MISSENSE MUTATION OF KCNQ4 GENE ASSOCIATED TO DFNA2: VIRAL VECTOR TO RECOVER HEARING FUNCTIONS

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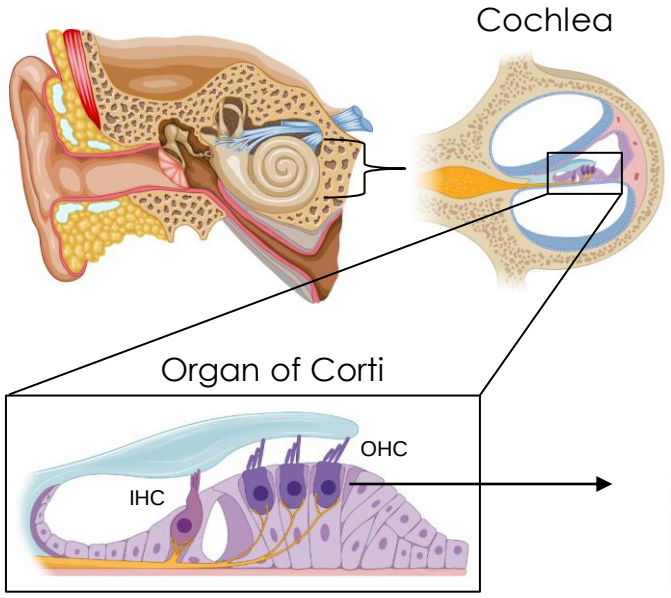
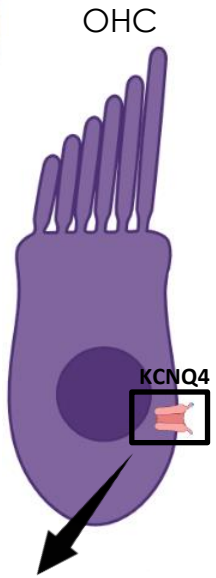
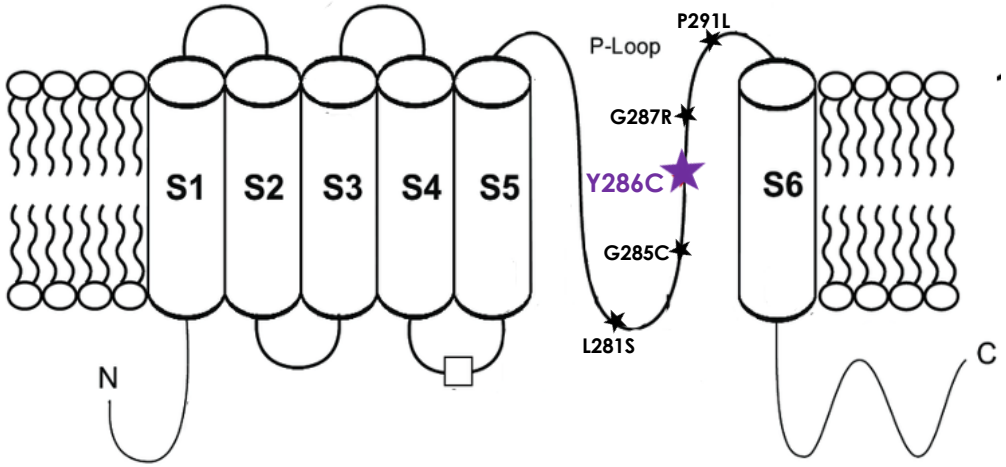
Background







KCNQ4 ASSOCIATED TO DFNA2

WHAT

Autosomal dominant disease due to **KCNQ4** channel mutation present on the **outer hair cells** in the inner ear

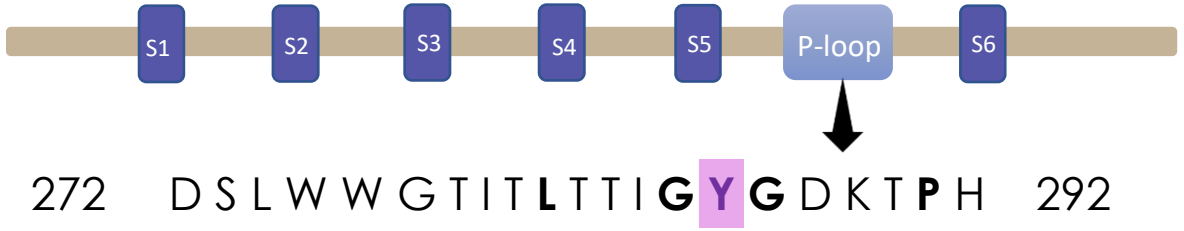
CAUSES

Gene nucleotide substitutions in the portion coding for the **P-loop** that generate **loss of function**

OUTCOME

- **cell death** due to cytotoxicity
- progressive **hearing loss** (**ipoacusie**)
- Worsens **every 10 years** till total compromise at 70

AGING

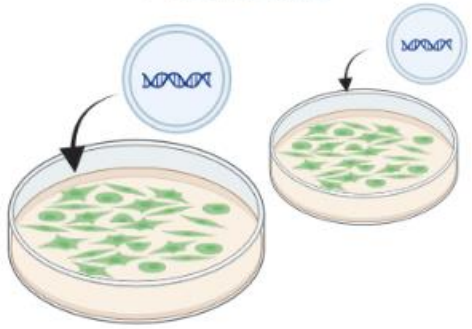


AIM OF THE PROJECT

IN VITRO

1

HEK transfection
- KCNQ4+
- KCNQ4Y286C



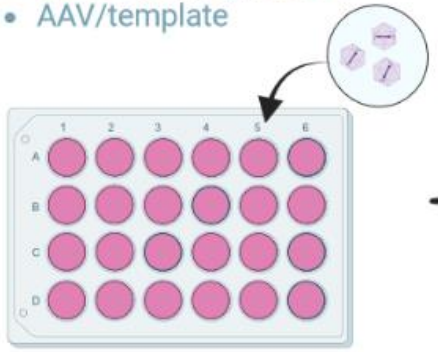
FACS

WB

Whole-cell patch clamp

2

- AAV/**Cas9n**/sgRNA5+6
- AAV/template



FACS

WB

Whole-cell patch clamp

CIRCLE-seq

IN VIVO

1

ABR

4 - 8 - 12 weeks

2

- AAV/**Cas9n**/sgRNA5+6
- AAV/template

OHCs explant

Thallium-based imaging

Whole-cell patch clamp

NGS

ABR

4 - 8 - 12 weeks

Vector design

AAV9 Cas9n

AAV9 template

Possible sgRNAs

CCGCCCCTGCAGACTTCATCGTGTTCGTGGCCTCGGTGGCCGTCATCGCCGCGGGTACCCAGG

GCAGACTTCATCGTGTTCGTGGCCTCGGTGGCCGTCATCGCCGCGGGTACCCAGGGCAACATCTTCGC

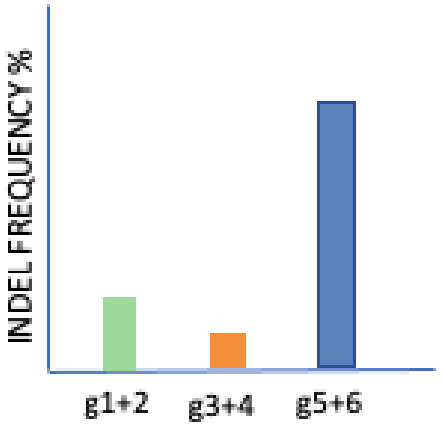
CCGCCCCGCCCCTGCAGACTTCATCGTGTTCGTGGCCTCGGTGGCCGTCATCGCCGCGGGTACCCAGGG

PAM CGG

g1 + g2

g3 + g4

g5 + g6 ←

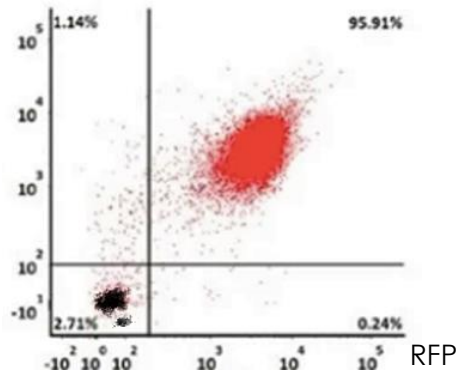


MODEL CONSTRUCTION *IN VITRO*

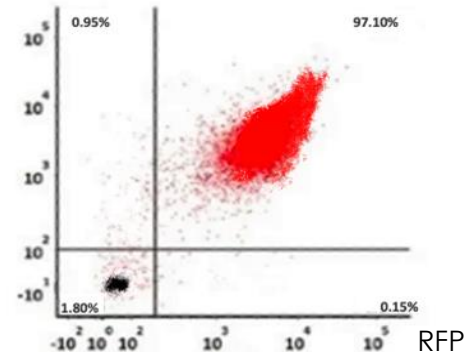
1 Creation of **HEK293** cell lines: **WT** and **KCNQ4$_{Y286C}$**

FACS

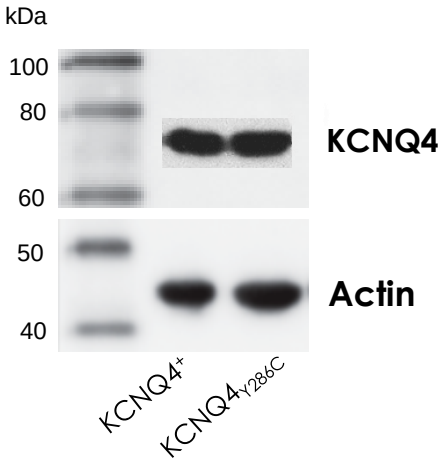
HEK293 cells+RFP labelled **KCNQ4$^+$**

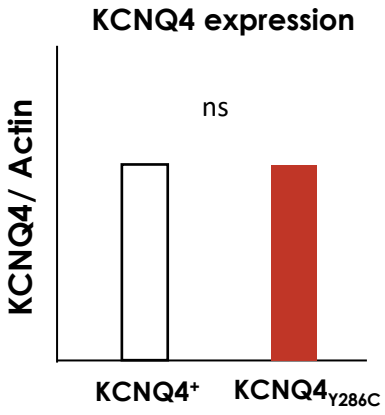


HEK293 cells+RFP labelled **KCNQ4$_{Y286C}$**

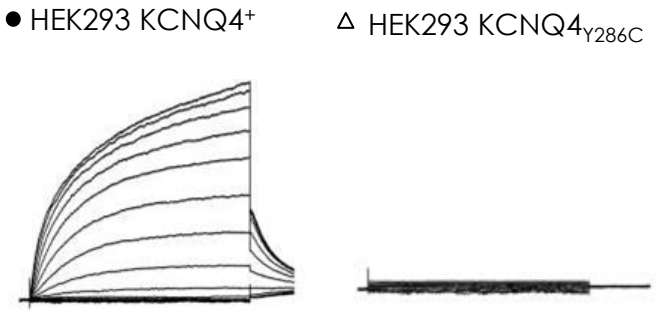


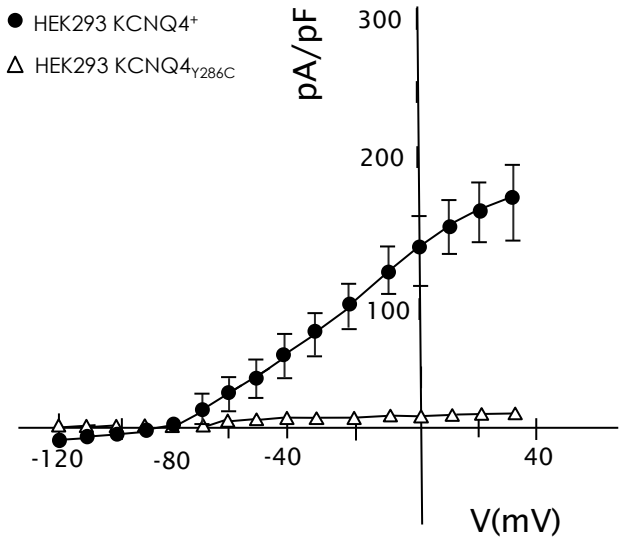
WB





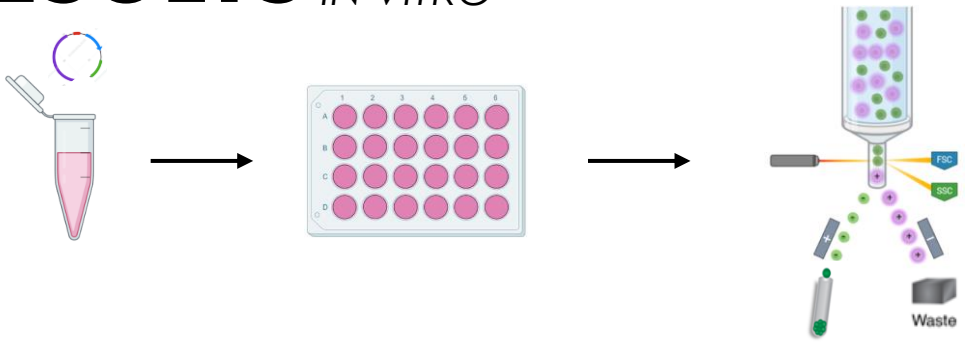
Whole-cell patch clamp





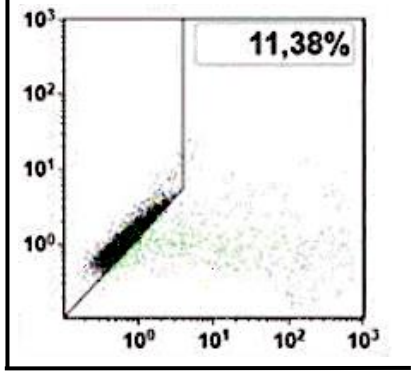
EXPECTED RESULTS *IN VITRO*

② **HEK KCNQ4$_{Y286C}$** trasduction with **AAV** vectors



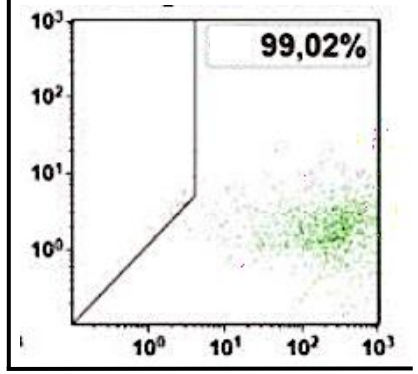
FACS

Cas9n/gRNA5+6+template



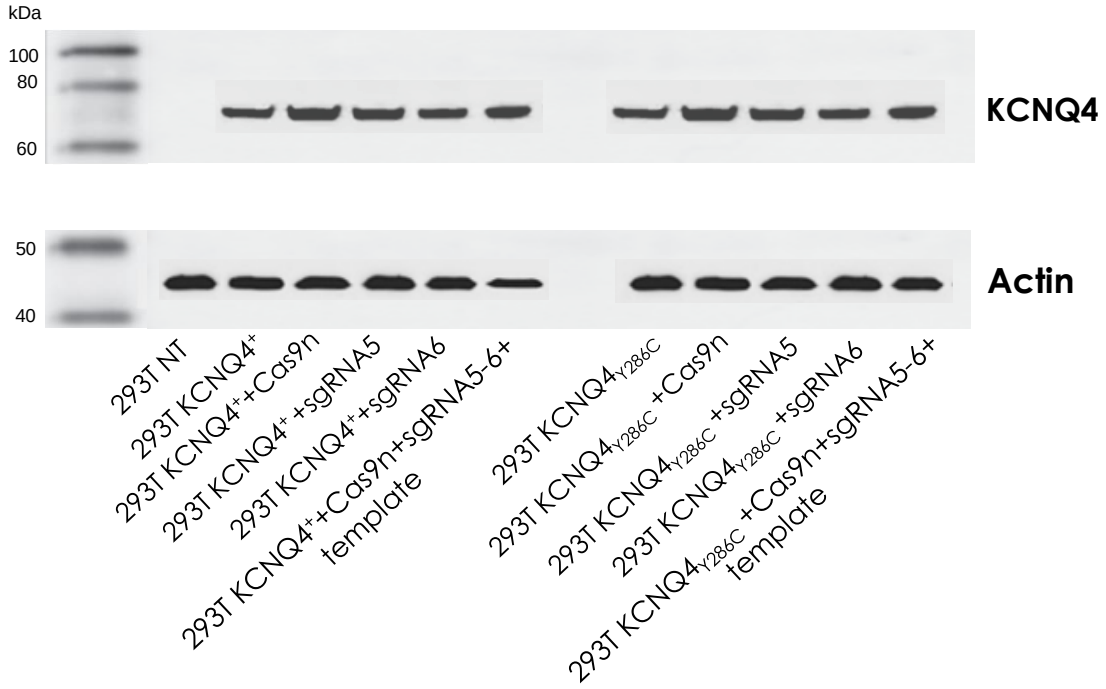
GFP – one day post transduction

Cas9n/gRNA5+6+template



GFP – 14 day post transduction

WB



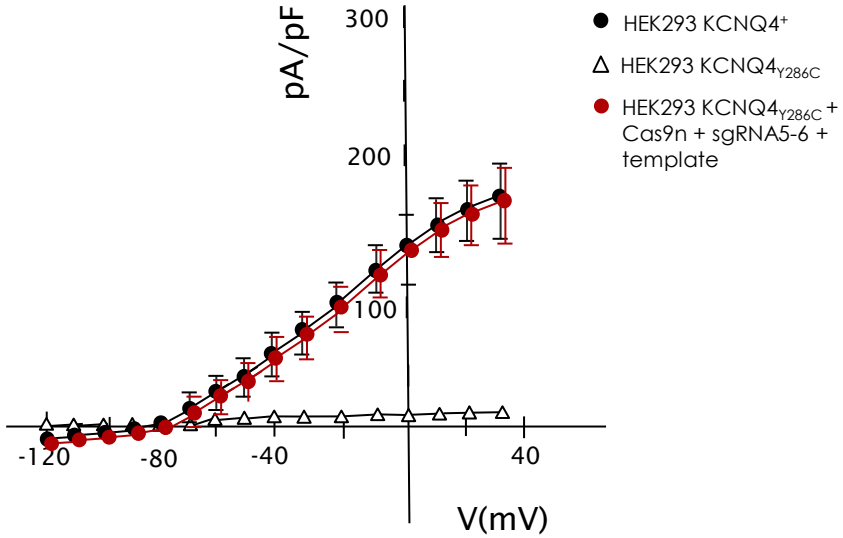
EXPECTED RESULTS *IN VITRO*

Whole-cell patch clamp

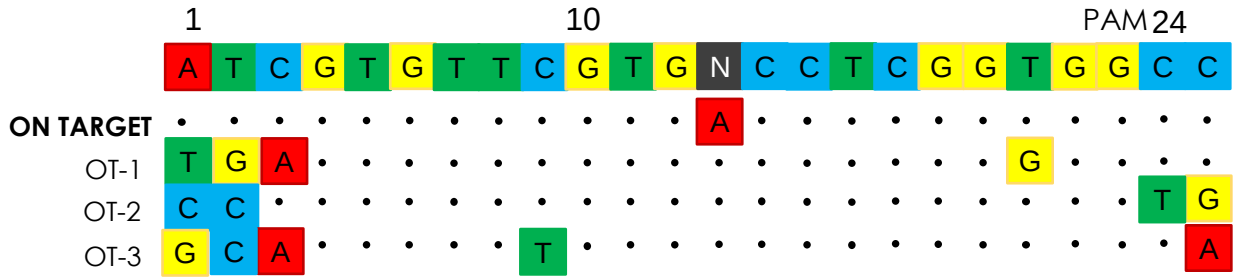
● HEK293 KCNQ4^{+}

△ HEK293 KCNQ4$_{Y286C}$

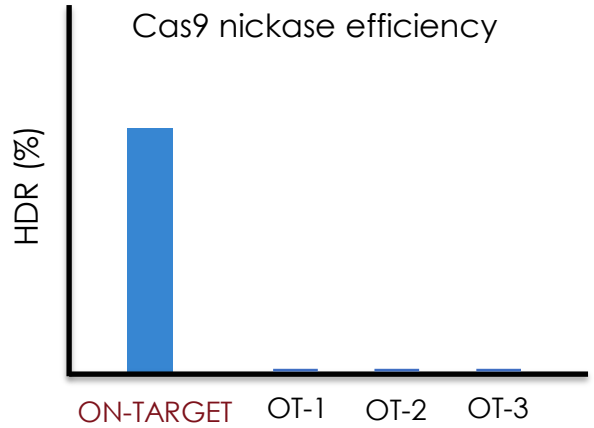
● HEK293 KCNQ4$_{Y286C}$ + Cas9n + sgRNA5+6 + template



CIRCLE-seq

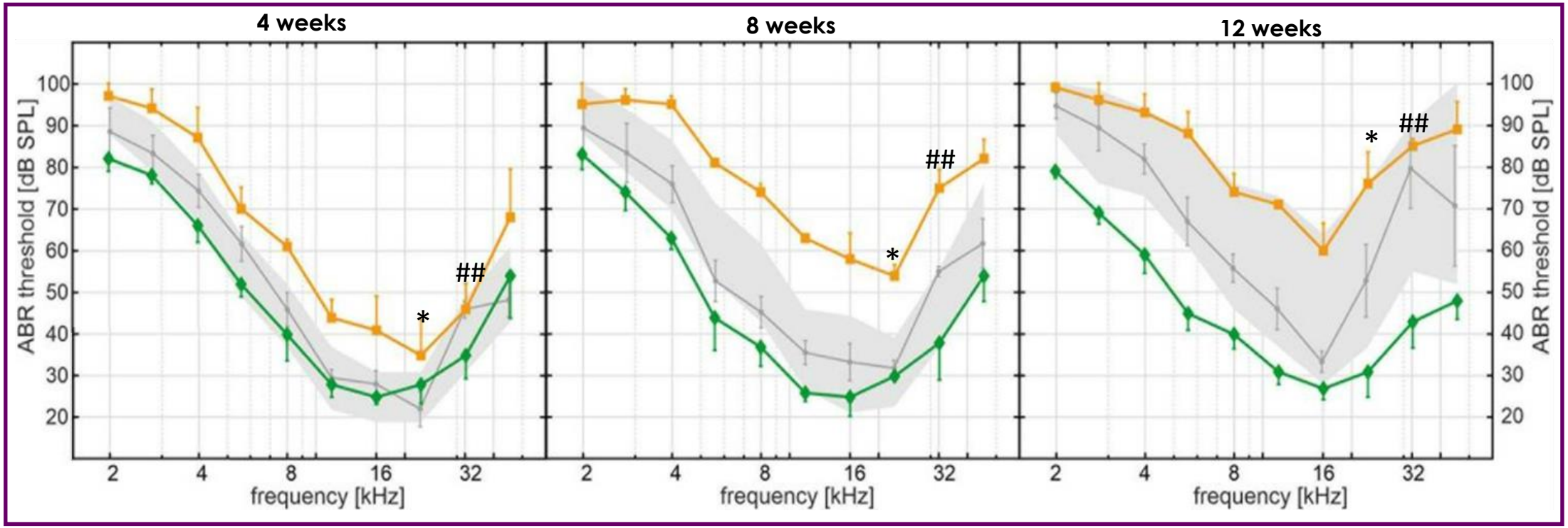


OT= OFF TARGET



EXPECTED RESULTS *IN VIVO*

1 **ABR** in **WT** e **KI** before AAV injection

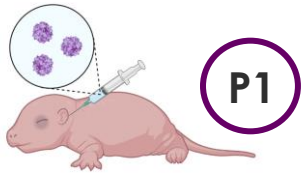


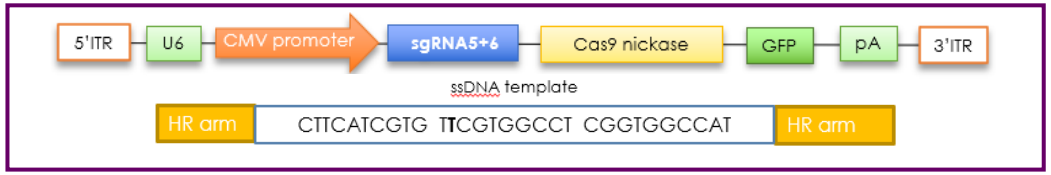
- ABR → KI (C57BL/6-tg KCNQ4^{Y286C}) n=7
- ABR → WT (C57BL/6) n=8
- Baseline threshold

* $p < 0.05$ WT vs KI, two-way ANOVA
$p < 0.05$ KI 4 weeks vs KI 8 weeks vs KI 12 weeks, two-way ANOVA
n obtained with G*power

EXPECTED RESULTS *IN VIVO*

② **AAV** intracochlear injection into **KI** (C57BL/6-tg KCNQ4^{Y286C})



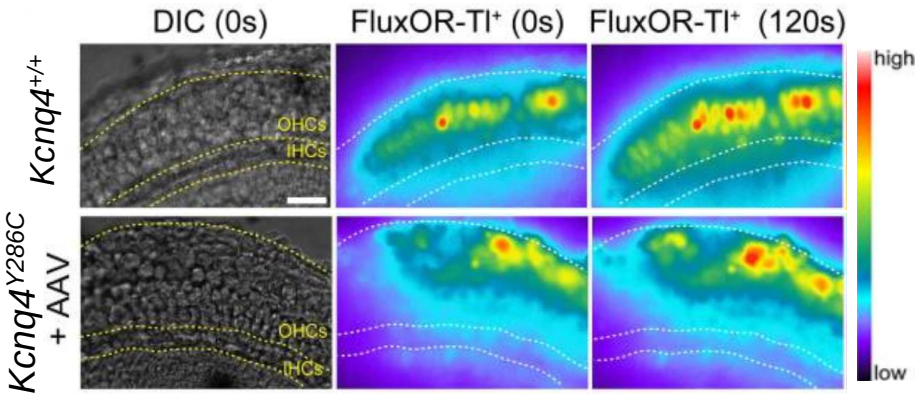


EXPERIMENTAL GROUP:
1. KI (C57BL/6-tg KCNQ4^{Y286C}) AAV double system injected (n=17)

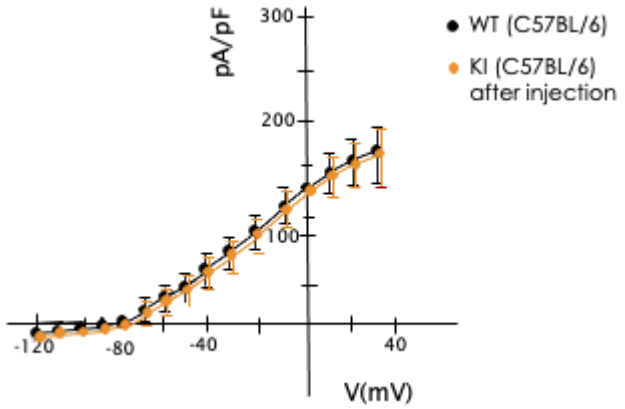
CONTROL GROUPS:
1. WT (C57BL/6) (n=12)
2. KI (C57BL/6-tg KCNQ4^{Y286C}) blank vector injected (n=8)

OHCs explant

Thallium-based imaging



Whole-cell patch clamp

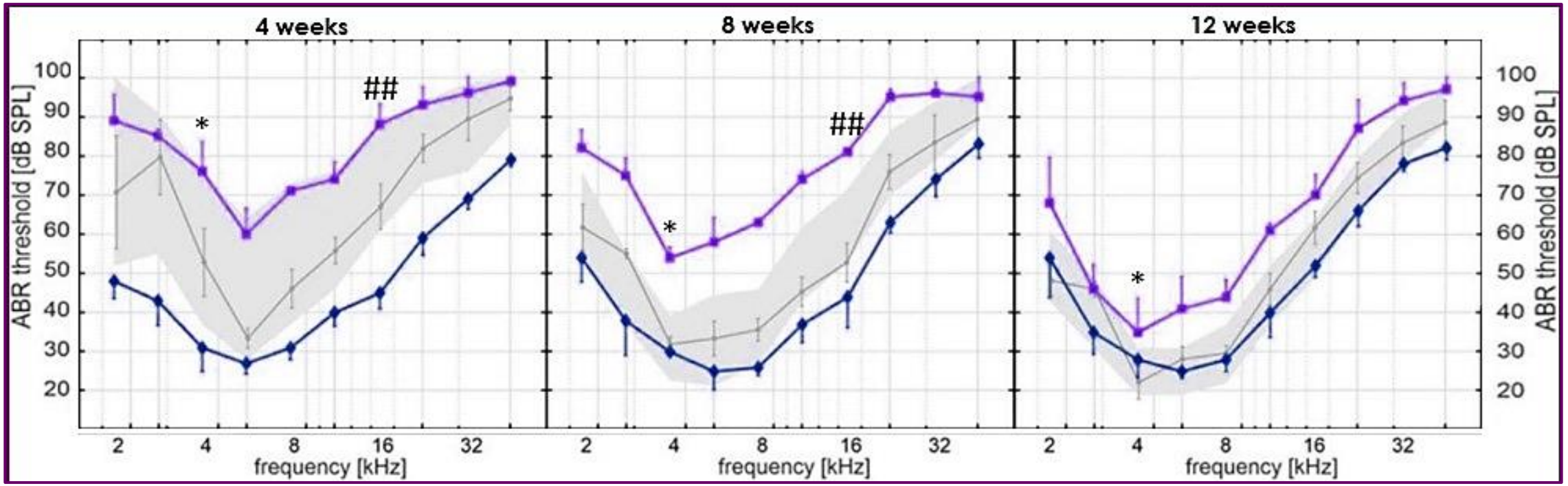


NGS

1 week after the injection:
Genotyping of OHC
- 68,8% efficiency of AAV double-system integration
- No Off-Target effects

EXPECTED RESULTS *IN VIVO*

ABR → evaluation of hearing restoration



■ KI +AAV (n=12)

♦ WT (n=8)

p<0.05 KI vs WT
n obtained with G*power
***** p<0.05 4 weeks vs 8 weeks vs 12 weeks, two-way ANOVA

CONCLUSIONS

AAV/**Cas9n**/sgRNA5+6+DP transduction allowed:

high HDR efficiency in HEK293 and OHC KCNQ4*857A>G showed by the **electrophysiological response increase**

to use the same therapy for **5 mutations** in P-loop

C57BL/6-tg KI KCNQ4*857A>G **hearing restoration**

PITFALLS

Invasive intracochlear injection

Limitation on **bp amount** between PAMs to have a good HDR

SOLUTIONS

Finding a high specific OHC promotor to somministrate vectors **orally** or **sistemically**

Improve the Cas9 use to allow an efficient HDR for a longer template to have a **larger scale therapy**

BUDGET AND MATERIALS → 2 YEARS

In Vitro	Cell line HEK293 + culture medium supplements (DMEM, FBS, GlutaMAX, Penicillin + Strepromicine, ViraDuctin AAV Transduction Kit + PEI)	3.495€
	WB Analysis kit + Antibody (Anti-KCNQ4, Anti-IgG, Anti Actin)	312€ (1y) + 125€ (1y) + 550€ = 3.5000€
	AssayLite Mutli-color Conjugated Antibodies Flow Cytometry (FACS Analysis kit)	595€
In Vivo	Mice: C57BL/6 C57BL/6-tg(KCNQ4*857A>G)	22€ (ca)= 220€ 540€ (ca) = 5.400€
	Animal Housing	10.000€ (1y) = 20.000€
	AAV9 + packaging Lipofectamine 2000 tranfection reagent Crispr/Cas9 Nickase sgRNAs Cas9 protein	1200€ 479€ 1500€ 400€ 200€
	ABR kit	350€
OHC Analysis	Culture medium OHC (DMEM, FBS, N2 supplement, B27 supplement)	635€
	NGS kit	1.000€ (1y) = 2000€
	Staff Salary:1 PI, 2 PhD, 1 Technician	120.000€ (1y) = 240.000€

TOT: 279.662€

References

KCNQ4 MUTATION

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