



Cystine transporter expression is a marker to identify a subpopulation of canine adipose-derived stem cells

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ABSTRACT. Adipose-derived stem cells (ADSCs) are promising cell sources for regenerative medicine due to the simplicity of their harvest and culture; however, their biological properties are not completely understood. Moreover, recent murine and human studies identified several functional subpopulations of ADSCs varying in differentiation potential; however, there is a lack of research on canine ADSCs. Cystine transporter (xCT) is a stem cell marker in gastric and colon cancers that interacts with CD44 to enhance cystine uptake from the cell surface and subsequently accelerates intercellular glutathione levels. In this study, we identified a ~5% functional subpopulation of canine ADSCs with xCT⁺ expression (xCT^{hi}). Compared with those of the xCT⁻ subpopulation (xCT^{lo}), the xCT^{hi} subpopulation showed a significantly higher proliferation rate, higher expression of conventional stem cell markers (*SOX2*, *KLF4*, and *c-Myc*), and higher expression of adipogenic markers (*FABP4* and *PPAR γ*). By contrast, the xCT^{lo} subpopulation showed significantly higher expression of osteogenic markers (*BMP1* and *SPP*) than xCT^{hi} cells. These results suggest xCT as a candidate marker for detecting a functional subpopulation of canine ADSCs. Mechanistically, xCT could increase the adipogenic potential while decreasing the osteogenic differentiation potential, which could serve as a valuable target marker in regenerative veterinary medicine.

KEY WORDS: adipogenic differentiation, adipose-derived stem cell, cystine transporter, flow cytometry, osteogenic differentiation

J. Vet. Med. Sci.

82(6): 713–720, 2020

doi: 10.1292/jvms.19-0373

Received: 8 July 2019

Accepted: 20 March 2020

Advanced Epub:

9 April 2020

Adipose-derived stem cells (ADSCs) are mesenchymal stem cells (MSCs) from adipose tissues that function as progenitor cells of adipocytes. Recent studies confirmed the multipotent potential of ADSCs to differentiate into adipocytes, osteocytes, chondrocytes, and vascular cells [22]. Compared with bone marrow-derived stem cells (BMSCs) obtained from the bone marrow stroma, ADSCs are more suitable cell sources for research and clinical applications of stem cells, as they can be obtained in higher quantities using a less invasive procedure. Indeed, a recent study demonstrated that a higher number of ADSCs can be obtained per volume than BMSCs [21]. Human clinical studies have been performed on the use of ADSCs in the treatment of cardiovascular disease, spinal cord injury, cirrhosis, renal insufficiency, and skin fistula with Crohn's disease, as well as for breast reconstruction after mastectomy [1]. However, standardized treatment procedures using ADSCs have not been established mainly due to non-uniform therapeutic effects observed among patients, resulting in hesitation for insurance-approved treatments. To address these issues, new strategies for regenerative medicine with more effective outcomes using ADSCs are continuously being explored.

Despite the rapid progress in human medicine, regenerative veterinary medicine using ADSCs remains in the early research phase. In particular, there are essentially no clinical studies using ADSCs in veterinary medicine for small animals. However, based on the success of regenerative medicine studies using ADSCs in human medicine and clinical trials, there have been additional attempts to use ADSCs for the treatment of intractable diseases of dogs and cats, including spinal cord injury and osteoarthritis

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[3, 13]. Nevertheless, current applications of ADSCs for regenerative veterinary medicine are limited due to a lack of basic information regarding their biology and the low objective validity when using ADSCs clinically. Therefore, there is an immediate need to accumulate basic information regarding ADSC use in veterinary medicine.

Several recent studies suggest that ADSCs represent a heterogeneous cell type, with functionally distinct subpopulations identified by surface antigen markers. For example, the CD90⁺ murine ADSC subpopulation shows high angiogenic potential [17] and induces pluripotent stem cell induction ability [9], whereas the CD90⁻ subpopulation shows high adipogenic potential [17]. Other studies revealed a CD105⁻ human ADSC subpopulation with high osteogenic differentiation potential [10] and a CD73⁺ subpopulation with high cardiomyogenic differentiation potential [11]. These findings motivated efforts to establish more effective regenerative medicine strategies using an appropriate subpopulation of ADSCs according to the purpose; however, the function of these markers (CD90 and CD73) and their roles in cell differentiation and proliferation remain poorly understood. Moreover, to the best of our knowledge, no study has investigated the relationship between cell function and surface antigen markers, and there is only one report on a subpopulation of canine ADSCs identified by aldehyde dehydrogenase expression [7].

CD44 is a surface antigen marker commonly used to detect cancer stem cells of several cancer types, including breast, prostate, and colon cancers [1, 2, 4]. Ishimoto *et al.* [5] revealed that CD44 expression relies on its interaction with cystine transporter (xCT) to regulate intercellular glutathione (GSH) levels and contribute to defend against the accumulation of reactive oxygen species in gastric cancer stem cells. This finding represented the first instance of a surface antigen marker capable of detecting cancer stem cells along with an antioxidant function involved in maintaining stemness, with a recent study reporting an attempt to treat cancer by targeting xCT through cancer stem cell-specific treatment [12].

Nevertheless, the relationship between xCT and MSCs remains unclear. A previous study showed that environmental glutamine affects xCT expression and inhibits osteoblast differentiation in murine BMSCs [18], and another study detected xCT expression in the mouse osteoblast precursor cell line MC3T3-E1 [19]. However, there is no information on xCT expression in canine ADSCs, and it remains unknown whether xCT is a specific marker of an ADSC subpopulation in any species. By contrast, CD44 is reportedly a cancer stem cell marker and an ADSC-specific marker in mice, humans, and canines [14, 16, 20].

In this study, we elucidated the interaction between xCT expression and ADSC differentiation potential by investigating xCT expression in canine ADSCs and investigating the adipogenic and osteogenic differentiation potential of each subpopulation.

MATERIALS AND METHODS

Animals

Adipose tissue was obtained from the cervical back region of two clinically healthy laboratory beagles (aged 6–8 years). Anesthesia was induced in the animals with 7 mg/kg propofol (Intervet, Tokyo, Japan) and maintained with 1.3% isoflurane (DS Pharma Animal Health Co., Osaka, Japan) in oxygen. Analgesia was performed with 20 µg/kg buprenorphine (Otsuka Pharmaceutical, Tokyo, Japan) and 0.2 mg/kg meloxicam (Boehringer Ingelheim, Tokyo, Japan). The animal experiments were approved by the institutional animal experiment ethics committee and conducted in accordance with the institutional guidelines of Yamaguchi University (Approval number: 394).

ADSCs isolation

Isolation of ADSCs was performed according to our previous report [6]. Briefly, ~2 g to 3 g of adipose tissue was resected, washed with Dulbecco's phosphate-buffered saline (DPBS; Wako, Osaka, Japan), and cut into fine pieces, which were incubated at 37.5°C for 1 hr with shaking in high-glucose Dulbecco's modified Eagle medium (DMEM; Wako) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA), penicillin (100 U/ml)/streptomycin (100 µg/ml), amphotericin B (0.25 µg/ml) (100× antibiotic–antimycotic mixed stock solution; Nacalai Tesque, Kyoto, Japan), and collagenase type I (1.0 mg/ml; Sigma-Aldrich). The digested tissue was filtered through a sterile 100-µm nylon mesh (EASYstrainer, 100 µm; Greiner Bio-one Japan, Tokyo, Japan), followed by centrifugation at 775 g for 5 min in 30 ml of DPBS with 1% FBS and 1 mM EDTA·3Na (Wako) (FACS buffer). The pellet was resuspended in DMEM and seeded on culture plates with a 10-cm diameter (Corning, Corning, NY, USA). Upon reaching 80% to 90% confluence, ADSCs were passaged on two culture plates (10-cm diameter) using trypsin/EDTA (0.05% w/v Trypsin-0.53 mmol/l EDTA 4Na Solution with Phenol Red; Wako) after confirming the lack of bacterial contamination. Four cell passages were performed with the same protocol, with cultures subsequently reaching 80% to 90% confluence in a total of eight dishes.

Flow cytometry analysis and cell sorting

Adherent ADSCs from passage four were dissociated using 1 ml/50 cm² trypsin/EDTA after confirming the lack of bacterial contamination, and 1 × 10⁶ cells were resuspended in 100 µl FACS buffer and incubated for 5 min on ice with 2 µl of anti-mouse CD16/32 rat monoclonal antibody (BioLegend, San Diego, CA, USA). The cells were stained with 1 µl of viability probe (Zombie NIR; Biolegend) for 20 min at room temperature to stain dead cells and then washed in FACS buffer and centrifuged. To analyze xCT expression, resuspended cells were incubated for 60 min with 100 µl of FACS buffer and 2 µl of anti-xCT polyclonal antibody (xCT antibody PE; Biorbyt, Cambridge, UK). The resuspended cells were subjected to flow cytometric analysis and cell sorting using an Accuri C6 system (BD Bioscience, San Jose, CA, USA). After excluding dead cells, the baseline was established based on the negative and isotype controls (Rabbit IgG Isotype Control PE; Southern Biotech, Birmingham, AL, USA).

To analyze CD44⁺xCT⁺ subpopulations, resuspended cells were incubated for 60 min on ice with 100 µl of FACS buffer and

5 μ l of anti-mouse/human CD44 monoclonal antibody (FITC anti-mouse/human CD44 antibody; BioLegend). After washing with FACS buffer and centrifugation, the cells were incubated for 60 min with 100 μ l of FACS buffer and 2 μ l of anti-xCT polyclonal antibody. The resuspended cells were subjected to flow cytometric analysis and cell sorting using an Accuri C6 system (BD Bioscience) and an SH800 cell sorter (Sony, Tokyo, Japan), respectively. After excluding dead cells, the baseline was established based on the negative control, and positive cells were identified as those with numbers exceeding the baseline. Flow cytometric data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

Measurement of proliferation potential

To assess the viability of the ADSC subpopulations, we used a cell WST-8 assay (Cell Counting Kit-8; Dojindo Laboratories, Kumamoto, Japan) according to manufacturer instructions. Briefly, sorted xCT^{Hi} and xCT^{Lo} subpopulations were seeded in 96-well plates at a density of 3×10^3 cells/well. After a 12 hr pre-incubation, 100 μ l of fresh medium containing 10 μ l of CCK-8 solution was added to each well, followed by incubation at 37°C for 1 hr (0 hr), with the same procedure repeated at 12 hr, 24 hr, 48 hr, and 72 hr. The absorbance of each well at 450 nm was measured using an Epoch microplate spectrophotometer (BioTek Instruments, Winooski, VT, USA), with six replicates prepared for each group.

Adipogenic differentiation and immunofluorescence staining

The adipogenic differentiation potential of ADSCs was determined using a cell differentiation kit (mouse mesenchymal stem cell functional identification kit; R&D Systems, Minneapolis, MN, USA) according to manufacturer instructions. Briefly, cells (3×10^3 cells/well) sorted using the method presented above were cultured at 37°C with 5% CO₂ in a 96-well plate in 100 μ l of adipogenic differentiation medium comprising 5 ml of α -minimal essential medium (MEM) with 10% FBS, 1% plasma-stimulated medium, L-glutamine, and Phenol Red (Wako) (α -MEM basal medium) supplemented with 50 μ l of adipogenic supplement (containing hydrocortisone, isobutylmethylxanthine, and indomethacin). The medium was replaced every 3 to 4 days for 15 days. To detect adipogenic differentiation by immunocytochemistry, cells were fixed for 20 min in 4% paraformaldehyde phosphate buffer solution (Wako), washed three times with DPBS, and blocked with DPBS supplemented with 0.3% Triton X-100 non-ionic surfactant (Sigma-Aldrich) and 10% FBS for 45 min. The cells were then incubated for 1 hr in DPBS containing 10 μ g/ml of goat anti-mouse fatty acid-binding protein 4 (FABP4) polyclonal antibody (accessories of mouse mesenchymal stem cell functional identification kit, lot no. IOG0516031; R&D Systems) to label adipocytes. A negative control was included using DPBS with no primary antibody. The cells were washed with DPBS and incubated for 1 hr in DPBS containing phycoerythrin (PE)-conjugated secondary antibody [rabbit F (ab')₂ anti-goat IgG H&L (PE), pre-adsorbed; Abcam Japan, Tokyo, Japan]. After washing with DPBS, cells were mounted with a solution containing 5 μ g/ml Hoechst 33342 (Dojindo Laboratories) to label the nuclei. Photographs were obtained and analyzed using a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan) and its analysis software.

Osteogenic differentiation and immunofluorescence staining

The osteogenic differentiation potential of ADSCs was determined using a cell differentiation kit (mouse mesenchymal stem cell functional identification kit; R&D Systems) according to manufacturer instructions. Briefly, cells (3×10^3 cells/well) sorted using the method provided above were cultured at 37°C with 5% CO₂ in a 96-well plate in 100 μ l of osteogenic differentiation medium comprising 5 ml of α -MEM basal medium containing 250 μ l of mouse/rat osteogenic supplement (with ascorbate-phosphate, β -glycerophosphate, and recombinant human bone morphogenetic protein-2). The medium was replaced every 2 to 3 days for 15 days. To detect osteogenic differentiation by immunocytochemistry, cells were fixed for 20 min in 4% paraformaldehyde, washed three times with DPBS, and blocked for 45 min in DPBS supplemented with 0.3% Triton X-100 and 10% FBS. The cells were then incubated for 1 hr in DPBS containing 10 μ g/ml goat anti-mouse osteopontin polyclonal antibody (accessories of mouse mesenchymal stem cell functional identification kit, lot no. BDO0616031; R&D Systems) to label osteocytes. A negative control was included using DPBS without the primary antibody. After washing with DPBS, cells were incubated for 1 hr in DPBS containing PE-conjugated rabbit anti-goat secondary antibody, washed with DPBS, and mounted as described. Photographs were obtained and analyzed using a fluorescence microscope (BZ-9000; Keyence) and its analysis software.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from canine ADSCs using an RNA isolation kit (RNeasy Mini kit; QIAGEN, Hilden, Germany), and RNA concentration and purity were determined using a Nano Drop 8000 spectrophotometer (Pierce Thermo Fisher Scientific, Waltham, MA, USA). cDNA was synthesized using a ReverTra Ace qPCR kit (TOYOBO Co., Ltd., Osaka, Japan). qRT-PCR was performed with the SYBR Green assay using SYBR Premix Ex Taq II (TAKARA Bio, Kusatsu, Japan) combined with a real-time PCR detection system (Thermal Cycler Dice Real Time System Lite TP700; TAKARA Bio), with *hypoxanthine-guanine phosphoribosyltransferase 1* used for normalization. The primers used in this study are shown in Table 1.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (v.6.01 for Windows; GraphPad Software, La Jolla, CA, USA). Data are expressed as the mean \pm standard error. Two groups were compared using the independent *t* test, and data were considered statistically significant at a $P \leq 0.05$.

Table 1. Primers used in this study

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Sox2	GTGAGCGCCCTGCAGTACAA	GCGAGTAGGACATGCTGTAGGTG
Klf4	GATGTGACCCACACTGCCAGA	TGTTGGAACTTGACCATGATTGTA
c-Myc	GATCTCCTCCGGAGAGTGGAAAC	CACCGAGTCGTAGTCGAGGTCAT
FABP4	GATGAAGTTACTGCGGATGACAGAA	CCTGTACCAGGCACCTCCATCTA
PPAR γ	GGACCCGATGGTTCGAGATTA	CGGCATTCAATTGCCATGAG
BMP1	TGGAGCGCACTGATGAGGAC	CTCATGGACAACAATGCCGAAC
SPP1	TTCCCACTGACATTCCAGCAAC	GGACCTCAGTCCATAAGCCACAC
HPRT1	GGAGCATAATCCAAAGATGGTCAA	TCAGGTTATAGCCAACACTTCGAG

RESULTS

xCT expression on canine ADSCs

Canine ADSCs passaged four times were marked by xCT antibodies, with flow cytometry showing a positive shift relative to isotype and negative controls (Fig. 1A). Additionally, canine ADSCs were identified using anti-CD44 and -xCT antibodies, with flow cytometry showing a ~5% (5.21 ± 0.30) proportion of CD44⁺xCT⁺ cells (Fig. 1B).

Comparison of proliferation rate

To assess the difference in the proliferation potential between the xCT^{Hi} and xCT^{Lo} subpopulations, the proliferation rate was measured in each subpopulation using the WST assay. The xCT^{Hi} subpopulation showed a significantly higher proliferation rate than the xCT^{Lo} subpopulation at 12 hr, 24 hr, and 72 hr (Fig. 2A).

Stem cell marker expression in xCT^{Hi} and xCT^{Lo} subpopulations

CD44⁺ cells were sorted into xCT⁺ (xCT^{Hi}) and xCT⁻ (xCT^{Lo}) subpopulations, with the expression of conventional stem cell markers *SOX2*, *KLF4*, and *c-Myc* higher in xCT^{Hi} cells than in xCT^{Lo} cells (Fig. 2B).

Adipogenic and osteogenic differentiation in xCT^{Hi} and xCT^{Lo} subpopulations

After 15 days of culture in adipogenic differentiation medium, ADSCs appeared as large, round cells with lipid-rich cytoplasmic vacuoles, and both xCT^{Hi} and xCT^{Lo} subpopulations were FABP4⁺, indicating adipogenic differentiation (Fig. 3A). After 15 days of culture in osteogenic differentiation medium, ADSCs appeared spindle-shaped with cytoplasmic granules, and both xCT^{Hi} and xCT^{Lo} subpopulations were osteopontin⁺, indicating the potential for osteogenic differentiation (Fig. 3B).

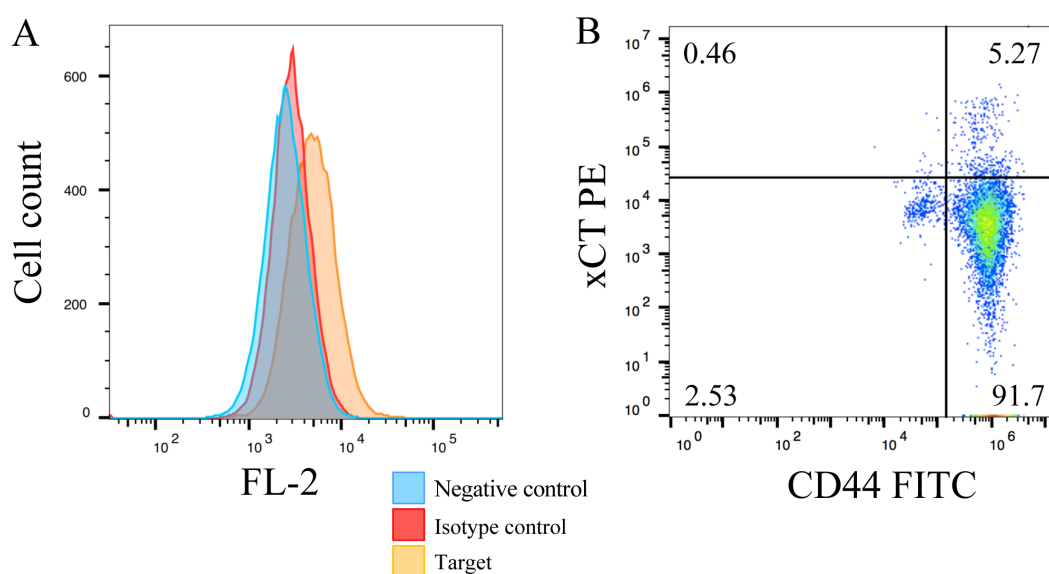


Fig. 1. Cystine transporter (xCT) and CD44 expression on canine adipose-derived stem cells (ADSCs). (A) Canine ADSCs passaged four times were identified using xCT antibodies. Flow cytometry showed a positive shift relative to isotype and negative controls. (B) ADSCs were identified using anti-CD44 and -xCT antibodies, with flow cytometry revealing that ~5% (5.21 ± 0.30) of ADSCs were CD44⁺xCT⁺. Values are expressed as the mean \pm standard error ($n=6$).

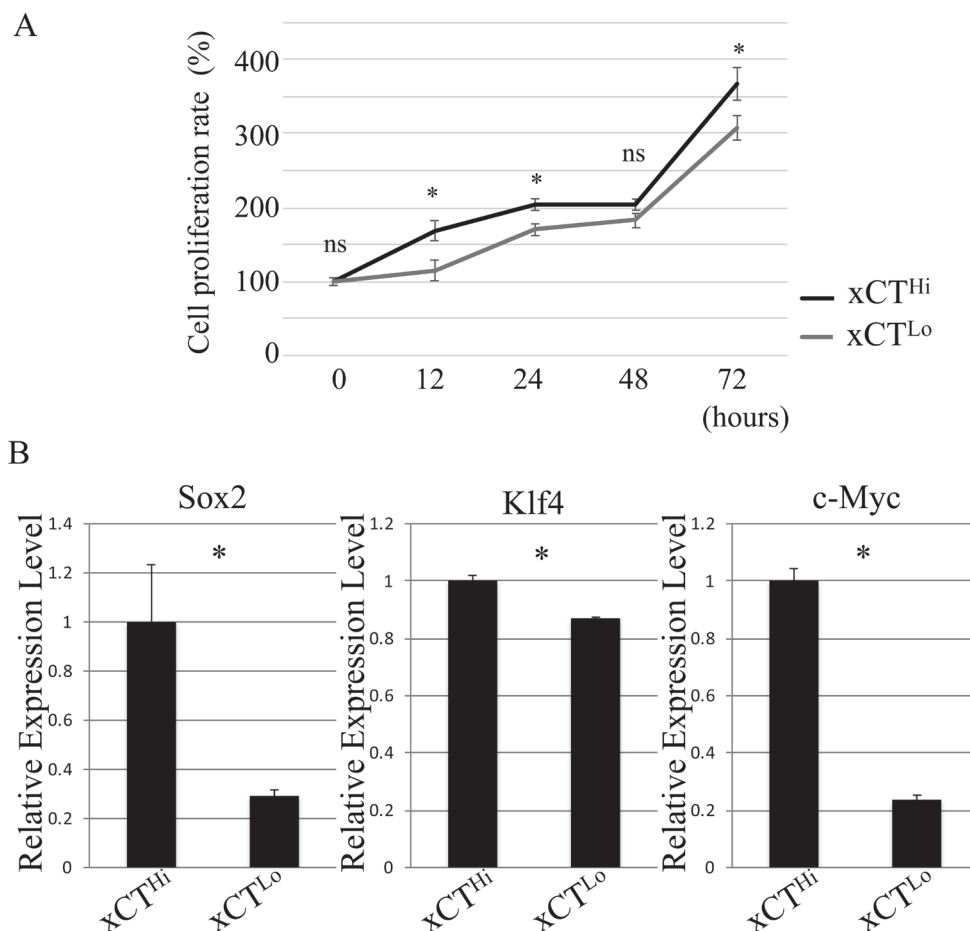


Fig. 2. Evaluation of the proliferation potential and stem cell marker expression in the CD44⁺xCT⁺ subpopulation (xCT^{Hi}) and CD44⁺xCT⁻ (xCT^{Lo}) subpopulations. (A) The xCT^{Hi} subpopulation showed significantly higher proliferation potential at 12 hr, 24 hr, and 72 hr. (B) Expression of stem cell markers *SOX2*, *KLF4*, and *c-Myc* in the xCT^{Hi} and xCT^{Lo} subpopulations. Values are expressed as the mean \pm standard error ($n=6$). * $P<0.05$.

Adipogenic and osteogenic differentiation-marker expression in xCT^{Hi} and xCT^{Lo} subpopulations

qRT-PCR results showed that the xCT^{Hi} subpopulation exhibited significantly higher expression of the adipogenic differentiation markers *FABP4* and *peroxisome proliferator-activated receptor gamma (PPAR γ)* (Fig. 4A). By contrast, higher mRNA levels of the osteogenic differentiation markers *bone morphogenic protein (BMP)1* and *signal peptide peptidase (SPP)* in the xCT^{Lo} subpopulation (Fig. 4B).

DISCUSSION

We demonstrated that cultured canine ADSCs express xCT and further identified a small subpopulation of CD44⁺xCT⁺, which allowed sorting of xCT^{Hi} and xCT^{Lo} subpopulations. Although xCT expression has been detected by western blot in murine ADSCs [8] and MC3T3-E1 cells [19], this is the first report of xCT expression on canine ADSCs and the first identification of a functional MSC subpopulation according to xCT expression in any species.

Although the detailed function of xCT in ADSCs remains to be elucidated, the physiological function of xCT is reportedly involved in enhanced resistance against intracellular oxidative stress accompanied by increased cellular cystine intake and GSH formation [15]. Because we were unable to determine all functions of xCT, this will represent the focus of future investigations.

In this study, xCT^{Hi} showed higher expression of adipogenic differentiation markers than xCT^{Lo}. Consistent with this finding, a previous report revealed that murine BMSCs with xCT expression downregulated by small-hairpin RNA (shRNA) showed decreased levels of PPAR γ , an adipogenic differentiation marker [8]. However, Takarada-Iemata *et al.* [18] reported that GSH supplementation in C3H10T1/2 cells, a murine MSC line, increased their osteogenic differentiation potential but did not significantly affect adipogenic differentiation [18]. They suggested that GSH supplementation negatively affected xCT function as a cystine–glutamine antiporter and increased osteogenic differentiation. This contrasts with the results of the present study; therefore, further investigation of the molecular biological effects of xCT on adipogenic differentiation is warranted.

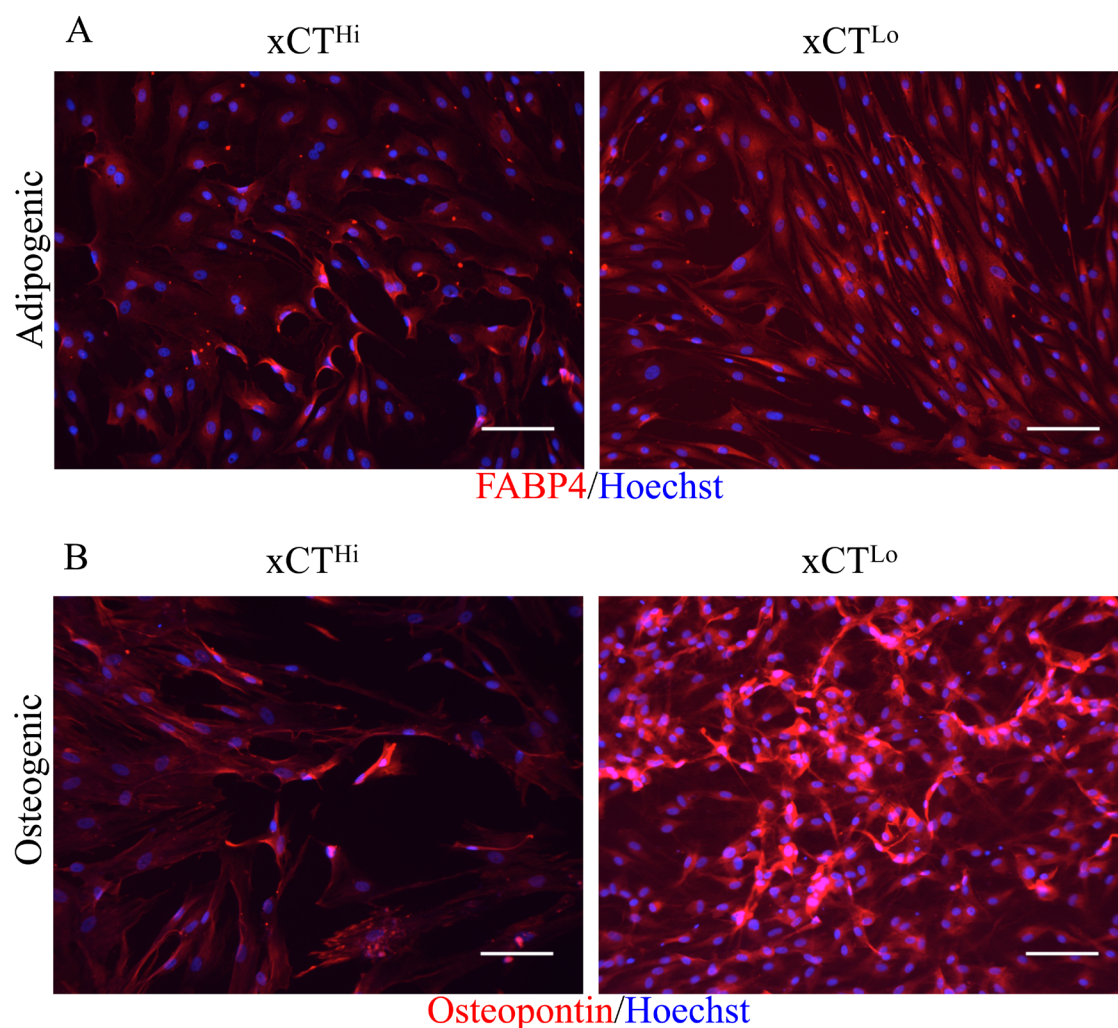


Fig. 3. Immunofluorescence analysis of adipogenic and osteogenic differentiation. (A) After 15 days of culture in adipogenic differentiation medium, adipose-derived stem cells (ADSCs) appeared as large, round cells with lipid-rich cytoplasmic vacuoles, with both xCT^{Hi} and xCT^{Lo} subpopulations fatty acid-binding protein 4 (FABP4)⁺, indicating adipogenic differentiation. (B) After 15 days of culture in osteogenic differentiation medium, ADSCs appeared spindle-shaped with cytoplasmic granules, with both xCT^{Hi} and xCT^{Lo} subpopulations osteopontin⁺, indicating osteogenic differentiation.

Uno *et al.* [19] reported that MC3T3-E1 cells transfected with the *xCT* gene showed decreased Runt-related transcription factor 2 levels and osteoblast differentiation. Another report indicated that murine BMSCs with *xCT* levels downregulated by shRNA showed increased levels of osteogenic differentiation markers, such as BMP2/4 [8]. In the present study, xCT^{Lo} cells showed higher osteogenic differentiation potential than xCT^{Hi} cells. The findings in these previous studies agree with those of the present study and suggest *xCT* as a functional marker of decreased osteogenic differentiation.

Recent studies reveal that cancer stem cells acquire anti-oxidative stress capacity according to *xCT* status [5]. In the present study, we detected higher stem cell marker expression and proliferation potential in the xCT^{Hi} subpopulation relative to the xCT^{Lo} subpopulation. Interestingly, the xCT^{Hi} subpopulation did not show high differentiation potential for each cell type, as xCT^{Hi} cells showed higher expression of adipogenic differentiation markers, whereas xCT^{Lo} cells showed higher expression of osteogenic differentiation markers. *xCT* is a functional marker involved in resistance to oxidative stress and a contributor to elevated stem cell marker expression and proliferation potential; however, it remains unclear how *xCT* affects adipogenic and osteogenic differentiation. Therefore, further studies are needed to investigate the mechanism by which *xCT* decreases osteogenic differentiation.

In conclusion, these findings indicated that canine ADSCs include xCT^{Hi} and xCT^{Lo} subpopulations and that xCT^{Hi} cells showed higher differential potential to adipocytes than xCT^{Lo} cells. By contrast, xCT^{Lo} cells showed higher osteogenic differentiation potential than xCT^{Hi} cells. These results suggest *xCT* as a marker for detecting a functional subpopulation of canine ADSCs. Accordingly, our findings suggest the potential for achieving effective bone-regenerative medicine using an xCT^{Lo} subpopulation of ADSCs for the treatment of intractable canine diseases, such as bone nonunion.

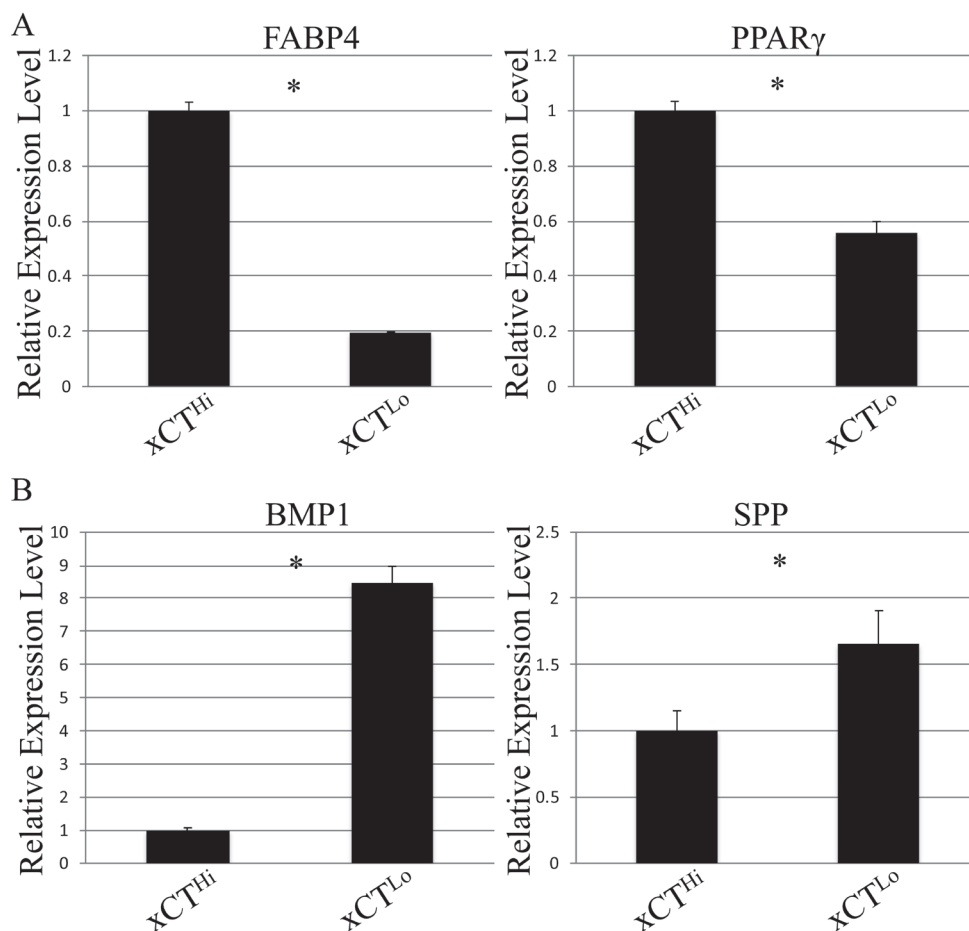


Fig. 4. Comparison of the expression of adipogenic and osteogenic differentiation markers. (A) Levels of adipogenic differentiation-specific mRNA (*FABP4* and *PPAR γ*) for xCT^{Hi} and xCT^{Lo} subpopulations determined by Quantitative reverse transcription polymerase chain reaction (qRT-PCR). xCT^{Hi} showed significantly higher marker levels than xCT^{Lo}. (B) Levels of osteogenic differentiation-specific mRNA (*BMP1* and *SPP*) in xCT^{Hi} and xCT^{Lo} subpopulations detected by qRT-PCR. xCT^{Lo} showed significantly higher marker levels than xCT^{Hi}. Values are expressed as the mean \pm standard error ($n=6$). Scale bar: 100 μ m. * $P<0.05$.

ACKNOWLEDGMENT. This work was partly supported by JSPS KAKENHI Grant Number 26893172.

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