ABSTRACT
The facial neural crest (FNC), a pluripotent embryonic structure forming craniofacial structures, controls the activity of brain organisers and stimulates cerebrum growth. To understand how the FNC conveys its tropic effect, we have studied the role of Smad1, which encodes an intracellular transducer, to which multiple signalling pathways converge, in the regulation of Foxg1. Foxg1 is a transcription factor essential for telencephalic specification, the mutation of which leads to microcephaly and mental retardation. Smad1 silencing, based on RNA interference (RNAi), was performed in pre-migratory FNC cells. Soon after electroporation of RNAi molecules, Smad1 inactivation abolished the expression of Foxg1 in the chick telencephalon, resulting in dramatic microcephaly and partial holoprosencephaly. In addition, the depletion of Foxg1 activity altered the expression Otx2 and Foxa2 in di/mesencephalic neuroepithelium. However, when mutated forms of Smad1 mediating Fgf and Wnt signalling were transfected into FNC cells, these defects were overcome. We also show that, downstream of Smad1 activity, Dkk1, a Wnt antagonist produced by the FNC, initiated the specification of the telencephalon by regulating Foxg1 activity. Additionally, the activity of Cerberus in FNC-derived mesenchyme synergised with Dkk1 to control Foxg1 expression and maintain the balance between Otx2 and Foxa2.

KEY WORDS: Neural crest, Telencephalic hemispheres, RNAi, Holoprosencephaly, Electroporation, Chick

INTRODUCTION
The telencephalon is the most evolutionarily recent part of the central nervous system (CNS), and represents a salient trait of the vertebrate phylum. The specification of the telencephalon primarily relies on the transcription factor Foxg1 (Tao and Lai, 1992; Xuan et al., 1995; Hanashima et al., 2004; Martynoga et al., 2005; Manuel et al., 2010), which is expressed in the prosencephalic neuroepithelium and is involved in segregating the telencephalon from the diencephalon. Investigations directed towards understanding forebrain regionalisation have demonstrated the importance of the anterior neural ridge (ANR) as the ‘prosencephalic brain organiser’, through the production of Fgf8 signal (Shimamura and Rubenstein, 1997; Houart et al., 1998; Shammugalingam et al., 2000; Storm et al., 2006). Removal of ANR completely inhibits Foxg1 expression and leads to the loss of telencephalic structures. Deficits can be overcome by supplying an exogenous source of Fgf8 (Shimamura and Rubenstein, 1997). Other brain-organising centres secreting Wnt and Shh molecules along the dorsal and ventral midlines are also required for brain development (McMahon and Bradley, 1990; Kim et al., 2001; Chang et al., 2004; Danesin et al., 2009; Vieira et al., 2010). Collectively, these factors generate a matrix of combinatorial morphogenetic factors, which governs planar specification and imparts a certain degree of molecular regionalisation to the cerebral neuroepithelium (Echevarria et al., 2003; Hoch et al., 2009).

Embryological studies have shown that the cephalic neural crest, which develops concomitantly to the formation of the CNS in vertebrates, is essential for brain development (Creuzet, 2009; Le Douarin et al., 2012). Neural crest cells originating from the posterior diencephalon down to rhombomere 2 (r2) provide the skeletogenic cells that build the facial structures and generate the musculo-connective cells lining the endothelium of blood vessels of the face and forebrain. This domain is here designated as FNC (facial neural crest). Absence of FNC prevents the formation of facial structures and also results in anencephaly: agenesis of telencephalic vesicles, thalamic and pre-tectal nuclei ensue (Creuzet et al., 2004, 2006). Brain defects occurring in the absence of the FNC stem from perturbations in the morphogenetic activities of secondary brain organisers: FNC ablation is promptly followed by loss of Fgf8 in the ANR, loss of Wnt activity dorsally, and expansion of Shh ventrolaterally (Creuzet et al., 2006). It turns out that, during their migration, FNC cells shield and protect the brain against the detrimental effects of bone morphogenetic proteins (Bmps) secreted by neighbouring tissues, and promote Fgf8 activity in ANR (Creuzet, 2009). However, to a large extent, the repertoire of the FNC-produced molecules required for brain patterning remain unknown.

To explore this mechanism further, we have focused our interest on Smad1, which is expressed in pre-migratory FNC cells. Smad1 acts like a ‘platform’ upon which multiple signals converge: it is a transducer of the Bmp pathway and it mediates Fgf and Wnt signalling (Gont and Lough, 2000; Fuentealba et al., 2007). Here, we show that Smad1 silencing in chick FNC cells inhibits Foxg1 activity in the telencephalon and causes a severe microcephaly and partial holoprosencephaly. At the di/mesencephalic level, Smad1 silencing reduces Otx2 expression and triggers a concomitant expansion of Foxa2 ventrolaterally, molecular changes that are attributable to the loss of Foxg1 in the telencephalon. We have sought downstream effectors and identified two modulators of the Wnt pathway, Dkk1 and Cerberus. Analysis of their epistatic relationships shows that these molecules, produced by the migratory FNC cells, cooperate under the control of Smad1 activity to control Foxg1 expression in the telencephalon and regulate the balance between Otx2 and Foxa2 at the di/mesencephalic level.

RESULTS
Smad1 is expressed in pre-migratory FNC cells
Smad1 gene expression pattern was analysed by whole-mount in situ hybridisation in chick embryos from the neurula stage.
Smad1 expression was rapidly switched off in FNC cells that had emigrated from the cephalic NF (Fig. 1D). Caudally, where the neural plate was still open, the accumulation of Smad1 transcripts was intense. Later on, Smad1 expression was observed in the FNC-derived mesenchymal cells that had colonised naso-frontal and maxillo-mandibular buds at 26ss.

**Smad1 silencing in FNC cells results in morphological defects of brain development**

To unravel the role of Smad1 expression in FNC cell on brain development, double-stranded RNA molecules were designed against chick Smad1 (dsSmad1), and bilaterally electroporated into the FNC cells at 4ss, when Smad1 expression is maximal in the cephalic NF. FNC cells were specifically transfected, as attested by the co-electroporation of Rhodamine Dextran and a GFP construct (Fig. 1E-H). At 8ss, FNC cells have migrated laterally, but the telencephalic territory is devoid of GFP labelling (Fig. 1I). At 12ss, the transfected FNC cells that have emigrated from the neural plate border are GFP positive, but the roof plate and the alar plate of the brain are GFP negative (Fig. 1J). The efficacy of Smad1 silencing was tested by whole-mount hybridisation (Fig. 1K-N). Ninety minutes after electroporation (i.e. 5ss), Smad1 expression was abolished in FNC cells (Fig. 1K,L), but the expression of other Smad genes, such as Smad5, were unaffected (Fig. 1M,N). At 22ss, GFP-labelled FNC cells have colonised the naso-frontal and maxillo-mandibular buds (Fig. 1O). Co-immunostaining with HNK1 and anti-phosphorylated histone3 (pH3) antibodies to demonstrate FNC cell migration and cell proliferation, respectively, confirmed that Smad1 silencing did not affect the capacities of FNC cells to populate the facial processes (Fig. 1P,Q).

![Smad1 expression diagram](image)

The morphological changes after Smad1 silencing were observed at 26ss. Compared with controls, we noted that repression of Smad1 activity in FNC cells caused severe fore- and midbrain malformations (n=76; Fig. 2A,B). Cephalic vesicles were atrophied and the subdivision of prosencephalon into tel- and diencephalon was no longer visible (Fig. 2B, arrowheads). At embryonic day (E) 4 (n=14), head morphogenesis in experimental embryos was affected by severe telencephalic atrophy: the development of the nasofrontal and mandibular processes was reduced. Notably, the telencephalic territory displayed a partial holoprosencephaly (Fig. 2C,D, asterisk). To characterise brain defects further, morphometric analysis was performed by measuring the impact of Smad1 silencing on the growth...
of tel-, di- and mesencephalic vesicles (Fig. 2E). The microcephalic phenotype was particularly pronounced anteriorly, where the telencephalic hemispheres only represented 40% of the normal size (Fig. 2C). In addition, the optic vesicles were also hypoplastic in dsSmad1-electroporated embryos.

Induction of long-term defects in cephalic development by Smad1 loss of function in FNC cells at E8 (Fig. 2F,G) were examined through structural staining of coronal sections (Fig. 2H,I). Telencephalic hemispheres exhibited severe disorganisation: the lateral and third ventricles were collapsed and the choroid plexi were hypoplastic (Fig. 2H), and the septal region was atrophied (Fig. 2I, asterisk). Para-sagittal sections revealed that the ventrolateral part of the pre-otic brain, from the thalamus down to the level of the anterior rhombencephalon was considerably expanded in dsSmad1-treated embryos compared with controls (Fig. 2J,K, asterisk). In addition, on mesial sections, in the olfactory bulbs, the olfactory nerves (Fig. 2L, arrows) were missing (Fig. 2M, asterisk). Altogether, these observations showed that repressing Smad1 activity in FNC cells had adverse effects on brain development.

To show that the morphological defects were specifically due to Smad1 inactivation, we tried to rescue cephalic development by co-electroporating dsSmad1 with a construct driving the expression of a wild-type form of human SMAD1 (hSmad1). We first tested the innocuity of the hSmad1 construct on cephalic development by transfecting this plasmid alone. We observed that hSmad1 did not cause any alteration of head morphogenesis at 24ss (dsSmad1/hSmad1; n=22). In a next step, hSmad1 construct was co-electroporated with dsSmad1 in the FNC at 4ss (n=42). Twenty-four hours post-transfection, dsSmad1/hSmad1 embryos showed a significant rescue of their cephalic development compared with dsSmad1-electroporated embryos (Fig. 3). Hence, the heterospecific restoration of Smad1 activity was sufficient to overcome defects resulting from Smad1 silencing.
**Smad1 silencing in FNC cells entails molecular changes in brain development**

In our experiments, perturbations of brain development in dsSmad1-depleted embryos were first observed at 26ss. To elucidate the molecular mechanisms involved in cephalic hypoplasia, expression of transcription factors involved in brain patterning were analysed at 26ss. Foxg1, which is the earliest marker of telencephalic vesicles (Xuan et al., 1995; Chapman et al., 2002), was strongly expressed in control embryos (Fig. 3A). Knocking down Smad1 abrogated its expression (Fig. 3B, red arrowhead; n=30), but the co-electroporation of a dsSmad1 and hSmad1 construct normalised Foxg1 activity and forebrain development (Fig. 3C; n=25).

Otx2 expression, which characterises the anterior neural plate at neurula stage (Acampora et al., 1998; Plouhinec et al., 2005), is detected in the telencephalon, and in the alar plate of the di- and mesencephalon at 26ss (Fig. 3D). In absence of Smad1 in FNC cells, Otx2 domain expression was reduced and limited at the dorsal midline of the di- and mesencephalon (Fig. 3E; n=20). Strikingly, Otx2 expression was completely lost in the telencephalic vesicles of Smad1-silenced embryos. Electroporation of the hSmad1 construct tended to normalise Otx2 activity in the fore- and midbrain (Fig. 3F; n=15). We also looked at the expression of Foxa2, a marker of di- and mesencephalic basal plate (Ruiz i Altaba et al., 1995) (Fig. 3G). In dsSmad1-electroporated embryos, Foxa2 expression was considerably expanded dorsally (Fig. 3H; n=18), and also upregulated in foregut endoderm. When Smad1 activity was restored by the co-electroporation of hSmad1, its expression was restricted to the basal plate (Fig. 3I; n=16). Six3 is strongly
expressed along the anteroventral midline of the telencephalon in normal embryos (Geng et al., 2008). Six3 expression was not significantly perturbed by the loss of function of Smad1 in FNC cells (in comparison with the absence of Foxg1 activity) or by the co-electroporation of hSmad1 (Fig. 3J-L). We also looked at the expression of Pax6, as a marker of the retinal primordium (Waltier and Gruss, 1991), transcripts of which are accumulated in the optical vesicles and in the epithalamus (Fig. 3M). In the absence of Smad1, the hypoplasia of developing eyes coincided with severe reduction of Pax6 (Pax6Δ=11%), Foxg1 (Foxg1Δ=75%) and Otx2 (Otx2Δ=55%). By contrast, in Smad1-silenced embryos, Foxa2 expression was significantly increased up to 1.5-fold compared with controls. As expected from whole-mount hybridisations, in embryos co-electroporated with a dsSmad1 and hSmad1 construct, gene expression was restored to the levels detected in controls. To see if cellular events could account for the molecular changes, whole-mount detection of cell death was performed. In controls, punctate Lysotracker Red (LTR) labelling was scarcely observed in the optic vesicles and in the epithalamus (Fig. 3N; n=12), but this default was rescued by co-electroporation hSmad1 (Fig. 3O; n=10). Molecular changes occurring in cephalic vesicles after Smad1 silencing in FNC cells were quantified by qRT-PCR performed on developing heads 20 h post-electroporation (Fig. 3P). The quantification of gene expression revealed that dsSmad1 altered the activity of Pax6 (Pax6Δ=11%), Foxg1 (Foxg1Δ=75%) and Otx2 (Otx2Δ=55%). By contrast, in Smad1-silenced embryos, Foxa2 expression was significantly increased up to 1.5-fold compared with controls. As expected from whole-mount hybridisations, in embryos co-electroporated with a dsSmad1 and hSmad1 construct, gene expression was restored to the levels detected in controls. To see if cellular events could account for the molecular changes, whole-mount detection of cell death was performed. In controls, punctate Lysotracker Red (LTR) labelling was scarcely observed in the retroocular region, where FNC cells migrate (Fig. 3Q). In dsSmad1-treated embryos, LTR staining was considerably expanded (Fig. 3R), but electroporation with hSmad1 alleviated cell death (Fig. 3S). Whatever the series, the dorsal aspects of the expanded (Fig. 3R), but electroporation with dsSmad1 retroocular region, where FNC cells migrate (Fig. 3Q). In

Changes in signalling triggered by Smad1 silencing

Perturbations in the expression of transcription factors suggested that crucial changes in the morphogenetic activity of ‘secondary brain organisers’ had occurred in absence of Smad1 activity in FNC cells. Production of Fgf8 in the ANR has been shown to play a leading role in telencephalic patterning (Shimamura and Rubenstein, 1997; Houart et al., 1998; Shanmugalingam et al., 2000). In controls, Fgf8 domain of expression is maximal at 26ss. In dsSmad1-electroporated embryos, a weak reduction of its domain of expression was observed (Fig. 4A-C; n=15). Bmp4 expression, normally present in the superficial naso-frontal ectoderm surrounding the ANR (Fig. 4D), was upregulated in the retroocular (i.e. presumptive maxillary) region after dsSmad1 silencing (Fig. 4E,F; n=10). In the basal plate, Shh is the major signal to be produced and has a deep impact on brain patterning (Vieira and Martinez, 2006). In dsSmad1-treated embryos, Shh expression was not reduced, but was maintained in proportion with the global prosencephalic hypoplasia (Fig. 4G-I; n=12).

At the dorsal midline, Wnt signalling is essential for fore- and midbrain patterning (Erter et al., 2001; Braun et al., 2003; Creuzet et al., 2004). Wnt1, which is expressed at the dorsal midline and by a strand of cells adjacent to the isthmus, was reduced dorsally in dsSmad1-electroporated embryos (Fig. 4J-L, arrowheads). Wnt3a, which has an overlapping domain of expression with Wnt1 at the midline, was absent in dsSmad1-electroporated embryos (Fig. 4M-O, arrowheads; n=13). Wnt8b, expression of which dorsally straddles...
the anterior diencephalon and posterior telencephalon, was decreased in both vesicles after Smad1 silencing (Fig. 4P-R; n=12). Changes in gene expression were quantified by qRT-PCR (Fig. 4S): Smad1 silencing significantly affected the activity Fgf8 (Fgf8Δ=60%), Wnt1 (Wnt1Δ=40%), Wnt3a (Wnt3aΔ=80%) and Wnt8b (Wnt8bΔ=30%). Therefore, these results indicated that Smad1 silencing primarily affected Fgf and Wnt production in the developing brain.

Foxg1 loss of function reproduces Smad1 silencing in the FNC

To explore whether the loss of Foxg1, on its own, can have an effect on the expression of Otx2 and Foxa2, Foxg1 expression was silenced in the telencephalon, specifically, leaving FNC cells unperturbed. dsRNA designed against Foxg1 (dsFoxg1) was bilaterally electroporated at 5ss (Fig. 5A). At 26ss, Foxg1 activity was abrogated in the prosencephalon (Fig. 5D,E; n=18); additionally, Otx2 expression was limited to the roof-plate (Fig. 5H,I, red brackets; n=12) and Foxa2 expression expanded in the lateral aspects of the di- and mesencephalon (Fig. 5L,M, red brackets; n=13). So, Foxg1 silencing in telencephalon interfered with the patterning of more caudal structures, the thalamus and the optic tectum through the regulation of Otx2 and Foxa2. This condition virtually recapitulated the molecular changes resulting from Smad1 inactivation in FNC cells (compare with Fig. 3). These observations indicated that, in normal development, the regulation of Foxg1 activity is central to the molecular patterning of the pre-otic brain.

Dissecting the interactions involved in Foxg1 expression

Current understanding of prosencephalon development suggests a direct relationship between Fgf8 activity from the ANR and the induction of Foxg1 expression in the ANR and its expansion to the entire telencephalon (Shimamura and Rubenstein, 1997; Houart et al., 1998; Hoch et al., 2009; Anderson et al., 2002). Therefore, we tested if supplying Fgf8 could bypass the effect of Smad1 silencing and rescue Foxg1 expression in dsSmad1-electroporated embryos. Beads soaked with Fgf8 recombinant protein (0.125 µg/µl) were implanted in contact with the ANR in a 4ss embryo subjected to Smad1 silencing (Fig. 5B). This concentration has been previously shown to restore cephalic development efficiently when endogenous expression of Fgf8 in ANR was abolished after FNC ablation (Creuzet et al., 2006). Here, despite Fgf8 supplementation, no restoration of telencephalic development occurred and Foxg1 expression was absent, as in Smad1-silenced embryos (Fig. 5D,F; n=9). In addition, the domain of Otx2 expression exhibited no restoration and remained similar to that in dsSmad1-electroporated embryos, and Foxa2 expression did not
tend to normalise (Fig. 5H,I,L,N; n=9). Altogether, our results indicated that Foxg1 activity in the telencephalon does not solely depend on the production of Fgf8 in ANR, but relies on a more complex mechanism, which involves FNC cells, for its induction and expansion.

**Stimulating Smad1 activity in FNC cells can rescue Foxg1 expression in the telencephalon**

To decipher the mechanisms responsible for Foxg1 expression in the telencephalon, we performed surgical ablation of the ANR at 5ss, before FNC cell migration (Fig. 5C,C). At 26ss, the morphology of the telencephalon was atrophied, and Foxg1 expression was abolished in ANR-ablated embryos (Fig. 5G), as previously reported (Shimamura and Rubenstein, 1997). Additionally, Otx2 and Foxa2 expressions were perturbed and displayed a pattern similar to that observed in dsSmad1- and dsFoxg1-electroporated embryos (Fig. 5I,K,M,O; compare with Fig. 3E,H).

To examine if FNC cells could regulate Foxg1 expression in the telencephalon independently of the production of Fgf8 in the ANR, we transfected mutated forms of hSmad1 in FNC cells to rescue the molecular patterning of the pre-otic brain in ANR-ablated embryos (Fig. 5P). These constructs have been previously designed to mimic constitutive activation in the linker region for the Mapk (hSmad1-MM) and Gsk3 (hSmad1-GM) pathways, respectively (Fuentealba et al., 2007). When electroporated in FNC cells of ANR-ablated embryos, these constructs generated a potent restoration of Foxg1 activity in the telencephalon (Fig. 5Q,R). Hence, the regulation of Smad1 activity by the Fgf and Wnt pathways in FNC cells is essential to establish the domain of Foxg1 expression in the developing prosencephalon.

**Smad1 regulates the activity of Bmp antagonists in FNC cells**

Previously, we have demonstrated that FNC cells play a crucial role in brain development by counteracting Bmp activity through the production of Bmp inhibitors (Creuzet, 2009). Noggin, a potent antagonist of Bmps is expressed in FNC cells before their migration and in FNC-derived mesenchyme of the naso-frontal and the retroocular region (Tzahor et al., 2003). In Smad1-silenced embryos, a strong reduction of Noggin activity was shown by RT-PCR at 26ss (Fig. 6A,B). This change was correlated with the loss of Foxg1 and the misexpression of Otx2 and Foxa2 (Fig. 6B). RT-PCR and whole-mount hybridisation also revealed that Smad1 silencing affected Noggin activity: its expression was downregulated as early as 5ss (2 h after dsSmad1 electroporation; Fig. 6C,D). In the same way, Gremlin, another potent Bmp inhibitor (Merino et al., 1999), was also downregulated after Smad1 silencing (Fig. 6E,F). Altogether, these observations showed that Smad1 expression in FNC was crucial for the regulation of Bmp inhibitors from neurula stage up to 26ss.

To test the possible role of Bmp signalling in regulating Foxg1 expression, the activity of Noggin and Gremlin was simultaneously silenced (dsNoggin/dsGremlin) in the FNC at 4ss. The joint inactivation of Noggin and Gremlin resulted in loss of Foxg1 expression, together with misexpression of Otx2 and Foxa2 (Fig. 6G-N; n=9). These experiments indicated that loss of Noggin and Gremlin expression in FNC cells are involved in the molecular changes consecutive to Smad1 silencing, therefore showing that Smad1 expression controls the expression of Bmp antagonists produced by migratory FNC cells.

We then tried to rescue Smad1 silencing with the joint electroporation of retroviral RCAS constructs driving Gremlin and Noggin expression (Fig. 6O). As a consequence, the cephalic
Brain development, and act downstream of this process. Another consequence of Dkk1 expression in the FNC is subjected to silencing. Altogether, these experiments bring compelling evidence (Fig. 6P,Q; holoprosencephalic vesicle, but developed as two hemispheres development tended to normalise: the telencephalon was no longer a cephalic structures (Mukhopadhyay et al., 2001). In chick, Wnt inhibitor, is required for the development of the anterior arches, which were left unperturbed difference in the level of expression of Dkk1, a potent Wnt inhibitor, is required for the development of the anterior cephalic structures (Mukhopadhyay et al., 2001). In chick, Dkk1 transcripts were detected in the NF and emigrating FNC cells (Fig. 7B-E), then became evenly distributed in the FNC-derived mesenchyme populating facial processes, thus leading to homogeneous staining of the developing head at 26ss (Fig. 7F). In Smad1-silenced embryos, increased activity of Dkk1 was also demonstrated by in situ hybridisation: embryos exhibited a sharp difference in the level of Dkk1 expression between the pre-otic head and the more caudal branchial arches, which were left unperturbed after Smad1 silencing (Fig. 7G, dashed line; n=7). The upregulation of Dkk1 was further confirmed by qRT-PCR analysis at 26ss (Dkk1Δ=1.8-fold higher than in control; Fig. 7I).

To understand the functional significance of Dkk1 upregulation for brain patterning, a construct driving Dkk1 expression (Dkk1-RCAS) was transfected into FNC cells at 5ss. Under this condition, Dkk1 expression was further confirmed by qRT-PCR analysis at 26ss (Dkk1Δ=1.8-fold higher than in control; Fig. 7I).

To explore the epistatic relationship between Smad1 and Dkk1 activity, we tried to restore brain patterning by forcing the expression of hSmad1 in combination with Dkk1-RCAS in FNC cells at 5ss. When analysed at 26ss, embryos showed a significant rescue of Foxg1, Otx2 and Foxa2 expression (Fig. 6 and Fig. 7L; n=9,7,8, respectively) as those triggered by Smad1 silencing, thus indicating that changes in Dkk1 activity in dsSmad1 embryos accounted for the defects in brain patterning.

Fig. 7. Control of Dkk1 expression by Smad1 is essential for telencephalic development. (A) RT-PCR analysis of Dkk1, Dkk2, Dkk3 gene expression in control and dsSmad1-treated embryos. (B-E) Dkk1 is expressed in the head process at 2ss (B) and in the FNC and surface ectoderm at 5ss (C). Dkk1 is expressed in migratory FNC cells at 7ss (D; arrow) and in FNC-derived mesenchyme at 26ss (E). (F-H) Lateral view showing hybridisation for Dkk1 in control (F), dsSmad1-transfected (G) and dsSmad1/hSmad1-transfected (H) embryos. Dashed line in G delineates the distinct levels of Dkk1 expression in pre-otic versus post-otic territories. (I) Quantification of Dkk1 expression in control (blue), dsSmad1-treated (red) and dsSmad1+hSmad1-treated (green) embryos (mean±s.e.m.; *P<0.0125). (J) RT-PCR showing expression of Foxg1, Fgf8, Noggin, Otx2 and Foxa2 in control and dsSmad1-, dsSmad1+hSmad1-, Dkk1-RCAS-transfected embryos. Gapdh is used as a normalizing gene. (K-P) Side view of hybridisation for Foxg1 (K,N), Otx2 (L,O) and Foxa2 (M,P) in Dkk1-RCAS-electroporated (K-M) and Dkk1-RCAS/hSmad1-electroporated (N-P) embryos. (Q-S) Para-sagittal sections of Dkk1-RCAS/hSmad1-co-electroporated embryos showing rescued brain at E8. (R) The olfactory bulb (OB) and nerve (ON) have developed. (S) Along the diencephalic roof plate, choroid plexi (Ch Pi) and epiphysis (Ep) have formed. LatV, lateral ventricle; 3°V, third ventricle.
Regulation of Cerberus expression by Dkk1 is required for Otx2 and Foxa2 expression

In Smad1-depleted embryos, the activity of Cerberus, which is known to interfere with Bmp and Wnt signalling (Bouwmeester et al., 1996), was examined at 26ss; its transcripts are normally accumulated in FNC cells that populate the retro- and peri-ocular region (Fig. 8A). After Smad1 silencing, Cerberus activity was totally inhibited (Fig. 8B; n=10). Similarly, when Dkk1 was over-expressed in FNC cells, the expression of Cerberus totally disappeared from the retroocular region (Fig. 8C; n=8). Together, these experiments indicated that Smad1 expression in FNC impinges on Dkk1 activity, which, in turn, interferes with Cerberus expression.

To see if the loss of Cerberus activity can have an effect on brain patterning, we tried to rescue the defects generated by Smad1 silencing on brain development by injecting Cerberus protein. After electroporation of dsSmad1 molecules at 4ss (Fig. 8D), we injected a solution of Cerberus recombinant protein (0.1 µg/µl) contrasted with Rhodamine Dextran at 12ss (Fig. 8D,E). Injection was performed bilaterally in a sub-ectodermal location in order to deliver Cerberus protein into the migration routes of FNC cells.

Supplying dsSmad1-treated embryos with Cerberus resulted in a potent rescue of the morphological defects: development of the pre-otic vesicles was significantly restored. At 26ss, the expression of Foxi1 tended to normalise (Fig. 8F,G; n=9). Furthermore, Otx2 and Foxa2 expression nearly recovered a normal pattern in the di- and mesencephalon (Fig. 8H-K). Hence, Cerberus activity in FNC cells is required for Foxi2 regulation in the telencephalon and maintains Otx2 and Foxa2 expression at di/mesencephalic level.

Fig. 8. Cerberus supplementation overcomes brain defects of Smad1 silencing. (A-C) Side view of hybridisation for Cerberus in control, dsSmad1- and Dkk1-RCAS-transfected embryos at 26ss. (A,”A”) Vibratome section showing Cerberus expression in peri-ocular mesenchyme at 26ss. (D) Smad1 silencing in the FNC at 4ss followed by bilateral injection of Cerberus protein into FNC-derived mesenchyme at 12ss. (E,E’) Cerberus protein contrasted with Rhodamine Dextran. (F-K) Hybridisation for Foxi1 (F,G), Otx2 (H,J) and Foxa2 (J,K) in control and dsSmad1-transfected+Cerberus-injected embryos. Note the significant rescue of the gene expression in the developing forebrain after Cerberus injection. (L) Model of interactions adapted from Shimamura and Rubenstein (Shimamura and Rubenstein, 1997) suggesting a direct induction of Foxi1 in the neurepithelium by Fgf8 from the ANR. (M) Proposed model for the regulation of Foxi1 expression by the FNC cells. For clarity, anatomical structures are on the left and interactions are on the right. Fgf8 from the ANR interacts with Smad1 in FNC cells, which controls Dkk1 expression, acting upstream of Noggin and Gremlin for the induction of Foxi1 expression in the telencephalon. Foxi1 expression maintains the balance between Otx2 and Foxa2. Dkk1 also controls Cerberus activity in the retroocular region, which regulates Otx2 and Foxa2 expression in the di- mesencephalon. Positive and negative regulations are indicated with solid and dashed lines, respectively. Regulations originating from the neurepithelium are in dark blue; regulations by the FNC cells are in red. ANR, anterior neural ridge; AP, alar plate; BP, basal plate; Di, diencephalon; FNC, facial neural crest; Gremlin, Gremlin; Mes, mesencephalon; Noggin, Noggin; not, notochord; pcp, pre-chordal plate; Pro, prosencephalon; Rh, rhombencephalon; Tel, telencephalon.

DISCUSSION

In this work, we have explored the role of the FNC in brain development by knocking down the expression of a signalling transducer, Smad1, which complexes with specific DNA-binding proteins to regulate gene expression. Smad1 belongs to a large family of cytoplasmic mediators for which translocation to the nucleus is triggered by the activity of Tgf𝛽 signalling. Smad1 expression, strongly detected in the FNC at the neural plate border, declines while FNC cells migrate, then increases in intensity and persists in the FNC-derived mesenchyme populating facial processes until E5.

To investigate the molecular mechanisms by which Smad1 expression in the FNC affects the developmental programme of cephalic vesicles, we silenced its activity, when its expression is maximal in FNC cell population. dsRNA-driven silencing had efficiently inhibited Smad1 activity in FNC cells by 1.5 hours following electroporation, and generated conspicuous defects in brain development 24 h later (at 25ss). These defects included microcephaly, which encompasses fore- and midbrain but was particularly pronounced at telencephalic level. The telencephalon dramatically drops to 40% of its normal size. Hemispheres display partial holoprosencephaly, with a vestigial septal region and atrophied choroid plexi. Additionally, Smad1-depleted embryos display severe microphthalmia.

These early morphological defects coincide with the downregulation of Fgf8 in the ANR and the complete loss of Foxi1 expression in the prosencephalon. Foxi1 is an early marker of cephalic development, which features the anterior neural plate at gastrula stage in chordates (Merino et al., 1999; Toresson et al., 1998). However, the expansion of its expression to the entire telencephalon is specific to vertebrates.

The effect of ANR in telencephalic specification was first investigated by explants and microsurgical experiments showing the leading role played by this discrete territory in forebrain
specification (Shimamura and Rubenstein, 1997) (Fig. 8L). The morphogenetic action of what was then referred to as the ‘prosencephalic organiser’ was assigned to the production of *Fgf8* at this level (Shimamura and Rubenstein, 1997; Houchart et al., 1998; Shammugalingam et al., 2000; Storm et al., 2006). The cooperation between *Fgf8* and *Foxg1* allows for the telencephalon to become secondarily specified from the primary prosencephalon (Rubenstein et al., 1998; Rubenstein and Beachy, 1998; Vieira and Martinez, 2006).

The cerebral defects encountered in our *Smad1*-depleted embryos virtually reproduce the anomalies encountered in *Foxg1* mutants (Xuan et al., 1995). However, in *Smad1*-deprived embryos, supplementation with *Fgf8* recombinant protein has no effect on *Foxg1* expression and telencephalic development. This indicates that the morphogenetic regulation exerted by FNC cells is crucial for specification of the rostral prosencephalon by stimulation of *Foxg1* expression.

To unravel the involvement of the FNC in the regulation of *Foxg1*, we performed ablation of the ANR at 5ss, before the onset of FNC cell migration: the predictable loss of *Foxg1* expression ensued. By contrast, when mutated forms of Smad1, which mimic the activation of the *Fgf8* or Wnt pathways, were transfected into the FNC cells at the time of ANR ablation, a potent rescue of telencephalic development occurred together with restoration of *Foxg1* expression.

Additionally, misregulation of *Foxg1* in the telencephalon was accompanied by perturbations in *Otx2* and *Foxa2* activity in thalamus and tectum, where their expression pattern characterises the alar and basal plate, respectively. *Otx2* is an important marker of dorsal patterning of the fore- and midbrain (Kimura et al., 2001, 2005). Its activity is antagonised by the expression of *Foxa2* in the di/mesencephalic basal plate (Weinstein et al., 1994; Nakano et al., 2000).

In the absence of the FNC, a considerable expansion of *Shh* expression occurs along with a conspicuous ventralisation of the cephalic neuroepithelium (Creuzet et al., 2004). Similarly in the present experiments, *Smad1* silencing also results in a potent ventralisation of the pre-otic brain, as attested by the thickening of the ventral structures at E8. This early phenotype coincides with the lateral expansion of *Foxa2* at the expense of *Otx2* expression, dorsally. Surprisingly, the domain of *Shh* expression remains unchanged whatever the experimental conditions examined. Hence, *Smad1* depletion in FNC decouples *Foxa2* and *Shh* expression in the di- and mesencephalic floor plate, and unmask the divergent regulation of these two ‘basal’ markers.

Compared with the phenotype of *dsSmad1*-treated embryos, the outcome of cerebral structures in *Foxg1*-depleted embryos was similar, indicating that the molecular changes in *Otx2* and *Foxa2* primarily stem from the downregulation of *Foxg1*. So, our data uncover an unappreciated role of *Foxg1* in the patterning of the thalamus and tectum through the regulation of *Otx2* and *Foxa2*. These observations reveal that these two genes are subjected to regulation exerted by FNC cells is crucial for specification of the rostral prosencephalon by stimulation of *Foxg1* expression.

In *in ovo* electroporation, Electroporation was achieved using a triple electrode system placed on the vitelline membrane with the two cathodes flanking the developing head with a gap of 5 mm, and the anode facing the anterior neuropore (Creuzet et al., 2002). Before electroporation, nuclear acids were contrasted with a solution of 0.01% Fast Green FCF (Sigma) to control the site of injection, then headed for the targeted tissues by a series of five square pulses of 27 V (T830 BTX, Genetronics). After electroporation, eggs were sealed and incubated at 38°C until reaching the stage required for analysis.

### Nucleic acid preparation

Gene silencing was performed by electroporating dsRNA. In mammals, dsRNA yields a non-specific blockade of protein synthesis (Billy et al., 2001; Elbashir et al., 2001). However, in chick, no such effects were ever detected; dsRNA electroporation resulted in highly specific gene inactivation (Pekarik et al., 2003; Stoeckl, 2003). For each series, efficiency of the silencing was analysed *in situ* hybridisation, RT-PCR and qRT-PCR.

Sense and antisense strands of RNA were synthesised from the cDNA encoding for the targeted gene (Pekarik et al., 2003). After elimination of the cDNA template, single strands were purified and annealed for 5 min at 95°C. dsRNA against *Smad1*, *Foxg1*, *Noggin* and *Gremlin* (Gont and Lough, 2000; Bardot et al., 2001; Li and Vogt, 1993; Osorio et al., 2009) were used at 0.33 µg/µl, 0.4 µg/µl, 0.25 µg/µl and 0.25 µg/µl, respectively. In the control series, solutions of non- annealed sense and anti-sense RNA strands of the targeted genes were transfected at the same concentration as in the experimental series.

For gain-of-function experiments, the following plasmid and RCAS constructs were used: pCAGOS-GERP (Momose et al., 1999), *kSmad1* (human *SMAD1* wild type) at 1.1 µg/µl, *kSmad1-MM* (human *SMAD1* mutated in the three MAPK sites of the linker region) at 1.25 µg/µl, *hSmad1-GM* (human *SMAD1* mutated in the four GSK3 sites of the linker region) at 1.25 µg/µl (Fuentelba et al., 2007), *Dkk1-RCAS* at 1.1 µg/µl (Chang et al., 2004), *Gremlin* and *Noggin-RCAS* at 1 µg/µl (Creuzet, 2009). RCAS constructs used in this study allow for the overexpression of the insert but do not produce viral particles. The ectopic gene activity is therefore restricted to the FNC cells and their progenies.
Supplementation with recombinant proteins

Depending on the series, protein supplementation of dsSmad1-electroporated embryos was delivered by either injecting a solution or implanting soaked beads. A solution of Cerberus protein (0.100 µg/µl in PBS; R&D Systems) contrasted with 0.01% Rhodamine Dextran (Sigma) was bilaterally injected into the migration routes of FNC cells, adjacent to the di- and mesencephalic vesicles. For Fgf8 supplementation, beads were soaked in a solution of Fgf8 recombinant protein (0.125 µg/µl; R&D Systems) overnight at 4°C, then bilaterally implanted in contact with the ANR. This concentration has been shown to induce a potent rescue of head and brain development after FNC ablation (Creuzet et al., 2004).

In situ hybridisation

Whole-mount in situ hybridisations (Creuzet et al., 2006) were performed using the following probes: Bmp4 (Francis et al., 1994), Cerberus (provided by Dr Ricardo Garecz, Institut de Neurobiologie, France), Smad1, Smad5 (Gont and Lough, 2000), Fosq1 (Chapman et al., 2002), Sic3 (Geng et al., 2008), Pax6 (Walther and Gruss, 1991), Foam2 (Ruiz i Altavía et al., 1995), Fgf8 (Crossley et al., 1996), Gremlin, Noggin (Connolly et al., 1997), Ot2x (Bonaccielli et al., 1993), Shh (Riddle et al., 1993), Wnt1, Wnt3a (Hollyday et al., 1995), Wntbb (Garcia-Lopez et al., 2004).

Whole-mount staining and immunochemistry

FNC cell migration and cell proliferation were demonstrated by immunocytochemistry with monoclonal antibodies against HNK1 (CD57; Santa Cruz Biotechnology) and pH3 (Sigma), and detected with Alexa Fluor 488- and Alexa Fluor-594-labelled secondary antibodies (Life Technology, Invitrogen). Cell death was detected by Lysotracker Red (LTR; Invitrogen) staining prior to fixation (Garcez et al., 2014). Quantification of cell proliferation and death was performed on vibratome sections using ImageJ software.

Embryos were fixed in a solution of 60% ethanol, 30% formaldehyde (37%, stock solution) and 10% acetic acid (100%, stock solution), for 24 h, dehydrated in ethanol, permeabilised in toluene and embedded in paraffin. Cresyl Violet staining was performed on 7-µm-thick sections.

RT-PCR analysis and Dkk1 probe construction

Total RNA was isolated from 25ss pre-otic heads in Trizol Reagent (Invitrogen). cDNA was obtained with MMLV reverse transcriptase (Invitrogen) and oligoDT12-18 primers. Amplification was performed from 1 µg cDNA, using specific primer pairs for Fgf8 (For: cctctgcgggtgaaggtga; Rev: gcctgcgccgctcaagga), Foxg1 (For: cctcgctaccaatgcagg; Rev: gcggctgtgctgctatgg), Ot2x (For: acacaacgcttcggga; Rev: cccagacgcttttacacta), Fox2 (For: ccctgtacgccgcttggt; Rev: aaggaacagacggcagac), Noggin (For: agctgcaagaggaaggct; Rev: acatgcacgggccaacag), Dkk1 (For: gcacacgtggctgccaggt; Rev: cctcagcggagggctggt), Dkk2 (For: aggtta-
ctcgtcggctggt; Rev: aaggaacagacggcagac), Noggin (For: gcacacgtggctgccaggt; Rev: cctcagcggagggctggt), Dkk1 (For: gcacacgtggctgccaggt; Rev: tgcagcgtggcagccctga).

Quantitative real-time PCR (qRT-PCR) analysis of gene expression

Quantifications were performed on seven control, six dsSmad1-treated and seven rescued embryos. Primer pairs (Invitrogen) were designed specifically for each gene: Smad1 (For: acatactgacgccgctaat; Rev: gatctgctgg-
agaggcgct), Fgf8 (For: gcgtgctgggtacatgg; Rev: ggtgtggaagtttgcagtg), Foxg1 (For: ccacgcctccgctgtgct; Rev: tggagagtgaggtggtc), Ot2x (For: acacaacgcttcggga; Rev: cccagacgcttttacacta), Fox2 (For: ccctgtacgccgcttggt; Rev: aaggaacagacggcagac), Noggin (For: agctgcaagaggaaggct; Rev: acatgcacgggccaacag), Dkk1 (For: gcacacgtggctgccaggt; Rev: cctcagcggagggctggt), Dkk2 (For: aggtta-
tcgtcggctggt; Rev: aaggaacagacggcagac), Noggin (For: gcacacgtggctgccaggt; Rev: tgcagcgtggcagccctga).


Morphometric analysis

Brain growth was estimated on E4 embryos by measuring the distance from the optic stalk to the top of each cephalic vesicle: telencephalon, thalamus and optic tectum. Data from control, dsSmad1-electroporated and dsSmad1/ hSmad1-co-transfected embryos were compared using one-way ANOVA and analysed with the GraphPad Prism 4.0 software.

Competing interests

The authors declare no competing financial interests.

Author contributions

D.P.A. performed experiments and data analysis, and prepared the manuscript; S.G. performed experiments and data analysis; S.C. developed concepts and approaches, performed experiments and data analysis, and prepared the manuscript.

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