Human Sternal Mesenchymal Stem Cells: Isolation, Characterization and Cardiomyogenic Differentiation

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Background: Bone marrow mesenchymal stem cells (MSCs) have multiple potentials and represent an attractive cell population for regenerative medicine. Here, we isolated human sternal MSCs (hMSCs) from patients undergoing cardiac surgery and investigated their growth properties, immortalization and the cardiomyogenic differentiation.

Methods: Bone marrow from sternum during cardiac surgery was collected (n = 20). hMSCs were isolated through immuno-depletion, density gradient centrifugation and selective adhesion. Their growth properties were determined. Sternal hMSCs were characterized by surface antigens, immortalized by HPV16 E6/E7 retroviral vector and then exposed to 5-azacytidine.

Results: Sternal hMSCs showed a gradual loss of ability to proliferate during cell passage, accompanied by a morphological conversion of senescence tendency. They uniformly lacked antigens typically identifying haematopoietic cells. They were positive for CD90, CD44, CD29 and other MSCs markers (SH2, SH3 and SH4). The HPV16 E6/E7 retroviral vector successfully immortalized the sternal hMSCs. After induction of 5-azacytidine, primary culture, early passage and immortalized E6/E7 hMSCs, but not late passaged cells, demonstrated the aborted cardiomyogenic differentiation by activating a cardiac-specific gene, cardiac troponin-I without a recognizable morphologically differentiated phenotype.

Conclusion: Expanded sternal hMSCs from patients undergoing cardiac surgery showed senescent tendency with decreased ability of proliferation and differentiation in cardiomyogenic lineage. Specifically immortalized hMSCs may regain the ability of cardiomyogenic differentiation and serve as a useful cell source for elucidation of cardiomyogenic differentiation.

Key Words: Cardiomyogenic differentiation • Immortalization • Mesenchymal stem cells • Regenerative medicine

INTRODUCTION

Since the discovery of somatic stem cells different to hematopoietic stem cells, the concept of post-natal cell therapy has evolved. Among these stem cells, bone marrow mesenchymal stem cells (MSCs) have been shown to have multiple differentiation potential to bone, cartilage, lung, muscle and nerves in in-vitro and in-vivo settings and represent a particularly attractive cell population for new applications in cell therapy and regenerative medicine to repair various tissues and organs after injury. We and others have demonstrated in the rodent model that bone marrow MSCs can differentiate into cells expressing phenotypes of cardiomyocytes including cardiac-specific contractile protein and gap junction proteins when implanted into injured heart.
even improve heart function.\textsuperscript{11} These findings suggested a wide variety of possibilities to use autologous MSCs for myocardial regeneration.

However, there are several obstacles before we can fully explore the potential of human MSCs (hMSCs) for myocardial repair in the clinical setting. In spite of the attention given to the study of MSCs derived from the bone marrow of humans and other species, there is still a lack of full understanding about hMSCs regarding the site of isolation, methods to isolate and expand and their characteristics and growth properties.\textsuperscript{12,13} A defined methodology for the culture of hMSCs, as well as a comprehensive understanding of their biology, will make the development of cellular and genetic therapy protocols in human models possible.

Furthermore, damaged myocardium or myocardial scar, which is the target area for myocardial regeneration, may lack the signal of cardiomyogenic differentiation for MSCs.\textsuperscript{14} For clinical application of cell therapy for myocardial regeneration, the pre-transplant cardiomyogenic programming to hMSCs may be advantageous and necessary. 5-azacytidine is a cytosine analogue and has been reported to cause phenotype changes in a number of cell types by activating novel gene expressions both in vitro and in vivo.\textsuperscript{15,16} However, the ability of 5-azacytidine to induce MSCs in vitro to differentiate along the cardiomyogenic lineage is still being debated regarding different species and cell preparation.\textsuperscript{17,18}

On the other hand, it has been estimated that more than 99% of implanted cells died within one week after transplantation.\textsuperscript{19,20} Poor viability of implanted cells may explain the reasons in part for marginal improvement of the cardiac function. Thus, cell therapy to replace lost myocardium is now limited by the inability to deliver large numbers of cells that resist graft cell death. Strategies to either prevent cell death or enhance cell proliferation are clearly necessary.

In this study, we demonstrated that hMSCs could be isolated from bone marrow aspiration of sternum in patients undergoing cardiac surgery based on immuno-depletion, cell density and selective adhesion. Expanded hMSCs showed senescent tendency with decreased ability to proliferation, and differentiation in cardiomyogenic lineage after induction by 5-azacytidine. Meanwhile, in our previous report,\textsuperscript{21} sternal hMSCs immortalized successfully by HPV E6/E7 gene transduction regained the ability for cardiomyogenic differentiation. Our findings suggest that these specifically immortalized hMSCs may serve as a useful cell source for elucidation of cardiomyogenic differentiation.

**METHODS**

**Isolation and culture of sternal hMSCs**

The study was approved by the Institutional Review Board of the Taipei Veterans General Hospital. Twenty patients (M/F: 12/8) from 45 to 78 years old (mean age 60.5 ± 12.3 years) were enrolled in this study with informed consent. They were victims of coronary artery disease or valvular heart disease. Left ventricular ejection fraction was 53.5 ± 6.7% by radionuclide ventriculography. Angiotension-converting enzyme inhibitors (13/20), calcium channel blocker (12/20), \(\beta\)-blockers (8/20) and nitroglycerin (15/20) were the drugs they took pre-operatively.

Twenty ml of heparinized human marrow blood from each sternum during cardiac surgery was collected. Mononuclear cells, first immuno-depletive of CD3, CD14, CD19, CD38, CD66b, and glycophorin-A positive cells using a commercially available kit (Rosette-Sep\textsuperscript{®}, StemCell Technologies), were obtained after Ficoll-Paque (Amersham-Pharmacia) density gradient centrifugation (1.077 g/cm\(^3\)). Cells were plated in non-coated tissue culture flasks with complete medium and allowed to adhere overnight. Non-adherent cells were washed out with medium changes the next day, and medium changes were carried out twice weekly thereafter.

Once adherent cells (hMSCs) reached approximately 60% confluent, they were detached with 0.25% trypsin-EDTA (Gibco), washed twice with PBS and re-plated at 1:3 under the same culture conditions. Complete medium consisted of Dulbecco’s Modified Eagle Medium (DMDM, Gibco) and 10% fetal bovine serum (FBS, Gibco) supplemented with antibiotics (100 U/mL penicillin G and 100 \(\mu\)g/mL streptomycin, Gibco)

**Quantification of the growth of sternal hMSCs**

To determine the initial density of hMSCs in primary culture, non-adherent cells were discarded by washing three times with PBS at 24 hours after seeding. Cells were detached with 0.25% trypsin and counted
with a hemocytometer under a phase-contrast microscope. 1.0 ± 0.03 × 10^3 cells/well (24-well plate) was taken as the initial density of hMSCs in primary culture and the seeding density for following subcultures. This cell counting procedure was repeated every 48 hours in triplicate (3 wells/24-well plate) until the cells almost ceased proliferation. The growth curves and properties in the primary culture (P0) and successive subculture (P1, P3 and P5) were determined.

**Antibodies**

The monoclonal antibodies (mAbs) to human CD29, CD34, CD51, and CD105 were from Ancell Corporation; CD44, and CD45 were from Caltag Laboratory; AC133 was from Miltenyi Biotec; and CD7 was from Becton Dickinson; platelet-derived growth factor α (PDGFRα) was from R&D Systems; CD90 was from Biosource; SH3 and SH4 were from each hybridoma cell line (American type culture collection).

**Flow cytometry**

The expanded hMSCs were characterized at passage 1 by flow cytometric analysis of specific surface antigens. The cells were harvested from the culture dishes by treatment of 5 mM EDTA in PBS. The cells were stained for 30 minutes on ice with fluorescein isothiocyanate-(FITC)- or phycoerythrin-(PE)-conjugated anti-marker mAbs (in optimal concentrations) in 50 μl washing buffer (PBS/1% FBS/0.1% NaN₃) at 0-4 °C for 30 min. Tested markers included hematopoietic lineage markers (CD7, CD34, CD45, CD 90 and AC133), matrix receptors (CD44 and CD105), integrins (CD29 and CD51), factor receptor (PDGFRα), and MSC markers (SH3, SH4). The cell mixture was then washed twice with washing buffer and fixed in 1% paraformaldehyde (in PBS). Cells were analyzed using a fluorescence-activated cell sorter (FACS Vantage SE; Becton Dickinson) using a 525 nm bandpass filter for green FITC fluorescence and a 575 nm bandpass filter for red PE fluorescence.

**Retroviral vector transduction**

The PA317 cell line producing the HPV16 E6/E7 retroviral vector (LXS1N6E6E7)[22] was originally provided by Dr. D.A. Galloway (University of Washington, Seattle, WA) and expanded in HAT (hypoxanthine, aminopterin, and thymidine)-containing growth medium to prepare stock in a large number of aliquots for storage in a liquid nitrogen freezer. To harvest the vector virus, 100-mm dishes of near-confluent monolayer cell cultures grown from a stock aliquot were each replenished with 5 ml of fresh HAT medium additionally containing 0.5 μM hydrocortisone and 0.1 mM/ml of insulin, which was collected at 24-hr intervals. The 24-hr medium was pooled, filtered through 0.45 μm membranes and stored in aliquots at -80 °C. After thawing, harvested medium usually gave a titer of 1-3 × 10⁶ neo° U/ml in NIH3T3 cells but contained no detectable replication-competent retroviruses using SC-1 cells, M. dunni cells or S+L-cells for the assay. For retroviral vector transduction, 10⁵ hMSCs were trypsinized and suspended in 1 ml of the filtered 24-hr medium adjusted to contain 8 μg/ml of polybrene and seeded directly onto a well of 6-well plate. After overnight incubation in a 37 °C, 5% CO₂ incubator, 3-ml of DMEM-LG was added. Cells were trypsinized and split 2 days later. Transduced cells were fed on alternate days by changing fresh growth medium and maintained by continuous passages, never allowing cell growth beyond confluence, usually at 1:5 every week.

**In-vitro cardiomyogenic induction of sternal hMSCs by 5-azacytidine**

To evaluate whether 5-azacytidine could induce the differentiation of sternal hMSCs into cardiomyocytes, sternal hMSCs in primary culture, subsequent passages (P1, P3 and P5) and E6/E7 transduced cells were exposed to 5 μM of 5-azacytidine (Sigma) in complete medium with 2% FBS for 48 hours on day 3 of culture. Then the cultured cells were washed with PBS twice and cultured in the complete medium thereafter. The dynamic changes in morphology and the growth properties were compared with the untreated cultures at indicated time points.

**RT-PCR**

Total RNA was extracted from hMSCs at different passages, E6/E7-transduced cells at passages 15 and 26 and tissues from human ventricles by Trizol Reagent (Life Technologies, Bethesda, MD). cDNA was synthesized from total RNA (1 μg) using M-MuLV reverse transcriptase, and PCR was performed using cDNA as
the template in a 30-μl reaction mixture containing a specific primer pair of each cDNA according to the published sequences (Table 1). The reaction mixture was incubated initially at 95 °C for 1 min, followed by 25-35 cycles of denaturation at 95 °C for 30 s, annealing at 53-60 °C for 1.5 min and 72 °C for 1 min with additional 7-min incubation at 72 °C after completing the last cycle (number of PCRs optimized in each case to ensure that the intensity of each product fell within the linear phase of amplification). DNA product was separated by electrophoresis in 1% agarose gel, stained and photographed under UV light illumination. β-actin performed as the internal control.

**Immunocytochemistry**

hMSCs and E6/E7-transduced cells at different passages were grown on glass coverslips and permeabilized with ice acetone for 10 minutes. Immunocytochemical staining was performed with antibodies for cardiac-specific proteins troponin-I (cTn-I) (Serotec), α sarcomeric actinin (αSA) (Sigma) and connexin-43 (Sigma). Fluorescein isothiocyanate-(FITC)-conjugated secondary antibody (Chemicon) and propidium iodide (PI) for nuclear counterstain were used.

**RESULTS**

**Morphology and growth properties of sternal hMSCs in culture**

After discarding the non-adherent cells by the first medium change and by washing with PBS three times at 24 h of primary culture, hMSCs were seen to attach to culture dishes sparsely, and the majority of the cells displayed a spindle-like shape. These cells began to proliferate at about day 4 (Figure 1A), and gradually grew to form small colonies (Figure 1B). By day 7, the number of cellular colonies with different size had obviously increased (about 6-10 colonies/well, 24-well plate). In large colonies, cells were more densely distributed and showed a spindle or triangle shape (Figure 1B). As growth of cells continued, colonies gradually expanded in size, with the adjacent ones interconnected with each other. Cells in the center of the colonies had formed several overcrowded layers (Figure 1C) and nearly ceased proliferation at this time.

Subcultured sternal hMSCs behaved similarly to those in primary cultures initially. However, the cells were larger in size and more heterogeneous in morphology and growth properties (Figures 1D to 1F). hMSCs in subcultures could be divided into two types: small spindle- or triangle-like, and broad flattened cells (Figure 1E). The flattened cells seldom proliferated and were gradually surrounded by the small spindle- or triangle-like cells that replicated faster and were more inclined to form colonies. It seems that the spindle- and triangle-like MSCs gradually transformed into broad flattened cells with passages. At the end of the first passage, about 16.46 ± 1.39% of cells were flattened, but at the end of passage 5 (Figure 1F), as many as 73.66 ± 2.55% of cells were broad flattened. This was accompanied by a progressive decrease in hMSCs proliferation and in the tendency of the cells to form colonies.

The growth curves of hMSCs in primary culture and in the three different passages are shown in Figure 2. For primary cultures, the cells remained quiescent during the first 4 days of culture, and then quickly replicated until day 10, when the average cell number reached 15 × 10⁴ cells/well. The number of cells did not significantly increase during the following 4 days of culture (days 11-14), although the cells had not yet reached confluence over the culture dishes. The patterns of the growth curves of hMSCs in passages 1 and 3 were similar to those of primary cells. However, the cells showed a

<table>
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<tr>
<th>Gene</th>
<th>Product size (bp)</th>
<th>Sequence (sense and antisense)</th>
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<tr>
<td>β-actin</td>
<td>515</td>
<td>5'-GCACCTCTCCAGCCTTCCCTCCT-3'</td>
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<tr>
<td></td>
<td></td>
<td>5'-TCACCTTTCCAGCCTTCCCTCCT-3'</td>
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<tr>
<td>E6/E7</td>
<td>628</td>
<td>5'-ATGCATAGTATATAGAGATGGAAT-3'</td>
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<td>5'-CTGCAGGATCATGCGAGTGA-3'</td>
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<td>C Troponin-I</td>
<td>227</td>
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<td>5'-TGCAATTGCCAAGCAAGAG-3'</td>
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shortened quiescent period (2 days) before proliferation and the cell number kept increasing significantly for a shorter period (days 4-8). The increase of average cell numbers was considerably slowed in passage 5. To determine the changes in precise growth properties of sternal hMSCs with passages, a comparison of the length of lag and log phases, number of cell doublings and doubling time in different passages are shown in Table 2.

Figure 1. Phase-contrast micrographs of sternal hMSCs in different passages. Day 4 (A), day 7 (B) and day 14 (C) of primary culture, and day 4 of passage 1 (D), passage 3 (E) and passage 5 (F). Colonies composed of spindle (arrows)-and triangle (arrowheads)-shaped sternal hMSCs in primary cultures (B). Cells in subculture could be divided into two types: small spindle- or triangle-like (arrows), and broad flattened (arrowheads) cells in E. Scale bars, 50 μm.

Figure 2. Growth curves of sternal hMSCs in different passages (P0, P1, P3 and P5). Results are presented as mean ± SD.
Characteristics of sternal hMSCs

Flow cytometric analyses indicated that sternal hMSCs were different in size. However, the surface marker expression of hMSCs was single peaked for all 12 antibodies we used (Figure 3). The result is summarized in Table 3.

E6/E7 expression in sternal hMSCs transduced by retroviral vector

At the 3rd passage of in vitro culture, hMSCs derived from the 61-year-old female donor were inoculated with replication-defective amphotropic retroviral vector LXSN16E6E7 at a multiplicity of infection (MOI) > 10 for mouse NIH3T3 cells. In subsequent culture in regular DMEM-LG without G418 selection, the vector-inoculated sternal hMSCs showed predominately in small spindle and triangle shapes (Figures 4A to 4C), with an obvious increase in proliferating activity and mitotic figures. After three weeks, the actively proliferating cells became the predominant cell population, could be easily detached from the culture dish by mild trypsinization for subcultivation, and were designated as E6/E7-hMSCs. The E6/E7-hMSCs were propagated by weekly 1:5 split passages, while the parental hMSCs by 1:3 split passages, with two changes a week of the DMEM medium, thus keeping both cell cultures in active proliferation but preventing growth beyond confluence or piling up of the cells. The E6/E7-hMSC cultures continued to proliferate after passage 36 (Figure 4C), indicating that E6/E7 transduction had conferred the property of an increased life span or immortalization to sternal hMSCs. RT-PCR analysis showed that specific HPV16 E6/E7 RNA transcripts were present in both the early and high passage E6/E7-hMSCs but not in the

| Table 2. Growth properties of sternal hMSCs in primary and passaged cultures |
|---------------------------------|---|---|---|---|
| Passages of hMSCs               | P0  | P1  | P3  | P5  |
| Lag phase (days)                | 4   | 2   | 2   | 2   |
| Log phase (days)                | 6   | 4   | 4   | 2   |
| Numbers of cell Doubling        | 7.29 ± 0.60 | 6.37 ± 0.38 | 6.22 ± 0.48 | 4.46 ± 0.38 |
| Doubling time of log phase (days)| 0.69 ± 0.026 | 0.90 ± 0.035 | 0.87 ± 0.036 | 0.82 ± 0.077 |

Figure 3. Characterization of surface molecules on hMSCs. Cytofluorimetric profiles of passage 1 hMSCs reacted first with (solid line) or without (broken line) mouse MAb specific for each marker and washed and second with fluorescein-labeled antimouse Ig antibody.
parental hMSCs (Figure 4D). Furthermore, E6/E7-hMSCs showed no signs of neoplastic transformation as examined by soft-agar anchorage-independent growth and NOD-SCID mouse tumorigenicity assays, which were demonstrated in a previous report.\(^{21}\)

In vitro cardiomyogenic differentiation after the induction of 5-azacytidine

Sternal hMSCs in primary culture (P0), subsequent passages (P1, P3 and P5) and E6/E7-hMSCs exposed to 5 µM of 5-azacytidine were observed under the same culture condition for control cells. Treatment of 5-azacytidine did not affect the growth properties of hMSCs in different settings. Throughout the 14-day period of observation, there were no spontaneously beating cells, myotubes, and typical ball-or stick-like cells, which had been suggested to be the myotube-forming cells. Nevertheless, P0, P1 and E6/E7-hMSCs demonstrated differentiation into cardiomyogenic lineage by activating gene of cardiac-specific protein, cTn-I (Figures 5A and 5B), but αSA and connexin-43 could not be detected. However, at P3 and P5, cTn-I, αSA and connexin-43 could not be detected by immunocytochemistry in 5-azacytidine-treated hMSCs (data not shown). These findings suggest that late-subcultured hMSCs may have lost their ability for induction to cardiomyogenic lineage and immortalization seem reverse this ability. In addition, the aborted cardiomyogenic differentiation of hMSCs in vitro falls short from progressing to a recognizable morphological and functionally differentiated phenotype.

DISCUSSION

We have shown here that hMSCs can be isolated...
from bone marrow aspiration of sternum in patients undergoing cardiac surgery based on immuno-depletion, cell density and selective adhesion. Expanded hMSCs expressed characteristic surface markers and progressively lost their ability to proliferate with senescent morphology and to differentiate in cardiomyogenic lineage after the induction of 5-azacytidine. They were amenable to be transfected by HPV E6/E7 gene to become immortalized and resume cardiomyogenic differentiation.

In previous investigations, hMSCs were isolated mostly from the iliac bones. Here, we tried to explore the feasibility of sternum in patients receiving cardiac surgery as another source for hMSCs. Sternum, iliac bones and femur are recommended sites for histologic evaluation of bone marrow cellularity in the hematology examinations. Studies have shown that the distributions of marrow cells in each of the phases of the cell cycle and their proliferative characteristics are compatible in iliac bones and sternum. They also demonstrated the successful growth of hematopoietic precursor cells from human sternal marrow. In the consideration of isolating hMSCs in patients during cardiac surgery, the approach from sternum has advantages of avoiding another anesthesia and discomfort. Furthermore, the isolation procedure from the same incision of cardiac surgery avoids possible contamination from another draping.

A wide array of cytokines and isolation techniques has been used to isolate and expand MSCs. However, the main obstacles to our understanding of the full potential of MSCs are what specifically defines MSCs and how they should be isolated and grown in vitro. Here, we first used immuno-depletion method to deplete the bone marrow of specific cell populations. After density gradient centrifugation, hMSCs preferentially adhered to the plastic plates. Using intricate antibody cocktails and relatively simple characteristic, adherence, we could isolate and enrich hMSCs from patients with different cardiac diseases and various ages. In addition, although no specific constellation of surface markers has been agreed on for these cells, hMSCs isolated and expanded by this strategy shared some similar patterns of surface markers reported in recent studies (Table 3). Here, hMSCs uniformly lacked antigens that typically identify hematopoietic cells. They were positive for CD90 (Thy-1), CD44 (hyaluronate), CD29 (β1-integrins) and other MSCs markers (SH2, SH3 and SH4).

The growth kinetics and the expandability of MSCs in vitro varied significantly among species, sources and conditions for isolation and plating of the cells. Here, we showed that sternal hMSCs demonstrated a slowing of cell proliferation as a function of increasing passages, in agreement with results of isolating cells from iliac bone. They report a faster proliferation rate compared with ours, but this may reflect different culture conditions, whereas the trend toward decreasing replication potential with increasing cumulative doublings is in con-

![Figure 5](image-url). In vitro cardiomyogenic differentiation of sternal hMSCs after the induction of 5-azacytidine. RT-PCR of cardiac specific troponin-I (cTn-I) in different passages and E6/E7 hMSCs. hMSCs(1) and hMSCs(2) in B were from two different patients. Cell line of fibroblasts (CCD-966SK) as a negative control. Human ventricles as a positive control. ß-actin as a loading internal control.
cordance with our data. In addition, the gradually loss of the ability to proliferate was accompanied by a gradual morphological conversion of the small spindle- or triangle-like MSCs into the broad flattened cells. The former grew rapidly, whereas the later grew slowly and increased in numbers as the cells were passaged. Previous studies have shown that this morphological change is one of the signs regarding MSCs’ commitment and senescence in the culture. Together with evidence from several other laboratories, this indicates that the in vitro expandability of hMSCs as a whole population is quite limited.

Another important question is whether cultured hMSCs are ready to be induced to differentiate into cardiomyocytes. Investigators have demonstrated the ability of 5-azacytidine to induce rodent MSCs in vitro to differentiate along the lineages of skeletal or cardiac muscles with expression of myogenic genes and specific contractile proteins. In our study, we could not identify any morphologic changes of hMSCs nor the contractile proteins immunologically, which suggested the myogenic differentiation after the induction of 5-azacytidine during the 2-week period of observation. However, primary culture (P0) and early passage (P1) of hMSCs did demonstrate differentiation through myogenic lineage by activating gene of cardiac specific protein, cTn-I. The reason for this aborted cardiomyogenic differentiation is unknown. However, it may point to malfunction of mechanism of some protein translation. Furthermore, the ability of cardiomyogenic differentiation from different patients may be variable, as Figure 5B shows. The reason may be due to individual difference, including age, healthy condition, diseases and drugs. It may need more patients to be enrolled, so we did not explore the reason for the difference in this study.

The specificity and reliability of the detection techniques in our study were confirmed using tissues from human ventricular myocardium as positive control. It is also known that variations in the isolation techniques and culture media used to grow MSCs may have a significant impact regarding the differentiation potential of these cells. Further studies about specific cardiomyogenic induction conditions need to be conducted. In addition, major species difference observed in the biomedical studies may also be influential on the present contradictory results. However, the lack of full understanding of stem cell regulation by the surrounding microenvironment may be the most important reason for this aborted differentiation. In the studies of cell transplantation for myocardial repair, we and others have shown that in-vivo cardiomyogenic differentiation of rodent and human MSCs and injury, especially ischemia, seems to be an important trigger for their cardiomyogenic differentiation. After excluding the possibility of cell fusion, these contradictory in-vitro and in-vivo results may imply the impact of microenvironment on stem cells’ growth and differentiation.

Fukuhara et al. evaluated the cardiomyogenic differentiation of murine MSCs by co-culturing with neonatal cardiomyocytes to simulate the cardiac environment in vitro. They demonstrated direct cell-cell interaction with cardiomyocytes was important for MSCs to differentiate into cardiomyocytes. In another study, Badorff et al. showed the transdifferentiation of human endothelial progenitor cells to functionally active cardiomyocytes when cocultivated with rat neonatal cardiomyocytes. These results further supported the speculation that aborted cardiomyogenic differentiation in our study was due to inadequate induction stimulus in the culture conditions.

A unique feature of tissue stem cells is that they occupy niches, a specific microenvironment that instructs and supports stem cell self-renewal, proliferation, and differentiation by providing specific cellular neighbors, signaling molecules, and extracellular matrix components. Further studies to identify the molecular basis underlying the interaction between stem cells and the niches will contribute to an understanding of the molecules involved in one of the most important issues in regenerative medicine, that is, regulation of stem cell activity.

In this study, we also demonstrated the loss of differential ability as the cells are replicated and passaged in the culture. As recently demonstrated by Satomura and Kuznetsov, adult stem cells including MSCs are not equally multipotent among the series passage. Some retain their multipotency through a number of passages, whereas others either become committed to a specific differentiation pathway or begin to exhibit senescence. This orderly loss of differentiation potential, as shown in our and other studies, strongly points to a progressive
commitment of the population as a whole and to the inability, in in-vitro conditions, of the adult stem cells in the expanding population to self-renew and maintain the capability of multipotent differentiation. This finding may have important implications for use of expanded cultures of hMSCs for cell and gene therapy. Further exploration of the molecular mechanism related to the senescence is necessary to fully understanding of hMSCs.

However in our study, in order to deal with cell senescence and loss of differentiation potential, we evaluated the possibility to reverse these undesired effects by immortalizing hMSCs transduced with HPV16 E6/E7. Immortalized hMSCs regain the ability of differentiation through cardiomyogenic lineage after the induction of 5-azacytidine, which was shown in the primary and early passaged hMSCs, with the expression of cardiac-specific contractile protein, cTnI. At the same time, this strategy may also have the chance to alleviate the undesired result of the poor viability and proliferation of implanted cell in the ways to use hMSCs as source for cell therapy, although the risk of tumorigenicity and other undesired effects of genetically modified cells need further evaluation.

We have isolated and immortalized hMSCs from sternal bone marrow aspiration during cardiac surgery. We speculate that future therapy for myocardial infarction with scar formation and deterioration of cardiac function may involve transplantation of genetically modified hMSCs to facilitate the repair of the damaged heart. These immortalized hMSCs may regain the ability of cardiomyogenic differentiation and serve as a useful tool for elucidation of cardiomyogenic differentiation.

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