Independent regulation of Sox3 and Lmx1b by FGF and BMP signaling influences the neurogenic and non-neurogenic domains in the chick otic placode

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The development of neural tissue starts with the activation of early neural genes such as the SoxB1 transcription factors, which are expressed in response to signaling molecules. Neural progenitors in the inner ear are only generated in the anterior placodal domain, but the mechanisms that determine when and how otic neural fate is acquired are still unknown. Here, we show that Sox3 expression becomes restricted to the anterior territory of the chick otic field and that misexpression of Sox3 induces Sox2 and Delta1 in the non-neurogenic otic territory. This suggests that Sox3 plays a central role in the establishment of an otic neural fate. Furthermore, Sox3 down-regulates the expression of Lmx1b, a marker of the posterior non-neurogenic otic epithelium. The expression of Sox3 is maintained by the positive action of FGF8 derived from the otic ectoderm. On the contrary, BMP signaling does not have a major influence on neural commitment but instead regulates Lmx1b expression in the otic placode. Together, the data support the notion that Sox3 is critical for the development of the neural elements of the inner ear, and they highlight the importance of localized signaling from the ectoderm in establishing the neurogenic vs. non-neurogenic anteroposterior asymmetry that characterizes the early otic placode.

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Introduction

The acquisition of neural fate is a fundamental step for the development of sensory organs and cranial ganglia. One such organ is the inner ear, which is responsible for the senses of hearing, balance and detection of acceleration. Sensory neurons, supporting cells and hair cells are the three different cell types that build up the sensory apparatus of the inner ear. They derive from the otic placode, a thickening of the embryonic ectoderm adjacent to the hindbrain (Kelley, 2006; Torres and Giráldez, 1998). Neural specification in the inner ear takes place only in the anterior part of the placode (Adam et al., 1998; Alsina et al., 2004; Hemond and Morest, 1991). The first patterning event during ear development is the establishment in the otic placode of an anterior neurogenic domain characterized by the expression of Sox3, FGF10, Neurogenin1, Delta1 and Hes5, and a posterior non-neurogenic domain expressing Tbx1 and Lmx1b, as well as components of the Notch signaling pathway such as Serrate1 and Hes1 (Abelló et al., 2007; Alsina et al., 2004; Cole et al., 2000; Giráldez, 1998; Raft et al., 2004; Vitelli et al., 2003). Although inhibition of Notch signaling affects some aspects of otic regionalization, the Notch pathway is dispensable for the early establishment of the neurogenic territory in amniotes (Abelló et al., 2007; Daudet et al., 2007). This raises the questions of when and where neural fate is acquired in the otic placode, and how this early anteroposterior (AP) patterning is established.

SoxB1 proteins (Sox1, Sox2, Sox3) belong to the HMG-domain family of transcription factors related to the testis determining gene, SRY (reviewed in Wegner, 1999; Wegner and Stolt, 2005). In evolutionarily diverse animal species, SoxB1 expression occurs first in neural competent ectoderm and is subsequently restricted to cells that adopt a neural fate (Penzel et al., 1997; Pevny et al., 1998; Rex et al., 1994; Wood and Episkopou, 1999). Although the different SoxB1 genes can compensate for each others functions (Overton et al., 2002), individual genes exhibit similar but not identical temporal and spatial expression patterns, suggesting that each member may have also specific functions (Rex et al., 1994; Uchikawa et al., 1999). For instance, during chick neural plate formation, Sox3 precedes Sox2 expression (Rex et al., 1994) and in mice only Sox3, but not Sox2 is expressed in the posterior embryonic ectoderm (Wood and Episkopou, 1999). In the chick inner ear, both Sox3 and Sox2 are expressed in the neurogenic region, but only Sox2 is maintained later on in the sensory precursors, where it is necessary for sensory development (Dabdoub et al., 2008; Kiernan et al., 2005; Neves et al., 2007). Mice mutant for Sox3 are affected by hypopituitarism and craniofacial defects (Rizzotti et al., 2004), as are human patients carrying Sox3 mutations (Laumonnier et al., 2002; Rizzotti and Lovell-Badge, 2007). A duplication of 7.5 Mb that encompasses or disrupts the SOX3 gene has been associated with hearing impairment in several females from the same family

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(Stankiewicz et al., 2005). A role for Sox3 in epibranchial placode formation and neurogenesis has already been demonstrated in zebrafish and chick, respectively (Abu-Elmagd et al., 2001; Nikaido et al., 2007; Sun et al., 2007). Yet, the function and regulation of Sox3 in ear development is unknown.

FGF signaling has been long recognized to have neural-inducing potential (Stern, 2005), and it has been directly linked to the expression of the SoxB1 genes in several vertebrate species. During neural plate formation and epibranchial development, FGF8 signaling regulates Sox3 expression (Nikaido et al., 2007; Streit et al., 2000; Sun et al., 2007), and the N1 enhancer of the Sox2 locus contains FGF response binding sites (Takemoto et al., 2006). Several members of the FGF family are expressed in the developing inner ear and in surrounding tissues, and they regulate otic induction, neuronal production, and morphogenesis (Alsina et al., 2004; Hatch et al., 2007; Ladher et al., 2000; Léger and Brand, 2002; Mansour et al., 1993; Pirvola et al., 2000; Vendrell et al., 2000; Wright and Mansour, 2003; Zelarayan et al., 2007). However, it remains unclear whether FGFs play a direct function on Sox3 and neural specification, or they reflect secondary events during neurogenesis, growth and survival.

BMP signaling, on the other hand, has been shown to repress Sox3 expression. In Xenopus laevis, targets of the BMP such as Vent2 can inhibit Sox3 expression during neural plate induction (Rogers et al., 2008). Similarly, BMP or the BMP effector Smad1 can repress Sox3 expression at midgastrula stage in zebrafish (Dee et al., 2007). In contrast, in chick, BMP signaling mainly inhibits Sox2 expression with only a late effect on Sox3 (Linker and Stern, 2004). The role of the BMP pathway in the initial expression of Sox3 in neural competent tissues, therefore, requires further study. Moreover, in epibranchial development BMP activity promotes their development and positively influences neurogenesis (Begbie et al., 1999; Kriebitz et al., 2009). Both in mouse and chick, BMP4 signaling plays a prominent role in the development of sensory organs (Chang et al., 1999, 2008; Li et al., 2005; Pujades et al., 2006). But, at placodal stages, BMP4 and BMP5 are expressed in the dorsal neural tube, and BMP7 is already expressed in the posterior otic territory (Liem et al., 1995; Oh et al., 1996), with a possible influence on early neural commitment.

Here, we identify Sox3 as a key specification factor of the neurogenic domain of the chick otic placode. Our results show that ectopic Sox3 expression in the preotic field is sufficient to promote the development of neuronal precursors in the non-neurogenic domain of the otic placode. The restriction of Sox3 to the anterior otic field parallels the expression of Fgf8 in the otic ectoderm and FGF activity is both required and sufficient to promote otic Sox3 expression. This suggests that sustained FGF signaling in the otic territory is the driving force that maintains high levels of Sox3 expression. On the other hand, gain or loss of BMP activity in the otic ectoderm does not have a major influence on neural commitment, but BMP signaling is required for the regulation of Lmx1b expression, suggesting a novel role for BMP signaling in early otic development. Together, the results stress the importance of signals that pattern the otic placode in the AP axis.

Material and methods

Embryos and staging

Fertilized hens’ eggs (Granja Gibert, Tarragona, Spain) were incubated in a humidified atmosphere at 38 °C for designated times (Covatutto incubators). Embryos were staged according to Hamburger and Hamilton (1992).

Whole embryo explants in Matrigel matrix

10 μl of Matrigel matrix (Gibco-Invitrogen, 354234) ice–cold solution was added per well making a round-shaped layer and left 10 min at room temperature until solid. Whole dissected embryos kept in M199 medium (Gibco-Invitrogen) were placed dorsal up onto the Matrigel dome and left with little medium to attach to the Matrigel matrix for 10 min. Then 250 μl of DMEM with 1% antibiotic solution (15140-122), 4 mM glutamine and 1% fetal calf serum were added together with inhibitors and incubated at 37.5 °C in a water-saturated atmosphere containing 5% CO2. To block FGF signaling, SU5402 inhibitor (Calbiochem, 572630) which inhibits tyrosine phosphorylation of the FGFR1 was used at 25 μM in DMSO on embryos from HH8–11. To inhibit BMP activity, Dorsomorphin compound (BML-275, Biomol) that blocks ALK2, ALK3 and ALK6 (Yu et al., 2008) was used at 10 μM in DMSO on embryos from HH9.

Overexpression experiments by in ovo electroporation

EGFP-Sox3 cloned in C2-EGFP; pCDNA-Sox3; pCIG-caAlk3-RES-nEGFP (gift from T. Schultheiss), pCIG-nEGFP (gift from E. Marti) and pCS2-mFGF8 (gift from T. Schimmang) and N2-EGFP (Clontech) were electroporated into the otic territory of HH9 embryos as described (Abelló et al., 2007).

Whole-mount in situ hybridization and immunohistochemistry

Whole-mount in situ hybridization was carried out with DIG-labeled RNA probes and alkaline-phosphatase-coupled anti-DIG antibody, which was then detected with NBT/BCIP according to Nieto et al., (1996). HMG-devoid Sox2 probe was digested with Sacl. Whole-mount immunohistochemistry after in situ hybridization was used to detect GFP protein (Clontech, 632460; 1:50), Islet1 (39.4D5; DSHB; 1:200) and HNK1 epitope (347390; Becton Dickinson; 1:50) as described (Abelló et al., 2007).

TUNEL assay

Distribution of apoptotic cells in the otic vesicles was determined by TdT-mediated dUTP nick-end labeling (TUNEL) of the fragmented DNA. Briefly, fixed whole-mount embryos were incubated with 10 μg/ml proteinase K (Sigma-Aldrich, Spain) for 15 min at room temperature and post fixed with 4% paraformaldehyde (in 0.1%PBST). After that, embryos were submerged for 1 h in TUNEL Label Mix (Roche-Applied Science) at 37 °C. TUNEL enzyme (Roche-Applied Science) was added to the embryos in proportion 1:9 with regard to TUNEL Label Mix, for 3 h at 37 °C. After being washed with 0.1% TBST, embryos were blocked with 10% NGS/TBST for 2 h at room temperature, and incubated overnight at 4 °C with anti-fluorescein-AP (Fab Fragments, GmbH, Germany) diluted in blocking solution 1:2000. Signal was detected using NBT/BCIP (Roche).

Results

Sox3 expression in the preotic-epibranchial field defines prospective neural domains

We analysed in detail the onset of Sox3 expression in the preotic-epibranchial ectoderm and compared it with that of Pax2, which is a typical early otic gene. Since at the stages under study Sox3 is also expressed in the endoderm, the underlying mesoderm and endoderm tissues were removed in order to allow the visualization of the Sox3 expression in the ectoderm. In some instances, MafB and Krox20 gene expression was also monitored for rhombomere positioning at the hindbrain, the latter also being expressed after 12 ss in the neural crest tissues as migrating to the ectoderm (Nieto et al., 1995). At 5–6 ss, Sox3 was expressed in a broad area of the ectoderm adjacent to hindbrain, within the Pax2-positive domain (compare Fig. 1A with 1D). Low levels of Tbx1 and Lmx1b transcripts were also detected, but only in few scattered cells of the presumptive otic region (Figs. 1G and J). Tbx1-positive cells were confined to the ectoderm domain at the
level of the posterior hindbrain, while Lmx1b-expressing cells were distributed throughout the medial ectoderm adjacent to the hindbrain. From 7–8 ss onwards, the initially broad expression of Sox3 became progressively refined and enhanced in the more anterior part of the preotic territory (Figs. 1E and F). By 9–10 ss, Sox3 was expressed in an oblique medial-lateral band that included the domain of the geniculate placode (Fig. 1F). Beyond 7 ss, Tbx1 was strongly expressed in the posterior domain of the presumptive otic ectoderm, at the level of rhombomeres 5 and 6 (r5–r6) (Figs. 1H, I). This was also the case for Lmx1b only after 9–10ss (Fig. 1L, and transverse sections of Figs. 1M–P). These results show first, that the asymmetric expression of transcription factors between neurogenic and non-neurogenic domains is established at the onset of otic placode formation. And secondly, that, in contrast to Tbx1, Sox3 and Lmx1b were initially expressed within a broad and overlapping region, to become progressively restricted to their final expression domains.

Ectopic expression of Sox3 induces Delta1 and Sox2 expression in non-neurogenic epithelium but ectopic neurons fail to differentiate

Otic neurogenesis begins at 14 ss, as revealed by the presence of a few scattered Delta1-expressing cells in the anterolateral otic epithelium (Alsina et al., 2004). In order to address whether restricted Sox3 expression was able to confer neurogenic fate to the anterior otic ectoderm, we overexpressed a construct of chick Sox3 N-terminally fused to GFP in the whole prospective otic ectoderm prior to the formation of the otic placode (5–9 ss). Forced expression of Sox3 showed a strong induction of Delta1 after 6 h throughout the whole otic territory and in the adjacent ectoderm (Figs. 2A, B and E–G; n=29/29). Delta1-positive cells were found in the posterior otic region, where cells do not undergo neurogenesis under normal conditions (Figs. 2E–G, posterior sections). Induction of Delta1 occurred mainly in a cell-autonomous manner (Fig. 2K, white arrowhead). However, in several cases GFP was barely detectable in cells with high levels of Delta1 transcripts (red arrowhead in Fig. 2K), either due to blue precipitate masking GFP after in situ hybridization or to endogenous expression. On no occasion was Delta1 induction observed after control EGFP vector overexpression (Figs. 2C, D; n=0/11). Ectopic Delta1-expressing cells were still observed 20 h post-electroporation, although only in few cells (n=4/8, Figs. 2H–J).

Sox3 precedes Sox2 expression in the chick neural plate (Rex et al., 1994), and both in zebrafish and Xenopus laevis misexpression of Sox3 ectopically induces Sox2 (Dee et al., 2008; Rogers et al., 2009). We explored whether this was also the case in the otic placode. Embryos were electroporated with Sox3 at 5–8 ss and, since the HMG-domains of Sox3 and Sox2 show high sequence identity, embryos were analysed for Sox2 expression with a probe lacking the HMG-domain. In normal conditions Sox3, is barely expressed in the otic placode, its expression being detected at the otic cup stage (Neves et al., 2007). Sox3 induced the expression of Sox2 in a cell-autonomous manner, and this occurred as early as 3 h post-electroporation (Figs. 2L and M, n=4/4). This effect was even stronger after 6 h (Figs. 2P–T). Embryos electroporated...
with an empty vector did not show induction of Sox2 (Figs. 2N and O; n = 0/7). Unlike Sox2, Delta1 was not induced when checked only 3 h after electroporation (n = 0/5, data not shown), albeit GFP-Sox3 protein was visible already 1–2 h after post-electroporation. This suggests that the effect of Sox3 on Delta1 may require intermediate steps which are not necessary for Sox2 induction.

Sox3 gain of function also induced in some cases ectodermal thickenings that protruded inside the lumen of the otic cup, with Delta1-expressing cells (Fig. 2F, Supplementary Fig. 1B' in Supplementary Material), similarly to the effects previously reported in epibranchial placodes (Abu-Elmagd et al., 2001). After Sox3 electroporation at 5–9 ss near the optic field, ectopic thickenings were also induced (Supplementary Fig. 1 in Supplementary Material). However, when expression of Pax2 was analysed in those ectopic thickenings no induction of Pax2 was observed (arrow in Supplementary Fig. 1E' in Supplementary Material). In contrast, in medaka fish ectopic vesicles generated by overexpression of Sox3 were able to ectopically express Pax2 (Koster et al., 2000).

In the neural tube, overexpression of Sox1–3 inhibits neuronal differentiation (Bylund et al., 2003). In the otic cup, expression of NeuroD is initiated at HH12 and it follows that of Ngn1 and Delta1 (Adam et al., 1998; Alsina et al., 2004). In order to assess whether
ectopic Delta1-positive cells were able to progress into differentiation, electroporated embryos were analysed for the expression of NeuroD, 20 and 40 h after Sox3 electroporation. After 20 h, otic cups showed only few ectopic NeuroD-positive cells in the non-neurogenic territory (n = 4/10, Figs. 3A and B), and after 40 h still some neuronal cells were detected in the posterior HNK1-positive region (Figs. 3C–F; n = 1/5). We also analysed Islet1, a transcription factor expressed in neuroblasts just prior to delamination and maintained in the cochlear-vestibular (CVG) neurons (Adam et al., 1998; Camarero et al., 2003). After 40 h post-electroporation, none of the electroporated embryos exhibited ectopic expression of Islet1 protein, suggesting that ectopic Delta1-expressing cells did not progress into a full differentiation program (Figs. 3G–J; n = 0/8). We then checked whether ectopic neuronal cells unable to fully differentiate entered apoptosis. Indeed, we found an increase of apoptotic cells detected by TUNEL assay in the posterior electroporated territory, suggesting that blockade of neuronal differentiation can lead cells to undergo apoptosis (Figs. 3K and L).

**Lmx1b, but not Tbx1, is down-regulated by Sox3 overexpression in the non-neurogenic otic epithelium**

During the process of otic patterning and morphogenesis, the interactions between sensory and non-sensory epithelium seem to be crucial for proper development of sensory organs (Nichols et al., 2008; Pauley et al., 2003). We decided to explore the possible effects of Sox3 overexpression. As Lmx1b displays a complementary pattern to Sox3 expression at otic cup stage (Fig 1; Alsina et al., 2004), we reasoned that Sox3 may influence Lmx1b regionalization. Forced expression of Sox3 resulted in a reproducible inhibition of Lmx1b in the posterior and dorsal part of the otic cup/vesicle, after 20 h of incubation (Figs. 4A–F; n = 10/10), but no effect was observed after 6 h (n = 4/4, data not shown). Close analysis of sections of electroporated otic vesicles revealed that down-regulation of Lmx1b expression was cell-autonomous (Fig. 4F). The delay in otic cup closure is sometimes detected but might be due to indirect effects of electroporating at preotic stages. Tbx1 expression is also confined to the non-neurogenic territory (Fig. 1), and in mice, Tbx1 negatively regulates otic neurogenesis (Raff et al., 2004; Vitelli et al., 2003; Xu et al., 2007). We explored also the possible effects of Sox3 on Tbx1 expression, but Sox3 electroporation showed little effects on the expression of Tbx1 (Fig. 4G–I, n = 3/3), suggesting that different posterior genes are regulated independently.

**The FGF dependence of otic Pax2 and Sox3 expression can be dissociated temporally**

During the induction of the otic placode, a cascade of FGF signaling from surrounding tissues leads the ectoderm adjacent to r4–6 to adopt an otic fate (reviewed in Ohyama et al., 2007; Schimmang, 2007). On the other hand, FGF signaling activates Sox3 gene during the induction of the neural plate and epibranchial placodes (Streit et al., 2000; Sun et al., 2007). With this in mind, we investigated whether, in addition to its role in otic placode induction, FGF signaling was also required for Sox3 expression in the otic field, and whether or not the processes of otic specification and neural commitment were coupled. Whole chick embryos were explanted and cultured on Matrigel for 6 h in the presence of the FGFR inhibitor, SU5402 (25 μM), or with DMSO as a control. Incubation with SU5402 inhibited the induction of Pea3, a well-established target of FGF signaling, in the ectoderm, caudal hindbrain and presomitic mesoderm, confirming its ability to block FGF signaling (n = 9/9; Fig. S2 Supplementary Material). As expected from the role of FGF signaling in chick otic induction (Ladher et al., 2000; Vendrell et al., 2000), 3–6 ss embryos
treated for 6 h with SU5402 did not develop otic placodes as revealed by absence of ectodermal thickening of the ectoderm, nor did they express Pax2 (Figs. 5A and B; n = 24/24, and compare Fig. 5A’ with Fig. 5B’; n = 40/40). This was accompanied by loss of Sox3 (Figs. 5C and D; n = 14/16). After 24 h of incubation, embryos treated with SU5402 did not develop otic cups neither they expressed Pax2, indicating the inability of the ectoderm to acquire the otic fate after FGF signaling blockade (Figs. 5E and F; n = 44/44). In contrast, when the experiment was performed in 7–8 ss embryos, neither Pax2 expression nor the typical ectodermal thickening of the otic placode was disturbed after treatment with SU5402 for 6 h (Figs. 5G and H; n = 8/8 and compare Fig. 5G’ with 5H’; n = 60/60). However, those embryos showed inhibition of Sox3 expression in the otic ectoderm (Figs. 5I and J; n = 10/10). This indicates that FGF signaling is required for both Sox3 and Pax2 expression, but that the two processes occur independently and sequentially. In SU5402 treated embryos, Sox3 was also absent in the epibranchial placodes, as reported in zebrafish (Nikaido et al., 2007; Sun et al., 2007), but remained unaltered in the neural tube (Figs. 5I and J; n = 5/5). After 24 h incubation, 7–8ss embryos treated with SU5402 showed a strong reduction or complete absence of Ngn1 expression in the otic territory and brain (Figs. 5K and L; n = 8/8). Embryos treated with the FGF inhibitor also showed an anterior expansion of Lmx1b expression into the prospective neurogenic domain (Figs. 5M and N). Since inhibition of FGF signaling resulted in loss of Sox3 expression, the observed Lmx1b expansion further suggests that, in addition to promoting neurogenesis, Sox3 is able to inhibit a non-neurogenic gene, although direct regulation of Lmx1b by FGF signaling cannot be ruled out. In order to test whether the expression of Lmx1b throughout the otic placode is due to a loss of anterior tissue, we checked for other posterior markers, such as Irx1 and Tbx1. We found that, in this case, they did not respond to FGF blockade in the same manner. Irx1 expression barely changed (Figs. 5O and P). In contrast, Tbx1 expression was strongly down-regulated in the otic placode and underlying mesoderm (Figs. 5Q and R). Moreover, we performed TUNEL assay after 24 h of FGF inhibition in culture and we did not observe a massive induction of apoptosis in the otic placode. Few scattered apoptotic cells were detected in the otic placode (red arrows in Fig. 5T) but increased apoptosis was mainly found in the ectoderm adjacent to the placode (compare black arrows Fig. 5S with 5T). Together, these data indicate that the expansion of Lmx1b to the anterior domain is not due to a loss of anterior territory, nor to a complete transformation of anterior fate to posterior.

During more advanced stages of development, between 9 and 12 ss, blockade of FGF signaling with SU5402 for 6 or 24 h did not affect the expression of Sox3, suggesting that the maintenance of Sox3 expression was no longer dependent on FGFs (n = 6/20 otic cups; data not shown). A similar effect has been recently reported during neural induction, suggesting a restricted temporal requirement for FGF signaling in neural specification (Stavridis et al., 2007).

Fgf8 is a candidate for the induction of Sox3 in the otic placode

Our results so far indicate that Sox3 expression is progressively enhanced in the anterior otic region at 7–10 ss and that Sox3 expression is dependent on FGF signaling. Next, we sought to identify a putative source of FGF along the AP axis that could account for Sox3 regionalization. This was done by exploring the expression pattern of different FGFs (Fig. 8, and 19) which are present during otic induction (Karabagli et al., 2002). At 5–6 ss, none of those FGFs exhibited an AP asymmetry in their expression similar to that of the

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**Fig. 4.** Ectopic expression of Sox3 in the non-neurogenic territory down-regulates Lmx1b but not Tbx1. (A–C) Sagittal sections of non-electroporated (A) and EGFP-Sox3 electroporated otic cup/vesicles (B) showing down-regulation of Lmx1b expression after 20 h (black arrows). (C) Anti-GFP immunofluorescence, (D–F) Transverse posterior sections showing down-regulation of Lmx1b expression in the electroporated side. (F) Merge image showing colocalization of EGFP-Sox3 and Lmx1b down-regulated cells. (G–I) Coronal sections showing that misexpression of EGFP-Sox3 into the Tbx1 domain did not result in a reproducible down-regulation of Tbx1 expression. A, anterior; L, lateral; D, dorsal.
Sox3 (Figs. 6A, C, and E). However, at 7–8 ss scattered Fgf8-expressing cells were found in the anterior preotic ectoderm (red arrow in Fig. 6F). To better analyse the expression of Fgf8 in the ectoderm, embryos were flat mounted and the underlying endomesoderm removed. The results show that in 7 to 10 ss embryos, Fgf8 was expressed in an oblique band that included the anterior preotic territory and the presumptive geniculate placode region (Fig. 6G), resembling the expression pattern of Sox3 at 10 ss (Fig. 1F). At 13 ss, Fgf8 was down-regulated in the otic territory but remained expressed in the epibranchial placodes and endoderm (Fig. 6G). These results suggest that Fgf8 may account for the progressive enhanced expression of Sox3 in the anterior otic domain. However, it should be kept in mind that Sox3 expression initially appears in a broader domain, suggesting that the initial regulation of Sox3 might be mediated by other Fgf signals present in the endomesoderm or neural tube.

We next examined the direct influence of Fgf8 on Sox3 expression. A mouse Fgf8 construct was electroporated into the ectoderm at 5–9 ss. After 6 h of incubation, the electroporated region displayed enhanced expression of Sox3 in the ectoderm and sometimes in the endoderm as compared with the left non-electroporated side (arrow in Figs. 7A and C, n = 10/10). Sprouty2 transcripts were used as readout of FGF activity and they were strongly induced in the electroporated ectoderm but also in the neural tube and mesoderm adjacent to the electroporated cells (Fig. 6E, n = 7/7). When electroporated embryos were incubated for 24 h and expression of neuronal markers analysed, an expansion of NeuroD-positive cells in the posterior otic epithelium was observed (Figs. 7G–I), without any major effect on Lmx1b expression (Figs. 7J–L). In summary, in chick, Fgf8, but not other FGFs, is expressed in the otic–epibranchial ectoderm concomitantly with Sox3 regionalization, and Fgf8 overexpression is enhancing Sox3 expression and induces ectopic neurogenesis, suggesting that Fgf8 might be regulating Sox3 in the otic ectoderm.

**BMP signaling does not affect Sox3 expression and neurogenic fate, but it regulates Lmx1b expression**

In order to investigate the function of the BMP pathway in otic neural commitment, we performed gain and loss of function experiments. Incubation of 5–9ss explants with the BMP receptor inhibitor Dorsomorphin for 10–20 h did not alter Sox3 expression in the otic field, which remained confined to the anterior otic territory (Figs. 8A–C, n = 4/4). Similarly, constitutive activation of BMP signaling in the neurogenic domain with the caAlk3-IRES-nGFP vector resulted in no inhibition on the expression of Sox3 (Figs. 8D–F). Cells overexpressing caAlk3, were able to delaminate into the CVG neuroblasts and coexpressed Islet1 protein (Figs. 8G–I, white arrows),
indicating that high levels of BMP signaling did not abrogate the establishment of neurogenic tissue.

In the ectoderm adjacent to the hindbrain, Lmx1b is initially expressed in a medial row of cells adjacent to the neural tube and its expression is progressively enhanced in the dorsomedial and posterior otic territory. BMP7 is also expressed in the posterior otic placode (Oh et al., 1996) and from 7 ss onwards, its expression is highly similar to Lmx1b (Supplementary Fig. 3 in Supplementary Material). During the establishment of the roof plate of the neural tube, Lmx1b expression depends on BMP signaling (Chizhikov and Millen, 2004). We reasoned that Lmx1b expression in the otic territory could be also regulated by local BMP signaling. Incubation of 5–9ss embryos with Dorsomorphin produced a strong down-regulation of Lmx1b, in both the neural tube and otic placode (compare Fig. 8J with K and L; n=4/4). In contrast, overexpression of caAlk3 at the same stages induced an expansion of Lmx1b into the prospective neurogenic epithelium 24 h post-electroporation as shown by two examples (Figs. 8M–Q; n=5/5). In some cases but not always, a reduction of otic vesicle size was very apparent (compare Figs. 8P and Q). The differences on the effects on growth could be due to differences in the initial somite stage of electroporation that ranged from 5 to 9 ss. Moreover, we confirmed that this was specific to the overexpression of caAlk3 since very little effect on growth was observed after electroporation of an empty vector (pCIG-nGFP) (Supplementary Fig. 4A and B in Supplementary Material). An induction of Lmx1b in the cells electroporated with an empty vector was not detected either (Figs. S4C–E in Supplementary Material).

### Discussion

Anteroposterior regionalization into neurogenic and non-neurogenic territories takes place at the onset of otic formation

Several genes are regionally expressed at otic cup/vesicle stages, and their role in the development of specific parts of the adult inner ear have been studied (Fekete, 1999). In a previous study, we showed that the otic placode/cup is patterned along the AP axis into an anterior neurogenic domain and a posterior non-neurogenic domain, each territory exhibiting a specific combination of transcription factors and signaling molecules (Abelló et al., 2007; Alsina et al., 2004). Here, we show that Sox3 and Lmx1b are initially expressed in a broad area of the otic ectoderm, to become progressively enhanced in the anterior and in the posterior preotic field, respectively. In contrast, Tbx1 is already restricted to the posterior ectodermal territory when it is first expressed. Cell movements may allocate Sox3 and Lmx1b cells to their final positions (Streit, 2002). But alternatively, or simultaneously, cells in the preotic field may not be yet committed to neurogenic or non-neurogenic fates, which may be specified by differential gene regulation. This latter possibility seems to be favoured by our results, because signals like Fgf8 and BMPs are able to differentially induce Sox3 or Lmx1b expression in the cranial ectoderm, resulting in their asymmetric expression in the otic placode. This early prepatternning, however is probably not fixed until HH12, as revealed by the plasticity shown by otic cup rotations in the AP axis (Bok et al., 2005).

**Fig. 6.** Fgf8 is expressed in the anterior otic ectoderm at 7–8 ss. (A and B) Transversal sections at the level of r4 and r5 showing Fgf3 expression in the endoderm (black arrows) and r5 hindbrain at 5 ss and 7 ss embryos. (C and D) Transversal sections at the level of r4 and r5 showing Fgf19 expression in the mesoderm, endoderm and neural tube at 5 ss and 7 ss (black arrows). (E and F) Transversal sections at the level of r4 and r5 showing Fgf8 expression in the endoderm in the endoderm at 5 ss (black arrows) and in the endoderm and ectoderm (red arrow) at the level of r4 in 7 ss. The embryos were also stained for Krox20 that is faintly visible in r5 at 7 ss. Dorsal is up in all images. (G) Dorsal views of otic regions with the mesendodermal tissue removed in embryos from 7 to 13 ss and hybridized for Fgf8 and MafB/Krox20. The expression of Fgf8 in the otic ectoderm is restricted to the anterior otic ectoderm. Dorsal is up in transverse sections. nt, neural tube; e, endoderm; m, mesoderm; et, ectoderm; r, rhombomere; ss, somite stage; L, lateral; A, anterior.
Overexpression of Sox3 can respecify the non-neurogenic otic territory

Sox1-3 gene function is associated with self-renewal and neural commitment of ectodermal cells in response to inductive signals (Pevny et al., 1998; Rex et al., 1994; Uwanogho et al., 1995). Sox3 is expressed early in the otic neurogenic domain together with Sox2 (Neves et al., 2007). Our results show that overexpression of Sox3 in the otic ectoderm promotes Delta1 expression in the otic fated ectoderm, and timing suggests that it does so through the induction of Sox2. Recently, injection of Sox3 mRNA in early zebrafish and Xenopus embryos induced ectopic neuroectoderm providing direct evidence for a role for Sox3 in neuralization (Dee et al., 2008; Rogers et al., 2009). The overexpression of SoxB1 proteins in the vertebrate CNS inhibits neuronal differentiation, yet early markers of neuronal fate are barely affected (Bylund et al., 2003; Graham et al., 2003; Schlosser et al., 2008). In zebrafish and Xenopus, overexpression of Sox3 before neural induction delays neuronal differentiation by keeping cells in a precursor undifferentiated state but it does not override the neurogenic program, although this lack of full repression may be due to the lability of the overexpressed Sox3 (Dee et al., 2008; Rogers et al., 2009). Our results show that the forced expression of Sox3 induces ectopic Delta1 expression, but the ectopic cells do not transit into full differentiation as measured by neuronal markers such as NeuroD and Islet1. Consequently these cells die by apoptosis. A similar phenomenon was observed by Vázquez-Echeverría et al. (2008) in Kreisler mutants, where ectopic neuroblasts generated in the non-neurogenic epithelium were shown to die during development. This may be caused by the lack of a proper environment and trophic support and/or differentiation signals in the non-neurogenic domain. In this connection, it has been shown that FGF signaling is required for otic neuronal differentiation (Alsina et al., 2004). Also, as the expression of Tbx1, a well-known repressor of otic neurogenesis (Raft et al., 2004), was not inhibited after Sox3 misexpression, it is possible that proper neuronal differentiation may be impaired by contradictory transcriptional Sox3/Tbx1 inputs.

FGF signaling is required for otic neural commitment and can be dissociated from otic placode induction

Placodal specification is a multistep process which involves sequential signaling events (Litsiou et al., 2005; Martin and Groves, 2006). In the present study, we examined whether an additional FGF-dependent step is required for neural commitment. Here we show that Pax2 and Sox3 expression can be uncoupled by blocking FGF signaling at specific developmental stages. Otic thickening and invagination can take place in the absence of Sox3 expression and neurogenesis after FGF signaling blockade, indicating that the process of otic induction per se does not lead by default to neural commitment. Classical graft experiments showed that presumptive otic ectoderm can develop ectopic otic vesicles without neurons (Noden and Van De Water, 1986). More recently, Groves and Bronner-Fraser (2000) showed that epiblast grafted into the presumptive otic region of young hosts (3–10 ss) express Pax2 and Sox3, but only Sox3 was expressed in grafts performed at later stages (11–21 ss), suggesting that Pax2-inducing signals are lost before those required for Sox3 induction. Taken together, the evidence suggests that acquisition of neurogenic competence is a step that follows specification of other otic characters, including expression of non-neurogenic markers and the invagination of the otic placode.
Fig. 8. BMP signaling regulates Lmx1b expression but not Sox3 expression in the otic placode. (A–C) Whole-embryo explant cultures of 7–9 ss embryos treated with 10 μM Dorsomorphin for a period of 12–20 h did not affect the expression of Sox3 in the otic placode (brackets) shown in dorsal views. (D and E) Sagittal sections of otic vesicles hybridized for Sox3. (F) Anti-GFP immunofluorescence. caAlk3-IRES-nGFP electroporation at 7–9 ss did not affect the expression pattern of Sox3. (G–I) High magnification images of a sagittal section with caAlk3 electroporated cells in the epithelium (continuous line) and in the CVG (discontinuous line). (G) Electroporated cells detected by anti-GFP staining. (H) Islet1 protein staining in neuroblasts from the CVG. Overlay image showing electroporated cells also expressing Islet1 protein in the CVG (white arrows). (J–L) Whole-embryo explant cultures of 7–9 ss embryos treated with 10 μM Dorsomorphin for a period of 12–20 h resulted in strong reduction of Lmx1b expression in the neural tube and otic placode (brackets) shown in dorsal views. Whole-embryo explant cultures of 7–9 ss embryos treated with DMSO (J) or 10 μM Dorsomorphin (K and L) for 12 h exhibiting reduced levels of Lmx1b expression in the neural tube and the otic cup. (M and N) Coronal sections of Lmx1b expression in non-electroporated vesicle (M) and caALK-IRES-nGFP electroporated vesicle (N). (P and Q), Sagittal sections of otic vesicles showing induction of Lmx1b expression in the neurogenic domain (brackets). (O and R) Overlay images of whole embryos stained for Lmx1b expression and GFP fluorescence. A, anterior; L, lateral; D, dorsal.
We propose that sustained FGF activity in the anterior otic field is a fundamental requirement for neural commitment and that Fgf8 is a good candidate to mediate this effect, since it foreshadows the regionalized expression of Sox3 within the otic ectoderm, and it is sufficient to induce the expression of Sox3 in the otic region. Fgf8 is known to induce Sox3 expression in the chick epiblast (Streit et al., 2000) and Sox2 in the posterior neural plate through the specific N-1 enhancer (Takemoto et al., 2006). It is tempting to think that a similar regulation operates on the Sox3 promoter region. In zebrafish, however, it is unclear whether otic-epibranchial Sox3 expression depends exclusively on FGF8 signaling (Nikaido et al., 2007; Sun et al., 2007) and no direct information is available in mouse.

In sum, our results place the FGF signaling emanating from the otic ectoderm as the major inductive source for the restriction of the neurogenic domain to the anterior otic placode. The relevance of ectodermal signals for early otic patterning is also supported by a recent report showing that Tbx1 expression and neural fate depend on a RA gradient imposed on the otic ectoderm (Bok and Wu, 2008, ARO, Bok and Wu, personal communication 2009). How these signals interact to result in the final specific gene expression patterns exhibited by the otic placode deserves further study.

The role of BMP in the regulation of early AP otic patterning

In addition to FGF signaling, several models propose a central role for BMPs in inhibiting neural fate (reviewed in Munoz-Sanjuan and Brivanlou, 2002). In the Xenopus CNS, the restriction of Sox3 to the presumptive neural plate depends on consensus binding sites for Vent, a BMP-regulated transcriptional repressor (Rogers et al., 2008). However, in chick and zebrafish, misexpression of BMP4 in the prospective neural plate only inhibits late Sox3 expression (Linker and Stern, 2004; Dee et al., 2007), and the induction of posterior neuroectoderm is independent of Smad signaling (Rentzsch et al., 2004).

At the stages of otic placode specification, BMP4 is expressed in the most lateral neuroectoderm, and becomes progressively concentrated to the dorsal portion of the neural tube (Liem et al., 1995). BMP7 is expressed in the posterior otic field and, at otic cup stages, together with BMP5, is expressed in the posterior and dorsomediial epithelium (Oh et al., 1996). Thus, in principle, BMPs from the neural tube or from the ectoderm could influence the otic ectoderm at the time of otic neural commitment. Our results show that inhibition of BMP signaling did not expand Sox3 expression into the non-neurogenic ectoderm, indicating that in normal development BMP signaling does not interfere with Sox3. Accordingly, neuroblasts develop in otic vesicles after overexpression of caAlk3 in the ectoderm, further showing that high levels of BMP activity do not block early otic neural fate acquisition. Thus, although not explored here, BMP activity might instead of inhibiting neuronal fate positively regulate neurogenesis as shown for the epibranchial placode development (Begbie et al., 1999; Kriebitz et al., 2009). However, our data show that otic Lmx1b expression is tightly regulated by BMP signaling. Lmx1b is a transcription factor of the LIM family, homologous to the Drosophila apterous gene. Apterous/Lmx1 genes are involved in the specification of the dorsal limb character in Drosophila and vertebrates (Blair, 1995; Riddle et al., 1995) and roof plate and isthmus development in mice (Adams et al., 2000; Chizhikov and Millen, 2004). Gain of function of BMP results in the expansion of Lmx1b expression, whereas Lmx1b is down-regulated by blockade of BMP activity by Dorsomorphin. The ectopic induction of Lmx1b in the neurogenic territory does not prevent neuroblast formation. This indicates that neither Lmx1b expression nor high levels of BMP activity can suppress the neurogenic genetic program. This is in agreement with previous results in which expansion of Lmx1b into the neurogenic territory after Notch blockade did not suppress neurogenesis (Abelló et al., 2007). However, it still remains unclear why Lmx1b has to be excluded from the neurogenic domain in normal conditions.

In summary, taking together the new observations and previous work discussed above, we like to propose a model for the early anteroposterior specification of neurogenic and non-neurogenic commitment of the otic placode. As illustrated by Fig. 9, one early step in otic development is the induction of a broad otic-epibranchial Pax2 and Sox3 territory mediated by FGF signaling from underlying mesendodermal tissue (Ladher et al., 2000, 2005). In a following step, sustained FGF signaling, mediated by Fgfr emanating from the anterior otic-epibranchial ectoderm provides the driving force for maintaining high levels of Sox3 in the anterior otic-epibranchial territory, thus restricting Sox3 to the anterior otic field. Sox3 in turn would promote neural character by inducing Sox2 and Delta1, resulting in the neurogenic activity of the otic placode. In parallel, BMP signaling, either BMP4/5 from the dorsal neural tube and/or BMP7 from the medial and posterior ectoderm could be regulating Lmx1b expression in the otic ectoderm, although it has not been yet

Fig. 9. Model illustrating the early anteroposterior patterning and the specification of the neurogenic domain. Sequential steps of early otic development are represented from left to right in dorsal and transverse views of the otic field. Pax2 expression labels a broad otic–epibranchial domain that is induced by FGF signals from the mesendoderm and neuroectoderm initially located at the r4 r5 level. At this stage BMP4 and BMP5 are also expressed in the dorsal neural tube. At 6–7 ss, Sox3 expression appears in a broad domain within the Pax2 territory and it is dependent on FGF signaling. From 7 ss to 10 ss, expression of Sox3 becomes regionalized to the anterior otic ectoderm due to a positive local action of FGF8 in the anterior otic–epibranchial field. In parallel, BMP7 and Lmx1b expression overlap in the medial ectoderm, Lmx1b expression being dependent on BMP signaling. As the otic placode starts to invaginate, Sox3 restriction to the anterior otic placode is able to promote neural fate in the anterior placode and induce the expression of Sox2 and Delta1. In the posterior otic placode, Lmx1b expression overlaps with BMP7. As the neurogenic domain is established, Sox3 activity inhibits Lmx1b expression refining its final pattern, directly or indirectly through Notch.
directly tested which of them can perform this function. In a last step, further confinement of Lmx1b expression in the non-neurogenic domain would result from down-regulation of Lmx1b levels in the neurogenic territory by Sox3 activity and/or as previously shown by Notch pathway (Abelló et al., 2007). Finally, the otic and geniculate neurogenic domains segregate. In summary, our results highlight the pivotal function of Sox3 in the early specification of the neurogenic elements of the inner ear, and the weight of local ectodermal signaling for the regulation of expression of neurogenic and non-neurogenic genes and the establishment of the early AP patterning of the otic placode.

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Appendix A. Supplementary data


References


