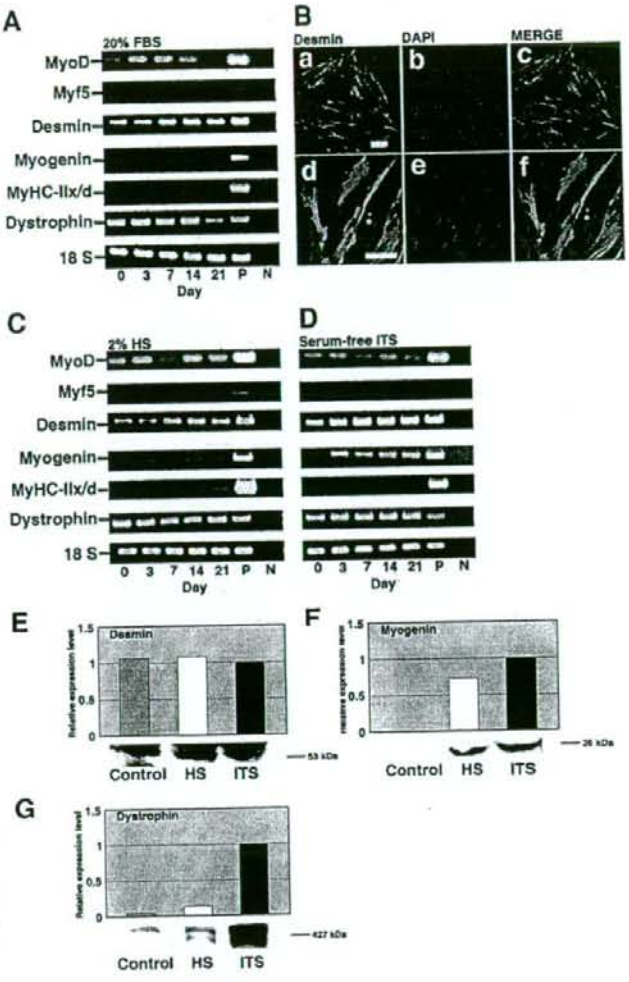

Figure 4. Expression of myogenic-specific genes in differentiated menstrual blood–derived cells. Menstrual blood–derived cells were cultured in DMEM supplemented with 20% FBS, 2% HS, or serum-free ITS medium. (A) RT-PCR analysis with PCR primers that allows amplification of the human MyoD, Myf5, desmin, myogenin, MyHC-IIx/d, and dystrophin cDNA (from top to bottom). RNAs were isolated from menstrual blood–derived cells in DMEM supplemented with 20% FBS at the indicated day after treatment with 5 μM 5-azacytidine for 24 h. RNAs from human muscle and H_2O served as positive (P) and negative (N) controls. Only the 18S PCR primer reacted with the human and murine cDNA. (B) Immunocytochemical analysis using an antibody to desmin (a–f) was performed on the menstrual blood–derived cells at 2 wk after exposure to 5 μM of 5-azacytidine for 24 h. The desmin-positive cells are shown at higher magnification (d–f). Merge of a and b is shown in c, and merge of d and e is shown in f. The images were obtained with a laser scanning confocal microscope. Scale bars, 200 μm (a–c) and 75 μm (d—f). (C and D) RT-PCR analysis of menstrual blood–derived cells on DMEM supplemented with 2% HS (C) or serum-free ITS medium (D) at the indicated day after exposure to 5 μM 5-azacytidine for 24 h. (E–G) Western blot analysis was performed on the cells cultured in myogenic medium indicated for 21 d. The blot was stained with desmin (E), myogenin (F), and dystrophin (G) antibodies followed by an HRP-conjugated secondary antibody.

EGFP-positive myotubes were detected. Multinucleated myotubes were revealed by the presence of specific human dystrophin (Figure 6, B and C) and myosin heavy chain (Figure 6D). Dystrophin was detected in cytoplasm in culture condition (Figure 6, B and C) despite evidence of cell surface localization in vivo. Human dystrophin and human nuclei were unequivocally identified by staining with antibodies to human dystrophin and human nuclei, whereas the numerous mouse nuclei present in this field, as shown by DAPI staining, are negative (Figure 6, B and C).

DISCUSSION

Skeletal muscle has a remarkable regenerative capacity in response to an extensive injury. Resident within adult skeletal muscle is a small population of myogenic precursor cells (or satellite cells) that are capable of multiple rounds of proliferation (estimated at 80–100 doublings), which are able to reestablish a quiescent pool of myogenic progenitor cells after each discrete regenerative episode (Mauro, 1961; Schultz and McCormick, 1994; Seale and Rudnicki, 2000; Hawke and Garry, 2001). Although muscle regeneration is a highly efficient and reproducible process, it ultimately is exhausted, as observed in senescent skeletal muscle or in patients with muscular dystrophy (Gussoni *et al.*, 1997; Cossu and Mavilio, 2000). In the present study, we investigated the myogenic potential of human endometrial tissue-derived immortalized EM-E6/E7/TERT-2 cells and primary cells derived from human menstrual blood. Human menstrual blood–derived cells proliferated over at least 25 PDs (9 passages) for more than 60 d and stopped dividing before 30 PDs. This cessation of cell division is probably due to replicative senescence or shortening of telomere length. Cell life span of menstrual blood cells is relatively short when compared with human fetal cells (Imai *et al.*, 1994; Terai *et al.*, 2005), and this shorter cell life span may be attributed to shorter telomere length of adult cells (i.e., endometrial stromal cells) at the start of cell cultivation, as is the case with hematopoietic stem cells (Suda *et al.*, 1984).

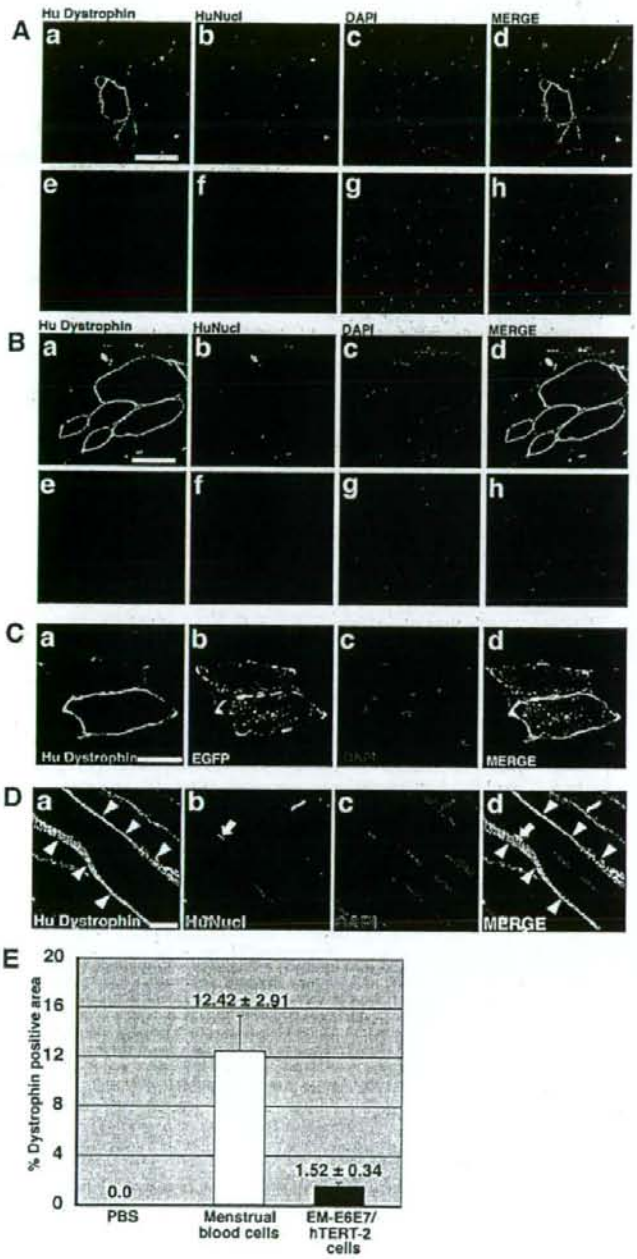

Figure 5. Conferral of dystrophin to mdx myocytes by human endometrial cells. (A and B) Immunohistochemistry analysis using an antibody against human dystrophin molecule (green), human nuclei (HuNucl, red), and DAPI staining (blue) on thigh muscle sections of mdx-scid mice after direct injection of EM-E6/E7/hTERT-2 cells (A) or menstrual blood–derived cells (B) without any treatment or induction. (C) EGFP-labeled EM-E6/E7/hTERT-2 cells without any treatment or induction were directly injected into the thigh muscle of mdx-scid mice. Immunohistochemistry revealed the incorporation of implanted cells into newly formed EGFP-positive myofibers, which expressed human dystrophin 3 wk after implantation. (A and B) As a methodological control, the primary antibody to dystrophin was omitted (e and f). (D) Immunohistochemistry analysis using an antibody against human dystrophin molecule (green, arrowheads), human nuclei (HuNucl, red, arrow), and DAPI staining (blue) on thigh muscle sections of mdx-scid mice after direct injection of human EM-E6/E7/hTERT-2 cells without any treatment or induction. (A and B) Merge of a–c is shown in d, and merge of e–g is shown in h. (C and D) Merge of a–c is shown in d. Scale bars, 50 μm (A and B), 20 μm (C and D). (E) Quantitative analysis of human dystrophin-positive myotubes. Menstrual blood–derived cells or EM-E6/E7/hTERT-2 cells without any treatment or induction were directly injected into thigh muscle of mdx-scid mice. The percentage of human dystrophin–positive-myofiber areas was calculated 3 wk after implantation of the EM-E6/E7/hTERT-2 cells or menstrual blood–derived cells. Injection of PBS without cells into mdx-scid myofibers was used as a control.

Menstrual blood–derived cells had a high replicative ability similar to progenitors or stem cells that display a long-term self-renewal capacity and had a much higher growth rate in our experimental conditions than marrow-derived stromal cells (Mori *et al.*, 2005). In addition, the myogenic potential of menstrual blood–derived cells, i.e., a high frequency of desmin-positive cells after induction, is much greater than expected. The higher myogenic differentiation ratio can be explained just by alteration of cell characteristics from epithelial and mesenchymal bipotential cells or heterogeneous populations of cells to cells with the mesenchymal phenotype in our cultivation condition, as determined by

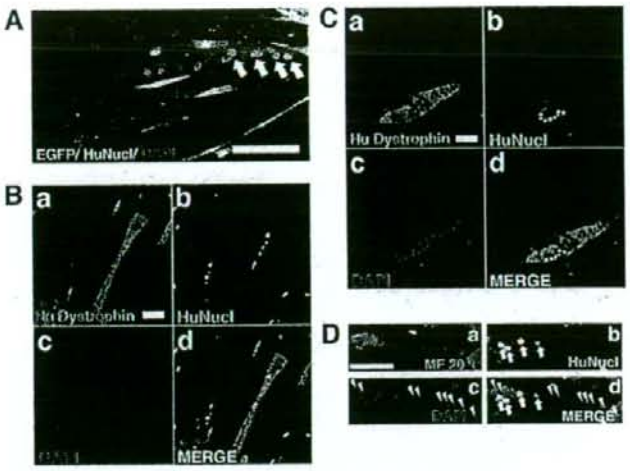

Figure 6. Detection of human endometrial cell contribution to myotubes in an in vitro myogenesis model. EGFP-labeled EM-E6/E7/hTERT-2 cells (A) or EM-E6/E7/hTERT-2 cells (B) or menstrual blood–derived cells (C and D) were cocultured with C2C12 myoblasts for 2 d under conditions that favored proliferation. The cultures were then changed to differentiation media for 7 d to induce myogenic fusion. (A) Myotubes were revealed by EGFP (green); human nuclei were detected by antibody specific to human nuclei (HuNucl, red, arrows). (B–D) Myotubes were revealed by specific human dystrophin mAb NCL-DYS3 (B and C, red) or anti-myosin heavy chain mAb MF-20 (D, red). (D) Human nuclei were detected by antibody specific to human nuclei (HuNucl, green, arrows). Total cell nuclei in the culture were stained with DAPI (blue, arrowheads). (B–D) Merge of a–c are shown in d. The cultures were then changed to differentiation media for 7 d to induce myogenic fusion. Scale bars, 100 μm (A–D).

cell surface markers (Figure 1, C–E). MyoD-positive cells are present in many fetal chick organs such as brain, lung, intestine, kidney, spleen, heart, and liver (Gerhart *et al.*, 2001), and these cells can differentiate into skeletal muscle in culture. Constitutive expression of MyoD, desmin, and myogenin, all markers for skeletal myogenic differentiation in both immortalized EM-E6/E7/hTERT-2 cells and menstrual blood–derived cells, implies either that most of these cells are myogenic progenitors or that these cells have myogenic potential. Expression of MyoD, one of the basic helix-loop-helix transcription factors that directly regulate myocyte cell specification and differentiation (Edmondson and Olson, 1993), occurs at the early stage of myogenic differentiation, whereas myogenin is expressed later, related to cell fusion and differentiation (Aurade *et al.*, 1994).

Acquisition or recovery of dystrophin expression in dystrophic muscle is attributed to two different mechanisms: 1) myogenic differentiation of implanted or transplanted cells and 2) cell fusion of implanted or transplanted cells with host muscle cells. Recovery of dystrophin-positive cells is explained by muscular differentiation of implanted marrow stromal cells and adipocytes (Dezawa *et al.*, 2005; Rodriguez *et al.*, 2005). In contrast, implantation of normal myoblasts into dystrophin-deficient muscle can create a reservoir of normal myoblasts that are capable of fusing with dystrophic muscle fibers and restoring dystrophin (Mendell *et al.*, 1995; Terada *et al.*, 2002; Wang *et al.*, 2003; Dezawa *et al.*, 2005; Rodriguez *et al.*, 2005). In this study using menstrual blood–derived cells, our findings—that the implantation of immortalized EM-E6/E7/hTERT-2 cells and menstrual blood–derived cells improved the efficiency of muscle regeneration and dystrophin delivery to dystrophic muscle in mice—is explained by both possibilities or the latter possibility alone, because cells expressing human dystrophin had both murine and human nuclei, located in the center and periphery of dystrophic muscular fiber, respectively (Figures 5D, in vivo, and 6, A–D, in vitro).

DMD is a devastating X-linked muscle disease characterized by progressive muscle weakness attributable to a lack of dystrophin expression at the sarcolemma of muscle fibers (Mendell *et al.*, 1995; Rodriguez *et al.*, 2005), and there are no effective therapeutic approaches for muscular dystrophy at present. Human menstrual blood–derived cells are obtained by a simple, safe, and painless procedure and can be expanded efficiently in vitro. In contrast, isolation of mesenchymal stem cells/mesenchymal cells from other sources, such as bone marrow and adipose tissue, is accompanied by a painful and complicated operation. Efficient fusion systems of our immortalized human EM-E6/E7/hTERT-2 cells and menstrual blood–derived cells with host dystrophic myocytes may contribute substantially to a major advance toward eventual cell-based therapies for muscle injury or chronic muscular disease. Finally, we would like to reemphasize that human menstrual blood–derived cells possess high self-renewal capacity, whereas biopsied myoblasts capable of differentiating into muscular cells are poorly expandable in vitro and rapidly undergo senescence (Cossu and Mavilio, 2000).

ACKNOWLEDGMENTS

We express our sincere thanks to J. Hata for support throughout this work, to H. Abe for providing expert technical assistance, to K. Saito for secretarial work, and to A. Crump for reviewing the manuscript. This study was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan; the Ministry of Health, Labor, and Welfare Sciences Research Grants; by a research grant on Health Science focusing on Drug Innovation from the Japan Health Science Foundation; by the program for promotion of Fundamental Studies in Health Science of the Pharmaceuticals and Medical Devices Agency; by a research grant for Cardiovascular Disease from the ministry of Health, Labor, and Welfare; and by a grant for Child Health and Development from the Ministry of Health, Labor, and Welfare.

REFERENCES

Aurade, F., Pinset, C., Chafey, P., Gros, F., and Montarras, D. (1994). Myf5, MyoD, myogenin and MRF4 myogenic derivatives of the embryonic mesenchymal cell line C3H10T1/2 exhibit the same adult muscle phenotype. Differentiation *55*, 185–192.

Bischoff, R. (1994). The satellite cell and muscle regeneration. In: Myology: Basic and Clinical, ed. A. Engel, C. Franzini-Armstrong, and D. A. Fischman, New York: McGraw-Hill, Health Professions Division, 97–118.

Cossu, G., and Mavilio, F. (2000). Myogenic stem cells for the therapy of primary myopathies: wishful thinking or therapeutic perspective? J. Clin. Invest. *105*, 1669–1674.

De Bari, C., Dell'Accio, F., Vandenabeele, F., Vermeesch, J. R., Raymackers, J. M., and Luyten, F. P. (2003). Skeletal muscle repair by adult human mesenchymal stem cells from synovial membrane. J. Cell Biol. *160*, 909–918.

Dezawa, M., Ishikawa, H., Itokazu, Y., Yoshihara, T., Hoshino, M., Takeda, S., Ide, C., and Nabeshima, Y. (2005). Bone marrow stromal cells generate muscle cells and repair muscle degeneration. Science *309*, 314–317.

Edmondson, D. G., and Olson, E. N. (1993). Helix-loop-helix proteins as regulators of muscle-specific transcription. J. Biol. Chem. *268*, 755–758.

Ervasti, J. M., and Campbell, K. P. (1991). Membrane organization of the dystrophin-glycoprotein complex. Cell *66*, 1121–1131.

Gerhart, J., Bast, B., Neely, C., Iem, S., Amegbe, P., Niewenhuis, R., Miklasz, S., Cheng, P. F., and George-Weinstein, M. (2001). MyoD-positive myoblasts are present in mature fetal organs lacking skeletal muscle. J. Cell Biol. *155*, 381–392.

Grounds, M. D., White, J. D., Rosenthal, N., and Bogoyevitch, M. A. (2002). The role of stem cells in skeletal and cardiac muscle repair. J. Histochem. Cytochem. *50*, 589–610.

Gussoni, E., Blau, H. M., and Kunkel, L. M. (1997). The fate of individual myoblasts after transplantation into muscles of DMD patients. Nat. Med. *3*, 970–977.

Hasty, P., Bradley, A., Morris, J. H., Edmondson, D. G., Venuti, J. M., Olson, E. N., and Klein, W. H. (1993). Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. Nature *364*, 501–506.

Hawke, T. J., and Garry, D. J. (2001). Myogenic satellite cells: physiology to molecular biology. J. Appl. Physiol. *91*, 534–551.

Hoffman, E. P., Brown, R. H., Jr., and Kunkel, L. M. (1987). Dystrophin: the protein product of the Duchenne muscular dystrophy locus. Cell *51*, 919–928.

Imai, S., Fujino, T., Nishibayashi, S., Manabe, T., and Takano, T. (1994). Immortalization-susceptible elements and their binding factors mediate rejuvenation of regulation of the type I collagenase gene in simian virus 40 large T antigen-transformed immortal human fibroblasts. Mol. Cell. Biol. *14*, 7182–7194.

Kyo, S., Nakamura, M., Kiyono, T., Maida, Y., Kanaya, T., Tanaka, M., Yatabe, N., and Inoue, M. (2003). Successful immortalization of endometrial glandular cells with normal structural and functional characteristics. Am. J. Pathol. *163*, 2259–2269.

Mauro, A. (1961). Satellite cell of skeletal muscle fibers. J. Biophys. Biochem. Cytol. *9*, 493–495.

Mendell, J. R. *et al.* (1995). Myoblast transfer in the treatment of Duchenne's muscular dystrophy. N. Engl. J. Med. *333*, 832–838.

Miyoshi, H., Blomer, U., Takahashi, M., Gage, F. H., and Verma, I. M. (1998). Development of a self-inactivating lentivirus vector. J. Virol. *72*, 8150–8157.

Miyoshi, H., Takahashi, M., Gage, F. H., and Verma, I. M. (1997). Stable and efficient gene transfer into the retina using an HIV-based lentiviral vector. Proc. Natl. Acad. Sci. USA *94*, 10319–10323.

Mori, T. *et al.* (2005). Combination of hTERT and bmi-1, E6, or E7 induces prolongation of the life span of bone marrow stromal cells from an elderly donor without affecting their neurogenic potential. Mol. Cell. Biol. *25*, 5183–5195.

Nabeshima, Y., Hanaoka, K., Hayasaka, M., Esumi, E., Li, S., Nonaka, I., and Nabeshima, Y. (1993). Myogenin gene disruption results in perinatal lethality because of severe muscle defect. Nature *364*, 532–535.

Rodriguez, A. M. *et al.* (2005). Transplantation of a multipotent cell population from human adipose tissue induces dystrophin expression in the immunocompetent mdx mouse. J. Exp. Med. *201*, 1397–1405.

Rudnicki, M. A., Schnegelsberg, P. N., Stead, R. H., Braun, T., Arnold, H. H., and Jaenisch, R. (1993). MyoD or Myf-5 is required for the formation of skeletal muscle. Cell *75*, 1351–1359.

Sabourin, L. A., and Rudnicki, M. A. (2000). The molecular regulation of myogenesis. Clin. Genet. *57*, 16–25.

Schultz, E., and McCormick, K. M. (1994). Skeletal muscle satellite cells. Rev. Physiol. Biochem. Pharmacol. *123*, 213–257.

Seale, P., and Rudnicki, M. A. (2000). A new look at the origin, function, and "stem-cell" status of muscle satellite cells. Dev. Biol. *218*, 115–124.

Sicinski, P., Geng, Y., Ryder-Cook, A. S., Barnard, E. A., Darlison, M. G., and Barnard, P. J. (1989). The molecular basis of muscular dystrophy in the mdx mouse: a point mutation. Science *244*, 1578–1580.

Suda, J., Suda, T., and Ogawa, M. (1984). Analysis of differentiation of mouse hemopoietic stem cells in culture by sequential replating of paired progenitors. Blood *64*, 393–399.

Terada, N., Hamazaki, T., Oka, M., Hoki, M., Mastalerz, D. M., Nakano, Y., Meyer, E. M., Morel, L., Petersen, B. E., and Scott, E. W. (2002). Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. Nature *416*, 542–545.

Terai, M., Uyama, T., Sugiki, T., Li, X. K., Umezawa, A., and Kiyono, T. (2005). Immortalization of human fetal cells: the life span of umbilical cord blood-derived cells can be prolonged without manipulating p16INK4a/RB braking pathway. Mol. Biol. Cell *16*, 1491–1499.

Wang, X., Willenbring, H., Akkari, Y., Torimaru, Y., Foster, M., Al-Dhalimy, M., Lagasse, E., Finegold, M., Olson, S., and Grompe, M. (2003). Cell fusion is the principal source of bone-marrow-derived hepatocytes. Nature *422*, 897–901.

Review Article

Two MSCs: Marrow stromal cells and mesenchymal stem cells

Akihiro Umezawa and Masashi Toyoda

Department of Reproductive Biology, National Institute for Child Health and Development

Marrow stromal cells (MSC1) are able to generate a series of terminally-differentiated cells *in vitro*. Most experiments are performed with heterogeneous stromal cells obtained by adherence to plastic culture dishes. Since bone marrow-derived stromal cells are purified to a homogeneous population meeting the criteria for non-hematopoietic stem cells, these cells have been termed "mesenchymal stem cells" and have the capability of generating an array of cells. However, "mesenchymal stem cells" (MSC2) are also actual multi-purpose cells capable of differentiating into cells of mesoderm-origin regardless of cell sources. MSC2 can be recovered from a variety of other tissues, such as fat, muscle, menstrual blood, endometrium, placenta, umbilical cord, cord blood, skin, and eye. The terms "mesenchymal stem cell" and "marrow stromal cell" have been used interchangeably in emerging literature to describe cells that can be used in regenerative medicine, thereby introducing a degree of confusion. In this review, we re-organize the understanding of the two MSCs, describe their biology and differentiate between the two.

Rec./Acc.1/5/2007, pp28-36

Correspondence should be addressed to:
Akihiro Umezawa, Department of Reproductive Biology, National Institute for Child Health and Development, 2-10-1 Okura, Setagaya, Tokyo 157-8535, Japan. Phone: +81-3-5494-7047, e-mail: umezawa@1985.jukuin.keio.ac.jp

Key words transdifferentiation, celltherapy, epigenetics, senescence

Introduction

Two MSCs, i.e., marrow stromal cells (MSC1) and mesenchymal stem cells (MSC2), are attracting a great deal of attention, as they represent a valuable source of cells for use in regenerative medicine, as well as offering an excellent model of cell differentiaton in biology. However, confusion exists in the literature due to poor application or misuse of the terms and nomenclature.

In general, mesenchymal stem cells are multi-potential stem cells that can differentiate into a variety of cell types (ref. http://en.wikipedia.org/wiki/Mesenchymal_stem_cell). They have been shown to differentiate, *in vitro* or *in vivo*, into osteoblasts, chondrocytes, myocytes, adipocytes and neuronal cell among others. Mesenchymal stem cells have traditionally been obtained from bone marrow, and have commonly been referred to as "marrow stromal cells"(MSC1).

While the terms"marrow stromal cell"(or"stromal cell") and "mesenchymal stem cell" have frequently been used interchangeably, they are increasingly recognized as separate entities as:

1. Stromal cells (MSC1) are a highly-heterogenous cell population, usually derived from bone marrow, consisting of multiple cell types with different potentials for proliferation and differentiation.

2. Mesenchymal stem cells (MSC2) encompass cells derived from other non-marrow tissues, such as fat, muscle, menstrual blood, endometrium, placenta, umbilical cord, cord blood, skin, and eye.

Bone marrow-derived mesenchymal stem cells or bone marrow stromal cells (MSC1) were discovered by Friedenstein in 1976, who described clonal, plastic-adherent cells from bone marrow that were capable of differentiating into osteoblasts, adipocytes, and chondrocytes. More recently, investigators have demonstrated that mesenchymal stem cells (MSC2) *per se* can be recovered from a variety of adult tissues and have the capacity to differentiate into a variety of specialist cell types. This review describes the recent advances in understanding of the two MSC cells, their biology and ongoing investigation and use.

Somatic stem cells

Somatic stem cells have been identified in hematopoietic[1], hepatic[2], epidermal[3], gastrointestinal[4], neural[5,6], muscle[6], and bone marrow[6-8] tissues. Many researchers have since demonstrated the developmental pluripotency of these cells. Bone marrow-derived stem cells can be transdifferentiated into multilineage cells, such as muscle[9] of mesoderm, lung[10] and liver[10,11] of endoderm, and brain[12-15] and skin[16] of ectoderm. Somatic stem cells are more desirable than embryonic stem (ES) cells for cell therapeutics because of ethical considerations and the possible immunologic rejection of ES cells. Mesenchymal stem cells have become the most popular somatic stem cells in medicine and biology, not least because of their high reproductive capability *in vitro*.

Bone marrow stromal cells (MSC1)

The existence of non-hematopoietic cells in bone marrow was first suggested by Cohnheim about 130 years ago[16]. Bone marrow-derived stromal cells (MSC1) can differentiate into most somatic cells, including osteoblasts, chondrocytes, myoblasts, cardiomyocytes[17-21], and adipocytes, when placed in appropriate *in vitro*[20] and *in vivo* environments[22], and thus are a useful cell source for regenerative medicine[23]. Recent studies suggest that MSC1 can also differentiate into a neuronal lineage[24], and murine bone marrow-derived adult progenitor cells can differentiate into dopaminergic neuronal cells[25,26]. Since the use of MSC1 entails no ethical or immunological problems, and bone marrow aspiration is an established routine procedure, these cells provide a useful and almost routine source of material for transplantation and tissue repair or regeneration (Fig.1).

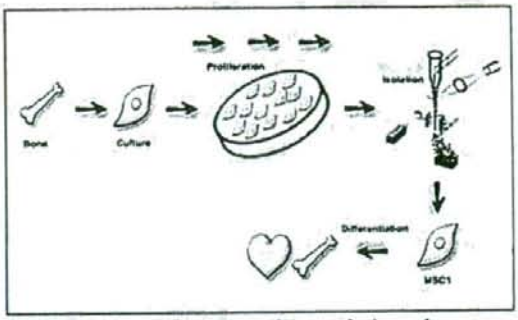

Fig.1 Development and differentiation of mesenchymal stem cells derived from bone marrow

1) Osteogenesis

KUSA-A1 cells, a murine marrow stromal cell line, are capable of generating mature bone *in vivo*[27]. They are a unique, mature osteoblast cell line and serve as a very suitable model for *in vivo* osteogenesis. Bone forms in subcutaneous tissue after subcutaneous injection of the cells into mice. The osteogenesis by KUSA-A1 is not mediated by chondrogenesis and thus is considered to be membranous ossification. Follow-up study on the fate of bone by immortalized osteoblasts shows that the ectopically-generated bone keeps its size and shape for 12 months[28]. Furthermore, the implanted cells do not metastasize like tumor cells. These unique characteristics of KUSA-A1 cells provide an opportunity to analyze the process of membranous ossification in detail.

2) Chondrogenesis

Chondrocytes differentiate from mesenchymal cells during embryonic development[29] and the phenotype of the differentiated chondrocyte is characterized by the synthesis, deposition, and maintenance of cartilage-specific extracellular matrix molecules, including type II collagen and aggrecan[29-31]. The phenotype of differentiated chondrocytes is rapidly lost since it is unstable in culture[32-35]. This process is referred to as "dedifferentiation" and is a major impediment to use of mass cell populations for therapy or tissue engineering of damaged cartilage. When isolated chondrocytes are cultured in a monolayer at low density, the typical round chondrocytes morphologically transform into flattened fibroblast-like cells, with profound changes in biochemical and genetic characteristics, including reduced synthesis of type II collagen and cartilage proteins[36]. When cultured

three-dimensionally in a scaffold such as agarose, collagen, and alginate, redifferentiated chondrocytes re-express the chondrocytic differentiation phenotype.

KUM5 mesenchymal cells, a MSC1 line, generate hyaline cartilage *in vivo* and exhibit endochondral ossification at a later stage after implantation[37]. OP9 cells, another MSC1 line, derived from macrophage colony-stimulating factor-deficient osteopetrotic mice, and also known to be niche-constituting cells for hematopoietic stem cells, express chondrocyte-specific or -associated genes, such as type II collagen β1, Sox9, and cartilage oligomeric matrix protein at an extremely high level, as do KUM5 cells. OP9 micromasses exposed to TGF-β3 and BMP2 form type II collagen-positive hyaline cartilage within two weeks *in vivo*. The unique characteristics of KUM5 and OP9 cells provide an opportunity to analyze the process of endochondral ossification.

3) Cardiomyogenesis

It has been generally accepted that cardiac myocytes are unable to divide once cell proliferation ceases shortly after birth in the mammalian heart, because mitotic figures have not been detected in myocytes[38]. Cardiomyocytes induce DNA synthesis *in vivo* and *in vitro*[39,40]. Adult hearts often exhibit a polypoid structure, which results from stochastic accumulation of mutations as cells pass through cell-cycle checkpoints[41]. Bone marrow-derived stromal cells (MSC1) are able to differentiate into cardiomyocytes *in vitro* and *in vivo*[19,20,42,43] and a hierarchical model has been proposed for this *in vitro* cardiomyogenic differentiation. MSC1 in culture include a mixture of at least three types of cells, i.e., cardiac myoblasts, cardiac progenitors and multi-potential stem cells, and a follow-up study of individual cells suggests that commitment of a single-cell-derived stem cell toward a cardiac lineage is stochastic[44]. Furthermore, MSC1 over-expressing well-known master transcription factors, i.e., Csx/Nkx2.5 and GATA4, unavoidably undergo cardiomyogenic fate and behave like transient amplifying cells. MSC1 also transdifferentiate into cardiomyocytes in response to humoral factors, such as demethylation of the genome, in addition to environmental factors (See the chapter "Epigenetic modifier as a differentiating inducer".

4) Neurogenesis

MSC1 can exhibit neural differentiation when exposed to demethylating agents[14]: the cells differentiating into three types of neural cells, i.e., neurons, astrocytes, and oligodendrocytes. With exposure to basic fibroblast growth factor, nerve growth factor, and brain-derived neurotrophic factor, the transdifferentiation of human stromal cells is limited to neurons[14]. The change in gene expression during differentiation is global and drastic[45]: the differentiated cells no longer exhibit the profile of stromal cells or the biphenotypic pattern of neuronal and stromal cells. Osteoblasts capable of intra-membranous ossification are likely to differentiate into neuronal lineages, but adipocytes do not[14]. Interestingly, the cranio-facial membranous bones develop from the neural crest, which is of ectodermal origin. Development naturally progresses from neural crest cells to terminally-differentiated osteoblasts[46]. The finding of *in vitro* differentiation from mesoderm- to ectoderm-derived cells is thus the opposite of the developmental process, i.e., from ectoderm- to mesoderm-derived cells. Converting differentiated osteoblasts or MSC1 to neuronal cells, a key future task for any cell-based therapy, would thus oppose the usual direction of cell differentiation. This can now be achieved by exposing stromal cells to neurotrophic factors, at least *in vitro*.

Dopaminergic neuron-associated genes, such as nurr1 and wnt-5a, are induced at an extremely high level in the neuronally-differentiated stromal cells. Wnt5a and nurr1 are involved in the differentiation of mid-brain precursors into dopaminergic neurons[25,26]. It is quite significant that dopaminergic neurons can be generated from MSC1, since they are one of the key targets for regenerative medicine.

Epigenetic modifier as a differentiating inducer

The demethylating agent, 5-azacytidine, is a cytosine analog that has a remarkable effect on transdifferentiation of cells and has been shown to induce differentiation of stromal cells into cardiomyocytes, skeletal myocytes, adipocytes, and chondrocytes[19,42,47]. The effect of this low-molecular substance is not surprising, since it is incorporated into DNA and has been shown to cause extensive demethylation. The demethylation is attributable to covalent binding of DNA methyltransferase to 5-azacytidine in the DNA[48], with subsequent reduction of enzyme activity in cells resulting in dilution-out and random loss of methylation at many sites in the genome. This may, in turn, account for the reactivation of cardiomyogenic "master" genes, such as MEF-2C, GATA4, dHAND, and Csx/Nkx2.5, leading to stochastic transdifferentiation of MSC1 into cardiomyocytes. Use of 5-azacytidine is beneficial, but since it may have drawbacks, i.e., gene activation leading to oncogenesis and undesired differentiation, care must be exercised before using it to induce cells to differentiate into target phenotypes. Immortalized cells, including marrow stromal cells, have specific patterns of DNA methylation. The established methylation pattern of cells is maintained

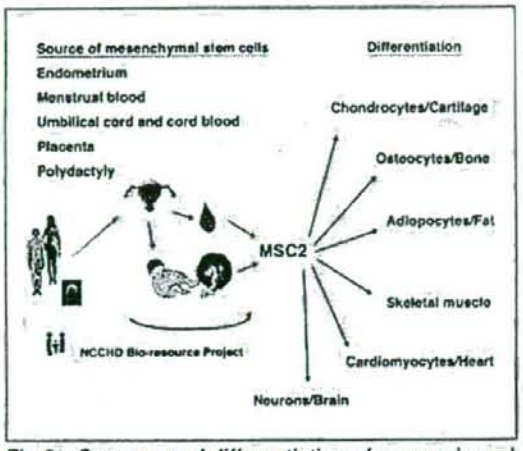

Fig.2 Sources and differentiation of mesenchymal stem cells

with considerable fidelity and silenced genes are stably inherited throughout the culture period[49-51]. The demethylating agent induces differentiation by altering the original methylated pattern and reactivating the silenced genes.

Mesenchymal stem cells (MSC2)

Tissues originating in the mesoderm include blood cells, blood vessels, heart, bone, cartilage, fat, skeletal muscle, tendon, and tissue mesenchyme. Blood cells in bone marrow are the elements that create the concept of stem cells, but bone marrow includes another cell group, i.e., mesenchymal stem cells (MSC2), which possess adherent properties. These cells have the ability to differentiate into a variety of cells and may have an organ maintenance mechanism that serves as back-up. Human mesenchymal stem cells (MSC2) are a useful source of cells for transplantation for several reasons: they have the ability to proliferate and differentiate into mesodermal tissues and they entail no ethical or immunological problems. MSC2 have been studied extensively over the past three decades and numerous independent research groups have successfully isolated them from a variety of sources, most commonly from bone marrow[19,22,52-55]. Yet, in addition to bone marrow, almost all human tissues or organs can be a source of mesenchymal stem cells, since they all have stroma or mesenchyme as well as parenchyma or epithelium.

Available mesenchymal cell lines and mesenchymal cells in culture

MSC2 have been extracted from fat, muscle, menstrual blood, endometrium, placenta, umbilical cord, cord blood, skin, and ey (Fig.2). Moreover, the source tissues can be obtained witho difficulty from resected tissues at surgery and from birth deliv eries (http://www.nch.go.jp/reproduction/cellbank2.htm an http://www.nch.go.jp/reproduction/cells/primary.html); men strual blood can be provided from volunteers. The placenta i composed of amniotic membrane, chorionic villi and decidu each of which can be a source of different types of MSC2. Larg numbers of MSC2 can be easily obtained because the placenta usually provided for research purposes. Menstrual blood als contains a large number of MSC2, although it is usually regarde as waste material.

We have also isolated many specific cell lines from adherin cells of mouse bone marrow (http://www.nch.go.jp/reproductio cellbank2.htm) as follows:

a. Multi-potential stem cell line: 9-15c cells (originally KUM cells) have multi-potential allowing differentiation into bon fat, skeletal muscle, and myocardial cells through conti ued passage;
b. Oligo-potential cell lines: KUM9 cells that lose the abili to differentiate to myocardial cells but retain differentiatio to bone, fat, and skeletal muscle and NRG cells that lose th capability to differentiate into myocardial cells and skelet myocytes but retain differentiation to bone and fat;
c. Bi-potential cells: KUSA-O cells are capable of differen ating into osteoblasts and adiopocytes;
d. Precursor cells: KUSA-A1 and H-1/A are osteoblasts an preadipocytes, respectively. Adipogenic 3T3-L1[56], oste genic MC3T3-E1[57], and chondrogenic ATDC5 cells[58] ha been isolated from stem cells of a mesenchymal nature.

Focusing on human MSC2 derived from umbilical cord blo (UCBMSC) as an example, isolation, characterization, and d ferentiation of clonally-expanded UCBMSCs have been r ported[59,60], and UCBMSCs have been found to have mul potential[61]. Most of the surface markers are the same as tho detected in their bone marrow counterparts[62], with both UC and bone marrow-derived cells being positive for CD29, CD4 CD55, and CD59, and negative for CD34 and CD117. Signi cantly, the differentiation capacity of UCB-derived cells is un fected during establishment of a plate-adhering population cells from UCB.

Life span of MSC1 and MSC2

Marrow stromal cells (MSC1) and mesenchymal stem ce (MSC2) are useful for cell transplantation. However, it is dif cult to study and apply them because of their limited life spa

One of the reasons for this is that normal human cells undergo a limited number of cell divisions in culture and then enter a non-dividing state called "senescence"[62,63]. Human cells reach senescence after a limited number of cell replications, and the average number of population doublings (PDs) of marrow-derived mesenchymal stem cells has been found to be about 40[42], implying that it would be difficult to obtain enough cells to restore the function of a failing human organ. Large numbers of cells must be injected into damaged tissues to restore function in humans, and cells sometimes need to be injected throughout entire organs.

A system that allows human cells to escape senescence by using cell-cycle-associated molecules may be used to obtain sources of material for cell therapy[64,65]. Both inactivation of the Rb/p16INK4a pathway and activation of telomerase are required for immortalization of human epithelial cells, such as mammary epithelial cells and skin keratinocytes. Human papillomavirus E7 can inactivate pRb, and Bmi-1 can repress p16INK4a expression. Inactivation of the p53 pathway is also beneficial, even if not essential, to extension of the life span[66]. Human marrow stromal cell strains with an extended life span can be generated by transduction of combination of TERT, and Bmi-1, E6 or E7[45]. Cells with extended life span grow *in vitro* for over 80 PDs, and their differentiation potential is maintained. Transfection of TERT alone is insufficient to prolong the life span of marrow stromal cells, despite TERT having been reported to extend the life span of cells beyond senescence without affecting their differentiation ability[67]. Human stromal cells transfected with TERT and Bmi-1, E6 or E7 do not transform according to the classical pattern: they do not generate tumors in immunosuppressed mice; they do not form foci *in vitro*; and they stop dividing after confluence. The possibility that gene-transduced stromal cells might become tumorigenic in patients several decades after cell therapy therefore cannot be ruled out. Nevertheless, these gene-modified stromal cells may be used to supply defective enzymes to patients with genetic metabolic diseases, such as neuro-Gaucher disease, Fabry disease, and mucopolysaccharidosis, which have a poor prognosis and are sometimes lethal. The "risk versus benefit" balance is essential when applying these gene-modified cells clinically, and the "risk" or "drawback" in this case is transformation of implanted cells. These marrow stromal cells (MSC1) with prolonged life span also provide a novel model for further study of cancer and stem cell biology.

Differentiation of mesenchymal stem cells

Retroviral labeling of individual cells is a useful clonal assay to monitor lineage commitment at the single cell level. At present, several models have been proposed in which hematopoietic lineage determination is driven intrinsically[68], extrinsically[69], or both[70]. The issue of the mechanism and the extent of cellular differentiation that occurs when stem cells begin to differentiate is the area of furthest advanced research. Two models have been proposed: a deterministic model, in which differentiation is governed by the microenvironment (including growth factors and cytokines), and a stochastic model, in which differentiation, self-replication and the direction of differentiation emerge somewhat randomly (Fig.3A,B). The different models arise from different conceptions of mesenchymal stem cells. The mesenchymal stem cell (MSC2) line is stochastically committed toward the cardiac lineage, and following this commitment, they proliferate as transient amplifying cells and differentiate into cardiac myocytes (Fig.3C).

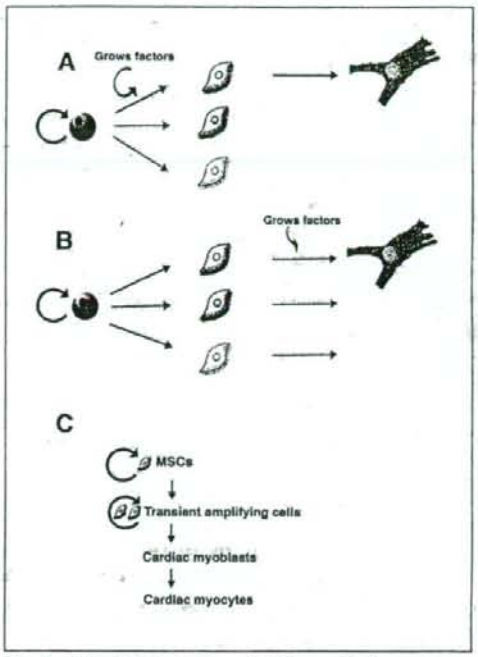

Fig.3 Model of stem cell differentiation
A. Deterministic model.
B. Stochastic model.
C. Differentiation model of mesenchymal stem cells.

Considering stem cell transplant as a therapy, when mature cells arising from hematopoietic stem cells are needed, as in marrow transplant, there are no problems attending cellular dif-

ferentiation. However, in the case of cells that serve to originate cells of several different organs, as in the case of mesenchymal stem cells, there is a possibility for differentiation to cells not needed in the treatment. Ectopic tissue may therefore emerge from implanted mesenchymal stem cells, especially where the buffering system from a given site is lost and the stem cells begin to differentiate randomly into cells differing from the implanted site, thereby creating unwanted ectopic tissue.

Conclusion

Mesenchymal stem cells can be isolated from bone marrow by standardized techniques and expanded in culture through many generations, while retaining their capacity to differentiate along set pathways when exposed to appropriate conditions. This property opens up therapeutic opportunities for the treatment of lesions in mesenchymal tissues, and protocols have been devised for the treatment of defects in articular cartilage[71], bone[72], tendon[73], and meniscus[74] and for bone marrow stromal recovery[75] and osteogenesis imperfecta[76].

In this context, we prefer to use the word "stroma" rather than "mesenchymal stem cells" for accuracy and to avoid confusion. In the field of hematopoiesis, marrow stroma were originally treated as "second class citizens"[77], and represented a niche field. Today, marrow stroma are a "major player" in regenerative medicine and stem cell biology and are no longer viewed as a peripheral field of research. In addition, there is also a rapidly growing body of research into the biology and potential use of true "mesenchymal stem cells" derived from other human tissues, which are showing significant promise for future therapy, reparation or regeneration of human tissues and organs.

Clearly, this field is in its relative infancy, our understanding is at present limited but the potential benefits are great. We should perhaps, therefore, remember that the unexpected and unrivalled potential of MSCs to differentiate into a wide variety of cells represents a gift not a privilege and, with respect to the two MSCs, we should recognise and welcome their role in medicine with the words "with great power comes great responsibility".

References

1) Bryder D, Rossi DJ, Weissman IL: Hematopoietic stem cells: the paradigmatic tissue-specific stem cell. Am J Pathol, 169: 338-346, 2006.
2) Alison M, Sarraf C: Hepatic stem cells. J Hepatol, 29: 676-682, 1998.
3) Watt FM: Epidermal stem cells: markers, patterning and the control of stem cell fate. Philos Trans R Soc Lond B Biol Sci, 353: 831-837, 1998.
4) Potten CS: Stem cells in gastrointestinal epithelium: numbers, characteristics and death. Philos Trans R Soc Lond B Biol Sci, 353: 821-830, 1998.
5) Gage FH: Mammalian neural stem cells. Science, 287: 1433-1438, 2000.
6) Jiang Y, Vaessen B, Lenvik T, Blackstad M, Reyes M, Verfaillie CM: Multipotent progenitor cells can be isolated from postnatal murine bone marrow, muscle, and brain. Exp Hematol, 30: 896-904, 2002.
7) Fridenshtein A: Stromal bone marrow cells and the hematopoietic microenvironment. Arkh Patol, 44: 3-11, 1982.
8) Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC, Largaespada DA, Verfaillie CM: Pluripotency of mesenchymal stem cells derived from adult marrow. Nature, 418: 41-49, 2002.
9) Ferrari G, Cusella-De Angelis G, Coletta M, Paolucci E, Stornaiuolo A, Cossu G, Mavilio F: Muscle regeneration by bone marrow-derived myogenic progenitors. Science, 279: 1528-1530, 1998.
10) Krause DS, Theise ND, Collector MI, Henegariu O, Hwang S, Gardner R, Neutzel S, Sharkis SJ: Multi-organ, multilineage engraftment by a single bone marrow-derived stem cell. Cell, 105: 369-377, 2001.
11) Petersen BE, Bowen WC, Patrene KD, Mars WM, Sullivan AK, Murase N, Boggs SS, Greenberger JS, Goff JP: Bone marrow as a potential source of hepatic oval cells. Science, 284: 1168-1170, 1999.
12) Sanchez-Ramos J, Song S, Cardozo-Pelaez F, Hazzi C, Stedeford T, Willing A, Freeman TB, Saporta S, Janssen W, Patel N, Cooper DR, Sanberg PR: Adult bone marrow stromal cells differentiate into neural cells in vitro. Exp Neurol, 164: 247-256, 2000.
13) Woodbury D, Schwarz EJ, Prockop DJ, Black IB: Adult rat and human bone marrow stromal cells differentiate into neurons. J Neurosci Res, 61: 364-370, 2000.
14) Kohyama J, Abe H, Shimazaki T, Koizumi A, Nakashima K, Gojo S, Taga T, Okano H, Hata J, Umezawa A: Brain from bone: efficient "meta-differentiation" of marrow stroma-derived mature osteoblasts to neurons with Noggin or a demethylating agent. Differentiation, 68: 235-244, 2001.
15) Kim BJ, Seo JH, Bubien JK, Oh YS: Differentiation of adult bone marrow stem cells into neuroprogenitor cells in vitro. Neuroreport, 13: 1185-1188, 2002.

16) Cohnheim J: Uber Entzfindung und Eiterung. Virchows Arch, 40: 1-79, 1867.
17) Allan EH, Ho PW, Umezawa A, Hata J, Makishima F, Gillespie MT, Martin TJ: Differentiation potential of a mouse bone marrow stromal cell line. J Cell Biochem, 90: 158-169, 2003.
18) Imabayashi H, Mori T, Gojo S, Kiyono T, Sugiyama T, Irie R, Isogai T, Hata J, Toyama Y, Umezawa A: Redifferentiation of dedifferentiated chondrocytes and chondrogenesis of human bone marrow stromal cells via chondrosphere formation with expression profiling by large-scale cDNA analysis. Exp Cell Res, 288: 35-50, 2003.
19) Makino S, Fukuda K, Miyoshi S, Konishi F, Kodama H, Pan J, Sano M, Takahashi T, Hori S, Abe H, Hata J, Umezawa A, Ogawa S: Cardiomyocytes can be generated from marrow stromal cells in vitro. J Clin Invest, 103: 697-705, 1999.
20) Gojo S, Gojo N, Takeda Y, Mori T, Abe H, Kyo S, Hata J, Umezawa A: In vivo cardiovasculogenesis by direct injection of isolated adult mesenchymal stem cells. Exp Cell Res, 288: 51-59, 2003.
21) Ochi K, Chen G, Ushida T, Gojo S, Segawa K, Tai H, Ueno K, Ohkawa H, Mori T, Yamaguchi A, Toyama Y, Hata J, Umezawa A: Use of isolated mature osteoblasts in abundance acts as desired-shaped bone regeneration in combination with a modified poly-DL-lactic-co-glycolic acid (PLGA)-collagen sponge. J Cell Physiol, 194: 45-53, 2003.
22) Umezawa A, Maruyama T, Segawa K, Shadduck RK, Waheed A, Hata J: Multipotent marrow stromal cell line is able to induce hematopoiesis in vivo. J Cell Physiol, 151: 197-205, 1992.
23) Alhadlaq A, Mao JJ: Mesenchymal stem cells: isolation and therapeutics. Stem Cells Dev, 13: 436-448, 2004.
24) Jiang Y, Henderson D, Blackstad M, Chen A, Miller RF, Verfaillie CM: Neuroectodermal differentiation from mouse multipotent adult progenitor cells. Proc Natl Acad Sci USA, 100(Suppl 1): 11854-11860, 2003.
25) Kim JH, Auerbach JM, Rodriguez-Gomez JA, Velasco I, Gavin D, Lumelsky N, Lee SH, Nguyen J, Sanchez-Pernaute R, Bankiewicz K, McKay R: Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. Nature, 418: 50-56, 2002.
26) Zetterstrom RH, Solomin L, Jansson L, Hoffer BJ, Olson L, Perlmann T: Dopamine neuron agenesis in Nurr1-deficient mice. Science, 276: 248-250, 1997.
27) Matsumoto S, Shibuya I, Kusakari S, Segawa K, Uyama T, Shimada A, Umezawa A: Membranous osteogenesis system modeled with KUSA-A1 mature osteoblasts. Biochim Biophys Acta, 1725: 57-63, 2005.
28) Solursh M: Differentiation of cartilage and bone. Curr Opin Cell Biol, 1: 989-994, 1989.
29) Hauselmann HJ, Fernandes RJ, Mok SS, Schmid TM, Block JA, Aydelotte MB, Kuettner KE, Thonar EJ: Phenotypic stability of bovine articular chondrocytes after long-term culture in alginate beads. J Cell Sci, 107(Pt 1): 17-27, 1994.
30) Reginato AM, Iozzo RV, Jimenez SA: Formation of nodular structures resembling mature articular cartilage in long-term primary cultures of human fetal epiphyseal chondrocytes on a hydrogel substrate. Arthritis Rheum, 37: 1338-1349, 1994.
31) Archer CW, McDowell J, Bayliss MT, Stephens MD, Bentley G: Phenotypic modulation in sub-populations of human articular chondrocytes in vitro. J Cell Sci, 97(Pt 2): 361-371, 1990.
32) Benya PD, Padilla SR, Nimni ME: Independent regulation of collagen types by chondrocytes during the loss of differentiated function in culture. Cell, 15: 1313-1321, 1978.
33) Benya PD, Shaffer JD: Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell, 30: 215-224, 1982.
34) Lefebvre V, Peeters-Joris C, Vaes G: Production of collagens, collagenase and collagenase inhibitor during the dedifferentiation of articular chondrocytes by serial subcultures. Biochim Biophys Acta, 1051: 266-275, 1990.
35) Bonaventure J, Kadhom N, Cohen-Solal L, Ng KH, Bourguignon J, Lasselin C, Freisinger P: Reexpression of cartilage-specific genes by dedifferentiated human articular chondrocytes cultured in alginate beads. Exp Cell Res, 212: 97-104, 1994.
36) Yoon YM, Kim SJ, Oh CD, Ju JW, Song WK, Yoo YJ, Huh TL, Chun JS: Maintenance of differentiated phenotype of articular chondrocytes by protein kinase C and extracellular signal-regulated protein kinase. J Biol Chem, 277: 8412-8420, 2002.
37) Sugiki T, Uyama T, Toyoda M, Morioka H, Kume S, Miyado K, Matsumoto K, Saito H, Tsumaki N, Takahashi Y, Toyama Y, Umezawa A: Hyaline cartilage formation and enchondral ossification modeled with KUM5 and OP9 chondroblasts. J Cell Biochem, 2006.
38) Karsner KW, Saphir O, Todd TW: The state of the cardiac muscle in hypertrophy and atrophy. Am J Pathol, 1: 351-371, 1925.

39) Kirshenbaum LA, Schneider MD: Adenovirus E1A represses cardiac gene transcription and reactivates DNA synthesis in ventricular myocytes, via alternative pocket protein- and p300-binding domains. J Biol Chem, 270: 7791-7794, 1995.

40) Soonpaa MH, Koh GY, Pajak L, Jing S, Wang H, Franklin MT, Kim KK, Field LJ: Cyclin D1 overexpression promotes cardiomyocyte DNA synthesis and multinucleation in transgenic mice. J Clin Invest, 99: 2644-2654, 1997.

41) Soonpaa MH, Field LJ: Survey of studies examining mammalian cardiomyocyte DNA synthesis. Circ Res, 83: 15-26, 1998.

42) Takeda Y, Mori T, Imabayashi H, Kiyono T, Gojo S, Miyoshi S, Hida N, Ita M, Segawa K, Ogawa S, Sakamoto M, Nakamura S, Umezawa A: Can the life span of human marrow stromal cells be prolonged by bmi-1, E6, E7, and/or telomerase without affecting cardiomyogenic differentiation? J Gene Med, 6: 833-845, 2004.

43) Hakuno D, Fukuda K, Makino S, Konishi F, Tomita Y, Manabe T, Suzuki Y, Umezawa A, Ogawa S: Bone marrow-derived regenerated cardiomyocytes (CMG Cells) express functional adrenergic and muscarinic receptors. Circulation, 105: 380-386, 2002.

44) Yamada Y SK, Takeda Y, Gojo S, Umezawa A.: Single-cell-derived mesenchymal stem cells overexpressing 3 Csx/Nkx2.5 and GATA4 undergo the stochastic 4 cardiomyogenic fate and behave like transient amplifying cells. Exp Cell Res, (in press).

45) Mori T, Kiyono T, Imabayashi H, Takeda Y, Tsuchiya K, Miyoshi S, Makino H, Matsumoto K, Saito H, Ogawa S, Sakamoto M, Hata J, Umezawa A: Combination of hTERT and bmi-1, E6, or E7 induces prolongation of the life span of bone marrow stromal cells from an elderly donor without affecting their neurogenic potential. Mol Cell Biol, 25: 5183-5195, 2005.

46) Olsen BR, Reginato AM, Wang W: Bone development. Annu Rev Cell Dev Biol, 16: 191-220, 2000.

47) Taylor SM, Jones PA: Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine. Cell, 17: 771-779, 1979.

48) Santi DV, Norment A, Garrett CE: Covalent bond formation between a DNA-cytosine methyltransferase and DNA containing 5-azacytosine. Proc Natl Acad Sci USA, 81: 6993-6997, 1984.

49) Kochanek S, Toth M, Dehmel A, Renz D, Doerfler W: Interindividual concordance of methylation profiles in human genes for tumor necrosis factors alpha and beta. Proc Natl Acad Sci USA, 87: 8830-8834, 1990.

50) Behn-Krappa A, Holker I, Sandaradura de Silva U, Doerfler W: Patterns of DNA methylation are indistinguishable in different individuals over a wide range of human DNA sequences. Genomics, 11: 1-7, 1991.

51) Umezawa A, Yamamoto H, Rhodes K, Klemsz MJ, Maki RA, Oshima RG: Methylation of an ETS site in the intron enhancer of the keratin 18 gene participates in tissue-specific repression. Mol Cell Biol, 17: 4885-4894, 1997.

52) Owen M: Marrow stromal stem cells. J Cell Sci Suppl, 10: 63-76, 1988.

53) Jaiswal N, Haynesworth SE, Caplan AI, Bruder SP: Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. J Cell Biochem, 64: 295-312, 1997.

54) Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR: Multilineage potential of adult human mesenchymal stem cells. Science, 284: 143-147, 1999.

55) Sekiya I, Larson BL, Vuoristo JT, Cui JG, Prockop DJ: Adipogenic differentiation of human adult stem cells from bone marrow stroma (MSCs). J Bone Miner Res, 19: 256-264, 2004.

56) Green H, Meuth M: An established pre-adipose cell line and its differentiation in culture. Cell, 3: 127-133, 1974.

57) Sudo H, Kodama HA, Amagai Y, Yamamoto S, Kasai S: In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. J Cell Biol, 96: 191-198, 1983.

58) Shukunami C, Shigeno C, Atsumi T, Ishizeki K, Suzuki F, Hiraki Y: Chondrogenic differentiation of clonal mouse embryonic cell line ATDC5 in vitro: differentiation-dependent gene expression of parathyroid hormone (PTH)/PTH-related peptide receptor. J Cell Biol, 133: 457-468, 1996.

59) Goodwin HS, Bicknese AR, Chien SN, Bogucki BD, Quinn CO, Wall DA: Multilineage differentiation activity by cells isolated from umbilical cord blood: expression of bone, fat, and neural markers. Biol Blood Marrow Transplant, 7: 581-588, 2001.

60) Lee OK, Kuo TK, Chen WM, Lee KD, Hsieh SL, Chen TH: Isolation of multipotent mesenchymal stem cells from umbilical cord blood. Blood, 103: 1669-1675, 2004.

61) Terai M, Uyama T, Sugiki T, Li XK, Umezawa A, Kiyono T: Immortalization of human fetal cells: the life span of umbilical cord blood-derived cells can be prolonged without manipulating p16INK4a/RB braking pathway. Mol Biol

Cell, 16: 1491-1499, 2005.
62) Hayflick L: The cell biology of human aging. N Engl J Med, 295: 1302-1308, 1976.
63) Campisi J: The biology of replicative senescence. Eur J Cancer, 33: 703-709, 1997.
64) Kiyono T, Foster SA, Koop JI, McDougall JK, Galloway DA, Klingelhutz AJ: Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells. Nature, 396: 84-88, 1998.
65) Romanov SR, Kozakiewicz BK, Holst CR, Stampfer MR, Haupt LM, Tlsty TD: Normal human mammary epithelial cells spontaneously escape senescence and acquire genomic changes. Nature, 409: 633-637, 2001.
66) Rheinwald JG, Hahn WC, Ramsey MR, Wu JY, Guo Z, Tsao H, De Luca M, Catricala C, O'Toole KM: A two-stage, p16(INK4A)- and p53-dependent keratinocyte senescence mechanism that limits replicative potential independent of telomere status. Mol Cell Biol, 22: 5157-5172, 2002.
67) Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu CP, Morin GB, Harley CB, Shay JW, Lichtsteiner S, Wright WE: Extension of life-span by introduction of telomerase into normal human cells. Science, 279: 349-352, 1998.
68) Ogawa M: Stochastic model revisited. Int J Hematol, 69: 2-5, 1999.
69) Watt FM, Hogan BL: Out of Eden: stem cells and their niches. Science, 287: 1427-1430, 2000.
70) Tenen DG, Hromas R, Licht JD, Zhang DE: Transcription factors, normal myeloid development, and leukemia. Blood, 90: 489-519, 1997.
71) Wakitani S, Goto T, Pineda SJ, Young RG, Mansour JM, Caplan AI, Goldberg VM: Mesenchymal cell-based repair of large, full-thickness defects of articular cartilage. J Bone Joint Surg Am, 76: 579-592, 1994.
72) Bruder SP, Kurth AA, Shea M, Hayes WC, Jaiswal N, Kadiyala S: Bone regeneration by implantation of purified, culture-expanded human mesenchymal stem cells. J Orthop Res, 16: 155-162, 1998.
73) Young RG, Butler DL, Weber W, Caplan AI, Gordon SL, Fink DJ: Use of mesenchymal stem cells in a collagen matrix for Achilles tendon repair. J Orthop Res, 16: 406-413, 1998.
74) Walsh CJ, Goodman D, Caplan AI, Goldberg VM: Meniscus regeneration in a rabbit partial meniscectomy model. Tissue Eng, 5: 327-337, 1999.
75) Koc ON, Gerson SL, Cooper BW, Dyhouse SM, Haynesworth SE, Caplan AI, Lazarus HM: Rapid hematopoietic recovery after coinfusion of autologous-blood stem cells and culture-expanded marrow mesenchymal stem cells in advanced breast cancer patients receiving high-dose chemotherapy. J Clin Oncol, 18: 307-316, 2000.
76) Horwitz EM, Prockop DJ, Fitzpatrick LA, Koo WW, Gordon PL, Neel M, Sussman M, Orchard P, Marx JC, Pyeritz RE, Brenner MK: Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. Nat Med, 5: 309-313, 1999.
77) Gerson SL: Mesenchymal stem cells: no longer second class marrow citizens. Nat Med, 5: 262-264, 1999.

EXPERIMENTAL CELL RESEARCH 313 (2007) 698–706

available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/yexcr

Research Article

Single-cell-derived mesenchymal stem cells overexpressing Csx/Nkx2.5 and GATA4 undergo the stochastic cardiomyogenic fate and behave like transient amplifying cells

Yoji Yamada[a], *Kazuhiro Sakurada*[a,1], *Yukiji Takeda*[b,2], *Satoshi Gojo*[b,3], *Akihiro Umezawa*[b,*]

[a]BioFrontier Laboratories, Kyowa Hakko Kogyo Co. Ltd., 3-6-6 Asahi-machi, Machida-shi, Tokyo 194-8533, Japan
[b]National Research Institute for Child Health and Development, 2-10-1 Okura, Setagaya-ku, Tokyo 157-8535, Japan

ARTICLE INFORMATION

Article Chronology:
Received 22 June 2006
Revised version received
31 October 2006
Accepted 15 November 2006
Available online 30 November 2006

Keywords:
Mesenchymal stem cells
Cardiomyocytes
Transient amplifying cells
Csx/Nkx2.5
GATA4

ABSTRACT

Bone marrow-derived stromal cells can give rise to cardiomyocytes as well as adipocytes, osteocytes, and chondrocytes in vitro. The existence of mesenchymal stem cells has been proposed, but it remains unclear if a single-cell-derived stem cell stochastically commits toward a cardiac lineage. By single-cell marking, we performed a follow-up study of individual cells during the differentiation of 9-15c mesenchymal stromal cells derived from bone marrow cells. Three types of cells, i.e., cardiac myoblasts, cardiac progenitors and multipotent stem cells were differentiated from a single cell, implying that cardiomyocytes are generated stochastically from a single-cell-derived stem cell. We also demonstrated that overexpression of Csx/Nkx2.5 and GATA4, precardiac mesodermal transcription factors, enhanced cardiomyogenic differentiation of 9-15c cells, and the frequency of cardiomyogenic differentiation was increased by co-culturing with fetal cardiomyocytes. Single-cell-derived mesenchymal stem cells overexpressing Csx/Nkx2.5 and GATA4 behaved like cardiac transient amplifying cells, and still retained their plasticity in vivo.

Introduction

Cell-based therapy is a novel therapeutic strategy, based on the concept of the cell-mediated restoration of damaged or diseased tissue. Candidate cell sources include embryonic stem (ES) cells, hematopoietic stem cells (HSCs), neural stem cells (NSCs), mesenchymal stem cells (MSCs) [1], and so on. Clinical trials with MSCs have been performed in patients with graft-versus-host disease through immunomodulatory effects [2], and osteogenesis imperfecta [3,4], and MSCs are expected to be one of the most available cells. The source of MSCs includes bone marrow [5], adipose tissue [6], umbilical cord [7] and placenta [8].

Bone marrow-derived stromal cells [9] can differentiate into mesenchymal progenitors, including osteoblasts [10], chondroblasts [11], skeletal myoblasts [12], adipoblasts [13],

* *Corresponding author.*
E-mail address: umezawa@1985.jukuin.keio.ac.jp (A. Umezawa).
[1] Present address: Research Center, Nihon Schering K.K., 1-5-5 Minatojima-minamicho, Chuo-ku, Kobe-shi, Hyogo 650-0047, Japan.
[2] Present address: Department of General Medicine and Clinical Investigation, Nara Medical University, 840 Shijo-cho, Kashihara-city, Nara 634-8522, Japan.
[3] Present address: Department of Cardiovascular Surgery, Saitama Medical Center, 1981 Kamoda, Kawagoe, Saitama 350-8550, Japan.

0014-4827/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.yexcr.2006.11.012

and neurons [14,15] when placed in appropriate in vitro and in vivo environments. We have shown that bone marrow-derived stromal cells are also able to differentiate into cardiomyocytes in vitro and in vivo [13,14,16,17]. However, the characteristics of the cells that can differentiate into cardiomyocytes are poorly understood, and how the progeny of multipotent cells adopt one fate among several possible fates remains a fundamental question.

Hematopoietic stem cells are defined as cells that are capable of self-renewal to maintain a long-term supply of progeny and are capable of differentiating into multiple hematopoietic lineages [18]. Retroviral labeling of individual cells is one of the useful clonal assays to monitor lineage commitment at the single cell level [16,17,19]. At present, several models have been proposed in which hematopoietic lineage determination is driven intrinsically [20], extrinsically [21], or both [22]. We therefore performed retroviral labeling experiments of bone marrow-derived stromal cells to investigate whether cardiomyocytes are generated from committed cardiac precursor cells or uncommitted stem cells.

In the present study, we provide evidence that cardiomyocytes are stochastically differentiated from MSCs, and we demonstrate that forced expression of cardiomyocyte-specific transcription factors, i.e., Csx/Nkx2.5 and GATA4, destined these MSCs to a cardiomyocytic lineage.

Materials and methods

Cell culture

9-15c cells were used as a source of uncommitted stem cells in this study [23,24]. 9-15c cells are available through one of the cell banks (JHSF cell bank: http://www.jhsf.or.jp/English/index_gc.html; RIKEN cell bank: http://www.brc.riken.go.jp/lab/cell/english/guide.shtml). 9-15c cells were cultured using methods described previously [25]. The cells were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% fetal bovine serum and penicillin (100 μg/ml)/streptomycin (100 μg/ml)/amphotericin B (250 ng/ml) at 33°C with 5% CO_2.

Primary cultures of cardiac myocytes were prepared from the hearts of 16-day-old fetal C3H/HeJ mice (CLEA Japan, Inc., Tokyo, Japan) according to the method of Simpson et al. [26] with minor modifications. In brief, cardiomyocytes were dissociated into single isolated cells by trypsinization and the cells were plated in culture medium (IMDM with 20% fetal bovine serum).

Cloning of Csx/Nkx2.5 and GATA4 cDNAs

The full open reading frames of mouse Csx/Nkx2.5 and GATA4 cDNAs were cloned by RT-PCR from poly(A) RNA obtained from the hearts of fetal mice using the following primers: Csx/Nkx2.5, sense: 5′-TGAAACCTGCGTCGCCACCATGT-3′, antisense: 5′-GGCTCTTTCCCTACCAGGCTCGG-3′; GATA4, sense: 5′-TAGTTCTTGTCTGCCTCGTGCTCA-3′, antisense: 5′-GGCGCTGATTACGCGGTGATTATG-3′. The PCR products were subcloned into pGEM-T vector (Promega). DNA sequencing confirmed that the plasmids contained the full-length fragments of the mouse Csx/Nkx2.5 and GATA4 coding regions.

Retroviral transduction

The retroviral vectors pCLNCX (Imgenex), pCLPCX and pCLHCX were used. pCLPCX was constructed from pCLNCX by replacing the neomycin resistance gene with a puromycin resistance gene (pPUR; CLONTECH). pCLHCX was constructed from pCLNCX by replacing the neomycin resistance gene with a hygromycin resistance gene (pcDNA3.1/Hygro(+); Invitrogen). Fragments containing the EGFP, Csx/Nkx2.5, and GATA4 genes were cloned into pCLNCX, pCLPCX, or pCLHCX. Each of these DNAs and pCMV-Eco (kindly provided by Nikunj Somia) were transfected into the producer cells (293 gag pol; kindly provided by Nikunj Somia) using TransFast (Promega). Two days after the transfection, the culture supernatant was filtered through a 0.45-μm filter. 9-15c cells were treated with viruses and hexadimethine bromide (polybrene) (Sigma) (8 μg/ml) for 4–6 h. To generate stably expressing cells, 9-15c cells were cultured in the presence of 300 μg/ml G418, 300 ng/ml puromycin or 300 μg/ml hygromycin. The mixtures of drug-resistant clones were used to average the clonal variation of the transfected gene expression.

Cardiomyogenic induction

To induce differentiation, cells were initially plated at a density of 2×10^4 cells/ml. The cells were treated with 3 μM 5-azacytidine (Sigma) for 24 h the next day. In some experiments, PDGF-BB (Peprotech) and retinoic acid (Sigma) were added to the culture dish coated with fibronectin (BD Biosciences) to give a final concentration of 10 ng/ml and 1 nM, respectively, for 6 days. Total number of beating cells was estimated under phase contrast microscopy.

RT-PCR

Total RNA was extracted from adult mouse hearts, skeletal muscles and cultured cells with an RNeasy kit (QIAGEN), and cDNA was made using the SuperScript First-strand Synthesis System (Invitrogen) from 1 μg of total RNA. First-strand cDNA was diluted 20 fold and 1 μl of cDNA was used for each PCR reaction. The following primer sets for cardiomyocyte-associated genes were used: atrial natriuretic peptide (ANP), sense: 5′-TTCCTCGTCTTGGCCTTTTGG-3′, antisense: 5′-GCTGGATCTTCGTAGGCTCCG-3′; cardiac troponin I (cTnI), sense: 5′-GATCCTGTTCTCTGCCTCTGGA-3′, antisense: 5′-TCATCCACTTTGTCCACCCGAG-3′; fast troponin I (fTnI), sense: 5′-GAAGCGCAACAGGGCCATCACG-3′, antisense: 5′-CCACGTCACGCAGGTCCCGTTC-3′; Csx/Nkx2.5, sense: 5′-TGGCGTCTGGGGACCTGTCTG-3′, antisense: 5′-GAGTCTGGTCCTGCCGCTGTC-3′; GATA4, sense: 5′-TACATGGCCGACGTGGGAGCA-3′, antisense: 5′-TGGAGTTACCGCTGGAGGCAC-3′; exogeneous GATA4, sense: 5′-CCAGAAAACGGAAGCCCAAGAA-3′ (the sequence derived from mouse GATA4 gene), antisense: 5′-GCTTGCCAAACCTACAGGTGGG-3′ (the sequence derived from pCLPCX vector); adiponectin, sense: 5′-CTGAAGAGCTAGCTCCTGCTTTG-3′, antisense: 5′-GAAGAGAACGGCCTTGTCCTTC-3′; glyceraldehyde-

3-phosphate dehydrogenase (G3PDH), sense: 5′-CCCATCAC-CATCTTCCAGGAGC-3′, antisense: 5′-TTCACCACCTTCTT-GATGTCATCATA-3′. G3PDH was used as an internal control. PCR was performed with TaKaRa Ex-Taq (TAKARA SHUZO CO., LTD) for 30–35 cycles, with each cycle consisting of 94°C for 1 min, 61–68°C for 1 min, and 72°C for 2 min, with an additional 7 min incubation at 72°C after completion of the final cycle.

RT-PCR samples were electrophoresed through agarose gels and stained with ethidium bromide and visualized through a UV light digital imaging system. Densities of electrophoresis bands were analyzed using ScnImage software (Scion Corporation).

Western blot analyses

Western blots were performed using whole-cell extracts according to the standard protocol [27]. Aliquots (30 μg) of whole-cell extracts were electrophoresed in SDS-polyacrylamide gels and transferred onto Immobilon-P polyvinylidene difluoride membrane (Millipore) by electroblotting. After treatment in blocking buffer, membranes were sequentially probed with the antibodies against Nkx2.5 (sc-8697, Santa Cruz) or Gata4 (sc-9053, Santa Cruz), and then with HRP-conjugated anti-goat or rabbit IgG. The bands were revealed using the ECL Plus standard protocol (Amersham Pharmacia Biotechnology).

Cellular transplantation

Following priming by 5-azacytidine for 24 h, the cells were cultured for an additional 3 days. Then the cells were harvested with 0.05% trypsin and 0.25 mM EDTA, and suspended as single cells at a concentration of 1×10^5 cells/μl with PBS. The cell viability in suspension, determined by 0.05% erythrosine dye exclusion, was 90% to 95%. After general anesthesia of the recipient mice by an intraperitoneal injection of 0.05 mg/g body weight pentobarbitone, cell transplantation was performed into the quadrant muscles of syngeneic adult recipient C3H/HeJ mice (CLEA Japan, Inc., Tokyo, Japan), aged 8 to 10 weeks old at a dose of 1×10^6 and 1×10^8 cells per mouse. All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by Keio University School of Medicine and the National Research Institute for Child Health and Development, and the experimental procedures were approved by the Laboratory Animal Care and Use Committee of Keio University School of Medicine.

Histological analyses

Tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. Tissue sections (6 μm) were mounted on poly-L-lysine-coated slides. After deparaffinization with xylene, tissues were rinsed in acetone or ethanol. Slides were incubated in 0.3% H_2O_2 for 30 min. After washing in PBS, tissues were preblocked for 30 min with 5% normal swine serum. They were incubated overnight at 4°C with mouse monoclonal antibody against recombinant GFP (CLONTECH Laboratories, Inc.) diluted 1:500. After rinsing in PBS, the slides were incubated with horseradish peroxidase-conjugated swine anti-mouse immunoglobulin diluted 1:100 with 1% BSA in PBS, and washed in cold PBS. Staining was developed using a solution containing DAB and 0.01% H_2O_2 in 0.05 M Tris–HCl buffer, pH 6.7. Slides were counterstained with hematoxylin. Slices with positive signals for EGFP were further stained

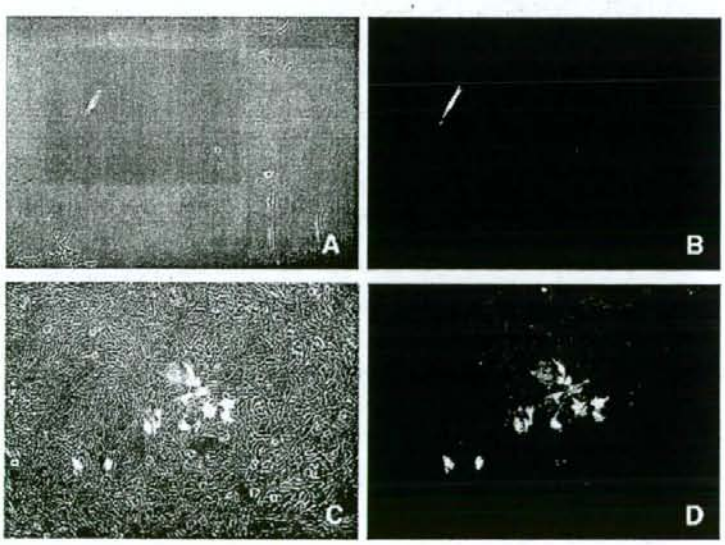

Fig. 1 – Single cell marking by infection of retrovirus carrying EGFP. Phase contrast photomicrograph (A, C) and fluorescent photomicrograph (B, D) of 9-15c cells 1 day (A, B) or 7 days (C, D) after infection with retroviruses carrying EGFP. EGFP-positive single cell-derived cells were clustered.

with anti-CD31 (PECAM-1) antibody (M-20, Santa Cruz Biotechnology, Inc, California, USA).

Frozen sections (6 μm) of the samples were used to detect the donor cells and the differentiation status by examination under a fluorescence microscope. After fixation with acetone and blocking with PBS containing 5% rabbit serum, anti-CD31 or anti-desmin (Bio-Science Products AG, Switzerland) antibodies was used as the first antibody, and rat anti-mouse IgG antibody conjugated with tetramethylrhodamine isothiocyanate (T4280, Sigma, Missouri, USA) and goat anti-mouse IgG antibody conjugated with rhodamine (M116, Leinco Technology, Inc., MO, USA) were used as the second antibody, respectively.

Results

Single-cell marking of 9-15c cells

9-15c cells are mesenchymal stem cells [23,24] capable of differentiating into cardiomyocytes in vitro with the use of 5-azacytidine. To determine if cardiomyocytes were generated from committed cardiac precursor cells or uncommitted stem cells during the differentiation of 9-15c cells, we carried out a single-cell marking experiment. Following retrovirus-mediated EGFP gene infection, a single EGFP-labeled cell could be detected at Day 1 after infection (Figs. 1A, B). The fate of

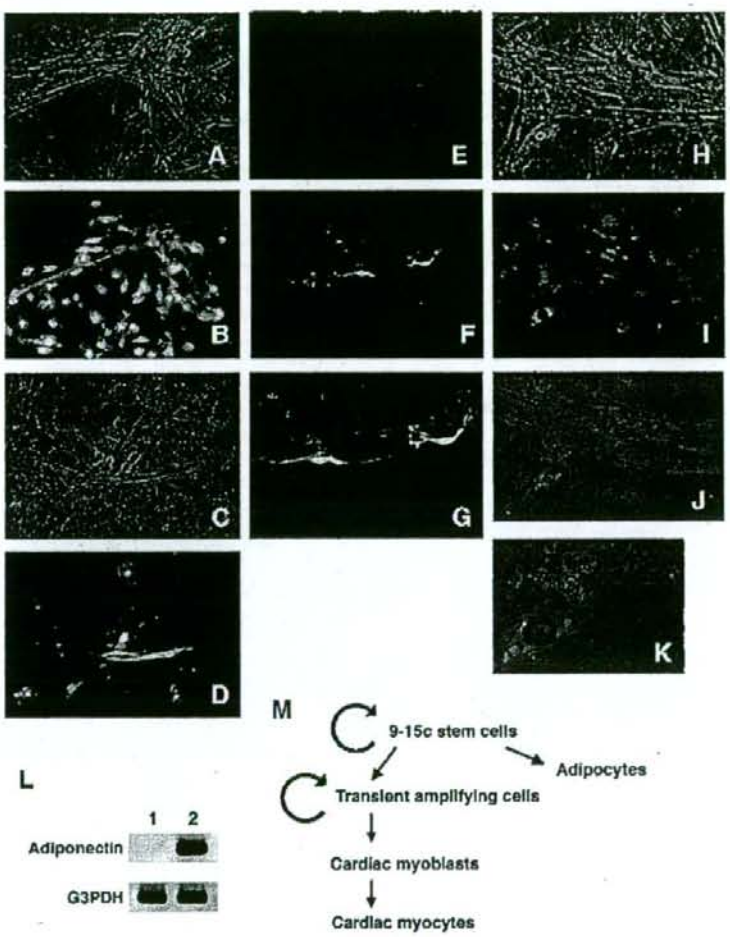

Fig. 2 – Bipotency, i.e., cardiomyogenic and adipogenic differentiation, of single cell-derived cells. Single-cell-derived 9-15c cells marked by EGFP exhibited cardiomyogenic and adipogenic differentiation after exposure to 5-azacytidine. (A–B) Cardiomyogenic and undifferentiated EGFP-marked, single-cell-derived 9-15c cells; (C–G) Cardiomyogenic differentiation of EGFP-marked, single-cell-derived 9-15c cells; (H–J) Cardiomyogenic and adipogenic differentiation of EGFP-marked, single-cell-derived 9-15c cells. (A, C, E, H, J) Phase contrast photomicrographs; (B, D, F, G, I) fluorescent photomicrographs. (K) Enlargement of the panel J. (L) RT-PCR analysis of the adiponectin and G3PDH genes in 9-15c cells at the growing phase without any treatment (lane 1) and 4 weeks after exposure to 5-azacytidine (lane 2). (M) Scheme of 9-15c cell differentiation.

retrovirally tagged 9-15c cells could be traced by monitoring EGFP throughout the differentiation process after exposure to 5-azacytidine. Seven days later, the EGFP-positive, single-cell-derived cells were clustered (Figs. 1C, D). Four weeks after 5-azacytidine treatment, the EGFP-positive cells were examined for differentiated phenotypes. We identified beating cells as cardiomyocytes and oil-red-positive cells as adipocytes. Three kinds of cell populations were observed: a) a cell population in which cardiomyocytes and undifferentiated stem cells were EGFP-positive (Figs. 2A, B); b) a cell population in which all the EGFP-positive cells were cardiomyocytes (Figs. 2C–G); c) a cell population in which cardiomyocytes, adipocytes and undifferentiated stem cells were EGFP-positive (Figs. 2H–K). RT-PCR analysis shows that these cells express adiponectin (Fig. 2L), suggesting the presence of adipocytes among the differentiated population. These results imply that cardiomyocytes are generated from uncommitted stem cells (Fig. 2M).

9-15c multipotent cells were preferentially destined to generate cardiomyocytes by forced expression of transcription factors Csx/Nkx2.5 and GATA4

In order to elucidate the roles of Csx/Nkx2.5 and GATA4 in 9-15c cell differentiation, we infected 9-15c cells with retroviruses carrying Csx/Nkx2.5 and GATA4. We detected Csx/Nkx2.5 and GATA4 gene expression in the infected cell by RT-PCR and Western blotting (Figs. 3A and B). GATA4 gene was originally expressed in 9-15c; we detected the GATA4 transgene with specific primers, but not the endogenous GATA4 gene (Fig. 3A).

Four weeks after the induction of differentiation by 5-azacytidine treatment, we examined the efficiency of cardiomyogenic differentiation or the expression of cardiomyogenic markers. The expression of the ANP and cTnI genes was up-regulated in 9-15c cells overexpressing Csx/Nkx2.5 and GATA4 (9-15c-CG cells) compared to the uninfected 9-15c cells (Fig. 3C, lanes 5 and 9). When 9-15c-CG cells were treated with PDGF and retinoic acid on dishes coated with fibronectin in addition to 5-azacytidine, the expression of the ANP and cTnI gene was further up-regulated (Fig. 3C, lane 10).

Cell implantation into immunodeficient mice

To investigate whether 9-15c-CG cells differentiate in vivo, the cells treated with 10 μM 5-azacytidine for 24 h were injected into immunodeficient mice (Figs. 4A–F). The donor cells clearly formed striated muscles without a branched structure as well as undifferentiated cells 81 days after implantation. The implanted 9-15c-CG cells clearly expressed desmin (Fig. 4G). The grafted cells also generated neovascularization near the injected site 1 month after injection; the EGFP-positive donor cells could be identified as the endothelium of these vessels (Fig. 4H). Immunohistochemistry with an antibody against CD31, a marker for endothelium, confirmed that the donor cells of the newly formed vessels had differentiated into endothelium (Fig. 4Hb). Engrafted donor cells appeared to maintain the characteristics of stem cells, that is, they continued to produce progeny, i.e., differentiated endothelial cells in this case.

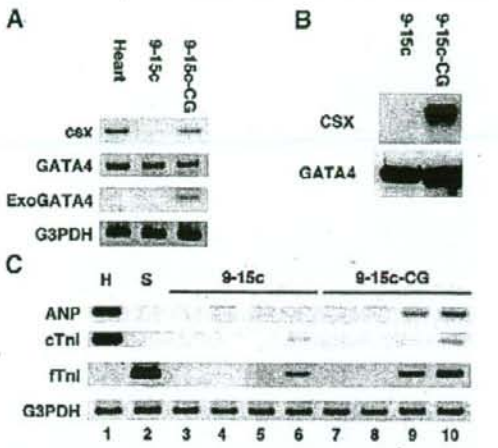

Fig. 3 – Expression of cardiomyocyte-specific or associated genes in 9-15c cells. A: RT-PCR analysis of the Csx, GATA4, exogeneous GATA4 and G3PDH genes (from top to bottom) in adult mouse heart, 9-15c cells and 9-15c cells overexpressing the Csx and GATA4 genes (9-15c-CG cells). B: Western blotting analysis of the Csx and GATA4 proteins in 9-15c cells and 9-15c-CG cells. C: RT-PCR analysis of the ANP, cTnI, and G3PDH genes (from top to bottom) in 9-15c cells (lanes 3–6) and 9-15c-CG cells (lanes 7–10). 9-15c cells (lane 3) and 9-15c-CG cells (lane 7) were cultured without any treatment (lanes 4 and 8) or with exposure to 5-azacytidine alone (lanes 5 and 9), or 5-azacytidine, PDGF, retinoic acid, and fibronectin coating on a dish (lanes 6 and 10) for 4 weeks. Heart (lane 1: H) and skeletal muscle (lane 2: S) served as controls.

Enhancement of cardiomyogenic differentiation by the co-cultivation with cardiomyocytes

We co-cultured EGFP-labeled 9-15c-CG cells with cardiomyocytes of fetal mice in vitro. Four weeks after 5-azacytidine treatment, EGFP-positive beating cardiomyocytes were increased (Figs. 5A, B). To determine whether factors secreted from the cultured cardiomyocytes promoted cardiomyocytic differentiation, 9-15c cells and 9-15c-CG cells were cultured in growth medium supplemented with conditioned medium from cardiomyocyte cultures. The expression of the ANP and cTnI genes was up-regulated in both 9-15c cells and 9-15c-CG cells with exposure to the conditioned medium of cardiomyocyte cultures (Fig. 5C, lanes 3 and 7). Furthermore, treatment with PDGF and retinoic acid, and fibronectin coating on a dish enhanced cardiomyogenic marker expression in both 9-15c cells and 9-15c-CG cells (Fig. 5C, lanes 4 and 8).

Discussion

Different models arise from different conceptions of the MSCs as in hematopoietic stem cells' differentiation [28,29]. A hierarchical model of MSCs has been proposed based on the in vitro differentiation potential of human MSCs as observed

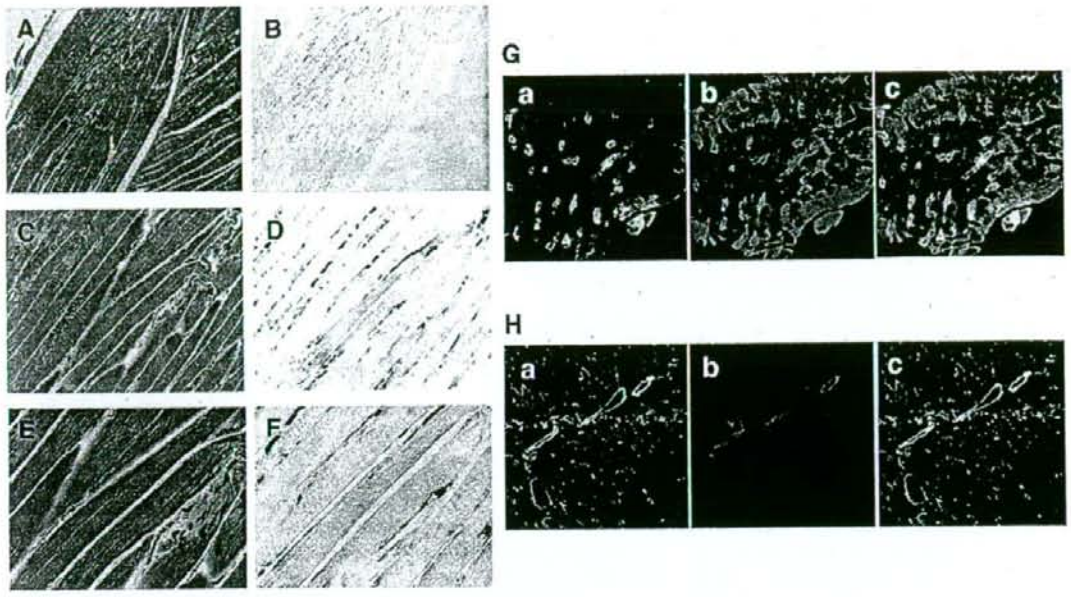

Fig. 4 – Myogenic differentiation of the EGFP-labeled 9-15c-CG cells into the quadriceps femoris muscle. EGFP-labeled 9-15c-CG cells could be recognized morphologically as the skeletal myocytes in the quadriceps femoris muscle 3 months after transplantation (A, C, E: HE staining; B, D, F: immunohistochemistry using anti-GFP antibody). The EGFP-positive donor cells exhibited skeletal myocyte-specific features such as multiple nuclei in the periphery of the cells and striation. Generation of myocytes (G) and endothelial cells (H) by the EGFP-labeled 9-15c cells. The injected donor 9-15c cells labeled with EGFP were detected by green fluorescence. (Ga, Ha) Green fluorescence of EGFP-labeled donor cells. (Gb, Hb) Immunohistochemistry for desmin (Gb, red) or CD31 (Hb, red). (Gc, Hc) The merged images of green fluorescence of injected 9-15c cells and rhodamine of desmin or CD31 clearly demonstrated that 9-15c cells differentiated into myocytes or endothelium. A–F: Longitudinal section; G, H: Cross section.

by clonal analysis [30]. In the present study using single-cell marking, we found that 9-15c cells in culture consisted of a mixture of at least three types of cells, i.e., cardiac myoblasts, cardiac progenitors and multipotent stem cells. Cardiac myoblasts are defined as cells which can differentiate into only cardiac myocytes and have low proliferative potential; cardiac progenitors have proliferative capability and the ability to become cardiomyocytes; multipotent stem cells have both proliferative capability and multipotency. The results obtained in the present study suggest that 9-15c cells are stochastically committed toward the cardiac lineage, and that following this commitment they proliferate as transient amplifying cells and differentiate into cardiac myocytes through the differentiation process, and the hierarchical model applies in the case of 9-15c multipotent cells.

In the present study, we used 5-azacytidine to induce differentiation. 5-azacytidine is a cytosine analog that causes extensive demethylation. The demethylation is attributable to covalent binding of DNA methyltransferase to 5-azacytidine in the DNA [31], with the subsequent reduction of enzyme activity in cells resulting in random loss of methylation at many sites in the genome. Previously, it has been thought that 5-azacytidine activates cardiomyogenic master genes, such as Nkx2.5/Csx, GATA4, and MEF-2C, leading to stochastic transdifferentiation of MSCs into cardiomyocytes [32,33]. This concept is difficult to account for the existence of cardiac progenitors and multipotent stem cells we identified, and we propose two possibilities how 5-azacytidine works. First, treatment of 5-azacytidine modulates heterochromatin remodeling and leads to dedifferentiation of 9-15c cells. Second, 9-15c cells are stochastically committed toward the cardiac lineage, being independent of treatment of 5-azacytidine. At this time we cannot conclude which is feasible, but it is certain cardiomyocytes are not only transdifferentiated by treatment of 5-azacytidine.

Csx/Nkx2.5 and GATA4 are two cardiac-enriched transcription factors that are expressed in precardiac mesoderm from the very early developmental stage [34,35]. In the present study, increased frequency of cardiomyogenic differentiation of 9-15c cells was successfully achieved in vitro by forced expression of Csx/Nkx2.5 and GATA4. These results are consistent with a report showing that both Csx/Nkx2.5 and GATA4 are required for the cardiac differentiation of P19CL6 cells derived from embryonic teratocarcinoma cells [36]. Cardiomyogenic differentiation, however, could proceed only after treatment with 5-azacytidine in our experimental setting, implying that Csx/Nkx2.5 and GATA4 are required but not sufficient for cardiac differentiation. Unknown factors

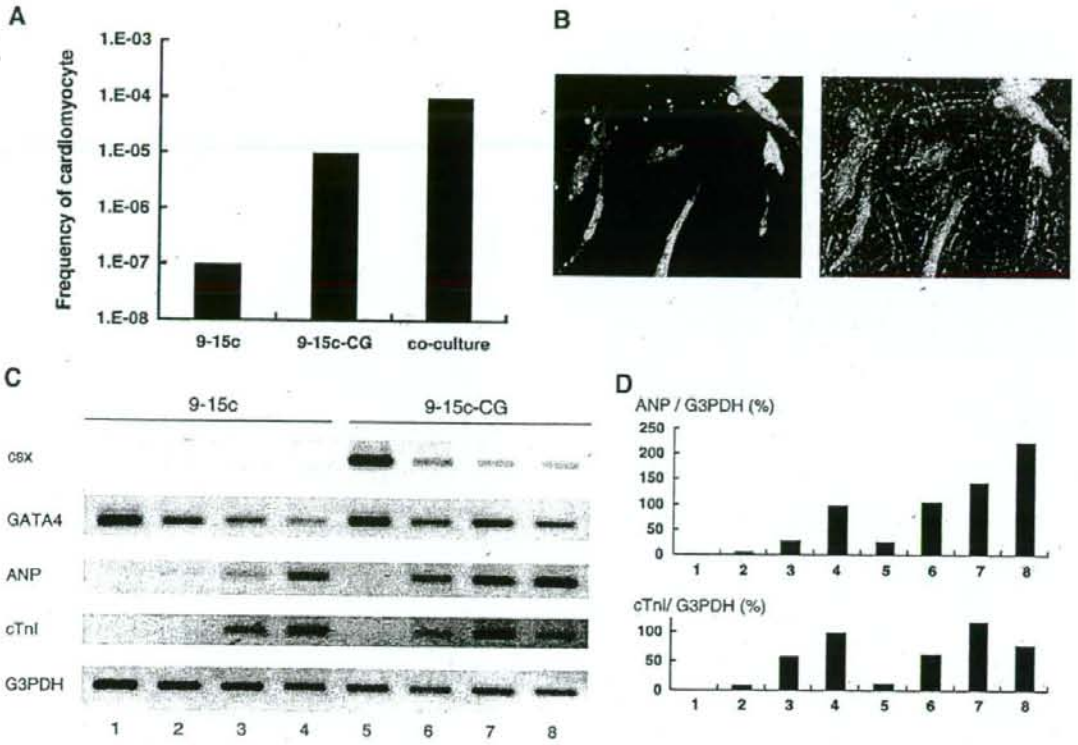

Fig. 5 – Enhancement of cardiomyogenic differentiation of 9-15c cells by co-cultivation with murine fetal cardiomyocytes. A: Frequencies of cardiomyogenic differentiation in 9-15c cells, 9-15c cells overexpressing the Csx and GATA4 genes (9-15c-CG cells), and 9-15c-CG cells co-cultured with murine fetal cardiomyocytes. B: Cardiomyogenic differentiation of EGFP-positive 9-15c-CG cells co-cultured with murine fetal cardiomyocytes. Left: Green fluorescence of EGFP-positive 9-15c-CG cells. Right: Same field visualized by phase-contrast microscopy merged with fluorescence image. C: RT-PCR analysis of the Csx, GATA4, ANP, cTnI and G3PDH genes in 9-15c cells (lanes 1–4) and 9-15c-CG cells (lanes 5–8). 9-15c cells (lane 1) and 9-15c-CG cells (lane 5) were cultured with exposure to 5-azacytidine alone (lanes 2 and 6) or 5-azacytidine and conditioned medium of cardiomyocyte cultures (lanes 3 and 7), or 5-azacytidine, conditioned medium of cardiomyocyte cultures, PDGF, retinoic acid, and fibronectin coating on a dish (lanes 4 and 8) for 4 weeks. D: Ratio mRNA expression level of ANP and cTnI to G3PDH in C. The mRNA level of 9-15c cells (lane 4) was regarded as equal to 100%.

induced by 5-azacytidine or microRNAs, whose key roles in stem cell biology are just emerging [37], also seem to be needed.

Adipogenic 3T3-L1 [38], osteogenic MC3T3-E1 [39], and chondrogenic ATDC5 cells [40] have been isolated from stem cells with a mesenchymal nature. In addition, cardiomyogenic precursors may be obtained from stem cells such as cardiac stem cells, embryonic stem cells, and mesenchymal stem cells. Fetal cardiomyocytes are differentiated cardiomyocytes, but not stem cells that can proliferate in vitro. Recently, cardiac stem cells capable of clonogenically self-renewing have been isolated from the adult heart [41–43]. Some cardiac stem cells also retain plasticity. The retention of plasticity, i.e., the ability to transdifferentiate into skeletal myocytes and endothelium, of 9-15c cells overexpressing Csx/Nkx2.5 and GATA4 supports the idea that these cells may be considered cardiac stem or amplifying cells in terms of differentiation and self-renewal. On the other hand, Csx/Nkx2.5 inhibits the myogenic differentiation of C2C12 cells and promotes neuronal differentiation [44]. This unexpected effect of Csx/Nkx2.5 may be due to differential effects of the gene in different cell types, or of transient versus constitutive expression of the infected gene; dependency of the differentiated phenotypes on the gene expression period is observed for the Notch gene [45,46] and noggin gene [47].

Cell transplantation has been attempted to improve cardiac function in severe heart failure; MSCs have been transplanted to functionally restore damaged or diseased tissue in animal models, and mononuclear cells or myoblasts have been injected into ischemic hearts clinically. MSCs are capable of differentiating into many types of cells, and 'cardiomyogenic master genes' are able to enhance the commitment or determine the path to cardiomyogenic differentiation of these MSCs. The stemness of MSCs determined by single-cell