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Research article

Expression of prestin in OHCs is reduced in *Spag6* gene knockout mice

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HIGHLIGHTS

- SPAG6 exists in cochlear outer hair cells.
- The expression of prestin in outer hair cells is significantly reduced in *Spag6* gene knockout mice.
- SPAG6 is indispensible for the stability of outer hair cells.
- *Spag6* gene is essential for mechanosensory function of outer hair cells.

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ABSTRACT

Sperm-associated antigen 6 (*Spag6*) gene, which encodes an axonemal protein (SPAG6), ubiquitously expresses in tissue and organs containing ciliated cells. The present work was to investigate whether SPAG6 expressed in cochlear hair cells and, if so, to explore the presumable correlations between prestin and SPAG6. The distribution of SPAG6 in organ of Corti and the morphological features of hair cells in basilar membrane were investigated by immunofluorescent staining. The amount of prestin in *Spag6* mutant mice was measured by Western blotting and real-time PCR, respectively. Additionally, co-immunoprecipitation tests were performed to confirm the presumed interaction between prestin and SPAG6. We observed that SPAG6 expressed in the cuticular plate in outer hair cells (OHCs) and prestin in the lateral wall of OHCs that located along with SPAG6 at this site. In comparison to *Spag6* +/+ mice, *Spag6* −/− mice showed apparent morphological abnormity of OHCs and lower intensity of prestin fluorescence. The expression of prestin in *Spag6* −/− mice reduced significantly at both protein and mRNA levels. Moreover, co-immunoprecipitation tests demonstrated the interaction between prestin and SPAG6. Taken together, these data indicate that SPAG6 is indispensible for the stability of OHCs by maintaining the normal expression of prestin, which implies that *Spag6* gene is essential for mechanosensory function of OHCs.

1. Introduction

Sperm-associated antigen 6 (*Spag6*) gene encodes an axonemal protein (SPAG6) within the sperm flagella, which is firstly found on infertile males [1] and essential for sperm motility and male fertility [2,3]. SPAG6 ubiquitously expresses in tissue and organs containing ciliated cells, such as central nervous system [4], respiratory system [3]. Deficiencies of this gene render severe diseases, highlighting that SPAG6 is significant for microtubule-related ciliated cells whereby the corresponding organs function normally.

As it is well-known, hair cells in organ of Corti are fundamental for hearing generation [5]. The cylindrical outer hair cell (OHC) is able to alter its length and stiffness in response to acoustic mechanical stimulation. These mechanical changes derive from a putative molecular motor designated prestin [6,7], which is absolutely required for electromotility and for the cochlear amplifier [8]. Nonetheless, the amount of prestin is impressionable to different conditions, such as the application of ototoxic drugs, noise exposure and interactions with specific biomolecules [9–11]. Till now, a diversity of proteins has been proved to interact with prestin *in vivo* [12–14].

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Noticeably, patients afflicted with primary ciliary dyskinesia often have hearing impairment simultaneously [15], which hints that some genes encoding microtubule-related protein are essential for auditory function. On the basis that SPAG6 widely distributes in ciliated cells and potentially involves in inner ear development [16], it is reasonable to hypothesize that this protein expresses in cochlear hair cells. Moreover, the cortical cytoskeleton, constituted by intracellular microtubules and actins, facilitates the transformative ability of OHCs. Therefore, if SPAG6 expresses in OHCs, it possibly associates with the process of electromotility and correlates to prestin. In this regard, the present works were designed to determine whether SPAG6 existed in cochlear hair cells and, if so, to study the presumable correlations between prestin and SPAG6.

2. Materials and methods

2.1. Genotyping and animal preparation

Spag6 mutant mouse models were generated previously [4]. Neonates were born by the intercross of *Spag6* +/− male and female mice, which were kindly provided by Zhang et al. [2]. For genotype identification, DNA was abstracted with a Tissue DNA Kit (D3396-02, OMEGA) and, all the procedures of PCR were complied with previous study [4]. All experimental procedures were conducted in accordance with the policies of the Animal Care Committee of Shandong University, Ji'nan, PR China.

2.2. Preparation of cochleae samples

The dissection and preparation of osseous labyrinths were conducted in accordance to previous research [7]. Basilar membrane was carefully peeled off and Reissner's membrane and tectorial membrane were removed simultaneously.

2.3. Immunofluorescent staining and image analysis

Immunofluorescent staining procedures were performed as antecedent description [7]. The primary antibodies were rabbit anti-prestin, (sc-30163, Santa Cruz), goat anti-SPAG6 (sc-165529, Santa Cruz) and rabbit anti-myosinVIIa (PA1-936, Thermo Scientific Pierce Antibodies). The secondary antibodies were Alexa Fluor 488 donkey anti-rabbit IgG (A-21206, Molecular Probes) and Alexa Fluor 647 donkey anti-goat IgG (A-21447, Molecular Probes). The cell nucleus and the F-actins (cilia bundles) were stained by DAPI (D9542, SIGMA) and FITC-Phalloidin (P5282, SIGMA), respectively.

Specimens were observed under a laser scanning confocal microscope (TSC SPE, LEICA). The 488 nm laser was used for the visualization of Alexa Fluor 488 and FITC-Phalloidin staining. The 635 nm laser was used for the visualization of Alexa Fluor 647. DAPI staining was watched under UV light, the 405 nm laser.

For hair cells counting, we used the cell counter tool in Image J software to accumulate the myosinVIIa-positive hair cells within the 400 μm length in the middle turn of the cochlea [17]. As for the quantification of fluorescence intensity of prestin, we also performed as previous study [17]. The fluorescence intensity ratio of *Spag6* −/− to *Spag6* +/+ mice in different time points were calculated.

2.4. Protein extraction and Western blotting

The isolated basilar membrane was dissociated by RIPA lysis buffer (P0013B, Beyotime Institute of Biotechnology) and then was centrifuged to harvest the crude protein. Crude protein was separated by SDS-PAGE electrophoresis and subsequently transferred onto PVDF membrane (Immobilon-FL, Millipore). The primary antibodies in Western blotting were rabbit anti-prestin (sc-22692, Santa Cruz), mouse anti-beta actin (TA-09, ZSGB-BIO), rabbit anti-myosinmyosinVIIa (PA1-936, Thermo Scientific Pierce Antibodies). The relative intensity values of the grayscale images were calculated by Image J software.

2.5. RNA extraction and real-time PCR

Total RNA of the basilar membrane was eluted with the RNA extraction kit (RNeasy Mini QIAcube Kit QIAGEN). Then cDNA was synthesized with the RevertAid First Strand cDNA Synthesis Kit (K1622, Thermo scientific). Three pairs of primers used in real-time PCR were as follows: prestin, forward primer: 5′-CGTCAAGGACAAAGTCACAGAG-3′, reverse primer: 5′-CCCGAGACCAAGTCACCTAA-3′; MoysinVIIa, forward promer: 5′-TGGTACACTTGACACTGAAGAAAAAGT-3′, reverse primer: 5′-CCATCGTTCAGCCTCTTGGT-3′; GAPDH, forward primer: 5′-AGGTCGGTGTGAACGGATTTG-3′, reverse primer: 5′-TGTAGACCATGTAGTTGAGGTCA-3′. Ingredients were 2 × SYBR Green Premix EX Taq 10 μL (RR42LR, Takara Biotechnology), forward primer 1 μL, reverse primer 1 μL, cDNA 1 μL, deionized water complemented for the rest part of the 25 μL system. The parameters of PCR program were 95 °C for 8 min (initial denaturation) followed by 40 cycles of 95 °C for 30 s (denaturation), 57 °C for 30 s (annealing) and 72 °C for 40 s (extention). Defined the relative expression of prestin mRNA in *Spag6* +/+ subjects as control, the $2^{-\Delta\Delta Ct}$ values of *Spag6* +/− and *Spag6* −/− mice [18] in each age group were calculated. The same measurements were applied to moysinVIIa.

2.6. Co-immunoprecipitation tests

Spag6 +/+ and *Spag6* −/− mice were sacrificed for co-immunoprecipitation tests. A moderate dosage of protein extracted from cochlear basilar membrane (300–500 μg) was incubated with 2 μg primary antibody for 2 h. The primary antibodies were rabbit anti-prestin (sc-30163, Santa Cruz), goat anti-SPAG6 (sc-165529, Santa Cruz) as well as the nonspecific normal rabbit IgG (sc-2027, Santa Cruz) and normal goat IgG (sc-2028, Santa Cruz), which acted as the negative control. Then 40 μL resuspended protein A/G PLUS-Agarose beads (sc-2003, Santa Cruz) was added into the mixture to bind the primary antibody at 4 °C overnight. Immunoprecipitate was collected by centrifugation and was stringently washed with RIPA lysis buffer. After washing, all supernatant was discarded and the beads were resuspended in electrophoresis sample buffer. Subsequently, Western blotting was performed to assay the pulled down proteins. Moreover, to avoid the interferential results, the host species of antibodies used in immunoprecipitation tests was different with that used in Western blotting.

2.7. Statistical analysis

Measurement data conformed to Gaussion distribution were analyzed by one-way analysis of variance (ANOVO), which was followed by Student-Newman–Keuls test for multiple comparisons. $P < 0.05$ was considered statistical significance.

3. Results

3.1. Genetic traits and gross features of the Spag6 gene knockout mice

For genotype identification of the neonates, the PCR results were completely consistent with the previous study [4]. As for the gross appearance of *Spag6* −/− mice, besides the antecedent

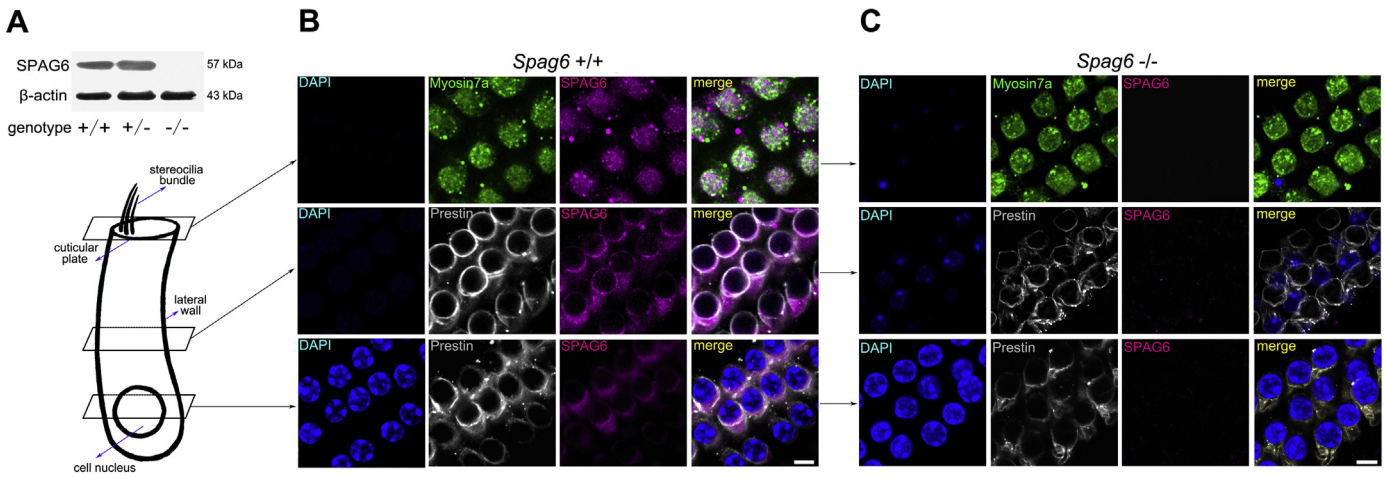

Fig. 1. The traits of SPAG6 distribution in OHCs. (A) SPAG6 exists in cochleae of *Spag6* +/+ and *Spag6* +/− mice. (B) In *Spag6* +/+ mice, SPAG6 localizes in cuticular plate and co-localizes with prestin in the lateral wall of OHCs. MoysinVIIa (light green) marks the cuticular plates of OHCs while prestin (light gray) specifically identifies the lateral wall of OHCs. No differences of fluorescence distribution patterns have been observed between *Spag6* +/+ and *Spag6* +/− mice, for which the latter is omitted. (B) SPAG6 signals cannot be detected in *Spag6* −/− mice. All scale bars represent 5 μm. Mice age in this figure is postnatal days 30 (P30). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

description [4], we also found that *Spag6* −/− mice appeared a frequently occurred fur-loss and exhibited abnormal behaviors, such as clumsy motion and continuous head tossing in comparison to their corresponding *Spag6* +/+ and *Spag6* +/− littermates.

3.2. Co-localization of prestin and SPAG6 in OHCs

SPAG6 do existed in basilar membrane, which was demonstrated by Western blotting. A 57-kDa band, the predicted size, was detectable in cochleae of *Spag6* +/+ and *Spag6* +/− mice but was absent in that of *Spag6* −/− mice (Fig. 1A). SPAG6 immunolabeling clearly marked cuticular plate in OHCs. Moreover, prestin and SPAG6 immunolabeling simultaneously localized in lateral wall of OHCs, in which site the distribution range of these two proteins widely overlapped (Fig. 1B). Yet the SPAG6 signals could not be detected in OHCs of *Spag6* −/− mice (Fig. 1C).

3.3. Morphological abnormalities of OHCs in Spag6 −/− mice

During the dissection of cochlea, we found certain distinctions of the osseous labyrinths between adult mice (≥P45) of different

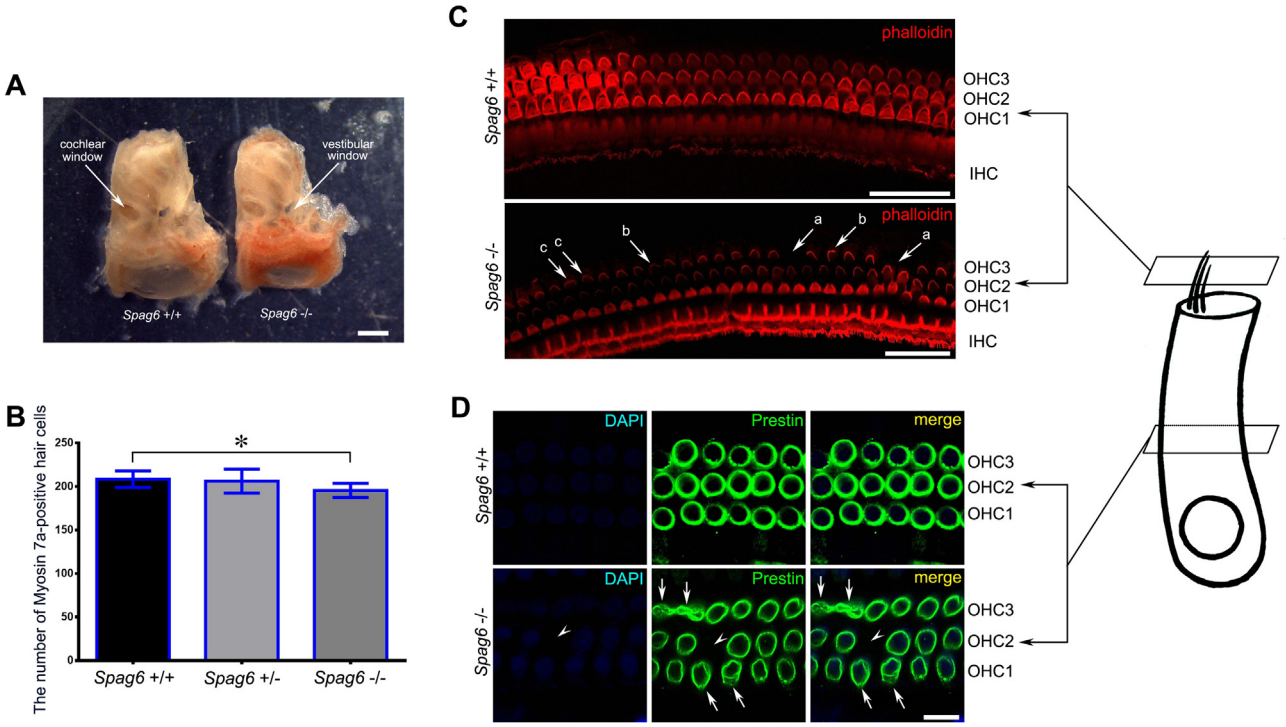

Fig. 2. Morphological abnormalities of OHCs in *Spag6* −/− mice. (A) Gross appearances of osseous labyrinths of *Spag6* −/− and *Spag6* +/+ mouse. (B) MoysinVIIa-positive hair cells counts per 400 μm length in cochlear middle turn. (C) Hair cells identified by phalloidin. Arrows show different morphological abnormalities of stereocilia bundles and the scattered OHC loss. (D) The lateral wall of OHCs identified by prestin. Arrows show the irregular lateral wall of OHCs and arrowhead shows OHC loss. Data shown here are the mean ± S.E.M., $^{*}P < 0.05$; Scale bars: 1 mm in A, 30 μm in C and 10 μm in D; IHC: inner hair cell; mice age: P45.

genotypes. The osseous labyrinths of *Spag6* −/− mice appeared smaller, worse ossified and more fragile to external force, which differentiated from osseous labyrinths of their *Spag6* +/+ littermates, indicating the possibility of delayed inner ears development (Fig. 2A).

In comparison to *Spag6* +/+ mice, hair cells in *Spag6* −/− mice appeared multiple morphological abnormalities, including the loss of single OHC as shown by the absent fluorescence of cilia bundles (Fig. 2C, a arrows), the disturbed orientation and the irregular shape of cilia bundles (Fig. 2C, b and c arrows). The intensive prestin immunolabeling appeared in *Spag6* +/+ mice (Fig. 2D, upper row), which was in contrast with the obscure and irregular immnolabeling in *Spag6* −/− mice (Fig. 2D, lower row, arrows). Prominently, the unexpected scattered OHCs loss occurred frequently in *Spag6* −/− mice (Fig. 2D, lower row, arrowhead). Subsequently, we quantified the

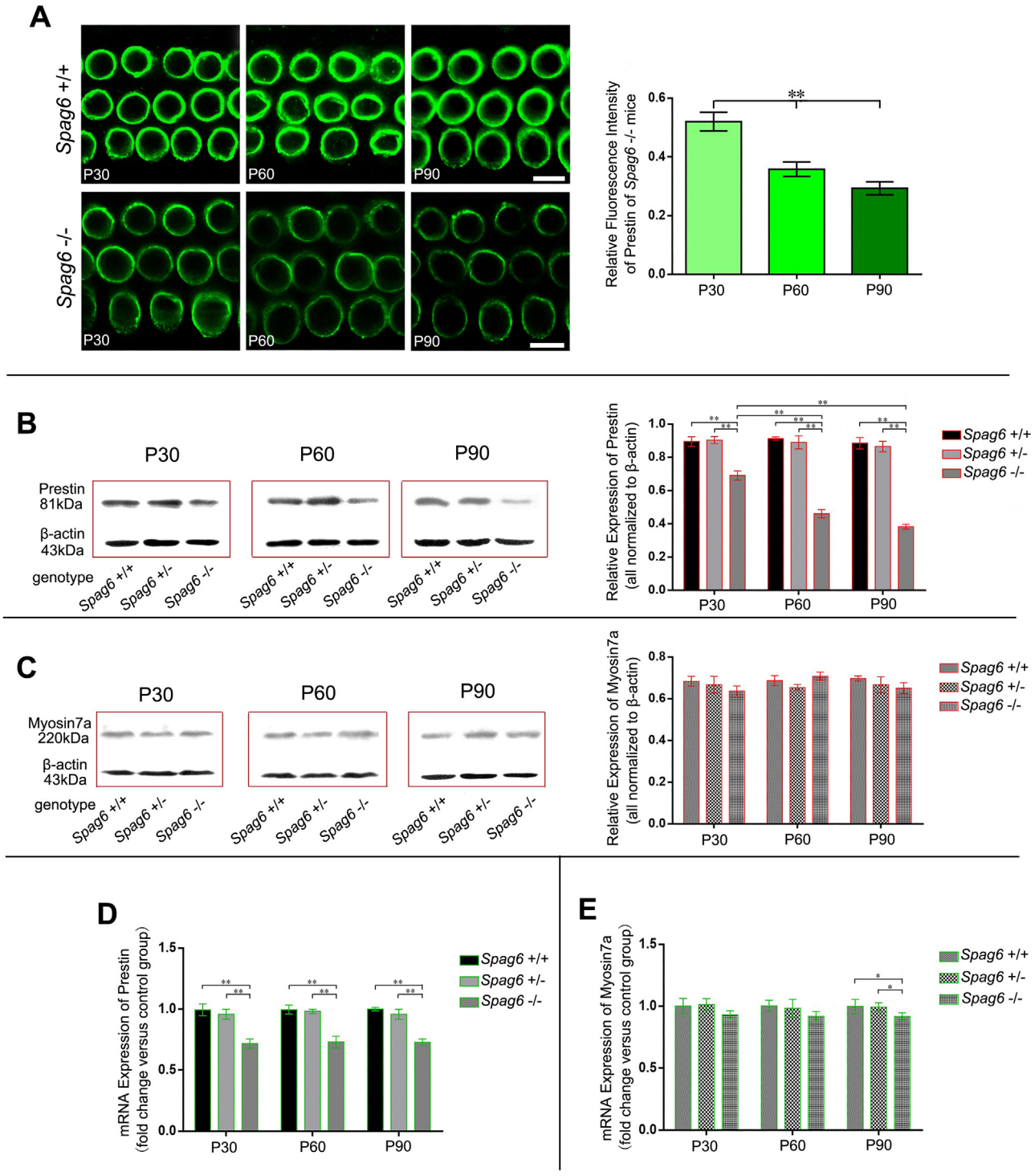

Fig. 3. Expression of prestin in different genotypic mice. (A) Immunofluorescent staining of prestin in *Spag6* +/+ and *Spag6* −/− mice during development and the corresponding statistical analysis of fluorescence intensity. Mice ages are shown by the corner marks. (B and C) Western blotting analyses of prestin and moysinVIIa in mice of different genotypes. The multiple comparisons are shown by the bars above the related columns. (D and E) Real-time PCR analyses of prestin and moysinVIIa in mice of different genotypes. Data shown here are the mean ± S.E.M., $^{*}P < 0.05$, $^{**}P < 0.01$; Scale bars in A: 5 μm.

moysinVIIa-positive hair cells in the middle turn of cochlea to measure the hair cell loss (Fig. 2B). The specific loss of OHCs was statistical significant between *Spag6* −/− and *Spag6* +/+ mice ($P < 0.05$) and there was no IHC loss, illuminating that the impairment of OHCs potentially resulted from the deletion of SPAG6.

3.4. Expression of prestin in different genotypic mice

The expression of prestin was tested in mice of different genotypes during their development by immunofluorescent staining, Western blotting and real-time PCR, respectively. Meanwhile, the expression of moysinVIIa also was measured as an internal control to prestin. We found, in comparison to *Spag6* +/+ mice, the corresponding *Spag6* −/− subjects showed weaker intensity of prestin fluorescence at the simultaneous time point (Fig. 3A left panel). The relative prestin fluorescence intensity of *Spag6* −/− mice in all age groups was calculated, which demonstrated a progressive decrease of prestin quantity after the auditory maturity ($P < 0.01$, Fig. 3A right panel).

The relative prestin expression ratio of *Spag6* −/− mice revealed a significant decrease trend during development (Fig. 3B, $P < 0.01$), which was consistent with the immunofluorescence data (Fig. 3A). In addition, no significant variation of moysinVIIa expression was detected (Fig. 3C). The expression of prestin mRNA in *Spag6* −/− mice markedly reduced in all time groups in comparison to those of *Spag6* +/+ and *Spag6* +/− littermates ($P < 0.01$) and the corresponding $2^{-\Delta\Delta Ct}$ values of *Spag6* −/− mice showed no statistical differences during the time course (Fig. 3D). As for moysinVIIa, distinct decrease of mRNA just appeared in time group P90 (Fig. 3E, $P < 0.05$). Given the mismatch of prestin and moysinVIIa data in both Western blotting and real-time PCR analyses, we considered that the expression of prestin is specifically reduced in *Spag6* −/− mice.

3.5. The interaction between prestin and SPAG6 in OHCs

In regard of all the aforementioned results, we speculated that an interaction between prestin and SPAG6 might exist. Co-immunoprecipitation tests showed that, in *Spag6* +/+ mice, prestin and SPAG6 could bind to each other in a reciprocal way (Fig. 4A and B). But in *Spag6* −/− mice, tests yielded no positive result (Fig. 4C).

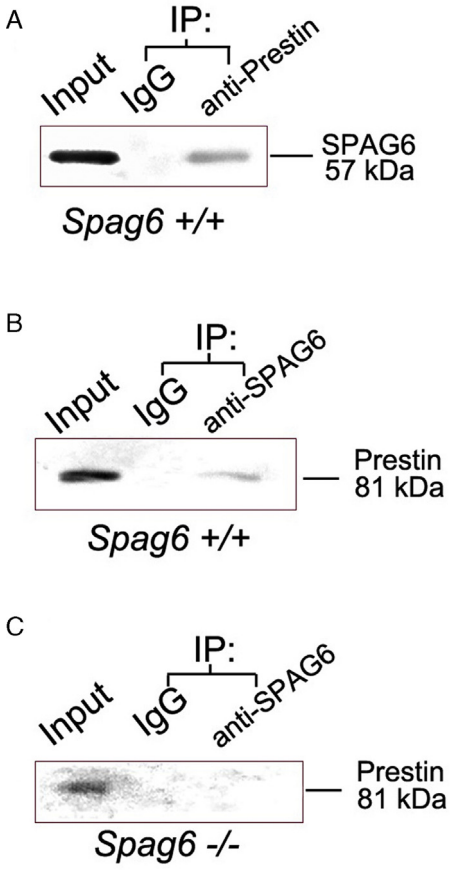

Fig. 4. Reciprocal co-immunoprecipitation tests between prestin and SPAG6. (A) Prestin pulls down SPAG6. (B) SPAG6 pulls down prestin. (C) Prestin cannot be detected by SPAG6 immunoprecipitation in *Spag6* −/− mice. Lane input: the crude protein extracted from basilar membrane, representing the positive control.

4. Discussion

In present study, neonatal mice were bred by the intercross of *Spag6* +/− male and female mice available from Zhang et al. [3]. The genetic traits and gross appearances of these neonates were consistent with the previous findings [4], which suggested that this mice strain was successfully reproduced in our lab, thereby laying a feasible foundation for the following experiments.

It has been confirmed that, rootlets of stereocilia and kinocilia anchor into cuticular plate, which is significant for the mechanosensory function of cilia bundles [5]. In this work, we found that SPAG6 localized in cuticular plate and, also, in lateral wall of OHCs in *Spag6* +/+ and *Spag6* +/− mice. The extensive distribution of SPAG6 in OHCs suggests that this protein is a crucial component of these specialized ciliated cells. Moreover, the highly overlapped distribution range of prestin and SPAG6 implies that these two proteins have a close infinity.

In following works, we found that *Spag6* −/− mice appeared visible abnormalities in arrange patterns of stereocilia bundles, as well as scattered OHCs loss and irregular shape of lateral wall of OHCs. These findings clearly demonstrate that SPAG6 helps to maintain the stability of plasma membrane in OHCs. However, it has not been confirmed the mechanism responsible for the irregular stereocilia bundles in *Spag6* −/− mice. As a microtubule-related protein within cuticular plate, SPAG6 is likely to regulate planar cell polarity of the cilia. Notably, the indispensible role of SPAG6 for hair cells also deciphers the abnormal behavior of *Spag6* −/− mice. The underlying impairment of hair cells in vestibular apparatus, which senses head movement and position, results in the equilibrium dysfunction in *Spag6* −/− mice. Therefore, the clumsy mobility and the continuous head tossing of *Spag6* −/− mice aggravates their developmental retardation because of the weakened ability of scrambling for food, as evidenced by their abnormal gross features [4] and the possible retardation of inner ear development.

It is reported that, tubulin acts as one constitution of cytoskeleton and possibly relates in docking molecules to the membrane of hair cells [5,19]. *Spag6* −/− mice displayed a visible and specific diminution of prestin density in lateral wall, revealing that, similar to tubulin, SPAG6 might also participate in the constitution of plasma membrane and maintaining the transmembrane proteins of OHCs. Akin to other proteins [14], a latent dependency relationship among SPAG6 and prestin was evidenced by the progressive decrease of prestin in *Spag6* −/− mice. Because prestin is a principal component of the cell's lateral wall [8], it is not surprising that the deletion of SPAG6 severely impairs the stability of cellular membrane and damages the mechanosensory function of OHCs. However, mechanism by which prestin mRNA are down-regulated in *Spag6* −/− mice is still unclear. Certain transcription factors, such as GATA-3, and Pou4f3 [20,21], may play different roles in transcription progress in *Spag6* −/− mice. Furthermore, some researchers note that *Spag6* gene likely involves in the chromatin assembly [22], indicating that SPAG6 potentially correlates with prestin transcription, and even with moysinVIIa transcription.

Available data show that prestin interacts with other proteins [10]. Prominently, one research reports that Microtubule-associated Protein 1S (MAP1S) binds to prestin *in vivo* and could augment the activity of prestin [12]. In the current study, the deduced interaction between prestin and SPAG6 was confirmed by the reciprocal co-immunoprecipitation tests. We conclude, therefore, that SPAG6 binds to prestin *in vivo* and contributes to fix prestin biomolecules in lateral plasma membrane of OHCs. Yet prestin is not completely eliminated in *Spag6* −/− mice, which implies that various factors and signaling pathways, together with SPAG6, may be responsible for maintaining this crucial transmembrane protein.

5. Conclusion

Taken together, we show, for the first time, that SPAG6 exists in OHCs inside the cochlea. Moreover, SPAG6 is found to be indispensible for the stability of OHCs by maintaining the normal expression of prestin, which implied that *Spag6* gene is essential for mechanosensory function of OHCs.

Acknowledgements

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