causally related to the loss of proliferation of cardiomyocytes during terminal differentiation. Inconsistent with this, we observed that fetal rat cardiomyocytes, which decrease cell growth capacity but are still capable of proliferating, exhibited the intermediate activity of the *in vitro* Skp2 ubiquitylation and p27 expression between neonatal cardiac cells and REF52 fibroblasts.[2] More recently, it has been reported that Skp2 and its cofactor Cks1 proteins are degraded by the ubiquitin ligase APC/Ccdh1 (anaphase-promoting complex/cyclosome and its activator Cdh) (31, 32). Detailed analysis of APC expression and its Skp2 degrading activity in cardiomyocytes may clarify the mechanism of terminal differentiation and such an investigation is now in progress.

Finally, we demonstrated that p27 is a strong cell cycle barrier of terminally differentiated cardiomyocytes, and its down-regulation by a combination of p27 siRNA or Skp2 and D1NLS/CDK4 significantly overrode the limited cell proliferation of cardiac cells. The impaired Skp2/p27 regulation may represent one of the molecular mechanisms by which not only cardiomyocytes but also other terminally differentiated cells lose the capacity to proliferate.

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A New Method for Manufacturing Cardiac Cell Sheets Using Fibrin-Coated Dishes and Its Electrophysiological Studies by Optical Mapping

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Abstract: We developed a novel simple method for making functional myocardial cell sheets that may be used as transplants. Polymerized human fibrin-coated dishes were prepared with fibrinogen monomers mixed with thrombin. Neonatal rat cardiomyocytes cultured on these dishes formed myocardial cell sheets within 4 days. These cell sheets were easily dissociated intact from the polymerized fibrin layer, because the fibrin had been digested by intrinsic protease. Two overlaid myocardial cell sheets exhibited synchronized spontaneous beating and captured artificial pacing. Optical mapping confirmed that the conduction of the action potential between two partially overlaid myocardial cell sheets was established, and the action potential propagated across the junction without any delay. Transplanted three-layered myocardial cell sheets exhibited strong spontaneous beating and showed well-differentiated striations and an increase in cell size. This simple method of cell sheet engineering may also be applicable for various other cell types. **Key Words:** Tissue engineering—Cell sheet—Polymerized fibrin—Cardiomyocyte—Optical mapping—Electrical connection.

The use of organ transplantation, a powerful treatment for patients with severely damaged organs, remains limited by the shortage of donors. Recent advances in the field of regenerative medicine now promise possible alternative sources of organ grafts. The regeneration of cardiomyocytes from various stem cells, such as embryonic stem cells (1,2) and bone marrow-derived stem cells (3,4), has been observed in vitro, and a number of cell transplantation therapies have restored the function of damaged cardiac tissues. A clinical trial involving the myocardial injection of autologous myoblasts has produced a limited but important recovery of impaired cardiac function (5,6). However, the direct delivery of isolated cells that were used in these studies can induce some aggregation and necrosis of the grafted cells, and it remains difficult to transplant a sufficient number of cells to significantly improve cardiac function (7,8).

The challenge in tissue engineering has been the production of functional heart grafts with a three-dimensional structure. To maintain the three-dimensional structure of tissues, different scaffolds composed of extracellular matrix and artificial polymers have been tested (9–13). Okano et al. described a unique cell-manipulation technique that could be used to construct three-dimensional myocardial tissues by layering two-dimensional cell sheets. To obtain myocardial cell sheets, they created temperature-sensitive culture dishes by grafting temperature-responsive polymer (poly-N-isopropylacrylamide; PIPAAm) onto the surface of the dish. At 37°C, the surface is hydrophobic, and cells can attach to the dishes. However, when the temperature is dropped to 32°C or below, the surface becomes hydrophilic and the grafted polymer rapidly hydrates. The

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grafted polymer changes to expand, which causes cells to detach from the surface (14,15). This enables the generation of a free, confluent, thin cell sheet just by cooling the dishes to room temperature. Moreover, in contrast to enzymatic digestion, both adhesive proteins and cell–cell junctions between the confluent cells are perfectly preserved with this method, enabling generation of a three-dimensional functional tissue that lacks any scaffold.

We have developed a very simple new cell sheet engineering method using thin biodegradable polymerized fibrin-coated dishes. Here, we describe this new tissue engineering technique, and the histological and electrophysiological characteristics of the myocardial cell sheets. Electrical disconnection between the myocardial cell sheets or between a myocardial cell sheet and a host cardiac tissue may cause fatal arrhythmias (16), so we evaluated the electrical synchronization of engineered myocardial tissue with a high-resolution optical mapping system using a voltage-sensitive dye.

MATERIALS AND METHODS

Preparation of myocardial cell sheets

All experimental procedures and protocols were approved by the Animal Care and Use Committee of Keio University and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Tissiel was purchased from Baxter (Vienna, Austria), and its constitution was changed to 90 mg of human fibrinogen, 20 mg of human serum, 0.4 U of thrombin, 0.59 mg of $CaCl_2$ ($2H_2O$), and 3000 U of aprotinin. The solution was diluted with 16 mL of saline, and 0.3 mL was spread rapidly onto 35-mm culture dishes (Becton Dickinson and Company, Bedford, MA, U.S.A.). Two hours later, the polymerized fibrin-coated dishes were obtained, and they were stored at 4°C. Primary cultures of cardiomyocytes were prepared from the ventricles of 1 day-old neonatal Wistar rats (Japan CLEA, Tokyo, Japan) as described previously (17) and were plated on the dishes ($2.8 \times 10^5/cm^2$).

Transplantation of myocardial cell sheets onto adult rat subcutaneous space

The dorsal skin of male F344 nude rats (Japan CLEA) (8 weeks of age, $n = 10$), which were anesthetized by inhalation of diethyl ether (Wako, Osaka, Japan) and subcutaneous injection of 1% procaine-HCL (5–10 mL) (AstraZeneca, London, U.K.), was cut and opened. Then, trilayered myocardial cell sheets were transplanted into the subcutaneous tissue.

Histological analysis

Immunostaining was performed as described previously (18) by using antifibrin (Monosan, Uden, the Netherlands), anti-α-actinin (Sigma, St. Louis, MO, U.S.A.) monoclonal antibodies, and anticonnexin43 (Sigma) polyclonal antibodies. The samples were incubated with either Alexa488-labeled antimouse IgG antibody (Molecular Probes, Eugene, OR, U.S.A.), TRITC-labeled antirabbit IgG antibody (Dako, Tokyo, Japan) or Alexa594-conjugated phalloidin (Molecular Probes Europe BV, Leiden, the Netherlands). Nuclei were stained with TOTO-3 (Sigma). They were observed under confocal laser microscope (LSM510, Carl Zeiss International, Jena, Germany).

Electro-optical analysis of the myocardial cell sheets

For analysis of electrical communication, two myocardial cell sheets were overlapped by 2 mm at the edges and cocultured for 1, 2, and 3 days on culture dishes pretreated with laminin (Roche, Mannheim, Germany) as described previously (19). Extracellular electrical potentials at both ends of each sheet were recorded with a pair of contact bipolar electrodes. The optical mapping system was applied by using a membrane voltage indicator, di-4-ANEPPS (Molecular Probes), to monitor two-dimentional action potential propagation and the electrical connection between the two cocultured myocardial cell sheets. Di-4-ANEPPS stock solution (20 mM) was freshly prepared with DMSO (Sigma) solution containing 20% pluronic F-127 (P-3000, Molecular Probes) and added to the culture media to give a final concentration of 10 μM di-4-ANEPPS. The samples were exposed to the dye at 37°C for 30 min. The plates were then washed in Tyrode's solution consisting of (in mM) 140 NaCl, 4 Cl, 0.5 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES, 55 D-glucose (pH adjusted to 7.4 with NaOH), and 100 mg/L bovine serum albumin (Sigma). Glucose was then added to adjust the osmotic pressure of Tyrode's solution to the culture media. The myocardial cell sheets were kept in an originally made temperature-controlled chamber (37°C) on the fluorescence microscope (BX50WI, Olympus, Tokyo, Japan). Signals were monitored through a high-resolution CCD-camera system (MiCAM01, 192 × 128 points, 3.5 ms time resolution, Brain Vision, Tokyo, Japan) at an emission wavelength of greater than 610 nm and an excitation wavelength of 520 nm. The sheets were immobilized by cytochalasin-D (25 μM) (Sigma). Action potentials were observed in the case of spontaneous excitation or bipolar pacing via the contact Ag–AgCl electrode. The obtained data were processed accord-

ing to the original procedure (20) using commercial Igor Pro software (Wavemetrics, Inc., Lake Oswego, OR, U.S.A.).

RESULTS

Generation of myocardial cell sheets

The primary cell cultures contained approximately 78% desmin- and connexin43-double-positive cardiomyocytes. After the cells were plated on fibrin polymer-coated dishes (Fig. 1A,B,G), the fibrin layer was gradually degraded by proteases secreted from the cultured cells (Fig. 1C). After 4 days, the myocardial cell sheets were detached from the plates with a cell scraper (Fig. 1D,H), laid flat in culture media (medium 199/DMEM supplemented with 10% FBS) (Fig. 1E,I), then trimmed to a square shape and overlaid for subsequent experiments (Fig. 1F,J).

Histological analysis of myocardial cell sheets obtained from the polymerized fibrin-coated dishes

To assess the myocardial cell sheets obtained by this method, we collected the cells with a scraper at various time points after plating of the cardiomyocytes on the polymerized fibrin-coated dishes (Fig. 2). When the myocardial cell sheets were peeled off from the dishes after 4 days, the detached myocardial cell sheets decreased in diameter by $38 \pm 3.6\%$ ($n = 30$). Myocardial cell sheets could not be obtained by using noncoated, gelatin-, laminin-, or fibronectin-coated culture dishes (data not shown). We defined the time from the primary culture to the fabrication of the myocardial cell sheets as PX days, and the time from the fabrication to the experiment as SX days (Fig. 2A). Residual fibrin polymer was observed at the bottom of the sheet in the sample obtained on day 4 (P4–S0) (Fig. 2B), but was not detected in the sample taken on day 6 (P6–S0) (Fig. 2C). Fibrin polymer was still visible in the interstitial space in the P4–S1 sheet (Fig. 2D,F) but was completely digested in the P4–S3 sheet (Fig. 2E,G). When aprotinin (serine protease inhibitor) was added (600 KIU/mL) to the samples after separation of the cell sheets, a considerable amount of fibrin polymer was observed to have remained undigested (Fig. 2H–K).

Characteristics of the myocardial cell sheet made using the fibrin-coated dish

To investigate the optimal time to harvest the myocardial cell sheets after primary culture, we collected the cells with a scraper at various time points after plating the cardiomyocytes on the polymerized fibrin-coated dishes ($n = 12$ each, Fig. 3A). The success rate for obtaining myocardial cell sheets increased after 3 days and peaked at day 4 (100% success rate).

The percentage of spontaneous beating of the myocardial cell sheets was then taken at different time intervals ($n = 12$ each). We first altered the duration of PX days while keeping SX days fixed to 3 (S3 day). The percentage of beating myocardial cell

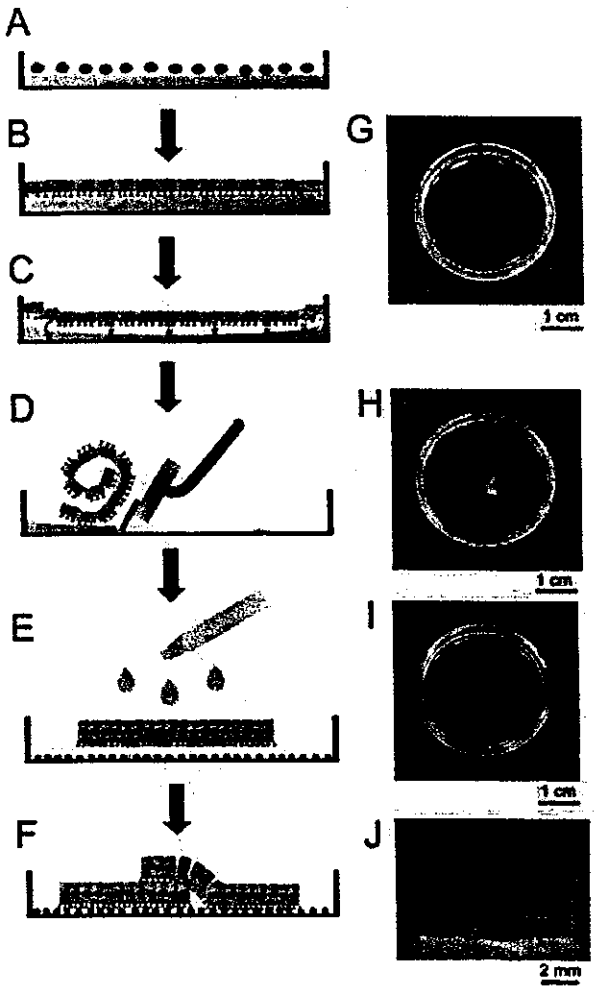

FIG. 1. Representative schema of the manipulation of myocardial cell sheets using polymerized fibrin-coated dishes. (A) Primary cultured neonate rat cardiomyocytes were spread onto the polymerized fibrin-coated dishes. (B,G) Cardiomyocytes became confluent. (C) In 4 days, the fibrin polymer had been degraded by proteases secreted from cardiomyocytes. (D) Cells were gently raked from the edge toward the center of the dishes so as not to tear the myocardial cell sheets with the cell scraper. (H) Shrunken myocardial cell sheets were obtained. (E,I) A few drops of culture media were applied to the shrunken sheets to unfold them. (F,J) The edges of the flattened myocardial cell sheets were trimmed into a square shape by using a blade. In some experiments, two myocardial cell sheets were overlaid at the margin with 2-mm width and co-cultured on laminin-coated culture dishes.

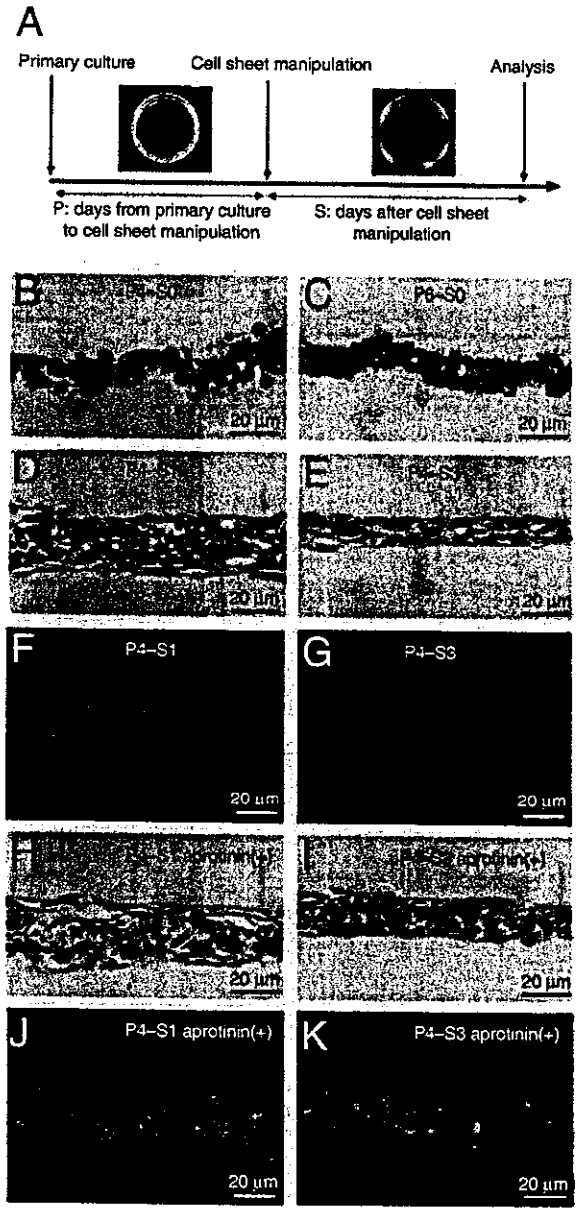

FIG. 2. Histological analysis of the myocardial cell sheets. (A) Protocol for making the myocardial cell sheets and definition of PX and SX days. (B–E,H,I) Hematoxylin and eosin staining of the myocardial cell sheets. (F,G,J,K) Immunofluorescent staining of the myocardial cell sheets. Red: F-actin; Green: fibrin; Blue: TOTO-3 indicating nuclei. Protocols and scale bars are indicated in the figure inset.

sheets showed that the difference in PX days did not affect the percentage (Fig. 3B).

Next, we fixed the PX to 4 days (P4 day), changed the length of SX days, and again measured the percentage of spontaneous beating of the cell sheets ($n = 30$). The percentage of spontaneous beating began to increase significantly from S2 day and reached 100% at S6 day (Fig. 3C). Furthermore, the percentage of myocardial cell sheets that captured artificial pacing increased from S2 day and reached 100% at S5 day (Fig. 3D).

Figure 3(E) shows the beating rate of the myocardial cell sheets from S0 day. It increased rapidly to S3 day and continued to increase at a slower rate thereafter ($n = 6$).

Inasmuch as we could reach 100% of the success rate on P4 day and P5 day, and responsiveness to artificial pacing increased remarkably for the first 3 days, the P4–S3 sheet appeared to have established relatively stable electrical nature.

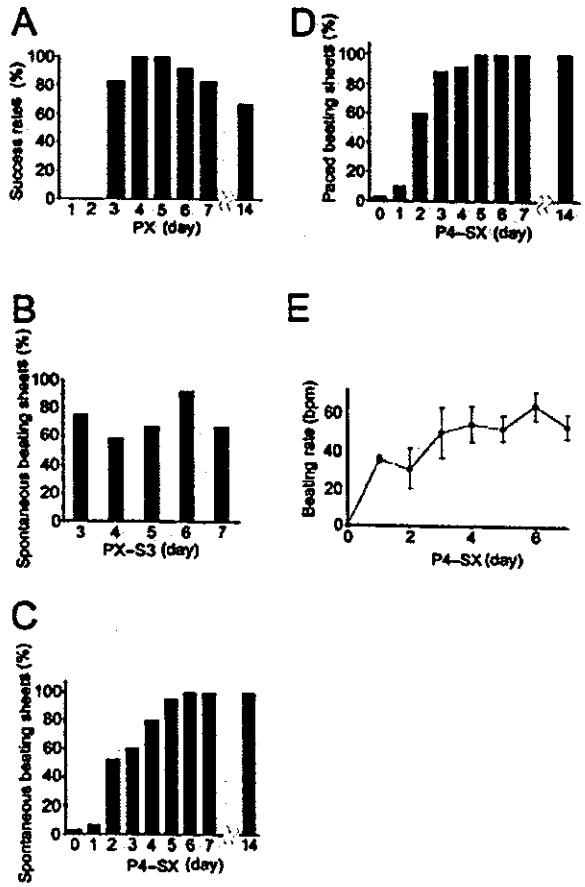

FIG. 3. Characterization of the myocardial cell sheets. Abbreviations in the figures (PX, PX–S3, P4–SX) were explained at Fig. 2(A). (A) Rate of success of obtaining myocardial cell sheets in the PX day sample. (B) Percentage of spontaneously beating myocardial cell sheets in the PX–S3 day samples. (C) Percentage of spontaneously beating sheets in the P4–SX day samples. (D) Percentage of myocardial cell sheets that captured artificial pacing in the P4–SX day samples. (E) The rate of beating in the P4–SX day samples. Values are presented as mean ± SEM.

Comparison of optical mapping with contact bipolar electrodes to analyze action potential propagation

We examined the action potential propagation within the myocardial cell sheets to determine whether an electrical connection was established between the two myocardial cell sheets. Recordings of extracellular electrical potentials at both ends of the sheets using a pair of contact bipolar electrodes were compared with the data obtained using the optical mapping system. With the use of bipolar electrodes, electrical activation was detected simultaneously on sheets A and B (Fig. 4A,C). The electrical spikes in sheets A and B were synchronous, which suggested that the two myocardial cell sheets had established an electrical connection. Subsequent optical mapping (Fig. 4D) performed on the same samples (Fig. 4B) demonstrated that the action potential arose from the lower left of sheet A, conducted to the upper right before passing through the upper junction and spreading to sheet B, meaning that action potential propagation did not follow a direct route. Thus, the optical mapping system was the more effective means of electrophysiological analysis of the action potential propagation and electrical communication between the myocardial cell sheets.

Constitution process of the electrical connection between the two cell sheets

To investigate when the electrical connection between the two myocardial cell sheets was constituted, we performed optical mapping on days 1 and 3. The action potential did not propagate from one myocardial cell sheet to the other in the P4–S1 samples (Fig. 5A–C). The contour map of propagation of the action potential (Fig. 5E) revealed that the action potential was blocked at site t. These data suggested that electrical connection was not established in the P4–S1 samples.

In contrast, the action potential propagated from sheet A to sheet B without conduction delay in the P4–S3 myocardial cell sheets (Fig. 6). All P4–S3 tissue samples had sufficient electrical communication between the two myocardial cell sheets (10/10) without any delay in conduction speed.

Histological evidence of communication between two myocardial cell sheets in vitro

Immunofluorescent staining of the P4–S3 myocardial cell sheets (Fig. 7) showed that cardiomyocytes had well-organized sarcomeres, and connexin43 was localized at the junctions between cardiomyocytes. The two-layered myocardial cell sheets were approximately 15 ± 2 μm thick, and the two layers

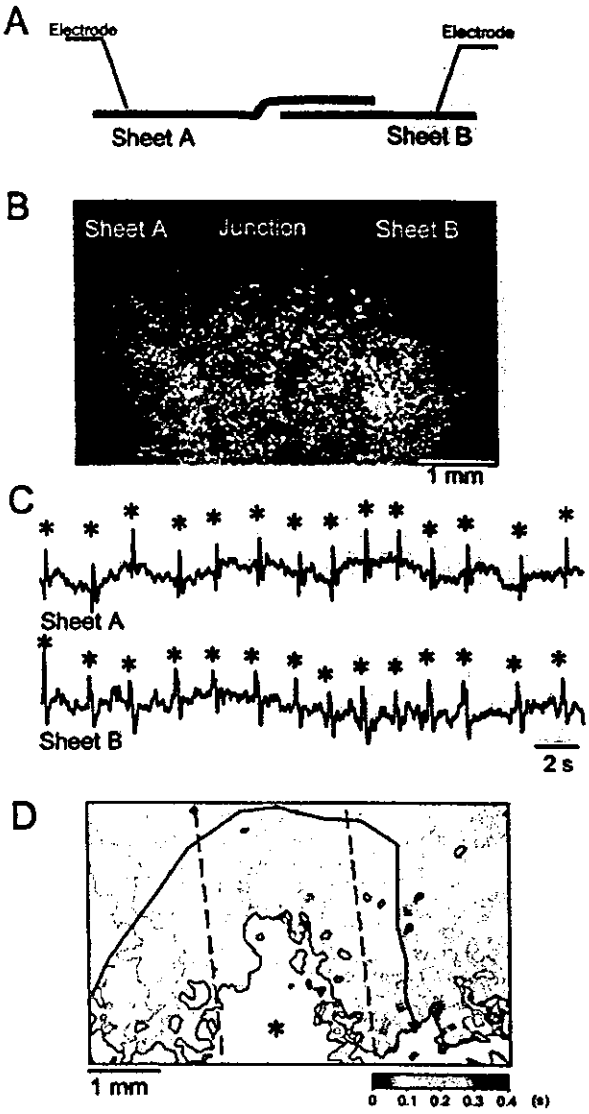

FIG. 4. Comparison of optical mapping and extracellular electrical potential recoding using a pair of contact bipolar electrodes for analysis of electrical connection between the two overlaid myocardial cell sheets. (A) Schema of the two overlaid myocardial cell sheets and position of contact bipolar electrodes, and (B) representative microscopy. (C) Extracellular electrical potentials obtained from each myocardial cell sheet, which was beating spontaneously, showing synchronization. (D) Optical image of the action potential was recorded for same sample, and an activation map was drawn from the recorded action potentials. The interval between each isochronal line was 35 ms. The spontaneous excitation originated from the left lower site of sheet A, went around the lower half of the junction which was an electrically unexcitable area (*), and propagated to sheet B via the upper half of the junction. The wave front of the action potential proceeded along the black curved line with arrowed head.

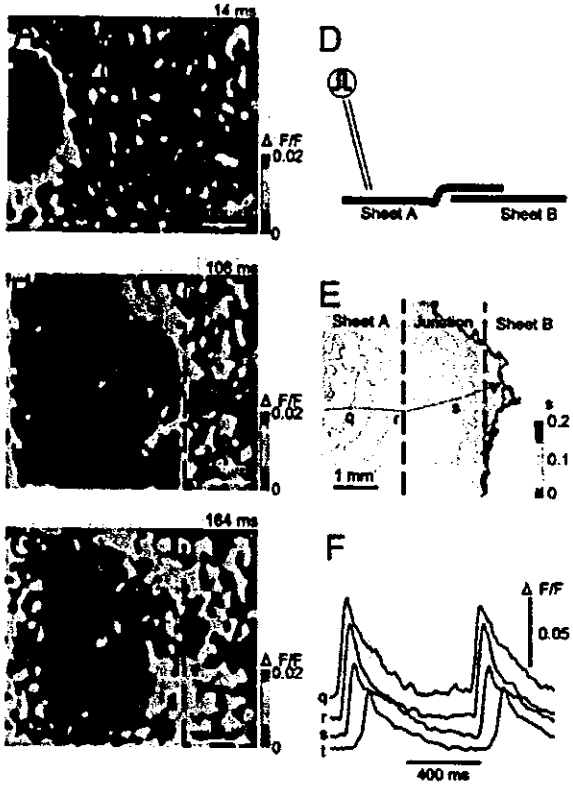

FIG. 5. Optical mapping of the action potential propagation and electrical connection of the overlaid myocardial cell sheets at day 1. Representative data at day 1. (A–C) Optical images obtained at 14, 108, and 164 ms after pacing at the left margin of sheet A, respectively. (D) Cross-sectional schematic image of the myocardial cell sheets and the site of pacing. (E) The activation map, in which the interval between each isochronal line is denoted 7 ms. There was crowding of the isochronal line at the left margin of the junction, suggesting significant conduction delay. Action potential propagation was blocked at the end of the junction. The front of the action potential proceeded along the curved line with arrowed head. (F) The action potential traces along the excitation wave front were superimposed. The character corresponds to the position of the myocyte from which the action potential was recorded.

were completely connected and indistinguishable (Fig. 7A).

Transplantation of the trilayered myocardial cell sheets in vivo

Three-layered myocardial cell sheets transplanted onto the subcutaneous tissue of nude rats showed strong rhythmical beating on posttransplant day 14 (Fig. 8A) (see supplemental Movie 3). Hematoxylin–eosin and Azan staining showed that the attached myocardial cell sheets were sandwiched between the host-derived connective tissues (Fig. 8B,C). The layers of the myocardial cell sheets were 102 ± 11 μm thick (Fig. 8E), and confocal laser microscopy showed that the length of the cardiomyocytes in vivo was greater than that of the cardiomyocytes in the cell sheets in vitro (compare Fig. 7A,B and 8E). In addition, some layers of transplanted myocardial cell sheets had rich neovascularization, not only at the capillary level but also of vessels 10–25 μm in diameter (Fig. 8D). The sarcomere of the cardiomyocytes was well organized and oriented in the same direction (Fig. 8E). These findings indicate that myocardial cell sheets obtained from the polymerized fibrin-coated dishes remained functional in vivo.

DISCUSSION

Since PIPAAm-coated dishes were first reported, significant advances have been made in cell sheet engineering of various organs, with the generation of two- and three-dimensional tissues. Cell sheet engineering has now become an important tool in the field of regenerative medicine. Cell sheets have been

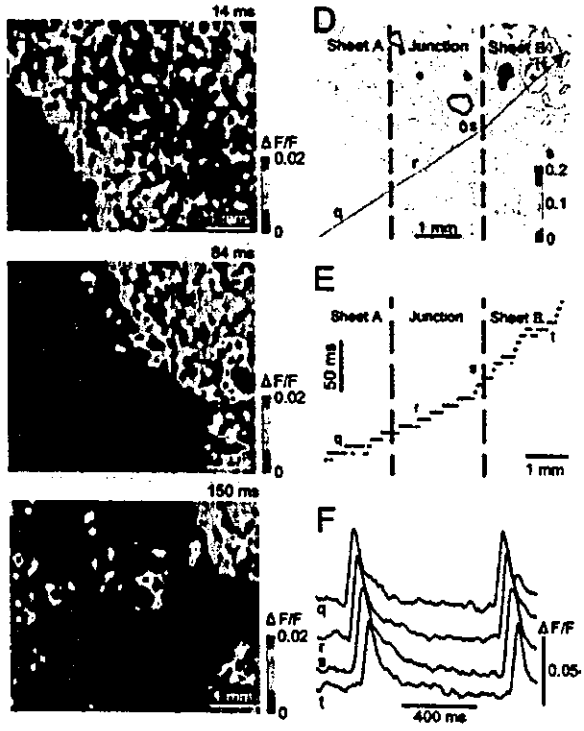

FIG. 6. Optical mapping of the action potential propagation and electrical connection of the overlaid myocardial cell sheets at day 3. Representative data at day 3. (A–C) Optical mapping images obtained at 14, 84, and 150 ms after pacing at the left margin of sheet A. (D) The calculated activation map suggested that the propagation of action potential was quite smooth with no delay between the two myocardial cell sheets. (E) The impulse propagation sequence along the excitation wave front suggested the formation of tight electrical communication between the two myocardial cell sheets. (F) The action potential traces along the excitation wave front were superimposed.

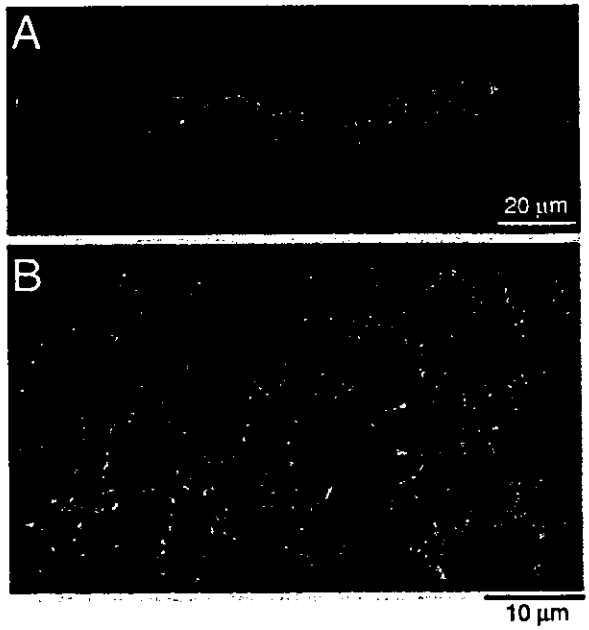

FIG. 7. Histological evidence of electrical communication between overlaid cardiomyocyte cell sheets in vitro. Laser confocal microscopy of the overlaid myocardial cell sheets (day 3), which was triple stained with antiactinin (green), anticonnexin43 (red) antibody, and TOTO-3 to stain the nucleus. (A) Side view, (B) Top view. Note that the cardiomyocytes formed a confluent sheet, and that connexin43 was clearly present at the cell junctions.

created by using temperature-sensitive culture dishes with vascular endothelial cells (14), hepatocytes (21), renal epithelial cells (22), and corneal epithelial cells.

The new method we describe here has several characteristics that were different from previous approaches using the temperature-responsive dishes. First, it does not require any specialized equipment, using only widely available fibrin polymers, nor any high level of technical expertise. Second, sheets can be generated with almost any cell type, because almost all cells will attach to polymerized fibrin-coated dishes, even those that do not attach readily to noncoated or fibronectin-coated dishes. Third, it is quick and easy to harvest the cell sheets.

Other more specialized approaches have been used to successfully generate functional tissue grafts. Li et al. used a piece of gelform, a biodegradable gelatin mesh, to form cardiac grafts from primary cultured cardiomyocytes that were transplanted into myocardial scar tissue in a cryoinjured heart (9). The cells attached to the gelatin mesh grew in three dimensions to form a beating cardiac graft, and when grafted formed cardiac tissue that contracted spontaneously. Zimmerman et al. reported that engineered heart tissue could be reconstituted by mixing cardiomyocytes from neonatal rats with liquid collagen type I, called matrigel, and that engineered heart tissue might serve as graft material to repair diseased myocardium (13). These pioneering studies, which greatly advanced research in bioengineering of cardiac grafts, both used biodegradable scaffold or collagen from other species. A scaffold can help form grafts in an appropriate shape, but they may also limit the function of the graft because of inflammation, inhibition of mechanical movement, and

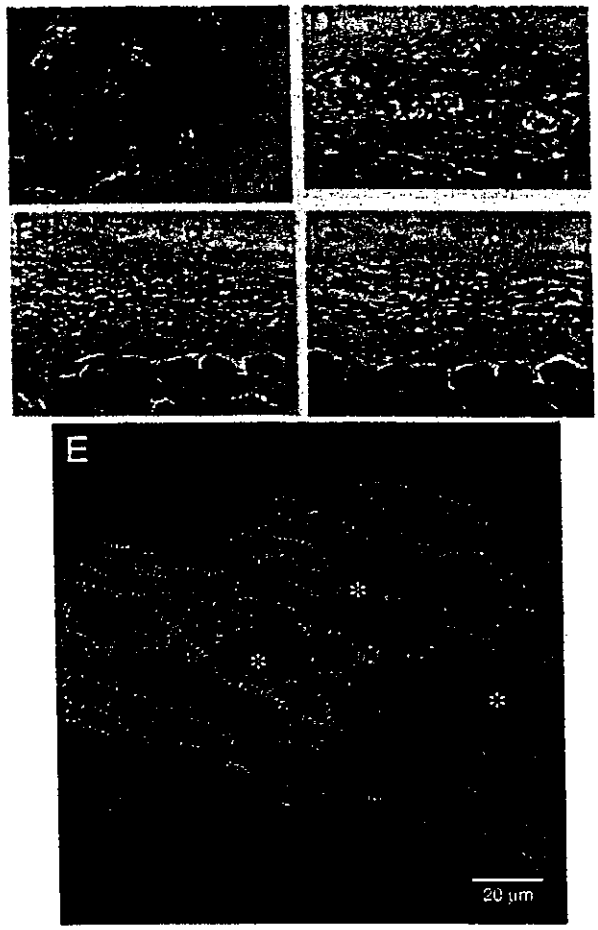

FIG. 8. Transplantation of trilayered myocardial cell sheets in vivo. The tri-layers of myocardial cell sheets were transplanted into the subcutaneous tissue of nude rats, and the samples were observed at day 14. (A) The transplanted area (black dotted line) showed rhythmical spontaneous beating (200 bpm). (B) Hematoxylin and eosin staining of the cross-sectional view of the trilayered myocardial cell sheet graft. Sk, skeletal muscle of the host rat; Ct, connective tissue; Cs, grafted trilayered myocardial cell sheets. (C) Azan staining of serial sections. (D) Note that microvessels are apparent in the grafted myocardial cell sheets. *Microvessels. (E) Triple staining of the grafted trilayered myocardial cell sheets, as described in Fig. 7. Note that the grafted cardiomyocytes show a well-organized sarcomere with a coincident direction of orientation.

adverse effects on organ function. A graft of cardiomyocytes must contract smoothly and form electrical connections with the recipient heart tissue, which a long-lived scaffold may interfere with. This is one potential advantage of our method, which has no scaffold.

We concluded that the attachment of the myocardial cell sheets to the dishes was probably loosened as fibrin was steadily degraded by proteases secreted from the grafted cardiomyocytes, as the addition of aprotinin to culture media blocked the digestion of fibrin polymers. Proteolytic degradation of fibrin probably began from the beginning of primary culture, and we observed that the residual fibrin had disappeared 6–7 days after plating of the cardiomyocytes. Loss of fibrin may vary between different cell types depending on the proteases secreted and on cell density. Plasmin is the most potent protease for fibrin in vivo, and is secreted from the liver (23). Therefore, even if the fibrin polymer remains undigested in the myocardial cell sheets after detachment from the plate, plasmin in host tissue might digest residual fibrin following transplantation. Therefore, the polymerized fibrin-coated dishes that we used provide a practical and convenient method for cell sheet engineering.

The use of a cell scraper to detach the cell sheet from the dishes did not cause any significant damage to cells. We found that cell damage at detachment was mainly dependent upon the strength of attachment of the cells to the dishes. We tested combinations of various concentrations of fibrinogen and thrombin solution and different harvest times and cell density, to find an optimal combination that left the myocardial cell sheet only very loosely attached to the dish at the time of harvest. This meant that the cell sheet could be detached with minimal cell damage or loss by just touching the margin of the dishes with a cell scraper. Moreover, in some experiments we counted the average number of cardiomyocytes in a cell sheet as 1.69×10^6 from 2.0×10^6 of primary cultured cardiomyocytes plated into a polymerized fibrin-coated dish (precise data not shown). This estimate confirmed that there was no marked loss of cells with scraping.

Finally, the conduction and propagation of action potential within and between the myocardial cell sheets is critical for successful cardiac tissue engineering. We found that measurement of P4–S3 samples by optical mapping was an effective way to evaluate conduction. This approach showed that even if the two overlaid myocardial cell sheets seemed to beat synchronously under microscopic observation, the propagation of action potential followed an indirect route via the narrowed conduction pathway. We found that 3 days are necessary to establish a sufficient electrical connection between the two myocardial cell sheets without any conduction disturbance. Thus, we think that optical mapping analysis will provide important basic data for the transplantation of myocardial cell sheets in future studies.

In the present study, we used primary cultured neonatal cardiomyocytes as the cell source, but this may not be possible for clinical use because of ethical issues. However, we hope that multipotent stem cells may, with some methodological advances, provide a sufficient source of cardiomyocytes in the near future, thereby obviating this problem.

CONCLUSIONS

We have developed a new method for generating myocardial cell sheets using polymerized human fibrin-coated dishes. Intact cell sheets of primary cultured rat cardiomyocytes were easily obtained by scraping confluent cells after the polymerized fibrin had been digested by intrinsic proteases. Two overlaid and cocultured myocardial cell sheets exhibited synchronized spontaneous beating and captured artificial pacing. Optical mapping revealed that an action potential was propagated from the junction without any delay in the two partially overlaid cell sheets. Transplanted three-layered myocardial cell sheets exhibited strong spontaneous beating, well-differentiated striations, and an increase in cell size. This technique promises to be a useful and convenient tool for generation of sheets of myocardial cells and possibly various other cell types.

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Basic Science

心筋細胞の再生
Current topics and future in cardiomyocyte regeneration

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Key Words bone marrow, mesenchymal stem cell, cardiomyocyte, regenerative medicine, ES cell, adult stem cell, cell transplantation

Summary

Recent studies revealed that cardiomyocytes can be regenerated from both embryonic- and adult bone marrow-stem cells in animal experiment. We have established a cardiomyogenic (CMG) cell line from mouse bone marrow stromal cells that can be induced to differentiate into cardiomyocytes *in vitro* by 5-azacytidine treatment. A number of lines of evidence confirm the cardiomyocyte characteristics of CMG cells. These cells could be transplanted into the recipient heart, and survive for long period. Clinical application of these cells had some problems at present. Embryonic stem cells had ethical problems, and had a possibility of making teratoma when undifferentiated cells could not be eliminated. Adult stem cells had a difficulty in isolation and culture expansion, since they were not yet well characterized. However, transplantation of regenerated cardiomyocytes would become a future method for the treatment of severe heart failure.

Key Sentence

Recent studies revealed that cardiomyocytes can be regenerated from stem cells in animal experiment.

はじめに

従来心筋細胞は出生直後よりその増殖能を失うため，心臓は再生しない臓器と考えられていた。しかし近年における再生医学研究の目覚ましい発達により，心筋再生は実現可能なものとなりつつある。胚性幹細胞(ES細胞)や骨髄体性幹細胞を用いた心筋細胞の再生が現実のものとなり，再生心筋細胞移植により心機能が改善された，とする報告も出始めている。また幹細胞を壊死した心筋梗塞領域周辺に注入すると，心筋細胞へと分化しうることも報告されている。また，いまだ動物実験の段階ではあるものの，再生心筋細胞を利用した心不全治療への試みも始まっている。近年心臓内にも心筋幹細胞といえるような，side population(SP)細胞の存在に関する報告と，これらを用いた心筋再生による心機能の改善も報告されており，心臓再生における研究は，将来の臨床応用に向けて着実に前進しつつある。

本稿では，心臓再生の現状として現在研究されているいくつかの方法について，これらの心機能回復への応用法や問題点，さらに将来の展望について紹介していく。

I 心筋細胞再生に関する最近の話題

心筋細胞は胎生期には増殖能を有し細胞分裂を行っているが，生後まもなく最終分化してからは増殖能を失い，細胞分裂を停止すると考えられてきた。しかし近年，割合は非常に低いものの，心筋梗塞巣周囲のごく一部の心筋細胞が細胞分裂していることが報告されたため，既存の概念に対する再検討が必要となってきた[1)-4)]。この分裂像を示す細胞の性質は明らかになっていないが，あまりにわずかな細胞であり失われた心機能は代償されない。心筋細胞の増殖能を制御する研究は以前より行われてきたが，最近になって*in vitro*の実験ではあるが，細胞周期調節蛋白の1つであるcyclin D1やサイクリン依存性キナーゼCDK4の発現を制御することにより，最終分化した心筋細胞を増殖させたという報告がされ[5)]，今後の発展が期待される。

また，心臓移植後の症例の病理学的検討から興味深い報告がなされている[6)]。女性ドナーから男性レシピエントに移植された心臓に，Y染色体をもつ心筋細胞が認められた，というものである。この事実から，Y染色体をもつ心筋細胞は心臓以外の部位から幹細胞，あるいは前駆細胞として心臓に到達した，と推測されている。またヒトの骨髄移植患者4例を検討した最近の報告[7)]ではそれらの細胞の由来を骨髄と考えているが，今後さらなる検討が必要であろう。

心筋細胞の再生には幹細胞を用いる方法以外に，非心筋細胞を心筋細胞に形質転換させる方法も以前から研究されてきた。骨格筋のマスター遺伝子であるmyoDを線維芽細胞に遺伝子導入すると，その細胞が骨格筋細胞になるためのすべての遺伝子を発現し，骨格筋細胞としての性質をもつようになる。心臓でも同様なマスター遺伝子の単離が試みられてきた。心筋特異的に発現する転写因子はNkx2.5，GATA4，dHAND，eHAND，HRTなど10個以上単離されているが，マスター遺伝子は現在のところ同定されていない。

以上に挙げられた方法も試みられているが，現在最も有望であると考えられているのは，ES細

Interface

Key Sentence

Cardiomyogenic (CMG)-cell line was established from mouse bone marrow stromal cells that can be induced to differentiate into cardiomyocytes *in vitro* by 5-azacytidine treatment.

胞(embryonic stem cell)や体性幹細胞を利用した心筋細胞再生であろう。

II ES細胞を用いた心筋再生

ES細胞は受精早期の胚盤胞由来の細胞で，内・中・外胚葉のいずれにも分化することができ，かつ大量に増殖することができる。ES細胞は，生体のすべての細胞に分化できる万能性幹細胞と呼ばれ，現在この細胞を用いて血液，血管内皮，神経，心筋，インスリン分泌細胞などの再生が行われている。以前からマウスでは，ES細胞を浮遊培養し，細胞を凝集させた胚様体(embryoid body)を形成させると，一部の細胞が心筋細胞となり拍動を開始することが知られていた。ヒトES細胞からもこの方法により心筋細胞が得られたと報告された[8]。

ES細胞を用いた再生にはいくつかの問題点がある。第1に，胚様体から心筋細胞が得られる確率が低く，特定の分化誘導方法が定まっていないこと。第2に，ES細胞から分化させた細胞を実際に移植する際に，未分化状態の細胞が混入すると奇形腫を形成してしまうこと。第3に，ES細胞は第三者の細胞であるため，移植後に免疫抑制剤の投与が必要となることである。以上の問題点はあるが，国内においてもヒトのES細胞作製が開始されており，さらなる発展が期待される。

III 体性幹細胞を用いた心筋再生

近年の研究により，これまで再生能をもたないと考えられていた神経や心臓にも幹細胞が存在することが明らかにされた。中胚葉由来の臓器では幹細胞は骨髄に存在すると考えられている[9]が，骨髄は元来造血の場であり造血幹細胞から血球系細胞の増殖分化が起きている。しかし，骨髄には骨髄間質細胞や造血支持細胞と呼ばれる血球系以外の細胞も存在する。骨髄間質細胞は多彩なサイトカインや細胞増殖因子を分泌し，血球系細胞の再生増殖分化を維持しており，骨芽細胞，軟骨芽細胞，脂肪細胞などに分化することが知られていた。現在では骨髄間質細胞のうち，間葉系幹細胞と呼ばれる一部の細胞が多分化能を有することが知られ，中胚葉由来の多くの細胞の幹細胞となりうると考えられている。

間葉系幹細胞は骨髄中にわずかに存在する細胞で，ヒト新生児骨髄中の細胞の10,000個に1個しか存在せず，その数は出生後急速に減少し高齢者では新生児の200分の1程度に減少するといわれている。間葉系幹細胞の同定には表面抗原が用いられているが，報告者により異なっており[10][11]，今後の研究が必要であろう。

IV 間葉系幹細胞を用いた心筋再生

われわれは骨髄間葉系幹細胞が中胚葉のさまざまな臓器の細胞に分化することより，同じ中胚葉由来の心筋細胞にも分化するのではないかと考え，間葉系幹細胞に各種の分化誘導剤を投与する実験を施行した。その結果，自己拍動をする心筋細胞に分化することを明らかにした[12]。まずマウス骨髄初代培養を行い付着系の細胞である骨髄間質細胞を分離し，長期培養することで不死化した細胞株を作製した。この多クローン細胞株にDNA脱メチル化剤である5-azacytidineを負荷しさらに2週間程度培養を続けると，非常に少ない確率ではあるが自己拍動する細胞が得られた。こ

Key Sentence

Clinical application of these stem cells had some problems at present.

の周辺の細胞を採取し同様の操作を繰り返すと，自己拍動を開始した細胞自体は継代不可能であるが心筋芽細胞と考えられる細胞は分裂，増殖を繰り返すことができる。自己拍動する割合の高いクローンを最終的にCMG(cardiomyogenesisより命名)細胞株として樹立した。CMG細胞は5-azacytidineにより最終的に分化誘導を行うと心筋細胞の表現型を獲得するが，最終分化誘導後に自己拍動を開始する比率は約30%であった。CMG細胞は培養条件下において毎分120～250程度の速さで規則的に収縮した。電顕では典型的な横紋構造に加え，心房顆粒を多数認めた。心筋細胞に分化したCMG細胞の収縮蛋白(α-アクチン，ミオシン重鎖，ミオシン軽鎖)のアイソフォームを表1に示す。心筋細胞は胎仔期，新生仔期，成獣期および心房，心室で異なる収縮蛋白のアイソフォームを示すが，CMG細胞のアイソフォームを解析すると，胎仔型心室筋に一致した表現型を取ることが明らかとなった。

ガラス微小電極によりCMG細胞の活動電位を記録すると，洞結節細胞型と心室筋細胞型の2種類が観察された(図1)。両者に共通した活動電位の特徴は，①活動電位持続時間が長いこと，②比較的浅い静止期電位をもつこと，③ペースメーカー細胞にみられる静止期電位の緩やかな脱分極が認められることであった。また，心室筋細胞型では活動電位はpeak & dome型を呈した。分化誘導後早期(2～3週間)にはすべての細胞で洞結節型が記録されたが，分化誘導後後期(4週後)には心室筋細胞型が観察され，次第に増加した。

CMG細胞の心筋細胞としての表現型を解析するため，心筋細胞特異的蛋白質の発現を調べた。CMG細胞では心房利尿ホルモンANPおよびBNPを発現していた。心筋分化に関与する転写因子としてNkx2.5，GATA4，TEF-1，eHAND，HRTなどが発現していた。MEF2 familyではMEF2A，MEF2C，MEF2Dの発現が観察された。しかし，その発現時期は3者で異なり，MEF2Cは分化誘導前で発現が認められたが，MEF2A，MEF2Dは分化誘導後に発現していた(図2)。

生体内の心筋細胞はカテコラミンα_1受容体，β受容体，アセチルコリンのムスカリン受容体が発現し，心拍数や心収縮力，興奮伝導速度などの調節を行っている。CMG細胞ではα_1受容体の3つのサブクラス(α_{1A}，α_{1B}，α_{1D})を発現し，α_1

表1　心筋収縮蛋白のアイソフォームからみたCMG細胞の表現型

	心房			心室			CMG細胞
	胎仔型	成獣型		胎仔型	新生仔型	成獣型	
α-アクチン	skeletal	cardiac	skeletal＞cardiac	skeletal	cardiac	skeletal＞cardiac	
ミオシン重鎖	α型＞β型	α型	β型＞α型	α型＞β型	α型	β型＞α型	
ミオシン軽鎖	2a	2a	2v	2v	2v	2v	

Key Sentence

Embryonic stem cells had ethical problems, and had a possibility of making teratoma when undifferentiated cells could not be eliminated.

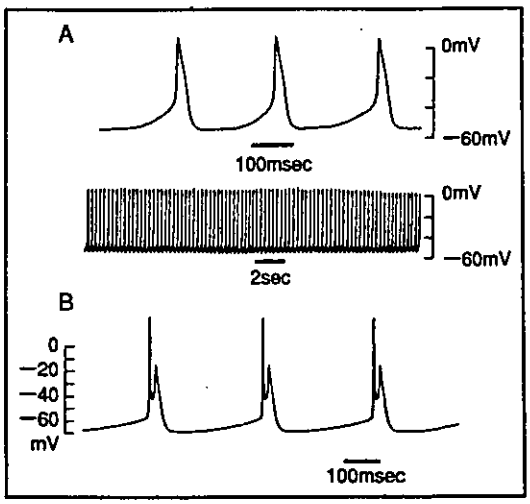

図1　骨髄由来心筋の活動電位
A：洞結節型，B：心室筋細胞型

刺激薬フェニレフリンで刺激すると，シグナルの活性化と細胞肥大が観察された[13]。一方，β受容体はβ1，β2ともに発現し，β刺激薬イソプロテレノールで刺激するとセカンドメッセンジャーのcAMPの上昇と拍動数の増加，収縮速度・収縮距離の増加が観察された。ムスカリン受容体はM1～M5まで5種類あることが知られているが，CMG細胞では本来の心筋細胞と同様にM1，M2の発現が認められ，ムスカリン受容体刺激薬カルバコールで刺激するとセカンドメッセンジャーのIP3が増加した(表2)。これらの性質はCMG細胞が心筋としての特徴をほぼ有していることを意味

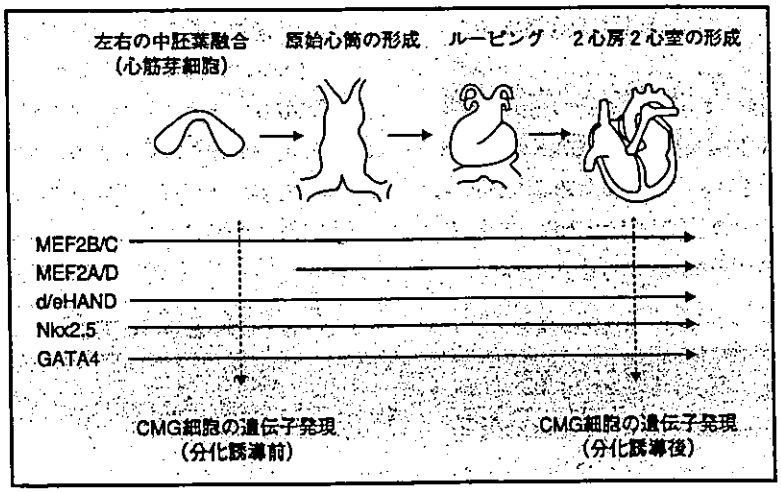

図2　心筋細胞の分化過程における転写因子の発現とCMG細胞の関係
前外側板中胚葉から心筋前駆細胞に分化した状態では，Nkx2.5, GATA4, TEF-1, HAND遺伝子などの心筋分化に必要な転写因子は発現している。CMG細胞も心筋分化に必要な遺伝子は最終分化誘導前にすでに発現していることがわかる。

Key Sentence

Adult stem cells had a difficulty in isolation and culture expansion.

している。

V 再生心筋細胞を利用した心不全治療の試み

心筋再生を利用した治療法は，幹細胞を直接壊死した心臓内に注入してその部位で心筋細胞へと分化させる方法と，*in vitro*で幹細胞を心筋細胞に分化させ，それを心臓に注入する方法の2つに大別される(図3)。前者は現在実験動物において用いられているが，ヒトを対象として心機能の回復を目指すには，より多くの細胞が必要であると予想される。また，注入した細胞が確実に心筋

表2　CMG細胞の受容体発現と受容体刺激による効果

受容体の種類	受容体のサブクラス	受容体の発現時期	シグナル伝達の確認	確認できた作用
α_1受容体	α_{1A}	最終分化誘導前より(漸増)	ERK活性化	
	α_{1B}	最終分化誘導前より(不変)	ERK活性化	細胞肥大
	α_{1D}	最終分化誘導前より(漸増)	ERK活性化	
β受容体	β_1	最終分化誘導後1週より	cAMP上昇	拍動数増加，収縮力増強
	β_2	最終分化誘導後1週より	cAMP上昇	
ムスカリン受容体	M_1	最終分化誘導後1週より	IP_3上昇	
	M_2	最終分化誘導後1週より	IP_3上昇	

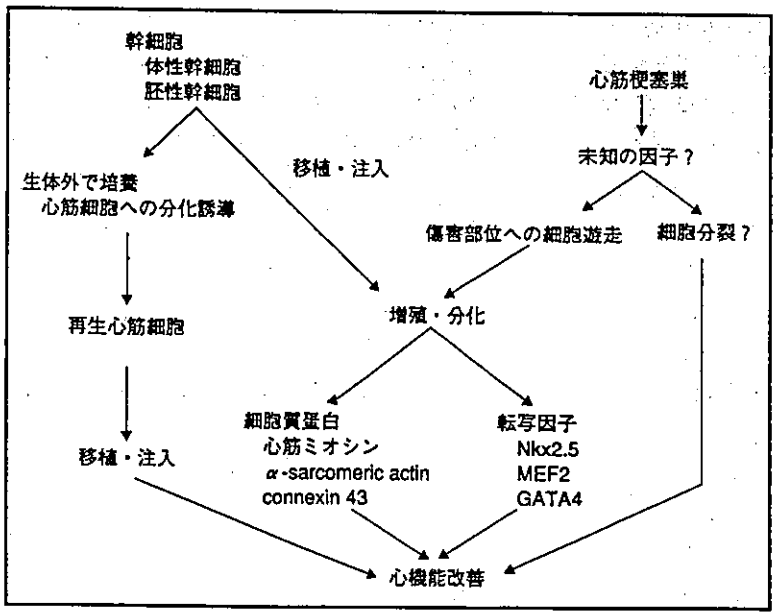

図3　心筋細胞再生による心機能改善の模式図

Key Sentence

Transplantation of regenerated cardiomyocytes would become a future method for the treatment of severe heart failure.

細胞へと分化し，もともと存在する細胞と同期して機能的に収縮するかなどの問題点も多い。後者の方法では生体外で幹細胞を分化させて移植に用いることになるが比較的実現しやすいと考えられる。しかしこの場合にはある程度の大きさの細胞集団として移植を行うので，移植細胞塊の強度や細胞塊内部への血流の確保などの問題点が生じてくることが予想される。また再生心筋細胞が生着し心機能の改善が得られたとしても，この細胞が新たな致死性不整脈の原因となる可能性もある。臨床応用に至るまでには，これらの問題点を1つずつ克服していくことが必要である。

また再生心筋細胞を臓器として機能させるにはtissue engineering(組織工学)の考え方が不可欠である。再生細胞を培養するスカフォールド(鋳型)の開発，異なった種類の細胞の配列，血管系・神経系の構築など解決すべき課題は多い。

これまでに再生心筋により傷害心筋の機能改善を認めた例として，ES細胞[14]，骨髄間葉系幹細胞[15][16]，造血幹細胞[17][18]，血管内皮前駆細胞[19]などが報告されている。残念ながらいずれの報告も移植した細胞の心筋細胞への分化を免疫染色で確認しているのみであり，実際そのようにみえる細胞が機能的・電気生理学的に心筋細胞となっているかに関しても，今後検証していく必要がある。さらに最近Cre/lox組み換えに基づく方法を用いて，骨髄由来の細胞が*in vivo*で心筋細胞およびプルキンエ神経細胞と融合する，つまり骨髄細胞のもつ広い分化能，可塑性がtransdifferentiation(分化転換)ではなくcell fusion(細胞融合)による，とする報告もされている(図4)[20]が，一概にそうとはいい切れず，今後のさらなる詳細な検討が必要である。

図4　心筋細胞と融合している骨髄由来の細胞（巻頭カラーグラビア：写真1参照）
Nature 425 : 968-973, 2003より抜粋

おわりに

心筋細胞の再生の材料として，胚性幹細胞と体性幹細胞のどちらを選択するかについては，今後さらに検討が行われるであろう。また体性幹細胞より多能性を有し上位に存在すると考えられている，multipotent adult progenitor cellの利用も期待される。

日常の臨床において重症心不全患者を救命できない状況を目前にすると，新たなより効果的，根本的な治療法の必要性を痛感させられる。再生心筋細胞を用いた治療が重症心疾患治療の選択肢の1つとなることが期待される。

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日外会誌 105（8）：454～458，2004

特　集

外科領域における再生医療の現況と展望

6. 心筋細胞の新生，再生療法の現況と展望

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キーワード　心筋細胞，分化誘導，ES細胞，間葉系幹細胞，細胞移植

I. 内容要旨

近年の分子発生生物学の発展により，神経細胞など従来は不可能と考えられていた細胞の再生も可能となってきた．心筋細胞もその1つで胚性幹細胞（Embryonic stem cell，ES細胞），骨髄間葉系幹細胞，心臓幹細胞などから再生出来ると報告されている．またG-CSFなどのサイトカインを用いてこれら幹細胞を効率的に動員させる試みもなされている．実験動物レベルでは心筋梗塞巣周囲に幹細胞を注入・移植すると，再生心筋細胞や新生血管が認められ，心機能の改善が得られる．しかしながら再生心筋細胞の数はごくわずかであり，再生心筋細胞によって心機能の改善が得られたとは言い難い．また幹細胞から心筋細胞へと分化誘導される詳細な機序についても解明されていない．ただ，自家骨髄単核球細胞を用いた重症狭心症治療など臨床応用を目指した試験も行われ，少数例ではあるが好ましい結果が得られている．循環器領域における再生医学は，臨床応用可能な再生医療に向けて着実に前進している．

II. はじめに

1997年10月16日に臓器移植法（正式名称は「臓器の移植に関する法律」）の施行以来，29例の脳死判定が行われ，28例の脳死臓器摘出がなされている．このうち心臓移植が行われたのは19例にとどまっている（2004年3月末現在）．この一方で，2004年3月末現在の心臓移植希望登録患者数（心肺同時移植を除く）は73人（日本臓器移植ネットワークホームページ http://www.jotnw.or.jp/datafile/index.html）となっており，臓器移植を待ちながら死を迎える患者がいる．臓器提供意志表示カード保有者数は増加してきているが，臓器移植が一般的な治療法として浸透するにはまだ時間がかかるものと思われる．このような背景の中で再生医療に対する期待が高まっている．

ES細胞や骨髄体性幹細胞を用いた心筋細胞の再生が現実のものとなり，再生心筋細胞を利用した心機能の改善に関する報告もある．また幹細胞を壊死した心筋梗塞領域周辺に注入すると，心筋細胞へと分化しうることも報告されている．いまだ実験動物段階ではあるものの，再生心筋細胞を利用した心不全治療への試みも開始されている．近年心臓内にも心臓幹細胞のようなSP細胞（side population cell）の存在に関する報告も出されている．これらを用いた心筋再生による心機能の改善も報告され，心臓領域における再生医学は，臨床応用に向けて着実に前進している．

本稿では心筋細胞再生方法のいくつかを紹介し，これらの心機能回復への応用法，問題点，ならびに将来の展望を概説する．

III. 心筋細胞再生に関する最近の知見

心筋細胞は胎生期には細胞分裂を行うが，生後まもなく最終分化して細胞増殖を停止するものと考えられてきた．一方で頻度は低いものの，心筋梗塞巣周囲のごく一部の心筋細胞が細胞分裂することが報告されており，既存の概念に対する再検討が必要となってきた[1)～3)]．この分裂像様の形態を示す細胞の起源は明確に

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なっていないが，これのみで生理学的に心機能の代償を期待することは出来ない．in vitro の実験ではあるが，細胞周期調節蛋白の1つであるサイクリンやサイクリン依存性キナーゼを制御することにより，最終分化した心筋細胞を細胞分裂させたという報告があり[4]，今後の発展が期待される．

心臓移植後の症例検討から興味深い報告がなされている[5]．女性ドナーから男性レシピエントに移植された心臓内に，Y染色体を有する心筋細胞が認められたというものである．このY染色体を有する心筋細胞は心臓以外の部位から幹細胞あるいは前駆細胞のような形で心臓に到達したと推測されている．ヒトの骨髄移植患者4例を検討した最近の報告[6]ではその由来を骨髄としているが，今後詳細な検討が必要である．

心筋細胞の再生には幹細胞を用いる方法以外に，非心筋細胞を心筋細胞に形質転換させる方法も以前から検討されていた．骨格筋のマスター遺伝子である *myoD* と同様な"心臓版 *myoD*" 遺伝子の単離が試みられた．しかし *myoD* のように単一遺伝子で他の細胞を心筋細胞に形質転換しうる遺伝子は現在のところ同定されていない．

以上のような取り組みは精力的になされているものの，近年最も注目されているのは，幹細胞を利用した心筋細胞再生であろう．幹細胞はES細胞と体性幹細胞に大別される．これら2種類を用いた心筋細胞再生の現状を紹介する．

IV. 胚性幹細胞を用いた心筋再生

ES細胞は受精直後の早期胚（胚盤胞の内部細胞塊）から取り出された細胞である．そして，すべての臓器・組織に分化しうる多分化能を有している．またES細胞をin vitroで大量に増殖させることが可能となっており，多分化能を維持しながら培養する技術も確立している．現在，ES細胞を用いて血液，血管内皮，神経，心筋，インスリン分泌細胞などの再生が行われている．ES細胞を培養条件下で未分化状態を維持するには，マウスではLIF（白血病抑制因子）を培養液中に入れておくだけでよいが，ヒトES細胞ではLIFに依存せず，マウス胎児フィーダー細胞との共培養が必要となる．ES細胞を心筋細胞に分化させるにはLIFを除去し，細胞を凝集塊（胚様体）にして培養すると一部の細胞が心筋細胞となり，拍動を開始する．ヒトES細胞から心筋細胞が分化できるとの報告が既になされている[7]．

ES細胞を用いた再生にはいくつかの問題点がある．第1にES細胞は第三者の細胞であるため，移植後に免疫抑制剤の投与を必要とすることである．第2に，ES細胞から分化させた細胞を実際に移植する際に，未分化状態の細胞が混入すると奇形腫を形成してしまう点である．第3に，発生段階の早期に分化してくる細胞は得やすいが，発生後期に出現する細胞を得るのは難しいことである．問題点はあるものの，国内においてもヒトのES細胞が作製されており，さらなる発展が期待される．

V. 体性幹細胞を用いた心筋再生

近年の研究により，これまで再生能力がないと考えられていた神経や心筋細胞にも生体内にある程度の幹細胞が存在することが明らかにされた．中胚葉由来の臓器では幹細胞は骨髄に存在すると考えられている[8]．骨髄は元来造血の場であり，そこには造血幹細胞を頂点とした血球系細胞の増殖分化が営まれている．しかし，骨髄には骨髄間質細胞や造血支持細胞と呼ばれる血球系以外の細胞も存在する．骨髄間質細胞は多彩なサイトカインや細胞増殖因子を分泌し，血球系細胞の再生増殖分化を維持しており，その一部が骨や軟骨にも分化しうることは以前より知られていた．現在は骨髄間質細胞すべてが多分化能を持つのではなく，これらに含有される間葉系幹細胞と呼ばれる一部の細胞が多分化能を有することが知られ，中胚葉由来の多くの細胞の幹細胞となりうると考えられている．

間葉系幹細胞は骨髄中にわずかに存在する細胞で，ヒト新生児骨髄中の細胞の10,000個に1個が間葉系幹細胞であり，その頻度は出生後急速に減少し高齢者では新生児の200分の1程度に減少する．従来，間葉系幹細胞の同定は骨髄間質細胞に種々の方法により分化誘導を行い，他の細胞に分化すればretrospectiveに間葉系幹細胞が含有されていたのであろうとされてきた．間葉系幹細胞の同定は，現在ではある程度表面抗原が同定されてきたが，報告者により異なっており[9][10]，さらなる研究が必要とされる．

VI. SP細胞からの心筋再生

成体の心臓内に心筋幹細胞のような細胞が存在することが示されている[11]．造血幹細胞はHoechst33342という蛍光のDNA結合色素を強く排出する性質を有しており，flow cytometerで解析するとほとんど蛍光を発しない稀な細胞集団として検出される．図にプロットすると横に突き出ていることからSide Population