein. Meanwhile, treatment with taurine trigger ferroptosis and ferritinophagy to eliminate activated HSCs characterized by iron overload, lipid ROS accumulation, glutathione depletion, and lipid peroxidation. Furthermore, bioinformatics analysis demonstrated that taurine have a direct targeting effect on NCOA4, exhibiting the best average binding affinity of -20.99 kcal/mol.

Research conclusions

Taurine inhibited autophagy of hepatic stellate cells, promoted ferroptosis and ferritinophagy of hepatic stellate cells.

Research perspectives

Ferritinophagy was regarded as a cell-selective autophagy mediated by nuclear receptor coactivator 4 (NCOA4), and was involved in iron metabolism related-pathophysiologic

process. In future study, we would carry out the experimental verification of NCOA4 gene knockout and overexpression.

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