Circulation

RESEARCH LETTER

Efficient In Vivo Homology-Directed Repair Within Cardiomyocytes

Yanjiang Zheng, PhD*; Nathan J. VanDusen, PhD*; Catalina E. Butler, BS; Qing Ma, MD; Justin S. King, BS; William T. Pu, MD

Downloaded from http://ahajournals.org by on March 10, 2022

CRISPR/Cas9-based genome editing technologies provide powerful tools for genetic manipulation. Cas9 in vivo genome editing of cardiomyocytes through nonhomologous end joining efficiently creates insertion–deletion mutations at guide RNA–targeted sites.[1] However, precise addition of new genetic information, or mutation correction, requires alternate strategies. Delivery of Cas9 and a homology directed repair (HDR) template using adeno-associated virus (AAV) was recently shown to enable creation of precise genomic edits, even within postmitotic cells.[2] We studied CRISPR/Cas9 and AAV-based homology directed repair (CASAAV-HDR) in cardiomyocytes. Animal experiments were approved by the Institutional Animal Care and Use Committee and adhered to institutional guidelines. The authors will provide data, results, and reagents on reasonable request.

We studied CASAAV-HDR at *Myl2* (*MLC2v*), a highly expressed, ventricle-specific sarcomere gene. We constructed AAV9 with an *Myl2*-specific guide RNA and a promoterless HDR template that replaces the native stop codon with self-cleaving 2A peptide followed by mScarlet, a red fluorescent protein (Figure [A]). Subcutaneous delivery of the AAV to postnatal day 0 mice with cardiac-restricted Cas9 expression (*Tnnt2Cre;Rosa26$^{fsCas9\text{-}P2A\text{-}GFP}$*) yielded strong mScarlet expression in postnatal day 7 ventricular cardiomyocytes (Figure [Bi and C]). mScarlet expression required Cas9 and *Myl2* homology arms (Figure [Bii and Biii]). AAV-delivered Cas9 successfully directed mScarlet expression, albeit with reduced efficiency compared with *Rosa26$^{fsCas9\text{-}P2A\text{-}GFP}$* (Figure [Biv]). Guide RNAs targeting sequences on either side of the stop codon had equivalent performance (data not shown). We did not detect mScarlet in atrial cardiomyocytes, consistent with the targeted *Myl2* allele retaining its expression pattern (Figure [C], top). Parallel experiments targeting atrial-specific *Myl7* (*MLC2a*) resulted in mScarlet expression within atrial but not ventricular cardiomyocytes (Figure [C], bottom). Flow cytometry quantification of *Myl2* knockin efficiency after systemic AAV injection showed that ≈45% of ventricular cardiomyocytes displayed strong fluorescence, with steep dose response (Figure [D]). In atrial cardiomyocytes, *Myl7* knockin efficiency at the cellular level was 20% and had similar dose response (Figure [D]).

We quantified mutations created during the CASAAV-HDR DNA repair process (Figure [E]). The 5′ and 3′ junctions between the inserted template and the endogenous *Myl2* or *Myl7* sequences were amplified from RNA and deeply sequenced. We also quantified mutations in alleles that did not contain an inserted template by amplifying and sequencing cardiomyocyte genomic DNA flanking the guide RNA target site. For *Myl2*, 95.9% and 99.4% of transcripts containing the inserted template had the expected 5′ or 3′ junction sequences, respectively, whereas 11.3% of alleles lacking an insert contained a mutation, reflecting nonhomologous end joining (Figure [E]). For *Myl7*, these numbers were 85.8%, 97.8%, and 27.1%, respectively (Figure [E]). These data indicate that CASAAV-HDR insertion is precise and that a subset of alleles without repair template integration contained nonhomologous end joining–induced mutations. Integration of AAV-inverted terminal repeats at *Myl2* or *Myl7* was detected in <2% of sequences (Figure [E]). Inverted ter-

Key Words: CRISPR-associated protein 9 ■ genome editing ■ homology-directed dsDNA break repair ■ myocytes, cardiac

Correspondence to: Nathan J. VanDusen, PhD, or William T. Pu, MD, 300 Longwood Ave, Boston, MA 02115. Email nathan.vandusen@childrens.harvard.edu or william.pu@cardio.chboston.org

*Y. Zheng and N.J. VanDusen contributed equally.

For Sources of Funding and Disclosures, see page 789.

© 2022 American Heart Association, Inc.

Circulation is available at www.ahajournals.org/journal/circ

Nonstandard Abbreviations and Acronyms

AAV	adeno-associated virus
CASAAV	CRISPR/Cas9/AAV9-based somatic mutagenesis
HDR	homology directed repair

minal repeat sequencing found inverted terminal repeat integration elsewhere in the genome 4- to 28-fold less frequently than at *Myl2*.

Although HDR has been thought to be limited to proliferating cells,[3] CASAAV-HDR occurred in postmitotic neurons and in postmitotic adult cardiomyocytes.[2,4] We assessed the effect of cardiomyocyte proliferation on CASAAV-HDR efficiency by measuring CASAAV-HDR at *Myl2* at different developmental stages. CASAAV-HDR efficiency at the cellular level was comparable when AAV was delivered to fetal, neonatal, or mature mice (Figure [F]). Because of technical limitations, we were unable to quantify the fraction of cardiomyocyte genomes successfully modified by HDR and make comparisons among AAV delivery times at the genome level.

We targeted 7 additional loci—*Yap1*, *Tmem43*, *Nfatc3*, *Bdh1*, *Mkl1*, *Ttn*, and *Pln*—fusing either an HA tag or mScarlet to each. Insertion efficiency varied dramatically between loci, with HDR efficiency at the cellular level generally correlating with target gene

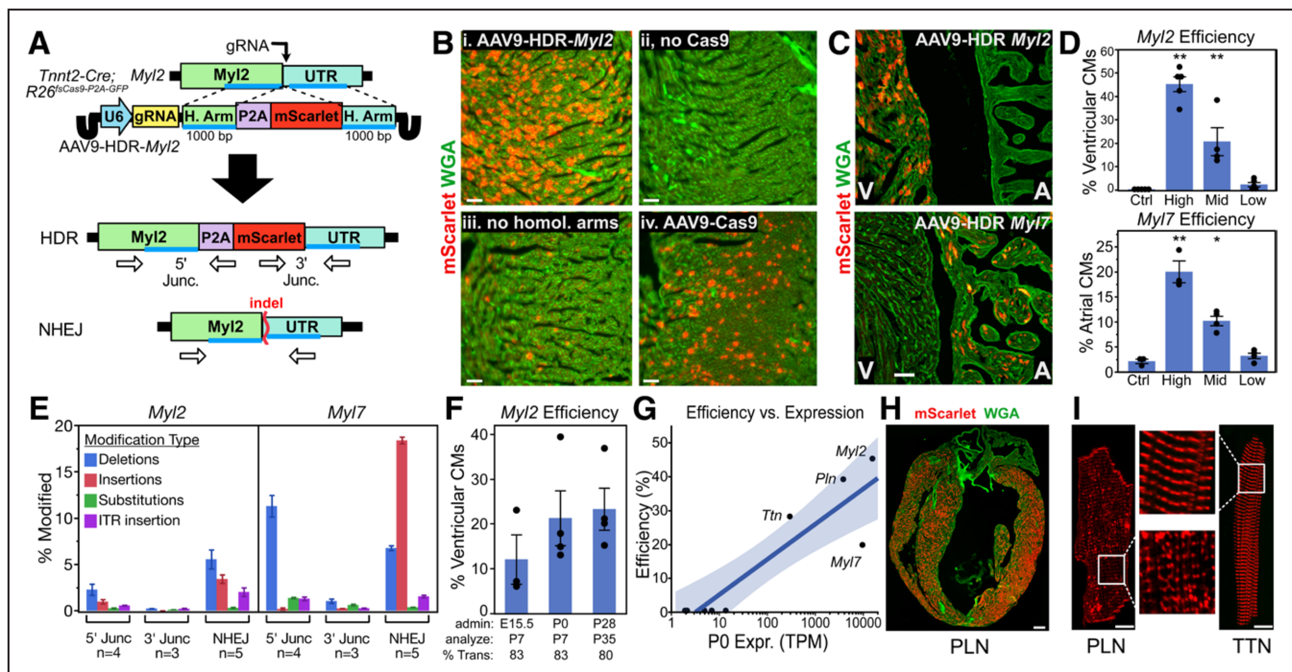

Figure. CASAAV-HDR targeted transgene insertion is highly efficient in neonatal and terminally differentiated cardiomyocytes in vivo.

Unless noted otherwise, adeno-associated virus (AAV) was delivered subcutaneously at postnatal day 0 (P0) and hearts were analyzed at postnatal day 7 (P7). **A**, CRISPR/Cas9/AAV9-based somatic mutagenesis (CASAAV)–homology directed repair (HDR) strategy to insert P2A-mScarlet at the stop codon of endogenous *Myl2*. Cas9 was expressed specifically in cardiomyocytes in *Tnnt2Cre;Rosa26*$^{fsCas9\text{-}P2A\text{-}GFP}$ mice. Cas9-induced double strand breaks can be repaired by HDR or nonhomologous end joining (NHEJ). Blue lines, homology arms; open arrows, primers used for amplicon sequencing. Unless noted otherwise, AAV9 was delivered subcutaneously at P0 and hearts were analyzed at P7. **B**, CASAAV-HDR integration of P2A-mScarlet at *Myl2*. i, AAV9-HDR-*Myl2*, CASAAV-HDR vector targeting *Myl2*, was delivered to *Tnnt2Cre;Rosa26*$^{fsCas9\text{-}P2A\text{-}GFP}$ mice. ii, No Cas9 vector was delivered without Cas9 expression. iii, AAV similar to AAV9-HDR-*Myl2* but lacking homology arms was delivered to *Tnnt2Cre;Rosa26*$^{fsCas9\text{-}P2A\text{-}GFP}$ mice. iv, AAV-Cas9, AAV9-HDR-*Myl2*, plus AAV9-Tnnt2-Cas9 was delivered to wild-type mice. Scale bar, 50 µm. **C**, CASAAV-HDR vectors targeting *Myl2* or *Myl7* resulted in mScarlet expression specifically in ventricular or atrial chambers. Scale bar, 50 µm. **D**, CASAAV-HDR dose response. AAV was administered at high, middle, and low doses (5×10^{11}, 5×10^{10}, and 5×10^{9} vg/g, respectively), resulting in ≈96%, 83%, and 51% myocardial transduction, respectively. Cardiomyocytes, dissociated by Langendorff perfusion, were analyzed for GFP expression by flow cytometry. **E**, Quantification of mutations induced by high-dose CASAAV-HDR insertion of P2A-mScarlet into the C terminus of *Myl2* or *Myl7*. The junctions between inserted sequence and endogenous sequence were amplified from cDNA. Primers are illustrated in **A**. For alleles lacking an insert, a fragment was amplified from DNA using primers flanking the guide RNA target site. Amplicons were deeply sequenced and analyzed for the indicated types of modifications. **F**, *Myl2* HDR efficiency in fetal, neonatal, or mature cardiomyocytes. AAV was administered at equivalent middle dose (5×10^{10} vg/g; E15.5 embryo = 0.6 g) at each stage, resulting in 80% to 83% myocardial transduction. mScarlet-expressing cardiomyocytes were quantified by flow cytometry. Differences between groups were not significant. **G**, Summary of HDR efficiency at 9 different loci, as a function of gene expression level in P0 ventricular cardiomyocytes, or atrial cardiomyocytes in the case of *Myl7*. Homology arms were ≈1 kb long. Shading indicates 95% CI for fitted line. **H**, Adult heart with insertion of mScarlet into the *Pln* locus after P0 administration of CASAAV-HDR vector. Scale bar, 200 µm. **I**, In situ imaging showing localization of mScarlet fused to *Pln* or *Ttn* in mature cardiomyocytes. Scale bar, 10 µm. **Dunnett $P<0.001$. *$P<0.01$. Error bars reflect standard error.

Downloaded from http://ahajournals.org by on March 10, 2022

expression (Figure [G]). The 5 lowly expressed genes (<5 transcripts per million) had low HDR efficiency, whereas ≥20% of cardiomyocytes were edited in each of the 4 robustly expressed genes (>100 transcripts per million; Figure [G and H]). TTN-mScarlet and mScarlet-PLN fusion proteins localized to the sarcomere and sarcoplasmic reticulum, respectively, consistent with the localization of the endogenous proteins (Figure [I]).

Systemic delivery of CASAAV-HDR vectors achieved efficient and precise in vivo somatic genome modification that did not require cardiomyocyte proliferation. Efficiency correlated with expression level of the target gene and in the best case reached remarkably high levels (45% of cardiomyocytes). While this article was in preparation, limited success with CASAAV-HDR in the heart was reported, although HDR efficiency was low and required direct intramyocardial injection because systemic delivery was unsuccessful.[5] We successfully used CASAAV-HDR to monitor protein localization and anticipate it will be useful for many other applications, such as precise introduction of mutations to model disease or probe gene function. CASAAV-HDR may also enable efficient, permanent, and precisely targeted delivery of therapeutic transgenes to validated loci. We envision future studies will further expand research and translational applications by identifying attributes of loci that make them amenable to efficient HDR.

Downloaded from http://ahajournals.org by on March 10, 2022

ARTICLE INFORMATION

Affiliations

Department of Biochemistry, West China School of Basic Medical Sciences & Forensic Medicine, Sichuan University, Chengdu, China (Y.Z.). Department of Cardiology, Boston Children's Hospital, MA (Y.Z., N.J.V., C.E.B., Q.M., J.S.K., W.T.P.). Harvard Stem Cell Institute, Cambridge, MA (W.T.P.).

Sources of Funding

Dr Zheng was funded by the Chinese Scholarship Council. Drs VanDusen (grant K99HL143194) and Pu (grants 2UM1 HL098166 and R01 HL146634) were supported by the National Institutes of Health/National Heart, Lung, and Blood Institute.

Disclosures

None.

REFERENCES

1. Guo Y, VanDusen NJ, Zhang L, Gu W, Sethi I, Guatimosim S, Ma Q, Jardin BD, Ai Y, Zhang D, et al. Analysis of cardiac myocyte maturation using CASAAV, a platform for rapid dissection of cardiac myocyte gene function in vivo. *Circ Res.* 2017;120:1874–1888. doi: 10.1161/CIRCRESAHA.116.310283
2. Nishiyama J, Mikuni T, Yasuda R. Virus-mediated genome editing via homology-directed repair in mitotic and postmitotic cells in mammalian brain. *Neuron.* 2017;96:755–768.e5. doi: 10.1016/j.neuron.2017.10.004
3. Yeh CD, Richardson CD, Corn JE. Advances in genome editing through control of DNA repair pathways. *Nat Cell Biol.* 2019;21:1468–1478. doi: 10.1038/s41556-019-0425-z
4. Ishizu T, Higo S, Masumura Y, Kohama Y, Shiba M, Higo T, Shibamoto M, Nakagawa A, Morimoto S, Takashima S, et al. Targeted genome replacement via homology-directed repair in non-dividing cardiomyocytes. *Sci Rep.* 2017;7:9363. doi: 10.1038/s41598-017-09716-x
5. Kohama Y, Higo S, Masumura Y, Shiba M, Kondo T, Ishizu T, Higo T, Nakamura S, Kameda S, Tabata T, et al. Adeno-associated virus-mediated gene delivery promotes S-phase entry-independent precise targeted integration in cardiomyocytes. *Sci Rep.* 2020;10:15348. doi: 10.1038/s41598-020-72216-y