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

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P0109

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 encoding the protein connexin 26 (CX26), underlie 50% congenital deafness and are mostly associated with autosomal recessive non-syndromic DFNB1A. In inner ear supporting cells, CX26 oligomerizes into hemichannel, or connexons, which are ultimately inserted in the plasma membrane. Juxtaposed hemichannels from adjacent cells dock to form gap junctions (GJs), allowing intercellular connections. GJ intercellular communication (GJIC) is crucial for potassium ion (K+) recycling, regulates intercellular ATP exchange 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 gene therapy vector, SENS-601, and designed in vitro assays to assess the functionality and potency of manufactured vector batches.

First, we developed the Hemichannel Potency Assay (HPA), which exploits the patency of functional CX26 hemichannels to propidium iodide (PI), a membrane impermeant molecular tracer. Intracellular PI fluorescence, measured by flow cytometry, increased in a dose-dependent manner in human cell lines (HeLa DH) transduced with SENS-601. PI uptake was abolished in SENS-601 transduced cells pretreated with CX26 inhibitors and was comparable to non-transduced cells lacking CX26 expression. Using the HPA on genetically engineered HeLa DH cells expressing human pathogenic variants of GJB2 (c.35delG; p.Val37Ile, p.Met34Thr), we showed that **PI uptake is strongly reduced in these cells, consistent with the impaired hemichannels assembly, and that SENS-601 restores hemichannel function in these pathogenic contexts**. Taken together, these data demonstrate the specificity and quantitative nature of the HPA in support of SENS-601 development.

As a secondary approach to assess SENS-601 ability to form functional GJs, we developed a method to track the intercellular diffusion of a fluorescent GJ-permeant dye (Lucifer Yellow, LY) in SENS-601 treated HeLa DH cells by real-time imaging. Microinjection of LY into a single transduced cell resulted **in the rapid diffusion of the dye to neighboring cells. Importantly, dye diffusion only occurs in cells which shares a CX26 GJ channel with the microinjected cell.** In contrast, no dye diffusion was observed in non-transduced cells or in SENS-601 transduced cells pre-treated with CX26 inhibitors, demonstrating the assay specificity.

In summary, we have developed sensitive, quantitative and specific orthogonal assays to measure the SENS-601 functionality in vitro. Using these methods, we confirmed that **SENS-601 transduction leads to functional CX26 localization to the plasma membrane permitting functional GJIC assembly and rescue of deficient CX26 function.** Together with our comprehensive in vivo data package demonstrating efficacy and safety of SENS-601 in several preclinical models, this work supports the clinical development of SENS-601 to treat patients suffering from non-syndromic recessive GJB2-related hearing loss.

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

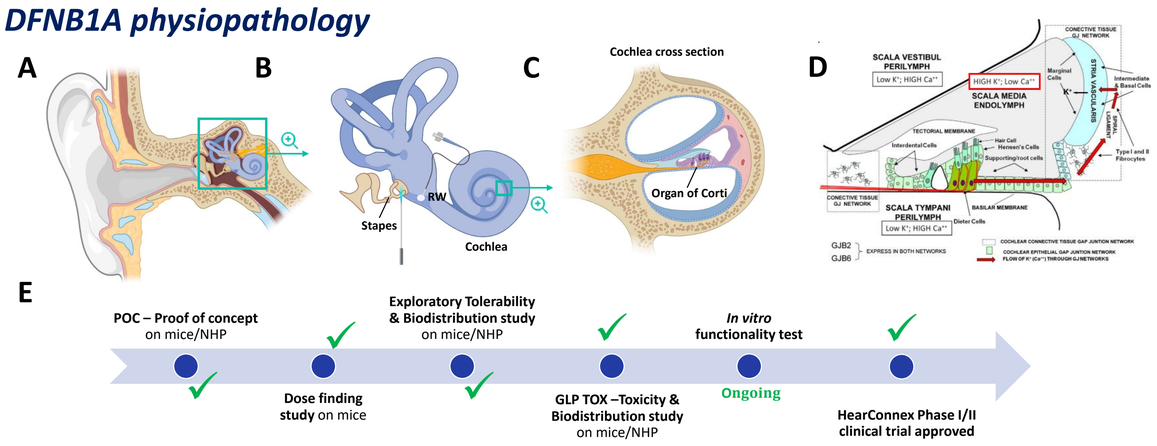


Figure 1 : A. The human inner ear is good target for gene therapy, as it is an isolated, enclosed organ allowing limited biodistribution outside the ear, composed by non-dividing cells. A safe routine surgery allows the access to critical inner ear compartments for precise injection. **B, C.** For SENS-601 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 SENS-601 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 (from Xu and Nicholson 2013). **E.** Main preclinical program milestones. HearConnex Phase I/II clinical trial is approved in France, first patient dosing anticipated in early 2027. NHP=Non-Human Primate, GLP TOX=Good laboratory practice toxicology.

2. CX26 is expressed and accurately trafficked to the cell membrane of HeLa DH cells

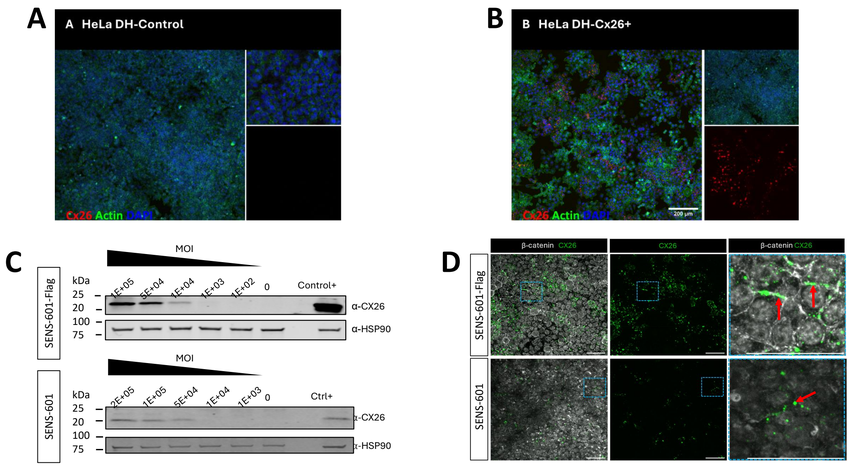


Figure 2 : A. Immunolabelling performed in HeLa DH-Control (**A**) and (**B**) cells suggests an **accurate localization of CX26 in/along the cell membrane forming dot-like structures and potential mature GJ plaques connecting adjacent cells**. CX26 is illustrated in red, actin in green, and DAPI in blue. Similarly, Western Blot analysis shows that Hela DH cells transduced with SENS-601 or SENS-601-Flag manifest **increasing levels of CX26 expression with increasing MOI** for each construct (**C,** upper panel SENS-601-Flag, lower panel SENS-601). While expression levels were observed with a MOI of 1E05, 5E04 and 1E04 in SENS-601-Flag-transduced HeLa DH cells, in SENS-601-transduced HeLa DH cells similar levels were obtained at a minimal MOI of 1E05. Immunolabelling of SENS-601- (**D**, lower panel) or SENS-601-Flag-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 of SENS-601 demonstrated via an hemichannel permeability assay

Efficient propidium iodide (PI) dye uptake via CX26-formed hemichannels

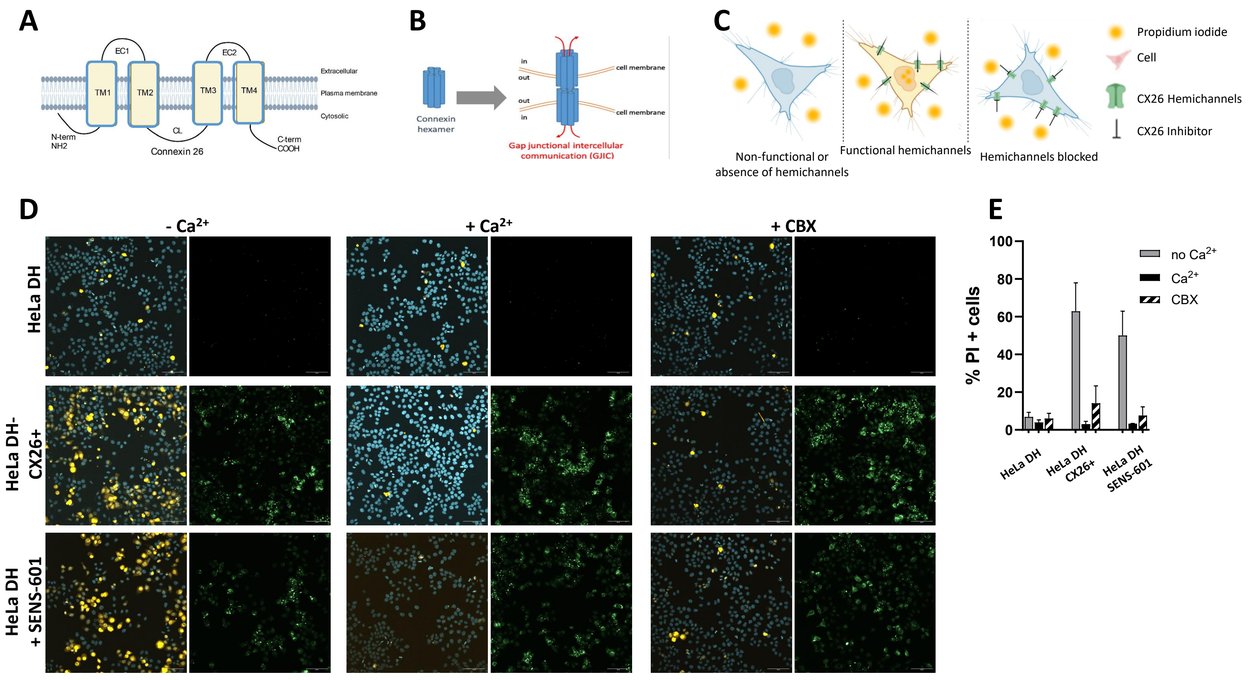


Figure 3 : A. Schematic of the CX26 transmembrane protein (Mao *et al.*, 2023). **B**. Six CX26 proteins assemble into a hemichannel (connexon), which then form GJs spanning from one cell to another. **C.** Schematic of PI action in HeLa DH cells (Smith 2025). **D.** HeLa DH cells (parental cell line), stable *GJB2*-expressing HeLa DH cells, or HeLa DH cells transduced with SENS-601 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 SENS-601 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), which induces channel closure, CX26 protein is detected in HeLa DH cells transduced with SENS-601, 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 SENS-601 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

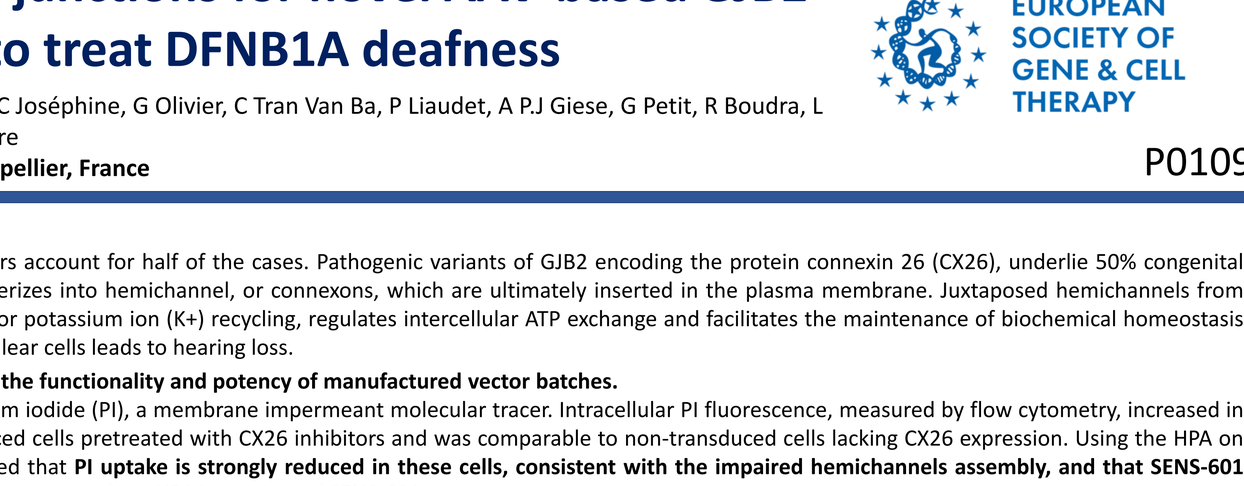


Figure 4 : A-D. Quantitative approach for the hemichannel permeability assay was validated using immunolabelling (A, B) and fluorescence flow cytometry (C, D). A. HeLa DH cells transduced with SENS-601 at increasing MOI (0–250K) were immunostained for PI. Representative images show an increase in the percentage of PI-positive cells with increasing MOI. CX26 is illustrated in green, PI in orange. **B.** Quantification of PI-positive cells by fluorescence microscopy. **C,D.** Correspondingly, flow cytometry implemented on SENS-601-transduced HeLa DH cells offered an automated detection, counting, and sorting of PI-positive cells, validating a high-throughput analysis of our product SENS-601. **D.** Represents the quantitative analysis of the flow cytometry results. **Taken together, the flow cytometry analysis allows us to run automated time-saving analyses to evaluate different SENS-601 batches.**

5. SENS-601-mediated restoration of protein expression and function in a CX26 pathogenic context

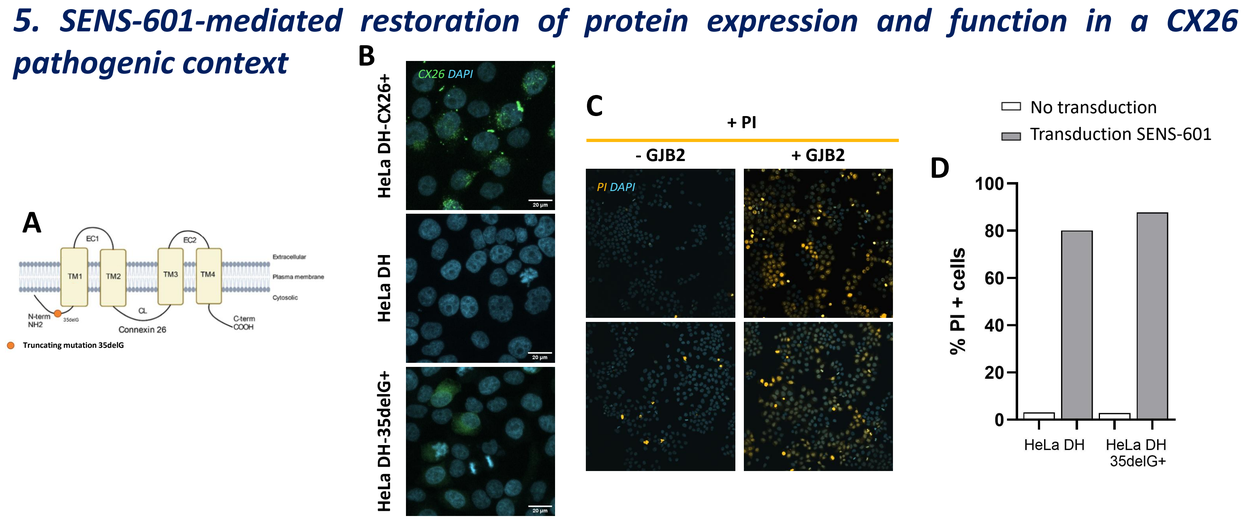


Figure 5 : A,B. The 35delG mutation is a common mutation in the *GJB2* gene, which represents a truncating mutation resulting in severe-to-profound hearing loss. **C.** Here, the PI hemichannel permeability assay was used to investigate whether our SENS-601 product can rescue the 35delG-related mutant CX26 profile in HeLa DH-35delG+ cells by re-introducing functional SENS-601-produced CX26 in HeLa DH cells. While HeLa DH cells (parental cell line, left image in middle panel in **B**) serve as the negative control and cells as the positive control (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.** SENS-601 transduction in HeLa DH cells resulted as expected in PI uptake (**C**, top panel right image, yellow staining). In contrast, in the absence of SENS-601 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 SENS-601 in these mutant cells caused a PI dye uptake in the cells, suggesting that **our SENS-601 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

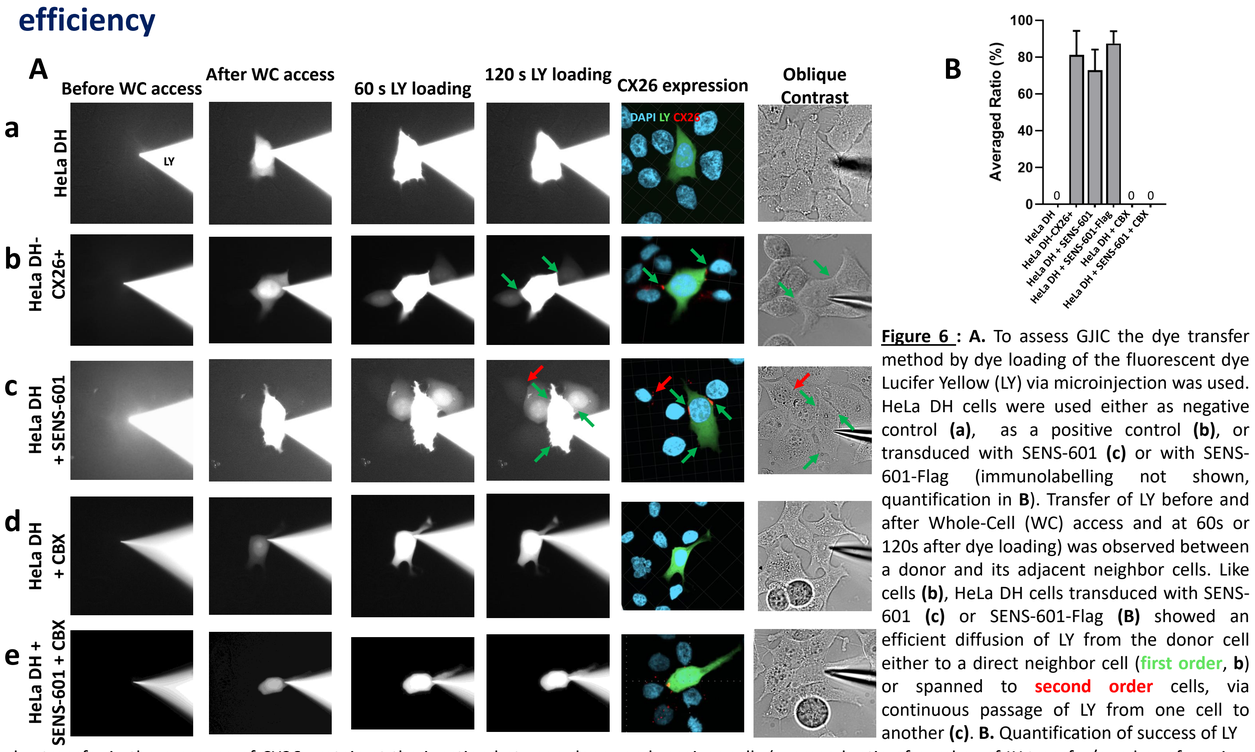


Figure 6 : A. To assess GJIC the dye transfer method by dye loading of the fluorescent dye Lucifer Yellow (LY) via microinjection was used. HeLa DH cells were used either as negative control **(a)**, as a positive control **(b)**, or transduced with SENS-601 **(c)** or with SENS-601-Flag (immunolabelling not shown, quantification in **B**). Transfer of LY before and after Whole-Cell (WC) access and at 60s or 120s after dye loading) was observed between a donor and its adjacent neighbor cells. Like cells **(b)**, HeLa DH cells transduced with SENS-601 **(c)** or SENS-601-Flag **(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 SENS-601 transduced HeLa DH cells. In conclusion, these preliminary results indicate that SENS-601 is capable of adequately forming functional CX26-formed GJs between adjacent cells.

Conclusions

- SENS-601 leads to the expression of a functional CX26 protein, which is accurately targeted to the cell membrane.
- The hemichannel permeability assay is a robust *in vitro* test confirming the functionality of SENS-601 by allowing the diffusion of PI into cells.
  The use of the flow cytometry approach allows a rapid and automated analysis and test validation (linearity, specificity, etc.) is ongoing.
- CX26-formed hemichannels are blocked by a known GJ blocker carbenoxolone and by increased levels of Ca^{2+}, indicating that SENS-601 transduction leads to efficient assembly of CX26 into hemichannels.
- Interestingly, SENS-601 transduction of HeLa DH cells expressing a truncated version of CX26 (35delG) can restore GJIC functionality.
- The GJ functionality & GJIC efficiency assay using LY microinjection was successful to confirm results obtained with the PI assay.
- Efficacy was replicated across multiple research and development manufacturing batches of SENS-601.