The bHLH transcription factor Hand2 regulates the expression of nanog in ANS differentiation

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Abstract

The basic helix-loop-helix transcription factor Hand2 is induced by bone morphogenetic proteins (BMPs) in neural crest-derived precursor cells during the early stage of development of the autonomic nervous system (ANS). Previous studies showed that Hand2 was essential for the ANS differentiation. However, regulatory mechanism of pluripotent genes has not been elucidated in ANS differentiation. Here, we show that Hand2 regulated *nanog* expression in ANS differentiation. Our studies demonstrated that the forced expression of Hand2 promoted the ANS differentiation program in P19 embryonal carcinoma (EC) cells without aggregation. Furthermore, our results suggested that Hand2 bound to the promoter of *nanog*, a gene required for embryonic stem cells self-renewal, and suppressed *nanog* expression after Hand2 induction. The rapid down-regulation of *nanog* mRNA during ANS differentiation correlated with the Hand2 transcriptional activity and *nanog* promoter methylation. These findings are evidence for a presence of the novel regulatory mechanism of *nanog* in ANS differentiation.

Keywords

Hand2, Nanog, P19 EC cells, autonomic nervous system

Introduction

The development of sympathetic neurons is initiated by bone morphogenetic proteins (BMPs). BMPs control the expression of a group of transcription factors, including *ascl1*, *phox2b*, *phox2a*, *hand2*, *gata2/3* that play important roles in sympathetic neuron specification and differentiation [1,2]. However, it is not clarified how pluripotent genes are regulated in ANS differentiation.

The bHLH Hand2 (previously called dHand) is expressed in a number of tissues during development, including heart, limbs and crest-derived tissues, and has been shown to regulate the development of these tissues [3,4,5]. In the autonomic nervous system (ANS) of chicks and mice, Hand2 is expressed by sympathetic neurons, adrenal chromaffin cells and enteric neurons [6,7]. During development, *hand2* expression is induced by BMPs but is first observed after the onset *ascl1* and *phox2b* expression in chick sympathetic ganglion primordia [8]. This finding, together with the lack of *hand2* expression in the *phox2b* knockout and maintained expression in the *gata3* knockout [9], implicates a function of *hand2* downstream of *phox2b* and upstream of *gata2/3* in the group of BMP induced factors. Hand2 overexpression was found to induce the generation of catecholaminergic neuron from neural crest precursor cells and P19 embryonal carcinoma cells [1,8], acting in concert with *phox2b*, *phox2a* and *ascl1*, which are ectopically expressed after *hand2* overexpression. Also upon forced *phox2a* expression, *ascl1* and *phox2b* are induced, suggesting cross-regulation between these transcription factors, which are thus considered to be a network rather than a regulatory cascade [10,11].

Nanog, a homeodomain protein exclusively expressed in embryonic stem cells (ESCs), is required to maintain self-renewal and the undifferentiated state of ESCs. *nanog* expression is rapidly down-regulated during ESC differentiation and the constitutive expression of *nanog* inhibits ESC differentiation[12,13].

To address the role of Hand2 in ANS differentiation, we established that the exogenous expression of Hand2 could be initiated by the withdrawal of doxycycline (Dox) in a P19-derived cell line. In the results, Hand2 expression activated the ANS differentiation program in P19-derived cell line. Furthermore, our data suggested that Hand2 bound to the promoter of *nanog*, a gene required for embryonic stem cells self-renewal, and suppressed *nanog* expression after Hand2 induction. Our studies revealed a novel mechanism in which Hand2 bound to the E-box in the promoter region of *nanog* which is one of a pluripotent master gene, and thereby promoted the ANS differentiation.

Materials and Methods

Cell culture and construction of the Tet-controlled Hand2 expression system

P19 EC cells were cultured in α -MEM with 10% fetal bovine serum (FBS; Japan Bioserum Co., Ltd., Nagoya, Japan), 100 U/ml penicillin and 100 g/ml streptomycin (Gibco). Tet-controlled Hand2 expression in the P19 cells was performed using the Tet-offTM gene expression system (Clontech). As a first step, the P19 cells were transfected with a pTet-Off vector using DOTAP and cultivated in the presence of 400 g/ml G418 (Wako, Tokyo, Japan) for selection. The resulting G418-resistant colonies were then screened by transient transfection with a pTRE2-Luc vector (Clontech) to isolate P19 Tet-off cells exhibiting a low background and high Tet-dependent induction of Luciferase by the withdrawal of Tet. The selected P19 Tet-off cell line was designated R13. At the second step, R13 cells were transfected with pTRE2-Hand2 and cultivated in the presence of 400 g/ml hygromycin (Invitrogen) and 2 g/ml doxycycline(Clontech) for selection. Hygromycin-resistant

colonies were then screened for the induction of Hand2 mRNA in the α -MEM with 10% Tet system approved FCS (Clontech) by RT-PCR.

Transient transfection

P19 EC cells were maintained in α -MEM with 10% FCS. One day prior to transfection, the cells were plated in 10 cm dishes at a density of 4×10^6 cells per plate.

A total of 12 μ g /plate plasmid was transfected with P19 EC cells using Lipofectamine 2000 (Invitrogen, CA, USA). The cells were harvested after 48 h.

Bisulfate genomic sequencing

Bisulfite treatment was performed using the EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA) according to the manufacturer's instructions. The PCR primers used to amplify nanog promoters are indicated in Supplementary Table 2. PCR products were cloned into pGEM-T easy vector (Promega, Madison, WI), and 11 randomly selected clones for each gene were sequenced with T7 forward and SP6 reverse primers.

Immunocytochemistry

Live cells were incubated with 5 μ g/ml Hoechst 33258 (Molecular Probes, Eugene, OR) for 20 min, rinsed in PBS, fixed in 100% ethanol for 10 min at -20°C, incubated in 100% acetone for 1 min at -20°C, and washed three times with PBS. The cells were incubated with blocking solution (10% rabbit serum in PBS) for 1 h at room temperature, then incubated with primary antibody in 4% rabbit serum in PBS for 1 h. After washing, the cells were incubated in Alexa Fluor 488 conjugated anti-mouse and Alexa Fluor 594 conjugated anti-rabbit secondary antibodies (Invitrogen) for 30 min. The antibodies were Peripherin (sc-7604; Santa Cruz Biotechnologies) and TuJ1 (MMS-435P; Covance, Berkeley, CA).

***nanog* promoter construct and luciferase assays for promoter activity**

A 1.2-kb genomic DNA encompassing the promoter region of *nanog* was cloned into a promoterless luciferase construct, pGL3 Basic vector (Promega). Luciferase assays were performed using the Dual Luciferase system (Promega). Cells were plated at a density of 5×10^4 cells per well of a 24-well plate. *nanog* promoter luciferase constructs were transfected into the cells with a control vector by Lipofectamine according to the manufacturer's instructions (Invitrogen). 24 hours after transfection, the cells were replated in a 96-well plate with dox free medium. 24 hours after replating, the luciferase activities were analyzed using the Dual Luciferase system.

Western blot analysis, quantitative RT-PCR (qRT-PCR), ChIP assay were described in "Supplementary materials and methods"

Results

1. Forced expression of Hand2 induced differentiation of PNS in P19 EC cells without aggregation

To investigate whether the forced expression of Hand2 activated the peripheral nervous system (PNS) differentiation program, we established a cell line from P19 EC cells in which the exogenous expression of Hand2 could be initiated by the removal of Dox. Briefly, a pTRE2-Hand2 construct was transfected into the P19 EC cell line which harbored the pTet-Off

vector (termed R13). The resulting stable transformants should have expressed Hand2 (termed P19-Hand2 cells) upon removal of Dox. To examine whether the expression of exogenous Hand2 could be efficiently induced by the removal of Dox, we analyzed the expression level of the gene in the Tet-free medium. We detected the maximal protein expression of Hand2 after 48 h (Fig. 1A). However, the expression of Hand2 was not detected in P19 EC cells (data not shown). Using these stable transformants, we investigated whether Hand2 induces the differentiation of the PNS in P19 EC cells without aggregation by an immunocytochemical analysis (Fig. 1). In the presence of Dox (0 h), none of the transformants expressed either the pan-neuronal marker TuJ1 or the PNS marker peripherin (Figs. 1C and D). In contrast, when Hand2 was compulsorily expressed in the absence of Dox without aggregation, all transformants could differentiate into TuJ1 and peripherin-positive neurons at 96 h after removal of Dox, with an efficiency of above 80% (Fig. 1F-I and R). Neurons that use the same transmitter have many features in common, but they are operationally defined by the presence of the stored neurotransmitter. So, noradrenergic (NA) neurons are generally defined as neurons that express Th and Dbh [14]. Accordingly, we analyzed the expression of Th and Dbh after 72 h by immunocytochemistry stain analysis, and both noradrenergic neuron markers were detected (Fig. 1 J-Q). In conclusion, these neurons were determined to be NA neurons. These data suggested that the forced expression of Hand2 induced the differentiation of the peripheral nervous system in P19 EC Cells.

2. Forced expression of Hand2 activated the ANS differentiation program

Previous studies have shown that the activation of the ANS differentiation program is accompanied by the activation of *phox2a* and *phox2b* as well as *mash1* [8,15]. To demonstrate that Hand2 induction activates the ANS differentiation program, we examined the changes in the expression level of the following genes during the PNS differentiation of P19 EC cells: *phox2a*, *phox2b*, *mash1*, the noradrenergic markers *th* and *dbh*, *nanog*, the ectodermal marker *otx2*, the neural crest marker *sox10* and the PNS marker *prph1*. Total RNAs were extracted from P19-Hand2 cells at various time points and were analyzed by qRT-PCR. *Nanog* is a recently discovered homeodomain transcription factor which is both sufficient and necessary for maintenance of the pluripotency of mouse ES cells and of mouse epiblasts [12,13]. *nanog* mRNAs decreased steeply after the induction of Hand2's expression (Fig. 2A), and *otx2* and *sox10* mRNAs increased after the induction of Hand2 (Figs. 2B and C); thereafter, the *sox10* mRNAs decreased to the basal level. The expression level of *sox10* was changed in a manner that was antiparallel to that of *prph1* (Fig. 2D). *phox2b*, *mash1* and *dbh* mRNA increased after the expression of Hand2 (Fig. 2E-G). *phox2a* mRNA increased after the expressions of *phox2b* and *mash1* mRNA were induced (Fig. 2H). *th* mRNA was expressed, but not changed (data not shown). Taken together, these results indicate that, while the Hand2 expression inhibited genes necessary for the maintenance of pluripotency, it activated genes which are necessary for ANS differentiation. Thus, these results indicate that the forced expression of Hand2 activated the ANS differentiation programs.

3. Hand2 regulated the *nanog* transcription

As *nanog* expression is immediately downregulated by the induction of Hand2 expression in ANS differentiation, we examined whether Hand2 regulated expression of *nanog*. First, we examined the changes in the expression level of the following genes which were related to *nanog* expression during the ANS differentiation of P19 EC cells: *p53*, *tcf3*, *foxd3*, *oct4*, and *sox2*. The tumor suppressor p53 can bind to the *nanog* promoter as suggested by both *in vitro* and *in vivo* evidence. During ES cell differentiation, the rapid downregulation of *nanog* correlates with the induction of the transcriptional activity [16]. Tcf3, a transcription factor that functions downstream of the Wnt pathway, is highly expressed in undifferentiated

mouse ES cells. Tcf3 binds to a regulatory region on the *nanog* promoter and represses the promoter activity in ES cells [17]. FoxD3, a forkhead family transcription factor, is highly expressed in mouse ES cells and in pluripotent cells of the early embryo [18]. FoxD3 null mouse embryos die shortly after implantation with a loss of epiblast, a phenotype similar to Nanog deficient embryos, and FoxD3 is an activator of *nanog* [19,20]. The Oct4/Sox2 motif localized -180 bp upstream of the transcription start site was found to be important for *nanog* regulation. Recent studies have suggested that Oct4/Sox2 acts to promote *nanog* transcription in pluripotent cells [21,22]. However, the expression level of these genes showed no change in ANS differentiation (Fig. 3A). Thus, we think that Hand2 regulates *nanog* transcription directly. To understand the molecular mechanism underlying Hand2-mediated suppression of *nanog* expression during ANS differentiation, we identified three putative Hand2-binding sites within the *nanog* promoter region (Fig. 3B). The first of these sites is denoted E1, and is located at -1255 to -1249 relative to the putative initiation ATG; the second site, denoted E2, is located at -975 to -969; and the third site, denoted E3, is located at -545 to -539. We carried out a luciferase promoter assay as well as ChIP analysis of these Hand2-binding sites and bisulfate sequence analysis in order to examine the extent of methylation in the *nanog* promoter region. Moreover, we were not able to detect the recruitment of the co-activator CBP which is well known to increase transcription activity with Hand2 [23] by ChIP assay. *nanog* expression was significantly reduced in P19-Hand2 cells (Fig. 3C). Moreover, Hand2 and Dnmt3, which is the genomic DNA methyltransferase, bound to the *nanog* promoter region (Fig. 3D and Supplementary Figure 1). In addition, the *nanog* promoter [-265 to +15] that was bound to the *nanog* transcription activator FoxD3 and Oct4/Sox2 was 13% methylated (Fig.3E), and the *nanog* first exon was hypermethylated after 48 h in P19-Hand2 cells (data not shown). These data suggested that Hand2 has the capability of regulating *nanog* transcription.

Previous studies have reported that *nanog* is located in the Wnt-signaling pathway [24,25]. In P19 EC cells, it has also been reported that the Wnt-signaling pathway is activated by aggregation [26]. Accordingly, we further analyzed whether Wnt1 could repress the *nanog* expression. The results suggested that neither Wnt1 activation by aggregation nor Wnt1 transient expression in P19 EC cells repressed *nanog* transcription (Fig. 3 F).

Discussion

Previous studies have shown that Hand2 overexpression itself could not activate the ANS neuronal developmental program in P19 EC cells, but could activate the development program if the transient expression was performed in conjunction with aggregation of the cells [15,27]. However, it seemed difficult to identify the target genes of Hand2 because the expression of numerous genes may well be altered by aggregation. To identify target genes for Hand2, we established a P19 derived cell line which exogenously expresses Hand2 upon removal of Dox. We also attempted to induce the differentiation of ANS in P19 EC cells without aggregation using this cell line, and succeeded in this attempt. On the other hand, as the transient Hand2 expression could not induce ANS differentiation, it suggests that the continuously elevated expression of Hand2 is required for ANS differentiation.

Our study showed that Hand2 induced ANS differentiation without aggregation. The role of the aggregation process in the differentiation has previously been discussed from various points of view. The neuronal differentiation of P19 EC cells is dependent on cell aggregation during the induction period [28]. Conditions that reduce adhesion in the aggregates inhibit the formation of neurons [29]. Although aggregation is clearly essential, little is known about the mechanism by which it promotes differentiation. Our present data suggested that *Wnt-1*, the vertebrate counterpart of the *Drosophila wingless* gene, is up-regulated by aggregation alone in P19 EC cells, as has been demonstrated previously [26]. Wnt-1 signals through a receptor complex composed of members of the Frizzled and low density lipoprotein families and

activates the β -catenin/transcriptional T-cell factor signaling pathway (known as the canonical Wnt pathway) [30]. Pluripotent master genes, such as *nanog*, *oct-3/4* and *rex-1* are located at the downstream of the Wnt-signaling pathway [24,25]. Thus, we predicted that activation of Wnt signaling pathway suppressed the transcription of *nanog* in P19 EC cells. However, our results suggested Wnt1 activation alone is not sufficient for suppression of *nanog* transcription. Therefore, these data suggested that Hand2 regulated the transcription of *nanog* through another signaling pathway. In our study employing a ChIP assay and luciferase promoter assay, it was suggested that the downregulation of *nanog* by expression of Hand2 induced the recruitment of Dnmt3 to the *nanog* promoter region. Consequently, the *nanog* promoter region, which is a binding site of the *nanog* transcription activators FoxD3 and Oct4/Sox2, was methylated by Hand2 induction. These data suggests that Hand2 regulates the *nanog* transcription by methylation of the *nanog* promoter in ANS differentiation. Our findings propose a novel regulatory mechanism of *nanog* transcription in ANS differentiation by Hand2. Previous studies have been focused on the genes which have a role to promote ANS differentiation. However, we found a novel regulatory mechanism of *nanog*, as follow, Hand2 repressed the *nanog* transcription by induction of methylation in *nanog* transcription activator sites with Dnmt3 during ANS differentiation. After the *nanog* repression, Hand2 activated the ANS-related genes. Finally, Hand2 induced the DBH, and P19EC cells differentiated into the ANS neurons (Fig. 3G).

It has already been shown that the exogenous expression of a couple of transcription factors converted fully differentiated mouse cells to iPS cells which can be differentiated to a variety of tissues [31]. This finding has stimulated the hunt for master gene(s) which regulate the differentiation of various stem cells. Our study has shown that the exogenous expression of one transcription factor (Hand2) activated the ANS differentiation program. 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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Figure legends

Fig.1 Forced expression of Hand2 induced differentiation of NA neurons

The ability of Hand2 to activate the neuronal program was examined by the forced expression of Hand2 without aggregation. (A) A P19-derived cell line in which the exogenous expression of Hand2 could be initiated by the withdrawal of Dox was established. To examine whether the expression of exogenous Hand2 could be induced by the removal of Dox, the time-dependent expression level of Hand2 was analyzed by Western blotting. (B-I): Cells were cultured for 96 h with Dox (B-E) or without Dox (F-Q) and analyzed for the expression of Tuj1 (pan-neuronal marker) or peripherin (peripheral neuronal marker). (B, F, J, N) Hoechst 33258, (C, G, K, O) Tuj1, (D, H) peripherin, (L) TH, (P) DBH, (E, I, M, Q) merge. (R): Ratio of Tuj1 (gray) or peripherin (white) -positive cells to Hoechst-labeled cells. Each value is the mean $\pm$ S.E. of triplicate chamber slides; * p <0.0003, ** p <0.00003 compared with the P19-H2 cells cultured with Dox.

Fig.2 Forced expression of Hand2 activated the ANS differentiation program

(A-H) The expression levels of the genes that may be involved in ANS differentiation were characterized by qRT-PCR after induction of the Hand2 gene expression. The quantities of mRNAs were determined by comparing the E11.5 embryo total RNA with a known amount of internal control RNA copies. (A) *nanog*, (B) *otx2*, (C) *sox10*, (D) *prph1*, (E) *dbh*, (F) *mash1*, (G) *phox2b*, (H) *phox2a*. The expression level of beta actin mRNA was used as an internal control. Each value is the mean $\pm$ S.E. of triplicate independent experiments in two clones.

Fig.3 Hand2 regulated the *nanog* transcription

(A) RT-PCR was performed to examine the expression of the genes regulating *nanog* transcription. Samples were collected at the indicated time after withdrawal of Dox. The relative ratios of the mRNA expression levels were estimated compared with the 0 h expression levels in each gene. The expression level of beta actin mRNA was used as an internal control. (B) *nanog* promoter region sequences. (C) At 24 h after withdrawal of Dox, the cells were lysed and assayed for luciferase activity. The relative luciferase activity of *nanog*-Luc compared with the promoterless vector is shown. Each value is the mean $\pm$ S.E. of triplicate independent experiments; * p <0.0002, ** p <0.0007. (D) ChIP analysis was performed with anti-Hand2 antibody and-CBP antibody or with Protein G Dynal magnetic beads as a negative control. (E) At 48 h after withdrawal of Dox, bisulfite genomic sequencing was performed to examine the DNA methylation profiles for individual CpG sites in the *nanog* promoter regions in P19 EC cells, R13 cells P19-Hand2 cells. The CpG dinucleotide locations are indicated. 11 clones were

sequenced and are shown for each site. *Open circles* represent unmethylated cytosines, and closed circles represent methylated cytosines. (F) RT-PCR was performed to examine the expression of *nanog* or *wnt1* mRNA in wild type P19 EC cells, pcDNA3.1-Wnt1 transfected P19 EC cells, or P19 EC cells with aggregation (Agg.) over days 0-2 or 0-4. (G) Model for the transcriptional cascade that regulates the NA neuron differentiation. At first, Hand2 expression is induced in P19 EC cells. This expression repressed the *nanog* transcription by induction of methylation in *nanog* transcription activator sites. On the other hand, Hand2 induction results in the independent activation of *phox2b* and *mash1*. The function of *mash1* remains unclear, but it appears to promote the survival of neuronal progenitors and possibly also their differentiation. *phox2b* is required for the generation and maintenance of all noradrenergic cells. *mash1* and *phox2b* expression is required for *phox2a* and the genes encoding TH and DBH to be expressed. Thus, Hand2 induced the ANS differentiation by down-regulation of *nanog* transcription instead of aggregation and activated the ANS related genes.

Figures

Figure 1

Figure 2

Figure 3

A

B

D

E

F

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