主論文の要旨

NeuroD1 promotes neuroblastoma cell growth by inducing the expression of ALK

（NeuroD1はALKの発現誘導を介して神経芽腫細胞の増殖を促進する）

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Introduction

Neuroblastoma (NB) is the most frequently occurring of the extracranial pediatric solid tumors derived from the sympathetic neuronal lineage of neural crest cells. It accounts for around 15% of pediatric cancer-related deaths, and its prognosis still remains poor. MYCN transgenic (Tg) mice have been utilized as a useful and reliable model of NB. Through the analysis of MYCN Tg mice, we previously identified NeuroD1 as a novel gene involved in NB. NeuroD1 is a transcription factor containing a basic helix-loop-helix motif, and has been shown to be involved in neurogenesis. In Xenopus, ectopic NeuroD1 can convert ectoderm into neurons. In mice, NeuroD1-knockout has been shown to result in severe neuronal deficit in the granule layers of the cerebellum and hippocampus. In addition to the functional expression of NeuroD1 in normal development, NeuroD1 was found to be anomalously upregulated in several aggressive neural and neuroendocrine cancers, including small-cell lung cancer, medulloblastoma, gastric and prostate cancers. In aggressive neuroendocrine lung tumors, NeuroD1 promotes both tumor cell survival and metastasis. In our previous report, we identified that NeuroD1 was highly expressed in the tumor cells of MYCN Tg mice. Knockdown of NeuroD1 in NB cell lines was accompanied by the induction of Slit2 expression, and resulted in motility inhibition. 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, IMR-32 and SK-N-AS cells were cultured with DMEM supplied with 10% heat-inactivated FBS in an incubator with humidified air at 37°C with 5% CO_2.

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 129^{+Ter}/SvJcl mice (CLEA, Tokyo, Japan).

Luciferase reporter assay. 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, USA). Total amounts of plasmid DNA per well were kept constant by adding empty plasmid pcDNA3.1. The firefly luminescence signals were normalized by those of renilla.

ChIP assay. ChIP assay with SH-SY5Y cells was carried out as previously described. The following primers are 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. Around 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, St. Louis, MO) for 15 min, and the digestion was stopped by adding trypsin inhibitor (Sigma). The supernatant was collected into a new tube and centrifuged. The collected cells were cultured in a petri dish with DMEM/F12 HAM (Sigma) plus EGF 10 ng/ml, bFGF 15 ng/ml (Peprotech, Rocky Hill, NJ), 2% B27 supplement, 1% penicillin/streptomycin (GIBCO, Rockville, MD), 15% FBS, 1% non-essential amino acid (NEAA), 1% sodium pyruvate, and 55 μM β-mercaptoethanol. The hippocampus region of an embryonic mouse was isolated and minced. These cells were cultured in DMEM/F12 HAM supplemented with B27, EGF, bFGF and penicillin/streptomycin (Invitrogen, Carlsbad, CA) in a 37°C, 5% CO_2 tissue culture incubator.

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 with Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions.

Results

In previous study, we identified NeuroD1 as a novel gene involved in cell motility of NB. 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. Furthermore, we found that the growth inhibition of neuroblastoma cells induced by knockdown of NeuroD1 was accompanied by a reduction of ALK expression. Because the phenotype resulting from knockdown of NeuroD1 was suppressed by forced expression of ALK, NeuroD1 appears to act mainly via ALK to promote the proliferation of neuroblastoma cells. In addition, our results show that NeuroD1 directly bound to the promoter region of ALK gene, and the particular E-box in the promoter was turned out to be 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*. Taken together, our finding provides the evidence that NeuroD1 could play important role during NB tumorigenesis through regulating expression of ALK.

Discussion

NeuroD1 is a transcription factor involved in neuronal differentiation. We previously identified NeuroD1 as a NB-related gene that promotes cell motility by downregulating Slit2 expression. 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. In this report, we found that NeuroD1 binds to the promoter region of ALK, and indentified the particular E-box, which is involved in the expression of ALK. ALK has been shown to be an important predisposition gene, whose mutation or amplification has been identified in NB patients. 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. The simultaneous promotion of migration and proliferation by NeuroD1 might be important for tumorigenesis in NB.

Conclusion

We concluded that the novel mechanism would regulate the expression of ALK in neuroblastoma and that NeuroD1 should be significantly involved in neuroblastoma tumorigenesis.