BMP-4 Upregulates Kit Expression in Mouse Melanoblasts prior to the Kit-Dependent Cycle of Melanogenesis

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Genes encoding Kit and the Kit ligand (KL) play essential roles in the differentiation of melanoblasts. We previously established three immortal but distinct cell populations of mouse neural crest (NC) cells. NCCmelb4M5 cells do not express Kit and grow independently of KL; they have the potential to differentiate into NCCmelb4 cells, which are Kit-positive melanocyte precursors. NCCmelan5 cells show the characteristics of differentiated melanocytes. All three cell lines demonstrated bone morphogenetic protein (BMP) receptor expression. BMP-4 upregulated Kit protein and mRNA expression in most immature NCCmelb4M5 cells. Noggin, a BMP-4 antagonist, dramatically decreased the Kit expression induced by BMP-4. Western blot analysis revealed that extrinsic BMP-4 leads to the phosphorylation of Smads in NCCmelb4M5 cells. Using transfected Kit-promoter reporter, we showed BMP-4 could activate Kit promoter in transfected NCCmelb4M5 cells. We conclude that BMP-4 is active and is involved in the regulation of Kit expression on most immature melanocyte precursors. We further investigated the influence of BMP-4 *in vitro* using primary NC cells cultured from wild-type mice. Addition of BMP-4 to the medium increased the number of Kit-positive cells compared to diluent-treated controls. We have identified BMP-4 as an important factor for prenatal Kit-negative melanoblasts just prior to entering the Kit-dependent cycle of melanogenesis.

Journal of Investigative Dermatology (2008) **128,** 1220–1226; doi:10.1038/sj.jid.5701136; published online 25 October 2007

INTRODUCTION

Neural crest (NC) cells can differentiate into numerous derivatives including melanocytes, neurons, and peripheral glia. These multiple cell types arise from pluripotent precursors, whose existence has been demonstrated in avian and in rodent NC at the premigratory and migratory stages (Baroffio *et al.*, 1988; Bronner-Fraser and Fraser, 1988; Stemple and Anderson, 1992). In mouse embryos, melanoblasts, the precursors of skin melanocytes, originate from NC cells in the neural tube. Kit/Kit ligand (KL) signaling is required for the survival of melanocyte precursors during their migration toward the skin (Nishikawa *et al.*, 1991; Murphy *et al.*, 1992; Ito *et al.*, 1999). Activation of the Kit receptor in the presence of KL suggests that KL is essential to melanocyte differentiation during the early stages of development (Scott *et al.*, 1994; Hachiya *et al.*, 2001). It has been observed that while mouse melanocyte precursors migrate from the NC to the skin, they first become Kit-positive, and then differentiate into L-3,4-dihydroxyphenylalanine-positive, mature melanocytes. However, the factors that control melanoblast differentiation to the Kit expressing stage are not known.

We previously established an immortal cell population of NC cells from WB mice, named NCCmelb4 (Ito *et al.*, 2004). They appear to be immature melanocytes, as they are positive for tyrosinase-related protein 1, L-3,4-dihydroxyphenylalaninechrome tautomerase (Dct), and Kit, but are negative for tyrosinase and for L-3,4-dihydroxyphenylalanine reactivity. In addition, they contain only stage I melanosomes without any melanosomes in more advanced stages. They differentiate into tyrosinase-positive melanocytes having stages III–IV melanosomes after treatment with all-*trans* retinoic acid or 1,25-dihydroxyvitamin D_3 (Watabe *et al.*, 2002a, b). NCCmelb4 cells have the potential to differentiate into mature mouse melanocytes, but since they express melanocyte markers, such as tyrosinase-related protein 1, L-3,4-dihydroxyphenylalaninechrome tautomerase, and Kit, they are considered to be immature melanocytes, not pluripotent precursors that can differentiate into neurons or glia. To study the differentiation of the melanocyte lineage, we obtained an immortal cell line from NCCmelb4 cells by removing KL from the culture medium to establish NCCmelb4M5 cells that do not express Kit and grow

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Abbreviations: BMP, bone morphogenetic protein; BMPR, BMP receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KL, Kit ligand; NC, neural crest; p-Smad, phosphorylated Smad; RT, room temperature

Received 29 May 2007; revised 14 August 2007; accepted 20 August 2007; published online 25 October 2007

independently of KL (Kawa *et al.*, 2005). NCCmelb4M5 cells show stable and immortal growth in a medium without KL. Electron microscopy revealed that some stage I melanosomes are produced in NCCmelb4M5 cells. These findings suggest that NCCmelb4M5 cells belong to the mouse melanocyte lineage, but are less differentiated than NCCmelb4 cells. We have also established an immortal cell population of NC cells, cultivating them with KL and endothelin 3, named NCCmelan5 (Ooka *et al.*, 2001). NCCmelan5 cells were positive for Kit, tyrosinase-related protein 1,, L-3,4-dihydroxyphenylalaninechrome tautomerase, tyrosinase, and could oxidize L-3,4-dihydroxyphenylalanine to form melanin. These findings strongly suggest that NCCmelan5 cells demonstrate the characteristics of differentiated melanocytes. We consider these three cell lines to be ideal for studying factors that affect melanocyte development and melanogenesis.

Bone morphogenetic proteins (BMPs), members of transforming growth factor-β superfamily, signal in many cells including neural precursors. BMPs bind two transmembrane receptors, type II (BMPRII) and type I (BMPRIA, BMPRIB). Activation of type II and type I receptors leads to the phosphorylation of receptor-activated Smads (Graff *et al.*, 1996). Among the receptor-activated Smads, Smad1, Smad5, and Smad8 are the preferential substrates for BMPRIA and BMPRIB. Noggin is a secreted BMP antagonist that competes for binding to BMP receptors. BMP-4 has been identified as a novel signaling pathway that influences melanogenesis in human skin (Yaar *et al.*, 2006). We have previously showed that transforming growth factor-β can upregulate Kit expression in NCCmelb4 cells (Kawakami *et al.*, 2002). Using the NCCmelb4M5, NCCmelb4, and NCCmelan5 cell lines, we investigate the effects of BMP-4 on these immature melanocyte precursors.

RESULTS

BMPR expression in NCCmelb4M5, NCCmelb4, and NCCmelan5 cells

To investigate whether BMPRs are expressed by NCCmelb4M5, NCCmelb4, and/or NCCmelan5 cells, western blot analysis was performed. BMPRIA, BMPRIB, and BMPRII were detectable in all three cell lines (Figure 1).

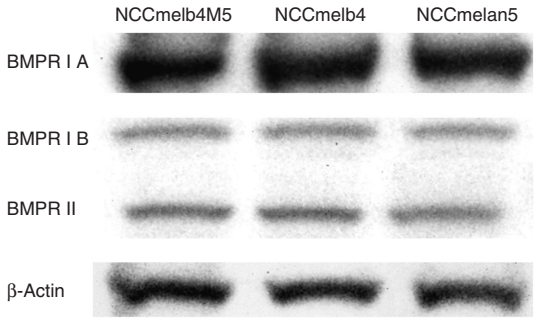

Figure 1. BMPR expression in NCCmelb4M5, NCCmelb4, and NCCmelan5 cells. Western blot analysis of BMPRIA, BMPRIB, and BMPRII expression in all three cell lines compared to β-actin expression as a control (lower panel).

Effects of BMP-4 on proliferation of NCCmelb4M5, NCCmelb4, and NCCmelan5 cells

We investigated effects of BMP-4 on proliferation of three cell lines using Alamar Blue fluorescence assay (data not shown). Alamar Blue fluorescence assay was performed as described previously (Kawakami *et al.*, 2002; Kawa *et al.*, 2005). When BMP-4 was added to the medium of NCCmelb4M5 cells, this growth was significantly retarded after 4 days in the presence of 100 ng ml^{-1} BMP-4 ($P<0.01$). In contrast, there was no evidence of BMP-4-induced suppression in the NCCmelb4 and NCCmelan5 cells. To investigate further the effects of extrinsic BMP-4, we examined whether growth inhibition in NCCmelb4M5 cells was recovered by the addition of an overdose of noggin to the culture medium, as noggin binds and effectively inactivates BMP-4. The addition of noggin led to a recovery in the decrease in cell proliferation by BMP-4. The BMP-4-induced reduction was completely negated when the noggin concentration was increased to 100 ng ml^{-1} against 10 ng ml^{-1} BMP-4.

Western blot analysis of Kit expression in NCCmelb4M5 cells incubated in BMP-4-enhanced medium

Since Kit is a receptor that mediates differentiation of melanocytes through its ligand KL, we investigated whether Kit is induced by BMP-4. After treatment for 3 days with BMP-4 at various concentrations (0, 1, 10, or 50 ng ml^{-1}), NCCmelb4M5 cell extracts were prepared and analyzed by western blotting for Kit content (Figure 2a). As positive controls, NCCmelb4 and NCCmelan5 cells were incubated in basic medium. Kit protein expression in NCCmelb4M5 cells showed a dose-dependent increase up to a concentration of 50 ng ml^{-1} (Figure 2b). We further examined the time-dependent relationship between BMP-4 treatment and Kit expression in NCCmelb4M5 cells. Kit expression increased in a time-dependent manner following 1, 2, or 3 days of incubation with 10 ng ml^{-1} BMP-4 (Figure 2c). Kit expressions on 50 ng ml^{-1} BMP-4 were similar to that seen in 10 ng ml^{-1} (data not shown). We then examined the effect of an excess of extrinsic noggin on the increase in Kit protein expression induced by BMP-4 (Figure 2d). Using 10 times (100 ng ml^{-1}) noggin concentration against an extrinsic BMP-4 (10 ng ml^{-1}), BMP-4-induced Kit expression was suppressed by the addition of a high dose of noggin (Figure 2e).

Real-time PCR analysis of Kit mRNA expression in NCCmelb4M5 cells

We examined the expression of Kit mRNA transcripts in NCCmelb4M5 cells treated for 3 days with 1, 10, or 50 ng ml^{-1} BMP-4 compared to normal controls (Figure 3). Significant induction of Kit mRNA was observed after treatment with 10 and 50 ng ml^{-1} BMP-4, but not with 1 ng ml^{-1}. Kit expression showed a dose-dependent increase up to a concentration of 50 ng ml^{-1} BMP-4 at both the protein and mRNA levels after 3 days. Real-time PCR results after 3 days suggest that BMP-4 may also have indirect activation mechanism for the Kit expression.

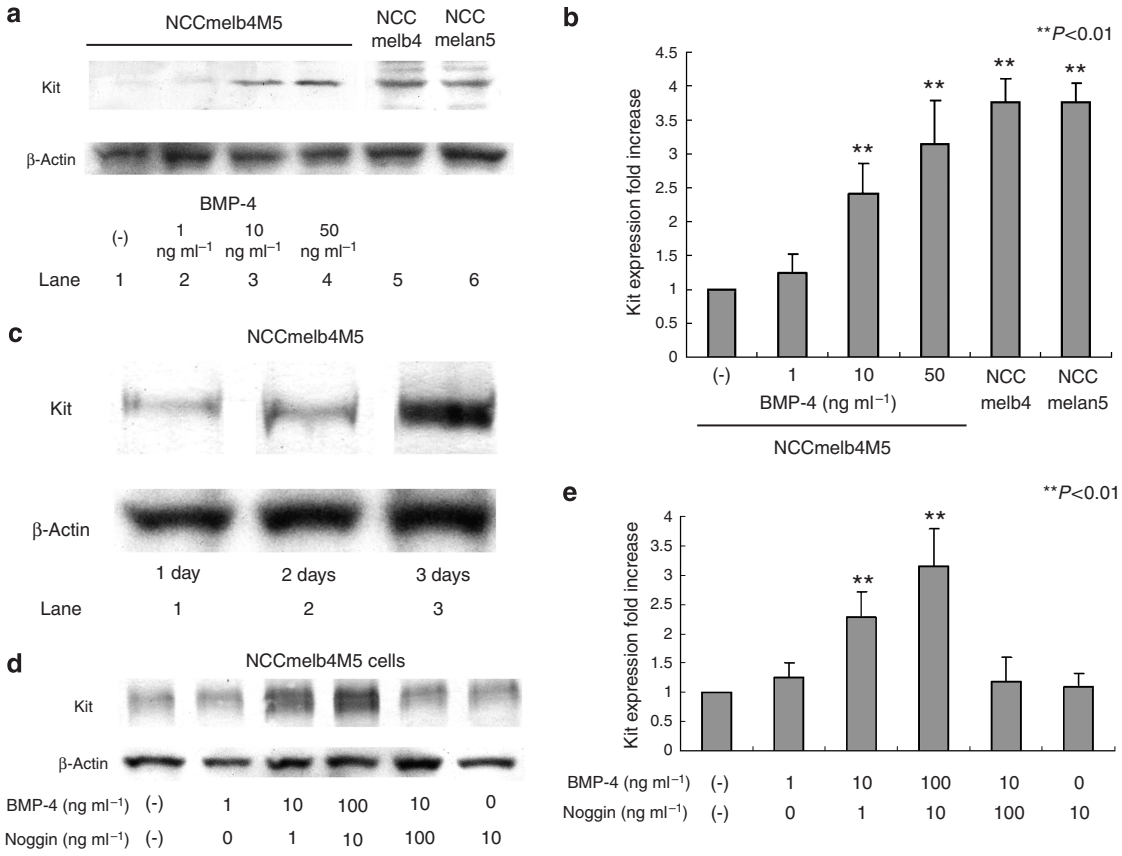

Figure 2. Western blot analysis of Kit expression by BMP-4-treated NCCmelb4M5 cells. (**a**) NCCmelb4 and NCCmelan5 cells were incubated in basic medium as positive controls (lanes 5 and 6). NCCmelb4M5 cells were incubated for 3 days with BMP-4 (lane 1: no BMP-4, lane 2: 1 ng ml^{-1}; lane 3: 10 ng ml^{-1}; lane 4: 50 ng ml^{-1}). (**b**) Kit expression levels were normalized to the amount of β-actin and are shown relative to expression levels. Kit protein expression in NCCmelb4M5 cells was highest in the presence of 50 ng ml^{-1} BMP-4. $^{**}P<0.01$. (**c**) We analyzed the time-response of treatment with BMP-4. Cultures were incubated in 10 ng ml^{-1} BMP-4. BMP-4 was added to the basic medium for varying incubation times (lane 1: 1 day; lane 2: 2 days; lane 3: 3 days). Kit protein expression increased in a time-dependent manner during the first 72 hours. (**d**) Cells were incubated for 72 hours in various amounts of BMP-4 and/or noggin. The increased Kit expression at a concentration of 10 ng ml^{-1} BMP-4 was significantly reduced by an excess amount of extrinsic noggin at 100 ng ml^{-1}. (**e**) Kit expression levels were normalized to the amount of β-actin and are shown relative to expression levels.

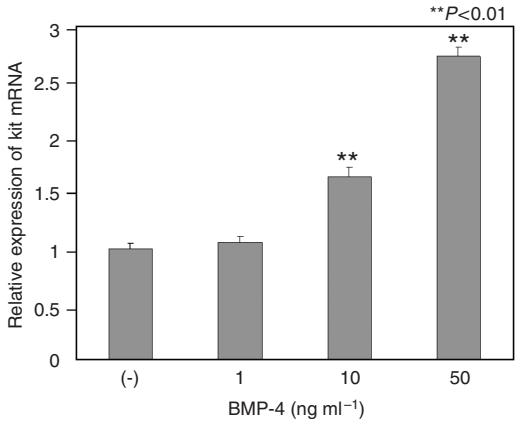

Figure 3. Real-time PCR analysis of Kit mRNA expression induced by BMP-4. Subconfluent cultures of NCCmelb4M5 cells were treated with 1, 10, or 50 ng ml^{-1} BMP-4 for 3 days. Total RNA was extracted from the NCCmelb4M5 cells, reverse transcribed into cDNA, and then subjected to real-time PCR analysis, as described in the Materials and Methods section. Expression levels were normalized to the amount of GAPDH mRNA and are shown relative to GAPDH expression. $^{**}P<0.01$.

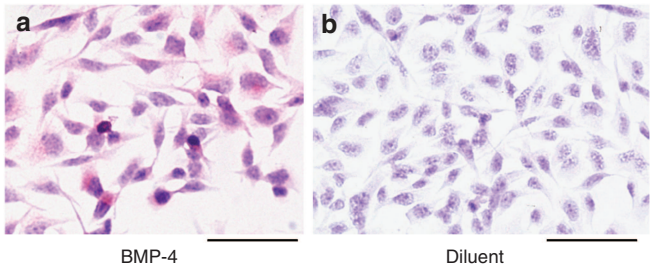

Figure 4. Kit immunostaining of NCCmelb4M5 cells induced by BMP-4. Cells were immunostained for Kit. NCCmelb4M5 cells were incubated with 10 ng ml^{-1} (**a**) BMP-4 or (**b**) diluent alone for 3 days. Bar = 50 μm.

Kit immunostaining induced by BMP-4 in NCCmelb4M5 cells

We examined changes in NCCmelb4M5 cells on addition of BMP-4. After incubation with 10 (Figure 4a) or 50 ng ml^{-1} (data not shown) BMP-4 for 72 hours, NCCmelb4M5 cells became Kit-positive. In diluent-treated cultures, NCCmelb4M5 cells did not reveal Kit-positive (Figure 4b).

Variation of p-Smad 1/5/8 expression in BMP-4-treated NCCmelb4M5 cells

To understand the precise mechanisms involved in BMP signaling, we tested Smad protein expression for Smad state of phosphorylation (Figure 5). Smad activation of BMP signaling was detected using an antibody against phosphorylated Smad (p-Smad) 1/5/8. BMP-4 was added to the medium and incubated for 3 days at varying concentrations (0, 1, 10, or 50 ng ml^{-1}). p-Smad 1/5/8 levels increased in a dose-dependent manner in Kit-negative NCCmelb4M5 melanoblasts treated with BMP-4. The BMP-4-induction at 10 ng ml^{-1} was reduced by the addition of an excess of noggin (100 mg ml^{-1}).

Effect of BMP-4 on Kit-promoter activity in NCCmelb4M5 cells

We investigated the effect of BMP-4 on Kit-promoter activity in a transfection assay based on the dual luciferase reporter system. By inserting Kit promoter into the luciferase plasmid, we found that the addition of the BMP-4 to the Kit-promoter system significantly increased relative luciferase activity compared to untreated cells (Figure 6).

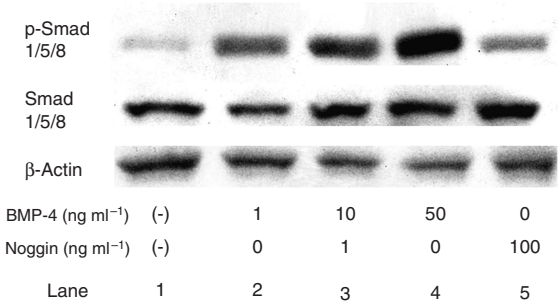

Figure 5. p-Smad expression in NCCmelb4M5 cells incubated in BMP-4-enhanced medium. BMP-4 induced a significant increase in the p-Smad 1/5/8 protein in a dose-dependent manner. The increased p-Smad expression at a concentration of 10 ng ml^{-1} (lane 3) was reduced by an excess amount of extrinsic noggin at 100 ng ml^{-1} (lane 5).

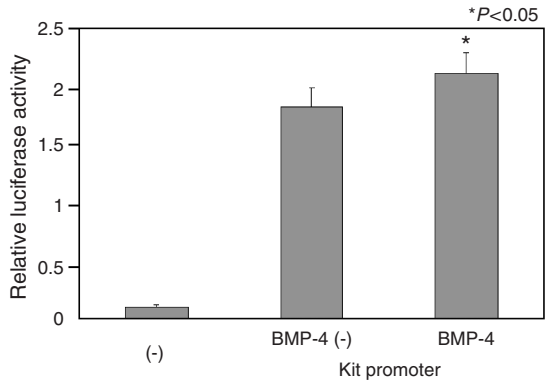

Figure 6. Kit-promoter luciferase activity induced by BMP-4 using the luciferase reporter assay. We generated correct constructs by placing a Kit promoter fragment into the pGL4 *luc2* vector. We found that the addition of the BMP-4 to the Kit promoter system increased significantly relative luciferase activity compared to untreated cells (*$P<0.05$).

Kit-positive melanocytes induced by BMP-4 in *in vitro* primary cultures of NC cells

NC cell explants in culture medium supplemented with KL were fixed at day 9 and were subjected to immunohistochemical staining for BMP receptors. Almost all NC cell explants were positive for BMPRIA, BMPRIB, and BMPRII (data not shown). To investigate whether BMP-4 induces Kit expression in *in vitro* primary cultures, we treated NC cultures in which neural tubes remained for the entire culture period with 1 ng ml^{-1} BMP-4 and investigated the emergence of Kit-positive melanocyte precursors. More Kit-positive cells were detected in NC cells incubated with BMP-4 than in the diluent-treated controls (Figure 7). At a concentration of 10 or 50 ng ml^{-1} BMP-4, NC cell explants did not survive and were completely abolished by day 6 (data not shown).

DISCUSSION

We have examined the effect of BMP-4 combined with Kit expression on melanocyte precursors. Western blot analysis revealed that Kit protein expressed by NCCmelb4M5 cells increased in proportion to BMP-4 incubation. This same pattern was also evident at the mRNA level using real-time PCR. We found that exposure of most immature melanocytes to exogenous BMP-4 leads to an upregulation of Kit expression. These observations led us to hypothesize that extrinsic BMP-4 combined with Kit/KL plays a contributory role as an active factor in mouse melanocyte development. Noggin binds BMP-4 with a 10–15 times higher affinity than BMPRs and also neutralizes its activity (Groppe *et al.*, 2002). We tested whether the addition of extrinsic noggin would neutralize the effect of BMP-4 on Kit protein expression. We noted a decrease in Kit protein expression induced by BMP-4 addition following treatment with an excess of noggin during incubation. Extrinsic BMP-4 was able to induce Kit expression in Kit-negative melanoblasts, thus conferring KL sensitivity to these cells. The results suggest that BMP-4 might selectively enhance the proliferation of Kit-expressing cells. Western blot analysis revealed an apparent dose-dependent induction of p-Smad1/5/8 protein levels in proportion to the BMP-4 concentration within NCCmelb4M5 cells. Luciferase promoter assay showed that BMP-4 treatment of NCCmelb4M5 cells increased transcription of the Kit-promoter gene. It has been shown that transforming

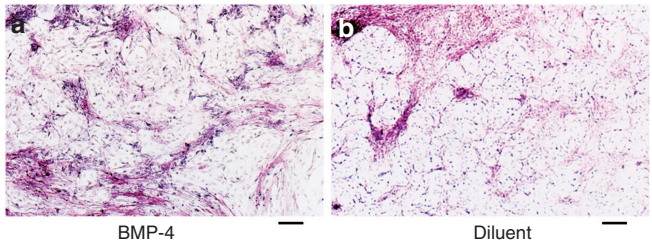

Figure 7. Kit staining of *in vitro* primary cultures of NC cells. NC cell explants were cultured on cover glasses in sixwell plates and then were immunohistochemically stained, as described in the Material and Methods section. Cultured from day 3 to day 9 in the presence of BMP-4 (**a**: 1 ng ml^{-1}), the number of Kit-positive cells increased compared to (**b**) diluent alone control. Bar = 100 μm.

growth factor-β can upregulate Kit expression in T-leukemia cells and in melanoblasts (Tomeczkowski *et al.*, 1998; Kawakami *et al.*, 2002). Pellegrini *et al.* (2003) found that exposure of spermatogonia to exogenous BMP-4 was able to upregulate Kit expression both at the RNA and protein level, conferring KL responsiveness to spermatogonia at 4-day postnatal, when the majority of germ cells are Kit-negative. These findings indicate that the BMP-4 could be active and involved in the regulation of Kit expression in germ cell.

At NCC lineage segregation in the mouse neural tube, mouse melanocyte precursors are Kit-positive and their binding to the KL results in a signal that is required for their survival and differentiation into the next stage (Wilson *et al.*, 2004). It appears that BMP signaling can control various aspects of NC development through regulation of the epithelial/mesenchymal transformation that allows NC cells to separate from the neural tube (Selleck and Bronner-Fraser, 1996; Sela-Donenfeld and Kalcheim, 1999). Bilodeau *et al.* (2001) demonstrated that BMP-2 and cAMP signaling similarly exert a combinatorial effect on melanocyte development. BMP-2 treatment of NC cells increased melanogenesis by promoting the synthesis of melanin. BMP-4 is expressed in the dorsal neural tube throughout the time of NC cell migration, but is decreased coincident with the timing of melanoblast migration (Jin *et al.*, 2001). We detected the presence of BMP receptors in NC cells *in vitro* primary cultures by immunohistochemical staining. The number of Kit-positive cells increased in the presence of BMP-4, indicating that the ability to express Kit in NC cells is selectively induced in the presence of exogenous BMP-4. These results show that BMP-4 can play an important role in relation to Kit signaling in the differentiation and migration of mouse NC cells *in vitro*.

In conclusion, we suggest that BMP-4 is a requirement for Kit-negative melanoblasts, before entering the Kit-dependent cycle of melanogenesis. BMP-4 may further be one of the signals involved in the very early stages of melanocyte development, leading melanoblasts to express Kit.

MATERIALS AND METHODS

Mice

C57BL/6 mice obtained from Japan SLC Co. Ltd (Hamamatsu, Japan) were used at 9.5 days post-coitum and were mated in our laboratory; the midnight before the presence of a vaginal plug was termed E0. This study was approved by the Animal Care and Use Committee of St Marianna University, School of Medicine.

Cells and culture conditions

NCCmelb4 cells were grown in a humidified atmosphere of 5% CO_2 and 95% air at 37°C in Eagle's modified minimal essential medium supplemented with 5% fetal bovine serum and 50 ng ml^{-1} recombinant mouse KL (Kirin Brewery, Tokyo, Japan). NCCmelb4M5 cells were cultured in Eagle's modified minimal essential medium/5% fetal bovine serum without KL. The culture medium was changed every 3 days, and cells were subcultured when they became confluent. Various concentrations of BMP-4 and noggin (R&D Systems Inc., Minneapolis, MN) were added to some of the cultures as noted in the text.

NC cell culture

NC cell cultures were established as described by Ito and Takeuchi (1984). Trunk regions posterior to the forelimb buds were dissected from E9.5 embryos using tungsten needles. The trunks were individually treated with 1% trypsin (1:250; Difco, Detroit, MI) in Tyrode's solution for 20 minutes at 4°C. Trypsinization was terminated by washing with Tyrode's solution containing 10% fetal bovine serum. The tissues were gently pipetted using small-pore Pasteur pipettes to separate the neural tubes from other components of the trunk. Eagle's modified minimal essential medium containing 15% fetal bovine serum and 50 ng ml^{-1} soluble KL was used as the basic medium. Neural tubes were explanted individually into 12-well plates (Falcon, Oxnard, CA) with 1 ml of medium per well. One half of the volume of culture medium in each well was changed every 3 days.

Immunostaining

The mAb to mouse Kit (ACK2), which was generated as described previously (Nishikawa *et al.*, 1991), was a generous gift from Dr Nishikawa (Riken Center for Developmental Biology, Kobe, Japan). The cells were fixed with 95% ethanol for 1 minute (for polyclonal antibodies) or for 1.5 minutes (for mAbs) at room temperature (RT). The cells were then treated with normal goat serum (KPL, Gaithersburg, MD) for 15 minutes at RT. For Kit staining, cells were treated with ACK2 (20 μg ml^{-1}) for 1 hour at RT. Color was developed using the new Fuchsin Substrate System (DAKO Corp, Carpinteria, CA), and a levamisole solution was used as an inhibitor of internal alkaline phosphatase.

Western blotting

NCCmelb4M5 and NCCmelb4 cells were cultured in sixwell plates at a density of 5×10^4 cells well^{1}. BMP-4 and noggin was added to the medium and cells were cultured for 24, 48, or 72 hours. Cell extracts were collected by scraping and centrifugation. Cells were extracted at 4°C for 2 hours. Samples were placed on ice and analyzed for protein concentrations using the DC Protein Assay (Bio-Rad Laboratories, Tokyo, Japan). Aliquots of proteins, 20 μg each, were separated on Tris-glycine gels (Bio-Rad Ready Gels J; Bio-Rad Laboratories). After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (Immobilon-p; Millipore, Bedford, MA) and incubated with antibodies to BMP-4, BMPRIA, BMPRIB, BMPRII, Smad 1/5/8 (Santa Cruz Biotechnology, Santa Cruz, CA), p-Smad 1/5/8 (Cell Signaling Technology, Beverly, MA) overnight at RT. Horseradish peroxidase donkey anti-rabbit IgG, anti-mouse IgG, or anti-goat IgG (Amersham Biosciences, Piscataway, NJ) was used as a secondary antibody. The antibody to mouse β-actin was obtained from Abcam Limited (Cambridgeshire, UK). Visualization of immunocomplexes was performed by enhanced chemiluminescence using the ECL kit (Amersham Pharmacia Biotech, Dübendorf, Switzerland), followed by exposure to Hyperfilm ECL (Amersham Biosciences). To detect Kit expression, the cell pellets were treated with a solubilization buffer, and the cell extract was obtained following centrifugation at 1,000 r.p.m. for 10 minutes. Aliquots of samples were separated on 10% SDS-polyacrylamide gels. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (Immobilon-p) and incubated with an anti-Kit mAb (ACK2) overnight at RT. Alkaline phosphatase-labeled anti-rat IgG (Southern Biotechnology Associates, Birmingham, LA)

was used as a secondary antibody. Immunoreactive proteins were detected using nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate disodium salt (Sigma Chemical Co, St Louis, MO).

Real-time PCR

One microgram of total RNA was reverse transcribed into cDNA using the first Strand cDNA Synthesis Kit for RT-PCR (Roche Diagnostics Co., Indianapolis, IN) following the manufacturer's instructions. Target genes were amplified with each specific primer and SYBR Premix Ex Tag (Perfect Real Time; Takara Bio Inc., Ohtsu, Japan) using a LightCycler (Roche Diagnostics Co.). PCR conditions were 10 minutes at 95°C, followed by 40 cycles at 95°C for 10 seconds, at 55°C for 10 seconds (GAPDH and Kit), and at 72°C for 14 (GAPDH) or 21 seconds (Kit). Sequences used were as follows: Kit sense primer: 5′-CGACTGCCCGTGAAGTGGA-3′; Kit antisense primer: 5′-GCCAGAAGGACGGGGTCGG-3′; GAPDH sense primer: 5′-YGCCTGCTTCACCACCTTC-3′; GAPDH antisense primer: 5′-TG CMTCCTGCACCACCAACT-3′; LightCycler Software Ver. 3.5 was used to extract the PCR data, which were then exported to Excel (Microsoft, Redmond, WA) for further analysis.

Cloning of mouse Kit gene promoter

The 670 bp mouse Kit gene promoter DNA sequence corresponding to nucleotides −625 to +45 was amplified by PCR from mouse genomic DNA template. The nucleotides are numbered based on the sequence of the mouse Kit gene promoter published previously (Tsujimura *et al.*, 1996). The PCR primers are 5′-GATGGTACC CAGCTGTATTCTTAC-3′ (forward) and 5′-CTCTCCTCGAGTCTCA GATC-3′ (reverse). The Kit promoter nucleotide sequence was confirmed by DNA sequencing and cloned into pGL4 *luc*2 basic luciferase vector (Promega Corp., Madison, WI) to generate reporter plasmid.

Transient transfection and luciferase reporter assay

Lipofection was carried out using *Trans*IT®-LT1 Transfection Reagent (Mirus, Madison, WI) according to the manufacturer's protocol. Cells were seeded at 6×10^4 cells well^{1} (NCCmelb4M5) in sixwell plates 24 hours before transfection. The medium was then replaced with fresh culture medium with or without 100 ng ml^{-1} BMP-4, and cells were harvested 48 hours after transfection for luciferase assay. Luciferase activity was measured sequentially from a single-cell lysate on a Lumicounter 700 (Microtec nition, Chiba, Japan) using the dual luciferase reporter assay system (Promega Corp.).

Statistical analysis

Statistically significant differences were evaluated by the Mann–Whitney *U*-test. The level of significance was set at $P<0.05$ in all cases. All experiments were repeated at least four times.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We sincerely thank Dr Hearing (NIH, Bethesda, MD) for providing us with the antibodies for Tyrp1, Dct, and tyrosinase. We also sincerely thank Dr Nishikawa (Riken Center for Developmental Biology, Kobe, Japan) for providing us with the antibody for Kit (ACK2). We also sincerely thank KIRIN Co. Ltd (Tokyo, Japan) for providing KL. This work was supported by grants from the Scientific Research Fund of the Ministry of Education, Science, Sports and Culture, Japan (Grant-in-Aid for Scientific Research, Grant nos. 16591211 and 18591261).

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