Int J Clin Exp Pathol 2012;5(7):634-641
www.ijcep.com /ISSN:1936-2625/IJCEP1205011

Original Article

Sema3F downregulates p53 expression leading to axonal growth cone collapse in primary hippocampal neurons

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Received May 25, 2012; Accepted July 26, 2012; Epub September 5, 2012; Published September 15, 2012

Abstract: Hippocampal nerve growth is regulated by the coordinated action of numerous external stimuli, including positively acting neurotrophin-derived growth cues and restrictive semaphorin cues, however the underlying cellular mechanisms remain largely unclear. We examined the potential cellular mechanism of Semaphorin3F (Sema3F) in cultured primary hippocampal neurons. We show that Sema3F can down-regulate p53 expression in primary hippocampal neurons, thereby contributing to growth cone collapse. Sema3F suppressed p53-induced pathways, which we show to be required to maintain growth cone structure. Sema3F-induced growth cone collapse was partially reversed by overexpression of p53, which promoted growth cone extension. Inhibition of p53 function by inhibitor, siRNAs, induced axonal growth cone collapse, whereas p53 over-expression led to larger growth cones in cultured primary hippocampal neurons.These data reveal a novel mechanism by which Sema3F can induce hippocampal neuron growth cone collapse and provide evidence for an intracellular mechanism for cross talk between positive and negative axon growth cues.

Keywords: Sema3F, p53, hippocampal neurons, collapse, transfection, growth cone

Introduction

Several families of guidance molecules, including netrins, slits, ephrins, and semaphorins, guide neural axons to their targets during both vertebrate and invertebrate development [1, 2]. Semaphorins are a large family, comprising 19 secreted and membrane bound molecules in vertebrates [3]. Within this family, the six secreted proteins in class 3 are the best studied, and Sema3F is particularly well-characterized for its effects on developing axons, particularly axon repulsion. The ratio of collapsed growth cones is a good index to assess the efficacy of repulsion [4-6]. Growth cones consist of lamellipodia and filopodia; the lamellipodia contain cross-linked networks of actin filaments, while filopodia contain bundled filamentous actin (F-actin). Advancement and retraction of growth cone movement depend on balanced regulation of actin polymerization and depolymerization in lamellipodia and filopodia [7]. Semaphorin3F repels axons and collapses growth cones by depolymerizing actin [8].

The transcriptional activity of p53 is increased in murine neuronal precursors, but reduced in cells undergoing terminal differentiation [9, 10]. However, high levels of p53 mRNA have also been detected in the developing brain in areas showing little or no apoptosis [11], and p53-deficient mice exhibit high-frequency neuronal abnormalities, particularly defects in neural tube closure [12]. In addition, studies of neuronal-like cells have suggested that p53 plays a role in cell survival following NGF(Nerve Growth Factor) administration in PC-12 cells, and that the interaction of p53 with neuron-specific transcription factors, such as Brn-3a, may promote neuronal survival [13]. Thus, due to its dual role in regulating both the cell cycle and cell death, p53 is a crucial and

versa-tile coordinator of cellular growth and differentiation.

P53 was highly and specifically expressed in axons and axonal growth cones in primary hippocampal neurons, and inhibition of p53 function by inhibitors, small interfering RNAs, or introduction of dominant-negative mutant forms, induced axonal growth cone collapse, whereas p53 overexpression led to larger growth cones [14]. Sema3F repels axons and collapses growth cones. However, though Sema3F and p53 both affect the growth cones of primary hippocampal neurons, the relationship between these factors during this process is unknown.

Here, we report a novel mechanism by which Sema3F regulates axonal growth cone motility through down-regulation of P53 expression. These findings reveal a novel mechanism that could play a critical role in axonal wiring and in regeneration after neuronal injury.

Materials and methods

Neuronal culture, immunofluorescence, image analysis and growth cone measurement

Hippocampal neurons were dissociated from postnatal day 0 rats [15] and cultured in Neurobasal medium (Invitrogen) supplemented with 10% bovine serum albumin (BSA), 2% B27, and 1% glutamine. For immunofluorescence analyses, cells were fixed with 4% paraformaldehyde in phosphate buffer, pH7.4, for 15min. After washing with 1×PBS, cells were permeabilized with 0.05% Triton X-100 in 1×PBS for 15min, and incubated with preblock buffer (3% BSA, 0.02% Triton X-100 in 1×PBS) for 15min before being stained for 1h in the dark with Rhodamine-conjugated phalloidin (Sigma) . After 6×10min washes with 1×PBS at room temperature, cells were incubated with Hoechst 33258 (1:10000) for 5min to stain nuclei. Immunofluorescence signal was detected using a Nikon confocal microscope. Quantification of growth cone morphology was performed with confocal images taken with a 60×objective. Approximately 30–40 images were randomly selected from each culture dish (20mm in diameter); at least six to eight dishes from three to six individual culture preparations were used for each experimental group. Quantification of growth cone morphology was done blindly by 3 independent investigators. Growth cones with ≤ 3 filopodia were considered collapsed [16]. Image J software was used to quantify growth cone areas. Only the growth cone of the longest neurite, the axon, was scored. The area of the growth cones was measured by framing the actin-positive extensions using the freehand modus. Axonal length was determined by measuring the longest process from the soma of the neuron to the distal tip of the growth cone [14]. Results were expressed as means ± SEM, and p value was determined by one-way ANOVA followed by *post hoc* analysis; values of $p < 0.05$ were considered statistically significant.

The experimental procedures and the animal use and care protocols were approved by the Committee on Ethical Use of Animals of Shengjing Hospital of China Medical University.

Chemicals and antibodies

Sema3F protein (R&D) was dissolved in sterile deionized water and diluted in culture medium. Pifithrin-α (0.5–1 μM) was first dissolved in 10% DMSO and diluted in culture medium. The final DMSO concentration was < 0.01%.

Time lapse imaging

Cells were washed once with 1×PBS and placed in pre-warmed (37°C) imaging buffer (100 mM NaCl, 3 mM KCl, 10 mM HEPES, 2 mM CaCl2, 2 mM MgCl2, 10 mM glucose, and 2% B27 dissolved in 1xPBS). The culture dish was transferred to an imaging chamber and the temperature was maintained at 37°C. Images were acquired with a Nikon microscope. Parameters for image acquisition were kept constant between different treatments.

SiRNA-mediated gene transfer and electroporation transfection

The newly extracted rat hippocampal neurons were suspended in 400μL sterile serum-free Dulbecco's modified Eagle's medium (DMEM) after filteration. Hippocampal neurons at a density of approximately 2-3×10^6/ml were mixed with 40 pmol siRNA in a 0.4 cm electroporation cell (P53-siRNA: Sense 5'-GCAUGAACCGCCGGCCCAUTT-3', Anti-Sense 5'-AUGGGCCGGCGGUUCAUGCTT-3'; Negative control: Sense 5'-UUCUCCGAACGUGUCACGUTT-3', Anti-Sense 5'-ACGUGACACGUUCGGAGAATT-3'). The

Table 1. Growth cone collapse rate after sema3F treatment (%)

Concentration	Time		
	5 min	15 min	30 min
1% BSA	8.61 ± 1.57	8.33 ± 2.22	8.89 ± 1.85
1 ng/ml	7.52 ± 0.83	17.78 ± 1.85	43.89 ± 9.62
5 ng/ml	16.33 ± 2.21	31.39 ± 3.80	78.33 ± 1.11
10 ng/ml	22.33 ± 5.56	46.11 ± 6.30	98.87 ± 1.85
20 ng/ml	28.01 ± 1.57	60.56 ± 7.40	99.44 ± 7.4
50 ng/ml	43.055 ± 1.57	79.44 ± 5.19	99.72 ± 4.6

electroporation cell was placed in the electroporation device (Bio-RAD, Gene Pulser Xcell, USA) after 10min on ice. The cells were electroporated with a single pulse in square wave mode at 200V for 20ms. After electroporation, the hippocampal neurons were suspended in 400 μL 37°C serum-free, sterile antibiotic-free DMEM, put in complete medium and cultured at 37°C and 5% CO_2.

Lentivirus-mediated gene transfer

The lentivirus used for expression of green fluorescent protein (GFP) and GFP-p53 were obtained from Sunbio (Shanghai, China). For transfection, the lentivirus was mixed with rat hippocampal neurons in 2% B27 neural medium, which had been cultured for 24h. 12-18h later, the neural medium was replaced with new 2% B27 neural medium after three PBS washes. Transfection efficiency was evaluated 24, 48, and 72h after transfection via an inverted fluorescence microscope. The lentivirus application in neuronal cultures was supported by NeuronBiotech (Shanghai, China).

Immunoblotting procedures and real-time RT-PCR

Immunoprecipitates were isolated by incubation with protein A/G-agarose beads for 3h at 4°C. After several washes, the beads were resuspended in 2×SDS sample buffer (4% SDS, 100 mM Tris-HCl, pH 6.8, 10% β-mercaptoethanol, 20% glycerol, and 0.2% bromophenol blue) and boiled for 10min. The resulting proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes for immunoblotting using previously described protocols [17].

Real-time RT-PCR

Total RNA extraction and quantitative RT-PCR was performed as previously reported [18]. Briefly, total RNA was extracted (RNA Purification System; Invitrogen) and 3 μmoles of total RNA was subjected to RT (SuperScript III First-Strand Synthesis System; Invitrogen). First strand cDNAs were amplified using a real-time PCR thermal cycler (ABI7700; Applied Biosystems, Inc. [ABI]) with a PCR core kit (SYBR Green; Applied Biosystems). For relative comparison of each gene, we analyzed the Ct values of the real-time PCR data with the $^{\Delta\Delta}$Ct method (P53 primers: F, 5'-CGACGACATTCGGATAAG-3'; R, 5'-TTGCCAGATGAGGGACTA-3'; GAPDH primers: F, 5'-CGGCAAGTTCAACGGCACAG-3'; R, 5'-CGCCAGTAGACTCCACGACAT-3'), according to the manufacturer's instructions (ABI). To normalize the amount of sample cDNA added to each reaction, the Ct value of the endogenous control (18rRNA) was subtracted from the Ct value of each target gene.

Statistical analysis

Statistical analyses were performed using SPSS-12.0 software. All results are expressed as mean ± standard error. Each experiment was repeated 3times, unless otherwise indicated. P values were determined by one-way ANOVA followed by post hoc analysis; P values less than 0.05 were considered statistically significant.

Results

Sema3F inhibits p53 expression in primary hippocampal neurons

We found that the shape of the primary rat hippocampal neuronal growth cones was changed

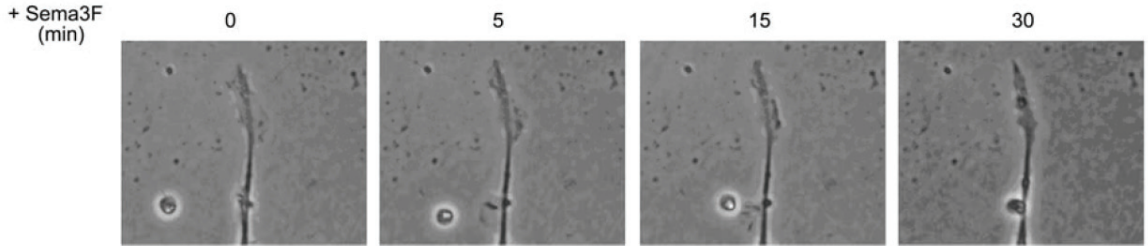

Figure 1. Growth cone collapse（10 ng/ml）observed with delay camera technology.

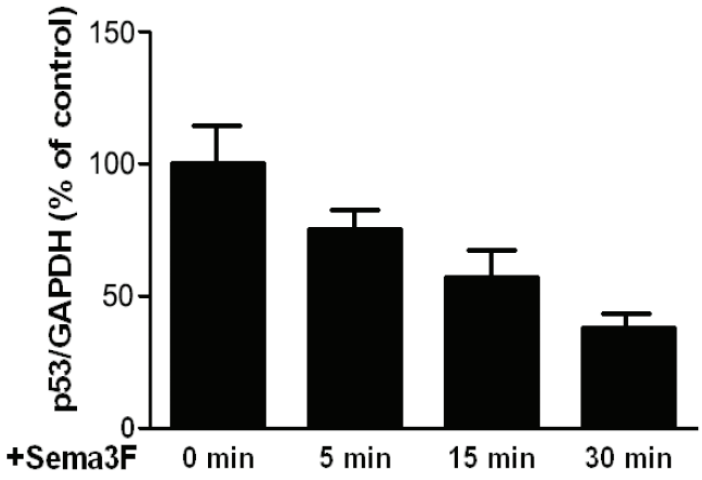

Figure 2. p53 mRNA expression was inhibited by Sema3F（10ng/ml） in primary hippocampal neurons.

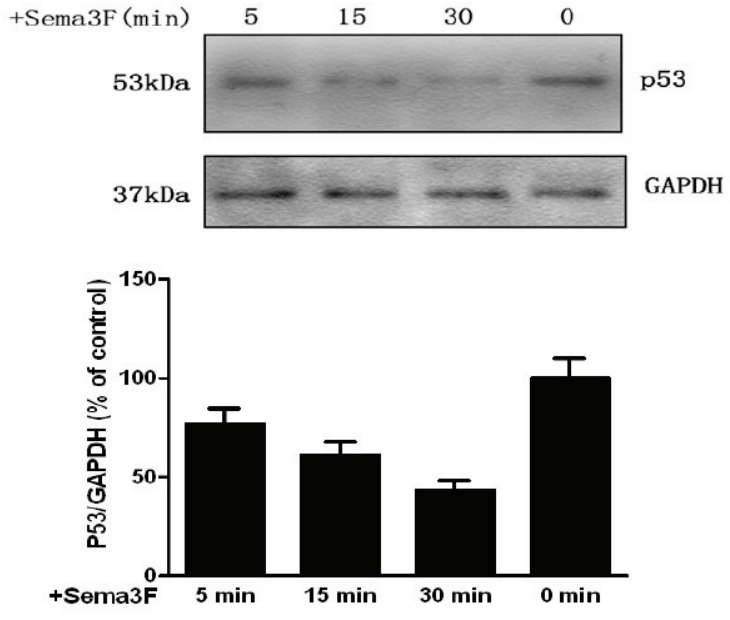

Figure 3. Expression of p53 was inhibited by Sema3F（10ng/ml）in primary hippocampal neurons.

by Sema3F. Specifically, primary rat hippocampal neuronal growth cones collapsed in the presence of Sema3F in a concentration and time-dependent manner (**Table 1**). Further experiments were performed using 10ng/ml of Sema3F as the final concentration in the neural medium. Growth cone collapse after Sema3F treatment was observed with delay camera technology (**Figure 1**).

Expression of p53 protein and mRNA in primary hippocampal neurons was detected with western blotting and real-time PCR, respectively. Neurons were treated with 10ng/ml Sema3F for 5, 15 or 30. Sema3F treatment caused decreased expression of p53 mRNA and protein in these cells (**Figure 2, 3**).

Knock-down of p53 in primary hippocampal neurons led to axonal growth cone collapse

Growth cone collapse rates were evaluated 72h after electroporation of siRNA targeting p53 as well as negative control siRNA. We found that the collapse rate for the p53-siRNA group was 62.22 ± 26.30%, whereas neurons electroporated with control siRNA exhibited a collapse rate of 13.33 ± 7.78%. These results were statistically significant ($p < 0.01$, **Figure 4A**).

In addition, treatment with pifithrin-α, which is p53 inhibitor,

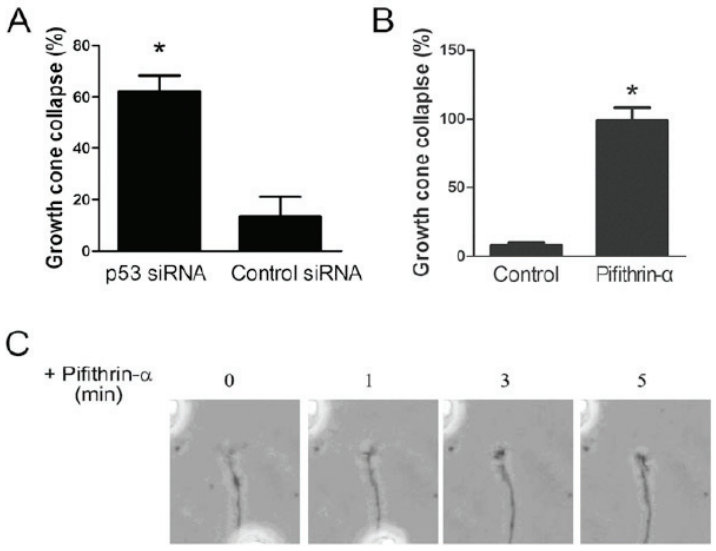

Figure 4. A: The growth cone of primary hippocampal neurons was collapsed after p53-siRNA electroporation 72h (n = 60, *p < 0.01). B: Primary hippocampal neuron growth cones were collapsed after pifithrin-α treatment (n=60, * p < 0.01). C: Growth cone collapse after pifithrin-α treatment was observed with delay camera technology.

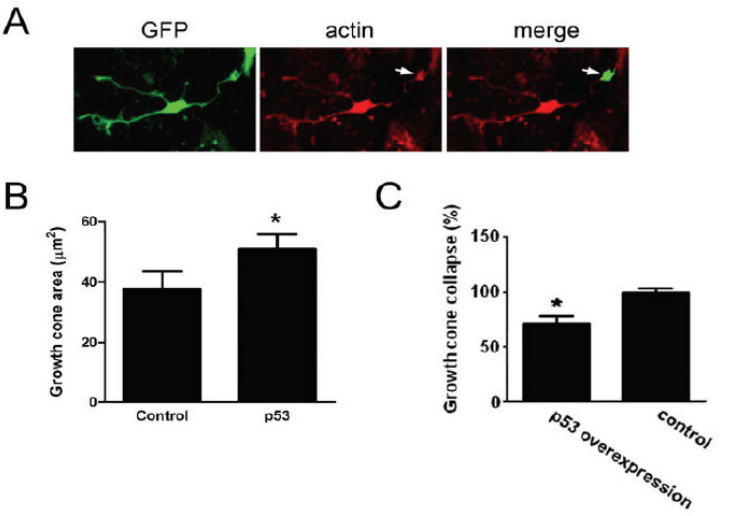

Figure 5. A: Measurement of growth cone area（arrows indicate axonal growth cone). B: Overexpression of p53 promoted the growth cone extension of primary hippocampal neurons（n = 60, *p < 0.01). C: Overexpression of p53 attenuated Sema3F-induced axonal growth cone collapse(n = 60, *p < 0.01).

also resulted in growth cone collapse. Primary hippocampal neurons were treated with 1 μmole pifithrin-α or 0.1% DMSO for 5min and the collapse. rates of the axonal growth cones were evaluated. The collapse rate of the pifithrin-α group was 99.17 ± 9.4%, while the control group had a collapse rate of 8.05 ± 1.60%. These results were statistically significant (p < 0.01, **Figure 4B**). The growth cone collapse induced by Pifithrin-α was observed with delay camera technology (**Figure 4C**).

Overexpression of p53 attenuates Sema3F-induced axonal growth cone collapse

Primary hippocampal neurons were co-cultured with p53-GFP-lentivirus and GFP-lentivirus for 48h and growth cone areas were measured (**Figure 5A**). the area of growth cones in the p53-GFP-lentivirus-treated group was 51.01 ± 4.8μm2, while the area of the GFP-lentivirus-treated control group, was 37.80 ± 5.79μm2 (p < 0.01, **Figure 5B**).

In addition, we cultured primary hippocampal neurons that had been transfected with p53-GFP or GFP-lentivirus for 48h in 10ng/ml Sema3F for 30min, and growth cone collapse rates were evaluated. The collapse rate of the p53-GFP-lentivirus group was 71.11 ± 6.96%, while the collapse rate of the GFP- lentivirus control group was 99.17 ± 3.94 (p < 0.01, **Figure 5C**).

Discussion

In the present study, we showed that Sema3F induced growth cone collapse and decreased p53 expression in primary hippocampal neurons. Inhibition of p53 expression or activity induced growth cone collapse, while overexpression of p53 promoted growth cone extension in primary rat hippocampal neurons. Overexpression of p53 attenuated Sema3F-induced axonal growth cone collapse. Thus, we conclude that Sema3F inhibits p53 expression to cause axnal growth cone collapse in primary rat hicampal neurons.

In part, Sema3F induced axonal growth cone collapse via downregulation of p53 expression. Many signaling proteins have been shown to participate in Sema3F-induced growth cone collapse, including Rho kinase, GTP kinase and cGMP [19]. Semaphorin-induced growth cone collapse can be reversed by inhibitors of Rho signaling proteins, such as Rho kinase and LIM kinase inhibitors or through Cofilin structure [20-22]. Sympathetic neurons activate PI3-K and MEK signaling via one or more pathways to maintain the growth cone, while Gab-1 reverses Semaphorin-mediated axon collapse through high levels of PI3-K and MEK expression [19]. These mechanisms include the Rac1, CasL, and PKA pathways [22-25]. PI3-K and MEK regulate growth cone structure in neurons. Specifically, PI3-K changes the dynamics of actin filaments to promote the formation of new actin filaments in the nucleus and mediates cell chemotaxis [26-28]. PI3-K also mediates the actin network through the inhibitory effect of Rac and GSK-3 [29, 30]. The ERK pathway mediates microtubule changes through phosphorylation of microtubule-associated protein and nerve fiber protein, which in turn regulate growth cone dynamics [31, 32]. EphB2-induced axonal collapse occurs via down-regulation of the Ras-MEK–ERK pathway in the NG108 neuroblastoma cell line [33, 34]. In addition, both the PI3-kinase and MEK pathways may represent common molecular points of convergence for different growth cone regulating factors [19]. The PI3-K-Akt-MEK pathway participates in Sema3F-induced collapse of the growth cone in neurons via NGF signalling [19].

P53has previously been shown to promotegrowth cone extension in neurons [35-41]. For instance, downregulation of p53 inhibits NGF-induced neuronal differentiation and nerve growth in rat PC12 cells. Coronin 1b, small GTPase and Rab13 all participate in p53-induced nerve growth in PC12 cells and cortical neurons [40]. In primary neurons, modification of p53 in the growth cone is correlated with growth cone collapse [42]. In primary neurons, treatment with pifithrin-α and pifithrin-μ, both inhibitors of p53, results in rapid growth cone collapse, and blocking nuclear export of p53 in primary neurons without inhibiting p53 also results in growth cone collapse [42]. Furthermore, decreacing in phosphorylated p53 levels in axons and growth cones involved in Semaphorin 3A-induced rapid growth cone collapse in cultured hippocampal neurons[43]. These studies suggest that p53 promotes growth cone extension in neurons.

In primary hippocampal neurons, Sema3F downregulates p53 expression to induce growth cone collapse. pathHowever, the ways by which Sema3F exerts these effects on p53 are unknown.

In this article, we report a novel mechanism for Sema3F-mediated regulation of axonal growth cone motility that acts through downregulation of P53 expression. Futhermore, we find that downregulation of p53 participates in Sema3F-induced growth cone collapse in primary hippocampal neurons. These molecular mechanisms could play a critical role in axonal wiring and regeneration after neuronal injury.

Acknowledgement

We thank Medjaden Bioscience Limited for assisting in the preparation of this manuscript.

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