Original Article

Targeted over-expression of FGF in chick embryos induces formation of ectopic neural cells

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ABSTRACT Fibroblast growth factors (FGFs) are known to be involved mainly in mesoderm formation in *Xenopus* embryos but their participation in other inductive mechanisms such as neural induction has not been clearly established and is now under study. Here, we provide evidence that targeted over-expression of members of this family of growth factors in the periphery of full-length primitive streak chick embryos produces the formation of ectopic neural cells that are able to differentiate into neurons. The supernumerary neural plate obtained derives from the epiblast layer of the blastoderm and show signs of neural differentiation 24 h after the application of FGF. We have used cell labeling and have examined the expression of mesodermal markers to ascertain how this expansion of the neural forming region of the epiblast takes place. We conclude that the new neural cells formed are originated in the region of the epiblast fated to be epithelia and that the induction of the ectopic neural tissue is not mediated by an increase, migration or new formation of axial mesoderm. This strongly suggests that FGF is acting directly on epiblast cells, changing their fate from epidermal ectoderm to neural ectoderm. Therefore, our results show that FGF can induce neural ectoderm when acting on still uncommitted cells and, therefore, it is a putative candidate for acting in normal neural induction during development.

KEY WORDS: *chick embryo, epiblast, neural plate, ectoderm, FGF, competence, induction*

Introduction

The cellular and molecular processes implicated in early regional patterning of the nervous system are now the subject of intensive studies (Lumsden and Krumlauf, 1996; Tanabe and Jessell, 1996). However, the mechanisms participating in the establishment of the early neural plate are still largely unknown. Transplantation of the dorsal blastopore lip in amphibians (Spemann and Mangold, 1924) and Hensen's node (HN) in amniotes (Waddington, 1932; see Dias and Schoenwolf, 1990, Storey *et al.*, 1992, and Beddington, 1994, for recent work) to ectopic positions in host embryos induces the formation of a secondary neuraxis, suggesting that signals emanating from these centers (organizers) mediate the specification of neural fate (see Harland, 1994 and Ruíz i Altaba, 1994, for reviews). Recent studies have shown that other signals that mainly belong to the BMP family determine ectodermal fate in *Xenopus* embryos (Liem *et al.*, 1995; Schmidt *et al.*, 1995; Wilson and Hemmati-Brivanlou, 1995). In the chick, the cells that produce these ectoderm-forming signals are probably localized in the periphery of the embryo (border between area opaca and area pellucida) because this region can induce dorsalization of neuroepithelial cells (Dickinson *et al.*, 1995). Collectively, this would support a dual system of signals (HN and periphery) for establishing the neural plate-epidermal border (Sasai *et al.*, 1995). Fate mapping studies in chick embryos (Schoenwolf and Alvarez, 1989, 1991; García-Martínez *et al.*, 1993) have identified the region of the epiblast that will form the neural plate (prospective neural fate) and surface epithelium (prospective epidermal fate) at the full-length primitive streak stage. This information, in addition to the ability to manipulate young chick embryos in whole-embryo culture, makes this system suitable for exploring the mechanisms of how the neural plate is induced by organizer centers.

Although the type and number of signaling molecules that can participate in instructive processes during development are still being revealed, growth factors are clearly involved in induction processes. Since their characterization by their ability to promote cell division in culture, the fibroblast growth factor family (FGFs)

Abbreviations used in this paper: FGF, fibroplast growth factor; HN, Hensen's node; BMP, bone morphogenetic protein; PBS, phosphate buffered saline; T, Brachyury.

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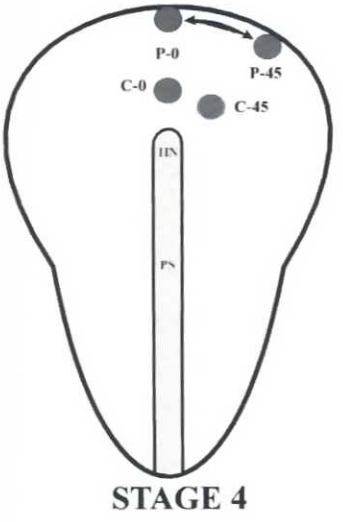

Fig. 1. Schematic drawing of a chick embryo at the full-length primitive streak stage (Stage 4 of Hamburger and Hamilton, 1951), showing the positions selected for the targeted application of FGF soaked beads. *Four positions are demarcated: Periphery with 0 degrees (P0), that is, the region at the border between the area opaca and the area pellucida that lies at the end of an imaginary line extending rostrally from the primitive streak, periphery with 45 degrees (P45), that is, the region of the epiblast that borders the area opaca and forms an angle of 45 degrees with the rostrocaudal line demarcated by the primitive streak, C0 (C for center of the anterior epiblast) situated in the midline between P0 and the Hensen's node and C45 that is an intermediate position of the epiblast situated at 45 degrees with respect to the imaginary line demarcated by the primitive streak. Some other positions have also been tested in the area ranging between P0 and P45 (double headed arrow).*

has been shown to play important roles in development. The phenotypes of homozygous null mutations, the construction of dominant–negative receptors and over–expression experiments have all proved that growth factors are necessary for a variety of developmental processes throughout almost all stages of development (Baird, 1994; Doniach, 1995; Wilkie *et al.*, 1995; Yamaguchi and Rossant, 1995; Mason, 1996; Smallwood *et al.*, 1996). During gastrulation, FGFs have been implicated mainly in mesoderm induction, and their possible roles in other processes (including neural induction) have been somewhat ignored. Nevertheless, there is evidence that strongly suggests that FGFs are involved in the establishment of neural fate in blastoderm cells. For example, when *Xenopus* animal caps are dissociated and treated with FGF they can form neurons and neural crest cells instead of adopting the mesodermal fate that they acquire when undissociated animal caps are treated with FGF (Kengaku and Okamoto, 1993, 1995). Moreover, the expression of a truncated FGF receptor in Xenopus blocks neural induction by Noggin (Lunay *et al.*, 1996). Also in support of this role of FGFs, the expression pattern of some members of this family (FGF– 3, FGF–4 and FGF–8) provides evidence that these growth factors can be released by HN and axial mesoderm (the most plausible candidates for inducing the neural plate) during the course of neural induction (Kalcheim and Neufeld, 1990; Niswander and Martin, 1992; Crossley and Martin, 1995; Mahmood *et al.*, 1995; Bueno *et al.*, 1996).

Targeted expression of FGFs using heparin acrylic beads can yield information about the biological function of the FGFs during development. This system has been used recently in gastrulating chick embryos (Levin *et al.*, 1995), to explore the influence of FGF–8 in neural tube development (Crossley *et al.*, 1996) and extensively in the formation of the limb bud (Cohn *et al.*, 1995). We have used this approach (i.e., targeted over–expression of FGFs with soaked beads) to evaluate which processes of early neural patterning can be regulated by the accessibility of FGF–2 (bFGF) and FGF–4 to epiblast cells. These members are representative of the FGF family, and have been used previously in induction experiments in *Xenopus* (Thompson and Slak, 1992) and chick embryos (Mitrani and Shimoni, 1990; Cooke and Wong, 1991; Cooke, 1993). Surprisingly, over–expression of FGF in the periphery of the embryo at the full–length primitive streak stage, but not in other regions or stages, elicits partial duplications of the neural plate. Moreover, we show that FGF induction of new neural cells is not mediated by modification of the endogenous axial mesoderm or formation of new mesodermal cells, suggesting a direct mechanism of actuation on still pluripotent epiblast cells.

Results

Chick embryos can respond to ectopically released FGFs

PBS soaked beads placed in the different positions described (Fig. 1) do not produce major alterations in the development of the embryo (Figs. 2A–B), excluding any mechanical interaction in the response of epiblast cells that will be described below. Instead, beads soaked with FGF–2 or FGF–4 at the concentrations tested and targeted to the periphery (P) of gastrulating chick embryos developing in New culture cause the formation of ectopic structures derived from the epiblast cell layer. This effect is independent of the host's left–right symmetry because the same results were obtained with bead implantation on the left and right sides. Overall, the morphology of the alterations produced depends on the stage and position at which the bead was implanted (see below), ranging from small embryo–like structures without connection with the main axis (Figs. 2C–D) to lateral extensions of the main neuraxis (Fig. 2E). Similar structures have been described previously as secondary supernumerary axial structures (Fig. 7 in Roberts *et al.*, 1991). Histological sections of these embryos show that the new tissues formed resemble neuroepithelium (Fig. 2F).

Interestingly, this general effect on the epiblast does not depend upon the type of FGF utilized (FGF–2 or FGF–4) although when the accessibility of FGF is expanded by the placement of two beads or by using the highest concentrations, the response seems to be more robust. Nevertheless, when the number of beads is more than three, an inhibition of the growth of the embryo occurs, probably because of a toxic effect of the growth factor or a mechanical effect of the beads. When FGF soaked beads are applied to the central region of the anterior epiblast (position C45) after 24 h of development, the embryos exhibit in whole-mount an essentially correct neuraxis but inhibition of somitogenesis and other mesodermal alterations occurs in about half of the cases (Fig. 2G).

Chick embryos respond to FGF forming neural tissue in a stage and position dependent basis

The competence of epiblast cells to form supernumerary structures under the influence of FGFs is restricted both spatially and temporally. Before stage 3 (15 cases), the ectopic application of FGF

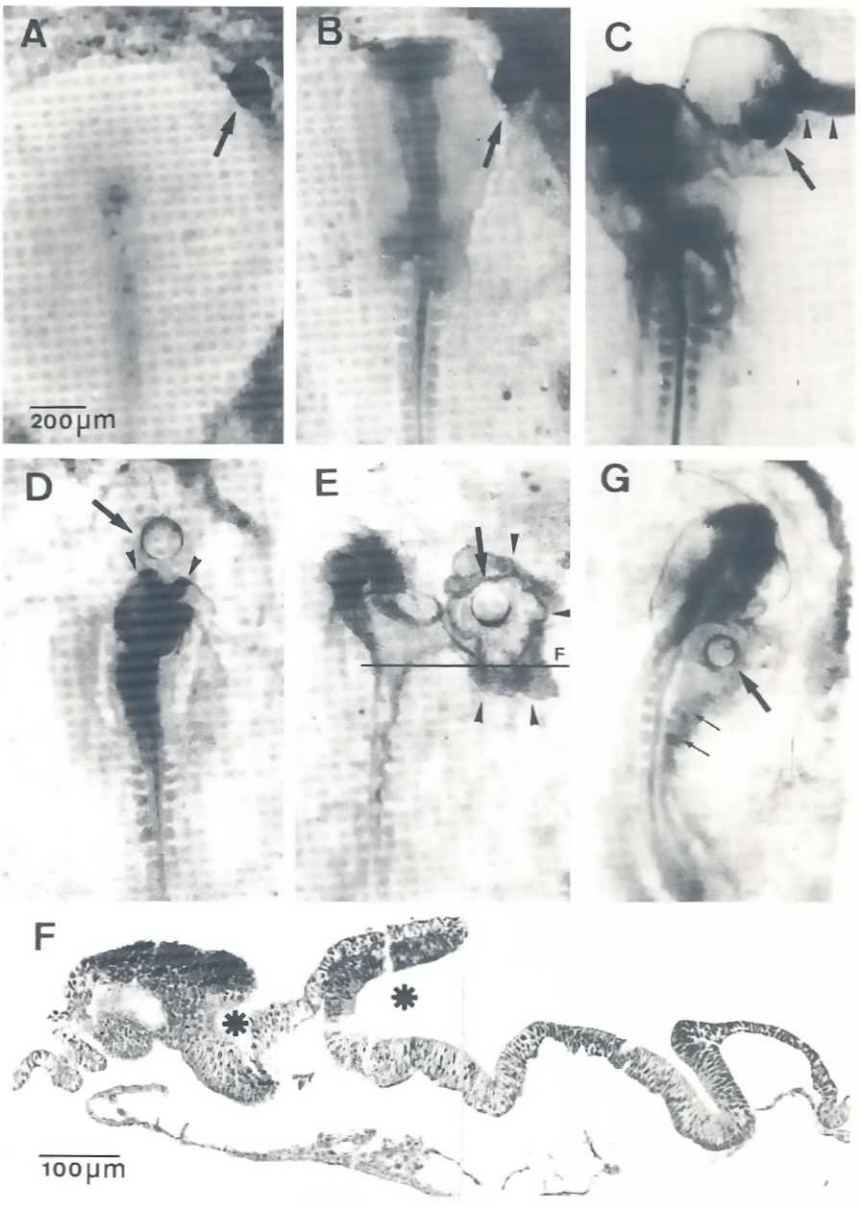

Fig. 2. Ectopic tissues obtained after the implantation of FGF soaked beads.(A) *Image that shows an embryo 2 h after the implantation of the bead at P45. The hole made in the endoderm to introduce the bead between the epiblast and the endoderm is already healed. Note the position of the bead with an angle of 45° with respect to Hensen's node.* **(B)** *Control embryo developed for 24 h with a bead soaked with PBS. The morphology of the embryo is roughly normal despite of the large size of the bead implanted. Figures* **(C, D and E)** *are ventral views of chick embryos in New culture treated at stage 4 with FGF–4 soaked beads at 1 mg/ml that show examples of the morphology obtained when FGF–soaked beads are placed in different positions with respect to Hensen's node at the full–length primitive streak stage according the nomenclature described in (Fig. 1C). Embryo treated with a FGF–2 soaked bead at 1 mg/ml in position P45 and allowed to develop for 36 h. The bead has produced an ectopic structure (arrowheads) that resembles a small ectopic neural tube.* **(D)** *Beads implanted rostral to Hensen's node at P0 induce ectopic tissues (arrowheads) that seem to be independent of the main axis of the embryo.* **(E)** *Beads implanted at P45 cause expanded neural plates (arrowheads), often connected with the main neuraxis.* **(F)** *Mirror image of an histological section of the embryo shown in 2E obtained at the level indicated by the line. The epiblast at this level has acquired along all its width the morphology of a neuroepithelium and the ectopic tissue (asterisks) is almost indistinguishable from the original neural plate.* **(G)** *When the bead is implanted in position C45 the neuraxis shows almost normal morphology, and only mesodermal derivatives such as the somites (thin arrows) and the heart are affected. All embryos in this figure were stained with 0.5% eosin for 10 min after fixation. Arrows always indicate the bead. Magnification is the same for Figs A to E and G.*

at the highest concentration tested, arrested development, although the embryos did not die (data not shown). FGF soaked beads placed at P45 or C45 from stage 5 onwards (15 cases) produce alterations in the mesoderm and the adjacent endoderm with formation of extra cardiac loops, double hearts and loose of the appropriate cardiac left–right symmetry (in preparation). Therefore there is a narrow time window for the epiblast cells to form ectopic structures that coincides with the full–length primitive streak stage (late stage 3, stage 4 and early stage 5 of Hamburger and Hamilton, 1951). When FGF is applied to the periphery of the blastoderm rostral to the Hensen's node (position P0), ectopic tissue is obtained that is independent of the original embryonic axis (Fig. 2D), whereas the application of FGF in more lateral regions (P45) often results in extra tissue that is usually connected to the main axis, a condition that we have termed bifurcation (Fig. 2E). To further elucidate the competence of the epiblast cells at the full–length primitive streak stage to form neural tissues by FGFs, we applied FGF soaked beads at the periphery of the embryo at positions located between P0 and P45 and in a position that corresponds to the midpoint between Hensen's node and P0 (C0). All of these positions are marked in Figure 1. 24 h after the application of the beads we observed the morphology of the tissue obtained. Although there is some variability, probably due to slight differences in the stage of development, most of the embryos in which the bead was implanted in any position between P0 and P45 (10 cases) and in position C0 (6 cases) produced ectopic tissue that is independent of the original neuraxis. Therefore we can conclude that at stage 4 the epiblast tissue surrounding the anterior border between area opaca and area pellucida and the region rostral to the node is competent to form tissue that resembles neuroepithelium in structure.

We have verified the neuroepithelial character of these extra tissues by immunodetection of an antigen specific for early differ-

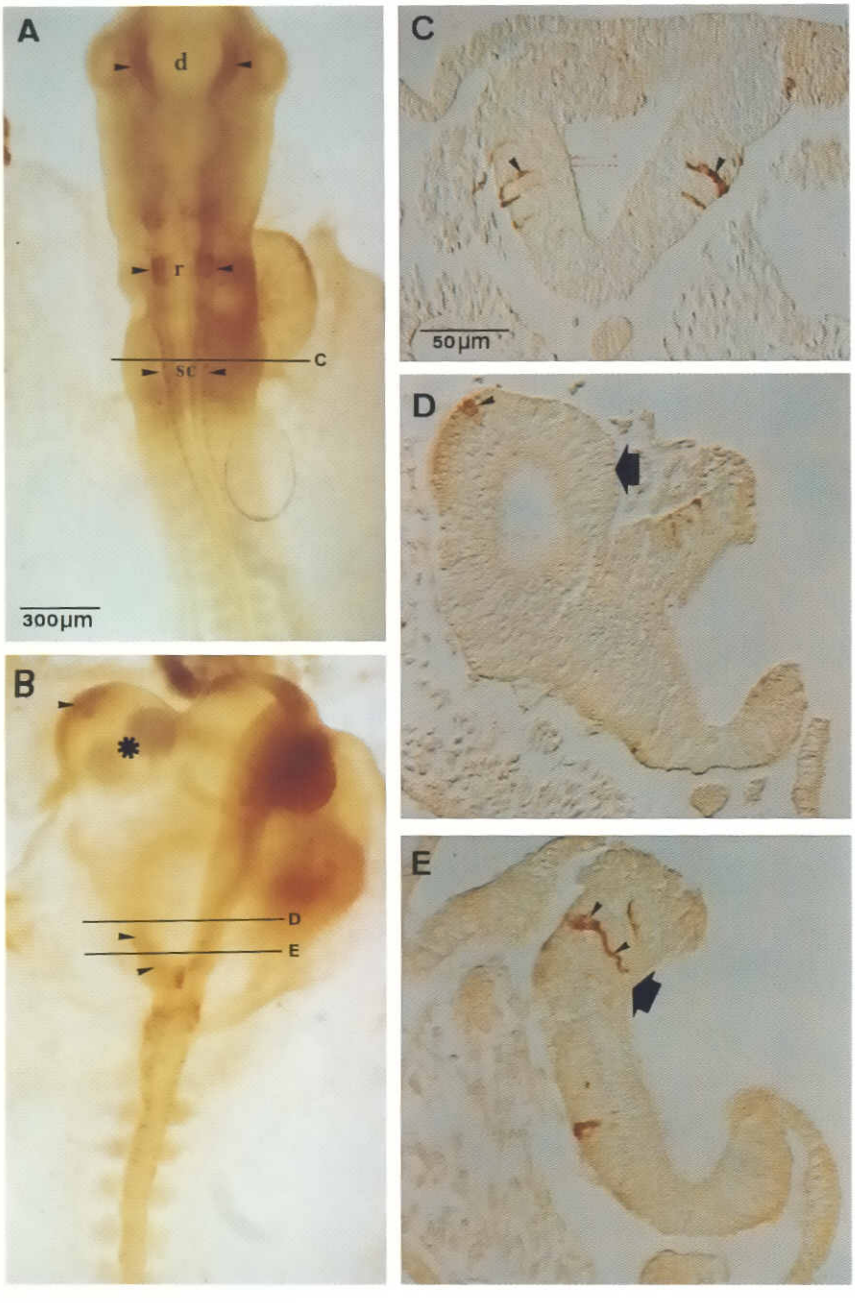

Fig. 3. Neurofilament-associated protein (NAP) positive cells induced by FGF. (A) *Whole-mount immunocytochemistry showing the expression of NAP in an stage 12 control embryo. The normal pattern of differentiating neurons (arrowheads) can be seen in the diencephalon (d), the rhombencephalon (r) and the spinal cord (sc). In embryos treated with FGF* **(B)** *NAP positive cells can be seen in the ectopic neural-like structure developed (arrowheads).* **(C–E)** *High magnifications of sections obtained at the level indicated by the lines in A and B. The neurons (arrowheads) can be distinguished clearly both in the control embryos* **(C)** *and in the ectopic neuroepithelium obtained with the application of FGF (thick arrows). Asterisk indicates bead. Magnification is the same in A–B; and in C–E.*

entiating neurons (Dodd *et al.*, 1988) as a neurofilament associated protein (NAP) expressed in a specific pattern in the developing chick nervous system (Fig. 3A). After FGF treatment some scattered positive cells can be detected in the ectopic tissue formed (Fig. 3B) and histology of these embryos confirmed the presence of cells completely marked in the extra neural tissue developed (Figs. 3C–E). Therefore, FGF–2 and FGF–4 are able to induce the formation of new neural cells that can differentiate into neurons.

Fate of labeled epiblast cells under the influence of FGF

One can argue, in the case of the bifurcations, that the extra neural tissue obtained is not forming in the epiblast region committed to form epithelia (induction) but it becomes from the original neural plate that has been extended under the influence of the FGF on already existing neural cells. To gain insight in the mechanism in the formation of the bifurcations (to distinguish between a real neural induction an expansion of the original neural plate) we conducted the following experiments. First, we labeled a region of the epiblast that will form under normal circumstances lateral neural plate and checked the destine of these cells with or without FGF targeted in the periphery (Fig. 4). Embryos with labeled cells in this position of the epiblast without treatment or with implantation of PBS soaked beads show after 24 h in culture a band of labeled cells in the lateral neural tube (Figs. 4A–B). The same cells in embryos that have been treated with FGF (two beads) at P45 (five cases) did not show in any case alteration in the fate of the labeled cells (Figs. 4C–D) meaning that no incorporation of neural plate cells to the forming bifurcation (asterisk in 4D) has occurred. Second, we performed similar experiments but labeling the cells of the ectoderm forming region of the epiblast that is close to the site of implantation of the bead (Fig. 5). In untreated embryos, labeled cells in this position end up in the extraembryonic ectoderm of the embryo (Figs. 5A–B) according to already existing fate maps. The same initial labeling at stage 4 in embryos treated with FGF at P45 (five cases) can be observed after 24 h in the bifurcation formed and also some cells in the endogenous neural tube (Figs. 5C–D) meaning a change in fate of epithelial cells to neural (that incorporate to the bifurcation). Taken together, these results demonstrate that epiblast cells at the periphery of the area opaca–area pellucida can change their fate from ectodermal to neural under the influence of FGFs and that there is no incorporation of cells to the ectopic tissues from the original neural plate (no expansion of the neural forming territory).

The extra neural plate induced by FGF is not determined by expansion of axial mesoderm or transdetermination of other mesodermal cells

FGF is well know by its ability to induce mesoderm and mesoderm cells are (in part) responsible for neural induction under normal circumstances. Then, two possibilities arise for explaining

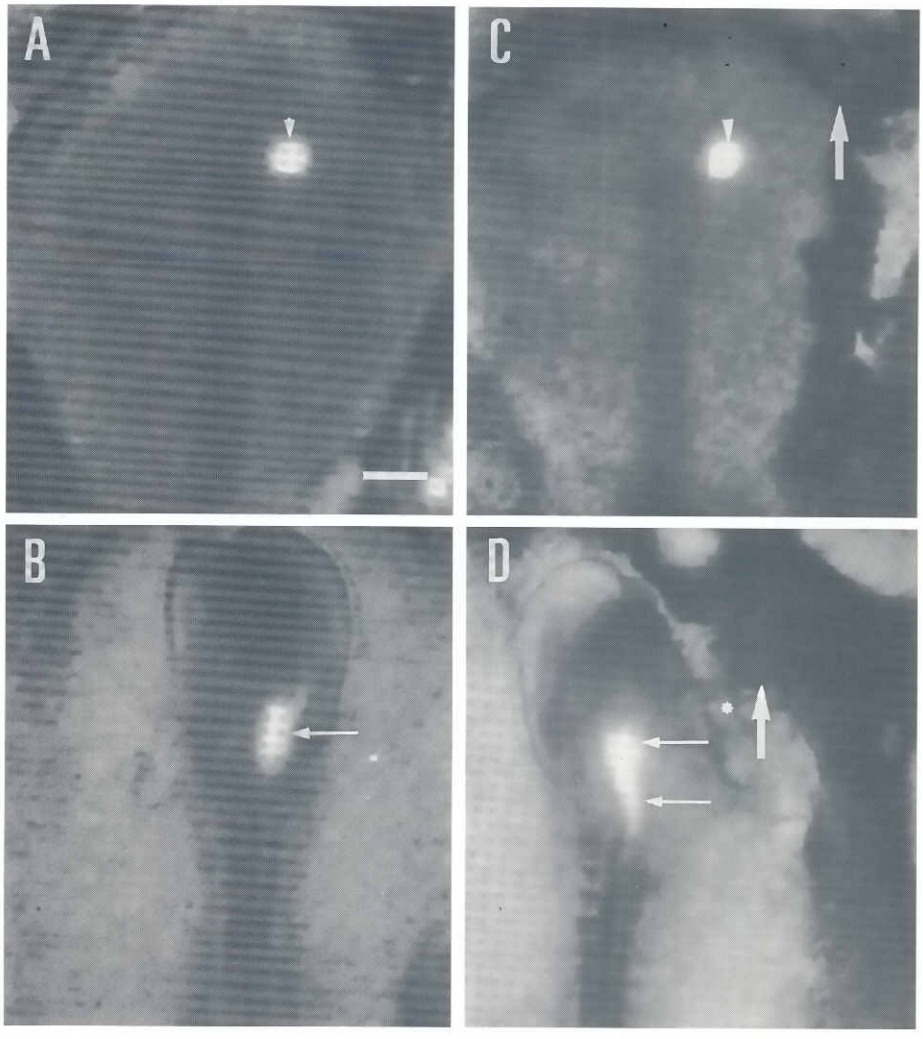

Fig. 4. Cell labeling experiments in the neural plate. *CSFE labeling of cells at stage 4 in the neural plate territory (arrowheads) and their fate (thin arrows) after 24 h of development in embryos without FGF* **(A and B)** *and in embryos treated FGF* **(C and D)**. *In both cases labeled cells are located in the main neural tube at the rombencephalon level, without incorporation of neural fated cells to the bifurcation (asterisk) formed. This means that FGF has not produced an expansion of the original neural plate. Thick arrows indicate the position of FGF soaked beads. Magnification is the same in all cases. Bar in A, 250 μm.*

the formation of new neural cells with FGF: i) epiblast cells receive directly the FGF signal and become neural cells or ii) FGF produce new axial mesoderm that then induces neural cells. To ascertain how the induction of neural cells by FGF takes place and to distinguish between these two possibilities, we conducted similar labeling experiments to those described above and checked the expression of mesodermal markers in FGF treated embryos. When a labeled piece of epiblast that includes the HN of a stage 4 embryo is transplanted to the same position of an unlabeled host embryo of the same age (fig 6A), the explanted cells will form mainly axial mesoderm and 24 h later grafted cells form a rostrocaudal stripe along the midline of the embryo (Fig. 6B). When the same graft was made in an embryo in which two beads soaked with FGF were implanted (five cases), the disposition of the cells 24 h later is similar to untreated embryos (Figs. 6C–D). Therefore, the pattern of formation of axial mesoderm in FGF–treated embryos is not altered by the growth factor, without expansion or migration of mesodermal cells derived from the HN. This experiment confirms the observation that embryos treated with FGF did not show a significant increase in the amount of mesoderm compared with control embryos as observed by histology (see Figure 2F). Nevertheless, we asked whether FGF could induce the expression of axial mesodermal markers in other mesodermal cells formed beneath the ectopic neural cells induced and then eliminate any possibility of secondary induction throughout newly axial mesodermal cells. In situ hybridization showed that the pattern of expression of *Brachyury* (*T*) a marker for axial mesoderm, is almost not altered in FGF treated embryos (Fig. 7). Embryos treated with FGF–2 or FGF–4 showed RNA transcripts for this gene along the rostrocaudal axis in the midline without positive cells following the bifurcation produced.

Discussion

FGF can induce ectopic neural tissues when emulating a signaling center

Overall treatment of cultured chick embryos with members of the FGF family has already shown that epiblast cells can respond to these growth factors by producing severe alterations in the pattern of development (Cooke and Wong, 1991; Cooke, 1993). Although the mechanism of action is not well understood, the application of peptides using acrylic beads has the advantage of targeting the growth factors to specific locations, and this method has been effectively used in gastrulating chick embryos (Levin *et*

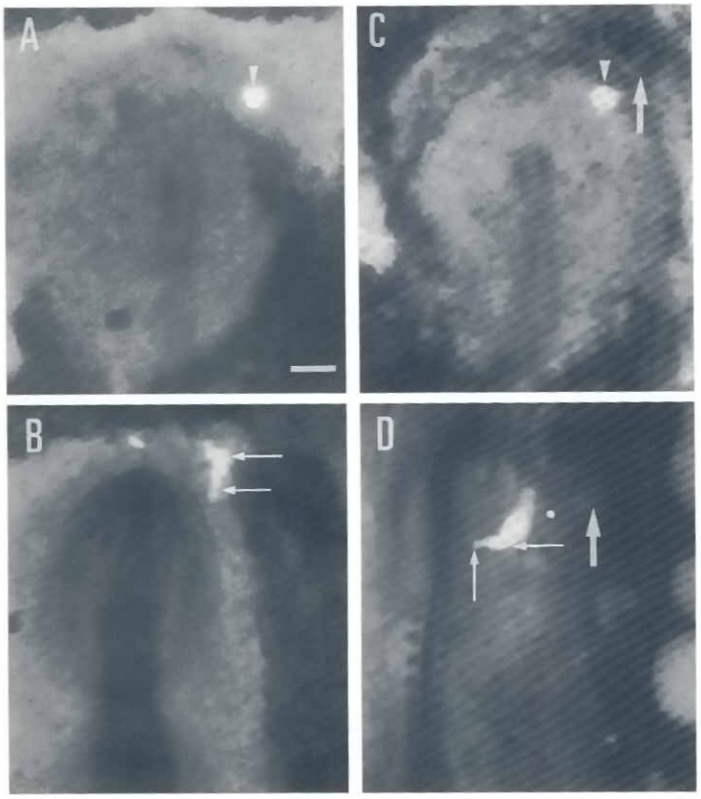

Fig. 5. Cell labeling experiments in the ectoderm. *Similar experiment to that described in figure 5 but the labeled cells in this case are at the periphery of the embryo (arrowheads). In control embryos* **(A and B)** *these cells will form extraembryonic ectoderm (thin arrows in B) ending up far away of the neural tube. When two beads soaked with FGF are placed at P45* **(C and D)** *the embryo produces a bifurcation (asterisk) and the fluorescent cells incorporate to the new neural tissue formed (horizontal thin arrow in D) and in fact, some cells end up in the main neural tube (vertical thin arrow in D). Thick arrows point out the FGF soaked beads. Bar in A, 250 μm and magnification for the four pictures is the same.*

al., 1995) and other systems. In fact, the release from localized embryonic regions (organizers) and the subsequent formation of a concentration gradient is the way by which most of the known signaling molecules seem to act in the embryo. Targeted expression using soaked beads emulates the way of action of such signaling centers. In early chick embryos, at least one of such signaling center has been extensively studied and corresponds to the rostral end of the primitive streak or Hensen's node (HN). This center releases a plethora of signaling molecules that have neural–inducing capabilities (Sokol *et al.*, 1990; Lamb *et al.*, 1993; see Harland, 1994, for a review; Stein and Kessel, 1995) and when transplanted ectopically to ectodermal regions can induce extra neural tubes (Kintner and Dodd, 1991). This evidence favors the hypothesis that HN and/or cells derived from it are responsible for the induction of the neural plate from epiblast cells (Harland, 1994). Several lines of evidence show that members of the FGF family are produced by HN at gastrulation (Bueno *et al.*, 1996; Smallwood *et al.*, 1996) and, consequently, are also potential candidates for neural induction. Nevertheless, during gastrulation, FGF has been tested mainly in *Xenopus* animal caps and only in whole expression or repression systems, namely, through direct addition of the growth factor to the culture medium or using of dominant–negative receptors (see Slack, 1994, for a review of FGF roles in development). In these experimental systems the main consequence is the alteration of the normal pattern in the formation of mesodermal derivatives. It has been assumed that the results obtained in these experimental systems can be inferred to normal embryonic development and that are universal for different organisms and, therefore, FGF is widely considered one of the major mesodermal inducers.

The results described in this paper show that appropriate targeted expression of FGF–2 or FGF–4 using acrylic beads (i.e., emulating signaling centers) is able to induce ectopic tissue that shows expression of neuronal markers. Therefore this family of growth factors may have neural inducing capability in chick embryos. Our results are in concordance with several lines of evidence that recently showed that FGF can induce neural tissue in *Xenopus* embryos (Cox and Hemmati–Brivanlou, 1995; Kengaku and Okamoto, 1995; Lamb and Harland, 1995; Lunay *et al.*, 1996). Both members of the FGF family used in this study are very similar in amino acid structure, although FGF–2 lacks the signal peptide necessary for *in vivo* secretion. This difference seems not to be important for our expression system because probably both factors can be released in a similar way by the bead. The different concentrations tested did not either show differences in the competence of the response of epiblast cells (in all the cases we were able to produce new neural cells) meaning that the range of concentration for both FGF tested (from 0.1 to 1 mg/ml) has passed the threshold for neural induction. This hypothesis is in agreement with the differences observed in the effects of FGF depending on time and position of bead implantation (see below).

Induction of neural tissue by FGF is dependent on the competence state of the epiblast cells

The narrow interval in time and space during which epiblast cells can respond to the growth factor by forming an ectopic neuraxis points out that the competence state (determined by age and position) of the epiblast cells determines their responsiveness to instructive signals. Using transplantation experiments it has been already shown that the competence of epiblast cells to appropriately respond to instructive signals from the HN is maximum near stage 4 (Dias and Schoenwolf, 1990; Storey *et al.*, 1992; Streit *et al.*, 1997). Our results confirm these data because the application of FGF before or after the establishment of the full–length primitive streak does not produce ectopic neural plate formation, although other alterations in development are produced. Besides, at the full–length primitive–streak stage the ability to form ectopic neural plate cells is greater in cells at the periphery of the embryo than near the node (P>C). In fact, the cells that are forming the ectopic neural plate are located relatively far away from the cells that will normally form neural plate (Schoenwolf and Alvarez, 1991; García–Martínez *et al.*, 1993). This result suggests that when appropriately targeted, FGFs can change the fate of otherwise ectodermal cells and has been confirmed by the incorporation of ectodermal cells to the ectopic bifurcation of the main neuraxis formed (Fig. 5). This means that at stage 4 epiblast cells located at the border between the area opaca and the area pellucida are still competent to form neural plate, but that they are too far from the HN to receive the

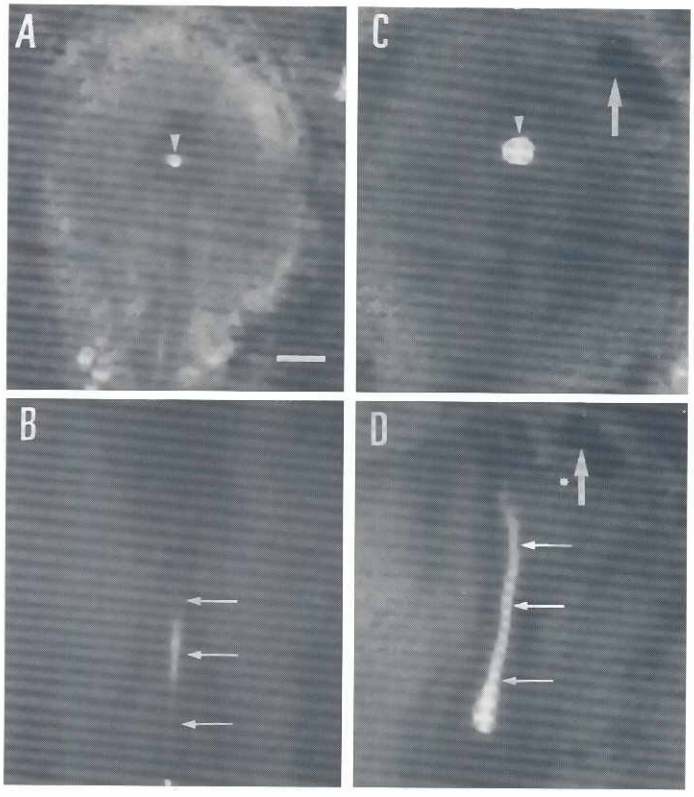

Fig. 6. Cell labeling experiments in the HN. *In untreated embryos* **(A–B)** *cells derived from the fluorescently labeled graft (arrowhead) form a stripe that runs along the rostrocaudal axis of the embryo that corresponds to the axial mesoderm (thin arrows in B). When the same type of graft is made in embryos to which two FGF–4 soaked beads* **(C–D)** *had been implanted, the distribution of labeled cells (thin arrows in D) was not altered, and the formation of an extension of the axial mesoderm, which could be responsible for the formation of ectopic neural plate (asterisk), failed to occur. Thick arrows indicated beads. Magnification is the same in all figures. Bar in A, 300 μm.*

neural inducing signal. When appropriately delivered, a neural–inducing signal, such as FGF, causes the change of fate from epithelial to neural. When targeted to C45 or other regions that are close to the endogenous neural plate territory, the cells that receive the growth factor are already committed to neural plate and no additional neural cells are produced. Nevertheless, neuroepithelial cells already committed at stage 4 are receptive to the new source of FGF. Preliminary results show that, when targeted to already formed neural cells, FGFs are able to change the rostro–caudal character of neuroepithelial cells. In this way, positional markers for precise regions of the neural tube express in more caudal segments of the neural tube after FGF application, meaning that they are acting as posteriorizing agents (in preparation). This role of FGFs is in agreement with data that show a posteriorization of neural structures after treatment with FGF–8 (Crossley *et al.*, 1996).

As mentioned, the bead must be placed in the mesenchymal compartment of the embryo in order to obtain ectopic neural cells. One possibility that must be considered is that FGF can act only over mesodermal cells and then these cells are responsible for inducing an ectopic neural plate. As mentioned, axial mesoderm can induce neural plate formation, and some molecules responsible for such mesodermal induction have been identified in the head process and notochord (Slack, 1994). Additional proliferation induced in these cells by FGF or acquisition of inducing properties by non–axial mesoderm cells could explain the results obtained by means of extending the neural–inducing mesoderm. Our results support a direct action of FGFs on epiblast cells because the pattern of growth and development of axial mesoderm is not altered by FGF as shown by cell labeling experiments (Fig. 6) and the expression pattern of *T* (Fig. 7). Nevertheless, double labeling experiments with neural and axial markers and checking of more specific markers for axial mesoderm will be necessary before concluding that there is no participation of mesoderm in the neural induction mediated by FGF. In this way, we are now currently studying the expression pattern after FGF treatment of Not–1, a marker for notochord cells (Rodríguez–Gallardo *et al.*, 1996) and *Sonic hedgehog*.

If FGF is not acting over the mesoderm we can assume that the necessity of actuation in the mesenchymal compartment is because the responding molecules to FGF in the epiblast cells are

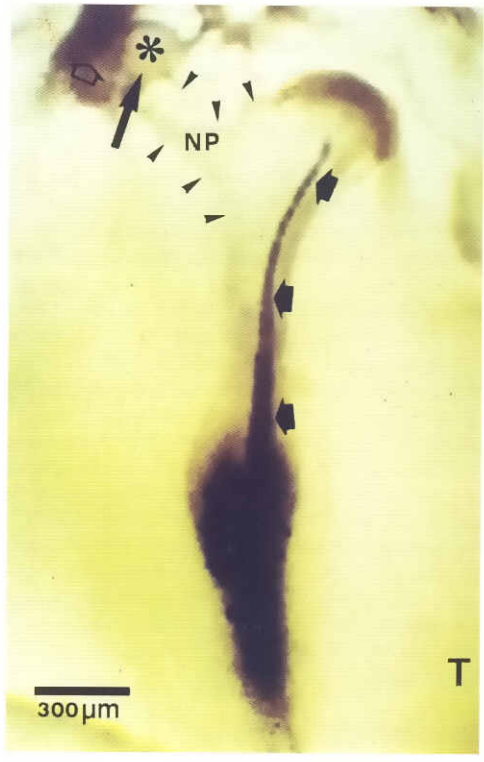

Fig. 7. Identification of axial markers after FGF treatment. *Whole mount in situ hybridization for Brachyury (T) in an embryo treated with a FGF–4 soaked bead (asterisk) implanted at P45 position (arrow). The endogenous expression (thick arrows) of T can be observed in the original axis. There is no new T positive cells running along the new neuraxis (NP) formed as bifurcation (delimited by arrowheads). Open arrow indicates background.*

located in the basal part of the epithelium. This is consistent with the presence of both low and high affinity FGF receptors at the basal surface of the cells (Schlessinger *et al.*, 1995). A better knowledge of the location and role of the different FGFs receptors in the developing chick blastoderm and the early nervous system will help us to understand the mechanism of action of FGF on epiblast and neural cells (Godsave and Durston, 1997). Nevertheless, other possibilities such as FGFs acting on the endodermal layer that has also been proposed as a neural inducer (Bouwmeester *et al.*, 1996), could be considered.

Materials and Methods

Targeted application of FGF in chick embryos

Chick embryos ranging from stages 3 to 6 of Hamburger and Hamilton (1951) were prepared for New culture (New, 1955) as described previously (Schoenwolf and Alvarez, 1989). Heparin acrylic beads (Sigma) approximately 150–250 μm in diameter were soaked for at least 2 h at room temperature in FGF–2 or FGF–4 (R&D Systems) and placed in different positions (see below) of the chick blastoderm. In most cases the beads were inserted between the endoderm and the epiblast layers by making a hole in the endoderm (facing up in New culture). Control experiments were carried out with beads soaked in PBS for 2 h.

Four main positions were chosen for ectopic expression based on their relative position with respect to Hensen's node and the border between the area opaca–area pellucida. Positions C (for center) are situated at the central region of the anterior epiblast and positions P (for periphery) at the region close to the border between the area opaca and the area pellucida. Moreover, for positions C and P we chose the sublocations called C0, C45, P0 and P45 respectively, depending of the angle formed with the middleline. The location of all of these positions in a stage 4 chick embryo is schematically shown in Figure 1 and an example of an embryo with a bead at P45 is shown in Figure 2A. Embryos were videotaped and/or photographed at the moment of transplantation (0 h), 4 h later to ensure that the bead retained the position selected for implantation and when collected 18–36 h later. After removing the embryos from the culture dish, they were fixed and processed for histology, immunocytochemistry or *in situ* hybridization (see below).

A total of 318 embryos were treated with FGF as described above and analyzed for this study. Of these, 97 were either treated with beads soaked with PBS (control embryos) or were not at the full–length primitive streak stage; the remaining 221 were considered as early neurulating embryos (late stage 3, stage 4 and early stage 5) and were used for a more detailed analysis according to the position were the bead was placed and the concentration of FGF used. Roughly half of the embryos were treated with FGF–2 at three concentrations (0.2, 0.4 and 1 mg/ml) and the other half with FGF–4 also with three concentrations (0.1, 0.5 and 1 mg/ml). About one fourth of the embryos were treated with two beads (instead of one) that were placed together (i.e., at the same position). Because of the large number of embryos used, we ensured at least 10 cases for each position/growth factor/concentration at the full–length primitive streak stage. Embryos were treated randomly on the left or right side of the embryo with a final percentage close to 50% for each side.

Fate mapping experiments

Cell labeling experiments were performed by covering donor chick embryos set up in New culture with a 0.01% solution of CFSE (Molecular Probes) in PBS for 1 h (stock solution of CFSE was 0.5% in DMSO). After generous washing, the desired pieces of the blastoderm were removed and transferred to an unlabeled embryo of the same age, to which the homotopic region had been previously removed. After incorporation of the labeled graft in the host embryo (1–2 h), one or two beads soaked with FGF–4 at 0.5 mg/ml were placed as described above in position P45. The course of the transplanted graft was followed over time by periodic examinations of the embryo with a fluorescent microscope and videotape documentation. Control transplantations consisting of fluorescent grafts transplanted to embryos without beads or to embryos with beads soaked in PBS, were used for comparison with treated embryos.

Immunohistochemistry

For immunodetection of neurofilament associated protein (NAP) antibodies in whole-mount, embryos were fixed in 4% paraformaldehide (PFA) overnight at 4°C, washed with phosphate–buffered saline (PBS) and permeabilized with a 0.1% solution of Triton X–100 in PBS. After repeated washing and blockage with 1% NGS in PBS for 1 h, embryos were left overnight in supernatant containing the monoclonal antibody (dilution 1:1). Afterwards, the embryos were thoroughly washed with PBS and incubated with the secondary antibody (anti–mouse IgG peroxidase–conjugated at 1:200 obtained from Sigma). The presence of peroxidase was detected by diaminobenzidine (DAB) precipitation using a 0.1% solution of DAB and 0.025% hydrogen peroxidase in PBS. After observation and photography, selected embryos were then processed for histology and serially sectioned transversely.

In situ hybridization (ISH)

For detection of *Brachyury* (*T*) RNA, control and experimental embryos were fixed in 4% PFA overnight and processed for ISH as described by Nieto *et al.* (1996). After hybridization with the *T* anti–sense probe (kindly provided by J.C. Izpisúa–Belmonte) and binding with the antidigoxigenin antibody, gene expression was detected with the alkaline phosphatase chromogenic reaction and the embryos cleared with glycerol before photography.

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