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CARDIACO Development of Regenerative Cardiomyocytesfrom Mesenchymal Stem Cells for Cardiovascular Tissue Engineering

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Abstract: We have isolated a cardiomyogenic (CMG) cell line from murine bone marrow stroma. Stromal cells were immortalized, treated with 5-azacytidine, and spontaneous beating cells were repeatedly screened for. The cells showed a fibroblast-like morphology. However, this morphology changed after 5-azacytidine treatment in about 30% of the cells, which connected with adjoining cells after 1 week, formed myotube-like structures and began spontaneous beating after 2 weeks, and beat synchronously after 3 weeks. These cells expressed atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP). Electron microscopy revealed a cardiomyocyte-like ultrastructure including typical sarcomeres and atrial granules. They had sinus node-like or ventricular cell-like action potentials. Analysis of the isoform of contractile protein genes, such as myosin and α-actin, indicated that their phenotype was similar to fetal ventricular cardiomyocytes. These cells expressed Nkx2.5, GATA4, TEF-1, and MEF2-C mRNA before 5-azacytidine treatment, and expressed MEF2-A and MEF2-D after treatment. This new cell line provides a powerful model for the study of cardiomyocyte transplantation. **Key Words:** Bone marrow stroma—Mesenchymal stem cell—Cardiomyocyte—Differentiation—5-azacytidine.

Cardiomyocytes do not regenerate after birth, and they respond to mitotic signals by cell hypertrophy rather than by cell hyperplasia (1). Loss of cardiomyocytes leads to regional contractile dysfunction, and necrotized cardiomyocytes in infarcted ventricular tissues are progressively replaced by fibroblasts to form scar tissues. A recent study showed that transplanted fetal cardiomyocytes can survive in this heart scar tissue and that these transplanted cells limit scar expansion and prevent postinfarction heart failure (2). The transplantation of cultured cardiomyocytes into the damaged myocardium has been proposed as a future method for the treatment of heart failure (3,4). However, this revolutionary idea remains unfeasible in the clinical setting because it is difficult to obtain donor fetal heart. A cardiomyogenic cell line potentially could substitute for fetal cardiomyocytes in this therapy. Therefore, both developmental biologists and cardiologists have eagerly awaited the development of a cardiomyogenic cell line.

Recent reports demonstrated the existence of pluripotent stem cells in adult tissues. Figure 1 shows the classification of pluripotent stem cells in adult tissues. Roy et al. reported the existence of neural stem cells in the brain, which can differentiate into neurons, oligodendrocytes, and astrocytes in vitro (5), and marrow stromal cells were shown to have many characteristics of mesenchymal stem cells (6). Pluripotent progenitor marrow stromal cells may differentiate into various types of cell types including bone (7,8), muscle (9), fat (10), tendon, and cartilage (11). Based on these findings, we hypothesized that marrow stromal cells also might differentiate into cardiomyocytes and repeatedly screened marrow stromal cells which began spontaneous beating after exposing them to 5-azacytidine, a cytosine analog capable of altering expression of certain genes that may regulate differentiation. We finally established a cell line which differentiates into cardiomyocytes in vitro, named CMG (cardiomyogenic), from adult marrow stromal cells (12). The use of adult tissues as a source of cardiomyocytes makes this system par-

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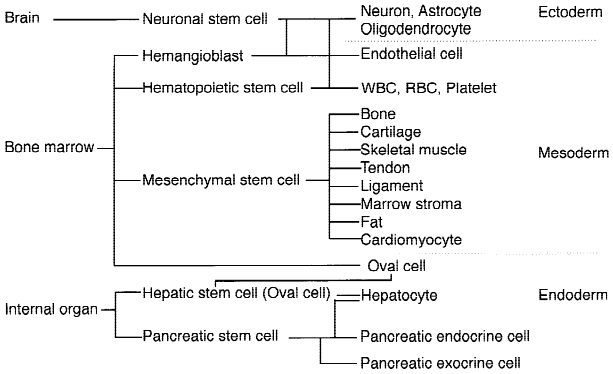

FIG. 1. The classification of pluripotent stem cells in adult tissues is shown. Bone marrow contains various kinds of stem cells. Mesenchymal stem cells may differentiate into various mesoderm-derived cells such as osteoblasts, chondroblasts, adipocytes, skeletal muscle cells, and possibly cardiomyocytes.

ticularly appropriate for the development of gene therapy strategies for heart disease. In this article, I introduce the characteristics of bone marrow-derived cardiomyocytes and discuss the possibility of the use of these cells for cardiovascular tissue engineering.

MATERIALS AND METHODS

Cell culture

Female C3H/He mice (n = 10) were anesthetized with ether, thighbones were excised, and bone marrow cells were obtained. The procedures were performed in accordance with the guidelines for animal experimentation of Keio University. Primary culture of the marrow cells was performed according to Dexter et al.'s method as described (13). Cells were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% fetal bovine serum and penicillin (100 μg/ml)/streptomycin (250 ng/ml)/amphotericin B (85 μg/ml) at 33°C in humid air with 5% CO_2. After a series of passages, attached marrow stromal cells became homogeneous and were devoid of hematopoietic cells. The marrow stromal cells basically did not require co-culture of blood stem cells. Immortalized cells were obtained by frequent subculture for more than 4 months. Cell lines from different dishes were subcloned by limiting dilution. To induce cell differentiation, cells were treated with 3 μmol/L of 5-azacytidine for 24 h. Subclones that included spontaneous beating cells were screened by microscopic observation (first screening), and cells surrounding spontaneous beating cells were subcloned by cloning syringes. Subcloned cells were maintained and exposed to 5-azacytidine again for 24 h, and clones that showed spontaneous beating most frequently were screened (second screening). The clonal cell line thus obtained was named the CMG cell.

Immunostaining

Monoclonal antibody (MF20) to sarcomeric myosin was obtained from American type culture collection (ATCC). Cells grown on glass coverslips were permeabilized in 1% formaldehyde/phosphate buffered saline (PBS) for 10 min. After blocking with 5% bovine serum albumin in PBS for 1 h at 23°C, the cells were incubated with primary antibodies. After 3 washes in PBS for 5 min each, the biotinylated-conjugated antimouse IgG was applied for 30 min at a dilution of 1:400. Visualization was achieved through the streptavidine-biotin horseradish peroxidase detection system.

Transmission electron microscopy

Cells were washed 3 times with PBS (pH 7.4). The initial fixation was done in PBS containing 2.5% glutaraldehyde for 2 h. The cells were embedded in epoxy resin. Ultrathin sections cut horizontally to the growing surface were double stained in uranyl acetate and lead citrate, and viewed under a JEM-1200EX transmission electron microscope.

Action potential recording

Electrophysiological studies were performed in IMDM containing (mmol/L) $CaCl_2$, 1.49; KCl, 4.23; and HEPES, 25 (pH 7.4). Cultured cells were placed on the stage of an inverted phase contrast optic (Diaphoto-300, Nikon, Tokyo, Japan) at 23°C. Action potentials were recorded by conventional microelectrode. Intracellular recordings were taken from 2 to 5 week cultured cells. Glass microelectrodes filled with KCl (3 mol/L) having a DC resistance of 15 to 30 MΩ were selected. Membrane potentials were measured by means of current clamp mode (Nihon Kohden, MEZ-8300) with a built-in 4 pole Bessel filter set at 1 kHz. Data were recorded on a thermal recorder (Nihon Kohden, RTA-1100M, Tokyo, Japan) and stored on a digital magnetic tape (Sony Magnescale; frequency range 0–20 kHz).

Ribonucleic acid extraction and RT-PCR-Southern blot analysis

Total ribonucleic acid (RNA) was extracted from fetal, neonatal, and adult mouse heart, skeletal muscle, and differentiated CMG cells by TRIzol Reagent (Gibco BRL, Rockville, MD, U.S.A.). Reverse transcription-polymerase chain reaction (RT-PCR) of cardiomyocyte-specific genes including atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), α-myosin heavy chain (MHC), β-MHC, α-skeletal actin, α-cardiac actin, myosin light chain (MLC)-2a, MLC-2v, Nkx2.5, GATA-4, TEF-1, MEF2-A, MEF2-C, and MEF2-D was performed

as described previously (12). In some experiments, the gels were transferred to nylon membranes (Hybond N), and UV-crosslinked. The Southern blot hybridization was performed using a ^{32}P end-labeled internal 25-bp fragment. For detection of α-skeletal and α-cardiac actin expression, Northern blot analysis was used as described previously (12). Complementary deoxyribonucleic acids (cDNAs) for α-skeletal and α-cardiac actin were obtained by RT-PCR from mouse heart RNA and cloned into the pCR II plasmid. All cDNAs were confirmed by sequencing.

RESULTS

CMG cells form myotubes and show spontaneous contraction

By repeated rounds of limiting dilution, we isolated 192 single clones, several of which could differentiate into cardiomyocytes and show spontaneous beating. These experiments were reproducible, but the percentage of cardiomyocyte differentiation was distinct among these clones.

Phase contrast photography and/or immunostaining with antisarcomeric myosin antibodies was used to determine the morphological changes in CMG cells (Fig. 2). CMG cells showed a fibroblast-like morphology before 5-azacytidine treatment (Week 0), and this phenotype was retained through repeated subculturing under nonstimulating conditions. After 5-azacytidine treatment, the morphology of of the cells gradually changed. Approximately 30% of the CMG cells gradually increased in size, formed a ball-like appearance, or lengthened in one direction, and formed a stick-like morphology at 1 week. They connected with adjoining cells after 2 weeks and formed myotube-like structures at 3 weeks. The differentiated CMG myotubes maintained the cardiomyocyte phenotype and beat vigorously for at least 8 weeks after final 5-azacytidine treatment and did not dedifferentiate. Most of the other nonmyocytes had an adipocyte-like appearance.

CMG cells were stained with MF20 at 1, 2, 3, and 4 weeks after 5-azacytidine treatment (Fig. 3). Myosin-positive cells gradually joined with neighboring myosin-positive cells and formed a myotube-like appearance. The maximum length of the myotubes ranged from 1,000 to 3,000 μm. Most of the cells were mononuclear, some were binuclear, but a few were multinucleated (3–10/cell). Cells were connected to each other via intercalated discs and formed myotubes.

CMG cells have a cardiomyocyte-like ultrastructure

Representative transmission electron microscopy photographs are shown in Fig. 4. A longitudinal section of the differentiated CMG myotubes clearly revealed the typical striation and pale-staining pattern of the sarcomeres. CMG myotube nuclei were positioned in the center of the cell, not beneath the sarcolemma. The most conspicuous feature of the differentiated CMG myotubes was the presence of membrane-bound dense secretary granules measuring 70 to 130 nm in diameter. These granules were

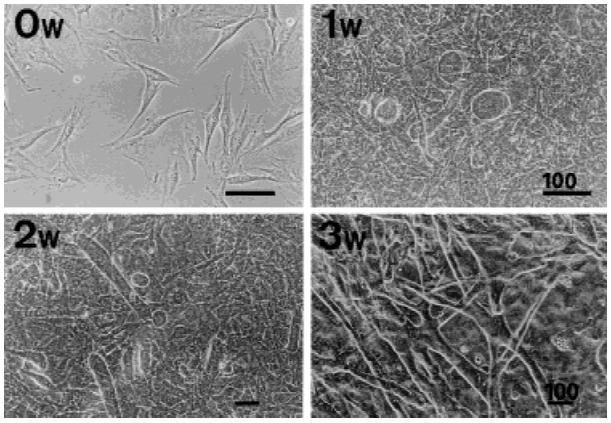

FIG. 2. The phase contrast photographs depict CMG cells before and after 5-azacytidine treatment. CMG cells have a fibroblast-like morphology before 5-azacytidine treatment (Week 0) **(upper left)**. One week after treatment, some cells gradually increased in size and formed a ball-like or stick-like appearance **(upper right)**. These cells began spontaneous beating thereafter. Two weeks after treatment, ball-like or stick-like cells connected with adjoining cells and began to form myotube-like structures **(lower left)**. Three weeks after treatment, most of the beating cells were connected and formed myotube-like structures **(lower right)**. Bars indicate 100 μm.

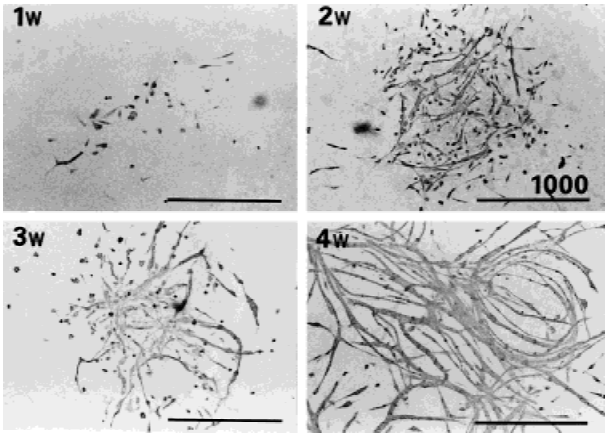

FIG. 3. Immunostaining of CMG cells with antisarcomeric myosin antibody after the 5-azacytidine treatment is shown. Myosin-positive cells could be observed 1 week after the 5-azacytidine treatment. Myosin-positive cells gradually joined to neighboring myosin-positive cells and formed myotube-like structures. Bars indicate 1,000 μm.

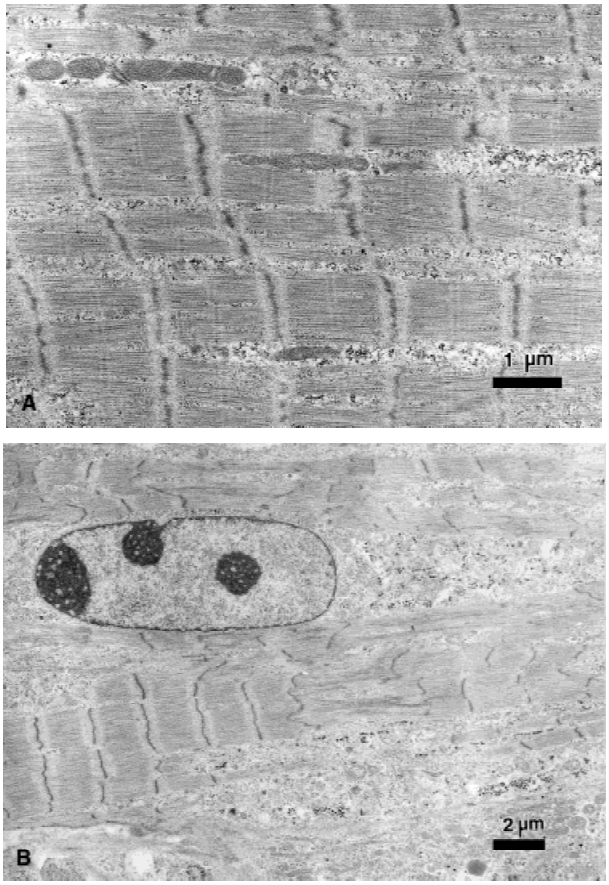

FIG. 4. The transmission electron micrograph depicts CMG myotubes. Differentiated CMG myotubes had well organized sarcomeres. Rich glycogen granules and a number of mitochondria were observed. Bar indicates 1 μm **(A)**. Ultrastructural analysis revealed that nuclei were oval and positioned in the central part of the cell, not immediately beneath the sarcolemma. Atrial granules measuring 70 to 130 nm in diameter are observed in the sarcoplasm and are concentrated especially in the juxtanuclear cytoplasm. Bar indicates 2 μm **(B)**.

thought to be atrial granules. They were especially concentrated in the juxtanuclear cytoplasm, but some also were located near the sarcolemma. These findings indicated that CMG cells had a cardiomyocyte-like rather than skeletal muscle ultrastructure.

CMG myotubes have several types of action potential

An electrophysiological study was performed on differentiated CMG cells at 2 to 5 weeks after 5-azacytidine treatment. There were at least 2 types of distinguishable morphological action potentials: sinus node-like potentials (Fig. 5A) and ventricular myocyte-like potentials (Fig. 5B). The sinus node-like action potential showed a relative shallow resting membrane potential with late diastolic slow depolarization, like a pacemaker potential. Peak and dome-like morphology were observed in ventricular myocyte-like cells. The cardiomyocyte-like action potential recorded from these spontaneous beating cells was characterized by a relatively long action potential duration or plateau, a relatively shallow resting membrane potential, and a pacemaker-like late diastolic slow depolarization. Fig. 5C shows a time course of the percentage of the sinus node-like and ventricular myocyte-like action potentials. All the action potentials recorded from the CMG cells until 3 weeks revealed sinus node-like action potential. The ventricular myocyte-like action potentials could be recorded after 4 weeks, and the percentage of these action potentials gradually increased thereafter. It is possible that the percentage of the ventricular myocyte-like action potentials at 5 weeks was underestimated. Most of the action potentials recorded from differentiated CMG myotubes had a ventricular myocyte-like appearance, but the action potential of the differentiated CMG myotubes was difficult to record. The glass microelectrode was frequently damaged because the spontaneous contraction of the differentiated myotube at 5 weeks was too large.

Cardiomyocyte-specific gene expression

RT-PCR or Northern blot analysis was performed to detect the expression of cardiomyocyte-specific genes in differentiated CMG cells. Total RNA obtained from cardiomyocytes (in vivo heart) and skeletal muscles (soleus muscle) were used as positive and negative controls, respectively. Differentiated CMG myotubes expressed both ANP and BNP genes (data not shown).

Table 1 summarizes the expression of cardiac contractile protein isoforms. Fetal, neonatal, and adult ventricle and atrium were used as controls. Both α- and β-MHC expression could be detected by RT-PCR in differentiated CMG cells, but β-MHC expression was overwhelmingly stronger than that of α-MHC. CMG cells expressed both α-cardiac and α-skeletal actin. On Northern blot analysis, the α-skeletal actin gene was expressed at markedly higher levels than the α-cardiac actin gene in CMG cells. Interestingly, CMG cells expressed MLC-2v but not MLC-2a. These patterns of gene expression in the differentiated CMG cells were consistent with a fetal ventricular phenotype.

Figure 6 shows the time course of the expression of cardiomyocyte-specific transcription factors in fetal developing heart and CMG cells. Nkx2.5, GATA4, HAND1/2, and MEF2-B/C genes were expressed in the early stage of heart development while MEF2-A and MEF2-D were expressed in the middle stage. CMG cells already expressed GATA4,

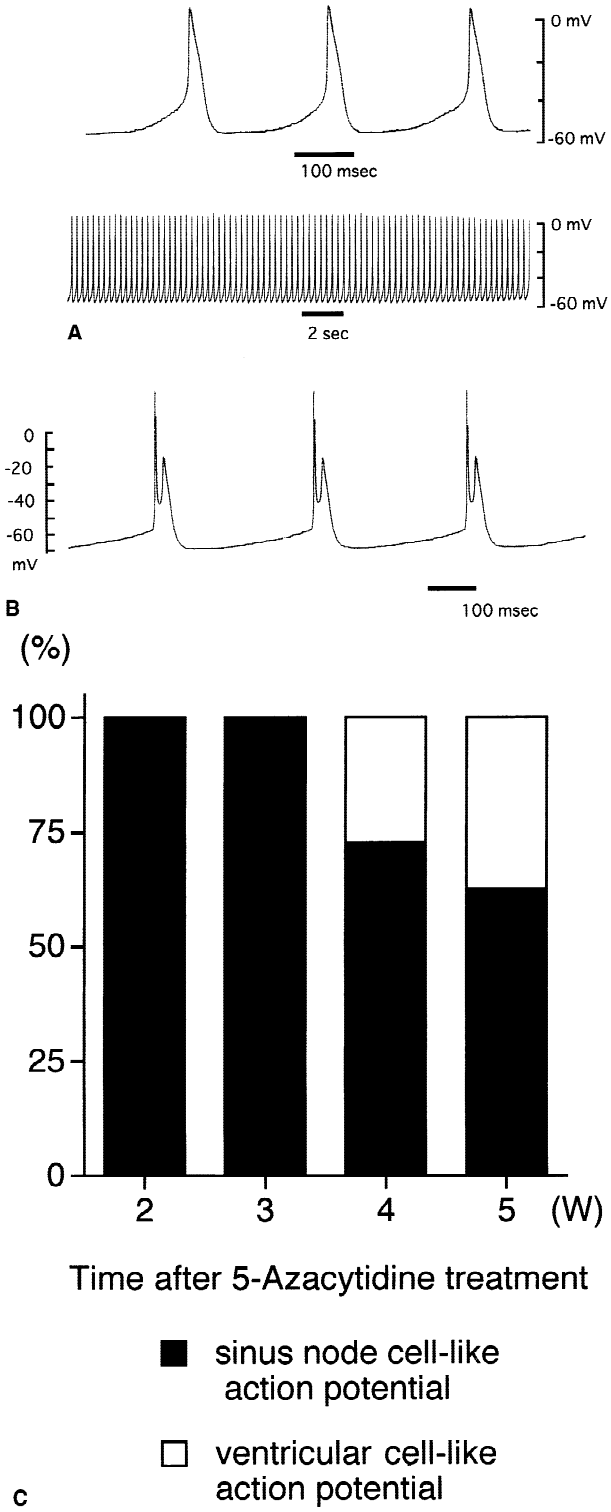

FIG. 5. The representative tracing of the action potential of CMG myotubes is shown. Action potential recordings were obtained from the spontaneous-beating cells at Day 28 after the 5-azacytidine treatment using a conventional microelectrode. We categorized these action potentials into 2 groups: a sinus node-like action potential **(A)** or a ventricular cardiomyocyte-like action potential **(B)**. The percentage of CMG cells with a sinus node-like or ventricular cardiomyocyte-like action potential is shown after the 5-azacytidine treatment. A ventricular cardiomyocyte-like action potential was first recorded 4 weeks after the 5-azacytidine treatment and then rapidly became more prevalent **(C)**.

TEF-1, Nkx2.5, HAND, and MEF2-C before the final 5-azacytidine treatment, and it expressed MEF2-A and MEF2-D after the 5-azacytidine treatment. These patterns of gene expression indicated that the developmental stage of the undifferentiated CMG cells is near to the cardiomyoblasts or the early stages of heart development.

DISCUSSION

Development of cardiomyocytes in vitro

We have established a cardiomyogenic 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 confirmed the cardiomyocyte characteristics of these CMG cells. These cells expressed a number of cardiomyocyte-specific genes including ANP, BNP, GATA4, and Nkx2.5. In ventricular muscle of small mammals, there is a developmental switch from expression of β-MHC, which is the predominant fetal form, to that of α-MHC around the time of birth. There also is a developmental switch from the expression of α-skeletal actin, which is the predominant fetal and neonatal form, to that of α-cardiac actin, the predominant adult form. Differentiated CMG cells mainly expressed β-MHC and α-skeletal actin. Expression of α-MHC and α-cardiac actin was detected, but only at low levels. MLC-2 genes are specifically expressed in the chamber. MLC-2v is specifically expressed in ventricular cells while MLC-2a was specifically expressed in atrial cells. Differentiated CMG cells expressed MLC-2v but not MLC-2a. Moreover, skeletal muscle cells do not express α-MHC or MLC-2v. These results indicated that differentiated CMG cells had the specific phenotype of fetal ventricular cardiomyocytes.

Differentiated CMG cells expressed Nkx2.5, GATA4, TEF-1, and MEF2-C before the final 5-azacytidine treatment. The MEF2-A and MEF2-D genes were expressed after the final 5-azacytidine treatment. This pattern of gene expression in CMG cells was similar to that of in vivo developing cardiomyocytes (14). These results indicated that the stage of differentiation of CMG cells is between cardiomyocyte-progenitor and differentiated cardiomyocytes.

Differentiated CMG myotubes have a cardiomyocyte-like ultrastructure including atrial granules. Tagoe et al. (15) reported that the most common size of atrial granules observed in the adult mice atrium was 150 to 200 nm in diameter, but they also found that approximately 35% of the atrial granules in the adult mice atrium ranged between 50 to 150 nm in diam-

TABLE 1. *Isoform of the contractile proteins in differentiated CMG cells and in vivo cardiomyocytes at different developmental stages*

Protein	Atrium		Ventricle			CMG
	Fetus	Adult	Fetus	Neonate	Adult	
α-actin	skeletal	cardiac	skeletal > cardiac	skeletal	cardiac	skeletal > cardiac
Myosin heavy chain	α > β	α	β > α	α > β	α	β > α
Myosin light chain	2a	2a	2v	2v	2v	2v

CMG: cardiomyogenic.

eter. The atrial granules observed in the differentiated CMG myotubes were 70 to 130 nm in diameter. A previous report found that almost all atrial myocytes expressed ANP in the fetal heart whereas in the ventricular wall, cells containing immunoreactive granules were scattered (16). The high density granules observed in the differentiated CMG cells might correspond to those in fetal ventricular cardiomyocytes.

CMG myotubes have either sinus node-like or ventricular myocyte-like action. Although action potentials can be seen in nonmyocyte cells such as skeletal muscle cells or nerve cells, the action potential in CMG cells is characterized by duration (17,18). The duration of action potentials in skeletal muscle cells or nerve cells is less than 5 ms (19,20). The most diastolic potential, action potential amplitude, and the overshoot potential of the sinus-nodal-like CMG cells were close to the equivalent values reported for in vivo rabbit sinus nodal cells (21). In rabbit ventricular cells, the most diastolic potential and action potential amplitude were reported to be –90 to –95 mV and 120 mV, respectively. Although the most diastolic potential and action potential amplitude of the ventricular cardiomyocyte-like CMG cells was slightly shorter than these values, the shape of the action potential was very close to that of in vivo ventricular cardiomyocytes. The observation of several distinct patterns of action potential in CMG cells may reflect different developmental stages.

Future directions

CMG cells provide a powerful tool for further investigation of cardiomyocyte differentiation and cardiomyocyte transplantation. We already have transplanted these cells into the normal adult mice heart and observed that the transplanted cell could survive in the recipient heart for at least several weeks. Cell transplantation into scar tissue of the in vivo heart caused by experimental myocardial infarction was initially performed using fibroblasts, smooth muscle cells, or skeletal muscle cells. Transplantation of these cells into the scar tissue might improve cardiac remodeling or diastolic function, but most likely will not improve systolic function. Transplantation of cardiomyocytes, however, could potentially rescue systolic function. The only potential sources of regenerated cardiomyocytes to date are embryonic stem (ES) cells and mesenchymal stem cells. ES cells differentiate into cardiomyocytes in vitro and have both advantages and disadvantages for cardiomyocyte regeneration. A major advantage of ES cells is

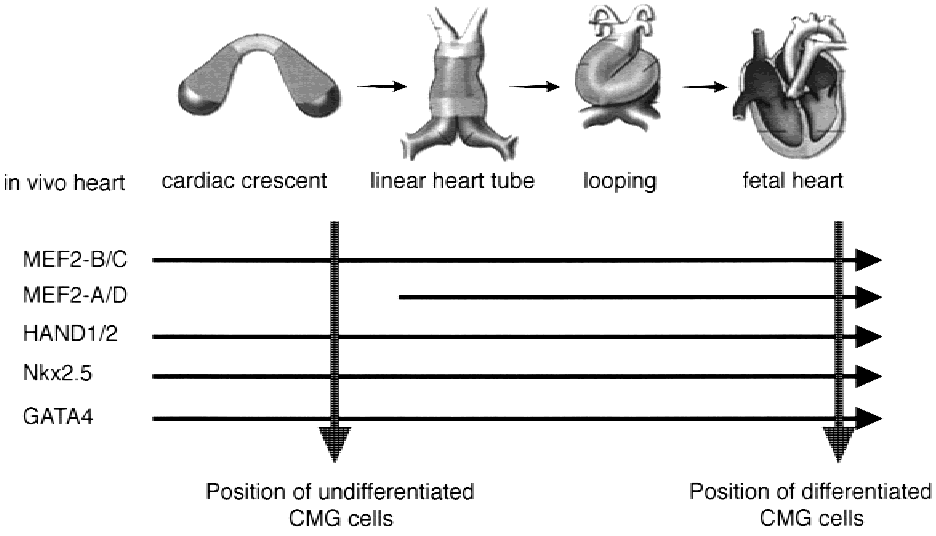

FIG. 6. Shown is the expression of cardiac-specific transcription factors in the developing heart and CMG cells. The horizontal arrows indicate the time course of the expression of cardiac specific transcription factors in the fetal developing heart. The dotted vertical arrow indicates the expression of these factors in undifferentiated and differentiated CMG cells. CMG cells expressed MEF2-A and MEF2-D after the 5-azacytidine treatment when they obtained a cardiomyocyte phenotype.

that the method for induction of differentiation into cardiomyocytes already is well established. However, transplanted ES cells can potentially form teratomas if some undifferentiated totipotent cells are still present. In addition, recipients must receive immunosuppressants because ES cells are allogeneic. In contrast, mesenchymal stem cells do not carry any inherent risks of tumor formation and are syngeneic. However, there is a need to both improve the current methods of identification and culture of mesenchymal stem cells and of induction of CMG cell differentiation, which remain inefficient and slow. The identification of specific growth factors, cytokines, or extracellular matrix factors that regulate cardiomyocyte differentiation may make this process faster and more efficient.

The number of candidates for heart transplantation remains much larger than the number of donor hearts world-wide, but the possibilities of transplantation of regenerated cardiomyocytes may provide a promising option to current therapies.

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