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Original Article

Expression patterns of fibroblast growth factor 8 in the spiral ganglion of rat cochlea

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Abstract: Fibroblast Growth Factor 8 (FGF8) plays a critical role in promoting the growth of axons in the brain, and it may also be a potentially important regulatory factor in the auditory system. However, no report has yet studied the expression patterns of FGF8 in spiral ganglions. Therefore, we evaluated the expressions of FGF8 and its receptors in the spiral ganglion of rats' cochleae using immunocytochemistry and Western Blot. FGF8 was found expressed in adult rats spiral ganglion of both upper and basal turn of the cochleae, specifically in the cytoplasm of the spiral ganglion neurons (SGNs), while it was not observed in the nuclei and glial cells. FGFR1 but not FGFR2 expression was present in the spiral ganglion of adult rats, suggesting that FGF8 might function through FGFR1 to exert its effects. Moreover, FGF8 protein reached its maximal expression at early stages of postnatal development and decreased in adulthood. Collectively, our study provides preliminary proof for the presence of FGF8 in the primary auditory neurons, indicating a potential role for FGF8 in modulating neuronal functions within the spiral ganglion.

Keywords: FGF8, FGFR1, spiral ganglion, spiral ganglion neurons, cochlea

Introduction

Spiral ganglion is the peripheral sensory ganglion located in Rosenthal's canal, and consists of spiral ganglion neurons (SGNs), glial cells, and connective tissues. The function of spiral ganglion is to receive electric signals from the cochlea and transfer it to the central nervous system (CNS). Based on morphological and physiological differences, SGNs can be categorized into type I and type II SGNs. Type I SGNs are myelinated and connected to the inner hair cells (IHC), in which sound signals of different frequencies are edited. Type II SGNs are not myelinated and connected to outer hair cells (OHC) [1]. Noise, ototoxic medicine, genetic mutations and aging can damage SGNs and subsequently lead to cell degeneration and death [2, 3]. Because SGNs are the primary neurons in the auditory pathway responsible for the development and maintenance of language, it is crucial to prevent damages to them and promote SGNs regeneration. Recent studies showed that fibroblast growth factors (FGFs) can delay SGNs degeneration and promote neuronal processes regeneration [4].

Previous studies indicated that FGFs have a pivotal role in the development, metabolism, and regeneration of primary auditory neurons [5-7]. There are at least 22 structurally related members of the FGF family, which bind to four high affinity, ligand-dependent FGF receptors (FGFR1-4) to exert their effects [8]. In addition, FGFs play an important role in the development of the inner ear. Indeed, Pirvola et al. reported that FGF1 is expressed in the rat inner ear [9], while Luo et al. reported FGF1 expression during the development of spiral ganglions, with an increased expression from basal turn to apex turn [10]. In addition, Mckay et al. found that FGF3 is required to induce otocyst formation during development [11]. Importantly, FGF8 is involved in the brain development during embryo developmental stage, since it promotes axon growth in the CNS [12-14]. However, despite its important role, few studies have investigated FGF8 role in the auditory system.

Leger et al. demonstrated that FGF8 is required for zebrafish inner ear patterning [15], while Ladher et al. reported that FGF8 plays a critical upstream role in an FGF signaling cascade that initiated oticplacode induction in chicks and mice [16]. Furthermore, Jacques et al. reported that FGF8 regulates the differentiation of pillar cells, suggesting a role in regenerative therapies [17]. More recently, Garcia et al. suggested that FGF8 may promote the *in vitro* SGNs regeneration in mice [4].

Taken together, these studies indicate that FGF8 might play an important role in the auditory system. However, no evidence is available regarding FGF8 expression in the spiral ganglion. On the basis of the above evidence, we hypothesize that FGF8 and its receptors might be expressed in the spiral ganglion of the cochlea. Thus, we evaluated the distribution of FGF8 and its receptors in the spiral ganglions of postnatal and adult rats.

Materials and methods

Animals

A total number of 30 Sprague-Dawley male rats at the age of 7, 14, 28 postnatal days (P7, P14, P28, respectively) and 10 adults (P32) were provided by the Animal Experimental Center of the Xi'an Jiaotong University School of Medicine, Xi'an, China. Rats use and euthanasia were performed in accordance with the institutional guidelines of the Xi'an Jiaotong University School of Medicine.

Rats' cochlear tissue collection and processing

After anesthesia by 10% pelltobarbitalumnatricum, the rats were decapitated using surgical scissors and the skulls were opened from the midsagittal plane. The cochlea was obtained by microdissection of the temporal bone of each rat. Cochlear samples were washed in ice-cold phosphate buffered saline (PBS) and prepared for further studies. Due to the specific anatomy of the cochlea in which peripheral tissues, such as the organ of Corti, striavascularis, as well as spiral ligaments, need to be dissected from the modiolus tissue (containing the spiral ganglion), it is crucial to involve an experienced technician to separate the border between spiral limbus and spiral ganglion [18, 19].

Immunocytochemistry

Immunocytochemistry was used to evaluate FGF8 expression in the adult rat's spiral ganglion (P32). Hoechst stain, FGF8 antibody, and Tubulin B-III (TuJ1) were used to mark the nucleus, FGF8, and SGNs, respectively. The frozen cochlear tissues of adult rats were cut into sections and prepared for immunocytochemistry staining as follows: cochlear samples were fixed with 4% paraformaldehyde (PFA) solution (PFA in 0.1 M PBS, pH 7.2) and stored in 4°C for 12 h. Then, the samples were immersed in 10% sucrose cryoprotection solution for 4 h, followed by 30% sucrose for 24 h. Next, all cochleae were embedded in OCT compound (Sakura Finetek, USA) frozen at -20°C and cut into 20 μm thick midmodiolar cross-sections using a cryostat microtome (Leica CM1850, Germany) and placed on poly-l-lysine-coated slides. Immunocytochemistry staining was performed as follows: after rinsing with 0.01 M PBS (pH 7.4), slides were treated with PBS containing 0.1% Triton X-100 for 20 min. Next, slides were rinsed with 0.01 M PBS, and blocked with 5% normal donkey serum (NDS, Jackson ImmunoResearch, USA) for 30 min at 37°C and incubated overnight at 4°C with monoclonal mouse anti-FGF8 antibody (R&D Systems, USA), polyclonal mouse anti-Tubulin-III antibody (TuJ1, R&D Systems), and either polyclonal rabbit anti-FGFR1 or anti-FGFR2 antibody (both 1:250; Boster Bio, China) in PBS. After thoroughly rinsing, the specimens were treated with Alexa Fluor 594-conjugated donkey anti-mouse IgG (1:500; Invitrogen, USA), DyLight 488-conjugated goat anti-rabbit IgG and Cy3-conjugated goat anti-rabbit IgG (both 1:300; Boster Bio, China) antibody solution at room temperature for 1 h. Each experiment also included a negative control in which PBS replaced the primary antibody. After rinsing, the sections were examined under a fluorescent microscope (Olympus, Japan). All images were saved as TIFF files and processed with Adobe Photoshop CS6 (Adobe Systems, USA) for adjustments. Cochlea nuclei emitted blue fluorescence by ultraviolet excitation under fluorescent microscope, SGNs emitted green fluorescence by blue-ray excitation, FGF8 emitted red fluorescence by green-ray excitation, FGFR1 emitted green fluorescence by blue-ray excitation, and FGFR2 emitted red fluorescence by green-ray excitation.

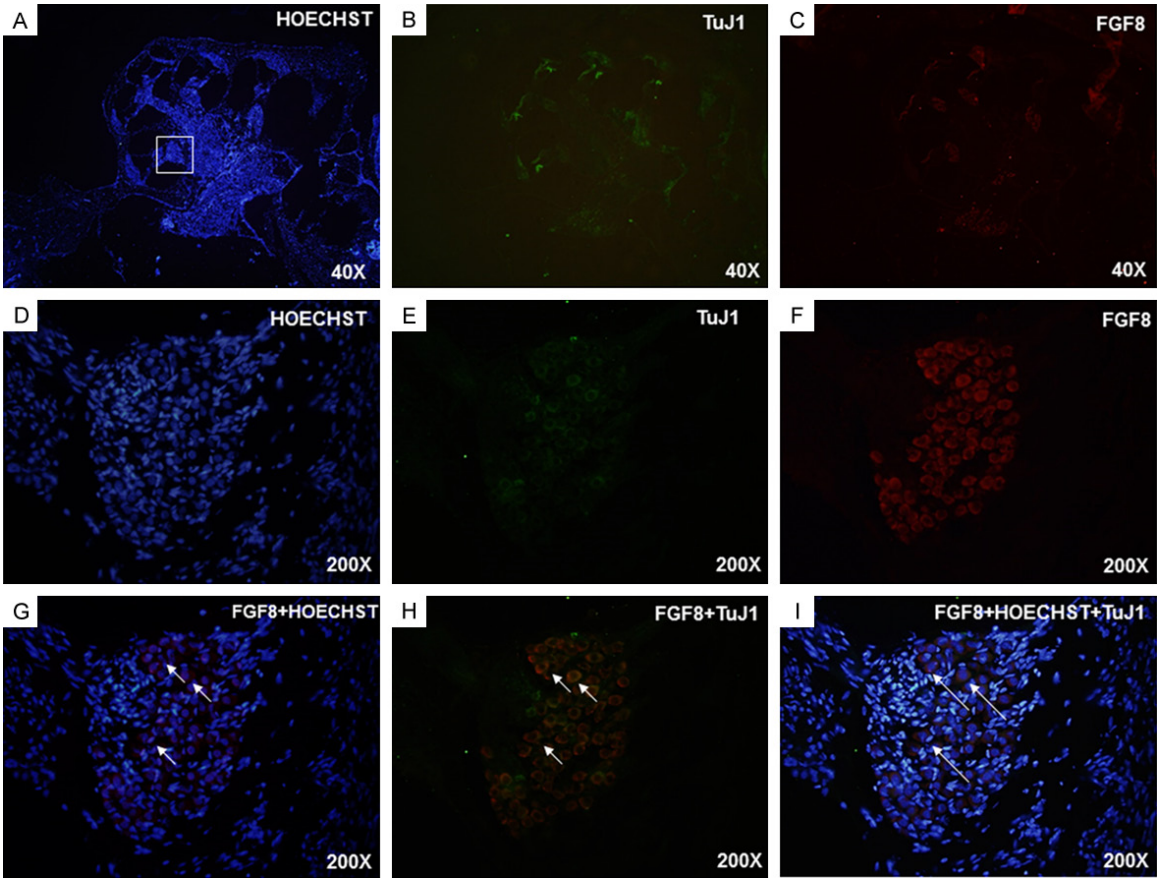

Figure 1. The distribution of FGF8 in the spiral ganglions of adult rats. (A) Low magnification image of adult rat cochlear sections triple labeled with Hoechst, TuJ1, and FGF8 showed the nuclei of cochlea (Hoechst, blue, 40×). (B) SGNs in the spiral ganglions (TuJ1, green, 40×). (C) FGF8 in the spiral ganglions (Red, 40×). (D) The magnification of the rectangle highlighted in (A) shows the nuclei of spiral ganglion cells (Hoechst, blue, 200×). (E) SGNs in the spiral ganglion (TuJ1, green, 200×). (F) FGF8 is evenly distributed in the spiral ganglion (Red, 200×). (G) Merged image of (D and F), short arrows indicate FGF8 distributed around the nuclei of the spiral ganglion cells. (H) Merged image of (E and F), short arrows show the co-localization of FGF8 and SGNs, indicating FGF8 is distributed in the SGNs. (I) Merged image of (D-F), long arrows indicate FGF8 is distributed in the cytoplasm of SGNs.

Western blot

Western blot assay was used to validate whether FGF8 protein was also present in postnatal rat's spiral ganglion (P7, P14, and P28). The modiolus tissues of postnatal rats cochlea, at P7, P14, and P28 containing the spiral ganglion tissues, were used. Total protein extracts of spiral ganglions were obtained from modiolus tissue by a micro tissue grinder with 0.1% sodium dodecyl sulfate (SDS), and lysis buffer containing 20 mM Tris-HCl, 0.5% sodium deoxycholate, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, a protease inhibitor cocktail (Sigma, USA), and 1 mM phenylmethylsulphonyl fluoride. Next, the specimens were crushed, sonicated, and centrifuged for 25 min at 14,000 rpm at 4°C. Protein concentration in the supernatant was evaluated with a spectrophotometer (NanoDrop 2000c; Thermo Fisher Scientific, USA). Protein samples (30 μg) were separated by 10% SDS-PAGE (Bio-Rad, USA) and transferred onto a polyvinylidene fluoride membrane (PVDF; Millipore, USA) using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad). The blots were incubated in blocking buffer containing 5% nonfat dry milk in PBS-T (0.1% Tween 20 in PBS) for 1 h and then incubated overnight with primary antibody (monoclonal mouse anti-FGF8, 1:500; R&D Systems) diluted in blocking buffer at 4°C. The blots were washed in PBS-T and incubated at room temperature for 2 h with the correspondent peroxidase-conjugated secondary antibody (1:800, Santa Cruz, USA), and developed using an enhanced chemiluminescence reagent (Thermo Fisher Scientific, USA). Immuno-

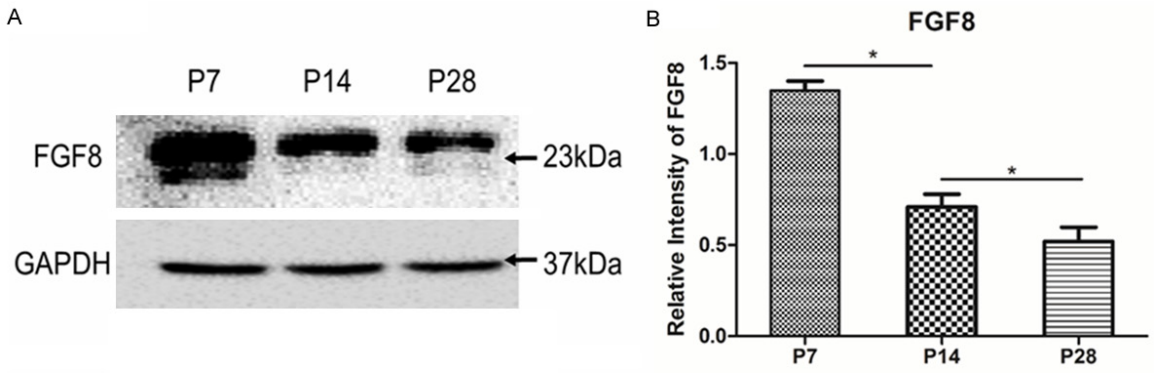

Figure 2. Expression patterns of FGF8 in the postnatal rat spiral ganglion. A. Western blot assay for FGF8 and GAPDH in the rat spiral ganglion during cochlear development. There are two bands of FGF8 at P7, the upper strong band (23 kDa) is the band for FGF8, while the lower one is nonspecific. B. Relative quantification of expression patterns of FGF8 in the rat spiral ganglion using GAPDH as an endogenous housekeeping control protein. $^{*}P < 0.05$, P14 versus P7, P28 versus P14.

reactive bands were visualized on Kodak LS film (Kodak Eastman, USA). Bands relative density was measured by Image Pro Plus software (IPP, version 6.0, Media Cybernetics, USA). FGF8 corresponded to a band of approximately 23 kDa, while GAPDH band was at 37 kDa. FGF8 expression at different developmental stages was normalized using GAPDH as reference protein. Western blot was performed at least three times.

Statistical analysis

All data were expressed as the mean ± standard deviation (SD). According to the distribution of the data by normality tests, Student's *t*-test or Wilcoxon rank-sum test were used to compare differences in the relative protein expression levels analyzed as a continuous variable among groups. All tests were two-sided and *P* value less than 0.05 was considered statistically significant. All statistical analyses were performed with SAS software (version 9.4; SAS Institute, Inc., Cary, NC).

Results

FGF8 distribution in spiral ganglion

Cells with negative TuJ1 staining were considered glia cells in the spiral ganglion. As shown in **Figure 1A-C**, FGF8 was evenly distributed in rat cochleae spiral ganglions, and no distinct differences in FGF8 expression were found between cochlear apical turn and basal turn. As shown in **Figure 1G**, FGF8 was observed around the nucleus of the spiral ganglion cells, indicating that FGF8 was distributed in the cytoplasm of the cells. Moreover, FGF8 was co-localized with TuJ1-positive SGNs (**Figure 1H**), indicating that FGF8 was present in SGNs, but not in glial cells. Overall, FGF8 was mainly distributed in the cytoplasm of SGNs, as shown in **Figure 1I**.

FGF8 expression in postnatal rat spiral ganglions

As shown in **Figure 2**, FGF8 protein was expressed at P7, P14, and P28. In normality tests, the relative protein expression levels at P7, P14, and P28 were all normally distributed. In Student's *t*-test for differences in relative protein expression levels between different stages, the highest expression level was observed at P7 (1.35 ± 0.05), but decreased significantly at P14 (0.70 ± 0.07, $P < 0.001$), and further reduced significantly to the minimum level at P28 (0.52 ± 0.08, $P < 0.001$).

FGFR1 and FGFR2 expression in spiral ganglions

As shown in **Figure 3A-F**, FGFR1 expression was evenly distributed in both the cytoplasm and nuclei of the spiral ganglion cells. However, we did not observe FGFR2 expression in the rat cochlear spiral ganglions under fluorescent microscope, as shown in **Figure 3G-I**.

Discussion

Our results provide preliminary proof for the presence and synthesis of FGF8 in primary auditory neurons during cochlear development,

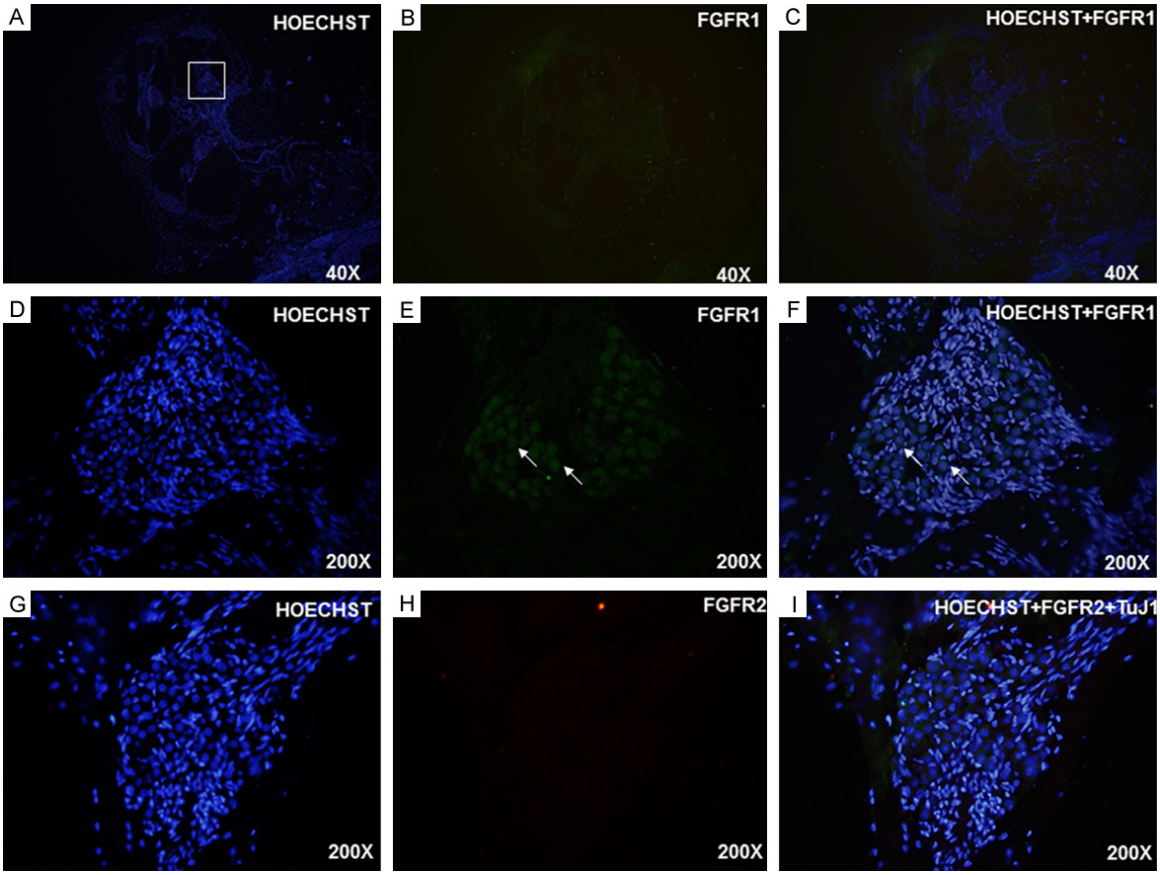

Figure 3. The expression of FGFR1 and FGFR2 in the spiral ganglions of adult rats. (A) Low magnification image of adult rat cochlear sections double labeled with Hoechst and FGFR1 showed the nuclei of cochlea (Hoechst, blue, 40×). (B) FGFR1 in the spiral ganglions (Green, 40×). (C) Merged image of (A and B). (D) The magnification of the rectangle highlighted in (A) shows the nuclei of the spiral ganglion cells (Hoechst, blue, 200×). (E) FGFR1 in the spiral ganglion (Green, 200×). (F) Merged image of (D and E), short arrows indicate FGFR1 is expressed in the spiral ganglion cells. (G) High magnification image of adult rat cochlear sections triple labeled with Hoechst, TuJ1 and FGFR2 shows the nuclei of the spiral ganglion cells (Hoechst, blue, 200×). (H) FGFR2 was not present in the spiral ganglion. (I) Merged image of (G and H), FGFR2 was not present in the spiral ganglion cells.

indicating a potential role of FGF8 in modulating SGN functions.

Increasing evidences have shown that FGF8 is widely expressed in human tissues during embryo development, with key roles in morphogenesis of the CNS, limb development, and the circulatory system [20-22]. Circumstantial evidence also indicated that FGF8 might promote the CNS axon regeneration, suggesting FGF8 might function to reconnect neural synapses after neural damage [13]. However, the role of FGF8 in the auditory system is not well documented as it is for the other FGFs' members. In the auditory system, FGF8 is required for cochlear development, in particular in the induction of the oticplacode, pillar cells differentiation, and patterning of the organ of Corti [15-17, 23]. Importantly, Garcia et al. demonstrated that FGF8 could promote the *in vitro* outgrowth of mouse SGNs [4]. However, no studies are available demonstrating FGF8 presence in spiral ganglion. On the basis of the above evidences, our results suggested that FGF8 might modulate synaptic transmission, neuronal development and neuronal regeneration in spiral ganglions, making it a promising target for novel therapies to prevent or deal with sensorineural hearing loss.

Previous studies showed that mammalian SGNs originate from an ectoderm thickening called the oticplacode. This then detaches from the ectoderm to form an ovoid otic vesicle. The inner ear neuron in the otic vesicle leaves the epithelium and proliferates to form the cochle-

ar-vestibule ganglion (CVG), and then part of the neurons from the CVG divide and segregate along Rosenthal's canal to form spiral ganglions [24, 25]. In the current study, we selected three key time points during the cochlear development of postnatal rats to measure the FGF8 protein expression. P7 is when the SGN soma undergoes myelination, P14 is when mature IHC is formed, and P28 represents rat maturation (rats older than P28 are considered as adult rats) [24]. Thus, according to the evidences described above, our results showing differential patterns of FGF8 protein expression in the spiral ganglion during postnatal development, suggesting the involvement of FGF8 in spiral ganglionneural development.

Previous studies found that FGF8 binds to FGFR1-4 to exert its functions [8]. In the auditory system, FGFR1-3 are present in the epithelium of the cochlear duct, whereas FGFR4 is restricted to the mesenchyme surrounding the duct [26]. Studies have shown that FGFR1 may play an important role in the auditory epithelium during development, and that blocking FGFR1 may cause up to 85% hair cell reduction [27]. Other studies found that FGFR1 is required in the signaling pathway involved in the differentiation of sensory progenitors during hair cell development [28]. In addition, Pirvola et al. reported that FGFR2 mRNA is expressed in the non-sensory epithelium of the otocyst, which later differentiates into endolymphatic and semicircular ducts [23]. Furthermore, FGFR3 can modulate the differentiation of supporting cells and thus influence the width of the auditory epithelium [29]. In the current study, we evaluated FGFR1 and FGFR2 expressions by immunocytochemistry, but not FGFR3 because a suitable antibody for FGFR3 is not available. Previous studies have provided direct evidence for the role of FGFR1-2 in the sensory compartment of the cochlea, suggesting a function for FGFR1-2 in the morphogenesis of the inner ear and modulation of progenitor cell differentiation. However, no study has illustrated the presence and function of FGFR1-2 in SGNs. Our results demonstrated the presence of FGFR1, but not FGFR2, in the cochlear spiral ganglion. Therefore, we assumed that FGF8 might signal via FGFR1 to modulate SGNs neuronal function and protect SGNs from neuronal damages.

In conclusion, our results indicated that FGF8 might participate in the regulation of neuronal function within the spiral ganglion by signaling through its receptors during development. The functions of FGF8 in modulating progenitor cell differentiation, synaptic transmission, as well as neuronal regeneration suggest it is an important regulator of auditory development, synaptic communication, and neuronal regeneration.

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Disclosure of conflict of interest

None.

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