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IGF2 Regulates Neuronal Differentiation of Hippocampal Radial Glial Cells *In Vitro*

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Abstract

We previously observed that radial glial(RG)-like cells showed larger soma, thicker and longer neuritis in vivo(i.e., in rats) after fimbria-fornix (FF) transection, and in vitro RG-like cells showed the same results after the application of the extract from FF-transected hippocampus. In the present study, RG-like cells cultured in 24-well plates supplemented with 5% extract from FF-transected hippocampus were more likely to differentiate into neurons, compared with a normal group. After receiving the insulin-like growth factor 2 (IGF2) gene and knockdown or over expression viruses, the influence of IGF2 on the differentiation of RG-like cells into neurons and the expression of IGF2 were measured. Then, real-time polymerase chain reaction (real-time PCR), enzyme-linked immunosorbent assay (ELISA), and immunofluorescence were used to detect the differentiation of the RG-like cells into neurons. ELISA and real-time PCR showed that the expression of IGF2 in the IGF2 over expression (IGF2 OE) group was significantly higher compared to the normal group. However, the expression of IGF2 in the FF+IGF2 knockdown (FF+IGF2 KD) group was significantly lower than in the FF group. Moreover, the number of MAP2-positive neurons produced in the FF+IGF2 KD group, which expressed less IGF2, was significantly lower compared with the FF group, but the number of MAP2-positive neurons was significantly higher in the IGF2 OE group. The differentiation of RG-like cells into neurons was correlated with a significant increase in the expression of IGF2, indicating that IGF2 was an important regulatory factor for the extract to stimulate the differentiation of RG-like cells into neurons.

Keywords: Radial glia-like cells (RG-like cells); IGF2; Hippocampus; Neurons

Introduction

The hippocampal formation, including the dentate gyrus (DG), hippocampus and subicular complex is an important structure for learning, memory, and other cognitive functions. Cholinergic neuronal fibers of the medial septal nucleus (MS) and the nucleus of the vertical limb of the diagonal band (VDB) project to the DG mostly through the fornix hippocampal fimbria, if FF was transected, the animals would appear cognitive dysfunctional [1,2]. Many clinical diseases, such as traumatic brain injury [3,4], cerebral ischemia and hypoxia [5,6], hypoglycemia [7], epilepsy and multiple sclerosis [8], were not only occurred degeneration, apoptosis or necrosis of neurons in hippocampal formation, but also associated with nerve regeneration in hippocampal dentate gyrus.

The hippocampus of vertebrates and invertebrate is abundant with radial glial cells (RGCs) during embryonic development, as are the cerebral cortex and cerebellum, which together form a temporary network of RGCs. RGCs themselves, which have been suggested to be neural stem cells (NSCs) [9-12], are a special type of cells with protrusions that are thin, long and unbranched [10,11]. RGCs display astrocyte characteristics [13] and also express various astroglia markers, such as brain lipid binding protein (BLBP) and/or vimentin. Increasing evidence has shown that RGCs: (a) are the stent for the neuronal migration of embryonic cells; (b) have a NSCs-like effect in the late embryonic stage and/or after birth; and (c) can differentiate into neurons [14,15]. Since RGCs have some astroglia and stem/progenitor properties, they can generate new cells, such as astrocytes and progenitor cells, which can differentiate into neurons [16,17].

A recent study reported that proliferation of NSCs can be inhibited by insulin-like growth factor (IGF), even though IGF also can promote the differentiation of NSCs into neurons and promote the maturation of neurons [18]. Despite these and related functions, little research has been done on the effect of IGF2 in the central nervous system. IGF2 is an important signaling molecule of autocrine and paracrine in the central nervous system. Therefore, the effect of IGF2 and its receptor IGF2R on the differentiation of RGCs into neurons is worthy of investigation.

In previous research on hippocampal neurogenesis, we observed that NSCs differentiated into neurons in vivo (i.e., in rats) after fimbria-fornix transection [19]. We also observed that the number of RG-like cells in the DG increased, their somas were larger, and their neuritis were thicker and longer compared to their normal size in vivo. RG-like cells that were applied extract from FF-transected hippocampus in vivo also showed the same morphological results [20]. The RG-like cells in this environment exhibited changes in gene expression in addition to their morphological changes, including an 11-fold increase in IGF2 compared to the normal group, according to the results of microarray detection.

NSCs can differentiate into glial cells or neurons. However, under normal conditions, NSCs cultured in vitro, without extract, mainly give rise to glial cells and seldom give rise to neurons [21,22]. Therefore, investigating a method to control the differentiation of NSCs into neurons has become a focal point in neuroscience research. This study investigates the effect of IGF2 expression on the differentiation of RGCs into neurons.

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Materials and Methods

Animals and operations

Bilateral FF transections were performed on adult female Sprague-Dawley (SD) rats (approximately 220 g), a total of 6, as described by Hefti [23]. After anesthesia with chloral hydrate (2 ml/kg body weight, ip), the rats were transferred to a stereotaxic apparatus. When expose the anterior fontanelle, its coordinate of sagittal axis (A), coronal axis (L), vertical axis (V) was recorded. And then a hole was drilled in the skull between A_1=A–1.4 mm, L_1=L–4.0 mm and A_2=A–1.4 mm, L_2=L+4.0 mm according to atlas by Paxinos and Watson [24]. A wire-knife was lowered to a depth $V_{1\text{-}2}$=V+5.4 mm ventral to the dura and shifted laterally in the opening 3 times before the knife was slowly withdrawn from the brain. Fourteen days later, we extracted the hippocampus and made the transected hippocampal extract [25]. All experiments were carried out in accordance with the animal protection law.

IGF2 small interfering RNAs and Plasmids assay

Based on IGF2 gene sequences and the principle of siRNA sequence selection, small interfering RNAs (siRNAs) against rat IGF2 were designed and synthesized by Shanghai GenePharma Technology Co., Ltd., and its fragment sequences was 5′-GCTTCTACTTCAGCAGGCCTT-3′. And the NC-siRNA (no homology with IGF2 gene sequences) as a negative control also purchased from GenePharma.

About IGF2 overexpression virus, we used a GV287 lentiviral expression system (GenePharma company, Shanghai, China) to acquired lentivirus LV-IGF2 as manufacturer's instruction. The full length of IGF2 cDNA (NM_001190162) was obtained by RT-PCR using RNA isolated from the E16 rat hippocampus. The amplified product was inserted into the lentiviral vector GV287, then, the vector was transfected into 293T cells line. Two days later, viral supernatant LV-IGF2 was harvested and titer was determined (2×10^8 TU/ml). The negative control lentiviral vector LV-NC was also acquired from GenePharma company.

Cell culture

Pregnant SD rats were purchased from the experimental animal center of Nantong University, Nantong, China. We isolated neural stem cells (NSCs) from the hippocampus of fetal SD rats on embryonic day 16. After anesthesia with chloral hydrate (2 ml/kg body weight, ip), embryos were taken from the pregnant rats and the embryonic brains were isolated. After removal of the meningeal membranes and arteries the fetal hippocampus was isolated, and the tissue was triturated into single-cell suspensions by mechanical dissociation. The cells were then filtered through a 40 μm cell strainer (Biologix Research Company, KS). After that, the filtered cells were suspended at a density of 1×10^5 cells/ml in Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco, USA) containing 2% B27 (Gibco, Rockville, MD). The medium was supplemented with 20 ng/ml epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) (Sigma, China) as an NSC expansion medium at 37°C in a 5% CO_2/95% air humidified incubator (Jouan, Winchester, VA). Three to five days later, the neurospheres were formed and then subcultured with trypsin (Beyotime, China).

After expansion for 2 passages, the neurospheres were rinsed in DMEM/F12, then gently triturated into single cell suspensions with trypsin (Beyotime, China) and re-plated at a density of 1×10^5 cells per well onto poly-lysine-coated coverslips of NSCs expansion medium in 24-well plates (adherent conditions). The density for enzyme-linked immunosorbent test (ELISA), or real-time polymerase chain reaction (real-time PCR) should be 1×10^6 cells/well, counted by TC10™ Automated Cell Counter (Biologix Research Company, KS).

The molecular mass of IGF2 is very light, only 7.5 KD, and it is a secreted factor. Even after several preliminary experiments, it was difficult to detect the expression of IGF2 by western blot, so, ELISA was used to detect the proteins of IGF2, instead of the western blot.

After culture in an NSC expansion medium for 3 days, the RG-like cells were transferred to 6 different media: a DMEM/F12 medium supplemented with 5% extract from FF-transected hippocampus, 2% B27 and 2% fetal bovine serum (FBS) as the FF group; a DMEM/F12 medium supplemented with 5% extract from FF-transected hippocampus, 2% B27, 2% FBS and 8×10^5 transducing units (TU) IGF2 empty virus (GenePharma, Shanghai) as the FF+B group; and a DMEM/F12 medium supplemented with 5% extract, 2% B27, 2% FBS, and 8×10^5 TU IGF2 knockdown virus (GenePharma, Shanghai) as the FF+IGF2 KD group. Three other groups were as follows: DMEM/F12 medium supplemented with 2% B27, 2% FBS as the normal group; DMEM/F12 medium supplemented with 2% B27, 2% FBS and 8×10^5 TU IGF2 empty virus (GenePharma, Shanghai) as the blank group; and DMEM/F12 medium supplemented with 2% B27, 2% FBS and 8×10^5 TU IGF2 overexpression virus (GenePharma, Shanghai) as the IGF2 OE group. Half of the medium was replaced by fresh medium every 3 days. Ten days later, the RG-like cells were processed for PCR or immunofluorescence.

Real-time polymerase chain reaction (real-time PCR)

Total RNA was isolated and purified from the RG-like cells cultured in 24-well plates using UNIQ-10 Spin Column RNA Purified Kit (Sangon Biotech, shanghai). The first strand cDNA was synthesized according to the manufacturer's instructions of RevertAid First Stand cDNA Synthesis Kit (Thermo, Lithuania). The specific methods are as follows: add the following regents into a sterile, nuclease-free tube on ice: Total RNA (1 μg), Oligo(dT)18 Primer (1 μL), nuclease-free water (add to 12 μL), total volume is 12 μL. And then, add the following components to the tube in order: 5X Reaction Buffer (4 μL), RiboLock RNase Inhibitor (1 μL), 10 mM dNTP Mix (2 μL), RevertAid M-MuLV Reverse Transcriptase (1 μL), total volume is 20 μL. The next process carried out using PTC-100™ Peltier Thermal Cycler (MJR).

Then, the PCR amplification was performed as follows: add the following regents into a sterile, nuclease-free tube on ice: the sense and antisense primers (1 μL each), cDNA from control RT reaction (1 μL), FastStart Universal SYBR Green Master (Rox) (USA) (12 μL), nuclease-free water (10 μL). Total volume is 25 μL. Immediately after this, perform PCR in a thermal cycle: initial denaturation was carried out for 5 min at 95°C, and then, denaturation at 95°C for 15 sec, annealing at 52°C for 30 sec and extension at 72°C for 40 sec, for 40 cycles in order to achieve specific amplified products.

The sense and antisense primers were synthesized

as follows:

GAPDH 5′-GCAAGTTCAACGGCACAG-3′,

5′-GCCAGTAGACTCCACGACAT-3′;

IGF2 5′-CCTTCGCCTTGTGCTGCAT-3′,

5′-ACGGTTGGCACGGCTTGAA-3′;

IGF2R 5′-CCGTGTATCCTGAGCACTGAAA-3′,

5′-GTGAGGAGGTGGAGTAGGGAGA-3′. The PCR results were analyzed by software Primer5.0.

Enzyme-linked immunosorbent test (ELISA)

Protein that was isolated from the RG-like cells was cultured in 24-well plates using pancreatic digestive cells (Beyotime, China). Then, a BCA protein concentration measurement kit (Enhanced resistance) was used for protein quantification, and a Synergy2 multifunctional microplate reader was used to calculate protein concentration. Next, we used an IGF2 enzyme-linked immunosorbent assay kit (Shanghai enzyme-linked biological supplies Ltd.) for protein analysis. Firstly, the standard products should be diluted, and then the control wells, standard wells and the sample wells were set. In standard plates, added 50 μL Standard products; in sample wells, added 40 μL dilution and 10 μL test samples (the samples were diluted five-fold). Secondly, the wells were incubated for 30 min at 37°C, and then washing 3 times except the blank wells. The third step was added 50 μL ELISA reagent to each well, and then the wells were also incubated for 30 min at 37°C. After washed 3 times, adding color reagent A and B, the wells were incubated for 10 min at 37°C in dark conditions before adding the stop solution. Finally, the OD value was measured with a microplate reader at a wavelength of 450 nm.

Immunofluorescence assays

On the first day, the expansion medium was removed from the 24-well plates and washed once or twice for 3 min in phosphate-buffered saline (PBS). After this process, the RG-like cells were fixed in 4% paraformaldehyde for 20 min and then blocked with 10% normal goat serum for 2–4 h, before incubating the primary antibodies overnight at 4°C. On the second day, the cells were washed 3 times (10 min/time) in PBS before they were incubated with secondary antibodies conjugated to fluorescein 488 and 594 for 2 h. The RG-like cell nuclei were counterstained with Hoechst (Sigma, China) to count the total number of cells. The primary antibodies used to analyze the cells were rabbit anti-MAP2 (1:1,000, Millipore), and mouse anti-vimentin (1:200, Millipore). However, if the primary antibodies were omitted in immunocytochemistry, no immunoreactivity was detected. The cells were counted by morphological image analysis system version 1.0 provided by JeDa Technology.

Image processing and statistical analysis

Images were processed using Leica Qwin image processing and analysis software (Leica Imaging Systems, Cambridge, UK). Fluorescent signals were detected by Leica Qwin software (Leica, Germany). On each well, three visions were selected randomly, and the positive cells per field were counted. The statistical analysis was performed using GraphPad Prism 6. Comparison of differences between the experimental groups was performed using Student's t-test, and $P<0.05$ was considered statistically significant.

Results

The extract from FF-transected hippocampus can promote the differentiation of RGCs into neurons

In order to confirm the effects of the extract on the differentiation of the RG-like cells, the cultured hippocampal glial cells were seeded in 24-well culture plates, and then divided into the normal group and the FF group. After 2 weeks of cultivation, immunofluorescence was used to detect their differentiation. The number of MAP2-positive neurons in the cells that received the extract from FF-transected hippocampus was significantly higher compared with the normal group (*$P<0.05$) (Figure 1). The results suggest that after the FF was transected, some changes in the microenvironment of the hippocampus have a role in promoting the differentiation of RGCs into neurons.

IGF2 reduces the effectiveness of the extract to promote the differentiation of RG-like cells into neurons

We isolated NSCs from the hippocampus of 16-day-old fetal SD rats and formed floating proliferation neurospheres in vitro. In the 3 days after seeding 24-well plates, the RG-like cells exhibited the morphological features of RGCs [26].

First, the efficiency of infection was detected, using immunofluorescence. The highest efficiency of infection appeared when the virus titer was 8×10^5 TU, whether it was a knockdown virus or an overexpression virus. The efficiency of infection was more than 90% (Supplementary Figure 1). All of the experiments used this concentration.

Since the extract from FF-transected hippocampus could promote the differentiation of RGCs into neurons in vitro, and IGF2 was 11-fold increased in the extract. Then, what will be happened when IGF2 was been silent. We added 8×10^5 TU IGF2 knockdown virus to detect

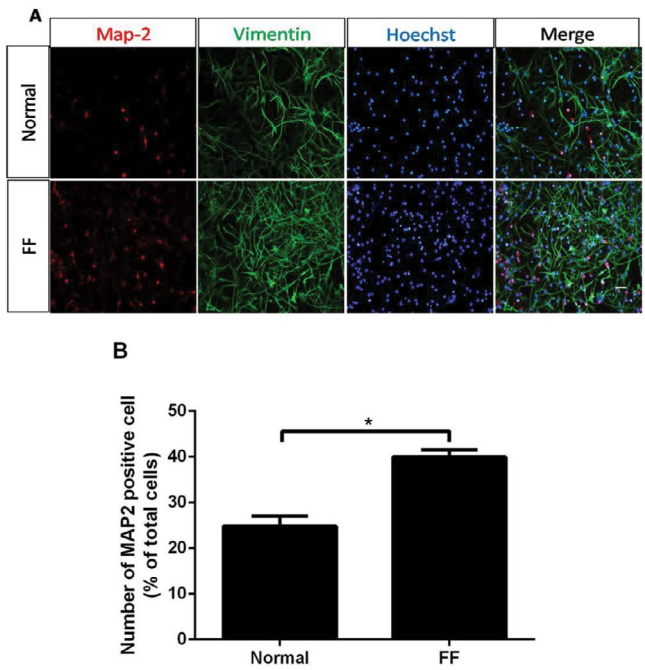

Figure 1: Differentiation of RG-like cells into neurons after adding the extract from FF-transected hippocampus. More MAP2-positive neurons occurred in the FF group compared with normal group: (A) quantification analysis of the number (B) of MAP2-positive neurons in the 2 groups (*P<0.05). Scale bar, 50 μm. apanicolaou-stained cells by LBC
Non-cancer cells: NHDF (a), COS-7 (b); cancer cells: LS-180 (c), MCF-7 (d), A549 (e), Kato-III (f).
The color of the nucleus of each cell, the intensity of purple was stronger in cancer cells than in non-cancer-derived cells. Regarding the color tone to distinguish non-cancer and cancer cells, it is difficult to express the cytological findings with purplish-red and bluish-purple tones, other than purple. Non-cancer-derived and cancer cells were similar with regard to many of the points in the above cellular findings.

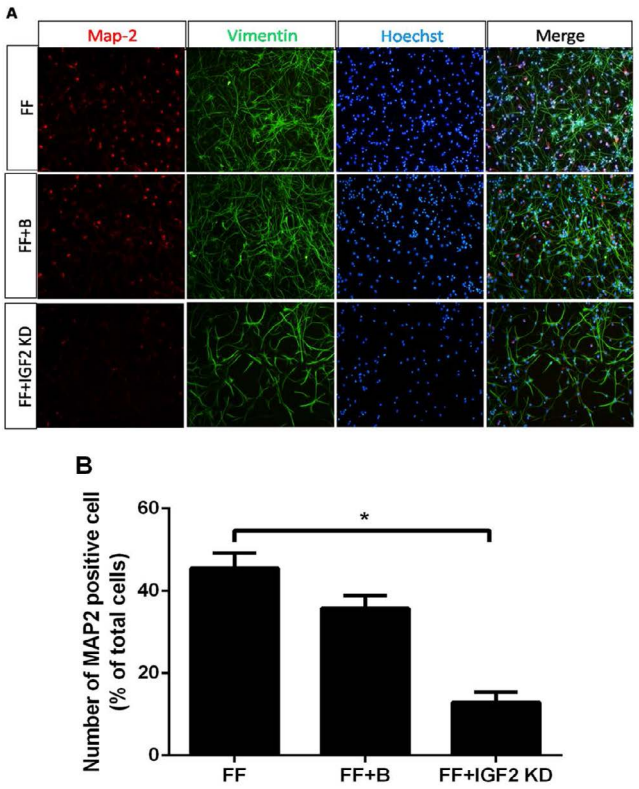

Figure 2: Differentiation of RG-like cells into neurons after adding IGF2 knockdown virus. Few MAP2-positive neurons were detected in the FF+IGF2 KD group compared to the FF group (A). The MAP2-positive cells were quantified on statistics, and the results showed that the number of MAP2-positive cells in the FF+IGF2 KD group was lower than in the FF group (*P<0.05) (B). Scale bar, 50 μm.

the differentiation again. Firstly, we used real-time PCR and ELISA analyses to detect the relative expression of IGF2 in each group. The results indicated that the expression of IGF2 in the FF+IGF2 KD group whether mRNA or protein was obviously lower than the FF group. However, the mRNA and protein expression of IGF2 in the FF+B group did not change compared with the FF group (Supplementary Figure 2).

Then, to determine the role of IGF2 on the differentiation of RGCs, immunofluorescence was used, and the results showed that the number of MAP2-positive neurons in the cells that expressed less IGF2 was significantly reduced compared to the FF group (*P<0.05) (Figure 2).

The ability of RG-like cells to differentiate into neurons was increased when IGF2 was overexpressed

The experimental results, so far, indicate that reduced expression of IGF2 can attenuate the differentiation of RG-like cells. However, it is not apparent whether differentiation of RG-like cells will be affected if IGF2 is overexpressed.

The real-time PCR and ELISA analyses showed that the mRNA and protein levels of IGF2 were significantly increased in IGF2 OE group compared to the normal group (Supplementary Figure 3). In contrast to the IGF2 knockdown experiments, RG-like cells in the IGF2 OE group showed a significant increase in MAP2-positive neurons (*P<0.05) (Figure 3).

Discussion

Most central nervous system (CNS) diseases, such as Alzheimer disease, are caused by the degeneration and eventual death of neurons, and the reduced ability of nerves to regenerate [27]. Previous studies indicated that embryonic RGCs can play a role as progenitors for various types of neurons in vitro [14,15]. Therefore, stem cell therapy offers a possible way to replace the neurons lost by injury or disease. Techniques used to isolate RGCs include fluorescence-activated cell sorting (FACS) [9] embryonic stem (ES) cell induction [28,29] and adherent culture. When cultured under adherent conditions and exposed to both EGF and bFGF, NSCs can differentiate into RGCs and resemble ES-cell-derived RGCs [30]. Although no specific marker of RGCs has yet been identified, these cells express multiple markers, such as vimentin and BLBP [31-33]. Research has shown that RGCs maintain a vimentin+ radial fiber throughout each stage of cell division [34] and that the expression of vimentin can be detected from E13 to P21 [35]. Therefore, we cultured RGCs in vitro under adherent conditions, and cells positive for vimentin that presented characteristic morphological features were identified as RGCs in this study.

Although the number of cells per well is the same at the beginning, the Hoechest test results are not the same. Because after adding extract, it has an effect of proliferation to RGCs [17], and IGF2 highly expressed in the extract could regulate proliferation of NSCs in vitro via AKT signaling [36]. Moreover, after adding the virus, it also has a certain impact on cell survival.

We extracted RNA from the RG-like cells in the FF group and the normal group, and then sent it to Biochip at Shanghai National Engineering Research Center for microarray detecting. The results

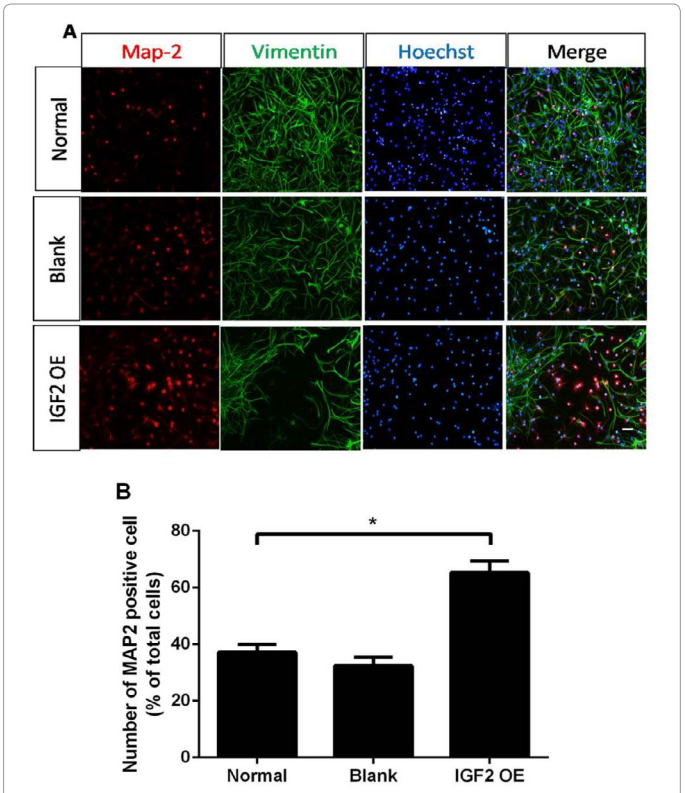

Figure 3: Differentiation of RG-like cells into neurons after adding IGF2 overexpressed virus. More MAP2-positive neurons occurred in the IGF2 OE group (A), quantification analysis of the number of MAP2-positive neurons in 3 groups (B). Scale bar, 50 μm.

showed that in the up-regulated genes, IGF2 related to the increased differentiation of NSCs into neurons and helped them mature, with the neuron count of the FF group being 11 times higher than the normal group. IGF2, which is composed of a single chain of 67 amino acids, is a central nervous system autocrine and paracrine signaling molecule. IGF2 is a multifunctional regulator of cell proliferation, and it plays an important role in promoting cell differentiation, the growth of embryos, and the proliferation of tumor cells [37].

In this study, we demonstrated that the extract from FF-transected hippocampus can significantly improve the potential of RG-like cells to differentiate into neurons, in vitro. However, when IGF2 in the cells was silent, this differentiation declined. In contrast, after making a simple overexpression of IGF2, we observed that more RG-like cells differentiated into neurons. Together, these results suggest that IGF2 was an important regulatory factor for the extract to stimulate the differentiation of the RG-like cells into neurons. However, further research is needed to clarify the mechanism by which IGF2 produces this effect.

Conclusion

We conclude that IGF2 expressed in the RG-like cells played a critical role in promoting the differentiation of RG-like cells into neurons, as well as the extract from FF-transected hippocampus in vitro. However, further studies are needed to determine how IGF2 promotes the differentiation of RG-like cells into neurons.

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