Function of autophagy on differentiation of rat bone marrow mesenchymal stem cells into neurons

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ABSTRACT: Autophagy refers to an evolutionarily conserved catabolic process in which long-lived proteins and damaged organelles are sequestered in double membrane vesicles and degraded upon fusion with lysosomal compartments.And cells can generate metabolic precursors for macromolecular biosynthesis or ATP generation by autophagy. Recent studies shows autophagy autophagy plays a critical role in a variety of cell differentiation processes, we found that autophagy activity was activated during the neural differentiation of rat bone marrow mesenchymal stem cells (MSCs), autophagy activator Rapamycin could promote neural differentiation, and autophagy inhibitor inhibited neural differentiation. Our results indicate that autophagy may involved in the process of cell differentiation, and increased autophagic activity may improve neural differentiation efficiency of rat MSCs.

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1. Introduction

Autophagy is a non-selective lysosome-dependent degradation process by which cytoplasmic components are degraded. can be activated under different stimuli, such as nutrient deficiency, hypoxia and so on [1,2]. More and more evidences indicate autophagy plays an important role in stem cell maintainance and differentiation [3]. However, the function of autophagy in the neural differentiation of Rat MSCs is still unknown. In the present study, we determined that autophagy might promote the differentiation of MSCs into neurons.

2. Materials and Methods

2.1 cell culture and grouping

MSCs were isolated from femurs and tibias of rat,then the cells were maintained in a complete medium consisting of Dulbecco's modified Eagle's medium(DMEM, Invitrogen), 10% fetal bovine serum (FBS, Invitrogen),and incubated in a 37℃, 5% CO2 incubator.

2.2 Cell grouping and differentiation into neurons

MSCs were divided into 3 groups: control group, 3-MA group and Rapamycin group. When MSCs grew to 50%~70%confluence, cells were cultured in DMEM containing 10% fetal bovine serum and 1 mmol/L β-mercaptoethanol (β-ME) for 24 h.After that, MSCs from control group were transferred to serum-free medium containing 10 mmol/L β-ME for 6h [4], for the 3-MA group, 5 mmol/L 3-MA was added into the medium, and for the Rapamycin group, 20 μmol/L rapamycin was added into the medium as mentioned above.

2.3 Assay of cell viability

The 3-(4,5-dimethylthizol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to assess cell viability. MSCs were transferred to 96-well plates, treated with MTT (5.0 mg/ml)and then centrifuged. The supernatant was removed, and 200 μl dimethyl sulfoxide was added. Using an enzyme linked immunosorbent assay, the absorbance (A) at 490 nm was determined.

2.4 Immunocytochemistry

Cells were fixed for 2h at room temperature in fresh 4% parafor-maldehyde, followed by maintaining in blocking buffer, and subquently incubated with primary antibodies for 24 h, including LC3B (Cell Signaling), MAP-2 (Santa Cruz), NSE （Santa Cruz) at 4℃. After three washes in PBS, the cells were incubated with the secondary antibody (anti-Ig-G-Cy3 goat anti-rabbit, Santa Cruz) at room temperature for 2 h. The cells were visualized using an inverted fluorescence microscope.

2.5 Western blot analysis

Cell lysates were subjected to SDS polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore Corp).The membrane were then blocked with TBST containing 5% non-fat milk, followed by incubation with primary antibodies: LC3B (Cell Signaling), MAP-2 (Santa Cruz), NSE (Santa Cruz) at 4 °C overnight, then incubated with horseradish peroxidase-conjugated (HRP) anti-rabbit IgG (Santa Cruz). Detection of reactive antigens were performed using an ECL kit (Santa Cruz).

2.6 Statistical analysis

All data are expressed as means ± SD. To identify significant differences, the results of different groups were compared using Student's t-test.

The differences were considered significant if $P<0.05$.

3. Results

3.1 Autophagy activator increased cell viability

MTT analysis showed that the cell survival rate of all the groups decreased after induction, while Rapamycin group had higher cell survival rate than the other groups ($P<0.05$; Fig. 1).

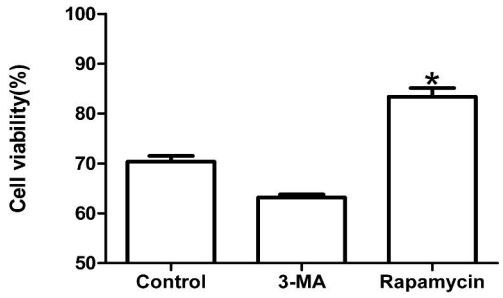

Fig.1 Vitality of MSCs after induction, ($\bar{x} \pm s$, n=6). Rapamycin *vs.* other groups, *$P<0.05$.

3.2 Autophagic activity increased during neural differentiation of MSCs

Western blot and immunocytochemistry staining were used to evaluate the autophagy activity at 0, 3, 6h after induction. By immunocytochemistry staining, few fluorescent LC3 dots in the perinuclear cytoplasm of MSCs could be observed before induction. And Western blot analysis also showed that there were no expression of LC3-II before induction. After induction, LC3-II and the ratio of LC3-II/LC3-I increased,and fluorescent LC3 dots also increased (Fig. 2A,B and C), which indicated autophagic activity increased after MSCs were induced to differentiate to neural cells.

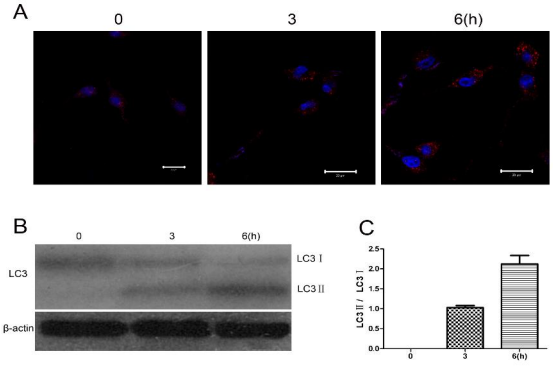

Fig. 2 Autophagy activity increased during during neural differentiation of MSCs: (A) LC3-positive dots (in red) in MSCs at 0, 3, 6h after induction(scale bar: 20 μm), . Autophagy-dependent expression of LC3-II in MSCs, protein levels of LC3 at 0, 3, 6h after induction were analyzed with western blot (B,C).

3.3 Increased autophagic activity promotes the differentiation of MSCs into neurons

MSCs were induced by β-ME for 6h, cells contracted their cytoplasm into globular or spindle-shaped bodies and emitted cellular processes during the induction. Immunocytochemistry and Western blotting were applied to measure the neural differentiation efficiency. These two methods showed that there were higher expressions of the neuron-specific markers, MAP-2 and NSE, in Rapamycin group than in the other groups ($P<0.05$; Fig. 3A,B and C). And the expression of MAP-2 and NSE were much lower in 3-MA group than in the other groups ($P<0.05$; Fig. 3A,B and C).

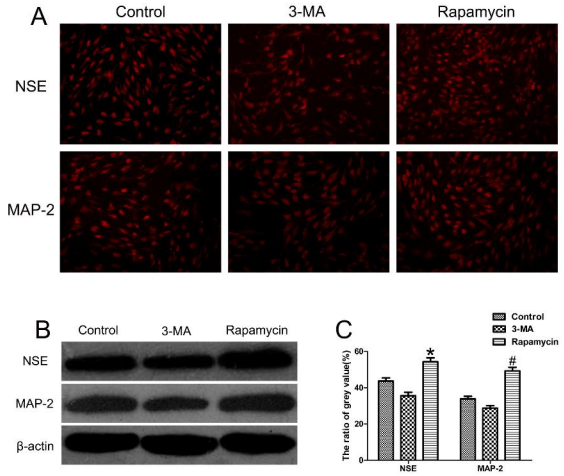

Fig. 3 The expression of NSE and MAP-2 6 h after induction with β-ME. (A) The expression of MAP-2 and NSE by immunocytochemical staining is shown. The stronger fluorescence of NSE and MAP-2 was exhibited in Rapamycin group. (B) The expression levels of NSE and MAP-2 in MSCs 6 h after induction with β-ME as analyzed by Western blot(C). The quantification of the expression levels of NSE and MAP-2, (mean ± SD; n=6). Rapamycin *vs.* other groups, *$P<0.05$, $^{\#}P<0.05$.

4. Discussion

Autophagy is very few in normal condition. When autophagy is induced by all kinds of stressors, breakdown products derived from autophagy were recycled to yield adenosine triphosphate (ATP), by which cells can survive in these conditions [5]. Zhang et al found autophagy was induced in MSCs during both ischemia and hypoxia/serum deprivation (H/SD), and the autophagy inducer, rapamycin, could enhance MSCs survival during H/SD [6]. In this study, we found cells from rapamycin group had higher cell survival rate than the other groups after induction, it indicated that increased autophagy might help MSCs

to survive during the induction.

In addition to maintain cell homeostasis, autophagy is also involved in cell differentiation [3,7]. Zhao et al found autophagy activity markedly increased after glioma stem/progenitor cells (GSPCs) were induced to differentiate by fetal calf serum (FCS), and autophagy activator could effectively promote differentiation of GSPCs [8]. Magri L et al found mTOR pathway, the negative regulator of autophagy, was hperactivated in the neural stem cells (NSCs) of Tuberous Sclerosis patients,which reduced the differentiation efficiency of NSCs to mature neurons [9].In this study, we found autophagy activity increased during neuronal differentiation, and the autophagy activator, rapamycin, could promote MSCs differentiation, while the autophagy inhibitor, 3-MA effectively inhibited the induced differentiation. These indicated autophagy might involved in the process of neuronal differentiation, and increased autophagy activity might promote neural differentiation of MSCs. Differentiated cells generally acquire new morphology and functions, and they involve significant morphological and structural changes, so it requires large amounts of ATP, breakdown and recycling of cellular components [3,10]. Autophagy is an evolutionarily conserved process of bulk degradation. It involves sequestration of their own cytoplasm and organelles within a double membrane structure termed autophagosome and consume them in lysosomes, and the breakdown products were recycled to generate energy and to synthesize new proteins and membranes[11,12]. Therefore, our study provides evidence that autophagy may play an important role in the process of cell differentiation.

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