Original Article
doi: 10.5115/acb.2011.44.1.69
pISSN 2093-3665 eISSN 2093-3673
Anatomy & Cell Biology acb

Identification of genes concordantly expressed with *Atoh1* during inner ear development

Heejei Yoon, Dong Jin Lee, Myoung Hee Kim, Jinwoong Bok
Department of Anatomy, Brain Korea 21 Project for Medical Science, College of Medicine, Yonsei University, Seoul, Korea

Abstract: The inner ear is composed of a cochlear duct and five vestibular organs in which mechanosensory hair cells play critical roles in receiving and relaying sound and balance signals to the brain. To identify novel genes associated with hair cell differentiation or function, we analyzed an archived gene expression dataset from embryonic mouse inner ear tissues. Since atonal homolog 1a (Atoh1) is a well known factor required for hair cell differentiation, we searched for genes expressed in a similar pattern with *Atoh1* during inner ear development. The list from our analysis includes many genes previously reported to be involved in hair cell differentiation such as *Myo6*, *Tecta*, *Myo7a*, *Cdh23*, *Atp6v1b1*, and *Gfi1*. In addition, we identified many other genes that have not been associated with hair cell differentiation, including *Tekt2*, *Spag6*, *Smpx*, *Lmod1*, *Myh7b*, *Kif9*, *Ttyh1*, *Scn11a* and *Cnga2*. We examined expression patterns of some of the newly identified genes using real-time polymerase chain reaction and *in situ* hybridization. For example, *Smpx* and *Tekt2*, which are regulators for cytoskeletal dynamics, were shown specifically expressed in the hair cells, suggesting a possible role in hair cell differentiation or function. Here, by re-analyzing archived genetic profiling data, we identified a list of novel genes possibly involved in hair cell differentiation.

Key words: Inner ear, Hair cell, Atoh1, Differentiation

Received October 8, 2010; Revised December 2, 2010; Accepted December 15, 2010

Introduction

The mammalian inner ear is a highly intricate organ composed of a cochlear duct and vestibular organs, responsible for hearing and balance, respectively (Fig. 1A). The hair cells residing in each sensory organ of the inner ear play a primary role in sensory transduction by converting sound and balance information into electrical signals, which are then transmitted to their associated neurons to convey the signals to the brain. In humans, these hair cells can easily be damaged by various causes including noise, drugs, aging, or genetic mutations. The damage is irreversible in most cases, leading to permanent hearing loss [1]. Thus far, efforts to preserve or regenerate hair cells using stem cells or gene therapy have met with only limited success [2-4]. A better understanding of normal hair cell development may help overcome such limitations.

Inner ear hair cells are formed via a series of cell fate decision and differentiation processes which are tightly regulated by a combination of extrinsic and intrinsic factors [5]. Among such factors, the basic helix-loop-helix transcription factor, Atoh1 (Atonal homolog, also known as Math1), has been shown to be a major determinant in hair cell differentiation. In *Atoh1* knockouts, both cochlear and vestibular hair cells failed to differentiate [6], while delivering the *Atoh1* gene induced formation of ectopic hair cells in tissue cultures as well as animal models [3, 7, 8]. These results indicate a crucial role of Atoh1 in hair cell differentiation, yet how Atoh1 functions to dictate hair cell differentiation remains poorly understood.

Corresponding author:
Jinwoong Bok
Department of Anatomy, Brain Korea 21 Project for Medical Science, College of Medicine, Yonsei University, 134 Sinchon-dong, Seodaemun-gu, Seoul 120-752, Korea
Tel: +82-2-2228-1650, Fax: +82-2-365-0700, E-mail: bokj@yuhs.ac

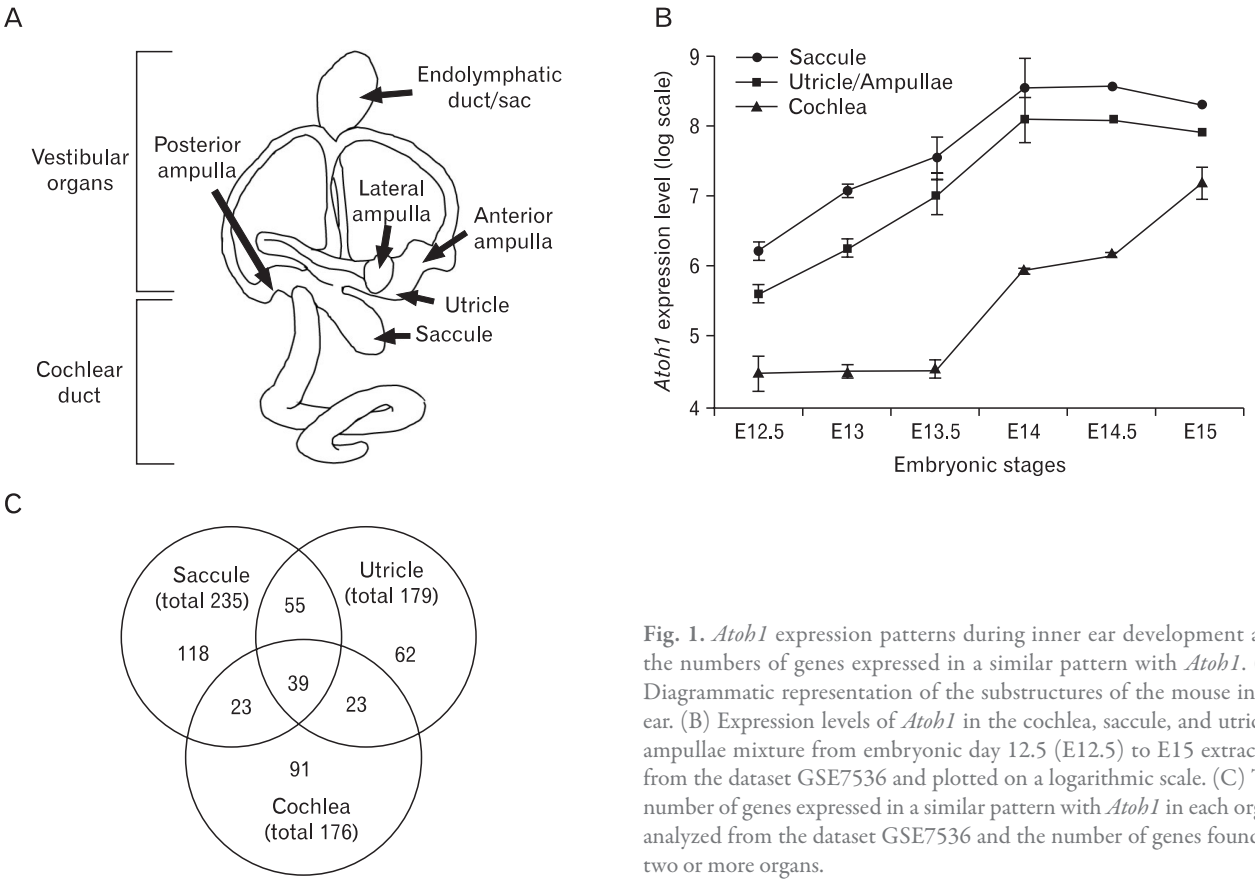

Fig. 1. *Atoh1* expression patterns during inner ear development and the numbers of genes expressed in a similar pattern with *Atoh1*. (A) Diagrammatic representation of the substructures of the mouse inner ear. (B) Expression levels of *Atoh1* in the cochlea, saccule, and utricle/ampullae mixture from embryonic day 12.5 (E12.5) to E15 extracted from the dataset GSE7536 and plotted on a logarithmic scale. (C) The number of genes expressed in a similar pattern with *Atoh1* in each organ analyzed from the dataset GSE7536 and the number of genes found in two or more organs.

In this study, to identify novel genes that play a role in hair cell differentiation, we analyzed an archived gene expression dataset, generated by extensive microarray analysis using mouse inner ear tissues at several embryonic stages [9]. Since Atoh1 plays a key role in hair cell differentiation [5], we assumed that genes involved in hair cell differentiation would be regulated directly or indirectly by Atoh1 and their expression patterns would be closely associated with that of *Atoh1* during inner ear development. For example, expression patterns of *Gfi1*, *Pou4f3*, and *Myo7a*, which are important for hair cell differentiation, have been shown to resemble that of *Atoh1* during inner ear development [10, 11]. We identified several genes that showed similar expression patterns to that of *Atoh1* during inner ear development, some of which were confirmed as being specifically expressed in developing hair cells.

Materials and Methods

Data analysis

We analyzed a gene expression dataset (NCBI Gene Expression Omnibus; GSE7536), generated by microarray analysis (mouse MOE430A_2 gene chip; Affymetrix, Santa Clara, CA, USA) using inner ear tissues from CBA/J mouse embryos, which were micro-dissected at half-day intervals from embryonic day 9 (E9) to E15 [9]. The experimental procedures and statistical analysis used for the GSE7536 dataset have been described in detail in the original paper [9]. In particular, we focused our analysis on expression profiles between E12.5 to E15, when *Atoh1* expression begins and rapidly increases. In the dataset, the cochlea, saccule, and utricle/ampullae mixtures were divided and profiled separately [9]. In addition, non-ear tissues were also isolated from each stage and profiled [9]. We first applied Pearson's correlation to the dataset to eliminate genes exhibiting low degrees of correlation ($-0.5<r<0.5$) compared to the *Atoh1* expression pattern. We then segregated the profiles into two groups by developmental stage according to *Atoh1* expression

levels (E12.5, E13, E13.5 vs. E14, E14.5, E15) for cochlear samples and (E12.5, E13 vs. E14, E14.5, E15) for utricle and saccule samples. Significant analysis of microarrays [12] was applied to identify genes differentially expressed between the two groups with at least two-fold changes and an estimated false discovery rate of ≤1%. We particularly focused on genes positively correlated with *Atoh1* expression patterns. Information regarding gene ontology was extracted from the Mouse Genome Informatics (MGI) database (http://www.informatics.jax.org/).

Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR)

Timed pregnant C57BL/6 mice were euthanized at E13.5, E15.5, and E17.5 at which time the embryos were collected in ice-cold phosphate buffered saline (PBS). Embryonic inner ear tissues from each stage were micro-dissected under a stereomicroscope (SZX10, Olympus, Tokyo, Japan) and the vestibular and cochlear tissues were collected separately. Total RNAs were isolated from each tissue sample using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). RNA quality was assessed by agarose gel electrophoresis of an aliquot of total RNA. The cDNA was synthesized from each RNA sample using poly(dT)$_{20}$ and a Superscript III RT kit according to the manufacturer's instructions (Invitrogen). Real-time PCR was performed using SYBR Green PCR kits and an ABI 7500 machine (Applied Biosystems, Carlsbad, CA, USA). Primers used for real-time PCR are listed in Table 1. Relative expression levels in each stage were determined by comparative methods.

In situ hybridization

In situ hybridization was performed as previously described [13]. Briefly, embryos were fixed overnight in 4% paraformaldehyde in PBS, dehydrated in 30% sucrose, and embedded in O.C.T. (Tissue-Tek, Sakura Finetechnical Co., Tokyo, Japan). Embryos were then sectioned at a thickness of 12-μm onto Superfrost slides (VWR Scientific, West Chester, PA, USA). The slides were dried at room temperature

Table 1. Primers used in real-time polymerase chain reaction

Genes	Forward primer	Reverse primer
Atoh1	TTTCCCCAACTGCTTGAGAC	TGCATTGGCAGTTGAGTTTC
Myo7a	GATCCCAGAACCAAGGACATC	TCTGGCTGATGTAGGAAGTCAG
Myo6	CATCCTTCTTGTAGCTGGCAAG	GAGCAGGTTCTGCAGCATG
Smpx	TCCAGAGAGCAGGGCTAAGAC	AGGAGCTCCCTCCTCAGTTTC
Tekt2	AGCTTGGCACAGGCCAAAG	CAATGACTTCCACCTCTTTCAG
Gnmt	GGCTCTCCTGGCTTCAGTAAG	TCAGTCTGTCTTCTTGAGCAC
Kif9	GCCTGCTCATGGAATTTGAC	AAGACCTTCAGGCAGGACAG
Lmod1	CTTAAGCAGCTGAAGAAGGTAGAG	AGGGAGAAGAGATGAGGTGTC
Cldn8	TTTGCAGCCTCGAGGAGTAAG	GCATTTGAACAATCCCTTGCATG
Rorc	TTCTCATCAA TGCCAACCGTC	CTGAAGAGTTCCTTATAGAGTG
Spag6	AGTTCAGTAAGGTGCTGCCAC	AGAAGTGTATCGGAGTATCCAG
Lrrtm1	CACTCGCTGGAAAAGGATTTC	CTGTTGTAGCGCAAAGACAAG
Esrrb	CCCTCGCCAACTCAGATTC	CTGCAGTTTCACACTGTAGAAG
Calml4	CTCACTCACAAGGAAGTGGATG	AGGCTCTCATCTGCTCTCTTG
Fbxo32	CAGAGAGGCAGATTCGCAAG	AAAGTGAGACGGAGCAGCTC
Ptgds	CAATCTCACCTCTACCTTCCTC	AGTCTGGGTTCTGCTGTAGAG
Tmod1	AACAACGACCTTGTGAGGAAG	AACCTCAGTGCACAAAGAACTG
Nrcam	GACGTTTGGAGAATACAGTGATG	TTCCCTCTGCTGGCTCTTTC
ActB	CATGTTTGAGACCTTCAACACCCC	GCCATCTCCTGCTCGAAGTCTAG

doi: 10.5115/acb.2011.44.1.69

Table 2. Examples of the genes expressed concordantly with *Atoh1* during inner ear development

Cochlea			Saccule			Utricle		
Gene symbol	Gene description	Fold Δ	Gene symbol	Gene description	Fold Δ	Gene symbol	Gene description	Fold Δ
Kcnn4	K+ intermediate/small conductance Ca2+-activated channel, ubfamily N, number 4	20.9	*Smpx*	Small muscle protein, X-linked	41	*Smpx*	Small muscle protein, X-linked	32.5
1500015 O10Rik	RIKEN cDNA 1500015O10 gene	19.2	*Il33*	Interleukin 33	25	*Cars*	Cysteinyl-tRNA synthetase	30.8
Dkk2	Dickkopf homolog 2	11.4	*Spag6*	Sperm associated antigen 6	21.7	*Ptgds*	Prostaglandin D2 synthase	21.1
Cryab	Crystallin, alpha B	10	*Ap3b2*	Adaptor-related protein complex 3, beta 2 subunit	18.2	*Gfi1*[a)]	Growth factor independent 1	15.7
Enpep	Glutamyl aminopeptidase	9.3	*Tectb*[a)]	Tectorin beta	17.9	*Calml4*	Calmodulin-like 4	12.7
Otor[a)]	Otoraplin	9	*Ttr*	Transthyretin	16.2	*Esrrb*[a)]	Estrogen related receptor, beta	10.3
Tectb[a)]	Tectorin beta	7.3	*Ptgds*	Prostaglandin D2 synthase	14.6	*Tekt2*	Tektin 2	9.6
Esrrb[a)]	Estrogen related receptor, beta	7	*Otor*[a)]	Otoraplin	13.6	*Enpep*	Glutamyl aminopeptidase	9.2
Efhd1	EF hand domain containing 1	6.6	*Gfi1*[a)]	Growth factor independent1	10.6	*Kazald1*	Kazal-type serine peptidase inhibitor domain 1	9.1
Fbxo32	F-box protein 32	6.4	*Cnga2*	Cyclic nucleotide gated channel alpha 2	9.4	*Calb1*[a)]	Calbindin 1	8.6
Nfkb2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, p49/p100	6.3	*Kazald1*	Kazal-type serine peptidase inhibitor domain1	8.8	*Kcnn4*	K+ intermediate/small conductance Ca2+-activated channel, subfamily N, number 4	8.5
Kazald1	Kazal-type serine peptidase inhibitor domain 1	6.3	*Ttyh1*	Tweety homolog 1	8.2	*Gnb3*	Guanine nucleotide binding protein (G protein), beta 3	8.1
Gsn[a)]	Gelsolin	6.1	*Cryab*	Crystallin, alpha B	7.8	*Spag6*	Sperm associated antigen 6	7.8
Epyc	Epiphycan	5.7	*Tmprss3*[a)]	Transmembrane protease, serine 3	7.6	*Chgb*	Chromogranin B	7.6
Igf1[a)]	Insulin-like growth factor 1	5.7	*Pou2af1*	POU domain, class 2, associating factor 1	7.6	*Cryab*	Crystallin, alpha B	7.5
Ttr	Transthyretin	5.7	*Cdh23*[a)]	Cadherin 23 (otocadherin)	7.5	*Fbxo32*	F-box protein 32	7.4
Tecta[a)]	Tectorin alpha	5.6	*S100a1*[a)]	S100 calcium binding protein A1	7.3	*Hes5*[a)]	Hairy and enhancer of split 5 (Drosophila)	7.2
Slc44a3	Solute carrier family 44, member 3	5.5	*Gnb3*	Guanine nucleotide binding protein (G protein), beta 3	7	*Hsd3b4*	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 4	7.2
Sfi1	Sfi1 homolog, spindle assembly associated	5.4	*Hsd3b4*	Hydroxy-δ-5-steroid dehydrogenase, 3 δ- and steroid β-isomerase 4	6.9	*Mia1*	Melanoma inhibitory activity 1	6.9
Atoh1[a)]	Atonal homolog 1 (Drosophila)	5.2	*Mal*	Myelin and lymphocyte protein, T-cell differentiation protein	6.8	*Dkk2*	Dickkopf homolog 2 (Xenopus laevis)	6.6
Doc2b	Double C2, beta	5.2	*Tekt2*	Tektin 2	6.4	*Mal*	Myelin and lymphocyte protein, T-cell differentiation protein	6.5
Calb1[a)]	Calbindin 1	5.1	*Tecta*[a)]	Tectorin alpha	6.4	*Wnt7a*[a)]	Wingless-related MMTV integration site 7A	6.3

Table 2. Continued.

Cochlea			Saccule			Utricle		
Gene symbol	Gene description	Fold Δ	Gene symbol	Gene description	Fold Δ	Gene symbol	Gene description	Fold Δ
Itga8[a]	Integrin alpha 8	4.8	*Myo7a*[a]	Myosin VIIA	5.8	*Tecta*[a]	Tectorin alpha	4.6
Ly6h	Lymphocyte antigen 6 complex, locus H	4.7	*Atp6v1b1*[a]	ATPase, H+ transporting, lysosomal V1 subunit B1	3.8	*Ush1c*[a]	Usher syndrome 1C homolog (human)	4.3
Grik1	Glutamate receptor, ionotropic, kainate 1	4.7	*Atoh1*[a]	Atonal homolog 1 (Drosophila)	3.5	*Atoh1*[a]	Atonal homolog 1 (Drosophila)	4.2
Ttyh1	Tweety homolog 1 (Drosophila)	4.5	*Myo6*[a]	Myosin VI	3.5	*Cdh23*[a]	Cadherin 23 (otocadherin)	3.8
Smpx	Small muscle protein, X-linked	2.8	*Slc26a4*[a]	Solute carrier family 26, member 4	3.4	*Myo7a*[a]	Myosin VIIA	3.6
Tekt2	Tektin 2	2.6	*Pou4f3*[a]	POU domain, class 4, transcription factor 3	2.4	*Myo6*[a]	Myosin VI	3.3
Myo6[a]	Myosin VI	2.3	*Cacna1d*[a]	Ca2+ channel, voltage-dependent, L type, α1D	2.2	*Jag2*[a]	Jagged 2	2.6

[a]Genes previously associated with inner ear development or deafness.

overnight. Before in situ hybridization, slides were rehydrated, post-fixed, and permeabilized using 10 μg/ml proteinase K for 2-5 minutes. Hybridization was performed in Seal-a-Meal bags (Kapak, Minneapolis, MN, USA). Each bag contained four slides and 5-7 ml of hybridization solution with a DIG-labeled RNA probe. RNA probes for *Tekt2* were generated from a fragment of mouse *Tekt2* cDNA containing −46 to +531 nucleotides (GenBank accession number: NM_011902.2), and for *Smpx*, from a 664 base pair (bp) mouse *Smpx* cDNA fragment containing the entire open reading frame as well as 112 bp 5' and 294 bp 3' untranslated regions (GenBank accession number: NM_025357.2).

Results

Search for genes concordantly expressed with *Atoh1* in the developing inner ear

To identify genes involved in differentiation of mechanosensory hair cells, we took advantage of the vast amount of gene expression profiles available in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). We chose dataset GSE7536, in which mouse inner ear tissues from E9 to E15 had been micro-dissected at half-day intervals and subjected to microarray analysis [9]. This dataset provides comprehensive gene expression profiles for substructures of the inner ear tissues including the cochlea, saccule, and utricle/ampullae mixture ("utricle" hereafter) during critical periods of hair cell differentiation.

Hair cells in the vestibular organs such as saccule and utricle differentiate earlier than those in the cochlea. Consistently, *Atoh1* expression in the vestibular organs is obvious around E12.5, but not in the cochlea until E14.5 [14, 15]. Our analysis of the GSE7536 dataset confirmed such expression patterns of *Atoh1* during inner ear development (Fig. 1B). While *Atoh1* expression levels in the vestibular organs (utricle and saccule) gradually increased from E12.5 and plateaued at E14, cochlear *Atoh1* expression remained low until E13.5 and began to increase at E14 (Fig. 1B). In order to identify genes exhibiting expression patterns similar to *Atoh1* during these developmental stages, we divided the expression profiles into two groups by developmental stage according to *Atoh1* expression levels. For cochlear samples, expression profiles of E12.5, E13, and E13.5 were combined and designated as 'low Atoh1 stages' and those of E14, E14.5, and E15 were combined and designated as 'high Atoh1 stages.' For saccule and utricle samples, expression profiles of E12.5 and E13 were combined as 'low Atoh1 stages' and those of E14, E14.5, and E15 were combined as 'high Atoh1 stages.' The mean expression levels of *Atoh1* were at least three fold higher in the 'high Atoh1 stages' compared to the 'low Atoh1 stages,' indicating that expression levels of *Atoh1* significantly differ between the two groups.

By applying Pearson's correlation and comparing fold-change differences, we searched for genes whose expression patterns positively correlated with that of *Atoh1* and showed at

doi: 10.5115/acb.2011.44.1.69

least a two-fold difference between 'low Atoh1 stages' and 'high Atoh1 stages' from each organ sample. A total of 176 cochlea, 179 utricle, and 235 saccule genes were identified from each organ, and 140 genes were found in at least two organs and 39 genes were found in all three organs (Fig. 1C). Some examples exhibiting high-fold changes from each organ are listed in Table 2. The list contains several genes shown to be involved in inner ear development, hair cell differentiation or hereditary deafness such as *Myo6*, *Tecta*, *Tmprss3*, *Atp6v1b1*, *Slc26A4*, *Pou4f3*, *Gfi1*, *Tecta*, *Tectb*, *Hes5* and *Jagged 2* [16-20].

Genes not previously associated with hair cell differentiation or hearing loss

The fact that many genes previously associated with hair cell differentiation or hearing loss are included in our list (Table 2) demonstrates the validity of our methodology and also suggests that novel genes previously unrecognized, yet playing important roles in hair cell differentiation, may be included in the list.

To increase our chance of identifying novel inner ear genes, we removed the genes previously associated with inner ear development such as *Atoh1*, *Myo6*, *Myo7a*, *Wnt7a*, and *Gfi1*, and also excluded genes that are abundantly expressed in non-inner ear tissues. Thus, the resulting list contains genes that are expressed in a similar pattern with *Atoh1* but have not previously been associated with inner ear development or function (Table 3). These genes include *Tekt2* and *Spag6*, which are implicated in ciliary or flagellar motility [21-23], *Smpx*, *Lmod1*, *Myh7b*, and *Kif9*, implicated in muscle contraction or cytoskeleton dynamics [24-28], transmembrane ion transporters *Ttyh1*, *Scn11a*, and *Cnga2* [29-33], and transcription factors such as *Rorc* and *Pou2af1* [29-33].

Real-time PCR analyses for the selected genes

To confirm that the genes included in Tables 2 and 3 are indeed expressed in a similar pattern with *Atoh1* during inner ear development, we dissected mouse inner ear tissues at E13.5, E15.5, and E17.5 and performed quantitative RT-PCR (Fig. 2). Since the gradual increase of *Atoh1* expression levels was more obvious in the cochlea (Fig. 1), we used the cochlear tissues for our RT-PCR experiments. The examined genes included some known hair cell-specific genes as controls (*Atoh1*, *Myo7a*, *Myo6*), genes associated with cytoskeletal structures or regulation (*Smpx*, *Tekt2*, *Kif9*, *Lmod1*, *Spag6*, *Fbxo32*, *Tmod1*), and genes encoding nuclear proteins (*Rorc*,

Table 3. Examples of genes concordantly expressed with *Atoh1* in ear tissues but not in non-ear tissues

Gene symbol	Description	Fold change		
		Sa	Ut	Co
Smpx	Small muscle protein, X-linked	41	32.5	2.8
Tekt2	Tektin 2	6.4	9.6	2.6
Cnga2	Cyclic nucleotide gated channel alpha 2	9.4	6	2.7
Cryab	Crystallin, alpha B	7.8	7.5	9.9
Ttyh1	Tweety homolog 1 (Drosophila)	8.2	2.2	4.5
Pld1	Phospholipase D1	5	4.1	2.4
Atoh1[a)]	Atonal homolog 1 (Drosophila)	3.5	4.2	5.2
Aldoc	Aldolase C, fructose-bisphosphate	3.7	3.4	2.8
Agr3	Anterior gradient homolog 3 (Xenopus laevis)	2.1	4.8	4.2
Masp1	Mannan-binding lectin serine peptidase 1	3.6	3.3	2.2
Rorc	RAR-related orphan receptor gamma	2.7	4	2.5
Spag6	Sperm associated antigen 6	21.7	7.8	1.2
Gnb3	Guanine nucleotide binding protein (G protein), beta 3	7	8.1	1
Hsd3b4	Hydroxy-delta-5-steroid dehydrogenase, 3 β- and steroid delta-isomerase 4	6.9	7.1	1.1
Mal	Myelin and lymphocyte protein, T-cell differentiation protein	6.8	6.5	1.4
Dnajb13	DnaJ (Hsp40) related, subfamily B, member 13	7.3	4.8	1.3
Pou2af1	POU domain, class 2, associating factor 1	7.6	3.4	1
Tctex1d1	Tctex1 domain containing 1	5.5	4.5	1.1
Ccdc96	Coiled-coil domain containing 96	6.1	3.3	1.3
Kif9	Kinesin family member 9	6.2	2.8	1.3
Muc15	Mucin 15	5.4	2.9	1.9
Ecel1	Endothelin converting enzyme-like 1	4.3	3.7	1.5
Plscr2	Phospholipid scramblase 2	5.3	2.6	1.5
Lmod1	Leiomodin 1 (smooth muscle)	3.9	3.9	1.5
Cyp2s1	Cytochrome P450, family 2, subfamily s, polypeptide 1	3.2	4.6	1.9
Gnmt	Glycine N-methyltransferase	3.8	3.5	1.9
Scn11a	Sodium channel, voltage-gated, type XI, alpha	3	3.5	1.7
Myh7b	Myosin, heavy chain 7B, cardiac muscle, beta	2.1	2.1	1.4

Some seemingly interesting genes that were changed <two-fold in the cochlea were also included. [a)]Genes previously associated with inner ear development or deafness were excluded from the list, except for *Atoh1*.

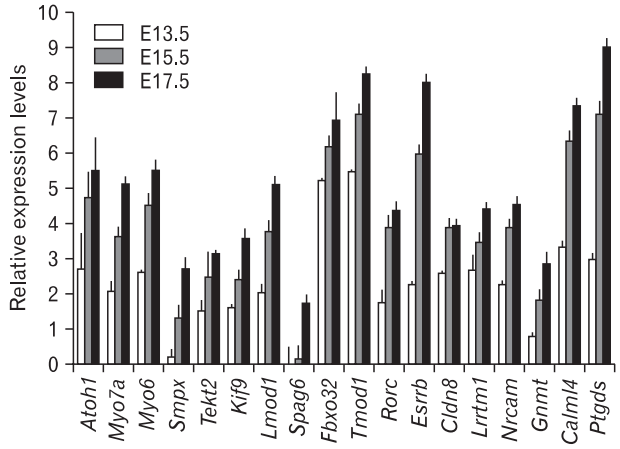

Fig. 2. Real-time polymerase chain reaction analysis of the selected genes. Total RNAs isolated from mouse cochlear tissues at E13.5, E15.5, and E17.5 subjected to quantitative real-time polymerase chain reaction. Expression levels of the genes examined gradually increased similar to that of *Atoh1*.

Esrrb), membrane proteins (*Cldn8*, *Lrrtm1*, *Nrcam*), and various other functional proteins (*Gnmt*, *Calml4*, *Ptgds*).

Real-time PCR results confirmed that genes associated with hair cell differentiation such as *Myo7a* and *Myo6* showed similar expression patterns with *Atoh1* during inner ear development (Fig. 2). Other genes tested also gradually increased similar to *Atoh1*, although relative expression levels of each gene were different from each other to some extent. These results demonstrate that the microarray results from the GEO dataset, GSE7536, closely represent gene expression patterns *in vivo* (Fig. 2).

Expression of *Smpx* and *Tekt2* in developing hair cells

We next examined whether the genes confirmed by real-time PCR are expressed specifically in the developing hair cells by *in situ* hybridization. As examples, we chose a few genes associated with cytoskeletal dynamics or architecture which would be directly related to differentiation or maturation of the hair cells.

Small muscle protein, X-linked (*Smpx*) showed the highest fold changes in both saccule and utricle during inner ear development (Table 3). *Smpx* expression was observed in the hair cells of the vestibular organs including utricle, saccule, and all three cristae at E15.5 (Fig. 3a-c) and continued to be present at least up to P5 (data not shown). In the cochlea, *Smpx* expression was barely detected at E15.5 (data not shown), probably because differentiation of the cochlear hair cells occurs later than that of the vestibular hair cells [14, 15]. Thus, we compared the cochlear expression patterns of *Smpx* in later stages with *Atho1* as well as *Myo15*, which is known to be essential for hair cell maturation [34]. At P1, *Atoh1* expression was detected in all cochlear turns from the base to apex (Fig. 3e, arrow), but *Myo15* expression was only detected up to the mid-apical turn (Fig. 3f, arrows), showing the wave of hair cell differentiation in a base to apex progression [14, 35]. At this stage (P1), *Smpx* expression was clearly found in the base and mid-base of the cochlear duct (Fig. 3g, arrows) and faintly in the mid-apex (Fig. 3g, arrowhead), indicating that the onset of *Smpx* expression is slightly later than that of *Myo15*. By P5, *Smpx* was strongly expressed in the hair cells of all cochlear turns (Fig. 3h, i).

Another gene found to be expressed in the hair cells is *Tekt2*, which encodes Tektin 2, a member of the Tektin family of proteins [21, 22]. Tektins are known as constitutive proteins of microtubules contributing to the stability and structural complexes of axonemal microtubules [22]. *Tekt2* expression was observed in the hair cells of all vestibular organs from E15.5 to P5 (Fig. 3d) (data not shown), whereas its expression in the cochlea was observed faintly in the basal and mid-basal turns at E15.5 and barely in the neonatal cochlea (data not shown). These expression patterns of *Tekt2* may be related to the transient appearance of the microtubule-based kinocilium in the cochlear hair cells, in contrast to the vestibular hair cells that retain kinocilium [36].

Discussion

To identify novel genes playing a role in hair cell differentiation, we re-analyzed the gene expression dataset GSE7536 [9] and searched for genes concordantly expressed with *Atoh1* during hair cell differentiation. As expected, the list includes many genes previously associated with inner ear development, hair cell differentiation or hereditary deafness (Table 2). For example, *Myo6*, *Tecta*, and *Tmprss3* have been associated with autosomal recessive or dominant deafness [16-18]. Also included were *Atp6v1b1* and *Slc26A4*, which are involved in maintenance of pH and ion composition in the inner ear fluids [19, 20]. Several other genes, such as transcription factors *Pou4f3* and *Gfi1*, extracellular matrix (tectorial membrane) components *Tecta* and *Tectb*, and components of the Notch signaling pathway *Hes5* and *Jagged 2* were also included.

Interestingly, some genes known to be important for

doi: 10.5115/acb.2011.44.1.69

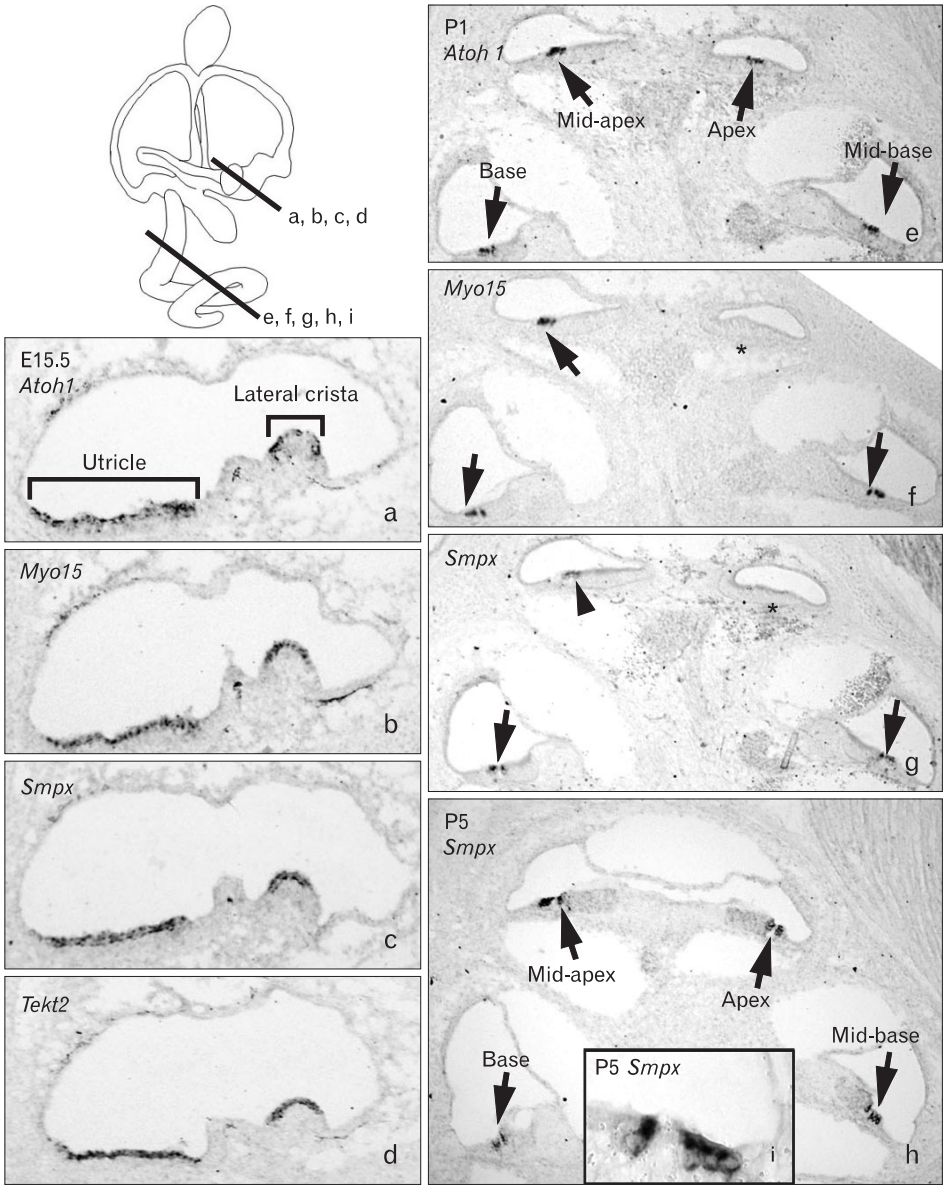

Fig. 3. Expression patterns of *Smpx* and *Tekt2* in the developing inner ear. Expression patterns of *Smpx* and *Tekt2* in the developing inner ear analyzed with *in situ* hybridization at E15.5 (a-d), P1 (e-g), and P5 (h, i). Lines in the inner ear diagram show the levels of sections. *Atoh1* (a, e) and *Myo15* (b, f), well known markers for hair cells, were used to locate the hair cells in the inner ear tissues. At E15.5, expressions of *Smpx* and *Tekt2* were found in the developing hair cells of the vestibular organs and continued up to P5 (a-d; data not shown), but were barely detected in the cochlea at this stage (data not shown). At P1, *Atoh1* expression was found in the hair cells of all cochlear turns (e, arrows) and *Myo15* expression was found in the base, mid-base, and mid-apex of the cochlea (f, arrows), but not yet in the apex (f, asterisk). *Smpx* expression was found in hair cells of basal and mid-basal turns (g, arrows) and weakly in the mid-apical turn (g, arrowhead). At P5, *Smpx* expression was detected in hair cells of all cochlear turns (h, i, arrows).

shaping the stereocilia of the hair cells such as *Myo7a*, *Cdh23*, and *Ush1c* were identified as being concordantly expressed with *Atoh1* only in the vestibular organs but not in the cochlea from the evaluated dataset. This suggests that since the dataset used in this study covers expression profiles only up to E15 [9], the genes included in the dataset do not accurately represent gene expression changes in later stages (i.e., later than E15) and may not include some important genes involved in maturation of the hair cells, especially in the cochlea. Thus, careful attention should be paid when comparing the genes identified from the vestibular organs and the cochlea.

From the list of genes concordantly expressed with *Atoh1*, we further excluded ones previously associated with inner ear as well as ones abundantly expressed in non-inner ear tissues to increase our chance to identify novel genes important for hair cell differentiation (Table 3). However, not all genes in the list were expressed in the hair cells but were in other parts of the inner ear such as cochleovestibular ganglion neurons, supporting cells, or mesenchyme-derived structures (data not shown). This is because hair cell differentiation dictated by Atoh1 occurs in harmony with development of the entire inner ear as a whole. Thus, specific expression domains in the developing inner ear should be carefully examined for each candidate.

As examples, we found that *Smpx* and *Tekt2* were specifically expressed in the hair cells of developing inner ear.

Smpx encodes an 85-amino acid protein, which is associated with actin and focal adhesion complexes and functions as a regulator of cytoskeletal dynamics [25, 27]. The onset of *Smpx* expression in the developing hair cells appeared to be slightly later than *Myo15* (Fig. 3), suggesting a possible role of *Smpx* in maturation or maintenance, rather than early specification, of hair cells. *Tekt2* encodes Tektin 2, also known as Tektin-t. It has been shown that *Tekt2* is expressed in sperm flagellum and the loss of *Tekt2* leads to a defect in the dynein arms of flagellar axonemal microtubules, resulting in immotile sperm and male infertility [21, 22, 37]. Interestingly, male infertility is often associated with hearing loss, especially in patients suffering from primary ciliary dyskinesia [38]. Since our observation of *Tekt2* expression in the developing hair cells suggests a possible role of *Tekt2* in hair cell differentiation, especially in kinocilium mainly composed of microtubules, it will be interesting to examine whether the loss of *Tekt2* causes hearing loss in addition to male infertility.

Taking advantage of gene expression profiles that are readily available in the GEO database [9], we identified several genes not previously associated with, yet possibly involved in hair cell differentiation. Further investigation of the genes identified in this study will enhance our understanding of how the hair cells normally develop and function.

Acknowledgements

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science, and Technology (KRF-2008-331-E00010 and 2009-0066470).

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