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A novel function of transcription factor α-Pal/NRF-1: Increasing neurite outgrowth ☆

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Abstract

α-Pal/NRF-1 is a critical regulator of the promoter of human IAP/CD47 gene, a gene related to memory formation in rodents. However, its function in neurons was unknown. We found that stable or transient expression of full-length α-Pal/NRF-1 in human neuroblastoma IMR-32 cells significantly induced neurite outgrowth and increased the length of neurites both in medium containing 10% fetal bovine serum and in serum-free medium. In contrast, the dominant-negative mutant of α-Pal/NRF-1 inhibited the induction and extension of neurites. Ectopic expression of full-length α-Pal/NRF-1 also increased the induction of neurite outgrowth in primary mouse cortical neurons. The IAP antisense cDNA significantly inhibited the increase of neurite outgrowth by α-Pal/NRF-1. These findings indicate that a novel function of α-Pal/NRF-1 is to regulate neuronal differentiation, and that this function is mediated partly via its downstream *IAP* gene.

Keywords: α-Pal/NRF-1; IAP/CD47; Neurite outgrowth; Dominant-negative mutant; Serum deprivation; Antisense cDNA; Neuroblastoma; Primary cortical neurons

α-Pal was first identified on the *eukaryotic initiation factor 2α* (*eIF2α*) gene, the product of which is the rate-limiting enzyme of protein synthesis [1]. Nuclear respiratory factor 1 (NRF-1) was identified as a nuclear transcription factor that activates the promoter of many genes involved in mitochondrial function and biogenesis; for example, *cytochrome c* and *NADH dehydrogenase* [2,3]. We previously found that α-Pal/NRF-1 is a critical transcriptional regulator of the human *IAP* (or *CD47*) gene [4]. *IAP* is a memory-related gene that is up-regulated in the rat hippocampus during the processes of memory formation [5]. Knock out of the *IAP* gene in mice impaired memory performance and maintenance of long-term potentiation [6]. IAP is a membrane protein containing an Ig-V-like domain in the extracellular region and shares the characteristics of cell adhesion molecules [7]. IAP and its heterophilic binding partner P84 (also known as SHPS-1, BIT, and SIRP) are expressed in the synapse-rich region in the central nervous system, suggesting their roles in synapse formation [8,9]. In primary cortical neurons, *IAP* gene promoter is up-regulated by α-Pal/NRF-1 [4]. The functional role of α-Pal/NRF-1 in neurons, however, is unknown.

☆ *Abbreviations:* α-Pal/NRF-1, α-Pal/nuclear respiratory factor-1; IAP, integrin-associated protein; FBS, fetal bovine serum; GFP, green fluorescence protein; NFM, neurofilament M; SHPS-1, SH2-domain-containing protein tyrosine phosphatase substrate 1; BIT, brain immunoglobulin-like molecule with tyrosine-based motifs; SIRP, signal-regulated protein.

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α-Pal/NRF-1 contains a putative basic leucine zipper DNA-binding domain in its N-terminal domain and an activation domain in the C-terminal half [10,11]. The DNA-binding domain of α-Pal/NRF-1 shows strong homology with two invertebrate genes, sea urchin P3A2 and *Drosophila* erect wing gene (*ewg*) [10,12], which have been implicated in embryonic or larval development [13,14]. Furthermore, α-Pal/NRF-1 shows 91% identity to not really finished (*nrf*) in zebrafish [15]. The protein encoded by *nrf* is located primarily in the brain of zebrafish and is associated with the development of the central nervous system [15]. These studies suggest the implication of α-Pal/NRF-1 in neuronal development. In this study, we explored the function of α-Pal/NRF-1 in neuronal differentiation in cultured neuroblastoma cells and cortical neurons. We proposed that overexpression of α-Pal/NRF-1 increases neurite outgrowth and that this function is mediated, at least in part, via its downstream *IAP* gene.

Materials and methods

Cell culture. Human neuroblastoma IMR-32 was purchased from Culture Collection and Research Center, Food Industry and Development Institute, Hsinchu, Taiwan. N56R5 and N56R6 were stable cell lines overexpressing α-Pal/NRF-1 obtained in this study. These cells were grown in minimum essential medium Eagle with Earle's salt base (Sigma–Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) and cultured at 37 °C in a humidified chamber containing 5% CO_2.

Isolation of stable cell lines. α-Pal/NRF-1 cDNA was inserted in the sense orientation under the regulation of the CMV promoter in the mammalian expression vector pcDNA3.1, which contains the neomycin resistance gene. The construct was named as pcDNA3.1-NRF-1 [4]. IMR-32 cells (1.5×10^5/well) were cultured in a six-well plate and transfected with 2 μg of pcDNA3.1-NRF-1. Stable transfectants were selected by adding G418 to a final concentration of 1 mg/ml in the culture medium. Individual clones were isolated after 20–35 days of selection.

RT-PCR. For the analysis of α-Pal/NRF-1 transcripts, the total RNA was isolated from cells using TRIzol reagent (Invitrogen, Carlsbad, CA) and used for cDNA synthesis. The resultant cDNA was amplified using PCR with different pairs of primers. To confirm the expression of the exogenous construct, the PCR was performed with a forward primer in the coding sequence of α-Pal/NRF-1 (AN-1, 5′-GAG TCC AAG ATG CTA ATG) and a reverse primer in the vector sequence (BGH, 5′-TAG AAG GCA CAG TCG AGG) to avoid amplifying endogenous α-Pal/NRF-1 mRNA. The endogenous α-Pal/NRF-1 mRNA was amplified using the primer pairs of AN-1 and AN-2 (5′-GAA AAT CAA GAG TGG TGC). Total α-Pal/NRF-1 mRNA was amplified using the primer pair of AN-1 and AN-7 (5′-GACTCGAG TCA CTG TTC CAA TGT CAC CA). The PCR was performed in 20 μl of 1× PCR buffer containing 1 μl of RT products, 1 U of AmpliTaq DNA polymerase (Roche Applied Sciences, Indianapolis, IN), 200 μM dNTP, 1.5 mM $MgCl_2$, and 0.4 μM primer pair. PCR parameters were: 94 °C for 3 min, 94 °C for 15 s, 48 °C for 15 s, and 72 °C for 15 s, for 30 cycles, and 72 °C for 7 min.

Plasmid constructs. For the GFP constructs, pCMS-EGFP (Clontech, BD Biosciences, Palo Alto, CA) was used as the vector. The full-length and truncated α-Pal/NRF-1 cDNA fragments were amplified using primer pair AN-12 (5′-CTAGCTAGC GCG CAG CCG CTC TGA GGA A) and AN-7 and primer pair AN-12 and AN-11 (5′-GACTCGAG TCA CTG TGA TGG TAC AAG ATG AGC), respectively, with pcDNA3.1-NRF-1 as the template. cDNA fragments were cut with *Nhe*I and *Xho*I, and ligated into linearized pCMS-EGFP. The correct constructs were confirmed using DNA sequencing, and were named pCMS-α-Pal/NRF-1-FL and pCMS-α-Pal/NRF-1-DN. For the construction of IAP antisense cDNA, IAP cDNA was amplified using the primer pair hIAPF2 (5′-TTTGCGGCCGC AGA CAC CTG CGG CGG CGG CGG) and hIAPR1 (5′-GCTCTAGA TAC TTT TCT TGT TTC TTC TCC CCA), digested by *Not*I and *Xba*I, and inserted in a reverse orientation into pCMS-EGFP vector. The correct construct was named pCMS-AS-IAP. In these constructs, the α-Pal/NRF-1 or IAP antisense cDNA was under the control of CMV promoter, and the GFP cDNA was under the control of SV40 promoter.

Transient transfection. The plasmids pCMS-α-Pal/NRF-1-FL, pCMS-α-Pal/NRF-1-DN, and pCMS-AS-IAP were transfected into various cell lines using the calcium phosphate precipitation method [16]. Cells (1.5×10^5) were cultured on 4 cm dishes for 24 h. Then, the medium was exchanged with 2 ml of fresh medium and cells were incubated for additional 1 h for the subsequent transfection. Plasmid DNA (1 μg) was mixed with 5 μl of 2.5 M calcium chloride in a volume of 50 μl containing 0.1× TE buffer. The mixture was then mixed with one volume of 2× Hepes buffer and incubated for 1 min at room temperature. The mixture was added to the cells for transfection. After 12 h of transfection, the medium was exchanged with fresh medium and the cells were grown for neurite measurement or immunostaining on the following days.

Primary cortical culture. C3H/HeN mice pregnant for 15 days were anesthetized with pentobarbital, and their embryos were removed for primary cortical culture as described previously [4]. Primary cortical cells (4×10^5) were plated onto a 12 mm coverslip coated with poly-L-lysine in a 24-well plate for 4 h and transfected with 1 μg plasmids using the calcium phosphate precipitation method. After 12 h of transfection, the medium was exchanged with fresh medium and the cells were grown for immunostaining and neurite measurement on the following days.

Immunofluorescence microscopy. For the primary cortical culture, cells were washed with PBS and fixed with 4% formaldehyde for 10 min at room temperature. After a 1 h blocking with 10% normal goat serum and 0.2% Triton X-100 in PBS at room temperature, cells were incubated with monoclonal anti-neurofilament M-160 kDa protein (NFM) antibody (Zymed Laboratories, San Francisco, CA; diluted 1:150 in PBS) at 4 °C overnight. The cells were washed with PBS and incubated for 1 h at room temperature with Alexa-Fluor 488 conjugated anti-GFP antibody and Alexa-Fluor 594 conjugated goat anti-mouse antibody (Molecular Probes, Eugene, OR; diluted 1:400 and 1:200, respectively, in blocking solution). For the N56R5 cells transfected with pCMS-AS-IAP, the cells were fixed with 100% methanol at −20 °C for 10 min, and then exposed to 1.25% normal rabbit serum in PBS for 1 h after washing with PBS. IAP immunostaining was performed at 4 °C overnight with goat anti-CD47 (S-19) antibody (Santa Cruz Biotechnology, Santa Cruz, CA; diluted 1:100 in blocking solution), followed by 1 h exposure to Alexa-Fluor 594 conjugated rabbit anti-goat antibody (Molecular Probes; diluted 1:200 in blocking solution) at room temperature. After washing with PBS, the intracellular GFPs were detected with Alexa-Fluor 488 conjugated anti-GFP antibody for 1 h at room temperature. Following a final wash, the cells cultured on coverslips or dishes were mounted with mounting medium (VectaMount Permanent Mounting Medium; Vector Laboratories, Burlingame, CA) and sealed with nail polish. Cells were observed under fluorescent microscopes. The intensity of IAP immunoreactivity was quantified using 1-D advanced Universal Software (American Applied Biotechnology).

Measurement of neurite outgrowth. Induction of neurite outgrowth was examined by measuring the percentage of neurite-bearing cells. GFP-positive cells were observed under the fluorescent microscope

(Leica). The length of the longest process for each of >100 cells was determined for each sample using Metamorph Software (Universal Imaging Corporation, Downingtown, PA). A process, which is longer than 25 μm in IMR-32 and primary neurons, is defined as a neurite. The cell numbers with various neurite lengths were measured and plotted in the cumulative curve as percentages of cells bearing various lengths of neurite to the sum of its predecessors.

Statistical analysis. All results were expressed as means ± SEM. Graphs were drawn using GraphPad Prism 3.0 Software (Cadmus Professional Communications, Richmond, VA). Statistical analysis between two groups was performed using unpaired *t* tests. Statistical significance was set at $p < 0.05$.

Results

Stable overexpression or ectopic expression of α-Pal/NRF-1 increased neurite outgrowth in IMR-32 cells

To investigate the function of α-Pal/NRF-1 in neurons, we overexpressed α-Pal/NRF-1 in human neuroblastoma IMR-32 cells. Stable cell lines that had expressed exogenous α-Pal/NRF-1 were obtained, and two of them, N56R5 and N56R6, were chosen for further examination. N56R5 and N56R6 cells showed extended neurites when cultured with 10% FBS. Under the same conditions, the parental IMR-32 cells showed no neurites or short neurites (Fig. 1A). IMR-32 cells grew long neurites when cultured in serum-free medium; however, in such serum-free medium, the growth of neurites in N56R5 and N56R6 cells was potentiated (Fig. 1A). To rule out that these stable cell lines were spontaneous mutant cells, exogenous expression of α-Pal/NRF-1 was determined using RT-PCR analyses. While the levels of endogenous α-Pal/NRF-1 mRNA in all cell lines and wild-type cells were similar, the exogenous α-Pal/NRF-1 mRNA was significantly expressed in four selected stable cell lines. Total α-Pal/NRF-1 mRNA levels in the four cell lines were about two to three times that in the parental IMR-32 cells (Fig. 1B). These results suggested that overexpression of α-Pal/NRF-1 increased neurite outgrowth in neuroblastoma cells.

To further confirm and characterize the effect of α-Pal/NRF-1 on neurite outgrowth, we introduced the α-Pal/NRF-1-GFP constructs into IMR-32 cells using transient transfection. The transfectants were visualized as GFP-positive cells under the fluorescent microscope. Again, we observed that IMR-32 cells transfected with full-length α-Pal/NRF-1 cDNA had longer neurites in the medium containing 10% FBS. Neurite outgrowth was more prominent in serum-free medium (Fig. 2A). Two parameters were used to quantify the growth of neurites. First, the percentage of cells bearing neurites (over 25 μm in length; ⩾1.5 times the diameter of the cell body) was used to measure the induction of neurites. One day or two days after transfection, the percentage of neurite-bearing cells transfected with full-length

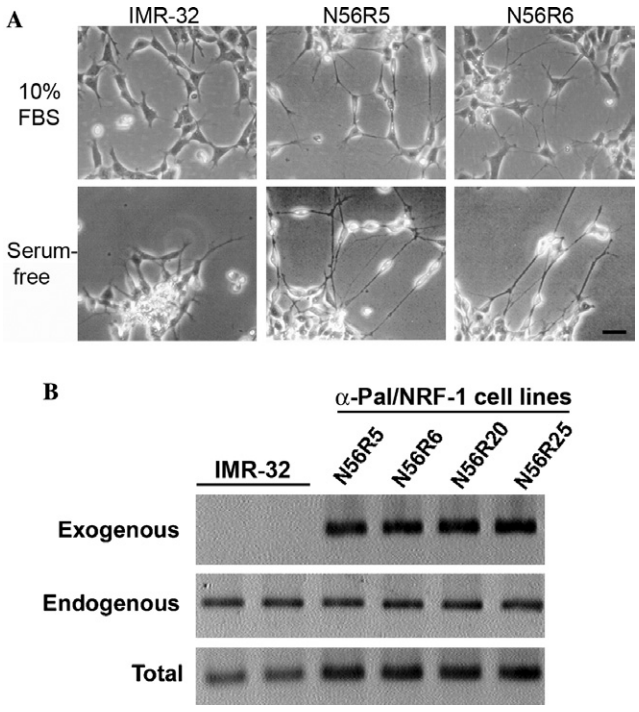

Fig. 1. Increases of neurite outgrowth in stable cell lines overexpressing α-Pal/NRF-1. (A) Morphological changes of IMR-32 cells and stable cell lines N56R5 and N56R6 overexpressing α-Pal/NRF-1 grown in medium containing 10% FBS or in serum-free medium for 3 days. Bar scale, 25 μm. (B) Detection of exogenous α-Pal/NRF-1 transcripts using RT-PCR followed by gel electrophoresis. The mRNA was prepared from IMR-32 cells and four stable cell lines (N56R5, N56R6, N56R20, and N56R25) overexpressing α-Pal/NRF-1. The cDNAs that represented the exogenous, endogenous, and total α-Pal/NRF-1 transcripts were amplified using three different pairs of primers as described under Materials and methods.

α-Pal/NRF-1 was significantly increased both in culture containing 10% FBS and in serum-free culture (Fig. 2B). Second, the curve of the cumulative percentage of cells with neurites of varying lengths was used to measure the length of neurites. The curve shifted to the right in cells transfected with full-length α-Pal/NRF-1 cDNA, both in medium containing 10% FBS and in serum-free medium, suggesting that α-Pal/NRF-1 increased the average length of neurites (Fig. 2C). These results revealed that α-Pal/NRF-1 not only induced neurite outgrowth but also increased the length of neurites in human neuroblastoma IMR-32 cells.

Dominant-negative α-Pal/NRF-1 inhibited neurite outgrowth

If α-Pal/NRF-1 enhances neurite outgrowth, competition of endogenous α-Pal/NRF-1 by dominant-negative mutant will inhibit the effect of enhancement. We, therefore, transfected the dominant-negative α-Pal/NRF-1 cDNA into IMR-32 and N56R5 cells. Although no significant decrease in the percentage of neurite-bearing cells was observed on day one after transfection of dom-

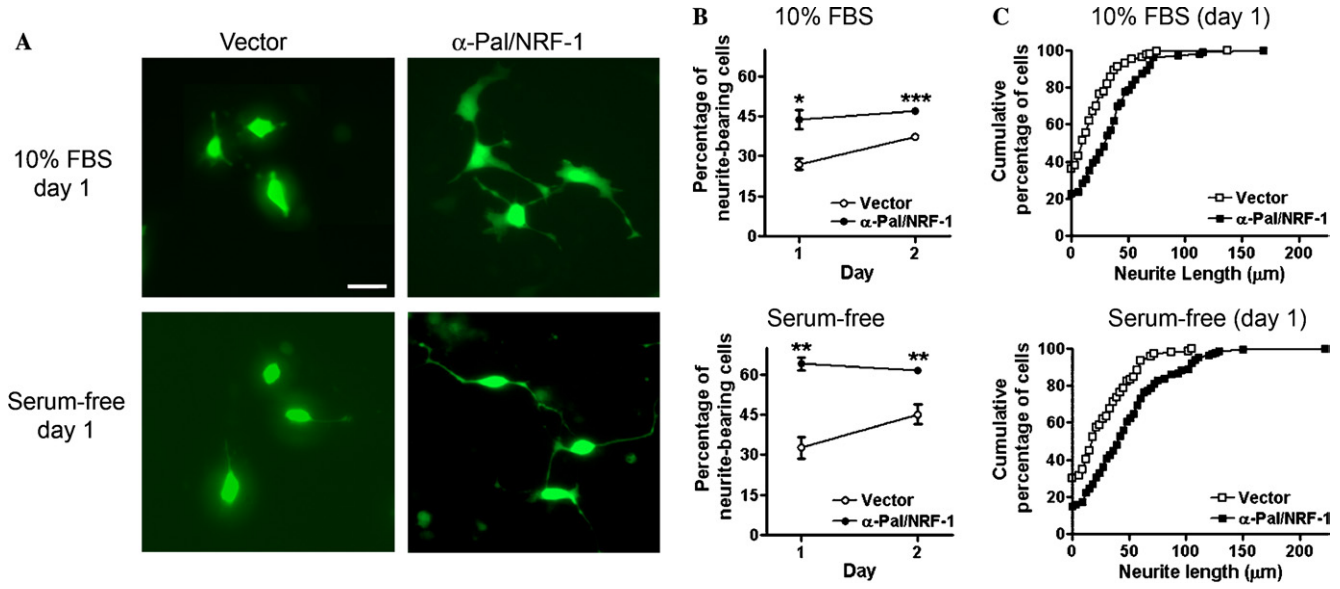

Fig. 2. The ectopic expression of α-Pal/NRF-1 increased neurite outgrowth in IMR-32 cells. (A) The morphology of IMR-32 cells transfected with empty vector or full-length α-Pal/NRF-1 cDNA. IMR-32 cells were transfected with GFP-empty vector (1 μg) or vector containing full-length α-Pal/NRF-1 cDNA (1 μg) for 12 h. After transfection, cells were cultured for 1 day in the medium containing 10% FBS or in serum-free medium. GFP-positive cells were observed under a fluorescent microscope. Bar scale, 25 μm. (B) Quantification of neurite-bearing cells. One or two days after transfection, cells carrying extended neurites (>25 μm) were counted. Values are averages of three counts on more than 100 GFP-positive cells. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; unpaired t test. (C) The cumulative curve for percentage of cells bearing various lengths of neurites in a representative experiment. The cumulative percentage was calculated by adding the percentage of cells bearing specific lengths of neurites to the sum of its predecessors.

inant-negative α-Pal/NRF-1 cDNA, the significant effect was observed on day 2 after transfection (Fig. 3). In medium containing 10% FBS, the percentage of neurite-bearing cells was significantly decreased in IMR-32 cells as well as in N56R5 cells transfected with dominant-negative α-Pal/NRF-1 cDNA on day two after transfection (Fig. 3A). The curve of the cumulative percentage of cells with neurites of varying lengths shift-

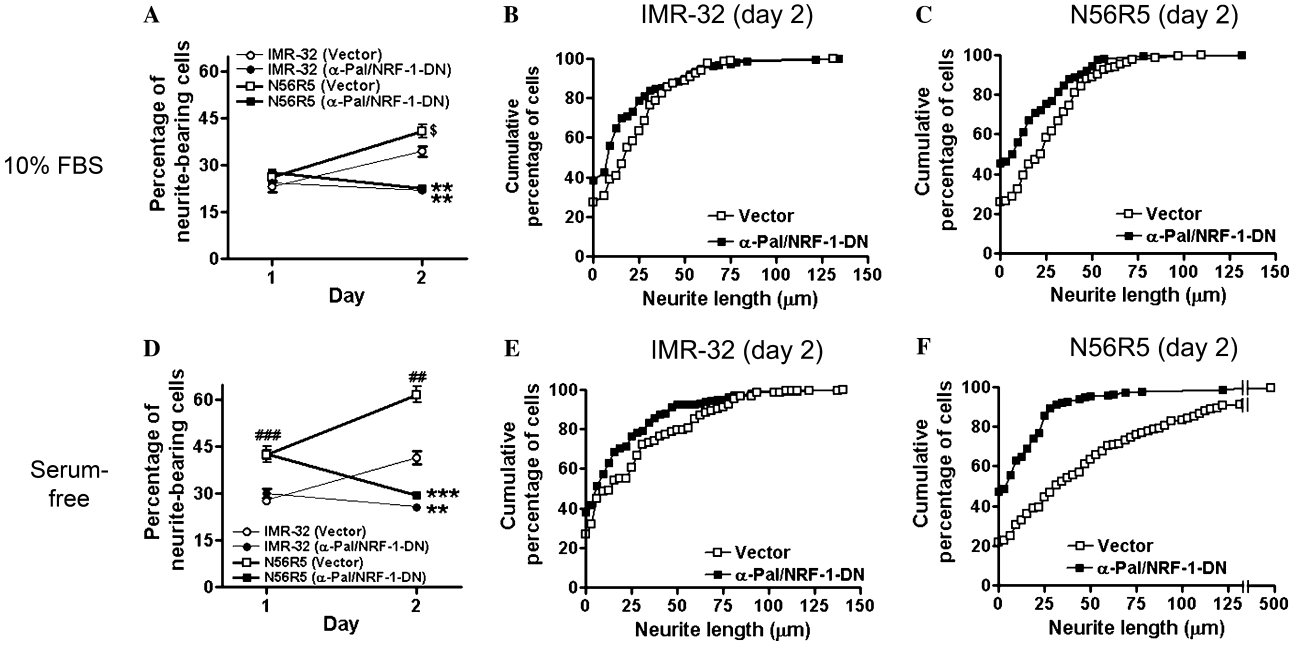

Fig. 3. Dominant-negative α-Pal/NRF-1 inhibited neurite outgrowth in IMR-32 and N56R5 cells. IMR-32 and N56R5 cells were transfected with GFP-empty vector (1 μg) or vector containing truncated α-Pal/NRF-1 cDNA (1 μg) for 12 h. Cells were then cultured for 1 or 2 days in medium containing 10% FBS (A,B,C) or serum-free medium (D,E,F). (A,D) Quantification of cells carrying extended neurites ($n = 3$–4). $^{**}p < 0.01$; $^{***}p < 0.001$ compared with the vector control; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$; $^{\$}p = 0.07$ compared with IMR-32 cells transfected with the empty vector. (B, C, E, and F) Examples of the cumulative percentages of cells bearing various lengths of neurites in different experimental conditions.

ed to the left in these two cell lines cultured with 10% FBS (Figs. 3B and C). In serum-free medium, the differences were more prominent. Serum-free medium significantly increased the number of cells bearing neurites in N56R5 cells transfected with the empty vector as compared with IMR-32 cells transfected with the empty vector. However, transfection of the dominant-negative α-Pal/NRF-1 cDNA significantly abolished the enhancement of neurite outgrowth by serum deprivation as well as by overexpression of α-Pal/NRF-1 in N56R5 cells (Fig. 3D). The curve of the cumulative percentage of cells with neurites of varying lengths also shifted to the left in both IMR-32 and N56R5 cells cultured in serum-free medium (Figs. 3E and F). These results revealed that blockage of the function of endogenous α-Pal/NRF-1 decreased the induction and extension of neurites in IMR-32 cells.

α-Pal/NRF-1 regulated neurite outgrowth in primary cortical cells

To determine whether α-Pal/NRF-1 is involved in neurite outgrowth in normal immature neurons, we next examined the effects of wild-type and dominant-negative α-Pal/NRF-1 on cultured mouse cortical cells. Mouse E15 cortical cells containing neural progenitor cells and immature neurons were transfected with the empty GFP vector, the GFP constructs containing full-length or truncated α-Pal/NRF-1 cDNA. The neuronal marker NFM was used to identify neurons. The morphology of

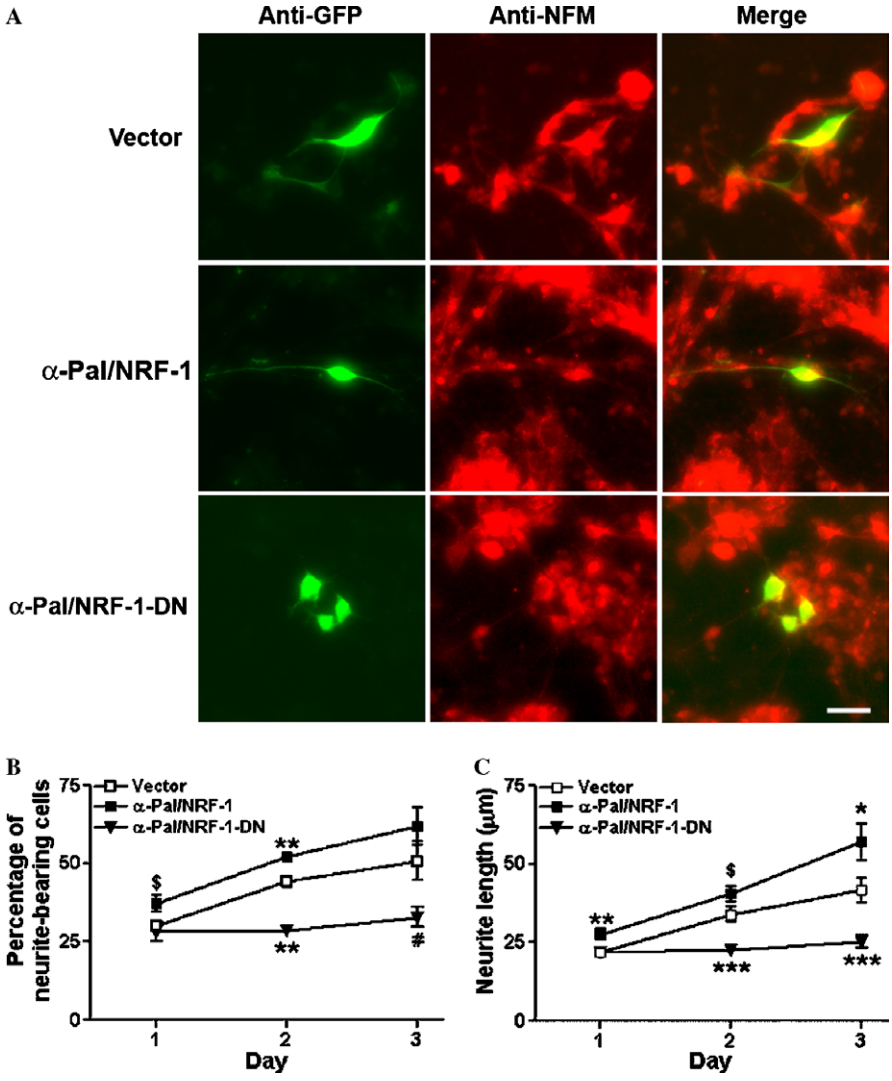

Fig. 4. α-Pal/NRF-1 regulated neurite outgrowth in primary cortical cells. Cells cultured from E15 mouse cortex were transfected with 1 μg of empty vector, vector containing full-length, or truncated α-Pal/NRF-1 cDNA. Cultured cells were immunostained with antisera against GFP (green) and NFM (red) on the following 3 days. (A) Examples of the morphology of GFP-and NFM-positive mouse cortical neurons on the second day. Left, GFP; middle, NFM; right, merged images. Bar scale, 25 μm. (B) Percentage of neurite-bearing (>25 μm) GFP- and NFM-positive cells, calculated by the proportion of neurite-bearing GFP-positive cells in more than 100 cells ($n = 3$–4). (C) The mean values of neurite length of all GFP- and NFM-positive cells in B ($n = 300$–500). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; $^{\#}p = 0.06$; $^{\$}p = 0.07$; unpaired t test.

NFM-positive neurons was observed under a fluorescent microscope (Fig. 4A). The percentage of neurite-bearing NFM-positive cells significantly increased on days 1 and 2, but not on day 3, after transfection of wild-type α-Pal/NRF-1 (Fig. 4B). The neurite length significantly increased on days 1, 2, and 3 (Fig. 4C). Overexpression of dominant-negative α-Pal/NRF-1 did not influence the morphology of transfected neurons on day 1, but the percentage of neurite-bearing cells and neurite length significantly decreased on days 2 and 3 (Figs. 4B and C). These results suggested that α-Pal/NRF-1 is involved in the neurite outgrowth of immortal and normal neuronal cells.

Antisense IAP cDNA suppressed an α-Pal/NRF-1-induced increase of neurite outgrowth

To further confirm that IAP mediates the function of α-Pal/NRF-1 in neurite outgrowth, we used the strategy of antisense cDNA to knock down the expression of the *IAP* gene in N56R5 cells. Results of immunocytochemistry demonstrated that antisense IAP cDNA significantly decreased the intensity of IAP immunoreactivity in transfected cells as compared with that in non-transfected cells (Figs. 5A and B). Antisense IAP cDNA significantly reduced the percentage of cells bearing neurites in N56R5 cells when cultured with 10% FBS (Figs. 5C and D, upper panels) or in serum-free medium (Figs. 5C and D, lower panels) 1, 2, and 3 days after transfection. These results suggested that IAP is one of the α-Pal/NRF-1 downstream genes involved in neurite outgrowth.

Discussion

In this study, we revealed a novel function of α-Pal/NRF-1 in neurons; i.e., increasing neurite outgrowth. The wild-type α-Pal/NRF-1 increased neurite outgrowth

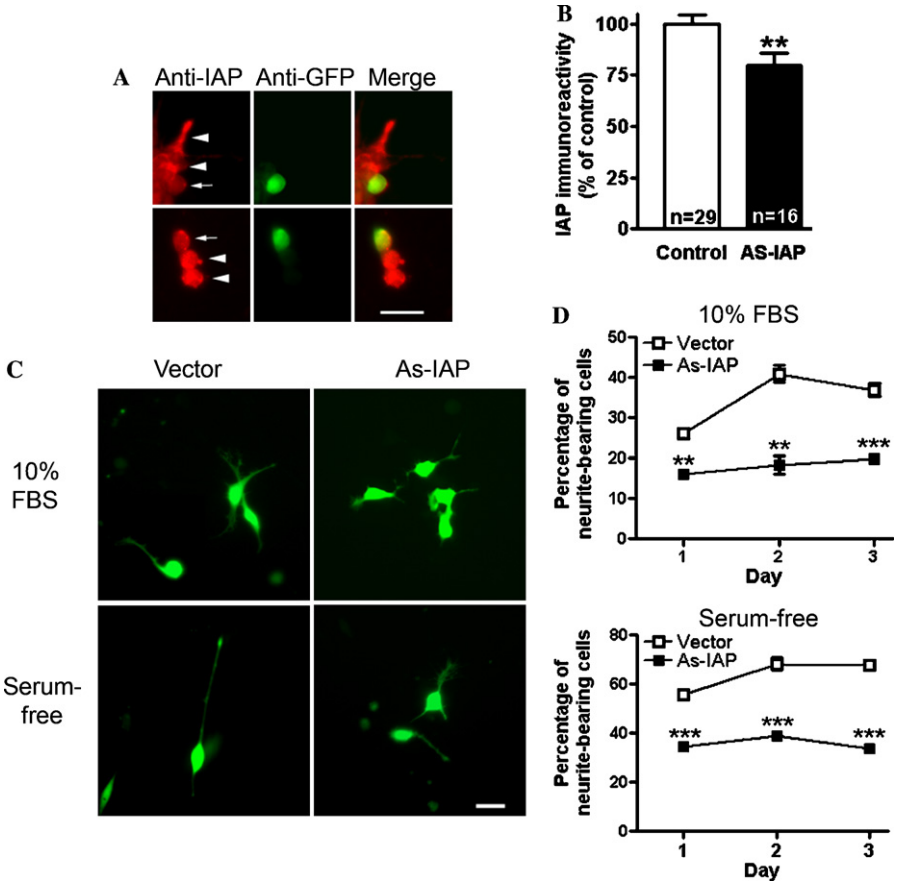

Fig. 5. IAP mediated the function of α-Pal/NRF-1 in neurite outgrowth. (A) Effects of antisense IAP cDNA treatment on the IAP protein immunoreactivity. IMR-32 cells were transfected with the construct of pCMS-AS-IAP (1 μg). IAP immunoreactivity was detected with the anti-IAP antibody and the secondary antibody conjugated with Alexa-Fluor 594 (left panels). Transfected cells were then detected with the Alexa-Fluor 488 conjugated anti-GFP antibody (middle panels). Transfected and GFP-positive cells are indicated by arrows. Non-transfected cells, which were GFP-negative, are indicated by arrow heads. Right panels are the merged image. Bar scale, 25 μm. (B) Antisense IAP cDNA significantly decreased IAP immunoreactivity. Non-transfected cells adjacent to cells transfected with the antisense IAP cDNA (AS-IAP) were used as the controls. $^{**}p < 0.05$. (C) Morphology of N56R5 cells transfected with antisense IAP cDNA and grown with or without 10% FBS serum for 2 days. Bar scale, 25 μm. (D) Antisense IAP cDNA inhibited neurite outgrowth in N56R5 cells ($n = 4$). After being grown in the presence or absence of 10% FBS for 1, 2, or 3 days, the cells bearing neurites were counted under the fluorescence microscope. $^{**}p < 0.01$; $^{***}p < 0.001$; unpaired t test.

in human neuroblastoma cells and in mouse primary cortical neurons. In contrast, a dominant-negative mutant of α-Pal/NRF-1 reduced neurite production in these cells. We also found that *IAP* is a downstream gene mediating the function of α-Pal/NRF-1 in neurite outgrowth.

α-Pal/NRF-1 is a transcription factor that regulates a variety of genes. In our previous report [4], we found that α-Pal/NRF-1 is a critical positive regulator of the human *IAP* gene. In this study, we have demonstrated that IAP mediates the function of α-Pal/NRF-1 in neurite outgrowth of human neuroblastoma IMR-32 cells and primary cortical neurons. It is plausible that α-Pal/NRF-1 upregulates the expression of the *IAP* gene in neuroblastoma cells and neurons, hence increasing neurite outgrowth. This idea is supported by our findings that serum deprivation, which increases neurite outgrowth, increased α-Pal/NRF-1 binding to the recognition site in the *IAP* promoter region and the *IAP* mRNA level in IMR-32 cells (data not shown). Most importantly, antisense cDNA in α-Pal/NRF-1-overexpressing cells reduced expression of the *IAP* gene and significantly impaired the increase of neurite outgrowth. Thus, one of the significant roles of α-Pal/NRF-1 and its downstream *IAP* gene in neurons is to increase neurite outgrowth.

Our findings that the *IAP* gene mediates the function of α-Pal/NRF-1 in neurite outgrowth are supported by two recent and elegant studies. Miyashita et al. [17] reported that IAP promoted neurite and filopodium formation in mouse N1E-115 neuroblastoma cells. They have demonstrated that the interaction of the extracellular domain of IAP with its heterophilic ligand SHPS-1 is sufficient to induce neurite formation. In their report, it has been shown that the interaction of SHPS-1 with IAP promotes neurite formation through the activation of two small G proteins, Rac and Cdc42, and that integrins containing the β3 subunit participate in the effect of IAP on neurite formation. The neuronal roles of IAP were extensively investigated by Numakawa et al. [18]. Using IAP-overexpressing (form 4) cortical neurons, the most abundant isoform expressed primarily in the brain, through virus-mediated gene transfer, these authors demonstrated that dendritic outgrowth was increased, synaptic proteins were up-regulated, and glutamate release and Ca^{2+} oscillation were reinforced. Taken together, these studies suggested that activation of α-Pal/NRF-1 will increase the amount of IAP protein on the membrane, which will in turn promote neurite outgrowth through the interaction with SHPS-1 or other adhesion molecules and downstream signaling molecules [18].

It is also possible that α-Pal/NRF-1 increases neurite outgrowth through other downstream genes. A putative α-Pal/NRF-1 site was found in the promoter region of the *synapsin I* gene [10–12]. Using a gel electrophoresis mobility assay, we have demonstrated that the α-Pal/NRF-1 site on the *synapsin I* gene is functional (unpublished observations). Overexpression of synapsin I accelerated synaptogenesis in embryonic cell cultures [19], whereas reduction of synapsin I led to delayed neuronal differentiation, axonal outgrowth, and synaptogenesis [20]. Therefore, synapsin I could be a candidate gene that mediates the function of α-Pal/NRF-1 as well.

From the shifting of the cumulative curve of neurite length in neurite-bearing cells, we believe that one of the effects of α-Pal/NRF-1 on neurites is to extend the length of neurites. This effect might result from the interaction of IAP with its ligands described above. We also found that overexpression of α-Pal/NRF-1 significantly increased the number of cells bearing neurites, indicating that more cells were undergoing differentiation instead of proliferation. Thus, it is possible that α-Pal/NRF-1 increases neurite outgrowth through the regulation of genes related to repression of the cell cycle. Overexpression of α-Pal/NRF-1 retards cell proliferation in erythroleukemia cells, which is caused by the down-regulation of E2F-1 [21]. Down-regulation of E2F-1 inhibited cell proliferation and increased neurite outgrowth in mouse neuroblastoma N1E-115 cells [22]. Therefore, it is likely that overexpression of α-Pal/NRF-1 in IMR-32 cells results in down-regulation of E2F-1, repression of the cell cycle, and induction of neurite outgrowth.

We have shown that α-Pal/NRF-1 increases neurite outgrowth not only in human neuroblastoma cells, but also in immature and normal cortical neurons. It is proposed that α-Pal/NRF-1 and its downstream *IAP* gene promote neuronal differentiation during embryonic development as well as in the neurogenesis of the adult hippocampus. The homolog of α-Pal/NRF-1 in zebrafish has been shown to be involved in the development of the central nervous system [15]. It is possible that α-Pal/NRF-1 and *IAP* gene are also involved in the development of the mammalian brain. Neurogenesis in the adult hippocampus has been correlated with learning and memory in some hippocampus-dependent behavioral tests [23,24]. We previously found that increase of the IAP mRNA level in the hippocampus is related to an inhibitory avoidance learning task in adult rats [5]. An increase of IAP mRNA by the activation of α-Pal/NRF-1 and hence increase of neuritogenesis in the adult hippocampus may explain its role in memory formation.

In conclusion, we have demonstrated a novel function of α-Pal/NRF-1 in neurons; that is, an increase in neurite outgrowth. This function is, at least in part, mediated via its downstream *IAP* gene.

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