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別添4

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CHAPTER 8. REGENERATION OF CARDIOMYOCYTES FROM MESENCHYMAL STEM CELLS AND ITS APPLICATION TO CELL TRANSPLANTATION THERAPY

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ABSTRACT
We have isolated a cardiomyogenic cell line (CMG cell) from murine bone marrow. mesenchymal stem cells. The cells showed a fibroblast-like morphology, but the morphology changed after 5-azacytidine exposure. They began spontaneous beating after 2 weeks, and expressed ANP and BNP. Electron microscopy revealed a cardiomyocyte-like ultrastructure. These cells had several types of action potentials; sinus node-like and ventricular cell-like action potentials. The isoform of contractile protein genes indicated that their muscle phenotype was similar to fetal ventricular cardiomyocytes. They expressed α_{1A}, α_{1B}, α_{1D}, β_1, and β_2 adrenergic and μ_1 and μ_2 muscarinic receptors. Stimulation with phenylephrine, isoproterenol and carbachol increased ERK phosphorylation and second messengers. Isoproterenol increased the beating rate, which was blocked with CGP20712A (β_1-selective blocker). These findings indicated that cell transplantation therapy for the patients with heart failure might possibly be achieved using the regenerated cardiomyocytes from autologous bone marrow cells in the near future.

KEY WORDS
bone marrow stroma, mesenchymal stem cell, cardiomyocyte, differentiation, regenerative medicine, adrenergic receptor, muscarinic receptor.

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1. INTRODUCTION

Heart transplantation is the ultimate therapy for the treatment of severe heart failure. However, it has not been widely examined, because it requires donor hearts. The inadequate supply of donor hearts is often a major problem everywhere in the world. As a result, the current challenge in cardiology is how to reserve pump failure by cell transplantation or regenerative medicine. Recent studies have shown that transplanted fetal cardiomyocytes can survive in heart scar tissue and that the transplanted cells limit scar expansion and prevent post-infarction heart failure. Transplantation of cultured cardiomyocytes into damaged myocardium has been proposed as a future method of treating heart failure[1,2]. This revolutionary concept remains unfeasible in clinical settings because of the difficulty of obtaining donor fetal hearts. Thus, a cardiomyogenic cell line has long been awaited, and such a line might be capable of substituting for fetal cardiomyocytes in this therapy.

Various studies have demonstrated that cardiomyocytes can differentiate from multipotent stem cells such as embryonic stem (ES) cells[3] and embryonic carcinoma (EC) cells[4]. ES cells are an attractive cell source in regenerative medicine, but the recipients must take immunodepressant drugs throughout their lives because the transplanted ES cells are allogeneic. Use of these reagents impairs the quality of life of the recipients, and transplantation of undifferentiated ES cells often causes teratocarcinoma. In addition, the establishment of human ES cells involves ethical problems and is not allowed in every country. Because of these circumstances, the regeneration of cardiomyocytes from adult autologous stem cells has been awaited.

Recent reports have demonstrated the existence of pluripotent stem cells in adult tissues. Roy *et al.* reported the existence of neural stem cells

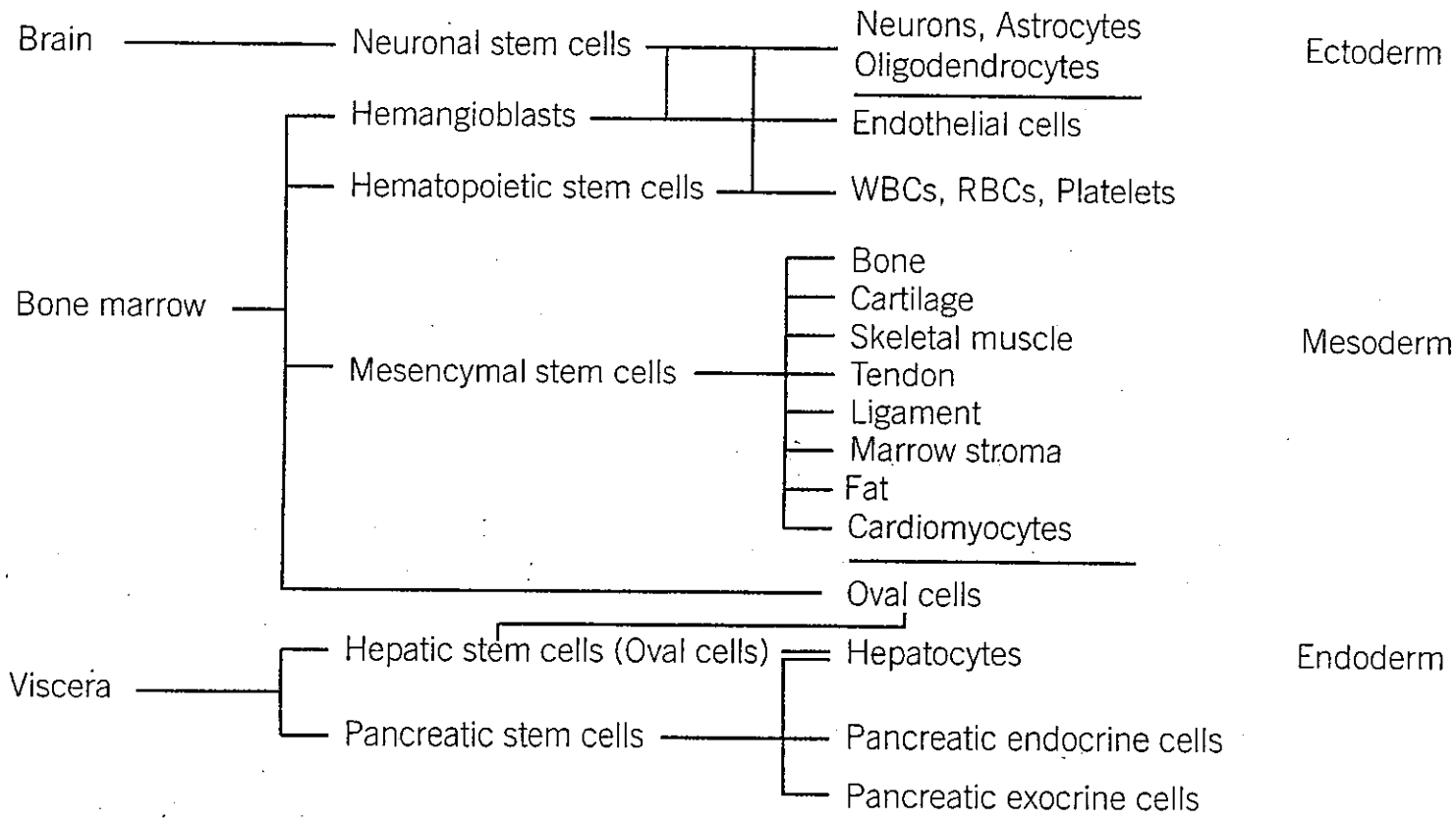
in the brain that can differentiate into neurons, oligodendrocytes, and astrocytes *in vitro*[5]. Marrow stromal cells have been shown to possess many characteristics of mesenchymal stem cells[6], and pluripotent progenitor marrow stromal cells can differentiate into various types of cell types, including osteoblasts[7,8], myocytes[9], adipocytes, tenocytes, and chondroblasts[10]. We recently reported the differentiation of mesenchymal stem cells into cardiomyocytes after exposure to 5-azacytidine and the establishment of cell line CMG (cardiomyogenic) that differentiates into cardiomyocytes *in vitro*[11]. CMG cells exhibit spontaneous beating and express atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), and they may provide a useful and powerful tool for cardiomyocyte transplantation after further characterization of their cardiomyocyte phenotype.

This paper describes the characteristics of bone marrow-derived regenerated cardiomyocytes and discusses the possibility of using them for cardiovascular tissue engineering. The expression and function of adrenergic and muscarinic receptors in CMG cells is also described, because these receptors play a critical role in modulating cardiac function[12].

2. MESENCHYMAL MARROW STEM CELLS AS A POSSIBLE SOURCE OF CARDIOMYOCYTES: THE CARDIOMYOGENIC (CMG) CELL?

Fig. 1 shows the classification of the stem cell system of adults[13]. Bone marrow stromal cells were previously used as a feeder layer to culture hematopoietic stem cells, and are known to be of mesodermal origin and produce various cytokines and growth factors. In late 1990's, a number of papers reported that bone marrow stromal cells contain multipotent stem cells for non-hematopoietic tissues, called «marrow mesenchymal stem cells», that could differentiate into osteoblasts, chondroblasts, and adipocytes. All of these cells were known to be of mesodermal origin. If mesenchymal stem cells are multipotent, we hypothesized that they might have the ability to differentiate into cardiomyocytes and instituted this study. We also thought that bone marrow cells could be obtained from patients themselves and that autologous cells would not be rejected after cell transplantation.

FIGURE 1. Classification of pluripotent stem cells in adult tissues



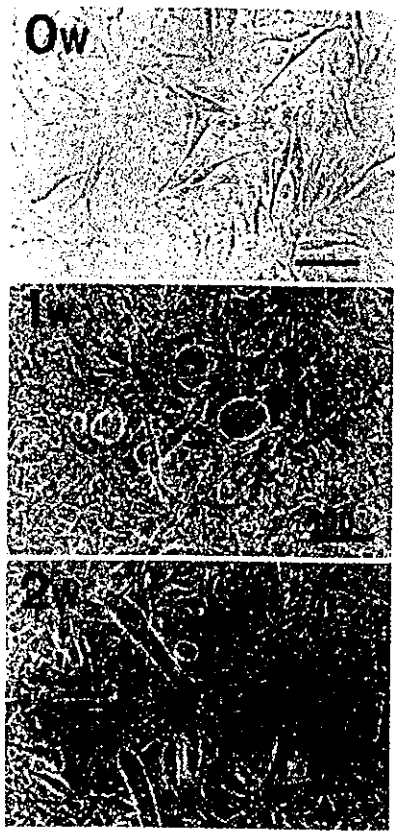
Bone marrow contains various kinds of stem cells. Mesenchymal stem cells may differentiate into various mesoderm-derived cells, such as osteoblasts, chondroblasts, adipocytes, skeletal muscle cells and possibly cardiomyocytes.

3. METHOD OF ESTABLISHING BONE-MARROW DERIVED CARDIOMYOCYTES

Female C3H/He mice were anesthetized with ether, their femora were excised, and primary culture of the marrow cells was performed according to Dexter's method. 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 at 33°C in humid air containing 5% CO_2. After a series of passages, 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 mmol/L of 5-azacytidine for 24 hours. Subclones that included spontaneously beating cells were screened by microscopic observation (first screening), and cells surrounding spontaneous beating cells were subcloned with cloning syringes. Subcloned cells were maintained, exposed to 5-azacytidine again for 24 hours, and clones that showed spontaneous beating most frequently were screened (second screening). The clonal cell line thus obtained was named the CMG cell.

As a result of repeated rounds of limiting dilution, we succeeded in isolating 192 single clones, several of which differentiated into cardiomyocytes and showed spontaneous beating. The experiments were reproducible, but the percentage of cells that differentiated into cardiomyocyte differentiation was specific to each clones. Phase-contrast photography and/or immunostaining with anti-sarcomeric myosin antibodies were used to identify the morphological changes in the CMG cells. CMG cells showed a fibroblast-like morphology before 5-azacytidine treatment (0 week), and this phenotype was retained through repeated subculturing under non-stimulating conditions. After 5-azacytidine treatment, however, the morphology of the cells gradually changed (Fig. 2). Approximately 10-30% of the CMG cells gradually increased in size at 1 week, and they formed ball-like appearance, or had lengthened in one direction to exhibit a stick-like morphology. Most of the other non-myocytes had an adipocyte-like appearance.

FIGURE 2. Phase-contrast photographs of CMG cells before and after 5-azacytidine exposure



(Upper panel) CMG cells have a fibroblast-like morphology before 5-azacytidine exposure (0 week). (Middle panel) One week after exposure, some cells gradually increased in size, and developed a ball-like or stick-like appearance. (Lower panel) Two weeks after exposure, the ball-like or stick-like cells began spontaneous beating. Bars indicated 100 μm.

4. REGENERATED CARDIOMYOCYTES DISPLAY A FETAL VENTRICULAR PHENOTYPE

Various cardiac contractile protein isoforms are differentially expressed in cardiomyocytes at different developmental stages and in different chambers. At around the time of birth there is a developmental switch in the ventricular muscle of small mammals from expression of β-myosin heavy chain (MHC), which is the predominant fetal form, to expression of α-MHC. There is also a developmental switch from expression of α-skeletal actin, which is the predominant fetal and neonatal form, to that of α-cardiac actin, the predominant adult form. We investigated the contractile protein isoforms of bone marrow-derived CMG cells to characterize their phenotype as cardiomyocytes. Table 1 summarizes the results. Fetal, neonatal, and adult ventricle and atrium were used as controls[13]. Expression of both *α-* and *β-MHC* was detected in differentiated CMG cells by RT-PCR, but *β-MHC* expression was overwhelmingly greater than that of *α-MHC*. CMG cells expressed both α-

TABLE 1. Isoforms of the contractile proteins in differentiated CMG cells

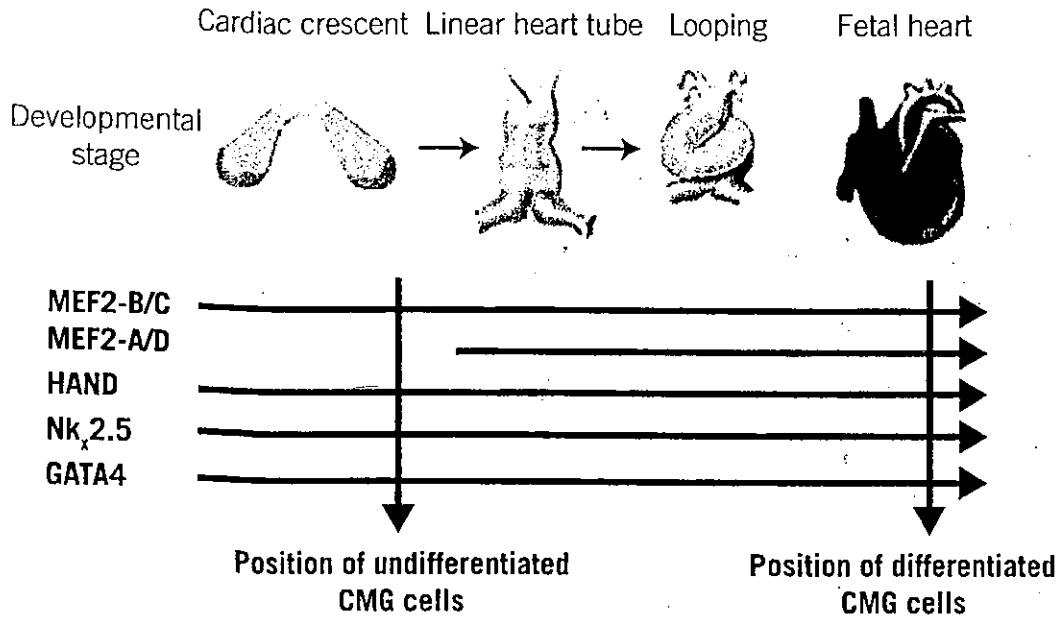
Developmental stage	Atrium		Ventricle			CMG
	Fetus	Adult	Fetus	Neonate	Adult	
α-actin	skeletal	cardiac	skeletal>cardiac	skeletal	cardiac	skeletal>cardiac
myosin heavy chain	α>β	α	β>α	α>β	α	β>α
myosin light chain	2a	2a	2v	2v	2v	2v

cardiac and *α-skeletal actin*, but the *α-skeletal actin* gene was expressed at markedly higher levels than the *α-cardiac actin* gene. Interestingly, CMG cells expressed the *myosin light chain (MLC)-2v* gene, but not the *MLC-2a* gene. *MLC-2v* is specifically expressed in ventricular cells, while *MLC-2a* is specifically expressed in atrial cells. Skeletal muscle cells do not express either *α-MHC* or *MLC-2v*. These results indicated that differentiated CMG cells possess the specific phenotype of the fetal ventricular cardiomyocytes[11].

5. DEVELOPMENTAL STAGE OF UNDIFFERENTIATED AND DIFFERENTIATED CMG CELLS

Various cardiac specific transcription factors have been cloned, and their genes are serially expressed in the developing heart during myogenesis and morphogenesis. Fig. 3 shows the time course of the expression of cardiomyocyte-specific transcription factors in fetal developing heart and CMG cells. The genes coding $Nk_x2.5$[14](homeobox

FIGURE 3. Expression of cardiac-specific transcription factors in the developing heart and in CMG cells



The horizontal arrows indicate the time course of the expression of cardiac-specific transcription factors in the developing fetal heart. The dotted vertical arrows indicate the expression of these factors in undifferentiated and differentiated CMG cells. CMG cells expressed MEF2-A and MEF2-D after 5-azacytidine exposure, when they acquired a cardiomyocyte phenotype.