Purification and long-term expansion of multipotent endothelial-like cells with potential cardiovascular regeneration

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SHORT TITLE:

Endothelial-like cells for cardiovascular repair

Abstract

***Aims*—**Endothelial progenitor cells (EPC) represent a relatively rare cell population, and expansion of sufficient cell numbers remains a challenge. Nevertheless, human adipose-derived stem cells (hASC) can be easily isolated and possess the ability to differentiate into endothelial cells. Here, we propose the isolation and characterization of multipotent endothelial-like cells (ME-LC) with the capacity to maintain their vascular progenitor properties for long periods of time.

***Methods and Results*—**hASC were isolated from lipoaspirates and cultured through distinct consecutive culture stages for two months to enrich ME-LC: first in DMEM-FBS (stage I), followed by a stage of culture in absent of FBS (stage II), a culture in SFO3 medium (stage III) and finally, the culture of ME-LC into collagen IV-coated flasks in EGM-2 medium (stage IV). ME-LC display increased expression levels of endothelial and hematopoietic lineage markers (CD45, KDR and CXCR4) and EPC markers (CD34 and CD133) whereas the expression of CD31 was barely detectable. RT-PCR assays showed expression of genes involved in early stages of EPC differentiation and decreased expression of genes associated to differentiated EPC (*TIE-2*, *DLL4* and *FLT-1*). ME-LC formed capillary-like structures when grown on Matrigel, secreted increased levels of SDF-1 and showed the ability to migrate attracted by SDF-1, VEGF and HGF cytokines. Importantly, ME-LC retained the capacity to differentiate into cardiomyocyte-like cells.

***Conclusions*—**We present a simplified and efficient method to generate large numbers of autologous ME-LC from lipoaspirates-derived hASC, opening up potential cell-based therapies for cardiovascular regenerative medicine.

Keywords

Multipotent endothelial-like cells (ME-LC), endothelial progenitor cells (EPC), cardiovascular diseases, cell therapy, human adipose-derived stem cells (hASC).

Introduction

In the adult, different mesodermal stem/progenitor cells have been identified including endothelial progenitor cells (EPC) which are involved in the angiogenesis and vasculogenesis processes [1]. Upon vascular injury, EPC are mobilized from the bone marrow (BM) by cytokine secretion such as vascular endothelial growth factor (VEGF) and SDF-1 in order to migrate and regenerate the damaged tissue [2].

EPC are commonly characterized by the expression of the surface markers CD34 and CD133, lack of the hematopoietic marker CD45, co-expression of CXCR4 and VEGFR2 (KDR/flk-1) [2,3]. EPC differentiation into mature endothelial cells is accompanied by a loss of expression of CD133 and a concomitant increase in CD31 expression, CD144 (VE-cadherin) and other markers [4]. Furthermore, expression of certain genes is also used during the characterization of *bona fide* endothelial cells. For instance, *FLT-1*, *TIE-2*, *CCR7* and *C-KIT* are expressed on EPC, among others cell types, while *CDK2*, a cyclin dependent kinase, is overexpressed on later stages of EPC differentiation and absent on early phases of EPC differentiation [5].

Blood-derived EPC or BM-derived stem cells have been used to improve myocardial perfusion and contractile function and enhance limb perfusion in patients [6,7]. However, there are still some drawbacks for their clinical utility such as the extremely low number of EPC in the bloodstream and the low availability and harvesting difficulties of BM-derived stem cells. This may be severely hampered in elderly patients or due to high morbidity associated with vascular disease [8].

In contrast human adipose-derived stem cells (hASC) can be isolated in a greater number through a safe non-invasive routinely liposuction procedure. These hASC can also be expanded in culture and differentiate into different cell types, including endothelial cells [9]. However, in order to use these progenitor cells clinically in regeneration of vascular and/or heart lesions it is necessary to develop reliable and reproducible methods to isolate and expand these cells [10].

In the present study we propose a new approach of easy-to-derive large number of multipotent endothelial-like cells (ME-LC) from human adipose tissue with the capacity to display endothelial and cardiomyocytes-like properties in culture for long periods of time.

Methods

Isolation and culture of hASC from human adipose tissue

Subcutaneous adipose tissue was obtained from 15 different patients by a minimally invasive procedure after signed informed consent from all patients and approval from the Ethics Committee of the Clinic University Hospital of Málaga (Spain). This study conformed to the principles outlined in the Declaration of Helsinki. In each experiment we used at least four lipoaspirates. Isolation and culture of hASC was performed as described previously [11,12]. hASC were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma, St Louis, MO) containing 10% fetal bovine serum (FBS; Lonza, Basel, Switzerland) and 1% Penicillin-Streptomycin solution (Sigma) (DMEM-FBS).

Differentiation assays of hASC

hASC were plated at 2 x 10^3 cells/cm2 in DMEM-FBS and were allowed to adhere for 24 hours. Culture medium was then replaced with specific differentiation inductive media. For adipogenic and osteogenic differentiation, cells were cultured for 2 weeks in Adipogenic Mesenchymal Stem Cells (MSC) Differentiation Bullet Kit and Osteogenic MSC Differentiation Bullet Kit (Lonza), respectively. For chondrogenic differentiation, cells growing in monolayer were cultured in NH ChondroDiff Medium (Miltenyi Biotec, Auburn, CA) for 3 weeks. Recent articles published by our group have demonstrated the capability of chondrogenic differentiation of hASC in monolayer cultures [12,13]. Differentiated cell cultures were stained with Oil Red O (Amresco, Solon, OH, USA) for adipogenic differentiation, Alizarin Red (Lonza) for osteogenic differentiation or Toluidine Blue (Sigma) for chondrogenic differentiation [14].

ME-LC isolation and expansion

hASC were split and seeded at 3 x 10^6 cells/ T-75 tissue culture flask (BD Falcon, Franklin Lakes, NJ) in DMEM-FBS (stage I). After 3rd or 4th cell-culture passage (2 weeks), the culture medium was replaced by serum-free medium (DMEM) to induce the development of multicellular aggregates

which were termed sphere cluster formations (SCF) (stage II) (Figure 1). SCF were scraped off after 3 weeks and seeded in a 6-well plate at a concentration of 10-15 SCF per well in DMEM-FBS for 48 h. Isolated cells were then subcultured in medium SFO3 [RPMI-1640: DMEM: F12, 0.1% bovine serum albumin (BSA), 50 µM 2-mercaptoethanol and 1% Penicillin-Streptomycin (Sigma)] (stage III) [15]. Within the next 3 weeks, cells were scraped off and seeded into collagen IV-coated flasks and grown in endothelial cell medium, endothelial basal medium-2 (EBM-2, Lonza) containing 5% FBS, and human recombinant vascular endothelial growth factor (VEGF), hydrocortisone, human recombinant epidermal growth factor (rhEGF), human recombinant long R insulin-like growth factor-1 (R3-IGF-1), ascorbic acid, human recombinant basic fibroblast growth factor (rhFGF) and gentamicin sulfate-amphotericin-B (EGM-2, Lonza) (stage IV) (Figure 1). Cells obtained from stages III and IV were considered as ME-LC. Human umbilical vein endothelial cells (HUVEC) were also cultured in EGM-2 medium as a control.

Flow cytometry

Cells were trypsinized, washed and resuspended in phosphate buffered saline (PBS) with 2% BSA (Sigma), and 2 mM ethylenediaminetetraacetic acid (EDTA, Sigma). Cells were incubated in the dark at 4º C for 45 minutes with the following fluorochrome-conjugated monoclonal antibodies: CD133-PE (Miltenyi), CD105-APC, CD90-FITC (eBioscience Inc., San Diego, CA), KDR-APC (R&D System, Minneapolis, MN), CD34-FITC, CD45-APC-Cy7, CD 73-PE and CXCR4-PE (BD Biosciences, San Jose, CA). Cells were then washed in PBS and analyzed in a FACS Canto II cytometer equipped with the FACS Diva analysis software (BD Biosciences) [16]. Data obtained were expressed as mean ± standard error (SE) from four independent experiments performed in triplicate ($P<0.05$).

Gene expression profile

For RT-PCR analysis, total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer´s instructions. The cDNA-reaction was performed using 0.5-2 µg of total RNA with primers from SuperScript II-kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Forward and reverse primer sequences and expected PCR product

sizes for each specific gen are shown in Table 1. The PCR reaction was performed with Reddy Mix PCR Master Mix (Thermo Fischer Scientific, Epsom, UK). After initial denaturation (2 min at 94 °C) 35 cycles were performed (20 sec 94 °C, following by 1 min annealing and 51 °C for *FLT-1*, *TIE-2*, *DLL4*, *CDK2* and β-actin and 43 °C for CXCR4, CD133 and Ccr-7 followed by 1 min extension at 72 °C). The PCR products were run on 1% agarose gel and photographed under UV light [17,18].

Functional capillary formation assays

The ability to form capillaries in semisolid medium was tested by culturing trypsinized cells on Matrigel™-coated 96-well plates (BD Biosciences) in EGM-2 medium. Matrigel™ was thawed, used to cover the culture plastic (50 µL per well of a 96-well plate) and allowed to solidify for 1 h at 37º C. Cells from stage I to stage IV cultures were independently seeded. Outgrowths obtained from cultures at different stages of the endothelial isolation process were seeded on Matrigel™-containing plates at 5 to 20 x 10^3 cells per well and cultured in EGM-2 medium for 7 days. Four h, 24 h and 7 days after the initial plating photographs were taken using a Leica DM 5500B (Leica, Solms, Germany) microscope equipped with the Meta Systems software [19]. Figures were processed with Adobe Photoshop 7.0. Cells were counted for the formation of capillary structures. The number of capillary-like structures was measured after 24 hours and each cord portion between the ramifications was considered one capillary unit. Mean ± SE values were obtained by evaluating the whole cultures of each well under the same conditions from three independent experiments performed in duplicate. A semi-quantitative measurement of capillary formation on Matrigel™ was performed as described elsewhere (capillary formation index) [20,21], using HUVEC like control.

Cytokine determination

The production of the chemokine SDF-1 in different cultures was assayed by ELISA. Firstly, serum and other supplements were removed from the culture medium to avoid potential interference with the measurements. Cell culture supernatants were collected 24 hours later and were used for the assay. ELISA was performed using the Human SDF-1 Kit (R&D System), according to the manufacturer's protocol, and the measurements of emitted signal at 450 nm were taken with the ELx800TM

microplate absorbance reader (Bio-Tek Instruments GmbH, Bad Friedrichshall, Germany). All data about SDF-1 secretion were compared taking account the same number of cells in each culture stage (5×10^5 cells) and were obtained from four independent experiments performed in duplicate.

Migration assays

To determine cell migration, a modified Boyden chamber assay was performed using a 24-well microchemotaxis chamber (BD Biosciences). 10^5 cells growing in collagen IV-coated flasks in EGM-2 medium (stage IV) were seeded onto the upper Boyden chamber in EBM-2 medium supplemented with 10% FBS. In the lower chamber, a culture medium containing 50 ng/mL of VEGF, 25 ng/mL of HGF and 100 ng/mL of SDF-1 was added. Cells were labelled with 5 µM of calcein AM (Invitrogen) and they were photographed using a confocal microscope (Leica DMI6000). The non-migrating cells in the upper chamber were scraped off using blunt-ended forceps and swabs, and washed with PBS. Moreover, after 1 to 12 h incubation at 37 °C in a 5% CO2 atmosphere, the cells were fixed in 2% paraformaldehyde for 5 min and washed in PBS. The fluorescence from the cells migrated to the lower chamber was measured using a fluorescence microplate reader (FLx800, Bio-Tek Instruments, Inc., Winooski, VT) from the bottom at 485/535 nm wavelength. The migrated cells were represented by the ratio of fluorescence as compared to the control.

Cardiac differentiation

ME-LC (stages III and VI) were seeded at 5-20 x 10^3 cells per well of a Matrigel™-coated 96 well plate in EGM-2 medium. Culture medium was replaced two weeks later by EBM-2 containing 5-10 or 15 µM of 5-azacytidine (5-aza) for 24-48 hours. Culture medium was changed back to EGM-2 and cells were cultured for 3-4 weeks. Cells were then detached from Matrigel™ with dispase (BD Biosciences) and seeded in a 8 well chamber slide (Nunc, Rochester, NY) at 5-10 x 10^3 cells per well for 4 days in EGM-2 for immunofluorescence analysis.

Immunofluorescence

Cells were washed three times with PBS and fixed with 4% paraformaldehyde in PBS for 30 minutes at room temperature. Then, cells were permeabilized with 0.1% Triton X-100 for 15 minutes, washed three times with PBS and blocked in 2% blocking buffer solution (Roche, Barcelona, Spain) for 1 hour at room temperature. Cells were then incubated overnight with primary antibodies diluted 1:100 in blocking buffer solution at 4ºC, washed three times in PBS and then incubated for 2 hours with secondary (FITC- or TRITC-conjugated) antibodies diluted 1:200 in blocking buffer solution. Afterwards, they were washed three times in PBS and slides were mounted with DAPI-containing mounting solution (Ultra Cruz TM Mounting Medium, Santa Cruz Biotechnology, Santa Cruz, CA). Cells treated in the same way but incubated with isotype matched control antibodies were used like negative control. Antibodies used were as follows: human desmin (Goat monoclonal; Sigma); human cardiac-specific troponin T (mouse monoclonal; Research Diagnostics, Flanders, NJ); and human sarcomeric α-actinin (mouse monoclonal; Sigma). Photographs were taken with a Leica DM 5500B fluorescent microscope equipped with Meta Systems software.

Statistical analysis

Statistical analysis was performed using the non parametric Kruskal-Wallis H test for independent experiments. Significant differences between groups were estimated using the Mann–Whitney U-test. For the statistical analysis SPSS 15.0 software program was used. Data are presented as mean ± standard error (SE). $p<0.05$ was considered as statistically significant.

Results

hASC obtained from lipoaspirates possess mesenchymal stem cells properties

hASC isolated from human lipoaspirates were maintained in culture for >10 weeks with no signs of senescence. FACS characterization showed that *ex-vivo* cultured hASC expressed the surface markers CD105 (>99%), CD90 (>90%) and CD73 (>99%) while lacked expression for both hematopoietic and endothelial cell markers CD45, CD34, CD133, CXCR4 and KDR (Figure 2A). Cells cultured in

adipogenic medium acquired typical morphology of lipid-laden cells containing intracellular lipid-filled droplets which stained positive for Oil Red O. Alizarin Red S staining demonstrated the presence of osteogenic differentiation, with the presence of mineralized nodules as shown in Figure 2B. Chondrogenic differentiation was confirmed by toluidine blue staining showing accumulation of proteoglycans (Figure 2B).

ME-LC isolation and expansion

ME-LC were isolated through a series of consecutive stages I to IV detailed in the methods section (see Figure 1 for details). hASC obtained from lipoaspirates grew as a monolayer and displayed a fibroblast-like and spindle-shaped morphology when they were cultured in DMEM-FBS (stage I, Figure 3A). When the culture medium was replaced by DMEM without serum, the cells began to form three dimensional cellular aggregates (termed SCF) which increased in size over time (stage II; Figure 3B). We obtained between 50-70 SCF in each T-75 tissue culture flask. When these SCF were scraped-off and seeded in SFO3 medium, SCF began to connect each other and showed a variable morphology (stage III; Figure 3C and 3D). At the end of stage III were obtained $3.9 \pm 2.1 \times 10^5$ cells in each well-plate from 10-15 SCF. For the stage IV, cells scraped-off from two wells were seeded in a T-75 tissue culture flask. Cells displayed an elongated morphology and were arranged in parallel or grew attached surrounding the SCF (stage IV; Figure 3E and 3F). At this stage the doubling time of ME-LC was shorter than hASC, 2.7 ± 0.1 days and 3.8 ± 0.3 days, respectively.

ME-LC isolated after several culture stages express markers associated to vascular progenitors coupled to a decreased expression of the mesenchymal marker CD90

Flow cytometry was carried out in each of these culture stages to assess the presence or absence of hematopoietic, endothelial and mesenchymal markers. The expression of the hematopoietic markers CD133, CD34, CD45, KDR and CXCR4 significantly varied throughout the isolation process ($P<0.05$, Mann-Whitney U-test) (Figure 4A). Initially, the cultures were negative for all markers during stages I and II. When the cells were cultured in SFO3 medium (stage III), they slightly upregulated these markers to a some extend: 14.1±1.7% for CD133, 18.3±4.9% for CD34, 21±3.7%

for CD45, 27.3±5.5% for KDR and 13±1.65% for CXCR4. In stage IV, when the cells had been cultured in EGM-2 medium, the expression of these markers dropped (Figure 4A).

High levels of expression of the MSC markers CD105, CD73 and CD90 were observed throughout the different stages of the ME-LC isolation procedure (Figure 4B). A Mann-Whitney U-test indicates significant (P<0.05) differences among the expression of the three markers (Figure 4B). Interestingly, CD90 expression decreased significantly in cells cultured in SFO3 medium (stage III: 41±15.8%) in comparison with cells in the stage I (94.1±2.2%), stage II (80±8.6) and stage IV (87.5±2.7%).

Figure 4C shows a comparison between marker expression of cells at stages I, III and IV versus HUVEC, which were used as control of mature endothelial cells. HUVEC and ME-LC showed expression of the progenitor markers (CXCR4 and KDR). CD133 and CD34 were found expressed in ME-LC but not in HUVEC. Expression of the mesenchymal stem cell marker CD90 decreased in both ME-LC cultured in SFO3 and HUVEC as compared with cells at stage I. Finally, the endothelial marker CD31 was highly expressed in mature HUVEC (63±11.4%) but it was barely expressed in stages I, III and IV (Figure 4C). All together, these data suggest that ME-LC isolated from hASC cultures express markers resembling a vascular progenitor phenotype.

Expression levels of genes related with EPC were assessed by RT-PCR. Cells cultured from stage I to IV maintained the expression of genes such as *CD133, CXCR4*, *CDK2* and *FLT1*. A weak expression of *CCR7* and *CDK2* genes was detected in cells at stage III (Figure 4D). Expression of *TIE2* was only detected at stage I, but not in subsequent stages. Finally, expression of *DLL4* (a Notch ligand) was found highly expressed at stage I. Its expression, however, decreased at from stage II onwards and was completely lost at stages III and IV (Figure 4D). These gene expression data suggest the endothelial progenitor phenotype of ME-LC.

ME-LC enhance functional capillary-like structures ~~tubes~~ formation in a matrigel assay

As shown in Figure 5A, cells from cultures at stage I and II were not able to form any capillaries over a 7 days period. On the other hand, cells previously grown in SFO3 or EGM-2 (stages III and IV, respectively) displayed a large number of capillary-like structures as early as 4 hours after being

seeded on Matrigel™ and the appearance of capillary ~~-tube-~~ like structures increased overtime. After 7 days in culture a well-established cellular network was present in all the cultures (Figure 5A). As a positive control, the capability of HUVEC to form capillary-like structures in Matrigel™ was also assessed. The results showed the appearance of these capillary-like structures after 4 and 24 hours in Matrigel™. However, these structures disappeared when HUVEC were cultured for 7 days likely due to their very mature nature (Figure 5A).

The number of capillary-like structures was counted in every Matrigel™-coated well after 24 hours of culture such as is described in methods section. As the hASC were cultured throughout the distinct stages they gradually gained ability to form capillary-like structures ($P<0.05$). By stage IV, the number of capillary-like structures was similar in comparison with the data rendered by HUVEC, used as control (Figure 5B).

Increased release of the angiogenic cytokine SDF-1 by ME-LC

The presence of the SDF-1 cytokine in the medium was analyzed by ELISA at different time points. There was a significantly increased of SDF-1 ($P<0.05$) in supernatants harvested at stage III (583±67 pg/mL) and IV (1420±225 pg/mL) in comparison with the SDF-1 levels at stages I and II (148±20 pg/mL and 148±37 pg/mL, respectively). Moreover, when cells at stage IV were maintained for further 2 weeks in EGM-2 medium, the concentration of SDF-1 increased drastically (4113±170 pg/mL) (Figure 6A).

Finally, in order to determine the influence of endothelial growth factors on ME-LC included in a model of extracellular matrix we cultured ME-LC for 2 weeks on Matrigel™-coated plates on EGM-2 medium versus EBM-2 medium supplemented with 5% FBS. The SDF-1 levels in the supernatants were 148±15 pg/mL in cells cultured in EBM-2 supplemented with 5% FBS and 568±40 pg/mL for cells cultured in EGM-2 (Figure 6B).

ME-LC are able to migrate in response to angiogenic cytokine stimuli

To investigate the cell migratory response of ME-LC towards different angiogenic cytokines such as SDF-1, VEGF and HGF, we used a modified Boyden chamber with cells cultured in EBM-2 medium supplemented with 10% FBS for 12 hours (Figure 7A). SDF-1, VEGF or HGF cytokines induced cell migration of ME-LC from the upper compartment through the pores of the membrane into the lower compartment (Figure 7B and 7C). Negative controls were performed adding cytokine-free medium into the lower compartment.

ME-LC have the ability to differentiate into a cardiac phenotype

Finally, we tested the potential of the ME-LC to differentiated toward a cardiomyocyte phenotype following exposure to 5-aza [12]. Morphological changes and expression of cardiac specific markers were determined after three weeks of culture. Upon 5-aza treatment, ME-LC changed their morphology. Treated ME-LC were wider and displayed branching fibers easily observed by phase contrast microscopy (Figure 8A). Immunocytochemistry analysis revealed the expression of typical cardiomyocyte markers such as Troponin-T, Desmin and α-Actinin in the cytoplasm of the 5-aza treated cells. Interestingly, these markers colocalized (Figure 8B). Moreover, cells which stained positive for these cardiac markers showed a parallel and interconnected distribution with the presence of bi- and multinucleated cells. Control ME-LC non-treated with 5-aza were negative for all the cardiomyocyte markers examined (Figure 8C).

Discussion

Cardiovascular diseases, such as the ischemic heart disease and peripheral arterial occlusive disease, cause an elevated morbidity and mortality in developed countries. Currently, several clinical trials use different strategies for cell delivery and a diverse cell sources for transplantation [6-8]. In this respect, regenerative therapy to treat endothelial tissue damage has focused on the use of autologous stem cells, mainly EPC harvested from blood, BM or umbilical cord blood [22]. Nevertheless, the scarcity of EPC in adult tissues makes its therapeutic use a challenge. As an alternative, the endothelial

differentiation potential of MSCs has been explored by stimulating MSCs with angiogenic growth factors and it was proved that the differentiated MSCs were able to integrate into new blood vessels *in vivo* [23].

Recently, it has been shown that adipose tissue contains a population of adult multipotent cells with extensive proliferative capacity *in vitro* which are able to differentiate into several lineages, including endothelial cells, smooth muscle cells and cardiomyocytes [13,24,25]. In fact, there are preclinical studies supporting the capability of MSCs obtained from BM, umbilical cord blood or adipose tissue to differentiate into endothelial mature cells [26,27]. However, it was reported that mature endothelial cells can proliferate *in vitro* although they gradually loss their proliferative potential hampering their clinical application [28]. From a therapeutic standpoint, it becames necessary the isolation of sufficient numbers of progenitor cells capable of maintaining their angiogenic potential *in vitro* for long periods. Here, we present a simple and reproducible approach to maintain ME-LC isolated from subcutaneous adipose tissue. Moreover, we tried to induce cardiomyocytic differentiation to demonstrate the capacity of ME-LC to differentiate into both endothelial and cardiomyocyte-like cells, which could have advantages in the stem cell based cardiovascular therapy.

Phenotypic characterization of hASC isolated from lipoaspirates showed a high expression of mesenchymal-specific surface markers such as CD105, CD73 and CD90, and barely expressed hematopoietic stem cells (HSC) or EPC markers (CD45, CD34, CD133, CXCR4 or KDR). Moreover, hASC possessed the ability to differentiate into various lineages as previously has been shown [29]. hASC were cultured along several stages (stage I to IV, Figure 1). Culture in serum-free media for three weeks resulted in the appearance of SCF that increased in number and size throughout the subsequent culture stages. Previously, Hirashima et al., 2003 [15] demonstrated that a chemically defined serum-free culture system, including 2-mecarptoethanol, had the ability to support the proliferation of endothelial cells and their progenitors from mesoderm cells. When cells were cultured in SFO3 medium (stage III) and in endothelial cell medium (EGM-2; stage IV), both termed ME-LC, increased their expression of EPC and hematopoietic markers (CD34, CD133, KDR, CXCR4 and CD45) [30]. The coexistence of hematopoietic and endothelial markers in the ME-LC is indicative of a phenotype resembling early vascular progenitors since it has been reported the existence of a bipotent

precursor cell, termed the hemangioblast, capable of giving rise to both hematopoiesis and vascular endothelium [31]. In contrast, mature endothelial cells HUVEC were negative for CD34 and CD133 progenitor markers and strongly positive for CD31, a mature endothelial marker. These antigens (CD34, CD133) are lost upon differentiation of endothelial progenitors to endothelium [30]. CD34 expression in hASC is correlated with replicative capacity, differentiation potentials, expression profiles of angiogenesis-related genes, and immaturity or stemness of these cells [32]. Similar results were obtained by Howsonet al. [33] using postnatal aorta to develop culture conditions for the isolation of non-endothelial mesenchymal cells with long-term maintenance in an undifferentiated state. Under serum-free conditions vascular progenitor cells obtained were CD34+/CD31-, grew forming spheroids and were identified as pericyte progenitor cells [33]. However, another study showed that using the same serum-free media, cells obtained from lipoaspirates had increased proportion of Flk-1+ (KDR) marker and were negative for CD34, CD45 and CD133. When these cells were seeded in endothelial cell (EC) differentiation medium for three days the expression of Flk-1+ decreased over time while the expression of mature endothelial cell markers increased [27]. In contrtast to our study they cultured the cells during shorter period of time in serum free-media and in EC medium, likely accelerating endothelial maturation due to serum components, which have been proved to induce cell maturation [34].

Interestingly, both ME-LC and HUVEC expressed high levels of CXCR4 and KDR. Phenotypically, MSCs are identified by the absence of CD45, CD34 and other hematopoietic-associated markers [3]. In addition, the mesenchymal stem cell marker CD90, which expression was practically absent in HUVEC, was the marker whose expression most significantly decreased when ME-LC were cultured in SOF3 medium (stage III), suggesting endothelial differentiation. In agreement, it has been shown a lost of CD90 antigen expression on mesenchymal stromal cells by angiogenic stimulation *in vitro*[35]. In contrast, CD90 positive cells recovered at stage IV in which cells reached a high rate of proliferation. It has been showed that the expression of CD90 on EPC and pericytes may be indicative of their angiogenic potential and capacity for proliferation [36].

Gene expression profile confirmed the endothelial progenitor phenotype with the expression and maintenance of specific genes involved in self-renewal, cell cycle promotion and antiapoptotic such as

has been recently showed in cord blood-derived EPC [5]. Both CXCR4 and CD133 vascular genes were expressed at similar levels throughout the distint culture stages. Nevertheless, genes expressed in differentiated EPC such as *Cdk2* and *Flt-1*[5] showed a weak expression level in ME-LC cultured in SOF3 medium. Another interesting result was the disappearance of *Tie-2* gene expression, which has been previously reported as angiogenic factor clearly induced in the differentiated endothelial cells [37]. Constitutive Ang1–Tie2 signaling is thought to maintain the quiescent endothelial phenotype *in vivo* [38]. In addition to endothelial cells, Tie2 is expressed in a subpopulation of HSC being, in part, responsible of maintaining a quiescent state in the bone marrow niche [39]. Also, our results showed that the expression of the Notch ligand, delta-like 4 (DLLl4), was down-regulated in ME-LC, which correlated with the early formation of large number of capillary-like structures and the late formation of a vascular network. Recent studies have demonstrated that DLLl4 limits the angiogenic potential in developing blood vessels and the loss of DLL4 results in an arterial hyperbranching phenotype [40]. Interestingly, in contrast to HUVEC, only ME-LC cultured in SFO3 (stage III) and EGM-2 (stage IV) media were able to strongly enhance capillary tubes formation after 7 days. It has been shown that VEGF stimulation of HUVEC induces Dll4 expression which reduced vessel sprout length in a 3D tubulogenesis assay confirming that DLL4 signaling inhibits angiogenesis. DLL4 expression seems to acts as a switch blocking endothelial cell proliferation and allowing induction of a more mature differentiated phenotype [41].

Several studies have identified several molecules such as VEGF and SDF-1 as key regulators of the proliferation, chemotaxis towards ischemic tissues and differentiation of EPC [42]. hASC secrete multiple potentially synergistic proangiogenic growth factors including VEGF, HGF and chemokine SDF-1 which are likely to play a pivotal role for the hASC-mediated angiogenesis [43]. In fact, SDF-1 has been shown to enhance neovascularization by accelerating EPC recruitment into ischemic foci [42]. In our study, ME-LC showed an increased secretion of SDF-1 in comparison with hASC, even when they were seeded into Matrigel™. In addition, the role of SDF-1 to induce migration of CD133+/CD34+/KDR+ cells has been shown [30]. In the present study, we demonstrate how the ME-LC, which express the SDF-1 receptor, CXCR4, were able to migrate towards a SDF-1 gradient. These data suggest the potential homing of ME-LC to sites of vascular injury for tissue repair.

Most importantly, we show that following exposure to 5-azacytidine ME-LC retained the capacity to differentiate into cardiomyocyte-like cells with the acquisition of a cardiogenic phenotype and the expression of cardiomyocyte-specific markers. Morphological changes consisted in the appearance of wide, branching and multinucleated cells resembling cardiac muscle cells [44]. The expression of cardiomyocyte markers such as troponin-T and α-sarcomericactinin [44] and the presence of a desmin filaments network, which take part in regulating the mesodermal specification into cardiomyocytes [45], support the cardiomyocyte differentiation from ME-LC.

In summary, our studies indicate that subcutaneous adipose tissue may be a useful source of autologous ME-LC with the capacity to maintain their vascular progenitor properties. The culture method described in the `present study may be used to isolate, maintain, and propagate these cells with an increased expression of specific endothelial progenitor markers. This methodology could be applied to MSCs from other origins such as bone marrow derived stromal/stem cells. However, the interest of our study was the use of hASC isolated from liposuction which is a less invasive method than bone marrow aspiration and allows the collection of a high rate of progenitor cells. This can overcome the limited proliferation potential of mature endothelial cells and EPC, which hampers their clinical use. ME-LC increased the secretion of SDF-1, formed vascular-like structures and displayed the ability to migrate towards a cytokine gradient. Moreover, ME-LC retained the capacity to differentiate into cardiomyocyte-like cells, showing expression of typical cardiomyocyte markers. This property suggests the potential of ME-LC to recellularize damaged tissue or strengthen the post-infarct scar as well as inducing neovascularization of the affected area, which could have advantages in the stem cell based cardiovascular therapy. It has been demonstrated the ability of cardiac stem cells to differentiate into the endothelial cells, contributing to neovascularization in the process of tissue remodeling and/or regeneration [46]. Furthermore, recent studies showed interest in the cardiac and endothelial capacity of stem cell providing important tools for the study of differentiation *in vitro* and future stem cell therapy for ischemic cardiomyopathy [47-48]. Despite future studies should explore further the functional *in vitro* and *in vivo* mechanisms of ME-LC in cardiovascular diseases along with their potential therapeutic impact, our experimental data suggest that these cells may prove to be a valuable tool for vascular and cardiac repair.

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Conflict of Interest: none declared

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Figure 1. Cartoon representing the ME-LC isolation and purification procedure

hASC were isolated by enzymatic digestion from lipoaspirates obtained from patients by a minimally invasive surgery. Stage I: cells cultured in DMEM-FBS for 10-14 days; stage II: cells cultured in DMEM-FBS for 10-14 days and then 2-3 weeks in DMEM; stage III: cells scrapped off from the stage II and seeded in SFO3 for about 3 weeks; stage IV: cells scrapped off from the SFO3 culture medium and cultured into collagen IV-coated flasks in EGM-2 for at least 1 week.

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(A) Flow cytometry characterization of hASC. Grey histograms identify the isotype controls (negative). **(B)** Differentiation potential of hASC towards adipogenic, chondrogenic and osteogenic lineage. Adipogenesis was confirmed by Oil Red O staining, chondrogenesis by Toluidine Blue staining and osteogenesis by Alizarin Red S staining. Upper pictures show negative controls, hASCs cultured in normal medium for 2 weeks and then histochemically stained. Scale bar = 200 μm.

Figure 3. Changes in cellular morphology and distribution in different culture stages

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Figure 4. FACS and RT-PCR analysis of endothelial, hematopoietic and mesenchymal markers throughout ME-LC isolation stages

(A) hASC cultured in different media were tested for hematopoietic and endothelial markers (CD133, CD34, KDR, CD45 and CXCR4) and **(B)** mesenchymal surface markers (CD105, CD73 and CD90) by flow cytometry. **(C)** Comparative expression of surface antigens determined between HUVEC (grey bars), ME-LC cultured in EGM-2 (stage IV; striped bars) and SFO3 (stage III; black bars), and hASC cultured in DMEM-FBS (stage I; white bars) by flow cytometry. All data are expressed as mean

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Figure 5. Capillary network formation assay

(A) Representative light microscopy analysis of cells at different culture stages and HUVEC grown on Matrigel™- coated wells with EGM-2 medium. Pictures were taken at 4 hours, 24 hours and 7 days of culture. Pictures from one representative experiment of three independent experiments are shown. Scale bar = 200 μm. **(B)** Semi-quantification of the capillary formation index. Bars correspond to the percentage of the number of capillary-like structures comparatively to control (HUVEC) measured after 24 hours of culture on Matrigel™. All data from three independent experiments performed in duplicate are expressed as mean ± SE (** P<0.05 vs. HUVEC).

Figure 6. SDF-1 detection in culture supernatants

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Figure 7. Migration capacity of ME-LC

(A) Illustrative cartoon of the modified Boyden chamber experiment. **(B)** Confocal microscopy image of calcein AM-labeled cells that migrated through the filter using SDF-1 as chemoactractive cytokine after 12 hours. Scale bar = 100 μm. **(C)** Migratory effect of SDF-1, VEGF and HGF cytokines in ME-LC after 1 and 12 hours. The migration index was estimated dividing fluorescence data of ME-LC migration toward different cytokines respect to the control, which represent ME-LC migration when

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Figure 8. Cardiomyocyte differentiation potential of ME-LC

(A) Phase contrast microscopy of ME-LC treated with 10 µM of 5-azacytidine for 24 hours and then cultured in EGM-2 for 3 weeks. Image shows a cell with an increased size and the presence of branching fibers which are characteristics of myotube-like cells. Scale bar = 100 µm. **(B)** Immunofluorescence staining for the expression of cardiac markers in cells cultured in EGM-2 for 3 weeks after induction with 5-azacytidine. Top panels show the cytoplasmatic expression of ~~double staining for~~ Troponin-T-TRICT (red) and Desmin-FITC (green) in treated cells. Scale bar = 50 µm. Botton panels show a double staining with the cardiac markers α-Actinin-TRICT (red) and Desmin-FITC (green) which colocated in the cytoplasm. Scale bar = 50 µm. **(C)** Parallel and interconnected distribution of cardiomyocyte-like cells stained with different antibodies displaying the presence of bi and multinucleated cells. The small picture in the upper right corner represents the negative control. Nuclei are stained with DAPI (blue). Scale bar = 50 µm.

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	Rev: 5´-AAG TCA TCT TCC GAG CTT GG-3´	
CXCR4	Fw: 5´-AGA ACC AGC GGT TAC CAT-3´	174
	Rev: 5´-ATG CCA GTT AAG AAG ATG AT-3´	
Dll4	Fw: 5´-ACT ACT GCA CCC ACC ACT CC-3´	359
	Rev: 5´-CCT GTC CAC TTT CTT CTC GC-3´	
Cdk2	Fw: 5´-CCT GGC ACT GAG ACT GAG GG-3´	516
	Rev: 5´-CTC AGA ATC TCC AGG GAA CAG G-3´	
Flt-1	Fw: 5´-CAC CAA GAG CGA CGT GTG-3´	196
	Rev: 5´-TTT TGG GTC TCT GTG CCA G-3´	
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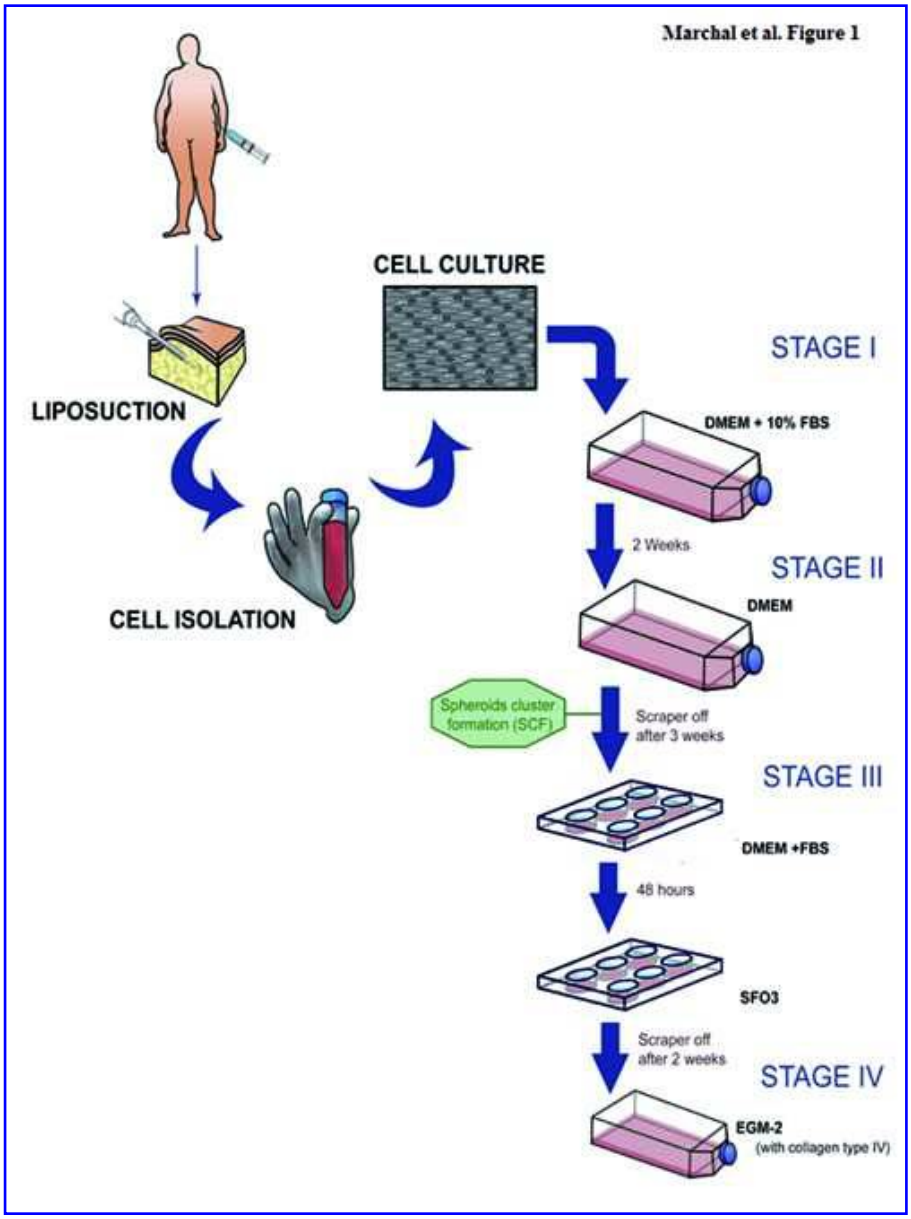

Cartoon representing the ME-LC isolation and purification procedure
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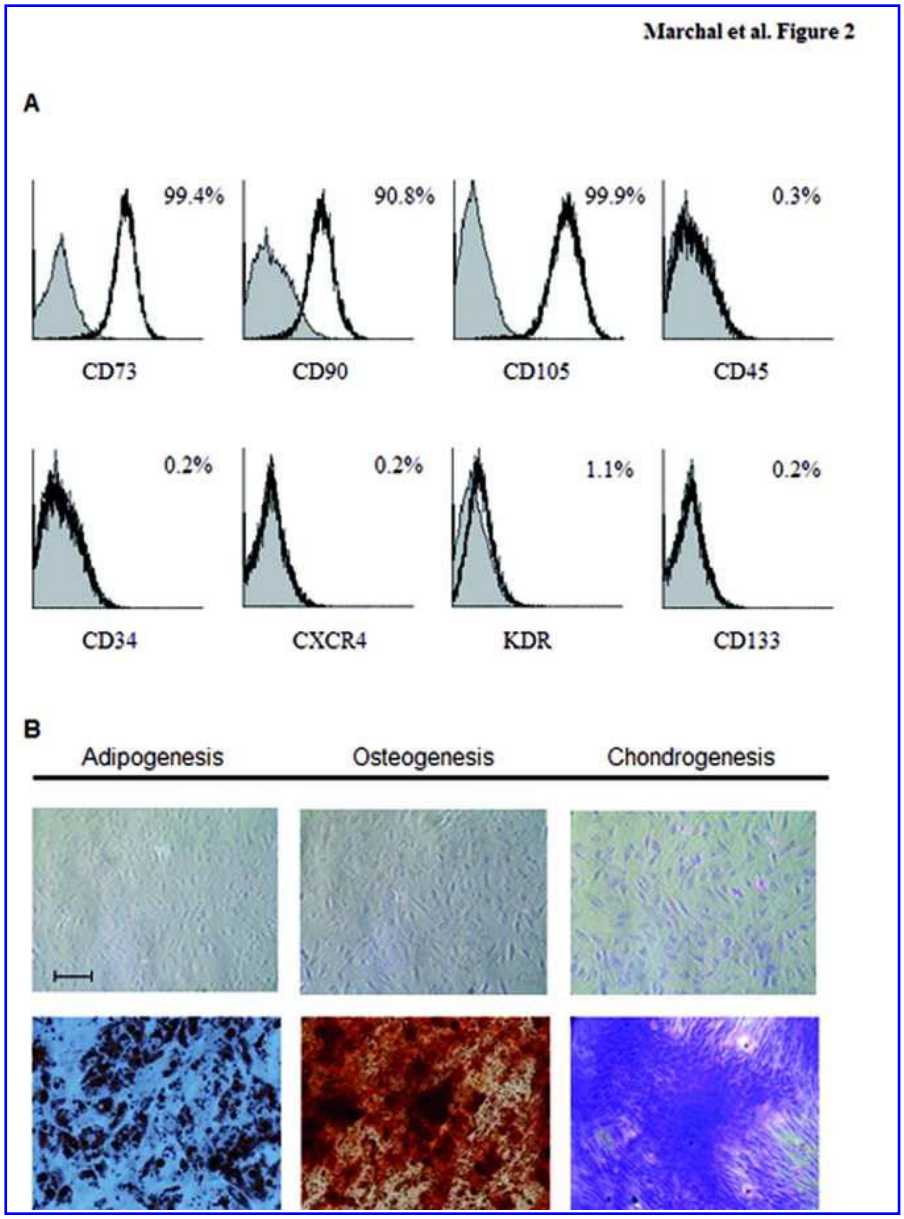

Figure 2. Phenotypic characterization and differentiation potential of hASC
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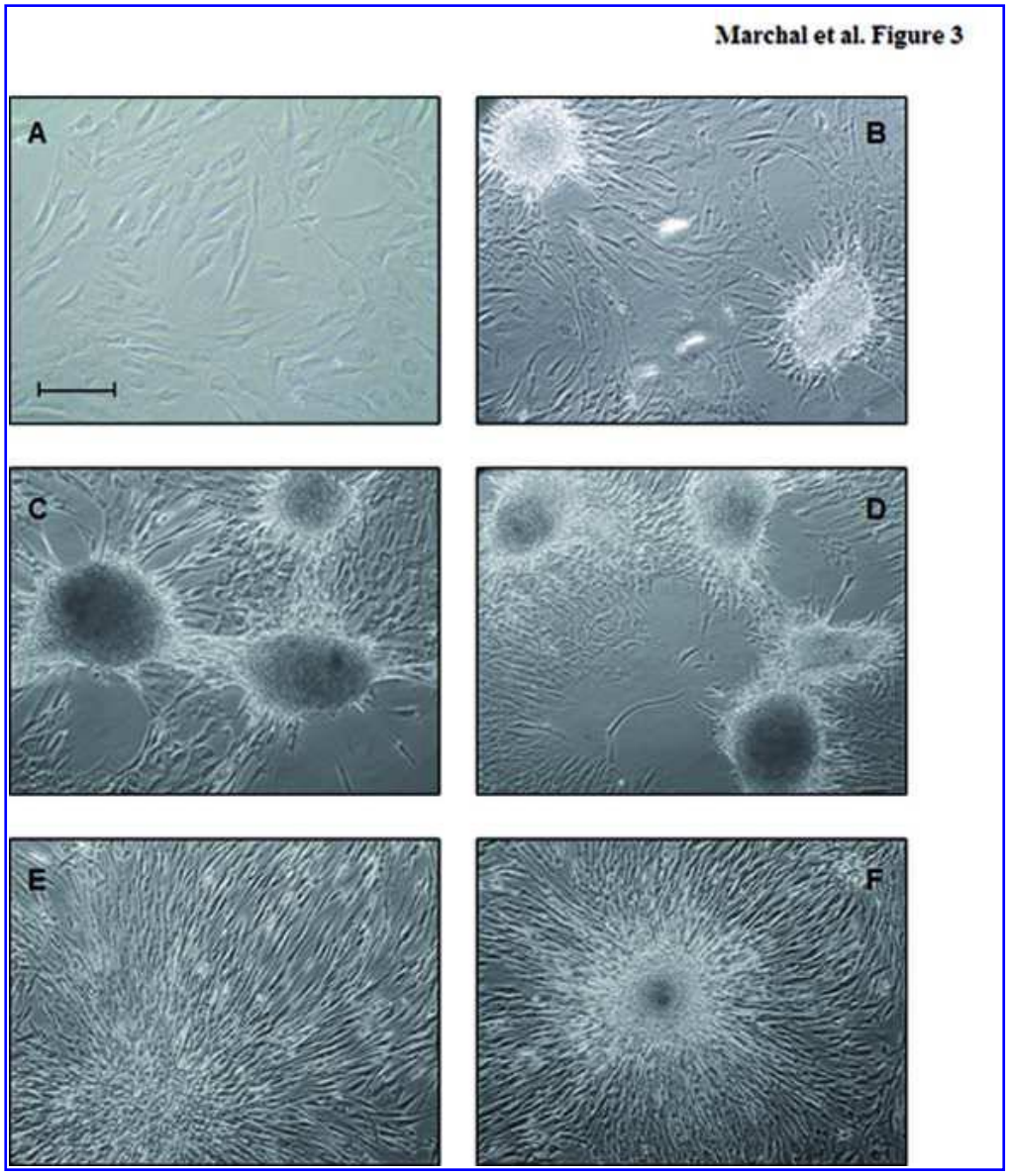


Figure 3.Changes in cellular morphology and distribution in different culture stages.
24x28mm (600 x 600 DPI)

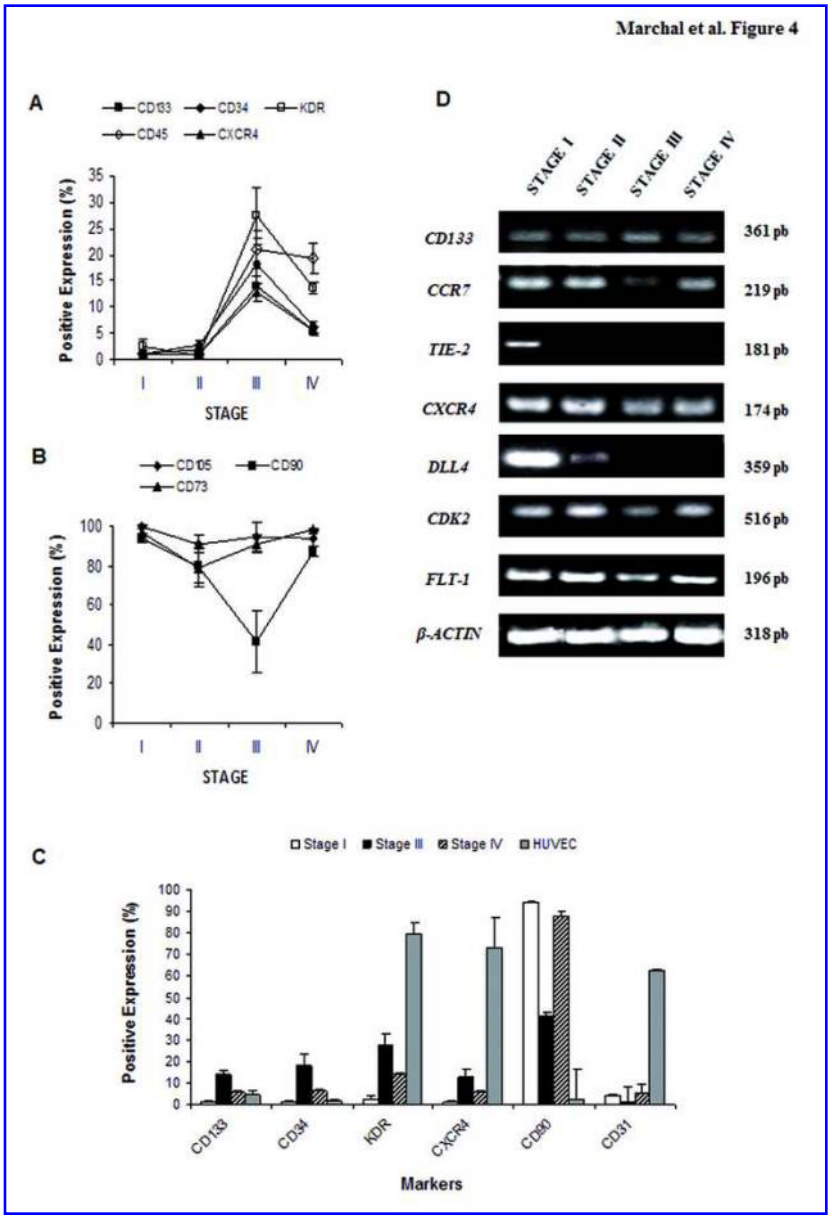


Figure 4.FACS and RT-PCR analysis of endothelial, hematopoietic and mesenchymal markers throughout ME-LC isolation stages
31x45mm (600 x 600 DPI)

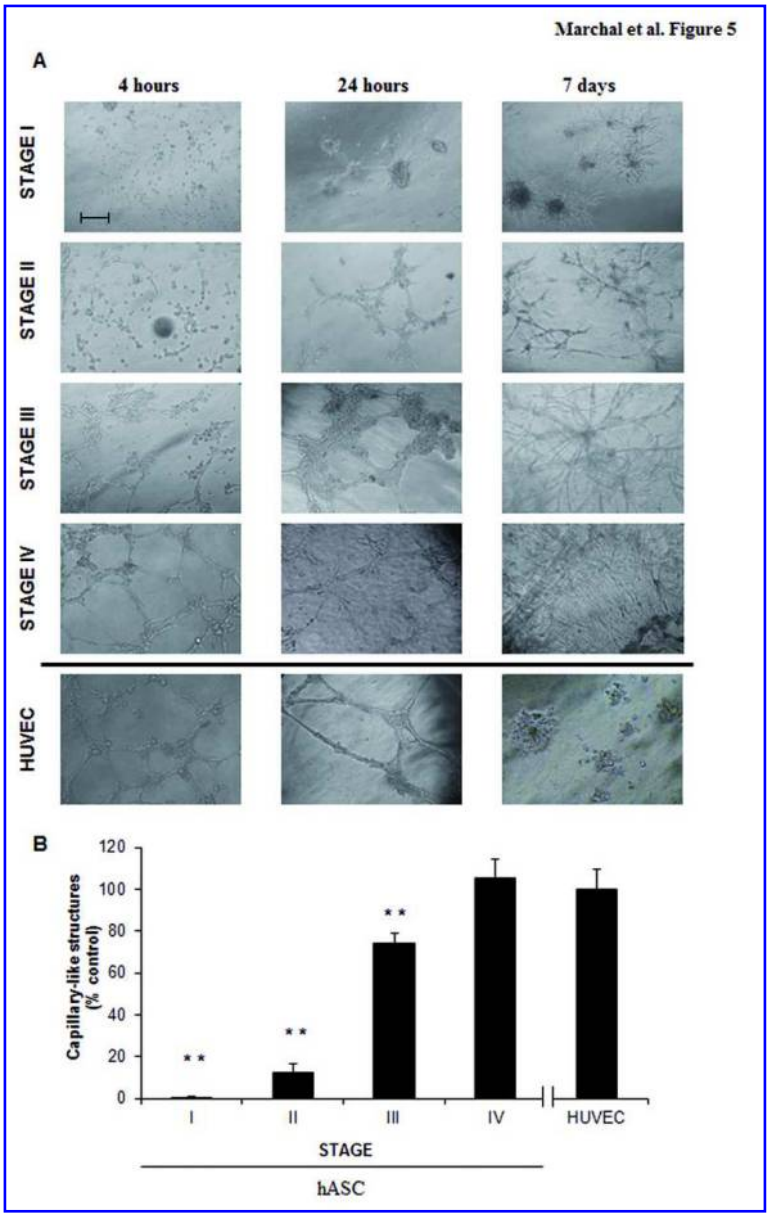
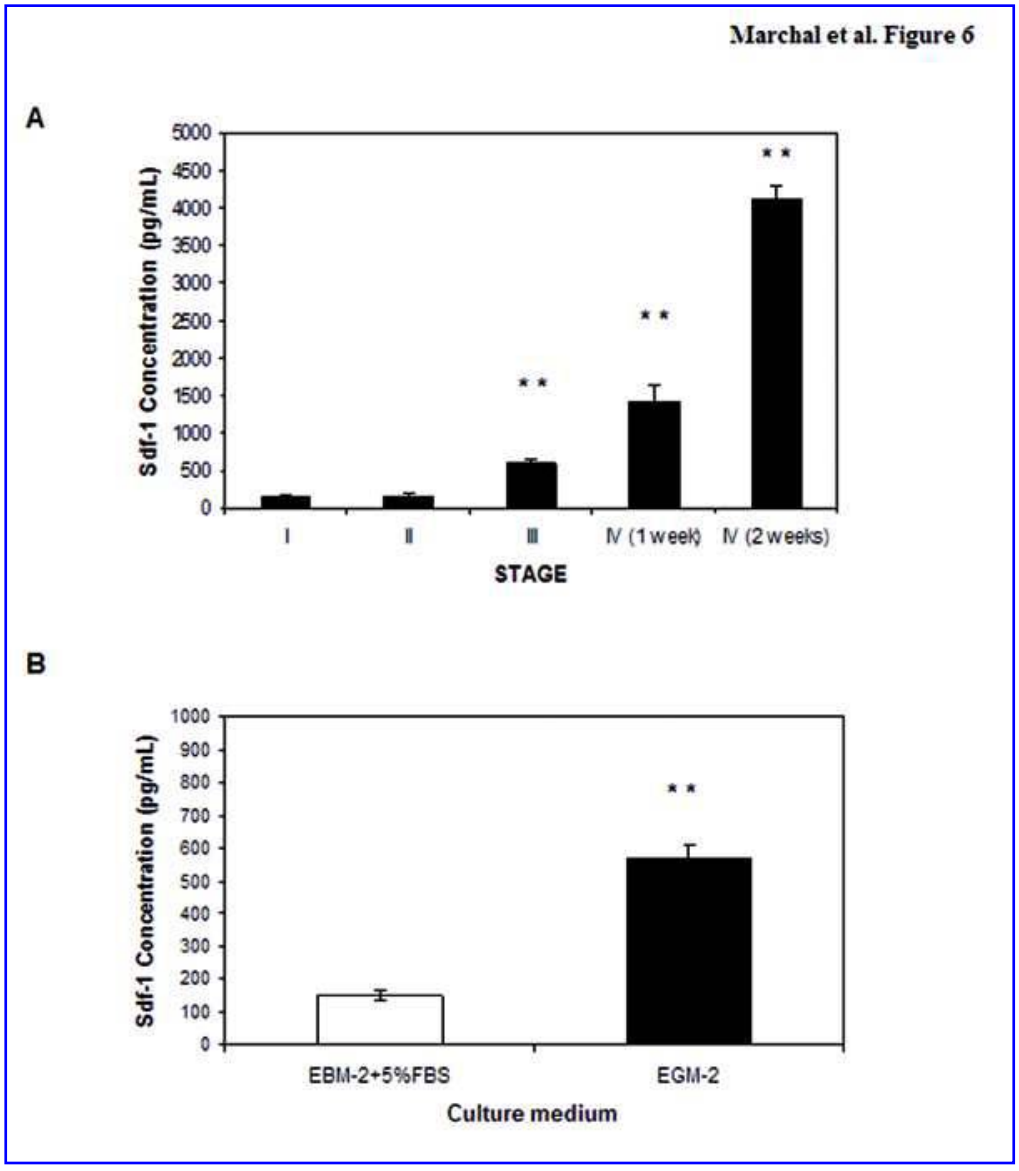


Figure 5. Capillary network formation assay
33x53mm (600 x 600 DPI)



SDF-1 detection in culture supernatants
25x29mm (600 x 600 DPI)

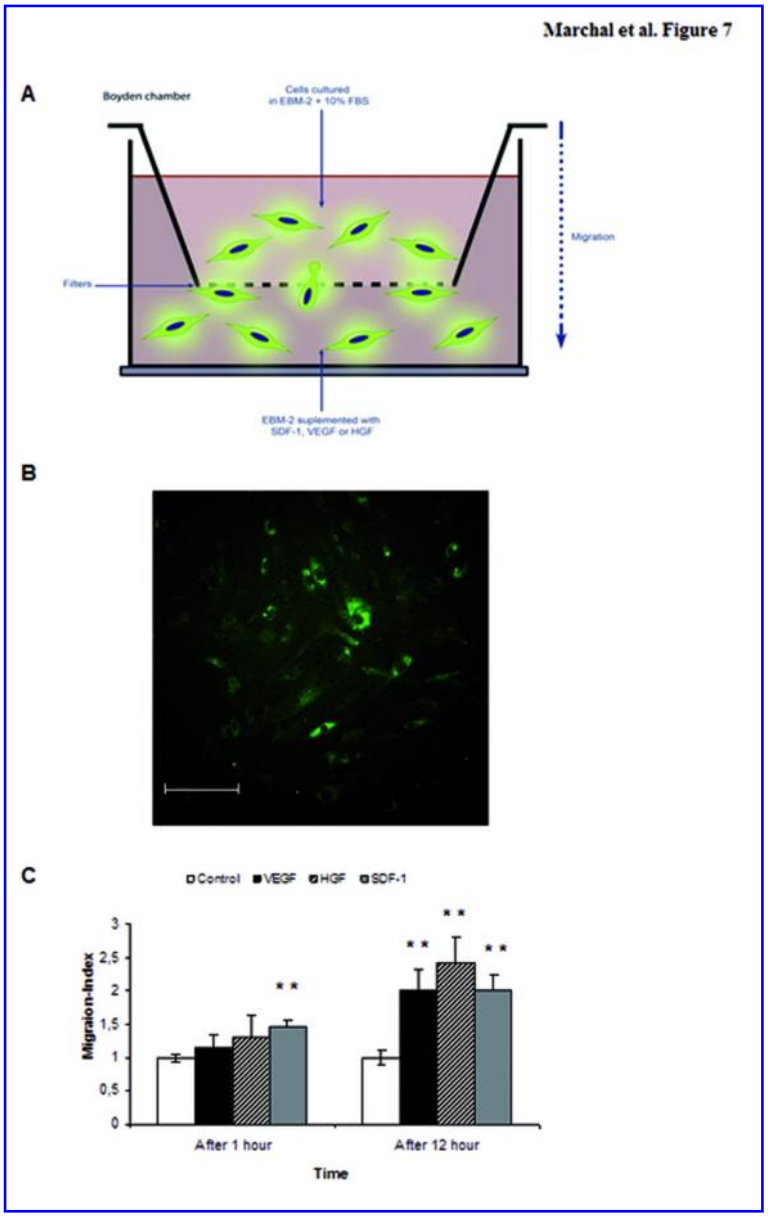
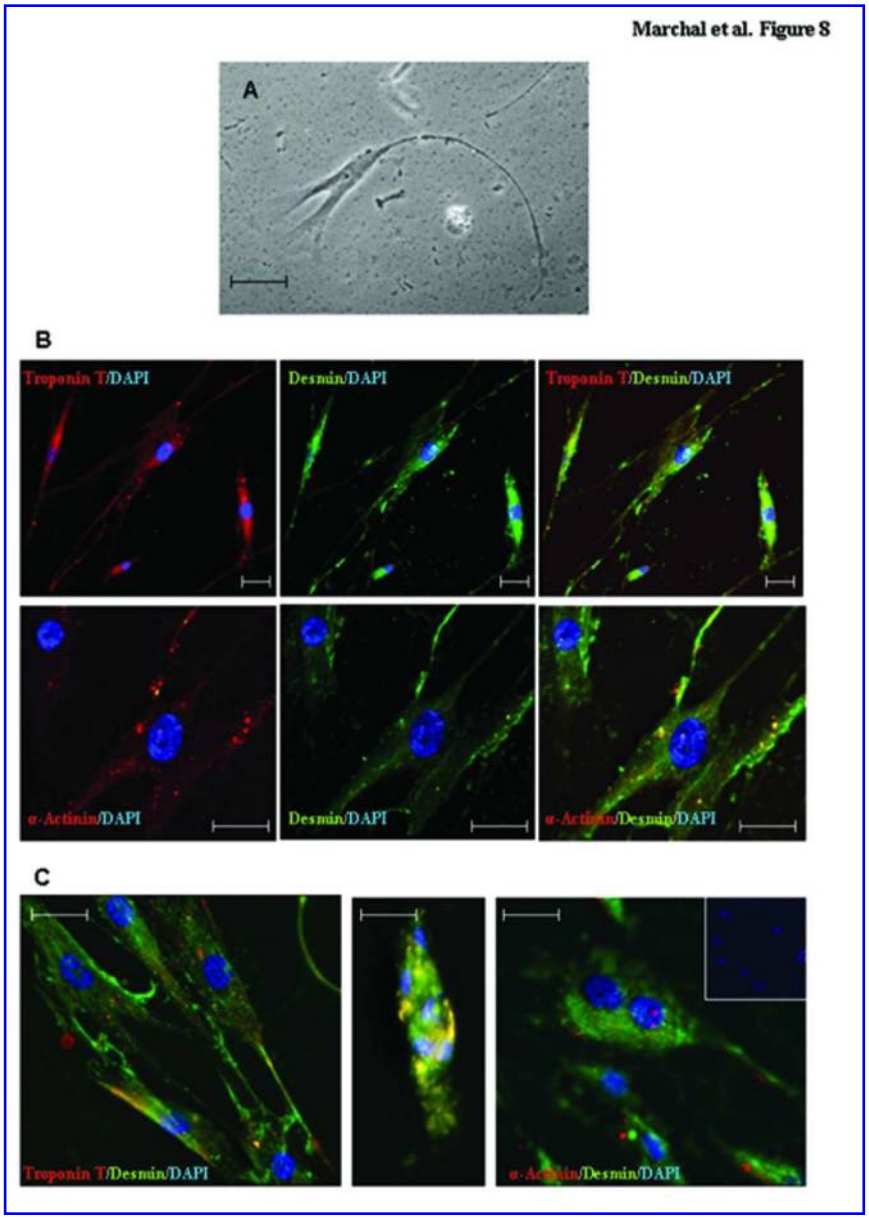


Figure 7. Migration capacity of ME-LC
33x54mm (600 x 600 DPI)



Cardiomyocyte differentiation potential of ME-LC
84x119mm (300 x 300 DPI)