

## Review

### Heart regeneration using reprogramming technology

By Masaki IEDA<sup>\*1,\*2,\*3,†</sup>

(Communicated by Hiroo IMURA, M.J.A.)

**Abstract:** Loss of terminally differentiated cardiomyocytes due to heart disease is irreversible and current therapeutic regimes are limited. Cell therapy using stem cell-derived cardiomyocytes is an attractive option to repair injured hearts. The discovery of direct reprogramming of fibroblasts into induced pluripotent stem cells (iPSCs) and successful differentiation of iPSCs into cardiomyocytes provided a revolutionary paradigm in heart regenerative research. During the past decades, significant advances in stem cell culture, differentiation and purification protocols, as well as in cell transplantation methodologies, have been achieved. On the other hand, recent studies demonstrated that a somatic cell could be converted into an alternative differentiated cell type without first becoming a stem cell by overexpression of lineage-specific factors. We found that functional cardiomyocytes can be directly induced from fibroblasts by a combination of three cardiac transcription factors, Gata4, Mef2c and Tbx5, *in vitro* and *in vivo*. I will review the perspectives of heart regeneration using reprogramming technology.

**Keywords:** heart, heart regeneration, iPSC cell, induced cardiomyocyte, reprogramming

#### Introduction

Heart disease is a leading cause of death worldwide. As adult cardiomyocytes have little regenerative capacity following injury, their malfunction or loss due to heart disease may lead to lethal consequences. Heart transplantation is an established therapy for heart failure, but is limited due to the number of donor organs available.<sup>1)</sup> Thus, new cardiac regeneration therapy is demanded, and cell replacement treatment might be an attractive option to repair injured myocardium. In particular, advances in stem cell research during the past decades have brought a tremendous amount of excitement about the possibility of using stem cells to repair damaged cardiac tissues.<sup>2)</sup>

Embryonic stem cells (ESCs) remain undifferentiated and propagate infinitely in culture, while maintaining their pluripotency to differentiate into all three embryonic germ layers and their derivatives, including cardiomyocytes. Therefore, it is possible to generate a large number of cardiomyocytes from differentiated cultures of ESCs.<sup>3)–5)</sup> Moreover, with the recent discovery of induction of pluripotency with Yamanaka's four factors, researchers can make cardiomyocytes from patients' somatic cells through induced pluripotent stem cell (iPSC) generation.<sup>6),7)</sup> Recent findings demonstrated that human pluripotent stem cells (PSCs) can be efficiently differentiated into cardiomyocytes using small molecules and growth factors.<sup>8),9)</sup> Moreover, to reduce the risk of tumor formation by contamination of residual PSCs, procedures involving cell sorting with specific cell-surface markers and a mitochondrial dye have been developed for cardiomyocyte enrichment.<sup>10)–12)</sup> Electrical coupling of human PSC-derived cardiomyocytes and the suppression of arrhythmias in injured hearts has been reported in guinea-pig recently.<sup>13)</sup> Thus, cell therapy using iPSC technology might be a potential regenerative strategy for patients with heart disease in the future.

<sup>\*1</sup> Department of Clinical and Molecular Cardiovascular Research, Keio University School of Medicine, Tokyo, Japan.

<sup>\*2</sup> Department of Cardiology, Keio University School of Medicine, Tokyo, Japan.

<sup>\*3</sup> JST, CREST, Tokyo, Japan.

<sup>†</sup> Correspondence should be addressed: M. Ieda, Department of Cardiology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan (e-mail: mieda@z8.keio.jp).

We and others recently reported that a somatic cell could be directly reprogrammed into an alternative differentiated cell type without first becoming a stem/progenitor cell.<sup>14)–18)</sup> We found that a combination of three cardiac transcription factors, Gata4, Mef2c, and Tbx5, rapidly and efficiently induced cardiomyocyte-like cells (iCMs) from mouse cardiac fibroblasts.<sup>14)</sup> The induced cardiomyocytes were similar to neonatal cardiomyocytes and could be useful as a source of cardiomyocytes for regenerative purpose.<sup>14)</sup> More recently, we and others have demonstrated that direct gene transfer of cardiac reprogramming factors in mouse infarct hearts converted resident cardiac fibroblasts into cardiomyocyte-like cells.<sup>19)–21)</sup> However, compared with the iPSC field, the direct lineage reprogramming has just emerged and more refinements and investigations will be necessary for future regenerative applications. In this article, I will review the ESC/iPSC-based cardiac regeneration and a new emerging technology, direct cardiac reprogramming.

#### Generation of cardiomyocytes from pluripotent stem cells

ESCs are undifferentiated, pluripotent and self-renewing cells in appropriate culture conditions. In their differentiated state, they can give rise to cells of all three embryonic germ layers. *In vitro* ESC differentiation requires an initial step of forming three-dimensional aggregates called embryoid bodies (EBs). EBs mimic the development of the embryos and they can differentiate into a wide variety of cell types including cardiomyocytes. The development and gene expression profiles of ESC-derived cardiomyocytes correlate well with those of developing mammalian hearts, becoming mature with time. Thus, the most successful differentiation approaches of ESCs are those that recapitulate the regulatory pathways that control cardiac differentiation in the early embryo.<sup>9)</sup> Using cues from developmental biology, significant advances have been made in cardiac differentiation from mouse and human PSCs.

Findings from serial studies demonstrated that one of the earliest stages of differentiation, the induction of cardiac progenitor cells, can be monitored by the expression of cell surface markers, vascular endothelial growth factor receptor-2 (KDR/Flk-1) and platelet-derived growth factor receptor (PDGFR)- $\alpha$ , or by the expression of transcription factors, *isl-1* and *Nkx2.5*, in the cells.<sup>22)–24)</sup> Stage-specific induction of mouse and human ESCs with chemically-defined media containing activin A, bone

morphogenetic protein-4, basic fibroblast growth factor, vascular endothelial growth factor and temporal modulation of canonical Wnt signaling results in generation of abundant KDR/PDGFR- $\alpha$  double-positive cardiac progenitor cells which can efficiently differentiate into cardiomyocytes *in vitro*.<sup>9),22)</sup> On the other hand, *mesp1* is one of the earliest transcription factors in cardiac progenitor cells and critical for heart development.<sup>25),26)</sup> *Mesp1* overexpression in mouse ESCs activated cardiac transcriptional regulators, Gata4, Mef2c, Tbx5, myocardin, and *Nkx2.5*, and differentiated the ESCs into the cardiovascular lineage.<sup>27)</sup> Further modifications of these strategies may enable induction of a large cardiac progenitor cell pool, which lead to generation of highly enriched cardiomyocytes (Fig. 1).

Generation of patient-specific cells for autologous transplantation should be pursued to avoid immune rejection of the transplanted cardiac grafts. Takahashi and Yamanaka discovered that four ESC-enriched transcription factors, Oct4, Sox2, Klf4 and c-Myc, can reprogram mouse fibroblasts into ESC-like cells, iPSCs, in 2006.<sup>7)</sup> Just one year later, human iPSCs were generated by the same set of transcriptional factors, as well as another set of factors (Fig. 1).<sup>28),29)</sup> Although there has been some concern that human iPSCs are not identical to ESCs, iPSCs are very similar to human ESCs morphologically and they have been shown to differentiate into cell types of all three germ layers, as well as cardiomyocytes.<sup>29)</sup> Initially, iPSCs were made from somatic cells by retroviral or lentiviral transduction of the required transcription factors. Transgenes are inserted randomly into the host genome by this method and may therefore disrupt normal gene expression. To avoid insertional mutagenesis, iPSCs were recently generated by transduction of the reprogramming factors without genome integration, using plasmids, mRNAs, proteins and removable transposons as well as Sendai viruses.<sup>30),31)</sup> Although there is variability among cell lines, iPSCs can be differentiated into cardiomyocytes with the same cardiac differentiation protocol originally developed for ESCs. Patient-specific iPSC-derived cardiomyocytes could be obtained for cell therapy in the future, thereby possibly avoiding the immune rejection problems. Moreover, iPSCs may offer the possibility of generating gene-corrected, patient-specific cardiac cells from individuals who have genetic diseases.<sup>32)</sup> Appropriate evaluation of safe iPSC clones as a cell source for cardiomyocytes might be critical for future applications in heart regeneration therapy.<sup>33)</sup>

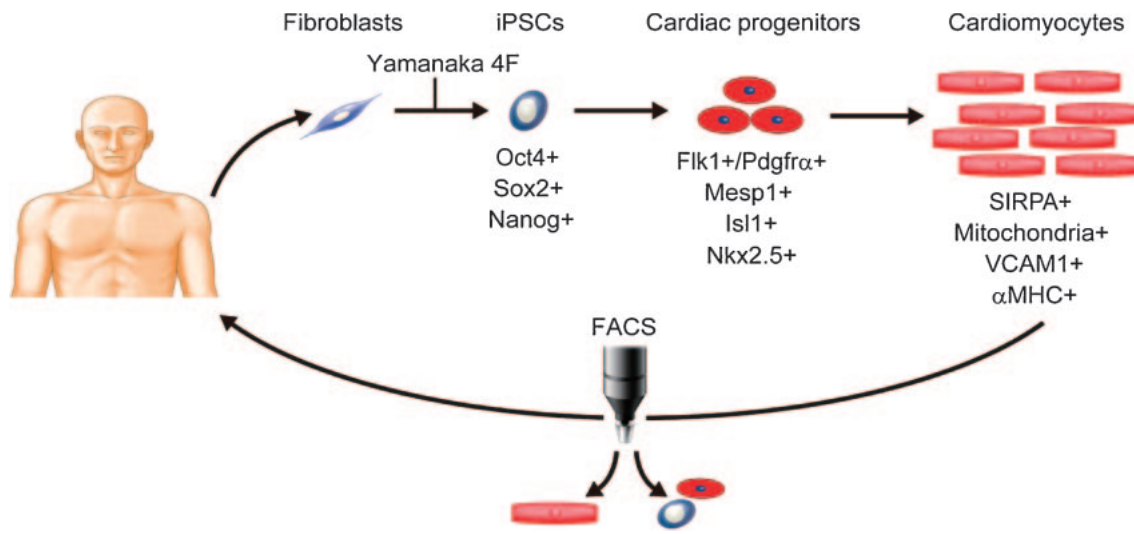


Fig. 1. Cardiac regenerative strategy through human iPS cell generation. Fibroblasts are obtained from patients, and reprogrammed into iPSCs by transduction of Yamanaka's 4 factors. The iPSCs differentiate into cardiac progenitors and cardiomyocytes, which express specific genes and markers. Pure cardiomyocytes can be sorted by FACS for cell treatment.

#### Heart repair using pluripotent stem cell-derived cardiomyocytes

As cardiomyocytes are terminally differentiated cells, myocardial tissue recovery after injury is particularly limited. The transplantation of cardiomyocytes derived from PSCs into the infarcted heart has been reported to improve cardiac function in animal models.<sup>3),5)</sup> Laflamme *et al.* reported an improvement in cardiac function 4 weeks after coronary artery ligation and injection of human ESC-derived cardiomyocytes in immunodeficient rats.<sup>3)</sup> In contrast, van Laake *et al.* reported that improvement of heart function was observed at 1 month but not at 3 months after myocardial infarction and injection of ESC-derived cardiomyocytes in mouse hearts.<sup>5)</sup> The precise mechanisms mediating the beneficial effect of transplanted ESC-derived cardiomyocytes are currently unclear, as the majority of the grafted cardiomyocytes disappeared gradually after direct injection of cells into the heart.<sup>3),34)</sup> Thus, to enhance the effect of PSC-based cardiac repair, it is important to improve the survival of grafted cells. The administration of prosurvival factors with the grafted cells may improve the survival of transplanted PSC-derived cardiomyocytes.<sup>3)</sup> The formation of aggregates of PSC-derived cardiomyocytes through cell-cell adhesion may improve their survival in the heart through auto- and paracrine factors.<sup>11)</sup> Recently, Shiba *et al.* reported that human ESC-derived cardiac grafts in injured

hearts protected against arrhythmias and contracted synchronously with host muscle in guinea-pig hearts at least till 1 month.<sup>13)</sup> Injured hearts with cardiac grafts showed improved mechanical function and a significantly reduced incidence of both spontaneous and induced ventricular tachycardia. It would be necessary to carry out safety and efficacy studies with longer follow-up durations in a large animal model in the future.

The transplantation of undifferentiated PSCs may result in the formation of teratomas. Thus, it is critical to purify cardiac cells and remove contaminated immature cells from the cardiac grafts to eliminate the risk of tumor formation. Cardiomyocyte-specific gene reporter systems can be used for the high-grade purification of cardiomyocytes, but this method requires genetic modifications.<sup>35),36)</sup> Hattori *et al.* reported that a fluorescent dye that labels mitochondria can be used to selectively mark ESC/iPSC-derived cardiomyocytes.<sup>11)</sup> They demonstrated that highly pure cardiomyocytes could be obtained by fluorescence-activated cell sorting (FACS) with this dye. Dubois *et al.* used a surface protein, signal-regulatory protein alpha (SIRPA), as a cardiac-specific marker and purified cardiomyocytes (up to 98% purity) via FACS.<sup>12)</sup> Tohyama *et al.* have reported a simple medium-exchanging procedure that enabled cardiomyocyte purification of up to 99%, based on the marked biochemical differences in glucose and lactate metabolism between cardiomyocytes and noncardiomyocytes.<sup>37)</sup>

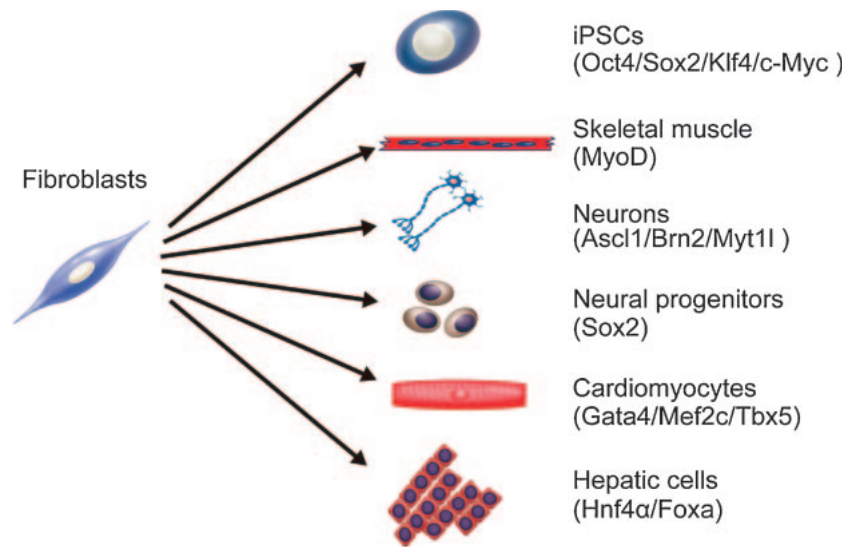


Fig. 2. Overexpression of specific reprogramming factors reprograms fibroblasts into other type of cells. Fibroblasts can be converted into iPSCs,<sup>7)</sup> skeletal muscle cells,<sup>38)</sup> neurons,<sup>17)</sup> neural progenitors,<sup>39)</sup> cardiomyocytes,<sup>14)</sup> and hepatocytes<sup>15)</sup> by transduction of lineage-specific transcription factors (parentheses).

Such non-genetic methods might be powerful tools for future clinical applications.

#### Direct reprogramming of fibroblasts into cardiomyocytes by defined factors

It is possible to avoid tumor formation by cell therapy, if target cells can be obtained directly from somatic cells without passing through stem cell generation (Fig. 2).<sup>14)–17),29),38),39)</sup> Zhou *et al.* reported that the combination of pancreatic transcription factors, Neurogenin 3, Pdx1 and Mafa, can efficiently reprogram pancreatic exocrine cells into functional  $\beta$ -cells *in vivo*.<sup>18)</sup> Vierbuchen *et al.* reported that neural-specific transcription factors, Ascl1, Brn2 and Myt11, convert dermal fibroblasts into functional neurons.<sup>17)</sup> We first found that a minimum cocktail of three cardiac-specific transcription factors, Gata4, Mef2c and Tbx5 (GMT), directly induced cardiomyocyte-like cells from mouse fibroblasts *in vitro*.<sup>14)</sup>

In our study, we used cardiac fibroblasts (CFs) as a cell source for cardiac reprogramming. CFs are found throughout cardiac tissues along with cardiomyocytes, and account for more than half of the cells in the heart.<sup>40)–42)</sup> In diseased hearts, fibroblasts proliferate and secrete extracellular matrix and growth factors which induce fibrosis and myocardial remodeling, leading to heart failure.<sup>43)</sup> To determine the candidate factors of cardiac reprogramming, we identified the genes which are specifically expressed

in embryonic cardiomyocytes. We developed a novel cell purification system in which embryonic cardiomyocytes and CFs can be purified using FACS.<sup>41)</sup> Using this system, we selected 14 factors as candidates for cardiac reprogramming, which are specifically expressed in cardiomyocytes and are critical for heart development, demonstrated in knockout mouse studies.

We next developed a screening system in which the induction of mature cardiomyocytes from fibroblasts could be analyzed quantitatively by FACS. We generated  $\alpha$  myosin heavy chain ( $\alpha$ MHC) promoter-driven EGFP-IRES-Puromycin transgenic mice ( $\alpha$ MHC-GFP), in which only mature cardiomyocytes expressed the green fluorescent protein (GFP).<sup>14),44),45)</sup> Transduction of all 14 factors into fibroblasts induced 1.7% of GFP<sup>+</sup> cells, and serial removal of individual factors demonstrated that a combination of three factors (Gata4, Mef2c and Tbx5) were sufficient for efficient GFP<sup>+</sup> cell induction (around 15%) from CFs. The three cardiac reprogramming factors, Gata4, Mef2c and Tbx5, are core cardiac transcription factors during early heart development<sup>46)–48)</sup> and are known to interact with each other, coactivate cardiac gene expression and promote cardiomyocyte differentiation.<sup>49)–51)</sup> We designated these GFP<sup>+</sup> cardiomyocyte-like cells induced cardiomyocytes (iCMs). The iCMs expressed several cardiac marker proteins, such as sarcomeric  $\alpha$ -actinin, cardiac troponin T and atrial natriuretic

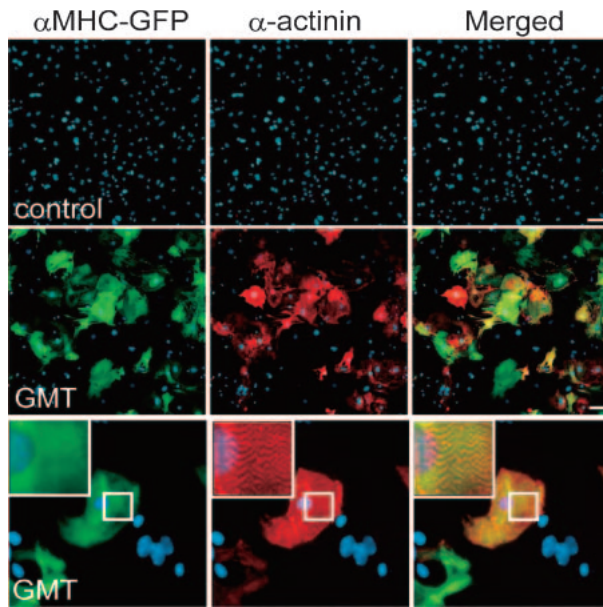
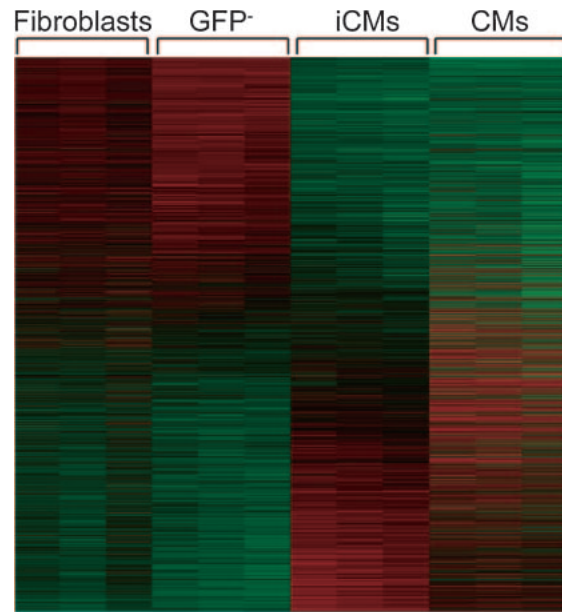


Fig. 3. Gata4/Mef2c/Tbx5 Induce cardiac gene expression in fibroblasts. Immunofluorescent staining for  $\alpha$ MHC-GFP (green),  $\alpha$ -actinin (red) and DAPI (blue). In the negative control (control), cardiac genes were not induced. The combination of three factors, Gata4/Mef2c/Tbx5 (GMT), induced abundant  $\alpha$ MHC-GFP and  $\alpha$ -actinin expression in fibroblasts. High-magnification views in insets showed sarcomeric organization.<sup>14)</sup>

factor (Fig. 3). They also had well-defined sarcomeric structures similar to neonatal cardiomyocytes. The global gene expression profile of GFP<sup>+</sup> iCMs is not identical but similar to neonatal cardiomyocytes, and different from original CFs (Fig. 4). We also found that functionally important cardiac genes, such as ion channel genes and sarcomere genes were upregulated more in 4-week iCMs than in 2-week iCMs, suggesting full maturation occurred over several weeks. The chromatin state of iCMs was also similar to cardiomyocytes but different from fibroblasts in histone modifications and DNA methylation patterns.<sup>14)</sup> These results suggest that iCMs are cardiomyocyte-like cells in gene expression and epigenetic states. We also demonstrated that iCMs possessed functional properties which are characteristic of cardiomyocytes. The iCMs exhibited intracellular Ca<sup>2+</sup> transient and action potentials after 2–4 weeks of culture. In addition, CF-derived iCMs contracted spontaneously.

To determine if the iCMs could arise from cells in other organs, we transduced the three factors into mouse tail-tip fibroblasts (TTFs). We found that TTF-derived GFP<sup>+</sup> cells expressed cardiac markers



FDR-adj  $p < 0.0001$  in at least one comparison

Fig. 4. Global gene expression pattern of induced cardiomyocytes is similar to cardiomyocytes. Heatmap image of microarray data illustrating differentially expressed genes among fibroblasts,  $\alpha$ -MHC-GFP<sup>-</sup> cells (GFP<sup>-</sup>), iCMs ( $\alpha$ -MHC-GFP<sup>+</sup> cells sorted 4 weeks after GMT transduction) and cardiomyocytes (CMs) ( $n = 3$  in each group). FDR-adj  $p < 0.0001$  in at least one comparison was shown. Global gene expression pattern of iCMs is not identical but similar to cardiomyocytes.<sup>14)</sup>

and spontaneous intracellular Ca<sup>2+</sup> transient, while the percentage of cardiac troponin T<sup>+</sup> cells was 50% less than that of CF-derived iCMs. The TTF-derived iCMs had some functional characteristics of cardiomyocytes, such as spontaneous intracellular Ca<sup>2+</sup> transients, but we did not observe cellular contraction, suggesting that TTFs are more resistant to cardiac reprogramming by GMT. These findings exclude the possibility that iCMs arise from contamination of cardiomyocytes or cardiac progenitors in the fibroblast population.<sup>52),53)</sup>

While direct conversion of somatic cells to  $\beta$ -cells and neurons has been reported, the “route” of cell fate alteration is not clear. There are two possibilities for the reprogramming of fibroblasts into differentiated cardiomyocytes. One is a direct conversion of fibroblasts into differentiated cardiomyocytes and the other is that fibroblasts first revert to a cardiac progenitor/stem cell fate before further cardiac differentiation. We were able to genetically test these two possibilities by using the mice expressing Isl1-Cre-yellow fluorescent protein (YFP) and Mesp1-





Fig. 5. Gata4/Mef2c/Tbx5 Injection into mouse infarct hearts. Strategy for *in vivo* cardiac reprogramming by Gata4, Mef2c and Tbx5. Myocardial infarction was generated by coronary artery ligation in mouse. A pool of Gata4, Mef2c and Tbx5 was directly injected into the mouse hearts immediately after coronary artery ligation. Gene transfer of reprogramming factors induced new cardiomyocyte generation in a subset of fibroblasts in infarcted hearts.

Cre-YFP obtained by crossing *Isl1-Cre* or *Mesp1-Cre* mice with R26R-EYFP mice.<sup>54)</sup> *Isl1* and *Mesp1* are transiently expressed in early cardiac progenitor cells before further cardiac differentiation.<sup>25),55)</sup> We found that iCMs induced from *Isl1-Cre-YFP* and *Mesp1-Cre-YFP* fibroblasts did not express YFP, suggesting the fibroblasts directly reprogrammed into differentiated cardiomyocytes without passing through a cardiac progenitor cell state, with GMT transduction. We next investigated whether GMT-transduced CFs can be transplanted *in vivo* and be reprogrammed into cardiomyocytes within the heart. CFs infected with GMT expressed  $\alpha$ -MHC GFP and sarcomeric markers, and converted into cardiomyocyte-like cells in the mouse hearts after 2 weeks of cell transplantation.

Following our initial report, other groups also reported generation of cardiomyocyte-like cells from mouse fibroblasts based on the same factors or microRNAs. Song *et al.* reported that adding *Hand2* to GMT converted adult CFs and TTFs into functional cardiomyocyte-like cells more efficiently than GMT alone.<sup>20)</sup> Protze *et al.* found that the combination of *Mef2c*, *Myocardin* and *Tbx5* upregulated a broad spectrum of cardiac genes and induced functional cardiomyocytes from mouse embryonic fibroblasts and CFs.<sup>56)</sup> Jayawardena *et al.* reported that a combination of muscle-specific microRNAs, *mir-1*, *133*, *208* and *499*, can convert CFs into functional cardiomyocyte-like cells.<sup>57)</sup> Thus, both GMT and other combinations of factors can induce cardiac reprogramming, which is similar to the experience in the iPSC field. Although the induction of fully matured cardiac cells remains low in culture and further refinements are needed, these results suggest that large amounts of an individual's own fibroblasts can be transduced with the cardiac

reprogramming factors *ex vivo* and be delivered to damaged myocardium for regenerative purposes.

#### Induction of cardiomyocyte-like cells *in vivo* by gene transfer of cardiac reprogramming factors

Based on the findings of direct cardiac reprogramming *in vitro*, we investigated whether gene transfer of GMT into mouse injured hearts could similarly induce cardiomyocyte generation (Fig. 5).<sup>21)</sup> We first confirmed that *in vitro* transduction of GMT retrovirus converted adult cardiac fibroblasts from the mouse infarct region into iCMs with cardiac-specific gene expression and sarcomeric structures. Injection of GMT retrovirus into  $\alpha$ MHC-GFP transgenic mouse hearts induced the expression of  $\alpha$ MHC-GFP, a reporter of cardiomyocytes, in 3% of virus-infected cells. A mixture of GMT injection into the immunosuppressed mouse hearts induced cardiac protein expression in retrovirus-infected cells within 2 weeks with a conversion rate of approximately 1%, although few cells showed striated muscle structures. Next, to transduce GMT more efficiently *in vivo*, we developed a polycistronic retrovirus vector expressing GMT separated by 2A "self-cleaving" peptides (3F2A). *In vivo* gene transfer of 3F2A into infarct hearts resulted in generation of induced cardiomyocyte-like cells in fibrotic tissues, which expressed sarcomeric  $\alpha$ -actinin, cardiac troponin T, and several cardiac-specific genes, and had clear cross striations (Fig. 6). These results suggest that 3F2A-iCMs were more mature cardiomyocytes and that the polycistronic vector can be used for cellular reprogramming *in vivo*.

In parallel with us, two other groups also published independently *in vivo* cardiac reprogramming in mouse infarct hearts. Qian *et al.* found that non-myocytes in infarcted hearts were converted into

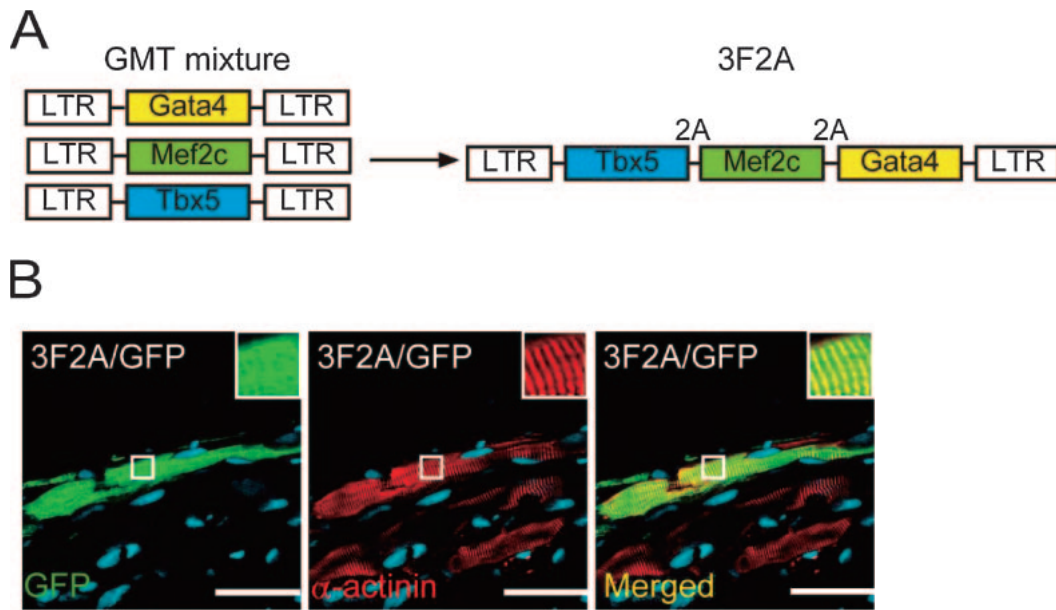


Fig. 6. Induction of cardiomyocyte-like cells in infarct hearts by a single polycistronic vector of Gata4/Mef2c/Tbx5. (A) We developed a polycistronic retrovirus expressing GMT at near equimolar levels from the same promoter using “self-cleaving” 2A peptides (3F2A). Schematic representation of the 3F2A retrovirus containing Gata4, Mef2c and Tbx5 (GMT).<sup>14)</sup> (B) 3F2A retrovirus induced  $\alpha$ -actinin expression and cross striations. White boxes are enlarged in the insets.<sup>14)</sup>

cardiomyocyte-like cells by GMT retroviral gene transfer.<sup>19)</sup> Song *et al.* reported that adding Hand2 to GMT converted CFs into functional cardiomyocyte-like cells more efficiently than GMT alone *in vivo*.<sup>20)</sup> Both studies demonstrated that the *in vivo* iCMs had well-organized sarcomeric structures and exhibited functional characteristics of adult ventricular cardiomyocytes, including cellular contraction, electrophysiological properties and functional coupling to other cardiac cells. They also demonstrated that retroviral gene transfer of reprogramming factors into infarct hearts significantly improved cardiac function and reduced fibrosis 2 and 3 months after myocardial infarction. Although all three studies, including us, demonstrated *in vivo* cardiac reprogramming, the approaches used to address this issue differ. The other two groups used mainly fibroblast-lineage tracing mice to demonstrate cardiac conversion from CFs, while we took an alternative approach of co-transduction with GMT plus marker genes (GFP or DsRed) to demonstrate cardiac induction in the infarct regions using  $\alpha$ MHC-GFP transgenic or nude mice. It is possible that the different mouse lines and viral titers used may result in different outcomes among the three studies. Although it is not clear how many newly generated iCMs remained in the injured hearts in the longer-term follow-up and to what extent the iCMs

contributed to the improvement in cardiac function, these findings might inform new regenerative strategies for repairing injured hearts. Further works in larger animals, generation of human iCMs and more efficient protocol for direct cardiac reprogramming are needed in the future research.

### Conclusions

The work of numerous laboratories has led to significant therapeutic and scientific advances in stem cell applications to cardiac regenerative medicine. Nevertheless, the issues relating to cardiac differentiation efficiency, risk of tumor formation and the time-consuming steps required to generate a large number of cardiomyocytes through iPSCs need to be solved before clinical applications. The new direct cardiac reprogramming technology, in which target cardiac cells can be generated from fibroblast cells by defined factors without reverting to stem cells, may overcome these issues.<sup>14)</sup> Although much refinement of the reprogramming procedures will be necessary, this new technology raises the possibility of reprogramming endogenous cardiac fibroblasts into functional cardiomyocytes. Studies in human cells, the safe and efficient delivery system of defined factors *in vivo*, and an understanding of the molecular mechanisms involved in direct cardiac reprogramming are necessary. A combination of iPSC-based

cardiomyocyte therapy and direct cardiac reprogramming may facilitate the translation of heart regenerative research into clinical applications in the future.

### Acknowledgements

This study was supported in part by research grants from the JST CREST, JSPS, The Mitsubishi Foundation, AstraZeneca, Takeda Science Foundation, Banyu Life Science, The Uehara Memorial Foundation, Kimura Memorial Heart Foundation, Japan Research Foundation for Clinical Pharmacology, Nateglinide Memorial Toyoshima Research and Education Fund, and SENSHIN Medical Research Foundation.

### References

- 1) Taylor, D.O., Edwards, L.B., Aurora, P., Christie, J.D., Dobbels, F., Kirk, R., Rahmel, A.O., Kucheryavaya, A.Y. and Hertz, M.I. (2008) Registry of the international society for heart and lung transplantation: Twenty-fifth official adult heart transplant report–2008. *J. Heart Lung Transplant.* **27**, 943–956.
- 2) Mummery, C., Ward, D., van den Brink, C.E., Bird, S.D., Doevendans, P.A., Opthof, T., Brutel de la Riviere, A., Tertoolen, L., van der Heyden, M. and Pera, M. (2002) Cardiomyocyte differentiation of mouse and human embryonic stem cells. *J. Anat.* **200**, 233–242.
- 3) Laflamme, M.A., Chen, K.Y., Naumova, A.V., Muskheli, V., Fugate, J.A., Dupras, S.K., Reinecke, H., Xu, C., Hassanipour, M., Police, S., O’Sullivan, C., Collins, L., Chen, Y., Minami, E., Gill, E.A., Ueno, S., Yuan, C., Gold, J. and Murry, C.E. (2007) Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. *Nat. Biotechnol.* **25**, 1015–1024.
- 4) Nussbaum, J., Minami, E., Laflamme, M.A., Virag, J.A., Ware, C.B., Masino, A., Muskheli, V., Pabon, L., Reinecke, H. and Murry, C.E. (2007) Transplantation of undifferentiated murine embryonic stem cells in the heart: Teratoma formation and immune response. *FASEB J.* **21**, 1345–1357.
- 5) van Laake, L.W., Passier, R., Doevendans, P.A. and Mummery, C.L. (2008) Human embryonic stem cell-derived cardiomyocytes and cardiac repair in rodents. *Circ. Res.* **102**, 1008–1010.
- 6) Zhang, J., Wilson, G.F., Soerens, A.G., Koonce, C.H., Yu, J., Palecek, S.P., Thomson, J.A. and Kamp, T.J. (2009) Functional cardiomyocytes derived from human induced pluripotent stem cells. *Circ. Res.* **104**, e30–e41.
- 7) Takahashi, K. and Yamanaka, S. (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**, 663–676.
- 8) Lian, X., Hsiao, C., Wilson, G., Zhu, K., Hazeltine, L.B., Azarin, S.M., Raval, K.K., Zhang, J., Kamp, T.J. and Palecek, S.P. (2012) Robust cardiomyocyte differentiation from human pluripotent stem cells via temporal modulation of canonical wnt signaling. *Proc. Natl. Acad. Sci. U.S.A.* **109**, E1848–E1857.
- 9) Kattman, S.J., Witty, A.D., Gagliardi, M., Dubois, N.C., Niapour, M., Hotta, A., Ellis, J. and Keller, G. (2011) Stage-specific optimization of activin/nodal and bmp signaling promotes cardiac differentiation of mouse and human pluripotent stem cell lines. *Cell Stem Cell* **8**, 228–240.
- 10) Uosaki, H., Fukushima, H., Takeuchi, A., Matsuoka, S., Nakatsuji, N., Yamanaka, S. and Yamashita, J.K. (2011) Efficient and scalable purification of cardiomyocytes from human embryonic and induced pluripotent stem cells by vcam1 surface expression. *PLoS ONE* **6**, e23657.
- 11) Hattori, F., Chen, H., Yamashita, H., Tohyama, S., Satoh, Y.S., Yuasa, S., Li, W., Yamakawa, H., Tanaka, T., Onitsuka, T., Shimoji, K., Ohno, Y., Egashira, T., Kaneda, R., Murata, M., Hidaka, K., Morisaki, T., Sasaki, E., Suzuki, T., Sano, M., Makino, S., Oikawa, S. and Fukuda, K. (2010) Nongenetic method for purifying stem cell-derived cardiomyocytes. *Nat. Methods* **7**, 61–66.
- 12) Dubois, N.C., Craft, A.M., Sharma, P., Elliott, D.A., Stanley, E.G., Elefanty, A.G., Gramolini, A. and Keller, G. (2011) Sirpa is a specific cell-surface marker for isolating cardiomyocytes derived from human pluripotent stem cells. *Nat. Biotechnol.* **29**, 1011–1018.
- 13) Shiba, Y., Fernandes, S., Zhu, W.Z., Filice, D., Muskheli, V., Kim, J., Palpant, N.J., Gantz, J., Moyes, K.W., Reinecke, H., Van Biber, B., Dardas, T., Mignone, J.L., Izawa, A., Hanna, R., Viswanathan, M., Gold, J.D., Kotlikoff, M.I., Sarvazyan, N., Kay, M.W., Murry, C.E. and Laflamme, M.A. (2012) Human ES-cell-derived cardiomyocytes electrically couple and suppress arrhythmias in injured hearts. *Nature* **489**, 322–325.
- 14) Ieda, M., Fu, J.D., Delgado-Olguin, P., Vedantham, V., Hayashi, Y., Bruneau, B.G. and Srivastava, D. (2010) Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. *Cell* **142**, 375–386.
- 15) Sekiya, S. and Suzuki, A. (2011) Direct conversion of mouse fibroblasts to hepatocyte-like cells by defined factors. *Nature* **475**, 390–393.
- 16) Pang, Z.P., Yang, N., Vierbuchen, T., Ostermeier, A., Fuentes, D.R., Yang, T.Q., Citri, A., Sebastiano, V., Marro, S., Sudhof, T.C. and Wernig, M. (2011) Induction of human neuronal cells by defined transcription factors. *Nature* **476**, 220–223.
- 17) Vierbuchen, T., Ostermeier, A., Pang, Z.P., Kokubu, Y., Sudhof, T.C. and Wernig, M. (2010) Direct conversion of fibroblasts to functional neurons by defined factors. *Nature* **463**, 1035–1041.
- 18) Zhou, Q., Brown, J., Kanarek, A., Rajagopal, J. and Melton, D.A. (2008) *In vivo* reprogramming of



- adult pancreatic exocrine cells to  $\beta$ -cells. *Nature* **455**, 627–632.
- 19) Qian, L., Huang, Y., Spencer, C.I., Foley, A., Vedantham, V., Liu, L., Conway, S.J., Fu, J.D. and Srivastava, D. (2012) *In vivo* reprogramming of murine cardiac fibroblasts into induced cardiomyocytes. *Nature* **485**, 593–598.
  - 20) Song, K., Nam, Y.J., Luo, X., Qi, X., Tan, W., Huang, G.N., Acharya, A., Smith, C.L., Tallquist, M.D., Neilson, E.G., Hill, J.A., Bassel-Duby, R. and Olson, E.N. (2012) Heart repair by reprogramming non-myocytes with cardiac transcription factors. *Nature* **485**, 599–604.
  - 21) Inagawa, K., Miyamoto, K., Yamakawa, H., Muraoka, N., Sadahiro, T., Umei, T., Wada, R., Katsumata, Y., Kaneda, R., Nakade, K., Kurihara, C., Obata, Y., Miyake, K., Fukuda, K. and Ieda, M. (2012) Induction of cardiomyocyte-like cells in infarct hearts by gene transfer of Gata4, Mef2c, and Tbx5. *Circ. Res.* **111**, 1147–1156.
  - 22) Kattman, S.J., Huber, T.L. and Keller, G.M. (2006) Multipotent flk-1<sup>+</sup> cardiovascular progenitor cells give rise to the cardiomyocyte, endothelial, and vascular smooth muscle lineages. *Dev. Cell* **11**, 723–732.
  - 23) Qyang, Y., Martin-Puig, S., Chiravuri, M., Chen, S., Xu, H., Bu, L., Jiang, X., Lin, L., Granger, A., Moretti, A., Caron, L., Wu, X., Clarke, J., Taketo, M.M., Laugwitz, K.L., Moon, R.T., Gruber, P., Evans, S.M., Ding, S. and Chien, K.R. (2007) The renewal and differentiation of *Isl1*<sup>+</sup> cardiovascular progenitors are controlled by a wnt/ $\beta$ -catenin pathway. *Cell Stem Cell* **1**, 165–179.
  - 24) Wu, S.M., Fujiwara, Y., Cibulsky, S.M., Clapham, D.E., Lien, C.L., Schultheiss, T.M. and Orkin, S.H. (2006) Developmental origin of a bipotential myocardial and smooth muscle cell precursor in the mammalian heart. *Cell* **127**, 1137–1150.
  - 25) Bondue, A., Lapouge, G., Paulissen, C., Semeraro, C., Iacovino, M., Kyba, M. and Blanpain, C. (2008) *Mesp1* acts as a master regulator of multipotent cardiovascular progenitor specification. *Cell Stem Cell* **3**, 69–84.
  - 26) David, R., Brenner, C., Stieber, J., Schwarz, F., Brunner, S., Vollmer, M., Mentele, E., Muller-Hocker, J., Kitajima, S., Lickert, H., Rupp, R. and Franz, W.M. (2008) *MesP1* drives vertebrate cardiovascular differentiation through Dkk-1-mediated blockade of Wnt-signalling. *Nat. Cell Biol.* **10**, 338–345.
  - 27) Lindsley, R.C., Gill, J.G., Murphy, T.L., Langer, E.M., Cai, M., Mashayekhi, M., Wang, W., Niwa, N., Nerbonne, J.M., Kyba, M. and Murphy, K.M. (2008) *Mesp1* coordinately regulates cardiovascular fate restriction and epithelial-mesenchymal transition in differentiating ESCs. *Cell Stem Cell* **3**, 55–68.
  - 28) Yu, J., Vodyanik, M.A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J.L., Tian, S., Nie, J., Jonsdottir, G.A., Ruotti, V., Stewart, R., Slukvin, I.I. and Thomson, J.A. (2007) Induced pluripotent stem cell lines derived from human somatic cells. *Science* **318**, 1917–1920.
  - 29) Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K. and Yamanaka, S. (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**, 861–872.
  - 30) Okita, K. and Yamanaka, S. (2010) Induction of pluripotency by defined factors. *Exp. Cell Res.* **316**, 2565–2570.
  - 31) Seki, T., Yuasa, S., Oda, M., Egashira, T., Yae, K., Kusumoto, D., Nakata, H., Tohyama, S., Hashimoto, H., Kodaira, M., Okada, Y., Seimiya, H., Fusaki, N., Hasegawa, M. and Fukuda, K. (2010) Generation of induced pluripotent stem cells from human terminally differentiated circulating T cells. *Cell Stem Cell* **7**, 11–14.
  - 32) Tiscornia, G., Monserrat, N. and Belmonte, J.C. (2011) Modelling long QT syndrome with iPS cells: Be still, my beating heart... *Circ. Res.* **108**, 648–649.
  - 33) Tsuji, O., Miura, K., Okada, Y., Fujiyoshi, K., Mukaino, M., Nagoshi, N., Kitamura, K., Kumagai, G., Nishino, M., Tomisato, S., Higashi, H., Nagai, T., Katoh, H., Kohda, K., Matsuzaki, Y., Yuzaki, M., Ikeda, E., Toyama, Y., Nakamura, M., Yamanaka, S. and Okano, H. (2010) Therapeutic potential of appropriately evaluated safe-induced pluripotent stem cells for spinal cord injury. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 12704–12709.
  - 34) Zhang, M., Methot, D., Poppa, V., Fujio, Y., Walsh, K. and Murry, C.E. (2001) Cardiomyocyte grafting for cardiac repair: Graft cell death and anti-death strategies. *J. Mol. Cell. Cardiol.* **33**, 907–921.
  - 35) Caspi, O., Huber, I., Kehat, I., Habib, M., Arbel, G., Gepstein, A., Yankelson, L., Aronson, D., Beyar, R. and Gepstein, L. (2007) Transplantation of human embryonic stem cell-derived cardiomyocytes improves myocardial performance in infarcted rat hearts. *J. Am. Coll. Cardiol.* **50**, 1884–1893.
  - 36) Huber, I., Itzhaki, I., Caspi, O., Arbel, G., Tzukerman, M., Gepstein, A., Habib, M., Yankelson, L., Kehat, I. and Gepstein, L. (2007) Identification and selection of cardiomyocytes during human embryonic stem cell differentiation. *FASEB J.* **21**, 2551–2563.
  - 37) Tohyama, S., Hattori, F., Sano, M., Hishiki, T., Nagahata, Y., Matsuura, T., Hashimoto, H., Suzuki, T., Yamashita, H., Satoh, Y., Egashira, T., Seki, T., Muraoka, N., Yamakawa, H., Ohgino, Y., Tanaka, T., Yoichi, M., Yuasa, S., Murata, M., Suematsu, M. and Fukuda, K. (2013) Distinct metabolic flow enables large-scale purification of mouse and human pluripotent stem cell-derived cardiomyocytes. *Cell Stem Cell* **12**, 127–137.
  - 38) Davis, R.L., Weintraub, H. and Lassar, A.B. (1987) Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell* **51**, 987–1000.
  - 39) Ring, K.L., Tong, L.M., Balestra, M.E., Javier, R., Andrews-Zwilling, Y., Li, G., Walker, D., Zhang, W.R., Kreitzer, A.C. and Huang, Y. (2012) Direct

- reprogramming of mouse and human fibroblasts into multipotent neural stem cells with a single factor. *Cell Stem Cell* **11**, 100–109.
- 40) Baudino, T.A., Carver, W., Giles, W. and Borg, T.K. (2006) Cardiac fibroblasts: Friend or foe? *Am. J. Physiol. Heart Circ. Physiol.* **291**, H1015–H1026.
- 41) Ieda, M., Tsuchihashi, T., Ivey, K.N., Ross, R.S., Hong, T.T., Shaw, R.M. and Srivastava, D. (2009) Cardiac fibroblasts regulate myocardial proliferation through  $\beta 1$  integrin signaling. *Dev. Cell* **16**, 233–244.
- 42) Camelliti, P., Borg, T.K. and Kohl, P. (2005) Structural and functional characterisation of cardiac fibroblasts. *Cardiovasc. Res.* **65**, 40–51.
- 43) Weber, K.T. and Brilla, C.G. (1991) Pathological hypertrophy and cardiac interstitium. Fibrosis and renin-angiotensin-aldosterone system. *Circulation* **83**, 1849–1865.
- 44) Ieda, M., Kanazawa, H., Kimura, K., Hattori, F., Ieda, Y., Taniguchi, M., Lee, J.K., Matsumura, K., Tomita, Y., Miyoshi, S., Shimoda, K., Makino, S., Sano, M., Kodama, I., Ogawa, S. and Fukuda, K. (2007) Sema3a maintains normal heart rhythm through sympathetic innervation patterning. *Nat. Med.* **13**, 604–612.
- 45) Gulick, J., Subramaniam, A., Neumann, J. and Robbins, J. (1991) Isolation and characterization of the mouse cardiac myosin heavy chain genes. *J. Biol. Chem.* **266**, 9180–9185.
- 46) Zhao, R., Watt, A.J., Battle, M.A., Li, J., Bondow, B.J. and Duncan, S.A. (2008) Loss of both GATA4 and GATA6 blocks cardiac myocyte differentiation and results in acardia in mice. *Dev. Biol.* **317**, 614–619.
- 47) Srivastava, D. (2006) Making or breaking the heart: From lineage determination to morphogenesis. *Cell* **126**, 1037–1048.
- 48) Olson, E.N. (2006) Gene regulatory networks in the evolution and development of the heart. *Science* **313**, 1922–1927.
- 49) Ghosh, T.K., Song, F.F., Packham, E.A., Buxton, S., Robinson, T.E., Ronksley, J., Self, T., Bonser, A.J. and Brook, J.D. (2009) Physical interaction between TBX5 and MEF2C is required for early heart development. *Mol. Cell. Biol.* **29**, 2205–2218.
- 50) Garg, V., Kathiriya, I.S., Barnes, R., Schluterman, M.K., King, I.N., Butler, C.A., Rothrock, C.R., Eapen, R.S., Hirayama-Yamada, K., Joo, K., Matsuoka, R., Cohen, J.C. and Srivastava, D. (2003) GATA4 mutations cause human congenital heart defects and reveal an interaction with TBX5. *Nature* **424**, 443–447.
- 51) Morin, S., Charron, F., Robitaille, L. and Nemer, M. (2000) GATA-dependent recruitment of MEF2 proteins to target promoters. *EMBO J.* **19**, 2046–2055.
- 52) Andersen, D.C., Andersen, P., Schneider, M., Jensen, H.B. and Sheikh, S.P. (2009) Murine “cardiospheres” are not a source of stem cells with cardiomyogenic potential. *Stem Cells* **27**, 1571–1581.
- 53) Davis, D.R., Zhang, Y., Smith, R.R., Cheng, K., Terrovitis, J., Malliaras, K., Li, T.S., White, A., Makkar, R. and Marban, E. (2009) Validation of the cardiosphere method to culture cardiac progenitor cells from myocardial tissue. *PLoS ONE* **4**, e7195.
- 54) Srinivas, S., Watanabe, T., Lin, C.S., William, C.M., Tanabe, Y., Jessell, T.M. and Costantini, F. (2001) Cre reporter strains produced by targeted insertion of *EYFP* and *ECFP* into the *ROSA26* locus. *BMC Dev. Biol.* **1**, 4.
- 55) Laugwitz, K.L., Moretti, A., Lam, J., Gruber, P., Chen, Y., Woodard, S., Lin, L.Z., Cai, C.L., Lu, M.M., Reth, M., Platoshyn, O., Yuan, J.X., Evans, S. and Chien, K.R. (2005) Postnatal *Isl1*<sup>+</sup> cardioblasts enter fully differentiated cardiomyocyte lineages. *Nature* **433**, 647–653.
- 56) Protze, S., Khattak, S., Poulet, C., Lindemann, D., Tanaka, E.M. and Ravens, U. (2012) A new approach to transcription factor screening for reprogramming of fibroblasts to cardiomyocyte-like cells. *J. Mol. Cell. Cardiol.* **53**, 323–332.
- 57) Jayawardena, T.M., Egemnazarov, B., Finch, E.A., Zhang, L., Payne, J.A., Pandya, K., Zhang, Z., Rosenberg, P., Mirotsov, M. and Dzau, V.J. (2012) MicroRNA-mediated in vitro and in vivo direct reprogramming of cardiac fibroblasts to cardiomyocytes. *Circ. Res.* **110**, 1465–1473.

(Received Dec. 4, 2012; accepted Jan. 17, 2013)

## Profile

Masaki Ieda was born in 1971 and graduated from Keio University School of Medicine in 1995 to obtain M.D. He performed residency and worked as a medical staff in Internal Medicine, Keio University School of Medicine (1995–1999). He received Ph.D. from Keio University School of Medicine in 2005 for the study of the molecular mechanisms of heart development and disease under the direction of Prof. Satoshi Ogawa and Keiichi Fukuda. Dr. Ieda was an instructor in Keio University School of Medicine and continued his research (2005–2007). He moved to the Gladstone Institute of Cardiovascular Disease, University of California, San Francisco, U.S.A., as a Postdoctoral Fellow (2007–2009), and was promoted to be a research scientist in the same institute (2009–2010). After coming back to Japan, he was promoted to the Research Director in Japan Science and Technology Agency (JST), Core Research of Evolutional Science & Technology (CREST), and the Project Assistant Professor in Keio University School of Medicine. He performed a pioneering work in direct reprogramming, which is altering one mature cell type into another without first becoming stem cells. He demonstrated that overexpression of three cardiac-specific transcription factors, *Gata4*, *Mef2c* and *Tbx5*, induced cardiomyocyte-like cells from mouse fibroblasts *in vitro* (Ieda *et al.* (2010) Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. *Cell*. **142**, 375–386). This is the first demonstration of generating cardiomyocytes from non-myocytes by defined factors and has brought a tremendous amount of excitement about the possibility of using direct reprogramming technology in regenerative medicine. Subsequently, he and other researchers conducted *in vivo* direct cardiac reprogramming to regenerate mouse ischemic hearts (Refs. 19–21). He was awarded Young Investigator's Award (2006), Yagi's Award (2007), Cardiovascular Regeneration Medical Science Award (2011) from Japanese Circulation Society, and Louis N. & Arnold M. Katz Young Investigator Award (2008) from American Heart Association. He was given the Medical Research Encouragement Prize from The Japan Medical Association in 2011, and the JSPS PRIZE from JSPS for his study in the Mechanisms of Heart Development and Regeneration in 2012. He is now working in Keio University School of Medicine as the Project Assistant Professor, and in JST CREST as the Research Director.

