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Osaka University
Doctoral Dissertation

Formation mechanisms of mouse neural
crest-derived stem cells

（マウス神経冠細胞由来幹細胞の形成機構）

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Department of Biological Sciences
Graduate School of Science
Osaka University
2015
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<th>Definition</th>
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<tbody>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>α-MEM</td>
<td>α-modified minimum essential medium</td>
</tr>
<tr>
<td>μChIP</td>
<td>micro chromatin immunoprecipitation</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>μm</td>
<td>micrometer</td>
</tr>
<tr>
<td>μM</td>
<td>micromolar</td>
</tr>
<tr>
<td>BIO</td>
<td>6-Bromoindirubin-3’-oxime</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-bromo-2'-deoxyuridine</td>
</tr>
<tr>
<td>CEE</td>
<td>extract of day-11 chick embryos</td>
</tr>
<tr>
<td>CHD7</td>
<td>chromodomain helicase DNA-binding protein 7</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DN</td>
<td>dominant-negative</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DRG</td>
<td>dorsal root ganglia</td>
</tr>
<tr>
<td>E</td>
<td>embryonic day</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>EMT</td>
<td>epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>ES</td>
<td>embryonic stem</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>TSS</td>
<td>transcriptional start sites</td>
</tr>
<tr>
<td>Twist1</td>
<td>twist basic helix-loop-helix transcription factor 1</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
<tr>
<td>Zic1</td>
<td>zinc finger protein of the cerebellum 1</td>
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General Introduction

The neural crest is a transient embryonic structure unique to the vertebrates. Neural crest cells are multipotent and give rise to a diverse array of cell types, such as peripheral neurons and glia, smooth muscle cells, bones and cartilage of the head, melanocytes, and adipocytes [1, 2]. Neural crest cells are generated at the neural fold, the junction of the neural plate and the ectoderm, in response to BMPs/Wnts signaling [3-5]. These signals promote the expression of neural crest inducer genes including Msx1, Pax3/7, and Zic1 [6]. Neural crest inducer genes activate the expression of neural crest specifier genes, such as FoxD3, Sox9, Sox10, Slug, and Twist1 [7, 8]. Especially, Sox10 has been known to be an important factor for the maintenance of the undifferentiated state of neural crest cells [9]. After neural crest specification, neural crest cells undergo an EMT, delaminate from the dorsal aspect of the neural tube, and migrate throughout the embryo. Some neural crest cells maintain the multipotency and form NCSCs [10-13]. NCSCs are found in various adult tissues, such as DRG [14, 15], sciatic nerve [16, 17], and bone marrow [15]. NCSCs are typical tissue-specific stem cells with self-renewal capability and express p75 [16, 18, 19], a marker of undifferentiated neural crest cells [20]. However, little is known about the formation mechanisms of these cells.

Recently, CHD7 has been shown to play important roles in neural crest induction [21]. CHD7 is a member of ATP-dependent chromatin remodeling CHD family and characterized by the presence of two chromodomains, centrally located helicase domains [22]. In the neural crest induction, CHD7 activates the neural crest specifier genes, Sox9, Twist1, and Slug by binding to their enhancer elements marked with H3K4me1 [21]. CHD7 has been also shown to promote the migration of Xenopus neural crest cells [21].
Mutation of CHD7 gene causes CHARGE syndrome, a multiple anomaly condition characterized by coloboma, heart defect, atresia choanae, retarded growth and development, genital abnormality, and ear abnormality [22-24]. This syndrome is due to abnormal development of the neural crest. In mouse ES cells, furthermore, CHD7 co-localizes with Oct3/4, Sox2, and Nanog at active gene enhancer elements showing high levels of H3K4me1/2 and act on the maintenance of the pluripotency of ES cells [25]. In the cardiomyocyte cells, CHD7 shows the direct binding to G/S and AR2 enhancers of Nkx2.5 in a BMP-dependent manner [26]. In addition, CHD7 interacts with Sox2 and coordinately regulate the common target genes, such as Jagged1 and Mycn, in neural stem cells [27]. CHD7 has been also known to be a critical regulator of the quiescence of neural stem cells in the adult hippocampus [28]. These findings show that CHD7 plays multiple roles in the regulation of transcriptional activities in various cell types.

In present studies, we analyzed the maintenance mechanisms of the multipotency of mouse trunk neural crest cells, leading to the formation of mouse NCSCs. CHD7 expression was observed in early migratory and undifferentiated mouse trunk neural crest cells expressing Sox10 and p75. The treatment with BMP2 and Wnt3a or the overexpression of CHD7 maintained the expression of CHD7, Sox10, and/or p75 in the undifferentiated neural crest cells. Furthermore, the inhibition of CHD7 expression significantly suppressed the maintenance of the undifferentiated state of these cells. These data suggest that this chromatin remodeler as well as BMP2/Wnt3a signaling play essential roles in the maintenance of the undifferentiated state of mouse trunk neural crest cells. In addition, clonal analysis of the neural crest cells showed that BMP2/Wnt3a signaling and CHD7 promote the maintenance of the multipotency of these cells. Thus, it is possible that BMP2/Wnt3a signaling and CHD7 maintain the multipotency of mouse trunk neural crest cells and lead to the formation of mouse NCSCs. Furthermore, we analyzed a
regulatory gene cascade in the formation of mouse NCSCs. The inhibition of FoxD3 expression significantly suppressed the expression of Sox10, which is an indispensable transcription factor for mouse NCSC formation, in the presence of BMP2/Wnt3a. CHD7, Oct3/4, Sox2, and Nanog bound to the cis-regulatory regions of mouse FoxD3 (mE1, mE2, and mE3) in a BMP2/Wnt3a-dependent manner. In addition, these cis-elements contained the binding sites of CHD7, Oct3/4, Sox2, and Nanog. Furthermore, siRNAs of CHD7, Oct3/4, Sox2, and Nanog significantly suppressed the expression of FoxD3 and Sox10. The inhibition of histone H3K4 mono- or tri-methylation also repressed FoxD3 expression. The present data suggest that CHD7, Oct3/4, Sox2, and Nanog directly induce FoxD3 expression when stimulated by BMP2/Wnt3a signaling, that FoxD3 promotes Sox10 expression, and that histone H3K4 methylation plays important roles in this process of mouse NCSC formation. Thus, the formation mechanism of mouse NCSCs is dramatically different from the induction mechanisms of neural crest cells.
Chapter I

Roles of chromatin remodeler in maintenance mechanisms of multipotency of mouse trunk neural crest cells in the formation of neural crest-derived stem cells

Introduction

The neural crest is a transient embryonic structure in vertebrates. Neural crest cells arise within the neural fold, migrate ventrally and laterally (Fig. 1.1A), and contribute to a wide variety of cell types, such as peripheral neurons and their supportive cells, pigment cells, skeletal derivatives, adipocytes, endocrine cells, and smooth muscle cells [1, 2] (Fig. 1.1B). Recent studies have revealed that some neural crest cells maintain multipotency and form NCSCs [10-13]. NCSCs have been shown to exist in late embryonic and adult tissues such as DRG [14, 15], sciatic nerve [16, 17], gut [18, 19], heart [29], hair follicle [30], and bone marrow [15] (Fig. 1.2). However, little is known about the formation mechanisms of NCSCs.

It has been shown that BMP and Wnt are involved not only in neural crest induction in chick and Xenopus [3-5] but also in the maintenance of the undifferentiated state of neural crest cells in mouse and rat [31]. Moreover, a recent study has elucidated that CHD7, one of chromatin remodelers, plays important roles in neural crest induction by controlling the expression of neural crest specifier genes [21]. CHD7 also interacts with three master regulators of multipotency, Oct3/4, Sox2, and Nanog, in ES cells [25]. The mutation of this remodeler gene causes the CHARGE syndrome by abnormal neural crest development.
These findings suggest that CHD7 as well as BMP/Wnt signaling may be involved in maintaining of the multipotency of neural crest cells and conduct them to mouse NCSCs. In the present study, therefore, we analyzed roles of CHD7 and BMP/Wnt signaling in maintaining the multipotency of mouse trunk neural crest cells, leading to the formation of mouse NCSCs.

**Fig. 1.1 Development of the neural crest.** (A) The neural crest is induced at the neural plate border (green), the boundary of the neural ectoderm (blue) and the epidermis (white). After EMT, neural crest cells delaminate from dorsal aspects of the neural tube and migrate throughout the embryo. (B) Neural crest cells differentiate into diverse cell types, such as melanocytes, peripheral neurons and glia, chondrocytes, endocrine cells, and adipocytes. Figure 1.1A redrawn after Green et al. (2015) [32].
Fig. 1.2  **Distribution of NCSCs.** NCSCs are present in various adult tissues including dorsal root ganglion, gut, cornea, heart, bone marrow, and skin. Figure redrawn after Shakhova and Sommer (2010) [11].
Materials & Methods

Primary and clonal cultures

Primary cultures of mouse trunk neural crest cells were prepared from ddY mouse embryos at E9.5 (24- to 29-somite stages) [33-35]. Neural tubes were isolated from the dorsal trunk region at the last six-somite levels of E9.5 embryos. Neural tubes were explanted into 35-mm culture dishes coated with collagens gel (PureCol). The explants were scraped away with a tungsten needle after 48 h in culture, leaving the emigrated trunk neural crest cells on the substratum.

Clonal cultures of the trunk neural crest cells were performed by a modification of methods described previously [33, 36]. Neural crest cells were resuspended by trypsinization after 6 days in culture. This essentially single cell suspension (> 90% single cells) was diluted with culture medium, and 1 ml aliquots of this cell suspension were seeded at 500 cells/ml onto 35-mm culture dishes coated with a collagen gel and conditioned with culture medium containing 10 µg/ml plasma fibronectin (Itoham). The clone founder cells were identified at 8 h after seeding cells. The efficiency of clone formation was 5.5 (± 1.5)%.

The culture medium consisted of 85% α-MEM (Sigma), 10% FBS (GE Healthcare Life Sciences), 5% CEE, and 50 µg/ml gentamicin (Sigma). The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO₂. The culture medium was changed every other day. BMP2 (R&D Systems), Wnt1 (PEPROTECH), and Wnt3a (R&D Systems) were added to the medium at a concentration of 10 or 100 ng/ml. FGF2 (R&D Systems) was added to the medium at a concentration of 10 ng/ml. Chordin (R&D Systems)
Systems), Noggin (R&D Systems), and BIO (Calbiochem) were added at concentrations of 1 µg/ml, 100 ng/ml, and 1 µM, respectively.

Transfection of expression vectors

Mouse trunk neural crest cells were transfected with 1 µg of expression vectors for 24 h immediately after the removal of the neural tubes, using Lipofectamine 2000 (Invitrogen). Expression vectors are listed in Table 1.1. pcDNA3.1 (Invitrogen) was used as the control vector. The transfection efficiency of the vectors encoding a myc epitope or a Flag tag was estimated by immunostaining using mouse anti-c-myc or mouse anti-Flag. The transfection efficiency was presented as the proportion of cells expressing the myc epitope or the Flag tag per total cells in a neural crest cell colony. The percentage of cells expressing the myc epitope or the Flag tag reached 52.7 (± 3.5) or 72.2 (± 2.8)%.

Table 1.1 List of expression vectors

<table>
<thead>
<tr>
<th>Expression Vectors</th>
<th>Source</th>
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<tr>
<td>pcDNA3 encoding the human DN TCF and a myc-tag</td>
<td>A gift from Dr. Jane B. Trepel [37, 38]</td>
</tr>
<tr>
<td>pcDNA4 encoding the mouse DN β-catenin and a His-tag</td>
<td>A gift from Dr. Jane B. Trepel [37, 38]</td>
</tr>
<tr>
<td>pcDNA3.1 encoding the human WT CHD7 and a Flag-6×His-tag</td>
<td>A gift from Dr. J. Wysocka [21]</td>
</tr>
<tr>
<td>pcDNA3.1 encoding the human DN CHD7 and a Flag-6×His-tag</td>
<td>A gift from Dr. J. Wysocka [21]</td>
</tr>
</tbody>
</table>

CHD7 siRNA preparation

CHD7 siRNA was prepared as described previously [39, 40]. The siRNA duplex for CHD7 were designed on the basis of CHD7 sequence published online [GenBank Accession
No. NM001081417]. The sequence corresponded to bases 4481-4506 of the open reading frame of CHD7 mRNA: 5’-CAGGCTCAAGCTAGATGCCACAGAA (prepared by Invitrogen). Stealth™ RNAi Negative Control Medium GC Duplex #2 (Invitrogen) was used as the control for CHD7 siRNA. Using Lipofectamine 2000, mouse trunk neural crest cells were transfected with 40 nM CHD7 siRNA or RNAi Negative Control for 24 h immediately after the removal of the neural tubes. The transfection efficiency was examined by means of BLOCK-iT Fluorescent Oligo (Invitrogen). The percentage of cells showing a fluorescent signal per neural crest cell colony reached 98.4 ± 0.49%.

**Immunostaining**

Primary cultures of mouse trunk neural crest cells were fixed with 4% PFA for 1 h on ice. The cultures were immunostained with the primary antibodies for 16 h at 4°C and with the secondary antibodies for 1 h at room temperature.

E9.5 mouse embryos, DRG dissected from 11- to 12-week-old ddY mice, and sciatic nerve from L3 to L5 in 11- to 12-week-old ddY mice were fixed with 4% PFA for 1 or 2 h on ice. The fixed tissues were immersed in gradually increasing concentrations of sucrose solution and embedded in OCT compound (Miles). Cryostat sections were cut at 10 μm and mounted on albumin-coated glass slides. The sections were stained using the primary antibodies. Primary antibodies were applied for 16 h at 4°C. The specimens were treated with the secondary antibodies at room temperature for 1 h. Finally, the cultures and sections were nuclear-stained with 0.1 μg/ml DAPI (Dojindo). DAPI nuclear staining was particularly important for counting the exact number of immunoreactive cells in the neural crest cell cultures and for judging cell death. All antibodies are listed in Table 1.2.
<table>
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<th>Antigen/Conjugation</th>
<th>Dilution</th>
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<tr>
<td>Sox10 (rabbit polyclonal antibody)</td>
<td>1:100 (IF)</td>
<td>MILLIPORE (AB5727)</td>
</tr>
<tr>
<td></td>
<td>1:200 (IHC)</td>
<td></td>
</tr>
<tr>
<td>CHD7 (goat polyclonal antibody)</td>
<td>1:100 (IF)</td>
<td>Santa Cruz (sc-79207)</td>
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<tr>
<td></td>
<td>1:50 (IHC)</td>
<td></td>
</tr>
<tr>
<td>p75 (rabbit polyclonal antibody)</td>
<td>1:100 (IF)</td>
<td>Promega (G323A)</td>
</tr>
<tr>
<td></td>
<td>1:100 (IHC)</td>
<td></td>
</tr>
<tr>
<td>NF (rabbit polyclonal antibody)</td>
<td>1:500</td>
<td>MILLIPORE (AB9568)</td>
</tr>
<tr>
<td>SMA (1A4, mouse monoclonal IgG2a)</td>
<td>1:800</td>
<td>Sigma (A2547)</td>
</tr>
<tr>
<td>GFAP (rabbit polyclonal antibody)</td>
<td>1:100</td>
<td>Dako (Z0334)</td>
</tr>
<tr>
<td>BrdU (B44, mouse monoclonal IgG1)</td>
<td>1:10</td>
<td>Becton Dickinson (347580)</td>
</tr>
<tr>
<td>Caspase-3 p11 (mouse monoclonal IgG1)</td>
<td>1:50</td>
<td>Santa Cruz (sc-271759)</td>
</tr>
<tr>
<td>Alexa Fluor 488 (Donkey anti-Goat IgG)</td>
<td>1:500</td>
<td>Invitrogen (A-11055)</td>
</tr>
<tr>
<td>Alexa Fluor 488 (Donkey anti-Mouse IgG)</td>
<td>1:500</td>
<td>Invitrogen (A-21202)</td>
</tr>
<tr>
<td>Alexa Fluor 555 (Donkey anti-Rabbit IgG)</td>
<td>1:500</td>
<td>Invitrogen (A-31572)</td>
</tr>
</tbody>
</table>

**Measurement of cell proliferation and apoptosis**

BrdU-labeling experiments were performed to estimate cell proliferation in mouse trunk neural crest cell cultures. BrdU (Wako) was prepared as a 1 mM stock solution in α-MEM and added to the cultures at a final concentration of 50 µM. The cultures were exposed to BrdU for 90 min and subsequently fixed in 4% PFA on ice for 1 h. BrdU-incorporated cells were detected with anti-BrdU. Furthermore, we characterized the developmental cell death of the trunk neural crest cells by measuring the expression of the apoptotic marker, caspase-3. The cultures were fixed in 4% PFA on ice for 1 h and double-stained with anti-Sox10 and anti-caspase-3 or with anti-p75 and anti-caspase-3. The cultures were nuclear-stained with 0.1 µg/ml DAPI for the total cell count.
Results

**CHD7 expression in trunk neural crest cells of mouse embryos**

Since there have been no reports of CHD7 expression in mouse neural crest cells, we examined the expression patterns of CHD7 in trunk neural crest cells of mouse embryos. We performed the double-stainings using anti-CHD7 and antibodies against Sox10, which is known to be involved in the maintenance of the undifferentiated state of neural crest cells [9], or against p75, another marker of undifferentiated neural crest cells [20]. No expression of CHD7, Sox10, and p75 were observed at the unsegmented levels of the 28-somite stage embryos (Fig. 1.3A-J). At 26-somite levels of the 26-somite stage embryos, CHD7 expression was found in early migratory neural crest cells containing Sox10 or p75 (arrows in Fig. 1.3L-O and Q-T). Furthermore, cells that express both CHD7 and Sox10 were observed in the dorsal neural tube containing premigratory neural crest cells (arrowheads in Fig. 1.3L-O). In addition, there existed CHD7-expressing cells with both Sox10 and p75 at the 25-somite levels of the 28-somite stage embryos (data not shown). We also examined the expression of CHD7, Sox10, and p75 during late migratory stages of the trunk neural crest cells. CHD7-expressing cells containing Sox10 and/or p75 were observed (arrows in Fig. 1.3V-Y and A’-D’). These results indicate that undifferentiated mouse trunk neural crest cells express CHD7 as well as Sox10 and p75.
Fig. 1.3  Expression patterns of CHD7, Sox10, or p75 during mouse trunk neural crest development. The top, bottom, left, and right of each photograph correspond to the dorsal, ventral, proximal, and distal side of the embryo, respectively. (A-J) Transverse sections of a 28-somite stage embryo at the unsegmented level. (A-E) and (F-J) are neighboring sections. (A) Bright-field image. (B) Expression pattern of CHD7 in the same field as (A). (C) Expression pattern of Sox10 in the same field as (A). (D) DAPI nuclear stain of the same field as (A). (E) Merged image of (B-D). (F) Bright-field image. (G) Expression pattern of CHD7 in the same field as (F). (H) Expression pattern of p75 in the same field as (F). (I) DAPI nuclear stain of the same field as (F). (J) Merged image of (G-I). (K-T) Transverse sections of a 26-somite stage embryo at the 26-somite level. (K-O) and (P-T) are neighboring sections. (K) Bright-field image. (L) Expression pattern of CHD7 in the same field as (K). (M) Expression pattern of Sox10 in the same field as (K). (N) DAPI nuclear stain of the same field as (K). (O) Merged image of (L-N). (P) Bright-field image. (Q) Expression pattern of CHD7 in the same field as (P). (R) Expression pattern of p75 in the same field as (P). (S) DAPI nuclear stain of the same field as (P). (T) Merged image of (Q-S). (U-D') Oblique sections of a 26-somite stage embryo at the 4-somite level. (U-Y) and (Z-D') are neighboring sections. (U) Bright-field image. (V) Expression pattern of CHD7 in the same field as (U). (W) Expression pattern of Sox10 in the same field as (U). (X) DAPI nuclear stain of the same field as (U). (Y) Merged image of (V-X). (Z) Bright-field image. (A') Expression pattern of CHD7 in the same field as (Z). (B') Expression pattern of p75 in the same field as (Z). (C') DAPI nuclear stain of the same field as (Z). (D') Merged image of (A'-C'). Arrows indicate the neural crest cells expressing both CHD7 and Sox10 or both CHD7 and p75. Arrowheads show a cell expressing both CHD7 and Sox10 in the dorsal neural tube that the premigratory neural crest cells exist. NP; neural plate, NT; neural tube.

Scale Bars = 20 μm.
Expression of CHD7, Sox10, and p75 in mouse trunk neural crest cell cultures

Mouse trunk neural crest cells undergo temporally regulated differentiation even in cultures [33, 34]. Therefore, to examine the temporal changes in CHD7 expression, we used mouse trunk neural crest cell cultures. After 2, 4, or 6 days in culture, we performed immunocytochemical analysis using anti-CHD7 and anti-Sox10. The percentage of cells expressing CHD7 and/or Sox10 per neural crest cell colony (each colony of neural crest cells was derived from a neural tube explant) was significantly lower at 6 days in culture than at 2 days in culture (Fig. 1.4A-M). Experiments using anti-CHD7 and anti-p75 also showed the same results (Fig. 1.4N-Z).

Actions of BMP and Wnt signaling on the maintenance of the undifferentiated state of mouse trunk neural crest cells

It has been known that the concerted activity of BMP2 (10 ng/ml) and Wnt1 (unknown concentrations) maintains the undifferentiated state of neural crest cells [18]. Therefore, we examined whether these factors not only participate in the maintenance of the undifferentiated state of mouse trunk neural crest cells under our experimental conditions but also affect CHD7 expression in these cells. BMP2 and Wnt1 were added to the medium from 2 days to 6 days in culture (Fig. 1.5A). When Wnt1 was used at 10 ng/ml, the expression of CHD7 and/or p75 was significantly maintained on culture day 6 (Fig. 1.5G and O-Q). The maintenance of Sox10 expression was less pronounced (Fig. 1.5H). However, in the treatment with 100 ng/ml Wnt1 and 10 ng/ml BMP2, Sox10 expression drastically decreased (Fig. 1.5H). Furthermore, the percentage of double-positive cells expressing Sox10 and CHD7 was also suppressed (Fig. 1.5I). Therefore, we used Wnt3a, which is
Fig. 1.4  Expression patterns of CHD7, Sox10, or p75 in mouse trunk neural crest cell cultures. Immunostaining with anti-CHD7, anti-Sox10, and anti-p75 was performed on culture day 2, 4, and 6.  (A and F) Bright-field images.  (B and G) Anti-CHD7-positive cells in the same fields as (A) and (F), respectively.  (C and H) Anti-Sox10-positive cells in the same fields as (A) and (F), respectively.  (D and I) DAPI nuclear stains of the same fields as (A) and (F), respectively.  (E) Merged image of (B-D).  (J) Merged image of (G-I).  (K) Percentage of cells expressing CHD7 per total cells in a neural crest cell colony (each colony was derived from a neural tube explant).  (L) Percentage of cells expressing Sox10 per total cells in a neural crest cell colony.  (M) Percentage of cells expressing both CHD7 and Sox10 per total cells in a neural crest cell colony.  (N and S)
Bright-field images. (O and T) Anti-CHD7-positive cells in the same fields as (N) and (S), respectively. (P and U) Anti-p75-positive cells in the same fields as (N) and (S), respectively. (Q and V) DAPI nuclear stains of the same fields as (N) and (S), respectively. (R) Merged image of (O-Q). (W) Merged image of (T-V). (X) Percentage of cells expressing CHD7 per total cells in a neural crest cell colony. (Y) Percentage of cells expressing p75 per total cells in a neural crest cell colony. (Z) Percentage of cells expressing both CHD7 and p75 per total cells in a neural crest cell colony. *, $p < 0.05$ (Student’s $t$-test) compared to culture day 2. Data in (K-M and X-Z) are expressed as mean ± SEM of separate counts of 5-11 colonies (the number in a parenthesis on each bar). Scale Bars = 50 µm.

Expressed in the dorsal neural tube in which neural crest cells are formed, similar to Wnt1 [41]. The treatment with 10 ng/ml BMP2 and 10 ng/ml Wnt3a promoted the expression of CHD7, Sox10, or p75. The effects were unchanged even when Wnt3a was used at 100 ng/ml (Fig. 1.5). These results suggest that Wnt3a is more effective for the maintenance of the undifferentiated state of mouse trunk neural crest cells. Therefore, we examined the effects of BMP2, Wnt1, and Wnt3a in more detail. BMP2 or Wnt1 alone had almost no effects on CHD7 expression or inhibitory effects on Sox10 expression (Fig. 1.6B-D). These inhibitory effects may cause the decrease of Sox10 expression in BMP2/Wnt1 treatment, leading to the reduction of the proportion of cells expressing both Sox10 and CHD7. On the other hand, the treatment with Wnt3a alone, especially with 100 ng/ml Wnt3a, produced effects similar to those of the 10 ng/ml BMP2 and 10 ng/ml Wnt3a treatments on the maintenance of the undifferentiated state (Fig. 1.6B-D). We prevented BMP activities with two BMP inhibitors, noggin and chordin, to exclude the possibility that the effects of Wnt3a alone were caused by the BMP activities in FBS and/or CEE in the culture medium [42].
These inhibitors had no effects on the maintenance of CHD7 or Sox10 expression (Fig. 1.6E-G). These data suggest that the Wnt signaling is indispensable for the maintenance of the undifferentiated state of mouse trunk neural crest cells and BMP signaling plays supportive roles in the maintenance of the undifferentiated state of these cells.

We tried to identify the Wnt signaling pathway involved in the maintenance of the undifferentiated state. When the neural crest cell cultures were treated with 10 ng/ml BMP2 and BIO, a reagent that activates the Wnt/β-catenin pathway [43], effects similar to those of the 10 ng/ml BMP2 and 10 ng/ml Wnt3a treatments were produced (Fig. 1.6H-J). In addition, the treatment with the DN β-catenin expression vectors or the DN TCF expression vectors drastically inhibited the effects of 10 ng/ml BMP2 and 10 ng/ml Wnt3a on CHD7 or Sox10 expression (Fig. 1.6H-J). Thus, Wnt/β-catenin pathway may play important roles in the maintenance of the undifferentiated state of mouse trunk neural crest cells.

Previous studies have shown that the treatment with 10 ng/ml BMP2 [44] or unknown concentrations of Wnt1 [45, 46] promotes sensory neurogenesis of neural crest cells. Therefore, we investigated the effects of BMP2, Wnt1 and/or Wnt3a on sensory neurogenesis of mouse trunk neural crest cells under the present culture condition. The treatment with 10 ng/ml BMP2, Wnt1 (10 ng/ml or 100 ng/ml), and/or Wnt3a (10 ng/ml or 100 ng/ml) promoted the differentiation of sensory neurons containing Brn3a (Fig. 1.7), similar to previous reports. However, no synergistic effects of the treatment with both 10 ng/ml BMP2 and 10 ng/ml Wnt3a were found. These data suggest that the coordinated activity of BMP2 and Wnt3a acts on the maintenance of the undifferentiated state of mouse trunk neural crest cells rather than promotes differentiation of these cells into sensory neurons.
Fig. 1.5  Effects of BMP and Wnt on the maintenance of the undifferentiated state.  (A) After removal of neural tube explants, mouse trunk neural crest cells were exposed to BMP2/Wnt3a or BMP2/Wnt1 from 2 days to 6 days in culture.  Immunostaining with anti-CHD7 and anti-Sox10 or with anti-CHD7 and anti-p75 was performed on culture day 6.  (B) Bright-field image in the presence of BMP2 and Wnt3a.  (C) Anti-CHD7-positive cells in the same field as (B).  (D) Anti-Sox10-positive cells in the same field as (B).  (E) DAPI nuclear stain of the same field as (B).  (F) Merged image of (C-E).  (G) Percentage of cells expressing CHD7 per total cells in a neural crest cell colony (each
colony was derived from a neural tube explant). (H) Percentage of cells expressing Sox10 per total cells in a neural crest cell colony. (I) Percentage of cells expressing both CHD7 and Sox10 per total cells in a neural crest cell colony. (J) Bright-field image in the presence of BMP2 and Wnt3a. (K) Anti-CHD7-positive cells in the same field as (J). (L) Anti-p75-positive cells in the same field as (J). (M) DAPI nuclear stain of the same field as (J). (N) Merged image of (K-M). (O) Percentage of cells expressing CHD7 per total cells in a neural crest cell colony. (P) Percentage of cells expressing p75 per total cells in a neural crest cell colony. (Q) Percentage of cells expressing both CHD7 and p75 per total cells in a neural crest cell colony. *, p < 0.05 (Student's t-test) compared to the untreated cultures. Data in (G-I and O-Q) are expressed as mean ± SEM of separate counts of 5-12 colonies (the number in a parenthesis on each bar). Scale Bars = 50 µm.
Fig. 1.6  Roles of Wnt/β-catenin pathway in the maintenance of the undifferentiated state.

(A) After removal of neural tube explants, mouse trunk neural crest cells were exposed to BMP2, Wnt1,
Wnt3a, noggin, chordin and/or BIO from 2 days to 6 days in culture. The DN β-catenin expression vectors, the DN TCF expression vectors, or the control vectors were applied from 2 days to 3 days in culture. Immunostaining with anti-CHD7 and anti-Sox10 was performed on culture day 6. **(B)** Percentage of cells expressing CHD7 per total cells in a neural crest cell colony (each colony was derived from a neural tube explant). **(C)** Percentage of cells expressing Sox10 per total cells in a neural crest cell colony. **(D)** Percentage of cells expressing both CHD7 and Sox10 per total cells in a neural crest cell colony. *, p < 0.05 (Student’s t-test) compared to the untreated cultures. †, p < 0.05 (Student’s t-test) compared to the BMP2/Wnt3a-treated cultures. **(E)** Percentage of cells expressing CHD7 per total cells in a neural crest cell colony. **(F)** Percentage of cells expressing Sox10 per total cells in a neural crest cell colony. **(G)** Percentage of cells expressing both CHD7 and Sox10 per total cells in a neural crest cell colony. **(H)** Percentage of cells expressing CHD7 per total cells in a neural crest cell colony. **(I)** Percentage of cells expressing Sox10 per total cells in a neural crest cell colony. **(J)** Percentage of cells expressing both CHD7 and Sox10 per total cells in a neural crest cell colony. *, p < 0.05 (Student’s t-test) compared to the BMP2/Wnt3a-treated cultures. †, p < 0.05 (Student’s t-test) compared to the cultures treated with the control vectors. Data are expressed as mean ± SEM of separate counts of 5-9 colonies (the number in a parenthesis on each bar).
Fig. 1.7  Sensory neurogenesis in mouse trunk neural crest cell cultures. Immunostaining with anti-Brn3a was performed on culture day 6.  

((A), (E), and (I)) Bright-field images.  

((B), (F), and (J)) Anti-Brn3a-positive cells in the same fields as (A), (E), and (I), respectively.  

((C), (G), and (K)) DAPI nuclear stains of the same fields as (A), (E), and (I), respectively.  

(D) Merged image of (B) and (C).  

(H) Merged image of (F) and (G).  

(L) Merged image of (J) and (K).  

(M) Percentage of cells expressing Brn3a per total cells in a neural crest cell colony (each colony was derived from a neural tube explant). * , p < 0.05 (Student’s t-test) compared to the untreated cultures.  

Data are expressed
as mean ± SEM of separate counts of 5-11 colonies (the number in a parenthesis on each bar).

Scale Bars = 50 µm.

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**Actions of CHD7 on the maintenance of the undifferentiated state of mouse trunk neural crest cells**

In this study, we showed that BMP/Wnt signaling not only maintained the undifferentiated state of mouse trunk neural crest cells but also activated CHD7 expression. It is possible that CHD7 plays important roles in the maintenance of the undifferentiated state of mouse trunk neural crest cells. To examine this possibility, the neural crest cell cultures were treated with the WT CHD7, DN CHD7 expression vectors, or CHD7 siRNA (Fig. 1.8A and Fig. 1.9A). The number of CHD7-positive cells was dramatically increased by the addition of the WT CHD7 expression vectors (Fig. 1.8C, G, K, and O). This showed that the WT CHD7 expression vectors were effectively introduced to the neural crest cells. Furthermore, the WT CHD7 expression vectors maintained the expression of Sox10 or p75 (Fig. 1.8D, H, L, and P) and the co-expression of CHD7 and Sox10 (Fig. 1.8F and I) or of CHD7 and p75 (Fig. 1.8N and Q). When the DN CHD7 expression vectors were added, the number of CHD7-positive cells increased (Fig. 1.9B and E), indicating that the anti-CHD7 antibody used in this study recognizes the mutant CHD7, because this mutant protein changed only lysine 998 in the ATPase domain of CHD7 to arginine. However, treatment with the DN CHD7 expression vectors significantly suppressed the expression of Sox10 and p75 (Fig. 1.9C and F). This treatment also suppressed the co-expression of CHD7 and Sox10 or of CHD7 and p75 (Fig. 1.9D and G). Treatment with CHD7 siRNA dramatically suppressed the expression of CHD7, Sox10, and/or p75 (Fig. 1.9B-G). These results show
that CHD7 is a key requisite for the maintenance of the undifferentiated state of mouse trunk neural crest cells.
Fig. 1.8 Effects of the WT CHD7 expression vectors on the maintenance of the undifferentiated state.  

(A) After removal of neural tube explants, mouse trunk neural crest cells were exposed to the WT CHD7 expression vectors from 2 days to 3 days in culture. Immunostaining with anti-CHD7 and anti-Sox10 or with anti-CHD7 and anti-p75 was performed on culture day 6.  

(B) Bright-field image in the presence of the WT CHD7 expression vectors.  

(C) Anti-CHD7-positive cells in the same field as (B).  

(D) Anti-Sox10-positive cells in the same field as (B).  

(E) DAPI nuclear stain of the same field as (B).  

(F) Merged image of (C-E).  

(G) Percentage of cells expressing CHD7 per total cells in a neural crest cell colony (each colony was derived from a neural tube explant).  

(H) Percentage of cells expressing Sox10 per total cells in a neural crest cell colony.  

(I) Percentage of cells expressing both CHD7 and Sox10 per total cells in a neural crest cell colony.  

(J) Bright-field image in the presence of the WT CHD7 expression vectors.  

(K) Anti-CHD7-positive cells in the same field as (J).  

(L) Anti-p75-positive cells in the same field as (J).  

(M) DAPI nuclear stain of the same field as (J).  

(N) Merged image of (K-M).  

(O) Percentage of cells expressing CHD7 per total cells in a neural crest cell colony.  

(P) Percentage of cells expressing p75 per total cells in a neural crest cell colony.  

(Q) Percentage of cells expressing both CHD7 and p75 per total cells in a neural crest cell colony.  

*, p < 0.05 (Student’s t-test) compared to the untreated cultures.  

†, p < 0.05 (Student’s t-test) compared to the BMP2/Wnt3a-treated cultures.  

Data are expressed as mean ± SEM of separate counts of 5-11 colonies (the number in a parenthesis on each bar).  

Scale Bars = 50 µm.
Fig. 1.9 Effects of the DN CHD7 expression vectors or CHD7 siRNA on the maintenance of the undifferentiated state. (A) After removal of neural tube explants, mouse trunk neural crest cells were exposed to BMP2 and Wnt3a from 2 days to 6 days in culture. The DN CHD7 expression vectors or CHD7 siRNA were applied from 2 days to 3 days in culture. Immunostaining with anti-CHD7 and anti-Sox10 or with anti-CHD7 and anti-p75 was performed on culture day 6. (B) Percentage of cells expressing CHD7 per total cells in a neural crest cell colony (each colony was derived from a neural tube explant). (C) Percentage of cells expressing Sox10 per total cells in a
neural crest cell colony.  (D) Percentage of cells expressing both CHD7 and Sox10 per total cells in a neural crest cell colony.  (E) Percentage of cells expressing CHD7 per total cells in a neural crest cell colony.  (F) Percentage of cells expressing p75 per total cells in a neural crest cell colony.  (G) Percentage of cells expressing both CHD7 and p75 per total cells in a neural crest cell colony.  *, $p < 0.05$ (Student's $t$-test) compared to the untreated cultures.  †, $p < 0.05$ (Student's $t$-test) compared to the BMP2/Wnt3a-treated cultures.  Data are expressed as mean ± SEM of separate counts of 4-11 colonies (the number in a parenthesis on each bar).

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**Proliferation and apoptosis of mouse trunk neural crest cells by treatment with BMP2/Wnt3a or the WT CHD7 expression vectors**

Since the number of the undifferentiated neural crest cells was significantly increased by treatment with BMP2 (10 ng/ml)/Wnt3a (10 ng/ml) or the WT CHD7 expression vectors, we examined the effects of these factors on the proliferation of mouse trunk neural crest cells using BrdU incorporation method.  The percentage of BrdU-incorporated cells was increased by treatment with BMP2 (10 ng/ml)/Wnt3a (10 ng/ml) or the WT CHD7 expression vectors on culture day 6 (Fig. 1.10K).  Furthermore, these treatments increased the proportion of BrdU-incorporated cells per total Sox10- or p75-expressing cells (Fig. 1.10A-E and L).  The DAPI nuclear-staining experiments showed that almost no cell death occurs under treatment with BMP2 (10 ng/ml)/Wnt3a (10 ng/ml) or the WT CHD7 expression vectors.  Therefore, we measured apoptosis in these culture conditions on culture day 6.  No significant differences of the proportion of apoptotic cell death were found among the culture conditions examined (Fig. 1.10M).  Moreover, no cells expressing both caspase-3 and Sox10 (Fig. 1.10F-J) or both caspase-3 and p75 were observed under the
culture conditions examined. BMP2/Wnt3a signaling or CHD7 is likely to promote the proliferation of undifferentiated mouse trunk neural crest cells.

![Image](image_url)

**Fig. 1.10  Proliferation and apoptosis of mouse trunk neural crest cells.** After removal of neural tube explants, mouse trunk neural crest cells were exposed to BMP2 and Wnt3a from 2 days to 6 days in culture. The WT CHD7 expression vectors were applied from 2 days to 3 days in culture. BrdU incorporation and the double-labeling with anti-Sox10 and anti-BrdU or with anti-p75 and anti-BrdU were carried out on culture day 6. The double-staining with anti-Sox10 and anti-caspase-3 or with anti-p75 and anti-caspase-3 was also performed on culture day 6. (A) Bright-field image in the presence of the WT CHD7 expression vectors. (B) BrdU-labeled cells in the same field as (A). (C) Anti-Sox10-positive cells in the same field as (A). (D) DAPI nuclear stain of the same field as (A). (E) Merged image of (B-D). (F) Bright-field image of an untreated culture. (G) Caspase-3-labeled...
cells in the same field as (F).  (H) Anti-Sox10-positive cells in the same field as (F).  (I) DAPI nuclear stain of the same field as (F).  (J) Merged image of (G-I).  (K) Percentage of BrdU-incorporated cells per total cells in a neural crest cell colony (each colony was derived from a neural tube explant).  (L) Percentage of anti-Sox10- or anti-p75-positive cells that incorporated BrdU per total Sox10- or p75-expressing cells in a neural crest cell colony.  (M) Percentage of anti-caspase-3-positive cells per total cells in a neural crest cell colony.  *, p < 0.05 (Student's t-test) compared to the untreated cultures.  Data are expressed as mean ± SEM of separate counts of 5-19 colonies (the number in a parenthesis on each bar).  Scale Bars = 50 µm.

**Actions of CHD7 and BMP/Wnt signaling on the maintenance of the multipotency of mouse trunk neural crest cells**

We showed here that CHD7 was expressed in undifferentiated mouse trunk neural crest cells and involved in the maintenance of the undifferentiated state.  Since undifferentiated neural crest cells are likely to be multipotential, CHD7 and BMP2/Wnt3a may participate in maintaining the multipotency of mouse trunk neural crest cells.  It has been known that multipotent trunk neural crest cells can differentiate into smooth muscle cells, neurons, and glia [47].  Therefore, we investigated whether or not the activation of CHD7 or BMP2/Wnt3a signaling triggers the increase in the number of multipotent mouse trunk neural crest cells that differentiate into smooth muscle cells, neurons, and glia.  The trunk neural crest cells were treated with the WT CHD7 expression vectors or with BMP2 (10 ng/ml) /Wnt3a (10 ng/ml) and clonal cultures were subsequently performed in the presence or absence of 10 ng/ml FGF2 (Fig. 1.11A).  FGF2 was required for glial differentiation in clonal cultures [40, 44].  Four types of clones appeared in the absence of FGF2: clones
containing both smooth muscle cells and neurons, clones containing either smooth muscle cells or neurons, and clones containing neither smooth muscle cells nor neurons (Fig. 1.11B-D). Furthermore, three types of clones were observed in the presence of FGF2: clones containing both smooth muscle cells and glia, clones containing smooth muscle cells only, and clones containing neither smooth muscle cells nor glia (Fig. 1.11F and G). The addition of WT CHD7 expression vectors or BMP2 (10 ng/ml) /Wnt3a (10 ng/ml) significantly increased the proportion of clones consisting of both smooth muscle cells and neurons or both smooth muscle cells and glia that originated from the multipotent trunk neural crest cells (Fig. 1.11E and H). These results suggest that BMP2/Wnt3a signaling or CHD7 profoundly participate in the maintenance of the multipotency of mouse trunk neural crest cells. Thus, cells expressing CHD7 and Sox10 or expressing CHD7 and p75 observed in mouse trunk neural crest cell cultures, the number of which was increased by BMP2/Wnt3a treatment, may be NCSCs.

**Expression of CHD7 in mouse adult DRG and adult sciatic nerve**

Adult DRG and adult sciatic nerves have been known to contain NCSCs expressing Sox10 and/or p75 [14-17]. Therefore, we analyzed the expression of CHD7, Sox10, and p75 in adult DRG and adult sciatic nerves. Cells expressing CHD7, Sox10, or p75 were observed in the adult DRG (Fig. 1.12A-J) and in the adult sciatic nerves (Fig. 1.12K-T). Moreover, the double-positive cells were found in any of tissues examined (arrowheads in Fig. 1.12).
Fig. 1.11  Effects of the WT CHD7 expression vectors and BMP2/Wnt3a on the maintenance of the multipotency.  (A) After removal of neural tube explants, mouse trunk neural crest cells were exposed to BMP2 and Wnt3a from 2 days to 6 days in culture.  The WT CHD7 expression vectors were applied from 2 days to 3 days in culture.  The clonal cultures were started on culture day 6.  Immunostaining with anti-SMA and anti-NF or with anti-SMA and anti-GFAP was performed on culture day 11.  In clonal cultures, FGF2 was added to the medium to stimulate the differentiation of anti-GFAP-positive glial cells.  (B) A clone containing both anti-SMA (green)- and anti-NF (red)-positive cells in the presence of BMP2 and Wnt3a.  (C) A clone containing anti-SMA-positive cells only in the presence of BMP2 and Wnt3a.  (D) A clone containing anti-NF- positive cells only in the presence of BMP2 and Wnt3a.  (E) Percentage of clones containing neuron+ smooth muscle cell+ and neuron/- smooth muscle cell-.  (F) Percentage of clones containing neuron+ smooth muscle cell+ and neuron/- smooth muscle cell-.  (G) Percentage of clones containing neuron+ smooth muscle cell+ and neuron/- smooth muscle cell-.  (H) Percentage of clones containing neuron+ smooth muscle cell+ and neuron/- smooth muscle cell-.
cells expressing SMA and/or NF per total number of neural crest cell clones under various culture conditions.  (F) A clone containing both anti-SMA (green)- and anti-GFAP (red)-positive cells in the presence of WT CHD7 expression vectors.  (G) A clone containing anti-SMA-positive cells only in the presence of WT CHD7 expression vectors.  DAPI nuclear stain (blue).  (H) Percentage of clones containing cells expressing SMA and/or GFAP per total number of neural crest cell clones under various culture conditions.  *, $p < 0.05$ (Student's t-test) compared to the untreated cultures.  Data are expressed as mean ± SEM of separate counts of 3 independent experiments (the number in a parenthesis on each bar).  Scale Bars = 50 µm.
Fig. 1.12  Expression patterns of CHD7, Sox10, or p75 in DRG or sciatic nerve of adult mice.

(A-J) Transverse sections of DRG in a 12-week old mouse.  (A) Bright-field image.  (B) Expression pattern of CHD7 in the same field as (A).  (C) Expression pattern of Sox10 in the same field as (A).  (D) DAPI nuclear stain of the same field as (A).  (E) Merged image of (B-D).  (F) Bright-field image.  (G) Expression pattern of CHD7 in the same field as (F).  (H) Expression pattern of p75 in the same field as (F).  (I) DAPI nuclear stain of the same field as (F).  (J) Merged image of (G-I).  (K-T) Transverse sections of sciatic nerve in a 12-week old mouse.  (K) Bright-field image.  (L) Expression pattern of CHD7 in the same field as (K).  (M) Expression pattern of Sox10 in the same field as (K).  (N) DAPI nuclear stain of the same field as (K).  (O) Merged image of (L-N).  (P) Bright-field image.  (Q) Expression pattern of CHD7 in the same field as (P).  (R) Expression pattern of p75 in the same field as (P).  (S) DAPI nuclear stain of the same field as (P).  (T) Merged image of (Q-S).

Arrowheads show cells expressing both CHD7 and Sox10 or both CHD7 and p75.  Scale Bars = 20 µm.
Discussion

In the present study, we analyzed roles of a chromatin remodeler, CHD7 [25, 48, 49] and BMP/Wnt signaling in maintaining the multipotency of mouse trunk neural crest cells. Chromatin remodeling plays important roles in development and differentiation [50]. It has been shown that CHD7 controls the expression of genes that characterize ES cells [25] and is involved in the induction of neural crest cells [21], which show multipotency like ES cells. Immunohistochemistry using mouse embryos showed that CHD7 was expressed in undifferentiated trunk neural crest cells containing Sox10, which participates in the maintenance of the undifferentiated state of neural crest cells [9], and p75, another marker of undifferentiated neural crest cells [20]. Further, CHD7 expression was found in the dorsal neural tube. These in vivo data are consistent with the results of a previous study [21].

In mouse trunk neural crest cell cultures, the number of Sox10- or p75-positive undifferentiated neural crest cells decreased as the culture progressed. Similarly, CHD7 expression was also suppressed over time. These results suggest the hypothesis that CHD7 may participate in the maintenance of the undifferentiated state of neural crest cells. This hypothesis was examined by experiments using BMP and Wnt proteins. Kléber et al. (2005) [31] has shown that undifferentiated neural crest cells expressing Sox10 and p75 are maintained by combined treatment with BMP2 (10 ng/ml) and Wnt1 (unknown concentrations). In this study, BMP/Wnt treatment maintained CHD7 expression as well as Sox10/p75 expression. Thus, it is conceivable that CHD7 not only affects neural crest induction but also plays important roles in the maintenance of the undifferentiated state of mouse trunk neural crest cells.
Wnt1 and Wnt3a are expressed in partially overlapping domains in the dorsal neural tube in which neural crest cells are formed [41]. When Wnt3a was added to mouse trunk neural crest cell cultures with BMP2, the expression of CHD7, Sox10, and p75 was maintained more effectively than the addition of Wnt1. Moreover, a high concentration of Wnt3a (100 ng/ml) independently maintained the expression of CHD7, Sox10, and p75, similar to the case with 10 ng/ml BMP and 10 ng/ml Wnt3a. This effect was unchanged even in the presence of noggin or chordin. These data suggest that the activation of Wnt signaling is a key requisite for the maintenance of CHD7 expression and thus for the maintenance of the undifferentiated state of neural crest cells, and that BMP signaling plays supportive roles in the maintenance of the undifferentiated state of these cells, as shown in the maintenance of the undifferentiated state of ES cells [51-53]. The addition of BIO, instead of Wnt, to neural crest cell cultures also maintained the undifferentiated state. Furthermore, the effects of BMP2 and Wnt3a were inhibited by the DN β-catenin or DN TCF expression vectors. These results suggest that the Wnt/β-catenin pathway is essential to the maintenance of the undifferentiated state of mouse trunk neural crest cells.

Wnt signaling plays important roles in neural crest induction [54]. Neurons derived from the proximal ganglion of cranial nerve IX are absent in the mutant mouse embryos lacking both Wnt1 and Wnt3a [55]. Further, β-catenin mutant mouse embryos lack melanocytes and sensory neurons [56]. These in vivo data have shown that the Wnt/β-catenin pathway participates in neural crest induction and differentiation [57]. In addition, it has been known that BMP signaling is essential for neural crest induction [4] and BMP2 is especially required for migration of mouse neural crest cells [58]. In the present study, BMP2 and/or Wnts also promoted sensory neurogenesis of mouse trunk neural crest cells. Thus, it is possible that BMP/Wnt signaling affect multiple aspects of neural crest
development such as induction, migration, differentiation, the maintenance of the undifferentiated state.

When mouse trunk neural crest cell cultures were treated with the WT CHD7 expression vectors, the undifferentiated state of the trunk neural crest cells was maintained, similar to the case with BMP (10 ng/ml) /Wnt3a (10 ng/ml) treatment. The treatment with the DN CHD7 expression vectors or CHD7 siRNA significantly inhibited the effects of BMP2/Wnt3a. It is conceivable that CHD7 participates in the maintenance of the undifferentiated state of mouse trunk neural crest cells as well as in neural crest induction [21], similar to the case with BMP/Wnt signaling.

BrdU-incorporated experiments showed that the proliferation of undifferentiated mouse trunk neural crest cells were significantly promoted by the treatment with BMP2/Wnt3a or WT CHD7 expression vectors. On the other hand, any selective cell death was not found in these treatments. Thus, the maintenance of the undifferentiated state of the trunk neural crest cells may be due to the promotion of the proliferation of the undifferentiated neural crest cells by BMP2/Wnt3a signaling and the chromatin remodeler.

The maintenance of the undifferentiated state of mouse trunk neural crest cells may enable the maintenance of the multipotency of these cells. In fact, the results of clonal cultures showed that multipotent cells were increased by the addition of WT CHD7 expression vectors or BMP2/Wnt3a. Furthermore, CHD7-containing cells expressing Sox10 or p75 were observed in the DRG and sciatic nerves of adult mice that had NCSCs [14, 15]. These results suggest that CHD7 as well as Sox10 participates in the maintenance of the multipotency of neural crest cells and conduct them to mouse NCSCs expressing p75. Taken together, the present results suggest the possibility that BMP2/Wnt3a signaling and CHD7 play essential roles in the maintenance of the multipotency of mouse trunk neural crest cells, leading to the formation of mouse NCSCs (Fig. 1.13).
Fig. 1.13 Involvement of CHD7 and BMP2/Wnt3a signaling in the formation of mouse NCSCs.

CHD7, which is activated by BMP2/Wnt3a signaling, maintains the multipotency of mouse neural crest cells through the promotion of Sox10 expression and leads to the formation of mouse NCSCs expressing p75.
Chapter II

Direct control of FoxD3 expression by CHD7, Oct3/4, Sox2, and Nanog in the formation of mouse neural crest-derived stem cells

Introduction

Neural crest induction is initiated by BMPs/Wnts signaling at the neural plate border [3]. These signaling molecules induce the expression of neural crest inducer genes, Msx1, Zic1, and Pax3/7 [6]. Furthermore, these transcriptional factors activate neural crest specifier genes such as FoxD3, Sox9, Sox10, and Twist1 [7, 8]. Neural crest specifier genes participate in the generation of multipotent neural crest cells [47, 59, 60]. FoxD3 is also indispensable for the maintenance of pluripotency in ES cells [61, 62].

As described in Chapter I, some neural crest cells maintain their multipotency and form NCSCs in various tissues, including DRG, sciatic nerve, epidermis, cardiac outflow tract, and bone marrow [10-13]. We have shown that CHD7 as well as Sox10 plays important roles in the formation of mouse NCSCs and the activities of CHD7 and Sox10 are promoted by BMP2/Wnt3a treatment [63].

CHD7 has been known to interact with histone H3 methylated at lysine 4 in enhancer regions of target genes via its chromodomains [64, 65]. CHD7 regulates the activities of Sox9 and Twist1 by binding to H3K4me1 in the enhancer regions of the genes of these transcription factors in neural crest induction [21]. In addition, CHD7 and the products of the pluripotent stem cell-related genes, which contain Oct3/4, Sox2, and Nanog, colocalize at
CHD7 binding sites of target gene enhancers marked with H3K4me1 in ES cells [25].

Furthermore, it has been shown that neural crest cells and neural crest-derived cells express Oct3/4, Sox2, and Nanog [66, 67].

The data described above suggest that the interaction among CHD7, the neural crest inducer genes, the neural crest specifier genes, and the pluripotent stem cell-related genes may play essential roles in the formation of NCSCs (Fig. 2.1). However, nothing is known about their interactions in the formation of NCSCs. In the present study, therefore, we analyzed the roles of CHD7, neural crest inducer genes, neural crest specifier genes, and pluripotent stem cell-related genes in the formation of NCSCs. Data show that FoxD3 activation by CHD7 and the products of pluripotent stem cell-related genes are required for the formation of mouse NCSCs.

**Fig. 2.1  Hypothesis for a regulatory gene cascade in the formation of mouse NCSCs.** We have shown that BMP2/Wnt3a signaling activates CHD7 and Sox10 and that activation of these factors leads to the formation of mouse NCSCs [63]. Now, we propose a hypothesis that the products of neural crest specifier genes promote the expression of CHD7 and/or Sox10 and that neural crest inducer genes and/or pluripotent stem cell-related genes play important roles in the NCSCs formation through the activation of neural crest specifier genes.
Materials & Methods

Primary cultures

Primary cultures of mouse trunk neural crest cells were performed by using procedures described in Chapter I. Neural tubes isolated from E9.5 embryos were explanted into collagen-coated 35-mm culture dishes. The explants were scraped away with a tungsten needle after 48 h in culture, leaving the emigrated trunk neural crest cells on the substratum. The culture medium consisted of 85% α-MEM (Sigma), 10% FBS (GE Healthcare Life Sciences), 5% CEE, and 50 µg/ml gentamicin (Sigma). BMP2 (R&D Systems) and Wnt3a (R&D Systems) were added to the medium at a concentration of 10 ng/ml.

Transfection of CHD7 expression vector or siRNAs

Mouse trunk neural crest cells were transfected with 1 µg of pcDNA3.1 encoding the human WT CHD7 and a Flag-6×His tag (a gift from Dr. J. Wysocka, Stanford University) [21] or with siRNAs for 24 h immediately after the removal of the neural tubes, using Lipofectamine 2000 (Invitrogen). The siRNA duplexes for FoxD3, Sox9, Twist1, CHD7, Oct3/4, Sox2, Nanog, Setd7, MLL1, Setd1a, ESET, and Suv39h1 were designed on the basis of their sequences published online [GenBank Accession Nos. NM01425, NM011448, NM011658, NM001081417, NM 001252452, NM011443, AB093574, NM080793, NM001081049, NM178029, NM018877, NM011514]. All siRNA sequences are listed in Table 2.1. pcDNA3.1 (Invitrogen) and Stealth™ RNAi Negative Control Medium GC Duplex #2 (Invitrogen) was used as the control.
Table 2.1. List of siRNA sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FoxD3</td>
<td>UUCACCAGGCUGUUCUUGGCUUGU</td>
</tr>
<tr>
<td>Sox9</td>
<td>CCAGCAAGAACAACGCACACGUCAA</td>
</tr>
<tr>
<td>Twist1</td>
<td>CCAGGUACAUCCUCGUACUACCA</td>
</tr>
<tr>
<td>CHD7</td>
<td>CGGCUACUCGUAGAUCACAGAA</td>
</tr>
<tr>
<td>Oct3/4</td>
<td>CCGGAAGAGAAAGCAGAUCUAGCAU</td>
</tr>
<tr>
<td>Sox2</td>
<td>UUAUAUUCCCAGGUGCUCCUUGAUG</td>
</tr>
<tr>
<td>Nanog</td>
<td>UUAUGCUCCAGUCCAGAAUGGAG</td>
</tr>
<tr>
<td>Setd7</td>
<td>AAUGGAUCCAACACACUGUGUC</td>
</tr>
<tr>
<td>MLL1</td>
<td>UUAUUUGCGCCACCAAACUUGG</td>
</tr>
<tr>
<td>Setd1a</td>
<td>UUGGCUGCAUCCUGGAUGGCUG</td>
</tr>
<tr>
<td>ESET</td>
<td>CCAAGCAGCUGACACUGAAAGCUUA</td>
</tr>
<tr>
<td>Suv39h1</td>
<td>AUUCAAUACGGACUCGUUCUGG</td>
</tr>
</tbody>
</table>

All siRNA were designed by BLOCK-iT™ RNAi Designer (http://rnaidesigner.thermofisher.com/rnaiexpress/).

Immunofluorescence

Immunostainings were performed as described in Chapter I. Primary cultures of mouse trunk neural crest cells were fixed with 4% PFA for 1 h on ice. The cultures were immunostained with the primary antibodies for 16 h at 4°C and with the secondary antibodies for 1 h at room temperature. The antibodies are listed in Table 2.2. Finally, the cultures were nuclear-stained with 0.1 μg/ml DAPI (Dojindo). DAPI nuclear staining was important for counting the exact number of immunoreactive cells in the cultures and for judging cell death.
μChIP-qPCR

μChIP was performed by using a modification of the method described by Dahl and Collas (2008) [68] (Fig. 2.2). Mouse trunk neural crest cells were cross-linked by adding FA for 8 min at room temperature. The final concentration of FA was 1%. Cross-linked cells were washed with PBS containing 20 mM NaBu. The cell pellets were resuspended and lysed in lysis buffer [1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0, 20 mM NaBu, and protease inhibitor tablet (Complete Protease Inhibitor Mini EDTA-free, Roche)] for 5 min on ice. The lysate was diluted 5-fold with RIPA buffer (0.1% SDS, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5 mM EGTA, 0.1% Na-deoxycholate, 1% Triton X-100, and protease inhibitor) and sonicated using Advanced Sonifier 250A at output 2, duty cycle 60% for 6×10 s pulses each with a 2 min pause between pulses on ice. Chromatin fragments were incubated with 10 µl Protein G-coated paramagnetic beads for 2 h at 4°C that had been preincubated with 5-10 µg of the appropriate antibodies. Antibodies are listed in Table 2.2. The complexes were washed twice with RIPA buffer and once with TE, and were eluted in elution buffer (1% SDS, 20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 5 mM EDTA, and 20 mM NaBu) from the beads by heating at 65°C with occasional vortexing. The cross-linking was reversed by incubation at 65°C overnight. DNA was purified by treatment with Proteinase K (0.2 mg/ml) and phenol/chloroform/isoamyl alcohol. Real-time PCR reactions were carried out using ABI7300. The primer sequences used for PCR are shown in Table 2.3.
Fig. 2.2 Schematic diagram of the μChIP assay. (A) Agarose gel electrophoresis assessment of chromatin fragmentation. Chromatin derived from mouse trunk neural crest cells was fragmented by sonication. DNA isolated from these chromatin fragments was resolved in 1.5% agarose and stained with ethidium bromide. Most of the DNA fragments concentrated in the 200-700 bp.

Public data

ChIP-seq raw data were downloaded from Chromatin Regulator Cistrome (http://cistrome.org/cr/index.php) using accession GSM558674 [25] and from NCBI GEO (www.ncbi.nlm.nih.gov/gds) using accessions GSM1082340, GSM1082341, GSM1082342, GSM1082343, GSM307137, GSM307138, GSM307140, and GSM307155 [69, 70].
### Antibodies used for Immunofluorescence

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<th>Antigen/Conjugation</th>
<th>Dilution</th>
<th>Catalog number</th>
</tr>
</thead>
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<tr>
<td>Sox10 (rabbit polyclonal antibody)</td>
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<td>MILLIPORE (AB5727)</td>
</tr>
<tr>
<td>CHD7 (goat polyclonal antibody)</td>
<td>1:100</td>
<td>Santa Cruz (sc-79207)</td>
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<tr>
<td>FoxD3 (5G9, mouse monoclonal IgG1)</td>
<td>1:300</td>
<td>Sigma (SAB5300098)</td>
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<tr>
<td>Alexa Fluor 488 (Donkey anti-Goat IgG)</td>
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<td>Invitrogen (A-11055)</td>
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<tr>
<td>Alexa Fluor 488 (Donkey anti-Mouse IgG)</td>
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<td>Invitrogen (A-21202)</td>
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<tr>
<td>Alexa Fluor 555 (Donkey anti-Rabbit IgG)</td>
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<td>Invitrogen (A-31572)</td>
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### Antibodies used for µChIP

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Amount</th>
<th>Catalog number</th>
</tr>
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<tbody>
<tr>
<td>CHD7 (rabbit polyclonal antibody)</td>
<td>10 µg</td>
<td>Abcam (ab31824)</td>
</tr>
<tr>
<td>Oct3/4 (rabbit polyclonal antibody)</td>
<td>5 µg</td>
<td>Santa Cruz (sc-9081 X)</td>
</tr>
<tr>
<td>Sox2 (goat polyclonal antibody)</td>
<td>5 µg</td>
<td>Santa Cruz (sc-17320 X)</td>
</tr>
<tr>
<td>Nanog (D1G10, rabbit monoclonal IgG)</td>
<td>5 µl</td>
<td>Cell Signaling (8785)</td>
</tr>
<tr>
<td>Pax3/7 (rabbit polyclonal antibody)</td>
<td>5 µg</td>
<td>Santa Cruz (sc-25409 X)</td>
</tr>
<tr>
<td>Msx1 (rabbit polyclonal antibody)</td>
<td>5 µg</td>
<td>Santa Cruz (sc-15395 X)</td>
</tr>
<tr>
<td>Zic1 (rabbit polyclonal antibody)</td>
<td>5 µg</td>
<td>Sigma (HPA004098)</td>
</tr>
</tbody>
</table>

Table 2.2. Antibodies used for immunostaining and µChIP
### Identification of highly conserved genomic regions of mouse FoxD3

UCSC Genome Browser (http://genome.ucsc.edu) was used to identify highly conserved genomic regions of mouse FoxD3. The sequence reads were aligned to the mm9 assembly of the mouse genome. A BigWig file of the CHD7 ChIP-seq data set (GSM558674) was created by using SraTailor software (www.devbio.med.kyushu-u.ac.jp/sra_tailor/) [71] and the file was converted to WIG with the bigWigToWig program from UCSC. WIG files (GSM558674, GSM1082340,
GSM1082341, GSM1082342) were visualized by using the UCSC Genome Browser. We identified three highly conserved genomic regions of mouse FoxD3, mE1, mE2, and mE3. The conserved region in which the ChIP-seq peaks of CHD7, Oct3/4, Sox2, and Nanog overlapped was named mE1. mE2 and mE3 are homologous with chick FoxD3 enhancer elements NC1 and NC2 [72], respectively. Highly conserved cis-regulatory regions of mouse Sox9 and Sox10 were visualized by using the UCSC Genome Browser.

**Binding motif analysis**

MEME-ChIP (http://meme-suite.org/tools/meme-chip) [73] was used to find *de novo* binding motifs of CHD7 with default parameters. We obtained ChIP-seq peak regions from a BED file of ChIP-seq data (GSM558674) and analyzed 500 bp of the genomic sequence centered on each peak declared by Schnetz et al. (2010) [25]. Nanog binding motifs were obtained by using the MotIV program contained in the ChIP-seq pipeline of the National Institute of Genetics (https://cell-innovation.nig.ac.jp/members/index_en.html). Binding motifs of Oct3/4, Sox2, Pax3/7, Msx1, and Zic1 were obtained from the JASPAR database (http://jaspar.genereg.net/cgi-bin/jaspar_db.pl). Based on the information of these binding motifs, binding sites of CHD7 and Nanog were predicted by using FIMO (http://meme-suite.org/tools/fimo) and of Oct3/4, Sox2, Pax3/7, Msx1, and Zic1 were anticipated by using the JASPAR database.
Results

FoxD3 and Sox9 control Sox10 expression

Since it has been known that Sox10, which is required for the formation of mouse NCSCs by BMP2/Wnt3a signaling [63], interacts with FoxD3, Sox9, and Twist1 in neural crest induction [8], we examined whether or not FoxD3, Sox9, and Twist1 affect Sox10 expression to form NCSCs. Mouse trunk neural crest cells were treated with FoxD3, Sox9, and Twist1 siRNA (Fig. 2.3A). Whereas Sox10 expression was significantly suppressed by knocking down FoxD3 and Sox9 even under BMP2/Wnt3a-treated conditions (Fig. 2.3B-E, G), Twist1 siRNA had no effect on Sox10 expression (Fig. 2.3G). Furthermore, no effects on CHD7 expression were evident in any of the siRNAs examined (Fig. 2.3F). These results indicate that FoxD3 and Sox9 promote Sox10 expression in the formation of mouse NCSCs.

CHD7, Oct3/4, Sox2, and Nanog directly bind to the highly conserved genomic regions of FoxD3 in a BMP2/Wnt3a-dependent manner

The neural crest inducer genes Pax3/7, Msx1, and Zic1 control the expression of neural crest specifier genes [6-8]. Furthermore, FoxD3 has been shown to maintain the pluripotency of ES cells by interacting with the pluripotent stem cell-related genes Oct3/4, Sox2, and Nanog [74, 75]. Therefore, we examined the binding of the neural crest inducer genes and the pluripotent stem cell-related genes to the cis-elements of Sox9, Sox10, and FoxD3 by μChIP-qPCR analyses. We focused on two cis-regulatory elements (E1 and E3)
Fig. 2.3 Effects of FoxD3, Sox9, or Twist1 siRNA on Sox10 and CHD7 expression in mouse trunk neural crest cells. (A) After removal of neural tube explants, mouse trunk neural crest cells were exposed to BMP2 and Wnt3a from 2 days to 6 days in culture. Sox9, FoxD3, or Twist1 siRNA were applied from 2 days to 3 days in culture. Immunostaining with anti-CHD7 and anti-Sox10 was performed on culture day 6. (B) Bright-field image in the presence of BMP2 and Wnt3a. (C) Anti-CHD7-positive cells in the same field as (B). (D) Anti-Sox10-positive cells in the same field as (B). (E) DAPI nuclear stain of the same field as (B). (F) Percentage of cells expressing CHD7 per total cells in a neural crest cell colony (each colony was derived from a neural tube explant). (G) Percentage of cells expressing Sox10 per total cells in a neural crest cell colony. *, p < 0.05 (Student's t-test) compared to the untreated cultures. †, p < 0.05 (Student's t-test) compared to the
BMP2/Wnt3a-treated cultures. Data are expressed as mean ± SEM of separate counts of 5-11 colonies (the number in a parenthesis on each bar). Scale Bar = 50 µm.

of Sox9 [21, 76] and three cis-regulatory elements (D6, D7, and U3) of Sox10 [77, 78], which are active in mouse neural crest development (Fig. 2.4B, C). However, no regulatory elements have been identified in mouse FoxD3. Using the UCSC Genome Browser, therefore, we examined the genomic regions of FoxD3 conserved among vertebrates including mouse, rat, human, opossum, chick, X_tropicalis, and zebrafish. Three regions, localized at 14 kb, 54 kb, and 143 kb upstream of the FoxD3 gene, were found and were named mE1, mE2, and mE3, respectively (Fig. 2.4A). The mE2 and mE3 regions are homologous to the cis-regulatory regions, NC1 and NC2, identified in the chicken [72]. Previous ChIP-seq analyses using mouse ES cells [25, 69] showed direct binding of CHD7, Oct3/4, Sox2, and Nanog to the mE1 (Fig. 2.4A).

We performed μChIP-qPCR assays in mouse trunk neural crest cell cultures toward these eight cis-elements, using CHD7, Oct3/4, Sox2, Nanog, Pax3/7, Msx1, and Zic1 antibodies. μChIP assays indicated noticeable BMP2/Wnt3a-dependent enrichment of CHD7, Oct3/4, Sox2, and Nanog at mE1, mE2, and/or mE3 of FoxD3 (Fig. 2.5). However, no significant enrichment was detected at the Sox9 and Sox10 cis-elements for the products of pluripotent stem cell-related genes (Fig. 2.6B-D and 2.7B-D). CHD7 bound to the Sox9 and Sox10 cis-elements in a BMP2/Wnt3a-independent manner (Fig. 2.6A and 2.7A). Whereas Pax3/7 bound to the cis-elements of FoxD3 under the control condition only (Fig. 2.8A), this transcription factor showed binding activities to the Sox9 and Sox10 cis-elements regardless of the presence or absence of BMP2/Wnt3a (Fig. 2.9A and 2.10A). No binding activities of Msx1 (Fig. 2.8B, 2.9B, and 2.10B) and Zic1 (Fig. 2.8C, 2.9C, and 2.10C) were found in any
of the cis-elements examined. These data suggest that CHD7, Oct3/4, Sox2, and Nanog bind directly to the FoxD3 conserved elements in mouse NCSC formation (Fig. 2.11).

Based on the previous data of ChIP-seq, we found de novo binding motifs of CHD7 and Nanog. Using MEME-ChIP, six binding motifs were found in the CHD7 data set shown by Schnetz et al. (2010) [25] (Fig. 2.12A, 2.13A, and 2.14A). Using MotIV program, three binding motifs were obtained in the Nanog data set shown by Marson et al. (2008) [69] (Fig. 2.12A, 2.13A, and 2.14A). Binding motifs of Oct3/4, Sox2, Pax7, Msx1, and Zic1 were obtained from the JASPAR database. Judging from the information on binding motifs, we defined putative binding sites of CHD7, Sox2, Nanog, and Pax7 within cis-elements of FoxD3, Sox9, and Sox10 (Fig. 2.12B-D, 2.13B, C, and 2.14B, C) and binding sites of Oct3/4, Msx1, and Zic1 within FoxD3 and Sox10 cis-elements (Fig. 2.12B-D and 2.14B, C). These data are consistent with the present results of the μChIP-qPCR assays.
Fig. 2.4  Highly conserved genomic regions of mouse FoxD3, Sox9, and Sox10.  (A) Schematic diagram showing comparative genomic analysis and ChIP-seq signals around mouse FoxD3 using the UCSC genome browser.  Rat, human, opossum, chicken, X_tropicalis, zebrafish FoxD3 genomic sequences were compared.  Three conserved regions (yellow) were named mE1, mE2, and mE3, respectively.  (B) Schematic diagram showing comparative genomic analysis around mouse Sox9.  Sox9 cis-regulatory elements, E1 and E3, were indicated by yellow.  (C) Schematic diagram showing comparative genomic analysis around mouse Sox10.  Sox10 cis-regulatory elements, D6, D7, and U3, were indicated by yellow.

Fig. 2.5  Binding patterns of CHD7, Oct3/4, Sox2, and Nanog to the highly conserved genomic regions of mouse FoxD3.  (A) μChIP-qPCR analysis using CHD7 antibodies.  The Y-axis represents percentage of co-immunoprecipitated DNA over input.  (B) μChIP-qPCR analysis using Oct3/4 antibodies.  (C) μChIP-qPCR analysis using Sox2 antibodies.  (D) μChIP-qPCR analysis using Nanog antibodies.  Data are presented as mean ± SEM of 2 to 5 independent experiments.
Fig. 2.6  Binding patterns of CHD7, Oct3/4, Sox2, and Nanog to the cis-regulatory elements of mouse Sox9.  (A) μChIP-qPCR analysis using CHD7 antibodies.  The Y-axis represents percentage of co-immunoprecipitated DNA over input.  (B) μChIP-qPCR analysis using Oct3/4 antibodies.  (C) μChIP-qPCR analysis using Sox2 antibodies.  (D) μChIP-qPCR analysis using Nanog antibodies.  Data are presented as mean ± SEM of 2 to 4 independent experiments.
Fig. 2.7  Binding patterns of CHD7, Oct3/4, Sox2, and Nanog to the cis-regulatory elements of mouse Sox10.  (A) μChIP-qPCR analysis using CHD7 antibodies.  The Y-axis represents percentage of co-immunoprecipitated DNA over input.  (B) μChIP-qPCR analysis using Oct3/4 antibodies.  (C) μChIP-qPCR analysis using Sox2 antibodies.  (D) μChIP-qPCR analysis using Nanog antibodies.  Data are presented as mean ± SEM of 2 to 5 independent experiments.
Fig. 2.8  Binding patterns of Pax3/7, Msx1, and Zic1 to the highly conserved genomic regions of mouse FoxD3.  (A) μChIP-qPCR analysis using Pax3/7 antibodies. The Y-axis represents percentage of co-immunoprecipitated DNA over input.  (B) μChIP-qPCR analysis using Msx1 antibodies.  (C) μChIP-qPCR analysis using Zic1 antibodies. Data are presented as mean ± SEM of 2 to 3 independent experiments.
Fig. 2.9  Binding patterns of Pax3/7, Msx1, and Zic1 to the cis-regulatory elements of mouse Sox9.  (A) μChIP-qPCR analysis using Pax3/7 antibodies.  The Y-axis represents percentage of co-immunoprecipitated DNA over input.  (B) μChIP-qPCR analysis using Msx1 antibodies.  (C) μChIP-qPCR analysis using Zic1 antibodies.  Data are presented as mean ± SEM of 2 to 3 independent experiments.
Fig. 2.10  Binding patterns of Pax3/7, Msx1, and Zic1 to the cis-regulatory elements of mouse Sox10.  (A) μChIP-qPCR analysis using Pax3/7 antibodies. The Y-axis represents percentage of co-immunoprecipitated DNA over input.  (B) μChIP-qPCR analysis using Msx1 antibodies.  (C) μChIP-qPCR analysis using Zic1 antibodies. Data are presented as mean ± SEM of 2 to 4 independent experiments.
Fig. 2.11  Summary for binding patterns of CHD7, Pax3/7, Msx1, Zic1, Oct3/4, Sox2, and Nanog to the cis-elements of neural crest specifier genes. Solid lines indicate the direct binding in BMP2/Wnt3a-dependent manner. Dotted lines show the BMP2/Wnt3a-independent binding.
Fig. 2.12  Binding sites of CHD7, Pax3/7, Msx1, Zic1, Oct3/4, Sox2, and Nanog in the 
cis-elements of mouse FoxD3.  (A) Enrichment of CHD7 and Nanog consensus DNA motifs in ChIP-seq peaks [20, 66] observed in the cis-elements of mouse FoxD3.  (B-D) Binding sites of CHD7, the products of pluripotent stem cell-related genes, and the products of neural crest inducer genes in mE1 (B), mE2 (C), and mE3 (D) of mouse FoxD3.
Fig. 2.13  Binding sites of CHD7, Pax3/7, Msx1, Zic1, Oct3/4, Sox2, and Nanog in the *cis*-regulatory elements of mouse *Sox9*.  (A) Enrichment of CHD7 and Nanog consensus DNA motifs in ChIP-seq peaks [20, 66] observed in the *cis*-regulatory elements of mouse *Sox9*.  (B and C) Binding sites of CHD7, the products of pluripotent stem cell-related genes, and the products of neural crest inducer genes in E1 (B) and E2 (C) of mouse *Sox9*. 
Fig. 2.14  Binding sites of CHD7, Pax3/7, Msx1, Zic1, Oct3/4, Sox2, and Nanog in the cis-regulatory elements of mouse Sox10.  (A) Enrichment of CHD7 and Nanog consensus DNA motifs in ChIP-seq peaks [20, 66] observed in the cis-regulatory elements of mouse Sox10.  (B and C) Binding sites of the products of pluripotent stem cell-related genes, and the products of neural crest inducer genes in D6/D7 (B) and U3 (C) of mouse Sox10.  The regions of D6 and D7 are shown by the pink or red underlines, respectively (B).

FoxD3 expression is suppressed by the knockdown of CHD7, Oct3/4, Sox2, or Nanog

Our present data strongly suggest that the formation of mouse NCSCs by BMP2/Wnt3a is promoted by the direct regulation of FoxD3 expression by CHD7, Oct3/4, Sox2, and Nanog.  Therefore, we examined the effects of CHD7, Oct3/4, Sox2, and Nanog on FoxD3 expression in mouse trunk neural crest cell cultures.  Overexpression of CHD7 or
BMP/Wnt3a treatment significantly stimulated FoxD3 and Sox10 expression (Fig. 2.15A-H). These treatments also increased the proportion of cells expressing both FoxD3 and Sox10 (Fig. 2.15I). In contrast, the expression of both FoxD3 and Sox10 was severely decreased by the knockdown of CHD7, Oct3/4, Sox2, or Nanog despite the presence of BMP2 and Wnt3a (Fig. 2.15G-H). Moreover, these siRNA treatments decreased the percentage of cells expressing both FoxD3 and Sox10 (Fig. 2.15I). These results show that the activation of FoxD3 by CHD7, Oct3/4, Sox2, and Nanog promote Sox10 expression, leading to the formation of mouse NCSCs. This regulatory gene cascade differed from that in the neural crest induction mechanisms.

**Fig. 2.15** Effects of CHD7, Oct3/4, Sox2, or Nanog siRNA on Sox10 and FoxD3 expression in mouse trunk neural crest cell cultures. (A) After removal of neural tube explants, the WT CHD7 expression vectors were applied from 2 days to 3 days in culture. CHD7, Oct3/4, Sox2, or Nanog
siRNA were applied from 2 days to 3 days in culture. Immunostaining with anti-Sox10 and anti-FoxD3 was performed on culture day 6. **(B)** Bright-field image in the presence of the WT CHD7 expression vectors. **(C)** Anti-FoxD3-positive cells in the same field as (B). **(D)** Anti-Sox10-positive cells in the same field as (B). **(E)** DAPI nuclear stain of the same field as (B). **(F)** Merged image of (C–E). **(G)** Percentage of cells expressing FoxD3 per total cells in a neural crest cell colony (each colony was derived from a neural tube explant). **(H)** Percentage of cells expressing Sox10 per total cells in a neural crest cell colony. **(I)** Percentage of cells expressing both Sox10 and FoxD3 per total cells in a neural crest cell colony. *, p < 0.05 (Student's t-test) compared to the untreated cultures. †, p < 0.05 (Student's t-test) compared to the BMP2/Wnt3a-treated cultures. Data are expressed as mean ± SEM of separate counts of 5-9 colonies (the number in a parenthesis on each bar). Scale Bar = 50 µm.

**Histone H3K4 methylation promotes FoxD3 and Sox10 expression**

Since H3K4me3 occurs in the promoter regions of transcriptionally active genes [79, 80] and CHD7 binds mainly to H3K4me1 [25], we examined the effects of H3K4 methylation on the expression of FoxD3 and Sox10. We inhibited H3K4 methylation by using siRNAs of Setd7 (the H3K4 mono-methylation specific histone methyltransferase) [81] and of MLL and Setd1a (H3K4 tri-methylation specific histone methyltransferases) [82, 83]. These siRNA treatments suppressed FoxD3 and Sox10 expression despite the presence of BMP2/Wnt3a or CHD7 overexpression (Fig. 2.16A, C). CHD7 overexpression expectedly stimulated CHD7 expression (Fig. 2.16B). It is conceivable that Setd7, MLL, and Setd1a play important roles in the formation of mouse NCSCs. Interestingly, siRNA of ESET, an H3K9 tri-methylation specific histone methyltransferase
[84], inhibited Sox10 expression, although siRNA of Suv39h1, another H3K9 tri-methylation specific histone methyltransferase [84], had no effect on Sox10 expression (Fig. 2.16C). ESET may be involved in the formation of mouse NCSCs by repressing transcription of some differentiation-related genes.

Fig. 2.16 Effects of siRNAs of histone methyltransferase genes on FoxD3, Sox10, and CHD7 expression in mouse trunk neural crest cell cultures. (A) Percentage of cells expressing FoxD3 per total cells in a neural crest cell colony (each colony was derived from a neural tube explant). *, p < 0.05 (Student's t-test) compared to the untreated cultures. †, p < 0.05 (Student's t-test) compared to BMP2/Wnt3a-treated cultures. Data are expressed as mean ± SEM of separate counts of 5-7 colonies (the number in a parenthesis on each bar). (B) Percentage of cells expressing CHD7 per total cells in a neural crest cell colony. (C) Percentage of cells expressing Sox10 per total cells in a neural crest cell colony. *, p < 0.05 (Student's t-test) compared to the untreated cultures. †, p < 0.05 (Student's t-test) compared to the cultures transfected with WT CHD7 expression vector. Data are expressed as mean ± SEM of separate counts of 5-10 colonies (the number in a parenthesis on each bar).
Discussion

In the present study, we showed a mechanism underlying mouse NCSC formation (Fig. 2.17). Although BMP2/Wnt3a signaling and CHD7 are implicated in neural crest induction, the regulatory gene cascade in NCSC formation mechanism was drastically different from that in the neural crest induction mechanisms. CHD7, Oct3/4, Sox2, and Nanog stimulated by BMP2/Wnt3a signaling directly induce FoxD3 expression. In turn, FoxD3 promotes the expression of Sox10, which is an indispensable transcription factor for mouse NCSC formation [9, 63]. The products of neural crest inducer genes such as Pax3/7, Zic1, and Msx1 have been known to regulate FoxD3 expression in neural crest induction [6]. However, our μChIP-qPCR assays showed that CHD7, Oct3/4, Sox2, and Nanog stimulated by BMP2/Wnt3a signaling directly bind to FoxD3 conserved regions in mouse NCSC formation. On the other hand, Pax3/7, Zic1, and Msx1 did not bind to the FoxD3 conserved genomic regions in a BMP2/Wnt3a-dependent manner. The inhibition of the expression of CHD7, Oct3/4, Sox2, and Nanog suppressed FoxD3 expression. Furthermore, FoxD3 siRNA repressed Sox10 expression. This latter result is consistent with the observation that FoxD3 binds to the U3 enhancer element of Sox10 and activates Sox10 [78]. Thus, these data suggest that the binding of CHD7, Oct3/4, Sox2, and Nanog to FoxD3 cis-regulatory elements in a BMP2/Wnt3a-dependent manner plays important roles in the formation of mouse NCSCs expressing Sox10. Schnetz et al. (2010) [25] have reported that CHD7, Oct3/4, Sox2, and Nanog colocalize at active gene enhancer elements in ES cells. Furthermore, Oct3/4, Sox2, Nanog, and FoxD3 interact with each other to maintain the pluripotency of ES cells [85]. A physical association between Oct3/4 and FoxD3 in ES cells was also reported [86]. Therefore, the mechanism underlying NCSC formation
presented in this study may be similar to that underlying the maintenance mechanism of the pluripotency of ES cells.

*Sox9* siRNA suppressed Sox10 expression in the presence of BMP2/Wnt3a. Since Sox9 has been known to bind to the U3 enhancer element of *Sox10* [78], Sox9 as well as FoxD3 may control mouse NCSC formation. However, our μChIP-qPCR assays showed no enrichment of CHD7, Oct3/4, Sox2, Nanog, Pax3/7, Zic1, or Msx1 at *Sox9* enhancer elements E1 and E3 in a BMP2/Wnt3a-dependent manner. It is conceivable that other factors and/or other regulatory elements participate in Sox9 activation, leading to mouse NCSC formation.

The present data revealed that mono- and/or tri-methylation of H3K4 is necessary for the expression of Sox10 and FoxD3. It has been reported that CHD7 binds to the enhancer elements marked with H3K4me1 in neural crest induction [21]. The BMP2/Wnt3a-dependent binding of CHD7 to the mE2 regulatory region of FoxD3 may be due to the mono-methylation of H3K4 in mE2. Since the binding of CHD7 to enhancer elements promotes gene activation [26, 27], it is conceivable that the binding of this chromatin remodeler to mE2 stimulates FoxD3 expression in mouse NCSC formation. Sox10 expression was suppressed by the inhibition of H3K4 mono-methylation despite CHD7 overexpression. As the predominant binding of CHD7 to *Sox10* regulatory regions was not observed, the inhibition of FoxD3 expression might cause this suppression. Moreover, H3K4 is tri-methylated in TSS of transcriptionally active genes [79, 87]. It is possible that the tri-methylation of H3K4 in TSS of *FoxD3* and *Sox10* activates these genes and leads to the formation of mouse NCSCs. ESET, an H3K9 tri-methylation specific histone methyltransferase, was involved in mouse NCSC formation, although Suv39h1, another H3K9 tri-methylation specific histone methyltransferase, had no effect on NCSC formation. ESET has been shown to repress the expression of trophoectoderm-associated
genes *Cdx2*, *Gata2*, and *Hand1*, and thereby to participate in the maintenance of the pluripotency of ES cells [88]. These transcriptional factors act on neural crest development [89, 90]. Furthermore, Fei et al. (2015) [91] have reported that the interaction between ESET and PRC2 maintains the pluripotency of ES cells. Thus, the downregulation of differentiation-related genes by ESET may be crucial for maintaining the multipotency of mouse neural crest cells and for the formation of mouse NCSCs.

**Fig. 2.17 Model for a regulatory gene cascade in the formation of mouse NCSCs.** The multipotency of neural crest cells is maintained by BMP2/Wnt3a signaling. CHD7, Oct3/4, Sox2, and Nanog activated by BMP2/Wnt3a directly bind to the *FoxD3* cis-regulatory elements and activate this gene. *FoxD3* promotes Sox10 expression and leads to mouse NCSC formation.
Conclusion

1. Undifferentiated mouse trunk neural crest cells express Sox10, which is indispensable for the maintenance of the undifferentiated state of neural crest cells, and p75. We have examined the expression of CHD7, one of chromatin remodelers, in mouse trunk neural crest cells. CHD7 expression was found in the early migratory neural crest cells containing Sox10 or p75. Moreover, co-expression of CHD7 and Sox10 was observed in the dorsal neural tube containing premigratory neural crest cells. These data suggest that undifferentiated mouse trunk neural crest cells express CHD7 as well as Sox10 and p75.

2. We have examined the temporal change of CHD7 expression in mouse trunk neural crest cell cultures. The number of the undifferentiated neural crest cells expressing Sox10 or p75 decreased from culture day 2 to culture day 6. Furthermore, the expression of CHD7 was also suppressed with time.

3. The treatment of 10 ng/ml BMP2 and 10 ng/ml Wnt3a promoted the expression of CHD7, Sox10, or p75 on culture day 6. High concentration of Wnt3a (100 ng/ml) alone showed the similar effects. When BMP activities in FBS and/or CEE was prevented by noggin and chordin, these inhibitors had no effects on CHD7 or Sox10 expression in the presence of BMP2/Wnt3a or 100 ng/ml Wnt3a. These data suggest that the activation of Wnt signaling is a key requisite for the maintenance of CHD7 expression and thus for the maintenance of the undifferentiated state of neural crest cells, and that BMP signaling plays supportive roles in the maintenance of the undifferentiated state of these cells. Further, when we used BIO instead of Wnt3a, BIO treatment promoted the expression of CHD7 and/or Sox10. The addition of the DN β-catenin or the DN TCF expression vectors in the
presence of BMP2 and Wnt3a significantly suppressed the expression of CHD7 and/or Sox10. Thus, Wnt/β-catenin pathway may play important roles in the maintenance of the undifferentiated state of mouse trunk neural crest cells.

4. The addition of WT CHD7 expression vectors significantly promoted the expression of Sox10 or p75 and the co-expression of CHD7/Sox10 or CHD7/p75. On the other hand, cells expressing CHD7, Sox10, and/or p75 were significantly suppressed by the treatment of DN CHD7 expression vectors or CHD7 siRNA in the presence of BMP2 and Wnt3a. These results show that CHD7 is required for the maintenance of the undifferentiated state of mouse trunk neural crest cells.

5. Since undifferentiated neural crest cells may be multipotential, CHD7 and BMP2/Wnt3a may participate in maintaining the multipotency of mouse trunk neural crest cells. It has been known that multipotent trunk neural crest cells can differentiate into smooth muscle cells, neurons, and glia. Therefore, we investigated whether or not the activation of CHD7 or BMP2/Wnt3a signaling increases the number of multipotent mouse trunk neural crest cells that differentiate into smooth muscle cells, neurons, and glia. The addition of WT CHD7 expression vectors or BMP2/Wnt3a significantly increased the proportion of clones consisting of both smooth muscle cells and neurons or both smooth muscle cells and glia. These data suggest that BMP2/Wnt3a signaling and CHD7 plays essential roles in the maintenance of the multipotency of mouse trunk neural crest cells. Moreover, adult mouse DRG and sciatic nerves contained Sox10- and/or p75-positive NCSCs expressing CHD7. These results suggest that CHD7 as well as Sox10 participates in the maintenance of the multipotency of neural crest cells and conduct them to mouse NCSCs expressing p75.
6. BMP2/Wnt3a signaling and CHD7 maintain the multipotency of mouse trunk neural
crest cells and lead to the formation of mouse NCSCs. Since it has been shown that these
factors activate neural crest specifier genes, such as Sox9, Sox10, FoxD3, in neural crest
induction, we examined roles of these genes in the formation of mouse NCSCs.
Knockdown of Sox9 or FoxD3 significantly suppressed Sox10 expression even under
BMP2/Wnt3a-treated conditions. Data indicate that FoxD3 and Sox9 promote Sox10
expression in the formation of mouse NCSCs.

7. It has been known that the products of neural crest inducer genes, such as Pax3/7, Zic1,
and Msx1, activate FoxD3 and Sox9 in neural crest development. Moreover, CHD7 has
been known to interact with the pluripotent stem cell-related genes, such as Oct3/4, Sox2,
and Nanog, in ES cells. Therefore, we examined the binding of neural crest inducer genes
and pluripotent stem cell-related genes to the cis-elements of Sox9, Sox10, and FoxD3 by
µChIP-qPCR assays. CHD7, Oct3/4, Sox2, and Nanog bound to the FoxD3 cis-elements
only in a BMP2/Wnt3a-dependent manner. In addition, we defined putative binding sites
of CHD7, Oct3/4, Sox2 and Nanog within FoxD3 cis-elements. These results suggest that
CHD7, Oct3/4, Sox2, and Nanog bind directly to the FoxD3 cis-elements in mouse NCSC
formation.

8. Since BMP2/Wnt3a-dependent direct binding of CHD7, Oct3/4, Sox2 and Nanog to
FoxD3 cis-elements may be essential for the formation of mouse NCSCs, we examined the
effects of CHD7, Oct3/4, Sox2, and Nanog on FoxD3 expression in mouse trunk neural crest
cell cultures. The addition of WT CHD7 expression vectors or BMP2/Wnt3a treatments
significantly increased the number of cells expressing FoxD3 and/or Sox10. On the other
hand, knockdown of CHD7, Oct3/4, Sox2, or Nanog drastically decreased the number of cells
expressing FoxD3 and/or Sox10 despite the presence of BMP2/Wnt3a. These data suggest that the activation of FoxD3 by CHD7, Oct3/4, Sox2, and Nanog promoted the Sox10 expression, leading to the formation of mouse NCSCs. This regulatory gene cascade was dramatically different from that in the mechanisms of neural crest induction.

9. Since CHD7 binds mainly to H3K4me1 regions in cis-elements of target genes and H3K4me3 occurs in the promoter regions of transcriptionally active genes, we examined the effects of H3K4 methylation on the expression of FoxD3 and Sox10. Treatments with siRNAs of Setd7 (the H3K4 mono-methylation specific histone methyltransferase), MLL1, and Setd1a (H3K4 tri-methylation specific histone methyltransferases) significantly suppressed FoxD3 and Sox10 expression despite the presence of BMP2/Wnt3a or overexpression of CHD7. These data show that Setd7, MLL, and Setd1a play important roles in the formation of mouse NCSCs. siRNA of ESET, a H3K9 tri-methylation specific histone methyltransferase, inhibited Sox10 expression. ESET may be implicated in the formation of mouse NCSCs by repressing transcription of some differentiation-related genes.
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Research achievement

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