In Vitro Fabry Disease Correction in Patient iPSC-Derived Cardiomyocytes and Endothelial Cells Using an Evolved and Optimized AAV Gene Therapeutic (4D-310)

Chris Schmitt, Jessica Sepulveda, Roxanne Croze, Austin Klein, Paul Szymanski, Kevin Whittlesey, Gabriel Brooks, Melissa Kotterman, Peter Francis, David Schaffer, David Kirn

4D Molecular Therapeutics, 5980 Horton St #460, Emeryville, CA 94608

Purpose

Fabry disease is an X-linked lysosomal storage disorder in which mutations in the *GLA* gene (encoding α-galactosidase A, AGA) result in reduced or absent AGA enzyme activity and consequent accumulation of globotriaosylceramide (Gb3). Gb3 is considered cytotoxic to cardiomyocytes and endothelial cells (as well as kidney, heart, & neurons) resulting in significant morbidity and shortened life expectancy. Administration of recombinant AGA (ERT) primarily clears endothelial cells of Gb3, however Gb3 clearance of other cell types, for example cardiomyocytes, is attenuated. Reduced effectiveness of ERT to treat the heart may explain persistent cardiomyopathy in patients with Fabry and why cardiovascular disease and arrhythmia remain the most prevalent cause of death in these patients. Thus, there is a compelling need for a durable treatment such as a single administration intravenous gene therapeutic targeted to key tissues that express *GLA* cell-autonomously, reducing Gb3 and thereby improving clinical outcomes.

Using an industrialized directed evolution approach ("Therapeutic Vector Evolution") performed exclusively in nonhuman primates, we have identified and characterized an AAV capsid variant that can efficiently target key organs in Fabry disease, particularly the heart. We engineered this capsid variant to carry and express the *GLA* gene (4D-310). A key component of translating 4D-310 into clinical development is to evaluate its tropism, expression and function in human cell models of Fabry-diseased cardiomyocytes and endothelial cells.

Here, we present 4D-310 transduction data in Fabry diseased iPSC-derived cardiomyocytes and endothelial cell models We show that transduction led to rapid, dose-dependent AGA protein expression and activity, well above basal levels. In addition, transduction resulted in efficient clearance of accumulated Gb3 in Fabry cardiomyocyte and endothelial cell models.

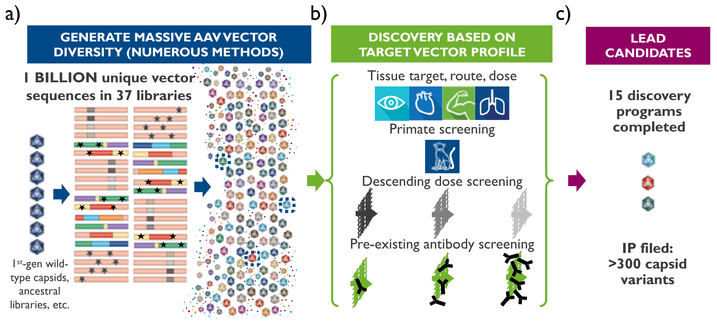

Figure 1: ***Schematic of Therapeutic Vector Evolution.*** *a) a viral capsid library comprising 37 proprietary combinations of DNA mutation techniques and cap genes is created viruses are then packaged such that each particle is composed of a mutant capsid surrounding the cap gene encoding that capsid and purified; b) enrichment of successful clones through repeated selection using in vivo primate delivery and in vitro cell models to iteratively increase viral fitness. Variants identified as hits during Vector Selection are manufactured as recombinant AAV vectors and characterized for the level of transduction of target cell types and tissues. c) TVE has resulted in 15 active discovery programs and IP filed on >300 capsid variants.*

Methods

We aimed to generate *in vitro* human cell models carrying a Fabry patient genetic background. Fibroblasts (Coriell #GM02769 harboring the W162X pathogenic mutation in the *GLA* gene that typically results in absent AGA protein activity) were reprogrammed into induced pluripotent stem cells (iPSCs. Fibroblasts were reprogrammed through a single non-integrative RNA transfection (Simplicon). After robust characterization of Fabry iPSCs by karyotype and germ layer differentiation capacity, cells were differentiated into cardiomyocytes and endothelial cells. Cardiomyocytes were differentiated using Wnt modulation and glucose deprivation. Endothelial cells were differentiated through sequential Wnt inhibition and VEGF stimulation followed by purification through $CD144^+$ magnetic cell sorting.

To analyze the ability of our AAV capsid carrying a codon optimized *GLA* gene, 4D-310, to correct the Fabry disease *in vitro* phenotype cultured Fabry patient-derived iPSC cardiomyocytes and endothelial cells, were transduced with different MOIs of 4D-310. Cell models were transduced at various MOIs with 4D-310. For cardiomyocytes, cells were processed for assays at four days post-transduction. 4D-310 was washed out at 48 hours for cardiomyocytes and 24 hours for endothelial cells. AGA expression was detected by flow cytometry using a custom PE-conjugated antibody from Abnova (#H00002717-801p) to analyze overall transduction efficiency. The same antibody was also utilized for immunofluorescence. Activity of AGA was assayed using an optimized AGA fluorometric activity assay that measures enzymatic glycolipid substrate cleavage (BioVision). Gb3 accumulation, a clinical hallmark pathology of Fabry disease, was assessed by immunocytochemistry using CD77 antibody (BD 563632). Western blot analysis was used to determine the expression of intracellular AGA protein using Atlas rabbit polyclonal antibody (HPA000237).

Results

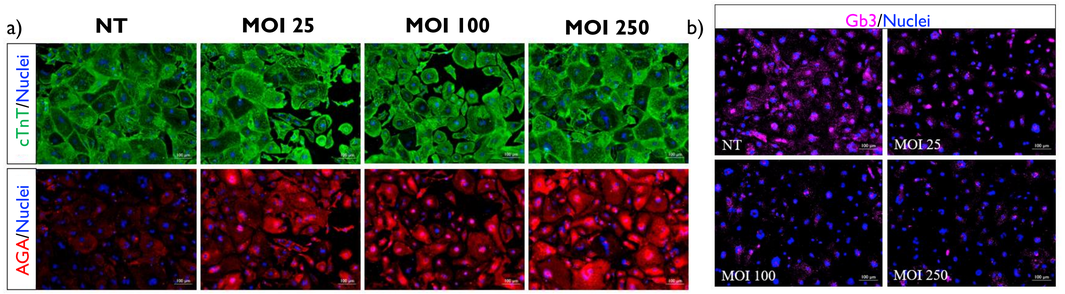

Figure 2: ***4D-310 transduces patient iPSC-derived cardiomyocytes and reduces intracellular Gb3.*** *(a) Immunocytochemistry of Fabry patient-derived cardiomyocytes for cTnT (green), exogenous AGA protein (red) and Hoechst (blue). (b) Immunocytochemistry for Gb3 (CD77) (magenta). Scale bars = 100 μM; NT = not transduced.*

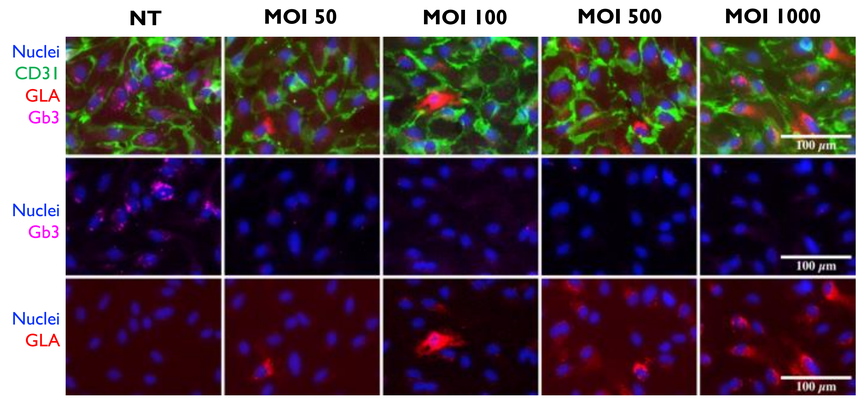

Figure 3: ***4D-310 transduces patient iPSC-derived endothelial cells and reduces intracellular Gb3.*** *(a) Immunocytochemistry of Fabry patient-derived endothelial cells for CD31 (green), Gb3 staining (magenta), exogenous AGA protein (red), and DAPI (blue). Scale bars = 100 μM*

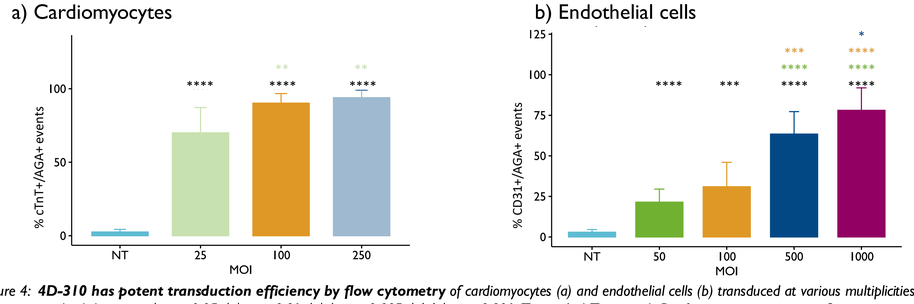

Figure 4: ***4D-310 has potent transduction efficiency by flow cytometry*** *of cardiomyocytes (a) and endothelial cells (b) transduced at various multiplicities of infection. Mean + standard deviation; * = p<0.05; ** = p<0.01; *** = p<0.005, **** = p<0.001, Two-tailed T-test with Bonferroni correction, n = 3 experimental replicates, 3 technical replicates; Black asterisks are comparisons with NT, light green with MOI 25, green with MOI 50, gold with MOI 100, and blue with MOI 500.*

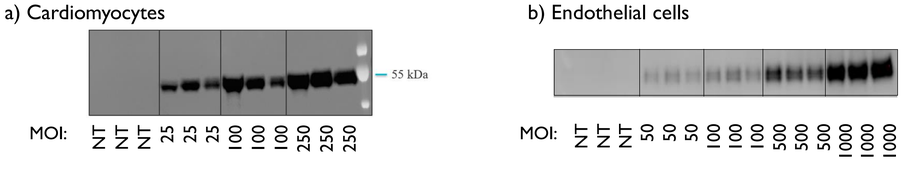

Figure 5: ***Detection of AGA protein by Western blot analysis.*** *(a) 4D-310 transduced Fabry patient-derived cardiomyocytes and (b) endothelial cells Expected dose-dependent increase in AGA protein was seen at the expected 55 kDA size.*

Results (cont.)

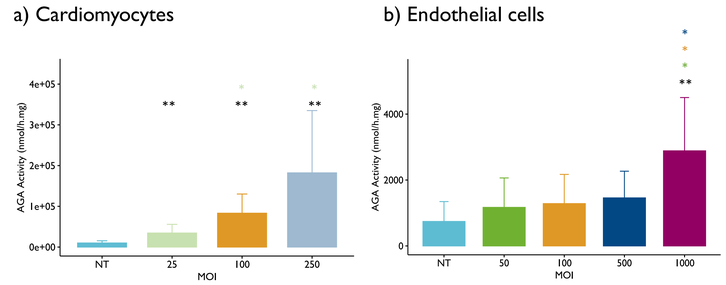

Figure 6: ***Quantification of AGA enzymatic activity after 4D-310 transduction*** *of cardiomyocytes (a) and endothelial cells (b) transduced at various multiplicities of infection. Mean + standard deviation; * = p<0.05; * * = p<0.01; * * * = p<0.005, Two-tailed T-test with Bonferroni correction; n = 3 experimental replicates, 3 technical replicates; Black asterisks are comparisons with NT, light green with MOI 25, green with MOI 50, gold with MOI 100, and blue with MOI 500.*

Conclusions

- Recombinant 4D-310 demonstrated potent transduction of human cell types derived from Fabry patient iPSCs, evidenced by intracellular AGA protein and activities.
- We developed several assays to evaluate the expression and functional activity of the AGA transgene product.
- Significant expression of AGA protein is detectable at lowest MOIs; 25 vg/cell in cardiomyocytes and 50 vg/cell in endothelial cells.
- 4D-310-delivered AGA protein is enzymatically active by cleavage of a synthetic fluorescent substrate in the AGA activity assay from Biovision.
- Robust and concomitant reduction in Gb3 accumulation was seen in both cell types by immunocytochemistry, indicating that 4D-310-delivered AGA is active intracellularly and is active in the lysosomal clearance of Gb3, the etiological cause of Fabry disease.
- These data demonstrate that this next-generation evolved capsid is highly tropic towards human cardiomyocytes and endothelial cells *in vitro*. 4D-310 results in rapid cell-autonomous, dose-related AGA activity intracellularly, resulting in clearance of Gb3, the accumulation of which is considered central to the pathogenesis of Fabry disease in humans.

References

Baig, S., Edward, N. C., Kotecha, D., Liu, B., Nordin, S., Kozor, R., … Steeds, R. P. (2018). Ventricular arrhythmia and sudden cardiac death in Fabry disease: a systematic review of risk factors in clinical practice. Eur Soc Cardiol, 20, 153–61.

Banikazemi, M., Bultas, J., Waldek, S., Wilcox, W. R., Whitley, C. B., McDonald, M., … Welch, K. (2007). Agalsidase-beta therapy for advanced fabry disease: A randomized trial. Ann Intern Med. 146(2):77-86.

Bartel, M. A., Weinstein, J. R., & Schaffer, D. V. (2012). Directed evolution of novel adeno-associated viruses for therapeutic gene delivery. Gene Ther, 19(6),694–700.

Lian X, Hsiao C, Wilson G, Zhu K, Hazeltine LB, Azarin SM, Raval KK, Zhang J, Kamp TJ, Palecek SP (2012). Robust cardiomyocyte differentiation from human pluripotent stem cells via temporal modulation of canonical Wnt signaling. Proc Natl Acad Sci. 109(27):E1848-57.

Lian X, Zhang J, Azarin SM, Zhu K, Hazeltine LB, Bao X, Hsiao C, Kamp TJ, Palecek SP. (2013). Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/β-catenin signaling under fully defined conditions. Nat Protoc. 8(1):162-75.

Maheshri N, Koerber JT, Kaspar BK, & Schaffer DV. (2006). Directed evolution of adeno-associated virus yields enhanced gene delivery vectors. Nat Biotechnol, 24(2), 198–204.

Meylan, L, Patsch, C., Thoma, E. (2015) Endothelial cells differentiation from hPSCs. Nat Prot Exch, DOI:10.1038/protex.2015.055

Murray GJ, Anver MR, Kennedy MA, Jane M., & Schiffmann R. (2007). Cellular and Tissue Distribution of Intravenously Administered Agalsidase Alfa. Mol Genet Metab, 90(3):307–12.

Thurberg B, Fallon JT; Mitchell R, Aretz T, Gordon R, O'Callaghan MW (2009). Cardiac Microvascular Pathology in Fabry Disease Evaluation of Endomyocardial Biopsies Before and After Enzyme Replacement Therapy Circulation. 2009;119:2561-256

Declaration of Interest

4D Molecular Therapeutics is responsible for the study design, data collection, analysis of all data presented https://www.4dmoleculartherapeutics.com;