In vitro assessment of functional gap junctions for novel AAV-based GJB2 gene therapy developed to treat DFNB1A deafness

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Abstract

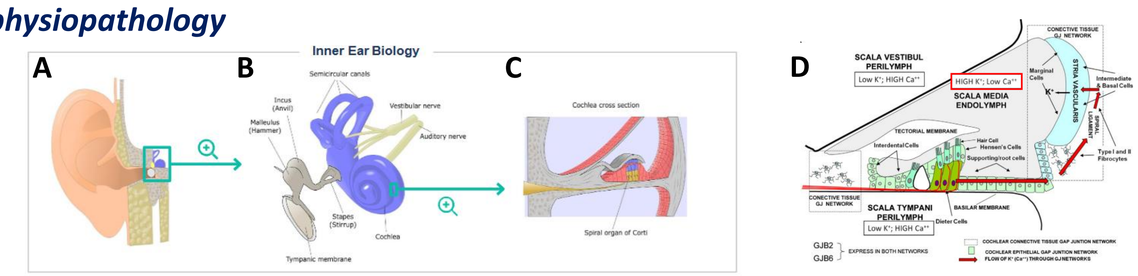
In the world, the estimated prevalence of severe or profound deafness in human is 1 out of 1000 neonates, and genetic factors account for half of the cases. Pathogenic variants of GJB2, the gene encoding for the gap junction (GJ) protein connexin 26 (Cx26), are involved in 50% of congenital deafness and are mostly associated with an autosomal recessive non-syndromic DFNB1A. Cx26 is expressed in inner ear supporting cells to post-translationally oligomerize to form hemichannel assemblies, which are ultimately inserted in the plasma membrane. Membranous Cx26-formed hemichannels are pore-forming structures that, when juxtaposing one another, build GJs allowing intercellular connexions. GJ intercellular communication (GJIC) is crucial for potassium ion (K^+) recycling, regulates intercellular ATP circulation and facilitates the maintenance of biochemical homeostasis required for normal hearing. It is hypothesized that in the absence of Cx26, disrupted potassium recycling and energy supply in cochlear cells leads to hearing loss.

In this perspective, we developed a GJB2-adeno associated vector-based gene therapy (GJB2-GT) and designed several preclinical *in vitro* assays to assess Cx26 functionality of our GJB2-GT product. The primary test explored the functionality of Cx26 hemichannels in the plasma membrane of a cell to form functional pores (Cx26-Hemichannel Permeability Assay) using propidium iodide uptake and entrapment. To assess the full functionality of GJs made up of juxtaposing Cx26 hemichannels ("Parachute" Dye-Coupling Assay), a GJ-permeant dye (Calcein AM) combined with a GJ-non-permeant dye (Vybrant DiD) was used to evaluate a dye transfer from cell to cell. Similarly, a dye transfer method by dye loading via cell microinjection of a fluorescent GJ-permeant dye (Lucifer Yellow) was developed to trace GJIC and allow to follow the diffusion of the dye in real time. All functionality tests were performed on HeLa DH cells -exempt of Cx26 under wild-type conditions- commonly used for connexin transfection assays. As positive control, HeLa DH cells, engineered to stably express Cx26, showed functional Cx26 protein localized to the plasma membrane.

GJB2-GT transduced cells displayed typical dot-like structures or Cx26-assembled GJ plaques between adjacent cells. While the **Cx26-Hemichannel Permeability Assay showed a significant dye accumulation in HeLa DH cells expressing GJB2**, addition of the widely used GJ blocker, carbenoxolone (CBX), prevented PI from crossing the hemichannels into the cytoplasm of single cells. Both dye transfer assays -the "Parachute" Dye-Coupling Assay and the microinjection-dye-loading assay, where dye was passed from donor to receiver cells, evidenced **direct passage of small dye molecules into the cytoplasm of neighbouring cells**. **This intercellular dye transfer relies on accurately and functionally assembled GJs between adjacent cells and demonstrates functional GJIC.**

Taken together, our GJB2-GT product leads to functional Cx26 localization to the plasma membrane permitting functional GJIC assembly and deficient Cx26 rescue. Additional ongoing experiments to assess the impact of our GJB2-GT product on cell physiology and efficacy in preclinical GJB2 models will complete the preclinical package paving the way to use GJB2-GT as therapeutic approach for DFNB1A patients.

1. Inner ear as an ideal target for gene therapy - facilitating the study of DFNB1A physiopathology



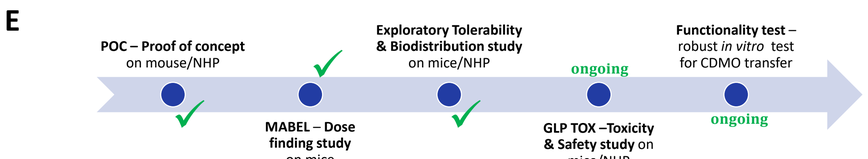


Figure 1 : A. The human inner ear is good target for gene therapy, as it is an isolated enclosed organ limiting off-target effects. Improved, safe routine surgery allows the access to critical inner ear compartments to permit precise injection of medicinal products for the treatment of human DFNB1A. **B,C**. For GJB2-GT gene therapy treatment, a transcanal round window (RW) approach is performed following a stapedotomy. An in-house designed catheter connected to a proprietary medical injection device is inserted in the RW membrane and the GJB2-GT vector injected into the cochlea, diffusing along the tonotopic axis and reaching target cells. **D.** Schematic of the cochlea, with its various compartments and the **essential role of Cx26 - K^+ ion recycling** - depicted by red arrows. **E.** Our current pipelines are covering three indications with all milestones progressing toward a timely CTA/IND application. NHP=Non-Human Primate, MABEL=Minimum anticipated biological effect level; GLP TOX=Good laboratory practice toxicology; CDMO=Contract Development Manufacturing Organization

2. Cx26 is expressed and accurately trafficked to the cell membrane of HeLa DH cells

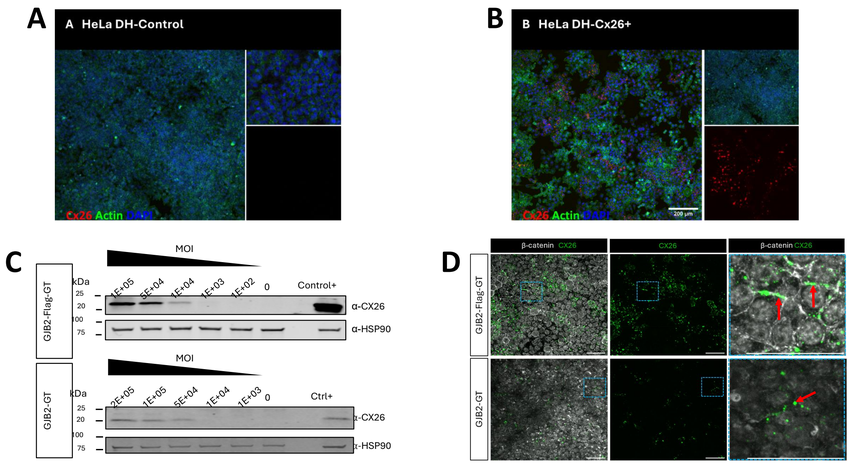


Figure 2 : A. Immunolabelling performed in HeLa DH-Control (**A**) and (**B**) cells suggests an **accurate localization of Cx26 in/along the cell membrane forming dot-like structures and potential mature GJ plaques connecting adjacent cells**. Cx26 is illustrated in red, actin in green, and DAPI in blue. Similarly, Western Blot analysis shows that Hela DH cells transduced with GJB2-GT or GJB2-Flag-GT manifest **increasing levels of Cx26 expression with increasing MOI** for each construct (**C,** upper panel GJB2-Flag-GT, lower panel GJB2-GT). While expression levels were observed with a MOI of 1E05, 5E04 and 1E04 in GJB2-Flag-GT-transduced HeLa DH cells, in GJB2-GT-transduced HeLa DH cells similar levels were obtained at a minimal MOI of 1E05. Immunolabelling of GJB2-GT- (**D**, lower panel) or GJB2-Flag-GT-transduced (**D**, upper panel) HeLa DH cells (at a MOI of 1E05) indicate the **presence of Cx26 plaques/hemichannels (red arrows), consistent with the formation of gap junctions.** Scale bar 50 µm. Cx26 in green and β-Catenin in grey for membrane labeling.

3. In vitro functionality demonstrated via the hemichannel permeability assay

Efficient propidium iodide (PI) dye uptake via Cx26-formed hemichannels

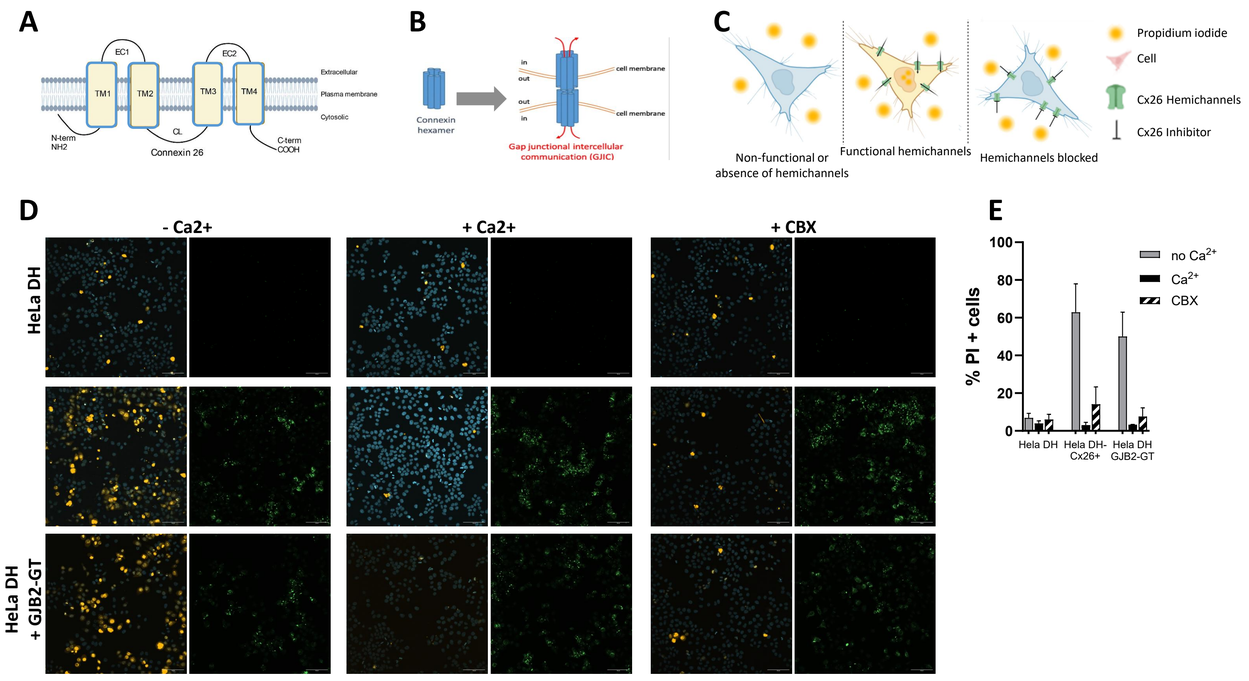


Figure 3 : A Schematic of the Cx26 transmembrane protein (Mao *et al.*, 2023)**. B**. Six Cx26 proteins assemble into a hemichannel (connexon), which then form GJs spanning from one cell to another. **C.** Schematic of PI action in HeLa DH cells (Smith 2025). **D.** HeLa DH cells (parental cell line), stable GJB2-expressing HeLa DH cells, or HeLa DH cells transduced with GJB2-GT vector were incubated with PI prepared in HBBS without Ca^{2+}, containing 1 mM EGTA and 1 mM $MgCl_2$. PI in yellow, DAPI in blue, Cx26 in green. In HeLa DH control cells, which are exempt of connexin proteins, no PI dye transfer is observed, while **HeLa DH cells constitutively expressing Cx26 or transduced with the transgene GJB2-GT both demonstrated PI loading (yellow staining) in the presence of Cx26 protein** (green staining; left panel). When cells are incubated in the presence of high levels of Ca^{2+} (middle panel), Cx26 protein is detected in HeLa DH cells transduced with GJB2-GT, but PI dye transfer is absent. Equally, the preincubation with carbenoxolone (CBX; 100 µM; right panel), a known GJ blocker, prevents the passage of PI into Cx26-positive HeLa DH cells, without affecting the presence of the Cx26 protein. **E.** Quantification of PI-positive cells under the various conditions. **Taken together, the hemichannel permeability assay confirms that our GJB2-GT transgene is expressed and assembled correctly into connexons in the cell membrane, which are permeable to the PI dye.**

4. The in vitro hemichannels permeability assay allows a quantitative approach to evaluate Cx26 functionality

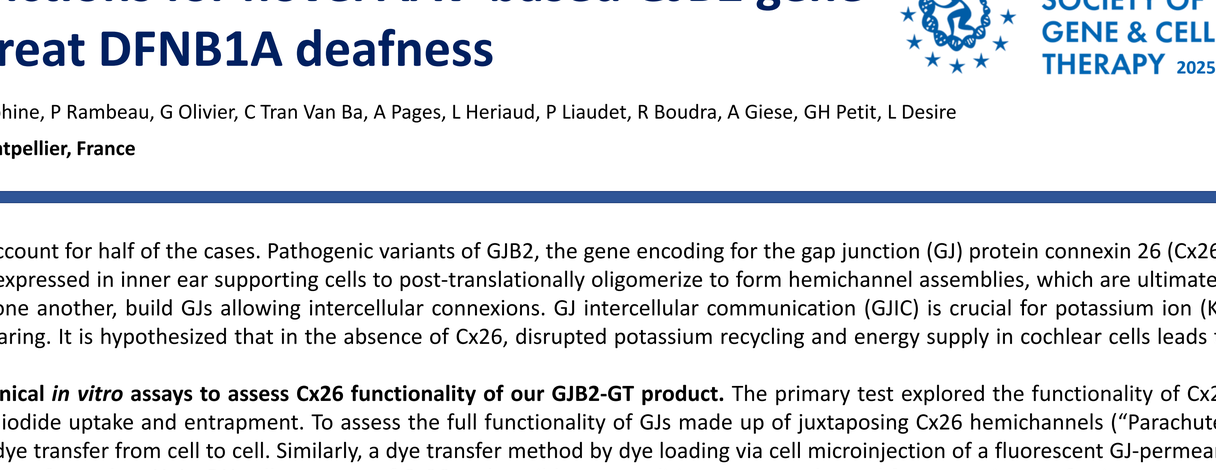


Figure 4 : A Quantitative approach for the hemichannel permeability assay was validated using immunolabelling (A, B) and fluorescence flow cytometry (C, D). A, B. Immunolabelling performed in GJB2-GT-transduced HeLa DH cells reflected an increase in PI-positive cells (PI+ cells) in a concentration-dependent manner (increase in MOI from 0 to 250 k) (**A**). **B.** represents the quantitative analysis of the immunofluorescence results. Cx26 is illustrated in green, PI in orange. **C,D.** Correspondingly, flow cytometry implemented on GJB2-GT-transduced HeLa DH cells offered an automated detection, counting, and sorting of PI-positive cells, validating a high-throughput analysis of our product GJB2-GT. **D.** represents the quantitative analysis of the flow cytometry results. **Taken together, the flow cytometry analysis allows us to run automated time-saving analysis and evaluate the different transgene R&D batches.**

5. GJB2-GT-mediated rescue of protein expression and function in a Cx26 pathogenic context

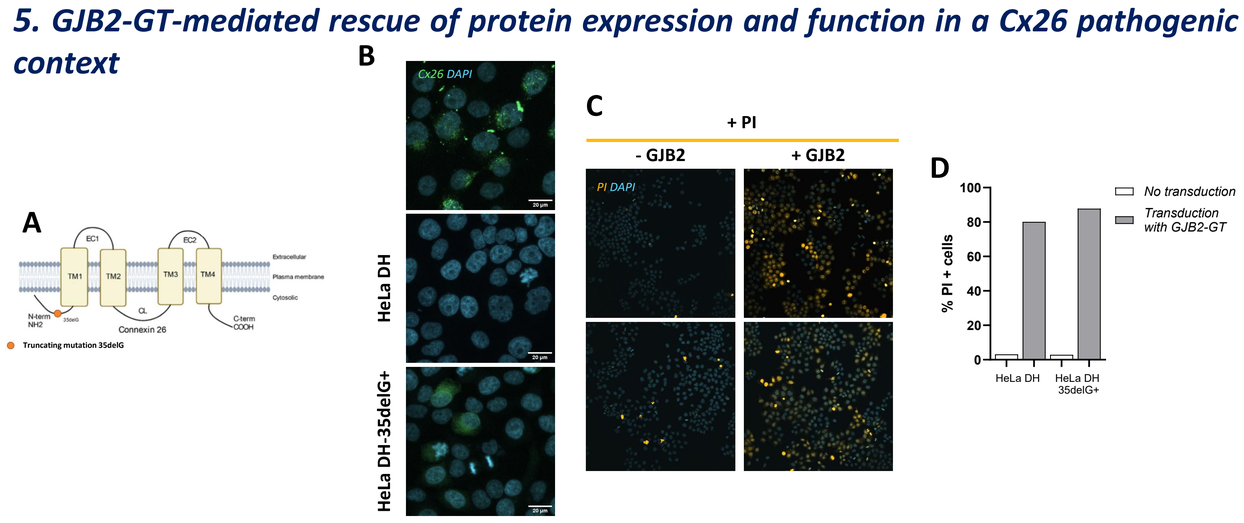


Figure 5 : A,B. The 35delG mutation is a common mutation in the GJB2 gene, which represents a truncating mutation resulting in severe-to-profound hearing loss. **C.** Here, the PI hemichannel permeability assay was used to investigate whether our GJB2-GT product can rescue the 35delG-related mutant Cx26 profile in HeLa DH-35delG+ cells by re-introducing functional GJB2-GT-produced Cx26 in HeLa DH cells. While HeLa DH cells (parental cell line, left image in middle panel in **B**) serve as the control negative and cells as the control positive (top image in top panel in **B**), another HeLa DH cell line was generated in-house to stably express the 35delG product (HeLa DH-35delG+, left image in lower panel in **B**). **C.** GJB2-GT transduction in HeLa DH cells resulted as expected in PI uptake (**C**, top panel right image, yellow staining). In contrast, in the absence of GJB2-GT no PI uptake was observed in HeLa DH-35delG+ cells, indicating that this **truncating mutation did not lead to the production of functional hemichannels** (**C**, lower panel left image). Interestingly, addition of GJB2-GT in these mutant cells caused a PI dye uptake in the cells, suggesting that **our GJB2-GT product can rescue the mutant 35delG profile by expressing functional Cx26 and might be efficient in different Cx26 pathogenic contexts**. Scale bar 20 µm. **D.** Quantification of PI-positive cells under the two conditions.

6. Exploratory methods for the in vitro assessment of Cx26 & GJ functionality & GJIC efficiency

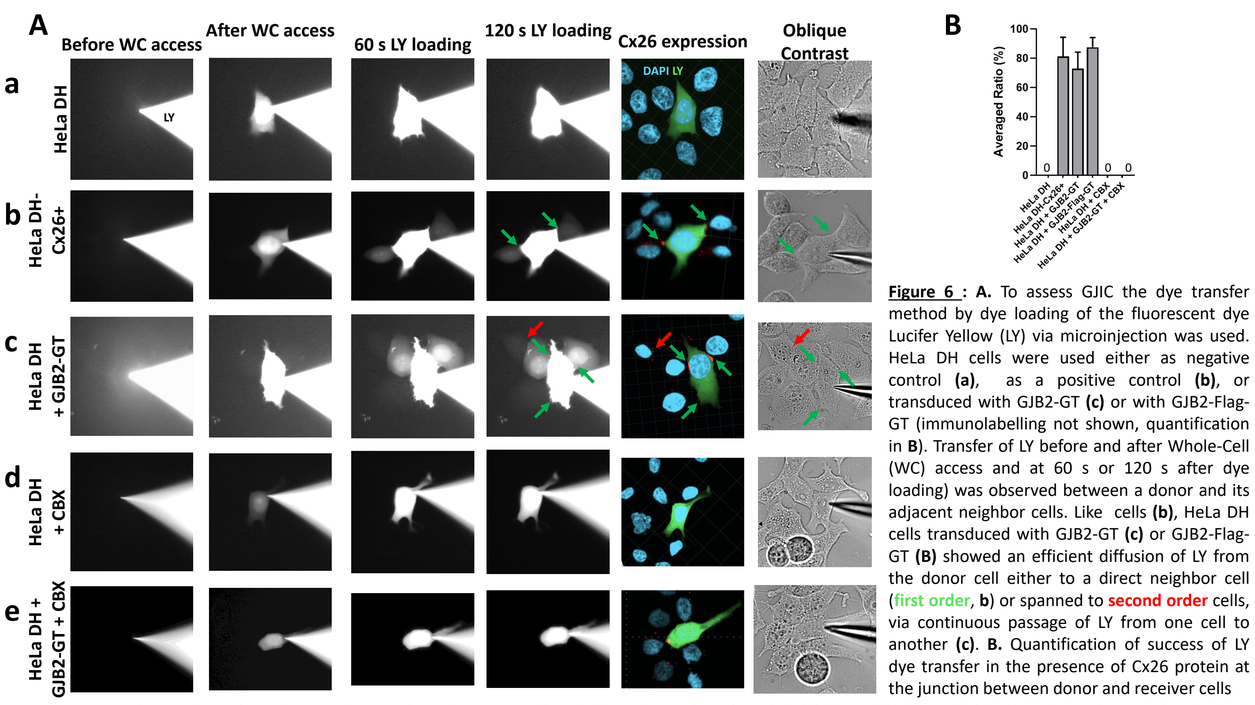


Figure 6 : A. To assess GJIC the dye transfer method by dye loading of the fluorescent dye Lucifer Yellow (LY) via microinjection was used. HeLa DH cells were used either as negative control **(a)**, as a positive control **(b)**, or transduced with GJB2-GT **(c)** or with GJB2-Flag-GT (immunolabelling not shown, quantification in **B**). Transfer of LY before and after Whole-Cell (WC) access and at 60 s or 120 s after dye loading) was observed between a donor and its adjacent neighbor cells. Like cells **(b)**, HeLa DH cells transduced with GJB2-GT **(c)** or GJB2-Flag-GT **(B)** showed an efficient diffusion of LY from the donor cell either to a direct neighbor cell (first order, **b**) or spanned to **second order** cells, via continuous passage of LY from one cell to another **(c)**. **B.** Quantification of success of LY dye transfer in the presence of Cx26 protein at the junction between donor and receiver cells (Averaged ratio of number of LY transfer/number of receiver cells + Cx26 in %). **A. (d) and (e)** GJ inhibition with CBX (a GJ blocker) showed a complete lack of LY transfer in GJB2-GT transduced HeLa DH cells. In conclusion, these preliminary results indicate that GJB2-GT is capable of adequately forming functional Cx26-formed GJs between adjacent cells.

Conclusions

- Our product GJB2-GT leads to the expression of a functional CX26 protein, which is accurately presented at the cell membrane.
- The hemichannel permeability assay is a robust *in vitro* test confirming the functionality of GJB2-GT by allowing the diffusion of PI into cells.
  The use of the flow cytometry approach allows a rapid and automated analysis compatible with a method for a potency test.
- CX26-formed hemichannels are blocked by a known GJ blocker carbenoxolone and by increased levels of Ca^{2+}, indicating that GJB2-GT transduction leads to accurate assembly of CX26 into hemichannels.
- Interestingly, GJB2-GT transduction of HeLa DH cells expressing a truncated version of Cx26 (35delG) can rescue the pathological phenotype.
- The GJ functionality & GJIC efficiency assay using LY microinjection was successful to confirm results obtained with the PI assay.
- Several R&D batches of GJB2-GT have demonstrated activity in these assays.
- These tests have the potential to evolve into potency assays pending validation (linearity, specificity, etc.).