Characterization of a safe and functional GJB2-GT vector for the treatment of DFNB1A hearing loss

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Abstract

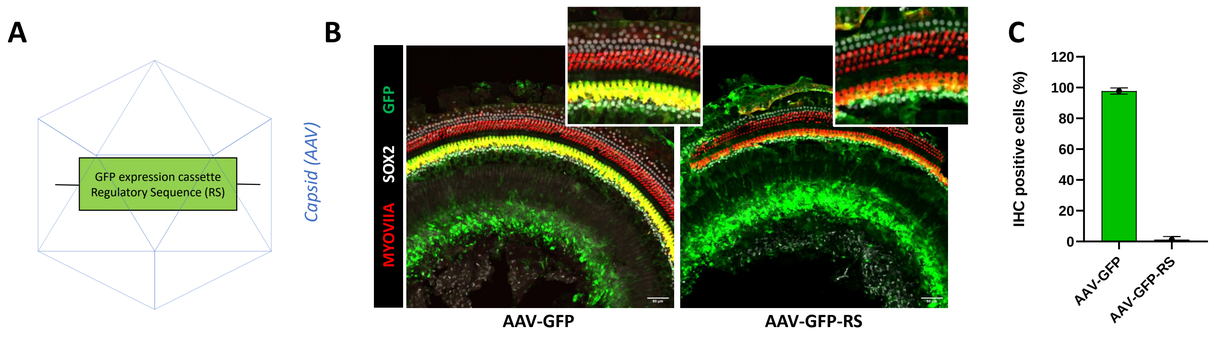
The estimated prevalence of severe to profound deafness is 1 in 1000 neonates worldwide, with genetic factors accounting for half of the cases. Pathogenic variants of *GJB2*, the gene encoding for Connexin 26 (CX26 also known as Gap Junction protein Beta 2), are involved in 50% of congenital deafness and are mostly associated with an autosomal recessive non-syndromic form, DFNB1A. No specific treatment exists for these patients besides cochlear implantation, which provides restorative benefits but does not fully replicate the clarity of normal hearing. Gene therapy is a promising therapeutic avenue as it aims for long lasting restoration of natural hearing function.

Connexin 26 hexamers oligomerize into hemichannels or connexons that connect head-to-head to form an axial channel or gap junction between adjacent cells, thereby allowing the exchange of small molecules such as nutrients, ions, and signaling molecules. GJB2 plays an important role in the recycling of potassium ions, which is critical for the maintenance of endocochlear potential and cochlear functions. In the cochlea, *GJB2* is largely expressed in the supporting cells (SCs) of the inner and outer sulcus as well as in fibrocytes and lateral wall regions associated with the stria vascularis. Importantly, sensory hair cells (HCs) are devoid of connexin 26, and the ectopic expression of this protein has been reported to be toxic. A major challenge for successful GJB2 gene therapy is therefore the development of a vector allowing precise expression of the transgene in the intended cells with very limited off-target. Here, we combined an AAV with strong tropism towards a broad range of cochlear target cells and a cassette that includes proprietary cis-regulatory elements to prevent transgene expression in sensory hair cells. *In vitro*, our therapeutic vector, hereby referred to as GJB2-GT, efficiently transduces human cell lines to produce CX26 protein that is adequately addressed to the plasma membrane. Using an adapted dye transfer assay, we show that the transgenic CX26 protein is functional and permits the passage of the fluorescent tracer to neighboring cells. Intracochlear injection of GJB2-GT in mice and NHP results in transgene expression in most cells that naturally express *GJB2* along the tonotopic axis, with good local and systemic tolerability. In all analyzed cochleae, no sensory hair cells expresses the transgene, confirming the specificity of our cassette. Long term safety studies conducted in wild type mice demonstrate prolonged persistence of transgene expression with no impact on auditory function and maintenance of the cochlear cytoarchitecture. Additionally, intravenous injection of GJB2-GT in mice does not induce any adverse events or changes in behavior, vital functions, hepatic damage or inflammatory biomarkers in the tested animals.

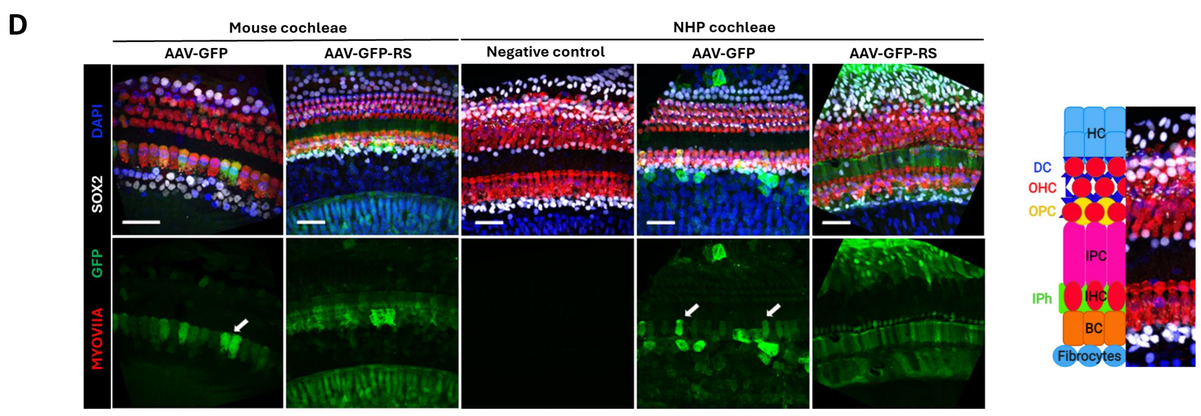
In conclusion, we have developed a specific AAV vector/expression cassette combination that leads to strong and durable expression of CX26 in cells of interest in the cochlea, with virtually no off-target detected. The vector is safe over an extended time after intracochlear administration in mouse and NHP models and represents therefore an excellent candidate for Sensorion's GJB2 gene therapy program.

1. The combination of the candidate AAV capsid and a proprietary cis-regulatory sequence (RS) allows for widespread transduction of cochlear cells while preventing transgene expression in sensory hair cells

Sensorion's proprietary RS effectively prevents transgene expression in cochlear sensory hair cells in mice



Intracochlear injection of AAV-GFP results in targeted transgene expression in cochlear supporting cells in mice and NHP

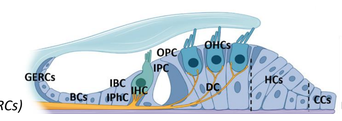


E

Cell types	Mice	NHP
Inner hair cells	-	-
Outer hair cells	-	-
Deiters cells	-	-
Inner phalangeal cells	++	++
Pillar cells	+	+
Outer sulcus (SC)	+++	+++
Inner sulcus	+	++
Interdental cells	++	+

F

Inner Pillar cell (IPC)
Outer Pillar cell (OPC)
Inner phalangeal cell (IPhC)
Inner border cells (IBC)
Inner hair cells (IHC)
Great epithelial ridge cells (GERCs)

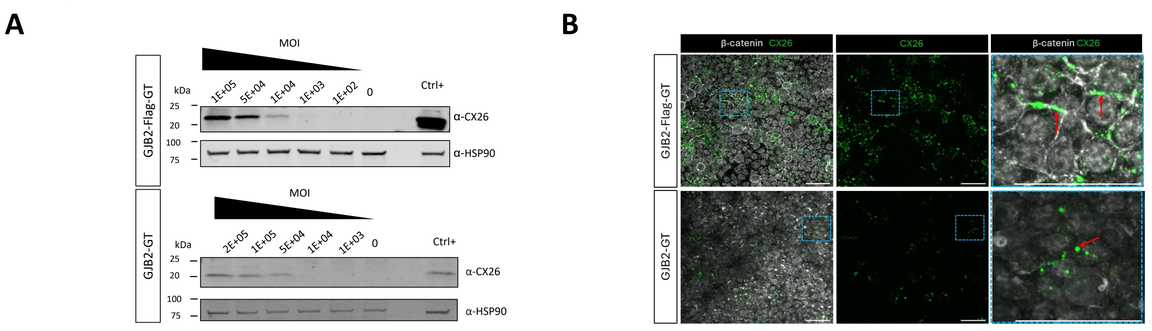


Lateral epithelial ridge cells (LERCs)
Hensen cells (HCs)
Claudius cells (CCs)
Deiters cells (DCs)
Outer hair cells (OHCs)

Figure 1: A. Schematic representation of the candidate AAV vector with a GFP expression cassette. The RS is added to prevent transgene expression in sensory hair cells. **B.** Whole mount imaging of organs of Corti (OoC) immunostained with the indicated antibodies. Mice were injected at post natal day (P) 15 with AAV-GFP or AAV-GFP-RS through the round window membrane (RWM). Scale bar 40 µm. **C.** Quantification of GFP$^+$ IHC. **Addition of the RS strongly prevent transgene expression in IHC D.** Whole mount imaging of OoC from mice and NHP injected with AAV-GFP or AAV-GFP-RS. Arrows indicate transduced inner hair cells (IHC) Scale bar 40 µm. **The RS efficiently inhibit transgene expression in IHC of both mouse and primates E.** GFP Expression profile in mice and NHP cochleae after AAV-GFP-RS injection. **F.** AAV-GFP-RS covers a **wide range of cells that normally express GJB2 in the cochlea, including the inner sulcus region with IPhC (Inner Phalangeal cells), IPC (Inner Pillar cells), BC (Border cell) and outer sulcus region including Hensen cells (HC), Claudius cells (CC).**

2. In vitro transduction of HeLa-DH cells with GJB2-GT vector results in the production of a functional Cx26 protein localized to the plasma membrane

CX26 protein levels and localization in Hela DH cells transduced with GJB2-GT



Both GJB2 and GJB2-Flag transgenes allow for functional assembly of gap junctions in HeLa-DH cells

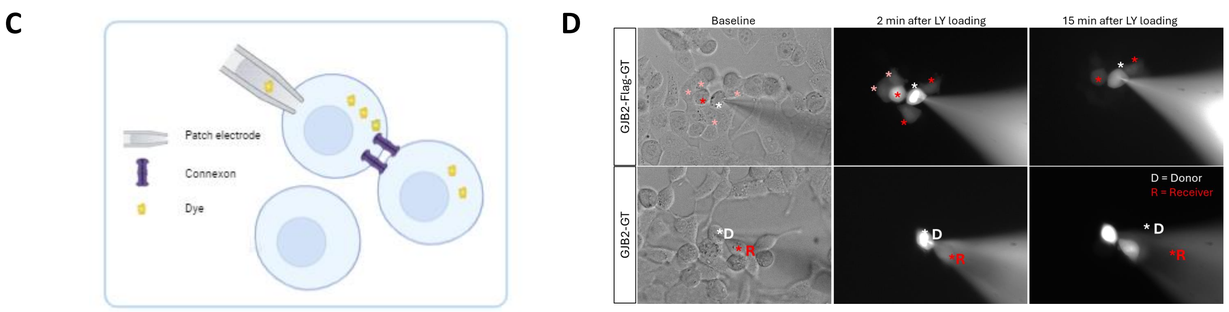


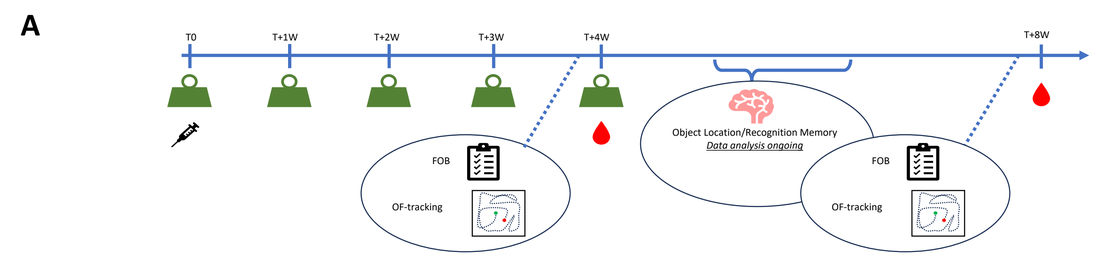
Figure 2: A. Western blot analysis of CX26 proteins levels in Hela-DH cells transduced with GJB2-GT or GJB2-FLag-GT at the indicated multiplicity of infection (MOI). HSP90 is used as a loading control. **B**. Hela DH cells transduced with GJB2-GT or GJB2-Flag-GT (MOI 1E+05) were immuno-stained with antibodies 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 C.** Schematic representation of the dye transfer assay**.** A gap-junction permeable fluorescent dye (Lucifer Yellow, LY) is micro-injected into transduced HeLa-DH cells using a patch electrode. The presence of functional gap junctions is revealed by the spread of LY to neighboring cells. **D. LY spread to adjacent cells when injected in HeLa-DH transduced with GJB2-GT (lower panel) or GJB2-Flag-GT (upper panel), indicating the assembly of functional gap junctions**.

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

GJB2-GT administration does not affect cochlear cytoarchitecture, sensory hair cell survival and hearing thresholds

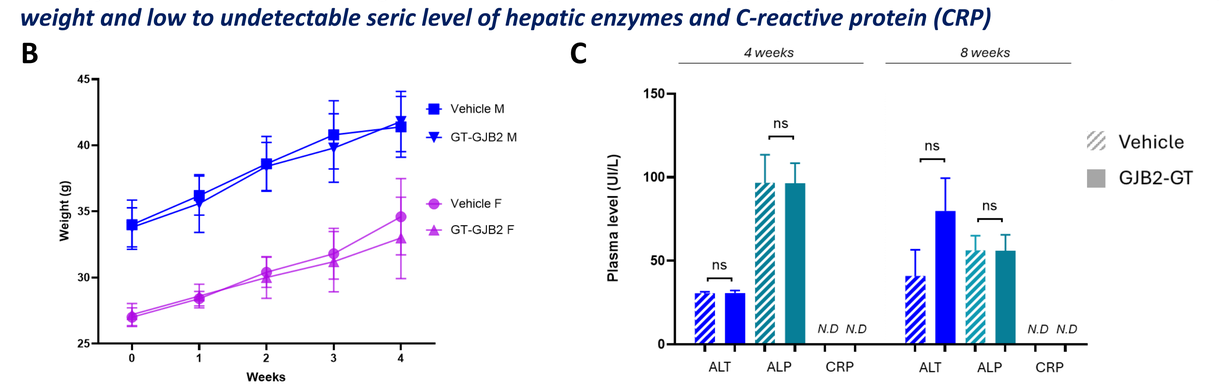
Figure 3: A. P0-P2 mice were injected through the RWM with either vehicule (n=3) or GJB2-GT vector (n=5) at the dose 3X. 3 months after injection, temporal bones were dissected and whole mount imaging of OoC were immunostained with indicated antibodies. A cochleogram analysis was performed to map the different area of the OoC with the corresponding tonotopic frequency. **Cochlear cytoarchitecture was not impacted by GJB2-GT injection. B.** Cryosections of mouse temporal bones injected with vehicle or GJB2-Flag-GT immunostained with the indicated antibodies. **Expression profile of GJB2-GT-Flag is similar to endogenous CX26 protein (vehicle injected cochlea) C.** Quantitative analysis of OHC and IHC loss at each frequency. **No significant loss of IHC/OHC was observed at all frequencies in both vehicle and GJB2-GT injected groups. D**. Auditory Brainstem Response (ABR) was measured up to 11 weeks post-injection using a close-field set-up. **ABR thresholds of GJB2-GT injected ears are similar to vehicle-injected ears indicating that GJB2-GT does not alter cochlear function.**

4. High dose intravenous (IV) injection of GJB2-GT is safe in mice



GJB2-GT is intended to be administered through intra-cochlear injection. Side effects may however arise from unwanted exposure through the blood stream. Therefore, high dose intravenous (IV) injection of GJB2-GT was conducted as an exploratory IV toxicity study.

Mice injected with GJB2-GT through the IV route display normal (sensory) motor activity, coordination, behavior, weight and low to undetectable seric level of hepatic enzymes and C-reactive protein (CRP)



No significant impact on exploratory behavior 3 and 7 weeks after GJB2-GT IV injection

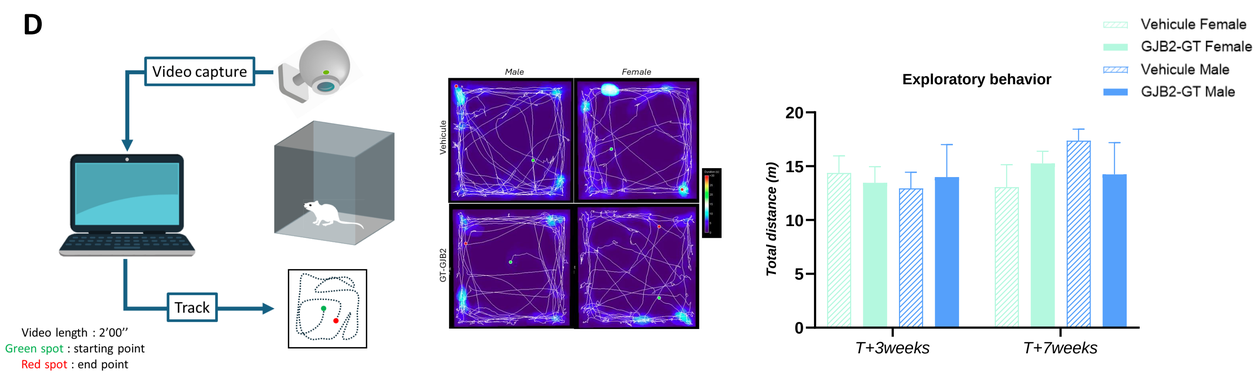


Figure 4: A. Safety study design. Two groups of adult CD® Crl:CD1 (ICR) mice received a single intravenous injection of either vehicle (n=10) or GJB2-GT (n=10). The GJB2-GT dose injected per animal corresponds to 15-fold the minimal efficacious dose in mice. B. Weight monitoring (once a week up to 4 weeks) was performed as well as FOB battery test and clinical chemistry analysis at necropsy including ALT/ALP and CRP evaluation. **B,C. No significant difference was observed in terms of weight gain (B) and hepatic enzyme levels (C) between the vehicle group and the one injected with GJB2-GT regardless of sex. Protein levels of inflammation biomarker CRP (C) was below detection threshold in the two groups and at the different timepoints tested. D**. The open field test (OF tracking) was performed to evaluate the effects of GJB2-GT on mice spontaneous exploratory behavior (level of anxiety-like behaviors and general locomotor activity). **No significant changes was noticed between vehicle and GJB2-GT injected groups, regardless of sex. Functional Observational Battery (FOB) and object location memory/object recognition memory assays analysis are ongoing. Preliminary results indicate no significant differences in neuromuscular, sensory, and autonomic functions between GT-GJB2 and vehicle injected groups.**

Conclusions

- **Targeted expression of transgene in supporting cells of the cochlea is achieved with our specific combination of capsid, promoter and regulatory sequence**
- **GJB2-GT encodes for Cx26 protein that is appropriately localized to the plasma membrane in the cochlea and functional in vitro**
- **Two safety studies conducted in mice demonstrate:**
  - ✓ **no impact of therapeutic vector on cochlea cytoarchitecture and auditory response for at least 3 months after intracochlear injection of GJB2-GT**
  - ✓ **no adverse effects regarding hepatic function, inflammation and behavior after an IV injection of GJB2-GT**
- **GJB2-GT is progressing into IND/CTA enabling studies with Clinical Trial Applications planned in 2025**