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Mir-27a promotes apoptosis of cochlear sensory epithelium in Cx26 knockout mice

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TABLE OF CONTENTS

1. ABSTRACT

To investigate the underlying molecular mechanism for connexin 26 (Cx26) knockout-induced apoptosis, we performed TUNEL assays to detect apoptosis in the cochlear sensory epithelium in Cx26 knockout mice. We also compared the miRNA expression profiles of Cx26 knockout and wild-type mice using microarray technology and bioinformatic analyses. Real-time PCR, luciferase reporter gene assays, and scala media microinjections were performed to identify the effect of a specific miRNA and its targets. The results showed that apoptosis increased in the cochlear sensory epithelium of Cx26 knockout mice. The abnormal expression of mir-27a and *sgk1* in Cx26 knockout mice was verified with real-time PCR. Luciferase reporter gene assays showed that overexpression of mir-27a significantly decreased *sgk1* reporter gene activity; an inhibitor of mir-27a blocked the effect. Mir-27a lentivirus also inhibited *sgk1* expression in cultured cochlear tissue. Mir-27a shRNA treatment inhibited Cx26 knockout-induced apoptosis in the cochlear sensory epithelium of mice and increased the expression of *sgk1* mRNA. Thus, mir-27a was identified as an apoptotic molecule that participates in Cx26 knockout-induced apoptosis in the cochlear sensory epithelium of mice by downregulating *sgk1* expression.

2. INTRODUCTION

Non-sensory cells in the cochlea are connected via gap junctions (GJs), which facilitate intercellular ionic and biochemical coupling. Connexin 26 (Cx26), a subunit in the Cx family, is a prominent member of most cochlear GJ assemblies (1,2). Cx26 mutations are the most common cause of human nonsyndromic hereditary deafness (3). It has been reported that deafness in mice resulting from Cx26 mutation is related to cell death within

the organ of Corti (4). In our previous study, we found that postnatal development of the organ of Corti was stalled in Cx26 knockout mice; the tunnel of Corti and Nuel's space never opened (5). Thereafter, massive cell death occurred in the middle turn and gradually spread to the basal turn, resulting in the secondary degeneration of spiral ganglion neurons in the corresponding cochlear locations (5). Although apoptosis has been identified as a cause of cell death, the mechanisms underlying Cx26 knockout induced-apoptosis have not been elucidated (6).

MicroRNAs (miRNAs) are a class of small non-coding RNAs of approximately 22 nucleotides (7). To date, more than 800 miRNAs have been identified in animal cells. MiRNAs play important regulatory roles in various biological processes, including proliferation, differentiation, and apoptosis, by targeting mRNAs for degradation or translational repression (7,8). A relationship between miRNAs and cell apoptosis has been identified. MiRNA-708 induces apoptosis and suppresses tumorigenicity in renal cancer cells (9). In an experimentally induced osteoarthritis model, overexpression of miRNA-146a upregulates vascular endothelial growth factor expression and increases chondrocyte apoptosis by inhibiting Smad4 in cartilage (10). Herein, we hypothesized that a miRNA mediates apoptosis in the cochlear sensory epithelium in Cx26 knockout mice. In the present study, we performed microarray-based high-throughput miRNA expression profiling to identify the candidate miRNA, and we analyzed the target genes responsible for Cx26 knockout-induced apoptosis.

3. MATERIALS AND METHODS

3.1. Animal studies

Conditional Cx26 knockout mice (Pax2-cCx26KO) were provided by Emory University and fed at the Experimental Animal Center of Shanghai Medical College, Fudan University. Wild-type mice were used as controls. The animals were housed under conventional conditions at 22 ± 1°C with a 12-h light/12-h dark cycle and free access to food and water. The experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All animals used in the study received humane care. The protocol for the generation and genotyping of Pax2-cCx26KO mice was described previously (5,11).

3.2. Cochlea dissection, cryosectioning, and culture

The mice were decapitated, the cochlea was quickly excised, and the bony shell of the cochlea was removed. The basilar membrane, spiral ligament, and stria vascularis were separated carefully. Detailed cochlear dissection and cryosectioning procedures have been described in our published papers (12,13). Cochlea tissues from wild-type mice (either sex) were used in the cultures. In brief, cochleae were dissected in ice-cold Hanks' buffered salt solution (HBSS; Invitrogen) at postnatal day 1 (P1). The glass coverslips were coated with 500 g/ml poly-d-lysine (Invitrogen) and 50 g/ml laminin (Invitrogen) for the cochlear culture. The culture medium contained Dulbecco's modified Eagle medium (DMEM; Sigma), 1 mM gentamicin (Sigma), 25 mM HEPES (Sigma), 6 mg/ml glucose, and 10 ng/ml N2 supplement (Invitrogen). The culture incubator was set at 37°C and 6.5% CO_2. The culture medium was replaced every 24 h. The volume of the culture medium was 1 ml. Samples cultured for 72 h were imaged and used for western blotting. The cultures were divided into two groups (n = 5 in each group), which were treated with either 300 nM mir-27a or defined medium only with no supplement.

3.3. Microarray

We separated the basilar membrane, spiral ligament, and stria vascularis from the cochlea of wild-type (n = 3) and knockout mice (n = 3) at P1. Total RNA was extracted using TRIzol reagent (Invitrogen) and labeled with the Hy3 fluorescent label as described previously (14). Microarray analysis of the miRNA and mRNA profiles was performed by KangChen Bio-tech (Shanghai, China) using the Exiqon Array and Agilent array platforms, respectively. To ensure the repeatability and reliability of the results, each group included at least three samples. Differential expression of miRNA/mRNA was defined as a fold change of >1.5 between wild-type mice and Cx26 knockout mice.

3.4. Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed to validate the differentially expressed miRNA and mRNA, according to a study described previously (15). For miRNA detection, a polyA tail was added to RNase-free total RNA using *Escherichia coli* polyA polymerase (NEB) to convert mature miRNA into cDNA. For mRNA detection, reverse transcription was carried out with a first strand cDNA synthesis kit (FSK-100) (Toyobo). Real-time PCR was performed using an Applied Biosystems 7900 Real-Time PCR System with SYBR Green Master Mix (Takara) and standard thermocycler conditions. All primer sequences are listed in Table 1.

3.5. Plasmids construction and transfection

The luciferase reporter plasmids used in the present study were constructed as previously described (15). To obtain the mir-27a expression vector, the DNA fragment encoding pre-mir-27a was PCR-amplified from the genomic DNA of mouse cochlear sensory epithelium and inserted into the lentiviral expression vector pCDH-EF1-MCS-T2A (Systems Biosciences). The lentivirus was packaged using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Table 1. Primers for qRT- PCR analysis

Primers	Primer sequence
Mir-27a F	GTGCTGAGGGCTTAGCTGCTTGTGAG
U6 F	CTCGCTTCGGCAGCACA
U6 R	AACGCTTCACGAATTTGCGT
Universal rev primer	CCAGTCTCAGGGTCCGAGGTATTC
Sgk1 F	AGTGGCCTCTGAAAGTGGG
Sgk1 R	GGAACGCTGAGCATTTCTTG
GAPDH F	GTCGGTGTGAACGGATTTGG
GAPDH R	TCCTGGAAGATGGTGATGGG

To construct a recombinant adenoviral vector with which to inhibit mature mir-27a expression, we synthesized three duplicated fragments, fully complementary to the mir-27a sequence, and cloned them into the shuttle vector pAdTrack-CMV (SinoGenoMax) using miRNA sponge technology. To overexpress *sgk1 in vivo*, the complete coding sequence of *sgk1* was subcloned into the BamHI and EcoRI sites of the pShuttle-CMV vector. The successfully constructed plasmids were linearized and introduced into BJ5183 bacterial cells with the pAdEasy-1 vector for homologous recombination. The recombinant adenovirus plasmids were then transfected into 293A cells.

3.6. Cell culture and luciferase assay

HEK-293 and 293A cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 g/ml streptomycin at 37°C and 5% CO_2. The luciferase assays were conducted as previously described (16). HEK293 cells were seeded at a density of 50% in 6-well plates. After 12 h, the cells were co-transfected with the luciferase vector, the mir-27a expression vector or inhibitor, and a psiCHECK-2 vector containing the 3′ untranslated region (UTR) or the 3′UTR-mut fragment of *sgk1* using Lipofectamine 2000 (Invitrogen). The luciferase activity was measured 36 h after transfection using the Dual Luciferase Reporter Assay System (Promega).

3.7. Microinjections

In order to avoid adverse effects on the hearing sensitivity, P1 mice were used for microinjections. The scala media of the right cochlea was injected. To detect apoptosis in the cochlear epithelium, we chose P8 as the time point. Adenovirus carrying mir-27a or a control vector (200 nl) was injected into the scala media of the cochlea of Cx26 knockout mice at P1. Samples were harvested for cryosectioning at P8. The details and instruments used in surgery were described previously (16).

3.8. Apoptosis assays

For the TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay, cryopreserved cochlear sensory epithelium samples from Cx26 knockout and wild-type mice were fixed in freshly prepared 4% paraformaldehyde in PBS (pH 7.4) for 20 min at room temperature. The fixed tissue sections were then washed with PBS for 30 min and incubated in permeabilization solution for 2 min on ice. The TUNEL reaction was performed in microcentrifuge tubes (1.5 ml) using the In Situ Cell Death Detection Kit (catalog #11684817910; Roche) according to the manufacturer's instructions. As a nuclear counterstain, 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI, 1 μg/ml; Sigma) was added, followed by the TUNEL reagents. Each experiment included negative and positive controls. DAPI-stained and TUNEL-positive nuclei were observed under a confocal laser scanning microscope. The experiment was repeated at least three separate times.

3.9. Western blotting

The protein levels of candidate genes were determined with western blot. Total protein was isolated from cochlear tissues with ice-cold lysis buffer (50 mM Tris HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100) containing a protease inhibitor cocktail (Roche). The proteins extracted from Cx26 knockout mice and the controls were separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Millipore). The membranes were blocked with 5% skim milk, incubated overnight at 4°C with primary antibodies against sgk1 (polyclonal, 1:500; BioWorld), and reacted with the corresponding secondary antibodies at room temperature. Antibodies were detected with enhanced chemiluminescence, and the results were recorded on X-ray film.

3.10. Statistical analysis

All results were expressed as the mean ± SD. The statistical significance was determined using the SPSS 11.0 statistical program. One-way ANOVA was performed for multiple comparisons followed by Fisher's LSD post-hoc comparison test. Differences were deemed significant if the calculated P-value was <0.0.5.

4. RESULTS

4.1. Cx26 knockout-induced apoptosis in the cochlear sensory epithelium in mice

As shown in Figure 1, apoptosis in the cochlear sensory epithelium was higher in Cx26 knockout mice than in wild-type mice.

4.2. Identification of apoptosis-associated molecules in Cx26 knockout mice

To identify candidate molecules related to apoptosis in the cochlear sensory epithelium, microarray-based high-throughput miRNA expression profiling was performed. We analyzed miRNA expression in three Cx26 knockout mice and three wild-type mice. To identify miRNAs that were differentially expressed in Cx26

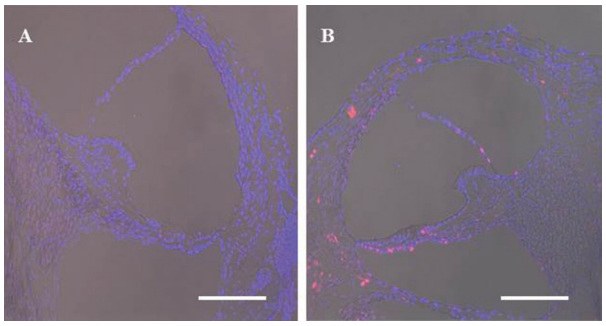

Figure 1. TUNEL assay. Apoptosis of cochlear sensory epithelium in Cx26 knockout mice was obviously increased compared with that in the wild type mice. (A) The wild type mice. (B) Cx26 knockout mice. Nuclei were stained with DAPI (blue). Scale bars: 200μm.

knockout and wild-type mice, we performed a statistical comparison of the two groups of samples (1.5-fold change and P < 0.0.5). Thirty miRNAs were differentially expressed in Cx26 knockout mice, of which 18 were increased and 12 were decreased, relative to levels in wild-type mice.

The potential target genes of the differentially expressed miRNAs were identified based on the overlap between two prediction programs, TargetScan and Miranda. For the 30 differentially expressed miRNAs, we hypothesized that the mRNA levels of their target genes were negatively regulated. Therefore, the predicted target genes that were also negatively regulated in our mRNA microarray analysis were selected as candidate targets.

To assess the possible biological effects of the 30 differentially expressed miRNAs in Cx26 knockout mice, we performed pathway enrichment analysis with the predicted targets of the 30 miRNAs (587 genes). As shown in Figure 2A, the targets were enriched for apoptosis-related functions, such as anti-apoptosis, induction of apoptosis, and negative regulation of protein biosynthesis. Furthermore, we constructed a co-expression network using the k-core algorithm to determine which genes might play pivotal roles in apoptosis in Cx26 knockout mice, based on an analysis of Gene Ontology (GO) terms. We obtained 39 genes (Figure 2B).

4.3. Confirmation of mir-27a and *sgk1* expression in Cx26 knockout mice

To identify a specific miRNA and the corresponding targets that mediate apoptosis in Cx26 knockout mice, we compared the expression of the 30 candidate miRNAs and 39 genes in the cochlear sensory epithelium of Cx26 knockout (n = 12) and wild-type (n = 10) mice using real-time PCR. Some miRNAs and mRNAs were differentially expressed in Cx26 knockout and wild-type mice (data not shown). Luciferase assays were then performed to validate the selected miRNA-mRNA pairs. The role of mir-27a and its target gene, *sgk1* (serum- and glucocorticoid-inducible kinase 1), in apoptosis was characterized. In the apoptosis co-expression network, *sgk1* showed the highest degree, correlating with seven miRNAs, including mir-27a, whose alteration was most prominent. Real-time qRT-PCR analysis showed that the expression of mir-27a increased ~1.7-fold and the expression of *sgk1* decreased ~0.7-fold in Cx26 knockout mice, when compared with expression in wild-type mice (Figure 3), consistent with the results obtained from microarray. Therefore, we hypothesized that mir-27a plays an important role in Cx26 knockout-induced apoptosis in cochlear sensory epithelial cells, with *sgk1* as its target.

4.4. Mir-27a directly targets the 3′UTR of *sgk1*

Next, we determined whether there was a direct relationship between mir-27a and *sgk1* by using the luciferase reporter gene assay. An *sgk1* reporter vector was transfected into HEK-293 cells with or without the mir-27a expression plasmid, pSilencer4.1-miR-27a. As expected, the luciferase activity of the wild-type *sgk1* 3′UTR vector was significantly inhibited when overexpressed with mir-27a. However, when the seed regions of the *sgk1* 3′UTR were mutated (Figure 4A), the luciferase activity was not affected by mir-27a overexpression. Furthermore, a mir-27a inhibitor blocked the decrease in *sgk1* reporter gene activity induced by the overexpression of mir-27a (Figure 4B).

4.5. Mir-27a lentivirus inhibits the expression of sgk1 in cultured cochlea tissue

We demonstrated that mir-27a directly downregulated *sgk1* reporter gene activity. However, whether overexpression of mir-27a also decreased the expression of sgk1 was not yet clear. Therefore, cultured cochlear tissue was infected with lentivirus encoding mir-27a-GFP. The results showed that mir-27a-GFP was extensively expressed in the cultured cochlear tissue (Figure 5A). Western blot showed that overexpression of mir-27a reduced the expression of sgk1 in cultured cochlea tissue (Figure 5B).

4.6. Mir-27a sponge inhibits Cx26 knockout-induced apoptosis in the cochlear sensory epithelium in mice

In the previous experiments, mir-27a was identified as a mediator of Cx26 knockout-induced apoptosis in the cochlear sensory epithelium of mice. However, whether mir-27a participated in Cx26 knockout-induced apoptosis required verification. Therefore, adenovirus carrying mir-27a sponge was injected into the scala media of the cochlea of Cx26 knockout mice. A TUNNEL assay showed that mir-27a sponge decreased Cx26 knockout-induced apoptosis in the cochlear sensory epithelium (Figure 6A). Furthermore,

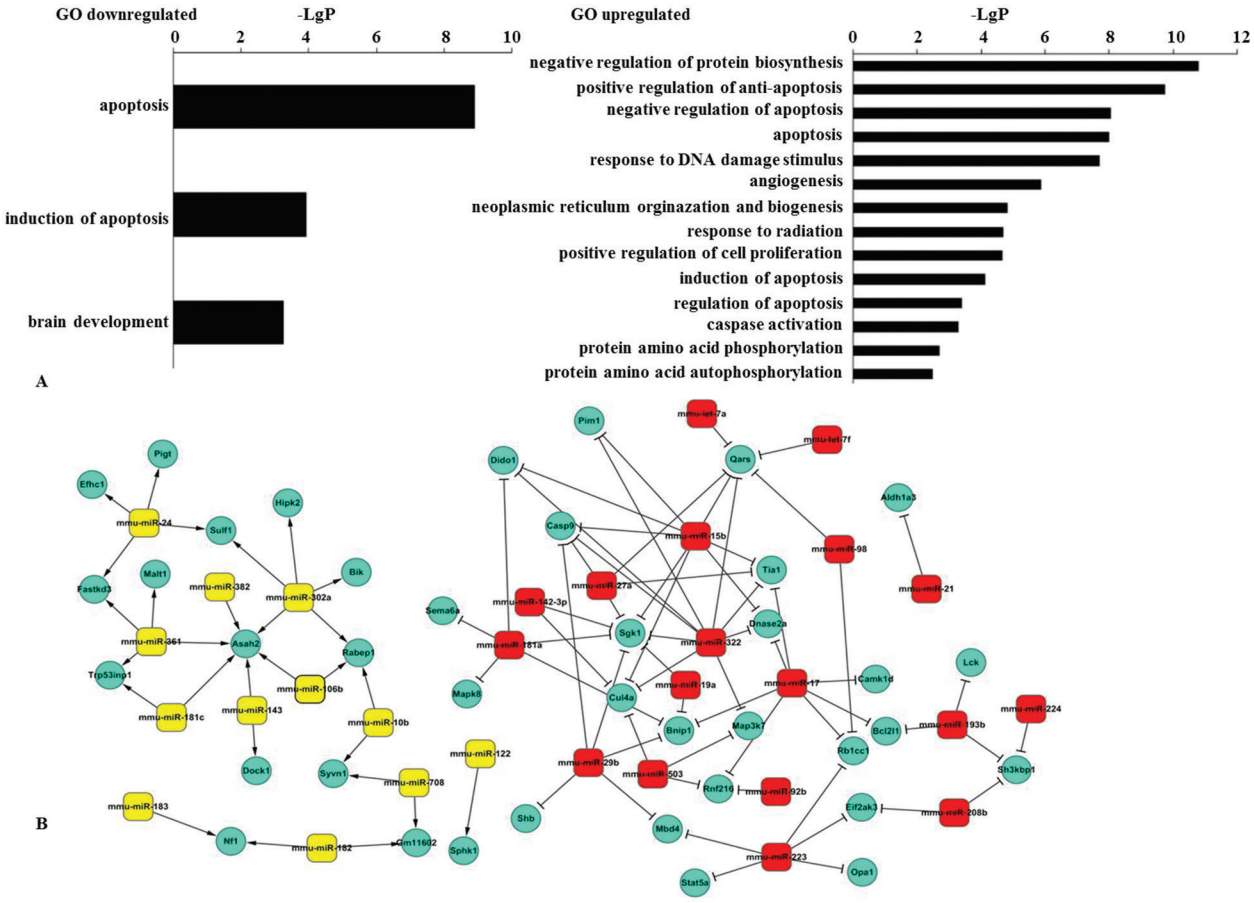

Figure 2. Identification of apoptosis-associated molecules in Cx26 knockout mice. (A) GO enrichment analyses based on the predicted targets of differentially expressed miRNAs. These targets were enriched for apoptosis-dominant functions. (B) A network of putative interactions between differentially expressed miRNAs and their targets. Yellow indicates down-regulated miRNAs (n=12); red indicates up-regulated miRNAs (n=18); light blue indicates target genes that were also negatively regulated in our mRNA microarray. Node size represents the node degrees.

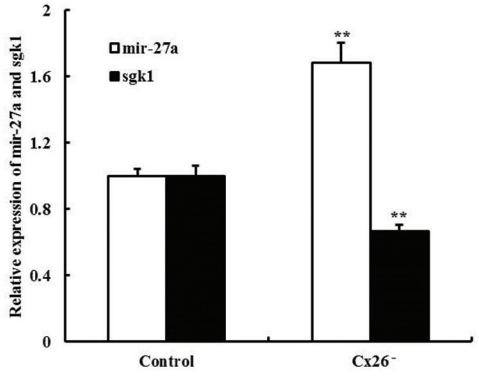

Figure 3. The expression of mir-27a and sgk1 mRNA in Cx26 knockout mice. qRT-PCR analysis showed that mir-27a was significantly increased and sgk1 was significantly decreased in Cx26 knockout mice (n=12) compared with those in wild type mice (n=10). Each bar represents the mean ± SD. ** P<0.01.

the *sgk1* mRNA level was higher in the mir-27a sponge group than in the control group (Figure 6B).

4.7. Sgk1 upregulation protects the cochlear sensory epithelium from Cx26 knockout-induced apoptosis

To clarify the molecular mechanisms of Cx26 knockout-induced apoptosis, adenovirus carrying *sgk1* was injected into the scala media of the cochlea of Cx26 knockout mice. A TUNNEL assay showed that *sgk1* overexpression inhibited Cx26 knockout-induced apoptosis in the cochlear sensory epithelium, when compared with apoptosis in the control group (Figure 7).

5. DISCUSSION

Identification of miRNAs, prediction of their targets, and inference of their functions are critical for understanding the roles of miRNAs in normal biological processes and disease development (17). In the present study, we found that conditional Cx26 knockout induced apoptosis in the cochlear sensory epithelium in mice. Mir-27a was identified as a mediator of Cx26 knockout-induced apoptosis, based on microarray data, and *sgk1* was identified as a predicted target

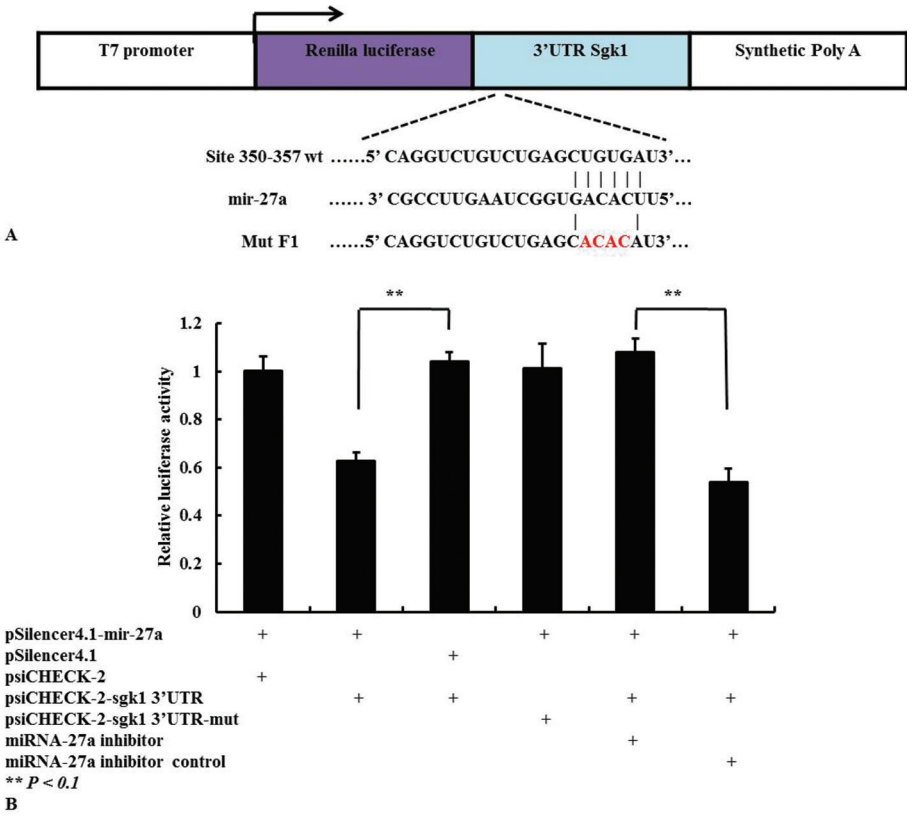

Figure 4. Mir-27a directly binds to 3'UTR of sgk1. (A) The conserved binding sites for mir-27a-sgk1 pairing is indicated. To generate a sgk1 3'UTR-Mut construct, the seed sequence was mutated from CUGUGA to CACACA. (B) Luciferase activity in HEK-293 cells co-transfected with mir-27a, mir-27a inhibitor and different luciferase-3'UTR constructs. Each bar represents the mean ± SD (n=3). ** P<0.01.

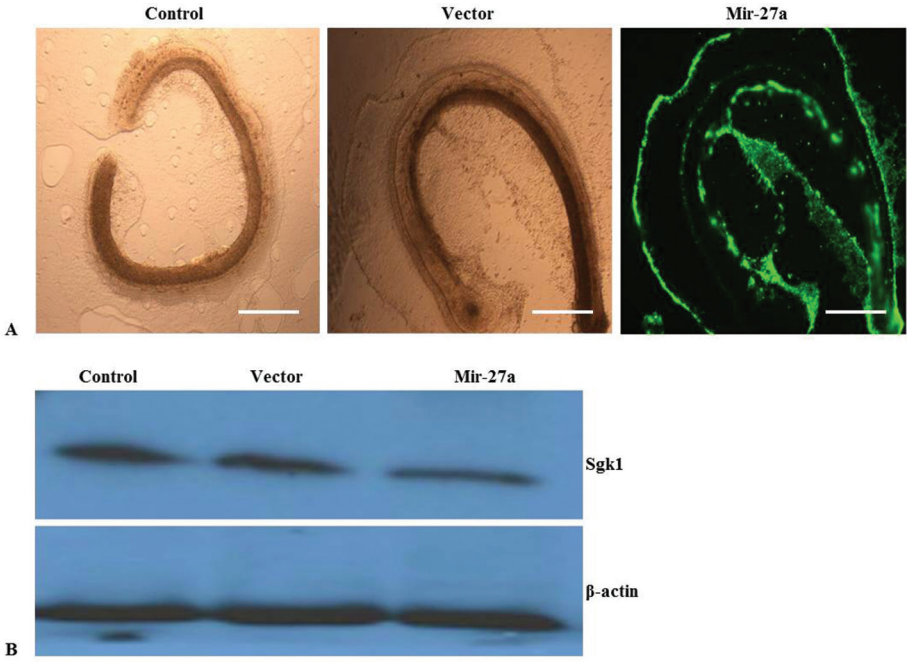

Figure 5. Mir-27a overexpression inhibited the expression of sgk1 in cultured cochlea tissue. (A) Mir-27a-GFP was extensively expressed in the cultured cochlea tissue. (B) Western blot showed that over-expressed mir-27a obviously reduced the expression of sgk1 in cultured cochlea tissue. Scale bars: 200μm.

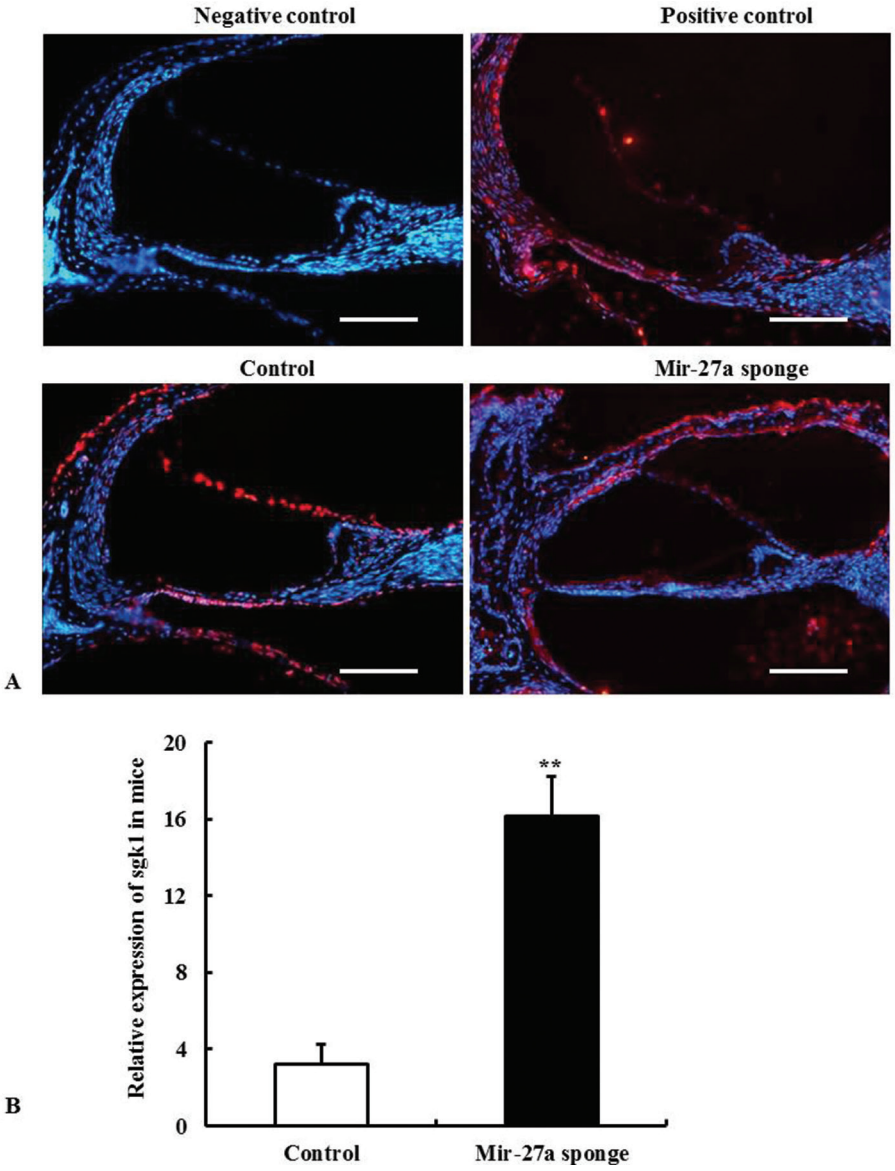

Figure 6. Effect of mir-27a sponge on Cx26 knockout-induced apoptosis of cochlear sensory epithelium and sgk1 mRNA expression in mice. (A) TUNNEL assay showed that apoptosis of cochlear sensory epithelium after mir-27a sponge treatment was significantly inhibited in Cx26 knockout mice. (B) The relative mRNA levels of sgk1 in the cochlear sensory epithelium after mir-27a sponge treatment in Cx26 knockout mice. Each bar represents the mean ± SD (n=3). ** P<0.01. Scale bars: 200μm.

gene of mir-27a. Real-time qRT-PCR confirmed the upregulation of mir-27a and the downregulation of *sgk1* in Cx26 knockout mice. The results of reporter gene assays and western blot established a direct correlation between mir-27a and *sgk1*. Additionally, inhibition of mir-27a significantly reduced Cx26 knockout-induced apoptosis in the cochlear sensory epithelium in mice, indicating that mir-27a plays an important role in Cx26 mutation-induced apoptosis in the sensory epithelium of the cochlea and in deafness. To our knowledge, this is the first study to investigate the molecular mechanism of Cx26 knockout-induced apoptosis in the cochlear sensory epithelium.

Apoptosis plays an important role in Cx26 conditional knockout-induced cochlear cell death (4,6). However, the mechanism by which Cx26 knockout induces cochlear cell apoptosis has not been investigated. To understand the underlying molecular mechanisms, we analyzed differences in miRNA expression in Cx26 knockout mice and wild-type mice. Several differentially expressed miRNAs that might be involved in apoptosis in the cochlear sensory epithelium were identified. MiRNAs regulate their targets by directly inducing mRNA degradation or by inhibiting protein synthesis. Bioinformatics analyses have estimated that over one-third of human genes are miRNA targets (18).

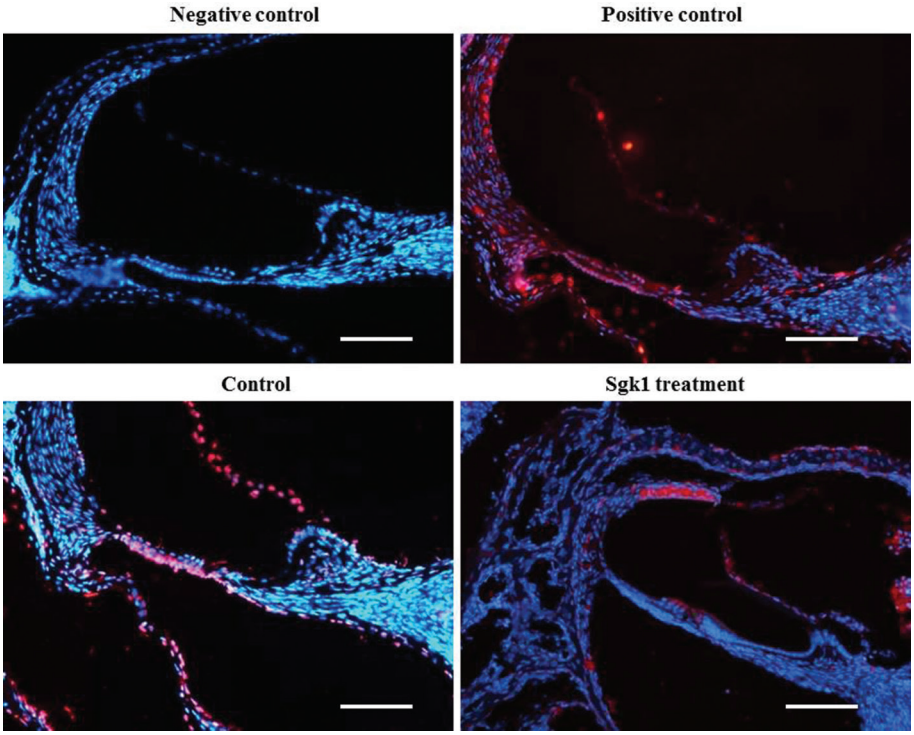

Figure 7. Apoptosis dection of cochlear sensory epithelium after adenovirus carrying sgk1 injected into the scala media of the Cx26 knockout mice cochlea. Scale bars: 200μm.

However, the predicted target genes are numerous, and experimentally validating each one is not practical. Therefore, the predicted target genes that were also negatively regulated in our mRNA microarray analysis were selected as candidate targets because miRNAs usually negatively regulate their targets (19,20). This process might increase the precision of target prediction. To identify the exact miRNA and the corresponding target genes involved in apoptosis in cochlear cells, we performed a GO analysis with the predicted targets. Interestingly, the targets were enriched for apoptosis-related functions. In the apoptosis-related target genes, *sgk1* was notable because it interacted with seven miRNAs in our co-expression network. We chose mir-27a for further investigation because it was upregulated the most. We predicted that mir-27a mediated Cx26 knockout-induced cochlear cell apoptosis, with *sgk1* as its target gene.

Because microarray data are not always reliable, they must be validated using other methods. Thus, real-time qRT-PCR was performed to confirm the expression of mir-27a and *sgk1*. Consistent with findings from the microarray analysis, real-time qRT-PCR showed that mir-27a was upregulated and *sgk1* was downregulated in Cx26 knockout mice. Next, we examined whether *sgk1* was a target of mir-27a using reporter gene assays and western blot. Our data showed that overexpression of mir-27a inhibited *sgk1* reporter gene activity and protein expression. Furthermore, when another reporter vector containing an *sgk1* 3′UTR mutant was used in the fluorescent reporter assay, miR-27a overexpression did not affect the luciferase activity. Moreover, a mir-27a inhibitor also abolished the inhibition of *sgk1* reporter gene activity. These findings indicate that mir-27a directly regulates *sgk1* expression.

Sgk1 is a ubiquitously expressed serine/threonine kinase that participates in a wide variety of cellular biological processes, including cell proliferation, renal sodium retention, and sodium homeostasis (21). The anti-apoptotic effect of sgk1 is known to play an important role in cell survival (22), and the expression of sgk1 is regulated by a variety of stimuli, including numerous hormones and cytokines (23). Upregulation or activation of sgk1 inhibits apoptosis and promotes survival by regulating transcription factors such as FOXO3A (24,25). Inhibition of miRNAs might rescue mRNA targets from degradation (26). The most effective route for expressing exogenous genes in the sensory epithelium of the cochlea is to inject virus directly into the scala media of the cochlea. Injecting lentivirus into the scala media of the cochlea did not induce cell degeneration and damage in the cochlea of mice at P1 (16). Therefore, we packaged adenoviruses encoding mir-27a sponge or sgk1 and injected them into the cochlea of conditional

Cx26 knockout mice. TUNEL assays showed that apoptosis in the cochlear sensory epithelium decreased. Interestingly, inhibition of mir-27a *in vivo* also increased the level of sgk1. Because the upregulation of sgk1 also protected the cochlear sensory epithelium from Cx26 knockout-induced apoptosis, our data suggest that mir-27a targets *sgk1* to mediate apoptosis in the cochlear sensory epithelium in Cx26 knockout mice.

In conclusion, our study suggests that downregulation of *sgk1* by mir-27a mediates Cx26 knockout-induced apoptosis in the sensory epithelium. However, other differentially expressed miRNAs identified in our miRNA microarrays might also regulate the expression of *sgk1* and thereby contribute to apoptosis. Furthermore, mir-27a has other targets, in addition to *sgk1*, that might participate in the process. Identification of other miRNA-mediated pathways will provide a more comprehensive understanding of the molecular mechanism of Cx26 knockout-induced sensory epithelium apoptosis.

6. ACKNOWLEDGEMENTS

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Abbreviations: Cx26: connexin 26; sgk1: serum and glucocorticoid-inducible kinase 1; GJs: gap junctions; miRNAs: MicroRNAs; FBS: fetal bovine serum; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling

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