Precision Editing of *PLN-R14del* Mutation Using a Self-Inactivating, All-in-One AAV Vector to Rescue PLN-R14del-Associated Cardiomyopathy

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PLN-R14del Gene Editing Program

▶The Arg14del (R14del) mutation in the *Phospholamban* (*PLN*) gene is a genetic cause of familial dilated or arrhythmogenic cardiomyopathy. PLN-R14del-associated cardiomyopathy has a poor prognosis, high adolescent mortality, limited therapeutic options other than heart transplantation, and currently available therapies do not address the underlying genetic cause of disease. Genome editing, particularly with the CRISPR-Cas9 system, has opened avenues for modifying genes associated with genetic disorders, ushering in a potential era of curing and preventing human genetic diseases. *In vivo* delivery of CRISPR-Cas9 components holds promise for treating PLN-R14del-associated cardiomyopathy by specifically addressing the R14del mutation.

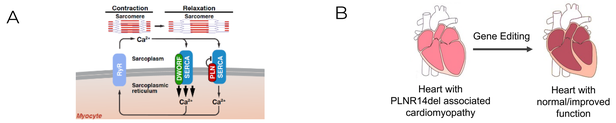


Figure 1. Tenaya's *PLN-R14del* Gene Editing Program.

(A) PLN negatively regulates SERCA, essential for intracellular calcium homeostasis. The *PLN-R14del* mutation causes misfolded protein aggregation, disrupting heart function. **(B)** Gene editing offers hope for treating genetic disorders by targeting specific mutations like *R14del* in PLN-R14del-associated cardiomyopathy through in vivo delivery.

First-Gen *PLN-R14del* Edit Vector, TNGE101, is Highly Effective in A Severe *Pln-R14del* Model

▶We previously demonstrated the efficacy of gene editing approach in the PLN-R14del mouse model, using an All-In-One AAV vector.

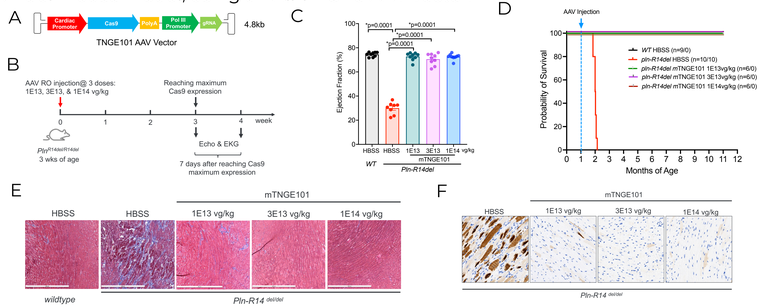
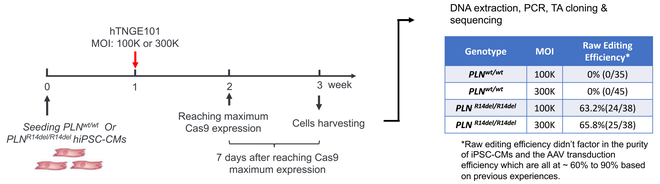


Figure 2. *In Vivo* Efficacy of First-Generation *PLN-R14del* Gene Editing Vector TNGE101 in the *Pln-R14del* Mouse Model.

(A) Tenaya's all-in-one adeno-associated virus (AAV) therapy gRNA is optimized for specific *Pln-R14del* targeting while avoiding *Pln-WT* locus. Cas9 is driven by a cardiac-specific promoter for tissue specificity. **(B)** Experimental design illustrating the *in vivo* gene editing efficacy using first generation vector TNGE101 with mouse *Pln-R14del* gRNA (mTNGE101) in *Pln-R14del* homozygote mice. Retro-orbital (RO) injection was employed to deliver mTNGE101 at three different doses. **(C)** TNGE101 preserved heart function even at the lowest dose of 1E13 vg/kg, as measured by ejection fraction (EF). **(D)** TNGE101 at doses as low as 1E13 vg/kg resulted in 100% survival. **(E)** Trichrome staining of hearts reveals a notable decrease in cardiac fibrosis following treatment with mTNGE101 at various doses compared to the vehicle control (HBSS). **(F)** DAB staining of the hearts demonstrates a significant decrease in PLN protein aggregation following treatment with mTNGE101 compared to the control.

TNGE101 Efficiently and Specifically Edits *PLN-R14del* but not *PLN-WT* Locus

▶We previously tested our gene editing therapy in patient-specific human iPSC-derived cardiomyocytes (iPSC-CMs) and found that hTNGE101 precisely and efficiently edited the *PLN-R14del* allele without affecting the *wild-type PLN* allele.



Genotype	MOI	Raw Editing Efficiency*
***PLN*^wt/wt**	100K	0% (0/35)
***PLN*^wt/wt**	300K	0% (0/45)
***PLN*^R14del/R14del**	100K	63.2%(24/38)
***PLN*^R14del/R14del**	300K	65.8%(25/38)

*Raw editing efficiency didn't factor in the purity of iPSC-CMs and the AAV transduction efficiency which are all at ~ 60% to 90% based on previous experiences.

Figure 3. Efficient *PLN-R14del* Gene Editing with Locus Specificity in Human *Wildtype* (*WT*) or *PLN-R14del* iPSC-CMs Model.

Experimental design depicting the gene editing process using TNGE101 with gRNA targeting the human *PLN-R14del* locus (hTNGE101) in both *PLN WT/WT* and *R14del/R14del* human induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs). Gene editing results demonstrate the efficient and selective targeting of the *PLN-R14del* allele by hTNGE101, while leaving the PLN WT allele unaffected.

Second-Gen Vectors Integrates Cas9 Self-inactivation to Bolster Safety Measures

▶A significant challenge in gene editing applications is the immunogenicity arising from the bacteria-originated gene editing machinery, along with concerns about off-target events. This becomes pronounced when employing an AAV vector for genome editing, as it results in the continuous expression of the therapeutic transgene. To overcome this challenge, we designed a Cas9 self-inactivating editing system, that achieves high editing efficiency even with transient Cas9 protein expression.

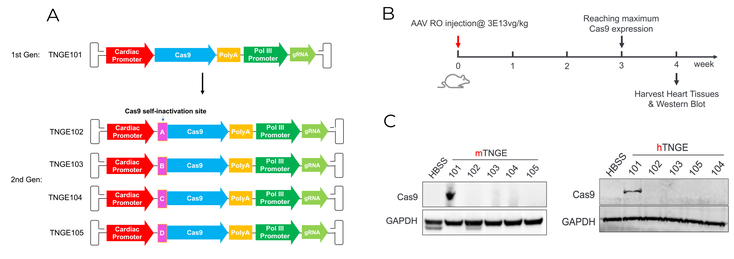


Figure 4. Designs for Cas9 Self-Inactivation Effectively Diminished Cas9 Expression Within One Month after AAV Injection

(A) Second-generation Cas9 self-inactivating cassettes designs. **(B)** Experimental design for testing the *in vivo* Cas9 expression for first- and second- generation vectors in wild type mice. Retro-orbital (RO) injection was employed to deliver AAVs at a dose of 3E13 vg/kg. **(C)** Cas9 self-inactivation cease Cas9 expression within one month after AAV injection.

Self-inactivating Vectors Preserve Cardiac Function and Ensure 100% Survival

▶Self-inactivating vectors at various doses in *PLN-R14del* mice revealed a rescue of heart function to near wild-type levels, along with a significant prolongation of survival. Notably, these positive effects were observed even at low doses in the range of E13 vg/kg

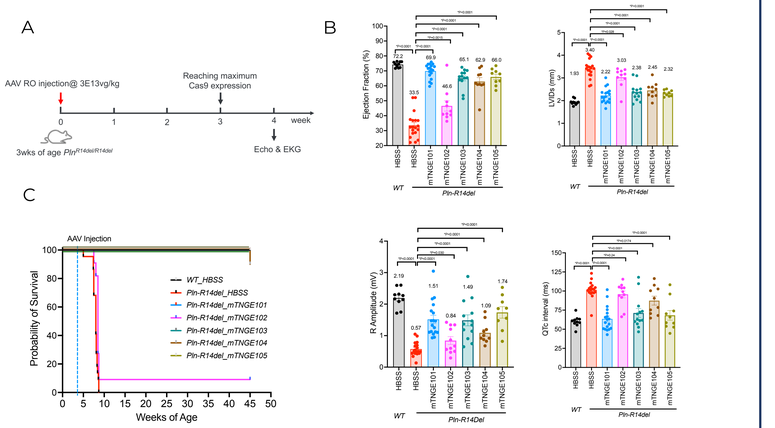


Figure 5. *In Vivo* Efficacy of Second-Generation Self-inactivating *PLN-R14del* Gene Editing Vectors in the *Pln-R14del* Mouse Model.

(A) Experimental design for comparing first and second-generation gene editing vectors in *Pln-R14del* homozygote mice. **(B)** Most self-inactivating gene editing vectors, except for TNGE102, preserved heart function even at the lowest dose of 1E13 vg/kg, as measured by ejection fraction (EF), left ventricular internal dimension in systole (LVIDs) and R amplitude. **(C)** Self-inactivating gene editing vectors, TNGE103 and TNGE105, resulted in 100% Survival at doses as low as 1E13 vg/kg.

CONCLUSION

- ***PLN-R14del* gene editing demonstrates efficacy even at low doses (1E13 vg/kg), achieving 100% survival and notable improvement in cardiac parameters. Histology analysis reveals significant reductions in cardiac fibrosis and PLN protein aggregation across all doses.**
- **Cas9 self-inactivation effectively diminished expression within one month post-AAV injection.**
- **The self-inactivating vectors preserve cardiac function and ensure 100% survival.**
- **These preclinical results suggest that *PLN-R14del* gene editing holds promise as an approach for PLN-R14del-associated cardiomyopathy.**