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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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ABSTRACT

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.

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 unit of the inner ear. They derive from the otic placode, a thickening of 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 (Rizzoti et al., 2004), as are human patients carrying *Sox3* mutations (Laumonnier et al., 2002; Rizzoti 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 (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% CO_2. 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-IRES-nEGFP (gift from T. Schultheiss), pCIG-nGFP (gift from E. Martí) 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 SacI. Whole-mount immunohistochemistry after in situ hybridization was used to detect GFP protein (Clontech, 632460; 1:500), 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%PBT). 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 cells 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