Molecular Therapy
Nucleic Acids
Commentary

Leveraging cardiac reprogramming with CRISPRa-SAM technology

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https://doi.org/10.1016/j.omtn.2025.102532

The need for novel approaches for cardiomyocyte generation to treat a diseased heart has led to a considerable research in the field of direct cardiac reprogramming. This domain focuses on generating cardiomyocytes, bypassing the pluripotent state, aided by multiple viral and non-viral reprogramming approaches.[1] Researchers are not only focused on finding ways to directly convert resident fibroblasts in a post-infarct heart into functional cardiomyocytes but are also exploring more precise, versatile, and advanced techniques, such as the use of CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats associated with CRISPR-associated protein 9), to further enhance the process. In a recent study published in *Molecular Therapy Nucleic Acids*, Huang et al. has aimed to endogenously activate cardiac-specific core transcription factors (TFs) using a modified CRISPR-dCas9 (dead Cas9) strategy, thereby bringing about the generation of induced cardiomyocytes (iCMs) from both mouse and human fibroblasts.[2] The endogenous activation of *GATA4* gene assisted by single guide RNAs (sgRNAs) combined with exogenous expression of *MEF2C* and *TBX5* genes shed light on the possible use of CRISPR-dCas9 in the reprogramming paradigm, circumventing the concerns of virus-based reprogramming approaches (Figure 1).

The majority of conventional reprogramming techniques rely on the use of lentiviral or retroviral transduction to overexpress cardiac-specific TFs like GATA4, MEF2C, and TBX5 (G/M/T). Although successful to a large extent, this comes with its own set of challenges like insertional mutagenesis, unelicited immune response, and viral toxicity. In this issue of *Molecular Therapy Nucleic Acids*, Huang and colleagues have efficiently aimed to address these challenges by using CRISPR-Cas9 technology, hoping to reduce the dependency on the use of viral vectors for cellular reprogramming. To be more precise, the authors have utilized a second generation CRISPR activation system (CRISPRa-SAM [Synergistic Activation Mediator]) consisting of a dCas9-VP64 fusion, a sgRNA containing two MS2 RNA aptamers, and an MCP-p65-hsf1 (MPH) activation helper protein to endogenously activate the aforementioned TFs.

After identifying the promoter and enhancer regions for the three genes, multiple sgRNAs were designed and screened for their ability to overexpress G/M/T using the SAM system in comparison to the first generation dCas9-VP64 CRISPRa system. Among the three target genes, the sgRNAs designed against *Gata4* gene demonstrated the highest efficiency, resulting in a 280-fold increased expression using a single sgRNA. The efficiency was further enhanced to as high as 500-fold with multiple sgRNAs used simultaneously. A noteworthy observation here was the varied degree of gene activation among different cell types, with significant upregulation in mouse embryonic fibroblasts (MEFs) compared to the negligible fold increase in cardiac fibroblasts (CFs), highlighting the fact that CRISPRa-based gene activation may actually be influenced by the basal level gene expression in a given cell line. In contrast, in spite of extensive testing with numerous sgRNAs, MEF2C and TBX5 presented a markedly different scenario and proved to be less effective compared to the successful upregulation of GATA4 expression. Interestingly, the combined use of multiple sgRNAs for TBX5 had an antagonistic effect on the expression level compared to GATA4 and MEF2C, shedding light on the importance of empirical validation for every factor in an experimental setup.

Considering the efficiency of the SAM-based CRISPRa system for G/M/T, the authors proceeded with the CRISPR-mediated activation of *GATA4* gene combined with the exogenous activation of *MEF2C* and *TBX5* genes for cardiac reprogramming in MEFs and human H9-derived fibroblasts (H9Fs). In the case of MEFs, this resulted in a significant upregulation of cardiac-specific markers like *Myh6*, *cTnT* and a simultaneous downregulation of fibroblast-associated genes like *Postn*, *Col1a1/2* in comparison to the retroviral control group. Further, the same strategy when applied to H9Fs, aided by a precursor miRNA, miR-133a2, resulted in a 14-fold increase in MYH6 (although insignificant TNNT2 expression) in the resultant iCMs. These results underscore the possibility of using sgRNA/SAM-based endogenous activation of *GATA4* to replace retroviral transduction in enhancing the efficiency of cardiac reprogramming in both mouse and human fibroblasts.

The authors also attempted to utilize a CRISPR-based gene delivery method entirely, eliminating the need of retroviral delivery of G/M/T in the reprogramming process. The fold increase in the expression of G, M, and T was evident, yet lower when compared to the retroviral delivery of the same. In terms of cardiac markers, TNNT2, SCN5A, and NPPB were significantly upregulated than their respective negative controls. Nevertheless, the low

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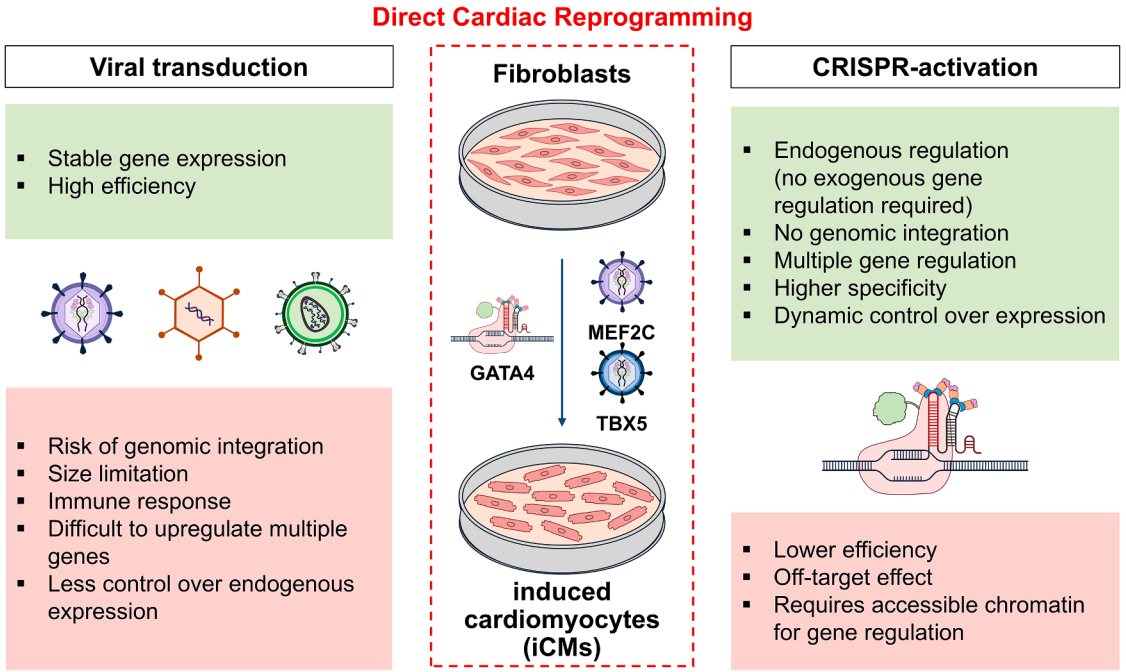

Figure 1. Direct cardiac reprogramming of fibroblasts to induced cardiomyocytes (iCMs) using viral and CRISPR-based methods

The study by Huang et al. demonstrated the use of CRISPRa-based endogenous upregulation of *GATA4* gene along with the retrovirus-based exogenous upregulation of *MEF2C* and *TBX5* genes, throwing light on the possible use of CRISPRa in the field of cardiac reprogramming. The figure compares the pros and cons of both the methods used in the study.

expression of cTnT and α-actinin confirms the limited efficiency of iCM generation using the CRISPRa-SAM-based method. These findings are in line with another recent study that has used a similar strategy activating cardiac-specific TFs, viz., *Gata4*, *Nkx2.5*, and *Tbx5*, to activate a positive feedback loop, thereby generating cardiac progenitor cells from MEFs.[3] The same CRISPRa-SAM system has also been employed to reprogram human fibroblasts into induced cardiac progenitor cells using GATA4, HAND2, MEF2C, and TBX5 under defined conditions.[4] Interestingly, the conventional CRISPRa-dCas9 system was able to activate the endogenous *Gata4*, *Mef2C*, *Tbx5*, and *Hand2* genes, but it was unable to reprogram mouse fibroblasts into cardiomyocyte-like cells.[5] This study highlighted that the conventional CRISPRa-dCas9 system may not be suitable for reprogramming hard-to-reprogram cells like fibroblasts, suggesting that novel CRISPRa-dCas9 approaches such as CRISPR-SAM system should be explored for more efficient reprogramming. The feasibility of using the CRISPRa-SAM system in hepatocyte generation was also very recently explored by Li et al. wherein the endogenous activation of two TFs, *Gata4* and *Foxa3*, successfully transformed MEFs into functional induced hepatocyte-like cells (iHeps).[6]

While Huang and colleagues have presented a promising approach in the field of direct cardiac reprogramming, the study has opened multiple avenues that intrigue us into further exploration and investigation. The possibility that the epigenetic landscape may have affected the CRISPR-based activation of *Mef2c* and *Tbx5* genes is very likely and can be investigated further. Using epigenetic modifiers or other gene editing technologies to render the target loci easily accessible or using modified Cas9 to more effectively target these chromatin regions may help overcome the said barriers.[7] Strategically considering the distance of the enhancer regions from the transcription start sites while designing gRNAs may also help to enhance the efficiency of the CRISPRa system. The generation of iCMs can also be enhanced by the CRISPR interference (CRISPRi)-based downregulation of fibroblast-specific genes simultaneously with the CRISPRa-based upregulation of cardiac-specific genes, ensuring a seamless transition towards the cardiac lineage.[8,9] The process of delivery of the CRISPR machinery may be refined further via ribonucleoprotein loaded nanoparticles, making it a safer and a more targeted approach to treat the diseased fibroblasts in a post-infarct heart.[7]

Despite multiple facets requiring further refinement, it is evident that the second generation of CRISPRa-SAM had a superior efficiency than the CRISPR-Cas9 system in activating endogenous GATA4 expression. Given the promising results of CRISPRa-SAM-based reprogramming so far, research in the field of direct cardiac reprogramming appears to be advancing in the right trajectory. Huang et al.'s study is thus a compelling proof that endogenous activation of TFs can indeed be bought about using CRISPRa-SAM technology, reducing our

reliance on viral gene delivery for cellular reprogramming. With further extensive research in this field, it is only a matter of time for this gene editing technology to be able to entirely reprogram diseased cardiomyocytes, bringing cardiac regeneration closer to clinic.

ACKNOWLEDGMENTS

This work is supported by grants to R.P.T. and A.B. funded by Science and Engineering Research Board (SERB) and the Prime Minister's Research Fellowship (PMRF) program, respectively. A.B. and K.N.V. are supported by fellowships from Indian Institute of Technology Guwahati, Assam, India.

AUTHOR CONTRIBUTIONS

R.P.T. and A.B. wrote the manuscript, and K.N.V. created the artwork.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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