nature portfolio

Peer Review File

Cell-specific delivery of GJB2 restores auditory function in mouse models of DFNB1 deafness and mediates appropriate expression in NHP cochlea

Corresponding Author: Dr David Corey

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript presents a well-designed and innovative study, demonstrating the use of gene regulatory elements to drive cell-type-specific expression of GJB2 in the cochlea.
GJB2-associated deafness is the most common form of hereditary hearing loss worldwide. The discovery of the pathogenic variants in this gene in 1997 changed the way diagnostics and genetic counseling is done for deafness, and paving the way for gene therapy. However, given that Gjb2 mutations are often lethal in mice, this commonly used model for human deafness was far from ideal. The lack of appropriate AAV that were compatible with connexin 26 expression, encoded by the GJB2 gene, further complicated attempts to develop gene therapy.

The authors of this manuscript chose to identify gene regulatory elements specific for connexin 26, to help circumvent previous hurdles. HA-tagged GJB2 partially rescued hearing and preserved cochlear structure in a conditional mouse mutant. Expression of these GREs was validated in nonhuman primate cochleas.These findings support the potential of GRE-driven GJB2 delivery as a promising strategy for treating DFNB1 in humans.

Comments:
1. This study used two different gene regulatory elements to drive cell-type-specific expression of Gjb2. Was the effect of each GRE tested separately? If so, what were the differences?

2. Figure 3 does not include SEM images at P30. Are hair cells absent across all regions of the cochlea?

3. The authors mentioned that “GJB2 has a critical developmental role as early as P0, and that vector delivered at P1 might not produce GJB2 protein until P2 or P3.” scAAV transduces cells more rapidly and is suitable for small genes like GJB2. Have the authors considered trying scAAV?

4. How do the authors explain the differing effects of the HA tag in the Cre+ mouse model compared to the Gjb2 partial knockdown mouse?

5. In figures 4 and 7, how many mice were tested within each group for ABR/DPOAE?

6. In figure 7, the ABR results are presented in different titer concentrations. Which concentration was used for the cross sections and SEM images in Figure 5?

7. Do the different vector titers result in variations in expression levels or morphological changes over time, as presented in the ABR and DPOAE measurements at P60 and P90?

Minor:
1. The figures are not presented in the same order in which they are mentioned in the text, which makes it difficult to follow the results. I recommend reordering some panels of the figures.

2. Line 128 refers to Figures 1g and h? These panels do not appear in the figure.

Reviewer #2

(Remarks to the Author)
This is an excellent report describing the development of reagents that are successful in preventing deafness resulting from Gjb2 mutations using targeted regulatory elements to express the gene in the correct cell types in mouse and non-human primates. GJB2 mutations are an important cause of deafness in childhood and this report has strong translational relevance. I have a few minor comments to improve clarity.
Line 81: Please define CBA here.
Fig 1c: Please label GRE1 and GRE2 on the diagram.
Fig 2b: It is difficult to distinguish the colours used for phalloidin and Gjb2 in the sections, particularly the lower middle panel, and confusing that the same colour has been used for phalloidin and Gjb2 in the upper and lower panels respectively. Could the colours be changed to distinguish the two markers more clearly?
Fig 2c,d: Please define PV and BIODIPY in the legend and explain what they are labelling.
Line 163: Just a matter of interest – why Pfl?
Line 200: Point out labelling of GJB2 in the basal cell region of the stria vascularis.
Line 248-249: Was the improvement in ABR thresholds after injection of the human GJB2 consistent with a lack of normal OHC function? Were there any other measures of OHC function?
Line 301: Should this be “preservation” instead of “restoration”?
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Fig 6: It is not possible to see the thickened tectorial membrane, reduced scala media area, or other features mentioned in these images. A standard H&E or similar image would help.
Fig 7: It may just be my screen, but I found it quite difficult to distinguish the colours in this figure. Would the authors consider more contrasting colours? In particular the dark blue and black were difficult.
Line 522: Do you mean stereotypes or serotypes?
Discussion: This is essentially a summary of the main findings of the project, which is fine but it would be helpful to include discussion of other aspects and put the research into a broader context. For example, why do the human GREs seemed to work better than mouse GREs in the mouse? What do the findings tell us about the role of Gjb6 in hearing – is it redundant? Why do the authors think that the HA tag allows Gjb2 to function in one mutant but not in the other mutant?
Line 609-615: Could the genetic background of each mouse line be added to the methods? Presumably it is mixed but it would be helpful to know if the two Gjb2 mutants had different backgrounds that may contribute to their phenotypes.
Line 633: What is meant by “as described above prior to fixation”? Presumably these samples were not fixed?

Reviewer #3

(Remarks to the Author)
GJB2 encodes connexin26. Recessive variants of human GJB2 are associated with clinically heterogeneous deafness and are common in many countries. A founder variant of GJB2 accounts for the majority of nonsyndromic deafness in the Ashkenazi Jewish population. Gene therapy for GJB2 deafness is on the horizon and looks more hopeful considering the new data in the manuscript from Ivanchenko and co-authors entitled “Cell-specific delivery of GJB2 restores auditory function in mouse models of DFNB1 deafness and mediates appropriate expression in NHP cochlea”. The authors describe an AAV vector with two cell-specific regulatory cis-acting sequences (GREs) driving the expression connexin26 in the appropriate inner ear cell types. Their new AAV vector can significantly restore hearing in an otherwise deaf mouse and in a partially deaf mouse model. The manuscript is nicely crafted, the figures are of good quality and the data is robust and convincing.

There is one concerning issue that hopefully can be resolved so that the data can be reproduced by other investigators. A list below of minor edits are recommended.

Major issue: Uncontrolled GJB2 expression using a ubiquitous promoter is lethal, as the authors clearly point out and reference. To spatially control GJB2 expression, the authors performed ATAC-seq and identified two chromosomal regions described in the text and in figure1. The two “GREs” are referred to as GRE1 and GRE2. Unless this reviewer missed the information, the authors did not provide the exact nucleotide sequence of GRE1 and GRE2. On line 112, GRE1 is described as including noncoding exon 1 and ~200 bp of upstream sequence. That’s vague. GRE2 is described as “3’ noncoding segment of exon 2.” That’s entirely inexact. Without the nucleotide sequence included in the manuscript for GRE1 and GRE2, the data and observations by Ivanchenko and co-authors cannot be independently reproducible by other scientists, should they wish to pursue this topic. The Nature Communications editors should decide if this is acceptable secrecy by the authors although it is understandable but not appropriate. Some of the authors have equity in Skylark Bio as indicated on line 1017 (Competing Interests) and likely these authors want to move their observations into the clinical for gene therapy for GJB2-associated human deafness without giving commercial competitors an advantage. That too is understandable from a business point of view. But in a high tier science publication, is it proper to intentionally withhold critical information?

Minor edits:

Abstract, lines 24. “partially” and “partial” are vague and not scientific. Please provide some actual data.

Abstract and Introduction, lines 26 and 70, name the nonhuman primate.

Introduction, line 31, Many authors state that hearing loss is the most common sensory deficit. Perhaps. But color blindness is 1 in 12 men and 1 in 200 women.

Introduction, line 43, The authors state that “GJB2 [expression] is absent from the sensory hair cells…”. Literally, as stated, the Gjb2 gene is not absent. GJB2 expression, according to data in gEAR, is also not absent from hair cells but is expressed at a low level compared to support cells. Which is it? The data in gEAR about Gjb2 expression or is it a myth about GJB2 not being present in hair cells? Please clarify.
Line 228, define “vgc”.
Line 249, provide the genotype of the “control mice”.
Line 461, Please clarify the statement “A well-developed exogenous GJB2.HA network was also see….” What is meant by an “exogenous … network”? Clarify please.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
All requests have been adequately addressed.

Reviewer #2

(Remarks to the Author)
The authors have dealt with all of my comments on the previous version in a satisfactory way.

Reviewer #3

(Remarks to the Author)
The authors have revised the manuscript and am satisfied with their edits.

RESPONSE TO REVIEWERS

Reviewer #1 (Remarks to the Author):

The manuscript presents a well-designed and innovative study, demonstrating the use of gene regulatory elements to drive cell-type-specific expression of GJB2 in the cochlea. GJB2-associated deafness is the most common form of hereditary hearing loss worldwide. The discovery of the pathogenic variants in this gene in 1997 changed the way diagnostics and genetic counseling is done for deafness, and paving the way for gene therapy. However, given that Gjb2 mutations are often lethal in mice, this commonly used model for human deafness was far from ideal. The lack of appropriate AAV that were compatible with connexin 26 expression, encoded by the GJB2 gene, further complicated attempts to develop gene therapy.

The authors of this manuscript chose to identify gene regulatory elements specific for connexin 26, to help circumvent previous hurdles. HA-tagged GJB2 partially rescued hearing and preserved cochlear structure in a conditional mouse mutant. Expression of these GREs was validated in nonhuman primate cochleas. These findings support the potential of GRE-driven GJB2 delivery as a promising strategy for treating DFNB1 in humans.

Comments:

1. This study used two different gene regulatory elements to drive cell-type-specific expression of Gjb2. Was the effect of each GRE tested separately? If so, what were the differences?

We tested about 30 different constructs, encoding GFP, mmGJB2 or hsGJB2. Seven of them did not include the second GRE, and they had lower efficacy, so we did not pursue them. Those experiments were exploratory, so we did not have sufficient numbers to make a quantitative comparison to vectors with the second GRE. We feel it is best not to include partial data in the paper.

2. Figure 3 does not include SEM images at P30. Are hair cells absent across all regions of the cochlea?

Gjb2$^{Pfl/Pfl}$, *Cre+* cochleas showed degeneration of the sensory epithelium across all cochlear turns, as revealed by immunofluorescence on cryosections (new Supplementary Fig. 1), resulting in a flattened epithelium. At this stage, SEM imaging is not informative, as the sensory epithelium is lost and no structures remain for visualization. It is also very difficult, as the thin basilar membrane tends to break.

3. The authors mentioned that "GJB2 has a critical developmental role as early as P0, and that vector delivered at P1 might not produce GJB2 protein until P2 or P3." scAAV transduces cells more rapidly and is suitable for small genes like GJB2. Have the authors considered trying scAAV?

We did, but the numbers don't work. The CDS plus GREs plus WPRE plus bGH sequences add up to 3741 bp, too much to allow additional reverse complement sequence for an scAAV.

4. How do the authors explain the differing effects of the HA tag in the Cre+ mouse model compared to the Gjb2 partial knockdown mouse?

The differing effects of the HA tag in the *Gjb2*$^{Pfl/Pfl}$, *Cre+* mouse model compared to the *Gjb2*$^{Gjb6-}$ mouse suggest that, although GJB2.HA is strongly expressed and properly trafficked to the cell membrane in both models, it may not be fully functional. In the *Gjb2*$^{Pfl/Pfl}$, *Cre+* mice, the presence of GJB6 may partially compensate for the impaired function of GJB2.HA, preserving essential ion or metabolite transport. In contrast, when GJB2.HA

is expressed in the absence of GJB6 (as in *Gjb2*$^{Gjb6-}$ mice), it leads to detrimental effects in the inner ear, likely due to the loss of GJB2 and GJB6 gap junction function.

5. In figures 4 and 7, how many mice were tested within each group for ABR/DPOAE?

We have now specified the number of mice tested for each condition in the figure legends for Figures 4 and 7, as well as in the corresponding source data files.

6. In figure 7, the ABR results are presented in different titer concentrations. Which concentration was used for the cross sections and SEM images in Figure 5?

The cross-section immunofluorescent images and SEM images in Figure 5 were obtained from ears injected with the medium dose (7.0×10^{10} vg/ear). We have now clarified this in the figure legend.

7. Do the different vector titers result in variations in expression levels or morphological changes over time, as presented in the ABR and DPOAE measurements at P60 and P90?

While we did not assess morphological changes at P60 and P90, we observed sustained functional hearing rescue with both high (1.4×10^{11} vg/ear) and medium (7.0×10^{10} vg/ear) doses through P90, suggesting stable expression and a lack of progressive degeneration. Given these consistent functional outcomes, we believe that additional morphological analysis at later time points would be unlikely to provide further meaningful insights. In contrast, the low-dose (3.0×10^{10} vg/ear) group exhibited a gradual elevation in ABR thresholds over time, which may reflect suboptimal GJB2 expression and potentially lead to degenerative changes. However, we agree that this could be an interesting direction for future studies focused on long-term structural effects.

Minor:

1. The figures are not presented in the same order in which they are mentioned in the text, which makes it difficult to follow the results. I recommend reordering some panels of the figures.

In general, the figures are presented in the order mentioned. Some subpanels of a figure are mentioned after earlier subpanels of a subsequent figure, but the panels are grouped into figures based on the experimental methods, so some out-of-order discussion is unavoidable.

2. Line 128 refers to Figures 1g and h? These panels do not appear in the figure.

The reference in line 128 should be to Fig. 2c,d (insets), not Fig. 1g,h. We have corrected this in the revised manuscript. Thanks for catching this.

Reviewer #2 (Remarks to the Author):

This is an excellent report describing the development of reagents that are successful in preventing deafness resulting from Gjb2 mutations using targeted regulatory elements to express the gene in the correct cell types in mouse and non-human primates. GJB2 mutations are an important cause of deafness in childhood and this report has strong translational relevance. I have a few minor comments to improve clarity.

Line 81: Please define CBA here.

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Fig 1c: Please label GRE1 and GRE2 on the diagram.

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Fig 2b: It is difficult to distinguish the colours used for phalloidin and Gjb2 in the sections, particularly the lower middle panel, and confusing that the same colour has been used for phalloidin and Gjb2 in the upper and lower panels respectively. Could the colours be changed to distinguish the two markers more clearly?

We have updated the figure to use distinct colors for phalloidin and GJB2 to improve clarity and avoid confusion between panels. The revised version now ensures better visual distinction between the two markers.

Fig 2c,d: Please define PV and BIODIPY in the legend and explain what they are labelling.

Done

Line 163: Just a matter of interest – why Pfl?

This allele was first used in Paris. We have two other floxed alleles (not described here) and needed to distinguish them.

Line 200: Point out labelling of GJB2 in the basal cell region of the stria vascularis.

Done

Line 248-249: Was the improvement in ABR thresholds after injection of the human GJB2 consistent with a lack of normal OHC function? Were there any other measures of OHC function?

We assessed OHC function using distortion product otoacoustic emissions (DPOAEs). No improvement in DPOAE responses was observed following injection of either hsGJB2.HA or untagged hsGJB2 in *Gjb2*$^{Pfl/Pfl}$, *Cre+* mice. However, we did observe a significant improvement in ABR thresholds with the untagged hsGJB2, suggesting that the functional rescue occurred despite the absence of detectable OHC function recovery. This clarification has now been added to the text.

Line 301: Should this be "preservation" instead of "restoration"?

Done

Line 309: Typo bottom

Done

Fig 6: It is not possible to see the thickened tectorial membrane, reduced scala media area, or other features mentioned in these images. A standard H&E or similar image would help.

We have now added standard H&E-stained images to Fig. 6 to better illustrate the structural changes mentioned in the text.

Fig 7: It may just be my screen, but I found it quite difficult to distinguish the colours in this figure. Would the authors consider more contrasting colours? In particular the dark blue and black were difficult.

Thanks for pointing this out. We have expanded the color range.

Line 522: Do you mean stereotypes or serotypes?

Thanks; fixed

Discussion: This is essentially a summary of the main findings of the project, which is fine but it would be helpful to include discussion of other aspects and put the research into a broader context. For example, why do the human GREs seemed to work better than mouse GREs in the mouse?

We did not test the endogenous mouse GREs in our experiments, as the selected human GREs effectively restricted GJB2 expression in the mouse cochlea. We prioritized human GREs from the outset to align with future translational applications and observed that they yielded a spatial expression pattern consistent with endogenous GJB2 expression in mouse cochlear supporting cells and fibrocytes. While we cannot conclude that human GREs 'work better' than mouse GREs in this context, our findings showed that human regulatory sequences can function appropriately in the mouse inner ear, supporting their potential use in cross-species preclinical studies.

We have added a paragraph to the discussion to put the findings into a broader context, commenting on the different roles for GJB2 in different cells at different times, and thus the differing ability of our vectors to compensate for GJB2 loss.

What do the findings tell us about the role of Gjb6 in hearing – is it redundant? Why do the authors think that the HA tag allows Gjb2 to function in one mutant but not in the other mutant?

Regarding the role of GJB6, our findings indicate that it is not redundant with GJB2 but plays a complementary role in supporting normal auditory function. The severe phenotype observed when GJB2.HA was overexpressed in *Gjb2*$^{Gjb6-}$ mice, suggests that, in the absence of GJB6, the precise balance and composition of connexin proteins becomes critical, and that altered GJB2 alone cannot compensate.

As for the differential effect of the HA tag, we propose that GJB2.HA retains partial function in the *Gjb2*$^{Pfl/Pfl}$, *Cre+* model because GJB6 is still present to support gap function. In contrast, in *Gjb2*$^{Gjb6-}$ mice, the absence of GJB6 and the presence of the GJB2.HA together resulting in structural abnormalities and no functional rescue.

Line 609-615: Could the genetic background of each mouse line be added to the methods? Presumably it is mixed but it would be helpful to know if the two Gjb2 mutants had different backgrounds that may contribute to their phenotypes.

Gjb2$^{Pfl/Pfl}$, *Cre+* or *Gjb2*$^{Pfl/Pfl}$, *Cre-* mice were maintained on a C57BL/6J background. *Gjb2*$^{Gjb6-}$mice were maintained on mixed C57BL/6J–CBA genetic backgrounds. We added this to the text.

Line 633: What is meant by "as described above prior to fixation"? Presumably these samples were not fixed?

Corrected

Reviewer #3 (Remarks to the Author):

GJB2 encodes connexin26. Recessive variants of human GJB2 are associated with clinically heterogeneous deafness and are common in many countries. A founder variant of GJB2 accounts for the majority of nonsyndromic deafness in the Ashkenazi Jewish population. Gene therapy for GJB2 deafness is on the horizon and looks more hopeful considering the new data in the manuscript from Ivanchenko and co-authors entitled "Cell-specific delivery of GJB2 restores auditory function in mouse models of DFNB1 deafness and mediates appropriate expression in NHP cochlea". The authors describe an AAV vector with two cell-specific regulatory cis-acting sequences (GREs) driving the expression connexin26 in the appropriate inner ear cell types. Their new AAV vector can significantly restore hearing in an otherwise deaf mouse and in a partially deaf mouse model. The manuscript is nicely crafted, the figures are of good quality and the data is robust and convincing. There is one concerning issue that hopefully can be resolved so that the data can be reproduced by other investigators. A list below of minor edits are recommended.

Major issue: Uncontrolled GJB2 expression using a ubiquitous promoter is lethal, as the authors clearly point out and reference. To spatially control GJB2 expression, the authors performed ATAC-seq and identified two chromosomal regions described in the text and in figure1. The two "GREs" are referred to as GRE1 and GRE2. Unless this reviewer missed the information, the authors did not provide the exact nucleotide sequence of GRE1 and GRE2. On line 112, GRE1 is described as including noncoding exon 1 and ~200 bp of upstream sequence. That's vague. GRE2 is described as "3' noncoding segment of exon 2." That's entirely inexact. Without the nucleotide sequence included in the manuscript for GRE1 and GRE2, the data and observations by Ivanchenko and co-authors cannot be independently reproducible by other scientists, should they wish to pursue this topic. The Nature Communications editors should decide if this is acceptable secrecy by the authors although it is understandable but not appropriate. Some of the authors have equity in Skylark Bio as indicated on line 1017 (Competing Interests) and likely these authors want to move their observations into the clinical for gene therapy for GJB2-associated human deafness without giving commercial competitors an advantage. That too is understandable from a business point of view. But in a high tier science publication, is it proper to intentionally withhold critical information?

Yes, we withheld the nucleotide sequences of the GREs in the review process but always understood that they would be necessary for final publication. They are now included in the Supplementary Table 1.

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Minor edits:

Abstract, lines 24. "partially" and "partial" are vague and not scientific. Please provide some actual data.

We have added the degree of hearing restoration in decibels.

Abstract and Introduction, lines 26 and 70, name the nonhuman primate.

Done.

Introduction, line 31, Many authors state that hearing loss is the most common sensory deficit. Perhaps. But color blindness is 1 in 12 men and 1 in 200 women.

Corrected.

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Based on our SHIELD database, there is indeed some *Gjb2* mRNA in cochlear hair cells in neonatal mice, and the gEAR database shows sparse mRNA in hair cells. However we did not detect GJB2 *protein* in hair cells with antibodies (Fig. 3a), suggesting post-transcriptional down-regulation.

Line 228, define "vgc".

Done

Line 249, provide the genotype of the "control mice".

Done

Line 461, Please clarify the statement "A well-developed exogenous GJB2.HA network was also see…." What is meant by an "exogenous … network"? Clarify please.

Corrected.