**Int. J. Dev. Biol. 60:** 21-28 (2016)
doi: 10.1387/ijdb.160040mk

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

# Bone morphogenetic protein 4 promotes craniofacial neural crest induction from human pluripotent stem cells

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**ABSTRACT Neural crest (NC) cells are a group of cells located in the neural folds at the boundary between the neural and epidermal ectoderm. Cranial NC cells migrate to the branchial arches and give rise to the majority of the craniofacial region, whereas trunk and tail NC cells contribute to the heart, enteric ganglia of the gut, melanocytes, sympathetic ganglia, and adrenal chromaffin cells. Positional information is indispensable for the regulation of cranial or trunk and tail NC cells. However, the mechanisms underlying the regulation of positional information during human NC induction have yet to be fully elucidated. In the present study, supplementation of bone morphogenetic protein (BMP) 4 in defined serum-free culture conditions including fibroblast growth factor-2 and Wnt3a from day 8 after NC specification induced the expression of cranial NC markers, *AP2*α, *MSX1*, and *DLX1*, during NC cell differentiation from human pluripotent stem cells. On the other hand, the proportion of cells expressing p75$^{NTR}$ or HNK1 decreased compared with that of cells cultured without BMP4, whereas gene expression analysis demonstrated that the expression levels of cranial NC-associated genes increased in BMP4-treated NC cells. These BMP4-treated NC cells were capable of differentiation into osteocytes and chondrocytes. The results of the present study indicate that BMP4 regulates cranial positioning during NC development.**

KEY WORDS: *cranial neural crest, BMP signaling, homeobox gene, human embryonic stem cell, defined culture condition*

## Introduction

Neural crest (NC) cells are a group of cells located in the neural folds at the boundary between the neural and epidermal ectoderm. In vertebrates, they delaminate from the dorsal ridges of the neural tube and then migrate extensively within the developing embryo during the process of neurulation (Morikawa *et al.*, 2009). NC cells in the cranial region migrate to the branchial arches and give rise to the majority of cranial mesenchyme that eventually differentiates into odontoblasts, cartilage, craniofacial bone, and connective tissue; a subset of these cells differentiate into cranial ganglia. Cardiac NC cells contribute to heart development and vagal NC cells contribute to the development of enteric ganglia of the gut. Trunk NC cells migrate along the dorsal and lateral pathway and give rise to melanocytes, sympathetic ganglia, and adrenal chromaffin cells (Santagati and Rijli, 2003).

Several studies have demonstrated that neural and NC induction in various species, such as fish and amphibians, is regulated by several signaling pathways, such as TGFβ signaling, including Activin/Nodal, fibroblast growth factor (FGF) signaling, Wnt signaling, and bone morphogenetic protein (BMP) signaling (Baker *et al.*, 1999, Furue and Asashima, 2004, Patthey *et al.*, 2009). In mice, we previ-

*Abbreviations used in this paper:* BMP, bone morphogenetic protein; BSA, bovine serum albumin; ECM, extra cellular matrix; ESCs, embryonic stem cells; FGF, fibroblast growth factor; *HOX* gene, *Homeobox* gene; hESCs, human embryonic stem cells; NC, neural crest; NCSCs, neural crest stem cells; qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction.

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**Supplementary Material** (one figure and three tables) for this paper is available at: http://dx.doi.org/10.1387/ijdb.160040mk

*Accepted:* 21 January 2016; *Edited by:* Makoto Asashima

ISSN: Online 1696-3547, Print 0214-6282
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Printed in Spain

ously reported that culture in the presence of BMP4 in combination with FGF-2 induces the up-regulation of NC marker genes, including AP2α and P0 protein, during the differentiation of mouse embryonic stem cells (ESCs) into NC cells under chemically defined conditions (Aihara *et al.*, 2010, Furue, 2012). Because culture systems for the stable expansion of human pluripotent stem cells had been developed (Amit *et al.*, 2000, Furue *et al.*, 2008, Kinehara *et al.*, 2013), *in vitro* studies producing NC cells from human pluripotent stem cells have been reported (Fukuta *et al.*, 2014, Lee *et al.*, 2010, Menendez *et al.*, 2013, Menendez *et al.*, 2011). Dalton et al. (Menendez *et al.*, 2013, Menendez *et al.*, 2011) have demonstrated that a combination of Wnt activation and Smad inhibition under serum-free culture conditions directs the differentiation of human ESCs (hESCs) and induced pluripotent stem cells into NC stem cells (NCSCs).

BMP signaling is well known to inhibit early neural induction. Therefore, the inhibition of BMP signaling, particularly by extracellular antagonists, such as Noggin and Chordin, is usually used for neural induction (Piccolo *et al.*, 1996, Zimmerman *et al.*, 1996). However, Dalton et al. (Menendez *et al.*, 2011) demonstrated that active suppression of BMP signaling by the addition of Noggin is not required for NCSC induction because of the low level of basal BMP-dependent Smad 1, 5, and 8 activity in undifferentiated and differentiating ESCs. On the other hand, we previously demonstrated that AP2α, a premigratory and migratory NC cell marker (Gajavelli *et al.*, 2004), was up-regulated in cells cultured in the presence of recombinant BMP4 for 10 days following culture with recombinant FGF-2 for 4 days in a study using mouse ESCs (Aihara *et al.*, 2010). Sasai et al. (Kamiya *et al.*, 2011) reported that late exposure to BMP4 induces neural ectodermal differentiation of mouse ESCs. However, the directed induction of cranial NC cells, which contribute to the development of craniofacial bone and connective tissue, has yet demonstrated. Whereas positional information is considered to be indispensable for NC development *in vivo*, its importance during human NC induction *in vitro* is currently unknown.

The present study describes the effect of BMP4 on NC induction *in vitro* and the development of an efficient protocol for the induction of cranial NC cells from hESCs, building on the achievements of previous studies (Aihara *et al.*, 2010, Furue, 2012, Menendez *et al.*, 2013, Menendez *et al.*, 2011). Cranial NC cells were induced by late exposure to recombinant BMP4 in combination with recombinant FGF-2 followed by NC specification. Gene expression induced in the cells by our protocol was comprehensively analyzed, which indicated high expression of migratory cranial NC/pharyngeal arch mesenchyme markers in induced cells. We further confirmed the osteogenic and chondrogenic potential of induced cranial NC cells. The results of the present study demonstrate the specific induction of cranial NC *in vitro* by BMP4 treatment in a finely timed manner.

## Results

### *MSX and DLX gene expression in response to BMP4 treatment during NC induction*

To examine the effect of BMP4 on the induction of NC from hESCs, we tested the NCSC induction method previously reported by Dalton et al. (Menendez *et al.*, 2013, Menendez *et al.*, 2011). NCSC induction was driven by treatment with recombinant FGF-2, TGF-β/Activin inhibitor SB431542, and recombinant Wnt-3a. We previously reported that late exposure to BMP4 induces the neural ectodermal differentiation of mouse ESCs. From this finding, we hypothesized that the initiation timing of BMP4 treatment affects positional information. To monitor the effects of BMP4 during NC cell differentiation from hESCs, treatment with recombinant BMP4 was initiated at various time points (Fig.1 A) and the expression of *AP2α* was assessed on day 12 by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) (Fig.1 B). Expression levels of *AP2α* were higher in the cells treated with BMP4 compared with those treated without BMP4 on day 12.

We also examined the effect of BMP4 on the expression of the positional information genes, *MSX1* and *DLX1*, in cells cultured under the conditions described above. The expression of *MSX1* and *MXS2* genes, in addition to *AP2α*, has been reported in both premigratory and migratory craniofacial NC cells and the craniofacial/pharyngeal arch mesenchyme (Ishii *et al.*, 2005). *DLX* genes are expressed in the developing cranial NC cell derivatives, particularly

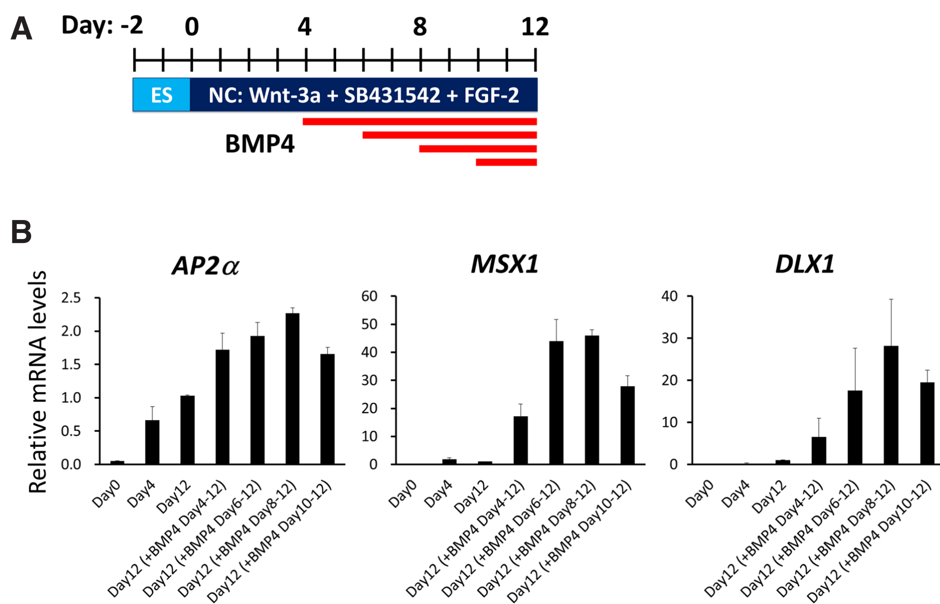


**Fig. 1. Altered neural crest (NC) gene expression in response to BMP4 treatment during NC induction. (A)** *BMP4 was added to cultures at the indicated time points during NC differentiation. ES, hESF9 ESC culture medium; NC, NC differentiation medium.* **(B)** *Relative mRNA expression levels of* AP2α, MSX1, *and* DLX1 *on culture days 0, 4, and 12 were analyzed using qRT-PCR. Expression levels were normalized against the housekeeping gene GAPDH. Samples indicated by day 0, day 4, and day 12 were cultured without BMP4. Day 12 samples were cultured with BMP4 during the periods shown in (A). The relative expression level of each gene is shown with values of cells cultured in NC (without BMP4) on day 12 defined as 1.0. Values are presented as mean ± SD (n = 3).*

in the first and second pharyngeal arches, but not in premigratory or migratory NC cells (Park *et al.*, 2004). In the absence of BMP4, although *AP2α* expression increased during culture, no changes in the expression of *MSX1* or *DLX1* were observed during the 12-day culture (Fig.1 B). On the other hand, on days 4–12, treatment with BMP4 considerably increased the expression of *MSX1* or *DLX1*, and on days 6–12, that of *MSX1*, *DLX1*, and *AP2α* considerably increased. Furthermore, on days 8–12, *DLX1* and *AP2α* expression greatly increased compared with the other time points. The effect of BMP4 treatment on days 10–12 was comparable with those on days 4–12. Treatment with BMP4 on days 8–12 induced higher gene expression of *AP2α*, *MSX1*, and *DLX1* than that during other days. These results indicate that BMP4 treatment on days 8–12 in combination with FGF-2 and Wnt-3a induces the generation of NC cells with cranial positional information from hESCs. We designated this culture condition as NC-B culture condition.

### *Characterization of cells induced by activation of BMP4 signaling during NC cell differentiation*

To verify the induction of cranial NC cells from hESCs by NC-B culture condition described above, we next examined the expression profiles of NC markers in induced cells compared with those of cells cultured in the NC culture condition (Fig.2 A). First, the expression of the classical NCSC markers, p75$^{NTR}$ (Gajavelli *et al.*, 2004) and HNK1 (Nagase *et al.*, 2003), by induced cells was examined by immunocytochemistry (Fig.2 B). The NC culture condition promoted robust specification of p75$^{NTR}$ and HNK1-expressing NCSCs, as described by Menendez et al. (Menendez *et al.*, 2011). In contrast, major popu-

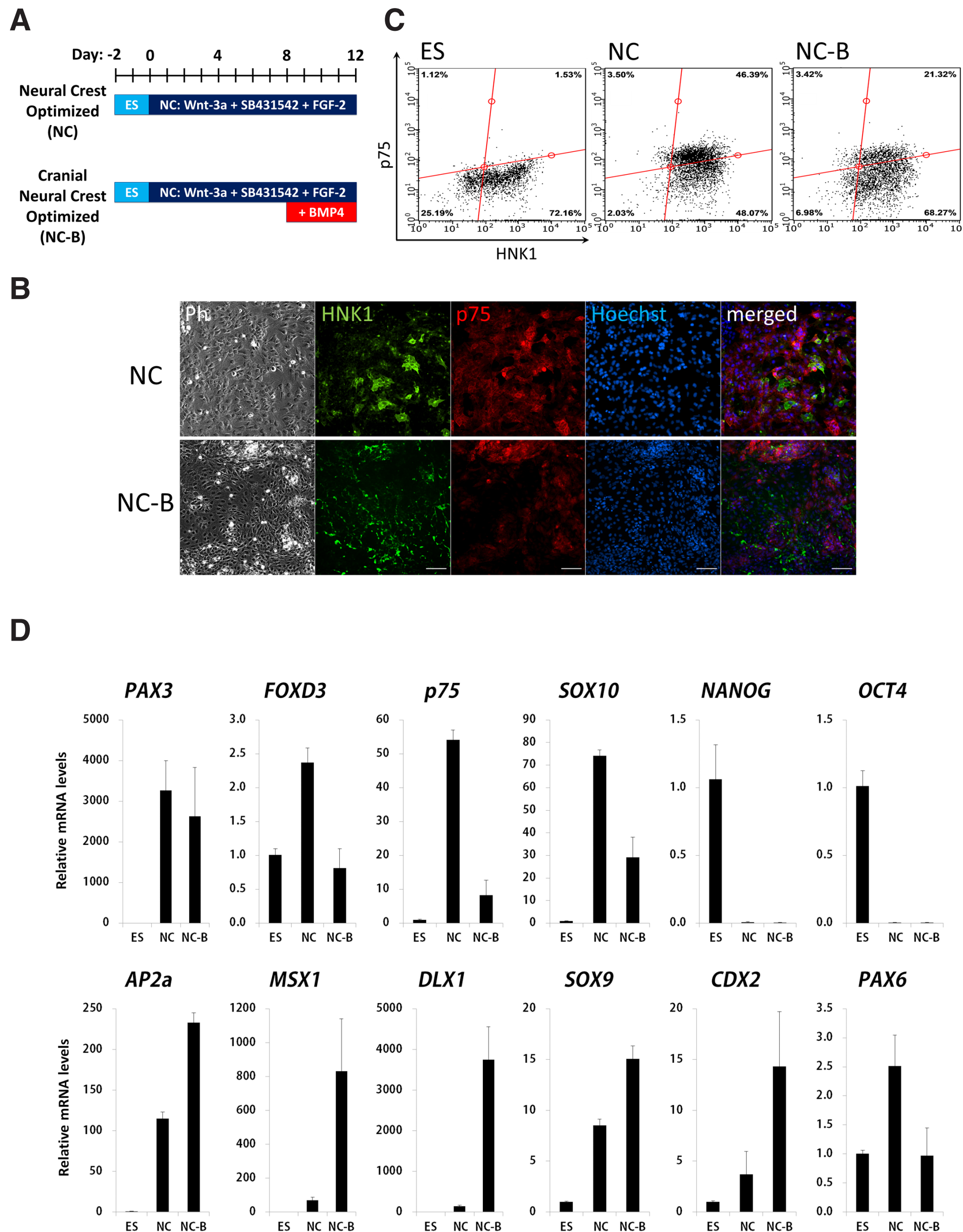


**Fig. 2. Characterization of cells induced by the modified neural crest differentiation protocol. (A)** *Scheme of the neural crest differentiation and the modified cranial neural crest differentiation (NC-B) protocols.* **(B)** *H9 human embryonic stem cells were cultured according to the NC or NC-B protocols. Cells were then fixed on day 12 and stained with antibodies for HNK1 and p75. Nuclei were detected by staining with Hoechst33342. Scale bar, 100 mm.* **(C)** *Flow cytometry analysis of undifferentiated H9 (ES, left) and neural crest cells derived from H9 cells cultured in NC (middle) and NC-B (right) culture conditions. Flow cytometry was performed using HNK1/p75 antibodies. The percentage of cells in each quadrant is indicated.* **(D)** *Relative gene expression levels in NC cells derived from H9 cells cultured in NC and NC-B culture conditions. Expression levels were normalized against the housekeeping gene GAPDH. The relative expression level of each gene is shown with values of cells cultured in NC (without BMP4) on day 12 defined as 1.0. Values are presented as mean ± SD (n = 3).*

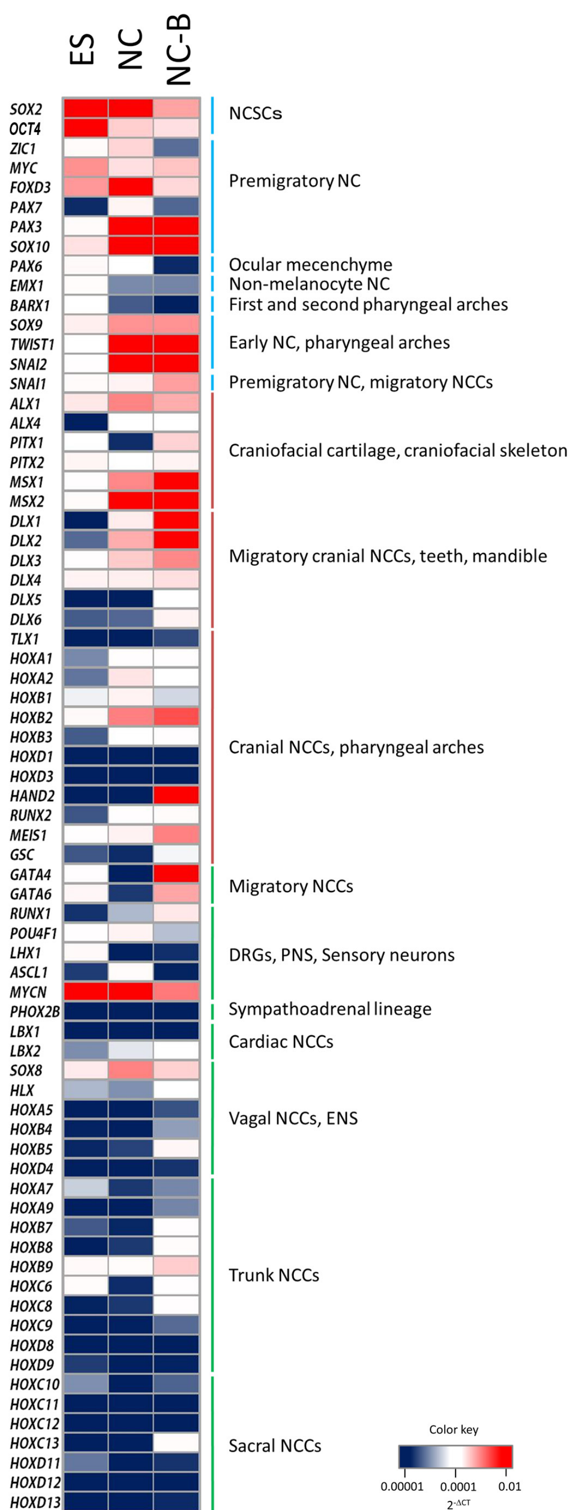


**Fig. 3. Microarray analysis demonstrating the up-regulation of cranial neural crest genes in cells cultured in neural crest differentiation medium containing BMP4 (NC-B).** *Heat map of the expression profiles of 72 genes in undifferentiated H9 cells (ES, n = 3), and NC cells derived from H9 cells cultured in NC (n = 3) and NC-B (n = 6) conditions (Fig. 2A). Average delta CT values are shown in Table S3. Blue, red, and green lines indicate early NC, cranial NC, and migratory and posterior neural crest marker genes, respectively.*

lations of cells induced by NC-B culture condition expressed p75$^{NTR}$ or HNK1 at low levels and displayed different morphology compared with that of p75$^{NTR+}$/HNK1$^{+}$ cells cultured in the NC culture condition. To quantify the population of p75$^{NTR+}$/HNK1$^{+}$ cells, flow cytometry of cells cultured in each condition was performed (Fig.2 C). The flow cytometric profile of p75$^{NTR}$ and HNK1 expression in day 12 cells induced by NC-B culture condition was compared with that by NC culture condition, indicating that NC-B culture condition decreased the yield of p75$^{NTR+}$/HNK1$^{+}$ cells, whereas the HNK1 single-positive (p75$^{NTR-}$/HNK1$^{+}$) cell population was increased.

p75$^{NTR}$ and HNK1 have been used to enrich human ES-derived multipotent NCSC cells because these cell surface markers are convenient and useful for staining and sorting living cells (Lee *et al.*, 2010, Menendez *et al.*, 2013, Menendez *et al.*, 2011). However, a previous study reported that both p75$^{NTR-}$ and p75$^{NTR+}$ cells express other NC markers and are able to differentiate into NC cell derivatives *in vitro* (Curchoe *et al.*, 2010). A further study of migratory NC cells in early human embryos demonstrated that *in vivo* p75$^{NTR+}$/HNK1$^{+}$ cells were localized to a small subset of migratory NC cells and cells of the ventral neural tube *in vivo* (Betters *et al.*, 2010). It is likely that additional markers are required for definitive characterization of NC cell subsets derived hESC cultures. Therefore, we further examined the expression levels of the following genes by qRT-PCR analysis (Fig.2 D): well-established NC markers, *PAX3*, *FOXD3*, *p75$^{NTR}$*, *SOX10*, *SOX9*, *AP2*$\alpha$, *MSX1*, and *DLX1*; the neuroectoderm/forebrain and ocular marker, *PAX6*; the hindbrain/posterior neural tube marker, *CDX2*; and markers of undifferentiated ES cell state, *NANOG* and *OCT4*. *NANOG* and *OCT4* expression levels were significantly decreased in cells induced by both NC and NC-B culture conditions. It was also confirmed that *PAX3*, *FOXD3*, *p75$^{NTR}$*, and *SOX10* were prominently expressed in cells induced by NC condition, corroborating the study by Menendez et al. demonstrating the abundance of premigratory NCSCs (Menendez *et al.*, 2011). On the other hand, in the cells induced by NC-B culture condition, *PAX3* expression was maintained at a high level, whereas *SOX10* expression was lower than that in NC condition, and *FOXD3* and *PAX6* expression levels were the same as those observed in undifferentiated ES cells. In agreement with the above immunostaining results, cells induced by NC-B culture condition expressed significantly lower levels of *p75$^{NTR}$* mRNA than those by NC culture condition. Cells induced by NC-B culture condition expressed high levels of *SOX9*, *AP2*$\alpha$, and *CDX2*. Moreover, we observed robust expression of the cranial NC markers *MSX1* and *DLX1*, specifically in cells induced by NC-B culture condition (Figs.1 B & 2 D). These results demonstrate that our NC-B culture condition induced a different subset of NC cells, which expressed cranial NC markers, compared with cells induced by NC culture condition.

### *Analysis of anterior–posterior identities of the NC cells induced by BMP4 activation*

Furthermore, gene expression was evaluated in hESC derived NC cells to define anterior–posterior positional identities using a customized qRT-PCR array set of 156 genes including 76 NC-related genes and 80 *HOX* genes (Table S2, S3, Fig.S3). The 72 NC-related and *HOX* genes were transcription factors with defined expression profiles and functions in NC cells (Curchoe *et al.*, 2010, Nelms and Labosky, 2010). Comparison of expression profiles of the 72 genes in NC cells induced by NC or NC-B culture condition demonstrated that both conditions induced the expression of *AP2*$\alpha$, *MSX1*, and

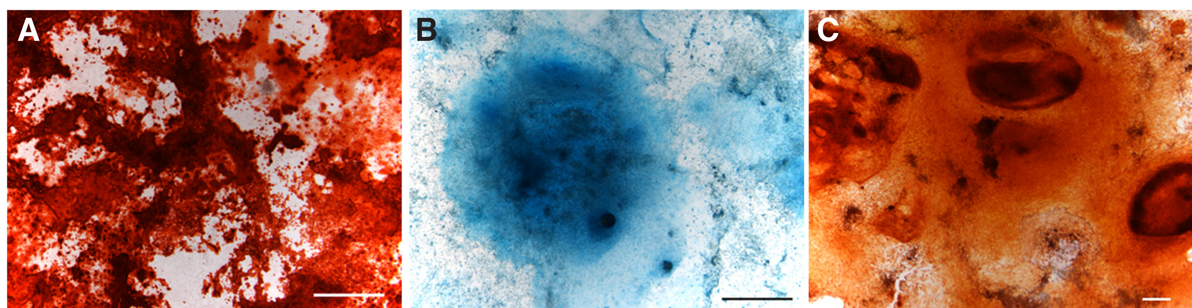


**Fig. 4. Differentiation of neural crest cells cultured in BMP4-containing medium. (A)** *Osteocytes analyzed by alizarin red staining.* **(B)** *Chondrocytes analyzed by alcian blue staining.* **(C)** *Chondrocytes analyzed by safranine O staining. Scale bars, 1 mm.*

*MSX2* genes, which are abundant in both early NC and mesenchymal NC cells in pharyngeal arches (Betters *et al.*, 2010, Brunskill *et al.*, 2014, Nelms and Labosky, 2010) (Fig.3, Table S3). Cells induced by the NC culture condition had high expression of the early NC markers, *FOXD3* and *PAX3*, in addition to *PAX7*, *PAX8*, *SOX10*, and *SNAI2* (Fig.3, Table S3). Moreover, the expression of posterior *HOX* genes was not observed in cells induced by the NC culture condition; however, the expression of anterior *HOX* genes, *OTX1* and *OTX2*, was observed (Table S3). Both of *SOX2* and *OCT4* are observed in numerous NCSC lines and commonly used as NCSC markers as well as pluripotency markers. It is known that migratory NC and postmigratory NC cells maintain a low *SOX2* expression level (Nelms and Labosky, 2010). The expression level of these NCSC markers was high in induced cells by NC culture condition and reduced in NC-B culture condition (Fig.3, Table S3). On the other hand, NC-B culture condition promoted substantially increased activation of craniofacial NC gene subsets, including *DLX1*, *DLX2*, *DLX3*, *DLX4*, *DLX5*, *DLX6*, *PITX1*, *PITX2*, and *HAND2*, which are abundantly expressed in migratory and postmigratory NC cells in pharyngeal arches (Brunskill *et al.*, 2014, Nelms and Labosky, 2010) (Fig.3, Table S3). Migratory NC cell markers, *GATA4* and *GATA6*, were prominently expressed in the cells induced by NC-B culture condition (Fig.3, Table S3). A part of posterior *HOX* genes containing *HOXB9* and *HOXC13* were slightly activated; however, the expression levels of these genes were relatively lower than anterior *HOX* genes/cranial NC genes (Fig.3, Table S3).

These gene expression profiles indicate that the NC culture condition induced anterior premigratory NC cells and NCSCs and that NC-B culture condition induced pharyngeal arch NC cells and migratory NC cells. Thus, late exposure to BMP4 may have an important role in the transcriptional regulation of pharyngeal mesenchymal NC development.

### *Differentiation of the induced NC cells into osteocytes and chondrocytes in vitro*

Cranial NC cells give rise to the majority of the craniofacial bone and cartilage (Santagati and Rijli, 2003). Therefore, we further evaluated the osteogenic and chondrogenic potential of NC cells induced by NC-B culture condition by further culturing for 21 days in an osteogenic or chondrogenic medium, respectively. Cells cultured in the osteogenic medium were stained deeply by alizarin red (Fig.4 A). Those cultured in the chondrogenic medium were stained deeply by both alcian blue (Fig.4 B) and safranine O (Fig.4 C). These results demonstrate that NC cells induced by NC-B culture condition are capable of differentiating into osteocytes and chondrocytes, indicating the NC-B culture condition induced a cranial mesenchyme population. It had been demonstrated that NCSCs induced without BMP4 were differentiated into mesenchymal stem cells by culturing in the medium containing 10%(v/v) FBS, and the mesenchymal stem cells were further differentiated over 3 or 4 weeks to osteocytes or chondrocytes by Dalton et al. (Menendez *et al.*, 2013, Menendez *et al.*, 2011). These results suggested that NC cells induced from human ESCs in NC and NC-B culture condition included anterior NCSCs and cranial mesenchyme in cranial regions.

## Discussion

In the present study, we demonstrate that timely treatment with BMP4 after NC specification is able to induce a distinct subset of NC cells which express cranial positional information and pharyngeal mesenchymal genes *in vitro*. NC cells treated with BMP4 were capable of differentiating into both osteocytes and chondrocytes, indicating the successful induction of cranial NC. Thus, we describe the development of a differentiation protocol for cranial mesenchymal NC induction from hESCs, modifying the previous NCSC induction protocol using chemically-defined culture conditions.

Our differentiation strategy, late exposure to BMP4, coincides with the current model of intracellular and extracellular signaling during NC induction (Stuhlmiller and Garcia-Castro, 2012). Previous studies in vertebrates have identified numerous signaling factors, including FGF, Wnt, and BMP, involved in the specification and fate determination of NC cells in a different manner during gastrulation and neurulation. The canonical Wnt signaling is critical for the specification of NC during gastrulation (Garcia-Castro *et al.*, 2002). Wnt signaling can inhibit *BMP4* expression at early gastrula stages in *Xenopus* (Baker *et al.*, 1999). Although there is no direct evidence that FGF signaling is involved in NC induction during mouse development, there is accumulating evidence for the role of FGF signaling during NC induction in *Xenopus*, chicken, and zebrafish (Stuhlmiller and Garcia-Castro, 2012). The activation of FGF signaling in the early embryo up-regulates both BMP and Wnt signaling (Garnett *et al.*, 2012). We previously reported that Wnt signaling induces the development of anterior and posterior primitive streak cells from both mouse and hESCs under defined culture conditions (Nakanishi *et al.*, 2009). Furthermore, BMP signaling is temporally activated during NC induction in *Xenopus* and chick embryos, with the inhibition of BMP signaling at the gastrula stage and activation of BMP and Wnt signaling at the neurula stage required (Patthey *et al.*, 2009). These studies indicate that inhibition

of BMP signaling is required for the specification of early NC and that BMP and Wnt signaling are required for the maintenance of NC population and further differentiation. We believe the following two-step NC induction method developed in the present study is compatible with these models: (1) activation of Wnt and FGF signaling from day 0 to day 8 for NC specification and (2) activation of BMP in combination with Wnt and FGF signaling from day 8 to day 12. Activating BMP4 signaling at specific time points appears critical for the induction of cranial mesenchyme from early NC cells.

Heterogeneity among p75$^{NTR+}$ cells in induced NC populations has been reported *in vitro*, with both *SOX10* expression and the ability to differentiate into NC derivatives (Curchoe *et al.*, 2010). The expression of *PAX3*, *SOX9*, and *SOX10* in premigratory NC cells and that of *PAX7*, *SOX9*, *AP2*$\alpha$, and *p75*$^{NTR}$ in migratory NC cells has been demonstrated in addition to the expression of *SOX10* in human embryos (Betters *et al.*, 2010). Overall, these results indicate that *SOX10* and other NC marker genes, in addition to *p75*$^{NTR}$ and *HNK1*, are required for the definitive characterization of NC cells *in vivo* and *in vitro*. Moreover, recent studies have reported the *in vivo* NC expression profiles of transcription factors and other related genes at both the mRNA and protein level in mouse and human embryos (Betters *et al.*, 2010, Brunskill *et al.*, 2014). Gene expression profiles in induced cells *in vitro* should be comprehensively analyzed and compared with recent *in vivo* findings. In the present study, the cranial mesenchyme NC population induced by BMP4 in the NC induction culture condition demonstrated a lower proportion of p75$^{NTR+}$ cells compared with those induced without BMP4 in the NC induction culture condition. Induced cranial mesenchyme NC cells had substantial up-regulation of genes related to NC maintenance, migration, craniofacial positioning, and tooth development, whereas small changes in genes expressed in posterior NC positions, such as the vagal, trunk, or sacral NC, were observed. To the best of our knowledge, we were first to demonstrate high levels of *MSX* and *DLX* expression in NC cells directly induced from hESCs. The expression of anterior *HOX* genes is affected by retinoic acid treatment during NC induction (Fukuta *et al.*, 2014). These findings indicate the potential utility of modulating signaling factors step-by-step in controlling regional characteristics of NC cells during directed induction *in vitro*.

In order to efficiently induce specific NC populations, such as cranial NC cells *in vitro*, the sequential gene expression changes occurring *in vivo* during embryonic development are required to be accurately replicated. Temporal prescription of extra cellular matrixes (ECM), growth factors and/or small molecules, which act as inhibitors or activators of signaling cascades, can control gene expression during directed differentiation. We previously demonstrated the induction of jaw cartilage with the expression of maxillofacial regional marker genes from *Xenopus* undifferentiated presumptive ectoderm *in vitro* using Activin A and a sandwiched explant culture system (Furue *et al.*, 2002). Furthermore, we also reported the induction of tooth development from mixed cultures of Activin A-treated dissociated cells and untreated cells of *Xenopus* undifferentiated presumptive ectoderm (Myoishi *et al.*, 2004). In the present study, we induced cranial NC, particularly craniofacial mesenchyme, from hESCs. The combination of the methodology used in these culture systems and recent developments in the understanding of gene expression profiles in the early human embryo will facilitate the development of novel directed differentiation methods and promote an increased understanding of the mechanisms underlying the regulation of cranial NC development in humans. Although various methods for efficient directed differentiation of human pluripotent stem cells have been reported, numerous limitations remain. Almost all induction methods reported till date do not allow the analysis of the effects of ECM, growth factors, or small molecules because currently-used methods utilize undefined components, such as Matrigel and conditioned medium, or BSA at high concentrations. In order to analyze extracellular and intracellular signaling during differentiation more accurately, the use of completely chemically-defined culture conditions is preferable. Accordingly, we are currently developing a new defined culture method to control the induction of NC cells with the expression of positional information genes.

## Materials and Methods

### *Cell culture*

The human ES cell line, H9 (WA09, WISC Bank, WiCell Research Institute, Madison, WI, USA) was routinely maintained on mouse embryo fibroblast feeder cells inactivated by $\gamma$-irradiation (MEF CF-1, Applied StemCell, Inc., CA, USA) in a hESC expansion medium (KSR-based medium) consisting of DMEM/F12 medium (Life Technologies, CA, USA) supplemented with 20% KSR (Life Technologies Ltd, Paisley, UK), 0.1 mM 2-mercaptoethanol (Sigma, St. Louis, MO, USA), 2 mM L-glutamine (Gibco, NY, USA), 0.1 mM non-essential amino acids (Gibco), and 5 ng/ml human recombinant FGF-2 (Katayama Kagaku Kogyo, Japan) as previously described (Kinehara *et al.*, 2013). The cells were split at a ratio of 1:8–1:10 every 6–7 days. Culture medium was replaced with hESF9, a growth-factor defined serum-free ESC culture medium, which we previously developed (Furue *et al.*, 2008) at least 24 h before re-seeding for differentiation. The hESF9 medium consisted of ESF basal medium (Wako Pure Chemicals, Japan) supplemented with five factors (10 $\mu$g/ml of human recombinant insulin, 5 $\mu$g/ml of human transferrin, 10 $\mu$M of 2-mercaptoethanol, 10 $\mu$M of 2-ethanolamine, 20 nM of sodium selenite), 9.4 $\mu$g/ml of oleic acid conjugated with fatty acid-free bovine serum albumin (BSA), 100 ng/ml of bovine heparan sulfate sodium salt (all from Sigma), 0.1 mg/ml of L-ascorbic acid 2-phosphate, and 10 ng/ml of human recombinant FGF-2. Cells were checked for mycoplasma infection every month using a MycoAlert Mycoplasma Detection Kit (Lonza). Identification of cells used in the present study confirmed the H9 cell line against the JCRB Cell Bank (National Institutes of Biomedical Innovation, Health and Nutrition, Japan). Human ESCs were used following the Guidelines for the utilization of hESCs of the Ministry of Education, Culture, Sports, Science and Technology of Japan after approval by the institutional ethical review board at National Institutes of Biomedical Innovation, Health and Nutrition.

### *Differentiation of hESCs into NC cells in monolayer culture*

H9 cells were cultured under the modified protocol reported by Menendez et al. (Menendez *et al.*, 2013, Menendez *et al.*, 2011) for the differentiation of hESCs into NC cells. H9 cells at a cell density of 1 × $10^4$ cells were dissociated into single cells by Accutase (EMD Millipore) and seeded on Matrigel (BD)-coated wells of 6-well plates in hESF9 medium and cultured for 1–2 days. Cells were then cultured in NC differentiation medium. NC differentiation medium consisted of DMEM/F12 (Cat. No. 10505, supplemented with GlutaMAX-I, Invitrogen) containing 2% BSA (Cat. No. 82-067-3, EMD Millipore), 1× MEM non-essential amino acids (Invitrogen), 1× trace element A (Cellgro), 1× trace element B (Cellgro), 1× trace element C (Cellgro), 0.1 mM 2-mercaptoethanol, 10 $\mu$g /ml apo-transferrin (Sigma), 50 $\mu$g/ml (+)-sodium L-ascorbate (Sigma), 10 ng/ml NRG1/HRG1 (R&D), 200 ng/ml LONGR$^3$ IGF-1 (Sigma), 8 ng/ml FGF-2, 25 ng/ml Wnt-3a (R&D), and 20 $\mu$M SB431542 (Tocris). Culture medium was replaced every day during differentiation. From day 8 to day 12, cells were cultured in NC differentiation medium supplemented with 100 ng/ml of BMP4 (NC-B condition) or without BMP4 (NC condition).

***Differentiation of hESC-derived NC cells into osteocytes and chondrocytes***

Osteogenic or chondrogenic differentiation protocols were performed using osteogenic induction medium (StemPro Osteogenesis Differentiation kit, Life technologies) or chondrogenic induction medium (StemPro Chondrogenesis Differentiation kit, Life technologies), respectively. Alizarin Red (Wako) was used to detect osteocytes. Safranine O (Wako) and Alcian Blue solution (Sigma) were used to detect chondrocytes. Images of stained cells were obtained using a stereoscopic microscope (MZ12, Leica).

***Immunocytochemistry and flow cytometry***

Immunocytochemistry and flow cytometry were performed as previously described (Menendez *et al.*, 2013). Monoclonal anti-HNK-1/N-CAM antibody (mouse IgM clone VC1.1, C6680, Sigma) and monoclonal anti-p75/NGFR antibody (mouse IgG clone ME20.4, AB-N07, Advanced Targeting Systems, San Diego, CA, USA) were used as primary antibodies. Alexa Fluor 488 labeled goat anti-mouse IgM antibody and Alexa Fluor 647 labeled goat anti-mouse IgG antibody were used for fluorescent detection. Nuclei were detected by Hoechst33342 staining. Images were obtained by fluorescent microscopy (Ti, Nikon). Flow cytometry data were acquired using a guava easyCyte™ 8HT Flow Cytometer (EMD Millipore).

***Gene expression analysis***

Total RNA was extracted from undifferentiated hESCs or hESC-derived NC cells using AllPrep DNA/RNA Mini kits (QIAGEN), treated with RNase-free DNaseI (Invitrogen), and reverse transcribed with SuperScript VILO cDNA synthesis Kits (Invitrogen), according to the manufacturer's instructions. Synthesized cDNA was amplified using RT$^2$ Profiler™ PCR Arrays (Human Homeobox (HOX) Genes and custom designed array, QIAGEN) or gene-specific primers (Invitrogen). The primers used in the present study are shown in Supplementary Table S1. Genes analyzed in the array are listed in Supplementary Table S2. Array data were analyzed using web-based software for cataloged and custom arrays (RT$^2$ Profiler ™ PCR Array Data Analysis Ver. 3.5, QIAGEN).

*Author Contributions*

*M.K.F. and H.N. conceived and supervised this study. S.M., M.S., K.O. and M.K. performed all cell culture experiments and related analyses. M.S. and M.K.F. supervised and performed the microarray analyses. All authors contributed to manuscript preparation.*

*Acknowledgments*

*We thank Jim Hackland, Christian Unger, and Peter W. Andrews for helpful discussion. We thank Haruka Asano, Mari Wakabayashi, and Naoko Ueda for excellent technical support and Takayuki Fukuda for excellent support and discussion. The authors would like to thank Enago (www.enago.jp) for the English language review. This study was supported by grants-in-aid from the Ministry of Health, Labor and Welfare of Japan to M.K.F. and the Ministry of Education, Culture, Sports, Science and Technology of Japan to M.K.F. and M.S.*

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