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# Phenotypic and functional properties of feline dedifferentiated fat cells and adipose-derived stem cells

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ABSTRACT

It has been reported that mature adipocyte-derived dedifferentiated fat (DFAT) cells show multilineage differentiation potential similar to that observed in mesenchymal stem cells. Since DFAT cells can be prepared from a small quantity of adipose tissue, they could facilitate cell-based therapies in small companion animals such as cats. The present study examined whether multipotent DFAT cells can be generated from feline adipose tissue, and the properties of DFAT cells were compared with those of adipose-derived stem cells (ASCs). DFAT cells and ASCs were prepared from the floating mature adipocyte fraction and the stromal vascular fraction, respectively, of collagenase-digested feline omental adipose tissue. Both cell types were evaluated for growth kinetics, colony-forming unit fibroblast (CFU-F) frequency, immunophenotypic properties, and multilineage differentiation potential.

DFAT cells and ASCs could be generated from approximately 1 g of adipose tissue and were grown and subcultured on laminin-coated dishes. The frequency of CFU-Fs in DFAT cells (35.8%) was significantly higher than that in ASCs (20.8%) at passage 1 (P1). DFAT cells and ASCs displayed similar immunophenotypes ($CD44^+$, $CD90^+$, $CD105^+$, $CD14^-$, $CD34^-$ and $CD45^-$). Alpha-smooth muscle actin-positive cells were readily detected in ASCs (15.2 ± 7.2%) but were rare in DFAT cells (2.2 ± 3.2%) at P1. Both cell types exhibited adipogenic, osteogenic, chondrogenic, and smooth muscle cell differentiation potential in vitro. In conclusion, feline DFAT cells exhibited similar properties to ASCs but displayed higher CFU-F frequency and greater homogeneity. DFAT cells, like ASCs, may be an attractive source for cell-based therapies in cats.

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## Introduction

Cell-based therapies that aim to repair and replace lost or damaged tissues offer a promising therapeutic approach in both human and veterinary medicine (Fortier and Travis, 2011; Stoltz et al., 2012). Mesenchymal stem cells (MSCs) are multipotent stromal cells capable of differentiating to mesenchymal lineages, including tissues such as adipose tissue, bone, cartilage and muscle (Pittenger et al., 1999; Salem and Thiemermann, 2010). MSCs are increasingly applied in cell-based therapies in humans (Ren et al., 2012), since sufficient cells can be prepared from a patient's own tissues and transplanted safely.

MSCs were originally isolated from bone marrow but have also been found in many other connective tissues, such as adipose tissue, synovial membranes, and embryonic tissue (Orbay et al., 2012). MSCs derived from adipose tissue, currently referred to as adipose-derived stem cells (ASCs) (McIntosh et al., 2006), are considered to be a valuable source for cell-based therapies since adipose tissue can be harvested less invasively and contain more stem/progenitor cells than bone marrow (Zuk et al., 2002). In small companion animals, such as cats and dogs, autologous transplantation of MSCs/ASCs is possible but relatively difficult to perform, since the number of cells harvested is much smaller than in humans (Spencer et al., 2012; Van de Velde et al., 2013). In addition, considerable individual differences in MSC numbers and growth have been reported in cats and dogs (Neupane et al., 2008; Quimby et al., 2011; Spencer et al., 2012). Therefore, alternative cell sources that can be isolated and stably expanded from small samples are of great interest in these animals.

Mature adipocytes can be cultured and dedifferentiated into fibroblast-like cells with an in vitro processing technique known as the ceiling culture (Sugihara et al., 1986). Our group has established a preadipocyte cell line, dedifferentiated fat (DFAT) cells, from murine mature adipocytes using the ceiling culture method (Yagi et al., 2004). Subsequently, we were able to show that porcine and human DFAT cells have multilineage differentiation potential similar to MSCs, including adipogenic, osteogenic, chondrogenic

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differentiation (Matsumoto et al., 2008). DFAT cell transplantation has the potential to replace or repair lost or damaged tissues such as bone, heart (Jumabay et al., 2009), spinal cord (Ohta et al., 2008), bladder (Sakuma et al., 2009), kidney (Nur et al., 2008), and urethral sphincter (Obinata et al., 2011). Because DFAT cells can be obtained and expanded from small amounts (approximately 1 g) of adipose tissue, these cells are expected to facilitate cell-based therapies primarily in cats.

There is currently limited information available regarding feline MSCs (Martin et al., 2002; Jin et al., 2008; Iacono et al., 2012) and ASCs (Webb et al., 2012). Furthermore, there are no existing reports concerning feline DFAT cells. In the present study, we examined whether DFAT cells could be generated from feline adipose tissue, and compared the phenotypic properties and differentiation potential of the resulting cells with feline ASCs.

## Materials and methods

### *Cell cultures*

The study was performed in accordance with Institutional and National Institutes of Health regulations governing the treatment of vertebrate animals and following Institutional Animal Research and Care Committee approval. Samples of omental adipose tissue (approximately 1 g) were collected from female cats (1–3 years old, $n = 24$) during ovariectomy or ovariohysterectomy in private veterinary hospitals. Informed consent was obtained from all cat owners.

Isolation of mature adipocytes and preparation of DFAT cells was performed as described previously (Matsumoto et al., 2008) with minor modifications. Briefly, adipose tissue was minced and digested in 0.1% (w/v) collagenase type II solution (Sigma–Aldrich) at 37 °C for 30 min. After filtration and centrifugation at 135 *g* for 1 min, the floating uppermost layer (containing the mature adipocytes) was collected. The cells ($5 \times 10^4$) were then placed in 12.5 $cm^2$ culture flasks filled completely with Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 20% fetal bovine serum (FBS, JRH Bioscience). The flasks were inverted immediately prior to incubation and the cells of interest were incubated at the top inner surface (ceiling) of the flask. After 7 days, the medium was changed to 10% FBS/DMEM, and the flasks were once again inverted, so that the cells of interest were located at the bottom (floor) of the flask. Cells were trypsinized and subcultured on regular tissue culture dishes or laminin-coated dishes (BD bioscience). Cells were used for experiments before they reached passage 3 (P3).

Preparation of cultured ASCs was performed as described previously (Webb et al., 2012) with minor modifications. Briefly, the stromal vascular fraction (SVF) was isolated as the pellet fraction from collagenase-digested adipose tissue by centrifugation at 380 *g* for 5 min after the collection of the floating uppermost layer as described above. The SVF cells were placed in 12.5 $cm^2$ culture flasks with 20% FBS/DMEM at concentrations of $2 \times 10^6$ cells/flask. After 4 days, SVF-derived ASCs were trypsinized and subcultured on regular tissue culture dishes or laminin-coated dishes. Cells were used for experiments before they reached P3. In the comparison experiments, ASCs and DFAT cells were prepared from the same samples and used at the same passage.

### *Microscopy*

Cells of the floating uppermost layer were fixed in 4% paraformaldehyde for 15 min, washed once in phosphate-buffered saline (PBS), and incubated for 10 min with AdipoRed (Cambrex) and 5 μg/mL Hoechst 33342 (Sigma–Aldrich) to visualize lipid droplets and nuclei respectively. Images of staining were captured under immunofluorescence microscopy (Nikon Eclipse TE 2000-U, Nikon).

### *Growth kinetics*

P1 DFAT cells (at 7 days in culture) and ASCs (at 4 days in culture) from the same samples were plated at a concentration of $1 \times 10^4$ into 35 mm dishes and incubated for 10 days. Cells were counted using a hemocytometer every 2 days and growth curves were plotted. Doubling times for each cell type were calculated in triplicate dishes.

### *Colony-forming unit fibroblast (CFU-F) assay*

P1 DFAT cells and ASCs from the same samples were cultured at a density of 50 cells/35 mm dish in NH CFU-F medium (Miltenyi Biotec) for 14 days. After fixing with 4% paraformaldehyde, cells were stained with 0.5% crystal violet in methanol for 5 min, washed twice in distilled water, and subsequently photographed. Cell clusters of 50 or more cells were considered as a colony and the number of colonies was counted in triplicate dishes.

### *Flow cytometry*

P1 DFAT cells and ASCs from the same samples ($n = 4$) at approximately 80% confluence were suspended in PBS containing 0.2% bovine serum albumin (Sigma–Aldrich) and 1 mM EDTA. After blocking, cell aliquots ($1 \times 10^5$ cells per sample) were incubated with primary antibodies for 30 min on ice. The following monoclonal antibodies were used: anti-feline CD14 (1:200, clone:CAM36A, VMRD), anti-human CD34 (1:10, clone:581, Beckman Coulter), anti-feline CD44 (1:200, clone:BAG40A, VMRD), anti-feline CD45 (1:200, clone:25-2C, VMRD), anti-human α-smooth muscle actin (α-SMA, 1:200, clone:1A4, Dako), Phycoerythrin (PE)-conjugated anti-human CD90 (1:200, clone:5E10, BD Bioscience), and PE-conjugated anti-human CD105 (1:20, clone:SN6, eBioscience). Non-labeled antibodies were visualized using a secondary PE-conjugated goat-anti-mouse IgG antibody (BD Bioscience). Cells were analyzed with a FACSCalibur flow cytometer using CellQuest software package (Becton Dickinson). Positive cells were counted and compared with the signal of corresponding immunoglobulin isotypes. To verify cross reactivity of the above antibodies, cultured human DFAT cells and feline leukocytes were analyzed as control. A minimum of $1 \times 10^4$ events were recorded for each sample and analysis was repeated at least three separate times for each condition tested.

### *Differentiation assays*

Differentiation assays for adipocytes, osteoblasts, chondrocytes, and smooth muscle cells (SMCs) were performed as previously described (Matsumoto et al., 2008; Sakuma et al., 2009) with minor modifications. Briefly, for adipogenic differentiation, confluent cells (P2) were cultured for 14 days in 10% FBS/DMEM supplemented with 1 μM dexamethasone, 0.5 mM 3-isobutyl-L-methylxanthine (Sigma–Aldrich) and 5% insulin–transferrin–selenium-X (Invitrogen). Cells were fixed and stained with oil red O (Sigma–Aldrich) for 20 min.

For chondrogenic differentiation, P1 cells were seeded at a density of $2 \times 10^6$ cells per pellet in 15 $cm^3$ conical tubes. Cells were centrifuged at 300 *g* for 3 min to allow cells to collect at the bottom of each tube, facilitating the formation of a cell pellet. Cells were maintained at 37 °C with 5% $CO_2$ in NH ChondroDiff medium (Miltenyi Biotec) for 21 days. The pellets were fixed, paraffin-embedded, sectioned (5 μm thick), and stained with hematoxylin and eosin (HE), Masson's trichrome, and toluidine blue. For immunohistochemistry, the sections were fixed in 4% paraformaldehyde, permeabilized in 0.2% Triton X-100, blocked with normal goat serum, and incubated with rabbit anti-human collagen type II antibody (1:50, Abcam) followed by Alexa 594 goat anti-rabbit IgG antibody (1:500, Invitrogen). After staining of nuclei with Hoechst 33342, samples were examined using a confocal laser scanning microscope (Olympus FluoView FV10i, Olympus).

For osteogenic differentiation, P1 cells ($1 \times 10^5$) were seeded on β-tricalcium phosphate (TCP)/collagen sponges ($5 \times 5 \times 2$ mm, β-TCP diameter 100–300 μm; dry weight ratio of β-TCP:collagen = 10:1, Olympus Terumo Biomaterials) and incubated for 24 h. The cells were then cultured in 10% FBS/DMEM supplemented with 100 nM dexamethasone, 50 μM L-ascorbic acid, and 10 mM β-glycerophosphate (Sigma–Aldrich) for 21 days. Medium was changed and β-TCP/collagen sponges were inverted twice per week. Samples were fixed in 4% paraformaldehyde for 60 min and stained with 1% alizarin red S (Sigma–Aldrich) to detect calcium deposits. After photography under a stereomicroscope (VB-7000, Keyence), samples were paraffin-embedded and sectioned (10 μm thick) for examination by light microscopy (Olympus BX50).

For SMC differentiation, P1 cells ($5 \times 10^4$) were seeded on 35 mm dishes and incubated with 5% FBS/DMEM supplemented with 5 ng/mL TGF-β1 (R&D Systems) for 7 days. Cells were fixed in 4% paraformaldehyde, permeabilized in 0.2% Triton X-100, blocked with normal goat serum, and incubated with mouse anti-human α-SMA antibody (1:200) followed by Alexa 594 goat anti-mouse IgG antibody (1:500, Invitrogen). After staining nuclei with Hoechst 33342, the samples were examined using an immunofluorescence microscope (Eclipse TE 2000-U, Nikon).

### *Statistical analysis*

Data are shown as means ± standard deviation (SD). Statistical analysis was performed using SPSS 16.0 software package (SPSS). The Mann–Whitney *U* test was used for intergroup comparisons. $P < 0.05$ was considered statistically significant.

## Results

### *Successful preparation of DFAT cells from feline adipose tissue*

DFAT cells and ASCs were prepared from feline omental adipose tissue. After collagenase treatment and centrifugation of adipose tissue, the majority of cells (⩾95%) in the floating top layer were monovacuolar adipocytes with a single nucleus (Fig. 1A). During ceiling culture, approximately 50% of isolated cells adhered to the ceiling of the flask and exhibited extended cytoplasm by day 3 (Fig. 1B). The adherent cells divided asymmetrically and generated

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