response to increased blood pressure may explain the aberrant production of cardiomyocytes in the *hoz* ventricle.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2010.01.014.

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Strategies for ensuring that regenerative cardiomyocytes function properly and in cooperation with the host myocardium

Fumiyuki Hattori[1,2,3] and Keiichi Fukuda[1,3]

[1]Division of Cardiology
Department of Medicine
Keio University School of Medicine
Tokyo, Japan
[2]Asubio Pharma Co., Ltd.,
Osaka, Japan
[3]Corresponding authors: E-mail, hattori@sc.itc.keio.ac.jp (F.H.);
E-mail, kfukuda@sc.itc.keio.ac.jp (K.F.)
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Abbreviations: EGFP, enhanced green fluorescent protein; EPC, endothelial progenitor cell; ES cells, embryonic stem cells; iPS cells, induced pluripotent stem cells

Abstract

In developed countries, in which people have nutrient-rich diets, convenient environments, and access to numerous medications, the disease paradigm has changed. Nowadays, heart failure is one of the major causes of death. In spite of this, the therapeutic efficacies of medications are generally unsatisfactory. Although whole heart transplantation is ideal for younger patients with heart failure, many patients are deemed to be unsuitable for this type of surgery due to complications and/or age. The need for therapeutic alternatives to heart transplantation is great. Regenerative therapy is a strong option. For this purpose, several cell sources have been investigated, including intrinsic adult stem or progenitor cells and extrinsic pluripotent stem cells. Most intrinsic stem cells seem to contribute to a regenerative environment *via* paracrine factors and/or angiogenesis, whereas extrinsic pluripotent stem cells are unlimited sources of cardiomyocytes. In this review, we summarize the various strategies for using regenerative cardiomyocytes including our recent progressions: non-genetic approaches for the purification of cardiomyocytes and efficient transplantation. We expect that use of intrinsic and extrinsic stem cells in combination will enhance therapeutic effectiveness.

Keywords: embryonic stem cells; guided tissue regeneration; heart; induced pluripotent stem cells; myocytes, cardiac; transplants

Introduction

The heart is designed as a blood pump that works from the earliest organogenetic stage until death. Cardiomyocytes lose the ability to undergo cytokinesis soon after birth, which means that instead they hypertrophy in line with the increased demand for blood supply; which may be an evolutionary strategy to avoid tumorigenesis and achieve long-term stable and high-power pumping functions. However, once a certain number of cardiomyocytes is lost, the functional loss is compensated by pathological hypertrophic remodeling of the remaining cardiomyocytes, which have limited capacity. The excessive remodeling and overload of cardiomyocytes causes their sequential death and an irreversible circle of degeneration.

Although whole heart transplantation is the optimal treatment for a severely failing heart, the numbers of donor hearts are limited. Furthermore, many patients who suffer heart failure are excluded from transplantation therapy due to complications and/or age. Therefore, revolutionary therapeutic alternatives to heart transplantation that can be applied to patients with general congestive heart failure are urgently needed. Heart regenerative therapy is a strong candidate for this type of therapy.

For the proper regeneration of heart functions, it is necessary to have a clear understanding of the nature of this organ. The heart is a complex organ that consists of various types of cells, including cardiomyocytes and non-cardiomyocytes. Cardiomyocytes can be categorized as atrial, ventricular, pacemaking, and purkinje. Furthermore, ventricular cardiomyocytes are functionary sub-divided into M-cell, sub-endocardial, and sub-epicardial cardiomyocytes. Non-cardiomyocytes include fibroblasts, endocardial and epicardial cells, vascular endothelial and smooth muscle cells, sympathetic and parasympathetic cells, valvular and chordal cells, and cardiac-resident immune cells. These cell types work co-operatively to create the physiologic heart. Therefore, to ensure that regenerative cardiomyocytes function properly, provision of the appropriate regenerative environment is critical.

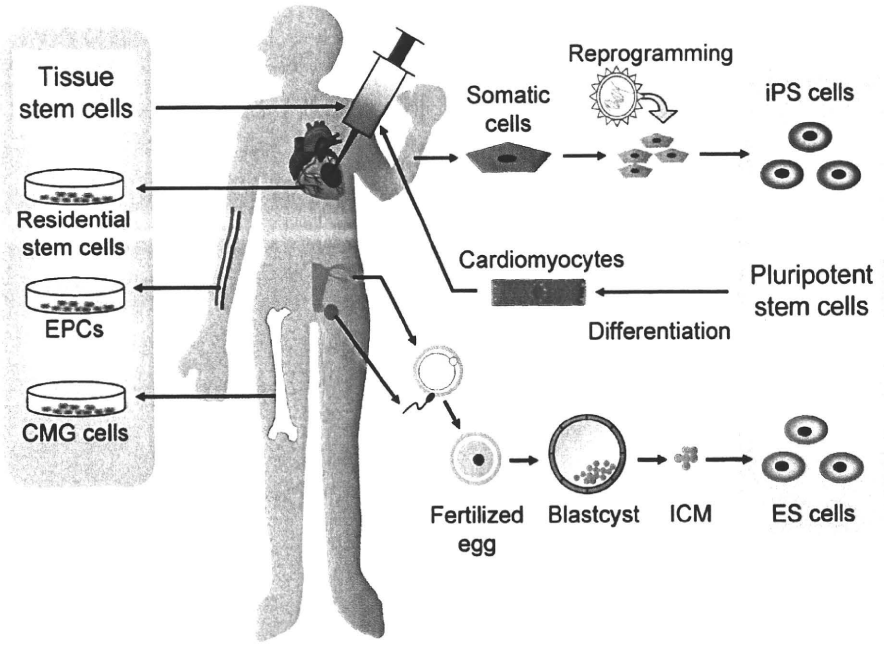

Figure 1. Sources of cells for regenerative therapies.

There are many candidate cell sources for heart regenerative therapies, such as cardiac-resident, bone marrow, and peripheral blood-derived stem cells. However, no adult stem cell-based strategy has achieved the production of sufficient numbers of cardiomyocytes to enable functional recovery of the failing heart. It is clear that the primary role of some adult stem cells is the creation of the regenerative environment rather than regeneration of cardiomyocytes. Therefore, cardiomyocyte administration/substitution from outside sources, e.g., human embryonic and induced pluripotent stem cells, appears to be promising (Figure 1).

In the present report, we introduce strategies for the preparation and delivery into the host myocardium of stem cells. The ultimate goal is to ensure that regenerative cardiomyocytes work properly and co-operatively with the host myocardium. Although different strategies are being tested, we believe that these studies will generate safe and effective therapies in the future.

Selection of cell sources

Tissue stem cells

In 1999, we demonstrated that bone marrow mesenchymal stem cells, which we term cardiomyogenesis cells, transdifferentiated into cardiomyocytes after treatment with 5-azacytidine (Makino *et al.*, 1999). This finding and previously obtained information on heart-derived cardiomyocytes (Soonpaa *et al.*, 1994) ushered in the concept of "heart regeneration therapies with own cardiogenic stem cells". Furthermore, we demonstrated the transplantation of cardiomyogenesis cells into the heart and observed that the integrated cardiomyogenesis cells had features of the adult myocardium (Hattan *et al.*, 2005). However, the cardiomyogenesis-based therapy has two major drawbacks: (1) the need to use the teratogenic reagent 5-azacytidine; and (2) low efficiency in terms of the establishment of cardiogenic cell lines from primary cultures of bone marrow adhesive cells. Despite intensive investigations, we have not yet overcome these obstacles.

5-Azacytidine is known to be an inhibitor of DNA methyltransferase (Kiefer, 2007). Recent publications have revealed the relationships between epigenetic modifications and the developmental specification of cell fates (Kiefer, 2007). In this context, we believe that cardiomyogenesis cells represent dedifferentiated cardiogenic stem cells. Other stem cells that have been identified for cardiomyocyte generation include endothelial progenitor cells (EPCs) (Asahara, 2007), c-kit (Beltrami *et al.*, 2003), sca-1 (Oh *et al.*, 2004), isl-1 (Laugwitz *et al.*, 2005), and neural crest stem cells (Tomita *et al.*, 2005). Asahara's group first reported the existence in the peripheral blood of multipotent stem cells, which are recruited into injured tissues and contribute to healing. They showed that EPCs not only differentiate into vessel endothelial and smooth muscle cells, but also cardiomyocytes,

albeit with very low efficiency. The c-kit-positive cells were identified as residential stem cells by Anversa's group (Beltrami *et al.*, 2003); these cells can repopulate cardiomyocytes under both normal and pathologic conditions. They reported that transplantation of collected and concentrated c-kit-positive cells dramatically improved cardiac function and inhibited remodeling. Moreover, they developed a method for expanding c-kit-positive stem cells *in vitro* (Beltrami *et al.*, 2003). The cardiogenic potential of sca-1-positive stem cells was first reported by Oh and colleagues (Oh *et al.*, 2004). These authors showed that sca-1-positive cells have high telomerase activity and can be differentiated into cardiomyocytes *in vitro* through treatment with 5-azacytidine. They also indicated that sca-1-positive cells have the potential to promote regenerative healing *in vivo* through both fusion and non-fusion cardiogenic transdifferentiation mechanisms.

Isl1 cells were discovered as cardiac-lineage progenitor cells by Chien's group (Laugwitz *et al.*, 2005). They reported the expression of transcription factor Isl1 in secondary cardiogenic cells at the cardiac crescent stage. From lineage-tracing studies, Isl1-positive cells have been found to contribute to atrial and right ventricular construction. Few Isl1-positive cells are observed in the neonatal atrial right ventricular, and they are absent from the adult heart. Isl1-positive cells can be isolated from murine embryonic stem cells, and they can be expanded and differentiated into vascular endothelium, vascular smooth muscle, and cardiomyocytes. Tomita *et al.* (2005) have shown that mammalian neural crest-derived cells have the potential to differentiate into cardiomyocytes, and they regard neural crest stem cells as new residential multipotential progenitor cells.

Pluripotent stem cells

Embryonic stem cells: Embryonic stem (ES) cells can be produced from the blastocyst inner cell mass. Therefore, the production of human ES cells from embryos raises ethical concerns. This is the major drawback to the use of human ES cells for research and therapeutics. Differences in the differentiation abilities of several ES cell lines have been reported (Moore *et al.*, 2008). Some researchers have attributed this to differences in epigenetic modifications or the accumulation of certain mutations. Since the differentiation efficiencies are generally low, the enhancement of differentiation efficiency has been studied extensively. ES cells have the ability to produce teratomas upon transplantation into immunodeficient animals (Prokhorova *et al.*, 2008). Therefore, in applications involving cells differentiated from ES cells, undifferentiated ES cells and unwanted cells must be excluded. Some studies have reported the susceptibility of human ES cells to become 'cancer ES cells' through the accumulation of mutations and genomic rearrangements (Harrison *et al.*, 2007). Although ES cells can theoretically proliferate indefinitely, many researchers believe that ES cells that have undergone more than 30 passages should not be used even for research purposes. The methodologies used for culturing and expanding ES cells should be stringently verified with respect to genomic and epigenetic stability. For ES cells used in therapies, an animal cell-free culture system should be used. Recently, improved systems have been reported, and some of these are already commercially available.

Induced pluripotent stem (iPS) cells: Recently, Takahashi *et al.* revealed that induced pluripotent stem (iPS) cells could be generated not only in mice (Takahashi and Yamanaka, 2006), but also in humans (Takahashi *et al.*, 2007); this may allow us to obtain individual ES-like cells. In our hands, the established murine iPS and ES cells and human iPS and ES cells are similar in terms of cell morphology, stem cell marker expression, and teratoma formations. However, cardiogenic differentiation properties tend to be lower than ES cells. The greatest advantage of iPS cells for stem cell researchers is that they do not have the ethical issues of ES cells, as they are derived from non-embryonic sources, although there are many unrevealed cocerns in iPS cells.

Mass production of ES cell-derived cardiomyocytes

For the eventual application of ES/iPS cell-derived cardiomyocytes, there are two major prerequisites: 1) improvement of the efficiency of differentiation into cardiomyocytes; and 2) efficient mass production of the differentiated cells. Many attempts have been made to improve cardiogenic differentiation efficiencies (Wobus *et al.*, 1997; Sauer *et al.*, 2000; Pandur *et al.*, 2002; Paquin *et al.*, 2002; Choi *et al.*, 2004; Kanno *et al.*, 2004; Passier *et al.*, 2005; Stary *et al.*, 2005; Yuasa *et al.*, 2005; Zhu and Lou 2005; E *et al.*, 2006; Hosseinkhani *et al.*, 2007a, 2007b; Roggia *et al.*, 2007; Singh *et al.*, 2007). However, inter-species, inter-strain, inter-laboratory, and even intra-laboratory variabilities have been problematic, and differentiation efficiencies $>30\%$ (cell-based counting) have not been achieved

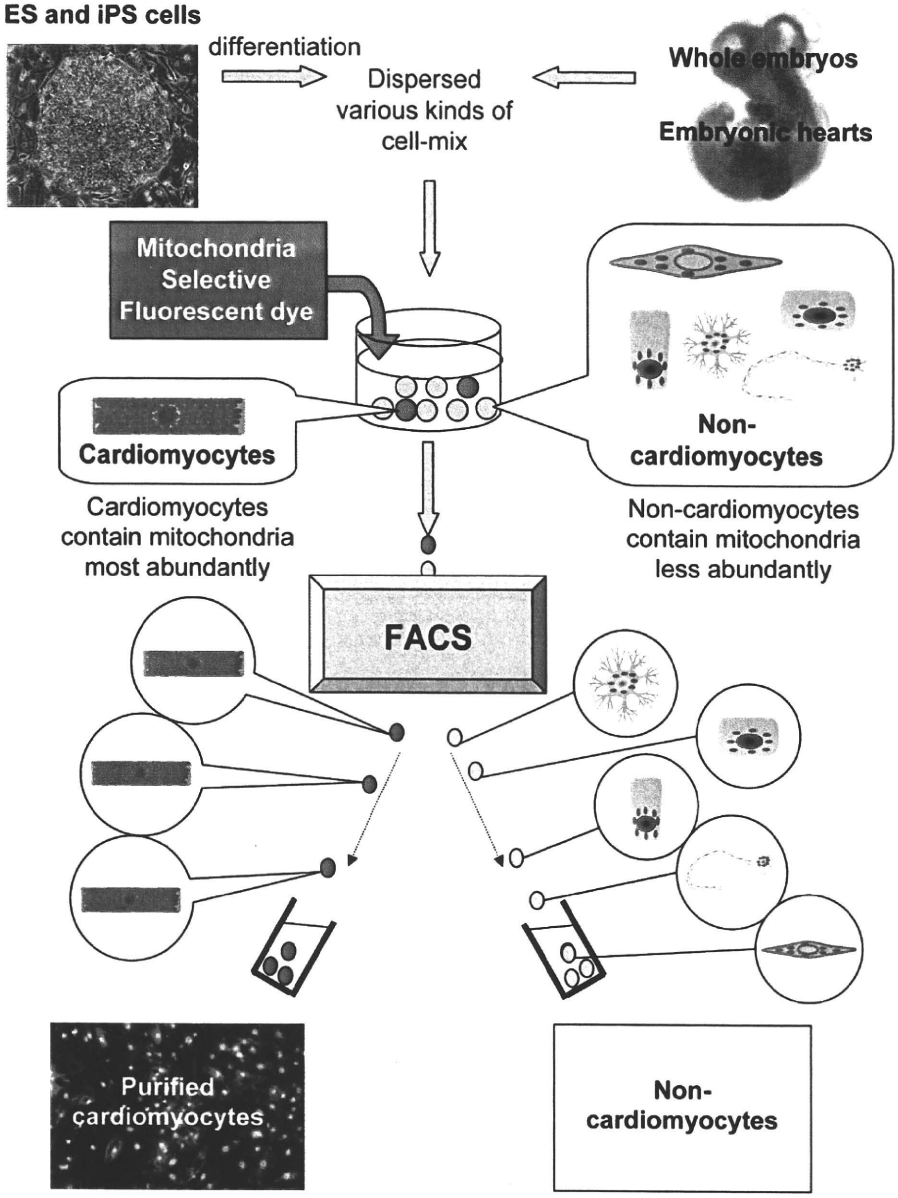

Figure 2. Scheme for the purification of cardiomyocytes from cell mixtures using a nongenetic method.

without enrichment. This may reflect susceptibilities to epigenetic fluctuations during maintaining cultivations and the difficulties experienced in controlling and unifying the individual states of differentiating cells due to cell-cell interactions. The scaling up of cultures is not easy. For this purpose, several groups have used the combination of micro-carriers and spinner flasks (Bauwens *et al.*, 2005; Schroeder *et al.*, 2005; Rourou *et al.*, 2007). In the next step of large-scale culturing, some groups have applied gene modification-based enrichment methods for murine embryonic stem cells, and obtained high numbers of enriched cardiomyocytes (Bauwens *et al.*, 2005).

Enrichment and purification of cardiomyocytes

Purification of ES-CM cells was first reported by Klug and colleagues in 1996 (Klug *et al.*, 1996), who established murine ES cell lines by permanent gene transfection of the aminoglycoside phosphotransferase (neo) gene driven by the β-myosin heavy chain promoter, and obtained highly enriched ES-CM cells (> 99% pure). Thereafter, several

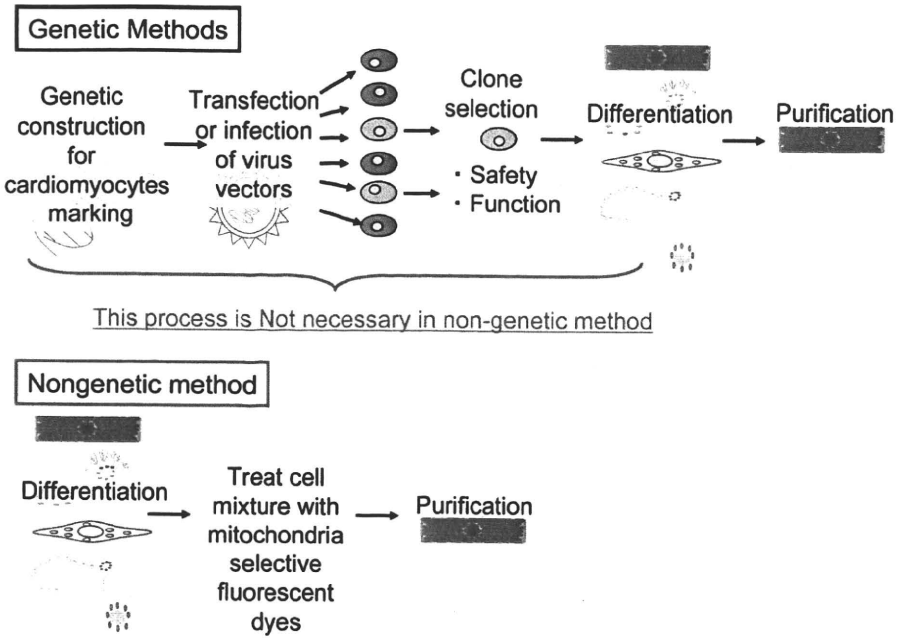

Figure 3. Comparison of a simple nongenetic method and genetic methods.

studies tested various combinations of cardiomyocyte-specific promoters and reporters to obtain pure ES-CM cell populations, including the stable transfection of the enhanced green fluorescent protein (EGFP)-tagged myosin light chain-2v promoter (Muller *et al.*, 2000), EGFP knock-in to the Nkx2.5 locus (Hidaka *et al.*, 2003), stable transfection of the Na^{+}/Ca^{2+} exchanger promoter (Fijnvandraat *et al.*, 2003), and stable transfection of the EGFP-tagged atrial natriuretic peptide promoter (Gassanov *et al.*, 2004). Anderson *et al.* (2007) demonstrated the enrichment of human ES-CM cells (> 93%) utilizing genetic modifications. Recently, we developed a widely applicable enrichment method that gives the highest level of cell purity (Hattori *et al.*, 2010). Initially, we applied the fluorescent mitochondrion-selective indicator MitoTracker Red to neonatal rat heart-derived cells, which contained cardiomyocytes and non-cardiomyocytes, and found that the dye selectively accumulated within the cardiomyocytes. This observation led us to hypothesize that mitochondrial dyes might be useful in cardiomyocyte purification (Figure 2). We validated this hypothesis using embryonic heart and whole embryo-derived cells. Next, we successfully purified mouse, marmoset (monkey), and human ES cells and mouse and human iPS cell-derived cardiomyocytes from their respective embryoid bodies. The purities of these cell populations were verified by sequential immunofluorescence FACS analysis. The expression of several cardiomyocyte marker genes was detected, whereas that of non-cardiomyocyte marker genes was not detected by PCR amplification of reverse-transcribed mRNAs extracted from the purified human ES cell-derived cardiomyocytes. Finally, we transplanted 1.9×10^5 purified mouse ES cell-derived cardiomyocytes into immunodeficient mouse testes, and confirmed the absence of teratoma formation (Hattori *et al.*, 2010). Our method for cardiomyocyte isolation has two advantages. First, it does not require genetic modification of the cells. Genetic modifications using non-viral or viral systems have several disadvantages, i.e., extrinsic genes may be silenced, the number of integration events in a single cell is difficult to control, targeted integration is not straightforward, and line selection and the verification of proper expression of extrinsic genes are time-consuming. Furthermore, genetic modification entails certain risks, such as tumor formation. Second, our method is likely to be widely applicable. We demonstrate here that it may be used to purify ESC-derived cardiomyocytes from four species, including humans, and that it is also applicable to murine and human iPSCs. An abundance of cellular mitochondria is likely to be a common characteristic of cardiomyocytes, irrespective of species. In contrast, most genetic modifications require species-specific constructs. The ESC-derived cardiomyocytes purified using our method did not induce teratoma formation in either the heart or testes (Figure 3). Although for clinical safety, further studies using large animal

models with much higher numbers of ESC-derived cardiomyocytes will be required, we believe that our purification method has significant advantages over existing methods in terms of eventual clinical applications.

Transplantation strategies

Direct injection

Direct injection of heart-derived cardiomyocytes: Soonpaa *et al.* first reported intramyocardial injection of embryonic cardiomyocytes and nascent intercalated disk formation (Soonpaa *et al.*, 1994). Regarding injection into an infracted heart Leor *et al.* reported the injection of cultured fragments of human fetal ventricles or rat fetal ventricles into the scar of a 7-24-day-old reperfused myocardial infarction in a rat (Leor *et al.*, 2000). Scorsin *et al.*, reported injecting cultured neonatal rat cardiomyocytes into the border zone of a myocardial infarction created permanent coronary occlusion (Scorsin *et al.*, 1996). The viability of the graft was demonstrated up to 48 h post-transplantation. Reinecke *et al.* demonstrated the transplantation of fetal, neonatal, and adult cardiomyocytes into normal and cryoinjured hearts (Reinecke *et al.*, 1999). They observed that neonatal rat cardiomyocytes hypertrophied to close to the size of adult cardiomyocytes by 8 weeks. Watanabe *et al.*, demonstrated the transplantation study using porcine myocardial infarction model (Watanabe *et al.*, 1998). They transplanted fetal and neonatal pig cardiomyocytes into the hearts with 4 to 5-week-old infarctions, and failed to show the presence of grafted fetal or neonatal pig cardiomyocytes. The discrepancies among these two reports are likely due to differences in the species studied and/or how recently the injuries occurred.

Direct injection of ES cell-derived cardiomyocytes: Klug *et al.* first reported the transplantation of murine ES cell-derived cardiomyocytes into the heart of a mouse (Klug *et al.*, 1996). They highly enriched cardiomyocytes using genetic engineering (> 99%) and transplanted these cells, although the transplanted cardiomyocyte number was very few (1×10^4). They extracted DNA from the heart which had been received the transplantation seven weeks before, and amplified the graft cell-specific DNA sequence by PCR and detected that by Southern blotting. The successful engraftment using highly enriched cardiomyocytes (> 99%) have not been reported after Klug's reports, although many studies using various genetic techniques for cardiomyocyte marking were reported. Kollosov *et al.* reported that the co-transplantation of highly enriched cardiomyocytes (1×10^5 cells) with fibroblasts resulted in good survival of the donor cardiomyocytes (Kolossov *et al.*, 2006). With regard to human ES cells, Kehat *et al.* reported that human ES cell-derived cardiomyocytes could normalize complete electrical heart block through the injection of beating embryoid bodies (Kehat *et al.*, 2004). Laake *et al.* achieved long-term engraftment of human ES cell-derived cardiomyocytes in the infracted hearts of immunodeficient mice, and showed that a transient improvement was effected by engrafted cardiomyocytes, as compared with engrafted non-cardiomyocytes. However, they reported that the improvement conferred by cardiomyocytes dropped to the same level as that produced by the engraftment by non-cardiomyocytes (van Laake *et al.*, 2007). Laflamme *et al.* transplanted cardiomyocyte-containing embryoid bodies into rat myocardium (Laflamme *et al.*, 2005), reported the introduction of enriched human ES cell-derived cardiomyocytes (10×10^6 cells) into infracted myocardium, and confirmed histologically the survival of these cells (Laflamme *et al.*, 2005). Van Laake *et al.* (van Laake *et al.*, 2007) histologically analyzed engrafted human ES cell-derived cardiomyocytes and speculated that the reason why the cardiomyocytes did not confer long-term improvement was that the engrafted cardiomyocytes produced human extracellular matrix, which hampered their electrical and functional connections and hindered co-operation with the host cardiomyocytes. They also stated that in the current strategies, the cardiomyocytes derived from ES cells could not functionally integrate with the host cardiomyocytes.

Survival of transplanted cardiomyocytes: The numbers of cells remaining in the myocardium after transplantation were investigated by Muller-Ehmsen *et al.* (Muller-Ehmsen *et al.*, 2002). They reported that almost 80% of the injected cells were lost between 1 day and 4 weeks post-injection, irrespective of the number of injected cells, whereas in the short period of time (1 h) after transplantation, there was a negative relationship between the number of lost cells and the number of injected cells. Dow *et al.* injected neonatal rat cardiac cells (5×10^6) directly into the free wall of the left ventricle at either 15 min post-reperfusion or 75 min after permanent occlusion (Dow *et al.*, 2005). Histological analysis of the transplanted cells revealed that the cardiac blood vessels contained cardiomyocytes. PCR analysis revealed that 100% of the animals (5 out of 5) in both groups had cells in their hearts and lungs, 40% of the reperfusion

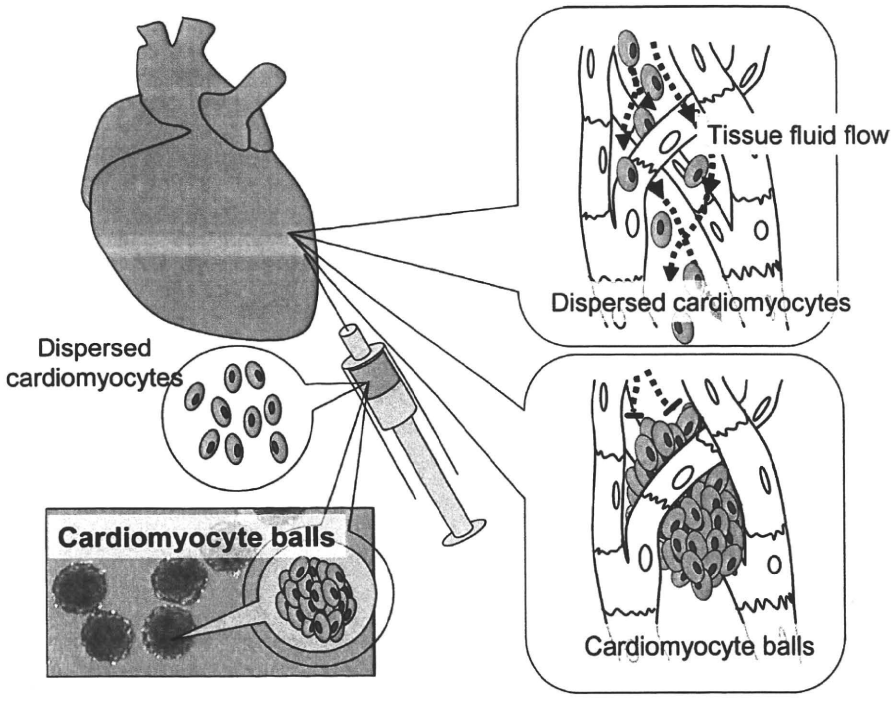

Figure 4. Schematic of the re-aggregation method for the efficient transplantation of FACS-purified cardiomyocytes.

group and 60% of the permanent occlusion group demonstrated cells in the liver and kidneys, and 40% of the permanent occlusion group had cells in the spleen. These results suggest that suspended cardiomyocytes are washed out into the circulation and spread throughout the body. Kollosov *et al.*, reported that genetically highly enriched mouse ES cell-derived cardiomyocytes did not survive in the heart (Kolossov *et al.*, 2006). Co-transplantation with fibroblasts synergistically enhanced the survival of these cells in the host myocardium. When we transplanted highly purified ES cell-derived cardiomyocytes and mouse embryonic fibroblasts into mouse hearts, histologic analysis showed that $< 1\%$ of the cardiomyocytes and 50% of the fibroblasts remained in the myocardium after 24 h (Hattori *et al.*, 2010). These results indicate that differences in adhesive abilities may be crucial. Furthermore, to investigate the possibility of rapid washing out via the coronary circulation, we injected purified and labeled cardiomyocytes into an *ex vivo* perfused heart. The drainage fluids from the coronary sinus were collected, and the labeled cardiomyocytes were counted in a hemocytometer. In seven individual experiments, 30% to 50% of the injected cardiomyocytes were ejected from the heart within 10 min of injection. These results clearly show that the direct injection of suspended cardiomyocytes carries the risk of dispersing the cardiomyocytes throughout the whole body (Hattori *et al.*, 2010).

To improve the survival rate of cardiomyocytes, Kollosov *et al.* co-transfected fibroblasts with purified cardiomyocytes (Kolossov *et al.*, 2006). In this strategy, the fibroblasts may act as bridges to facilitate the adhesion of cardiomyocytes to the myocardium and may exert a 'packing effect' to prevent the cardiomyocytes being washed out. For this approach, appropriate cell types, ideally cardiac lineages, should be used in future studies. To improve the survival rate of injected cardiomyocytes, Laflamme *et al.* (Laflamme *et al.*, 2005) applied a prosurvival cocktail, which included Matrigel (a cell-permeable peptide from Bcl-XL that blocks mitochondrial death pathways), cyclosporine A (to attenuate cyclophilin D-dependent mitochondrial pathways), pinacidil (to open ATP-dependent K^+ channels so as to mimic ischemic preconditioning), IGF-1 (to activate Akt pathways and the caspase inhibitor ZVAD-fmk), and obtained improved survival. We encountered the same drawback, and investigated the mechanism underlying the reduced survival of injected cardiomyocytes. We studied the *in vitro* adhesive properties of purified cardiomyocytes and confirmed that they were less adhesive. From the above studies, it is clear that suspended cardiomyocytes are vulnerable to washing out and anoikis. To overcome these problems, we developed a "re-aggregation method", which simply means that hundreds to thousands of

purified cardiomyocytes are aggregated in cell-non-adhesive round-bottomed 96-well plates. In this method, >90% of the injected cardiomyocytes survive and hypertrophy in a time-dependent manner (Figure 4). The sarcomeric structures of the donor cardiomyocytes were aligned along the host myocardium. Similar experiments were successfully carried out with human ES-derived cardiomyocytes (Hattori *et al.*, 2010). Finally, we studied the mechanism of re-aggregated cardiomyocyte or "cardiomyocyte ball" survival *in vitro* and *in vivo*. When we added several growth factors and measured the cardiomyocyte ball diameters, we identified that ET-1, EGF, bFGF, and PDGF-BB as possible autocrine/paracrine factors (Hattori *et al.*, 2010).

Myocardial cell sheets

In 1999, the group of Okano invented the temperature-sensitive culture dish (Kaneko *et al.*, 1999). This enabled the fabrication of cardiomyocyte cell sheets and opened up the possibility of heart regeneration using cell sheets. The Okano group demonstrated the subcutaneous transplantation of triple-layered cardiomyocyte sheets (Shimizu *et al.*, 2006). The ectopically transplanted cell sheet could be visibly observed beating rhythmically. Subsequently, they succeeded in transplanting the cardiomyocyte sheets onto hearts (Masuda *et al.*, 2008). However, they were unable to demonstrate a functional benefit of cardiomyocyte sheet transplantation. They commented in their papers that a triple-layered cardiomyocyte sheet is not sufficient to improve the cardiac function of damaged hearts; therefore, a multi-layered sheet with functional vasculature should be fabricated. We developed an alternative method to produce functional myocardial cell sheets using a thin scaffold composed of human fibrin and thrombin. This system has the advantage that scaffold degradation can be controlled by inhibiting internal proteinases. We also constructed triple-layered myocardial cell sheets, and performed subcutaneous and on-heart transplantations (Itabashi *et al.*, 2005). Furthermore, we showed that an arrhythmogenic re-entry circuit caused by cryo-injury could be fixed by myocardial sheet transplantation (Furuta *et al.*, 2006). Recently, we investigated the combination therapy of myocardial cell sheet transplantation and omentopexy, which is a surgical procedure whereby the omentum is attached to another organ for the purpose of increasing arterial circulation. This strategy synergistically ameliorated cardiac function and dilating remodeling in a long-term (8-week) study (Suzuki *et al.*, 2009). We evaluated the mechanism underlying this synergy, and found: (1) improvement of the blood supply by OM-derived vascular and neo-vascular development in the infracted region; (2) reduction of the infracted region; (3) mechanical support of the infracted region, preventing dilation; and (4) enhancement of the long-term survival and maturation of the transplanted CM.

3D Tissue engineering

Cardiac tissue engineering was first reported by Leor *et al.* in 2000 using an alginate porous scaffold (Leor *et al.*, 2000). The isolated cardiac cells were seeded at a concentration of 3×10^5 cells per scaffold within cylindrical alginate scaffolds (6 mm in diameter × 1.0 mm in height). The scaffolds has an average pore diameter of 100 μm. Biograft transplantation was performed 7 days after MI. Histologic examination identified well-formed myofibers with striation, cellular gap junctions, and newly formed capillaries. Typical fibroblasts, macrophages, and lymphocytes were also found in the grafts. The beneficial effect of the biografts on LV remodeling was translated into the prevention of LV function deterioration, as reflected in the preservation of FS after implantation (53 ± 4% versus 47 ± 5%, $P = 0.52$). Over the past few years, Eschenhagen's group have developed a different technique that uses liquid collagen I instead of preformed scaffolds to reconstitute embryonic chicken or neonatal rat cardiomyocytes on three-dimensional cardiac grafts (Fink *et al.*, 2000). The technique, which has been further proven by Zhao *et al.*, with a minor modification, uses collagen I to support the endogenous capability of immature cardiac cells to form a heart tissue-like structure *in vitro* (Zhao *et al.*, 2005). Isolated cardiomyocytes are mixed with freshly neutralized collagen type I, Matrigel, and culture medium. This cell matrix mixture is pipetted into casting molds of the desired size and shape. After 7 days in the casting molds, engineered cardiac tissues (ECTs) are transferred to a stretching device and subjected to phasic stretching by 10% for an additional 5-7 days. The contractile activity of the constructs is superb, and the method seems to be highly reproducible. Success in heart tissue repair using ECTs has been reported recently by Zimmermann *et al.* (2006). They derived large (1-4 mm in thickness × 15 mm in diameter), force-generating engineered heart tissue from neonatal rat heart cells. The engineered heart tissue formed thick cardiac muscle layers when implanted into the myocardial infarcts of immunosuppressed rats.

When evaluated 28 days later, the engineered heart tissue showed undelayed electrical coupling to the native myocardium without evidence of arrhythmia induction. Moreover, the engineered heart tissue prevented further dilation, induced systolic wall thickening of infarcted myocardial segments, and improved the fractional area shortening of the infarcted hearts, as compared with the controls (sham-operated and noncontractile constructs). On the basis of this method, Guo *et al.* (2006) created cardiac tissues using cardiomyocytes derived from mouse ES cells (mESCs) (Guo *et al.*, 2006). In that study, they enriched cardiomyocytes from mESCs using Percoll density gradients. The cells were then mixed with liquid collagen to construct cardiac tissue. The engineered cardiac tissue was mechanically stretched *in vitro*, and was found to resemble both structurally and functionally neonatal native cardiac muscle.

Future prospective

Mummery *et al.* made a long-term comparison of cardiomyocyte-transplanted and non-cardiomyocyte-transplanted samples in myocardial infarction models (Passier *et al.*, 2008). They found that the cardiac functions of both groups were significantly improved in the short-term and long-term, as compared with the non-treated group. In the short-term, the cardiac functions of the animals treated with cardiomyocytes were significantly superior to those treated with non-cardiomyocytes; however, the long-term outcomes were not significant. Mummery *et al.* discussed how this might be related to an inability of the cardiomyocytes transplanted into the myocardium to work co-operatively with the host cardiomyocytes. We observed weak expression of connexin 43 protein in 2-month-transplanted mouse ES cell-derived cardiomyocytes, which was much lower than the levels in the host cardiomyocytes, suggesting that the transplanted cardiomyocytes were not fully matured. It is possible that critical points on the pathway towards full maturation of pluripotent stem cell-derived cardiomyocytes are not passed. We suspect the absence of factors that induce cardiomyocytes to mature and connect functionally with the host myocardium, i.e., humoral factors, extracellular matrixes, interactions with non-cardiomyocytes, and mechanical stress. We also postulate the existence of inhibitory mechanisms in the host heart direct against the maturation and integration into the myocardium of transplanted ES cell-derived cardiomyocytes. We further speculate that the adult myocardium has lost the ability to accept cardiomyocytes that are newly supplied during development. For the realization of the goal of "regeneration of heart using pluripotent stem cells-derived cardiomyocytes", we need to overcome these drawbacks and facilitate the process of functional integration into the host myocardium.

Tissue stem cell research has progressed in discovering intrinsic healing mechanisms and possible ways for enhancing these pathways through *ex vivo* stem cell expansion and the use of certain drugs. We expect that in the future these regenerative strategies will be combined with the administration of ES cell- and iPS cell-derived cardiomyocytes, which will synergistically enhance therapeutic effectiveness.

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Creating frog heart as an organ: *in vitro*-induced heart functions as a circulatory organ *in vivo*

MASAYOSHI KINOSHITA[1,2,#], TAKASHI ARIIZUMI[3,#], SHINSUKE YUASA[1,2], SHUNICHIROU MIYOSHI[2], SHINJI KOMAZAKI[4], KEIICHI FUKUDA[1] and MAKOTO ASASHIMA[3,5,*]

[1]Department of Regenerative Medicine and Advanced Cardiac Therapeutics, [2]Division of Cardiology, Department of Internal Medicine, Keio University School of Medicine, Tokyo, [3]Department of Life Sciences (Biology), Graduate School of Arts and Sciences, The University of Tokyo, Tokyo, [4]Department of Anatomy, Saitama Medical School, Saitama and [5]Organ Development Research Laboratory, National Institute of Advanced Industrial Science and Technology (AIST), Ibaraki, Japan.

ABSTRACT Cardiomyocytes have been induced from various pluripotent cells, such as embryonic stem cells and myeloid stem cells; however, the generation of cardiac tissues beyond two-dimensional cell-sheets has not been reported. Creating higher order, three-dimensional structures that are unique to heart is the long-awaited next step in realizing cardiac regenerative medicine. We have previously shown that cardiomyocytes can be induced *in vitro* from undifferentiated cells (animal caps) excised from *Xenopus* embryos. Cardiomyocytes were induced by first dissociating the animal caps and then reaggregating them following treatment with activin. Here, we describe an interesting method for creating a complete ectopic heart *in vivo*, involving the introduction of *in vitro*-created tissue during early embryogenesis. Thus, animal cap reaggregates were transplanted into the abdomen of late-neurula-stage embryos, resulting in two-chambered hearts being formed. The dual-heart larvae matured into adult animals with transplanted hearts intact. Involvement of transplanted hearts in systemic circulation was demonstrated. Moreover, the ectopic hearts possessed higher order structures such as atrium and ventricle, and were morphologically, histologically, and electrophysiologically identical to original hearts. This system should facilitate the study of heart organogenesis and may promote a shift from tissue to organ engineering for clinical applications.

KEY WORDS: *activin, animal cap, cardiogenesis, organ engineering, Xenopus laevis*

Introduction

The African clawed frog *Xenopus laevis* develops outside the maternal corpus, making development easy to observe. This organism also follows the same developmental pattern as humans, thus offering a very useful model for early organogenesis and particularly those aspects related to cardiac research (Ariizumi and Asashima 2001; Warkman and Kreig 2007; Asashima *et al.* 2009). A wealth of research is ongoing into myocardial regeneration using cell types such as embryonic stem (ES) cells (Fukuda and Yuasa 2006; Asashima *et al.* 2008) and myeloid stem cells (Dengler and Katus 2002). More recently, induced pluripotent stem (iPS) cells have emerged as a potent candidate for myocardial regeneration (Mauritz *et al.* 2008). Numerous studies have transplanted myocardial cells generated from such cell types into hearts that have undergone myocardial infarction to improve function (Caspi *et al.* 2007). It is apparent from the collective results that cells used to induce myocardial cells have now been sourced from higher-order animals ranging from mice to humans, and that cardiac tissue engineering has already entered a mature stage, with breakthrough experimental systems under development. An important question regarding cardiac regeneration is

Abbreviations used in this paper: ES, embryonic stem; iPS, induced pluripotent stem (cell).

***Address correspondence to:** Makoto Asashima. Department of Life Sciences (Biology), Graduate School of Arts and Sciences, The University of Tokyo, Tokyo, Japan. Fax: +81-3-5454-6998. e-mail: asashi@bio.c.u-tokyo.ac.jp

#Note: M.K. and T.A. contributed equally to this work.

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whether an entire heart could be regenerated rather than simply aggregating cells that exhibit cardiac function. We attempted to address this issue using *Xenopus laevis* as an animal model of cardiogenesis. Success in this endeavour would represent a shift from tissue to organ engineering.

Undifferentiated cells collectively known as the animal cap are present in the blastula of *Xenopus laevis*. This region comprises approximately 1,000 cells and is capable of inducing differentiation of myocardial cells following activin activation (Ariizumi *et al.* 1996) or overexpression of factors such as GATA4 (Latinkic *et al.* 2003) and Wnt11 (Pandur *et al.* 2002). In all such investigations, however, the induction rate for myocardial cells was by no means high, and induced tissues did not structurally resemble the heart as an organ. We recently established an experimental system that induces myocardial cells with near to 100% probability, using a novel procedure for temporarily dissociating cells before the animal cap is treated with activin (Ariizumi *et al.* 2003). When these cells are cultivated, they do not simply form a mass of myocardial cells, but rather take on a tubular structure. In the present investigation, animal caps for which cardiac differentiation was induced were transplanted ectopically into other neurulae. The transplanted frogs were then examined for one year after transplantation for the presence of higher order heart structures and for the potential function of such ectopic organs in the systemic circulation. Analysis involved immunohistochemical, electron microscopic, echocardiographic, and electrophysiological examinations.

Results

In vitro *cardiomyocyte induction and* in vivo *ectopic heart formation*

We first induced *in vitro* heart formation using *Xenopus* animal cap cells (Fig. 1A). Blastula animal caps did not differentiate into beating cardiomyocytes after treatment with 100 ng/ml of activin for 5h; however, upon dissociation and subsequent treatment with activin to reassemble the animal cap cells, nearly all reaggregates (94.7%, 36 of 38 cases) began beating on culture day 2, which is comparable timing-wise to the initiation of heart beating in normal embryos (st. 35; Nieuwkoop and Faber 1956).

To investigate whether the activin-treated reaggregates (cardiomyocyte primordium) could form heart *in vivo*, these primordia were transplanted into the abdomen of 182 neurulae (Fig. 1A). Ectopic heart was formed in 138 recipients (75.8%) and beating of the abdominal heart-like tissue began 1 day after transplantation, simultaneously with the developing host heart (Fig. 1B). At 5 days after transplantation, erythrocytes began flowing into the transplanted heart chambers, with flow readily observed through the transparent epidermis (Supplemental movie 1). The new heart created in the posterior abdomen comprised at least two chambers and participated in the systemic circula-

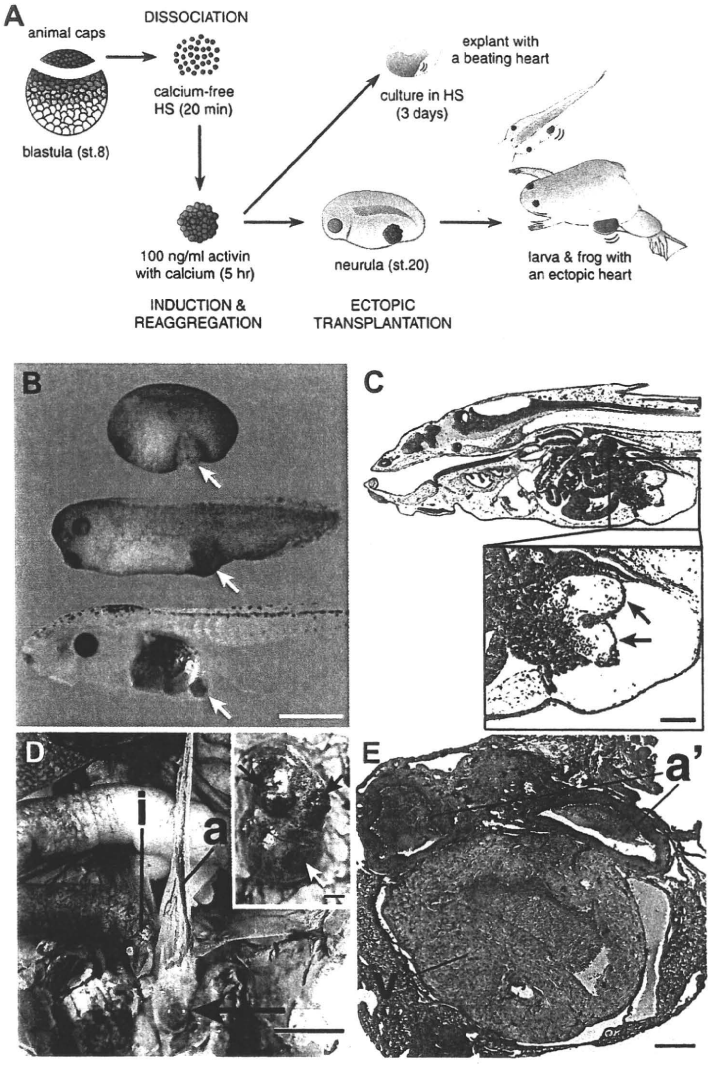

Fig. 1. *In vitro* cardiomyocyte induction and *in vivo* ectopic heart formation. (A) *Experimental protocol of the* in vitro *cardiomyocyte induction and ectopic heart transplantation. For further details, see Materials and Methods.* **(B)** *External morphology of embryos that received the transplant in the abdomen: top, immediately after transplantation (st. 20); middle, 1 day after transplantation (st. 35); bottom, 5 days after transplantation (st. 46). Transplanted* in vitro*-induced cardiac primordium started beating on day 1 after transplantation (middle), and erythrocytes were present in the ectopic heart at day 5 (bottom). Arrows indicate the transplants. Scale bar, 1 mm. Also see Supplemental movie 1.* **(C)** *Sagittal section of larva at day 5 after transplantation (st. 46). Erythrocytes are visible in the ectopic heart, which is clearly divided into two chambers (arrows). HE staining. Scale bar, 100 μm.* **(D)** *Necropsy photograph of frog abdomen at one year after heart transplantation. Ectopic heart (arrow) is surrounded by epicardium and is present between the intestine (i) and anterior abdominal vein (a). Blood vessels appear black because India ink was injected into the original host heart. Scale bar, 10 mm. Contour of excised ectopic heart (right upper corner). At least 3 chambers can be observed from outside the heart. Contractions start in the upper two chambers (black arrows) and continue to the lower chamber (white arrow). Scale bar, 1 mm.* **(E)** *Histological appearance of the excised ectopic heart, showing section of same heart shown in (D). One of the three chambers comprises a thick and deeply penetrating layer of myocardium with ventricle-like morphology (v'). The remaining two chambers are surrounded by a thin layer of myocardium and exhibit atrium-like morphology (a'). HE staining. Scale bar, 200 μm.*

tion. Histological examination of 20 recipients confirmed the presence of erythrocytes inside the heart chambers formed in the abdomen (Fig. 1C). Approximately 60% (68 of 118) of recipients developed normally and metamorphosed into adult frogs within the normal time frame of 2 months. The ectopic hearts continued to beat in the adults, with cardiac contraction observable from the abdominal surface (Supplemental movie 2). Further morphological and physiological analyses were carried out on 24 of the 68 adult recipients (aged approximately 1 year), selected at random. Laparotomized frogs were injected with India ink into the ventricle of the host heart. This showed blood flow into the ectopic heart through the mesenteric arteries of the small intestine and out of the ectopic heart to the anterior abdominal vein (Fig. 1D). Excision and macroscopic observation of ectopic hearts revealed chambers with two atrium-like sections and one ventricle-like part, as typical of a normal frog heart (Fig. 1D right-upper corner). Cardiac contractions appeared to originate from the atrium-like sites and continue toward the ventricle-like site. Histological sections showed multiple chambers in the ectopic hearts: a ventricle-like chamber comprising a thick and deeply penetrating layer of myocardium, and two atrium-like chambers surrounded by a thin layer of myocardium (Fig. 1E).

Physiological analyses and echocardiography of the dual-heart frogs

Electrocardiography (ECG) recordings were obtained from the body surface of frogs to detemine actual rhythms of the host and ectopic hearts and how such rhythms were related. The ECG of normal hearts displayed p-wave (representative of an atrial contraction) and narrow QRS (representative of a ventricular contraction) complexes (Fig. 2A). The ectopic hearts showed no apparent p-waves and wide QRS (QRS' in Fig. 2A) complexes, which persisted after elimination of the host heart (Fig. 2B). The ectopic heart rhythms were regular, with a relatively long cycle, and independent of the host rhythms. Monophasic action potentials from the excised ectopic hearts revealed three different action potentials, resembling those of the sinus nodes, atria, and ventricles of normal heart (Fig. 2C). Two-dimensional echocardiography of the ectopic heart revealed two different chambers with muscular layers of differing thickness, and valve-like structures separating the chambers. Blood flow was observed in each chamber under color Doppler echocardiography (Fig. 3, Supplemental movie 3).

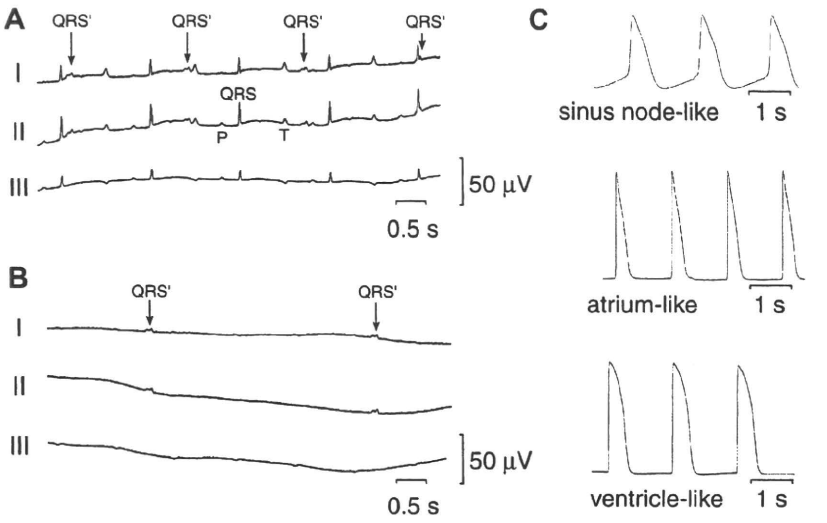

Fig. 2. Physiological analysis of ectopic heart developed from *in vitro*-induced cardiac primordium. (A) *ECG of frog with two hearts. P and QRS indicate host atrial and ventricular potentials, respectively. QRS' indicates ectopic ventricular potential. T is host ventriclar repolarization.* **(B)** *ECG after elimination of the host heart. QRS' waveforms remained apparent in the ectopic heart.* **(C)** *Action potential in the excised ectopic heart. By changing the measurement site, three types of action potentials were recorded, resembling sinus node, atrial, and ventricular action potentials.*

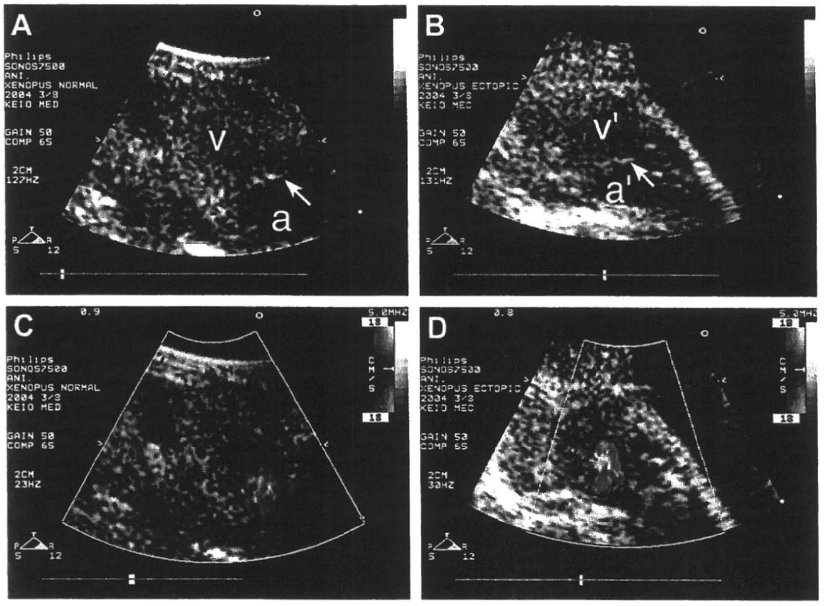

Fig. 3. Echocardiography of the dual-heart frogs. (A) *Two-dimensional echocardiogram of normal heart, showing atrium (a) and ventricle (v). Valve-like tissue (arrow) is visible between these two chambers.* **(B)** *Two-dimensional echocardiogram of the ectopic heart. As with the normal heart, two chambers are apparent and valve-like tissue (arrow) is visible between the atrium-like (a') and ventricle-like (v') structures. Also see Supplemental movie 3.* **(C)** *Color Doppler echocardiography of the normal heart.* **(D)** *Color Doppler echocardiography of the ectopic heart. Also see Supplemental movie 3. Blood flow between the two chambers is indicated by color (red, blood flow towards probe; blue, flow in the opposite direction).*

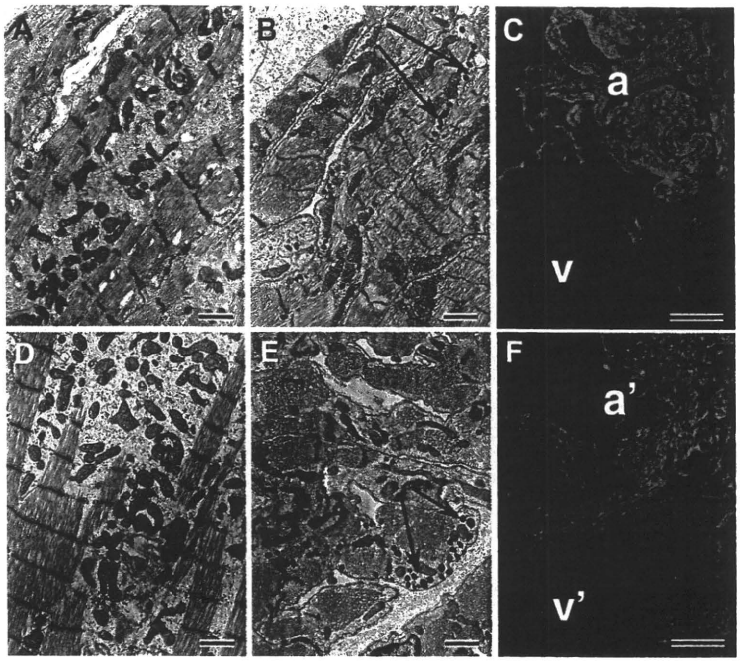

Fig. 4. Electron microscopy and immunohistochemical observations of the heart chambers. (A,D) *Electron micrographs of ventricular myocardium in the normal and ectopic hearts. Ectopic hearts showed similar structures to those observed in normal hearts. No ANP granules are apparent in either image.* **(B,E)** *Electron micrographs of atrial myocardium in the normal and ectopic hearts. Numerous ANP granules (arrows) are present in both.* **(C,F)** *Immunostaining of normal and ectopic hearts with anti-ANP antibody showed strong ANP expression in the atrium (a) and atrium-like region (a'), but not in the ventricle (v) or ventricle-like region (v'). Scale bars are 1 μm in (A,B,D and E); 200 μm in (C,F).*

Electron microscopy and immunohistochemical observations of the heart chambers

Electron microscopy revealed characteristic cardiac structures in the ectopic hearts including sarcomeres and intercalated discs. Numerous atrial natriuretic peptide (ANP) vesicles were clearly apparent in the atrium-like parts of ectopic hearts, but not in the ventricular myocardial cytoplasm (Fig. 4). These findings strongly indicated that the ectopic heart had two different chambers, atrium and ventricles. Immunofluorescent staining revealed contractile proteins including actin and troponin in the ectopic hearts as in the host organs (data not shown). ANP was more strongly expressed at the atrial myocardium than at ventricles in both the ectopic and normal hearts (Fig. 4).

Discussion

The successful induction of cardiomyocytes from ES cells sparked vigorous efforts to develop clinical applications. Difficulties persist, however, in creating higher order cardiac structures such as atria and ventricles from ES cells. Current methods to regenerate myocardium involve selecting only those stem cells that have differentiated into cardiomyocytes and transplanting them into infarcted hearts (Caspi *et al.* 2007; Reinecke *et al.* 2008). Despite innovations such as transplantation of cardiomyocyte sheets (Shimizu *et al.* 2009), these efforts remain in the realm of tissue engineering. Successful regeneration of a functioning organ requires a means of differentiating cells into a heart complete with higher order structures such as atria and ventricles.

The present investigation assessed an unique induction method developed by us to reliably induce cardiomyocytes with high frequency (Ariizumi *et al.* 2003). Specifically, the animal cap is dissociated once then reaggregated by treatment with activin. Beating tissues were exclusively formed when myocardial cells induced using this method were cultured. We also recently reported the transient expression of BMP inhibitor noggin in precardiac mesoderm, and showed that the consequent inhibition of BMP signaling efficiently induced cardiomyocytes from murine ES cells (Yuasa *et al.* 2005). We postulated that the molecular mechanism of specific cardiomyocyte induction at play in our protocol involved the transient and strong inhibition of intrinsic BMP signals that activate cardiomyocyte induction. The dissociation of animal caps would dilute intrinsic BMP signaling pathways by disrupting cell-cell contacts, followed by the reaggregation and stimulation with activin.

The *Xenopus* cardiomyocyte induction method might be more widely applicable for analyzing cardiogenesis in vertebrates at the molecular level. It will enable the identification of new genes involved in the earliest stages of cardiogenesis, for which analysis has been difficult in previous experimental systems that use the presumptive cardiac region as source material. The system will also permit more detailed analyses of the roles of growth factors and transcription factors in cardiogenesis (Marvin *et al.* 2001; Schneider and Melcola, 2001; Flaherty and Dawn 2008; Zhu *et al.*, 2008).

Transplantation of the *in vitro*-derived cardiac primordia into neurula-stage embryos resulted in complete ectopic heart formation at the abdominal site. The temporal and spatial environment of the recipient tissue is thought to be critical in accepting and maturating the cardiac primordium into a viable ectopic heart. The abdominal site might be successful because (1) the inhibitory signals for cardiomyocyte differentiation were not expressed in this area, (2) major vessels that could connect to the ectopic heart are numerous in this area, (3) space was available in the abdomen for the ectopic heart to establish and grow, and (4) the ectopic heart did not interfere with the host circulatory systems. Transplanted cardiac primordium not only differentiated into a beating heart, but this heart also communicated with the vascular system of the host and was incorporated into the systemic circulation. This system may thus be useful for analyzing the mechanisms of communication between the heart and vasculature. Moreover, transplanted cardiac primordia could be manipulated to undergo morphogenetic processes such as looping and separation of atria and ventricles, and such studies would advance our understanding of inductive interactions with surrounding tissues (Melcola 1999; Takano *et al.*, 2007; Wagner and Siddiqui 2007 a,b).

In this investigation, use of a special method involving transplantation into embryos was attempted. Although this has no immediate clinical applications, the finding that a heart induced *in vitro* can form higher-order structures in the body and function as a circulatory organ seems to represent a basic research finding

that will prove important to the advancement of heart regeneration research.

Materials and Methods

In vitro *cardiomyocyte induction from animal caps*

Cardiomyocyte differentiation was induced *in vitro* using animal caps of *Xenopus* blastulae as described previously (Fig. 1A; Ariizumi *et al.* 2003). Briefly, embryos were cultured and prepared in Holtfreter's saline as medium (HS: 60 mM NaCl, 0.7 mM KCl, 0.9 mM $CaCl_2$, 4.6 mM HEPES, 0.1 g/l kanamycin sulfate, 0.1% BSA [A-7888, Sigma-Aldrich, St. Louis, MO], pH 7.6). Human recombinant activin A, a peptide growth factor (a gift from Dr. Y. Eto [Central Research Laboratories, Ajinomoto Co, Japan]), was dissolved in HS and used as an inducer. At the mid-blastula stage (st. 8; Nieuwkoop and Faber 1956), 0.8 mm x 0.8 mm of the animal cap region was excised using tungsten needles. Animal caps from 5 blastulae were pooled, placed in 100 µl of Ca^{2+}-free HS per well of 96-well plates with round bottoms (MS-309UR, Sumitomo Bakelite, Tokyo, Japan) and left to stand for 20 min to loosen intercellular adhesions. The solution was then substituted with 100 µl of HS (Ca^{2+}-plus) containing 100 ng/ml of activin A and the cells were dissociated by gentle pipetting before being treated in the activin solution for 5 h. Reaggregates formed during this period were cultured in HS.

In vivo *transplantation of cardiomyocyte primordium for ectopic heart formation*

For the ectopic transplantation experiment (Fig. 1A), reaggregates were split into appropriate sizes (20-25% of original) after 1 day of culture. The smaller pieces of reaggregate were then transplanted into an incision made in the abdomens (immediately anterior to the cloaca) of neurulae (st. 20) from the same parent. Embryos that received transplants were allowed to develop and metamorphose into frogs over approximately 2 months, and were then maintained for one year. Beating ectopic (secondary) hearts in the transplanted embryos were subjected to morphological and electrophysiological analysis.

Action potential recording, electrocardiography, two-dimensional, and Doppler echocardiography

Action potentials in the ectopic hearts were recorded at multiple sites in water at 22°C using the patch-clamp method. The heart contractions were too strong to obtain stable recordings using glass microelectrodes, thus action potentials were obtained using suction-type recordings of monophasic action potentials. For electrocardiography, four needle electrodes were inserted into the limbs (left arm, right arm, left leg, and one electrode in the right leg for earthing the current), and limb-lead electrocardiography was conducted at room temperature. Two-dimensional, continuous-wave, and color Doppler echocardiography was performed from the abdominal surface using an Image point 1500 (Philips, USA) with a 15-MHz transducer.

Histological, electron microscopic, and immunohistochemical observations

Ectopic hearts excised from 1-year-old frogs were fixed in Bouin's fluid, and 6-µm-thick paraffin sections were prepared using standard procedures. Sections were stained with haematoxylin and eosin to assess tissue differentiation. For electron microscopy, the excised normal and ectopic hearts were fixed using 3% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), and then post-fixed in 1% OsO_4 in cacodylate buffer. Specimens were dehydrated and embedded in epoxy resin, sectioned, and double-stained using uranyl acetate and lead citrate, for observation under a transmission electron microscope (JEM-100C, JEOL, Tokyo, Japan). For immunohistochemical analysis, excised normal and ectopic hearts were fixed in 4% paraformaldehyde buffered with PBS (pH 7.0) at 4°C overnight and placed in PBS in which the sucrose concentration was increased to 20% in a stepwise fashion. Tissues were subsequently embedded in OCT compound, and a cryostat was used to prepare frozen sections at 10-µm thickness. Sections were blocked with 10% goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA) in PBS and incubated with primary antibody dissolved in 10% goat serum in PBS overnight at 4°C. A rabbit polyclonal antibody specific for atrial natriuretic peptide (ANP) (AB5490, Chemicon) was used at a dilution of 1:250. After two washes with PBS, sections were incubated for 1 h at room temperature with Alexa Fluor® 488 goat anti-rabbit IgG (A-11008, Molecular Probes) secondary antibody at a dilution of 1:500. Antibody binding was observed using a confocal laser scanning microscope (Radiance 2100, Japan Bio-Rad Laboratories, Tokyo, Japan).

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Case Report

Too friable to treat?

Kensuke Kimura, Miho Sakai-Kimura, Ryuichi Takahashi, Atsushi Watanabe, Makio Mukai, Shigetaka Noma, Keiichi Fukuda

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Department of Regenerative Medicine and Advanced Cardiac Therapeutics, Keio University School of Medicine (K Kimura MD, Prof K Fukuda MD), **Division of Diagnostic Pathology, Keio University Hospital** (M Mukai MD), **Shinjuku-ku, Tokyo, Japan; Department of Rheumatology and Infectious Diseases, Kitasato University School of Medicine, Sagamihara, Kanagawa, Japan** (M Sakai-Kimura MD); **Department of Cardiovascular Surgery** (R Takahashi MD) **and Department of Cardiology** (S Noma MD), **Saiseikai Utsunomiya Hospital, Utsunomiya, Tochigi, Japan; and Division of Clinical Genetics, Nippon Medical School Hospital, Bunkyo-ku, Tokyo, Japan** (A Watanabe MD).

Correspondence to:
Dr Kensuke Kimura, Department of Regenerative Medicine and Advanced Cardiac Therapeutics, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan
kimuken@cpnet.med.keio.ac.jp

In February, 2002, a 24-year-old woman was admitted to our hospital with severe chest pain. The pain developed when she was cycling. Nitrates administrated by her general practitioner effectively improved both the symptoms and the ST-segment elevations in leads V1 to V5 of the electrocardiogram. In view of probable acute coronary syndrome, she was admitted to our hospital and underwent immediate cardiac catheterisation. Although coronary angiography showed an intact right coronary artery (RCA) and left circumflex coronary artery (LCX), diffuse stenosis without a radiolucent flap was detected from the mid to distal segments of the left anterior descending coronary artery (LAD) (figure A). Despite spontaneous reperfused LAD flow, the narrowed lumen was unresponsive to either isosorbide dinitrate or nicorandil infusion. The left-ventricular ejection fraction was 48%, and maximum serum concentrations of creatine kinase and the MB isozyme were 2069 IU/L and 186 μg/L respectively (normal range 32–180 IU/L and <5·0 μg/L) 6 h after the onset of chest pain. The patient had no family history of cardiovascular disease, atherosclerotic risk factors, predisposition for hypercoagulability (lupus anticoagulant-negative; anti-cardiolipin-β2-GP1 antibody <1·2 IU/mL; no use of oral contraceptives), or indications of inflammatory disease (antinuclear antibody <×40; antineutrophil cytoplasmic antibodies negative). Refractory vascular spasm or spontaneous coronary artery dissection was therefore considered as the diagnosis. We started treatment with aspirin, an angiotensin receptor blocker, diltiazem, and heparin.

Her clinical course was stable and coronary angiography was done to follow up the LAD lesion 26 days after onset. Surprisingly, despite the spontaneously repaired LAD, both the RCA and LCX had intimal tears that were accompanied by luminal irregularities, indicating several coronary dissections (figure B). The procedure was momentarily paused while treatment options were discussed; at this point, the patient developed chest pain. Coronary angiography showed that the dissection had extended from the LCX to the LAD via the left main trunk, and that the LAD was totally occluded in its proximal segment (figure C). Emergency aorto-coronary artery bypass graft surgery using saphenous vein grafts was done. Histopathological examination of the excised aorta showed disorganised media with fragmented elastic fibres, indicating friable vessels. Soon after the patient was discharged, her 21-year-old brother suffered a gastrointestinal rupture and was diagnosed with vascular-type Ehlers-Danlos syndrome. Eventually our patient was shown to have the same mutation as her brother—Gly220Trp in the (Gly-X-Y)n repeat of the triple-helical domain of type III procollagen (COL3A1).[1] Subsequently, the patient's aunt was also suspected of having vascular-type Ehlers-Danlos syndrome. When seen in December, 2009, the patient was doing well.

Vascular-type Ehlers-Danlos syndrome is an autosomal dominant connective tissue disorder caused by abnormal type III collagen, which is the result of mutations in the COL3A1 gene. Because type III collagen is a major component of blood vessels, viscera, and the uterus, affected patients frequently suffer life-threatening ruptures of the bowel and arteries.[2] Although vascular-type Ehlers-Danlos syndrome is difficult to diagnose in a patient who does not have any of the associated physical features, clinicians should consider the possibility of the syndrome when multiple vascular dissections are present, especially in young patients. Of note, angiography can damage the delicate vessels.[3] The friability of the vessels and associated complications have received much attention in surgical publications.[4] However, the optimum treatment for patients with vascular-type Ehlers-Danlos syndrome remains unclear.

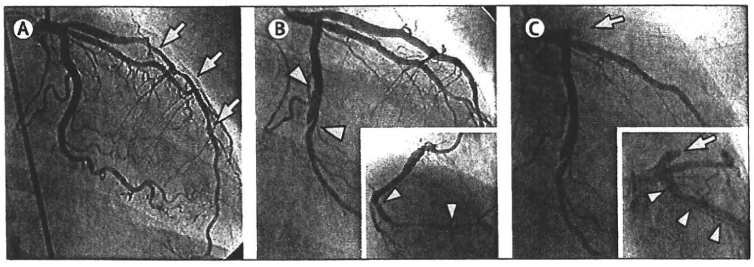

Figure: **Angiography**
(A) LAD showing diffuse stenosis (arrows) on admission. (B) Follow-up 26 days later shows tearing of the RCA (inset) and LCX (main). (C) After the development of chest pain, the dissection of the LCX has extended to the left main stem (inset, arrow heads) and the LAD is totally occluded (arrow).

Contributors
All authors contributed to patient management and writing the report. KK and MS-K contributed equally to this work. Written consents to publish were obtained.

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Norepinephrine-induced nerve growth factor depletion causes cardiac sympathetic denervation in severe heart failure

Kensuke Kimura *, Hideaki Kanazawa, Masaki Ieda, Haruko Kawaguchi-Manabe, Yoshiko Miyake, Takashi Yagi, Takahide Arai, Motoaki Sano, Keiichi Fukuda

Department of Regenerative Medicine and Advanced Cardiac Therapeutics, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan

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Norepinephrine

ABSTRACT

In severe congestive heart failure (CHF), sympathetic overactivity correlates with the exacerbation of cardiac performance. To test the hypothesis that the cardiac sympathetic nerve density dramatically changes with the acceleration of circulating norepinephrine (NE) concentration, we investigated the temporal association of nerve growth factor (NGF) expression in the heart and cardiac sympathetic nerve density during the development of CHF in the continuous NE-infused rats. The animals were analyzed at 0-, 1-, 3-, 7-, 14-, and 28-day after implantation of osmotic pump at a rate of 0.05 mg/kg/hr. The cardiac performance was temporally facilitated in NE-exposed rats at 3-day in accordance with the sympathetic hyper-innervation induced by the augmentation of NGF mRNA expression in the heart. In NE-treated rats, left ventricular end-diastolic pressure was significantly increased after 7-day and marked left ventricular hypertrophy and systemic fluid retention were observed at 28-day. CHF-induced sympathetic overactivity further increased plasma NE concentration in NE-treated rats and finally reached to 16.1 ± 5.6 ng/ml at 28-day (control level was 0.39 ± 0.1 ng/ml, $p<0.01$). In the decompensated CHF rats at 28-day, the NGF mRNA expression was conspicuously reduced concomitant with the obvious nerve fiber loss confirmed by the immunostaining of nerve axonal marker, PGP9.5 and sympathetic neuron marker, tyrosine hydroxylase. This resulted in the attenuated tissue NE contents and the exacerbating cardiac performance. The cardiac sympathetic fiber loss was also confirmed in NE-exposed DBH (dopamine β-hydroxylase)-Cre/Floxed-EGFP (enhanced green fluorescent protein) mice with severe CHF, in which sympathetic nerve could be traced by EGFP. Our results suggest that the cardiac sympathetic nerve density is strictly regulated by the NGF expression in the heart and long-exposure of high plasma NE concentration caused myocardial NGF reduction, following sympathetic fiber loss in severe CHF animals.

1. Introduction

It is well known that plasma norepinephrine (NE) concentration is high in the patients with congestive heart failure (CHF) due to the extreme activation of sympathetic nervous system (SNS), which is progressively augmented corresponding to the severity of CHF (Thomas and Marks, 1978). Moreover, the activation of the cardiac SNS in CHF correlates with the adverse outcome (Cohn et al., 1984). Recent clinical studies have shown that the administration of β-adrenergic receptor blockers improve cardiac performance and reduce cardiac mortality (Packer et al., 1996). NE spillover from sympathetic neurons and the impaired neuronal reuptake have been considered as the major causes of the high plasma concentration in CHF (Hasking et al., 1986; Himura et al., 1993). However, the reduced gene expression of NE synthetic enzyme and NE transporter in the innervated nerves resulting depletion of NE in the failing myocardium cast doubt whether the cardiac sympathetic neurons are still activating in severe CHF (Pool et al., 1967; Eisenhofer et al., 1996). Although the existence of the cardiac-innervated sympathetic neurons which are responsible for synthesizing and secreting NE seems to be of little significance under the circumstance of highly augmented plasma NE level, the innervation anatomy and the source of increased NE overflow remains unexplained so far.

Nerve growth factor (NGF) is a prototypic member of the neurotrophin family, which is critical for the differentiation, maturation, survival, and synaptic activity of the peripheral sympathetic and sensory nervous sytem (Snider, 1994). Expression levels of NGF within innervated tissues roughly correspond to innervations density (Heumann et al., 1984). Some recent studies focused on the decreased myocardial NGF expression in CHF, however the direct evidence of the anatomically "denervated" sympathetic fibers lacked in severe heart failure (Kaye et al., 2000; Qin et al., 2002). We recently reported that the augmentation of NGF expression causes cardiac sympathetic hyperinnervation in the compensated cardiac hypertrophy (Kimura et al., 2007).

* Corresponding author. Tel.: +81 353633874; fax: +81 353633875.
E-mail address: kimuken@cpnet.med.keio.ac.jp (K. Kimura).

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