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Insulin-like growth factor-binding protein 7 is up-regulated during EAE and inhibits the differentiation of oligodendrocyte precursor cells

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ABSTRACT

Oligodendrocyte precursor cells (OPCs) differentiation failure is one of the leading causes for remyelination defects in the demyelinating lesions of multiple sclerosis (MS). In this study, we explored the roles of insulin-like growth factor-binding proteins 7 (IGFBP-7) on OPCs differentiation during experimental autoimmune encephalomyelitis (EAE). We first investigated the expression pattern of IGFBP-7 by real-time PCR and immunofluorescence staining. It showed that IGFBP-7 was expressed in astrocytes (ACs), oligodendrocytes (OLs) and neurons both in vitro and in vivo. The mRNA and protein level of IGFBP-7 was also increased in the spinal cord from mice at the peak of EAE disease. Next we found that IGFBP-7 acted as a negatively regulator of the OPCs differentiation. Together, these data suggest that IGFBP-7 was up regulated during EAE and inhibit the transition from OPCs to mature OLs, implying its use as a potential therapeutic target for the treatment of inflammatory demyelinating diseases.

1. Introduction

Oligodendrocytes are the myelin-forming cells in the central never system (CNS). Myelin damage caused by various internal and external factors may lead to demyelination and causes neuronal axon degeneration with consequent dysfunction [9,17]. Differentiation of oligodendrocyte precursor cells (OPCs) is a prerequisite for remyelination. Recent studies have shown that in many demyelinating diseases, including multiple sclerosis (MS), the impedance of OPCs differentiation and maturation is the leading cause of remyelination failure [2,19]. However, the precise underling mechanisms are not fully understood.

A number of extracellular signals and intracellular pathways have been reported in regulating OPCs differentiation [12]. Among them, insulin-like growth factor 1 (IGF-1) is one of the most important factors crucial for CNS development and OPCs maturation [3,22]. The bioavailability of the IGF-1 is partly controlled by the family of secreted insulin-like growth factor-binding proteins (IGFBPs), which can bind to IGFs therewith regulating the interaction of these ligands to their receptors [16]. Unlike the others (IGFBP-1-6) which are so called high-affinity IGF binders, IGFBP-7, whose affinity for IGF-1 is at least 100-fold lower [1], may be involved in functions independent of IGFs rather than regulating IGF activity. There is evidence that in several types of cancer, such as breast, brain, colon, lung, liver and pancreatic cancers IGFBP-7 usually act as a tumor suppressor [7,15]. However, IGFBP-7 presents a cell type-dependent diversity in function which can be either positive or negative on regulation of cells growth and proliferation. For example, IGFPB7 can promote anchorage-independent growth in malignant mesenchymal cells and in epithelial cells with an epithelial-to-mesenchymal transition phenotype [14], while it also induces senescence and apoptosis of human primary fibroblasts and melanocytes by inhibiting BRAF-MEK-ERK signaling pathway [18]. Weather IGFBP-7 is actively involved in the demyelination disease and regulating OPCs differentiation remains unknown. In the present study, an animal model of MS, experimental autoimmune encephalomyelitis (EAE) was introduced to investigate IGFBP-7 expression pattern. Furthermore in vitro pharmacological method was utilized to exam the effects of IGFBP-7 on OPCs differentiation.

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2. Materials and methods

2.1. Cells culture

CNS mixed glial cell cultures were generated and cultured in DMEM/F12 containing 10% fetal bovine serum (FBS, GBICO) (D10) and an antibiotic mixture (1% penicillin/streptomycin) (GIBCO) at 37 °C and 5% CO2 for 10 days. For the purification of microglia, cultures were shaken for 6 h at 180 rpm at 37 °C to collect microglia.

Purification and culture of OPCs were performed as described previously [21]. Briefly, mixed cortical glial cell were obtained from the cerebral cortex of newborn rat and cultured in DMEM with 10% FBS (D10). DMEM, Neurobasal medium and B27 were purchased from Gibco/Invitrogen. After 10–12 days microglia (MG) was removed by shaking the flasks on a rotary shaker at 160 rpm for 1 h at 37 °C. The supernatant was discarded and the flasks with fresh D10 were shaken at 200–210 rpm for an additional 16–18 h at 37 °C to separate OPCs from the cell monolayer. The OPCs were purified from contaminating microglia by seeding cell suspension and leaving it to adhere in uncoated petri dishes for 1 hat 37C and 5% CO2, and then cultured in Poly-L-lysin (sigma)-coated24-well plates. Six hours later, the culture medium was changed into neurobasal medium supplemented with 2% B27 (differentiation medium). To keep OPCs undifferentiated, differentiation medium was supplemented with 30% B104 conditioned media (proliferation medium). For differentiation assay, OPCs were first plated in proliferation medium for 1 day, and then in differentiation medium with IGFBP-7 (0, 50, 100, 200 ng/ml) or other cytokines premixed for 3 days before analysis. Serving as positive control in differentiation assay, 40 ng/ml triiodothyronine (T3) and 40 ng/ml thyroxine (T4) were added into the medium.

2.2. Immunocytofluorescence staining

For immunocytofluorescence analysis, OPCs or tissue slices on coverslips were washed with PBS and fixed with 4% paraformaldehyde (PFA) for 20 min, followed by permeabilization with 0.4% Triton X-100 in 0.1M PBS for 10 min. After blocking the nonspecific binding with 10% normal goat serum in 0.1 M PBS, cells were incubated with primary antibodies against primary antibodies at 4 °C overnight. Anti-IGFBP-7 antibody (Abcam), anti-GFAP antibody (Sigma), anti-MAG2 antibody (Sigma), anti-myelin basic protein antibody (Millipore). After washing, cells were incubated with second antibodies (Jackson immune Research laboratories, Inc.) and counterstain with Hoechst33342 (Sigma). Fluorescence images were captured by fluorescence (DXM1200,Nikon), and quantified with image-pro Plus (Media cybernetics).

2.3. Western blot analysis

Western blot was performed by standard protocol with proper antibodies. Briefly, OPCs were allowed to differentiate for 3 days before whole cell lysates were harvested. Cell lysates were subjected to western blot using primary antibodies as previously described. To control for differences in protein loading, membranes were also incubated with anti-GAPDH antibody (Sigma). After incubated with HRP-conjugated secondary antibodies, immunoreactive bands were visualized by chemiluminescence reagents (BIORAD).

2.4. TUNEL assays

Programmed cell death *in situ* was analyzed by specific labeling of nuclear DNA fragementation (TUNEL) using the *In Situ* Cell Death Detection Kit (Roche) according to the manufacturer's protocol. Briefly, the sample prepared as described for immunocytofluorescence were immersed in TUNEL reaction mixture and incubated at 37 °C for 60 min. Quantification of cell apoptosis was accomplished by counting the number of TUNEL and NG2-positive cells under a fluorescence microscope.

2.5. Real-time RT-PCR

For RT-PCR, total RNA was extracted from cultured cells using Trizol RNA isolation reagent (Invitrogen) according to the manufacturer's protocol. RNA from each sample was incubated with reverse transcriptase according to the manufacturer's protocol using random hexamer primers. Real-time PCR was done using IQ5 sequence detection systems (BioRad). Expression of GAPDH in cDNA samples was used to control for differences in the extraction and reverse transcription of total RNA. Each reaction was performed in 20 μl with 50% Sybgreen PCR MIX (TOYOBO), 400 nM each of the forward and reverse primer, and 200 nM probe. Condition for the PCR was 2 min at 50 °C, 10 min at 95 °C, and then 30 cycles, each consisting of 15 S at 95 °C, and 1 min at 60 °C. Relative expression values were calculated by dividing the expression level of the target gene *(IGFBP-7)* by the expression level of *GAPDH*. The following primers were used for real-time RT-PCR:

IGFBP-7 forward primer (TGGTTGATGCCATACACGAAA) and reverse primer (ATGTATTCGCAGGTTTATAGCTGA).

GAPDH forward primer (CCATCAACGACCCCTTCATT) and reverse primer (ATTCTCAGCCTTGACTGTGC).

MBP forward primer (ATCTCAGCATTTTGAAAGTTTAGGC) and reverse primer (ACCCAAAGTTAACAAGTACAGG).

HES1 forward primer (CTTCTGACGGACACTAAATACGAA) and reverse primer (CAACAAAGTAACCACCGACT).

HES5 forward primer (CCGCATCAACAGCAGCATTGAGCA) and reverse primer (TGCAGGCACCACGAGTAACCC).

Smad1 forward primer (GCGTTTACAATACAGACATAGGC) and reverse primer (TGCGAATAATGAACAGAGTTACCAG).

Smad4 forward primer (TGGCCTGATCTACACAAGAACGA) and reverse primer (ATATGGGTTCACACAGACGCTA).

Smad8 forward primer (TTGAATTGGTCCCCACTGCTT) and reverse primer (CAGGTCAGCGGCAAGTATCT).

2.6. Induction of EAE

C57BL/6J female (8–10 weeks) mice were subcutaneous immunization with 100 μg/mouse MOG35–55 (GLBiochem) in complete Freund's adjuvant containing heat-killed Mycobacterium tuberculosis (H37Ra strain; Difco). Mice were subsequently administered 500 ng pertussis toxin (Difco Laboratories) in PBS immediately after induction and again 48 h later.

2.7. Statistical analysis

All of the data are from at least three independent experiments and presented as the mean standard error. Statistical analysis was made using ANOVA or Student's t-test. All significant differences were considered at $P < 0.05$.

3. Results

3.1. Constitutive expression of IGFBP-7 in astrocytes, oligodendrocytes and neurons

To define the effect of IGFBP-7 on oligodendrocytes, the expression pattern of IGFBP-7 in CNS was examined. Analyses of IGFBP-7 expression in different cultured cells from CNS by Real-time PCR showed high expression in astrocytes and

oligodendrocytes, lower expression in neurons, and a lack of expression in microglia (Fig. 1*A*). Similar results were obtained by immunohistochemistry in sections derived from adult mice spinal cord (Fig. 1*B*). This was confirmed by immunohistochemistry using specific antibodies of astrocytes, oligodendrocytes and neurons double-labeled with IGFBP-7 (Fig. 1*C*). These results suggested that IGFBP-7 were expressed in the CNS.

3.2. Up-regulation of IGFBP-7 after EAE and cytokine stimulation

In the next step, we introduced an inflammatory demyelination model, Experimental Autoimmune Encephalomyelitis (EAE), to further study the expression of IGFBP-7 proteins in demyelination process by immunohistochemistry (Fig. 2*A*) followed by fluorescence intensity quantitative analysis (Fig. 2*B*). As we can see, the intensity of fluorescence (Fig. 2*A*) was much greater and stronger in acute active demyelinating lesions of EAE compared with the normal and chronic demyelinating spinal cord. Real-time PCR also showed that as the progress of demyelination IGFBP-7 was up-regulated, with highest mRNA level expressed at the peak of the disease (Fig. 2*C*). Interesting, this specifically up-regulation of IGFBP-7 came back to normal level during later phase. To assess whether this alteration was mediated by the inflammatory micro-environment, we performed a disparate cell cultures treated with different pro-inflammatory cytokines and tested the expression of IGFBP-7 after stimulation for 8 h. In astrocytes cultures, treatment with either LPS (100 ng/ml) or IFN-γ (100 ng/ml) significantly up-regulated IGFBP-7 mRNA expression (Fig. 2*D*). Additional, this increase of IGFBP-7 was also observed in neuron group treated with IFN-γ (Fig. 2*E*). In contract, it seems there were no significant changes in IGFBP-7 mRNA level when oligodendrocytes were treated with LPS or IFN-γ (Fig. 2*F*). These results were consistent with our hypothesis that the increased expression of IGFBP-7 was a result of inflammatory factor stimulation.

3.3. IGFBP-7 plays a negative role in regulating OPCs differentiation in vitro

To study IGFBP-7 function on OPCs progression, firstly we plated purified OPCs on coverslips in differentiation medium, and various

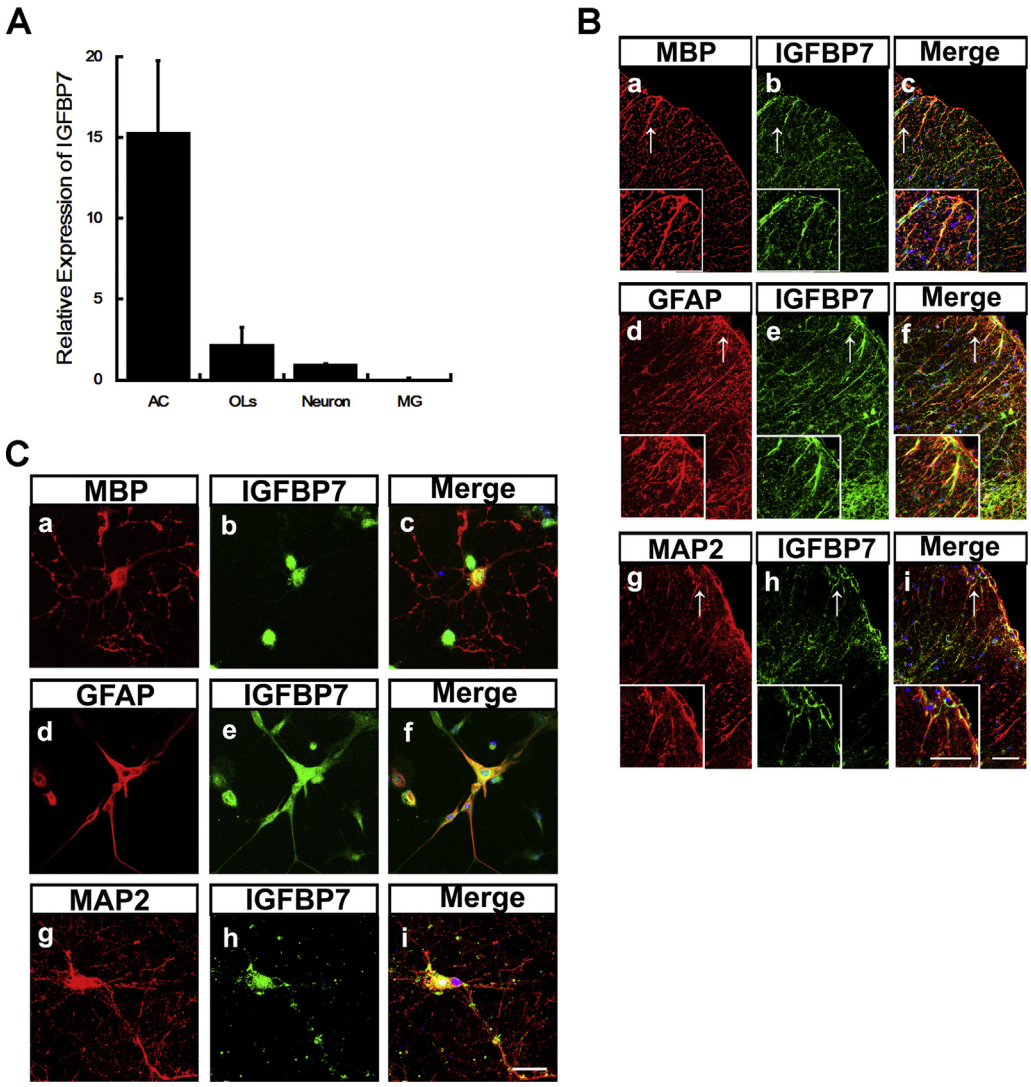

Fig. 1. IGFBP-7 is expressed in different lineage cell of CNS in vitro and in vivo. (A) Real-time PCR analyses in vitro show IGFBP-7 is expressed in different cultured cells of CNS. The expression in neurons is treated as an control(n = 5) (B) IGFBP-7 is expressed on MBP positive OLs (a–c), MAP2 positive neurons (d–f), and GFAP positive ACs (g–i) in the normal spinal cord by immunohistochemistry. Arrows indicate cells double-labeled by indicated marker. The square area at the bottom left corner is the enlargement of the arrow-pointed area. Scale bar, 50 μm(C) Immunohistochemistry of IGFBP-7 on MBP positive OLs (a–c), GFAP positive ACs (d–f), and MAP2 positive neurons (g–i) in vitro. Scale bar, 25 μm.

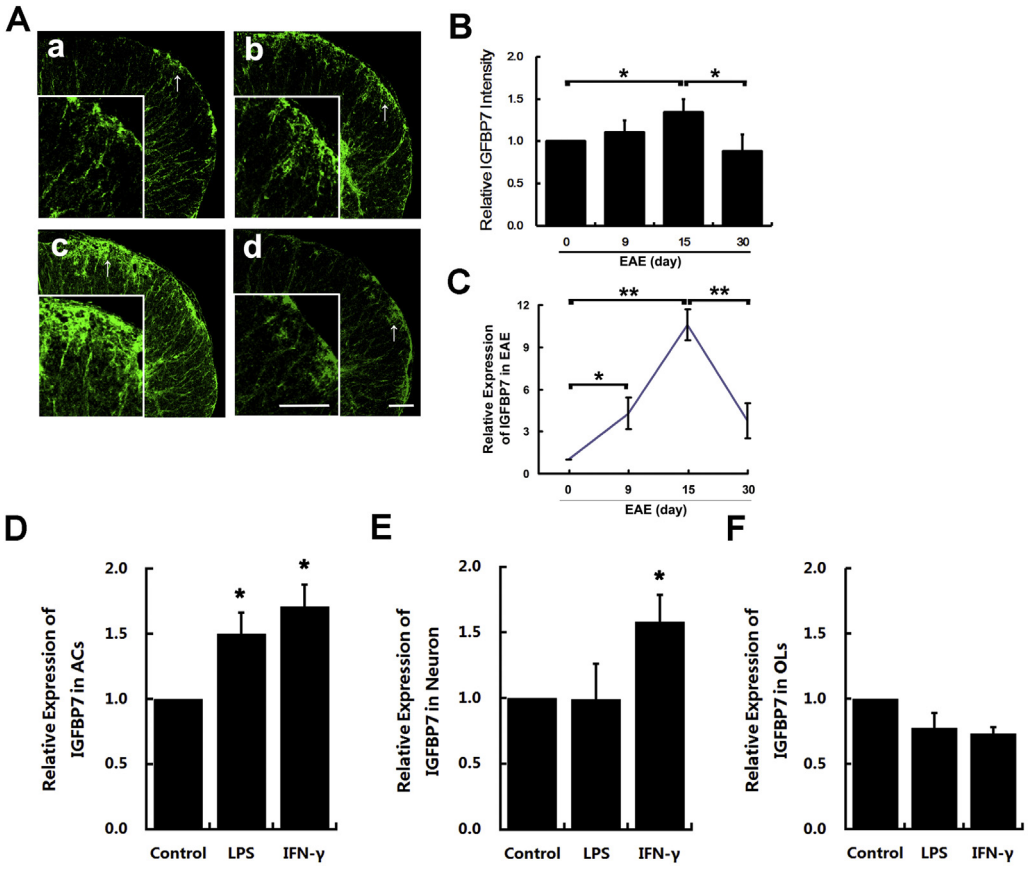

Fig. 2. IGFBP-7 expression is up-regulated during demyelination course of EAE. (A) Representative image of IGFBP-7 staining from different time-point of EAE. Image (a)–(d) represent day0, day9, day15 and day30 respectively. The square area at the bottom left corner is the enlargement of the arrow-pointed area. Scale bar, 50 μm. Arrows indicate cells labeled by IGFBP-7 (B) The quantification of relative IGFBP-7fluorescence intensity at the indicated time after of EAE (C) IGFBP-7 mRNA expressions within the spinal cord at the indicated time after EAE. RT-PCR show that the treatment of different inflammatory cytokines dramatically regulated IGFBP-7 expression in vitro (D)-(F)Represent ACs, neurons and OLs (n = 5). *P < 0.05; **P < 0.01 versus control.

concentrations (0, 50, 100, 200 ng/ml) of IGFBP-7 or T3/T4 were applied. After 3 days of differentiation, quantitative analysis of MBP protein by Western blot showed that IGFBP-7 treatment significantly decreased MBP expression compared with control group (Fig. 3*A,B*). To examine whether IGFBP-7 regulate the morphological progression of OPCs during differentiation in vitro, we performed an immunohistochemistry study on the differentiating OPCs. Consistent with the western blot analysis, after 48 h cultures, addition of 200 ng/ml IGFBP-7 resulted in less highly-differentiated oligodendrocytes demonstrated by the decrease in the percentage of MBP + oligodendrocytes, which was 30% lower compared with the controls (Fig. 3*C,D*). These results indicated that IGFBP-7 acted as a negatively regulator of the oligodendrocyte differentiation.

To test if IGFBP-7 could possible affect the survival of oligodendrocytes during the differentiation, TUNEL staining experiment was performed. Treatment of IGFBP-7 in different concentrations did not show statistically significant changes in the percentage of Tunel + cells (Fig. 3*E,F*). Thus, our data suggested that IGFBP-7 did not affect OPCs survival.

3.4. IGFBP-7 up regulated the expression of Hes5, an inhibitor in OPCs differentiation

To further investigate the cellular mechanism by which IGFBP-7 regulates OPCs differentiation, we examined the expression of several of transcription factors that have been reported to play negative effect on OPCs differentiation process. As shown (Fig. 4), IGFBP-7 treatment dramatically increased the expression of the Hes5 which has been reported to inhibit OPCs differentiation and myelin gene expression [10]. However, IGFBP7 has no significant effect in moderating the expression of other transcription factors, such as Smad1, Smas4, Smad5, Smad8, Hes1, ID2 and ID4.

4. Discussion

The aim of our study was to clarify the role of IGFBP-7 in the CNS remyelination, which is to the best of our knowledge, the first study on IGFBP-7 in demyelinating diseases.

Previous studies have demonstrated that IGFBPs were present in the brain such as in the choroid plexus [6], in which IGFBP-1 were predominantly localized on astrocytes while IGFBP-2 and IGFBP-3 were expressed in microglia [5]. In our research, we further showed that IGFBP-7 was expressed in three kinds of primary cells in the CNS. The great majority of IGFBP-7 came from astrocytes, followed by oligodendrocytes and neuron. We also detected the existence of IGFBP-7 in GFAP+, MBP+, and MAP2+ cells in the spinal cord in vivo. These results suggested that IGFBP-7 was broadly expressed in the CNS and provide an extracellular microenvironment for OPCs.

Demyelination is caused by various reasons such as genetics, infections, autoimmune reactions and other unknown factors [4]. Previous studies have demonstrated an up-regulation in cellular distribution of IGFBP-1 and IGFBP-2 in MS lesions and EAE model [11], while only minor difference of IGFBP-3 has been observed [5].

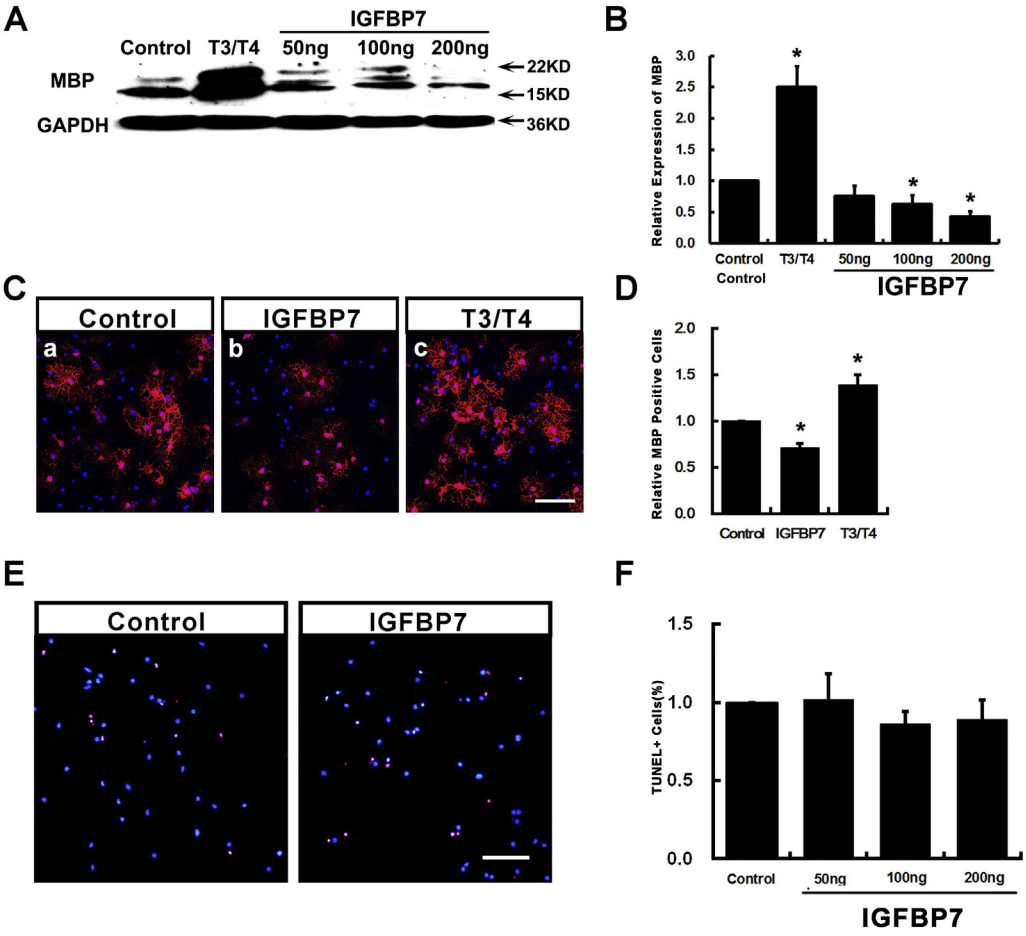

Fig. 3. IGFBP-7 prohibits myelin proteins expression during OPCs differentiation while does not affect the survival of OPCs in vitro. (A) Western blot analysis of the protein expression of MBP in the OPCs cultured 48 h in indicated condition. GAPDH expression was used as an internal control (B) Histogram shows the relative expression of MBP in indicated situation. Scanning densitometry was quantified and normalized to control(n = 3) (C) Representative MBP positive cells (red) in control group and IGFBP-7 treatment group cultured for 3 d. Scale Bars, 5 μm.The nuclei were stained by Hoechst (blue) (D) Quantification of the percentage of MBP positive cells in each category. Control group N = 5670 cells, IGFBP-7 treatment group N = 5675 cells, T3/T4 treatment group N = 6082 cells (E) Representative images showing the TUNEL + cells (red) in control (a) or IGFBP-7 (b) treated condition. The nuclei were stained by Hoechst (blue). The concentration of IGFBP-7 is 200 ng/ml, Scale bar, 5 μm(F) Quantification of the percentage of TUNEL + cells in indicated conditions. Control group N = 4987 cells, IGFBP-7 treatment group N = 5162 cells. Results were showed from 5 independent experiments. Cells were counted in at least 10 randomly selected fields from one coverslip and 3 coverslips for each group. *P < 0.05; **P < 0.01 versus control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

In the present study, we demonstrated that IGFBP-7 expression was increased during the period of inflammatory demyelination by quantitative analysis of immunofluorescence of IGFBP-7 in spinal cord from different phase of disease. The quantitative RT-PCR analysis also confirmed this alteration of IGFBP-7. These data suggested that IGFBP-7 expression levels might be correlated with the degree of inflammatory demyelination in vivo.

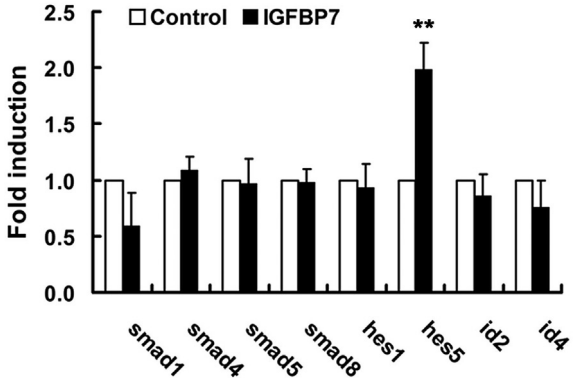

Fig. 4. Hes5 is up-regulated by IGFBP-7 treatment. Real-time PCR demonstrated the disparate effect of IGFBP-7 on the expression of different transcription factors during OPCs differentiation process (n = 3). *P < 0.05; **P < 0.01 versus control.

IGFBP-1, IGFBP-2 and IGFBP-6 have been shown to inhibit OPCs survival and differentiation in IGF-1-dependent or IGF-1-independent ways [11]. In the present study, by western blot analysis, we found that IGFBP-7 stimulation could significantly decrease the expression level of MBP protein. Consistently, there was also a reduction in the percentage of MBP + oligodendrocytes compared to the control showed by immunofluorescence staining. Furthermore, IGFBP-7 was found to have no significant effect on the survival of OPCs. Taken together, our result provides evidence that IGFBP-7 could suppress OPCs differentiation without affecting the survival.

What molecular mechanisms underlie the effects of IGFBP-7on OPCs? We proposed that IGFBP-7 could modulate transcription of target genes that regulate the differentiation and maturation of OPCs. Upon examination of the expression profile of large numbers of transcriptional factors been implicated in maintaining OPCs differentiation *(Samd1, Samd4, Samd5,Samd8, ID2, ID4, Hes1 and Hes5)*, we demonstrated that IGFBP-7 treatment could significant increase the expression of Hes5 in mRNA level. HES family

members (Hes1, Hes3 and Hes5) have been shown to be involved in modulating neural [13] and astrocytes differentiation [20] during embryonic development in mammalian cells. *Hes5* is induced by the ligand Jagged1 via activation of Notch1 receptors on oligodendrocyte precursors [10]. It has been reported Jagged1 is expressed at high levels by astrocytes, which can further induce the activation of Notch1 and Hes5 localized to OPCs leading to an immature oligodendrocyte phenotype in MS [8]. Here we proposed that there may exist an extra signaling pathway that local inflammation up regulated IGFBP-7 level in astrocytes and neurons then increased Hes5 expression in OPCs and inhibit oligodendrocyte maturation in inflammatory demyelinating diseases.

Thus we show here that IGFBP-7 is up-regulated in inflammatory demyelinating CNS and play a negative role in regulating OPCs differentiation, which maybe one of the reasons that causes remyelination defect in the inflammatory demyelinating lesions. In addition, it is unlikely that IGFBP-7 regulates oligodendrocytes survival. The genes that hinder oligodendrocytes-dependent myelination are various, and finally, the up-regulation of Hes5 implies that IGFBP-7 might inhibit OPCs differentiation via enhancing Dealt/Notch signaling. As such, the next challenge will be to identify the precise molecular mechanism involved in this process.

Conflict of interest

None.

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Transparency document

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