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Human Adipose CD34$^+$CD90$^+$ Stem Cells and Collagen Scaffold Constructs Grafted In Vivo Fabricate Loose Connective and Adipose Tissues

Giuseppe A. Ferraro,[1] Francesco De Francesco,[1,2] Gianfranco Nicoletti,[1] Francesca Paino,[2] Vincenzo Desiderio,[2] Virginia Tirino,[2*] and Francesco D'Andrea[1]

[1] *Dipartimento di Scienze Ortopediche, Riabilitative, Traumatologiche e Plastico-Ricostruttive, Seconda Università degli Studi di Napoli, Italy*

[2] *Dipartimento di Medicina Sperimentale, Sezione di Istologia ed Embriologia Medica, Tissue Engineering and Regenerative Laboratory (TERM), Seconda Università degli Studi di Napoli, Italy*

ABSTRACT

Stem cell based therapies for the repair and regeneration of various tissues are of great interest for a high number of diseases. Adult stem cells, instead, are more available, abundant and harvested with minimally invasive procedures. In particular, mesenchymal stem cells (MSCs) are multi-potent progenitors, able to differentiate into bone, cartilage, and adipose tissues. Human adult adipose tissue seems to be the most abundant source of MSCs and, due to its easy accessibility; it is able to give a considerable amount of stem cells. In this study, we selected MSCs co-expressing CD34 and CD90 from adipose tissue. This stem cell population displayed higher proliferative capacity than CD34$^-$CD90$^-$ cells and was able to differentiate in vitro into adipocytes (PPARγ$^+$ and adiponectin$^+$) and endothelial cells (CD31$^+$VEGF$^+$Flk1$^+$). In addition, in methylcellulose without VEGF, it formed a vascular network. The aim of this study was to investigate differentiation potential of human adipose CD34$^+$/CD90$^+$ stem cells loaded onto commercial collagen sponges already used in clinical practice (Gingistat) both in vitro and in vivo. The results of this study clearly demonstrate that human adult adipose and loose connective tissues can be obtained in vivo, highlighting that CD34$^+$/CD90 ASCs are extremely useful for regenerative medicine. J. Cell. Biochem. 114: 1039–1049, 2013.

KEY WORDS: HUMAN ADIPOSE STEM CELLS; COLLAGEN SCAFFOLD; LOOSE CONNECTIVE TISSUE; ADIPOSE TISSUE

Recently, the role of stem cells in tissue formation and regenerative medicine attracted considerable attention as a possible source of differentiated cells. In addition, the use of stem cells with biomaterial scaffolds and growth factors represents a suitable strategy for clinical therapies.

Stem cells are undifferentiated cells characterized by their ability to self-renew and to differentiate into multiple lineages [Zech et al., 2011]. In contrast to embryonic stem cells (ESCs), adult stem cells are immune-compatible and there are no ethical issues related to their use [Brignier and Gewirtz, 2010]. Initially, adult stem cells from bone marrow (BM-MSCs) have been studied as an alternative source [Conget and Minguell, 1999; Wang et al., 2011]. These cells can differentiate into adipocytes, chondrocytes, osteoblasts, myoblasts, and neurons, among others [Ferrari et al., 1998; Woodbury et al., 2000; Schilling et al., 2007]. However, the clinical use of BM-MSCs presented several problems, including pain, morbidity, and low cell number upon harvest. Therefore, many researchers investigated alternative sources of MSCs in different tissues such as dental pulp [Graziano et al., 2008], Wharton jelly [Romanov et al., 2003], amniotic fluid [Roubelakis et al., 2011], and adipose tissue [Zuk et al., 2002].

Adipose tissue derived from mesenchyme represents an optimal source of adult stem cells, sharing many characteristics with bone marrow, including extensive proliferation and ability to undergo multi-lineage differentiation [Witkowska-Zimmy and Walenko, 2011]. It contains numerous cells types, including adipocytes, preadipocytes, and vascular endothelial cells. In our previous experiments, using human liposuction or lipectomy, we have

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*Correspondence to: Virginia Tirino, PhD, Section of Histology and Embryology, Faculty of Medicine and Surgery, Department of Experimental Medicine, 5 via L. Armanni, Naples 80138, Italy. E-mail: virginia.tirino@unina2.it

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identified a stem cell population that expressed MSCs markers, including CD34, CD90, CD29, CD44, and CD105, but they do not express the hematopoietic marker CD45. Cloning studies have shown that this cells have multi-lineage differentiation potential [Halvorsen et al., 2001; Zuk et al., 2001; Erickson et al., 2002]. Moreover, we have demonstrated that $CD34^+/CD90^+$ stem cell population is capable of: (i) differentiating into endothelial cells and forming capillary-like structures in a semisolid medium with or without the use of angiogenic factors; (ii) forming sphere clusters in a serum-free medium supplemented with bFGF and EGF; (iii) showing higher telomerase activity than differentiated cells; and (iv) displaying a side population phenotype [De Francesco et al., 2009]. The combination of these properties, and the large quantity of cells that can be obtained from fat, suggest that this tissue will be a useful tool in biotechnology and regenerative medicine.

Currently, plastic surgeon uses autologous tissue grafts, including dermal and fat grafting, free and pedicle flap or synthetic implants for the reconstruction and/or augmentation of soft tissue [Rosson et al., 2010; Ferraro et al., 2011]. Various levels of clinical success have been reported with the use of autologous soft tissue. However, donor-site morbidity remains the principal liability. In addition, autologous tissue grafts have a tendency to lose volume with an average of 40–60% reduction in tissue volume gradually. Engineered soft tissue from stem cells has the potential to overtake most of lack associated with autologous soft-tissue grafts [Stosich and Mao, 2005]. Biodegradable scaffolds should ideally provide the initial required blood flow, and then gradually degrade to leave a graft composed only of new tissue. Researchers have developed several studies of adipose tissue engineering by using BM-MSCs and ASCs. Various scaffold materials have been analyzed both in vitro and in vivo, using synthetic or natural prefabricated scaffolds such as poly(lactic-*co*-glycolic acid) [Morgan et al., 2009], poly(ethylene glycol)-based hydrogel [Stosich and Mao, 2007], silk fibroin scaffold [Mauney et al., 2007], hyaluronic acid [Hemmrich et al., 2008], and collagen [Von Heimburg et al., 2001, 2003], but, up to now, obtained data are still not suitable for clinical application.

The ideal scaffold must have certain characteristics: (i) easy to shape it in the tissue defect; (ii) good capacity of growing and maturating stem cells; and (iii) reabsorption time compatible with the time required for tissue formation.

Type I collagen, the main fibre of the extra-cellular matrix in connective tissues, has shown a high affinity for the living organism and has also been extensively used as scaffold for adipose tissue engineering [Rubin et al., 2007; Itoi et al., 2010].

Therefore, the aim of this study was to evaluate the distribution in vitro of cultured $CD34^+CD90^+$ ASCs into commercial solid collagen sponges, and study the differentiation potential in vivo of the grafted ASCs loaded on these scaffolds.

MATERIALS AND METHODS

IN VITRO TISSUE CULTURE

Subcutaneous adipose tissue from abdomen and mammary region was obtained with informed consent, approved by our Internal Ethical Committee (Second University Ethical Committee) from 52 female donors aged 18–43 years (mean years 30.5) that had endured elective procedures for plastic surgery. Adipose tissue was obtained by lipectomy or liposuction in the Plastic and Reconstructive Surgery Clinic of the Second University of Naples. The adipose tissue was placed in a physiological solution (0.9% NaCl), washed twice in PBS (phosphate saline buffer: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4), scraped, and placed in a digestion solution: collagenase type I (3 mg/ml) and dispase (4 mg/ml) in PBS at 37°C in agitation for 60 min. The digestive solution was filtered through 70 μm filters (Becton & Dickinson, Sunnyvale, CA).

After filtration and washing, the pellet was re-suspended in erythrocyte lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.3) for 10 min at room temperature. The cell suspension was centrifuged at 1,300 rpm for 7 min and the pellet re-suspended in 5 ml Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin and seeded in 25 cm^2 flasks. Flasks were incubated at 37°C under 5% CO_2 and the medium changed twice a week. Cells reached confluence in 5–7 days. Experiments were performed in quadruplicate.

Cells were detached using trypsin-EDTA (200 mg/L EDTA, 500 mg/L trypsin; Cambrex). At least 200,000 cells were incubated with primary antibody for 30 min at 4°C, washed twice in PBS, and incubated with a secondary antibody. Alternatively, cells were incubated directly with fluorescent-conjugated antibodies for 30 min at 4°C, washed, and re-suspended in 0.6 ml PBS. Samples were analyzed at Day 0 (day of surgery), Days 7, 15, and 30 by flow cytometry using a FACS Aria II (Becton & Dickinson, Mountain View, CA). The antibodies used in this study were: anti-CD117 PE (c-kit; Miltenyi–Biotech, Calderara di Reno, Bologna, Italy); anti-CD34 FITC and PE (Miltenyi–Biotech); anti-CD90 FITC (BD Pharmingen, Buccinasco, Milano, Italy); anti-CD105 FITC (Santa Cruz, CA); anti-CD29 PE (Miltenyi–Biotech); anti-CD31 FITC (Miltenyi–Biotech); anti-CD133 PE (Miltenyi-Biotech); anti-hVEGF (Santa Cruz); anti-VEGFR-2 (Santa Cruz); anti-CD54 PE (Miltenyi-Biotech); anti-CD44 FITC (Miltenyi-Biotech), anti-CD45 Cy and PE (BD Pharmingen); and anti-CD14 PE (Miltenyi-Biotech). $CD34^+/CD90^+$ cells were sorted for experiments. The purity of sorted populations was routinely of 90%.

GROWTH CURVE

$CD34^+CD90^+$, $CD34^-CD90^-$, and unsorted cells were plated at a density of 8.0×10^4 cells/well in 6-well plates. Every 12 h, cells were harvested and re-suspended in PBS for 7 days. An aliquot of cell suspension was counted under a microscope at 20× magnification. The number of viable cells for each experimental condition was counted and represented on a linear graph. The doubling time (DT) was determined from the growth curves or by using the formula:

$$DT = \frac{(t - t_0)\log 2}{\log N - \log N_0}$$

where t and t_0 were the times at which the cells were counted, and N and N_0 were the cell numbers at times t and t_0, respectively.

IN VITRO ADIPOGENIC DIFFERENTIATION OF ASCs

Cells were induced in the following adipogenic medium for 2–3 weeks: DMEM supplemented with 10% FBS plus dexamethasone

(1 μM; Sigma), human recombinant insulin (10 μM; Sigma), indomethacin (200 μM; Fluka), and 3-isobutyl-1-methyl-xantine (IBMX; 0.5 mM; Sigma). Cells cultured in basal medium (see above) were used as controls.

IN VITRO ANGIOGENIC DIFFERENTIATION OF ASCs

To analyze in vitro capillary-like morphology, 2×10^5–5×10^5 cells/ml were plated in 24-well plates in a semisolid growth medium that consisted of 0.9% methylcellulose in DMEM, 30% FBS, 1% bovine serum albumin (BSA), 10^{-4} mol/L mercaptoethanol, and 2 mmol/L L-glutammine. In parallel experiments, cultures were stimulated in addition with vascular endothelial growth factor (VEGF, 50 ng/ml). All cultures were performed in triplicate, incubated at 37°C under 5% CO_2 and left for 7 days to develop a capillary-like morphology.

PREPARATION OF COLLAGEN SPONGES FOR IN VITRO ANALYSES AND IN VIVO TRANSPLANTATION

After trypsinization with 0.05% trypsin–EDTA, either unsorted ASCs or ASCs $CD34^+/CD90^+$, at a density of 2×10^5 cells/injection were seeded onto collagen sponge of 10 mm × 10 mm size and cultured in DMEM at 10% FBS. Gingistat (GabaVebas, San Giuliano Milanese, Italy) used in this study, is a commercial type 1 collagen, made of native lyophilized collagen from animal origin (equine) with interconnected pores with an average size of 300 μm. Collagen sponges were cut in 10 mm × 10 mm squared pieces and then sterilized under UV exposure. These constructs were incubated for 48 h at 37°C to allow the cells to attach to the scaffolds. For in vitro examinations of cell adherence and proliferation on carriers, sponges were analyses after 7 and 14 days, and histologically evaluated. Cell viability was routinely checked by calcein AM at the concentration of 250 μM in culture medium for 30 min at 37°C. This dye passes through the cell membrane of viable cells. After calcein permeates into the cytoplasm, it is hydrolyzed by esterases to calcein, which remains inside the cell. The excitation and emission wavelengths of calcein were of 490 and 515 nm, respectively. For in vivo studies, the construct was left undisturbed in DMEM in the incubator for 48 h until implantation.

IN VIVO EXPERIMENTAL MODEL FOR GRAFTING

For in vivo experiments, we have used ASCs of subcutaneous adipose tissue from abdomen and breast of two healthy female donors (mean years 30) with a normal range of BMI. We have previously obtained informed consent, approved by our Internal Ethical Committee (Second University Ethical Committee).

Twenty-four hours after seeding of the cells to the scaffold, unsorted ASCs and $CD34^+/CD90^+$ ASCs (at a density of 2×10^5 cells/injection)/sponge matrix constructs were engrafted subcutaneously to the right and left scapular area of 4-week-old immune-compromised athymic nude mice (NU/NU nude mice, Charles River, Ballina, Italy) according to the scheme reported in Table I. Mice were operated under aseptic conditions and inhalation anesthesia. In each of these animals, the cell-loaded sample was transplanted to the right and the control scaffold to the left scapular area through separate incisions. After 4 weeks, the animals were killed by an overdose of gaseous anesthetic and the graft was explanted. The experimental protocols have been evaluated and approved by our Internal Animal Ethic Committee (Second University Animal Ethic Committee). Explanted tissue volume was calculated by the formula $(l \times w^2)/2$. The injection experiments were made in triplicates.

HAEMATOXYLIN & EOSIN AND MALLORY'S TRICHROMIC STAINING

Constructs derived from implantations in mice were fixed with 4% paraformaldehyde for 24 h at 4°C, washed in PBS, incubated in 30% sucrose for 3 days, washed in PBS and then embedded in an optimal cutting temperature (OCT-purchased from Bio-Optica, Milan, Italy). Finally, they were stored at −80°C for Mallory's trichromic and hematoxylin and eosin (H&E) staining. The frozen sections in the OCT were cut into 5 μm thick slices, washed in distiller water for 10 min. For H&E staining, sections were placed in haematoxylin for 5 min. After three washes by distilled water, samples were placed in spring water for 20 min. Then, the samples were placed in eosin acidified with acetic acid for 30 s. Finally, they were placed in alcohol 95°, 75°, and 95° and mounted with DPX. For Mallory's trichromic stain, the sections were stained in acid fuchsine for 1 min, methylene blue-orange G and oxalic acid solution for 25 min, and phosphotungstic acid for 15 min; fibrils of collagen are blue, nuclei red, and cytoplasm is orange. Finally, they were placed in alcohols 95°, 75°, and 95° and mounted with DPX.

IMMUNOFLUORESCENCE AND IMMUNOHISTOCHEMISTRY

Frozen sections in OCT were cut into 5 μm thick slices, washed in distiller water for 10 min. For immunofluorescence, tissue sections were incubated in PBS at 5% milk for 60 min at room temperature. After a double washing in PBS for 10 min at room temperature, cells were incubated overnight at 4°C with monoclonal anti-human antibodies (diluted 1:100 in PBS). Sections were washed in PBS three times for 10 min at room temperature and incubated for 90 min at 4°C with the secondary FITC- or PE-conjugated antibody (diluted 1:200 in PBS; AbCam, Cambridge, UK). Moreover, sections were stained with DAPI (4′,6-diamidino-2-phenylindole; Invitrogen, San

TABLE I. In Vivo Construct Implantation Protocol

	Right side	Left side	Scaffold size	Cell number
Nude mouse 1	Unsorted ASCs/collagen	ASCs $CD34^+CD90^+$/collagen	10 mm × 10 mm	2×10^5 cells
Nude mouse 2	Unsorted ASCs/collagen	ASCs $CD34^+CD90^+$/collagen	10 mm × 10 mm	2×10^5 cells
Nude mouse 3	Unsorted ASCs/collagen	ASCs $CD34^+CD90^+$/collagen	10 mm × 10 mm	2×10^5 cells
Nude mouse 4	Unsorted ASCs/collagen	ASCs $CD34^+CD90^+$/collagen	10 mm × 10 mm	2×10^5 cells
Nude mouse 5	Unsorted ASCs/collagen	ASCs $CD34^+CD90^+$/collagen	10 mm × 10 mm	2×10^5 cells
Nude mouse 6	Unsorted ASCs	ASCs $CD34^+CD90^+$	0	2×10^5 cells
Nude mouse 7	Unsorted ASCs	ASCs $CD34^+CD90^+$	0	2×10^5 cells
Nude mouse 8	Collagen	Collagen	10 mm × 10 mm	0
Nude mouse 9	Collagen	Collagen	10 mm × 10 mm	0

Giuliano Milanese, Milan, Italy) diluted 1:10,000 (5 μg/ml) in PBS for 7 min at room temperature. Tissue sections incubated for 90 min at 4°C only with conjugated secondary antibodies were used as negative control. The sections were then observed under a fluorescence microscope (Nikon Instruments Italia, Calenzano, Firenze, Italy). Immunohistochemical analyses were performed with a DAKO CYTOMATION kit (En Vision + System-HRP-AEC, Dako Italia, Milan, Italy). Antibodies used were: I class HLA (AbCam), anti-adiponectin (AbCam), Glut4 (AbCam), PPARγ (AbCam), collagen type I (AbCam), and VEGF (AbCam).

RNA ISOLATION AND POLYMERASE CHAIN REACTION

Frozen tissues were homogenized in TRIzol® lysis buffer using a handheld homogenizer for at least 45 s. Then, the samples were centrifuged at 12,000*g* for 10 min at 4°C. The supernatant was processed for the phase separation in according with the manufacturer's instructions. RNA concentration and purity were determined by A_{260} and A_{260}/A_{280} ratios, respectively. The integrity of total RNA was assessed on standard 1% agarose/formaldehyde gels. The RNA samples were treated with DNase I to remove residual traces of DNA. cDNA was obtained from 1 μg of total RNA, using reverse transcriptase (Promega Italia srl, Milan, Italy) and random primers (Promega) in a final volume of 20 μl. cDNAs (1 μl for each sample) were amplified by PCR using the primer sequences as follows:

GADPH: fw5′-AGCCGCATCTTCTTTTGCGTC-3′, rw 5′-TCATATTTGGCAGGTTTTTCT-3′;
humanadiponectin: fw 5′-CAACATTCCTGGGCTGTACT-3′, rw 5′-CCTGTGAAGGTGGAGTCATT-3′;
human PPAR-γ: fw 5′-ACAGCAAACCCCTATTCCATGC-3′, rw: 5′-ATTACGGAGAGATCCACGGAGC-3′;
humanVEGF: fw5′-TGACAGGGAAGAGGAGGAGA-3′, rw5′-CGTCTGACCTGGGGTAGAGA-3′;
humanfibronectin: fw5'-CTGGGATGCTCCTGCTGTCAC-3', rw 5'-CTGTTTGATCTGGACCTGCAG-3';
human collagen type I: fw 5′-TACCATGACCGAGACGTGTG-3′, rw 5′-ATAAGACAGCTGGGGAGCAA-3′.

The RT-PCR products were separated by 2% agarose gel electrophoresis, stained with ethidium bromide, and photographed under UV illumination.

STATISTICAL ANALYSIS

Student's *t*-test (two-tailed) was used for statistical evaluation. Level of significance was set at $P < 0.05$.

RESULTS

IN VITRO EXPERIMENTS

In vitro characterization of ASCs. Adipose tissue was obtained by lipectomy or liposuction. After enzymatic digestion, samples of 1,000,000 cells were collected from cell suspension and cultured. After 3 days of culture, adherent cells acquired fibroblast-like or polygonal shape morphology. Cells were analyzed by flow-cytometry at different times. At Day 0, as previously demonstrated by us [De Francesco et al., 2009], we detected stromal cells co-expressing CD117 (~5%) and CD34 (~7%), mesenchymal stem cells, co-expressing CD29 (β1-integrin; ~72%), CD34 (~25%), CD90

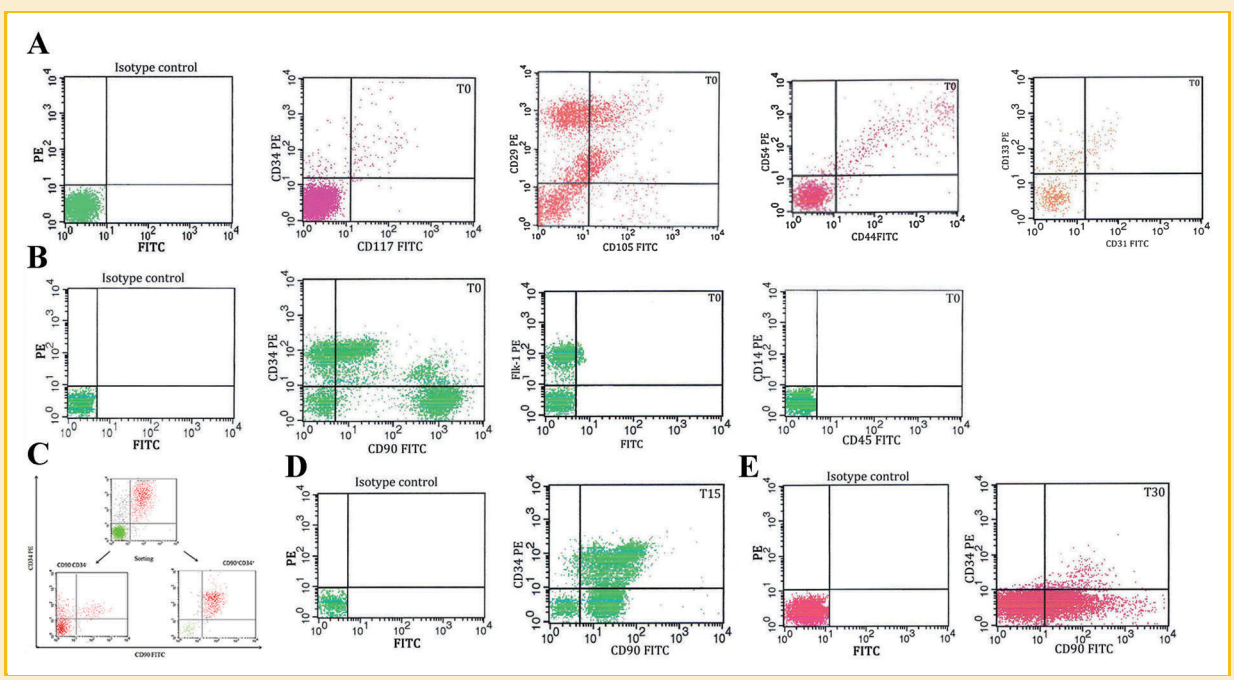

Fig. 1. Representative image of flow cytometric analyses performed at Days 0, 15, and 30 of culture. A,B: At Day 0, a significant percentage of ASCs are clearly positive for stromal markers, including CD117 (5%), CD34 (7%); for mesenchymal markers, including CD29 (72%), CD105 (5%), CD34 (25%), CD90 (30%), and for endothelial markers, including CD31 (6%), CD133 (8%), CD44/CD54 (5%), and Flk-1 (45%). C: Sorting image for $CD34^{+}CD90^{+}$. D: At Day 15 co-expression of CD34 and CD90 markers was 80%. E: After 30 days of culture, co-expression of CD34 and CD90 was 8%, while only CD90 expression was 80%.

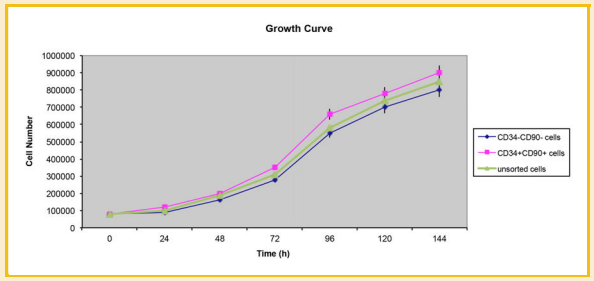

Fig. 2. Representative image showing growth curves of $CD34^+/CD90^+$ cells with respect to $CD34^-/CD90^-$ and unsorted cells displaying a different mean doubling time.

(Thy-1; ~30%), and CD105 (endoglin; ~5%) and endothelial stem cells, co-expressing CD31 (~6%), CD133 (~8%), $CD44^+CD54^+$ (~5%), and Flk-1 (VEGFR-2; ~45%; Fig. 1A,B). Only $CD34^+CD90^+$ cells (~25%) were sorted (Fig. 1C) and cultured for both in vitro and in vivo experiments. Co-expression of CD34 and CD90 markers was ~53% and ~80%, at 7 and 15 days (Fig. 1D), respectively while after 30 days, their expression was ~8%. CD90 expression was 80% (Fig. 1E). $CD34^+CD90^+$ cells exhibited: (i) a higher proliferative capacity than $CD34^+CD90^+$ cells with a mean DT of 72 h with respect to 86 and 100 h of unsorted cells and $CD34^-CD90^-$ cells, respectively (Fig. 2); (ii) in adipogenic medium, some unsorted cells acquired the morphology of fibroblasts, whereas other acquired the morphology of multi-vacuolar adipocytes (Fig. 3A), positive for adiponectin, (Fig. 3B). On the contrary, $CD34^+CD90^+$ cells acquired the typical morphology of adipocytes (Fig. 3C) positive, for adiponectin and PPAR-γ already after 24 h of incubation at the immunohistochemistry (Fig. 3D,E); (iii) in methylcellulose without VEGF, they formed a vascular network (Fig. 3F) while in standard medium, they acquired an endothelial morphology and expressed high levels of endothelial markers such as CD90 (~97%), CD44 (~90%), CD54 (~90%), VEGF (~70%), CD133 (~18%), and Flk-1 (~60%; Fig. 3G).

Cell attachment to the scaffold and in vitro observations. Each scaffold was loaded with 2×10^5 sorted ASCs ($CD34^+CD90^+$), placed in the incubator for 48 h and analyzed afterwards for cell attachment. The range of unattached $CD34^+CD90^+$ cells was approximately 5% in all sponges, as well as for unsorted cells. Adherence and viability of $CD34^+CD90^+$ ASCs to sponges was high, as shown by calcein staining (Fig. 4A,B). In order to evaluate cell proliferation, constructs were analyzed by light-inverted microscopy (Fig. 4C,D) and by histological staining after 7 and 14 days (Fig. 4E,F). Light-inverted microscopy and H&E staining on serial sections of ASCs seeded onto the Gingistat collagen sponge demonstrated that the cells were homogeneously distributed throughout the whole sponge. Moreover, at Day 14, a strong spreading and the proliferation of ASCs was observed with respect to Day 7.

IN VIVO EXPERIMENTS

In vivo adipose tissue and loose connective formation. Unsorted ASCs or ASCs $CD34^+/CD90^+$, at a density of 2×10^5 cells/injection were seeded onto collagen sponge of 10 mm × 10 mm size (Fig. 5A). After 30 days of implantation, we extracted the constructs sorted ASCs/collagen from athymic nude mice (Fig. 5B,C; mean tissue volume $125.94 \pm 1\ mm^3$), unsorted ASCs/collagen (mean tissue volume $119.52 \pm 0.8\ mm^3$; Table II). Collagen without cells used as controls, was not detectable on the dorsum of mice because it has been completely reabsorbed (Fig. 5D) as well as unsorted and sorted ASCs. The grafts with unsorted ASCs showed formation of adipose tissue appearing almost avascular (Fig. 6A). All collagen sponges with ASCs $CD34^+CD90^+$ showed a thin layer of macroscopically yellow tissue and new vessels on the top. This layer was tightly connected to the sponge.

H&E staining showed that, within the sorted ASCs/collagen constructs, stem cells differentiated towards adipocytes (Fig. 6B). Neo-formed adipose tissue was made of fat cells arranged into groups or lobules separated by collagenic septa. Moreover, each individual fat cell was surrounded by a network of loose connective tissue (Fig. 6C). The formation of this connective tissue surrounding the adipose tissue was observed in all histological serial slides. The fibres interlaced themselves loosely to form a network with interstices or spaces between them. The tissue was characterized by cellular, fibrous, and amorphous components. Collagenic fibres predominated and constituted the bulk of the loose connective tissue as shown by Mallory's tri-chromic staining (Fig. 6D). Amorphous material was transparent and homogenous under the optical microscope and occupied numerous small areas (areolas) in which no structures were orderly seen.

Class I HLA positive staining (Fig. 6E) clearly demonstrated the human origin of the adipose tissue obtained excluding any murine contamination. In addition, the tissue showed a strong positivity for adiponectin (Fig. 6F), Glut-4 (Fig. 6G), PPAR-γ (Fig. 6H), collagen type I (Fig. 6I) that are specific markers of both adipose and loose connective tissues. Moreover, the positivity of some areas for the endothelial markers such as VEGF, demonstrated that the tissue was well vascularized: in fact, a rich network of blood capillaries in and among the lobules was evidenced (Fig. 6J).

Expression of adipogenic and loose connective differentiation-associated genes. RT-PCR analyses for adipose differentiation-associated genes in sorted ASCs grown on collagen sponge, after 30 days from implantation, revealed that PPAR-γ and adiponectin expressions were detectable. Moreover, VEGF levels were highly expressed in all the samples, confirming the neo-angiogenesis of the neo-formed adipose tissue. In addition, expression of mRNA for fibronectin and collagen type I showed the generation of loose connective tissue associated to neo-formed adipose tissue (Fig. 7).

DISCUSSION

Regenerative medicine and tissue engineering both harness the potency of human cells to repair, regenerate, and even recreate tissue and organs with the goal of restoring their architecture and functionality. Soft-tissue loss can be caused by trauma, tumor resection, extensive deep burns, as well as congenital or acquired anomalies and it presents an ongoing challenge in plastic and reconstructive surgery. Standard approaches to soft-tissue

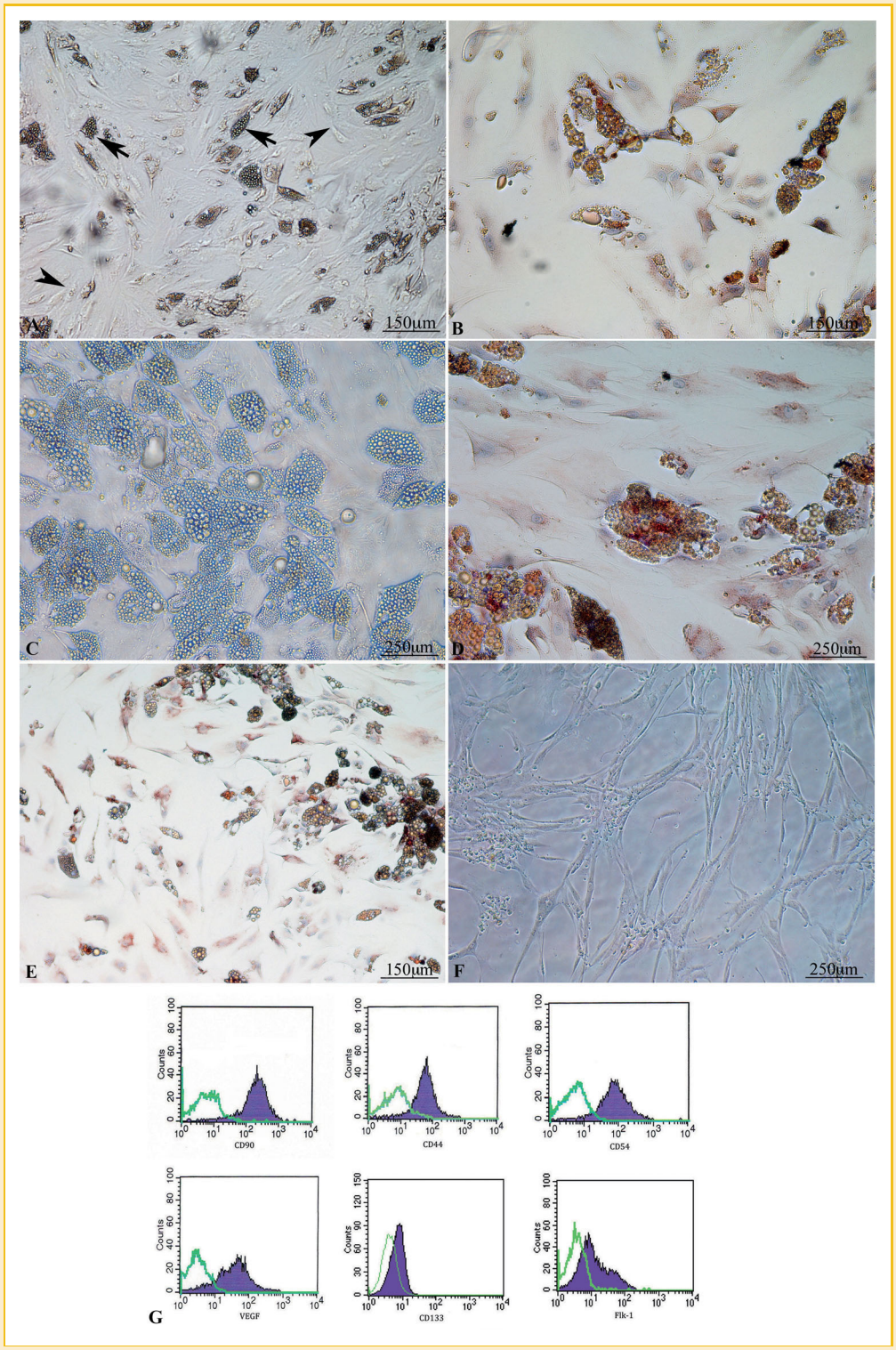

Fig. 3. Image showing (A) unsorted cells in adipogenic medium, exhibiting the morphology of fibroblasts (arrowheads), whereas other cells exhibiting the morphology of multi-vacuolar adipocytes (arrows; Scale bar: 150 μm); (B) some unsorted cells in adipogenic medium, displaying positivity for adiponectin by immunohistochemistry (Scale bar: 150 μm); (C) $CD34^+/CD90^+$ ASCs in adipogenic medium, exhibiting the characterictic adipocyte morphology (Scale bar: 250 μm); (D) $CD34^+/CD90^+$ ASCs in adipogenic medium, displaying positivity for adiponectin by immunohistochemistry (Scale bar: 250 μm); (E) $CD34^+/CD90^+$ ASCs in adipogenic medium, showing positivity for PPAR-γ by immunohistochemistry (Scale bar: 150 μm). (F) Differentiated endothelial cells that, after Day 7 of culture in presence of methylcellulose, formed a rather extended intercellular tube network (Scale bar: 250 μm). G: Flow cytometry demonstrating that a significant number of ASCs are positive for endothelial markers, including CD90 (97%), CD44 (90%), CD54 (90%), VEGF (70%), CD133 (18%), and Flk-1 (60%). Control FITC-conjugated and PE-conjugated isotypes were in green.

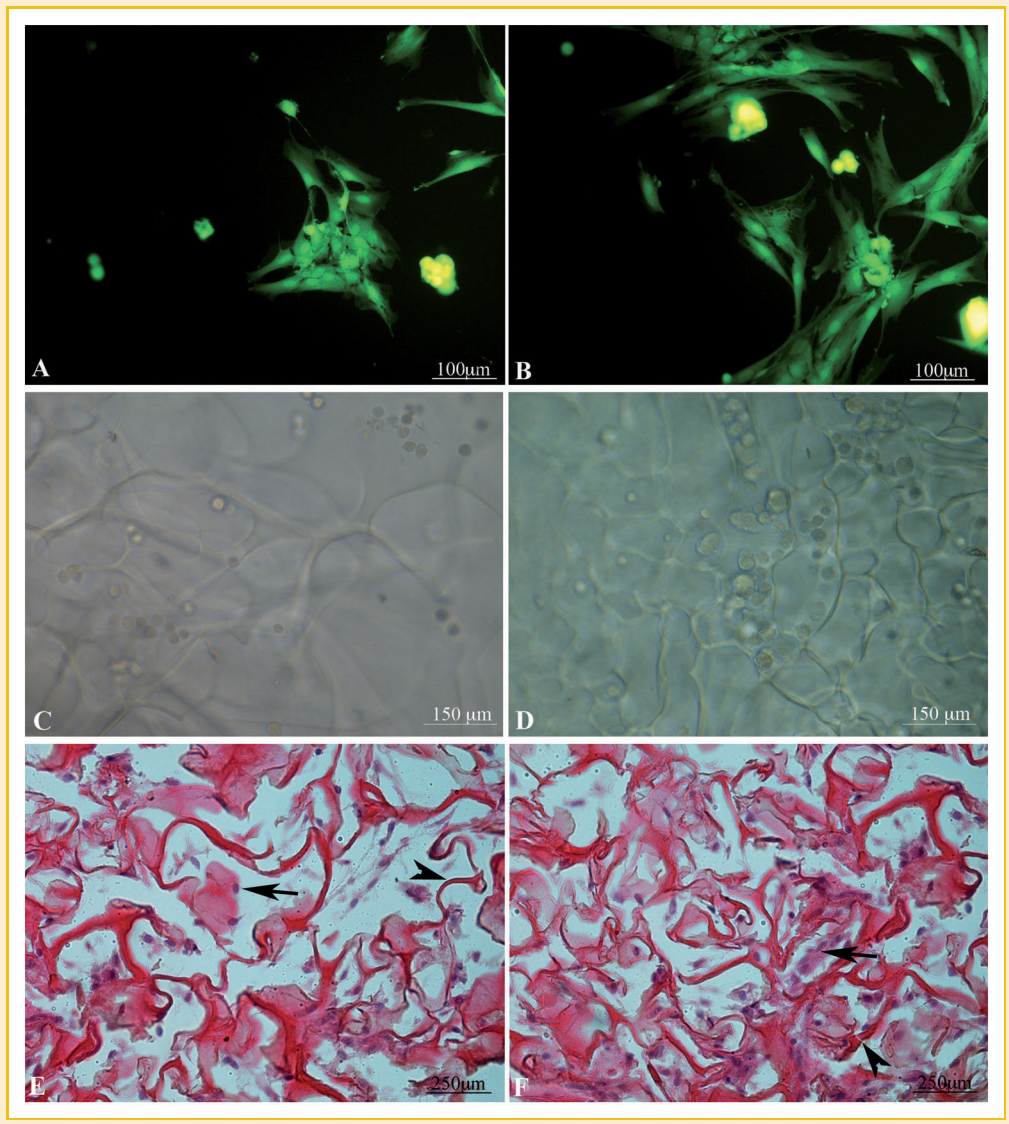

Fig. 4. Figure showing the viability of sorted ASCs in (A) controls (Scale bar: 100 μm) and (B) when loaded onto the scaffold (Scale bar: 100 μm). In both cases calcein staining demonstrates a high viability, also after adherence to the scaffold in (B). In order to evaluate cell proliferation, constructs were analyzed by inverted microscope after 7 (C) and 14 day (D) and by histology after 7 (E) and 14 day (F) (Scale bar: 250 μm). Light-inverted microscopy and H&E staining on serial sections of ASCs seeded onto the Gingistat collagen sponge demonstrated that the cells were homogeneously distributed throughout the whole sponge. Moreover, at Day 14, either a strong spreading or ASCs proliferation was observed with respect to Day 7 (arrowheads indicate the scaffold and the arrows indicate the cells).

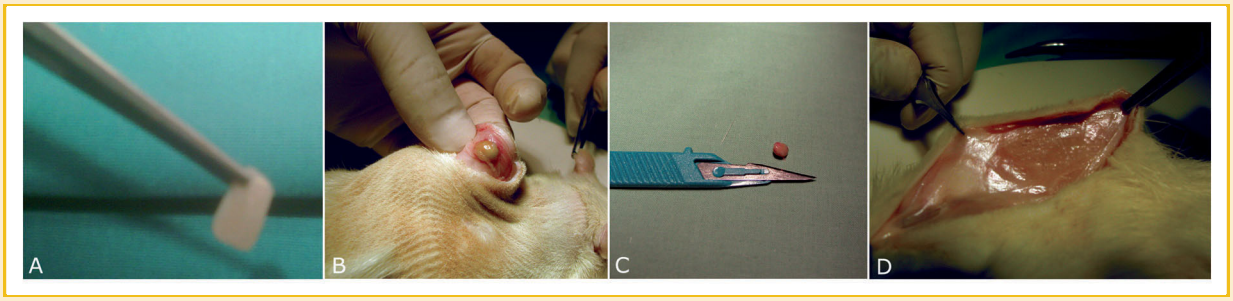

Fig. 5. Figure showing in (A) sorted ASCs/collagen construct before implantation; (B) sorted ASCs/collagen construct injected into nude mice and withdrawn after 30 days of implantation; (C) sorted ASCs/collagen construct explanted from a nude mouse; (D) collagen scaffold, grafted without cells (used as control) which was not detectable on the dorsum of mice, because completely reabsorbed.

TABLE II. The In Vivo Construct Explantation

	Right side	Tissue volume (mm^3)	New tissue formed	Left side	Tissue volume (mm^3)	New tissue formed
Nude mouse 1	Unsorted ASCs/collagen	85.2 ± 1.1	Adipose tissue	ASCs $CD34^+CD90^+$/collagen	152.3 ± 1.4	Adipose and loose connective
Nude mouse 2	Unsorted ASCs/collagen	129 ± 0.8	Adipose tissue	ASCs $CD34^+CD90^+$/collagen	102.5 ± 0.6	Adipose and loose connective
Nude mouse 3	Unsorted ASCs/collagen	135 ± 1.5	Adipose tissue	ASCs $CD34^+CD90^+$/collagen	95.6 ± 1.1	Adipose and loose connective
Nude mouse 4	Unsorted ASCs/collagen	100.8 ± 0.3	Adipose tissue	ASCs $CD34^+CD90^+$/collagen	132.8 ± 0.8	Adipose and loose connective
Nude mouse 5	Unsorted ASCs/collagen	147.6 ± 0.5	Adipose tissue	ASCs $CD34^+CD90^+$/collagen	146.5 ± 1.2	Adipose and loose connective
Nude mouse 6	Unsorted ASCs	Reabsorbed	Reabsorbed	ASCs $CD34^+CD90^+$	Reabsorbed	Reabsorbed
Nude mouse 7	Unsorted ASCs	Reabsorbed	Reabsorbed	ASCs $CD34^+CD90^+$	Reabsorbed	Reabsorbed
Nude mouse 8	Collagen	Reabsorbed	Reabsorbed	Collagen	Reabsorbed	Reabsorbed
Nude mouse 9	Collagen	Reabsorbed	Reabsorbed	Collagen	Reabsorbed	Reabsorbed

reconstruction include autologous tissue flaps, autologous fat transplantation and alloplastic implants [Bucky and Percec, 2008; Megerle and Sauerbier, 2011]. All of these approaches have disadvantages, including donor-site morbidity, implant migration and absorption, and foreign body reaction. ASCs are currently used for the engineering of substitutes that will ultimately be utilized in several fields of reconstructive surgery.

The availability of progenitors within the adipose stromal fraction ($CD34^+/CD90^+$ stem cells) for building neo-tissue types and the understanding of vectors that govern their differentiation emerge as important factors for successful neo-tissue construction. To successfully create a 3D structure capable of supporting tissue formation, several key roles of tissue engineering need to be considered, including pores with interconnected architecture; material degradability; biocompatibility with the seeded cells to enhance cellular activity [Kuberka et al., 2002; O'Brien et al., 2005]. Therefore, the formation of a new tissue in the 3D matrix structure is highly influenced by the chemical composition of the scaffold [Cassell et al., 2001; Liu et al., 2011]. The extracellular matrix (ECM) is recognized as a highly hydrated network that comprises several main effectors. Its architecture provides not only an instructive cue for the conversion and spatial orientation of precursor cells ($CD34^+/CD90^+$ stem cells) into a functional tissue but also functional properties including osmosis, molecular, and nutrient transport. Collagen, being a major protein of the natural ECM, has been commonly used in the form of a gel for tissue engineering in spite of limitations principally related to cell-mediated interactions [Natesan et al., 2010]. It is a physiological component, with a fast biodegrading rate and a low mechanical strength. In fact, many surgeons often add other components to collagen in order to improve its stability [Labbè et al., 2011].

Multiple independent groups have examined the surface immunophenotype of ASCs isolated from human [Gronthos et al., 2001; Mitchell et al., 2006; Yoshimura et al., 2006]. Our previous studies have evidenced that adipose tissue houses $CD34^+CD90^+$ multi-potent stem cells [D'Andrea et al., 2008; De Francesco et al., 2009]. No significant difference between subcutaneous and omental adipose tissue is demonstrated concerning the expression of these markers. There is some controversy in the literature regarding expression of CD34 by ASCs. CD34 is used as marker of hematopoietic stem and vascular endothelial cells. Our data suggest that expression of this molecule is present in cultured ASCs [De Francesco et al., 2009] but, by contrast, Zuk et al. [2002] and De Ugarte et al. [2003], have both reported low-to-absent levels of CD34 expression. In our studies, $CD34^+/CD90^+$ stem cells, within human adipose tissue, are numerous. In vitro, this cells are able to proliferate and differentiate into mesodermal (such as osteogenic, chondrogenic, adipogenic, and myogenic) and non-mesodermal (such as endothelial cells, epithelial cells) lineages, under appropriate stimuli. Moreover, this cells population are capable of differentiating into endothelial cells with or without the use of angiogenic factors, then $CD34^+/CD90^+$ stem cells are a endothelial commitment [Miranville et al., 2004; De Francesco et al., 2009].

On the basis of our in vitro studies of ASCs, tissue regeneration and differentiation experiments using $CD34^+/CD90^+$ stem cells with appropriate scaffold have been performed in vivo. In this study, we describe the in vivo formation of both loose connective and adipose tissues, starting from ASCs co-expressing CD34 and CD90 markers and seeded on a biocompatible commercial scaffold of type I collagen (Gingistat). This lyophilized collagen sponge is widely used as a scaffold for spontaneous bone regeneration, mainly in periodontal diseases. Therefore, its wide use reflects the good histo-compatibility of this material with cells. The results of our study suggest that ASCs/collagen sponge constructs better support in vivo engraftment, as indicated by vascularization after in vivo transplantation. In addition, ASCs/collagen sponge better support formation of both loose connective and adipose tissues, as indicated by higher expression levels of specific markers.

Studies by Von Heimburg et al. [2001] have demonstrated that the transplantation of isolated and cultured preadipocytes seeded on freeze-dried collagen scaffolds resulted in well-vascularized adipose-like tissue. Here, we used $CD34^+/CD90^+$ ASCs demonstrating that these stem cells are easily absorbed and distributed among the whole collagen scaffold. The structural characteristics and architecture of the collagen sponge with interconnected pores sustained and enhanced both cell proliferation and spreading without lose so facility to differentiate. Biodegradable scaffold used in the experiments was designed to meet tissue engineering imitating the in vivo three-dimensional extracellular architecture and to provide to precursors the signals for develop in desirable phenotypes. In the xenograft experiments, analyses of the new-formed tissue through histological staining revealed that the collagen, on which $CD34^+/CD90^+$ ASCs were loaded, induced the adipose differentiation with complete absorption of scaffold and loose connective tissue, suggesting $CD34^+/CD90^+$ ASCs have high affinity for the type I collagen sponge. In addition, we found an

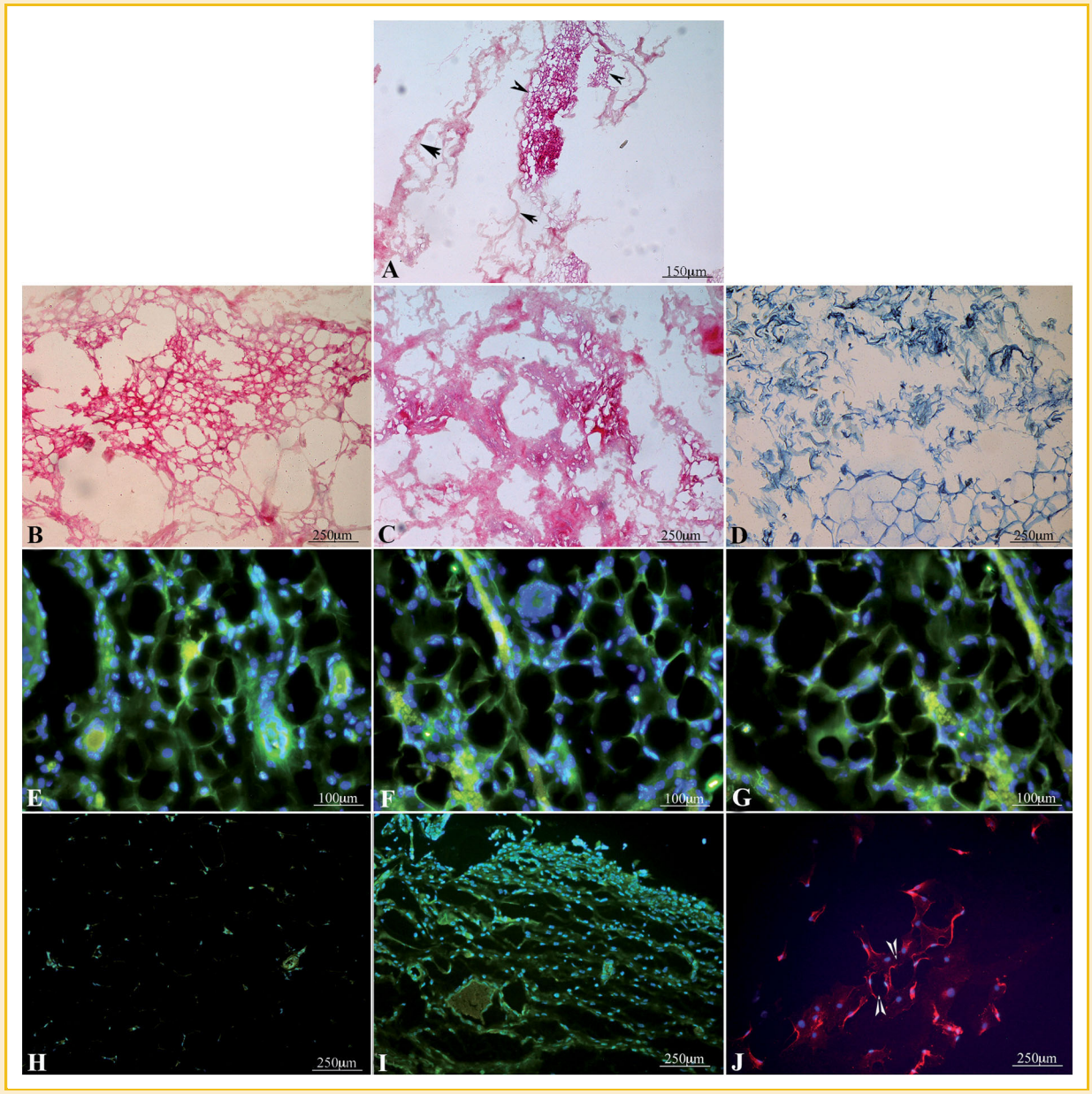

Fig. 6. Figure showing: (A) H&E stain displaying that, within the unsorted ASCs/collagen constructs, stem cells only differentiated towards adipocytes (arrowheads indicate the scaffold and the arrows indicate the adipocytes; Scale bar: 150 μm). B: H&E staining displaying that, within the sorted ASCs/collagen constructs, stem cells differentiated towards adipocytes (Scale bar 250 μm). C: H&E staining shows that fat cell are often surrounded by a network of loose connective tissue (Scale bar 250 μm). D: Collagenous fibres predominated and constituted the bulk of the loose connective tissue (Mallory's Trichrome staining; Scale bar: 250 μm). E: Class I HLA positive staining clearly demonstrated the human origin of the adipose tissue obtained excluding any murine contamination (Scale bar 100 μm). F: Immunofluorescence staining for adiponectin (Scale bar 100 μm). G: Immunofluorescence staining for Glut-4 (Scale bar: 100 μm). H: Immunofluorescence staining for PPAR-γ (Scale bar: 250 μm). I: Immunofluorescence staining for collagen type I (Scale bar 250 μm). J: Immunofluorescence staining for VEGF (arrowheads indicate the capillary-like cells network). (Scale bar: 250 μm).

extensive formation of new, tightly integrated vessels throughout the neo-formed tissue. By the contrast, in xenografts in which non-sorted ASCs were used, only adipose tissue was obtained.

Investigators have postulated a number of mechanisms through which MSCs can be used to repair and regenerate tissues. We may hypothesize that $CD34^{+}CD90^{+}$ ASCs may secrete cytokines and growth factors that stimulate recovery in a paracrine manner. They could modulate the "stem cell niche" of the host by stimulating the recruitment of endogenous stem cells to the site and promoting their differentiation along the required lineage pathway [Gimble et al., 2007]. In a related manner, they might provide anti-oxidants chemicals, free radical scavengers, and chaperone/heat shock proteins at an ischemic site. As a result, toxic substances released into the local environment would be removed, thereby promoting recovery of the surviving cells. Exciting studies have suggested that transplanted bone marrow-derived MSCs can deliver new mitochondria to damaged cells, thereby rescuing aerobic metabolism [Spees et al., 2006]. It may develop that similar studies in ASCs will

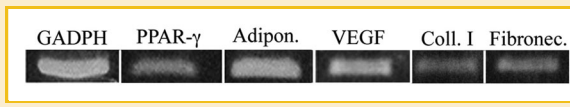

Fig. 7. RT-PCR analyses. Figure showing adipose differentiation-associated genes in sorted ASCs grown on collagen sponge, after 30 days from implantation. Analyses revealed that PPAR-γ and adiponectin expressions as well as VEGF were highly expressed, confirming the adipose tissue formation with neo-angiogenesis.

uncover a comparable ability to contribute mitochondria. Therefore many and different mechanisms highlight the great potentiality of $CD34^+CD90^+$ ASCs versus unsorted ASCs in transplantations.

Shape and dimensions are of critical importance to plastic surgery applications of engineered soft tissue. Creation and maintenance of the shape and dimensions of the engineered soft tissue are perhaps best performance by scaffold biomaterials. Collagen scaffold is biodegradable and fully absorbable when transplanted subcutaneously. Moreover, for effective MSC seeding and proliferation needed to generate new tissue, the scaffold must be porous and three-dimensional. The structure of collagen sponge used in this study confirmed their porous 3D architecture. Pores of the collagen matrix have an average size of 300 μm. In this study, analysis of $CD34^+$/$CD90^+$ cells by light-inverted microscopy confirmed their satisfactory seeding, proliferation and differentiation within pores.

This is the first time that adipose and loose connective tissues have been obtained, and that a complete physiological and functional reconstruction of tissues has been generated reinforcing the hypothesis that $CD34^+/CD90^+$ ASCs could be used in regenerative medicine. Moreover, the use of the collagen as scaffold with $CD34^+$/$CD90^+$ ASCs is advantageous for different motivations (i) commercial available; (ii) easily obtainable; (iii) avoid donor morbidity; and (iv) easily formed into the desired shape and volume. For the repair of tissue defects, especially after cancer ablation, typically it is essential to restore both the volume and shape. Therefore, to correct such defects is crucial to recreate a three-dimensional multi-layer especially for the tissue shape. In an in vivo study of engineering adipose tissue using MSC from human bone marrow, Stosich and Mao [2007] have used a poly(ethylene glycol) scaffold to maintain a stable shape; instead Lin et al. [2008] have used non-biodegradable polyglycolic acid scaffold to keep the predefined shape. Other researchers have used natural scaffolds such as fibrin [Mauney et al., 2007] and gelatin sponges [Kimura et al., 2003]. The main drawback of these scaffolds is the fast resorption rate that does not allow the achievement of an adequate volume so as not allow the organization of an appropriate shape.

The novelty of this research is represented by the formation not only of adipose tissue starting from ASCs, differently from Von Heimburg et al. [2001] who used preadipocytes, but also by the formation of loose connective tissue, as shown in this study using E&E and Mallory's trichromic stainings, RT-PCR and immunofluorescence analyses for adiponectin, PPAR-γ, Glut-4, fibronectin, and collagen I. In particular, we have reconstructed both connective and adipose tissues from cultured human ASCs differentiating into adypocytes, endoteliocytes, and connective cells, able to secrete cellular matrix components, including collagen fibres, fibronectin, and amorphous substance.

In our study, we used a biodegradable and bio-absorbable collagen matrix, so that both the volume and the shape of the defect can be reconstructed using a predefined scaffold at the outset. The slow biodegradation of the scaffold allows maintenance of the scaffold volume of adipose tissue newly generated by SVF.

On the basis of both in vitro and in vivo experiments and on the basis of preclinical studies available in the literature [Mizuno, 2009], ASCs/collagen construct could be applied to various clinical fields, such as treatment of chronic ulcers, cancer ablation, post-traumatic calvarial bone defect, but also could be an alternative to soft tissue augmentation surgery including cosmetic breast augmentation.

In conclusion, with this study we provided evidence that ASCs differentiate in vivo into both adipose and loose connective tissues, when loaded on collagen scaffold. This demonstrates the feasibility of collagen scaffold with sorted ASCs ($CD34^+/CD90^+$) as transplantation vehicle for adipose and loose connective tissue engineering and its employment for reconstruction in plastic and reconstructive surgery. Collagen scaffolds were demonstrated to be suitable materials for soft-tissue regeneration; they maintained volume when seeded with sorted ASCs. The material had a progressive rate of biodegradation, lacked cytotoxicity and did not induce a systemic immune response or chronic inflammation in this human in vivo model. The observed tissue integration is promising and in addition of this the angiogenic formation suggest that collagen scaffold would be an efficient biomaterial for adipose and loose connective growth and function.

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