Isolation and Characterization of a Spontaneously Immortalized Multipotent Mesenchymal Cell Line Derived from Mouse Subcutaneous Adipose Tissue

Running title: A spontaneously immortalized mesenchymal cell line

Isolation and characterization of a spontaneously immortalized multipotent mesenchymal cell line derived from mouse subcutaneous adipose tissue

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A B S T R A C T

The emerging field of tissue engineering and regenerative medicine is a multidisciplinary science, based on the combination of a reliable source of stem cells, biomaterial scaffolds and cytokine growth factors. Adult mesenchymal stem cells are considered to be important cells for applications in this field and adipose tissue has revealed to be an excellent source of them. Indeed, Adipose derived Stem Cells (ASCs) can be easily isolated from the stromal vascular fraction (SVF) of adipose tissue. During the isolation and propagation of murine ASCs we observed the appearance of a spontaneously immortalized cell clone, named m17.ASC. This clone has been propagated for more than 180 passages and stably expresses a variety of stemness markers, such as Sca-1, c-kit/CD117, CD44, CD106, islet-1, nestin and nucleostemin. Furthermore these cells can be induced to differentiate towards osteogenic, chondrogenic, adipogenic and cardiogenic phenotypes. m17.ASC clone displays a normal karyotype and stable telomeres, does not proliferate when plated in soft agar, nor give rise to tumors when injected subcutaneously in NOD/SCID–γ^{null} mice. The analysis of gene expression highlighted transcriptional traits of SVF cells. m17.ASCs were genetically modified by lentiviral vectors carrying GFP as a marker transgene and efficiently engrafted in the liver, when injected in the spleen of NOD/SCID–γ^{null} monocrotaline-treated mice. These results suggest that this non-tumorigenic spontaneously immortalized ASC line may represent a useful tool (cell model) to the study differentiation mechanisms involved in tissue repair as well as a model for pharmacological/toxicological studies.

INTRODUCTION

Adult stem cells are present in most of the tissues and are able of self-renewal and multipotency, contributing to tissue homeostasis. Among adult stem cells, the mesenchymal ones have a greater availability and plasticity, likely candidating them for applications in regenerative medicine [1]. These cells were originally isolated from bone marrow and more

recently, from the stromal vascular fraction (SVF) of adipose tissue [2]. This is an attractive stem cell source because of the simple and repeatable access to adipose tissue, the basic enzyme-based isolation procedures, and the relatively larger available stem cell amounts compared to bone marrow [3,4,5,6]. Several comparative studies have shown that Adipose-derived Stem Cells (ASCs) and those from bone marrow display similar properties concerning cell-surface expression profile, differentiation potential, and therapeutic efficacy [7,8,9,10,11]. The SVF at early passages is composed by a heterogeneous cell population consisting, besides stem cells, also of endothelial, smooth muscle cells, pericytes, fibroblasts, mast cells, and pre-adipocytes [3,4,5,12]. Noteworthy, within few *in vitro* passages a relatively homogeneous population of ASCs can be isolated. Although antigenic identity of this cell population remains controversial, there is a general consensus for the expression of a set of surface markers, such as Sca-1, c-kit/CD117, CD44, CD90, CD73, CD29, CD105 and the absence of CD31 [3,4,5,10,13].

Despite ASCs have been studied since more than 10 years, some aspects for their use in a safe regenerative medicine still require further investigation [5,15,16]. In particular, because of their stem nature, concerns have arisen on their possible tumorigenic potential [17,18,19], which can be unleached if cells stand long-term culture, and is generally accompanied by chromosomal instability [20,21,22,23]. It has been suggested that this risk could be reduced, or even abolished, when stem cells are induced towards differentiation [24]. Although many *in vitro* differentiation protocols have been established, at present this field is still under active investigation. For example, stem cells can now be used in cell therapy applications to obtain cell sheets or 3D proto-tissues under controlled conditions [25,26,27,28,29,30]. The latter are dictated by different local environment factors, such as growth-differentiation factors, adhesive molecules within the extracellular matrix, the properties of a possible supporting scaffold, as well as genetic programming and stochastic events [31,32,33,34,35,36,37,38,39,40,41]. All these studies may require a significant amount of

primary cells and an extensive *in vitro* expansion, but ASCs generally undergo "replicative senescence" and irreversible growth arrest after a few replication cycles [42]. Alternatively, these cells can stand long-term culture, but are affected by chromosomal instability and loose multipotency, making them not suitable for regenerative medicine application [43]. The availability of a stable cell line model could facilitate studies aimed to describe the contribution of different factors in tissue plasticity and would add knowledge in the field of multipotent stem cell differentiation and moreover could also be used to investigate its pharmacological modulation.

Herein, we report the characterization of a spontaneously immortalized cell line, named m17.ASC, derived from adipose tissue of adult FVB/N mice. This cell line expresses stemness markers, has a normal karyotype, is devoid of transforming and tumorigenic potential and, most interestingly, is multipotent. When compared with SVF, m17.ASCs display similar gene expression profile. Furthermore, when GFP-transduced m17.ASC cells were injected in the spleen of syngeneic mice, they efficiently engrafted in the liver of transplanted mice, candidating this cell line as a valuable stemness model.

MATERIALS AND METHODS

Cell isolation, cloning, culturing and transduction

SVF was isolated from minced s.c. and epididymal/parametrial fat pads of 11-week-old mice (FVB/N strain, Charles River, Calco, Italy) and digested with 0.1% type I collagenase (Worthington Biochemical, Lakewood, NY) in PBS at 37°C for 1h. After filtration through 30-μm strainers (MiltenyiBiotec GmbH, Bergisch Gladbach, Germany) and centrifugation for 10 min at 1500 rpm at 4 °C, floating adipocytes were removed, the SVF pellet was treated with erythrocyte lysis buffer (154 mM NH 4 Cl, 20 mMTris pH7.5). Cells were sorted by immunomagnetic procedure with Sca-1 antibodies (MiltenyiBiotec) and plated in Claycomb medium (Sigma-Aldrich St. Louis, MO, USA), supplemented with 2mM L-glutamine, 10%

fetal bovine serum, (FBS, Lonza Ltd, Basel, CH), 100 IU/ml penicillin, and 100 µg/ml streptomycin. Cells were regularly splitted when subconfluent at a ratio 1/3, and medium was changed every 2-3 days. After the second passage some cells were plated at low density (50-200 cells/ cm^2). A single cell colony was detected after 1 week by microscope inspection, which was then grown and cloned twice. This cell line (m17.ASC) can be passaged 1/8-1/10 every 3 days. Population doubling time, calculated by using the formula from Cristofalo [44], as well as telomeres length were evaluated in a 32 days time span. For these analysis m17.ASC ($1x10^5/cm^2$) were sequentially transplanted every three days. In parallel the same procedure was adopted for freshly isolated primary cells at p1, which were used as reference. m17.ASCs were transduced with a Lentiviral Vector (LV) for the expression of Green Fluorescent Protein (GFP) under the control of the phosphoglycerate kinase promoter. L929 fibroblasts (ATCC CRL-2148), NIH-3T3 (ATCC, CRL-1658^{TM}), NIH-3T3-MET-EC$^-$ were also used [45].

Cytofluorimetric analysis

Cells, detached with 5 mM EDTA, were incubated for 20 min on ice with PE-labelled anti-Sca-1, anti-c-kit (anti-CD117) (Biolegend, San Diego, USA), anti-CD90 (ImmunoTools, Friesoythe, Germany) or FITC-labelled anti-CD44, CD106, CD-31, CD34, CD45, F4/80 antibodies (Biolegend), as suggested by manufacturer's indications. Cells were fixed in buffered 1% paraformaldehyde (PAF), 2% FBS and analyzed in a FACScalibur flow cytometer (BD Biosciences, Buccinasco, Italy).

RT-PCR analysis

Total cell RNA was extracted in Trizol® reagent (Invitrogen, Monza, MB, Italy), followed by DNase treatment (DNAse I, Fermentas, St. Leon-Rot, Germany). Then, 1 µg of RNA was retrotranscribed in cDNA with the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas) using oligo(dT) primers. PCR reactions were performed using the PCR Master

Mix 2x kit (Fermentas) in a final volume of 25 µl containing 50 ng cDNA and 200 nmol/l primers (listed in Table 1). PCR conditions were the following: 94°C 2 minutes, 35 cycles 94°C for 30 seconds, specific annealing temperature for 30 seconds (see Table 1), 72°C for 30 seconds and 72°C for 7 minutes. Twentyfive cycles were performed for GAPDH. The amplified products were resolved by 2% agarose gel electrophoresis, stained with ethidium bromide and documented with GelDoc system (Bio-Rad Laboratories, Milan, Italy). The quantitative RT-PCR was performed on the cDNA from control and differentiated cells in a 20-µl total volume containing 1X SYBR green PCR master mix (PROMEGA), 0,5 ìM forward and reverse primers 50 ng of cDNA. Quantitative PCR were performed by incubation at 95°C for 3 minutes and 40 amplification cycles of 95°C for 15 seconds and then 60°C for 1 minute.

Telomere length determination

Genomic DNA was extracted with Gres whole blood DNA extraction kit (InCura, Cremona, Italy). Real-time PCR was used to assess average telomere length ratio. Telomeric DNA amount was normalized to that of the acidic ribosomal phosphoprotein PO (36B4), as house-keeping gene. The analysis was based on Callicot method [46]. Briefly, telomere assay was performed in triplicate for each treatment in a 25 µl reaction volume containing 20 ng genomic DNA, 12.5 µl EVA Green SMX (BioRad), 300 nM each forward and reverse primers for telomeres and 300 nM and 500 nM forward and reverse primers for 36B4, in an automated CFX96 thermocycler (BioRad). The reaction conditions for telomere were 95 °C for 10 min followed by 30 cycles of data collection at 95 °C for 15 seconds and a 56 °C anneal–extend step for 1 min. For the 36B4 portion, the reaction conditions were set at 95 °C for 10 min followed by 35 cycles of data collection at 95 °C for 15 s, with 52 °C annealing for 20 s, followed by extension at 72 °C for 30 s.

Results were analyzed with BioRad CFX Manager and exported to Excel (Microsoft, Redmond, WA) for calculation. For each data point T/S value was calculated: threshold cycle values (Ct) were determined from semilog amplification plots (log increase in fluorescence versus cycle number) for telomere (T) and 36B4 gene (S). The relative ratio of telomere

repeat copy number to the 36B4 single copy gene copy number (T/S ratio) for each time point was calculated as:

$[2^{Ct(telomere)} / 2^{Ct(36B4)}]^{-1} = 2^{-\Delta Ct}$, and related to the primary cell line telomere T/S at passage 3 by using the formula: $2^{-(\Delta Ct\ m17.ASC\ -\ \Delta Ct\ primary)} = 2^{-\Delta\Delta Ct}$ (Figure 5c; $p>0.1$). Samples with a T/S >1.0 had an average telomere length ratio (ATLR) greater than that of the primary cell line.

Differentiation protocols

Cells were plated onto 35 mm dishes ($2x10^4$ cells/cm^2) and cultured in adipogenic medium (Lonza), or osteogenic medium (DMEM, FBS 10%, 50 µg/ml ascorbic acid, 10 mMβ-glicerophosphate, 10 nM dexamethasone), which were changed every 3 days. After 14-20 days, cells were washed in cold PBS, fixed with 4% PFA in PBS and stained with Adipored (Lonza) or 40 mM Alizarin Red S, pH 4.1 [47]. The presence of lipid vacuoles was visualized under fluorescence microscope, while the production of calcium deposits was examined in light microscopy. For chondrogenic differentiation, $2.5x10^5$ cells were cultured as "pellet" for 40 days in 15 ml centrifuge tubes in Chondrogenic Differentiation Medium (Lonza). Medium was changed every second day. Cells were then washed, fixed as above, included in OCT (Fisher, Hampton, NH, USA) and frozen at -80° C. Five µm sections were cut, fixed again as before, washed, stained with 1% Alcian Blue in 3% acetic acid, pH 2.5 for 30 min and observed at light microscope. Sections were also fixed in 3% PAF for 20 minutes, stained with goat antibody against collagen II (Santa Cruz Biotechnology Inc., California, USA 1:200), in PBS, 1% bovine serum albumin (BSA), 4% FBS for 2 h at room temperature, followed by secondary Alexa Fluor® 546 donkey-anti-goat-IgG antibody (Invitrogen, 1:500) for 45 minutes at RT. Nuclei were stained with TO-PRO3 (Invitrogen, 1:100) and observed by Leica SP2 laser scanning confocal microscope. For cardiomyogenic differentiation m17.ASC were co-cultured with neonatal cardiomyocytes (nCMs) isolated from hearts of 1-3-day-old FVB/N mice, as in the manufacturer's instructions (kit by Worthington Biochemical

Corp). Briefly, immediately after isolation, cells were pre-plated for 2 h 30 min to recover the non-adherent-enriched fraction of nCMs, which were then seeded on fibronectin (2 μg/mL, Sigma-Aldrich), laminin (0.2%), gelatin (0.02%) pre-coated plates or pre-coated glass chamber slides (BD Biosciences). m17.ASC, labeled with DIIC12(3) fluorescent dye (BD Bioscences), were seeded directly onto neonatal cardiomyocytes (1:10 ratio) in complete medium and co-cultures were carried on for 7 days. m17.ASC and nCMs alone were used as negative controls. Co-cultures were also set up with cells isolated from the SVF at their second passage and nCMs. Cells were fixed in 3% PAF for 20 minutes, permeabilized in 0.2% Triton X 100 in PBS for 10 minutes, and incubated with rabbit antibodies against GATA-4 (1:400), MEF2c (1:100) (Abcam, Cambridge, UK) or monoclonal antibodies against Troponin T and alpha sarcomeric actinin (Abcam, 1:100), in PBS, 1% bovine serum albumin (BSA), 4% FBS for 2 hours at room temperature, followed by secondary Alexa Fluor® 488 goat-anti-rabbit-IgG antibody or secondary goat-anti-mouse fluorescein isothiocyanate (FITC)-labeled antibodies (Abcam, 1:500) for 45 minutes at room temperature. Nuclei were counter-stained with DAPI (Sigma-Aldrich, 1:200) and observed by Leica DMI6000B microscope. Positive cells were counted in at least 12 independent fields against total nucleated cells.

Karyotype analysis

Chromosomal analysis of m17.ASCs was carried out starting from passage 10, every 10 passages up to passage 108, as previously described [48]. Briefly, cells undergoing active division were blocked at metaphase by colchicine and actinomycin D, detached, centrifuged and the pellet was resuspended in hypotonic solution (0.075 M potassium chloride). Cells were then fixed in Carnoy-fixative and stained with 0.06% Wright's stain (Sigma-Aldrich) pH 6.8. Unmounted slides were examined using Nikon Eclipse 1000 light microscopy and photographed with Genicon (San Diego, CA, USA) software. Thirty high-quality G-banded

metaphases were selected each time. The chromosomes were classified according to the Standard Karyotype of the Mouse (Committee On Standardized Genetic Nomenclature For Mice, 1972)[49].

Analysis for anchorage-independent proliferation

Cells ($5x10^3$/well) were seeded in 12-well plates in semisolid medium (0.3% agar - Agar Noble, Sigma-Aldrich, in DMEM 2% FCS). NIH-3T3 and NIH-3T3-Met^{EC-} cells were used as negative and positive controls [45]. Medium was replaced weekly. After 3 weeks, the colonies were stained with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma-Aldrich], photographed with Versadoc imager (Biorad, USA) and counted with Quantity One colony counting software (Biorad).

Gene Expression Analysis

Total RNA was extracted using miRNeasy kit (QIAGEN, Milan, Italy) and quality controlled using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). Microarray analysis was performed using MOUSEWG-6_V2 Beadchips (Illumina, San Diego, CA), according to standard protocols. Raw data were processed and cubic-spline normalized using the GenomeStudio software (Illumina, San Diego, CA). Analysis for selection of differentially expressed genes and dot plot generation was carried out with Excel (Microsoft, Redmond, WA).

***In vivo* experiments**

For the tumorigenicity assay 5×10^6 GFP-m17.ASC cells at passage 120 were injected subcutaneously into the posterior flank of 5 NOD/SCID-γ^{null} female mice. Two $x10^6$ of B16 murine melanoma cells, and of A549 human lung adenocarcinoma cells were injected as positive controls. Mice were inspected twice a week for up to 10 weeks.

For the engraftment experiments six-seven week old NOD/SCID-γ^{null} male mice were treated with monocrotalin (200 mg/Kg, i.p. injection; Sigma-Aldrich), a toxic pyrrolizidine alkaloid of plant origin causing lung and liver endothelial injury, and next day they 2 x 10^6 GFP-m17.ASC were infused by intrasplenic injection [50]. Mice received cyclophosphamide (20 mg/Kg twice a week, Sigma-Aldrich) to blunt immune response to GFP expressed in transplanted cells. The animals were observed daily, and killed at different time points (from 1 week up to 6 weeks) after cell injection. Livers were fixed in 4% PAF, embedded in OCT, and frozen in liquid nitrogen-precooled isopenthane. Cryostat sections (5 µm thick) were postfixed with 4% PAF, blocked with 5% goat serum (Vector Laboratories, Burlingame, CA), 1% BSA, 0.1% Triton X-100 in PBS, and incubated with rabbit anti-GFP (Invitrogen, 1:400), and rat anti-CD31 antibodies (BD Pharmigen, BD Biosciences, Milan, Italy;1:50), followed by Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 546 goat anti-rat IgG (Invitrogen, 1:500) secondary ones respectively, and TO-PRO3 (Invitrogen). Tissue sections were observed at Leica SP2 laser scanning confocal microscope. All procedures were carried out in accordance with the European Community Directive for Care and Italian Laws on animal experimentation (Law by Decree 116/92).

Statistical analysis

The results are shown as mean ± standard deviation (SD) and significance was calculated by unpaired *t*- test. The values are considered significantly different when $P < 0.05$. The number of replicated experiments performed is given as *n*.

RESULTS

Characterization of the m17.ASC clone

Cells isolated from the SVF of s.c. adipose tissue after immunomagnetic selection for Sca-1 appeared as a homogeneous population highly positive for Sca-1 in immunofluorescence.

Sca-1 expression in this cell population declined after few passages and cells generally displayed morphological signals of senescence and stopped to proliferate after 7-9 passages. At the second passage cells were plated sparsely (50-200 cells/cm^2 for a total of 180 cm^2) and a colony emerged from a single cell. This original colony was cloned twice and it has been propagated since then for more than 2 years of continuous culture and named m17.ASC. It displays fibroblast-like morphology (Figs. 1a). m17.ASCs were constantly analyzed for the expression of different stemness markers, namely Sca-1, c-kit, Islet 1, nestin, and nucleostemin in RT-PCR (Fig 1b) and no differences were observed at the different passages. They were constantly positive for all these markers. By contrast, cells from primary culture, which at the beginning expressed the same markers, although some (nestin and nucleostemin) at lower levels, displayed the tendency to lose their expression from the very first passages. The expression of the embryonic stemness cell markers Nanog, Oct4, Sox2, Klf4 and Myc was found to be negative (Nanog, Oct4), very low (Sox2), or medium-low (Klf4, Myc) both with RT-PCR and microarray analysis (data not shown). Cytofluorimetric analysis confirmed the expression of Sca-1 and c-kit/CD117 in m17.ASC cells (Fig. 1c-d). Moreover these cells expressed also the mesenchymal markers CD44, CD106 at high levels. They were negative for the endothelial marker CD31, the leukocyte markers CD34 and CD45 and the macrophage marker F4/80. The L929 fibroblasts, used as control, do not express any of the stemness markers, except nucleostemin (Nst).

The m17.ASC line displays multipotency and can be driven towards the cardiomyogenic phenotype

This spontaneously immortalized cell line displayed the multilineage potential of MSC. Indeed, when cultured in appropriate differentiation media, m17.ASCs acquired features of the osteogenic, adipogenic, and chondrogenic phenotypes, as they were specifically stained with alizarin red, adipo-red and Alcian blue and the tissue-specific collagen II antibodies,

respectively (Fig. 2a,). Moreover, quantitative RT-PCR analysis showed that the respective lineage markers osteocalcin, adiponectin and aggrecan were up-regulated upon the differentiation treatments (Fig. 2b).

In view of the fact that in regenerative medicine cells with cardiomyogenic properties may be particularly interesting, we focused on the possibility of driving m17.ASC multipotency towards a cardiomyogenic phenotype. When co-cultured with murine neonatal cardiomyocytes, DIIC12(3)-labelled m17.ASCs displayed a marked up-regulation of GATA-4 and de novo expression of Mef2c (Fig. 2c, d). Also adherent cells from the SVF of adipose tissue, from which the m17.ASC clone was derived, became positive for the expression of GATA-4 and MEF2c, when co-cultured with neonatal cardiomyocytes with similar efficiency (Fig. 2c). In the same kind of experiment few co-cultured DilC12(3)-labelled m17.ASCs acquired the expression of alpha-sarcomeric actin and troponin T (Fig. 2d). All together these results indicate that m17.ASC have the same multipotency as the bulk population from which they were derived.

Karyotypic analysis of the m17.ASC line

m17.ASC G-banding analysis did not revealed any macroscopic chromosome alterations of cultured cells overtime, if compared with normal mouse karyotype. Cell karyotype analysis performed on two different *in vitro* passages (p10 and p73) are shown (Fig. 3a). Analysis at passage 108 gave identical results (not shown).

Cell duplication and telomere length

Population doubling time and telomeres length were evaluated in m17.ASC sequentially at several times. Both the clone, which was analyzed from passage 82 to passage 91 (p82-p91) and the primary cultures, which were analyzed from p1 to p10, displayed a constant doubling time of 32 hours up to the fifth passage (Fig. 3b). After this passage, doubling time of

m17.ASC remained stable, while primary cells showed a progressive increase. In this sequence of passages (p82-91) of m17.ASC cells, as well as in others (from passage 27 to passage 32, from passage 43 to passage 49, from passage 107 to passage 113) telomere length was evaluated and compared to that of the primary cells (SFV) at passage 1. Telomeres displayed a variable length, but the overall mean length was rather constant throughout p27 to p113, displaying a variability within +/- 40%, when compared to reference SVF at p1. This behaviour is in line with what reported also in other systems, such as germinal cells with a modulated telomerase activity.

The m17.ASCs maintain the properties of a normal immortalized cell line, and do not display a transformed or a tumorigenic phenotype

Cell immortalization is often accompanied to transformation and acquisition of a tumorigenic phenotype. m17.ASC cells were tested for the ability to grow in anchorage-independent conditions and thus plated in soft agar. Cultures were monitored for 21 days: the monitored m17.ASC colonies were in the same range of those obtained from the NIH-3T3 negative control (Fig. 3d). By contrast transformed NIH-3T3-Met-EC^{-}, which were used as positive controls, gave a significantly higher number of colonies. To further corroborate this *in vitro* finding, cells were tested for their ability to form tumors in NOD/SCID-γ ^{null} mice, which are immunocompromised allowing the growth of allo- and xeno-tumors. In none of the five animals the GFP-m17.ASC injected cells could induce a tumor in a period of 10 weeks. By contrast B16 murine melanoma cells and A549 human lung adenocarcinoma cells induced the formation of tumors within 2 weeks after cells injection. In these cases animals had to be killed for ethical reason before 4 weeks (tumors bigger than 2 cm in the two measured directions) (data not shown). It can thus be concluded that this cell line behaves as a normal immortalized cell line and is devoid of transforming or oncogenic potential.

Global gene expression

Global gene expression profiles obtained with DNA microarrays revealed that m17.ASCs and SVF cells have closely related transcriptomes ($R^2 = 0.805$). However, a fraction of genes displayed cell-specific expression, defined as significant detection p-value only in one of the two cell types, and different thresholds of fold-change. When the threshold was set at a threefold gain, out of 45,281 probe sequences analyzed, 414 gave signal only in SVF cells and 235 only in m17.ASC, which became 82 and 23 respectively for a threshold of tenfold change. Additional probes gave detectable signal in both cells but at different levels: 481 with more than 3-fold change (238 up- and 243 down-regulated in m17.ASCs) and 41 with more than 10-fold change (23 up- and 18 down-regulated in m17.ASCs; see Supplementary Table for full gene lists). The four categories of differentially expressed genes are illustrated in Figure 5a. From the above data it can be concluded that about 2.5% of the genes underwent an expression change or gain/loss of > 3-fold, and only 0.4% of the genes was more than 10-fold changed. The fact that more genes were lost than gained is not surprising since the SVF is more heterogeneous than the clonal m17.ASC line.

m17.ASCs were found to up-regulate or ex-novo acquire the expression of genes positively regulating cell growth or other activities usually found associated to stem and progenitor cells, such as migration, morphogenesis (i.e., UP: Histones Egln3, Hist1h2an, Hist1h2ak, Hist1h2ao, Mdk, Rpl29, Tgfbi; GAINED: Esm1, 9230117N10Rik, Mcm6, Cxcl12, Ercc5, Gcnt1, Enpp2). Conversely these cells were found to down-regulate or lose the expression of genes negatively regulating the cell cycle (i.e., DOWN: nhba, Cryab, Gadd45g (-8.96), Il11, LOST: IL-11, Cxcl14, Gas6, H19, cdkn1c). Moreover m17.ASCs down-regulated or lost the expression of genes involved in specific cell differentiated phenotypes, such as the keratins and different junctional proteins typical of epithelial tissues (Krt1-14, Krt1-17, krt7, cldn4, Gjb3) or axon guidance and typical of nervous tissues (Sema7a, Ncam).

In-depth gene analysis was carried on to disclose whether genes related to immortalization, such as c-myc, bcl2, cyclin D, telomerase, were up-regulated in m17.ASC cells as well. However, no evidences emerged in favour of this possibility, their expression level being the same in the three cell types tested (Ccnd1: high; c-Myc: medium-low; bcl-2:low; mTert: negative) (data not shown). A similar analysis was performed relative to the up-regulation of oncogenes (Wnt family, Fzd family, Stat family, HIF1) and the down-regulation of tumour suppressor genes (Rb, p53) and also in this case no clear evidences were found for changes between the three cell types, because oncogenes were generally found to be expressed from very low to medium levels and tumour suppressor genes were found always expressed at medium-low or medium-high levels (data not shown).

Due to the relevance in obtaining cells to be adopted for cardiac tissue engineering, and the difficulty in culturing adult mesenchymal stem cells, SVF were cultured in a medium suited for cardiomyocytes. On this basis we expected that the clone could acquire signatures of cardiac cells. We thus carried out expression profiles also of neonatal cardiomyocyte (nCM) preparations for comparison. Indeed, the m17.ASC transcriptome turned out to be more correlated to nCMs (R^2 = 0.87) than to SVF (R^2=0.80). Interestingly, as illustrated in Figure 5b, expression changes between m17.ASC and SVF cells were found to be highly correlated to expression changes observed between nCMs and SVF, in particular when genes with more than 10-fold change between m17.ASC and SVF were selected (R^2=0.73; Figure 5b top). These data indicate that long-term culture in specific conditions can drive partial commitment towards the cardiomyocyte phenotype, although further stimuli are required for a complete differentiation.

The m17.ASC line can engraft in NOD/SCID-γ^{null} mice

In regenerative medicine the ultimate goal is to transplant cells capable to engraft *in vivo*, proliferate and provide a therapeutic benefit. In order to explore the *in vivo* properties of GFP-

m17.ASC, NOD/SCID-γ ^{null} mice, in which adaptative immune response lacks and innate immune response is impaired and thus GFP positive cells should not rejected by the immune system, were transplanted with these cells by intrasplenic injection. At different time points animals were killed, and their liver sections processed for immunofluorescence staining with anti-GFP and anti-CD31 antibodies. Cells did engraft and persisted until 6 weeks, the longest time tested , and could be found in the liver parenchyma and in the proximity of vessels (Fig. 5), a location which correspond to their original location in the stromal adipose tissue from where they originated. Few cells were found to be positive for CD31, a recognized endothelial cell marker. It can thus be concluded that m17.ASC cells can engraft in vivo, persist there for at least 6 weeks and can start to differentiate towards an endothelial phenotype.

DISCUSSION

Adult stem cells are a key component for regenerative medicine. The aim of the researchers involved in regenerative medicine is to reproduce the right environment, mimicking as much as possible the *in vivo* situation of a living organism, where tissues are formed and renewed under the influence of spatially and temporally orchestrated physical and chemical stimuli [25].

In vitro tissue engineering is a relatively young discipline, and, although great advances have been obtained, many investigations are going on to refine and improve these complex biomimetic constructs. Many materials are being developed for scaffolds for structural substrate and able to release mechanical and structural signals. The role of scaffold design is particularly evident when stem/progenitor cells are used to fabricate architecturally complex tissues, such as the myocardium [26,27,33]. It is thus clear the advantage to have a standardized and easily available source of stem cells endowed of multipotency, which can be unlocked and guided towards specific histotypes by the physical and chemical cues provided by the environment in which they are cultured *in vitro*. The studies involving stem cells

usually require a significant amount of primary cells and an extensive *in vitro* expansion. Adult stem cells are particularly difficult to expand and keep in culture for long time, even those from adipose tissue, and although different culture conditions have been suggested by many researchers [51,5], no continuous lines with these properties have been established. Herein the properties and some of the possible applications of a cell line, named m17.ASC, stabilized with appropriate culture conditions from a clone of spontaneously immortalized murine adult mesenchymal stem cells, are reported. The m17.ASCs originated from cultures of the stromal vascular fraction (SVF) of adipose tissue, selected for the expression of the stemness marker Sca-1. This cell line has been fully characterized and both *in vitro* and *in vivo* behaviours have been described. The peculiar characteristics of m17.ASC have been analysed in their complex in order to prove its potential and safety. The main peculiarities of m17.ASC described are their stemness properties, i.e. indefinite self-renewal and multipotency. Stem cells from murine adipose tissue, as well as from bone marrow, usually undergo senescence within few culture passages. To overcome this limit, some Authors have proposed to immortalize cells from bone marrow with mTERT, thus inducing constitutive telomerase activity and telomere elongation [52,53,54]. However, these cells acquired also transformed and tumorigenic activities, when inoculated in syngeneic recipient animals [17,19,55]. By contrast m17.ASCs behave as normal, as testified by their inability to proliferate in anchorage-independent conditions or as tumors in NOD/SCID-γ^{null} mice, although they are able to maintain a stable proliferation, probably sustained by a substantial telomere stability. Moreover both the karyotype and gene expression analyses did not demonstrate any typical tumor-associated alteration. In line with the not transformed nor tumorigenic properties of m17.ASC cells, global gene expression analysis revealed that no up-regulation of oncogenes, neither down-regulation of tumor suppressor genes were detectable. These data strongly exclude the above mechanisms as causative of m17.ASC immortalization.

Although m17.ASCs did not grow as tumors in NOD/SCID-γ ^{null} mice, they could engraft in the liver when transplanted in vivo through intraspleenic injection and persisted there for at least 6 weeks, the last time point examined, in which the number of transplanted cells increased with some level of proliferation. Few cells appeared to have undertaken the differentiation pathway towards endothelial cells, since they expressed the CD31 marker. Thus the m17.ASC maintained the multipotency typical of the SVF from which it originated, being able to differentiate in osteogenic, chondrogenic, adipogenic and cardiogenic phenotypes *in vitro*, and endothelial phenotype *in vivo*.

In order to propose this cell line as a good source of adult stem cells able to stably replicate, we also analyzed if long term culture (to date more than two years of continuous culture) or cryopreservation would modify its phenotype. The results confirmed that m17.ASC maintained a stable expression of different stemness markers and substantially maintained the transcriptional features of the original SVF cells. Only a minor fraction of the sequences analyzed underwent qualitative or quantitative changes, with an increased or de novo expression of genes positively regulating cell growth or other activities usually found associated to stem/progenitor cells, such as migration and morphogenesis, and a decreased or lost expression of genes involved in specific differentiated cell phenotypes. As already discussed above, no evidence of up-regulation of oncogenes, nor down-regulation of tumor suppressor genes was detected. Telomere length displayed some variability, which, however, was in the range of the one observed in the SVF at their first passages. This finding is congruent with what is observed in stem or progenitor cells, such as germinal cells, and is indicative of the telomere maintenance [56]. On the contrary in most cancer cells with high or low proliferation rate telomere length is greatly reduced or significantly elongated respectively, due to a constitutive expression of telomerase [57].

The successful stabilization of the clone might also be due to the particularly rich culture medium used, originally developed for cardiomyocytes. For this reason cells acquired an

expression profile somehow closer to neonatal cardiomyocytes, still maintaining, however, their multipotency.

m17.ASC displays the typical fibroblastoid morphology and expresses the stemness markers Sca-1, c-Kit/CD117, nestin, nucleostemin, CD44, CD106, klf-4, E ALTRI (Sox2, Nanog, Myc??. Oct4 è negative). Due to its many properties, m17.ASC may represent a valuable tool for a number of applications in *ex vivo* tissue engineering, namely aimed at analyzing physical (e.g. electrical, mechanical, topographical) or biochemical parameters (selected growth factors, hormones and other biologically active molecules and possibly inhibiting drugs, their combination or timing) with the advantage that it does not require repeated animal sacrifice.

CONCLUSION

As such, this cell line is an excellent candidate to investigate the requirements in terms of biomolecules, their combination or timing, as well as of scaffolds in tissue engineering and regenerative medicine. Moreover, it can be used as a platform to test the effects of drugs in the differentiation induction process. Future studies would investigate if m17.ASC could be induced to osteoblastic differentiation when plated on titanium scaffolds. Indeed preliminary data suggest that they can be suitable to test unconventional cell culture substrates for tissue engineering.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

M. Prat, S. Pietronave, A. Zamperone, research agreement with the Università del Piemonte Orientale, and Patent Appl. No. US 13/309,132 filed on Dec. 1, 2011 related to US Provisional application No. 61/344,973, filed on Dec. 1, 2010. Thanks the cooperation of MITO Technology srl, it has been signed a Deposit License Agreement with Health Protection Agency Culture Collection (HPACC) on November 15, 2011 for the preservation and distribution of the new cell line.

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LEGENDS TO FIGURES

Fig 1: Properties of the clonal cell line m17.ASC. Morphology of the m17.ASC clone, which was generated from Sca-1+ cell cultures plated at low density (200 cells/cm^2) at the phase contrast microscope at two different enlargement (a). Phenotype of m17.ASCs, characterized in RT-PCR (b) and cytofluorimetry (c and d) for different stemness markers at different passages. The clone has a fibroblast-like aspect and continues to express stemness markers with time. Representative experiments out of the three performed are reported.

Fig. 2: The m17.ASCs are multipotent can be induced towards a muscle/cardiac phenotype. a) Cells were cultured in normal expansion medium (left) or in appropriate media per specific differentiation (from top to bottom) towards osteogenic (alizarin red), adipocytic (Adipored), and chondrogenic (Alcian blue and collagen II) phenotypes (right); b) quantitative RT-PCR was performed for differentiation markers (osteocalcin, adiponectin, aggrecan) respectively; c) DiIC12(3)-labelled m17.ASCs (red) were co-cultured with neonatal cardiomyocytes for 7 days, then fixed, permeabilized, immunostained for GATA-4 or MEF2c (left panels: green), counter-stained with DAPI (blu) and examined at a fluorescence microscope, singly or after merging (right panels). Quantification of DiIC12(3)-labelled m17.ASCs and SVF differentiated cells after 7 day co-culture. The results are shown as mean ± standard deviation (SD) and are obtained counting 12 indipendent fields at 10X magnification (n =3). At least 500 DIIC12(3)-labelled cells were counted in each of the three experiments performed. $p< 0.05$, Student's *t* test. d) DiIC12(3)-labelled m17.ASCs (red) were co-cultured, fixed, permeabilized as above, and immunostained for alpha-cardiac actinin or cardiac Troponin T (left panels: green) or DAPI (blu) and examined at a fluorescence

microscope, singly or after merging (right panels). Representative experiments out of the three performed are reported.

Fig. 3: m17.ASCs have a normal immortal phenotype. a) Karyotype analysis: G-banding chromosome of m17.1ASC cells at two passages (p 10 and p73), which was normal if compared with normal mouse karyotype (19, XX). b) Population doubling time. Every three days $1x10^5/cm^2$ of m17.ASC (from p82 to p91) and primary cells (from p1 to p10) were passaged sequentially (named p1-10 in abscissae for both cell types). c) Telomere length was measured in this series of passages, as well from p27 to p32, from p43 to p49 and from p107 to p113 and was compared to the telomere length of SFV at p1. d) m17.ASCs do not display transforming activity, as assessed in a soft agar assay, which allows only anchorage-independent cell proliferation. In these conditions m17.ASCs produced even a lower number of colonies relative to NIH-3T3 cells used as negative control. By contrast transformed NIH-3T3-Met-EC$^-$ gave a high number of colonies. Representative experiments out of the three performed are reported.

Fig. 4. Gene expression profile. a) Genes displaying gain (■, left), loss (■, right) or differential expression (□, up-regulated on the left and □, down-regulated on the right) in m17.ASC cells (vertical axis) compared to the stromal vascular fraction derived from subcutaneous adipose tissue (SVF, horizontal axis) by microarray. The scatterplot shows normalized microarray datasets of m17.ASC and SVF. All 45,281 gene probes are represented in this plot. b) Gene expression changes in m17.ASC and neonatal cardiomyocytes (nCM) versus SVF are correlated. Genes previously selected for up- and down-regulation in m17.ASCvs SVF cells are plotted for their m17.ASC/SVF log2 ratio expression on the x-axis, and their nCM/SVF Log2 ratio expression on the y-axis.

Fig. 5. m17.ASCs engraft in NOD/SCID-γ^{null} mice. GFP-m17.ASCs in liver sinusoids after intrasplenic injection. Liver sections from mice killed after different time points were stained for GFP (green), TO-PRO3 (blue) and CD31 (red) and analyzed by confocal microscopy. Cell

engraftment was analysed at 1 week, 3 weeks and 6 weeks after transplantation and are reported at two different enlargements (a: original magnification 400x; b: original magnification 950x). One representative experiment out of 3 performed is reported (2 animals per group per experiment).