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In vivo cardiac reprogramming using an optimal single polycistronic construct

Direct cardiac reprogramming holds great promise for cardiac regeneration and disease modelling.^{1–15} We recently reported that stoichiometry of Gata4 (G), Mef2c (M), and Tbx5 (T) influences the efficiency and quality of induced cardiomyocyte (iCM) reprogramming.¹⁶ We generated a full set of polycistronic vectors to manipulate the relative levels of G, M, and T protein expression. Among the six combinations, MGT that expresses a relative high expression of M and low expressions of G and T resulted in the most efficient *in vitro* iCM reprogramming.¹⁶ Here, we performed genetic lineage tracing in a murine myocardial infarction (MI) model to determine whether MGT could improve *in vivo* iCM reprogramming efficiency and result in a further improvement in ventricular contractile function compared with the traditional separate G, M, and T (G/M/T) delivery.

We took advantage of the unique feature that retrovirus infects only dividing cells to deliver MGT, G/M/T, or dsRed into actively dividing cardiac fibroblasts, but not CMs, in MI hearts.³ We prepared retrovirus encoding the single-triplet MGT as well as G/M/T and dsRed control viruses. These viruses were subjected to ultracentrifugation and were concentrated at 1×10^{10} p.f.a./mL. According to the previously established protocol,³ 8–12 week old male mice were treated with 10 μ L of ultra-high-titre retrovirus ($\sim 10^{10}$ p.f.a./mL) by injection into the myocardial wall immediately following coronary artery ligation.

To determine whether MGT enhances reprogramming of non-myocytes into iCMs *in vivo*, we performed genetic lineage tracing experiments using Periostin-Cre; R26R-lacZ mice³ to quantify the number of iCMs that had a fibroblast origin. Consistent with previous reports,³ we did not detect any β -galactosidase activity in the CMs of control dsRed retrovirus-infected MI hearts (Figure 1A). However, 4 weeks after MI and retroviral delivery of G/M/T and MGT, we found β -gal⁺ cells that were also α -Actinin⁺ in the injured area of the heart (Figure 1B and C). Quantification revealed that MGT resulted in an increase in the number of β -gal⁺ α -Actinin⁺

reprogrammed iCMs compared with separate G/M/T delivery (Figure 1G).

We next determined whether MGT could improve structural maturation of *in vivo* reprogrammed iCMs. Morphologically, most G/M/T- and MGT-induced β -gal⁺ α -Actinin⁺ cells closely resembled endogenous CMs and assembled sarcomere structures (Figure 1B–F). We evaluated the quality of sarcomeric structure as previously described³ to determine the full spectrum of maturation status of the reprogrammed cells induced by either MGT or G/M/T. In contrast to our *in vitro* finding¹⁶ that MGT improved both efficiency and quality of iCM reprogramming, we did not find a significant difference in reprogramming quality between the two groups (Figure 1G, table). Since cardiac fibroblasts are more fully reprogrammed *in vivo* in their native environment, we speculate that the native microenvironment could play a more significant role than MGT in enhancing reprogramming quality.

Next, we performed high-resolution two-dimensional echocardiography to determine whether an increased number of iCMs could translate into further improvement of heart function after MI (Figure 1H–P). MGT-, G/M/T-, or dsRed- injected mice underwent serial high-resolution echocardiography 1 day before, and 4 and 8 weeks after MI followed by blinded measurement and calculation. All mice had a similar reduction in left ventricular function after coronary artery ligation (data not shown). Eight weeks after injection, the fraction of blood ejected from the left ventricle with each contraction (ejection fraction, EF) and fractional shortening (FS) of the ventricular chamber were improved in mice injected with MGT, compared with dsRed-injected controls (Figure 1J–P). Importantly, MGT-treated mice exhibited a further improvement in heart function compared with the G/M/T-treated group (Figure 1J–P).

Cardiac fibrosis and scar formation often result from ischaemia and shortage of oxygen in MI hearts. Based on previous findings that iCM reprogramming resulted in a decrease in scar size,^{3,6} we tested if MGT delivery could lead to a further reduction in scar size. To this end, we performed Masson's trichrome staining on sections from multiple layers of left ventricles to delineate both viable myocardium and scar area (Figure 1Q–S). After blinded quantification on six sections from each of the four layers spanning left ventricles from control and treated mice, we found a reduction in scar area in reprogramming mice compared with controls, and an MGT group exhibited a further reduction compared with the G/M/T group

(Figure 1Q–T). We also performed Sirius Red (SR) staining in parallel to further evaluate the effect of G/M/T and MGT delivery on scar size. SR staining is commonly used in the histological visualization of collagen I and III fibres in addition to muscle in infarcted heart by differentially staining collagen (red), cytoplasm (yellow), and muscle fibres (yellow; Figure 1U–W). Consistently, we found that both G/M/T- and MGT-treated hearts showed a decrease in SR-positive area, and the reduction was more significant with MGT delivered as a single vector (Figure 1U–X). Taken together, our data suggest that direct reprogramming with single-triplet MGT causes less scarring than using the pooled separate G/M/T viruses.

Here, we show that delivery of a single polycistronic vector MGT upon cardiac injury resulted in an increased number of *in vivo* reprogrammed iCMs compared with separate G/M/T delivery. In contrast to its effect on *in vitro* reprogramming, MGT appears to only influence the efficiency but not the quality of *in vivo* cardiac reprogramming. This difference may be due to an effect of the cytokines, growth factors, and mechanical cues in the native microenvironment that could more efficiently enhance functional maturation of *in vivo* reprogrammed iCMs. The further improved cardiac function upon delivery of single MGT vs. separate G/M/T after MI may be attributable to the increase in the number of reprogrammed iCMs in combination with MGT overexpression in non-myocytes. Ultimately, our results demonstrate the advantage of MGT single triplet over traditional G/M/T in reprogramming non-myocyte to iCM. This proof of principle promises to improve flexibility and consistency of iCM production and may reduce technical barriers to future applications. Similarly, we are working to generate a genetic mouse model where an inducible MGT cassette is targeted to endogenous fibroblast locus for conditional and inducible generation of iCMs.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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Figure 1 *In vivo* cardiac reprogramming using MGT. (A–G) Lineage tracing using Periostin-Cre:R26RlacZ mice 4 weeks post-MI. Quantification of the number of iCMs per section (averaged from six sections per heart, 3–5 hearts per group) is shown in G. As previously published,³ the quality of iCMs was assessed by the sarcomere structures of the *in vivo* iCMs labelled by α -Actinin. Percentages of cells showing different phenotypes were calculated out of all α -Actinin, β -Gal, and DAPI triple-positive cells. Class I: no α -Actinin expression. Class II: low α -Actinin expression and partial sarcomere assembly (only in the boxed area, < 1/3 of the cells). Class III: sarcomere assembly was observed in majority part of the cell (> 2/3). Class IV: α -Actinin expression and pattern closely resembled those of endogenous CMs. *n*, number of iCMs from 3–5 hearts per group. (H–P) Echocardiography to measure ventricular contractility 4 and 8 weeks (4w and 8w) post-MI. Quantification of 8w EF and FS as absolute values are shown and labelled in (P). dsRed *n* = 9, G/M/T *n* = 8, and MGT *n* = 8. Other parameters and all raw numbers are summarized in Supplementary material online, Table S1. (Q–T) Masson's trichrome staining of control and reprogrammed hearts 4 weeks post-MI with quantification for percentage of scar (blue) area shown in (T). (U–X) Sirius Red staining of control and reprogrammed hearts 4 weeks post-MI with quantification for percentage of scar (red) area shown in X. dsRed *n* = 9, G/M/T *n* = 8, and MGT *n* = 8. Error bars represent SEM. **P* < 0.05; ***P* < 0.01.

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