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Forskolin Effect on the Lineage Specification of Trunk Neural Crest Cells *in Vitro*

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Recent evidence has suggested that trunk neural crest cell generally assumed to have equivalent differentiation potentials, demonstrate differentiation bias along the anterior/posterior axis. In amphibian and fish, neural crest cells give rise to three chromatophore types, melanophores, xanthophores, and iridophores. Each pigment cell type has distinct characteristics but there is speculation about the cellular plasticity that exists among them. Neural crest cells migrate along specific routes, ventromedially and dorsolaterally. Neural crest cells that travel dorsolaterally are the first cells to begin migration in the axolotl and are the major contributors to the visible pigment pattern. Many factors and mechanisms that are responsible for guiding migratory neural crest cells along potential pathways or determining their fate remain unknown. A single lineage of the crest, which becomes restricted to one of the three pigment cell types, gives us the opportunity to examine the existence of neural crest stem cell populations and cellular plasticity. Study presented here showed results from recent in vitro studies designed to identify parameters influencing differentiation events of individual neural crest-derived pigment cell lineages. Melanophore production from neural crest explants originating from different levels along the anterior/posterior axis of wild type-axolotl embryos were compared and demonstrate that the differentiation of melanophores is enhanced in subpopulation of neural crest treated with forskolin. Forskolin (an adenylate cyclase activator) increases intracellular cAMP concentration and eventually activates the protein kinase-A signaling pathway. Melanophore number, melanin content, and tyrosinase activity in explants taken from the anterior-most region of the crest increased significantly in response to forskolin treatment. This study suggests implications of region specific influences and developmental regulation in the development of pigment pattern.

Early embryonic cells are totipotent, but as development proceeds cells give rise to specific cell and tissue types. Genetic mechanisms that control cell fate decisions made by developmentally equivalent cells are being identified (Ghysen et al., 1993), but those controlling the differentiation of specific cell types from pluripotent stem cells are less well understood. The neural crest gives rise to a diversity of cell types that share a common embryonic origin and exhibit signs of cellular plasticity. Cells of the neural crest arise early during embryogenesis when neural folds are closing. These cells form a ridge atop the developing neural tube, begin migrating to locations sometimes far from their origin, and gradually take up residence in positions conducive to their differentiation into an appropriate cell type. Derivatives of the neural crest include cells of the cranio-facial structures, peripheral nerves, various endocrine glands, and all dermal and epidermal pigment cells (Le Douarin, 1982). However, the specific factors and mechanisms that are responsible for guiding these migratory cells along their pathways or determining their fate remain unclear. Previous studies have demonstrated that neural crest cells can indeed be instructively influenced to differentiate along specific cell lineages (Stemple and Anderson, 1992; Thibaudeau and Frost-Mason, 1992). The Mexican axolotl (*Ambystoma mexicanum*), a neotenic aquatic salamander, provides a number of experimental opportunities for examining neural crest cell behavior. Wild-type axolotl pigmentation results from the presence of three pigment cell types: melanophores (melanin-containing cells), xanthophores (yellow pteridine-containing cells), and iridophores (reflective purine-containing cells). In wild-type axolotl larvae, pigment cell numbers continue to increase, eventually producing a mottled olive gray pigment pattern (Frost et al., 1984). Observations of pigment-mutant phenotypes and alteration of pigmentation that occurs after

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drug treatment in axolotls in vivo suggest considerable plasticity in the pigment cell lineage of the neural crest (Frost and Bagnara, 1979; Frost et al., 1987; Frost, 1988; Thibaudeau and Holder 1998). Thibaudeau et al. (1998) suggested the existence of spatial and temporal differences among premigratory trunk neural crest cells under minimal culture conditions and in response to guanosine treatment.

This study presents results from recent in vitro experiments designed to identify parameters influencing differentiation events of individual neural crest-derived pigment cell lineages and also discusses implications of forskolin (an adenylate cyclase activator) effect on and exploitation of region specific influences and responses.

Material and Methods

Axolotl embryos obtained from the Indiana University Axolotl Colony were reared in Holtfreters solution until they reached the desired developmental stage of Bordzilovskaya and Detlaff (1979). Each clutch of wild-type embryos was raised to stage 24, a time when the crest has just formed. Operations were carried out under standard sterile conditions. A strip of dorsal ectoderm was removed with tungsten microneedles to expose the neural crest located head to tail on top of the neural tube. The neural crest corresponding to the level of somites 2-9 was removed from stage-24 embryos. This strip of neural crest was divided into four explants of equal size, approximately two somites long. Of the four explants, only the most anterior and most posterior explants were transferred to fibronectin-coated wells of a 24-well tissue culture plate containing 0.5 mL of culture medium. The standard culture media used was 50% Leibovitz-15, supplemented with antibiotics. For studies of melanophore differentiation, an equal number of explants were cultured in this medium in the absence or presence of 2 µM forskolin (Jin and Thibaudeau, 1999). The origin of each explant was designated as the anteriormost explant (A; crest at the level of somites 2-3) and a posteriormost explant (P; crest at the level of somites 8-9). For each embryo, anteriormost and posteriormost regions were cultured in a similar fashion. Counts of melanophores and the total number of cells that migrated away from the explants were recorded at 1, 3, and 5 d after cultures were started. Statistical analysis was performed using Fishers-protected LSD ($p < 0.05$). Different letters represent a significant difference.

In order to observe differences in the responses of anterior versus posterior explants under control conditions and after forskolin treatment, neural crest cultures were also analyzed for melanin content and tyrosinase activity. In preparation for melanin content analysis, cells of forskolin-treated and control cultures were digested with 0.5 mL of sodium hydroxide after 3 or 5 d of culture. Optical densities were determined at 414 nm 24 h after cultures were digested. A standard curve of synthetic melanin was run to calculate melanin content and make appropriate comparisons. In preparation for tyrosinase activity assays, 3 or 5 d post culture forskolin-treated and control cultures were washed with TBS and lysed in 1% Triton X-100/TBS with agitation. Five microliter of 10 mM L-DOPA were added to each lysate. Following incubation at 37℃ for 30 min, the absorbance was measured at 475 nm. Data were standardized with mushroom tyrosinase to calculate tyrosinase activity. The experiments were repeated 5 times and 12 explants for each experimental group were used for each experiment ($n = 60$ explants per each experimental group).

Results

In the experiment presented here neural crest cultures originating from different axial levels responded differently depending on their original location along the anterior/posterior axis and also responded differently to forskolin treatment. Such differences were most evident when explants were cultured under serum-free conditions in the presence of forskolin. Explants from the anteriormost and posteriormost regions of stage-24 embryos underwent similar migratory activities and both demonstrated an ability to differentiate melanophores. The posterior region however produced significantly more melanophores compared to anterior regions (compare Fig. 1A and 1C). Anterior and posterior regions also differed in their response to forskolin treatment. Anterior regions produced more melanophores in response to forskolin treatment by 5 d post culture when compared to controls (compare Fig. 1A and 1B). The increase in gross pigmentation of treated cultures was dramatic. A similar comparison could not be made in explants from posterior regions. That is, explants from the posteriormost region of the crest displayed no differences in pigment cell composition compared to their appropriate untreated control cultures (compare Fig. 1C and 1D) in response to the forskolin treatment.

Results presented in Fig. 2 demonstrate no significant differences in total cell numbers between control explants taken from the anteriormost and those taken from posteriormost regions of the crest at day 1, 3, or 5 after explantation. Similarly, no significant differences in total cell numbers were observed in the forskolin treated cultures at day 1, 3, or 5 post explantation. The conclusion therefore was that the increase in gross pigmentation in forskolin-treated anterior cultures was not simply an increase in total cell number, but must be due to an increase in the number of differentiated melanophores or in melanization.

Although the total cell numbers were similar, the cellular composition of anterior vs. posterior cultures was significantly different. That is, a much larger percent of the cells that had migrated away from

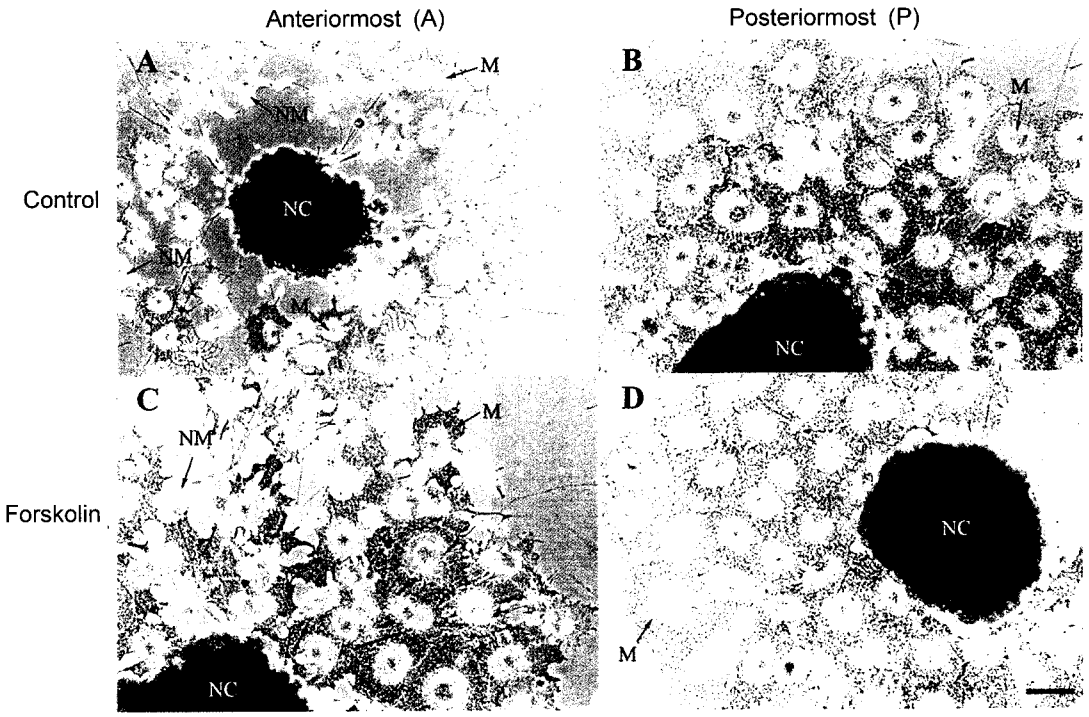

Fig. 1. Representative photos of explant culture on 5 days after forskolin treatment (C, D) compared to appropriate control (A, B). Under normal condition, premigratory trunk neural crest form different regions of origin showed differences in differentiation potentials such that posteriormost (P) regions of the crest consistently gave rise to more melanophores than anteriormost (A) regions (A, B). However, anteriormost regions produced more melanophores in the response to forskolin treatment at 5 days of culture (C) compared to proper control (A). M; melanophore, NM; non melanocytic cell, NC; neural crest. Scale bar = 100 μm.

posteriormost explants gave rise to melanophores compared to anteriormost explants (Fig. 3). Under control culture conditions, explants from the posteriormost region of the crest produced significantly more melanophores by day 3 and 5 post culture compared to explants from the anteriormost region. A dramatic increase in melanophore number was seen in anteriormost cultures in response to forskolin. These data indicated that the presence of forskolin in serum-free cultures had significant effects on melanophore differentiation/proliferation and/or melanization. Melanization of forskolin-treated cultures consistently increased by day 5 after explantation. This demonstrated a definite influence of forskolin on the differentiation of melanophores in neural crest cells *in vitro*. Furthermore, this influence was realized only in cells from particular axial

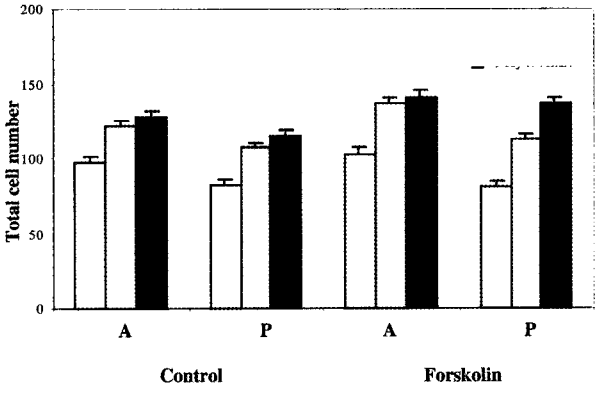

Fig. 2. There is no significant differentces in total cell numbers between control and forskolin treated explants. Representative total cell number counts between control and forskolin treated explants take for them anteriormost (A) and form posteriormost (P) regions of the crest at day 1 (□), 3 ( ), and 5 (■) after explantation.

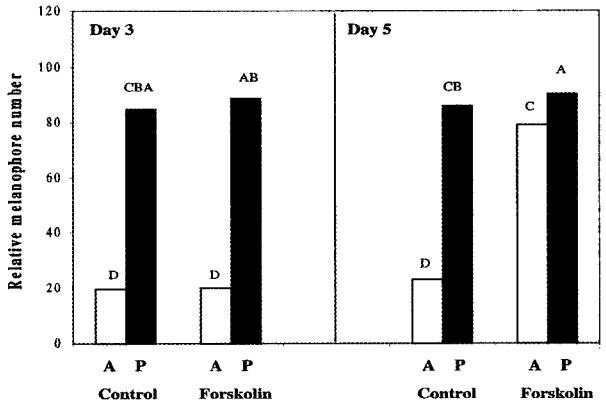

Fig. 3. Representative relative melanophore numbers between control and forskolin treated explants taken from anteriormost (A) and from posteriormost (P) regions of the crest. Melanophore numbers in cultures of anteriormost (A) region showed a dramatic increase in the melanophore numbers in response to forskolin compared to control at day 5 of culture. The percentage of melanophores form the explants were counted and analyzed by Fishers protected LSD.

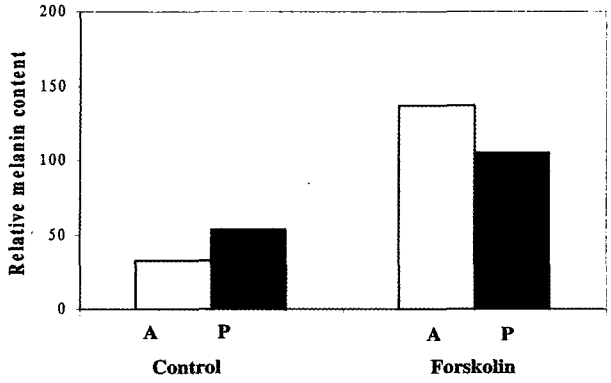

Fig. 4. There is a significant increase in the melanin content of explants taken from anteriormost (A) regions of the crest in response to forskolin treatment. Representative relative melanin content between control and forskolin treated explants taken form the anteriormost (A) and form posteriormost (P) regions of the crest at day 5 of cultures.

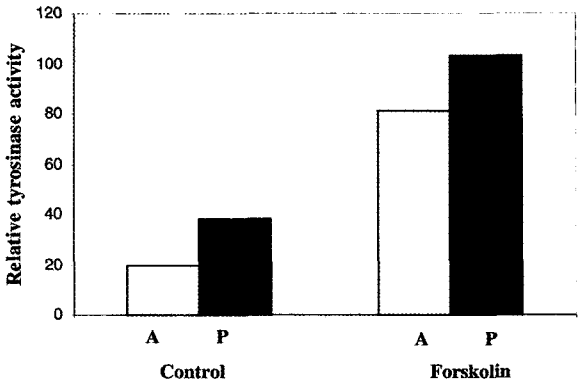

Fig. 5. There is a significant increase in the tyrosinase activity of explants taken from anteriormost (A) regions of the crest in response to forskolin treatment.Representative relative tyrosinase activity between control and forskolin treated explants taken form the anteriormost (A) and form posteriormost (P) regions of the crest at day 5 of cultures.

regions of the neural crest.

In addition to cell counts and culture composition studies, the anterior and posterior cultures were analyzed for melanin content and tyrosinase activity. Results of the melanin content analyses for both forskolin-treated explants and control explants taken from the anteriormost and posteriormost region of the crest are presented in Fig. 4. Compared to untreated controls, the melanin content in forskolin-treated explants increased significantly on day 5 after explantation. There was also a significant increase in melanin content of explants taken from both anteriormost regions and posteriormost regions of the crest in response to forskolin on day 5 post culture.

Fig. 5 demonstrates results from the tyrosinase activity assays of control and forskolin-treated explants taken from the anteriormost and posteriormost regions of the crest. Results were similar to the results of the melanin content analyses. By 5 d of culture, a significant increase in tyrosinase activity was observed in explants from the posteriormost region of the crest compared to those taken from the anteriormost region. In addition, by day 5 after explantation both anteriormost and posteriormost explants receiving forskolin treatment showed significant increases in tyrosinase activity ($P < 0.05$) compared to the corresponding controls. These results indicated an influence of forskolin in the increase of tyrosinase activity in neural crest cells *in vitro*.

Discussion

Although premigratory cells of the cranial and trunk neural crest exhibit differences in their differentiation potentials, premigratory trunk neural crest cells are generally assumed to have equivalent developmental potentials. Pigment cell precursors of the embryonic axolotl give rise to melanophores, xanthophores and/or iridophores. The decision to follow one of these chromatophore lineages must be a major decision made by the neural crest-derived pigment cell precursors and there are surely many intrinsic and extrinsic cues involved in these decision-making processes (Henion and Weston, 1997). Mechanisms and factors leading to the differentiation of these various chromatophores, however, and the cell signaling mechanisms related to the development of pigmentation remain obscure. Although differentiated states are presumed to be quite stable, plasticity does exist among and within neural crest and pigment-cell lineages (Kajishima, 1958; Thibaudeau and Holder, 1998). The degree of flexibility and potential influences that are capable of altering differentiated states however remain unknown. Ide (1973, 1974, 1978, 1986) and Kondo and Ide (1983) documented transdifferentiation events among pigment cell types from tadpole skin. Thibaudeau et al. (1998) demonstrated that axolotl neural crest cells from different regions of origin, different stages of development, and challenged with different culture media possess spatial and temporal differences concerning differentiation preferences. These differences found along the anteroposterior axis of the developing embryo influence developmental potentials and diminish the equivalency of axolotl trunk neural crest cells. Similar nonequivalent differentiation potentials have been found in fish (Kajishima, 1958) and avian (Artinger and Bronner-Fraser, 1992; Reedy et al., 1997) neural crest cells.

In the study presented here, Axolotl neural crest cells were cultured under various culture conditions in order to examine the mechanisms involved in chromatophore differentiation and pigment cell signaling. Explants taken from the most posterior sites of the axolotl neural crest exhibited preferential differentiation of melanophores compared to explants taken from the anterior sites of the crest. This preference held true in the absence or presence of forskolin although to a

lesser degree in the presence of forskolin. To discern at what level melanophore development was being effected, (1) the number of melanophores in control and experimental cultures 1, 3 and 5 d post-culture were counted, and (2) tyrosinase activity and melanin content at 1, 3 and 5 d post-culture were measured. Overt pigmentation increased dramatically in forskolin-treated cultures taken from anterior regions of the crest, which has capacity to differentiate into non-melanocytes. Cell counts indicated that this effect on pigmentation was not necessarily due to an increase in the proliferation of melanophores but was possibly through an increase in recruitment of melanophores (differentiation) from precursors and/or an increase in melanin synthesis/deposition in premelanophores or newly recruited premelanophores.

Study presented here propose that posterior-most neural crest cells may be specified as melanophore-restricted precursors prior to or at the time migration commences. Cellular restrictions are known to exist prior to migration. For example, a restrictive cell division has been proposed to occur for most trunk neural crest cells in zebrafish even before cells begin to migrate away from the neural keel (Raible and Eisen, 1994). There is also evidence for temporal differences in melanoblast differentiation in the mouse (Morrison-Graham and Weston, 1993; Yoshida et al., 1996). Preferential representation of the pigment cell lineage in a specific region of the axolotl neural crest in vivo would have implications in patterning events of the pigment system and the neural crest cell population in general.

In the presence of minimal environmental cues cell populations from the posterior-most region of crest produce significantly more melanophores than those from more anterior regions (Thibaudeau and Holder, 1999). These posterior cell may be precusor cells already restricted to a melanophore lineage as mentioned above or they may be stem cells or undifferentiated progeny that are located posteriorly along the crest and produce melanophores *in vitro* by entering the default pathway of melanogenesis. Studies involving the development of the myotome in the mouse by Nicolas et al. (1996) set a precedent for the existence of such a mechanism. Ongoing studies are designed to identify whether such a mechanism is at work in the axolotl neural crest.

Enhancement of melanophore differentiation by forskolin may reflect a developmental decision/restriction event whereby melanophore precursors either respond to extrinsic melanophore-differentiation cues supplied by the chemical or respond to intrinsic melanophore-differentiation factors. Forskolin may encourage specification of relatively undifferentiated cells as premelanophores or may turn on the melanin synthesis machinery in specified premelanophores. In any case mentioned the posterior sites of the neural crest seem to possess a larger and/or more responsive population of pigment-cell precursors on which forskolin may act. Again, the fact that there is no change in total cell number and a corresponding increase in the relative number of melanophores suggest that forskolin acts by increasing melanin synthesis and/or increases cell recruitment and not proliferation. Further, the observation that no increase in relative melanophore number is seen in posterior explants receiving forskolin treatment compared to non-treated controls may reflect a saturation of premelanophores in the posterior region. Likewise, a significant increase in melanophores in forskolin-treated anterior cultures compared to the corresponding non-treated anterior controls would indicate more available precursors of the crest capable of recruitment in response to the presence of appropriate cues. Of course, cell autonomous mechanisms surely play important roles in regulating how cells respond to extrinsic inductive signals. Elegant studies of Nakagawa et al. (1996) demonstrated that cell autonomously regulated region-specific genes could be maintained in cells cultured *in vitro*. The cells of the embryonic neural crest are required to use spatially restricted instructive signals restricted to given embryonic location to guide their migration and differentiation (Yamada, 1993). Data presented here show that neural crest cells from different axial levels and challenged with different culture media have different characteristics regarding differentiation of the individual neural crest-derived pigment cell lineages.

Although the precise mechanisms of differential responses reported here remain unknown, the existence of region-dependent preferential differentiation among trunk neural crest cells *in vitro* is clear. Collectively, the results support the hypothesis that spatial differences among premigratory trunk neural crest cells found along the anteroposterior axis render axolotl trunk neural crest cells not equivalent.

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