chapter **FOUR**

FRESHLY ISOLATED STROMAL CELLS FROM THE INFRAPATELLAR FAT PAD ARE SUITABLE FOR A ONE-STEP SURGICAL PROCEDURE TO REGENERATE CARTILAGE TISSUE

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ABSTRACT

Background aims Stem cell therapies are being evaluated as promising alternatives for cartilage regeneration. We investigated whether stromal vascular fraction cells (SVF) from the infrapatellar (Hoffa) fat pad are suitable for a one-step surgical procedure to treat focal cartilage defects.

Methods SVF was harvested from patients undergoing knee arthroplasty (*n* = 53). Colony-forming unit (CFU) assays, growth kinetics and surface marker profi les were determined, and the chondrogenic differentiation capacity of freshly isolated SVF was assessed after seeding in three-dimensional poly (L-lactic-co-ε-caprolactone) scaffolds.

Results SVF yield per fat pad varied between 0.55 and 16 x 10^6 cells. CFU frequency and population doubling time were 2.6 ± 0.6% and ± 2 days, respectively. Surface marker profi es matched those of subcutaneousderived adipose-derived stem cells (ASC). CFU from Hoffa SVF showed differentiation toward osteogenic and adipogenic lineages. Cartilage differentiation was confirmed by up-regulation of the cartilage genes sox9, aggrecan, collagen type II and cartilage oligomeric matrix protein (COMP), collagen II immunostaining, Alcian Blue staining and glycosaminoglycan production. Compared with passaged cells, SVF showed at least similar chondrogenic potential.

Conclusions This study demonstrates that SVF cells from the infrapatellar fat pad are suitable for future application in a one-step surgical procedure to regenerate cartilage tissue. SVF shows similar favourable characteristics as cultured ASC, and chondrogenic differentiation even appears to be slightly better. However, because of variable harvesting volumes and yields, SVF from the infrapatellar fat pad might only be applicable for treatment of small focal cartilage defects, whereas for larger osteoarthritic defects subcutaneous adipose tissue depot would be preferable.

INTRODUCTION

Full-thickness articular cartilage defects have limited regenerative potential. Eventually these defects can result in the development of osteoarthritis with serious complaints of pain and physical discomfort. Current therapies aimed at improving these symptoms include drilling microfractures[1–3], autologous chondrocyte therapy (ACT)[4–7] and replacement of the diseased joint with a prosthesis. However, these therapies suffer from serious drawbacks, such as donor site and joint morbidity, the presence of alloplastic material, suboptimal long-term outcome and even structural failure[8]. Therefore, alternative treatment modalities are being studied to offer a therapy that regenerates the defect without the drawbacks of current therapies.

Tissue engineering is a promising treatment modality in modern medicine. The combination of cells and biologic factors, which are put together in a scaffold material, should ideally result in the regeneration of lost or damaged tissue. Recently some products have entered the market that combine autologous chondrocytes with a collagenous or hyaluronic acid scaffold material for the treatment of cartilage defects (e.g. Chondrogide® and Hyaff-11®)[9–11]. Up to now results are promising, although long-term results are not yet clear[4,11–14]. However, the use of fully differentiated cells such as chondrocytes has some disadvantages, for example (1) harvesting of chondrocytes gives rise to additional articular cartilage damage, (2) limited availability, thus it is only applicable for small defects otherwise culture expansion is imperative, and (3) upon culturing chondrocytes can show dedifferentiation[15–17]. These problems can be overcome by the use of (adult) stem cells.

Besides bone marrow (BM), stem cells have been isolated from other sources, such as muscle, periosteum, cord blood and adipose tissue[18–20]. Adipose tissue is an attractive source of adult stem cells because it is abundant, easy accessible by liposuction or resection, and contains a large number of stem cells[21]. These stem cells reside in the stromal vascular fraction (SVF) of the adipose tissue and are referred to as adipose-derived stem cell (ASC). In earlier studies, these ASC have shown their proliferative capacity and multipotency toward various cells of the mesodermal lineage, for example adipocytes, chondrocytes, osteocytes and (cardio)myocytes[21–27]. Both the high yield and multipotency of these ASC warrant the development of a one-step surgical procedure (OSP) in which ASC are obtained and given back to the patient in one procedure, thereby avoiding the need for lengthy and costly tissue-culture expansion, which would subject the patient to a second procedure to re-implant the induced cells[28] The choice of the infrapatellar fat pad as source for SVF cells would be beneficial to the patient, as with a single incision both harvesting of regenerative cells and treatment of the chondral defect could be realized by application of a cell-loaded scaffold.

The purpose of this study was to determine whether SVF cells from the infrapatellar fat pad are a suitable cell source for application in an OSP for the regeneration of focal cartilage defects. Development of this OSP would require: (1) rapid cell procurement, (2) a high yield of cells for regenerative therapy, (3) cells with stem cell characteristics[29] and (4) a chondrogenic differentiation capacity of the cells in the scaffold material. These requirements were specifically addressed in this study.

METHODS

Donors

Human Hoffa fat pads (*n*=53, age range 43–89 years, median age 72 years) were obtained, with informed consent of the patients, as waste material after surgical resection *en block* under general anesthesia and used by the Department of Orthopedic Surgery, VU University Medical Center, Amsterdam, the Netherlands.

Cell isolation and storage

Isolation of the SVF from adipose tissue was performed as has been described previously[30]. The isolation protocol included a Ficoll density-centrifugation step to remove contaminating erythrocytes. After isolation, cells were used immediately for colony-forming unit (CFU) assays, fluorescence-activated cell sorting (FACS) analysis and culture passaging. For storage, 1 10^6 SVF cells/mL were resuspended in a mixture (1:1) of Dulbecco's modified Eagle medium (DMEM) and cyroprotective medium (Freezing Medium; BioWhittaker, Cambrex, Verviers, Belgium), frozen, and stored in the vapor phase of liquid nitrogen under good manufacturing practice (GMP) conditions.

CFU assays

To assess the frequency of ASC in the SVF of adipose tissue from the infrapatellar fat pad, and to determine the frequency of ASC capable of osteogenic and adipogenic differentiation, CFU-fibroblast (F), -alkaline phosphatase (ALP), -osteogenic (Ost) and -adipogenic (Ad) assays were performed as described elsewhere[25]. In brief, fresh SVF cells were resuspended in normal culture medium, consisting of DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine (Invitrogen, Gibco, Carlsbad, CA, USA). Four six-well plates were prepared in which the SVF was diluted 10-fold across both rows, resulting in an upper row containing 10^4 and a lower row containing 10^3 nucleated SVF cells, respectively.

For the CFU-F assay, cells were grown for 10–14 days, fixed in 4% paraformaldehyde and stained with a 0.2% toluidine blue solution in borax buffer. The frequency of ASC within the SVF was expressed as the percentage of seeded cells.

Cells of the remaining triplicate six-well plates were submitted to a CFU-ALP/Ost/Ad assay. Cultures were performed in normal medium for 7–14 days in order to obtain colonies of at least 16 cells (four population doublings) and to get a homogeneous population of ASC. These colonies were subsequently stimulated by osteogenic or adipogenic medium[31] for 2–3 weeks. Following this period, cells in all three plates were rinsed with phosphate-buffered saline (PBS), fixed in 4% formaldehyde and stained to determine the amount of colonies able to differentiate into the osteogenic or adipogenic lineage. For osteogenic differentiation, cells were stained with Alizarin Red, to visualize extracellular matrix calcification, and with nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP; Roche, Mannheim, Germany), for alkaline phosphatase activity, according to the manufacturer's protocol, whereas adipogenic differentiation was confirmed by staining lipid vacuoles with 0.5% Oil Red O.

Culture and growth kinetics of ASC

Culture and determination of the growth kinetics of single-cell suspensions of fresh SVF cells were performed as described elsewhere[25]. In brief, SVF cells were seeded at $2.0x10^5$ nucleated cells/cm^2 in normal culture medium. The cultures were maintained in a 5% CO_2 incubator at 37°C in a humidified atmosphere. The medium was changed twice a week. When reaching 80–90% confluency, cells were detached with 0.5 mm EDTA/0.05% trypsin (Invitrogen) for 5 min at 37°C and replated. Cell viability was assessed using a trypan blue exclusion assay. Growth kinetics of cultured ASC were determined by seeding $1x10^5$ cultured ASC (passage 2 or 3) in 12 T25 flasks per donor, harvesting cells from two duplicate flasks at increasing time-points (after 2, 4, 7, 9, 11 and 14 days) and counting the cells, as described previously[25].

Flow cytometry

Single-cell suspensions of freshly isolated adipose tissue-derived SVF cells from the Hoffa fat pad, as well as cultured cells, were characterized phenotypically using FACS (FACSCalibur; Becton Dickinson, New Jersey, USA), as has been described previously for subcutaneous SVF [32]. Stem cells in the freshly isolated stromal fraction were selected based on the presence of the early marker CD34 and the absence of the endothelial marker CD31. Furthermore, the most common populations in the stroma were determined as described previously[32]. All monoclonal antibodies (MAb) were of the immunoglobulin G1 (IgG1) isotype. Cells were stained with fluorescently labeled antibodies (fluorescein isothiocyanate, phycoerythrin and allophycocyanin conjugated) against CD31, CD34, CD45, CD54, CD90, CD106, HLA-DR and HLA-ABC (BD Biosciences, San José, CA, USA), CD29 (R&D Systems Inc., Minneapolis, MN, USA), CD166 (RDI Research Diagnostics, Flanders, NJ, USA), CD105 (Caltag Laboratories, Burlingame, CA, USA), CD117 (PharMingen, San Diego, CA, USA), and CD146 (Chemicon, Temecula, CA, USA). Non-specific fluorescence was determined by incubating cells with conjugated MAb anti-human IgG1 (Dako Cytomation, Glostrup, Denmark).

Flow cytometry data were analyzed using CELLQUEST software (Becton Dickinson). Fluorescent signals up to two times the IgG signal were considered negative, signals between two and four slightly positive, and above four times positive.

Multidifferentiation capacity

The chondrogenic differentiation capacity of SVF cells from the infrapatellar fat pad (n=5) was studied. Selective attachment of ASC from the heterogeneous SVF was accomplished by seeding into a 70:30 poly(l-lactic-co-ε-caprolactone) (PLA-CPL) scaffold (kindly provided by Dr K. A. Thomas (Cytori Therapeutics, San Diego, CA, USA), which has been shown to select mainly for the ASC in the SVF[33,34]. Pore sizes of this scaffold varied between 250 and 425 µm and porosity between 85% and 90%. In all studies, scaffolds of 6 mm diameter and approximately 1 mm height were used. Before use, the scaffolds were rinsed with 70% ethanol, washed with PBS and incubated overnight in serum containing medium in a humidified incubator (37°C and 5% CO_2), using a procedure developed by Ishaug-Riley *et al*.[35]. Prior to seeding, cells were stained with CM-DiI (Molecular Probes, Eugene, OR, USA) to evaluate attachment to the scaffold, seeded onto the scaffold by dropping 30 µL of a

solution of $5.0x10^5$ cultured cells or $2.0x10^6$ freshly isolated SVF cells onto the scaffolds, and allowed to attach to the scaffold for 1 h. The amount of seeded cells was chosen based on the minimal yield of isolated SVF cells (i.e. $2x10^6$ SVF cells) and the percentage of those cells that were able to adhere to the scaffold[33], being about 25% of SVF cells (i.e. 5 10^5 cultured ASC). Then 1 mL chondrogenic medium [DMEM containing ITS™ Premix (BD, New Jersey, USA), 10 ng/nL transforming growth factor- β1 (TGF-β1; Biovision, ITK-Diagnostics, Mountain View, CA, USA), 1% fetal calf serum (FCS), 25 µm ascorbate-2-phosphate (Sigma, St Louis, MO, USA), 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine] was overlaid gently. Cells were maintained in a 5% CO_2/1% O_2 custom-designed hypoxia workstation (TCPS Rotselaar, Belgium) at 37°C in a humidified atmosphere. Chondrogenic media were changed every other day. In addition, the cell-loaded scaffolds were centrifuged at 700 *g* for 5 min when the medium was changed, in order to subject them to forces mimicking those generated in the intra-articular environment[36].

Osteogenesis and adipogenesis were induced as has been previously described[31]. Briefly, for osteogenic differentiation, passaged cells were seeded at 5000 cells/cm^2 and cultured in a monolayer in osteogenic medium [DMEM supplemented with 10 mM β-glycerol phosphate, 50 µg/mL ascorbate-2-phosphate and 100 ng/mL bone morphogenetic protein 2 (BMP-2; Peprotech EC Ltd, London, UK)].

Adipogenesis was induced by culturing passaged cells in a monolayer at a concentration of $2.0x10^4$ cells/cm^2 in adipogenic medium [DMEM supplemented with 10% FCS, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine (Gibco, Invitrogen, Paisley, UK), 10 µM insulin (Sigma, Zwijndrecht, the Netherlands), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma), 1 µM dexamethasone (Sigma), and 200 µM indomethacin (Merck, Whitehouse Station, NJ, USA)] for 3 weeks. Media were changed twice a week.

The multidifferentiation capacity was analyzed by expression of lineage-specific genes at increasing time points and (immuno)histologic and biochemical analysis of extracellular matrix formation after 21 days of induction.

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

RNA isolation was performed using an RNeasy® kit (RNeasy Mini Kit; Qiagen, Valencia, CA, USA) according to the manufacturer's protocol, and cDNA synthesis was performed as described previously[30]. Real-time PCR reactions were performed using a SYBRGreen reaction kit according to the manufacturer's instructions (Roche Diagnostics, Almere, the Netherlands) in a LightCycler 480 (Roche Diagnostics). cDNA (approximately 5 ng) was used in a volume of 20 µL PCR mix (LightCycler DNA Master Fast Start Kit; Roche Diagnostics) containing a final concentration of 0.5 pmol of primers. Relative housekeeping gene expression of 18S, HPRT and YWHAZ, and relative target gene expression of aggrecan (AGG), collagen type IIb (COL2B), collagen type X (COL10a1), cartilage oligomeric matrix protein (COMP) and early transcription factor SOX-9 for chondrogenic differentiation, collagen type I (COL1A) for osteogenic differentiation and peroxisome proliferator-activated receptor gamma (PPARγ) for adipogenic differentiation, were determined.

Primers (Invitrogen, Carlsbad, CA, USA) used for real-time PCR are listed in Table I .They were designed using the Clone Manager Suite software program version 6 (Scientific & Educational Software, Cary, NC, USA). The amplified PCR fragment extended over at least

Table 1: *PCR primer sets used for reverse transcription PCR*

Gene	Primer sets	Accession number, product length (bp)
18S	Forward -5′ GTAACCCGTTGAACCCCATT- 3′	Human, NM_10098,
	Reverse -5′ CCATCCAATCGGTAGTAGCG 3′	151 bp
HPRT	Forward-5′ GCTGACCTGCTGGATTACAT-3′	Human, NM_000194,
	Reverse-5′ CTTGCGACCTTGACCATCT-3′	260 bp
YWHAZ	Forward-5′ CACTACCCAACACCAAGACA-3′	Human, NM_003406,
	Reverse-5′ CTGGTTTCCCTACAGCTGAT-3′	229 bp
AGG	Forward -5′CAACTACCCGGCCATCC 3′	Human, NM_001135,
	Reverse -5′GATGGCTCTGTAATGGAACAC 3′	160 bp
COL1αI	Forward –5′ AAGCCGAATTCCTGGTCT 3′	Human, NM_000088,
	Reverse –5′ TCCAACGAGATCGAGATCC 3′	195 bp
COL2B	Forward -5′ AGGGCCAGGATGTCCGGCA 3′	Human, NM_033150,
	Reverse -5′ GGGTCCCAGGTTCTCCATCT 3′	195 bp
COL10a1	Forward -5′ CACTACCCAACACCAAGACA 3′	Human, NM_000493,
	Reverse -5′ CTGGTTTCCCTACAGCTGAT 3′	225 bp
COMP	Forward -5′ AACGCGGCGCTGCAGGAC 3′	Human, NM_000095
	Reverse -5′ CGAGCCGTTGCCCGTGAAG 3′	246 bp

one exon border, based on the homology in conserved domains between human, mouse, rat, dog and cow, except for the 18S gene, which is encoded by one exon only. For real-time PCR, the values of relative target gene expression were normalized for geometric means of relative 18S, HPRT and YWHAZ housekeeping gene expression.

With the LightCycler software (version 4), the crossing points were assessed and plotted versus the serial dilution of known concentrations of the standards derived from each gene using the fit points method. PCR efficiency was calculated by LightCycler software and the data were used only if the calculated PCR efficiency was between 1.85 and 2.0.

(Immuno)histochemistry

(Immuno)histochemistry was performed as described before[30]. Cell-loaded scaffolds cultured under chondrogenic conditions were stained with Alcian Blue (Sigma) at acidic pH for detection of proteoglycans. For the detection of collagen type II, sections were stained with mouse MAb II-II6B3 (1:50; Developmental Studies Hybridoma Bank, Iowa City, IA, USA) against human collagen type II in PBS containing 1% bovine serum albumin (BSA). Induced and non-induced pellet cultures served as positive and negative controls (incubated with mouse IgG1), respectively.

Cells that were induced into the osteogenic lineage for 21 days were examined by Alizarin Red staining to prove the formation of a calcified matrix, which is typical for mature osteoblasts. Calcified extracellular matrix was visualized as red spots. Alkaline phosphatase activity of these cells was determined by staining the induced cells with

NBT/BCIP. Adipogenesis was confirmed by staining cells with Oil Red O after 21 days to visualize lipid vacuoles formed after adipogenic induction, as described previously[31].

Extracellular matrix biosynthesis

To quantify the deposit of extracellular sulfated glycosaminoglycans (GAG), the chondrogenically induced cells were harvested after 3 weeks of differentiation in the scaffold. The tissue formed in the scaffold was digested in 3% papain solution buffer overnight at 60°C. GAG was measured using a Blyscan kit according to the manufacturer's protocol (Biocolor Ltd, Carrickfergus, UK). Results were depicted as the total GAG production per scaffold, and corrected for the amount of cells (DNA) in the pellet using CyQUANT® (Molecular Probes, Leiden, the Netherlands) analysis, according to the manufacturer's protocol.

Statistics

Kolmogorov–Smirnov tests were used to determine the normalcy of measurements and, if appropriate, their logarithmics. For evaluation of gene expression, repeated-measures anova was used to determine significant differences when increasing time-points in one donor within one variable were compared. Induction of specific genes in SVF and ASC were compared using the Student's *t*-test with Welch correction. If levels of gene expression were below the detection limit (0.05), values were set at 10^{-2} (or log level at –2). All statistical tests used a significance level of α=0.05.

RESULTS

Hoffa fat pad contains ASC in the SVF

After being excised during total knee arthroplasties, human Hoffa fat pads (*n*=53) were obtained weighing 15.1 ± 5.8 g (range 8.7–26.3 g). The total isolation process was completed in about 2.5 h maximum. The number of SVF cells in the fat pads averaged $4.0x10^6 \pm 4.45x10^6$ (range $0.55–16x10^6$ cells). This meant the Hoffa fat contained *c.* 440 000 cells/g (range 75 397–3 040 000 SVF cells/g).

SVF cells were cultured in normal medium and, depending on the donor, formed colonies between 7 and 14 days, the so-called CFU-F. The frequency of the CFU was on average 2.6 ± 0.6 % (mean ± SD; Table II).

Stromal cells from the infrapatellar fat pad show stem cell characteristics

Freshly isolated stromal cells from the infrapatellar fat pad of five donors were characterized phenotypically. The frequency of the most common populations in the infrapatellar fat pad is depicted in Table III .

In the SVF, the $CD34^+CD31^-$ subpopulation constituted 30 ± 5.7% (mean ± SD) of the total amount of cells. Cells gated for the $CD34^+CD31^-$profile were characterized further and shown to be additionally positive for the stem cell-associated markers CD29, CD54, CD90, CD105, CD166 and HLA-ABC and negative for hematopoietic/ leucocytic/ endothelial markers such as CD45, CD106 and CD146. Furthermore, they were slightly positive for CD117 and HLA-DR (Table IV).

Table 2: *CFU assays*

	Donors (n)	Frequency (mean ± SD)	% CFU-F
CFU-F	42	2.6 ± 0.6	100
CFU-ALP	3	1.3 ± 0.1	50
CFU-Ost	2	2.1 ± 0.3	77
CFU-Ad	7	1.6 ± 0.2	62

Table 3: *Putative cell populations in the stroma of Hoffa's fat pad (n=5)*

Phenotype	Frequency (%)	Cell type
CD 34brightCD31-CD146-	30.0 ± 15.7	ASC-like
CD 34brightCD31+CD146+	3.0 ± 2.4	Endothelial cell
CD 34dimCD31-CD146+	8.3 ± 4.4	Vascular smooth muscle cell/pericyte
CD 45+CD34+	2.2± 0.9	HSC-like
CD 45+CD34-	8.9 ± 4.1	Leucocyte

Cell populations in the stromal vascular fraction of Hoffa's fat pad of 5 donors were phenotypically characterized in triple stainings, using flow cytometry. Results are depicted as mean ± SD.

Table 4. *Marker profile of freshly isolated and cultured ASCs*

Cell surface marker	Freshly isolated ASCs (n = 5) §	Cultured ASCs passage 3 (n = 3)
CD 34	251.5 ± 109	1.8 ± 0.1
CD 31	1.1 ± 0.2	1.0 ±0.1
CD 105	4.3 ± 2.0	15.4 ± 0.8
CD 166	1.0 ± 0.3	10.1 ± 6.5
CD 90	54.1 ± 46.3	94.9 ± 81.9
CD 45	1.6 ± 0.8	1.1 ± 0.1
CD 117	2.3 ± 0.9	3.8 ± 0.6
CD 146	1.5 ± 0.6	1.5 ± 0.6
CD 106	1.2 ± 0.6	1.6 ± 0.8
CD 54	8.2 ± 2.2	7.9 ± 4.3
CD 29	54.4 ± 34	51.5 ± 39.8
HLA-ABC	24.9 ± 15.3	21.5 ± 16.7
HLA-DR	3.7 ± 2.2	4.1 ± 1.8

Results are depicted as mean fluorescence compared with an isotype control (mean ± SD).
§ *Fluorescence of specific markers on freshly isolated ASC was determined after gating the $CD34^{+}CD31^{-}$ subpopulation in the SVF of Hoffa's fat pad.*

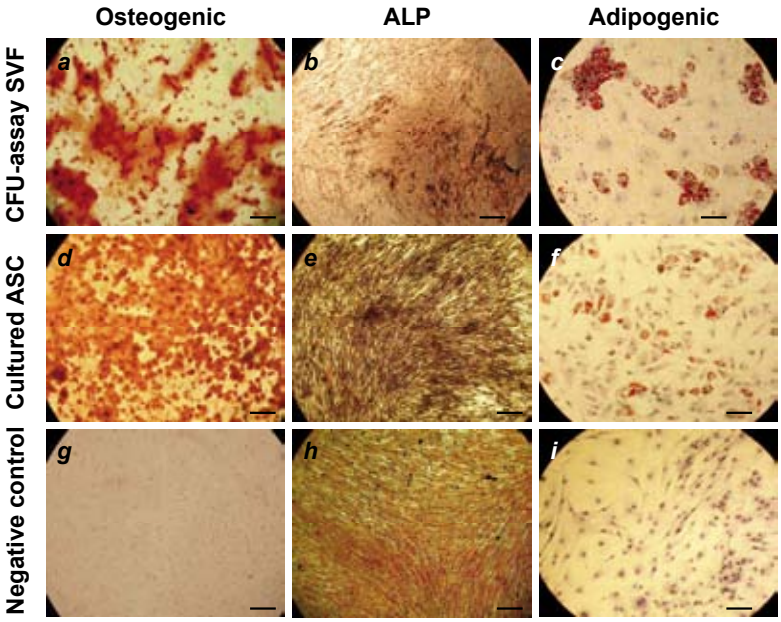

Figure 1. *Expression of extracellular matrix deposition (column 1), intracellular enzyme activity (column 2) and fat droplets (column 3) in both fresh SVF cells (first row) and cultured ASC (second row) after inducing cells into the osteogenic and adipogenic lineage. Fresh SVF cells were induced into the osteogenic and adipogenic lineage using colony-forming unit assays, and subsequently stained for calcification of extracellular matrix with alizarin red (A,D,G, 10x), alkaline phosphatase activity with NBT/BCIP (B,E,H, 10x) and lipid droplets with Oil Red O (C,F,I, 20x). The second row proofs retainment of multilineage differentiation capacity of cultured cells by specific intracellular or extracellular matrix deposition (D-F). Uninduced cells served as negative controls (G-I).*

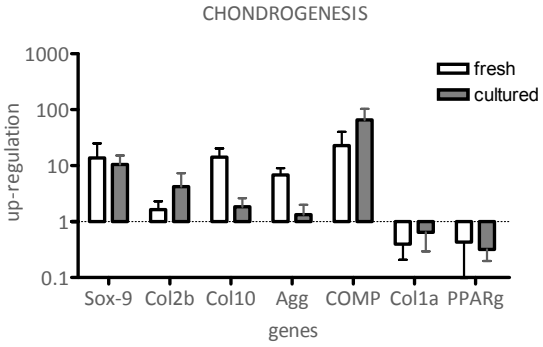

Figure 2. *Up-regulation of chondrogenic genes of fresh SVF cells and cultured ASC after inducing cells in chondrogenic medium in a 3-D PLA-CPL scaffold (mean ± SEM). Chondrogenic genes were significantly up-regulated in both SVF and ASC, whereas the osteogenic gene COL1a and the adipogenic gene PPARγ were both down-regulated. No significant differences could be detected in gene induction between SVF cells and cultured cells.*

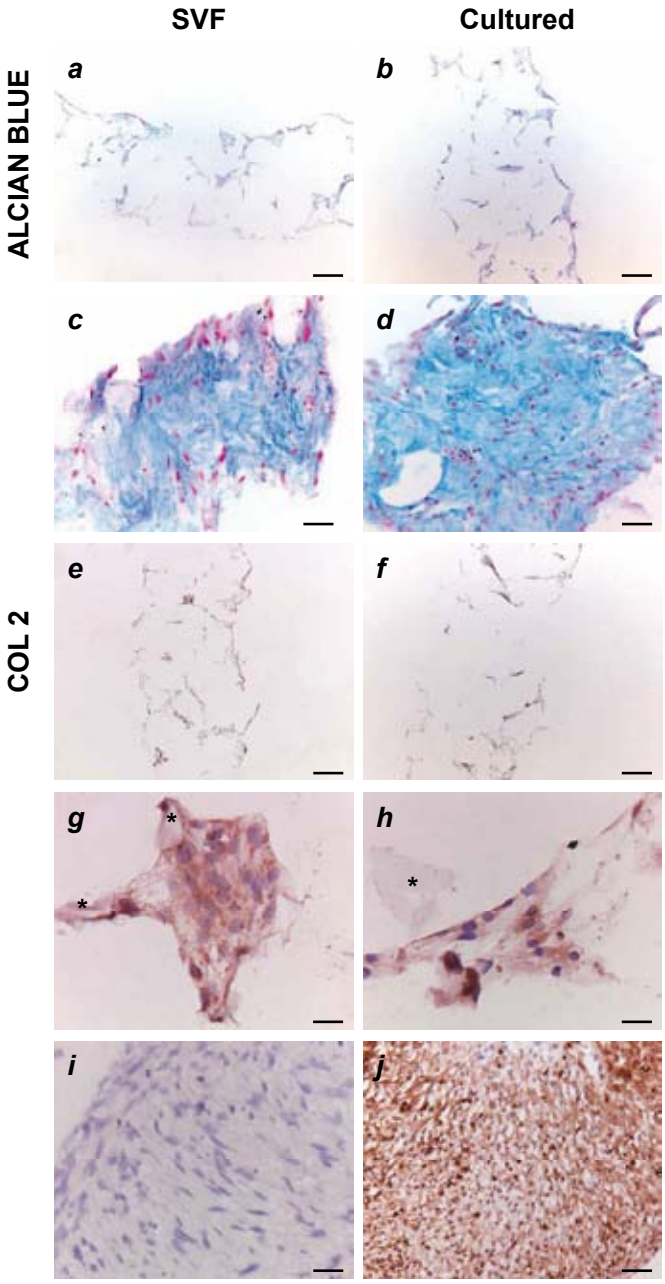

Figure 3. Expression of extracellular matrix in SVF and ASC after inducing cells into the chondrogenic lineage in a 3-D PLA-CPL scaffold. Both SVF and ASC stained positive for (A-D) proteoglycans (Alcian Blue) at low (A-B, 25x) and high (C-D, 200x) magnification and (E-H) for collagen type II at low (E-F, 25x) and high (G-H, 400x) magnification. Asterisks represent scaffold material. Pellet cultures served as positive (I, 200x) and negative controls (J, 400x).

Freshly isolated stromal cells from Hoffa's fat pad show multilineage differentiation

The multilineage differentiation capacity of freshly isolated Hoffa cells was assessed in CFU-F assays and subsequently osteogenic (CFU-ALP/Ost) and adipogenic (CFU-Ad) differentiation (Figure 1). Table II lists the results for the amount of SVF cells able to differentiate into the specific lineages.

After seeding freshly isolated and cultured cells to the PLA-CPL scaffold, both attached to the scaffold, as visualized with CM-DiI staining (data not shown).

RNA expression in human fat pad-derived stromal cells was determined at several time-points using qRT-PCR. The kinetics of specific gene expression varied between donors, but most donors showed increased expression of chondro-specific genes such as SOX-9, AGG, COL2 and COMP (Figure 2). The hypertrophic marker COL10 was only expressed to a small extent and limited to early time-points. Furthermore, both the osteogenic marker COL1a and the adipogenic marker PPARγ were down-regulated. No significant differences could be detected in quantitative up-regulation of any marker between freshly isolated SVF cells and cultured ASC from Hoffa's fat pad (Figure 2).

When cultured with chondrogenic medium, both fresh SVF cells and cultured ASC clearly synthesized a metachromatic extracellular matrix. Alcian Blue staining confirmed the formation of proteoglycans, whereas immunohistochemical analysis revealed that collagen type II was synthesized by cells that were grown for 21 days under chondrogenic stimulation (Figure 3).

Quantification of GAG formation showed an increased expression after 21 days of induction in both SVF and cultured ASC (Figure 4). A significant difference could be detected between the total amount of GAG production produced by SVF cells and cultured ASC (P=0.021; Figure 4A). When corrected for the total amount of cells in the scaffold material, this difference lost significance but a clear trend was still seen (Figure 4B).

Cultured cells retain stem cell characteristics and differentiation potential

SVF cells were cultured in normal culture expansion medium and after 2–3 weeks cells formed a homogeneous population of ASC-like cells. To determine the growth kinetics of these cultured ASC from the infrapatellar fat pad, the population doubling time from passage 2 to 3 was determined.

When ASC numbers were monitored over time, a growth curve was obtained showing an exponential growing phase, after which the cells reached confluency. The mean population doubling time of ASC in the exponential growing phase was about 2 days (1.8 ± 0.3 days, mean ± SD; data not shown).

Cultured ASC (passage 2–3) retained their stem cell phenotype, with only small changes compared with freshly isolated cells. Cultured ASC demonstrated homogeneous populations staining positive for the same cell surface marker profile, except for the early marker CD34, which was lost upon proliferation. On the other hand, CD166 and CD105 expression was increased upon culturing of ASC (Table IV).

Histochemical analysis of the cultured cells revealed extracellular matrix formation after specific induction. Osteogenically induced cells stained positive for alkaline phosphatase and Alizarin Red, indicating formation of a calcified matrix (Figure 1D–E). Adipogenesis

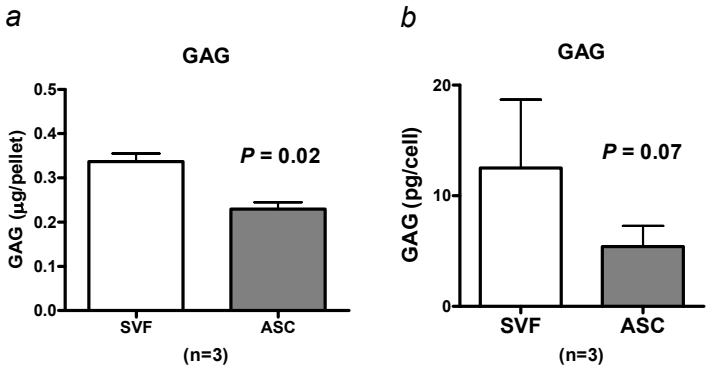

Figure 4. *Production of glycosaminoglycans in SVF and ASC after inducing cells into the chondrogenic lineage in a 3-D PLA-CPL scaffold. (A) A significant difference could be detected in GAG production per scaffold between SVF and ASC (P=0.02). (B) When corrected for the number of cells in each scaffold, still a clear trend was visible in favor of the SVF (P=0.07, n=3).*

was confirmed by Oil Red O staining of the lipid droplets (Figure 1F). Induction into the chondrogenic lineage was realized by seeding the cells into the three-dimensional (3-D) PLA-CPL scaffold as mentioned above. Cells stimulated in this way showed extracellular deposition of both proteoglycans (Figure 3B, D) and collagen type II (Figure 3F, H).

DISCUSSION

In this study we investigated whether freshly isolated stromal cells from the infrapatellar fat pad are a suitable cell source for future application in an OSP for the regeneration of cartilage tissue. We have confirmed four requirements for the development of an OSP. First, we demonstrated that SVF cells can be isolated from the infrapatellar fat pad within a short time frame. Second, we showed that SVF can be harvested in clinically relevant quantities from the infrapatellar fat pad for treatment of focal cartilage defects. Third, we showed this SVF contained a population of cells resembling ASC regarding growth kinetics, cell marker profile and multidifferentiation capacity using CFU assays. Finally, freshly isolated SVF cells seeded in a 3-D PLA-CPL scaffold were able to differentiate into the chondrogenic lineage, as demonstrated by up-regulation of chondro-specific genes, formation of chondrogenic extracellular matrix and production of GAG.

The first requirement for the development of an OSP includes a limited time frame for cell procurement. In this regard, resection of the Hoffa's fat pad and manual processing of the SVF cells took a maximum of 2.5 h. This implies a relative long (and expensive) surgery, with the joint opened for a prolonged period. With the advancing technologies of automated adipose tissue harvesting, it should be possible to reduce the time span of the procedure to 1.5–2.0 h[37]. This fits within the time frame of other knee surgery procedures, such as total knee arthroplasty[38].

The second requirement for the development of an OSP is a high cell yield. Depending on the depth, (mean 9600 cells/mm^3, 1.5–2.0 volume%)[39]. Once damaged, regeneration toward qualitatively good cartilage probably requires at least this cell density, possibly higher. Having shown that the infrapatellar fat pad contains $0.55x10^6$– $16x10^6$ stromal cells and that on average 2.6% of these cells show multipotency, this would imply that only moderate cartilage defects from 1.5 mm^3 up to 4.3 mm^3 could be treated. However, additional considerations may challenge these one-to-one extrapolations: (1) ASC will still proliferate in the scaffold material, as shown in earlier studies[33,34]; (2) a higher yield of ASC might be obtained from patients suffering from focal cartilage damage[40–42]; (3) the quality and history of the cells implanted may influence regeneration outcomes, which is, for example, indicated by this study, where SVF appears to display stronger regenerative potential than cultured ASC, which might be because of minimal manipulation of these cells before administration; and (4) optimal cell-seeding densities for cartilage tissue engineering still have to be determined, as contradictory results have been reported so far [43–46]. Therefore, *in vivo* studies are ongoing and urgently required to address the feasibility of this particular application.

Concerning the third requirement of stem cell characteristics, Wickham *et al.*[47] were the first to describe the existence of stromal cells in the infrapatellar fat pad, and the ability of these cells to differentiate into chondrocytes, osteoblasts or adipocytes after culture passaging, which was later confirmed by others[36,48,49] and this study. However, while in the other studies only culture passaged cells were used, we also tested the multidifferentiation capacity of freshly isolated, minimally manipulated SVF cells in CFU assays for the intended use in an OSP to treat focal cartilage damage. We found that the frequency of the cells able to differentiate into specific lineages was interchangeable with ASC from other subcutaneous tissue-harvesting sites[25].

Both freshly isolated and cultured ASC from the Hoffa fat pad were characterized by cell-marker profile, and cultured cells were also characterized by growth kinetics. Both of these characteristics were in concordance with ASC from the abdomen and hip/thigh regions[25,32]. Others have also characterized the phenotype of Hoffa ASC[47–49] but only looked into culture passaged cells. Thereby certain markers such as CD34 were found to be negative, whereas in fact they are present on the surface in freshly isolated cells and lost upon culturing, as has been shown before by our group[32] and others[21,50,51].

Cartilage tissue is well known for its avascular (and aneural) nature. This would imply that cell sources used for regenerative strategies should include as few as possible cells related to angiogenesis. Remarkably, when comparing subpopulations in the SVF of Hoffa fat pad, the percentage of endothelial cells, marked as $CD34^{bright}CD31^+CD146^+$, was somewhat lower than in the abdomen and hip/thigh regions[32]. This can be advantageous regarding the use of this stroma in an OSP for cartilage regeneration, as the formation of cartilage tissue might be favored, when the endothelial potential of this stroma is diminished.

The fourth requirement includes chondrogenic differentiation potential of the cells. Chondrogenic differentiation was analyzed by studying the expression of chondro-specific genes with qRT-PCR. Both freshly isolated SVF cells and culture-passaged ASC showed up-regulation of these genes. No significant differences could be detected in the induction of

specific genes between SVF and ASC. Furthermore, no conclusive remarks could be made regarding the translation into extracellular matrix deposition, as no clear differences in histologic stainings were observed. However, a clear trend was seen in the induction of the chondrogenic gene aggrecan between fresh SVF and cultured ASC (P=0.07). Surprisingly, the production of GAG was also significantly different between SVF and ASC (P=0.02). This underlines a correlation between gene expression and matrix production, in favor of the SVF cells.

Histologic analysis after chondrogenic induction of both SVF and cultured ASC revealed cells producing abundant extracellular matrix specific for chondrogenic differentiation. Although some (immature) tissue was formed within the scaffold material after 21 days of induction, maturation of the tissue could not be expected within this short time frame. For further maturation into hyaline-like cartilage additional biochemical and biophysical cues are required, which implies the need for a more natural environment, such as *in vivo* models, or at least systems in which these parameters can be controlled better.

Some drawbacks of the current study design can be identified. First, the designated patient population consisted of relatively young patients with focal cartilage damage. However, it is ethically unacceptable to obtain material from this (healthy) patient population for research purposes. Therefore, we had to use Hoffa fat pads resected during total knee arthroplasties, which were removed to visualize the knee joint and prevent impingement of the fat by the prosthesis[52,53]. The use of these fat pads from osteoarthritic joints implies that the quality of the obtained tissue might be inferior compared with that of patients with focal cartilage damage[54,55]. Thus the results of this study might even underestimate the chondrogenic differentiation potential of freshly isolated SVF cells.

Second, complete removal of the Hoffa fat pad for regenerative therapies results in loss of a structure containing a dense network of nerve fibers, which might play a role in quadriceps stability[56–58] and also might have other functions, such as the production of surface-zone proteins. On the other hand, (partial) removal of the fat pad and its nerve fibers by resection could result in diminished pain sensation because of removal of the nerve fibers and concomitant increased stability of the knee joint by the quadriceps muscle resulting from the absence of pain sensation. Moreover, not removing the fat pad can result in a preponderance of sensory over sympathetic innervation in the infrapatellar fat pad, which possibly leads to aggravation and continuation of anterior knee pain and local inflammation[59]. Furthermore, the production of the superficial-zone proteins by the mesenchymal stem cells in the fat pad is not lost but, even better, concentrated to the region of interest by transplanting the superficial zone protein-producing ASC[60] to the damaged site. Other structures in the knee (e.g. synovium and meniscus) will still produce these proteins. Therefore, these functions should definitely be taken into account, but in our opinion do not interfere with partial resection of the infrapatellar fat pad for regenerative therapies.

In conclusion, our *in vitro* data on freshly isolated SVF cells look promising for the development of an OSP to regenerate cartilage tissue. Four requirements were addressed and met: (1) cells can be obtained within a short time frame, (2) no culture expansion steps are required (because of the high yield of SVF cells), (3) the cells show optimal stem cell characteristics as a result of minimal manipulation, and (4) SVF cells show at least

similar chondrogenic differentiation capacity compared with cultured ASC. Whether this concept is specifically applicable to SVF cells from the infrapatellar fat pad is debatable because of the limited and variable availability of these cells, and removal of a healthy structure involved in stabilization and innervation of the knee joint. Therefore, SVF from the infrapatellar fat pad might only be applicable for treatment of small focal cartilage defects, whereas for larger osteoarthritic defects subcutaneous adipose tissue depot would be preferable. Further *in vivo* studies are warranted to assess the feasibility of the proposed concept.

REFERENCES

1. Bartha L, Vajda A, Duska Z, Rahmeh H, Hangody L., 2006. Autologous osteochondral mosaicplasty grafting. J Orthop Sports Phys Ther ;36:739-50.
2. Hangody L, Fules P, 2003. Autologous osteochondral mosaicplasty for the treatment of full-thickness defects of weight-bearing joints: ten years of experimental and clinical experience. J Bone Joint Surg Am; 85-A Suppl 2:25-32.
3. Kish G, Modis L, Hangody L, 1999. Osteochondral mosaicplasty for the treatment of focal chondral and osteochondral lesions of the knee and talus in the athlete. Rationale, indications, techniques, and results. Clin Sports Med Jan;18:45-66, vi.
4. Bartlett W, Skinner JA, Gooding CR, Carrington RW, Flanagan AM, Briggs TW, et al,2005. Autologous chondrocyte implantation versus matrix-induced autologous chondrocyte implantation for osteochondral defects of the knee: a prospective, randomised study. J Bone Joint Surg Br ; 87:640-645.
5. Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L, 1994. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N Engl J Med 6: 889-95.
6. Peterson L, Minas T, Brittberg M, Nilsson A, Sjogren-Jansson E, Lindahl A, 2000. Two- to 9-year outcome after autologous chondrocyte transplantation of the knee. Clin Orthop Relat Res May;374:212-34.
7. Zheng MH, Willers C, Kirilak L, Yates P, Xu J, Wood D, et al, 2007. Matrix-induced autologous chondrocyte implantation (MACI): biological and histological assessment. Tissue Eng; 13:737-46.
8. Niemeyer P, Pestka JM, Kreuz PC, Erggelet C, Schmal H, Suedkamp NP, et al, 2008. Characteristic complications after autologous chondrocyte implantation for cartilage defects of the knee joint. Am J Sports Med ; 36:2091-9.
9. Haddo O, Mahroof S, Higgs D, David L, Pringle J, Bayliss M, et al, 2004. The use of chondrogide membrane in autologous chondrocyte implantation. Knee Feb; 11:51-5.
10. Manfredini M, Zerbinati F, Gildone A, Faccini R, 2007. Autologous chondrocyte implantation: a comparison between an open periosteal-covered and an arthroscopic matrix-guided technique. Acta Orthop Belg Apr; 73:207-18.
11. Marcacci M, Berruto M, Brocchetta D, Delcogliano A, Ghinelli D, Gobbi A, et al, 2005. Articular cartilage engineering with Hyalograft C: 3-year clinical results. Clin Orthop Relat Res ; 435:96-105.
12. Gobbi A, Kon E, Berruto M, Francisco R, Filardo G, Marcacci M, 2006. Patellofemoral full-thickness chondral defects treated with Hyalograft-C: a clinical, arthroscopic, and histologic review. Am J Sports Med; 34:1763-73.
13. Kon E, Gobbi A, Filardo G, Delcogliano M, Zaffagnini S, Marcacci M, 2009. Arthroscopic second-generation autologous chondrocyte implantation compared with microfracture for chondral lesions of the knee: prospective nonrandomized study at 5 years. Am J Sports Med; 37:33-41.
14. Visna P, Pasa L, Cizmar I, Hart R, Hoch J, 2004. Treatment of deep cartilage defects of the knee using autologous chondrograft transplantation and by abrasive techniques--a randomized controlled study. Acta Chir Belg ;104:709-14.
15. Brittberg M, 2008. Autologous chondrocyte implantation--technique and long-term follow-up. Injury ;39 Suppl 1:S40-S49.

16. Caplan AI, Elyaderani M, Mochizuki Y, Wakitani S, Goldberg VM, 1997. Principles of cartilage repair and regeneration. Clin Orthop Relat Res; 342:254-69.
17. Solchaga LA, Goldberg VM, Caplan AI, 2001. Cartilage regeneration using principles of tissue engineering. Clin Orthop Relat Res Oct;391 Suppl:S161-S170.
18. Asakura A, Komaki M, Rudnicki M, 2001. Muscle satellite cells are multipotential stem cells that exhibit myogenic, osteogenic, and adipogenic differentiation. Differentiation Oct; 68:245-53.
19. Nakahara H, Goldberg VM, Caplan AI, 1991. Culture-expanded human periosteal-derived cells exhibit osteochondral potential in vivo. J Orthop Res; 9:465-76.
20. Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, et al, 2002. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell; 13:4279-95.
21. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, et al, 2001. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Engr; 7:211-28.
22. Erickson GR, Gimble JM, Franklin DM, Rice HE, Awad H, Guilak F, 2002. Chondrogenic potential of adipose tissue-derived stromal cells in vitro and in vivo. Biochem Biophys Res Commun 18:763-9.
23. Guilak F, Lott KE, Awad HA, Cao Q, Hicok KC, Fermor B, et al, 2006. Clonal analysis of the differentiation potential of human adipose-derived adult stem cells. J Cell Physiol; 206:229-37.
24. Rangappa S, Fen C, Lee EH, Bongso A, Sim EK, 2003. Transformation of adult mesenchymal stem cells isolated from the fatty tissue into cardiomyocytes. Ann Thorac Surg ; 75:775-9.
25. Jurgens WJ, Oedayrajsingh-Varma MJ, Helder MN, Zandiehdoulabi B, Schouten TE, Kuik DJ, et al, 2008. Effect of tissue-harvesting site on yield of stem cells derived from adipose tissue: implications for cell-based therapies. Cell Tissue Res ; 332:415-26.
26. Knippenberg M, Helder MN, Zandieh DB, Wuisman PI, Klein-Nulend J, 2006. Osteogenesis versus chondrogenesis by BMP-2 and BMP-7 in adipose stem cells. Biochem Biophys Res Commun 14:902-8.
27. van Dijk A, Niessen HW, Zandieh DB, Visser FC, van Milligen FJ, 2008. Differentiation of human adipose-derived stem cells towards cardiomyocytes is facilitated by laminin. Cell Tissue Res ; 334:457-67.
28. Helder MN, Knippenberg M, Klein-Nulend J, Wuisman PI, 2007. Stem Cells from Adipose Tissue Allow Challenging New Concepts for Regenerative Medicine. Tissue Eng 22:1799-808.
29. Dominici M, Le BK, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, et al, 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy; 8:315-7.
30. Oedayrajsingh-Varma MJ, van Ham SM, Knippenberg M, Helder MN, Klein-Nulend J, Schouten TE, et al, 2006. Adipose tissue-derived mesenchymal stem cell yield and growth characteristics are affected by the tissue-harvesting procedure. Cytotherapy; 8:166-77.
31. Kroeze KL, Jurgens WJ, Doulabi BZ, van Milligen FJ, Scheper RJ, Gibbs S, 2009. Chemokine-mediated migration of skin-derived stem cells: predominant role for CCL5/RANTES. J Invest Dermatol Jun; 129:1569-81.
32. Varma MJ, Breuls RG, Schouten TE, Jurgens WJ, Bontkes HJ, Schuurhuis GJ, et al, 2007. Phenotypical and functional characterization of freshly isolated adipose tissue-derived stem cells. Stem Cells Dev; 16:91-104.
33. Hoogendoorn RJ, Lu ZF, Kroeze RJ, Bank RA, Wuisman PI, Helder MN, 2008. Adipose stem cells for intervertebral disc regeneration: current status and concepts for the future. J Cell Mol Med ; 12:2205-16.
34. Kroeze RJ, Breuls RG, Schouten TE, Smit TH, van Milligen FJ, Helder MN, 2008. Rapid and Selective Attachment of Adipose Stem cells to a Bioresorbable Scaffold: Possible Implications for a One-step Surgical Procedure. Transactions of the 53th Annual Meeting, Orthopaedic Research Society Mar 2-5; San Francisco, USA. abstract no. 129
35. Ishaug-Riley SL, Okun LE, Prado G, Applegate MA, Ratcliffe A, 1999. Human articular chondrocyte adhesion and proliferation on synthetic biodegradable polymer films. Biomaterials ; 20:2245-56.
36. Dragoo JL, Samimi B, Zhu M, Hame SL, Thomas BJ, Lieberman JR, et al, 2003. Tissue-engineered cartilage and bone using stem cells from human infrapatellar fat pads. J Bone Joint Surg Br ; 85:740-7.
37. Lin K, Matsubara Y, Masuda Y, Togashi K, Ohno T, Tamura T, et al, 2008. Characterization of adipose tissue-derived cells isolated with the Celution system. Cytotherapy; 10:417-26.
38. Yasunaga H, Tsuchiya K, Matsuyama Y, Ohe K, 2009. Analysis of factors

affecting operating time, postoperative complications, and length of stay for total knee arthroplasty: nationwide web-based survey. J Orthop Sci; 4:10-6.

39. Hunziker EB, Quinn TM, Hauselmann HJ, 2002. Quantitative structural organization of normal adult human articular cartilage. Osteoarthritis Cartilage; 10:564-72.
40. iba-Kojima E, Tsuno NH, Inoue K, Matsumoto D, Shigeura T, Sato T, et al, 2007. Characterization of wound drainage fluids as a source of soluble factors associated with wound healing: comparison with platelet-rich plasma and potential use in cell culture. Wound Repair Regen; 15:511-20.
41. Song HY, Jeon ES, Jung JS, Kim JH, 2005. Oncostatin M induces proliferation of human adipose tissue-derived mesenchymal stem cells. Int J Biochem Cell Biol; 37:2357-65.
42. Yu S, Cho HH, Joo HJ, Bae YC, Jung JS, 2008. Role of MyD88 in TLR agonist-induced functional alterations of human adipose tissue-derived mesenchymal stem cells. Mol Cell Biochem; 317:143-50.
43. Bernstein P, Dong M, Graupher S, Corbeil D, Gelinsky M, Gunther KP, et al, 2008. Sox9 expression of alginate-encapsulated chondrocytes is stimulated by low cell density. J Biomed Mater Res A 18.
44. Hui TY, Cheung KM, Cheung WL, Chan D, Chan BP, 2008. In vitro chondrogenic differentiation of human mesenchymal stem cells in collagen microspheres: influence of cell seeding density and collagen concentration. Biomaterials 29:3201-12.
45. Kelly DJ, Crawford A, Dickinson SC, Sims TJ, Mundy J, Hollander AP, et al, 2007. Biochemical markers of the mechanical quality of engineered hyaline cartilage. J Mater Sci Mater Med; 18:273-81.
46. Mauck RL, Wang CC, Oswald ES, Ateshian GA, Hung CT, 2003. The role of cell seeding density and nutrient supply for articular cartilage tissue engineering with deformational loading. Osteoarthritis Cartilage; 11:879-90.
47. Wickham MQ, Erickson GR, Gimble JM, Vail TP, Guilak F, 2003. Multipotent stromal cells derived from the infrapatellar fat pad of the knee. Clin Orthop Relat Res ; 412:196-212.
48. English A, Jones EA, Corscadden D, Henshaw K, Chapman T, Emery P, et al, 2007. A comparative assessment of cartilage and joint fat pad as a potential source of cells for autologous therapy development in knee osteoarthritis. Rheumatology (Oxford); 46:1676-83
49. Khan WS, Adesida AB, Hardingham TE, 2007. Hypoxic conditions increase hypoxia-inducible transcription factor 2alpha and enhance chondrogenesis in stem cells from the infrapatellar fat pad of osteoarthritis patients. Arthritis Res Ther; 9:R55.
50. Katz AJ, Tholpady A, Tholpady SS, Shang H, Ogle RC, 2005. Cell surface and transcriptional characterization of human adipose-derived adherent stromal (hADAS) cells. Stem Cells Mar; 23:412-23.
51. Gronthos S, Franklin DM, Leddy HA, Robey PG, Storms RW, Gimble JM, 2001. Surface protein characterization of human adipose tissue-derived stromal cells. J Cell Physiol; 189:54-63.
52. Krebs VE, Parker RD, 1994. Arthroscopic resection of an extrasynovial ossifying chondroma of the infrapatellar fat pad: end-stage Hoffa's disease? Arthroscopy; 10:301-4.
53. Kumar D, Alvand A, Beacon JP, 2007. Impingement of infrapatellar fat pad (Hoffa's disease): results of high-portal arthroscopic resection. Arthroscopy; 23:1180-6.
54. Barry FP, 2003. Mesenchymal stem cell therapy in joint disease. Novartis Found Symp; 249:86-96.
55. Murphy JM, Dixon K, Beck S, Fabian D, Feldman A, Barry F, 2002. Reduced chondrogenic and adipogenic activity of mesenchymal stem cells from patients with advanced osteoarthritis. Arthritis Rheum; 46:704-13.
56. Buma P, 1994. Innervation of the patella. An immunohistochemical study in mice. Acta Orthop Scand; 65:80-6.
57. Krenn V, Hofmann S, Engel A, 1990. First description of mechanoreceptors in the corpus adiposum infrapatellare of man. Acta Anat (Basel); 137:187-8.
58. Wojtys EM, Beaman DN, Glover RA, Janda D, 1990. Innervation of the human knee joint by substance-P fibers. Arthroscopy ;6:254-63.
59. Lehner B, Koeck FX, Capellino S, Schubert TE, Hofbauer R, Straub RH, 2008. Preponderance of sensory versus sympathetic nerve fibers and increased cellularity in the infrapatellar fat pad in anterior knee pain patients after primary arthroplasty. J Orthop Res; 26:342-50.
60. Lee SY, Nakagawa T, Reddi AH, 2008. Induction of chondrogenesis and expression of superficial zone protein (SZP)/lubricin by mesenchymal progenitors in the infrapatellar fat pad of the knee joint treated with TGF-beta1 and BMP-7. Biochem Biophys Res Commun 7:148-53.