A Simple Method for Cat Bone Marrow-derived Mesenchymal Stem Cell Harvesting

Guang Zhen Jin[1], Young Soo Lee[1], Eugene Choi[1], Kyu Woan Cho[2] and Il Keun Kong[1,3,*]

[1]Division of Applied Life Science (BK21), [3]Institute of Agriculture and Life Science,
[2]Department of Veterinary Internal Medicine, Institute of Animal Medicine, College of Veterinary Medicine,
Graduate School of Gyeongsang National University, Jinju 660-701, Korea

ABSTRACT

Bone marrow (BM) cell harvesting is a crucial element in the isolation of mesenchymal stem cells (MSCs). A simple method for harvesting cat BM cells is described. The results show that a large number of BM cells can rapidly be harvested from the cat by this simple procedure. MSCs prepared by density-gradient method were spindle-shaped morphology with bipolar or polygonal cell bodies and strongly positive for CD9 and CD44 and negative for CD18 and CD45-like. They were capable of differentiation to adipocytic and osteocytic phenotypes when exposed to appropriate induction media. The advantages of this method are its rapidity, simplicity, low invasiveness, and low donor attrition and good outcome.

(Key words : bone marrow, cat, harvesting, mesenchymal stem cell)

INTRODUCTION

Adult bone marrow (BM) contains two types of stem cells: hematopoietic stem cells and mesenchymal stem cells (MSCs) (Prockop, 1997). Many recent studies have shown that MSCs derived from adult BM have the potential to differentiate into osteocytes, chondrocytes, adipocytes (Pittenger *et al.*, 1999), and neural cells (Brazelton *et al.*, 2000). Therefore, MSCs may be candidates for the treatment of neurodegenerative disease.

BM cell harvesting is the first and crucial step in MSC isolation. There are diverse methods for BM cell harvesting depending on the animal species; e.g., the extrusion method for *ex vivo* mouse bone (Kabos *et al.*, 2002; Sanchez-Ramos *et al.*, 2000) and the perfusion or aspiration methods for *in vivo* pet (feline or canine), monkey, and human bones (Kushida *et al.*, 2000; Martin *et al.*, 2002; Suter *et al.*, 2004; Tao *et al.*, 2005). The cat is phylogenetically closer to humans than rodents or other laboratory mammals (Menotti-Raymond *et al.*, 1999). It is an important model for acquired immunodeficiency diseases, inherited human disease, and nervous system diseases (Malik, 2001; Rohn *et al.*, 1996; Wade *et al.*, 2001). We have harvested BM cells from 8 different cats with similar results. In the present study, we describe a simple method, which is rapidity, low invasiveness, and low donor attrition and good outcome, for cat BM cell harvesting. Representative results from 1 cat are described.

* Correspondence : E-mail : ikong@gnu.kr

MATERIALS AND METHODS

1. Animals

Male cats were housed in stainless-steel cages measuring 0.9 m × 0.7 m × 0.65 m and provided with dry food and water *ad libitum*. All experiments were performed according to the guidelines of Gyeongsang National University for animal care and ethics.

2. Materials

Ⅰ. One male domestic cat

Ⅱ. Reagents:

i. Dulbecco's modified Eagle's high-glucose medium (DMEM; Gibco-BRL, USA) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL), penicillin (100 U/ml)/streptomycin (100 mg/ ml) (Sigma, USA) and 2 mM L-glutamine (Sigma, USA).

ii. 0.25% trypsin/1 mM EDTA (Gibco-BRL), dimethyl sulfoxide (DMSO; Sigma, USA)

iii. Ethanol (70%)

Ⅲ. Tissue culture supplies including 90 mm dishes, T75 flasks, 5 and 10 ml pipettes, and sterile 15 ml centrifuge tubes, cryopreservation ampoules (NUNC)

Ⅳ. Two 20 ml syringes, two 18 gauge needles, one #20 scalpel blade

Ⅴ. Sterilized instrument pack consisting of the following items in a metal bowl wrapped inside a 24"×24" drape: one small hemostat, one large needle holder, one scalpel handle, one pair of scissors, ten 2"×2" gauze squares

3. BM Harvesting

The cat was anesthetized by subcutaneous injection with acepromazine maleate (0.025 mg/kg; Sedaject; Samwoo, Republic of Korea) and ketamine (5 mg/kg; Daesung, Republic of Korea). The fur (5 cm×5 cm) surrounding the left greater trochanter was shaved with electric clippers (Fig. 1A). The skin was sterilized with a swab soaked in 70% ethanol. The 24"×24" drape became the sterile field for operating on the cat. Using the scalpel, a 0.5 cm transversal incision was made at a 1 cm site from the greater trochanter toward the femoral neck (Fig. 1B) and a small port was constructed on the subcutaneous fascia with the blade tip. An 18 gauge needle connected to a 20 ml syringe was screwed into the femoral medullary cavity through the base of the neck of the femur. The needle direction followed the shaft of femur (Fig. 2). This was very important for successfully obtaining BM. When the needle was within the medullary cavity, the shaft of the femur moved concomitantly with the needle. The BM was withdrawn slowly, adjusting the direction of the eye of the needle and the depth of the needle body. Three ml of BM was usually obtained in this way. The BM was placed into a heparin-coated tube and shaken to prevent coagulation. Rarely, the needle became obstructed with bone debris and needed to be changed. After the BM abstraction, the syringe was removed from the shaft of the femur, and the wound was cleaned and sutured with a needle. BM was harvested from the other side using the same procedure. No special post-operative care was required.

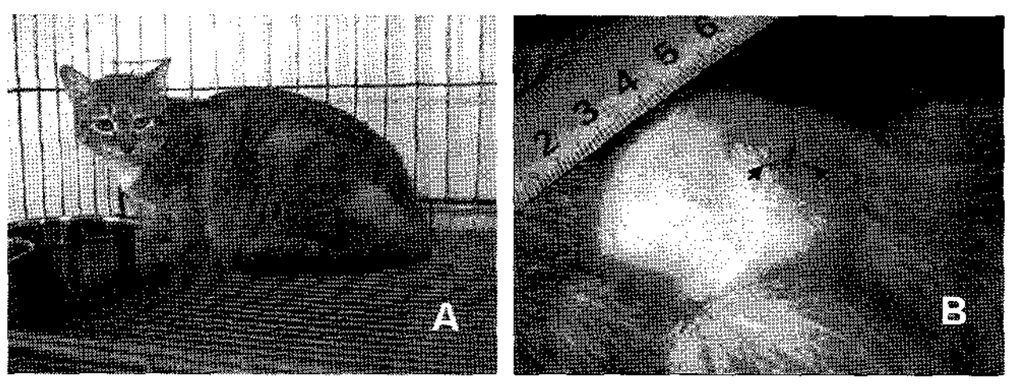

Fig. 1. At 1 week post-BM harvesting (A); 5 cm × 5 cm operating field (B). Dotted line and arrow in B indicate the 0.5-cm incision and the post-suture trace, respectively.

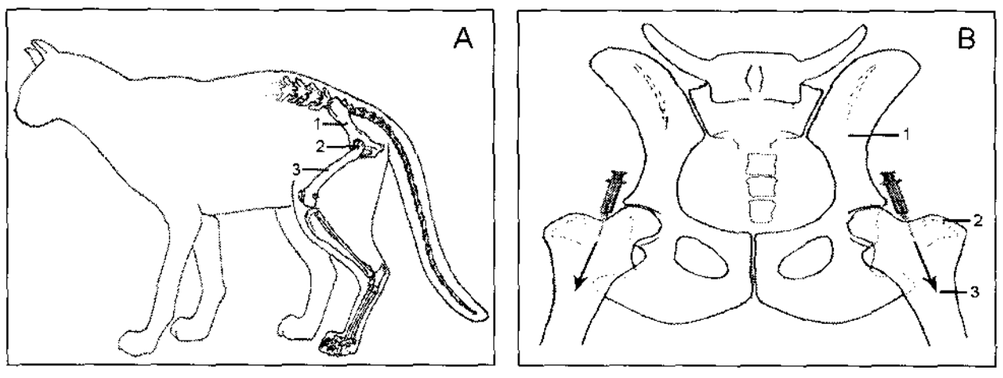

Fig. 2. Lateral view (A) and anterior view (B) of the cat leg bone. 1, hip bone; 2, greater trochanter; 3, femur; arrows, direction of the needles.

4. Preparation of MSCs

The nucleated-cell fraction of the BM was isolated by the Ficoll® 400 (1.077 g/ml, Sigma, USA) density-gradient method. The cells at the medium-Ficoll interface were collected, rinsed twice with phosphate-buffered saline, and plated at a density of 1×10^6 cells/cm^2 in 90 mm dishes in 10 ml of medium consisting of DMEM, 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine. The cells were incubated at 37℃ in a humidified atmosphere with 5% CO_2 until they reached 80~90% confluence. The cells were harvested with 0.25% trypsin/1 mM EDTA and diluted 1:3 for passage.

5. Cell Cryopreservation

Cells were resuspended in DMEM containing 40% (v/v) FBS and 10% DMSO at a concentration of 1×10^6 cells/ml and loaded into cryopreservation ampoules in 0.5 ml aliquots. The cells were frozen in liquid nitrogen at −196℃.

6. Analyses of Cell-Surface Antigens

MSCs were obtained from passage 4. Cell-surface antigens on the BMCs were determined using primary cat-specific antibodies against CD9 (Serotec, Raleigh, NC, USA), CD18, CD44, and CD45-like (VMRD, Pullman, WA, USA) and fluorescein isothiocyanate-coupled secondary antibodies (IgG or IgM). Labeled cells were detected by flow cytometry.

7. Osteogenic and Adipogenic Potential of MSCs

The osteocytic phenotype was induced by seeding cells at 6,000~8,000/cm^2, followed 1 day later by incubation in DMEM containing 10% FBS, 1 μM dexamethasone (Sigma, USA), 10 mM β-glycerophosphate (Sigma, USA), and 0.25 mM L-ascorbic acid (Aldrich, USA). Differentiation proceeded for 3 weeks with media changes twice weekly. Induced cells were stained for calcium with Alizarin Red S (2%, pH 4.2; Fluka, USA).

Adipocytic differentiation was achieved by allowing cells to remain at confluency for 3 to 7 days, followed by incubation in DMEM supplemented with 10% FBS, 1 μM dexamethasone (Sigma, USA), and 10 μg/ml insulin (Fluka, USA). After 7 days, cells were stained for lipid droplets with Oil Red O (0.3% in isopropanol; Sigma, USA).

RESULTS

1. Growth Characteristics of MSCs

The cat MSCs were spindle-shaped and tightly attached to the culture dish 5 days after initial seeding (Fig. 3A). After 12 days of primary cultivation, cells were nearly 90% confluent (Fig. 3B).

2. Cell Cryopreservation

After three passages, as many as 30 million cells were generated from 5~6 ml of cat BM and were frozen in liquid nitrogen in 0.5 ml aliquots in 24 cryopreservation ampoules.

3. Cell-surface Antigen Profile

The MSC surface-antigen profile was determined by staining with cat-specific monoclonal antibodies followed by flow cytometry, as described previously (Jin *et al.*, 2007). Cat MSCs were strongly positive for CD9 and CD44 and negative for CD18 and CD45-like (Fig. 4).

4. MSCs Exhibit Multilineage Differentiation Potential

Mineralized matrix of MSCs under osteogenic conditions was evidenced by staining with Alizarin-red S solution (Fig. 5B) at day 21. Upon induction in adipogenic media, neutral lipid vacuoles were noticeable as early as 7 days and visualized by staining with oil red O (Fig. 5D).

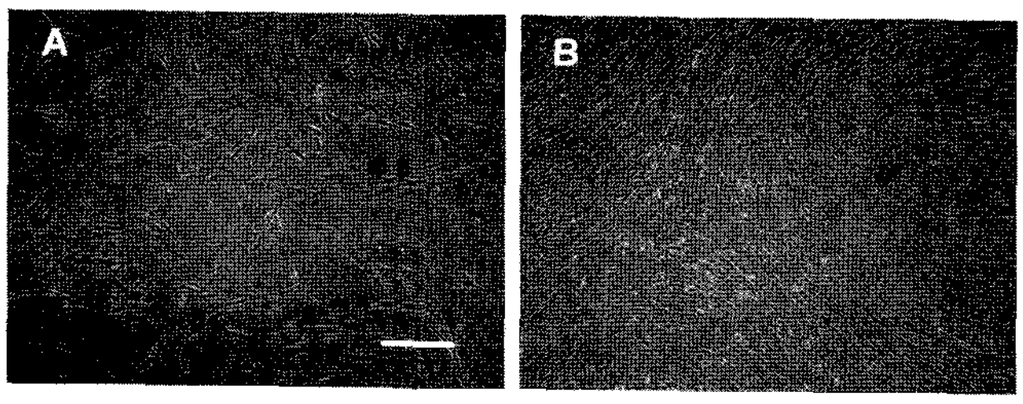

Fig. 3. Cat MSCs in primary culture. After 5 days of culture, adherent spindle-shaped MSCs could be clearly observed (A). After 12 days, MSCs had proliferated to 90% confluence (B). Scale bar: 200 μm.

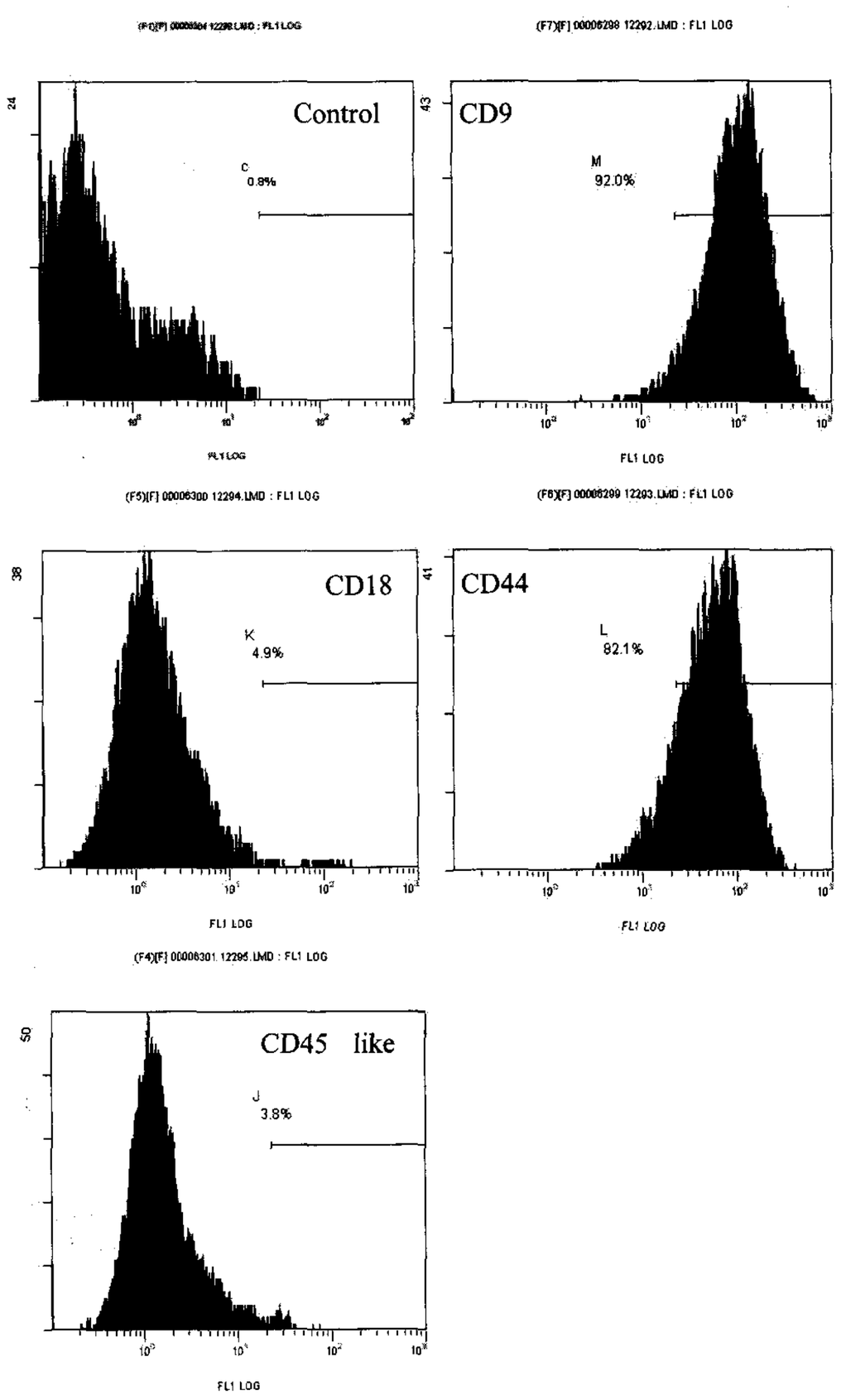

Fig. 4. Flow cytometric determination of cell-surface antigen profile for cat MSC. Cat bone marrow MSCs were stained with cat specific antibodies to a variety of cell-surface antigens. The percentage of cells positive for each antibody is listed in the corresponding histogram. An isotype control is used to identify background fluorescence. MSC from 3 separate cats were tested with similar results. Representative results from 1 cat are shown.

DISCUSSION

The present study demonstrates that a large number of BMCs can be rapidly harvested from the cat by our simple procedure. This procedure may satisfy the requirements of many MSC studies.

One of the advantages of our method is low donor attrition and good outcome. This makes it possible for the donor to be reused and to be the recipient of autogenic MSCs. The proce-

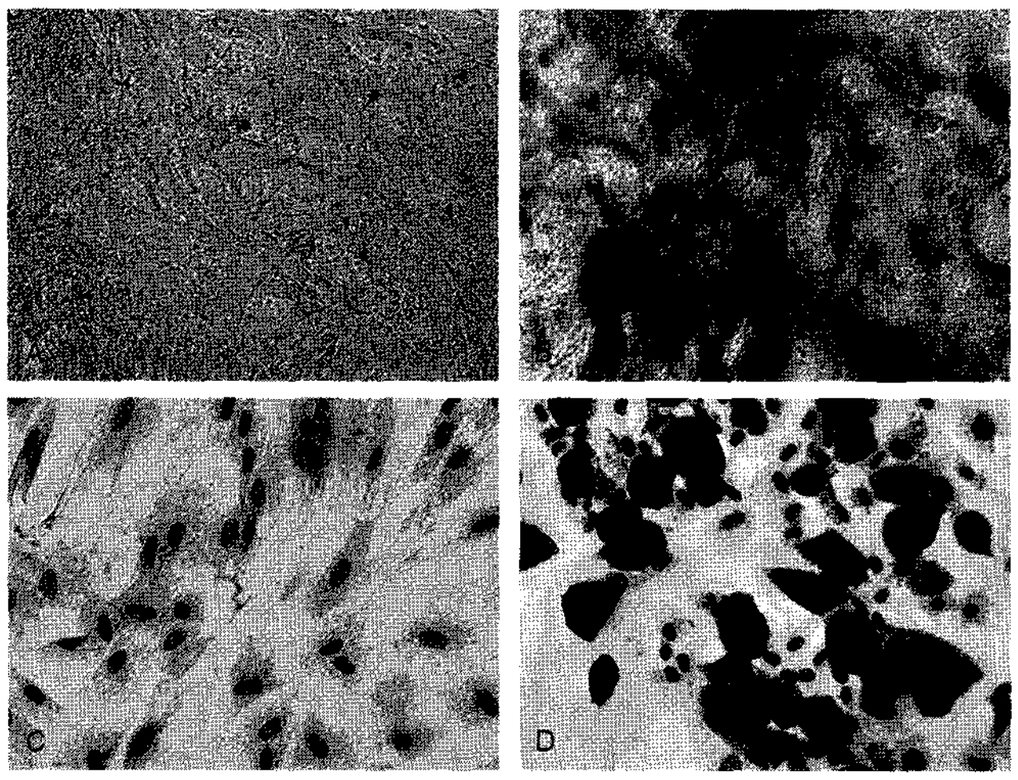

Fig. 5. Osteocyte and adipocyte differentiation of feline MSC. After an incubation in osteocyte induction media for 21 days (A and B) or in adipocyte differentiation media for 7 days (C and D), the cells were stained with Alizarin Red S or Oil Red O, respectively. (A) Undifferentiated control cells and (B) differentiated osteocytes. (C) Uninduced cells and (D) induced adipocytes. All images are shown at a magnification of ×400.

dure is completed within 5~10 min after the anesthetization. This may reduce the burden on the donor and meet criteria for the care and use of laboratory animals. We have performed the procedure on 8 cats with similar results. We have chosen a puncture point at the base of the neck of the femur under the greater trochanter. Because this site is comprised of cancellous bone which is readily punctured (Sartoris *et al.*, 1986). A needle is readily screwed into the femoral medullary cavity and no drilling is required.

MSCs are referred to stem cells originated mainly from the mesoderm, embedded in mesenchymal tissues and BM, and giving rise to cells of connective tissues, such as bone, cartilage, fat, and tendon (Friedenstein *et al.*, 1974; Owen, 1988; Pittenger *et al.*, 1999). The differentiation potential of BM-derived MSCs has been extensively investigated, and is now known to extend beyond cells of a mesodermal layer to nonmesenchymal neuronal and glial cells, as supported by a sizable body of *in vitro* and *in vivo* studies (Mezey *et al.*, 2003; Hermann A *et al.*, 2004; Cizkova *et al.*, 2006; Suon *et al.*, 2006). We also showed here that MSCs were induced not only cells of connective tissues (Fig. 5) but also neural cells (data not shown). Therefore, MSCs prepared by us were demonstrated to posses the multipotential of differentiation. Morphologically, cat MSCs appeared very similar to MSCs of rodent and human (Pittenger *et al.*, 1999; Woodbury *et al.*, 2000).

Here, we select four representative surface antigens, CD9, CD18, CD44, and CD45, to test the isolated cells. CD9 and CD44 are surface antigens on mesenchymal cells, whereas CD18 and CD45 are on the hemopoietic cells in bone marrow (Koda *et al.*, 2005). Our results show that among the four surface antigens only the markers of the mesenchymal cells express positively. Thus, they retain the MSC phenotype.

Similar procedures may apply equally well to other medium-sized and small animals, such as dogs, so that MSC investigations can be easily carried out in other species using our method. We believe that this method will contribute to the progress toward the ultimate application of MSCs to human health problems.

ACKNOWLEDGEMENTS

We thank Eun-Ill WANG for his help in constructing Fig. 2. This work was supported by a grant from the Korea Science and Engineering Foundation (KOSEF) (#M10525010001-05N2501-00110), funded by the Korean government (MOST). Guang Zhen Jin, Young Soo Lee and Eugene Choi were supported by scholarships from the Post BK21 Program.

REFERENCES

Brazelton TR, Rossi FM, Keshet GI and Blau HM. 2000. From marrow to brain: Expression of neuronal phenotypes in adult mice. Science 290:1775-1779.

Cizkova D, Rosocha J, Vanicky I, Jergova S and Cizek M. 2006. Transplants of human mesenchymal stem cells improve functional recovery after spinal cord injury in the rat. Cell Mol. Neurobiol. 26:1167-80.

Friedenstein AJ, Deriglasova UF, Kulagina NN, Panasuk AF, Rudakowa SF, Luriá EA and Ruadkow IA. 1974. Precursors for fibroblasts in different populations of hematopoietic cells as detected by the *in vitro* colony assay method. Exp. Hematol. 2:83-92.

Hermann A, Gastl R, Liebau S, Popa MO, Fiedler J, Boehm BO, Maisel M, Lerche H, Schwarz J, Brenner R and Storch A. 2004. Efficient generation of neural stem cell-like cells from adult human bone marrow stromal cells. J. Cell Sci. 117:4411-22.

Jin GZ, Yin XJ, Yu XF, Cho SJ, Lee HS, Lee HJ and Kong IK. 2007. Enhanced tyrosine hydroxylase expression in

PC12 cells co-cultured with feline mesenchymal stem cells. J. Vet. Sci. 8:377-382.

Kabos P, Ehtesham M, Kabosova A, Black KL and Yu JS. 2002. Generation of neural progenitor cells from whole adult bone marrow. Exp. Neurol. 178:288-293.

Koda M, Okada S, Nakayama T, Koshizuka S, Kamada T and Nishio Y. 2005. Hematopoietic stem cell and marrow stromal cell for spinal cord injury in mice. Neuroreport 16:1763-1767.

Kushida T, Inaba M, Ikebukuro K, Ngahama T, Oyaizu H, Lee S, Ito T, Ichioka N, Hisha H, Sugiura K, Miyashima S, Ageyama N, Ono F, Iida H, Ogawa R and Ikehara S. 2000. A new method for bone marrow cell harvesting. Stem Cells 18:453-456.

Malik R. 2001. Genetic diseases of cats. J. Feline Med. Surg. 3:109-113.

Martin DR, Cox NR, Hathcock TL, Niemeyer GP and Baker HJ. 2002. Isolation and characterization of multipotential mesenchymal stem cells from feline bone marrow. Exp. Hematol. 30:879-886.

Menotti-Raymond M, David VA, Lyons LA, Schaffer AA, Tomlin JF, Hutton MK and O'Brien SJ. 1999. A genetic linkage map of microsatellites in the domestic cat (*Felis catus*). Genomics 57:9-23.

Mezey E, Key S, Vogelsang G, Szalayova I, Lange GD and Crain B. 2003. Transplanted bone marrow generates new neurons in human brains. Proc. Natl. Acad. Sci. U. S. A. 100:1364-1369.

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

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

Prockop DJ. 1997. Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 276:71-74.

Rohn JL, Gwynn SR, Lauring AS, Linenberger ML and Overbaugh J. 1996. Viral genetic variation, AIDS, and the multistep nature of carcinogenesis: the feline leukemia virus model. Leukemia 10:1867-1869.

Sanchez-Ramos J, Song S, Cardozo-Pelaez F, Hazzi C, Stedeford T, Willing A, Freeman TB, Saporta S, Janssen W, Patel N, Cooper DR and Sanberg PR. 2000. Adult bone marrow stromal cells differentiate into neural cells *in vitro*. Exp. Neurol. 164:247-256.

Sartoris DJ, Andre M, Resnik CS and Resnick D. 1986. Trabecular bone density in the proximal femur: Quantitative CT assessment. Work in progress. Radiology 160: 707-712.

Suon S, Yang M and Iacovitti L. 2006. Adult human bone marrow stromal spheres express neuronal traits *in vitro* and in a rat model of Parkinson's disease. Brain Res. 1106:46-51.

Suter SE, Gouthro TA, McSweeney PA, Nash RA, Haskins ME, Felsburg PJ and Henthorn PS. 2004. Isolation and characterization of pediatric canine bone marrow $CD34^+$ cells. Vet. Immunol. Immunopathol. 101:31-47.

Tao H, Rao R and Ma DD. 2005. Cytokine-induced stable neuronal differentiation of human bone marrow mesenchymal stem cells in a serum/feeder cell-free condition. Dev. Growth Differ. 47:423-433.

Wade TV, Rothblat DS and Schneider JS. 2001. Changes in striatal dopamine D3 receptor regulation during expression of and recovery from MPTP-induced parkinsonism. Brain Res. 905:111-119.

Woodbury D, Schwarz EJ, Prockop DJ and Black IB. 2000. Adult rat and human bone marrow stromal cells differentiate into neurons. J. Neurosci. Res. 61:364-370.

(접수일: 2008. 6. 12 / 채택일: 2008. 6. 20)