

The differentiation *in vitro* of the neural crest cells of the mouse embryo

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SUMMARY

A culture method for neural crest cells of mouse embryo is described. Trunk neural tubes were dissected from 9-day mouse embryos and explanted in culture dishes. The developmental potential of mouse neural crest *in vitro* was shown to be essentially similar to that of avian neural crest. In the mouse, however, melanocytes always appeared in association with the epithelial sheet close to the explant. Neural crest cells surrounding the epithelial sheet, which probably migrated from the neural tubes in the early culture phase, never differentiated into melanocytes. The bimodal behaviour of mouse crest cells seems to be due to the heterogenous potency of the crest cells and the interaction of these cells with the surrounding microenvironment. This culture system is well suited for various experiments including the analysis of gene control on the differentiation of neural crest cells.

INTRODUCTION

In vertebrates, neural crest is the embryonic precursor of a diversity of cell types, including pigment cells, and the neurons and glial cells of the peripheral nervous system. Neural crest migrates from the roof of the neural tube to various sites in the embryo during early embryogenesis, and differentiates in various ways according to their location sites.

Hitherto, many embryologists have been interested in these characteristics of the neural crest, and have performed intensive studies (Hörstadius, 1950; Weston, 1970). Recently, in investigations using avian neural crest, it has been suggested that differentiation and migration of these cells were affected by the surroundings. In particular, by establishing a culture system for quail neural crest (Cohen & Konigsberg, 1975), it has been shown that the developmental potential of individual crest cells is heterogenous and can be altered by environmental factors.

Despite abundant information on the developmental behaviour of crest cells, gene control of the interaction between neural crest and the surrounding environment has not yet been satisfactorily established. In mouse, it is well known that there are many mutations which influence differentiation of the

neural crest (Silvers, 1961, 1979). The use of these mutations, combined with *in vitro* culture systems, will enable us to obtain more information on the differentiation of the crest cells.

The objective of the present study is to establish a culture system for neural crest in mouse and to describe the behaviour of mouse crest cells *in vitro* from wild-type and mutant mice.

MATERIALS AND METHODS

C57BL/6J mice were originally obtained from the Jackson Laboratory (Bar Harbor, Maine), and further maintained in our laboratory by inbreeding. Mice of C3HB/St strain were provided by Dr. W. C. Quevedo, Jr., Brown University.

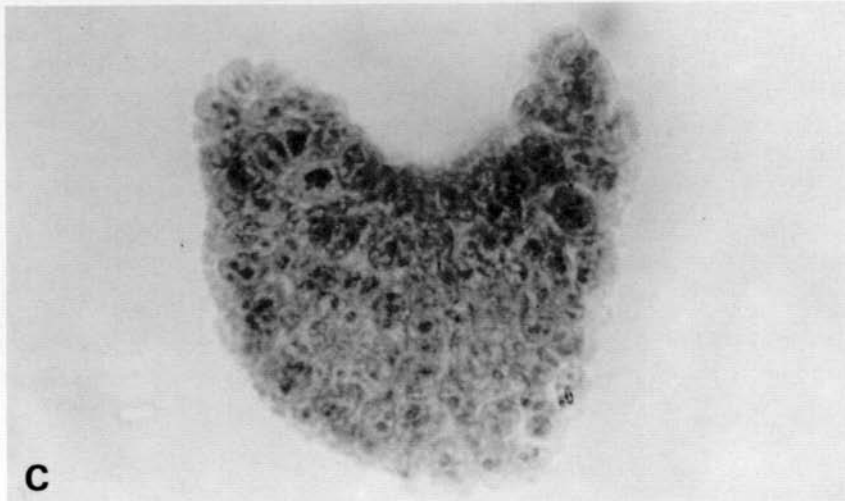
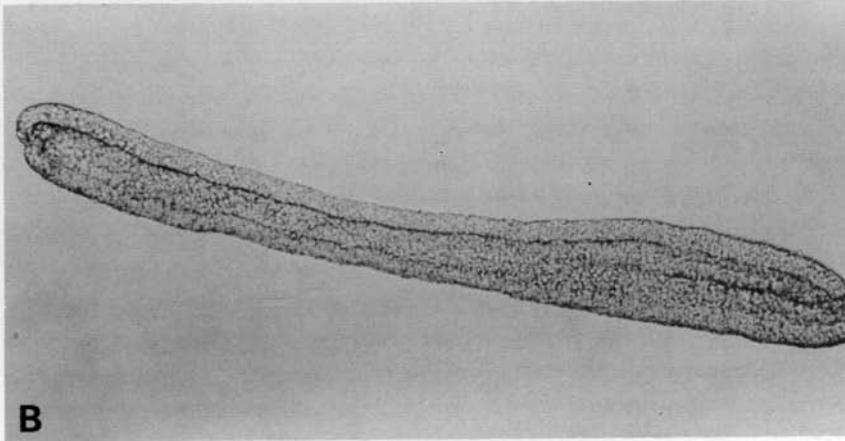
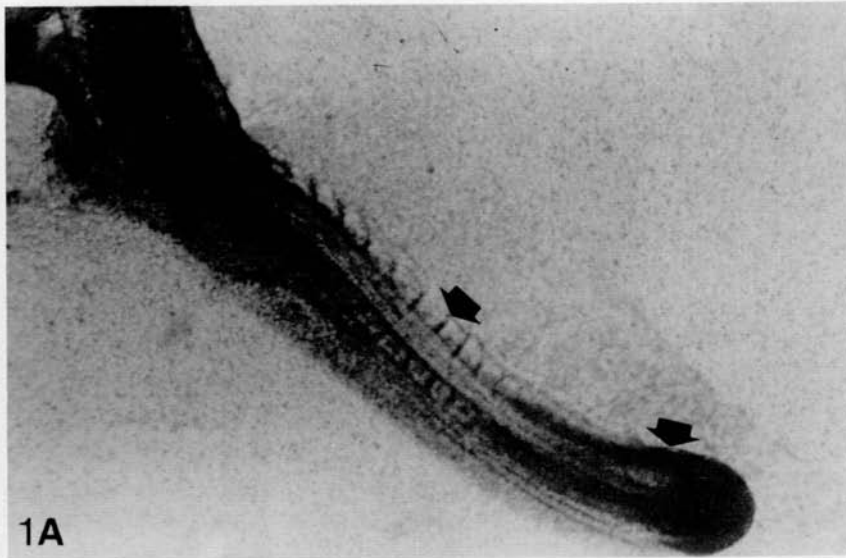
Culture methods

9-day mouse embryos were used. Embryos were obtained from timed mating; the day vaginal plugs were observed was defined as day 0 of pregnancy.

The dorsal trunk region posterior to the hind limb buds (Fig. 1A) was dissected out by means of sharpened tungsten needles. The trunk was treated with 1% trypsin (Difco, 1 : 250) in Tyrode solution for 20 min at 4°C. Trypsinization was terminated by replacing the enzyme with a 10% solution of foetal bovine serum (FBS, Flow) in Tyrode solution, and the tissue was then gently pipetted with a small pore Pasteur pipette to separate the neural tube from other components of the trunk (Cohen & Konigsberg, 1975).

Careful visual inspection indicated that mouse neural tubes prepared in this manner were free of their surrounding tissues (Fig. 1B, C). Three or four neural tubes were explanted in a 35 mm tissue culture dish (Falcon) containing 1.5 ml of culture medium (see below). In some cases, the neural tubes were carefully detached from the dishes with tungsten needles and discarded after 2 days in culture. The cells that had emigrated from the explants were fed with 1.5 ml fresh medium. The culture was incubated at 37°C in a humidified atmosphere of 5% CO₂ in air for 10 to 15 days. One-half of the culture medium was changed every third day.

Fig. 1. (A) The posterior trunk region of a 9-day embryo. Neural tube was isolated from the section between arrows. $\times 32$. (B) A neural tube isolated from the posterior trunk region. The neural fold has not yet fused at this stage. $\times 80$. (C) Transverse section of the isolated neural tube. The neural tubes were fixed in Bouin's fluid, and then embedded in paraffin. Sections were cut at 10 μm , and stained with haematoxylin and eosin. $\times 700$.



Culture medium

Culture medium consisted of Eagle's minimum essential medium (MEM, Nissui), FBS, and chick embryo extract (CEE). By examining the effect of the concentrations of FBS and CEE on the appearance of melanocytes (data not shown), the concentrations of 15% FBS and 2–4% CEE were selected. In some experiments, the cultures were treated with 0.1 $\mu\text{g/ml}$ α -melanocyte stimulating hormone (α -MSH, Ciba Geigy) in Tyrode solution.

CEE was prepared from the bodies of 10-day chick embryos. After washing the embryos in Tyrode solution, they were homogenized, and then mixed with an equal volume of Tyrode solution. The mixture was freeze-thawed three times, and centrifuged twice at 3000 r.p.m. for 15 min. The resulting supernatant was used as CEE.

DOPA reaction

The cultures were rinsed in Tyrode solution, and fixed at 4°C in periodate-lysine-paraformaldehyde fixative (McLean & Nakane, 1974) for 2 h. After being washed in 0.1 M-phosphate-buffered saline (PBS, pH 7.4), the cultures were incubated at 36°C in 0.1% 2- β (3,4-dihydroxyphenyl) alanine (DOPA) solution in PBS for 3 h. The DOPA reaction was terminated by rinsing the cultures several times in PBS.

The cultures to which the DOPA reaction had been performed were post-fixed in 1% OsO_4 at room temperature in PBS for 30 min. These samples were routinely dehydrated and embedded in Epon 812. The sections were cut with a LKB ultramicrotome (8800), stained with uranylacetate and lead citrate, then examined in a Hitachi electronmicroscope (H-500).

Formaldehyde-induced fluorescence

The cultures were examined for intracellular catecholamine (CA) by formaldehyde-induced fluorescence (FIF) histochemistry (Falck & Owman, 1965). They were washed with Tyrode solution, and then quenched in isopentane cooled with liquid nitrogen. The samples were dried in a lyophilizer (Refrigeration For Science Inc., Island Park, NY) for 1 h, and exposed to formaldehyde gas at 75–85°C for 1 h. CA fluorescence was viewed with Olympus fluorescence microscope (BH2-RFL, excitation filter V (BG-3 + IF405), barrier filter Y475).

RESULTS

Appearance of neural crest cells

Within 24 h, the explants of neural tubes adhered to the bottom of the culture dish. Shortly after the adhesion, a population of small stellate cells was

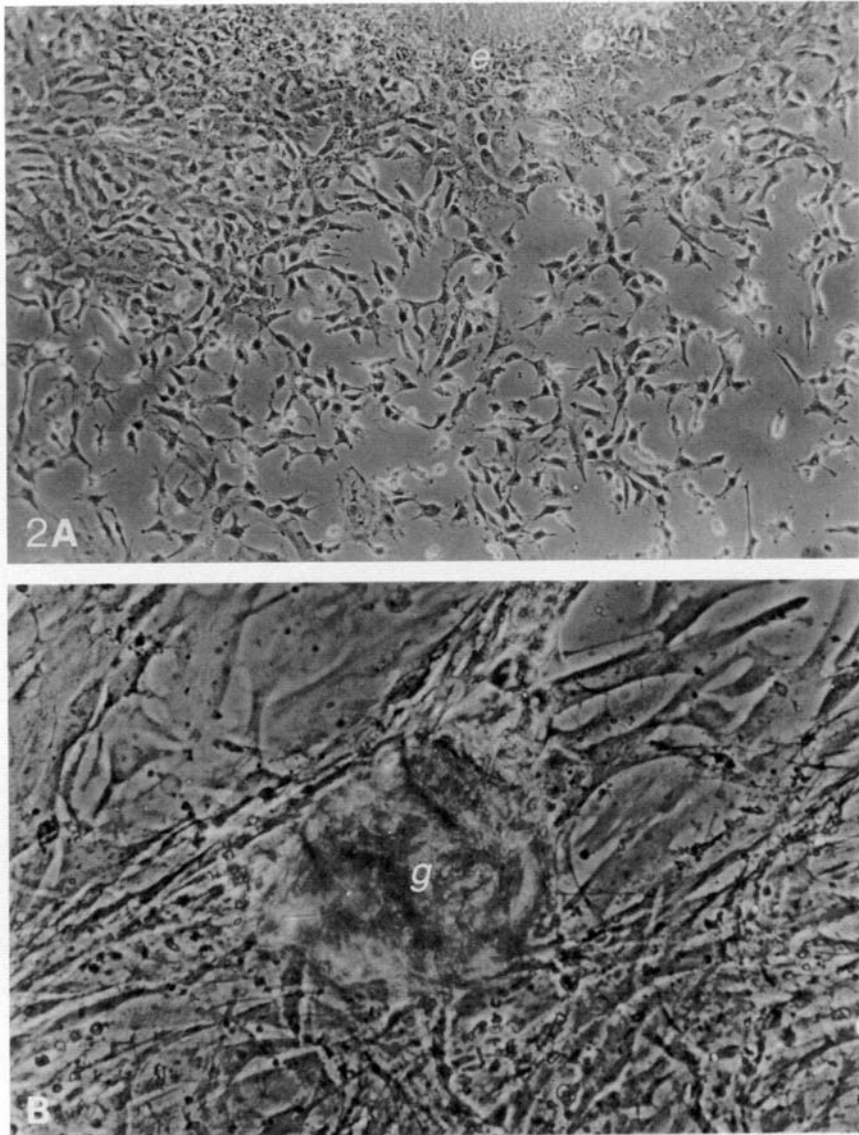


Fig. 2. (A) The crest cell outgrowth around the explant (e), after 53 h in culture. $\times 80$. (B) Phase-contrast micrograph of ganglion-like structure (g) in culture for 15 days. $\times 300$.

observed in the area surrounding the explants (Fig. 2A). These cells rapidly increased in number in the early stage of culture. By 6 days in culture, randomly distributed cell clumps were beginning to be seen in this area (Fig. 2B). As the culture in progresses the margin of the tube flattened to form an epithelial sheet.

Differentiation into melanocytes

In experiments with neural crest from 2-day quail embryos, some of the crest cells differentiated into melanocytes under the conditions used in this study. Cluster formation, which was reported in the study of Loring, Glimelius, Erickson & Weston (1981) with quail embryos, also occurred. In cultures of neural tube from mouse embryos, however, fully developed melanocytes never appeared (Fig. 3A). Therefore, in order to detect immature melanocytes, the DOPA reaction (Ito & Takeuchi, 1981) was performed. After 10 to 15 days in culture, cells positive to the reaction were always found in association with epithelial sheet around the explants (Fig. 3B). These DOPA-positive cells were of dendritic morphology, characteristic of melanocytes derived from the neural crest (Fig. 3C). When the cells were examined with an electron microscope, melanosomes of various stages were observed (Fig. 4).

On the other hand, fully melanized cells appeared after the addition of α -MSH to the culture medium (Fig. 5). These cells began to appear by 12 days in culture, and, like the DOPA-positive melanocytes, were always in association with epithelial sheet around the explants. DOPA-positive melanocytes that were likely to be in their migrating phase from explants to epithelial sheet were also observed (Fig. 6A, B). The cell population that proliferated in the area surrounding the explants failed to undergo melanogenesis under the culture conditions used.

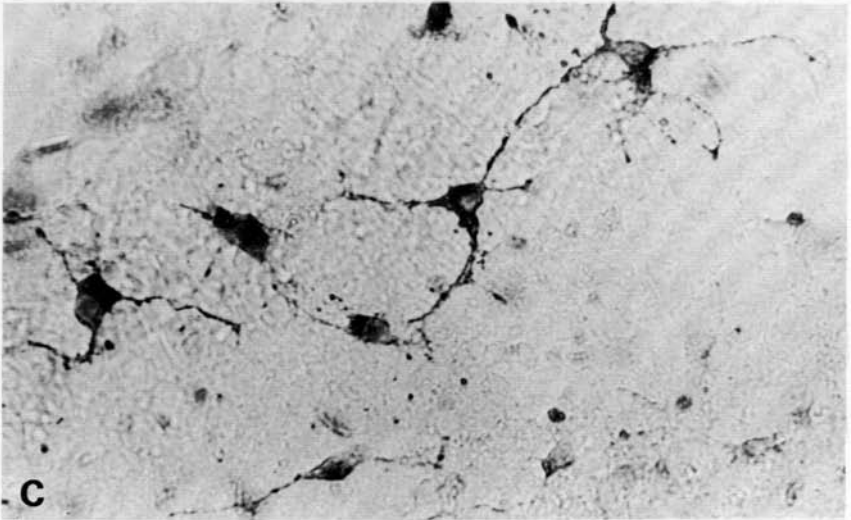
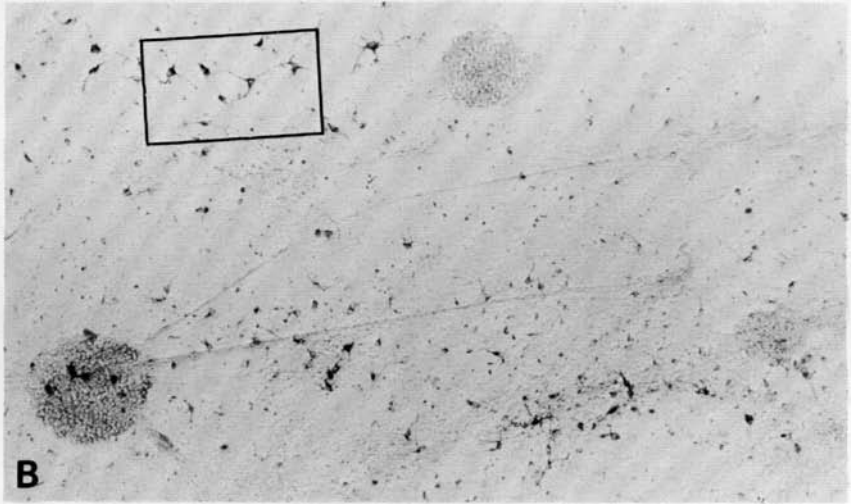
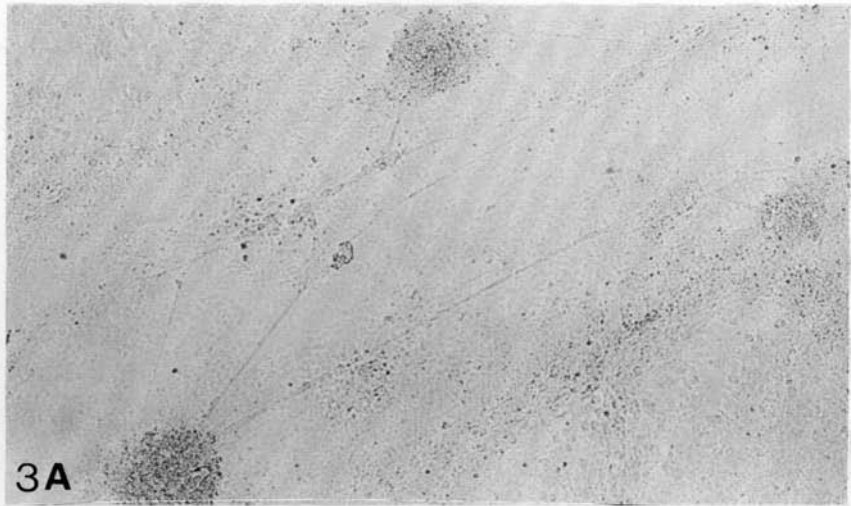
Differentiation into adrenergic cells

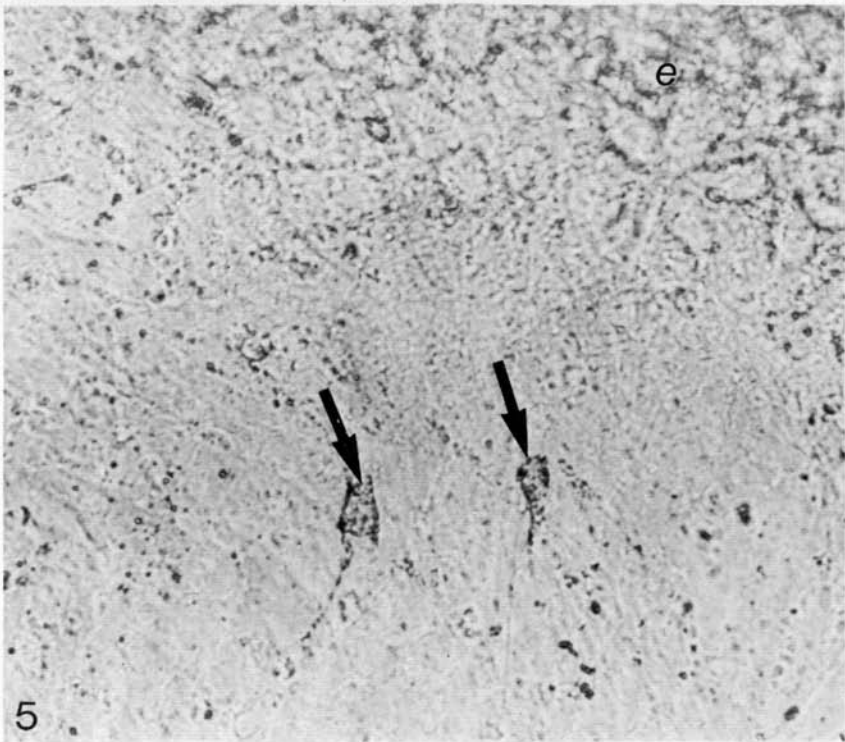
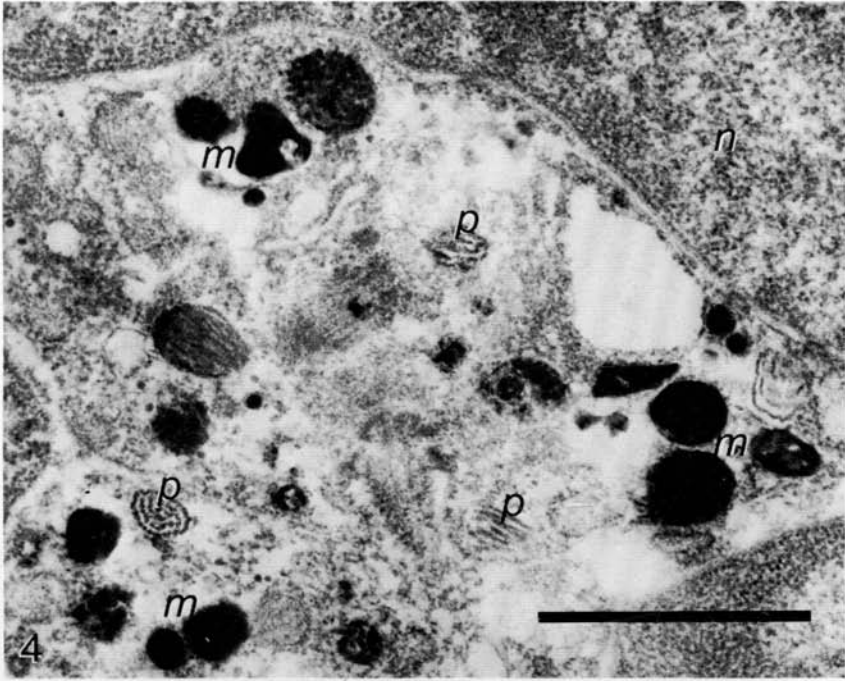
In avian embryos, the expression of CA-positive adrenergic phenotype *in vitro* in neural crest cells was reported (Norr, 1973; Cohen, 1977; Sieber-Blum & Cohen, 1980; Sieber-Blum, Sieber & Yamada, 1981; Loring, Glimelius & Weston, 1982). In mouse neural crest, the cell clumps (Fig. 2B) that appeared by 6 days in culture were particularly CA-positive (Fig. 7A, B). As culture proceeded, the number of CA-positive clumps increased. Some of the clumps possessed axon-like processes. The ganglion-like structures randomly distributed on the epithelial sheet and in the surrounding outgrowth. The differentiation into adrenergic cells also occurred in the culture in which neural tubes were detached and discarded after 2 days in culture (Fig. 7C).

The culture of neural crest in mutant mice (mi^{bw}/mi^{bw})

Mice of C3HB/St strain are homozygous for mi^{bw} (*black-eyed white* allele at the *microphthalmia* locus), one of *white spotting* mutations. Mutations at the

Fig. 3. (A) The epithelial sheet around the explant, after 15 days in culture. $\times 80$. (B) The same epithelial sheet in (A) after the DOPA reaction. The DOPA-positive melanocytes are observed. $\times 80$. (C) Enlargement of box (B). Dendritic melanocytes are seen. $\times 320$.





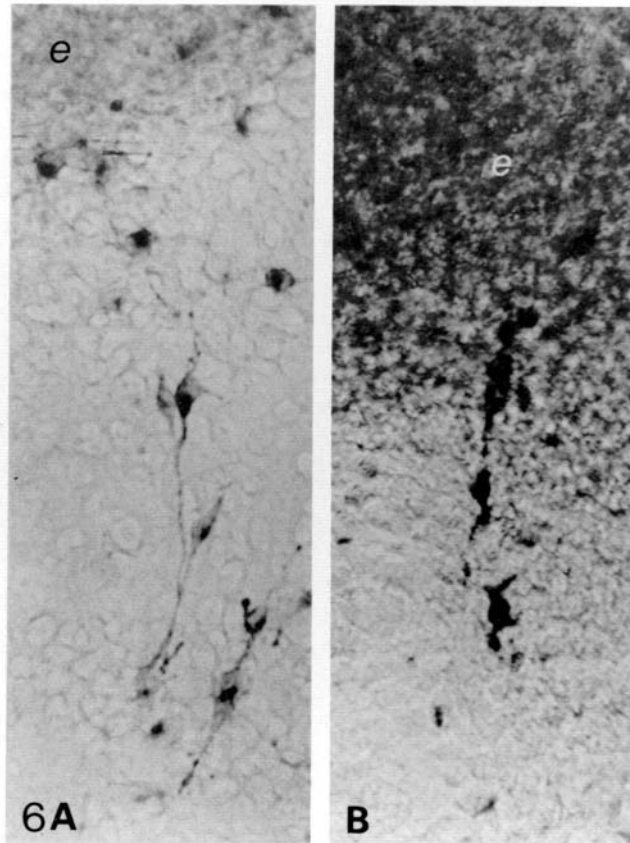
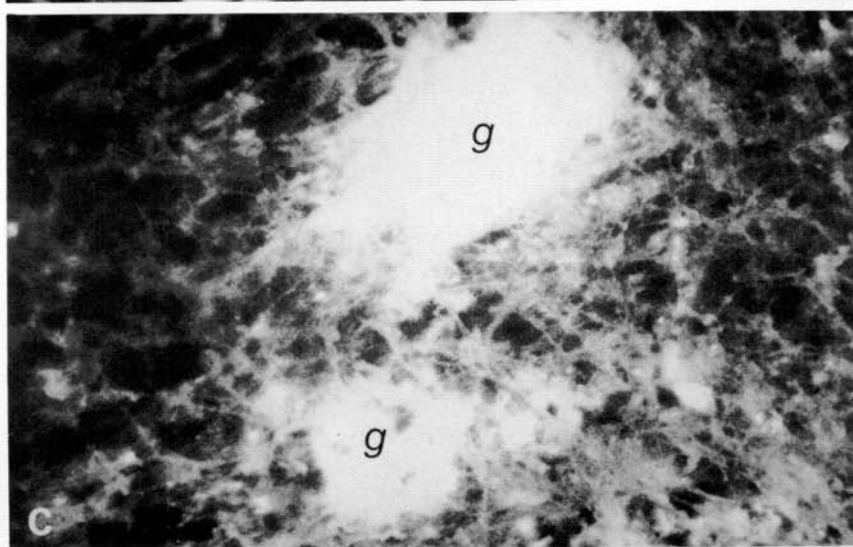
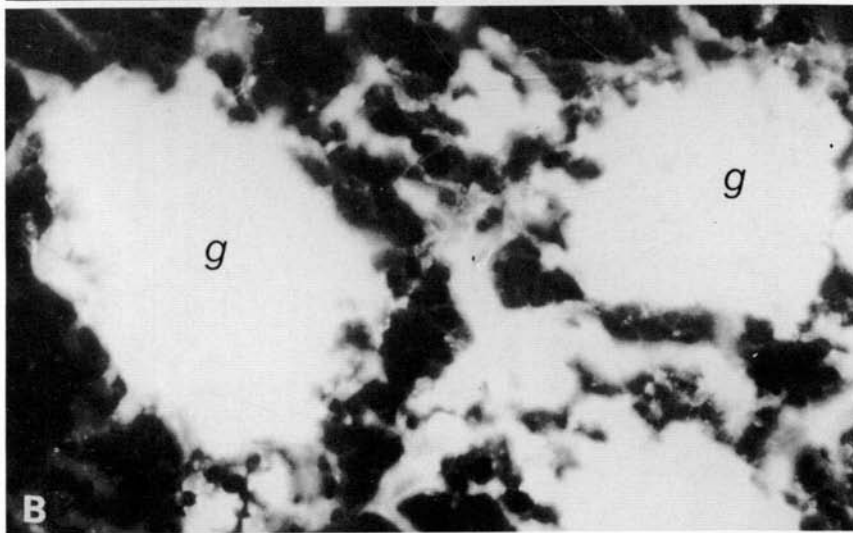
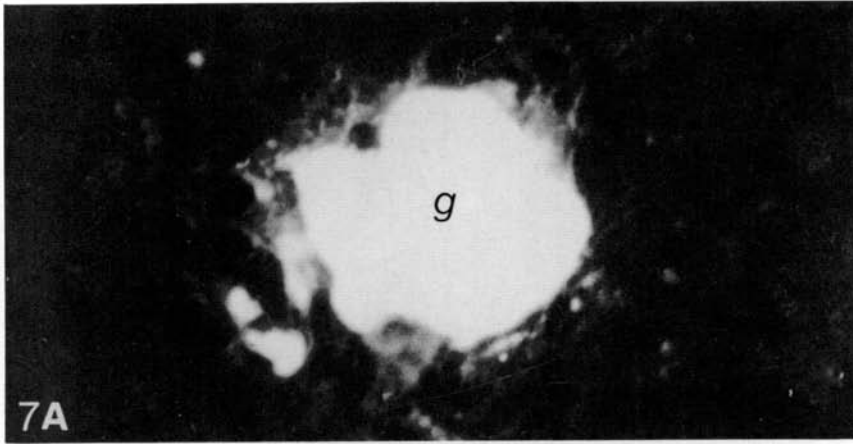


Fig. 6. DOPA-positive melanocytes, likely to be migrating from the explants(*e*). The DOPA reaction was performed after 10 days (A) or 15 days (B) in culture. $\times 250$.

mi-locus usually result in depigmentation in the coat but not eyes which remain black. Therefore, it is suggested that this gene acts during the process of differentiation of neural crest. When neural tube culture was performed with mutant embryos, differentiation into melanocytes has never been found (Fig. 8). No DOPA-positive melanocytes appeared even when $0.1 \mu\text{g/ml}$ α -MSH was added to the culture medium (Table 1).

Fig. 4. Electronmicrograph of a DOPA-positive cell cultured for 15 days. Melanosomes of various stages are observed. *m*, mature melanosome; *p*, pre-melanosome; *n*, nucleus. Scale bar = $1 \mu\text{m}$.

Fig. 5. Fully melanized cells (arrows) in a culture to which α -MSH was added. The culture was incubated for 15 days. (*e*) indicates explant. $\times 320$.



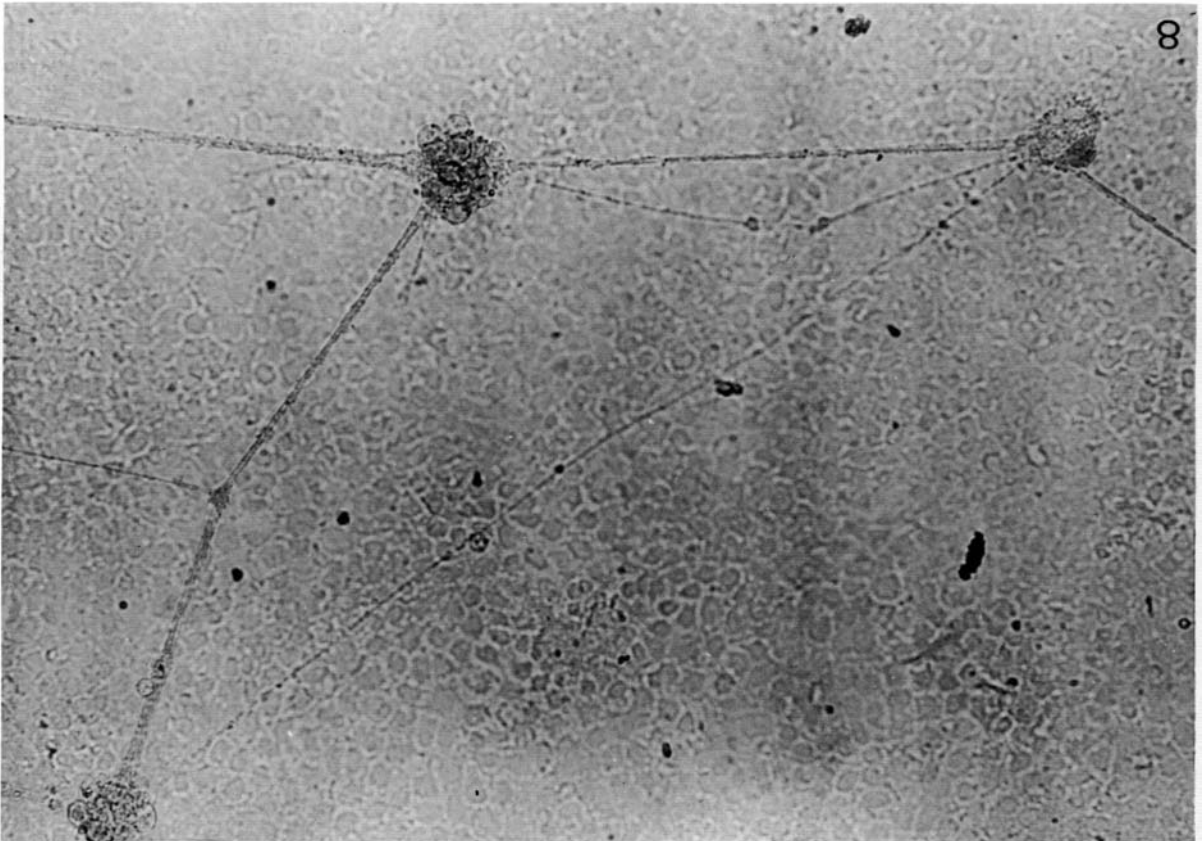


Fig. 8. The epithelial sheet around the explant after 15 days in culture, from C3HB mutant (mi^{bw}/mi^{bw}) mouse embryo. Although the DOPA reaction was performed, DOPA-positive melanocytes have never been found. $\times 250$.

Table 1. Differentiation potency of neural crest cells into melanocytes in C3HB/St mutant (mi^{bw}/mi^{bw}) mice.

Experiments	Number of explants with DOPA-positive cells/Total explants			Total
	1	2	3	
15.4*	0/3	0/2	0/2	0/7
15.4.M†	0/2	0/4	-	0/6

*Cultured in MEM + 15% FBS + 4% CEE
 †Cultured in MEM + 15% FBS + 4% CEE + 0.1 $\mu\text{g/ml}$ α -MSH

Fig. 7. FIF of CA-positive cells after 12 days (A) and 15 days (B, C) in culture. In (C), the explanted neural tube was detached and discarded after 2 days in culture. Ganglion-like cell clumps (g) are particularly CA-positive. $\times 220$.

DISCUSSION

In the present study, culture of trunk neural tube of mice gave rise to the appearance of a population of small stellate cells. Judging by their characteristic morphology, it seems that these cells are neural crest cells (Cohen & Konigsberg, 1975; Maxwell, 1976). In contrast to quail neural crest, differentiation of mouse crest cells into melanocytes has never been found previously since explants were discarded at an early stage of culture. All of the observed melanocytes appeared in association with epithelial sheet which seemed likely to be derived from the neural tube. The melanocytes (from neural crest) can be identified by the typically dendritic morphology.

The behaviour of mouse neural crest demonstrated in the present study seems to suggest the following.

(1) The developmental potential of mouse trunk crest cells *in vitro* seems to be essentially similar to that of avian trunk crest cells.

(2) In the mouse, the epithelial sheet provides the microenvironment to support the differentiation of the crest cells into melanocytes. In the study using quail embryos, Weston and his collaborators (Loring *et al.*, 1971; Glimelius & Weston, 1981) reported that part of the neural crest cells formed clusters on the explanted neural tube, and that all cluster cells differentiated into melanocytes *in vitro*. They further suggested that there is a close association of the clusters with neural tube and that cell aggregation may be necessary for the crest cells to become melanocytes *in vitro*. In mouse, it is likely that the association between neural crest cells and epithelial sheet from the neural tube is required for the differentiation of crest cells into melanocytes.

It has been shown that migration and differentiation of neural crest depend on the surrounding environment (Le Douarin, Renaud, Teiller & Le Douarin, 1975; Le Douarin, 1980). A series of recent studies (Sieber-Blum & Cohen, 1980; Sieber-Blum *et al.*, 1981; Loring *et al.*, 1982; Pratt, Larsen & Johnston, 1975; Newsome, 1976; Derby, 1978; Pintar, 1978; Newgreen & Thiery, 1980; Mayer, Hay & Hynes, 1981; Greenberg, Seppä, Seppä & Hewitt, 1981; Bronner-Fraser, 1982) have indicated the importance of the interaction between neural crest and extracellular matrix. The behaviour of mouse crest cells observed in the present study may also result from the interaction of these cells with the surrounding environment.

(3) Several investigators (Cohen & Konigsberg, 1975; Sieber-Blum & Cohen, 1980; Ziller *et al.*, 1983; Kahn & Sieber-Blum, 1983) have suggested that the developmental potential of individual crest cells may be heterogeneous. Mouse crest cells which are already committed to the melanogenic pathway might leave the neural tube during the late stage rather than the early stage under the culture condition used in the present study. This possibility is indicated by the appearance of DOPA-positive melanocytes on the epithelial

sheet. They are likely to be migrating from explant to the epithelial sheet.

In the cultures using mutant mice, crest cells failed to undergo melanogenesis under any tested culture conditions. This result suggests that *mi^{bw}* gene functions within neural crest cells. In the previous study, we proposed the idea that *mi^{bw}* gene may function through the interaction between melanoblasts and skin environment (Ito & Takeuchi, 1981). The effect of this mutant gene during melanocyte differentiation may be similar to that of piebald mutation, *s*, another white spotting gene. Mayer (1965, 1967a,b, 1977) has suggested that the primary site of function of *s* gene is within melanoblasts and that the function is also dependent on the skin environment.

Although intensive efforts have been made by several investigators to study the differentiation of mouse neural crest (Rawles, 1947; Mayer, 1965, 1967a,b, 1977), there have been some technical restrictions: (1) the experimental manipulation was rather complicated. (2) In the culture of mouse tissues in chick coelom, it has been difficult to perform an artificial treatment during the culture period. (3) Differentiated cell types detected under their conditions were solely pigment cells.

Our culture system seems to overcome these difficulties. (1) The experimental operation is much simpler. (2) It is possible to perform an artificial treatment during culture period. (3) The differentiated cell types were not only pigment cells but also adrenergic cells. This culture system, combined with the mutation affecting the differentiation of the neural crest, is well suited for the study of the gene control on neural crest development.

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REFERENCES

- BRONNER-FRASER, M. (1982). Distribution of latex beads and retinal pigment epithelial cells along the ventral neural crest pathway. *Devl Biol.* **91**, 50–63.
- COHEN, A. M. (1977). Independent expression of the adrenergic phenotype by neural crest cells *in vitro*. *Proc. natn. Acad. Sci., U.S.A.* **74**, 2899–2903.
- COHEN, A. M. & KONIGSBERG, I. R. (1975). A clonal approach to the problem of neural crest determination. *Devl Biol.* **46**, 262–280.
- DERBY, M. A. (1978). Analysis of glycosaminoglycans within the extra-cellular environments encountered by migrating neural crest cells. *Devl Biol.* **66**, 321–336.
- FALCK, C. & OWMAN, C. (1965). A detailed methodological description of the fluorescence method for the cellular demonstration of biogenic monoamines. *Acta Univ. Lund, Sect. 2*, **7**, 5–23.
- GLIMELIUS, B. & WESTON, J. A. (1981). Analysis of developmentally homogeneous neural crest cell population *in vitro*. III. Role of culture environment in cluster formation and differentiation. *Cell Differ.* **10**, 57–67.
- GREENBERG, J. H., SEPPÄ, S., SEPPÄ, H., & HEWITT, A. T. (1981). Role of collagen and fibronectin in neural crest cell adhesion and migration. *Devl Biol.* **87**, 256–266.
- HÖRSTADIUS, S. (1950). *The Neural Crest*. New York and London: Oxford Univ. Press.
- ITO, K. & TAKEUCHI, T. (1981). Melanocyte differentiation in black-eyed white mice. In

- Pigment Cell 1981: Phenotypic Expression in Pigment Cells*, pp. 185–188. Tokyo: Univ. of Tokyo Press.
- KAHN, C. R. & SIEBER-BLUM, M. (1983). Cultured quail neural crest cells attain competence for terminal differentiation into melanocytes before competence to terminal differentiation into adrenergic neurons. *Devl Biol.* **95**, 232–238.
- LE DOUARIN, N. M. (1980). The ontogeny of the neural crest in avian embryo chimeras. *Nature* **286**, 663–669.
- LE DOUARIN, N. M., RENAUD, D., TEILLET, M. A. & LE DOUARIN, G. H. (1975). Cholinergic differentiation of presumptive adrenergic neuroblasts in interspecific chimeras after heterotopic transplantation. *Proc. natn. Acad. Sci., U.S.A.* **72**, 728–732.
- LORING, J., GLIMELIUS, B., ERICKSON, C. & WESTON, J. A. (1981). Analysis of developmentally homogenous neural crest populations *in vitro*. *Devl Biol.* **82**, 86–94.
- LORING, J., GLIMELIUS, B. & WESTON, J. A. (1982). Extracellular matrix materials influence quail neural crest cell differentiation *in vitro*. *Devl Biol.* **90**, 165–174.
- MAYER, T. C. (1965). The development of piebald spotting in mice. *Devl Biol.* **11**, 319–334.
- MAYER, T. C. (1967a). Pigment cell migration in piebald mice. *Devl Biol.* **15**, 521–535.
- MAYER, T. C. (1967b). Temporal skin factors influencing the development of melanoblasts in piebald mice. *J. exp. Zool.* **166**, 397–404.
- MAYER, T. C. (1977). Enhancement of melanoocyte development from piebald neural crest by a favorable tissue environment. *Devl Biol.* **56**, 255–262.
- MAYER, Jr. B. W., Hay, E. D. & Hynes, R. O. (1981). Immunochemical localization of fibronectin in embryonic chick trunk and *area vasculosa*. *Devl Biol.* **82**, 267–286.
- MAXWELL, G. D. (1976). Cell cycle changes during neural crest cell differentiation *in vitro*. *Devl Biol.* **49**, 66–79.
- McLEAN, I. W. & NAKANE, P. K. (1974). Periodate-lysine-paraformaldehyde fixative: a new fixative for immunoelectron microscopy. *J. Histochem. Cytochem.* **22**, 1077–1083.
- NEWGREEN, D. & THIERY, J. -P. (1980). Fibronectin in early embryos: synthesis and distribution along the migration pathways of neural crest cells. *Cell Tissue Res.* **211**, 269–291.
- NEWSOME, D. A. (1976). *In vitro* synthesis of cartilage in embryonic chick neural crest cells by products of retinal pigmented epithelium. *Devl Biol.* **49**, 496–507.
- NORR, S. C. (1973). *In vitro* analysis of sympathetic neuron differentiation from chick neural crest cells. *Devl Biol.* **34**, 16–38.
- PINTAR, J. E. (1978). Distribution and synthesis of glycosaminoglycan during quail crest morphogenesis. *Devl Biol.* **67**, 444–464.
- PRATT, R. M., LARSEN, M. A. & JOHNSTON, M. C. (1975). Migration of cranial neural crest cells in a cell-free hyaluronate-rich matrix. *Devl Biol.* **44**, 298–305.
- RAWLES, M. E. (1947). Origin of pigment cells from the neural crest in the mouse embryo. *Physiol. Zool.* **20**, 248–266.
- SIEBER-BLUM, M. & COHEN, A. M. (1980). Clonal analysis of quail neural crest cells: they are pluripotent and differentiate *in vitro* in the absence of noncrest cells. *Devl Biol.* **80**, 96–106.
- SIEBER-BLUM, M., SIEBER, F. & YAMADA, K. M. (1981). Cellular fibronectin promotes adrenergic differentiation of quail neural crest cells *in vitro*. *Expl Cell Res.* **133**, 285–295.
- SILVERS, W. K. (1961). Genes and the pigment cells of mammals. *Science* **134**, 368–373.
- SILVERS, W. K. (1979). *The Coat Colors of Mice: a Model for Mammalian Gene Action and Interaction*. New York, Heidelberg and Berlin: Springer-Verlag.
- WESTON, J. A. (1970). The migration and differentiation of neural crest cells. *Adv. Morphogenesis* **8**, 41–114.
- ZILLER, C., DUPIN, E., BRAZEAU, P., PAULIN, D. & LE DOUARIN, N. M. (1983). Early segregation of a neuronal precursor cell line in the neural crest as revealed by culture in a chemically defined medium. *Cell* **32**, 627–638.

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