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A New Nonenzymatic Method and Device to Obtain a Fat Tissue Derivative Highly Enriched in Pericyte-Like Elements by Mild Mechanical Forces From Human Lipoaspirates

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Adipose tissue contains multipotent elements with phenotypic and gene expression profiles similar to human mesenchymal stem cells (hMSCs) and pericytes. The chance of clinical translation of the multilineage potential of these cells is delayed by the poor/negligible cell survival within cryopreserved lipoaspirates, the difficulty of ex vivo expansion, and the complexity of current Good Manufacturing Practice (cGMP) requirements for expanded cells. Hence, availability of a minimally manipulated, autologous, hMSC/pericyte-enriched fat product would have remarkable biomedical and clinical relevance. Here, we present an innovative system, named Lipogems, providing a nonexpanded, ready-to-use fat product. The system uses mild mechanical forces in a completely closed system, avoiding enzymes, additives, and other manipulations. Differently from unprocessed lipoaspirate, the nonexpanded Lipogems product encompasses a remarkably preserved vascular stroma with slit-like capillaries wedged between adipocytes and stromal stalks containing vascular channels with evident lumina. Immunohistochemistry revealed that Lipogems stromal vascular tissue included abundant cells with pericyte/hMSC identity. Flow cytometry analysis of nonexpanded, collagenase-treated Lipogems product showed that it was comprised with a significantly higher percentage of mature pericytes and hMSCs, and lower amount of hematopoietic elements, than enzymatically digested lipoaspirates. Differently from the lipoaspirate, the distinctive traits of freshly isolated Lipogems product were not altered by cryopreservation. Noteworthy, the features of fresh product were retained in the Lipogems product obtained from human cadavers, paving the way to an off-the-shelf strategy for reconstructive procedures and regenerative medicine. When placed in tissue culture medium, the Lipogems product yielded a highly homogeneous adipose tissue-derived hMSC population, exhibiting features of hMSCs isolated from other sources, including the classical commitment to osteogenic, chondrogenic, and adipogenic lineages. Moreover, the transcription of vasculogenic genes in Lipogems-derived adipose tissue hMSCs was enhanced at a significantly greater extent by a mixture of natural provasculogenic molecules, when compared to hMSCs isolated from enzymatically digested lipoaspirates.

Key words: Adipose tissue; Lipoaspirates; Stromal vascular architecture; Stem cells; Nonenzymatic isolation

INTRODUCTION

Human mesenchymal stem cells (hMSCs) have been proposed as an attractive cell source for regenerative medicine in different contexts, including bone and cartilage repair, as well as cardiac, vascular, neuronal, and endocrine rescue (6,25,46). These cells are able to self-renew with a high growth rate and possess multipotent differentiation properties. We have recently shown that hMSCs can be obtained from the dental pulp (hDMSCs), fetal membranes of term placenta (hFMSCs), and adipose tissue (hASCs) and can be used to afford remarkable vasculogenesis and cardiovascular differentiation in vitro, as well as myocardial repair in vivo after exposure to synthetic agents (58), or improve islet graft revascularization and function in diabetic rats after preconditioning with natural molecules (8). These results are proof of principle

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that, independently from tissue source, the attainment of a vasculogenic lineage and vascular repair in vitro and in vivo is a common hMSC hallmark proving highly effective in tissue repair. Regardless of source, hMSCs of different origin also share the common feature of harboring a "secretome" encompassing multiple trophic mediators that act in a paracrine fashion within the recipient tissue to elicit angiogenic, antiapoptotic, and antifibrotic responses (7,27,32,58). Although bone marrow has been used as the main source of hMSCs (hBMSCs), the harvest of bone marrow is a relatively invasive and painful procedure and alternative sources for hMSCs should be investigated. Moreover, the use of hBMSCs is potentially associated with a high degree of viral infection and significant decline in cell viability and differentiation with donor age (51). Obtaining hBMSCs remains a cumbersome and invasive approach. Although there is also evidence that hMSCs may be nonimmunogenic or hypoimmunogenic (10), allogeneic transplantation of hFMSCs remains to be unequivocally established as a safe procedure and it is not readily envisionable in humans.

To this end, an ideal hMSC source should (i) be found in abundant quantities, (ii) be harvested by a minimally invasive procedure, and (iii) provide a hMSC population retaining a good viability and differentiating potential with donor's age (57). In the last few years, adipose tissue has been identified as possessing a population of multipotent adipose-derived stem cells (hASCs) (17,52,57). Over 300,000 liposuction surgeries are performed in the US each year and can yield anywhere from <30 ml to >6 L of lipoaspirated tissue. This material is routinely discarded even if it is now often reutilized in lipofilling procedure for which the Coleman method has been the standard of care (57).

hASCs exhibit phenotypic and gene expression profiles similar to hMSCs obtained from bone marrow (11,12) and other alternative sources and can be expanded in culture for extended periods (63). hASCs are a promising tool for regenerative therapies, since they have been used in vivo in animal models of acute myocardial infarction (26,34,50,53,60). Accordingly, cultured monolayer of hASCs repaired scarred myocardium in infarcted rat hearts, acting as trophic mediators for paracrine angiogenic pathways (34). hASCs can also be committed to both endothelial (5,15,33,37,40,41,48) and smooth muscle cell lineages (1,4,16,22,23,30,31). There is a growing body of experimental evidence from both in vitro and in vivo studies demonstrating the multipotentiality of ASCs isolated from humans and other species. These include the adipocyte (19,47,63,64), chondrocyte (14,59,63,64), hematopoietic supporting (9), hepatocyte (49,54,55), neuronal-like (24,28,43–45,63), osteoblast (18,20,21,63,64), pancreatic (56), and skeletal myocyte (29,35,63,64) pathways.

Human subjects have abundant subcutaneous fat deposits and hASCs can easily be isolated by enzymatic digestion of lipoaspirates, thus overcoming the tissue morbidity associated with bone marrow aspiration. Furthermore, the frequency of hMSCs in bone marrow is between 1 in 25,000 and 1 in 100,000 cells (2,13,36), whereas hASCs constitute approximately 2% of lipoaspirate cells (53).

Despite the advances in isolating hASCs, a number of hurdles still need to be overcome, including the poor/negligible cell survival following cryopreservation and thawing of lipoaspirates, the difficulty of ex vivo expansion, the poor delivery efficiency (less than 5% of transplanted cells are retained after transplantation), and uncertain fate in vivo (3).

These issues minimize the advantages achieved by cell expansion itself. Moreover, the chance of translation into clinical settings for stem cells subjected to extensive manipulation, including ex vivo expansion, is remarkably delayed due to requirements for compliance with "cell manufacturing" in accordance with current Good Manufacturing Practice (cGMP) Guidelines (42). However, these restrictions are not applied in the case of minimal manipulation [Regulation (EC) No. 1394/2007 of the European Parliament and of the Council; http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2007:324:0121:0137:en:PDF]. Therefore, developing processing technologies to obtain suitable autologous hASC products with minimal manipulation, which could be readily used, cryopreserved, or further expanded, would be highly desirable for clinical applications.

Toward this goal, in the current study, we have developed an innovative enzyme-free technology to process variable amounts of lipoaspirates, resulting in a non-expanded adipose tissue product that contains hASCs. This technology works through a mild mechanical tissue cluster size reduction in a full immersion, closed system, avoiding the use of any enzyme, and the additional processing and equipment requirements (i.e., centrifugation and subfractional harvesting). Here, we describe the methodological details of the system and provide the phenotypic characterization of the system product, both fresh and cryopreserved.

MATERIALS AND METHODS

A Novel System for Lipoaspirate Processing

The currently described system, named Lipogems (PCT/IB2011/052204), has been developed to harvest, process, and reinject human (or animal) lipoaspirates. Such a system has been conceived for being used by any trained doctor, keeping all surgical procedures as simple and effective as possible. Its "core" is a simple and disposable device (Fig. 1) that progressively reduces in size the clusters of adipose tissue (from spheroidal clusters with a diameter of 1–3.5 mm to clusters of 0.2–0.8 mm), while

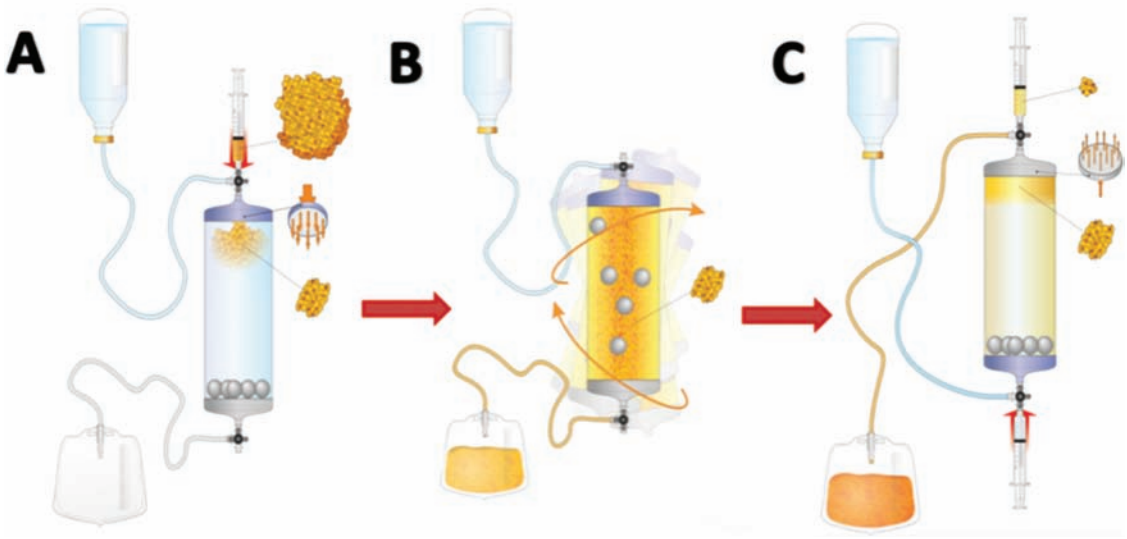

Figure 1. Schematic representation of the Lipogems device. In this completely closed system, the original lipoaspirate is processed by mild mechanical forces without using collagenase or other enzymes/additives. In the Lipogems device, the lipoaspirate is initially subjected to a first cluster reduction (A), obtained by pushing the aspirated fat from the syringe into the device through the large filter (blue end), and allowing the corresponding quantity of saline to exit towards the wasting bag. Stainless steel marbles contained in the device are essential to obtain a temporary emulsion between oil, blood, and saline, which can be washed away against density following the current of saline moved by gravity (B) (for details, see the Materials and Methods section). After this washing step (the flowing solution appears clear and the lipoaspirate yellow), the saline flux is stopped and the device is reversed (gray cap up), leading to the second adipose cluster reduction (C). Such reduction is obtained by pushing the floating adipose clusters through the second cutting hexagonal filter, pushing fluid from below with a 10-ml syringe. The reduced clusters pass in another 10-ml syringe placed above (C).

eliminating oil and blood residues. The entire procedure occurs in a full immersion system to minimize any traumatic action on the cell products (Fig. 1). With a standard 225 ml Lipogems device (Lipogems International S.R.L., Milan, Italy), the procedure takes less than 20 min. This allows one to process about 100–130 ml of lipoaspirate to obtain approximately 60–100 ml of final tissue product.

The original objective of the Lipogems system was to improve the classical Coleman lipofilling technique (57) by providing transplantable clusters of lipoaspirate with reduced size to improve their posttransplant engraftment. The importance of the tissue thickness to optimize engraftment is well known in skin grafting. Nonetheless, in classical lipofilling techniques, this goal could only be achieved by reducing the caliber of the aspirating cannula and/or their openings. However, this strategy imposes limitations, due to a significant increase in the tissue harvesting time and a decreased quality of the lipoaspirate cell product (57). The size reduction of the adipose tissue clusters obtained by other mechanical means, such as rotating blades (e.g., blenders) is very traumatic to cells, and produces large amounts of oil residues and cellular debris (57).

Surgical Procedure to Obtain Lipoaspirates Suitable for Lipogems Processing

The surgical procedure, used for both living and cadaveric donors, requires two steps: infiltration and aspiration. In the infiltration step, adrenalin in a saline solution (Galenica Senese S.r.l., Monteroni D'Arbia, Italy; 2 μg/ml final concentration) is infiltrated using a 19-cm specially designed disposable blunt cannula (Finella Medical, Bollate, Italy) inserted following puncture with an 18-gauge needle (Becton Dickinson, San Jose, CA, USA). The vasoconstriction together with the blunt point of the cannula avoids any accidental intravascular injection and facilitates the subsequent lipoaspiration. Adding very diluted lidocaine (Galenica Senese S.r.l.; 0.02%) to the mixture is an option to provide local anesthesia, requiring a waiting time of a minimum of 7 min before aspiration. Three hundred to 500 ml are usually injected in the chosen area for fat harvesting (usually the lower abdomen), making the tissue really "filled" with the injecting solution. An infiltration kit with a specially designed spring syringe and valve can be directly attached to the infusion sac to facilitate the infiltration step while providing a closed system ideal for outpatient office procedure. The aspiration step (lipoaspirate) is performed by a 10-cc luer lock syringe (Becton Dickinson) connected to a disposable 19-cm blunt cannula (3 mm OD), with 5 oval holes (1×2 mm). A few strokes using a standard liposuction technique are enough to harvest 6–10 ml of fat tissue. Vacuum while aspirating can be obtained manually or by clamping the syringe plunger with a clamp instrument. Up to 1,000 ml can be harvested in less than 15 min, and 1–2 min are enough

to harvest the 100–150 ml usually needed. The harvested lipoaspirate can be progressively put into the device using multiple 10-cc syringes.

Lipogems Processing of Lipoaspirate

Between 40 and 130 ml of lipoaspirate (ideally 100 ml) are processed at each time in the standard 225-ml device. To avoid cell damage, no air should be in the device during all procedural steps and the device should be prefilled with saline before beginning the processing. The aspirated fat should be always surrounded by a liquid environment: this is essential to obtain healthy smaller fat clusters instead of oil and adipose tissue debris. The first cluster reduction was obtained by pushing the aspirated fat from the syringe into the device and through the first size reduction filter while allowing the corresponding quantity of saline to exit towards the waste bag (Fig. 1A). When the desired amount of lipoaspirate tissue was placed in the device, kept vertically with first size reduction filter (Fig. 1A) on top, the floating layer of aspirated fat tissue should occupy no more that the half upper portion of the device. Five stainless steel marbles were used inside the device that was shaken to emulsify oil residues which were subsequently removed together with contaminating blood components by a gravity counterflow of saline solution, while the washed reduced adipocyte clusters migrated to the top of the Lipogems device (Fig. 1B). When the solution inside the device appears clear and the lipoaspirate yellow, the saline flow is stopped and the device turned upside-down (180°, gray cap up). The second adipose cluster reduction was obtained by passing the floating adipose clusters through the second size reduction filter by pushing additional fluid from the lower opening of the device using a 10-cc syringe (Fig. 1C). The final Lipogems product is then collected into 10-ml syringes connected to the upper opening of the device. The final Lipogems product is now ready for the desired clinical or banking application.

Histology and Immunohistochemical Analysis

Subcutaneous fat tissue harvested according to conventional and Lipogems methods were fixed in 10% buffered formalin (Carlo Erba Reagents, Milan, Italy) and then embedded in paraffin; 4-μm-thick sections were used for histological and immunohistochemical analysis. For histological analysis, each section was stained with hematoxylin and eosin (Carlo Erba Reagents) and observed under a light microscope. For immunohistochemical studies antigen–antibody reactions were developed with a non-biotin-amplified method (NovoLink Polymer Detection System, Novocastra Laboratories, Newcastle Upon Tyne, UK) for visualizing antigens in tissue sections according to manufacturer's protocol. Briefly, the samples were dewaxed, rehydrated through ethanol (from 100% to 70%), and rinsed in distilled water. Antigen retrieval was performed with citrate buffer (Sigma-Aldrich, Milan, Italy), pH 6, at 120°C, 1 atm for 21 min; endogenous peroxidase activity was quenched using 3% hydrogen peroxide (Sigma-Aldrich) in absolute methanol (Carlo Erba Reagents) for 5 min at room temperature. A panel of monoclonal and polyclonal antibodies was used to evaluate in the samples mature and immature fat cells and microvascular cells. Primary antibodies were diluted in 1% bovine serum albumin (BSA; Sigma-Aldrich) in phosphate-buffered saline (PBS; Sigma-Aldrich) overnight at 4°C using the appropriate dilutions. Sections were stained with the following monoclonal antibodies, anti-cluster of differentiation 34 (CD34; 1:80, clone QBEND-10, Dako Cytomation, Glostrup, Denmark), and anti-α-smooth muscle actin (ASMA, 1:9000, clone 1A4, Sigma-Aldrich). Polyclonal antibodies were used to detect CD146 (1:100, clone EPR3208, Abcam, Cambridge, UK) and S-100 protein (1:200, Dako Cytomation). After immunostaining, the sections were exposed to the substrate/chromogen 3,3′-diaminobenzidine (DAB; Molecular Probes, Milan, Italy), counterstained with hematoxylin, dehydrated, coverslipped, and observed with a Leitz Diaplan light microscope (Wetzlar, Germany) equipped with a video camera (JVC, 3CCD, KY-F55B, Jokohama, Japan). Digitalized images were analyzed using the Image Pro Plus 6 software (Media Cybernetics, Bethesda, MD, USA). Negative controls were performed by omitting incubation with primary antibodies. Quantitative immunohistochemical analysis was performed on digitalized light microscopic images randomly taken at 100× (CD34, ASMA, and CD146) and 250× (S-100 protein). CD34, ASMA, and CD146 immunostaining from triplicate experiments were measured, and the total stained areas were calculated using Image Pro Plus measurement tool. Reference area was 300 mm^2. For S-100 protein cell evaluation, cells were considered positive and counted if the cells expressed S-100 in the nuclear and cytoplasmic areas. *t* Test statistical analysis was performed using Prism 4 software (GraphPad Software, Inc., San Diego, CA, USA).

Flow Cytometry Analysis and Phenotypic Characterization

For flow cytometry analysis, cells, obtained by digestion of Lipogems or lipoaspirate products with collagenase I or from in vitro culture of cadaveric Lipogems product, were incubated with 1 μg/10^6 cells of fluorescent antibodies for 40 min at 4°C in the dark. The trypan blue (Sigma-Aldrich) exclusion test was used to determine the number of viable cells present in the cell suspension after collagenase digestion. The antibodies used for flow cytometry were anti-CD19, anti-CD31, anti-CD90, anti-CD105, anti-CD146, anti-HLA-DR (all from BioLegend, San Diego, CA, USA),

anti-CD14, anti-CD29, anti-CD34, anti-CD44, anti-CD45, anti-CD73, and anti-CD166 (all from BD Biosciences, San Jose, CA, USA). After washing, cells were analyzed on a flow cytometer (FACSAria, BD Biosciences) by collecting 10,000 events, and the data were analyzed using the FACSDiva Software (BD Biosciences).

Isolation and Expansion of Lipoaspirate and Lipogems-Derived hASCs

According to the policies approved by the institutional review boards for human studies of local ethical committees, all tissue samples were obtained after informed consent. Human subcutaneous adipose tissue samples were obtained from lipoaspiration/liposuction procedures described above. The nomenclature of MSCs from adipose tissue varies widely, but for the purposes of this article, we will use ASCs to identify that the cells being studied were derived from adipose tissue.

A part of the sample has been processed as described with the Lipogems device, the rest has been washed and digested in collagenase A type I solution (Sigma-Aldrich) at a final concentration of 0.05%, under gentle agitation for 1 h at 37°C, and centrifuged at 650×*g* for 10 min to separate the stromal vascular fraction (SVF) from adipocytes. If necessary, the SVF was treated with red blood cell lysis buffer (Becton Dickinson) for 5 min at room temperature, protected from light, and then centrifuged again. The supernatant was discarded, and the cell pellet was resuspended and seeded in culture flasks in minimum essential medium with α modification (α-MEM) supplemented with 20% heat-inactivated fetal bovine serum (FBS), antibiotics (200 units/ml penicillin, 100 µg/ml streptomycin), L-glutamine (1%), and incubated at 37°C in a humidified atmosphere with 5% CO_2 (all reagents from Lonza, Basel, Switzerland). Lipogems (1.5 ml; adipose tissue clusters) product was cultured in a T-75 flask (Corning, Milan, Italy), in the same culture medium used for the SVF. Medium was changed every 4 days, but the nonadherent fraction of Lipogems product was removed from the culture only after 2 weeks. At confluence, cells were detached by treatment with trypsin-EDTA (Sigma-Aldrich), characterized by flow cytometry and subcultured. For cryopreservation, the Lipogems product was suspended in culture medium containing 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich) and frozen at –180°C under liquid nitrogen. To expand the cryopreserved product, it was thawed at 37°C and cultured as for the fresh product.

Adipogenesis, Osteogenesis, and Chondrogenesis in Culture

To induce adipogenic differentiation, 10×10^3 cells/cm^2 were cultured in an adipogenesis induction medium (Chemicon Int., Millipore, Billerica, MA, USA) composed of Dulbecco's modified Eagle's medium (DMEM)-low glucose supplemented with 10% FBS, 0.5 mM isobutylmethyl xanthine (IBMX), 200 µM indomethacin, 1 µM dexamethasone, and 10 µg/ml insulin in a 24-well microplate (Greiner bio-one GmbH, Frickenhausen, Germany), replacing the medium every 2–3 days. After 2–3 weeks of culture, the cells were fixed in 10% formalin and stained with fresh oil red-O solution (Millipore).

To induce osteogenic differentiation, 10×10^3 cells/cm^2 were plated in 24-well microplate in DMEM-low glucose supplemented with 10% FBS, 10 mM β-glycerophosphate, 0.2 mM ascorbic acid, and 10 nM dexamethasone (Mesenchymal Stem Cell Osteogenesis Kit, Millipore) and cultured for 3–4 weeks, replacing the medium every 2–3 days. To demonstrate osteogenic differentiation, the cultures were fixed and stained with Alizarin red solution (Millipore).

To induce chondrogenic differentiation, aliquots of 5×10^5 cells were pelleted in polypropylene conical tubes in 0.5 ml of complete chondrogenic medium (Lonza) containing chondrogenic basal medium, supplements and growth factors (ITS + supplement, dexamethasone, ascorbate, sodium pyruvate, proline, penicillin/streptomycin, L-glutamine), and 10 ng/ml transforming growth factor-β3 (TGF-β3; R&D Systems, Minneapolis, MN, USA). This medium was replaced every 3–4 days for 3–4 weeks. Pellets were formalin-fixed, embedded in paraffin, examined morphologically, and immunostained for type II collagen (Chemicon Int., Temecula, CA, USA), using Vectastain elite ABC kit (Vector Laboratories, Burlingame, CA, USA).

Gene Expression Analysis

Total RNA was extracted using RNeasy Microkit (Qiagen, Milan, Italy), and 1 µg was reverse-transcribed into cDNA in a 21-µl reaction volume with SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). To assess gene expression, 2 µl of cDNA were used for real-time PCR performed with a Lightcycler system (Roche Diagnostics) and with the SYBR Green I FastStart kit (Lightcycler® FastStart DNA MasterPLUS SYBR Green I; Roche) following the manufacturer's instructions.

Primers (0.25 µM) used were human QuantiTect Primer Assay (Qiagen) for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), vascular endothelial growth factor (VEGF), kinase insert domain receptor (KDR), and hepatocyte growth factor (HGF). Data were normalized using GAPDH as an index of cDNA content after reverse transcription. Samples were run in duplicate, and the average threshold cycle (Ct) value was used for calculations. Relative quantification of mRNA expression was calculated with the comparative Ct method using the "delta-delta method" for comparing relative expression results between treatments in real-time PCR (39).

Data Analysis

The statistical analysis of the data was performed by using a one-way analysis of variance and the Bonferroni test, assuming a value of $p < 0.05$ as the limit of significance.

RESULTS

Histological and Immunohistochemical Analysis of the Lipoaspirate and the Lipogems Product

Histologically the Lipogems-treated samples showed a better maintained vascular stroma consisting of slit-like capillaries wedged between adipocytes and stromal stalks containing vascular channels with evident lumina; on the contrary, conventionally treated fat tissue showed compressed and distorted microchannels (Fig. 2A). To disclose quantitative differences in the stromal vascular tissue component an immunohistochemical panel against endothelial cells (CD34 and CD146), mural cells (CD146 and ASMA), adipocytes, and preadipocytes (S-100 protein) was performed. CD34 quantitative expression did not show a significant difference (Fig. 2B); CD146 expression was significantly increased in the Lipogems-treated samples (Fig. 2C); because CD146 is coexpressed by endothelial cells and pericytes while CD34 is a marker of endothelial cell differentiation, these results indicate that pericytes, a mesenchymal cell that is supposed to have stem-like properties, contribute significantly to the increased expression of CD146 found in the Lipogems-treated samples. In agreement with this finding, the Lipogems treatment also increased the expression of ASMA (Fig. 2D), a well-established marker of mural cells. The number of cells expressing S-100 protein was similar in both conditions (Fig. 2E).

Flow Cytometry Analysis of Cellular Composition of the Lipogems Product

Freshly obtained Lipogems product, the product previously stored at 4°C for 24 h, or the product thawed after 7 days of cryopreservation at –180°C under liquid nitrogen, was processed through collagenase digestion to release the stromal vascular fraction and to remove adipocytes. In all samples, a cellular viability close to 100% was observed, as inferred by the trypan blue dye exclusion test, with no differences between groups (not shown). Results from comparative flow cytometry analyses of selected stem cell markers in nonexpanded cellular components of lipoaspirate and of Lipogems product are reported in Table 1. Interestingly, the Lipogems SVF differed significantly from the SVF of the lipoaspirate (Fig. 3). The expression pattern $CD146^+/CD90^+/CD34^-$, identifying cells with pericyte identity (38), was significantly higher in the Lipogems product than in the lipoaspirate cellular product (Fig. 3). The SVF of the Lipogems product also exhibited a significantly higher proportion of $CD146^+/CD34^+$ elements, compared to the lipoaspirate product (Fig. 3). This expression pattern has been found to identify a pericyte subset that may be transitional between pericytes and supra-adventitial adipose stromal cells, and/or a set of endothelial (progenitor) cells (61,62). The percentage of hMSCs was higher in the Lipogems product, when compared to the lipoaspirate. In particular, the amount of $CD90^+/CD29^+/CD34^-$ elements, which unambiguously identify a mesenchymal population, was more than double in the Lipogems product than in the lipoaspirate (Fig. 3). Compounding these differences among the two cellular products, the percentage of hematopoietic-like elements positive for CD14, CD34, and CD45 was also significantly reduced in the Lipogems compared to the lipoaspirate products (Fig. 3). All these distinctive traits were remarkably retained in the Lipogems product thawed after cryopreservation.

Lipogems-Derived hASCs Can Be Easily Expanded in Culture

We provide evidence that the Lipogems product can be simply transferred without any manipulation into tissue culture. hASCs slipped out from the tissue cluster product, starting after day 2–3, attached to the tissue culture plastic, and reached 70–80% confluence in 7–12 days (Fig. 4). Therefore, even in a GMP setting, the Lipogems product can be immediately transferred to a tissue culture environment for expansion, while in the same setting, the enzymatic processing and related washing of blood and oil contaminants from a lipoaspirate would require considerably longer periods and additional manipulation (usually 40–50 min per sample), prior to placing the released cells into culture. hASCs were also readily expanded from the cryopreserved Lipogems product from living donors (data not shown). Conversely, the release of viable hASCs from cryopreserved lipoaspirates was a rare, low-yield, and nonreproducible phenomenon (data not shown).

Obtaining Cadaveric Lipogems Product

The ability to use cadaveric tissue for the isolation of hASCs by the Lipogems method was also evaluated. In cadaveric tissue (≤30 h postmortem), there were approximately 75% fewer total viable cells present in the SVF after either enzymatic digestion or the Lipogems processing compared with tissue obtained from live donors ($n = 4$ and 5, respectively). The number of cells released from the cell clusters after enzymatic digestion are similar in magnitude to the number of cells obtained by direct treatment of the lipoaspirate with collagenase, indicating that the Lipogems procedure did not affect cell recovery.

To expand the hASC fraction from the cell clusters obtained by the Lipogems procedure, the cell clusters were placed in tissue culture flasks to allow viable cells to

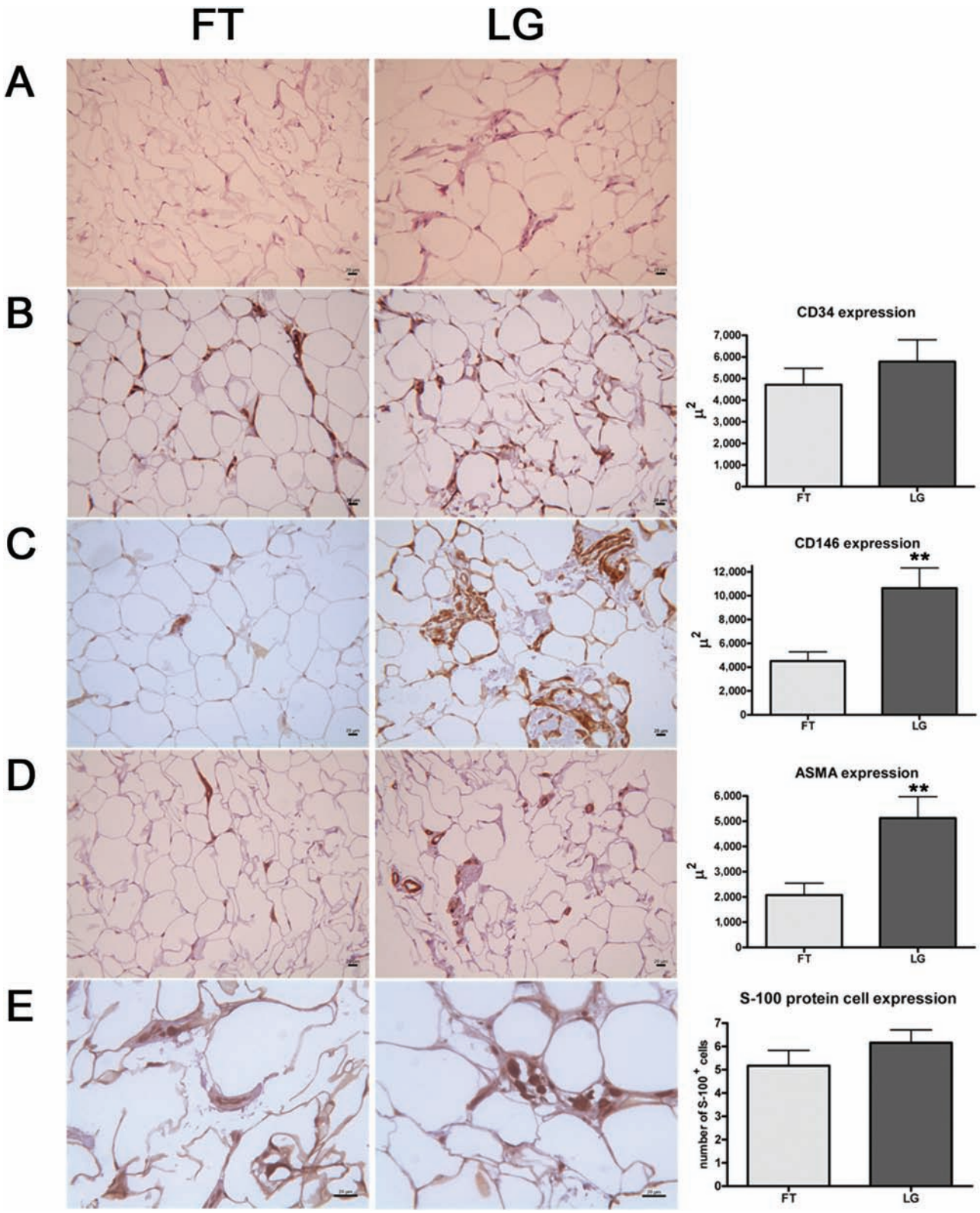

Figure 2. Histological and immunohistochemical analysis of the fat tissue lipoaspirate (FT) and freshly isolated Lipogems product (LG). (A) comparative histological analysis. Comparative immunohistochemical analyses were performed to assess the abundance of cells expressing cluster of differentiation 34 (CD34) (B), CD146 (C), α-smooth muscle actin (ASMA) (D), and S-100 protein (E). Scale bar: 20 μm. Representative of four separate experiments. Panels to the right show total area of staining for each marker, $^{**}p < 0.05$.

Table 1. Comparative Analysis of Stem Cell Markers in the Lipoaspirate and the Lipogems Product After Enzymatic Digestion to Obtain a Stromal Vascular Fraction

	Lipoaspirate	Lipogems
CD146	50.5 ± 4.6	54.5 ± 13.9
CD45	19.93 ± 6.0	9.7 ± 3.2
CD14	8.8 ± 1.8	3.1 ± 1.7
CD34	34.8 ± 9.6	18.1 ± 4.0
CD105	19.2 ± 8.0	20.5 ± 10.0
CD73	26.3 ± 10.6	7.1 ± 3.3
CD44	38.8 ± 15.6	19.3 ± 14.2
CD166	1.5 ± 1.4	2.1 ± 3.0
CD90	43.4 ± 8.7	45.2 ± 9.3
CD29	72.5 ± 7.9	73.0 ± 14.4
$CD105^+/CD73^+/CD45^-$	4.8 ± 1.1	2.2 ± 1.6
$CD146^+/CD90^+/CD34^-$	13.9 ± 2.0	23.2 ± 1.8
$CD90^+/CD29^+/CD34^-$	11.6 ± 0.3	29.4 ± 12.7
$CD146^+/CD34^+$	8.3 ± 1.7	17.8 ± 0.5

Mean ± SE (n = 4 different donors). CD, cluster of differentiation.

grow and expand. After 2–4 weeks in culture, the explants reached confluence and were used for flow cytometry analysis. The data show that after culture the number of cells that express markers expected for hASCs are seen in ~80% of the cell population, and the data obtained from the cadaveric tissue was similar to data obtained from cells isolated from a living donor (Table 2).

Lastly, studies were performed to demonstrate that the cell clusters obtained by the Lipogems procedure could be cryopreserved as a source of banked tissue that could be used for future isolation and expansion of hASCs. Lipogems cell clusters were washed and suspended in cryopreservation media (culture medium containing 10% DMSO) and frozen to –80°C at a controlled rate of freezing then transferred to and stored in the vapor phase of a liquid nitrogen freezer. After 10 days, the cell clusters were thawed and cultured to allow for the growth and expansion of any viable cells. Figure 5 shows that after 10 days in culture, cells were able to grow and expand from the cryopreserved tissues.

Lipogems-Derived hASCs Can Be Committed to Classical Mesenchymal-Derived Lineages

Lipogems-derived hASCs were cultured under specific conditions for targeted commitments, including osteogenic, chondrogenic, and adipogenic lineages, demonstrating that these cells exhibit the typical developmental potential of hMSCs (Fig. 6). Adipogenic differentiation showed multiple adipocytic multivacuolar cells, the size increasing with the time of induction (Fig. 6A). Osteogenic

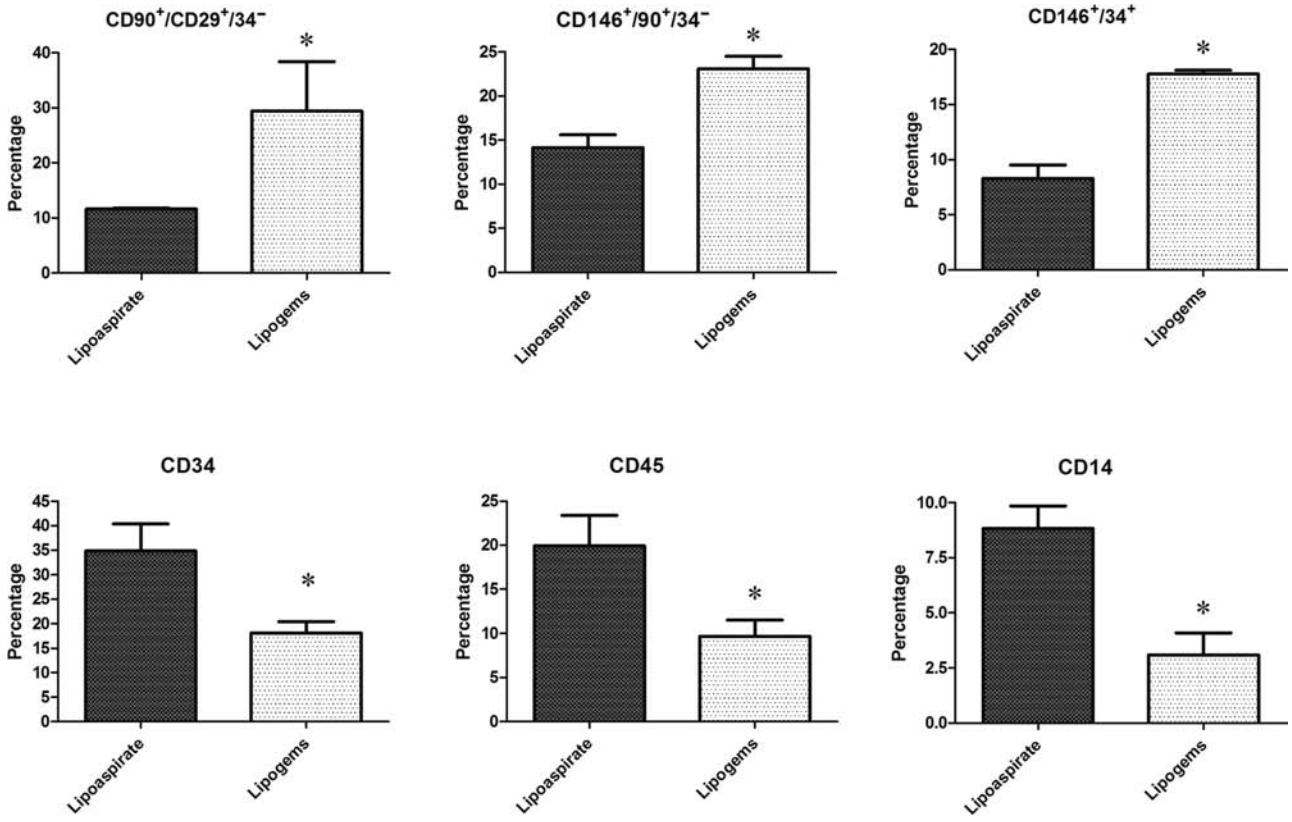

Figure 3. Differential expression of selected markers in the lipoaspirate and in the Lipogems product. Flow cytometry analysis was performed as described in Materials and Methods. Mean ± SE (n = 4). *p < 0.05 significantly different from the percentage in the lipoaspirate.

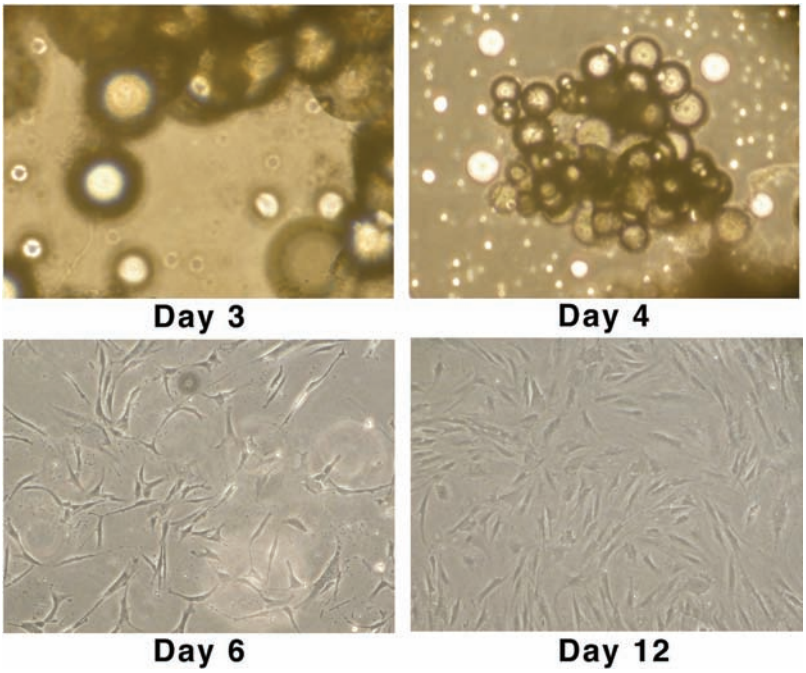

Figure 4. Expansion of Lipogems-derived hASCs. The Lipogems product (1.5 ml) was simply placed into the culture medium and seeded in culture for the indicated times to allow released cells to adhere and proliferate to confluence. Representative of five separate experiments (original magnification: 100×). hASCs, human adipose-derived stem cells.

differentiation was revealed as early as the first week of induction by morphological changes and, at the end of the induction period, by the formation of mineralized matrix. Cells became flattened and showed calcium deposits as demonstrated by Alizarin red staining (Fig. 6B). Chondrogenic differentiation was inferred after 3-week induction by the appearance of abundant extracellular matrix. Such a conclusion was strengthened by immunohistochemical analysis, which showed the presence of human type II collagen (Fig. 6C).

Table 2. Flow Cytometric Immunophenotype Analysis of Cells Expanded From the Lipogems Product Obtained From Cadaveric or Live Donors

		% Frequency	
Marker	Specificity	Cadaveric Donor (n=2)	Live Donor (n=3)
CD90	MSCs	31.1	79.6
CD105	MSCs	79.4	84.8
CD73	MSCs	78.7	84.2
CD44	MSCs	76.2	82.6
CD146	Pericytes/MSCs	17.4	16.4
CD34	Endothelial cells	9.9	5
CD19	Hematopoietic cells	0	0.9
CD14	Hematopoietic cells	0.2	0.6
CD45	Hematopoietic cells	3.1	0.3
CD31	Endothelial cells	15.6	18
HLA-DR	MHC CLASS II	1.1	19.1

Data are expressed as means of the percent of total viable cells positive for the indicated markers. HLA, human leukocyte antigen; MSCs, mesenchymal stem cells; MHC, major histocompatibility complex.

Lipogems-Derived hASCs Express Noticeable Vasculogenic Properties

Both Lipogems-derived hASCs and hASCs resulting from enzymatic digestion of lipoaspirates spontaneously expressed a set of vasculogenic genes, including VEGF, KDR, encoding a major VEGF receptor, and HGF (Fig. 7). We have previously shown that the expression of these genes can be remarkably enhanced following the exposure of hASCs isolated with conventional collagenase-based digestion to a mixture of natural molecules including hyaluronan (H), butyric (B), and retinoic acids (R) (8). Here, we show that following a 24- to 72-h exposure to a mixture containing H (2 mg/ml), B (5 mM), and R (1 μM) the mRNA levels of VEGF, KDR, and HGF were significantly higher in Lipogems-derived hMSCs than in hMSCs obtained from enzymatically digested lipoaspirates (Fig. 7).

DISCUSSION

Lipogems product is a fat tissue derivative with the characteristics of a minimally manipulated product that can be readily injected in an autologous fashion in the

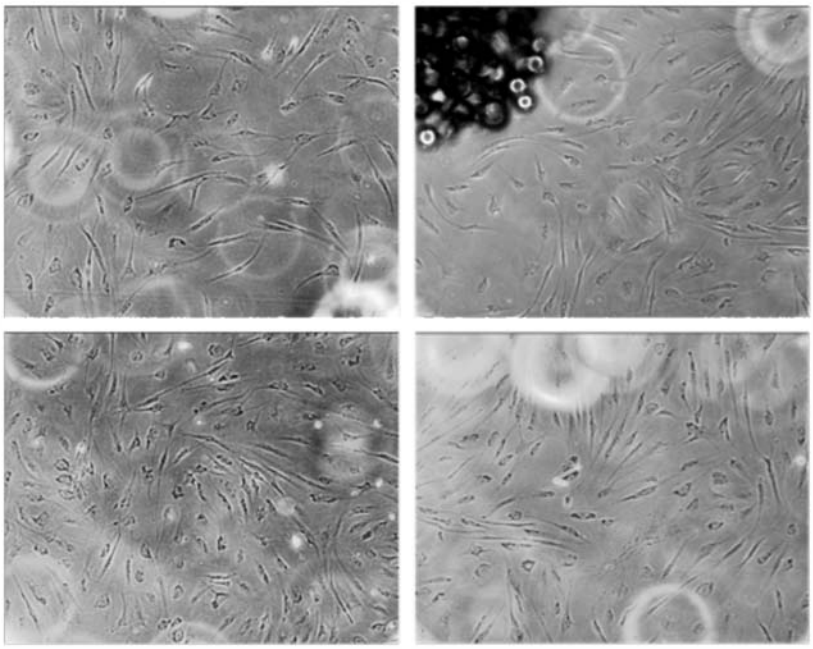

Figure 5. Photomicrograph of expanded cells obtained from the Lipogems cell clusters after cryopreservation and culture for 10 days. The Lipogems product isolated from a cadaveric donor (1.5 ml) was cultured for the indicated times to allow released cells to adhere and proliferate to confluence. Representative of two separate experiments (original magnification: 40×, random fields selected).

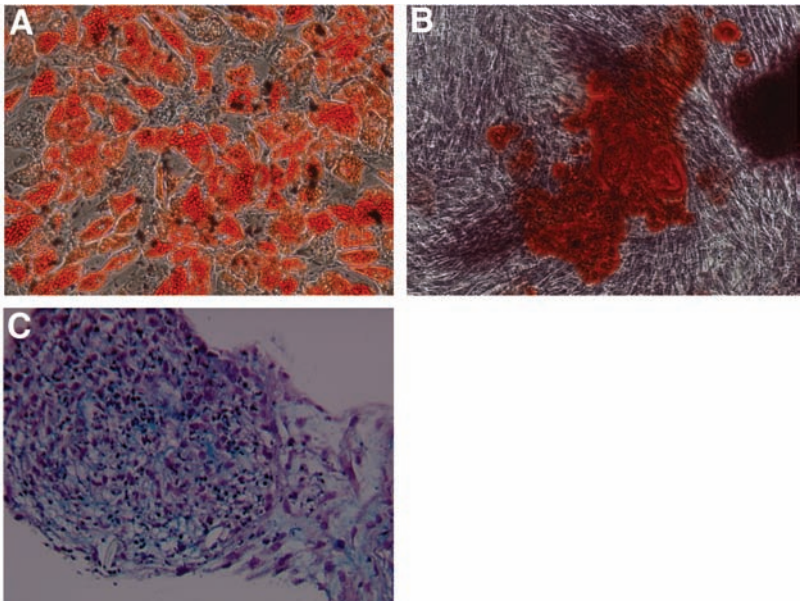

Figure 6. Multilineage differentiation of the Lipogems product in vitro. Adipogenic differentiation (A) was revealed by Oil Red-O staining for neutral lipids (original magnification: 100×). Osteogenic differentiation (B) was evidenced by the formation of mineralized matrix as shown by Alizarin red staining (original magnification: 100×). Chondrogenic differentiation (C) was revealed by immunohistochemical stain for collagen II (original magnification: 40×). Representative of four separate experiments.

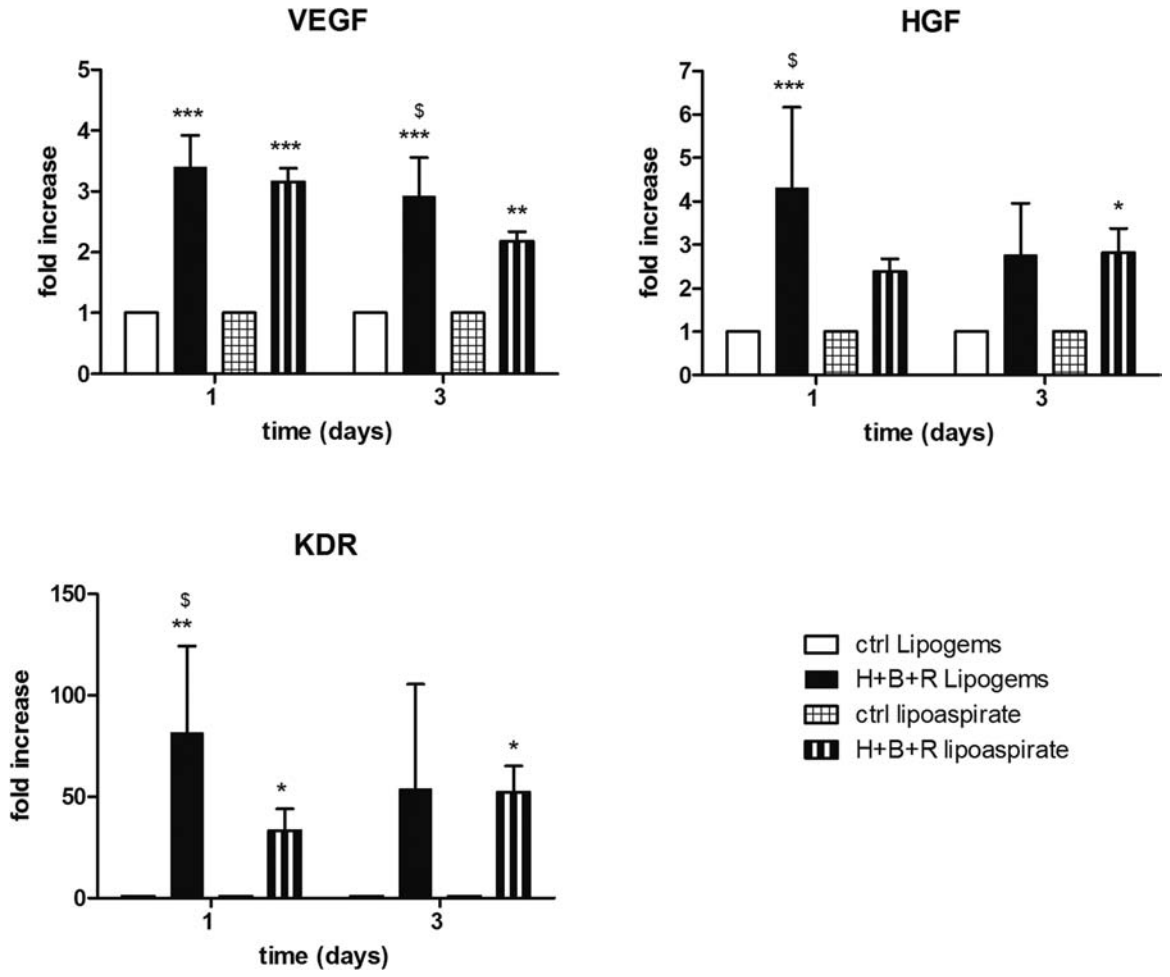

Figure 7. Comparative analyses of gene expression patterning of vasculogenic genes in hMSCs derived from the Lipogems product and enzymatically digested lipoaspirates. At the indicated times, cells were exposed in the absence or presence of a mixture containing hyaluronan (H, 2 mg/ml), butyric (B, 5 mM), and retinoic acids (R, 1 μM). White bars and black bars indicate untreated and mixture exposed Lipogems-derived human mesenchymal stem cells (hMSCs), respectively. Cross-hatched and black-dashed bars indicate unexposed and mixture-exposed hMSCs obtained from enzymatically digested lipoaspirates, respectively. *,**,***Significantly different from untreated ($p < 0.05$, 0.01, or 0.001, respectively). §Significantly different from enzymatically derived hMSCs. Mean ± SE ($n = 4$). VEGF, vascular endothelial growth factor; HGF, hepatocyte growth factor; KDR, kinase insert domain receptor (VEGF receptor).

donor subject. The overall procedure is very fast and safe; it does not require stem cell expansion or manipulation, and therefore, it is not subjected to the regulatory restrictions imposed by cGMP Guidelines.

Differently from the lipoaspirate that undergoes a widely distributed derangement of the cytoarchitectonics, the Lipogems product exhibited a remarkably preserved vascular/stromal architecture, retaining elements with pericyte/perivascular identity that were evident at a significantly higher yield than in the lipoaspirate. Interestingly, the mechanical procedure executed through the Lipogems device yielded a stem cell population with clearly distinctive traits when compared to the classical lipoaspirate. In particular, our results indicate that the Lipogems product can be considered as a readily transplantable fat tissue derivative essentially comprised with high percentages of mature pericytes and hMSCs, with a low amount of hematopoietic-like elements. Noteworthy, these traits were retained in the Lipogems product harvested from human cadavers and were not altered by cryopreservation. When the Lipogems product was subjected to tissue culture, it yielded a virtually pure population of hMSCs, exhibiting the same features of hMSCs isolated from other sources, including the classical commitment to osteogenic, chondrogenic, and

Table 3. Summary of the Distinctive Features Between the Lipogems Product and the Lipoaspirate

	Lipogems Product	Lipoaspirate
Stromal vascular architecture	Highly preserved	Largely deranged
Pericytes/MSCs	High yield	Comparatively lower yield
Pericyte/MSC harvesting from cadaveric donors	Remarkably affordable	Rare/unaffordable
Chance for allogenic use	High	Low
Cell culture	No enzymatic digestion required	Enzymatic processing required
Cell expansion after cryopreservation	Highly affordable	Unaffordable
Response of expanded cells to vasculogenic molecules	Remarkable	Comparatively lower

adipogenic lineages. Like hMSCs obtained from other sources, Lipogems-derived hASCs retained the ability to express a set of genes, including VEGF, KDR, and HGF, involved in the orchestration of vasculogenesis and proper capillary formation. Interestingly, the transcription of these genes was significantly more enhanced in Lipogems-derived than in enzymatically digested hASCs following cell exposure to a mixture of natural molecules that was previously shown to enhance hASC vasculogenesis in vitro and improve pancreatic islet revascularization and function in vivo by preconditioned hASCs transplanted in diabetic rats (8). The exact mechanism(s) accounting for the higher degree of vasculogenic potential in Lipogems-derived hMSCs remain to be established. However, digestive enzymes are known to degrade cell glycocalyx, a carbohydrate-rich layer lining cell plasma membranes and the vascular endothelium, encompassing several "backbone" molecules, mainly proteoglycans and also glycoproteins. Glycocalyx physiological functions include restricting molecules from reaching the cell surface, enhancing cell–cell adhesion, decreasing cell membrane permeability, controlling extravasation of intracellular colloids and fluids, modulating inflammatory responses by attenuating the binding of cytokines to cell surface receptors, and providing the cell better rheologic characteristics. A cell defective of glycocalyx is a weaker, impaired cell, with much less adhesion properties, and a minor propension to adapt to novel environments and respond to molecules orchestrating complex developmental decisions. It is likely that lipoaspirate processing through the Lipogems device, avoiding the use of collagenase and other enzymes, may have preserved the cell surface environment and glycocalyx composition better than other methods based on enzymatic dissociation. Studies are in progress to comparatively assess the glycocalyx features in Lipogems-derived and enzymatically obtained hASCs.

Differently from the lipoaspirate, the Lipogems product can be stored frozen without losing the ability to release highly functional and viable hMSCs after thawing. This implies the availability of a fat tissue product that can be cryopreserved and banked without the needs of prior manipulation and cell expansion.

Even within a GMP setting requiring stem cell culture and expansion, the Lipogems product has several advantages compared with the standard enzymatic processing of lipoaspirates. In fact, the only action required will simply be a direct transferring of the Lipogems product to the tissue culture environment without any additional step.

Of substantial impact for future developments is the possibility to harvest a highly viable Lipogems product from human cadavers. In fact adipose-derived biomaterials have been shown to be biocompatible and hMSCs have exhibited remarkable tollerogenic cues. Hence, the availability of the human cadaveric Lipogems product may provide future off-the-shelf and large-scale approaches for reconstructive procedures and regenerative medicine.

In conclusion, we have developed a product for autologous use, with remarkable distinctive features compared to fat lipoaspirates (Table 3) that can potentially pave the way for novel strategies and paradigms in the rescue of diseased tissues, due to its minimally manipulated derivation and the chance of transfer into a clinical setting.

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