Otx2 is a target of N-myc and acts as a suppressor of sensory development in the mammalian cochlea

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ABSTRACT

Transcriptional regulatory networks are essential during the formation and differentiation of organs. The transcription factor N-myc is required for proper morphogenesis of the cochlea and to control correct patterning of the organ of Corti. We show here that the Otx2 gene, a mammalian ortholog of the Drosophila orthodenticle homeobox gene, is a crucial target of N-myc during inner ear development. Otx2 expression is lost in N-myc mouse mutants, and N-myc misexpression in the chick inner ear leads to ectopic expression of Otx2. Furthermore, Otx2 enhancer activity is increased by N-myc misexpression, indicating that N-myc may directly regulate Otx2. Inactivation of Otx2 in the mouse inner ear leads to ectopic expression of prosensory markers in non-sensory regions of the cochlear duct. Upon further differentiation, these domains give rise to an ectopic organ of Corti, together with the re-specification of non-sensory areas into sensory epithelia, and the loss of Reissner’s membrane. Therefore, the Otx2-positive domain of the cochlear duct shows a striking competence to develop into a mirror-image copy of the organ of Corti. Taken together, these data show that Otx2 acts downstream of N-myc and is essential for patterning and spatial restriction of the sensory domain of the mammalian cochlea.

KEY WORDS: Inner ear, Cochlea, Otx, Myc, Organ of Corti, Mouse

INTRODUCTION

Otx2, a murine ortholog of the Drosophila orthodenticle (otd; ocelliless – FlyBase) gene, encodes a transcription factor required for early specification of the brain and for development of sensory organs, including the inner ear (Cat-Yablonski, 2011). In humans, heterozygous mutations of OTX2 lead to eye and pituitary gland defects (Henderson et al., 2007, 2009) and have been also reported to cause hearing loss (Ragge et al., 2005). During mouse inner ear development, Otx2 is initially expressed in the ventral portion of the otic vesicle, which gives rise to the cochlea. Later on, it is expressed in the non-sensory area of the roof of the cochlear duct corresponding to the future Reissner’s membrane, which separates the scala media from the scala vestibuli (Morsli et al., 1999). Mouse mutants of the related Otx1 gene display shortening of the cochlea, loss of the lateral semicircular canal and fusion of sensory epithelia (Morsli et al., 1999). Additional loss of one Otx2 allele in an Otx1 null background results in a more severe phenotype, especially in the cochlea. However, owing to the early lethality of Otx2 mutant mice (Acampora et al., 1995; Ang et al., 1996; Matsuo et al., 1995), its role during inner ear development has not been addressed.

Inactivation of N-myc (Mycn – Mouse Genome Informatics) in the mouse inner ear affects proliferation, morphogenesis and differentiation of the inner ear (Domínguez-Frutos et al., 2011; Kopecky et al., 2011). Besides an overall size reduction of the inner ear, N-myc mouse mutants show more complex phenotypes affecting morphogenesis and patterning, including loss of the lateral semicircular canal and fusion of sensory epithelia. The cochlea is shortened and characterized by an increased number of hair cells in its apical part (Domínguez-Frutos et al., 2011; Kopecky et al., 2011).

In the present work, we show that in the course of a microarray-based screen designed to identify N-myc target genes in the cochlea, we found Otx2 as a candidate to mediate some of the functions of N-myc during cochlear development. Otx2 is one of the genes expressed in the roof of the cochlear duct that is most strongly downregulated in N-myc mutants. Conversely, N-myc misexpression in the chick inner ear leads to ectopic activation of Otx2 expression, and we provide evidence that distal enhancer elements located within the 3′ genomic regulatory region of the Otx2 gene are directly responsive to N-myc. To study the effects of Otx2 further, we generated conditional Otx2 mutant mice and showed that Otx2 inactivation in the inner ear results in re-patterning of the cochlea. Otx2 ablation leads to the ectopic expression of several key genes involved in formation of the prosensory domain in the prospective non-sensory domain normally defined by Otx2 expression and that gives rise to Reissner’s membrane (Morsli et al., 1999). Accordingly, Reissner’s membrane is absent in Otx2 mutant cochleas, which exhibit a striking mirror-image duplication of the organ of Corti. Our results suggest that Otx2 acts downstream of N-myc as a suppressor of sensory fate and that it is required for correct patterning of the non-sensory region that will give rise to Reissner’s membrane.

RESULTS

A screen for N-myc-regulated genes reveals downregulation of genes in the non-sensory region of the roof of the cochlear duct

Loss of N-myc in the mouse cochlea leads to its shortening and to an increased number of differentiating sensory hair cells in the apex (Domínguez-Frutos et al., 2011; Kopecky et al., 2011). In order to identify potential target genes of N-myc in the mammalian cochlea, we performed a microarray-based screen for differential gene expression in N-myc mutant versus wild-type cochleas (for details, see Materials and Methods). We used whole cochleas at embryonic day (E) 15 when N-myc expression is detected in the floor of the cochlear duct, including differentiating hair cells (Domínguez-
null allele in heterozygosity on an Otx1 null background leads to enhancement of the Otx1 mutant inner ear phenotype, consisting of loss of the lateral semicircular canal, fusion of sensory epithelia and shortening of the cochlea (Morsli et al., 1999). These alterations are very similar to those reported in N-myc mutant mice (Dominguez-Frutos et al., 2011; Kopecky et al., 2011), suggesting that Otx2 may be instrumental for N-myc function in the ear. The onset of Otx2 expression during inner ear development occurs in the otic vesicle and is lost in N-myc mutants (Fig. 1J,K). These results prompted us to analyze further the relationship between Otx2 and N-myc during inner ear development.

**Misexpression of N-myc induces expression of Otx2 in the otic vesicle**

In order to study the regulation of Otx2 by N-myc, we misexpressed N-myc in the chicken otic placode by electroporation of a cDNA encoding for N-myc together with a GFP reporter (see Dominguez-Frutos et al., 2011). The expression of Otx2 in the otic vesicle was examined 1 day after electroporation using riboprobes specific for chicken Otx2 (cOtx2). Like in the mouse, endogenous expression of cOtx2 occurs in the ventral portion of the otic vesicle (Fig. 2A). However, after N-myc electroporation, ectopic cOtx2 expression was detected in the anterior (Fig. 2B) and dorsal (Fig. 2C; supplementary material Fig. S1) portions of the otic vesicle (n=4/4; for details, see Materials and Methods). Interestingly, the ectopic cOtx2 expression domain was always broader than GFP reporter activity, suggesting non-cell-autonomous interactions.
In the developing mouse embryo, Otx2 expression is controlled by specific enhancers located 5′ and 3′ of its coding region (Kurokawa et al., 2004). A 1.2-kb fragment in the 3′ enhancer region of Otx2 has been shown to drive expression of a lacZ reporter gene in the otic vesicle (Kurokawa et al., 2004). Although this Otx2lacZ reporter shows a broader expression than the endogenous Otx2 gene, it nevertheless provides a tool to test potential transcriptional regulators that drive Otx2 expression in the otic vesicle. Electrodeposition of the Otx2lacZ reporter construct confirmed the activity of this enhancer region in the otic vesicle of chicken embryos (Fig. 2D). Moreover, the co-electrodeposition of the Otx2lacZ reporter together with N-myc showed an increased activity of the reporter compared with the Otx2lacZ reporter alone (compare Fig. 2E and 2F). In summary, the data show that N-myc is sufficient to drive Otx2 expression in the otic vesicle and increases the efficiency of transcription from the 3′ Otx2 enhancer.

To explore whether N-myc and Otx2 cross-regulate each other, we misexpressed Otx2 in the otic placode of chicken embryos. After Otx2 electroporation no ectopic expression of N-myc was observed and its endogenous pattern was unaffected (data not shown).

**Formation and patterning of the otic vesicle is unaffected in conditional Otx2 mutant mice**

The exact role of Otx2 during inner ear development has not yet been addressed because of the early lethality of Otx2 mutant mice (Acampora et al., 1995; Ang et al., 1996; Matsuo et al., 1995). To overcome this problem, we generated conditional Otx2 mutant mice in which tissue-specific deletion of the essential Otx2 exon 2 flanked by loxP sites (Fossat et al., 2006) was achieved using a Cre line driven by Pax2 regulatory sequences. The Pax2Cre line has been used to inactivate floxed alleles during initial stages of inner ear development (Ohyama and Groves, 2004). Loss of Otx2 in the otic vesicle of homozygous Pax2Cre-Otx2 mutants, at the onset of Otx2 expression, was confirmed by whole-mount RNA in situ hybridization (Fig. 3A). Additionally, loss of Otx2 expression was observed in other Pax2-expressing tissues, such as the optic vesicle and the mesencephalon. As previously shown, loss of Otx2 caused a severe reduction or absence of these structures (Acampora et al., 1995; Ang et al., 1996; Matsuo et al., 1995) (Fig. 3A) and homozygous Pax2Cre-Otx2 mutants showed early postnatal lethality within the first week after birth.

The size of the otic vesicle in Pax2Cre-Otx2 mutants was similar to that of control littermates (Fig. 3A), and the specification of its neurosensory domain was unaffected (Fig. 3B-K). This region develops in an anterior-medial position flanking the Otx2 expression domain and is characterized by the expression of Ngn1 (Ng1; Neurog1 – Mouse Genome Informatics), NeuroD (Fritzsche et al., 2010), lunatic fringe (LFinf) (Morsli et al., 1998) and Fgf3 (Hatch et al., 2007). Whole-mount in situ hybridization of wild-type and Pax2Cre-Otx2 mutant embryos revealed correct localization of mRNA for these genes in the otic vesicle (Fig. 3B-K). Moreover, NeuroD was expressed in the otic ganglion suggesting that neuroblast delamination was unaffected by the loss of Otx2 (Fig. 3J,K). Finally, to investigate a potential cross-regulation between Otx2 and N-myc, we examined N-myc expression in Otx2 mutant otic vesicles. N-myc is initially expressed throughout the otic vesicle and, later on, becomes restricted to the prosensory regions (Dominguez-Frutos et al., 2011). Expression of N-myc was unaffected in the otic vesicle of Otx2 mutants (Fig. 3LM; data not shown), suggesting that Otx2 expression is not required for the formation and early patterning of the otic vesicle.

**Loss of Otx2 expression leads to ectopic expression of prosensory markers in the cochlear duct**

The HMG-box transcription factor Sox2 and the Notch ligand Jag1 are necessary for the specification of sensory progenitors in the cochlear duct (Dabdoub et al., 2008; Kiernan et al., 2005, 2006) and both are initially expressed in the ventral part of the cochlea (Ohyama et al., 2012). Later on, Sox2 becomes restricted to the prosensory domain and to the region of the Kölliker’s organ that gives rise to the inner sulcus (Fig. 4A), whereas Jag1 is progressively restricted to Kölliker’s organ (Fig. 4C). The prosensory region is characterized by the expression of p27kip1 (Cdkn1b – Mouse Genome Informatics) (Fig. 4E), which labels postmitotic hair and supporting cell progenitors (Lee et al., 2006). Expression of these markers was normal in the basal portion of the cochlea of Pax2Cre-Otx2 mutants at E14 (data not shown). However, the midbasal and apical regions showed a dramatic expansion of the Sox2 expression domain towards the roof and the abneural portions of the cochlear duct (Fig. 4B). Sox2 expression was accompanied by an apparent thickening of the cochlear epithelium, a feature normally observed only in the floor of the cochlear duct but not in the non-sensory region of its roof. In parallel, and next to their normal regions of expression, Jag1 showed an additional ectopic expression domain in the neural portion of the
cochlear duct (Fig. 4D, asterisk) whereas p27kip1 was present both in the neural region and the adjacent portion of the cochlear roof (Fig. 4F, asterisk). Finally, we examined Fgf10, a marker for the prospective Kölliker’s organ (Ohyama et al., 2012) (Fig. 4G) and N-myc, which is restricted to the floor of the cochlear duct (Dominguez-Frutos et al., 2011) (Fig. 4I). The expression domains of both genes were expanded to the roof of the cochlear duct (Fig. 4H,J). Taken together, these results indicate that loss of Otx2 in the non-sensory region of the cochlear duct leads to re-patterning of the mouse cochlea with ectopic expression of markers normally restricted to the prosensory domain and the neighboring Kölliker’s organ.

Otx2 mutant mice lack Reissner’s membrane and instead form an ectopic organ of Corti

In order to determine the consequences of the abnormal patterning of the cochlear duct in Otx2 mutants, we next examined the early postnatal period when the organ of Corti fully develops. At postnatal day (P) 0, the different subcompartments of the cochlea can be distinguished in clarified wild-type inner ears: the scala media and scala vestibuli separated by Reissner’s membrane, and the scala tympani (Fig. 5A,B). Strikingly, in Pax2Cre-Otx2 mutants, no Reissner’s membrane could be detected (Fig. 5A,C). Histological sections confirmed the absence of Reissner’s membrane (Fig. 5D,E), and indicated the formation of ectopic hair cells on the neural side of the scala media (Fig. 5F,G). This was confirmed by whole-mount immunostaining with a myosin VIIA antibody (Fig. 5H-K). Pax2Cre-Otx2 mutant cochleas revealed the presence of two parallel myosin VIIA-positive stripes (Fig. 5I). Further dissection of the sensory regions revealed that the stripe of hair cells present in the normotopic position of the cochlear duct of Pax2Cre-Otx2 mutants was similar to that observed in the sensory epithelia from wild-type controls: one row of inner and three rows of outer hair cells (Fig. 5H,J). By contrast, the ectopic stripe of sensory
epithelia located in the roof and abneural portion of the cochlear
abneural side of the organ of Corti (Fig. 6G,H).

Finally, we examined the expression of markers of non-sensory
epithelia showed a poorly organized pattern that lacked the
ordered rows of inner and outer hair cells (Fig. 5K). Moreover, the
number of hair cells within the sensory epithelium of mutants was
higher than that observed in the sensory epithelium of control ears.
This phenotype was especially apparent in the apical region, which
exhibited a club-shaped ending containing numerous myosin VIIA-
positive cells (Fig. 5K, insets).

To characterize these changes further, we used a series of markers
providing information on the differentiation of specific subtypes of
cells, such as hair cells, nerve fibers, supporting cells and non-
sensory cells of Reissner’s membrane or the stria vascularis. The
presence of inner hair cells was confirmed using calretinin antibodies (Dechesne et al., 1994) (Fig. 6A). Calretinin-positive cells were detected in the normotopic organ of Corti and also on the
neural side of the ectopic patch of hair cells in Pax2Cre-Otx2
mutants (Fig. 6B). Neurofilament antibodies labeled nerve fibers
that reached both the normotopic organ of Corti and the ectopic hair
cells present in Pax2Cre-Otx2 mutant mice (Fig. 6C,D). We next
examined the presence of supporting cells that underlie or flank hair
cells within the organ of Corti. Upon differentiation of hair cells,
Sox2, which is initially expressed throughout the developing organ
of Corti, becomes restricted to supporting cells (Dabdoub et al.,
2008) (Fig. 6E). Ectopic sensory regions of Pax2CRE-Otx2 mice also showed a Sox2 staining pattern that was very similar to the
normotopic organ of Corti, confirming the presence of supporting
cells (Fig. 6F). Ectopic hair cells in Pax2CRE-Otx2 mutants displayed a faint immunoreactivity for Sox2, probably indicating
delayed differentiation. To specify further the supporting cell types
present in the Pax2CRE-Otx2 mutant, we examined the expression of the low-affinity neurotrophin receptor p75 (Ngfr – Mouse Genome
Informatics), which shows a highly characteristic expression in the
apical cell membranes of the inner pillar and Claudius cells during
differentiation of the organ of Corti (Mueller et al., 2002; Shim
et al., 2005) (Fig. 6G). As in control and in the normotopic sensory
epithelium of Pax2CRE-Otx2 mutants, p75 labeled the apical cell
membrane of the ‘inner pillar cell head’ in ectopic hair cell clusters
(Fig. 6G,H, arrows). In the vicinity of these cells, another group of
cells also showed their apical sides strongly labeled with p75. These
probably correspond to ectopic Claudius cells usually found on the
abneural side of the organ of Corti (Fig. 6G,H).

In summary, the results show that Otx2 is required for the correct
patterning of the cochlea (Fig. 7). Otx2 directs the formation of
Reissner’s membrane, which in Pax2CRE-Otx2 mutants is replaced
by an ectopic cochlear sensory domain consisting of ectopic hair cells
that are innervated and surrounded by supporting cells. The
staining pattern of calretinin, together with the characteristic
localization of the supporting cell markers Sox2 and p75 indicates
that the loss of Otx2 causes a mirror image duplication of the organ

Fig. 6. Differentiation of different cell types in the cochlear duct of Otx2 mutants. (A–L) Detection of the indicated proteins in the
cochlear duct of wild type (wt) and Otx2 mutants at P0. (A,B) Calretinin antibodies preferentially label normotopic (arrows
in A,B) and ectopic (asterisk in B) inner hair cells. (C,D) Nerve fibers labeled with antibodies against neurofilaments (nf200) are found
underneath the normotopic (s) and ectopic sensory region (s* in F). (E,F) Sox2 expression is detected in supporting cells underlying the
normotopic (s) and ectopic sensory epithelium (s* in F). (G,H) p75 immunoreactivity is found in the apical portions of normotopic pillar
cells (arrows) and Claudius cells (c), and in the ectopic pillar and Claudius cells (arrow* and c* in H). (I–L) Pax2 and Lrp2 are
expressed in Reissner’s membrane (rm) and the stria vascularis (sv). Note the absence of Reissner’s membrane and the expansion of the
striata vascularis in Otx2 mutants. Scale bars: in A, 50 μm for A, B,E–H; in C, 50 μm for C,D,I–L.

Fig. 7. Expression domains of genes involved in otic patterning during
development upon loss of Otx2 or N-myc. At the otic vesicle stage of wild-
type embryos, N-myc is initially broadly expressed, whereas Sox2 is found in
the antero-ventral neurosensory region which abuts with the more posterior
localized Otx2 domain. In otic vesicles of N-myc mutants Otx2 expression is
absent. The loss of Otx2 expression in the otic vesicle and/or the non-sensory
regions of the roof of cochlear duct leads to the ectopic expression of Sox2,
Jag1, p27Kip1, Fgf10 and N-myc, and to the formation of an ectopic prosensory
region and differentiation of hair cells. A, anterior; D, dorsal; P, posterior;
V, ventral.
of Corti across the neural-abneural axis of the cochlear duct. The ectopic expression of Fgf10, Sox2 and Jag1 during patterning of the prosensory region further suggests that this duplication also includes Kölliker’s organ.

**DISCUSSION**

The relevance of the interaction between transcription factors has recently been underlined by the ENCODE project, which has revealed how analysis of transcriptional regulatory networks is crucial for understanding human biology and disease (Gerstein et al., 2012). Some of the regulatory interactions that occur during the formation of the inner ear are starting to be clarified (Raft and Groves, 2015; Schimmang, 2013). For instance, a transcriptional complex composed of Sox2, Six1 and Eya1 has been shown to bind directly to the Atoh1 promoter, thereby promoting hair cell fate (Ahmed et al., 2012; Neves et al., 2012). Yet, the interactions between different transcription factors during inner ear formation remain largely unknown.

Transcriptional profiling of Myc-regulated processes has led to the discovery of a large number of genes controlled by the Myc gene family (Conacci-Sorrell et al., 2014). However, unlike c-myc (Myc – Mouse Genome Informatics), information on targets of N-myc remains scarce (Beltran, 2014). Our microarray-based screening has identified Otx2 as one of the genes for which expression depends on N-myc during inner ear development. Somehow surprisingly, the downregulation of Otx2 expression in the N-myc mutant cochlea was observed at E15, when the expression of both genes does not overlap. At this stage, Otx2 expression is restricted to non-sensory regions of the inner ear, whereas N-myc is detected in the prosensory regions and cochlear hair cells (Dominguez-Frutos et al., 2011; Kopecky et al., 2011). However, the expression domains of both genes do coincide earlier in development, at the onset of Otx2 expression in the most ventral part of the otic vesicle (Dominguez-Frutos et al., 2011; Kopecky et al., 2011; Morsli et al., 1999) (Fig. 1J; Fig. 7). Indeed, our results show that N-myc is required for Otx2 expression at this stage (Fig. 1K). This suggests that the expression of N-myc at the otic vesicle stage is crucial for the initiation and maintenance of Otx2 expression throughout later stages of inner ear development.

Experiments in the chick suggest that N-myc can activate Otx2 directly. First, ectopic Otx2 expression is observed following N-myc overexpression in the otic vesicle. Second, further experiments showed that, as in the mouse (Kurokawa et al., 2004), the 3’ Otx2 enhancer is likely to be responsible for this activation. *In silico* analysis of the 3’ Otx2 enhancer reveals the presence of several putative N-myc binding sites that may account for this activation.

The inner ear phenotype of N-myc mutants shows some interesting features in common with Otx1 mutants (Dominguez-Frutos et al., 2011; Kopecky et al., 2011; Morsli et al., 1999). By contrast, unlike N-myc or Otx1 knockout mice, the major defect of Otx2 mutants consists of a duplication of the organ of Corti. Why then, if N-myc is upstream Otx2, does the loss of N-myc not result in a similar mutant phenotype? The loss of N-myc leads to a truncated cochlea caused by a lack of proliferation and an overall alteration of morphogenesis and patterning (Dominguez-Frutos et al., 2011; Kopecky et al., 2011). This suggests the possibility that N-myc knockout animals do not develop the Otx2 mutant phenotype (duplication of the organ of Corti) because of the severity of the N-myc phenotype, which, besides Otx2, affects several other genes (supplementary material Table S1). Another surprising difference between N-myc and Otx2 mutants is the fact that N-myc mutants maintain Reissner’s membrane (Kopecky et al., 2011) whereas it is lost in Otx2 mutants. In this case, we can only speculate that, although loss of Otx2 leads to the conversion of the non-sensory region, destined to give rise to Reissner’s membrane, into an ectopic prosensory region, the deregulation of additional genes, most likely acting upstream of Otx2, prevents this effect in the N-myc mutants and somehow rescues the formation of Reissner’s membrane.

Interestingly, however, similarly to the Otx2 mutant, the loss of N-myc also leads to the formation of ectopic hair cells in the apical portion of the cochlea (Dominguez-Frutos et al., 2011; Kopecky et al., 2011), which indicates that both N-myc and Otx2 suppress the formation of hair cells. Further evidence for a suppressive activity of Otx2 on sensory differentiation comes from mouse mutants for the Gbx2 (Lin et al., 2005) and the Kreisler (Muñb – Mouse Genome Informatics) (Choo et al., 2006) genes. Loss of either of these genes leads to ectopic expression of Otx2 and suppression of sensory development in the cochlear duct.

Otx2 expression is associated with non-sensory regions of the developing cochlea (Morsli et al., 1999) and, in the otic vesicle, it abuts the neurosensory region (Sánchez-Calderón et al., 2007). We have examined several markers for the neurosensory region, which might be altered by loss of the neighboring Otx2 expression domain. However, at the otic vesicle stage, these markers are unchanged in the Otx2-deficient otic vesicles. The first signs of an alteration in gene expression are detected at E14, during the formation of the prosensory region. At this stage, an ectopic prosensory region is initiated, as indicated by the presence of p27kip1 on the neural side of the roof of the cochlear duct, where Otx2 is normally expressed (Morsli et al., 1999). Ectopic p27kip1 expression is also accompanied by a broad expansion of Sox2 and by an ectopic patch of Jag1. Sox2 and Jag1-mediated Notch signaling are both required for sensory lineage formation during normal development (Kierman et al., 2005, 2006), and their ectopic activation throughout the cochlear duct induces ectopic sensory patches (Hartman et al., 2010; Pan et al., 2013, 2010), which is similar to what we report on Pax2Cre-Otx2 mutants. The most likely explanation for this effect is that Otx2 either suppresses Sox2, Notch signaling, or both, thereby preventing the development of sensory fate in the roof of the cochlear duct. However, misexpression of Otx2 during chicken inner ear development does not downregulate Sox2 or affect sensory development (G.A., H.G., A.F.-R. and F.G., unpublished), suggesting that Otx2 is necessary but not sufficient to prevent sensory fate. Therefore, rather than Otx2 and Sox2 mutually repressing each other, the initial widespread expression of Sox2 throughout the ventral part of the otic vesicle first appears to repress Otx2, but the subsequent downregulation of Sox2 then may allow Otx2 to appear in a ventro-lateral domain (supplementary material Fig. S2).

The suppressor activity of Otx2 has been previously documented in the cerebellum and the retina, and during myogenesis and neurogenesis (Bai et al., 2012; Nishida et al., 2003; Puelles et al., 2006, 2003). Sox2 and Otx2 are able to interact directly via their HMG and homeobox DNA-binding domains, respectively (Danno et al., 2008), and Otx2 has been shown to suppress the expression of Sox2 during retinal differentiation (Nishihara et al., 2012). The widespread ectopic expression of Sox2 in the cochlear duct of Pax2Cre-Otx2 mutant mice may suggest that it acts as a permissive prosensory factor allowing ectopic expression of Jag1 and p27kip1, which eventually generate an ectopic organ of Corti in the non-sensory region usually characterized by Otx2 expression. However, ectopic activation of Notch in non-sensory regions of the cochlea has been shown to be more effective than Sox2 in promoting...
sensory fate (Pan et al., 2013). Therefore, it is possible that the combination of Sox2 and Notch activity underlies the generation of an ectopic organ of Corti in Pax2Cre-Otx2 mutants.

So far only a few examples of mirror duplications of the organ of Corti have been reported. In the Jackson circler mouse mutant, loss of the transcriptional repressor Jxcl (Sobp – Mouse Genome Informatics) leads to a partial mirror duplication of the organ of Corti and ectopic hair and pillar cells directly flanking the lateral side of the native sensory epithelium (Chen et al., 2008). More recently, a complete mirror image duplication of the organ of Corti has been shown to occur upon deletion of the transcriptional regulator Lmo4 (Deng et al., 2014). Similar to the Pax2Cre-Otx2 mutant, ectopic formation of the sensory epithelium in the Lmo4 mutant is presaged by ectopic domains of Sox2, Jag1 and p27kip1 in the cochlear duct. Until E14, we noted no ectopic expression of p27kip1 in Pax2Cre-Otx2 mutants. The onset of p27kip1 expression in ectopic patches is therefore delayed with respect to the normotopic prosensory region (Lee et al., 2006). A similar delay of p27kip1 expression was reported for the Lmo4 mutants (Deng et al., 2014). However, eventually the differentiation of the ectopic sensory regions is accelerated because this delay disappears at later developmental stages in Pax2Cre-Otx2 or Lmo4 mutants (Deng et al., 2014). Further understanding of the basis of the unexpected plasticity of non-sensory regions of the cochlea may prove useful for defining pathways for the regeneration of hair cells by the manipulation of expression of transcriptional regulators and their associated networks.

**MATERIALS AND METHODS**

**Transgenic mice**

Generation and genotyping of mice carrying the Pax2Cre transgene (Ohyama et al., 2012), and the floxed N-myc (Dominguez-Frutos et al., 2011) and Otx2 alleles (Fossat et al., 2006) have been described previously. Experiments conformed to the institutional and national regulatory standards concerning animal welfare.

**Screening for differentially regulated genes in N-myc mutants**

RNA was isolated from E15 cochleas of wild type and Pax2Cre-N-myc mutants using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. RNA integrity was assessed using Agilent 2100 Bioanalyzer (Agilent). Labeling and hybridizations were performed according to protocols from Affymetrix. Briefly, 100-300 ng of total RNA were amplified and labeled using the WT Expression Kit (Ambion) and then washed and scanning were performed using the Affymetrix GeneChip System (GeneChip Hybridization Oven 640, GeneChip Fluidics Station 450 and GeneChip Scanner 7G). The Robust microarray analysis algorithm was used for background correction, intra- and inter-microarray normalization, and expression signal calculation. The absolute expression signal for each gene was calculated in each microarray and significance analysis of microarrays was applied to calculate differential expression and find the gene probe sets that characterized the highly metastatic samples. The method uses permutations to provide robust statistical inference of the most significant genes and provides \( P \) values adjusted to multiple testing using false discovery rate. Probe synthesis, hybridizations and microarray data analysis were performed by the Genomics facility of the Centro de Investigación del Cancer (Salamanca, Spain). The microarray data from this screen have been deposited at GEO with accession number GSE61406. Genes downregulated twofold or greater were examined for their expression in the cochlea (Diez-Roux et al., 2011) and are listed in supplementary material Table S1.

**Histology and RNA in situ hybridization**

Preparation of histological sections stained with Hematoxylin and Eosin, \( \beta \)-galactosidase staining, sectioning of stained embryos and RNA whole-mount in situ hybridization have been described previously (Alvarez et al., 2003). Riboprobes were generated for detection of murine \( N\text{-}myc \) (Dominguez-Frutos et al., 2011), Otx2 (Fossat et al., 2006; Morsli et al., 1999), Sox2 (Pan et al., 2010), Fgf5 (Alvarez et al., 2003), lunatic fringe (Lfng), neurogenin 1 (Ngn1) and NeuroD (Vázquez-Echeverría et al., 2008), Fgf10 (Ohyama et al., 2012) and chicken otx2 (Hidalgo-Sánchez et al., 2000) and \( N\text{-}myc \) (Khudaykov and Bronner-Fraser, 2009). The Ecel1 riboprobe was generated from its cDNA (Genbank reference BC057569.1) corresponding to nucleotides 760-1222.

**Immunohistochemistry**

For immunohistochemistry, cryostat sections were prepared and processed using standard protocols. The following primary antibodies were used: Pax2 (PRB-276F; Covance; 1:200), p27kip1 (RB-9019-P0, Thermo Scientific; 1:1000), myosin VIIA (25-6790, Proteus; 1:50), calretinin (7699/3H, Swant; 1:1000), p75 (AB1554, Millipore; 1:200), NF 200 (N414, Sigma; 1:500), Lrp2/megalin (sc-16478, Santa Cruz Biotechnology; 1:200), Sox2 (sc-17320, Santa Cruz Biotechnology; 1:50) and Jag1 (sc-6011, Santa Cruz Biotechnology; 1:50). An antigen retrieval step consisting of incubation in 1 mM sodium citrate and 0.005% Tween 20, pH 6.0, at 98°C for 20 min was required for p27kip1, Jag1 and calretinin antibodies. The corresponding secondary antibodies used were Alexa 488-conjugated donkey anti-goat (1:400) and Alexa 568-conjugated goat anti-rabbit (1:1000) from Invitrogen. Sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Whole-mount immunolabeling with myosin VIIA antibody, dehydration and clearing of inner ears was performed as published (MacDonald and Rubel, 2008). Images of embryos, inner ears, sections and whole mounts of inner ear sensory epithelia were captured with a DFC 490 camera (Leica) on a Labophot-2 (Nikon) or MZ16FA fluorescence stereomicroscope (Leica). Immunofluorescence images of cochlear sections were taken with a Leica SP2 confocal microscope and processed using Adobe Photoshop.

**N-myc and Otx2 gain of function**

For in ovo electroporation, fertilized chicken eggs were incubated until embryos reached stage HH 12-14 (Hamburger and Hamilton, 1992). An expression vector carrying either murine \( N\text{-}myc \) (2 μg/μl) under the control of the CMV promoter or a vector carrying a 1.2 Kb fragment of the 3′ enhancer of the Otx2 gene (0.5 μg/μl, Otx2lacZ) or both, together with pEGFP-C1 (0.2 μg/μl, Clontech), were injected into the right otic cup by gentle air pressure through a micropipette. The platinum electrode was placed next to the otic cup and the anode electrode parallel to it on the other side of the embryo. Square pulses (eight pulses of 10 V, 50 Hz, 250 ms) were generated by a CUY-21 square wave electroporator (BEX, Tokiwa-saensisu, Japan). The left otic vesicle was not injected and was always used as control. Electroporated embryos were collected 24 h post electroporation and selected for high GFP expression in the otic vesicle and further processed for whole-mount RNA in situ hybridization or detection of \( \beta \)-galactosidase activity. Ectopic expression of Otx2 was detected after a short term exposure of 30 min to nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3′-indolophosphate (BCIP), which serve as chromogenic substrates for alkaline phosphatase coupled to an RNA probe that hybridizes to chick Otx2. Longer exposures of 2 h lead to detection of the endogenous ventrally localized chick Otx2 expression domain. Presence of \( \beta \)-galactosidase activity was detected using standard protocols (Alvarez et al., 2003). After a short term exposure of 30 min to the chromogenic substrates, embryos electroporated with \( N\text{-}myc \) and Otx2lacZ reporter (n=6) always showed a widespread blue precipitate within the electroporated vesicle whereas embryos electroporated with only the reporter showed no or few lacZ-positive cells (n=4). When the latter embryos were developed for 2 h, Otx2lacZ reporter activity was detected throughout the electroporated otic vesicle. Misexpression of Otx2 using an expression vector carrying murine Otx2 (2 μg/μl) under the control of the CMV promoter in the otic cup was performed as described above. Expression of \( N\text{-}myc \) was monitored by RNA whole-mount in situ hybridization using a chicken \( N\text{-}myc \) probe (Khudaykov and Bronner-Fraser, 2009). Images were obtained by conventional fluorescence microscopy (Leica DMRB) with Leica CCD camera DC300F and images were processed with Adobe Photoshop. The images are representative of the original data.
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Competing interests
The authors declare no competing or financial interests.

Author contributions

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Supplementary material
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References


