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NeuroD1 promotes neuroblastoma cell growth by inducing the expression of ALK

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Key words

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Neuroblastoma is derived from the sympathetic neuronal lineage of neural crest cells, and is the most frequently observed of the extracranial pediatric solid tumors. The neuronal differentiation factor, NeuroD1, has previously been shown to promote cell motility in neuroblastoma by suppressing the expression of Slit2. Here we report that NeuroD1 is also involved in the proliferation of neuroblastoma cells, including human cell lines and primary tumorspheres cultured from the tumor tissues of model mice. Interestingly, the growth inhibition of neuroblastoma cells induced by knockdown of NeuroD1 was accompanied by a reduction of ALK expression. ALK is known to be one of the important predisposition genes for neuroblastoma. The phenotype resulting from knockdown of NeuroD1 was suppressed by forced expression of ALK and, therefore, NeuroD1 appears to act mainly through ALK to promote the proliferation of neuroblastoma cells. Furthermore, we showed that NeuroD1 directly bound to the promoter region of ALK gene. In addition, the particular E-box in the promoter was responsible for NeuroD1-mediated ALK expression. These results indicate that ALK should be a direct target gene of NeuroD1. Finally, the expressions of NeuroD1 and ALK in the early tumor lesions of neuroblastoma model mice coincided *in vivo*. We conclude that the novel mechanism would regulate the expression of ALK in neuroblastoma and that NeuroD1 should be significantly involved in neuroblastoma tumorigenesis.

Neuroblastoma (NB) is the most frequently occurring of the extracranial pediatric solid tumors derived from the sympathetic neuronal lineage of neural crest cells.[1] It accounts for around 15% of pediatric cancer-related deaths, and its prognosis remains poor. The oncogenic transcription factor MYCN is considered to be one of the most important genes involved in the tumorigenesis of NB. Amplification of the MYCN gene is a strong prognostic factor. MYCN transgenic (Tg) mice have been used as a useful and reliable model of NB.[2] Through the analysis of MYCN Tg mice, we previously identified NeuroD1 as a novel gene involved in NB.[3] NeuroD1 is a transcription factor containing a basic helix-loop-helix motif,[4] and has been shown to be involved in neurogenesis. In Xenopus, ectopic NeuroD1 can convert ectoderm into neurons.[5] In mice, NeuroD1-knockout has been shown to result in severe neuronal deficit in the granule layers of the cerebellum and hippocampus.[6,7] NeuroD1 is also essential for the development of inner ear sensory neurons,[8,9] retinal development,[10] and the maturation and survival of adult-born neurons in the hippocampus and olfactory bulb.[11,12] In addition to the functional expression of NeuroD1 in normal development, NeuroD1 has been found to be anomalously upregulated in several aggressive neural and neuroendocrine cancers, including small-cell lung cancer, medulloblastoma, and gastric and prostate cancers.[13] In aggressive neuroendocrine lung tumors, NeuroD1 promotes both tumor cell survival and metastasis. In our previous report,[3] we identified that NeuroD1 was highly expressed in the tumor cells of MYCN Tg mice. In addition, knockdown of NeuroD1 in NB cell lines was accompanied by the induction of Slit2 expression, and resulted in motility inhibition. This inhibition was cancelled by simultaneous knockdown of Slit2, which indicated the significance of Slit2 as a downstream factor of NeuroD1 to regulate cell motility. Here we report the involvement of NeuroD1 in the proliferation of NB cells. Our results show that NeuroD1 directly induces the expression of ALK, a crucial predisposition gene for NB, and promotes cell proliferation.

Materials and Methods

Cell culture. The human NB cell line SH-SY5Y was obtained from the American Type Culture Collection (Manassas, VA, USA). IMR-32 and SK-N-AS cells were procured from RIKEN (Tsukuba, Japan). They were cultured with DMEM supplied with 10% heat-inactivated FBS in an incubator with humidified air at 37°C with 5% CO_2. On receiving the cell lines, we prepared the frozen stock within one to two passages. Every 1 to 2 months, we thawed the stock to maintain the original condition. The stocks were routinely authenticated on the basis of viability, growth rate and morphology by microscopic examination.

Animals. MYCN Tg mice were maintained in our animal facility under a controlled environment and fed with standard nourishment and water. They were backcrossed with

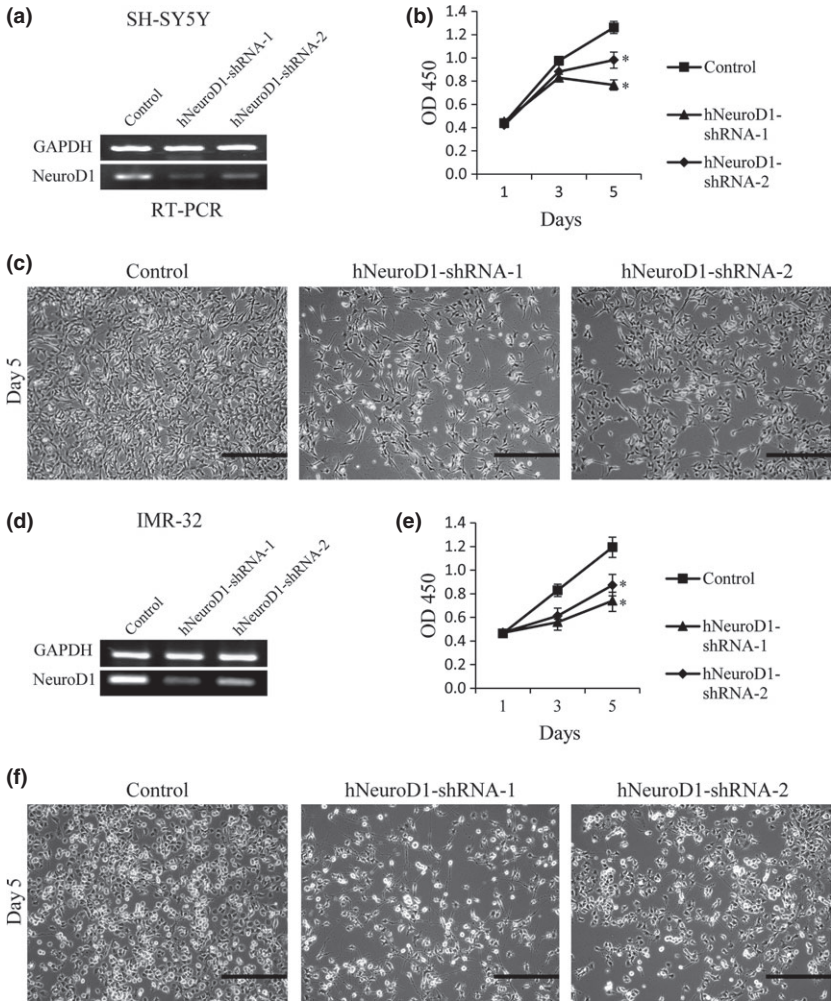

Fig. 1. Knockdown of NeuroD1 suppresses the proliferation of neuroblastoma (NB) cell lines. (a) Two independent shRNA were used to knockdown NeuroD1 in SH-SY5Y cells. NeuroD1 mRNA levels were confirmed by RT-PCR. (b) The proliferation of NeuroD1-knockdown cells was examined. At day 0, SH-SY5Y cells were infected with shRNA-expressing lentivirus. $*P < 0.001$. (c) The phase contrast images of cells at day 5 in (b). Scale bar, 200 μm. (d–f) The same experiments with IMR-32 cells as (a), (b) and (c), respectively.

129^{+Ter}/SvJcl mice (CLEA, Tokyo, Japan). The present study was approved by the Animal Care and Use Committee of the Nagoya University Graduate School of Medicine.

RNA interference. Non-targeting shRNA (Sigma, St. Louis, MO, USA) and shRNA specific for human or mouse NeuroD1 were used. The shRNA sequences are listed in Supplementary Table S1. The lentivirus packaging was performed as previously described.[3]

RT-PCR and real-time PCR. RT-PCR and real-time PCR were carried out as previously described.[3] The primer sequences and annealing temperatures are listed in Supplementary Table S2. The expression levels of each gene were normalized by GAPDH.

Immunohistochemistry and western blot analysis. Immunohistochemistry and western blot were carried out according to the previous report.[3] For western blot analysis, anti-NeuroD1 (1:500; Cell Signaling Technology, Danvers, MA, USA), anti-Akt (1:500; Cell Signaling Technology), anti-P-Akt (1:500; Cell Signaling Technology) and anti-ALK (1:500; Cell Signaling Technology) antibodies were used.

Plasmids. The expression vector of human NeuroD1 was previously constructed.[3] The CMV-ALK-tGFP plasmid was purchased from Thermo Scientific (Waltham, MA, USA).

Luciferase reporter assay. The ALK promoter region from −216 to +30 amplified from genomic DNA of 293 cells was cloned into pGL4.74 (Promega, Madison, Wisconsin USA). E-box3 (−44, cagctg), E-box4 (−38, caagtg) and E-box5 (−19, cagatg) were mutated as (cggatc), (gaattc) and (ccgcgg), respectively.

293T cells (5×10^4) were seeded in 24-well cell culture plates and allowed to adhere overnight. Cells were then co-transfected with NeuroD1 expression plasmid, pGL4.74 (firefly) reporter and pRL-CMV (renilla) control reporter by using FuGENE HD (Promega). Total amounts of plasmid DNA per well were kept constant by adding empty plasmid pcDNA3.1. A total of 48 h after transfection, cells were lysed, and both firefly and renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay system (Promega), according to the manufacturer's instructions. The firefly luminescence signals were normalized by those of renilla.

ChIP assay. ChIP assay with SH-SY5Y cells was carried out as previously described.[3] The following primers were used: F1: 5′-aatctatgtctgccacttcc-3′, R1: 5′-aacaaacctgcacattgtgc-3′, F2: 5′-tatgagttctgtgttggcag-3′, R2: 5′-gaatggtgtctgaacatgtg-3′, F3: 5′-tgcataggagccgatcgagc-3′, R3: 5′-agagccgctggatcgcatct-3′.

Primary culture of tumorspheres and neurospheres. The previous procedure was slightly modified as follows.[3] Approximately 0.5 cm^3 of tumor tissue was dissected from primary tumors of MYCN Tg mice. After washing, the tissue was minced and digested with 0.25% trypsin (Sigma) for 15 min, and the digestion was stopped by adding trypsin inhibitor (Sigma). The cells were centrifuged and washed twice. The

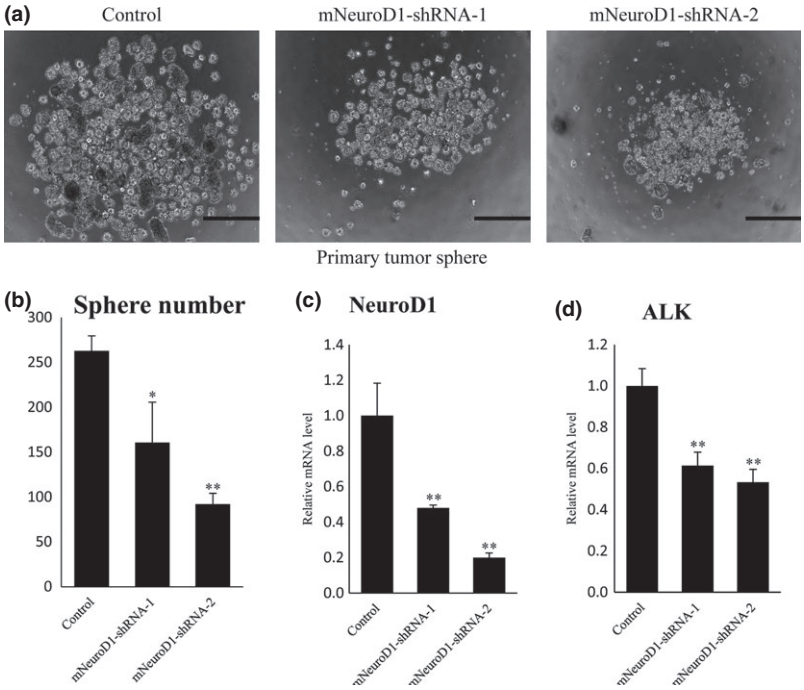

Fig. 2. NeuroD1 is involved in the proliferation of tumorsphere cells cultured from MYCN Tg mice. (a) NeuroD1 in tumorsphere cells cultured from tumor tissues of MYCN Tg mice was knocked down by two independent shRNA. Sphere cells were infected with shRNA-expressing lentivirus, and were dissociated into single cells. 4 days later, the phase contrast pictures were taken. Scale bar, 200 μm. (b) The sphere numbers in (a) were counted. (c) The mRNA levels of NeuroD1 examined by real-time PCR. (d) The mRNA levels of ALK examined by real-time PCR. $*P < 0.01$. $**P < 0.001$.

supernatant was collected into a new tube and centrifuged. To eliminate red blood cells, the pellet was treated with RBC lysis buffer (Biolegend, San Diego, CA, USA) according to the manufacturer's instructions. The collected cells were cultured in a Petri dish with DMEM/F12 HAM (Sigma) plus 10 ng/mL EGF, 15 ng/mL bFGF (Peprotech, Rocky Hill, NJ, USA), 2% B27 supplement, 1% penicillin/streptomycin (GIBCO, Rockville, MD, USA), 15% FBS, 1% non-essential amino acid (NEAA), 1% sodium pyruvate and 55 μM β-mercaptoethanol. The tumorspheres were digested with trypsin and dissociated into single cells by pipetting with a 1-mL tip and were passaged every 3–4 days. Embryonic neural stem cells were prepared from an E13.5 ICR mouse (Japan SLC, Hamamatsu, Japan). Briefly, the hippocampus of an embryonic mouse was isolated and minced. Then, the tissue was washed with HBSS and centrifuged twice. The tissue was then re-suspended with HBSS, plated and left to stand for 10 min. The supernatant was collected and centrifuged. The cell pellet was collected and used as embryonic neural stem cells. These cells were cultured in DMEM/F12 HAM supplemented with B27, EGF, bFGF and penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) in a 37°C 5% CO_2 tissue culture incubator. Successfully formed neurospheres were dissociated with a NeuroCult Chemical Dissociation Kit

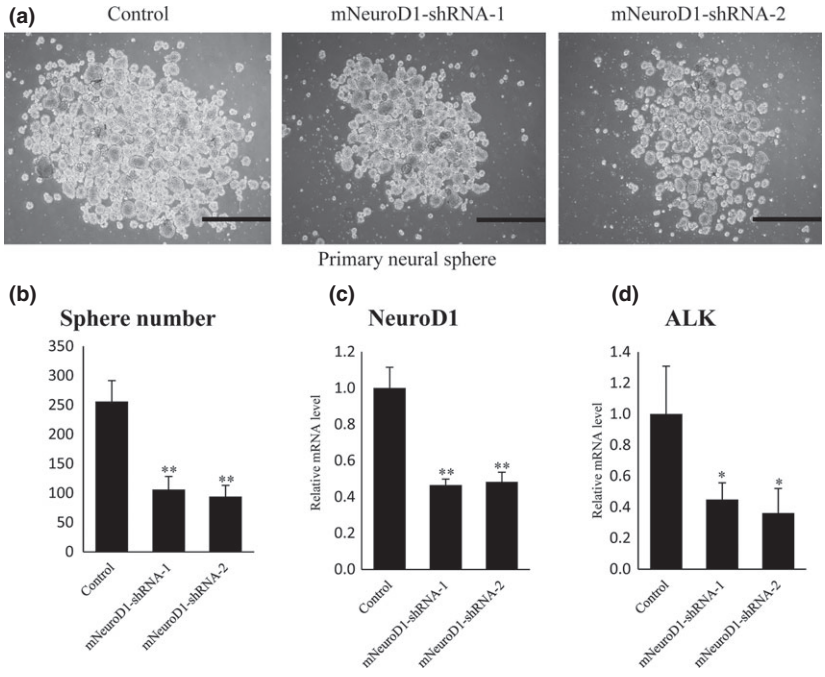

Fig. 3. Relationship of NeuroD1 and ALK in neurospheres. (a) NeuroD1 in neurosphere cells cultured from the hippocampus region of wild-type E13.5 embryos was knocked down by two independent shRNA. 4 days later, the phase contrast pictures were taken. Scale bar, 200 μm. (b) The sphere numbers in (a) were counted. (c) The mRNA levels of NeuroD1 were examined by real-time PCR. (d) The mRNA levels of ALK were examined by real-time PCR. $*P < 0.05$. $**P < 0.01$.

(Stem Cell Technologies, Vancouver, BC, Canada) into single cells according to the manufacturer's instructions and were passaged every 3–4 days.

Cell proliferation assay. Cells (5×10^3) were seeded in 96-well cell culture plates and allowed to adhere overnight. At each time point, the cell number was estimated using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions.

Results

Knockdown of NeuroD1 suppresses the proliferation of NB cell lines. We examined whether the proliferation of NB cells was affected after knockdown of NeuroD1. When NeuroD1 was knocked down by two different shRNA (Fig. 1a), the proliferation of SH-SY5Y cells was significantly suppressed (Fig. 1b,c). This growth suppression was observed 5 days after infection with shRNA-expressing lentivirus. The same results were obtained with IMR-32 cells (Fig. 1d–f). The oncogenic transcription factor MYCN gene is amplified and highly expressed in IMR-32 cells, but is not amplified and is rarely expressed in SH-SY5Y cells. These results indicate that NeuroD1 is required for the proliferation of NB cells regardless of their MYCN status.

NeuroD1 is involved in the proliferation of tumorsphere cells cultured from MYCN Tg mice. To further evaluate the involvement of NeuroD1 in the proliferation of NB cells, we next examined the tumorsphere cells cultured from MYCN Tg mice. Spheroid culture from tumor tissues is considered to be a method for selectively culturing tumor-initiating cells.[14] It has been reported that tumorsphere cells cultured from the tumor tissues of MYCN Tg mice efficiently developed subcutaneous tumors in nude mice.[3,15] The characterization of those tumorsphere cells could be helpful in addressing the molecular mechanism of NB tumorigenesis. Here we knocked

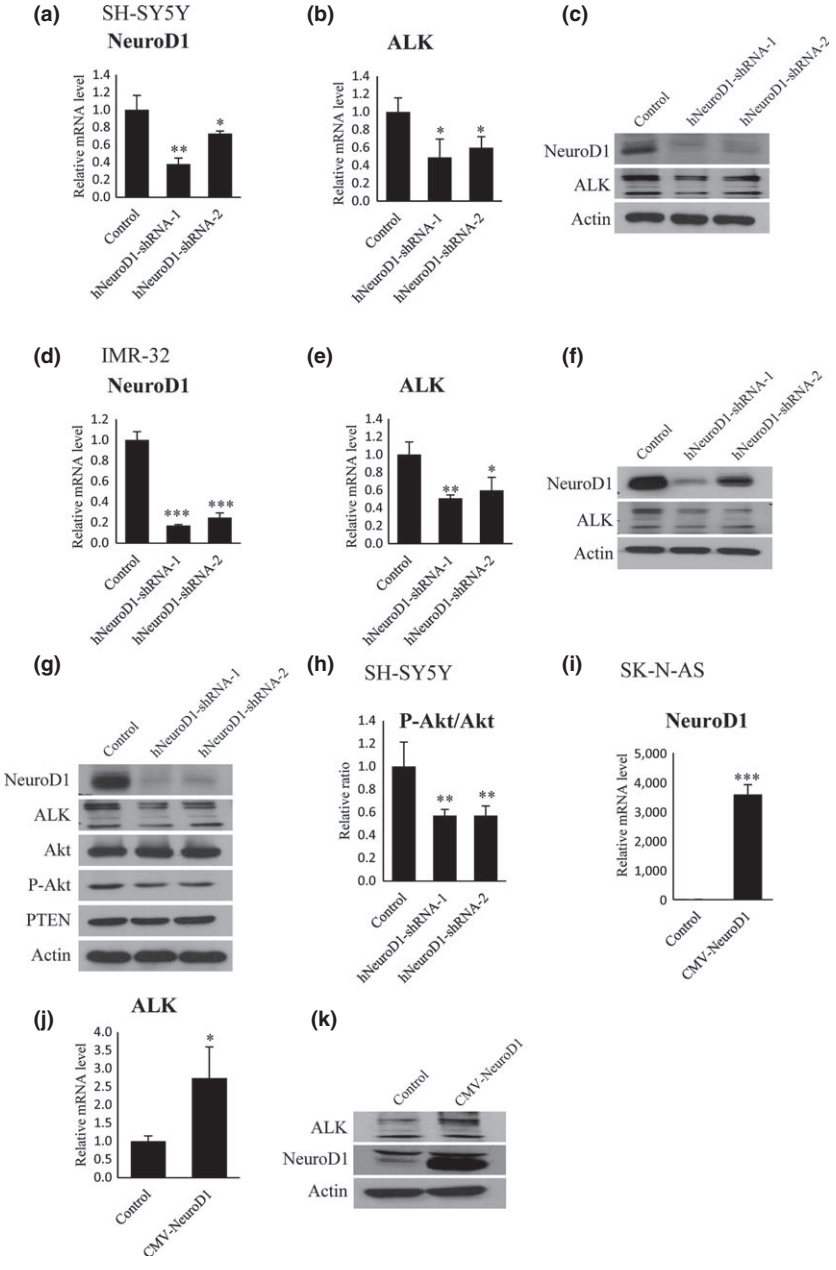

Fig. 4. Relationship of NeuroD1 and ALK in human NB cell lines. (a) NeuroD1 was knocked down in SH-SY5Y cells, and the expression level of NeuroD1 was examined by real-time PCR. (b) The expression level of ALK was examined by real-time PCR. (c) The protein levels of NeuroD1 and ALK were examined by western blotting. (d–f) The same experiments with IMR-32 cells as (a), (b) and (c), respectively. (g) NeuroD1 was knocked down in SH-SY5Y cells, and phosphorylated Akt (P-Akt) was detected by western blotting. (h) The ratio between P-Akt and Akt was measured based on densitometry by ImageJ. (i–k) NeuroD1 was overexpressed in SK-N-AS cells. The mRNA and protein levels of Neurod1 and ALK were examined by real-time PCR (i, j) and western blotting (k), respectively. $*P < 0.05$. $**P < P < 0.01$. $***P < 0.001$.

down NeuroD1 in tumorsphere cells with two different shRNA (Fig. 2c), and examined the effect on the sphere formation. The results showed that knockdown of NeuroD1 was associated with a significant reduction in the sphere numbers (Fig. 2a,b). The self-renewing ability of tumorsphere cells might be impaired in the absence of NeuroD1 expression. Interestingly, we found that ALK expression was also diminished in the NeuroD1-knockdown tumorsphere cells (Fig. 2d). ALK is one of the important predisposition genes for NB. The germ line and somatic hyperactive mutations have been identified and shown to be involved in the tumorigenesis of NB.[16–19] Our result that ALK expression was concomitantly reduced in response to the knockdown of NeuroD1 in tumorsphere cells suggests that there is some relationship between ALK and NeuroD1 in NB cells.

Relationship of NeuroD1 and ALK in neurospheres. In NeuroD1-knockout mice, severe neuronal deficits appear in the hippocampus.[6,7] We investigated the involvement of NeuroD1

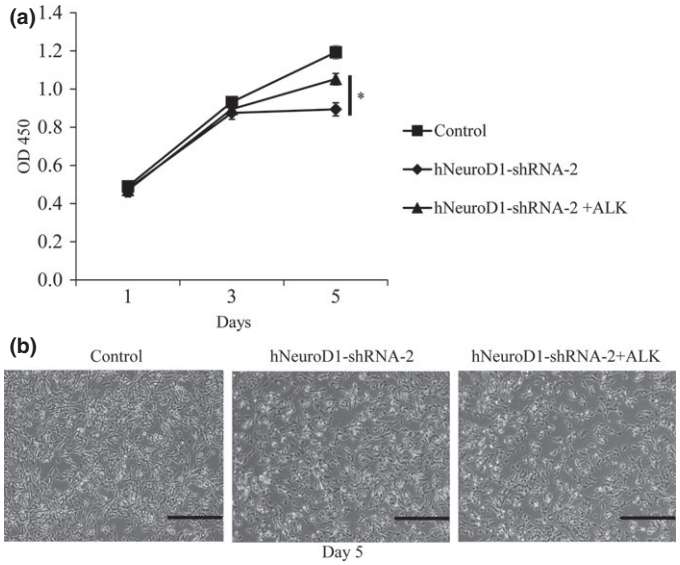

Fig. 5. Restoration of ALK expression can rescue NeuroD1 deficiency. (a) SH-SY5Y cells were infected with the lentiviruses expressing shRNA-2 against NeuroD1 and exogenous ALK, respectively. Then, the cell proliferation was assayed. *$P < 0.001$. (b) The phase contrast images of cells at day 5 in A. Scale bar, 200 μm.

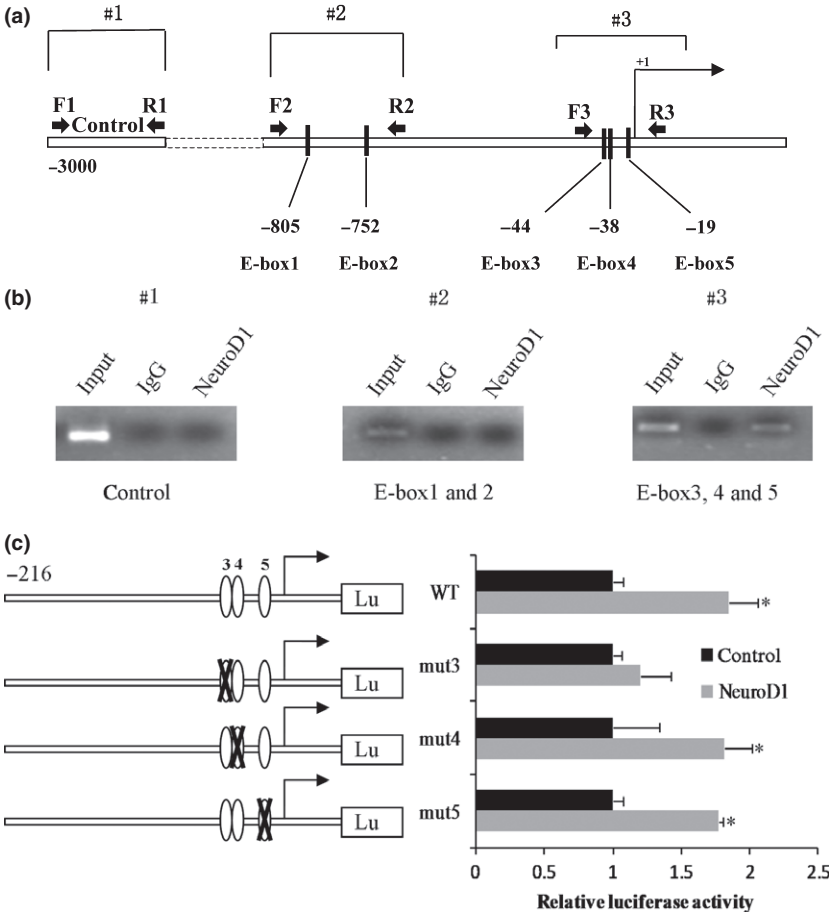

Fig. 6. NeuroD1 binds to the promoter region of ALK to induce its expression. (a) E-boxes in the promoter region of the ALK gene. There are five possible E-boxes (1–5). The black arrows are the primers used for ChIP assay. (b) ChIP assay with three sets of primers (#1–#3). The #3 PCR amplifying the region containing E-box3–5 exhibited the positive result. (c) The ALK promoter region containing E-box3, 4 and 5 was cloned into luciferase reporter construct (WT). Each E-box was mutated in mut3, mut4 and mut5 constructs, respectively. 293T cells were transfected with WT or mutated versions (mut3, 4, 5) of luciferase reporter constructs together with NeuroD1 expression vector. The graphs show the relative luciferase activity. *$P < 0.01$.

in neurospheres cultured from the hippocampus region of E13.5 embryos. Because NB would arise from immature neuronal progenitor cells, it is likely that a similar molecular mechanism exists to maintain both neurospheres and tumorspheres. Indeed, we found that the knockdown of NeuroD1 in neurospheres (Fig. 3c) resulted in suppression of both sphere formation (Fig. 3a,b) and ALK mRNA level (Fig. 3d), which is consistent with the results in tumorspheres (Fig. 2). NeuroD1, and its relationship to ALK, might be necessary for the maintenance of tumor and neurosphere cells.

Relationship of NeuroD1 and ALK in human NB cell lines. Next, we confirmed the molecular relationship between NeuroD1 and ALK in human NB cell lines. In SH-SY5Y cells (Fig. 4a–c) and IMR-32 cells (Fig. 4d–f), the knockdown of NeuroD1 by two different shRNA (Fig. 4a,d) suppressed the ALK expression at both the mRNA (Fig. 4b,e) and protein levels (Fig. 4c, f). In addition, the activity of Akt, the downstream molecule of ALK signaling, was simultaneously attenuated (Fig. 4g,h). In contrast, the ectopic expression of NeuroD1 in SK-N-AS cells (Fig. 4i), which show very low endogenous expression of NeuroD1, increased the ALK expression (Fig. 4j,k). These results indicated that NeuroD1 induced the expression of ALK not only in the spheroid cells cultured from the mouse model but also in human NB cells.

Restoration of ALK expression can rescue NeuroD1 deficiency. Because the knockdown or overexpression of NeuroD1 was accompanied by a decrease or an increase in ALK expression, respectively (Fig. 4), ALK would function in the region downstream of NeuroD1. Next, therefore, we investigated the significance of ALK as a downstream factor of NeuroD1 signaling. As shown in Figure 1, the knockdown of NeuroD1 in NB cell lines resulted in significant growth suppression. In contrast, when ALK was exogenously expressed in the NeuroD1-knockdown NB cells, their proliferations were significantly restored (Fig. 5a,b). These results suggested that ALK could be a significant downstream factor of NeuroD1 signaling involved in promoting cell proliferation.

NeuroD1 directly binds to the promoter region of ALK and induces its expression. As NeuroD1 is a bHLH transcription factor, it can bind to E-box in the promoter region of target genes. In the promoter region of ALK, we found five possible E-boxes (E-box1 to 5 [Fig. 6a]). In order to examine the binding of NeuroD1 to those E-boxes, we performed a ChIP assay. The results suggested that NeuroD1 could bind to the region containing E-box3, 4 and 5 (Fig. 6a,b). Next, for the purpose of addressing the involvement of these E-boxes, we carried out a reporter assay. The ectopic NeuroD1 induced the luciferase reporter expression driven by the WT promoter containing three intact E-boxes (E-box3, 4 and 5 [Fig. 6c]). The mutation in E-box3 (mut3) resulted in impairment of NeuroD1-induced reporter expression (Fig. 6c). In contrast, mutations in E-box4 (mut4) or E-box5 (mut5) showed no significant effects. These results suggest that NeuroD1 binds to E-box3 and induces the expression of ALK.

Expression patterns of NeuroD1 and ALK in MYCN Tg mice. Finally, we investigated the physiological evidence supporting a molecular relationship between NeuroD1 and ALK in NB tumorigenesis. As we previously reported, NeuroD1 was highly expressed in the early lesions of NB in MYCN Tg mice (Fig. 7a).[3] Here we found that the expression pattern of ALK was consistent with those of NeuroD1 (Fig. 7b). These expression patterns *in vivo* support the notion that NeuroD1 induces the expression of ALK and promotes the proliferation of NB cells.

Discussion

NeuroD1 is a transcription factor involved in neuronal differentiation. We previously identified NeuroD1 as an NB-related gene that promotes cell motility by downregulating Slit2 expression.[3] Here we report another function of NeuroD1 in NB. NeuroD1 is required for the proliferation of both human NB cell lines and tumorspheres cultured from MYCN Tg mice. In addition, ALK would be a critical and direct downstream factor of NeuroD1 signaling to promote proliferation. Seo *et al.*[20] previously identified ALK as a candidate direct transcriptional target of NeuroD1. They first screened the genes whose expression was induced by NeuroD1, and determined

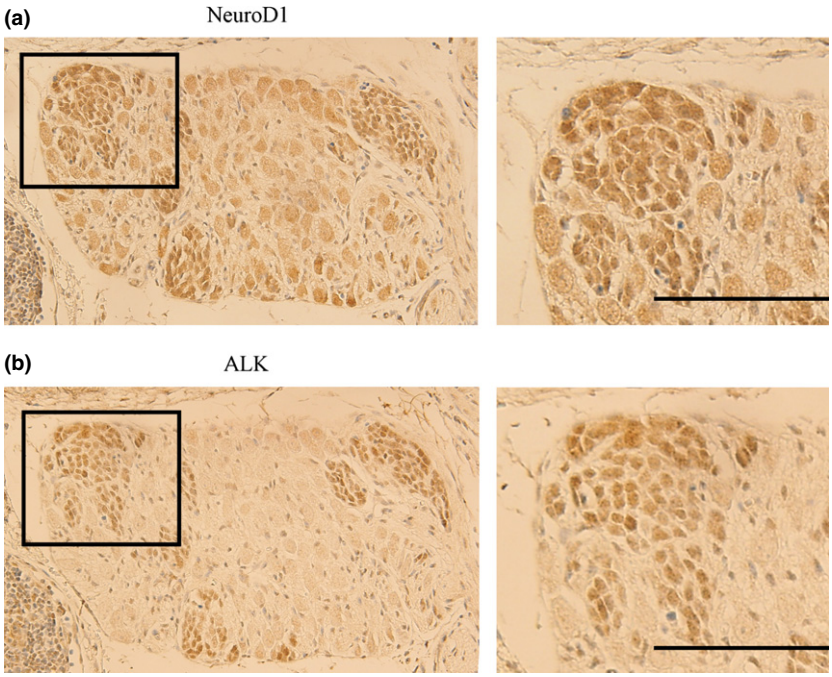

Fig. 7. The expression patterns of NeuroD1 and ALK in MYCN Tg mice. (a) The superior mesenteric ganglion from 2-week-old MYCN Tg mice was embedded in paraffin and sectioned, then subjected to immunohistochemistry with anti-NeuroD1 antibody. The boxed region in left was magnified in the right picture. The results are the representatives from those of five independent mice. Scale bar, 100 μm. (b) Immunohistochemistry with anti-ALK antibody. Scale bar, 100 μm.

the consensus E-box sequence recognized by NeuroD1. Based on the consensus sequence, they further screened putative target genes whose promoters contained the consensus E-box. As a result, ALK was included in the list of putative candidate genes. This report is consistent with our present data. We revealed that NeuroD1 binds to the promoter region of ALK, and indentified the particular E-box that is involved in the expression of ALK.

ALK is an important predisposition gene, whose mutation or amplification has been identified in NB patients. In terms of the mechanism for the expression of ALK in NB cells, it has recently been reported that MYCN directly induces the transcription of ALK to promote NB tumorigenesis.[21] Because both MYCN and NeuroD1 are considered to recognize and bind to the same DNA sequence, E-box, it is likely that these two transcription factors independently regulate the transcription of ALK. Addressing their contribution to the ALK expression in NB cells is an important research subject.

For the purpose of understanding the molecular mechanism of NB tumorigenesis, it is thought to be important to investigate the mechanism of normal development of sympathetic neurons. The tumorigenesis and normal development should mainly be regulated by common molecular machinery, because NB specifically arises from immature progenitor cells that belong to the sympathoadrenal lineage of neural crest cells. A particular failure in the machinery of normal development should be a trigger of NB tumorigenesis. So far, although we have reported the involvement of NeuroD1 in NB tumorigenesis, its role in the normal development of sympathetic neurons has not been elucidated. Because ALK could be a potent molecular target of NB therapy, elucidation of the NeuroD1-ALK axis should be beneficial for clinical purposes. In NB patients, a high expression of NeuroD1 is closely related to poor prognosis.[3] The simultaneous promotion of migration and proliferation by NeuroD1 might be important for tumorigenesis in NB.

Acknowledgments

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Disclosure Statement

The authors have no conflict of interest to declare.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Table S1. Sequences of shRNA for target genes

Table S2. Sequences of primer for PCR experiments