The distribution changes and significance of mTOR in the differentiation of rat bone marrow mesenchymal stem cells into neurons

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ABSTRACT: Bone marrow mesenchymal stem cells (MSCs) are known to differentiate into neurons in vitro. However, the mechanism underlying MSCs differentiation remains controversial. mTOR (the mammalian target of rapamycin), has emerged as a major effector of cell growth and proliferation via the regulation of protein synthesis.This study is to investigate the distribution changes and significance of mTOR in the differentiation of (MSCs) into neurons. We found that during the induction of MSCs to neurons, mTOR transfered into cytoplasm from nuclear, and the activity of mTOR declined. mTOR might play an important role in regulating MSCs differentiation into neurons.

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KEYWORDS: Bone marrow mesenchymal stem cells; Induction; Neurons; mTOR; Rapamycin

1. Introduction

TOR (target of rapamycin) which was found in yeast at first, is a kind of very conserved protein kinase family in evolutionary , and exists widely in all kinds of biological cells. Mtor, a serine/threonine kinase, can regulate the cell growth, proliferation and some cells activities, such as mRNA transcription and translation, autophagy and cytoskeleton structure reassembly. Bone marrow mesenchymal stem cells (MSCs) have extensive capacities for multidirectional differentiation and can differentiate into neurons in particular conditions [1]. The aim of this study is to explore whether the expression of mTOR changed during the neuronal differentiation of MSCs and what changes have taken place.

2. Materials and Methods

2.1 Cell culture

MSCs were obtained from the femurs and tibias of Wistar rats, aged 6-8 weeks. The cells were cultured in complete medium consisting of Dulbecco's modified Eagle's medium (Invitrogen), 10% fetal bovine serum (Gibco), and 0.3 mg/ml geneticin in a 5% CO2 atmosphere at 37 ℃.The cells were cultured for 10 passages before being used for assays.

2.2 Cell grouping

MSCs were divided into five groups: MSCs blank control group, Dimethyl sulfoxide(DMSO) negative control group,β-ME induced group, different concentrations rapamycin intervention group, different concentrations rapamycin intervention group + β-ME induced group. The concentrations of rapamycin include: 10μmol/L, 20μmol/L, 30μmol/L.

2.3 Neuronal differentiation of MSCs in vitro

To induce neuronal differentiation, the subconfluent cultures were treated with the pre-induction medium, which consisted of DMEM, 10% fetal bovine serum and 1 mM β-mercaptoethanol (β-ME) for 24h. After the pre-induction, the cells were washed with PBS, and the induction medium was replaced with serum-free medium with 10 mM β-ME for 5h. The morphology of the MSCs was evaluated before and after the induction using an inverted microscope.

2.4 MTT assay

MTT assay3 - (4,5 –dimethylthizol -2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used to assess cell viability. MSCs in each group were transferred to 96-well plates, treated with MTT (5.0 mg/ml) for 4 h, 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. The cell survival (%) for each group was calculated by comparing it to the control.

2.5 Immunocytochemistry

After washing with PBS, the cells were fixed at 4 ℃ in 4% paraformaldehyde for 20 min, permeabilized with 0.1% Triton X-100 for 10 min and the blocked with 10% borine serum albumin for 1h. The cells were then incubated with an antibody to one of the following proteins: MAP-2(rabbit, 1:200, Santa Cruz), Tau (rabbit, 1:200, Santa Cruz), mTOR (rabbit, 1:200, Cell Signaling Technology) at 4℃ overnight. After being washed with PBS, the cells were incubated with the secondary antibody (anti-Ig-G-Cy3 goat anti-rabbit, 1:500, Santa Cruz) at room temperature for 1 h. The cells were visualized using an inverted fluorescence microscope.

2.6 Western blot analysis

For Western blot analysis, cell lysates (100μl) were collected from each group. Twenty milligrams of total protein of each lysate was subjected to SDS polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore Corp). The membranes were blocked in 5% non-fat milk for 2 h and incubated with an antibody to one of the following proteins: MAP-2(rabbit, 1:1000, Santa Cruz), Tau (rabbit, 1:1000, Santa Cruz), mTOR (rabbit, 1:1000, Cell Signaling Technology), p-mTOR (rabbit, 1:1000, Cell Signaling Technology), p-p70s6k (rabbit, 1:1000, Cell Signaling Technology), p-4EBP1 (rabbit, 1:1000, Cell Signaling Technology), β-actin (rabbit, 1:1000, Santa Cruz) at 4℃ overnight. The membranes were then incubated with second antibody (1:3000) for 2h at room temperature and visualized by enhanced chemiluminescence (ECL Western blotting detection reagents; Santa Cruz).

2.7 Statistical analysis

All data are expressed as the means±SD. To determine whether a difference was significant, variance analysis was performed between groups, and the results of the different groups were compared using Student's t-test. The differences were considerd significant if $P<0.05$.

3. Results

3.1 Cell viability in groups

The MTT results showed that in rapamycin 10μmol/L group， the cell viability was the highest and after induction, the cell viability reduced comparing with before induction(Table 1).

Table1. the results of cell viability in groups ($\bar{x} \pm s$. n = 5) *P<0.05 vs other groups.

Group	0μmol/L	10μmol/L	20μmol/L	30μmol/L	50μmol/L
Non- inductin	95.72±0.41*	96.54±0.25*	90.96±0.43*	78.88±0.40*	28.06±0.82*
After induction	89.52±0.40*	90.32±0.43*	88.62±0.41*	69.52±0.40*	10.62±0.36*

3.2 Cytoplasmic-nuclear shuttling of mTOR

We used the immunocytochemistry method to observe the distribution of mtor in groups. We found that the fluorescence signal of mTOR mainly distributed in the nuclear before induction. After induction, the fluorescence signal in the nuclear was weakened (Fig1). In rapamycin groups, the signal in the nuclear was weakened comparing the control group and with the concentration of mTOR (10-30μmol/L) continuously improving, the signal in the nuclear abated more obviously.

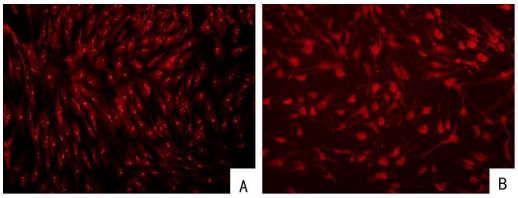
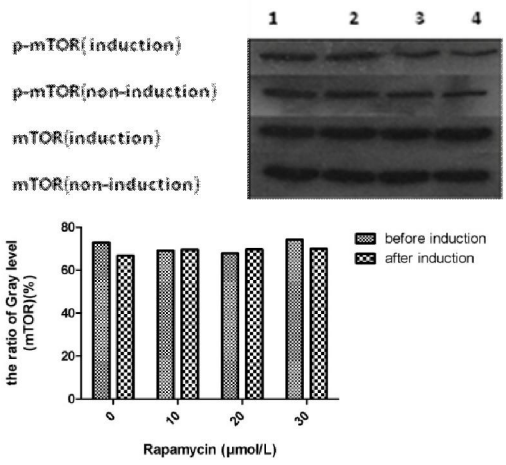


Figure 1. β-Mercaptoethanol induces MSCs into neurons， the expression of mTOR by Cy3(×200).A: before induction; B: after induction.

3.3 The activity of mTOR was declined

Considering the cytoplasmic-nuclear shuttling of mTOR, we asked the question what changes about the activity of mTOR have taken place in this process. Phosphorylation mTOR (p- mTOR) is the activated marker of mTOR. The expression level of p- mTOR can response mTOR activity directly. mTOR can phosphorylate two characterized substrates including 70kD S6 kinase (p-70S6K) and eukaryotic initiation factor 4E binding protein 1(4EBP1).

The Western blot results showed that before induction, the expression of p- mTOR, its downstream products p-p70S6K and p-4EBP1 were declining with the concentration of rapamycin (10-30μmol/L) continuously improving. The expression of total mTOR was unchanged. After induction, the expression of total mTOR was unchanged. The expression of p- mTOR, p-p70S6K and p-4EBP1 declined comparing before induction. With the concentration of rapamycin (10-30μmol/L) continuously improve, The expression of p- mTOR, p-p70S6K and p-4EBP1 declined more obviously (Fig2, Fig3).



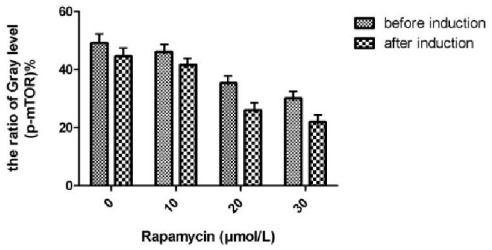


Figure2. the expression of mTOR and p-mTOR protein. 1: Rapamycin 0μmol/L group; 2: Rapamycin 10μmol/L group; 3: Rapamycin 20μmol/L group; 4: Rapamycin 30μmol/L group.

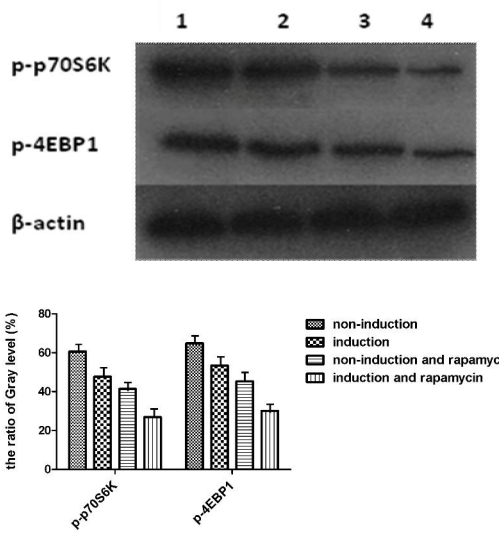


Figure 3. the expression of p-p70S6K and p-4EBP1 protein. 1: non-induction group; 2: induction group; 3: non induction group and rapamycin group; 4: induction and rapamycin group.

3.4 The efficiency of neuronal differentiation

When we used rapamycin to regulate the activity of mTOR, we found the efficiency of neuronal differentiation changed. To evaluate the efficiency of neuronal differentiation, we analyzed two neuronal markers, microtubule-associated protein 2 (MAP-2) and Tau protein. We used the immunocytochemistry and western blot (Fig 4) mehthods and the results showed that the expression of MAP-2 and Tau protein was the highest in rapamycin 20μmol/L group comparing with other groups.

4. Discussion

In the study of human embryonic kidney (HEK) 293 cells and monkey kidney epithelial CV-1cells, Kim found mtor is a cytoplasmic-nuclear shuttling protein which may be relative to mitogen-stimulated rapamycin-sensitive mTOR signaling pathway and protein translation initiation[2]. In the explore of normal mouse fibroblasts (MEF3T3), the results showed the shuttling of the mTOR protein from its normal, predominantly mitochondrial location to the cell nucleus [3]. Since then, there are a number of studies to show the nuclear shuttling of mTOR under certain conditions. In this study we found mTOR shuttled into cytoplasmic from the nuclear during the neuronal differentiation.

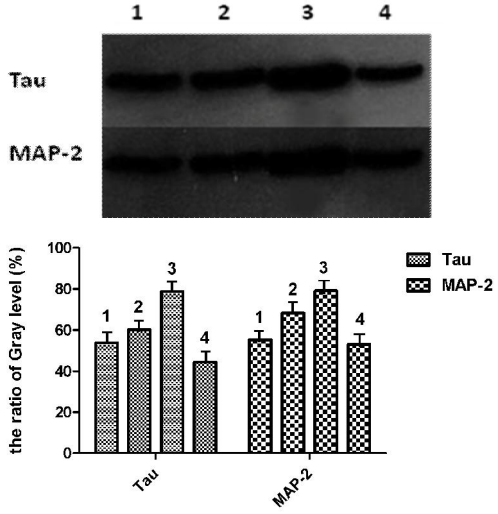


Figure 4.the expression of Tau and MAP-2 protein. 1: rapamycin 0μmol/L group; 2: rapamycin 10μmol/L group; 3: rapamycin 20μmol/L group; 4: rapamycin 30μmol/L group.

mTOR plays an important role in the progress of controlling neuronal differentiation and synaptic plasticity [4]. Axon growth and navigation, dendritic arborization, as well as synaptogenesis, depend on proper activity of mTOR [5]. After severe spinal cord injury [6] and inflammatory stimulation [7], inhibiting mTOR activity exerts obvious nerve protection function and axon-growth-promoting effects. In some neurodegenerative disorder treatment process, decrease the normal neural cell mTOR activity can reach the purpose to alleviate disease progression [8]. Too low or too high mTOR activity impairs cell differentiation. The application of a high concentration rapamytin to neuronal differentiation can reduce nerve swelling branch, cell size and neural markers of immune activity [9]. In the aging rat hypothalamus, black prime source neurons study show that improving mTOR activity can cause neurons hypertrophy [10]. In this study, after induction, the activity of mTOR declined during the neuronal differentiation. And in process, the expression of MAP-2 and Tau protein was the highest in rapamycin 20μmol/L group.

mTOR signaling pathway can be activated by amino acid, insulin and growth factor [11]. mTOR

is enabled into its activated form phosphor-mTOR which can response mTOR activity directly. mTOR is mainly through two phosphorylation means to regulate protein synthesis[12-15]. One way is to activate S6 kinase (S6K). Another way is to inactivate translation inhibitor 4 E-BP1 (eukaryotic cell 4 E binding protein 1). The phosphorylation level of S6K and 4 E-BP1 responses the mTOR activity indirectly [16]. In this study, before induction, the expression of p- mTOR, its downstream products p-p70S6K and p-4EBP1 were declining with the concentration of rapamycin (10-30μmol/L) continuously improving. The expression of total mTOR was unchanged. After induction, the expression of total mTOR was unchanged. The expression of p-mTOR, p-p70S6K and p-4EBP1 declined comparing before induction. With the concentration of rapamycin(10-30μmol/L) continuously improve, The expression of p- mTOR, p-p70S6K and p-4EBP1 declined more obviously. The results suggested during the neuronal differentiation of MSCs, the activity of mTOR declined.

5. Conclusion

During the neuronal differentiation of MSCs, mtor shuttled into cytoplasmic from the nuclear, and the activity of mtor was declined. It indicated mtor might involve in the process of neuronal differentiation of MSCs.

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