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Methods for Differentiation of Bone-Marrow-Derived Stem Cells into Myocytes

Shinji Makino and Keiichi Fukuda

Abstract Although heart transplantation is the ultimate therapy for severe heart failure, it is not widely used owing to the inadequate supply of donor hearts. Therefore, cell-based therapies for the prevention or treatment of cardiac dysfunction have attracted significant interest. Since we first reported (in 1999) that bone marrow (BM) mesenchymal stem cells (MSCs) could differentiate into cardiomyocytes in vitro [1], research on regenerative medicine has advanced dramatically [2, 3]. In addition to BM MSCs, embryonic stem cells, cardiac tissue stem cells, adipose tissue stem cells, and induced pluripotent stem cells undergo myocardial differentiation; additional cell types may also prove to have cardiac cell differentiation abilities. An early-phase clinical trial involving the direct infusion of BM mononuclear cells and peripheral blood mononuclear cells into coronary arteries and the myocardium has been undertaken. However, there is a vast gap between demonstrating that a cell type can differentiate into myocardium and translating this result into clinical practice. The major challenges for the therapeutic use of stem cells include the effective harvesting and in vitro expansion of cells to ensure sufficient numbers and purity of the cells. This chapter focuses on methods for the differentiation of BM-derived stem cells into myocytes.

Keywords Bone marrow stem cells • Mesenchymal stem cells • Cardiac stem cells • Hematopoietic stem cells • Endothelial progenitor cells • Cell transplant • Myocardial infarction • Myocytes

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S. Makino (✉)
Center for Integrated Medical Research,
and
Department of Cardiology,
KEIO University School of Medicine, Tokyo, Japan
e-mail: koshinji@sc.itc.keio.ac.jp

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1 Bone-Marrow-Derived Stem Cells

Stem cells are clonogenic cells that are capable of both self-renewal and differentiation into more specialized progeny. Traditionally, stem cells have been divided into two broad categories: adult stem cells and embryonic stem cells. Adult stem cells are derived from postnatal somatic tissues and are considered to be multipotent, meaning they can give rise to multiple differentiated cell types. Embryonic stem cells, which are derived from the inner cell mass of blastocyst-stage embryos, are pluripotent, meaning they can give rise to all the differentiated cell types of the postnatal organism. Differentiated somatic cell types can also be reprogrammed into a pluripotent state similar to that of embryonic stem cells via the forced expression of stem-cell-related genes, which represents the basis for a recent report on induced pluripotent stem cells [4].

Approximately one decade ago, several studies challenged the long-held view that adult stem cells give rise to only a restricted set of differentiated cell types. These reports described "transdifferentiation" events, whereby adult stem cells differentiated into unexpected cell types, and even across embryonic germ layer boundaries. Cardiac differentiation has been reported for a variety of expected and unexpected stem cell types. These manifestations of transdifferentiation continue to be sources of controversy.

The bone marrow (BM) is a very heterogeneous compartment that contains multiple stem cell populations with putative cardiac potential, e.g., hematopoietic stem cells (HSCs) [5], mesenchymal stem cells (MSCs) [1, 6–11], very small embryonic-like stem cells [12], and multipotent adult progenitor cells (MAPCs) [13]. In this chapter, we focus on these BM-derived progenitors, which have attracted considerable attention.

1.1 Mesenchymal Stem Cells

Friedenstein et al. first reported the existence of MSCs in the BM in 1966, terming them "bone formation progenitors" [14]. Subsequently, MSCs were reported to constitute 0.001–0.01% of the total nucleated cell population in the BM, which is far lower than the content of HSCs in the BM [15, 16]. BM-MSCs were initially believed to be the stem cells that gave rise to osteoblasts, chondroblasts, adipocytes, and connective tissues [17, 18]. Recent studies have demonstrated that BM-MSCs can also differentiate into neurons [19], skeletal muscle cells [20], and cardiomyocytes [1, 21, 22], both in vitro and in vivo. BM-MSCs are found in the stromal cell fraction, which can be easily separated from hematocytes in culture. These stem cells were initially isolated from the BM stromal cells on the basis of their characteristic proliferative activities and multipotencies. Cell-surface markers that can be used to isolate MSCs have yet to be determined. CD29, CD44, CD105, and Sca-1 (only in the mouse) are widely accepted cell-surface markers for MSCs, whereas the value of other markers is debated among researchers. In 1999, we [1, 22]

observed that the exposure of immortalized murine MSCs to 5-azacytidine (5-AzaC), which demethylates methylcytosine and induces the transcription of critical transcription factors by demethylating the CpG islands in the promoter regions, resulted in the appearance of spontaneously beating foci. We have termed these cell lines "CMG" (cardio*m*yo*g*enic), as they are from adult BM stromal cells. Through repeated limiting dilutions, we isolated hundreds of clones, and we identified several clones that could differentiate into cardiomyocytes that exhibited spontaneous beating. These experiments were repeatable and reproducible, although the percentages of cardiomyocyte differentiation varied among these clones. Phase-contrast photography revealed that the CMG cells had a fibroblast-like morphology before 5-AzaC treatment (week 0), and this phenotype was retained through repeated subcultures under nonstimulating conditions. After 5-AzaC treatment, the morphology of the cells gradually changed. Approximately 30% of the CMG cells increased gradually in size, attaining a ball-like appearance or lengthening in one direction, and showed a sticklike morphology after 1 week. These cells connected with adjoining cells after 2 weeks, and formed myotube-like structures at 3 weeks (Fig. 1). The differentiated CMG myotubes retained the cardiomyocyte phenotype and beat vigorously for at least 8 weeks after the final 5-AzaC treatment.

The cardiac phenotype of the treated cells was confirmed by a variety of techniques, including reverse transcription PCR (for the markers of atrial natriuretic peptide, myosin light chain 2a and myosin light chain 2v, GATA4, and Nkx2.5), immunocytochemistry (for the markers of sarcomeric myosin heavy chain (MHC) and α-actinin), and electron microscopy. An electrophysiology study was performed on the differentiated CMG cells 2–5 weeks after 5-AzaC treatment. Two types of morphologic action potentials were distinguishable: sinus-node-like potentials (Fig. 2a); and ventricular-myocyte-like potentials (Fig. 2b). All the action potentials recorded for the CMG cells until 3 weeks of 5-AzaC treatment were sinus-node-like action potentials. Ventricular-myocyte-like action potentials were recorded after 4 weeks, and the percentage of these action potentials gradually increased thereafter.

This outcome was surprising because at the time BM cells were thought to form only blood cell lineages or bone cells. This finding was followed up using a variety of approaches, revealing the potential of BM cells to differentiate into a variety of tissues, including cardiomyocytes. Although similar findings with 5-AzaC have been reported by others [6], some investigators have suggested that this type of cardiac induction requires "immortalized" MSCs [23]. Currently, less is known about methods for the specific induction of differentiation than is known about embryonic stem cells.

Shim et al. [7] isolated MSCs from the BM of human patients who were undergoing coronary artery bypass surgery, and treated the cells with insulin, dexamethasone, and ascorbic acid. The authors reported that the treated cells immunostained positively for α-MHC, β-MHC, and GATA4, but not for skeletal muscle markers, such as skeletal MHC and MyoD. However, the efficiency of cardiogenesis achieved using this approach appeared to be poor. The resultant "cardiomyocyte-like" cell cultures lacked appreciable spontaneous contractile activity, and only a

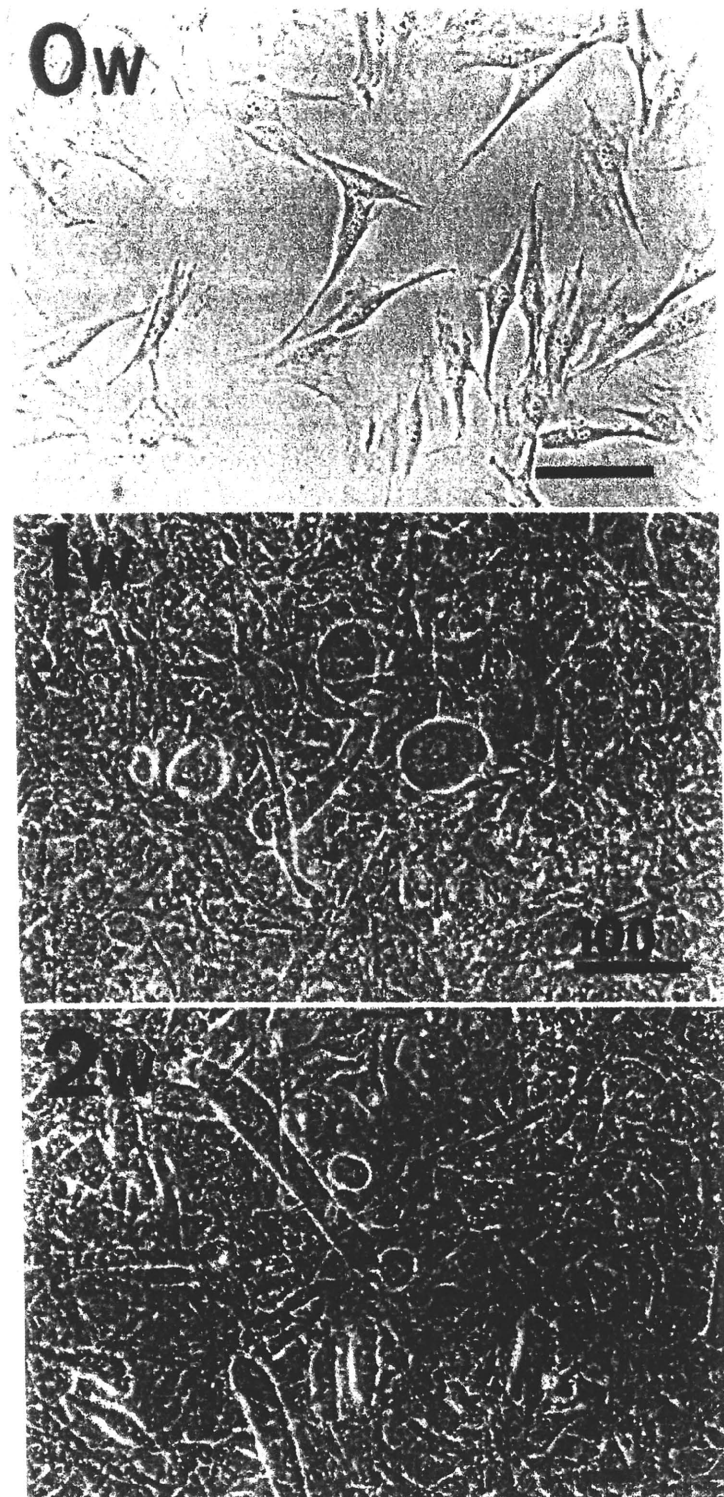

Fig. 1 Phase-contrast micrographs of CMG cells before and after 5-azacytidine (5-AzaC) treatment *Top*: CMG cells show fibroblast-like morphology before 5-AzaC treatment (week 0). *Middle*: CMG cells 1 week after treatment. Some of the cells have increased in size, assuming a ball-like or sticklike appearance. These cells began beating spontaneously thereafter. *Bottom*: CMG cells 2 weeks after treatment with 5-AzaC. Ball-like or sticklike cells are connected to adjoining cells, and are beginning to form myotube-like structures. *Bars* 100 μm

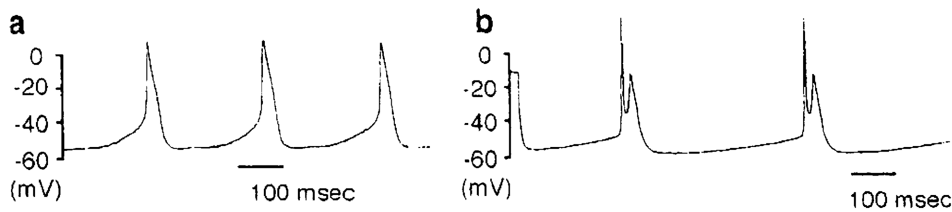

Fig. 2 Representative tracing of the action potential of CMG myotubes Action potential recordings were obtained for the spontaneously beating cells on day 28 after 5-AzaC treatment using a conventional microelectrode. These action potentials are categorized as a sinus-node-like action potential (**a**) and a ventricular-cardiomyocyte-like action potential (**b**)

small subset of the cells exhibited α-actinin-positive cross-striations. More recently, Shiota et al. have reported the cardiac induction of MSC-like progenitors derived using a complex culturing protocol that involves the formation of spheres by BM-derived adherent cells [24]. After treatment with 5-AzaC, the spheres showed spontaneous beating activity, as well as immunoreactivity for cardiac markers, including Nkx2.5 and myosin light chain 2v. The authors tested the capacity of these preparations to mediate cardiac repair in a murine infarct model. They reported functional improvements following the transplantation of green fluorescent protein (GFP)-tagged, sphere-derived cells, although the degree of remuscularization was extremely low. The latter study is one of many preclinical studies that assert beneficial effects for contractile function following the transplantation of MSCs in models of cardiac injury. Some [6, 8, 11], but not all [25], of these studies conclude that MSCs transdifferentiate into cardiomyocytes in vivo. In general, reports favoring myocardial repopulation by MSCs have shown only rare clusters of cells that lack the typical cardiomyocyte morphology but that immunostain positively for one or more cardiac markers.

In 2001, Beltrami et al. observed cardiomyocyte mitotic figures in human hearts after myocardial infarction (MI) [26]. In 2009, Bergmann et al. reported that cardiomyocytes undergo renewal, with a gradual decrease in annual turnover from 1% at 25 years of age to 0.45% at 75 years of age, according to carbon-14 measurements. Fewer than 50% of cardiomyocytes are exchanged during a normal life span [27]. Their report, which sparked controversy regarding cardiomyocyte induction, investigated the following possibilities: (1) whether the cells, which were thought to be terminally differentiated, had acquired the ability to proliferate; (2) whether immature cardiomyocytes differentiated from stem cells into cardiomyocytes and then began to proliferate; or (3) whether mature cardiomyocytes acquired the ability to proliferate by fusing with cells that had retained the proliferative capabilities of stem cells.

1.2 *Hematopoietic Stem Cells*

Recent advances in fluorescently activated cell sorting (FACS) techniques have enabled the prospective isolation of HSCs on the basis of their cell-surface antigen expression patterns and fluorescent dye efflux characteristics [28–30].

The FACS-derived CD34⁻ c-kit⁺ Sca-1⁺ Lin⁻ tip side population (SP) cell fractions contained the HSC population in mice; [30] c-kit is a stem cell factor receptor, Sca-1 is a stem cell antigen that is specifically expressed in various stem cells (only in the mouse), and Lin is a mixture of antibodies against lineage markers for hematocytes (in mouse, Gra-1, Mac-1, B220, CD3, and Ter119; in human, CD3, CD4, CD8, CD19, CD33, and glycophylin A). In 2001, Orlic et al. reported cardiomyocyte differentiation following the transplantation of c-kit⁺ Lin⁻ BM cells into peri-infarct tissue after MI [26]. They demonstrated directly that BM cells become cardiomyocytes in vivo. However, c-kit⁺ Lin⁻ BM cells are predominantly HSCs, and even if BM cells differentiate into a variety of cells, including cardiomyocytes, controversy persists regarding, for example, whether HSCs transdifferentiate or MSCs differentiate. Moreover, in 2002, fluorescent in situ hybridization analysis revealed the presence of numerous cardiomyocytes that seemed to be recipient-derived after human heart transplantation [31]. In contrast, in 2003, numerous BM-derived cardiomyocytes were shown to be present in the recipient heart after BM transplantation [32]. In an experiment that gave very different results, Wagers et al. examined a variety of organs after transplanting GFP-labeled single HSCs (c-kit⁺, Lin⁻, Sca-1⁺) into irradiated mice, and they concluded that if HSC transdifferentiation does occur, it is extremely rare, and that cardiomyocyte differentiation does not occur as a result of MI or induced injury [33]. Goodell et al. transplanted highly enriched HSCs into lethally irradiated mice, which were subsequently rendered ischemic by coronary artery occlusion for 60 min, followed by reperfusion; they reported that the transplanted BM cells differentiated into cardiomyocytes in the peri-infarct region at a prevalence of 0.02% [34]. In 2004, Balsam et al. investigated whether the c-kit⁺ HSCs in BM are capable of differentiating into cardiomyocytes [35], by directly injecting BM cells into myocardial tissue instead of transplanting BM cells after irradiation as other groups had done. Importantly, they conducted their study to exclude irradiation, given the possibility that invasive treatment, including irradiation, contributes to a fusion phenomenon. They concluded that c-kit⁺ HSCs do not include cells that are capable of differentiating into cardiomyocytes. Murry et al. investigated this differentiation ability in a similar manner, by directly infusing c-kit⁺ Lin⁻ HSCs into the heart [36] and, as expected, they found that the HSCs were unable to differentiate into cardiomyocytes. In the same year, we examined the differentiation capabilities of HSCs using a c-kit⁺ Sca-1⁺ CD34⁻ Lin⁻ SP (CD34⁻KSL-SP) of HSCs [37]. When we transplanted whole BM cell populations, which included both HSCs and MSCs, from GFP-transgenic mice into lethally irradiated mice and subsequently induced MI, we found very few GFP⁺ (BM-derived) cardiomyocytes. Interestingly, granulocyte colony stimulating factor (G-CSF) increased the number of GFP⁺ cardiomyocytes and nonmyocytes in the infarcted or border zone area. In contrast, when we performed HSC transplantation followed by induction of MI and administration of G-CSF, cardiomyocytes were rarely found in the group that was transplanted with HSCs alone, although fibroblast-like cells were observed, and G-CSF increased their number. Moreover, we confirmed the predominance of MSC-derived GFP⁺ cardiomyocytes in the group that was transplanted with cardiomyogenic cells, i.e., purified MSCs. It should be emphasized that in this type of BM

transplantation experiment the dosage of radiation must be carefully determined, as the sensitivity to radiation of MSCs is much higher than that of HSCs. We propose that the differentiation by whole BM cells into organs (cells) other than hematopoietic populations is attributable to MSCs rather than HSCs, and that MSCs are mobilized from the BM into the bloodstream, in similarity to HSCs.

Nonetheless, the cardiac potential of HSCs remains controversial. The authors of the original study by Orlic et al. recently revisited this issue, and they concluded once again that in a mouse infarct model, the c-Kit$^+$ BM cells transdifferentiated following transplantation and formed extensive replacement myocardium [38].

1.3 BM-Derived Endothelial Progenitor Cells

Endothelial progenitor cells (EPCs) should be viewed as both circulating and BM stem cell types, since they are known to reside in both compartments. In 1997, Asahara and colleagues described the phenotype of EPCs, which proliferate in response to tissue ischemia, home to areas of injury, and either incorporate within or otherwise promote neovascularization [39, 40]. EPCs express the markers of Flk-1, CD34, and CD133, and can differentiate into definitive endothelial cells [39, 41–43]. Initial interest in the application of EPCs to cardiac repair was naturally focused on their angiogenic properties. The capacity of EPCs to transdifferentiate into cardiomyocytes was first reported by Dimmeler and colleagues in 2003 [44]. In that study, CD34$^+$ human EPCs were obtained from peripheral blood mononuclear cells of healthy adults or from patients with coronary artery disease. After coculture with neonatal rat cardiomyocytes, EPCs were reported to transdifferentiate into cardiomyocytes on the basis of morphology, α-sarcomeric actinin immunoreactivity (as assessed by flow cytometry), and the expression of other cardiac markers (as assessed by immunostaining or reverse transcription PCR with species-specific probes). Furthermore, the EPCs showed calcium transients that synchronized with adjacent rat cardiomyocytes, suggesting communication with the host myocardium through gap junctions. Coculturing experiments with paraformaldehyde-fixed cardiomyocytes revealed that cell fusion was not required for EPCs to acquire the cardiac phenotype [44–47]. However, the efficiency of cardiac induction by EPCs was very low; even after enhancement through inhibition of Notch signaling, less than 1% of the EPCs expressed α-sarcomeric actinin [47]. Asahara and colleagues reported even lower rates of cardiac transdifferentiation in vitro following coculturing of EPCs with the rat heart-derived H9C2 cell line [48]. The latter authors also reported the in vivo cardiac differentiation of a related preparation of human circulating cells following transplantation into a rodent infarct model. However, this conclusion is complicated by the definitive demonstration of cell fusion between host myocytes and graft cells, using species-specific fluorescent in situ hybridization probes [49]. Moreover, Gruh et al. were unable to confirm the in vitro cardiac differentiation of EPCs following coculturing with primary myocytes [50]. These authors found no expression of human cardiac transcripts,

and they concluded that the rare, ostensibly transdifferentiated EPCs observed by FACS or epifluorescence microscopy were artifacts that resulted from overlying cells and/or autofluorescence. Thus, although the cardiac potential of EPCs remains a source of controversy, the report of Gruh et al. underscores the challenges inherent to interpreting coculture experiments.

1.4 Very Small Embryonic-Like Stem Cells

In 2006, employing multiparameter sorting, Kucia and colleagues identified in murine BM populations a homogenous population of rare (approximately 0.02% of BM mononuclear cells) Sca-1^+ Lin^- $CD45^-$ cells that express SSEA-1, Oct-4, Nanog, and Rex-1 [51]. These cells are very small and display several features that are typical of primary embryonic stem cells. In vitro cultures of these cells are able to differentiate into all three germ layer lineages, including cardiomyocytes. For cardiac differentiation, GFP^+ Sca-1^+ Lin^- $CD45^-$ or Sca-1^+ Lin^- $CD45^+$ cells together with unpurified GFP^- BM cells were plated in Dulbecco's modified Eagle's medium that was supplemented with 10% fetal bovine serum, 10 ng/ml basic fibroblast growth factor, 10 ng/ml vascular endothelial growth factor, and 10 ng/ml transforming growth factor β_1. Growth factors were added every 24 h, and the medium was replaced every 2–3 days.

Dawn et al. have reported that the transplantation of a relatively low number of very small embryonic-like stem cells is sufficient to improve left ventricular function and to alleviate myocyte hypertrophy after MI [12]. In that report, 10,000 very small embryonic-like stem cells in a 50-μl volume were injected intramyocardially using a 30-gauge needle.

1.5 Multipotent Adult Progenitor Cells

In 2002, Jiang et al. reported on pluripotent BM-derived cells, which they referred to as multipotent adult progenitor cells (MAPCs) [52]. When transplanted into blastocysts, MAPCs had the potential to differentiate into the three germ layers both in vitro and in vivo. These MAPCs were maintained using a low-density culture method, making independent corroboration of the findings by other laboratories rather difficult. In 2006, Zeng et al. showed that MAPCs could be derived from both postnatal and fetal swine BM. Swine MAPCs are negative for CD44, CD45, and major histocompatibility complex classes I and II, express octamer-binding transcription factor 3a messenger RNA and protein at levels close to those seen in human embryonic stem cells, and have telomerase activity, which prevents telomere shortening.

Transplantation of MAPCs (injected directly into heart at ten million cells per location diluted in 400 μl of saline) at the time of coronary artery ligation resulted in improved infarct zone contractile function and prevented peri-infarct border zone bioenergetic deterioration [13]. The left ventricular chamber response to cell transplantation resulted from the beneficial effects of sparing myocytes and

increasing revascularization in both the infarct zone and the peri-infarct border zone. A direct structural contribution of the engrafted cells to cardiomyocyte regeneration appears to be unlikely.

2 Other BM-Derived Cells and Cell Fusion

In 2005, Yoon et al. identified a subpopulation of human BM stem cells (hBMSCs) that did not belong to the previously described class of BM-derived stem cells [53]. These cells were $CD29^-$, $CD44^-$, $CD73^-$, demonstrating minimal expression of CD90, CD105, and CD117, and could differentiate into the three germ layers. Intramyocardial transplantation of hBMSCs after MI resulted in robust engraftment of transplanted cells, which exhibited smooth muscle cell identity and colocalization with markers of cardiomyocytes and endothelial cells, which is consistent with the differentiation of hBMSCs into multiple lineages in vivo. Coculturing of hBMSCs with cardiomyocytes revealed that phenotypic changes in the hBMSCs result from both differentiation and fusion. Other laboratories have identified additional multipotent, $CD45^-$, nonhematopoietic BM-derived cells [40, 54, 55]. In some cases, it is likely that similar or overlapping populations of primitive stem cells in the BM detected using various experimental strategies have been assigned different names. The relationships among the BM-derived stem cells reported from different laboratories need to be clarified.

In 2002, Terada et al. suggested that a cell fusion phenomenon had to be considered with regard to the plasticity of the BM cells reported thus far [56]. Their coculture of adult animal BM cells with embryonic stem cells induced cell fusion naturally in the presence of interleukin-3, and although the karyotype was tetraploid, the cells acquired pluripotency and proliferative ability. More recently, the transplantation of whole BM cells into lethally irradiated mice resulted in fused cardiomyocytes but no transdifferentiation [57]. In addition, the same study aimed to identify the cell lineages in whole BM populations that are responsible for cell fusion, by transplanting CD45-Cre mouse BM into R26R mice. Fused cardiomyocytes were observed in this experimental system, and BM-derived leukocyte lineage cells were found to be responsible for the fusion. The lack of a clear definition for cell plasticity has led to confusion, with several reports failing to demonstrate that a single cell can indeed differentiate into multiple lineages at significant levels.

Studies using the Cre-lox recombination system revealed only rare MSC-derived cardiomyocytes, nearly all of which resulted from cell fusion [58].

3 Specific Culture Method for Cardiac Differentiation and Cell Fusion

Another obstacle to cell therapy is that specific culture methods for differentiating BM cells are only available for some target cells. Specific differentiation is achievable for osteoblasts, chondroblasts, and adipocytes. The use of 5-AzaC is effective for cardiomyocyte differentiation but it is clinically toxic. For cardiomyocytes,

no methods have been established that use physiologic growth factors, cytokines, or nontoxic chemical compounds. Perhaps the most studied strategy to date with adult stem cells is the effect of 5-AzaC, a DNA demethylation reagent, on cardiac protein expression in MSCs [1, 59]. Several studies have demonstrated an increase in cardiac protein expression after treatment of MSCs with 5-AzaC [1]. Importantly, studies have consistently demonstrated improvement in cardiac function after the transplantation of 5-AzaC-treated MSCs, as compared with the transplantation of control MSCs [59–61]. As we begin to define the pathways, we can attempt to optimize further cardiac differentiation and functional effects [61]. For the further development of this field, it is necessary to find the small molecule and to elucidate the epigenetic status that can enhance cardiac differentiation from these stem cells [62].

Recently, Ge et al. reported the cardiomyocyte differentiation of rat BM-MSCs by treating the stem cells under conditions similar to those seen during MI [63]. The extract from the infarcted rat myocardium contained the same biochemical factors that arise after MI. Ge et al. found that the extract of infarcted myocardium could induce cardiomyocyte differentiation of BM-MSCs, as shown by the expression of cardiomyocyte-specific genes, including those for α-actin, connexin 43, Nkx2.5, MEF2c, GATA4, α-MHC, and troponin I. This approach could represent an alternative means of inducing cardiomyogenic differentiation in that it does not rely on gene demethylation or the use of viral vectors. The findings of that study appear to support the use of autologous extracts for the induction of stem cell differentiation and may have clinical implications for cardiac cell therapy.

Significant work has been performed to further understand the regulatory pathways involved in embryonic stem cell differentiation to cardiac myocytes [64–66]. These studies have suggested potential pathways that could be activated in adult stem cells so as to induce them to take on a cardiac phenotype [64, 66, 67].

Another approach that is being developed to direct the cardiac differentiation of adult stem cells is the delivery of chimeric proteins that encode cell-penetrating peptides (CPPs) and cardiac-specific transcription factors [68, 69] CPPs cause nonsecreted proteins to be secreted and to be internalized by surrounding cells. Bian et al. have demonstrated that the transplantation into the myocardium of cells that are genetically enhanced to express a CPP-GFP protein results in GFP expression in native cardiac myocytes [69]. To deliver functional transcription factors to the myocardium, Bian et al. developed a CPP-GATA4 construct and transplanted cardiac fibroblasts that were stably transfected with the CPP-GATA4 construct, 1 month after MI in the Lewis rat. The infarct border zones of the animals that received CPP-GATA4 demonstrated increased expression of cardiac myosin and Bcl-2 [69]. The modulation of GATA4-responsive gene expression led to hypertrophy of the cardiac myocytes at the infarct border zone and a global improvement in cardiac function [69]. These findings suggest that combining genetic enhancement of stem cells to deliver CPP–transcription factor chimeric proteins together with either stem cell homing agents or additional stem cells could lead to an increase in cardiac protein expression in the stem cells, cardiac myocyte regeneration, and further improvements in cardiac function.

4 Cardiospheres and Cardiac Extracts for Cardiomyogenesis

In 2004, Messina et al. described a novel technique for isolating resident cardiac progenitors from murine hearts, as well as subcultures of human atrial or ventricular specimens [70]. Mild enzymatic digestion of the tissue specimens yielded small, round, phase-bright cells that clustered together in suspension. These sphere-generating cells were allowed to adhere to poly(L-lysine)-coated plates, and were cultured in a medium that was supplemented with cytokines (epidermal growth factor, basic fibroblast growth factor, cardiotrophin-1, and thrombin). These "cardiosphere"-derived cells were self-renewing, clonogenic, and expressed both endothelial markers (KDR in human, flk-1 in mouse cells, and CD31) and stem cell markers (CD34, c-Kit, and Sca-1). Murine cardiosphere-derived cells showed spontaneous contractile activity, whereas human cardiosphere cells did so only after 24 h of coculturing with postnatal rat cardiomyocytes. The cardiosphere-derived cells from both human and mouse demonstrated trilineage differentiation into cardiomyocytes and endothelial and smooth muscle cells. However, quantitative data on the frequencies of these events were not reported. Cardiosphere-derived cardiomyocytes express cardiac markers, including cardiac troponin I, atrial natriuretic peptide, and cardiac MHC. In vivo, cardiosphere-derived cells have been reported to regenerate the infarcted mouse heart [70]. Subsequently, Smith et al. expanded on these findings by isolating cardiosphere-forming cells from human biopsy specimens [71]. These human cardiospheres, which were successfully isolated from 69 of the 70 biopsies tested, consistently expressed c-Kit but not the multidrug resistance gene MDR1, indicating that these cells were phenotypically distinct from the resident cardiac progenitors previously identified in situ (c-Kit$^+$, MDR1$^+$) [31, 72]. Consistent with the findings of Messina et al. [70] human cardiosphere-derived cells did not spontaneously contract, whereas coculturing with neonatal rat cardiomyocytes evoked calcium transients in synchrony with neighboring cardiomyocytes, action potentials, and fast inward sodium currents. Smith et al. also injected lentivirally transduced LacZ$^+$ human cardiosphere-derived cells into the border zones of infarcted SCID beige mice [71]. Twenty days later, the cardiosphere-derived cells were detected throughout the border regions of the mouse hearts, and occasional donor cells were immunostained for α-sarcomeric actin and von Willebrand factor. Echocardiography showed improvements in global left ventricular function, although given the apparently limited cardiomyocyte repopulation by LacZ$^+$ cells, these functional effects were attributed to a combination of regeneration and paracrine effects. On the basis of these studies, explant-derived cardiospheres appear to have cardiomyogenic potential and considerable promise for cardiac repair.

The differentiation of human adipose tissue stem cells to take on cardiomyocyte properties occurs following transient exposure to a rat cardiomyocyte extract [73–75]. Adult cardiomyocytes retain the capacity to induce cardiomyogenic differentiation of adult human MSCs. This approach could represent an alternative strategy to induce cardiomyogenic differentiation that does not rely on gene demethylation or the use of viral vectors.

5 Conclusions

Advances in stem cell and developmental biology have resulted in the identification of numerous candidate stem cell types with putative cardiogenic potential. The ideal cell type remains to be confirmed, despite all claims to the contrary. The cardiogenic potentials of BM-derived and circulating stem cells appear limited, whereas other candidates, including pluripotent stem cells, are clearly capable of more efficient cardiogenesis. We are optimistic that research into cell-based cardiac repair will eventually yield effective myogenic therapies, although success in this area will require rigorous cardiac phenotyping, cell fate mapping, and preclinical and clinical testing.

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5. 心不全における自律神経系の適応機構

慶應義塾大学医学部循環器内科　金澤英明
同　教授　福田恵一

key words　cardiac sympathetic nerve, heart failure, cholinergic differentiation, rejuvenation, nerve growth factor, leukemia inhibitory factor

動　向

心不全の治療戦略において重要なのは，病態解明の進歩である．心不全における心臓交感神経異常については，これまでにも様々な原因，機序が説明されてきたが，その詳細な病態解明や意義については明確な結論が出ていないのが現状である．

近年，心筋細胞と心臓交感神経の間には様々な液性因子を介したクロストークが存在することが明らかとなってきた．さらに，心不全の病態生理に関わる交感神経線維の軸索伸張，除神経，機能変化といった現象も分子生物学的に解明されつつあり，心不全における自律神経系の適応機構に関する新たな概念が展開されている．

本稿では，心不全に関わる心臓交感神経異常の新しい概念と心不全の病態との関連について解説する．

A. 心不全における交感神経異常とその原因，循環調節機構と中枢の関与

心不全では，交感神経活動の亢進が認められる．これは破綻してゆく循環動態を維持するための重要かつもっとも基本的な代償機転であるが，その一方で心筋に対しての負荷の増大を招き，最終的には心不全の進展を助長することになる．

心不全における交感神経活動亢進のメカニズムとしてこれまでに説明されてきた機序のひとつは，動脈圧受容体や心肺圧受容体の異常である．圧受容体で感知されたシグナルは，求心性神経を介して中枢に伝達され，交感神経活性を抑制すべく中枢から遠心性神経を介して交感神経活動を抑制するものである．しかし，心不全では圧受容体のNa^+-K^+ ATPase活性が亢進し，受容体膜が過分極に傾き，受容体の感受性が低下しているため，交感神経の抑制が起こらないとされている[1]．また，低酸素血症や高炭酸ガス血症を感知する化学受容体反射を介する機序も交感神経活性亢進の一因とされている[2]．さらには心臓からの交感神経求心路を介して反射性に交感神経活性を亢進するcardiac sympathetic afferent reflexの関与も示唆されている[3]．しかし，これらの自律神経による循環調節機構だけでは，心不全時の交感神経機能亢進の機序は解明されず，中枢神経系の関与に関する研究も進んできた[4]．特に中枢におけるレニン・アンギオテンシン系の亢進や脳内のNO産生低下が中枢性交感神経活性の亢進に重要な働きをしていることが明らかとなってきた．また，

最近では低分子量G蛋白であるRho/Rho-kinase系の活性化も心不全における交感神経活性化を修飾していることが報告されている[5]．中枢神経と交感神経の関係は密接かつ重要であり，今後の研究の成果が期待される領域である[6]．

B. 心臓交感神経と液性因子のクロストーク

心不全では，レニン・アンギオテンシン系の賦活化，エンドセリン（ET-1）やサイトカインの活性化などが起こり，神経体液性因子のクロストークという複雑な関連により，心臓交感神経活性化とともに心不全の病態を修飾していると考えられている．

交感神経誘導因子として知られている神経成長因子 nerve growth factor（NGF）は，ニューロトロフィンファミリーに属し，神経の分化，生存，シナプス形成に重要な働きをしている[7]．そして，標的臓器におけるNGFの発現が，交感神経密度を規定していると考えられている[8]．心肥大でET-1の発現が亢進することはよく知られているが，心筋細胞ではこのET-1によってNGFが特異的に誘導されることから，ET-1 / NGF pathwayは心臓の発生における心臓交感神経支配に重要であることが示されている[9]．

さらに，ラット右室肥大モデルによる検討では，右室におけるET-1のmRNAの発現亢進にともなってNGFの発現が蛋白レベルでも亢進し，右室の交感神経密度も著明に増加していることが報告され[10]，ET-1 / NGF pathwayが心臓の発生のみならず，病的状態においても重要であると考えられている．一方，高濃度ノルエピネフリン norepinephrine（NE）に長期間暴露された重症末期心不全モデルでは，心筋でのNGFの発現は低下し，交感神経密度が減少することが示された[11]．

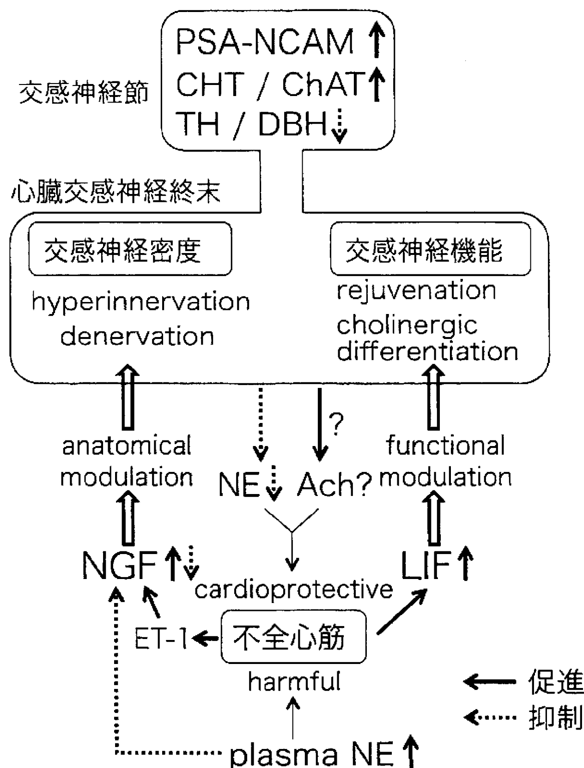

図1　心筋と心臓交感神経の液性因子を介するクロストーク

この心不全におけるNGF発現の調節機構はいまだ不明であるが，最近では心筋細胞に対する機械的伸展刺激およびα1受容体刺激がCalcineurin-NFAT（nuclear factor of activated t-cells）シグナルを介してNGFの発現を減弱させる可能性が報告されている[12]．

以上より，心臓交感神経密度はET-1の発現およびNE暴露の影響による心臓NGFの発現によって厳格に規定されており，様々な病態下において解剖学的心臓神経支配が変化することが明らかとなった（図1）．

C. 心不全における心臓交感神経の幼若化

心不全病態下では，心筋細胞においてβ型ミオシン重鎖，骨格筋型α-アクチン，ANP，BNPなどの胎児型遺伝子が発現してくることが広く知られている．この現象は，そのアイソフォームを変

化させることにより，何らかの生体防御機構を働かせているものとも考えられている．近年では心臓のみならず，神経系においても細胞障害に対する生体の反応過程として，胎児型遺伝子が発現してくることが知られるようになってきた．

心臓交感神経における胎児性遺伝子の発現に関する研究は，ラットにモノクロタリンを投与することにより肺高血圧を惹起した右室肥大・右心不全モデルの検討により報告された[10]．肥大心では心筋由来NGFの発現が亢進し，心臓交感神経が過剰分布（hyperinnervation）したものの，逆にNE合成酵素であるTH（tyrosine hydroxylase），DBH（dopamine β-hydroxylase）活性は低下し，NE再吸収などの交感神経機能は低下の方向を示した．さらに，これらの交感神経はPSA-NCAM（highly polysialylated neural cell adhesion molecule）といった幼若神経のマーカーの発現を確認した．以上の結果から，心不全における心臓交感神経は幼若化（rejuvenation）を惹起し，交感神経機能低下の一因となりうることが明らかとなった（図1）．

D．心臓交感神経終末の異常と機能転換

心不全病態下では，交感神経終末においても様々な異常が生じ，持続的な交感神経活性の亢進に伴って，心筋におけるNE含有量は減少し枯渇するとされている．これは，交感神経終末においてNEのturnoverが亢進し，過剰放出（spilloverの亢進）や再吸収障害（uptake1の障害）などによりNEの保持能力が低下するためとされている[13]．さらに，NEの前駆体であるドーパミン，およびNE律速合成酵素であるTHの発現も低下し，解剖学的交感神経の除神経（denervation）そのものが原因とされ[14]，臨床的にはMIBGの欠損像としてとらえられてきた．

一方では，心不全動物モデルにおいてTHの発現低下が認められるものの，交感神経線維の量的変化は認められず，機能的な変化がその主体であるとする報告もある[15]．

以上のように，これまでのさまざまな研究から心不全における交感神経終末の異常が示されてきたが，交感神経活性の亢進しているにもかかわらず，なぜTHの発現が低下するのかなどの矛盾点も多く，その病態生理は十分に解明されていない．

しかし，最近の研究では心臓交感神経の可塑性の観点から，この機序が解明されつつある[16]．Dahl食塩感受性ラットを用いた心不全モデル，および心不全患者の剖検例の検討では，左室，星状神経節のTH陽性神経は減少しているものの，副交感神経マーカーであるCHT（choline transporter），ChAT（choline acetyltransferase）陽性の神経細胞が多数認められた．さらに，その一部は交感神経マーカーと副交感神経マーカーの両方の性質を有している神経の存在も確認された（図2）．この現象は，心不全の心筋から分泌されたIL-6関連のサイトカインの作用により，心臓交感神経が副交感神経（コリン作動性神経）に分化転換（cholinergic transdifferentiation）する現象と考えられた．これまで，vitroの研究では培養交感神経細胞に白血病阻止因子 leukemia inhibitory factor（LIF）を添加することによって，アドレナリン作動性神経がコリン作動性神経に転換する（cholinergic differentiation）ことが知られていた[16,17]が，心不全などのvivoにおける病態生理学的な意義は不明であった．

さらに，このサイトカインの受容体の共通のサブユニットであるgp130遺伝子を交感神経特異的に破壊した遺伝子改変マウス（$gp130^{DBHCre}$マウス）を用いた解析から，この現象がgp130受容体を介した現象であることが確認された．また，交感神経特異的gp130欠損マウス（$gp130^{DBHCre}$マウス）の低酸素刺激右心不全モデルでは，その生存率が対照マウス（$gp130^{flox/flox}$マウス）に比

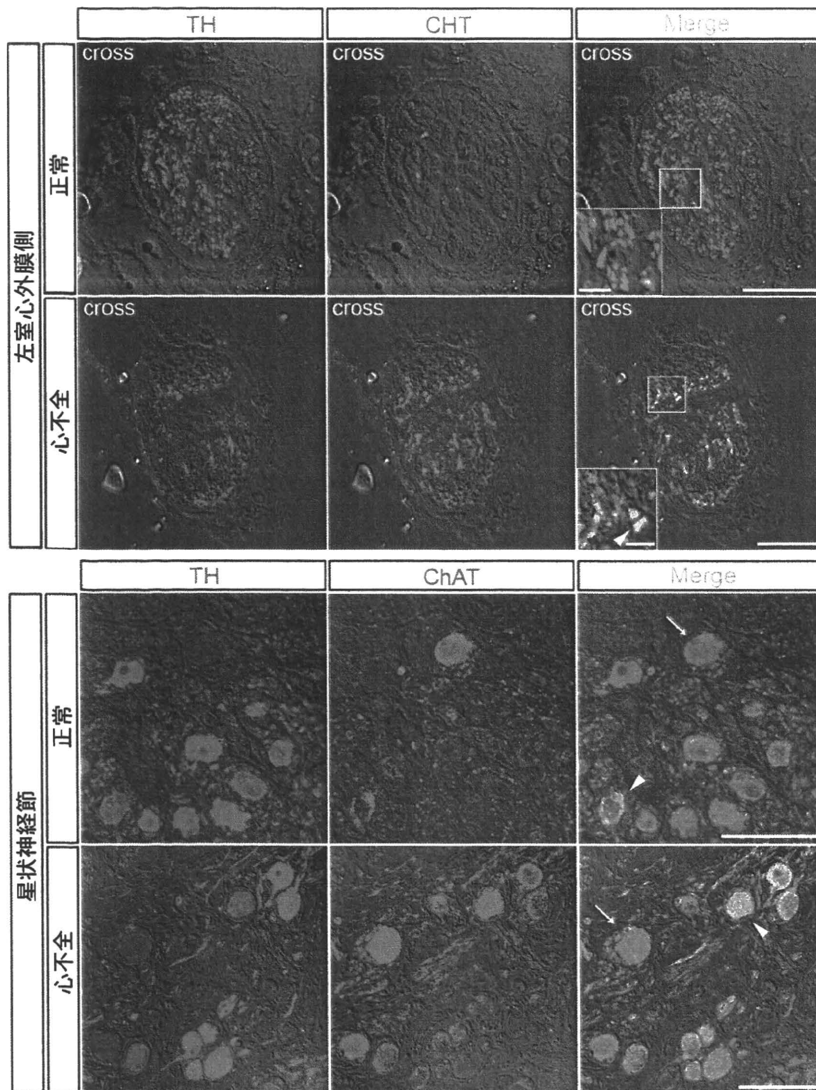

図2 ヒト剖検例における左室および星状神経節の二重蛍光免疫染色[18]

上段：左室心外膜側，TH（赤：カテコラミン作動性神経），CHT（緑：コリン作動性神経）．心不全ではCHT陽性神経線維が増加し，一部はTHおよびCHTの共染（矢印頭）を認める．

下段：星状神経節．TH（赤：カテコラミン作動性神経），ChAT（緑：コリン作動性神経）．心不全では，ChAT陽性細胞（矢印）が数多く認められる．一部はTHおよびChATの共染（矢印頭）を認める．

較して有意に低下したことから，この現象が心筋保護的に作用している可能性が示唆された（図3）．

以上の結果より，不全心筋から分泌されるサイトカインのgp130受容体を介した作用によって心臓交感神経の機能的変化が惹起されていることが明らかとなった（図1, 2）．

E. 心不全と心臓交感神経の可塑性

心臓交感神経と副交感神経は，発生学的にその起源は同一で，神経堤細胞に由来することが知られているが，この心不全時に観察された心臓交感神経の機能的変化はその可塑性に基づく多能性（脱分化と分化転換）を捉えたものである可能性