Mutation in *Phex* Gene Predisposes BALB/c-*Phex*<sup>Hyp-Duk</sup>/<sup>Y</sup> Mice to Otitis Media

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**Abstract**

Genetic susceptibility underlying otitis media (OM) remains to be understood. We show in this study that mutation in *Phex* gene predisposes the BALB/c-*Phex*<sup>Hyp-Duk</sup>/<sup>Y</sup> (abbreviated Hyp-Duk/Y) mice to OM, which occurs at post-natal day 21 (P21) with an average penetrance of 73%. The OM was identified by effusion in the middle ear cavity and/or thickening of middle ear mucosa, and was characterised by increase in goblet cells, deformity of epithelial cilia and higher expression of proliferating cell nuclear antigen (PCNA) in cells of the middle ear mucosae. Moreover, the transcription levels of Tlr2, Tlr4, Nfkb1, Ccl4, Il1b and Tnfα in the ears of the Hyp-Duk/Y mice at P35 were significantly upregulated, compared to those of the controls. Higher expression levels of TLR2, TLR4, NF-κB and TNF-α in the middle ears were demonstrated by immunohistochemistry (IHC). However, the OM in the mice was not prevented by azithromycin administration from gestational day 18 to P35. Further study showed that, in contrast to the low mRNA levels of *Phex* gene in the ears of the Hyp-Duk/Y mice, the mRNA level of Fgfl3 was significantly elevated at P9, P14, P21 and P35. Meanwhile, mRNA levels of EP2 (PGF2 receptor), which expressed in the middle ear epithelia as demonstrated by IHC, were already increased at P14 even before the occurrence of OM, indicating that PG2E, an inflammatory mediator, is involved in the OM development in the mutants. Taking together, *Phex* mutation primarily up-regulates gene expression levels in FGF23 mediated pathways in the middle ears, resulting in cell proliferation and defence impairment at the mucosae and subsequently bacterial infection. The Hyp-Duk/Y mouse is a new genetic mouse model of OM.

**Materials and Methods**

**Mice and animal care**

The BALB/cAnBomUrd-*Phex*<sup>Hyp-Duk</sup> mice were originally housed in The Jackson Laboratory (Bar Harbor, ME, USA) research facilities and were relocated to Case Western Reserve University and maintained through sibling inbreeding. Mainly male mice, mutants (*Hyp-Duk*/<sup>Y</sup>, n = 122) and controls (+/<sup>Y</sup>, n = 109) ranging in age from post-natal day 9 (P9) to 20 weeks, were included in this study. All mice were genotyped by PCR using conditions and primers described previously [6]. Care and use of animals were approved by the Institutional Animal Care...
Sequencing Phex cDNA from ears of Hyp-Duk/Y mice

Four Hyp-Duk/+ mice and 4+/Y littermates were randomly chosen. Mice were euthanised under anaesthesia (avertin 5 mg/10 g), and ears were quickly removed. Total RNA (DNA-free) was prepared using Pure-Link™ Micro-to-Midi Total RNA Purification System (Invitrogen, Carlsbad, CA). cDNA was synthesised using SuperScript™ First-Strand Synthesis System (Invitrogen). RT-PCR for Phex used primer pairs spanning the deletion sites for both transcripts: PhexF (TTCCCCAAAAGAAAGGCTTC, in exon 16) and PhexR1 (ATGGATGCAGGGACAAAAAG, in exon 12) and PhexR2 (TTCCCCAAAAGAAAGGCTTC, in exon 15) or PhexR02 (TTCCCCAAAAGAAAGGCTTC, in exon 16). Amplification conditions were 94°C, 2 min; followed by 28 cycles of 94°C for 30 s, 60°C for 40 s, and 72°C for 50 s; extension for 5 min, 72°C. Ten μl of PCR products were visualised on 2% agarose. Gapdh was amplified as positive control [7]. PCR products were sequenced on ABI Applied Biosystems 3730 DNA Analyzer (Life Technologies Corp., Carlsbad, CA).

Haematoxylin/Eosin staining

Fifty-five mutant and 43 control mice from 2 to 20 weeks of age were anaesthetised and perfused through the heart left ventricle with PBS followed by Bouin’s (haematoxylin/eosin or H&E staining) or 4% paraformaldehyde (for all others) fixative. Bullae were dissected, perfused with fixative, immersed in same for 14 days, embedded in paraffin, serially sectioned at 7 μm thickness, and stained with H&E for light microscopy (Leica DM4500 B, Leica Microsystems, Wetzlar, Germany) at 5 to 63× final magnification.

Scanning electronic microscopy (SEM)

Bullae were isolated from skulls of 4 control (+/Y) and 4 mutant (Hyp-Duk/Y) mice at P21 or P130. Samples were placed in 2.5% glutaraldehyde in cacodylic acid in 0.1 M phosphate buffer (pH 7.2) for 36 hours. After decalcification in 10% EDTA for 4–7 days, middle ear cavities were dissected from bullae and dehydrated in serial solutions of ethanol (60%, 70%, 80%, 95%, 100%) for 15 minutes each, with a second soak in 100% ethanol. Each middle ear cavity was subjected to CO2 critical point drying, sputter-coated with 60/40 gold-palladium, and viewed under high resolution scanning electron microscopy (Hitachi S-4500, Japan).

Table 1. Sequence of RT-PCR primers.

| ID     | Sequence                          | Product Size (bp) | Reference |
|--------|-----------------------------------|-------------------|-----------|
| PhexF1 | 5′-AAGCTGGACCAAGCAACACT-3′ (in exon 5) |                   |           |
| PhexR1 | 5′-TCCGTTCTACTGTTGGAATC-3′ (in exon 8) | 213              |           |
| Phex2F | 5′-AGGCCATACACTTACCAACA-3′ (in exon 20) |                   |           |
| Phex2R | 5′-TACCAGATGCGAGGAGGTACCC-3′ (in exon 22) | 228              |           |
| Fgf23F | 5′-ACCTGTGGGAGAAGACTC-3′              |                   |           |
| Fgf23R | 5′-GTGGGGAACAGTTGAAA-3′               | 143              |           |
| Ep2F   | 5′-TCCGCAGAGGAAGGAGAGAG-3′           |                   |           |
| Ep2R   | 5′-GGGGCTAAAGTAGGGCAA-3′              | 224              |           |
| Tlr4F  | 5′-CAGTTGGTCAGGAACAGAAGC-3′            |                   |           |
| Tlr4R  | 5′-GACAATGAAGATGATGAGAGGC-3′           | 540              | [38]      |

Figure 1. Open reading frame (ORF) shift in Phex gene of Hyp-Duk/+ mice. (A) 2% agarose gel electrophoresis of RT-PCR products from ear tissue of mutants and controls. Gapdh (upper panel) was amplified as a control for cDNA quality to allow comparison of expression level in the mutant and control mice. Middle (labeled exons 12–15) and lower (labeled exons 12–16) panels show the RT-PCR expression level in the mutant and control mice. Middle (labeled exons 12–15) and lower (labeled exons 12–16) panels show the RT-PCR products using primers spanning exons 13 and 14 in two control (+/Y, lanes 1 and 2) and two mutant (Hyp-Duk/Y, lanes 3 and 4) samples. RT-PCR products from control mice were 252 bp and 319 bp, respectively, in the middle and lower panels; whereas, those from the mutant were 70 bp and 137 bp. Only a single band from each sample was amplified. M indicates 100 bp DNA ladders. (B) Schematic diagramme of the sequence of exons 12–16 from the wild-type mouse Phex gene (+/Y, upper panel) and the Hyp-Duk/Y mutant gene (lower panel). Exons 13 and 14 were missing in the Hyp-Duk/ mouse compared with those of the control, leading to the ORF shift that occurs after aa 468 (underlined) in exon 15 and the resultant downstream stop codon at aa 520 (underlined).

doi:10.1371/journal.pone.0043010.g001
Mayer’s mucicarmine method

Goblet cells in the epithelium of the middle ear mucosae were identified by Mayer’s mucicarmine staining, following the protocol of Electron Microscopy Sciences (Hatfield, PA). Briefly, sections from both ears of mice at age 12 weeks (5 mutants and 4 controls, prepared as in the previous paragraph) were deparaffinised in xylene and hydrated with distilled water. Sections were first stained in Weigert’s haematoxylin, approximately 7 min and washed in running water for 10 min. Second, sections were stained in mucicarmine for 60 min at room temperature and rinsed quickly in distilled water. Thirdly, sections were counterstained in metanil yellow, rinsed in distilled water for 2–4 sec and dehydrated in 95% and 100% ethanol, sequentially. After clearing in xylene, sections were mounted with coverslips using permount for microscopic observation.

Proliferating cell nuclear antigen (PCNA) immunostaining

Specimens were prepared as described above (Mayer’s Mucicarmine Method). Immunohistochemistry was performed according to standard protocols. From 4 mutant and 4 control mice at

| Age      | No. of mice | No. of OM cases | Rates of OM |
|----------|-------------|-----------------|-------------|
| 2 weeks  | 7           | 0               | 0%          |
| 3 weeks  | 9           | 4               | 44%         |
| 5 weeks  | 11          | 9               | 82%         |
| 8 weeks  | 8           | 6               | 75%         |
| 12 weeks | 8           | 6               | 75%         |
| ≥16 weeks| 12          | 10              | 83%         |

doi:10.1371/journal.pone.0043010.t002

doi:10.1371/journal.pone.0043010.g002

doi:10.1371/journal.pone.0043010.g003
age 12 weeks, sections from both ears were deparaffinised for 15 minutes in xylene, put through two changes of 100% ethanol, and then immersed in 0.3% H2O2 in methanol for 30 min. After being washed in PBS, sections were placed in 0.2% Triton-X/PBS for 30 min. Sections were washed in PBS and blocked in 5% milk powder in PBS for 5 min. Primary antibodies (P8825, Sigma Biotechnology, 1:3000 dilution) were applied to the slides at 4°C overnight. Anti-goat secondary antibody was applied (Sigma Biotechnology, 1:500 dilution, incubated at room temperature for 1 h) and slides were DAB-stained (3, 3′-diaminobenzidine, Sigma) until brown cells were apparent. Reaction was quenched in PBS and sections were counterstained with haematoxylin. Slides were photographed using light microscopy (Leica, Wetzlar, Germany).

Bacterial identification and antibiotic prophylaxis

Four 5-week-old mice from each genotype (Hyp-Duk/Y and +/Y) were randomly chosen and anaesthetised. Mice were euthanised by CO2 asphyxiation. Under sterile conditions, bullae were removed; middle ears were isolated and washed with sterile PBS. One hundred μl of each PBS lavage were inoculated onto individual BBLTM TrypticaseTM Soy Agar plates with 5% sheep blood (TSA II, Fisher, Pittsburgh, PA) and plates were incubated at 37°C, 5% CO2 for 18 h. Colonies were sorted, counted, and further identified. *Staphylococcus* were identified by standard microbiologic features, with coagulase-negative *Staphylococcus* (CNS) differentiated from *S. aureus* by positive tube-coagulase test. Azithromycin (50 mg/kg/d) was therefore added to drinking water (final concentration of 0.25 g/l) [8] for PheX*Hyp-Duk*+/+ mice (n = 6, mated to +/Y mice) at gestational day 18 (G18) and drug treatment was maintained through lactation, after which the drug was given to the litters in drinking water. At P35, antibiotic-treated control (+/Y, n = 4) and mutant (Hyp-Duk/Y, n = 5) litters were sacrificed and the bullae of ears were isolated for H&E staining and microscopy, as described in histological preparation.

Semi-quantitative RT-PCR for evaluation of gene transcription levels in the ears

Four to five Hyp-Duk/Y mutant and 3–4+/Y wild-type male littersmates ranging in age from P9 to P35 were used for the experiments. Mouse treatment and total RNA preparation from the middle and inner ears were performed following the methods described previously and the mRNA levels of specific genes were evaluated by semi-quantitative RT-PCR [7]. Additional RT-PCR primers were listed in Table 1.

Fluorescence immunohistochemistry (IHC) assays in middle ears

For detection of PHEX, TLR2, TLR4, NF-κB and TNF-α protein expression in the middle ears of Hyp-Duk/Y mice, cryosections were used and the detail methods were described in the supplementary section. To localize EP2 expression in the middle ear, paraffin sections were used following the procedure described [7]. Goat anti-EP2 polyclonal antibody (sc-22195, 200 mg/ml, Aanta Cruz Biotechnology, INC.) was used at 1:200 dilution. Donkey anti-goat secondary antibody conjugated to Alexa Fluor-568 was used at 1:400.

Figure 4. SEM observation of the cilia in the mucociliary epithelia of the middle ears from mice at P21 and P150. Wild-type littermate control (+/Y) mice displayed a thick lawn of morphologically normal, evenly distributed cilia in the mucociliary epithelia of the middle ears at both time points (A, C). The cilia of the middle ear epithelia in the Hyp-Duk/Y mice (B, D) were morphologically thinner than that of the +/Y mice at P21 and P150 and were irregularly distributed as a consequence of “mucin-like” material (B) or patches of “effusion-like” material on the top of the cilia, as indicated by arrow heads. The results indicate that Hyp-Duk/Y mice have difficulty clearing the effusion from the middle ears. Images are representative of each group (n = 4) at each time point. Scale bars: 15 μm.

doi:10.1371/journal.pone.0043010.g004
were not significantly altered in the mutant mice. Error bars represent (n = 4) and control (dark bars). mRNA levels from genes, mainly in the TLR-mediated pathways (Table 2). However, only two of 43 control mice at ages 3 weeks and 20 weeks, respectively, presented with OM by histological examination. In OM mice, mucosae of the middle ears and Eustachian tubes showed general thickening with proliferation of epithelial cells and lamina propria connective tissue (Figure 2). The effusion was serous and contained variable numbers of inflammatory cells, mainly consisting of polymorphonuclear cells.

Figure 5. Upregulated mRNA levels of pro-inflammatory cytokines in the ears of Hyp-Duk/Y mice. mRNA accumulation levels from genes, mainly in the TLR-mediated pathways, were measured by semi-quantitative RT-PCR in the ear of Hyp-Duk/Y mutant (n = 4) and control (+/Y, n = 4) mice at P35. In contrast to the downregulated mRNA levels of Phex gene, transcription levels of genes in TLR2- and TLR4-mediated pathways (Nfkb1, Ikkg, Ccl4, Ccl2, Il1b and Tnfa) in the ears of Hyp-Duk/Y mice (dark bars) were significantly higher than levels in +/Y mice (light bars). The mRNA levels of Myd88 and Nod2 were not significantly altered in the mutant mice. Error bars represent the s.e. from the mean for each sample. (** P<0.01; * P<0.05).

doii:10.1371/journal.pone.0043010.g005

Statistical method

ANOVA test was used for statistical analysis. P<0.05 was considered to be significant.

Results

Exon deletion yields functionally null PHEX protein in Hyp-Duk/Y mouse ears

The RT-PCR products from 2 samples in each group were randomly chosen for agarose gel electrophoresis (Figure 1A), in which one single band from each sample was amplified. Sizes of Phex-specific RT-PCR products from Hyp-Duk/Y samples were smaller (70 bp and 137 bp) than products from control mice (252 bp and 319 bp) for both primer pairs. Sequencing of RT-PCR products confirmed that exons 13 and 14 were deleted from the Phex cDNA, leading to an open reading frame shift after aa 468 and introducing a stop codon at aa 520 (Figure 1B). The deletion in the Phex gene does not alter splicing of mRNA from the mutant gene. This is the first time the Hyp-Duk/Y mutation has been sequenced at cDNA level to determine the specific nature of the mutation in the ears.

PHEX protein expression in the middle ears of Hyp-Duk/Y and control mice (P35) was determined by immunostaining with two antibodies (PHEX-H-176 and PHEX-C-13) that recognise peptides flanking the altered sequence (aa 468 to 520) in the PHEX protein from aa positions 151 to 326 (H-176) and 530 to 560 (C-13), respectively. The antibody H-176 gave strong signals, mainly in middle ear mucosal cells, in both mutant and control mice. However, C-13 only gave signals in middle ears (mainly epithelial cells in mucosae and osteocytes in the bony wall) of control mice, and failed to stain any proteins in corresponding structures in mutant mice, indicating absence of the C-terminal portion of the PHEX protein, resulting from truncation in the mutant (Figure S1).

Histopathologic evidence of inflammation in the middle ears of Hyp-Duk/Y mice

Ear sections from 55 Hyp-Duk/Y mice and 43 age-matched littermate control (+/Y) mice from 2 to 20 weeks of age were examined for histopathology. Overall, OM with effusion in the middle ear space and/ or thickened mucosae occurred in at least one ear of Hyp-Duk/Y mice at (or after) 3 weeks of age with an average incidence rate of 73% (35/48, Table 2). However, only two of 43 control mice at ages 3 weeks and 20 weeks, respectively, presented with OM by histological examination. In OM mice, mucosae of the middle ears and Eustachian tubes showed general thickening with proliferation of epithelial cells and lamina propria connective tissue (Figure 2). The effusion was serous and contained variable numbers of inflammatory cells, mainly consisting of polymorphonuclear cells.

Increase in goblet cells and mucosal cell proliferation in the middle ears of Hyp-Duk/Y mice

Sections of each ear from mutant (Hyp-Duk/Y) mice and control (+/Y) mice at age 12 weeks were stained by the Mayer’s mucicarmine method to visualise goblet cells (Figure 3A, 3B), which have been demonstrated at high density in secretory OM [9]. In control mice, goblet cells were rare, but in Hyp-Duk/Y mutants, goblet cells were found scattered among other cells in the middle ear cavity epithelium, especially in cuboidal ciliated epithelium. PCNA is a reliable marker of cells undergoing active proliferation [10]. Strong PCNA staining was detected in the middle ear mucosae of Hyp-Duk/Y mutant mice, shown in Figure 3D, while epithelial cells of the middle ears in control mice were virtually PCNA negative (Figure 3C).

Irregular cilia on epithelial cells in middle ears of Hyp-Duk/Y mice

SEM images of cilia on epithelial cells in the middle ears at P21 and P150 are shown (Figure 4). The control (+/Y) mice displayed morphologically normal, evenly distributed cilia in the mucociliary epithelia of the middle ears (A, C). The cilia of middle ear epithelia in the Hyp-Duk/Y mice (B, D) were morphologically thinner than those of the +/Y mice and were irregularly distributed at both time points. Mutation in the Phex gene impairs ciliary development and clearance function.

Uregulation of pro-inflammatory cytokine expression in the middle ears of Hyp-Duk/Y mice

Inflammation caused by bacterial infection is mediated by TLR signaling pathways and is characterised by high level production of pro-inflammatory cytokines which contribute to otitis media pathogenesis [11]. Therefore, transcription levels of Tbl2, Tir4 and genes (Ccl4, Ccl2, Il1b and Tnfa) encoding pro-inflammatory cytokines were examined by semi-quantitative RT-PCR in mutant (Hyp-Duk/Y) and control (+/Y) mice. At P35, mRNA levels of the above genes were significantly upregulated (P<0.05 or 0.01) in the ears of mutant mice compared to those of controls (Figure 5). Expression of TLR2, TLR4, NF-kB and TNF-α was also...
examined by in situ fluorescent IHC staining of sections from middle ears of +/Y and Hyp-Duk/Y mice at age P35 (Figure S2). The four proteins were mainly expressed in the middle ear mucosal layers of both mutant and control mice, whereas the staining intensity of antibodies for each of the four proteins was much stronger in middle ear mucosae of Hyp-Duk/Y mice than that in the middle ears of +/Y mice. The results indicate that TLR-mediated signaling pathways were activated in the middle ear immune response in OM.

**Pathogenic bacteria isolation from middle ears of the Hyp-Duk/Y mice**

To investigate the causative agent of OM infection in Hyp-Duk/Y mice, we cultured middle ear lavage samples from 5-week-old mutant and control mice. Under microaerophilic conditions, the only type of bacteria isolated from middle ears of mutant (Hyp-Duk/Y) mice were coagulase-negative Staphylococci (CNS), a Gram-positive bacterium which has been frequently isolated from middle ear effusions in human otitis media patients [12,13]. Though CNS were also isolated from wild-type (+/Y) littermates, the mean
Phex gene mutation in Hyp-Duk/Y mice

↓

Function null Phex protein in the ears

↓

Transcription of FgF23 gene up-regulated

↑

Increase in FgF23/PGE2 levels

↑

Cell proliferation in middle ears

↑

Impaired epithelial cilia defense

↑

Abnormal in Eustachian tube

↑

Effusion and epithelial cell proliferation

↑

Colonization and infection of bacteria

↑

Cytokines (IL-1β, TNF-α) and chemokine (PGE2)

↑

Activation of TLR-mediated pathways

Figure 7. Schematic diagramme showing the hypothesised mechanism of OM development in the Hyp-Duk/Y mice. Possible elements or pathways involved in the OM development are illustrated. Solid lines show pathways for which there is direct evidence. Dashed lines are proposed links.

doi:10.1371/journal.pone.0043010.g007

Our further study implicates PHEx-FGF23 pathways in OM pathology of Hyp-Duk/Y mice. PHEx has been identified as a causative agent of XLH and autosomal dominant hypophosphatemic rickets/osteomalacia (ADHR) [3,20]. The native PHEx protein was reported to inactivate a phosphaturic factor, which is considered to be fibroblast growth factor 23 (FGF23) [21]. Circulatory FGF23 is elevated in most patients with XLH [22], and high level of intact FGF23 despite hypophosphatemia is a clinical indicator in XLH diagnosis [23]. While PHEx is expressed primarily in cells of bone lineage, the main protein effects on renal phosphate wasting and impaired vitamin D metabolism occur in the kidney. FGF23 regulates urinary phosphate excretion to maintain systemic phosphate homeostasis. The exact mode of action of the phosphaturic effects of FGF23 is not fully understood and is an intense area of research [24]. PHEx and the sibling protein DMP1 (dentin matrix protein 1) regulate growth factor FGF23 expression in osteocytes by controlling the pathway involving FGF receptor (FGFR) signaling [25]. In cultured kidney cells, FGF23 inhibits phosphate transport [26], probably via an FGF receptor 3c which is linked to the MAPK pathway [27]. FGF23 and alpha-klotho, a transmembrane protein, together stimulate osteoblast proliferation [28]; they also increased phosphorylation of ERK1/2, P38, JNK, AKT, IκB and GSK-3β and BrdU incorporation in renal and intestinal epithelial cell lines from human [29]. Activation of FGF receptor and Wnt/β-catenin is a newly identified molecular pathway stimulated by inactivation of PHEX in osteoblasts from Hyp mice [30]. The above studies suggest that FGF23, by interacting with FGF receptors, can initiate downstream signaling events by multiple pathways.

FGF23 increases urinary and renal production of PGE2, which inhibits proximal tubule phosphate transport in Hyp mice [14]. PGE2 in different tissues may have different functions. We therefore tested whether increased FGF23 can induce production of PGE2 in the ears of Hyp-Duk/Y mice. Direct determination of PGE2 expression in mouse ears is not feasible; thus, transcription of PGE2 receptor (EP2) was measured. mRNA levels of Ep2 were significantly upregulated in the ears of Hyp-Duk/Y mice not only at P21 and P35, but also at P14 (before the OM occurs), indicating that PGE2 would likely show a correspondent increase in the time course with OM development. As upregulation of Fgf23 mRNA levels in the ears of Hyp-Duk/Y mice was correlated with the levels of EP2 from P14 to P35, we postulate that OM in Hyp-Duk/Y mice is primarily mediated by FGF23/PGE2 pathways.

PGE2 can be released from various cell types (such as macrophages and endothelial cells) and mediates inflammation through its potent vascular permeabilization activity [31]. High concentration of PGE2 in middle ear effusions has been documented and correlated with the degree of inflammation during OM in humans [32]. PGE2 increases transepithelial ion transport rate by increasing intracellular cAMP content in a middle ear cell line (MESV); such a mechanism could diminish the periciliary sol layer to impair mucociliary clearance in OM [33]. In addition, PGE2 can cause production of TNF and interleukin IL-1β, which direct neutrophil trafficking into the middle ear cavity [34]. These cytokines can exacerbate OM in Hyp-Duk/Y mice, as in humans, by inflammation and tissue destruction [33].

Bacterial infection is a secondary factor which augments middle ear pathology. Coagulase-negative Staphylococcus (CNS) is currently a major cause of nosocomial and health-care related infections [36]. This bacterium was isolated from the middle ears of an OM mouse model of Down syndrome in our previous study [37]. The higher
CNS density in the middle ears of Hyp-Duk/Y mice may trigger, at least partially, elevation of mRNA levels of pro-inflammatory cytokines in TLR2-mediated pathway. The increased TLR4 levels in the ears of Hyp-Duk/Y mice were probably caused by infection of Gram-negative bacteria that were unable to grow at the culture conditions. As application of azithromycin from G18 to P35 failed to prevent OM in Hyp-Duk/Y mice, we concluded that bacterial infection was secondary to the primary pathological alteration mediated by FGF23/PGE2 in middle ears.

In summary, Phex gene mutation induced primary structural and functional middle ear abnormalities and, with secondary bacterial infection, resulted in OM in the Hyp-Duk/Y mice (Figure 7). Chronic inflammation could damage the fragile structures of the middle ear thus causing conductive and even sensory hearing loss. The Hyp-Duk/Y mouse is therefore a promising model for studying the molecular mechanism of OM and for testing and targeting drugs to prevent OM.

Supporting Information

Figure S1 Deletion of exons 13–14 leading to a functionally null PHEX protein in the ears of Hyp-Duk/Y mice. Representative middle ear sections from control and Hyp-Duk/Y mice stained with anti-PHEX-H-176 and anti-PHEX-C-13 antibodies detect regions of the PHEX protein preceding and following, respectively, the deletion and consequent stop codon (revealed by Alexa Fluor 488, green). The antibody H-176 (indicated as PHEX1 above panels) revealed expression of the N-terminal region of the PHEX protein in the middle ears of both the mutant and control mice, with stronger staining in the epithelial cells of the middle ear mucosae as indicated by arrows (A and B or C and D). However, C-13 (indicated as PHEX2 above panels) showed strong positive staining in mucosal epithelial cells (indicated by the arrow at the bottom of E or F) and osteocytes (indicated by arrow in the middle of E or F) in the bony structures of the middle ears of +/+ mice, and failed to stain the corresponding structures in Hyp-Duk/Y mutant mice, indicating loss of the C-terminal portion of the PHEX protein, downstream of exon 16 (G). Panels B, D, F and H are the merged images obtained by PI (Propidium iodide, red) and antibody (green) staining. Scale bars: 20 μm. (TIF)

Figure S2 Representative IHC staining the sections of middle ears from +/+ and Hyp-Duk/Y mice at the age of 5 weeks. (A to D) Anti-TLR2-FITC staining of middle ears from mice of +/+ (A), Hyp-Duk/Y (B), Hyp-Duk/Y with primary antibody omission (C) and TLR2R−/− (D). (E, F) Anti-TLR4-FITC staining of the middle ears from mice of +/+ (E) and Hyp-Duk/Y (F); (G, H) are enlarged area from (F) to show TLR4 expression in PMNs in the middle ear indicated by arrows. (I and J, K and L) Anti-NFκB-FITC and anti-TNFα-FITC staining of middle ear from +/+ and Hyp-Duk/Y mice, respectively. Overall, TLR2, TLR4, NF-kB and TNF-α were expressed in the middle ear mucosae of both control and mutant mice and the staining intensity of the 4 antibodies was much stronger in mucosae of the Hyp-Duk/Y mice than in the middle ears of the +/+ mice as indicated by the arrows. Scale bars: 100 μm in (A–F, I–J) and 20 μm in (G, H). (TIF)

Materials and Methods S1

(DOC)

Acknowledgments

We thank Dr. Michael Jacobs in the Department of Pathology at Case Western Reserve University for his help with the bacterial identification. We thank Christopher M. McCarty and Leah Rae Donahue in The Jackson Laboratory for technical support and Dr. Cindy Benedict-Alderfer for editing this manuscript.

Author Contributions

Conceived and designed the experiments: QYZ FH. Performed the experiments: FH HY PL JZ CT HL QYZ. Analyzed the data: FH HY PL JZ CT HL QYZ. Contributed reagents/materials/analysis tools: FH QYZ. Performed the bacterial identification: AB. Contributed reagents/materials/analysis tools: AB AB. Wrote the paper: FH JZ QYZ.

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