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ORIGINAL ARTICLE

AAV-Mediated Delivery of Plakophilin-2a Arrests Progression of Arrhythmogenic Right Ventricular Cardiomyopathy in Murine Hearts: Preclinical Evidence Supporting Gene Therapy in Humans

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BACKGROUND: Pathogenic variants in PKP2 (plakophilin-2) cause arrhythmogenic right ventricular cardiomyopathy, a disease characterized by life-threatening arrhythmias and progressive cardiomyopathy leading to heart failure. No effective medical therapy is available to prevent and arrest the disease. We tested the hypothesis that adeno-associated virus vector–mediated delivery of the human *PKP2* gene to an adult mammalian heart deficient in PKP2 can arrest disease progression and significantly prolong survival.

METHODS: Experiments were performed using a PKP2-cKO (cardiac-specific, tamoxifen-activated deletion of plakophilin-2). The potential therapeutic, adeno-associated virus vector of serotype rh.74 (AAVrh.74)-PKP2a (PKP2 variant A; RP-A601) is a recombinant AAVrh.74 gene therapy viral vector encoding the human PKP2a. AAVrh.74-PKP2a was delivered to adult mice by a single tail vein injection either before or after tamoxifen-activated PKP2-cKO. PKP2 expression was confirmed by molecular and histopathologic analyses. Cardiac function and disease progression were monitored by survival analyses, echocardiography, and electrocardiography.

RESULTS: Consistent with prior findings, loss of PKP2 expression caused 100% mortality within 50 days after tamoxifen injection. In contrast, AAVrh.74-PKP2a–mediated PKP2a expression resulted in 100% survival for >5 months (at study termination). Echocardiographic analysis revealed that AAVrh.74-PKP2a prevented right ventricle dilation, arrested left ventricle functional decline, and mitigated arrhythmia burden. Molecular and histological analyses showed AAVrh.74-PKP2a–mediated transgene mRNA and protein expression and appropriate PKP2 localization at the cardiomyocyte intercalated disc. Importantly, the therapeutic benefit was shown in mice receiving AAVrh.74-PKP2a after disease onset.

CONCLUSIONS: These preclinical data demonstrate the potential for AAVrh.74-PKP2a (RP-A601) as a therapeutic for PKP2-related arrhythmogenic right ventricular cardiomyopathy in both early and more advanced stages of the disease.

Key Words: arrhythmogenic right ventricular dysplasia ■ cardiomyopathies ■ death, sudden ■ genetic therapy ■ plakophilins

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Nonstandard Abbreviations and Acronyms

AAV	adeno-associated virus vector
AAVrh.74	adeno-associated virus vector of serotype rh.74
ACM	arrhythmogenic cardiomyopathy
ARVC	arrhythmogenic right ventricular cardiomyopathy
FB	formulation buffer
hTnT	human troponin T
LV	left ventricle
PKP2	plakophilin-2
PKP2a	plakophilin-2 transcript variant A
PKP2-ACM	plakophilin-2 arrhythmogenic cardiomyopathy
PKP2-cKO	cardiac-specific, tamoxifen-activated deletion of plakophilin-2
RV	right ventricle

Pathogenic variants in the *PKP2* gene, coding for the protein PKP2 (plakophilin-2), are the primary cause of gene-positive arrhythmogenic right ventricular cardiomyopathy (ARVC) in humans, a progressive disorder with autosomal dominant inheritance.[1,2] ARVC is a condition that falls within the umbrella term of arrhythmogenic cardiomyopathy (ACM), as defined by Towbin et al.[3] For simplicity, in the present document, we refer to PKP2-related ARVC as PKP2-ACM. The average age at first presentation is estimated between the second decade and the fourth decade, and the disease is symptomatic more commonly in males.[2,4] Pathogenic variants in PKP2 account for 20% to 45% of all cases of ACM cases, with an estimated prevalence between 1:1000 and 1:5000 in Europe and North America.[1] ARVC is characterized by a high risk of life-threatening arrhythmias before other clinical manifestations (ie, during the concealed phase of the disease), followed by a stage of clinically overt loss of myocardial mass and the presence of fibrofatty infiltrates.[2] The cardiomyopathy phenotype presents first in the right ventricle (RV), later progressing to a biventricular disease.[1,2] ARVC is a disease without a cure. Conventional heart failure therapies can be used; however, as is the case for other cardiomyopathies, they often fail to arrest disease progression and the heart advances to end-stage failure, leaving cardiac transplant as the only option to prevent death.

Adeno-associated virus vector of serotype rh.74 (AAVrh.74)-PKP2a (plakophilin-2 transcript variant A; RP-A601) is a recombinant AAVrh.74 gene therapy viral vector encoding the human PKP2a being developed to treat patients with ARVC caused by pathogenic variants in *PKP2*. The purpose of the present study was to examine the hypothesis that AAVrh.74-mediated delivery of an exogenous PKP2a gene in the setting of PKP2 deficiency in an adult mammalian heart can arrest the progression of the arrhythmogenic and cardiomyopathic components of the disease and significantly prolong life expectancy. For this purpose, we utilized a previously characterized PKP2-cKO (cardiac-specific, tamoxifen-activated deletion of PKP2).[5] Previous studies in these animals have shown that loss of PKP2 expression in adult mice leads to an ACM of RV predominance 21 days after tamoxifen injection, a decrease in left ventricular (LV) systolic function by 28 days after tamoxifen injection, and progression to end-stage heart failure and death by the sixth week after tamoxifen injection. As such, this animal model presents, in a compressed time span, the various stages of human disease.[2,5] The purpose of the present study was to assess whether AAVrh.74-mediated delivery of PKP2a in the PKP2-cKO mouse model can arrest the progression of the arrhythmia burden and cardiomyopathy components of the disease while significantly prolonging survival. These data provide preclinical support to the notion that gene replacement therapy effectively interrupts the progression of an otherwise deadly condition, paving the way for future translation to a carefully selected human patient population that could benefit from a gene therapy approach.

METHODS

An expanded methods section is provided in the Supplemental Material. The data that support the findings of this study are presented in the article and the Supplemental Material. Additional information can be made available from the corresponding author upon reasonable request. Procedures conformed with the Guide for Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the NYU-IACUC Committee (160726-03).

RESULTS

Initial experiments sought to establish that AAVrh.74-mediated PKP2a expression driven by a cardiac-selective hTnT (human Troponin T promoter; schematic presented in Figure SI) could prevent the ARVC phenotype in PKP2-cKO mice. All mice received a single tail vein injection of either AAVrh.74-PKP2a, at the specified dose, or formulation buffer (FB) alone. For the first set of experiments, all mice were then injected with tamoxifen 28 days after the injection of AAVrh.74-PKP2a or FB. Four groups of male mice, 3 to 4 months of age, were studied: (1) control (ie, PKP2 flox/flox Cre-negative) injected with FB, (2) PKP2-cKO (flox/flox Cre-positive) injected with FB, (3) PKP2-cKO injected with AAVrh.74-PKP2a at a dose of 3×10^{13} vg/kg, and (4) PKP2-cKO injected with AAVrh.74-PKP2a at a dose of 6×10^{13} vg/kg (Figure SIIA). Hearts were collected 28 days after tamoxifen injection (ie, 56 days after injection of either AAVrh.74-PKP2a or FB). The presence and abundance of adeno-associated virus vector (AAV) DNA, transgene mRNA transcripts, and PKP2 protein in

the heart lysates were determined by ddPCR, RT-ddPCR, and Western blot, respectively. Cumulative results are shown in Figure 1A and 1C. Examples of Western blots are presented in Figure SIII. Based on the ratio of mRNA to DNA copies, we found that human PKP2a expression is enriched in the heart by more than 300-fold compared with skeletal muscle and liver (Table SII). The results demonstrate efficient transduction and subsequent enriched expression of human PKP2a (hPKP2a) transgene mRNA and PKP2 protein in the heart in all AAVrh.74-PKP2a–injected mice.

Immunofluorescence images acquired from fixed sections of heart tissue are shown in Figure 2. Figure 2A shows the immunolocalization of native PKP2 in the heart of a control (Cre-negative, tamoxifen-injected) mouse. The image shows a clear immunoreactive PKP2-positive signal, visualized as well-defined plaques that align perpendicular to the direction of the fibers, as expected for an intercalated disc protein (see arrows in Figure 2A). Figure 2B and 2C shows cardiac sections of PKP2-cKO mice euthanized 28 days after tamoxifen injection and previously injected (28 days before tamoxifen injection) either with FB (Figure 2B) or with FB+AAVrh.74-PKP2a (Figure 2C). Immunolabeled PKP2 signal is notably absent in the heart of PKP2-cKO mice injected with FB alone (Figure 2B). However, dense PKP2 signals oriented perpendicular to the fiber orientation are present in the heart that received the AAVrh.74-PKP2a therapy (Figure 2C; arrows). These results were confirmed in 4 mice per group. Overall, the data show that the exogenous PKP2 gene was transcribed and translated, and the expressed protein was properly localized to the subcellular domain expected for the native PKP2 protein.

We examined whether the expression of the exogenous protein prevented the cardiomyopathic phenotype. Assessment of cardiac function in vector-treated mice by echocardiography was performed 28 days after tamoxifen injection. Figure 3 shows echocardiographic images (Figure 3A) and cumulative data (Figure 3B and 3C) obtained from the hearts of mice injected with FB alone (control, black bar; PKP2-cKO, red bar) or with AAVrh.74-PKP2a (PKP2-cKO+3×10^{13} vg/kg AAVrh.74-PKP2a, blue; PKP2-cKO+6×10^{13} vg/kg AAVrh.74-PKP2a, purple bar). As shown in Figure 2, AAVrh.74-PKP2a or FB was injected 56 days before recording and 28 days before tamoxifen injection (Figure SIIA). As expected, the loss of PKP2 expression caused a significant drop in the LV ejection fraction (red dotted line and red bar in Figure 2B) and an increase in RV area (red dotted line and red bar in Figure 2C). In contrast, AAVrh.74-mediated expression of hPKP2a mitigated or prevented the loss of contractile function in the LV and the increase in RV area in a dose-dependent manner.

Cardiac fibrosis is a common feature in PKP2-deficient hearts. As shown in Figure 4A and 4C, visualization and quantification of the extent of collagen abundance in the ventricular free walls revealed the presence of extensive fibrosis in PKP2-cKO animals (red bars), which was significantly mitigated by AAVrh.74-PKP2a administration (blue and purple bars), particularly at the higher dose (purple bar).

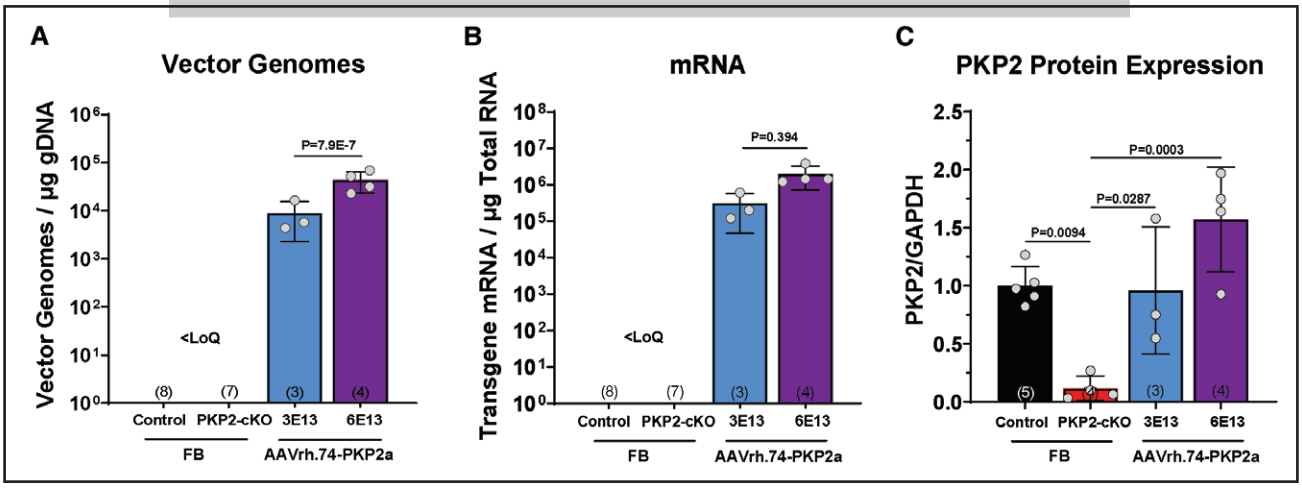

Figure 1. Adeno-associated virus vector of serotype rh.74 (AAVrh.74)-PKP2a (plakophilin-2 transcript variant A)–mediated expression in PKP2-cKO (cardiac-specific, tamoxifen-activated deletion of plakophilin-2) mouse heart.
Dose-dependent detection of (**A**) vector genomes (ddPCR), (**B**) transgene PKP2a mRNA (RT-ddPCR), and (**C**) PKP2 protein expression (Western Blot) in cardiac tissue from AAVrh.74-PKP2a–injected animals relative to controls. Data are presented as mean±SD. Black bars, control mice treated with formulation buffer (FB); red bars, PKP2-cKO mice treated with FB; blue bars, PKP2-cKO mice treated with AAVrh.74-PKP2a 3×10^{13} vg/kg; and purple bars, PKP2-cKO mice treated with AAVrh.74-PKP2a 6×10^{13} vg/kg. The number of mice studied was noted in corresponding bars (N). Data in (**A**) and (**C**) passed a test for normal distribution (Shapiro-Wilk), and significance was assessed by 1-way ANOVA followed by Tukey post hoc analyses. For (**B**), statistical significance was assessed using a nonparametric test (Kruskal-Wallis followed by Dunn post hoc analyses). Considering the small number of samples per group, the assessment of significance was repeated for data in (**A**) and (**C**) using a nonparametric test. The results are presented in Table SI. LoQ indicates less than the limit of quantitation.

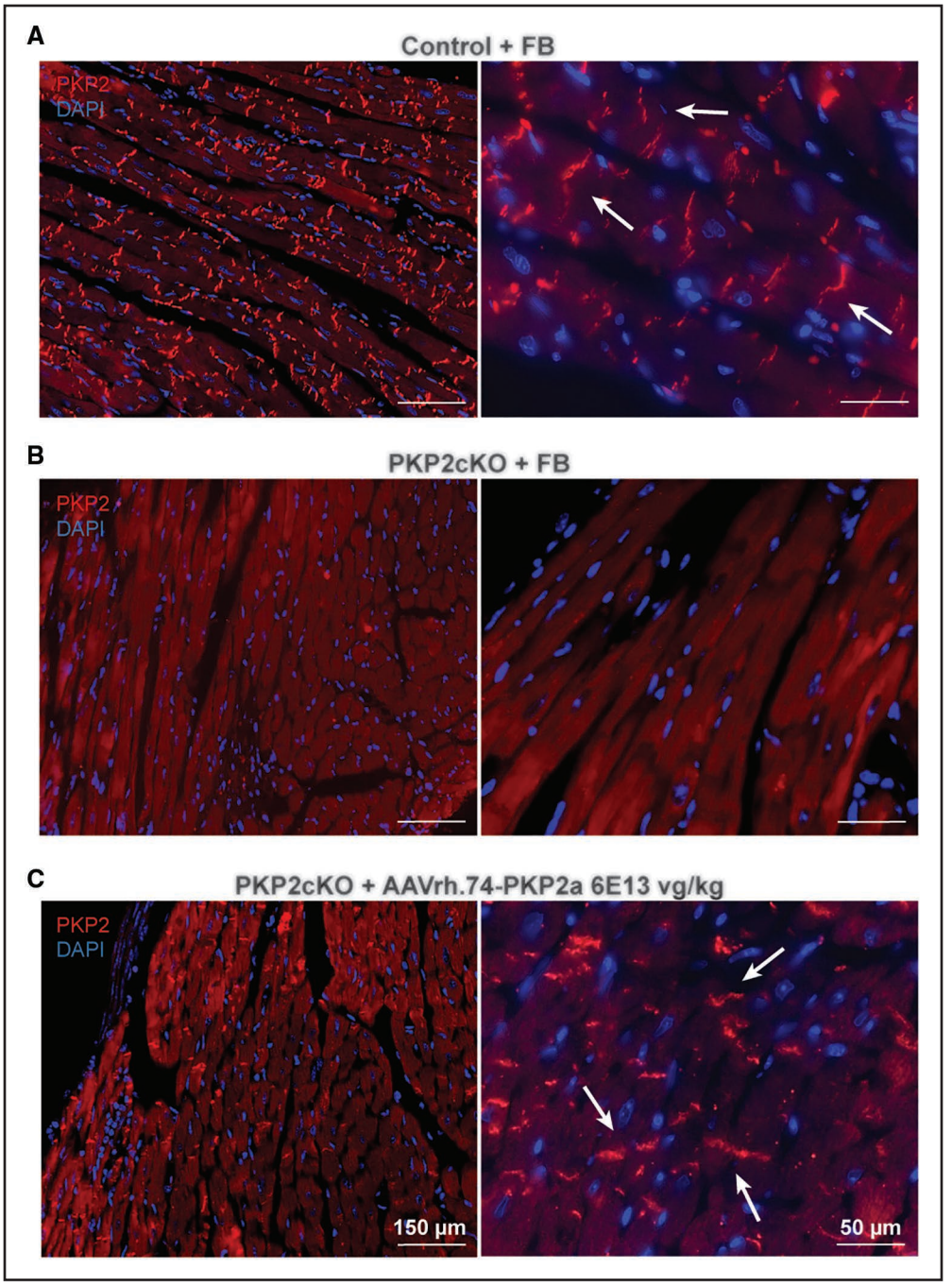

Figure 2. Cardiac expression of PKP2 (plakophilin-2) in adeno-associated virus vector of serotype rh.74 (AAVrh.74)-PKP2a (plakophilin-2 transcript variant A)–treated PKP2-cKO (cardiac-specific, tamoxifen-activated deletion of plakophilin-2) mice. Representative images of immunofluorescence staining for PKP2 (in red) and nuclei (DAPI; in blue) in hearts from (**A**) control mice treated with formulation buffer (FB), (**B**) PKP2-cKO mice treated with FB, and (**C**) PKP2-cKO mice treated with AAVrh.74-PKP2a 6×1013 vg/kg. Mice were treated with FB or AAVrh.74-PKP2a 28 days before tamoxifen injection. White arrowheads in (**A**) and (**C**) highlight PKP2 localization at the intercalated disc. Scale bar panels on the left, 150 µm. Scale bar panels on the right, 50 µm.

The data presented in the previous figures demonstrated that AAVrh.74-PKP2a treatment before tamoxifen-mediated knockout of PKP2 could mitigate the development of the cardiomyopathic phenotype in the PKP2-cKO mice. To evaluate whether AAVrh.74-PKP2a could arrest the progression of the ARVC phenotype when delivered after tamoxifen-mediated disease induction, we studied PKP2-cKO mice injected with AAVrh.74-PKP2a 7 or 14 days after tamoxifen injection (Figure SIIB) at doses of 6×10^{13} or 2×10^{14} vg/kg. In addition to echocardiography analyses, the mice were followed for long-term survival. As illustrated by the Kaplan-Meier curve in Figure 5, PKP2-cKO animals injected with FB only died between 30 and 50 days after

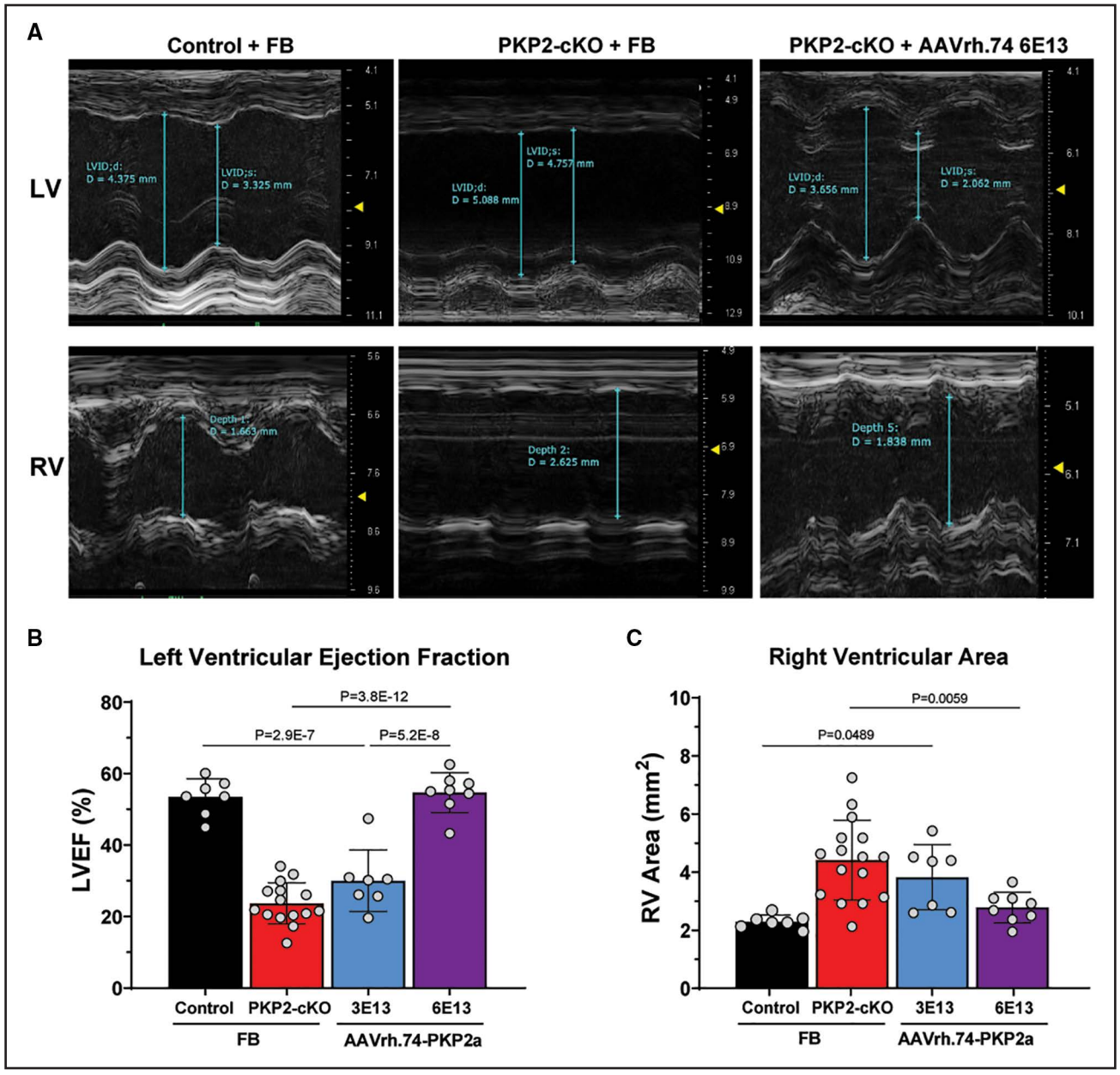

Figure 3. Cardiac contractility in adeno-associated virus vector of serotype rh.74 (AAVrh.74)-PKP2a (plakophilin-2 transcript variant A)–treated PKP2-cKO (cardiac-specific, tamoxifen-activated deletion of plakophilin-2) mice.
A, Representative images of left ventricular (LV) and right ventricular (RV) echocardiography to assess contractility in control and PKP2-cKO mice treated with formulation buffer (FB) or in PKP2-cKO mice treated with AAVrh.74-PKP2a 6×1013 vg/kg, 28 days before tamoxifen injection. Measurements were collected 28 days after tamoxifen injection. **B**, Quantification of LV ejection fraction (LVEF) measured by long-axis B-mode echocardiography and (**C**) RV area measured by modified long-axis B-mode echocardiography, as described in Cerrone et al.[5] Data are presented as mean±SD. Black bars, control mice treated with FB; red bars, PKP2-cKO mice treated with FB; blue bars, PKP2-cKO mice treated with AAVrh.74-PKP2a 3×10^{13} vg/kg; and purple bars, PKP2-cKO mice treated with AAVrh.74-PKP2a 6×10^{13} vg/kg. Data distribution passed a test of normality (Shapiro-Wilk and Kolmogorov-Smirnov), and significance was evaluated by 1-way ANOVA followed by Tukey post hoc analyses. LVID: Left ventricular internal diameter.

tamoxifen injection, consistent with previous reports.[5] In contrast, all but 1 of the mice injected with AAVrh.74-PKP2a survived for 5 months (155 days after tamoxifen injection), at which time the animals were euthanized to examine protein expression and cardiac structure. Furthermore, as shown by the representative images and cumulative data in Figure 6A and 6B, trichrome staining analyses revealed that the percent of the free wall of the LV and the RV occupied by collagen in animals injected with the higher dose of AAVrh.74-PKP2a (2×10^{14} vg/kg, orange bar) was similar (though trending toward higher values) compared with that observed in control animals injected with FB (black bar). The abundance of collagen in hearts from mice that received the lower dose of

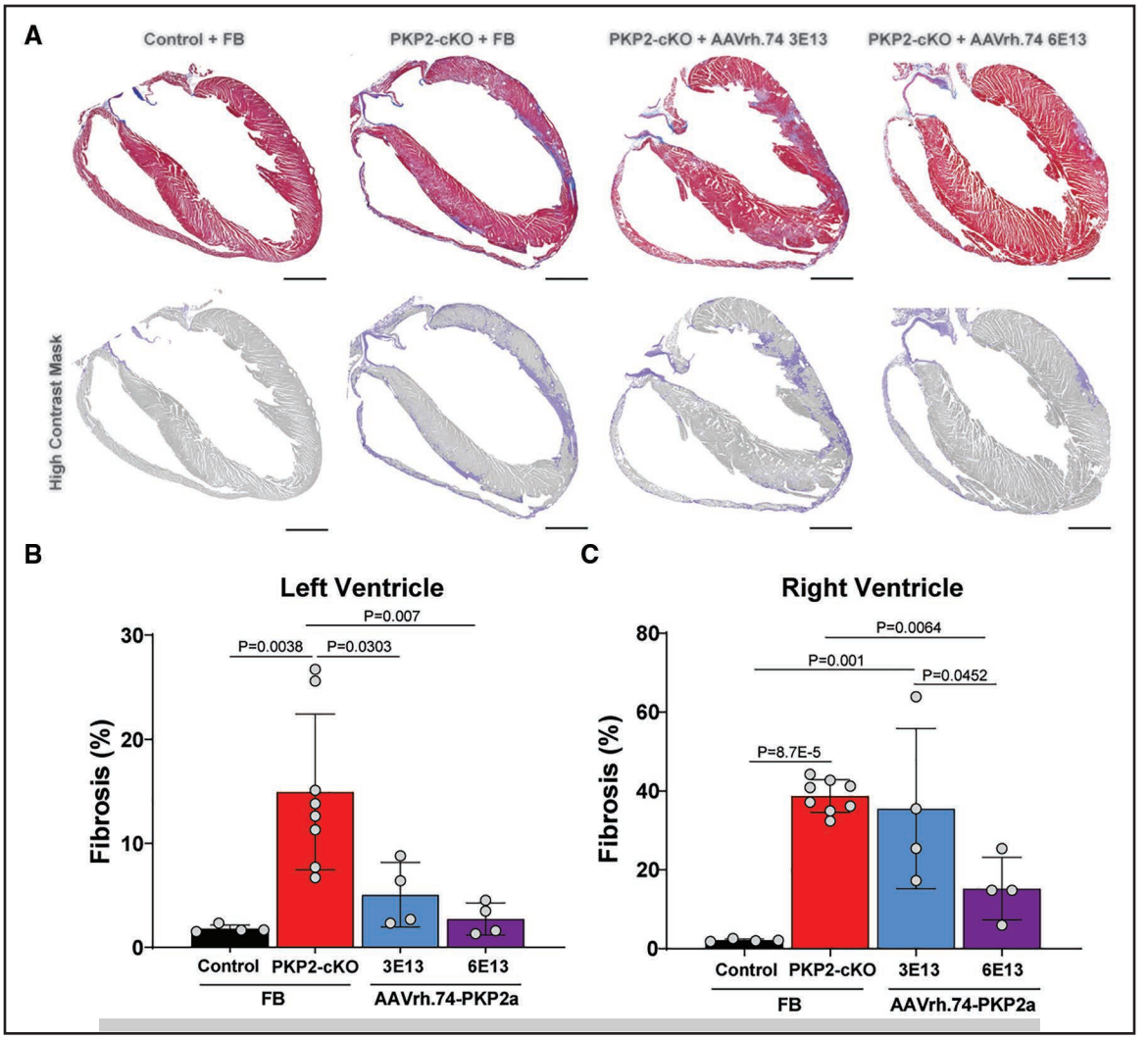

Figure 4. Cardiac fibrosis in adeno-associated virus vector of serotype rh.74 (AAVrh.74)-PKP2a (plakophilin-2 transcript variant A)–treated PKP2-cKO (cardiac-specific, tamoxifen-activated deletion of plakophilin-2) mice.
A, Top, Representative images of Masson trichrome staining of longitudinal heart sections of control and PKP2-cKO mice treated with formulation buffer (FB) and PKP2-cKO mice treated with AAVrh.74-PKP2a, 28 days before tamoxifen injection. **Bottom**, High contrast mask of the same sections emphasizing collagen deposition in blue. Scale bar, 1 mm for all images. Hearts were extracted 28 days after tamoxifen injection. **B** and **C**, Quantification of the percentage of left ventricular fibrosis (**B**) and right ventricular fibrosis (**C**) in hearts of the 4 different groups. Data are presented as mean±SD. Black bars, control mice treated with FB; red bars, PKP2-cKO mice treated with FB; blue bars, PKP2-cKO mice treated with AAVrh.74-PKP2a 3×10^{13} vg/kg; and purple bars, PKP2-cKO mice treated with AAVrh.74-PKP2a 6×10^{13} vg/kg. Data distribution passed a test for normality (Shapiro-Wilk and Kolmogorov-Smirnov tests). Assessment of statistical significance was performed using 1-way ANOVA followed by Tukey post hoc analyses. Considering the small number of samples per group, we also assessed significance via a nonparametric test (Kruskal-Wallis test, followed by the Dunn post hoc analysis). Data are presented in Table SI.

AAVrh.74-PKP2a (6×10^{13} vg/kg, purple bar) was higher than in control animals. Importantly, a comparison to collagen abundance in PKP2cKO animals at the same time point was not possible because of the early lethality in that group. However, the abundance of collagen in the AAVrh.74-PKP2a–treated animals was clearly less than what was observed in PKP2-cKO mice treated with FB at 28 days after tamoxifen injection (as presented by the red dotted line in Figure 6B) and previously published data on fibrosis in this model.[5] Finally, cumulative data obtained from echocardiographic analysis of hearts from mice injected with AAVrh.74-PKP2a 7 or 14 days after tamoxifen are presented in Figure 6C. Hearts from FB-injected PKP2-cKO mice presented a drastic reduction in LV ejection fraction (left in Figure 6C) and an increase in RV area (right in Figure 6C) 28 days after tamoxifen injection (compare red bar with black bar in Figure 6C), consistent with previous results.[5] These changes were mitigated by AAVrh.74-PKP2a, even when injected 14 days after tamoxifen, and the beneficial effects persisted up to 5 months after tamoxifen injection, the longest time point evaluated.

Previous studies have documented that a bolus injection of isoproterenol 3 mg/kg leads to premature

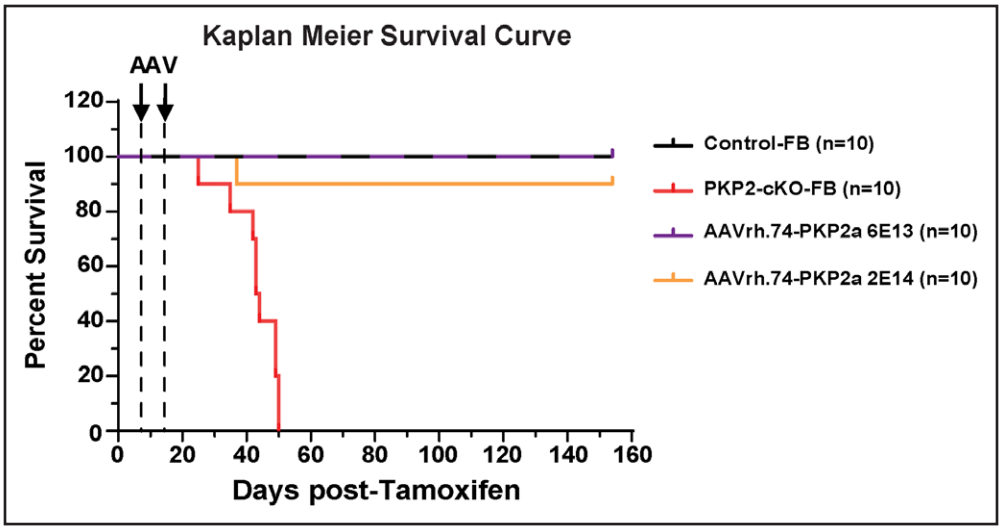

Figure 5. Survival following adeno-associated virus vector of serotype rh.74 (AAVrh.74)-PKP2a (plakophilin-2 transcript variant A) injection in PKP2-cKO (cardiac-specific, tamoxifen-activated deletion of plakophilin-2) mice at 7 or 14 days after tamoxifen injection.

The Kaplan-Meier curve depicting the long-term survival of PKP2-cKO mice following AAVrh.74-PKP2a (6×10^{13} vg/kg, 7 days after tamoxifen injection or 2×10^{14} vg/kg, 14 days after tamoxifen injection) administration compared with control and PKP2-cKO mice injected with formulation buffer (FB).

ventricular contractions in anesthetized PKP2-cKO mice 21 days after tamoxifen injection.[5,6] Therefore, we used the isoproterenol challenge protocol to determine whether AAVrh.74-PKP2a delivered 14 days after tamoxifen can mitigate arrhythmia burden in PKP2-cKO animals. Representative ECG traces are shown in Figure 7A. FB-injected PKP2-cKO animals presented multiple premature ventricular contractions, and 6 out of 10 animals showed >100 premature ventricular contractions within the 30 minutes of recording after isoproterenol injection (Figure 7B) with a total count of ≈300 premature ventricular contractions on average (Figure 7C). In contrast, both parameters of arrhythmia burden were drastically reduced by administration of AAVrh.74-PKP2a at both doses tested (6×10^{13} and 2×10^{14} vg/kg purple and orange bars, respectively, in Figure 7B and 7C). Overall, our data show that gene therapy is a viable way of limiting the impact of loss of the native PKP2 gene on cardiac mechanical and electrical functions, with no overt toxicity at clinically applicable doses.

DISCUSSION

The present study provides experimental evidence indicating that delivery of the human PKP2 gene into cardiomyocytes following administration of an AAVrh.74-PKP2a vector by a single intravenous injection can arrest the progression of an otherwise lethal ACM of RV predominance caused by loss of expression of the native PKP2 gene. Our results demonstrate that PKP2 gene therapy can drastically improve clinical outcomes in an animal model of ARVC. The results enable the potential for a cautious pursuit of studies that can determine the utility of PKP2-based gene therapy for human patients affected with ARVC consequent to PKP2 deficiency.

Early evidence that exogenous genes can be introduced into cardiac cells via viral particles with the purpose of affecting cardiac electrophysiology was provided by Leor et al[7] and later by Donahue et al.[8] Those attempts highlighted both the potential of the methodological principle and the hurdles associated with it. Bongianino et al[9] were the first to demonstrate that AAV-mediated gene delivery can restore function in an animal model of an inheritable arrhythmia disease, a demonstration later expanded upon by Liu et al.[10] These 2 proof-of-principle studies focused on rare non-RyR2 mutations causative of catecholaminergic polymorphic ventricular tachycardia. Recently though, the group of Ackerman has implemented a dual method to repress the expression of an endogenous, mutated, gene while expressing a gene with a native coding sequence.[11,12] Their experiments, using cellular models of long-QT syndrome, have been successful and provide a solid and encouraging framework for future implementation of this approach in the patient population. Of note, this method is a major breakthrough for the potential of gene therapy under conditions in which the mutated protein acts as a dominant negative component that disrupts function. Clinically relevant conditions resulting from strict loss-of-function mutations would not require repression of the native, mutated, gene for the replacement therapy approach to be successful. Any potentially translated protein fragment produced from a truncated variant of PKP2 is likely to be degraded and, therefore, unable to localize and remain in the intercalated disc[13] such that there is a low likelihood of negative competition against the therapeutic protein.

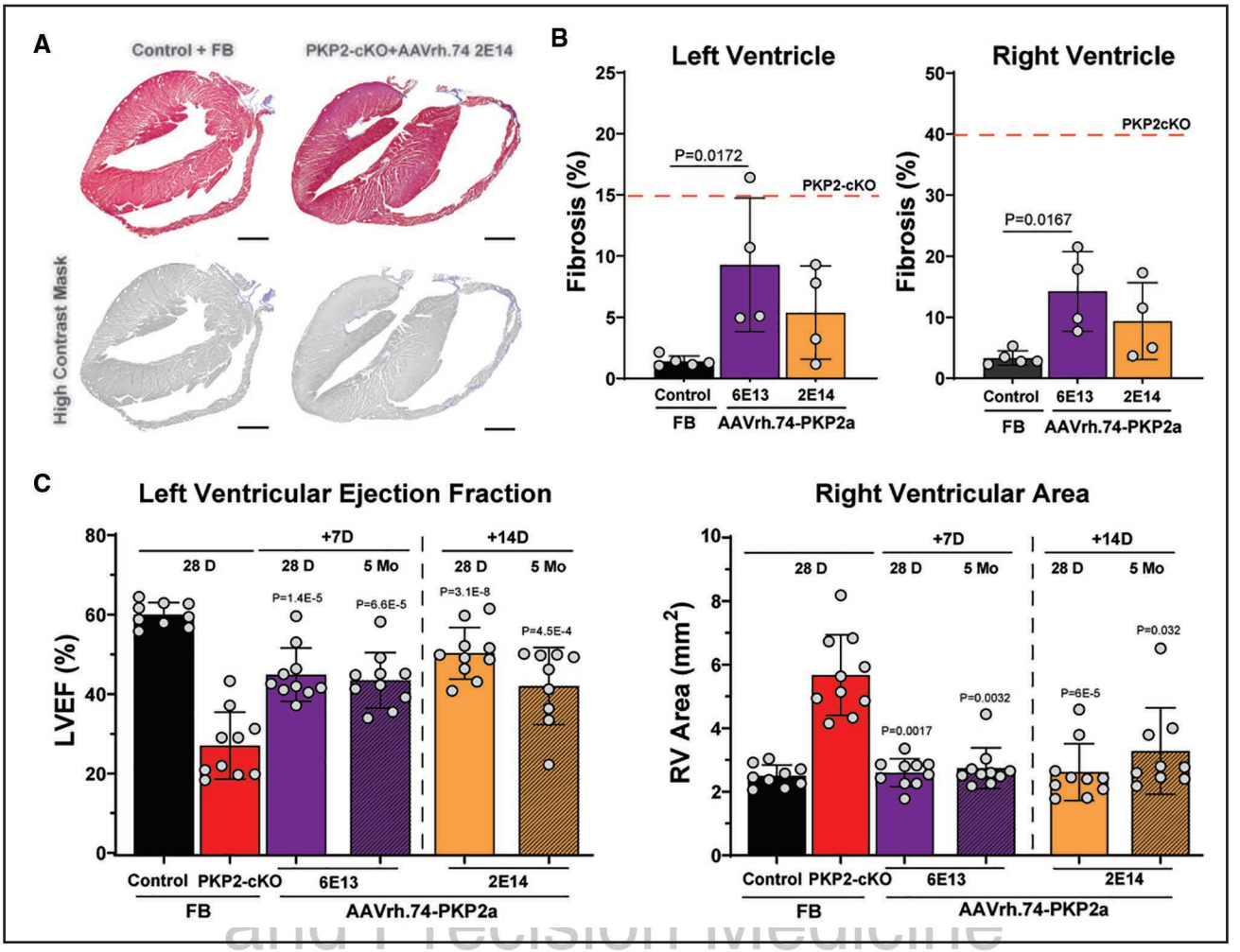

Figure 6. Disease progression in PKP2-cKO (cardiac-specific, tamoxifen-activated deletion of plakophilin-2) mice upon adeno-associated virus vector of serotype rh.74 (AAVrh.74)-PKP2a (plakophilin-2 transcript variant A) treatment at 7 or 14 days after tamoxifen injection.

A, **Top**, Representative images of Masson trichrome staining of longitudinal heart sections of control mice treated with formulation buffer (FB) and PKP2-cKO mice treated with AAVrh.74-PKP2a, 2×10^{14} vg/kg 14 days after tamoxifen injection. Hearts were extracted 5 mo after tamoxifen injection. **Bottom**, High contrast mask of the same sections emphasizing collagen deposition in blue. Scale bar, 1 mm for all images. **B**, Quantification of the percentage of left ventricular fibrosis (**left**) and right ventricular fibrosis (**right**) by Masson trichrome staining of longitudinal heart sections in control mice treated with FB and PKP2-cKO mice treated with AAVrh.74-PKP2a, 7 days (6×10^{13} vg/kg) or 14 days (2×10^{14} vg/kg) after tamoxifen injection. Hearts were extracted 5 mo after tamoxifen injection. The Red dashed line indicates the mean value of percent fibrosis recorded in PKP2-cKO mice 28 days after tamoxifen injection, injected with FB (as reported in Figure 4). Data passed the Shapiro-Wilk and Kolmogorov-Smirnov tests for normal distribution. Statistical significance was evaluated using 1-way ANOVA followed by Tukey post hoc analyses. **C**, Quantification of left ventricular ejection fraction (LVEF; **left**) and right ventricular area (RV area; **right**) across time in PKP2-cKO mice treated with AAVrh.74-PKP2a 7 or 14 after tamoxifen injection with the dose indicated at the bottom of the bars. Echocardiography was performed at 28 days and at 5 mo after tamoxifen injection. Echocardiography for control and PKP2-cKO mice treated with FB was performed at 28 days after tamoxifen injection only. Data are presented as mean±SD. Black bars, control mice treated with FB; red bars, PKP2-cKO mice treated with FB; purple bars, PKP2-cKO mice treated with AAVrh.74-PKP2a 6×10^{13} vg/kg; and orange bars, PKP2-cKO mice treated with AAVrh.74-PKP2a 2×10^{14} vg/kg. Data from the treated groups were compared with PKP2cKO-FB. Data on LVEF (left side of **C**) passed the Shapiro-Wilk and Kolmogorov-Smirnov tests for normal distribution. Significance was evaluated using 1-way ANOVA followed by the Tukey post hoc analysis. Data on the RV Area (right side of **C**) did not pass the normality test. Significance was evaluated by the Kruskal-Wallis test followed by the Dunn post hoc analyses.

Strong support for the hypothesis that AAV-mediated gene therapy may confer benefit in cardiomyopathy can be found in translational studies for Danon disease, an X-linked autophagic vacuolar myopathy, in which LAMP2B gene transfer has been found to improve metabolic and physiological functions in a preclinical animal model[14] and has subsequently been found in clinical trials to result in confirmed transgene protein expression in the heart with improvements in key cardiac biomarker and functional measures in this patient population.[15]

In the adult mammalian heart, PKP2 is densely localized in the intercalated disc, where it integrates into

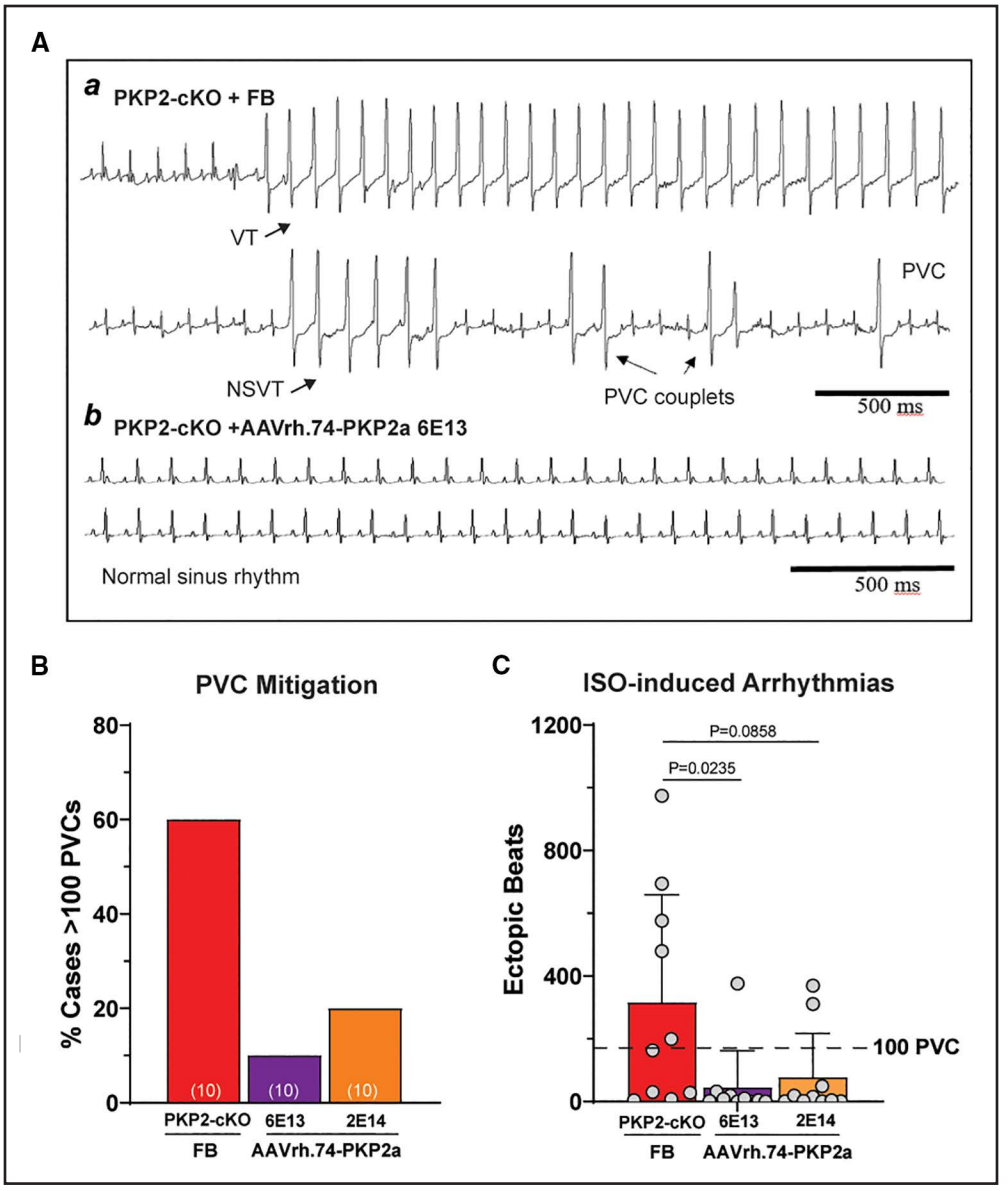

Figure 7. Isoproterenol-induced arrhythmias in PKP2-cKO (cardiac-specific, tamoxifen-activated deletion of plakophilin-2) hearts treated with adeno-associated virus vector of serotype rh.74 (AAVrh.74)-PKP2a (plakophilin-2 transcript variant A). **A**, Representative ECG traces from PKP2-cKO mice treated with (**A**) formulation buffer (FB) and (**B**) AAVrh.74-PKP2a 6×10^{13} vg/kg 14 days after tamoxifen injection. **B**, Percentage of mice that presented with >100 premature ventricular contractions (PVCs) after isoproterenol (ISO). **C**, Number of ISO-induced ectopic beats in PKP2-cKO mice treated with FB or AAVrh.74-PKP2a. The black dashed line represents the cutoff value of 100 PVCs. Data in (**B**) and (**C**) were quantified over a period of 30 min after ISO injection, and ECGs were recorded 21 days after tamoxifen injection. Data presented as mean±SD. Red bars, PKP2-cKO mice treated with FB; purple bars, PKP2-cKO mice treated with AAVrh.74-PKP2a 6×10^{13} vg/kg; and orange bars, PKP2-cKO mice treated with AAVrh.74-PKP2a 2×10^{14} vg/kg. Data did not pass the normality test. Determination of statistical significance was performed using the Kruskal-Wallis test followed by the Dunn post hoc analysis.

structural complexes necessary for intercellular adhesion (desmosomes and area composita).[16–18] Our data show that this precise localization is retained for the AAV-mediated expression of the PKP2 protein, thus indicating that trafficking mechanisms necessary for adequate localization of PKP2 to its functional site remain available for the exogenous protein. Although detailed future studies will be necessary, it is important to note that we did not observe diffuse or erroneous cellular localization of PKP2 in AAVrh74-injected PKP2-cKO mouse myocytes. The latter suggests that, at least at the doses injected, the cellular machinery was not overwhelmed by the presence of the exogenous gene and associated therapeutic hPKP2a protein expression.

Western blot analysis revealed the persistence of a light PKP2-immunoreactive band in heart lysates obtained from PKP2-cKO mice. This result, consistent with data previously published,[5] is likely consequent to the fact that PKP2 is also expressed in nonmyocyte cells in the heart such as in epicardial cells.[19] In accordance

with the latter, we did not detect PKP2 in cardiac myocytes in histological sections of hearts from PKP2-cKO mice injected with FB alone though PKP2 expression was observed in the AAV-injected animals (Figure 2).

The murine model utilized in the present study has allowed us to learn about the importance of PKP2 in the heart and its role in maintaining structural and functional homeostasis. Yet, we readily acknowledge that this is an imperfect model of disease. In fact, one could argue that none of the murine or cell-based models of desmosomal deficiency accurately recapitulates all aspects of ARVC. All of these models are, however, useful as entry points in the exploration of disease mechanisms and for the exploration of potential therapeutic strategies. Of importance is the absence of native protein in the PKP2-cKO mice versus the clinical setting in which patients often harbor a single-allele mutation of PKP2. This raises the question of whether adding copies of the full-length gene will overcome the functional deficiency associated with the presence of a pathogenic PKP2 variant in the genome of the patient. Though a firm answer will have to await an actual clinical trial, studies in experimental models provide a reason for optimism. Indeed, there is no evidence indicating that PKP2 mutant proteins can exert dominant negative functions, and murine models in which a PKP2 mutation has been knocked in strongly suggest that the resulting phenotype is consequent to the lack of the normal allele and not to a dominant effect of the mutation per se.[13,20] Additionally, a majority of mutations underlying the clinical disorder are truncating variants,[21,22] unlikely to generate deleterious aberrant proteins. Overall, the data suggest that PKP2-related ARVC is a loss-of-function disease, and exogenous introduction of the full-length gene would have the potential to address the loss of function. Importantly, AAV-mediated delivery of the PKP2 gene in a normal murine heart did not lead to a pathogenic phenotype,[23] thus addressing concerns about whether excessive expression of PKP2 may have adverse consequences in the treated patient population.

An additional limitation in our study is the fact that we only modify PKP2 expression in cardiac myocytes; yet, in humans, the PKP2 mutation would be present in all PKP2-expressing cells. Importantly, in humans, the heart is the only affected organ. It is theoretically possible that nonmyocyte cardiac–resident (or cardiac-interacting) cells may play a role in ARVC pathogenesis. Nonetheless, animal models of desmosomal deficiency have not provided strong evidence for the potential of other cell types to generate the full cardiomyopathic and arrhythmogenic phenotypes, and studies on human-induced pluripotent stem cell-derived epicardial cells[24] have not been confirmed in disease models involving fully differentiated adult hearts. Furthermore, even the inflammatory component of the disease, originally thought of as a consequence of the interaction with nonmyocyte cells, has been shown to originate, at least in part, in the myocytes themselves.[25,26] Overall, the data indicate that PKP2 deficiency in fully differentiated cardiomyocytes is a necessary condition for the disease phenotype.

All limitations notwithstanding, our results support the notion that gene therapy may be effective in treating PKP2-related ARVC in appropriately selected patients. It is likely that a future clinical trial will exclude patients with antibodies against the AAV serotype used to deliver the gene.[27] In the present study, we used an AAVrh.74. This vector, originally isolated from rhesus monkeys, effectively transduces myocytes.[28] Recent results (albeit preliminary in the context of all populations) suggest that preexisting antibodies to AAVrh.74 may be present at a relatively low prevalence in the human population.[29] More data will be necessary to determine if the use of AAVrh.74 increases the pool of candidates for gene therapy based on serotype and immunogenic potential. Yet, the successful results with AAVrh.74 in the present preclinical study support its implementation in a future clinical trial.

We observed a functional dose-dependence, whereby 3×10^{13} vg/kg of AAVrh.74 PKP2a restored protein expression, but full functional restoration required a higher dose. It is possible that the protein synthesis and trafficking of the exogenous PKP2 are less effective versus that of the native gene and that although a lower dose may yield an identifiable Western Blot band and immunofluorescence-detected spots at the intercalated disc, protein production may still be below the level of critical abundance necessary to completely restore function. Notably, the identified effective doses are consistent with those that can be clinically administered. Furthermore, the dose required to mitigate the disease phenotype in this homozygous mouse model may require transgene protein levels exceeding those required to ameliorate the disorder arising from monoallelic mutations. Based on the data from this study and additional formal safety/toxicology studies, it is generally anticipated that clinically relevant doses for AAVrh74-PKP2a will be in the high 10^{13} or low 10^{14} vg/kg range.

ARVC is a disease with incomplete penetrance.[2] While significant progress has been made in risk stratification of patients suspect of ARVC,[30–32] it remains unpredictable whether or when the disease will present in an asymptomatic gene carrier.[2] As such, any future implementation of gene therapy in PKP2 pathogenic variant carriers will likely not be in an asymptomatic individual as a preventive measure, but as a therapeutic procedure for a patient with an overt disease. In that regard, we note that gene replacement in our model was effective even when the AAVrh.74-PKP2a was injected 14 days after tamoxifen injection, a time point at which no PKP2 can be detected in the cardiomyocytes and an increase in RV area can already be detected by echocardiography.[5] It is also important to highlight that the reintroduction of the PKP2 gene arrested the progression of the

cardiomyopathy but did not revert the functional damage. This is consistent with the notion that gene therapy in cardiomyopathy associated with loss of muscle mass (such as ARVC) would not be expected to restore the muscle mass but only to prevent further loss as the disease progresses.

Recent studies have highlighted the importance of an inflammatory and innate immune response in PKP2-deficient hearts and myocytes.[25,33] We speculate that as the AAVrh.74-mediated delivery of PKP2 arrests disease progression, it may also arrest the inflammatory process, but additional experiments will be necessary to address this important question.

The present study focused on PKP2, the desmosomal gene most commonly associated with a gene-positive ACM of RV predominance. Recent studies suggest that the disease process consequent to variants in other desmosomal genes may follow a course different from that of PKP2, making it attractive to move to a nomenclature system in which the affected gene is represented in the name of the disease (eg, a desmoplakin cardiomyopathy[34]). Along those lines and to emphasize the heavy arrhythmogenic component that is often present in patients with PKP2 pathogenic variants, we propose to use the term PKP2-ACM as an entity of its own, one that, among the desmosomal cardiomyopathies, offers a feasible target for gene therapy.

In conclusion, we have conducted proof-of-principle preclinical studies to determine the potential for gene therapy in the setting of an ACM associated with PKP2 loss of function in the murine heart. Our results indicate that a single intravenous injection of AAVrh.74-PKP2a delivering the PKP2 gene leads to the production and proper localization of PKP2 in adult cardiac myocytes, arrests the progression of the disease, and drastically shifts survival, from a condition leading to 100% lethality to 100% survival in the treated population. Our data represent 1 component of the necessary body of evidence to support the implementation of gene therapy in a carefully selected fraction of patients. While the treatment was well tolerated and effective in the experimental model, data from extensive preclinical safety studies are also needed to ensure safe and successful implementation in the treatment of patients in need.

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