

CLAIMS

What is claimed is:

1. An isolated nucleic acid comprising an expression cassette, wherein the expression cassette comprises a Gap Junction beta 2 (*GJB2*) gene regulatory element (GRE), and a nucleotide sequence encoding a GJB2 protein.
2. The isolated nucleic acid of claim 1, wherein:
 - a) the GJB2 protein is a human GJB2 protein;
 - b) the GJB2 protein comprises an amino acid sequence at least 80% identical to SEQ ID NO: 1; and/or
 - c) the nucleotide sequence encoding the GJB2 protein comprises a nucleotide sequence at least 80% identical to SEQ ID NO: 2.
3. The isolated nucleic acid of claim 1 or 2, wherein the expression cassette further comprises a promoter operably linked to the nucleotide sequence encoding a GJB2 protein, optionally wherein:
 - a) the promoter is a human *GJB2* promoter;
 - b) the promoter comprises 500 nucleotides of a human *GJB2* promoter;
 - c) the promoter comprises a nucleic acid sequence at least 80% identical to SEQ ID NO: 5; and/or
 - d) the promoter comprises a nucleic acid sequence at least 80% identical to SEQ ID NO: 102, optionally 100% identical to SEQ ID NO: 102.
4. The isolated nucleic acid of any one of claims 1-3, wherein the *GJB2* GRE comprises a nucleotide sequence encoding a 5' UTR, optionally wherein:
 - a) the 5' UTR is positioned between the promoter and the nucleotide sequence encoding a GJB2 protein;
 - b) the 5' UTR comprises about 300 nucleotides of a human *GJB2* gene 5' UTR; and/or

c) the promoter and the 5' UTR comprises a nucleotide sequence at least 80% identical to SEQ ID NO: 30.

5. The isolated nucleic acid of any one of claims 1-4, wherein the *GJB2* GRE comprises an enhancer, optionally wherein:

- a) the enhancer is positioned 5' to the promoter;
- b) the enhancer is normally present within approximately 200 kb upstream or downstream of a *GJB2* gene;
- c) the enhancer is normally present within approximately 95 kb of a *GJB2* gene
- d) the *GJB* GRE comprises more than one enhancer, optionally wherein the more than one enhancers are the same enhancers or different enhancers;
- e) the enhancer comprises a nucleotide sequence at least 80% identical to a nucleotide sequence or a fragment thereof as set forth in any one of SEQ ID NOs: 6 to 29;
- f) the enhancer comprises a nucleotide sequence at least 80% identical to a *GJB2* enhancer as set forth in any one of SEQ ID NOs: 37-46; and/or
- g) the enhancer comprises a nucleotide sequence at least 80% identical to SEQ ID NO: 42.

6. An isolated nucleic acid comprising an expression cassette, wherein the expression cassette comprises a Gap Junction beta 2 (*GJB2*) promoter, and a nucleotide sequence encoding a *GJB2* protein.

7. The isolated nucleic acid of claim 6, wherein:

- a) the *GJB2* promoter comprises a nucleic acid sequence at least 80% identical to SEQ ID NO: 102, optionally 100% identical to SEQ ID NO: 102;
- b) the nucleotide sequence encoding the *GJB2* protein comprises a nucleic acid sequence at least 80% identical to SEQ ID NO: 2;
- c) the expression cassette further comprises a 5' UTR, optionally wherein the 5' UTR comprises:
 - a first nucleic acid sequence at least 80% identical to SEQ ID NO: 103, optionally 100% identical to SEQ ID NO: 103; and/or

- a second nucleic acid sequence at least 80% identical to SEQ ID NO: 104,
optionally 100% identical to SEQ ID NO: 104; and/or
- d) the isolated nucleic acid comprises a nucleic acid sequence at least 80% identical to
SEQ ID NO: 105, optionally 100% identical to SEQ ID NO: 105.
8. The isolated nucleic acid of any one of claim 1-7, wherein the isolated nucleic acid is
capable of expressing GJB2 in cells that normally express the *GJB2* gene.
9. The isolated nucleic acid of any one of claims 1-8, wherein the isolated nucleic acid is
capable of expressing GJB2 in cochlear connective tissue cells and supporting cells of the organ
of Corti, optionally wherein:
- a) the supporting cells of the organ of Corti are pillar cells, Deiters' cells, Hensen's cells,
Claudius cells, inner phalangeal cells, and/or border cells; and/or
- b) the cochlear connective tissue cells are strial intermediate cells, fibrocytes of the lateral
wall and supracochlear zone, basal cells of the stria vascularis, fibrocytes in the spiral ligament,
fibrocytes in the spiral limbus, mesenchymal cells lining the bony otic capsule facing the scala
vestibuli, and/or supralimbal dark cells.
10. The isolated nucleic acid of any one of claims 1-9, wherein the expression cassette is
flanked by two adeno-associated virus inverted terminal repeats (ITRs), optionally wherein:
- a) the AAV ITR is from a serotype selected from the group consisting of AAV1 ITR,
AAV2 ITR, AAV3 ITR, AAV4 ITR, AAV5 ITR, and AAV6 ITR;
- b) the AAV ITR is AAV2 ITR; and/or
- c) the expression cassette is flanked by:
- a 5' ITR having a nucleotide sequence at least 80% identical to SEQ ID NO: 106,
optionally 100% identical to SEQ ID NO: 106; and/or
- a 3' ITR having a nucleotide sequence at least 80% identical to SEQ ID NO: 107,
optionally 100% identical to SEQ ID NO: 107.
11. The isolated nucleic acid of any one of claims 1-10, wherein the expression cassette
further comprises a Woodchuck Hepatitis Virus (WHP) Posttranscriptional Regulatory Element

(WPRE) 3' to the nucleotide sequence encoding the GJB2 protein, optionally wherein the WPRE comprises a nucleotide sequence at least 80% identical to SEQ ID NO: 108, optionally 100% identical to SEQ ID NO: 108.

12. The isolated nucleic acid of any one of claims 1-11, wherein the expression cassette further comprises a nucleotide sequence encoding a 3' UTR located 5' of the WPRE, optionally wherein:

a) the 3' UTR is a GJB2 3' UTR; and/or

b) the GJB2 3' UTR comprises a nucleotide sequence at least 80% identical to SEQ ID NO: 32.

13. The isolated nucleic acid of any one of claims 1-12, wherein the expression cassette further comprises a poly A signal, optionally wherein:

a) the poly A signal is a bovine growth hormone poly A signal; and/or

b) the poly A signal comprises a nucleotide sequence at least 80% identical to SEQ ID NO: 109, optionally 100% identical to SEQ ID NO: 109.

14. The isolated nucleic acid of any one of claims 1-13, comprising a nucleotide sequence at least 80% identical to SEQ ID NO: 110 or 111, optionally 100% identical to SEQ ID NO: 110 or 111, optionally wherein the isolated nucleic acid is a vector, optionally wherein the vector is a plasmid or a viral vector, further optionally wherein the viral vector is an AAV vector.

15. A vector comprising the isolated nucleic acid of any one of claims 1-14, optionally wherein the vector is a plasmid or a viral vector, further optionally wherein the viral vector is an AAV vector.

16. A vector comprising from 5' to 3':

(a) a GJB2 promoter, or a basal GJB2 promoter sequence thereof comprising SEQ ID NO: 102;

(b) a GJB2 5' UTR comprising a first nucleic acid sequence comprising SEQ ID NO: 103 and a second nucleic acid sequence comprising SEQ ID NO: 104;

- (c) a nucleotide sequence encoding a GJB2 protein, wherein the nucleotide sequence comprises SEQ ID NO: 2;
 - (d) a GJB2 3' UTR comprising SEQ ID NO: 32;
 - (e) a Woodchuck Hepatitis Virus (WHP) Posttranscriptional Regulatory Element (WPRE) comprising SEQ ID NO: 108; and
 - (f) a bovine growth hormone poly A signal comprising SEQ ID NO: 109.
17. The vector of claim 16, further comprising:
- a 5' ITR located 5' of the GJB2 promoter or basal GJB2 promoter sequence thereof, wherein the 5' ITR comprises SEQ ID NO: 106; and
 - a 3' ITR located 3' of the bovine growth hormone poly A signal, wherein the 3' ITR comprises SEQ ID NO: 107.
18. A recombinant adeno-associated virus (rAAV) comprising:
- (i) a capsid protein; and
 - (ii) the isolated nucleic acid of any one of claims 1-14, or the vector of any one of claims 15-17.
19. The rAAV of claim 18, wherein the rAAV has tropism for a subset of cochlear cells that normally express the *GJB2* gene, optionally wherein the rAAV has tropism for cells of the inner ear.
20. The rAAV of claim 18 or 19, wherein the capsid protein is an AAV1 capsid protein, an AAV2 capsid protein, an AAV5 capsid protein, an AAV7 capsid protein, an AAV8 capsid protein, an AAV9 capsid protein, an AAV-S capsid protein, or a variant thereof, optionally wherein the AAV capsid is AAV9.PHP.B, AAV9.PHP.eB, or AAV-S, further optionally wherein the AAV capsid protein is AAV-S.
21. A cell comprising the isolated nucleic acid of any one of claims 1-14, the vector of any one of claims 15-17, or the rAAV of any one of claims 18-20.

22. A pharmaceutical composition comprising the isolated nucleic acid of any one of claims 1-14, the vector of any one of claims 15-17, the rAAV of any one of claims 18-20, or the cell of claim 21, optionally wherein the pharmaceutical composition further comprises a pharmaceutically acceptable carrier.

23. Use of the isolated nucleic acid of any one of claims 1-14, the vector of any one of claims 15-17, the rAAV of any one of claims 18-20, the cell of claim 21, or the pharmaceutical composition of claim 22 in the manufacture of a medicament for specifically expressing GJB2 in cells in a subject that normally express the *GJB2* gene.

24. Use of the isolated nucleic acid of any one of claims 1-14, the vector of any one of claims 15-17, the rAAV of any one of claims 18-20, the cell of claim 21, or the pharmaceutical composition of claim 22 in the manufacture of a medicament for treating Non-syndromic Hearing Loss and Deafness (DFNB1) in a subject in need thereof.

25. Use of the isolated nucleic acid of any one of claims 1-14, the vector of any one of claims 15-17, the rAAV of any one of claims 18-20, the cell of claim 21, or the pharmaceutical composition of claim 22 in the manufacture of a medicament for treating a GJB2-associated disease in a subject in need thereof.

26. The isolated nucleic acid of any one of claims 1-14, the vector of any one of claims 15-17, the rAAV of any one of claims 18-20, the cell of claim 21, or the pharmaceutical composition of claim 22 for use as a medicament.