Introduction

The inner ear is derived from a simple patch of otic placode adjacent to the hind brain. After formation of the otic cup and vesicle, otic neuroblasts delaminate from the otic epithelium around E9.0 by initiating neurogenic gene-mediated programs, such as neurogenin1. These neural precursors generate otic neurons, which are also known as cochleovestibular ganglion (CVG) cells [1]. After CVG complexes are separated into the spiral and vestibular ganglion, developing spiral ganglion neurons (SGNs) promote neuronal outgrowth between E12.5 and E15.5, and regulate peripheral axon guidance to synapse with their target hair cells [2,3]. This process of auditory neurogenesis depends on well-organized complex signaling networks comprised of trophic factors such as phosphatidylinositol 3 kinase (PI3K)/Akt and insulin-like growth factor I (IGF-I), as well as morphogens, including the Wnt family, cell adhesion molecules and transcriptional regulators [4–8]. Several studies of knockout mice and in vitro cultures have provided evidence of their important roles in neural survival, neurite outgrowth and nerve innervations to target hair cells of the inner ear [6,9,10]. However, spatiotemporal gene expression and the complex molecular networks in neuronal development in the inner ear are not yet fully understood.

Phosphatase and tensin homolog (PTEN), a lipid phosphatase, is negatively regulated by PI3K signaling and contributes to cellular processes including proliferation, differentiation and migration [11–14]. Many studies have investigated the function of Pten loss in mice, which causes profound alterations in the regulation of cellular maintenance in a cell-type specific manner in various organs [15–17]. Recently, we characterized the phenotype of inner-ear-specific Pten conditional knockout (cKO) mice, which demonstrated abnormal phenotypes (e.g., ectopic hair cells in the cochlear sensory epithelium and neuronal defects) [15]. In particular, mouse inner ear lacking Pten had neuronal deficits such as disorganized nerve fibers with apoptosis of spiral ganglion. Thus, Pten is believed to be one of the functional regulators that maintain differentiation of SGNs during inner ear development.

Understanding of the signaling networks during inner ear development may provide molecular information regarding the pathways underlying the maintenance of sensory cells and neurons to prevent hearing impairment. Microarray analysis may provide information that allows prediction of novel signaling networks by analyzing the spatiotemporal pattern of gene expression during inner ear neurogenesis [18–20]. Thus, analysis of changes in gene expression profiles and signaling networks obtained from Pten mutants may identify potential novel targets and regulatory mechanisms associated with neuronal maintenance during inner ear development.
tissue dissection and RNA extraction

The generation and characterization of inner ear-specific \textit{Pten} cKO (\textit{Pten}^{loxP/loxP}; \textit{Pten}^{Cre+}) and wild-type (\textit{Pten}^{+/+} or \textit{Pten}^{+/loxP}) mice was described previously [15]. \textit{Pten} cKO and littermate wild-type mice were used on E14.5 (60 embryos from each group). The entire inner ear tissues including the cochlea and vestibule, as well as the surrounding otic capsule, were micro-dissected in sterile, chilled phosphate-buffered saline (PBS) under a stereomicroscope (Olympus SZ61, Olympus Corporation, Tokyo, Japan). Three independent pools of inner ear tissues from each group were homogenized with a tissue grinder (Kimble Chase, Vineland, NJ, USA). Total RNA from three independent pools of inner ears was extracted with TRIzol following the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). To eliminate DNA contamination, total RNA was treated with DNase I (Roche Applied Science, Mannheim, Germany) before use in the microarray analysis or real-time polymerase chain reaction (RT-PCR). The concentration and purity of extracted total RNA were measured using both the spectrophotometric method at 260 and 280 nm, and RNA electrophoresis.

Microarray data analysis

Gene expression profiles were generated using the Illumina MouseRef-8 version 2.0 Expression BeadChip Illumina, Inc., San Diego, CA, USA. Three biological replicates (three chips for wild-type samples and three chips for \textit{Pten} cKO samples) were performed for microarray hybridization experiments. Biotinylated cRNA was prepared from 550 ng total RNA using the Illumina TotalPrep RNA Amplification kit (Ambion, Austin, TX, USA). Following fragmentation, 750 ng of cRNA was hybridized to the Illumina MouseRef-8 version 2.0 Expression Beadchip according to the manufacturer’s instructions. Array chips were scanned using the Illumina Bead Array Reader Conofocal scanner. Microarray data were analyzed using Illumina GenomeStudio Gene expression Module (version 1.5.4) and deposited in NCBI Gene Expression Omnibus Database (GEO, http://www.ncbi.nlm.nih.gov/geo/; #GSE49562) in agreement with the MIAME requirements. The significance analysis microarrays (SAM) software was used with the false-discovery rate (FDR) set at 0 or 0.05. SAM (FDR = 0) allowed the identification of genes whose expression varied significantly between the wild-type and \textit{Pten} cKO groups [21]. Hierarchical clustering was carried out using the R software [22]. Ingenuity Pathway Analysis (IPA; Ingenuity Systems, http://www.ingenuity.com) tools were used to analyze possible functional relationships between selected differentially expressed genes (DEGs).

Quantitative reverse-transcription PCR

Quantitative real-time PCR (qRT-PCR) was performed to validate the microarray data. Each pooled RNA sample was converted to cDNA using random hexanucleotide primers with a High Capacity cDNA Reverse Transcription kit according to the manufacturer’s instructions (Applied Biosystems, Carlsbad, CA, USA). The list of PCR primer sequences for selected genes is provided in Table S1. 18S rRNA was used as an endogenous control for normalization. The PCR reaction was performed in quadruplicate using SYBR Green PCR Master Mix and an ABI 7500 machine with the version 2.0.6 software under the following conditions (Applied Biosystems): denaturation at 95°C for 10 min followed by 40 cycles of amplification (95°C for 15 sec, 60°C for 1 min). The relative expression level of each target gene in an experimental sample compared with the wild-type sample was analyzed using SDS Relative Quantification (RQ) Manager software as described by the manufacturer (Applied Biosystems). RQ levels were calculated using the comparative C<sub>T</sub> (2<sup>ΔΔC<sub>T</sub></sup>) method [23]. Relationships between the microarray data and qRT-PCR were analyzed using Pearson’s correlation coefficient (\(r\)) from GraphPad Prism (GraphPad Software, http://www.graphpad.com).

In Situ hybridization

For E14.5 embryos, pregnant mice were sacrificed by decapitation and fixed in 4% paraformaldehyde in PBS overnight at 4°C, dehydrated in 30% sucrose in PBS overnight at 4°C, placed in embedding medium (Tissue Tek OCT compound; Torrance, CA, USA), and stored at −80°C until use. Tissues were sectioned at 10-μm thickness for in situ hybridization, which was performed as described previously, with minor modifications [24]. At least three embryos were tested for each selected gene at E14.5. Sense RNA probes were also included as controls, which showed no signal in the inner ear. All primers for RNA probes for otoancorin (\textit{Otoa}), β-tectorin (\textit{Tectb}), parvalbumin (\textit{Pvalb}), \textit{Spp1}, and \textit{Rgs4} are listed in Table S1.

Results and Discussion

Identification of genes differentially expressed between wild-type and \textit{Pten} cKO mice at E14.5

Recently, we reported that \textit{Pten} cKO mice showed severe abnormalities in neuronal maintenance with increased production of hair cells during inner ear development [15]. To identify the changes caused by \textit{Pten} deficiency-induced regulation of genes in the developing inner ear, we analyzed DEGs within inner ears at E14.5. Using SAM analysis, we identified a total of 46 transcripts with an FDR = 0 that significantly distinguished the wild-type and \textit{Pten} cKO groups. Among the transcripts, 45 genes were upregulated and one was downregulated in \textit{Pten} cKO mice, and are listed in Table 1. While the patterns of gene expression between \textit{Pten} cKO and wild-type samples were highly similar according to pair-wise comparisons with correlation coefficients (data not shown), 46 DEGs were significantly selected, and their segregation was clearly shown by clustering analysis of a heat map (Fig. 1).

Validation of the microarray by quantitative RT-PCR

Among the DEGs, 16 candidate genes were selected to validate by qRT-PCR; the DEGs were chosen for either their fold changes (>1.5) and/or potential roles associated with inner ear development (Table 2). These genes included \textit{Tectb}, \textit{Otoa}, and \textit{Esrrb}, the mutations of which are associated with hearing loss [25–30]. In addition, peptide YY (\textit{Ppy}) and integrin beta 6 (\textit{Itgb6}) were
Table 1. Differentially expressed genes in wild-type and Pten cKO mice at E14.5.

| Target ID   | Gene symbol | Definition                                      | Fold change |
|-------------|-------------|-------------------------------------------------|-------------|
| ILMN_2443330 | Ttr         | transthyretin                                    | 3.94        |
| ILMN_2754364 | Ltf         | lactotransferrin                                  | 2.28        |
| ILMN_2710905 | S100a8      | S100 calcium binding protein A8 (calgranulin A)  | 2.00        |
| ILMN_1260585 | Stfa2       | stefin A2                                        | 1.89        |
| ILMN_1259546 | Pyy         | peptide YY                                       | 1.87        |
| ILMN_2803674 | S100a9      | S100 calcium binding protein A9 (calgranulin B)  | 1.85        |
| ILMN_2690603 | Spp1        | secreted phosphoprotein 1                        | 1.83        |
| ILMN_2634484 | Tectb       | tectorin beta                                    | 1.71        |
| ILMN_2988931 | Stfa1       | stefin A1                                        | 1.70        |
| ILMN_2735754 | Otoa        | otocancorin                                      | 1.67        |
| ILMN_2596522 | Mt1         | metallothionein 1                                | 1.67        |
| ILMN_2712075 | Lcn2        | lipocalin 2                                      | 1.65        |
| ILMN_2805372 | Itgb6       | integrin beta 6                                  | 1.64        |
| ILMN_2648669 | Gpmb        | glycoprotein (transmembrane) mmb                 | 1.64        |
| ILMN_1251894 | Dct         | dopachrome tautomerase                           | 1.57        |
| ILMN_1244081 | Rgs4        | regulator of G-protein signaling 4               | 1.56        |
| ILMN_1228497 | Esrb        | estrogen related receptor, beta                  | 1.56        |
| ILMN_1244169 | Sftpd       | surfactant associated protein D                  | 1.52        |
| ILMN_2933022 | Plekhb1     | plekstrin homology domain containing, family B   | 1.52        |
| ILMN_1226157 | Pik3r3      | phosphatidylinositol 3 kinase, regulatory subunit| 1.52        |
| ILMN_1244829 | Hap1        | huntingtin-associated protein 1                  | 1.51        |
| ILMN_2955694 | Spag1       | sperm associated antigen 1                       | 1.49        |
| ILMN_2995688 | EG433016    | predicted gene, EG433016                         | 1.46        |
| ILMN_1213954 | Sgk1        | serum/glucocorticoid regulated kinase 1          | 1.45        |
| ILMN_2769777 | Msc         | musculin                                         | 1.45        |
| ILMN_2629112 | Ash3l       | N-acylphosphogine amidohydrolase 3-like          | 1.44        |
| ILMN_1258853 | Lgsf1       | immunoglobulin superfamily, member 1, transcript variant| 1.42 |
| ILMN_2768972 | Fam107a     | family with sequence similarity 107, member A    | 1.41        |
| ILMN_2826110 | Cat         | catalase                                         | 1.41        |
| ILMN_2625893 | Ces3        | carboxylesterase 3                               | 1.40        |
| ILMN_2766004 | Camp        | cathelicidin antimicrobial peptide               | 1.40        |
| ILMN_1229131 | Wfdc3       | WAP four-disulfide core domain 3                 | 1.40        |
| ILMN_2718589 | Fcna        | ficolin A                                        | 1.40        |
| ILMN_1220193 | Slc26a4     | solute carrier family 26, member 4               | 1.39        |
| ILMN_2941888 | Gna4l4      | gene model 414                                   | 1.39        |
| ILMN_2668093 | Rec8        | REC8 homolog (yeast)                             | 1.38        |
| ILMN_1254295 | Sox21       | SRY-box containing gene 21                       | 1.38        |
| ILMN_3091003 | Ms4a7       | membrane-spanning 4-domains, subfamily A, member7, transcript variant| 1.37 |
| ILMN_2667829 | Pkqc        | protein kinase C, theta                          | 1.37        |
| ILMN_2776034 | Gal         | galanin                                          | 1.37        |
| ILMN_2651582 | 9630031F12Rik| RIKEN cDNA 9630031F12 gene                       | 1.35        |
| ILMN_1229763 | Dmkn        | dermokine, transcript variant 2                  | 1.34        |
| ILMN_1236758 | Wfdc2       | WAP four-disulfide core domain 2                 | 1.33        |
| ILMN_2715840 | C1qc        | complement component 1, q subcomponent, C chain | 1.32        |
| ILMN_2593774 | 1190002H23Rik| RIKEN cDNA 1190002H23 gene                       | 1.31        |
| ILMN_1218223 | Pvalb       | parvalbumin                                      | 1.62        |

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Figure 1. Microarray analysis identifies novel Pten targets. Heat maps for relative gene expression of interest (FDR = 0) obtained from three microarrays comparing Pten cKO to wild-type embryos. Green and red indicate decreased and increased expression, respectively, in Pten cKO mice. doi:10.1371/journal.pone.0097544.g001
Table 2. Genes selected for validation of microarray data by qRT-PCR.

| Gene     | Accession #   | Microarray | qRT-PCR |
|----------|---------------|------------|---------|
| Ttr      | NM_013697.3   | 3.94       | 15.53   |
| Ltf      | NM_008522.3   | 2.28       | 5.40    |
| S100a8   | NM_013650.2   | 2.00       | 6.21    |
| Pyy      | NM_145435.1   | 1.87       | 4.52    |
| S100a9   | NM_009114.1   | 1.85       | 7.09    |
| Spp1     | NM_009263.1   | 1.83       | 3.62    |
| Tectb    | NM_009348.3   | 1.71       | 6.64    |
| Otoa     | NM_139310.1   | 1.67       | 3.02    |
| Mt1      | NM_013602.2   | 1.67       | 4.73    |
| Itgb6    | NM_021359.2   | 1.64       | 6.42    |
| Dct      | NM_010024.2   | 1.57       | 3.99    |
| Bsg4     | NM_009062.3   | 1.56       | 3.24    |
| Esrb     | NM_011934.3   | 1.56       | 4.43    |
| Pik3r3   | NM_181585.5   | 1.52       | 3.58    |
| Hap1     | NM_010404.2   | 1.51       | 2.58    |
| Pvalb    | NM_1218223    | −1.62     | 0.40    |

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identified; these have not been previously reported in the mammalian inner ear. For all analyzed upregulated genes in Pen cKO compared to wild-type mice, the average fold change from the qRT-PCR results showed a significant correlation of gene expression changes, as revealed by the microarray data (Pearson’s correlation coefficient, $r = 0.876$). This result indicates that changes in the expression of selected DEGs were validated by qRT-PCR while confirming the gene expression results obtained by microarray analysis.

In situ expression patterns for selected candidates

To confirm the changes in expression of DEGs in the inner ear, we performed in situ hybridization for the selected DEGs, i.e., Otoa, Tectb, Pvalb, Spp1, and Rgs4 (Figs. S1A–D). Higher expression of Otoa and Tectb was observed in the cochlea of Pen cKO mice than in the cochlea of wild-type mice (Fig. S1A–D). Many studies have reported that mutations in Otoa and Tectb cause hearing loss [25,26,28–30]. Inner ear-specific Otoa is reportedly expressed on the surface of the spiral limbus and greater epithelial ridge in the cochlea. Mutant mice lacking Otoa showed that otoancorin is required for the attachment of the tectorial membrane (TM) to the surface of the spiral limbus [28,29]. The TM is composed of collagen proteins, and other non-collagen proteins such as α-tectorin and β-tectorin, and all essential for auditory function. Tectb-null mutant mice develop deafness as well as mutation of Tecta [30,31]. Further functional characterization is needed to determine whether a Pen deficiency-induced upregulated pattern of Otoa and Tectb expression leads to abnormal function of the TM.

In particular, changed expression levels of several genes were detected in the Pen-deficient SGNs; i.e., Pvalb, Spp1, and Rgs4. We found that the levels of Pvalb, a neuronal marker [32], were downregulated (Fig. S1E, F). Reduced levels of Pvalb expression may be explained by the loss of Pvalb-expressing neurons in Pen-deficient mice. We observed increased levels of Spp1 (also known as osteopontin, Opa) and Rgs4 expression in Pen-deficient SGNs compared to the wild-type (Fig. 2). In the cochlea and vestibular dark cells, Spp1 may be responsible for regulation of ions in the inner ear fluid. The role of Spp1 in SGNs may be associated with regulation of nitric oxide production, which is considered to be associated with auditory neurotransmission in adenosine triphosphate (ATP)-induced Ca2+ signaling [33,34]. Functionally, several lines of evidence have shown that Spp1 may play a role in neurodegeneration [35,36]. Upregulation of Spp1 was detected in lesions or within the cerebral or spinal fluid in patients with neurodegenerative conditions such as Alzheimer’s and Parkinson’s diseases. Spp1-knockout mice showed reduced neurodegeneration induced by MPTP [37]. Following crush injury to the optic nerve, strongly expressed Spp1 by macrophages may have inhibitory effects on axon growth [38]. Therefore, inhibition of axon outgrowth described in Pen cKO mice (i.e., shortened length of spiral ganglion toward the modiolus) may be at least partly explained by the dysregulation of Spp1 expression in SGNs.

Inhibitory regulators of G protein signaling 4 (RGS4), a schizophrenia susceptibility gene, is one of the RGS that includes the Gαi/o and Gαq families and is required for modulation of neurotransmission in the nervous system [39,40]. In mice, the expression of Rgs4 is observed in peripheral and central neuronal precursors [41,42]. In the chicken spinal cord, Rgs4 has been suggested to play a role in neuronal differentiation in cooperation with paired-like homeodomain protein PHOX2b and the basic helix-loop-helix protein MASH1 [41]. Thus, our data suggest that the increased expression of Rgs4 in the Pen-deficient SGNs compared to wild-type mice may play a role in neurogenesis.

Network analysis

To examine signaling networks during neuronal maintenance in the Pen-deficient inner ear, networks were subjected to IPA analysis with 82 DEGs (FDR $<0.05$) (Fig. 3). IPA analysis identified significant biological functions, including auditory disease, cell death and survival, and cellular movement (data not shown). Auditory diseases included Otoa, Tectb, estrogen-related
levels of Spp1 directly induces migration of human lung cancer cells (A549 cells) through activation of Rgs4 [48,49]. Although approximately 50% of neuroblasts after focal cerebral ischemia [46]. Furthermore, produced by macrophages and microglia induces lateral migration of SPP1 have been implicated in cellular migration; i.e., SPP1 are required to elucidate the mechanism by which altered expression induces disturbance of neuronal migration through Akt signaling in SGNs. Consistent with the microarray results, expression of the Rgs4-Akt signaling pathway in the developing SGNs is not fully understood, we suggest that Rgs4-Akt-mediated signaling networks may be associated with neuronal defects in the Pten-deficient SGNs (e.g., abnormal path-finding of neurites and irregularly gathered radial bundles).

Finally, IPA analysis revealed two core gene (Spp1; red line and Rgs4; blue line)-mediated networks in SGNs of the Pten-deficient inner ear (Fig. 3). These networks were also associated with the axonal guidance signaling pathway, which includes several mediators, such as G protein, frizzled homolog 6 (Drosophila Fzd6), protein kinase C (Pkc), Akt, PI3K, Erk1/2, Fak, and Pck theta (Pckq). Therefore, we suggest that partially modulated functions of the axonal guidance signaling pathway are involved in axonal development in Pten cKO mice [50–53].

Conclusions

In this study, we investigated profiles of significantly differentially expressed transcripts and their respective networks associated with Pten deficiency in the developing inner ear at E14.5. We suggest the presence of core signaling networks mediated by upregulated expression of Spp1 and Rgs4, which also include several key factors associated with apoptosis, cellular movement, and axon guidance. This may be explained in terms of phenotypic defects implicated in neuronal differentiation of Pten-deficient SGNs during inner ear development (e.g., neuronal apoptosis,
shortened axon length, abnormal cell movement, and irregular neurite path-finding of SGNs). Our gene expression profiles will facilitate understanding of the neuronal maintenance in developing spiral ganglion. However, the functional roles of these candidates should be examined in future studies.

Supporting Information

**Figure S1** Expression patterns of Otoa, Tectb, and Pvalb during inner ear development at E14.5. Expression levels of Otoa (A, B), Tectb (C, D), and Pvalb (E, F) were determined by in situ hybridization at E14.5. Otoa transcripts were identified on the surface of the spiral limbus and greater epithelial ridge in the cochlea (A, B). Expression domains of Tectb were observed in the sensory epithelium of the cochlea (C, D). The neuronal marker Pvalb was expressed in SGNs (E, F). Consistent with the microarray data, the expression levels of Otoa (B) and Tectb (D) were higher, and that of Pvalb (F) was lower, in Pten cKO mice than in wild-type mice. Scale bars: 100 μm.

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**Author Contributions**

Conceived and designed the experiments: HJK SKK. Performed the experiments: HJK JR HMW. Analyzed the data: HJK SSC MKS TP SKK. Contributed reagents/materials/analysis tools: MKS SCK MHP. Wrote the paper: HJK SKK.
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