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Inductive differentiation of two neural lineages reconstituted in a microculture system from *Xenopus* early gastrula cells

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Summary

Neural induction of ectoderm cells has been reconstituted and examined in a microculture system derived from dissociated early gastrula cells of *Xenopus laevis*. We have used monoclonal antibodies as specific markers to monitor cellular differentiation from three distinct ectoderm lineages in culture (N1 for CNS neurons from neural tube, Me1 for melanophores from neural crest and E3 for skin epidermal cells from epidermal lineages).

CNS neurons and melanophores differentiate when deep layer cells of the ventral ectoderm (VE, prospective epidermis region; 150 cells/culture) and an appropriate region of the marginal zone (MZ, prospective mesoderm region; 5–150 cells/culture) are co-cultured, but not in cultures of either cell type on their own; VE cells cultured alone yield epidermal cells as we have previously reported. The extent of inductive neural differentiation in the co-culture system strongly depends on the origin and number of MZ cells initially added to culture wells. The potency to induce CNS neurons is highest for dorsal MZ cells and sharply decreases as more ventrally located cells are used. The same dorsoventral distribution of potency is seen in the ability of MZ cells to inhibit epidermal differentiation. In contrast, the ability of MZ cells to induce melanophores shows the reverse polarity, ventral to dorsal. These data indicate that separate developmental mechanisms are used for the induction of neural tube and neural crest lineages.

Co-differentiation of CNS neurons or melanophores with epidermal cells can be obtained in a single well of co-cultures of VE cells (150) and a wide range of numbers of MZ cells (5 to 100). Further, reproducible differentiation of both neural lineages requires intimate association between cells from the two gastrula regions; virtually no differentiation is obtained when cells from the VE and MZ are separated in a culture well. These results indicate that the inducing signals from MZ cells for both neural tube and neural crest lineages affect only nearby ectoderm cells.

Key words: *Xenopus* embryo, neural induction, monoclonal antibodies, microculture.

Introduction

Ectoderm gives rise to three distinct lineages (neural tube, neural crest and epidermis) during vertebrate neurulation (Slack, 1983). In amphibians, the separation of the two neural lineages from the epidermal one appears to arise as consequence of determinative cellular interactions during gastrulation, which precedes neurulation. The ectoderm overlying the invaginated dorsal mesoderm forms the neural plate, which is delineated by the neural fold (Schroeder, 1970; Warner, 1985). The neural plate and a part of the neural fold produce the neural tube, which eventually differentiates into the central nervous system (CNS). The rest of the neural fold, which fails to be incorporated into the neural tube, gives rises to the neural crest. Its descendents eventually differentiate into the peripheral nervous system, melanophores and a variety of other derivatives. The ectoderm outside the neural fold forms epidermis and placodes for lens or sensory epithelia (Slack, 1983).

Experimental manipulations such as grafting or explanting of embryonic tissue have elucidated the developmental mechanisms underlying the lineage separation of gastrula ectoderm in amphibians (Holtfreter, 1938; Spemann, 1938). Ectoderm pursues the epidermal lineage autonomously, but inductive action involving the close association of the dorsal mesoderm is required to produce the neural lineages. These classical observations have recently been confirmed molecularly with the aid of a variety of specific markers such as monoclonal antibodies (mAbs) or monospecific

antiserum that can distinguish the three ectoderm lineages (Akers *et al.* 1986; Jacobson and Rutishauser, 1986; Mitani, 1989; Jones and Woodland, 1989).

In spite of intensive research after the discovery of the neural induction (Spemann and Mangold, 1924), it is still not known how the inducing signal(s) is transmitted from mesoderm to ectoderm cells, nor what the nature of this signal is (Warner, 1985). Much less attention has been given to the question of the separation of the neural crest and neural tube lineages (Nieuwkoop, 1985; Mitani, 1989; Moury and Jacobson, 1989). One approach to these problems is to use a culture system of early gastrula cells in which the inductive differentiation of neural lineages can be obtained reproducibly from undetermined ectoderm cells. We recently developed a microculture system of early gastrula cells from *Xenopus laevis* (Mitani and Okamoto, 1989*a*) in which the autonomous differentiation of epidermal and muscular lineages proceeds from cells of the ventral ectoderm (VE) and the marginal zone (MZ), respectively. Here we report the application of this microculture method to the co-culturing of cells from these two regions of the gastrula to analyze the inductive differentiation of the neural lineages in a quantitative way.

Cells from the dorsal MZ (DMZ), which give rise to the middle part of the dorsal mesoderm, have the highest activity to induce neurons of neural tube origin (CNS neurons), whereas cells from the remaining MZs, which contribute the marginal part of the dorsal mesoderm in *Xenopus* (Keller, 1976), show a higher activity to induce melanophores than those from DMZ. Close association between VE and MZ cells is required for both types of inductive cellular differentiation and the inducing signals appear to be locally effective. These results suggest that neural tube and neural crest lineages are induced in normal development separately by a localized action of the different parts of the dorsal mesoderm that underlie the prospective neural plate and neural fold regions of ectoderm, respectively. Preliminary accounts of the present study appeared in an abstract form (Okamoto and Mitani, 1987; Mitani and Okamoto, 1989*b*).

Materials and methods

Animal care

Methods for keeping frogs and for obtaining embryos have been described previously (Mitani and Okamoto, 1988).

Microculture system for early gastrula cells

Early gastrula embryos of *Xenopus laevis* (stage 10¼ or 10½ according to Nieuwkoop and Faber, 1967) were used. Methods for culturing early gastrula cells were essentially as described before (Mitani and Okamoto, 1989*a*) and are summarized below. Up to 40 dejellied embryos were dissected in a modified Barth solution (MBS; Gurdon, 1977) to isolate the ventral ectoderm (VE) and portions of the marginal zone (MZ). These fragments were disaggregated by incubating in Ca^{2+}, Mg^{2+}-deficient MBS containing 1% bovine serum albumin (BSA, Fraction V, Wako) and 20 $\mu g\,ml^{-1}$ gentamycin, both being included in all subsequent media. After the outer pigmented layer of each fragment was removed and the remaining cells were thoroughly dissociated and resuspended in standard MBS (pH 7.25), the desired number of cells (5–150 in 10–20 μl) from each gastrula region were distributed separately or in combination (co-culture) into each microculture well of Terasaki plates (total 200–400 wells for a series of experiments). Unless otherwise indicated, the plates were centrifuged at a low speed (max. 600 revs min^{-1}) to facilitate reaggregation of cells in each microculture well. The cells were washed with 20 μl of fresh MBS (pH 7.75) over a period of 3 to 4 h. After the final centrifugation, each culture well contained 12 μl of culture medium (standard culture condition). For culture periods longer than 24 h, half the culture medium was substituted by 67% RPMI 1640 supplemented with 10 mM $NaHCO_3$ after around 20 h of culture and subsequently a 1:1 mixture of this medium and standard MBS was used for substitution of half the culture medium every other day.

Staining of cultured cells

Cultures were terminated by the addition of 20 μl of fixative (0.5% paraformaldehyde in BSA-free MBS containing 0.05 to 0.09 mg ml^{-1} poly-L-lysine) After approximately 1 h incubation on ice, the same volume was removed from each well and fresh 20 μl fixative was added. After fixation for further 9 to 17 h on ice, culture plates were washed by gentle dipping into PBS, PBS containing 50 mM glycine and Tris-buffered saline (TBS) successively over at least 2 days in a refrigerator.

For indirect immunofluorescence, each culture well received 10 μl of a first layer mAb containing 1% (for E3, specific for epidermis; Mitani and Okamoto, 1989*a*), 0.25% (for Mu1, specific for myotomal muscle; Mitani and Okamoto, 1989*a*), 0.1% (for Me1, specific for melanophores and retinal pigment cells; Mitani, 1989) or none (for N1, specific for CNS neurons; Mitani and Okamoto, 1988) of NP-40 After incubation for 2 h at room temperature, the plates were washed by gentle dipping into TBS solution. The washing solution was changed several times over 4 h (for E3, Me1 and Mu1) or overnight (for N1) at 4°C. The plates were finally removed from the TBS solution and excess TBS in the plates was aspirated. Each well then received 10 μl of FITC-conjugated second layer antibody (MBL) at an appropriate dilution: affinity-purified rabbit anti-mouse IgG (specific for H+L-chains) containing 0 25% (for E3 and Mu1) or 0.1% (for Me1) NP-40, or rabbit anti-mouse IgM (specific for μ-chain) for N1. After 1 h incubation at room temperature and washing as before, DAPI-staining and photographic recording were done as previously described (Mitani and Okamoto, 1989*a*).

When cultured cells were doubly stained with mAbs (N1–Me1, N1–E3, Me1–E3 or Mu1–E3 combinations), they were first stained with N1, Me1 or Mu1 and the staining recorded. The cultures were then stained with the second mAb (Me1 or E3). This reiterative double staining was carried out because all mAbs belong to the same immunoglobulin class (IgG) except for N1 which belongs to IgM class. FITC-conjugated rabbit anti-mouse IgG was used as second layer antibody in both the first and second stainings for Me1–E3 and Mu1–E3 combinations, whereas FITC-conjugated rabbit anti-mouse IgM for the first N1 and rhodamine-conjugated goat anti-mouse IgG (Cappel) for the second Me1 or E3 staining were used in N1–Me1 and N1–E3 combinations, respectively.

Results

Reconstitution of neural induction in a microculture system

In the previous study, we used a microculture system to demonstrate the autonomous differentiation of ventral ectoderm (VE) cells and marginal zone (MZ) cells from early gastrula (Mitani and Okamoto, 1989*a*). In this report, we show that the co-culture of these cells results in the induction of neural cells; during co-culture the undifferentiated ectoderm cells give rise to neural cells instead of epidermal cells.

The MZ (prospective mesoderm region) of early gastrula (stage 10½) was divided operationally into 4 portions along the dorsoventral axis of embryo as illustrated in Fig. 1A. Fate mapping study has shown that all these 4 portions contribute more or less subregions of the dorsal mesoderm (Keller, 1976). The potency of cells from each region of MZ to support the differentiation of CNS neurons in the co-culture with VE cells from stage 10¼ was examined using an mAb N1 as a probe. The spatiotemporal specificity of this antibody has been extensively studied in *Xenopus* larvae (Mitani and Okamoto, 1988); it shows a high affinity for neurons of neural tube origin both *in situ* and *in vitro*. The results are shown in Fig. 1B (some examples of typical cultures and their staining) and Fig. 1C (cumulative data). Reproducible neuronal

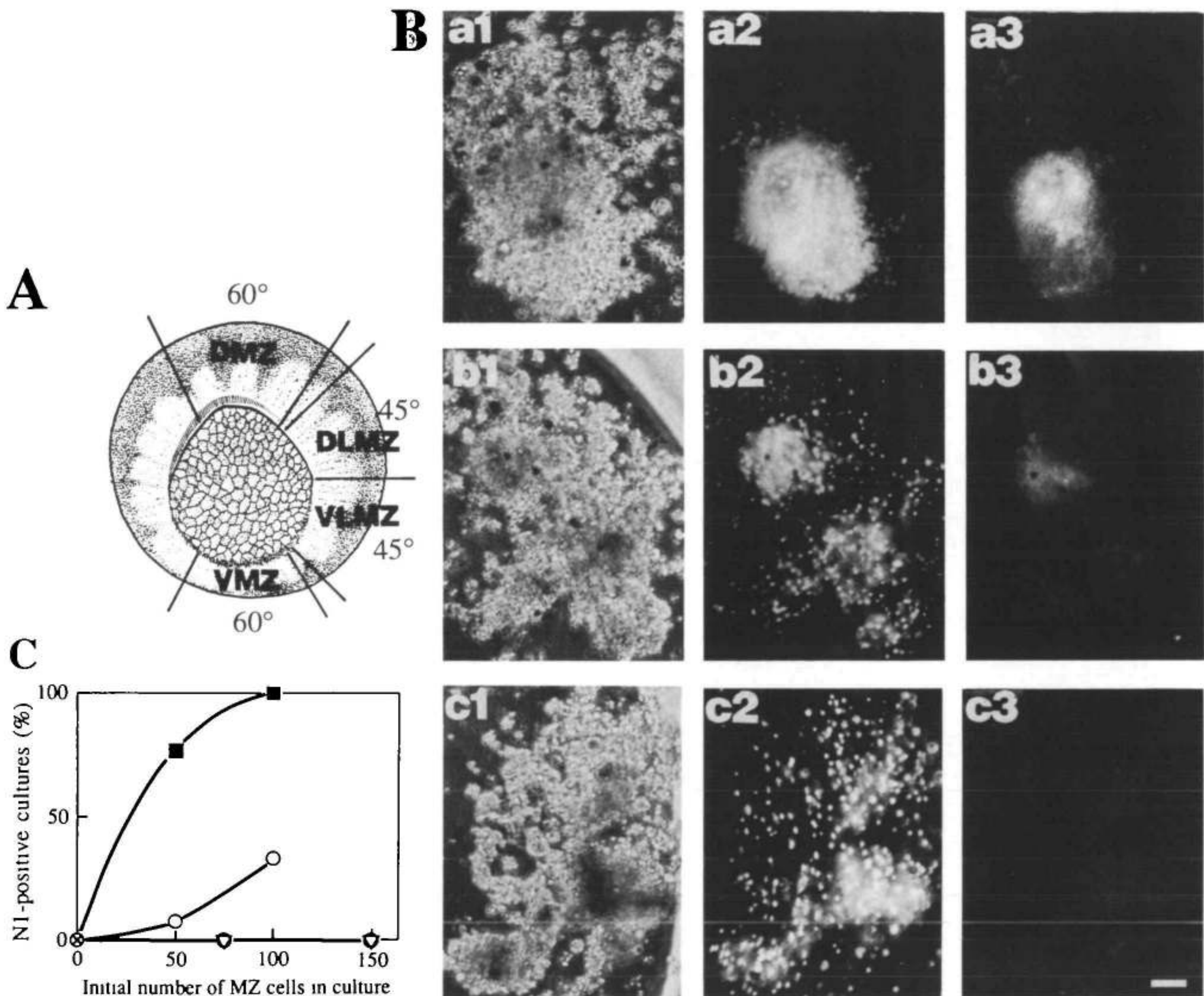


Fig. 1. Inductive differentiation of neuronal cells in a microculture system. (A) Portions of the marginal zone (MZ) used in our experiments. Vegetative view of an early gastrula (stage 10½). DMZ, dorsal marginal zone; DLMZ, dorsolateral marginal zone; VLMZ, ventrolateral marginal zone; VMZ, ventral marginal zone. (B) Neuronal cell differentiation in co-cultures. Deep layer cells from the ventral ectoderm (VE, 150 cells) were cultured with those from DMZ (100 cells, a1–a3), DLMZ (100 cells, b1–b3) or VLMZ (150 cells, c1–c3) These cultures were fixed after incubation for 138 h and examined for the expression of N1 antigen (a3–c3). (a1–c1) Phase-contrast appearance; (a2–c2) nuclear staining with DAPI. Bar=50 μm (C) Dorsoventral distribution of MZ potency to induce neuronal cell differentiation. Data were collected from a single series of experiments. The proportion of positive culture for the N1 antigen is plotted against initial number of cells in culture from the four regions of MZ indicated in A; ■, DMZ, ○, DLMZ; ●, VLMZ; ▽, VMZ. 12–26 culture wells were examined for each point.

differentiation is obtained only in co-cultures of cells from the VE and dorsal MZ (DMZ), being dependent on the initial cell number of the latter added in a culture well, as judged from the N1 antigen expression (Fig. 1C). Neither VE nor DMZ cells have detectable N1 binding when cultured on their own (data not shown). Cells from dorsolateral MZ (DLMZ) also induce N1 staining in co-cultures with VE cells, but the frequency (Fig. 1C) and extent of the antigen expression are considerably less than found in DMZ co-cultures (compare Fig. 1Ba3 with 1Bb3). There is evidently an inclined distribution of the activity to induce neuronal cell differentiation within the MZ of early gastrula. It is highest in the dorsal portion of MZ and decreases rather sharply towards the ventral side. Fig. 1C gives a quantitative measure of this inclined distribution.

To trace DMZ cells in co-cultures, they were labelled by tetramethyl rhodamine isothiocyanate (Heasman *et al.* 1984). When 20 to 80 of the labelled DMZ cells were co-cultured with 150 unlabelled VE cells, most of DMZ cells were seen to sort out as one or two clumps and extrude from a central mass of VE cells within 12 h of culture. Since N1 stain always resided in the differentiated VE cell mass, a large proportion of neuronal cells was certainly derived from VE cells. However, in most cases a small number of labelled DMZ cells remained in the VE cell mass until the end of culture. The possible contribution of these DMZ cells themselves to the neuronal cell population, though little if any, is not totally excluded at the moment (Okamoto, unpublished observation).

We next analyzed the potency of each region of MZ to suppress epidermal differentiation in the co-culture system with an mAb E3. This antibody is highly reactive with differentiated epidermal cells both *in situ* and in cultures composed of VE cells alone (Mitani and Okamoto, 1989a and also Fig. 2Aa3). Cells from DMZ inhibit E3 antigen expression partially (Fig. 2Ab3) or completely (Fig. 2Ac3) when co-cultured with VE cells, whereas cells from other sections of the MZ to do so to a slight extent (exemplified in Fig. 2Ad3) or actually promote the expression (data not shown). A quantitative analysis reveals an inclined distribution of activity

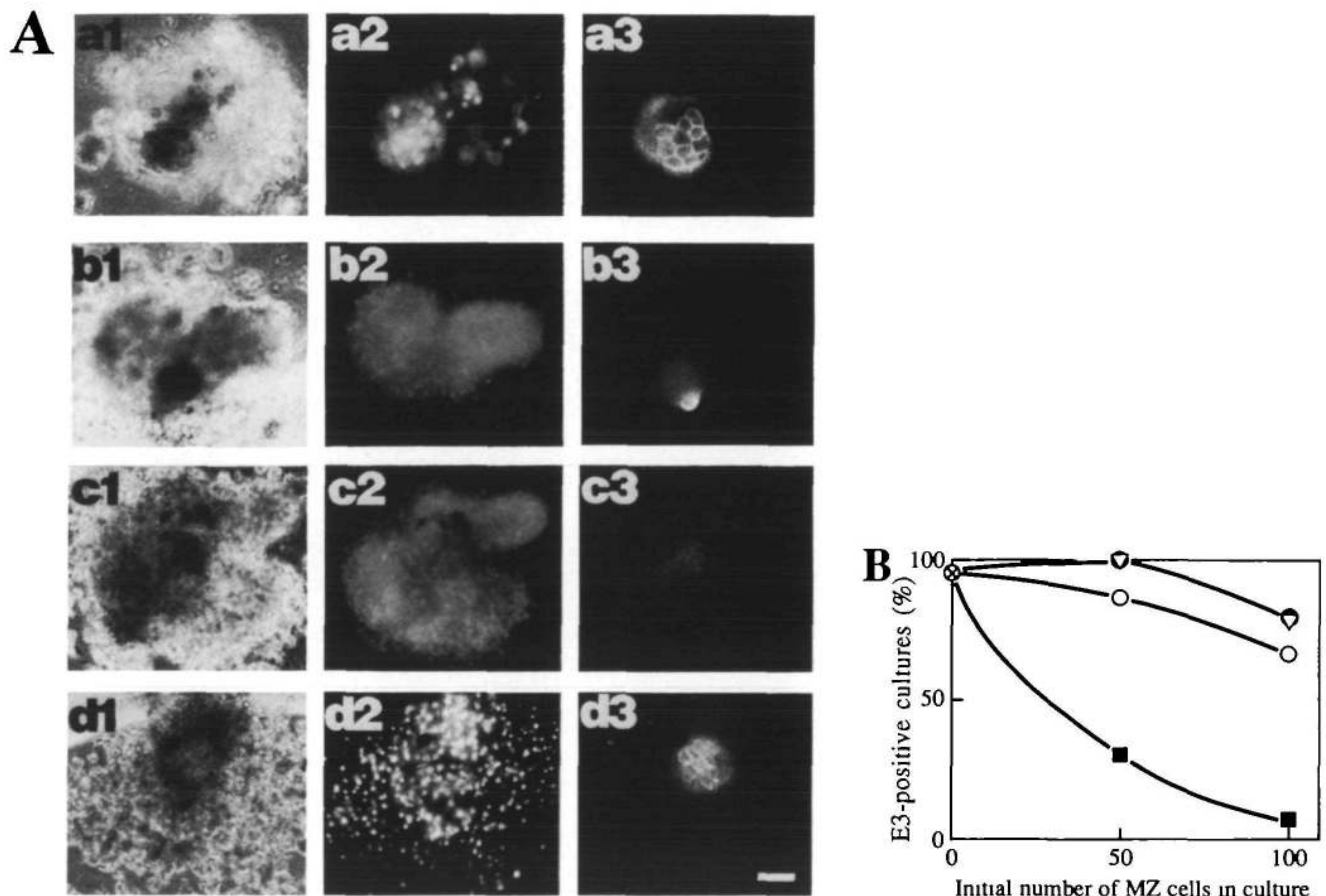


Fig. 2. Differentiation of epidermal cells in a microculture system. (A) Epidermal differentiation in culture. Deep layer cells from the VE were cultured alone (150 cells, a1–a3) or in combination with those from DMZ (50 cells, b1–b3 and c1–c3) or VMZ (50 cells, d1–d3). Cultures were fixed after incubation for 40 h and examined for the expression of E3 antigen (a3–d3). (a1–d1) Phase-contrast appearance; (a2–d2) nuclear staining with DAPI Bar=50 μm. (B) Dorsoventral distribution of MZ potency to suppress epidermal differentiation Data were collected from a single series of experiments. The proportion of cultures positive for the E3 antigen is plotted against initial number of cells in culture from the four MZ regions; ■, DMZ; ○, DLMZ, ●, VLMZ; ∇, VMZ. 13–21 culture wells were examined for each point.

within the MZ again (Fig. 2B). The potency to suppress epidermal differentiation is highest for dorsal MZ and sharply decreases in more ventral MZ. The polarity and extent of the inclination seem fairly parallel with those observed for the MZ ability to induce neuronal cell differentiation.

DMZ is the source of the middle part of dorsal mesoderm (Keller, 1976), which is known to be responsible for the induction of the neural tissue from the overlying ectoderm in normal development. This part of ectoderm could differentiate into epidermis, when experimentally freed from the influence of the dorsal mesoderm (Holtfreter, 1938; Kintner and Melton, 1987). The data described so far, thus, strongly suggest that our co-culture system can reconstitute normal neural induction *in vitro*.

Inductive differentiation of melanophores in co-culture

During the present study, we frequently observed pigmented cells differentiating in co-cultures, not necessarily with DMZ cells but with other MZ cells. These pigmented cells were thought to be melanophores, which are one of the derivatives of neural crest. We then examined whether the neural crest lineage is also induced by MZ from undifferentiated ectoderm and which part of MZ has the highest activity to induce the lineage. An mAb Mel was used as a marker for this purpose, which has a high affinity for melanophores of neural crest origin and retinal pigment cells in *Xenopus* embryos (Mitani, 1989).

Typical staining patterns of various co-cultures are shown in Fig. 3Aa to d and 3Ba3. Mel-positive cells have a star-like appearance and dispersed throughout the culture well. Most of these cells contain black pigment (Fig. 3Ba1). All these traits are characteristics of neural crest-derived melanophores *in situ*. Cells from neither the VE nor the MZ cultured on their own yield Mel-positive cells, as exemplified in Fig. 3Bb3 and c3. The degree of melanophore differentiation depends on the number of MZ cells initially added to culture wells as judged from the proportion of positive wells for the Mel-antigen expression (Fig. 4A) or from the mean number of Mel-positive cells per well directly counted (Fig. 4B). When the initial number of VE cells is 150, about 20 VMZ or VLMZ cells are sufficient to induce melanophores in every culture well examined, yielding an average of 25 Mel-positive cells per well. These results strongly suggest that the differentiation of melanophores also needs inductive cellular interaction between ectoderm and MZ, as in case of that of CNS neurons.

The potency of cells from the MZ to induce melanophores is highest for ventral tissue and gradually decreases as more dorsal tissue is used (Fig. 4). This polarity is opposite to that observed for the potency to induce CNS neurons (Fig. 1C) or to inhibit epidermal cell differentiation (Fig. 2B). These results indicate the difference in developmental mechanisms for the induction of neural crest and neural plate lineages and raise the interesting possibility that a significant portion of the neural crest lineage (probably for its trunk to tail region) is induced by the action of mesoderm other than the chordamesoderm which is derived from DMZ and is mainly responsible for the induction of neural tube lineage (see **Discussion**).

Increasing the number of DMZ cells in co-culture sometimes results in the production of a large aggregate of Mel-positive cells containing black pigment (Fig. 3Ca, c) instead of dispersed melanophores. These aggregates were seen only occasionaly in co-cultures with increased numbers of DLMZ cells, but never in co-cultures with VMZ or VLMZ cells. It is tempting to speculate that the aggregates contain retinal pigment cells that can also be recognized by Mel, a possibility that will be tested in future studies.

Co-differentiation of multiple lineages of ectoderm in a single culture well

Having reconstituted the inductive differentiation of neural lineages in the co-culture system, we investigated the cellular mechanisms underlying the induction processes, in particular, whether the inducing signal(s) from MZ cells readily diffuses throughout the culture medium, affecting all of the ectodermal cells in the well, or is locally active and affects only some of the cells.

Our data support the hypothesis of a localized action of the inducing signal(s). Cells derived from neural tube and epidermal lineages, for instance, can be seen in the same culture well, though at separate sites within a cell aggregate as demonstrated by double staining (Fig. 5Ac, d). However, often the E3-positive epidermal cells had mostly disappeared by the time the N1-positive neuronal cells were detectable, possibly because of the lack of support from connective tissue cells. To avoid this difficulty, we tested for multiple cell types in duplicate sets of co-cultures. A combination of 25 DMZ and 150 VE cells yields N1 antigen expression in all wells examined in one set and E3 antigen expression in 80 % wells in the other set of co-cultures (Fig. 5C); we may expect co-differentiation of CNS neurons and epidermal cells in a single culture well at the rate of 80 %. In another series of experiments shown in Fig. 5B, co-culturing of DMZ and VE cells in the same cell number combination results in the expression of both N1 and E3 antigen in about 70 % culture wells in duplicate sets of co-cultures; we may expect a rate of co-differentiation of at least 40 % in this series. About 5 DMZ cells are sufficient to activate N1 antigen expression in more than 20 % of E3-positive cultures (Fig. 5B), whereas even 100 DMZ cells are not enough to inhibit E3-antigen expression in all of N1-positive cultures (Fig. 5C). These results suggest that CNS neurons co-differentiate with epidermal cells in a single culture well more or less over a wide range of numbers of inducing DMZ cells. Further, changing culture volume per well (6 μl to 24 μl) in the DMZ cell titration experiment (Fig. 5B) does not appear to affect significantly the titration profile.

We also expected the coexistence of cells derived from neural crest and epidermal lineages from a comparison of titration curves shown in Fig. 2B and 4. Indeed, cultures with VMZ, VLMZ or DLMZ cells,

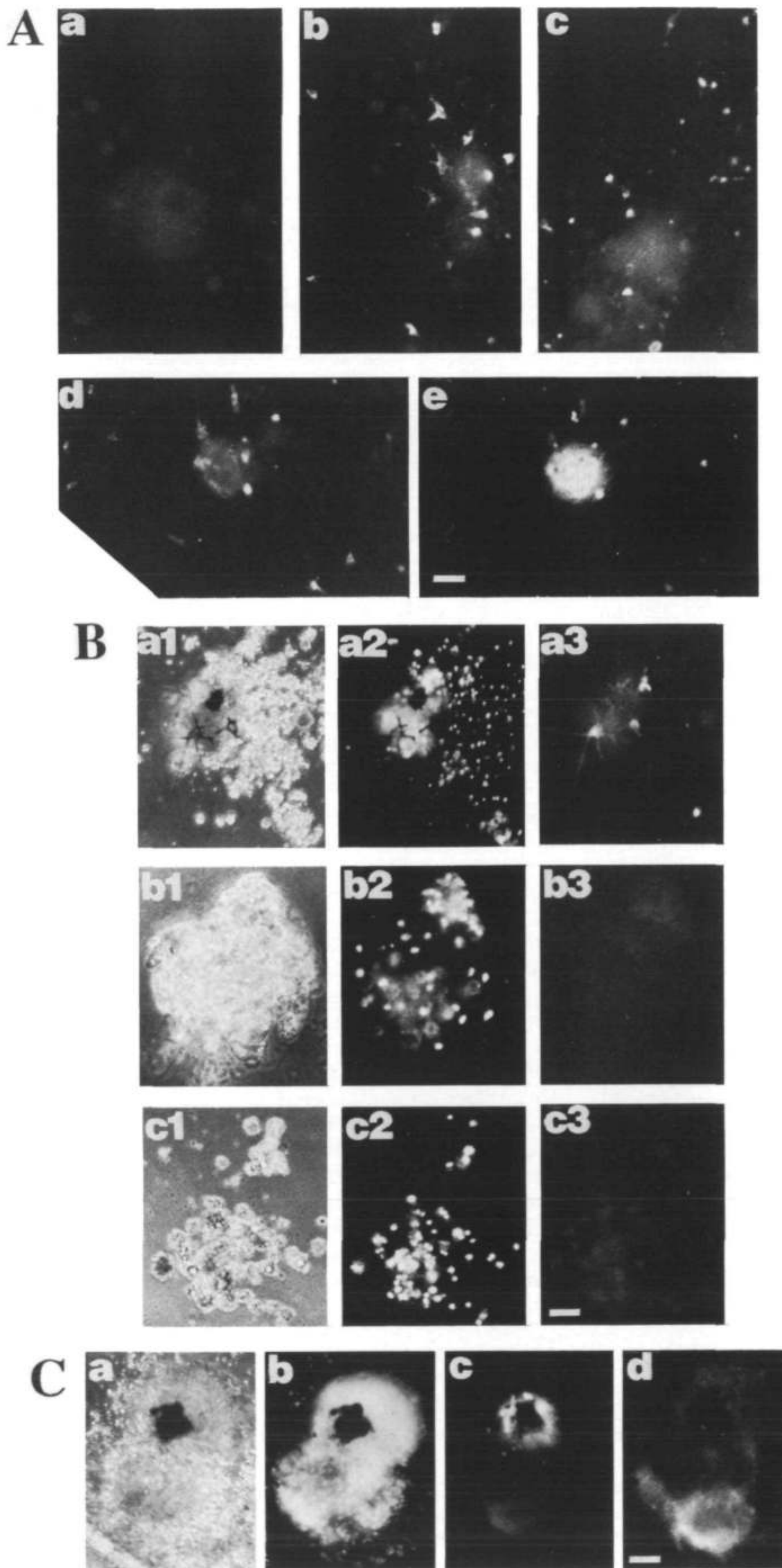


Fig. 3. Inductive differentiation of melanophores in a microculture system. (A) 150 VE cells were co-cultured with cells from DMZ (a), DLMZ (b), VLMZ (c) or VMZ (d). After incubation for 62 h, cultures were fixed and examined for the expression of Me1 antigen The culture shown in d was successively stained with E3 to show the presence of an epidermal cell aggregate (e). The focal planes in d and e are slightly different. (B) 150 VE cells and 20 VMZ cells were co-cultured (a1–a3) or cultured on their own (VE cells alone, b1–b3; VMZ cells alone, c1–c3). Incubation time was 62 h. (a1–c1) Phase-contrast appearance; (a2–c2) nuclear staining with DAPI; (a3–c3) Me1 staining. (C) 150 VE cells and 50 DMZ cells were co-cultured. After incubation for 130 h the culture was fixed and doubly stained with Me1 (c) and N1 (d). (a) Phase-contrast appearance; (b) nuclear staining with DAPI. Bar=50 μm in A–C

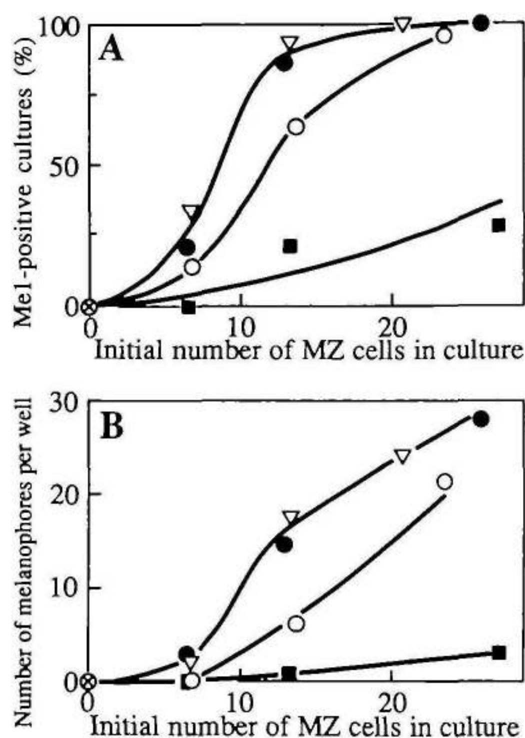


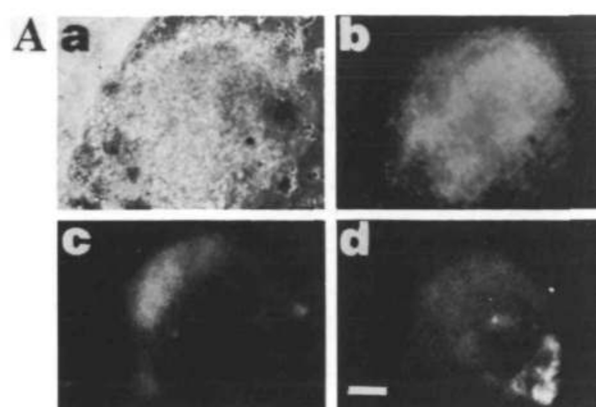
Fig. 4. Ventrodorsal distribution of MZ potency to induce melanophore differentiation Data were collected from the experiments in Fig. 3A, B. The proportion of Mel-positive cultures (A) or the mean number of Mel-positive melanophores per culture well (B) is plotted against initial number of MZ cells added to the culture: ■, DMZ; ○, DLMZ; ●, VLMZ, ▽, VMZ. 12–21 culture wells were examined for each point.

which stain with the melanophore-specific antibody Mel (Fig. 3Ab to d), also have E3-positive epidermal cells (Fig. 3Ae). Double staining with Mel and N1 antibodies revealed coexistence of the two neural lineages in a single culture well composed of VE and DMZ cells (data not shown), though its frequency was low as expected from titration profiles (Fig. 4 and Fig. 5B). When the large Mel-positive aggregates are seen (Fig. 3C), some regions are Mel-positive (Fig. 3Cc) and others are N1-positive (Fig. 3Cd).

The most straightforward explanation of the above data is that the inducing signals from MZ cells for the two neural lineages are effective only over a short distance within a cell aggregate in culture.

Requirement of intimate cell association for inductive action of MZ cells

By aggregating cells by centrifugation, we have also demonstrated that close association of VE and MZ cells is required for the induction of neural lineages. When VE and appropriate MZ cells are aggregated, both CNS neurons and melanophores differentiate (exp. 1 in Fig. 6A, B). However, when MZ cells are preaggregated about 30 min before the addition of dissociated (exp. 2) or preaggregated (about 30 min, exp. 3) VE cells, far fewer CNS neurons (Fig. 6A) and melanophores (Fig. 6B) arise. Neuronal or melanophore differentiation is restored when the two aggregates are brought into close contact by recentrifugation (exp. 4 in Fig. 6A, B).



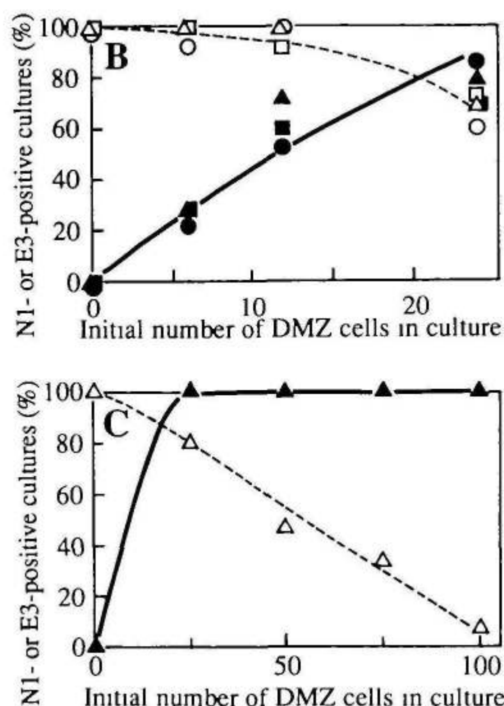


Fig. 5. Co-differentiation of neuronal and epidermal cells in a single culture well (A) 150 VE cells were co-cultured with 24 DMZ cells. After 130 h incubation they were doubly stained with N1 (c) and E3 (d). (a) Phase-contrast appearance; (b) nuclear staining with DAPI. Bar=50 μm. (B and C) 150 VE cells were co-cultured with increasing numbers of DMZ cells (two independent series of experiments differing in the number of DMZ cell added). Half of the cultures in each series were fixed after 40 h incubation and examined for the expression of E3 antigen, whereas the rest were fixed after 130 h incubation and examined for the presence of N1 antigen. The proportion of E3-positive (○, △, □), or N1-positive (●, ▲, ■) wells is plotted against initial number of DMZ cells in culture. Culture volume was 6 (○, ●), 12 (△, ▲) or 24 (□, ■) μl well^{-1}. 13–20 culture wells were examined for each point.

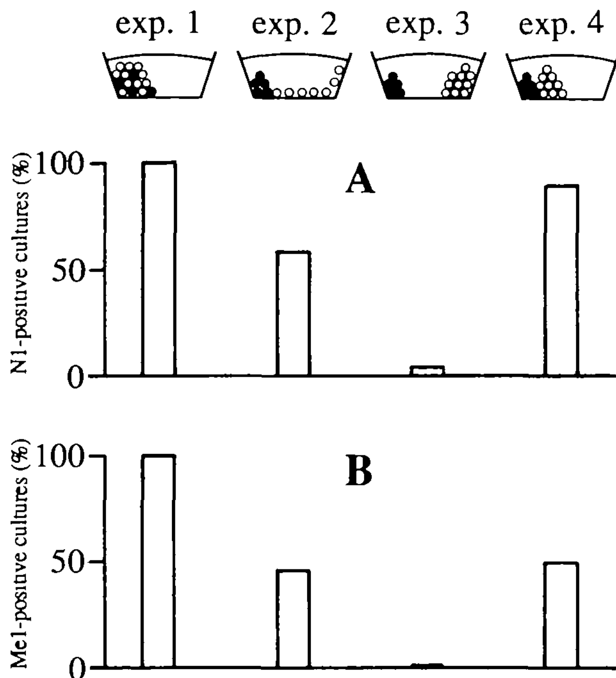


Fig. 6. Dependence of neuronal cell and melanophore differentiation on intimate cell association. 150 VE cells were co-cultured with 100 DMZ cells for 129 h (A) or with 50 lateral marginal zone (DLMZ+VLMZ) cells for 61 h (B) according to the four experimental protocols illustrated at the top of the figure (exp. 1 to 4; ○, VE cell; ●, MZ cell; see text for detail). Columns; proportion of N1-positive (A), or Mel-positive (B) cultures. 12–20 culture wells were examined for each column.

Discussion

One of the main results of the present study is the successful reconstitution of inductive differentiation of not only neural tube but also neural crest lineages from early gastrula cells in a microculture system. Together with our previous demonstration of the autonomous differentiation of the epidermal lineage in culture (Mitani and Okamoto, 1989*a*), we can now follow the differentiation of all three major lineages arising from *Xenopus* gastrula ectoderm in a microculture system.

Inductive differentiation of neural tube lineage in a microculture system

A sharp dorsoventral distribution of potency is seen in the ability of MZ cells to induce neuronal cells of neural tube origin (Fig. 1C). This distribution is in agreement with both classical studies and more recent reports that indicate that DMZ cells can serve as a strong organizer for the CNS (Spemann, 1938; Akers *et al.* 1986; Jacobson and Rutishauser, 1986; Kintner and Melton, 1987; Sharpe *et al.* 1987; Jones and Woodland, 1989). VE cells never give rise to CNS neurons when cultured alone (Fig. 1C) and lose their competence to respond DMZ cells to yield CNS neurons in the course of isolated culture (data not shown). Furthermore our dye marking experiments in culture suggest that a large proportion of CNS neurons arise from VE cells, but not from DMZ cells. Thus, our culture system of dissociated and reaggregated cells appears to support normal neural induction.

Deuchar (1970) co-cultured explants and groups of cells from *Xenopus* early gastrula and showed that for success of neuralization a certain number of ectoderm cells, but not of dorsal lip cells is critical. Although quantification of cell number and neural differentiation was not necessarily thorough in the study, her observation appears to be fairly consistent with ours (Fig. 5B and data not shown). On the other hand, some results of previous reports (Barth and Barth, 1959; Godsave and Slack, 1989) apparently contradict the present ones. They observed that neural cells differentiated from cultured amphibian ectoderm cells in the absence of MZ cells. However, Barth and Barth (1963) showed later that substance(s) released from the agar substrate at the bottom of operating dish had the inducing activity to *Rana pipiens* ectoderm cells. They also obtained the neural induction from *Rana* ectoderm (clumps of about 125 cells) exposed to various salt solutions (Barth and Barth, 1963, 1969), but the high level of Ca^{2+}, Mg^{2+} or Mn^{2+} in these solutions seems rather unphysiological. Godsave and Slack (1989) cultured *Xenopus* embryonic cells in microculture wells as in our experiments. There are, however, some differences in culture conditions between the two studies. Godsave and Slack used animal cap cells from stage 8 (age 5 h) instead of VE cells from stage $10\frac{1}{4}$ (age 10 h) as in our experiments, culture wells coated with laminin and fibronectin instead of uncoated wells and γ-globulin (fraction II) from bovine plasma in culture medium instead of bovine serum albumin. Among these, the difference of age of embryos used seems most important; the state of commitment of the animal cap cells is likely to be unstable, whereas that of the VE cells is already biased toward the epidermal differentiation. Isolated culture of the animal cap cells in the presence of γ-globulin and some extracellular materials may favor their commitment to the neural direction.

Since the number of cells and extent of neural differentiation can be quantified and also the manner of cellular interactions can be easily modified, we believe that the culture system described in the present study opens up the possibilities of analysing the *normal* course of neural induction quantitatively under a variety of experimental conditions, including that for testing inducing substances.

Inductive differentiation of neural crest lineage in a microculture system

We have shown that cells from a broad MZ sector support melanophore differentiation of neural crest origin in co-cultures with VE cells (Fig. 4). Cells from either the VE or MZ alone yield no melanophores in culture. Further, preculturing of VE cells on their own, which leads to the autonomous epidermal differentiation, suppresses their competence to respond to MZ cells to produce melanophores as in case of neuronal cell induction (data not shown). These results suggest that separation between epidermal and neural crest

lineages is brought about by the inductive action of MZ cells on undetermined ectoderm cells. However, it is also shown that ventral and not dorsal MZ cells best support melanophore differentiation. This difference in the induction of melanophores and CNS neurons may reflect differences in the inducing mechanisms of neural crest and neural tube lineages, as previously suggested (Mitani, 1989). Mitani showed that melanophores differentiated abundantly even in the larvae whose CNS was severely damaged by the injection of heparin or dextran sulfate into the blastocoel cavity of *Xenopus* early embryos.

Developmental mechanism for the formation of neural crest.

Neural crest cells are known to arise from a part of the neural fold that delineates the neural plate in vertebrate development (Slack, 1983). Both the neural plate and fold structures are induced from the part of ectoderm that is underlaid by the dorsal mesoderm during gastrulation. Since we have shown that close association between cells from VE and MZ (prospective mesoderm region) is required for the induction of both neural crest and tube lineages, it is highly likely that the marginal part of dorsal mesoderm that underlies the prospective neural fold region of ectoderm at late gastrula produces inducing signal(s) for the neural crest lineage; the signal is locally active and, thus, limits the spatial extent of the neural plate which is separately induced by the localized action of the remaining central part of dorsal mesoderm (Jones and Woodland, 1989).

We have obtained an unexpectedly high inducing activity of VMZ cells. However, understanding of the intricate morphogenetic movement that forms the dorsal mesoderm from MZs during *Xenopus* gastrulation, can explain our present results; in *Xenopus*, precursor cells for the dorsal mesoderm are present in a broad MZ sector (dorsal to ventralmost) of the early gastrula (Keller, 1976; Gerhart and Keller, 1986). Cells of the involuting MZ of early gastrula converge strongly toward the dorsal midline with concomitant extension in the animal–vegetal direction that corresponds to the anteroposterior axis of the late gastrula, after turning inside the gastrula (Gerhart and Keller, 1986). This *convergent extension* of MZ cells begins first and is most vigorous at the prospective dorsal midline of embryo and spreads laterally. As a result of these complicated cellular movements, the dorsal mesoderm of late gastrula is not necessarily derived solely from DMZ but also from other MZs of the early gastrula; the whole cephalic region and the central part of the trunk-to-tail region of the dorsal mesoderm arising from DMZ, whereas the remaining marginal part of the trunk-to-tail region of the dorsal mesoderm derives from other MZs including VMZ (Keller, 1976). Thus, if the marginal part of the dorsal mesoderm, which underlies the prospective neural fold region of ectoderm, induces the neural crest, as we have suggested, it is not surprising that cells from a broad MZ sector of early gastrula including VMZ are more or less capable of supporting melanophore differentiation. It is well known that head and trunk neural crest have a different set of derivatives (Hopwood *et al.* 1989). The ventrodorsal distribution in the potency of MZ cells that we have observed for melanophore differentiation may be due to this regional difference in the developmental fate of neural crest cells; more caudal neural crest cells would give rise to more melanophores. If DMZ cells exhibited their ability to induce neuronal cells in preference to melanophores, this would also contribute to the ventrodorsal distribution in the MZ cell potency.

There could, of course, be other explanations for the inducing potency of VMZ cells observed *in vitro*. VMZ cells for instance gain the inducing potency as a result of the *in vitro* operative procedures. Dale and Slack (1987) have presented evidence that ventrovegetal blastomeres from *Xenopus* embryos can have a dorsal inductive potency partially activated by microsurgery. However, they have also shown that by stage 7 these ventrovegetal blastomeres are less sensitive to the operative procedures. Furthermore, we explored the artificial effect of our *in vitro* procedures on MZ cells from stage $10\frac{1}{2}$ gastrula by examining the potency of these MZ cells to differentiate into muscle cells on their own. Cells (50/well) from each of the four MZ portions were cultured under the same conditions as described in this article and differentiated muscle cells were quantified using an mAb Mu1 as a specific probe (Mitani and Okamoto, 1989*a*). Mean numbers of Mu1-positive muscle cells per well were 7.1 ± 1.4 for VMZ cells, 7.6 ± 0.9 for VLMZ cells, 19.6 ± 2.0 for DLMZ cells and 0.36 ± 0.19 for DMZ cells (mean$\pm$s.e.). The relative ratio of these figures agrees with that expected from the fate map (Keller, 1976). Thus, although the possibility is not totally excluded that we overestimate the activity of VMZ cells to some extent due to our operative procedures, its degree is likely to be small. It is also possible that the ectoderm is sensitized to differentiate more readily into melanophores by the operative procedures. However, this mechanism of sensitization does not necessarily lead to overestimation of the activity of VMZ cells, as compared with those of cells from other MZs. When MZ cell activities were compared, cells from each of the four MZ portions were co-cultured with ectoderm cells that were derived from the same large pooled cell suspension.

There have been so far, at least, two different views on the neural crest formation from ours (Nieuwkoop, 1985; Moury and Jacobson, 1989). Nieuwkoop has suggested that the neural crest is formed in the peripheral region of the neural anlage when the neuroectoderm begins to lose its competence for the inducer that is propagating laterally from the chordamesoderm of the dorsal midline. According to this view, we could expect that the potency of MZ to support melanophore differentiation should be sharply biased toward a DMZ sector, because the chordamesoderm is mainly derived from the DMZ. However, the results we have obtained are totally inconsistent with this expectation. Since the view assumes that a rather weak interaction between the inducer and responding cells results in the neural crest formation, it also

predicts that a smaller number of DMZ cells would be needed for melanophore differentiation than for neuronal cell differentiation and that the preculturing of VE cells before addition of MZ cells, which has led to a loss of their competence to an appropriate extent, would facilitate melanophore differentiation. However, these are again not supported in our experiments.

Moury and Jacobson (1989) claim that the local interaction of neural and epidermal tissue is responsible for the formation of neural crest; when a lateral piece of neural plate was transplanted into the ventral epidermis, it gave rise to melanophores. However, other explanations could account for the observation. The neural plate transplants that they used may have already received the inducing signal locally from the underlying dorsal mesoderm. It would be, then, possible that these transplants, especially their lateral-most portions, autonomously produced the neural crest, when placed in the ventral epidermis and removed far from the influence of the chordamesoderm which strongly favors the induction of neural tube lineage. It is also noteworthy that according to this second view, we should obtain the greatest degree of differentiation of melanophores in co-cultures of VE cells with DMZ cells, because in this combination epidermal and neuronal cells co-differentiated most efficiently. However, the results we have obtained are inconsistent with this prediction.

Signal transmission in neural lineage induction

The second major result of this study is that close association between cells of the VE and MZ is essential for the induction of both neural tube and crest lineages (Fig. 6). Moreover, preliminary experiments to show an effect of conditioned culture medium from MZ cells on induction of neural lineages failed to do so (Okamoto, unpublished observation), in contrast to the previous result (Niu and Twitty, 1953). Together with our demonstration of localized action of the inducing signals within a cell aggregate, readily diffusible substances from MZ cells do not seem to play a major role in the induction processes.

The requirement of close cell contact for the 'neural induction' has also been indicated using embryonic pieces from *Xenopus laevis* (Tacke and Gruntz, 1988), or cleavage-arrested blastomeres from *Halocynthia roretzi*, a sea squirt (Okado and Takahashi, 1988). These and our data indicate that inducing signals are transmitted *via* either direct cell-to-cell interactions or substances that are diffusible but effective only over a short distance like neurotransmitters at synaptic junctions in the nervous system or some of growth factors that are sequestered to extracellular material. In the former case, molecules associated with outer surface of MZ cells, for instance, could bind to surface receptors on VE cells and initiate the induction. The transfilter experiments of Toivonen *et al.* (1975) seem to be in accord with the latter idea, although the extent of neural differentiation is pointed out to be poor (Warner, 1985). Examination of the inducing activities of killed MZ cells or membrane fractions from them will be helpful to decide between the two possibilities.

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