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RESEARCH ARTICLE

Comparison of infrapatellar and subcutaneous adipose tissue stromal vascular fraction and stromal/stem cells in osteoarthritic subjects

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Abstract

Since inflammatory mechanisms have been postulated to link obesity to osteoarthritis, the current study evaluated the ratio of immune cells to multipotent stromal cells within the infrapatellar fat pad (IPFP) and subcutaneous adipose tissue (SQ) of the knee; each depot has potential as a source of regenerative cells. The immunophenotypes of stromal vascular fraction (SVF) and adipose-derived stem cells (ASCs) of the IPFP and SQ were determined in tissues from osteoarthritic subjects ($n = 7$) undergoing total knee replacement. Based on a subset of surface antigens, the immunophenotype of ASCs from SQ of OA subjects was not significantly different from that of relatively healthy and leaner subjects undergoing elective liposuction surgery. Flow-cytometry comparison of SVF cell populations in the IPFP of OA subjects resembled those within the subject's own matched SQ, with the exception of the endothelial marker $CD31^+$, which was significantly greater in cells from SQ. In the OA subjects, lower numbers of capillary-like structures and higher numbers of stromal and alkaline phosphatase colony-forming units in the IPFP vs SQ were consistent with this finding; however, ASCs from both depots in OA subjects exhibited comparable adipogenic and osteogenic differentiation potential. Thus, the IPFP contains an ASC and immune cell population similar to that of donor-matched SQ, making it an alternative ASC source for tissue regeneration. Further studies will be needed to determine whether IPFP immune cell infiltrates play an aetiological role in osteoarthritis equivalent to that shown in diabetes associated with obesity.

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Supporting information may be found in the online version of this article.

Keywords **infrapatellar fat pad; subcutaneous adipose tissue; osteoarthritis; obesity; flow cytometry; immunophenotype**

1. Introduction

Adipose tissue is widely distributed throughout the human body. It not only functions as an endocrine organ but also has anti-inflammatory and immunomodulatory characteristics. Secretion of trophic factors, such as adiponectin and leptin, by adipose tissue influences other hormonal pathways in the body. In addition to its secretory activity, adipose tissue plays a role in regulating energy metabolism as well as neuroendocrine and immune functions via a feedback loop with the central nervous system. The stromal vascular fraction (SVF), an isolated cell pellet obtained after enzymatic digestion and centrifugation of a tissue sample,

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contains multiple cell lineages, including pre-adipocytes, endothelial cells, mesenchymal stem cells (MSCs) and leukocytes. T cells, a subset of leukocytes, recognize specific antigens with a specific cell surface antigen T cell receptor (TCR) (Liossis and Tsokos, 1998). This recognition by T cells leads to the activation, proliferation and secretion of cytokines and growth factors. These T cell populations reside in adipose tissue and contribute to the systemic inflammation observed with obesity.

The infrapatellar or Hoffa's fat pad (IPFP) is an intracapsular, extrasynovial structure within the knee that lies directly posterior to the patellar tendon (Saddik *et al*., 2004). It is routinely removed during knee replacement surgery, and may serve as a unique source of ASCs from previously discarded tissue. Unlike subcutaneous adipose tissue (SQ), which is viewed primarily as an endocrine and metabolic depot, the infrapatellar fat pad has a unique role as a mechanical fat depot (Clockaerts *et al*., 2010). In addition to the degenerative changes throughout the knee, osteoarthritis (OA) affects the infrapatellar fat pad via inflammation mediated by T cell-induced cytokine cascades (Clockaerts *et al*., 2010). T cells are believed to play a significant role in the onset and progression of knee OA through pro-inflammatory cytokine release and subsequent activation of macrophage populations within adipose tissue (Sakkas and Platsoucas, 2007; Yang *et al*., 2010). The T cells interact with and activate several cell types, including macrophages, monocytes, chondrocytes and osteoclasts, through either soluble mediators or direct interaction. T cell activation can also contribute to joint destruction in OA by both inducing the production of collagenase, an enzyme responsible for cleaving peptide bonds in collagen, and triggering apoptosis of chondrocytes in the knee cartilage (Yang *et al*., 2010).

Inflammatory changes in adipose tissue are not limited to the IPFP. Peripheral and visceral adipose depots exhibit inflammatory changes in the context of metabolic syndrome, obesity and diabetes (Apovian *et al*., 2008; Kloting *et al*., 2010; Strissel *et al*., 2007; Weisberg *et al*., 2003; Xu *et al*., 2003). Adipose tissue from obese subjects displays an increased density of macrophages or 'crown' cells (Apovian *et al*., 2008; Kloting *et al*., 2010; Strissel *et al*., 2007; Weisberg *et al*., 2003; Xu *et al*., 2003), as well as distinct T cell populations relative to lean individuals (Feuerer *et al*., 2009; Nishimura *et al*., 2009; Duffaut *et al*., 2009b; Winer *et al*., 2009; Yang *et al*., 2010). A growing body of literature supports a role for T cell-derived cytokines as causative agents in the onset and progression of metabolic syndrome locally within the adipose tissue (Duffaut *et al*., 2009a, 2009b; Yang *et al*., 2010).

We therefore asked: (a) whether osteoarthritis alters the subcutaneous adipose cell population as compared to a relatively healthy subject undergoing elective liposuction; and (b) whether the IPFP from osteoarthritic subjects would exhibit cell populations similar to that of donor-matched subcutaneous adipose tissue. To address these questions, the immunophenotype of the SVF cells and adherent, culture-expanded adipose-derived stromal/stem cells (ASCs) isolated from the IPFP and subcutaneous adipose tissue of patients undergoing total knee replacement were compared to each other and to liposuction aspirates from healthy individuals.

2. Methods

2.1. Institutional review board and patient evaluation

All specimens were collected under protocols reviewed and approved by institutional review boards at the Louisiana State University School of Medicine–New Orleans (LSUHSC-NO; Protocol No. 7248) and the Pennington Biomedical Research Center (PBRC; Protocols Nos 10007 and 23040). Studies were conducted in accordance with the 1964 Declaration of Helsinki and with US HIPAA requirements. Patients undergoing total knee replacement were evaluated with respect to their medical history and physical examination for evidence of diabetes and metabolic disease, hypertension and osteoarthritis. Radiological evaluation was used to grade for the Kellgren–Lawrence score, which measures joint narrowing, osteophyte formation and deformity of bone contours (Kellgren and Lawrence, 1957).

2.2. Serum analysis

Blood serum samples were collected from osteoarthritic subjects undergoing elective knee replacement ($n = 9$) or from subjects undergoing elective liposuction or bariatric surgery ($n = 30$) and transported to the clinical chemistry laboratory at PBRC for further analysis (Table 1). Of the osteoarthritic subjects, 33% of the donors were diabetic and 66% were hypertensive. The Homeostasis Model of Assessment – Insulin Resistance (HOMA-IR) was calculated as follows: fasting insulin (μU/ml) × fasting glucose (mg/dl)/405. All blood samples were collected from fasting glucose levels.

2.3. SVF cells and ASCs isolation and expansion

The samples were transported to Pennington Biomedical on the day of the procedure. Infrapatellar fat pad, periarticular

Table 1. Donor demographics and serum analysis data

Subjects	OA	Controls	*p*
Donor number	9	30	
Donor age	64.4 ± 18.0	43.8 ± 11.1	0.09
BMI	34.3 ± 8.7	25.8 ± 3.9	0.019
Kellergen–Lawrence score	3.4 ± 1.2	ND	
Glucose (mg/dl)	113.6 ± 21.0	79.6 ± 17.7	0.001
Cholesterol (mg/dl)	161.3 ± 21.0	179.1 ± 29.6	0.275
HDL (mg/dl)	46.1 ± 15.7	59.2 ± 12.9	0.042
LDL (mg/dl)	90.8 ± 27.6	101.3 ± 25.0	0.328
HOMA-IR	3.0 ± 2.9	1.0 ± 1.6	0.076
Insulin (μU/ml)	9.8 ± 6.8	4.9 ± 7.5	0.085
Triglyceride (mg/dl)	122.3 ± 43.1	93.1 ± 45.6	0.102

ND, not done; OA, osteoarthritis.

subcutaneous adipose tissue and blood serum samples were collected from osteoarthritic subjects ($n = 9$ total for serum analysis, $n = 6$–7 used in cell culture and flow-cytometry data analysis) undergoing total knee replacement. After weighing, the IPFP and SQ samples were minced, washed two or three times with phosphate-buffered saline (PBS) and digested in a volume of 2 ml collagenase digest: 1 g tissue (20 ml PBS, 20 μl 2 mM calcium chloride, 20 mg collagenase, 200 mg albumin) in a rocker dry oven at 37 °C for 1 h. Following digestion, the samples were centrifuged twice at $300 \times g$ for 5 min, filtered using a 100 μM filter and centrifuged again at $300 \times g$ for 5 min to obtain the SVF. In order to remove erythrocytes from the sample and decrease the total number of events in flow-cytometry analysis, 5 ml red blood cell lysis buffer (200 ml distilled water, 1.66 g ammonium chloride, 0.2 g potassium bicarbonate, 0.605 μl 0.25 M EDTA) was used; 20 ml PBS was added to terminate RBC lysis. After centrifugation at $300 \times g$ for 5 min, the supernatent was removed and the samples were resuspended in 5 ml stromal medium [DMEM/F-12, 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 1% antibiotic/antimycotic (MP Biomedicals, Solon, OH, USA)]. Following the processing procedure, 20 μl cell suspension from both samples was stained with trypan blue (Sigma-Aldrich, St. Louis, MO, USA) and counted using a haemocytometer. Cells from IPFP ($n = 7$) and SQ ($n = 6$) obtained from osteoarthritic subjects or subcutaneous lipoaspirate samples ($n = 3$) from patients undergoing elective cosmetic surgery were expanded and differentiated along the adipocyte and osteoblast pathways as previously described (Yu *et al*., 2010). Additionally, fibroblast (CFU-F) and osteoblast (CFU-ALP) colony-forming unit assays were performed in six-well plates, where individual wells were seeded with 50 or 100 passage 1 ASCs from IPFP or SQ from osteoarthritic subjects, as previously described (Staszkiewicz *et al*., 2010).

2.4. Flow cytometry

Flow-cytometry analysis (LSRII from BD Biosciences, San Jose, CA, USA) was performed according to published protocols (Yu *et al*., 2010). The SVF cells were analysed immediately following the initial collagenase digestion procedure, while the passage 2 ASCs were analysed following culture expansion and trypsin release; no cells were cryopreserved prior to analysis. If necessary, the samples were diluted to the required concentration of 1×10^6 cells/ml. The cells were washed in 10 ml PBS and centrifuged twice at $300 \times g$ for 5 min, then resuspended in 500 μl cold PBS. The sample was transferred to a 1.5 ml microcentrifuge tube containing the 87 μl mixture of the 13-fluorochrome panel (see Supporting information, Table S1). The stained samples were incubated in a dark environment at room temperature for 30 min. The cells were then washed with 1 ml PBS and 1% BSA and centrifuged at $300 \times g$ for 3 min, three times. They were resuspended in 500 μl 1% formaldehyde in PBS, stored at 4 °C, and transported to LSUHSC-NO within 48 h for flow-cytometry analysis.

2.5. Endothelial cell labelling

A 100–200 mg piece of tissue was sectioned from subcutaneous adipose tissue and infrapatellar fat pad samples ($n = 3$) and transferred to a 1.5 ml microcentrifuge tube; 1 ml stock solution of isolectin (500 mg/ml) was diluted to 10 μg/ml and transferred to each sample. The sections were then incubated in the dark for 1 h with lectin fluorescein isothiocyanate (FITC) conjugate from *Ulex europaeus* (Sigma-Aldrich) (Pasarica *et al*., 2009). Samples were then washed with PBS and analysed using a confocal microscope. Vessel density was determined by superimposing an image of each sample on a grid, using ImageJ software. Vessel density was defined as the number of times a vessel intersects a gridline within the specified area.

2.6. Statistics

Values are expressed as mean ± standard deviation (SD), where statistical significance is determined by p values 0.05, based on Student's t-test.

3. Results and discussion

Adipose tissue presents an accessible and abundant source of adult stromal/stem cells for regenerative medical therapies (Gimble *et al*., 2007). The majority of data on human SVF cells and ASCs is from subcutaneous adipose tissue isolated from relatively young and healthy subjects undergoing elective liposuction surgery (Aust *et al*., 2004; Gimble *et al*., 2007; Gronthos *et al*., 2001; McIntosh *et al*., 2006; Mitchell *et al*., 2006; Yu *et al*., 2010; Zuk *et al*., 2002); however, evaluation of cells isolated from subcutaneous adipose tissue of osteoarthritic subjects is limited at best. Therefore, the first research question was addressed by initial studies comparing the demographics, serum profile and SVF immunophenotype between osteoarthritic patients undergoing knee replacement and healthy elective liposuction subjects (Table 1; see also Supporting information, Table S2). The osteoarthritic population was significantly older than the healthy control population (mean age 64.4 vs 43.8 years). With one exception, all knee replacement patients were overweight or obese, with a mean BMI of 34.3 (range 22.3–53.8), significantly greater than that of the controls (mean BMI 25.8, range 20.9–34.5). Consistent with the fact that 33% of osteoarthritic subjects were diabetic, their fasting glucose levels were significantly elevated relative to controls, with a trend towards increased triglyceride levels, insulin levels and HOMA-IR, a derived value reflecting the level of insulin sensitivity. While HDL levels were significantly higher in the control relative to the osteoarthritic cohort, cholesterol and LDL levels were comparable. The knee replacement cohort displayed a mean Kellgren–Lawrence score of 3.4; this

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parameter was not evaluated in the liposuction cohort. The immunophenotype of the stromal vascular fraction (SVF) cells obtained from subcutaneous lipoaspirate samples donated by healthy liposuction subjects ($n = 3$) was similar to that from SQ of osteoarthritic knees, based on expression of a limited panel of endothelial (CD31), lymphoid (CD16, CD56), myeloid (CD14) and stromal (CD29) surface antigens (see Supporting information, Table S2). Although the current study examined only three liposuction donors in detail, the data obtained within this limited cohort were consistent with our own previously published studies (with $n = 5$–7 up to $n = 64$ subjects) (Aust *et al*., 2004; Gronthos *et al*., 2001; McIntosh *et al*., 2006; Mitchell *et al*., 2006; Yu *et al*., 2010) as well as that of others in the field (Katz *et al*., 2005; Maumus *et al*., 2011; Zuk *et al*., 2002). These data support the conclusion that subcutaneous adipose tissue has a similar potential as a stromal/stem cell source, independent of whether it is obtained from older, more obese osteoarthritic subjects with a higher incidence of diabetes or younger, leaner liposuction donors with no history of metabolic disease.

A number of studies in the literature have evaluated the differentiation potential and immunophenotype of ASCs isolated from the IPFP (Buckley *et al*., 2010a, 2010b; Dragoo *et al*., 2003; Khan *et al*., 2008, 2009, 2010; Wickham *et al*., 2003); however, only one study has performed a side-by-side comparison between these cells and bone marrow-derived MSCs (Vinardell *et al*., 2011) and none has compared them to subcutaneous adipose-derived ASCs. Therefore, the SVF cells and ASCs from matched IPFP and SQ adipose tissue from osteoarthritic subjects were compared in the second experiment of the current study. Flow-cytometric analyses were performed on the freshly isolated SVF cells from both depots, prior to plastic adherence or culture expansion using a wide panel of antibodies for lineage-specific antigens (Tables 2A and 2B). Compared to the IPFP, SQ SVF cells displayed significantly increased expression of the endothelial cell marker CD31 ($p = 0.02$), while other B cell (CD20), memory/effector/regulatory T cell (CD3, 4, 8, 25), myeloid (CD14), natural killer cell (CD56, CD66) and mesenchymal (CD29) antigens were comparable (Tables 2A and 2B; see also Supporting information, Figure S1, Table S2). Flow cytometry of trypsin-harvested passage 2 ASCs showed that the immunophenotype was not significantly different between matched IPFP and SQ depots of osteoarthritic donors for expression of haematopoietic (CD34, CD45) and stromal (CD29, CD44, CD73, CD90, CD105) antigens (see Supporting information, Table S2). Values were consistent with previous characterizations of passaged human ASCs from subcutaneous depots of healthy donors undergoing elective liposuction surgery (Gronthos *et al*., 2001; McIntosh *et al*., 2006; Mitchell *et al*., 2006; Yu *et al*., 2010).

Sakkas and Platsoucas (2007) and Sakkas *et al*. (1998) found T cell aggregates in 65% of patients with OA, and the T cells contribute to OA progression through release of cytokines such as interferon-γ and interleukin 2. The current study confirms and extends this finding, documenting the presence of a significant percentage of T cells within the IPFP stromal vascular fraction, where $CD3^+CD8^+$ T cells accounted for ~11% of the total population. With respect to lymphoid and myeloid cell markers, the composition of the IPFP was not significantly different from that of matched subcutaneous adipose tissue. While this suggests that the local microenvironments are comparable with respect to inflammation in both depots, additional evaluation of cytokines and inflammatory biomarkers should be evaluated in future comparisons of matched tissues.

Colony-forming unit assays compared passage 1 cells from matched IPFP and SQ depots ($n = 6$ donors) cultured under osteogenic (CFU-ALP) or undifferentiated conditions (CFU-F) (Figure 1 and Table 3). The CFU-F and CFU-ALP frequencies were significantly greater by 1.33-fold and 1.37-fold, respectively, in the IPFP relative to subcutaneous adipose tissue. Confocal photomicrographs of the lectin-stained adipose tissues examined vascularization of subcutaneous adipose tissue relative to the IPFP (Figure S4). Image analyses indicated that subcutaneous adipose tissue exhibited 22% more vessel intersections than IPFP (75.7 ± 12.1 vs 62.3 ± 14.6, $n = 3$ donors); however, these findings did not achieve significance ($p = 0.239$, one-tailed *t*-test). The ASCs

Table 2A. Comparison of the stromal vascular fraction cell immunophenotype in matched infrapatellar fat pad and subcutaneous adipose tissue obtained from subjects undergoing total knee replacement

Depot	Marker	Mean	*p*
Subcutaneous	CD14 %Parent	22.9 ± 8.2	0.39
fat pad	CD14 %Parent	17.9 ± 13.6	
Subcutaneous	CD29+ %Parent	42.6 ± 20	0.27
fat pad	CD29+ %Parent	34.1 ± 30.2	
Subcutaneous	CD31 %Parent	22.3 ± 7.8	0.02
fat pad	CD31 %Parent	9.9 ± 5.1	
Subcutaneous	CD56 %Parent	16.2 ± 12.1	0.29
fat pad	CD56 %Parent	7.2 ± 10.8	
Subcutaneous	CD3 %Parent	11.8 ± 9.6	0.28
fat pad	CD3 %Parent	6.6 ± 4.5	
Subcutaneous	$CD3^+CD8^+$ %Parent	12.8 ± 13.7	0.64
fat pad	$CD3^+CD8^+$ %Parent	10.7 ± 13.1	
Subcutaneous	CD16 %Parent	12.8 ± 9.6	0.50
fat pad	CD16 %Parent	8.6 ± 13.9	
Subcutaneous	CD66 %Parent	6.6 ± 12	0.25
fat pad	CD66 %Parent	0.4 ± 0.3	
Subcutaneous	CD20 %Parent	0.8 ± 1.7	0.32
fat pad	CD20 %Parent	0.2 ± 0.4	

Table 2B. Comparison of stromal vascular fraction immunophenotypes in subcutaneous adipose tissue obtained from subjects undergoing elective cosmetic surgery (lipoaspirate) or total knee replacement (subcutaneous)

Sample	Marker	Mean	*p*
Lipoaspirate	CD14 %Parent	26.1 ± 11.8	0.60
Subcutaneous	CD14 %Parent	22.1 ± 10.4	
Lipoaspirate	CD16 %Parent	8.9 ± 4.4	0.83
Subcutaneous	CD16 %Parent	10.7 ± 11.5	
Lipoaspirate	CD56 %Parent	8.8 ± 6.9	0.16
Subcutaneous	CD56 %Parent	20.2 ± 13.0	
Lipoaspirate	CD31 %Parent	26.7 ± 13.4	0.52
Subcutaneous	CD31 %Parent	20.7 ± 5.6	
Lipoaspirate	$CD29^+$ %Parent	45.8 ± 19.8	0.41
Subcutaneous	$CD29^+$ %Parent	31.9 ± 13.8	

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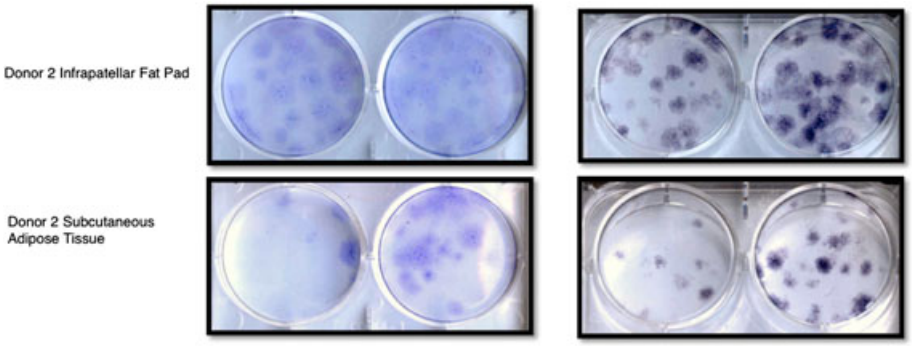

Figure 1. Colony-forming unit (CFU) assay and staining. IPFP and subcutaneous adipose tissue samples were diluted to concentrations of 50 (left wells) or 100 (right wells) cells/ml and plated in six-well plates. After 7 days in culture, the wells were either maintained in stromal medium (CFU-F, left panel) or induced with osteoblast differentiation medium (CFU-ALP, right panel). After 9 days of induction, the colonies were stained with toluidine blue (CFU-F) or alkaline phosphatase (CFU-ALP)

Table 3. Comparative analysis of colony-forming unit frequency in infrapatellar fat pad and subcutaneous adipose tissue

Tissue/CFU	CFU-F	CFU-ALP	p
IPFD	23.3 ± 4.9	25.7 ± 8.4	4×10^{-4}
SQ	17.5 ± 3.9	18.8 ± 6.0	3×10^{-3}

The colony forming unit-fibroblast (CFU-F) and alkaline phosphatase^{+} (CFU-ALP) were determined for 100 passage 1 ASCs from infrapatellar fat pad (IPFD) and subcutaneous (SQ) adipose tissue obtained from $n = 6$ donors. Values are expressed as the mean $\pm$ SD. p values were determined based on Student's t-test; no significant difference ($p < 0.05$) was determined between CFU-F and CFU-ALP values in a single tissue (IPFD, SQ).

from both depots differentiated along adipocyte and osteoblast lineages, based on oil red O or alizarin red histochemical staining (see Supporting information, Figure S2), confirming previous reports on the differentiation potential of IPFP- and subcutaneous-derived ASCs (Dragoo *et al*., 2003; Khan *et al*., 2008; Wickham *et al*., 2003; Yu *et al*., 2010).

We recognize certain limitations of this study. Ideally, IPFP samples from the patients undergoing lipoaspiration would have been the best control. However, harvesting this tissue would have been unethical. Second, increased sample size would allow for greater differentiation between cohorts and control for various disease states. Additionally, to obtain clinically relevant numbers of cells, the IPFP ASCs must be cultured beyond the current level of passage 2. While prior studies from our laboratory have examined subcutaneous ASCs from lipoaspirates up to passage 30, studies with IPFP at extended passages are needed (Izadpanah *et al*., 2006). Third, we did not compare the circulating systemic and tissue-specific adipokine profiles in matched IPFP and subcutaneous specimens. Nevertheless, these findings support the conclusion that the infrapatellar fat pad provides a potential source of ASCs for tissue engineering and regenerative medical applications comparable to that of subcutaneous adipose tissue (Dragoo *et al*., 2003; Khan *et al*., 2008; Wickham *et al*., 2003). The ASCs from both adipose depots are multipotent, capable of differentiating along the adipocyte and osteoblast lineage pathways. Likewise, the IPFP ASCs display an immunophenotype that is not significantly different from that of ASCs derived from donor-matched subcutaneous adipose tissue. The current data confirm and extend published studies documenting the multiple lineage differentiation potential of IPFP-derived ASCs (Dragoo *et al*., 2003; Erickson *et al*., 2002; Guilak *et al*., 2006; Halvorsen *et al*., 2001; Khan *et al*., 2008; Wickham *et al*., 2003; Zuk *et al*., 2001, 2002). With the growth of total knee replacements estimated to reach 3.5 million surgeries by 2030, the IPFP may serve as a safe, low-cost and unique ASCs source for orthopaedic procedures (Kurtz *et al*., 2007).

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Supporting information on the internet

The following supporting information may be found in the online version of this article:

Figure S1. (A, B) Subcutaneous adipose tissue and infrapatellar fat pad flow cytometric histograms for panel of surface antigens
Figure S2. Flow-cytometry analysis of IPFP in comparison to subcutaneous adipose tissue
Figure S3. ASCs differentiation and staining
Figure S4. Lectin staining of intact adipose tissue
Table S1. FACS staining fluorochrome panel
Table S2. Comparison of passage 2 ASCs marker expression of the infrapatellar fat pad and subcutaneous adipose tissue

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