Original Article

Differentiation Potentials of Canine Bone Marrow Mesenchymal Stem Cells

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Abstract

Mesenchymal stem cells (MSCs) are multipotent cells that have characteristics of self-renewal and differentiation into various specific cell types, in particular mesodermal lineages. This study aimed at isolating, identifying and examining the differentiation capability of canine MSCs. Bone marrow aspirates were obtained from 4 dogs. Putative MSCs were then cultured in MSC medium and subpassaged. At the 3rd to 5th passages, MSCs were examined for their morphology and doubling time. Two cell lines were examined for the expression of CD 34, CD 44 and CD 90, using flow cytometry. The *in vitro* differentiation of these MSCs into mesodermal lineages (bone, cartilage and adipose tissues) and ectodermal lineage (neuron) was performed using osteogenic, chondrogenic, adipogenic and neurogenic media, respectively. Histological examinations (Von Kossa and alcian blue staining) and mRNA expressions (GLA and COL1A1) were used to examine the bone and cartilage differentiation, while Oil red O staining was used to determine adipogenic differentiation.

Plastic-adhered MSCs had high potential for cell division, with a mean doubling time of 35.4±9.3 hours. These fibroblast-like MSCs expressed MSCs markers (CD 44 and CD 90), while fewer than 5% of these MSCs were tested positive to a hemopoietic stem cell marker (CD34). Based on the histological examinations and gene expressions, these cells demonstrated the ability to differentiate into bone, cartilage and adipose tissues. In conclusion, MSCs can be isolated from canine bone marrow and these cells are capable of *in vitro* differentiation into specific mesodermal lineages.

Keywords: bone marrow, canine, mesenchymal stem cells

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บทคัดย่อ

ประสิทธิภาพการเจริญเปลี่ยนแปลงของเซลล์ต้นกำเนิดมีเซนไคม์จากสุนัข

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เซลล์ต้นกำเนิดมีเซนไคม์ (MSCs) เป็นเซลล์ที่มีความสามารถในการเจริญเพิ่มจำนวนได้เองและสามารถพัฒนาเป็นเซลล์จำเพาะหลายชนิดโดยเฉพาะเซลล์ในกลุ่มเนื้อเยื่อชั้นกลาง การศึกษาครั้งนี้มีวัตถุประสงค์ เพื่อศึกษาวิธีการเก็บ การพิสูจน์ และการตรวจสอบคุณสมบัติการเจริญเปลี่ยนแปลงของเซลล์ต้นกำเนิดมีเซนไคม์ของสุนัข ภายหลังการเก็บของเหลวจากโพรงกระดูกสุนัข ทำการเลี้ยงเซลล์เพื่อเพิ่มจำนวนและทำการตรวจลักษณะรูปร่างและอัตราเร็วของการเพิ่มจำนวนเซลล์เป็นสองเท่า จากนั้นทำการตรวจการแสดงออกของโปรตีนบนผิวเซลล์ชนิด CD 34 CD 44 CD 90 ด้วยเทคนิคโฟลวไซโตเมตรี และตรวจคุณสมบัติของเซลล์ในการเจริญเปลี่ยนแปลงเป็นเซลล์ในกลุ่มเนื้อเยื่อชั้นกลาง (กระดูก กระดูกอ่อน และ ไขมัน) และเนื้อเยื่อชั้นนอก (เซลล์ประสาท) ใช้เทคนิคทางจุลพยาธิวิทยาร่วมกับการย้อมสี Von Kossa และ Alcian blue รวมถึงการแสดงออกของ mRNA ต่อยีน GLA และ COL1A1 ในการตรวจวินิจฉัยกระดูกและกระดูกอ่อนตามลำดับ และใช้ Oil Red O ในการตรวจเนื้อเยื่อไขมัน

เซลล์ MSCs ที่เกาะบนจานเพาะเลี้ยงพลาสติกมีคุณสมบัติในการแบ่งตัวโดยมีค่าเฉลี่ยของระยะเวลาการเพิ่มจำนวนเป็นสองเท่าเท่ากับ 35.4±9.3 ชั่วโมง เซลล์ MSCs มีลักษณะคล้ายเซลล์ไฟโบรบลาส ให้ผลบวกต่อการแสดงออกของโปรตีนจำเพาะต่อเซลล์ MSC (CD 44 และ CD 90) ในขณะที่เซลล์จำนวนน้อยกว่าร้อยละ 5 ให้ผลบวกต่อเซลล์ต้นกำเนิดเลือด (CD 34) จากการตรวจคุณสมบัติของเซลล์ด้วยเทคนิคทางจุลพยาธิวิทยาและการแสดงออกของยีน พบว่าเซลล์เหล่านี้สามารถเจริญพัฒนาเป็นเซลล์กระดูก กระดูกอ่อน และ เซลล์ไขมันได้ การศึกษาครั้งนี้สรุปว่าเซลล์ MSCs ที่เจาะเก็บจากโพรงกระดูกสุนัขมีความสามารถในการเจริญพัฒนาในจานเพาะเลี้ยงให้เป็นเซลล์ในกลุ่มเนื่อเยื่อชั้นกลางได้

คำสำคัญ: ไขกระดูก สุนัข เซลล์ต้นกำเนิดมีเซนไคม์

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Introduction

Stem cells have been intensively studied over the past two decades because these cells have a remarkable potential to develop into various specific cell lineages upon being cultured in appropriated conditions. Because of this, they have been considered as a powerful tool for cell- or tissue-based engineering in human and veterinary medicine (Barry et al., 2004; Bongso et al., 2008; Ribitsch et al., 2010). Stem cells are different from other cell types by two important characteristics (Wobus and Boheler, 2005), as they have the capability of self-renewal, while maintaining themselves in an undifferentiated stage. In specific condition, these quiescent stem cells, however, differentiate into specific cell types or tissues with special functions such as cardiomyocyte (Jing et al., 2008; Mayorga et al., 2009), bone (Barry and Murphy, 2004), cartilage (Kavalkovich et al., 2002) and neuron (Trzaska et al., 2007; Kim et al., 2009).

Stem cells are usually classified into three types (viz. embryonic, induced-pluripotent and adult stem cells), according to their origins and production techniques. Embryonic stem (ES) cells are pluripotent-specialized cells that are isolated from an inner cell mass of blastocyst-stage embryos (Evans and Kaufman, 1981; Martin, 1981). Induced-pluripotent stem cells (iPS) are also pluripotent stem cells that can be derived from genetic modification of non-pluripotent somatic cells (Takahashi and Yamanaka, 2006). Although these two types of ES cells are capable of unlimited cell division and differentiation into all three germ layers (endoderm, ectoderm and mesoderm), their clinical exploitation has been obscured by the possibility of tumorogenesis after transplantation *in vivo* (Arnhold et al., 2000; Reubinoff et al., 2000; Erdo et al., 2003). In contrast to the ES cells, adult stem cells have less ability of self-renewal, and their differentiation usually occurs within a cell lineage from which they originated. For example, mesenchymal stem cells (MSCs) can only differentiate

into mesodermal lineages, such as bone, cartilage and adipose tissues (Zuk et al., 2002). Interestingly, although these cells have been described as multipotent stem cells as differentiation potentials are essentially restricted to only mesodermal lineages, a recent report showed that they also have a potential to differentiate into other cell lineages, such as endoderm and ecdoderm origins (a process referred to as transdifferentiation) (Alaminos et al., 2010).

To date, host-specific MSCs are highly desired in regenerative medicine because they can be logically isolated and propagated from many tissue origins, such as bone marrow and adipose tissue. Canine MSCs have been demonstrated to have the potential for use in cell-based therapy, particularly for bone and soft tissue regeneration (Kraus and Kirker-Headm, 2006; Hiyama et al., 2008; Jang et al., 2008; Jung et al., 2009; Zucconi et al., 2010). It is commonly accepted that the identification of MSCs relies on the expressions of positive (Stro-1, CD 90, CD 105, CD 44, CD 73) and negative markers (i.e., markers for hemopoietic cells: CD 34 and 45). In dogs, only the attachment property of MSCs to plastic culture dishes is commonly-accepted method for the selection of canine MSCs, while the use of MSC markers for identification varies from one laboratory to the next.

The present study was aimed at evaluating the isolation and the identification techniques for canine mesenchymal stem cells (MSCs) that are derived from bone marrow aspirates and studying their differentiation potentials.

Materials and Methods

All chemicals were purchased from Sigma-Aldrich, St Louis, USA, unless otherwise specified.

Isolation of mesenchymal stem cells from bone marrow aspirates: Bone marrow aspirates were obtained from 4 healthy dogs. The procedure for obtaining these aspirates was reviewed and approved by the Ethical Committee for Animal Use, Faculty of Veterinary Science, Chulalongkorn University (Accession No. 0931055). In brief, the animals were premedicated intramuscularly with 0.1 mg/ml Acepromacine maleate (Vetranquil™; Ceva Sante animal, Libourne, France) and 0.25 mg/kg morphine sulphate (Food and Drug Administration, Bangkok, Thailand). After 15-20 min, anesthesia was induced intravenously with 4 mg/kg propofol (Fresenius Kabi Austria GmbH, Graz, Austria). The bone marrow contents were collected from either the humorous or iliac crest into a 10-ml heparinized syringe (containing 1000 IU heparin). The bone marrow aspirates were then transported to the laboratory (at 26°C) and processed within 4 hours after bone marrow aspiration. Upon arrival, the bone marrow aspirates were first layered onto gradient density (Histopaque®-1077 density 1.077 g/ml) and centrifuged at 26°C and 400g for 30 min. The mononuclear cells at the interface between each of the bone marrow aspirates and Histopaque® were used. Occasionally, remaining red blood cells were mixed and incubated with an equal volume of red blood cell lysis buffer for 5 min. The mixture was then centrifuged and resuspended with 1 ml of culture medium.

Culture of canine bone marrow mesenchymal stem cells: Following MSC isolation, presumptive MSCs were seeded into a 100 mm-Petri dishes (BD-Falcon™, Franklin Lake, NJ, USA), containing low glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (Invitrogen, Carlsbad, USA), 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. Non-adherent cells were removed by washing the culture dishes with Dulbecco's phosphate buffered saline (DPBS; Invitrogen) and the culture medium was changed every 2-3 days. Adherent cells were cultured (passage 0) until they reached approximately 70-80% confluence. To sub-passage, the adherent cells were washed twice with DPBS and then digested with 0.125% trypsin-EDTA (Gibco™, Invitrogen) for 2 min and the enzyme was inactivated with an excessive amount of fetal bovine serum in DMEM. The cell suspension was then centrifuged at 4°C and 1000 rpm for 5 min. If cryopreservation of cells were needed, a freezing medium containing 10% (v/v) dimethylsulphoxide (DMSO) and 90% (v/v) fetal bovine serum was added to the cells. The equilibrated cell suspension was added into a 1-ml cryovial (Corning, USA). The freezing rate was controlled at 1°C/min using a cryobox.

Cell morphology and population doubling time: MSCs were daily examined for cell morphology at 100 and 200x magnification using a phase contrast microscope (CKX41, Olympus, Japan). At the 3rd passage, the MSCs were plated into a 12-well plate at 20,000 cells/cm^2 (approximately, 40-50% confluence). The MSCs were then trypsinized with trypsin-EDTA, and the total number of cells in each culture well was counted using a hemocytometer at 24 hours interval for 3 consecutive days. The doubling time was calculated using the equation, ln(2)/growth rate, whereas the growth rate referred to the number of doublings that occurred per unit of time.

Flow cytometry analysis: Canine MSCs at the 3rd passage were immunologically examined for surface markers of MSCs. Because there is no universal marker that is specific to MSCs, identification of MSCs therefore relied on both positive and negative markers. To perform flow cytometry, the MSCs were first dissociated from the Petri dishes with Trypsin-EDTA and then centrifuged. A total of 200,000 to 300,000 cells were stained with each respective antibody. Rat monoclonal anti-canine CD 34 conjugated with fluorescein isothiocyanate (FITC) (a marker for hemopoietic stem cells) was used as the negative MSC marker. Rat monoclonal anti-canine CD 90 (AbD serotec, Kidlington, UK) with rabbit anti-rat FITC secondary antibody and monoclonal anti-canine CD44 conjugated with allophycocyanin (APC) (R&D system, Minneapolis, USA) were used as MSC positive markers. Fluorescently-labeled MSCs were finally washed once, fixed with 1% (w/v) paraformaldehyde in PBS and stored in the dark at 4°C until analysis. Non-staining MSCs and MSCs labeled with only the secondary antibody were used as controls. At least 20,000 MSCs were used to test the

presence of each cell surface marker, using flow cytometry (BD Biosciences, Franklin Lakes, USA).

Differentiation of MSCs: The MSCs derived from the canine bone marrow at the 3rd-5th passages from 2 dogs were used to demonstrate their differentiation potentials. MSCs were induced to differentiate into bone, cartilage and adipose tissues according to the methods as previously described (Bosch et al., 2006), with some modifications. For bone differentiation, MSCs were first sub-cultured to reach approximately 80% confluence, and the bone induction medium consisting of DMEM supplemented with 10% (v/v) FCS, 100 nM dexamethasone, 50 ng/ml ascorbic acid and 10 mM Beta-glycerophosphate was added into the Petri dishes. The bone induction medium was changed every 2-3 days. A three-dimensional culture system was used to induce cartilage differentiation. MSCs were dissociated and then transferred to a 15-ml cornical tube (BD-Falcon™, Franklin Lake, NJ, USA). After centrifugation, aggregated cells were re-suspended with the cartilage induction medium containing DMEM, 10 ng/ml TGF-β1, 100 nM dexamethasone and 50 ng/ml ascorbic acid 2-phosphate. The medium was changed every 2-3 days. The adipose tissue differentiation was performed with monolayer MSCs, as previously described (Zuk et al., 2002). The MSCs were treated with 4 cycles of adipogenic induction and maintenance (each cycle consisted of 3 days of adipogenic induction and 2 days of adipogenic maintenance). The adipogenic induction medium consisted of DMEM 10% (v/v) FCS, 0.1 mg/ml human recombinant insulin, 10 mM sodium pyruvate, 1 mM methyl isobutylxanthine (IBMX), 0.2 mM indomethacin and 1 μM dexamethasone. Adipogenic maintenance was prepared in a similar manner to adipogenic induction, but without IBMX, dexamethasone and indomethacin. Neurogenic induction was performed with DMEM supplemented with 200 μM butylated hydroxyanisole, 25 mM KCl, 2mM valproic acid, 10 μM forskolin, 1 μM hydrocortisone, 5 μg/ml Insulin and 2% (v/v) DMSO.

Assessment of MSC differentiation: After the fixation of MSC-derived bone tissues in 4% (w/v) paraformaldehyde, the tissues were embedded in paraffin for histological analysis. The histological sections of the tissues were stained with Von Kossa and alcian blue to detect the deposition of calcium phosphate and glycosaminoglycans enriched-matrix, indicating the successful differentiation of MSCs into bone and cartilage tissues, respectively. The presence of adipogenesis was confirmed by oil red O staining which detects intracellular neutral triglycerides and lipids.

Reverse transcription polymerase chain reaction (RT-PCR): Total RNA was extracted from undifferentiated MSCs that were cultured in monolayer and differentiated cell pellets collected on D9 and D21 of differentiation using Absolutely RNA® Nanoprep Kit (Stratagene, Agilent Technologies, USA). The contaminating genomic DNA was removed during the purification steps by DNase I treatment according to the manufacturer's instructions. Total RNA was eluted from the purification column with sterile distilled water and was quantified using a NanoDrop® 1000 spectophotometer (Thermo Scientific, USA). Gene-specific oligonucleotide primers were synthesized by BioDesign Co., Ltd. (Bangkok, Thailand). Two target genes reported to be involved in osteogenic lineage differentiation, i.e., collagen type I alpha I (COL1A1) and bone γ-carboxyglutamate protein (GLA), were selected for this experiment. A housekeeping gene, i.e., glyceraldehype 3-phosphate dehydrogenase (GAPDH), was included as the internal control and reference. Oligonucleotide sequences, PCR product size, Genebank accession number and references of primers are shown in Table 1.

cDNA was synthesized from an aliquot of 150 to 200 ng of total RNA using random hexamer primers and Omniscript® Reverse Transcription Kit (Qiagen, Hilden, Germany). One microliter of RT reaction was mixed with 12.5 μl of GoTaq® Green Master Mix (Promega, WI, USA), 6.25 μM each of forward- and reverse primers, and sterile distilled water to reach the final volume of 25 μl. PCR was carried out on an Amplitronyx™ thermocycler machine (Nyxtechnik, CA, USA). The suitable cycle condition was determined and applied for each primer pair as follows: GAPDH, 94°C for 2 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 45 sec, plus the final extension at 72°C for 5 min; COL1A1 and GLA, 94°C for 1 min, followed by 30 cycles of 94°C for 30 sec, 58°C for 30 sec, 72°C for 1 min, plus the final extension at 72°C for 10 min. PCR products were analyzed by electrophoresis on 2% ethidium bromide incorporated-agarose gels in Tris-Boric-EDTA (TBE) buffer. The images of agarose gels were taken using GeneFlash Gel Documentation (Syngene, Synoptic Ltd., Frederick, USA) and the calculation of relative intensity of target gene mRNA expression signal utilizing the expression intensity of GAPDH as the reference was carried out using the Scion Imaging program (Beta 4.0.3; Scion Corporation, MD).

Table 1 Oligonucleotide primers used in this study

Gene	Sequences	Size (bp)	Accession number	References
COL1A1	(F) 5′-CAC CTC AGGAGA AGG CTC AC–3′ (R) 5′-ATG TTC TCG ATC TGC TGG CT–3′	123	NM_001003090.1	Seo et al. (2009)
GLA	(F) 5′-GTG GTG CAA CCT TCG TGT C-3′ (R) 5′-GCT CGC ATA CTT CCC TCT TG-3′	131	XM_547536.2	Seo et al. (2009)
GAPDH	(F) 5′-GGA GAA AGC TGC CAA ATA TG-3′ (R) 5′-CAG GAA ATG AGC TTG ACA AAG TGG-3′	191	AB038241	Sano et al. (2005)

Results

Fibroblastic-like cells were isolated from canine bone marrow (Fig. 1). These cells adhered to the culture dish by 24 hours of culture. At passage 3, putative MSCs were cultured and analyzed for expression of MSC surface markers and population doubling time. These MSCs were expressed mesenchymal stem cells markers (CD 44 and CD 90) and also negative to hemopoietic stem cells marker (CD 34). Two canine MSCs (dog 3 and dog 4) strongly expressed CD 44 (99.9% and 86.7%) and CD 90 (92.5%, and 95.3%), respectively (Fig. 2). However, the growth rates of MSCs (population doubling time) were found to be different among putative MSCs from different dogs (46.1, 24.2, 38.8 and 32.4 hours).

We further demonstrated that these cells were also capable of differentiation into mesodermal lineages including bone, cartilage and adipose tissues. After 21 days of differentiation, differentiated MSCs were essentially positive to oil red O, Von kossa and alcian blue as indicators of fat, bone and cartilage formation respectively (Fig. 3). In addition, MSCs were also transdifferentiated into multiple-process neuron-like cells soon after treating MSCs with neurogenic medium (Fig. 3).

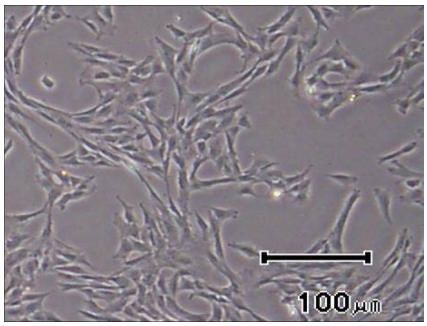

Figure 1 Fibroblastic-like mesenchymal stem cells derived from bone marrow aspirate

COL1A1 and GLA expression was detected in undifferentiated MSCs. The early stage of differentiation up-regulated the genes as seen in the increment of relative signal intensity on D9 post culture. COL1A1 mRNA increased continuously on D21 post differentiation, while the expression of GLA gene dropped below the starting point (Fig. 4).

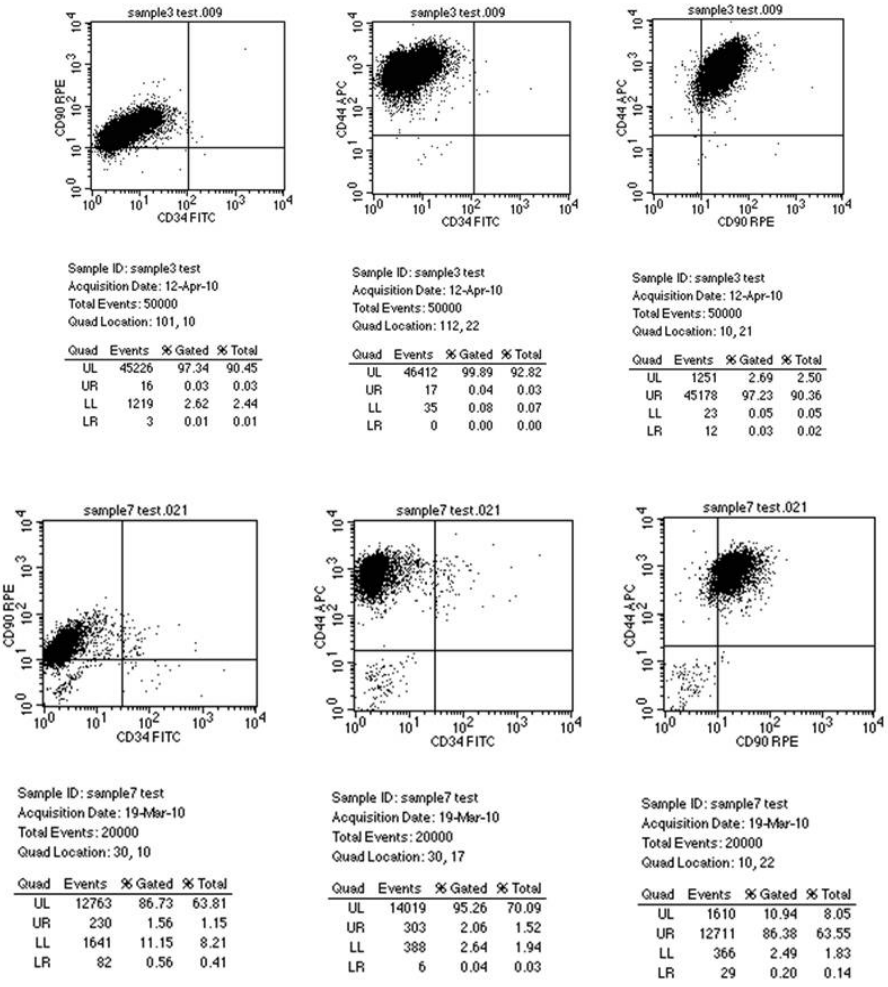

Figure 2 Expressions of CD 44 and CD 90 in canine MSCs. Samples from dog 3 (upper panel) and dog 4 (lower panel) were analyzed by flow cytometry.

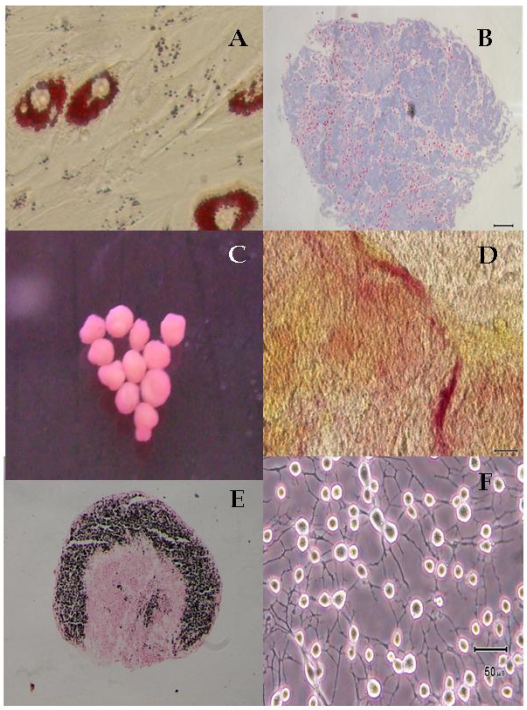

Figure 3 Differentiation potential of canine mesenchymal stem cell (A) adipose tissue; cartilage (B) bone (C and E) and neuron-like cells (F). Adipocytic cells accumulated with neutral lipids as shown by oil red O staining. Cartilagenous and bone tissues were confirmed by the presence of glucosaminoglycan enriched matrix, calcium phosphate and alkaline phosphatase activity (D) using alcian blue, Von kossa and leukocyte alkaline phosphatase kit, respectively.

Discussion

In the current study, we successfully isolated well-defined mesenchymal stem cells derived from canine bone marrow. These specialized cells are classified as multipotent stem cells because they have the capability of differentiation into mesodermal lineage. MSCs have been isolated from many tissues of the body such as bone marrow (Wagner et al., 2005; Kern et al., 2006), adipose tissue (Zuk et al., 2002), umbilical cord (Lee et al., 2004; Koch et al., 2007) and dental pulp (Jo et al., 2007; Waddington et al., 2009). In dog, the data in regard to the isolation, characterization and clinical use of canine MSCs have been limited. Until recently, canine MSCs have been isolated from bone marrow (Csaki et al., 2007; Jafarian et al., 2008), adipose tissue (Neupane et al., 2008; Vieira et al., 2010) and umbilical cord (Seo et al., 2009). Because there is no specific marker for the identification of canine MSCs, the isolation and identification technique has therefore been different among laboratories. MSCs from many species demonstrate antigen specific on cell membrane such as CD 29, CD 44, CD 90, CD 105 and Stro-1 (Martin et al., 2002; Bosnakovski et al., 2005; Csaki et al., 2007; Meirelles and Nardi, 2009; Rho et al., 2009). In the current study, only canine antibodies were proven a good candidate for MSC isolation when compared with antibodies from other animals such as anti-mouse Stro-1 and anti-human CD 105 (unpublished data), suggesting

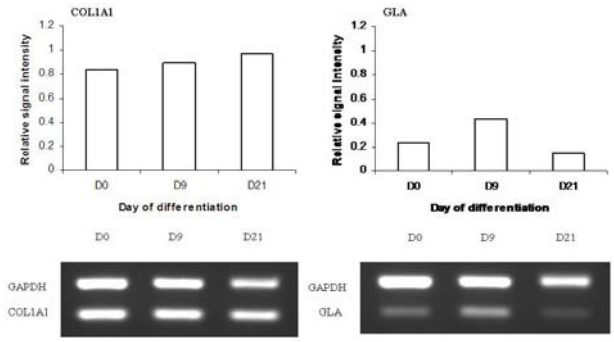

Figure 4 Relative signal intensity of COL1A1 and GLA expression compared with GAPDH expression on D0, D9 and D21 of dog MSCs bone differentiation *in vitro*.

the specificity of canine surface antigen. This phenomenon is one of the hallmarks affecting the exploitation of MSCs for clinical use since MSCs are located in the bone marrow with a mixed population of cells and it has been estimated that there are only 0.0001-0.01% MSCs in the nucleated cells of bone marrow aspirate (Pittenger et al., 1999). Most investigators have isolated MSC using their capacity to adhere to a plastic culture dish. However, macrophages, endothelial cells, lymphocytes, and smooth muscle cells can also adhere to culture plate and therefore contaminate the MSC preparations. The isolation, identification and purification of canine MSCs recently become an important issue for the clinical use of MSCs. Although legal regulation use of canine MSCs has yet to be discussed, the minimum requirements for clinical use of MSCs have been announced for human (Dominici et al., 2006). Human MSCs must demonstrate the fibroblast-like morphology and have the ability to adhere to a plastic culture dish, positively express (> 95%) cell surface receptors (e.g. CD 29, CD 44, CD 73, CD 105, CD 106, Stro-1, etc.) and negatively express (< 2%) the hematopoietic lineage markers (e.g. c-Kit, CD 14, CD 34, CD 45). More importantly, these cells must show a capability of differentiation into mesodermal lineage (bone, cartilage and adipose tissues). In the current study, we found that MSCs, at least under our conditions, were already committed to differentiate into bone and cartilage as they prematurely expressed GLA and COL1A1 (early markers for bone and cartilage differentiation) prior to differentiation induction. It is also possible that the MSCs used in this study had spontaneous differentiation during culture. Until recently, study of pathways regulating the bone and cartilage differentiation of canine bone marrow MSCs is still required in order to improve the efficiency of in vitro bone and cartilage differentiation for cell- or tissue-based engineering. Volk et al. (2005) reported that canine MSCs could be efficiently differentiated into bone using bone morphologic protein 2 (BMP-2) which was similar to other species (Li et al., 2007). In addition to the differentiation capability into mesodermal lineage, transdifferentiation of MSCs to neuron-like cells has been believed to hold a great promise in cell treatment therapy (Zipori, 2004; Krabbe et al., 2005; Bongso et

al., 2008). However, this transdifferentiation of MSCs into neuron-like cells has been contradictory especially the toxic effect of DMSO on actin microfilament during differentiation (Lu et al., 2004; Neuhuber et al., 2004). Interestingly, it has been shown that MSCs expressed neuronal specific genes even though they were not treated with neuronal differentiation medium (Yamaguchi et al., 2006; Kamishina et al., 2006). In this study, we only recorded morphological changes of canine MSCs after neuronal differentiation. The 'true' capability of canine MSCs in neuron differentiation has yet to be examined in the prospective study.

In conclusion, our study demonstrates that canine mesenchymal stem cells can be isolated from bone marrow, and these MSCs are capable of differentiation into specific mesodermal linage (bone, cartilage and adipose tissue) following passages and in vitro differentiation.

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