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Constitutive expression of FGF4 disrupts the development of the eye and the anterior CNS during mouse embryogenesis, but does not influence the expression of *shh* in these areas

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ABSTRACT. The biological consequences of constitutive *fibroblast growth factor-4 (fgf4)* expression have been analysed during anterior CNS development of mouse chimeric embryos. Severe mutant embryos exhibit exencephaly, absence of eye development and anomalous differentiation of the neuroepithelium. These embryos also show ectopic limb buds resembling the early phases of limb development. Because our results show that anterior CNS in those chimeric embryos does not express *shh* ectopically, we suggest that malformations may be due to interference between the ectopic expression of *fgf4* in the cephalic area and the receptors for the members of the FGF family that regulate brain and eye development, namely *fgf8*. If this is correct, the results indirectly support the crucial role of *fgf8* in patterning the anterior CNS.

KEY WORDS: Fibroblast Growth Factors, CNS development, eye development, mouse chimeric embryos, FGF4 gain of function.

INTRODUCTION

Inductive interactions are fundamental to the development of multicellular organisms. Among the different types of molecules involved in these interactions, the Fibroblast Growth Factor family (FGFs) has been shown to have multiple roles during embryogenesis, including the formation of skeletal structures and patterning of the limbs (reviewed by TABIN, 1995; and WILKIE et al., 1995) and brain (CROSSLEY et al., 1996; MEYERS et al., 1998; SHAMIM et al., 1999; LIU et al., 1999). The *fgf* gene family encodes a group of ligands that promote the growth and differentiation of many mesoderm and ectoderm cell types by binding to specific receptors (*fgfrs*) with a broad range of affinities (reviewed by BASILICO &MOSCATELLI, 1992; BAIRD, 1994; WILKIE et al., 1995).

Mutations and anomalous expression of both ligands and receptors in humans have been related to different syndromes that affect different structures including the head. The evidence for the distinct roles of FGFs in regulating the growth and patterning of the vertebrate embryo comes from their distinct patterns of expression during embryogenesis and from the generation of null mutations of several *fgfs* and their receptors in mice (MANSOUR et al., 1993; WERNER et al., 1993, 1994; HÉBERT et al., 1994; PETERS et al., 1994; FELDMAN et al., 1995; MEYERS et al., 1998) and experimental manipulations of chick limb buds (NISWANDER et al., 1993, 1994; COHN et al., 1995) and cephalic vesicles (CROSSLEY et al., 1996; LEE et al., 1997; SHAMIN et al., 1999).

An alternative approach to understanding *fgf* functions during mammalian development is to prepare chimeras between wild-type embryos and pluripotential embryonic stem cells (ES cells) that harbour regulatory mutations in the FGF system. One of the members of the *fgf* family with key roles in embryonic patterning is *fgf4*. The expression of *fgf4* and its putative receptors has been described during mouse embryogenesis (NISWANDER & MARTIN, 1992; ORR-URTREGER et al.,

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1991; YAMAGUCHI et al., 1992; ORR-URTREGER et al., 1993). For example, null mutations affecting *fgf4* are lethal in homozygotes just after implantation (FELDMAN et al., 1995), indicating the crucial role of this molecule during the first steps of mammalian development. On the other hand, ABUD et al. (1996) obtained chimeric mouse embryos constitutively expressing *fgf4* (gain of function). The most dramatic consequences of *fgf4* gain of function occur in the development of the limbs and anterior Central Nervous System (CNS). While ABUD et al. (1996) analysed the development of the limbs in these *fgf4* mouse chimeric embryos, the purpose of the experiments reported here was to describe the development of the CNS and to explain the malformations induced by *fgf4* constitutive expression.

METHODS

Generation of chimeras

The generation of the mutant chimeric mouse embryos constitutively expressing *fgf4* used in this work has been described elsewhere (ABUD et al., 1996). Briefly, they were generated using a ROSAβ-geo11 ES cell line (with constitutive expression of β-galactosidase; FRIEDERICH & SORIANO, 1991) transformed by electroporation with a PGKFGF4 expression construct, that contains a genomic DNA fragment including the entire mouse *fgf4* coding region under the control of mouse PGK-1 promoter and the SV40 small T antigen and SV40 polyadenylation sequences from PGK-o-term. PGKFGF4-ROSAβ-geo11 ES cells were injected into the blastocoel cavity of C57BL6/J blastocysts at day 3.5 post coitum (d.p.c.), and transferred to the uteri of random bred MF1 (purchased from Olac, U.K.) pseudopregnant recipients at day 2.5 p.c. The age of the embryos was calculated according to the date of mating of the mother (either pregnant or pseudopregnant foster mothers).

β-galactosidase staining

Chimerism was determined by staining for β-galactosidase activity as described by WHITING et al. (1991). Embryos dissected free of maternal decidual tissue were fixed for 1 h in 0.2% glutaraldehyde in a buffer containing 0.1 M potassium phosphate, 5 mM EGTA (Ethylene Glycol-bis(b-aminoethyl Ether) N,N,N',N'-Tetraacetic Acid; Sigma) and 2 mM $MgCl_2$ (pH 7.4). They were then washed for 3 x 20 min in the same buffer supplemented with 0.01% Na deoxycholate and 0.02% Nonidet P-40. The staining reaction was carried out overnight at 37ºC in the same buffer supplemented with 10 mM $K_3Fe(CN)_6$, 10 mM $K_4Fe(CN)_6$, and 0.5 mg/ml X-gal (Boehringer-Mannheim) dissolved in dimethylformamide. In cases where the embryo was to be used for *in situ* hybridisation, chimerism was determined by staining the yolk sac for β-galactosidase activity.

Haematoxylin-eosin staining

The embryos were fixed in Bouin fixative, dehydrated and embedded in Paraplast according to standard protocols. 5-7μm sections were stained with regular haematoxylin-eosin staining.

RNA in situ hybridisation

Double RNA in situ hybridisation was performed as described by BUENO et al. (1996a,b) using single stranded digoxigenin- and fluorescein-UTP labelled (Boehringer Mannheim) antisense RNA probes, detected sequentially with anti-digoxigenin and anti-fluorescein antibodies coupled to alkaline phosphatase. The first probe was detected by NBT/BCIP staining and visualised using transmitted light, and the second probe by ELF (Enzyme Labelled Fluorescence mRNA *in situ* hybridisation kit, Molecular Probes) substrate and UV light respectively. The *shh* (*sonic hedgehog*) probe was a 0.6 kb transcript generated by *Hin*dIII linearisation and T3 transcription (ECHELARD et al, 1993). The *fgf4* probe was a 0.6 kb transcript containing the full-length coding sequence and was generated by *Bam*HI linearisation and T3 transcription (HÉBERT et al., 1990).

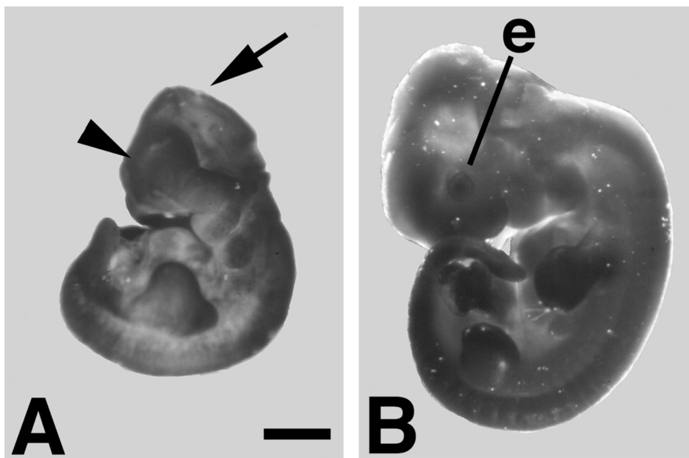
Photography

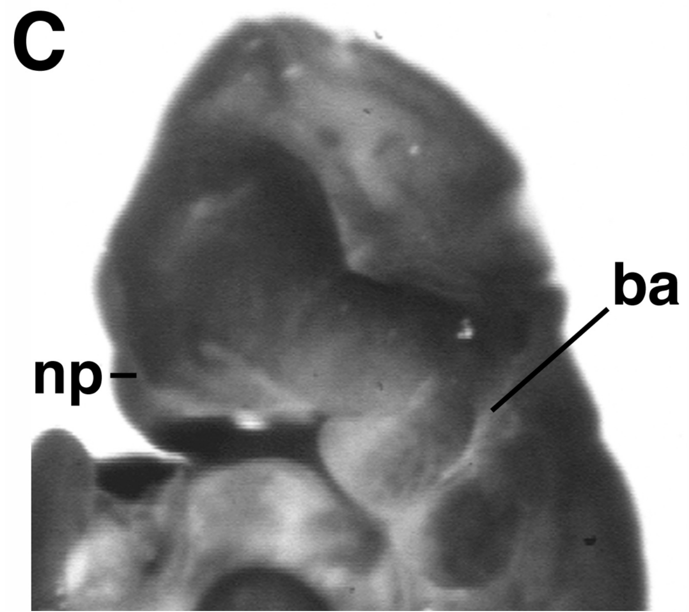
Photomicrographs of whole-mount embryos stained for b-galactosidase activity were taken on a Wild M8 photomicroscope on Agfa 64T film. Photomicrographs of sectioned embryos were taken using a Zeiss Axiophot microscope on Kodak 160T ASA film (NBT/BCIP or haematoxylin-eosin stained sections) or on Kodak 400 ASA film (ELF stained sections).

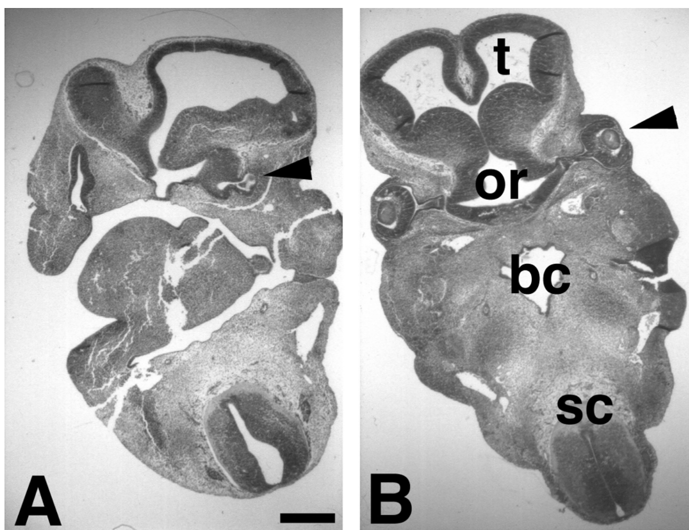
RESULTS AND DISCUSSION

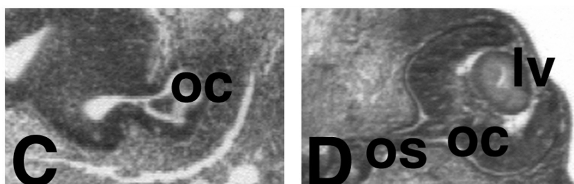
All chimeras that, on the basis of whole-mount staining for β-galactosidase, contained a substantial ES cell contribution (>50%) from PGKFGF4-ROSAβ-geo11 ES cells (33 chimeras analysed), showed anomalous development of the head regions upon external inspection (Fig. 1A, C). No phenotypic defects were detected in homozygous control embryos prepared with wild type ROSAβ-geo11 ES cells (Fig. 1B). Embryos younger than 9.5 d.p.c. did not show phenotypic defects in the anterior CNS upon external inspection (not shown), and embryos older than 14-14.5 d.p.c. with phenotypic defects were never recovered, and may have been reabsorbed.

Most embryos showed absence of eye development at 10.5-11.5 d.p.c. The embryos with the most severe phenotype also showed failure in neural tube closure in the midbrain area, exhibited exencephaly and a reduction of the diencephalon (Fig. 1A, C). The craniofacial area (including the brachial arches, the nasal prominence, the nasal pit and the region overlaying the telencephalic vesicle), which derives from the neural crest, appeared normal (Fig. 1C).









The internal structure of the head of some of the chimeras was examined histologically on series of sections at 10.5-12.5 d.p.c (Figs 2A, B). The neural tube of the mutant shown (Fig. 2A) is closed and, despite the angle of sectioning, the head is obviously asymmetrical. The hindbrain and telencephalon are visible but the structure of the diencephalon and midbrain is distorted (Figs 1A, 2A, and data not shown). Eye development is severely impaired in the mutant. The optic cups are visible in the centre of the head rather than contacting the surface ectoderm. These eyes consist of an optic cup but lack both optic stalk and lens structure (Figs 2C, D). The structure of the neuro-epithelium throughout the brain is distorted, resulting in a thinner neuro-epithelial layer. The central area of the head in the more severe mutants consists of a thin layer of neuro-epithelium, which had no differentiated internal structures (data not shown).

It has been reported that anterior structures, including eyes, are lost as a result of the ectopic expression of the homologue of *fgf4* in *Xenopus* embryos (e*fgf*; Isaacs et al., 1994). Our results suggest that eye development and CNS formation are disrupted by the activation of FGF-dependent signalling processes. The development of eyes and their lenses involves a series of complex interactions, and the failure of lens formation in the mutant chimeras may have resulted from the fail-

Fig. 1 (upper left). – Control and mutant embryos showing the malformations produced by FGF4 gain of function. (A) 11.5 d.p.c. mouse chimeric embryo prepared with PGKFGF4 ROSAβ-geo11 ES cells. Note the absence of an eye (arrowhead) and the exencephaly (arrow). Also note the malformations in the lateral ridge and limb buds compared with a control embryo. (B) Homozygous control embryo prepared with wild type ROSAβ-geo11 ES cells. (C) Magnification of the head region of (A). Note that the brachial arches and the nasal prominence are normal. Both embryos were stained for β-galactosidase activity to show the presence and extent of ROSAβ-geo11 ES cells derivatives. Bar: 1 mm (A, B); 0.25mm (C). Abbreviations: e, eye; ba, brachial arches; np; nasal prominence.

Fig. 2 (lower left). – Coronal sections through the head of chimera and control embryos at 11.5 d.p.c. stained with haematoxylin- eosin. (A) FGF4 mutant chimera. Note the position of the right eye structure (arrowhead). The opposite eye structure is in a contiguous serial section. (B) Control embryo. The position of the eyes is indicated by an arrowhead. (C) Defective eye of (A). (D) Right eye of (B). Sections are of 5 µm thick. Bar: 0.1 mm (A, B); 0.025 mm (C, D). Abbreviations: bc, buccal cavity; lv, lens vesicle; oc, optic cup, or; optic recess; os, optic stalk; sc, spinal chord; t, telencephalic vesicle.

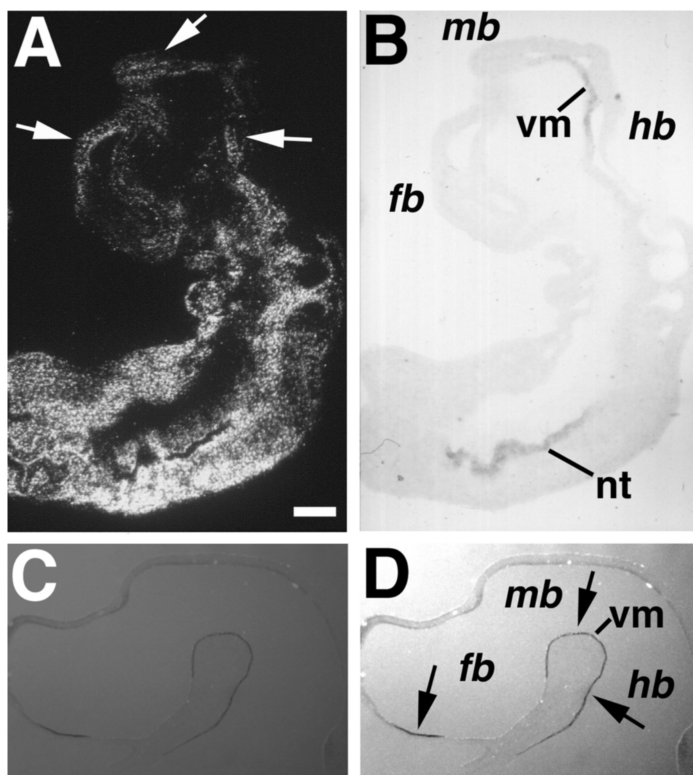


Fig. 3. – Sagittal sections of chimera and control embryos at 11.5 d.p.c. hybridised simultaneously to an antisense *fgf4* probe (DIG-labelled and ELF stained [in green]) and *shh* probe (FITC-labelled and NBT/BCIP stained [in blue]). (A) FGF4 mutant chimera showing ectopic expression of *fgf4*. The arrows indicate sites of *fgf4* ectopic expression in the developing brain. (B) Same section as (A) showing *shh* expression in the notochord (nt) and the ventral midline of the neuroectoderm (vm). The apparent absence of notochord in some areas of this section is due to the overall malformations of the embryo, which make it impossible to obtain a histological section that contains the entire notochord. (C) Sagittal section through the head of a control embryo at an equivalent level to (A) and (B) hybridised with *fgf4* antisense riboprobe. Note that *fgf4* expression is not detected. (D) Same section as (C) showing *shh* expression in the ventral midline of the neuroectoderm. Note that *shh* expression is detected at the ventral midline of the neuroectoderm (arrows) as in the mutant chimera. Sections are of 10 µm thick. Bar: 0.25 mm. Abbreviations: fb, forebrain; hb, hindbrain; mb, midbrain; nt, notochord; vm, ventral midline of neuroectoderm.

ure of the optic vesicle to reach the ectoderm (Saha et al., 1989). Normally, the optic vesicles develop from the diencephalon, and when they come into contact with the head ectoderm, the ectoderm thickens and initiates lens formation (reviewed by Saha et al., 1992).

At a molecular level, it has been previously shown that several members of the *fgf* family, namely *fgf2* and *fgf8*, function in eye development (Pittack et al., 1997; Desire et al., 1998; Picker et al., 1999; Heisenberg et al., 1999). Moreover, several FGFRs can bind FGF4 and other members of the FGF family, e.g. FGF8, with a similar specificity (MacArthur et al., 1995). It is tempting to speculate that the malformations in eye development observed here are due to interference between the ectopic expression of *fgf4* and the receptors for FGF8 and/or FGF2, that affects their binding and/or ectopically activates their receptors. However, as the eyes develop from the diencephalon (which is also severely affected in the chimeras) it is impossible to conclude from the current evidence whether the defects observed in eye development were the result of the *fgf4* ectopic expression or a secondary result due to disruption of the development of the anterior CNS.

The causes of the exencephaly, reduction of the diencephalon and anomalous differentiation of the neuroepithelium also remain unclear. It has been suggested that *shh* might play a role in the CNS phenotype of these mutant chimeras (Abud et al., 1996). This hypothesis is based on the fact that *shh* expression has been detected in areas adjacent to FGF expression domains in a number of anatomical sites during development, including the node, the floor plate, and the developing limb (Bueno et al., 1996b). Moreover, functional relationships between *fgf4* and *shh* have been described for the developing limb (Laufer et al., 1994; Niswander et al., 1994; Cohn et al., 1995; Yang & Niswander, 1995), and the expression of *shh* in ectopic limb buds has been reported in parallel mouse chimeric embryos (Abud et al., 1996).

In order to explore this possibility, we used double *in situ* hybridisation to analyse the expression of *fgf4* and *shh* in the cephalic region of mice embryos with severe mutant cephalic phenotypes. *Fgf4* gene expression was detected, at 10.5-11.5 d.p.c., in most tissues of the mutant embryos, including those of the cephalic area (Fig. 3A). In wild type embryos of this stage, the *fgf4* transcript was not detected in the tissues forming the cephalic vesicles (Fig. 3C). However, in the mutant embryos *shh* was detected only in the areas where it was expressed in wild type embryos (in the cephalic area, *shh* was expressed in the ventral midline of the neuroectoderm, Figs. 3B, D). Ectopic expression of *shh* was not detected either in the cephalic area or in the internal organs of the trunk or tail. Its absence may be due to the absence of the correct FGF4 receptors and signal transduction pathways in these regions that are necessary to activate *shh* expression (Laufer et al., 1994).

The absence of ectopic expression of *shh* in the heads of mutant mice indicates that this molecule may not be responsible for anterior CNS malformations. These malformations could be explained in other ways. Several FGFRs can bind FGF4 and other members of the FGF family (e.g. FGF8) with a similar specificity (MacArthur et al., 1995). Moreover, Crossley et al. (1996) identified FGF8 as an important signalling molecule for midbrain development, because it is expressed at discrete sites in the cephalic neuroectoderm during development (Crossley & Martin, 1995; Bueno et al.,

1996b). It is tempting to speculate that the reported malformations in the anterior CNS are due to interference between FGF4 ectopic expression and the receptors for FGF8. This would be consistent with recent reports (MEYERS et al., 1998; SHAMIN et al., 1999) showing that hypomorphic alleles, null mutants and ectopic expression of FGF8 in the midbrain and caudal forebrain cause exencephaly in mouse and chick embryos respectively, and showing that exogenous FGF4 can suppress anterior development in the mouse embryo during neurulation and early organogenesis (DAVIDSON et al., 2000). It would also explain the lack of malformations in the craniofacial area neural crest-derivatives. These exhibit a different set of FGFRs. If the hypothesis of FGF4 interference is correct, the results presented here would indirectly support a crucial role of *fgf*8 in patterning the anterior CNS and show once again the complexity of the FGF family in patterning embryonic structures.

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