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Characterization of the functional properties of the neuroectoderm in mo*use Cripto*$^{-/-}$ embryos showing severe gastrulation defects

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ABSTRACT During development of the mammalian embryo, there is a complex relation between formation of the mesoderm and the neuroectoderm. In mouse, for example, the role of the node and its mesendoderm derivatives in anterior neural specification is still debated. Mouse *Cripto*$^{-/-}$ embryos could potentially help settle this debate because they lack almost all embryonic endoderm and mesoderm, including the node and its derivatives. In the present paper, we show that *Cripto*$^{-/-}$ embryos can still form functional neural stem cells that are able to differentiate and maintain a neural phenotype both *in vivo* and *in vitro*. These data suggest that signals emanating from the mesoderm and endoderm might not be essential for the formation and differentiation of neural stem cells. However, we use grafting experiments to show that the *Cripto*$^{-/-}$ isthmus (the secondary organizer located at the midbrain-hindbrain boundary) loses its inductive ability. We further show that the *Cripto*$^{-/-}$ isthmus expresses lower amounts of the isthmic signalling molecule, Fgf8. Since nearby tissues remain competent to respond to exogenously added Fgf8, this reduction in *Fgf8* levels in the *Cripto*$^{-/-}$ isthmus is the potential cause of the loss of patterning ability in graft experiments. Overall, we interpret our data to suggest that the mammalian node and primitive streak are essential for the development of the regional identities that control the specification and formation of the secondary organizers within the developing brain.

KEY WORDS: *Cripto, mammalian node, neural differentiation, isthmic organizer, Fgf8, gastrulation defect*

Introduction

Neural induction is the process by which embryonic cells in the epiblast acquire a neural fate rather than give rise to other structures such as epidermis and mesoderm (Stern, 2006). The relation between mesoderm and neuroectoderm is complex. Recent studies emphasise that definition of the neural plate during normal development first requires establishing a boundary between the region of the epiblast destined to ingress into mesendoderm during gastrulation and the medial edge of the presumptive neural plate (Sheng *et al.*, 2003; Stern, 2005; Stern, 2006). On the hand, ingression of the epiblast inside the primitive streak to form mesoderm must be blocked in order to specify the presumptive neural plate; on the other hand mesoderm itself participates in neural induction and patterning.

Regarding mouse anterior neural development, a two-step model has been proposed, in which initiation of the anterior neural patterning is under the control of the anterior visceral endoderm (AVE), while node and its mesendoderm derivatives are required to maintain and/or refine the rostral neuroectoderm (Shawlot *et al.*, 1999; Simeone and Acampora, 2001; Thomas and Beddington, 1996). Then, at the neural plate stage, the secondary organizers, which are local signalling centres inside the neuroepithelium,

Abbreviations used in this paper: A-P, antero-posterior; AVE, anterior visceral endoderm; M-HB, mid-hindbrain boundary.

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refine the antero-posterior (A-P) specification of the three main domains in the brain primordium: the forebrain, the midbrain and the hindbrain. These secondary organizers are the anterior neural ridge (ANR) at the anterior end of the neural plate, the zona limitans intrathalamica (ZLI) in the middle of the diencephalon, and the isthmic organizer (IsO), located at the mid-hindbrain boundary (M-HB) (Echevarria *et al.*, 2003). However, evidence is accumulating against this two-step model. If the mouse primary organizer is ablated either surgically during gastrulation or genetically by deletion of *HNF3β* or *Cripto*, a neural plate still forms (Ang and Rossant, 1994; Davidson *et al.*, 1999; Ding *et al.*, 1998; Klingensmith *et al.*, 1999; Liguori *et al.*, 2003; Weinstein *et al.*, 1994). The mouse *Cripto* null mutant embryos are very informative. *Cripto* is the founding member of the *EGF-CFC* genes family, coding for membrane-anchored extracellular factors essential for vertebrate embryo development (Persico *et al.*, 2001). *Cripto* is expressed in embryonic stem (ES) cells and in the forming mesoderm, but has never been detected in the visceral endoderm (Dono *et al.*, 1993; Johnson *et al.*, 1994; Kimura *et al.*, 2001). As expected from this expression pattern, *Cripto* $^{-/-}$ embryos have the peculiar characteristics of losing almost all the embryonic endoderm and mesoderm, including the node and its derivatives, but the embryos still possess a functional AVE (Ding *et al.*, 1998; Liguori *et al.*, 2003). With these particular characteristics, *Cripto* null mutants constitute an extremely useful model to study the contribution of the node and primitive streak (including their derivatives) to early anterior neural plate development in mouse. We have previously analyzed the nature and topology of the neural tissues in *Cripto* $^{-/-}$ mutants, both *in vivo* and *in vitro*. We demonstrated that *Cripto* $^{-/-}$ embryos develop complex molecular patterns, suggesting that anterior neural fate and normal A-P regionalization do not require the presence of the node and primitive streak (Liguori *et al.*, 2003).

In the present study, we extend our analysis to the functional properties of the anterior neural cells in *Cripto* $^{-/-}$ embryos, with the aim of determining which aspects of the mutant neural epithelium are principally affected by the absence of signals emanating from the node and from the embryonic mesendoderm. Our data indicate that the loss of the primary organizer and of most all the embryonic mesoderm and endoderm does not affect the competence of neural cells to differentiate in response to surrounding stimuli. In other words, these results indicate that specification of neural lineages does not absolutely require signals from the mesoderm and endoderm. However, gastrulation failure specifically impairs the correct development of the regional identities that control the specification of the secondary organizers, that, in turn, are required for the definitive refining of anterior neural patterning.

Results

Cripto *is dispensable for neural differentiation both* in vitro *and* in vivo

Our previous analysis demonstrated that *Cripto* $^{-/-}$ embryos form anterior neural territories (Liguori *et al.*, 2003). In the present study, we plan to investigate the effect on neural progenitors of *Cripto* inactivation and the consequent loss of embryonic mesoderm and endoderm, by analysing whether *Cripto* $^{-/-}$ embryos develop neural progenitors and whether the competence of these progenitors is significantly affected. To this purpose, we derived *Cripto* $^{-/-}$ neural progenitors (Fig. 1A) by dissecting the embryonic region of 8.5 dpc *Cripto* null mutants, which is completely composed of anterior neuroectoderm (Liguori *et al.*, 2003). As a control, we dissected the corresponding region of the same stage wild type (wt) embryos, i.e. from the most rostral part of the forebrain until the otic vesicle (Fig. 1A). After three days in culture, both *Cripto* $^{-/-}$ and wt embryonic cells formed neurospheres (Fig. 1B, C). When the neurospheres were dissociated and the cells cultured in fresh medium, the isolated cells retain the ability to proliferate and to reform neurospheres. It was possible to maintain in culture and expand the neurospheres for at least one month, without any macroscopic difference in their number and morphology between *Cripto* $^{-/-}$ and wt cultures.

After 13 days of *in vitro* culture, a fraction of the *Cripto* $^{-/-}$ and wt neurospheres were fixed in PFA and analyzed by immunocytochemistry with anti-nestin antibody (an intermediate filament

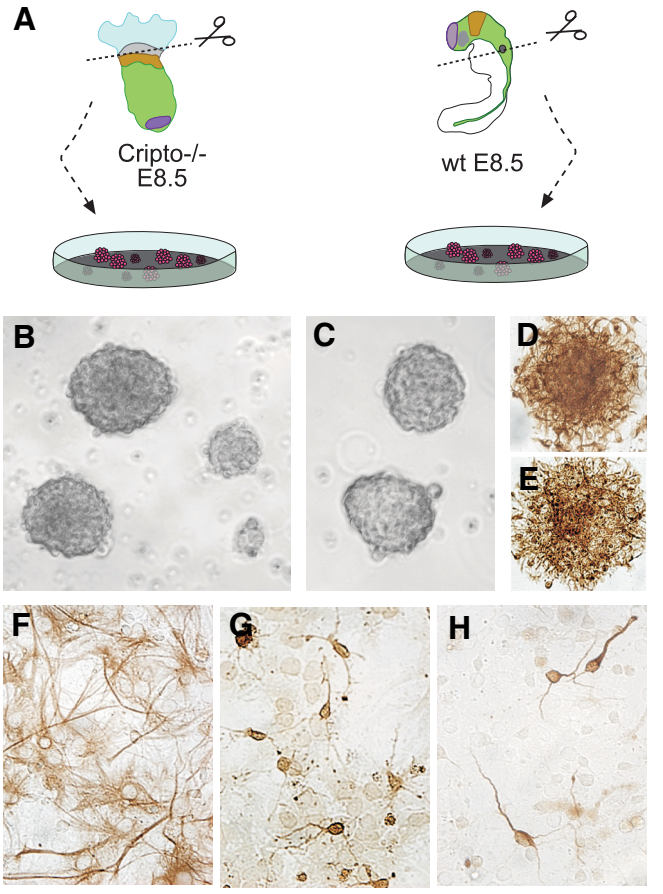

Fig. 1. Neurospheres derived from *Cripto* $^{-/-}$ embryos behave as wild type neurospheres *in vitro*. (A) *Schematic representation of experimental assays in which anterior neural tissues from* Cripto $^{-/-}$ *and wt (control) embryos were dissociated and allowed to develop* in vitro *(see Materials and Methods for details).* **(B,C)** *Brightfield image of* Cripto $^{-/-}$ *neurospheres (B) compared to wt neurospheres (C).* **(D,E)** *Nestin immunostaining of wt (D) and* Cripto $^{-/-}$ *(E) neurospheres.* **(F,G,H)** *Immunostaining of* Cripto $^{-/-}$ *neurospheres after differentiation (see Materials and Methods).* Cripto $^{-/-}$ *neurospheres are capable of differentiating into astrocytes (GFAP positive) (F), oligodendrocytes (NG2 positive) (G) and neurons (beta-tubulin positive) (H).*

expressed in undifferentiated neuroepithelial cells, Gritti *et al.*, 1996; Reynolds and Weiss, 1992; Tohyama *et al.*, 1992). The immunocytochemistry revealed that both *Cripto* $^{-/-}$ and wt neurospheres expressed nestin (Fig. 1D, E). The remainer of the cultures were grown following a protocol to cause neurosphere cells differentiation in both neural and glial cell types (adhesion to a Matrigel substrate, removal of growth factors) (Bonilla *et al.*, 2005). After another seven days in culture, the cells were fixed and analyzed by immunocytochemistry. The *Cripto* $^{-/-}$ differentiated cultures contained cells expressing the Glial Fibrillary Acidic Protein (GFAP) (astrocytes, Fig. 1F), cells expressing the β-tubulin III (neurons, Fig. 1H) and cells expressing NG2 (oligodendrocites both mature and precursors; Fig. 1G). We obtained the same results with *Cripto* $^{-/-}$ neurospheres after 26 days of *in vitro* culture (data not shown). Collectively, these data show that the neuroectoderm of *Cripto* $^{-/-}$ embryos contains neural progenitors, which are able to form neurospheres *in vitro*. Moreover, *Cripto* $^{-/-}$ neurospheres are able to differentiate into both neuronal and glial cell types. We were not able to detect any significant difference in the number and morphology between the *Cripto* $^{-/-}$ and wt cultures of both neurospheres and differentiated neural cells. However, we cannot exclude subcellular/structural or functional differences.

Finally, the embryonic region of *Cripto* null mutants at 8.5 dpc were dissected into equal parts and was grafted into the telencephalic region of newborn C3H/He mice (Fig. 2A). After five days, the mice were sacrificed and the brain was dissected, sectioned and analysed by immunohistochemistry. The H-2D^{b} antibody (specific for the C57Bl6/J background of the *Cripto* $^{-/-}$ embryos) revealed the presence of *Cripto* $^{-/-}$ cells inside the cortex of the host mice (Fig. 2B, E, H). Some *Cripto* $^{-/-}$ cells expressed nestin (Fig. 2C, D), while others were GFAP positive (Fig. 2F, G) being astrocytes. Finally, co-expression of H-2D^{b} (Fig. 2H) with anti β-Tubulin antibody (Fig. 2I, J) showed that some *Cripto* $^{-/-}$ cells also expressed neural markers. These results indicate that neuroepithelial cells from the *Cripto* $^{-/-}$ embryos can be incorporated into the brain tissue of a newborn mouse host. Moreover, the data suggest that *Cripto* $^{-/-}$ neuroepithelium is sufficiently competent to maintain its original fate as undifferentiated progenitors or is able to mature into neuronal and glial cells.

In conclusion, *in vivo Cripto* inactivation and the consequent lack of mesoderm and endoderm do not significantly affect the formation and the competence of neural progenitors inside the mouse embryo. These data are in agreement with previous analyses performed on *Cripto* $^{-/-}$ ES cells, demonstrating that *Cripto* is dispensable for neuronal differentiation both *in vitro* and *in vivo* (Minchiotti *et al.*, 2006; Parish *et al.*, 2005; Parisi *et al.*, 2003).

The mid-hindbrain boundary of Cripto $^{-/-}$ *embryos does not possess the functional properties of an organizer*

We next wished to determine how Cripto influences the functional activity of the most studied secondary organizer, the isthmus, which is located at the mid-hindbrain boundary (M-HB) and which controls anterior hindbrain and midbrain regionalization (Martinez, 2001). We have previously shown that *Cripto* null mutants do not form ANR or ZLI, but only develop the molecular pattern that characterizes the isthmic organizer (Liguori *et al.*, 2003; 2008). In the present study, we investigate whether this putative organizer located at the M-HB possesses properties of a true functional isthmic organizer. Grafting experiments have been classically made in chick. However, the development of an experimental technique based on explanting the mouse anterior neural tube (ANT) onto polycarbonate membrane has made possible short-term neuroepithelial grafting experiments also in mice (Echevarria *et al.*, 2001). This technique has allowed the use of wt mouse ANT as host tissue for a graft and then of tissues from knock-out mice as source for heterotopic and heterochronic

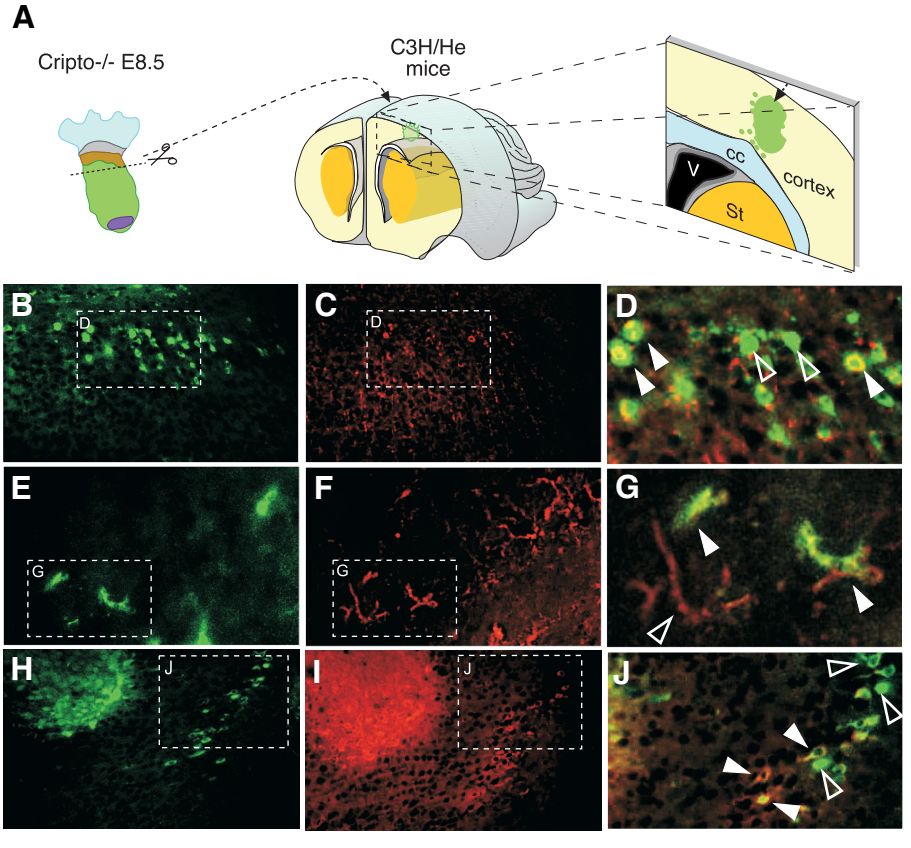

Fig. 2. *Cripto* $^{-/-}$ cells can be incorporated into the brains of newborn mice as both undifferentiated and differentiated neural cells. (A) *Schematic representation of experimental assays in which anterior neural tissues from* Cripto $^{-/-}$ *embryos were injected into newborn mouse cerebral cortex (see Materials and Methods for details).* **(B-I)** *Immunohistochemistry assays on brain sections of P5 C3H/He mice injected with 8.5 dpc* Cripto $^{-/-}$ *embryo tissues. The* Cripto $^{-/-}$ *cells are identified by the anti-H2Dd antibody* **(B,E,H)**. *Some of the* Cripto $^{-/-}$ *cells incorporated into the brain expressed nestin* **(C,D)**, *others expressed GFAP* **(F,G)** *and some β-tubulin* **(I,J)**. *Co-expressing cells are indicated by white arrowheads; non co-expressing* Cripto $^{-/-}$ *cells are pointed to by empty arrowheads.*

TABLE 1

SUMMARY OF THE GRAFT EXPERIMENTS

Graft	Host	Donor	Time in culture after the graft	Total number of grafts	*Fgf8* positive grafts	*En2* induction in the host
isthmus	9.5 dpc wt diencephalon	8.5 dpc *Cripto*$^{-/-}$ embryo	24 h	18	8	0
			48h	19	6	0
		8.5 dpc *Cripto*$^{-/-}$ explant cultured for 24h	48h	9	3	0
		8.5 dpc wt embryo	24h	18	8	0
			48h	19	6	3 (50%)
		9.5 dpc wt embryo	24h	18	8	4 (50%)

intraspecific transplants. Then, we grafted the M-HB of 8.5 dpc *Cripto*$^{-/-}$ embryos into the diencephalic-mesencephalic boundary region of 9.5 dpc ANT mouse explants (Fig. 3A). We cultured the explants *in vitro* for 24h after the graft and then analyzed, by double WISH, the expression profile of both *Fgf8*, coding for the isthmic signalling molecule, and *En2*, a known target of the Fgf8 signalling pathway, looking for ectopic induction of *En2* expression close to the *Fgf8* positive graft (Fig. 3C-C"). As control, we also grafted the isthmus of mouse wt embryos at both 8.5 and 9.5 dpc (Fig. 3A, F, F').

We found that approximately 50% of the explants showed *Fgf8* expression within the grafts (Fig. 3C, C', F), independent of whether the donor embryo was wt or *Cripto*$^{-/-}$. The grafts were labelled by DiI (Fig. 3C", F'). The detection of *Fgf8* expression confirmed that the region transplanted was at the M-HB of the donor embryo. However, while wt isthmic grafts (see Table 1) induce *En2* expression in the host tissue (Fig. 3F, F'), none of the *Cripto*$^{-/-}$ grafts were able to induce ectopic *En2* expression (Fig. 3C-C") (see Table 1). We cultured the *Cripto*$^{-/-}$ transplants also for 48h following the graft, but even in this case we could not detect any *En2* induction (data not shown; see Table1). Since the absence of inductive properties could reflect an immature condition of the isthmic neuroepithelium in *Cripto*$^{-/-}$ embryos, we explanted the *Cripto*$^{-/-}$ embryos at 8.5 dpc and let them develop *in vitro* for 24h in order to allow any potential stabilization of *Fgf8* expression (see also Liguori *et al.*, 2003). Then, we grafted the *Cripto*$^{-/-}$ M-HB in a wt host ANT at 9.5 dpc. The transplants were cultured for 24 or 48h but none showed ectopic *En2* induction (data not shown; Table 1).

We conclude that the capacity of the M-HB of the *Cripto*$^{-/-}$

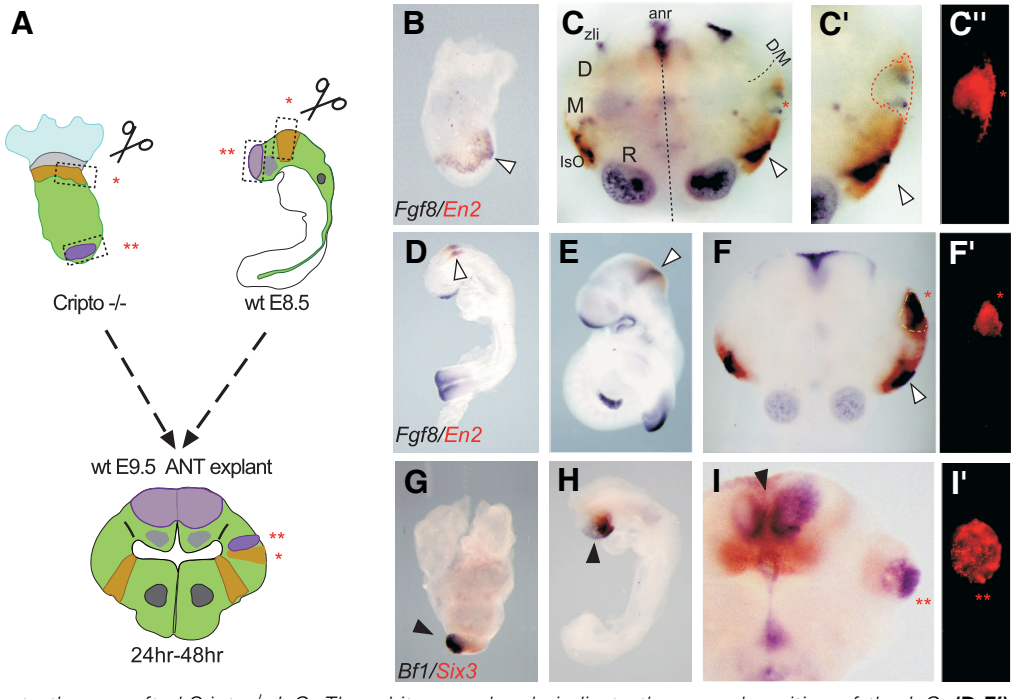

Fig. 3. *Cripto*$^{-/-}$ neural tissues are maintained in host tissue but *Cripto*$^{-/-}$ mid-hindbrain (M-HB) is unable to induce *En2* ectopic expression. (A) *Schematic representation of the embryological experimental manipulation of* Cripto$^{-/-}$ *and wt IsO (*) or ANR (**) into the mes/diencephalic boundary (m/d-B) region of wt anterior neural tube (ANT) explant culture.* **(B-C'')** *Experimental assays where the IsO from* Cripto$^{-/-}$ *animals was transplanted into the m/d-B of ANT explant culture.* **(B)** *Expression pattern of* Fgf8 *(blue) and* En2 *(red) in* Cripto$^{-/-}$ *embryos at 8.5 dpc.* **(C)** *Double WISH expression pattern with* Fgf8/ En2 *in a wt 9.5 dpc ANT explant after 24 h of graft incubation. The red asterisks indicate the engrafted* Cripto$^{-/-}$ *IsO. The white arrowheads indicate the normal position of the IsO.* **(C')** *Magnified area of the grafted region detected by double WISH with* Fgf8/En2. **(C'')** *The same area as (C') detected by fluorescence with the corresponding DiI excitation wavelength filter. The red asterisks indicate the engrafted* Cripto$^{-/-}$ *IsO. The white arrowheads indicate the normal position of the IsO.* **(D-F')** *Experimental assays where the IsO from a wt 9.5 dpc mouse embryo was transplanted into the m/d-B of wt ANT explant culture.* **(D,E)** *Double WISH expression pattern with* Fgf8/En2 *in wt 8.5 dpc* **(D)** *and 9.5 dpc* **(E)**. *The white arrowheads indicate the normal position of the IsO.* **(F)** *Double WISH expression pattern with* Fgf8/ En2 *in a wt 9.5 dpc ANT explant after 24 h of graft incubation. The red asterisk indicates the grafted wt IsO.* **(F')** *The same area as (F) detected by fluorescence with the corresponding DiI excitation wavelength filter.* **(G-I')** *Experimental assays where the most distal part (forebrain) of a* Cripto$^{-/-}$ *embryo was transplanted into the m/d-B of ANT explant culture.* **(G,H)** *Double WISH expression pattern with* Bf1 *(blue) and* Six3 *(red) in* Cripto$^{-/-}$ *(G) and wt (H) 8.5 dpc mouse embryos.* **(I)** *Double WISH expression pattern with* Bf1/Six3 *in a wt 9.5 dpc ANT explant after 24 h of graft incubation. The two red asterisks indicate the* Cripto$^{-/-}$ *forebrain grafted into the m/d-B. The black arrowhead indicates the normal position of the forebrain in the ANT explant.* **(I')** *The same area as detected by fluorescence with the corresponding DiI excitation wavelength filter. D, diencephalon; F, forebrain; M mesencephalon; R, rhombencepahlon; zli; zona limitans intrathalamic; anr anterior neural ridge; IsO Isthmic Organizer.*

embryos to induce *En2* ectopic expression is severely affected: the M-HB of the *Cripto* $^{-/-}$ embryos appears to loose its patterning properties and thus cannot be defined as a true organizer. Nonetheless, the transplanted region continues to express its characteristic markers (*Fgf8* and *En2*), at least for 48h after the graft, suggesting that the tissue inside the *Cripto* $^{-/-}$ M-HB is healthy and able to retain its molecular identity.

Finally, we performed an ectopic graft of the *Cripto* $^{-/-}$ distal region (corresponding to forebrain) dissected at 8.5 dpc into the diencephalon of 9.5 dpc wt embryos (Fig. 3A). We cultured the grafts for 24h and then analyzed their expression profile by double WISH. The chosen *Cripto* $^{-/-}$ region expressed forebrain markers, such as *Bf1* and *Six3* genes (Liguori *et al.*, 2003; Fig. 3G) and continued to express the same genes 24h after the graft (in three out of four cases) (Fig. 3I,I'). Therefore, *Cripto* null mutant tissues are not severely affected by the graft and *Cripto* $^{-/-}$ cells maintain their characteristic molecular pattern. Moreover, these data show that the ability to maintain specific gene expression (at least for 24h), when transplanted into an ectopic region, is a characteristic not only of the putative isthmus of the *Cripto* null mutants, but also a characteristic of other regions of the *Cripto* $^{-/-}$ embryo anterior neural plate.

Fgf8 signaling is active in* Cripto $^{-/-}$ *neuroectoderm, but the amount of* Fgf8 *mRNA is lower in the M-HB of* Cripto $^{-/-}$ *compared to wt embryos

To gain insight into the lack of patterning ability of the *Cripto* $^{-/-}$ embryo isthmus, we first verified that the Fgf8 signaling pathway is active in these embryos, in particular whether the *Cripto* $^{-/-}$ neuroectoderm can respond to the Fgf8 inductive stimulus. We implanted Fgf8b-coated beads (Crossley *et al.*, 1996; Garda *et al.*, 2001; Liu *et al.*, 1999; Shimamura and Rubenstein, 1997) in the *Cripto* $^{-/-}$ embryos explanted at 8.5 dpc, cultured the explants for 24h and then analyzed the expression of specific diagnostic markers by WISH (Fig. 4). In the *Cripto* $^{-/-}$ neuroectoderm Fgf8b induces the expression of the midbrain marker *En2* (Fig. 4A, B), the anterior hindbrain marker *Gbx2* (Fig 4C-F) and also the forebrain marker *Bf1* (Fig. 4G, H). The downstream modulator *Mkp3* gene (Echevarria *et al.*, 2005; Kawakami *et al.*, 2003) is also induced in *Cripto* $^{-/-}$ neural tissue after 24 hours exposure to the Fgf8b-soaked beads (Fig. 4I, J). Collectively, these data demonstrate that *Cripto* $^{-/-}$ neural cells are able to respond to the inductive stimulus provided by exogenous Fgf8b, indicating that Fgf8 signaling pathway is active in the *Cripto* $^{-/-}$ embryos.

Therefore, we hypothesised that the lack of inductive ability of the *Cripto* $^{-/-}$ M-HB might be due to weak expression of endogenous Fgf8 at this boundary. Thus, we compared the *Fgf8* expression level in the M-HB of wt and *Cripto* $^{-/-}$ embryos at 8.5 dpc by means of real-time PCR. We analysed separately the isthmus of five wt embryos and the M-HB of ten *Cripto* null mutants. We found that the M-HB of the *Cripto* $^{-/-}$ embryos expresses less *Fgf8* than the corresponding regions in the wt embryos. In particular, we identified two different groups of *Cripto* null mutants, the group A, composed by two out of ten embryos, which expresses very low doses of *Fgf8* mRNA and the group B, composed by the left eight, which express almost half of the level of *Fgf8* mRNA found in the wt (Fig. 4L). These results indicate that *Cripto* $^{-/-}$ embryos still express *Fgf8* at the *Otx2/Gbx2* molecular boundary, but that its level is at least half reduced, and in some cases dramatically reduced, respect to the wt embryos. These reduction of *Fgf8* expression level in the *Cripto* $^{-/-}$ M-HB could realistically be the cause of the loss of patterning abilities after grafting.

Discussion

The functional analysis we have performed on *Cripto* null mutants gives important information on the role that embryonic mesendoderm and the primary organizer play during neural dif-

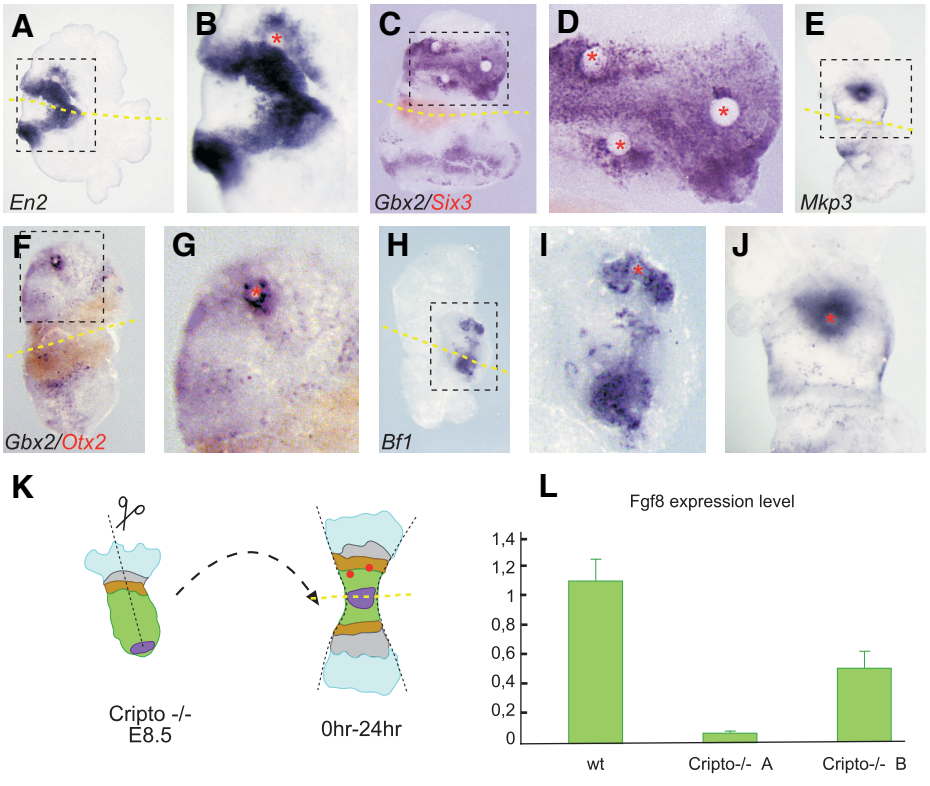

Fig. 4. *Cripto* $^{-/-}$ neuroectoderm is able to respond to the inductive stimulus provided by exogenous Fgf8b. *Implanting Fgf8b-coated beads into the* Cripto $^{-/-}$ *explants causes after 24 h of* in vitro *culture the expression of the midbrain marker* En2 **(A,B)**, *the anterior hindbrain marker* Gbx2 **(C,D,F,G)**, *the forebrain marker* Bf1 **(H,I)** *and the* Fgf8 *target* Mkp3 **(E,J)**. *The red asterisks indicate bead positions. The dashed squares indicate higher magnification view of examples (A,C,E,F,H) in (B,D,G,I,J) respectively. Dashed yellow lines indicate the midline of the* Cripto $^{-/-}$ *embryos flattened onto the polycarbonate membranes separating the experimental side from the control side (see Liguori* et al.*, 2003).* **(K)** *Schematic representation of* a Cripto $^{-/-}$ *explant.* **(L)** *Histograms represent the quantitative-PCR values of* Fgf8 *mRNA levels between wt and* Cripto $^{-/-}$ *mid-hindbrain cases (with standard error bars).*

ferentiation. The main conclusions of this work are essentially two.

First, in *Cripto* null mutants, differentiation of neural stem cells towards neuronal and glial fate is not significantly affected by the absence of signals emanating from the embryonic mesoderm and endoderm. These data suggest that mesendodermic signals are dispensable not only for neural stem cells to form, but also for their differentiation into both a neuronal and glial phenotype, possibly indicating that neuroectoderm itself provides all the signals necessary for neuronal and glial differentiation. This view reminds very close the default model of neural induction proposed by Hemmati-Brivanlou and Melton in 1997, according to which ectoderm cells have an inherent tendency towards the neural identity, but constitutive BMP signalling in the ectoderm prevents realization of the neural fate. Even though challenges to the default model emerged from experiments both in frog and other vertebrates, mainly in chick, showing that Fibroblast growth factors (FGF) and canonical Wnt signals have also been implicated in the neural induction process (Delaune *et al.*, 2005; Hongo *et al.*, 1999; Linker and Stern, 2004; Sheng *et al.*, 2003; reviewed in Stern, 2005, 2006), the findings that both FGF and Wnt signals can inhibit BMP signalling, might reconcile these apparent conflicting observations (Baker *et al.*, 1999; Pera *et al.,* 2003; Wessely *et al.*, 2001). Recently, inhibition of Activin/Nodal/Smad2 signaling has been also reported to be involved in neural induction (Chang and Harland, 2007; Smith *et al.*, 2008). We note that Nodal signalling is able to crosstalk with BMP signaling and induce *Bmp4* expression (Beck *et al.*, 2002; Ben-Haim *et al.*, 2006; Yeo and Withman, 2001). These data suggest that simultaneous suppression of BMP and Nodal-like signals, and then combined inhibitory rather than instructive signals, might be crucial for neural fate determination, Cripto has a key role in this scenario, being deeply involved in Nodal signalling (Gritsmann *et al.*, 1999). In fact, Cripto interacts with Alk receptors for the formation of the Nodal receptorial complex (Reissman *et al.*, 2001). Thus, neural differentiation might occur in the absence of Cripto, due to impairment in the Nodal signalling and consequently in the BMP signalling.

The second main conclusion of the paper is that the *Fgf8*-positive territory, corresponding to the putative isthmic organizer, in the neuroectoderm of *Cripto* null mutants does not develop the ability to redirect the diencephalon or rostral mesencephalon of a wt embryo explant towards a mid-hindbrain fate, as the wt isthmic region does. Our analysis also shows that the M-HB of *Cripto*$^{-/-}$ embryos expresses a lower amount of *Fg8* mRNA than the wt isthmus. Being Fgf8 be recognized as the principal isthmic signalling molecule, we hypothesise the M-HB of *Cripto*$^{-/-}$ embryos does not possess patterning activity in graft experiments, because of the reduction in the *Fgf8* expression level. It has been previously been suggested that achievement of a threshold level of Fgf8 is essential for induction of precise identity into neighbouring cells: Sato and Nakamura (2004) demonstrated that only a strong Fgf8 signal activates the Ras-extracellular signal-regulated kinase (ERK) pathway in the mesencephalon/metencephalon boundary and this is necessary and sufficient to induce cerebellar development (Sato and Nakamura, 2004). In contrast, a lower level of Fgf8 signalling seems to be required for midbrain development (Sato *et al.*, 2004; Sato and Nakamura, 2004; Basson *et al.*, 2008). Very recently, it has been demonstrated that transcriptional activation of *Wnt1* and *En1*, two targets of Fgf-signaling at the M-HB, requires different threshold levels of Ras–MAP kinase activity, thus supporting the idea that Fgf8 functions in a dose-dependent manner at the M-HB (Vennemann *et al.*, 2008). Furthermore, nonlinear dosage effects of *Fgf8* on the expression of a subset of genes, including *Bmp4* and *Msx1*, have been correlated with a holoprosencephaly phenotype and with the nonlinear expression of transcription factors that regulate neocortical patterning (Storm *et al.*, 2006). A nonlinear, threshold-like, epistatic response to an Fgf8 stimulus has also been observed during submandibular salivary gland morphogenesis (Jaskoll *et al.*, 2004). Overall, these data suggest that modifications in the relative strength of Fgf signaling can have profound effects on the relative size and nature of anterior neural tube subdivisions (Storm *et al.*, 2006). However, we note that most *Cripto*$^{-/-}$ embryos can still specify mesencephalic territories, as demonstrated by the expression of *Wnt1*, *Pax2*, *En1* and *En2* markers (Ding *et al.*, 1998; Liguori *et al.*, 2003 and present data), meaning that half *Fgf8* dosage remains sufficient to induce some territories. Interestingly, the distance of the cells from the Fgf8 sources might also play an important role. The amount of Fgf8 produced by the M-HB of *Cripto*$^{-/-}$ embryos might be sufficient to pattern the identity of the cells inside the graft, but not able to reach the cells of the host tissue, located outside the graft. However, we cannot exclude that other still unknown factors are affected in the *Cripto*$^{-/-}$ M-HB and that this uncharacterized defect might contribute to the phenotype we observe.

Finally, from the point of view of evolution, M-HB in *Cripto* null mutants might resemble the one found in some protochordates. In protochordates, the anterior part of the nerve cord is expanded into a cerebral vesicle, which is homologous to the forebrain and midbrain (Lacalli, 2006; Williams and Holland, 1998), or possibly just forebrain (Takahashi and Holland, 2004; Takahashi, 2005). Caudal to this region, the nerve cord expresses markers characteristic of the hindbrain and spinal cord (Holland and Holland, 1999). In the CNS of the cephalochordate amphioxus, *Gbx* and *Otx* genes are expressed in positions comparable to the Vertebrates, but *En, Pax2/5/8* and *Wnt1* are not expressed near the caudal limit of *Otx,* suggesting that the genetic machinery to position the M-HB was present in the protochordate ancestors of the vertebrates, but is insufficient for induction of organizer genes (Castro *et al.*, 2006). Therefore, the M-HB of *Cripto*$^{-/-}$ embryos might resemble this ancestral situation, possessing the M-HB molecular characteristics, but not fully acting as an organizer".

Experiments in a variety of organisms (mouse, frog, zebrafish) already showed that neural tissue might be specified without an absolute requirement for primary organizer (Klingensmith *et al.*, 1999; Liguori *et al.*, 2003; Saude *et al.*, 2000; Shih and Fraser, 1996; Wessely *et al.*, 2001). However, the present study is the first time that a functional analysis is performed on the neural tissues formed in absence of such an organizer. Our data propose that the reason for the node to be essential for the development and refining of the brain architecture is its requirement for the formation of the secondary organizers.

Materials and Methods

Neurosphere preparation and differentiation

The embryonic region of four *Cripto* null mutants at 8.5 dpc (completely composed of anterior neuroectoderm;(Liguori *et al.*, 2003) were dissected and collected in Hanks' balanced salt solution (Gibco BRL, Life Technologies). The most rostral part of the forebrain, up to the otic vesicle, of seven wt embryos at 8.5 dpc were dissected and collected in the same buffer

solution. Neurosphere preparation and differentiation have been performed according to Bonilla *et al.* (2005). Prior to immunochemical analysis, the cell cultures were fixed with 4% paraformaldehyde in PBS for 1h at 4°C.

Intracerebellar embryo grafts

Cripto $^{-/-}$ embryonic neuroectoderm pieces were dissected in cold DMEM solution and were injected into the telencephalon of cold-anaesthetized neonatal C3H/He mice. The *Cripto* $^{-/-}$ tissues were introduced in the brain, through whole in the cranium, using a 10 μl Hamilton syringe.

The injection was performed in the parietal area, 1mm caudal and lateral to the bregma point and 0.5 mm into the parenchyma from the dura mater (Franklin and Paxinos, 1997). Five days after tissue transplantation, experimental mice were anaesthetized with chloroform and were fixed by intracardiac perfusion with 4% paraformaldehyde in PBS. Dissected brains were postfixed overnight at 4°C in the same fixative. After washing in PBS, the fixed brains were cryoprotected for 8h at room temperature in 10% sucrose-PBS and overnight at 4°C in 20% sucrose–PBS for cryotome sections. Serial sections 20 μm thick were mounted in five parallel series.

Immunocytochemistry and immunohistochemistry

Immunochemical techniques were performed as described by Bonilla and coworkers (2005), using the following primary antibodies: anti-nestin monoclonal antibody (Chemicon, Temecula, CA), anti-NG2 polyclonal antibody (Chemicon, Temecula, CA), anti Beta-III-Tubulin monoclonal antibody (Eurogenec, Belgium), and anti-glial fibrillary acid protein (GFAP) monoclonal antibody (Calbiochem, San Diego, CA).

Grafting experiments

Neural tissue explant culture of 8.5-9.5 dpc wild-type ICR and 8.5 dpc *Cripto* $^{-/-}$ embryos were made according to Echevarria *et al.* (2001) and Liguori *et al.* (2003) respectively. For the grafting experiment, donor tissues were incubated with the lipophilic tracer DiI (100 ng/ml final concentration) (Honing and Hume, 1989) for 1h. After incubation, tissue was washed briefly in culture medium and prepared for microscopic dissection of neuroepithelium at the midbrain-hindbrain transition. Tissue grafting was performed using fine tungsten needles. The presumptive isthmus was dissected and isolated from the donor, transferred to culture medium by a glass pipette, and ectopically inserted in the host diencephalon into a previously made equivalent hole (heterotopic grafts). The grafts were *in vitro* cultured for 24-48h and then fixed with 4% paraformaldehyde in PBS overnight at 4°C. The isthmus was visualized by *Fgf8 in situ* hybridization.

Bead implantation

Heparin acrylic beads (Sigma) were rinsed four to six times in PBS and then soaked in 5 μl FGF8 solution (1 mg/ml; R&D), for 1h at 4°C. The beads were then rinsed three times in PBS 0.1 M and thereafter implanted in the neuroectoderm of *Cripto* $^{-/-}$ embryos. Control beads were soaked only in PBS and implanted in the same manner. After 24 hours of culture, explants were fixed in 4% paraformaldehyde in PBS and processed for successive *in situ* hybridization.

In situ *hybridization*

RNA antisense probes were synthesized from linearized plasmid in the presence of Digoxigenin-UTP (Boehringer Manheim) or Fluorescein-UTP (Boehringer Manheim). The *in situ* hybridization technique was performed according to Liguori and coworkers (2003).

Real time RT-PCR analysis

Wild-type and *Cripto* $^{-/-}$ embryos were used for analysis of mRNA quantities. The region situated at the midbrain-hindbrain boundary (M-HB) of five wt and ten *Cripto* $^{-/-}$ embryos at 8.5 dpc was dissected. In the wt embryo the M-HB is easily detectable by looking at the constriction ridge of the isthmus, due to lower proliferation rate than the surrounding tissues (Martinez *et al.*, 1991; 1995). On the other hand, in the *Cripto* $^{-/-}$ embryo the M-HB is at the boundary between the embryonic and extraembryonic regions, which have a different consistency and are separated by an embryonic constriction (Liguori *et al.*, 2003). The tissue was homogenized into Qiagen lysis buffer by passing it ten times through a 0.9 mm needle attached to a sterile plastic syringe. Total RNA from each M-HB was extracted using Rneasy Micro Kit (Qiagen) according to the manufacturer's instructions and quantitated by NanoDrop-1000 Spectrophotometer. cDNA synthesis was achieved by using the Quanti Tect Reverse Transcription kit (Qiagen). Real Time PCR was performed using two primer sets produced by Quanti Tect Primer Assay (Qiagen) (QT00108773 for *Fgf8*; QT00166768 for *Hprt*). The primers for Fgf8 (Qiagen) amplify a fragment of 114 bp spanning the exons 5 and 6, while the primers for *Hprt* amplify a fragment of 168 bp spanning exons 3, 4 and 5 of the gene. The reactions were conducted according to the protocol of the Fluo Cycle SYBR Green mix (Euro Clone). The PCR protocol involved a denaturation program (95° for 15 min), followed by an amplification and quantitation program repeated 34 times (95° for 15 sec, 58° for 20 sec, 72° for 20 sec), a melting curve program (65°C – 95°C, with a heating rate of 0.5°C per second and continuous fluorescence measurement). The relative quantitation of gene expression was analyzed by the 2-ddCt method. To normalize the output for each sample, the expression of *Fgf8* gene was divided by *Hprt* gene expression. The results are representative of three independent experiments. The one-way ANOVA test was used to analyze the data from all experiments and $P<0.0001$ was considered significant.

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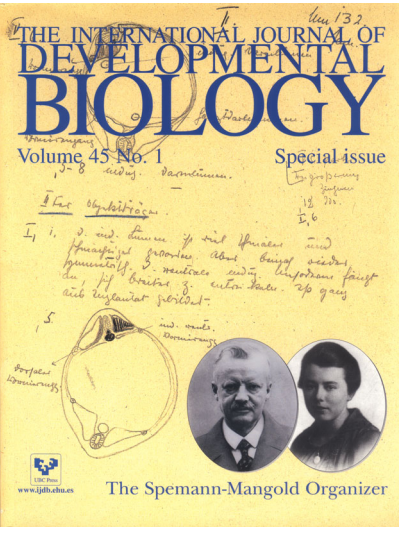

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