

AAV9-Mediated MYBPC3 Gene Therapy with Optimized Expression Cassette Enhances Cardiac Function and Survival in MYBPC3 Cardiomyopathy Models

Corresponding Author: Dr Laura Lombardi

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

General Comments:

The manuscript "Gene Therapy Mediates Therapeutic Improvement in Cardiac Hypertrophy and Survival in a Murine Model of MYBPC3-Associated Cardiomyopathy" by Xu et al. is a report from a biopharmaceutical firm that provides a detailed description of the pre-clinical evaluation of a gene therapy suitable for the treatment of MYBPC3-associated cardiomyopathy. The scientific approach is well-reasoned, the results are convincing and the report is well-written. Perhaps the most challenging question for the Editors is whether the current report represents a significant advance over previously-published studies using AAV9 to deliver gene therapy in human iPSC and/or in vivo murine models of MYBPC3-associated cardiomyopathy. On one hand, this report contains a complementary mix of experiments conducted in human MYBPC3-/- iPSC-CMs and in a rigorous mouse model of MYBPC3-associated cardiomyopathy. It also appears to be the most promising report to date with regard to therapeutic efficacy per unit dose AAV. Another unique feature is that it tackles the important question of whether mouse vs. human cDNA for MYBPC3 might provide divergent results in a mouse knockout model. On the other hand, it could be argued that the methodologic improvements are modest, and that similar work has been published previously, although separately, in both human iPSC-CMs and rodent models of MYBPC3 deficiency.

Specific Comments:

1) Page 1, lines 44-45: Please consider revising the first sentence of the Introduction: "Mutations in MYBPC3, the gene encoding cardiac myosin-binding protein C (MYBPC3 44 or cMyBP-C), are the most common genetic cause of HCM, an autosomal dominant condition¹." First, it is not entirely clear whether "autosomal dominant" refers specifically to MYBPC3 deficiency or HCM in general. Second, the content of Reference 1 does not directly address "autosomal dominant". Perhaps a more nuanced alternative would read something like: "Although HCM often demonstrates an autosomal dominant pattern of inheritance, many cases of non-familial HCM have been documented (PMID: 37301765). Variable penetrance and the potential for compound heterozygotes with pathogenic mutations in multiple sarcomeric genes can also lead to complex patterns of inheritance at the phenotypic level."

2) Page 4, lines 77-79: Please consider revising the following sentences: "Robust transgene expression is often dependent on the inclusion of two intact flanking ITR sequences, a promoter, intron, WPRE, and polyadenylation signal. Thus, standard cis-regulatory sequences used in AAV transgene expression cassettes amount to ~1.2 kb without the promoter." First, the literature does not support the contention that robust transgene expression is dependent on an intron or a WPRE. In fact, only a small minority of published AAV vectors contain either a WPE or an intron, and almost none contain both. An alternative approach might go something like: "Robust transgene expression from AAV transgene expression cassettes minimally requires the inclusion of two intact flanking ITR sequences, a promoter, a cDNA, and a polyadenylation signal. Accessory elements such as an intron or a WPE (intended to enhance expression) can bring the total up to ~1.2 kb in cis-regulatory sequences, even without the promoter."

3) Page 5, lines 100-101: Recommend adding the rationale for promoter selection between lines 100 and 101. Something like: "The different cardiac-specific promoters were all variations on the human cardiac troponin T (TNNT2) promoter used in

previous studies of cardiac-targeted gene therapy for MYBPC3 deficiency (34, 51, PMID 31796859)."

4) Page 8, lines 171-173: Recommend modifying the current sentence as follows to acknowledge closely related work: "To our knowledge, this experiment represents the first in vivo assessment of the human MYBPC3 activity in a mouse model, although the AAV-mediated expression of human MYBPC3 cDNA has previously been assessed in human iPSC-CMs (PMID: 28624223)."

5) Page 14, line 322: Upon introducing the "published" 5.4 kb cassette, the source where it was obtained should be also be disclosed (i.e., "kindly provided by XXX").

6) Page 18, line 397: It is recommended that the authors devise a clearer method for separating reference numbers from strain designations. IE, as written, it is not immediately clear that 46 is a reference for MyBP-C3t/t. Nor is it clear that 42 is a reference for cMyBP-C-/-.

7) Page 28, line 700: Upon first use, please define "vg/dg".

Reviewer #2

(Remarks to the Author)

This is a study of Tenaya product TN-201, an AAV9-based gene therapy for Mybpc3-deficient hypertrophic cardiomyopathy (HCM). A single dose of AAV9 encoding murine Mybpc3 in Mybpc3-deficient mice convincingly improved survival, cardiac systolic/diastolic function, and hypertrophy in a dose-dependent manner for out to 13 months. Sustained cardiac transgene expression (RNA and protein) was observed 20 months following gene therapy. This study underpins the recent approval for the use of TN-201 in clinical trials.

The main advance on a previous study using AAV-based gene replacement of Mybpc3 in a murine model of HCM (Nat Commun. 2014;5:551) is the refinement of the vector expression cassette to contain a minimal promoter and cis-regulatory elements to improve vector packaging and performance.

Comments:

The characterisation of the expression cassette design for superiority of TN-201 is scientifically superficial and does not readily support the conclusions made by the authors.

The authors need to show effects of deleting WPRE in a vector and cell specific context. For example: comparing the performance of AAV with card 0-2 promoters vs cardiac TnT promoter in relevant cell types (iPSC-CMs and cardiac fibroblasts) to be conclusive about omitting WPRE without consequence. A single or even two immunoblots is non-quantitative and inconclusive. It is unclear why these important studies were performed in cardiac fibroblasts.

Without knowing the patent disclosing the card family of promoters, it is difficult to assess the rationale behind their design and not possible to reproduce these studies. The authors need to give the sequence and maps (in the paper) of the promoter sequences used. A figure comparing promoters would be very helpful and orienting.

The assertion of improved packaging with the smaller expression cassette is plausible but needs to be addressed empirically. For example, were there differences in the vector titre achieved or the ratio of empty vs full capsids? Were the packaged AAVs with the smaller cassette sequences less prone to genome heterogeneity (see PMID: 35293222)?

Please elaborate on the AAV9 variant CR9-01: identity, reference and give reason why it performs better than wtAAV9 in human iPSCs.

Figure 1/S2. The sample size is remarkably small (n=1-3) for in vitro studies given the aim of this study was "to engineer an optimal AAV genomic cassette". It is not clear what 1K vs 3K is and the significance of the difference in results. Furthermore, the purity/heterogeneity of the iPSCs has not been quantified. What is the proportion of cardiomyocytes/other cell types and are the transduced GFP cells also the cardiomyocytes? This could easily be addressed using flow cytometry. As you move from 1K to 3K the number of positive cells does not increase substantially. Is transduction limited to a subset of cell types? This needs to be explored further given the importance of expression cassette design as the major novel development of this study.

Regarding the in vivo studies, the lack of a control vector such as AAV9 with a reporter or stuffer sequence is a major scientific limitation in this study. There is no control for the effects of the AAV capsid proteins or other components of the vector expression cassette on myocytes/non-myocytes following transduction.

Figure 3. Mouse vs human Mybpc3 – were there differences in the protein expression levels or subcellular localisation in the treated mouse hearts that could explain the transgene species difference in therapeutic effect?

Figure 4: As above: was there a relationship between Mybpc3 protein level/localisation and the size of the therapeutic effect for 1x10e3 vg/kg vs the two higher doses.

Figure 7; Given the disconnect between Mybpc3 RNA and protein (demonstrated in this paper) it would be more relevant to look at protein expression in these tissues to correlate level of transduction with size of therapeutic effect.

Could the author please compare/contrast their study to prior research exploring gene therapy for Mybpc3-deficient HCM.

Reviewer #3

(Remarks to the Author)

The manuscript by Greer-Short, Lombardi et al. describes the generation and validation of a novel AAV vector carrying a MYBPC3 transgene, to be developed for the treatment of HCM caused by haploinsufficiency-mutations of the MYBPC3 gene. The novel viral vector (TN-201) was tested in a transgenic MYBPC3-deficient mouse (MYBPC3-KO), which display a remarkable cardiomyopathy phenotype. Interestingly, the treatment led to a drastic reduction of the structural and functional expression of cardiomyopathy in treated transgenic mice, as evaluated with a large number of in vivo and in vitro investigations. The authors compared their viral vector (TN-201) with the construct that has been employed in a previously published study by L. Carrier et al., and found that their viral therapy is associated with a better outcome and improved cardiac function, when compared with the previous construct. The paper is remarkably well written, clear and comprehensive and the study has been conducted with exceptional care and precision. The results leave no doubt whatsoever on the very high efficiency and effectiveness of their viral vector. My only concerns are regarding the experimental model.

In general, mice are poor models for HCM in general, due to the different expression of myofilament protein isoforms in their ventricles. In particular, protein C mutations behave very differently in mice as compared with humans. While human disease is evident when MyBPC levels fall down to 50% of normal level (as a consequence of haploinsufficiency-mutations in heterozygosity), mice only show a phenotype when there is a complete lack of protein (i.e. in homozygosity). MYBPC3 homozygous mutations are not impossible in humans and are known to lead to a devastating disease leading to demise in the first months of life. Nonetheless, a viral gene-transfer therapy, such as the one tested here, would be clinically relevant if proven to be useful in a model carrying a MYBPC3 mutation in heterozygosity. Many works from several groups highlighted the high predictive value of cardiomyocytes and engineered tissues obtained through differentiation from patient-specific or CRISPR-mutated human induced pluripotent stem cells, as well-representative in vitro models of human HCM. Indeed, the authors of this paper have used hiPSC-CMs to test their construct. But did not test their viruses in hiPSC-CMs obtained from a patient carrying a MYBPC3 mutation in heterozygosity. Remarkably, a recent paper (Pioner et al., Circ Res 2023) showed that patient-specific hiPSC-derived cardiomyocytes and engineered heart tissues recapitulate the functional abnormalities found in human myocardium obtained from surgical samples of the same patient.

All in all, I believe that the authors should perform some tests demonstrating the efficacy of their approach in human iPSC-CMs carrying a heterozygous MYBPC3 mutation, showing that the treatment leads to restoration of a normal MyBPC protein expression and to a remarkable decrease of the typical functional abnormalities observed in mutated cells (that is, prolonged action potentials, altered calcium cycling, altered cell dimensions).

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have been responsive to review, particularly in clarifying the novelty of their experiments. The addition of the GFP control group in Fig S4 also strengthens the manuscript. However, the addition of the new "Ethical Statement" on page 21 prompts a suggestion. For consistency, one might first recommend making the statements in the main article and the supplemental methods identical; or perhaps deleting the copy in the supplemental methods. Second, it is noted that neither statement directly references the NIH Guide. If the following statement holds true, the authors might consider using it instead: "All animal experiments were pre-approved by the Institutional Animal Care and Use Committee of Tenaya Therapeutics (AUP: 2020.007 and 2023.007) and were performed in compliance with the "Guide for the Care and Use of Laboratory Animals, 8th edition", published by the US National Institutes of Health (ISBN-10: 0-309-15400-6)."

Another suggestion involves the "Editorial Policy Checklist" and Nature's adoption of the ARRIVE 2.0 guidelines. These guidelines recommend that the sex of the animals be disclosed along with the animal numbers as shown in Subitem 3c—Example 1 at <https://doi.org/10.1371/journal.pbio.3000411>. For example: "Group F contained 29 adult males and 58 adult females (n = 87)." It is not immediately clear whether this is an absolute requirement, but it is noted that the sex of the mice is never mentioned in the current manuscript. If all the mice were of the same sex, this should certainly be disclosed. If not, it is acknowledged that it would be cumbersome to describe the sex of every last mouse. However, the authors might consider doing so for all groups in the functional studies (ie, n=5 or more).

Finally, the authors are encouraged to be consistent about where and how they disclose the animal numbers. As indicated in the summary below, most animal numbers are indicated in the Figure legends, except for Figure 3, where the legend lacks any mention of "N" and the number is instead disclosed in the text of the main article:

Article:

Fig. 2: WT (n = 10), Mybpc3+/- (n = 7) and Mybpc3-/- (n = 12)
Fig. 3: Fig legend lacks any mention of "N". Text says (9-12/group)
Fig. 4: WT (n = 7), Mybpc3-/- Veh (n = 7), Mybpc3-/- 1E13 vg/kg (n = 4), Mybpc3-/- 3E13 vg/kg (n = 6), and Mybpc3-/- 1E14 vg/kg (n = 6)
Fig. 6: n = 9/group
Fig. 7: n = 7-10 mice/group
Fig. 8b and 8c: n = 6-8 mice/group
Fig. 8d and 8e: n = undefined / group
Fig. 9a and 9b: n = 8/group
Fig. 9c and 9d: n = 15-16/group
Fig. 9e and 9f: n = 8-10/group
Fig. 9g and 9h: n = 7-8/group

Supp Material:

Fig S1d (n = 5/group)
Fig S4: WT (n=6), Vehicle, Veh (n=9), AAV9:pCard1-GFP (n=9)
Fig S6: Vehicle, Veh (n=6), 3E13 vg/kg (n=5) and 3E14 vg/kg (n=6)

Reviewer #2

(Remarks to the Author)

The authors have satisfactorily addressed my previous comments. I have no new comments to make.

Reviewer #3

(Remarks to the Author)

I thank the authors for considering my comments and observations. The authors have addressed all my comments and requests in a very satisfactory manner. I believe that the many changes and amendments introduced by the authors have significantly improved the manuscript in terms of completeness, clarity and translational value. I have no additional comments or observations.

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Thank you so much for your consideration and your thorough and thoughtful assessment of our manuscript reporting the development of our gene therapy for patients with MYBPC3 mutations. Addressing your comments has vastly strengthened our paper.

We have generated the following key additional data:

1) To control for the effect of the AAV capsid proteins and other components of the vector expression cassette on the heart, AAV9 with the same cassette but encoding GFP (pCard1-GFP) was injected into KO mice at the efficacious dose of 3×10^{13} vg/kg. In contrast to AAV9:mMybpc3, echocardiographic and electrocardiographic assessments six weeks post-injection demonstrated no significant benefit of animals dosed with AAV9:pCard1-GFP compared to Vehicle-treated animals (New Fig. S4). This clearly shows that any beneficial effect observed is derived from the transgene itself.

2) We interrogated the utility of TN-201 in the heterozygous context using human iPSC-CMs. Despite the well-established variable penetrance of haploinsufficiency and contractile abnormalities in MYBPC3^{+/-} iPSC-CMs, we were able to demonstrate normalization of calcium handling abnormalities and relaxation defects in heterozygous iPSC-CMs upon transduction (New Fig. 9).

3) By performing additional reviewer-requested experiments, such as denaturing assessment of viral genomes, we were able to determine that the increased expression driven by pCard1-MYBPC3 virus appears to be due to a combination of increased potency of the cassette DNA sequence itself and less heterogeneity of viral packaging (New Fig. S2), strengthening the foundations of our claims.

The recommended changes to the text and figures suggested by you have enhanced the manuscript and clarified several key points to strengthen the conclusions drawn from our data.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

General Comments:

The manuscript "Gene Therapy Mediates Therapeutic Improvement in Cardiac Hypertrophy and Survival in a Murine Model of MYBPC3-Associated Cardiomyopathy" by Xu et al. is a report from a biopharmaceutical firm that provides a detailed description of the pre-clinical evaluation of a gene therapy suitable for the treatment of MYBPC3-associated cardiomyopathy. The scientific approach is well-reasoned, the results are convincing and the

report is well-written. Perhaps the most challenging question for the Editors is whether the current report represents a significant advance over previously-published studies using AAV9 to deliver gene therapy in human iPSC and/or in vivo murine models of MYBPC3-associated cardiomyopathy. On one hand, this report contains a complementary mix of experiments conducted in human MYBPC3^{-/-} iPSC-CMs and in a rigorous mouse model of MYBPC3-associated cardiomyopathy. It also appears to be the most promising report to date with regard to therapeutic efficacy per unit dose AAV. Another unique feature is that it tackles the important question of whether mouse vs. human cDNA for MYBPC3 might provide divergent results in a mouse knockout model. On the other hand, it could be argued that the methodologic improvements are modest, and that similar work has been published previously, although separately, in both human iPSC-CMs and rodent models of MYBPC3 deficiency.

Thank you for your thorough, insightful and fair assessment. The largest distinction between our work and that of other key contributors in the field is that our mouse AAV studies are performed on older mice with established cardiac disease symptoms (rather than perinatally, or virus injected into pups ≤ 36 hours of age PMID: 25463264, 32750038, 37067542). Demonstration of functional improvement upon dosing compared to symptomatic baseline, rather than prevention of decline upon presymptomatic dosing, is essential to vetting the potential clinical benefit of gene therapies. This had previously only been demonstrated with lentivirus (PMID: 22855556), which is of limited clinical relevance, or genetically using re-expression with an inducible TetOff system (PMID: 30573635). Thanks to your feedback, a sentence has been added to the Discussion section to highlight this advance. Further, as you note, the increased potency of our gene therapy enables restoration of wildtype protein levels unlike previous studies as recent as 2023 (PMID: 25463264, 32750038, 37067542) and at the clinically tenable dose of 3×10^{13} vg/kg.

Specific Comments:

1) Page 1, lines 44-45: Please consider revising the first sentence of the Introduction: "Mutations in MYBPC3, the gene encoding cardiac myosin-binding protein C (MYBPC3 44 or cMyBP-C), are the most common genetic cause of HCM, an autosomal dominant condition¹." First, it is not entirely clear whether "autosomal dominant" refers specifically to MYBPC3 deficiency or HCM in general. Second, the content of Reference 1 does not directly address "autosomal dominant". Perhaps a more nuanced alternative would read something like: "Although HCM often demonstrates an autosomal dominant pattern of inheritance, many cases of non-familial HCM have been documented (PMID: 37301765). Variable penetrance and the potential for compound heterozygotes with pathogenic mutations in

multiple sarcomeric genes can also lead to complex patterns of inheritance at the phenotypic level."

Thank you for this excellent feedback. Your point is well-taken. We have removed the phrase "autosomal dominant condition" in that sentence in favor of a subsequent sentence where it is clearly describing MYBPC3-associated HCM.

2) Page 4, lines 77-79: Please consider revising the following sentences: "Robust transgene expression is often dependent on the inclusion of two intact flanking ITR sequences, a promoter, intron, WPRE, and polyadenylation signal. Thus, standard cis-regulatory sequences used in AAV transgene expression cassettes amount to ~1.2 kb without the promoter." First, the literature does not support the contention that robust transgene expression is dependent on an intron or a WPRE. In fact, only a small minority of published AAV vectors contain either a WPE or an intron, and almost none contain both. An alternative approach might go something like: "Robust transgene expression from AAV transgene expression cassettes minimally requires the inclusion of two intact flanking ITR sequences, a promoter, a cDNA, and a polyadenylation signal. Accessory elements such as an intron or a WPE (intended to enhance expression) can bring the total up to ~1.2 kb in cis-regulatory sequences, even without the promoter."

This is an excellent point. We have made your requested revisions: "Transgene expression is minimally dependent on the inclusion of two intact flanking ITR sequences, a promoter, and polyadenylation signal. With additional accessory elements such as an intron and WPRE, standard cis-regulatory sequences used in AAV transgene expression cassettes can amount to ~1.2 kb without the promoter."

3) Page 5, lines 100-101: Recommend adding the rationale for promoter selection between lines 100 and 101. Something like: "The different cardiac-specific promoters were all variations on the human cardiac troponin T (TNNT2) promoter used in previous studies of cardiac-targeted gene therapy for MYBPC3 deficiency (34, 51, PMID 31796859)."

Thanks, we have added citations to manuscripts using sequences from the human promoter. The Stelzer lab constructs are based on the chicken gene sequence.

4) Page 8, lines 171-173: Recommend modifying the current sentence as follows to acknowledge closely related work: "To our knowledge, this experiment represents the first in vivo assessment of the human MYBPC3 activity in a mouse model, although the AAV-mediated expression of human MYBPC3 cDNA has previously been assessed in human iPSC-CMs (PMID: 28624223)."

Added, thanks.

5) Page 14, line 322: Upon introducing the “published” 5.4 kb cassette, the source where it was obtained should be also be disclosed (i.e., “kindly provided by XXX”).

This cassette was independently synthesized from the published patent and sequence verified.

6) Page 18, line 397: It is recommended that the authors devise a clearer method for separating reference numbers from strain designations. IE, as written, it is not immediately clear that 46 is a reference for MyBP-C3t/t. Nor is it clear that 42 is a reference for cMyBP-C-/-.

Thanks, we have added a space to increase readability.

7) Page 28, line 700: Upon first use, please define “vg/dg”.

Done, thanks.

Reviewer #2 (Remarks to the Author):

This is a study of Tenaya product TN-201, an AAV9-based gene therapy for Mybpc3-deficient hypertrophic cardiomyopathy (HCM). A single dose of AAV9 encoding murine Mybpc3 in Mybpc3-deficient mice convincingly improved survival, cardiac systolic/diastolic function, and hypertrophy in a dose-dependent manner for out to 13 months. Sustained cardiac transgene expression (RNA and protein) was observed 20 months following gene therapy. This study underpins the recent approval for the use of TN-201 in clinical trials.

The main advance on a previous study using AAV-based gene replacement of Mybpc3 in a murine model of HCM (Nat Commun. 2014;5:551) is the refinement of the vector expression cassette to contain a minimal promoter and cis-regulatory elements to improve vector packaging and performance.

Comments:

The characterisation of the expression cassette design for superiority of TN-201 is **scientifically superficial** and does not readily support the conclusions made by the authors.

Thank you very much for your thoughtful and thorough feedback. Thanks to your critical feedback, we performed multiple additional experiments and determined that indeed the

increased expression of the pCard1 virus appears to be due to a combination of increased potency of the cassette DNA sequence itself and less heterogeneity of viral packaging.

Consistent with superior expression, the increased potency of our gene therapy enables restoration of wildtype protein levels unlike previous works as recent as 2023 (PMID: 25463264, 32750038, 37067542) and at the clinically tenable dose of 3E13 vg/kg.

The authors need to show effects of deleting WPRE in a vector and cell specific context. For example: comparing the performance of AAV with card 0-2 promoters vs cardiac TnT promoter in relevant cell types (iPSC-CMs and cardiac fibroblasts) to be conclusive about omitting WPRE without consequence. A single or even two immunoblots is non-quantitative and inconclusive. It is unclear why these important studies were performed in cardiac fibroblasts.

Our apologies for the confusion. All studies were performed in iPSC-CMs or cardiac tissue with the exception of the original content of Figure S1. Thanks for highlighting this, as we have indeed gone on to show that there are no detrimental effects of WPRE deletion in the context of this cassette in vivo in mouse cardiac tissue based on GFP protein expression (n = 5/group). This has now been added as Fig. S1c&d.

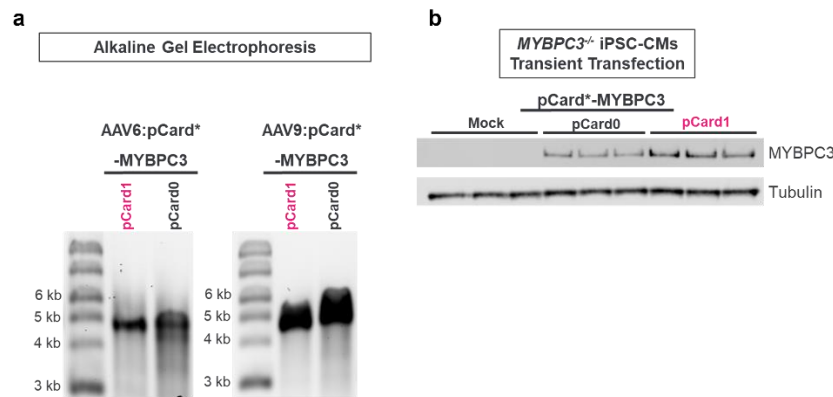
Without knowing the patent disclosing the card family of promoters, it is difficult to assess the rationale behind their design and not possible to reproduce these studies. The authors need to give the sequence and maps (in the paper) of the promoter sequences used. A figure comparing promoters would be very helpful and orienting.

All promoter and cassette sequences are clearly delineated and listed in full in the publicly available patent: Lombardi, L. Gene therapy vectors for treating heart disease (U.S. Patent No.: US 2023/0372541 A1). U.S. Patent and Trademark Office (2023). This is now clearly cited in the references. Additionally, for ease, we have added the requested sequences to the Supplement under "Sequences."

The assertion of improved packaging with the smaller expression cassette is plausible but needs to be addressed empirically. For example, were there differences in the vector titre achieved or the ratio of empty vs full capsids? Were the packaged AAVs with the smaller cassette sequences less prone to genome heterogeneity (see PMID: 35293222)?

Thanks to your excellent point we went back and thoroughly vetted this hypothesis and determined that indeed the increased expression of the pCard1 virus appears to be due to a combination of increased potency of the promoter DNA sequence itself and less heterogeneity of viral packaging. Specifically, based on the very helpful provided reference, we performed

denaturing gel analysis of extracted AAV vector genomes across multiple pCard1 and pCard0 viral preparations and observed pCard0 genomes did demonstrate greater heterogeneity than those of the pCard1 cassette (New Figure S2a). Further, we went back and performed additional matched transient transfections of MYBPC3^{-/-} iPSC-CMs and observed decreased protein expression of the pCard0 cassette compared to pCard1 (New Figure S2b). Thus, the pCard1 advantage appears to be a composite effect of increased promoter activity of the DNA sequence itself and less heterogeneity of viral packaging. This has been clarified in the text.



Please elaborate on the AAV9 variant CR9-01: identity, reference and give reason why it performs better than wtAAV9 in human iPSCs.

This point has been addressed in the revised manuscript. The AAV9 variant, CR9-01, has a higher iPSC-CM transduction efficiency than AAV9, as described in Cheng, Z. and Reid, C. A. Adeno-associated virus with engineered capsid (U.S. Patent No.: US 2023/0220014 A1). U.S. Patent and Trademark Office (2023). As described in the patent, CR9-01 contains a SQVNGRPRD substitution in the VR-IV site of AAV9 and was discovered by directed evolution selection screening in human iPSC-CMs. The receptor mapping of this has modification has not been performed, but its superiority to AAV9 for human iPSC-CM transduction has been confirmed by us and others (Wu et al., Commun Med (Lond) 2024; PMID: 38499690.) Our apologies for this oversight, we have now included these references in the manuscript.

Figure 1/S2. The sample size is remarkably small (n=1-3) for in vitro studies given the aim of this study was "to engineer an optimal AAV genomic cassette". It is not clear what 1K vs 3K is and the significance of the difference in results. Furthermore, the purity/heterogeneity of the iPSCs has not been quantified. What is the proportion of cardiomyocytes/other cell types and are the transduced GFP cells also the cardiomyocytes? This could easily be addressed using flow cytometry. As you move from 1K to 3K the number of positive cells does not increase substantially. Is transduction limited to a subset of cell types? This needs

to be explored further given the importance of expression cassette design as the major novel development of this study.

Thank you for this feedback. All our differentiations undergo quality and purity assessments by flow cytometry. Please find these data provided as new Fig. S9. With respect, to "1K v. 3K," our apologies for the confusion, this indicates the multiplicity of infection and has been clarified in the figure legend.

Regarding the in vivo studies, the lack of a control vector such as AAV9 with a reporter or stuffer sequence is a major scientific limitation in this study. There is no control for the effects of the AAV capsid proteins or other components of the vector expression cassette on myocytes/non-myocytes following transduction.

Thank you for this critical feedback. To control for the effect of the AAV capsid proteins and other components of the vector expression cassette on the heart, AAV9 with the same cassette but encoding GFP (pCard1-GFP) was injected into KO mice at the efficacious dose of 3E13 vg/kg. In contrast to AAV9:mMybpc3, echocardiographic and electrocardiographic assessments six weeks post-injection demonstrated no significant benefit of animals dosed AAV9:pCard1-GFP compared to Vehicle-treated animals in LV mass normalized to body weight, EF, Left ventricular posterior wall thickness in diastole (LVPW;d), and QT interval (Fig. S4 and below). This clearly shows that any beneficial effect observed is derived from the transgene itself.

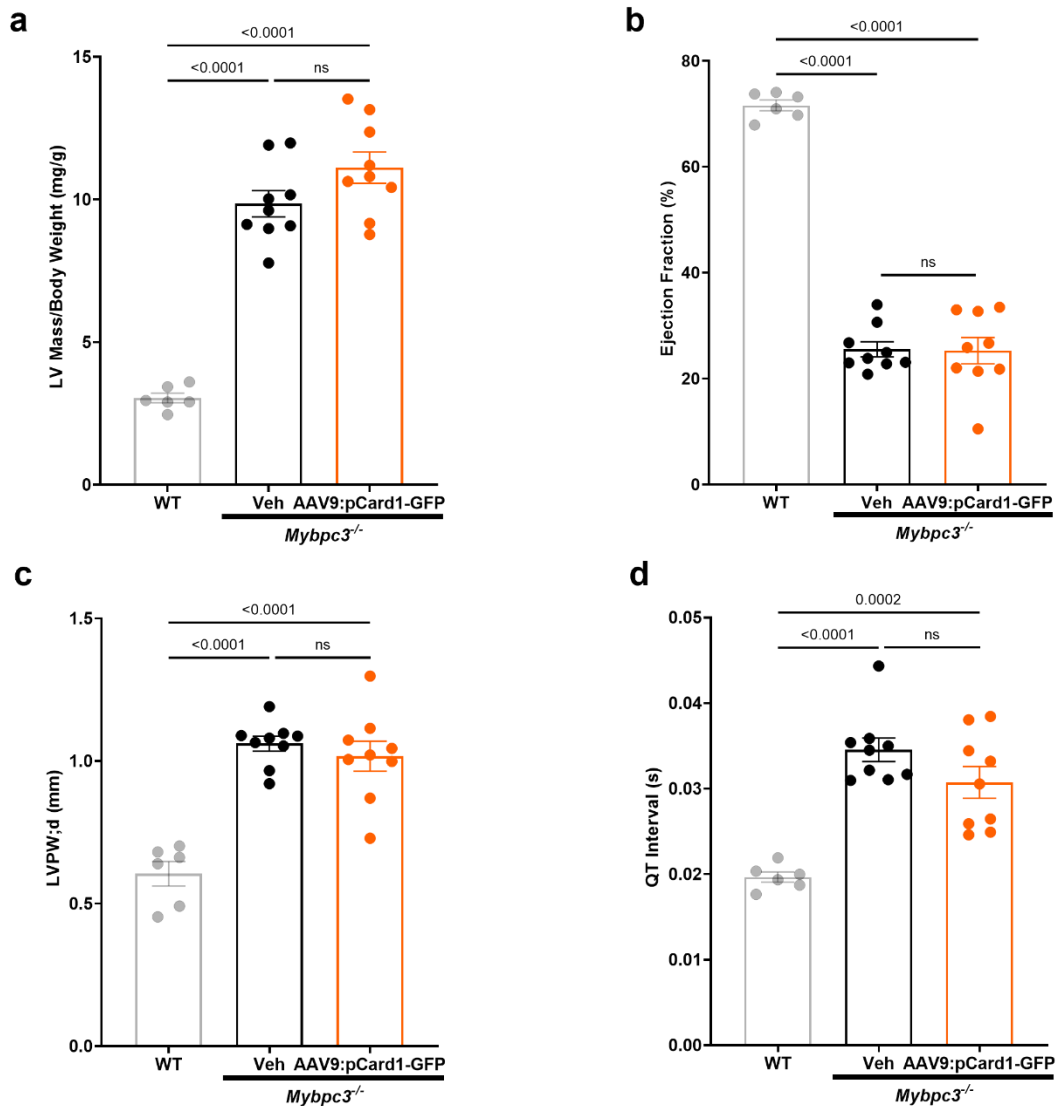


Figure 3. Mouse vs human Mybpc3 – were there differences in the protein expression levels or subcellular localisation in the treated mouse hearts that could explain the transgene species difference in therapeutic effect?

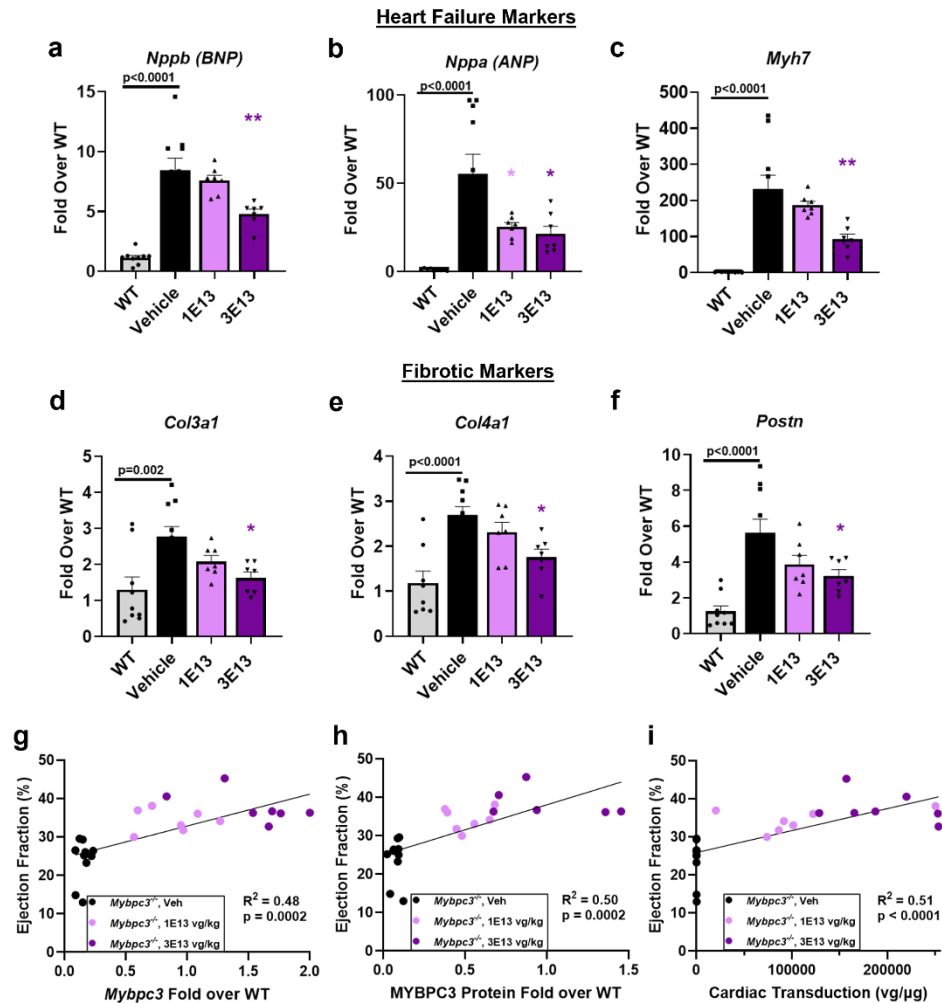
This is an excellent point which is the basis of further study. The empirical testing of the difference in protein stability of the orthologs is quite difficult given that we cannot rule out the confounding influence of antibody differences in affinity between the orthologs. In order to test this in an unbiased fashion, we would need to perform LC-MS to assess the abundance of each ortholog in the KO heart. Given this limitation, we have added this caveat to the discussion.

Figure 4: As above: was there a relationship between Mybpc3 protein level/localisation and the size of the therapeutic effect for 1x10³ vg/kg vs the two higher doses.

Thanks for this feedback. We have added this analysis for 1E13 and 3E13 vg/kg as requested into Figure 7. A dose of 3E13 vg/kg, resulted in MYBPC3 transgene expression equivalent to WT (Figure 7h, Figure 7i and Figure 5a). Further, consistent with the submaximal efficacy of 1E13 vg/kg (Figure 4), protein restoration was equivalent to 0.51 ± 0.05 with respect to wildtype.

Figure 7; Given the disconnect between Mybpc3 RNA and protein (demonstrated in this paper) it would be more relevant to look at protein expression in these tissues to correlate level of transduction with size of therapeutic effect.

Thank you so much for the excellent point! We have added this analysis as Fig. 7h. The disconnect observed between Mybpc3 RNA and protein is predominant once protein is saturated at WT levels. In the dynamic range, a dose-dependent increase in Mybpc3 RNA and MYBPC3 protein expression was observed at the level of cardiac tissue from individual treated mice, and this transgene expression was significantly correlated with the extent of cardiac improvement observed (Figures 7g and 7h). Similarly, cardiac transduction exhibited dose-dependency which was positively correlated with EF (Figure 7i). Importantly, this demonstrated that consistent with analysis of the TN-201 cassette in human iPSC-CMs (Figure 1f through 1i), transduction of ≥ 1 vector genome per diploid genome (or $\geq 1.6E5$ vg/ μ g DNA), as achieved with a dose of 3E13 vg/kg, resulted in MYBPC3 transgene expression equivalent to WT (Figure 7h, Figure 7i and Figure 5a). Further, consistent with the submaximal efficacy of 1E13 vg/kg (Figure 4), protein restoration was equivalent to 0.51 ± 0.05 with respect to wildtype.



Could the author please compare/contrast their study to prior research exploring gene therapy for Mybpc3-deficient HCM.

Of course, thank you for asking. Mybpc3 gene replacement therapy has previously been performed using both the AAV and the less clinically relevant lentiviral platforms in Mybpc3-deficient mouse models. Treatment of adult mice with lentivirus led to a partial recovery of heart function (PMID: 22855556), similar to our findings with treatment in adult mice. As discussed in our manuscript, the Mybpc3-deficient model we used has more severe and rapid heart failure progression compared to other models and therefore our therapy must overcome a higher bar to have significant improvements in heart function.

The more clinically relevant AAV9:Mybpc3 gene therapy has been used in prevention mode (i.e. virus injected in pups ≤36 hours of age) for Mybpc3-deficient mice (PMID: 25463264, 32750038, 37067542). Full length and smaller Mybpc3 peptides were found to inhibit the progression of heart failure, with full length Mybpc3 having the largest preventative effect. To

our knowledge, our manuscript demonstrates the first successful attempt to treat Mybpc3-deficient mice after the onset of heart failure with an AAV:Mybpc3 gene therapy. Additionally, we have demonstrated the longest duration of efficacy (54 weeks vs 34 weeks) and, critically, an improvement in survival. Furthermore, effective doses in the previous studies ranged from 2E14 to 3E15 vg/kg (provided by Stelzer paper, estimated from Carrier paper), which are very high AAV doses that could have deleterious impact in patients (PMID: 33309881). In contrast, we were able to demonstrate that the clinically relevant 3E13 vg/kg dose with our therapy was able to significantly improve heart function. Thanks to your feedback, a sentence has been added to the Discussion section to highlight this advance.

Reviewer #3 (Remarks to the Author):

The manuscript by Greer-Short, Lombardi et al. describes the generation and validation of a novel AAV vector carrying a MYBPC3 transgene, to be developed for the treatment of HCM caused by haploinsufficiency-mutations of the MYBPC3 gene. The novel viral vector (TN-201) was tested in a transgenic MYBPC3-deficient mouse (MYBPC3-KO), which display a remarkable cardiomyopathy phenotype. Interestingly, the treatment led to a drastic reduction of the structural and functional expression of cardiomyopathy in treated transgenic mice, as evaluated with a large number of in vivo and in vitro investigations. The authors compared their viral vector (TN-201) with the construct that has been employed in a previously published study by L. Carrier et al., and found that their viral therapy is associated with a better outcome and improved cardiac function, when compared with the previous construct. The paper is remarkably well written, clear and comprehensive and the study has been conducted with exceptional care and precision. The results leave no doubt whatsoever on the very high efficiency and effectiveness of their viral vector. My only concerns are regarding the experimental model.

In general, mice are poor models for HCM in general, due to the different expression of myofilament protein isoforms in their ventricles. In particular, protein C mutations behave very differently in mice as compared with humans. While human disease is evident when MyBPC levels fall down to 50% of normal level (as a consequence of haploinsufficiency-mutations in heterozygosity), mice only show a phenotype when there is a complete lack of protein (i.e. in homozygosity). MYBPC3 homozygous mutations are not impossible in humans and are known to lead to a devastating disease leading to demise in the first months of life. Nonetheless, a viral gene-transfer therapy, such as the one tested here, would be clinically relevant if proven to be useful in a model carrying a MYBPC3 mutation in heterozygosity. Many works from several groups highlighted the high predictive value of cardiomyocytes and engineered tissues obtained through differentiation from patient-specific or CRISPR-

mutated human induced pluripotent stem cells, as well-representative in vitro models of human HCM. Indeed, the authors of this paper have used hiPSC-CMs to test their construct. But did not test their viruses in hiPSC-CMs obtained from a patient carrying a MYBPC3 mutation in heterozygosity. Remarkably, a recent paper (Pioner et al, Circ Res 2023) showed that patient-specific hiPSC-derived cardiomyocytes and engineered heart tissues recapitulate the functional abnormalities found in human myocardium obtained from surgical samples of the same patient.

All in all, I believe that the authors should perform some tests demonstrating the efficacy of their approach in human iPSC-CMs carrying a heterozygous MYBPC3 mutation, showing that the treatment leads to restoration of a normal MyBPC protein expression and to a remarkable decrease of the typical functional abnormalities observed in mutated cells (that is, prolonged action potentials, altered calcium cycling, altered cell dimensions).

Thank you for your thorough and thoughtful assessment. Your point is well-taken. In the timespan allotted, we were able to observe in our hands multiple abnormalities of MYBPC3^{+/-} iPSC-CMs despite the well-established variable penetrance of haploinsufficiency and contractile abnormalities in MYBPC3^{+/-} iPSC-CMs (PMID: 31877118, 30586709, 36893011, 36744470). We observed the previously noted (PMID: 38642550, 30586709) increased Ca²⁺ amplitude of MYBPC3^{+/-} iPSC-CMs compared to isogenic WT cells (New Figure 9a). This increase in calcium amplitude upon pacing was coupled with increased fluorescence rise rate (Figure 9b). Importantly, addition of TN-201 virus was able to normalize this increase in fluorescence amplitude and rise rate when assessed one week post-transduction (Figure 9c and 9d). Additionally, the mild decrease in MYBPC3 protein levels observed, as per PMID: 31877118, 30586709, 36893011, was rescued based on immunoblotting and ELISA analysis (Figure 9e and 9f). Importantly, utilizing engineered heart tissues (EHTs), we were able to observe expected defects in relaxation (PMID: 36744470, 30554920) which were corrected upon transduction (Figure 9g and 9h). Thus, despite the limitations of the MYBPC3^{+/-} iPSC-CMs model, we were able to demonstrate rescue of the HCM hallmark relaxation defects.*

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have been responsive to review, particularly in clarifying the novelty of their experiments. The addition of the GFP control group in Fig S4 also strengthens the manuscript. However, the addition of the new "Ethical Statement" on page 21 prompts a suggestion. For consistency, one might first recommend making the statements in the main article and the supplemental methods identical; or perhaps deleting the copy in the supplemental methods. Second, it is noted that neither statement directly references the NIH Guide. If the following statement holds true, the authors might consider using it instead: "All animal experiments were pre-approved by the Institutional Animal Care and Use Committee of Tenaya Therapeutics (AUP: 2020.007 and 2023.007) and were performed in compliance with the "Guide for the Care and Use of Laboratory Animals, 8th edition", published by the US National Institutes of Health (ISBN-10: 0-309-15400-6)."

Thank you for this feedback. For consistency we have removed the statement in the supplement and, as it indeed applies, we have updated our statement to your excellent suggestion.

Another suggestion involves the "Editorial Policy Checklist" and Nature's adoption of the ARRIVE 2.0 guidelines. These guidelines recommend that the sex of the animals be disclosed along with the animal numbers as shown in Subitem 3c—Example 1 at <https://doi.org/10.1371/journal.pbio.3000411>. For example: "Group F contained 29 adult males and 58 adult females (n = 87)." It is not immediately clear whether this is an absolute requirement, but it is noted that the sex of the mice is never mentioned in the current manuscript. If all the mice were of the same sex, this should certainly be disclosed. If not, it is acknowledged that it would be cumbersome to describe the sex of every last mouse. However, the authors might consider doing so for all groups in the functional studies (ie, n=5 or more).

Thank you. We have provided in the "Source Data" file an Excel tab delineating the precise animal number by sex for each study. Our research findings apply to both sexes.

Finally, the authors are encouraged to be consistent about where and how they disclose the animal numbers. As indicated in the summary below, most animal numbers are indicated in the Figure legends, except for Figure 3, where the legend lacks any mention of "N" and the number is instead disclosed in the text of the main article:

Article:

Fig. 2: WT (n = 10), Mybpc3^{+/-} (n = 7) and Mybpc3^{-/-} (n = 12)

Fig. 3: Fig legend lacks any mention of "N". Text says (9-12/group)

Fig. 4: WT (n = 7), Mybpc3^{-/-} Veh (n = 7), Mybpc3^{-/-} 1E13 vg/kg (n = 4), Mybpc3^{-/-} 3E13 vg/kg (n = 6), and Mybpc3^{-/-} 1E14 vg/kg (n = 6)

Fig. 6: n = 9/group

Fig. 7: n = 7-10 mice/group

Fig. 8b and 8c: n = 6-8 mice/group

Fig. 8d and 8e: n = undefined / group

Fig. 9a and 9b: n = 8/group

Fig. 9c and 9d: n = 15-16/group

Fig. 9e and 9f: n = 8-10/group

Fig. 9g and 9h: n = 7-8/group

Supp Material:

Fig S1d (n = 5/group)

Fig S4: WT (n=6), Vehicle, Veh (n=9), AAV9:pCard1-GFP (n=9)

Fig S6: Vehicle, Veh (n=6), 3E13 vg/kg (n=5) and 3E14 vg/kg (n=6)

This is a great point. We have carefully gone through all figure legends to ensure that the precise "n" per group is indicated in all studies where the "n" is not equivalent across all groups. Specifically, the ranges previously provided in Fig. 1e-h, 5b-c, 5f-g, 7, 8b-c and 9c-h have been removed and the precise "n" per group is indicated. The regretful omissions for Fig 3 and 8d-e legends have been corrected, thanks. Further, we have ensured that the "n" is clearly discernible from all graphs with clear individual data points, as provided in full in the "Source Data."

Reviewer #2 (Remarks to the Author):

The authors have satisfactorily addressed my previous comments. I have no new comments to make.

Reviewer #3 (Remarks to the Author):

I thank the authors for considering my comments and observations. The authors have addressed all my comments and requests in a very satisfactory manner. I believe that the many changes and amendments introduced by the authors have significantly improved the

manuscript in terms of completeness, clarity and translational value. I have no additional comments or observations.