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BRIEF COMMUNICATION

Epidermal Growth Factor (EGF) Promotes the In Vitro Differentiation of Neural Crest Cells to Neurons and Melanocytes

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Abstract Proliferation of neural crest (NC) stem cells and their subsequent differentiation into different neural cell types are key early events in the development of the peripheral nervous system. Soluble growth factors present at the sites where NC cells migrate are critical to the development of NC derivatives in each part of the body. In the present study, we further investigate the effect of microenvironmental factors on quail trunk NC development. We show for the first time that EGF induces differentiation of NC to the neuronal and melanocytic phenotypes, while fibroblast growth factor 2 (FGF2) promotes NC differentiation to Schwann cells. In the presence of both EGF and FGF2, the neuronal differentiation predominates. Our results suggest that FGF2 stimulates gliogenesis, while EGF promotes melanogenesis and neurogenesis. The combination of both growth factors stimulates neurogenesis. These findings suggest that these two growth factors may play an important role in the fate decision of NC progenitors and in the development of the peripheral nervous system.

Keywords Neural crest · Glia · Neuron · Melanocyte · EGF · Stem cells · FGF2 · Peripheral nervous system

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Introduction

The neural crest (NC) is formed by the lateral borders of the neural primordium during neurulation. After extensive migration through embryonic tissues, NC cells differentiate into a large variety of cell types, including neurons and glial cells of the peripheral nervous system, melanocytes, and some endocrine cells. Cephalic NC cells yield adiposities, chondrocytes, osteocytes, forebrain meninges, connective tissue cells in gland and muscles, the cardiac outflow tract and the vascular smooth muscle cells associated with the vessels derived from the aortic arches and those that supply blood to the face and forebrain (Le Douarin and Kalcheim 1999; Le Douarin et al. 2008). The existence of a high number of multipotent NC progenitors endowed with both chondrogenic and neural potentials has been recently described (Calloni et al. 2007). In addition, both avian and mammalian NC cells are able to self-renew in vitro, demonstrating that subsets of NC cells display true stem cell properties (Stemple and Anderson 1992; Trentin et al. 2004).

Soluble growth factors play a role in NC development. Among these growth factors, fibroblast growth factor 2 (FGF2) has been demonstrated to promote the survival and proliferation of NC cells (Kalcheim 1989; Murphy et al. 1994) and to regulate their migration (Kubota and Ito 2000). In addition, FGF2 has been reported to promote chondrogenesis, skeletogenesis, and gliogenesis in neural

crest of both quail and mouse embryos, and at cephalic and trunk levels (Sarkar et al. 2001; Petiot et al. 2002; Ido and Ito 2006; Ota and Ito 2006; Ijuin et al. 2008). Epidermal growth factor (EGF) has also been suggested to play a role in quail NC production of proteoglycans and proliferation in vitro (Erickson and Turley 1987). However, the role of EGF in NC differentiation has never been investigated. In this paper, for the first time, we show that EGF promotes the differentiation of quail trunk NC cells to the neuronal and melanocytic phenotypes. In addition, we further demonstrate the effects of FGF2 in stimulating NC differentiation to Schwann cells.

Methods

Neural tubes obtained from quail embryos (18–25 somite stage) were dissected at the trunk level and plated in uncoated plastic culture dishes (Corning) in the basal medium α-minimum essential medium (α-MEM; Gibco-BRL), enriched with 10% fetal bovine serum (FCS, Cultlab) and 2% chicken embryonic extract (EE). After 24 h, emigrated NC cells were harvested for secondary plating in mass cultures (400 cells per well of a 96-well plate). Plastic dishes for secondary culture were coated with type I collagen (Sigma). Cultures were maintained for an additional 6 days in a complex medium containing basal medium enriched with growth factors and hormones (Trentin et al. 2004). The treatment with human recombinant FGF-2 (20 ng/ml, Sigma) and/or mouse submaxillary gland EGF (10 ng/ml, Sigma) was added during the primary culture (first 24 h of culture).

Cell phenotypes were analyzed using lineage-specific markers. Mature melanocytes were identified by the presence of melanin, smooth muscle cells by immunoreactivity to mouse α-smooth muscle actin (αSMA) mAb (Sigma), glial cells by staining with Schwann cell myelin protein (SMP) mAb (Dulac et al. 1988), and neurons by staining with tyrosine hydroxylase (TH) mAb (Fauquet and Ziller 1989), βIII-Tubulin (βIII-Tub) mAb (Promega). Detailed procedures are described elsewhere (Dupin et al. 2000; Trentin et al. 2004). Cell nuclei were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma). Fluorescent labeling was observed under an epifluorescence microscope (Olympus IX71). Pigmented cells were quantified in macroscopic views by evaluation of the intensity of melanin expression as arbitrary densitometric units (Scion, Image, National Institutes of Health) (Fig. 1). Quantification of phenotypic markers was performed at day 6 of culture by direct cell counting of positive cells in relation to total cell population (Fig. 2).

Statistical differences were evaluated by means of one-way ANOVA with Turkey's post test using the GraphPad Prism software (GraphPad Software, Inc.). The level of significance was set at $P < 0.05$ in all cases.

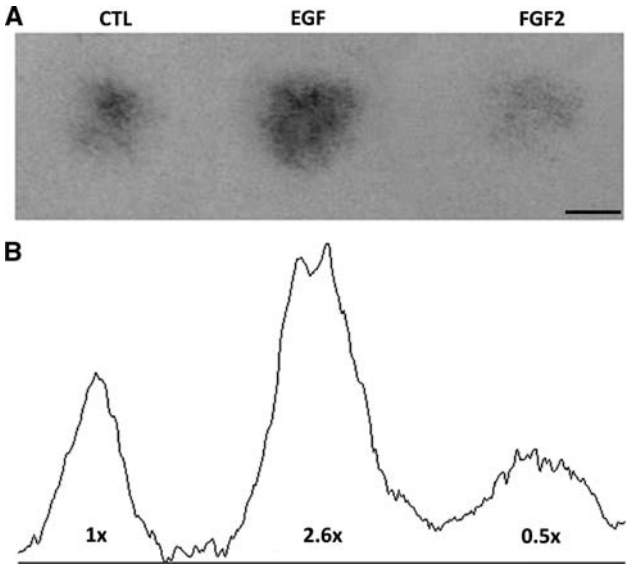

Fig. 1 Macroscopic views (**a**) and quantification (**b**) of the pigmented cells (melanocytes) in 6-day NC secondary cultures. Cultures were maintained in basic medium (CTL, control) or treated with FGF2 or EGF during the 24 h of primary culture, and then in the complex medium during the 6 days of secondary culture as described in "Methods". Pigmented cells were quantified in macroscopic views by evaluation of the intensity of melanin expression as arbitrary densitometric units. *Bar* = 5 mm

Results

Macroscopic views of NC cultures revealed that EGF treatment increased the number of pigmented cells (melanocytes) by 2.6-fold relative to the control. In contrast, the number of melanocytes was reduced by twofold after FGF2 treatment (Fig. 1). Figure 2 shows representative pictures of melanocytes (melanin-expressing cells), smooth muscle cells (αSMA^+cells), Schwann cells (SMP^+cells), and neurons ($\beta IIItub/TH^+$cells), maintained in control conditions, or treated with EGF, FGF2 or a combination of both (EGF + FGF2). Confirming the results from the macroscopic views, quantitative analysis of cells positive for these phenotypic markers revealed that melanocytes represented 13% of EGF-treated cells, about 2.8-fold more than the percentage obtained in control conditions, after treatment with FGF2 or EGF + FGF2 (Fig. 3a). In contrast, the proportion of smooth muscle cells was similar in all culture conditions (about 2% of the total cell population) (Fig. 3b). The highest proportion of Schwann cells was observed in FGF2-treated cultures (33%), an increase of approximately twofold when compared with all other

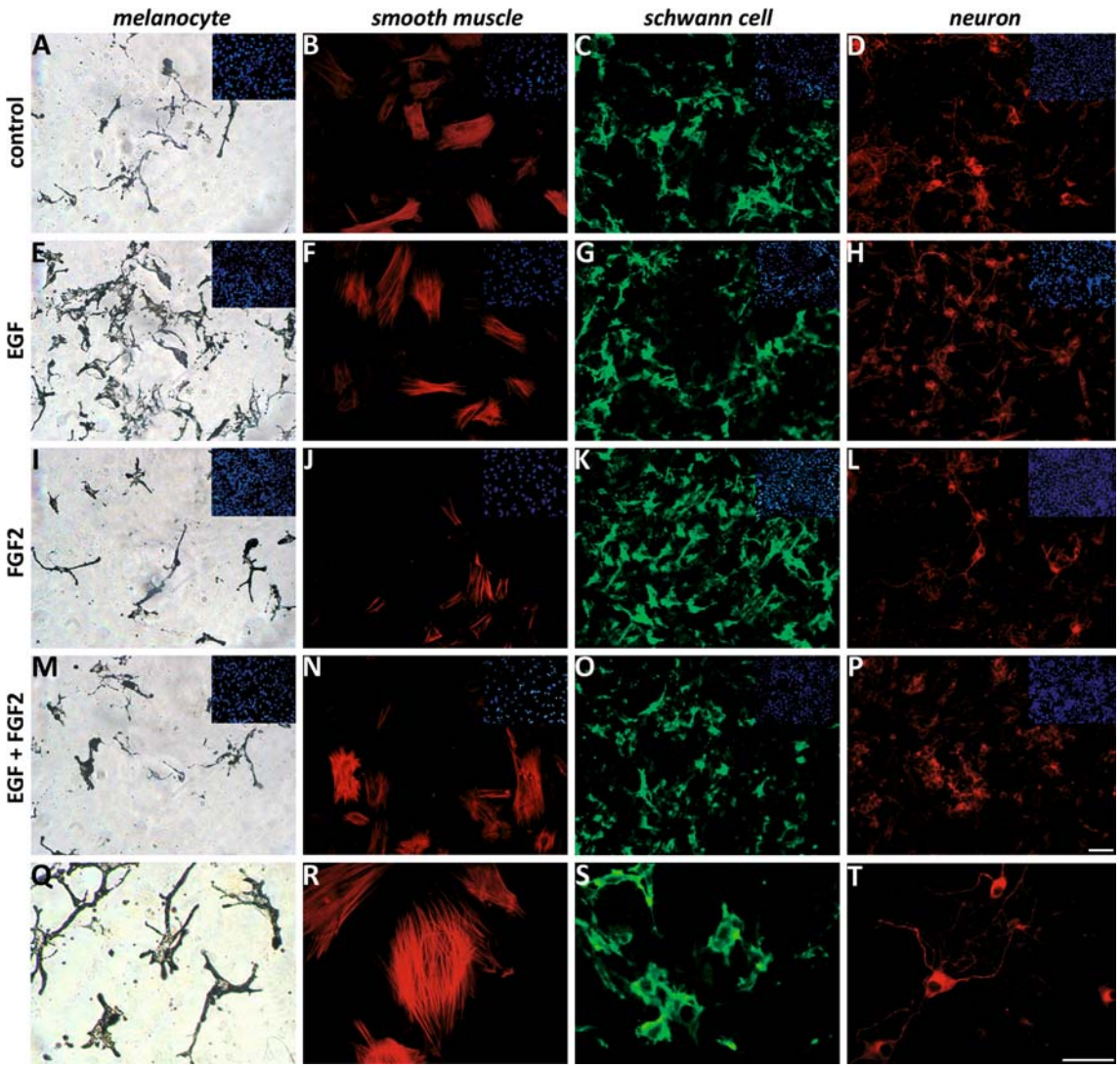

Fig. 2 Phenotypic analysis of cultured NC cells at day 6 of secondary culture. NC cells were grown and treated in primary cultures as described in Fig. 1 and in "Methods". Control cells (**a**–**d**), EGF (**e**–**h**), FGF2 (**i**–**l**), and EGF + FGF2 (**m**–**p**). Cell phenotypes were identified by the presence of specific cell markers: melanin for melanocytes (**a**, **e**, **i**, **m**, **q**), αSMA for smooth muscle cells (**b**, **f**, **j**, **n**, **r**), SMP for Schwann cells (**c**, **g**, **k**, **o**, **s**), and β-III-Tub and TH for neurons (**d**, **h**, **l**, **p**, **t**). Higher magnification pictures of melanocytes, smooth muscle cells, glial cells and neurons (**q**, **r**, **s**, **t**, respectively). *Inserts* represent the nuclei staining with DAPI. *Scale bar* = 50 μm

culture conditions (Fig. 3c). Neurons comprised about 3.1–3.4% of the total cell population after the EGF and EGF + FGF2 treatments, about 3.8-fold and 4-fold, respectively, more than in the control and FGF2-treated cultures (Fig. 3d). In our culture conditions, every neuron was positive for βIII-tub, and some of them also expressed TH. Neurons were observed for at least 10 days in culture. Our experiments also revealed that EGF treatment during the first 24 h of primary culture was critical for the observed increase in the percentage of neurons and pigmented cells, while treatment during the secondary culture period (24 h to 6 days) did not affect the proportion of these cell types relative to control (data not shown).

Discussion

In the present work, we provide the first evidence that EGF is an important soluble factor involved in the development of the peripheral nervous system. We show, for the first time, that this growth factor promotes the neuronal differentiation of embryonic NC cells, and also stimulates melanogenesis. In contrast, FGF2 stimulates gliogenesis by NC cells.

Soluble growth factors present at the sites where NC cells migrate are critical to the development of NC derivatives in each part of the body. Such growth factors include bone morphogenetic protein (BMP) 2/4, which drive NC cells to adopt autonomic sympathetic-like neuronal fate

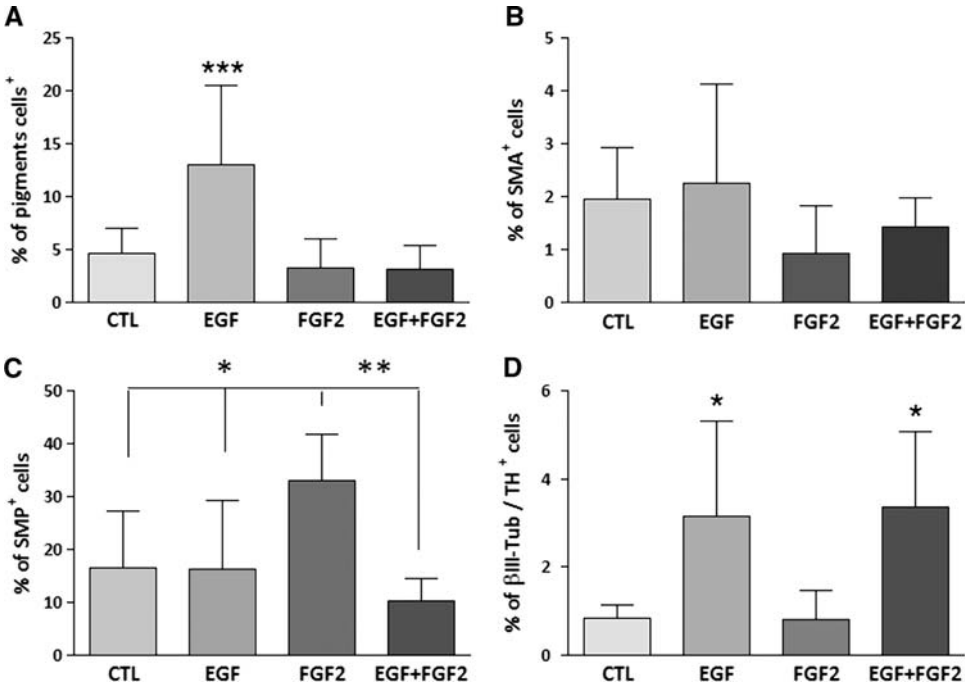

Fig. 3 Quantification of phenotypic markers at day 6 of secondary cultures. NC cells were grown and treated in primary cultures as described in Fig. 1 and in "Methods". (**a**) Melanocytes, (**b**) smooth muscle cells, (**c**) Schwann cells, and (**d**) neurons. Values were obtained from the analysis of 70 random fields of six independent experiments on each culture condition. Results are expressed as the mean ± SEM. $*P < 0.01$, $**P < 0.01$, $***P < 0.001$ by one-way ANOVA with Turkey's post hoc test

(Shah et al. 1996; Shah and Anderson 1997), neuregulin-1 and Notch ligands which favor gliogenesis (Morrison et al. 2000; Ijuin et al. 2008), and endothelin-3 (ET3) which promotes survival and proliferation of glial-melanocytic (GM) bipotent precursors as well as committed melanocytic and glial cells (Lahav et al. 1996, 1998; Trentin et al. 2004). Interestingly, human NC cells show a molecular profile similar to embryonic stem cells when cultured in the presence of EGF and FGF2 (Thomas et al. 2008). The multipotent NC progenitor that gives rise to glial cells, neurons, melanocytes, smooth muscle cells, and chondrocytes, which is stimulated by sonic hedgehog (Shh) (Calloni et al. 2007), which may act by affecting the proliferation of this progenitor. Interestingly, FGF2 treatment inhibits the proliferative effect of Shh on cerebellar granule cell precursors (Fogarty et al. 2007). Furthermore, FGF2 may play a role in regulating the survival, proliferation, and migration of NC cells, in addition to promoting gliogenesis, chondrogenesis, and skeletogenesis (Kalcheim 1989; Murphy et al. 1994; Kubota and Ito 2000; Sarkar et al. 2001; Petiot et al. 2002; Ota and Ito 2006; Ijuin et al. 2008).

Our results reinforce these findings, and further demonstrate the role of FGF2 in stimulating the differentiation of NC progenitors to Schwann cells. Our results also reveal the important role of EGF in promoting NC differentiation to the neuronal and melanocytic fates. The present data demonstrate that EGF treatment during the first 24 h, when most of the NC cells are undifferentiated (Nakanishi et al. 2007), is critical for the observed effect, suggesting that EGF acts at the early stages of neurogenesis and melanogenesis. FGF2 and EGF seem to have a preventive effect on melanogenesis and gliogenesis, respectively, since in the combined treatment condition, FGF2 inhibited the positive effect of EGF on melanocyte differentiation, whereas EGF inhibited the positive effect of FGF2 on Schwann cell differentiation. However, FGF2 did not alter the positive effect of EGF on neurogenesis, suggesting that this differentiation pathway might predominate in the presence of both growth factors (Figs. 2, 3).

Since NC is comprised progenitors endowed with different potentialities, including the oligopotent glial/neuronal/melanocytic (GNM) progenitor (Trentin et al. 2004), our results suggest a possible involvement of EGF and FGF2 in the fate decision of NC stem cells. FGF2 might stimulate the differentiation of the GNM progenitor into the glial phenotype, whereas EGF may promote entry into the neuronal and melanocytic pathways. However, in the presence of both growth factors, neuronal differentiation seems to predominate.

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References

Calloni GW, Glavieux-Pardanaud C, Le Douarin NM, Dupin E (2007) Sonic Hedgehog promotes the development of multipotent

neural crest progenitors endowed with both mesenchymal and neural potentials. Proc Natl Acad Sci USA 104:19879–19884

Dulac C, Cameron-Curry P, Ziller C, Le Douarin NM (1988) A surface protein expressed by avian myelinating and nonmyelinating Schwann cells but not by satellite or enteric glial cells. Neuron 1:211–220

Dupin E, Glavieux C, Vaigot P, Le Douarin NM (2000) Endothelin 3 induces the reversion of melanocytes to glia through a neural crest-derived glial-melanocytic progenitor. Proc Natl Acad Sci USA 97:7882–7887

Erickson CA, Turley EA (1987) The effects of epidermal growth factor on neural crest cells in tissue culture. Exp Cell Res 169:267–279

Fauquet M, Ziller C (1989) A monoclonal antibody directed against quail tyrosine hydroxylase: description and use in immunocytochemical studies on differentiating neural crest cells. J Histochem Cytochem 37:1197–1205

Fogarty MP, Emmenegger BA, Grasfeder LL, Oliver TG, Wechsler-Reya RJ (2007) Fibroblast growth factor blocks Sonic hedgehog signaling in neuronal precursors and tumor cells. Proc Natl Acad Sci USA 104:2973–2978

Ido A, Ito K (2006) Expression of chondrogenic potential of mouse trunk neural crest cells by FGF2 treatment. Dev Dyn 235:361–367

Ijuin K, Nakanishi K, Ito K (2008) Different downstream pathways for Notch signaling are required for gliogenic and chondrogenic specification of mouse mesencephalic neural crest cells. Mech Dev 125:462–474

Kalcheim C (1989) Basic fibroblast growth factor stimulates survival of nonneuronal cells developing from trunk neural crest. Dev Biol 134:1–10

Kubota Y, Ito K (2000) Chemotactic migration of mesencephalic neural crest cells in the mouse. Dev Dyn 217:170–179

Lahav R, Ziller C, Dupin E, Le Douarin NM (1996) Endothelin 3 promotes neural crest cell proliferation and mediates a vast increase in melanocyte number in culture. Proc Natl Acad Sci USA 93:3892–3897

Lahav R, Dupin E, Lecoin L, Glavieux C, Champeval D, Ziller C, Le Douarin NM (1998) Endothelin 3 selectively promotes survival and proliferation of neural crest-derived glial and melanocytic precursors in vitro. Proc Natl Acad Sci USA 95:14214–14219

Le Douarin NM, Kalcheim C (1999) The neural crest. Cambridge Universtiy Press, Cambridge

Le Douarin NM, Calloni GW, Dupin E (2008) The stem cells of the neural crest. Cell Cycle 7:1013–1019

Morrison SJ, Perez SE, Qiao Z, Verdi JM, Hicks C, Weinmaster G, Anderson DJ (2000) Transient Notch activation initiates an irreversible switch from neurogenesis to gliogenesis by neural crest stem cells. Cell 101:499–510

Murphy M, Reid K, Ford M, Furness JB, Bartlett PF (1994) FGF2 regulates proliferation of neural crest cells, with subsequent neuronal differentiation regulated by LIF or related factors. Development (Cambridge, England) 120:3519–3528

Nakanishi K, Chan YS, Ito K (2007) Notch signaling is required for the chondrogenic specification of mouse mesencephalic neural crest cells. Mech Dev 124:190–203

Ota M, Ito K (2006) BMP and FGF-2 regulate neurogenin-2 expression and the differentiation of sensory neurons and glia. Dev Dyn 235:646–655

Petiot A, Ferretti P, Copp AJ, Chan CT (2002) Induction of chondrogenesis in neural crest cells by mutant fibroblast growth factor receptors. Dev Dyn 224:210–221

Sarkar S, Petiot A, Copp A, Ferretti P, Thorogood P (2001) FGF2 promotes skeletogenic differentiation of cranial neural crest cells. Development (Cambridge, England) 128:2143–2152

Shah NM, Anderson DJ (1997) Integration of multiple instructive cues by neural crest stem cells reveals cell-intrinsic biases in relative growth factor responsiveness. Proc Natl Acad Sci USA 94:11369–11374

Shah NM, Groves AK, Anderson DJ (1996) Alternative neural crest cell fates are instructively promoted by TGFbeta superfamily members. Cell 85:331–343

Stemple DL, Anderson DJ (1992) Isolation of a stem cell for neurons and glia from the mammalian neural crest. Cell 71:973–985

Thomas S, Thomas M, Wincker P, Babarit C, Xu P, Speer MC, Munnich A, Lyonnet S, Vekemans M, Etchevers HC (2008) Human neural crest cells display molecular and phenotypic hallmarks of stem cells. Hum Mol Genet 17:3411–3425

Trentin A, Glavieux-Pardanaud C, Le Douarin NM, Dupin E (2004) Self-renewal capacity is a widespread property of various types of neural crest precursor cells. Proc Natl Acad Sci USA 101:4495–4500