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Basic FGF and TGF-β1 influence commitment to melanogenesis in neural crest-derived cells of avian embryos

KATE M. STOCKER, LARRY SHERMAN, SEAN REES and GARY CIMENT*

Department of Cell Biology and Anatomy, Oregon Health Sciences University, 3181 S.W. Sam Jackson Park Road, Portland, Oregon 97201-3098, USA

* Author for correspondence

Summary

In previous studies, we showed that neural crest (NC)-derived cells from embryonic quail dorsal root ganglia (DRG) and peripheral nerve (PN), which do not normally give rise to melanocytes, become committed to melanogenesis following treatment in culture with the phorbol ester drug 12-O-tetradecanoyl phorbol-13-acetate (TPA). These and other observations support the notion that melanocytes and Schwann cells are derived from a common bipotent intermediate in the neural crest lineage – the melanocyte/Schwann cell progenitor. In this study, we test the possibility that peptide growth factors found in the embryonic environment might act similarly to TPA to influence the fates of these cells. DRG and PN explants were cultured in medium supplemented with a variety of growth factors, and then the cultures were examined for the presence of pigment cells. We found that basic fibroblast growth factor (bFGF), but not various other growth factors, induced pigmentation in about 20 % of these cultures. When low concentrations of TPA were included in the culture medium, bFGF augmented the TPA-induced pigmentation, significantly increasing the proportion of pigmented cultures. These effects of bFGF were age-dependent, and could be blocked by addition of a bFGF-neutralizing antibody to the culture medium. In contrast to these stimulatory effects of bFGF, transforming growth factor-β1 (TGF-β1) was found to inhibit the TPA- or bFGF-induced pigmentation of DRG cultures. These data suggest, therefore, that at least some NC-derived cells are responsive to bFGF and TGF-β1, and that these growth factors may play an important role in the control of NC cell fate.

Key words: Neural crest, fibroblast growth factor, transforming growth factor-beta, melanocyte, Schwann cell, cell lineage.

Introduction

The neural crest (NC) is a transient embryonic structure that arises during neurulation in all vertebrates. Following closure of the neural plate to form the neural tube, NC cells migrate from the dorsal neural tube, localize at characteristic sites in the embryo, and eventually give rise to diverse cellular phenotypes. These cell types include the melanocytes of the integument and iris, sensory and autonomic neurons, Schwann cells, neurosecretory tissues in the adrenal medulla, and connective tissue of the head and face (see Le Douarin, 1982).

Although the extent to which individual NC cells are multipotent is unclear (reviewed by Anderson, 1989), at least some NC cells can have multiple fates. Individual NC cells in culture have been shown, for example, to give rise to colonies containing subpopulations of cells that express different sets of phenotypic markers (Sieber-Blum and Cohen, 1980; Baroffio *et al.* 1988). *In vivo*, individual NC cells injected with a lineage tracer dye have been shown to produce clones of cells that localize at multiple sites and express various phenotypic markers (Bronner-Fraser and Fraser, 1989).

There is also reason to believe that the process of NC commitment may occur as a series of events, rather than as a single, determinative step. Various early NC derivatives have been isolated, for example, and found to give rise to some, but not all, NC derivatives in heterospecific grafting experiments (Le Lievre *et al.* 1980; Nakamura and Ayer-Le Lievre, 1982; Ciment and Weston, 1985; Fontaine-Perus *et al.* 1988). These studies suggest that partially restricted subpopulations of NC-derived cells exist transiently at the time of NC cell migration, and that these subpopulations undergo subsequent commitment as the result of environmental influences. One such putative intermediate is the sympathoadrenal progenitor, which is believed to give rise to the neurons of sympathetic ganglia and adrenal chromaffin cells (Landis and Patterson, 1981; Anderson and Axel, 1986). This notion is based on the observations that nerve growth factor (NGF) induces

sympathetic neuronal development in chromaffin cell precursors (Aloe and Levi-Montalcini, 1979), whereas glucocorticoids induce expression of the chromaffin cell phenotype (Unsicker *et al.* 1978).

A similar type of bipotent progenitor cell may also exist transiently during NC cell migration and give rise to the melanocytes of the skin and Schwann cells associated with peripheral nerves and ganglia. This putative cell type – the melanocyte/Schwann cell progenitor – was first proposed by Nichols and Weston (1977), and its existence is inferred from various pieces of evidence [reviewed by Ciment (1990)]. For example, dorsal root ganglia, which contain Schwann cells and sensory neurons, but which normally do not contain melanocytes, give rise to pigmented cells when cultured in the presence of the phorbol ester 12-O-tetradecanoyl phorbol-13-acetate (TPA). Cultures of peripheral nerves, which consist largely of Schwann cell precursors (c.f., Bunge *et al.* 1989) but contain no neurons, also readily undergo pigmentation in response to TPA (Ciment *et al.* 1986). The evidence that these TPA-induced pigmented cells are *bone fide* melanocytes is based on observations that they could migrate into the overlying epidermis following grafting into host chicken embryos *in ovo* and contribute to the pigmentation of feathers. These results suggest, therefore, that TPA can reverse the developmental restriction of melanogenesis which normally occurs during early NC development, causing some Schwann cell precursors to become melanocytes.

Since various peptide growth factors have been shown to influence developmental decisions among a number of early embryonic cell types (see Aloe and Levi-Montalcini, 1979; Smith, 1989; Raff, 1989), we investigated whether such growth factors could also influence developmental decisions of early NC-derived cells and, in particular, the developmental restriction of melanogenesis in cultured quail embryonic dorsal root ganglion (DRG) and peripheral nerve (PN) cells. We report that basic fibroblast growth factor (bFGF) induced adventitious pigmentation in DRG and PN cultures, and that transforming growth factor-β1 (TGF-β1) inhibited the appearance of pigment cells. These data suggest that growth factors encountered by NC cells in the NC migratory space may play an important role in controlling the differentiative fate of NC cells.

Materials and methods

Embryos

Fertile quail (*Coturnix coturnix japonica*) eggs were obtained from the Poultry Sciences Department at Oregon State University (Corvalis, OR). Eggs were incubated in a humidified incubator at 38°C, and the embryos were staged using the criteria of Hamburger and Hamilton (1951).

Growth factors

Recombinant human bFGF and NGF were obtained from Collaborative Research (Lexington, MA). Recombinant human aFGF (Merck, Rahway, NJ), PDGF (Zymogenetics, Seattle, WA), and TGF-α (Genentech, San Francisco, CA) were the generous gifts of Drs Kenneth Thomas, Felix Eckenstein and Rik Derynck, respectively. TGF-β1, purified from human blood platelets, and EGF were the generous gifts of Dr Bruce Magun. Insulin, transferrin, selenium, α-MSH and TPA were all purchased from Sigma Chemical Co. (St Louis, MO).

Anti-human placental bFGF neutralizing antiserum

Rabbit anti-human placental bFGF antiserum was purchased from American Diagnostica Inc. (Greenwich, CT). This antiserum has been shown to neutralize bFGF activity in several assay systems (Sato and Rifkin, 1988; Broadley *et al.* 1989; Mignatti *et al.* 1989), and to cross-react with murine, bovine and chick homologues of bFGF, but not aFGF, at the concentrations used in these studies.

Tissue culture

DRG or PN were dissected from the brachial, thoracic and lumbosacral levels of quail embryos, cut into halves or quarters, and cultured as explants in 96-well Primaria plates (Falcon Plastics, Ventura, CA) in a humidified 5% CO_2–95% air atmosphere at 37°C. Tissue explants were used because previous studies had shown that explant cultures produced more reproducible pigmentation than did dissociated cell cultures of DRG and PN. Complete medium (CM) consisted of Hepes-buffered Ham's F-12 medium (Sigma, St. Louis, MO) supplemented with 0.03% L-glutamine, 100 i.u. ml^{-1} penicillin G, 100 $\mu g\,ml^{-1}$ streptomycin, 10% chick embryo extract (prepared by the method of Loring *et al.* 1982) and 20% fetal bovine serum (Hyclone, Logan, UT). For the defined medium (DM), the embryo extract and serum were replaced with 1 $mg\,ml^{-1}$ bovine serum albumin, 5 $ng\,ml^{-1}$ selenium, 1 $\mu g\,ml^{-1}$ transferrin, 1 $\mu g\,ml^{-1}$ insulin, and 100 $ng\,ml^{-1}$ α-MSH. Media were further supplemented with various growth factors and/or TPA (made from a 1000× stock solution in ethanol). Culture medium without TPA contained 0.1% ethanol as a vehicle control. Half of the medium was replaced with fresh medium and supplements after 5 days.

Assay for melanogenesis

Live cultures were examined at various times for the presence of pigmented cells, using a Nikon TMS inverted microscope with bright-field illumination. Wells were scored as positive when they contained one or more pigmented cells. Care was taken to distinguish melanocyte-like cells (cells with typically dendritic morphology containing dark, ovoid melanosomes) from brownish-yellow degenerating cells, containing irregularly-shaped granulations. 'Percent pigmentation' was calculated by dividing the number of positive wells by the total number of wells containing viable DRG or PN explants. Each point represents the data from 48 wells, unless otherwise noted. Statistical analysis involved the Newman Keuls multicomparison test, with $P<0.05$ defined as statistically significant.

Photomicroscopy

All photomicrographs were taken with a Zeiss IM-35 inverted microscope, with bright-field illumination using Kodak Plus-X panchromatic black-and-white film.

Results

Effects of various growth factors on adventitious pigmentation in embryonic quail DRG cultures

In previous work, we found that the phorbol ester TPA

induced adventitious pigmentation in a variety of NC-derived tissues (Ciment *et al.* 1986; Sears and Ciment, 1988). That is, TPA induced the appearance of pigmented cells in NC derivatives that normally do not give rise to melanocytes. To determine whether various peptide growth factors might mimic this effect of TPA, we screened a number of growth factors, using embryonic quail DRG as a source of NC-derived cells. In some cases, the culture medium also contained a low concentration of TPA, which caused an intermediate level of pigmentation. This allowed us to screen for both inhibitory as well as stimulatory effects of these growth factors.

DRG from 7 day (stage 32–33) quail embryos were dissected, cultured as explants in CM supplemented with 3.0 nM TPA in the presence or absence of growth factors, and then examined over the course of 10 days for the presence of pigmented cells. These growth factors included basic fibroblast growth factor (bFGF), acidic fibroblast growth factor (aFGF) (in the presence or absence of 10 mg ml^{-1} of heparin), epidermal growth factor (EGF), transforming growth factor-alpha (TGF-α), transforming growth factor-beta (TGF-β1), platelet-derived growth factor (PDGF) and nerve growth factor (NGF). These growth factors were tested at various concentrations. Fig. 1A contains data from a representative experiment and shows that bFGF, but not various other growth factors, augmented the proportion of cultures containing pigmented cells, as compared to cultures treated with TPA alone (broken line). This augmented TPA-induced pigmentation was characterized by both an increased rate of pigmentation, as well as an increase in the final percentage of pigmented cultures. This experiment was performed

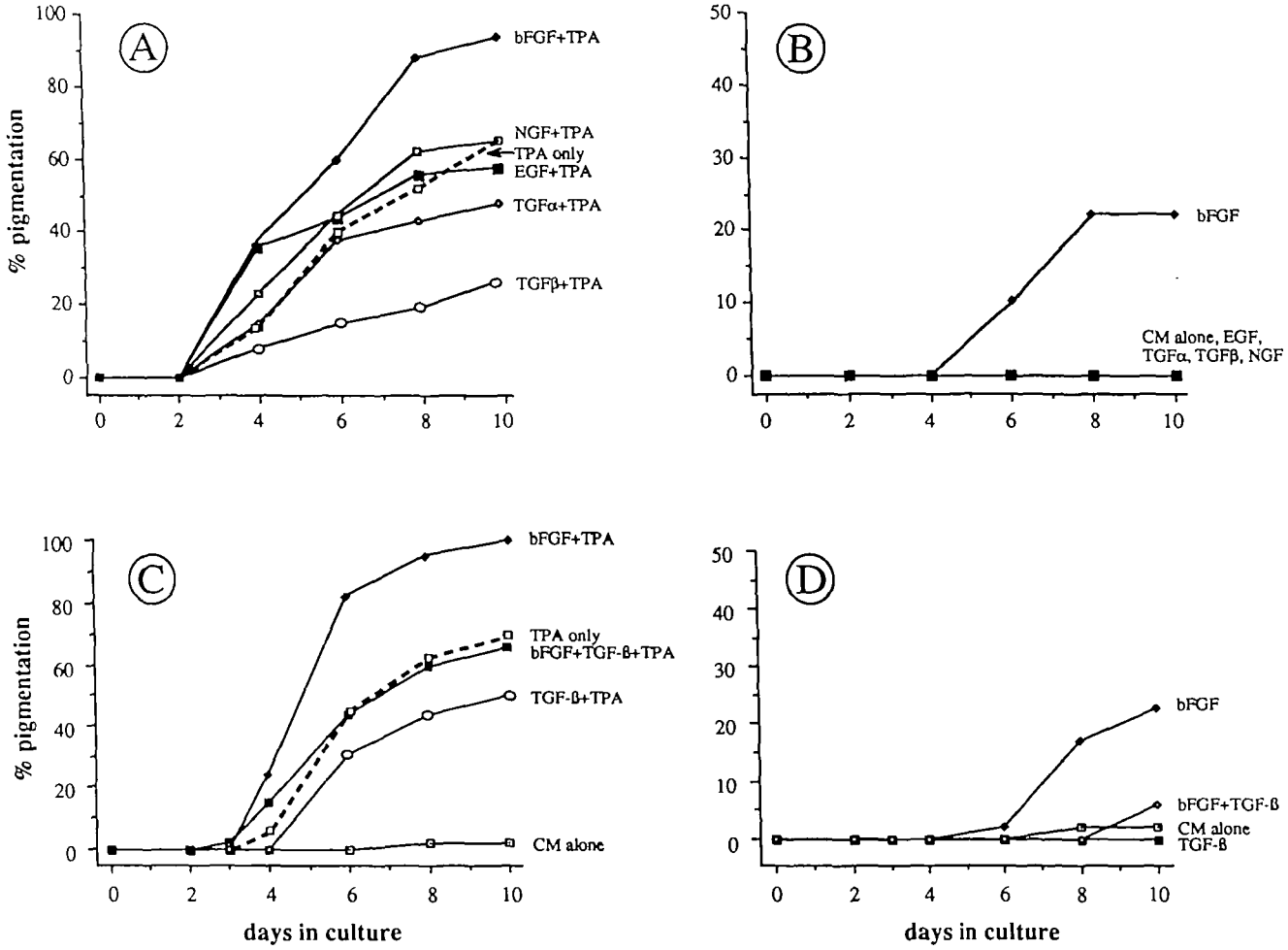

Fig. 1. Effects of growth factors on adventitious pigmentation of E7 DRG cells in CM. (A) DRG explants from E7 (stage 32–33) quail embryos were cultured in CM in the presence of 3.0 nM TPA and 10 ng ml^{-1} of either bFGF, NGF, EGF, TGF-β or TGF-α, and then the cultures were examined at various times for pigment cells. Note that bFGF, but not the various other growth factors, augmented the TPA-induced pigmentation. Note also that TGF-β1 inhibited this pigmentation. (B) DRG explants were cultured in the presence of each of the growth factors mentioned in A, but without TPA. Note that only bFGF induced adventitious pigmentation. (C) DRG explants were cultured in CM in the presence of 3.0 nM TPA and either 10 ng ml^{-1} bFGF alone, 10 ng ml^{-1} TGF-β1 alone, or both bFGF and TGF-β1. Note that TGF-β1 blocked the bFGF-induced pigmentation. (D) DRG explants were cultured in CM without TPA, but supplemented with either bFGF alone, TGF-β1 alone, or both bFGF and TGF-β1. Note that TGF-β1 reduced the bFGF- induced adventitious pigmentation in the absence of TPA.

three times and, although the absolute values varied between experiments, the same qualitative differences were consistently observed.

To determine whether bFGF or other growth factors could influence pigmentation in the absence of TPA, DRG explants were also cultured in medium supplemented only with the growth factors mentioned above. Fig. 1B shows that bFGF induced pigmentation in 23% of these cultures, but that none of the other growth factors tested induced any pigmentation. The effects of bFGF and TPA appeared to be additive, rather than synergistic, since the sum of pigmented cultures treated with either bFGF alone (Fig. 1B) or TPA alone (Fig. 1A) did not exceed the number of pigmented cultures treated simultaneously with both bFGF and TPA (Fig. 1A).

In contrast to the stimulatory effects of bFGF, TGF-β1 was found to antagonize the TPA-induced pigmentation in these experiments. Fig. 1A shows that addition of TGF-β1 decreased the extent of pigmentation in TPA-supplemented medium from 67% to 23% of the cultures after 10 days in culture. This inhibition by TGF-β1 was found, moreover, to be dose-dependent, with 10.0 ng ml^{-1} TGF-β1 producing a greater extent of inhibition than 1.0 ng ml^{-1} (data not shown). To determine whether the stimulatory and inhibitory effects of TGF-β1 and bFGF were subtractive, DRG explants were cultured in the presence of either bFGF alone, TGF-β1 alone, or with both TGF-β1 and bFGF. Fig. 1D shows that TGF-β1 inhibited the bFGF-induced pigmentation, reducing the proportion of pigmented cultures at day 10 from 27% to 6%. In the presence of TPA, TGF-β1 was also found to antagonize the stimulatory effects of bFGF on E7 DRG cells, decreasing the proportion of pigmented cultures to that of TPA alone (Fig. 1C). These experiments were performed four times with similar results.

Age-dependent effects of bFGF and TGF-β1 on adventitious pigmentation in DRG cultures

We had previously found that the ability of TPA to induce adventitious pigmentation of DRG cells was age-dependent (Ciment *et al.* 1986). To determine whether the bFGF-induced pigmentation showed a similar age-dependency, DRG from E4 (stage 23–24), E5 (stage 26–27), E6 (stage 29–30) and E7 (stage 32–33) quail embryos were cultured in CM in the presence or absence of bFGF. Fig. 2A shows that bFGF extended the developmental stages at which adventitious pigmentation occurred in DRG explants. In one experiment, for example, the addition of exogenous bFGF to the culture medium of E6 DRG explants increased the proportion of pigmented cultures from 32% to 77%. It should be noted that during normal quail development, pigmentation of the skin begins around embryonic day 7 (Padgett and Ivey, 1960), suggesting that the loss of bFGF-responsiveness in these NC-derived cells occurs at roughly the same time that pigmentation normally begins *in vivo*. This experiment was performed three times with similar results.

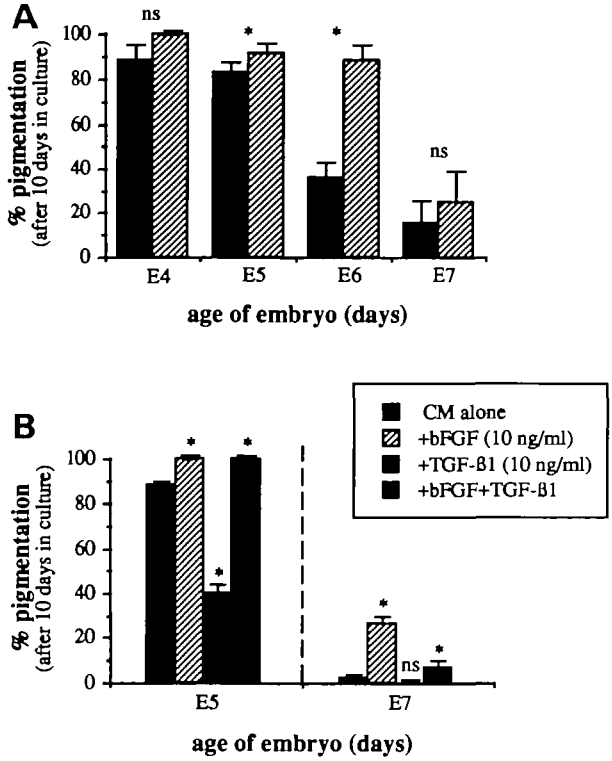

Fig. 2. Age-dependent differences in the abilities of bFGF and TGF-β1 to induce pigmentation in DRG cells. (A) DRG explants from E4 (stage 23–24), E5 (stage 26–27), E6 (stage 29–30), and E7 (stage 32–33) quail embryos were cultured for 10 days in CM in the presence or absence of 10 ng ml^{-1} bFGF, and then the proportion of pigmented cultures was determined and statistical analysis performed. Data represent the means±standard errors of the mean for three experiments. Note that there were statistically significant differences (i.e. $P<0.05$) in the extent of pigmentation between CM- and CM/bFGF-treated cultures from E5 and E6 embryos. (B) DRG explants from E5 and E7 quail embryos were cultured for 10 days in CM or in CM supplemented with either 10 ng ml^{-1} bFGF, 10 ng ml^{-1} TGF-β1, or both bFGF and TGF-β1, and then the proportion of pigmented cultures was determined and statistical analysis performed. In E5 cultures, note that bFGF-, TGFβ1- and bFGF/TGFβ1-treated cultures all differed significantly from CM-only cultures. In E7 cultures, note that bFGF- and bFGF/TGFβ1-treated cultures differed significantly from CM-only cultures, and that bFGF- and bFGF/TGFβ1-treated cultures differed significantly from one another.

The ability of TGF-β1 to inhibit pigmentation in the absence of TPA was also found to be age-dependent. Fig. 2B compares the effects of bFGF and TGF-β1 on E5 and E7 quail DRG explants, and shows that TGF-β1 had no significant effect on bFGF-induced pigmentation in E5 embryos (i.e. compare light-striped with dark-striped bars), but inhibited such pigmentation in E7 DRG explants. This figure also shows that TGF-β1 inhibited the spontaneous pigmentation seen in E5 DRG cultured in the absence of bFGF (i.e. compare black with stippled bars). This experiment was performed three times with similar results. These data

indicate that the growth factor-responsiveness of these NC-derived cells may change with developmental age.

DRG cultured in defined medium

To determine whether these effects of bFGF and TPA depended on other, unknown factors present in CM, experiments were also performed using a serum-free and embryo extract-free defined medium (DM), consisting of F-12 medium supplemented with insulin, transferrin, α-MSH, selenium and bovine serum albumin. Therefore, DRG explants from 7-day embryonic DRG were cultured in DM in the presence of either TPA alone, bFGF alone (at concentrations ranging from 1 to 50 ng ml^{-1}), or a combination of these two agents, and then the proportion of pigmented cultures was determined over the course of 10 days. In these experiments, TPA was used at 100 nM, which was determined to be the concentration that elicited optimal pigmentation in this medium (data not shown). Fig. 3 shows that neither DM alone, nor DM plus various concentrations of bFGF, were able to induce pigmentation. In contrast, DM supplemented with TPA alone induced pigmentation in 17% of the cultures by day 10, and cultures treated with both bFGF (50 ng ml^{-1}) and TPA induced even higher levels of pigmentation. The ability of bFGF to stimulate pigmentation in these cultures was dose-dependent, moreover, with 1 ng ml^{-1} inducing pigmentation in 50% of the cultures and 50 ng ml^{-1} inducing pigmentation in 86% of the cultures. Note that the onset of pigmentation in these DM cultures is delayed compared to CM cultures (see Fig. 1A). Similar results were obtained in three separate trials of this experiment. These results suggest that bFGF requires additional factors to induce pigmentation under these conditions.

Fig. 4 consists of bright-field photomicrographs from these studies and shows that bFGF may also influence

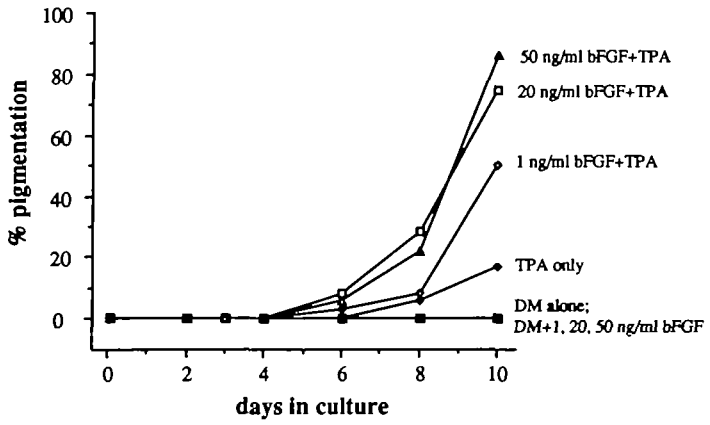

Fig. 3. Basic FGF- and TPA-induced pigmentation of E7 DRG cells cultured in defined medium. DRG explants from E7 quail embryos were cultured in defined medium (DM) supplemented with TPA and/or bFGF at various concentrations, and then cultures were examined at various times and the proportion of pigmented cultures determined. Note that bFGF alone did not induce pigmentation; that TPA alone induced pigmentation in only 18% of the cultures; but that bFGF acted synergistically with TPA to induce pigmentation.

the survival of non-neuronal cells in these cultures. Cultures in DM without bFGF, for example, often contained cells that were poorly attached to the substratum and contained vacuoles, suggesting that bFGF is required for the optimal health of these cultures (compare panel B with panel A). In the presence of TPA, DRG cultures in DM still contained a large proportion of non-attached or vacuolated cells (compare panels C and D), although the presence of TPA caused the appearance of many more neurites (compare panel D with panel B). This would suggest that TPA cannot substitute for bFGF in these culture conditions. When DRG were cultured in DM supplemented with bFGF, the cells appeared healthy, and both neurites as well as numerous non-neuronal cells could be seen (compare panel B with panel F). In the presence of both bFGF and TPA, many of these DM cultures also contained pigmented cells (panel H).

In contrast to these studies with DM, DRG cultured in CM plus TPA and/or bFGF contained large numbers of non-neuronal cells and pigmented cells (compare panel A with panels C,E,G). No significant cell death was observed, moreover, in explants cultured in CM alone (compare panel A with panel B). These results suggest, therefore, that bFGF may also promote the survival of non-neuronal cells in DM, but requires the presence of some unknown serum factor (which can be replaced by TPA) to induce adventitious pigmentation.

The effects of TGF-β1 on DRG explant pigmentation were also tested in DM. In three experiments, however, E7 DRG consistently demonstrated poor cell viability when cultured in DM supplemented with TGF-β1 (at either 1 or 10 ng ml^{-1}) both in the presence and absence of bFGF (10 ng ml^{-1}) and/or TPA (100 nM), making conclusions about the effects of TGF-β1 on pigmentation in DM difficult.

Effects of basic FGF-neutralizing antibodies on adventitious pigmentation in DRG explants

Since E5 DRG cultures underwent pigmentation in the absence of exogenously added bFGF, it is possible that CM contains a low level of bFGF, presumably from the chick embryo extract (e.g. Seed *et al.* 1988; Kimura *et al.* 1989). To determine whether this bFGF may have been responsible for pigmentation of some DRG cells from younger embryos, studies were performed utilizing neutralizing anti-bFGF antibodies. DRG from 5- and 7-day quail embryos were cultured in the presence or absence of this neutralizing antiserum in either CM, CM plus TPA, or CM plus bFGF. Fig. 5 shows that E5 DRG explants underwent adventitious pigmentation in CM alone, but that a 1:500 dilution of bFGF-neutralizing antibodies blocked this pigmentation (panel A). In the presence of antiserum and exogenous bFGF (10 ng ml^{-1}), however, the proportion of pigmented wells at 10 days increased from 0% to 100% of the cultures (compare panels A and E). When bFGF was added to CM containing a 1:250 dilution of bFGF-neutralizing antiserum, no pigmentation was observed after 10 days in culture (panel G). Addition of an excess of bFGF (50 ng ml^{-1}) to cultures treated with the 1:250

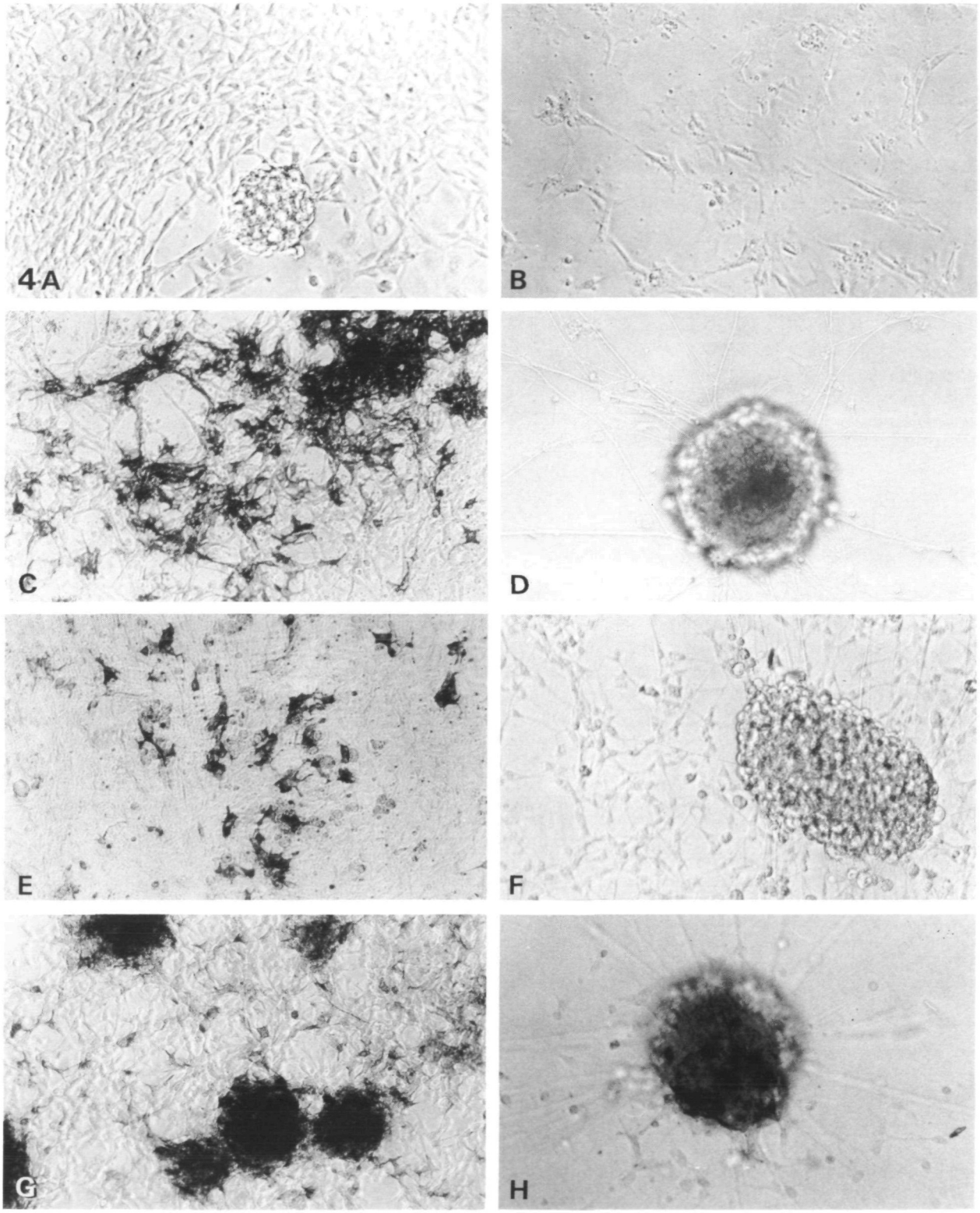

Fig. 4. Bright-field photomicrographs of E7 DRG explants in CM or DM supplemented with bFGF and/or TPA. DRG explants from E7 quail embryos were cultured in either: (A) CM alone; (B) DM alone; (C) CM in the presence of 3.0 nM TPA; (D) DM in the presence of 0.1 μM TPA; (E) CM supplemented with 10 ng ml^{-1} bFGF; (F) DM supplemented with 10 ng ml^{-1} bFGF; (G) CM containing both 3.0 nM TPA and 10 ng ml^{-1} bFGF; or (H) DM containing both 0.1 μM TPA and 10 ng ml^{-1} bFGF. Photographs were taken on the sixth day in culture. Note that in the absence of bFGF (i.e. B and D), viability of non-neuronal cells in the DM cultures was generally poor.

dilution of the neutralizing antibodies overcame this inhibition (data not shown). These data indicate, therefore, that the melanogenesis-promoting factor in CM is likely to be bFGF or a closely related growth factor. It should also be noted that E5 cultures containing neutralizing antiserum demonstrated poor cell viability, with most of the surviving cells having fibroblast-like or neuronal morphologies (data not shown).

The bFGF neutralizing antiserum also inhibited pigmentation in E7 DRG cultures. As with the E5 explants, the addition of bFGF (10 ng ml^{-1}) to E7 DRG blocked the effects of the neutralizing antiserum diluted 1:500 (panel F), but failed to block bFGF-neutralizing

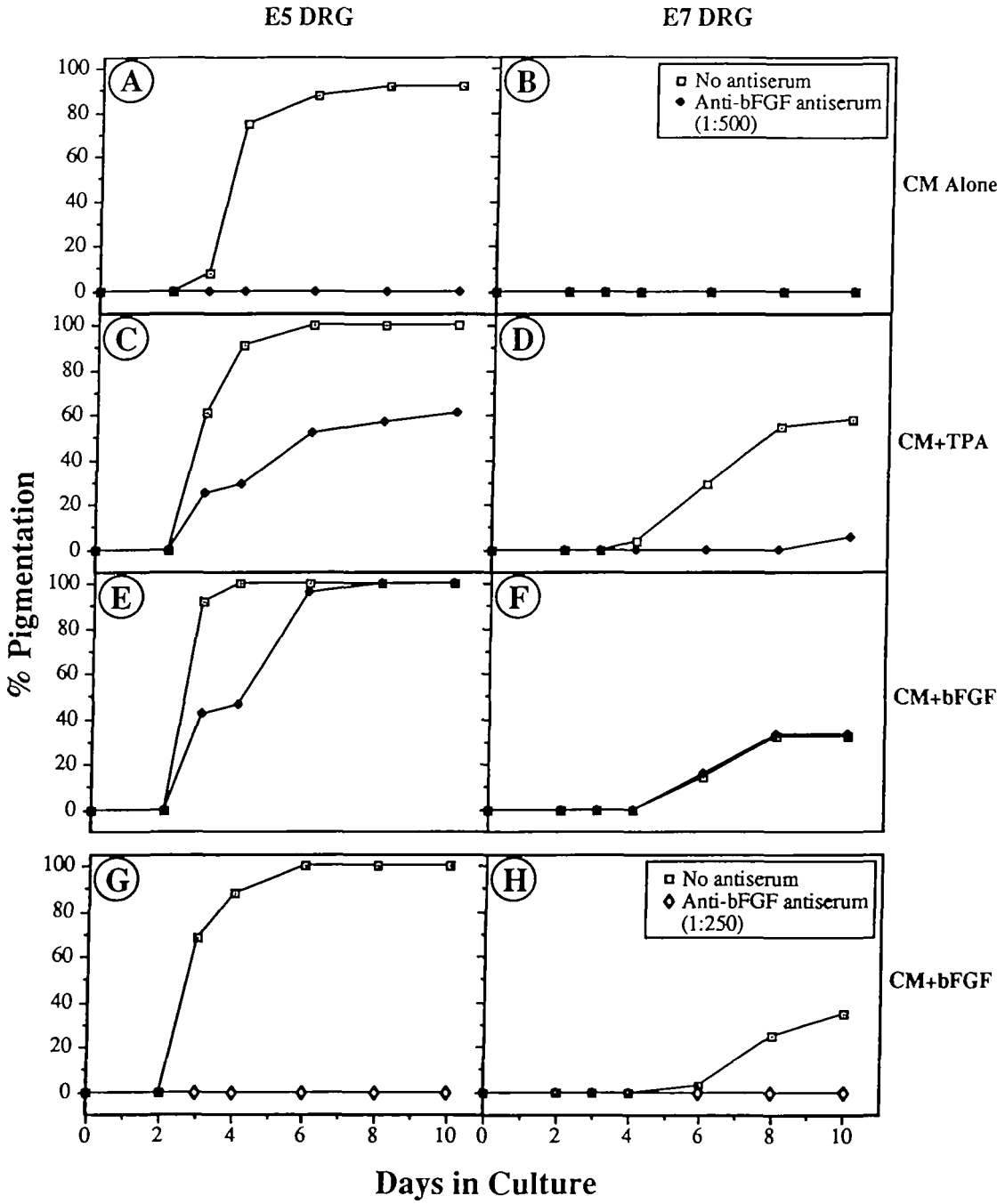

Fig. 5. Effects of bFGF-neutralizing antibodies on pigmentation of E5 and E7 DRG cells cultured in CM. DRG explants from E5 (A,C,E,G) and E7 (B,D,F,H) quail embryos were cultured in the absence (open squares) or presence (filled diamonds) of a 1:500 dilution of a bFGF-neutralizing antiserum in either: (A,B) CM with no supplements; (C,D) CM supplemented with 3.0 nM TPA; (E,F) CM supplemented with 10 ng ml^{-1} bFGF; (G,H) CM supplemented with 50 ng ml^{-1} bFGF. Each data point represents 24 culture wells. Note that bFGF-neutralizing antibody completely inhibited the spontaneous pigmentation of E5 DRG cells (A) and that an excess of bFGF reversed this inhibition (G). Note also that bFGF-neutralizing antibody partially inhibited the TPA-induced pigmentation of E5 DRG cells (B).

activity at a higher concentration of the antiserum (panel H). In contrast to E5 DRG cultures, however, the cell viability in E7 cultures appeared unaffected by the antiserum, supporting the notion that bFGF may also act as a survival factor for early (i.e. E5) DRG cells.

Interestingly, the bFGF-neutralizing antibody also inhibited TPA-induced pigmentation in DRG cultures. With E5 DRG, for example, the proportion of pigmented cultures decreased from 100% to 61% in the presence of neutralizing antibody (panel C), whereas with E7 DRG, the proportion dropped from 58% to 5% of the cultures (panel D). These experiments were performed three times with similar results.

Effects of bFGF on pigmentation in peripheral nerve cultures

Although DRG provide a relatively abundant source of NC-derived cells, these ganglia contain both neuronal and Schwann cell precursors, complicating the interpretation of these data. Previous studies had shown that peripheral nerve explants, which do not contain neuronal precursors, do contain cells that readily undergo adventitious pigmentation in response to TPA (Ciment *et al.* 1986). Therefore, to determine whether bFGF would induce pigmentation in cells that would normally give rise to Schwann cells, peripheral nerves were dissected from 7-day quail embryos and cultured in CM in the presence of TPA and/or bFGF. These cultures were then examined over the course of 10 days for the appearance of pigmented cells. Fig. 6 shows that none of the cultures in CM alone underwent adventitious pigmentation, but about 63% of the bFGF-treated cultures did contain pigmented cells and, in some cultures, 100% of the non-fibroblastic cells contained pigment granules (data not shown). And as seen before, TPA induced pigmentation in 100% of the cultures (Ciment *et al.* 1986). Qualitatively similar results were obtained in three separate trials of this experiment. These studies suggest, therefore, that at least some of the bFGF-responsive cells in these experiments would normally have given rise to Schwann cells.

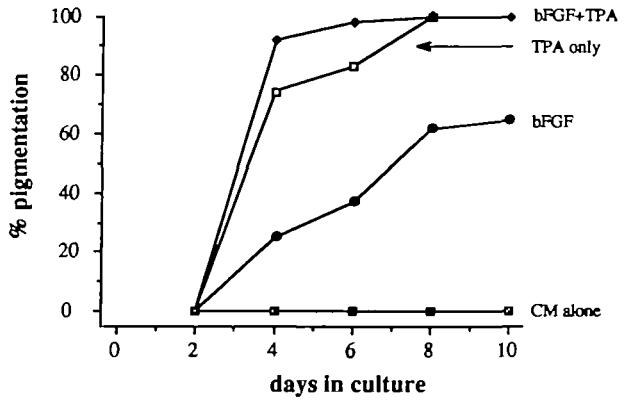

Fig. 6. Peripheral nerve cells also undergo pigmentation in response to bFGF. Distal portions of spinal nerves from the thoracic region of E7 quail embryos were cultured in CM supplemented with either 3.0 nM TPA, 10 ng ml^{-1} bFGF, or both bFGF and TPA. Each data point represents the percentage pigmentation of 24 culture wells. Note that bFGF induced pigmentation in these peripheral nerve cultures.

Discussion

In previous work, we found that the NC-derived cells of embryonic quail DRG and peripheral nerves underwent pigmentation following treatment in culture with the phorbol ester drug TPA (Ciment *et al.* 1986; Sears and Ciment, 1988). When TPA-treated DRG explants were backgrafted into the NC migratory space of host chicken embryos *in vivo*, they were found to give rise to melanocytes, but not other NC-derived cell types (Sears and Ciment, 1988). These and other observations support the hypothesis that melanocytes and Schwann cells are derived from a common bipotent intermediate cell type in the NC lineage – the melanocyte/Schwann cell progenitor (see Nichols and Weston, 1977; Ciment, 1990). In this study, we examined the possibility that growth factors present in the embryonic microenvironment may also influence the commitment of this putative bipotent progenitor.

Basic FGF induces pigmentation in various NC-derivatives, probably acting on Schwann cell precursors

We report here that bFGF, but not various other growth factors, induced the appearance of pigmented cells in DRG and peripheral nerve cultures from E7 quail embryos. This effect of bFGF could be blocked by the addition of bFGF-neutralizing antibodies, and the effects of these neutralizing antibodies could be overcome, in turn, by additional bFGF. This last observation suggests that this antibody preparation is not cytotoxic, but instead, produces its effects *via* binding bFGF. Further evidence for a role of bFGF in this adventitious pigmentation comes from the defined medium experiments, in which bFGF induced pigmentation in E7 DRG explants in a dose-dependent fashion. It may be noteworthy that TPA was also necessary for pigmentation in the DM studies, acting synergistically with bFGF to induce higher levels of pigmentation. In CM, in contrast, the effects of bFGF and TPA seemed to be additive. These observations suggest, therefore, that other factors present in CM may also influence melanogenesis.

Pigmentation was also observed, however, in E5 DRG explants cultured in CM in the absence of exogenous bFGF. This spontaneous pigmentation may also have been due to bFGF, probably present in the chick embryo extract which is added to CM (see Seed *et al.* 1988; Kimura *et al.* 1989). This conclusion is based on the observations that neutralizing antibodies against bFGF were able to block the spontaneous pigmentation

seen in E5 DRG cultures, and that this inhibition could again be overcome by adding higher concentrations of bFGF.

The observation that peripheral nerve cells underwent bFGF-induced pigmentation in these experiments supports the notion that the bFGF-responsive cells would normally have given rise to Schwann cells, rather than neurons. Schwann cells make up the majority of the cells within peripheral nerve, although some perineurial fibroblasts are also present (e.g. Bunge *et al.* 1989). Since these fibroblasts are not of NC origin (Le Douarin, 1982), however, it seems unlikely that this small subpopulation of cells would be responsible for the appearance of melanocytes in either bFGF-or TPA-treated peripheral nerve explants. In some peripheral nerve cultures, moreover, most or all of the non-fibroblastic cells underwent pigmentation in response to bFGF. It is possible, therefore, that Schwann cell precursors found in various NC-derived neural tissues retain the developmental capability to undergo a transformation into melanocytes in response to bFGF.

Basic FGF may also support the survival of a subpopulation of DRG cells

An alternative explanation for the effects of bFGF on DRG and PN pigmentation is that bFGF promotes the survival and/or mitogenesis of melanocyte precursors or other uncommitted NC-derived cells (see Halaban *et al.* 1987; Schubert *et al.* 1987; Unsicker *et al.* 1987; Eckenstein *et al.* 1990). We found, for example, that the viability of E5 DRG cultures grown in CM in the presence of bFGF-neutralizing antibodies or in DM without bFGF was consistently poor, with the few surviving cells having a fibroblast-like morphology. Recently, Kalcheim (1989) has also provided evidence that bFGF may promote the survival of a subpopulation of NC-derived cells within embryonic chicken DRG *in vivo*. In these experiments, impermeable barriers implanted between the spinal cord and nascent DRG were found to cause a decrease in the number of HNK-1 immunoreactive cells [i.e. NC-derived cells (Bronner-Fraser, 1986)] within the DRG, whereas bFGF-coated barriers did not cause such a decrease. Since the embryonic spinal cord contains high concentrations of bFGF at these developmental stages (Kalcheim and Neufeld, 1990), the interpretation is that bFGF released from the spinal cord supports the survival of a subpopulation of NC-derived cells.

It seems unlikely, however, that proliferation of committed melanoblasts can fully explain the bFGF-induced pigmentation seen in our cultures. Basic FGF induces pigmentation in a large proportion of E7 DRG cells, for example, and yet there is very little cell death in cultures treated with bFGF-neutralizing antibodies. If bFGF acted principally as a survival and/or a mitogenic factor, one might have expected to see significant cell death in such cultures. It seems likely, therefore, that at least some of the effects of bFGF are to reverse the developmental restriction of melanogenesis in these NC-derived cells (see Ciment *et al.* 1986).

TGF-β1 inhibits the adventitious pigmentation of DRG cells

In contrast to the stimulatory effects of bFGF, we found that TGF-β1, but not various other growth factors, decreased the extent of pigmentation in E7 DRG explants cultured in the presence of TPA, and prevented the bFGF-induced increase in pigmentation. TGF-β1 inhibited, moreover, the pigmentation that normally occurs in DRG from E5 quail embryos cultured in unsupplemented CM. TGF-β1 seems to antagonize, therefore, the ability of bFGF to promote adventitious pigmentation of DRG cells. It is intriguing to speculate that TGF-β1 may act to promote commitment of the putative melanocyte/Schwann cell progenitor to the Schwann cell lineage or, at least, to prevent melanocyte development within peripheral ganglia. It is interesting to note, therefore, that immunoreactivity for various members of the TGF-β gene family is present in the region of the embryonic chicken DRG at these developmental stages (S. Jakolew, personal communication). The recent findings that several members of the TGF-β gene family can act as inducers of mesoderm during *Xenopus* gastrulation (Weeks and Melton, 1987; Smith *et al.* 1989) support the notion that members of this family may play major roles in the process of cell commitment during early development.

The melanocyte/Schwann cell progenitor

These data support the notion of a transient subpopulation of NC-derived cells that can give rise to either melanocytes or Schwann cells, depending on various environmental factors. The existence of such a bipotent progenitor is also suggested by a variety of other circumstantial evidence. First, melanocytes and various melanoma cell lines express a number of 'cell type-specific' phenotypic markers in common with Schwann cells, but not other NC derivatives, including the S100 protein (Stefansson *et al.* 1982) and the RN2 antigen (Dippold *et al.* 1980). Second, clonal cultures of mesencephalic NC cells have been shown to give rise to colonies containing both melanocytes and cells expressing Schwann cell markers (Dupin *et al.* 1990). Finally, melanosome-bearing Schwann cells have been observed in a number of pathological conditions, including experimental murine melanotic tumors (Kanno *et al.* 1987), primary melanotic Schwannomas (Napoli and Domenico, 1987) and von Recklinghausen's neurofibromatosis (Pleasure *et al.* 1986).

The notion that the melanocyte/Schwann cell progenitor is likely to be responsive to growth factors in its environment makes specific predictions about the localization of TGF-β1 and bFGF in the NC pathway. In the mouse, TGF-β1 immunoreactivity has been localized to the somitic sclerotome, but not the myotome (Heine *et al.* 1987), suggesting that this growth factor may be present at this site of Schwann cell development in mammals. This is in contrast to bFGF, which has been localized in the chicken to the myotome, but not the rest of the somite, at this developmental stage (Joseph-Silverstein *et al.* 1989). It

is intriguing, moreover, that Kalcheim and Neufeld (1990) found bFGF immunoreactivity in the extracellular matrix associated with the mesenchyme just dorsal to the spinal cord anlagen of E4 quail embryos. This location is part of the migratory route through which NC cells enter the dermis (Weston, 1963; Teillet and Le Douarin, 1970), indicating that the NC cells giving rise to melanocytes are exposed to bFGF during their early migration into the skin. Although bFGF immunoreactivity can also be found in DRG, most of this immunostaining appears to be located intracellularly in neurons (Kalcheim and Neufeld, 1990). Since the bFGF mRNA transcript lacks a consensus signal sequence (Abraham *et al.* 1986), bFGF may not be released by these neurons, making it unclear whether the non-neuronal cells present in the DRG are exposed to bFGF. It is of interest to note, therefore, that the period of neuronal cell death (Hamburger and Levi-Montalcini, 1949) follows the ages at which non-neuronal cells in the DRG and peripheral nerves lose their ability to undergo bFGF-induced melanogenesis (i.e. E7). It seems reasonable to conclude, therefore, that the timing of this loss of bFGF-responsiveness is critical to prevent the ectopic appearance of melanocytes in these various neural tissues.

These data suggest, therefore, that the fate of the putative melanocyte/Schwann cell precursor may be influenced by specific environmental cues. Basic FGF, or some other member of this growth factor family, may support melanogenesis, while TGF-β may support commitment to the Schwann cell lineage, or at least, antagonize the effects of bFGF. While the existence of the melanocyte/Schwann cell precursor cell remains to be demonstrated directly, our observations of PN explants suggest that Schwann cell precursors transform into melanocytes in the presence of bFGF in culture. Localization studies of bFGF and TGF-β during the period of NC migration may provide further evidence for a role of these growth factors in the process of NC cell committment *in vivo*.

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References

Abraham, J. A., Mergia, A., Whang, J. L., Tumolo, A., Friedman, J. L., Hjerrild, K. A., Gospodarowicz, D. and Fiddes, J. C. (1986). Nucleotide sequence of a bovine clone encoding the angiogenic protein, basic fibroblast growth factor. *Science* **233**, 545–548.

Aloe, L. and Levi-Montalcini, R. (1979). Nerve growth factor-induced transformation of immature chromaffin cells *in vivo* into sympathetic neurons: Effect of antiserum to nerve growth factor. *Proc. natn. Acad. Sci. U.S.A.* **76**, 1246–1250.

Anderson, D. J. (1989). The neural crest cell lineage problem: Neuropoiesis? *Neuron* **3**, 1–12.

Anderson, D. J. and Axel, R. (1986). A bipotential neuroendocrine precursor whose choice of cell fate is determined by NGF and glucocorticoids. *Cell* **47**, 1079–1090.

Baroffio, A., Dupin, E. and Le Douarin, N. M. (1988). Clone-forming ability and differentiation potential of migratory neural crest cells. *Proc. natn. Acad. Sci. U.S.A.* **85**, 5325–5329.

Broadley, K. N., Aquino, A. M., Woodward, S. C., Buckley-Sturrock, A., Sato, Y., Rifkin, D. B. and Davidson, J. M. (1989). Monospecific antibodies implicate basic fibroblast growth factor in normal wound repair. *Lab. Invest.* **61**, 571–575.

Bronner-Fraser, M. (1986). Analysis of the early stages of trunk neural crest migration in avian embryos using monoclonal antibody HNK-1. *Devl Biol.* **115**, 44–55.

Bronner-Fraser, M. and Fraser, S. (1989). Developmental potential of avian trunk neural crest cells *in situ*. *Neuron* **3**, 755–766.

Bunge, M. B., Wood, P., Tynan, L., Bates, M. and Sanes, J. (1989). Perineurium originates from fibroblasts: demonstration *in vitro* with a retroviral marker. *Science* **243**, 229–231.

Ciment, G. (1990). The melanocyte/Schwann cell progenitor: A bipotent intermediate in the neural crest lineage. *Comments Devl Neurobiol.* **1**, 207–223.

Ciment, G., Glimelius, B., Nelson, D. and Weston, J. A. (1986). Reversal of a developmental restriction in neural crest-derived cells of avian embryos by a phorbol ester drug. *Devl Biol.* **118**, 392–398.

Ciment, G. and Weston, J. A. (1985). Segregation of developmental abilities in neural crest-derived cells: Identification of partially restricted intermediate cell types in the branchial arches of avian embryos. *Devl Biol.* **111**, 73–83.

Dippold, W. G., Lloyd, K. O., Li, L. T. C., Ikeda, H., Oettgen, H. F. and Old, L. J. (1980). Cell surface antigens of human malignant melanoma: Definition of six antigenic systems with mouse monoclonal antibodies. *Proc. natn. Acad. Sci. U.S.A.* **77**, 6114–6118.

Dupin, E., Baroffio, A., Dulac, C., Cameron-Curry, P. and Le Douarin, N. M. (1990). Schwann-cell differentiation in clonal cultures of the neural crest, as evidenced by the anti-Schwann cell myelin protein monoclonal antibody. *Proc. natn. Acad. Sci. U.S.A* **87**, 1119–1123.

Eckenstein, F. P., Esch, F., Holbert, T., Blacher, R. W. and Nishi, R. (1990). Purification and characterization of a trophic factor for embryonic peripheral neurons: Comparison with fibroblast growth factors. *Neuron* **4**, 623–631.

Fontaine-Perus, J., Chanconie, M. and Le Douarin, N. M. (1988). Developmental potentialities in the nonneuronal population of quail sensory ganglia. *Devl Biol.* **128**, 359–375.

Halaban, R., Ghosh, S. and Baird, A. (1987). bFGF is the putative natural growth factor for human melanocytes. *In Vitro Cell. Devl Biol.* **23**, 47–52.

Hamburger, V. and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morph.* **88**, 49–92.

Hamburger, V. and Levi-Montalcini, R. (1949). Proliferation, differentiation and degeneration in the spinal ganglia of the chick embryo under normal and experimental conditions. *J. exptl Zool.* **111**, 457–501.

Heine, U. I., Munoz, E. F., Flanders, K. C., Ellingsworth, L. R., Lam, H.-Y. P., Thompson, N. L., Roberts, A. B. and Sporn, M. B. (1987). Role of transforming growth factor-β in the development of the mouse embryo. *J. Cell Biol.* **105**, 2861–2876.

Joseph-Silverstein, J., Consigli, S. A., Lyser, K. M. and VerPault, C. (1989). Basic fibroblast growth factor in the chick embryo: Immunolocalization to striated muscle cells and their precursors. *J. Cell Biol.* **108**, 2459–2466.

Kalcheim, C. (1989). Basic fibroblast growth factor stimulates survival of non-neuronal cells developing from trunk neural crest. *Devl Biol.* **134**, 1–10.

Kalcheim, C. and Neufeld, G. (1990). Expression of basic fibroblast growth factor in the nervous system of early avian embryos. *Development* **109**, 203–215.

Kanno, J., Matsubara, O. and Kasuga, T. (1987). Induction of melanogenesis in Schwann cell and perineurial epithelium by 9, 10-dimethyl-1,2-benzanthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA) in BDF1 mice. *Acta pathol. Jpn.* **37**, 1297–1304.

KIMURA, I., GOTOH, Y. AND OZAWA, E. (1989). Further purification of a fibroblast growth factor-like factor from chick embryo extract by heparin-affinity chromatography. *In Vitro Cell Devl. Biol.* **25**, 236–242.

LANDIS, S. C. AND PATTERSON, P. H. (1981). Neural crest cell lineages. *Trends Neurosci.* **4**, 172–175.

LE DOUARIN, N. M. (1982). *The Neural Crest.* Cambridge, UK: Cambridge University Press.

LE LIEVRE, C. S., SCHWEIZER, G. G., ZILLER, C. M. AND LE DOUARIN, N. M. (1980). Restrictions of developmental capabilities in neural crest cell derivatives as tested by *in vivo* transplantation experiments. *Devl Biol.* **77**, 362–378.

LORING, J., GLIMELIUS, B. AND WESTON, J. A. (1982). Extracellular matrix materials influence quail neural crest cell differentiation *in vitro*. *Devl Biol.* **90**, 165–174.

MIGNATTI, P., TSUBOI, R., ROBBINS, E. AND RIFKIN, D. B. (1989). *In vitro* angiogenesis on the human amniotic membrane: Requirement for basic fibroblast growth factor-induced proteinases. *J. Cell Biol.* **108**, 671–682.

NAKAMURA, H. AND AYER-LE-LIEVRE, C. S. (1982). Mesectodermal capabilities of the trunk neural crest of birds. *J. Embryol. exp. Morph.* **70**, 1–18.

NAPOLI, P. AND DE DOMENICO, P. (1987). Primary melanotic Schwannoma of the spinal cord. *Appl. Pathol.* **5**, 253–260.

NICHOLS, D. H. AND WESTON, J. A. (1977). Melanogenesis in cultures of peripheral nervous tissue. I. The origin and prospective fate of cells giving rise to melanocytes. *Devl Biol.* **60**, 217–225.

PADGETT, C. S. AND IVEY, W. D. (1960). The normal embryology of the Coturnix quail. *Anat. Record* **137**, 1–12.

PLEASURE, D., KREIDER, B., SOBUE, G., ROSS, A. H., KOPROWSKI, H., SONNENFELD, K. H. AND RUBENSTEIN, A. E. (1986). Schwann-like cells cultured from human dermal neurofibromas: Immunohistological identification and response to Schwann cell mitogens. *Ann. N.Y. Acad. Sci.* **486**, 227–240.

RAFF, M. C. (1989). Glial cell diversification in the rat optic nerve. *Science* **243**, 1450–1455.

SATO, Y. AND RIFKIN, D. B. (1988). Autocrine activities of basic fibroblast growth factor: Regulation of endothelial cell movement, plasminogen activator synthesis, and DNA synthesis. *J. Cell Biol.* **107**, 1199–1205.

SCHUBERT, D., LING, N. AND BAIRD, A. (1987). Multiple influences of a heparin-binding growth factor on neuronal development. *J. Cell Biol.* **104**, 635–643.

SEARS, R. AND CIMENT, G. (1988). Changes in the migratory properties of neural crest and early crest-derived cells *in vivo* following treatment with a phorbol ester drug. *Devl Biol.* **130**, 133–143.

SEED, J., OLWIN, B. B. AND HAUSCHKA, S. D. (1988). Fibroblast growth factor levels in the whole embryo and limb bud during chick development. *Devl Biol.* **128**, 50–57.

SIEBER-BLUM, M. AND COHEN, A. M. (1980). Clonal analysis of quail neural crest cells: They are pluripotent and differentiate *in vitro* in the absence of non-crest cells. *Devl Biol.* **80**, 96–106.

SMITH, J. C. (1989). Mesoderm induction and mesoderm-inducing factors in early amphibian development. *Development* **105**, 665–677.

SMITH, J. C., COOKE, J., GREEN, J. B., HOWES, G. AND SYMES, U. (1989). Inducing factors and the control of mesodermal pattern in *Xenopus laevis*. *Development* **107**, 149–159.

STEFANSSON, K., WOLLMAN, R. AND JERKOVIC, M. (1982). S-100 protein in soft-tissue tumors derived from Schwann cells and melanocytes. *Am. J. Path.* **106**, 261–268.

TEILLET, M.-A. AND LEDOUARIN, N. M. (1970). La migration des cellules pigmentaires étudiée par la méthode des greffes hétérospécifiques de tube nerveux chez l'embryon d'Oiseau. *C.r. hebd. Séanc. Acad. Sci. Paris* **270**, 3095–3098.

UNSICKER, K., KRISCH, B., OTTEN, J. AND THOENEN, H. (1978). Nerve growth factor-induced fiber outgrowth from isolated rat adrenal chromaffin cells: impairment by glucocorticoids. *Proc. natn. Acad. Sci. U.S.A.* **75**, 3498–3502.

UNSICKER, K., REICHERT-PREIBSH, H., SCHMIDT, R., PETTMANN, B., LABOURDETTE, G. AND SENSENBRENNER, M. (1987). Astroglial and fibroblast growth factors have neurotrophic functions for cultured peripheral and central nervous system neurons. *Proc. natn. Acad. Sci. U.S.A.* **84**, 5459–5463.

WEEKS, D. L. AND MELTON, D. A. (1987). A maternal mRNA localized to the vegetal hemispheres in *Xenopus* eggs codes for a growth factor related to TGF-β. *Cell* **51**, 861–867.

WESTON, J. A. (1963). A radioautographic analysis of the migration and localization of trunk neural crest cells in the chick. *Devl Biol.* **6**, 279–310.

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