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Insights into Stem Cell Factor chemotactic guidance of neural crest cells revealed by a real-time directionality-based assay

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ABSTRACT

The extracellular environment through which neural crest cells (NCCs) translocate and differentiate plays a crucial role in the determination of cell migration and homing. In the trunk, NCC-derived melanocyte precursor cells (MPCs) take the dorsolateral pathway and colonize the skin, where they differentiate into pigment cells (PCs). Our hypothesis was that the skin, the MPCs' target tissue, may induce a directional response of NCCs toward diffusible factor(s). We show that the treatment of in vitro NCCs with skin extract (SE) or Stem Cell Factor (SCF) contributes to maintaining proliferative activity, accelerates melanocyte differentiation, and guides a subpopulation of NCCs by chemotaxis toward the gradient source of these factors, suggesting that they may represent the MPCs' subpopulation. Current data on stimulated directional persistence of NCCs supports the participation of diffusible molecules in the target colonization mechanism, guiding MPCs to migrate and invade the skin. Our results show similar effects of SE and SCF on NCC growth, proliferation and pigment cell differentiation. Also, the use of a proven real-time directionality-based objective assay shows the directional migration of NCCs toward SE and SCF, indicating that the epidermal SCF molecule may be involved in the chemotactic guidance mechanism of in vivo NCCs. Although SCF is the strongest candidate to account for these phenomena, the nature of other factor(s) affecting NCC-oriented migration remains to be investigated. This data amplifies the functional scope of trophic factors by involving them in new cell behaviors such as molecular guidance in the colonization mechanism of embryonic cells.

Introduction

In recent years, growing knowledge about cell communication at a distance has enabled the re-discovery of chemotactic phenomena, conceived as directional cell migration induced by a concentration gradient of soluble factors segregated by "target" fields. This molecular mechanism of cell orientation is well known in various biological systems such as flagellated bacteria (Chen et al., 2003), amoebas (van Haastert et al., 2007), leukocytes (Gómez-Moutón et al., 2004), neurons (Ng et al., 2005; Paratcha et al., 2006), and axonal growth cones (Charron and Tessier-Lavigne, 2005; Mortimer et al., 2008; von Philipsborn and Bastmeyer, 2007). In our laboratory, we made one of the first descriptions of the chemotaxis of mammal sperm toward the ovular region (Fabro et al., 2002; Giojalas and Rovasio, 1998; Giojalas et al., 2004a,b; Marín et al., 1995; Oliveira et al., 1999; Rovasio et al., 1994; Sun et al., 2003), with progesterone signals being the strongest attractant responsible (Guidobaldi et al., 2008; Teves et al., 2006). On the other hand, it is surprising that the embryonic cells, paradigmatic of accurately moving cells, have practically not been dealt with yet from a "chemotactic point of view" except in a few well documented systems on mouse embryos (Belmadani et al., 2005; Kubota and Ito, 2000; Natarajan et al., 2002; Young et al., 2004) and avian ambryos (Kasemeier-Kulesa et al., 2010; Tosney, 2004).

It is known that neural crest cells (NCCs), after segregating from the closing neural tube as a multipotent cell population, migrate along defined pathways and colonize precise sites giving rise to a variety of cell types, such as neurons, glia, cartilage and pigment cells (PCs) (Le Douarin and Kalcheim, 1999). In the modulation of such complex behaviors, the balance between the signals of a "central program" and those coming from the near milieu is not yet clear (see reviews by Kulesa et al., 2010; Krispin et al., 2010; Lock et al., 2008). However, there is growing evidence that indicates that the efficient migration and distribution of NCCs depends on coordinated genetic and epigenetic factors, specially triggered by micro-environmental signals (Kee et al., 2007; Le Douarin et al., 2007; Lock et al., 2008; Kulesa et al., 2010; Matthews et al., 2008a,b; Rovasio et al., 1983; Sauka-Spengler and Bronner-Fraser, 2008; Teddy and Kulesa, 2004; Wehrle-Haller et al., 2001). However, these factors are not sufficient to fully explain the oriented migration of NCCs. Moreover, the haptotaxis and galvanotaxis mechanisms lack sufficient supporting evidence

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(Le Douarin and Kalcheim, 1999), and the rejection of a chemotactic mechanism claimed by some authors is not endorsed by rigorous experimental trials (Carmona-Fontaine et al., 2008; Erickson, 1985; Matthews et al., 2008a,b).

In the trunk, NCCs migrate in a dorsoventral pathway between the neural tube and the cephalic half of the somitic mesoderm (Le Douarin and Dupin, 1993), and a second wave between the somites and ectoderm is taken by the subpopulation of melanocyte precursor cells (MPCs) homing to skin (Erickson and Goins, 1995). The PCs are one of the most extensively studied NCC derivatives, since the pigment itself constitutes a unique differentiation marker. Moreover, it is known that the relationships between MPCs and their developmental milieu, and their timing, play key roles in the definition of PC distribution (Faas and Rovasio, 1998; Fukuzawa et al., 1995; Thibodeau and Frost-Mason, 1992).

In the avian embryo, MPCs colonize the skin between day 3 and 3.5 of development (Richardson and Sieber-Blum, 1993), and massively invade from day 5 to 6, when they proliferate (6th–8th day) and then differentiate into PCs from day 9 of development (Teillet, 1971). In a previous report, we showed that the spatial distribution of MPCs varies according to both the axial level and the developmental stage of the embryo; furthermore, the observed general pattern of the centrifugal double wave of cell distribution may be attributed to a different timing of cell differentiation, closely related to their migratory behavior (Faas and Rovasio, 1998). Considering the intrinsic motion capacity of NCCs together with the precisely timed colonization in the epidermis by MPCs, in both avian and mammalian embryos, it was suggested that the mechanism involved could be through a chemoattractant activity of the skin, its target tissue (Le Douarin and Kalcheim, 1999; Tosney, 2004). Thus, as with some other developmental systems, such as angiogenesis (Czirok et al., 2008), lymphocyte transmigration and homing (Cinamon et al., 2001), or genital ridge colonization by primordial germ cells (Boldajipour and Raz, 2007), the skin's product(s) could be involved in the regulation of PC colonization and development. Although the molecular cues to account for the proposed directional mechanism are not known, one of the strongest candidates is Stem Cell Factor (SCF), considering its in vivo expression in the target region (epidermis) of the NCC-derived MPCs, as well as the expression of its C-Kit receptor on this cell population (Lahav et al., 1994; Lecoin et al., 1995; see also Tosney, 2004). These molecular facts display a spatiotemporal coherence with the NCC colonization of the skin as well as their involvement with the differentiation of the pigment phenotype (Kunisada et al., 1998).

In this context, the present work reports the first direct evidence about the influence of embryo skin diffusible factors and SCF on the growth, proliferation, melanocyte differentiation and chemotactically oriented migration of the NCC subpopulation. These findings support the idea that SCF may serve as a specific chemoattractant for in vivo guidance of NCCs as well as having its canonic growth factor activity, amplifying the functional scope of trophic factors by involving them in new activities as molecular guides for embryonic cells. Last, but not least, the results here reported on turning responses of NCCs, in particular extracellular gradients of SCF, were obtained using a computer-based real-time video system and a software based on strictly proven objective directional criteria (Fabro et al., 2002; Giojalas and Rovasio, 1998; Ming et al., 1997, 1999; Rovasio et al., 1994; Song et al., 1997, 1998; Zheng et al., 1994).

Materials and methods

Skin extract

Quail embryos (Coturnix coturnix japonica) at 6–6.5 days of development (stages 22–23 of Zacchei, 1961) were incubated in deionized MilliQ water (Millipore Corp., Billerica, MA, USA) at 37 °C for 20 min, and the dorsal skin was mechanically removed using fine tweezers. The skin obtained from 63 embryos was centrifuged for 2 min at 5600 × *g* to remove the excess water, resuspended in N2 basal medium (Bottenstein and Sato, 1979) (50% DMEM and 50% F12 media, plus 15 mM sodium bicarbonate, 15 mM HEPES buffer, 50 IU/ml G sodium penicillin and 50 μg/ml streptomycin sulfate) without serum and homogenized in an ice bath. The homogenate was centrifuged for 15 min at 170,000 × *g* to remove cell debris, and the supernatant obtained was sterilized by filtration (0.22 μm, Renner GMBH, Dannstadt, Germany), aliquoted and stored at −40 °C until use. The total protein concentration was determined on the skin extract (SE) by Bradford's method (Bradford, 1976) and was taken as reference for the dilutions utilized. SDS-PAGE and Western blot analyses were performed using standard protocols on SE following conventional electrophoresis on 15% SDS-polyacrylamide gels. After transfer to polyvinylidene difluoride membranes (Millipore Corp., Billerica, MA, USA), primary antibody against SCF (rabbit polyclonal ab9753; 1 μg/ml; Abcam Inc., Cambridge, MA, USA) was used. Rat brain tissue lysate (Cat.# 1463; 20 μg per lane; ProSci Inc., Poway, CA, USA) and SCF (S7901; 1 per lane; Sigma Chem. Co., St Louis, MO, USA) were running as positive controls, all according to the indicated manufacturer's instructions.

Cell culture

Primary cultures of NCCs were made from mesencephalic and trunk levels of chick embryos (Gallus gallus, Cobb line) incubated at 38 ± 1 °C in a humidified atmosphere up to stage 11–14 HH (Hamburger and Hamilton, 1951; Rovasio and Battiato, 2002; Jaurena et al., 2011). Briefly, after cutting and opening the ectoderm, mesencephalic NCCs were carefully obtained by microdissection from the mass of NCCs bilateral to the neural tube, transferred to coverslips precoated with fibronectin (Rovasio et al., 1983), and incubated in 35 mm Petri dishes (Sigma Chem. Co., St Louis, MO, USA) with 2 ml of N2 defined medium (N2 basal medium plus 5 μg/ml insulin, 100 μg/ml transferrin, 20 nM progesterone, 100 μM putrescine and 30 nM selenium in 100 ml of medium) (Bottenstein and Sato, 1979) supplemented with 10% fetal calf serum (FCS) (Sigma Chem. Co., St Louis, MO, USA) during 24 h at 37 ± 0.2 °C in 5% CO_2 in air. Trunk explants corresponding to the last five to seven somite pairs were cut away and incubated with 200 μg/ml of dispase (Calbiochem Corp., San Diego, CA, USA) in N2 defined medium without serum for 15 min at room temperature. The segments of neural tube were isolated, washed with N2 medium plus 10% FCS, transferred to coverslips and incubated as explained. After 24 h, the neural tube explant was removed from the trunk NCC cultures to discard the possible source of undesired diffusible molecules, then the cultures were treated with 100 μl SE (or N2 defined medium = control), changing 50% of the culture medium every day during the time indicated below and in the Results section. Equivalent cultures, after neural tube explant removal, were utilized in chemotactic experiments.

Applying the careful microdissection technique described above, the degree of purity of NCC cultures was constantly near 100%, without neural tube, ectoderm and/or mesoderm contaminants. If some culture contained tissue contaminants, they were detected by phase contrast microscopy and NC-1 immunolabeling (Rovasio et al., 1983; Rovasio and Battiato, 1996), and consequently discarded. To verify for equal numbers of cells in control and SE/SCF-treated conditions at the beginning of the experiments, an estimation of NCC population considered the total cell number per explant at the start of the corresponding treatments (see Table 1 and Fig. 2, insets). Assuming that, operatively and based on embryo age, the initial size of the explants could be considered constant, we calculated the cell number per explant as statistically