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Interaction of Hand2 and E2a is important for transcription of *Phox2b* in sympathetic nervous system neuron differentiation

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ABSTRACT

Transcription factors play a crucial role in the development of various tissues. In particular, the transcription factors of the basic helix-loop-helix (bHLH) family are crucial regulators of neurodifferentiation. Previous studies suggested that the bHLH transcription factor Hand2 is essential for sympathetic nervous system neuron differentiation *in vivo*, but the molecular mechanisms involved have not been well elucidated. It is important for understanding their mode of action in cellular differentiation to clarify how these bHLH factors regulate distinct transcriptional targets in a temporally and spatially controlled manner. Recent reports on ES cell differentiation suggested that its molecular mechanism mimics that of *in vivo* neurogenesis. However, the diverse nature of ES cell populations has prevented efficient analysis. To address this issue, we previously established a cell line in P19 embryonal carcinoma (EC) cells. Efficient sympathetic nervous system (SNS) neuron differentiation is induced in the cell line. Using this cell line, we succeeded in showing that the interaction of bHLH transcription factor Hand2 with E2a is required for transcription of *Phox2b*, which is essential for autonomic nervous system neuron development, and this binding activates this expression in SNS differentiation. Moreover, we also demonstrated that Hes5 regulated the transcription of *Phox2b* as a negative regulator and it inhibited the SNS differentiation. These findings have enabled us to determine the novel regulatory mechanism of *Phox2b* in SNS differentiation.

1. Introduction

The development of peripheral neurons is initiated by the action of bone morphogenetic proteins (BMPs). BMPs have the ability to control the expression of a group of transcription factors, including Mash1, Phox2b, Phox2a and Hand2. These factors are known to play important roles in the differentiation process of peripheral neurons (Fig. 1A) [1,2]. Moreover, a previous study reported that BMPs exposure was also essential for ES cells to differentiate into peripheral neurons [3]. Therefore, understanding how these transcription factors regulate distinct transcriptional targets in a temporally and spatially controlled manner is critical for clarifying their activity in the process of cellular differentiation. To obtain insight on such a regulatory mechanism using a well-defined model system is the major goal of this study.

The method for generating neural crest-derived peripheral neurons from mouse and primate embryonic stem cells has previously been developed and this method provided the strategy for elucidating the regulatory mechanism of peripheral neuron differentiation [3]. However, it is difficult to understand the molecular mechanism involved during sympathetic nervous system (SNS) differentiation because the ES cells differentiate into multiple directions upon the beginning of cell to cell interaction and a culture of ES cells inevitably contains various kinds of differentiated cells. To address this issue, we have recently established a particular cell line from P19 embryonal carcinoma (EC) cells, which has an ES-like phenotype and a more homogeneous phenotype than ES cells. In this cell line, differentiation to SNS neurons is efficiently induced upon induction of gene expression of Hand2, which is one of the transcription factors [4]. It has another advantage. The differentiation to the SNS neurons in the cell line occurs without aggregation, which is normally required for neural differentiation in P19 EC cells. By constructing this cell line, we have successfully simplified the signaling pathway in SNS differentiation by removing the aggregation step, which should contain complex and multiple signal transduction processes.

During development, the bHLH Hand2 is known to be expressed in a number of tissues, including heart, limbs and crest-derived tissues; it regulates the development of these tissues [5–7]. In the autonomic nervous system (ANS) of chick and mouse, Hand2 isexpressed in sympathetic neurons, adrenal chromaffin cells and

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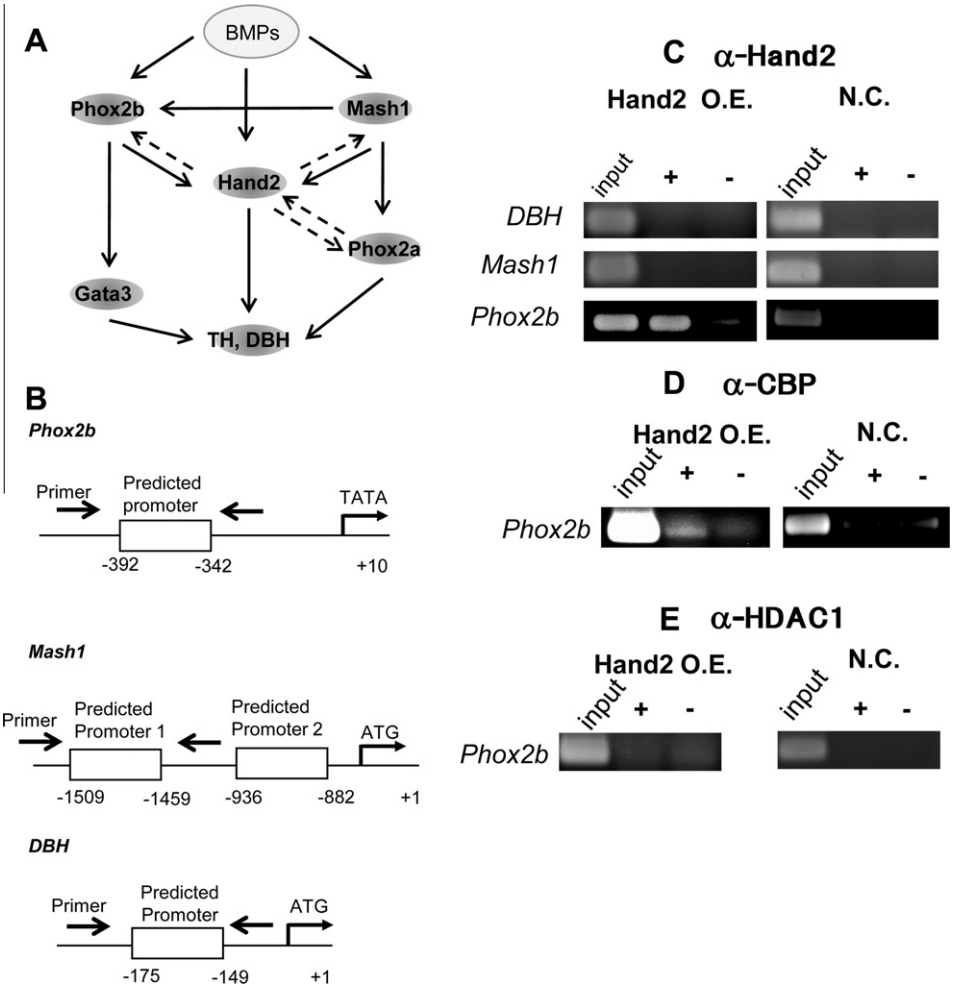

Fig. 1. Hand2 binds to the *Phox2b* promoter proximal region (A) Regulatory network controlling sympathetic neuron differentiation. Solid arrows indicate that the regulation has been shown by loss- and gain-of-function experiments; stippled arrows show that it has been deduced from gain-of-function experiments and might signify a maintenance rather than an inductive function. (B) SNS differentiation-related gene's promoter proximal region. All of the regions characterized in these experiments were located within 1 kb of the putative initiation site of RNA transcription. (C–E) ChIP analysis was performed with anti-Hand2 antibody, anti-CBP antibody, anti-HDAC1 antibody or with Protein G Dynal magnetic beads as a negative control in the transient overexpression of Hand2 in P19 EC cells. These cells were collected at 48 h after transfection or pcDNA3.1 vector was transfected as a negative control. The DNA templates were as follows: input, chromatin DNA as a positive control; +, immunoprecipitates with anti-Hand2 (C), anti-CBP (D) or anti-HDAC1 (E); -, immunoprecipitates with Protein G Dynal magnetic beads as a negative control.

enteric neurons [8,9]. Recently, an analysis of SNS development in Zebrafish carrying the *hand2* mutation "*hands off*" showed that the expression of the noradrenergic marker genes such as *tyrosine hydroxylase* (*Th*) and *dopamine β-hydroxylase* (*Dbh*) is strongly reduced [10,11]. A function of Hand2 in the control of Dbh expression has been suggested by the finding that Hand2 cooperates with Phox2a in activating transcription from the *dbh* promoter in experiments carried out with reporter constructs [1,5,8,12,13]. There is also *in vivo* evidence implicating Hand2 in the maintenance of *Th* and *Dbh* expression in autonomic neurons [14]. However, the roles of Hand2 in SNS differentiation remain unclear.

Using this cell line, we tried to findthe novel regulatory mechanism of *Phox2b* transcription in SNS differentiation. Previous studies have shown that the transient expression of NeuroD2, Mash1, Ngn1 or related bHLH proteins with their putative dimerization partner E12 can convert P19 EC cells into neurons, but not into SNS [15]. On the other hand, our results suggested that Hand2, which is one of the bHLH proteins, interacted with E-protein, E2a, and bound to the *Phox2b* promoter region in SNS differentiation.

The activation of Notch signaling is known to suppress the differentiation of neural crest cells from mouse trunk into autonomic neurons [16,17]. However, these mechanisms are poorly understood. Previous studies showed that Hes5, a major downstream effector for Notch signaling, inhibits neurogenesis by forming a heterodimer with E proteins, but its mechanism has yet to be elucidated in SNS differentiation [18]. We have successfully demonstrated that Hes5 interacted with E2a by competing with Hand2 and inhibited the transcription of *Phox2b* in SNS differentiation. These findings revealed the novel regulatory mechanism of *Phox2b* transcription in SNS differentiation.

2. Materials and methods

2.1. Phox2b promoter construct and luciferase assay for promoter activity

A 1 kb genomic DNA encompassing the promoter region of *Phox2b* was cloned into a promoterless luciferase construct, pGL3 basic vector (Promega). Cells were plated at a density of 5×10^4 cells per well of a 24-well plate. *Phox2b* promoter luciferase constructs were transfected into the cells with a control vector by Lipofectamine according to the manufacturer's instructions

(Invitrogen). Twenty-four hours after transfection, the cells were replating, the luciferase activities were analyzed using Dual Luciferase system (Promega).

2.2. qRT-PCR

RNA was isolated using an RNeasy kit (Qiagen, Valencia, CA). Primers were designed to produce amplicons of 100–150 bp length with a melting temperature of 60 °C. The primer sequences are listed in S-Table 1. All primers were synthesized by Primer3. The reactions were run on an ABI PRISM 7900 HT (Applied Biosystems, Foster City, CA). The cycle conditions consisted of 10 s at 95 °C, followed by 40 cycles at 95 °C for 5 s, and 60 °C for 35 s. The data were normalized relative to beta actin cDNA.

2.3. Short interfering (si) RNA

Stealth select RNAi was designed against the *e2a* transcript and synthesized by Invitrogen. siRNA was transfected using Lipofectamine 2000 (Invitrogen). The siRNA sequences are listed in S-Table 2. Negative control RNAs, designed to minimize sequence homology to any known vertebrate transcript, were purchased from Invitrogen (12935–300, Invitrogen).

Other methods were described in "Supplementary materials and methods".

3. Results

3.1. Hand2 activates the transcription of Phox2b

Previous studies showed Hand2 bound to *Phox2a*, *Phox2b* and *Dbh* promoter region in SH-SY5Y neuroblastoma cells. Thus, these genes may regulate these genes in SNS differentiation. To address this issue, we previously established a cell line from P19 EC cells in which the exogenous expression of Hand2 is initiated by the withdrawal of doxycycline (Dox) [4]. This cell line began a process of differentiation toward SNS upon Hand2 expression without aggregation. Removal of the aggregation process should be favorable for analyzing the molecular mechanism of differentiation. Because the aggregation process will trigger multiple signal transduction pathways and the obtained results will be complicated. At first, we tried to identify the target genes for Hand2 by focusing on genes whose expression levels were increased during SNS differentiation by qRT-PCR. This idea is consistent with results described in our previous report. We showed that *Phox2b*, *Mash1* and *Dbh* expression levels were increased in SNS differentiation by the induction of Hand2 [4].

To identify genes that lie under the control of Hand2, we hypothesized that Hand2 binds to the regulatory region of the target genes during SNS differentiation at a relatively early stage in P19 EC cells considering the expression profile of Hand2. For this purpose, we focused on the genes (*Dbh*, *Mash1* and *Phox2b*) the expression levels of which increased during SNS differentiation, and we carried out a ChIP assay for these genes (Fig. 1). We constructed primers in order to amplify the proximal region of the candidate genes' promoters (Fig. 1B). These promoter regions were predicted by the Neural Network Promoter Prediction program [19]. This program has been widely used, and has been shown to have a higher ability to predict promoter regions than other programs [20,21]. As is indicated in the results, Hand2 bound to the *Phox2b* promoter (from −342 to −390) but not to *Dbh* and *Mash1* (Fig. 1C).

Moreover, we analyzed the recruitment of the co-activator CBP/p300 and the co-repressor Hdac1 to the *Phox2b* promoter. CBP and p300 are highly homologous co-activator proteins that regulate all known pathways of gene expression in multi-cellular organisms via interaction with DNA-bound transcriptional regulatory proteins [22,23]. Hyper-acetylation of histone is strongly associated with actively transcribed genes [24]. Previous studies have shown that Hand2 interacts with CBP/p300, and this interaction has been shown to increase transcription activity [25,26]. Conversely, the removal of acetyl groups leads to chromatin compaction and thereby transcriptional repression. Such deacetylation is mediated by histone deacetylases, such as Hdac1 [27]. Our ChIP analysis suggested that CBP but not Hdac1 bound to the *Phox2b* promoter and proximal E-Box (Fig. 1D and E, Supplementary Fig. 1). Moreover, the luciferase promoter assay for *Phox2b* indicated that *Phox2b* promoter activity was increased by Hand2 induction (Fig. 2 A and B). These results suggest that the transcription of *Phox2b* is activated by Hand2.

3.2. Hand2 and E2a complex is required for activation of Phox2b transcription

It is well known that all members of the bHLH family heterodimerize with ubiquitously expressed bHLH E proteins, such as E2a, through their HLH domain [28]. Therefore, it is possible that Hand2 associates with E2a in SNS differentiation. To identify Hand2/E2a interaction during SNS differentiation, we performedco-immunoprecipitation experiments in P19 EC cells, which are transfected with pcDNA3.1-Hand2 plasmid vector. In the results, we could detect the interaction of Hand2 and E2a (Fig. 2C, lane 3). These results imply that Hand2 actually associates with E2a during SNS differentiation.

Since our data suggested that Hand2 associates with E2a during SNS differentiation, we then evaluated the role of E2a in the process, especially with respect to the actual role of E2a in the transcriptional complex.

For this purpose, we performed knockdown experiments using siRNA for e2a. P19-Hand2 cells were transfected with *e2a* siRNA to decrease the *e2a* transcript, cultured for 24 h, and then cultured for an additional 6 h in the Tet-free medium. Using quantitative RT-PCR (qRT-PCR), we analyzed the expression levels of the candidate genes (*Mash1*, *Phox2b*, *Dbh*, *Th*) during SNS differentiation of P19 EC cells The expression levels decreased to 0.1–0.5-fold of that of the negative control when one *e2a* siRNA-1 and one *e2a* siRNA-2 were administered (Fig. 2D). Our results also indicated that *Phox2b* mRNAs were remarkably suppressed by *e2a* knockdown (Fig. 2E) but other genes' mRNAs (*Mash1*, *Phox2a*, *Dbh*, *Th*)were not suppressed (data not shown). These results indicate that E2a is somehow involved in the molecular mechanism of activation of *Phox2b* transcription.

3.3. The interaction of Hes5 and E2a inhibited transcriptional activation of Phox2b by Hand2

The activation of Notch signaling is known to prevent neurogenesis [29]. Furthermore, previous study has shown that FGF-2 suppresses sensory neurogenesis by means of activation of Notch signaling [16]. Moreover, the suppression of Phox2b expression by FGF-2 recovers on the inhibition of Notch-1 expression [17]. This result suggests that FGF-2 prevents autonomic neurogenesis through the activation of Notch signaling. Hes family transcription factors are downstream effectors of Notch signaling. One of a Hes family transcription factor, Hes5, heterodimerized with the ubiquitously expressed bHLH E protein E2a, and inhibit the expression of target genes [18]. Moreover, previous study showed that Hes5 plays a crucial role in peripheral nervous system development [30]. However, this mechanism has not yet been well characterized. Therefore, we thought that it was possible that Hes5 associated with E2a and inhibited the interaction of Hand2 and E2a. To

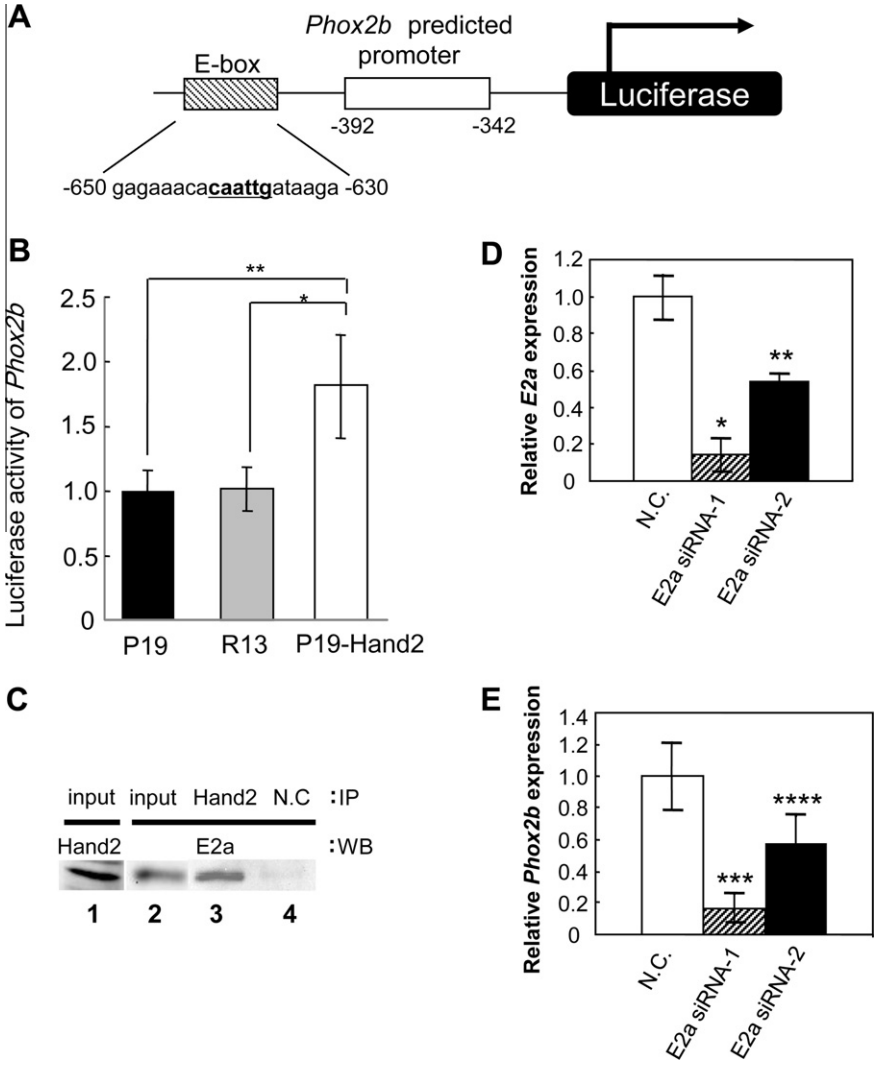

Fig. 2. Interaction between Hand2 and E2a is required for *Phox2b* transcription (A) *Phox2b* promoter region and E-box sequences. (B) Luciferase activity of *Phox2b* promoter region in P19-Hand2 cells. At 48 h after withdrawal of Dox, the cells were lysed and assayed for luciferase activity. Relative luciferase activity of phox2b-Luc compared with the promoterless vector is shown. Each value is the mean ± S.E. of triplicate independent experiments, $^{*}p < 0.03$, $^{**}p < 0.04$. (C) Analysis of the interaction between Hand2 and E2a by co-immunoprecipitation experiments using P19 EC cells in which Hand2 was transiently overexpressed. Samples were prepared from the cells that were cultured for 48 h after transfection. The cell lysates were prepared as described in the Section 2, immunoprecipitated with anti-Hand2 antibody, or Protein G Dynal magnetic beads as a negative control (NC), and finally subjected to Western blotting with the anti-E2a antibody. (D-E) E2a expression in Hand2 stable transformants was inhibited by each of two Stealth RNAi constructs for targeting E2a. E2a-targeted E2a siRNA-1, E2a siRNA-2 and negative control (NC) stealth RNAi. These siRNA were transfected using Lipofectamine 2000 into cells in which Hand2 expression had been induced by removing the Dox in the medium. siRNAs were transfected into the cells and incubated for 24 h with Dox, and samples were also prepared from the cells incubated for 6 h without Dox. Expression levels of the following genes were determined by qRT-PCR: (D) *E2a*, (E) *Phox2b*. The expression level of beta actin mRNA was used as an internal control. Each value is the mean ± S.E. of triplicate independent experiments; $^{*}p < 0.005$, $^{**}p < 0.05$, $^{***}p < 0.0005$, $^{****}p < 0.005$ compared with each cell transfected with siRNA as a negative control.

investigate whether Hes5 inhibits the interaction of Hand2 and E2a, we carried out co-immunoprecipitation experiments. P19 EC cells were transfected with two constructs (pcDNA3.1-Hand2 and pcDNA3.1-Hes5) in order to transiently over-express Hand2 and Hes5. The cells were then cultured for 48 h. Co-immunoprecipitation experiments were performed with the extract from the cells in conjunction with the anti-Hand2 and anti-Hes5 antibodies. Western blot analysis was carried out with the anti-E2a antibody (Fig. 3A). We detected the interaction of Hes5 and E2a (lane 6), but not that of Hand2 and E2a (lane 4). These results clearly indicated that Hes5 inhibits the interaction of Hand2 and E2a. We next focused on the target genes (*Mash1*, *Phox2b*, *Dbh*) whose expression increased during SNS differentiation by qRT-PCR, and analyzed them by ChIP assay using P19 EC cells in which Hand2 and Hes5 genes were transiently over-expressed. In these experiments, Hand2 did not bind the promoter region of all the candidate genes in which the expression levels increased during SNS differentiation in our previous qRT-PCR experiments (Fig. 3B). In addition, Hes5 down-regulated the expression of *Phox2b* in P19 EC cells in which Hand2 and Hes5 genes were transiently over-expressed (Fig. 3C). Moreover, expression of Hes5 strongly reduced the number of cells that were positive for expression of Tuj1 and Peripherin (Fig. 3D and E). These data suggested that Hand2 could not bind to the E-box of the *Phox2b* promoter proximal region and that this phenomenon was caused by the formation of the Hes5/E2a complex.

4. Discussion

4.1. Expression of Phox2b is required for Hand2 and E2a complex during SNS differentiation in P19 EC cells

Previous studies have shown that Hand2 and E-protein (E2a) complex binds to a subset of E-boxes, and it regulates the

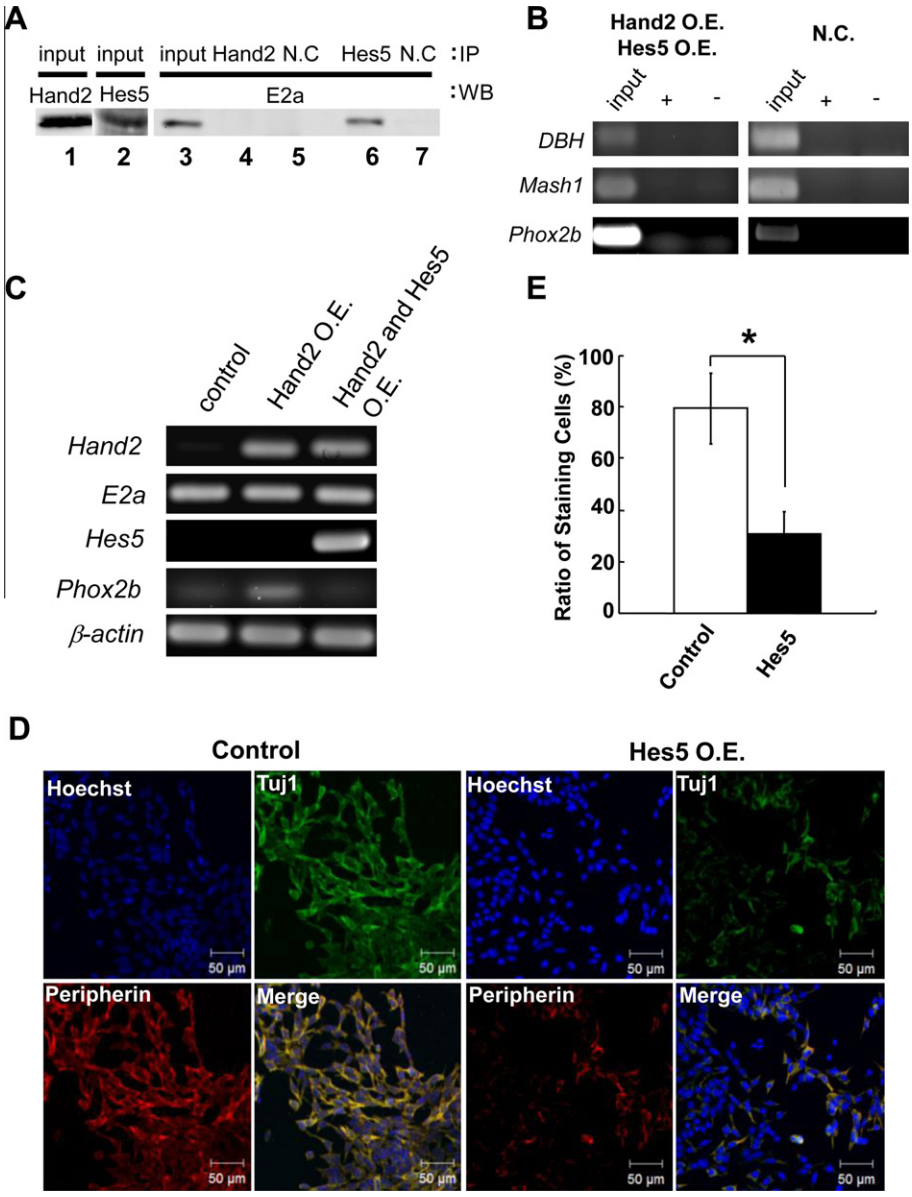

Fig. 3. Hes5 inhibits the SNS differentiation by interaction with E2a (A) Analysis of the interaction among Hand2, E2a and Hes5 by co-immunoprecipitation experiments in the P19 EC cells in which both Hand2 and Hes5 were transiently overexpressed. Samples were prepared from the cells that were cultured for 48 h after transfection. The cell lysates were prepared, immunoprecipitated with anti-Hand2 or anti-Hes5 antibody, and subjected to western blotting with anti-E2a antibody. The cell lysates were then immunoprecipitated with Protein G or anti-rabbit IgG Dynal magnetic beads as a negative control (NC). (B) Chromatin immuno-precipitation experiments using anti-Hand2 antibody. ChIP analysis was performed with anti-Hand2 antibody in P19 EC cells in which both Hand2 and Hes5 were transiently overexpressed or pcDNA3.1 vector was transfected as a negative control. Samples were prepared from the cells at 48 h after transfection. Protein G Dynal magnetic beads were used as a negative control. Chromatin was incubated with anti-Hand2 or Protein G Dynal magnetic beads, and the regulatory fragments were amplified by PCR from the immunoprecipitates. The DNA templates were as follows: input, chromatin DNA as a positive control; +, immunoprecipitates with anti-Hand2; -, immunoprecipitates with Protein G Dynal magnetic beads as a negative control. DBH, dopamine β hydroxylase. (C) RT-PCR analysis in P19 EC cells that transiently overexpressed Hand2 and Hes5. Cells were collected at 48 h after transfection. The cDNA templates were as follows: control, pcDNA3.1 vector was transfected in P19 EC cells; Hand2 O.E., pcDNA-Hand2 and pcDNA3.1 vector were transfected in P19 EC cells; Hand2 and Hes5 O.E., pcDNA-Hand2 and pcDNA-Hes5 were transfected in P19 EC cells. (D) Immunocytochemical staining was performed in P19-Hand2 cells that overexpressed Hand2 and Hes5. The control vector was transfected with P19-H2 cells, and these cells were cultured 96 h in dox-free medium. The pcDNA3.1-Hes5 vector was transfected with P19-H2 cells, and these cells were cultured in dox-free medium. (E) Ratio of Tuj1, peripherin double-positive cells to Hoechst. Each value is the mean ± S.E. of triplicate chamber slides; $p < 0.006$ compared with pcDNA3.1-transfected cells.

transcription of the target gene in the development of branchial arches and limb buds [25,31]. In neuronal differentiation, the bHLH transcription factor *Mash1* and all the members of the bHLH family heterodimerize with ubiquitously expressed E-proteins through their HLH domain and activate the transcription of genes that have an E-box in their promoter region [28]. Therefore, we speculated that Hand2 formed a complex with E2a, and bound to the E-box in the target gene promoter region. Importantly, there have been no reports indicating the formation of the Hand2/E2a complex in neurogenesis. In this study, we have successfully shown that Hand2 interacts with E2a in the differentiation of SNS in P19 EC cells. Furthermore, ChIP analysis and E2a knockdown studies suggested that the Hand2/E2a interaction is essential for the expression of *Phox2b* in SNS differentiation of P19 EC cells.

However, previous studies have shown that *Hand2* expression is induced by BMPs during development but it is first observed after the onset of *Mash1* and *Phox2b* expression in chick sympathetic ganglion primordia [31]. On the other hand, it was reported that

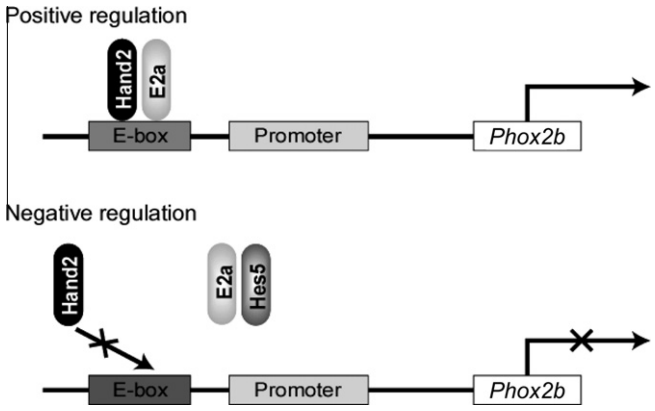

Fig. 4. Predicted model for the expression mechanism of Phox2b by Hand2 during mammalian SNS differentiation The bHLH transcription factor Hand2 is induced by BMPs, and Hand2 interacts with E2a. After that, the complex activates the transcription of *Phox2b* by binding to the E-box in the *Phox2b* promoter proximal region (upper panel). The bHLH transcription factor Hes5 is induced by the Notch signaling pathway, and Hes5 interacts with E2a. Hes5 competes with Hand2 and forms the Hes5/E2a complex, which does not have the ability to activate the *Phox2b* gene expression, and then downregulates the transcription of *Phox2b* by the formation of this complex (lower panel).

the number of cells expressing *Phox2b* was significant decreased in the sympathetic chain ganglia of Hand2 mutant embryos [11]. Therefore, these studies suggested that expression of Hand2 might be required for keeping the expression of *Phox2b* at a certain level, and maintained the generation of SNS neurons.

4.2. The interaction of Hes5 and E2a inhibited transcription of Phox2b in SNS differentiation

Previous study has shown that FGF2 suppresses ANS differentiation by means of the activation of Notch signaling [16]. Moreover, Hes1/Hes5 double knockout, major downstream effectors for Notch signaling, influenced for *Phox2b* expression pattern in mice. However, these mechanisms are poorly understood. Therefore, we focused on Hes5, and analyzed the mechanism of *Phox2b* transcription by Hes5 in SNS differentiation.

In the results, the formation of Hand2/E2a complex was inhibited by interaction of Hes5 with E2a in P19 EC cells in which Hand2 and Hes5 genes were over-expressed. Furthermore, ChIP analysis and RT-PCR studies suggested that Hand2 could not bind to the Phox2b promoter region and inhibited the SNS differentiation of P19 EC cells. These results suggested that Hes5 interacts with E2a by competing with Hand2, and regulates the expression of *Phox2b*. We considered that this negative control mechanism may play a crucial role in the differentiation of SNS (schematically illustrated in Fig. 4).

In this report, we demonstrate the novel regulatory mechanism of Phox2b transcription in SNS differentiation. This finding will stimulate the hunt for master gene(s) that regulate the differentiation of various stem cells. Our study has shown that the exogenous expression of Hand2 can activate the SNS differentiation program by induction of SNS-related genes, including *Phox2b*, *Mash1*, *Dbh* and *Th*. Identification as well as analysis of the transcriptional regulators will be increasingly important for clarifying the molecular mechanism of such a differentiation program.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.03.113.

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