# TFE2 and GATA3 enhance induction of POU4F3 and myosin VIIa positive cells in nonsensory cochlear epithelium by ATOH1

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## ARTICLE INFO

## ABSTRACT

Transcription factors (TFs) can regulate different sets of genes to determine specific cell types by means of combinatorial codes. We previously identified closely-spaced TF binding motifs located 8.2–8.5 kb 5′ to the ATG of the murine *Pou4f3* gene, a gene required for late hair cell (HC) differentiation and survival. These motifs, 100% conserved among four mammalian species, include a cluster of E-boxes preferred by TCF3/ATOH1 heterodimers as well as motifs for GATA factors and SP1. We hypothesized that these factors might interact to regulate the *Pou4f3* gene and possibly induce a HC phenotype in non-sensory cells of the cochlea. Cochlear sensory epithelium explants were prepared from postnatal day 1.5 transgenic mice in which expression of GFP is driven by 8.5 kb of *Pou4f3* 5′ genomic DNA (*Pou4f3*/GFP). Electroporation was used to transfect cells of the greater epithelial ridge with multiple plasmids encoding human ATOH1 (hATOH1), hTCF3 (also known as E2A or TEF2), hGATA3, and hSP1. hATOH1 or hTCF3 alone induced *Pou4f3*/GFP cells but hGATA3 and hSP1 did not. hATOH1 but not hTCF3 induced conversion of greater epithelial ridge cells into *Pou4f3*/GFP and myosin VIIa double-positive cells. Transfection of hATOH1 in combination with hTCF3 or hGATA3 induced 2–3X more *Pou4f3*/GFP cells, and similarly enhanced *Pou4f3*/GFP and myosin VIIa double-positive cells, when compared to hATOH1 alone. Triple or quadruple TF combinations were generally not more effective than double TF combinations except in the middle turn, where co-transfection of hATOH1, hE2A, and hGATA3 was more effective than hATOH1 plus either hTCF3 or hGATA3. The results demonstrate that TFs can cooperate in regulation of the *Pou4f3* gene and in the induction of at least one other element of a HC phenotype. Our data further indicate that combinations of TFs can be more effective than individual TFs in the inner ear.

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## Introduction

The hair cell (HC) is a critical cell type of the inner ear, required for normal auditory and vestibular function, and exquisitely specialized for mechanotransduction and communication with the nervous system. HCs do not regenerate in the mammalian inner ear and loss of these cells in the cochlea or in the vestibule results in permanent hearing loss or vestibular deficit. Gene therapy has been proposed as a means of stimulating HC regeneration or of preventing HC loss due to genetic or environmental factors (e.g. Kawamoto et al., 2003; Ono et al., 2009; Zheng and Gao, 2000).

A promising approach to HC regeneration has been to introduce regulators of HC development into other types of inner ear cells. In particular, the Class II basic helix-loop-helix (bHLH) transcription factor (TF) ATOH1 (also known as Math1) is required for HC fate determination, survival and differentiation. Atoh1 deletion leads to perinatal lethality, with some poorly differentiated cells in the organ of Corti expressing the early HC marker myosin 7A (Myo7A) (Bermingham et al., 1999). Conditional deletion of Atoh1 in the inner ear leads to the death of most cells in the organ of Corti prior to birth, but a few surviving cells in the organ express Myo7A at later stages (Pan et al., 2011). Premature termination of inner ear Atoh1 expression that has begun normally leads to the death of most partially differentiated HCs (Pan et al., 2012). However, forced Atoh expression can induce ectopic HC formation, in which nonsensory cochlear epithelial cell types adopt a HC-like phenotype (Gubbels et al., 2008; Izumikawa et al., 2005, 2008; Kawamoto et al., 2003; Woods et al., 2004; Zheng and Gao, 2000). However, despite

advances in identifying TFs critical for inner ear development and applying them to HC replacement, there has been less progress in understanding how these factors exert their specific effects in HCs. For example, ATOH1 is also expressed in several other cell types with very different phenotypes, playing a significant role in the development of, for example, Merkel cell cutaneous touch receptors (Leonard et al., 2002), spiral ganglion and cochlear nucleus cells (Maricich et al., 2009), cerebellar granule cells (Gazit et al., 2004), and secretory epithelial cells in the colon (Yang et al., 2001), as well as regulating mucin gene expression in various mucosal epithelia (Sekine et al., 2006). Why then does ATOH1 expression induce a HC phenotype in inner ear epithelial cells, but not in these other cells? A potential explanation is that ATOH1 interacts with other transcriptional regulators to generate cellular specificity. There is extensive evidence that TFs can combine with distinct TF partners in different cell types, in a combinatorial code, which regulates different sets of genes and contributes to the determination of cell phenotype. This concept was originally developed to explain conserved, modular elements in an Arabadopsis gene (Curie et al., 1993) and then applied to mammalian Hox gene regulation (Shashikant et al., 1995). Recently, such combinatorial coding was found to regulate expression of ATOH1 itself (Ahmed et al., 2012). TF interactions are highly likely to occur physically, on the regulatory DNA of genes. If so, in the case of HC genes regulated by ATOH1, this may be apparent in conserved binding motifs for TFs in the regulatory DNA of ATOH1 target genes expressed in this cell type. One potential target is the gene encoding the POU domain TF POU4F3 (also known as Brn-3.1, Brn-3c).

POU4F3 is required for late HC differentiation, including their transduction and synaptic specializations, and for HC survival. Deletion of POU4F3 leads to failure of HC differentiation and the death of most HCs (Erkman et al., 1996; Xiang et al., 1997, 2003), although some undifferentiated HCs are retained into adulthood (Xiang et al., 1997, 2003; Pauley et al., 2008). Cochlear POU4F3 expression is initiated around embryonic day 13 (E13), just after that of ATOH1 and preceding myosin VIIa expression, another HC specific marker, which begins on E15 (Bermingham et al., 1999; Chen et al., 2002; Pan et al., 2012; Sage et al., 2006; Woods et al., 2004; Xiang et al., 1997, 2003). It is associated with the regulation of the motor protein prestin in outer HCs in newborn rodents (Gross et al., 2011), and continues to be expressed into adulthood. It is the gene mutated in DFNA 15, a form of dominant, progressive hereditary hearing loss (Vahava et al., 1998, Collin et al., 2008). Like ATOH1, POU4F3 is also expressed in other developing and adult sensory or neural populations. For example, it is expressed in Merkel cells (Leonard et al., 2002), trigeminal neurons (Artinger et al., 1998) and a subset of retinal ganglion cells (Erkman et al., 1996), which have quite different specializations and do not express HC-specific genes. There is also evidence that different members of the Pou4f family (1–3) are combinatorially expressed and cross-regulate in retinal and somatosensory neurons (Badea et al., 2012).

The upstream regulation of the Pou4f3 gene is not well characterized. However, using bioinformatic analysis, we previously identified several closely-spaced motifs for TF binding located 8.2–8.5 kb 5′ to the ATG of the murine Pou4f3 gene (Masuda et al., 2011). These motifs are 100% conserved among four mammalian species: mouse, human, dog, and cow (Fig. 1). They include three E-boxes (CAGCTG × 2, CACCTG) to which Class I/Class II bHLH heterodimers such as TCF3/ATOH1 can bind with high affinity (Akazawa et al., 1995; Ledent et al., 2002; Massari and Murre, 2000; Scheffer et al., 2007b). Two of these are consistent with the motifs known to be activated by ATOH1 (Klisch et al., 2011). We also demonstrated that ATOH1 directly binds to this conserved region using chromatin
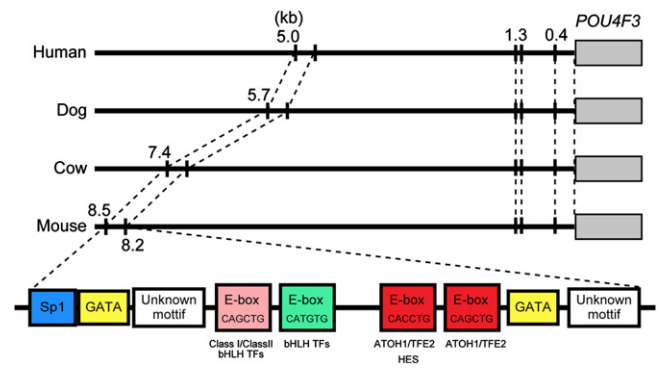


**Fig. 1.** Conserved 5′ TF biinding sites in the Pou4f3 gene. A region that is conserved across four mammalian species is located 8.2–8.5 kb 5′ to the ATG of Pou4f3 in the mouse, where it supports ATOH1 binding (Masuda et al., 2011). Clustered E-boxes (CANNTG) are conserved at this site, including two of a type that is activated by ATOH1 (red boxes) (Klisch et al., 2011). The E-boxes are located in close proximity to conserved binding motifs for GATA and SP1.

immunoprecipitation (Masuda et al., 2011). The remaining E-box (CATGTG) is typically preferred by Class III bHLH and Class IV bHLH factors (Fisher et al., 1992; Hamid and Kakar, 2004). In addition, preferred binding sites for SP1 and GATA3 are present (Ko and Engel, 1993; Masuda et al., 2011; Merika and Orkin, 1993; Wierstra, 2008). Moreover, co-transfection of a reporter construct in which 8.5 kb of 5′ Pou4f3 DNA drives eGFP with an expression construct encoding ATOH1 enhanced eGFP expression in HEK293 and VOT-E36 cells, when compared to transfection with the transgene alone (Masuda et al., 2011).

We speculated that TCF3, GATA3, and/or SP1 might act cooperatively at this conserved cluster of TF binding sites to control Pou4f3 gene expression. We further speculated that these factors might interact to induce a HC-like phenotype in non-sensory cells of the cochlea. To test these hypotheses, we evaluated whether these TFs, alone or in combination, enhance the ability of ATOH1 to induce ectopic inner ear Pou4f3 and/or Myo7a gene expression. Electroporation was used to transfect cells of the greater epithelial ridge (GER) of postnatal day 1.5 (P1.5) cochlear epithelial explants from transgenic mice (Pou4f3/GFP mice) in which expression of GFP is driven by 8.5 kb of 5′ Pou4f3 genomic DNA (Masuda et al., 2011). We demonstrate that ATOH1 can act in a combinatorial fashion with TCF3 or GATA3 to enhance both Pou4f3-promoter-induced eGFP and myosin VIIa expression in nonsensory cells of the GER.

## Materials and methods

*Animals.* P1.5 wildtype CBA/J or Pou4f3/GFP mice on a CBA/J background were used. In the transgenic mice, robust GFP (Pou4f3/GFP) expression is noted in neonatal cochlear and vestibular HCs, and not in other cochlear cells (Fig. 2). Preliminary studies showed that transfection of P1.5 sensory epithelia was less robust than at P0.5. P1.5 was chosen so that any combinatorial enhancement of ATOH1 effects might be detected more readily.

The local animal subjects committee of the VA San Diego Healthcare System approved all procedures in accordance with the guidelines laid down by the National Institutes of Health regarding the care and use of animals for experimental procedures.

*Plasmid DNA preparation.* All plasmids were prepared with the EndoFree Plasmid Maxi Kit (Qiagen, Valencia, CA) following the manufacture's protocol. Plasmids were resuspended to 3 μg/μl in HBSS (without calcium, magnesium and phenol red), aliquotted