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Do mesenchymal stem cells play a role in vocal fold fat graft survival?

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Abstract. *Objectives*: Adipose tissue in vocal fold lipoinjection is currently used to treat patients affected by laryngeal hemiplegia or anatomical defects. The aim of this study has been to evaluate the efficacy of this clinical strategy, by long-term follow-up of the patients and to investigate whether the fat samples used to treat them contain a stem cell population with a wide differentiation potential. *Materials and methods*: Fat samples harvested from 12 patients affected by severe breathy dysphonia who had undergone vocal fold lipoinjection were analysed by immunocytochemistry, by flow cytometry and reverse transcription-polymerase chain reaction, and the isolated adipose derived mesenchymal stem cells (ADMSCs) were evaluated in order to define their ability to produce soluble factors possibly involved in tissue regeneration, and to differentiate towards different lineages. *Results*: ADMSCs were efficiently and successfully isolated from all of the samples. They were positive for SSEA-4, an embryonic marker recently identified on bone marrow MSCs and which could explain their high differentiation plasticity. Molecular analysis showed that these cells also expressed Oct-4, Runx-1 and ABCG-2, which characterize the stem cell state, and a number of other specific lineage markers. Flow cytometry revealed mesenchymal markers expressed on ADMSCs and identified a subpopulation characterized by $CD146^{+}/34^{-}/45^{-}$ cells consistent with perivascular/pericyte-like cells. Osteogenic, adipogenic and endothelial tissue differentiation were obtained. *Conclusions*: Our results confirmed the therapeutic efficacy of this clinical approach and showed that adipose tissue, administered to patients in order to restore glottic competence, contains mesenchymal stem cells.

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INTRODUCTION

Mesenchymal stem cells (MSC) are an adherent, fibroblast-like cell population found in bone marrow (BM) as well as blood, muscle, dermis and adipose tissue. In this regard, fat is a source of uncommitted MSCs that can be easily expanded and, over the last few years, autologous fat has been used in some clinical applications for novel cell-based therapies (Zuk *et al*. 2002; Barry & Murphy 2004; Schäffler & Buchler 2007).

The main characteristic of adult stem cells is to give rise to specialized cell types of the tissue in which they reside, but they can also form specialized cells of other tissues. A number of studies have recently compared the properties of MSCs collected from BM, adipose tissue and cord blood (De Ugarte *et al*. 2003; Wagner *et al*. 2005; Kern *et al*. 2006). Adipose tissue is an ideal source of autologous stem cells, particularly in comparison with the traditional BM procurement procedure, as it is easily obtainable by lipoaspiration under local anesthesia with minimal discomfort for the patient, and its MSC content is adequate for clinical-grade cell manipulation in regenerative medicine.

One interesting clinical application is treatment of vocal fold scarring, which disrupts the viscoelastic layered structure of the lamina propria, increases stiffness of the vibratory structure and glottic incompetence, and is the most frequent cause of a poor voice after vocal fold injury. A number of new therapeutic strategies has been developed in animal models, including the use of synthetic extracellular matrix components, hydrogel constructs and hyaluronic acid, although the results obtained in vocal fold medialization are quite controversial and not completely satisfactory, mainly because changes in these molecules affect the viscoelasticity of scarred vocal folds (Hirano 2005). However, other strategies (including cell therapy) seem to have considerable therapeutic potential and, over the last 10 years, adipose tissue has been used to treat vocal fold medialization in cases of glottic incompetence mainly due to laryngeal hemiplegia (LH) (Shindo *et al*. 1996; McCulloch *et al*. 2002; Laccourreye *et al*. 2003; Cantarella *et al*. 2005).

The aim of this study was to explore whether fat tissue used to treat patients affected by LH or anatomical defects of the vocal folds contains a stem cell population that could be thought to be participating in long-term fat graft survival and in improvement in glottic competence, obtained in these patients after lipoinjection.

MATERIALS AND METHODS

Patients

The study involved 12 subjects aged 16–66 years, who were affected by severe breathy dysphonia due to LH ($n = 9$) or bilateral vocal fold defects (one case of scarring and two of sulcus glottidis). It was approved by our hospital's ethics committee, and all of the subjects gave their informed consent.

Surgical technique

Under strictly sterile conditions after the donor site had been infiltrated with lidocaine and epinephrine 1 : 100 000, fat tissue was harvested from the lower abdomen by means of curetting during suction under moderate negative pressure, using a 50-mL disposable syringe connected to a 2-holed 4.0 mm blunt cannula. Contents of the syringe were poured into one or two 10-mL test tubes (depending on the amount of fat), which were then centrifuged at 956 ***g*** for 3 min in

order to separate blood and liquid fat from the fat cells. The material forms three layers: an oily upper layer of ruptured adipocytes, a middle layer of fat cells and a lower layer of blood and lidocaine. The oily layer was gently aspirated with a needle, whereas the middle layer was collected and placed in the barrel of a pistol with a bayonet needle of 1 mm in diameter. Some of the fat was used for autologous injection into one to four sites of the vocalis muscle, under direct microlaryngoscopy, and some of the processed tissue was used for isolation and characterization of adipose derived mesenchymal stem cells (ADMSCs).

Evaluation of voice outcome

All patients underwent preoperative videolaryngoscopy, and a perceptual voice evaluation was made using yarameters of G (grade of dysphonia), R (roughness), B (breathiness), A (astenicity) and S (strain).

Patients also underwent measurement of maximal phonation time and multidimensional voice program acoustic voice analysis (MDVP), and were administered the voice handicap index questionnaire. The considered acoustic parameters were jitter (percentage), shimmer (percentage) and noise-to-harmonic ratio. All of the measurements were repeated 1 month after surgery and then at 3-month intervals; the mean duration of follow-up was 21 months (range 13–30).

ADMSC culture and expansion

Samples of lipoaspirate obtained from patients who received autologous transplantation were used for laboratory studies. Raw lipoaspirates were extensively washed with sterile phosphate-buffered saline (PBS; Invitrogen, Carlsdad, CA, USA) in order to remove contaminating debris and red blood cells, and then treated with 0.075% collagenase (type A; Roche, Mannheim, Germany) in PBS for 30 min at 37 °C with gentle agitation. Collagenase was inactivated by an equal volume of Dulbecco's modified Eagle's medium-low glucose (DMEM-LG) (Invitrogen) supplemented with 20% foetal bovine serum (FBS) (Biochrom AG, Berlin, Germany), and the suspension was centrifuged at low speed for 10 min. The stromal vascular fraction was re-suspended overnight in DMEM-LG/10% FBS/1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO, USA), after which the non-adherent fraction was removed, and adherent cells were cultured for 2 weeks (Zuk *et al*. 2002). Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2, and were passaged at 60–70% confluence. At passage 3, the ADMSCs were extensively characterized by flow cytometry, immunofluorescence and reverse transcription-polymerase chain reaction (RT-PCR), and their potentiality was evaluated by their ability to differentiate towards different lineages.

We also tested how different media influenced the fate of ADMSCs. After initial centrifugation, the lower-density solid phase was collected and digested at 37 °C for 45 min with 0.075% w/v collagenase. After neutralization, the stromal-vascular fraction was cultured in the presence of high-DMEM + 20% FBS (A) or EGM-2 (Cambrex, Walkersville, MD, USA) (B) for 1 week. The non-adherent fraction was removed after 24 h. After initial expansion, (A) cells were kept under the same culture conditions, whereas (B) cells were split and kept in EGM-2 (B) or cultured in the presence of high-DMEM + 20% FBS (C). In order to evaluate different commitment obtained using mesenchymal versus endothelial differentiation media, co-expression of *Ulex europaeus* agglutinin I (UEA) and CD146 was detected by immunocytochemistry.

RNA isolation and RT-PCR

Total RNA from 1×10^6 ADMSCs before and after differentiation was extracted using the RNeasy Mini Kit (Qiagen AG, Hilden, Germany), and contaminating genomic DNA was further eliminated by DNase (Qiagen) digestion according to the manufacturer's instructions. Total

Table 1. Primer sequences used for RT-PCR. The primers were constructed on the basis of published human sequences, and selected using version 1.5 of Primer Express software available from Applied Biosystems

Gene	Primer sequence	Gene	Primer sequence
Osteocalcin	F – gcagagtccagcaaaggtg	PDX1	F – ctgcctttcccatggatgaa
	R – atgtggtcagccaactcgtc		R – aagttcaacatgacagccagc
PPARgamma2	F – gctgaatccagagtccgctg	ABCG2	F – gcttgcaacaaccatgacgaa
	R – caaactcaaacttgggctcc		R – gccagttgtaggctcatccaa
MyoD	F – agcactacagcggcgact	PAX7	F – ctttgccgctaccaggagac
	R – gcgactcagaaggcacgtc		R – tcgatgctgtgtttggcct
Myf–5	F – cagtcctgtctggtccagaa	von Willebrand	F – tgtcaagigacgtgicagca
	R – gaactagaagcccctggag		R – gcagtagaaatcgtgcaacg
Myogenin	F – cagcgaaigcagctctcaca	Ve Cadherin	F – tgtctgtttgttgaggaccc
	R – agttgggcatggtttcatctg		R – aagtggtagaaaggctgctg
MRF4	F – ggctctcctttgtatccagg	CXCR4	F – aggtagcaaagtgacgccga
	R – cctlagccgttatcacgagc		R – aagtaccagtttgccacggc
Desmin	F – gatggaatacgacaccaga	Rex-1	F – aacatgagccagcaactgaag
	R – ggtaggtggcaatctccaca		R – agaaatcatcccctccgagag
CK18	F – gctggagagcaaaatccgg	FGF-4	F – ctactgcaacgtgggcatc
	R – cctccttgagagcctcgaict		R – acatgccggggtacttgtag
CK19	F – gaactccaggattgtcctgca	hTert	F – gagaacaagctgtttgcggg
	R – aaccaggcttcagcatccttc		R – ggcatctgaacaaaagccgt
alfa Feto Protein	F – ttccagaacctgtcacaagctg	Sox-2	F – accagaaaaacagcccgga
	R – tctcctctgcaacagtgctcat		R – tcatgagcgtcttggttttcc
Albumin	F – ggttgatgtgatgtgcactgc	Runx-1	F – tcactgtgatggctggcaat
	R – tcccttcatcccgaagttcat		R – ctgcatctgactctgaggctga
CYP1B1	F – gcctttatcctctctgcggaa	Oct-4	F – acatgtgtaagctgcggcc
	R – acaaagctggagaagcgcat		R – gttgtgcatagtcgctgcttg
CYP2B6	F – atggaaaccgctggaaggi	GAPDH	F – gcttgtcatcaatggaaatccc
	R – gccccaggaaagiatttcaaga		R – tccacacccatgacgaacatg

RNA was eluted in a final volume of 40 μL and its quality, integrity and size distribution was assessed by optical density (absorbance at 260/280 nm and ratio of >1.8). RT-PCR was performed using 800 ng of total RNA and GoTaq DNA polymerase (Promega, Madison, WI, USA), and 4 ng of cDNA were used for each PCR assay of different adipogenic (PPARgamma2), osteogenic (osteocalcin), myogenic (MyoD, myogenin, MRF4, Myf-5, desmin), hepatic (alfa Feto protein, albumin, cytokeratin (CK) 18, CK19, CYP1B1, CYP2B6), endothelial (VE-cadherin, von Willebrand factor) and stem cell markers (Oct-4, Runx-1, Rex-1, Sox-2, hTert, FGF-4, Pax-7, PDX-1, ABCG2, CXCR4); positive controls were obtained from the corresponding foetal tissues. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a normalizing housekeeping gene. Primers were constructed on the basis of published human sequences, and were selected using version 1.5 of Primer Express software available from Applied Biosystems (Foster City, CA, USA; Table 1). Each set of oligonucleotides was designed to span two different exons. The samples were loaded on 1.5% agarose gel.

Flow cytometric analysis

ADMSCs were extensively characterized by flow cytometry at passage 3, before starting any other characterisation or differentiation steps. They were washed in PBS solution and stained for 20 min at room temperature in the dark with the following directly coupled mouse–antihuman antibodies: CD45 PC7 (Beckman Coulter, Fullerton, CA, USA), CD34 PE (Becton Dickinson,

San Jose, CA, USA), CD146 FITC (Biocytex, Marseille, France), CD90 FITC (Becton Dickinson), α-SMA FITC (Sigma-Aldrich), NG2 PE (Immunotech, Marseille, France), CD133 PE (Miltenyi, Bergisch Gladbach, Germany), CD56 PE (Chemicon, Temecula, CA, USA), CD44 FITC (Sigma), CD105 PE (Biocytex), CD73 PE (Becton Dickinson), CXCR4 APC (Becton Dickinson), LNGFR PE (Becton Dickinson), HLA-ABC FITC (Becton Dickinson) and HLA-DR (Becton Dickinson). Cells were also incubated with the following primary mouse antihuman antibodies: BB9 (Becton Dickinson), Desmin (Dako Cytomation, Carpinteria, CA, USA) and SSEA-4 (Chemicon; 1 : 25) and, after washing with a secondary rat–antimouse antibody, IgG1 PE and FITC (Exalpha, Carlsbad, CA, USA). Isotype immunoglobulins IgG1 PE-FITC (Chemicon), IgG1 PC7 (Beckman Coulter), IgG1 APC (Becton Dickinson) were used as negative controls under the same conditions. When required, cells were permeabilized with 0.1% Triton X-100 (Sigma). After staining, they were washed once with PBS containing 0.1% of bovine serum albumin (Sigma). For each sample, at least 50 000 list mode events were acquired using a Cytomics FC500 (Beckman Coulter); all plots were generated using CXP analysis software.

Immunofluorescence assay

Passage 3 ADMSCs were plated on a Laboratory-Tek chamber slide (Nalge Nunc Int., Rochester, NY, USA), and were fixed with absolute methanol (–20 °C) for 5 min. After washing with PBS (Gibco, Grand Island, NY, USA), they were permeabilized with 0.1% Triton X-100 when required, blocked for 30 min at room temperature with PBS containing 2% of bovine serum albumin, and then incubated for 1 h in the dark with unconjugated primary mouse–antihuman monoclonal antibodies SSEA-4 (Chemicon; 1 : 25), von Willebrand factor (vWF, Clone F8/86, Dako Cytomation; 1 : 25) and with *Ulex europaeus* agglutinin I (Vector Laboratories, Burlingame, CA, USA; 1 : 50), and then conjugated with a secondary rat–antimouse antibody IgG1 FITC or PE (Exalpha). The same population was also stained with conjugated mouse–antihuman antibodies VE-cadherin FITC (Chemicon; 1 : 25) and CD146 FITC (Chemicon; 1 : 100). Cells were counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (Roche Diagnostic Corporation, Indianapolis, IN, USA) for 15 min in the dark at room temperature. Moreover, after endothelial differentiation and after culture with different media, they were characterized for von Willebrand factor and VE-cadherin, or for CD146 and UEA. FITC- and PE-conjugated goat antimouse IgG secondary antibodies (Chemicon, 1 : 1000) were used for the negative controls.

After incubation, coverslipped slides were examined using a fluorescence microscope (Eclipse 80i, Nikon, Tokyo, Japan), and micrographs were taken using DS camera control (DS-5 M, Nikon). Images were acquired using a Nikon Digital Slide DS-L1, and were merged and analysed using Adobe Photoshop 5.5 software (Microsoft Corporation, Washington DC, USA).

Multiplexed sandwich ELISA

A multiplexed sandwich ELISA that allows quantitative chemiluminescent measurement of seven proteins per well (HB-EGF, TPO, PDGF, KGF, HGF, FGF, ANG2; their lower detection limit was 1.8; 5.9; 1.0; 1.0; 3.1; 2.0; 4.9 pg/mL, respectively) (SearchLight proteome array, Pierce-Endogen Boston Technologies Center, Woburn, MA, USA) was used in order to evaluate ADMSCs' production of soluble factors. Briefly, supernatants were collected from confluent ADMSC cultures at passage 3 immediately stored at –20 °C until evaluation. Each well of the microplate was pre-spotted with target protein-specific antibodies and 50 µL of standards, and samples were added to the plate for one hour at RT with shaking at 200 r.p.m. After washing away unbound proteins, 50 µL of biotinylated antibodies were added to the plate for 30 min at RT with shaking at 200 r.p.m. Excess biotinylated antibodies was washed away, and 50 µL of

streptavidin-horseradish peroxidase (SA-HRP) was added to the plate for 30 min at room temperature with shaking at 200 r.p.m.

In order to identify the signal, we used SuperSignal ELISA femto chemiluminescent substrate (Pierce, Rockford, IL, USA); the luminescent signal was recorded within 10 min using a cooled CCD camera and amount of each target protein was analysed using Microsoft Excel 2000 (Microsoft Corporation) as recommended by the manufacturers. The culture medium, DMEM + 10% FBS, was also tested as a blank and the value obtained was subtracted from the amount of factor present in cell-conditioned medium.

Cell lineage differentiation

ADMSCs were differentiated into adipogenic, osteogenic and endothelial cell lineages. For differentiation experiments, passage 3 ADMSCs were cultured in different media, which were replaced twice a week.

In order to promote adipogenic differentiation, ADMSCs were plated at 20×10^3 cells/cm^2, and were cultured for 2 weeks in the presence of human MSC adipogenic induction medium (Cambrex). Medium was replaced every 3–4 days for 15 days. Cells were stained with Oil red O solution (Sigma) (three parts of stock solution 0.5% in isopropanol, and two parts distilled water), in order to detect the presence of lipid vacuoles.

For osteogenic differentiation, 20×10^3 cells/cm^2 were grown for 2 weeks in the presence of human MSC osteogenic medium (Cambrex). Osteocytes were stained with alizarin red in order to detect presence of calcium deposits.

For endothelial differentiation, 5×10^3 cells/well were cultured for 10 days on fibronectin chamber slides in the presence of DMEM (Invitrogen) and human vascular endothelial growth factor (PeproTech EC; 10 ng/mL), after which medium was replaced by EGM-2 medium (Cambrex) for 3 weeks. Immunofluorescence and RT-PCR procedures were performed after differentiation.

RESULTS

Clinical outcome

Videolaryngoscopy demonstrated improved glottic closure in all 12 patients and voice perceptual evaluation showed significant improvement in all three parameters of the GRBAS score: G ($P = 0.0197$), R ($P = 0.0115$) and B ($P = 0.0437$).

MDVP acoustic analysis revealed reduction in the mean values of jitter (from 4.48 ± 3.73% preoperatively to 2.16 ± 1.32%) and shimmer (from 11.01 ± 8.42% to 5.99 ± 3.07%), and mean noise-to-harmonic ratio changed from 0.18 ± 0.06 to 0.15 ± 0.05, thus indicating greater voice signal stability and reduced noise. Maximal phonation time improved in all cases, with mean values increasing from 7.08 ± 3.60 to 13.00 ± 5.62 s ($P = 0.0023$), and mean voice handicap index score significantly decreased from 67.08 ± 29.47 to 35.08 ± 24.41 ($P = 0.0004$).

All patients showed significant voice improvement. Results remained stable during the long-term follow-up (mean 21 months; range 13–30).

ADMSC characterization before differentiating treatments

ADMSC morphology

After expansion, all culture samples gave rise to MSCs with spindle-shaped morphology in confluent wave-like layers, which could be replated for at least 12 passages (Fig. 1a).

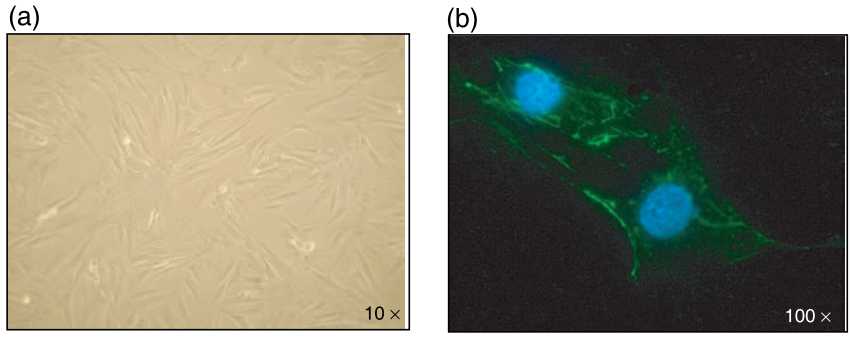

Figure 1. (a) Morphological appearance of human ADMSCs at passage 3: a heterogeneous population of cells with spindle-shaped morphology (×10 magnification). (b) Fluorescence image of ADMSCs after immunostaining with mouse–antihuman monoclonal antibody SSEA-4 (green). Nuclei counterstained with DAPI (blue) (×100 magnification).

Molecular profile

Reverse transcription-polymerase chain reaction analysis showed that ADMSCs were positive for desmin, osteocalcin, PPARgamma2, CK18 and CK19, CYP1B1, CYP2B6, von Willebrand factor and VE-cadherin, and negative for MyoD, myogenin, MRF-4, Myf-5, alphafoeto protein, albumin, PDX1, PAX7, CXCR4, FGF4, hTERT and SOX2. They also expressed Oct-4, Runx-1, Rex-1 and ABCG-2, which characterize the undifferentiated stem cell state (Fig. 2).

ADMSC phenotype

Interestingly, ADMSCs were positive for the embryonic marker SSEA-4, confirmed by flow cytometry, (Fig. 1b), and negative for endothelial markers such as von Willebrand factor and VE-cadherin.

Moreover, flow cytometry showed that they were positive for CD44, CD90, CD105, CD73, SSEA4 and HLA-ABC; weakly positive for CD34; and negative for CD56, CD133, HLA-DR, CXCR4, LNGFR, BB9 and desmin (Fig. 3). They also expressed CD146 (around 4%), α-SMA but not CD45 nor NG2, which characterize a multipotent adult stem cell population consistent with a perivascular/pericyte-like phenotype. The high level of α-SMA (Fig. 3) confirmed the ability of these cells to differentiate towards smooth muscle cell lineage, as recently reported by Lee *et al*. (2006b).

Multiplexed sandwich ELISA

ADMSCs secreted high levels of TPO, substantial amounts of KGF and HGF and only minimal amounts of HB-EGF, FGF and PDGF. No ANG2 was secreted (Fig. 4).

ADMSC expansion in the presence of different media

The aim of this part of the study was to determine how different media influence the fate and expansion of stem cells present in adipose tissue. To this end, the stromal-vascular fraction was cultured in the presence of: (A) DMEM; (B) EGM2 for 1 week, and then DMEM; or (C) EGM2. After 3 weeks, all cells were stained for CD146 and UEA in order to investigate possible differences of commitment. A few (A) cells were weakly positive for CD146 (Fig. 5aII) but negative for *Ulex* (Fig. 5aIII), and showed typical mesenchymal morphology (Fig. 5aI). A large

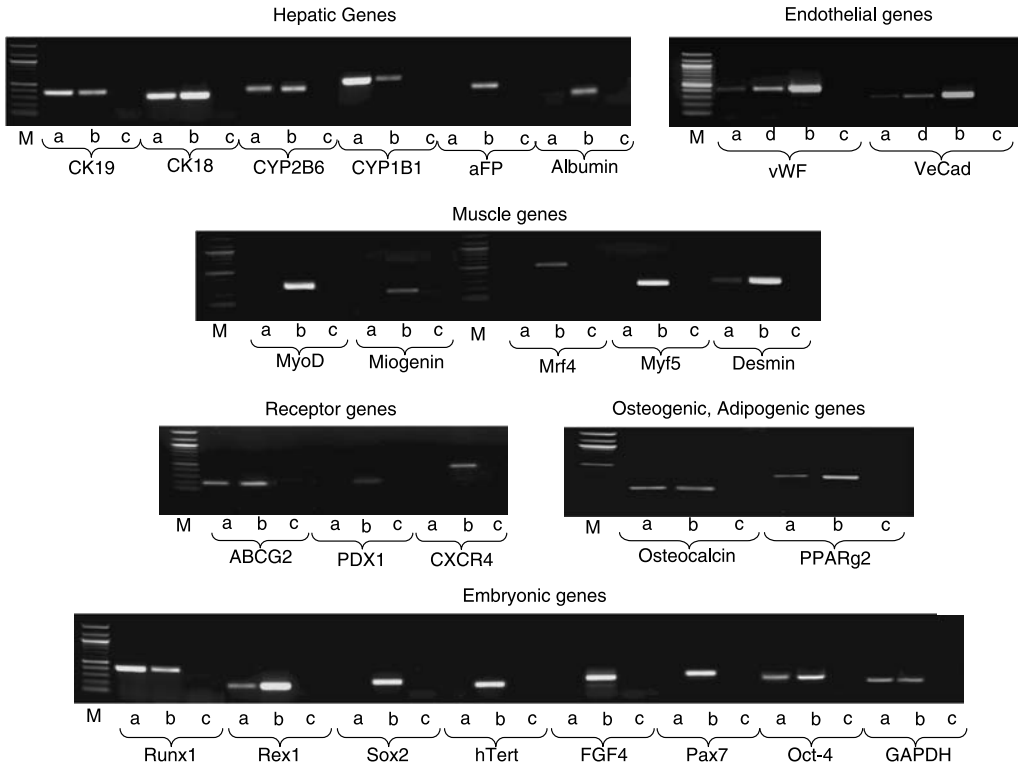

Figure 2. RT-PCR on total RNA. ADMSCs were characterized for expression of several hepatic, endothelial, muscle, receptor, osteogenic, adipogenic and embryonic genes. (a) Passage 3 ADMSCs. (b) Positive control obtained from corresponding foetal tissues. (c) Negative control. (d) ADMSCs after endothelial differentiation. M, Marker.

majority of (B) cells were strongly positive for CD146 (Fig. 5bII), negative for UEA (Fig. 5bIII) and their morphology was consistent with mesenchymal features (Fig. 5bI). Most of the (C) cells co-expressed CD146 and UEA (Fig. 5cII and 5cIII), and had typical endothelial morphology (Fig. 5cI).

ADMSC characterization after differentiating treatments

The potential of ADMSCs isolated from adult subcutaneous adipose tissue was demonstrated by culturing the cells in media promoting their differentiation into osteogenic, adipogenic and endothelial lineages.

Osteogenic differentiation

During osteogenic differentiation, ADMSCs showed extensive areas of mineralization indicated by staining with the calcium-specific marker alizarin red (Fig. 6a). RT-PCR showed that the cells were osteocalcin-positive.

Adipogenic differentiation

ADMSCs were induced to accumulate lipid vacuoles that stained positive with the triglyceride-specific dye oil red O and could be detected by light microscopy (Fig. 6b).

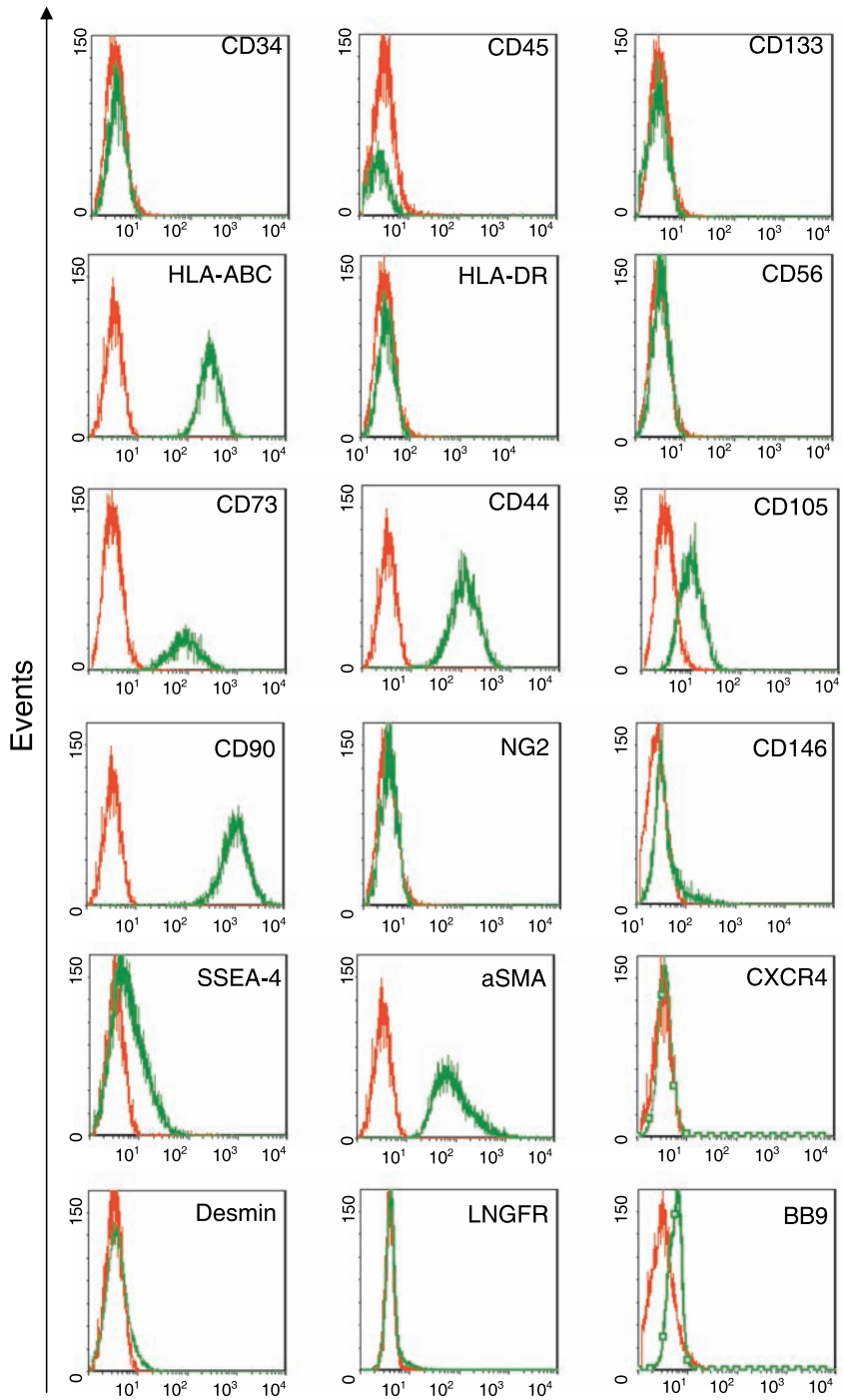

Figure 3. Flow cytometicy analysis. ADMSCs were extensively characterized by flow cytometry at passage 3, before starting any other characterization or differentiation.

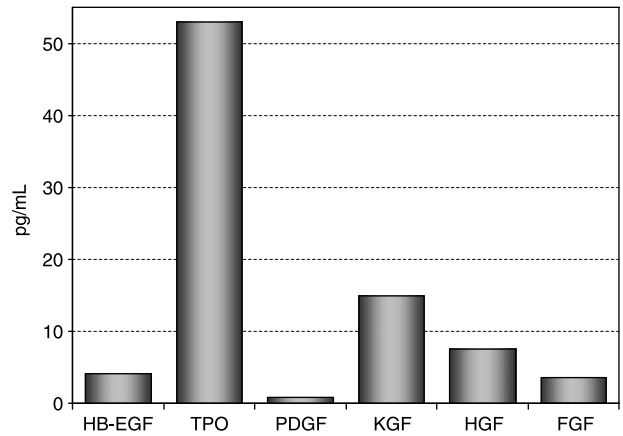

Figure 4. Secretion of HB-EGF, TPO, PDGF, KGF, HGF and FGF by ADMSCs at passage 3 was measured using a multiplexed sandwich ELISA.

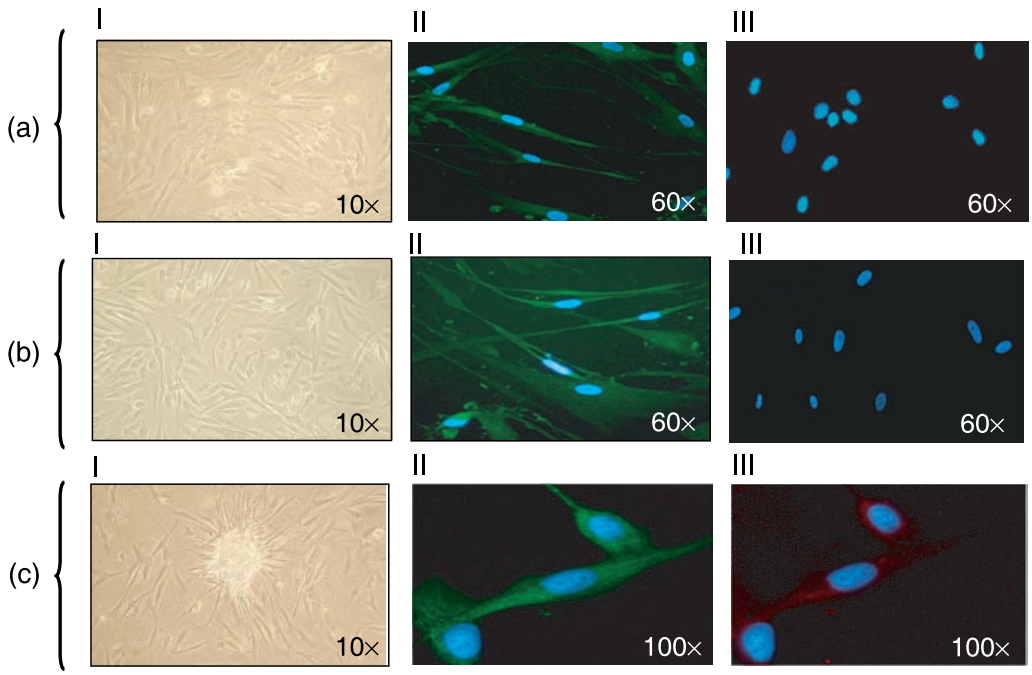

Figure 5. After culturing the stromal-vascular fraction for three weeks in the presence of different media, cells showed differing commitments. (a) A few cells were weakly positive for CD146 (Fig. 5aII, green) but negative for UEA (Fig. 5aIII, red) upon immunofluorescence (×60 magnification), and showed typical mesenchymal morphology (Fig. 5aI) (×10 magnification). (b) A large number of cells were strongly positive for CD146 (Fig. 5bII, green) and negative for UEA (Fig. 5bIII, red) on immunofluorescence (×60 magnification), and their morphology showed mesenchymal features (Fig. 5bI) (×10 magnification). (c) Most cells co-expressed CD146 and UEA (Fig. 5cII, green, and Fig. 5cIII, red) on immunofluorescence (×60 magnification), and had typical endothelial morphology (Fig. 5cI) (×10 magnification). Nuclei counterstained with DAPI (blue).

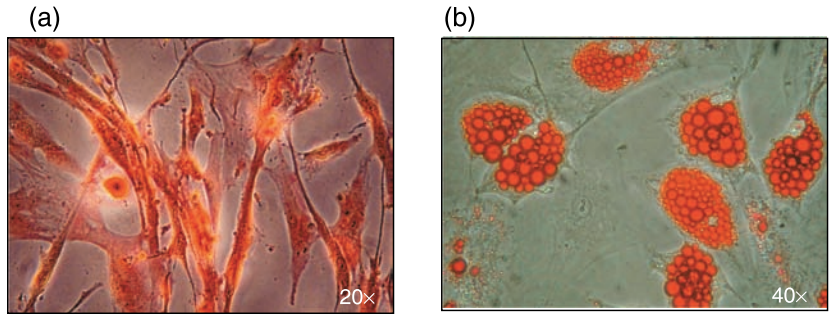

Figure 6. (a) Alizarin red staining showing an area of mineralization on ADMSCs cultured in osteogenic differentiating medium (×20 magnification). (b) Oil red O staining showing accumulation of lipid vacuoles in ADMSCs after adipogenic treatment (×40 magnification).

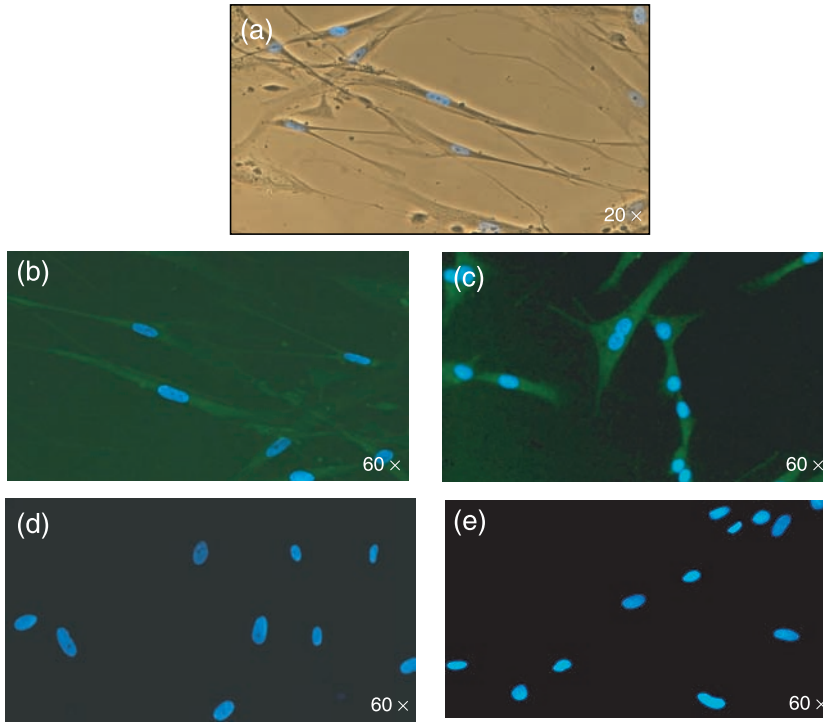

Figure 7. (a) Morphology of ADMSCs after exposure to endothelial conditioning medium (×20 magnification). Fluorescence image of ADMSCs after immunostaining with mouse–antihuman monoclonal antibodies for von Willebrand factor (b) and VE-cadherin (c). (d) and (e) are the negative controls. Nuclei counterstained with DAPI (blue) (×60 magnification).

Endothelial differentiation

After being cultured with specific endothelial differentiation media, ADMSCs showed typical endothelial morphology (Fig. 7a). Immunofluorescence indicated that they were positive for von

Willebrand factor and VE-cadherin, which are endothelial markers (Fig. 7b,c), thus confirming committed differentiation.

DISCUSSION

The aim of this study was to investigate whether adipose tissue in vocal fold lipoinjections used to treat patients affected by LH or anatomical defects, contains a stem cell population that may play a role in long-term fat graft survival. To this end, we isolated a stem cell population with mesenchymal features from adipose tissue samples of patients undergoing vocal fold repair, and explored its characteristics and multilineage potential.

First, we confirmed the presence of MSCs in the adipose tissue in accordance with recent findings (Zuk *et al*. 2002; Strem *et al*. 2005), and showed that the isolated cells expressed many genes typical of mesodermal and non-mesodermal lineages (Fig. 2) and a number of mesenchymal markers (Fig. 3), before the differentiation treatments. In particular, we identified a $CD146^{+}/34^{-}/45^{-}$subpopulation consistent with perivascular/pericyte-like cells, thus showing that adult human ADMSCs include a multipotential stem cell population that can be intimately associated with the cells surrounding blood vessels (Zannettino *et al*. 2008). It is worth noting that ADMSCs found in fat tissue were positive for some genes characteristic of embryonic and adult stem cells (Oct-4, Runx-1 and ABCG-2), which underline their possible multipotency (Fig. 2).

We also found that ADMSCs were positive for SSEA-4, an early embryonic glycolipid antigen that is commonly used as a marker of undifferentiated pluripotent human embryonic stem cells and embryos at the cleavage to blastocyst stage, and which have recently been identified in an adult BM mesenchymal stem cell population (Gang *et al*. 2007). This could explain their high differentiation plasticity (Figs 1a and 3).

Capacity for multilineage differentiation of the expanded human ADMSCs was also tested. After being cultured under specific osteogenic, adipogenic and endothelial conditions, they showed morphological and phenotypical characteristics of different mesodermal and non-mesodermal cell lineages. We also found that they secrete multiple soluble angiogenic factors involved in haematopoiesis, vasculogenesis and epithelial formation. This secretory profile shows that human ADMSCs offer an appealing cell-based approach that may have therapeutic effects in various clinical settings, as recently proposed and demonstrated in animal models (Nakagami *et al*. 2005; Rehman *et al*. 2007; Wu *et al*. 2007).

These results demonstrated that patients affected by LH or laryngeal defects and treated with this clinical approach based on fat tissue administration, showed restoration of normal voice levels confirmed by long-term follow-up.

Second, our findings demonstrated that all samples administered to these patients, contained MSCs exhibiting a high proliferative potential, very primitive stemness characteristics and an interesting secretion profile. Some recent *in vivo* animal model studies confirm that damaged tissues such as injured vocal folds can be regenerated by MSC tissue engineering (Kanemaru *et al*. 2003, 2005; Hertegard *et al*. 2006; Lee *et al*. 2006a). These recent data and our findings, taken together, could let us hypothesize that these ADMSCs found in fat tissue could play a role even in human tissue regeneration.

However, it is crucial to investigate appropriate MSC culture conditions (i.e. media, sera and further parameters) for clinical application very carefully (Mannello & Tonti 2007), as we have demonstrated that different culture reagents can give rise to different cell phenotypes. Given all

of the above, human ADMSCs are a highly flexible and very promising potential source of multipotent cells that can be used for regenerative somatic cell therapy in a variety of vocal fold disorders, as well as for tissue engineering.

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