Keratinocyte Growth Factor Stimulates Growth of p75⁺ Neural Crest Lineage Cells During Middle Ear Cholesteatoma Formation in Mice

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During development, cranial neural crest (NC) cells display a striking transition from collective to single-cell migration and undergo a mesenchymal-to-epithelial transformation to form a part of the middle ear epithelial cells (MEECs). While MEECs derived from NC are known to control homeostasis of the epithelium and repair from otitis media, paracrine action of keratinocyte growth factor (KGF) promotes the growth of MEECs and induces middle ear cholesteatoma (cholesteatoma). The animal model of cholesteatoma was previously established by transfecting a human KGF-expression vector. Herein, KGF-inducing cholesteatoma was studied in Wnt1-Cre/Floxed-enhanced green fluorescent protein (EGFP) mice that conditionally express EGFP in the NC lineages. The cytokeratin 14 expressing NC lineage expanded into the middle ear and formed cholesteatoma. Moreover, the green fluorescent protein expressing NC lineages comprising the cholesteatoma tissue expressed p75, an NC marker, with high proliferative activity. Similarly, a large number of p75-positive cells were observed in human cholesteatoma tissues. Injections of the immunotoxin murine p75-saporin induced depletion of the p75-positive NC lineages, resulting in the reduction of cholesteatoma in vivo. The p75 knockout in the MEECs had low proliferative activity with or without KGF protein in vitro. Controlling p75 signaling may reduce the proliferation of NC lineages and may represent a new therapeutic target for cholesteatoma.


Approximately 15% of American adults (37.5 million) aged 18 and over report some trouble hearing (https://www.nidcd.nih.gov/health/statistics/quick-statistics-hearing, last updated March 25, 2021, last accessed August 18, 2022). Chronic otitis media is one of the risk factors in childhood and adult hearing loss.¹ One-fourth (about 5 million) of the adults with trouble hearing have cholesteatomatous chronic otitis media, so-called middle ear cholesteatoma (cholesteatoma). The annual incidence of cholesteatoma is reported at 3 per 100,000 children and 9.2 per 100,000 adults.² Cholesteatoma is a pathologic condition associated with otitis media; it is a gradually expanding destructive epithelial tumor within the middle ear and the temporal bone.³ The understanding of the molecular mechanism underlying the pathogenesis of cholesteatoma is limited. However, active proliferation of epithelial cells under inflammation is known to generate irreversible change.⁴ In a recent study, an increased number of stem/progenitor cells were detected in the epithelial layer of cholesteatoma tissue in both the animal model and human samples.⁵ However, the origin of cholesteatoma’s stem cells is not fully understood. Current therapies for inflammatory diseases, based largely on targeting the growth factor pathway, show limited clinical benefits, thus necessitating the discovery of alternative targets. To identify the cell types that comprise cholesteatoma is needed for establishing a new therapy.

Neural crest (NC) cells are multipotent, migratory cells unique to vertebrates and are generated transiently during embryonic development.⁶ Of these, cranial NC cells are


Disclosures: None declared.
capable of differentiating into bone and cartilage tissues and form most craniofacial structures. During mouse middle ear development, NC cells also form the structures of the ossicles and epithelium of the middle ear cavity. In 2013, Thompson and Tucker showed that the epithelium lining the mammalian middle ear cavity is roughly half NC and half endodermal in mouse genesis. They concluded that the NC lineage epithelium controls the homeostasis of the middle ear mucosa and plays a role in repairing otitis media. However, it is difficult to identify these NC lineage epithelial cells in human cholesteatoma specimens because of the lack of specific cell markers for NC lineage cells in adults.

In previous studies, the expression of inflammatory cytokines was analyzed in cholesteatoma tissues. Keratocyte growth factor (KGF) is a mesenchymal cell–derived paracrine growth factor that specifically stimulates epithelial cell growth, thought to be secreted primarily from stromal fibroblasts and binds to the KGF receptor (KGFR), which is present on the membrane of epithelial cells. KGF is associated with the recurrence of human cholesteatoma. An animal model of cholesteatoma was previously established by human KGF (hKGF) expression vector transfection. The objectives of this study were to identify the role of NC lineage epithelial cells during cholesteatoma formation and to detect specific cell markers to identify these NC lineage epithelial cells in human cholesteatoma tissues. Herein, mouse genetics was used to determine the NC lineages at the origin of cholesteatoma. Using Wnt1-Cre/Flxed-enhanced green fluorescent protein (EGFP) mice (Wnt1-Cre/EGFP mice) conditionally expressing EGFP in the NC lineages, hKGF cDNA was transfected in the ear skin epidermis and cholesteatoma was induced. Next, RT-PCR analysis of the NC markers and double-immunofluorescence staining of the green fluorescent protein (GFP) and NC markers were performed in the cholesteatoma specimens of this mouse model. A large number of cytokeratin (CK) 14–positive NC lineages were detected in the KGF-inducing cholesteatoma in vivo, and almost all of them expressed p75, an NC marker. Similarly, a large number of p75-positive cells were also detected in the section of human cholesteatoma tissue.

Recently, murine p75-saporin (mu-p75-SAP), a selective immunotoxin of nerve growth factor receptor, was used to eliminate cells expressing p75 neurotrophin receptor in a mouse model. The current study, injecting mu-p75-SAP into the cholesteatoma tissue in vivo eliminated p75-positive NC cells and diminished cholesteatoma. NC cells expressed p75 protein and had a high in vitro proliferative activity with KGF protein administration. The p75 knockout (KO) NC cells had a low proliferative activity with or without KGF protein administration in vitro. These studies demonstrate that NC lineages are the cellular origin of cholesteatoma and that p75 transcription under KGF is required for the development of cholesteatoma. Controlling p75 signaling may reduce the proliferation of NC cells and may represent a new therapeutic target for cholesteatoma.

Materials and Methods

Animals

Wnt1-Cre/EGFP mice (aged 3 to 6 weeks; male; n = 22; body weight, 25 to 37 g) with normal ears were used in this study. Transgenic Wnt1-Cre mice expressing Cre recombinase under control of the Wnt1 promoter/enhancer [B6.Cg-Tg (Wnt1-Cre) 11Rth Tg (Wnt1-GAL4) 11Rth] were mated with indicator mice (CAG-CAT-loxP/loxP-EGFP) to obtain the Wnt1-Cre/EGFP mice. The Wnt1-Cre/EGFP mice expressed EGFP in all of the NC cells and their derivatives. When a vaginal plug appeared, the day was designated 0.5 days post-coitum. Wnt1-Cre/EGFP embryos were selected on the basis of EGFP expression. Adult Wnt1-Cre/EGFP mice were selected for PCR-based genotyping with primers for the Cre and CAT genes. The embryos used for the early developmental stage (E10.0).

KGF-Inducing Cholesteatoma of the Wnt1-Cre/EGFP Mouse

All experiments were conducted according to the principles and procedures outlined in the guidelines for animal experimentation of Jikei University (Tokyo, Japan) with the approval of the Institutional Animal Care and Use Committee (numbers 2015-139C6, 2018-055C1, D2020-059, and D2022-018). The 3X Flag hKGF cDNA expression vector for the cording region was kindly provided by Dr. Jeffrey Rubin (National Cancer Institute, Bethesda, MD). The p3X Flag–CMV14 vector was purchased from Sigma Chemical Co. (St. Louis, MO). Flag-hKGF DNA plasmid driven by a CMV14 promoter (0.5 μg/μL; KGF group, n = 8) or an empty plasmid driven by a CMV14 promoter (0.5 μg/μL; control group, n = 8) was transfected into the Wnt1-Cre/EGFP mice’s ear skin for five times every fourth day with a NEPA21 Electroporator (NEPA GENE Co Ltd, Chiba, Japan), according to the protocol of the previous article. The hKGF cDNA expression vector was successfully transfected, and KGF protein was expressed after hKGF expression vector transfection, with the same results as described previously (data not shown). Keratin accumulation within the hyperproliferative epidermis, and thickened mesenchyme and pseudostratified mucosal epithelium were induced by repetitive KGF vector transfection in all Wnt1-Cre/EGFP mice (KGF-1 and KGF-2). All mice were euthanized at 7 days after the fifth vector transfection, and their temporal bones were collected. The temporal bones of three mice were used for RT-PCR. The temporal bones of five mice were decalcified after fixation and embedded in paraffin using standard methods. Sections (5 μm thick) were prepared, and hematoxylin and
Administration of mu-p75-SAP in Vivo

Twelve male Wnt1-Cre/EGFP mice (aged 6 weeks; \( n = 12 \); 24 ears) with or without cholesteatoma were used in this experiment. The cholesteatoma was induced by repetitive hKGF expression vector transfection (0.5 \( \mu \)g/\( \mu \)L; KGF group, \( n = 12 \)) in the right mouse ears, as had been done previously.\(^{11}\) Twelve left ears with repetitive transfection of an empty plasmid driven by a CMV14 promoter (0.5 \( \mu \)g/\( \mu \)L; control group, \( n = 12 \)) were used as a control. After being anesthetized intraperitoneally with a mixture of medetomidine (0.5 mg/kg), midazolam (5 mg/kg), and fentanyl (50 \( \mu \)g/kg), 10 \( \mu \)L mu-p75-SAP (Advanced Targeting Systems, Carlsbad, CA; 0.2 or 0.6 mg/mL; \( n = 4 \) each) was injected into the cholesteatoma region or tympanic membrane (TM) locally in 24 ears (KGF group, 12 ears; control group, 12 ears) one time, as described previously.\(^{23}\) At 7 days after the mu-p75-SAP or PBS administration, all mice were euthanized, the temporal bone tissues were removed, and the paraffin sections were prepared as described above for histopathologic analysis.

Table 1
Analysis of the Pathologic State of Middle Ears under Administration of p75-SAP in an \textit{in Vivo} Model

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cholesteatoma (+)</th>
<th>Cholesteatoma (−)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hKGF vector transfection (( n = 4 ))</td>
<td>4/4</td>
<td>0/4</td>
</tr>
<tr>
<td>Empty vector transfection (( n = 4 ))</td>
<td>0/4</td>
<td>4/4</td>
</tr>
<tr>
<td>hKGF vector transfection + mu-p75-SAP 0.2 mg/mL (( n = 4 ))</td>
<td>3/4</td>
<td>1/4</td>
</tr>
<tr>
<td>hKGF vector transfection + mu-p75-SAP 0.6 mg/mL (( n = 4 ))</td>
<td>0/4</td>
<td>4/4</td>
</tr>
<tr>
<td>hKGF vector transfection + PBS (( n = 4 ))</td>
<td>4/4</td>
<td>0/4</td>
</tr>
</tbody>
</table>

hKGF, human keratinocyte growth factor; mu-p75-SAP, murine p75-SAP; PBS, phosphate-buffered saline; SAP, saporin.

Mouse Otoendoscopic Examination

In the present study, cholesteatoma formation was evaluated by otoendoscopy. At day 7 after the final transfection in the KGF-inducing cholesteatoma model and 7 days after the mu-p75-SAP injection experiments, each mouse underwent otoendoscopic examination with a rigid rod \( 0^\circ \) otoendoscope (AVS Co, Tokyo, Japan) at each time point.\(^{11,23}\) The findings were scored cholesteatoma positive (+) or negative (−) (Table 1).

p75KO in Vitro

Middle ear epithelial cells (MEECs) for the inhibition analysis of p75 were prepared from male Wnt1-Cre/EGFP mice (aged 3 to 5 weeks; \( n = 3 \); six ears) by the primary explant culture described previously.\(^{22}\) The Wnt1-Cre/EGFP MEECs [wild-type (WT) cells] subcultured up to the third passage were used. Nerve growth factor receptor (NGFR) p75 clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) KO plasmid (m) and NGFR p75 homology-directed repair (HDR) plasmid (Santa Cruz Biotechnology Inc., Dallas, TX) were stably transfected into WT cells, according to the manufacturer’s instructions, by using a NEPA 21 Electroporator, as previously described.\(^{24}\) The clones were selected with 1 \( \mu \)g/mL of puromycin, according to the manufacturer’s instructions.\(^{25}\) These p75KO Wnt1-Cre/EGFP MEECs refer to p75KO cells in the text. The p75KO cells or WT cells were plated on 12-well plates or chamber slides with bronchial epithelial cell growth medium (Lonza, Basel, Switzerland)/Dulbecco’s modified Eagle’s medium (DMEM)/20% fetal bovine serum (FBS) at a subconfluent concentration (60%) and incubated at 37°C until they attached to the plating surface.\(^{26}\) The same as for the \textit{vivo} model, KGF protein was administrated to the cells for the cholesteatoma model \textit{in vitro} described previously.\(^{27}\) The medium was replaced with low-glucose (1000 mg/L) DMEM containing 0.1% FBS (DMEM/0.1% FBS) for 2 days. Subsequently, recombinant human KGF/fibroblast growth factor (FGF) 7 (rhKGF) (10 ng/mL; R&D Systems, Minneapolis, MN) in PBS or PBS (\( n = 3 \) each) was added, and the cells were incubated overnight.
Expression Assay in Vitro

Construction of Human Constitutive Active p75 Expression Vector

pEGFP-N1 vector (Clontech, Takara Bio, Shiga, Japan) was digested by NotI and BamHI to remove an EGFP fragment. Kusabira-Orange 1 (KO1) was amplified by KOD One (Toyobo, Osaka, Japan) from phKO1-MC1 vector (AM-V0045; MBL, Tokyo, Japan) with forward primer 5'-CGCGGGCCCGGGATCCATGGTGAGCGTGATCAAGC-3' and reverse primer 5'-TCTAGAGTCGCGGCCGCTAGCAGTGGGCCACG-3'; then, a fragment was digested by BamHI and NotI (Takara Bio). Both digested vector and fragment were ligated together by Mighty Mix (Takara Bio) and transformed into DH5α, and pKusabira-N1 was cloned. Human p75 fragment was amplified by KOD One from cDNA of HEK293 with forward primer 5'-GGAATTCACCATGGGGGCAGGTGCCAC-3' and reverse primer 5'-CGGGATCCGGATGTGGCAGTG-GACTCAC-3'. A amplified fragment and a pKusabira-N1 were digested with EcoRI and BamHI (Takara Bio). Both digested fragment and vector were ligated together by Mighty Mix and transformed into DH5α, and pKO1-p75 was cloned. Previously, it was shown that p75 nerve growth factor receptor T248C mutants display constitutive and ligand-independent activity. Using pKO1-p75 as a template, PCR was done with forward primer 5'-GGCACCTGCGACAACCTCATCCCTGTC-3' and reverse primer 5'-GTTGTCGCAGGTGCCTCGGTCACCAC-3' to change the amino acid sequence to T248C. PCR fragment was transformed into DH5α, and pKO1-constitutive active p75 (p75CA) was cloned.

p75CA Expression Vector Transfection

The Wnt1-Cre/EGFP MEECs (WT cells) subcultured up to the third passage were used. pKO1-p75CA expression vector or pKO1 expression vector was stably transfected into WT cells, according to the manufacturer’s instructions, by using a NEPA 21 Electroporator. The cells were plated on 12-well plates or chamber slides with bronchial epithelial cell growth medium/DMEM/20% FBS at a subconfluent concentration (60%) and incubated at 37°C until they attached to the plating surface. The medium was replaced with low-glucose (1000 mg/L) DMEM/0.1% FBS, and the cells were incubated for 2 days.

### Table 2 Antibodies Used for Immunostaining or Western Blot Analysis

<table>
<thead>
<tr>
<th>Antigen, clone name or immunogen</th>
<th>Manufacturer, species, catalog no.</th>
<th>Dilution used</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>KGF, human FGF7 aa 179-182</td>
<td>Thermo Fisher Scientific (Hudson, NH), rabbit, PA5-49715</td>
<td>1:100</td>
<td>Primary</td>
</tr>
<tr>
<td>FGFR2, human FGF7 aa 362-374</td>
<td>Abcam (Cambridge, UK), rabbit, ab10668</td>
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<td>Primary</td>
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<tr>
<td>GFP, recombinant full-length protein corresponding to GFP</td>
<td>Abcam, rabbit, ab6556</td>
<td>1:100</td>
<td>Primary</td>
</tr>
<tr>
<td>GFP, recombinant full-length protein corresponding to GFP</td>
<td>Aves Labs (Davis, CA), chick, GFP-1010</td>
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<td>Primary</td>
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<td>Wnt1, human Wnt1 aa 50-150</td>
<td>Abcam, rabbit, ab15251</td>
<td>1:100</td>
<td>Primary</td>
</tr>
<tr>
<td>K14, Poly19053</td>
<td>BioLegend (Cambridge, UK), rabbit, RB-155P</td>
<td>1:4000</td>
<td>Primary</td>
</tr>
<tr>
<td>K10, Poly19054</td>
<td>BioLegend, rabbit, RB-159P</td>
<td>1:4000</td>
<td>Primary</td>
</tr>
<tr>
<td>Acetylated α-tubulin, 6-11B-1</td>
<td>Abcam, rabbit, ab24610</td>
<td>1:100</td>
<td>Primary</td>
</tr>
<tr>
<td>Snail, human SNAIL1 aa 215-264</td>
<td>LSBio (Seattle, WA), rabbit, LS-B11351</td>
<td>1:50</td>
<td>Primary</td>
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<tr>
<td>p75, Mouse p75 NGFR aa 43-181</td>
<td>Abcam, rabbit, ab8874</td>
<td>1:100, 1:400</td>
<td>Primary</td>
</tr>
<tr>
<td>NGFR p75 (B-1), human p75 aa 393-427</td>
<td>Santa Cruz Biotechnology, Inc. (Dallas, TX), mouse, sc-271708</td>
<td>1:500</td>
<td>Primary</td>
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<tr>
<td>Ki-67, mouse Ki-67 aa 1850-1950</td>
<td>Novus Biologicals, Inc. (Littleton, CO), rabbit, NB110-89717</td>
<td>1:400</td>
<td>Primary</td>
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<tr>
<td>Kusabira-Orange, recombinant full-length protein corresponding to Kusabira-Orange</td>
<td>MBL (Nagoya, Japan)</td>
<td>1:500</td>
<td>Primary</td>
</tr>
<tr>
<td>β-Actin, 13E5</td>
<td>Cell Signaling Technology (Danvers, MA), rabbit, 4970</td>
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<td>Primary</td>
</tr>
<tr>
<td>HRP-goat anti-mouse IgG</td>
<td>Cell Signaling Technology, mouse, 7076</td>
<td>1:10,000</td>
<td>Secondary</td>
</tr>
<tr>
<td>HRP-goat anti-rabbit IgG</td>
<td>Cell Signaling Technology, rabbit, 7074</td>
<td>1:10,000</td>
<td>Secondary</td>
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<td>Alexa Fluor 488—goat anti-chicken IgY</td>
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<td>Alexa Fluor 488—goat anti-mouse IgG</td>
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<td>Alexa Fluor 555—goat anti-rabbit IgG</td>
<td>Thermo Fisher Scientific, goat, A-21428</td>
<td>1:500</td>
<td>Secondary</td>
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</tbody>
</table>

Aa, amino acid; FGF, fibroblast growth factor; FGFR, FGF receptor; GFP, green fluorescent protein; HRP, horseradish peroxidase; KGF, keratinocyte growth factor; NGFR, nerve growth factor receptor; SNAIL, Snail-1.
Western Blot Analysis

For the detection of p75 and β-actin, a Western blot analysis was performed, as described previously. β-Actin protein was used as a loading control. All of the antibodies used in this study are listed in Table 2. The cells (5.0 × 10^5 cells) were washed with PBS and lysed using buffer (2 × Laemmli buffer and 2-mercaptoethanol, a protein/phosphatase inhibitor). Proteins were then separated by polyacrylamide gel and transferred electrophoretically onto polyvinylidene difluoride membranes. The membranes were blocked with 5% skim milk in 0.1% Tween 20 in tris-phosphate inhibitor). Proteins were then separated by horseradish peroxidase goat anti-mouse IgG or rabbit IgG (1:10,000 dilution). The signals were visualized using the ECL advance antibody detection kit (GE Healthcare, Little Chalfont, UK) and LAS 4000 mini (Fujifilm, Tokyo, Japan).

Immunohistochemistry

For the detection of Wnt1, GFP, CK14, CK10, acetylated α-tubulin, p75, Snail, KGF, FGFR receptor 2, and Ki-67 on the paraffin sections or for the detection of GFP, Ki-67, p75, and KO1 in the primary cultured cells, fluorescence immunohistochemistry was performed, as described previously. All of the antibodies used in this study are listed in Table 2.

For the immunohistochemistry on the paraffin sections, the sections were deparaffinized with toluene and rehydrated with serially graded ethanol solutions. Antigen retrieval was performed by immersion in proteinase K (S3020; Dako, Carpinteria, CA) for Wnt1, immersion with 0.2% Triton X-100 (Sigma Aldrich, St. Louis, MO) in PBS for GFP, CK14, CK10, acetylated α-tubulin, p75, and FGFR receptor 2 or autoclaving in HistoVT one (Nakarai Tesque, Kyoto, Japan) at 90°C for 20 minutes for Ki-67. The slides were pre-incubated with 500 μg/mL of normal goat IgG in 1% bovine serum albumin in PBS for 1 hour to block any nonspecific reaction, and then the sections were reacted overnight with the primary antibodies (Table 2) in 1% bovine serum albumin in PBS. After they were washed with 0.05% Tween 20 in PBS, the slides were reacted with the appropriate secondary antibody (Table 2). After being washed with 0.05% Tween 20 in PBS, the slides were counterstained with DAPI and visualized by fluorescence conjugate. For the immunohistochemistry in the primary cultured cells, cells were fixed with 4% paraformaldehyde and permeabilized in 0.1% Triton X-100 in PBS, followed by blocking with Block-One blocking reagent (Nakarai Tesque) and incubation with primary antibodies. After washing in 0.1% Tween 20 in PBS, the cells were incubated with fluorescence-labeled secondary antibodies, followed by counterstaining with DAPI. For a negative control, normal mouse IgG (1:100) or normal rabbit IgG (1:100) was used instead of the primary antibodies, respectively, in every experiment.

RNA Isolation and RT-PCR

Wnt1-Cre/EGFP young adult mice (aged 6 weeks; n = 3; six ears), Wnt1-Cre/EGFP embryos (E10; n = 3), and male Wnt1-Cre/EGFP mice (aged 6 weeks) with repetitive hKGF expression vector transfection in the ears (KGF group; n = 3; three ears) or with repetitive empty vector transfection in the ears (control group; n = 3; three ears) were used in this study. The induction of cholesteatoma formation by repetitive hKGF expression vector transfection was confirmed in all of the ears, but all of the ears with repetitive empty vector transfection had a normal TM, as had been done previously (data not shown). The head of each mouse was dissected and skinned, and the skull cap was removed. After removing the brain, the temporal bones were dissected, and the bullae with the TM were separated from the surrounding tissue under microscope observation. The TMs and middle ear mucosa or cholesteatoma tissues were harvested, and total RNA was isolated using Takara reagent, according to the manufacturer’s instructions (NucleoSpin RNA; Takara Bio), and 1 μg total RNA was reverse transcribed using the PrimeScript II first-strand cDNA Synthesis Kit (Takara Bio). An RT-PCR was performed using KOD FX (Takara Bio), according to the manufacturer’s instructions, and the following primer sets: Wntl forward, 5'-CATCTTCGGCACAACCCTCAG-3'; Wntl reverse, 5'-GTGGGACATTG-CACCTTGG-3'; Snail forward, 5'-CCCCACTCGGATGTAAGAGATACC-3'; Snail reverse, 5'-ATG TGTCCAGTAACCACCCTGTCGT-3'; Slug forward, 5'-GGCTGCTTCAAGGACACATAGA-3'; Slug reverse, 5'-GGCTTCAAGGACACATAGA-3'; Twist forward, 5'-A CGAGTGCCTGAACGAGGC-3'; Twist reverse, 5'-GTACAGGAATGCTGATAC-3'; Sox9 forward, 5'-5' GGCTGTGTGTGCCGTGGATAG-3'; Sox9 reverse, 5'-CACGCCACAGCATGAGTAAGAGA-3'; Sox10 forward, 5'-AGCGACTGTAGGACAGCTTGG-3'; Sox10 reverse, 5'-ATGAGGTATTGCAACGGAACTGG-3'; Pax3 forward, 5'-AACAAGCTGAGCCAACATCAGT-3'; Pax3 reverse, 5'-CTGAGGTCTGTGGAGCCCGTGCT-3'; p75 forward, 5'-AGTGCTTCAAGGACACATAGA-3'; and p75 reverse, 5'-TGGCCCTACACGACGTCAAG-3'. This was normalized to the housekeeping gene Gapdh, as indicated. The primer sequences used in this study are listed in the previous studies.

Microscopy, Image Analysis, Cell Count, and Statistical Analysis

H&E staining images were captured using an Axio Cam camera and AxioVision software version 4.8 (Carl Zeiss, Jena, Germany) with light microscopy. The images of fluorescent staining were obtained using fluorescence confocal laser scanning microscopy (LSM 880; Carl Zeiss). For the
quantitative analysis, the images of enzyme immunohistochemistry were graded as positive or negative compared with the negative control, and the number of cell nuclei was counted at >1000 nuclei in three equal epithelial regions at ×400 magnification. Per each specimen of immunofluorescence staining, three 10,000-μm² areas (100 × 100-μm squares) for the equal epithelial regions were assumed with fluorescence confocal laser scanning microscopy (LSM 880) and Zeiss acquisition analysis software (Zen 2.1 black edition), and the number of positive cell nuclei was counted. The number of positive cells was expressed as a percentage of positive cells per total number of counted cells [labeling index (LI); means ± SD]. DAPI labeling was used to obtain the total cell number. Means and SDs were calculated from numerical data. Statistical significance of group differences was examined using one-way analysis of variance tests, followed by the unpaired t-test or Tukey post hoc tests for normally distributed data. P < 0.01 denoted a statistically

Figure 1  A: Otoendoscopic image of the Wnt1-Cre/EGFP mouse indicates normal tympanic membrane (TM). B: Hematoxylin and eosin staining of the Wnt1-Cre/EGFP mouse temporal bone. C: Schematic illustrating the middle ear cavity. The yellow dashed line shows the attic region (Attic), blue dashed line shows the TM region, magenta dashed line shows the optic capsule (OC) region, and green dashed line shows the ventral region (VT). D: Results of RT-PCR showing Wnt1 expression in the cells of middle ear of adult Wnt1-Cre/EGFP mice (aged 6 weeks: lanes 1, 2, and 3). Total RNA from a whole E10 embryo (lane 4) was used as a positive control. Gapdh was the reference gene. E: Immunofluorescence detection of green fluorescent protein (GFP; green) immunofluorescence in the middle ear of Wnt1-Cre/EGFP mice. The nuclei were stained with DAPI (blue). Images are high-power views of the insets in B. Schematic indicating key regions of interest in the TM. The cells in the Attic, TM, and OC were GFP positive (arrowheads; left panel), but the cells in the VT were GFP negative (right panel). The important areas in E (insets) are shown in high-power views (lower panels). F: The labeling index (LI) of GFP-positive cells in the TM, Attic, OC, and VT of Wnt1-Cre/EGFP mice (aged 6 weeks). All data are expressed LI as means ± SD (F, n = 4 Wnt1-Cre/EGFP mice (F). ****P < 0.0001 (one-way analysis of variance test, followed by the unpaired Tukey post hoc tests for normally distributed data). Scale bars: 1.0 mm (B); 100 μm (E: upper panel); 20 μm (E: lower panel). OE, outer ear; Os, ossicles; PF, pars flaccida; PT, pars tensa.
significant difference. All analyses were performed using a statistical software package (JMP version 13; SAS Institute Japan, Tokyo, Japan).

**Results**

Histologic Analysis and Detection of Neural Crest Lineages in the Temporal Bone of *Wnt1-Cre/EGFP* Mice

Under an otoscopic view, *Wnt1-Cre/EGFP* mice (aged 6 weeks) had a normal TM (Figure 1A). Histologic analysis with H&E staining of the temporal bone of the *Wnt1-Cre/EGFP* mice indicated a normal structure of the ossicles and middle ear (Figure 1, B and C). RT-PCR was performed to evaluate the expression of endogenous *Wnt1* in the middle ear of adult mice. Endogenous *Wnt1* transcript was detected in an E10 embryo, but not in 6-week-old mice (Figure 1D). *Gapdh* was detected in all extracts at almost equal levels (Figure 1D). To determine the localization of the NC lineages (GFP-positive cells) in the middle ear of the *Wnt1-Cre/EGFP* mice, immunohistochemistry was performed using anti-GFP antibody. Schematic diagram indicating key regions of interest in the TM is shown in Figure 1E. The TM is composed of an epidermal layer, mesenchyme region, and mucosal layer. GFP-positive cells were observed in the epidermal layer, mucosal layer, and mesenchyme region of the pars flaccida (PF) of the TM region (Figure 1, C and E), around the malleus (Figure 1, C and E), in the basal layer of the middle ear mucosa in the attic region (Attic) (Figure 1, C and E), and the optic capsule region (OC) (Figure 1, C and E) of middle ear cavity. However, the GFP-positive cells were not detected in the mucosa around the ventral region of the middle ear cavity (Figure 1, C and E). The LI of the GFP-positive cells in the ventral region was significantly lower compared with that of the TM, Attic, or OC (ventral region versus TM, Attic, and OC: 1.47 ± 0.94 versus 17.8 ± 3.27, 39.0 ± 1.88, and 34.4 ± 3.21, respectively; n = 4; P = 0.0001, Tukey post hoc tests) (Figure 1F). As shown in the results, the NC lineage cells were mainly lined on the epidermis of the TM, surface of ossicles, mucosa of the TM, Attic, and cochlea in the middle ear cavity. NC lineage cells were also localized in the mesenchyme region of the PF of the TM.

**GFP-Positive Cells Present in Cholesteatoma Formation in Mouse Middle Ears after KGF Gene Transfection**

Cholesteatoma formation was induced in the PF of the TM by repetitive KGF vector transfection in all of the mouse ears in the KGF group (Figure 2A and Table 1). Otoendoscopic findings of the control group indicated a normal TM (Figure 2A and Table 1). KGF gene transfection induced cholesteatoma with a hyperproliferative epidermis and thickened mesenchyme of the TM, as indicated by H&E staining (Figure 2B). Moreover, a pseudostratified mucosal epithelium was observed in the TM and OC compared with the empty vector–transfected ears (Figure 2B). An increased number of GFP-positive cells was observed within the epidermis, mucosa, and mesenchyme region of the cholesteatoma in the mice (Figure 2C) and within the pseudostratified mucosal epithelium and thickened mesenchyme of the OC (Figure 2C). The LI of the GFP-positive cells in the KGF group was significantly increased compared with that of the control group (KGF group, n = 5; versus control group, n = 5: 51.78 ± 8.04 versus 23.3 ± 5.81, respectively; P < 0.0001, t-tests) (Figure 2E). To evaluate the type of increased number of GFP-positive epithelial cells in mice, immunohistochemical analysis of the expression of CK14 (stem/progenitor cell marker),33 CK10 (differentiated cell marker),34 and acetylated α-tubulin (ciliated cell marker)35 was performed. A large number of GFP-positive/CK14-positive epithelial cells were observed in the basal and upper layer of the epidermis, pseudostratified mucosal epithelium of cholesteatoma, and pseudostratified mucosal epithelium of the OC (Figure 2C). Many GFP-negative/CK14-positive epithelial cells were observed in the basal and upper layer of the hyperproliferative epidermis of TM of cholesteatoma (Figure 2C). A small number of GFP-positive/CK14-positive epithelial cells were observed in the epithelial layer of TM but not observed in the middle ears of the control group (Figure 2C). The LI of GFP-positive/CK14-positive epithelial cells against the number of GFP-positive cells in the KGF group was significantly increased compared with that of the control group (KGF group, n = 5; versus control group, n = 5: 25.31 ± 1.03 versus 9.23 ± 1.865, respectively; P = 0.0001, t-tests) (Figure 2E). GFP-positive/CK10-positive cells were not detected in any region of the middle ears of the KGF group or control group (Figure 2C). The LI of GFP-positive/CK10-positive epithelial cells against the number of GFP-positive cells in the KGF group was almost the same as that of the control group (KGF group, n = 5; versus control group, n = 5: 1.17 ± 0.68 versus 1.10 ± 0.65, respectively; P = 0.827, t-tests) (Figure 2E). Some of these GFP-positive cells were also positive for acetylated α-tubulin (Figure 2C). Almost all GFP-positive epithelial cells in the Attic and OC of the control group expressed acetylated α-tubulin (Figure 2C). In contrast, GFP-positive/acetylated α-tubulin—positive cells were found in the upper layers of the mucosa in the Attic and OC of the KGF group (Figure 2C). The LI of GFP-positive/acetylated α-tubulin—positive epithelial cells against the number of GFP-positive cells in the KGF group was significantly lower than that of the control group (KGF group, n = 5; versus control group, n = 5: 9.60 ± 0.39 versus 52.55 ± 8.23, respectively; P < 0.0001, t-tests) (Figure 2E). These results indicated that many NC lineage epithelial stem/progenitor cells (GFP-positive/CK14-positive cells) were present in the KGF-inducing cholesteatoma tissues in mice. In the same region, the expression of KGF and KGFR was analyzed immunohistochemically. GFP-positive cells were observed in the basal and upper

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Figure 2  A: Otoendoscopic views of normal ears (Normal-1 and Normal-2), empty vector–transfected ears (Control-1 and Control-2), and keratinocyte growth factor (KGF) vector–transfected ears (KGF-1 and KGF-2). The dotted white and black lines: tympanic membrane, pars flaccida (PF), and pars tensa (PT); black arrowheads: cholesteatoma (Chole). B: Hematoxylin and eosin staining of temporal bones of the control group and KGF-1 and KGF-2. Cholesteatoma formation (asterisks). Pseudostratiﬁed mucosal epithelium (stars) was observed in the optic capsule (OC) in all Wnt1-Cre/EGFP mice (KGF-1 and KGF-2). Insets: High-power views. C: Double-immunofluorescence detection of green ﬂuorescent protein (GFP; green or red), cytokeratin (CK) 14 (red), CK10 (red), and acetylated α-tubulin (green) in the sections of control and KGF-1 and KGF-2. The nuclei were stained with DAPI (blue). GFP(+)/CK14(–) cells [green arrows: GFP(+)/CK14(–) cells; white arrows: GFP(+)/CK14(+) cells] were found to expand into the middle ear and form middle ear cholesteatoma. GFP(+)/CK14(+) cells (white arrows) were detected in the basal and upper layer of the epidermis, pseudostratiﬁed mucosal epithelium of cholesteatoma, and pseudostratiﬁed mucosa of the OC (top panel; white arrows). GFP(+)CK10(+) cells were scarcely detected, but GFP(+)CK10(–) cells (green arrows) were detected in both groups (middle panel). GFP(+)/α-tubulin (+) cells (white arrows) were detected in the epithelium of the attic region in both groups (bottom panel; white arrows). GFP(–)/α-tubulin (+) cells were detected in the epithelium of the tympanic membrane (TM) region and OC region in both groups (bottom panel; red arrows). Red arrows: CK14(+) cells or GFP(+) cells; green arrows: GFP(+); white arrows: double-positive cells. D: The images indicate the high-power view of the inset in KGF-2 in C. GFP(+)KGF(+) cells (white arrows) were detected in the basal and upper layer of the epidermis, pseudostratiﬁed mucosal epithelium, and mesenchymal cells of cholesteatoma (white arrows). GFP(+)KGF(+)/CK10(+) cells (white arrows) were observed in the basal and upper layer of the epidermis and pseudostratiﬁed mucosal epithelium. White arrows: double-positive cells. E: The labeling index (LI) of GFP(+) cells, GFP(+)CK14(+) cells, GFP(+)CK10(+) cells, and GFP(+)α-tubulin(+) cells in the control group versus the KGF group. All data are expressed LI as means ± SD (E). n = 5 for the control and KGF groups (E). ***P < 0.001, ****P < 0.0001 (one-way analysis of variance test, followed by the unpaired t-test for normally distributed data). Scale bars: 200 μm (B); 50 μm (C and D). ME, middle ear; OE, outer ear; Os, ossicles.
layers of the epidermis, mesenchyme of the cholesteatoma, and pseudostratified mucosal epithelium (Figure 2D). Almost all of the KGF-positive cells were GFP-positive (Figure 2D). The localization of GFP-positive/KGF-positive cells in the cholesteatoma was similar to that of GFP-positive/CK14-positive cells in the epidermis and mucosa of the cholesteatoma (Figure 2D). However, the GFP-positive/KGF-positive cells in the mesenchyme of the cholesteatoma were CK14-negative (Figure 2, C and D). KGFR-positive cells were observed in the basal and upper layers of the epidermis and pseudostratified mucosal epithelium (Figure 2D). Almost all of the KGFR-positive cells were GFP-positive (Figure 2D). The localization of the GFP-positive/KGFR-positive cells in the cholesteatoma was similar to that of the GFP-positive/CK14-positive cells in the epidermis and mucosa of the cholesteatoma (Figure 2, C and D).

GFP-Positive Cells in Cholesteatoma in Vivo Strongly Express NC Cell-Specific Cell Markers and Have Increased Proliferative Activity

RT-PCR was performed to quantify the expression levels of the NC-specific cell markers Wnt1, Snail, Slug, Twist, Sox9, Sox10, Pax3, and p75 in the cholesteatoma of the KGF group and in the middle ear mucosa of the control group (Figure 3, A and B). PCR-amplified products with p75 primer were detected only in the KGF group but not in the control group (Figure 3B). Moreover, the level of PCR-amplified products with Snail primer tended to be high in the extracts of the KGF group (Figure 3B). To evaluate the localization of GFP-positive/p75-positive cells in the mouse cholesteatoma tissues, double-immunofluorescence staining with anti-GFP antibody and anti-p75 antibody was performed. A large number of GFP-positive/p75-positive cells were observed in the epidermis, mucosa, and mesenchyme of the cholesteatoma tissue (Figure 3C). However, some GFP-positive/Snail-positive cells were observed in the epidermis and mucosa of the cholesteatoma tissue (Figure 3C). A small number of GFP-positive mucosal cells in the Attic and OC of the control group expressed p75 but not Snail. The LI of GFP-positive/p75-positive cells in the KGF group was significantly higher than that of the control group (KGF group, n = 5, versus control group, n = 5: 47.0 ± 5.63 versus 1.34 ± 0.31, respectively; P < 0.0001, t-tests) (Figure 3D). The LI of GFP-positive/Snail-positive cells in the KGF group was significantly higher than that of the control group (KGF group, n = 5, versus control group, n = 5: 14.9 ± 3.20 versus 3.19 ± 1.37, respectively; P < 0.0001, t-tests) (Figure 3D). Next, to evaluate the proliferative activity of GFP-positive NC cells, double-immunofluorescence staining with anti-GFP antibody and anti–Ki-67 antibody in the sections was performed. Ki-67–positive cells were detected in the epidermal and mucosal region of the cholesteatoma (Figure 3C) but scarcely detected in the section of the control group (Figure 3C). The LI of GFP-positive/Ki-67–positive cells in the KGF group was significantly higher than that of the control group (KGF group, n = 5, versus control group, n = 5: 33.2 ± 7.12 versus 5.52 ± 2.20, respectively; P < 0.001, t-tests) (Figure 3D).

p75-Positive Cells Observed in Human Cholesteatoma

To address the localization and expression of NC lineage cells in human cholesteatoma, immunohistochemical analysis of NC-specific cell markers, p75 and Snail, was performed in the section of the cholesteatoma and normal skin. Human cholesteatoma tissue is histologically made up of an epithelium (hyperproliferative stratified squamous epithelium) and a stromal region (inflamed subepithelial connective tissue). A large number of p75-positive cells were seen in the epithelium and stromal region in the section of cholesteatoma but were scarcely detected in the section of normal skin (Figure 4A). Several Snail-positive cells were seen in the stromal region of cholesteatoma but not in normal skin (Figure 4A). On the other hand, Wnt1-positive cells were not detected in either the section of cholesteatoma or the normal skin, mirroring the results of the mouse model (Figure 4A). The p75 LI of the cholesteatoma was significantly increased compared with that of the normal skin (cholesteatoma, n = 23, versus skin, n = 23: 43.2 ± 2.26 versus 2.76 ± 1.08, respectively; P < 0.0001, t-test) (Figure 4B). The Snail LI of the cholesteatoma was also significantly increased compared with that of the normal skin (cholesteatoma, n = 23, versus skin, n = 23: 8.18 ± 5.42 versus 2.94 ± 1.06, respectively; P < 0.0001, t-test) (Figure 4B). The proliferative activity of the cells of the cholesteatoma was addressed by immunohistochemical analysis using anti-Ki-67 antibody. A large number of Ki-67–positive cells were seen in the epithelial and stromal regions in the section of cholesteatoma, but a small number of Ki-67–positive cells were detected in the section of normal skin (Figure 4A). The Ki-67 LI of the cholesteatoma was as significantly increased as that of the normal skin (cholesteatoma, n = 23, versus skin, n = 23: 33.5 ± 7.30 versus 3.34 ± 1.53, respectively; P < 0.0001, t-test) (Figure 4B). The results in the human specimens were similar to that in the mouse model.

Mu-p75-SAP Reduces Cholesteatoma Tissue in Vivo

Mu-p75-SAP or PBS was injected at day 23 after cholesteatoma formation was evaluated by otoendoscopy (Figure 5A). Cholesteatoma formations were seen in the TMs of all of the KGF vector–transfected ears in vivo (Table 1 and Figure 5B), similar to the results of the previous study. To clarify the efficacy of mu-p75-SAP in suppressing cholesteatoma formation in vivo, the ears were analyzed by otoendoscopy and histology at day 30, as had been done previously (Figure 5A). At 7 days after treatment with 0.2 mg/mL mu-p75-SAP in the in vivo model,
A: Schematic description of the method for keratinocyte growth factor (KGF)—induced cholesteatoma. Human KGF (hKGF) expression vector or empty vector was transfected electroporatically five times every fourth day into the epithelial region of the ear. The animals were euthanized using an i.p. injection of 200 mg/kg pentobarbital, and their temporal bones were removed at day 23. B: RT-PCR showing mRNA expression of various neural crest—specific cell (NCSC) markers in the cholesteatoma tissues from the hKGF vector—transfected ear (KGF) and middle ear specimens from the empty vector—transfected ear (Control). Total RNA from a whole E10 embryo was collected as a positive control, and Gapdh was used as a reference gene. C: Double-immunofluorescence detection of green fluorescent protein (GFP; green) and p75 (red), Snail (red), or Ki-67 (red) in KGF-inducing cholesteatoma tissue (KGF; top panels) and empty vector—transfected ear (Control; bottom panels). A large number of GFP(+)p75(+) cells (KGF; left panel) and GFP(+)Ki-67(+) cells (KGF; right panel) were detected in the epithelial region, mainly in KGF-induced cholesteatoma. GFP(+)Snail(+) cells were detected in the stromal region in KGF-induced cholesteatoma (KGF; middle panel). The nuclei were stained with DAPI (blue). Arrows: positive cells. Insets: High-power views. D: Box plots showing the labeling index (LI) of GFP(+)p75(+) cells, GFP(+)Snail(+) cells, or GFP(+)Ki-67(+) cells in the empty vector—transfected ear group (Control) and the KGF-inducing cholesteatoma tissue group (KGF). All data are expressed LI as means ± SD (D). n = 5 in the Control and KGF groups (D). ***P < 0.001, ****P < 0.0001 (one-way analysis of variance test, followed by the unpaired t-test for normally distributed data). Scale bar = 20 μm (C).
cholesteatoma was reduced in one of four ears as indicated by otoendoscopic analysis (Table 1 and Figure 5B). A histologic examination indicated slightly thickened epidermal layers of the TM with debris in the tissue sections from the hKGF expression vector-induced cholesteatoma treated with 0.2 mg/mL mu-p75-SAP (Figure 5C). After treatment of the cholesteatoma with 0.6 mg/mL mu-p75-SAP, cholesteatoma was not detected in any of the ears (Table 1 and Figure 5B). In fact, a histologic examination indicated an almost normal structure of the TM and middle ear in the tissue sections from the hKGF expression vector-induced cholesteatoma treated with 0.6 mg/mL mu-p75-SAP.

**Figure 4** Analysis of human middle ear cholesteatoma (Chole). **A:** Hematoxylin and eosin (H&E) staining of Chole and normal ear skin (Skin). The thickened epithelium and stroma with many infiltrated cells is seen in Chole. Immunostaining for p75 (red), Snail (red), Wnt1 (red), and Ki-67 (green) in paraffin-embedded, formalin-fixed sections of Chole and Skin. The nuclei were stained with DAPI (blue). p75(+) cells are seen primarily in the epithelium and stroma, while Snail(+) cells are primarily seen in the stroma in Chole. A small number of p75(+) or Snail(+) cells are seen in the Skin section. Ki-67(+) cells (arrows) are detected in the basal and suprabasal layers of the epithelium and stroma of Chole, and the localization of Ki-67(+) cells is similar to that of p75(+) cells. A small number of Ki-67-positive cells are seen in the normal skin section. Mouse (Ms) IgG and rabbit (Rb) IgG were used as negative control. **B:** The labeling index (LI) of p75, Snail, and Ki-67 in Chole versus Skin. All data are expressed LI as means ± SD (B). n = 23 in Chole and Skin (B). ****P < 0.0001 (one-way analysis of variance test, followed by the unpaired t-test for normally distributed data). Scale bars = 20 μm (A). E, epithelial region; S, subepithelial region.
p75KO Reduces the Number of GFP-Positive/p75-Positive Cells and GFP-Positive/Ki-67—Positive Cells in Vitro

To analyze the role of p75 against the proliferative activity of NC lineage cells, the pK01-p75CA plasmid was transfected into WT cells (p75CA plasmid cells) and analyzed proliferative activity in vitro (Figure 6D). As a control, pK01 plasmid was transfected into WT cells (control cells). The expression level of p75 protein was analyzed in the whole cell lysates by Western blot analysis (Figure 6E). As a result, an increased number of GFP-positive/Ki-67—positive cells were detected in the section of the p75CA plasmid cells (Figure 6D). A small number of GFP-positive/Ki-67—positive cells were detected in the section of the control cells (Figure 6D). A high level of p75 expression was detected in the rhKGF-treated WT cells, but not in the p75KO cells, as indicated by Western blot (Figure 6B). The LI of the GFP-positive/p75-positive cells in the section of the rhKGF-treated WT cell group (23.7% ± 0.52%) was significantly higher than those of the other groups (PBS-treated WT cell group, 6.0% ± 1.36%; rhKGF-treated p75KO cell group, 0% ± 0%; PBS-treated p75KO cell group, 0% ± 0%; all, n = 3; P < 0.0001, Tukey post hoc test) (Figure 6C). The LI of the GFP-positive/Ki-67—positive cells in the section of the rhKGF-treated WT cell group (18.2% ± 1.86%) was significantly higher than those of the other groups (PBS-treated WT cell group, 4.7% ± 1.98%; rhKGF-treated p75KO cell group, 1.4% ± 0.41%; PBS-treated p75KO cell group, 1.4% ± 0.38%; all, n = 3; P < 0.0001, Tukey post hoc test) (Figure 6C).

Discussion

Cholesteatoma is a type of otitis media characterized by the presence of a keratinizing epithelium and inflamed stroma that is believed to have hyper-proliferative activity. It might be important to prove the existence of NC lineage cells in the cholesteatoma tissue, because NC lineage epithelium is known to control the homeostasis of middle ear mucosa and plays a role in repairing otitis media. However, it is difficult to identify these NC lineage epithelial cells in human cholesteatoma specimens because of the lack of specific cell markers for NC lineage cells in adults. Wnt1-Cre/EGFP mice conditionally express EGFP...
Figure 5  Effects of murine p75-saporin (mu-p75-SAP) against cholesteatoma (Chole) tissues in vivo. A: Schematic representation of the method for mu-p75-SAP injection in keratinocyte growth factor (KGF)–induced cholesteatoma. KGF vector or empty vector was transfected electroporatically five times every fourth day into the epithelial region of the outer ear. At day 23, mu-p75-SAP or phosphate-buffered saline (PBS) was injected locally. The animals were euthanized using an i.p. injection of 200 mg/kg pentobarbital, and their temporal bones were removed at day 30. B: Otoendoscopic views of the tympanic membrane (TM) with administration of 0.2 or 0.6 mg/mL mu-p75-SAP (KGF + SAP 0.2 or KGF + SAP 0.6, respectively) or control (KGF + PBS) at day 23 and day 30. Before the administration, Chole was detected in all of the ears. After administration of 0.2 mg/mL SAP in the ear, debris was detected on the TM. After administration of 0.6 mg/mL SAP in the ear, Chole was not detected. Dotted black lines: tympanic membrane. C: Hematoxylin and eosin (H&E) staining of the TM with KGF + PBS, KGF + SAP 0.2, or KGF + SAP 0.6 at day 30. D: Double-immunofluorescence detection of green fluorescent protein (GFP; green) and p75 (red) or Ki-67 (red). The nuclei were stained with DAPI (blue). GFP(+)/p75(+) cells and GFP(+)/Ki-67(+) cells were not detected in the section of KGF + SAP 0.6. Negative control was obtained by normal mouse (Ms) IgG and normal rabbit (Rb) IgG instead of primary antibodies. These images indicate the high-power views of the insets in C. Yellow arrows: double-positive cells; green arrows: GFP(+) cells; red arrows: p75(+) cells or Ki-67(+) cells. E: The labeling index (LI) of GFP(+)/p75(+) cells and GFP(+)/Ki-67(+) cells in KGF + PBS versus KGF + SAP 0.2 versus KGF + SAP 0.6. All data are expressed LI as means ± SD (E). n = 4 each for GFP(+)/p75(+) cells and GFP(+)/Ki-67(+) cells in KGF + PBS versus KGF + SAP 0.2 versus KGF + SAP 0.6 (E). ***P < 0.001, ****P < 0.0001 (one-way analysis of variance test, followed by the Tukey post hoc tests for normally distributed data). Scale bars: 200 μm (C); 50 μm (D). OE, outer ear; Os, ossicles.
in the NC lineages and are used to analyze the localization of NC lineages in many organs.\textsuperscript{13,30} Herein, it was shown, for the first time, the presence of a novel, stem cell—like NC lineage cell population in the KGF-inducing mouse cholesteatoma model and in human cholesteatoma.

First, the localization of NC lineage cells in a section of the middle ears of 6-week—old Wnt1-Cre/EGFP mice was analyzed. According to the results, NC lineage cells were detected in the epithelial and mucosal layers of TM and mucosal epithelium of the Attic and OC of the normal middle ears of the Wnt1-Cre/EGFP mice (Figure 1, E and F). The localization of NC lineage cells in a normal middle ear had been investigated in Wnt1creR26R mice previously. The results indicated that the epithelium of the TM was derived from endodermal lineage cells, whereas NC lineage cells lined the Attic.\textsuperscript{7} The discrepancies in the results may be attributed to the different strains of mice, but this needs to be further investigated.

There is clinical evidence for the transition from a retraction pocket of the PF of the TM into an active and expanding cholesteatoma.\textsuperscript{35} Furthermore, many studies have indicated that the stem/progenitor cells of the TM are localized in the dermis and mucosa of the PF and around the handle of the malleus based on lineage tracing experiments and proliferation assays.\textsuperscript{38,39} Based on these findings, it was postulated that NC lineage cells localized in the PF region might grow and comprise cholesteatoma. Next, the changes in the localization and the number of NC lineage cells in the cholesteatoma tissues induced by KGF expression vector transfection in the ears of Wnt1-Cre/EGFP mice were analyzed. As expected, KGF-inducing cholesteatoma formation was observed in the PF of the TM and Attic (Figure 2, A and B). In the immunohistochemical analysis of the NC lineage cells in the mouse section of cholesteatoma, a large number of GFP-positive cells (ie, NC lineage cells) were detected in the thickened epidermis, pseudostratified mucosa, and mesenchymal region of the PF of the TM and around the malleus (Figure 2C). KGF-inducing cholesteatoma was not observed in the three other well-documented pathways of cholesteatoma formation: pars tensa, two routes, and open cholesteatomas.\textsuperscript{3} In the animal models of cholesteatoma in rats and mice, the retraction of the PF of the TM occurs first, and then the growth of cholesteatoma is seen after KGF expression vector transfection.\textsuperscript{11,27} This indicates that epithelial cells that proliferate under the action of KGF are present in the PF of TM in rats and mice. It is possible that the localization of NC cells in the human and mouse middle ear may differ, as well as differences in anatomic structure. The current study explores only one possibility, and additional studies are needed to explore this further.

NC stem cells from the craniofacial region also have been described in several adult organs and tissues, such as the skin, corneas, hair follicles, periodontal ligaments, palate, and/or pulp of the teeth.\textsuperscript{40—46} In vitro, NC stem cells behave as multipotent, self-renewing stem cells/progenitors\textsuperscript{47} and have shown the ability to differentiate into multiple lineages.\textsuperscript{48,49} Murine tissue injury models indicate that NC-derived Schwann cells dedifferentiate after injury and reacquire a progenitor-like cell state by up-regulating NC stem cell—associated factors, such as the p75 nerve growth factor receptor.\textsuperscript{49,50} These observations led to the hypothesis that NC lineage epithelial cells could possibly change into epithelial stem/progenitor cells and have a proliferative activity under KGF signaling. Therefore, the type of NC lineage epithelial cells in cholesteatoma tissues induced by KGF expression vector transfection in the ears of Wnt1-Cre/EGFP mice was evaluated. Immunohistochemical analysis of the expression of CK14 (stem/progenitor cell marker),\textsuperscript{33} CK10 (differentiated cell marker),\textsuperscript{32} and acetylated α-tubulin (ciliated cell marker)\textsuperscript{35} was performed. A small number of GFP-positive/CK14-positive epithelial cells were observed in the epithelial layer of TM but not in the middle ears of the control group (Figure 2C). A large number of CK14-positive/GFP-positive epithelial cells were observed in the basal and upper layers of the thickened dermis and the basal layer of the pseudostratified mucosa in the KGF-inducing cholesteatoma (Figure 2C). Moreover, Ki-67—positive/ GFP-positive cells were observed in the same region as that of the CK14-positive/GFP-positive cells in the KGF-inducing cholesteatoma mouse model (Figure 2C). CK10-positive/GFP-positive cells were not observed and acetylated α-tubulin—positive/GFP-positive cells were observed in the upper layer of the pseudostratified mucosa in the KGF-inducing cholesteatoma (Figure 2C). GFP-positive cells or α-tubulin—positive/GFP-positive cells were observed mainly in the simple columnar mucosa of the PF in the normal mouse ear (Figure 2C). These observations indicate two possibilities. CK14-positive/GFP-positive NC lineage epithelial progenitor cells may exist in the epithelium of PF and around the malleus in the TM, and KGF may promote their proliferation. Or, NC lineage mucosal cells in the PF and around the malleus in the TM may change to stem/progenitor-like cells exhibiting a CK14 gene, have a proliferative activity under the KGF signaling, and form cholesteatoma. In fact, KGF-positive NC lineage mesenchyme and KGF-positive and/or KGFR-positive NC lineage epithelial cells were observed in the KGF-inducing cholesteatoma in this study (Figure 2C). FGF signaling, including KGF, is one of the important signaling pathways for initiating the beginning of NC formation during gastrulation\textsuperscript{51} and craniofacial development.\textsuperscript{52} Also, during the development of the thymus, a possible molecular link between the NC lineage mesenchyme and thymus epithelium is provided via KGF and KGFR, and defects in this signaling pathway disrupt thymus development.\textsuperscript{53,54} These studies support the possibility that KGF/KGFR pathway signaling is strongly activated in the NC lineage cells of several epithelia to initiate cell growth. K14-positive epithelial stem/progenitor cells were also observed, which proliferate and form KGF-inducing cholesteatoma (Figure 2C).
Figure 6  Effects of p75 knockout (KO) or constitutive active p75 (p75CA) plasmid transfection against Wnt1-Cre/EGFP middle ear epithelial cells (MEECs) in vitro.  A: Double-immunofluorescence detection of green fluorescent protein (GFP; green), p75 (red), and Ki-67 (red) in the sections of Wnt1-Cre/EGFP MEECs (wild type (WT)) or p75KO Wnt1-Cre/EGFP MEECs (p75KO) treated with recombinant human keratinocyte growth factor (10 ng/mL) in phosphate-buffered saline (PBS; keratinocyte growth factor positive (KGF+)) or PBS (PBS+) for 17 hours. The nuclei were stained with DAPI (blue). A small number of GFP(+)Ki-67(+) cells was detected in the section of p75KO cells.  Yellow arrows: double-positive cells; green arrows: GFP-positive cells; red arrows: Ki-67-positive cells.  B: Western blot analysis of p75 and β-actin protein levels in the cells of WT (left lane) or p75KO (right lane) treated with KGF in PBS or PBS (top lane, reacted with anti-p75 antibody; bottom lane, reacted with anti-β-actin antibody). β-Actin was used as a loading control.  C: The labeling index (LI) of GFP(+)p75(+) cells and GFP(+)Ki-67(+) cells in WT KGF+ versus WT PBS+ versus p75KO KGF+ versus p75KO PBS+. One-way analysis of variance (ANOVA) test was performed, followed by the Tukey post hoc tests for normally distributed data.  D: Triple-immunofluorescence detection of GFP (green) and Ki-67 (white) or Kusabira-Orange 1 (KO1; red) in the sections of pKO1-p75CA plasmid transfected Wnt1-Cre/EGFP MEECs (p75CA plasmid) or pKO1 plasmid transfected Wnt1-Cre/EGFP MEECs (control). The nuclei were stained with DAPI (blue). Many GFP(+)Ki-67(+)KO1(+) cells were detected in the section of p75CA plasmid.  White arrows: triple-positive cells.  E: Western blot analysis of p75 and β-actin protein levels in the cells of p75CA plasmid (left lane) or control (right lane) (top lane, reacted with anti-p75 antibody; bottom lane, reacted with anti-β-actin antibody). β-Actin was used as a loading control.  F: The LI of GFP(+)Ki-67(+) cells in p75CA plasmid versus control. One-way ANOVA test was performed, followed by the unpaired t-test for normally distributed data. All data are expressed LI as means ± SD (C and F). n = 3 each for GFP(+)p75(+) cells and GFP(+)Ki-67(+) cells in WT KGF+ versus WT PBS+ versus p75KO KGF+ versus p75KO PBS+ (C); n = 3 each for GFP(+)Ki-67(+) cells in p75CA plasmid versus control (F). **** P < 0.0001. Scale bars = 50 μm (A and D).
A previous study showed that proliferating stem/progenitor cells in the basal and upper layers of the OC of the middle ear occur through the induction of KGF and KGFR signaling. In the current study, GFP-positive pseudosтратified epithelium was observed in the OC after KGF vector transfection (Figure 2, B and C). Similar to these results, Tucker et al showed that the NC lineage middle ear epithelium that lined the promontory underwent hyperplasia in cases of otitis media in mouse genesis. They concluded that the increased expression level of keratin 5 (stem cell marker) and sex determining region Y-box 2 (Sox2) in the NC lineage cell was associated with an increase in the proliferation of the epithelium in response to the disease, which was particularly pronounced on the NC-derived promontory, as this tissue usually has an extremely low rate of proliferation. In the trachea, the Sox2-expressed cells have been shown to be important for maintaining proliferation and differentiation during normal homeostasis and following injury. These findings suggest that the expression of Sox2 in GFP-positive pseudostratified epithelium observed in the OC region should be analyzed in future studies.

Development of the NC is established by a pan-NC gene regulatory network that is composed of hierarchically organized modules of signaling molecules and transcription factors that dictate each stage of development. NC induction is thought to initiate by the actions of WNT1, leading to the establishment of the neural plate border, characterized by the expression of transcriptional factors, including paired box 3 (Pax3). These factors, in concert with signaling events, launch a distinct NC-specifier state marked by the expression of a suite of transcriptional factors at the neural folds, including Snail, Slug, Twist, and Sox9. Subsequently, NC cells undergo an epithelial-to-mesenchymal transition, acquire migratory capacity, and express transcriptional factors, including Sox10 and p75 nerve growth factor receptor. Therefore, the expression of NC-specific markers in the GFP-positive NC lineage cells in the KGF-inducing cholesteatoma tissues was examined (Figure 3). Comparison of transcription level of NC-specific markers in the KGF-induced cholesteatoma tissues with that of normal middle ear tissues by RT-PCR resulted in the detection of PCR-amplified products with p75 primer only in the KGF group but not in the control group (Figure 3B). Moreover, the level of PCR-amplified products with Snail primer tended to be high in the extracts of the KGF group (Figure 3B). Several p75-positive/GFP-positive cells were observed mainly on the upper and basal layers of the keratinizing epithelium and the mucosal layer of the cholesteatoma in vivo (Figure 3C). Some p75-positive/GFP-positive cells and Snail-positive/GFP-positive cells were observed on the stroma of cholesteatoma in vivo (Figure 3C). Based on these results, it was postulated that p75-positive NC lineage epithelial cells, p75-positive stromal cells, and Snail-positive stromal cells might constitute KGF-inducing cholesteatoma tissue. In fact, a large number of p75-positive cells were observed in epithelial and stromal regions, and some Snail-positive cells were observed in the stromal region in human cholesteatoma tissues (Figure 4). However, p75 is known as a marker of human oral keratinocyte stem/progenitor cells. Similar to the p75-positive human cholesteatoma cells, the lineage of these cells is not known, so further investigation is required.

Snail is a major determinant of the mesenchymal movement under epithelial-to-mesenchymal transition, which is crucial for the formation of many different tissues and organs during embryogenesis, such as for the development of the mesoderm in amniotes, the NC in all vertebrates, the heart cushions, and palate, among others. A recent study indicated that Snail levels direct and control the movement and the metastatic behavior of tumor cells. In the current study, Snail-positive/GFP-positive cells were observed on the stroma of cholesteatoma in vivo (Figure 3C); several Snail-positive cells were similarly observed in the stromal region of human cholesteatoma but not in normal skin (Figure 4A). Several growth factors, including KGF, epidermal growth factor, transforming growth factor-α, FGF1, and Scatter factor/hepatocyte growth factor, have been found to act as scatter factors and mitogens for epithelial-to-mesenchymal transition. As in the previous studies, KGF expression in the mesenchymal cells in KGF-inducing cholesteatoma might express Snail and thus direct and control the movement and metastatic behavior in the current study, but this needs further investigation.

p75 is known as an NC stem cell—specific marker; p75-positive NC cells are capable of self-renewal and multipotency in differentiating into neurons, glia, and smooth muscle cells. In mouse trachea and human airway epithelium, NGFR (p75)—positive and p63-positive (stem cell marker) epithelial basal cells control self-renewal during postnatal growth and epithelial repair in adults. It is also known that KGF/ KGFR signaling plays a role in epithelial cell growth during epithelial repair in the adult trachea. As expected, the p75-positive NC lineage cells observed mainly in the inner epithelial layer and stromal region of the PF of the TM in the control ears were increased in the upper and basal layers of the keratinizing epithelium and mucosal layer of the KGF-inducing cholesteatoma in this study (Figure 3C). Moreover, the localization of Ki-67—positive/GFP-positive cells overlapped with that of p75-positive/GFP-positive cells, indicating that p75-positive NC lineage epithelial cells had high proliferative activity under KGF signaling (Figure 3, C and D). p75 is the first growth factor receptor found to be expressed in fibroblasts of the dermal papilla and epithelial cells and serves to control stemness and proliferating activity at the development stage during hair follicle morphogenesis. In this study, the correlation of p75 expression and the KGF/KGFR signaling against hair follicle morphogenesis indicated a low expression of KGF during hair follicle development. These observations led us to speculate that the morphologic change of p75-positive NC lineage cells might depend on the expression of Snail and Snail-like factors.
level of KGF because the NC lineage cells of the KGF-inducing cholesteatoma model were exposed in a high expression level of KGF. Treatment of Wnt1-Cre/EGFP MEECs with KGF protein induced the expression of p75 protein and increased the proliferative activity of NC cells (Figure 6A–C). However, the precise characterization of NC lineage cells of cholesteatoma formation in KGF knockout mice or KGF transgenic mice should serve as the most appropriate proof for the validity of this hypothesis.

In melanoma, NC lineage cells have been found to dedifferentiate into mesenchymal-like cells exhibiting a mesenchymal-like gene expression profile with downregulated melanocyte-related genes and strongly upregulated NC cell marker genes, such as p75.70 Similarly, a small number of p75-positive NC lineage cells were observed in the mesenchyme of mouse cholesteatoma (Figure 3C), and a large number of p75-positive cells were detected in the stroma of human cholesteatoma tissue (Figure 4A). Because this study focused on the epithelial cell growth of cholesteatoma, the dynamics of mesenchymal cells were not examined. Analyzing the character and role of p75-positive mesenchymal cells will be the subject of a future study.

Because these results and analysis of human samples showed that p75-positive NC cell populations mainly form cholesteatoma (Figure 4), the aim was to control p75-positive NC lineage cells because of their high proliferative potential and immaturity. Finally, the elimination of p75-positive NC lineage cells was performed by injecting mu-p75-SAP into the cholesteatoma tissue in vivo. The number of p75-positive NC lineage cells in the cholesteatoma tissue decreased with a reduction in proliferative activity, and the cholesteatoma tissue disappeared (Figure 5D). In fact, a histologic examination indicated an almost normal structure of the TM and middle ear in the tissue sections from the hKGF expression vector-induced cholesteatoma treated with 0.6 mg/mL mu-p75-SAP (Figure 5C). These results indicate the possibility that regulating p75 signaling may suppress NC cell proliferation. To address the role of p75 against the proliferative activity of NC lineage MEECs, p75KO assay and p75CA plasmid transfected assay were performed in vitro (Figure 6). As result, p75KO NC lineage MEECs had low proliferative activity, whereas the ligand-independent activity of p75 in p75CA plasmid-transfected–NC lineage MEECs had a high proliferative activity (Figure 6, D and F). These results also indicate the possibility that p75-positive NC lineage cells might play a role not only in increasing the size of the cholesteatoma through proliferation, but also in maintaining the cholesteatoma tissue. Some studies indicate the self-renewal potential of p75-enriched NC cells.72,73 More recent work has indicated that human adult NC-derived stromal cells within the nasal cavity kept their expression of NC and stemness marker p75 and showed the capability of sphere formation and clonal growth, thus keeping their stem cell character in vitro.74 This hypothesis needs to be further investigated.

In conclusion, herein is the first demonstration that the NC lineage cells consist of cholesteatoma tissues in the mouse model. It was demonstrated that p75-positive NC lineage cells have stem/progenitor potential and showed proliferative activity under KGF signaling during cholesteatoma formation. The p75-positive NC lineage cells could be a therapeutic target for this disease, although further studies are necessary to elucidate the cell dynamics of these cells in the pathophysiology of cholesteatoma.

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