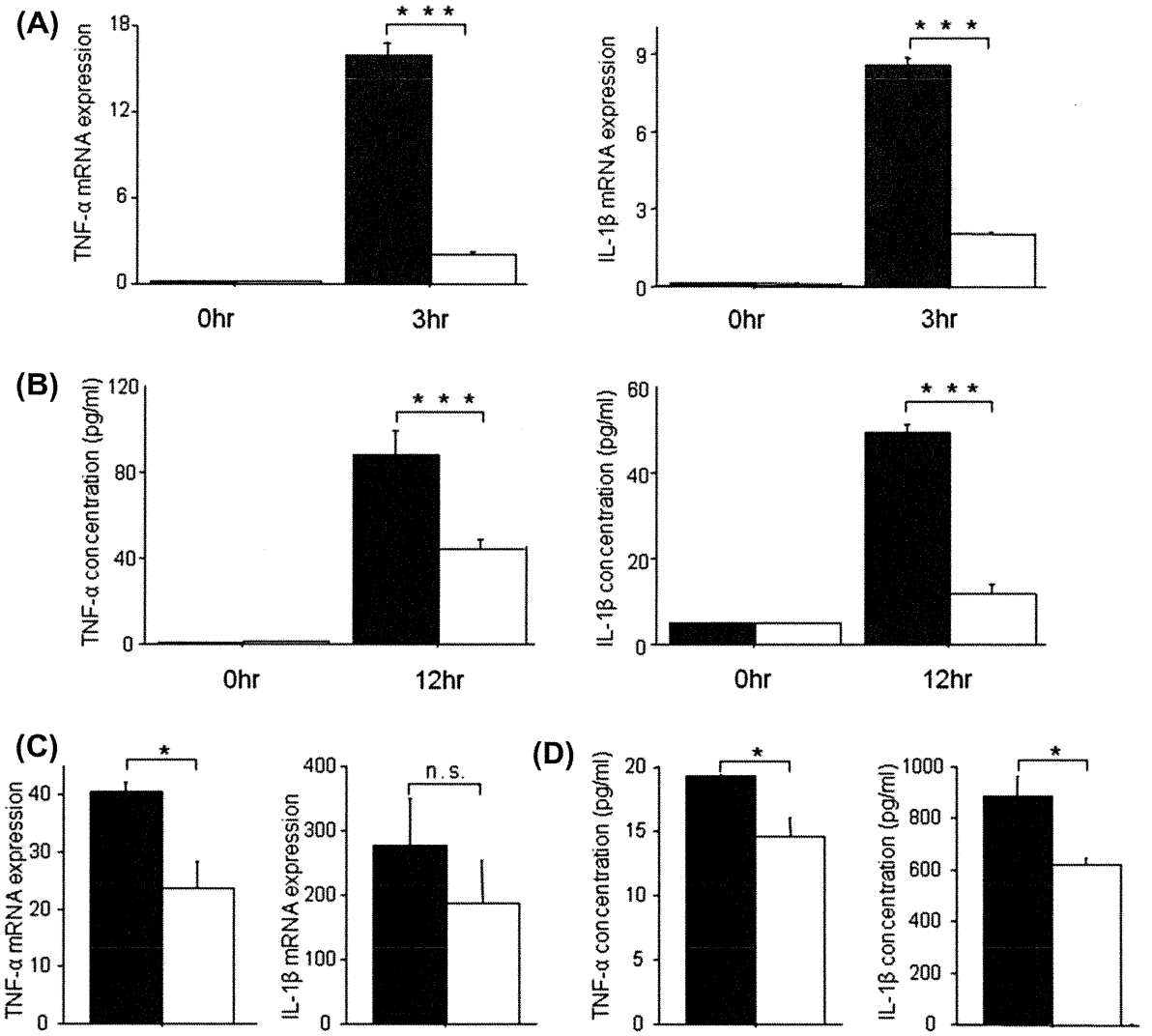

Fig. 3. Expression of proinflammatory cytokines in response to TLR ligand stimulation was decreased in human monocytes treated with tunicamycin. (A) THP-1 cells pretreated with tunicamycin (5 μg/ml) were cultured in AIM-V serum-free culture medium supplemented with LPS (1 μg/ml). After 3 h incubation with LPS, RNA was isolated from the THP-1 cells, and the expression levels of the TNF-α and IL-1β genes were analyzed by RTD-PCR. The expressions of these cytokines were down-regulated in THP-1 cells treated with tunicamycin. (B) THP-1 cells pretreated with tunicamycin were cultured in AIM-V serum-free culture medium supplemented with LPS (1 μg/ml) for 12 h and the concentration of TNF-α and IL-1β in the culture medium was measured by ELISA. The concentrations of these cytokines were decreased. (C) Primary human monocytes were cultured in AIM-V serum-free culture medium supplemented with LPS (1 μg/ml) and tunicamycin (5 μg/ml). After 4 h incubation, RNA was isolated from the cells, and the expression levels of the TNF-α and IL-1β genes was analyzed by RTD-PCR. The expression of IL-1β was down-regulated in cells treated with tunicamycin and TNF-α tended to be down-regulated. (D) Primary human monocytes were cultured in AIM-V serum-free culture medium supplemented with LPS (1 μg/ml) and tunicamycin (5 μg/ml) for 12 h, and the concentration of TNF-α and IL-1β in the culture medium was measured by ELISA. The concentration of these cytokines was decreased. Data are expressed as means ± SEM of four independent experiments. Filled bars; no treatment. Open bar; treatment with tunicamycin (5 μg/ml). $^{*}P < 0.05$, $^{***}P < 0.001$.

3.3. ER stress impaired monocyte differentiation into macrophages

Monocytes are progenitor cells that differentiate into macrophages and play an important role for inducing acquired immunity [16]. We tried to differentiate THP-1 cells and primary human monocytes into macrophages by PMA and GM-CSF treatment, respectively. When THP-1 cells were treated with PMA for 3 days, cells appeared morphologically mature, showing increased forward scatter (FSC) and side scatter (SSC) (Fig. 5A and B), and displayed enhanced expression of the macrophage-related markers CD11b and CD68 by flow cytometry (Fig. 5C). However, when THP-1 cells were pretreated with tunicamycin followed by PMA treatment, no alteration of FSC and SSC was observed (Fig. 5A and B) and the expression levels of CD11b and CD68 (Fig. 5C and D) were not enhanced. We confirmed that the ER stress markers CHOP and BiP were upregulated in the PMA-differentiated THP-1 cells with tunicamycin treatment (Fig. 5E). Furthermore, when primary human monocytes were treated with or without tunicamycin and cultured in media with GM-CSF for 4 days, tunicamycin-treated primary human monocytes showed less mature morphological appearance (Fig. 5F), lower FSC and SSC (Fig. 5G) and lower CD11b expression (Fig. 5H) compared to those of untreated primary human monocytes. In THP-1 cells, the differentiated cells under ER stress showed the same expression levels of TLR and MyD88 regardless of tunicamycin-induced ER stress (Fig. 6A and B). However, TNF-α and IL-1β expression levels were significantly decreased in differentiation-induced THP-1 cells under ER stress (Fig. 6C and D). These results suggest that tunicamycin-induced ER stress disturbed the differentiation capability as well as the signaling pathway of TLR4 in monocytes.

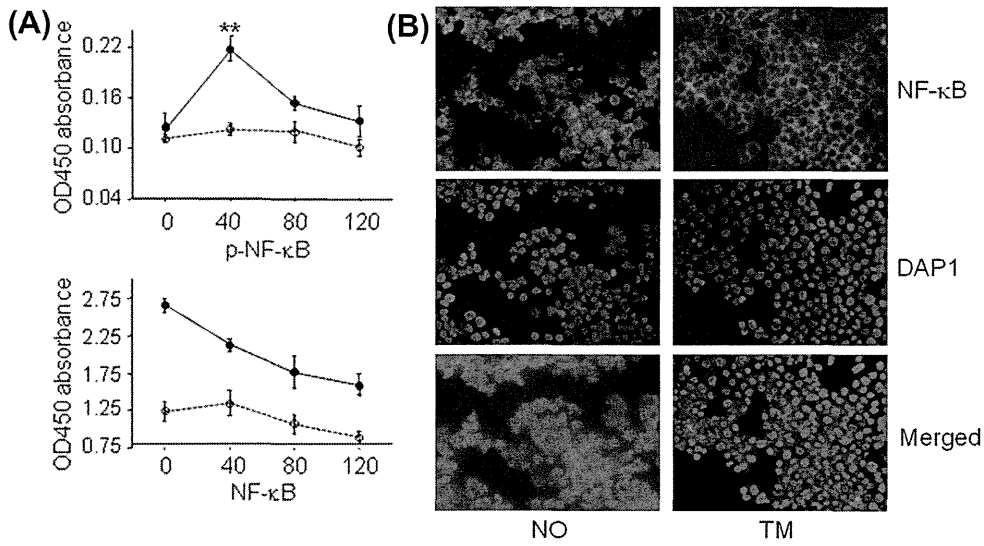

Fig. 4. NF-κB activation in response to LPS stimulation in human monocytes was attenuated under ER stress. (A) THP-1 cells treated with tunicamycin (5 μg/ml) for 12 h were cultured in AIM-V serum-free medium supplemented with LPS. Phosphorylated or total NF-κB quantity in THP-1 cells seeded on the culture plate was assessed by cellular activation of the signaling ELISA kit. Induction of phosphorylated NF-κB by LPS stimulation was not observed in THP-1 cells treated with tunicamycin. (B) Fluorescence microscopy examination of the tunicamycin-treated THP-1 cells followed by LPS stimulation. Cells were permeabilized and were stained with anti- NF-κB p65 and tetramethyl rhodamine isothiocyanate-goat anti-mouse IgG (red). The nucleus is stained with DAPI (4, 6-diamino-2-phenylindole; blue). The sustained localization of NF-κB p65 in the cytoplasm was observed in THP-1 cells treated with tunicamycin followed by LPS stimulation. Original magnification, ×400. Filled bars; no treatment. Open bar; treatment with tunicamycin (5 μg/ml). $^{*}P < 0.01$.

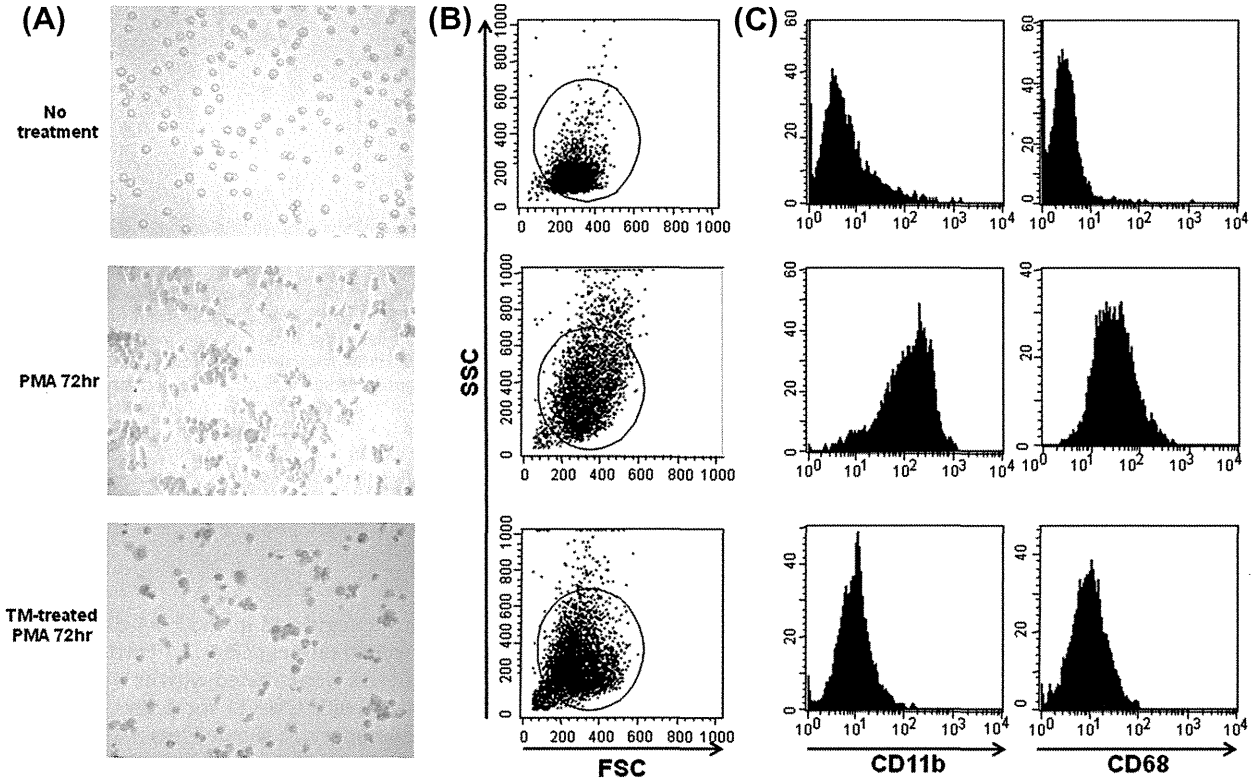

Fig. 5. *Human monocytes under ER stress demonstrated impaired capability for differentiation into macrophages.* (A–E) *Conditioning with tunicamycin* (5 μg/ml) THP-1 cells were treated with PMA (50 ng/ml) for 72 h. Cells were examined under microscopy for morphology and flow cytometry for surface molecules of macrophage markers CD11b and CD68. Gene expression of cells was also analyzed by RTD-PCR for CD11b, CD68, CHOP and BiP. Morphological analysis (A) and scatter and forward scatter by flow cytometry (B) PMA-differentiated THP-1 cells conditioned with tunicamycin were observed to be smaller and less granular compared to cells not treated with tunicamycin. Flow cytometry analysis (C) and gene expression analysis by RTD-PCR (D) for markers of macrophages, CD11b and CD68 showed decreased expression of these markers in THP-1 cells treated with tunicamycin and PMA. (E) Gene expressions of CHOP and BiP were increased in THP-1 cells conditioned with tunicamycin. (F–H) Primary human monocytes treated with tunicamycin (0.1 μg/ml) were cultured with GM-CSF (100 ng/ml) for 4 days for differentiation into macrophages. Cultured cells were examined by microscopy and flow cytometry. Tunicamycin-treated primary human monocytes were smaller (F) and less granular (G), and CD11b expression was repressed (H) after 4 days culture in media with GM-CSF. Data are expressed as means ± SEM of four independent experiments. Filled bars; no treatment. Open bar; treatment with tunicamycin (5 μg/ml). $^{**}P < 0.01$, $^{***}P < 0.001$.

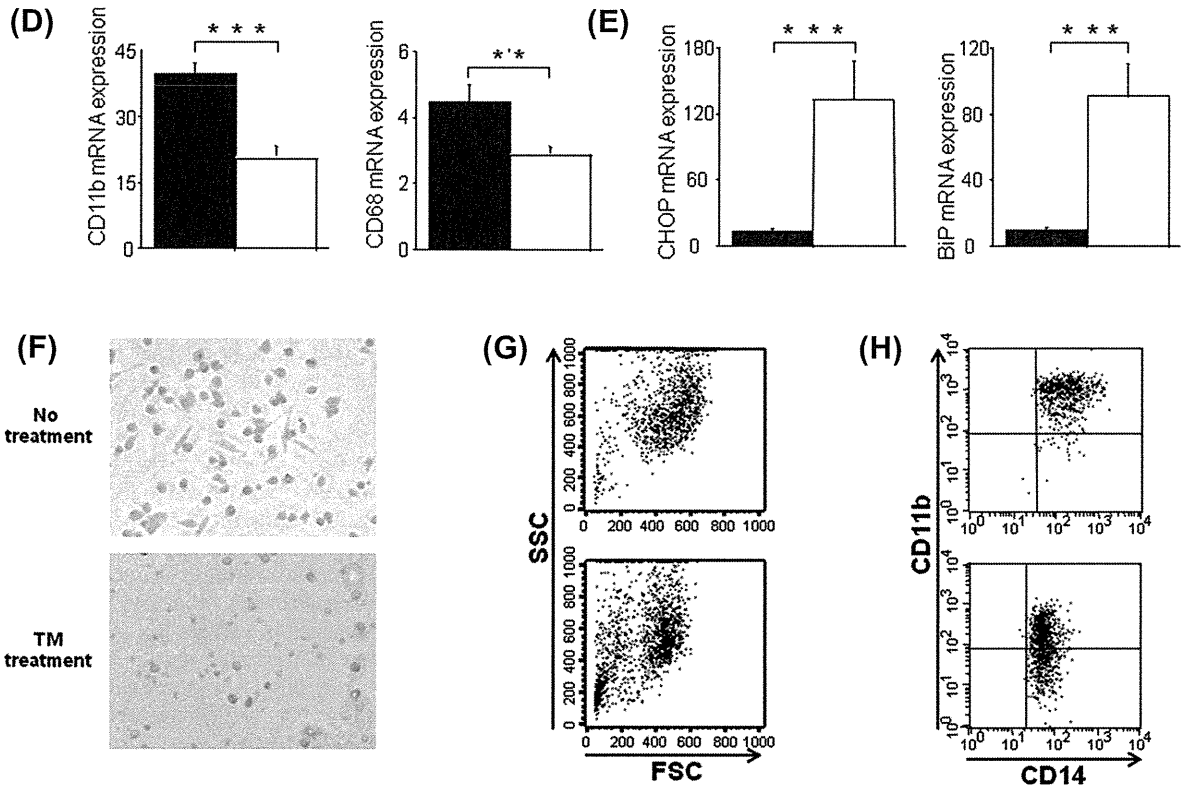

Fig. 5. (*continued*)

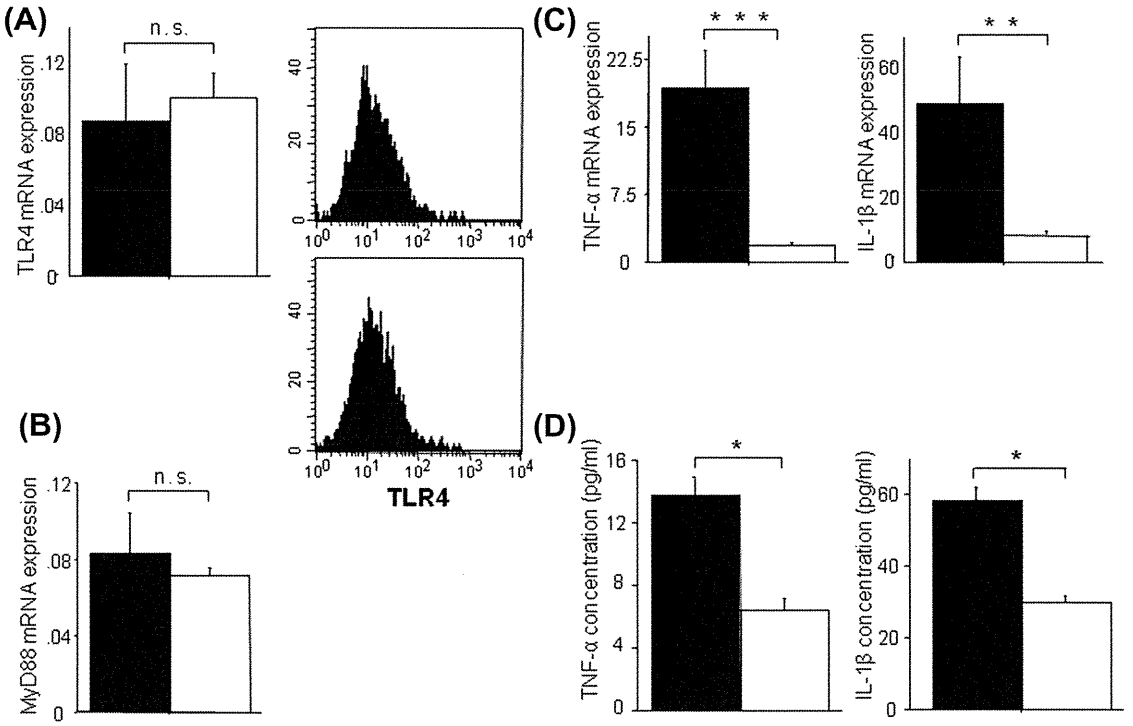

Fig. 6. The expression of proinflammatory cytokines in response to LPS stimulation was decreased in PMA-driven differentiated macrophage-like cells under ER stress without alteration of the expression of TLR4 and accessory molecules. THP-1 cells were treated with tunicamycin (5 μg/ml) followed by PMA treatment for differentiation induction into macrophages. Expression analysis by RTD-PCR (A) and flow cytometry (B) was performed for TLR4 and MyD88. Expressions of both molecules were not affected. (C–D) THP-1 cells were treated with tunicamycin (5 μg/ml) followed by PMA treatment for 72 h followed by LPS stimulation. Gene expression analysis (C) of cells stimulated with LPS for 3 h as well as ELISA quantification (D) of concentrations of cells stimulated with LPS for 12 h in culture medium were assessed. The expressions of TNF-α and IL-1β in response to LPS stimulation were significantly decreased in differentiated macrophage-like cells when they were under ER stress by tunicamycin. Filled bars; no treatment. Open bar; treatment with tunicamycin (5 μg/ml).

4. Discussion

We observed that ER stress induced susceptibility to apoptosis in THP-1 cells, a human monocytic cell line. Functionally, the response of LPS-stimulated human monocytes to express proinflammatory cytokines under ER stress was attenuated prior to the obvious appearance of apoptosis, with decreased activation of NF-κB. Furthermore, the differentiation capability of monocytes into macrophages was inhibited with a hypo-responsiveness to LPS under ER stress. These observations suggest that ER stress is an important pathological condition affecting a variety of monocyte functions.

The ER system is indispensable for synthesizing properly functioning proteins [1]. Under ER stress, cells malfunction due to the improper production or folding of proteins. Therefore, ER stress can result in the retention of harmful unfolded proteins as a consequence of impaired function of ER-related molecules such as protein kinase R-like ER kinase (PERK), inositol requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6), all of which are important molecules of the ER system [17,18]. In the immune system, an unfolded protein response (UPR) related to ER stress was observed in myeloma cells, malignant cells of plasma cells [19]. The affected immune response due to an UPR was also observed in the development of nonmalignant immunological disorders, such as rheumatoid arthritis and neurodegenerative disease [2]. UPRs affect the survival and function of DCs because of their pivotal capacity to process peptides for presentation and secrete cytokines [20]. These findings, as well as our previous observation that diabetic monocytes were under ER stress and were impaired [11], demonstrate that monocytes are an important subpopulation in immune cells in ER stress-related diseases.

Prolonged ER stress leads to apoptotic cell death, which is mediated by CHOP [21], a crucial specific molecule for ER stress-induced apoptosis. Apoptosis is also accompanied by alterations of the transcriptional expression of the BCL-2 gene family [22]. We observed that THP-1 monocytic cells under ER stress underwent apoptosis with decreased expression of the anti-apoptotic molecule BCL-2, indicating that ER stress induced THP-1 cell death using conventional apoptotic pathways [23,24].

The TLR pattern recognition by the innate immune system induces proinflammatory cytokines through the activation of NF-κB [25,26]. In the quiescent state, NF-κB remains inactive in the cytoplasm through binding to the inhibitory protein IκB. The phosphorylated IκB releases NF-κB to translocate into the nucleus where it mediates the transcription of its target genes [14]. We observed that the tunicamycin-induced ER stress diminished TLR4 signaling without altering the expressions of TLR4 and MyD88. With regard to the transcriptional factor, we observed a lack of the activated form of NF-κB and sustained cytoplasmic localization of NF-κB in LPS-stimulated THP-cells under ER stress. The activation of NF-κB is transient and cyclic upon continuous stimulation, which is due to specific negative feedback control systems such as the NF-κB inducible synthesis of IκB and A20 [27]. A previous report indicated that ER stress-induced A20, a deubiquitinating protease [28], acts as an inhibitor of NF-κB [29,30]. We observed that ER stress by tunicamycin treatment induced the expression of BiP, an important chaperone involved in quality control. BiP was previously reported to decrease the activation of NF-κB [31], and is a potent extracellular anti-inflammatory molecule [32]. Thus, these molecules may be related to the underlining mechanism of the attenuated TLR4 signaling by ER stress involving decreased activation of NF-κB. Although some molecules related to ER stress, such as A20 and BiP, were previously reported to be involved in decreased NF-κB activation, we were unable to identify a specific mechanistic ER stress pathway directly related to impaired TLR4-induced expression of TNF-α and IL-1β. Impaired TLR signaling in the presence of tunicamycin was not observed following treatment with specific inhibitors of IRE-1, ATF6, or PERK (data not shown), suggesting that multiple pathways regulate this effect. Further investigation of how ER stress-related molecules are involved in inhibition of NF-κB under ER stress in terms of cellular machinery is, therefore, warranted.

Diabetes is associated with chronic inflammation [33,34]. Although smoldering inflammation is a fundamental pathological condition of diabetic patients, a characteristic of their immunity is the hypo-responsiveness to pathogenic stimulation [35]. We observed that NF-κB was, to some extent, activated in THP-1 cells under ER stress induced by tunicamycin before TLR4 ligand stimulation, despite the attenuated signal transduction to TLR ligand stimulation in monocytes (Fig. 4A). ER stress slightly increased the activated form of NF-κB in the quiescent condition before external ligand stimulation. This finding may correspond to the smoldering inflammation of diabetes, whereas the hypo-responsiveness to impaired TLR4 signaling under ER stress is consistent with the impaired immunity of diabetes. Further investigations are needed to elucidate the details of the chronic inflammation status and the systemic immunity condition.

Monocytes are progenitor cells that can differentiate into mature resident macrophages in various human tissues and become important immune regulators that control the acquired immune response [36]. Monocyte differentiation to macrophages is characterized as a reduction in the nucleocytoplasmic ratio [37] and enhanced granularity [38], which was observed in THP-1 cells (Fig. 5A) and primary human monocytes (Fig. 5C). The morphological maturity of macrophage differentiation was not observed in cells under tunicamycin-induced ER stress. Furthermore, the characteristic change of surface markers in the monocyte/macrophage lineage was also affected, showing decreased expressions of macrophage differentiation markers. A previous report suggested that the inhibition of NF-κB activation is involved in the impairment of monocyte differentiation into macrophages in THP-1 cells [39]. Therefore, the affected NF-κB activation and subcellular localization under ER stress may explain, in part, the affected differentiation capability of monocytes into macrophages.

We observed that TLR4 signaling was impaired in differentiated macrophages when they were under tunicamycin-induced ER stress (Fig. 6). Therefore, ER stress may broadly affect the monocytic lineage cells that express the pattern recognition molecules related to innate immunity, implying that ER stress is also a significant condition that broadly impairs host immunity.

In conclusion, our findings demonstrate that ER stress affects the pathogenic ligand-induced TLR signaling as a consequence of attenuated activation of NF-κB, as well as the differentiation capability into macrophages, which are the important functional regulators of the immune reaction. Further investigations are needed to elucidate the mechanisms of ER stress-related perturbations of the immune reaction in association with various diseases.

Conflict of interest

The authors declare no competing financial interests.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cellimm.2013.04.006.

References

[1] B. Kleizen, I. Braakman, Protein folding and quality control in the endoplasmic reticulum, Curr. Opin. Cell Biol. 16 (2004) 343–349.

[2] M. Aridor, W.E. Balch, Integration of endoplasmic reticulum signaling in health and disease, Nat. Med. 5 (1999) 745–751.
[3] C. Xu, B. Bailly-Maitre, J.C. Reed, Endoplasmic reticulum stress: cell life and death decisions, J. Clin. Invest. 115 (2005) 2656–2664.
[4] Y. Ma, L.M. Hendershot, The role of the unfolded protein response in tumor development: friend or foe?, Nat Rev. Cancer 4 (2004) 966–977.
[5] U. Ozcan, Q. Cao, E. Yilmaz, A.H. Lee, N.N. Iwakoshi, E. Ozdelen, G. Tuncman, C. Görgün, L.H. Glimcher, G.S. Hotamisligil, Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes, Science 306 (2004) 457–461.
[6] G.K. Hansson, Inflammation, atherosclerosis, and coronary artery disease, N. Engl. J. Med. 352 (2005) 1685–1695.
[7] J.P. Taylor, J. Hardy, K.H. Fischbeck, Toxic proteins in neurodegenerative disease, Science 296 (2002) 1991–1995.
[8] S. Akira, S. Uematsu, O. Takeuchi, Pathogen recognition and innate immunity, Cell 124 (2006) 783–801.
[9] C. Pasare, R. Medzhitov, Toll-like receptors: linking innate and adaptive immunity, Microbes. Infect. 6 (2004) 1382–1387.
[10] S. Gordon, P.R. Taylor, Monocytes and macrophage heterogeneity, Nat. Rev. Immunol. 5 (2005) 953–964.
[11] T. Komura, Y. Sakai, M. Honda, T. Takamura, K. Matsushima, S. Kaneko, CD14^{+} monocytes are vulnerable and functionally impaired under ER stress in patients with type 2 diabetes, Diabetes 59 (2010) 634–643.
[12] M. Tateno, M. Honda, T. Kawamura, H. Honda, S. Kaneko, Expression profiling of peripheral-blood mononuclear cells from patients with chronic hepatitis C undergoing interferon therapy, J. Infect. Dis. 195 (2007) 255–267.
[13] S. Thoma-Uszynski, S. Stenger, O. Takeuchi, M.T. Ochoa, M. Engele, P.A. Sieling, P.F. Barnes, M. Rollinghoff, P.L. Bolcskei, M. Wagner, S. Akira, M.V. Norgard, J.T. Belisle, P.J. Godowski, B.R. Bloom, R.L. Modlin, Induction of direct antimicrobial activity through mammalian toll-like receptors, Science 291 (2001) 1544–1547.
[14] M.S. Hayden, S. Ghosh, Shared principles in NF-kappaB signaling, Cell 132 (2008) 344–362.
[15] F. Wong, C. Hull, R. Zhande, J. Law, A. Karsan, Lipopolysaccharide initiates a TRAF6-mediated endothelial survival signal, Blood 103 (2004) 4520–4526.
[16] N.V. Serbina, T. Jia, T.M. Hohl, E.G. Pamer, Monocyte-mediated defense against microbial pathogens, Annu. Rev. Immunol. 26 (2008) 421–452.
[17] D. Ron, P. Walter, Signal integration in the endoplasmic reticulum unfolded protein response, Nat. Rev. Mol. Cell Biol. 8 (2007) 519–529.
[18] B. Bukau, J. Weisseman, A. Horwich, Molecular chaperones and protein quality control, Cell 125 (2006) 443–451.
[19] D.T. Rutkowski, R.J. Kaufman, A trip to the ER: coping with stress, Trends Cell Biol. 14 (2004) 20–28.
[20] N.N. Iwakoshi, M. Pypaert, L.H. Glimcher, The transcription factor XBP-1 is essential for the development and survival of dendritic cells, J. Exp. Med. 204 (2007) 2267–2275.
[21] X.Z. Wang, D. Ron, Stress-induced phosphorylation and activation of the transcription factor CHOP (GADD153) by p38 MAP kinase, Science 272 (1996) 1347–1349.
[22] K.D. McCullough, J.L. Martindale, L.O. Klotz, T.Y. Aw, N.J. Holbrook, Gadd153 sensitizes cells to endplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state, Mol. Cell. Biol. 21 (2001) 1249–1259.
[23] T. Gotoh, K. Terada, S. Oyadomari, M. Mori, Hsp70-DnaJ chaperone pair prevents nitric oxide- and CHOP-induced apoptosis by inhibiting translocation of Bax to mitochondria, Cell Death Differ. 11 (2004) 390–402.
[24] I. Tabas, D. Ron, Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress, Nat. Cell Biol. 13 (2011) 184–190.
[25] A. Iwasaki, R. Medzhitov, Toll-like receptor control of the adaptive immune responses, Nat. Immunol. 10 (2004) 987–995.
[26] I. Sabroe, L.C. Parker, S.K. Dower, M.K. Whyte, The role of TLR activation in inflammation, J. Pathol. 214 (2008) 126–135.
[27] S.L. Werner, J.D. Kearns, V. Zadorozhnaya, C. Lynch, C. OLynch, M.P. Boldin, A. Ma, D. Baltimore, A. Hoffmann, Encoding NF-kappaB temporal control in response to TNF: distinct roles for the negative regulators IkappaBalpha and A20, Gene Dev. 22 (2008) 2093–2101.
[28] K. Hayakawa, N. Hiramatsu, M. Okamura, H. Yamazaki, S. Nakajima, J. Yao, A.W. Paton, J.C. Paton, M. Kitamura, Acquisition of anergy to proinflammatory cytokines in nonimmune cells through endoplasmic reticulum stress response: a mechanism for subsidence of inflammation, J. Immunol. 182 (2009) 1182–1191.
[29] K. Heyninck, R. Beyaert, A20 inhibits NF-κB activation by dual ubiquitin-editing functions, Trends Biochem. Sci. 30 (2005) 1–4.
[30] A. Krikos, C.D. Laherty, V.M. Dixit, Transcriptional activation of the tumor necrosis factor alpha-inducible zinc finger protein, A20, is mediated by kappa B elements, J. Biol. Chem. 267 (1992) 17971–17976.
[31] S. Nakajima, N. Hiramatsu, K. Hayakawa, Y. Saito, H. Kato, T. Huang, J. Yao, A.W. Paton, J.C. Paton, M. Kitamura, Selective Abrogation of BiP/GRP78 Blunts Activation of NF-κB through the ATF6 Branch of the UPR: Involvement of C/EBP and mTOR-Dependent Dephosphorylation of Akt, Mol. Cell. Biol. 31 (2011) 1710–1718.
[32] V.M. Corrigall, M.D. Bodman-Smith, M. Brunst, H. Cornell, G.S. Panayi, Inhibition of Antigen-Presenting Cell Function and Stimulation of Human Peripheral Blood Mononuclear Cells to Express an Antiinflammatory Cytokine Profile by the Stress Protein BiP, Arthritis. Rheum. 50 (2004) 1164–1171.
[33] K.E. Wellen, G.S. Hotamisligil, Inflammation, stress and diabetes, J. Clin. Invest. 115 (2005) 1111–1119.
[34] M. Stumvoll, B.J. Goldstein, T.W. van Haeften, Type 2 diabetes: principles of pathogenesis and therapy, Lancet 365 (2005) 1333–1346.
[35] C.P. Liang, S. Han, T. Senokuchi, A.R. Tall, The macrophage at the crossroads of insulin resistance and atherosclerosis, Circ. Res. 100 (2007) 1546–1555.
[36] G. Siamon, R.T. Philip, Monocyte and macrophage heterogeneity, Nat. Rev. Immunol. 5 (2005) 953–964.
[37] R.J. Sokol, G. Hudson, N.T. James, I.J. Frost, J. Wales, Human macrophage development: a morphometric study, J. Anat. 151 (1987) 27–35.
[38] K.C. McCullough, S. Basta, S. Knötig, H. Gerber, R. Schaffner, Y.B. Kim, A. Saalmüller, A. Summerfield, Intermediate stages in monocyte-macrophage differentiation modulate phenotype and susceptibility to virus infection, Immunology 98 (1999) 203–212.
[39] Y.S. Chuang, S.H. Yang, T.Y. Chen, J.H. Pang, Cilostazol inhibits matrix invasion and modulates the gene expressions of MMP-9 and TIMP-1 in PMA-differentiated THP-1 cells, Eur. J. Pharmacol. 670 (2011) 419–426.

Mesenchymal Stem Cells and Their Subpopulation, Pluripotent Muse Cells, in Basic Research and Regenerative Medicine

YASUMASA KURODA[1]* AND MARI DEZAWA[1,2]
[1]Department of Anatomy and Anthropology, Tohoku University Graduate School of Medicine, Sendai, Japan
[2]Department of Stem Cell Biology and Histology, Tohoku University Graduate School of Medicine, Sendai, Japan

ABSTRACT

Mesenchymal stem cells (MSCs) have gained a great deal of attention for regenerative medicine because they can be obtained from easy accessible mesenchymal tissues, such as bone marrow, adipose tissue, and the umbilical cord, and have trophic and immunosuppressive effects to protect tissues. The most outstanding property of MSCs is their potential for differentiation into cells of all three germ layers. MSCs belong to the mesodermal lineage, but they are known to cross boundaries from mesodermal to ectodermal and endodermal lineages, and differentiate into a variety of cell types both in vitro and *in vivo*. Such behavior is exceptional for tissue stem cells. As observed with hematopoietic and neural stem cells, tissue stem cells usually generate cells that belong to the tissue in which they reside, and do not show triploblastic differentiation. However, the scientific basis for the broad multipotent differentiation of MSCs still remains an enigma. This review summarizes the properties of MSCs from representative mesenchymal tissues, including bone marrow, adipose tissue, and the umbilical cord, to demonstrate their similarities and differences. Finally, we introduce a novel type of pluripotent stem cell, multilineage-differentiating stress-enduring (Muse) cells, a small subpopulation of MSCs, which can explain the broad spectrum of differentiation ability in MSCs. Anat Rec, 00:000–000, 2013. © 2013 Wiley Periodicals, Inc.

Key words: tissue engineering; stem cell; regenerative

INTRODUCTION

Our body is comprised of various kinds of tissues and cells, and all of their origins converge on a single cell, namely the zygote. The zygote undergoes cell division and develops into the blastocyst that contains the inner cell mass. Cells in the inner cell mass commit to any of the three germ layers, ectoderm (which mainly develops into the epidermis and nervous system), endoderm (including the liver, pancreas, and lung) or mesoderm (the remaining tissues including blood, bone, and bone marrow, adipose tissue, and connective tissues). Mesenchymal stem cells (MSCs), the topic of this review, belong to the mesodermal lineage and are tissue stem cells that reside in various kinds of mesenchymal tissues

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*Correspondence to: Yasumasa Kuroda, PhD, Department of Anatomy and Anthropology, Tohoku University Graduate School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan. Fax: +81-22-717-8030.
E-mail: y-kuroda@med.tohoku.ac.jp

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i.e., bone marrow, adipose tissue, the umbilical cord, dermis, dental pulp, and synovia. In this review, we focus on three representative MSC types derived from bone marrow, adipose tissue and the umbilical cord, which have been intensely studied both in basic research and clinical applications for the past decade.

MSCs are known to have pleiotropic actions. They exert trophic and anti-inflammatory effects on damaged tissues by producing a variety of factors and cytokines that act to protect tissues, but also modulate immunological reactions, which is the basis for their application in the treatment of graft-versus-host disease (GVHD) (Fang et al., 2007). Another important property of MSCs is the broad spectrum of differentiation beyond the boundaries between germ layer lineages (Prockop, 1997; Dezawa et al., 2001; Oyagi et al., 2006). Generally, tissue stem cells generate the cell types of the tissue in which they reside, and the range of their differentiation capabilities is limited. Hematopoietic stem cells generate blood cells, and neural stem cells generate neural lineage cells (Reynolds and Weiss, 1992; Osawa et al., 1996). In this context, the differentiation potential of MSCs is exceptional and has lead to a debate over the past decade concerning whether MSCs are pluripotent or not. Pittenger et al. (1999) showed that bone marrow derived-MSCs (BM-MSCs) are multipotent and able to differentiate into some kinds of mesodermal lineage cells such as osteoblastic, chondrocytic and adipocytic cells by treatment with certain cytokines and reagents. Because MSCs belong to the mesodermal lineage, this phenomenon appears to be reasonable. However, further studies revealed that, using cytokine induction and/or gene introduction, MSCs differentiate *in vitro* into cells of other lineages including endodermal (hepatocytes and insulin-producing cells) and ectodermal lineages (neuronal, peripheral glial and epidermal cells) (Prockop, 1997; Dezawa et al., 2001, 2004; Oyagi et al., 2006; Wu et al., 2006; Karnieli et al., 2007). In addition, a rare subpopulation of MSCs has been reported to spontaneously differentiate into mesodermal (cardiomyocytes), ectodermal (keratinocytes) and endodermal cells (hepatocytes) *in vivo* according to the local microenvironment in which they integrate to ultimately contribute to tissue repair (Terai et al., 2002; Orlic et al., 2003; Tamai et al., 2011). However, the important point is that the ratios of these differentiations are generally low and, therefore, putative pluripotent cells, if they exist among MSCs, are considered to correspond to a small number of MSCs.

Besides our basic understating of MSCs, they have gained a great deal of attention in the expectation of their contribution to regenerative medicine because of several beneficial aspects. MSCs can be collected from easily accessible tissues, such as bone marrow and the umbilical cord, and can be stably expanded to a large number of cells within a reasonable time period. Unlike embryonic stem (ES) or fetal stem cells, the collection of MSCs does not involve the use of fertilized eggs or aborted fetuses, respectively, which avoids ethical concerns. Most importantly, MSCs do not have a tumorigenic proliferative activity and, thus, they are considered one of the most suitable stem cell types for cell based-therapy. In fact, some clinical trials have been diligently conducted using MSCs (Horwitz et al., 1999; Terai et al., 2003; Gordon et al., 2006).

Three mesenchymal tissues, bone marrow, adipose tissue, and the umbilical cord, have their own distinct anatomical structures. Bone marrow is located inside of bones and consists of blood vessels, specialized vessels called sinusoids, and a sponge-like network of hemopoietic cells (Fig. 1A), and has two functional compartments, namely hematopoietic and nonhematopoietic compartments. Mesenchymal cells in the bone marrow (BM-MSCs) are located in the nonhematopoietic compartment. The umbilical cord is filled with Wharton's jelly, loose connective tissue, and has three blood vessels; two umbilical arteries and one umbilical vein. Umbilical cord-derived MSCs (UC-MSCs) are mainly collected from Wharton's jelly, but can be also collected from tissues around the blood vessels as well as umbilical cord blood (Fig. 1B). Adipose tissue is comprised mainly of adipocytes, blood capillaries and small volume of connective tissues (Fig. 1C), and adipose-derived MSCs (AD-MSCs) are considered to be localized in the connective tissue around blood capillaries.

While the anatomical structures are completely distinct among these tissues, each MSC type shares common characteristics. For example, all BM-MSCs, UC-MSCs, and AD-MSCs show similar morphology (Fig. 1D–F). In addition, they are known to provide trophic immunosuppressive and anti-inflammatory effects, and a broad spectrum of differentiation, which spans from mesodermal- to ectodermal- and endodermal-lineage cells. Despite these similarities, differences among BM-MSCs, UC-MSCs and AD-MSCs exist in their cell surface marker expression, responses to cytokines and reagents, and differentiation propensity (Pittenger et al., 1999; Zuk et al., 2002; Gimble et al., 2007, Troyer and Weiss, 2008).

In this review, we focus on the similarities and differences of these representative MSC types, and introduce the recently found pluripotent stem cell type, multilineage-differentiating stress-enduring (Muse) cells, which correspond to one to several percent of MSCs, and may explain the triploblastic differentiation of MSCs (Kuroda et al., 2010; Wakao et al., 2011). Finally, the perspective of MSCs and Muse cells for regenerative medicine is discussed.

Background of MSCs

BM-MSCs collected from bone marrow have been studied for a long time. Till and McCulloch first reported cloning of bone marrow cells in 1961 (Till and McCulloch, 1961). In the 1970s, Friedenstein et al. referred to cells that are adherent, clonogenic, non-phagocytic and fibroblastic as colony-forming unit-fibroblasts (CFU-Fs), and analyzed them ex vivo. The studies conducted by Friedenstein revealed that the cells were able to differentiate into mesodermal cells including osteocytes, chondrocytes and adipocytes (Friedenstein et al., 1970; Friedenstein et al., 1974; Friedenstein et al., 1976). In subsequent studies, these bone marrow cells were given many different names that caused confusion in this area. In 1987, Owen et al. named these cells "marrow stromal stem cells", and Caplan et al. used the term "mesenchymal stem cells" in 1991, which became the generally used term in later studies (Owen et al., 1987; Caplan et al., 1991).

The general method to collect BM-MSCs from bone marrow is simple. Either bone marrow aspirates or mononucleated cells isolated by a Ficoll gradient are suspended in culture medium containing 10% bovine serum

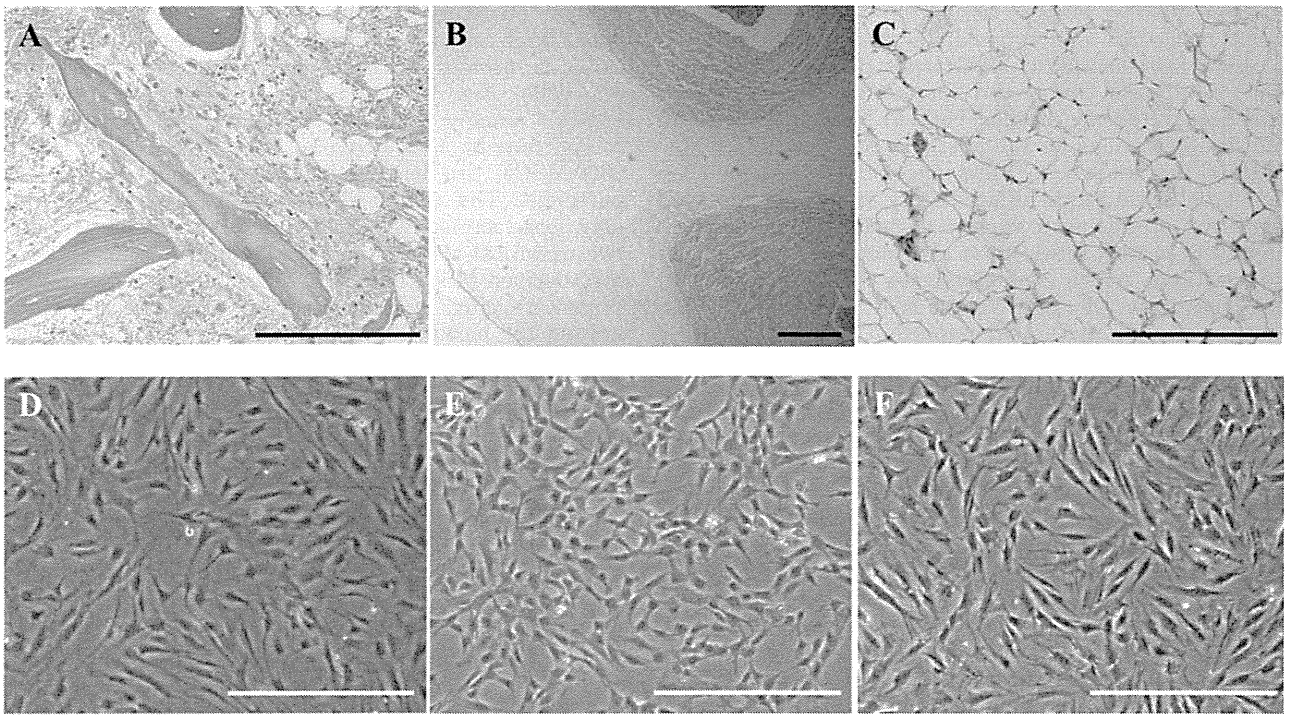

Fig. 1. Characteristic of human bone marrow, adipose tissue, and umbilical cord. (**A–C**) HE staining of human (A) bone marrow (B) umbilical cord and (C) adipose tissue. (**D–F**) Cell morphology of mesenchymal cells isolated from human (D) bone marrow (E) umbilical cord and (F) adipose tissue. Scale bar = 500 μm.

and then cultured adherently on plastic dishes. Hematopoietic stem cells, which are normally present in bone marrow aspirates, may contaminate the primary cultured cells. However, repeated washing and passaging usually eliminates hematopoietic stem cells that will not remain in adherent culture for a long period (Pittenger et al., 1999).

UC-MSCs have great advantages over other MSC types because they can be easily collected from donors during childbirth without harm, unlike bone marrow aspiration and liposuction. UC-MSCs were first isolated from umbilical cord blood (Ye et al., 1994), and in 2002, Hoerstrup et al. successfully established MSCs from Wharton's jelly (Hoerstrup et al., 2002). Since then, MSCs isolated from Wharton's jelly have been generally studied as UC-MSCs. Unlike MSCs from other tissues, UC-MSCs can be isolated from samples after 48 hr from collection at a high efficiency. Thus, the umbilical cord is very useful as a cell source for collection of MSCs. When collecting MSCs from umbilical cord blood, cells that adhere to a plastic dish are considered as UC-MSCs, as is the case with BM-MSCs. To isolate UC-MSCs from Wharton's jelly, minced tissues are placed on a plastic dish, and the cells that migrate from the tissues are considered as UC-MSCs (Troyer and Weiss, 2008).

Even though research has only begun recently, adipose tissue is the most notable tissue as a source of MSCs because adipose tissue is obtained easily. In the United States, liposuction surgery is performed more than 400,000 times per year, and adipose tissue is easily collectable from the 100 ml to 3 l per treatment (Katz et al., 1999), which is usually discarded. Collecting MSCs from these tissues was first reported by Zuk et al. They isolated fibroblast-like cells by treating liposuction aspirates or finely minced adipose tissue with collagenase, and then the isolated cells were shown to differentiate into adipocytes, osteocytes, chondrocytes, and myocytes *in vivo* (Zuk et al., 2001). After this report, these cells were named "adipose-derived stem cells" (Zuk et al., 2002), and the widely used method to collect AD-MSCs is based on the original method by Zuk et al.

Surface Marker Expression

Essentially, the formation of MSCs, collection efficiency and ratio of colony formation do not differ largely among BM-MSCs, UC-MSCs and AD-MSCs (Izadpanah et al., 2006; Kern et al., 2006). In terms of surface markers, MSCs from each tissue commonly express CD29, CD73, CD90 and CD105, and are negative for CD45 and CD56 (Table 1) (Pittenger et al., 1999; Zuk et al., 2002; Gimble et al., 2007, Troyer and Weiss, 2008). However, there are some differences among them. Whereas both BM-MSCs and UC-MSCs are positive for CD106, AD-MSCs are negative for this marker (Zuk et al., 2002; Kern et al., 2006). In contrast, AD-MSCs express CD34, whereas both BM-MSC and UC-MSCs are negative (Pittenger et al., 1999; Gimble et al., 2007; Troyer et al., 2008). The differences in surface marker expression can be explained by several factors. For example, slight differences in the collection method, quality of serum, methods of maintaining the cultured cells, and donors may cause differences in the composition and characters of MSC populations. In addition, the species may be a factor that causes differences in surface marker expression. In fact, human and rat BM-MSCs

TABLE 1. Comparison of the protein expression profile in human mesenchymal stem cells derived from bone marrow, umbilical cord, and adipose tissue.

		BM-MSCs	UC-MSCs	AD-MSCs
Common	CD10	○	○	○
	CD13	○	○	○
	CD29	○	○	○
	CD44	○	○	○
	CD49	○	○	○
	CD73	○	○	○
	CD90	○	○	○
	CD105	○	○	○
	MHC Class I	○	○	○
	SSEA-4	○	○	○
	CD14	×	×	×
	CD31	×	×	×
	CD45	×	×	×
	CD56	×	×	×
	CD144	×	×	×
	MHC Class II	×	×	×
Uncommon	CD9			○
	CD22	○		
	CD34			○
	CD51	○		
	CD54	○		○
	CD55			○
	CD59			○
	CD64a	○		
	CD71	○		○
	CD106	○	○	
	CD133	○		
	CD140b	○		
	CD146	○		○
	CD166	○		○
	CD271	○		
	CD340	○		
	CD349	○		
	ESG1		○	
	GD2 Syntase	○	○	
	LIFr		○	
	SCF		○	
	SSEA-1	○		
	Stro-1	○		
	Telomerase		○	
	Tra-1-60		○	

are negative for CD34, whereas mouse MSCs are positive. Human UC-MSCs express CD49e and CD105 at a high level in early passages, but this expression level decreases in later passages (Weiss et al., 2006). Therefore, the surface expression pattern of MSCs differs according to the species, origin, and various factors involving their collection, maintenance, and culture period. Furthermore, the expression pattern of markers analyzed *in vitro* does not always reflect the characteristics *in vivo*.

Trophic Effect

BM-MSCs, UC-MSCs, and AD-MSCs have been reported to show a profound effect on wound healing. One of the mechanisms of this effect is the secretion of various cytokines and trophic factors such as fibroblast growth factor (FGF)-2, vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), brain derived-neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), nerve growth factor (NGF), stromal cell derived factor (SDF)-1, interleukin (IL)-6, IL-8, and IL-11. However, the ability to secrete these factors is not equal among MSC types. For example, in contrast to BM-MSCs, UC-MSCs, and AD-MSCs can produce granulocyte-macrophage colony stimulating factor and granulocyte colony stimulating factor. In addition, the expression level of some factors, such as HGF, IL-6 and IL-8, in BM-MSCs is less than that in other MSC types (Crigler et al., 2006; Yoshihara et al., 2007; Fan et al., 2011). Several groups have examined whether undifferentiated or "naive" MSCs can promote wound healing. Transplantation of autologous BM-MSCs promotes wound healing in skin-incised mice (Falanga, 2007). MSCs also show a therapeutic effect in the functional recovery of central nervous system (CNS) damage such as cerebral infarction and spinal cord injury by secretion of neuroprotective tropic factors (Yoshihara et al., 2007; Kawabori et al., 2012). AD-MSCs combined with an atelocollagen matrix enhance the healing of rat skin damage induced by mitomycin C (Nambu et al., 2007). Moreover, AD-MSCs avoid skin flap necrosis caused by inadequate blood supply and improve the cell viability (Lu et al., 2008), as well as promote periodontal tissue regeneration (Tobita et al., 2008). Similar effects have been observed by the transplantation of AD-MSCs into a rat model of hindlimb ischemia (Miranville et al., 2004; Cao et al., 2005), a mouse model of myocardial infarction (Fraser et al., 2006), and skin ulcers in a diabetic mouse model (Nambu et al., 2009).

UC-MSCs are also reported to have a therapeutic trophic effect. For example, UC-MSCs derived from umbilical cord blood or Wharton's jelly are able to promote cutaneous wound healing (Luo et al., 2010; Zhang et al., 2012). Transplantation of umbilical cord blood cells that include UC-MSCs can improve neurological and motor deficits resulting from hypoxic brain ischemia (Rosenkranz et al., 2013). All of the above effects are attributable mainly to the trophic effects of transplanted MSCs.

Anti-Inflammatory and Immunosuppressive Effects

Recent reports have revealed that each MSC type shows not only promotion of wound healing but also anti-inflammatory effects by cytokine secretion. For example, BM-MSCs decrease inflammation by secretion of soluble tumor necrosis factor receptor 1 (sTNFR1) when they were transplanted into lipopolysaccharide-induced endotoxemic model rats (Yagi et al., 2010). Similarly, transplanted BM-MSCs suppress inflammatory reactions in ischemic cardiac muscle by the expression of TNF-α stimulated gene/protein 6 (TSG-6) (Wisniewski et al., 2004). Moreover, BM-MSCs can rescue damaged cells from apoptosis by secretion of stanniocalcin-1 (Block et al., 2009).

MSCs are also known to have immunosuppressive effects by secretion of anti-inflammatory cytokines such as IL-10. Proliferation of T cells in mixed lymphocyte culture has been shown to be suppressed by co-culture

TABLE 2. Differentiation potential of human mesenchymal stem cells derived from bone marrow, umbilical cord, and adipose tissue.

		BM-MSCs	UC-MSCs	AD-MSCs
Mesodermal cells	Osteoblasts	O	O	O
	Adipocytes	O	O	O
	Chondrocytes	O	O	O
	Endothelial cells	O	O	O
	Skeletal muscle cells	O	O	O
	Cardiac muscle cells	O	O	O
	Smooth muscle cells	O	O	O
	Epithelial cells	O	O	O
	Tenocytes	O		
Ectodermal cells	Neuronal cells	O	O	O
	Peripheral glia cells	O	O	O
Endodermal cells	Pancreatic cells	O	O	O
	Hepatocytes	O		O

with MSCs, even though they were not HLA- matched (Dao et al., 2011).

Taken together, the trophic, anti-inflammatory, and immunosuppressive effects by cytokine secretion give MSCs their remarkable indirect healing effects.

Differentiation Ability

In addition to cytokine effects, all BM-MSCs, UC-MSCs, and AD-MSCs can differentiate into various types of cells, and there are no obvious differences among their differentiation potentials (Izadpanah et al., 2006; Kern et al., 2006) (Table 2). Well-known differentiated cell types from the above types of MSCs are osteocytes, adipocytes and chondrocytes. Adipocytes are differentiated by treatment with 1-methyl-3-isobutylxanthine, dexamethasone, insulin and indomethacin, osteoblasts by dexamethasone, b-glycerol phosphate and ascorbate, and chondrocytes by TGF-β stimulation plus mechanical stimulation (Prockop, 1997; Kuznetsov et al., 1997; Bruder et al., 1997; Pittenger et al., 1997; Mackay et al., 1998; Izadpanah et al., 2006; Kern et al., 2006). Differentiation of these three cell types of the mesodermal lineage demonstrates the multipotency of MSCs. In addition, BM-MSCs can differentiate into other mesodermal cell types such as skeletal muscle cells by treatment with a combination of three kinds of cytokines followed by gene transfection with Notch intracellular domain (NICD) (Dezawa et al., 2005), smooth muscle cells by TGF-β treatment (Yan et al., 2011), endothelial cells by stimulation with VEGF, and cardiac muscle cells by 5-azacytidine treatment (Makino et al., 1999). BM-MSCs can also be induced into other germ layer cell types such as those of the ectodermal lineage. For example, neuronal cells are induced by gene transduction of NICD, and then treatment with three kinds of cytokines (Dezawa et al., 2004). Peripheral glial cells are induced by treatment with β-mercaptoethanol followed by retinoic acid, forskolin, basic (b) FGF, platelet-derived growth factor and heregulin (Dezawa et al., 2001). BM-MSCs can also differentiate into endodermal lineage cells as observed by hepatocyte differentiation using HGF (Oyagi et al., 2006) and pancreatic cell differentiation using platelet lysate, retinoic acid, activin, glucagon-like peptide I, and cytokines (Zanini et al., 2011).

AD-MSCs can differentiate into skeletal muscle cells by treatment with dexamethasone and hydrocortisone (Zuk et al., 2001), as well as smooth muscle cells (Rodriguez et al., 2006) and endothelial cells (Cao et al., 2005) using other combinations of cytokines. In addition, AD-MSCs can differentiate into epithelial cells using all-trans retinoic acid (Brzoska et al., 2005), neuronal cells using valproic acid, butylated hydroxyanisole, insulin and hydrocortisone (Safford et al., 2002), peripheral glial cells by factors basically identical to a cocktail reported by Dezawa et al. (Dezawa et al., 2001; Xu et al., 2008), hepatocytes using HGF, OSM and DMSO (Seo et al., 2005), and pancreatic cells using glucose, nicotinamide, activin, exendin and HGF (Timper et al., 2006).

UC-MSCs can also differentiate into neuronal cells and peripheral glial cells (Zhang et al., 2009) by cytokine treatment (Liang et al., 2012), hepatocytes using HGF and FGF-4 (Zhang et al., 2009), and pancreatic cells by transient transfection of *Pdx1* (Fedyunina et al., 2011).

Thus, the potential for triploblastic differentiation exists in BM-MSCs, UC-MSCs, and AD-MSCs.

Accessibility of MSCs

Gimble et al. suggested that stem cells for regenerative medicinal applications should ideally meet the following criteria (Gimble, 2003, 2007):

1. Can be found in abundant quantities (millions to billions of cells)
2. Can be harvested by a minimally invasive procedure
3. Can be differentiated along multiple cell lineage pathways in a regulated and reproducible manner
4. Can be safely and effectively transplanted into either an autologous or allogeneic host
5. Can be manufactured in accordance with current Good Manufacturing Practice guidelines

Previous studies indicate that MSCs meet these criteria. In the case of BM-MSCs, 10 ml of bone marrow aspirate contains approximately 7×10^5 cells expressing CD105 that is a general marker of MSCs including BM-MSCs (this quality corresponds to criterion 1). The collection method meets the safety requirements and bone marrow aspirates can be collected with comparative ease, so that BM-MSCs are easily accessible cells with abundant quantities within a reasonable time period (corresponding to criterion 2). BM-MSCs can differentiate into cells of all three germ layers as described above (corresponds to criterion 3), and they can be safely and effectively transplanted into autologous/allogeneic hosts (corresponds to criterion 4) (Wang et al., 2012).

For UC-MSCs, 80 ml of cord blood contains approximately 2.1×10^2 MSCs (corresponds to criterion 1), and the umbilical cord can be obtained during childbirth without harm, so that it is the most easily accessible source to obtain MSCs (corresponds to criterion 1).

AD-MSCs are yielded at only $\sim 5 \times 10^3$ cells from 1 g of adipose tissue (corresponds to criterion 1), but it is possible to obtain a sufficient amount of AD-MSCs in a single liposuction procedure that obtains 100 ml to 3 l of adipose tissue. Adipose tissue is easily collected by cosmetic surgery and liposuction, and these surgeries are performed more than 400,000 per year.

Both UC-MSCs and AD-MSCs can differentiate into various kinds of cells, as observed with BM-MSCs (corresponds to criterion 3), and can be transplanted into autologous/allogeneic hosts (corresponds to criterion 4).

Therefore, BM-MSCs, UC-MSCs, and AD-MSCs meet the criteria for application in regenerative medicine.

Clinical Trials of MSCs

Generally, MSCs have an immunosuppressive effect as mentioned above. Such properties of MSCs have led them to application in the treatment of GVHD. GVHD is a complication that accompanies allogeneic tissue transplantation such as bone marrow transplantation, and is caused by immunological attack of the host cells by the transplanted immune cells. In the case of bone marrow transplantation, GVHD is induced with a probability of approximately 80% in cases of a HLA mismatch. However, even if the HLA is matched, the risk of GVHD still remains at 30 to 40%. GVHD leads to the death of recipients in the worst cases and, thus, suppression and control of GVHD is an important urgent matter for bone marrow transplantation. Steroids are generally used for the treatment of GVHD patients, but some cases are resistant. In the latter cases, MSCs are administrated to patients for potent immunosuppressive effects. Fang et al. reported a complete reversal of GVHD by administration of AD-MSCs, and almost all patients who received AD-MSC treatment survived without any side effects after a follow-up period of 40 months (Fang et al., 2007). The notable point in this clinical trial is that HLA matching between donors and recipients was not performed for the trial and, thus, suppression of GVHD was achieved in HLA-mismatched allo-transplantation. The same effect is also expected for BM-MSCs and UC-MSCs and, therefore, already operating marrow and cord banks are expected to contribute to allo-transplantation of MSCs for the treatment of GVHD.

In addition to GVHD, MSCs are used for treating other diseases. BM-MSCs were the first MSCs to be used in clinical trials and have accumulated many promising results. In 1999, Horwitz et al. first applied allogeneic BM-MSCs to children with osteogenesis imperfecta, which led to the improvement of total-body bone mineral content and subsequent osteogenesis (Horwitz et al., 1999). Other groups used autologous BM-MSCs in clinical trials for treating patients with liver cirrhosis, which resulted in increases of hepatocyte proliferation and an improvement of bilirubin as well as albumin levels, and finally led to clinical improvement of the liver cirrhosis (Terai et al., 2002, 2003; Gordon et al., 2006). BM-MSCs have also been applied to acute myocardial infarction patients, resulting in efficient cardiac function recovery for up to 1 year (Misao et al., 2006; Schachinger et al., 2009). In 2005, Park et al. reported the first trial of autologous BM-MSCs in the treatment of spinal cord injury patients, and demonstrated an improvement in functional recovery (Park et al., 2005).

Clinical trials of UC-MSCs have been mainly performed for allo-transplantation of cord blood that includes UC-MSCs. Haller et al. reported that cord blood transplantation in patients with type 1 diabetes can attenuate the symptoms (Haller et al., 2008). In the case of UC-MSCs alone, transplantation into patients with decompensated liver cirrhosis was performed, resulting in an improvement of liver function and reduction of ascites in patients (Zhang et al., 2012).

The first clinical trials with AD-MSCs were performed in 2004. Autologous AD-MSCs were used to treat a 7-year-old girl with post-traumatic calvarial defects by infusion of AD-MSCs with an autologous fibrin glue (Lendeckel et al., 2004), resulting in almost complete calvarial continuity at 3 months post-transplantation. Autologous AD-MSCs have also been applied to patients with Crohn's disease (García-Olmo et al., 2005). In such cases, 75% of transplanted sites were repaired, and 30 months of follow-up demonstrated no obvious side effects. Furthermore, autologous AD-MSCs have been used to repair tracheomediastinal fistulas caused by cancer ablation (Alvarez et al., 2008). In these cases, no side effects or complications were observed during the follow-up period, and re-epithelialization and neovascularization led to successful closure of the fistula. Recently, AD-MSCs have been used in plastic surgery such as breast reconstruction and fat supplementation for patients with facial fat atrophy (Yoshimura et al., 2008).

Many clinical trials of patients with various diseases have been performed and no tumorigenesis has been reported thus far. This is a very important finding in terms of clinical application, because the generation of tumors has been observed after transplantation of fetal neural stem cells into a boy with ataxia telangiectasia (Amarglio et al., 2009). ES and induced pluripotent stem (iPS) cells are also expected to be used clinically, although their use in patients is limited by the fact that they are immortal cells with a serious risk of causing tumors and malignancies. The potential risks posed by the uncontrolled and unstable genomes of both ES and iPS cells have been emphasized by a recent study that demonstrated a large number of mutations acquired by the cells (Laurent et al., 2011). Because MSCs have a limited lifespan in culture, their use in patients presents a limited risk of tumorigenicity (Prockop et al., 2010). Therefore, MSCs have great advantages over fetal stem cells, as well as ES and iPS cells because of their non-tumorigenicity, ensuring that MSCs are applicable to patients.

Another issue is the effectiveness of MSCs for curing diseases in terms of tissue regeneration. As mentioned above, the regenerative effects exerted by BM-MSCs, UC-MSCs, and AD-MSCs are not always consistently successful. Many trials of MSCs have reported partial improvement, whereas some trials have shown no effect. Carrion et al. applied BM-MSCs to patients with human systemic lupus erythematosus, but no improvements were observed (Carrion et al., 2010). In this trial, no adverse effects or progression of disease activity were noted during the 14 weeks of follow-up, except one case that finally developed overt renal disease after infusion. The cause of the ineffectiveness of MSCs in such cases is still unclear. However, the accumulation of data may lead to an in-depth understanding of MSCs.

When naive MSCs are applied, a curative effect can be observed to some extent, which is attributed mainly to the trophic effect of MSCs. The cytokines and factors produced by transplanted MSCs rescue the damaged tissue. However, MSCs do not remain in the tissue for a long time unless they differentiate and integrate into the tissue. Therefore, such trophic effects do not last for many months. In addition to the trophic effect, there is evidence to support that a small number MSCs are pluripotent cells. When naive MSCs are infused into the blood stream, a very small number of them integrate into the damaged tissue and spontaneously differentiate

into tissue-specific cells. If such cells are identified, efficient regenerative treatment can be expected.

Muse Cells, Intrinsic Pluripotent Stem Cells that Reside Among MSCs

Besides trophic, immunosuppressive and anti-inflammatory effects, the scientific basis for the broad spectrum of differentiation by MSCs, which crosses the oligolineage boundaries between mesoderm and ectoderm or endoderm, is not yet clarified.

Several reports have claimed to discover pluripotent stem cells among bone marrow cells. Jiang et al. reported that MSCs derived from adult bone marrow, which they named multipotent adult progenitor cells (MAPCs), are pluripotent stem cells that show triploblastic differentiation (Jiang et al., 2002). However, the isolation of MAPCs has not been reproduced by other independent laboratories and, thus, their existence is now considered doubtful. Kucia et al. reported that they found pluripotent stem cells expressing some ES cell markers, stage specific embryonic antigen (SSEA)-1, Oct4, Nanog and Rex-1, in both adult mouse bone marrow and human cord blood, which they named very small embryonic-like (VSEL) stem cells (Kucia et al., 2006; Wojakowski et al., 2011). However, a recent report by Danova-Alt et al. demonstrated that the transcriptional profile of VSEL cells derived from human cord blood is clearly distinct from those of well-defined populations of pluripotent and adult stem cells, and mostly show an aneuploid karyotype, which questions the existence of pluripotent stem cells in umbilical cord blood (Danova-Alt et al., 2012). In both reports, there was no description of objective markers to isolate such cells and the methods that identified the cells were not specific enough to independently reproduce their data.

Even though the existence of MAPCs and VSEL stem cells is equivocal, these reports instigated the exploration of putative pluripotent stem cells that reside among MSCs. Many attempts have been made to identify pluripotent stem cells, but there are major problems to be overcome in MSC research.

First, the two main properties of pluripotent stem cells, namely triploblastic differentiation and self-renewal, need to be shown at a single cell level. Because MSCs are generally collected as adherent cells from bone marrow, adipose tissue, or the umbilical cord, they are crude heterogenous cell populations comprised of several kinds of cells. There are several reports showing the differentiation of crude bulk MSC populations into ectodermal, endodermal and mesodermal lineage cells. Because the cells were in crude populations, triploblastic differentiation from a single kind of cell could not be proven in the strict sense. Thus, from the viewpoint of basic science, there is a strong need to prove triploblastic differentiation and self-renewal at a single cell level.

Secondly, several reports have demonstrated that not all MSCs have a wide differentiation ability. For example, 10 to 25% of BM-MSCs can differentiate into alkaline phosphatase (ALP)-positive osteocytes (Birmingham et al., 2012), and differentiation across germ layers without gene transfer is much lower. Approximately 3% of MSCs differentiate into microtubule-associated protein 2 (MAP2)-positive neurons by co-culture with neurons (Hokari et al., 2008), and ~5% of MSCs differentiate into insulin-positive β cells by stimulation with cytokines such as bFGF and epidermal growth factor (EGF) under a high glucose condition (Gabr et al., 2013). These results suggest that not all MSCs participate in triploblastic differentiation and, therefore, only a small subpopulation of MSCs are suggested to possess pluripotency.

Another possibility is that MSC populations contain several kinds of unipotent/bipotent stem cells each responsible for ectodermal, mesodermal, and endodermal lineage differentiation. In this case, individual MSCs are not pluripotent, but whole MSC populations resemble a pluripotent population of cells.

While there are many debates regarding the pluripotency of MSCs, Kuroda et al. recently demonstrated that BM-MSCs and another type of mesenchymal cell, dermal fibroblasts, include pluripotent stem cells that can self-renew and individually differentiate into cells representative of all three germ layers. These cells were found by their stress resistance, and were named "multilineage-differentiating stress-enduring (Muse)" cells (Kuroda et al., 2010).

Unique Properties of Muse Cells

Muse cells were recently identified in adult human mesenchymal tissues such as bone marrow and the dermis, and also among cultured mesenchymal cells such as BM-MSCs and dermal fibroblasts (Kuroda et al., 2010; Wakao et al., 2011). Muse cells are legitimate mesenchymal cells, which exhibit a morphology identical to those of typical mesenchymal cells such as fibroblasts, and express common mesenchymal markers CD105, CD90, and CD29. However, Muse cells are unique because they also show pluripotent stem cell properties such as the expression of pluripotency markers, self-renewal and triploblastic differentiation, indicating that Muse cells are both pluripotent and mesenchymal cell-like. These properties can be demonstrated by their marker expression. Muse cells express both pluripotency and mesenchymal stem cell markers. They can be isolated from tissues or cultured cells as cells that are double positive for SSEA-3, a well-known marker of the undifferentiated state of human ES cells, and CD105, a mesenchymal stem cell marker (Kuroda et al., 2010) (Fig. 2).

Muse cells as Nontumorigenic Pluripotent Stem Cells

When Muse are isolated and maintained as a single cell-suspension culture, which is often used for tissue stem cell culture, they proliferate and form cell clusters that are very similar to ES cell-derived embryoid bodies. The clusters express pluripotency markers, Nanog, Oct3/4 and Sox2, and are positive for ALP. Importantly, a single cell-derived cluster differentiates into endodermal (α-fetoprotein and cytokeratin 7), ectodermal (neurofilament) and mesodermal (smooth muscle actin, and desmin) marker-positive cells when cultured on gelatin, demonstrating that the original single cell possesses a triploblastic differentiation ability. Furthermore, when single Muse cell-derived clusters are expanded, SSEA-3/CD105 double positive cells can be collected again and have been shown to differentiate into endodermal, ectodermal and mesodermal cells from single cells. This cycle can be repeated, demonstrating that Muse cells

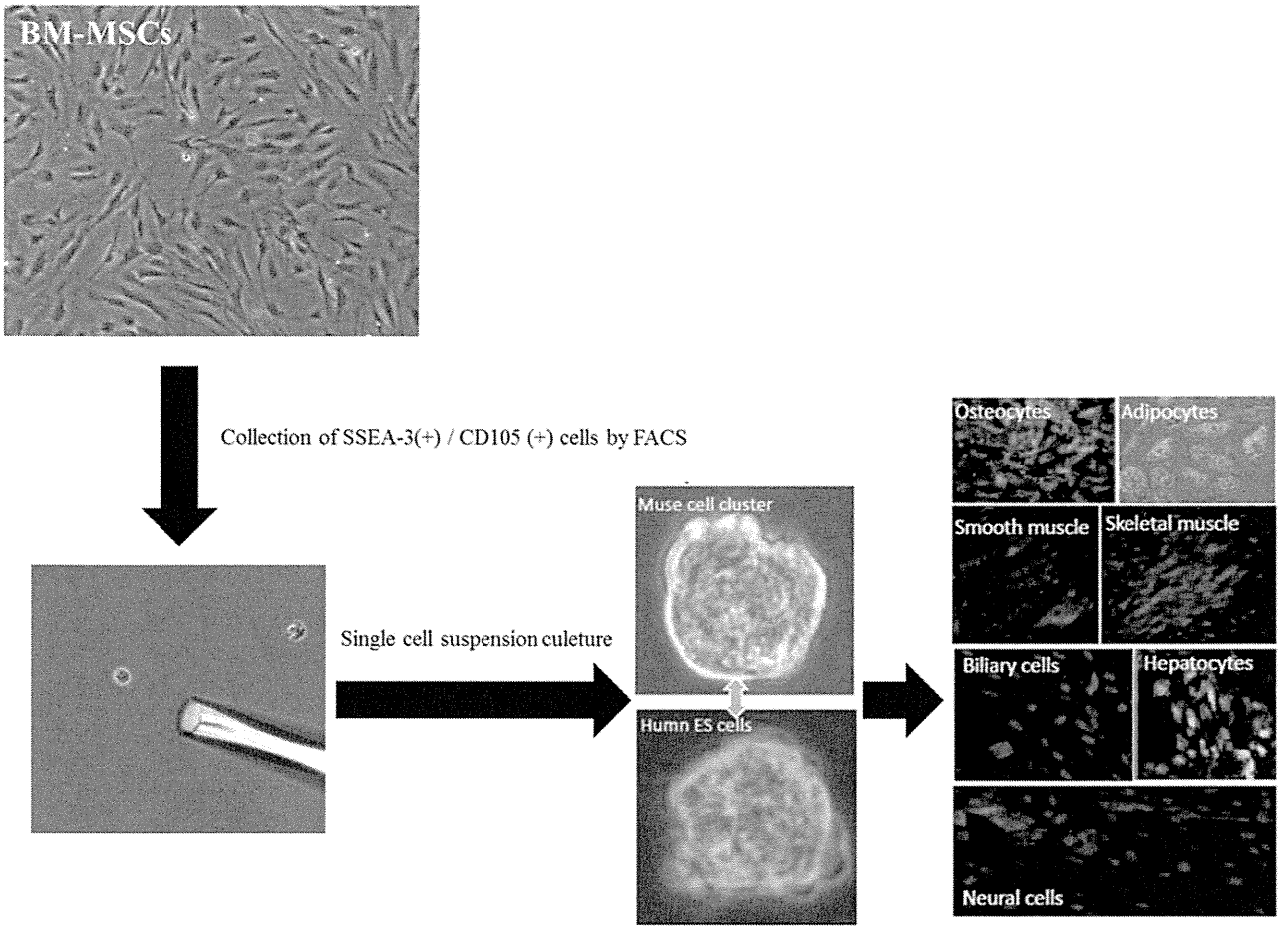

Fig. 2. Schema for isolation of Muse cells. Muse cells can be isolated from mesenchymal cells such as BM-MSCs and dermal fibroblasts as cells that are double positive for SSEA-3 and CD105. They can form clusters that are very similar to ES cell-derived embryoid bodies during maintained as a single cell-suspension culture, and they can differentiate into cells representative of three germ layers. Pictures adapted from Kuroda et al. (2010) with permission from The National Academy of Science, and adapted from Wakao et al. (2011) with permission from The National Academy of Science.

have a self-renewal ability (Kuroda et al., 2010). Thus, Muse cells are considered to correspond to putative pluripotent stem cells among BM-MSCs.

One of the great advantages of Muse cells is that they have no tumorigenicity when transplanted *in vivo*. ES and iPS cells form teratomas within 8 to 12 weeks when transplanted into the testes of immunodeficient mice, whereas Muse cell-transplanted immunodeficient mouse testes do not develop teratomas even after 6 months (Kuroda et al., 2010) (Fig. 3). Molecular analyses revealed that Muse cells have low levels of telomerase activity and gene expression related to cell-cycle progression compared with those in ES and iPS cells, and such activity and gene expression levels are at the same level as those in somatic cells such as fibroblasts (Wakao et al., 2011). Unlike popular pluripotent stem cells, namely ES and iPS cells, Muse cells are innate adult stem cells in our bodies, which is consistent with the fact that they are nontumorigenic. In addition, Muse cells have already been applied to leukemia patients as a subpopulation (~0.03%) of bone marrow mononucleated cells in bone marrow transplantation (Kuroda et al., 2010). Muse cells are pluripotent, but are non-tumorigenic, which are of great practical use for clinical application.

Stem cells are usually identified and isolated according to their surface antigens. However, Muse cells were initially found as stress-tolerant cells. Tissue stem cells are generally in a dormant state, but they are activated to repair tissues upon exposure to stress. For example, neural stem cells are usually dormant but are activated by stress such as ischemia, and enter into the cell cycle to generate neuronal cells (Ye et al., 2007). Based on this fact, pluripotent stem cells speculated to reside among BM-MSCs at a low frequency are expected to remain under a strong stress condition. In fact, when BM-MSCs are incubated with trypsin for 16 hr, the majority of cells do not survive and only a small number of cells remain alive. When these surviving cells are cultured in a single cell-suspension culture, they form clusters that are positive for pluripotency markers and are able to generate cells of all three germ layers. Because Muse cells are stress-enduring cells with a broad spectrum of differentiation, they were named "multilineage-differentiating stress-enduring" cells.

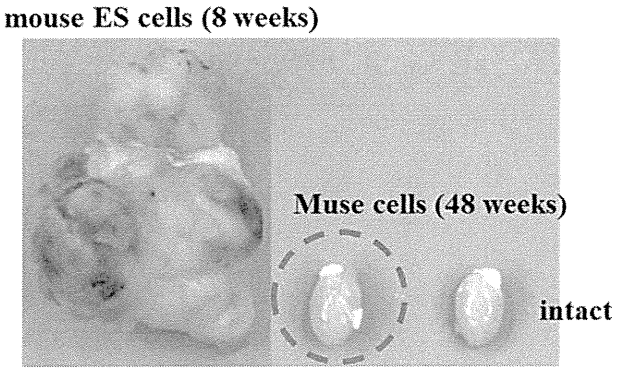

Fig. 3. Testes of immunodeficient mice injected with Muse cells. ES cells form teratomas within 8 weeks when transplanted into the testes of immunodeficient mice, whereas Muse cell-transplanted immunodeficient mouse testes do not develop teratomas even after 6 months. Pictures adapted from Kuroda et al. (2010) with permission from The National Academy of Science.

Muse cells can be directly isolated from cultured mesenchymal cells such as BM-MSCs and human dermal fibroblasts. Among human BM-MSCs, the percentage of Muse cells is around 1%, and the percentage of Muse cells among human dermal fibroblasts is several percent.

In bone marrow aspirates, as mentioned earlier, SSEA-3/CD105 double positive Muse cells are present at a ratio of 0.03% (1 in 3,000 mononucleated cells derived from bone marrow), indicating that nearly 10,000 Muse cells can be collected from 50 ml of bone marrow aspirate. Considering the doubling time of Muse cells, 1.3 days for one cell division, 10,000 freshly isolated Muse cells proliferate to more than 1 million in 8 days. Therefore, Muse cells are easily accessible pluripotent stem cells that can be obtained in large numbers for clinical applications.

Besides their broad potential for differentiation, the spontaneous differentiation rate of Muse cells *in vitro* on a gelatin-coated dish is not very high. For example, only 10 to 15% of Muse cells differentiate spontaneously into mesodermal lineage cells, and 3 to 4% of them are able to cross the boundaries between mesodermal and ectodermal or endodermal lineages to become liver or neuronal marker-positive cells. However, Muse cells show a very high differentiation rate when they are stimulated by certain combinations of cytokines and trophic factors (Fig. 4). For osteocyte or adipocyte differentiation, around 94 to 98% of Muse cells differentiate into these cells under proper induction systems. When Muse cells are treated with insulin-transferrin-selenium medium containing HGF, FGF-4 and dexamethasone, ~90% of the cells become positive for hepatocyte markers α-fetoprotein and human albumin. Similarly, approximately 90% of Muse cells differentiate into MAP-2- or

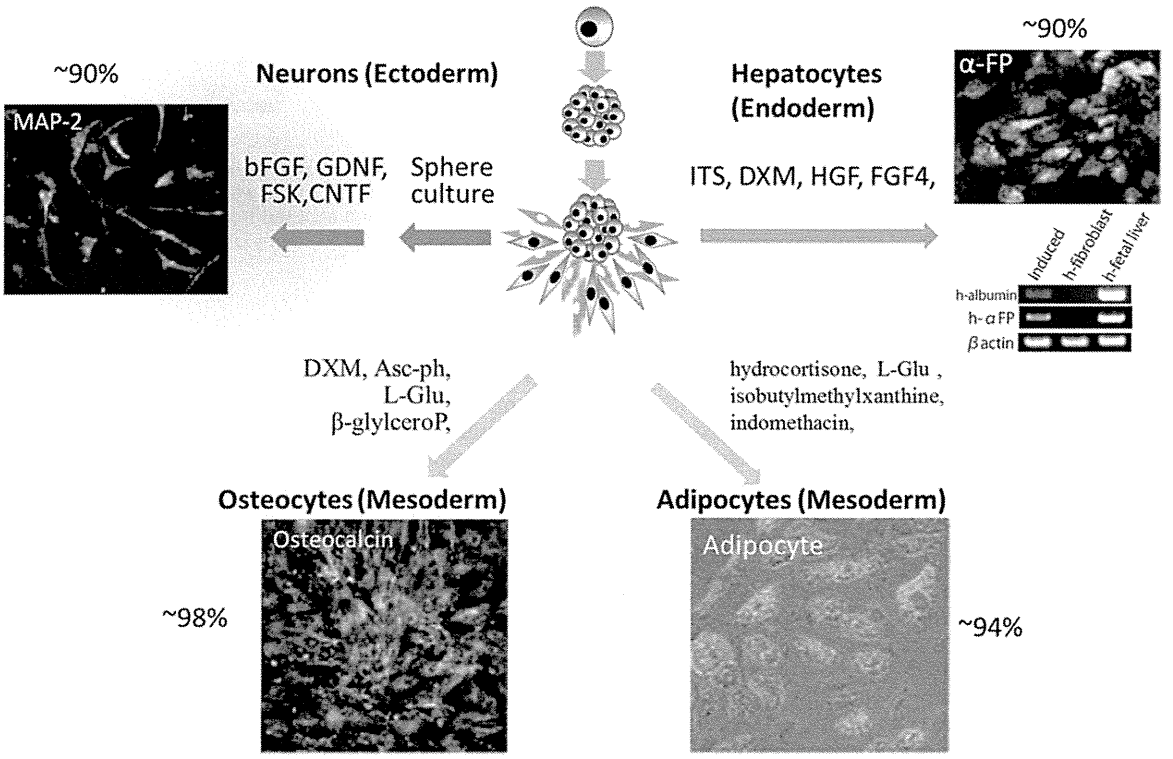

Fig. 4. Induction of Muse cells into neurons, osteocytes, adipocytes and hepatocytes. Muse cells can be directly converted into neurons, osteocytes, adipocytes and hepatocytes at very high efficiency with inductions. Pictures adapted from S. Wakao et al. (2011) with permission from The National Academy of Science.

neurofilament-positive neuronal cells when cultured in Neurobasal medium with B-27 supplement, followed by treatment with bFGF and EGF. Importantly, these inductions do not require any gene transduction, and are solely driven by cytokines and trophic factors.

Tissue Repair by Muse Cells *In Vivo*

Another attractive property of Muse cells is the ability to repair various tissues and organs that span all three germ layer lineages. When Muse cells from BM-MSCs or dermal fibroblasts are injected systemically, they recognize sites of damage, migrate, integrate into the tissue, and differentiate into cells that constitute the tissue to replace the lost cells. Such a repair effect has been shown in fulminant hepatitis (endodermal organ), muscle degeneration (mesodermal) and skin injury (ectodermal) in immunodeficient SCID mice by administration of human Muse cells. Muse cells were traced by lentivirus-GFP and human anti-golgi complex immunoreactivity, and demonstrated expression of human albumin and anti-trypsin in the integrated liver of the fulminant hepatitis model, human dystrophin in degenerated muscle, and cytokeratin 14 in injured skin. In the previous studies, a small number of Muse cells were trapped in the lung and spleen, but were rarely detected in the intact organs and tissues, suggesting that Muse cells recognize damage signals to repair the injured tissue.

Importantly, when non-Muse cells, i.e. the remaining MSC population after elimination of Muse cells, are injected systemically, they do not integrate or differentiate into functional cells in any of the above injury models. These results demonstrate that only Muse cells among MSCs can directly contribute to tissue repair, while non-Muse cells do not participate in this process.

In contrast to Muse cells, the role of non-Muse cells is complicated and multifaceted. As mentioned above, MSCs as a whole have various capabilities for cytokine secretion and immunosuppressive effects. While non-Muse cells do not have the triploblastic differentiation ability of Muse cells, they have trophic, anti-inflammatory and immunosuppressive effects that support the repair effect of Muse cells. However, such effects of non-Muse cells would be temporary because they do not remain in the tissue for a long period. Considering the differences in the actions and effects of Muse and non-Muse cells, both cell populations might be necessary for efficient tissue regeneration and, thus, there may be an optimal ratio of Muse to non-Muse cells for cell-based therapy.

Muse cells were initially identified among BM-MSCs and fibroblasts, as well as in bone marrow, suggesting that Muse cells potentially exist in other mesenchymal tissues such as adipose tissue and the umbilical cord. If so, their feasibility would be greatly extended for clinical and industrial uses.

Perspectives

Recently, MSCs have shown therapeutic effects in various kinds of tissues and organs, including promising results in clinical trials. However, the complex properties of MSCs remain unclear. Muse cells appear to explain the triploblastic differentiation and therapeutic effects of MSCs observed in various studies. Moreover, Muse cells can be isolated from various tissues including adipose tissue and the umbilical cord, but it is unclear whether Muse cells derived from each tissue are identical or not.

MSCs derived from bone marrow, adipose tissue and the umbilical cord are useful for cell-based therapy in humans because of their low risk of tumorigenesis and easy accessibility. Furthermore, MSCs that have long been debated to have pluripotency, because they show spontaneous differentiation into mesodermal, ectodermal and endodermal cells at a very low frequency, are known to home to sites of damage and contribute to tissue repair. Recently, we found pluripotent stem cells, namely Muse cells, which comprise ~1% of cultured MSCs and 0.03% of human bone marrow mononucleated cells, and show self-renewal, triploblastic differentiation and a tissue repair effect. Importantly, Muse cells do not form tumors when transplanted, and are expected to have a greater benefit in clinical applications.

REFERENCES

Alvarez PD, Garcia-Arranz M, Georgiev-Hristov T, Garcia-Olmo D. 2008. A new bronchoscopic treatment of tracheomediastinal fistula using autologous adipose-derived stem cells. Thorax 63:374–376.

Amariglio N, Hirshberg A, Scheithauer BW, Cohen Y, Loewenthal R, Trakhtenbrot L, Paz N, Koren-Michowitz M, Waldman D, Leider-Trejo L, Toren A, Constantini S, Rechavi G. 2009. Donor-derived brain tumor following neural stem cell transplantation in an ataxia telangiectasia patient. PLoS Med 6:e1000029.

Ashjian PH, Elbarbary AS, Edmonds B, DeUgarte D, Zhu M, Zuk PA, Lorenz HP, Benhaim P, Hedrick MH. 2003. In vitro differentiation of human processed lipoaspirate cells into early neural progenitors. Plast Reconstr Surg 111:1922–1931.

Banas A, Teratani T, Yamamoto Y, Tokuhara M, Takeshita F, Osaki M, Kawamata M, Kato T, Okochi H, Ochiya T. 2008. IFATS collection: In vivo therapeutic potential of human adipose tissue mesenchymal stem cells after transplantation into mice with liver injury. Stem Cells 26:2705–2712.

Banas A, Teratani T, Yamamoto Y, Tokuhara M, Takeshita F, Quinn G, Okochi H, Ochiya T. 2007. Adipose tissue-derived mesenchymal stem cells as a source of human hepatocytes. Hepatology 46: 219–228.

Birmingham E, Niebur GL, McHugh PE, Shaw G, Barry FP, McNamara LM. 2012. Osteogenic differentiation of mesenchymal stem cells is regulated by osteocyte and osteoblast cells in a simplified bone niche. Eur Cell Mater 23:13–27.

Block GJ, Ohkouchi S, Fung F, Frenkel J, Gregory C, Pochampally R, DiMattia G, Sullivan DE, Prockop DJ. 2009. Multipotent stromal cells are activated to reduce apoptosis in part by upregulation and secretion of stanniocalcin-1. Stem Cells 27:670–681.

Bruder SP, Jaiswal N, Haynesworth SE. 1997. Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. J Cell Biochem 64:278–294.

Brzoska M, Geiger H, Gauer S, Baer P. 2005. Epithelial differentiation of human adipose tissue-derived adult stem cells. Biochem Biophys Res Commun 330:142–150.

Cao Y, Sun Z, Liao L, Meng Y, Han Q, Zhao RC. 2005. Human adipose tissue-derived stem cells differentiate into endothelial cells in vitro and improve postnatal neovascularization in vivo. Biochem Biophys Res Commun 332:370–379.

Caplan AI. 1991. Mesenchymal stem cells. J Orthop Res 9:641–650.

Carrion F, Nova E, Ruiz C, Diaz F, Inostroza C, Rojo D, Monckeberg G, Figueroa FE. 2010. Autologous mesenchymal stem cell treatment increased T regulatory cells with no effect on disease activity in two systemic lupus erythematosus patients. Lupus 19:317–322.

Crigler L, Robey RC, Asawachaicharn A, Gaupp D, Phinney DG. 2006. Human mesenchymal stem cell subpopulations express a

variety of neuro-regulatory molecules and promote neuronal cell survival and neuritogenesis. Exp Neurol 198:54–64.

Cui X, Chopp M, Zacharek A, Dai J, Zhang C, Yan T, Ning R, Roberts C, Shehadah A, Kuzmin-Nichols N, Sanberg CD, Chen J. 2012. Combination treatment of stroke with sub-therapeutic doses of Simvastatin and human umbilical cord blood cells enhances vascular remodeling and improves functional outcome. Neuroscience 227:223–231.

Danova-Alt R, Heider A, Egger D, Cross M, Alt R. 2012. Very small embryonic-like stem cells purified from umbilical cord blood lack stem cell characteristics. Plos One 7:e34899.

Dao MA, Tate CC, Aizman I, McGrogan M, Case CC. 2011. Comparing the immunosuppressive potency of naive marrow stromal cells and Notch-transfected marrow stromal cells. J Neuroinflammation 8:133.

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

Dezawa M, Kanno H, Hoshino M, Cho H, Matsumoto N, Itokazu Y, Tajima N, Yamada H, Sawada H, Ishikawa H, Mimura T, Kitada M, Suzuki Y, Ide C. 2004. Specific induction of neuronal cells from bone marrow stromal cells and application for autologous transplantation. J Clin Invest 113:1701–1710.

Dezawa M, Takahashi I, Esaki M, Takano M, Sawada H. 2001. Sciatic nerve regeneration in rats induced by transplantation of in vitro differentiated bone-marrow stromal cells. Eur J Neurosci 14: 1771–1776.

Falanga V, Iwamoto S, Chartier M, Yufit T, Butmarc J, Kouttab N, Shrayer D, Carson P. 2007. Autologous bone marrow-derived cultured mesenchymal stem cells delivered in a fibrin spray accelerate healing in murine and human cutaneous wounds. Tissue Eng 13:1299–1312.

Fan CG, Zhang QJ, Zhou JR. 2011. Therapeutic potentials of mesenchymal stem cells derived from human umbilical cord. Stem Cell Rev 7:195–207.

Fang B, Song Y, Liao L, Zhang Y, Zhao RC. 2007. Favorable response to human adipose tissue-derived mesenchymal stem cells in steroid-refractory acute graft-versus-host disease. Transplant Proc 39:3358–3362.

Fedyunina IA, Rzhaninova AA, Kirienko EE, Goldshtein DV. 2011. Isolation of insulin-producing cells from different populations of multipotent stromal cells of the umbilical cord and human adipose tissue. Bull Exp Biol Med 151:114–120.

Fraser JK, Schreiber R, Strem B, Zhu M, Alfonso Z, Wulur I, Hedrick MH. 2006. Plasticity of human adipose stem cells toward endothelial cells and cardiomyocytes. Nat Clin Pract Cardiovasc Med 3 Suppl 1:S33–S37.

Friedenstein AJ, Chailakhjan RK, Lalykina KS. 1970. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Tissue Kinet 3:393–403.

Friedenstein AJ, Chailakhyan RK, Latsinik NV, Panasyuk AF, Keiliss-Borok IV. 1974. Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning in vitro and retransplantation in vivo. Transplantation 17:331–340.

Friedenstein AJ, Gorskaja JF, Kulagina NN. 1976. Fibroblast precursors in normal and irradiated mouse hematopoietic organs. Exp Hematol 4:267–274.

Fujimura J, Ogawa R, Mizuno H, Fukunaga Y, Suzuki H. 2005. Neural differentiation of adipose-derived stem cells isolated from GFP transgenic mice. Biochem Biophys Res Commun 333:116–121.

Gabr MM, Zakaria MM, Refaie AF, Ismail AM, Abou-El-Mahasen MA, Ashamallah SA, Khater SM, El-Halawani SM, Ibrahim RY, Uin GS, Kloc M, Calne RY, Ghoneim MA. 2013. Insulin-producing cells from adult human bone marrow mesenchymal stem cells control streptozotocin-induced diabetes in nude mice. Cell Transplant 22:133–145.

Garcia-Olmo D, Garcia-Arranz M, Herreros D, Pascual I, Peiro C, Rodriguez-Montes JA. 2005. A phase I clinical trial of the treatment of Crohn's fistula by adipose mesenchymal stem cell transplantation. Dis Colon Rectum 48:1416–1423.

Gimble JM. 2003. Adipose tissue-derived therapeutics. Expert Opin Biol Ther 3:705–713.

Gimble JM, Katz AJ, Bunnell BA. 2007. Adipose-derived stem cells for regenerative medicine. Circ Res 100:1249–1260.

Gordon MY, Levicar N, Pai M, Bachellier P, Dimarakis I, Al-Allaf F, M'Hamdi H, Thalji T, Welsh JP, Marley SB, Davies J, Dazzi F, Marelli-Berg F, Tait P, Playford R, Jiao L, Jensen S, Nicholls JP, Ayav A, Nohandani M, Farzaneh F, Gaken J, Dodge R, Alison M, Apperley JF, Lechler R, Habib NA. 2006. Characterization and clinical application of human CD34+ stem/progenitor cell populations mobilized into the blood by granulocyte colony-stimulating factor. Stem Cells 24:1822–1830.

Haller MJ, Viener HL, Wasserfall C, Brusko T, Atkinson MA, Schatz DA. 2008. Autologous umbilical cord blood infusion for type 1 diabetes. Exp Hematol 36:710–715.

Hoerstrup SP, Kadner A, Breymann C, Maurus CF, Guenter CI, Sodian R, Visjager JF, Zund G, Turina MI. 2002. Living, autologous pulmonary artery conduits tissue engineered from human umbilical cord cells. Ann Thorac Surg 74:46–52; discussion 52.

Hokari M, Kuroda S, Shichinohe H, Yano S, Hida K, Iwasaki Y. 2008. Bone marrow stromal cells protect and repair damaged neurons through multiple mechanisms. J Neurosci Res 86:1024–1035.

Horwitz EM, Prockop DJ, Fitzpatrick LA, Koo WW, Gordon PL, Neel M, Sussman M, Orchard P, Marx JC, Pyeritz RE, Brenner MK. 1999. Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. Nat Med 5:309–313.

Izadpanah R, Trygg C, Patel B, Kriedt C, Dufour J, Gimble JM, Bunnell BA. 2006. Biologic properties of mesenchymal stem cells derived from bone marrow and adipose tissue. J Cell Biochem 99: 1285–1297.

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. 2002. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 418:41–49.

Karnieli O, Izhar-Prato Y, Bulvik S, Efrat S. 2007. Generation of insulin-producing cells from human bone marrow mesenchymal stem cells by genetic manipulation. Stem Cells 25:2837–2844.

Katz AJ, Llull R, Hedrick MH, Futrell JW. 1999. Emerging approaches to the tissue engineering of fat. Clin Plast Surg 26: 587–603, viii.

Kawabori M, Kuroda S, Sugiyama T, Ito M, Shichinohe H, Houkin K, Kuge Y, Tamaki N. 2012. Intracerebral, but not intravenous, transplantation of bone marrow stromal cells enhances functional recovery in rat cerebral infarct: An optical imaging study. Neuropathology 32:217–226.

Kern S, Eichler H, Stoeve J, Kluter H, Bieback K. 2006. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem Cells 24:1294–1301.

Kucia M, Reca R, Campbell FR, Zuba-Surma E, Majka M, Ratajczak J, Ratajczak MZ. 2006. A population of very small embryonic-like (VSEL) CXCR4(+)SSEA-1(+)Oct-4+ stem cells identified in adult bone marrow. Leukemia 20:857–869.

Kuroda Y, Kitada M, Wakao S, Nishikawa K, Tanimura Y, Makinoshima H, Goda M, Akashi H, Inutsuka A, Niwa A, Shigemoto T, Nabeshima Y, Nakahata T, Fujiyoshi Y, Dezawa M. 2010. Unique multipotent cells in adult human mesenchymal cell populations. Proc Natl Acad Sci USA 107:8639–8643.

Kuznetsov SA, Krebsbach PH, Satomura K, Kerr J, Riminucci M, Benayahu D, Robey PG. 1997. Single-colony derived strains of human marrow stromal fibroblasts form bone after transplantation in vivo. J Bone Miner Res 12:1335–1347.

Laurent LC, Ulitsky I, Slavin I, Tran H, Schork A, Morey R, Lynch C, Harness JV, Lee S, Barrero MJ, Ku S, Martynova M, Semechkin R, Galat V, Gottesfeld J, Izpisua Belmonte JC, Murry C, Keirstead HS, Park HS, Schmidt U, Laslett AL, Muller FJ, Nievergelt CM, Shamir R, Loring JF. 2011. Dynamic changes in the copy number of pluripotency and cell proliferation genes in human ESCs and iPSCs during reprogramming and time in culture. Cell Stem Cell 8:106–118.

Lendeckel S, Jodicke A, Christophis P, Heidinger K, Wolff J, Fraser JK, Hedrick MH, Berthold L, Howaldt HP. 2004. Autologous stem cells (adipose) and fibrin glue used to treat widespread traumatic

calvarial defects: Case report. J Craniomaxillofac Surg 32:370–373.
Liang J, Wu S, Zhao H, Li SL, Liu ZX, Wu J, Zhou L. 2012. Human umbilical cord mesenchymal stem cells derived from Wharton's jelly differentiate into cholinergic-like neurons in vitro. Neurosci Lett 532:59–63.
Lopatina T, Kalinina N, Karagyaur M, Stambolsky D, Rubina K, Revischin A, Pavlova G, Parfyonova Y, Tkachuk V. 2011. Adipose-derived stem cells stimulate regeneration of peripheral nerves: BDNF secreted by these cells promotes nerve healing and axon growth de novo. Plos One 6:e17899.
Lu F, Mizuno H, Uysal CA, Cai X, Ogawa R, Hyakusoku H. 2008. Improved viability of random pattern skin flaps through the use of adipose-derived stem cells. Plast Reconstr Surg 121:50–58.
Luo G, Cheng W, He W, Wang X, Tan J, Fitzgerald M, Li X, Wu J. 2010. Promotion of cutaneous wound healing by local application of mesenchymal stem cells derived from human umbilical cord blood. Wound Repair Regen 18:506–513.
Mackay AM, Beck SC, Murphy JM, Barry FP, Chichester CO, Pittenger MF. 1998. Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. Tissue Eng 4:415–428.
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. 1999. Cardiomyocytes can be generated from marrow stromal cells in vitro. J Clin Invest 103:697–705.
Miranville A, Heeschen C, Sengenes C, Curat CA, Busse R, Bouloumie A. 2004. Improvement of postnatal neovascularization by human adipose tissue-derived stem cells. Circulation 110:349–355.
Misao Y, Takemura G, Arai M, Sato S, Suzuki K, Miyata S, Kosai K, Minatoguchi S, Fujiwara T, Fujiwara H. 2006. Bone marrow-derived myocyte-like cells and regulation of repair-related cytokines after bone marrow cell transplantation. Cardiovasc Res 69: 476–490.
Nambu M, Ishihara M, Nakamura S, Mizuno H, Yanagibayashi S, Kanatani Y, Hattori H, Takase B, Ishizuka T, Kishimoto S, Amano Y, Yamamoto N, Azuma R, Kiyosawa T. 2007. Enhanced healing of mitomycin C-treated wounds in rats using inbred adipose tissue-derived stromal cells within an atelocollagen matrix. Wound Repair Regen 15:505–510.
Nambu M, Kishimoto S, Nakamura S, Mizuno H, Yanagibayashi S, Yamamoto N, Azuma R, Kiyosawa T, Ishihara M, Kanatani Y. 2009. Accelerated wound healing in healing-impaired db/db mice by autologous adipose tissue-derived stromal cells combined with atelocollagen matrix. Ann Plast Surg 62:317–321.
Orlic D, Kajstura J, Chimenti S, Bodine DM, Leri A, Anversa P. 2003. Bone marrow stem cells regenerate infarcted myocardium. Pediatr Transplant 7(Suppl 3):86–88.
Osawa M, Hanada K, Hamada H, Nakauchi H. 1996. Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science 273:242–245.
Oswald J, Boxberger S, Jorgensen B, Feldmann S, Ehninger G, Bornhauser M, Werner C. 2004. Mesenchymal stem cells can be differentiated into endothelial cells in vitro. Stem Cells 22:377–384.
Owen ME, Cave J, Joyner CJ. 1987. Clonal analysis in vitro of osteogenic differentiation of marrow CFU-F. J Cell Sci 87:731–738.
Oyagi S, Hirose M, Kojima M, Okuyama M, Kawase M, Nakamura T, Ohgushi H, Yagi K. 2006. Therapeutic effect of transplanting HGF-treated bone marrow mesenchymal cells into CCl4-injured rats. J Hepatol 44:742–748.
Park HC, Shim YS, Ha Y, Yoon SH, Park SR, Choi BH, Park HS. 2005. Treatment of complete spinal cord injury patients by autologous bone marrow cell transplantation and administration of granulocyte-macrophage colony stimulating factor. Tissue Eng 11: 913–922.
Pastor D, Viso-Leon MC, Jones J, Jaramillo-Merchan J, Toledo-Aral JJ, Moraleda JM, Martinez S. 2012. Comparative effects between bone marrow and mesenchymal stem cell transplantation in GDNF expression and motor function recovery in a motorneuron degenerative mouse model. Stem Cell Rev 8:445–458.
Pittenger MF. 1998. Adipogenic differentiation of human mesenchymal stem cells. United States Patent 5:740.
Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. 1999. Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147.
Planat-Benard V, Silvestre JS, Cousin B, Andre M, Nibbelink M, Tamarat R, Clergue M, Manneville C, Saillan-Barreau C, Duriez M, Tedgui A, Levy B, Penicaud L, Casteilla L. 2004. Plasticity of human adipose lineage cells toward endothelial cells: physiological and therapeutic perspectives. Circulation 109: 656–663.
Prindull G, Zipori D. 2004. Environmental guidance of normal and tumor cell plasticity: Epithelial mesenchymal transitions as a paradigm. Blood 103:2892–2899.
Prockop DJ. 1997. Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 276:71–74.
Prockop DJ, Brenner M, Fibbe WE, Horwitz E, Le Blanc K, Phinney DG, Simmons PJ, Sensebe L, Keating A. 2010. Defining the risks of mesenchymal stromal cell therapy. Cytotherapy 12: 576–578.
Reynolds BA, Weiss S. 1992. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 255:1707–1710.
Rodriguez LV, Alfonso Z, Zhang R, Leung J, Wu B, Ignarro LJ. 2006. Clonogenic multipotent stem cells in human adipose tissue differentiate into functional smooth muscle cells. Proc Natl Acad Sci USA 103:12167–12172.
Rosenkranz K, Tenbusch M, May C, Marcus K, Meier C. 2013. Changes in Interleukin-1 alpha serum levels after transplantation of umbilical cord blood cells in a model of perinatal hypoxic-ischemic brain damage. Ann Anat 195:122–127.
Safford KM, Hicok KC, Safford SD, Halvorsen YD, Wilkison WO, Gimble JM, Rice HE. 2002. Neurogenic differentiation of murine and human adipose-derived stromal cells. Biochem Biophys Res Commun 294:371–379.
Safford KM, Safford SD, Gimble JM, Shetty AK, Rice HE. 2004. Characterization of neuronal/glial differentiation of murine adipose-derived adult stromal cells. Exp Neurol 187:319–328.
Schachinger V, Assmus B, Erbs S, Elsasser A, Haberbosch W, Hambrecht R, Yu J, Corti R, Mathey DG, Hamm CW, Tonn T, Dimmeler S, Zeiher AM. 2009. Intracoronary infusion of bone marrow-derived mononuclear cells abrogates adverse left ventricular remodelling post-acute myocardial infarction:insights from the reinfusion of enriched progenitor cells and infarct remodelling in acute myocardial infarction (REPAIR-AMI) trial. Eur J Heart Fail 11:973–979.
Seo MJ, Suh SY, Bae YC, Jung JS. 2005. Differentiation of human adipose stromal cells into hepatic lineage in vitro and in vivo. Biochem Biophys Res Commun 328:258–264.
Snykers S, De Kock J, Rogiers V, Vanhaecke T. 2009. In vitro differentiation of embryonic and adult stem cells into hepatocytes: State of the art. Stem Cells 27:577–605.
Tamai K, Yamazaki T, Chino T, Ishii M, Otsuru S, Kikuchi Y, Iinuma S, Saga K, Nimura K, Shimbo T, Umegaki N, Katayama I, Miyazaki J, Takeda J, McGrath JA, Uitto J, Kaneda Y. 2011. PDGFRalpha-positive cells in bone marrow are mobilized by high mobility group box 1 (HMGB1) to regenerate injured epithelia. Proc Natl Acad Sci USA 108:6609–6614.
Terai S, Sakaida I, Yamamoto N, Omori K, Watanabe T, Ohata S, Katada T, Miyamoto K, Shinoda K, Nishina H, Okita K. 2003. An in vivo model for monitoring trans-differentiation of bone marrow cells into functional hepatocytes. J Biochem 134:551–558.
Terai S, Yamamoto N, Omori K, Sakaida I, Okita K. 2002. A new cell therapy using bone marrow cells to repair damaged liver. J Gastroenterol 37(Suppl 14):162–163.
Till JE, McCulloch EA. 2012. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. 1961. Radiat Res 178:AV3–AV7.
Timper K, Seboek D, Eberhardt M, Linscheid P, Christ-Crain M, Keller U, Muller B, Zulewski H. 2006. Human adipose tissue-derived mesenchymal stem cells differentiate into insulin,

somatostatin, and glucagon expressing cells. Biochem Biophys Res Commun 341:1135–1140.
Tobita M, Uysal AC, Ogawa R, Hyakusoku H, Mizuno H. 2008. Periodontal tissue regeneration with adipose-derived stem cells. Tissue Eng Part A 14:945–953.
Troyer DL, Weiss ML. 2008. Wharton's jelly-derived cells are a primitive stromal cell population. Stem Cells 26:591–599.
Wakao S, Kitada M, Kuroda Y, Dezawa M. 2012. Isolation of adult human pluripotent stem cells from mesenchymal cell populations and their application to liver damages. Methods Mol Biol 826:89–102.
Wang Y, Han ZB, Song YP, Han ZC. 2012. Safety of mesenchymal stem cells for clinical application. Stem Cells Int 2012:652034.
Weiss ML, Medicetty S, Bledsoe AR, Rachakatla RS, Choi M, Merchav S, Luo Y, Rao MS, Velagaleti G, Troyer D. 2006. Human umbilical cord matrix stem cells: Preliminary characterization and effect of transplantation in a rodent model of Parkinson's disease. Stem Cells 24:781–792.
Wislet-Gendebien S, Hans G, Leprince P, Rigo JM, Moonen G, Rogister B. 2005. Plasticity of cultured mesenchymal stem cells: switch from nestin-positive to excitable neuron-like phenotype. Stem Cells 23:392–402.
Wisniewski HG, Vilcek J. 2004. Cytokine-induced gene expression at the crossroads of innate immunity, inflammation and fertility:TSG-6 and PTX3/TSG-14. Cytokine Growth Factor Rev 15:129–146.
Wojakowski W, Kucia M, Zuba-Surma E, Jadczyk T, Ksiazek B, Ratajczak MZ, Tendera M. 2011. Very small embryonic-like stem cells in cardiovascular repair. Pharmacol Ther 129:21–28.
Wu M, Yang L, Liu S, Li H, Hui N, Wang F, Liu H. 2006. Differentiation potential of human embryonic mesenchymal stem cells for skin-related tissue. Br J Dermatol 155:282–291.
Xu Y, Liu Z, Liu L, Zhao C, Xiong F, Zhou C, Li Y, Shan Y, Peng F, Zhang C. 2008. Neurospheres from rat adipose-derived stem cells could be induced into functional Schwann cell-like cells in vitro. BMC Neurosci 9:21.
Yagi H, Soto-Gutierrez A, Navarro-Alvarez N, Nahmias Y, Goldwasser Y, Kitagawa Y, Tilles AW, Tompkins RG, Parekkadan B, Yarmush ML. 2010. Reactive bone marrow stromal cells attenuate systemic inflammation via sTNFR1. Mol Ther 18:1857–1864.
Yan P, Xia C, Duan C, Li S, Mei Z. 2011. Biological characteristics of foam cell formation in smooth muscle cells derived from bone marrow stem cells. Int J Biol Sci 7:937–946.
Ye M, Wang XJ, Zhang YH, Lu GQ, Liang L, Xu JY, Sheng-Di C. 2007. Therapeutic effects of differentiated bone marrow stromal cell transplantation on rat models of Parkinson's disease. Parkinsonism Relat Disord 13:44–49.
Ye ZQ, Burkholder JK, Qiu P, Schultz JC, Shahidi NT, Yang NS. 1994. Establishment of an adherent cell feeder layer from human umbilical cord blood for support of long-term hematopoietic progenitor cell growth. Proc Natl Acad Sci USA 91:12140–12144.
Yoshihara T, Ohta M, Itokazu Y, Matsumoto N, Dezawa M, Suzuki Y, Taguchi A, Watanabe Y, Adachi Y, Ikehara S, Sugimoto H, Ide C. 2007. Neuroprotective effect of bone marrow-derived mononuclear cells promoting functional recovery from spinal cord injury. J Neurotrauma 24:1026–1036.
Yoshimura K, Sato K, Aoi N, Kurita M, Hirohi T, Harii K. 2008. Cell-assisted lipotransfer for cosmetic breast augmentation:supportive use of adipose-derived stem/stromal cells. Aesthetic Plast Surg 32:48–55; discussion 56–47.
Zanini C, Bruno S, Mandili G, Baci D, Cerutti F, Cenacchi G, Izzi L, Camussi G, Forni M. 2011. Differentiation of mesenchymal stem cells derived from pancreatic islets and bone marrow into islet-like cell phenotype. Plos One 6:e28175.
Zhang HT, Cheng HY, Zhang L, Fan J, Chen YZ, Jiang XD, Xu RX. 2009. Umbilical cord blood cell-derived neurospheres differentiate into Schwann-like cells. Neuroreport 20:354–359.
Zhang L, Ye JS, Decot V, Stoltz JF, de Isla N. 2012. Research on stem cells as candidates to be differentiated into hepatocytes. Biomed Mater Eng 22:105–111.
Zhang Y, Hao H, Liu J, Fu X, Han W. 2012. Repair and regeneration of skin injury by transplanting microparticles mixed with Wharton's jelly and MSCs from the human umbilical cord. Int J Low Extrem Wounds 11:264–270.
Zhang YN, Lie PC, Wei X. 2009. Differentiation of mesenchymal stromal cells derived from umbilical cord Wharton's jelly into hepatocyte-like cells. Cytotherapy 11:548–558.
Zhang Z, Lin H, Shi M, Xu R, Fu J, Lv J, Chen L, Lv S, Li Y, Yu S, Geng H, Jin L, Lau GK, Wang FS. 2012. Human umbilical cord mesenchymal stem cells improve liver function and ascites in decompensated liver cirrhosis patients. J Gastroenterol Hepatol 27(Suppl 2):112–120.
Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH. 2002. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 13:4279–4295.
Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH. 2001. Multilineage cells from human adipose tissue:implications for cell-based therapies. Tissue Eng 7:211–228.

The Laryngoscope

Functional Regeneration of Laryngeal Muscle Using Bone Marrow–Derived Stromal Cells

Shin-ichi Kanemaru, MD, PhD; Yoshiharu Kitani, MD, PhD; Satoshi Ohno, MD, PhD; Taeko Shigemoto, MS; Tsuyoshi Kojima, MD; Seiji Ishikawa, MD; Masanobu Mizuta, MD; Shigeru Hirano, MD, PhD; Tatsuo Nakamura, MD, PhD; Mari Dezawa, MD, PhD

Objectives/Hypothesis: To investigate the functional efficiency of skeletal muscles regenerated by transplantation of bone marrow–derived stromal cells (BSCs) or induced-muscle progenitor cells (IMCs) as assessed in the canine posterior cricoarytenoid (PCA) muscle injury model.

Study Design: Prospective animal experiment with control.

Methods: We performed BSC/IMC transplantation into injured canine PCA muscles. We investigated the capability of auto- and allo-BSC/IMC transplantation using a gelatin sponge scaffold to promote functional regeneration of PCA muscles. Transplantation was assessed by fiberscopic analysis of vocal fold movement. We also examined the histologic changes of the transplanted regions. As a control, a gelatin sponge scaffold without additional cells was transplanted into the injured area.

Results: Auto-BSC/IMC transplantation effectively restored vocal fold movement, whereas scaffold alone or allo-BSC/IMC transplantation did not. Histologic examination revealed that (in cases of good recovery) muscle regeneration occurred in the area of cell transplantation, and scar formation without muscle regeneration was observed under control conditions. The dogs with autologous transplantation of BSC had faster functional recovery than did dogs treated with autologous transplantation of IMC.

Conclusions: Functional efficiency was shown in skeletal muscles regenerated using BSCs and IMPs. Motor function recovery was observed using autologous transplantation of BSCs and IMCs. Minimal functional recovery was observed using allogeneic transplantation of these cells.

Key Words: Mesenchymal stem cells, muscle regeneration, induced muscle progenitor cells, posterior cricoarytenoid muscle, vocal fold.

Level of Evidence: NA.

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INTRODUCTION

When muscle injuries occur due to disease or trauma, they might not always be fatal but can lead to functional impairment. Conventionally, impaired motor function due to muscle injury has mainly been restored by rehabilitation and not tissue repair of the damaged site. However, if muscle tissue regeneration is possible and the original function can be restored, tissue repair is obviously superior to residual tissue assuming the function of the damaged tissue. In addition, tissue repair can potentially be applied clinically to treat incurable muscular degenerative diseases such as muscular dystrophy.

Dezawa et al. reported that mesenchymal stem cells (MSCs) can be induced to differentiate into muscle tissue under appropriate conditions.[1] Bone marrow–derived stromal cells (BSCs) consist primarily of MSCs. In previous animal experiments using a vocal fold injury model, we performed BSC transplantation and achieved regeneration of the vocal fold.[2,3] In our present study, functional efficiency was examined in skeletal muscles regenerated using BSCs.

Vocal folds were selected as the target organ to show the recovery of function of regenerated muscle. We focused on the posterior cricoarytenoid (PCA) muscle, the only glottal abductor among the four intrinsic laryngeal muscles. The PCA muscle originates from the posterior surface of the cricoid lamina, runs superiolaterally, and inserts into the muscular process of the arytenoid cartilage. The PCA muscle is innervated by the recurrent laryngeal nerve, and contraction of this muscle

From the Foundation for Biomedical Research and Innovation (S.-I.K.), Kobe, Japan, Department of Otolaryngology–Head and Neck Surgery, Medical Research Institute, Kitano Hospital (S.-I.K.), Osaka; Department of Otolaryngology–Head and Neck Surgery, Graduate School of Medicine (Y.K., S.O., T.K., S.I., M.M., S.H.), Kyoto University, Kyoto; Department of Stem Cell Biology and Histology, Tohoku University Graduate School of Medicine (T.S., M.D.), Sendai; and Department of Bioartificial Organs, Institute for Frontier Medical Sciences (T.N.), Kyoto University, Kyoto, Japan

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Send correspondence to Shin-ichi Kanemaru MD, PhD, Department of Otolaryngology, Head and Neck Surgery, Medical Research Institute, Kitano Hospital, 2-4-20 Ohgimachi, Kitaku, Osaka, 530-8480, Japan. E-mail: kanemaru@ent.kuhp.kyoto-u.ac.jp

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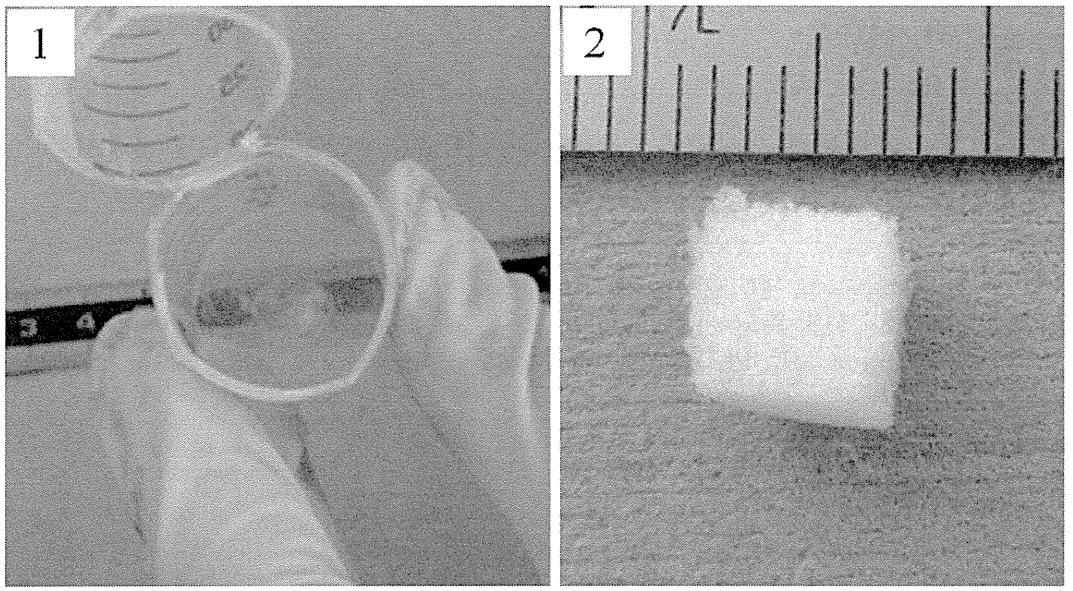

Fig. 1. (A) Transplanted bone marrow–derived stromal cells. (B) Gelatin sponge.

pulls the muscular process of the arytenoid cartilage. This action causes the arytenoid cartilage to rotate, displacing the vocal process laterally and resulting in the abduction of the glottis. Thus, resection of the PCA muscle belly prevents the abduction of the glottis without other effects. In addition, the PCA muscle is suitable for muscle regeneration because its anatomic positional relationship makes it difficult for the scaffold to shift. Because the PCA muscle is very small, the number of cells required for regeneration is relatively small. Regeneration can be directly examined by observation of vocal fold movements using a laryngeal fiberscope. Based on this rationale, we used the PCA muscle injury model. BSCs or induced-muscle progenitor cells (IMCs) were transplanted, and examination was performed to determine whether the regenerated PCA muscles functioned effectively to abduct the vocal fold.

This is the first report of functional regeneration of skeletal muscle in a large animal.

MATERIALS AND METHODS

This study was performed in accordance with the animal experiment manual of the Animal Research Committee of the Kyoto University.

Cells

One milliliter of bone marrow was harvested from the forelimb humerus of each adult beagle dog (body weight, 8–12 kg). Fetal bovine serum 10% (FBS; Invitrogen, Carlsbad, CA) was added to Dulbecco's modified Eagle's medium (D-MEM; Gibco, Life Technologies, Inc., Grand Island, NY) to yield 15 mL of culture medium. This medium was placed in a 200-mL culture flask and placed in an incubator (MCO-17AIC; Sanyo Co., Osaka, Japan) at 37°C in 5% CO_2 atmosphere for 48 hours. The culture medium was replaced with fresh medium while leaving the cells attached to the bottom surface of the flask. The medium was replaced every 3 days, and the level of cell proliferation was examined under an optical microscope (Olympus, IX70; Olympus Co., Tokyo, Japan). On day 14, the cells were confirmed to have completely covered the bottom of the flask. Two milliliters of trypsin/0.25% EDTA (EDTA; Invitrogen) was added to the culture medium, and the cells attached to the bottom surface of the flask were removed. Five minutes later, the trypsin was neutralized with 10 mL of D-MEM + 10% FBS. Subsequently, a cell counter was used to confirm that there were approximately 4×10^6 cells, and these cells were collected. The resulting cells were centrifuged at 3,500 rpm for 3 minutes (Fig. 1A). For direct transplantation, 5×10^5 of these cells were adsorbed to a scaffold of gelatin sponge and then transplanted.

When IMCs were used, centrifuged cells were collected, and the method of Dezawa et al.[1] was subsequently used to induce differentiation of BSCs. In the same way as in direct transplantation, transplantation was performed in the resected site of the PCA muscle belly. Both BSCs and IMCs were divided into two groups: a group with autologous (auto) transplantation and another group with allogeneic (allo) transplantation. Allogeneic transplantation was performed by similar methods but with use of cells from another beagle instead of autologous cells. No immunosuppressant was used. In the controls, a gelatin sponge scaffold was soaked with physiologic saline and transplanted.

Scaffold

The gelatin sponge (Spogel; Astellas Co., Tokyo, Japan) used as a scaffold is made by aqueous extraction with heating of crude collagen. This crude collagen is acid- or alkali-treated collagen from animal bones, skin, ligaments, or tendons. The resulting product is lyophilized and sterilized to yield a porous gelatin sponge. The triple helix structure of a collagen molecule unfolds due to thermal denaturation. The main ingredient of the gelatin sponge is a mixture of the denatured products (Fig. 1B).

Surgical Procedure and Cell Transplantation

Each beagle was placed under general anesthesia using intramuscular ketamine hydrochloride (50 mg/kg) (Ketaral; Sankyo Co., Tokyo, Japan) and xylazine (2.0 mg/kg) (Ceractal; Bayer, Tokyo, Japan). An incision of approximately 10 cm was made in the anterior neck to reach the larynx. The thyroid