Virally mediated Kcnq1 gene replacement therapy in the immature scala media restores hearing in a mouse model of human Jervell and Lange-Nielsen deafness syndrome

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Abstract

Mutations in the potassium channel subunit KCNQ1 cause the human severe congenital deafness Jervell and Lange-Nielsen (JLN) syndrome. We applied a gene therapy approach in a mouse model of JLN syndrome (Kcnq1⁻/⁻ mice) to prevent the development of deafness in the adult stage. A modified adeno-associated virus construct carrying a Kcnq1 expression cassette was injected post-natally (P0–P2) into the endolymph, which resulted in Kcnq1 expression in most cochlear marginal cells where native Kcnq1 is exclusively expressed. We also found that extensive ectopic virally mediated Kcnq1 transgene expression did not affect normal cochlear functions. Examination of cochlear morphology showed that the collapse of the Reissner’s membrane and degeneration of hair cells (HCs) and cells in the spiral ganglia were corrected in Kcnq1⁻/⁻ mice. Electrophysiological tests showed normal endocochlear potential in treated ears. In addition, auditory brainstem responses showed significant hearing preservation in the injected ears, ranging from 20 dB improvement to complete correction of the deafness phenotype. Our results demonstrate the first successful gene therapy treatment for gene defects specifically affecting the function of the stria vascularis, which is a major site affected by genetic mutations in inherited hearing loss.

Keywords  gene therapy; hearing restoration; Jervell and Lange-Nielsen syndrome; Kcnq1 null mice; virus
Subject Categories  Genetics, Gene Therapy & Genetic Disease; Neuroscience
DOI 10.15252/emmm.201404929 | Received 4 December 2014 | Revised 17 May 2015 | Accepted 21 May 2015 | Published online 17 June 2015
EMBO Mol Med (2015) 7: 1077–1086

Introduction

Deafness caused by genetic mutations, which are responsible for more than half of all cases of congenital permanent hearing loss, has a prevalence of about 1–2 in every 1,000 human births (Smith et al, 2005). Genetic predisposition is also a significant factor in age-dependent hearing loss (ADHL) (Yamasoba et al, 2013), a major form of adult-onset sensorineural hearing loss affecting tens of millions of people (Dobie, 2008; Yamasoba et al, 2013). More than 100 deafness genes have been identified. Also, both our knowledge of the molecular etiology of deafness and our capability to diagnose genetic mutations have been greatly improved (Brownstein et al, 2011). Nevertheless, biological interventions based on cellular and molecular mechanisms that correct the root genetic causes of sensorineural hearing loss are not yet available. Currently, the major therapeutic options for sensorineural hearing loss are hearing aids and cochlear implant prostheses.

Most hereditary hearing loss is caused by homozygous recessive mutations (Lenz & Avraham, 2011; Shearer et al, 2013). The deafness genotype and phenotype relations usually are tightly defined (Smith et al, 2005). A monogenic mutation affecting the function of hair cells, supporting cells, or the stria vascularis (SV) are three major types of mutations causing severe hearing loss (Hilgert et al, 2009; Avraham & Kanaan, 2012). This means that most cases of genetic hearing loss are potentially amenable to gene replacement or augmentation therapy by exogenous expression of a single wild-type (WT) protein (Akil et al, 2012; Sacheli et al, 2012). Multiple research groups in the hearing field have worked for years to introduce gene therapy into clinical applications for the treatment of deafness. Experiments have repeatedly demonstrated that exogenous reporter genes such as green fluorescent protein (GFP) are expressed with high transduction efficiency by various types of viral...
vectors in the inner ear (Raphael et al., 1996; Sacheli et al., 2012). Although recent studies have yielded promising results with regard to the use of gene therapy to treat defective hair cells (Akil et al., 2012), work is still needed to demonstrate the efficacy of virally mediated gene therapy in treating more common genetic deafness resulting from mutations affecting the function of either supporting cells (e.g. those caused by mutations in GJB2) or cells in the SV.

Mutations in the KCNQ1 gene (also known as KcLQT1 or Ko7.1) are associated with Jervell and Lange-Nielsen (JLN) syndrome (Jervell & Lange-Nielsen, 1957), the phenotypes of which include congenital deafness and long QT intervals in the cardiogram, as well as sudden infant death syndrome and Romano–Ward syndrome (Lee et al., 2000). KCNQ1 is widely expressed in cardiovascular muscle cells, the kidneys and stomach, and marginal cells in the SV of the inner ear. The KCNQ1 is a voltage-gated potassium channel; its protein has 676 residues. The KCNQ1 consists of a cytosolic N-terminal domain followed by the S1-S4 voltage sensor, a canonical pore (S5-P-S6) domain, and a long cytosolic C-terminus. At least 16 mutations in the KCNQ1 gene, typically recessive, cause JLN syndrome (Casimiro et al., 2001). The most common ones are missense mutations that result in single amino acid residue replacements. In the inner ear, KCNQ1 co-assembles with KCNE1 to play critical roles in the secretion of K⁺ into the endolymph and the establishment of the endocochlear potential (EP) (Lang et al., 2007).

The endolympathic space in the cochlear duct is bound by epithelial cells of the membranous labyrinth on three sides by the Reissner’s membrane, the reticular lamina, and the lateral wall (Fig 1A). Kcnq1 is expressed exclusively in the apical membrane of the marginal cells in the SV. SV in the inner ear generates a high concentration of K⁺ in the endolymph and high extracellular endocochlear potential (EP, 80 mV), both of which are crucial for the transduction of sound by HCs into neural signals (Lang et al., 2007). Inactivating the Kcnq1 in mice produces a completely deaf model of human JLN syndrome, although the cardiac phenotypes are less prominent (Lee et al., 2000; Casimiro et al., 2001). In this study, we injected a Kcnq1-expressing AAV1 viral construct into the endolymph of Kcnq1−/− mice in the early postnatal period. Results demonstrated for the first time that a gene therapy approach could be applied in a mouse model of JLN syndrome to successfully treat gene defects specifically affecting the functions of the SV.

**Results**

**Viral inoculation into the scala media (SM) resulted in on-target and extensive ectopic Kcnq1 expression in the cochlea**

Our immunolabeling data from WT cochlear sections (n = 5, see arrow in Fig 1B) showing a thin green line at the border of the SV confirmed that Kcnq1 is exclusively expressed by the marginal cells in the SV (Lang et al., 2007). The specificity of the immunoreactivity was supported by both the ultra-low background signal (Fig 1B) and the disappearance of the thin line in the cochleae of Kcnq1−/− mice (n = 6; see arrow in Fig 1C). Since one of the aims of this study was to re-establish the missing Kcnq1 expression (Fig 1C) in as many marginal cells as possible, we first compared the viral inoculation efficiency achieved by injections into the SM and scala tympani (ST). For either Kcnq1 (comparing panels E&F in Fig 1) or green fluorescent protein (GFP) (comparing panels A & B in Supplementary Fig S1) expression, virus injection into the SM was the only route that resulted in successful transduction of the marginal cells, as indicated by a single arrow in Fig 1D and F (and arrows in Supplementary Fig S1A). We never observed virally mediated Kcnq1 expression (arrow in Fig 1E) or GFP (arrow in Supplementary Fig S1B) in SV cells when injections were made into the ST (n = 5 in both cases).

These findings indicated that we need to inoculate viruses directly into the SM in order to re-establish missing Kcnq1 expression (Fig 1C) with high efficiency in the marginal cells. This choice of viral delivery route was also supported by our auditory brainstem response (ABR) test results, which showed that the correction of the deafness phenotype in Kcnq1−/− mice was not achieved with viral inoculation into the ST (Supplementary Fig S1B).

The fact that native Kcnq1 is found only at the apical membrane of the marginal cells (Fig 1B; Lang et al., 2007) also facilitated our studies of virally mediated ectopic Kcnq1 gene expression. When we compared immunolabeling results obtained in the injected cochlea of WT (Fig 1D) and Kcnq1−/− (Fig 1F) mice, we found that Kcnq1 was detectable not only in the marginal cells in the SV (see arrow, Fig 1D and F), but also ectopically in cells in the lateral wall, spiral ganglia (arrowheads, Fig 1D and F), and spiral limbs regions (double arrows, Fig 1D and F). Importantlly, ABRs obtained from WT mice injected with the Kcnq1-expressing viral construct (n = 6, Fig 5B, data points shown by filled circles) showed that ABR thresholds were indistinguishable from those of WT mice (n = 6, Fig 5B, data points shown with filled squares), indicating that strong, extensive ectopic Kcnq1 expression (Fig 1D) did not affect normal cochlear functions.

Kcnq1 is transported to apical side of the marginal cells (Lang et al., 2007). The polarized intracellular trafficking of native Kcnq1 protein was confirmed by our immunolabeling of WT cochlear sections (n = 5), as indicated by the big arrow in Fig 2A (small arrows shows the locations of nuclei of the marginal cells). In the marginal cells of treated Kcnq1−/− mice, similar polarized intracellular trafficking was observed for virally expressed Kcnq1 protein (in Fig 2B, an example is indicated by a big arrow. Smaller arrows show the locations of nuclei of the marginal cells). In contrast, ectopically expressed Kcnq1 in fibrocytes (arrowheads in Fig 2B and C) and in the cells of spiral ganglia (Fig 2D) showed prominent intracellular presence, but did not show polarized intracellular trafficking to any particular side of the cell membrane.

Immunolabeling using the flattened cochlear preparation (Chang et al., 2008) allowed us to quantify cellular Kcnq1 expression (Fig 3). The hexagonal cell membrane of individual marginal cells was outlined by labeling with phalloidin (labeled in red in Fig 3). In the untreated cochleae of Kcnq1−/− mice, we observed that the orderly hexagonal organization of the marginal cells (Fig 3A–C,E) was damaged (Fig 3F). We also found that, compared to the marginal cells in WT mice (Fig 3E), the sizes of the marginal cells in untreated Kcnq1−/− mice vary greatly. Moreover, many cells in untreated Kcnq1−/− mice had missing nuclei (arrows in Fig 3F), suggesting cellular degeneration or distress. Counting positively transduced marginal cells (Fig 3B and C) yielded viral transduction efficiencies ranging from 75 ± 5% (n = 6) to 71 ± 8% (n = 6) to 61 ± 10% (n = 6) for marginal cells, respectively, located in the basal, middle, and apical turns (black bars on the right of Fig 3D).
These values were slightly lower than those in WT counterparts (Fig 3D, gray bars), but were sufficient to prevent deafness (Fig 5B).

Comparing the native Kcnq1 (Fig 3A) and virally mediated Kcnq1 expressions (Fig 3B and C), we found interesting similarities and differences:

1. As in the cochlea of treated Kcnq1−/− mice, native Kcnq1 expression in a subgroup of marginal cells was always below the detection limit of immunolabeling in WT animals (Fig 3A). The percentage of Kcnq1 expression in the marginal cells was never 100% in both groups.

2. Judging by immunolabeling intensity, we found that Kcnq1 expression levels appeared to be more variable among WT marginal cells (Fig 3A) than marginal cells of treated Kcnq1−/− mice (Fig 3B and C).

3. Within individual marginal cells, virally mediated Kcnq1 expression was more homogeneous. This was in sharp contrast to the many WT marginal cells that showed non-uniform or even spotted immunoreactivity (arrows, Fig 3A), suggesting aggregation of the Kcnq1 potassium channels in the cell membrane.

Figure 1. Cellular locations of native and virally mediated Kcnq1 expression in the cochlea.

A  Diagram showing the major landmarks of the cochlear section to facilitate comparison of data shown in (B–F).
B–F  Immunolabeling results of Kcnq1 (green) in cochlear cryosections are shown for uninjected WT (B), untreated Kcnq1−/− (C), injected WT (D), and Kcnq1−/− mice given injections into the ST (E) or SM (F). Cell nuclei were outlined by counterstaining with either DAPI (B–D, F) or Qnuclear deep red (E). Scale bars represent approximately 100 μm. Meaning of white arrows are given in the text.
Morphological and functional changes in the cochlea of Kcnq1−/− mice in response to treatment

Consistent with previous reports (Lee et al, 2000; Casimiro et al, 2001), we found that untreated Kcnq1−/− mice had a collapsed Reissner’s membrane, which was adherent to the spiral ligament and the tectorial membrane, resulting in disappearance of the SM. We also observed degeneration of inner and outer HCs, as well as supporting cells in the organ of Corti (compare Fig 4A and B), and secondary degeneration of cells in the spiral ganglia (Fig 4B). The mesothelial cells on the scala tympani side of the basilar membrane were absent as well. In treated Kcnq1−/− cochleae, the collapse of the Reissner’s membrane was prevented (Fig 4C), as was the death of hair cells, mesothelial cells, and cells in the spiral ganglia (Fig 4C). EPs were 87.3 ± 5.7 (n = 5), 3.2 ± 0.2 (n = 5), and 85.3 ± 9.1 (n = 6) for WT, untreated Kcnq1−/−, and treated Kcnq1−/− mice, respectively.

Figure 5A shows examples of ABR waveforms from the three groups of mice, and Fig 5B and C shows summary of ABR thresholds. Similar ABR thresholds were obtained in WT mice treated or not treated by viral injection (Fig 5B, n = 6, comparing data points given by filled squares and filled circles, none of the data points showed statistically significant differences on the Student’s t-test).

Untreated Kcnq1−/− mice had ABR thresholds around 90 dB SPL (n = 6, Fig 5B; data points shown by open triangles). Injection of the Kcnq1-expressing viral construct into the endolymph of Kcnq1−/− mice led to significant hearing preservation (n = 14, Fig 5B; data shown by filled triangles). Differences between the average ABR thresholds obtained from treated and untreated mice were statistically significant (P < 0.05, Student’s t-test) for all frequencies tested between 4 and 32 kHz (Fig 5B). Five of fourteen treated mice had ABR thresholds (Fig. 5B, Students’ t-test, dashed plot connecting open-circle data points) that were indistinguishable from those of treated WT mice (filled circles). In contrast, when the same viral construct was injected into the ST through the RW membrane (n = 7), no injected mice showed significant hearing improvement (Supplementary Fig S2B). We have repeated viral inoculations using solutions independently made from three batches. Data obtained by four different experimenters showed essentially the same results, suggesting that the treatment protocol used in this study yielded stable results (Supplementary Fig S2A).

We examined the effect of hearing preservation in treated Kcnq1−/− mice for up to 30 weeks (Fig 5C). Click-ABR results (n = 5) demonstrated that the treatment effect was stable for the initial 18 weeks and then started to decline (Fig 5C) at a rate of

**Figure 2.** Polarized intracellular trafficking of native and virally expressed Kcnq1 was found specifically in the marginal cells. Kcnq1 and cell nuclei were, respectively, labeled green and purple. The SV consists of three layers of cells; marginal cells are the first layer of cells on the side of the endolymphatic space.

A Cryosection through the SV of WT mice. The large arrow points to the native Kcnq1 (labeled green) in WT mice. Smaller arrows show nuclei of the marginal cells.

B Cryosection through the SV of a Kcnq1−/− mouse injected with AAV expressing Kcnq1. Labeled in green (bigger arrow) is the AAV1-expressed Kcnq1, found only in the apical membrane of the marginal cells. Smaller arrows show nuclei of the marginal cells. Arrowheads show Kcnq1 immunolabeling in fibrocytes outside the SV.

C Cryosection through the lateral wall of Kcnq1−/− mice showing intracellular distribution of Kcnq1 immunolabeling (green) in fibrocytes.

D Cryosection through the spiral ganglia of cochlea of treated Kcnq1−/− mice showing immunolabeling (green) in the cells of spiral ganglia (arrows).

Data information: Purple staining in all panels is counterstaining with DAPI showing the locations of cell nuclei. Scale bars represent appropriately.
about 1.4 dB/week. By the end of 30 weeks, click-ABR thresholds in the treated mice increased by about 17 dB on average. However, the difference between treated and untreated ears was still statistically significant (Student’s t-test, \( P < 0.05 \)). In addition, we found that even in \( Kcnq1^{-/-} \) mice that had worsening click-ABR thresholds \((n = 3, \text{data not shown})\), the gross cochlear morphology was normal for hair cells and cells in the spiral ganglia, as well as the position of the Reissner’s membrane.

**Discussion**

Gene therapy studies in the hearing field have traditionally focused on the restoration of hearing by regenerating sensory hair cells (HCs) from surviving supporting cells, by using a cell replacement approach to restore normal cochlear function, or by expressing exogenous trophic factors (Raphael et al., 1996; Sacheli et al., 2012). Relatively few investigations have used genetically deaf mouse...
models to test treatment outcomes directly. Most studies have yielded either marginal hearing improvement (Maeda et al., 2009) or phenotype correction limited to morphological characteristics (Yu et al., 2013). Some successful cases have involved the use of technical approaches that are not directly applicable to humans (Maeda et al., 2005; Ahmad et al., 2007; Miwa et al., 2013). One exception has been a recent gene therapy study in which VGLUT3 knockout (KO) mice were treated (Akil et al., 2012). VGLUT3-expressing AAV1 was injected into the cochlea to replace the null VGLUT3. The treated VGLUT3 KO mice gained stable long-term hearing. When this study is compared to the current work, important similarities and differences emerge, as summarized in Table 1. When reviewing this table, one important difference needs to bear in mind is that the cellular targets for treatment in the two studies are hair cells in the organ of Corti and marginal cells in the SV, respectively, which are two different sites in the cochlea.

It is interesting to note that although the same AAV subtype and promoter were used in both studies, our injections into the ST generally failed to transduce any cells lining the endolymphatic space (Supplementary Fig S1). This study also yielded a number of novel findings, among them the extensive ectopic expression shown by virally mediated Kcnq1 in the cochlea (Figs 1 and 2). These results are in contrast to virally expressed VGLUT3, which was exclusively in 100% of the inner HCs (Akil et al., 2012). Considering

Figure 5. Comparison of the ABR data from WT, treated, and untreated Kcnq1−/− mice.
A Waveforms of ABRs are compared in WT, untreated Kcnq1−/−, and treated Kcnq1−/− mice, as labeled above data traces. Series of averaged ABR data traces were evoked from tone-burst sounds with intensities ranging from 20 to 90 dB SPL.
B Summary of averaged ABR thresholds at various frequencies for different groups of mice; untreated Kcnq1−/− (open triangles), treated Kcnq1−/− (filled triangles), injected WT (filled circles), and un.injected WT (filled squares). Plot legends are given in the figure. The plot with open circles connected with dashed lines represents average data from the five best cases of treated Kcnq1−/− mice. Error bars represent standard error of the mean.
C Click-evoked ABR thresholds of WT (filled squares), treated Kcnq1−/− mice (filled triangles), untreated Kcnq1−/− mice (open squares) measured 4–30 weeks after mice were born. Error bars represent standard error of the mean. Upward arrows indicate that click-ABR thresholds were at the maximal sound level that could be reliably measured by the system.
that other virally expressed exogenous proteins, including GFP (Akil et al., 2012; Wang et al., 2013) and connexin26 (Yu et al., 2013), all showed nonspecific expression, our results suggest that the cellular specificity achieved by VGLUT3 driven by a nonspecific promoter is an exception rather than a common phenomenon. The ectopic Kcnq1 expression demonstrated in this work also gave us an opportunity to investigate possible side effects of such expression outside the targeted cells. Since ectopic Kcnq1 expression in WT mice did not damage normal hearing (Fig 5B, data points shown by filled circles), we conclude that Kcnq1 expressed in the cells in the spiral ganglia, in fibrocytes of the lateral wall, and in interdental cells (Fig 1D and F) probably did not form K⁺ channels that are harmful to the function of those cells. This is not surprising since it is known that functional potassium channels in the marginal cells need the co-assembly of Kcnq1 and Kcn1 (Lang et al., 2007). In addition to the lack of proper intercellular trafficking of Kcnq1 to the cell membrane we have observed (Fig 2), the expression of Kcn1 may be lacking in ectopic cellular locations, thus preventing the formation of membrane channels.

Our results also showed for the first time that virally expressed exogenous protein was correctly trafficked intracellularly to its native membrane location (Fig 2). Kcnq1 encodes a potassium channel subunit that is known to be required for generation of the EP and the high K⁺ concentration, both of which are essential for auditory transduction (Barhanin et al., 1996; Sanguinetti et al., 1996). We found that exogenous Kcnq1 was correctly targeted to these apical membranes; this was in sharp contrast to the diffuse intracellular distribution of virally expressed GFP or VGLUT3 in inner HCs (Akil et al., 2012). Interestingly, ectopically expressed connexin26 (Cx26) in the marginal cells is not transported to the cell membrane (Yu et al., 2013), but virally expressed Cx26 in supporting cells was correctly targeted to the cell membranes to form gap junctions (Yu et al., 2013). These findings suggest that crucial endogenous protein regulatory mechanisms govern the transportation and assembly of virally expressed proteins. These proteins are able to be trafficked like native proteins and co-assembled with their native molecular partners (e.g., Kcn1, Cx30) to form functional membrane channels.

One of the important tasks in conducting preclinical trials in animal models, assuming that the time course of disease progression and the phenotype characteristics observed in animal models can be applied in humans, is to examine reasonable boundary conditions for optimal treatment options in humans. This study has established a few of these boundary conditions for the treatment of Kcnq1 null mutations:

1. We found that in order to have the hearing preserved in the Kcnq1<sup>−/−</sup> mice, the percentage of marginal cells expressing the Kcnq1 need not to be 100%. By immunolabeling criteria, the percentage of marginal cells expressed Kcnq1 after viral injections is in the range of 61–75% (Fig 3F). Whether higher transduction efficacy may give better or longer-lasting treatment effect is unknown.

2. Because of the collapse of the Reissner’s membrane and degeneration of the multiple types of cochlear cells observed in the mouse model (Fig 4B), it appeared that the optimal timing for the treatment of the Kcnq1 null mutation would be before these permanent histological changes happen. Any therapy implemented after malformation of the cochlea would be significantly more difficult. This finding may also serve as a guide to the treatment of other genetic deafness mutations that predominantly affect the morphological development of the cochlea.

3. With the viral type and promoter we tested in this study, it appears that the results of one-time treatment for mutations affecting the function of the SV are not permanent (Fig 5C). Thus, either a new viral type must be tested or Supplementary treatment be done for longer-term efficacy.

Injections directly into scala media often produce side effects such as the breaking of cochlear structure or mixing of the perilymph and endolymph, both of which invariably lead to severe hearing loss (Kawamoto et al., 2001; Shibata et al., 2009). We found that injections into the perilymph did not give meaningful transduction in the marginal cells, while viral inoculation into the endolymphatic space after P5 almost certainly causes permanent hearing loss due to surgical procedures (Wang et al., 2013), thus negating the original purpose of the therapy. These results are consistent with those of most studies, which have shown that injection directly into the endolymph is needed to yield high transduction efficiency for cells lining the endolymphatic space (Fig 1A) (Sacheli et al., 2012). A few authors have reported positive GFP labeling in the SV cells by injection into the ST. However, their results apparently lack the cellular resolution (Duan et al., 2002; Lei & Han, 2010) needed to determine whether or not marginal cells in the SV were transduced. By injecting in early postnatal (P0–P2) mice, when the cochlear bony shell is still soft, we avoided causing damage to cochlear morphology and to hearing sensitivities by the surgical procedures (Fig 5B, plot connected by filled circles; Wang et al., 2013; Yu et al., 2013). The same surgical procedure would be difficult to apply to
humans because the corresponding developmental period is embryonic. At that time, motivation to treat a condition that does not threaten life would not be high. Nonetheless, we have completed the first proof-of-principle study demonstrating that a gene therapy approach is effective for hearing preservation in a mouse model of gene defects specifically affecting the function of the SV. In the future, this approach may be used to test the feasibility of treating other inherited deafness cases in which the SV is the predominant site affected (e.g. mutations in KCNE1, CCDC50, DFNA5, MYH14, TFCP2L3, TMPRSS3 genes).

Gene augmentation or replacement therapy for multiple inherited retinal degeneration diseases (e.g. Leber congenital amaurosis, choroideremia, Stargardt’s disease, and retinoschisis) has advanced to clinical phase I or phase II trials (Smith et al, 2012; Dalkara & Sahel, 2014). Our results in the auditory system suggest that as long as noninnvasive gene delivery to the marginal cells in the scala media can be achieved in sufficient quantity and before the degeneration of cochlear cells, the human efficacy of such treatment suggested by the current study is optimistic. Recent advances in developing new viral vehicles aimed at effectively penetrating the blood–brain barrier (Foust et al, 2009; Manfredsson et al, 2009) or diffusing across dense tissue for gene delivery in the eyes (Dalkara et al, 2013) are promising candidates for further examining whether these methods could be used to treat genetic deafness in humans.

Materials and Methods

Preparation of viral constructs

Mouse Kcnq1 cDNA was purchased from Open Biosystems (Pittsburgh, PA). Sgfl and Fsei restriction sites were added to the 5’ and 3’ ends, respectively, of the Kcnq1 gene by a PCR-based procedure using primers: Kcnq1-F:5’-GGGATCCGCCATGGGACACGGCCTCGT-3’ and Kcnq1-R: 5’-GGCGCCGGCTCAAGAACCTCATCAG-3’. The PCR product and the PCR II plasmid (Life Technologies, Grand Island, NY) were digested by Sgfl and Fsei. The Kcnq1 was then incorporated into PCR II to form the PCR II-Kcnq1 plasmid. An Fsei restriction enzyme site was added to pENN.AAV.CB7.CL.RBG (Gene Therapy Program, University of Pennsylvania), using the primer: 5’-GGTACCAGCTAGCTTAAACGCGCGGCCCTCGAG-3’ to form the pAAV1-CB7-FseI plasmid. After digestion with EcoRI and Fsei, Kcnq1 was cloned into pAAV1-CB7-FseI to form pAAV1-CB7-Kcnq1 plasmid. This plasmid was verified by restriction digestion and immunolabeling. We used GFP-expressing viral constructs as controls; such constructs have been described in our publications (Wang et al, 2013; Yu et al, 2013).

Recombinant AAV particles were produced by double transfection of HEK293 cells with the AAV and AAV helper packaging plasmids pDP1rs expressing the AAV Rep2 and Cap1 genes (PlasmidFactory, Bielefeld, Germany). Recombinant AAV1 was harvested 72 h after transfection by three cycles of freezing and thawing. The crude viral lysate was then purified by fractionation by the glass micropipettes controlled by a micromanipulator (MP-285, Sutter Instrument, Novato, CA), back-filled with viral solution, and controlled by a Picospritzer III pressure microinjection system (Picospritzer III; Parker Hannifin, NY). The glass micropipettes were controlled to penetrate into either the scala media through the soft bony cochlear shell of early postnatal mice near the basal cochlear turn or the scala tympani through the round window membrane. We ejected approximately 0.5 µl of fluid out of the tip of glass pipettes by controlling the duration (12 ms) and the number of pressure pulses (set at 12). Fast green dye (Sigma-Aldrich, St Louis, MO), which is visible under bright-field illumination with a dissecting microscope (Stemi2000; Carl Zeiss, Oberkochen, Germany), was included in the solution to help visually confirm fluid ejection. After surgery, mice were allowed to recover on a 37°C heating pad (model TR-100, Fine Science Tool Inc., Foster City, CA) before returning to the animal housing facility. More details of surgical and injection procedures have been given previously (Wang et al, 2013; Yu et al, 2013).
Examination of cochlear morphology and immunolabeling of Kcnq1 expression

Anesthetized animals were perfused via cardiac catheter, first with 10 ml 1xPBS and then with 15 ml of a mixture of 2% paraformaldehyde and 2% glutaraldehyde. The experimenters were unaware of the genotype of the mice. After removal of the temporal bone, the inner ear was fixed in 4% PFA overnight at 4°C and decalcified in 10% EDTA for 3 days at 4°C. The tissues were postfixed with 1% osmium tetroxide for 1 h, dehydrated serially in 30, 50, 70, 95 and 100% ethanol, and then embedded in epoxy resin (Ted Pella Inc., Redding, CA). Cochlear sections were cut and stained with toluidine blue according to our previously published protocol (Sun et al., 2009). Immunolabeling was done using either cochlear cryosections or whole-mount preparations (Chang et al., 2008) of the SVs of adult mice (~45 days after birth). Dissected cochlear samples were fixed in 4% paraformaldehyde for 20 min, permeabilized in 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 30 min, and blocked in 10% goat serum in PBS for 1 h. Primary antibodies against Kcnq1 (Santa Cruz Biotechnology, Dallas, TX) were labeled first at 4°C overnight. The specificity of the Kcnq1 antibody was examined by Western blotting. Only a single band was observed on the gel (data not shown). After washing three times in PBS, samples were incubated with Alexa 488-conjugated secondary antibody for 1 h at room temperature. Counterstaining for cell nuclei was done with either 4’,6-diamidino-2-phenylindole (DAPI) or Quinacrine deep red (both from Life Technologies, Grand Island, NY). Cell membrane was stained with isothiocyanate-conjugated phalloidin (Sigma-Aldrich, St. Louis, MO). Samples were then mounted in fluoromount-G antifading solution and examined by confocal microscopy. To quantitatively assess gene transfection efficiency in the marginal cells, we calculated the percentage of transduced cells by counting the number of Kcnq1-positive cells (Fig 3) and then divided the number by the total number of marginal cells in the field of view. More details of the immunolabeling protocol have been described previously (Ahmad et al., 2007; Chang et al., 2008).

Functional assays for measuring ABRs, the EP, and vestibular responses

After viral inoculations, we measured ABRs in adult mice at time points given in the Results. Tone-burst ABR is an objective measure of the hearing threshold at specific frequencies. The ABR testers were unaware of the genotype of the mice. We presented sound stimuli to mice to test frequency-specific hearing thresholds at 4k-32k Hz (Fig 5). Click ABR was also used in long-term follow-up studies. Data were given as mean ± standard error of the mean (mean ± s.e.). During ABR tests, we anesthetized mice with a mixture of ketamine hydrochloride (80 mg/kg) and xylazine (10 mg/kg). A plastic tube connected to the speaker was inserted into one ear to deliver sound stimuli generated by the BioSig software package (Tucker-Davis Technologies, Alachua, FL). The contralateral (uninjected) ear was also tested as a control. Details of the ABR and EP testing methods are given in our published papers (Ahmad et al., 2007). We monitored circling behavior, head tilt, and swimming ability of the injected mice; these parameters reflect vestibular functions.

The paper explained

Problem
Hereditary hearing loss is one of the most common human genetic diseases. Genetic mutations are responsible for more than half of all human congenital permanent hearing loss cases. Biological interventions based on cellular and molecular mechanisms that correct the root genetic causes of sensorineural hearing loss are not yet available.

Results
We tested a gene therapy approach to the treatment of human congenital syndromic deafness resulting from the JLN syndrome using a mouse model (Kcnq1<sup>+/−</sup> mice). The treatment performed postnatally significantly corrected both morphological and deafness phenotypes. The amount of hearing improvement ranged from 20 dB to complete rescue of the deafness phenotype at various frequencies tested.

Impact
This is the first gene therapy study in successfully preventing the development of deafness in the adult stage of a mouse model of JLN syndrome caused by gene defects specifically affecting the function of the stria vascularis (SV). Since the SV is a common site affected by many hearing-related genes, the results shown here provide a proof of principle that other genetic mutations (e.g., mutations in KCNE1, CCDC50, DFNAS, MYH14, TFCP2L3, and TMRPSS3 genes) specifically affecting the function of the SV may also be treated by a similar approach. The improvement of noninvasive viral delivery methods developed for adult-stage cochlea could lead to future investigations of the therapeutic effectiveness of this gene therapy for this type of genetic deafness in humans.

Supplementary information for this article is available online:
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Acknowledgements
We thank Ms. Anne Lin for proofreading the manuscript. This study was supported by grants to XL from the National Institute on Deafness and Other Communication Disorders (RO1 DC006483 and RO1 DC010204). Huawei Li received grant support from the National Science Foundation of China (#30728029 and #301230019) and the 973 Program (2011CB504506). Lin and Li also received grant support from the National Science Foundation of China (30728029).

Author contributions
QC and JW conducted and designed part of the experiments, analyzed a subset of data, and wrote part of the paper. QL, YK and YW conducted part of the experiments and analyzed a subset of data. B2 conducted part of the experiments and maintained mouse breeding pairs. HL designed part of the experiments, analyzed all the experiments, analyzed data, and wrote the paper.

Conflict of interest
The authors declare that they have no conflict of interest.

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