Basic fibroblast growth factor induces differentiation of neural tube and neural crest lineages of cultured ectoderm cells from *Xenopus* gastrula

Mineko Kengaku and Harumasa Okamoto

Department of Neurobiology, Institute for Brain Research, Faculty of Medicine, University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113, Japan

SUMMARY

The vertebrate nervous system is initially induced from a section of dorsal ectoderm by signal(s) from the underlying dorsal mesoderm during gastrulation. In an effort to identify the neural inducing factor(s) emanating from the dorsal mesoderm, we have examined the inductive action of various growth factors by applying them to ectoderm cells from *Xenopus* gastrulae (8- to 12.5-hour age; embryonic stage 9+ to 11.5) in a microculture system. Monoclonal antibodies that specifically recognize cellular differentiation antigens from three distinct ectoderm lineages (N1 for CNS neurons from neural tube, Me1 for melanophores from neural crest and E3 for skin epidermal cells from epidermal lineages, respectively) and a mesoderm lineage (Mu1 for muscle cells) were used as markers to monitor the differentiation of cultured ectoderm cells.

We found that basic fibroblast growth factor (bFGF) was capable of specifically and reproducibly inducing gastrula ectoderm cells to produce CNS neurons and melanophores at concentrations as low as 5 pM, a value about 50-fold lower than that required to induce the formation of muscle cells from blastula animal cap cells (6-hour age; stage 8+). The induction of neural lineages by bFGF was correlated with a suppression of epidermal differentiation in a dose-dependent manner. bFGF never induced the formation of muscle cells from gastrula ectoderm cells even at concentrations as high as 5 nM.

The response of ectoderm cells to bFGF changed dramatically during gastrulation. Ectoderm cells from early (8- to 9-hour age; stage 9+ to 10) gastrula gave rise to CNS neurons, but yielded few melanophores. As ectoderm cells were prepared from gastrulae of increasing age, their competence to form neurons was gradually lost, whereas the production of melanophores was enhanced and peaked in 11-hour gastrula (stage 10). The ability to form both neurons and melanophores was substantially reduced in 12.5-hour gastrula (stage 11). By examining ectoderm cells from the ventral and dorsal sides independently, it was also shown that during gastrulation the change in response to bFGF of the ventral ectoderm preceded that of the dorsal ectoderm.

The state of competence of the ectoderm changed primarily due to intrinsic factors rather than by instruction from other parts of the gastrula embryo. This was shown by adding bFGF to cultures of ectoderm cells that were isolated at 9-hour (stage 10) and cultured for increasing periods to allow their autonomous development. The time course of both loss of neuronal competence and gain and loss of melanophore competence closely paralleled that observed in vivo during gastrulation. Further, we showed that the sensitivity of ectoderm cells to bFGF in the neuronal and melanophore induction processes decreased during later gastrula stages; i.e., higher doses of bFGF were required for older ectoderm cells to be fully induced. A possible role of bFGF in neural induction during normal development is discussed.

Key words: *Xenopus laevis*, basic fibroblast growth factor, neural induction, neural tube, competence, gastrula

INTRODUCTION

The initial stage in the development of the vertebrate nervous system is the formation of the neural plate from a section of dorsal ectoderm during neurulation. Lateral edges of the neural plate subsequently protrude to form the neural folds, which eventually fuse at the dorsal midline producing the neural tube. During this stage of morphogenesis, some neural fold cells migrate out of the neural tube dorsally and produce the neural crest beneath the future epidermis. The neural tube lineage gives rise to the central nervous system (CNS), whereas the neural crest lineage produces the peripheral nervous system (PNS) and other derivatives including melanophores.

Experimental manipulations such as transplantation or ablation of embryonic portions in amphibians have revealed that the instruction of dorsal mesoderm to overlying ectoderm during gastrulation is required for the formation of the neural plate and neural fold (Spemann, 1938). The part of ectoderm that escapes the influence of the dorsal
mesoderm undergoes epidermal differentiation, a process shown by explanting experiments to be autonomous (Holtfreter, 1938). Based on these classical observations and others that followed, it has long been believed that the change of developmental fate of the ectoderm from epidermis to neural tube or to neural crest lineage is brought about by inducing signal(s) emanating from the dorsal mesoderm at the appropriate time. However, in spite of intensive research on the molecular and cellular mechanisms underlying the neural induction, very little is yet understood about the nature of the specific signal(s) that emanate from dorsal mesoderm cells, and how these are transmitted to ectoderm cells (Warner, 1985).

An initial approach to these questions is to establish a culture system for early gastrula cells in which inductive differentiation of neural lineages can be reproducibly obtained and analyzed quantitatively under a variety of experimental conditions. For this purpose, we recently developed a microculture system for gastrula cells from *Xenopus laevis* and developed an immunocytochemical assay for quantitative examination of cellular differentiation in culture using monoclonal antibodies that distinguish several lineages of embryonic cells (Mitani and Okamoto, 1989, 1991). These studies have shown that gastrula ectoderm cells cultured alone autonomously differentiate into epidermal cells, whereas the same cells, when cocultured with cells from the marginal zone (MZ), produce a derivative of neural tube or neural crest lineage depending on the origin of MZ. Dorsal MZ (DMZ) cells, which give rise to the central part of the dorsal mesoderm, have the greatest ability to induce ectoderm cells to form CNS neurons of neural tube origin. By contrast, cells from the more ventral MZs, which give rise to the lateral marginal part of the dorsal mesoderm, show a greater ability to induce ectoderm cells to form melanophores of neural crest origin.

Reconstitution of neural induction in a microculture system enables us to identify candidates for the neural inducing factor from the dorsal mesoderm simply by screening substances that mimic the action of DMZ or other MZs to ectoderm cells in culture. In the present studies, we have paid special attention to growth factors, since some of them have been shown to induce blastula animal cap cells (presumptive ectoderm cells) to form mesoderm derivatives (Slack et al., 1987; Kimelman and Kirschner, 1987). We found that basic fibroblast growth factor (bFGF) reproducibly induced both neural tube-derived CNS neurons and neural crest-derived melanophores at picomolar concentrations. We also showed that the state of competence of the ectoderm to respond to bFGF changed during gastrulation. When applied to ectoderm cells of earlier gastrula, bFGF induced primarily the neural tube lineage with strong suppression of epidermal cell formation, thus mimicking the action of DMZ cells. By contrast, when applied to ectoderm cells of later gastrula, bFGF induced primarily the neural crest lineage with rather weak suppression of the epidermal lineage, thus mimicking the action of more ventral-side MZ cells. In addition, the change of ectoderm competence appeared to occur autonomously. These findings raise the possibility that the release of bFGF at appropriate times from the respective portions of MZ plays a crucial role in the neural induction process during normal development. A preliminary account of this study has been presented (Kengaku and Okamoto, 1991).

### MATERIALS AND METHODS

#### Materials

Tissue-specific monoclonal antibodies (E3 for epidermal cells, N1 for CNS neurons, Me1 for melanophores and Mu1 for myocytes) were produced as previously described (Mitani and Okamoto, 1988, 1989; Mitani, 1989). Recombinant bovine bFGF was purchased from Fogen Biotechnik, Heidelberg, and recombinant human transforming growth factor β (TGF-β) was from King Brewing Co., Ltd., Kakogawa-shi, Japan. Human platelet-derived growth factor (PDGF) and mouse epidermal growth factor (EGF) were obtained from Collaborative Research Inc., Bedford, MA.

#### Microculture of *Xenopus* embryonic cells

Embryos were staged according to Nieuwkoop and Faber (1967), but developmental stages are expressed both as ages in hours and as Nieuwkoop Faber stages throughout the manuscript. This is because the developmental stages of some of embryos that we used were not clearly indicated in the Nieuwkoop Faber stage table and also because we correlated the age of embryonic cells in vitro with that in vivo in some experiments. Dissociation and culture of ectoderm cells from gastrula embryos of *Xenopus laevis* were performed as previously described (Mitani and Okamoto, 1989, 1991). Briefly, animal pole tissue was dissected from dejellied embryos in modified Barth solution (MBS; Gurdon, 1977) and dissociated by incubating in Ca²⁺/Mg²⁺-deficient MBS containing 1% BSA at room temperature. The dispersed cells were suspended in standard MBS containing 1% BSA and inoculated into plastic culture wells of Terasaki plates (Nunc) at 120 cells/well. The plates were briefly centrifuged to facilitate reaggregation of cells and then cultured at 22.5°C in humidified air. After around 20 hours of incubation, the culture medium was removed and substituted with a 1:1 mixture of 67% RPMI 1640 supplemented with 10 mM NaHCO₃ and standard MBS containing 1% BSA. Cultures were maintained for 2 days for E3, Mu1 and Me1 staining, and 5 to 6 days for N1 staining. Basic FGF or other growth factors were included for 20 hours from the beginning of culture unless otherwise noted. 

#### Immunocytochemistry

The cells cultured in Terasaki plates were fixed in 0.45% paraformaldehyde with 70 µg/ml poly-L-lysine (Sigma Chemical Co., St Louis, MO) in MBS at 0°C for 12 to 17 hours. Then they were washed with PBS, 50 mM glycine in PBS and Tris-buffered saline (TBS) successively at 4°C over at least 2 days. The cells were incubated for 2 hours with primary antibodies diluted in MBS containing 0.2% bovine serum albumin (BSA) and appropriate concentration of NP-40 (Mitani and Okamoto, 1991), and then incubated for 1 hour with FITC-conjugated secondary antibodies. Plates were washed with TBS after each incubation. After nuclear staining with DAPI, individual wells were observed using a fluorescent microscope. 10 to 15 culture wells were examined for each data point to assess the experimental results quantitatively.

#### Explant culture of embryonic tissues

Ectoderm tissue was dissected from dejellied gastrula embryos in MBS and the pigmented outer cell layer was removed manually using a hair-loop instrument. Brief incubation in Ca²⁺/Mg²⁺-deficient MBS facilitated the operation, while keeping the inner cell layer largely intact. The isolated inner layer was then cultured in standard MBS as a cell aggregate explant in the presence or absence of bFGF. Cultures were maintained for 2 to 5 days and fixed with 0.45% paraformaldehyde in MBS at 0°C for about 16 hours.
Neural induction by bFGF

1069

Neural induction by bFGF

hours. Frozen sections were prepared and stained as previously described (Mitani and Okamoto, 1988).

RESULTS

Basic FGF induces neuronal differentiation of ectoderm cells from early gastrula

In an effort to search for substances that mimic the neural inducing action of dorsal mesoderm, we screened a number of growth factors that have been shown to influence the developmental fate of various embryonic cells (Cross and Dexter, 1991). Animal cap ectoderm fragments were dissected from 10-hour gastrulae (stage 10) and a small number of dissociated ectoderm cells were cultured in the presence or absence of growth factors after reaggregation in Terasaki plate wells (Mitani and Okamoto, 1989, 1991).

By monitoring neuronal differentiation in cultures with the aid of a mAb N1 that specifically binds to CNS neurons of neural tube origin (Mitani and Okamoto, 1988), we found that bFGF reproducibly induced differentiation of CNS neurons of cultured ectoderm cells at concentrations in the picomolar range. We obtained N1-positive signal in every culture well examined when bFGF was added at concentrations above 0.5 ng/ml (Fig. 1). By contrast, epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and transforming growth factor beta 1 (TGF-β1) never induced neuronal differentiation even at much higher concentrations (Fig. 1), nor did they affect the autonomous differentiation of ciliated epidermal cells, which were constantly observed in ectoderm cell cultures without growth factors (Mitani and Okamoto, 1989).

Among other growth factors tested, acidic FGF (aFGF) showed essentially a similar inducing effect on ectoderm cells as bFGF. TGF-β2 (200 ng/ml) and activin (1-10 ng/ml) occasionally induced N1-positive CNS neurons. However, compared to bFGF, we could not obtain reproducible results with these two agents and therefore did not further investigate their effects on ectoderm cells in the present experiments.

To ensure the specificity of the neuronal inducing action of bFGF, we examined native bFGF isolated from bovine pituitary in place of the recombinant bovine bFGF that was used for the experiment in Fig. 1. We found that the native bFGF was several-fold more potent than recombinant bFGF. Moreover, when native bFGF (3.3 ng/ml) was pretreated with the anti-bFGF neutralizing antibody (200 µg/ml), it showed almost no inducing activity for ectoderm cells in culture. Since much larger amounts of the recombinant bFGF were available, it was used throughout the following experiments.

The dose-response profile of the neuronal inducing action of bFGF is shown in Fig. 2. Both the extent (Fig. 2A, upper panels) and frequency (Fig. 2B, closed circles) of the N1 antigen expression were enhanced by increment in the bFGF dose. Significant induction was observed at concentrations as low as 0.1 ng/ml with maximal induction at 0.5 ng/ml bFGF. In contrast, differentiation of epidermal cells was inhibited in a dose-dependent manner by the addition of bFGF. This was shown by the staining with a mAb E3 that specifically binds to epidermal cells (Mitani and Okamoto, 1989). Both the extent (Fig. 2A, lower panels) and frequency (Fig. 2B, open circles) of the E3 antigen expression were suppressed by increasing the bFGF dose. An approximately

Fig. 1. Effects of growth factors on the differentiation of CNS neurons from gastrula ectoderm cells. Animal cap cells from 10-hour gastrulae (stage 10) were dissociated and cultured in the presence of either EGF, bFGF, PDGF or TGF-β1 at the concentrations indicated. Left panels show the expression of N1 antigen after 5 days of culture. Right panels show nuclear staining with DAPI. Note that bFGF, but not other growth factors, induced the differentiation of aggregated CNS neurons. Scale bar, 100 µm.
80% decrease in the frequency of E3-positive cultures was obtained by treatment with 2.5 ng/ml bFGF. Thus bFGF can substitute in a dose-dependent manner for the cell number-dependent neuronal-inductive and epidermal-suppressive actions of DMZ cells, which were previously shown in cocultures with ectoderm cells (Mitani and Okamoto, 1991).

When bFGF was added at concentrations below 0.5 ng/ml and washed away after about a 2 hours incubation with ectoderm cells, the extent of neuronal differentiation appeared comparable to that obtained without the washing procedure, as judged by N1 staining. This means that a comparatively short-term exposure to bFGF during an early embryonic stage is sufficient to obtain its inducing effect during a later larval stage. The requirement for only short-term exposure during an earlier stage is one of the characteristics of a variety of induction systems involving two embryonic tissues (Gurdon, 1987).

**State of competence of ectoderm cells to respond to bFGF changes during gastrulation**

Many reports have suggested that the ectoderm retains its ability to respond to the neural inducing signal(s) from the dorsal mesoderm (neural competence) for a limited period during development (Holtfreter, 1938; Gallera, 1952; Sharpe and Gurdon, 1990; Servetnick and Grainger, 1991). We then asked whether this temporal limit also exists for the neuronal competence of cultured ectoderm cells in the response to bFGF.

Ectodermal cells from late blastula (7-hour age; stage 9) and gastrulae of increasing ages (8- to 12.5-hour; stage 9+ to 11) were cultured with 1 ng/ml bFGF and examined for the expression of N1 antigen. N1-positive CNS neurons were first detected in cultures of about 8-hour ectoderm cells. Neuronal competence of ectoderm cells remained high during early gastrula stage, reaching a peak at 9- to 9.5-hour age and then was gradually lost by 12.5 hours (Fig. 3A, upper panels; Fig. 3B, open circles). N1 antigen expression induced in cultures of ectoderm cells from later gastrula is not only less frequent (Fig. 3B) but also substantially weaker (Fig. 3A) than that in cultures of early gastrula ectoderm cells. However, in cultures of later gastrula ectoderm cells treated with bFGF, we frequently observed the differentiation of pigmented cells instead of CNS neurons. These cells were confirmed to be melanophores of neural crest origin by their binding of a specific marker, mAb Me1 (Mitani, 1989).

We next examined the competency period of ectoderm cells for the induction of neural crest lineage by bFGF (Fig. 3A, lower panels; Fig. 3B, closed circles). Early gastrula ectoderm cells (8- to 9-hour ages; stage 9+ to 10) cultured with 1 ng/ml bFGF had little or no ability to differentiate into melanophores. However, as ectoderm cells were taken from older gastrulae, the ability to develop into melanophores rose, peaking at 11-hour (stage 10). Ectoderm cells retained the ability to develop into melanophore for 2-3 hours at mid-gastrula stage, then rapidly lost it by 12.5-hour age (stage 11). Ectoderm cells from embryos older than 12.5 hours had neither neural tube nor neural crest competence but autonomously differentiated into epidermal cells irrespective of the presence of bFGF.

The dose-response profile of the melanophore-inducing action of bFGF was examined in cultures of 11-hour (stage 10) ectoderm cells (Fig. 4). Maximal induction was obtained at about 0.5 ng/ml (Fig. 4B). This value is comparable to that obtained for the neuronal inducing action of bFGF in cultures of 10-hour (stage 10) ectoderm cells. However, the differentiation of E3-positive epidermal cells from 11-hour ectoderm cells appeared to be more resistant to the suppressive action of bFGF than that from 10-hour ectoderm cells: more than a 10-fold higher dose of bFGF was required for 11-hour ectoderm cells to obtain the same extent of inhibition of epidermal differentiation as for 10-hour ectoderm cells (Fig. 4B versus Fig. 2B). This characteristic profile of melanophore-inductive, epidermal-suppressive effects of bFGF on 11-hour ectoderm cells is reminiscent of a similar profile previously obtained for the
Neural induction by bFGF

It has been shown that bFGF can induce blastula animal cap cells (future ectoderm cells) to differentiate into mesoderm derivatives such as muscle and mesothelium (Slack et al., 1987; Kimelman and Kirschner, 1987). This result was reproduced in our microculture system. The expression of Mu1 antigen, a specific marker of myocyte

Fig. 3. Changes in the competence of gastrula ectoderm cells to respond to bFGF. (A) Neuronal and melanophore differentiation from ectoderm cells at various gastrula stages. Dissociated animal pole cells from embryos of indicated ages were cultured in the presence of 1 ng/ml bFGF, and then examined for the expression of N1 (upper panels) or Me1 (lower panels) antigen after incubation for 5 days or 2 days, respectively. Scale bar, 100 µm. (B) Quantitative assessment. The proportion of positive cultures for N1 antigen (○) and the mean number of Me1-positive melanophores per culture (●) are plotted against the ages of ectoderm cells examined. Data were collected from a single series of experiments.

Fig. 4. Dose dependency of the effect of bFGF on melanophore differentiation from gastrula ectoderm cells. (A) Melanophore and epidermal differentiation of ectoderm cells from 11h gastrula (stage 10). Dissociated animal cap cells were cultured in the presence of various concentrations of bFGF indicated (ng/ml) and then examined for the expression of Me1 or E3 antigen after incubation for 2 days. Scale bar, 100 µm. (B) Dose-response profiles. The mean number of Me1-positive melanophores per culture (●) and the proportion of E3-positive cultures (○) are plotted against the concentrations of bFGF added to the cultures. Data were collected from a single series of experiments.
differentiation (Mitani and Okamoto, 1989), was detected in cultures of 6-hour (stage 8+) animal cap cells in the presence of more than 5 ng/ml bFGF and the concentration of 10 ng/ml was required to obtain myocyte differentiation in every culture wells examined. We also found that myocytes were never induced from 8- to 11-hour (stage 9+ to 10+) gastrula ectoderm cells by bFGF, even at concentrations as high as 10-100 ng/ml. This finding is again consistent with previous reports that competence of animal cap cells to form mesoderm in response to bFGF was lost by about 8-hour age of early gastrula (Slack et al., 1988; Green et al., 1990). Mul and N1 antigens were not detectable in ectoderm cells from 7-hour (stage 9) blastula exposed to bFGF. Since these cells also failed to express the E3 antigen, they probably differentiated into an unidentified type of cell of mesodermal or neural origin.

Taken together with the results described above, these data suggest that animal cap cells dramatically change their state of competence with respect to bFGF during embryonic stage from mid-blastula to late gastrula. They are first competent to produce mesoderm, then neural tube and finally neural crest lineages. Ectoderm cells that have lost their neural crest competence at some point in late gastrula pursue autonomously the epidermal lineage thereafter.

Ectodermal competence autonomously changes during gastrulation

The response of ectoderm cells to bFGF changes during gastrulation from the production of neurons to the production of melanophores. This change in competence could result from events intrinsic to ectoderm cells themselves. It is also possible that the ectodermal competence is affected by signals from the marginal zone area, which spreads through the ectoderm plane during gastrulation period. To assess these alternatives, we isolated ectoderm cells at 9-hour age (stage 10) when they have only neuronal competence and cultured them on their own to allow autonomous development for increasing time. bFGF was then added to see whether the state of competence changed (Fig. 5A). The results are shown in Fig. 5B,C.

When bFGF was added within 1.5 hours from the start of culture, N1-positive neurons were induced in every well examined (Fig. 5C, open circles). When the ectoderm cells were cultured for longer periods before the addition of bFGF, N1-positive neurons decreased both with respect to the proportion of N1-positive wells (Fig. 5C) and the size of the signal in each well (Fig. 5B, upper panels). These data show that the loss of neuronal competence occurs autonomously in ectoderm cells in vitro.

Fig. 5. Autonomous changes in competence of cultured ectoderm cells to respond to bFGF. (A) Schedules of treatment of cultured cells. Animal cap ectoderm from 9-hour gastrula (stage 10) was dissociated, cultured in vitro for various periods of time indicated and then treated with 1 ng/ml bFGF. (B) Typical staining patterns against mAbs N1 (upper panels) and Me1 (lower panels). Scale bar, 100 µm. (C) Quantitative assessment. Neuronal or melanophore differentiation from cultured ectoderm was scored as described in Fig. 3 and plotted against preincubation periods before addition of bFGF. Data were collected from a single series of experiments.
By contrast, melanophore competence rose autonomously during the preculture period without bFGF, peaking in ectoderm cells cultured for 2 hours before the addition of bFGF (Fig. 5C, closed circles). Ectoderm cells cultured for longer than 2 hours prior to the addition of bFGF rapidly lost melanophore competence. The gain and loss of melanophore competence in cultured ectoderm cells changed roughly in parallel with those in vivo (cf. Fig. 3).

These results indicate that the state of ectodermal competence changes mainly by an intrinsic timing mechanism in the ectoderm rather than by the action of other parts of gastrula such as the marginal zone. Similar autonomous changes of competence of the gastrula ectoderm were recently reported for the transition from neural competence to lens-forming competence (Servetnick and Grainger, 1991).

Ventral precedes dorsal in changes in ectodermal competence

In the experiments described so far, we used cells isolated from ectoderm in the vicinity of the animal pole including both the dorsal and ventral sides. Recently, it has been shown using molecular markers that these two regions of animal hemisphere can be distinguished. The dorsal ectoderm is predisposed to neural differentiation (Sharpe et al., 1987), whereas the ventral ectoderm is predisposed to epidermal differentiation (London et al., 1988). Our culture system of ectoderm cells provides another opportunity to see whether the dorsal bias exists in neural competence by applying bFGF. For this purpose, cells from dorsal and ventral portions of ectoderm were independently cultured and their response to bFGF was examined. The results are shown in Fig. 6.

When we prepared ectoderm cells from 10.5-hour gastrulae (stage 10\(^+\)), which retain both neuronal and melanophore competence (Fig. 3), dorsal- and ventral-side cells were induced to differentiate into CNS neurons to a similar extent by 0.6 ng/ml bFGF. By contrast, melanophore differentiation at the same dose of bFGF was only observed in cultures of ventral ectoderm cells. It is possible that dorsal ectoderm cells differ from ventral ones in that the former have no melanophore competence. However, our studies described in preceding sections indicate another possibility that the phase of competence change in the dorsal ectoderm was shifted earlier than that in the ventral ectoderm. We surmised that, at the stage examined (10.5-hour), the dorsal ectoderm had not yet gained the melanophore competence, while the ventral ectoderm had already become competent.

If this were the case, we could expect that the dorsal ectoderm cells of a somewhat later stage would be capable of differentiating into melanophores in response to bFGF. The experiment presented in Fig. 6 supports this idea. When ectoderm cells were prepared from 11.5-hour gastrula (stage 11\(^+\)), the dorsal-side cells produced melanophores at the same level as the ventral-side ones. Both sides of ectoderm cells appeared to have lost their neuronal competence at this stage.

These results indicate that the difference between dorsal and ventral portions of gastrula ectoderm reflect the shift in developmental phase between the two regions; the ventral ectoderm precedes the dorsal one in the changes of competence during gastrulation.

Sensitivity of ectoderm cells to bFGF changes during gastrulation

The density of FGF receptors in animal cap cell surface has been reported to peak at mid-blastula stage (6-hour age; stage 8+) and decrease during the following embryonic period (Gillespie et al., 1989). If the decrease continued through gastrula stage, we could expect a successive change in the sensitivity of ectoderm cells to bFGF during this period, in addition to the change in the state of competence; higher doses of bFGF would be required for older age ectoderm cells to be induced.

To test this idea, we examined dose-response profiles for the inducing actions of bFGF in cultures of ectoderm cells of increasing age (Fig. 7). At 9-hour (stage 10), when ectoderm cells have only neuronal competence, CNS neurons were maximally induced by 0.1 ng/ml of bFGF (Fig. 7A). However, at 10.5-hour (stage 10\(^+\)) when neuronal and melanophore competence overlap, 0.5 ng/ml of bFGF was necessary for maximal induction of both CNS neurons and melanophores (Fig. 7B). It is noteworthy that, as the concentration of bFGF was further increased, the induction of melanophores was suppressed. By contrast, the induction of CNS neurons remained at its maximum level or was slightly enhanced as judged by the extent of N1 antigen expression (not shown). The inhibition of melanophore induction at higher doses of bFGF could barely be detected in cultures of ectoderm cells of somewhat later stage (Fig. 4). At 12-hour (stage 11\(^+\)), 15 ng/ml of bFGF was necessary to induce the maximum number of melanophores (Fig. 7C). The melanophore differentiation at this stage, however, was extensive compared to that...
observed at earlier stages; the maximum number of melanophores per culture well was more than 100 (nearly 90% of initially added cells), whereas it was on average less than 20 when cells from 10.5- to 11.5-hour gastrula (stage 10 to 11) were used. N1-positive CNS neurons were not induced by bFGF at concentrations up to 120 ng/ml at this later stage.

All the above findings support our idea that the sensitivity of ectoderm cells to bFGF in the neuronal and melanophore induction processes decreases during gastrula stages, possibly accompanied with the decrease of FGF receptor density in the ectoderm cell surface.

Neural-inducing effect of bFGF on gastrula ectoderm explant

It seems puzzling that FGF had no effect on explants of gastrula ectoderm when mesodermal-specific or neural-specific markers were examined (Slack et al., 1988; Green et al., 1990). A plausible explanation is that there is an upper limit to the number of cells that allow neural differentiation by bFGF in vitro, since an explant of gastrula ectoderm contains a much larger number of cells (500 to 1000) than used in the microculture study. We found, however, that bFGF was capable of inducing neural differentiation in cultures consisting up to 600 ectoderm cells (data not shown).

When we examined the effect of bFGF on gastrula ectoderm explants, we noticed that these explants became surrounded by the pigmented outer layer cells within 15 to 20 minutes after the start of culture. These outer layer cells are known to be impermeable to FGF (Darlington, 1989) as well as mesoderm-inducing factors (Cook et al., 1987), and were therefore intentionally excluded from our microculture system. We also found that gastrula ectoderm cells exposed to bFGF for 20 minutes in culture underwent a far weaker neural differentiation than those exposed for 2 hours. We then manually removed the pigmented outer cell layer from gastrula ectoderm explant (10- to 11-hour ages; stage 10 to 11) and cultured the remaining inner cell layer as an aggregate in the presence of bFGF. Although an extensive analysis as described in the previous sections for the microculture system has not yet been done, we found that at least 2.5 ng/ml bFGF was sufficient to induce both N1-positive and Me1-positive cells in these explants. An example is shown in Fig. 8, in which melanophores were shown to differentiate in an inner cell layer explant of 11-hour ectoderm cultured for 2 days (Fig. 8a3). There seems to be no muscle cell differentiation in neighbouring sections, as judged by staining with a mAb Mu1 (Fig. 8b3) that specifically binds to muscle cells in Xenopus embryos (Mitani and Okamoto, 1989). In the absence of bFGF, we obtain only epidermal cell differentiation as expected (Fig. 8c3). Since a mass effect of cell number in the explants is unlikely, it may be that the pigmented outer cell layer acts as a permeation barrier for bFGF thereby preventing the induction of neural differentiation of the inner cell layer in commonly used gastrula ectoderm explants. However, we cannot exclude the possibility of a direct inhibitory effect by the outer cell layer on neuralization of inner cell layer at present.

DISCUSSION

Since the discovery of the neural induction of ectoderm (Spemann and Mangold, 1924), identification of the inducing substance(s) in the dorsal mesoderm has been one of the leading themes in developmental biology. We still do not know what the nature of this substance is, however. The present study was undertaken to approach this question by extending our previous studies in which the inductive differentiation of CNS neurons of neural tube origin and melanophores of neural crest origin have been reconstituted in a microculture system from cells of the ectoderm and MZ (prospective mesoderm region) of Xenopus early gastrula (Mitani and Okamoto, 1989, 1991). Here we have demonstrated that bFGF, at picomolar concentration levels, mimics the action of MZ cells on gastrula ectoderm cells in culture to induce differentiation of the two neural lineages. We have also shown that the ability of the ectoderm to respond to bFGF actively changes during gastrulation from a neuron-producing state to a melanophore-producing state. Because several members of the FGF family (Kimelman et al., 1988; Slack and Isaacs, 1989; Shiurba et al., 1991; Isaacs et al.,
Neural induction by bFGF

1075

1992; Tannahill et al., 1992) and their receptors (Gillespie et al., 1989; Musci et al., 1990; Friesel and Dawid, 1991; Friesel and Brown, 1992) are known to be expressed in *Xenopus* early embryos, our findings raise the possibility that an FGF-like molecule is secreted from the dorsal mesoderm in an appropriate spatiotemporal pattern during gastrulation and induces the overlying ectoderm to give rise to the neural lineages in normal development.

Does bFGF directly induce the neural lineages?

It is widely accepted that bFGF shows a mesoderm-inducing activity when applied to animal cap fragments or cells from *Xenopus* blastula (Slack et al., 1987; Kimelman and Kirschner, 1987; Godsave and Slack, 1991). bFGF could directly induce a fraction of gastrula ectoderm cells in the microculture to form mesodermal cells, which in turn induce the remaining ectoderm cells to differentiate into neural lineages. However, several lines of evidence of ours and others argue against this idea.

We showed that the effective dose of bFGF for the neural induction of gastrula ectoderm was extremely low compared to that for the mesoderm induction of blastula ectoderm. Both CNS neurons and melanophores could be induced by 5 pM bFGF, a concentration about 50-fold lower than the dose required for the induction of myocytes, the main dorsal type derivative of mesoderm induced by bFGF (Godsave and Slack, 1991). We never observed ectoderm cells of early- to mid-gastrula in culture differentiating into myocytes even with as high as 5 nM bFGF. This is in good agreement with a previous report that the mesodermal competence of animal cap cells in response to bFGF was lost by the beginning of gastrulation (Slack et al., 1988). The effective dose for neural induction (5 pM) is within the range of $K_d$ values reported for the high affinity FGF receptors in mammalian cell lines (Klagsbrun and Baird, 1991), but considerably smaller than $K_d$ of 140 pM reported for a FGF receptor in animal cap cells of *Xenopus* blastula (Gillespie et al., 1989). A different type of FGF receptor with a higher affinity might be expressed in gastrula ectoderm cells to mediate the neural inducing action of bFGF.

Our previous study has shown that CNS neurons are predominantly induced by DMZ cells that give rise to the axial part of the dorsal mesoderm, whereas melanophores are induced by cells from the more ventral MZs that contribute the marginal part of the dorsal mesoderm (Mitani and Okamoto, 1991). If the neural induction of ectoderm cells in culture by bFGF occurred secondarily as a consequence of mesoderm induction, our results might be thought to suggest that gastrula ectoderm cells were first competent to produce DMZ-type cells and then turn competent to produce more ventral-type MZ cells. However, this possibility seems unlikely, because it was recently demonstrated that bFGF fails to induce the expression of *goosecoid* mRNA, which accumulates predominantly in DMZ during *Xenopus* normal development (Cho et al., 1991).

It has been shown that blastula animal cap cells (future ectoderm cells) can be neuralized by disaggregation alone (Godsave and Slack, 1991). However, we could not detect any spontaneous neuralization when fewer than 20 gastrula ectoderm cells were cultured under aggregated or dispersed conditions (data not shown). Reproducible neural differentiation induced by bFGF requires about 100 ectoderm cells that have been reaggregated by brief centrifugation. The same is true when MZ cells are used as the inducer. In a...
previous report, we showed that reproducible epidermal differentiation of gastrula ectoderm cells was also dependent on the reaggregation of dissociated cells and on the size of the resultant aggregate, which was determined by the number of cells initially added to culture wells (Mitani and Okamoto, 1989). It is likely that these characteristics are general features of the cellular differentiation of gastrula ectoderm cells in culture. A similar effect of aggregation of embryonic cells has been reported for the mesoderm induction and termed the ‘community effect’ (Gurdon, 1988), in which the ability of an animal cap cell to respond to induction by differentiating into muscle cell is enhanced by, or even dependent on, other neighbouring animal cap cells differentiating in the same way at the same time.

**Autonomous changes in the competence of ectoderm to respond to bFGF**

The second major result of our study is the demonstration of the transition in the competence of ectoderm cells to respond to bFGF during gastrulation and the autonomous nature of this transition. Ectoderm cells acquire neuronal competence during early (8- to 9-hour) gastrula stages (stage 9+ to 10) and subsequently lose it by the end of mid gastrula stage (12.5-hour age; stage 11). As the neuronal competence decreases, ectoderm cells become competent to differentiate into melanophores, although this period of competence is transient (10- to 12.5-hour). Since the changes of competence proceed in isolated ectoderm cells in culture with approximately the same time course as seen in vivo, an intrinsic timing mechanism must be present within ectoderm cells.

Does the change of ectodermal competence have any significance in normal development? According to a fate-mapping study of MZ (prospective mesoderm region), there may be a time lag between the induction of the neural tube and neural crest lineages by the axial and marginal parts of the dorsal mesoderm, respectively (Keller, 1976; Gerhart and Keller, 1986). This raises the possibility that ectoderm cells exposed to the same neural inducing signal give rise to different lineages depending on their age. This idea is consistent with a previous observation by Mitani (1989), which showed that, when the timing of gastrulation was retarded during the initial 2-3 hours by injecting heparin or dextran sulphate into the blastocoel cavity of *Xenopus* early embryos, the anterior part of CNS was severely damaged, whereas melanophores of neural crest origin were overproduced. Since our present experiment has shown that the response of ectoderm cells to bFGF changes with a similar time course of 2-3 hours from the production of CNS neurons to the production of melanophores, we must consider the possible significance of bFGF as a neural inducer in normal development, as further discussed in the next section.

We observed that both ventral and dorsal parts of ectoderm had neuronal and melanophore competence. This finding agrees with classical studies demonstrating that all regions of ectoderm can be induced to form neural tissues when conjugated to strongly inductive tissue such as the dorsal blastopore lip (e.g. Spemann and Mangold, 1924). In contrast, it has recently been reported that dorsal-side animal cap fragment of 10-hour embryo (stage 10½) is more easily induced to produce neural cells by a weaker neural inducer such as the anterior dorsal mesoderm (Sharpe et al., 1987). This apparent discrepancy can be explained by our data showing that the ventral ectoderm preceded the dorsal one in the change of the state of competence. Possibly, the ventral ectoderm of 10-hour gastrula has begun to lose neuronal competence and needs a higher dose of the inducing substance than the dorsal ectoderm.

**Is a FGF-like molecule the endogenous neural inducer secreted from the dorsal mesoderm?**

The present data suggest that bFGF could be an endogenous neural inducing factor and that regionalization of neural tube and neural crest occurs by temporal changes in ectodermal competence to respond to bFGF. However, this idea is apparently inconsistent with our previous conclusion that the origin of the inducing MZ cells is critical to decide which neural lineages differentiate. One way to avoid this difficulty is to assume that DMZ cells secrete larger amounts of bFGF and/or secrete it more rapidly than ventral-side MZ cells. If it were the case, the state of competence of ectoderm cells when co-cultured with VMZ cells would become more biased towards the melanophore-producing state before sufficient amounts of bFGF from VMZ cells accumulated to trigger the induction process. According to this idea, VMZ cells could induce CNS neurons when co-cultured with ectoderm cells as young as 8-hour old (stage 9+) and, conversely, DMZ cells could induce melanophores when co-cultured with ectoderm cells of as old as 12-hour (stage 11+). Preliminary results are promising. However, we cannot totally exclude at the moment the presence of factors that may help DMZ and VMZ cells preferentially to induce CNS neurons and melanophores, respectively.

It has been proposed that an inducing molecule is released by the DMZ and diffuses through the ectodermal plane where it stimulates neuralization (Doniach et al., 1992; Doniach, 1992). If this is the case, bFGF could act as a permissive inducer of ectoderm neuralization, supplementing the action of the postulated inducing molecule. We cannot exclude this alternative possibility.

Immunocytochemical studies show that bFGF is prepositioned in mesoderm-forming regions intracellularly throughout cleavage and gastrula stages and, during the gastrula stage, it is also detected extracellularly between embryonic tissues (Shiruba et al., 1991; Godsave and Shiruba, 1992). Recently, a novel FGF (XeFGF) was found in *Xenopus* embryo (Isaacs et al., 1992), which is another promising candidate for an endogenous inducing factor. There is an excess of XeFGF mRNA in dorsal quarters over ventral three-quarters of early gastrulae (Isaacs et al., 1992). Interestingly, in situ hybridization further demonstrates that the expression of XeFGF is first detected in DMZ region, then extends to more ventral MZ region during gastrulation. It is also shown that there are at least two different receptors for FGF in *Xenopus*, (Gillespie et al., 1989; Musci et al., 1990; Friesel and Dawid, 1991; Friesel and Brown, 1992). Involvement of FGF-like molecules in the neural induction process both in vitro and in vivo could be ascertained by the use of specific inhibitors of FGF action such as neutralizing antibodies against bFGF or its receptors.

Amaya et al. (1991) have shown that overexpression of a
dominant-negative form of FGF receptor inhibits the normal function of a fraction of FGF receptor in *Xenopus* early embryos and gives rise to embryos deficient in trunk axial structures such as notochord and spinal cord. This result is not necessarily inconsistent with the idea that an FGF-like molecule is an endogenous neural inducer, because it can play dual functions of mesoderm and neural inductions in early development. However, they also show that, in these trunk-deficient animals, the anterior head structure remains relatively undisturbed. This may mean that the anterior CNS is induced by factor(s) other than a FGF-like molecule, whereas the latter may be solely responsible for the induction of the trunk CNS (spinal cord). Alternatively, the types of FGF receptor might differ in the future head and trunk CNS regions of ectoderm and the function of only the trunk type receptor might be blocked by the dominant-negative form of FGF receptor used. It is also probable that the normal function of a fraction of FGF receptor was restored before the prospective head CNS region of ectoderm comes to be underlayed by the inducing anterior part of the dorsal mesoderm. This is possible because movement of the inducing MZ cells during gastrulation is oriented from the future posterior end to anterior end of the body and this allows a time lag between the induction processes for the head CNS and trunk CNS.

**Concluding remarks**

In this report, we have provided evidence that bFGF has a potential role in the neural induction process in *Xenopus* normal development. We cannot say at the moment whether bFGF is the sole neural inducing factor from the dorsal mesoderm. Nevertheless, the changes in ectodermal competence to respond to bFGF during gastrulation is possibly important for the regional specification of ectoderm, which gives rise to the neural tube and neural crest. Although many aspects of the function of bFGF in *Xenopus* early development are still unclear, our findings offer an important clue to understand the cellular and molecular mechanisms of the neural induction process.

Authors would like to thank Professor Kunitaro Takahashi for his invaluable advice and discussions on this study, and Dr David W. Saffen for his critical reading of the manuscript. This work was supported by grants in aid scientific research from the Ministry of Education, Science and Culture, Japan

**REFERENCES**


Nieuwkoop, P. D. and Faber, J. (1967). *Normal Table of *Xenopus* laevis (Daudin).* Amsterdam: North-Holland Publishing Company.


(Accepted 7 September 1993)