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

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18 **Abstract**

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

30

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

41

42 *Keywords:* Adipose-derived stem cell; Feline; Mature adipocyte; Mesenchymal stem

43 cell; Regenerative medicine.

44

45 **Introduction**

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

54
55 MSCs were originally isolated from bone marrow but have also been found in
56 many other connective tissues, such as adipose tissue, synovial membranes, and
57 embryonic tissue (Orbay et al., 2012). MSCs derived from adipose tissue, currently
58 referred to as adipose-derived stem cells (ASCs) (McIntosh et al., 2006), are considered
59 to be a valuable source for cell-based therapies since adipose tissue can be harvested
60 less invasively and contain more stem/progenitor cells than bone marrow (Zuk et al.,
61 2002). In small companion animals, such as cats and dogs, autologous transplantation of
62 MSCs/ASCs is possible but relatively difficult to perform, since the number of cells
63 harvested is much smaller than in humans (Spencer et al., 2012; Van de Velde et al.,
64 2013). In addition, considerable individual differences in MSC numbers and growth
65 have been reported in cats and dogs (Neupane et al., 2008; Quimby et al., 2011; Spencer
66 et al., 2012). Therefore, alternative cell sources that can be isolated and stably expanded
67 from small samples are of great interest in these animals.

68

69 Mature adipocytes can be cultured and dedifferentiated into fibroblast-like cells
70 with an in vitro processing technique known as the ceiling culture (Sugihara et al.,
71 1986). Our group has established a preadipocyte cell line, dedifferentiated fat (DFAT)
72 cells, from murine mature adipocytes using ceiling culture (Yagi et al., 2004).
73 Subsequently, we were able to show that porcine and human DFAT cells have
74 multilineage differentiation potential similar to MSCs, including adipogenic, osteogenic,
75 chondrogenic differentiation (Matsumoto et al., 2008). DFAT cell transplantation has the
76 potential to replace or repair lost or damaged tissues such as bone, heart (Jumabay et al.,
77 2009), spinal cord (Ohta et al., 2008), bladder (Sakuma et al., 2009), kidney (Nur et al.,
78 2008), and urethral sphincter (Obinata et al., 2011). Because DFAT cells can be obtained
79 and expanded from small amounts (approximately 1 g) of adipose tissue, these cells are
80 expected to facilitate cell-based therapies primarily in cats.

81

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

88

89 **Materials and methods**

90 *Cell cultures*

91 The study was performed in accordance with Institutional and National Institutes
92 of Health regulations governing the treatment of vertebrate animals and following

93 Institutional Animal Research and Care Committee approval. Samples of omental
94 adipose tissue (approximately 1 g) were collected from female cats (1-3 years old, $n=$
95 24) during ovariectomy or ovariohysterectomy in private veterinary hospitals. Informed
96 consent was obtained from all cat owners.

97

98 Isolation of mature adipocytes and preparation of DFAT cells was performed as
99 described previously (Matsumoto et al., 2008) with minor modifications. Briefly,
100 adipose tissue was minced and digested in 0.1% (w/v) collagenase type II solution
101 (Sigma-Aldrich) at 37 °C for 30 min. After filtration and centrifugation at 135 g for 1
102 min, the floating uppermost layer (containing the mature adipocytes) was collected. The
103 cells (5×10^4) were then placed in 12.5 cm² culture flasks filled completely with
104 Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 20%
105 fetal bovine serum (FBS, JRH Bioscience). The flasks were inverted immediately prior
106 to incubation and the cells of interest were incubated at the top inner surface (ceiling) of
107 the flask. After 7 days, the medium was changed to 10% FBS/DMEM, and the flasks
108 were once again inverted, so that the cells of interest were located at the bottom (floor)
109 of the flask. Cells were trypsinized and subcultured on regular tissue culture dishes or
110 laminin-coated dishes (BD bioscience). Cells were used for experiments before they
111 reached passage 3 (P3).

112

113 Preparation of cultured ASCs was performed as described previously (Webb et al.,
114 2012) with minor modifications. Briefly, the stromal vascular fraction (SVF) was
115 isolated as the pellet fraction from collagenase-digested adipose tissue by centrifugation
116 at 380 g for 5 min after the collection of the floating uppermost layer as described above.

117 The SVF cells were placed in 12.5 cm² culture flasks with 20% FBS/DMEM at
118 concentrations of 2 x 10⁶ cells/flask. After 4 days, SVF-derived ASCs were trypsinized
119 and subcultured on regular tissue culture dishes or laminin-coated dishes. Cells were
120 used for experiments before they reached P3. In the comparison experiments, ASCs and
121 DFAT cells were prepared from the same samples and used at the same passage.

122

123 *Microscopy*

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

129

130 *Growth kinetics*

131 P1 DFAT cells (at 7 days in culture) and ASCs (at 4 days in culture) from the same
132 samples were plated at a concentration of 1 x 10⁴ into 35 mm dishes and incubated for
133 10 days. Cells were counted using a hemocytometer every 2 days and growth curves
134 were plotted. Doubling times for each cell type were calculated in triplicate dishes.

135

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

137 P1 DFAT cells and ASCs from the same samples were cultured at a density of 50
138 cells/35 mm dish in NH CFU-F medium (Miltenyi Biotec) for 14 days. After fixing with
139 4% paraformaldehyde, cells were stained with 0.5% crystal violet in methanol for 5 min,
140 washed twice in distilled water, and subsequently photographed. Cell clusters of 50 or

141 more cells were considered as a colony and the number of colonies was counted in
142 triplicate dishes.

143

144 *Flow cytometry*

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

162

163 *Differentiation assays*

164 Differentiation assays for adipocytes, osteoblasts, chondrocytes, and smooth

165 muscle cells (SMCs) were performed as previously described (Matsumoto et al., 2008;
166 Sakuma et al., 2009) with minor modifications. Briefly, for adipogenic differentiation,
167 confluent cells (P2) were cultured for 14 days in 10% FBS/DMEM supplemented with
168 1 μ M dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich) and 5%
169 insulin-transferrin-selenium-X (Invitrogen). Cells were fixed and stained with oil red O
170 (Sigma-Aldrich) for 20 min.

171

172 For chondrogenic differentiation, P1 cells were seeded at a density of 2×10^6 cells
173 per pellet in 15 cm³ conical tubes. Cells were centrifuged at 300 g for 3 min to allow
174 cells to collect at the bottom of each tube, facilitating the formation of a cell pellet.
175 Cells were maintained at 37 °C with 5% CO₂ in NH ChondroDiff medium (Miltenyi
176 Biotec) for 21 days. The pellets were fixed, paraffin-embedded, sectioned (5 μ m thick),
177 and stained with hematoxylin and eosin (HE), Masson's trichrome, and toluidine blue.
178 For immunohistochemistry, the sections were fixed in 4% paraformaldehyde,
179 permeabilized in 0.2% Triton X-100, blocked with normal goat serum, and incubated
180 with rabbit anti-human collagen type II antibody (1:50, Abcam) followed by Alexa 594
181 goat anti-rabbit IgG antibody (1:500, Invitrogen). After staining of nuclei with Hoechst
182 33342, samples were examined using a confocal laser scanning microscope (Olympus
183 FluoView FV10i, Olympus).

184

185 For osteogenic differentiation, P1 cells (1×10^5) were seeded on β -tricalcium
186 phosphate (TCP)/collagen sponges (5 x 5 x 2 mm, β -TCP diameter 100-300 μ m; dry
187 weight ratio of β -TCP:collagen = 10:1, Olympus Terumo Biomaterials) and incubated
188 for 24 h. The cells were then cultured in 10% FBS/DMEM supplemented with 100 nM

189 dexamethasone, 50 μ M L-ascorbic acid, and 10 mM β -glycerophosphate
190 (Sigma-Aldrich) for 21 days. Medium was changed and β -TCP/collagen sponges were
191 inverted twice per week. Samples were fixed in 4% paraformaldehyde for 60 min and
192 stained with 1% Alizarin Red S (Sigma-Aldrich) to detect calcium deposits. After
193 photography under a stereomicroscope (VB-7000, Keyence), samples were
194 paraffin-embedded and sectioned (10 μ m thick) for examination by light microscopy
195 (Olympus BX50).

196

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

204

205 *Statistical analysis*

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

209

210 **Results**

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

212 DFAT cells and ASCs were prepared from feline omental adipose tissue. After

213 collagenase treatment and centrifugation of adipose tissue, the majority of cells ($\geq 95\%$)
214 in the floating top layer were monovacuolar adipocytes with a single nucleus (Fig. 1A).
215 During ceiling culture, approximately 50% of isolated cells adhered to the ceiling of the
216 flask and exhibited extended cytoplasm by day 3 (Fig. 1B). The adherent cells divided
217 asymmetrically and generated fibroblast-like DFAT cells (Fig. 1C). Following this, the
218 cells divided symmetrically, forming colonies by day 7. After inversion of the flasks, the
219 DFAT cells began to proliferate extensively, exhibiting spindle-shaped morphology (Fig.
220 1D).

221

222 We also successfully prepared ASCs from SVF cells of the same
223 collagenase-digested adipose tissue used for the DFAT cell preparation (Figs. 1 E-G).
224 Preparation of both DFAT cells and ASCs was successful from approximately 1 g or less
225 of adipose tissue in most of cats we examined ($n = 24$), and most initial ASC cultures
226 reached confluence in 5 days or less and then needed to be passaged approximately
227 every 5 days. We found that the optimal seeding density of adipocytes (5×10^4
228 cells/flask) for DFAT cell preparation was much lower than that of SVF cells for ASC
229 preparation (2×10^6 cells/flask). In these culture conditions, the number of DFAT cells
230 ($5.8\text{--}17.5 \times 10^6$) generated from the initial culture was higher than that of ASCs ($1.7\text{--}4.1$
231 $\times 10^6$), although the cell culture time to reach nearly confluence in DFAT cells (7 days)
232 was longer than that in ASCs (4 days) (Table 1).

233

234 *Feline DFAT cells show high proliferative activity and high CFU-F frequency*

235 Growth kinetics studies conducted at the time of the first passage suggested that
236 both DFAT cells and ASCs exhibited similar proliferative activity and morphology

237 during culture (Fig. 2A). The logarithmic phase of growth data was also similar between
238 both cell types (Fig. 2B). Mean population doubling times of DFAT cells and ASCs (at
239 P1) were $48 \text{ h} \pm 9 \text{ h}$ and $50 \text{ h} \pm 13 \text{ h}$, respectively. Both DFAT cells and ASCs appeared
240 to form nodular aggregates followed by detachment from the plastic flask surface when
241 the subcultured cells approached confluence. Culturing the cells on laminin-coated
242 dishes prevented formation of nodular aggregates and allowed maintenance of the
243 monolayer (Fig. 2C).

244

245 CFU-F colony formation was observed in both DFAT cell and ASC cultures even
246 at the low initial cell density of 50 cells/well (Fig. 3A). The CFU-F colony number in
247 P1 DFAT cells (17.9 ± 2.2 , frequency 35.8%) was significantly higher than that in P1
248 ASCs (10.4 ± 2.6 , frequency 20.8%; $P < 0.05$) from the same samples (Fig. 3B). These
249 results suggest that feline DFAT cells possess more clonogenic potential than feline
250 ASCs.

251

252 *Feline DFAT cells exhibit MSC-like phenotype with high purity*

253 Flow cytometry analysis showed that both P1 DFAT cells and ASCs from the same
254 samples were uniformly positive for the stromal markers CD44, CD90, and CD105, but
255 were negative for the hematopoietic markers CD14, CD34, and CD45 (Fig. 4A). Similar
256 results were obtained from samples of four different cats. A minor subpopulation of
257 α -SMA-positive cells, indicating contamination of vascular SMCs, was detected in
258 ASCs but was rare in DFAT cells at P1 (Fig. 4B). The percentage of α -SMA-positive
259 cells in ASCs at P1 ($15.2 \pm 7.2\%$) and P2 ($6.3 \pm 7.2\%$) was significantly higher than that
260 in DFAT cells at P1 ($2.2 \pm 3.2\%$) and P2 ($1.5 \pm 0.6\%$), respectively (each passage $n = 4$,

261 $P < 0.05$) (Fig. 4C). These results suggest that DFAT cells represent a more
262 homogeneous cell population than ASCs.

263

264 *Feline DFAT cells exhibit multilineage differentiation potential*

265 Under adipogenic differentiation conditions, both DFAT cells and ASCs exhibited
266 intracellular accumulation of oil red O-positive lipid droplets, a characteristic of mature
267 adipocytes (Fig. 5). Lipid droplets accumulation was not observed in either cell type
268 when cultured in control medium.

269

270 In chondrogenic pellet culture, both DFAT cells and ASCs formed morphologically
271 smooth and round cartilaginous nodules (Fig. 6A). These nodules expressed type II
272 collagen, a major component of the extracellular matrix found in articular cartilage. The
273 DFAT cell-derived nodules also showed extensive accumulation of extracellular matrix,
274 a morphological feature consistent with immature cartilage tissue, by HE and Masson's
275 trichrome staining (Fig. 6B). A hyaline cartilage-specific metachromasia was also
276 observed in a region of the nodules by toluidine blue staining (Fig. 6B).

277

278 Under osteogenic differentiation conditions, alizarin red S staining revealed
279 mineralized matrix formation in both DFAT cells and ASCs (Fig. 7, Induction +).
280 Histological analysis revealed that these cells were localized at collagen substrates close
281 to β -TCP and exhibited calcium deposits. The mineralized matrix formation was not
282 observed in either cell type when cultured in control medium, (Fig. 7, Induction -).

283

284 During SMC differentiation culture, both DFAT cells and ASCs expressed α -SMA

285 at a percentage > 50% (Fig. 8). Consistent with the findings of flow cytometry (Fig. 4B
286 and C), a certain number of α -SMA-positive cells were observed in ASCs but not in
287 DFAT cells before SMC induction. These findings indicate that feline DFAT cells
288 exhibit adipogenic, chondrogenic, osteogenic, and smooth muscular differentiation
289 potential in a manner similar to feline ASCs.

290

291 **Discussion**

292 In the present study, we successfully obtained DFAT cells and ASCs from
293 approximately 1 g or less of feline adipose tissue. We have previously reported that a
294 yield of $4-6 \times 10^6$ adipocytes can be consistently isolated from 1 g of human adipose
295 tissue and that approximately 40% of these adipocytes divided, generating DFAT cells
296 during ceiling culture (Matsumoto et al., 2008). In contrast, 1 g of human adipose tissue
297 contains approximately 5,000 ASCs (approximately 2% of SVF cells) (Lin et al., 2010).
298 Our data showed that the number of feline DFAT cells in the initial culture tended to be
299 higher compared to the number of ASCs obtained from the same samples, although
300 there were individual differences in cell yield. A higher number of original cells may
301 lead to the higher yield during feline DFAT cell preparation.

302

303 Feline ASCs and DFAT cells showed similar proliferative rates with doubling times
304 between 48-50 h, which bears a degree of similarity to human, canine and equine ASCs
305 (Izadpanah et al., 2006; Spencer et al., 2012; Vidal et al., 2007), and to human, rat and
306 rabbit DFAT cells (48-65 h) (Kikuta et al., 2013; Matsumoto et al., 2008; Sakuma et al.,
307 2009). CFU-F frequency in feline ASCs (20.8%) also corresponded to that in human
308 ASCs at P1 (12.5-20%) (Mitchell et al., 2006).

309

310 In the present study, both feline DFAT cells and ASCs formed nodular aggregates
311 followed by detachment when approaching confluence or osteogenic induction (data not
312 shown). This phenomenon was not observed in our previous studies on DFAT cells and
313 ASCs derived from humans, pigs, rabbits, rats, or mice, suggesting species-specific cell
314 adhesion properties. Both feline cell types retained cell adhesion and proliferation when
315 cultured on laminin-coated dishes, and could differentiate to exhibit osteogenic lineage
316 properties when cultured on a β -TCP/collagen matrix. Laminin coating of culture dishes
317 has been shown to prevent canine MSC aggregate formation during osteogenic
318 differentiation (Neupane et al., 2008). Laminin coating may be necessary for
319 applications/assays involving cell confluent cultures in certain species.

320

321 Flow cytometric analysis revealed that feline DFAT cells and ASCs displayed
322 similar immunophenotypes (CD44⁺, CD90⁺, CD105⁺, CD14⁻, CD34⁻ and CD45⁻),
323 mostly consistent with the minimal criteria for defining MSCs (Dominici et al., 2006).
324 The feline ASC population is comprised of approximately 15.3% (P1) and 6.2% (P2) of
325 α -SMA-positive vascular SMCs, which are rarer in DFAT cells, suggesting that DFAT
326 cells are a more homogeneous cell population than ASCs. Similar results were obtained
327 from each cell type derived from human subcutaneous adipose tissue (Matsumoto et al.,
328 2008). These differences between ASCs and DFAT cells most likely reflect the varying
329 degree of heterogeneity of the isolated cells prior to tissue culture.

330

331 In vitro differentiation assays revealed that feline DFAT cells and ASCs are similar
332 in multilineage differentiation potentials as reported in other species (Matsumoto et al.,

2008; Oki et al., 2008; Sakuma et al., 2009). Our group has reported that DFAT cell transplantation contributes to rabbit bone, rat heart, and rat bladder regeneration by in vivo differentiation of osteoblasts, cardiomyocytes and SMCs, respectively (Jumabay et al., 2009; Kikuta et al., 2013; Sakuma et al., 2009). MSCs and ASCs also have immunomodulatory, angiogenic, and anti-inflammatory properties, predominantly due to the secretion of a variety of cytokines (Keating, 2012). A recent study demonstrated that the cytokine profiles of human DFAT cells showed high similarity with those of bone marrow MSCs and ASCs (Kikuta et al., 2013). Since no information is currently available regarding the cytokine production or function of feline DFAT cells, further studies will be required to clarify these issues.

There are some limitations to the present study. Firstly, samples were only taken from omental adipose tissue in healthy young female cats. The preparation efficiency and functional properties of DFAT cells derived from different sites of adipose tissue from male cats, from various ages or from diseased cats need to be examined. Influence of donor age, sex, or collection method/site on the MSC/ASC number and function has been described (Baglioni et al., 2009; de Girolamo et al., 2009; Izadpanah et al., 2006; Schipper et al., 2008; Siegel et al., 2013). Secondly, in vivo transplantation studies are required to clarify the feasibility and safety of DFAT cell transplantation in cats.

Conclusions

Multipotent DFAT cells can be prepared from small amounts of feline adipose tissue. Like ASCs, DFAT cells exhibit high proliferative and multilineage differentiation potentials. In addition, DFAT cells exhibit higher CFU-F frequency (at P1) and greater

357 homogeneity (at P1 and P2) than ASCs derived from the same samples. Overall, these
358 findings suggest that not only ASCs, but also DFAT cells are a candidate cell source for
359 cell-based therapies in cats.

360

361

362 **Acknowledgements**

363 We acknowledge the support for this study by financial grants from the Ministry of
364 Education, Science, Sports and Culture of Japan (20590707, 23390190, and 23591428)
365 and the Strategic Research Base Development Program for Private Universities
366 subsidized by MEXT (S0801033) and by a financial grant from the Japan Science and
367 Technology Agency (08030216). The authors gratefully thank Chii Yamamoto and
368 Minako Kazama for their technical support of our experiments.

369

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527

528 **Table 1**

529 Comparison of cell number between dedifferentiated fat (DFAT) cells and
 530 adipose-derived stem cells (ASCs) derived from the same samples

531

Sample number	Tissue weight (mg)	Cell number upon harvest		Cell number and date after culture ^a		
Sample 1	620	Adipocytes	8.4 x 10 ⁵	→	DFAT cells	6.1 x 10 ⁶ at day 7
		SVF cells	27.9 x 10 ⁵	→	ASCs	4.1 x 10 ⁶ at day 4
Sample 2	412	Adipocytes	8.3 x 10 ⁵	→	DFAT cells	10.0 x 10 ⁶ at day 7
		SVF cells	28.2 x 10 ⁵	→	ASCs	1.7 x 10 ⁶ at day 4
Sample 3	400	Adipocytes	14.6 x 10 ⁵	→	DFAT cells	17.5 x 10 ⁶ at day 7
		SVF cells	33.3 x 10 ⁵	→	ASCs	3.5 x 10 ⁶ at day 4
Sample 4	520	Adipocytes	13.8 x 10 ⁵	→	DFAT cells	5.8 x 10 ⁶ at day 7
		SVF cells	26.1 x 10 ⁵	→	ASCs	2.1 x 10 ⁶ at day 4

532

533 ^aCell number of cultured DFAT cells/ASCs was measured at passage 0 (P0) just before
 534 passage.

535

536 **Figure legends**

537

538 Fig. 1. Morphological transformation of feline mature adipocytes into dedifferentiated
539 fat (DFAT) cells using the ceiling culture method. (A) Isolated mature adipocytes from
540 omental fat tissue were stained with AdipoRed and Hoechst33342. (B) On day 3 of
541 ceiling culture, adipocytes adhered to the flask ceiling and exhibited cytoplasmic
542 extension. (C) On day 5, fibroblast-like DFAT cells are generated from the adipocytes
543 and form a colony. (D) On day 7, DFAT cells exhibit spindle-shaped morphology. (E,
544 F) Isolated stromal vascular fraction (SVF) cells were cultured overnight and stained
545 with AdipoRed and Hoechst33342. A fluorescent image (E) and a phase-contrast image
546 (F) are shown. (G) Morphology of cultured adipose-derived stem cells (ASCs) at day 4
547 in adherent culture. Scale bars represent 100 μ m.

548

549 Fig. 2. Morphology and growth kinetics of cultured dedifferentiated fat (DFAT) cells
550 and ASCs at first passage (P1). (A) Representative photomicrographs of DFAT cells and
551 ASCs at day 2, 6 and 10 in adherent culture. DFAT cells closely resemble ASCs in
552 morphology. Scale bars represent 100 μ m. (B) Calibrated growth curves plotted for
553 DFAT cells and ASCs. Bars represent mean $\pm$ SD of triplicated dishes. (C)
554 Representative photomicrographs of DFAT cells at day 11 and 14 in adherent culture on
555 regular cell culture dish or on laminin-coated dish. Numbers in parentheses represent
556 the number of days in cell culture prior to P1. Scale bars represent 100 μ m.

557

558 Fig. 3. Colony forming unit-fibroblast (CFU-F) assay in dedifferentiated fat (DFAT)
559 cells and adipose-derived stem cells (ASCs). P1 DFAT cells and ASCs from the same

560 samples were cultured in NH CFU-F medium at a density of 50 cells/35mm dish. At 14
561 days after plating, cells were stained with 0.5% crystal violet. (A) Representative
562 CFU-F colonies of DFAT cells and ASCs. Scale bars, 10 mm. (B) The number of
563 CFU-F colonies was counted in triplicated dishes. Bars represent mean $\pm$ SD. *, $P <$
564 0.05 (Mann-Whitney U test)

565

566 Fig. 4. Immunophenotyping of dedifferentiated fat (DFAT) cells and adipose-derived
567 stem cells (ASCs). (A and B) Expression of cell surface markers (A) and α -smooth
568 muscle actin (α -SMA) (B) in DFAT cells and ASCs at passage 1 (P1) from the same
569 samples were analyzed by flow cytometry. The unfilled histograms represent isotype
570 control and filled represent expression of markers. A minimum of 1×10^4 events were
571 recorded for each sample. Data are representative of four samples and at least three
572 repeat experiments. (C) The percentage of α -SMA⁺ cells in DFAT cells and ASCs at P1
573 and P2 was quantified from four different cats. Bars represent mean $\pm$ SD. *: $P < 0.05$ vs.
574 DFAT (Mann-Whitney U test)

575

576 Fig. 5. Adipogenic differentiation of dedifferentiated fat (DFAT) cells and
577 adipose-derived stem cells (ASCs). Passage 2 (P2) cells were cultured in adipogenic
578 differentiation medium (Induction +) or growth medium (Induction -) for 14 days.
579 Intracellular lipids were visualized by staining with oil red O. Scale bars, 100 μ m.

580

581 Fig. 6. Chondrogenic differentiation of dedifferentiated fat (DFAT) cells and
582 adipose-derived stem cells (ASCs). Passage 1 (P1) cells were seeded in 15 cm³ conical
583 tubes and cultured in chondrogenic differentiation medium for 21 days. (A)

584 Morphology of cartilaginous nodules derived from DFAT cells and ASCs (left panels;
585 scale bars, 1 mm). Sectioned paraffin-embedded nodules were stained for type II
586 collagen (Col II) and counterstained with Hoechst 33342 (right panels; scale bars, 200
587 μm). (B) Sectioned nodules were also stained by hematoxylin and eosin (HE), Masson's
588 trichrome (MT), and toluidine blue (TB). Scale bars, 200 μm .

589

590 Fig. 7. Osteogenic differentiation of dedifferentiated fat (DFAT) cells and
591 adipose-derived stem cells (ASCs). Passage 1 (P1) cells were seeded on β -TCP/collagen
592 scaffolds and cultured in osteogenic differentiation medium (Induction +) or growth
593 medium (Induction -) for 21 days. The presence of calcium deposits was detected by
594 alizarin red S staining. Representative photomacroscopic (left panels) and
595 photomicroscopic (right panels) pictures are shown. Scale bars, 200 μm .

596

597 Fig. 8. Smooth muscle cell (SMC) lineage differentiation of dedifferentiated fat (DFAT)
598 cells and adipose-derived stem cells (ASCs). Passage 1 (P1) cells were cultured in SMC
599 differentiation medium for 7 days. Before and after smooth muscle cell induction, cells
600 were fixed and stained for α -SMA and counterstained with Hoechst33342. Scale bars,
601 100 μm .

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

A

DFAT

ASC

B

DFAT

Figure 7

Figure 8