Jagged 1 regulates the restriction of Sox2 expression in the developing chicken inner ear: a mechanism for sensory organ specification

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SUMMARY

Hair cells of the inner ear sensory organs originate from progenitor cells located at specific domains of the otic vesicle: the prosensory patches. Notch signalling is necessary for sensory development and loss of function of the Notch ligand jagged 1 (Jag1, also known as serrate 1) results in impaired sensory organs. However, the underlying mechanism of Notch function is unknown. Our results show that in the chicken otic vesicle, the Sox2 expression domain initially contains the nascent patches of Jag1 expression but, later on, Sox2 is only maintained in the Jag1-positive domains. Ectopic human JAG1 (hJag1) is able to induce Sox2 expression and enlarged sensory organs. The competence to respond to hJag1, however, is confined to the regions that expressed Sox2 early in development, suggesting that hJag1 maintains Sox2 expression rather than inducing it de novo. The effect is non-cell-autonomous and requires Notch signalling. hJag1 activates Notch, induces Hes/Hey genes and endogenous Jag1 in a non-cell-autonomous manner, which is consistent with lateral induction. The effects of hJag1 are mimicked by Jag2 but not by Dll1. Sox2 is sufficient to activate the Atoh1 enhancer and to ectopically induce sensory cell fate outside neurosensory-competent domains. We suggest that the prosensory function of Jag1 resides in its ability to generate discrete domains of Notch activity that maintain Sox2 expression within restricted areas of an extended neurosensory-competent domain. This provides a mechanism to couple patterning and cell fate specification during the development of sensory organs.

KEY WORDS: Sensory development, Sensory progenitor, Hair cell, Notch signalling, SoxB1. Chicken, Serrate 1

INTRODUCTION

Sensory organs of the inner ear originate from the otic placode and develop from a neurosensory-competent domain, with a stereotyped temporal and spatial pattern (Alsina et al., 2009; Bell et al., 2008; Fritsch et al., 2006; Raft et al., 2007; Satoh and Fekete, 2005). Sensory patches emerge at specific locations and they can be identified by the expression of prosensory genes that foreshadow and accompanies sensory development (Cole et al., 2000; Neves et al., 2007; Oh et al., 1996; Wu and Oh, 1996). Yet, the molecular mechanisms that couple patterning and cell fate in the sensory patches are unknown.

Notch signalling is important for sensory development, but its mechanism of action is far from simple. Notch activity is required for the specification of sensory organs and for the determination of hair cells (Brooker et al., 2006; Daudet et al., 2007). The latter function results from the well-known mechanism of lateral inhibition, whereby Notch ligands are expressed in hair cells and signal to neighbouring cells to prevent their differentiation (Brooker et al., 2006; Daudet and Lewis, 2005; Haddon et al., 1999; Kiernan et al., 2005a; Lanford et al., 1999). However, the role played by Notch in the specification of sensory fate is not well understood. Early blockade of Notch signalling results in the loss of sensory domains, and the overexpression of the intracellular domain of Notch (NICD) in the otic vesicle induces ectopic sensory patches. This has suggested that the specification of the sensory patches requires Notch signalling operating through lateral induction (Daudet et al., 2007; Daudet and Lewis, 2005; Eddison et al., 2000; Hartman et al., 2010; Pan et al., 2010). The Notch ligand jagged 1 [Jag1, also known as serrate 1 (Ser1)] is expressed in the prosensory patches (Adam et al., 1998; Cole et al., 2000; Morrison et al., 1999) and is a good candidate to drive the early prosensory function of Notch. In mice, the loss of function of Jag1 results in the disruption of the sensory epithelium and the loss of hair cells (Brooker et al., 2006; Kiernan et al., 2001; Kiernan et al., 2006; Pan et al., 2010; Tsai et al., 2001). Critical steps of this model are still missing, including whether Jag1 is sufficient for prosensory specification and what links Notch activity to sensory fate.

Sox2 is a high mobility group (HMG) box domain transcription factor that belongs to the B1 subfamily of Sox proteins (Uchikawa et al., 1999). In the developing inner ear, Sox2 is expressed in neurogenic and sensory progenitors, being downregulated in differentiated neurons and hair cells (Neves et al., 2007). Sox2 is necessary for sensory fate specification in the inner ear and Sox2 mutant mice show impaired sensory development with a reduced number of hair cells (Kiernan et al., 2005b). Sox2-deficient and Jag1-deficient mice show similar phenotypes, and Sox2 is reduced in Jag1 mutants or after pharmacological blockade of Notch. This suggests a common and hierarchical role of these genes in the specification of sensory fate (Dabdoub et al., 2008; Daudet et al., 2007; Kiernan et al., 2006). However, it is not known whether Jag1 is able to induce Sox2 expression and, if so, what the consequences are for cell fate.

This work addressed the role played by Jag1 in patterning and cell fate specification of the sensory organs and how this is related to the function of Sox2. The expression patterns of these two genes...
and the effects of their gain of function were examined. The results show that the prosensory function of Jag1 relies on its ability to maintain Sox2 expression within restricted domains of the otic epithelium. This occurs through a mechanism of Notch-mediated lateral induction and allows sensory patches to retain Sox2 function, which provides the competence to develop as sensory cells. This mechanism, patterning and cell fate determination are coupled so as to generate the sensory organs at the correct time, size and location.

MATERIALS AND METHODS

Chicken (Gallus gallus) embryos

Fertilised hens’ eggs (Granja Gilbert, Tarragona, Spain) were incubated at 38°C for the designated times and embryos were staged according to Hamburger and Hamilton (HH) (Hamburger and Hamilton, 1951). Embryos were dissected in cold phosphate-buffered saline (PBS, pH 7) and fixed overnight in 4% paraformaldehyde in PBS at 4°C.

Immunohistochemistry (IHC) in sections

Embryos were sectioned and processed according to Neves et al. (Neves et al., 2007). Blocking solution was 10% horse serum (Gibco) in PBST (PBS containing 0.1% Tween 20). Primary antibodies were as follows: goat polyclonal anti-Sox2 (Santa Cruz, Y-17; 1:400); rabbit polyclonal anti-Jag1 (Santa Cruz, H-114; 1:50); mouse monoclonal anti-Tuj-1 (Babco, 1:400); mouse monoclonal anti-Isl1 (Developmental Studies Hybridoma Bank (DSHB), 39.4D5; 1:400); rabbit polyclonal anti-MyoVIIA (Proteus BioSciences; 1:400); mouse monoclonal anti-MyoVIIA (DSHB, 39.4D5; 1:400); rabbit polyclonal anti-GFP (Clontech; 1:400); and mouse monoclonal anti-MyoVIIa (Proteus BioSciences; 1:400); mouse monoclonal anti-Sox2 (Santa Cruz, 1:400); mouse monoclonal anti-Sox2 (DSHB), 39.4D5; 1:400]; rabbit polyclonal anti-MyoVIIa (Proteus BioSciences; 1:400); and rabbit polyclonal anti-Sox2 (Santa Cruz, 1:400). Secondary antibodies were Alexa Fluor 488-, 555- or 594-conjugated anti-goat, anti-mouse or anti-rabbit (Molecular Probes, Invitrogen; 1:400). Sections were counterstained with DAPI (100 ng/ml, Molecular Probes) and mounted in Mowiol medium (Calbiochem). Images were obtained by conventional fluorescence microscopy (Leica DMRB fluorescence microscope fitted with a Leica DC300F CCD camera) or confocal microscopy (Leica DMRB fluorescence microscope fitted with a Leica DMI 6000B confocal microscope). Three-dimensional reconstructions and volume calculations were made from serial 20-μm sections using BioVis3D software.

In situ hybridisation (ISH) in whole-mount embryos

Embryos were processed according to Wilkinson and Nieto (Wilkinson and Nieto, 1993) using the automated system from InsitePro VS (Intavis, Bioanalytical Systems). Probes were: Sox2, Jag1, Notch1, Hairy1, Hes5.1 and Hey1 (Fior and Henrique, 2008; Henrique et al., 1995; Palmeirim et al., 1997; Rex et al., 1997).

Quantitative real-time PCR (qRT-PCR)

Otic vesicles were dissected out and total RNA isolated using the RNeasy Mini Kit (Qiagen). For each retrotranscription, 15 ng of purified mRNA was used to synthesise cDNA with Superscript III DNA polymerase (Invitrogen) and random primers (Invitrogen). Real-time PCR was carried out using SYBR Green Master Mix (Roche), 1 μl retrotranscribed cDNA and specific primer sets for each gene (Invitrogen) (see Table S1 in the supplementary material) in a LightCycler 480 (Roche). Gapdh was used as calibrator gene. For each relative quantification, three lots of five to ten otic vesicles generated in three independent experiments were used. Each of these samples was retrotranscribed three times and each retrotranscription was used as template for each pair of primers in a triplicate PCR reaction. Expression levels of each gene were normalised to Gapdh and then referred to the levels in control otic vesicles, which were arbitrarily set to 1.

Electroporation and vectors

HH12-14 embryos were electroporated into the right otic cup. The platinum cathode electrode was placed next to the right otic cup and the anode electrode placed parallel to it, on the other side of the embryo. The desired vector (1 μg/μl) mixed with pCIG vector (0.75 μg/μl) and Fast Green (0.4 μg/μl) was injected onto the otic vesicle by gentle air pressure through a fine micropipette. Square pulses (eight pulses of 10 V, 50 Hz, 250 milliseconds) were generated by a CUY-21 square wave electroporator (BEX, Tokiwasiensyu, Japan). Vectors used for electroporation were: pHRES2-EGFP-cSox2, pCMV-cSox2, pCMV-cSox2AHMG, pCIG-hJag1, pDsRed and 12xCSL-DsRed, pCIG-hJag2 and pCMV-cDelta1.

In vitro culture of otic vesicles

Electroporated and control otic vesicles were dissected from electroporated embryos, transferred into four-well culture plates (NUNC, Roskilde, Denmark) and incubated in DMEM at 37°C in a water-saturated atmosphere containing 5% CO2 as described (Pujades et al., 2006). Additions were 1% foetal bovine serum (FBS) (Bio Whittaker Europe) and DAPT (Calbiochem) at 20 μM.

Atoh1 enhancer activity assays

293T cells (~40,000 cells per well) were transiently transfected with Atoh1-BGZA or Atoh1-BG-EGFP (1.5 μg) alone or together with pCMV-cSox2 (0.1 μg) using a standard calcium phosphate precipitation method. Following transfection, cells were cultured in DMEM with 10% FBS for 36 hours. Cells were then harvested and β-galactosidase (β-gal) activity was determined. For each experimental group, three independent transfections were analysed in triplicate activity assays.

Statistics

Results are shown as averages ± s.e.m. of three independent experiments and Student’s t-test was applied to assess statistical significance.

RESULTS

Sox2 expression becomes restricted to Jag1 domains during prosensory patch formation

Sox2 and Jag1 are expressed in the prosensory patches of the developing inner ear of mouse and chick embryos (Adam et al., 1998; Cole et al., 2000; Hume et al., 2007; Mak et al., 2009; Morrison et al., 1999; Neves et al., 2007). The experiments that follow show that, before prosensory specification, the Sox2 expression domain is broader than that of Jag1, but as prosensory patches develop Sox2 persists only within the Jag1-positive domains. At embryonic day (E) 3, Sox2 was expressed throughout the otic vesicle, but at lower levels laterally (Fig. 1A-C). The most dorsal aspect of the otic vesicle was devoid of Sox2 expression (not shown, see diagram to the right in Fig. 1). Jag1 was expressed within the Sox2 domain but restricted to the anterior and posterior poles of the otic vesicle, connected through a domain of weaker expression that extended medially and ventrally. By E4, both Sox2 and Jag1 were detected in all the prospective sensory domains of the otocyst (Adam et al., 1998; Cole et al., 2000; Neves et al., 2007). Sox2 and Jag1 expression was restricted to the cristae (Fig. 1D), but in the prospective maculae and basilar papilla Sox2 expression still remained broader than that of Jag1 (Fig. 1E,F and diagram). Later in development (E7), the expression of Sox2 and Jag1 became confined to all sensory organs (Fig. 1G-I). Sox2 expression always extending a few cell diameters beyond the Jag1-positive domain. In summary, Jag1 was initially expressed within a larger Sox2 domain, but as development proceeded Sox2 expression was lost outside the Jag1-positive domains. This process followed a dorsal-to-ventral temporal sequence, mirroring the order of differentiation of the sensory organs (Bell et al., 2008; Wu and Oh, 1996). Taken together, this suggests that one function of Jag1 might be to maintain Sox2 expression in the prosensory patches.

hJag1 induces Sox2 expression outside the prosensory domains

In order to analyse the prosensory function of Jag1, we used a gain-of-function approach by means of the electroporation of full-length human jagged 1 (hJag1). The plasmid was able to drive the
expression of hJag1 protein transiently, with the maximal value reached 20 hours after transfection (see Fig. S1A in the supplementary material). Embryos were electroporated before prosensory specification at E2 (HH12-14), were allowed to develop in ovo for different periods and then selected by GFP expression. GFP protein stability allowed us to use it as a tracer of electroporated cells and their progeny even after transgene expression had been shut down. The results are shown in Fig. 2A, where the diagrams on the left depict the location of sensory patches and the level of the sections.

The ectopic expression of hJag1 resulted in the expression of Sox2 outside the prosensory patches (Fig. 2). This effect was not always visible 1 day after hJag1 transfection (Fig. 2A-a'; n=8 embryos, bar chart), but it was clearly present after 2 days (Fig. 2Ac-d' and bar chart). During normal development, the cristae are singled out at the anterior and posterior poles of the otocyst (see the two Sox2-positive patches in Fig. 2Ac and diagram on the left). Electroporated otocysts (Fig. 2Ad,d'), however, exhibited Sox2 expression in both medial (arrows) and lateral (arrowheads) aspects of the otic wall, which corresponded to the domains of expression of the transgene (n=11/12 ectopic Sox2 domains induced by hJag1). The ectopic expression of chicken Sox2 (cSox2) did not result in changes in the expression of Jag1 (see Fig. S1B in the supplementary material).

Confocal microscopy of the extended Sox2 domains revealed that the effect of hJag1 was both cell-autonomous and non-cell-autonomous (Fig. 2Ac). Almost all of the electroporated cells expressed Sox2 (yellow, 97±1%), but not all Sox2-positive cells were double labelled (red, 77.3±12.5%; n=8). No cells co-expressed GFP and Sox2 after pCIG (control) electroporation (Fig. 2Af,f'; n=3). In parallel experiments, otic vesicles were analysed by qRT-PCR, which confirmed that Sox2 mRNA levels increased 2 days after hJag1 electroporation (Fig. 2A, bar chart).

The ability of hJag1 to induce Sox2 was spatially restricted in the otocyst. Sox2 was only induced within the domains that had expressed Sox2 during earlier stages of development. The forced expression of hJag1 in the dorsal domain of the otocyst close to the origin of the endolymphatic sac was ineffective in inducing Sox2 expression (Fig. 2Ba-a'). Fate maps show that this domain derives from the dorsal and posterior otic cup (Abello et al., 2007; Bell et al., 2008; Brigande et al., 2000), which is devoid of neurosensory competence and does not express Sox2 (Neves et al., 2007) (Fig. 1). By contrast, as shown above (Fig. 2A), the forced expression of hJag1 resulted in the expression of Sox2 in electroporated domains at the level of, and ventral to, the cristae, which derive from the Sox2-positive, neurosensory-competent domain. This is summarised in Fig. 2B (diagram to right), which illustrates the fraction of ectopic hJag1 electroporations that were positive for Sox2 expression in the dorsal (A) or ventral (B) domains.

Between E3 and E4, the cristae become specified and the surrounding tissue downregulates Sox2 expression (Fig. 1A,D). We tested whether hJag1 was able to induce Sox2 in the vicinity of the sensory patches once Sox2 expression had been restricted, i.e. from those domains that had lost Sox2 expression. We targeted hJag1 electroporations to the presumptive cristae and surrounding domains at E3.5 and assayed for Sox2 (Fig. 2Bb-b'). In this case, hJag1 did not induce Sox2 expression in the epithelium neighbouring the cristae (n=0/6 electroporated domains).

Taken together, these results suggest that hJag1 is able to maintain the expression of Sox2 in those regions that initially expressed Sox2, but not to induce its expression de novo.

The effects of hJag1 on Sox2 expression require Notch activity

Jag1 is a ligand of the Notch receptor and Notch signalling is active in the prosensory patches (Murata et al., 2006). We tested whether hJag1 results in Notch activation in the otic vesicle and whether Notch activity is required for the effects of hJag1 on Sox2 expression.

hJag1 activated Notch in the otic epithelium, as shown by a fluorescent reporter assay of Notch activity in situ (Fig. 3A). Otic cups were co-electroporated with 12xCSL-DsRed (Hansson et al., 2006) and either NICD, hJag1-pCIG, or pCIG. Specimens were then sectioned and analysed for green and red fluorescence. Constitutively active Notch (NICD) activated DsRed expression from the reporter in all electroporated cells (Fig. 3Aa-a'), as did...
hJag1 (Fig. 3Ab-b'), whereas the pCIG control did not (Fig. 3Ac-c'). Note that the endogenous levels of NICD are insufficient to activate the 12xCSL-DsRed reporter (Hansson et al., 2006).

Hes/Hey genes are direct transcriptional targets of the Notch signalling pathway and are expressed in the prosensory patches of the mouse and chick otocyst (Hayashi et al., 2008; Murata et al., 2009). We tested whether they are downstream effectors of the Jag1-mediated activation of Notch. The relative mRNA levels of four Hes/Hey genes were analysed by qRT-PCR 1 and 2 days after hJag1 transfection (Fig. 3B). Hairy1, Hey1 and Hey2 mRNA levels were significantly increased after 1 day, Hex5 remaining unaltered. This effect was transient, paralleling the profile of transgene expression. These results were confirmed by ISH for Hairy1 and Hey1 (data not shown).

To test the requirement of Notch signalling for the regulation of Sox2 by hJag1, we combined the in ovo electroporation of hJag1 with the in vitro culture of explanted otic vesicles in the presence of DAPT (Fig. 3C). DAPT blocks Notch activation by inhibiting the γ-secretase activity required for the S3/S4 cleavage of NICD (Dovey et al., 2001; Geling et al., 2002). Otic vesicles were electroporated with hJag1 and allowed to develop for 1 day. Otic vesicles were then isolated and cultured with either DAPT or DMSO (carrier control) for an additional day, after which they were analysed by qRT-PCR for Sox2 expression (Fig. 3C, bar chart). The results show that Sox2 induction by hJag1 was blocked by DAPT, indicating that it requires active Notch signalling.

In summary, hJag1 activates Notch in the otic epithelium and this activation is required for hJag1-dependent Sox2 expression.

Jag1 operates through lateral induction
The results above show that the effects of hJag1 are mediated by Notch signalling and that the transfection of hJag1 results in coherent domains of Sox2 expression. The formation of such cooperative cell clusters has been associated with the mechanism of lateral induction mediated by Notch (Bray, 1998; de Celis and Bray, 1997; Lewis, 1998). By definition, lateral induction refers to the positive-feedback mechanism in which Notch activation in one cell induces the expression of the Notch-activating ligand in that cell (Bray, 1998).

We tested the ability of hJag1 to induce the expression of endogenous Jag1. Otic vesicles were electroporated with hJag1 and analysed for the expression of Jag1 with chick-specific probes and primers by ISH and qRT-PCR. Transcript levels of endogenous Jag1 significantly increased with respect to the control 1 day after
hJag1 transfection (Fig. 4A). This effect was transient and faded after 2 days, paralleling the temporal profile of transgene expression (see Fig. S1A in the supplementary material). hJag1 did not induce Notch1 or delta 1 (Dl1) transcription (Fig. 4A).

The induction of endogenous Jag1 by hJag1 was further confirmed by ISH (Fig. 4Ba-b'; n=3/3) and by double immunostaining for GFP and Jag1 (Fig. 4Bc). Immunostaining revealed that not all Jag1-positive cells were GFP positive. This
indicates that a fraction of the anti-Jag1 immunoreactivity was not driven by the hJag1 transgene, but corresponded to the endogenous Jag1 expression induced in neighbouring cells.

A crucial question is that of the specificity of the different Notch ligands in the processes described above. Are other Notch ligands equally efficient in lateral induction and in promoting Sox2 expression? We electroporated chick Dll1 (cDll1) and hJag2 under the same conditions as described above and analysed the expression of Jag1 and Sox2 (Fig. 4C). cDll1 was unable to mimic the effects of hJag1 (Fig. 4Ca-b; n=0/3), whereas the forced expression of hJag2 induced both Sox2 and Jag1 (Fig. 4Cc-d; n=3/3). This suggests that both lateral induction and Sox2 regulation are specific to the cellular response to Jag ligands.

In summary, the forced expression of hJag1 was able to activate Notch, to induce Notch targets and to induce Jag1 expression in a non-cell-autonomous manner, without affecting Notch1 expression. This strongly supports the notion that Jag1 operates by a mechanism of lateral induction that relies on a positive-feedback loop provided by ligand induction and receptor activation.

**hJag1-induced Sox2 patches develop as sensory organs**

Since hJag1 is able to extend Sox2 expression outside the prosensory patches, we examined whether this resulted in larger sensory organs. hJag1-transfected otic vesicles were analysed after 4 days of development in ovo, which is equivalent to E6, when nascent hair cells express differentiation markers such as MyoVIIa (Sahly et al., 1997) and supporting cells express Sox2 (Neves et al., 2007). Serial sections were used to reconstruct three-dimensional models of the otic vesicles to analyse the size and position of the sensory organs.

The expression of Sox2 induced by hJag1 was maintained after 4 days (Fig. 5Aa-b’). The high-magnification image in Fig. 5Ac shows Sox2-positive cells located at the basal layer of the sensory patch corresponding to the supporting cells (Neves et al., 2007). Transfected cells also differentiated as hair cells, as revealed by MyoVIIa staining (Fig. 5Ad-e’; arrowheads). Fig. 5B shows a dorsal view of a three-dimensional reconstruction of an otocyst that was electroporated in the region of the maculae (right), as compared with the corresponding contralateral control otocyst (left). The macular domain was expanded in the transfected otic vesicle and corresponded well to the GFP-positive region. The ability of hJag1 to induce sensory cells was restricted to the regions of the otocyst located at, or ventral to, the level of the cristae and GFP-positive cells dorsal to those domains did not express MyoVIIa or Sox2 (Fig. 5B, asterisks). This is also illustrated in Fig. 5Ca-a’, in which an ectopic hJag1-positive domain in the dorsal otocyst is not paralleled by MyoVIIa expression (arrowheads; arrow points to a normotopic crista). The results of several experiments are summarised in the
Sox2 is sufficient to induce neurosensory fate in the otic epithelium

The data described above suggest that the prosensory function of Jag1 relies on Sox2 to specify the sensory fate. Therefore, the question arises as to whether Sox2 is sufficient to specify sensory fate in the absence of Notch activation. We electroporated E2 otic vesicles with cSox2 and analysed hair cell differentiation. Electroporations outside sensory domains were selected by comparison with the untransfected contralateral otic vesicle (Fig. 6A-B', compare domains with arrows). The ectopic expression of Sox2 induced Islet1 and resulted in ectopic sensory and neurogenic patches (n=14/16 ectopic domains).

Fig. 6C-C' show a detail of one cluster of electroporated cells that delaminated from the otocyst and co-expressed GFP and Islet1. Ectopically delaminated GFP-positive cells also expressed Tuj1 (also known as Tubb3) (not shown). The volume of the CVG significantly increased in Sox2-transfected otocysts, as did the transcript levels of Ngn1 (see Fig. S2 in the supplementary material). This suggests that Sox2 is sufficient to specify neurogenic fate in the otic epithelium. This was not modified by hJag (see Fig. S2 in the supplementary material).

Domains transfected with cSox2 also expressed MyoVIIa, indicating that they differentiated as hair cells (n=14/16 ectopic domains electroporated with Sox2; Fig. 6D-E'). In contrast to hJag1, after the forced expression of Sox2 all regions of the otocyst were able to generate MyoVIIa-positive ectopic patches (Fig. 6F, diagram). Even the dorsal-most electroporations, targeted at the endolympathic duct, resulted in ectopic MyoVIIa-positive cells (Fig. 6E,E'). This suggests that Sox2 expression provides the competence to develop into sensory cells and that this is extended to the whole otocyst.

Atoh1 is a basic helix-loop-helix (bHLH) transcription factor that is expressed in sensory progenitors and behaves as a master gene for hair cell determination (Bermingham et al., 1999; Zheng and Gao, 2000). In order to further confirm the ability of Sox2 to induce sensory fate, we analysed its ability to activate Atoh1 expression. We made use of a reporter construct that contains either the lacZ or EGFP gene under the control of the murine Atoh1 regulatory region (Ebert et al., 2003; Helms et al., 2000; Timmer et al., 2001). This enhancer region resides ~3.4 kb 3' of the coding sequence and is sufficient to recapitulate the endogenous Atoh1 expression pattern in several species. We transfected human 293T cells with this reporter construct alone or together with Sox2, and analysed β-gal activity (Fig. 6G, bar chart) or EGFP expression (Fig. 6G, photomicrographs) 2 days after transfection. The results show that Sox2 induces a large increase in the activity of the Atoh1 reporter, strongly suggesting that Sox2 is able to promote Atoh1 expression.

DISCUSSION

The generation of prosensory patches remains one of the most intriguing questions in inner ear development. Its understanding requires deciphering how sensory potential is acquired and restricted to specific domains of the otic epithelium. The expression of Jag1 and Sox2 foreshadows the emergence of the sensory organs from early developmental stages (Adam et al., 1998; Cole et al., 2000; Neves et al., 2007) and these genes are necessary for correct sensory organ development (Brooker et al., 2006; Kierman et al., 2005b; Kierman et al., 2006). The data presented in this work dissect the mechanism that links Jag1, Notch activation and Sox2 during sensory specification. We propose a model in which Jag1 restricts Sox2 expression to prosensory domains through a mechanism that links Jag1, Notch activation and Sox2 during sensory specification. We propose a model in which Jag1 restricts Sox2 expression to prosensory domains through a mechanism that links Jag1, Notch activation and Sox2 during sensory specification. We propose a model in which Jag1 restricts Sox2 expression to prosensory domains through a mechanism that links Jag1, Notch activation and Sox2 during sensory specification. We propose a model in which Jag1 restricts Sox2 expression to prosensory domains through a mechanism that links Jag1, Notch activation and Sox2 during sensory specification. We propose a model in which Jag1 restricts Sox2 expression to prosensory domains through a mechanism that links Jag1, Notch activation and Sox2 during sensory specification.
This suggests that the restriction of Sox2 driven by Jag1 underlies the transition from a common and extended neurosensory-competent domain to discrete prosensory patches. Several studies have shown that Jag1 and Notch signalling are indeed necessary for Sox2 expression in the otic epithelium (Dabdoub et al., 2008; Daudet et al., 2007; Kiernan et al., 2006). Our results indicate that Jag1 is not sufficient to induce Sox2 expression de novo, but maintains its expression in those regions that already expressed Sox2 early in development.

The model proposed here suggests that all sensory organs develop from a common neurosensory domain, the singling out of sensory organs being an independent process that requires the local expression of Jag1. The neurosensory competence is dependent on Sox2 (Kiernan et al., 2005b) (this work) and is acquired early in development. The initiation of Sox2 expression and neurosensory competence in the inner ear do not depend on Notch, but on FGF signalling and Sox3 (Abello et al., 2010). It is only later in development that the initially broad and continuous Sox2 domain resolves into smaller and individual domains that correspond to prosensory patches. We propose that this patterning process depends on the activation of Notch by Jag1 and relies on the ability of Notch signalling to maintain Sox2 expression. This explains why ectopic activation of Notch is unable to generate ectopic hair cells in all domains of the otocyst (Daudet and Lewis, 2005; Hartman et al., 2010; Pan et al., 2010), but only within the neurosensory-competent domains of the otic placode.

Although the maintenance of Jag1 expression in the sensory patches is Notch dependent, its onset is not (Daudet et al., 2007). Thus, the regulation of initiation of Jag1 expression is a key event to complete an understanding of the patterning process. Jag1 is an evolutionarily conserved target of canonical Wnt signalling (Katoh, 2006) and there is evidence that during inner ear development, Jag1 is induced by Wnt (Jayasena et al., 2008). Interestingly, the gain of function of Wnt results in ectopic and fused sensory patches (Sienknecht and Fekete, 2008; Stevens et al., 2003). This suggests that Wnt signalling might regulate the expression of Jag1 and Sox2 in the prosensory patches.

**Jag1 functions through lateral induction**

The model discussed above requires Notch to be active in all the cells of the patch where Sox2 expression is to be maintained. This cannot result from lateral inhibition, which creates a salt and pepper pattern. On the contrary, it requires a mechanism in which Notch activation by a ligand results in the induction of that ligand and in consequence in a continuous domain of Notch activity. Such a positive-feedback mechanism is known as lateral induction (Bray, 1998; Lewis, 1998) and has been associated with the formation of morphological boundaries and spatial patterns in development (Baek et al., 2006; Cheng et al., 2004; de Celis and Bray, 1997).

Several studies have suggested that Jag1 expression in the otic epithelium is maintained by lateral induction (Daudet et al., 2007; Daudet and Lewis, 2005; Eddison et al., 2000; Hartman et al., 2010). However, there is no direct demonstration that the mechanism operates in a Jag1-dependent manner. Our results show that hJag1 activates Notch signalling and induces endogenous Jag1 expression in a non-cell-autonomous manner. Furthermore, we show that scattered ectopic transgene expression results in the generation of a coherent domain where all the cells express Jag1. Lateral induction and Sox2 expression are associated with Jagged-like ligand activity but not with Dll. Differential effects of Notch ligands on ear and neural development have been suggested previously, both in chick and mammals (Brooker et al., 2006; Daudet et al., 2007; Eddison et al., 2000; Ramos et al., 2010). Since active Notch mimics the effects of Jag1 in the expansion of prosensory patches (Daudet and Lewis, 2005; Hartman et al., 2010; Pan et al., 2010), it is likely that the differences between the effects of Jag1 and Dll arise from their ability to interact with the receptor, and Fringe proteins are likely candidates to mediate this selectivity (Fortini, 2009; Zhang et al., 2000).

**Sox2 and sensory fate specification**

Sox2 is sufficient to specify neurosensory fate (i.e. sensory and neuronal progenitors) in the otic epithelium. Other Sox2 genes are also sufficient to specify neuronal fate in the chick otic epithelium (Abello et al., 2010), and, in the course of our study, Puligilla et al. (Puligilla et al., 2010) showed that Sox2 is able to induce neuronal fate in the developing mammalian cochlea. Our work shows that Sox2 is also able to induce sensory markers and to activate the Atoh1 enhancer, i.e. to promote early steps in sensory commitment. This ability is independent of Notch activity, but it probably depends on other partners that provide context dependence (Kamachi et al., 2000). However, Sox2 is known to counteract Atoh1 function in the ear (Dabdoub et al., 2008). This seemingly contradictory function of Sox2 is reminiscent of that of Sox2B1 genes in the neural tube (Bylund et al., 2003; Pevny and Placzek, 2005). Sox2 expression in otic progenitors would define a population of cells that is committed to the neurosensory fate but prevented from differentiation. This ensures expansion of the neural-competent population and the generation of different cell types.

**Neuronal versus sensory specification in the inner ear**

In the amniote inner ear, the generation of neurons and of hair cells proceed sequentially, neurons being specified prior to sensory cells (Bell et al., 2008; Raft et al., 2007). Sox2 is expressed in neuronal and sensory progenitors and it is sufficient to specify both cell fates (Neves et al., 2007) (this work). Thus, it is tempting to suggest that Sox2 expression defines this dual competence. It is conceivable that Sox2-positive progenitors would generate neurons as an early fate and sensory cells as a late fate. By facilitating the persistence of Sox2 and neurosensory competence, Jag1-dependent Notch activity might allow the expression of late fates only within restricted domains. Notch activity would also allow the expansion of the progenitor pool, so that all cell types could be formed at the correct times. As a consequence, Sox2 expression associated with Jag1 would predict sensory fate, whereas Jag1-independent Sox2 expression would predict neurogenesis, and this corresponds well with the expression of Sox2, Jag1 and Dll during neurosensory development (Abello et al., 2007; Adam et al., 1998; Neves et al., 2007).

In summary, the present work provides evidence for a link between Jag1 and Sox2 functions during sensory organ development in the chick inner ear. Our model (Fig. 7) proposes that sensory organ generation would result from two processes – patterning and cell fate specification – that are regulated independently. First, a broad Sox2-positive domain would be set by FGF signals and confer neurosensory competence to a subdomain of the otic placode. This region would go through the stage of neurogenesis as an early fate of neurosensory-competent progenitors. Wnt activity would drive Jag1 expression, which, in turn, would maintain local Sox2, whereas Sox2 is switched off in neighbouring regions. As a result, progenitor cells residing within the Jag1-Notch patches would allow the expression of late fates and become hair and supporting cells. The model provides a
Jag1, Sox2 and otic sensory development

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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