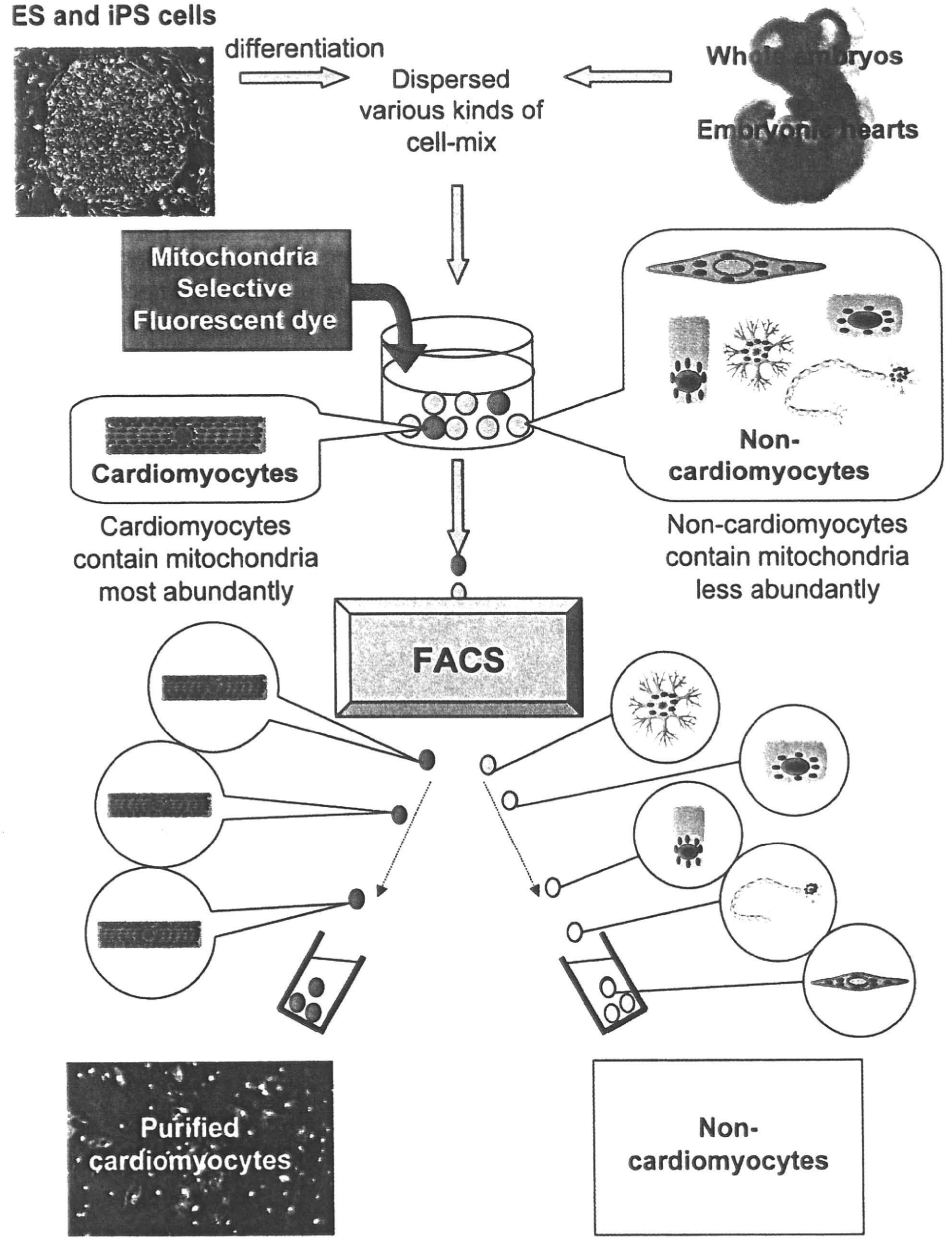

Figure 2. Scheme for the purification of cardiomyocytes from cell mixtures using a nongenetic method.

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; Rouro *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

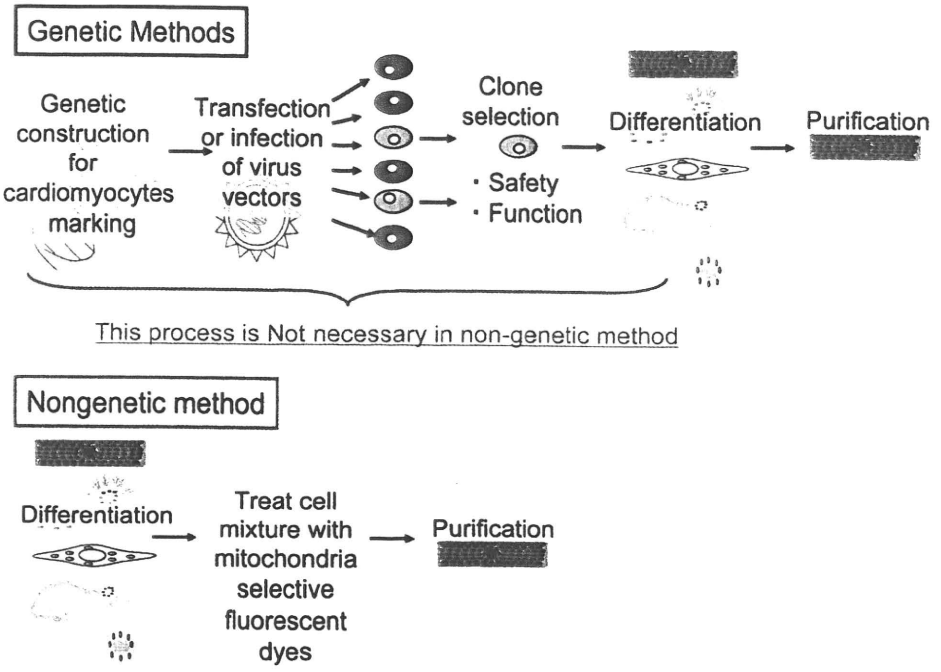

Figure 3. Comparison of a simple nongenetic method and genetic methods.

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

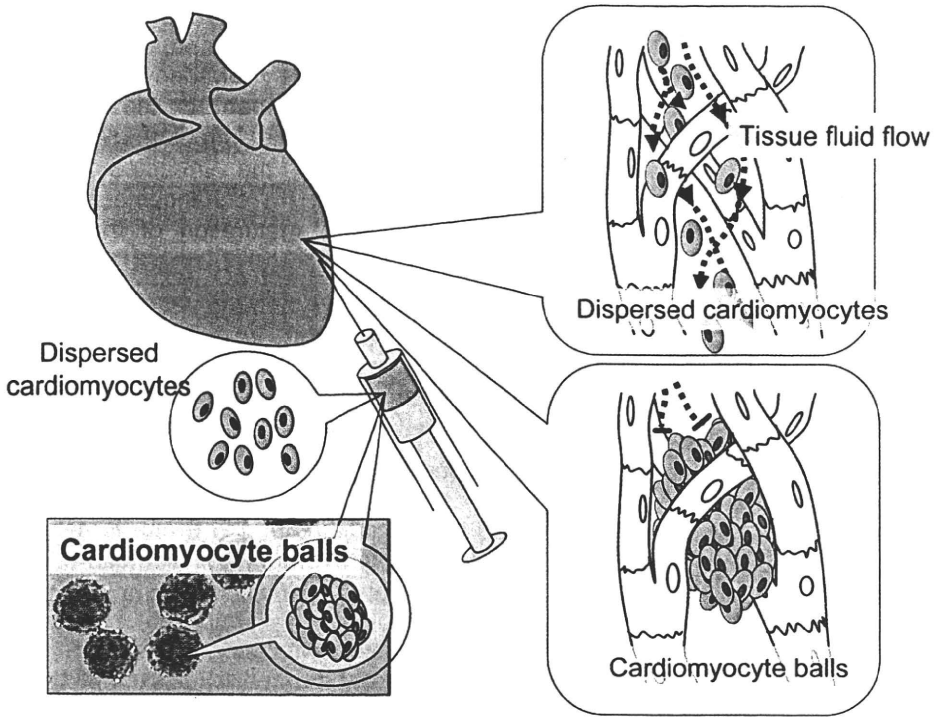

Figure 4. Schematic of the re-aggregation method for the efficient transplantation of FACS-purified cardiomyocytes.

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*

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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).

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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-

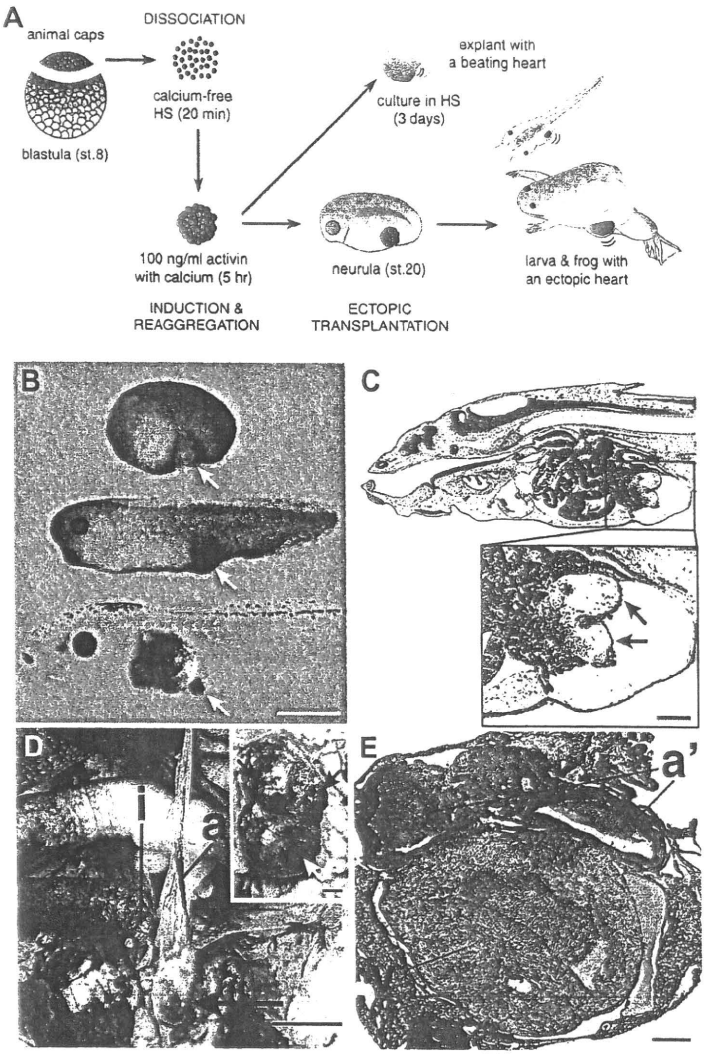

Fig. 1. *In vitro* cardiomyocyte induction and *in vivo* ectopic heart formation. (A) *Experimental protocol of the* in vitro *cardiomyocyte induction and ectopic heart transplantation. For further details, see Materials and Methods.* **(B)** *External morphology of embryos that received the transplant 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).

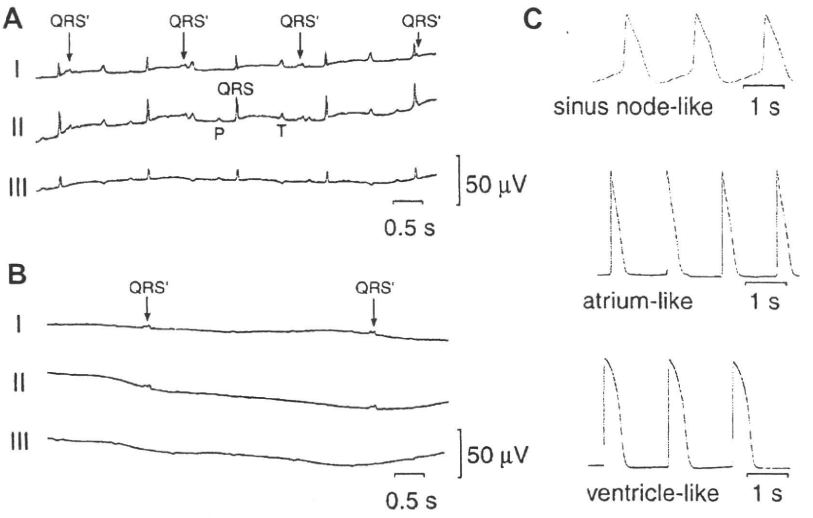

Fig. 2. Physiological analysis of ectopic heart developed from *in vitro*-induced cardiac primordium. (A) *ECG of frog with two hearts. P and QRS indicate host atrial and ventricular potentials, respectively. QRS' indicates ectopic ventricular potential. T is host ventriclar repolarization.* **(B)** *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.*

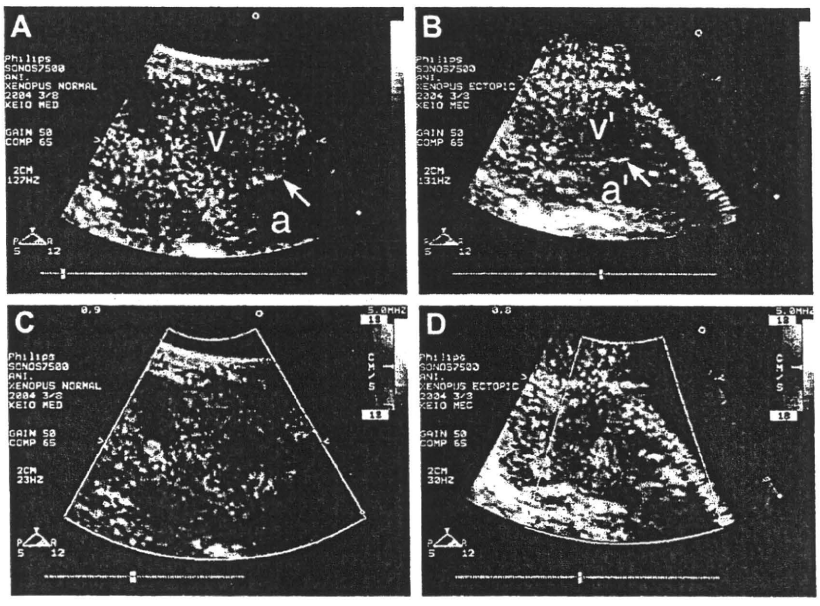

Fig. 3. Echocardiography of the dual-heart frogs. (A) *Two-dimensional echocardiogram of normal heart, showing atrium (a) and ventricle (v). Valve-like tissue (arrow) is visible between these two chambers.* **(B)** *Two-dimensional echocardiogram of the ectopic heart. As with the normal heart, two chambers 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).*

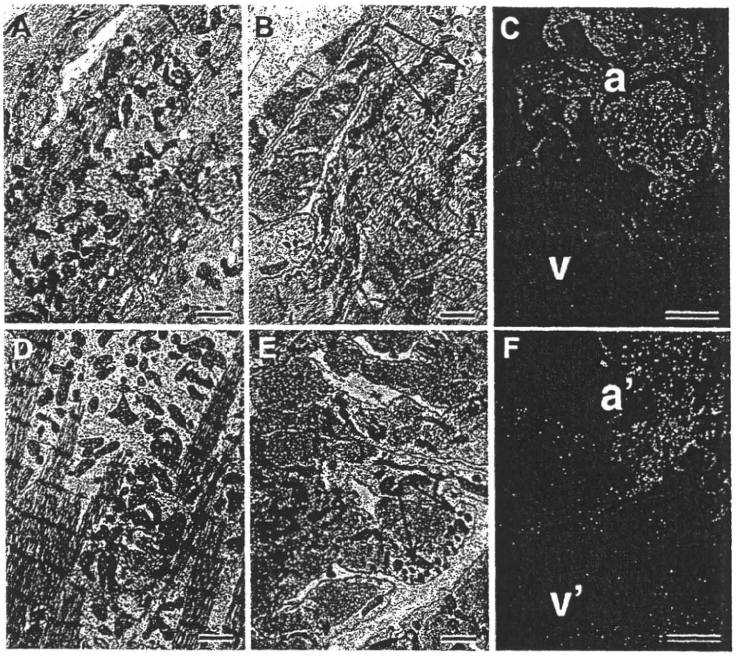

Fig. 4. Electron microscopy and immunohistochemical observations of the heart chambers. (A,D) *Electron micrographs of ventricular myocardium in the normal and ectopic hearts. Ectopic hearts showed similar structures to those observed in normal hearts. No ANP granules are apparent in either image.* **(B,E)** *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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肺高血圧

佐藤 徹

ポイント

- 肺動脈性肺高血圧症が，新しい血管拡張薬の基本的な適応疾患となる．
- 重症例には，エポプロステノールの投与を行う．
- 中等症以下では，内服薬であるボセンタン，シルデナフィルを投与する．
- 各薬剤の長所，短所を十分に検討して，投与薬剤を決定する．

肺高血圧症は，原因により表1のように分類される．このなかで分類2の左心性疾患，分類3の肺疾患に対しては，原病の治療が行われる．肺疾患で重症の肺高血圧症を合併すると肺高血圧症の血管拡張療法が適応されることがあるが，エビデンスはない．分類4の慢性肺血栓塞栓症の治療は，手術(肺動脈血栓内膜摘除術)が原則で手術療法の適応を決めることが最も重要となる．しかし，手術不能例と軽症例では分類1の肺動脈性肺高血圧症と同様に薬物療法を行う．分類1の肺動脈性肺高血圧症が最近開発された血管拡張薬の最もよい適応となる．本稿では，肺動脈性肺高血圧症の薬物療法について述べる．

肺動脈性肺高血圧症の薬物治療

表1には肺動脈性肺高血圧症の原因疾患が記載されている．原因疾患によって血管拡張薬に対する反応，進行のスピード，予後などが異なるため，正確な原因疾患の診断が重要といえる．図1に2007年にChest誌に発表されたACCP(American College of Chest Physician)のガイドラインを示す．現在，日本で使用可能な血管拡張薬を順に概説したい．いずれの治療薬も投与初期(エポプロステノールは増量期間中)には血圧が低下することがあり，患者に血圧測定を義務づけたほうがよい．

Caブロッカー

肺動脈性肺高血圧症のCaブロッカーの有用性については誤解がある．Caブロッカーが有効なのは，カテーテル検査中に急性血管反応性検査(肺血管拡張作用のあるどのような血管拡張薬でも構わないので，血圧低下が限界に達するまで投与量を増やして肺動脈圧の低下度をみる)を行い，急性血管拡張反応が陽性(肺動脈圧が前値の10%以上低下し，かつ平均肺動脈圧が40 mmHg以下となる)となる患者に限られる．図1に示すガイドラインで，一般的ケアのあと，急性血管反応性試験に進むフローチャ

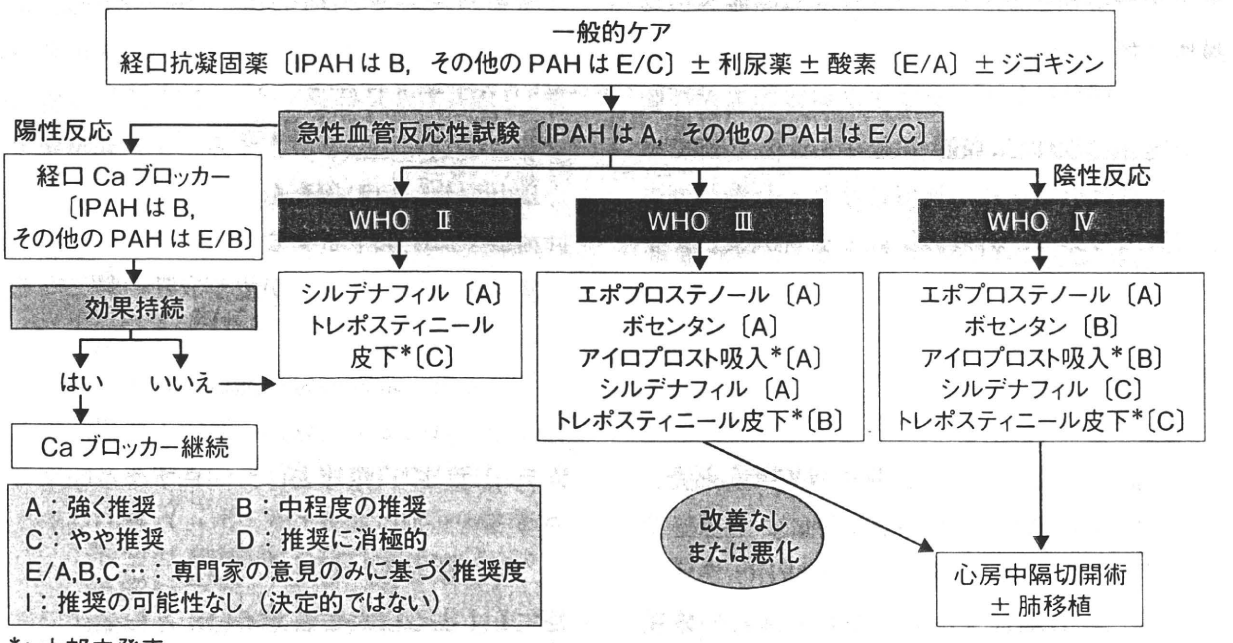
さとう とおる：慶應義塾大学医学部教育統轄センター 〒160-8582 東京都新宿区信濃町35

0025-7699/09/¥500/論文/JCLS

表1 肺高血圧症の分類（ヴェニス分類2003）

1. 肺動脈性肺高血圧症(PAH)
   1)特発性
   2)家族性
   3)各種疾患に伴う肺動脈性肺高血圧症
      ①膠原病性
      ②先天性心疾患
      ③肝臓病
      ④エイズ
      ⑤薬物と毒物
      ⑥その他：甲状腺疾患，糖原病，Gaucher病，遺伝性出血性毛細血管拡張症，異常ヘモグロビン症，骨髄増殖性疾患，脾摘出
   4)肺静脈および／または肺毛細管閉塞
      肺静脈閉塞性疾患(PVOD)，肺毛細血管腫症(PCH)
   5)新生児遷延性肺高血圧症
2. 左心性心疾患に伴う肺高血圧症
   1)左心の心房性，心室性心疾患
   2)左心の弁膜症
3. 肺疾患および／または低酸素血症に伴う肺高血圧症
   1)慢性閉塞性肺疾患
   2)間質性肺疾患
   3)睡眠障害呼吸
   4)肺胞低換気障害
   5)高所における慢性曝露
   6)発育障害
4. 慢性血栓性および／または塞栓性疾患による肺高血圧症
   1)近位肺動脈の血栓塞栓性閉塞
   2)末梢肺動脈の血栓塞栓性閉塞
   3)非血栓性肺塞栓症(腫瘍，寄生虫，異物)
5. その他の肺高血圧症
   サルコイドーシス，ヒスチオサイトーシスX，リンパ管腫症，肺血管の圧迫(リンパ節腫脹，腫瘍，線維性縦隔炎)

第3回世界シンポジウム，Venice, Italy, 2003



*：本邦未発売

図1 ACCPのガイドライン

ートを確認いただきたい．急性血管反応性検査が陽性だとCaブロッカーにより特発性肺動脈性肺高血圧症(idiopathic pulmonary arterial hypertension：IPAH，昔の原発性肺高血圧症)の5年予後は95%と報告されている[1]．欧米では全患者の10～20%に急性血管反応陽性者が存在するとされるが，本邦では5%以下といわれている．

エポプロステノール

適応

最も強力な治療薬で，使用当初から欧米では

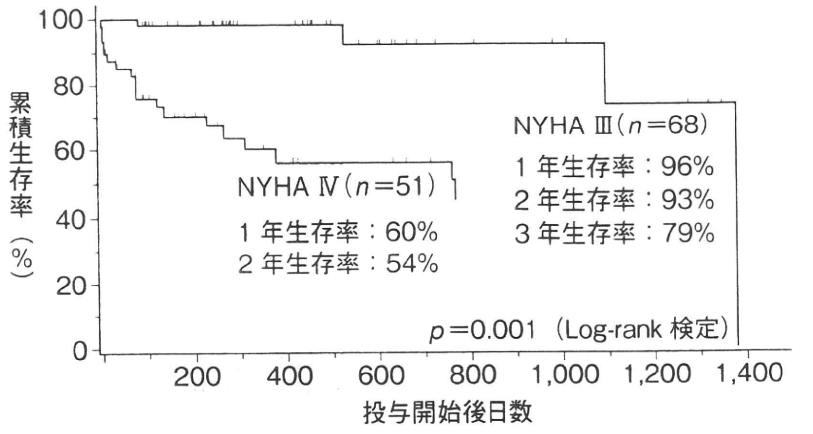


図2　IPAHの生存率

IPAHの5年生存を約30%から70%前後に改善した．**図1**のガイドラインでWHO Ⅳ(WHO分類はNYHA心機能分類とほぼ同様の基準で，症状は呼吸困難のみを対象とした臨床症状からの重症度分類．ちなみにNYHA心機能分類は胸痛，動悸など，ほかの心臓病の症状も対象としている)では，エポプロステノールのみが「強く推奨」とされている．すなわち，重症例ではエポプロステノールが適応となる．しかし，エポプロステノール治療は留置カテーテルが必要で患者にかなりの負担を強いることになるため，適応は慎重に考慮する必要がある．肺高血圧症の予後には，右心不全の程度が強く関連するが，以前のわれわれの検討では肺血管抵抗値20 Wood unitが右心不全出現の目安であった．また，実際にWHO ⅣとなるとⅢに比べて予後が不良であるため(**図2**；図はWHOではなくNYHAの分類によるものだが，両者の分類は同等のものである)，真に重症化する少し手前と考えて，肺血管抵抗値15 Wood unitあたりからエポプロステノールの適応を考えるようにしている．原因疾患も適応決定の考慮要素となるが，膠原病を原因とする患者ではエポプロステノールに対する反応がより良好で，逆にほかの内服の治療薬は，膠原病を原因とする肺高血圧症に対して効果がやや弱い傾向を示す．また，在宅持続点滴静注療法となるため，エポプロステノールの治療の自己管理が十分にできることが必要となる．

投与法

留置カテーテル挿入後，1 ng/m*l*/minより開始し，4日ごと程度で1 ng/m*l*/minずつ増量し，2〜3カ月経過したら7日ごとの増量とする．半年で増量を中止するか1年間増量するかは，カテーテル検査を施行して判断する．初回治療で血圧低下を生じない範囲で，スピーディに増量していくことが治療成功の鍵になる．

注意点

この治療の最大の合併症は，カテーテル挿入部の皮膚感染で，十分に予防する必要がある．カテーテルの自然抜去にも注意する．薬剤自身の副作用は頭痛，顔のほてり，皮膚の発赤，下痢，骨痛などで，頻度は低くないが対症的に対処する．投与前から右心不全がある症例では，エポプロステノールによる心拍出量増加の結果，右心不全が悪化することがあり，しっかり経過をみてゆく．

ボセンタン

適応

欧米の前向き研究では，WHO Ⅱ，Ⅲに対し，2年生存率89%で，コントロールの57%を大

幅に上回った．日本でもWHO Ⅱ，Ⅲの21例に対し，3カ月後，6分間歩行距離の86mの延長などが認められた．ACCPガイドライン(**図1**)でWHO Ⅲ，Ⅳが適応となる．最近WHO Ⅱに対しても有効との前向き研究が発表された．

投与法

1日量2錠(1錠62.5 mg)を分2で投与開始し，1カ月後副作用の出現がなければ，1日量4錠を1日2回投与する．1日量2錠よりも4錠のほうが効果が高いことがわかっている．

注意点

自覚的な副作用は頭痛，顔面紅潮，筋痛などで多くは自制できる．他覚的な副作用が問題で，肝機能検査を1カ月に一度施行し，AST，ALT値が正常値の3倍までは経過観察し，5倍まではボセンタン減量で対処する．副作用として3系統の血球のいずれも減少する可能性があり，1カ月に一度末梢血検査を行う．妊産婦・授乳婦への投与は禁止となる．シクロスポリン，タクロリムスはボセンタンの血中濃度を上昇させ，グリベンクラミドは肝障害を増悪させるなど，薬剤相互作用に注意する．

■ シルデナフィル

適応

2008年初頭に肺動脈性肺高血圧症治療薬として保険適用となった．ACCPガイドライン(**図1**)でWHO Ⅱ～Ⅳが適応となる．臨床的改善作用はエポプロステノールよりは劣るが，ボセンタンにほぼ匹敵すると感じている．しかし，薬剤に対する反応性は個人差が大きく，効果の予測は今後の課題と思われる．シルデナフィルは血管内皮でのcGMPを増量して効果を発揮するが，cGMPには血管拡張作用と独立して，後負荷増を原因とする心機能障害の強力な改善作用がある．肺高血圧症に伴う右心不全がまさにこれに相当し，右心不全を伴う肺高血圧症例では第一適応となる．われわれは右心不全を伴う重症肺高血圧症では，シルデナフィル投与後にエポプロステノールを導入するようにしている．

投与法

1日量3錠(一錠20 mg)の分3が標準量とされる．血圧が低い患者では1/2量，1/4量から開始して可能なら増量する．シルデナフィルは効果に用量依存性が少ないとされ，少量でも有効との意見もある．これに関する前向き試験も予定されている．

注意点

副作用は頭痛，顔面紅潮，下痢などでボセンタン同様自制可能な患者が多いが，なかにどうしても継続が難しい患者もいる．ボセンタンと併用するとボセンタンの血中濃度を上げてボセンタンの副作用が発現しやすくなる．50歳以上の男性で糖尿病，高血圧を有する患者にED(erectile dysfuncion)治療のために使用すると失明が多かったとの報告があるが，肺高血圧症では視力障害なども報告されていない．視力障害を認めたら報告するよう，念のため患者に伝えておく．

■ 長時間型ベラプロスト

従来使用されていたベラプロストを長時間型に改良した薬剤が本年初頭に発売された．前向き研究で6分間歩行距離などが改善している．従来のベラプロストは，6カ月までは有意に6分間歩行距離の改善効果があったが，9カ月で投与前に戻り長期効果がないことからアメリカでは認可とならなかった．軽症例には有効であることは示されており，今後，長時間型製剤の効果を確認していく必要がある．

文　献

1) Rich S, Kaufmann E : High dose titration of calcium channel blocking agents for primary pulmonary hypertension : Guidelines for short-term drug testing. J Am Coll Cardiol 18 : 1323-1327, 1991

2. 呼吸困難

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はじめに

呼吸困難とは，呼吸時にふだんは意識しない呼吸運動を意識して不快に感じることを指す．呼吸困難は急性発症と慢性発症に分類することができる．

本稿は救急疾患を対象とすることから急性発症の疾患，とくに心肺系疾患で頻度の多いものを中心に述べる．

原因疾患

急性呼吸困難の原因は**表1**のように分類される[1]．とくに頻度の多い重要な鑑別疾患は赤字で示した．

表1 呼吸困難をきたす疾患の鑑別

1. 呼吸器疾患	①喘　息 ②肺　炎 ③急性肺塞栓症 ④気　胸 ⑤上気道閉塞（誤嚥，窒息） ⑥下気道閉塞 ⑦成人呼吸窮迫症候群（ARDS）
2. 心臓血管疾患	①心不全 ②急性心筋虚血（ACS） ③心タンポナーデ ④急性心膜炎
3. 腹部疾患	機械的な横隔膜運動の障害
4. 代謝性疾患	①糖尿病性ケトアシドーシス ②腎不全 ③代謝性アシドーシス ④刺激物の吸入
5. 耳鼻科系疾患	喉頭炎
6. 血液系疾患	①CO中毒 ②貧　血
7. 神経筋疾患	①中枢系疾患 ②有機リン中毒
8. 精神神経疾患	不安神経症

問　診（表2）

1. 発　症

急性発症の呼吸困難をきたす疾患のなかでも，急性肺塞栓症，気胸，上気道閉塞，急性心筋虚血，

表2 呼吸困難をきたす疾患の問診による鑑別

		急激な発症	起座呼吸	発　熱	痰	血　痰
1. 呼吸器疾患	①喘　息		○		○	
	②肺　炎		○	○	○	○
	③急性肺塞栓症	○		○		○
	④気　胸	○				
2. 心臓血管疾患	①心不全		○			○
	②急性心筋虚血（ACS）	○				
	③心タンポナーデ					
3. 腹部疾患						
4. 代謝性疾患						
5. 耳鼻科系疾患	喉頭炎			○		
6. 血液系疾患						
7. 神経筋疾患			○			
8. 精神神経疾患						

表３　呼吸困難をきたす疾患の身体診察による鑑別

		頻呼吸	頻　脈	肺聴診	心　臓
1. 呼吸器疾患	①喘　息	○	○	乾性ラ音	
	②肺　炎	○	○	乾性・水泡性ラ音	
	③急性肺塞栓症	○	◎	胸膜摩擦音	右心負荷所見
	④気　胸	○	○	呼吸音低下	
2. 心臓血管疾患	①心不全	○	◎	水泡性ラ音	両心負荷所見
	②急性心筋虚血（ACS）	○	○		
	③心タンポナーデ	○	○		右心負荷所見
3. 腹部疾患		○			
4. 代謝性疾患		○			
5. 耳鼻科系疾患	喉頭炎	○		○	
6. 血液系疾患		○	○		
7. 神経筋疾患			○		
8. 精神神経疾患		△			

中毒性のものなどではとくに急激な発症をする．

2. 症状の特徴

起座呼吸は左心不全で多いが，呼吸器疾患や神経筋疾患でも横臥で横隔膜が上昇するため起座呼吸を訴えることがある．発熱は肺炎，喉頭炎などの感染症や急性肺塞栓症，急性心膜炎などの炎症を伴う疾患で見られる．痰は喘息，肺炎などの呼吸器疾患で多い．血痰は急性肺塞栓症で多いが，肺炎，心不全（ピンク状泡沫痰）などでも見られる．精神神経系疾患では不定愁訴や不安の訴えがある．上気道閉塞や中毒性のもの，事故の関係したものなどは，誘因を問診で明らかとし鑑別に結びつける．

3. 既往歴

原因疾患の既往を問診することは当然必要となる．急性肺塞栓症では長期臥床，下肢の整形外科的手術，妊娠などの有無に注意する．急性心筋虚血では狭心症の既往を聞く．心タンポナーデの原因は該当稿に譲る．

身体診察（表3）

肺疾患・心疾患では頻呼吸，頻脈となることが多い．

1. 肺疾患

喘息では種々の乾性ラ音が聞かれる．肺炎では，乾性・水泡性ラ音，呼吸音の低下，打診による濁音，清音伝導の亢進が見られることがある．急性肺塞栓症では，肺高血圧症によるSIIp亢進，右室拍動，肺野では胸膜摩擦音が聞かれることがある．片側性の下肢腫脹は原因となる下肢深部静脈血栓症を示唆していることがあるほか，足背を背屈させて下腿に痛みを生ずるホーマンズ徴候（Homans sign）も確認する．気胸では一側の呼吸音が低下する．

2. 心疾患

頸静脈が怒張し，SIII・SIVが出現することがあり，左心不全では肺野の水泡性ラ音が見られる．急性心膜炎では心膜摩擦音が聞かれる．