

# Human adipose-derived stem cells promote vascularization of collagen-based scaffolds transplanted into nude mice

**Aim:** After *in vivo* implantation of cell-loaded devices, only the cells close to the capillaries can obtain nutrients to maintain their functions. It is known that factors secreted by stem cells, rather than stem cells themselves, are fundamental to guarantee new vascularization in the area of implant. **Materials & methods:** To investigate this possibility, we have grafted mice with Bilayer and Flowable Integra® scaffolds, loaded or not with human adipose-derived stem cells. **Results:** Our results support the therapeutic potential of human adipose-derived stem cells to induce new vascular networks of engineered organs and tissues. **Conclusion:** This finding suggests that our approach can help to form new vascular networks that allow sufficient vascularization of engineered organs and tissues in cases of difficult wound healing due to ischemic conditions.

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**Keywords:** human adipose-derived stem cells • new vascularization • tissue engineering • ulcers • wound healing

It is widely accepted that tissue engineering and regenerative medicine are on the front line as potentially ideal approaches for the treatment of diseases as well as the repair of damaged tissues and organs [1]. Nevertheless, crucial obstacles persist for a wide application of these approaches. For example, after the *in vivo* implantation of cell-loaded devices, only the cells within a distance of 100–200  $\mu\text{m}$  to the nearest capillaries obtain a sufficient supply of oxygen and nutrients to maintain their metabolism and functions, while the cells in the core of the device are destined to die. This is more valid for large scaffolds where cell survival is largely hampered by the limited diffusion of oxygen and nutrients [2]. Supplying neovascularization could partially overcome these problems. New vessel formation can be enhanced by designing instructive materials, in other words, materials capable to impart instructions to cells with the aim of guiding their

fate [3], containing angiogenic growth factors or by the inclusion of endothelial cells [1,4]. In particular, endothelial progenitor cells are good candidates for vascularization in tissue engineering, as they are able to differentiate into mature endothelial cells both *in vitro* and *in vivo* [2,5–6].

The majority of materials and cell types screened so far in tissue engineering approaches have shown limited clinical relevance, due to the insufficient number of obtainable endothelial cells or to the use of animal-derived matrices [3,7]. Ingram *et al.* [8] identified a number and a hierarchy of endothelial progenitor cells in the vessel wall, providing a new framework for classification of cells supporting endopoiesis akin to that previously established for hematopoiesis. Endothelia may be derived from various sources including mesenchymal stem cells, also known as mesenchymal stromal cells (MSCs), a heterogeneous pop-

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ulation of multipotent adult stem cells of mesodermal origin. The principal source of MSCs is the adipose tissue from human adults, which is easily accessible in large quantities with minimal invasive harvesting procedure. A subset of the MSC fraction consists of the so-called adipose-derived stem cells (ASCs) that contains a high amount of stem cells, essential requirement for stem-cell-based therapies and tissue engineering [9]. MSCs have a variety of roles in tissue vascularization, both through direct contact and indirect signaling. Within this frame, it has been postulated that MSCs (and ASCs in particular) are a subset of pericytes or vascular stem/precursor cells at various stages of differentiation, located in the wall surrounding the vasculature [10]. It has also been hypothesized that, in virtually all organs and tissues, blood vessels harbor ubiquitous MSCs in their perivascular niche [11].

Biomaterial-induced auto-regeneration of functional tissues is based on the temporary substitution with an implanted material that will be later replaced by functional tissue [1,12]. During this time, which is characterized by cell growth, differentiation, invasion and angiogenesis, it is fundamental to ensure the presence of factors derived from MSCs rather than the MSCs themselves [13]. These secreted factors, mostly proteins, include numerous enzymes, growth factors, cytokines and hormones or other soluble mediators that are fundamental for regulating cell-to-cell and cell-to-extracellular matrix interactions [14].

To investigate the possibility to induce the angiogenic process, we have grafted athymic CD-1<sup>®</sup> nude male mice with collagen scaffolds (Integra<sup>®</sup> bilayer and Flowable Wound Matrix) loaded or not with human ASCs (hASCs). In clinical setting, the bilayer scaffold is commonly used for the management of ulcers and wounds, including the surgical or chronic wounds and burn reconstruction; instead, the Flowable Wound Matrix is more indicated in the case of a 'tunnel' wound into deep soft tissue characterized by an irregular geometry, when is necessary to fill the wound and achieve a contact with the wound bed, we have found that hASCs can induce potent vascularization of both supercharged scaffolds. This finding lets us to conclude that our approach could be used in situations where wound healing is difficult or even impossible because of ischemic condition.

## Materials & methods

All protocols were reviewed and approved by the 'University of Insubria' and 'Ospedale di Circolo Fondazione Macchi' ethic committees in accordance with the Declaration of Helsinki.

## Patient samples

The adipose tissue were obtained, after the informed consensus, by surgical intervention of female subjects ( $n = 5$ ), due to breast hypertrophy (gigantomastia). Their ages ranged from 21 to 58 years (mean  $\pm$  standard deviation [SD]:  $33.4 \pm 5.3$  years) and their BMI from 18.8 to 27.2 kg/m<sup>2</sup> (mean  $\pm$  SD:  $25.6 \pm 2.6$  kg/m<sup>2</sup>). All donors were in good health, had no history of diabetes or metabolic disorders, and were not receiving medications at the time of surgery. Gigantomastia allowed us to get large amount of adipose tissue from the same depot. Furthermore, the severe selection of the patients, young and healthy women who have not undergone to heavy weight loss diet, guaranteed adipose tissue of good quality rich hASCs which are characterized by low immunogenicity.

## Animals

4-week-old athymic CD1 nude mice (CrI:CD1-Foxn1<sup>tm</sup>086) were obtained from Charles River (Calco, LC, Italy).

The animal studies were conducted at the University of Insubria following international and national rules for animal care, and approved by the local Ethical Committee (Comitato Etico per la Sperimentazione Animale). In total, 25 animals have been used for experimental purposes.

## Materials

Integra bilayer and Flowable Wound Matrix were kindly provided and characterized by LifeSciences Corporation (Plainsboro, NJ, USA). The bilayer scaffold is an advanced wound care device comprised of porous matrix of crosslinked bovine tendon collagen, glycosaminoglycan and a semi-permeable polysiloxane layer. The meshed bilayer allows drainage of wound exudate and provides a flexible adherent covering for the wound surface. The collagen-glycosaminoglycan biodegradable matrix provides a scaffold for cellular invasion and capillary growth [15].

## Scaffold compatibility & cell viability

Integra bilayer was incubated in petri dish supplemented with culture medium (see below) for 24 h at 37°C in order to test its compatibility with hASCs. Petri dish was then observed by an inverted phase-contrast microscope Zeiss Axiovert 200 (Arese, MI, Italy) connected to an AxioCam ICm1 camera (Zeiss); photographs were acquired by the Axiovision Rel 4.8 software (Zeiss).

Cell viability was determined by the RealTime-Glo<sup>™</sup> MT assay (Promega, Milano, Italy) according to the manufacturer's instructions. Briefly, 200  $\mu$ l of hASCs suspension (containing about  $1 \times 10^3$  cells)

were seeded, together with the scaffold and the assay reagent, into 24-well assay plates and cultivated for 6 days at 37°C in 5% of CO<sub>2</sub>. Every 24 h luminescent signal has been recorded using the Infinite F200 plate reader (Tecan Group, Männedorf, Switzerland). The experiments were performed in triplicate and normalized compared with cell viability in absence of the scaffold. For all the experiments we have used hASCs at the passage 5.

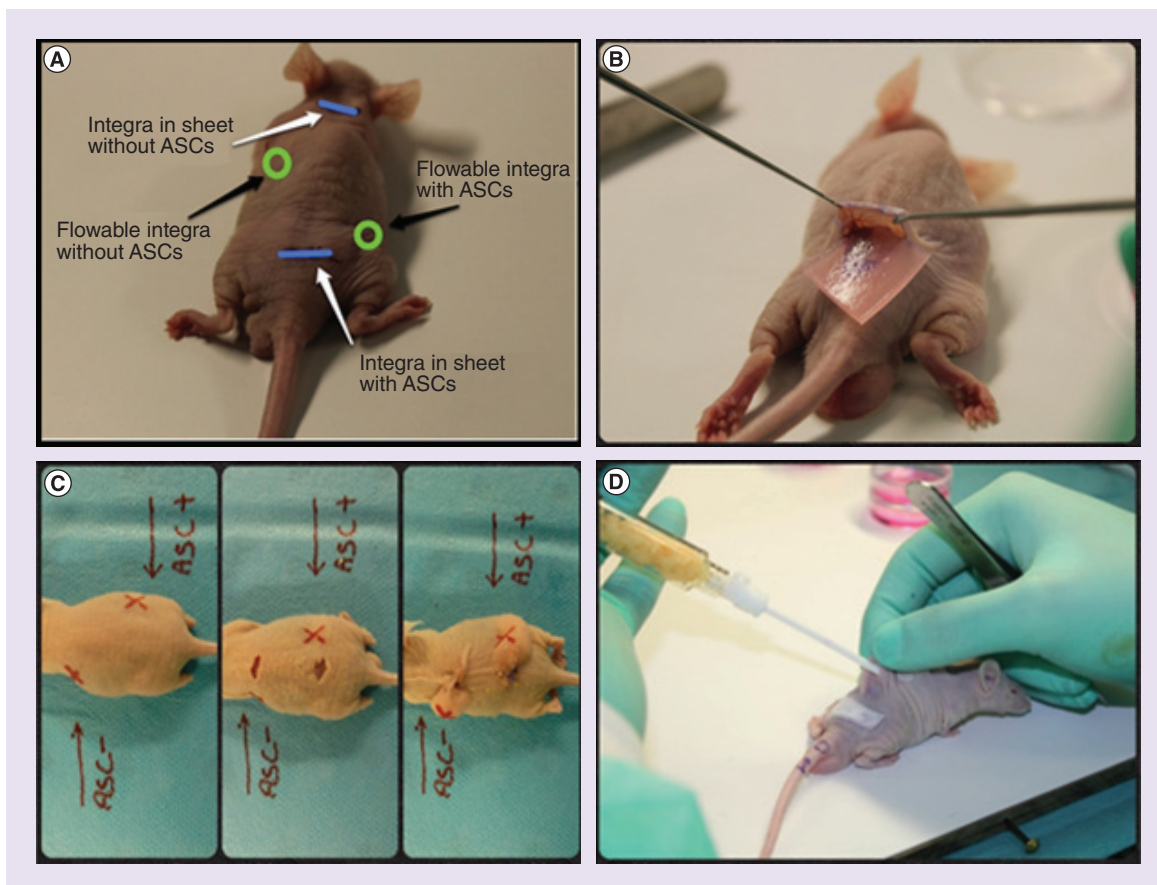
### hASCs isolation & culture

hASCs were isolated from human mammary adipose tissue after surgical intervention due to breast hypertrophy (gigantomastia). The stromal cellular fraction, obtained according to Gronthos and Zannettino protocol [16], was seeded in flask and maintained in 15 ml of complete 1:1 DMEM/DMEM-F12 medium containing 10% fetal bovine serum, 2–4 mM L-Glu, 1% penicillin/streptomycin, 0.1%

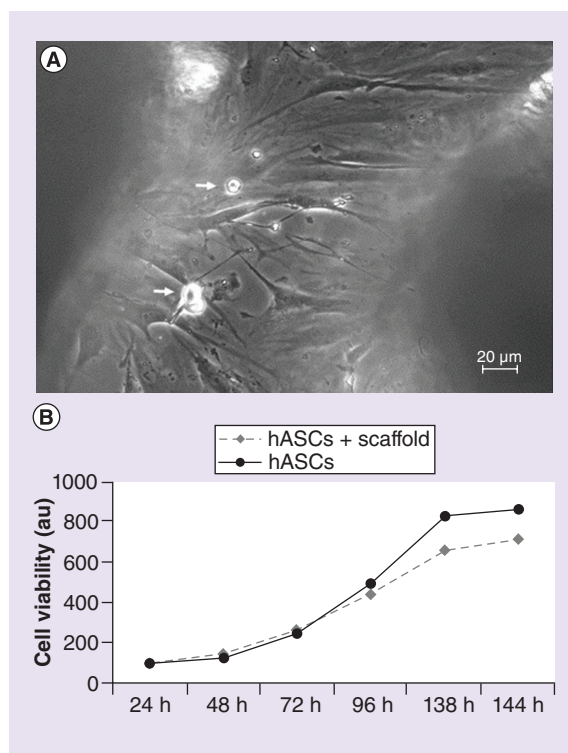
gentamicin and 0.5 mg fungizone. After 6 h, non attached cells were removed and adherent cells were fed with fresh medium.

### hASCs characterization by FACS analysis

Cell surface phenotype was assessed by immunofluorescence and cytofluorimetric analysis using a FACSAria II apparatus (BD, NJ, USA) and a series of monoclonal antibodies (mAb) specific for nominal staminal markers CD44, CD90, CD105, for the differentiation marker CD45 and for major histocompatibility molecules including HLA class I (HLA-A,B,C) and class II (HLA-DR). Anti-CD44, -CD45, -CD105 mAbs were fluorescein isothiocyanate labeled while anti-CD90 was PE labeled. Anti-HLA class I and class II mAbs were unlabeled and revealed by a second-step incubation with fluorescein isothiocyanate-labeled goat antimouse Ig. After antibody staining, cells were washed with PBS at 4°C and



**Figure 1. Xenogenic grafting of bilayer and flowable Integra® scaffolds.** (A) Photograph of the mouse posterior side. White arrows point to the position of Integra bilayer scaffold injections with (bottom) or without (upper) ASCs; black arrows point to the position of flowable Integra scaffold injection with (right) or without (left) ASCs. (B) Grafting of 1 × 1 cm<sup>2</sup> Integra bilayer scaffold in the lower back side of the mouse used for this experiment. (C) Position tracks and mice after injection of flowable Integra scaffolds. (D) Grafting of the flowable Integra scaffold in the left dorsum of the mice used for this experiment. ASC: Adipose-derived stem cell.



**Figure 2. Compatibility test of the scaffold.** (A) Picture, acquired by a camera connected to an inverted phase-contrast microscope, shows the presence of living cells seeded onto the Integra® bilayer and maintained in culture for 24 h. Arrows indicate cells in mitosis. (B) Cell viability of hASCs cultured in presence or not of Integra for 144 h. au: Arbitrary unit; hASC: Human adipose-derived stem cell.

fixed with 150 µl of PBS 4% paraformaldehyde for 30 min before cytofluorimetric analysis.

### hASCs differentiation

#### Xenogenic grafting

Integra bilayer scaffold (1 × 1 cm<sup>2</sup>) was left in culture medium for 24 h at 37°C and 5% CO<sub>2</sub>. Then, 3 × 10<sup>5</sup> ASCs were seeded onto the scaffold and maintained for 24 h before the grafting into the mice, pretreated with intraperitoneal injection of 60 mg/kg of ketamine and 5 mg/kg of midazolam. The grafting was accomplished by an incision of 1.5 cm in the lower backside of the mouse between muscle and subcutaneous layer. The control scaffold was grafted in the upper backside. The incision was sutured by a 4/0 polyamide monofilament nonabsorbable suture (Figure 1A & B).

The flowable Integra was grafted between muscle and subcutaneous layer into the left dorsum of mice by a syringe with a luor-lock connector. The injection system consisted of two separate syringes, one containing 1 ml of flowable Integra, and one containing 1 ml the ASC suspension (3 × 10<sup>6</sup>); as control, 1 ml of flowable Integra

and 1 ml of culture medium was used. A total volume of 200 µl was injected into the right backside of each animal (Figure 1C & D). The incision was sutured by a 4/0 polyamide monofilament as described above. During the first 3 days after the surgical procedure, a daily subcutaneous injection of carprofen (4 mg/kg) was performed.

### Macroscopic & microscopic scaffold analysis after animal sacrifice

After 30 days from the grafting, mice were sacrificed in a CO<sub>2</sub> chamber. The scaffold and the newly formed tissue specimens were harvested along with the attached skin. After gross observation by circular lens (Canon EOS 550 D) and image capture, the samples were fixed in 10% neutralized formalin solution, dehydrated with ethanol (70, 90, 95, 100%), then embedded in paraffin and sectioned. In order to analyze the newly formed tissue, some slices were stained with H&E solution following classical procedures. Induction of vascularization was evaluated by counting the number of capillaries present in the microscope field of three H&E stained tissue samples for each injected animal.

The presence of hASC was investigated by immunohistochemistry using MAB 1287, against a human small nuclear ribonucleoprotein, and MAB 1273, an antihuman mitochondrial antigen. Briefly, sections were immersed twice in xylene, rehydrated with a series of ethanol (95, 90, 70, 50, 30%), washed with distilled water, then the endogenous peroxidase activity was blocked with H<sub>2</sub>O<sub>2</sub> (Bio-Optica, Milano, Italy). The antigen retrieval was performed with 0.05% trypsin in a wet chamber at 37°C and 15 min. The sections were incubated, at room temperature, with 100 µl of 1:200 diluted primary antibody (MAB 1273 or MAB 1287) in a wet chamber for 1 h, rinsed twice, incubated with 100 µl of appropriately diluted biotinylated secondary antibody for 30 min, rinsed twice, and incubated with 100 µl appropriately diluted HRP Streptavidin (Bio-Optica) for 30 min. After washing, the color was revealed by incubation with freshly made DAB solution (Bio-Optica). Slides were counterstained with 30% hematoxylin to visualize nuclei for 4 min, washed with distilled water and dehydrated with ethanol (70, 90, 95, 100%), placed in xylene and mounted using Eukitt (Bio-Optica). The sections were then observed by an optic microscope Aristoplan (Leica Microsystems, Milano, Italy) equipped with a Nikon Coolpix 5400 camera.

### Scanning electron microscopy analysis

Samples were fixed in 1% Karnovsky solution in 0.1 M sodium cacodylate buffer (pH 7.4) for 6 h at 4°C and preserved in the same buffer. The specimens were dehydrated in graded ethanol and dried in hexamethyldisilazane (Sigma-Aldrich, Milano, Italy),

mounted on aluminium stubs, then covered with 8 nm of gold (Emitech K550) and observed with a Philips SEM-FEG XL-30 (Eindhoven, The Netherlands). The scaffold and the newly formed tissue specimens were harvested along with the attached skin.

### RNA extraction, retrotranscription & real-time PCR

Total RNA was isolated using the ReliaPrep™ FFPE (Promega) according to the protocol. The extracted RNA was quantified by the QuantiFluor® RNA System (Promega) and then retrotranscribed by the iScript™ cDNA Synthesis Kit (Bio-Rad, Segrate, Italy) according to the manufacturer's instructions. The generated cDNA was stored at -20°C.

The quantitative real-time PCR was performed by iTaq™ Universal SYBR® Green Supermix (Bio-Rad) technology using CFX Connect™ Real-Time PCR Detection System apparatus (Bio-Rad). Specific primers were designed by Beacon Designer Program (Bio-Rad). Mouse *β-Actin* was used as endogenous control gene whereas *Cd105*, *Cd31* and *Cd34* as angiogenic markers (Table 1).

PCR reaction contained 5 µl of SYBR Green Supermix (2×), 0.5 µl of primers mix (6 µM), 75 ng of cDNA and nuclease-free water to a total volume of 10 µl.

Thermocycler program included an initial hot start cycle at 95°C for 3 min followed by 50 amplification cycles consisting of a denaturation step at 95°C for 10 s and an extension phase at 60°C for 30 s. The temperature was raised from 65°C to 95°C, at 0.5°C/5 s, for Melt-curve analysis. For all samples, reactions were performed in triplicate.

Comparative cycle threshold ( $\Delta C_t$ ) method was used to normalize gene-expression levels among control and target genes. Results are expressed as mean  $\pm$  SD.

## Results

### Scaffold characterization & biocompatibility

Bilayer and flowable Integra have been made by Life-Sciences Corporation. Information about the structure and porosity characteristics are available at Integra web site [15]. As shown in Figure 2, the hASCs seeded on to the scaffold can survive and proliferate.

### hASC characterization

To assess the phenotype of *in vitro* isolated ASCs, morphology and cell surface markers were investigated. By phase contrast microscopy, the cells displayed a spindle-shaped aspect with fibroblast-like morphology (Figure 3, bottom panel). They grew attached to the culture plates and these characteristics were well preserved also after seeding on to the scaffold as highlighted in Figure 2. By immunofluorescence and cytofluorimetric analysis (Figure 3, histograms in the upper panel), ASCs expressed MSC markers such as CD44, CD90 and CD105, but not CD45, a cell surface molecule expressed on endothelium as well as on leukocytes. ASCs expressed HLA class I but not HLA class II (DR) cell surface molecules. Of notice, cell surface expression of CD44, CD90, CD105 and HLA class I markers was a characteristic of the entire ASC population, thus operationally defining the homogeneity of the cells under study.

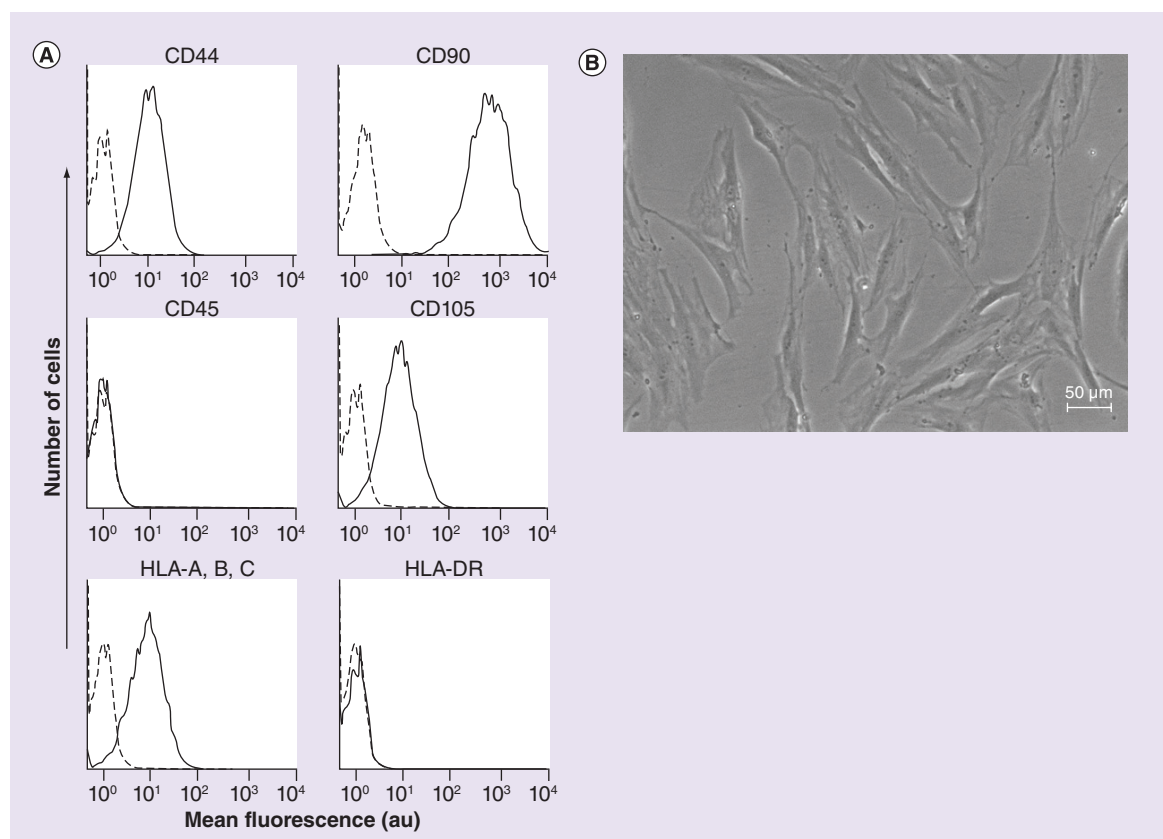
### Macroscopic scaffold analysis after animal sacrifice

Scaffolds injected with hASC were surgically implanted into immunodeficient CD1 mice as described in the 'Materials & Methods' section. 30 days after surgery the animals were sacrificed, the engrafted scaffolds removed and analyzed. Upon macroscopic inspection of both flowable and bilayer scaffolds, clear differences

Table 1. Primers used in this work.

Gene name	Sequence 5'–3'	Tm (°C)	NCBI accession number
<i>βActin</i>	Fw GCCCAGAGCAAGAGAGGTA	65	NM_007393.5
	Rv TAGAAGGTGTGGTGCCAGAT	64.9	
<i>Gapdh</i>	Fw ACCTGCCAAGTATGATGAC	61	NM_008084.3
	Rv GGAGTTGCTGTTGAAGTC	59.7	
<i>B2m</i>	Fw CTCGGTGACCCTAGTCTTTCT	64.9	NM_009735.3
	Rv AGTTCAGTATGTTCCGGCTTCC	64.2	
<i>Cd105</i>	Fw CGATAGCAGCACTGGATGAC	64.7	NM_001146350.1
	Rv TGGCAAGCACAAAGAATGGT	64.5	
<i>Cd31</i>	Fw AACAGAGCCAGCAGTATGA	62.6	NM_001305157.1
	Rv ATGACAACCACCGCAATG	62.5	
<i>Cd34</i>	Fw CTGCTCCGAGTGCCATTA	63.3	NM_001111059.1
	Rv CTCCTCACAACACTAGATGCTTCA	63.7	

Fw: Forward; NCBI: National Center for Biotechnology Information; Rv: Reverse; Tm: Melting temperature.



**Figure 3. Cell surface phenotype and morphology of primary adipose-derived stem cells.** Cell surface phenotype of primary adipose-derived stem cells was assessed by immunofluorescence and flow cytometry. **(A)** The various cell surface markers listed in the top of each histogram were assessed by using specific monoclonal antibodies either directly labeled with fluorochromes (fluorescein isothiocyanate-labeled anti-CD44, anti-CD45, anti-CD105 or PE-labeled anti-CD90), or unlabeled (anti-HLA-A, B, C and anti-HLA-DR) and revealed by a second step incubation with fluorescein isothiocyanate-labeled rabbit antimouse IgG. Specific fluorescence is represented by the bold histogram; negative isotype control is represented by the dashed histogram. Values are expressed in the abscissa as mean fluorescence in au and in the ordinate as number of cells analyzed. **(B)** Cell morphology of adipose-derived stem cell is shown in as assessed by inverted phase-contrast microscopy. The cells present a spindle-shaped aspect with fibroblast-like morphology. au: Arbitrary unit.

were seen between the systems loaded or not with hASCs (Figure 4). In particular, as shown in Figure 4A–C, vascularization did not occur in cell-free scaffolds, either bilayer or flowable. Conversely, in bilayer and flowable hASCs-enriched scaffolds (Figure 4D–G), the vascularization was evident. Note that the vascularization developed in a centripetal mode and branched within the scaffold, (Figure 4F & G). Moreover, after removal from the animal, the flowable ASCs system appeared yellow and soft on palpation. These characteristics have been observed for all the examined scaffolds.

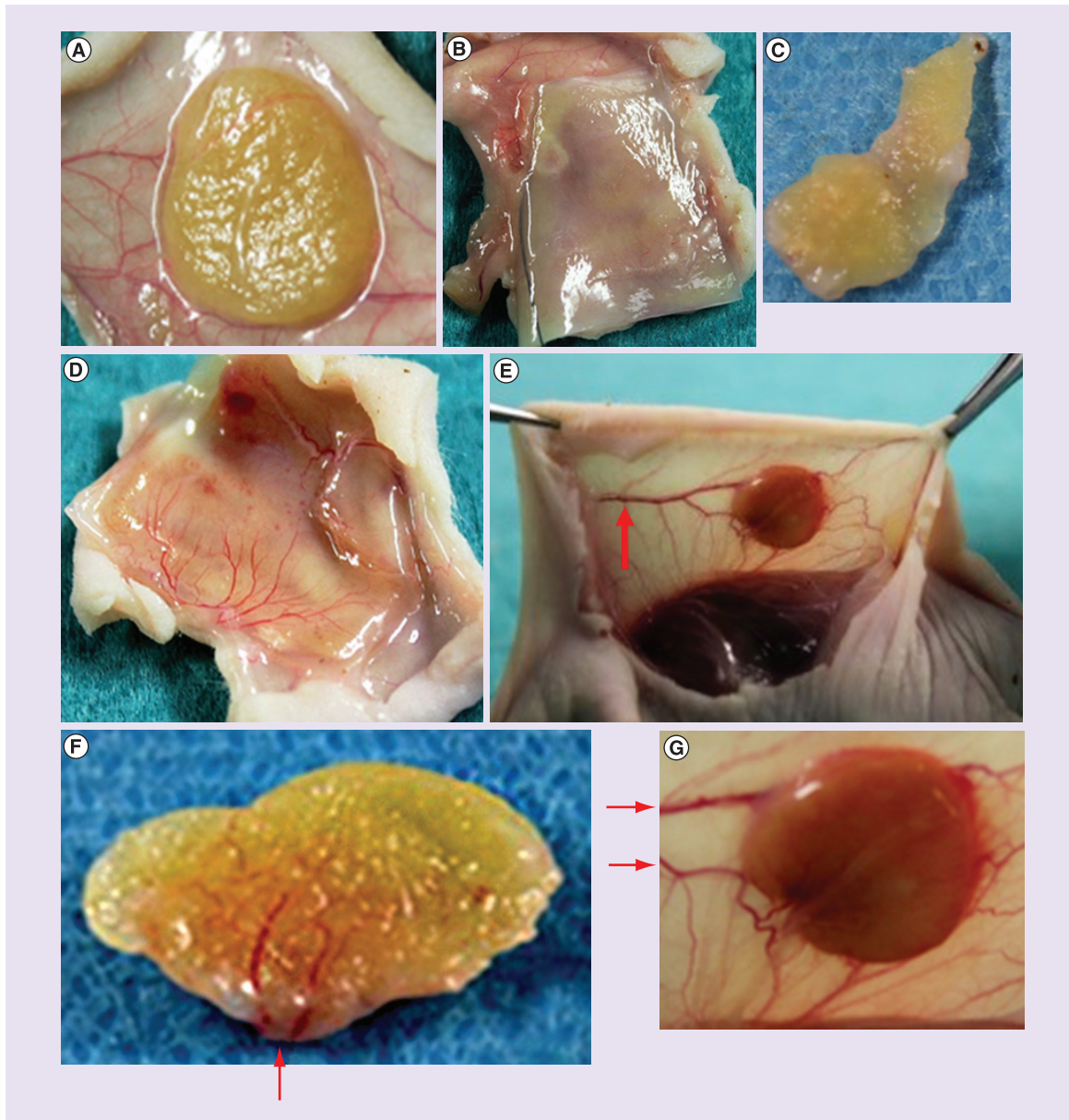
#### Microscopic scaffold analysis after animal sacrifice

To confirm that new vascularization, as well as fresh connective tissue, have been successfully induced only in the hASCs Integra system, all the scaffolds, removed 1 month after grafting, were subjected to

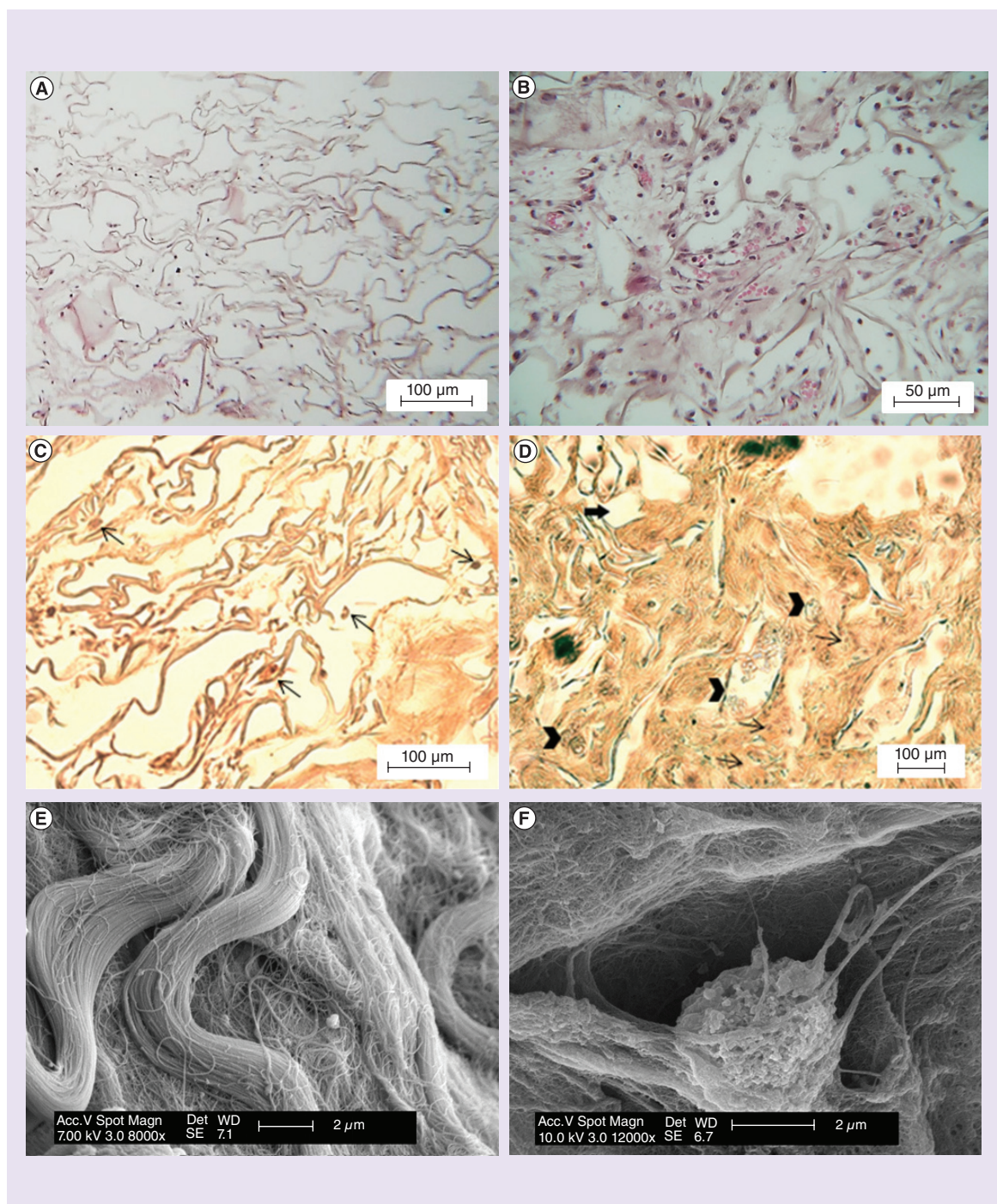
microscopic observation (representative photographs are reported in Figure 5). A simple H&E staining clearly showed the total absence of vascularization in the scaffold not loaded with hASCs (Figure 5A); conversely, numerous cell nuclei (probably of fibroblasts) and capillaries (full of erythrocytes) were present in the scaffold loaded with hASCs (Figure 5B). The immunohistochemistry, performed using MAB 1273 and MAB 1287, markers of human cells and human stem cells respectively, showed no positive reaction (Figure 5C & D). After immunohistochemistry, the same slides were counterstained with hematoxylin to relieve the presence of some nuclei. Also in this case, histological analysis of the Integra cell free system showed only the presence of rare connective tissue cells and the total absence of vascularization (Figure 5C). Conversely, similar analysis of the hASCs Integra system highlighted the presence of new connective

tissue, including the adipose tissue, and several capillaries containing erythrocytes (Figure 5D). The quantitative evaluation of *Cd105*, *Cd31* and *Cd34* mRNA strengthens the undeniable vascularization process in mice that were grafted with Integra-hASCs system (Figure 6). The number of vessels ranged from 2 to 13/mm<sup>2</sup>, depending of their diameter, with a mean of  $5 \pm 2.5$  without significant differences between the two types of scaffolds.

Scanning electron microscopy, performed 1 month after grafting, showed the presence of newly formed connective tissue (Figure 5E) and cells inside the scaffold. Cell colonization was represented by numerous rounded or ovoidal cells (Figure 5F). Tissue necrosis, cyst formation or fibrosis were not observed. For brevity, figures refer only to the results obtained on flowable scaffold, but the same outcomes have been seen in the Integra bilayer system.



**Figure 4. Representative macroscopic scaffold analysis after animal sacrifice.** (A) *In toto* bilayer and (B) flowable cell-free scaffold. The vascularization is absent. (C) Cell-free flowable scaffold, sectioned, after removal from the animal show that the vascularization is completely absent. *In toto* bilayer and flowable adipose-derived stem cell-enriched scaffolds (D & E), the neo-vessel component, developed in centripetal mode and branched within the scaffold, is evident (arrow). (E) Flowable adipose-derived stem cells system, sectioned after removal from mice shows the presence of vascularization. (F) Enlargement of panel (E). (G) The scaffold appears yellow and soft on palpation, the neo-vessel component is clearly developed inside (arrow).



**Figure 5. Microscopic analysis of scaffolds removed 1 month after grafting.** Microphotograph in (A) is a representative photograph of Integra® cell free system stained with hematoxylin and eosin: note the presence of collagen fibers and absence of vascularization. (B) is a representative photograph of Integra-adipose-derived stem cells system stained with hematoxylin and eosin: numerous cell nuclei (probably of fibroblasts) and capillaries (full of erythrocytes) were present. (C & D) show the immunohistochemistry analysis of Integra cell loaded system using the antibodies MAB 1287, against the human small nuclear ribonucleoprotein, and MAB 1273, an antihuman mitochondrial antigen stained with DAB and counterstained with hematoxylin. No signal for the presence of human cells was detected. In (C), note the presence of newly formed collagen fibers and several adipocytes (bold arrows). In (D), in addition to collagen fibers, note the presence of several fibroblast nuclei (thin arrows) and new vessels, which contain erythrocytes (arrowheads). (E) shows a scanning electron microscopy picture where newly formed collagen fibrils are evident. In (F), a cell, covered by some new formed collagen fibrils, appears to have colonized the scaffold.



## Discussion

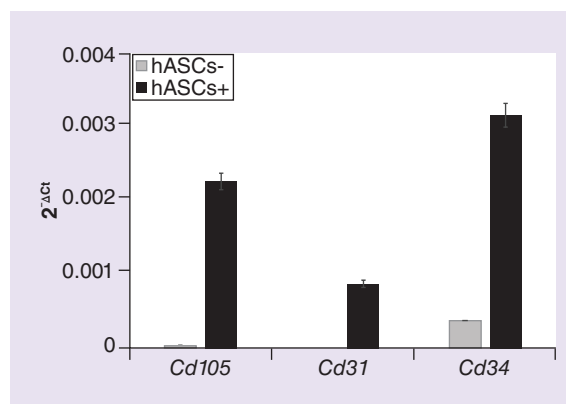
Soft tissue reconstruction is a challenge for regenerative medicine. Stem cell transplantation approaches have acquired increasing interest in this direction although the mechanisms by which stem cells of various origin actually operate as promoters and/or generators of new tissues are still poorly understood.

In this study, we investigated one fundamental aspect of tissue reconstruction related to the possibility that hASCs may promote neo-vascularization *in vivo* of a collagen scaffold implanted in immunodeficient mice and thus favor the correct microenvironment for neo-tissue generation.

The novelty of our approach resides in the simultaneous graft of two different scaffolds (Integra bilayer and Flowable Wound Matrix) loaded or not with hASCs, in the same animal. We demonstrated that, indeed, hASCs could recruit cells able to form new connective tissue (loose and adipose) and enhance the vascularity inside the grafted scaffold. Adult adipose tissue is one of the largest and most plastic tissues in the body and it is well recognized as a source of hormones, cytokines as well as the main reservoir of energy. These characteristics contribute in rendering the adipose tissue one of the most highly vascularized tissues in the body [17]. In adipose tissue, a very close anatomical and physiological relationship has been demonstrated between blood vessels, perivascular cells and the hASCs [18]. It has been reported that hASCs are capable of dividing and renewing for extended periods of time both *in vitro* and *in vivo* [19,20], moreover, *in vitro* they have the capability of differentiating into many different cell types, such as osteoblasts, chondrocytes, myocytes, neuronal-like cells, cardiovascular cells, hepatocytes and adipocytes [21]. Furthermore, a single hASC can undergo clonal expansion while maintaining multilineage differentiation potential. Indeed, our hASC population maintains the expression of adipose stem cell markers, while lacking markers of mature endothelium. Interestingly, hASCs express substantial amounts of HLA class I cell surface molecules, which are directly involved in the early critical events of human lymphocyte activation and proliferation [22] and may be expressed at low level in other pluripotent stem cells. Conversely, our hASCs do not express antigen-presenting cell surface HLA class II (DR) molecules, which are the most potent inducers of immune rejection responses in human and mouse transplantation. This could be an advantage for the potential use of hASCs in human tissue reconstruction, in particular when heterologous transplant is necessary.

Among the several animal models introduced in the last years to study the hASCs *in vivo*, nude mice have the advantages of accepting xenogeneic transplants and having a limited cost. Thus, they are amenable to

repeatable controlled experimentation. In our *in vivo* study, athymic CD1 nude mice have been grafted with two different collagen–chondroitin sulphate scaffolds (bilayer and flowable Integra) already used in reconstructive daily surgery. The flowable is more indicated in deep defect where a 3D reconstruction is needed, while the laminar is used for superficial defects where the dermal layer is missing. Collagen and chondroitin sulphate, used in scaffold formulation, have shown high biocompatibility, both *in vitro* and *in vivo*. Furthermore, collagen, in addition to being the most diffuse protein in the human body, is crucial in extracellular matrix organization, and for its potential of being vascularized, a fundamental step for the success of tissue reconstruction. Our results demonstrate that the combined use of hASCs and scaffold can result in a newly induced vascularization (Figures 4–6). At macroscopic level, the hASC-seeded scaffolds, removed 30 days after the graft, showed a structured and ordered diffusion of neo-angiogenesis toward a centripetal vascular orientation, as the new blood vessels were stimulated to grow inside of the tridimensional scaffold. At microscopic level, hematoxylin staining of the hASC-Integra system highlighted the presence, within the scaffold, of fibroblasts, unilocular adipose tissue and vessels containing erythrocytes; this last observation has been also confirmed by *Cd105*, *Cd31* and *Cd34* mRNA expression in the hASC-Integra system. The immunohistochemistry analysis has excluded the presence of human cells above and within the hASC-Integra system suggesting that the hASCs seeded onto the scaffold were not survived. Scanning electron microscopy analysis further substantiated the presence of newly formed collagen fibrils, probably induced by the presence of hASCs as observed *in vitro* by Tsuji *et al.* [23]. All together, these results, suggest that hASCs are



**Figure 6. Expression of *Cd105*, *Cd31* and *Cd34* in mice grafted with human adipose-derived stem cells-enriched or cell-free scaffold system.** Cytoplasmic  $\beta$ Actin has been used as reference gene. hASC: Human adipose-derived stem cell.

able to create a kind of Stem Bag system [24] inside the collagen scaffold, capable to release growth factors and cytokines to establish the correct microenvironment to attract new cells from the host tissue and support their differentiation during tissue regeneration.

All the characteristics just described were absent in the scaffolds not charged with hASCs. Another interesting aspect is that no difference was observed between the laminar and the flowable scaffold in their potential to generate neo-angiogenesis and new tissues in presence of hASCs. Thus, we may conclude that both scaffolds can be used in association with hASCs to successfully induce new vascularization and tissue regeneration.

Several studies reported the transfection of the hASCs to induce controlled differentiation and increment of growth factors secretion [19,25]. However, stem cells transfection is a major manipulation and, in a therapeutic prospective, it should be taken into consideration that in several countries the law forbids such major cell manipulations.

The capacity of inducing neovascularization in a paracrine fashion and the relatively short lifespan of hASCs are both crucial elements to favor their use in clinical settings [26]. Indeed, on one side, hASCs can provide the necessary physiological elements and microenvironment promoting neo-vascularization and, on the other side, their rapid disappearance might prevent a possible neoplastic transformation.

We think that this is another step ahead in the stem cells therapy, especially when the principal aim is not the direct differentiation of the staminal cells, but their use to promote the new tissue reconstruction by the cells already present in the host organism.

## Conclusion

In conclusion, the results of this work strongly support the therapeutic potential of using hASCs, to form new vascular networks that allow sufficient vascularization of engineered organs and tissues. The principal source of hASCs is the adipose tissue from human adults, which is easily accessible in large quantities with minimal invasive harvesting procedure and provides higher amount of stem cells than the bone marrow (500-times more).

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## Financial and competing interests disclosure

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## Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles

### Executive summary

#### Characteristics of human adipose mesenchymal stem cells

- Previous works have suggested that mammary adipose tissue, obtained after a gigantomastia intervention, is the best source to get human adipose-derived stem cells (hASCs) in comparison to abdominal, inguinal or thigh adipose tissue.
- hASC characterization has indicated that more than 90% of the population expressed stem cell markers.
- hASC seeded on to the scaffold can survive and proliferate *in vitro* for at least 6 days.

#### Grafting procedure

- Simultaneous graft of two different scaffolds (Integra® bilayer and Flowable Wound Matrix) loaded or not with hASCs, has been performed in the same animal.

#### *In vivo* performances of the scaffold adipose-derived stem cell system

- Both flowable and bilayer scaffolds hASCs enriched induce new vascularization and connective tissue.
- The immunohistochemistry, performed using markers of human cells and human stem cells, showed no positive reaction indicating that after 30 days the hASCs are no longer present.
- These results were confirmed both by macroscopic and microscopic analysis.

#### Conclusion

- The principal aim of the scaffold adipose-derived stem cells system is not the survival and differentiation of the staminal cells, but to promote the new tissue reconstruction by the cells already present in the host organism.
- The capacity of inducing neovascularization in a paracrine fashion and the relatively short lifespan of hASCs are both crucial elements to favor their use in clinical settings.

outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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