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Original Article

Katanin p60 promotes neurite growth and collateral formation in the hippocampus

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Abstract: Objective: This study aimed to investigate the effect of Katanin p60 on the neurite growth and collateral formation in the hippocampus. Methods: Gene cloning was performed to construct the Katanin p60 eukaryotic vector. The microtubule cutting effect and protein expression of Katanin p60 were investigated in 293T cells. Then, these vectors were transfected into hippocampal neurons of rats, and the effects of Katanin p60 on the neurite growth and collateral formation were observed. Results: In the present study, we successfully constructed Katanin p60-GFP recombinant plasmids. After transfecting into 293T cells, the Katanin p60 was over-expressed in these cells, the mesh-like structure of microtubules was disrupted, the residual microtubules circled the nucleus, the expression microtubule proteins reduced, and the tapered protrusions disappeared. In hippocampal neurons with Katanin p60 over-expression, the neural neurite growth was obvious, and a lot of dendrites arose from cell bodies. In cells without Katanin p60 expression, the neurites were small, and the number and length of dendrites reduced significantly when compared with Katanin p60 over-expressing cells ($P < 0.05$). In addition, in Katanin p60 over-expressing cells, the number of collaterals from the neurites and dendrites increased markedly when compared with cells without Katanin p60 expression ($P < 0.05$). Conclusion: Katanin p60 can promote the neurite growth and collateral formation of hippocampal neurons.

Keywords: Katanin, neurite, collateral, microtubule, neuron, hippocampus

Introduction

The sensation, movement and advanced functions (such as learning and memory) of the brain are dependent on the neurons and their branches as well as the neuronal network formed via the synapses [1]. The formation of neuronal network requires the neuronal migration, neurite growth, neuronal branching, and synaptic formation and remodeling [2, 3]. Neuronal precursor cells migrate into the predesigned site, the growth cone at the neurite terminal may guide the guide the growth of neurites into targeted region. When the neurites reach the site near the targeted region, some branches arise from the growth cone and form synaptic connection with one or more target cells [4]. The neurites grow continuously and branch, which are crucial for the formation of neuronal network. In these processes, microtubule severing is indispensable for the neurite growth and collateral formation [5]. Under normal conditions, there are continuous depolymerization and polymerization. However, the long microtubules are inconvenient to transport and migrate, and are required to be severed by specific severing proteins into small segments, which may migrate and switch the polymerization status [6]. To stabilize or depolymerize the microtubules may abolish the ability of neurons to branch and inhibit the neurite growth. Microtubule severing proteins are a group of proteins that can catalyze ATP hydrolysis and cut microtubules. They can cut the microtubules with the energy during the hydrolysis of ATP. Microtubule-severing proteins include Katanin, Spastin and Fidgetin [7, 8]. Katanin is a protein isolated and purified from sea urchin eggs in 1993 and can catalyze ATP hydrolysis, leading to the microtubule severing. This pro-

tein has ATPases associated with different cellular activities (AAA) domain, belongs to AAA protein superfamily and is widely distributed in eukaryotic cells [9]. Katanin is a heterodimer composed of p80 subunit with the molecular weight of 80 kDa and p60 with the molecular weight of 60 kDa. Only p60 has AAA domain and is able to severe microtubules [10-12]. Katanin was initially found to localize at the centrosome. Later, studies also revealed that it is widely distributed in the neurites, and is crucial for the number and length of neurites and collateral formation [13]. To confirm the effect of Katanin on the neurite growth and collateral formation, Katanin-p60 with AAA activity vector was constructed and transfected into hippocampal neurons, and the branching and length of neurites were observed, aiming to investigate the effect of Katanin-p60 on the neurite growth and collateral formation of hippocampal neurons.

Materials and methods

Material

SD rats (specific pathogen free) were purchased from the Experimental Animal Center of Sun Yat-Sen University. Plasmid vector pEGFP-C1 was kindly provided by Prof. Chen Y in the School of Medicine of Sun Yat-Sen University. TRIZOL, cDNA (Invitrogen), DNA polymerase, T4 DNA ligase (TaKaRa), BGI II and SaL I restriction endonucleases (Fermentas), DNA Marker, E coli. DH5A, gel recycling kit, plasmid extraction kit (TransGen Biotech), primers for PCR (Invitrogen, Guangzhou), DMEM/F12 (Invitrogen), Neurobasal medium, B27 supplement (Gibco), polylysine (Sigma), Ara-C (Invitrogen), fetal bovine serum (FBS; Gibco) and other domestic chemicals were used in the present study, Rabbit anti-GFP antibody was purchased from Abcam, mouse anti-tubulin from sigma and fluorescence secondary antibody from Jackson.

Construction of Katanin p60-GFP recombinant plasmids

According to the functional encoding sequence of Katanin p60 in the GenBank (No. AY621629) and the sequence of PEGFP-c1, primers for PCR were designed as follows: 5'-CACAGATCTATGAGTCTTCTAATGATTACTGAG-3' (forward), 5'-CACGTCGACGCATGATCCAAACTCAACTATCCA-3' (reverse). BGI II and SaL I restriction endonucleases were used. Total RNA was extracted from the brain of neonatal rats born within 24 h and cDNA was synthesized with total RNA according to the manufacturer's instructions. The conditions for PCR were as follows: pre-denaturation at 98°C for 2 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 45 s, and a final extension at 72°C for 10 min. Products were stored at 4°C. BGI II and SaL I restriction endonucleases were used to treat PEGFP-c1 plasmids and the recycled PCR products were used to harvest Katanin p60. In the presence of T4 DNA ligase, the Katanin p60 was connected at 16°C overnight and then transfected into E coli. Identification of these plasmids was done by digestion with restriction endonucleases and subsequent sequencing.

Culture and transfection of primary hippocampal neurons and 293T cells

SD rats born within 24 h were sacrificed and the hippocampus was collected under an aseptic condition. The hippocampus was cut into blocks which were then transferred into a 15-ml tube and treated with 0.125% trypsin at 37°C for 10 min in an environment with 5% CO_2. The reaction was stopped and the cells were pipetted more than 10 times, followed by centrifugation at 800 rpm for 5 min. The supernatant was removed, and the remaining cells were re-suspended in Neurobasal medium containing 10 serum and 2% B27. The cell suspension was filtered through the filter and the cell density was adjusted. These, these cells were seeded into PDL-coated cover-slips, and the medium was refreshed with serum free medium 24 h later. Then, half medium was refreshed once every 3 d. 293 T cells were routinely cultured. GFP and Katanin p60-GFP were used to transfect 293 T cells and neurons with calcium phosphate transfection method. Immunofluorescence staining and Western blot assay were performed.

Detection of protein expression by western blot assay

At 72 h after transfection with Katanin p60-GFP and GFP, 293 T cells were harvested, washed with cold PBS twice, lysed in lysis buffer on ice for 20 min, and then centrifuged at 12 000 r/min for 10 min at 4°C. The supernatant was collected and protein quantification was done by using Coomassie brilliant blue.

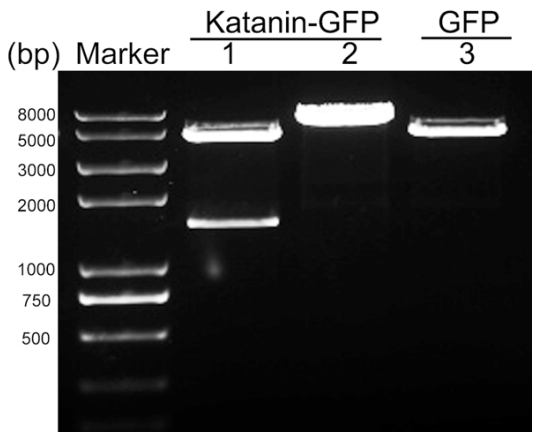

Figure 1. Identification of Katanin p60-GFP after digestion with restriction endonucleases. Marker: DNA marker; 1: Katanin p60-GFP after digestion with restriction endonucleases BGI II and Sal I; 2: Katanin p60-GFP after digestion with restriction endonuclease Sal I; 3: GFP after digestion with restriction endonuclease Sal I.

The protein concentration was calculated. Then, total proteins were subjected to 10% SDS-PAGE (50 μg/lane) and then transferred onto 0.45 μm NC membrane which was subsequently blocked, treated with anti-tubulin/GAPDH antibody and horseradish peroxidase conjugated secondary antibody. Visualization was done with ECL chemiluminescence, and protein bands were scanned.

Fluorescent immunocytochemistry

After calcium phosphate mediated transfection, cells were harvested, fixed in 4% paraformaldehyde, treated with perforating agent twice (5 min for each), and blocked in 5% donkey serum for 1 h at room temperature. Then, these cells were incubated with primary antibody at 4°C over night and washed with TTBS on the second day. Following incubation with secondary antibody, cells were washed with TTBS. After DAPI staining, the coverslips containing cells were air-dried and mounted, and then observed under a laser scanning confocal microscope. Image-Proplus software was used to detect the content of microtubules in 293 cells.

Detection of length and branches of neurites

Image-Proplus software was used to detect the length and branches of neurites. The total length of neurites and axon (the longest neurite), and the number of secondary branches of dendrites and neurons were calculated. The neurites with their length shorter than 2 times of neurite diameter were not included for counting.

Statistical analysis

SPSS version 17.0 was used for statistical analysis. Data are expressed as Mean ± Standard Deviation and comparisons were done with t test. A value of $P < 0.05$ was considered statistically significant.

Results

Construction, identification and sequencing of Katanin p60-GFP recombinant plasmids

The full length Katanin p60 and GFP vectors were digested with restriction endonucleases, and the resultant products were connected, transformed and cultured. The monoclonal colonies were selected for extraction of plasmids. Identification of these vectors was done by digestion with restriction endonucleases, followed by electrophoresis. Results showed the plasmids were observed, and the target bands had good specificity. The target segment was about 1500 bp in length, which was consistent with the anticipated (**Figure 1**). These plasmids after validation were subjected to sequencing in Invitrogen (Guangzhou), and results showed the sequence was consistent with the theoretical sequence (**Figure 2**).

Katanin p60 over-expression affects microtubules in 293T cells

293T cells were routinely cultured and transfected with GFP plasmids and Katanin p60-GFP plasmids, independently. In control group, cells were transfected with blank plasmids. In control group, cells had large nucleus, tapered protrusions of different sizes, and GFP expression in the cytoplasm, and fluorescence staining showed tubulin was expressed in the cytoplasm around the nucleus and neurites. After Katanin p60 transfection, the 293T cells shrunk, protrusions disappeared, tubulin was only found around the nucleus, and Katanin p60 and tubulin co-expressed in the cytoplasm (**Figure 3**). Fluorescence staining was done in these cells, and results showed the content of microtubules in Katanin p60 transfected 293T

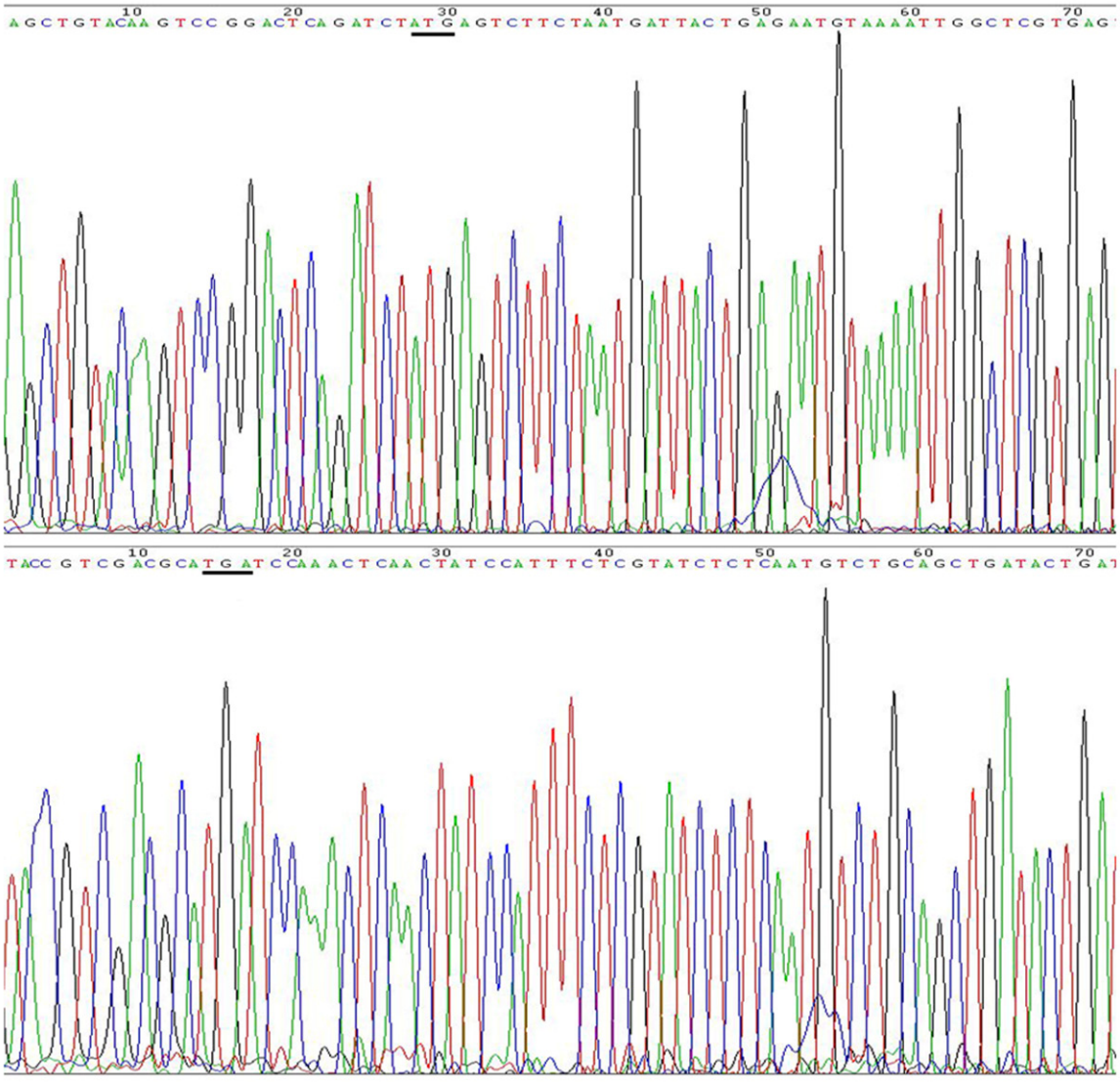

Figure 2. Sequencing of Katanin p60-GFP recombinant plasmids. Underline: ATG: initiation codon; TGA: termination codon.

cells was significantly smaller than that in control group (**Figure 4**). At 48 h after transfection, cells were lysed for detection of tubulin by Western blot assay with GAPDH as an internal reference. Results showed Katanin p60-GFP transfection significantly reduced the expression of tubulin.

Katanin p60 over-expression promotes branching of hippocampal neurons

After culture of primary neurons for 24 h, cells were transfected with GFP or Katanin p60-GFP with calcium phosphate method, aiming to investigate the effects of Katanin p60 on the neurite growth. At 48 h after transfection, cells were stained with GFP, tubulin and DAPI, and then observed under a confocal microscope. The cells transfected with blank vectors were equivalent to those at the developmental phase of 3 d, and their neurites began to differentiate. The long protrusions were axons and growth cone was observed at the terminal. The cell body arose different small dendrite-like protrusions. In Katanin p60 transfected cells, the axonal growth was obvious and collateral was observed. Dendrites arising from the cell body also increased, and were lengthened. These cells were equivalent to those at the developmental phase of 5-6 d (**Figure 5**). The length of neurites was measured. The total length, and the length of axons and neurites were 216.02 ± 37.01, 116.05 ± 31.46 and 99.97 ± 10.22, respectively in Katanin p60 transfected neu-

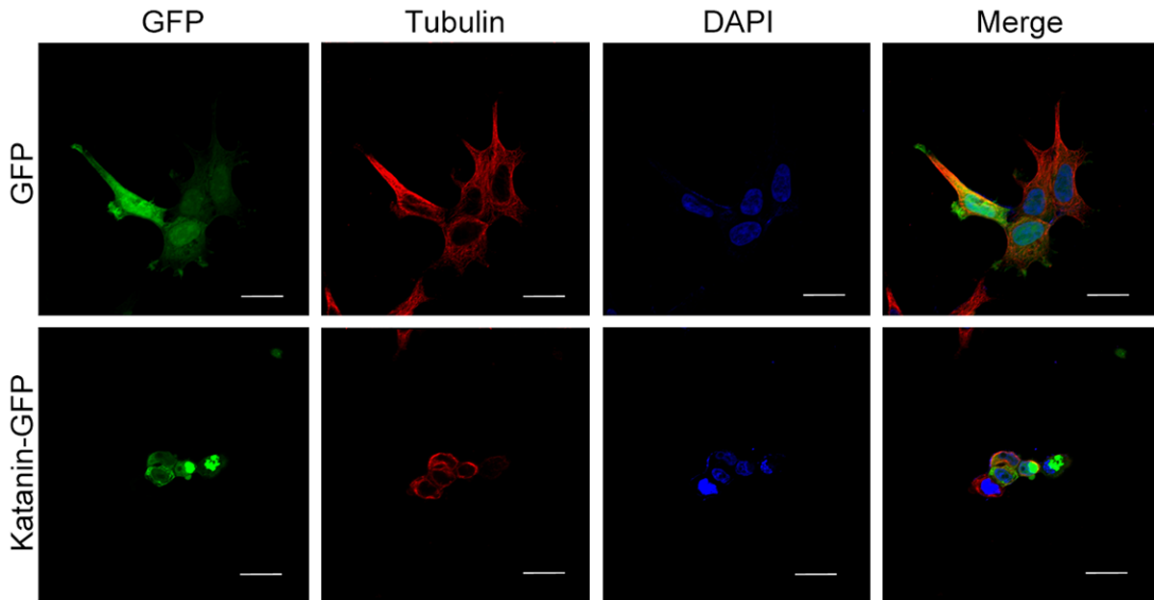

Figure 3. Detection of tubulin protein by fluorescent immunocytochemistry in transfected 293T cells. Anti-GFP antibody and anti-tubulin antibody were used to detect GFP and tubulin proteins, respectively, in 293T cells at 48 h after transfection with the Katanin p60-GFP plasmid or GFP. DAPI was used to stain the nuclei. GFP: green: nuclei: blue; microtubule: red. Scale bar, 20 μm.

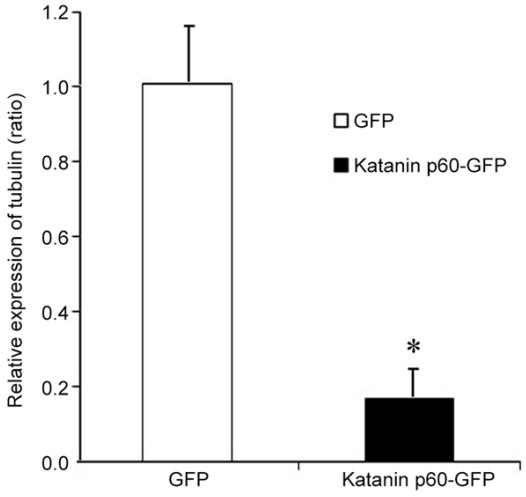

Figure 4. After transfection, the tubulin protein expression was detected in 293T cells. At 48 h after transfection with GFP or Katanin p60-GFP, immunocytochemistry was performed for tubulin, and the relative optical density was measured in 293T cells. T test was used for comparisons. N = 30 per group, and 3 detections were performed. *P < 0.05.

rons, which significantly increased when compared with control group (P < 0.05; **Figure 6**). The number of neurites arising from the cell body was counted and statistically analyzed. Results showed the number of neurites of Katanin p60 transfected cells was 7.11 ± 1.71, which was significantly larger than that in control group (1.89 ± 0.75; P < 0.05; **Figure 7**).

Discussion

Katanin protein is composed of 60-kDa and 80-kDa subunits. The p80 subunit has no severing activity, but acts to regulate the severing activity of p60. The p60 subunit is able to catalyze the ATP hydrolysis and severe microtubules [10-12]. To investigate the ability of Katanin to severe microtubules and its effects on the development and differentiation of neurites, total RNA was extracted from rat brain and used to expand Katanin 60 gene with RT-PCR. Then, the Katanin 60 gene was introduced into eukaryotic vector pEGFP-C1. Results showed the size of products after PCR were consistent with the anticipated. After connecting to vectors, the recombinant plasmids were digested with restriction endonuclease, and results revealed the target gene was connected to vectors. Sequencing indicated that the inserted target gene had no mismatching and mutations. Above findings suggest that Katanin p60-GFP eukaryotic vector is successfully constructed.

To confirm that this vector is able to correctly express Katanin p60 protein, we first observed

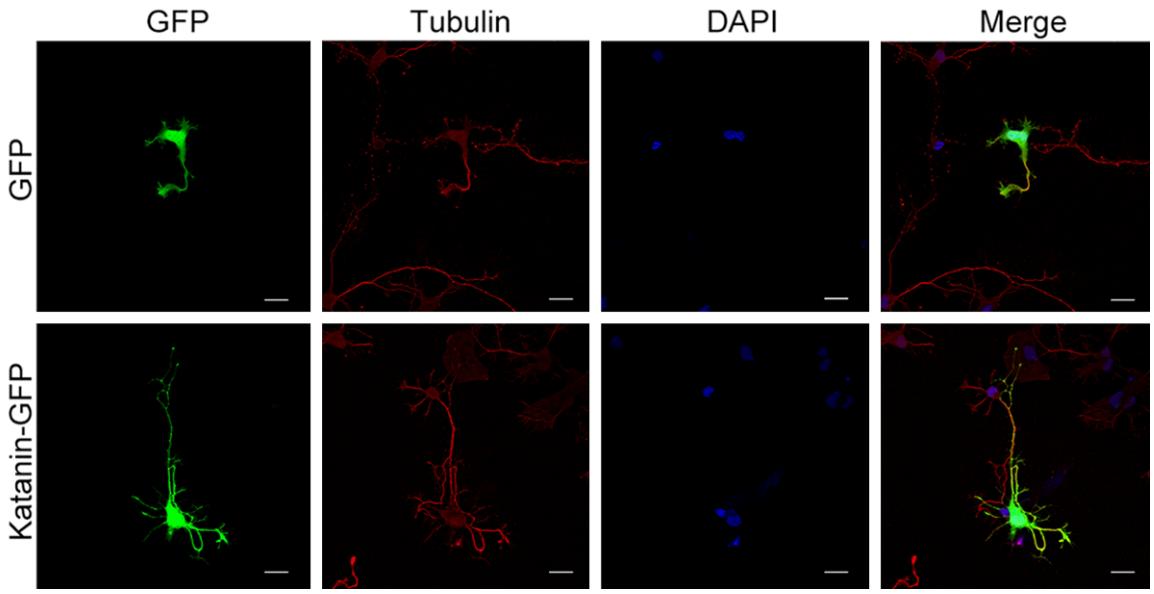

Figure 5. Neurite growth of hippocampal neurons after transfection with GFP or Katanin p60-GFP. At 48 h after transfection with Katanin p60-GFP or GFP, the protein expression of GFP and tubulin was detected in these hippocampal neurons. DAPI was used to stain the nuclei. Green: tubulin, blue: nuclei, red: tubulin. Scale bar, 20 μm.

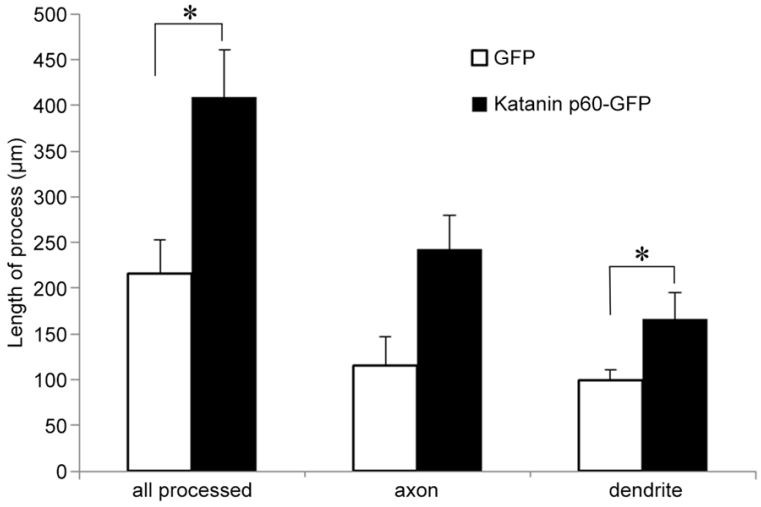

Figure 6. Length of neurites arising from the hippocampal neurons transfected with GFP or Katanin p60-GFP. Hippocampal neurons cultured for 24 h, and then transfected with Katanin p60-GFP or GFP. After culture for 48 h, neurons were fixed and the length of neuritis was measured. Comparisons were done with t test, n = 30 cells per group from 3 independent experiments, *P < 0.05.

the morphological change of 293T cells. Results showed the normal 293T cells were polygonal, had several small tapered protrusions, and were rich in microtubules in the cytoplasm which were distributed around the nucleus in a mesh-like manner. Cells with Katanin p60 overexpression had no protrusions, and the cytoplasmic microtubules were distributed mainly along the cell membrane and accumulated around the nucleus. This suggests that Katanin p60 severs a lot of cytoplasmic microtubules and residual microtubules were only found around the nucleus. The cell membrane shrunk and the microtubules also accumulated along the cell membrane. Zhang et al. postulated that, in cells with migrating activity, the microtubules showed a radial distribution in the cytoplasm, and severing proteins severed the microtubules along the borderline of nucleus. After severing, the resultant microtubules have no direction and then disappear. Intact microtubules curve along the cell membrane. The microfilaments beneath the cell membrane may initiate the cell migration and change the morphology of cell membrane due to the destabilization of cell membrane without the support from microtubules, resulting in occurrence of protrusions [14]. We further investigated whether Katanin p60-GFP fusion protein affect-

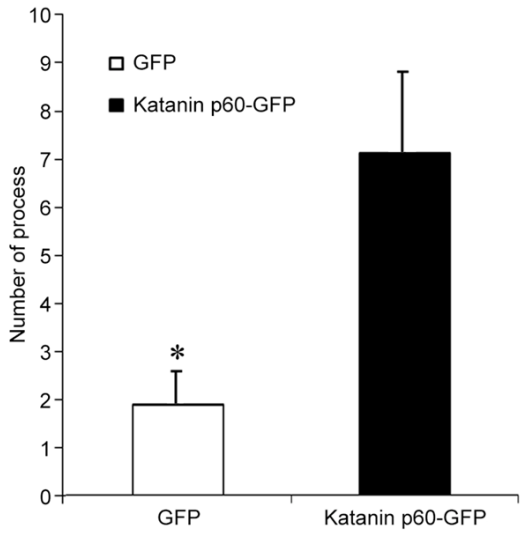

Figure 7. Number of secondary branches of hippocampal neurons transfected with GFP and Katanin p60-GFP. Hippocampal neurons were cultured for 24 h and then transfected with Katanin p60-GFP or GFP. After culture for 48 h, the number of secondary branches of hippocampal neurons was measured. Comparisons were done with t test, n = 30 cells per group from 3 independent experiments, *P < 0.05.

ed the tubulin expression by using western blot assay. Results showed, in cells trasnfected with Katanin p60-GFP, the tubulin expression reduced. This suggests that the structure and expression of microtubules change after introduction of exogenous Katanin p60 into 293T cells.

However, 293T cells may not reflect the growth and development of neurons. Thus, we further investigated the effects of Katanin p60 on the morphology of hippocampal neurons. Results showed neurons without transfection with exogenous Katanin p60 developed axons and detritus, long axons and small detritus arose from the cell body, the enlarged growth cone was also observed at the terminal of axons, but there were no obvious branches on these protrusions. These cells were similar to the normal neurons on day 3 in morphology [15]. The hippocampal neurons with Katanin p60 over-expression developed several neurites with different lengths, the axons further prolonged, the number and length of dendrites increased and small branches were also observed on these protrusions. Quantification showed neurons with Katanin p60 over-expression had a significantly increased length of cell protrusions when compared with cells in control group, and the number and length of branches were also markedly different between two groups. These findings suggest that Katanin p60 not only promote the neurite growth but facilitate the branching of these neurites. Why can Katanin alter the neurite growth of neurons? The neurite growth and branching of neurons are as a result of movement of cytoskeletons, especially the microtubules [16]. The microtubules in the axons and dendrites are arranged in a parallel and buddle like manner, and the polymerization end is at the terminal. However, the polymerization segments of microtubules in the dendrites extend toward distal or proximal end [17, 18]. Regardless of the direction of polymerization end, microtubules continuously polymerize to the distal end or the proximal microtubules are transported into the distal end. However, the long microtubules are inconvenient to transport, and the longer the microtubules, the more difficult the transportation of microtubules is [6]. However, Katanin can catalyze ATP to sever microtubules [19], which may promote the transportation of microtubules and benefit the prolongation of neurites. In the collateral formation, microtubules at the site of branching are severed into severe segments, and then the direction of microtubules is adjusted in the help of motor protein and kinesin. Subsequently, these microtubules migrate into the sites of protrusions due to microfilament movement, which may stabilize the branching [20]. Baas postulated that the stabilized microtubules may arrest the neurite growth, and to stabilize the microtubules at a status of small segments with Taxol might be helpful for the regeneration of neurites [21]. Our results showed the over-expressed Katanin could sever microtubules and more neurites occurred, which is beneficial for the formation of functional connections with adjacent neurons. Especially after neuronal injury, the focal neuronal network is disrupted or the connections among neurons are damaged, and thus, to promote the neurite growth and branching is beneficial for the repair of injured tissues.

Acknowledgements

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Disclosure of conflict of interest

None.

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