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ORIGINAL PAPER

Forced expression of NR4A3 induced the differentiation of human neuroblastoma-derived NB1 cells

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Abstract

Nuclear receptor subfamily 4, group A, member 3 (NR4A3) is a member of the NR4A subgroup of orphan nuclear receptors, implicated in the regulation of diverse biological functions, including metabolism, angiogenesis, inflammation, cell proliferation, and apoptosis. Although many reports have suggested the involvement of NR4A3 in the development and/or progression of tumors, its role varies among tumor types. Previously, we reported that DNA hypomethylation at NR4A3 exon 3 is associated with lower survival rate of neuroblastoma (NB) patients. As hypomethylation of this region results in reduced expression of NR4A3, our observations suggested that NR4A3 functions as a tumor suppressor in NB. However, the exact mechanisms underlying its functions have not been clarified. In the present study, we analyzed public databases and showed that reduced NR4A3 expression was associated with shorter survival period of NB in two out of three datasets. An in vitro study revealed that forced expression of NR4A3 in human NB-derived cell line NB1 resulted in elongation of neurites along with overexpression of GAP43, one of the differentiation markers of NB. On the other hand, siRNA-mediated knockdown of NR4A3 suppressed the expression level of GAP43. Interestingly, the forced expression of NR4A3 induced only the GAP43 but not the other molecules involved in NB cell differentiation, such as MYCN, TRKA, and PHOX2B. These results indicated that NR4A3 directly activates the expression of GAP43 and induces differentiated phenotypes of NB cells, without affecting the upstream signals regulating GAP43 expression and NB differentiation.

Keywords Neuroblastoma · NR4A3 · Differentiation · GAP43

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Introduction

Neuroblastoma (NB) is the most common extracranial solid malignant tumor in children, accounting for approximately 15% of pediatric cancer deaths [1]. NB arises from neural crest progenitor cells of the sympathetic nervous system, typically from the adrenal medulla or paraspinal ganglia. NB is characterized by marked heterogeneity of biological characteristics and clinical features. NBs occurring in patients younger than 12 months old usually show regression or maturation into benign ganglioneuroma spontaneously. However, most NBs show an aggressive phenotype and poor prognosis when they occur in patients at 18 months or older [1]. According to the International Neuroblastoma Risk Group (INRG) classification system, NB patients are classified into four categories, i.e., very low risk, low risk, intermediate risk, and high risk, based on a number

of factors, such as age at diagnosis, tumor stage, histology, differentiation grade, and copy number status at MYCN and chromosome 11q [2]. Although the treatment outcomes have improved remarkably in patients with lower grade NB, the 5-year survival rate of patients with high-risk NB is still < 40% [1].

Among the genetic aberrations observed in NBs, amplification of MYCN is strongly associated with poor prognosis, and NB patients harboring the amplification are classified as a high-risk group in the INRG system [2]. NBs with MYCN amplification show an aggressive phenotype and resistance to chemotherapy. Other genetic aberrations, such as gain of chromosome 17q [3] or deletion of chromosome 1p or 11q [4], have also been reported to be associated with poor prognosis of NB. Nevertheless, a significant number of malignant NBs do not harbor any of above-mentioned genomic abnormalities. To develop a more effective treatment strategy for malignant NB, it is necessary to determine the molecular mechanism underlying the development and progression of NB.

Nuclear receptor subfamily 4, group A, member 3 (NR4A3), also known as neuron-derived orphan receptor-1 (NOR1), is a member of the NR4A subgroup of orphan nuclear receptors. In mammals, NR4A1 (Nur77), NR4A2 (Nurr1), and NR4A3 belong to the NR4A subgroup, with high homology in their amino acid sequence. All of the members act as transcription factors, and bind to DNA as monomers, homodimers, and heterodimers [5]. They have been implicated in the regulation of diverse biological functions, including metabolism, angiogenesis, inflammation, cell proliferation, and apoptosis [6, 7].

Numerous studies have shown that expression of NR4A receptors is frequently altered in many types of tumors; however, the alteration patterns vary depending on receptor type and tumor type [6]. Overexpressions of NR4A1 and/or NR4A2 have been observed in various solid tumors, such as carcinomas in the colon, breast, pancreas, and bladder [8–12]. NR4A3 was also reported to be highly expressed in triple-negative breast cancer [13], salivary gland acinic cell carcinoma (AciCC) [14], and melanoma [15]. These observations suggest that NR4A3 has oncogenic roles; however, it has also been reported that NR4A1 and NR4A3 expressions are reduced in leukemic blasts in acute myeloid leukemia (AML) [16]. Moreover, NR4A1 and NR4A3 double-knockout mice were shown to develop AML [16].

Previously, we reported that DNA hypomethylation of CpG island at *NR4A3* exon 3 is associated with low *NR4A3* expression [17]. In addition, in an analysis of 20 NB patients, eight patients with hypomethylation of this region showed significantly lower survival rate than patients with hypermethylation [17]. These results indicated that low expression of NR4A3 is associated with poor prognosis of NB. However, the role of NR4A3 in NB has not been elucidated.

In the present study, we performed functional analysis of NR4A3 to elucidate its role in the development and/or progression of NB, and to understand the molecular mechanisms underlying its functions.

Materials and methods

Cell lines and culture conditions

The human NB-derived cell line NB1 was obtained from National Institutes of Biomedical Innovation, Health and Nutrition (Osaka, Japan). SK-N-SH, SK-N-AS, and SH-SY5Y were obtained from American Type Culture Collection (ATCC, Manassas, VA), NB69 was from Riken Cell Bank (Ibaraki, Japan), and Kelly was from DS Pharma Biomedical (Osaka, Japan). NB1, Kelly, and NB69 were cultured in RPMI-1640 (Nacalai Tesque, Kyoto, Japan) supplemented with heat-inactivated fetal bovine serum (FBS; Nichirei Bioscience, Tokyo, Japan) at a final concentration of 10% (NB1, Kelly) or 15% (NB69). SK-N-SH and SH-SY5Y were cultured in MEM supplemented with 10% FBS, 0.1 mM nonessential amino acids (Thermo Fisher Scientific, Waltham, MA), and 5 mM sodium pyruvate (Thermo Fisher Scientific). SK-N-AS cells were cultured in DMEM (Nacalai Tesque) supplemented with 10% FBS. All of the media contained 100 IU/ml of penicillin (Life Technologies, Carlsbad, CA) and 100 µl/ml of streptomycin (Life Technologies). Cells were maintained at 37 °C in a CO_2 incubator with a controlled humidified atmosphere composed of 95% air and 5% CO_2.

Establishment of cell lines stably expressing NR4A3

NB1 cells were transfected with pcDNA3.1(+) (Thermo Fisher Scientific) or pcDNA3.1(+) vector containing NR4A3 CDS (GeneScript, Piscataway, NJ) using Lipofectamine 3000 (Thermo Fisher Scientific) in accordance with the manufacturer's instructions. The transfected cells were cultured with culture medium containing 500 µg/ml G418 for more than 1 month, and clones expressing NR4A3 protein were selected.

Knockdown of NR4A3 using siRNA

NB1 cells were seeded at a density of 1×10^5 cells/ml, and immediately transfected with NR4A3 siRNA or control siRNA (Thermo Fisher Scientific) using Lipofectamine 3000 (Thermo Fisher Scientific) in accordance with the manufacturer's instructions. The target sequence of the NR4A3 siRNA was 5′-AGAGAACAGTGCAGAAAAA-3′. As a control siRNA, Silencer Select Negative Control #1

(Thermo Fisher Scientific), whose target sequence information is not available, was used.

Analysis of cell viability

NB1 clones stably expressing NR4A3 or stably transfected with control vector were seeded in 96-well plates at a density of 1×10^3 cells/well. For knockdown experiments, NB1 and SK-N-SH cells were seeded in 96-well plates at a density of 1×10^4 cells/well, and immediately transfected with control siRNA or NR4A3 siRNA. Viability of the cells was measured by WST8 assay using Cell Count Reagent CF (Nacalai Tesque) at the indicated time points.

Measurement of neurite length

Images of the cells were acquired using a CCD camera (DFC 300FX DP-SP; Leica, Wetzlar, Germany) and LAS v4.3 software (Leica) at × 200 magnification. The length of neurites was measured using a Lumina Vision image analysis system (Mitani, Tokyo, Japan). One hundred cells from four fields were analyzed for each sample.

Quantitative real-time PCR (qPCR)

Total RNA was extracted from cells using RNeasy mini kits (Qiagen, Valencia, CA) according to the manufacturer's instructions. For cDNA synthesis, 500 ng of total RNA was reverse transcribed using iScript cDNA synthesis system (Bio-Rad Laboratories, Hercules, CA). qPCR was performed using a SYBR Premix Ex Taq™ system according to the manufacturer's recommendations (Takara, Shiga, Japan). A mixture of cDNA derived from total RNA of NB1, SK-N-AS and SK-N-AS cells was used as a reference. A dilution series of the cDNA mixture was prepared and used in real-time PCR as the templates to obtain a standard curve for each gene. Three independent measurements were taken. Primer sets used for qPCR-based amplification were as follows: NR4A3, 5′-CTACGGCGTGCGAACCTG-3′ (sense) and 5′-TTCGACGTCTCTTGTCTACTGG-3′ (antisense); GAP43, 5′-GAGGATGCTGCTGCCAAG-3′ (sense) and 5′-GGCACTTTCCTTAGGTTTGGT-3′ (antisense); and GAPDH, 5′-TCACCAGGGCTGCTTTTAAC-3′ (sense) and 5′-TGACGGTGCCATGGAATTTG-3′ (antisense). The housekeeping gene GAPDH was used as an internal reference.

Immunoblotting

To obtain cell lysate, cells were lysed in RIPA buffer containing protease inhibitor cocktail (Nacalai Tesque) and phosphatase inhibitor cocktail (Nacalai Tesque), followed by sonication using BIORUPTER UDC250 (Cosmo Bio, Tokyo, Japan). Protein concentration of the lysates was measured using Bio-Rad DC kits (Bio-Rad, Hercules, CA). The lysates were separated by 4–12% SDS-polyacrylamide gel electrophoresis and then electroblotted onto Immobilon-P membranes (Millipore, Billerica, MA) by the wet transfer method. Membranes were blocked with Blocking-one (Nacalai Tesque) overnight at 4 °C, and incubated with mouse anti-NR4A3 monoclonal antibody (1E11; Sigma-Aldrich, St. Louis, MO), rabbit anti-GAP43 antibody (Abcam, Cambridge, UK), rabbit anti-MYCN antibody (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-TRKA antibody (Cell Signaling Technology, Beverly, MA), rabbit anti-PHOX2B antibody (Cell Signaling Technology), or with rabbit anti-GAPDH antibody (Abcam) at 4 °C. After 24 h of incubation, membranes were washed with Tris-buffered saline containing 0.1% Tween 20 (TBS-T), followed by incubation with horseradish peroxidase-conjugated secondary antibody for mouse or rabbit IgG (GE Healthcare Life Sciences, Buckinghamshire, UK), for 1 h at room temperature. The membranes were washed extensively with TBS-T, and treated with Chemi-Lumi-One Super (Nacalai Tesque) to visualize immunoreactivity using LAS4000 (Fujifilm, Tokyo, Japan).

Statistical analysis

Student's *t* test was applied to examine the significance of differences between paired data, and one-way ANOVA followed by post hoc Tukey test was used to examine significance among multiple data. All of the statistical analyses were performed using JMP software ver. 11.2 (SAS Institute, Inc., Cary, NC). Data are presented as means ± SD from at least three independent experiments. In all analyses, $P < 0.05$ was taken to indicate statistical significance.

To generate survival curves for overall survival of NB patients, the three independent microarray datasets, gse45547, gse49710, and gse16476 were obtained from the R2 platform (http://r2.amc.nl). Dataset gse45547 contains gene expression profiles from 649 NB samples generated using Agilent-020382 Human Custom Microarray 44k oligonucleotide microarrays. Gse49710 contains gene expression profiles from 498 NB samples generated by RNA sequencing as well as by microarray analyses using Agilent-020382 Human Custom Microarray 44k. In gse16476, gene expression profiles of 88 NB samples generated using Affymetrix Human Genome U133 Plus

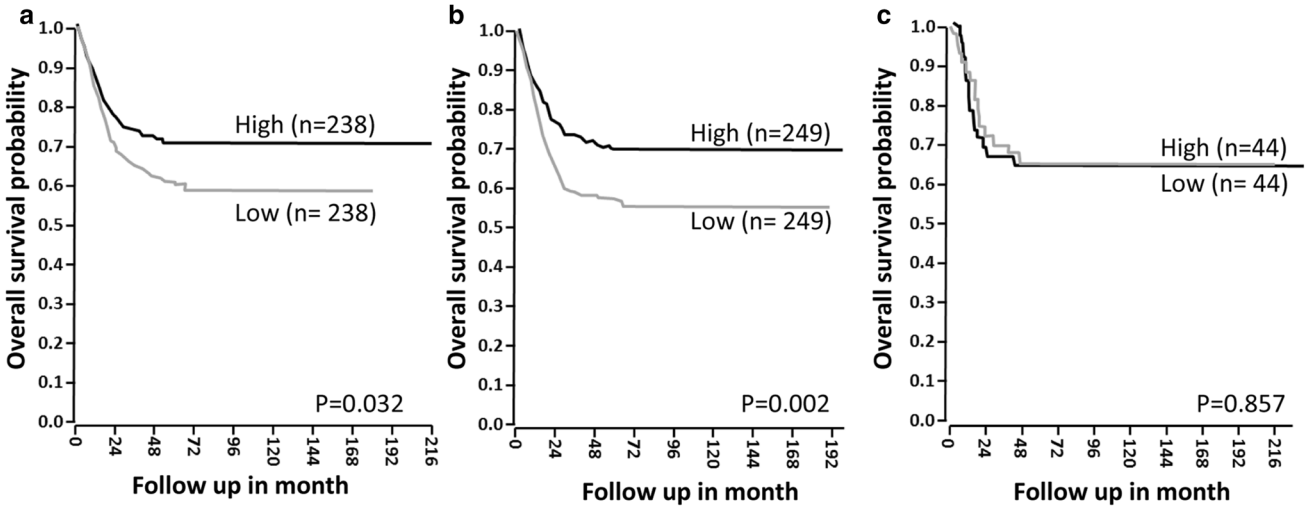

Fig. 1 Kaplan–Meier survival analyses based on three independent public microarray datasets. Elevated expression level of *NR4A3* was associated with favorable prognosis in NB patients in two datasets, gse45547 (**a**) and gse49710 (**b**), and but no significant association was observed in gse16476 (**c**)

2.0 Array are available. Overall survival was shown as a Kaplan–Meier plot and was analyzed statistically by the log-rank test followed by Bonferroni's test.

Results

Lower NR4A3 expression level is related to poor prognosis of patients with NB

To examine the clinical significance of NR4A3 in the development and/or progression of NB, Kaplan–Meier survival analysis was performed utilizing public microarray datasets. As shown in Fig. 1, lower NR4A3 expression level was significantly associated with shorter survival period in patients with NB, in datasets gse45547 and gse49710. No significant difference was observed in survival period between NB patients with higher expression of NR4A3 and with lower expression in the dataset gse16476. These results suggested that NR4A3 may have a suppressive effect on malignant progression of NB.

Stable expression of NR4A3 induces NB1 cell neurite elongation

To clarify its function in NB cells, we analyzed the expression levels of NR4A3 in NB-derived cell lines. The results indicated that the NR4A3 expression level varied among the six NB cell lines examined. SK-N-SH, SK-N-AS and SH-SY5Y showed significantly higher expression levels of NR4A3 mRNA than in NB1, Kelly, and NB69 cells (Fig. 2a). Similar results were obtained in analysis of the expression levels of NR4A3 protein

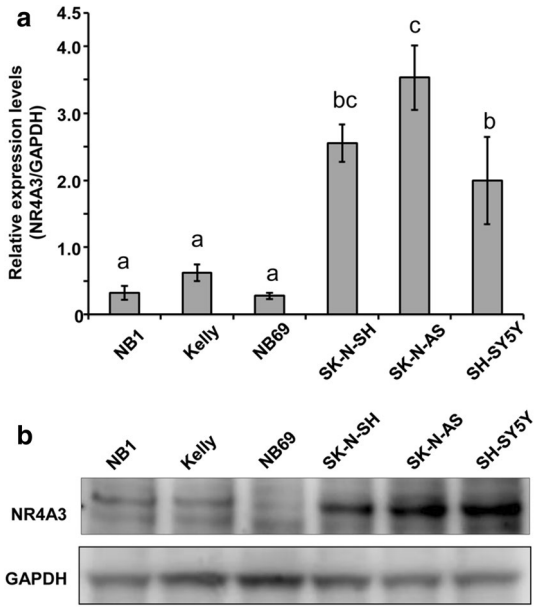

Fig. 2 Expression levels of NR4A3 varying among NB cell lines. **a** NR4A3 mRNA and **b** protein levels in NB cell lines were analyzed by real-time PCR and immunoblotting, respectively. Values represent means ± SD of measurements performed in triplicate. Statistical significance was analyzed by one-way ANOVA with Tukey's post hoc test. Values without same lower case letters are significantly different ($P < 0.05$)

(Fig. 2b). To investigate the function of NR4A3 in NB cells, we transfected NB1 cells with an expression vector and established clones stably expressing NR4A3. As controls, NB1 clones stably transfected with mock vector were established (Fig. 3a, b). When cell viability was analyzed, the growth

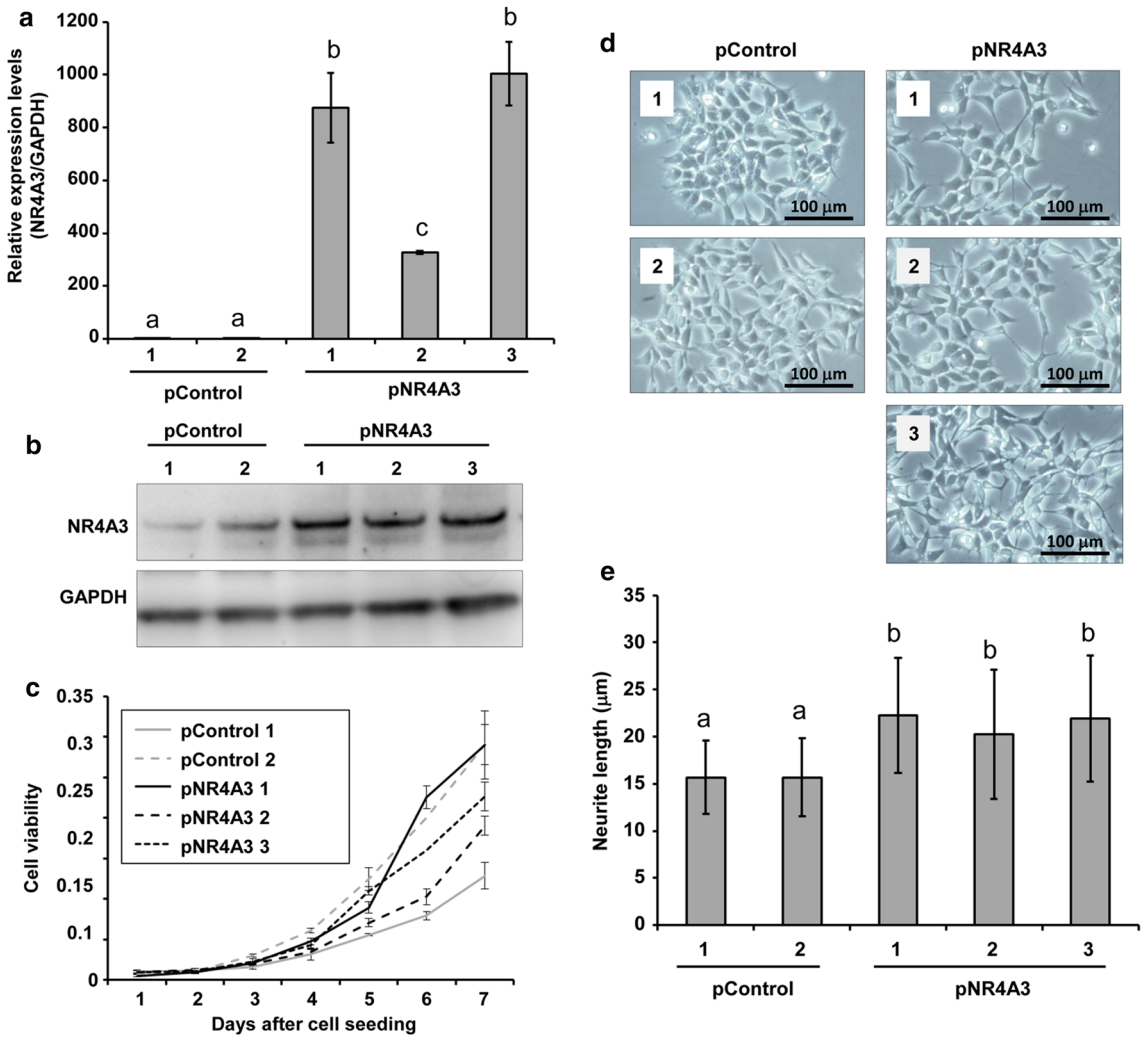

Fig. 3 Overexpression of NR4A3 inducing well-differentiated phenotypes of NB1 cells with elongated neurites. **a** NR4A3 mRNA and **b** protein expression levels in NB1 clones stably transfected with control vector or with the NR4A3 expression vector were analyzed by real-time PCR and immunoblotting, respectively. **c** Viability of NB1 clones was measured by WST8 assay at the indicated times after seeding. Values represent means ± SD of measurements performed in triplicate. **d** Representative images of NB1 clones. **e** Neurite length was measured for 100 cells in all NB1 clones. Values represent means ± SD of measurements of 100 cells. For **a** and **e**, statistical significance was analyzed by one-way ANOVA with Tukey's post hoc test. Values without same lower case letters are significantly different ($P < 0.05$)

rate varied among the clones, and no clear differences were observed between NR4A3-overexpressing clones and controls (Fig. 3c). On the other hand, all three NR4A3-overexpressing clones showed longer neurites than control cells (Fig. 3d). By measuring the lengths of individual neurites, we found that neurite outgrowth of NR4A3-overexpressing clones was significantly promoted compared with control clones (Fig. 3e).

Expression of GAP43 was upregulated in NR4A3-overexpressing NB1 cells

As extensive neurite outgrowth is a characteristic morphological change in differentiated NB, we analyzed the expression levels of molecules involved in the regulation of NB differentiation. As shown in Fig. 4a, no clear differences were observed in the expression levels of MYCN, TRKA, and PHOX2B between NR4A3-overexpressing clones and control clones. The expression levels of growth-associated protein 43 (GAP43), which is also known to be a marker of differentiated NB, were markedly higher in NR4A3-overexpressing clones compared to control clones (Fig. 4a). To examine whether expression of GAP43 is upregulated in NR4A3-overexpressing clones at the transcriptional level, the amount of GAP43 mRNA was measured by real-time PCR. As shown in Fig. 4b, the GAP43 mRNA expression levels were higher in all of the

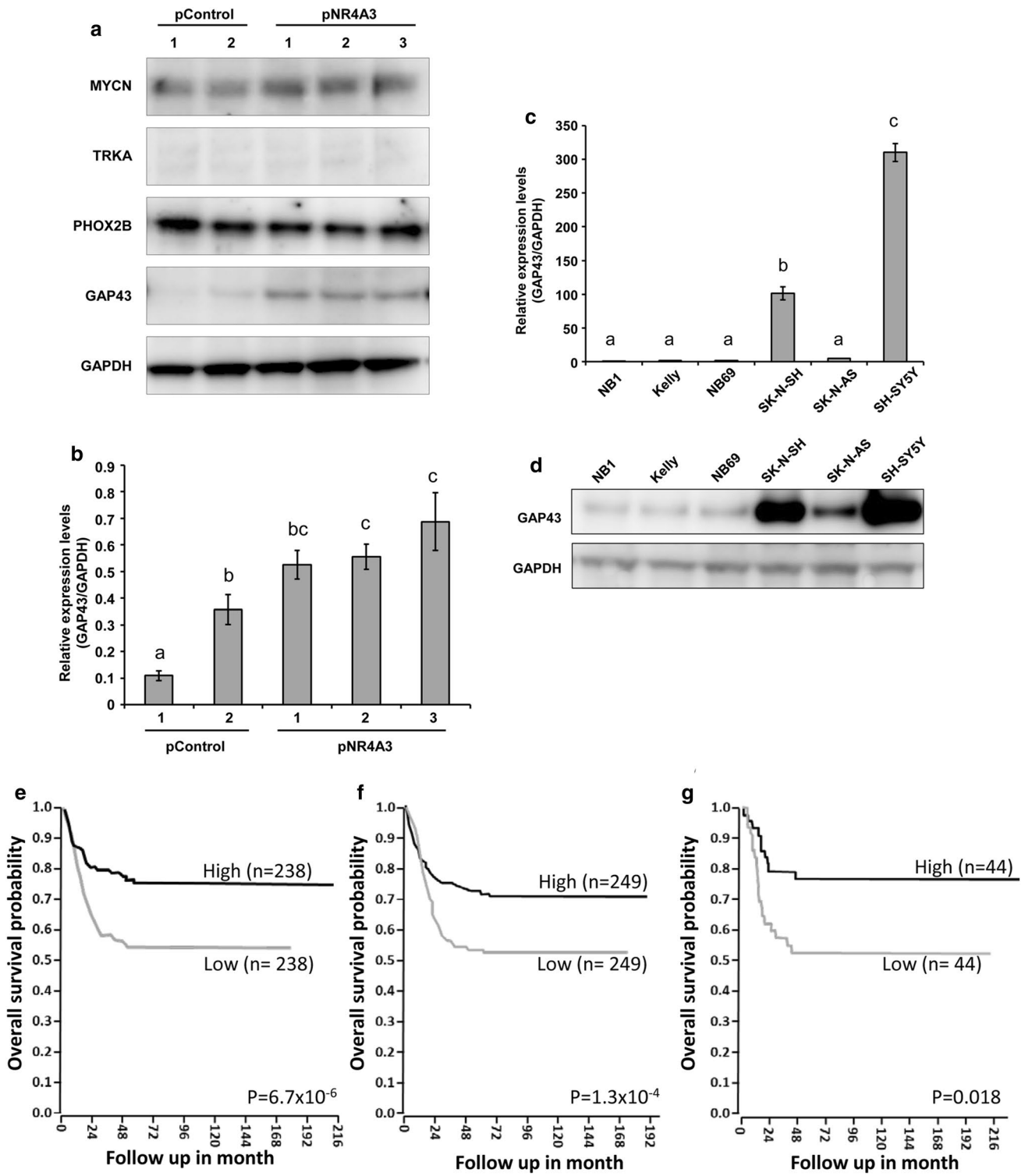

Fig. 4 Overexpression of NR4A3 upregulating the expression level of GAP43. **a** Protein expression levels of markers for neuroblastoma differentiation were analyzed by immunoblotting for NB1 clones stably transfected with control vector or with NR4A3 expression vector. **b** GAP43 mRNA in NB1 clones was analyzed by real-time PCR. **c** NR4A3 mRNA and **d** protein levels in NB1 clones stably transfected with control vector or with NR4A3 expression vector were analyzed by real-time PCR and immunoblotting, respectively. Values represent means ± SD of measurements performed in triplicate. Statistical significance was analyzed by one-way ANOVA with the Tukey's post hoc test. Values without the same lower case letters are significantly different ($P < 0.05$). **e**–**g** Kaplan–Meier survival analyses based on three independent public microarray data sets gse45547 (**e**), gse49710 (**f**) and gse16476 (**g**). Elevated expression level of GAP43 was associated with favorable prognosis in NB patients in all data sets

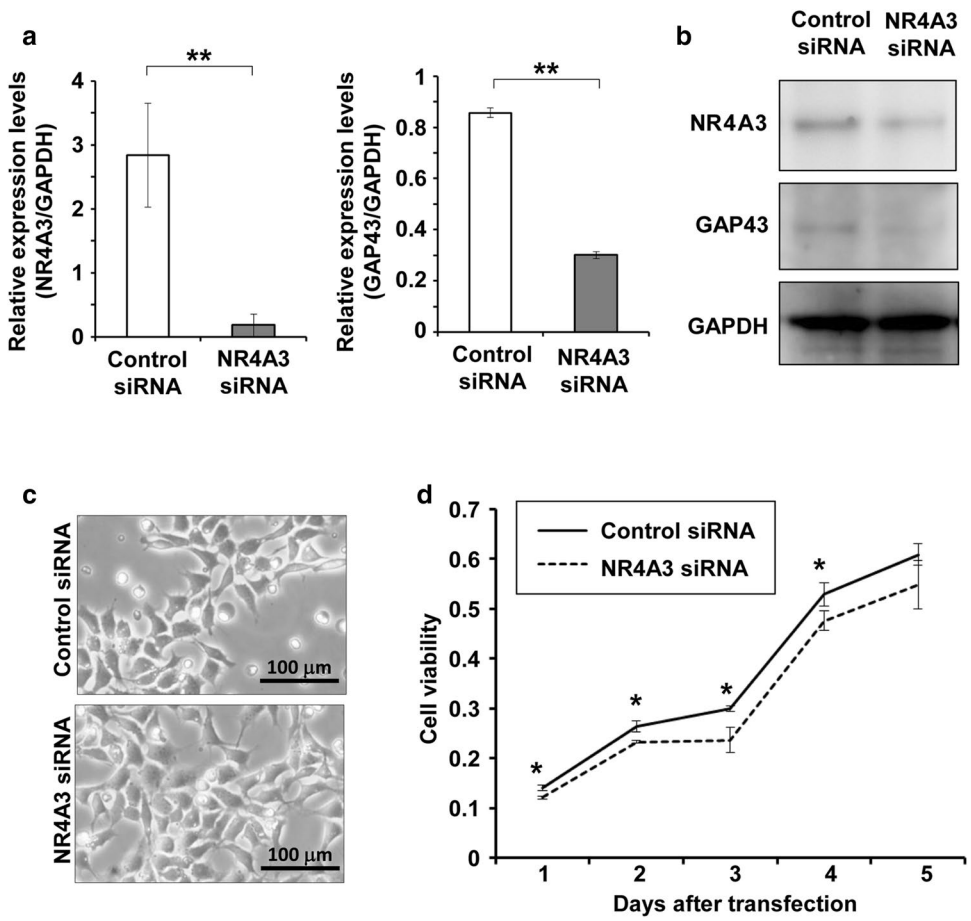

Fig. 5 Knockdown of NR4A3 downregulating the expression level of GAP43 in NB1 cells. NB1 cells were transfected with control siRNA or NR4A3 siRNA. Seventy-two hours after transfection, total RNA and cell lysate were prepared and analyzed by real-time PCR (**a**) or immunoblotting (**b**). **c** Representative images of NB1 cells transfected with control siRNA or NR4A3 siRNA are shown. **d** Cell viability was measured by WST8 assay at the indicated times after transfection with NR4A3 siRNA (dashed line) or control siRNA (solid line). The means ± SD of experiments performed in triplicate are presented. Statistical significance was analyzed by Student's *t* test. $^{*}P < 0.05$, $^{**}P < 0.01$

NR4A3-overexpressing clones than in control clones. All of the differences between control and NR4A3 overexpressing clones, except between control #2 and NR4A3 #1, were statistically significant.

We analyzed the expression levels of GAP43 in six NB cell lines to determine whether they were correlated with NR4A3 expression level. As shown in Fig. 4c, SK-N-SH and SH-SY5Y showed significantly higher levels of GAP43 mRNA expression than the other three cell lines, NB1, Kelly, and NB69, in which NR4A3 expression level was very low. SK-N-AS also showed significantly higher GAP43 expression level than NB1, Kelly, and NB69, on statistical analysis among NB cells other than SK-N-SH and SH-SY5Y. As the levels of GAP43 expression in SK-N-SH and SH-SY5Y were markedly higher than that in SK-N-AS, the difference between SK-N-AS and the three cell lines with low NR4A3 expression level was not determined as significant. As shown in Fig. 4d, GAP43 protein was highly expressed in SK-N-AS, SK-N-SH, and SH-SY5Y compared to the other three cell lines.

To examine the clinical significance of GAP43 in the prognosis of NB, Kaplan–Meier survival analysis utilizing public microarray datasets was performed. As shown in Fig. 4e–g, lower GAP43 expression level was significantly associated with shorter survival period in patients with NB, in all datasets analyzed.

siRNA-mediated knockdown of NR4A3 suppresses the expression of GAP43 in NB1 cells

To determine whether NR4A3 is involved in the regulation of GAP43 expression, we silenced NR4A3 in NB1 using siRNA. As shown in Fig. 5a, b, NR4A3 knockdown cells showed lower level of GAP43 expression compared to control cells. No obvious differences were observed in cell morphology between NR4A3 knockdown and control cells (Fig. 5c). NR4A3 knockdown cells showed significantly lower viability than control cells, but the difference was very small (Fig. 5d).

Discussion

The results of the present study showed that reduced expression of NR4A3 is associated with shorter survival period of NB in two out of three datasets in the public databases. An in vitro study showed that forced expression of NR4A3 in the human NB-derived cell line NB1 resulted in neurite

elongation along with overexpression of GAP43, a specific marker of differentiated NB. These results indicated that NR4A3 induces differentiation in NB cells.

NR4A3 is a member of the NR4A nuclear receptor family involved in a large number of biological functions, including metabolism, angiogenesis, inflammation, cell proliferation and apoptosis [6]. Although other members of NR4A subfamily, NR4A1 and NR4A2, have been implicated in the progression of cancer, several reports suggested that NR4A3 may act as a tumor suppressor [6, 7]. Reduced levels of NR4A1 and NR4A3 expression in leukemic blasts were observed in patients with AML [16], and mice deficient in both NR4A1 and NR4A3 were shown to develop AML [16]. Recently, it was reported that long noncoding RNA BREAS1 suppresses growth and survival of lung cancer cells by upregulating NR4A3 [18]. Analyses using breast cancer cell line, it was demonstrated that expression of NR4A3 could be activated by tumor suppressor p53 directly, and that NR4A3 suppresses proliferation and promotes apoptosis of cells by inducing the pro-apoptotic genes, PUMA and BAX [19]. These observations strongly suggested that NR4A3 has a tumor suppressor function. Meanwhile, Haller et al. reported that genome translocation in AciCC resulted in upregulation of NR4A3 expression, and NR4A3 could activate proliferation of salivary gland cells [14]. In the tissues of triple-negative breast cancer, which is prone to distant metastasis, expression level of NR4A3 was higher than that in the tissues of less-metastatic luminal subtypes [13]. These reports support the oncogenic functions of NR4A3.

The findings of the present study indicated that NR4A3 has a tumor suppressor function in NB. Although we could not clarify the effects of NR4A3 on cell viability because of the relatively huge variation of the results among the clones, we found that all three NR4A3-overexpressing clones showed significantly elongated neurites and upregulated expression of GAP43 compared to control clones. Interestingly, we did not observe any differences in expression levels of other differentiation markers between NR4A3-overexpressing and control clones. MYCN is a transcription factor that regulates target genes involved in fundamental cellular processes, such as cell proliferation, differentiation, and cell death [20]. MYCN amplification has been shown to be associated with poor prognosis of NB [21, 22]. PHOX2B is a transcription factor crucial for the development of the nervous system, and is known to promote proliferation of NB cells in vitro and in vivo [23]. TRKA is a member of the neurotrophic tyrosine kinase receptor family, which is known to be one of the receptors for nerve growth factor (NGF) and to play a crucial role in nervous system development [24]. High levels of TRKA expression in NB were reported to be associated with favorable prognosis [25]. Consistent with these observations, increased expressions of MYCN and PHOX2B, and reduced expression of TRKA were known to be associated with differentiated phenotypes of NB cells [26–28]. However, no clear differences in expression levels of these markers were observed between NR4A3 overexpressing clones and control clones.

Among the differentiation markers analyzed, only GAP43 was shown to be upregulated in all of the NR4A3-overexpressing clones. GAP43 also plays important roles in development and regeneration of neuronal networks via regulation of axonal outgrowth [29]. GAP43 is localized to the inner surface of the plasma membrane of growing axons in neuronal cells, especially at axon terminals [30]. When GAP43 is phosphorylated by PKC in the presence of a high concentration of Ca^{2+}, it interacts with cytoskeletal molecules, followed by axon elongation [31]. In NB, enhanced expression of GAP43 has been observed on differentiation of NB cells by treatment with retinoic acid [32] or transfection with RET gene [33]. The present observations in which forced expression of NR4A3 activated only GAP43 but not MYCN, TRKA, and PHOX2B, indicated that NR4A3 directly activates the expression of GAP43 and induces differentiated phenotypes of NB cells, without affecting the upstream signals regulating GAP43 expression and NB differentiation.

It has been reported that NR4A3 is also involved in the development of nervous system. NR4A3 knockout mice showed failure in postnatal development of hippocampus, caused by the abnormality in neuronal survival and axon guidance [34]. Previously, we have found that NR4A3 expression was increased during postnatal brain development in mice [17]. These observations suggest the possibility that NR4A3 activates neurite outgrowth in NB and normal neuron, at least in part through activating GAP43 expression. The observation that lower expression of GAP43 is associated with shorter survival period of NB in the public databases also indicates this possibility. Expression of GAP43 mRNA is regulated both transcriptionally and post-transcriptionally. For transcriptional regulation, the E-box located in the promoter region of GAP43 has been shown to play a pivotal role in regulation of axon outgrowth [35]. As posttranscriptional regulation, GAP43 is stabilized by the neuron-specific RNA binding protein HuD [36]. At present, we do not have data suggesting that NR4A3 could bind to the E-box or increase the expression level of HuD. Further analyses are needed to elucidate the precise mechanism by which NR4A3 upregulate GAP43.

In conclusion, the present study indicated that reduced expression of NR4A3 is associated with poor prognosis in NB patients, and that forced expression of this gene results in a differentiated phenotype, along with increased expression of GAP43. These data indicate that NR4A3 exerts its tumor suppressive effects by regulating GAP43 expression.

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Compliance with ethical standards

Conflict of interest None.

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