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FGF2 plays a key role in embryonic cerebrospinal fluid trophic properties over chick embryo neuroepithelial stem cells

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Abstract

During early stages of brain development, neuroepithelial stem cells undergo intense proliferation as neurogenesis begins. Fibroblast growth factor 2 (FGF2) has been involved in the regulation of these processes, and although it has been suggested that they work in an autocrine–paracrine mode, there is no general agreement on this because the behavior of neuroepithelial cells is not self-sufficient in explants cultured in vitro.

In this work, we show that during early stages of development in chick embryos there is another source of FGF2, besides that of the neuroepithelium, which affects the brain primordium, since the cerebrospinal fluid (E-CSF) contains several isoforms of this factor. We also demonstrate, both in vitro and in vivo, that the FGF2 from the E-CSF has an effect on the regulation of neuroepithelial cell behavior, including cell proliferation and neurogenesis.

In order to clarify putative sources of FGF2 in embryonic tissues, we detected by in situ hybridization high levels of mRNA expression in notochord, mesonephros and hepatic primordia, and low levels in brain neuroectoderm, corroborated by semiquantitative PCR analysis. Furthermore, we show that the notochord segregates several FGF2 isoforms which modify the behavior of the neuroepithelial cells in vitro. In addition, we show that the FGF2 ligand is present in the embryonic serum; and, by means of labeled FGF2, we prove that this factor passes via the neuroepithelium from the embryonic serum to the E-CSF in vivo.

Considering all these results, we propose that, in chick embryos, the behavior of brain neuroepithelial stem cells at the earliest stages of development is influenced by the action of the FGF2 contained within the E-CSF which could have an extraneural origin, thus suggesting a new and complementary way of regulating brain development.
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Introduction

In vertebrates, early brain development takes place at the expanded anterior end of the neural tube. At these early developmental stages, the brain wall is formed by a pseudo-stratified neuroepithelium, mainly built by pluripotent stem cells. This neuroepithelial wall also encloses a large cavity containing the embryonic cerebrospinal fluid (E-CSF).

The highly dynamic cellular behavior of neuroepithelial stem cells at these stages of development includes both an intense proliferation phase in which these cells increase in number to generate a large number of progenitor cells in the central nervous system (CNS) and the initiation of neurogenesis. The mechanisms involved in controlling in vivo replication and differentiation of neuroepithelial stem cells are not fully known. It has been suggested that proliferation and neurogenesis are sequentially driven during the ontogeny of neural cells, and that morphogenetic factors involved in the inducing the proliferation of neuroepithelial cells also promote their transition to the

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differentiation phase, thus initiating neurogenesis (Panchision and McKay, 2002). Although it has been proposed that in different species, several growth factors could be involved in the regulation of these processes through different sorts of interactions, several studies conducted in many different experimental conditions have shown that FGF2 and, subsequently, EGF are involved in regulating the proliferation and differentiation of neural precursors at early stages of developments (Tropepe et al., 1999; Vaccarino et al., 1999a; Junier, 2000; Ford-Perriss et al., 2001; Panchision and McKay, 2002; Dono, 2003). However, so as to understand what influences embryonal brain development, it is important to know of the origin and modus operandi of its regulating factors.

Regarding the role of FGF2 in neuroepithelial development, it has been suggested that it acts in an intracrin (Bikfalvi et al., 1995; Davis et al., 1997) and autocrine–paracrine manner (Vaccarino et al., 1999a,b; Panchision and McKay, 2002, among others). In this regard, it has been shown that FGF2 immunoreactivity (Kalcheim and Neufeld, 1990; Dono and Zeller, 1994; Raballo et al., 2000; Dono, 2003) or mRNA (Zúñiga Mejia Borja et al., 1993; Savage and Fallon, 1995; Ozawa et al., 1996; Han, 1997 Vaccarino et al., 1999a) is expressed from early stages of development in a developmentally regulated manner at numerous distinct sites, including the proliferative neuroepithelium in several species, specially in rodents. In addition, FGF2 signaling involves high affinity transmembrane tyrosine-kinase receptors (FGFR1 to FGFR4), which are also expressed at distinct sites during embryogenesis in a developmentally regulated manner, including the CNS (Fayein et al., 1992; Ozawa et al., 1996; Wilke et al., 1997; Okada-Ban et al., 2000; Reuss and von Bohlen und Halbach, 2003).

In the last few years, it has been shown that the intra-cavity E-CSF has an important effect on brain development. For instance, we have recently reported that E-CSF, which has a complex protein composition (Gato et al., 2004; Parada et al., 2005a), exerts a trophic action on the neuroepithelial cells, promoting their survival, proliferation and neurogenesis, and has a role in regulating the expression of some genes involved in the patterning of the CNS, i.e., *otx2* (Gato et al., 2005; Parada et al., 2005b). In this regard, it has been suggested that CSF is involved in the regulation of cortical histogenesis during fetal and early postnatal stages (Miyan et al., 2003). Moreover, the theory that FGF2 has an autocrine–paracrine action on neuroepithelial cells implies a tissue self-sufficiency in regulating cell behavior; we have also demonstrated that neuroepithelial explants cultured in vitro with a chemically defined medium are unable to self-induce a normal level of proliferation and neurogenesis of neuroepithelial cells, and that they require the action of specific E-CSF components on such cells (Gato et al., 2005).

However, there are many reports that enable us to presume that the FGF2 could act on the neuroepithelium by means other than those autocrine–paracrine previously proposed. FGF2 is known to be one of the prototypic heparin binding growth factors (HBGF) and may act over longer distances, as it has been reported that members of the HBGF family may be secreted at a certain distance from their target cells. It is also known that FGF2 normally travels via extracellular routes bound to heparan-sulphate proteoglycans (HSPG), facilitating the displacement of the growth factor, and protecting them from enzymatic degradation, as well as, in some cases, concentrating them near the target cells or working in collaboration with the FGF2-receptor union (Yayon et al., 1991, Nugent and Edelman, 1992, Joseph et al., 1996, Brickman et al., 1998). These findings indicate that during development FGF2 is a factor which can act at a distance between cells of the same or different structure. In this regard, it has been shown that proteoglycans are present inside the brain cavity of chick embryos during the period of maximum cerebral expansion. (Gato et al., 1993, Alonso et al., 1999).

Considering all this data, we hypothesize that a source of FGF2 other than the neuroepithelium itself could be necessary to maintain normal in vivo behavior of neuroepithelial cells, and that the E-CSF may provides the neuroepithelium with this function during early development.

In these work, we show that in chick embryos at early stages of CNS development, E-CSF contains FGF2, that this FGF2 is involved in regulating the behavior of neuroectodermal cells, including cell proliferation and neurogenesis, and our results suggest that the FGF2 of the E-CSF might come from the embryonic serum and may have, at least in part, an extraneural origin.

Material and methods

Obtaining E-CSF and embryonic serum

Fertile chicken eggs were incubated at 38°C in a humidified atmosphere to obtain chick embryos at developmental stage HH25 (Hamburger and Hamilton, 1951). To obtain E-CSF, after incubation, the extraembryonic membranes were removed, the embryos were rinsed twice in sterile saline solution and placed in a dry Petri dish, and surrounding fluid was aspirated to avoid CSF contamination. A glass microneedle (30-μm inner diameter at the tip) connected to a microinjector (Medical System PLI 100) was carefully placed on the middle of the mesencephalic cavity under dissecting microscope control. CSF was slowly aspirated, avoiding contact with the neuroepithelial wall so as to obtain samples not contaminated by neuroepithelial cells. A sufficient number of embryos were used to obtain an adequate amount of CSF. To minimize protein degradation, E-CSF samples were kept at 4°C during this procedure, and immediately afterwards, they were aliquoted, lyophilized, and frozen at −40°C until used.

Although the organotypic cultures in which this E-CSF was used were made with mesencephalic neuroectodermal tissue from embryos at HH19–20, to be cultured 24 h in vitro (up to the equivalent of stage HH23), the E-CSF was obtained from embryos at stage HH25 in order to obtain a reasonable amount of this fluid (approximately 5 μl/embryo). It has been reported that the protein composition of the E-CSF monitored by SDS-polyacrylamide gel electrophoresis and silver staining is not qualitatively modified between HH19 to 28 (Gato et al., 2004).

To obtain embryonic serum, blood from stage HH25 chick embryos was extracted "in vivo". A small window was made in the eggshell, the vitelline and amniotic membranes were cut and separated by microdissection. Subsequently a small support was placed under the embryo to facilitate holding in place and improve visualization. The blood was obtained by microaspiration in the outflow tract of the embryonic heart with a glass microneedle of 20-μm inner diameter connected to a microinjector (Medical System PLI 100). The blood from different embryos was pooled and immediately centrifuged, and the serum fraction aliquoted, lyophilized, and stored at −40°C until used.

Organotypic cultures of mesencephalic neuroectoderm

Organotypic cultures of mesencephalic neuroectoderm were performed as described in Gato et al. (2005). Briefly, this took place as follows: chick embryos at HH20 were dissected out of extraembryonic membranes, and the ectoderm and mesoderm covering the mesencephalic vesicle was removed using a tungsten needle. Following this, the mesencephalic roof of the neuroepithelium was cut with microscissors. The explants, after extensive washing in a serum-free medium, were placed on small pieces of Millipore filters (0.8-μm pore size) previously boiled in distilled and de-ionized water. After equilibrating the filters in a serum-free medium for 15 min, the explants were placed on the filter with the apical surface in close contact with the filter. To avoid the detachment of the explants, these were peripherically fixed to the filter with the tungsten needle. They were then transferred to a culture well containing a serum-free medium (DMEM F12, Sigma) supplemented with 1% ascorbic acid and cultured as a Trowell's tissue culture, as described in Brunet et al. (1993) for 24 h at 37°C and 5% CO_2 (which chronologically corresponds to stage HH23). Finally, the explants were fixed in Carnoy, embedded in paraffin and processed for histological sectioning.

For the different sets of experiments, cultures were made only in a serum-free medium (400 μl per dish) as negative controls, or alternatively they were supplemented with the following: (1) E-CSF from H.H. 25 chick embryos at 1/7 v/v, as a positive control; (2) E-CSF + 2 μl of FGF2 antibody, diluted 1/100 (Sigma F-3393), to gauge the effect of the specific immunodeprivation of FGF2 in the E-CSF; (3) E-CSF + 2 μl of FGF2 antibody diluted 1/100 (Sigma F3393), previously blocked with FGF2 at a 1/10 ratio (human recombinant Sigma F0291-25UG), to assess the blocking capacity of FGF2's biological activity by the FGF2 antibody employed; (4) 60 ng of FGF2 (human recombinant, Sigma F0291-25UG) in 400 μl culture medium in order to confirm the effect of this isolated factor; and (5) E-CSF + 2 μl of NGF antibody (Sigma N-6655) at 1/100 dilution, to discard any unspecific effects of the antibody added to the culture medium. It is important to note that according to the supplier, both antibodies block the biological activity of the recognized molecule. Occasionally we carried out neuroepithelial explants cultured with a medium previously conditioned by notochord. This took place as follows: fragments of cephalic notochord from 25 H.H. stage chick embryo were dissected with tungsten needles under microscope. Culture plates with 400 μl of specific medium (DMEM F12, supplemented with 1% ascorbic acid), plus 3 fragments of notochord per dish, were cultured for 24 h under the conditions explained above; after the notochord fragments were removed, use was made of the specific medium for neuroepithelium culture already described. Occasionally we did cultures with a notochord-conditioned medium + FGF2 antibody (2 μl of FGF2 antibody, diluted 1/100) to verify that the effect of the conditioned medium is due to the presence of this factor.

In ovo mesencephalic cavity microinjection

Chick embryos at HH20 were microinjected in ovo into the brain cavity, with 400 nl of the same antibodies to FGF2, or alternatively to NGF, as those employed in the in vitro study, both at 1/100 dilution, to compare the effects of the immunodeprivation with respect to the in vitro experiments (see above). Other embryos were microinjected with 400 nl of a solution of 50 ng of FGF2 ligand in 400 μl of Hank sterile, to compare with the experimental conditions cited above. Controls were performed by microinjecting the solvent of the antibodies, namely, PBS. Microinjection was performed with a glass microneedle (10-μm inner diameter at the tip) connected to a microinjector (Medical System PLI 100) into the mesencephalic cavity through a small opening made in the extraembryonic membranes with a tungsten needle. The microinjected embryos were reincubated in ovo for 24 h, and they were removed from the egg at HH23.

BrdU incorporation and tubulin immunostaining

In order to test the effect of the different experimental conditions on the behavior of the neuroepithelial cells, we selected the main parameters, cell replication and neurogenesis. Determination of BrdU incorporation into cell nuclei was performed by adding BrdU to the culture medium at just 1 h from the end of the culture, at a final concentration of 5 μM (for the organotypic culture), or by microinjecting 190 nl of BrdU in the outflow of the heart, once again at 1 h from the end of the culture (for in ovo experiments). Immediately after that, the explants or embryos were fixed in Carnoy for 20 min, dehydrated in an alcohol series, passed through xylene and embedded in paraffin. After transversal sectioning of the tissues, they were deparaffinated, and BrdU was detected following standard procedures. The sections were incubated in a solution containing a monoclonal antibody to BrdU (Dako) at 1/100 for 30 min. To detect the primary antibody, the avidin–extravidin system conjugated to peroxidase (mouse anti-rabbit 1/20 for 30 min and extravidin 1/20 for 10 min; Sigma) was used and staining was undertaken with DAB. We visualized and photographed the preparations using a Nikon microphot-FXA photomacroscope. A quantitative analysis of nuclear BrdU incorporation was performed by counting the number of BrdU-positive nuclei in 34 microscopic fields of 1400 μm^2 with 5 samples from 5 different explants. The average of each condition and the standard error were plotted, and their significance was tested by a two-tailed Student's *t* test.

We detected neuronal differentiation by β3-tubulin expression. After treatment, the explants or the embryos maintained in ovo from different experimental conditions were fixed in Carnoy and processed as described below for histological study. Sections were blocked in 1% BSA in PBS and subsequently incubated in a solution containing a monoclonal anti-tubulin antibody at 1/500 (BAbCO), and, after extensive washes, they were incubated with a solution containing an anti-mouse antibody conjugated to FITC at 1/64 (Sigma) for 1 h at room temperature. In order to facilitate identification of the β3-tubulin-positive cells, we used single plane images obtained by confocal laser microscopy in which cells showing a negative nucleus surrounded by a labeled cytoplasm were deemed positive (see Fig. 2G).

A quantitative analysis of β3-tubulin expressing cells in vitro and in ovo was performed by counting the number of neuroepithelial cells with immunostained cytoplasm in 20 microscopic fields of 1900 μm^2 from at least 4 different samples. In the whole of the analysis, the average of each condition and the standard error were plotted, and their significance was tested by a two-tailed Student's *t* test.

TUNEL assay

In order to rule out the possibility of the influence of the E-CSF FGF2 on replication and neurogenesis in the neuroepithelial explants being the result of a non-specific effect on cell survival, we assessed apoptosis by means of the TUNEL technique on paraffin sections from formalin-fixed explants. Apoptotic cells were detected using the Apoptosis Detection System Fluorescein Kit (Promega) following the manufacture's instructions. Visualization was made with a confocal microscope (Zeiss LSM-310). We performed quantitative analysis by counting the number of stained nuclei of neuroepithelial cells in 20 microscopic fields of 1900 μm^2 from at least 4 different samples. In the whole of the analysis, the average of each condition and the standard error were plotted and their significance was tested by a two-tailed Student's *t* test.

PC12 cells in vitro culture

In order to evaluate the capacity of our chosen anti-FGF2 antibody to block the biological activity of FGF2, we conducted a biological assay on in vitro cultured PC12 cells.

PC12 (8ATCC-CRL-1721) cells were cultured in DMEM + 6% horse serum + 6% fetal bovine serum + 1% glutamin + 1% penicillin/streptomycin, in a 5% CO_2 atmosphere for 5 days, with a supplement of 100 μg/ml FGF2 (Sigma F0291-25UG) or of the same amount of FGF2 blocked with 1.5 μl at 1/200 dilution of the anti-FGF2 antibody (Sigma F3393). For cell identification βIII tubulin immunostaining was performed as described previously and revealed with DAB. An evaluation of the effect of the FGF2 ligand was carried out by establishing the percentage of cells emitting neurites which doubled the diameter length of the cell body with respect to all the cells. The average of each condition and the standard error were plotted, and their significance was tested by a two-tailed Student's *t* test.

SDS-polyacrylamide gel electrophoresis, 2D electrophoresis and Western blot analysis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed under denaturing conditions according to the method of Laemmli (1979), with a Miniprotean II electrophoresis system (BioRad). Molecular mass standards of high and low range (BioRad) were also used. SDS-PAGE was performed in a discontinuous buffer system for 30 min at 200 V.

For 2D gel electrophoresis. The first dimension was run in a strip of 24 cm, pH 7–11 (Amersham Bioscience), in the IPGPhor system, within a gradient from 50 to 8000 V (accumulated voltage: 96,000 V/h). The second dimension was run in a 24 × 21 cm, 12,5% acrylamide (50% de Duracryl) gel electrophoresis by using the Ettan Dalt II system (Amersham Bioscience), at 3 w/gel for 30 min, followed by 19 w/gel for 4 h.

For Western blot analysis, proteins were electrotransferred from the electrophoresis gel to nitrocellulose membrane (Trans-Blot Transfer Medium, BioRad), for 1 h at 100 V (for SDS-PAGE) or overnight at 17 V (for 2D gel electrophoresis), using a basic transfer buffer following standard protocols. The immunoblotting was developed either with Opti-4CN (Sigma) or chemiluminescence (Biological Industries) on an X-ray film.

With a view to determining whether FGF2 is capable of passing from the embryonic fluid to the E-CSF, we conducted a Western blot SDS-PAGE of aliquots of 50 μl E-CSF from embryos at developmental stage HH25, 5 μl of a solution of 100 ng/μl of commercial FGF2 (Sigma F5542) linked to FITC, as we describe later or 5 μl of a solution of 100 ng/μl of glutathione-*S*-transferase–alcohol–dehydrogenase (GST-Adh) linked to FITC, as described below. Detection was made with antibody rabbit anti-FGF2 (Sigma F3393, 1/2000), mouse anti-GST (kindly provided by N. Cols, Dept. of Genetics, U. of Barcelona, Spain) 1/2000 or mouse anti-FITC (Sigma F5636) 1/2000 and subsequently with a secondary antibody anti-rabbit or anti-mouse IgG (Sigma) conjugated to peroxidase.

In order to rule out crossed reactions with other ligands, we performed a Western blot SDS-PAGE of aliquots of 0.5 μl of different solutions (100 μg/μl): EGF (Sigma, E6135), FGF-1 (Sigma, F5542), FGF2 (Sigma, F0291-25UG) and FGF8 (Sigma, F1802), which were exposed to the anti-FGF2 antibody (Sigma, F3393).

The presence of different isoforms of FGF2 in the E-CSF, embryonic serum and notochord-conditioned medium, were detected by a Western blot of bidimensional electrophoresis of E-CSF (250 μl) from 25 H.H. stage chick embryos, 150 μl of embryonic serum from 25 H.H. stage chick embryos and 250 μl notochord conditioned culture medium (see below). Immunostaining was with anti-FGF2 antibody (Sigma F3393) or anti-FGF2 antibody previously incubated with FGF2 factor (Sigma F0291-25UG) at 1/10 ratio.

FITC coupling, microinjection and detection

Commercial FGF2 stabilized with albumin (human recombinant, Sigma F0291-25UG) and a glutathione-*S*-transferase–alcohol–dehydrogenase (GST-Adh) fusion protein were coupled to fluorescein isothiocyanate Isomer I (FITC, Sigma). The GST-Adh fusion protein (kindly provided by N. Cols, Dept. of Genetics, U. of Barcelona, Spain) was a recombinant protein with a molecular mass of 38 kDa made from the recombination of the *Schistosoma japonicum GST* and the *Drosophila lebanonensis Adh* genes. This recombinant protein was used as a negative control as its amino acidic sequence did not show similarity with any known chick protein.

The coupling with FITC was performed as described in Harlow and Lane (1988) for antibodies. The unbounded dye was separated by gel filtration (Sephacryl S-200, Pharmacia). The final ratio of absorbance at 495/280 nm for the conjugated proteins was 0.81 for FGF2-FITC and 0.93 for GST-Adh-FITC.

500 nl of both conjugates at 0.2 mg/ml in NaCl physiological solution (9 g/l) were microinjected into the outflow of the heart of H.H.22 chick embryos with a glass microneedle (10-μm inner diameter at the tip) connected to a microinjector (Medical System PLI 100), and the E-CSF from these embryos was recovered after 24 h of in ovo development (i.e., HH25), as described above. To identify the conjugates, FGF2-FITC and GST-Adh-FITC in the E-CSF of the microinjected embryos, this fluid was run in a 12% SDS-PAGE and transferred to a nitrocellulose membrane; FGF-2, GSH and FITC were detected by Western blot immunolabeling as described in the previous section.

RNA in situ hybridization and semiquantitative PCR analysis

RNA in situ hybridization was performed as described by Bueno et al. (1996) for one probe using single-stranded digoxigenin–UTP-labeled (Boehringer Mannheim) antisense riboprobes. *Fgf2* riboprobe (*fgf2*-288; Savage and Fallon, 1995; EcoRI digestion and T3 transcription), recognizing all known *fgf2* isoforms, was used. Hybridization was developed with NBT/BCIP according to standard protocols for 6 to 36 h, depending on the embryo developmental stage. To avoid the trapping of reagents in the cephalic cavities, embryos older than HH18 were dissected in two symmetrical halves prior to whole-mount hybridization.

After probe detection, embryos were embedded in paraffin, sectioned at 10 μm and mounted on gelatine-coated slides. Photomicrographs were taken using a Stemi V6 stereomicroscope (Zeiss) and an Axiophot microscope (Zeiss). Images were digitalized by capturing them with a CCD Coopix 995 camera (Nikon), and they were assembled by using Photoshop software.

PCR analysis was made on several different embryonic tissues, dissected under the control of a dissecting microscope. The total mRNA was purified by using the RNeasy Mini Kit for RNA extraction (Qiagen). Primers used for FGF2 PCR amplification were as follows: forward, 5′ctgcagcttcaagcagaag3′, which amplifies all known isoforms; forward, 5′gcttccagccagccgaggg3′, which amplifies the Low Molecular Isoforms (LMW) isoform; forward, 5′ tggacggccgcggcaggg3′ and 5′gagcatcaccacgctgcc3′, which amplify different High Molecular Isoforms (HMW); and reverse, 5′, for all amplification reactions. Primers used for actin controls were the following: forward, 5′ tggatgatgatattgctgc3′; and reverse, 5′atcttctccatatcatccc3′. A regime consisting of 94°C for 2 min (1 cycle), 94°C for 1 min–45°C for 30 s–72°C for 45 s (35 cycles), followed by a single terminal extension step (72 C for 5 min) on an Eppendorf Mastercycler was used for all primer sets. Actin was used as an internal control for semiquantitative comparison. Sample dissection was independently performed twice, and each PCR experiment was performed with 4–5 separate RNA-cDNA collections.

Results

FGF2 is present in the chick E-CSF

First of all, in order to establish the basis for our theory that FGF2 acts on neuroepithelial cells from the E-CSF, it is necessary to test whether FGF2 is present in the E-CSF. Western blot analysis of E-CSF after 2D gel electrophoresis was performed (Fig. 1A) and the chemiluminescence of the Western blot showed the presence of immunoreactive spots located between p*I* 10 and p*I* 10.5, as expected for basic FGF. Four immunoreactive spots were clustered at a molecular mass between 17 and 24 kDa, and one other spot showed a molecular mass of approximately 34 kDa, as expected for the different low molecular weight (LMW) and high molecular weight (HMW) FGF2 isoforms cited in the literature. To ensure specificity in FGF2 ligand recognition by the antibody we employed, we conducted two complementary experiments: first, we checked (Fig. 1B) that previous exposure of this antibody to FGF2 eliminated its capacity to mark all the spots in the E-CSF; secondly, we performed a monodimensional Western blot with anti-FGF2 antibody on different growth factors (Fig 1D), which enabled us to verify that our anti-FGF2 antibody showed no crossed reactions with other members of the family of FGFs (particularly, FGF1 and FGF8) or other factors such as the EGF. These experiments allow us to assert that at the H.H. 25 developmental stage, E-CSF contains distinct FGF2 isoforms.

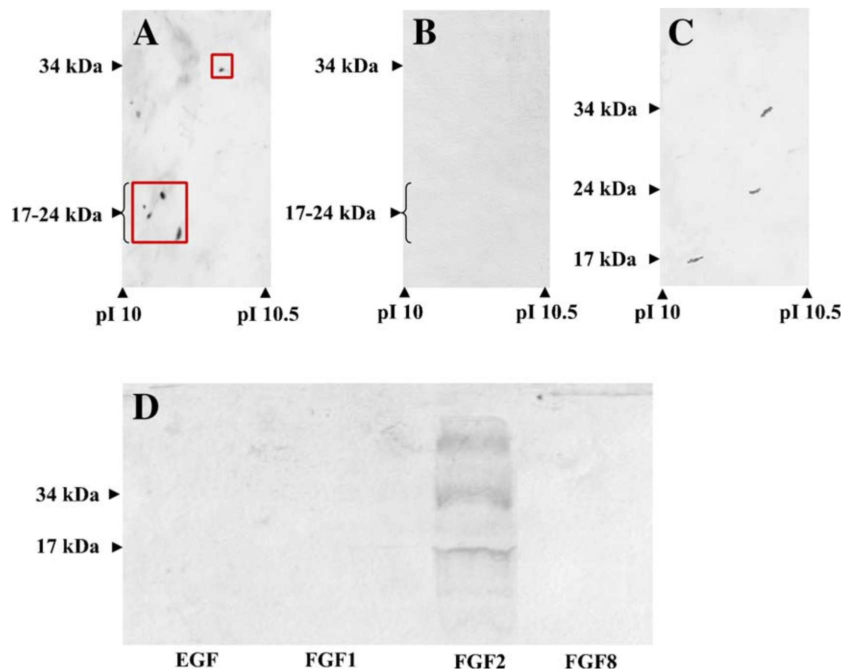


Fig. 1. Western blot analysis from a 2D gel electrophoresis (A, B and C) developed with chemiluminescence to increase sensibility. (A) E-CSF from chick embryos at stage HH25, labeled with monoclonal antiFGF2 antibody showing the presence of diverse isoforms of the FGF2 in E-CSF. (B) E-CSF from chick embryos at stage H.H. 25, labeled with monoclonal antiFGF2 antibody preabsorbed with FGF2 ligand showing the absence of spots labeled in panel A, corroborating the specificity of the label. (C) Embryonic serum from chick embryos at stage HH25, labeled with monoclonal antiFGF2 antibody showing the presence of diverse isoforms of the FGF2 in embryonic serum. (D) Western blot analysis from a SDS-PAGE electrophoresis of several growth factors (indicated at the bottom of the image) labeled with monoclonal antiFGF2 antibody showing no cross-reaction except with FGF2. Molecular weight is indicated on the left side and the isoelectric point (p*I*) appears at the bottom of the images. The red squares in panel A delimit the positive spots for FGF2 of the E-CSF in the different ranges of molecular weight.

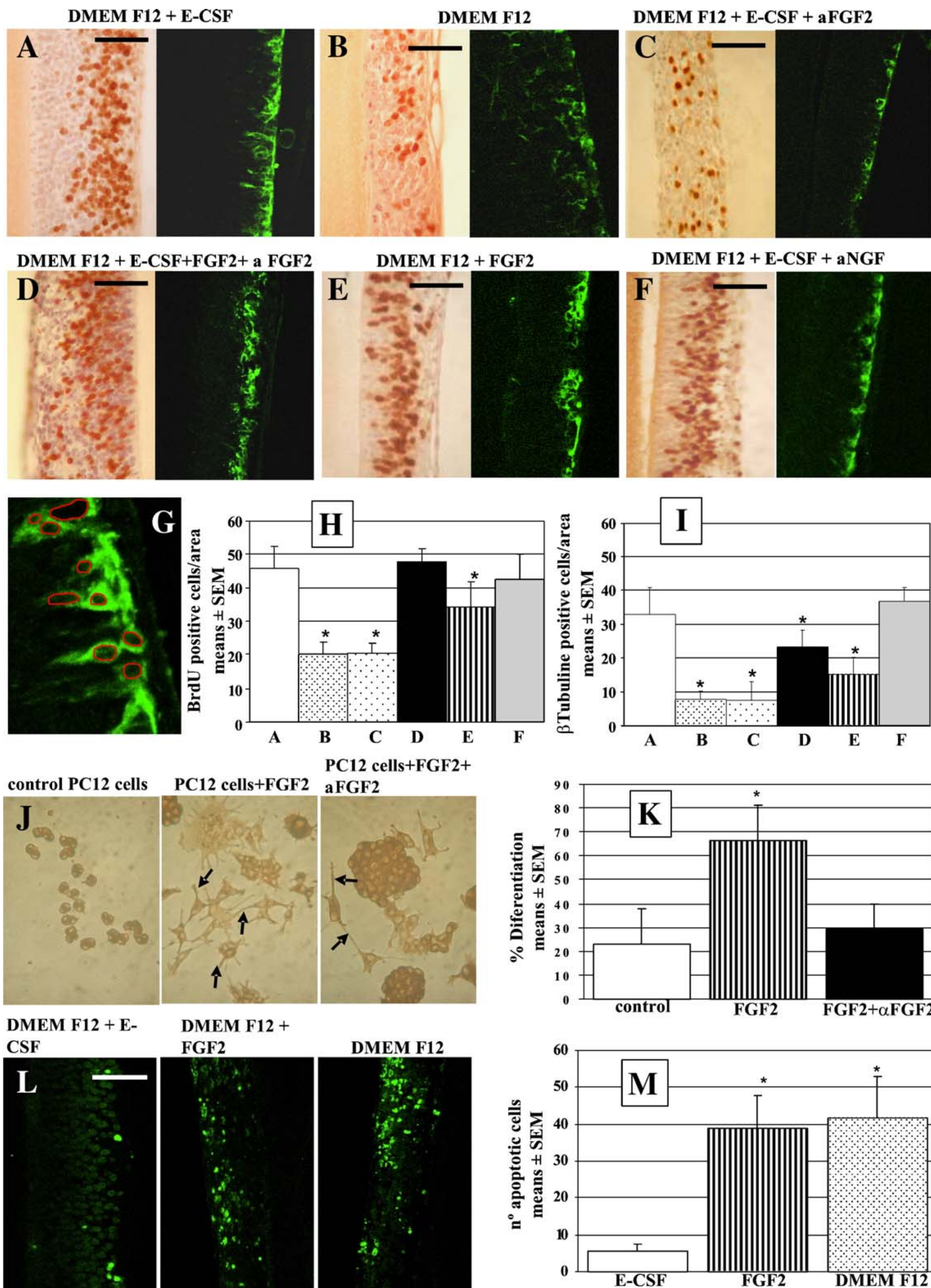
FGF2 contained within chick E-CSF is involved in regulating the behavior of neuroepithelial stem cells

To check whether E-CSF is an alternative source of FGF2 capable of influencing neuroectodermal cell proliferation and differentiation, and thus whether it is one of the active components of this fluid, responsible at least in part for its previously described trophic role, we performed two experiments.

Firstly, mesencephalic organotypic cultures were made in several different conditions (Fig. 2). As a positive control, we used explants cultured with whole E-CSF supplemented medium (Fig. 2A), and, as a negative control, we used explants cultured only in a chemically defined medium (Fig. 2B). As we described in the introduction, the presence of E-CSF in the culture, medium is capable of activating replication and neurogenesis in neuroepithelial cells. To test whether the FGF2 contained within the E-CSF was involved in regulating the behavior of neuroepithelial stem cells, mesencephalic explants were cultured with E-CSF in which this ligand had been specifically immunodeprived by the previous adding of the monoclonal antibody to FGF2. In these organotypic cultures, both BrdU incorporation and β3-tubulin expression

Fig. 2. Analysis of neuroepithelial cell proliferation and neuronal differentiation in transversal histological sections of chick mesencephalic neuroectoderm explanted at HH20 and cultured in vitro for 24 h (i.e., to a developmental stage equivalent to HH23). BrdU incorporation has been monitored with a specific antibody and labeled nuclei are shown in brown. Neuronal differentiation has been monitored by immunostaining with a commercial antibody to β-tubulin, a high magnification image of β-tubulin-positive cells (G) shows the identifying criteria of the β3-tubulin-positive cells which show a negative nucleus (red line) surrounded by a labeled cytoplasm. The different experimental conditions are as follows: (A) Explants cultured in serum-free medium supplemented with E-CSF. (B) Explants cultured in serum-free medium with no supplement; note an intense decrease in replication and neurogenesis with respect to panel A. (C) Explants cultured in serum-free medium supplemented with E-CSF and immunodeprived with an antibody to FGF2; note that immunoblocking of FGF2 in E-CSF also leads to an intense reduction in replication and neurogenesis. (D) Explants cultured in serum-free medium supplemented with E-CSF and with antibody to FGF2 previously saturated by FGF2 ligand; no reduction in replication and neurogenesis is observed showing that FGF2 antibody action (see C) is due to specific blocking of E-CSF FGF2 biological activity. (E) Explants cultured in serum-free medium supplemented with commercial FGF2 human recombinant; note the recuperation of replication and neurogenesis. (F) Explants cultured in serum-free medium supplemented with E-CSF and immunodeprived with a commercial antibody to NGF; this condition has no effect on replication and neurogenesis in neuroepithelial cells. Quantification of BrdU incorporation (H) and quantification of neuronal differentiation (I). The letters at the bottom of each bar correspond with those of the different experimental conditions. Standard error is shown. *Values that differ significantly ($P < 0.001$) from controls, according to the two-tailed Student's *t* test. Image J corresponds to a PC12 cells in vitro culture showing the inhibition of the neuritic growth induction (arrows) by FGF2 ligand, when an FGF2 antibody was added to the culture media. Quantification data are expressed in image K; experimental conditions are indicated at the bottom of each bar. Image L shows apoptotic cells detected by TUNEL method in mesencephalic explants. Note the low number of apoptotic cells in E-CSF supplemented explants and the increase of apoptotic cells in FGF2-supplemented and non-supplemented explants. This demonstrates that FGF2 is not involved in neuroepithelial cell survival. Image M shows the quantification of apoptosis data in different experimental condition (indicated at the bottom of each bar). Standard error is shown. *Values that differ significantly ($P < 0.001$) from controls, according to the two-tailed Student's *t* test. Scale bar in all images: 50 μm.

were drastically reduced, to approximately 50% and 25%, respectively (Figs. 2C, H and I), with respect to the positive control. Interestingly, the number of proliferating and differentiating cells in explants treated with E-CSF plus the anti-FGF2 antibody was similar to that in negative controls, suggesting that the FGF2 present in E-CSF plays a key role in E-CSF biological activity. In order to verify that anti-FGF2 antibody is capable of selectively blocking the biological activity of the FGF2 ligand, we repeated the previous experiment but adding to the E-CSF supplemented medium anti-FGF2 antibody which had been exposed beforehand to the exogenous FGF2 ligand (Fig. 2D); the results showed the recuperation of proliferation and neurogenesis values similar to those of the controls (Figs. 2H and I). Similarly, and so as to verify the specificity of the blocking of FGF2 biological activity by the anti-FGF2 antibody we employed, we performed complementary experiments with



in vitro cultured PC12 cells, as shown in Fig. 2J; the PC12 cells underwent differentiation in the presence of FGF2, triggering the emission of neurites, a process almost totally inhibited when the anti-FGF2 antibody was added to the FGF2-supplemented culture medium (Fig. 2K). These data allow us to affirm that the action of the anti-FGF2 antibody is attributable to its capacity to block the biological activity of the FGF2 ligand.

Subsequently, and to corroborate these results, mesencephalic neuroepithelial explants were cultured in serum-free medium supplemented with commercial FGF2 (Fig. 2E). The rate of BrdU incorporation and neuronal differentiation reached elevated values (Figs. 2H and I), albeit not so high as those of the positive controls; what is more, although the differences observed were statistically significant, the number of BrdU and β3-tubulin-positive cells was nearly double that of both explants cultured with the medium supplemented with E-CSF and immunodeprived with anti-FGF2 antibody, and negative controls (Figs. 2B and C).

To discard any unspecific effect due to the addition of the antibody to the culture medium, mesencephalic neuroepithelial explants were cultured in E-CSF supplemented medium to which a commercial antibody to NGF was added (Fig. 2F). We used this antibody as a negative control since it has been reported that NGF does not act on brain development at these developmental stages (Ford-Perriss et al., 2001; Panchision and McKay, 2002). In these organotypic cultures, the rate of BrdU incorporation and neuronal differentiation reached the same level as in positive controls (Figs. 2H and I).

Finally, to rule out the possibility of the influence of FGF2 on replication and differentiation of the neuroepithelial cells being due to an indirect action on cell survival, we conducted a study of the presence of apoptotic cells on the in vitro cultured neuroepithelial explants. The results demonstrate (Figs. 2L and M) that the addition of FGF2 to the culture medium does not significantly reduce the number of apoptotic cells in respect of the explants cultured in a defined medium; however, the presence of apoptotic cells was scarce in the explants with E-CSF added to the culture medium. These data indicate that FGF2 does not have a direct influence on cell survival, and that this might be controlled by other factors present in the E-CSF.

Secondly, to check whether FGF2 from the E-CSF is able to influence neuroectoderm proliferation and differentiation in vivo, 20 H.H. chick embryos were microinjected in the brain cavity with the monoclonal antibody to FGF2 to selectively block this ligand in vivo, with the FGF2 ligand and alternatively with a commercial antibody to NGF as a control for immunodeprivation (see above); following 24 h culture, no macroscopic malformations were appreciated in the embryonic brain under any experimental condition. Embryos microinjected with the antibody to FGF2 showed a reduction in the number of BrdU-positive nuclei to approximately 40% with respect to the controls, a statistically significant finding (Figs. 3A, B and J); however, the embryos microinjected with the FGF2 ligand and antibody to NGF did not show any significant variation in the number of BrdU-positive nuclei (Figs. 3C, D and J).

With respect to neurogenesis, embryos microinjected with the antibody to FGF2 showed a reduction in the number of β3-tubulin-immunoreactive cells to approximately 50% with respect to the controls, again statistically significant (Figs. 3E, F and K); yet embryos microinjected with FGF2 ligand presented a significant reduction with respect to the controls, but their values were higher tan those of the embryos microinjected with the anti-FGF2 antibody (Figs. 3G and K). The antibody to NGF did not show any significant reduction in the number of β3-tubulin-immunoreactive cells (Figs. 3H and K).

Finally, to test whether FGF2 immunodeprivation affects only the ligand contained within the E-CSF or also the ligand contained within the neuroepithelial tissue, the distribution of the antiFGF2 antibody microinjected into the brain cavity was analyzed 24 h after injection. As shown in Fig. 3I, the microinjected antibody to FGF2 was confined within the brain cavity, suggesting that FGF2 immunodeprivation only affects the ligand contained within the E-CSF.

These data indicate that, in chick embryos, the FGF2 contained within E-CSF exerts a mitogenic and differentiating role on the neuroectodermal cells both in vitro and in vivo.

Origin of E-CSF FGF2

To throw light on the embryological origin of the FGF2 detected within the chick E-CSF, i.e., to determine whether the neuroectoderm is the source of the FGF2 contained within E-CSF, or whether, instead, it comes at least in part from any other embryonic tissue or organ, we carried out a study of *fgf2 mRNA* expression by in situ hybridization at developmental stages HH13 to HH27, and semiquantitative PCR analyses of different embryonic tissues of H.H.25 chick embryos were performed.

Fgf2 in situ hybridization analysis showed that none of the known isoforms of this gene could be clearly detected at any site on the neuroectoderm in all developmental stages analyzed, i.e., from HH13 to 27 (Figs. 4A, D, G and data not shown). It is important to note that in some in situ hybridizations, a diffuse NBT/BCIP precipitate in the neuroepithelium was observed (not shown), although this was hard to interpret as a positive hybridization.

Despite this apparent low level of *fgf2* expression in the neuroectoderm, this gene was clearly expressed at other distinct sites of interest to us. At HH18, *fgf2* started to be expressed in the notochord, the rope-shaped structure that it is known to be responsible for the secretion of distinct signaling molecules (Figs. 4A, B and C). *Fgf2* expression in the notochord was detected from H.H.18 to H.H.27. The intensity of this structure's staining increased notably with growth, and at stage 23 H.H. it was much greater than that of other structures (Figs. 4D, E and F). *Fgf2* expression was also detected in the mesonephros from H.H.13 to H.H. 27 (Figs. 4C, F, and data not shown), and in a portion of the liver primordium, an organ that is known to supply different molecules and cells to the embryonic serum, in a location close to the ductus venosus, from HH22 to 27 (Fig. 4F, and data not shown). In addition, *Fgf2* expression was detected in other embryonic sites (data not

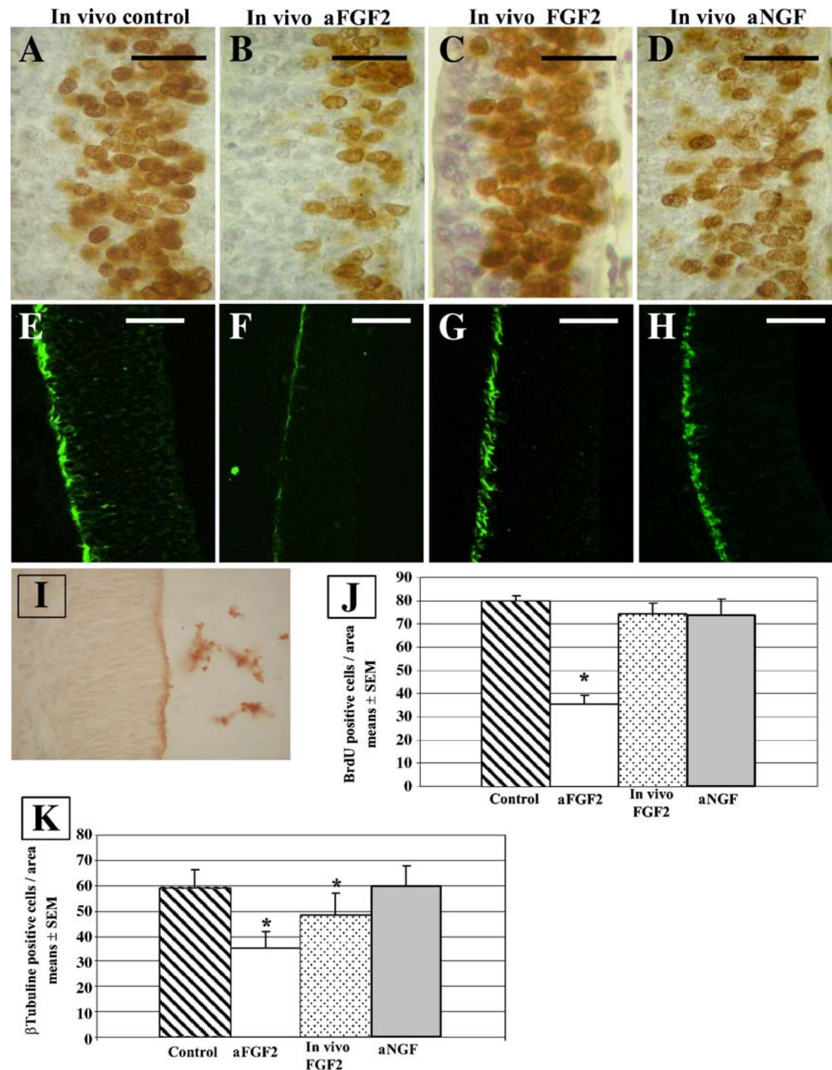


Fig. 3. Transversal histological sections and quantitative analysis of BrdU incorporation and neuronal differentiation in the mesencephalic neuroectoderm of embryos at HH23, maintained in ovo 24 h after microinjection in the brain cavity. BrdU incorporation has been monitored with a specific antibody and is shown in brown. Neuronal differentiation has been monitored by immunostaining with a commercial antibody to β-tubulin and is shown in green fluorescence. Experimental conditions are indicated at the top of the cultures or at the bottom of each bar plot. (A and E) In vivo control microinjected with Hank saline, (B and F) microinjection of anti-FGF2 antibody; (C and G) microinjection of FGF2 ligand and (D and H) microinjection of anti-NGF antibody. Note the decrease in BrdU incorporation and neuronal differentiation in the neuroepithelium of embryos after the immunoblocking of FGF2 in the E-CSF (B and F), while FGF2 ligand microinjection (C and G) or microinjection of another antibody does not produce any alteration. In panel G, it is shown as the anti-FGF2 antibody microinjected into the midbrain cavity, detected by immunolabeling with a secondary antibody marked with peroxidase; it is confined to the cavity without penetrating the neuroepithelium. Quantification of BrdU incorporation (J) and quantification of neuronal differentiation (K) Standard error is shown. *Values that differ significantly ($P < 0.001$) from controls, according to the two-tailed Student's t test. Scale bar in panels A, B, C and D: 30 μm. Scale bar in panels E, F, G and H: 50 μm.

shown): i.e., in the mesoderm close to the lens during eye development, from HH18 to 27, in the developing heart, in the foregut, and in the limb bud.

Due to the presence of very faint NBT/BCIP precipitate in the neuroectoderm in some hybridizations, and as other authors had reported the expression of *fgf2* in some neuroectodermal tissues, such as the mesencephalon and the spinal cord, when radiolabeled riboprobes were used, a semiquantitative PCR analysis of different portions of the neuroectoderm (i.e., mesencephalon and spinal cord) was performed for different *fgf2* isoforms. The result was then compared with a semiquantitative PCR analysis of the tissues where *fgf2* expression was clearly detected in our in situ hybridizations (i.e., the notochord and the liver and mesonephros area). This analysis clearly showed that different LMW and HMW *fgf2* isoforms were expressed in the notochord and in the liver and mesonephros area (Fig. 4H), as well as in the neuroectodermal tissue of the mesencephalon and the spinal cord. The relative level of *fgf2* expression in the tissues analyzed with respect to actin control detected by this semiquantitative PCR amplification allows us to suggest that, in chick embryos, *fgf2* is expressed at much higher levels in the notochord and in the liver and mesonephros area than in the neuroectoderm, which is in agreement with the results obtained by in situ hybridization.

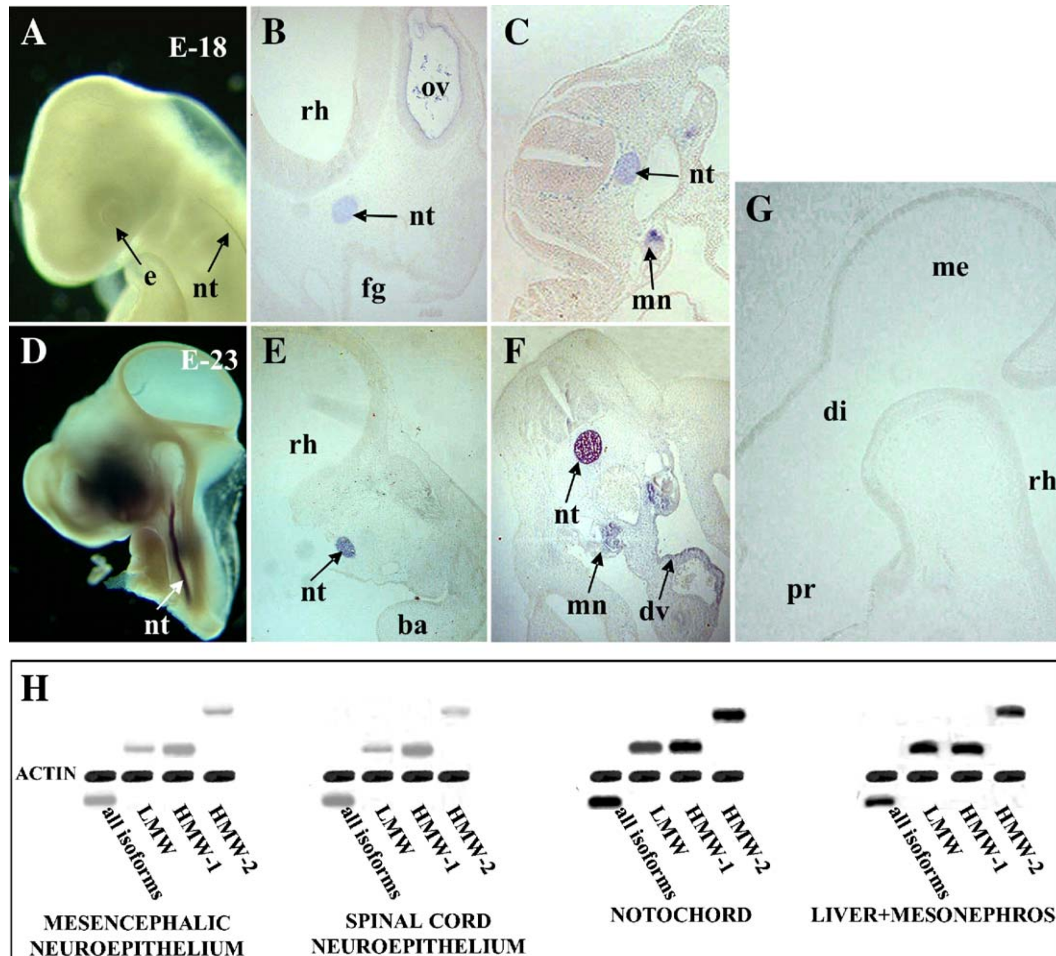


Fig. 4. *Fgf2* in situ hybridization on whole-mount chick embryos at different developmental stages. (A) Whole-mount embryo at H.H.18. (B) Transversal section of the embryo depicted in A at the level of the rhombencephalon and otic vesicle. (C) transversal section of the embryo depicted in panel A at the spinal cord level. (D) Whole-mount embryo at H.H.23. (E) Transversal section of the embryo depicted in panel D at the level of the rhombencephalon and first branchial arch. (F) Transversal section of the embryo depicted in panel D at the spinal cord level. (G) Sagittal section of an embryo at H.H. 23 stage showing the brain vesicles. Note the scarce or non-existent *mRNA* expression at different levels of neuroepithelium and the increase in *mRNA* expression of *fgf2*, with development particularly in the notochord, mesonephros and ductus venosus of the hepatic primordium. (G) *Fgf2 mRNA* semiquantitative PCR analysis on several different embryonic tissues: mesencephalon, spinal cord, notochord, and liver plus mesonephros, in order to show quantitative differences in the levels of *fgf2 mRNA* expression among the different tissues. *Actin mRNA* expression has been included as a reference parameter. Abbreviations: ba, branchial arch; dv, ductus venosus; e, eye; fg, foregut; pr, prosencephalon; di, diencephalon; me, mesencephalon; rh, rhombencephalon; mn, mesonephros; nt, notochord; ov, otic vesicle; LMW, low molecular weight isoform; HMW, high molecular weight isoforms.

The results obtained with neuroepithelial explants cultured with a chemically defined medium, together with those of immunodeprivation of FGF2 in the E-CSF, both in vitro and in vivo, and also the low *fgf2* expression detected in the neuroepithelium as compared with other embryonic tissues and organs, indicate that neuroepithelial FGF2 production is not able to self-induce a normal level of neuroepithelial cell proliferation and neurogenesis; in addition, these findings suggest that the FGF2 contained within the E-CSF may be produced at least in part by extraneural tissues and then shifted into the E-CSF via the embryonic serum. To support this hypothesis, we contemplate three conditions. (1) The extra-neural tissues which express *fgf2* should be capable of liberating soluble forms of FGF2. (2) FGF2 should be present in the embryonal serum. (3) FGF2 should be able to pass from the serum to the E-CSF via the neuroepithelium.

In order to test the first point, we made cultures of fragments of cephalic notochord from stage 25 H.H. chick embryo over a period of 24 h in a defined medium. A Western blot analysis of this medium (Fig. 5A) revealed the existence of diverse isoforms of FGF2 coinciding with the normal molecular weights, thus confirming that at least one of the tissues which expressed FGF2 is capable of liberating this as a soluble factor. In addition, and with the aim of checking whether this liberated FGF2 is able to directly influence the behavior of the neuroepithelial cells, cultures were made of neuroepithelial explants in a notochord-conditioned medium. Results demonstrated that the defined medium conditioned with notochord is able to activate neuroepithelial cell replication up to three times more than the value for the explants cultured in a medium not conditioned with notochord (Figs. 5B, D and H) and induce a high degree of neurogenic activity in the neuroepithelial cells,

five times superior to the value for the explants cultured in a non-conditioned medium (Figs. 5 C, E and I). Both effects drastically diminished when the FGF2 antibody was added to the conditioned medium (Figs. 5 F, G, H and I), as a result of which we conclude that this is due to the presence of FGF2.

As regards the second point, we performed a Western blot analysis of embryonic serum and E-CSF with an antibody to FGF2 after a bidimensional electrophoresis. This analysis showed the presence of 34, 24 and 17 kDa antiFGF2-immunoreactive spots in both the E-CSF and the embryonic serum (Figs. 1A and C).

To test the third point, commercial FGF2 was coupled to FITC and microinjected into the outflow of heart of chick embryos at HH24. The E-CSF from these embryos was recovered at HH27 and run in a SDS-PAGE.

Commercial FGF2 coupled to FITC before injection appears as a 34-kDa band on the Western blot recognized by both the anti-FGF2 antibody (Fig. 6A) and the anti-FITC antibody (Fig. 6B), which indicates that the FGF2-FITC union is stable.

In Fig. 6B appears another band of 66 kDa which corresponds to the bovine seroalbumin bound to FITC, used by the suppliers to stabilize the FGF2 ligand.

Twenty-four hours following intravascular microinjection of FGF2-FITC, the Western blot of the E-CSF revealed a band of 34 kDa labeled with both the anti-FGF2 antibody (Fig. 6C) and the anti-FITC antibody (Fig. 6D). This indicates that the FGF2-FITC injected in the vascular system of the embryo passes to the E-CSF, while a protein of higher MW (albumin) does not, suggesting that certain proteins could pass from the embryonic serum to the E-CSF in accordance with their molecular weight.

To analyze the effect of protein size on this favored uptake, a fusion protein not existent in chick but with a similar molecular mass to FGF2 was also coupled to FITC and microinjected into the outflow of heart of chick embryos at H.H.24 (GST-Adh, predicted MW = 38 kDa). Preinjection solution of GST/Adh-FITC was detected by Western blot as a band of 38 kDa both with the anti-GST antibody (Fig. 6E), and with the anti-FITC antibody (Fig. 6F). This indicates that the GST/Adh-FITC union was stable. Twenty-four hours after intravascular microinjection of the GST/Adh-FITC protein, the Western blot of the E-CSF showed no band with any of the two antibodies we employed (Figs. 6 G and H), suggesting that E-CSF uptake of this fusion protein from the embryonic serum was not favored.

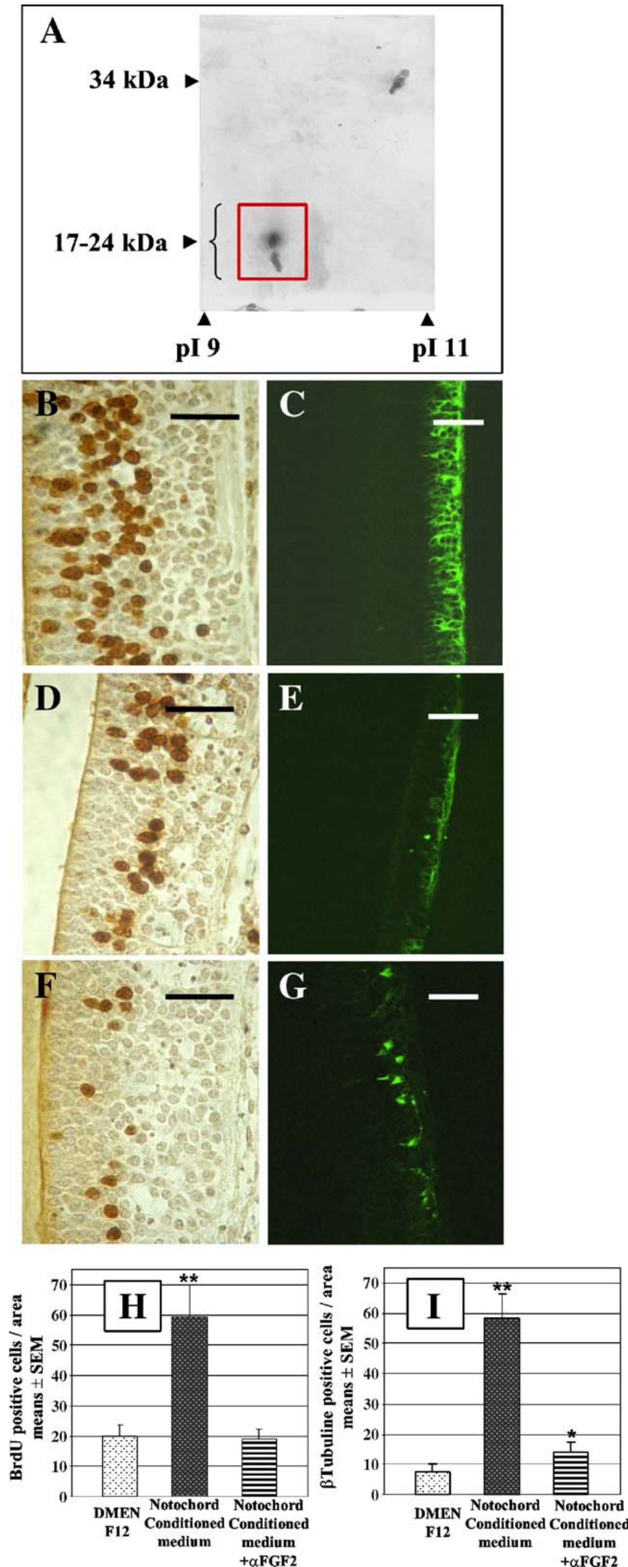


Fig. 5. (A) Western blot analysis of defined medium conditioned with notochord developed with monoclonal antiFGF2 antibody from 2D gel electrophoresis, chemiluminescence was used to increase sensibility. Molecular mass is indicated on the left-hand side, and the isoelectric point (p*I*) appears at the bottom of the image. Analysis of neuroepithelial cell proliferation and neuronal differentiation in transversal histological sections of chick mesencephalic neuroectoderm explanted at H.H.20 and cultured in vitro for 24 h (i.e., to a developmental stage equivalent to H.H.23). BrdU incorporation has been monitored with a specific antibody and labeled nuclei are shown in brown. Neuronal differentiation has been monitored by immunostaining with a commercial antibody to β-tubulin and positive cytoplasms are shown as green fluorescence. The different experimental conditions are as follows: (B and C) Explants cultured in a defined medium conditioned with notochord. (D and E) Controls explants cultured in a defined medium without supplement. (F and G) Explants cultured in a defined medium conditioned with notochord plus FGF2 antibody. BrdU-positive cells quantification (F) and neurogenesis quantification (G) has been plotted and experimental conditions are indicated at the bottom of each bar. Note the increase in proliferation and neurogenesis induced by the notochord-conditioned medium. Standard error is shown. Values that differ significantly ($*P < 0.05$, $**P < 0.001$) from controls, according to the two-tailed student's *t* test. Scale bar in panels B and D: 30 μm. Scale bar in panels C and E: 50 μm.

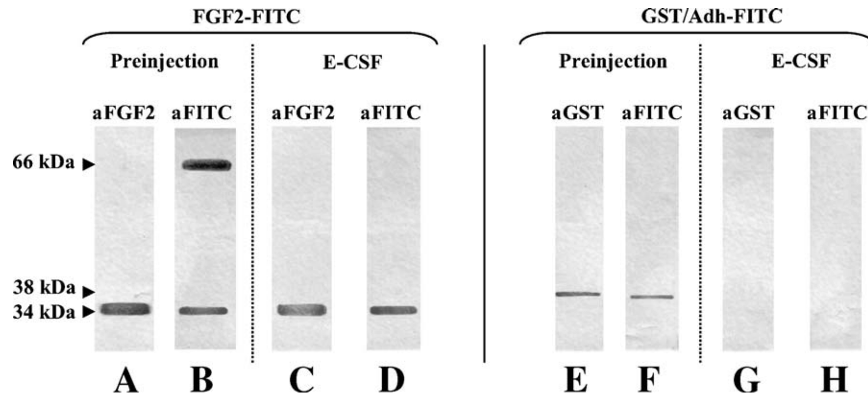


Fig. 6. Western blot analysis from SDS-PAGE electrophoresis of commercial FGF2 ligand and glutathione-*S*-transferase-alcohol–dehydrogenase linked to FITC. Both molecules were immunolabeled by an antibody against to specific protein or with an antibody against FITC either in solution (preinjection) or in E-CSF 24 h. after intravascular microinjection. The FGF2 antibody detected a 34-kDa band (A) and a similar band was also detected by the anti-FITC antibody (B) in FGF2 preinjection solution, showing that FGF2-FITC linkage was successful. A 66-kDa band was recognized by anti-FITC antibody (B) which corresponds to seroalbumin used to stabilize the commercial FGF2. After intravascular microinjection of FGF2-FITC a 34-kDa band was detected in E-CSF by both anti-FGF2 (C) and anti-FITC (D) antibodies, showing that FGF2-FITC, passes via the neuroepithelium from the serum to the E-CSF; however, seroalbumin does not. GST-Adh-FITC protein was labeled in preinjection solution by both, anti-GST (E) and anti-FITC (F) antibodies as a 38-kDa band, showing that the GST-Adh-FITC linkage was successful. After intravascular microinjection of GST-Adh-FITC protein, no band was detected in E-CSF either by the anti-GST (G) or the anti-FITC (H) antibodies, showing that proteins with a similar molecular weight to FGF2 does no pass from the serum to the E-CSF.

These findings suggest that the FGF2 is capable of selective incorporation from the embryonic serum to the E-CSF in chick embryos, whereas other proteins of a similar molecular weight are incapable of behaving in the same way.

Discussion

The results of our study demonstrate the following: (1) different isoforms of FGF2 are present in the CSF and serum of chick embryos in early stages of development; (2) in this species the FGF2 in E-CSF is directly involved in both the in vivo and in vitro proliferation and neural differentiation of neuroepithelial cells; (3) neuroepithelial cells express low levels of *fgf2 mRNA* and the FGF2 from serum is incorporated into the E-CSF, suggesting that some FGF2 contained within E-CSF may have an extraneural origin.

FGF2 is an important active component of E-CSF

Embryonal CSF has a complex protein composition which differs from that of the adult both in terms of quality and quantity; also, it has been suggested that it might play diverse biological roles during brain development (Birge et al., 1974; Fielitz et al., 1984; Checiu et al., 1984; Dziegielewska et al., 2000; Miyan et al., 2003; Gato et al., 2004; Parada et al., 2005b). In this regard, we have recently demonstrated that E-CSF exercises an important trophic effect in vitro on neuroepithelial cells during early chick embryo development (Gato et al., 2005), despite the fact that the molecules involved are still to be identified. In this paper, we show that chick embryo E-CSF contains different isoforms of FGF2 which could influence the behavior of the neuroepithelial cells; this factor is considered the main instrument involved in activating replication and neurogenesis from neural stem cells (Vaccarino et al., 1999b; Panchision and McKay, 2002). Flamme et al. (1919) and Ribatti et al. (1995), had previously described a similar role of FGF-2 via a liquid medium on chick embryo chorioallantoic fluid, in which it activates vasculogenesis of the chorioallantoic membrane. In addition, the presence of FGF-2 or FGF2-like activity has been described in adult CSF, where it has been attributed a role in neuronal survival and in neurogenesis from stem cells (Bruni, 1998; Nicholson, 1999; Reuss and von Bohlen und Halbach, 2003); also, alterations in the concentration and/or bioavailability of this factor in CSF have been cited in patients with neurodegenerative disorders or brain injuries (Patterson et al., 1993; Tooyama, 1993; Hanneken et al., 1995; Yosimoto et al., 1997; Stopa et al., 2001).

We have detected distinct isoforms of FGF2 (34 and between 17 and 24 kDa) in the E-CSF of chick embryos which coincide with the molecular weights described for this factor in CNS (Reuss and von Bohlen und Halbach, 2003). The presence of these molecules in E-CSF suggests soluble forms of this factor, and although it has been proposed that in the brain embryo the LMW forms of FGF2 act according to a paracrine mechanism while those of HMW follow an intracrine mechanism (Vaccarino et al., 1999b; Reuss and von Bohlen und Halbach, 2003), our results show that such isoforms may also work as soluble molecules from the E-CSF. Moreover, FGF2 is considered a heparin binding growth factor, and it is known that certain proteoglycans such as heparan sulphate (Ruoslahti and Yamaguchi, 1991; Nurcombe et al., 1993; Guimond and Turnbull, 1999; and Sperinde and Nugent, 2000) and chondroitin sulphate (Milev et al., 1998) have a direct influence on its level of biological activity, favoring extracellular transportation, and protecting it from degradation by means of proteases, as well as helping to activate receptors. In this regard, we have previously demonstrated the presence of diverse sulphated proteoglycans in E-CSF (Gato et al., 1993; (Alonso et al., 1998; Parada et al., 2005a), which suggests that the activity of the FGF2 from the E-CSF could have complex regulating

mechanisms in which other molecules such as proteoglycans play a role.

These results suggest that an extraneuroepithelial source of FGF2 might participate in regulating the behavior of neural stem cells during the development of the embryo.

The FGF2 in E-CSF is involved in controlling neuroepithelial replication and neurogenesis

In this study, we show that selective in vitro and in vivo immunoblocking of the biological activity of the FGF2 in E-CSF at early stages of development, notably reduces the trophic effect of this fluid on the neuroepithelium which we recently described (Gato et al., 2005), thereby significantly diminishing mitotic activity and neurogenesis. However, our neuroepithelial cell apoptotic study show that FGF2 does not play a key role in cell survival, which could be controlled by other E-CSF factors.

In order to demonstrate that these effects are not due to an immunoblocking of FGF2 biological activity in the neuroepithelium itself, we have verified that the anti-FGF2 antibody injected into the embryonal brain cavity does not penetrate the neuroepithelial tissue but remains confined to the cavity of the brain vesicles; this ensures the specificity of the antibody acting on the FGF2 of E-CSF. In this way, we show that, in vitro, disruption of replication and neurogenesis in the neuroepithelium cultured in a defined medium is to a large extent compensated for by the exogenous addition of FGF2. These data suggest, therefore, that the FGF2 is a key component in the trophic action of E-CSF on neuroepithelial stem cells.

In fact, FGF2 is considered a trophic factor involved in the behavior of CNS stem cells, on which it exerts a strong mitogenic and neurogenetic influence both in embryonal and adult stages. It has been demonstrated that during the development of the embryo (above all, in in vitro studies) FGF2 has an intense mitogenic effect on neuroepithelial stem cells and is capable of triggering neurogenesis (Murphy et al., 1990; Tropepe et al., 1999; Vaccarino et al., 1999b; Raballo et al., 2000; Panchision and McKay, 2002; Reuss and von Bohlen und Halbach, 2003). In this regard, our findings provide experimental evidence of these actions of FGF2 in vivo on the neuroepithelial cells of chick embryos in early stages of development, this accord with the intense reduction in replication and neurogenesis described in the neuroepithelium of FGF2 knockout mice (Raballo et al., 2000). Despite the references in these processes to other factors such as EGF and IGF1 (Drago et al., 1991; Tropepe et al., 1999), the general opinion is that during early developmental stages neuroepithelial stem cell proliferation and differentiation mainly depend on FGF2. Similar effects have been described for FGF2 on neural progenitor cells in postnatal and adult stages (Tao et al., 1996, 1997; Wagner et al., 1999; Cheng et al., 2001), although it has also been attributed a role as a neuroprotector, in the repair of CNS lesions (Patterson et al., 1993; Yoshimura et al., 2003; Reuss and von Bohlen und Halbach, 2003), and disruptions have been described of FGF2 levels in the cerebrospinal fluid among patients with neurodegenerative disorders (Stopa et al., 2001).

The existence of a specific spatio-temporal pattern of fgf2 expression in the neuroepithelium of rodent embryos has been shown in various studies (Vaccarino et al., 1999a,b; Dono et al., 1998; Dono, 2003). Our findings show that fgf2 mRNA expression in the CNS of chick embryos detected by means of in situ hybridization at the analyzed stages is much more lower than that detected in other embryonic structures, such as the notochord and the mesonephros. Such differences have been confirmed by semiquantitative PCR, which allows us to make a comparative estimation of the fgf2 expression levels. In fact, fgf2 expression by both in situ hybridization and immunochemistry on the neuroepithelium of avian embryos is controversial because, despite most authors have shown a very low fgf2 expression or not expression at all in the CNS (Han, 1997; Savage and Fallon, 1995, on chick embryos; and Kalcheim and Neufeld, 1990, on quail embryos), other authors have described the presence of FGF2 in the telencephalon and mesencephalon of chick embryos at HH17 (Dono, 2003). However, the lack of histological section in this latter report makes very difficult to evaluate the tissular location and the intensity of FGF2 immunochemistry. In this way, our data support the idea that in chick embryos fgf2 exhibits a low level of expression in the neuroepithelium, and thus, this difference with respect to rodents suggests the existence of phylogenetic variations.

The different members of the family of FGFs act via different types of receptors, although it is reported (Tropepe et al., 1999) that the action of FGF2 on the early development of the CNS is mediated mainly by FGFr1 and 2. These receptors are expressed differentially in the neuroepithelium of chick and rodent embryos, with FGFr1 presenting a more ubiquitous pattern than FGFr2 (Wilke et al., 1997; Walshe and Mason, 2000; Trokovic et al., 2005); however, certain authors have described a greater concentration of FGFr for both avian and rodent embryos in those regions of the neuroepithelium which are close to the ventricular cavities in contact with E-CSF (Heuer et al., 1990; Raballo et al., 2000).

The coincidence in FGF2 expression and that of its receptors in the neuroepithelium has led to a theory of autocrine–paracrine behavior on the part of FGF2 during the development of the CNS. However, previous studies we carried out (Gato et al., 2005) have demonstrated that mesencephalic neuroepithelia of chick embryos cultured in a chemically defined medium without external supplements are incapable of maintaining normal levels of proliferation and neurogenesis during a short 24-h period. This indicates that intrinsic factors in the developing brain are insufficient to maintain normal levels of proliferation and neurogenesis, suggesting that factors outside the neuroepithelium are involved in regulating these processes; this coincides with the findings of Drago et al. (1991) and Raballo et al. (2000), in that an exogenous contribution of FGF2 is required in the regulation of neuroepithelial cell behavior.

In this regard, our results show that in chick embryos E-CSF is an exogenous source of FGF2, and the specific immunoblocking of this factor's biological activity on E-CSF considerably reduces both the in vivo and in vitro rate of

replication and neuroepithelial neurogenesis, showing that the FGF2 of E-CSF has a direct influence on the behavior of neuroepithelial cells at early stages of development. These findings are coherent with the apical positioning of the FGF2 and its receptors described by Raballo et al. (2000), who suggest that these cells could incorporate FGF2 from the cerebrospinal fluid itself.

The abovementioned data suggest the existence of two simultaneous routes by which FGF2 acts on the neuroepithelium during chick brain development: one of an autocrine–paracrine mechanism within the neuroepithelium itself and the other an exogenous source in E-CSF. One possible explanation of this is given in the studies carried out by Qian et al. (1997) and Raballo et al. (2000), who suggest that neurogenesis from neuroepithelial progenitor cells requires low concentrations of FGF2; as a result, those cells influenced only by the low levels of FGF2 from inside the neuroepithelium, namely, those situated at its basal portion, would be subject to neural differentiation, while the cells capable of proliferation are situated near to the apical side, that is, next to the cerebral cavity, where they can receive the influence of the FGF2 from the E-CSF.

Finally, the presence of FGF2 in the cerebrospinal fluid is constant both during brain development and in the postnatal period, in which it plays an important physiological role and is involved in the physiopathology of certain neurodegenerative disorders. In this paper, we show that the FGF2 of embryonal cerebrospinal fluid also play a key role in the development of the CNS.

The extraneural origin of E-CSF in the embryonal cerebrospinal fluid

Taking into consideration both the fact that our results reveal low levels of *fgf2* expression in the neuroepithelium of the brain primordium of chick embryos, when compared with other extraneural structures, and, as we established previously, that the neuroepithelium seems insufficient to maintain normal levels of proliferation and neurogenesis, we suggest that the FGF2 from E-CSF might have an extraneural origin.

In order to test this hypothesis, we have attempted to check whether extraneural primordia (notochord) are capable of liberating diffusible and active forms of FGF2, if this factor is present in the serum of embryos during the stages we studied and if it is able to pass from the serum to E-CSF.

In this paper, we demonstrate that during the initial phases of chick embryo development, there are different structures, such as the hepatic primordium, the mesonephros and, above all, the notochord, which present much higher levels of FGF2 expression than the neuroepithelium itself. Moreover, we have seen that the notochord liberates different soluble isoforms of FGF2, which are capable of activating the replication and neurogenesis in vitro of neuroepithelial cells. Such findings are consistent with previous studies demonstrating the capacity of chick embryo notochord to produce FGF2 (Hebrok et al., 1998); what is more, ectopically implanted notochord induces in vivo an increase in the mitotic activity of the neuroblasts in chick spinal cord (van Straaten et al., 1989).

Secondly, we have observed that HMW and LMW isoforms of FGF2 are present in the serum of chick embryos at very early stages of development (E 25 H.H.), which demonstrates the existence of diffusible forms of FGF2 circulating, the origin of which could be in the structures with high levels of mRNA expression of *fgf2*, permitting it to act remotely on target cells.

Finally, we demonstrate that the FGF2 stained with exogenous FITC injected into the embryonic vascular system passes to the E-CSF in a short space of time, while other proteins of the same or greater molecular weight are incapable of this process. Considering that at these stages of development the cavity of the embryonic brain is a physiologically sealed system, surrounded exclusively by neuroepithelial cells in which the choroid plexuses are yet to develop, we may affirm that the FGF2-FITC has selectively traversed the neuroepithelium in a basal–apical direction. These data suggest that there might exist within the neuroepithelium some type of specific transportation mechanism for this molecule, a matter which requires further investigation. In adults, it has been proven that the choroid plexuses are responsible for regulating the concentration of FGF2 levels in cerebrospinal fluid (Stopa et al., 2001). However, in early phases of embryonic development, when the choroid plexus are undeveloped or not yet functional, the neuroepithelium itself might assume this function. In this regard, the existence has been shown of a mechanism that selectively internalizes specific proteins in the E-CSF of chick embryos (Moro and Uriel, 1981), which testifies to the transportation capacity of this epithelium.

This paper contributes new ideas regarding the regulation and differentiation of neuroepithelial cells in early stages of chick embryo development, confirming the important trophic role, previously described by us, of E-CSF in this species, and providing evidence that FGF2 plays a key role in this action.

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