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CRISPR/Cas9-Mediated Targeted Knockout of the Peroxidasin-Like Gene (PXDNL) in Rabbit Reveals a Novel Non-Rodent Model

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Introduction

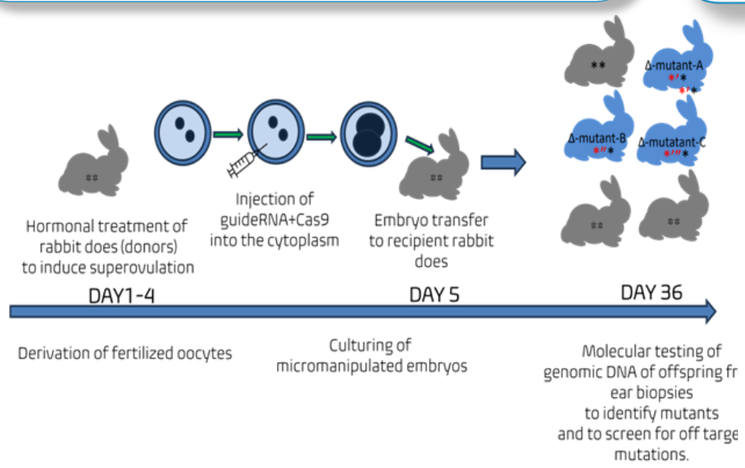
- Peroxidasin-like protein **(PXDNL)** is a **non-enzymatic peroxidase** homologue expressed almost exclusively in cardiomyocytes.
- Variants in ***PXDNL*** have been **associated** with dilated **cardiomyopathy**, yet its physiological role remains poorly understood.
- Rodent species lack the *Pxdnl* gene, limiting their suitability for functional studies.
- **Rabbits** provide a more relevant **model** due to their human-like cardiac physiology, anatomy, and early embryonic development, and because PXDNL is strongly expressed in rabbit heart tissue.

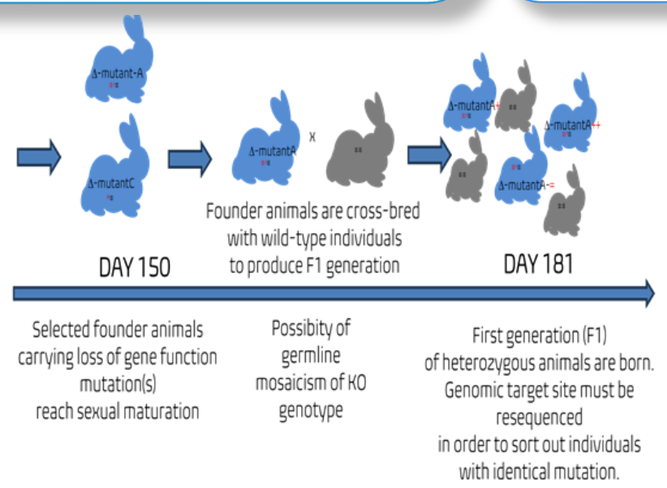
Objectives

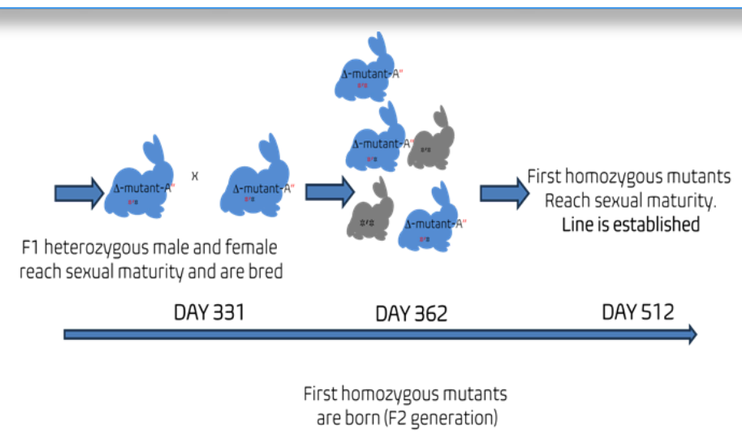
- Establishing a **PXDNL knockout (KO) rabbit line** to investigate the consequences of PXDNL loss of function and to clarify its potential role in cardiac development, remodeling, and disease.

Methods

- Designed single guide RNAs targeting the **first coding exon** of the rabbit *PXDNL* gene (GATGCTGGACCACATTCCTC).
- Performed **CRISPR/Cas9–sgRNA microinjection** into New Zealand White rabbit zygotes.
- Screened founder animals for mutations introduced by **error-prone non-homologous end joining (NHEJ)**.
- Identified and selected a founder carrying a **frameshift mutation predicted to generate an early stop codon**.
- Conducted **off-target analysis**, confirming the absence of detectable off-target edits.





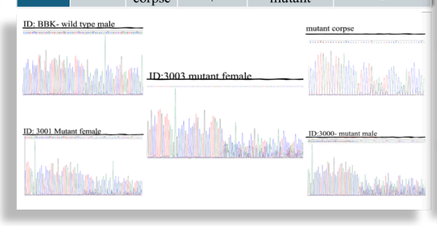


Results

- Successfully generated the **first non-rodent PXDNL knockout model**.
- A total of 6 founder individuals were born, 3 were found to be genetically modified.
- A founder with a disruptive allele was identified and used to establish a stable mutant line.
- Off-target screening confirmed **high editing specificity** with no unintended mutations.
- The line is now maintained and expanded for downstream studies.

Identification of genome-edited founder animals

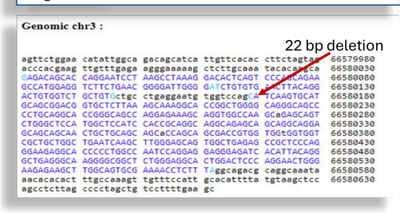
Animal ID	Animal Sex	Animal code	T7 assay result	Sequencing results	Proportion of heterozygotes in F1 offspring
3002	♀	JBK	Wild type	not sequenced	14%
BBK	♂	bbk	Wild type	Wild type	
3003	♀	BKK	+	Mutant	100%
3000	♂	jt	+	mutant	
3001	♀	bt	+	mutant	50%
3004	♀	jt	Wild type	not sequenced	-
		corpse	+	mutant	-



- Sanger sequencing of the CRISPR-targeted locus enabled the identification of genome-edited founder animals. The wild-type control (ID: BBK, wild-type male) displayed a clean, unaltered chromatogram with uniform peak spacing, confirming the intact reference sequence.
- In contrast, all edited founders exhibited clear signatures of CRISPR-induced mutagenesis: mixed and overlapping chromatogram peaks, frameshifted signal patterns, and disrupted base-calling profiles.
- These features are evident in the mutant females (ID: 3001 and ID: 3003), the mutant males (ID: 3000),and the mutant corpse sample, each showing distinct heterogenous peak structures consistent with mosaic indel formation.
- Together, these sequencing traces provide direct molecular evidence of successful genome editing in multiple founder animals and confirm the presence of independent mutation events across the cohort.

ID:3003 female produced 100% genome-edited F1 offspring.

- Since each offspring carried a different indel variant, we focused on the 22 bp deletion, which was suitable for line establishment.
- From this allele, we initiated the development of a stable knockout line using a male–female F1 pair, both carrying the same 22 bp deletion, ensuring a uniform genetic background for subsequent generations.



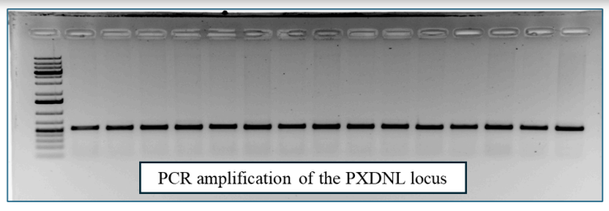
F1 Genotyping Summary of #3003 founder

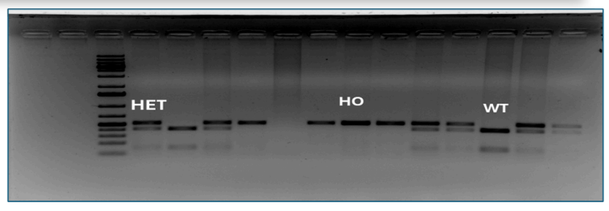
- The CRISPR-edited #3003 founder rabbit was mosaic, resulting in F1 offspring carrying multiple independent indel variants at the target locus. Across the seven genotyped F1 animals, deletions ranged from **2 bp to 29 bp**.
- For establishing a stable knockout line, we prioritized individuals harboring the **22 bp deletion** (IDs **3102** and **3105**), as this allele is suitable for consistent transmission and subsequent line development.

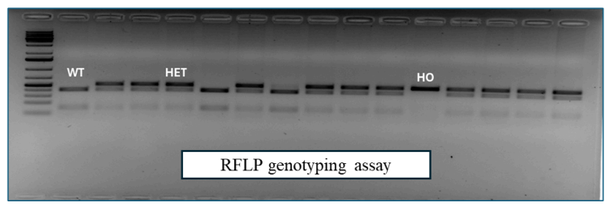
3101	KO 8bp-DEL
3102	KO 22bp-DEL
3103	KO 29bp-DEL
3106	KO 7bp-DEL
3105	KO 22bp-DEL
3104	KO 2bp-DEL
3107	4bp-DEL

RFLP-based genotyping for the 22 bp deletion allele

- To confirm and routinely identify the selected **22 bp deletion,** we established a targeted **RFLP genotyping assay**.
- The method consists of PCR amplification of the PXDNL locus using the PXDNL1ex F/R primers, followed by **AvaII digestion**, which specifically recognizes and cuts the wild-type sequence but not the 22 bp deletion variant.
- As a result, the digested band pattern allows clear discrimination between **wild-type**, **heterozygous**, and **homozygous knockout** genotypes.
- This AvaII-based RFLP assay is now used for all subsequent genotyping during line establishment.





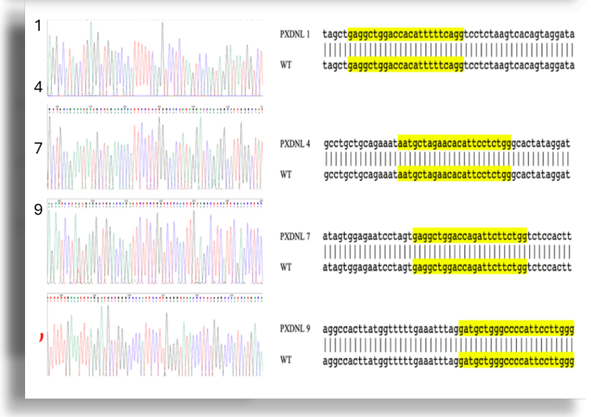


Off-Target Analyses

- The potential off-target sites for the CRISPR/Cas9 system used to generate the knockout founder rabbit (ID:3003) were predicted using the online tool Cas OFFinder (http://www.rgenome.net/cas-offinder/).
- This tool scanned the rabbit genome for sequences similar to the designed sgRNA. For the sgRNA sequence GATGCTGGACCACATTCCTC, Cas OF Finder identified 12 candidate off-target sites containing two to three mismatches.

Off-Target site	Chromosome	Related gene
PXDNL-OFF1	1	PCSK5
PXDNL-OFF2	11	No overlapping gene
PXDNL-OFF3	13	No overlapping gene
PXDNL-OFF4	13	NTNG1
PXDNL-OFF5	17	No overlapping gene
PXDNL-OFF6	19	No overlapping gene
PXDNL-OFF7	2	UNC5D
PXDNL-OFF8	6	No overlapping gene
PXDNL-OFF9	7	No overlapping gene
PXDNL-OFF10	8	No overlapping gene
PXDNL-OFF11	9	Gene: ENSOCUG00000036756
PXDNL-OFF12	chrUn0096	No overlapping gene

- Custom primers were designed to flank each site, and PCR amplification was used to isolate the corresponding genomic regions for sequencing.
- Of the twelve sites, four were successfully amplified and sequenced, while eight could not be amplified—likely due to inefficient primer design or non-optimal PCR conditions.
- These regions are currently being re-evaluated with redesigned primers, and PCR experiments are ongoing.



Conclusion

➢ The newly established PXDNL KO rabbit line provides a **unique and relevant in vivo model** to study PXDNL function. This model enables investigation of how PXDNL contributes to cardiac structure, function, and remodeling.

Future Directions

- Production of **homozygous knockout stock for characterization of the knockout phenotypes.**
- Development of **rabbit-specific PXDNL antibodies**.
- Comprehensive **molecular and cardiovascular phenotyping** to link gene disruption with functional cardiac outcomes.

Keywords

Peroxidasin-like protein; CRISPR/Cas9; rabbit model; genome editing

Acknowledgement

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