GJB2-GT, a Novel AAV-Based Gene Therapy as a Treatment for the Autosomal Recessive Non-Syndromic Deafness 1A (DFNB1A)

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In the world, the estimated prevalence of severe or profound deafness in human is 1 out of 1000 neonates, and genetic factors account for half of the cases. Pathogenic variants of GJB2, the gene encoding for Connexin 26 (Cx26), are involved in ~50% of congenital deafness and are mostly associated with autosomal recessive non-syndromic hearing loss (DFNB1A). In the cochlea, GJB2 is largely expressed in the supporting cells (SCs) of the sensory neuroepithelium, fibrocytes, basal and intermediate cells of stria vascularis but not in sensory hair cells. It is hypothesized that Cx26 is essential for the recycling of potassium, which is essential for the proper functioning of sensory hair cells, but in vivo studies also suggest that Cx26 deficiency leads to cochlear developmental disorders. Gene therapy is a promising therapeutic strategy for autosomal recessive forms of deafness, and Adeno-Associated Vectors (AAVs) are being developed to this aim.

Here, we have developed GJB2-GT, an AAV tailored for DNFB1A with proprietary cis-regulatory elements for a safe and targeted expression of the transgene in vivo. GJB2-GT efficiently transduces human cell lines to produce Cx26 protein that is adequately addressed to the plasma membrane. Using dye transfer assays, we show that the transgenic CX26 protein is functional and permits the passage of connexon-permeable fluorescent tracers into GJB2-GT transduced cells. Early tolerability and biodistribution evaluations of GJB2-GT or GJB2-FLAG-GT were conducted in mice and Non-Human Primates (NHP) species after injections through the round window (RW). Intracochlear injection in both species results in transgene expression in most cells that naturally express Gjb2 along the tonotopic axis of the cochlea, with good local and systemic tolerability. In all analyzed cochleae, no sensory hair cell express the transgene, confirming the specificity of the expression cassette. Importantly, GJB2-GT administration in NHP was performed using the surgical procedure and injection device intended for clinical use. Long-term safety studies conducted in wild-type mice demonstrate persistence of transgene expression with no impact on auditory function and maintenance of the cochlear cytoarchitecture. Additionally, high dose intravenous injection of GJB2-GT in mice does not induce any adverse events or changes in behavior, vital functions, increase in hepatic damage or inflammatory biomarkers. To circumvent embryonic lethality caused by complete loss of Gjb2 expression in mice, a conditional model was generated, resulting in a biallelic Gjb2 inactivation mimicking the most common forms of DFNB1A and severe/profound hearing loss. Intracochlear injections of GJB2-GT lead to improvement of hearing thresholds as early as 3 weeks post-injection in a dose dependent manner which was statistically significant across multiple frequencies. Microscopic examination of injected cochlea confirmed that GJB2-GT efficiently restores Cx26 expression in the sensory epithelium. Current dose-response experiments confirm that intracochlear GJB2-GT injection is safe over a wide range of doses administered and provide a significant therapeutic benefit in our DFNB1A mouse model. Altogether, these data support GJB2-GT development to restore physiological hearing in DFNB1A patients.

1. A proprietary regulatory sequence inhibits transgene expression in sensory hair cells

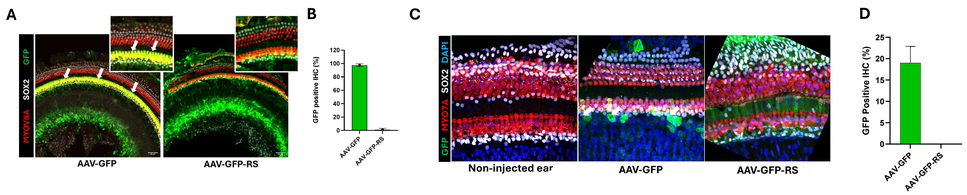
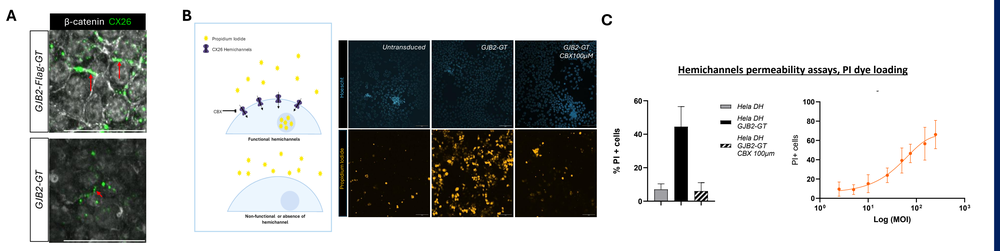


Figure 1: A. Whole mount imaging of organ of Corti from mice injected with AAV-GFP or AAV-GFP-RS. Arrows indicate transduced inner hair cells (IHC) Scale bar 40 µm. **B**. Quantification of GFP+ IHC in the organ of Corti represented in B. **C.** Whole mount imaging of organ of Corti from NHP injected with AAV-GFP or AAV-GFP-RS. Arrows indicate transduced inner hair cells (IHC). Scale bar: 50 µm. **D.** Quantification of GFP+ IHC in the organ of Corti represented in C. **Sensorion's proprietary regulatory sequence (RS) effectively abolish transgene expression in sensory hair cells of the cochlea.**

2. GJB2-GT drives the expression of functional CX26 connexons



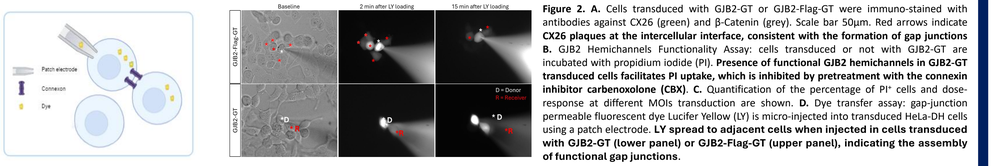
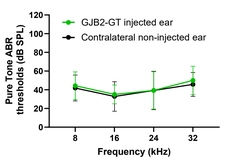
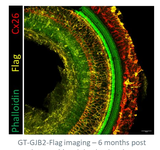


Figure 2. A. Cells transduced with GJB2-GT or GJB2-Flag-GT were immuno-stained with antibodies against CX26 (green) and β-Catenin (grey). Scale bar 50µm. Red arrows indicate **CX26 plaques at the intercellular interface, consistent with the formation of gap junctions B.** GJB2 Hemichannels Functionality Assay: cells transduced or not with GJB2-GT are incubated with propidium iodide (PI). **Presence of functional GJB2 hemichannels in GJB2-GT transduced cells facilitates PI uptake, which is inhibited by pretreatment with the connexin inhibitor carbenoxolone (CBX). C.** Quantification of the percentage of PI+ cells and dose-response at different MOIs transduction are shown. **D.** Dye transfer assay: gap-junction permeable fluorescent dye Lucifer Yellow (LY) is micro-injected into transduced HeLa-DH cells using a patch electrode. **LY spread to adjacent cells when injected in cells transduced with GJB2-GT (lower panel) or GJB2-Flag-GT (upper panel), indicating the assembly of functional gap junctions**.

3. Intracochlear and systemic injection of GJB2-GT is well tolerated in mice

1-year exploratory safety study: intracochlear injection





GT-GJB2-Flag imaging – 6 months post intracochlear injection in mice

Acute toxicity study: High dose IV injection

- Experiment performed in preparation of upcoming GLP-toxicity study in mice after IV injection
- GT-GJB2 does not interfere with normal growth and don't elicit elevated transaminase levels 4 and 8 weeks after injection
- Behavioral evaluation - Functional Observation Battery, exploratory behavior (videotracking), 3 and 7 weeks after injection: no findings

Figure 3: A. WT mice were injected with the indicated vectors through the RWM. Auditory brainstem response (ABR) were measured up to 1 year after vector administration. **GJB2-GT injected animals display normal ABR thresholds, consistent with good local tolerability of the vector B.** Whole-mount imaging of organ of Corti from WT mice injected with a flagged version of the GJB2-GT vector. The flag epitope is broadly detected in supporting cells, **indicating appropriate cellular tropism and maintenance of transgene expression up to 6 months post-injection. Injected cochleae displayed normal histology. No transgene expression was detected in hair cells**

4. Natural history of a new conditional mouse model for DFNB1A : *Gjb2*$^{cKO1/cKO1}$

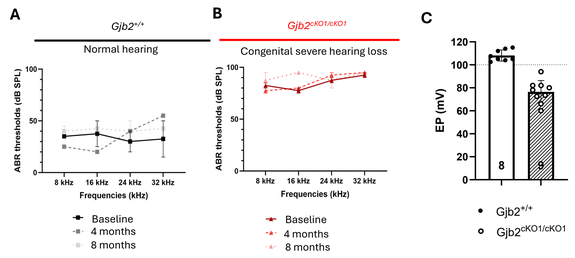


Figure 4: *Gjb2*$^{cKO1/cKO1}$ mutant mouse model is a useful tool to study DFNB1A physiopathology and to perform proof of concept (POC) studies since the homozygosity for *Gjb2*$^{KO/KO}$ allele is lethal during the embryonic development of the mouse. **A, B.** Assessment of the hearing function of *Gjb2*$^{cKO1/cKO1}$ mutant mice up to 8 months. Control mice from this strain show normal hearing and do not present age related hearing loss due to the absence of Cdh23AHL allele in the genetic background.Congenital severe hearing loss animals present ABR above 80dB thresholds from hearing onset. **Our ongoing efficacy studies currently focus on the congenital form.** **C.** Endocochlear Potential values of 1 month-old mice Gjb2$^{+/+}$ vs *Gjb2*$^{cKO1/cKO1}$. At one month the mutant mice display a deficit of EP in our model.

5. Dose studies in *Gjb2*$^{cKO1/cKO1}$ mutant mice : SENS-601 exhibits efficacy similar to GJB2-GT

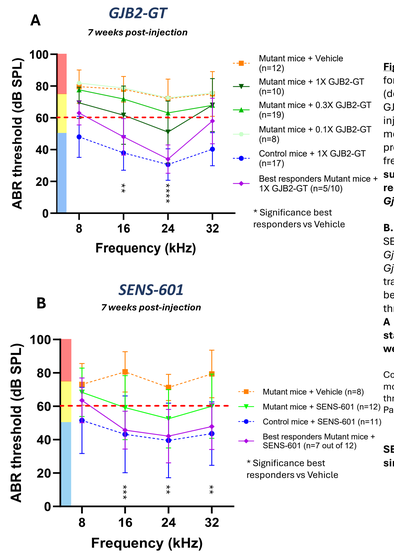


Figure 5: A. GJB2-GT dose study. Average ABR recordings for GJB2-GT 1X to 0.3X (dark to light green) or vehicle (dotted orange) injected *Gjb2*$^{cKO1/cKO1}$ mutant mice and for GJB2-GT injected *Gjb2*$^{+/+}$ control mice (blue) 7 weeks after injection. Purple traces represent average ABR measurements for the 5 best responder animals, presenting average ABR thresholds across tested frequencies ≤ 60 dB at dose 1X. **A 1X dose of GJB2-GT is sufficient to induce a statistically significant hearing recovery for at least 7 weeks after injection in *Gjb2*$^{cKO1/cKO1}$ mutant mice.**

B. SENS-601 dose study. Average ABR recordings for SENS-601 1X (green) or vehicle (dotted orange) injected *Gjb2*$^{cKO1/cKO1}$ mutant mice and for SENS-601 injected *Gjb2*$^{+/+}$ control mice (blue) 7 weeks after injection. Purple traces represent average ABR measurements for the 7 best responder animals, presenting average ABR thresholds across tested frequencies ≤ 60 dB at dose 1X. **A 1X dose of SENS-601 is sufficient to induce a statistically significant hearing recovery for at least 7 weeks after injection in *Gjb2*$^{cKO1/cKO1}$ mutant mice.**

Colored zones represent approximative profound (>70 dB, red), moderate (45-70 dB, yellow), and no (<45 dB, blue) hearing loss thresholds. **** p<0.0001 by two-way ANOVA followed by All Pairwise Multiple Comparison Procedures (Holm-Sidak method)

SENS-601, our future drug candidate, shows efficacy similar to GJB2-GT (R&D batch).

6. Evaluation of the transduction efficiency of GJB2-GT in the mouse cochlea of mutant mice after a RWI

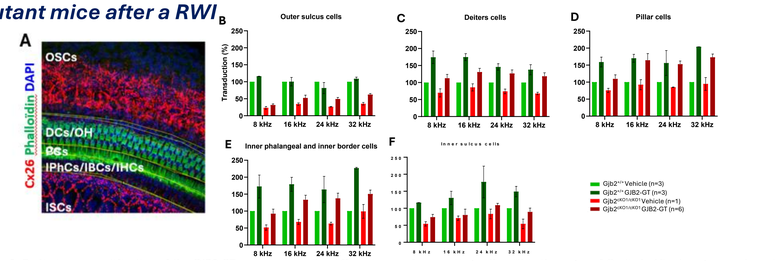
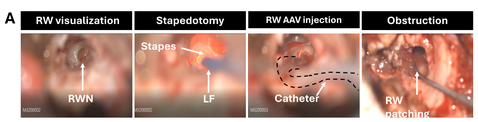
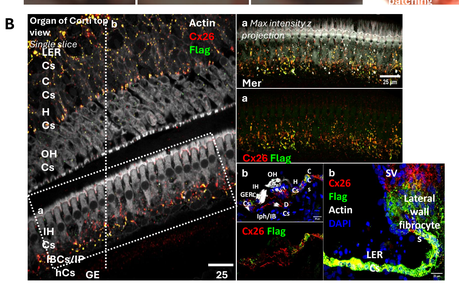
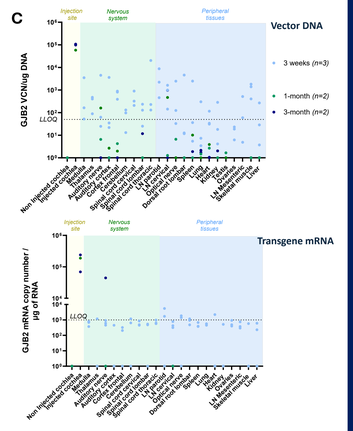


Figure 6: Preliminary quantification of the GJB2-GT expression in the different area of supporting cells within the organ of Corti. A. After being micro-dissected, organ of Corti were stained for Connexin 26 (red) and counterstained with phalloidin (green) and DAPI (blue) and processed in Whole-Mount. **B-F.** Quantification of Connexin 26 staining in different supporting cells areas. All values are normalized toward the Gjb2$^{+/+}$ vehicle-injected animal. **Injection of GJB2-GT restored expression of CX26 in Deiters' cells (C), Pillar cells (D), and Inner Phalangeal and Inner Border cells (E) and had no major effect on CX26 expression in Inner Sulcus cells (F).**

7. Intracochlear administration of SENS-601 is specifically targeted to the intended cells in NHP and is well tolerated







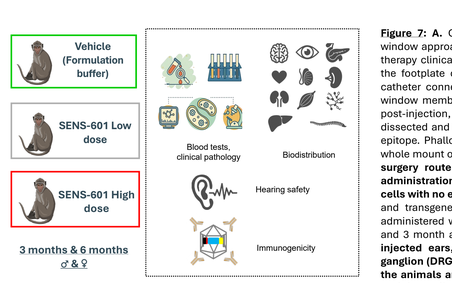


Figure 7: A. GJB2-GT-Flag administrations were performed in NHP using the round window approach and the injection medical device currently used in Sensorion's gene therapy clinical trial. Briefly, a transcanal approach is performed. After visualization of the footplate of the stapes, a stapedotomy is performed and an in-house designed catheter connected to a dedicated injection medical device is inserted in the round window membrane and the intracochlear administration performed. **B.** Three weeks post-injection, NHP were euthanized, the temporal bone collected. Cochleae were fine-dissected and immunostained using antibodies directed against CX26 and the Flag tag epitope. Phalloidin counterstaining is used to detect actin. Max intensity projection of whole mount organ of Corti (a) as well as cochlea cryosections (b) are shown**. Using the surgery route and injection device envisioned for human, GJB2-GT Flag vector administration result in correct expression of CX26 at the cell membrane of target cells with no expression in hair cells. C.** Biodistribution of vector genome (upper panel) and transgene mRNA (lower panel) in the cochlea and peripheral tissues of NHP administered with GJB2-GT. Biodistribution studies were conducted 3 weeks, 1 month and 3 month after vector administration. **The vast majority of the vector remains in injected ears, no dissemination observed in gonads, main organs, dorsal root ganglion (DRG). Overall, GJB2-GT intracochlear administration was well tolerated by the animals and did not induce any macroscopic/organ weight changes. There was no lab or clinical findings over the course of the study. D.** Design of ongoing **GLP toxicity and biodistribution study and readouts.**

Conclusions

- In GJB2-GT, the combination of an AAV serotype with high tropism toward supporting cells and specific regulatory element offers broad coverage of *Gjb2*-expressing cells of the inner ear in both mouse and NHPs, with very limited off target. No transgene expression was detected in sensory hair cells.
- GJB2-GT intracochlear administration efficiently restore hearing threshold in a new conditional DFNB1A mouse model.
- Biodistribution studies in NHP shows minimal level of VCN in peripheral tissues that decreases overtime. Strong detection of vector DNA and transgene derived mRNA is observed at the injection site (temporal bone) and transgene mRNA is almost undetectable in VCN-positive peripheral tissues and contralateral ears, demonstrating very low ectopic Cx26 expression outside the inner ear.
- SENS-601, restores efficiently hearing thresholds and demonstrates a safety and efficacy profile to support its development for therapeutic use in humans. The program is progressing as planned with CTA submission in Q1 2026.