

# Generation of sensory hair cells by genetic programming with a combination of transcription factors

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## ABSTRACT

Mechanosensory hair cells (HCs) are the primary receptors of our senses of hearing and balance. Elucidation of the transcriptional networks regulating HC fate determination and differentiation is crucial not only to understand inner ear development but also to improve cell replacement therapies for hearing disorders. Here, we show that combined expression of the transcription factors Gfi1, Pou4f3 and Atoh1 can induce direct programming towards HC fate, both during *in vitro* mouse embryonic stem cell differentiation and following ectopic expression in chick embryonic otic epithelium. Induced HCs (iHCs) express numerous HC-specific markers and exhibit polarized membrane protrusions reminiscent of stereociliary bundles. Transcriptome profiling confirms the progressive establishment of a HC-specific gene signature during *in vitro* iHC programming. Overall, this work provides a novel approach to achieve robust and highly efficient HC production *in vitro*, which could be used as a model to study HC development and to drive inner ear HC regeneration.

**KEY WORDS:** Atoh1, Gfi1, Hair cells, Cell type programming, Gene regulation

## INTRODUCTION

Sensory hair cells (HCs) of the inner ear are mechanoreceptors that convert the displacements of their specialized apical ‘hair’ bundle into electrochemical signals. The mammalian inner ear has a very limited capacity to replace lost or damaged HCs (Warchol, 2011), leading to permanent hearing and vestibular impairments for millions of people worldwide. Progress towards understanding the transcriptional networks involved in HC fate specification has led to new strategies for their replacement by gene or stem cell-based therapies. The basic helix-loop-helix (bHLH) transcription factor (TF) Atoh1 has received much attention because it has a key role in HC differentiation. *Atoh1* deletion in mice causes HC loss in all inner ear sensory organs (Bermingham et al., 1999; Cai et al., 2013), whereas its overexpression promotes the generation of ectopic HCs in the developing inner ear (Zheng and Gao, 2000; Woods et al., 2004; Kelly et al., 2012; Liu et al., 2012). However, *Atoh1* is also necessary for the specification of various subsets of neurons (Ben-Arie et al., 1997; Bermingham et al., 2001; Rose et al., 2009),

intestinal secretory cells (Yang et al., 2001) and Merkel cells (Maricich et al., 2009), implying that Atoh1 acts in combination with different TFs to activate lineage-specific differentiation programs.

Besides Atoh1, the zinc-finger TF Gfi1 and the POU-domain TF Pou4f3 are the only known transcriptional regulators essential for the proper differentiation and/or survival of all vestibular and auditory HCs (Xiang et al., 1997, 1998; Wallis et al., 2003). The expression of *Gfi1* and *Pou4f3* is initiated in nascent HCs soon after the onset of *Atoh1* upregulation (Wallis et al., 2003; Sage et al., 2006; Pan et al., 2012). These findings raise the hypothesis that Gfi1 and Pou4f3 function together with Atoh1 in determining HC fate in the inner ear.

Previous reports have described relatively complex protocols that are able to steer embryonic stem cell (ESC) differentiation towards HC fate by recapitulating *in vivo* HC development through the temporal control of defined signaling pathways (Oshima et al., 2010; Koehler et al., 2013). Although stepwise differentiation protocols can promote successful HC generation *in vitro*, the efficiency of HC production is relatively low and reproducibility is a potential issue. This limits the use of these methods for inner ear studies in which large cell numbers are needed, such as high-throughput drug screening or cell transplantation therapies. Here, we report a simple, relatively quick and highly efficient protocol to generate sensory HCs *in vitro* from mouse ESCs (mESCs) by simultaneous overexpression of Gfi1, Pou4f3 and Atoh1.

## RESULTS

### *In vitro* differentiation of mESCs into HC-like cells

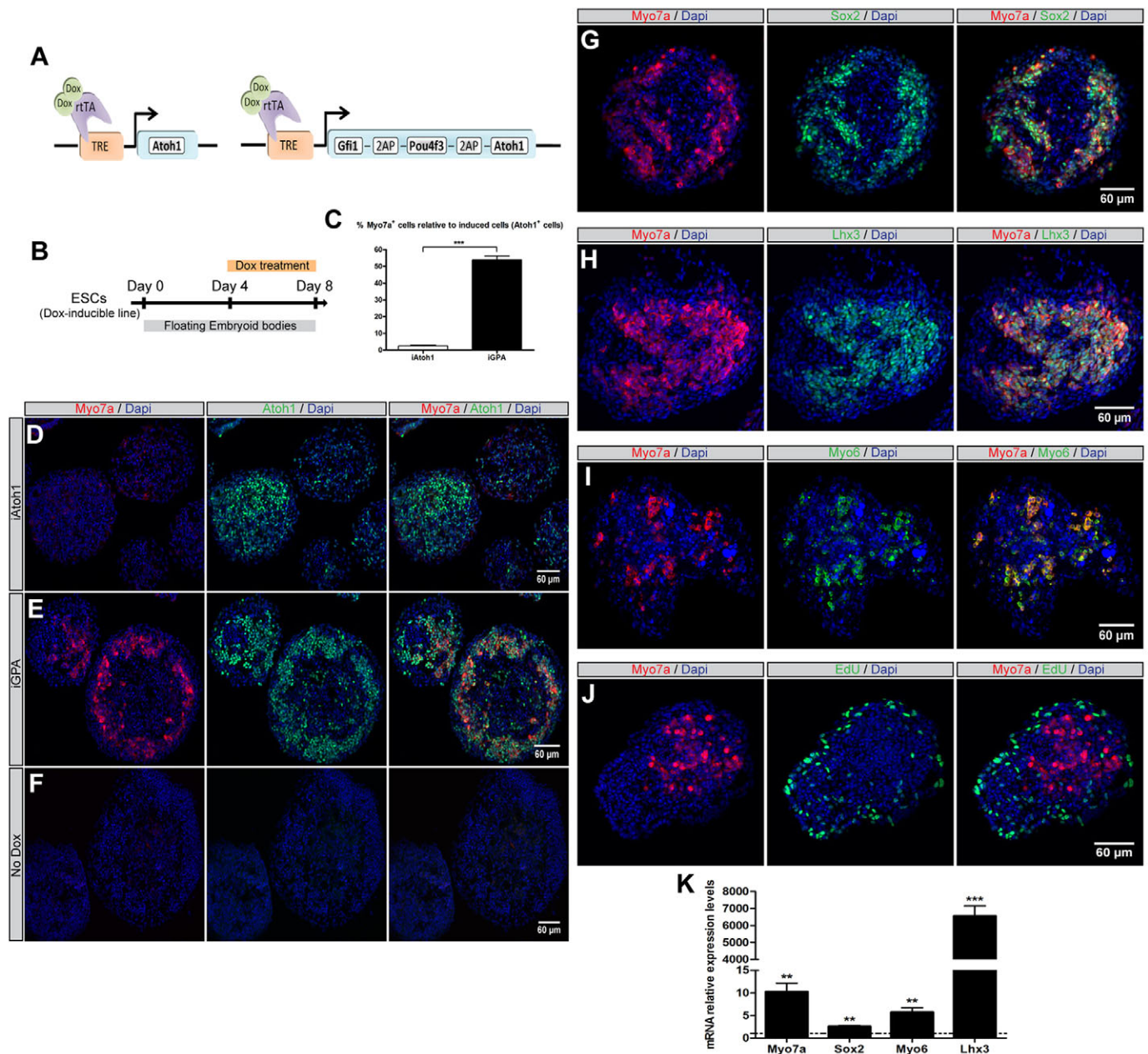
To determine whether Atoh1 or the combination of Gfi1, Pou4f3 and Atoh1 (GPA) can program mESC-derived progenitors into HCs, we first generated two mESC lines (iAtoh1 and iGPA) that enable doxycycline (Dox)-inducible expression of these TFs (Kyba et al., 2002). The iGPA line contains a polycistronic cassette in which Gfi1, Pou4f3 and Atoh1 are linked by 2A peptides (Fig. 1A; supplementary material Fig. S1A,B) to allow robust and balanced co-expression of all three TFs upon Dox addition (supplementary material Fig. S1C-F). In the absence of Dox, no significant expression of the inducible TFs was observed at the mRNA or protein level, whereas Dox treatment led to transgene expression in more than 60% of embryoid body (EB) cells (supplementary material Fig. S1C-F).

We subjected the iAtoh1 and iGPA mESCs to an EB-mediated differentiation protocol in which Dox treatment was initiated at day 4 and maintained during the following 4 days, until day 8, when EBs were collected for analysis (Fig. 1B). Immunostaining for the HC marker Myo7a (El-Amraoui et al., 1996) revealed striking differences between iAtoh1 and iGPA cells. Widespread upregulation of Myo7a was only detected in iGPA-derived EBs (54±2% of cells), and never in EBs derived from iAtoh1 cells, or in

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**Fig. 1. Inducible expression of *Gfi1*, *Pou4f3* and *Atoh1* promotes the differentiation of EB-derived progenitors towards a HC fate.** (A) The Dox-inducible *iAtoh1* (left) and *iGPA* (right) lines used in this study. 2AP, 2A peptide; TRE, tetracycline responsive element; rTA, reverse tetracycline transactivator. (B) mESC differentiation protocol describing the Dox treatment timeline. (C) The mean percentage of *Atoh1*<sup>+</sup> cells that are positive for *Myo7a* in *iGPA* and *iAtoh1* cells after 4 days of Dox treatment. (D–F) Immunostaining analysis of *Myo7a* and *Atoh1* expression in EBs harvested at day 8 from the *iAtoh1* (D) and *iGPA* (E,F) mESC lines. Strong upregulation of *Myo7a* is detected only in *iGPA*-derived *Atoh1*<sup>+</sup> cells (E). (G–J) Representative images obtained from immunostaining for *Myo7a/Sox2* (G), *Myo7a/Lhx3* (H), *Myo7a/Myo6* (I) and *Myo7a/EdU* (J) in *iGPA* EBs, analyzed at day 8 after 4 days of Dox exposure. (K) qRT-PCR analysis reveals upregulation of *Myo7a*, *Sox2*, *Myo6* and *Lhx3* in *iGPA*-derived EBs treated for 4 days with Dox. Relative expression of each transcript is presented as fold change normalized to the mean of untreated EBs (dotted baseline=1) at day 8. Results are mean±s.e.m. \*\**P*<0.01, \*\*\**P*<0.001 (*n*=3).

the absence of Dox (Fig. 1C–F). This suggests that the combined activities of the three TFs favor commitment towards a HC fate.

To investigate further the identity of induced *Myo7a*<sup>+</sup> (*iMyo7a*<sup>+</sup>) cells in *iGPA*-derived EBs, we examined the expression of markers known to be expressed at the onset of vestibular and auditory HC differentiation, such as *Sox2*, *Lhx3* and *Myo6* (Xiang et al., 1998; Hume et al., 2007). Notably, all *iMyo7a*<sup>+</sup> cells were found to co-express these markers (Fig. 1G–I). Quantitative reverse transcription PCR (qRT-PCR) analyses also confirmed the significant increase in their transcript levels, as compared with untreated EBs (Fig. 1K). By

contrast, forced expression of *Atoh1* alone in *iAtoh1* EBs never resulted in upregulation of HC markers (including *Gfi1*, *Pou4f3* and hair bundle markers) (supplementary material Fig. S2A–F).

We tested whether forced *Atoh1* expression might lead to cell death, as implied by Liu et al. (2012). Given the known role of *Gfi1* and *Pou4f3* in promoting cell survival, their activity might counteract *Atoh1* function and contribute to the differences in HC induction between *iAtoh1* and *iGPA* EBs. However, no significant differences were found in the percentage of apoptotic cells between *iAtoh1*<sup>+</sup> and *iGPA*<sup>+</sup> cells (supplementary material Fig. S3A–D),

indicating that the sustained expression of any of these TFs has no effect on the overall level of cell death in EBs, and that their differing effects in terms of HC induction are unlikely to be secondary to different impacts on cell viability.

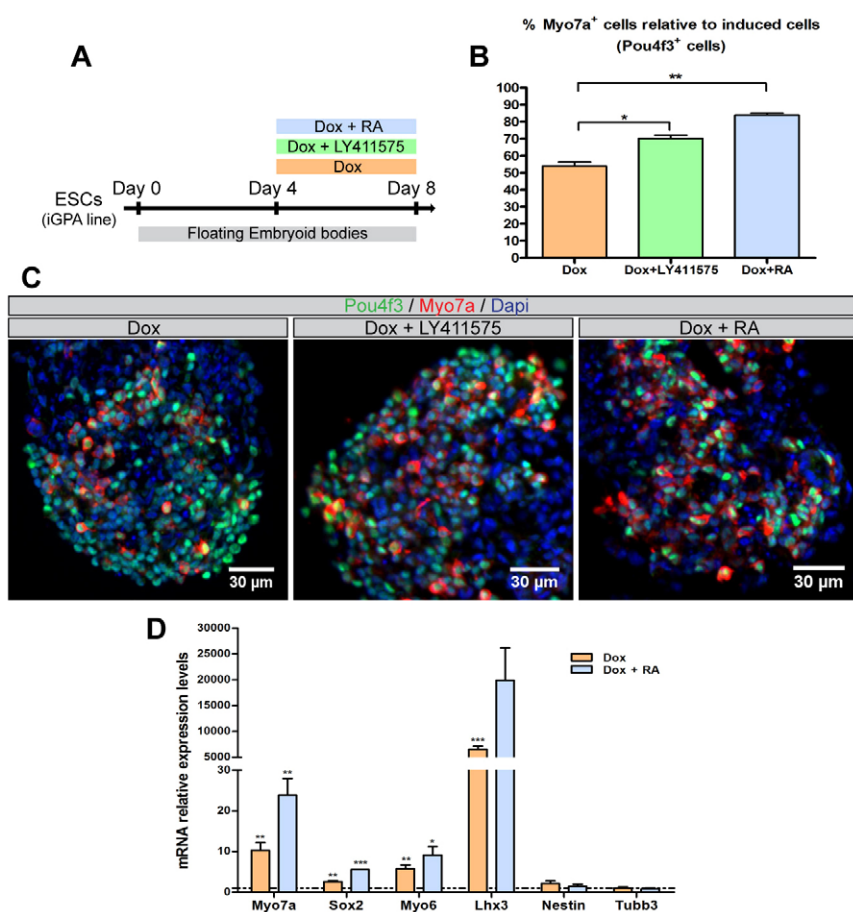
During inner ear development, Sox2 expression rapidly declines as HC differentiation progresses (Hume et al., 2007). Thus, the strong Sox2 upregulation that we observed in iMyo7a<sup>+</sup> cells suggests that these are at an initial phase of HC commitment. To assess their developmental status further, we next tested whether iMyo7a<sup>+</sup> cells are already postmitotic, as sensory progenitors first exit the cell cycle before initiating full differentiation as HCs (Ruben, 1967; Matei, et al., 2005; Chen et al., 2002). A 30 min pulse of 5-ethynyl-2'-deoxyuridine (EdU) incorporation was performed 4 days after initiating Dox treatment in iGPA-derived EBs. Immunodetection revealed a clear lack of colocalization between EdU<sup>+</sup> and iMyo7a<sup>+</sup> cells, indicating that induced cells have already exited the cell cycle by this stage (Fig. 1J). Altogether, these observations suggest that co-expression of Gfi1, Pou4f3 and Atoh1 induces a rapid conversion of EB cells into postmitotic cells with sensory HC characteristics, which we hereafter refer to as induced HC-like cells, or iHCs.

Previous studies have shown that Gfi1, Pou4f3 and Atoh1 are also expressed during development of the central and peripheral nervous systems (Ninkina et al., 1993; Xiang et al., 1995; Ben-Arie et al., 2000; Wallis et al., 2003) and that each of these TFs is necessary for proper differentiation of multiple neuronal cell types (Ben-Arie et al., 1997; Bermingham et al., 2001; Wang et al., 2002; Tsuda et al., 2005; Rose et al., 2009). We therefore tested whether expression of the three TFs, or of Atoh1 alone, might also promote neuronal differentiation during EB differentiation by examining the expression of neuronal marker

Tuj1 (Tubb3) and neural progenitor marker nestin, which are not expressed in mammalian HCs (Malgrange et al., 2002; Wallis et al., 2003). We found that iGPA-derived EBs treated with Dox for 4 days do not exhibit any increase of either Tuj1 or nestin expression (supplementary material Fig. S2G,H). By contrast, iAtoh1-derived EBs show strong Tuj1 expression in most Atoh1-overexpressing cells (supplementary material Fig. S2G). The absence of nestin expression in iAtoh1-derived EBs (supplementary material Fig. S2H) suggests that Atoh1 promotes direct conversion into neurons rather than inducing neural progenitors. These observations are consistent with previous *in vivo* studies showing that Atoh1 overexpression is sufficient to promote neural differentiation in non-neural ectoderm progenitors (Kim et al., 1997). Together, these results indicate that whereas Atoh1 is able to convert EB cells into neurons, the combination of Atoh1, Gfi1 and Pou4f3 drives progression towards a HC fate.

### iHC generation is enhanced by retinoic acid or inhibition of Notch signaling

In the embryo, HC differentiation is regulated by the Notch and retinoic acid (RA) signaling pathways, which can be manipulated to cause an increase in HC production: disruption of Notch signaling with gamma-secretase inhibitors leads to the overproduction of HCs at the expense of SCs (Kiernan, 2013), while RA supplementation of cultures of otic vesicles and sensory explants promotes the generation of extra HCs (Represa et al., 1990; Kelley et al., 1993). Hence, we tested whether the efficiency of GPA-induced HC programming could be enhanced in a similar way. We exposed iGPA-derived EBs to 4 days of Dox treatment combined with either the gamma-secretase inhibitor LY411575 or RA (Fig. 2A).



**Fig. 2. Enhancing HC programming efficiency by Notch inhibition or RA exposure.**

(A) iGPA EB differentiation protocols, including the combinatorial treatment with Dox plus LY411575 or RA. (B) Quantification of Myo7a<sup>+</sup> cells among cells expressing the three TFs (Pou4f3<sup>+</sup> cells) analyzed in 8 day EBs treated with Dox, Dox+LY411575 and Dox+RA. (C) Representative images of iGPA-derived EBs at day 8 obtained by immunostaining of Pou4f3 and Myo7a, showing the significant increase in Myo7a<sup>+</sup> cells upon combined treatment with Dox+LY411575 or with Dox+RA, as compared with Dox treatment alone. (D) qRT-PCR analysis shows higher expression levels of HC markers (*Myo7a*, *Sox2*, *Myo6* and *Lhx3*) but not neuronal markers (*nestin* and *Tubb3*) in EBs treated with Dox+RA, as compared with Dox treatment. Fold change was normalized to the mean of untreated EBs (dotted baseline=1). Results are mean±s.e.m. \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001 (*n*=3).



Remarkably, the proportion of iHCs increased significantly in the presence of LY411575 (70±2%) or RA (84±1%) when compared with the Dox treatment alone (54±2%) (Fig. 2B,C). In addition, mRNA expression levels of *Myo7a*, *Sox2*, *Myo6* and *Lhx3* were significantly elevated by RA treatment of iGPA EBs (Fig. 2D), confirming that RA enhances HC programming efficiency. We also examined the expression of nestin and *Tubb3*, as neuronal commitment can occur upon RA treatment in differentiating EBs (Li et al., 1998). However, no significant upregulation of either of these neural markers was detected (Fig. 2D), suggesting that the effect of RA on HC differentiation is specific.

### iHCs are able to develop hair bundle-like structures

To determine whether iHCs are capable of developing stereociliary bundles similar to those of sensory HCs *in vivo*, we examined the expression of espin, one of the major actin-bundling proteins of stereocilia (Zheng et al., 2000b), in iGPA-derived EBs (Fig. 3A-C'). Immunostaining revealed weak espin expression within GPA-expressing cells at day 8, after 4 days of Dox treatment (Fig. 3B). However, it is known that the onset of espin expression during normal inner ear development occurs later than that of *Myo7a* (Chen et al., 2002; Sekerkova et al., 2006), raising the possibility that day 8 iHCs were still at an initial stage of HC differentiation. We therefore extended the period of Dox treatment for an additional 4 days and examined EBs at day 12 (Fig. 3C). Notably, at this later time point, high levels of espin are present in polarized membrane projections emanating from iHCs (Fig. 3C). These espin-rich protrusions were observed in 55±3% of *Myo7a*<sup>+</sup> cells, a percentage that was not increased by RA treatment (55±6%). Another essential hair bundle protein, cadherin 23 (*Cdh23*) (Siemens et al., 2004), was also detected in the iHC protrusions at day 12 (supplementary material Fig. S4A). A significant increase in the expression of *Espin* and *Cdh23* at day 12 compared with day 8 was also found by qRT-PCR (Fig. 3D). The concurrent decline of *Sox2* levels in iHCs between day 8 and day 12 (Fig. 3D; supplementary material Fig. S4C) further supports the idea that iHCs progress towards a more mature HC phenotype (Fritzsch et al., 2015).

We noted that the espin-rich protrusions in iHCs show heterogeneous morphology and, although apparently polarized, do not exhibit consistent orientation (Fig. 3C'). We hypothesized that exposing iHCs to inner ear polarity cues might improve the morphological differentiation of their hair bundle-like structures (Oshima et al., 2010). To test this idea, we established a co-culture system in which iGPA-derived EBs were dissociated and plated on the surface of mitotically inactivated utricle mesenchymal cells for 6 days, in the presence of Dox and RA (Fig. 3E). Although we did not observe a clear epithelial organization with defined polarity among iHCs in these co-cultures, detailed confocal analysis revealed that the espin-rich projections are preferentially directed towards either the bottom or the top of the cell layer (Fig. 3F-I; supplementary material Fig. S4B). In addition, actin filaments were also present in these espin-rich protrusions (Fig. 3J), resembling normal HC stereocilia that contain a core of uniformly spaced actin filaments cross-linked with espin.

In agreement with the immunostaining data, scanning electron microscopy (SEM) showed that elongated membrane protrusions reminiscent of hair bundles are present at the apical surface of some iHCs (Fig. 3K). However, the vast majority of these putative bundles are poorly organized compared with normal HC stereociliary bundles, indicating that the maturation of hair bundle-like structures in iHCs is incomplete under these culture conditions.

### Combined expression of *Gfi1*, *Pou4f3* and *Atoh1* can induce HC specification and differentiation *in vivo*

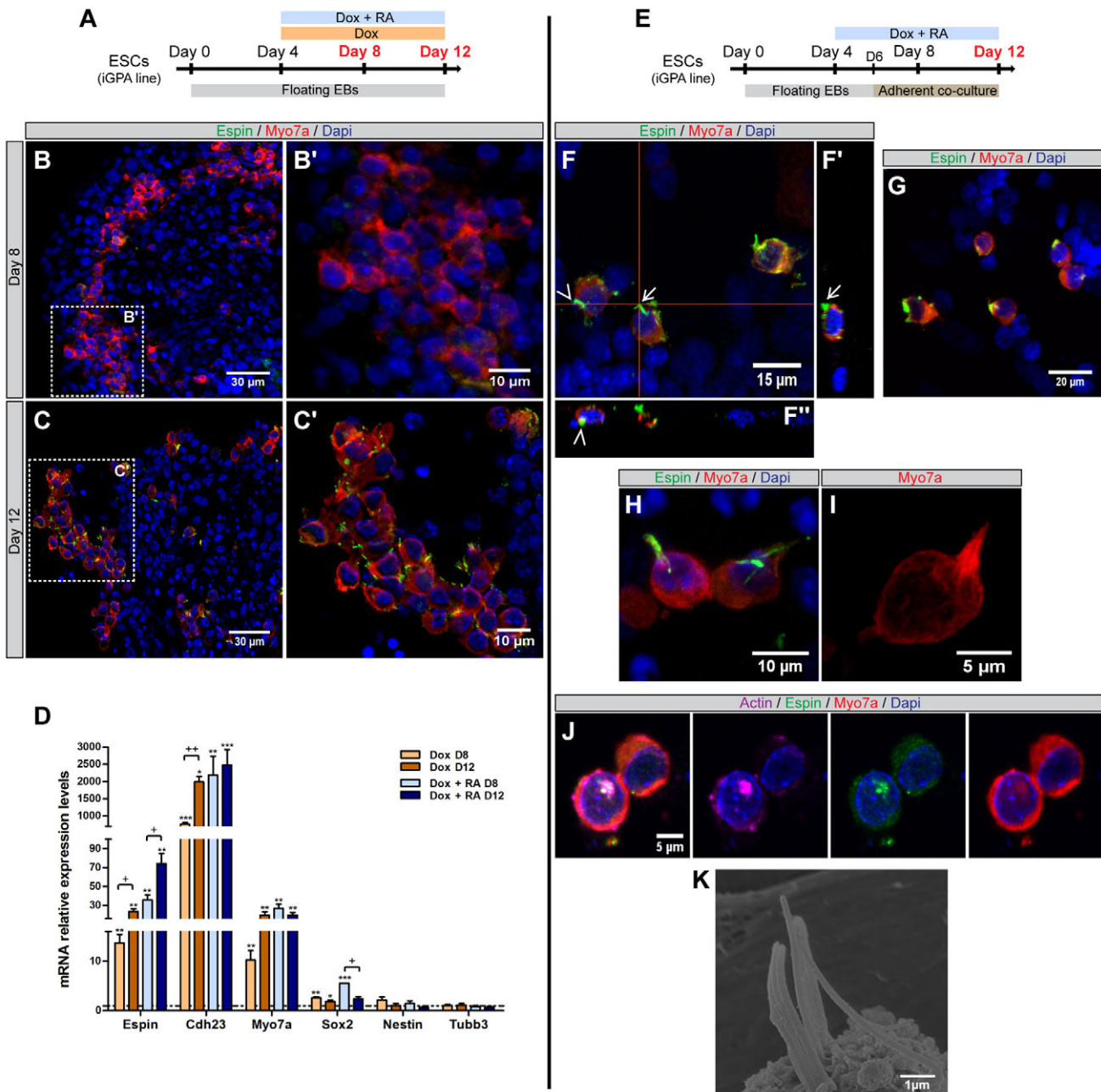
The lack of proper hair bundle maturation in iHCs could be due to inappropriate culture conditions, but it might also result from incomplete implementation of a HC differentiation program by *Gfi1*, *Pou4f3* and *Atoh1*. To address this question, we tested the effects of combined GPA expression *in vivo*, in the developing inner ear of chicken embryos, using a Tet-on inducible Tol2 transposon system that allows spatial and temporal control of transgene expression (Takahashi et al., 2008; Freeman et al., 2012). The otic cup was co-electroporated *in ovo* at E2 with the TRE:GPA-eGFP vector [which contains a bidirectional tetracycline-responsive element (TRE) that drives the expression of both *Gfi1*-*Pou3f4*-*Atoh1* and eGFP upon Dox treatment] and plasmids encoding the rtTA-M2 Tet-on activator and Tol2 transposase (Fig. 4A). Two days later, the embryos were treated with Dox and incubated for a further 2–4 days, until analysis at E6 or E8 (Fig. 4B). The results show that the combined activity of *Gfi1*, *Pou4f3* and *Atoh1* induces *Myo7a* expression in electroporated cells located in various regions of the developing inner ear ( $n=14/14$  embryos, Fig. 4C,D), including both sensory and non-sensory domains of the vestibular and auditory system. Ectopic *Myo7a*<sup>+</sup> cells could even be found in the epithelium of the endolymphatic duct (a non-sensory component of the endolymphatic system, Fig. 4F).

Among eGFP<sup>+</sup> cells analyzed at E6 in the vestibular and auditory regions, 77±2% ( $n=265$ ) and 76±6% ( $n=492$ ) showed *Myo7a* expression, respectively (four independent inner ear samples were analyzed at E6). These ectopic *Myo7a*<sup>+</sup> cells are preferentially located close to the luminal surface of the otic epithelium where HCs normally reside, and express other HC markers such as *Sox2*, *Myo6*, hair cell antigen (HCA), otoferlin (HCS-1) and parvalbumin (Fig. 4E–K). They are also correctly polarized, with HCA-positive stereociliary bundles at the luminal surface (Fig. 4H',I). The production of HCs is delayed in the auditory epithelium compared with the vestibular organs, and HC markers such as *Myo7a*, *Myo6*, HCA and HCS-1 are not yet detected in the basilar papilla at E6 (data not shown). Nonetheless, ectopic eGFP<sup>+</sup> iHCs located in the sensory and non-sensory regions of the cochlear duct express these HC markers already at E6 (Fig. 4H–J), suggesting that GPA expression can induce rapid commitment towards HC fate independently of the developmental stage and character of the transfected cells. Remarkably, *Tuj1*<sup>+</sup> nerve fibers originating from the otic ganglia appear to be recruited by ectopic eGFP<sup>+</sup> HCs at E8, even when these cells are present in non-sensory epithelia ( $n=3/5$ , Fig. 4L).

Altogether, these data show that GPA expression can efficiently induce the generation of differentiated HCs from various types of otic progenitors *in vivo*.

### Transcriptional profiling of iHCs reveals a specific HC signature

What is the precise genetic program induced by the combined activities of *Gfi1*, *Pou4f3* and *Atoh1*, and to what extent does it match the transcriptional signature of an HC? To answer these questions, we first sought to obtain homogeneous populations of iHCs for transcriptional profiling. A novel iGPA-derived mESC line was prepared that contains a *Myo7a* promoter-driven fluorescence reporter system to specifically label iHCs. This was based on an established *Myo7a* promoter that, along with a strong HC-specific enhancer located in intron 1 of *Myo7a*, has been shown to drive specific transgene expression in vestibular and auditory HCs (Boeda et al., 2001).



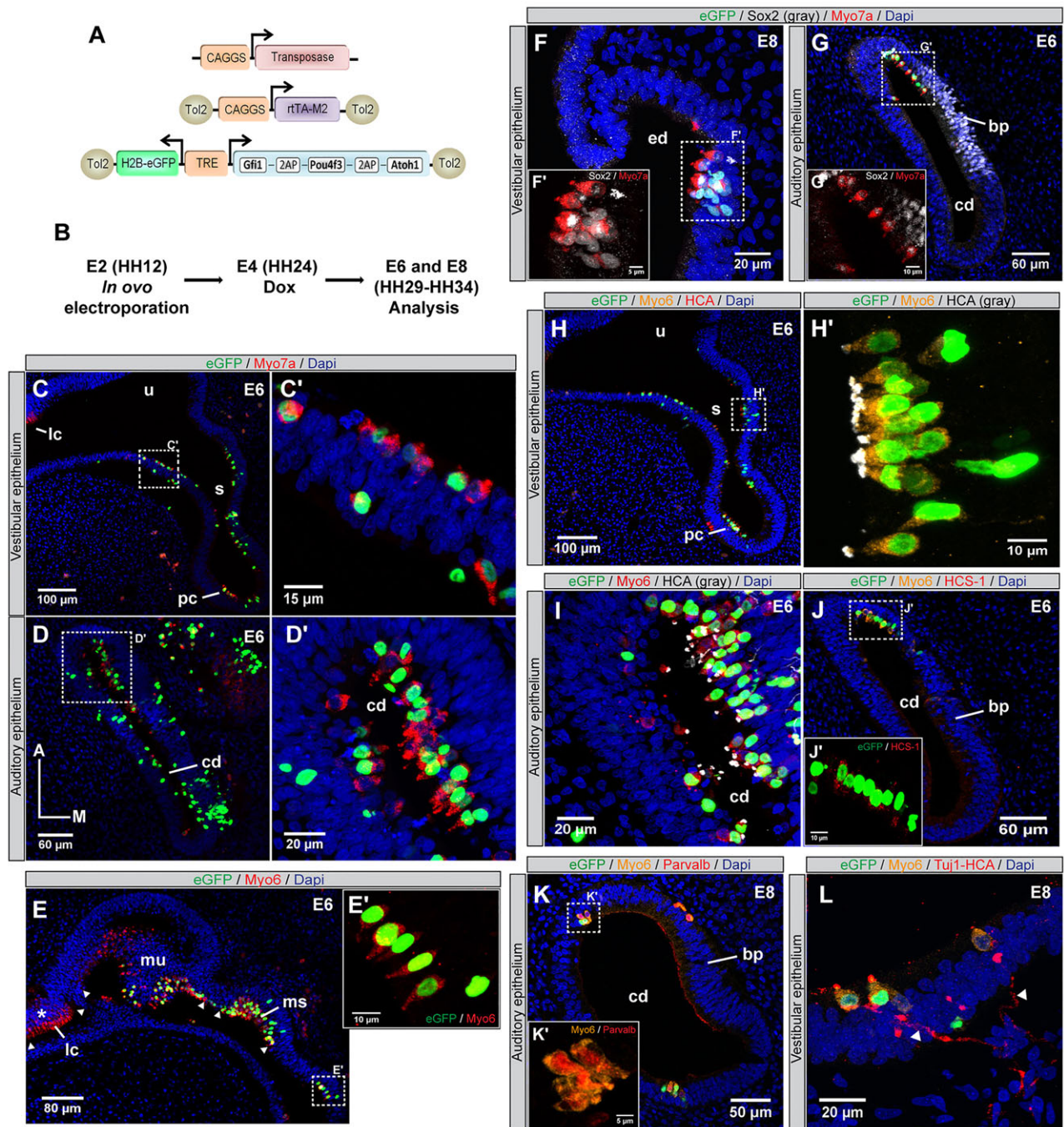
**Fig. 3. Morphological characterization of hair bundle-like structures in iHCs.** (A) iGPA EB differentiation protocol describing the Dox treatment timeline. (B,C) Immunostaining for espin and Myo7a in iGPA-derived EBs treated with Dox for 4 (B) or 8 (C) days reveals a strong and polarized espin<sup>+</sup> structure on the surface of iMyo7a<sup>+</sup> cells. These structures were absent in EBs exposed to Dox for only 4 days (B). (B',C') The boxed regions at high magnification. (D) The relative mRNA levels (presented as fold change normalized to the mean of untreated EBs at the corresponding time point; dotted baseline=1) of genes encoding hair bundle markers (*Espn* and *Cdh23*), HC markers (*Myo7a* and *Sox2*) and neuronal markers (*nestin* and *Tubb3*) in EBs treated with Dox or Dox+RA at day 8 and at day 12. (E) Dissociated EB co-culture differentiation protocol with mitotically inactivated chicken utricle periodic mesenchyme cells. (F) Confocal stacks of hair bundle-like protrusions labeled for Myo7a and espin in the adherent co-cultures at day 12. (F',F'') Orthogonal views showing espin<sup>+</sup> structures oriented towards the utricle mesenchyme layer (arrow in F') or in the opposite direction facing the cell surface (arrowhead in F''). (G-J) Confocal images showing representative espin<sup>+</sup> and Myo7a<sup>+</sup> protrusions in several iHCs grown in adherent co-cultures at day 12. Phalloidin immunostaining shows that polarized Myo7a<sup>+</sup>/espin<sup>+</sup> structures are F-actin-filled membrane protrusions (J). (K) Morphology of microvilli-like stereocilia protruding from the cell surface of an iHC, as observed by SEM. Results are mean±s.e.m. +, \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001 (*n*=3).

The new inducible reporter mESC line (iGPA-Myo7a:mVenus, Fig. 5A) showed comparable HC programming efficiency and RA sensitivity to the parental iGPA line (Fig. 5B). Although the Myo7a reporter exhibited weak activity in the absence of induction (Fig. 5B, C,E), addition of Dox and RA led not only to a strong increase in the total number of Venus<sup>+</sup> cells, but also to much higher fluorescence levels per cell (Fig. 5B-D,F). Furthermore, a high

degree of correlation between Venus and Myo7a expression was observed in these EBs by immunostaining or by flow cytometry analysis following intracellular antibody staining (Fig. 5F). These results indicate that the Myo7a:mVenus reporter provides an effective readout for *Myo7a* induction during HC programming.

We next used the iGPA-Myo7a:mVenus reporter line and FACS to isolate day 8 and day 12 iHCs from EBs that were treated with either

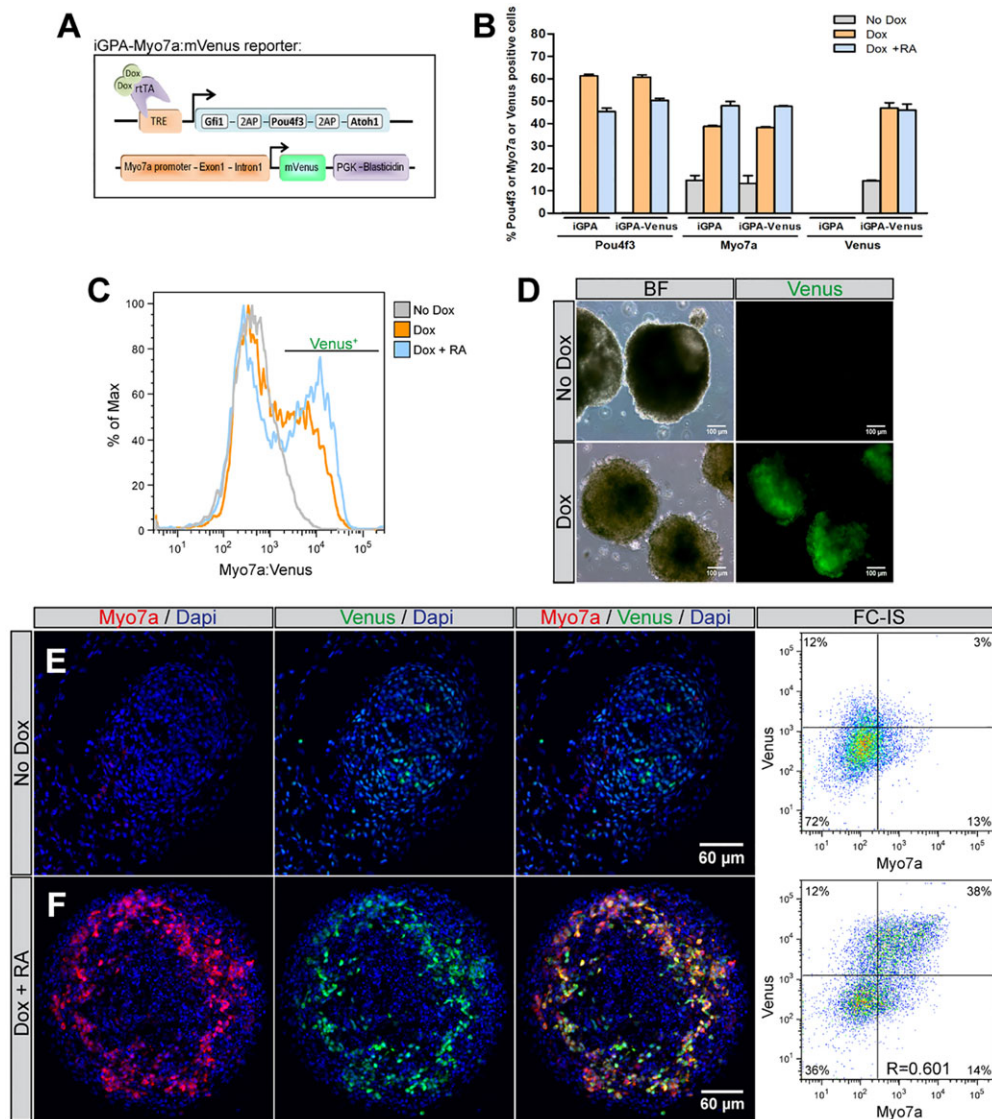




**Fig. 4. Induction of Gfi1, Pou4f3 and Atoh1 expression induces HC differentiation in various regions of the embryonic chick inner ear.** (A) Expression vectors used for Dox-inducible Gfi1-Pou4f3-Atoh1 and eGFP expression by *in ovo* electroporation. Note that eGFP is fused with histone 2B (H2B) for nuclear localization. (B) Experimental design to test the effects of Gfi1-Pou4f3-Atoh1 expression during inner ear development in the chick embryo. (C,D) Representative images of the vestibular (C) and auditory (D) epithelia showing Myo7a and eGFP immunofluorescence in E6 electroporated embryos. (C',D') The boxed regions at high magnification. (E) A section through the vestibular region shows electroporated eGFP<sup>+</sup> cells with Myo6 expression in various sensory patches (arrowheads), as well as in non-sensory domains of the otic epithelium (E'). Non-electroporated patches with Myo6-expressing HCs are also present (asterisk). (F,G) Immunostaining analysis for Myo7a/eGFP/Sox2 showing expression of Sox2 in ectopic Myo7a<sup>+</sup> cells (F',G'). (H,I) Immunostaining analysis for Myo6/eGFP/HCA in the vestibular (H) and auditory (I) epithelium shows polarized localization of HCA in the apical domain of ectopic iHCs (red in H, white in I, H'). (J,K) Analysis of electroporated eGFP<sup>+</sup> cells in the basilar papilla epithelium shows that ectopic iHCs express the HC markers HCS-1 (J) and parvalbumin (K). (L) Ectopic iHCs (expressing Myo6 and HCA) are innervated by Tuj1-positive neuronal extensions (arrowheads) that project from neurons at the otic ganglion. Some ectopic HCs are eGFP negative, possibly owing to eGFP decay at E8 (Dox was added only at E4). bp, basilar papilla; cd, cochlear duct; ic, lateral crista; u, utriculi; s, sacculi; pc, posterior crista; mu, macula utriculi; ms, macula sacculi; ed, endolymphatic duct. A, anterior; M, medial.

Dox alone or with Dox and RA. Unsorted EBs from the same reporter line, grown in the same conditions but without Dox or RA treatment, were used as controls. Independent RNA preparations from each of the

selected time points and treatments (three biological replicates for Dox or Dox+RA treatments, two for 'no treatment') were processed and hybridized on Affymetrix whole-transcript microarrays.

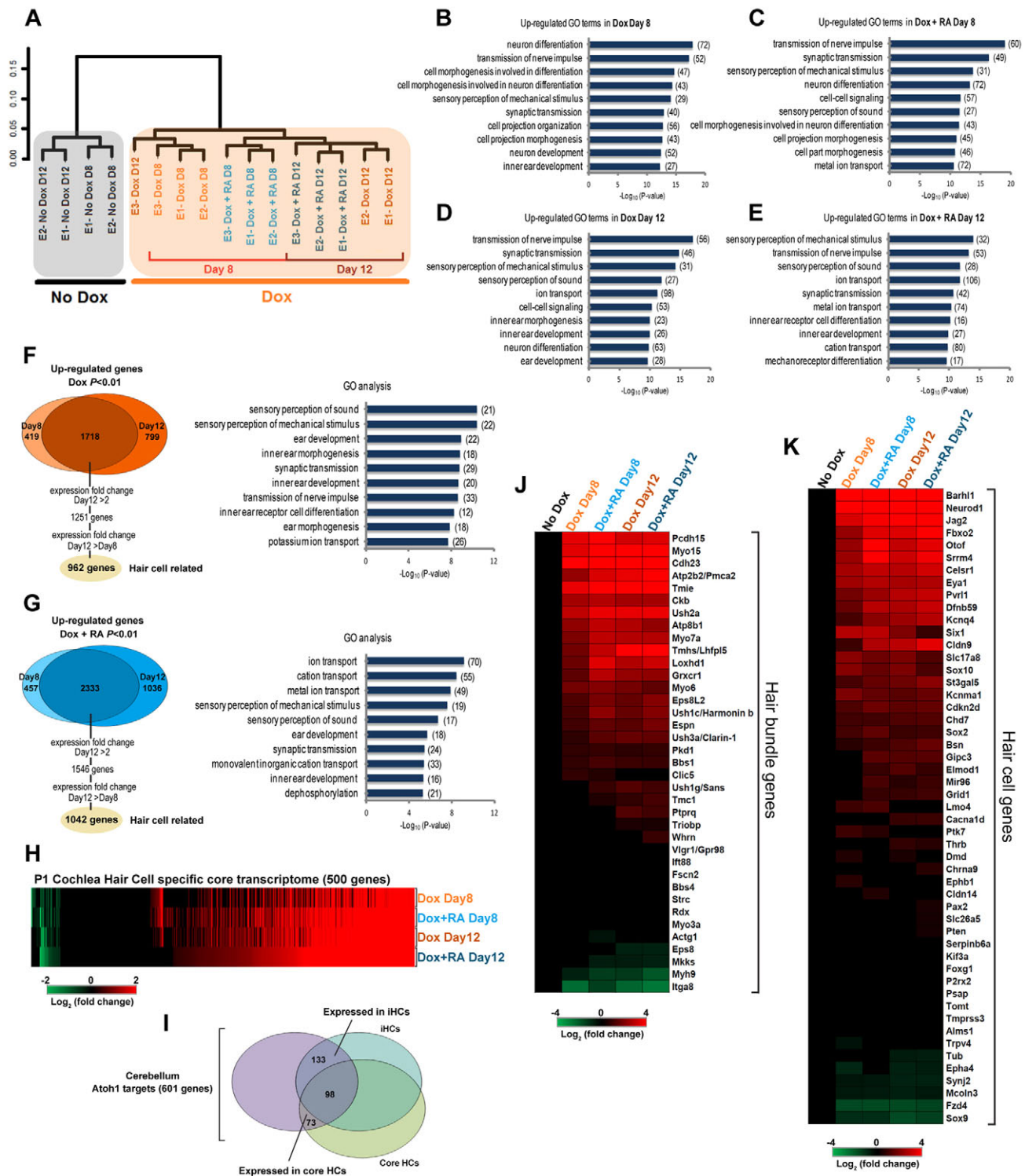


**Fig. 5. The iGPA-Myo7a:mVenus ESC line is an adequate fluorescence reporter for Myo7a expression.** (A) The iGPA-Myo7a:mVenus ESC line containing the mouse *Myo7a* regulatory regions driving transcription of a Venus fluorescent protein, followed by a selection cassette. PGK, phosphoglycerate kinase promoter; Blasticidin, blasticidin resistance gene. (B) Quantification of Pou4f3<sup>+</sup>, Myo7a<sup>+</sup> and Venus<sup>+</sup> cells relative to total cell numbers found within EBs grown in the absence or presence of Dox and Dox+RA at day 8. Cell counts were performed for EBs generated from the iGPA and iGPA:Myo7a:mVenus lines. No significant differences were found between these two lines regarding the mean percentage of total Pou4f3<sup>+</sup> and Myo7a<sup>+</sup> cells in the different treatments. (C) Representative histogram showing Venus expression in iGPA:Myo7a:mVenus-derived EBs that were untreated (14.5±0.4%), treated with Dox (46.9±4.1%) or Dox+RA (46±4.6%) at day 8. (D) Brightfield and fluorescence images of live floating iGPA:Myo7a:mVenus-derived EBs at day 8, showing weak Venus fluorescence levels in the absence of Dox, but high numbers of strongly fluorescent Venus<sup>+</sup> cells following Dox induction. (E,F) Immunostaining analysis for Myo7a and Venus in iGPA-Myo7a:mVenus-derived EBs, in untreated (E) or Dox+RA conditions (F) at day 8, showing a high degree of colocalization between the two proteins (79.5±0.3% and 54.4±1.7% of Venus<sup>+</sup> cells were Myo7a<sup>+</sup> in EBs treated with Dox+RA and Dox, respectively). Included are representative dot plots of intracellular staining for Myo7a and Venus proteins, as analyzed by flow cytometry (FC-IS, flow cytometry analysis following intracellular staining). Statistical analysis indicates a good correlation between the expression of both proteins (Pearson correlation=0.6). Results are mean±s.e.m. (n=3).

Hierarchical clustering of the transcriptome datasets revealed a clear segregation between ‘no Dox’ and ‘Dox’ samples (Fig. 6A). Interestingly, in the Dox branch, samples cluster in two groups: one containing day 8 iHCs treated with Dox, and the other composed of day 8 iHCs treated with Dox+RA, together with all the day 12 iHC samples (Fig. 6A). This suggests that day 8 iHCs treated with RA reach a differentiation stage similar to that of day 12 iHCs, indicating that RA treatment improves the efficiency of iHC programming by accelerating the initial differentiation steps. This would be consistent with the earlier onset of *Myo7a* expression in RA-treated iGPA EBs (supplementary material Fig. S5A-C).

We next analyzed the genes that were differentially expressed in iHC populations obtained with different treatments by comparison with control (‘no Dox’) cells at the same time point (supplementary material Table S1). Genes upregulated in day 8 iHCs were enriched in gene ontology (GO) functional groups related to neuronal differentiation and inner ear development (Fig. 6B,C). The set of upregulated genes in day 12 iHCs was highly enriched in genes involved in inner ear development and HC functions, such as sensory perception of sound/mechanical stimulus and synaptic transmission (Fig. 6D,E). Strikingly, analysis of the subset of day 8 upregulated genes that are further upregulated at day 12 revealed a





**Fig. 6. Analyses of iHC transcriptome profiles.** (A) Dendrogram showing hierarchical clustering of the various expression profiles obtained from iGPA-Myo7a: mVenus reporter-derived EBs (E1, E2 and E3 correspond to three biological replicates). (B-E) Gene ontology analysis performed using the DAVID functional annotation tool for genes significantly upregulated (fold change >2,  $P < 0.01$ ) in the four iMyo7a:Venus groups relative to uninduced cells. The number of upregulated genes included within each GO functional term is shown. (F,G) Venn diagram illustrating the overlap between the significantly upregulated genes identified in iMyo7a<sup>+</sup> cells at day 8 and day 12, as compared with uninduced cells. From the list of common upregulated genes in iGPA cells treated with Dox only, those that showed higher fold change at day 12 were selected. This list was subjected to a GO analysis using DAVID. (G) The same procedure was performed for the overlapping genes from iGPA cells cultured with Dox+RA. (H) Heat map depicting the relative fold changes in expression of 500 core HC genes (Cai et al., 2015) across the four iMyo7a groups, relative to uninduced cells ( $P < 0.05$ ). (I) Venn diagram illustrating the overlap between the transcriptome of the cochlear core HC signature and of iHCs ( $P < 0.05$ ) relative to the 601 cerebellum Atoh1 direct target genes previously identified by Klich et al. (2011). (J,K) Heat maps depicting the relative fold changes in expression of hair bundle (J) or HC (K) deafness-related genes across the four iMyo7a groups, relative to uninduced cells ( $P < 0.05$ ).



stronger enrichment in gene categories involved in HC functions (Fig. 6F,G). Furthermore, GO analysis showed that genes connected to the cell cycle and cell division are specifically repressed in iHCs (supplementary material Fig. S6A–D), consistent with the observation that these cells have ceased proliferation and exited the cell cycle. Altogether, this analysis indicates that the combined activity of *Gfi1*, *Pou4f3* and *Atoh1* is able to induce a bona fide HC developmental program. This conclusion is also supported by the significant overlap between the transcriptional profiles of iHCs and *Atoh1*-GFP-sorted HC populations from P1 mouse cochleas: out of a core HC signature of 500 genes defined for *Atoh1*-GFP HCs (Cai et al., 2015), 69% are upregulated in iHCs (Fig. 6H). By contrast, only 38% of the defined *Atoh1* gene targets in cerebellar granule neuron precursors (CGPs) (Klisch et al., 2011) are upregulated in iHCs (Fig. 6I).

Finally, we analyzed the transcriptome datasets for the expression of genes known to be functionally relevant for inner ear HC development/function. We first selected a list of 250 genes associated with hereditary forms of deafness in mouse or human ([http://hearingimpairment.jax.org/master\\_table1.html](http://hearingimpairment.jax.org/master_table1.html)), and further refined this gene set by selecting those that are known to be expressed in HCs (supplementary material Table S1, 88 genes). Analysis of their expression in iHCs at different stages showed that the majority of these 88 genes are significantly upregulated by the combined activity of the three TFs, in particular in day 12 iHCs, which exhibit a clear enrichment in expression of deafness genes known to participate in the formation of hair bundles (Fig. 6J,K).

Since multiple genes involved in the mechanoreception machinery are expressed in iHCs, we examined whether functional mechanotransduction channels are also present in these cells by performing an FM1-43 permeation assay (Gale et al., 2001). When co-cultures grown in the absence of Dox were exposed (for 60 s) to FM1-43, no labeled live cells were detected (supplementary material Fig. S7A). By contrast, in Dox-treated cells (day 12 with RA), ~25% of the Venus<sup>+</sup> population was labeled by FM1-43 (supplementary material Fig. S7B). The specific internalization of FM1-43 into Venus<sup>+</sup> cells suggests that they contain open and potentially functional mechanotransduction channels.

## DISCUSSION

We report here that a combination of three TFs (*Gfi1*, *Pou4f3* and *Atoh1*) is able to promote the direct conversion of somatic cells into HC-like cells, both *in vitro* and *in vivo*. Transcriptome profiling of iHCs at different stages reveals that a specific HC genetic program is activated in these cells. This program appears to recapitulate the normal progression of HC development: genes that are induced early (day 8) are known to participate in HC commitment, while genes encoding components of the mechanotransduction machinery are activated at a later stage of the process (day 12), coinciding with the appearance of polarized espin-rich hair bundle-like protrusions in iHCs. Although these bundles are less organized than native stereociliary bundles, some iHCs are able to rapidly incorporate the FM1-43 dye, which would be consistent with the presence of functional mechanoreceptor channels in these cells. Altogether, our data suggest that *Gfi1*, *Pou4f3* and *Atoh1* can activate the genetic program required for the specification and differentiation of functional HCs. However, complete maturation of iHCs is likely to be dependent on additional extrinsic and intrinsic cues.

### Combinatorial transcriptional control of HC formation

Our results show that *Gfi1*, *Pou4f3* and *Atoh1* are core TFs of the genetic network regulating HC differentiation. Although *Atoh1* has

been considered a ‘master’ gene for HC differentiation owing to its capacity to induce new HCs following ectopic expression in the inner ear (Zheng and Gao, 2000; Woods et al., 2004), our work shows that this pro-HC function is context dependent, as *Atoh1* is unable to induce a HC fate in EB cells, driving instead a neuronal differentiation program. By contrast, we show here that the combination of *Atoh1* with *Gfi1* and *Pou4f3* leads to the implementation of a HC differentiation program in EB cells, as well as in non-sensory otic epithelia. These findings unveil a novel regulatory layer in HC fate specification and provide a molecular basis to explain how *Atoh1* can induce different cell fates in the embryo – not only HCs in the inner ear, but also Merkel cells in the skin, secretory cells in the intestine, or granule neurons in the cerebellum (Mulvaney and Dabdoub, 2012).

What roles do *Gfi1* and *Pou4f3* play in this process? *Gfi1* is a known transcriptional repressor and previous studies indicate that it might contribute to diverting *Atoh1*-expressing cells from an exclusively neural differentiation program. For instance, HCs in the inner ear of *Gfi1* mutant mice exhibit abnormal *Tuj1* expression, suggesting a partial transformation into neurons (Wallis et al., 2003). Also, in the intestinal epithelium of *Gfi1* null mice, *Atoh1*-dependent mucous and Paneth cells acquire abnormal *Ngn3* expression and can convert to pro-enteroendocrine lineages (Bjerknes and Cheng, 2010). However, *Gfi1* may also act as a transcriptional coactivator to positively modulate *Atoh1* activity, by analogy with the functional interaction between their *Drosophila* homologs (*Senseless* and *Atonal*) during sensory precursor specification (Jarman and Groves, 2013). In this process, *Senseless* may function to increase or modify the E-box binding specificity of *Atonal*, modulating its proneural functions. Whether *Senseless/Gfi1* acts by direct physical interaction with *Atonal/Atoh1*, or by binding promoter regions adjacent to E-boxes to modulate *Atonal/Atoh1* transcriptional activity, remains to be investigated. However, in the case of HC induction discussed here, it is unlikely that the observed specificity can be ascribed only to the modulatory activity of *Gfi1*, as the *Atoh1/Gfi1* pair is also active during other cell fate decision processes, such as the determination of secretory cell identities in intestinal crypts (Shroyer et al., 2005; Bjerknes and Cheng, 2010).

Although we have not tested whether *Pou4f3* is absolutely required for HC induction in EBs, it is likely that this TF also plays an essential role in the process, not only by independently activating an additional set of HC differentiation genes, but also by modulating *Atoh1/Gfi1* activity to establish a specific HC genetic program. The first function is suggested by the crucial role of *Pou4f3* in ensuring the proper differentiation and survival of all vestibular and auditory HCs (Xiang et al., 1998). The second role of *Pou4f3* in contributing to the specificity of *Atoh1/Gfi1*-driven HC induction is suggested by the cooperation observed between *Pou3f2*, a related member of the Pou-HD family of TFs, and the bHLH TF *Ascl1* in the activation of a neurogenic program in the developing mouse central nervous system (Castro et al., 2006).

We should also note the remarkable similarity between the cocktail of TFs used to convert fibroblasts and hepatocytes into neurons [*Ascl1*, *Brn2* (*Pou3f2*) and *Myt11*] (Vierbuchen et al., 2010; Marro et al., 2011) and the three TFs used in our study, in both cases consisting of a bHLH TF, a Pou-HD TF and a zinc-finger TF. However, whereas the main contribution of *Brn2* and *Myt11* is to increase the neuronal reprogramming efficiency of *Ascl1*, our results show that *Gfi1* and *Pou4f3* are able to radically alter the *Atoh1* transcriptional program to promote a distinct HC differentiation program. The work described here thus offers a new model system with which to address the crucial question of how

similar sets of TFs can operate in different modes to implement unique cell fates.

### Similarities and differences in the transcriptional profiles of iHCs and native HCs

The ability to obtain purified populations of iHCs using the Myo7a:mVenus reporter line allowed us to generate highly reproducible gene expression profiles for these cells at various phases of their differentiation. Comparison of the iHC transcriptional signature with a core gene expression signature defined for cochlear HCs (500 genes; Cai et al., 2015) reveals that 69% of core HC genes are upregulated by the combined activity of Gfi1, Pou4f3 and Atoh1 in iHCs. When these HC signatures are compared with the set of Atoh1 target genes in cerebellar granule neurons (Klisch et al., 2011), the overlap is much smaller (28% for cochlear HCs and 38% for iHCs), supporting the conclusion that Gfi1, Pou4f3 and Atoh1 activate a HC-specific genetic program. This comparison also highlights the existence of a common set of Atoh1 targets between neurons and HCs, possibly underlying the similar capacity of these cell types to engage in neurotransmission.

The finding that 30% of core HC genes are not activated by the three TFs in iHCs correlates well with the relative immaturity of these cells in culture. In fact, among the ~30% of HC genes that are not upregulated in iHCs there are several ‘deafness’ genes encoding late-expressed hair bundle proteins, such as Slc9a9, Fscn2, Gpr98 (Adgrv1), Myo3a and Strc. It is possible that the lack of expression of these genes is due to a developmental delay, as day 12 iHCs (8 days after induction of the three TFs) are likely to be less advanced in development than the neonatal or postnatal day 0 cochlear Atoh1-GFP cells used to define the core HC signature. Another reason might be that the GPA combination can only induce a partial HC phenotype, lacking the activity of additional TFs that are crucial for late HC differentiation. By comparison with the Atonal-driven sensory program in *Drosophila* chordotonal (Ch) neurons (Newton et al., 2012), in which the TF Fd3F acts downstream of Atonal to regulate various genes required for the assembly of mechanosensory cilia, we note that various ‘missing’ iHC genes are homologs of Fd3F targets in *Drosophila*. These include *Tekt1*, *Wdr63*, *Dnahc6* (*Dnah6*), *Dnahc9* (*Dnah9*) and *Dynlrb2*, which are all involved in axonemal dynein assembly. These genes are also known to be direct or indirect targets of Foxj1, the vertebrate homolog of Fd3F (Stubbs et al., 2008; Jaquet et al., 2009). However, Foxj1 is induced in iHCs by the GPA combination, suggesting that the lack of expression of its targets is due to blockage of its activity in immature iHCs, preventing, for instance, the formation of a proper kinocilium.

We have also scrutinized the list of Atoh1 neuronal targets that are repressed in iHCs, and two genes are worthy of mention – *Gli2* and *Foxm1*, which are known to be crucial for Shh-driven proliferation of CGPs (Flora et al., 2009). Repression of these genes in iHCs illustrates how the combination of Atoh1 with Gfi1 and Pou4f3 leads to key differences in gene expression, with likely functional consequences: whereas Atoh1 induction of *Gli2* in CGPs allows these cells to proliferate in response to Shh (Flora et al., 2009), *Gli2* repression in iHCs might shield these cells from Shh and contribute to the rapid cell cycle exit that we observed after induction of the three TFs.

### Maturation of iHCs *in vitro* requires an adequate cellular environment

Despite their clear progression towards HC differentiation, iHCs failed to develop stereotypical hair bundles *in vitro*. The morphology

and length of the espin-rich projections of iHCs were heterogeneous and, although clearly polarized, their position and orientation were variable. This could be due to the inability of iHCs to form a coherent epithelium in culture, preventing the definition of apical-basal polarity required for hair bundle differentiation. By contrast, overexpression of Gfi1, Pou4f3 and Atoh1 in the embryonic chick inner ear induced ectopic but normally polarized HCs, indicating that this TF combination does not interfere with the normal progression of HC differentiation. These findings suggest that environmental factors and/or a proper cellular context are essential to achieve complete HC differentiation *in vitro*.

With regard to environmental factors, our data show that inhibition of Notch signaling or addition of RA can improve iHC differentiation. Notch activity is known to prevent HC specification in the inner ear (Kiernan, 2013), and our microarray data reveal that Notch signaling is active during iHC formation (supplementary material Fig. S8). The addition of a Notch inhibitor is therefore expected to facilitate iHC formation, and our results confirm this. The activity of RA was also expected to increase iHC differentiation, following previous findings that the addition of RA to chick otic vesicles or mouse organ of Corti explants results in early onset of HC differentiation and supernumerary HCs (Represa et al., 1990; Kelley et al., 1993). Little is known about how RA signaling leads to this effect, but our work might offer some cues as to the underlying molecular mechanisms. For instance, the finding that RA addition represses *Hes1* expression raises the hypothesis that this could relieve Atoh1 from its antagonizing activity (Zheng et al., 2000a) and lead to an increased efficiency of iHC generation.

Concerning the requirement for an adequate multicellular organization for iHC differentiation, we have used several strategies to address this issue, either by co-culturing iHCs with otic-derived mesenchymal cells or by using various synthetic scaffolds to grow dissociated EB cells, but never observed a proper epithelial organization of iHC aggregates *in vitro*. A possible explanation is the absence of supporting cells (SCs) in iHC cultures, which are necessary *in vivo* to establish specific cell-cell adhesion with HCs. In fact, our data suggest that GPA induction of iHCs does not lead to the concomitant induction of SCs. Although iHCs expressed Sox2, typical supporting cell markers such as Prox1 and E-cadherin (cadherin 1) were absent in Dox-treated EBs (data not shown), and analysis of iHC transcriptomes confirms the lack of expression of SC genes [*Gfap*, neurotrophin receptor P75 (*Ngfr*), *GLAST* (*Slc1a3*) and *Jag1*]. This suggests that the combined activation of Gfi1, Pou4f3 and Atoh1 promotes direct conversion to an HC fate, bypassing the bipotent progenitor cell state that normally precedes SC and HC formation *in vivo*.

In summary, we report the first successful and efficient method for direct conversion of mESC-derived progenitors into iHCs, providing a proof-of-concept for HC programming. This simple and rapid method offers an alternative approach to produce large numbers of HC-like cells *in vitro*. Further work will aim to investigate whether the forced expression of Gfi1-Pou4f3-Atoh1 could also direct other somatic cell types towards HC differentiation, and how these three TFs regulate HC commitment and differentiation.

## MATERIALS AND METHODS

### mESC maintenance and differentiation

Ainv15, iAtoh1, iGPA and iGPA-Myo7a:mVenus mESC lines were maintained on gelatin-coated dishes in DMEM (Invitrogen) supplemented with 10% ES-qualified FBS (Invitrogen), 1 mM 2-mercaptoethanol and 2 ng/ml LIF. For EB differentiation, mESCs were trypsinized using 0.25%



trypsin-EDTA (Invitrogen) and resuspended on bacterial-grade Petri dishes in the same medium without LIF. Medium supplementation with 2 µg/ml Dox, 1 µM RA and 10 nM LY411575 was performed as described in the figures. For further details, see the supplementary Materials and Methods.

### Generation of iAtoh1 and iGPA lines

Ain15 mESC cells ( $4 \times 10^6$ ) were electroporated (Gene Pulser II, Bio-Rad; 250 V, 500 µF) with 20 µg pTurbo-Cre and 20 µg Atoh1Plox or GPAPlox vectors (see supplementary Materials and Methods). Cells were subsequently plated on neomycin-resistant and mitotically inactivated MEF feeder cells in DMEM medium supplemented with 350 µg/ml G418. Individual colonies were picked 10–14 days after electroporation.

### RNA extraction, microarray analysis and quantitative PCR

Total RNA was extracted from  $10^6$  EB-derived cells subjected to different culture conditions using the High Pure RNA Isolation kit (Roche Diagnostics) for hybridization on Mouse Genome 2.1 ST Arrays Strip (Affymetrix). Log<sub>2</sub> expression values of transcripts were imported to Chipster 2.4 (Kallio et al., 2011) for data analysis. The GEO accession number for the mRNA microarray data is GSE60352. For further details of microarray sample preparation and data analysis see the supplementary Materials and Methods.

To perform quantitative real-time PCR, first-strand cDNA was synthesized from 1 µg total RNA using Superscript II reverse transcriptase (Invitrogen) and random hexamers. Real-time PCR was performed with SYBR Green and exon-spanning primers in 7500 and ViiA 7 real-time PCR systems (Applied Biosystems). Further details, including primer sequences, are provided in the supplementary Materials and Methods.

### Immunocytochemistry, imaging and cell counts

EBs and electroporated embryos were processed for immunocytochemistry as described in the supplementary Materials and Methods using the primary antibodies listed in supplementary material Table S2 and Alexa Fluor-conjugated secondary antibodies (Molecular Probes) and 0.15% DAPI (Sigma-Aldrich). EdU incorporation employed the Click-iT EdU Alexa Fluor 488 Flow Cytometry Assay Kit (Molecular Probes).

Fluorescent and bright-field images of fixed sections and cells were captured and processed as described in the supplementary Materials and Methods. The number of cells expressing Myo7a, espin, Pou4f3 or Venus was quantified among total induced cells or among the total number of cells in EBs in four to five randomly selected fields using Photoshop CS Cell Counter software (Adobe) as described in the supplementary Materials and Methods.

### Flow cytometry

Live cell analysis and intracellular staining of fixed cells were performed as described in the supplementary Materials and Methods using Myo7a and GFP primary antibodies (supplementary material Table S2) and Alexa Fluor-conjugated secondary antibodies (Molecular Probes). Data were analyzed with FlowJo software (Tree Star).

### FM1-43 uptake assay

iHC fast permeation to FM1-43 dye [*N*-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl)pyridinium dibromide] (Molecular Probes) was assessed as described in the supplementary Materials and Methods.

### Scanning electron microscopy

Co-cultures of EB-derived progenitors and mitotically inactivated chicken utricle mesenchyme treated with Dox and RA were processed for SEM using a JEOL 6700F microscope as described in the supplementary Materials and Methods.

### Chicken otic cup electroporation

Electroporations of the inner ear were performed at E2 using Electro Square Porator TM ECM830 (BTX) as described (Freeman et al., 2012). The Tol2 transposon vectors were electroporated at a final concentration of 1 µg/µl. For details, see the supplementary Materials and Methods.

### Statistics

All data are expressed as mean±s.e.m. and statistical significance was assessed using an unpaired Student's *t*-test. For all statistics, data from at least three biologically independent experiments were used. Data and graphs were tabulated and prepared using Microsoft Excel and GraphPad Prism software. *P*<0.05 was considered statistically significant.

### Acknowledgements

We thank Tiantian Cai and Andrew Groves for sharing unpublished RNA-seq data from Atoh1-GFP HCs; Jörg Becker/IGC Gene Expression Unit for technical support with microarray analysis; Andrew Forge and Telmo Nunes for SEM support; Sara Ferreira, IMM bioimaging and flow cytometry facilities for technical help; Fernando Giraldez for critical reading of the manuscript; and Filipe Vilas-Boas for helpful discussions.

### Competing interests

The authors declare no competing or financial interests.

### Author contributions

A.C. conceived, performed and analyzed the experiments and wrote the paper. L.S.-G. performed the chick electroporations. S.J. prepared the inactivated utricle periodic mesenchyme cells. J.E.G. and N.D. provided scientific and technical advice for some experiments. D.H. conceived and supervised the study and wrote the paper.

### Funding

This work was supported by Fundação para a Ciência e Tecnologia, Portugal [PTDC/SAU-NEU/71310/2006, SFRH/BD/38461/2007 to A.C.]. A.C. was also a recipient of an EMBO Short-Term Fellowship during her stay at the UCL Ear Institute. S.J. is funded by a UCL Impact Studentship to J.E.G. Work in N.D.'s lab is supported by the BBSRC [BB/L003163/1].

### Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.119149/-/DC1>

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