Derivation of cochlear cells from pathological or isogenic human iPSCs for modeling hereditary hearing loss.

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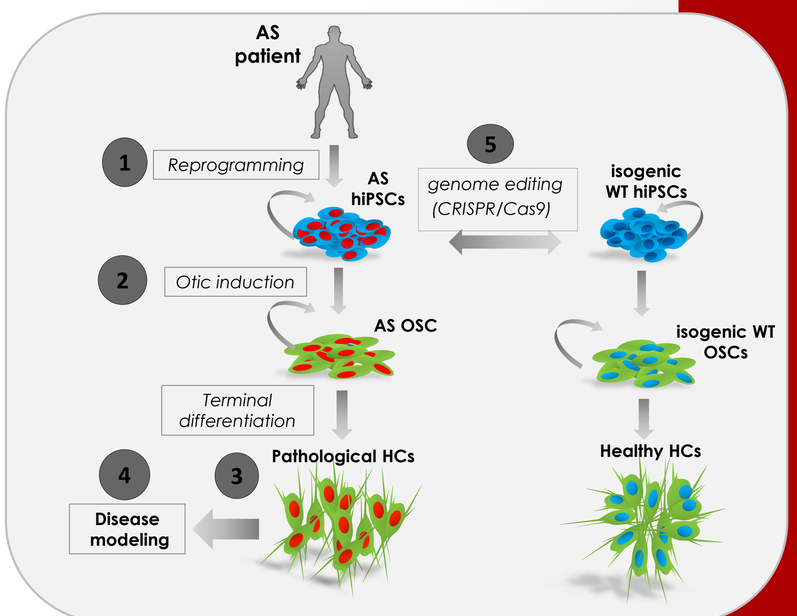
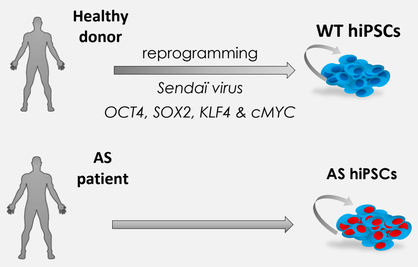
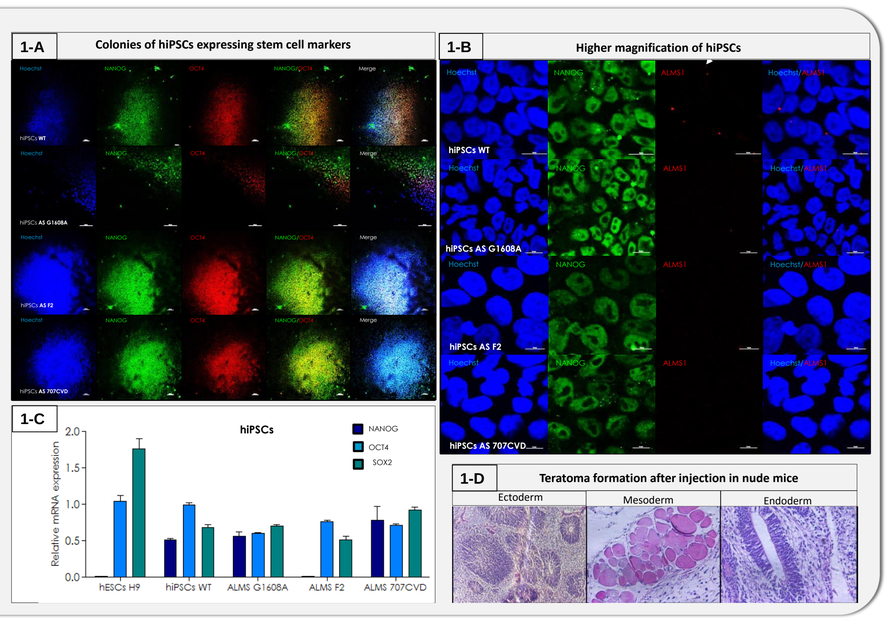
Alström Syndrome (AS) is a human autosomal recessive genetic disorder characterized by numerous clinical symptoms including deafness. AS is caused by mutations in the *ALMS1* gene encoding for ALMS1 protein which is expressed at the basal body and implicated in ciliogenesis, cell cycle and proliferation (Jagger et *al*., 2011; Zulato et *al*., 2011 & Shenje et *al*., 2014). We are interesting in understanding the unknown mechanisms involving this protein in the genetic deafness of AS patients. To develop a model as closer as possible to the human pathology, we are using human induced pluripotent stem cells (hiPSCs) generated from fibroblasts of healthy and AS patients. Using a stepwise protocol, we demonstrated that healthy hiPSCs (waiting for isogenic hiPSCs) can generate a population of cells with gene and protein expression patterns consistent with the ones of otic progenitor cells (OSCs). In these OSCs, we observed some proliferation defects and an increase in apoptosis tin the AS cells compared to the WT. When human OSCs are co-cultured with mouse feeder cells, they are able to differentiate into hair cells (HCs). We successfully differentiated AS hiPSCs into HCs. We are currently confirming gene expression pattern and testing HCs functionality. To exclude patient linked epigenetics and differentiation defects, we are correcting the genomic mutation in the AS hiPSCs to generate isogenic hiPSCs using the CRIPSR/Cas9 system. Thanks to the isogenic hiPSCs we will be able to confirm that these defects are well due to the *ALMS1* mutation.



1

Fibroblasts from healthy (WT) and deaf AS patients (AS) were reprogrammed into human induced pluripotent stem cells (WT and AS hiPSCs). Fibroblasts were infected by Sendaï virus particles containing the Oct4, Sox2, Klf4 and C-myc mRNAs. HiPSCs were then characterized before being differentiated into cochlear cell types. Reprogrammed hiPSCs expressed stem cell markers such as NANOG, OCT4, SOX2 and TRA-1-81 by immunofluorescence (1-A, 1-B) and qRT-PCR (1-C). (1-D) 8 weeks after injection in a nude mice, hiPSCs formed teratoma containing cells from the 3 germ layers.





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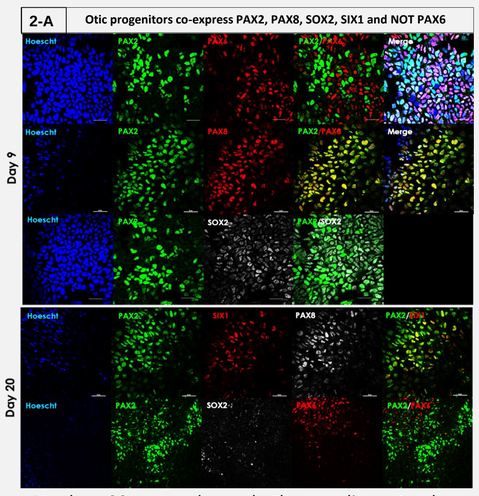
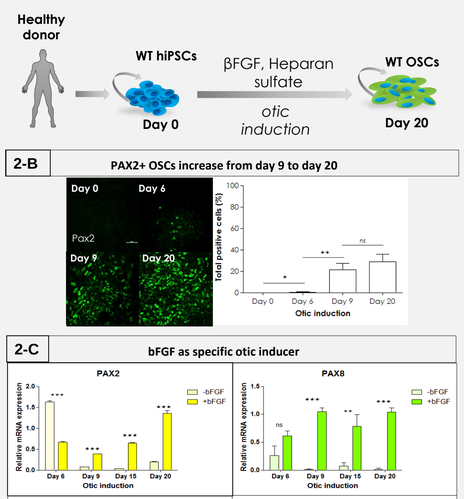
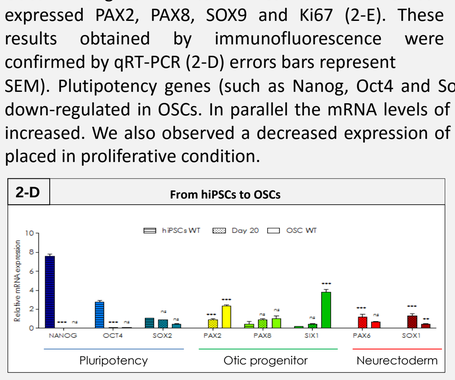
Induction of otic progenitor cells (OSCs)

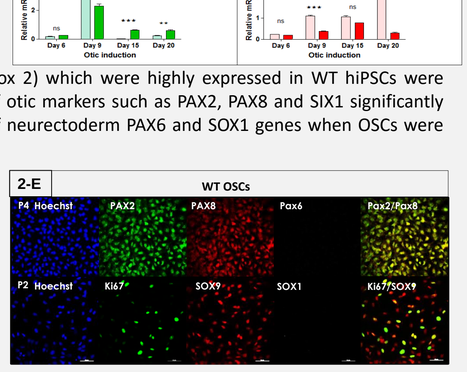
The stepwise guidance protocol is based on the inner ear embryonic development and adapted from Oshima et *al*, 2010 and Chen et *al*, 2012. Firstly we induced differentiation of hiPSCs into anterior ectoderm by blocking WNT and TGFb pathway and adding IGF1 to the culture medium. Ectodermal cells were then pushed toward an otic fate by high doses of bFGF until day 20. Around 20% of cells observed by immunofluorescence at day 9 were positive for PAX2 and the otic markers PAX8 and SIX1 meaning that these cells were OSCs. This number increased until day 20 to reach 30% (2-B). We further characterized the obtained OSCs by immunofluorescence (2-A) and qPCR (2-C). We measured the expression of PAX6, a transcription factor specifically expressed in optic progenitors and neurectoderm cells but not in OSCs as control. In presence of SU4802, a bFGF signaling inhibitor, OSCs were not induced as shown by IF and qRT-PCR.





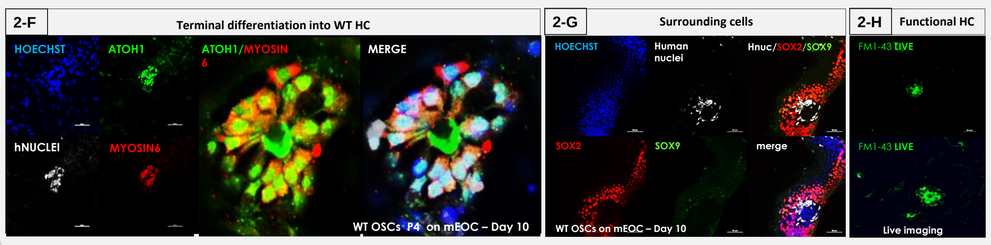
At day 20, we changed the medium to have proliferative conditions. This culture medium maintains OSCs in long term culture. These OSC cultures still expressed PAX2, PAX8, SOX9 and Ki67 (2-E). These results obtained by immunofluorescence were confirmed by qRT-PCR (2-D) errors bars represent SEM). Plutipotency genes (such as Nanog, Oct4 and Sox 2) which were highly expressed in WT hiPSCs were down-regulated in OSCs. In parallel the mRNA levels of otic markers such as PAX2, PAX8 and SIX1 significantly increased. We also observed a decreased expression of neurectoderm PAX6 and SOX1 genes when OSCs were placed in proliferative condition.





Hair cells (HCs) differentiation

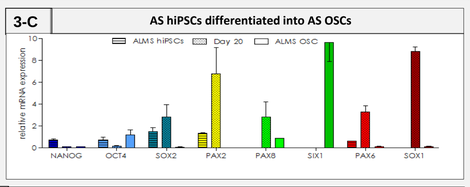
We cultured WT OSCs on dissociated mouse embryonic cochlear cells (from E14 embryos) which act as feeder cells to provide a kind environment for the terminal differentiation of the human OSCs into HCs. After 10 days of co-culture, we observed by IF some clusters of double positive ATOH1/MYOSIN6 cells (2-F). Using a specific antibody against human nuclei we confirmed that these cells were human OSCs differentiated and not feeder cells. HCs were surrounded by mouse cells co-expressing SOX9 and SOX2, two markers of supporting cells in the organ of Corti (2-G). Recently, we obtained interesting results regarding the functionality of differentiated HCs using FM1-43 (2-H). This dye has the property to enter specifically into HCs by the mechanical channels so to label only functional HC.

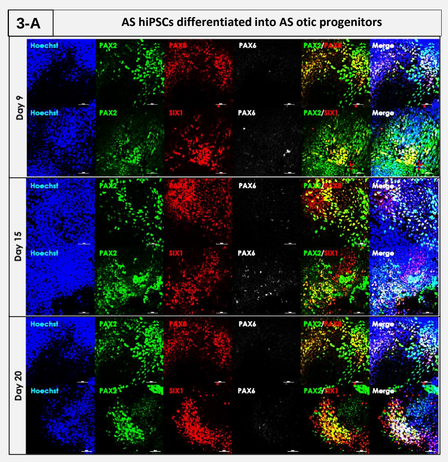


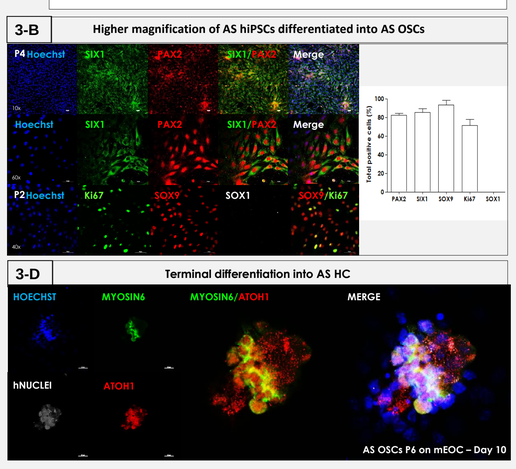
3

Differenciation of AS hiPSCs into HCs

AS hiPSCs are able to differentiate into pathological otic progenitors similarly to WT hiPSCs, as shown by IF (3-A, 3-B) and qRT-PCR (3-C). It seemed that terminal differentiation into HCs was not affected by the *ALMS1 mutation* (3-D). Functional analyses are still in progress.







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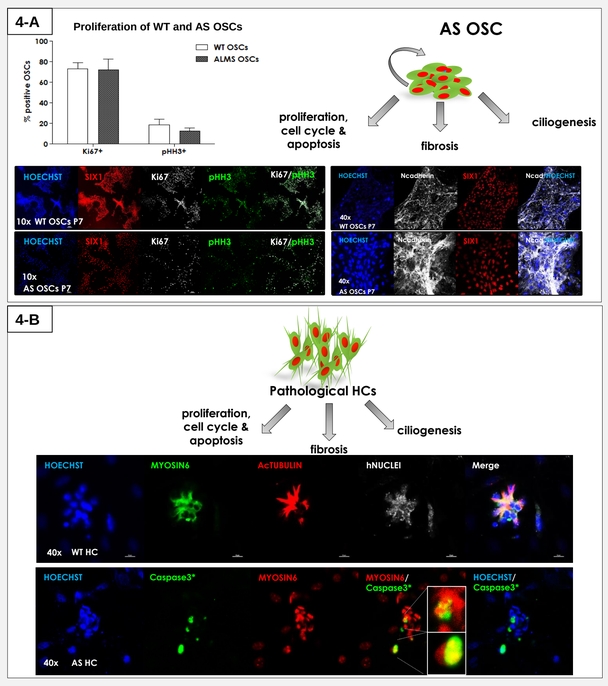
Modeling the Alstrom syndrome

Results obtained from the differentiation of AS hiPSCs into HCs suggest that the differentiation potential of these cells into cochlear cells is unaffected in AS patients. However, we observed some proliferation defects and increase in apoptosis between healthy and AS OSCs (4-A, 4-B).



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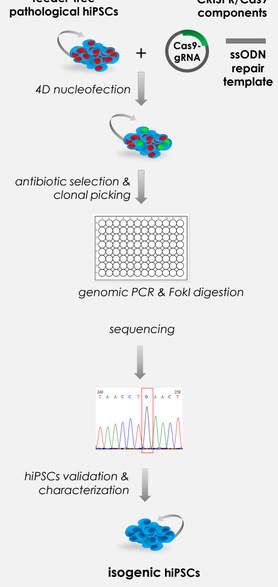
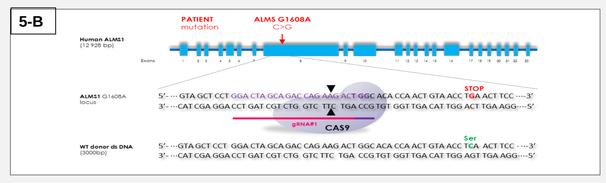


Isogenic hiPSCs

To be sure that observed phenotypes are only due to the *ALMS1* disease-causing mutation, we needed isogenic cells. The strategy used to obtain them is described in panel 5-A. We used the CRISPR/Cas9 genome editing to correct the AS hiPSCs STOP mutation. The Cas9 binding is guided by base-pair complementarities between the engineered single-guide RNA (gRNA) and a genomic DNA target sequence. We designed gRNAs complementary to the *ALMS1* genomic locus (5-B). Using the engineered gRNA, the Cas9 will induce specific double-stranded break (DSB), which can be repaired through the Homology Directed Repair (HDR) pathway. The HDR mechanism is induced by providing a single stranded oligo dinucleotide (ssODN) repair template to the AS hiPSCs. The ssODN contains a FokI site we will use to check homologous recombining events.



Conclusion

We developed a human model to study the impact of ALMS1 mutation. We also performed genome editing to obtain isogenic hiPSCs used as a reliable control. We obtained preliminary results indicating that ALMS1 mutation does not affect differentiation of hiPSCs into HCs but induces proliferative defects and an increase in apoptosis. Identification of molecular mechanisms implicated in these phenotypes is crucial to understand the hearing loss in Alström syndrome.

References

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