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Isolation and characterization of canine umbilical cord blood-derived mesenchymal stem cells

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Human umbilical cord blood-derived mesenchymal stem cells (MSCs) are known to possess the potential for multiple differentiations abilities *in vitro* and *in vivo*. In canine system, studying stem cell therapy is important, but so far, stem cells from canine were not identified and characterized. In this study, we successfully isolated and characterized MSCs from the canine umbilical cord and its fetal blood. Canine MSCs (cMSCs) were grown in medium containing low glucose DMEM with 20% FBS. The cMSCs have stem cells expression patterns which are concerned with MSCs surface markers by fluorescence- activated cell sorter analysis. The cMSCs had multipotent abilities. In the neuronal differentiation study, the cMSCs expressed the neuronal markers glial fibrillary acidic protein (GFAP), neuronal class III β tubulin (Tuj-1), neurofilament M (NF160) in the basal culture media. After neuronal differentiation, the cMSCs expressed the neuronal markers Nestin, GFAP, Tuj-1, microtubule-associated protein 2, NF160. In the osteogenic & chondrogenic differentiation studies, cMSCs were stained with alizarin red and toluidine blue staining, respectively. With osteogenic differentiation, the cMSCs presented osteoblastic differentiation genes by RT-PCR. This finding also suggests that cMSCs might have the ability to differentiate multipotentially. It was concluded that isolated MSCs from canine cord blood have multipotential differentiation abilities. Therefore, it is suggested that cMSCs may represent a be a good model system for stem cell biology and could be useful as a therapeutic modality for canine incurable or intractable diseases, including spinal cord injuries in future regenerative medicine studies.

Keywords: canine umbilical cord blood, differentiation study, mesenchymal stem cell, stem cell characterization

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Introduction

Mesenchymal stem cells (MSCs) are defined to be multipotent stem cells that can be differentiated into various type of cells such as, neuronal cells, chondrocytes, adipocytes, cardiomyocytes and osteoblasts *in vitro* and *in vivo* under controlled conditions [15,24,27]. These cells can be isolated from many kinds of tissues, including fat, skin, and even the brain [2,13,18,22]. However, the most common source to obtain these cells is bone marrow. Isolation and transplantation of hematopoietic stem cells (HSCs) from human bone marrow into the bone marrow of a leukemia patient is now a feature of stem cell therapy. To perform this therapy it is difficult to find an appropriate immune matched donor for the transplantation, and the therapy is still recognized to be intricate [5]. MSCs isolated from human umbilical cord blood represent an alternative source of HSCs. The dog has been considered an attractive animal model to evaluate new drugs or medical trials for preclinical purposes [23,30]. One advantage of using dogs is that canine model transplantation uses a large size animal [9].

The isolation and characterization of CD34+ cells from canine bone marrow to optimize the conditions for bone marrow derived CD34+ cells transplantation has been studied [31]. Bhattacharya and colleagues identified isolated CD34+ cells from canine bone marrow that had endothelialized into the grafted area [3]. However, there are few studies on canine umbilical cord blood derived MSCs. These cells should be of use for cell based therapies and tissue engineering which have been performed in trials to overcome the difficulties of gene based therapies and their medical limitations. The use of stem cell implantation has been increasing, and it is strongly suggested that its use may enable an improved treatment of some incurable diseases such as genetic disorders [26], spinal cord injuries [11] and bone fracture malignancies [25,35].

For the past few years, it has been clearly recognized that

MSCs possess immune regulatory properties [1,8]. Adult stem cells are known to have a limited differentiation potential while embryonic stem cells are totipotent. Multipotent stem cells were first isolated from adult bone marrow [17]. The multipotent stem cells have been isolated and characterized from other adult tissues by several investigators [32]. In the present study, we successfully isolated and characterized umbilical cord blood-derived multipotent stem cells from dogs. The characterization conditions and basic settings for the application of gene delivery were also investigated.

Materials and Methods

Cell isolation and culture

Canine umbilical cord blood (cUCB) and blood of the canine fetus heart using paracentesis was drawn and used for the isolation of mononuclear cells. The collected blood was delivered in tubes treated with EDTA as an anti-coagulant. Blood was diluted 1 : 1 with PBS (Cellgro, USA). A density gradient using Ficoll-paque (GE Healthcare, USA) was sperformed to collect the buffy coat layer. Mononucleated cells were seeded into T75 cell culture flasks (Nunc, USA) at 5×10^6 cells/mL. Three days after the cells were seeded, they were transferred to new flasks containing half the amount of Dulbeco's Modified Eagle's Medium (low glucose DMEM; Gibco BRL, USA). The adhered cells were trypsinized to maintain passage after 7 days that the primary cells were seeded.

Cell expansion

Cumulative population doubling level (CPDL) was calculated using the formula "$x = \{\log10(N_H) - \log10(N_1)\}\log10$" [6] where N_1 is the inoculum cell number and N_H is the cell harvest number. To yield the cumulated doubling level, the population doubling for each passage was calculated and then added to the population doubling levels of the previous passages. As the cell number of isolated cells of all three tissues could be determined for the first time at passage 1, the cumulative doubling number was first calculated for passage 1 for this result.

Neurogenic differentiation

The cUCB-MSCs were seeded into a low-glucose DMEM with 20% FBS to confluent population. Cells were preincubated for 24 h with 1 mM Beta-mercaptoethanol and 20% FBS. After preincubation, cells were transferred to induction medium constituted with 100 μM Docosahexaenoic (Sigma, USA), B27 supplement (Gibco, USA) and 1.5% Dimethyl sulfoxide (Sigma, USA) serum free for 2 days [19].

Osteogenic differentiation

Adherent cells were cultured in osteogenic medium composed of LG-DMEM supplemented with 10% FBS, 10 mM β-glycerophosphate, 0.1 μM dexamethasone (Sigma-Aldrich, USA), and 50 μM ascorbic acid-2-phophate for 30 days. Osteogenic differentiation was evaluated by calcium mineralization. Alizarin red S staining was used to determine the presence of calcium mineralization. For Alizarin red S staining, cells were washed with D.W 2 times and fixed in a solution of ice-cold 70% ethanol for 1 h. After carefully washing 7 times with D.W, cells were stained for 10 min with 40 mM Alizarin red S after washed with D.W for 2 times in room temperature [10,29].

Chondrogenic differentiation

Chondrogenic differentiation was followed as previously described [14,29]. Briefly, 5×10^5 cells were seeded in a 15-mL polypropylene tube and centrifuged to a pellet. The pellet was cultured at 37°C in a 5% CO_2 incubator in 1 mL of chondrogenic medium that contained 500 ng/mL bone morphogenetic protein-2 (BMP-2; R&D Systems, USA) for 3 weeks. The chondrogenic differentiation medium [DMEM with 10% FBS] was replaced every 3 days with fresh medium. The pellets were embedded in paraffin and cut into 3 μm sections. For histological evaluation, the sections were stained with toluidine blue following general precedures.

Fluorescence-activated cell sorter (FACS) analysis

Cultured canine cord blood derived mononucleated cells were collected from each passage, washed in PBS, counted and aliquots of approximately 1×10^6 cells for each antibody were obtained. Mouse anti-canine CD4, mouse anti-canine CD8a, mouse anti-canine CD10 (Serotec, USA), mouse anti-canine CD14, mouse anti-canine CD20, mouse anti-canine CD24, mouse anti-canine CD29, mouse anti-canine CD31, mouse anti-canine CD33, R-phycoerythrin-conjugated mouse anti-canine CD34 (BD Biosciences, USA), mouse anti-canine CD38, mouse anti-canine CD41a, mouse anti-canine MHC II (HLA-DR alpha), rat anti-mouse CD44 endothelium, mouse anti-canine CD45, mouse anti-canine 49b, mouse anti-canine CD 51/61, mouse anti-canine CD62p, mouse anti-canine CD73, mouse anti-canine CD90, mouse anti-canine CD105, mouse anti-canine CD133, mouse anti-canine CD133, mouse anti-canine CD184, Flurescein-labeled affinity purified antibody to rat IgG (H+L), Flurescein-labeled affinity purified antibody to mouse IgG (H+L) (KPL, USA) were used for cell surface antigen detection. Analysis was evaluated by the use of FACS Calibur (BD Biosciences, USA) and Cell Quest Pro (BD Biosciences, USA) software.

Immunostaining

Immunostaining was carried out as previously reported [16]. Antibodies used were rabbit anti-Nestin (Nestin; Santa Cruz Biotechnology, USA), mouse anti-glial fibrillary

acidic protein (GFAP; Chemicon, USA), rabbit anti-microtubule-associated protein 2 (MAP2; Chemicon, USA), mouse anti-neuronal class III β tubulin (Tuj-1; Covance, UK) and mouse anti-neurofilament M (NF160; Chemicon, USA). For immunostaining, cells were fixed in 4% paraformaldehyde for 15 min, and then permeabilized for 10 min at room temperature in 0.4% Triton-X 100 diluted in PBS. After washing 3 times, cells were blocked with normal goat serum overnight at 4°C. Cells were incubated with primary antibodies overnight at 4°C. After washing 3 times, the cells were incubated with secondary antibodies Alexa 488 & 594 (1 : 1,000; Molecular Probe, USA) for 1 h. Finally, for nuclear staining, Hoechst 33238 (1 mg/mL) was diluted 1 : 100 in PBS and loaded into samples for 15 min. Images were captured on a confocal microscope (Eclipse TE200; Nikon, Japan).

Reverse transcriptase polymerase chain reaction

Total RNA was isolated from the cUCB-MSCs using TRIzol (Invitrogen, USA). RNA concentrations were measured by absorbance at 260 nm with a spectrophotometer, and 2 μg total RNA was used for reverse transcription using Superscript II reverse transcriptase (Invitrogen, USA). The cDNA was amplified using Taq Platinum (Invitrogen, USA). The primers used were designed according to the following oligonucleotide primers: homeobox gene MSX2 (MSX2) (sense, 5′-TCCGCCAGA AACAATACCTC-3′; antisense, 5′-AAGGGTAGGACGCTCCGTAT-3′), collagen 1A1 (COL1A1) (sense, 5′-CACCTCAGGAGAAGGCTC AC-3′; antisense, 5′-ATGTTCTCGATCTGCTGGCT-3′), osteonectin (SPARC) (sense, 5′-TGAGAAGGTATGCAG CAACG; antisense, 5′-AGTCCAGGTGGAGTTTGTGG), vitamin D receptor (VDR) (sense, 5′-CCAATCTGGATCTG AGGGAA; antisense, 5′-TTCAGCAGCACAATCTGGTC-3′), and osteoclacin (BGLAP) (sense, 5′-GTGGTGCAAC CTTCGTGTC; antisense, 5′-GCTCGCATACTTCCCTCTT G-3′). Canine glyceraldehyde-3-phosphate dehydrogenase primers (sense, 5′-AACATCATCCCTGCTTCCAC-3′; antisense, 5′-TCCTTGGAGGCCATGTAGAC-3′) were used as internal control for polymerase chain reactions (PCRs). The RNA templates were amplified at 33 to 45 cycles of 94°C (30 sec), 58°C to 61°C (30 sec), 72°C (1 min), followed with 72°C for 10 min. PCR products were visualized with ethidium bromide on a 3% agarose gel.

Results

Cell culture & cell growth kinetics and CPDL

We isolated cUCB-MSCs from canine umbilical cord blood following to the cell isolation & culture method. The cUCB-MSCs (1×10^6) were collected and assessed in a T-25 cell culture flask. The passaged cells were collected every 2 days to count the cell number. The CPDL was measured and calculated and drawn as a graph. A consistently increasing rate of growth of the cumulative population was seen. Cells were cultured and maintained until passage 11. Small colonized populations were observed at the early stages of culture and dissociated for passaging. For each of the passages 1 to 11, cells were cryopreserved for further passaging and experiments (Fig. 1A). The morphology of cells was spindle-shape and typical fibroblast-like shape (Fig. 1B)

Immunophenotypical characteristics determined by FACS analysis

To detect surface markers and characterize the cUCB-MSCs, we performed FACS analyses of cUCB-MSCs at the passage 3, showing positive expressions for CD29, CD33, CD44, CD105, CD184 and Oct4, whereas the following were negatively expressed: CD4, CD8a, CD10, CD14, CD20, CD24, CD31, CD34, CD38, CD41a, CD45, CD49b, CD41/61, CD62p, CD73, CD90, CD133 and HLA-DR (Table 1). The expression patterns of the immunophenotyping with cUCB-MSCs revealed that the

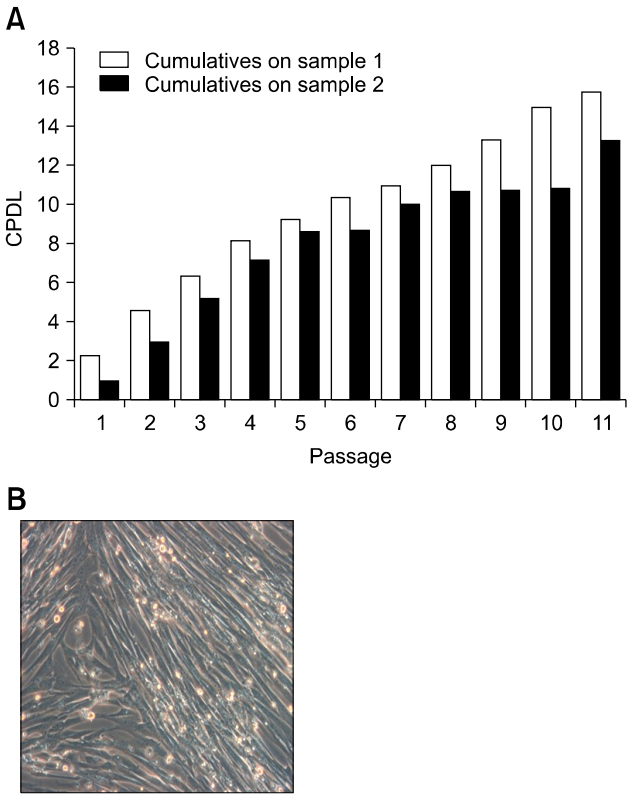

Fig. 1. Identification of the cumulative population doubling level (CPDL) and culture of canine umbilical cord blood (cUCB)-mesenchymal stem cells (MSCs). Cells were cultured in DMEM (with 20% FBS). A: Two bars in a graph indicate the CPDL increase. Both bars show a consistently increasing growth rate during the passages. Each bar increase originates from the CPDL cumulative values, which were two separated sampled cells. B: Phase-contrast image of cUCB-MSCs, ×200.

cells were positive for many common MSC markers [37] : CD29, CD44, CD105. Also the cUCB-MSCs strongly expressed the embryo stem cells associated surface marker [37]: Oct4. The cUCB-MSCs had negative expression patterns for the hematopoietic surface markers of CD14, CD34 and CD45.

Table 1. Fluorescence-activated cell sorter analysis of canine UCB-mesenchymal stem cells

Marker	Percentage	Marker	Percentage
CD4	14.5	CD44	69.79
CD8a	1.67	CD45	0.01
CD10	15.18	CD49b	11.65
CD14	0.73	CD51/61	20.96
CD20	1.31	CD62p	0.25
CD24	16.56	CD73	0.39
CD29	60.11	CD90	0.05
CD31	0.03	CD105	94.07
CD33	64.93	CD133	0.73
CD34	0.01	CD184	79.35
CD38	0.03	HLA-DR	0.15
CD41a	0.02	Oct4	99.72

Cells were identified for expression against a series of CD antibodies immune receptors. Grey boxes indicate positive expression markers.

Differentiation study of the neuronal induction

Neuronal differentiation was examined according to the neuronal induction method. The cUCB-MSCs showed basically neuronal associated protein markers in the basal culture status. In the undifferentiated condition, the cUCB-MSCs slightly expressed GFAP, Tuj-1, and NF160 neuronal cell protein markers. However, the cUCB-MSCs did not express about Nestin and MAP2 (Fig. 2A). When inducted with neuronal differentiation media, the cUCB-MSCs showed positive expression patterns for Nestin, GFAP, Tuj-1, MAP2 and NF160 (Fig. 2B). Compared to the basal culture condition, the cUCB-MSCs had positive for Nestin, MAP2 with neuronal induction, but were negative prior to differentiation. These data showed that cUCB-MSCs had the ability to be inducted into glial and neuron cells under differentiation conditions (Figs. 2A-H).

Differentiation study of osteogenic and chondrogenic induction

To show osteogenesis, the cUCB-MSCs were culture in the osteogenic induction media. Osteogenic induction

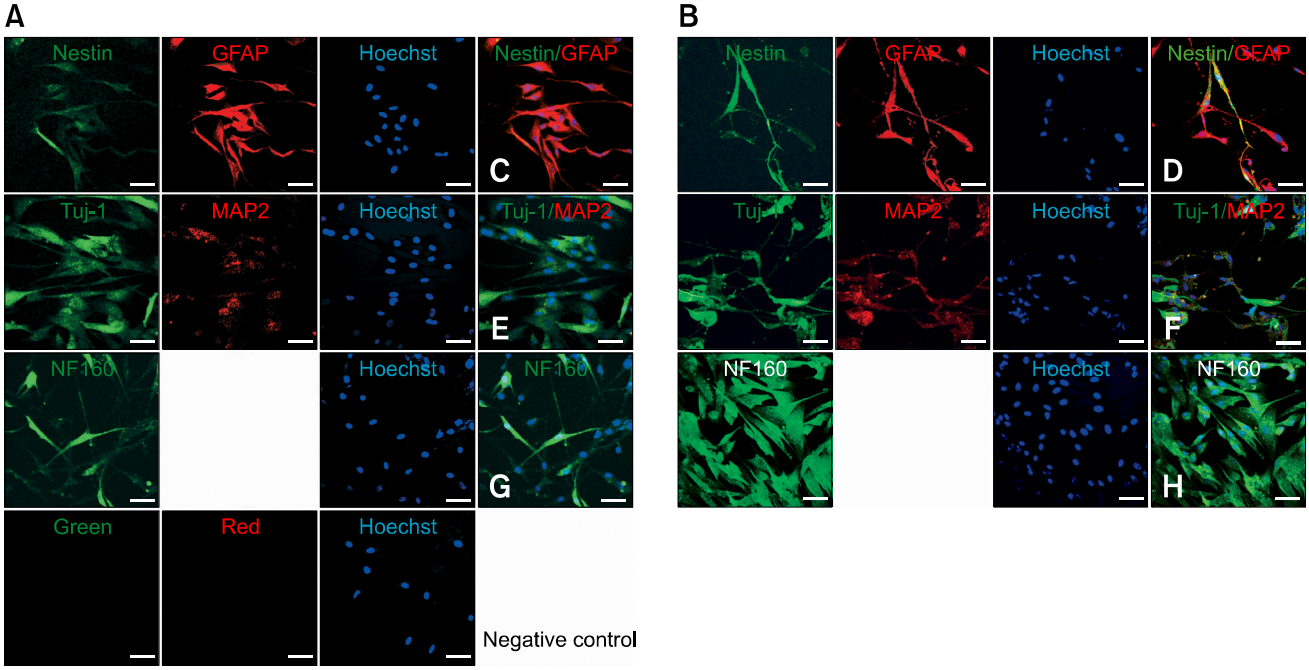

Fig. 2. Immunostaining of undifferentiated and neuronal differentiated cUCB-MSCs. cUCB-MSCs were immunostained with glial fibrillary acidic protein (GFAP), microtubule-associated protein 2 (MAP2), neuronal class III β tubulin (Tuj-1), Nestin and neurofilament M (NF160). Negative control was confirmed with Alexa 488 (green) and Alexa 594 (red). A: The cells were cultured with basal cultured media. B: The cells were cultured with neuronal differentiation media. C-H: Comparing to basal culture condition (undifferentiation) with neuronal differentiation condition. C, E and G: Undifferentiation; D, F and H: Neuronal differentiation. Nestin, Tuj-1 and NF160 were green. GFAP and MAP2 were red. Scale bars = 50 μm.

medium was changed every 3 days for 3 weeks. Calcium mineralization forms were detected on the induced cells to show a significant difference compare to the undifferentiated cells, which did not show any changes (Fig. 3A). Alizarin red staining was positive after 3 weeks under osteogenic induction media (Fig. 3B). Also, gene expression of markers associated with osteoblastic differentiation such as MSX2, COL1A1, SPARC, VDR and BGLAP was evident when compared to basal culture condition. The cUCB-MSCs had a strongly positive MSX2 expression. After osteogensis, osteoblastic gene markers such as COL1A1, SPARC, VDR and BGLAP were abundantly increased except MSX2, which was steadily expressed (Fig. 3C). However, other osteoblastic differentiation markers, alkaline phosphatase and osteopontin, did not appear both in the basal culture and osteogenic differentiation conditions (data not shown).

To investigate the chondrogenesis, the cUCB-MSCs were seeded into 15-mL polypropylene tubes and centrifuged to a pellet. The pellet was cultured at 37°C in a 5% CO_2 incubator in 1 mL of chondrogenic medium changed every 3 days for 2～3 weeks. The pellet was white in color and had atransparent structure. The pellet formed aggregates in the bottom of the tube (Fig. 3D), and positive to toluidine blue staining (Figs. 3E and F).

Discussion

Isolation and characterization of stem cells derived from various tissues and sources have been one very critical issue for stem cell therapy [12,28,33]. The purpose of this study was to isolate, characterize, and differentiate canine umbilical cord blood-derived mesenchymal stem cells. We cultured cUCB-MSCs with basal culture medium (DMEM with 20%FBS) for 11 passages to show that the cUCB-MSCs could be cultured successfully and expanded *in vitro*. The morphology of the cUCB-MSCs showed typical mesenchymal cells along with fibroblastoid and spindle shape, plastic-adherence character. The immunophenotype of cUCB-MSCs expressed mesenchymal stem cells surface markers such as CD29, CD44 and CD105. However, the cUCB-MSCs had negative expressions of hematopoietic surface markers of CD14, CD34 and CD45.

The cUCB-MSCs had the multipotent ability to differentiate into neuronal cells, osteocytes and chondrocytes. In our differentiation studies, we tried to induce adipogenesis with the cUCB-MSCs. However, the cUCB-MSCs did not appear to be able to differentiate into adipocytes, with non-morphological changes on containing oil droplets for 4 weeks (data not shown). The cUCB-MSCs were able to differentiate into neuronal cells, positively expressing neuronal protein markers such as GFAP, Tuj-1 and NF160. This observation and the reports that undifferentiated stem cells express neuron markers [7] explains the possibility of stem cells possessing a neural progenitor's characteristics. A similarity between various tissues has been observed in previous studies [20,21]. We also found the undifferentiated cells could be driven to osteogenic lineaged cells, with calcium deposition after differentiation induction. Also, cUCB-MSCs can undergo chondrogenic differentiation as shown in pellet formation and toluidine blue staining. In this study, we used cUCB-MSCs at 3～5 passage. Generally, increasing the passage number of adult stem cells often leads to a decline in the multipotent abilities [36]. Human mesenchymal stem cells could be proliferated and have differentiation abilities at least 15 passages [34].

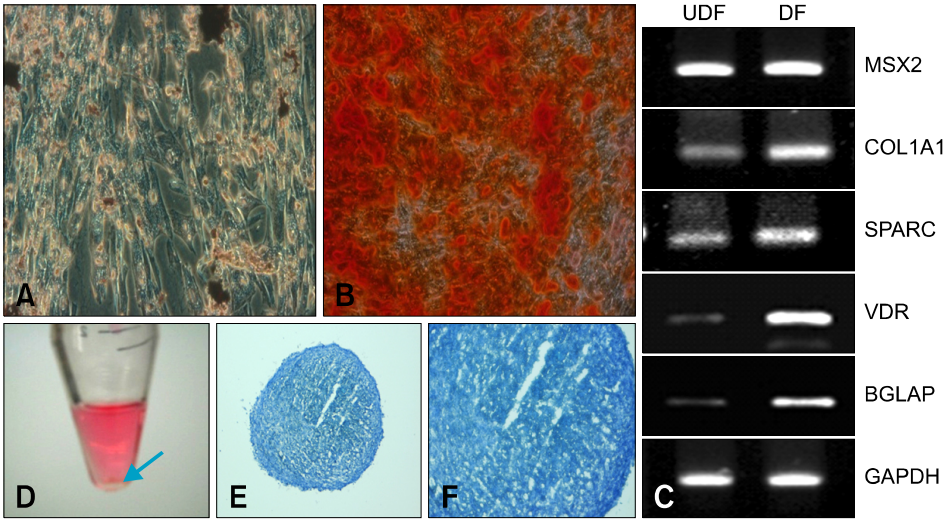

Fig. 3. Osteogenic and chondrogenic differentiation of cUCB-MSCs. A-C: Osteogenic differentiation. A and B: Alizarin red S staining. A: Undifferentiation (UDF), B: Differentiation (DF), C: RT-PCR. (D-F) Chondrogenic differentiation. D: Pellet formation; E and F: Toluidine blue stain. A: ×200, B: ×200, E: ×100, F: ×200.

A typical fibroblastoid morphology was observed in the isolated and maintained cultures, which is commonly observed in human umbilical cord blood derived MSCs [4]. A rapid growth rate is an intrinsic aspect of cultured cUCB-MSCs [4]. Cytotherapy using human umbilical cord blood stem cells frequently has encountered a number of obstacles with the number of available cells for analysis. There are large difficulties in isolating enough multipotent stem cells from human umbilical cord blood and maintaining cell culture for experimental analysis. A guarantee of enough numbers of multipotent stem cells out of a very small quantity of cord blood sample from the canine umbilical cord blood is attractive.

In conclusion, this study provides a simplified isolation and characterization procedure for mesenchymal stem cells derived from canine umbilical cord blood, which can differentiate into neuronal cells, osteocytes and chondrocytes. This study suggests that the cUCB-MSCs have the potential to be a resource for stem cell therapy and regenerative medicine in a canine animal model system.

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