Stoichiometry of Transcription Factors Is Critical for Cardiac Reprogramming

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Direct reprogramming of fibroblasts into cardiomyocytes holds great potential for cardiovascular disease research and treatment. This new technology may be used for patient-specific drug screening, cardiac disease modeling, and regenerative purposes. We reported first that a combination of 3 cardiac-specific transcription factors, Gata4 (G), Mef2c (M), and Tbx5 (T), constituted the minimum requirement to directly reprogram mouse fibroblasts (CFs) into induced cardiomyocytes (iCMs).1 Subsequently, multiple groups have achieved and improved direct cardiac reprogramming from mouse and human fibroblasts by overexpressing other combinations of cardiac transcription factors and cardiac-enriched miRNAs.2–9 Intriguingly, it was reported that gene delivery of reprogramming factors into mouse infarcted hearts converted endogenous CFs into functional iCMs, reduced scar size, and improved cardiac function after myocardial infarction.4,10,11 Although these recent achievements are promising, the low reprogramming efficiency of fully reprogrammed functional iCMs and the reproducibility of cardiac reprogramming continue to be controversial aspects of this technology. Chen et al12 showed that transduction of pooled lentiviruses expressing G, M, and T was insufficient to reprogram fibroblasts into a cardiac fate. We reported that a polycistronic vector and a mixture of individual vectors expressing G, M, and T could reprogram resident CFs into iCMs in vivo, but the efficiency of cardiac reprogramming in this study was lower than that in other reports.13 These findings suggest that slight technical or biological differences might result in variable reprogramming efficiency.14 Therefore, critical factors for cardiac reprogramming need to be identified, and standardized platforms for efficient and reproducible cardiac induction should be established to advance this field. In this issue, Wang et al15 generated a complete set of polycistronic constructs encoding G, M, and T with all possible splicing orders and analyzed the cardiac reprogramming efficiency using these vectors. They found that each polycistronic vector gave rise to distinct G, M, and T protein expression levels, and that an optimal balance of G, M, and T protein expression greatly improved both the efficiency and the quality of cardiac reprogramming.

Previous studies mainly used a mixture of viruses expressing individual factors for cardiac reprogramming.1–10,12,16 This approach has heterogeneous and uncontrollable ratios of reprogramming factor expression among infected fibroblasts, which may lead to variable and low reprogramming efficiency. To overcome this issue, we previously developed the polycistronic construct TMG to express each factor in a homogeneous ratio and to improve in vivo reprogramming, but the optimal stoichiometry of reprogramming factors has not been explored.13 In this study, Wang et al generated 6 polycistronic constructs with identical self-cleaving 2A peptides (P2A and T2A) to include all possible splicing orders of G, M, and T in a single transgene (ie, MGT, MTG, GMT, GTM, TMG, and TGM; Figure).15 The total and endogenous transcript levels of G, M, and T were not significantly different in the CFs transduced with the 6 polycistronic vectors, whereas the G, M, and T protein expression was high when the reprogramming factor was placed at the 5′ end of a polycistronic construct, upstream of the P2A peptide. This is consistent with the results of a previous study demonstrating that the P2A peptide is more efficiently cleaved than other 2A peptides for efficient protein translation from polycistronic vectors.17 The authors next determined the reprogramming efficiency in CFs by FACS (fluorescence activated cell sorting), immunohistochemistry, and Western blot analyses using α-myosin heavy chain-green fluorescent protein reporter mice. Transduction of the polycistronic vectors resulted in significantly different reprogramming efficiencies as indicated by variable induction of cardiac markers, including α-myosin heavy chain-green fluorescent protein, cardiac troponin T (cTnT), and α-actinin. Notably, only 2 vectors, MGT and MTG, which expressed a high level of Mef2c and low levels of Gata4 and Tbx5, enhanced cardiac reprogramming compared with separate G, M, and T vectors, whereas the other 4 vectors resulted in a diminished reprogramming efficiency. Next, a puro-MGT vector was generated by inserting a puromycin resistance gene into an MGT vector to increase cardiac reprogramming by purifying transduced CFs using antibiotics. Puro-MGT transduction and antibiotic selection promoted α-myosin heavy chain-green fluorescent protein and cTnT induction by 3- and 5-fold, respectively, and increased the number of beating iCM loci by >10-fold compared with a mixture of G, M, and T vectors. Molecular characterization revealed that optimal G, M, and T stoichiometry correlated with higher expression of mature cardiac genes. Thus, stoichiometry of reprogramming factors influences both the efficiency and the quality of iCM generation, and these newly developed polycistronic systems not only eliminate the need for multiple vectors.
but also provide a homogeneous gene expression stoichiometry for cardiac reprogramming that can serve as a valuable platform for future research.

Consistent with these results, it was reported that expression levels and stoichiometry of reprogramming factors used to generate induced pluripotent stem cells also affect the quality and efficiency of induced pluripotent stem cell generation. Carey et al reported that polycistronic vector–induced high expression of Oct4 and Klf4 with concomitant low expression levels of Sox2 and c-Myc produced high-quality induced pluripotent stem cells that efficiently generated tetraploid complementation. Okita et al generated 6 polycistronic constructs containing all possible splicing orders of Oct4 (O), Klf4 (K), and Sox2 (S) with identical 2A peptides and found that the OKS vector yielded a higher reprogramming efficiency than other constructs. These results suggest that the stoichiometry of transcription factors is critical for cellular reprogramming in general, which is consistent with the notion that precise dosage and temporal expression of transcription factors are important for cell fate specification and development. The inefficient cardiac reprogramming reported by Chen et al might be caused by the relatively high expression levels of Tbx5 and Gata4 because the analysis of cardiac induction was performed in Tbx5-expressing fibroblasts purified by antibiotic selection.

The present study by Wang et al provides important insights into cardiac reprogramming, but also raises several interesting questions. First, the obvious question is the molecular mechanism underlying efficient and high-quality iCM generation by an optimal stoichiometry of the reprogramming factors. Transcriptome and epigenetic analyses using their new polycistronic system may answer this important question in the future. Second, we have previously used the polycistronic vector TMG for in vivo cardiac reprogramming, but it remains unclear whether the MGT or MTG construct promotes in vivo cardiac reprogramming further. Third, it would be interesting to explore whether the stoichiometry of reprogramming factors has an influence on direct reprogramming of fibroblasts into specific cardiac cell types, including pacemaker-, atrial-, and ventricular-type cardiomyocytes. Nam et al recently demonstrated that a mixture of G, M, T, and Hand2 viruses generated all 3 types of cardiac cells from fibroblasts, but the mechanisms and effects of stoichiometry in cell fate specification remain unclear. Finally, low efficiency of cardiac reprogramming in human cells could be improved using optimal polycistronic constructs, although 5 or 6 reprogramming factors may be required. Nevertheless, Wang et al reveal the importance of reprogramming factor stoichiometry and provide a valuable platform for future cardiac reprogramming research. A better understanding of the molecular mechanisms of cardiac reprogramming as well as translational studies using human cells could further develop the potential of this new technology and may provide insights into the mechanisms of cardiac cell fate determination during development.

**Sources of Funding**

M. Ieda was supported by research grants from JST CREST, JSPS, Banyu Life Science, The Uehara Memorial Foundation, Takeda Science Foundation, and SENSIN Medical Research Foundation. N. Muraoka was supported by research grant from SENSIN Medical Research Foundation.

**Disclosures**

None.

**References**


**Key Words:** Editorials ■ cellular reprogramming ■ myocytes, cardiac
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Circ Res. 2015;116:216-218
doi: 10.1161/CIRCRESAHA.114.305696

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2015 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/116/2/216

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