REVIEW

Heart Development and Regeneration via Cellular Interaction and Reprogramming

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The heart consists of many types of cells, including cardiomyocytes, vascular cells, neural cells, and cardiac fibroblasts. Adult cardiomyocytes are terminally differentiated cells, and loss of cardiomyocytes as a result of heart damage is irreversible. To regenerate damaged hearts and restore cardiac function, understanding the cellular and molecular basis of heart development is of considerable importance. Although it is well known that heart function is tightly regulated by cell–cell interactions, their roles in heart development are not clear. Recent studies, including ours, identified important roles of cell–cell interactions in heart development and function. The balance between neural chemoattractants and chemorepellents secreted from cardiomyocytes determines cardiac nervous development. Nerve growth factor is a potent chemoattractant synthesized by cardiomyocytes, whereas Sema3a is a neural chemorepellent expressed specifically in the subendocardium. Disruption of this molecular balance induces disorganized cardiac innervation and may lead to sudden cardiac death due to lethal arrhythmias. Cardiac fibroblasts, of which there are large populations in the heart, secrete high levels of specific extracellular matrix and growth factors. Embryonic cardiac fibroblast-specific secreted factors collaboratively promote mitotic activity of embryonic cardiomyocytes and expansion of ventricular chambers during cardiogenesis. More recently, utilizing knowledge of the regulatory mechanisms of heart development, we found that cardiac fibroblasts can be directly reprogrammed into cardiomyocyte-like cells *in vitro* and *in vivo* by gene transfer of cardiac-specific transcription factors. Understanding the mechanisms of heart development and cardiac reprogramming technology may provide new therapeutic approaches for heart disease in the future. (doi: 10.2302/kjm.2012-0020-RE; Keio J Med 62 (4) : 99–106, December 2013)

***Keywords*: sympathetic nervous system, growth factor, fibroblasts, reprogramming, regeneration**

Introduction

Heart consists not only of cardiomyocytes, but also of many additional types of cells, including vascular cells, neural cells, and cardiac fibroblasts. Heart function is tightly regulated by the interactions between cardiomyocytes and other types of cells through the secretion of molecules and extracellular matrix (ECM). Understanding these cell–cell interactions and their molecular mechanisms during heart development might provide insight to new therapeutic approaches for heart disease.

Compared with other organs, heart is extensively innervated via the autonomic nervous system, which comprises sympathetic and parasympathetic nerves. The sympathetic nervous system produces norepinephrine, which increases the heart rate, conduction velocity of cardiac cell excitation, and myocardial contraction and relaxation. It is well known that sympathetic innervation

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density is strictly determined within the heart, and is high in the subepicardium and the central conduction system.[1] The regional differences in sympathetic innervation influence specific cardiac functions, effectively controlling heart rate and myocardial contraction. Despite the clinical importance of cardiac innervation density, little is known about the regulatory mechanisms underlying sympathetic nerve development.

Cardiac fibroblasts are found throughout cardiac tissues, along with cardiomyocytes, and account for more than half of the cells in the heart.[1,2] Under physiological conditions, fibroblasts provide a mechanical scaffold for cardiomyocytes and coordinate the pump function of the heart.[3] In diseased hearts, fibroblasts proliferate and secrete ECM and growth factors that promote cardiomyocyte hypertrophy, leading to myocardial remodeling and heart failure.[4,5] Although these findings demonstrate that cardiac fibroblasts are critical in adult heart function, little is known about their developmental roles in the embryonic heart.

In contrast to cardiac fibroblasts, post-natal cardiomyocytes have little or no regenerative capacity. Loss of terminally differentiated cardiomyocytes as a result of heart disease is irreversible; consequently, new therapeutic approaches are demanded. The large population of endogenous cardiac fibroblasts might be a potential source of cardiomyocytes for regenerative purposes, if it proves possible to directly convert resident fibroblasts into beating cardiomyocytes. The generation of induced pluripotent stem cells (iPSCs) by Dr. Yamanaka and colleagues suggested that a specific combination of defined factors could alter the global gene expression profile of a cell and allow greater plasticity of cell type than was previously appreciated.

We and others demonstrated that cellular interactions during development are critical for proper heart function, and that their disruption may lead to various kinds of heart disease. This article first reviews the molecular mechanisms of heart development, focusing on the crosstalk among cardiac sympathetic nerves, fibroblasts, and cardiomyocytes. Based on these studies, we recently found that overexpression of cardiac-specific transcription factors can convert cardiac fibroblasts into cardiomyocyte-like cells *in vitro* and *in vivo*.

Nerve Growth Factor is Critical for Development of the Cardiac Sympathetic and Sensory Nervous Systems

The heart is highly innervated by autonomic nervous systems, including sympathetic, parasympathetic, and sensory nerves, derived from neural crest cells. Cardiac sympathetic nerves extend from sympathetic neurons in the stellate ganglia, which are located bilateral to the thoracic vertebra. Sympathetic nerve fibers project from the base of the heart into the myocardium, and are located predominantly in the subepicardium of the ventricle.[6,7] The central conduction system, which includes the sinoatrial node, atrioventricular node, and bundle of His, is abundantly innervated compared with the working myocardium.[7–10] This regional difference in cardiac sympathetic innervation is highly conserved among mammals.[7,8,11,12] Cardiac sensory neurons, which are located in the dorsal root ganglia, are derived from trunk neural crest cells, just as sympathetic neurons are. The cardiac sensory nervous system is responsible for pain perception and for initiating a protective cardiovascular response during myocardial ischemia.

The growth-cone behavior of nerves is modulated by the balance between neural chemoattractants and chemorepellents synthesized in the innervated tissue. Nerve growth factor (NGF), a potent neural chemoattractant, is a prototypic member of the neurotrophin family, and the levels of NGF expression within innervated tissues correspond approximately to the levels of sympathetic innervation density. Indeed, the sympathetic ganglion volume is reduced by 80% at postnatal day 3 in mice with disruption of the NGF gene,[13] while in mice lacking the NGF receptor TrkA, no neurons remain at postnatal day 9.[14,15] Deletion of a single copy of the NGF gene results in a 50% reduction in sympathetic neurons, whereas overexpression of NGF in the heart causes cardiac hyperinnervation.[16,17] Despite the importance of NGF in sympathetic neural development, the upstream molecules that regulate NGF expression *in vivo* remain unknown.[18] Of the several cardiac hypertrophic factors tested, only endothelin-1 (ET-1) specifically upregulated NGF expression in primary cultured cardiomyocytes.[17] In addition, NGF expression and cardiac sympathetic innervation were reduced in ET-1-deficient mouse hearts, but not in the hearts of angiotensinogen-deficient mice (**Fig. 1**). In ET-1-deficient mice, the sympathetic stellate ganglia also exhibited excessive apoptosis and neuronal loss.[17,19] Moreover, we found that cardiac-specific overexpression of NGF in ET-1-deficient mice reversed the sympathetic nerve retardation. These findings indicated that ET-1 is a key regulator of NGF expression in cardiomyocytes, and that the ET-1/NGF pathway is critical for sympathetic innervation in the heart.[17]

In contrast to somatic tissues, visceral organs such as the heart are believed to be rich in autonomic efferent innervation, but poor in nociceptive afferent nerves.[20] Zahner et al. reported that vanilloid receptor-1-immunopositive sensory nerves are enriched in the epicardium, but scarce in the myocardium.[21] We reported that cardiac sensory innervation is rich both at epicardial sites and in the ventricular myocardium, and that sensory innervation increases with heart development.[22,23] In our screening of several neurotrophic factors, we found that cardiac sensory nerves develop in parallel with NGF synthesized in the heart.[23,24] Cardiac sensory nerves that are immunopositive for calcitonin gene-related peptide (the dorsal

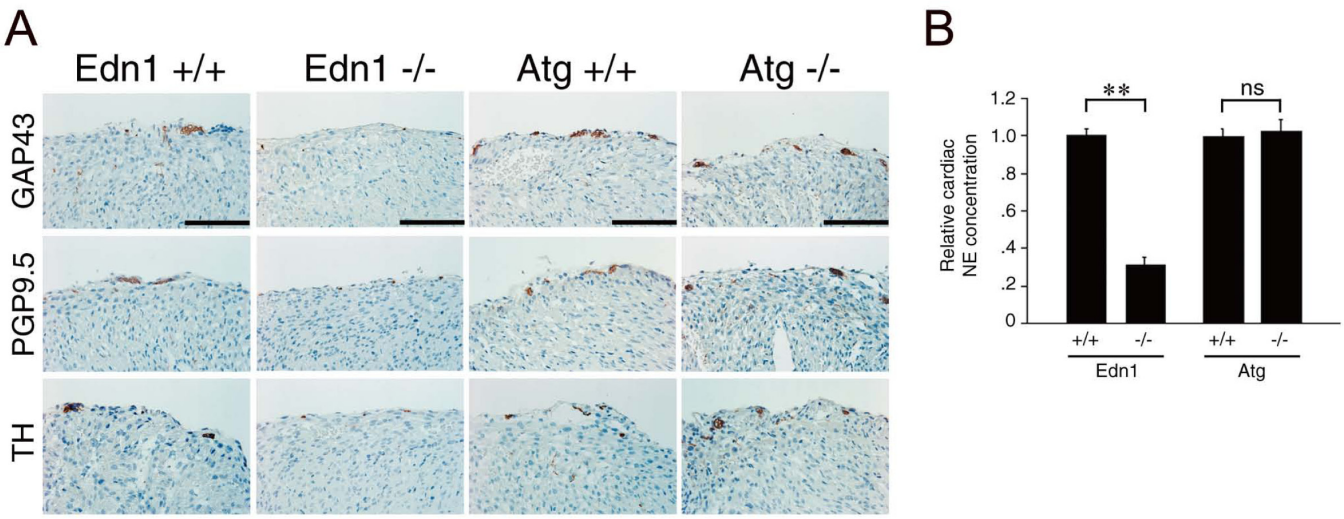

Fig. 1 Endothelin-1-deficient mouse hearts demonstrate reduced sympathetic innervation and downregulation of norepinephrine concentration.
(A) Immunostaining for nerve fibers with anti-GAP43, -PGP9.5, and -tyrosine hydroxylase (TH) antibodies in embryonic day 18.5 mouse hearts. Nerves were restricted to the epicardium in both endothelin-1 knockout ($Edn1^{-/-}$) and angiotensinogen knockout ($Atg^{-/-}$) mice , and levels of GAP43, PGP9.5, and TH were lower in $Edn1^{-/-}$ mice, but not in $Atg^{-/-}$ mice, compared with wild type littermates. (B) Cardiac norepinephrine (NE) concentrations were reduced in $Edn1^{-/-}$ mice.[17] $^{**}P<0.005$; ns, not significant.

root ganglia and the dorsal horn) were markedly retarded in NGF-deficient mice, whereas cardiac-specific overexpression of NGF reversed these deficits. Thus, NGF synthesis in the heart is also critical for the development of the sensory nervous system.[23,25] We also found that the reduced NGF expression in diabetic hearts might explain the cardiac sensory denervation and neuropathy in diabetic mice, and overexpression of NGF in the hearts can reverse sensory denervation and diabetic neuropathy in the mouse.[23,26]

Sema3a Determines Cardiac Sympathetic Innervation Patterning and Maintains Normal Heart Rhythm

As discussed above, NGF, a neural chemoattractant, plays critical roles in cardiac nerve development; however, no neural chemorepellent that induces growth-cone collapse and repels nerve axons has been identified in the heart. Sema3a is a class 3 secreted semaphorin that has been cloned and identified as a potent neural chemorepellent and directional guidance molecule for nerve fibers in the skin.[27–29] However, it was not known until recently whether cardiomyocytes produce Sema3a, and if so, whether this protein affects sympathetic neural patterning and cardiac performance.

We found that Sema3a is strongly expressed in the developing heart at embryonic day 12 in mice, and expression gradually fell with development.[12] By analyzing *Sema3a* knocked-in *lacZ* mice, we found *Sema3a* expression in the subendocardium, but not in the subepicardium of the atria and ventricles, the opposite pattern to the epicardial-to-endocardial gradient of sympathetic innervations.[12,30] These results indicate that the distribution pattern of Sema3a expression is the opposite that of sympathetic innervation in developing hearts, implicating Sema3a as a negative regulator of cardiac innervation. *Sema3a* knockout mice showed disrupted sympathetic innervation patterning and malformation of the stellate ganglia, which extend sympathetic nerves to the heart. Cardiac-specific *Sema3a*-overexpressing mice had reduced sympathetic innervation and attenuation of the epicardial-to-endocardial innervation gradient. Importantly, both types of mutant mice were susceptible to sudden death, and *Sema3a*-overexpressing mice were highly susceptible to ventricular tachyarrhythmias, whereas $Sema3a^{-/-}$ mice developed sinus bradycardia and sinus arrest. These results indicate that cardiomyocyte-derived Sema3a plays critical roles in cardiac sympathetic innervation patterning and the maintenance of arrhythmia-free hearts (**Fig. 2**).

Cardiac Fibroblasts Regulate Heart Development by Promoting Myocardial Proliferation through β1 Integrin Signaling

Cardiac ventricular formation involves growth of the heart muscle by proliferation of cardiomyocytes during

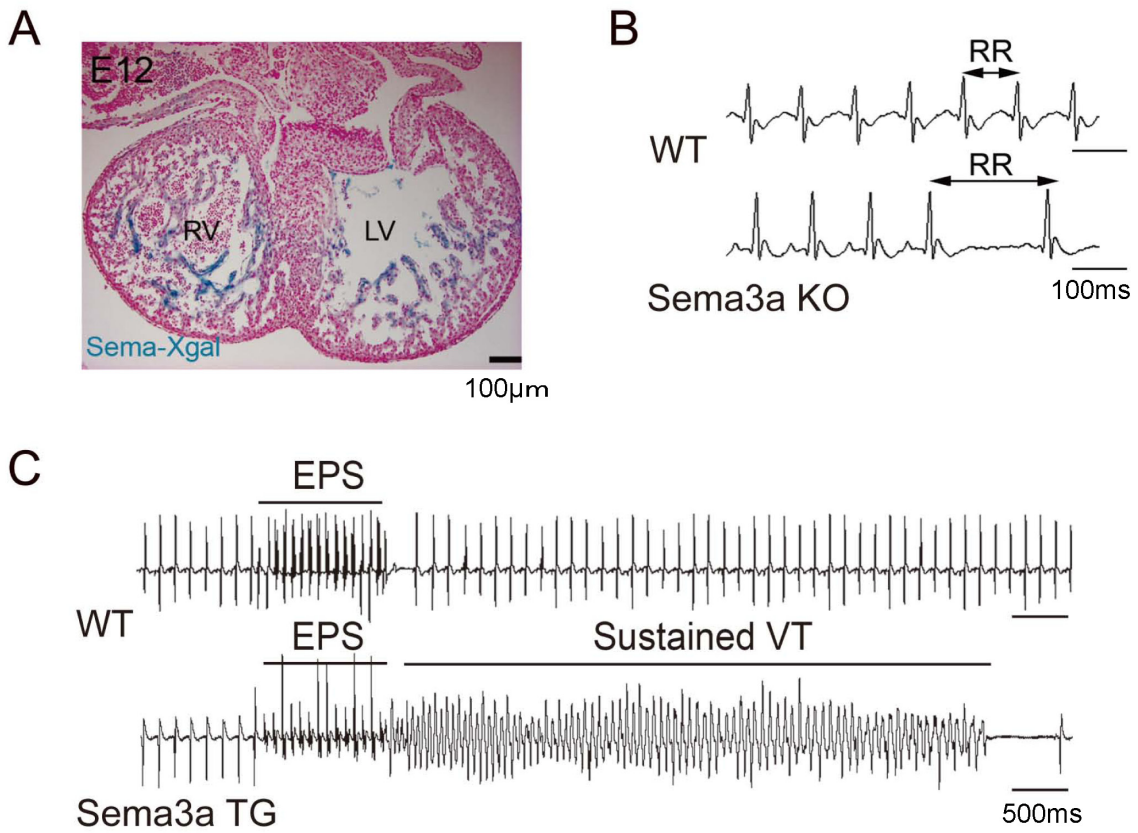

Fig. 2 Sema3a expression in the developing heart and various kinds of arrhythmias in Sema3a-mutated mice.
(A) X-gal staining (blue) of *Sema3a*$^{lacZ/+}$ hearts at embryonic day 12.5 (E12.5) demonstrates strong *Sema3a* expression in the subendocardium. Bar, 100μm. (B) Electrocardiogram (ECG) recordings from wild-type (WT) and Sema3a$^{-/-}$ (KO) mice. Sema3a-deficient mice display abrupt sinus slowing and bradycardia. Bars, 100ms. (C) ECG during programmed electrical stimulation (EPS) showing sustained ventricular tachyarrhythmias (VT) after administration of isoproterenol in cardiac-specific *Sema3a*-overexpressing (Sema3a TG) mice.[12] Appropriate Sema3a-mediated sympathetic innervation patterning is critical for the maintenance of an arrhythmia-free heart. Bars, 500ms.

embryogenesis. Signals from the endocardium and epicardium may influence cardiomyocyte proliferation in a paracrine fashion,[31,32] but how this proliferative activity of embryonic cardiomyocytes is regulated and subsequently terminated after birth remains unknown. Cardiac fibroblasts make up more than 50% of all the cells in the heart, but their function during cardiogenesis has not been determined. We demonstrated that embryonic cardiac fibroblasts develop coincident with expansion of the ventricular compact layer and regulate the proliferation of cardiomyocytes.[33] Embryonic cardiac fibroblasts secrete high levels of fibronectin, collagen, and heparin-binding epidermal growth factor-like growth factor (HB-EGF), which collaboratively interact and regulate mitotic activity of cardiomyocytes through β1 integrin signaling. β1 integrin is required for myocardial proliferation and ventricular compaction during development, as indicated by the smaller ventricles and interstitial fibrosis in the hearts of β1 integrin-deficient mice (**Fig. 3**). The bromodeoxyuridine labeling index was reduced in β1 integrin-deficient hearts, and microarray analyses revealed that the cell-cycle promoters *Ccnd1* and *Ccne1* were strongly downregulated in the mutant hearts. These results suggest that β1 integrin is required for cardiomyocyte proliferation and compact layer growth during embryogenesis, coincident with the development of cardiac fibroblasts. In contrast to embryonic fibroblasts, we found that adult cardiac fibroblasts had a unique function in promoting hypertrophy rather than proliferation of cardiomyocytes, suggesting that a fundamental switch in the cardiac fibroblast gene program may contribute to some of the physiological differences between embryonic and adult hearts.

Direct Reprogramming of Cardiac Fibroblasts into Cardiomyocyte-like Cells by Defined Factors

In contrast to fibroblasts, adult cardiomyocytes are terminally differentiated cells and their regenerative capac-

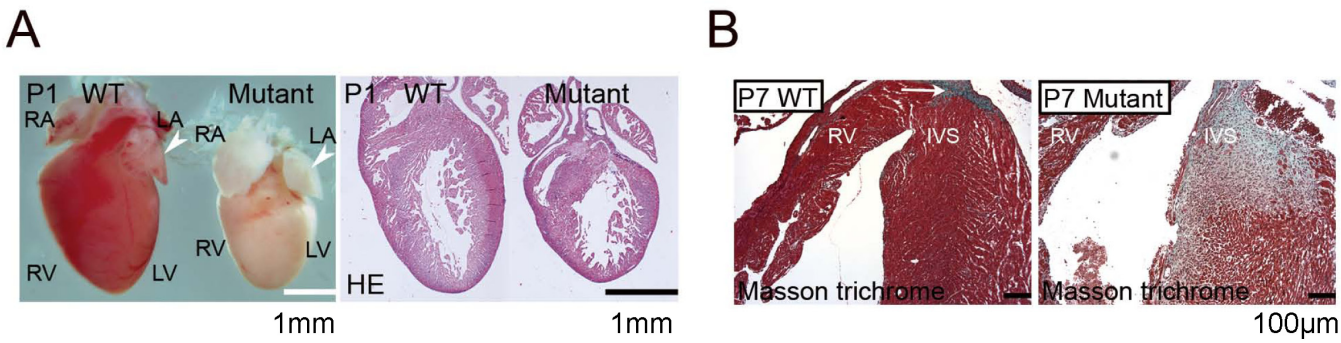

Fig. 3 β1 integrin signaling is critical for myocardial growth and muscle integrity.
(A) Wild-type and β1 integrin-deficient mouse hearts at postnatal day 1 (P1). Mutant ventricles were hypoplastic compared with wild type hearts. Bars, 1mm. (B) Masson-trichrome staining in postnatal day 7 (P7) wild-type and β1 integrin-deficient hearts. Fibrosis was detected in the mutant interventricular septum (IVS) and the right ventricular (RV) subendocardium.[33] The arrow indicates the bundle of His. Bars, 100μm.

ity following injury is very limited; this fact is a major contributor to mortality due to heart disease. Consequently, new cardiac regeneration therapy is demanded, and cell replacement treatment using stem cell-derived cardiomyocytes might be an attractive option to repair injured myocardium. The ability to reprogram fibroblasts into iPSCs using four defined factors might address some of the issues related to the cellular rejection and ethical concerns resulting from cell therapy, but generating sufficient numbers of iPSC-derived cardiomyocytes that are pure and mature and that can be delivered safely remains challenging. We hypothesized that if it were possible to directly convert resident cardiac fibroblasts into cardiomyocytes in situ, the large population of endogenous fibroblasts might be a potential source of new cardiomyocytes for regenerative purposes. To determine the candidate factors of cardiac reprogramming, we used a microarray to screen the genes that are specifically expressed in embryonic cardiomyocytes and selected 14 factors as candidates for cardiac reprogramming.

We generated α myosin heavy chain (αMHC) promoter-driven enhanced green fluorescent protein transgenic mice (αMHC-GFP), in which only mature cardiomyocytes expressed GFP, and used this mouse for screening.[12,34] Transduction of all 14 factors into fibroblasts induced 1.7% of GFP^+ cells, and serial removal of individual factors demonstrated that a combination of three factors [Gata4, Mef2c, and Tbx5, (GMT)] were sufficient for efficient GFP^+ cell induction (around 15%) from cardiac fibroblasts. We designated these GFP^+ cardiomyocyte-like cells induced cardiomyocytes (iCMs). These iCMs expressed several cardiac-specific marker proteins, such as sarcomeric α-actinin, cardiac troponin T, and atrial natriuretic factor, and had well-defined sarcomeric structures similar to neonatal cardiomyocytes (**Fig. 4**). The global gene expression profile of the iCMs was not identical to, but was similar to, neonatal cardiomyocytes, and was different from that of the original fibroblasts. The chromatin state of iCMs was also similar to that of cardiomyocytes but different from that of fibroblasts in terms of histone modifications and DNA methylation patterns in several cardiac gene promoters, at least.[35] A subset of iCMs possessed functional properties of cardiomyocytes, including intracellular Ca^{2+} transients and action potentials after 2–4 weeks of culture. We also found that iCMs could be derived from tail-tip fibroblasts, thereby excluding the possibility that the iCMs arise from contamination of cardiomyocytes or cardiac progenitors in the fibroblast population.[36,37] However, we also found that tail-tip fibroblasts are more resistant to cardiac reprogramming by GMT, suggesting that some epigenetic blocks may exist in some starting cell populations. We were also able to genetically map the "route" of cell fate alteration using a Cre-loxP system. We tagged cardiac progenitors and their derivatives with yellow fluorescent protein (YFP) using Isl1-Cre-YFP and Mesp1-Cre-YFP mice, and found that the generation of iCMs from fibroblasts was a direct process that did not pass through cardiac progenitor states.[38]

More recently, we investigated whether direct gene transfer of GMT into mouse hearts could similarly induce new cardiomyocyte generation from cardiac fibroblasts.[39] Retrovirus was used as a vector for gene delivery after myocardial infarction in the mouse because this type of virus infects mainly fibroblasts but not terminally differentiated cardiomyocytes. Injection of GMT retrovirus into αMHC-GFP transgenic mouse hearts induced expression of GFP, a reporter of cardiomyocytes, in 3% of virus-infected non-myocytes. GMT injection into the hearts of immunosuppressed nude mice induced cardiac protein expression in 1% of the transduced fibroblast cells, although few cells showed sarcomeric structures. We next developed a polycistronic vector expressing

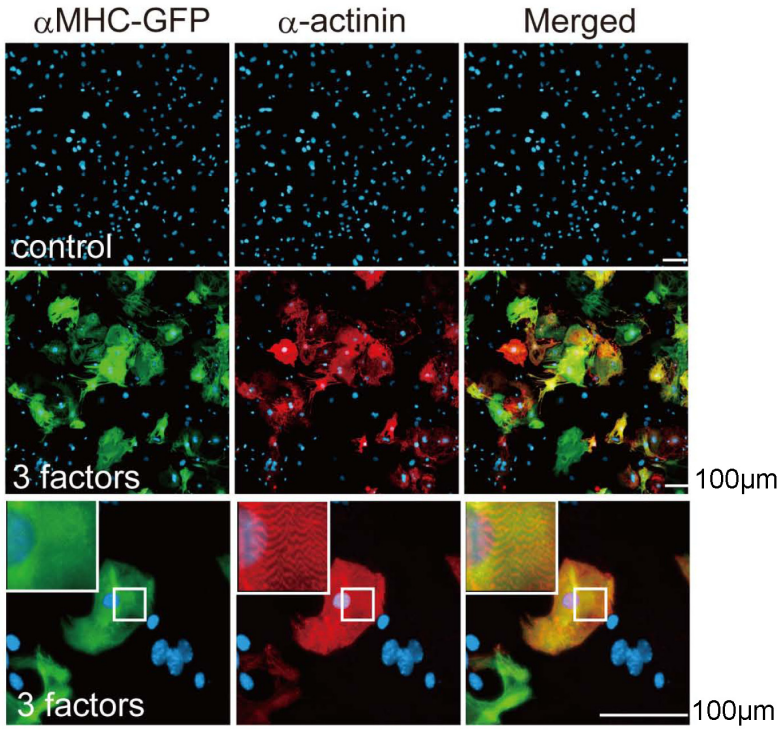

Fig. 4 Overexpression of Gata4, Mef2c, and Tbx5 induces cardiac gene expression and sarcomeric structures in fibroblasts. Immunofluorescent staining for αMHC-GFP, α-actinin, and nuclei. The combination of the cardiac-specific factors Gata4, Mef2c, and Tbx5 induced abundant αMHC-GFP and α-actinin expression in fibroblasts 2 weeks after transduction (middle row, bar 100μm). High-magnification views in insets show sarcomeric organization (bottom row, bar 100μm). Fibroblasts without transduction were used as controls.[38]

GMT separated by 2A "self-cleaving" peptides (3F2A) to improve reprogramming efficiency. Injection of this polycistronic retrovirus vector resulted in the generation in fibrotic tissues of iCMs that expressed sarcomeric α-actinin, cardiac troponin T, and several cardiac-specific genes. Importantly, more iCMs had well-defined sarcomeric structures by using this system. These results suggest that 3F2A-iCMs were more mature cardiomyocyte-like cells and that the polycistronic vector can be used for cellular reprogramming *in vivo*.

Following our first report of cardiac reprogramming, other groups also reported generation of cardiomyocyte-like cells from mouse fibroblasts based on the same factors or microRNAs *in vitro* and *in vivo*.[40–43] Although further work in larger animals is needed, as is the generation of iCMs from human fibroblasts and more efficient protocols of cardiac reprogramming, these reports demonstrate that this new direct reprogramming strategy might be a potential approach for heart regeneration in the future (**Fig. 5**).[44,45]

Conclusions

During heart development, many cell types coalesce, interact, and develop into mature organs. In this process, cell function, proliferation, and differentiation are tightly regulated by cell–cell interactions to insure that the heart attains the necessary structure and function. Cardiac sympathetic innervation patterning is strictly controlled by the balance between neural chemoattractants (e.g., NGF) and chemorepellents (e.g., Sema3a) derived from cardiomyocytes. Disruption of the sympathetic innervation patterning may eventually lead to fatal arrhythmias in both diseased and developing hearts. Better understanding of the mechanisms of cardiac sympathetic innervation patterning may represent an important approach for future therapies to avoid sudden cardiac death. Cardiac fibroblasts express specific ECM and growth factors that collaboratively promote proliferation of myocardial progenitors through β1 integrin signaling. Deficits of this interaction result in ventricular hypoplasia and heart failure. Identification of the molecular mechanisms involved in the interactions between cardiomyocytes and other type of cells may enhance our understanding of heart development, function, and disease. Direct reprogramming technology raises the possibility of converting endogenous cardiac fibroblasts in injured hearts directly into functional cardiomyocytes by gene transfer. Further

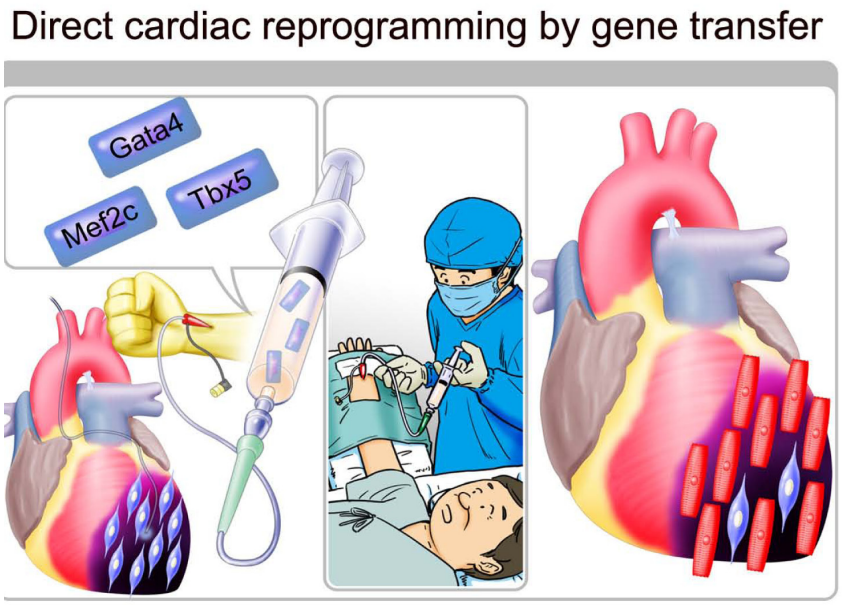

Fig. 5 Possible future regenerative therapy using direct cardiac reprogramming technology.
In the future, direct cardiac reprogramming may be used to regenerate damaged myocardium. Gene transfer of Gata4, Mef2c and Tbx5 into infarcted hearts may be able to convert endogenous cardiac fibroblasts into induced cardiomyocytes in situ. To achieve this goal, the following are needed: further work in larger animals, the generation of induced cardiomyocytes from human fibroblasts, and more efficient and safe protocols for cardiac reprogramming.

refinements and understanding of the molecular basis of cardiac reprogramming should be developed to enhance reprogramming efficiency and advance this new technology to clinical application.

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