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Fibroblast growth factor-2 up-regulates the expression of nestin through the Ras–Raf–ERK–Sp1 signaling axis in C6 glioma cells

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ABSTRACT

Nestin is a 240-kDa intermediate filament protein expressed mainly in neural and myogenic stem cells. Although a substantial number of studies have focused on the expression of nestin during development of the central nervous system, little is known about the factors that induce and regulate its expression. Fibroblast growth factor-2 (FGF-2) is an effective mitogen and stimulates the proliferation and differentiation of a subset of nestin-expressing cells, including neural progenitor cells, glial precursor cells, and smooth muscle cells. To assess whether FGF-2 is a potent factor that induces the expression of nestin, C6 glioma cells were used. The results showed that nestin expression was up-regulated by FGF-2 via *de novo* RNA and protein synthesis. Our RT-PCR results showed that C6 glioma cells express FGFR1/3, and FGFRs is required for FGF-2-induced nestin expression. Further signaling analysis also revealed that FGF-2-induced nestin expression is mediated through FGFR–MAPK–ERK signaling axis and the transcriptional factor Sp1. These findings provide new insight into the regulation of nestin in glial system and enable the further studies on the function of nestin in glial cells.

1. Introduction

Nestin, also known as IFAP-70/280 kDa for hamster nestin, is a type VI intermediate filament (IF) protein predominantly expressed in neurogenic and myogenic precursors [1–6]. During central nervous system (CNS) development, the expression of nestin in the gliogenic progenitors gradually decreases and is replaced by glial fibrillary acidic protein (GFAP) as they differentiate into mature astrocytes [7–9]. The expression of nestin ceases in the rat CNS within one week after birth and is absent in normal adult rat astrocytes, but it reappears in reactive astrocytes upon tissue injury [10–12]. Although nestin has been widely used as a marker for neural and muscular progenitor cells, its extracellular inducer and regulation mechanism in glial system have not been elucidated.

Our previous studies revealed that in primarily cultivated rat aortic smooth muscle cells (RASMC), nestin expression is induced by epidermal growth factor (EGF) and thrombin (Thr)-induced EGF receptor transactivation [13,14]. In addition, another research group showed that platelet-derived growth factor (PDGF) also induces the expression of nestin in RASMC [15]. In the current study, among the growth factors we tested, including fibroblast growth factor 2 (FGF-2), EGF, Thr, PDGF, and thrombomodulin (TM), FGF-2 was the most effective inducer of nestin expression in rat C6 cells, a widely used model cell line of astroglial cells. Herein, we showed that nestin expression is induced by FGF-2 through FGF receptors (FGFR) in C6 cells, which expressed FGFR1/3. In addition, the FGF-2-induced nestin expression requires both *de novo* RNA and protein synthesis. Further signaling analysis showed that blockage of the downstream Ras/Raf, ERK, or transcriptional factor Sp1 activity by their specific inhibitors abolished the FGF-2-induced nestin expression. This is the first report to resolve the inducing factor, FGF-2, of nestin in cells of glial lineage. In addition, the downstream signaling mechanism is deciphered. This report, together with previous studies in RASMC, indicates that nestin expression is regulated by different extracellular signaling molecules among various cell types.

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2. Materials and methods

2.1. Cell culture

C6 cells were cultivated in dulbecco's modified eagle medium (DMEM) (Gibco-Invitrogen, USA) supplied with 10% of fetal bovine serum (FBS) and 1% penicillin/streptomycin at pH 7.4. Cells were maintained at 37 °C in a humidified incubator supplied with 95% air and 5% CO_2.

2.2. Establishment of C6 glioma subclones by limiting dilution

Single-cell clones of C6 cells were established by serial dilution method. Homogeneity of each clone was confirmed by immunofluorescence microscopy using anti-nestin antibody (Pharmingen San Diego, CA).

2.3. Whole cell extraction and western blotting analysis

Whole cell lysates were obtained as previously described [14]. The denatured whole cell lysates (20 μg) were separated by SDS–polyacrylamide gel electrophoresis and processed for the immunoblotting assay with anti-nestin antibody and with anti-β-actin antibody as loading control.

2.4. Immunofluorescence microscopy

Immunofluorescence microscopy was performed as described with a few modifications [13,16]. The methanol-fixed cells were immunolabeled with anti-nestin (1:500). DAPI was used for counterstaining cell nuclei. The preparations were examined using a Zeiss Axioplan2 photomicroscope equipped with epifluorescence optics and photographed using an AxioCam HRm CCD (Carl Zeiss, Inc., Oberkochen, Germany).

2.5. RT-PCR

Total RNA was isolated using RNeasy® Mini kit (QIAGEN, Valencia, USA). The purified RNA (1 μg) was subjected to reverse transcription with RevertAid Premium reverse transcriptase (Fermentas). PCR with *Taq* DNA Polymerase Master Mix RED (Ampliqon) was conducted with specific sets of oligonucleotides (primer sequences are described in Supplementary Methods). The amplification was performed in 35 cycles at 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min.

2.6. Nuclear extraction and electrophoretic mobility shift assay (EMSA)

The EMSA assay was performed using a gel-shift kit (Panomics, Fremont, USA) according to the manufacturer's instructions. The sequence of double-stranded oligonucleotides probes for AP-1, Sp1, and Pit1 used was previously described [14].

2.7. Densitometry and statistical analysis

Data were collected using densitometric analysis of Quantity One quantification software package (Bio-Rad Laboratories, Inc., USA). In each sample, the intensity of the band corresponding to nestin was standardized to that of β-actin. All the assays were performed in at least three independent experiments. Data were expressed as mean ± standard error of the mean (SEM). Two-tailed, unpaired, Student's *t* test was used to evaluate the difference among the treatments or between the treatment and the control. A value of $p < 0.05$ was designated as $*$, $p < 0.01$ was designated as $**$, and $p < 0.001$ was designated as $***$ in figures, and $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Subclones of C6 cells were established based on their nestin expression levels

In native C6 glioma cells, various nestin expression levels were observed among different subpopulations (Supplementary Fig. 1A), suggesting that this cell line comprises a heterogeneous population with respect to the regulation of nestin expression. To obtain cells with homogenous nestin expression, single-cell subclones of C6 glioma cells were generated by limiting dilution cloning. Based on the results of immunofluorescence microscopy, three types of C6 clones were established, including two clones expressing high levels of nestin (B2 and B8), two clones expressing medium levels of nestin (D6 and B11), and one clone expressing low levels of nestin (C11) (Supplementary Fig. 1B–D).

3.2. FGF-2 induces nestin expression in serum-deprived C6 cells

Among these five clones, the expression of nestin is depleted in the D6 and C11 clones upon serum deprivation for 48 h (Fig. 1A, Supplementary Fig. 2D–F). Nestin expression decreased most drastically in cells of D6 clone, which expressed nestin at medium levels (Fig. 1A). In addition, further time-course experiments showed that nestin expression of cells of D6 clone gradually decreased upon serum deprivation, and its expression level was halved (0.47 ± 0.07 folds of 0 h) after treatment for 48 h (Fig. 1B), as revealed by immunoblotting assay. Cells of D6 clone were therefore selected as representative for the following experiments. It is interesting to note that the expression of nestin was not down-regulated upon serum deprivation in the clones expressing high levels of nestin (i.e., B2 and B8), as revealed by immunofluorescence microscopy and immunoblotting assay (Fig. S3A–F).

To identify the factors responsible for inducing nestin expression, D6 cells were subjected to serum deprivation for 24 h, followed by another 24 h treatment of FGF-2, EGF, PDGF, Thr, or TM. For control, D6 cells were subjected to serum deprivation for 48 h (designated as untreated, UT). Among all these treatments, FGF-2 was the most effective in inducing nestin expression (3.49 ± 0.42 folds of UT) (Fig. 1C, Supplementary Fig. 4 and 5). Furthermore, the induction of nestin expression by FGF-2 was dose dependent, as shown by the immunoblotting results (Fig. 1D). To further confirm that FGF-2 is responsible for the induction of nestin expression, serum-deprived D6 cells were pretreated with various concentrations of FGF-2 neutralizing antibody (1–20 μg/ml) for 1 h, followed by 24 h treatment of 10 ng/ml FGF-2. The immunoblotting results showed that the nestin expression of D6 cells was abolished by treatment of 20 μg/ml FGF-2 neutralizing antibody (Fig. 2A). In addition, nestin expression in the serum-deprived B11 and C11 cells was also induced by FGF-2, as shown by immunofluorescence microscopy and immunoblotting assay (Fig. S2A–F). It is interesting to note that FGF-2 did not affect the expression of nestin in the serum-deprived B2 and B8 cells, which express high levels of nestin (Fig. S3A–F).

3.3. FGF-2-induced nestin expression is mediated by fibroblast growth factor receptors

FGFs bind to FGFRs and initiate the downstream signaling cascade [17]. To confirm that the induction of nestin expression in D6 cells by FGF-2 is mediated through FGFRs, the pan-FGFR inhibitor (SU5402) was applied to the serum-deprived D6 cells. The

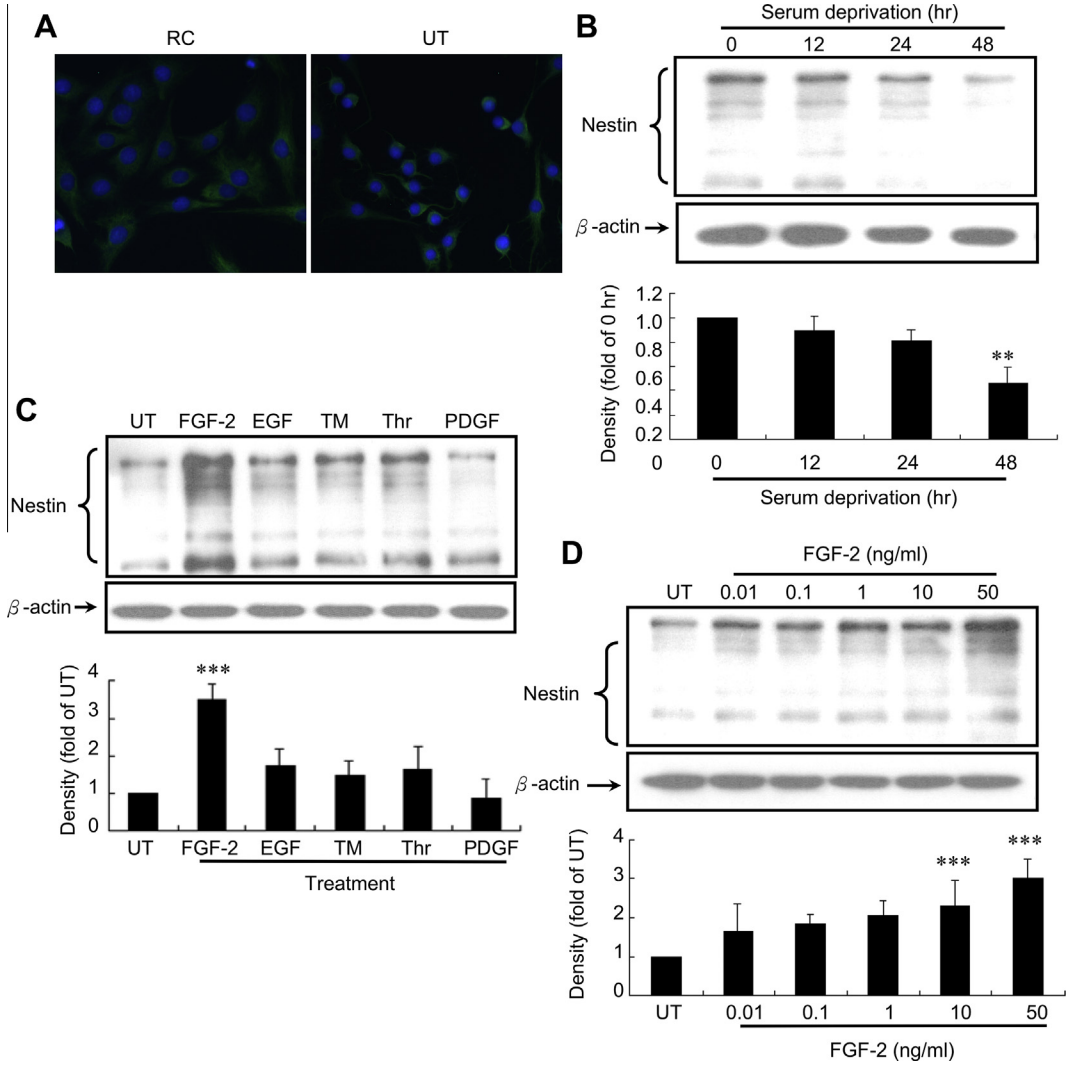

Fig. 1. Stimulation of nestin expression by FGF-2 treatment in D6 clone of C6 glioma cells. (A) Cells of D6 clone were cultivated in serum-containing medium (RC, left panel) or serum-deprived medium for 48 h (UT, right panel) and singly labeled with anti-nestin. (B) Cells of D6 clone were cultivated in serum-deprived medium for various durations (from 0 to 48 h). Data were shown as percentages of the serum-containing medium control (100% at 0 h). Results were shown as mean ± SEM (n = 3). (C) Cells of D6 clone were cultivated in serum-deprived medium for 24 h and followed by treatments for another 24 h in serum deprived medium supplied with different reagents, i.e., 50 ng/ml of FGF-2, 50 ng/ml of EGF, 1 μg/ml of TM EGF-like domain, 1 unit/ml of thrombin, or 1 nM of PDGF. Nestin expression was assessed by immunoblotting. Data were shown as folds of untreated control (1.0 for UT). Results were shown as mean ± SEM (n = 4). (D) Serum-deprived D6 cells were treated with various concentrations of FGF-2 in serum-deprived medium for 24 h, showing the FGF-2 dosage responses on nestin expression. UT was used as control. Results were shown as mean ± SEM (n = 3). For immunofluorescence, nuclei were counterstained with DAPI. Scale bar: 50 μm. RC, regular serum-containing condition; UT, untreated.

immunoblotting experiments showed that SU5402 at the concentration of 5 μM abolished the FGF-2-induced nestin expression of serum-deprived D6 cells (Fig. 2B). In addition, SU5402 also abolished the FGF-2-induced nestin expression of serum-deprived B11 and C11 cells (Fig. S2B, C, E, and F). To date, four FGFRs, namely FGFR1, FGFR2, FGFR3, and FGFR4, were shown to be activated by FGF-2 [18,19]. To examine the expression profile of FGFRs in D6 cells, RT-PCR was carried out with primer sets that specifically amplify each FGFR. The results showed that only the mRNAs of FGFR1 and FGFR3 were detected among the four FGFRs (Supplementary Fig. 6). This result suggested that the induction of nestin expression in D6 cells by FGF-2 is mediated through FGFR1/3.

3.4. FGF-2 induces nestin expression via de novo RNA and protein synthesis

To determine whether transcriptional and translational activities are necessary for the FGF-2-induced nestin expression, serum-deprived D6 cells were pretreated for 1 h with various concentrations of either Actinomycin D (Act-D), a transcriptional inhibitor, or cycloheximide (CHX), an inhibitor of protein synthesis. These cells were then treated with 10 ng/ml FGF-2 in serum-deprived medium for 24 h. Immunoblotting results showed that Act-D and CHX treatments inhibited the FGF-2-induced nestin expression at the concentration of 0.1 μg/ml and 1 μg/ml, respectively (Fig. 2C and D), indicating that the FGF-2-induced nestin expression requires both *de novo* RNA and protein synthesis.

3.5. FGF-2 induces nestin expression through the RasRaf–ERK signaling axis

It is known that the FGF-2-FGFR signal transduction leads to PI3K–Akt, PLCγ–PKC, and Ras–Raf pathways [17,20]. To determine the signaling pathways underlay the FGF-2-induced nestin expression, specific pharmacological inhibitors were used. The immunoblotting results showed that treatment with a Raf-1 inhibitor, GW5074, inhibited the FGF-2-induced nestin expression in a concentration-dependent manner, and at the concentration of 5 μM,

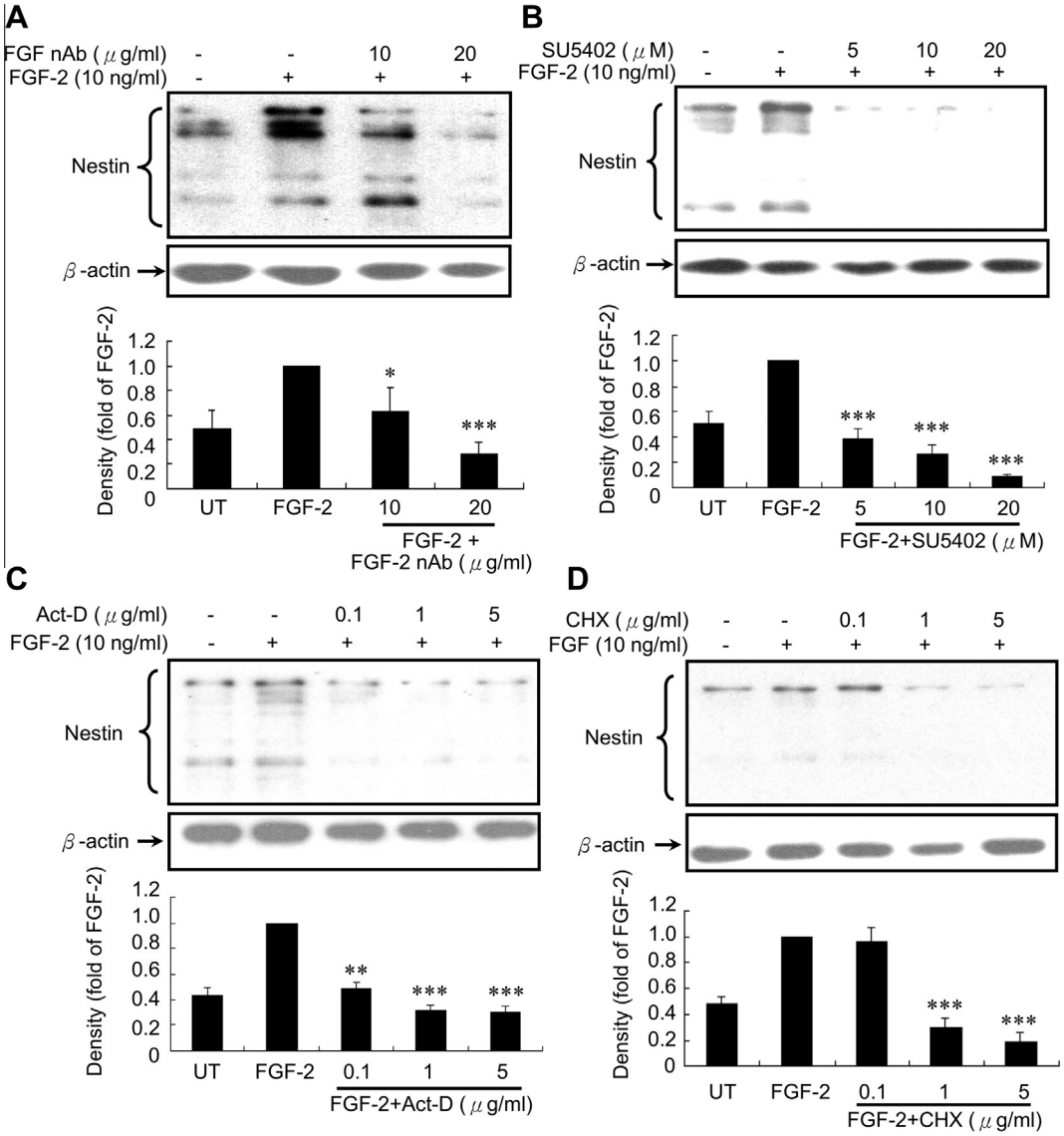

Fig. 2. FGF-2-induced nestin expression via FGF-2-FGFR and *de novo* RNA and protein synthesis. Cells of D6 clone were serum-deprived for 24 h, preincubated with various concentrations of either FGF-2 neutralizing antibody (A), pan-FGFR inhibitor SU5402 (B), mRNA synthesis inhibitor Act-D (C), or protein synthesis inhibitor CHX (D) for 1 h, and followed by cotreatment with 10 ng/ml FGF-2 for another 24 h. Nestin expression was assessed by immunoblotting with anti-nestin. Cells treated with only FGF-2 were used as control (set at 1.0). Data were shown as folds of control. Results were shown as mean ± SEM ($n = 3$). UT, untreated; FGF-2 nAb, FGF-2 neutralizing antibody; Act-D, actinomycin D; CHX, cycloheximide.

this inhibitor abolished the FGF-2 effects (Fig. 3A). In addition, the involvement of PLCγ–PKC and PI3K pathways was examined by applying their specific inhibitors U73122 and LY294002, respectively. The results showed that both U73122 and LY294002 did not attenuate the FGF-2-induced nestin expression, as revealed by immunoblotting assay (Fig. 3B and C). These results indicated that Ras–Raf is the main pathway involved in the FGF-2-induced nestin expression of D6 cells, but not PLCγ–PKC or PI3K–Akt pathways.

Previous studies revealed that Ras-activated mitogenesis is mediated through mitogen-activated protein kinases (MAPKs), including ERK1/2, p38, and JNK [21]. To clarify the involvement of each MAPK, specific pharmacological inhibitors were tested. Treatment with U0126, a MEK-1 inhibitor that blocks ERK1/2 activation, suppressed the FGF-2-induced nestin expression in a concentration-dependent manner and abolished FGF-2 effects at 10 μM, as shown by immunoblotting assay (Fig. 3D). On the other hand, the expression of nestin was not altered in the presence of SB203580, a p38 specific inhibitor, or SP600125, a JNK specific inhibitor (Supplementary Fig. 7A and B). Taken together, these results indicated that the FGF-2-induced nestin expression is mediated through the Ras–Raf–ERK signaling axis in D6 cells. It is of interest to note that the FGF-2-induced nestin expression was also attenuated by treatment with U0126 in the B11 and C11 clones of C6 cells (Fig. S2B, C, E, and F).

3.6. Sp1 is involved in the FGF-2-induced nestin expression

The promoter region of nestin was shown to contain several binding sites for various transcriptional factors (TFs), including Sp1, AP-1, and Pit-1 [22,23]. Our previous research showed that Sp1 is involved in the thrombin-induced nestin expression in RASMC [14]. To determine whether Sp1 participates in the FGF-2-induced nestin expression of D6 cells, nuclear proteins were extracted from FGF-2-treated D6 cells and subjected to electrophoretic mobility shift assay (EMSA) experiment using the biotinylated probe corresponding to the promoter binding sequence of Sp1. For control, nuclear proteins from D6 cells subjected to serum deprivation for 48 h were examined. The results showed that the shifted band was only observed in the

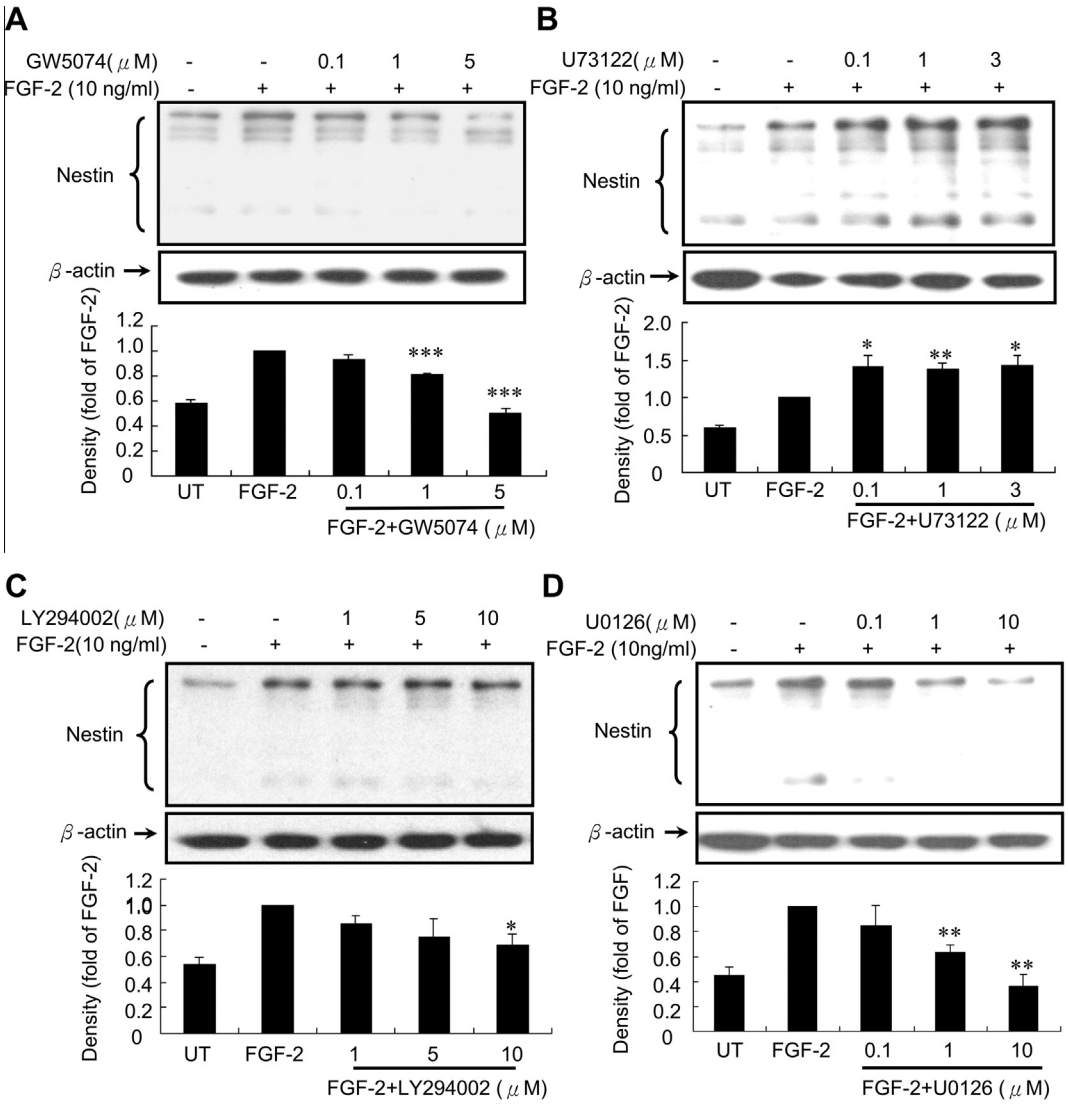

Fig. 3. FGF-2-induced nestin expression through Ras–Raf–ERK signaling pathways. Cells of D6 clone were serum-deprived for 24 h, pre-incubated with various concentrations of Raf-1 inhibitor GW5074 (A), PLCγ–PKC inhibitor U73122 (B), PI3K inhibitor LY294002 (C) or MEK-1 inhibitor U0126 (D) for 1 h, and followed by cotreatment with 10 ng/ml FGF-2 for another 24 h. Nestin expression was assessed by immunoblotting with anti-nestin. Cells treated with only FGF-2 were used as control (set at 1.0). Data were shown as folds of control. Results were shown as mean ± SEM ($n = 3$). UT, untreated.

FGF-2-treated samples. In addition, this FGF-2-induced Sp1-DNA binding was significantly attenuated by co-treating with the MEK-1 inhibitor, U0126 (Fig. 4A). The shifted bands were not observed with either the biotin-labeled AP1 or the Pit-1 probes following FGF-2 treatment (Fig. 4B).

To further confirm that Sp1 is required in the FGF-2-induced nestin expression, serum-deprived D6 cells were pre-treated for 1 h with Mithramysin A, an Sp1-specific inhibitor, followed by FGF-2 treatment for 24 h. The immunoblotting results revealed that Mithramycin A inhibited the FGF-2-induced nestin expression in a concentration-dependent manner and abolished the expression of nestin at 1000 nM (Fig. 4C). Taken together, these results indicated that FGF-2 induces nestin expression through the binding activity of Sp1. It is of interest to note that in clones of B11 and C11, the FGF-2-induced nestin expression was also abolished by Mithramycin treatment (Fig. S2B, C, E, and F).

4. Discussion

The intermediate filament cytoskeleton is composed of more than 70 subunit proteins which are expressed in a tissue-specific and differentiation stage-specific manner [24]. The main function of IFs is widely known for providing mechanical support to cells of multicellular organisms. Recently, studies have indicated a non-mechanical cytoprotective role of IFs in which they could act as tissue-specific scaffolds to organize and modify signaling pathways for cell survival, migration, and the sequestering of stress-activated kinases [25]. However, little is known about the gene regulation of IF proteins, which have been a focus of recent researches. We previously reported that nestin is induced extracellularly by EGF, as well as Thr via the EGFR transactivation, in RASMC [13,14]. A later study revealed that PDGF is another inducer of nestin expression in the same cells [15]. Still, the regulation of nestin expression in cells derived from neural tissue remains elusive. Herein, our results showed that FGF-2 is a potent factor in inducing nestin expression in C6 glioma cells. This current study is the first report that resolves the regulatory mechanism of nestin in glial cells.

Previous studies showed that nestin expression in RASMC is induced by EGF, PDGF, and Thr [13–15]. In this present study, the above-mentioned mediators, as well as TM and FGF-2 were applied to investigate their effects on nestin expression. The results re-

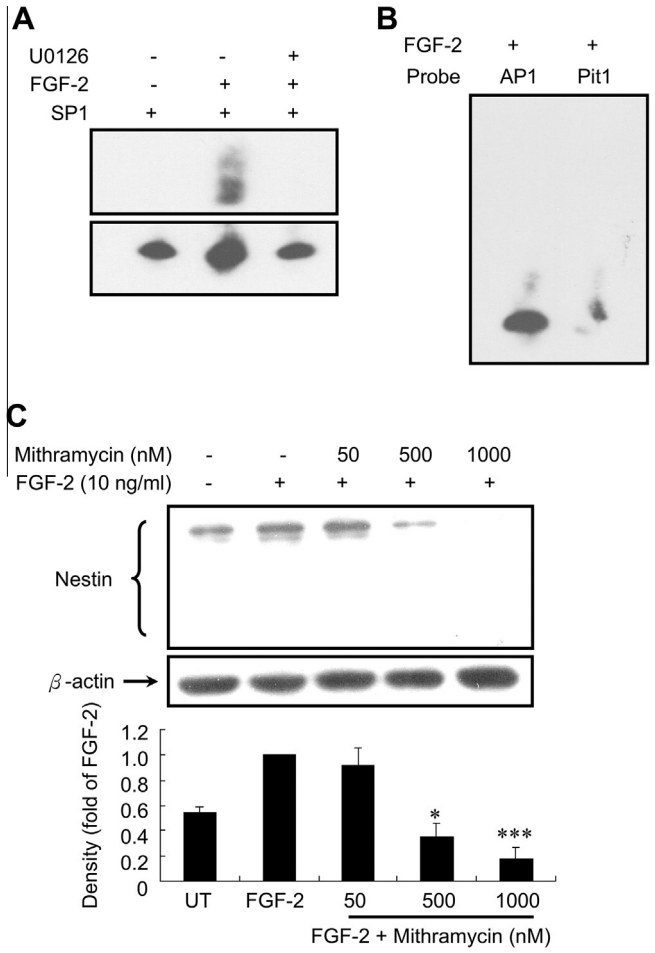

Fig. 4. FGF-2-induced nestin expression via ERK-Sp1. (A) Cells of D6 clone were serum-deprived for 48 h (lane 1), serum-deprived for 24 h followed by the treatment with 10 ng/ml FGF-2 for 24 h (lane 2), or serum-deprived for 24 h, preincubated with 10 μM U0126 followed by another 24 h of 10 ng/ml FGF-2 treatment (lane 3). Nuclear proteins were then extracted and analyzed by EMSA using the biotin-labeled Sp1 oligonucleotide probe. (B) Cells of D6 clone were serum-deprived for 24 h followed by the treatment with 10 ng/ml FGF-2 for 24 h. Nuclear proteins were then extracted and analyzed by EMSA using either the biotin-labeled AP-1 (lane 1) or Pit-1 (lane 2) oligonucleotide probe. (C) Cells of D6 clone were serum-deprived for 24 h, preincubated with various concentrations of mithramycin A for 1 h, and followed by cotreatment with 10 ng/ml FGF-2 for another 24 h. Nestin expression was assessed by immunoblotting with anti-nestin. Serum-deprived cells treated with FGF-2 were used as control. Data were shown as folds of control. The results were shown as mean ± SEM ($n = 4$). UT, untreated.

vealed that among these potential mediators, only FGF-2 significantly induced nestin mRNA and protein expression in serum-deprived C6 glioma cells (Supplementary Fig. 4 and Fig. 1C). This result is further confirmed by the fact that the FGF-2-induced nestin expression was abolished by the FGF-2 neutralizing antibody (Fig. 2A).

FGF-2 is one of the best characterized members of the FGF family in the CNS. It plays an important role in the proliferation, migration, and maturation of astrocytes [26–29]. It is well known that FGF-2 exerts its activity through FGFR, and the activation of FGFR leads to the phosphorylation of specific cytoplasmic tyrosine residues, which subsequently trigger the activation of cytoplasmic signal transduction pathways. FGFRs comprise a family of four receptor tyrosine kinases designated as FGFR1 to FGFR4 [17,30]. In the CNS, FGFR1-3 can be detected while FGFR4 is absent. Among FGFR1-3, astrocytes mainly express FGFR1 and FGFR3, whereas FGFR2 expression is restricted to cells of the oligodendroglial lineage. [31,32]. In agreement with these previous studies, our RT-PCR result revealed that mRNA of FGFR1 and FGFR3 were detected in C6 glioma cells (Supplementary Fig. 6). Treatment with the pan-FGFR inhibitor SU5402 in C6 cells abolished the FGF-2-induced nestin expression (Fig. 2B). In addition, our preliminary RNAi data revealed that knockdown of either FGFR1 or FGFR3 in C6 glioma cells attenuated the FGF-2-induced nestin expression (unpublished data). Our further examination showed that FGF-2-induced nestin expression in C6 glioma cells was abolished by treatment with Raf-1 inhibitor GW5074 and MEK-1 inhibitor U0126 (Fig. 4A and D). Taken together, these results confirmed that the FGF-2-induced nestin expression is mediated mainly through FGFR1/3 and the downstream Ras–Raf–ERK activation.

Cheng et al. reported that the minimal promoter of nestin gene resides in the region −11 to +183 of 5′ non-coding region, as shown by serial deletion of the nestin promoter [22]. In the same study, the EMSA experiment also demonstrated that the activation of nestin promoter requires two-adjacent Sp-1-binding sites, but not AP-1 or AP-2, in mouse embryonic carcinoma P19 cells [22]. In our current study, the EMSA results showed that the Sp1-DNA binding ability, but not AP-1 or Pit-1, increased upon the FGF-2 treatment, and this binding ability was prevented by treatment with MEK-1 inhibitor U0126 (Fig. 4A and B). Moreover, the FGF-2-induced nestin expression was abolished by treating with Sp1 inhibitor Mithramycin (Fig. 4C). These results are consistent with previous studies on RASMC, in which the nestin gene activation is mediated by the transcriptional factor Sp1 [14].

In conclusion, this present study showed that among the factors we examined, nestin expression in C6 glioma cells is up-regulated mainly by FGF-2, and this induction is mediated through Ras–Raf–ERK signaling axis and the transcriptional factor Sp1. Despite the fact that nestin expression is induced by different ligands among different cell types, these activations lead to the same downstream signaling pathway and transcriptional mechanism. Our novel findings may shed new lights on future studies regarding the regulation and functions of nestin expression in glial system.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.04.031.

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