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Generation of a *PKP2* heterozygous knockout pig model of arrhythmogenic cardiomyopathy

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ABSTRACT

Arrhythmogenic cardiomyopathy (ACM) confers elevated risk of ventricular arrhythmias and sudden cardiac death, yet limitations in early lesion sampling and model development continue to hinder mechanistic and translational research. Clinical, pathological, and mutational profiles were examined in 24 individuals with ACM harboring *PKP2* variants. Among these, a patient carrying the c.1132C>T mutation exhibited the earliest onset and presented both structural cardiac abnormalities and major adverse cardiovascular events. To facilitate disease modeling, the c.1147C>T variant—a previously reported pathogenic substitution located proximal to position c.1132 in *PKP2*—was selected to enhance the feasibility of generating a porcine model. The BE3 gene editing system was used to induce C>T mutation. Two single guide RNAs targeting the *PKP2* gene were designed (sgRNA1 for c.1132C>T and sgRNA2 for c.1147C>T), yielding editing efficiencies of 42.9% and 25.9%, respectively. SgRNA1 was used to generate *PKP2*^{+/-} porcine fetal fibroblasts. A total of 14 cloned piglets were produced, including 11 viable and three

stillborn *PKP2*^{+/-} individuals. By 24 months of age, *PKP2*^{+/-} pigs developed premature ventricular contractions and right ventricular dilatation. Histological analysis revealed adipocyte infiltration within the right ventricular wall, and electron microscopy demonstrated reduced desmosomal length and electron density consistent with desmosomal dysfunction. Transcriptomic profiling showed high expression of genes associated with lipid catabolic processes. This study established the first *PKP2*^{+/-} porcine model of ACM using BE3-mediated base editing, providing a valuable platform for elucidating early pathogenic mechanisms and evaluating therapeutic interventions.

Keywords: Arrhythmogenic cardiomyopathy; Pig model; BE3 gene-edit system

INTRODUCTION

Arrhythmogenic cardiomyopathy (ACM) encompasses a group

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of inherited myocardial disorders primarily characterized by right ventricular (RV) dysfunction and life-threatening ventricular arrhythmias (Corrado et al., 2017). The pathological hallmark involves progressive replacement of RV myocardium with fibrofatty tissue, contributing to conduction abnormalities and structural deterioration (Austin et al., 2019). ACM represents a leading cause of sudden cardiac death, especially in adolescents and athletes (Sharma et al., 2015). Among the genes implicated in desmosome-associated ACM, *PKP2* (plakophilin-2) accounts for more than 40% of genetically confirmed cases (Camors et al., 2022), and its disruption is strongly associated with early onset arrhythmias, RV dilation, contractile impairment, and fibrofatty infiltration (Smith et al., 2020). Carriers of *PKP2* mutations exhibit markedly elevated risk of ventricular arrhythmias and sudden cardiac death (Paldino et al., 2022). Despite recognition of *PKP2* as a central driver of ACM pathogenesis, no disease-modifying therapy has proven effective in delaying or reversing disease progression. Clinical management relies primarily on prophylactic implantation of cardioverter defibrillators (ICDs) to mitigate the risk of fatal arrhythmic events (Calkins et al., 2017), often at the cost of significant physical and psychological burdens on patients due to recurrent discharges and persistent arrhythmic activity.

Mechanistic insights derived from murine models, patient-specific induced pluripotent stem cell (iPSC)-derived cardiomyocytes, and explanted hearts from advanced ACM cases indicate that dysregulation of the Wnt/ β -catenin signaling cascade and persistent inflammatory activation are central to disease progression. These pathways regulate the balance between myogenesis and adipogenesis, and their modulation has yielded therapeutic benefit in mouse models of ACM (Asatryan et al., 2021; Austin et al., 2019). However, translation into clinical practice remains challenging due to the lack of reliable large animal models that faithfully reproduce the early-stage molecular and histopathological features of ACM. Addressing this limitation is critical for advancing early diagnostic strategies, improving risk stratification for sudden cardiac death, reducing arrhythmic burden, and enabling the development of interventions capable of modifying disease trajectory.

Pigs share close genetic, anatomical, and physiological similarities with humans, making them more suitable than rodents for modeling human diseases. More importantly, the availability of advanced genetic engineering tools has enabled the generation of porcine models that recapitulate a wide range of human pathologies (Yin et al., 2022). Compared to non-human primates, pigs also offer practical advantages, including shorter reproductive cycles and large litter sizes, allowing for more rapid and scalable model development.

To construct a *PKP2*-mutant pig model that mimics ACM, pathogenic variants were examined in 184 individuals diagnosed with ACM and subjected to genetic testing at Fuwai Hospital. Among these, 24 were found to carry 15 unique *PKP2* mutations. To enhance the likelihood of early phenotypic manifestation, the c.1132C>T variant—detected in the patient with the earliest clinical onset—was prioritized for model generation. The BE3 base-editing system was selected for targeted mutagenesis. This platform, which integrates cytidine deaminase and a uracil glycosylase inhibitor based on CRISPR/Cas9n, enables precise C-to-T or G-to-A nucleotide conversions without generating double-strand breaks (DSBs) or requiring DNA template (Komor et al., 2016), offering a

powerful approach for generating precise and stable disease models. To further improve editing success, a second nonsense mutation, c.1147C>T, was selected from the ClinVar database. Both substitutions introduce premature stop codons and are expected to truncate *PKP2* translation, thereby increasing the likelihood of generating a functionally relevant model. Editing efficiency was evaluated in the Diannan miniature pig system, which demonstrated higher efficiency for the c.1132C>T target using the BE3 system. Gene-edited *PKP2*^{+/-} fetal fibroblasts were subsequently used as donor nuclei for somatic cell nuclear transfer (SCNT), resulting in the successful generation of *PKP2*^{+/-} cloned pigs carrying the c.1132C>T mutation. Phenotype evaluation at 24 months of age revealed hallmark features of ACM, including interstitial adipocyte infiltration, lipid droplet deposition within cardiomyocytes, and RV dilatation. Transcriptomic profiling demonstrated elevated expression of genes associated with lipid catabolism, and immunofluorescence analysis confirmed increased expression of PLA2G5 in *PKP2*^{+/-} myocardium. This model accurately recapitulates the early natural progression of ACM and provides a robust platform for elucidating disease mechanisms, identifying early diagnostic markers, and supporting drug and therapeutic development.

MATERIALS AND METHODS

Study population and clinical characterization

A total of 184 individuals diagnosed with ACM at the National Center for Cardiovascular Diseases, Fuwai Hospital, between June 2004 and December 2019 were enrolled for comprehensive genetic screening and clinical assessment. Among these, 24 individuals were identified as carriers of pathogenic or likely pathogenic variants in *PKP2* and were included in this analysis. Diagnoses were established based on the revised 2010 Task Force Criteria for ACM (Marcus et al., 2010). Major arrhythmia cardiovascular events (MACE) were defined as the occurrence of ventricular fibrillation, persistent ventricular tachycardia, or cardiogenic syncope. Heart transplantation was designated as the endpoint outcome event.

Animals

All animal procedures were conducted with approval from the Bioethics Committee of Yunnan Agricultural University in China (No. 202303080). To control for sex-based physiological variability, only male pigs were utilized. Animals were maintained under standardized conditions with unrestricted access to food and water and monitored daily by trained veterinary staff to ensure optimal welfare. Euthanasia was performed under deep anesthesia via intravenous administration of high concentrations of potassium chloride, in accordance with humane endpoints. All efforts were made to minimize suffering and ensure ethical treatment throughout the study.

Genetic testing and variant analysis

Genomic DNA was isolated from peripheral blood using a commercial extraction kit (Blood DNA Extraction Kit, Enriching Biotechnology, China), following the manufacturer's protocols. Next-generation sequencing was performed using the Illumina HiSeq 2500 platform (USA) to sequence 14 ACM-related genes. This panel included five desmosomal genes: Plakophilin 2 (*PKP2*), Desmoplakin (*DSP*), Desmoglein 2 (*DSG2*), Desmocollin 2 (*DSC2*), and Junction Plakoglobin

(*JUP*), and nine non-desmosomal genes: Transmembrane Protein 43 (*TMEM43*), Phospholamban (*PLN*), Catenin Alpha 3 (*CTNNA3*), Lamin A/C (*LMNA*), Desmin (*DES*), transforming growth factor beta 3 (*TGFB3*), Sodium Voltage-Gated Channel Alpha Subunit 5 (*SCN5A*), Cadherin 2 (*CDH2*), Filamin C (*FLNC*), Titin (*TTN*), and Ryanodine receptor 2 (*RYR2*). Variant annotation was conducted using the following multistep pipeline: (1) Variants with a minor allele frequency (MAF) below 5% were retained based on population databases, including 1000 Genomes (1000g_all) (The 1000 Genomes Project Consortium, 2015), ESP6500 (esp6500siv2_all) (<http://evs.gs.washington.edu/EVS>), and gnomAD data (gnomAD_ALL and gnomAD_EAS) (Lek et al., 2016). (2) Only single nucleotide variants (SNVs) located in exonic or splicing junction regions (± 10 bp) were analyzed, as these were expected to result in coding changes. (3) Synonymous SNVs with no predicted effect on amino acid sequences were excluded unless flagged by dbSNV. Non-frameshift indels smaller than 10 bp located in repetitive elements defined by RepeatMasker were also discarded. (4) Functional predictions were performed using SIFT (Kumar et al., 2009), PolyPhen (Adzhubei et al., 2010), MutationTaster (Schwarz et al., 2010), and CADD (Kircher et al., 2014). Variants were retained if at least three of the four tools predicted a deleterious effect (Muona et al., 2015). Variants larger than 2 bp with no influence on alternative splicing were excluded. (5) For *TTN*, only truncated variants were considered in pathogenicity assessments.

Putatively pathogenic variants were validated by Sanger sequencing. Familial screening was also performed using Sanger sequencing where available. Variant classification followed the guidelines of the American College of Medical Genetics and Genomics (ACMG), with variants classified as pathogenic, likely pathogenic, uncertain significance, likely benign, or benign (Richards et al., 2015).

Pathological examination and quantification

Gross pathological evaluation was performed on explant hearts obtained from patients and pigs heterozygous for *PKP2* mutation. Following macroscopic assessment, six representative regions were dissected from the basal ventricular myocardium. The left ventricular (LV) region included the anterior wall (LVAW), lateral wall (LVLW), and posterior wall (LVPW), while the RV region included the anterior wall (RVAW), outflow tract (RVOT), and posterior wall (RVPW). The interventricular septum (IVS) was also sampled. All tissues were dehydrated, embedded in paraffin, and sectioned at a thickness of 3 μ m, then stained with hematoxylin and eosin (H&E) and Masson's trichrome. Whole-slide digital images were acquired using a high-resolution scanner. Prior to quantitative analysis, epicardial adipose tissue, including blood vessels and pericardial structures, was digitally removed using Adobe Photoshop CC 2018 (Adobe Systems, USA). In cases where endocardial thickening was detected, the endocardium was excluded from fibrosis analysis. Quantitative analysis of myocardial, fibrotic, and adipose tissue components was performed using Image Pro Plus v.6.0 (Media Controllers, USA). The ratio of LV myocardium, fibrosis, and adipose tissue was the average of the LVAW LVLW and LVPW, while the ratio of the RV was the average of the RVAW and RVPW.

SgRNA design and vector construction

The pCMV-BE3 plasmid was kindly provided by Professor

Xing-Xu Huang (Shanghai Tech University, China). Two single-guide RNAs (sgRNAs) targeting exons 4 and 5 of the porcine *PKP2* gene were manually designed based on BE3 editing principles and in reference to pathogenic human *PKP2* mutations at Q383* and Q412*. The sgRNA expression vector pGL3-U6-sgRNA-PGK-puromycin (Addgene plasmid #51133) was linearized with BsaI and gel-purified. Annealed and phosphorylated oligonucleotide pairs corresponding to the *PKP2* target sequences (Supplementary Tables S1) were ligated into the vector backbone, yielding the constructs pGL3-U6-*PKP2*-sgRNA1 and pGL3-U6-*PKP2*-sgRNA2.

Cell culture, transfection, and selection

Pig fetal fibroblasts (PFFs) were prepared following previously established protocols (Wei et al., 2013). Briefly, cells were thawed and cultured in a medium containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Upon reaching approximately 80% confluence, cells (7×10^5) were resuspended in 700 μ L of phosphate-buffered saline (PBS) and electroporated with 10.5 μ g of pCMV-BE3 plasmid and 10.5 μ g of either pGL3-U6-*PKP2*-sgRNA1 or pGL3-U6-*PKP2*-sgRNA2 plasmids using the 4D-Nucleofector system (Lonza, Switzerland) according to the manufacturer's protocols. Post-electroporation, cells were seeded in T25 flasks containing 5 mL of fresh DMEM with 10% FBS and 1 μ g/mL puromycin and incubated at 37°C for 48 h. Cells were digested with 0.25% trypsin-EDTA for 2 min, neutralized in complete medium, centrifuged at 1 500 rpm/min for 3 min at room temperature, and resuspended. Some cells were used to assess BE3 editing efficiency, while the remainder were subjected to limiting dilution to establish single-cell colonies. After 12–14 days, colonies were screened by polymerase chain reaction (PCR) using primer pairs F1/R1 or F2/R2 (Supplementary Tables S1), followed by genotyping via restriction endonuclease analysis and sequencing. Finally, positive fibroblast cell lines with mutations were selected as nuclear donors for SCNT.

BE3 editing efficiency assessment

Genomic DNA was extracted from ear tissue of newborn piglets and from partially screened cell populations using lysis buffer, as previously described (Zhu et al., 2009). Edited *PKP2* alleles were amplified by PCR using the same primer pairs as above. Amplified products were subjected to T7E1 analysis or sequencing after purification using a gel extraction kit (28704, Qiagen, Germany).

SCNT and embryo transfer

SCNT and embryo transfer were performed as previously described (Wei et al., 2013). Oocytes were harvested and subjected to *in vitro* maturation. After cumulus cell removal, mature oocytes were enucleated using a micromanipulation system. Donor fibroblasts were microinjected into the perivitelline space of enucleated oocytes. Reconstructed embryos were fused in an electrofusion medium using the LF201 Electro Cell Fusion Generator (Nepa Gene, Japan) at 200 V/mm for 20 μ s, followed by activation at 150 V/mm for 100 μ s. Embryos were then cultured in PZM-3 medium supplemented with 5 μ g/mL cytochalasin B for 2 h at 39°C in a humidified atmosphere of 5% CO₂, 5% O₂, and 90% N₂, then maintained in PZM-3 medium until embryo transfer.

Crossbred prepubertal gilts (Large White/Landrace/Duroc) weighing over 100 kg were selected as embryo recipients. Reconstructed embryos were surgically transferred into the

oviducts of surrogate gilts. Pregnancy was confirmed 23–29 days post-transfer via transabdominal ultrasonography (HS-101 V, Honda Electronics, Japan). Piglets were delivered naturally after a gestation period of approximately 114 days.

Immunofluorescence

Primary antibodies used included PKP2 (ab223757, Abcam, UK), α -actinin (ab9465, Abcam, UK), and PLA2G5 (bs-19624R, Bioss, China). Secondary antibodies were all sourced from Abcam. For immunofluorescence, heart tissues were fixed in 4% paraformaldehyde in 0.01 mol/L PBS for 48 h, embedded, and sectioned at 4 μ m. Slides were pre-blocked in 4% normal goat serum with 0.1% Triton X-100 in PBS for 30 min, followed by overnight incubation at 4°C with primary antibodies diluted in 1% bovine serum albumin (BSA) in PBS. After three PBS washes, secondary antibodies were applied. Nuclei were counterstained using mounting medium containing DAPI. Confocal imaging was performed using a Zeiss LSM 800 microscope.

Transmission electron microscopy

Ultrastructural analysis of ventricular myocardium was performed as previously described (Basso et al., 2006). Samples were fixed in 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.3), post-fixed in buffered 1% osmium tetroxide, dehydrated through a graded ethanol series, and embedded in Epon resin. Semi-thin sections were initially screened under light microscopy. Ultrathin sections were stained with uranyl acetate and lead citrate, then examined using a JEM-1400Flash transmission electron microscope (JEOL, Japan).

Catecholamine provocation testing

Isoproterenol was administered via continuous infusion at 45 μ g/min for 3 min, irrespective of baseline heart rate, following previously established protocols (Denis et al., 2014). The 12-lead electrocardiogram (ECG) was recorded continuously from the start of infusion to up to 10 min post-infusion.

Computed tomography (CT) and cardiac magnetic resonance (CMR) imaging

For CT, pigs were anesthetized and positioned prone on a scanner table to ensure full cardiac coverage within the field of view (FOV). Image reconstruction and cardiovascular analysis were performed using dedicated post-processing software (Comprehensive Cardiac Analysis).

For CMR, all studies were performed using a uMR 890 whole-body scanner (United Imaging, China). The protocol included standard segmented cine steady-state free-precession (SSFP) sequences for high-resolution anatomical imaging, as well as T1-mapping and SPectral Attenuated Inversion Recovery (SPAIR) sequences for tissue characterization.

AC16 cell culture

The AC16 human cardiomyocyte cell line (ZQ0960, Zqxbio, China) was cultured in DMEM/F12 supplemented with 10% FBS and 1% penicillin/streptomycin (ZM0960, Zqxbio, China). The medium was replaced every 48 h. All culture procedures were performed in a humidified incubator at 37°C with 95% air and 5% CO₂. Cells were treated with PLA2G5 protein (PA1000-7709, IPODIX, China) at the indicated concentration for 48 h.

TUNEL assay for apoptosis detection

AC16 cells were seeded in a 4-well chamber slide system

(177399PK, ThermoFisher, USA). After three PBS washes, cells were fixed in 4% paraformaldehyde and permeabilized with 0.3% TritonX-100, followed by additional PBS washes. Apoptosis was evaluated using a TUNEL cell apoptosis detection kit (C1091, Beyotime, China) in accordance with the manufacturer's protocols.

Quantitative real-time PCR analysis

Total RNA was extracted from cultured cells using TRIzol reagent (15596026, ThermoFisher, USA) and precipitated with isopropyl. Quantitative real-time PCR analysis was performed using the Q7 Real-Time PCR System (Applied Biosystems, USA) with SYBR Green Master Mix (A46109, ThermoFisher, USA). Gene expression levels were normalized against GAPDH using the $2^{-\Delta\Delta C_t}$ method. Primer sequences for PKP2 were as follows: Forward: GTACAGTTCCCAGAAGTCAGT GG; Reverse: GGCTTCTCTGGCTGTACTGGTG.

Western blot analysis

RV tissue was lysed using RIPA buffer supplemented with protease and phosphatase inhibitors (PPC1010, Sigma, Germany). Protein concentration was quantified by BCA assay (Pierce™, ThermoFisher, USA). Equal amounts of protein (20 μ g per lane) were resolved via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 4%–11% Tris-glycine gels (NuPAGE™, ThermoFisher, USA) under reducing conditions and transferred to polyvinylidene fluoride (PVDF) membranes (iBlot™, ThermoFisher, USA). Membranes were blocked with 5% non-fat milk in TBST for 1 h at room temperature, followed by overnight incubation at 4°C with primary antibodies: anti-PKP2 (ab223757, 1:250, Abcam, UK), anti-connexin 43 (71-0700, 1:80, ThermoFisher, USA), and horseradish peroxidase (HRP)-conjugated monoclonal mouse anti-GAPDH (Cat#KC-5G5, 1:5 000, Aksomics, China). After three washes with TBST (10 min each), membranes were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Protein bands were visualized using enhanced chemiluminescence (ECL) substrate (Pierce™ 32109, ThermoFisher, USA).

Transcriptome sequencing of cardiac tissue

Total RNA was extracted from RV tissue of PKP2^{+/-} and wild-type (WT) pigs using the RNeasy Mini Kit (74104, Qiagen, Germany). For each sample, 1 μ g of total RNA was used as input for library preparation. RNA sequencing (RNA-seq) libraries were generated using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (New England Biolabs, USA), following the manufacturer's recommendations. Index barcodes were incorporated to enable sample multiplexing. Libraries were sequenced on the Illumina NovaSeq platform (USA), generating 150 bp paired-end reads. The reference genome index was constructed using HISAT2 v.2.0.5., followed by alignment of paired-end clean reads to the reference genome. Differential gene expression analysis between groups was performed using DESeq2 in R v.1.16.1. Gene Ontology (GO) enrichment analysis of differentially expressed genes (DEGs) was conducted using clusterProfiler in R.

Statistical analysis

Two-group comparisons were evaluated using unpaired, two-tailed Student's *t*-tests. For multiple-group comparisons, one-way analysis of variance (ANOVA) was applied when data satisfied assumptions of normality and homogeneity of variance, otherwise non-parametric tests were used.

RESULTS

Clinical profiles of individuals harboring *PKP2* mutations

Twenty-four individuals carrying *PKP2* mutations and fulfilling the 2010 Revised Task Force Criteria for ACM were included in this analysis. Clinical and phenotypic characteristics are summarized in Table 1. The average age of initial symptom onset was 32.58 ± 14.42 years, with the earliest presentation occurring at 12 years of age. The cohort was predominantly males (79%, $n=19$), with females comprising 21% ($n=5$). Palpitations were the most frequently reported initial symptom (96%, $n=23$), followed by presyncope (83%, $n=20$) and syncope (58%, $n=14$). Functional classification based on the New York Heart Association (NYHA) criteria indicated that most individuals were class I–II (88%, $n=21$), with the remaining three patients (12%) classified as NYHA class III–IV.

Echocardiographic evaluation revealed preserved LV function, with a mean LV ejection fraction (LVEF) of $59.36 \pm 2.40\%$. The mean LV end-diastolic dimension (LVEDD) was 44.26 ± 1.12 mm, and the RV end-diastolic dimension (RVEDD) was 36.26 ± 2.00 mm. MACE occurred at a mean age of 30.27 ± 2.74 years, and ICDs had been implanted in 42% of patients. The mean follow-up duration from initial ACM diagnosis to endpoint events was 6 years. Life-threatening arrhythmias, including cardiogenic syncope, persistent ventricular tachycardia, and ventricular fibrillation, were observed in 96% of patients, underscoring the high arrhythmic burden within this mutation-defined population (Table 1).

Mutation loci in *PKP2* mutant patients

Details of the *PKP2* mutations identified in the 24 ACM patients are summarized in Table 2. All individuals harbored *PKP2* mutations exclusively, with no pathogenic or likely pathogenic variants detected in other ACM-associated genes. A total of 15 distinct *PKP2* mutations were identified, including

10 classified as pathogenic and five classified as likely pathogenic based on ACMG guidelines. Of these, 19 individuals carried previously reported pathogenic variants, while five patients harbored novel mutations. Among the identified variants, four were found to disrupt canonical splice sites and were predicted to induce aberrant RNA splicing, potentially leading to impaired *PKP2* protein synthesis and functional disruption. The remaining 11 mutations were localized to coding exons and primarily represented stop-gain substitutions or frameshift indels (Table 2). To illustrate inter-individual variation in disease trajectory, the clinical timelines of all 24 patients carrying *PKP2* mutations were compiled, highlighting age of onset and major cardiac events in relation to specific *PKP2* variants. Notably, Patient #23, who carried the c.1132C>T mutation, exhibited disease onset at 12 years of age, with both MACE and detectable structural abnormalities present at the time of clinical manifestation (Figure 1; Table 2). To accelerate phenotypic development in the porcine model, mutations associated with early-onset disease were prioritized. The c.1132C>T variant was selected for model generation due to its strong pathogenicity and early clinical presentation. This mutation involves C-to-T conversions, enabling precise modeling using the BE3 base-editing system. This tool can induce targeted C-to-T (or G-to-A) single-nucleotide conversions without generating DSBs or requiring donor DNA templates, with editing efficiency often exceeding 50% in mammalian cells without selection pressure (Fichter et al., 2023). In parallel, the previously reported pathogenic variant c.1147C>T, located proximally to c.1132 of *PKP2*, was selected as an alternative target to increase the likelihood of successful model construction. Both mutations introduce premature termination codons and are expected to disrupt *PKP2* translation, supporting their suitability for use in disease modeling.

Efficient induction of C-to-T base conversion in PFFs

To evaluate conservation of the *PKP2* gene, sequence homology was compared among different species. The *PKP2* gene in pigs exhibited greater similarity to the human sequence than that in mice, but lower than that observed in monkeys (Figure 2A). Given the logistical and reproductive constraints associated with non-human primates, pigs were selected as the preferred large-animal model due to their favorable reproductive characteristics, along with cardiovascular anatomy and physiology that closely approximate human features (Jia et al., 2024).

Two gRNAs were designed for base-editing the porcine *PKP2* locus, with sgRNA1 targeting the c.1132C>T mutation and sgRNA2 targeting the c.1147C>T mutation (Supplementary Tables S1). These C-to-T conversions are predicted to generate Q378* and Q383* nonsense mutations, respectively, introducing premature termination codons and mimicking clinically observed stop-gain variants (Figure 2B, C). Following transfection and T7E1 digestion, WT samples showed no cleavage, whereas both sgRNA1- and sgRNA2-edited samples exhibited distinct digestion bands, indicating successful editing activity (Figure 2D). Targeted sequencing revealed six distinct base substitutions and eight indels at the predicted editing sites (Figure 2E). Both sgRNAs demonstrated efficient targeting of the *PKP2* gene, with editing efficiencies of 42.9% and 25.9%, respectively (Table 3; Figure 2F).

Table 1 Demographics and presentation in study population

Variable	<i>PKP2</i> ($n=24$)
Age of onset (years)	32.58 ± 14.42
Male, n (%)	19 (79)
History	
Dyspnea, n (%)	6 (25)
Palpitation, n (%)	23 (96)
Presyncope, n (%)	20 (83)
Syncope, n (%)	14 (58)
Abdominal distension, n (%)	0 (0)
Bilateral oedema, n (%)	3 (13)
Adverse arrhythmic events, n (%)	22 (92)
NYHA, n (%)	
I–II	21 (88)
III–IV	3 (13)
Echo	
LVEF (%)	59.36 ± 2.40
LVEDD (mm)	44.26 ± 1.12
RVEDD (mm)	36.26 ± 2.00
Age of MACE	30.27 ± 2.74
ICD implantation, n (%)	10 (42)

Variables are expressed as frequency (percent), mean $\pm$ standard deviation (SD), or median (interquartile range). NYHA, New York Heart Association.

Table 2 *PKP2* mutation types in study population

Patient	Chr:pos	Location	Nucleotide change	Amino acid change	Type	Reported previously
1, 2	32945601	Exonic	c.2422delG	p.E808Nfs*79	Frameshift deletion	Reported
3, 4, 5	32949042	Splicing	c.2357+1G>A	N/A	N/A	Reported
6	32949219	Exonic	c.2180_2181del	p.L727Pfs*2	Frameshift deletion	Reported
7	32949234	Splicing	c.2168-2A>G	N/A	N/A	Novel
8	32955408	Exonic	c.2096delC	p.P699Qfs*3	Frameshift deletion	Novel
9, 10	32955433	Exonic	c.C2071T	p.R691X	Stop-gain	Reported
11	32955457	Exonic	c.A2047T	p.K683X	Stop-gain	Reported
12	32955490	Exonic	c.2014delA	p.M672Cfs*30	Frameshift deletion	Novel
13	32955491	Splicing	c.2014-1G>C	N/A	N/A	Reported
14	32975421	Exonic	c.C1819T	p.R607X	Stop-gain	Reported
15	32994054	Exonic	c.1464delC	p.I489Sfs*30	Frameshift deletion	Novel
16	33003785	Exonic	c.1292dupT	p.L431Ffs*5	Frameshift insertion	Novel
17, 18, 19	33021860	Splicing	c.1170+1G>A	N/A	N/A	Reported
20, 21, 22, 24	33049515	Exonic	c.148_151del	p.T50Sfs*61	Frameshift deletion	Reported
23	33021899	Exonic	c.1132C>T	p.Q378X	Stop-gain	Reported

N/A: Not applicable

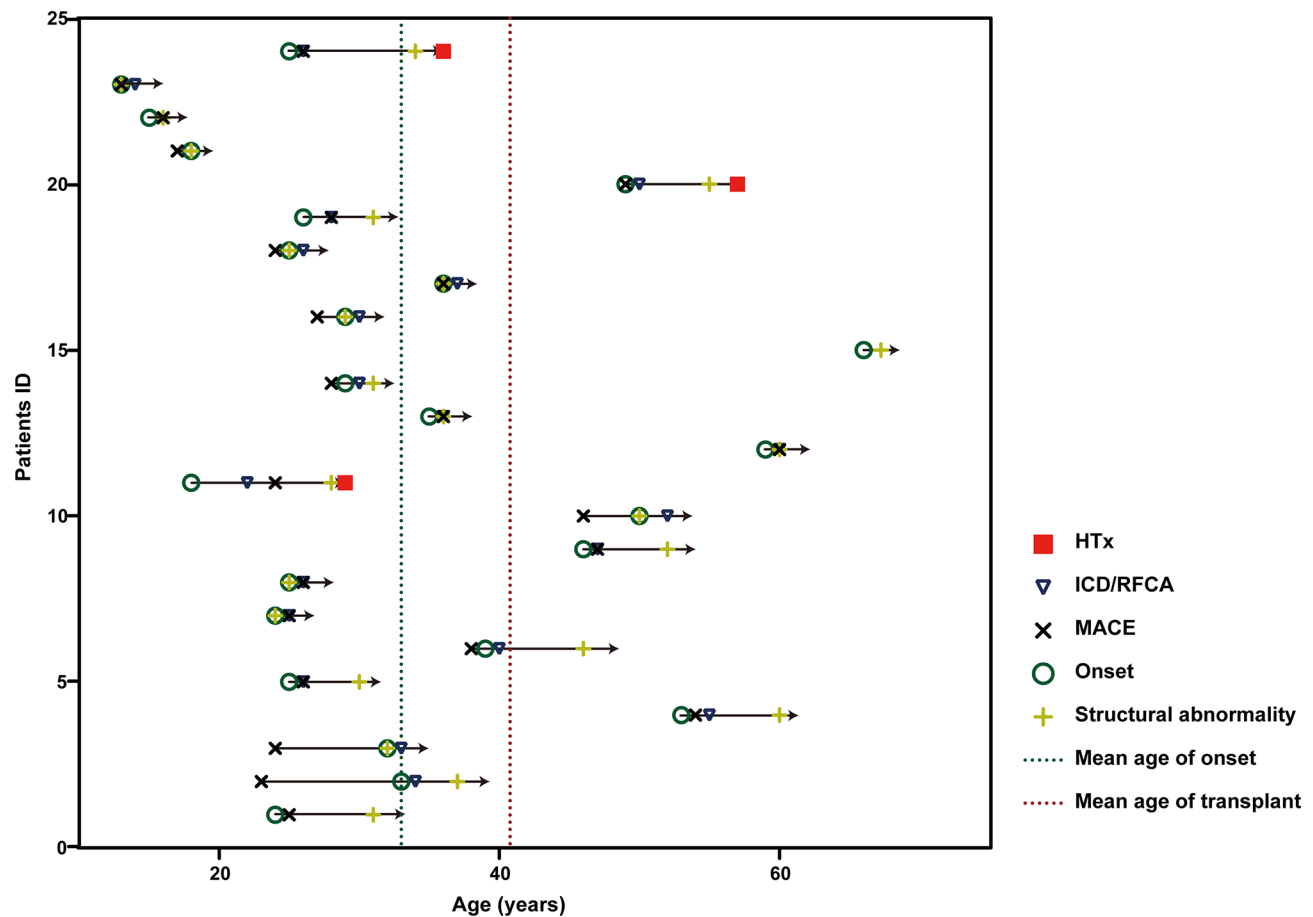


Figure 1 Natural history of 24 individuals with *PKP2* mutation-associated ACM

HTx, heart transplantation; ICD, implantable cardioverter-defibrillator. MACE, major adverse cardiovascular events. RFCA, radiofrequency catheter-based ablation.

Efficient generation of *PKP2* mutant PFFs via the BE3 system

Positive PFFs were screened using puromycin selection followed by clonal expansion. Among 25 single-cell clones derived from *PKP2*-sgRNA1-treated cells, 19 exhibited successful editing, yielding a base editing efficiency of 76.0%. Specifically, 14 clones carried monoallelic mutations and five

carried biallelic mutations. TA cloning combined with Sanger sequencing of 50 alleles from these clones identified 25 edited *PKP2* sequences. Of these, 24 sequences harbored a nonsense mutation (TTC to TTT, Phe to Phe) that introduced a premature stop codon (CAG to TAG), while one sequence only carried a nonsense mutation (TTC to TTT, Phe to Phe) (Figure 3A, B).

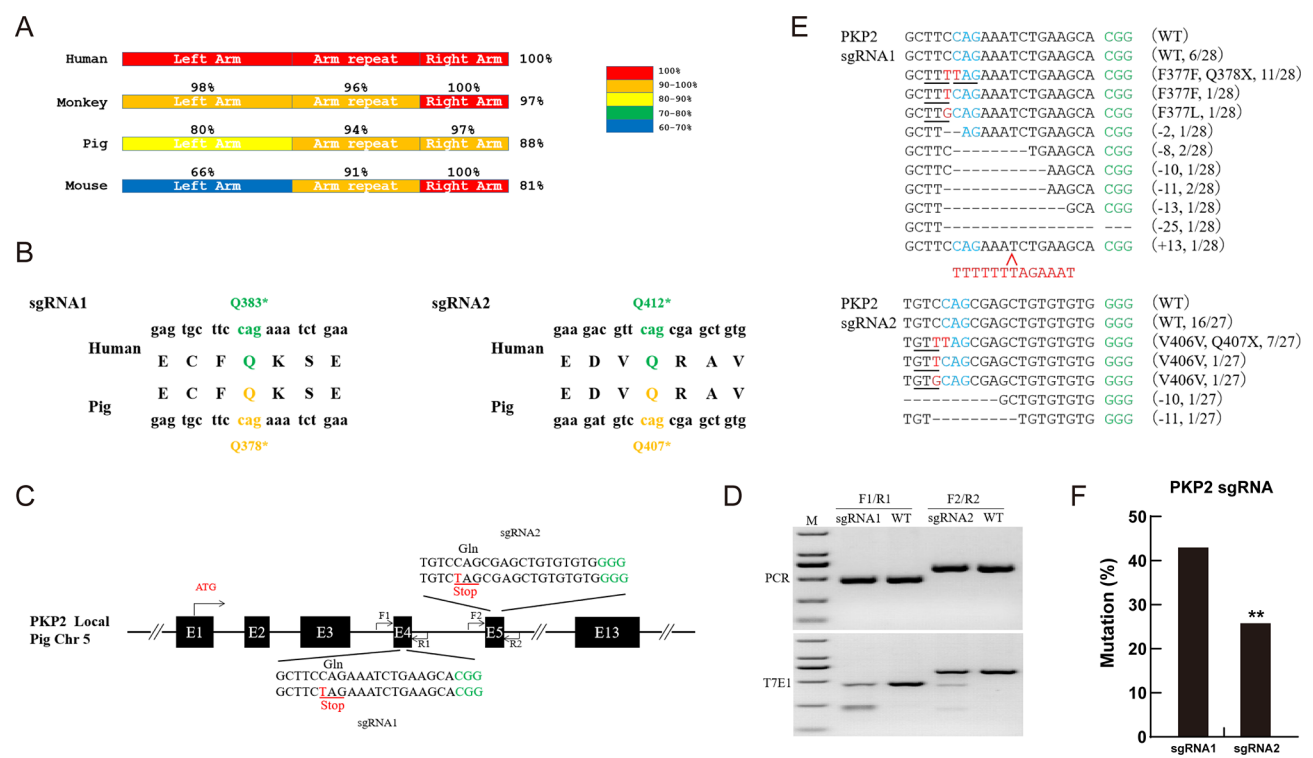


Figure 2 C-to-T conversion by the BE3 system and sgRNA targeting efficiency

A: Cross-species homology of *PKP2* gene. B: Schematic representation of *PKP2* mutations in humans and pigs. C: Targeted region within the *PKP2* locus. sgRNA sequences are in black; PAM sequences are in green; BE3-mediated nucleotide substitutions are in red and underlined. D: Detection of sgRNA1 and sgRNA2: BE3-mediated base editing of *PKP2* by PCR and T7E1 cleavage assay. M, DNA marker; sgRNA1, *PKP2*-sgRNA1; sgRNA2, *PKP2*-sgRNA2; WT, wild-type. E: Alignment of mutant sequences from targeted sequencing. Protospacer adjacent motif (PAM) sequences are in green; substitutions are in red; codons are underlined. Right column indicates amino acid mutation type, deletions (-), insertions (+), and proportion of positive colonies out of all sequenced colonies. F: Single base editing efficiency for *PKP2*-sgRNA1 and *PKP2*-sgRNA2 (C>T).

Table 3 Summary of base-editing efficiency using BE3 system

sgRNA	Total clones sequenced	Editing efficiency (%)			
		Mutant clones	C>T mutations	Indels	Non-C>T mutations
<i>PKP2</i> -sgRNA1	28	22 (78.6)	12 (42.9)	9 (32.1)	1 (3.6)
<i>PKP2</i> -sgRNA2	27	11 (40.7)	8 (25.9)	2 (7.4)	1 (3.7)
Total	55	33 (60.0)	20 (36.4)	11 (20.0)	2 (3.6)

Generation of *PKP2* mutant fetuses and piglets through SCNT and genotyping

Two donor cell colonies, C7# and C8#, were selected for SCNT based on robust proliferation. A total of 315 reconstructed embryos from C7# were transferred into recipient gilt p135, yielding six live piglets and one stillbirth after 33 days. Similarly, 280 reconstructed embryos from C8# were transferred into recipient gilt p110, yielding three stillbirths after 33 days (Supplementary Table S2). Sanger sequencing confirmed successful base editing at the target site in all 10 fetuses (Figure 3C, D). PFFs derived from the *PKP2* KO-7-1# fetus were used as nuclear donors for further SCNT. In this round, 1 996 reconstructed embryos were transferred into six recipient gilts, three of which became pregnant (Supplementary Table S3), producing 11 live piglets and three stillbirths (Figure 3E; Supplementary Table S4). Sanger sequencing confirmed that all 13 piglets (excluding one mummified fetus), carried genotypes consistent with the donor cell line (Figure 3F, G). These gene-edited pigs exhibited normal reproductive capacity, and three were randomly selected for mating with WT females. Among 19

offspring, 18 were genotyped, revealing 10 *PKP2* mutants and eight WT individuals (Figure 3H).

PKP2^{-/-} pigs display ventricular precontraction and RV dilation

All *PKP2*^{-/-} pigs showed normal growth, and catecholamine challenge was initiated at 24 months of age. Following isoproterenol administration, all *PKP2*^{-/-} pigs developed ventricular precontraction, a response absent in WT controls (Figure 4A; Supplementary Figure S1). Cardiac CT revealed mild RV dilation during both systole and diastole in the mutant pigs (Figure 4B, C), indicating early structural alterations associated with *PKP2* haploinsufficiency. To further characterize these changes, CMR imaging was performed. T1-weighted sequences showed increased signal intensity, while SPAIR sequences exhibited reduced intensity in the RV myocardium, consistent with intramyocardial fat infiltration (Figure 4D; MRI cine video in Supplementary Video S1). Functional assessment demonstrated elevated cardiac index (CI) in *PKP2*^{-/-} pigs compared to WT controls (Figure 4E). These early structural and functional changes may underlie the increased risk of arrhythmic events and sudden cardiac

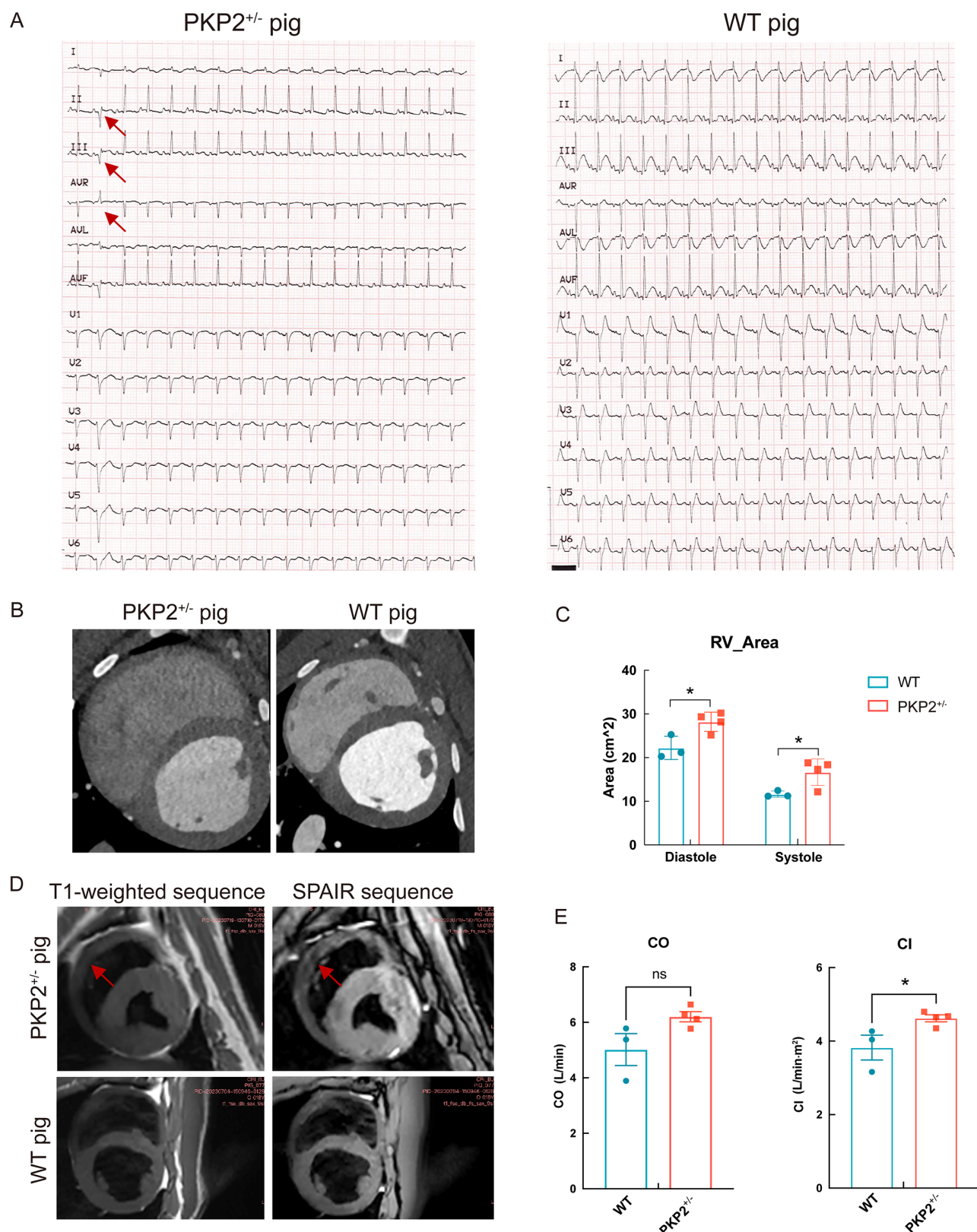


Figure 4 Electrocardiogram and imaging examination of *PKP2^{+/-}* knockout pigs

A: Electrocardiogram of *PKP2^{+/-}* and WT pigs after isoproterenol challenge. B: CT reconstruction of heart cross section in *PKP2^{+/-}* and WT pigs. C: Right ventricular (RV) area comparison between *PKP2^{+/-}* and WT pigs. D: MRI of heart cross-section in *PKP2^{+/-}* and WT pigs. Area indicated by the red arrow is a suspicious region of fat infiltration. E: Cardiac output (CO) and cardiac index (CI) comparison between *PKP2^{+/-}* and WT pigs. ns: Not significant; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; ****: $P < 0.0001$.

Transmission electron microscopy further revealed disrupted intercalated disc architecture, characterized by shortened desmosomal regions with diminished electron density. Pale

desmosomes were evident in *PKP2^{+/-}* myocardium, as indicated by white arrows (Figure 6D, F), consistent with ultrastructural abnormalities previously reported in human

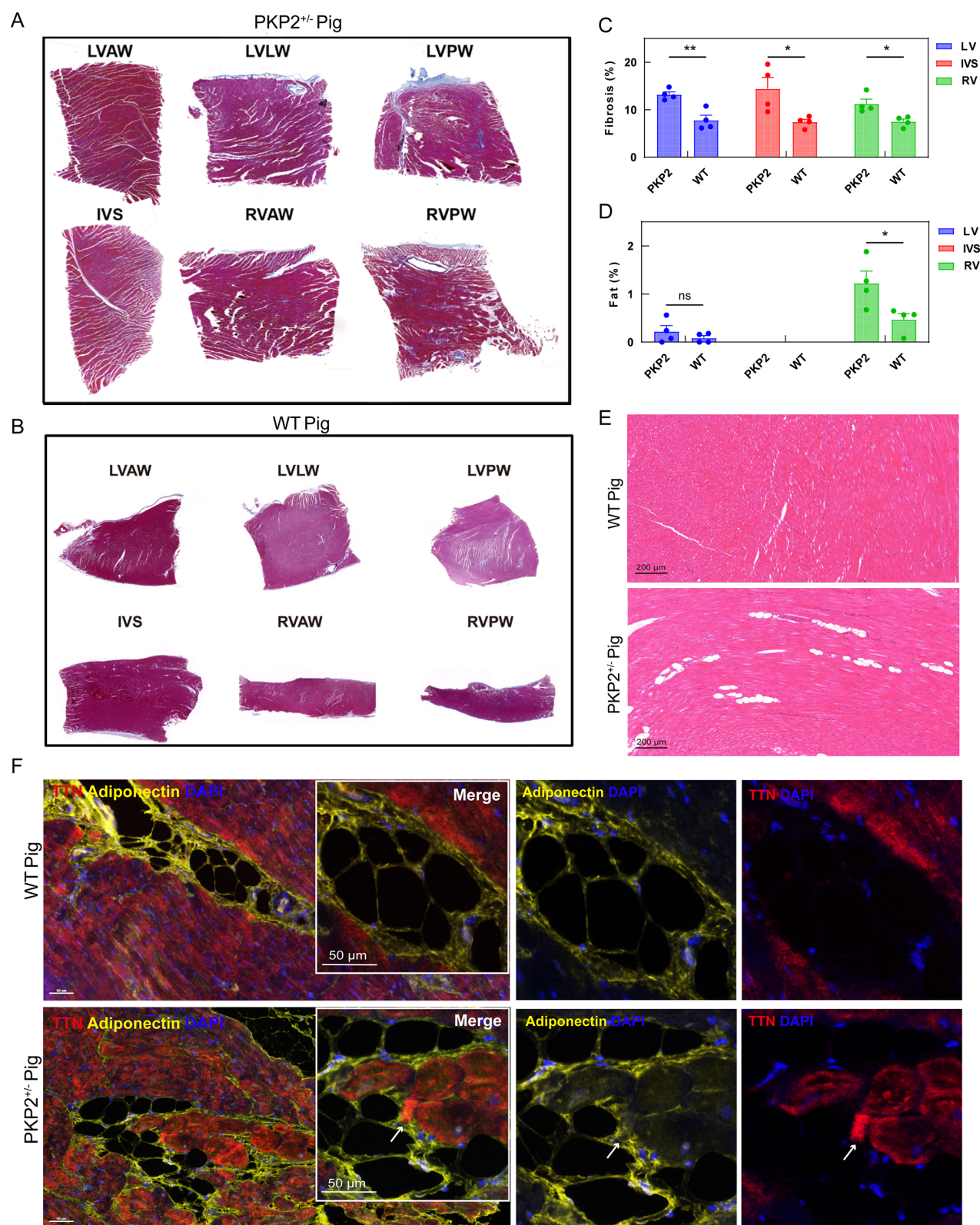


Figure 5 Comparison of cardiac pathology between ACM patients and PKP2^{+/-} pigs

A: Extensive fibrofatty replacement in the RV wall of PKP2^{+/-} pigs. B: Minimal fibrofatty tissue in WT pigs. C: Semi-quantitative analysis of fibrotic tissue content in PKP2^{+/-} pigs. D: Semi-quantitative analysis of fat tissue content in PKP2^{+/-} pigs. E: H&E staining of cardiac tissue from PKP2^{+/-} and WT pigs. F: Immunofluorescence staining of TTN and adiponectin in PKP2^{+/-} and WT pigs. White arrow indicates clear co-staining of TTN and adiponectin. LVAV: Left ventricle anterior wall; LVLW: Left ventricle lateral wall; LVPW: Left ventricle posterior wall; IVS: Interventricular septum; RVAW: Right ventricle anterior wall; RVPW: Right ventricle posterior wall. ns: Not significant; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; ****: $P < 0.0001$.

ACM patients (Basso et al., 2006). Despite the presence of the c.1132C>T nonsense mutation, qPCR analysis showed no change in PKP2 mRNA expression (Figure 6G). In contrast,

western blot analysis revealed a significant decrease in PKP2 protein levels (Figure 6H, I), with no truncated PKP2 fragments detected in unclipped blots (Supplementary Figure

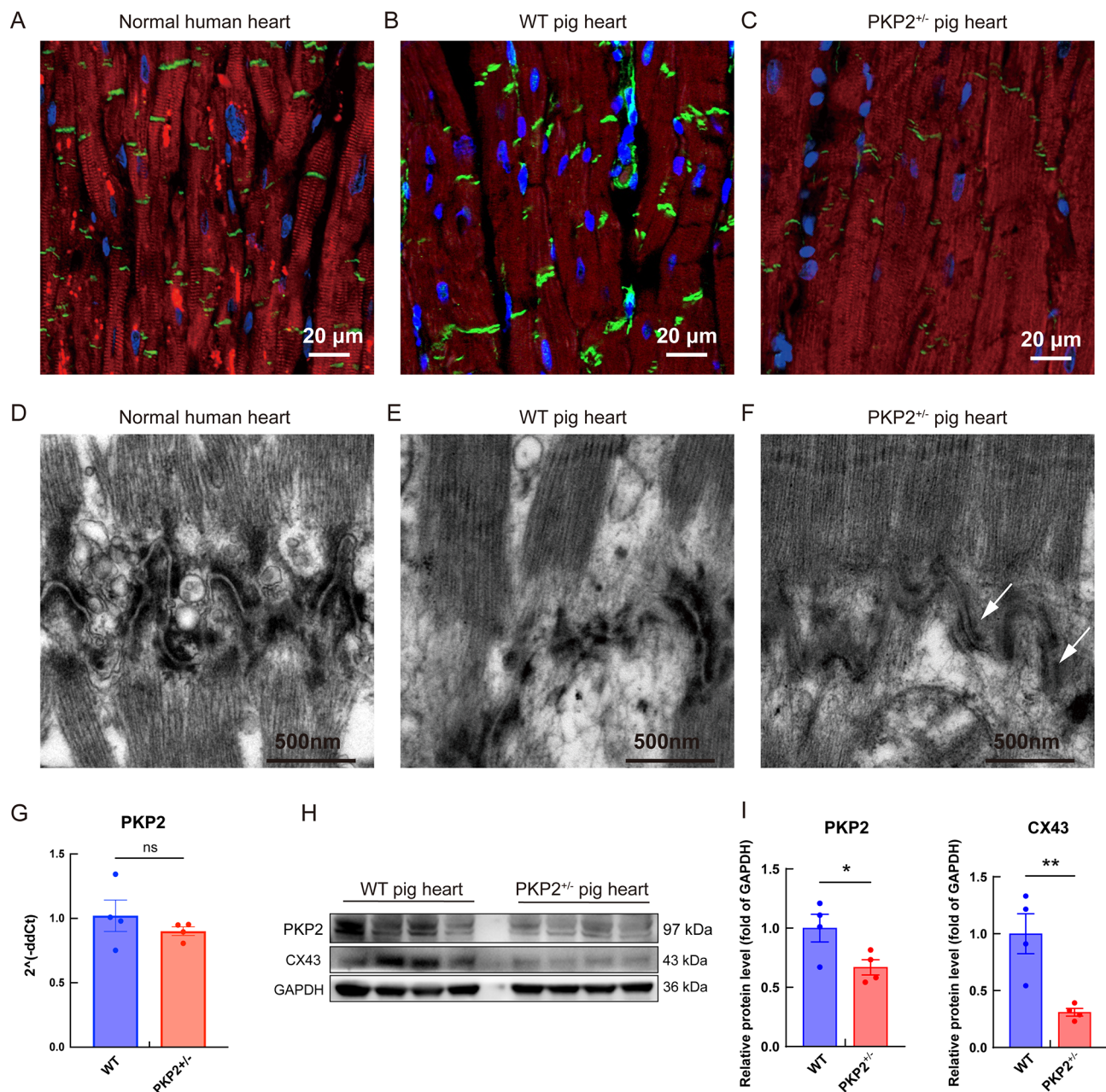


Figure 6 Comparison of desmosomal structure in ACM patients and *PKP2*^{+/-} pigs

A: Immunofluorescence staining of PKP2 (green), actin (red), and DAPI (blue) in normal human heart. B: Immunofluorescence staining of PKP2 (green), actin (red), and DAPI (blue) in WT pig heart. C: Immunofluorescence staining of PKP2 (green), actin (red), and DAPI (blue) in *PKP2*^{+/-} heart. D: Transmission electron microscopy of desmosomes in normal human heart. E: Transmission electron microscopy of desmosomes in WT pig heart. F: Transmission electron microscopy of desmosomes in *PKP2*^{+/-} pig heart. G: qPCR analysis showing no significant change in *PKP2* mRNA expression in *PKP2*^{+/-} pigs. H–I: Western blot analysis showing significantly reduced PKP2 and CX43 protein expression in *PKP2*^{+/-} pig. ns: Not significant; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; ****: $P < 0.0001$.

S4), suggesting premature translational termination and protein loss. Furthermore, PKP2 haploinsufficiency resulted in down-regulation of the gap junction protein CX43, indicating impaired gap junction assembly (Figure 6H, I). These findings suggest a reduction in the strength of intercellular connections between cardiomyocytes, not only in number but also in intercalated disc integrity.

Transcriptome sequencing implicates PLA2G5 in early myocardial lesions associated with *PKP2* mutation-induced ACM

Transcriptomic profiling of RV myocardium from *PKP2*^{+/-} pigs and WT controls revealed candidate mechanisms underlying

early adipocyte infiltration in ACM. Tissues from four *PKP2*^{+/-} pigs and four WT pigs were subjected to RNA-seq, and DEGs were categorized into three expression clusters using the Weighted Fuzzy C-Means Clustering (Mfuzz) algorithm (Figure 7A). Cluster 1 (C1), predominantly expressed in WT pigs, was enriched in pathways associated with adherens junction assembly, cell membrane protein complexes, and calcium ion binding. Clusters 2 (C2) and 3 (C3), exhibiting higher expression in *PKP2*^{+/-} pigs, were linked to cell membrane lipoprotein particle assembly and lipid metabolic processes (including lipid degradation, lipase activity, and triglyceride lipase activity), respectively.

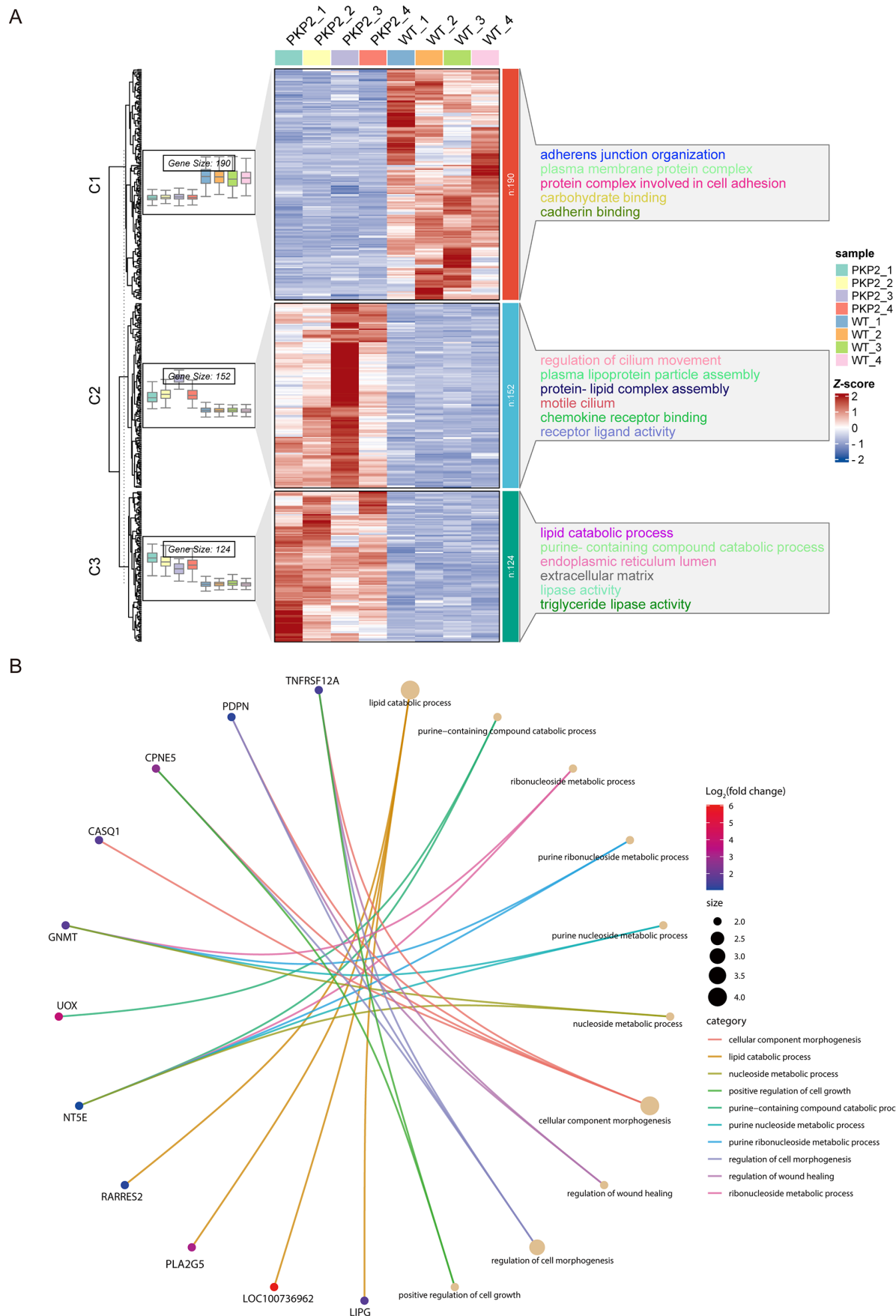


Figure 7 Transcriptomic profiling of right ventricular myocardial tissues from four *PKP2*^{+/−} pigs and four wild-type (WT) pigs

A: Weighted Fuzzy C-Means Clustering Algorithm (Mfuzz) analysis of differentially expressed genes between *PKP2*^{+/−} and WT pigs. B: Relationship between genes and Gene Ontology (GO) functional enrichment in Cluster 1.

Notably, PLA2G5 was among the most significantly up-regulated transcripts in *PKP2*^{+/-} pigs (Figure 7B). This phospholipase, known to hydrolyze membrane phospholipids and promote inflammatory signaling, was further validated by immunofluorescence staining, which confirmed its elevated expression in mutant myocardium compared to controls (Figure 8A–C). Functional validation was conducted in AC16 human cardiomyocytes, where recombinant human PLA2G5 protein induced a significant increase in apoptosis, implicating PLA2G5 in cardiomyocyte injury that drives disease pathogenesis (Figure 8D). In addition, immunohistochemical analysis revealed increased expression of inflammatory factors IL6 (*PKP2*^{+/-} vs WT, 1.57%±0.31% vs. 0.55%±0.06%, *P*=0.0181) and IL1β (*PKP2*^{+/-} vs WT, 3.09%±0.60% vs. 0.82%±0.12%, *P*=0.0100) in *PKP2*^{+/-} hearts compared to controls (Figure 8E, F; Supplementary Figure S5).

DISCUSSION

This study delineated the clinical trajectory of individuals harboring *PKP2* mutations over a 6-year observational period. *PKP2* haploinsufficiency predominantly resulted in fibrofatty remodeling of the RV, with minimal impact on LV function. Notably, 96% of affected individuals developed malignant arrhythmias during follow-up, consistent with previous findings that *PKP2*-linked ACM primarily manifests with RV involvement and arrhythmic phenotypes (Dewitt et al., 2019). These findings underscore the imperative to halt progression of lesions in the RV to prevent life-threatening arrhythmias. Murine models carrying *PKP2* mutations recapitulate some disease features, including reduced systolic performance and sudden cardiac death; however, they typically lack the hallmark fibrofatty infiltration observed in human ACM (Camors et al., 2022; Van Opbergen et al., 2022). With the caveat that mouse models of ACM generally do not have the robust fatty involvement seen in humans, their disease progression may differ from the human condition (Austin et al., 2019). In contrast, pigs exhibit close physiological and anatomical alignment with humans, including comparable heart size, coronary anatomy, ventricular pressure profiles, and baseline heart rates (Jia et al., 2024). As such, pigs have been extensively used as superior models for studying human cardiomyopathies and bridging preclinical research with clinical application. For example, (Del Rio et al., 2017) employed homologous recombination and SCNT to introduce the R403Q mutation into the porcine MYH7 gene, producing a heterozygous Yucatan miniature pig model of hypertrophic cardiomyopathy (HCM). These animals exhibited enhanced contractility, increased calcium sensitivity, decreased cardiac reserve, and elevated filling pressures, faithfully recapitulating human HCM pathophysiology. Given these findings, pigs were selected for genome editing to establish a robust ACM model based on *PKP2* haploinsufficiency, enabling investigation into early-stage pathogenic mechanisms with high translational relevance.

Considering the extended developmental timeline of pigs, the c.1132C>T variant associated with the earliest clinical onset was selected for model generation. The BE3 base-editing platform enables precise C-to-T nucleotide conversion in mammalian cells and has been reported to achieve editing efficiencies of up to 75% (Komor et al., 2016). This study represents the first application of BE3-mediated base editing to generate *PKP2*^{+/-} cell lines. Following SCNT, *PKP2*^{+/-} fetuses were obtained at day 33 of gestation and used to

establish fetus fibroblast lines for a second round of SCNT. This approach yielded 14 cloned pigs, including 11 live animals and three nonviable outcomes, one of which was a mummified fetus. Genotyping of the 13 available piglets confirmed successful introduction of the *PKP2*^{+/-} genotype in all cases. Alternative strategies combining prokaryotic microinjection with BE3-mediated editing have been applied to generate disease models without the need for somatic cell screening, often resulting in higher developmental and survival rates for naturally fertilized embryos compared with cloned embryos. However, prokaryotic microinjection frequently produces mosaic genotypes with unpredictable allelic distribution (Xie et al., 2019). Achieving sufficient numbers of genetically uniform disease models under such conditions typically requires increased embryo transfer numbers and, in some case, multiple rounds of mating and selection. While this approach is well-suited for small animals with short reproductive cycles, early sexual maturity, and low maintenance costs, its application to large animals such as pigs is less practical. Long reproductive cycles, delayed sexual maturity, and high breeding costs substantially extend experimental timelines and reduce overall efficiency.

This study provides the first demonstration that a large mammalian model can recapitulate selective adipocyte infiltration within the RV driven by *PKP2* haploinsufficiency, accompanied by pronounced RV dilatation by 24 months of age. ACM pathology has been proposed to progress through three pathological stages: an early concealed phase characterized by malignant ventricular arrhythmias and sudden cardiac death preceding overt remodeling; an electrical phase marked by fibrofatty replacement of a single ventricle, typically the RV; and a late phase involving biventricular fibrofatty infiltration (Camors et al., 2022). The present study showed that micro-scale adipocyte substitution within the cardiomyocyte network is already detectable during the early disease phase. In the *PKP2*^{+/-} pig model, the c.1132C>T nonsense variant did not result in detectable truncated *PKP2* protein but instead manifested primarily as reduced overall *PKP2* protein abundance. The antibody employed, targeting *PKP2* amino acids 50–200, would be expected to detect N-terminal fragments if truncation occurred downstream of this epitope, as predicted for the c.1132C>T mutation that introduces a stop codon after residue 378. However, conformational alterations of truncated proteins may impair antibody recognition, potentially obscuring detection. Previous work using human iPSC-derived cardiomyocytes carrying truncated *PKP2* variants demonstrated that plakophilin-2 truncation can disrupt sarcomere organization and impair contractile function (Zhang et al., 2021). While the present results do not exclude a contributory role for truncated *PKP2* protein in ACM pathogenesis, they establish that heterozygous *PKP2* c.1132C>T mutation in pigs is sufficient to induce canonical ACM lesions.

Transcriptomic profiling was performed to identify gene expression differences between *PKP2*^{+/-} and WT pigs, aiming to uncover early pathogenic mechanisms of ACM with *PKP2* mutations. Differential expression analysis revealed significant down-regulation of genes involved in adherens junction organization in *PKP2*^{+/-} pigs compared to WT pigs. Additionally, *PKP2*^{+/-} pigs showed elevated expression of genes involved in lipid catabolic metabolism, with PLA2G5 emerging as a prominently induced gene. PLA2G5 encodes group V secretory phospholipase A2 (sPLA2), a cardiac-

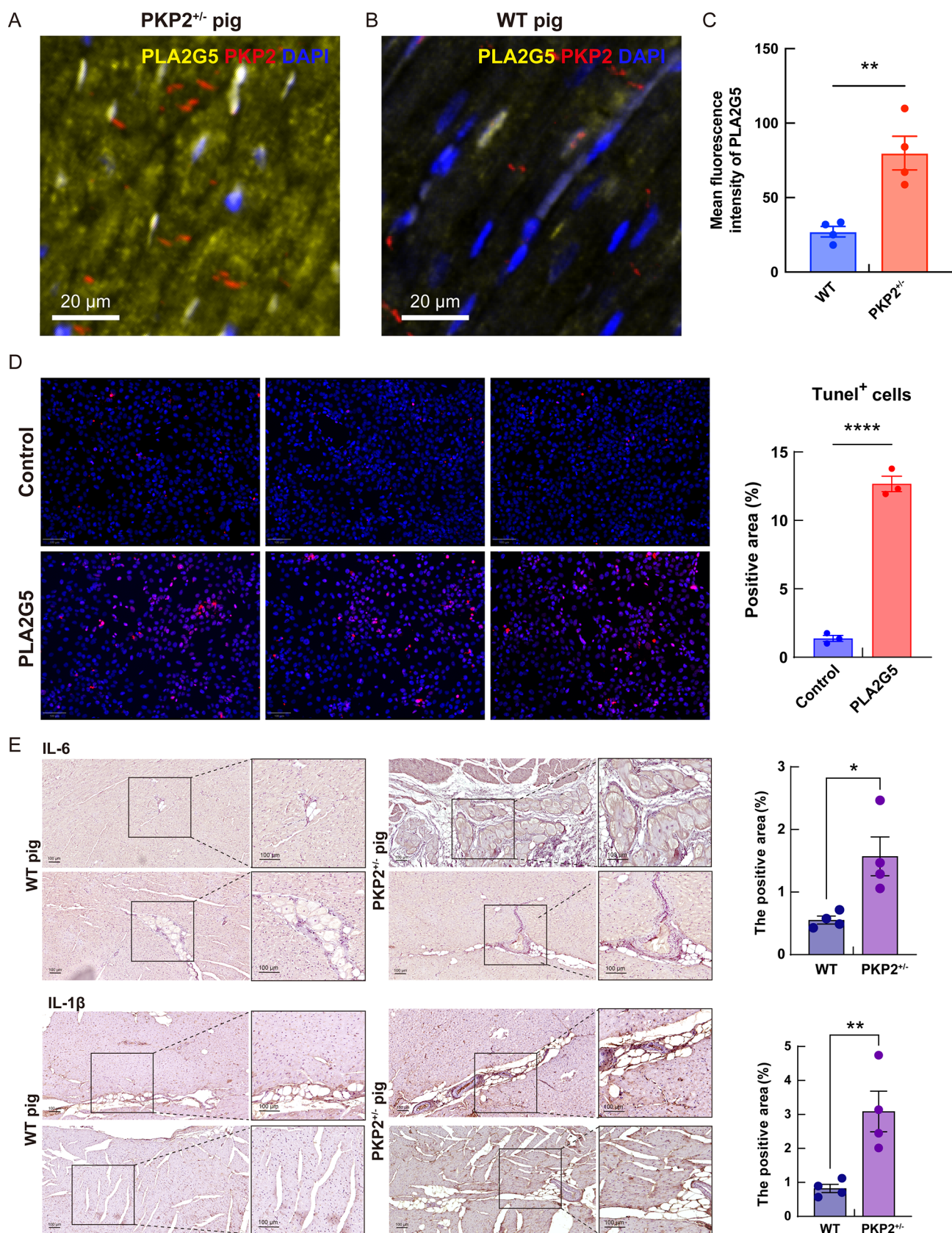


Figure 8 High expression of PLA2G5 in PKP2^{+/-} pigs

A: Immunofluorescence staining of PLA2G5, PKP2, and DAPI in right ventricle of PKP2^{+/-} pigs. B: Immunofluorescence staining of PLA2G5, PKP2, and DAPI in right ventricle of WT pigs. C: Fluorescence intensity of PLA2G5 between PKP2^{+/-} and WT pigs. D: PLA2G5 treatment promotes apoptosis in AC16 cells. Scale bars: 100 μ m. E: Immunohistochemical staining of inflammatory cytokine IL6 in PKP2^{+/-} and WT pig hearts, showing increased secretion of IL6 in PKP2^{+/-} pigs. F: Immunohistochemical staining of inflammatory cytokine IL-1 β in PKP2^{+/-} and WT pig hearts, showing increased secretion of IL-1 β in PKP2^{+/-} pigs. ns: Not significant; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; ****: $P < 0.0001$.

enriched isoform that hydrolyzes membrane phospholipids to yield lysophospholipids and free fatty acids such as arachidonic acid, thereby amplifying proinflammatory eicosanoid synthesis (Kishi et al., 2020). Notably, Group V secretory phospholipase A2 has been implicated in myocardial ischemia-reperfusion injury, showing that PLA2G5 knockout mice exhibited a 44% reduction in myocardial infarct size and a 100% improvement in fractional shortening (Yano et al., 2011). Further research has demonstrated that the phospholipase A2 inhibitor varespladib reduced atherosclerotic lesion area by 75% and improved plaque stability by increasing fibrous cap size by more than 200% in apolipoprotein E knockout mice (Shaposhnik et al., 2009). Despite these promising preclinical results, clinical trials targeting sPLA2 have failed to significantly reduce mortality or myocardial infarction rates in acute coronary syndrome, likely due to compensatory activation of parallel inflammatory pathways (Duarte, 2014). In the context of ACM, in which myocardial inflammation and ectopic lipid deposition are hallmark features, such therapeutic strategies remain unexplored. In this study, PLA2G5 overexpression in *PKP2*^{+/-} pigs was accompanied by elevated cardiac levels of IL1 β and IL6, implicating a potential role in inflammation-driven disease progression. However, it remains unclear whether PLA2G5 dysregulation is a key driver or a secondary consequence of PKP2 haploinsufficiency, which will require further research.

Previous research has demonstrated that elevated serum testosterone in males and reduced estradiol in females are independently associated with MACE in ACM and contribute directly to disease progression (Akdis et al., 2017). To mitigate potential confounding by sex hormones, this study included only male *PKP2*-mutant and WT pigs. Although this design improves internal consistency by reducing sex-based variation, it limits interpretability, as phenotypic manifestations in female *PKP2*^{+/-} pigs remain uncharacterized, thus precluding extrapolation to affected females. Additionally, this model was restricted to early-stage pathological alterations, with no longitudinal follow-up data available. Nonetheless, this large animal model offers several translational opportunities for future work, including: (1) establishing exercise safety thresholds based on cardiac remodeling dynamics; (2) accelerating gene therapy development through optimized adeno-associated viruses (AAV)-mediated delivery; and (3) validating biomarkers for clinical risk assessment.

DATA AVAILABILITY

The CT and MRI data generated in this study have been deposited in the Science Data Bank (<https://doi.org/10.57760/sciencedb.23736>). The RNA sequencing data generated in this study have been deposited in the GSA database (accession number PRJCA047620; <https://ngdc.cncb.ac.cn/gsa/browse/CRA035344>), NCBI database (GSE315016; BioProjectID PRJNA1394859), and Science Data Bank (<https://doi.org/10.57760/sciencedb.j00139.00300>).

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

H.J.W., J.P.S., and H.Y.Z. designed the research and supervised the study. H.H.L., J.X.G., T.Y.W., H.Z.W., D.L.J., K.X.X., H.Zhao., Y.B.Q., Y.H.S., B.Y.J., and H.J.W. constructed the *PKP2* mutant pigs. H.H.L., Y.C., Y.X.S.,

H.Zhang., H.M., P.Y.L., X.C., M.D.X., X.M.H., H.C., and H.J. performed the phenotype identification experiments. Y.C., H.J.W., and H.H.L. wrote the manuscript. H.J.W. and J.P.S. revised the manuscript. All authors read and approved the final version of the manuscript.

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