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High-Level Activation of Cyclic AMP Signaling Attenuates Bone Morphogenetic Protein 2-Induced Sympathoadrenal Lineage Development and Promotes Melanogenesis in Neural Crest Cultures

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The intensity of cyclic AMP (cAMP) signaling is a differential instructive signal in neural crest (NC) cell specification. By an unknown mechanism, sympathoadrenal lineage specification is suppressed by high-level activation of cAMP signaling. In NC cultures, high-level activation of cAMP signaling mediates protein kinase A (PKA)-dependent Rap1-B-Raf-ERK1/2 activation, leading to cytoplasmic accumulation of phospho-Smad1, thus terminating bone morphogenetic protein 2 (BMP2)-induced sympathoadrenal cell development. Concurrently, cAMP signaling induces transcription of the melanocyte-determining transcription factor Mitf and melanogenesis. dnACREB and E1A inhibit Mitf expression and melanogenesis, supporting the notion that CREB activation is necessary for melanogenesis. However, constitutively active $CREB_{DIEDML}$ without PKA activation is insufficient for Mitf expression and melanogenesis, indicating PKA regulates additional aspects of Mitf transcription. Thus, high-level activation of cAMP signaling plays a dual role in NC cell differentiation: attenuation of BMP2-induced sympathoadrenal cell development and induction of melanogenesis. We conclude the intensity of activation of signal transduction cascades determines cell lineage segregation mechanisms.

A central question in developmental biology is how pluripotent cells differentiate to diverse cell types. It is well accepted that gradients of microenvironmental signals are determinants in cellular differentiation (4, 24). However, the molecular mechanism(s) by which pluripotent cells recognize these gradients of microenvironmental signals and transduce this information to the nucleus to activate specific gene expression and cell fate determination is incompletely understood.

Neural crest (NC) cells are ideal for the study of cell lineage segregation mechanisms, since they differentiate to both neuronal cell types, i.e., sympathoadrenal (SA) and sensory neurons, and nonneuronal cell types, i.e., melanocytes and glia (39). NC cells are a transient, embryonic, stem-cell-like population located at the crest of the closing neural tube which migrate along defined routes within the developing embryo, giving rise to diverse cell types (5, 11, 22, 38). Under the instructive influence of microenvironmental signals secreted by tissues in proximity to their migratory path, NC cells from the trunk region of the neural tube differentiate to sympathetic and sensory neurons, adrenal medullary cells, glia, and melanocytes (28).

Extracellular signaling molecules that have been implicated to date in NC lineage segregation include the Wnt proteins (19, 20, 35), bone morphogenetic proteins (BMP2, -4, -7) (53, 65, 66, 67), transforming growth factors β1 to β3 (59), and glial growth factor 2 (60). Known mechanistic aspects of NC lineage segregation include the instructive role of BMPs in SA lineage development (56, 59) in mediating transcriptional induction of proneural transcription factors Ash1 and phox2a/2b (43, 61). Neuregulin2 (glial growth factor 2) also acts instructively in glial differentiation of rat neural crest stem cells (NCSCs) by suppressing neuronal differentiation and Ash1 expression (60) by a mechanism not yet understood. Regarding melanocyte development (reviewed in reference 27), the melanocyte determination transcription factor Mitf (51, 63) is transcriptionally regulated by Sox10 (52), Pax3 (35, 70), Lef1/TCF (64), and CREB (8). However, the extracellular instructive signal(s), the signaling cascade(s), and the transcription factor(s) initiating Mitf expression and melanogenesis in the developing embryo are unknown.

Many studies support the notion that cyclic AMP (cAMP) signaling is an important regulator of NC cell development, although the extracellular ligands activating cAMP signaling during NC cell development remain to be defined. Specifically, Maxwell and Forbes (48) reported that increased cAMP levels inhibited adrenergic neuron development while increasing the number of melanocytes. Lo et al. (42) have demonstrated that in murine NCSCs overexpression of Phox2a requires activation of cAMP signaling for SA cell development. Recent studies (25) also reported that in avian NC cultures increasing cAMP levels result in the augmentation of the melanocyte population and the concomitant reduction of the neuronal population. Furthermore, in our earlier studies we have investigated how cAMP signaling integrates with BMP2 signaling in NC cell development. Specifically, we have demonstrated that low-level activation of cAMP signaling synergizes with BMP2 in SA (neuronal) cell development, whereas high-level activation of cAMP signaling suppresses BMP2-induced SA cell development (9). Accordingly, these in vitro studies suggest that gradients of microenvironmental signals activating the cAMP

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pathway to differing extents determine NC lineage segregation in vivo.

Since the level of cAMP pathway activation is an instructive but differential signal in NC cell differentiation (9), we investigated the molecular mechanism by which high-level activation of cAMP signaling interfaces with BMP2 signaling, suppressing BMP2-induced SA cell development. We demonstrate that high-level activation of cAMP signaling promotes protein kinase A (PKA)-dependent Rap1 and B-Raf activation and sustained ERK1/2 activation. In turn, activated ERK1/2 mediates the cytoplasmic localization of phosphorylated Smad1, the transcriptional effector of BMP2, as it was shown to occur in response to epidermal growth factor (EGF) (36). This cytoplasmic localization of pSmad1 terminates Ash1 transcription and suppresses SA cell development. Interestingly, under these conditions, a concurrent activation of CREB initiates Mitf transcription, thus promoting the development of the melanocytic lineage. We conclude that the cAMP signaling network plays a dual role in NC cell differentiation, antagonizing BMP2-induced SA cell development and concurrently promoting melanogenesis. Considering that BMPs are expressed by the surface ectoderm (41, 58) at the junction of the developing epidermis and the uncommitted NC cells, the mechanism we report herein identifies the cAMP signaling network as a likely, physiologically relevant, instructive mechanism in melanogenesis.

MATERIALS AND METHODS

Growth medium and reagents. Growth medium contained 75 ml of Dulbecco modified Eagle medium–Ham's F-12 medium (Life Technologies), 15 ml of heat-inactivated horse serum (HyClone), 10 ml of day 9 chicken embryo extract, 10 mg of gentamicin sulfate, 10 mg of kanamycin sulfate, 1 ml of 7.5% sodium bicarbonate, 1 ml of 0.2 M L-glutamine, and 1 ml of vitamin mix. Vitamin mix contained 0.05 mg/ml 6,7-dimethyl-5,6,7,8-tetrahydropterine, 5 mg/ml L-ascorbic acid, and 0.25 mg/ml oxidized glutathione, pH 6.0. rhFGF2 (Promega) and BMP2 (generously provided by Wyeth Laboratory) were reconstituted in growth medium. Forskolin (Biomol), 3-isobutyl-1-methylxanthine (IBMX; Sigma), PD98059 (Biomol), and H89 (Biomol) were reconstituted in dimethyl sulfoxide.

Primary cultures of trunk NC cells were prepared from Japanese quail (*Coturnix coturnix*) embryos, stages 12 to 13, as described by Bilodeau et al. (9). Neural tube explants were incubated in pancreatin (Gibco), washed in growth medium, and plated in dishes coated with Vitrogen 100 (Collagen Corporation). Following NC cell migration for 42 h, neural tubes were removed, and cells were washed with calcium- and magnesium-free phosphate-buffered saline (PBS), pH 7.4, and harvested in growth medium with trypsin-EDTA. For mass cultures, NC cells were seeded at a density of 320 cells/mm^2 into dishes treated with bovine fibronectin (40 mg/ml; Sigma). For clonal cultures, cells were seeded at a density of 300 cells/ml in 35-mm dishes treated with bovine fibronectin. Following cell attachment for 2 h, the seeding medium was replaced with 2 ml of growth medium. Cells were fed by an exchange of 1 ml of growth medium on day 3 after passage into secondary culture and every other day thereafter.

Immunoblot. NC cells were harvested in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM sodium orthovanadate) and sonicated on ice for 10 seconds, and lysates were clarified by centrifugation. Cell lysates (20 μg) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane. Primary antibodies included monoclonal anti-TH (Developmental Studies Hybridoma Bank, University of Iowa); polyclonal anti-Tyrosinase M-19 (Santa Cruz); polyclonal anti-actin (Sigma); monoclonal anti-SMA, clone 1A4 (Sigma); monoclonal anti-GFAP (BD Biosciences); antiactive MAPK pAb (Promega); polyclonal anti-ERK1 K-23 (Santa Cruz); polyclonal anti-phospho-Smad1 (Ser463/465) (Upstate Biotechnology); and polyclonal anti-Smad1 T-20 (Santa Cruz). Detection was done with 1:5,000, 1:2,000, and 1:1,000 dilutions of horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (Jackson Laboratory), goat anti-rabbit IgG (Vector), and donkey anti-goat IgG (Santa Cruz), respectively, using the Amersham ECL (enhanced chemiluminescence) detection system.

Immunofluorescence. For pSmad1 localization, NC cells were grown in Lab-Tek chamber slides coated with bovine fibronectin. Serum-deprived (1 h) NC cells were preincubated for 10 min at 37°C with 20 μM PD98059 where indicated, followed by the addition of 100 μM forskolin or 100 μM IBMX for 10 min, followed in turn by the addition of 10 ng/ml BMP-2 and the continued incubation of all indicated reagents for an additional 30 min at 37°C. Cells were washed with PBS, fixed for 20 min in 4% paraformaldehyde, permeabilized in PBS containing 0.5% Triton X-100 for 10 min, and blocked for 1 h with 5% goat serum in PBS. Polyclonal phospho-Smad1 (Ser463/465) antibody (Upstate Biotechnology) diluted to a ratio of 1:500 in 3% goat serum was applied for 1 h. Following three washes in PBS, cells were incubated for 1 h with Alexa Fluor 488 goat anti-rabbit IgG (heavy plus light chains) (Molecular Probes) diluted to a ratio of 1:500 in 3% goat serum. Nuclei were stained with Hoechst. The slides were rinsed with distilled water and mounted using FluorSave reagent (Calbiochem) and a coverslip. Tyrosine hydroxylase (TH) staining of NC cells was performed as described by Bilodeau et al. (9).

Rap1 and Ras activation assays were performed employing pGEX-GST-(RalGDS)RBD (26) and pGEX-GST (Raf)RBD (17), respectively. The glutathione *S*-transferase (GST)-Rap1 binding domain (RBD) fusion protein was affinity purified on glutathione-Sepharose 4B beads. NC lysates (500 μg) were incubated with 5 μg of GST-RBD bound to glutathione-Sepharose 4B beads for 1 h at 4°C; the beads were washed three times with lysis buffer, and the GST-RBD bound fraction was fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane. A monoclonal Rap1 antibody (Transduction Laboratories) or a monoclonal Pan-Ras antibody (Calbiochem) was employed in Western blot analysis as described previously.

B-Raf kinase assay. NC lysates were prepared in RIPA buffer. Lysates (500 μg) were incubated with 2 μg B-Raf antibody (Serotec) for 1 h at 4°C; this incubation was followed by the addition of protein A/G agarose (Santa Cruz) and another incubation for 1 h at 4°C. Immunoprecipitates were washed three times with RIPA and once with kinase buffer (20 mM MOPS [morpholinepropanesulfonic acid], pH 7.2, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol). The reaction mixture was resuspended in 40 μl kinase buffer containing 25 mM $MgCl_2$, 100 μM ATP, 0.4 μg inactive MEK1 (Upstate Biotechnology), and 1 μg inactive Erk2 (Upstate Biotechnology) and incubated for 30 min at 30°C. Following centrifugation, 10 μl of supernatant was mixed with 30 μl kinase buffer containing 25 mM $MgCl_2$, 100 μM ATP, 10 μCi [γ-^{32}P]ATP (6,000 Ci/mmol; NEN), and 20 μg myelin basic protein (Upstate Biotechnology) and incubated for 10 min at 30°C. Reaction mixture (25 μl) was spotted onto P81 paper and washed three times with phosphoric acid and once with acetone; the activity was measured by scintillation counting.

Real-time PCR was performed as described in the work of Lee et al. (40) with 18S rRNA as an internal control. RNA was isolated from NC cultures using TRIZOL reagent (Life Technologies). Total RNA (2 μg) was treated with DNase I for 15 min and used for cDNA synthesis. Real-time PCRs were performed in a 20-μl reaction mixture containing 0.1 to 1 μg cDNA template, 1X SYBR green buffer (Applied Biosystems), and 1 μM of each primer. PCR conditions were as follows: 50°C for 2 min, 94°C for 10 min, and 40 cycles at 94°C for 15 sec and 60°C for 1 min. For Ash1, the forward primer was 5′-AAGAAG ATGAGCAAAGTGG-3′ and the reverse primer was 5′-GAGGAGTAGGAG GAGACG-3′. For Mitf, the forward primer was 5′-TTGATACCCAAGTCAA ATGACC-3′ and the reverse primer was 5′-GCGTTGCTGTTCTCTTTGC-3′.

RCAS virus infection. RCAS BP (A) vectors encoding CREB variants and E1A were transfected in chicken embryo fibroblast cells by the calcium phosphate method. Following transfection at 7 to 10 days, supernatants were collected and concentrated by ultracentrifugation (32, 49). Viral titers were determined by immunostaining for p19 gag protein with an ABC kit (Vector Laboratories). Typical titers for the four RCAS viral constructs were 10^9 IU/ml. NC cultures were infected at day 0 of the secondary culture with 10^6 IU per 10^5 cells.

RESULTS

High-level activation of cAMP signaling promotes cytoplasmic localization of pSmad. Our previous studies have demonstrated that low-level activation of cAMP signaling by the addition of IBMX or 1 μM forskolin, determined by measuring PKA activity, synergizes with BMP2 in SA cell development; by

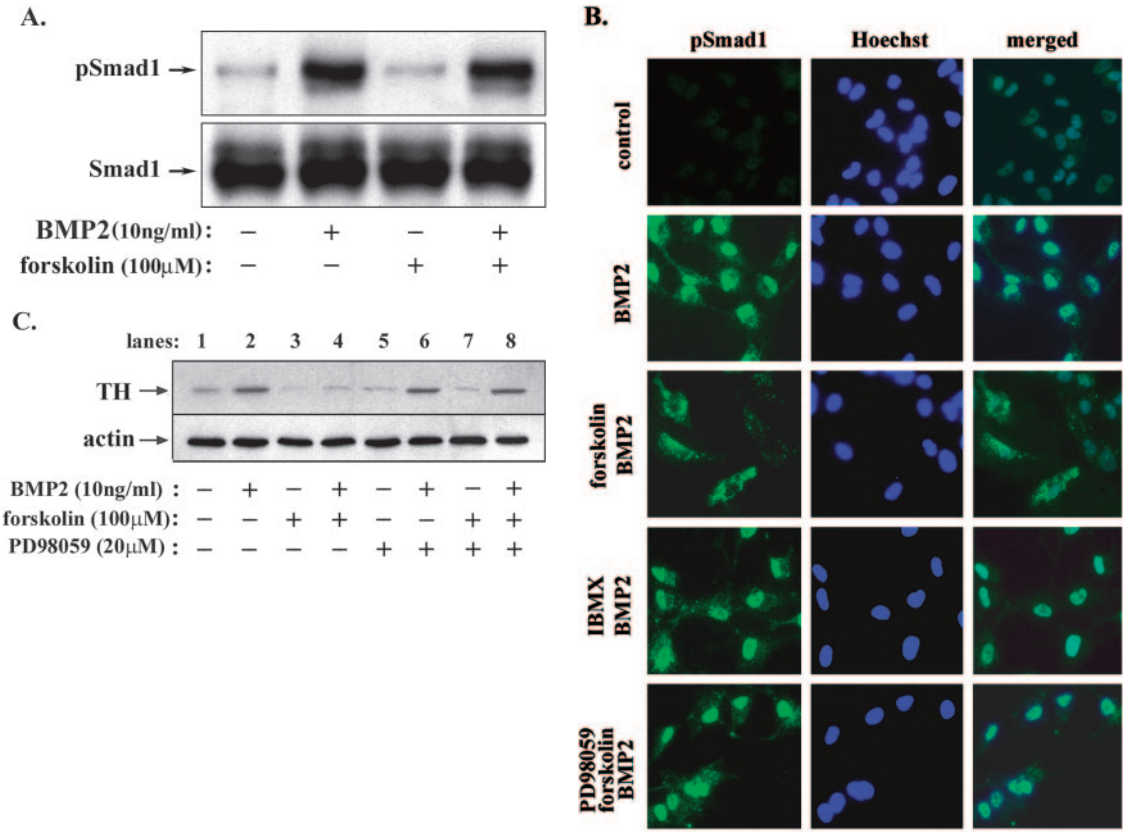

FIG. 1. High-level activation of cAMP signaling regulates pSmad1 subcellular localization. (A) Western blot analysis of pSmad1 in NC cells treated with 100 μM forskolin and 10 ng/ml BMP2. (B) Immunofluorescence microscopy of pSmad1 in NC cells as a function of 100 μM forskolin, 100 μM IBMX, 10 ng/ml BMP2, and 20 μM PD98059 treatment as indicated. Serum-deprived (1 h) NC cells were pretreated for 10 min with PD98059 where indicated, which was followed by a 10-min treatment with forskolin or IBMX followed in turn by the addition of BMP2 and the continued incubation of the indicated reagents for 30 min. Nuclei were visualized by staining with Hoechst. (C) Western blot analysis of TH expression in 5-day NC cultures treated with (+) or without (−) BMP2, forskolin, and PD98059 as indicated.

contrast, high-level activation of cAMP signaling by the addition of 100 μM forskolin or the nonhydrolyzable cAMP analog 8-Br-cAMP, determined by measuring PKA activity, suppresses BMP2-induced SA cell development (9). The molecular mechanism by which high-level activation of cAMP signaling exerts this antagonistic effect on BMP2 signaling is unknown. Accordingly, we investigated whether the BMP2 signal transduction pathway is regulated by cAMP signaling. BMPs act by binding to serine/threonine kinase receptors which phosphorylate the Smad family of transcription factors (46, 47). Phosphorylated Smads translocate to the nucleus and control gene expression. Therefore, we first examined whether the receptor-mediated Smad1 phosphorylation is impaired by high-level activation of cAMP signaling. Immunoblot analyses for pSmad1 indicated that activation of cAMP signaling had no effect on BMP2-stimulated Smad1 phosphorylation (Fig. 1A). To determine whether the pSmad1 nuclear accumulation is affected by 100 μM forskolin, we performed immunostaining of pSmad1 in NC cells. The results demonstrate that although high-level activation of cAMP signaling did not inhibit BMP2-induced Smad1 phosphorylation, it altered pSmad1 nuclear localization, resulting in its accumulation in the cytoplasm (Fig. 1B). By comparison, low-level activation of cAMP signaling (100 μM IBMX) did not alter the nuclear localization of pSmad1. These results suggest that in NC cells, 100 μM forskolin antagonizes BMP2 signaling by promoting pSmad1 cytoplasmic accumulation, thus inhibiting BMP2-induced SA cell development.

Earlier studies (36, 37) demonstrated that growth factor-mediated ERK1/2 activation attenuates agonist-induced nuclear accumulation of phosphorylated Smad1, -2, and -3. Furthermore, Vossler et al. (68) demonstrated with PC12 cells that cAMP signaling is a network involving not only PKA and CREB activation but also activation of ERK1/2. Accordingly, we investigated the involvement of ERK1/2 in cAMP regulation of pSmad1 nuclear accumulation. Addition of the MEK1 inhibitor PD98059 rescued the inhibition of pSmad1 nuclear accumulation by forskolin (Fig. 1B). Importantly, the addition of PD98059 to NC cultures rescued BMP2-induced SA development from the forskolin-mediated antagonism (Fig. 1C, compare lanes 2, 4, and 8) examined by monitoring TH protein expression. These observations suggest that high-level activation of cAMP signaling antagonizes BMP2 signaling and SA cell development via ERK1/2 activation.

High-level activation of cAMP signaling activates ERK1/2 in NC cells. The activation of ERK1/2 by 100 μM forskolin was assessed by immunoblotting for phospho-ERK1/2. High-level activation of cAMP signaling activated ERK1/2 in a sustained

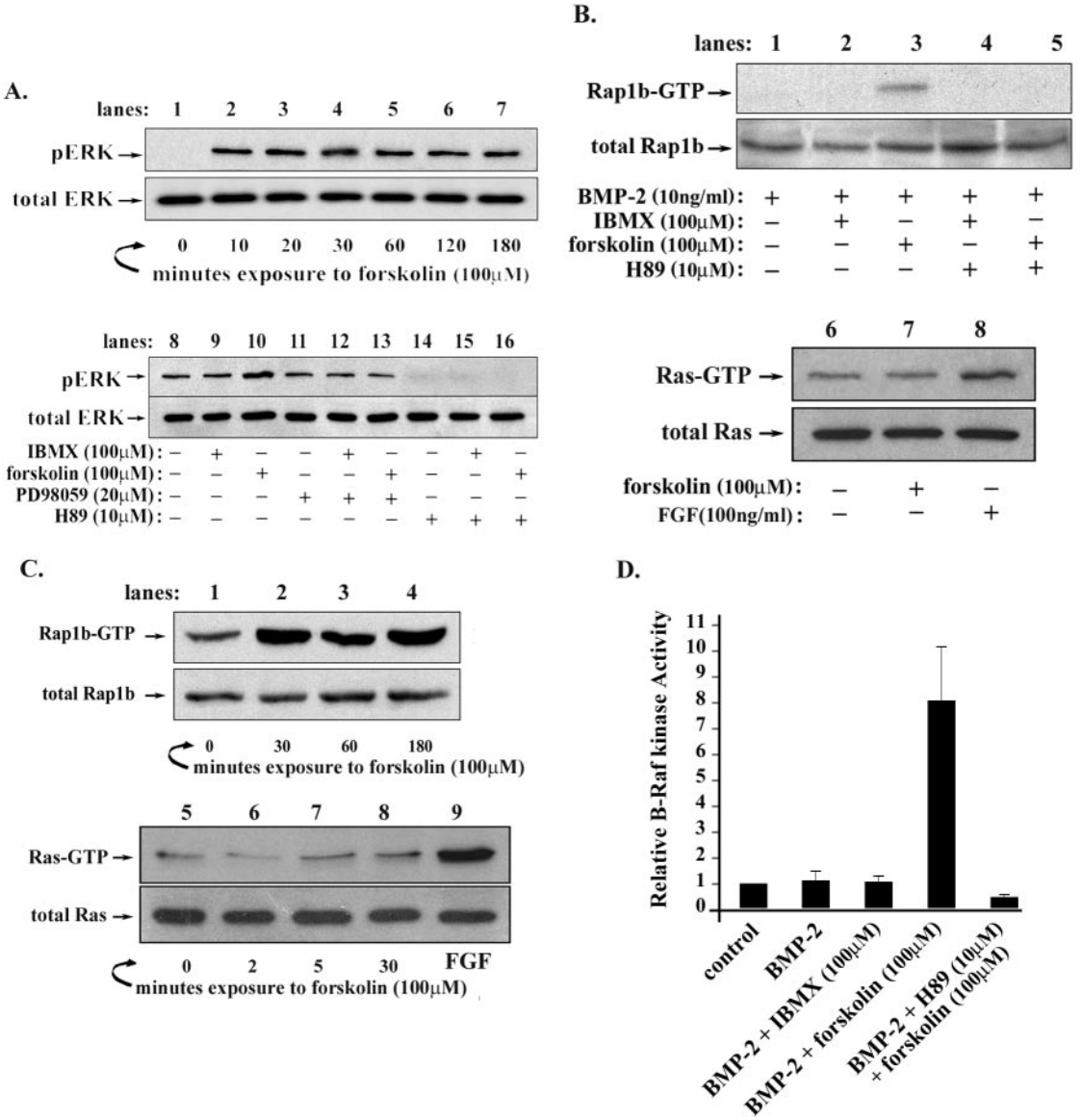

FIG. 2. High-level activation of cAMP signaling activates Rap1-B-Raf-ERK. (A) Upper panel, Western blot analysis monitoring phospho-ERK in extracts isolated from NC cultures treated with forskolin in an indicated time course. Lower panel, Western blot analysis of phospho-ERK using NC extracts treated with (+) or without (−) forskolin, IBMX, PD98059, or H89, as indicated. (B) Rap1 (upper panel) and Ras (lower panel) activation assays with lysates isolated from NC cultures treated with forskolin, IBMX, BMP2, H89, or FGF, as indicated. Lysates (500 μg) were bound to 5 μg GST-RBD coupled to glutathione-Sepharose 4B (17, 26). Active Rap1 (upper panel) or Ras (lower panel) obtained from the GST-RDB-bound fraction were detected by Western blot analysis. (C) Activation assay of Rap1 (upper panel) or Ras (lower panel) with NC lysates derived following an indicated time course of forskolin treatment. (D) In vitro B-Raf immunocomplex kinase assay, using NC cell lysates (500 μg) treated as indicated in a reaction mixture containing inactive MEK1 and ERK2 (Upstate Biotechnology) followed by the addition of myelin basic protein as its substrate and [γ-^{32}P]ATP. Activity was measured by scintillation counting. Data represent three independent experiments.

manner, lasting at least 3 h (Fig. 2A, lanes 1 to 7). This forskolin-mediated ERK1/2 activation is inhibited by the addition of the PKA inhibitor H89 (Fig. 2A, compare lanes 8 and 14 and 10 and 16), whereas low-level activation of cAMP signaling by 100 μM IBMX addition does not activate ERK1/2 (Fig. 2A, lanes 8 and 9).

To investigate the mechanism by which 100 μM forskolin induces ERK1/2 activation in NC cells, we initially examined the activation of Rap1b, according to the methods of previous studies with PC12 cells (68). The GTP-bound, activated form of Rap1 has higher affinity for the RBD of RalGDS than the GDP-bound, inactive form does (10). Thus, Rap1 activation in NC cells was determined by the GST-RBD protein-protein interaction assay followed by Rap1 immunoblot analysis (26). Activated Rap1b was not detected with the low level of activation of the cAMP pathway mediated by the addition of 100 μM IBMX (Fig. 2B, lanes 1 and 2). By contrast, 100 μM forskolin activated Rap1b in a PKA-dependent manner, based on the inhibition of Rap1b activation by the PKA-specific inhibitor H89 (Fig. 2B, lanes 1, 3, and 5). Importantly, this Rap1

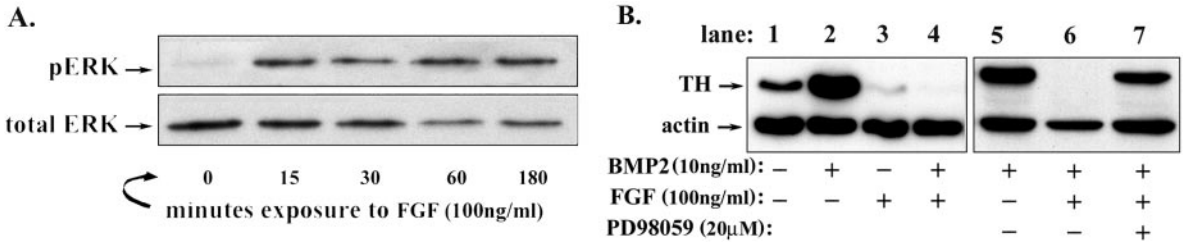

FIG. 3. FGF suppresses sympathoadrenal cell development in NC cultures. (A) Western blot analysis of NC lysates for phospho-ERK in an indicated time course following FGF (100 ng/ml) treatment. (B) Western blot analysis for TH protein expression in 5-day NC cultures with (+) or without (−) FGF, BMP2, and PD98059, as indicated.

activation was sustained, lasting for at least 3 h following forskolin stimulation (Fig. 2C, lanes 1 to 4).

In B16 melanoma cells and human melanocytes (13), cAMP activates ERK1/2 via Ras-dependent B-Raf activation. Accordingly, we examined whether high-level activation of cAMP signaling also mediates in NC cells Ras-dependent ERK1/2 activation. Employing the GST-(Raf) RBD assay (17) for detecting the GTP-bound active form of Ras (Fig. 2B, lanes 6 to 8), we demonstrated that 100 μM forskolin does not activate Ras. Likewise, a similar assay performed following a time course of forskolin stimulation did not result in Ras activation in NC cells (Fig. 2C, lanes 5 to 9).

To determine whether Rap1b activation by 100 μM forskolin leads to B-Raf activation, we performed in vitro kinase assays as described in Materials and Methods. As with Rap1b activation, B-Raf was activated only by 100 μM forskolin in a PKA-dependent manner and not by 100 μM IBMX (Fig. 2D), supporting the notion that high-level activation of cAMP signaling exerts distinct effects in NC cells.

FGF antagonizes BMP2-induced SA cell development via ERK1/2 activation. In PC12 cells, fibroblast growth factor (FGF) leads to sustained Rap1 activation and sustained ERK1/2 activation (71). Immunoblots indicated that FGF addition to NC cultures mediated sustained ERK1/2 activation (Fig. 3A). Thus, to further confirm our observations that ERK1/2 activation antagonizes BMP2-induced SA cell development, we investigated whether FGF exerts a similar effect on NC cell development. Indeed, FGF addition to NC cultures interfered with SA cell development, as monitored by TH protein expression (Fig. 3B, lanes 1 to 4). BMP2-induced SA cell development was rescued from FGF inhibition by the addition of PD98059 (Fig. 3B, lanes 5 to 7). These results support the notion that FGF inhibition of SA cell development is mediated by ERK1/2 activation and corroborate the inhibitory effects of ERK1/2 activation mediated by high-level activation of cAMP signaling (Fig. 1C).

High-level activation of cAMP signaling promotes melanogenesis in NC cultures. It is possible that cAMP signaling and its integration with BMP2 signaling regulate the differentiation of other NC cell lineages besides SA cells. Therefore, we examined the effect of 100 μM forskolin and BMP2 in regulating the differentiation of various NC derivatives. Specifically, we monitored the appearance of cellular markers for cells of the SA lineage, glial cells, smooth muscle cells, and melanocytes by assessing the relative expressions of TH, glial fibrillary acidic protein, smooth muscle actin, and tyrosinase, respectively.

Immunoblots of these cellular markers indicated that 100 μM forskolin increased the expression of the melanocytic marker tyrosinase (Fig. 4A, left panel), suggesting that cAMP signaling promotes the development of melanocytes. When NC cells were exposed to both 100 μM forskolin and BMP2, the expression of tyrosinase was induced, whereas the expression of TH remained at the unstimulated level, suggesting that the cAMP signal is dominant over BMP2. The expression of glial fibrillary acidic protein and smooth muscle actin was not influenced by BMP2, forskolin, or their combination (Fig. 4A, left panel).

To assess whether gradients of cAMP signaling differentially regulate the expression of TH versus that of tyrosinase, NC cultures were treated either with 100 μM IBMX, effecting low activation of cAMP signaling, or with increasing concentrations of forskolin (1 to 100 μM), as a function of BMP2 addition. Increased activation of cAMP signaling with or without BMP2 progressively increases tyrosinase expression (Fig. 4A, right panel). By comparison, low-level activation of cAMP signaling synergizes with BMP2 and increases TH expression, while forskolin concentrations ranging from 10 to 100 μM inhibit TH expression (Fig. 4A, right panel). These results agree with our earlier observations (9) and demonstrate that high-level activation of cAMP signaling is dominant relative to BMP2 signaling.

To confirm further these findings, we examined the expression of transcription factors Ash1 (61) and Mitf (27, 63), which regulate the lineage determinations of SA cells and melanocytes, respectively. Using real-time PCR, we quantified the effect of BMP2 and cAMP signaling on Ash1 and Mitf expression. In agreement with earlier studies (59), treatment of NC cultures with BMP2 induced Ash1 expression, whereas forskolin (100 μM) addition, with or without BMP2, suppressed Ash1 expression (Fig. 4B). By contrast, forskolin (100 μM) addition with or without BMP2 induced Mitf expression (Fig. 4C). Since Ash1 and Mitf are cell-lineage-determining factors, these results suggest that high-level activation of cAMP signaling regulates the early lineage determination steps involved in NC lineage segregation to SA cells and melanocytes. Importantly, the dominant effect of high-level activation of cAMP signaling relative to BMP2, observed during NC cell differentiation in vitro (Fig. 4A), regulates inversely the expression of Mitf and Ash1 in favor of melanogenesis (Fig. 4B and C).

High-level activation of cAMP signaling is instructive for melanogenesis. The effect of microenvironmental signals on NC cell differentiation is either selective, by influencing cell

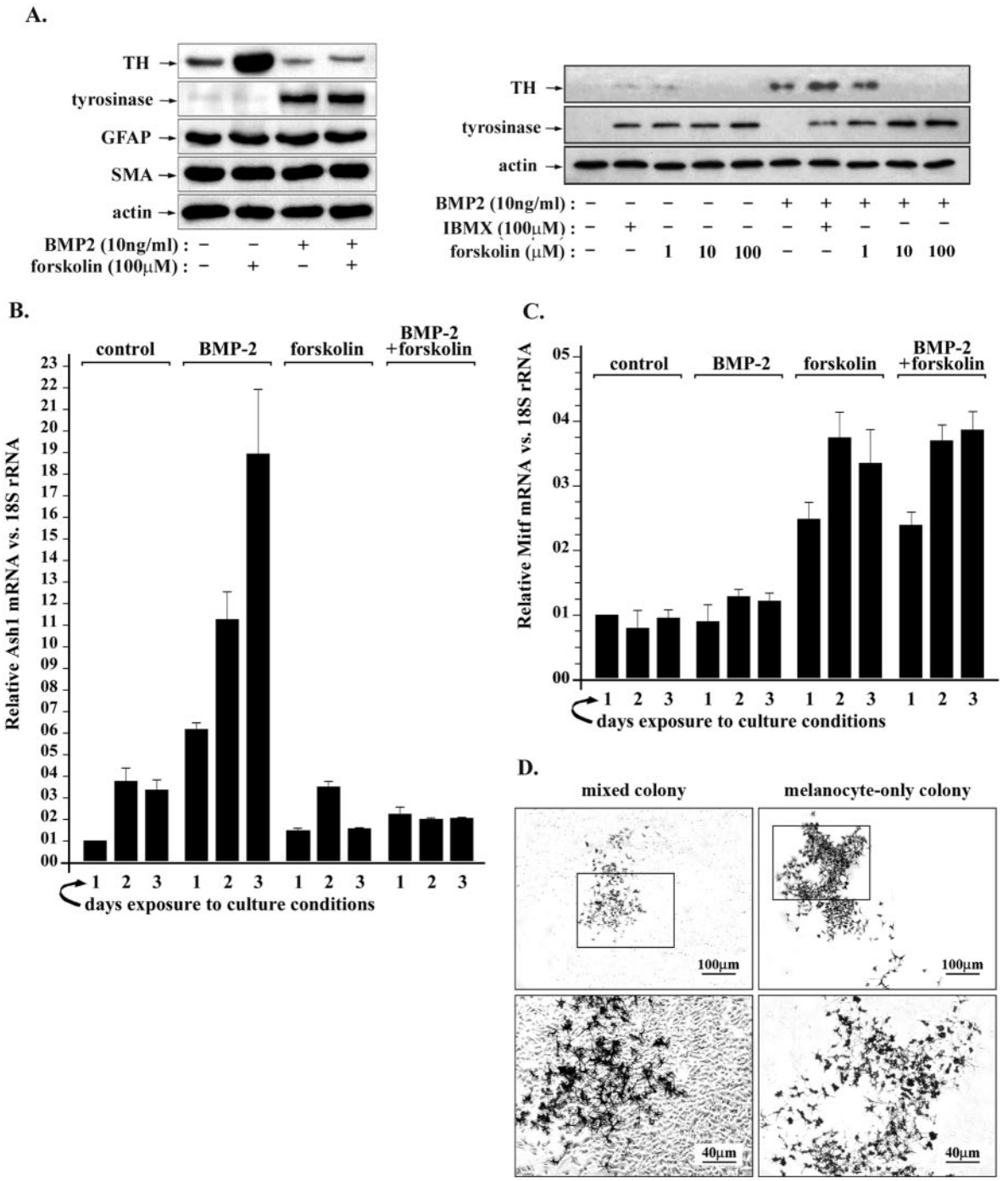

FIG. 4. High-level activation of cAMP signaling promotes melanogenesis. (A) Left panel, Western blot analyses of lysates from NC cultures grown for 5 days with (+) or without (−) forskolin and BMP2 as indicated. GFAP, glial fibrillary acidic protein; SMA, smooth muscle actin. Actin is the internal control. A representative experiment from three independent assays is shown. Right panel, Western blot analysis monitoring TH or tyrosinase expression in extracts isolated from NC cultures treated with (+) or without (−) forskolin (1 to 100 μM), IBMX (100 μM), or BMP2 (10 ng/ml) as indicated. (B and C) Quantifications of Ash1 and Mitf mRNA, respectively, employing real-time PCR with total RNA isolated at days 1 to 3 from NC secondary cultures grown as a function of 100 μM forskolin and/or BMP2 (10 ng/ml) addition, as indicated. Quantification is from three independent RNA preparations, expressed relative to 18S RNA as an internal control. Each RNA preparation was analyzed by real-time PCR employing identical triplicates. (D) Melanocyte-containing colonies, mixed (left panel) and homogeneous/pure (right panel), derived from clonal NC cell assays (Table 1) grown for 12 days in the presence of 100 μM forskolin and BMP2 (10 ng/ml).

TABLE 1. Effect of BMP2, forskolin, and FGF on NC cell growth[a]

Treatment	Colonies			
	Total no. (mean ± SE)	% TH positive (mean ± SE)[b]	% Melanin positive (mean ± SE)[c]	% Pure melanocyte (mean ± SE)
Control	31.7 ± 2.3	0	70 ± 8	14 ± 4
BMP2	32.2 ± 1.9	15 ± 2	34 ± 4	4 ± 2
Forskolin	31.0 ± 2.0	0	100	83 ± 4
BMP2 + forskolin	31.5 ± 1.8	0	100	76 ± 6
FGF	31.8 ± 1.6	0	13 ± 2	0
BMP2 + FGF	32.0 ± 2.2	0	9 ± 3	0

[a] NC cells were plated at clonal density, as described in Materials and Methods, and grown for 12 days in the presence of BMP2, forskolin (100 μM), or FGF, as indicated. Data represent three independent experiments.
[b] TH-positive colonies were assessed by immunofluorescence microscopy.
[c] Melanin-positive colonies were identified via melanin/pigment production.

proliferation or survival of a committed progenitor, or instructive, by restricting the pluripotent nature of NC cells. Studies of NCSCs support the notion that the influence of BMP2 on SA cell differentiation is instructive (59). To gain further insight into how cAMP signaling influences melanogenesis, we studied NC differentiation in cultures plated at clonal density. TH-positive cells were visualized by immunofluorescence, whereas melanocytes were observed directly, due to their production of melanin.

The total numbers of colonies generated in the assay were similar in all treatment groups (Table 1). TH-positive colonies appeared only with BMP2 treatment. By comparison, melanocyte colonies were formed with all treatments. The melanocyte colonies are of two distinct types: (i) mixed colonies containing both melanocytes and nonmelanocytes and (ii) pure melanocyte colonies (Fig. 4D). The results demonstrate that while 100 μM forskolin addition suppressed TH-positive colony formation without an effect on NC cell survival, it promoted by 83% the formation of pure melanocytic colonies (Table 1). This is a striking result, since only 4% and 14% of the colonies are of pure melanocyte composition in the BMP2 and control groups, respectively. These results support the notion that high-level activation of cAMP signaling is an instructive signal in promoting the differentiation of pluripotent NC cells into melanocytes. Furthermore, pure melanocyte colonies were composed of fewer cells in comparison to the cell numbers in the mixed colonies, suggesting that high-level activation of cAMP signaling inhibits the self-renewal of the founder cells by inducing their differentiation to melanocytes. The dominant effect of 100 μM forskolin was also observed in our clonal assays performed with combined BMP2 and forskolin treatment (Table 1), consistent with the results from mass cultures (Fig. 4).

CREB activation is necessary for Mitf transcription in NC cultures. The transcription factors implicated in the regulation of the Mitf promoter, based on studies of melanoma cell lines, include Pax3, CREB, Sox10, and Lef1/TCF (reviewed in reference 27). However, it is unknown which of these transcription factors initiates Mitf transcription during melanocyte specification of NC cells in the developing embryo. Since high-level activation of cAMP signaling promotes the formation of more-than-80%-pure melanocyte colonies (Table 1) and CREB mediates the transcriptional response of the cAMP transduction pathway (6), we investigated whether CREB activation is causally linked to cAMP-induced Mitf expression and melanogenesis in NC cultures.

Immunoblots of phospho-CREB indicated that 100 μM forskolin treatment activated CREB in NC cells (Fig. 5A). The activation of CREB lasted for at least 2 h. In PC12 cells, ERK1/2 activation stimulates CREB phosphorylation via $pp90^{rsk}$ (72). Thus, we asked whether ERK1/2 activation is sufficient to stimulate CREB phosphorylation in NC cells and whether ERK1/2 activation is involved in forskolin-stimulated CREB phosphorylation in NC cells. Immunoblots of phospho-ERK1/2 and phospho-CREB indicated that while FGF treatment activated ERK1/2 in a sustained manner (Fig. 3A), it did not activate CREB in NC cells (Fig. 5A). Furthermore, employing the PKA inhibitor H89 and the MEK1 inhibitor PD98059, we demonstrated that the forskolin-stimulated CREB phosphorylation is PKA dependent but not ERK1/2 dependent (Fig. 5A).

To demonstrate the direct involvement of CREB in melanogenesis, we employed avian RCAS viral vectors (49) and introduced into NC cultures via viral infection the dnACREB variant (2), the CBP-interfering adenovirus E1A (7, 44), and the constitutively active $CREB_{DIEDML}$ (14). To confirm that the RCAS virus-expressed $CREB_{DIEDML}$, ACREB, and E1A are functional, cAMP-responsive element (CRE)-luciferase expression was assessed in transient transfection assays in virus-infected NC cell cultures (Fig. 5B). The results clearly demonstrate the constitutive activity of $CREB_{DIEDML}$ as described by Cardinaux et al. (14) and the dominant interfering activity of ACREB (2) and E1A (7, 44) on CRE-driven transcription.

Following the infection of secondary cultures at day 0, we determined Mitf transcription and tyrosinase protein levels. Real-time PCR of Mitf (Fig. 5C) and immunoblots of tyrosinase (Fig. 5D) indicated that both ACREB and E1A blocked forskolin-induced Mitf and tyrosinase expression, supporting the involvement of CREB and CBP, respectively, in cAMP-induced melanogenesis. However, $CREB_{DIEDML}$, although functional in mediating CRE-luciferase expression (Fig. 5B) without forskolin addition, i.e., without PKA activation, was insufficient in inducing the expression of either Mitf (Fig. 5C) or tyrosinase (Fig. 5D). PKA inhibitor H89 repressed forskolin-induced Mitf and tyrosinase expression even in the presence of $CREB_{DIEDML}$ (Fig. 5C and D), suggesting that besides

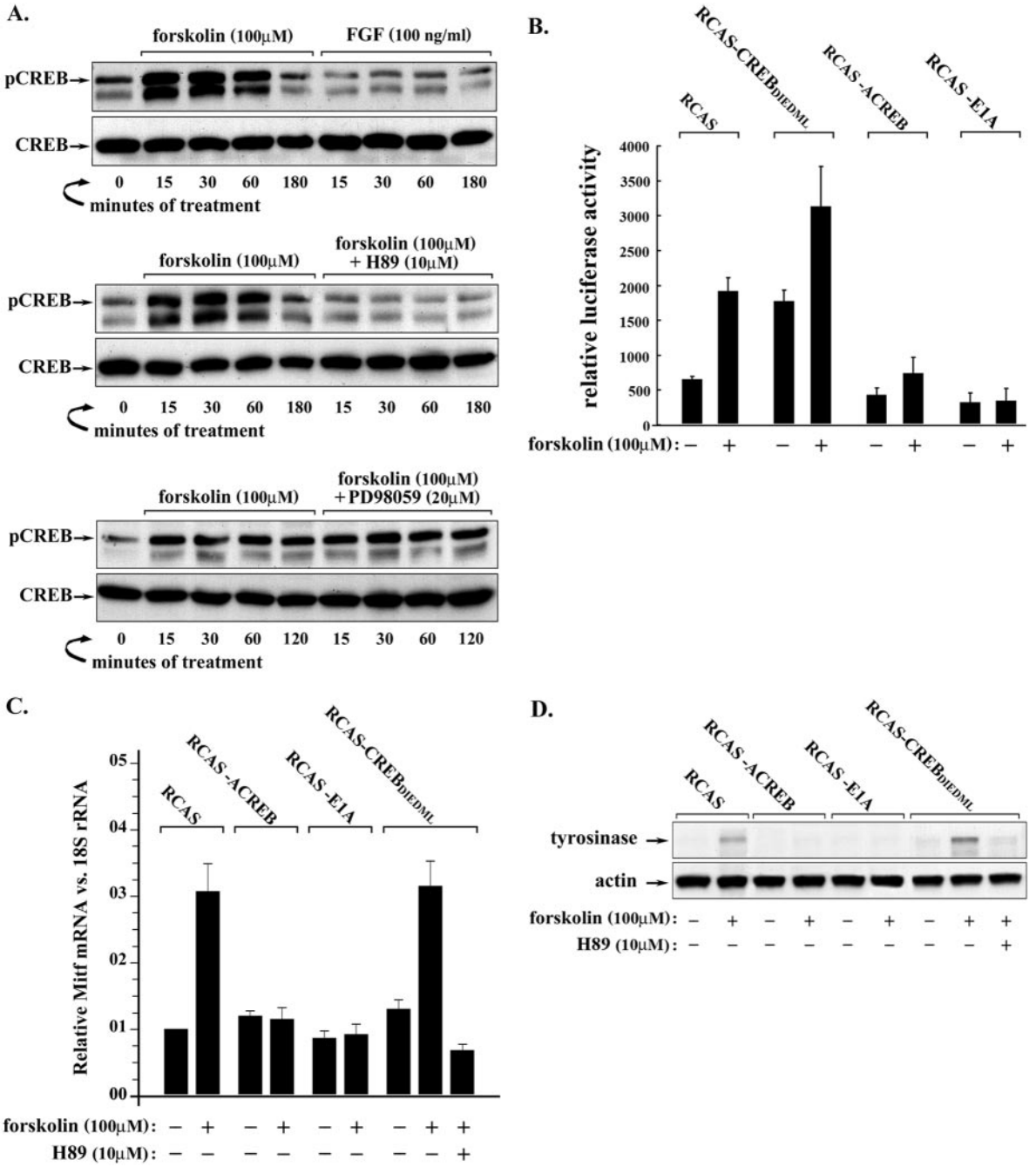

FIG. 5. CREB activation is necessary for melanogenesis. (A) Western blot analyses of phospho-CREB employing NC cell lysates derived from cultures treated with 100 μM forskolin or FGF (100 ng/ml) in the indicated time course with (+) PD98059 or H89. A representative assay is shown from three independent experiments. (B) NC cultures were infected at day 0 of secondary culture with RCAS-, RCAS-ACREB-, RCAS-E1A-, or RCAS-$CREB_{DIEDML}$-encoding viruses; 24 h after infection, cells were transiently transfected with 1 μg CRE-luciferase and 200 ng cytomegalovirus-β-gal reporters and grown with (+) or without (−) forskolin. Luciferase activity was quantified relative to β-gal expression, which was used as the transfection control. (C and D) Real-time PCR analyses of Mitf mRNA expression (panel C) and Western blot analyses of tyrosinase expression (panel D) in NC cultures infected with RCAS-, RCAS-ACREB-, RCAS-E1A-, or RCAS-$CREB_{DIEDML}$-encoding viruses, as indicated, with (+) or without (−) forskolin or H89 addition.

CREB phosphorylation, PKA activation regulates additional aspects of Mitf transcription and melanogenesis. We interpret these results to mean that CREB activation is necessary but not sufficient for melanogenesis and that PKA activation regulates additional aspects of Mitf transcription.

Both PKA and CREB activation are necessary in NC differentiation to melanocytes. Since high-level activation of cAMP signaling activates ERK1/2 via PKA-dependent Rap1-B-Raf activation (Fig. 2), we investigated whether the PKA dependence for the initiation of Mitf transcription and melanogen-

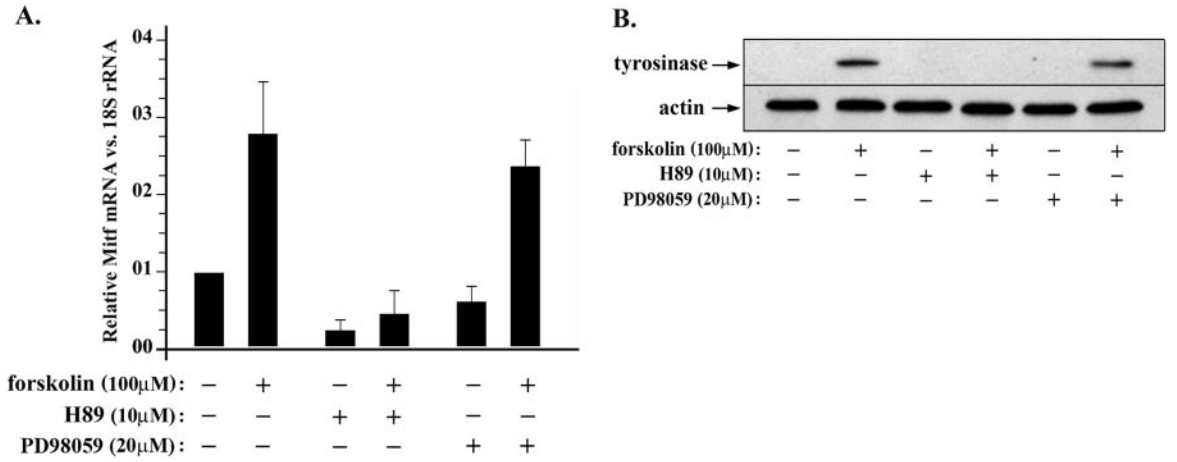

FIG. 6. PKA activation is required for melanogenesis. (A) Real-time PCR analysis of total RNA isolated from day 3 NC cultures treated with (+) or without (−) forskolin, H89, and PD98059, as indicated. (B) Western blot analysis of tyrosinase expression in day 5 NC cultures treated with (+) or without (−) forskolin, H89, and PD98059, as indicated.

esis (Fig. 5C and D) is mediated by ERK1/2. Following forskolin stimulation of NC cells, we demonstrated that both the transcription of Mitf (Fig. 6A) and the expression of tyrosinase (Fig. 6B) are inhibited by the PKA inhibitor H89 but not by the MEK1 inhibitor PD 98059. These results support the direct involvement of PKA in melanogenesis.

To exclude the possibility that the expression of the CREB variants and E1A affects NC survival, we examined their effect on clonal cultures. All treatment groups generated the same number of colonies (Table 2), supporting the notion that the expression of ACREB, $CREB_{DIEDML}$, and E1A do not affect the survival of the colony founder cells. Importantly, ACREB suppresses by 70% the formation of pure melanocyte colonies, E1A completely inhibits melanogenesis following forskolin (100 μM) treatment, and $CREB_{DIEDML}$ induces melanogenesis only with PKA activation (Table 2). These results identify PKA and CREB activation by high-level activation of cAMP signaling as necessary signaling components in melanogenesis.

FGF signaling does not promote melanogenesis in NC cultures. Although high-level activation of both cAMP signaling and FGF signaling antagonize BMP2-induced SA cell development, the clonal assays demonstrate that FGF signaling fails to promote melanogenesis (Table 1). This observation suggests that melanogenesis is not a default fate of NC cells when SA lineage differentiation is inhibited and that the suppression of SA lineage is not a secondary effect of melanogenesis. Thus, the cAMP signaling network plays a dual role in regulating NC cell differentiation, namely, the attenuation of BMP2-induced SA development via ERK1/2 activation and the promotion of melanogenesis via PKA and CREB activation (Fig. 7).

TABLE 2. Effect of CREB variants and E1A on NC cell growth[a]

Treatment	Total no. of colonies (mean ± SE)[a]	% Pure melanocyte colonies (mean ± SE)[b]
RCAS	33.0 ± 1.0	13 ± 2
RCAS + forskolin*	31.3 ± 2.3	78 ± 7
ACREB	33.3 ± 2.5	7 ± 3
ACREB + forskolin*	32.7 ± 0.6	22 ± 2
E1A	33.7 ± 1.2	0
E1A + forskolin*	32.7 ± 2.1	0
$CREB_{DIEDML}$	32.3 ± 2.5	17 ± 3
$CREB_{DIEDML}$ + forskolin*	31.3 ± 1.5	79 ± 2
$CREB_{DIEDML}$ + forskolin* + H89	32.7 ± 3.1	0

[a] Clonal NC cultures prepared as described in Materials and Methods were infected with the various RCAS viruses at day 0 of secondary culture. Total colonies and pure melanocyte colonies were quantified at day 12 of culture. Results are from three independent experiments.

[b] The asterisks indicate forskolin at 100 μM.

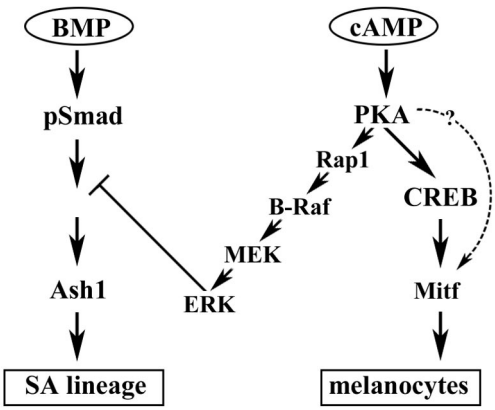

FIG. 7. The diagram shows the mechanism by which the cAMP signaling network suppresses sympathoadrenal cell development and promotes melanogenesis in NC cultures. High-level activation of cAMP signaling (9) activates ERK1/2 via PKA-mediated Rap1-B-Raf activation. In turn, activated ERK1/2 mediates cytoplasmic accumulation of phospho-Smad1, the termination of the BMP-2-induced Ash1 expression, and the suppression of SA cell development. Concurrent PKA-mediated CREB activation induces Mitf expression and melanogenesis. In addition to CREB activation, Mitf transcription requires activated PKA. The mechanism of the direct involvement of active PKA in Mitf transcription remains to be determined.

DISCUSSION

High-level cAMP signaling via PKA-dependent Rap1-B-Raf-ERK1/2 activation suppresses SA cell development. Herein, we have defined the molecular mechanism by which high-level activation of cAMP signaling suppresses SA lineage development and concomitantly induces melanogenesis. We demonstrate that the suppression of SA cell development by high-level activation of cAMP signaling involves the attenuation of BMP2 signaling and Ash1 expression by mediating the cytoplasmic accumulation of the BMP2-activated phospho-Smad1. The cytoplasmic localization of the activated phospho-Smad1 requires ERK1/2 activation, based on the reversal of this effect by the MEK1 inhibitor PD98059. This result agrees with the attenuation of BMP2 signaling in response to EGF-mediated activation of ERK1/2 occurring in keratinocytes, lung epithelia, and COS cells (36, 37). Our studies demonstrate for the first time that ERK1/2 activation antagonizes BMP2 signaling in primary NC cells and suggest that this mechanism for the attenuation of BMP signaling is functional during embryonic development. Importantly, this mechanism of antagonism of BMP2 signaling may account for the inhibitory effect of Neuregulin2 on SA cell development reported by Shah et al. (60). Neuregulin2, which suppresses neurogenesis and promotes glial differentiation in murine NCSCs (60), is a member of the EGF family, acts as an agonist of the ERB family of receptor tyrosine kinases, and is known to mediate ERK1/2 activation (12).

The activation of ERK1/2 by 100 μM forskolin is sustained and involves PKA-dependent activation of Rap1. This signaling network involving ERK1/2 activation via the cAMP pathway was shown to be operational in PC12 cells (68). Our studies demonstrate that this cAMP signaling network is also functional in primary NC cells (Fig. 3). Importantly, Rap1 and B-Raf activation does not occur with IBMX treatment, which is known to mediate low-level activation of cAMP signaling. This result explains why IBMX treatment does not antagonize BMP2-induced SA development but instead synergizes with BMP2 to enhance SA cell development (9). These results demonstrate for the first time with NC cells that the intensity of the activation of signal transduction cascades is a determining factor in cell lineage segregation mechanisms.

Considering that the in vitro conditions of low- or high-level activation of cAMP signaling may model in vivo gradients of extracellular signals activating cAMP signaling to differing extents, our results suggest a mechanism by which cells interpret signal gradients via differential activation of signal transduction cascades. Several studies suggest that concentration gradients of extracellular signals are distinguished at the level of cell surface receptors and at the level of transcription (1, 16, 23, 30, 45). Our studies clearly demonstrate that understanding mechanisms of cell lineage segregation requires a definition of both the signal transduction cascade(s) activated in response to specific microenvironmental signals and the intensity of its activation, ultimately determining how this signaling cascade(s) activates and/or silences specific gene expression patterns via the remodeling of chromatin architecture (3, 50, 54, 62). Importantly, the NC culture model system and its response to cAMP signaling is a physiologically and developmentally relevant model system that permits studies on chromatin remodeling mechanisms linking the segregation of SA and melanocyte lineages.

Melanogenesis is induced by the cAMP signaling network. It has been reported that addition of forskolin or 8-bromo-cAMP increases the number of melanocytes in NC cultures (25, 48) by an unknown mechanism. Herein, by quantifying the expression of the melanocytic lineage-determining factor Mitf (27), we demonstrate that high-level activation of cAMP signaling regulates the early lineage determination steps involved in NC lineage segregation to melanocytes. High-level activation of cAMP signaling is not a survival factor in melanogenesis, since in clonal assays it does not alter the survival of the colony founder NC cells (Table 1). This conclusion is further supported by the fact that 80% of the melanocyte-containing colonies formed in the presence of 100 μM forskolin are homogeneous, comprised of only melanocytes. Moreover, these pure melanocyte colonies are composed of fewer cells in comparison to the cell numbers of the mixed colonies, suggesting that the activation of cAMP signaling inhibits the self-renewal of the founder cells, inducing their differentiation to melanocytes. Thus, we conclude that high-level activation of cAMP signaling is an inductive signal in the terminal differentiation of the founder NC cells to melanocytes.

Physiologically relevant mechanisms likely mediating high-level activation of cAMP signaling include developmentally regulated expression of enzyme isoforms regulating the rate of synthesis versus that of the degradation of cAMP (reviewed in references 33 and 34), the spatial three-dimensional localization of PKA via anchoring proteins (AKAPs) generating signaling microdomains (15, 18, 31), and the occurrence of cross talk between different G protein-coupled receptors resulting in signal amplification (29, 57).

Transcription of the melanocyte-determining factor Mitf, based on the use of melanoma cell lines, is mediated by transcription factors Sox10, Pax3, CREB, and Lef1/TCF (27). However, the transcription factor initiating Mitf expression in vivo in the developing embryo or in NC cultures, leading to melanocyte differentiation, is unknown. Employing the constitutively active $CREB_{DIEDML}$, the dnACREB, and the CBP-interfering adenovirus protein E1A, we demonstrate that CREB activation is necessary for cAMP-induced Mitf expression and melanogenesis in NC cultures. These results suggest that CREB activation has the potential to initiate Mitf transcription in vivo, leading to melanocyte lineage determination.

However, CREB activation, although necessary, is insufficient for melanogenesis. The PKA inhibitor H89, but not the MEK-1 inhibitor PD 98059, repressed forskolin-induced Mitf and tyrosinase expression even in the presence of $CREB_{DIEDML}$, suggesting that in addition to CREB phosphorylation, PKA activation regulates additional aspects of Mitf transcription and melanogenesis. This result corroborates earlier observations that PKA further potentiates $CREB_{DIEDML}$ transcriptional activity (14). The mechanism of the PKA involvement in NC cell differentiation to the melanocytic lineage requires further investigation. Importantly, this requirement of PKA activation for melanogenesis supports the essential role of the cAMP signaling network in melanogenesis.

Interestingly, although FGF antagonizes BMP2-induced SA lineage development, it does not promote melanogenesis. This observation suggests, firstly, that melanogenesis is not a default

fate of NC differentiation occurring following inhibition of SA lineage development and, secondly, that the suppression of SA lineage is not an indirect effect of melanogenesis. Considering that in NC cells FGF activates ERK1/2 but does not activate CREB, the distinct effect of FGF supports the idea that the cAMP signaling network plays a dual role in regulating NC cell differentiation, i.e., the attenuation of BMP2-induced SA development via ERK1/2 activation and the induction of melanogenesis via PKA and CREB activation.

In the developing embryo, NC cells migrating dorsolaterally give rise to melanocytes (21). Although melanoblasts represent the majority in the dorsolateral migratory path, there also exist NC cells with neuronal potential (55, 69). Since both the epidermis and the dorsal neural tube express BMPs, this raises the question of why there are no cells of neuronal lineage along the dorsolateral path. Wakamatsu et al. (69) suggested that apoptosis is a mechanism by which improper cell types are removed from the dorsolateral path. Herein, our studies in primary NC cells suggest a mechanism by which NC cells escape BMP induction and differentiate to the melanocytic lineage. This mechanism derived from our in vitro studies needs to be tested in vivo and requires the discovery of the extracellular signals leading to cAMP signaling activation in the dorsolateral path.

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