INTERNATIONAL PhD IN

IMMUNOPHARMACOLOGY

XXVI CYCLE

Adipose stem cells on the basis of tumor transformation

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2013-2014-2015

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Summary

Mesenchymal stem cells thanks to their differentiated multipotent ability are considered the most promising candidate for tissue engineering and regenerative medicine. The source of mesenchymal stem cells up to about 20 years was represented by the bone marrow, but due to their limited amount of cells together with a very invasive and painfull surgical treatment, poorly accepted by patients, has led the scientific community to investigate an alternative mesenchymal stem cells source with similar properties. In particular, the adipose tissue has attracted the greatest interest thanks to its relative abundance, the easy of availability and the large amounts of mesenchymal stem cells which are obtenible. So this adipose stem cells subpopulation, once verified the same differentiation potential of them derived from bone marrow, represent the ideal type of adult mesenchymal stem cells useful for numerous regenerative and tissue engineering applications. In particular the bone regeneration, obtained after the lipotransfer of a specific stem cells subpopulation isolated from adipose tissue, should be considered one of the most successful applications in the field of regenerative medicine and tissue engineering**. (Chapter 1)**. A further widespread use of mesenchymal stem cells is to reconstructive medicine. In particular, most patients with breast cancer, whenever possible, based on the type and stage cancer disease **(Chapter 2**), first make use of a demolitive surgery, in order to completely remove the tumor mass presence, and then resort to a reconstructive plastic surgery procedure. In breast cancer patients it is often used the adipose tissue as autologous filler. Even though the surgical treatment does not represent a potential danger for the patient, many scientific works have highlighted how the use of these self transplantations constitute a great risk for a possible cancer relapse despite the absence of a minimal residual disease. The presence of a mature adipocytes population infact, is responsible of a specific microenvironment, composed by pro-inflammatory cytokines and paracrine signals, that induce a generalized inflammatory state and which stimulate a proliferative return of those few quiescent cancer cells that still remain in the implantation site. In particular we have seen that the action of adipokines and interleukins generated by adipose autologous filler works both on differentiated tumor cells, just in a active proliferation cell phase but also on the cancer stem cell population that instead reside in a quiescent cell cycle phase. **(Chapter 3).** Therefore it is essential develop a more accurate and specific lipofilling procedure, especially going to select an appropriate autologous filler, in which a particular adipose stem cell subpopulation, with great regenerating properties has to be chosen among the adipose populations already present in conventional fillers. (**Chapters 4).** So the goal of this study is to enable the development of new selective therapies useful to remove the cancer stem cells, real responsible for tumor relapse. One of the most promising cancer therapies among those currently in the testing

phase could be the differentiative therapy, and specifically one that uses the negative regulatory properties of the miRNAs **(Chapter 5)**.

Chapter| 1

Di Stefano et al., J Regen Med 2015, 4:2
http://dx.doi.org/10.4172/2325-9620.1000124

Journal of Regenerative Medicine

Research Article

A SCITECHNOL JOURNAL

Identification and Expansion of Adipose Stem Cells with Enhanced Bone Regeneration Properties

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Abstract

Introduction: Adipose tissue represents an abundant source of mesenchymal stem-like cells. Adipose-derived stem cell progenies have been investigated and used in regenerative medicine for decades. In the last few years, they have been used to "enrich" lipoaspirates in fat grafting techniques, in an attempt to boost the regenerative potential of adipose tissue when used as autologous "filler".

Materials and Methods: 50 adipose tissue samples from lipoaspirates and subcutaneous breast tissue biopsies were used to generate adipose floating spheroid cell (ASphC) lines. ASphC were characterized for the expression of putative mesenchymal stem cell markers and used *in vitro* to test their multilineage potential. Furthermore, ASphCs were seeded on dermal regeneration template (Integra®) and implanted into the T8 vertebral laminectomy site of immunocompromised mice.

Results: Here, we show that the majority of ASphCs are in a quiescent state and express the putative surface stem cell marker CD271. Unlike $CD271^-$ cells, $CD271^+$ ASphCs grew indefinitely *in vitro* as undifferentiated spheres in serum-free medium, maintaining their multilineage differentiation potential ability. Importantly, p107, a functional adipose stem cell marker, is strictly expressed in ASphCs and barely present in their differentiated mesenchymal lineages. These sphere cells display an enhanced ability *in vitro* to differentiate into distinctive end-stage cell types, such as osteoblasts, chondrocytes and adipocytes. Gene expression profiling analysis indicated that ASphCs are endowed with stem cell potential that is gradually lost during specific differentiation. Finally, ASphCs facilitate bone inter body repair and regeneration after laminectomy.

Conclusions: We conclude that ASphCs possess a pronounced *in vivo* activity to regenerate the bone injury. Overall, ASphCs represent a heterogeneous population of stem-like cells harbouring multilineage differential potential and representing a prospective promising tool in cell therapy and tissue engineering.

Keywords: Mesenchymal stem cells; Hematopoietic transplantation; Adipose tissue; Liposuction; Biocompatible scaffolds; Cell therapy.

Abbreviations: ABCB1: ATP-Binding Cassette sub-family B member 1; ADA: ADSC-Derived Adipocytes; ADC: ADSC-Derived Chondrocytes ; ADO: ADSC-Derived Osteoblasts; ADSC: human Adipose Derived Stem Cells; AEC: 3-Amino-9- Ethyl Carbazole; ALP: Alkaline Phophatase; ASphC: Adipose Sphere Cells; BAT: Brown Adipose Tissue; BCIP: 5-Bromo-4-Chloro-3'-Indoly-phosphate P-toluidine salt; bFGF: basic Fibroblast Growth Factor; BMP: Bone Morphonegic Protein; BRAF: Serine/Threonine-Protein Kinase B-Raf; DMEM: Dulbecco's Modified Eagle's Medium; EGF: Epidermal Growth Factor; FBS: Fetal Bovine Serum; FITC: Fluorescein Iso Thio Cyanato; GAPDH: Glyceraldehyde 3-Phosphate Dehydrogenase; H&E: Hematoxylin and Eosin; HRP: Horseradish Peroxidase; KRAS: K-Ras oncogene is a member of the Ras gene family; MSC: Mesenchymal Stem Cells; MTS: 3-(4,5-dimethylthiazol-2-Yl)-5-(3-Carboxymethoxyphenyl)-2-(4-Sulfophenyl)-2H-Tetrazolium; NBT: Nitro-Blue Tetrazolium chloride; NP40: Nonidet P-40; NRAS: N- oncogene is a member of the Ras gene family; NSG: Non-Obese Diabetic Severe Combined Immunodeficiency Gamma Mice; OPN: Osteopontin; PBS: Phosphate Buffered Saline; PE: Phycoerythrin; PFA: Paraformaldehyde; PI3K: Phosphatidylinositol-4,5-Bisphosphate 3-Kinase; POU5F1: POU Class 5 Homeobox 1; PPARγ: Peroxisome Proliferator-Activated Receptor Gamma; Rb: Retinoblastoma; RUNX2: Runt-Related Transcription Factor 2; SCM: Stem Cell Medium; SD: Standard Deviation; SDA: ASphC-Derived Adipocytes; SDC: ASphC-Derived Chondrocytes; SDO: ASphC-Derived Osteoblasts; SDS: Sodium Dodecyl Sulfate; SHAM: Placebo Surgery; SOX2: SRY Sex Determining Region Y Box2; SOX9: SRY Sex Determining Region Y Box 9; TP53: Tumor Protein P53; VEGF: Vascular Endothelial Growth Factor; WAT: White Adipose Tissue

Introduction

Mesenchymal stem cells (MSCs) are considered to be important and promising candidates with regard to tissue engineering and their applications as regenerative medicine. This is largely due to their multipotent differentiation ability as well as their immunosuppressive properties [1,2]. The main characteristics of MSCs were first described in bone marrow tissue [3]. MSCs have been used with varying degrees of success in treating neurological, cardiovascular and hematopoietic disorders as well as other related diseases [4]. MSCs can also be isolated in minimal quantities from other tissues among which liver, synovium, skeletal muscle, peripheral blood [5]. Over the last 15 years, however, regenerative medicine research was focused on finding an abundant valuable alternative source of multi-potent stem cells [6,7]. It is known that bone marrow represents a suitable source of mesenchymal stem cells, which are used routinely in clinical applications associated with hematopoietic transplantation, revealing a promising alternative source for further development in regenerative medicine [8,9]. However, the difficult accessibility and low volumes of tissue obtained *ex vivo,* limit the clinical use of bone marrow-derived stem cells. Their harvest requires a painful procedure (iliac crest or sternal tap), neither of which is free from complications and overall, with a low compliance of the patients. Recent studies have shown that mesenchymal stem cells derived from adipose tissue can differentiate into a variety of cell lines (cardiomyocytes, myocytes, neurons, adipocytes, osteocytes, chondrocytes) [10,11] and improve the neo-vascularization of ischemic tissue in limbs [12,13]. Adipose tissue is

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Received: July 30, 2015 **Accepted:** October 14, 2015 **Published:** October 20, 2015

considered an ideal source of MSCs in association with therapeutic needs, since their isolation is achieved by means of a minimal invasive surgery [14,15]. Liposuction is a safe and non-invasive procedure. It is routinely performed through a 2-3 mm skin incision, well-accepted by patients who normally undergo this procedure for cosmetic reasons [16,17]. Approximately 5×10^3 adipose-derived stem cells are contained in 1 gram of adipose tissue, approximately 500 times more than those retrieved from bone marrow, of which only 0.01–0.001% of nucleated cells present in its structure, are MSCs [18,19]. The aim of this study is to enhance regenerative processes through the use of autologous mesenchymal stem cells. Adipose tissue can be discerned into three main categories: the visceral and subcutaneous anabolic white adipose tissue (WAT) that stores excess energy, the catabolic brown adipose tissue (BAT) which is responsible of dissipation of energy through heat production, and "Brown and White" (brite or beige) adipose tissue. WAT is typically related to obesity given that it refers to both, adipose cell hypertrophy or their hyperplasia from committed progenitors. Whereas, BAT fat cells are highly oxidant with abundant mitochondria that oxidizes fatty acids and generate heat [20,21].

One of the master regulators of adipocyte lineage is p107, a member of the retinoblastoma protein (Rb) family, which was reported to be strictly expressed in white adipose stem cells, down-regulated during commitment toward the brown adipose lineage [22]. In particular, human adipose stem cells show high p107 expression levels, that progressively disappear after 7 days of differentiation [23,24].

Although it has been reported that human adipose-derived cells can be grown and expanded in serum-free medium as floating spheres harbouring a multilineage differentiation mesenchymal capacity [25], little is known about their phenotypic and molecular characterization. Several markers such as CD9, CD29, CD44, CD90, CD105 and CD271 were suggested to be implicated in the identification of adipose mesenchymal stem cells equipped with a pronounced self-renewal ability [18,26]. Nowadays, a unique marker that identifies adipose cells with stem cell features is still unknown. In recent years, there has been a considerable evolution in our understanding of the mechanisms underlying tissue regeneration by MSCs [11]. The current applicability of mesenchymal stem cells in regenerative medicine seems to be limited by the combinatorial use of specific adjuvants molecules (cytokines and growth factors) for specific lineage differentiation (i.e. the use of BMP-2 for bone formation promotion [27], and kartogenin for chondrocyte differentiation induction [28]). An innovative approach in the field of regenerative medicine consists in the use of biocompatible scaffolds in combination with living cells for damaged tissue repair [29]. Here we show that the majority of adipose sphere cells (ASphCs) express CD271, necessary for the differentiation toward adipogenic, chondrogenic and osteogenic lineages. This cell compartment shows an enhanced *in vivo* regeneration ability especially when sphere cells are seeded on a dermal regeneration template (Integra®), suggesting a future prospective in cell therapy.

Materials and Methods

Adipose tissue samples

Adipose tissue was extracted from a lipoaspirate and subcutaneous breast tissue biopsy of 50 patients (16 males; 34 females) in compliance with our Department's policy and following patient's written consent on adipose tissue harvest and its use for research purposes. Patients ranged from 20 to 65 years of age and all selected donors were healthy and without a prior history of malignancy. Tissues were obtained either from subcutaneous adipose tissue or during liposuction of the abdominal or inner thigh regions using Coleman's cannulas, following infiltration with Klein's solution (NaCl 0.9%; lidocaine 2%; epinephrine 1:1000; $NaHCO_3$ 8.45%). Approximately, 20 cc of lipoaspirate and 0.05±0.02 gr tissue biopsies were collected from each patient and directly transferred to tissue culture tubes for enzymatic digestion and stem cell purification.

Cell isolation and culture

Breast-derived adipose tissue and lipoaspirate samples were digested with collagenase (1.5 mg/ml, GIBCO) and hyaluronidase (20 mg/ml, Sigma) through gentle agitation for 30 minutes at 37°C. The digested sample was centrifuged at 1200 rpm for 5 min and the recovered cells (Freshly) were plated with serum-free stem cell-specific media as previously described [30]. Cells were plated in stem cell medium in presence of bFGF (10 ng/ml, Sigma) and EGF (20 ng/ml, Sigma), in ultra-low adhesion tissue culture flasks (Corning) and placed at 37°C in a 5% CO_2 humidified incubator. In these conditions, cells grew as floating spheroids (ASphCs). Part of the isolated cells were plated in adherent conditions (DMEM+FBS 10%) and were referred as primary culture (Primary). Conversely, we termed ADSCs the commercially available STEMPRO® Human Adipose-Derived Stem Cells, plated as recommended by the manufacturer (Invitrogen). To evaluate ASphC multilineage differentiation capacity, 5×10^3 single cells were diluted 1:4 in a cold Matrigel solution (growth factor reduced BD). This solution (50 µl/well) was dispensed into pre-warmed 24-wells plates and let polymerize for 30 minutes at 37°C. Finally, the wells were filled using 700 µl/well of basal culture medium.

In order to classify adipose spheres as small, medium and large, cells within spheres derived from abdomen, hips and breast adipose tissue, were cytospun, fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton X-100 (Sigma-Aldrich), and exposed to Toto3 iodide (642/660, Invitrogen). Nuclear immunofluorescence analysis was detected by confocal laser-scanning microscopy. Counting was performed blinded by two independent observers.

Flow cytometry

Cells were stained with conjugated antibodies against CD44-FITC (G44-26, mouse IGg2bk, BD), CD271-ALEXA FLUOR 647 (C40-145, mouse IgG1k, BD), CD90-PE (5E10, mouse IgG1k, BD), CD45-FITC (5B1, mouse IgG2a, Miltenyi), CD19-ALEXA FLUOR 488 (HIB19, mouse IgG1k) or with purified primary, CD29 (MAR4, IgG1k, BD), CD73 (AD2, mouse IgG1k, BD), CD9 (ML13, mouse IgG1k, BD). Then, cells were labeled with goat anti-mouse IgG FITC secondary antibody (Invitrogen). Specific corresponding isotype matched antibodies were used as negative controls. Samples were acquired using a FACS ARIA (BD Biosciences) flow cytometer. All data were analyzed using FlowJo software (Tree Star).

Gene expression

Total RNA was extracted by using a RNeasy Mini Kit (Qiagen) and 1 µg of each sample was retro-transcribed into cDNA using a high-Capacity cDNA Reverse Transcription Kit as recommended by manufacturer (Applied Biosystems). Real-Time PCR reactions were performed with Two-Step RT-PCR TaqMan Probes (Applied Biosystems) using this primer set: Hs00765700_m1 (RBL1) (Applied Biosystems). The relative quantification of gene expression was calculated on triplicate reactions using the comparative Ct method

(ΔΔCt). Experimental target quantities were normalized with the endogenous Hu GAPDH control (Applied Biosystems). Expression of mesenchymal stem cell genes was performed through RT^2 profiler PCR array (PAHS-082ZR, Qiagen), according to manufacturer's instructions. Arrays were performed for ASphCs, ADSCs and their differentiate cells. At least 2 replicates were run for each sample.

Cell cycle and proliferation assay

Cell cycle analysis was performed on dissociated cells by staining with 50 μg/ml propidium iodide (Sigma-Aldrich) dissolved in buffer 0.1% sodium citrate (Sigma-Aldrich), 0.1% Triton X-100, 10 μg/ml RNAse (Sigma-Aldrich) for 1h on ice. Samples were acquired through a FACS Calibur flow cytometer (BD Biosciences).

The proliferation assay was evaluated by using PKH26 dye (Sigma-Aldrich). 20×10^4 of dissociated cells were stained for 1 h at 37°C with PKH26 according to manufacturer's instructions, then washed extensively with PBS and cultured for 14 additional days. PKH26 red fluorescence was analyzed by FACS Aria flow cytometer. All data were analyzed using FlowJo software.

Evaluation of ASphC and ADSC proliferation was assessed using CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) according to the manufacturer's instruction.

Western blotting

Cells were re-suspended in ice-cold NP40 lysis buffer and fractioned on SDS-polyacrylamide gels and blotted on nitrocellulose membranes, as previously described [31]. Membranes were exposed to specific antibodies for p107 (C-18, rabbit IgG, Santa Cruz biotechnology) and β-actin (JLA20, mouse IgM; Calbiochem) and detected using HRP conjugated anti-rabbit or anti-mouse antibodies (Amersham). Chemioluminescent detection of protein expression was performed by Amersham Imager 600.

Osteogenic differentiation

Briefly, ASphCs and ADSCs were trypsinized 1 min at 37°C and plated into 24-well cell culture plates (50,000 cells/well). Cells were allowed to adhere and cultured in the STEMPRO® Osteogenesis Differentiation Kit (Invitrogen) up to 28 days. Cell viability, adhesion and differentiation were assessed by daily observation using an optical microscopy. Osteogenic differentiation was assessed by performing a staining for alkaline phosphatase activity (ALP) (BCIP/NBT alkaline phosphatase substrate kit, Vector Laboratories), osteopontin (OPN) (polyclonal rabbit, Sigma-Aldrich) or von Kossa for calcium deposition (Polysciences Inc.).

For ALP, cells were fixed in 80% methanol/20% citrate for 1 minute and then stained in the dark at room temperature for 2 hours. For OPN detection and von Kossa staining see below.

Chondrogenic differentiation

Cells were allowed to adhere and cultured in the STEMPRO® Chondrogenesis Differentiation Kit (Invitrogen) up to 28 days. Chondrogenic differentiation was assessed by alcian blue. Briefly, the sample was fixed in 2% PFA for 30 min at 37°C, washed in PBS and incubated with alcian blue for 30 minutes. Cells were then counterstained with nuclear fast red for 5 minutes.

Adipogenenic differentiation

Cells were allowed to adhere and culture in the STEMPRO® Adipogenesis Differentiation Kit (Invitrogen) up to 28 days. Phenotype change was assessed by adipored assay (Lonza) for 10 min at room temperature. Nuclei were counterstained with Toto3 and the staining was observed using a confocal microscope.

Animal model

NSG mice were purchased from Charles Rivers Laboratories (Milan, Italy) and maintained according to University of Palermo's institutional guidelines for animal care and use committee in animal house authorized by the Italian Ministry of Health (DGSAF#0020301-P-03102014).

In vivo laminectomy procedure was performed at the T8 vertebral level under aseptic conditions. 5×10^3 ASphCs and ADSCs, were seeded on 1 cm^2 of dermal regeneration template (Integra®) for 4 days in presence of SCM and then implanted into lesion whose the mice were subjected. Integra® (Integra Lifesciences Inc., Plainsboro, USA) is a bi-laminar skin replacement which comprises a layer composed by bovine collagen and shark glycosaminoglycans that facilitate the regeneration and a second layer composed of a synthetic polysilicoxane polymer that acts as a scaffold matrix.

After 60 days the Integra® and the surrounding bone were harvested, fixed, paraffin-embedded and sectioned in order to evaluate the bone regeneration.

Five-uM thick paraffin-embedded sections were heated with antigen retrieval in 10 μM sodium citrate (pH 6.0). After have been incubated for 5 minutes with 3% H_2O_2, sections were permeabilized for 10 minutes with 0.2% Triton X-100 in PBS on ice. Sections were exposed overnight at 4°C to a specific antibody for OPN (Sigma Aldrich) or isotype-matched control at appropriate dilutions. Subsequently, sections were exposed to biotinylated immunoglobulins and streptavidin-peroxidase following manufacturers' instructions (LSAB2 Kit, Dako). Staining was revealed using 3-amino-9-ethylcarbazole (AEC) substrate (red colour) and counter-stained with aqueous hematoxylin (blue color). The OPN positivity was calculated by observing at the red color staining and counting the percentage of positive cells in the regeneration area. Counting was accomplished blinded by two independent observers on four different areas per sample.

For H&E, paraffin-embedded sections were stained according to standard protocols. The regeneration area was measured by observing the H&E staining. The percentage of the sectional area occupied by ASphCs-derived differentiated cells was determined by computer-assisted analysis (Image ProPlus software). From each section, 8 consecutive fields were captured at 200 magnification and analyzed. Analysis was performed blinded by two independent observers on 3 samples per group derived from 3 independent ASphC cultures.

In order to detect calcium deposits in the area subjected to the laminectomy, the von Kossa method for calcium kit (Polysciences, Eppelheim, Germany) was adopted and performed according to manufacturer's directions. Hence, sections were placed in 3% Silver Nitrate Solution and exposed to UV light for 40 minutes. Then, cells were placed in 5% sodium thiosulfate for 2 minutes and counterstained with nuclear fast red for 5 minutes.

For DNA mutation analysis, DNA from paraffin-embedded sections was purified using QIAmp® DNA FFPE Tissue Kit (Qiagen). DNA samples were subjected to TruSeq Amplicon-Cancer Panel (FC-130-1008, Illumina) and analyzed by MiSeq Reporter (MSR) software.

Statistical Analysis

Data are explicited as mean ± standard deviation (SD). Statistical significance was calculated by applying Student's t-test. Significance levels were indicated as p values.

* indicates P<0.05, **indicate P<0.01 and ***indicate P<0.001.

Results

Adipose spheroids comprise stem-like cells

It was postulated that adipose tissue can be regarded as a source of quiescent mesenchymal stem-like cells, which give rise to a population of heterogeneous cells that exhibit diverse degrees of differentiation. In order to investigate if the adipose tissue is characterized by the presence of a small subset of cells with a stem cell phenotype, we initially evaluated the presence of cells positive for different surface molecules, which were previously identified as putative markers for mesenchymal stem cells in freshly purified adipose tissue cells and primary adherent cells after 10 days of culture in presence of FBS. The surface marker levels of CD29, CD44, CD73, CD9 and CD90 were uniformly expressed among freshly purified cells (Freshly), primary cells (Primary), anchorage-dependent adipose-derived stem cells (ADSCs) and adipose floating spheroid cells (ASphCs), with the exception of CD271 (Figure 1A and B).

Expression levels of CD271 were barely present on freshly purified cells (Freshly) and absent on primary cultures (Primary) (Figure 1A and C) thus, suggesting that this putative stem cell marker is lost in presence of high concentration of FBS, a well-known inducer of differentiation [30,32]. CD45 and CD19 markers were used to exclude the hematopoietic cells (Figure 1A). Although both ADSC and ASphC cultures showed high levels of CD271 expression, only $CD271^+$ ASphC fraction (Figure 1D) was endowed with a potent sphere forming capacity, which was indefinitely maintained during serial *in vitro* passages (Figure 1E). Contrarily, $CD271^-$ ASphC and the $CD271^+$ ADSC compartments failed to generate floating spheres when cultured in absence of serum and in presence of bFGF and EGF (data not shown and Supplementary Figure 1A and 1B). Moreover, $CD271^+$ ADSCs in non-adherent condition gradually died up to 10 days as compared with ASphCs which showed a significant growth (Supplementary 1C). Intriguingly, ASphCs, enriched in $CD271^+$ cells, showed a greater multilineage differentiation potential, being able to originate mature adipogenic, chondrogenic and osteogenic lineages when cultured with specific differentiation medium (Figure 1E).

ASphCs retain differentiation capacity

ASphCs propagation, regardless of the site of origin, led to the observation that these cells can grow either as larger adipose spheroids composed by about 100 cells, as medium spheroids, or as small spheroids composed by almost 30 cells (Figure 2A).

To determine whether ASphCs represent undifferentiated cells that have retained their capacity to differentiate toward adipogenic (SDA), chondrogenic (SDC) and osteogenic (SDO) lineages, spheroids were cultured in the presence of FBS or seeded under adherent conditions in presence of a specific differentiation medium for up to 28 days. Although small spheroids purified from abdomen, hips and subcutaneous breast tissue showed higher differentiation potential than the medium and large ones, those that were breast-derived, retained the highest differentiation capacity (Figure 2B). Of note, the best efficiency, in terms of absolute number of spheres obtained from various samples is represented by subcutaneous breast tissue-derived cultures (Table 1).

Although both ADSCs and ASphCs express high levels of CD271, only the ASphC cultures exposed to the specific differentiation medium, generated a progeny of propagating differentiated adipocytes, chondrocytes and osteoblasts up to 28 days (Figure 2C). ALP, OPN and von Kossa staining showed that ASphCs possess a strong potential to differentiate toward osteogenic lineages. Moreover, ASphCs grown in chondrogenic media were positive for alcian blue. Meanwhile, those grown in adipogenic media resulted positively stained for adipored. This suggests that these cells also display a high differentiation potential. Therefore, the ASphCs show higher differentiation potential compared to ADSCs grown in the same differentiation conditions (Figure 2D).

Among the different putative mesenchymal stem cell markers analysed by FACS, we found that expression levels of CD29, CD9 and CD90 are maintained at high levels by both ASphC- and ADSC-derived differentiated progenies (Figure 3A). In contrast, CD44 and CD73 expression showed a distinct expression profile in the differentiated ASphC and ADSC subset. While CD44 expression was significantly reduced in differentiated cells derived from ADSC as compared with ADSC (Figure 3A, upper panels and 3B), in ASphCs-derived differentiated progeny it remained highly expressed (Figure 3A, lower panels and 3B). Of note, CD271 was significantly reduced in both ADSC-derived adipocytes (ADA), chondrocytes (ADC) and osteoblasts (ADO) and SDA, SDC, SDO (Figure 3A and 3C).

ASphCs are quiescent and show mesenchymal traits

In order to determine whether ASphCs display stem cell peculiarities in a quiescent state, we explored the cell cycle in our spheroids and ADSCs and compared them with differentiated progeny. ASphCs are characterized by very low clonogenic activity and show a large G_0/G_1 phase, comparable to the one present in ADSCs. While the G_2/M and S phases in ADSC-derived adipocytes, chondrocytes and osteoblasts resulted unchanged with respect to the untreated cells, ASphC-derived cell lineages show a reduced G_0/G_1 phase and increased G_2/M phase. This indicates that these latter cells retain a high potential to differentiate and propagate (Figure 4A). Moreover, ASphC-derived differentiated cells showed a greater proliferation rate than ADSC-derived cells. In ASphC progeny, PKH26 loss was likely due to the asymmetric division induced by the non-specific differentiation induction of FBS (Figure 4B, upper panel). In contrast, ADSCs displayed unaltered proliferative behavior (Figure 4B, lower panel). This confirms their limited capacity to be serially passed *in vitro*.

Then, we sought to analyze the potential changes of mesenchymal gene expression profile in ASphCs and ADSCs, together with their relative differentiated counterparts (adipogenic, chondrogenic and osteogenic compartments). It was revealed that ASphCs possess a more pronounced mesenchymal stem cell trait which is lost upon 28 days of differentiation in specific differentiation media, as compared with ADSC compartment (Figure 4C). Of note, ASphCs showed higher expression levels of mesenchymal stem cell markers, such as *SOX2* (>5 fold regulation) and *POU5F1* (>2 fold regulation), when compared to ADSCs (Figure 4D, upper-left panel). The mRNA expression levels of *PPARγ* (>3 fold regulation), *SOX9* (>6 fold regulation), and *RUNX2* (>13 fold regulation), which are associated to specific differentiation in adipocytes, chondrocytes and osteoblasts respectively, resulted up-regulated in the differentiated ASphC progenies, compared with the ADSC compartment. Interestingly, when ADSCs are exposed to

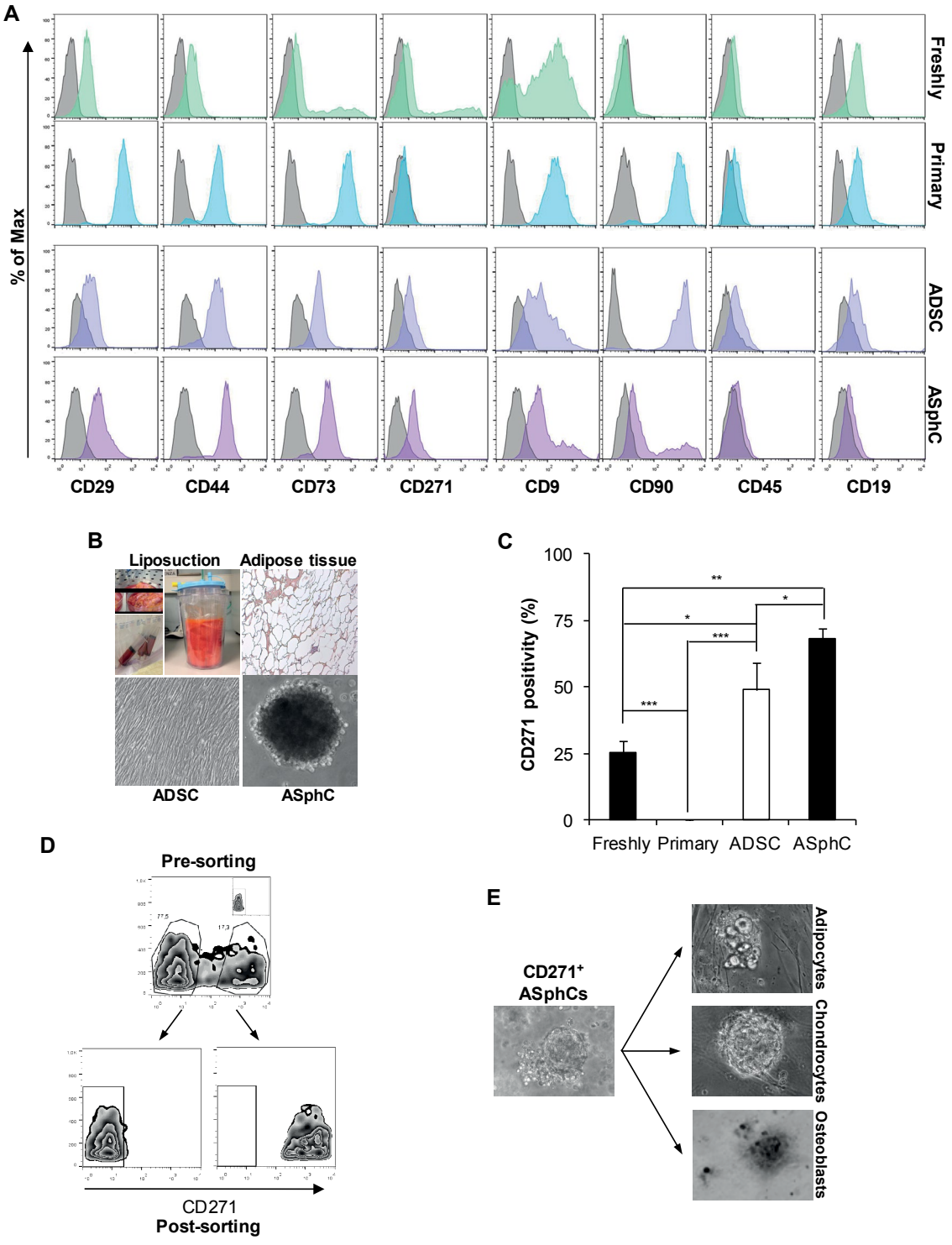

Figure 1: CD271⁺ cells retain sphere forming capacity.(A) Representative flow cytometry profiles of CD29, CD44, CD73, CD271, CD9, CD90, CD45 and CD19 on freshly purified (Freshly, green histograms), primary adipose cells (Primary, light blue histograms), adipose-derived stem cells (ADSC, purple histograms) and adipose spheroid cells (ASphC, pink histograms). The grey histograms represent the isotype-matched controls. (B) Fresh adipose tissue sample and its H&E staining on paraffin embedded sections (right panels). Phase contrast analysis of ADSC and ASphC cultures (lower panels). (C) Percentage of CD271 positivity in Freshly, Primary, ADSCs and ASphCs performed by flow cytometry. Data are mean ± S.D. of 5 independent experiments. (D) CD271 profiles performed on freshly, depleted and purified adipose cells performed by flow cytometry cell sorting. Data are representative of 5 independent experiments using cells from different healthy donors. (E) Sphere forming and ASphC-derived adipocytes, chondrocytes and osteoblasts of CD271⁺ enriched ASphCs.

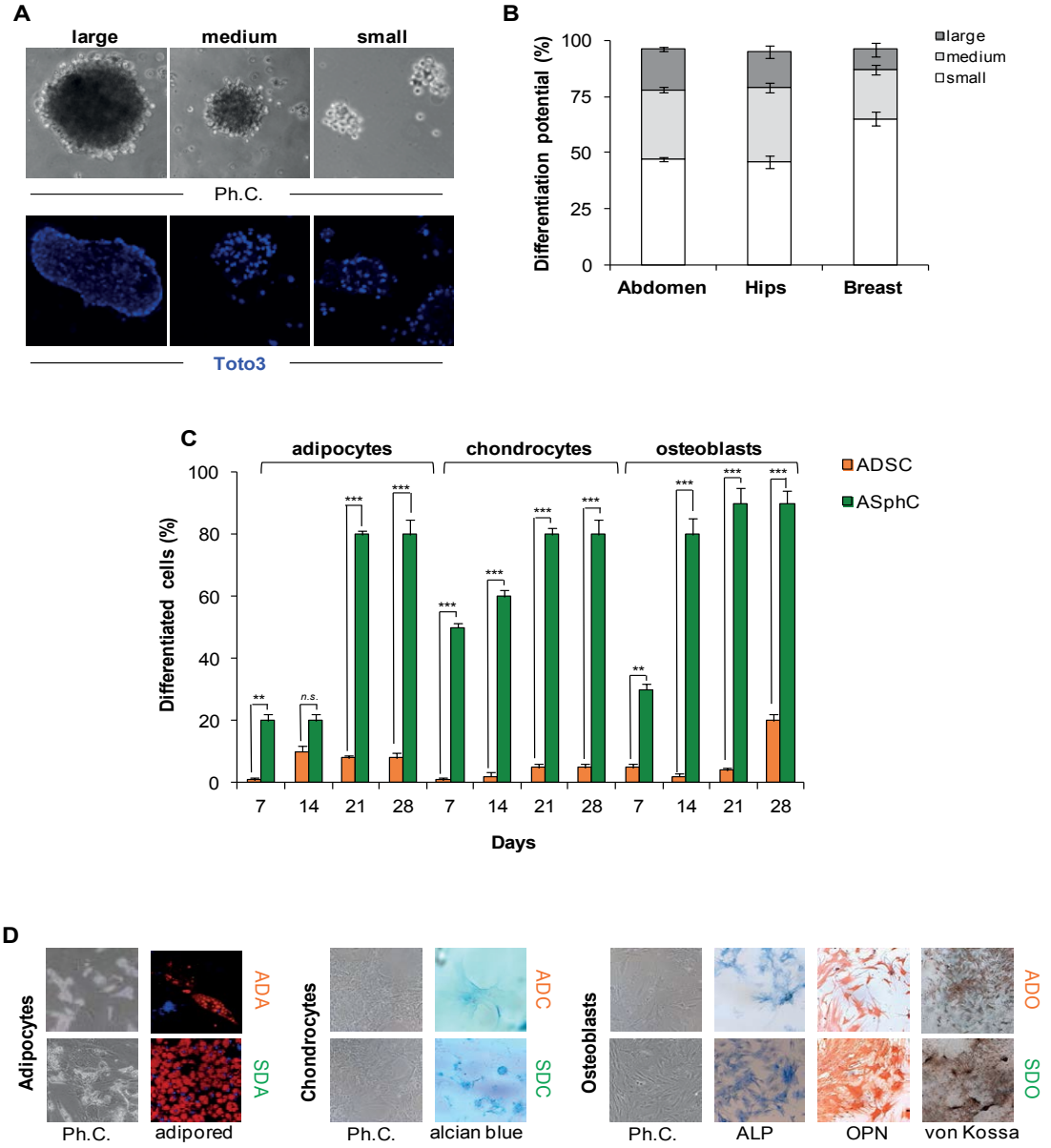

Figure 2: ASphCs display multi-lineage differentiation ability.(A) Representative phase contrast analysis (upper panels) and fluorescence analysis of Toto3 (blue color, lower panels) in large, medium and small adipose tissue-derived spheroids. Data are representative of 15 ASphC cultures. (B) Percentage of differentiation potential of large, medium and small spheroids purified from abdomen, hips and subcutaneous breast adipose tissues. Data are mean ± S.D. of 7 independent experiments. (C) Percentage of ADSC (orange bars) - and ASphC (green bars)- derived adipocytes, chondrocytes and osteoblasts at the indicated time points. Data are mean ± S.D. of 7 independent experiments. (D) Microscopy analysis of adipored, alcian blue, alkaline phosphatase (ALP), osteopontin (OPN) (red color) and von Kossa (brown color) in ADSC-derived adipocytes (ADA), chondrocytes (ADC) and osteoblasts (ADO) and ASphC-derived adipocytes (SDA), chondrocytes (SDC) and osteoblasts (SDO).

Table 1: Case descriptions and sphere forming.

Number of samples	Liposuction	Biopsy	Gender (M/F)	Spheroids/gr of adipose tissue
31	Abdomen		9/20	16.7 (± 2,121)
5	Hips		6/10	11.7 (± 1,931)
2	Gluteus		0/2	10.4 (± 0,282)
3		Breast	0/3	20.8 (± 0,2)
3	Breast		3/0	5.5 (± 1,95)

specific differentiated media they show a distinctive gene expression pattern, including the up-regulation of *BMP4* (>9 fold regulation), *BMP7* (>9 fold regulation) (ADA), and *ABCB1* (>5 fold regulation) (ADC) (Figure 4D).

These data suggest that ASphCs and ADSCs could reside at different levels within the mesenchymal stem hierarchy. This consideration is supported by our results in which, we compared the three differentiated progenies ASphCs- and ADSCs-derived, showing that the *PPARγ*, *SOX9*, and *RUNX2* are mostly up-regulated in the SDA, SDC, SDO.

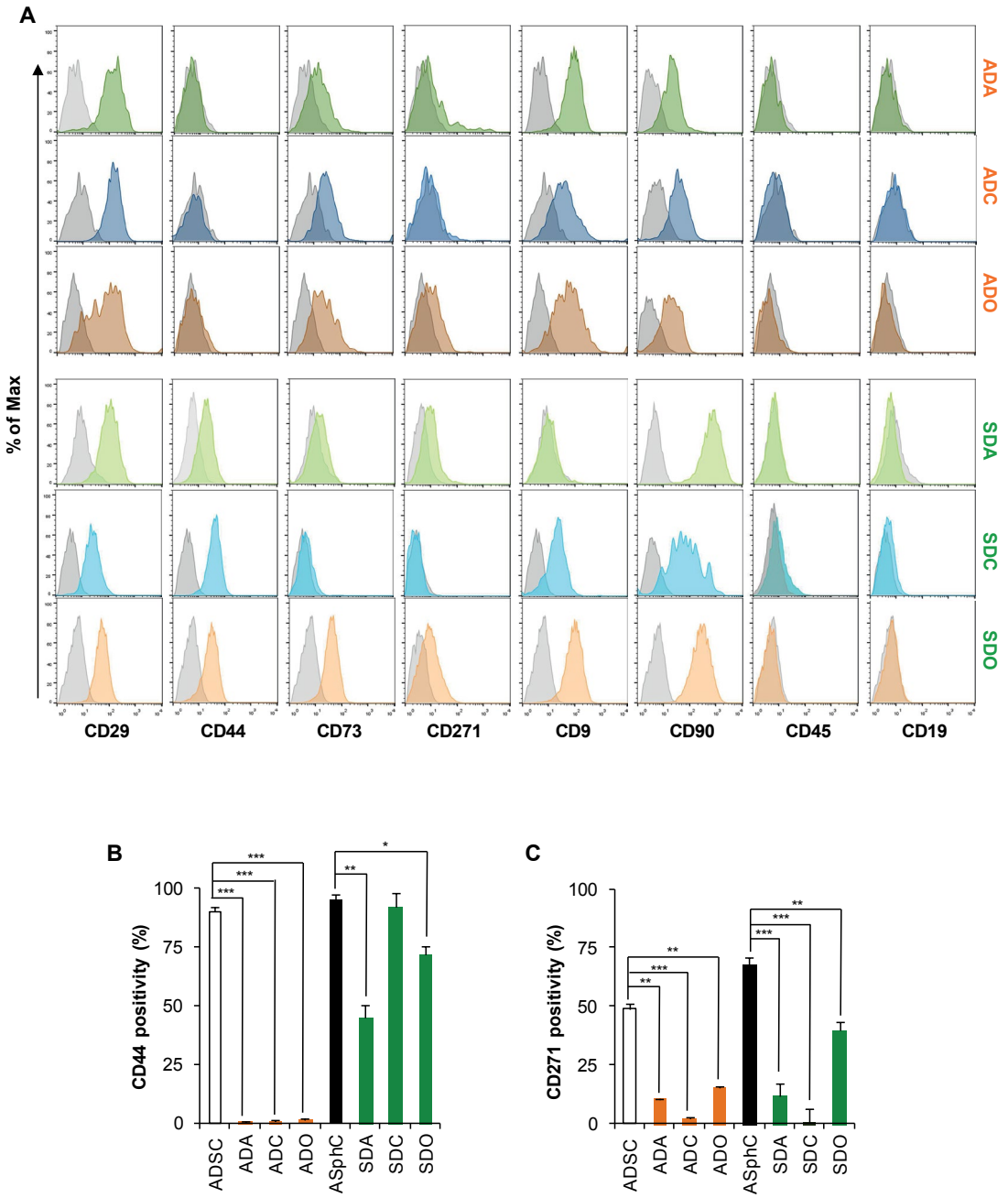

Figure 3: CD271 is gradually lost during ASphCs specific differentiation.(A) Representative flow cytometry profiles of CD29, CD44, CD73, CD271, CD9, CD90, CD45 and CD19 in ADA (green histograms), ADC (blue histograms) and ADO, (orange histograms) (upper panels) and SDA (light green histograms), SDC (light blue histograms) SDO (light orange histograms) (lower panels). The grey histograms represent the isotype-matched control. (B) Percentage of CD44 positivity, performed by flow cytometry, in ADA, ADC, ADO compared with ADSC and SDA, SDC, SDO compared with ASphC. Data are mean ± S.D. of 5 independent experiments. (C) CD271 positivity percentage as in (B).

p107, a marker that identifies the stem cell population of white adipose tissue and therefore, a master regulator of adipocytes differentiation, was significantly higher in ASphCs than in ADSCs. In ADSCs, both p107 mRNA and protein expression levels were comparable with those found in ASphC-derived differentiated cells. These data suggest that ADSCs are already programmed toward a terminal differentiation (Figure 4E and 4F).

ASphCs own regenerative ability

In order to evaluate ASphCs' ability to promote bone regeneration, we used an *in vivo* murine model, which harbours a laminectomy at the T8 vertebral level. We sought to investigate whether ASphCs were able to grow and differentiate in Integra® scaffolds up to 28 days in presence of osteogenic medium. Interestingly, ASphC-derived differentiated cells highly expressed OPN (Figure 5A) showing a

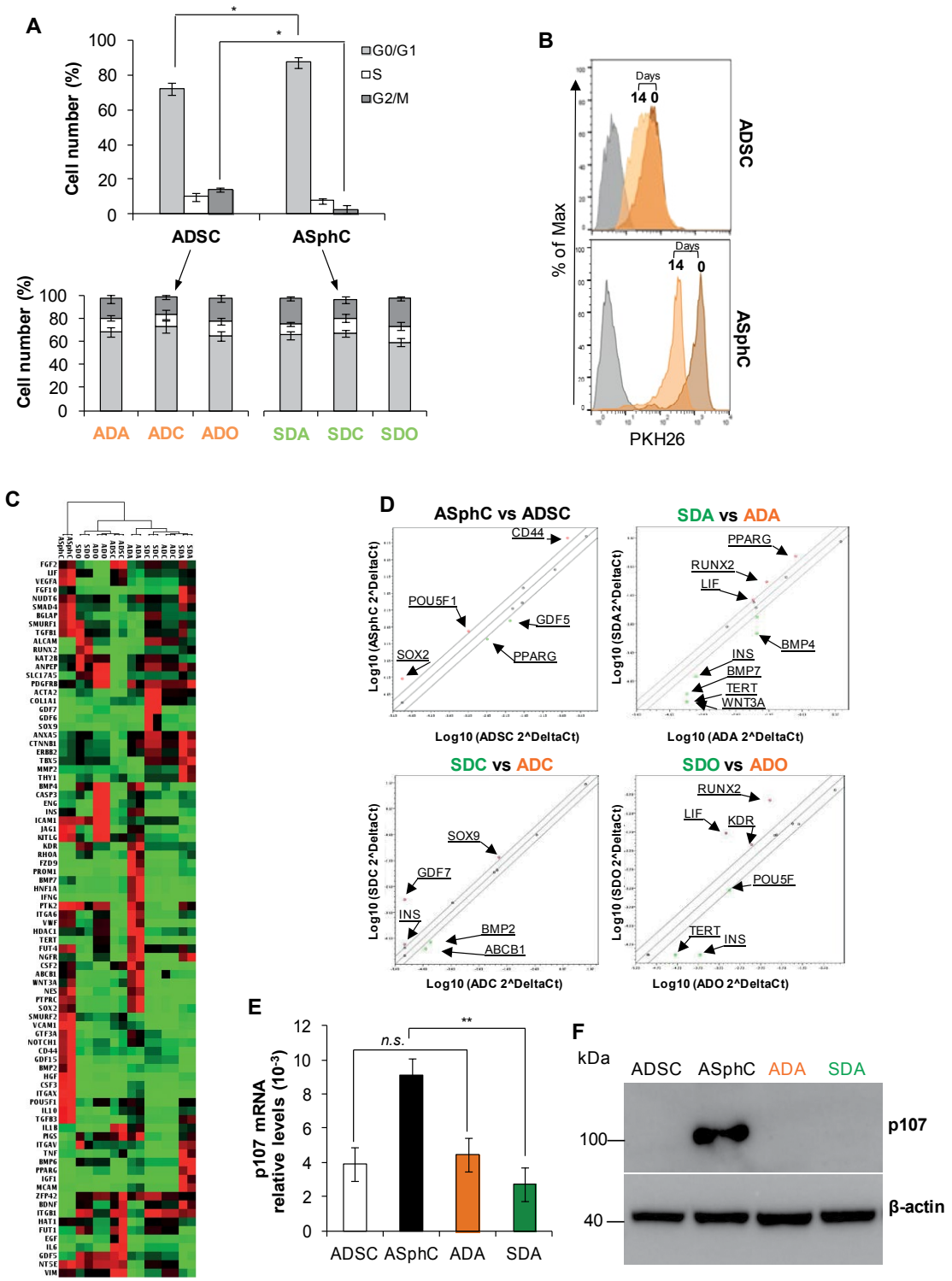

Figure 4: ASphCs express stem cell-related genes.(A) Cell cycle analysis performed in ADSCs and ASphCs and in their differentiated progenies ADA, ADC, ADO and SDA, SDC, SDO after 28 days exposure to specific differentiation media. Data are expressed as mean ± SD of 4 independent experiments. (B) Representative PKH26 flow cytometry analysis of ADSCs and ASphCs cultured in presence of FBS at day 0 and day 14. (C) Clustergrams of mesenchymal-related genes in ASphCs and ADSCs and in ADA, ADC, ADO and SDA, SDC, SDO. Gene expression profiles are derived from 2 independent experiments. (D) Scatter plot of mesenchymal-related genes as in (C). (E) Expression levels of p107 mRNA in ADSCs, ASphCs, ADA and SDA. GAPDH amplification was used as endogenous control. Results are shown as mean ± S.D. of 4 independent experiments. (F) Western blot analysis of p107 as in (E).

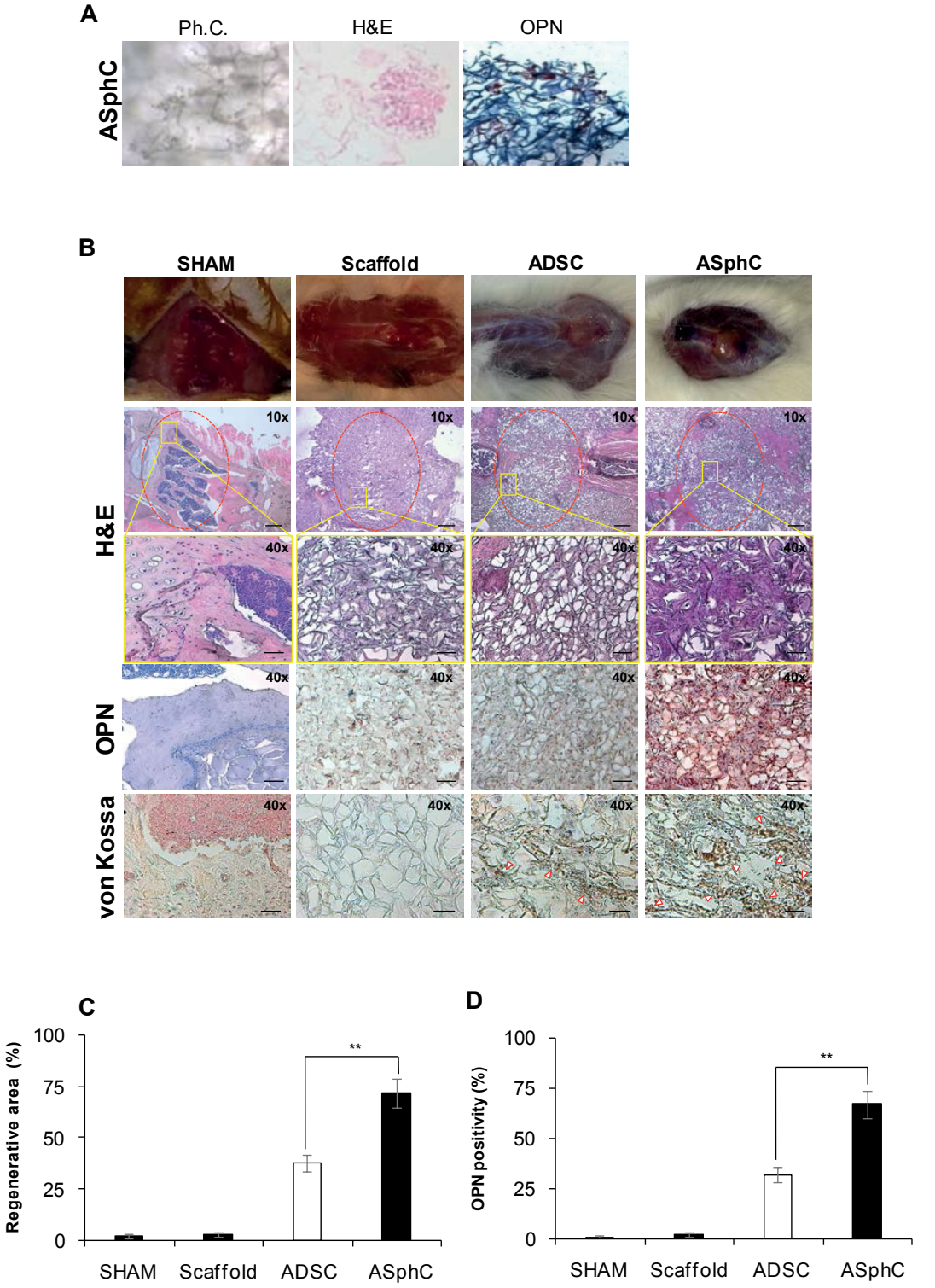

Figure 5: ASphCs repair bone tissue injury.(A) Phase contrast, H&E staining and OPN immunohistochemistry analysis in ASphCs seeded on Integra® and cultured in vitro for 28 days. (B) Macroscopic overview of mice subjected to laminectomy at day 60 (SHAM, untreated mice; Scaffold, mice treated with only Integra®; ADSC and ASphC, mice treated with Integra® plus seeded cells) (upper panels). H&E, OPN (red color) and von Kossa (brown) staining on paraffin embedded sections in SHAM, Scaffold, ADSCs and ASphCs. Red dotted circles emphasize injured/regenerated area. Yellow boxes indicates the magnification-matched area. Scale bars represent 150µm in 10x; 50µm in 40x. (C) Percentage of the regenerative area in the laminectomy site of mice treated as in (B). Data are expressed as mean ± SD of 3 independent experiments. (D) Percentage of OPN positivity as in (C). Data are expressed as mean ± SD of 3 independent experiments.

great *in vitro* differentiation potential on Integra®. To assess the specific osteogenic differentiation potential of ASphC and ADSC, we seeded cells on Integra®, cultured for 4 days and then implanted in the laminectomy site (Figure 5B). Following 60 days from surgery, the regenerative capacity, achieved by both ASphC and ADSC, was evaluated by histochemistry and immunohistochemistry. Hematoxylin/Eosin (H&E) staining demonstrated that the tissue repairing capacity promoted by ASphCs, was significantly greater than that shown in the xenotransplantation of ADSCs (Figure 5B middle panels and 5C). Likewise, immunohistochemistry analysis for osteopontin, highlighted the significant osteogenic differentiation determined by the injection of ASphCs as compared with the regeneration potential of ADSCs (Figure 5B and 5D). The osteogenic nature of the new generated tissue, performed by von Kossa staining, confirmed that ASphCs possess a pronounced bone regeneration capacity, which could be peculiar characteristics of adipose stem cells (Figure 5B lower panels). In order to exclude any possible aberrant cell proliferation, paraffin-embedded over spinal sections derived from mice implanted with ASphCs or ADSCs seeded on Integra® were subjected to a mutational panel analysis for vertebral tumours including osteosarcoma, chondrosarcoma and liposarcoma. The most related gene mutations involved in the initial event of transformation such as TP53, BRAF, NRAS, KRAS, HRAS, PI3K resulted wild-type.

Discussion

Here we provide evidence that spheroids derived from human adipose tissue are enriched with mesenchymal stem cells, which are an efficient source of cells, endowed with the capability of multilineage differentiation and repairing bone injury. Diverse approaches and several putative stem cell markers have been exploited to identify and purify the mesenchymal stem cells from adipose tissue. It was determined that CD9, CD29, CD44, CD90, CD105 or CD271, are cell surface molecules that are highly expressed in multipotent stem cell populations present within the adipose tissue [33,34]. It has already been reported that stem cells derived from adipose tissue can grow as floating spheres, maintaining the capacity to differentiate *in vitro* toward adipocytes, chondrocytes and osteoblasts [25,35,36]. It is likely that CD271 identifies both stem and progenitor cell subsets. Being that ADSC are cultured since the first passages in 2% FBS supplemented medium (which is known to drive stem cells toward differentiation), we can speculate that CD271 expressing ADSCs are non-clonogenic progenitors, which are poorly able to form floating spheroids and to sustain *in vitro* serial passages. Conversely, CD271 expressing ASphCs are mesenchymal stem-like subset endowed with a clonogenic activity that retains the multilineage differentiation capacity.

In mammals, white adipose tissue corresponds to the stem cell compartment and is mainly distributed among several visceral organs, central nervous system, subcutis and breast tissue whereas, brown adipose tissue is located in the scapulae and neck regions [22,37]. Within the white adipose tissue, there is a presence of mesenchymal stem cells that are associated with potent intrinsic peculiarities and were found located in the subcutaneous breast tissue, suggesting that the subcutaneous site is likely a major reservoir. Breast adipose tissue-derived ASphCs showed a prominent specific differentiation capacity toward adipocytes, chondrocytes and osteoblasts.

It was demonstrated that while the transcription factor SOX9 is required for chondrogenic differentiation, RUNX2, which activates osteocalcin, is essential in osteogenesis [38,39]. Several *in vivo* studies have shown that white adipose tissue development is strictly dependent on *PPARy* activity. It was ascertained that *PPARy* governs, through the regulation of PPARy ligands' endogenous production, the differentiation of mesenchymal stem cells toward the adipogenic lineage [40]. In line with previous reported data that highlight intrinsic stem cell properties, ASphCs are quiescent. Once they are forced to differentiate, they up-regulate the specific gene regulators that govern the maturation in the three different lineages. This subpopulation has characteristics which are inherent to mesenchymal stem cells that ADSCs lack on a functional level. Indeed, the ADSCs showed a limited capacity to be serially passed *in vitro,* progressively losing multilineage potential. Furthermore, their lineage acquisition seems to be governed by different pathways. So it is likely that ASphCs and ADSCs may reside at different levels of the stemness hierarchy.

The majority of mesenchymal stem cells often die quickly following an *in vivo* implantation [41]. Mesenchymal stem cells have been widely used in experimental models that are related to the treatment of bone diseases. To increase survival and ensure the differentiation in osteocytes, mesenchymal stem cells were previously genetically engineered and thus over-expressed AKT1, BMP2 and VEGF [27]. They were then transplanted into the fracture site. In this model, mesenchymal stem cells were not integrated into the newly generated bone, but they played a supportive role during the early stages of tissue formation. To circumvent these issues, an artificial scaffold has been used to prolong the mesenchymal stem cells' viability as well as enhancing their differentiation capacity. In our model, ASphCs cultured on Integra® and implanted into the laminectomy site, were able to directly participate in bone tissue regeneration. Our study supports that a stem cell population within adipose tissue that expresses CD271, possesses an *in vitro* multipotency and harbours the capacity to repair bone injury that could be exploited in regenerative medicine.

Acknowledgements

Authors are pleased to thank Tatiana Terranova for her editorial assistance. We thank F. Calò for help in figure preparation, A. Gorgone and G. Tomaselli for technical assistance. This work was funded by Associazione Italiana per la Ricerca sul Cancro (AIRC) IG 12819 to G.S., PON FESR 2010-2013 (PON01-01059 and PON01-01078) to G.S. and Progetto Giovani Ricercatori 2010 (GR-2010-2321017) to A.A.L.B. and M.T.

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Chapter| 2

The adipose tissue may be considered as a real endocrine organ. it acts as a reservoir of energy of the organism, storing energy as triglycerides in times of satiety, and mobilize energy in the form of fatty acids in case of need. Fatty acids and their metabolic products could be toxic, therefore are homeostatic mechanisms to manage the build up of lipids and their mobilization order to avoid accumulation of potentially toxic in peripheral organs [1]. The ability of the adipose organ to buffer variations in energy supply and demand is achieved by integrated endocrine and metabolic responses, as well as through dynamic changes in cellular composition [2]. The adipose tissue buffering capacity could be overcome during chronic overnutrition, resulting in overflowing of lipids from adipose tissue, causing a pathological accumulation in other key metabolic organs. Adipose tissue can be discerned into three main categories: the visceral and subcutaneous anabolic white adipose tissue (WAT) that stores excess energy, the catabolic brown adipose tissue (BAT) which is responsible of dissipation of energy through heat production, and the "Brown and White" (brite or beige) adipose tissue. White adipose tissue (WAT) can expand its energy-buffering capacity by fat cell hypertrophy and/or by hyperplasia from committed progenitors. In contrast to WAT, brown adipose tissue (BAT) is a highly oxidative tissue containing multilocular fat cells with abundant mitochondria that oxidize fatty acids and generate heat via uncoupling protein 1 (UCP1). WAT has been historically defined by anatomical location and the presence of parenchymal cells containing a single large lipid droplet. However, WAT can be remodeled under various physiological and pharmacological conditions to a more oxidative phenotype resembling BAT. Work in experimental models has demonstrated the remarkable plasticity of the adipose organ [3], and it is clear that beneficial effects can be achieved by promoting either anabolic or catabolic phenotypes. Whether the adipocyte phenotypes modulation should be designed as a therapeutical approach it becomes essential to better understanding of adipocyte cellular types and the relative extrinsic signals which contribute to its development and plasticity.

Adipose tissue composition. Adipose tissue is characterized by a marked cellular heterogeneity: among its cellular components, we can find adipocytes, preadipocytes, fibroblasts, endothelial cells and multipotent stem cells able to differentiate into several cell types. Overall, fat tissue consists of approximately one-third of mature adipocytes. The remaining two-thirds are a combination of small mesenchymal stem cells (MSCs), T regulatory cells, endothelial precursor cells, macrophages and preadipocytes in various stages of development. Preadipocytes have the ability to proliferate and differentiate into mature adipocytes, conferring adipose tissue a constant functional plasticity,which determines its ability to develop.[46]

Adipogenesis is a finaly regulate process that has been studied for the last 30 years,. It could be divided into different phases, characterized by different cellular composition and differentiation state. [4]. Adipose tissue appears first in the connective tissue that contain extracellular matrix and mesenchymal cells [5]. These vascular pre-structures seem to provide a framework for further recruitment of

progenitors and subsequent adipogenesis. In the process of adipose tissue maturation, the mesenchymal cells aggregate to form "lobules" possessing a reticulated structure. [5] In this process the most important steps are the vascularization and expansion of mesenchymal cells [7-9]. Finally, terminal differentiation and enlargement of individual adipocytes occur, which are the major modes of postnatal adipose tissue growth [10-11]. Application of the stem cell concept to adipose tissue biology launched an explosion of research into the molecular characteristics and differentiation potential of progenitor cells that reside in these tissue. However, the fact that adipose tissue depots appear at different times and possess distinct molecular characteristics suggests that there may be regional heterogeneity among Adipose Tissue depots, and in particularly in the subcutaneous and visceral ones. (Fig. 1)

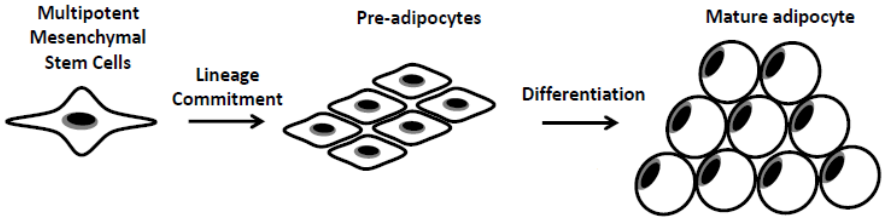

Figure 1. Representative image of the mesenchymal commitment toward mature adipocyte.

Potential adipocyte progenitor niches. Stem cells reside in specialized anatomical locations, known as stem cell niches, where adjacent cells or external signals from the microenvironment maintain their quiescence or recruitment [12-13]. The stem cell niche may be compartmentalized into distinct domains that enhance dormancy, or provide cues for proliferation and differentiation [13,15]. In particulary, preadipocytes have been localized to a perivascular niche during development [16,17-18]. It is important to note that the vast majority of adipose tissue cells are not adipose mature cells [19-20], indeed they are a dynamic mixture of cellular and non-cellular elements, including progenitors, resident/recruited immune cells, fibroblasts, blood vessels, lymphatic vessels, peripheral nerves, and extracellular matrix (ECM).

Adipose tissue macrophages. The adipose tissue macrophages are a critical cell type for the metabolic and endocrine adipose tissue functions [83,84]. The macrophages, based on their functional phenotypes, have been classified into two subpopulations: the M1 macrophages that are associated with the defense responses and pro-inflammatory immune, and the M2 macrophages that are involved in tissue repair [21-22]. Macrophages depletion impairs adipogenesis and macrophage-conditioned medium could inhibit preadipocyte apoptosis and promote survival [23-24]. In addition, macrophages are a major source of angiogenic factors and can indirectly mediate Adipose Tissue expansion [25]. However, every specific effects of macrophages have not been investigated. Pro-inflammatory M1 macrophages accumulate in adipose tissue during obesity and thereby could promote insulin resistance. [26-27]

Vasculature. Experimental data suggest that angiogenesis and adipogenesis are tightly coupled during development. *In vitro* studies suggest that signals derived from surrounding vascular units directly influence preadipocyte proliferation and differentiation. The vascular-derived signals remain poorly characterized [9,28-29]. Several studies have shown that overexpression of VEGFA improves metabolic profiles and prevents adipose tissue dysfunction during the early phase of obesity . In contrast, reducing vascularization during obesity may improve metabolic outcomes by inducing apoptosis in dysfunctional adipocytes [30].

The adipose extracellular matrix (ECM). It is an adipose stromal component that provides structural support and biochemical signals to maintain tissue function. Importantly, physical and biochemical elements of ECM can affect MSC lineage specification, proliferation, and differentiation.on the other hand the adipocytes progenitors play an important role in ECM remodeling. [31-33]. I.e, their differentiation rapidly upregulate expression of collagen IV and various laminin complexes. Contrary, fibronectin expression decreases during adipocyte differentiation [34-36]. Together, these findings suggest that ECM configuration undergoes a transition from a fibrillar to a laminar structure to accommodate adipocyte growth and fat storage [33]. ECM degrading enzymes, includes fibrinolytic enzymes and numerous matrix metalloproteinases (MMP), play a crucial role in modifying ECM structure creating a permissive environment for adipose tissue growth/remodeling. [37]. Dysregulation of ECM synthesis could lead to fibrosis and adipose tissue disruption. Fibrosis is a common pathophysiological response to chronic injury in which excess accumulation of fibrotic ECM distorts tissue architecture and impairs the function of parenchymal cells [38]. During obesity, metabolically-challenged adipose tissue increases fibrous collagen production and upregulates related genes. Adipose tissue macrophages, which are recruited in obese states, contribute to fibrosis by stimulating fibroblast ECM synthesis.

Pathological role of adipose tissue. Substantial evidence suggests that adverse consequences of obesity have their origins in the pathological remodeling of adipose tissue [39]. Obesity is a major disease in recent decades, it is associated with increased incidence of many diseases, including cancer. It is known that obesity is associated with a generalized inflammation, with an elevated expression of inflammatory cytokines and recruitment of macrophages, an endothelial dysfunction and a wide adipocyte death [40-43]. A chronic overnutrition, infact, increases the occurrence of hypertrophic fat cells, which become distressed and undergo necrosis/apoptosis reaching a critical size. Adipocyte cell death recruits macrophages and triggers a local inflammatory response [44-45]. Furthermore there are secreted molecules called adipocytokines, that exert a significant effect on every adjacent cells included the immune system. For these reasons adipose tissue could be considered not only a simple energy storage tissue system but it could be considered an important endocrine and immune organ, whose hormones, however, not yet fully characterized. Moreover recent studies on inflammatory cells infiltrating the adipose

tissue highlight that these cells generate a specific environment that perpetuates the inflammation within tissues and activate adipocytes to produce others inflammatory mediators and adipocytokines, creating a virous circle between inflammation and obesity [47]. A depth study of each molecule released from adipose tissue there are necessary. In particular in our study the key issue becomes identifying the main cytokines responsible of an inflammatory and pro proliferative microenvironment. The tumor and tissue microenvironment are indeed closely related. It is well established that the microenvironment plays a critical role in cancer development and metastasis formation. It has been demonstrated that obesity promotes significant changes in the biological properties of Adipose Stem Cells (ASC). [48]. Although current knowledge of the biological impacts of MSCs on tumor development is greatly improved, the underlying effects of MSCs from different origin remain controversial. Fact, adipose tissue adjacent to tumor is thought affect tumor progression, which may be the alternative mechanism of cancer incidence associated with obesity, since ASCs reveal tropism to inflammatory sites and tumor lesions, and could contribute to the tumor microenvironment, promote a higher cancer progression and also an invasion and migration of cancer cells. [49-50]. Accumulating evidence, indicates that ASCs promote tumor growth and metastasis of various cancers [51-54], whereas conflicting reports reveal the anti-tumorigenic potentials of ASCs such as anti-proliferation and pro-apoptosis [55-57]. MSCs have been proposed as an attractive candidate in the regenerative medicine for their ability to home into tumor sites and to secrete anti-tumor agents [58], but the recent findings suggest that the MSC-secreted soluble factors in a tumor microenvironment may contribute to a tumor progression [51,57]. (Fig. 2)

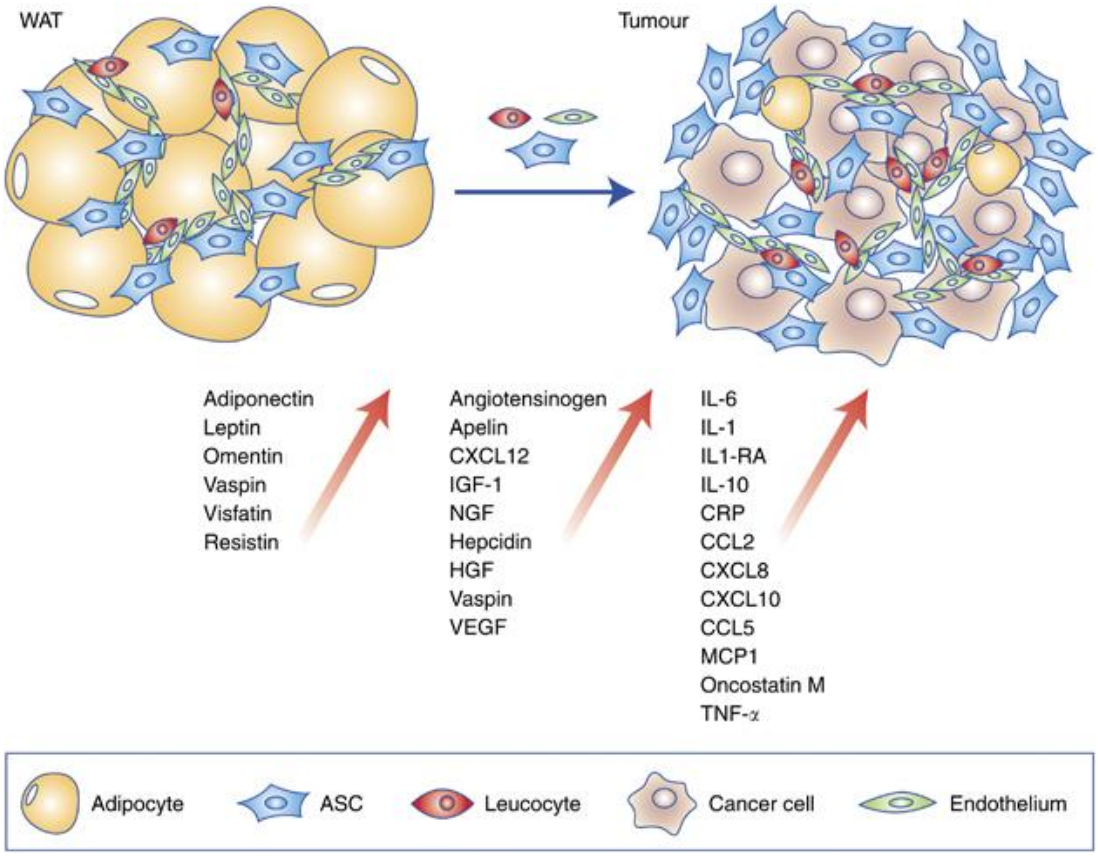

Figure 2. White adipose tissue presence in tumours.

The tumor and adipose tissue are closely related and constantly interacted. (*British Journal of Cancer* (2015) 112, 419–423. doi:10.1038/bjc.2014.657)

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Chapter| 3

Breast cancer

Breasts are equal and symmetrical glandular organs placed in the district subcutaneous of the front and upper female chest. The breast is a composed tubule-alveolar gland consists of 15-20 lobes plunged in the adipose tissue and sedimented of connective laminae. Each lobe consists of several lobules containing alveoli secreting glandular units, each of these posses its own excretory galattoforo duct which is divided into lobular ducts that continued in lobular alveolar ducts flowing into the alveoli terminals and finally converging into a single large duct in the nipple. Breast carcinoma represents the most common neoplasia in women with a ratio of 25%, responsible for over 400,000 deaths every year. The incidence of the disease varies widely in different geographical areas with a higher prevalence in industrialized countries. In Italy about 37,000 cases are diagnosed. The age in which there is the highest breast cancer incidence is between 45 and 50 years. The individual risk is indeed influenced by many different factors such as family history, age, lifestyle and environment. (Figure 1)

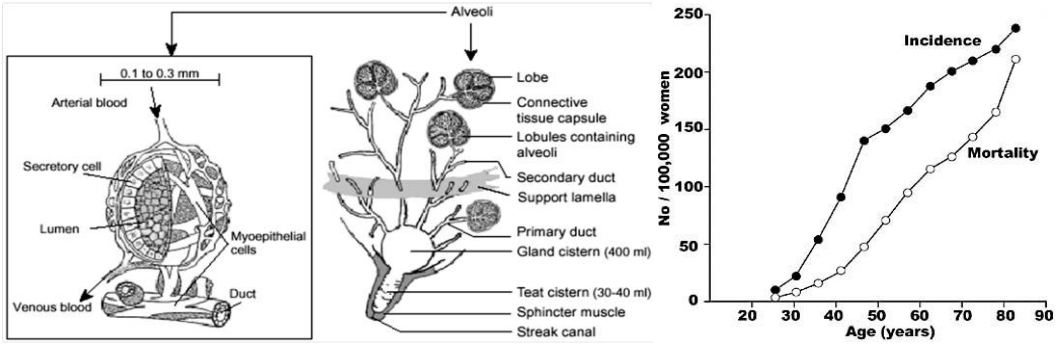


Figure 1. Breast anatomy and breast cancer incidency

The detected mutations of some tumor suppressor genes such as BRCA-1 (BRCA1) and Type 2 (BRCA2) in chromosome 17 or p53 mutations increase the breast cancer risk. The most widely accepted opinion, however, is that the breast cancer onset hormonal factors play the higher significant causative role[1].

The breast invasive carcinomas classification for many decades was done on the histologic type and grade basis

- Epithelial: derived from glandular epithelium, which lines the inside of the ducts and lobules.
- Fibroepiteliali: it is made of epithelial and mesenchymal or stromal components
- Mesenchymal: cancer type observed in so-called soft tissue, such as skin and subcutaneous tissue.

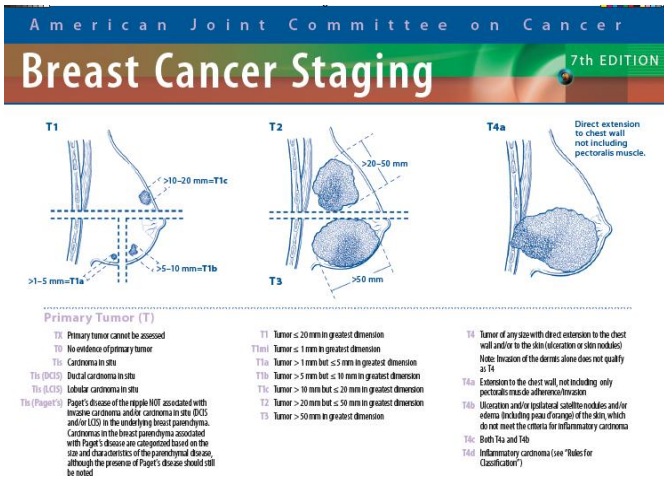
- Miscellaneous: rare cancers, which are not grouped in the previous classes.

The most common breast cancer type is the epithelial lesion, and the most frequent histotypes are ductal carcinoma and lobular that.

Ductal carcinoma develops in the galactophorous ducts while the lobular develops in the glands galattofore. The two histotypes may be "in situ", that is non-invasive, or "infiltrating". In the first case it is a tumor intraepithelial, that is, the surface in which there is no migration in the underlying tissues and not to be exceeded the basement membrane.

This neoplasia could be characterized either on the basis of different stages of the disease that in relation to the originating site and thus will distinguish a ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS).

When cancer cells beyond the surface epithelium and spread within the underlying tissues through the lymph vessels and blood, invading More distant organs giving rise new centers of the disease, in this case we talk about tumor infiltrating or invasive and the most common is ductal histology (DIC = Invasive ductal carcinoma), which represents about 75% of tumors infiltrating lobular while about 5%. Other histologies infiltrating less frequent are the medullary carcinoma (15%), colloid or mucinous (2%), the tubular (1-2%), to which are added several other rare forms. [2]:



ANATOMIC STAGE/PROGNOSTIC GROUPS			
Stage 0	Tis	N0	M0
Stage IA	T1*	N0	M0
Stage IB	T0	N1mi	M0
	T1*	N1mi	M0
Stage IIA	T0	N1**	M0
	T1*	N1**	M0
	T2	N0	M0
Stage IIB	T2	N1	M0
	T3	N0	M0
Stage IIIA	T0	N2	M0
	T1*	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
Stage IIIB	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
Stage IIIC	Any T	N3	M0
Stage IV	Any T	Any N	M1

Figure 2 Breast Cancer Staging on the basis of TNM classification.

Together with the histological characterization, there is a sub molecular classification on the basis of gene expression and identified five subtypes of breast cancer: luminal A, luminal B, HER2-enriched, basal-like and claudin-low.

Luminal A:

It is characterized by the expression (94%) of the receptors for the steroid hormone estrogen (ER). These tumors are typically positive for the progesterone receptor (PR), although there is a subtype of ER + / PR-, furthermore express other markers luminal epithelium-specific protein such as intermediate filament cytokeratin (CK18) and MUC1, and can be positive (9%) or negative (92%) for the receptor of the human epidermal growth factor 2 (HER2). According to this pattern genetic, luminal tumors are made up most part to differentiated cells sensitive to hormonal treatments, and therefore have a much more favorable prognosis than those with triple negative phenotype (NPT), which lack ER, PR and HER2 (4-5%), to which correspond phenotype local recurrence and dissemination to distant sites.[3-4]

The Basal-like (BL):

it is characterized by low or absent gene expression markers luminal: ER (91%), PR- / low, Hermann / low, in fact, this subtype is the high percentage (39-54%) of the NPT. Such class of breast tumors express low levels of other markers luminal as GATA3 and CK18 and 19 against and are highly expressed basal cytokeratins CK5, CK14, and CK17. As part of the markers of stem / progenitor the BL show high gene expression of CD44 molecules, CD49f and ALDH-1 and rather low expression of epithelial differentiation markers including CD24, EpCAM and MUC1. However, recent experimental evidence state that the BL levels of CD24 / EpCAM and ALDH-1 are not significantly lower and higher, respectively . Therefore within cancers basal can be found CD44 + CD24low CD49f + and + / high EpCAM + cell populations. Thompson and Snow have also shown that in these subtypes of tumors is elevated gene expression of markers of transaction epithelial mesenchymal (EMT) whose predominant function is to suppress the E-Cadherin (glycoprotein of calcium-dependent cell-cell adhesion) in directly through Snail1, Snail2, Zeb1, Zeb2, TCF3 and indirectly through TWIST1, Twist2, Id1, TCF4, FOX2 and NFkB . The molecular profile of the BL is therefore consistent with an enrichment of epithelial cells and undifferentiated tumor with mesenchymal characteristics, ie a high density of ICT / CST whose antigenic phenotype coincides with the expression pattern of the gene of the BL. The high percentage of SC cells / progenitors in BL would be the cause of the resistance to current hormone therapies and chemotherapy and is therefore associated with a poorer prognosis than luminal subtypes.[3,5-6]

The Claudin-low (CL)

It was first identified in 2007. The name comes from the low levels of expression of genes involved in the junctions occluding such as Claudina 3, 4, 7 and occludin. The pattern and the molecular phenotype of this cancer subtype is very similar to the subtype BL fact also has a high percentage (25-39%) of the NPT, negativity for cytokeratins CK18 luminal and 19 and high expression of basal cytokeratins CK5,

CK14, and CK17. CL also tumors have high density cell populations with phenotype CD44 + / CD24- high / low and CD49f + / high EpCAM- and high levels of ALDH-1 unlike the BL. Studies conducted by Thompson and Snow show that even in these tumor subtypes there is an expression of the marker gene EMT even higher than the BL; However, the same studies show that the distinct characteristics present in the mesenchymal cells of the CL as well as in BL, are only associated with epithelial tumor cells and not due to contamination from adjacent stroma [6]. E 'was also noted that in CL there is a high expression of genes involved in the immune system's response (CD79, CD14, Vav1), this data suggests a potential recruitment of different types of leukocytes in this type of tumors. The CL, in agreement with the high expression of mesenchymal markers / normal stem cells, presents a higher percentage of ICT normal stem cells / progenitors compared to BL which makes them slightly sensitive to chemotherapy (neo adjuvant anthracycline / taxane) but which total can not be eradicated and therefore are linked to a very poor prognosis.

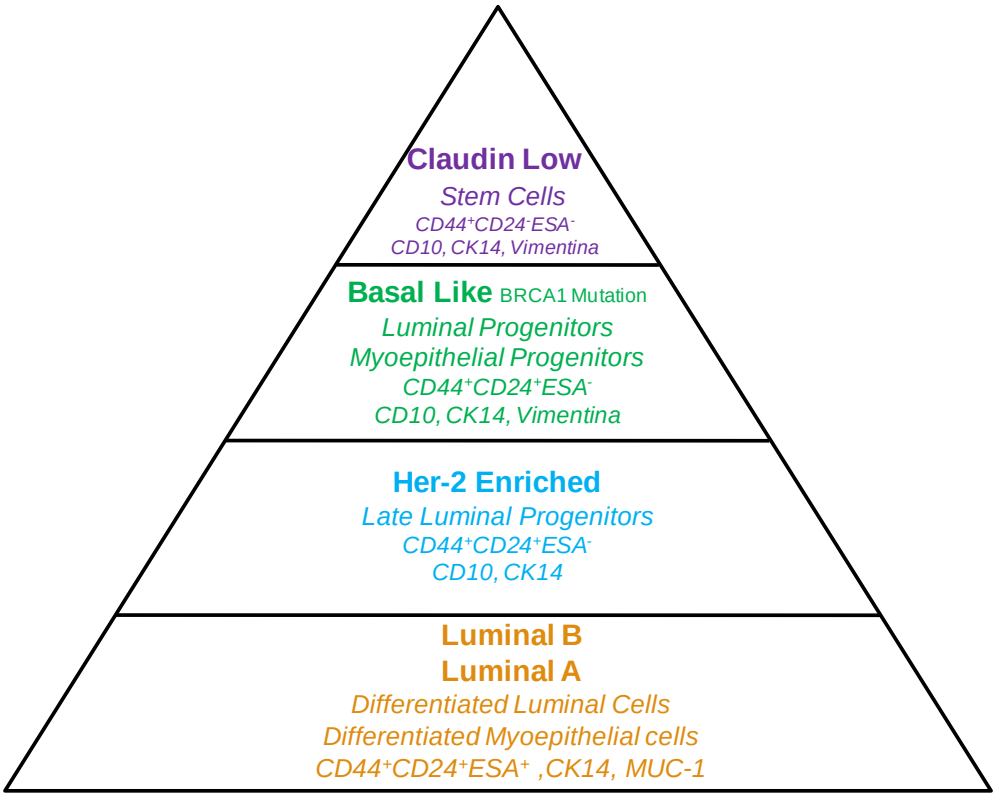
Luminal B

this class of breast cancer has a molecular profile and phenotypic HR + (ER + PR +) / HER2 +. The Ki-67 protein has been studied as a potential marker of immunohistochemistry (IHC) that could distinguish subtypes luminal B tumors than A. High expression of Ki-67 (therefore highly proliferative) were associated with luminal B unlike luminal Type A [7]. Kabose and Horwitz (2010) have shown that this type of breast cancer presents a certain heterogeneity. Studies show that the luminal B subtype is not only relatively (compared to the BL and HER2-enriched) chemo-insensitive (anthracycline / taxane) but also resistant to hormone therapy. The biology of the luminal B could be explained by the resistance mechanisms of hormone therapy including activating ligand-independent ER by the family of HER receptors, which could cause resistance to endocrine therapies in cancers basal ER + / HER2 + but also in ER + / HER2 thanks to an activation in an alternative signal of HER. Two clinical trials have tested this hypothesis by showing that a combination therapy, endocrine and anti-HER2 (trastuzumab), increased survival in breast cancer patients with HR + / HER2 + metastatic. However, many patients do not benefit from HER2 + therapy with trastuzumab [8-10].

The HER2-enriched

It is characterized by overexpression of the HER2 receptor. It also shows mainly negativity for ER (69%); The tumors belonging to this subtype exhibit the loss of expression of gene cluster and low basal expression of gene cluster luminal if compared to that of luminal A and B [3]. Cell populations belonging to this subtype have high transcriptional levels of CD24 [6]. Recent studies show a high chemo-sensitivity (anthracycline / taxane) subtype HER2-enriched and Basal-like (widely ER-negative), but despite the high response to chemotherapy, the occurrence of relapses due to the "minimal residual disease "greatly improves the survival free of disease progression in patients with ER + tumors (primarily luminal). The molecular

characterization and phenotypic then shows how the intrinsic subtypes of breast cancer could be made up of cells resembling those of normal tissue at different stages of differentiation as evidenced by recent studies Lim et al. (2009). On the origin of the development of the intrinsic subtypes have been several hypotheses; one of these, in line with the theory of the CST, said the SC could be the origin of any intrinsic subtype. In this context, the transformed cells SC are capable of experiencing division symmetric and asymmetric, the latter capable of generating differentiated cells that are stopped at a specific stage differentiation dependent on the particular cancer subtype. There was therefore a bulk tumor composed of cells more or less differentiated, with a subpopulation of cells with characteristics of CST / ICT. According to another hypothesis the different molecular subtypes may not originate directly from alterations in SC but CST derived from a de-differentiation of differentiated cells transformed into cancer. Other authors instead argue that the different subtypes could arise from transformed cells that are found at different levels of luminal differentiation of normal breast. In this context, the SC would give rise to tumors CL, luminal progenitors to BL and luminal cells mature tumors luminal A and B; However, this hypothesis contrasts with other studies (in support of the theory of CST) that a smaller percentage of ICT / CST (CD44 + / CD24-) is observed in all tumor subtypes. Numerous experimental evidence, however, have supported that the origin of CL and BL are stem cells, in contrast to the more differentiated subtypes (Luminale A, B and HER2 enriched). In this context Prat and Perou (2011) are more favorable to a combination of these theories: CL and BL originerebbero from the processing of a normal stem cells, with limited differentiation into progenitors in the case of luminal tumors BL, instead luminal tumors may arise from a transformation of differentiated cells [3].



Classificazione	Estrogeni	Progester.	EGFR (Her2)	indice di proliferaz.	Citocheratine e Marker	Fenotipo Antigene	Mutazione Principale	Prognosi
Luminal A (42-59%)	ER+	PR+/-	Her2 -	Ki67 Low	CK8+, CK18+ Muc1+	CD44+low CD24+ ESA+	Pi3K High p53 low MAPK+ V-High	Favorevole
Variante rara Luminal A	ER+	PR+/-	Her2 +	Ki67 Low	CK8+, CK18 + Muc1	CD44+low CD24+ ESA+	Pi3K High p53 low	Favorevole
Luminal B (6-19%)	ER+	PR+/-	Her2 -	Ki67 High	CK5+ , CK14+	CD44+low CD24+ ESA+	Pi3K Med p53 Med	Sfavorevole
Variante rara Luminal B Her2+	ER+	PR+/-	Her2 +	Ki67 High	CK 5-14	CD44+low CD24+ ESA+	Pi3K Med p53 Med	Sfavorevole
Her2 Arricchiti (non Luminali)	ER-	PR-/+	Her2 +	Ki67 High	CK 5-14	CD44+low CD24- (Stem) CD24+(Mioepit) CD10 ESA-	Pi3K Med p53 High	Infausta
Basal	ER-	PR-	Her2 -	Ki67 High	CK 5-14-17	CD44+low CD24- (Stem) CD24+(Mioepit) CD10 ESA-	Pi3K V-Low p53 V-High Mapk V-Low E-Caderina - Vimentina -	Infausta
Normal Like	ER-	PR-	Her2 -	Ki67 High	No-CKs			
Claudin Low	ER-	PR-	Her2 -	Ki67 High	CK 5-14-17	CD44+low CD24- (Stem) CD24+(Mioepit) CD10 ESA-	Pi3K V-Low p53 V-High	Infausta

Figure 3. Molecular Classification of Breast Cancer Subtypes (lower panel) and Pyramidal hierarchy of breast cancer subtypes, the apex we find the most undifferentiated and poor prognosis subtype to most differentiated and favorable prognosis (upper panel).

Stem cells

The adult stem cell is a cell highly undifferentiated with distinct replicative capacity and is capable of giving rise to all cell types of the organism through a process of differentiation. The classic definition of a stem cell gives it two essential properties: the self renewal and potency [11]. The self renewal or self-renewal is the ability to generate daughter cells with the same characteristics of the mother cell during each cell replication. Stem cells in fact undergo asymmetric cell division giving rise to two different daughter cells, one identical to the parent cell and another, called progenitor, with the properties of a cell more specialized and differentiated.

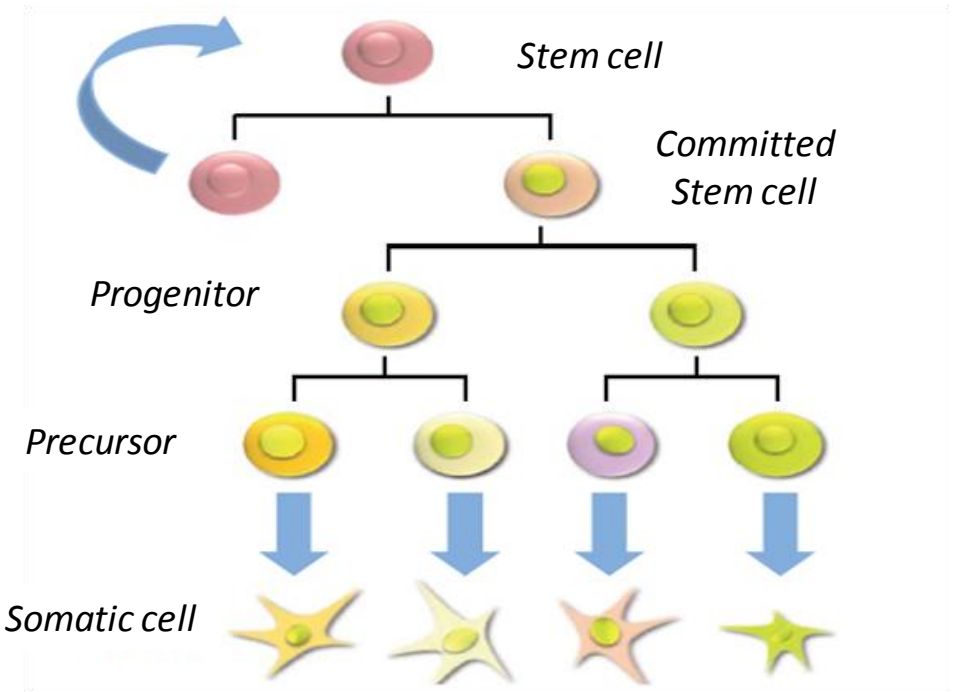


Figure.1: Representation of the properties of a stem cell to do a asymmetric division.

The potency represents the ability of the cell to differentiate into various specialized cell types. Stem cells have a great plasticity and differentiation based on this are classified into totipotent, pluripotent, multipotent and unipotent.

- Totipotent cells have the ability to reason a complete individual, retain the ability to differentiate into all cell types and fetal membranes and possess an unlimited proliferative capacity and multiplicative.
- The pluripotent cells have lost the ability to give rise to a complete individual, have multiplicative and proliferative capacity, maintain immortality and cell it remains unchanged the ability to differentiate into all cell types except the fetal membranes.

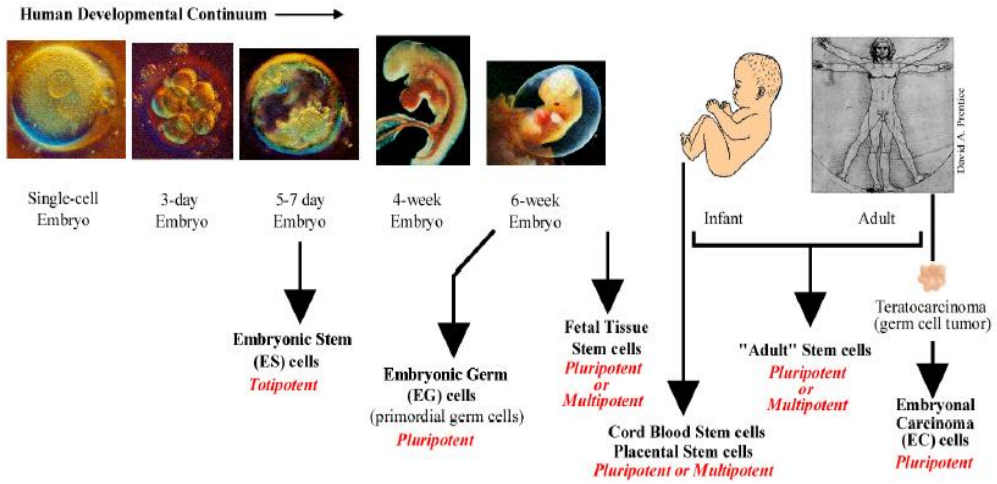
- Multipotent cells have lost the immortal cell but retain the ability to differentiate.[11]

Depending on their origin, the stem cells are also classified in embryonic, fetal and adult.

- The embryonic stem cells are pluripotent and totipotent. The first resident in the zygote and embryo until the third cell division, the latter are present in the embryo until the first week after fertilization. Cells are able to perform an unlimited number of symmetrical divisions without differentiating.

- The fetal stem cells are pluripotent and multipotent stem. They are present in the fetus during the developmental period that goes from the tenth week of gestation until birth and can be found in the umbilical cord, placenta and amniotic fluid.

- The adult stem cells are multipotent and unipotent. District are cells, undifferentiated and deputies to tissue regeneration. They are present in the bone marrow and other tissues such as the neural tissue, skin, adipose tissue and many other tissues. They have the ability to both self-renew is to differentiate into various specialized cell types of the tissue / organ of belonging and generate intermediate cells called undifferentiated progenitors.



The Cancer Stem Cell Concept

In recent years the views of the scientific community on cancer biology has drastically changed. In 1976 Nowell had described the stochastic model according to which the tumor originated from any cell of the body following the acquisition of multiple mutations borne by those genes that promote cell proliferation (oncogenes) or the genes responsible for the inhibition of cell cycle (tumor suppressor genes). According to this model the mutant progeny could acquire additional mutations generating a genetically heterogeneous tumor. So according to this model of clonal evolution, each cell could be the target of malignant transformation [12]. Based on this assumption, however, he would not find an explanation for the failure of current cancer therapies aimed at killing cells proliferating.

In recent years, therefore it has been proposed a different model of carcinogenesis, defined " hierarchical ", according to which a tumor would have originated from a small portion of cells called " cancer stem cells " (TSA). This different theory has taken shape by experimental evidence that, although the tumor originated from a clone, the cell population is not homogeneous as in the course of proliferation cells acquire new mutations. The hierarchical model therefore submits that the tumor mass would be composed of a large number of cells capable of proliferating in a limited manner and by a rare population of cells responsible for the growth and neoplastic progression, capable of proliferating in the long and maintain the tumor (Figure.5). The stochastic model was also refuted by experimental evidence that in the animal model of reference was necessary to inoculate a large number of tumor cells for the obtaining of a xenograft. As opposed to the model of CST it is seen that a small fraction cell is necessary and sufficient for the recapitulation of the tumor starting [13-15].

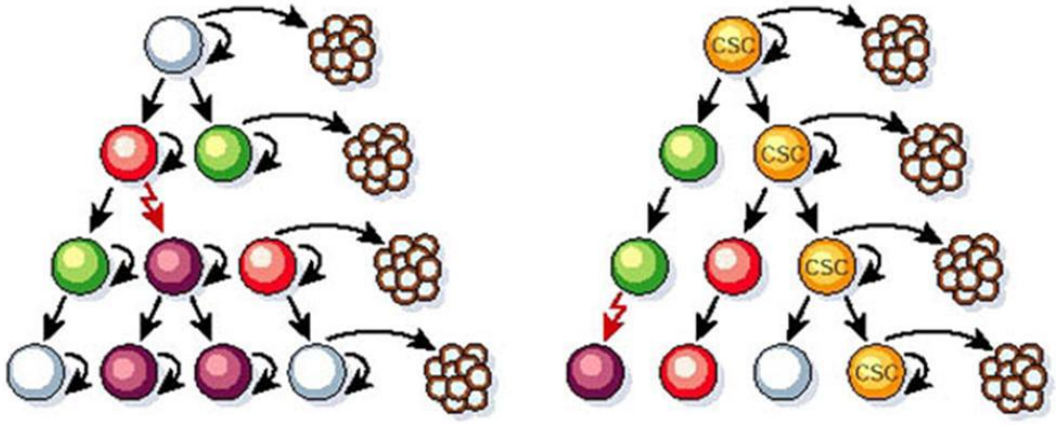


Figure. 5: Rappresentazione grafica dei differenti modelli di carcinogenesi. **A)** Modello stocastico, le cellule tumorali sono eterogenee e la maggior parte di esse può proliferare e generare nuovi tumori. **B)** Modello gerarchico, le cellule tumorali sono eterogenee e solo la sottopopolazione delle CST (Cellule Staminali Tumorali) possiede la capacità di proliferare ampiamente e formare nuovi tumori (Weissman et al., Nature, 2001).

The first demonstration of the existence of the CST was provided by Dick and colleagues at the end of the nineties, using as a model the acute myeloid leukemia (AML). Sorting by flow cytometry, it was isolated a subpopulation of CD34 + CD38- phenotype characterized. Although these represent only a small percentage of all leukemic cells, CD34 + CD38- were the only ones capable of giving rise to leukemia in mice immunocompromised NOD / SCID (Non-Obese Diabetic / Severe Combined immunodeficient) and to keep this potential following serial transplantation. Also obtained from the xenograft mouse model, it was the exact phenocopy parental tumor, confirming that the CD34 + CD38- fraction was able not only to self-renewal, but also to give rise to a more differentiated progeny [16]. An alternative method to flow cytometry sorting, for the isolation of the CST, is the sage of side population (SP), which refers to the high expression of membrane transporters ABC (ATP Binding Cassette) providing the stem cell's ability to mediate the outflow of the chemotherapeutic drug and some dyes such as Hoechst 33342 [17]. This method is now little used and supplanted by the identification of stem cell markers that is the subject of scientific debate. Some markers currently used to enrich the population of cancer stem cells are CD133 for glioblastoma, colon, pancreas, lung liver and prostate; and CD44, CD24 for the pancreas, ovary, head and breast [18]. For this reason the molecular targeting of such highly tumorigenic cells becomes fundamental to improve the efficacy of current anti-cancer strategies, aiming to sensitize tumours to conventional therapies thus definitely abrogate tumorigenesis.The main problem in CSC research is the identification and characterization of this cell subset, to study their biology and design new specific target therapy against them. In this regard the use of biomarkers, for CSC purification (most of the time by cell sorting, using membrane markers), has given a great contribute in the field. The panel of biomarkers used is different in different types of cancer, as shown in Table 1.

Table 1. CSC markers

Tumour type	CSC markers
Leukemia	CD34+ CD38- HLA-DR-CD71- CD90- CD117- CD123+
Multiple myeloma	CD138-
Breast cancer	ESA+ CD44+ CD24-/low ALDHhigh
Liver cancer	CD133+ CD49f+ CD90+
Brain cancer	CD133+ BCRP1+ A2B5+ SSEA-1+
Lung cancer	CD133+ ABCG2high
Colon cancer	CD133+ CD44+ CD166+ CD24+ EpCAM+
Prostate cancer	CD44+ CD133+ α2β1high
Pancreatic cancer	CD133+ CD44+ CD24+ EpCAM+
Head and neck cancer	CD44+
Melanoma	CD20+

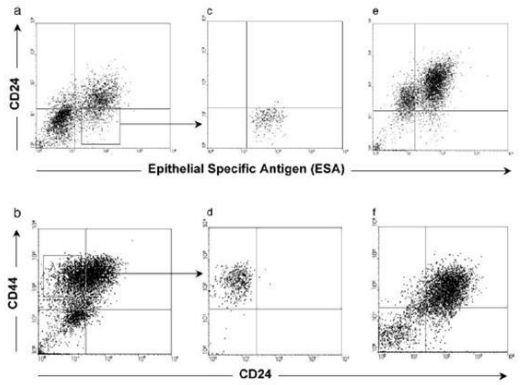
The specificity of each marker is often questioned by different research groups for several reasons, i.e. the sequential cell sorting purifying CSCs having all the CSC markers has never been reported in literature (it is not rare to find a CSC population expressing one marker and not another one) [19] and sometimes these markers are also good markers of normal tissue adjacent to the tumour [20].

CSC state is increasingly being seen as a flexible, rather than fixed, quality of tumour cells that can be lost and gained over time [21]). These events could lead to a spatial/temporal intra-tumour heterogeneity (ITH) that may contribute to some of the difficulties in validating biomarkers for clinical use, despite the continued discovery of potential novel biomarkers [22]. CSCs are indeed used nowadays as a marker of tumour aggressiveness and drug resistance, the CSC number and phenotype is also used by clinicians to decide the best treatment to apply. Despite the considerable progress made in cancer research, the majority of patients still do not show advantages by the use of a particular anti-cancer therapy, this phenomenon could be explained by ITH [23]. The ITH was initially proposed in 70's and justified by the continuous selection of tumour cells due to the applied regimen and by the CSCs differentiation. It was recently demonstrated that about two thirds of the mutations found in single biopsies of renal cell carcinoma were not expressed through all the sample regions of the patient's tumour [24]. An important role in the generation and maintenance of ITH is certainly played by the microenvironment (the tumour, such as the normal tissue, possess a variable architecture, in terms of vascularisation, infiltration degree and connective tissue components) and the chemo- or radio-treatment. Recent studies using sequential sequencing through different lines of therapy highlighted the effect of DNA damaging agents, leading to the expansion of resistant clones, or generating new cell populations as a result of new genomic alterations gained during the therapy [25-26]. According to the same model of spatial and temporal ITH, it was recent demonstrated that even if the metastatic lesions are related to primary tumours, they sometimes carry additional mutations in functionally important loci completely absent in the primary tumour [27-29], this discovery resulted fundamental since most of the therapeutic decisions are based on the primary tumor analysis.

Breast cancer stem cells

In an experimental study reported by Al-Hajj and colleagues in 2002 are identified by flow cytometric sorting " cells initiators breast cancer " based on the expression of surface antigens such as CD44, CD24, ESA [30-31] (Fig. 7). CD44 is the receptor activity iauloronico, but it can also interact with other ligands such as osteopontin, collagen, FGF and MMPs; CD24 is a glycoprotein that is part of the category of cell adhesion molecules; ESA (Epithelial Specific Antigen) is a transmembrane glycoprotein of 40kDa portion expressed in the basolateral surface of the epithelial cell.

Subsequently, additional markers were used for the characterization of these cells as CD133, a glycoprotein with five domains transmembranari and the ALDH1, the isoform 1 of the aldehyde dehydrogenase, an enzyme which oxidizes aldehydes intracellular detoxifying and converts retinol to retinoic acid. In particular a study by Ginestier and colleagues in 2007, it was assumed that ALDH1 was able to protect the cells from oxidative stress allowing a greater survival and even modulating the proliferation, with experiments conducted both in vitro and in vivo shows that epithelial cells breast, both normal and tumor cells with increased activity of the enzyme ALDH1, have characteristics of stem cell [32-33]. For which cells CD44 + CD24- and ALDH 1, possess tumorigenic capacity, being able to generate tumor in immunocompromised mice since the inoculation of 20 cells. In the same study it was also shown that cells CD44 +, CD24- and ESA + would generate cells CD44 + and CD24- additional, confirming the theory of asymmetric cell division, maintaining the characteristics of self-renewal and the generation of a population heterogeneous cell also not tumorigenic that constitute the so-called tumor mass. Recent work by Pitch and colleagues of 2010 also proposed, as a method for the isolation and purification of stem cell component from mammosfere cancer, FACS analysis (Fluorescence Activated Cell Sorter) by marking the cells with the vital fluorescent dye PKH-26 . In accordance with the fluorescence intensity it is then possible to select a population PKH-26 $^{+}$ " stem-like " (high intensity of dye) and a population PKH-26 $^{-}$ " dividing cells " (low intensity of dye). According to this profile is therefore possible to predict the biological and molecular characteristics of different mammary carcinoma: poorly differentiated or well differentiated.



	Tumors/Injections		
	8×10^5	5×10^5	2×10^5
Passaged T1			
CD44⁻	0/2	0/2	—
CD44⁺	2/2	2/2	—
B38.1⁻	0/2	0/2	—
B38.1⁺	2/2	2/2	—
CD24⁺	—	—	1/6
CD24⁻	—	—	6/6
Passaged T2			
CD44⁻	0/2	0/2	—
CD44⁺	2/2	2/2	—
B38.1⁻	0/2	0/2	—
B38.1⁺	2/2	2/2	—
CD24⁺	—	—	1/6
CD24⁻	—	—	6/6

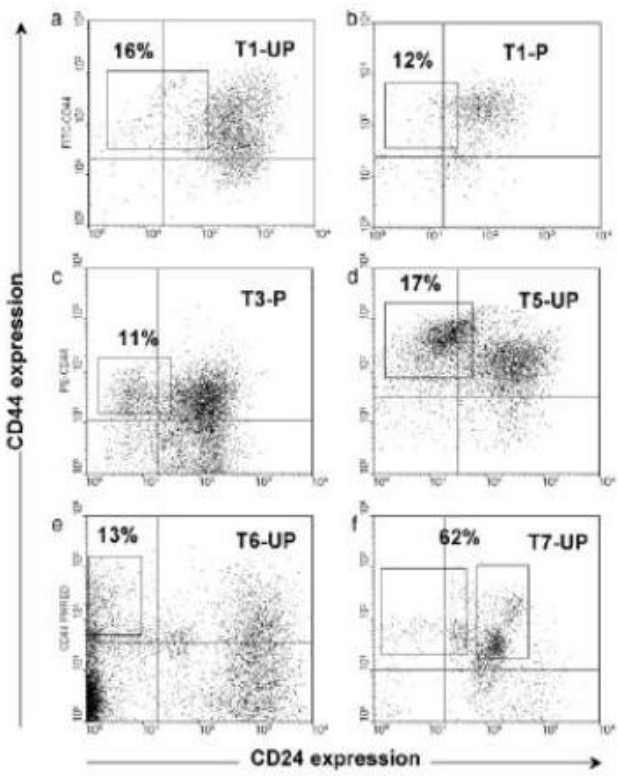


Figure. 6 Isolamento delle cellule iniziatrici il tumore al seno (Al-Hajj et al. , PNAS, 2002).

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Chapter| 4

The role of adipose-microenviroment in breast cancer relapse

Introduction

Mesenchymal stem cells were studied for the first time in the early 70s by Friedestein and colleagues [60]. The main characteristics of MSCs were first described in bone marrow tissue, that represents a suitable source of mesenchymal stem cells and which are used routinely in clinical applications, revealing a promising alternative source for further development in regenerative medicine [61-63]. Over the last 15 years, however, the attention was focused on finding an abundant valuable alternative source of multi-potent stem cells MSCs can also be isolated in minimal quantities from other tissues[64-65]. The difficult accessibility and low volumes of tissue obtained *ex vivo,* limit the clinical use of bone marrow-derived stem cells. Their harvest requires a painful procedure, neither of which is free from complications and with a low compliance of the patients. Recent studies have shown that mesenchymal stem cells derived from adipose tissue can differentiate into a variety of cell lines (cardiomyocytes, myocytes, neurons, adipocytes, osteocytes, chondrocytes) and improve the neo-vascularization of ischemic tissue.[66-68] Thus the adipose tissue is considered an ideal source of MSCs in association with therapeutic needs and in particular for the repair of defects in the regenerative medicine, since their isolation is achieved by means of a minimal invasive surgery.[69-71]

Surgical techniques: Mastectomy is the surgical breast resection, it is often the only possible therapy for the breast carcinoma, depending on the severity of the underlying disease, there is a single mastectomy, a radical mastectomy, a modified radical mastectomy or others. [74-75] Liposuction is a safe and non-invasive procedure, routinely performed through a 2-3 mm skin incision and well-accepted by patients who normally undergo this procedure. Lipofilling is surgical transfer of autologous fat tissue to areas of body that need to be filled. Over the last 15 years this method has been used just for purposes in partial reconstruction of the breast [76]. This technique consists in aspiring the fat from the patient, through micro cannulas, filtering and centrifugation process, after which the fat is reinserted in the breast. Lipofilling has been indicated for post mastectomy breast reconstruction. These surgical techniques have been considered safe or very low risk but around 1987 the American society of plastic and recostrutive surgeons began to raise doubts about the safecty of these.[72-73]

Cancer stem cells (CSCs) Recent experimental evidences have showed that the tumorigenic capacity resides only in a small fraction of cells able to sustain the tumor growth, called cancer stem cells (CSCs).[77-78] As well as normal tissues, tumors are constituted by a highly heterogenic cell population organized as unidirectional cellular hierarchies in which CSCs constitute a biologically unique subsets of cells, distinguished by their exclusive ability to perpetuate the growth of malignant cell

population. CSCs have share several important properties, among these we find include the capacity for self-renewal, the ability to differentiate, active telomerase expression, activation of anti-apoptotic pathways, increased membrane transporter activity and the ability to migrate and metastasize. One of the key early events in transformation may be the dysregulation of the normally highly regulated process of self-renewal. In the steady state, stem cells undergo to asymmetric divisions in which a stem cell is able to produce an exact copy of itself as well as a daughter cells that undergoes differentiation into lineages found in differentiated tissue.[79-81] Gene mutations responsible of this balance in normal tissues can give rise to the formation of partially differentiated cells constituting the heterogeneous tumor mass. Cancer cells in tumors undergo additional genetic or epigenetic changes and interact with thei microenvironment, leading to a constant changing variety of tumor cell types within a single neoplasm, for example the acquisition of migratory phenotype through the Epithelial-mesenchymal transition (EMT) or in the opposite direction through the the Mesenchymal to epithelial transition (MET). Within the primary tumor mass, CSCs through their ability to trans differentiate and their plasticity are able to move and migrate within the body, invading distant organs and forming metastases. [82]

Results

breast cancer stem popularion

We isolated the breast cancer stem cells (BCSCs) from fresh tissue derived from mastectomy patients that are undergone by enzymatic and mechanical digestion, as previously described [Todaro et al 2007]. The digest single cell suspension was plated in ultra low attached flasks and grown in stem cell medium supplemented with bFGF and EGF. So, we characterized our BCSCs, following the breast cancer stem guide lines established by scientific literature. We characterized the BCSCs by immunofluorescence and flow cytometry analysis. (Fig 1)

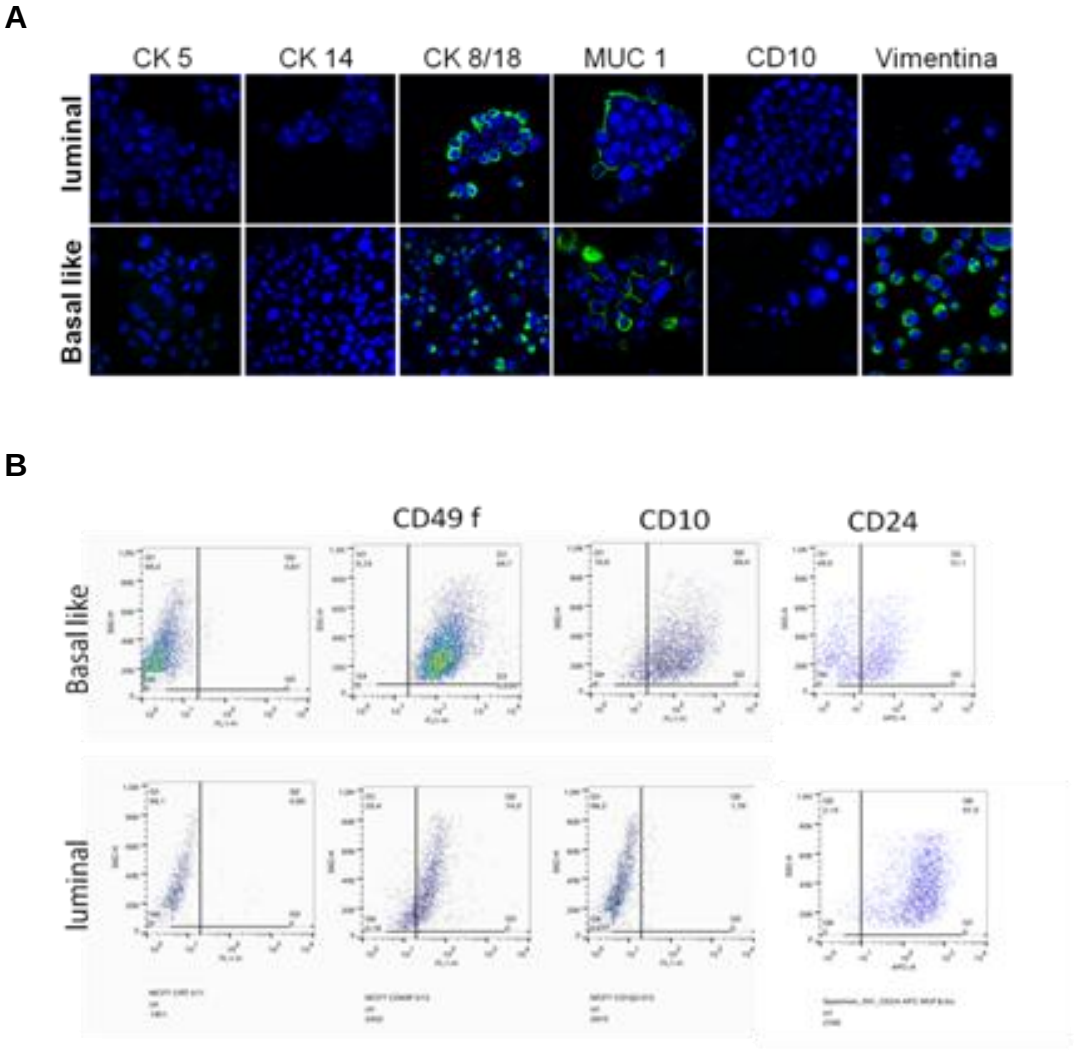


Figure 1. Breast cancer subtypes: morphologic and biologic characterization

(A) Representative confocal microscopy images of immunofluorescence (IF) analysis of Cytokeratins (CK5, CK14, CK8-18), MUC1 and Vimentin performed in luminal and Basal BCSCs. **(B)** Flow cytometry analysis of CD44, CD24, CD10, and CD49f expression in luminal and basal BCSCs.

Different adipose cells cultures release proinflammatory, pro angiogenic and pro metastatic cytokines.

To evaluate the adipocytokines secreted by the different adipose stem culture cells and their adipocytes derived we plated ADSCs, ASphCs and their derived cultured cells. Then the cell culturing media thus obtained was analyzed by a Elisa luminex analysis for the presence of specific growth factors or cytockines. The luminex system combine the flow cytometry with the xMAP® microspheres fluorescently dyed Technologies. The system enables simultaneously identify and measure up to 100 analytes in a single microplate well. The analysis showed that ASphCs and ASphC-Derived adipocytes compared to adipose tissue undigested (SVF) (routinely used in lipofilling techniques) possess a similar profile of secreted cytokines, even if at lower concentration. In particular they express a high concentration of all those proinflammatory adipokines, as IL8, IL6, IL12, IFNγ, TNFα, MCP1and RANTES, and moreover they secreted an high levels of proliferative cytokines such as LIF and GCSF. The analysis then showed how there is even a considerable production of those cytokines which can promote angiogenesis and metastasis such as VEGF, SCF ,PDGF and HGF,MIF and SDF. Contrary in the medium secreted from adipose cell cultures the anti-inflammatory cytokines are barely expressed, such as the IL10, IL13, IL1ra and IL4. (Fig 2)

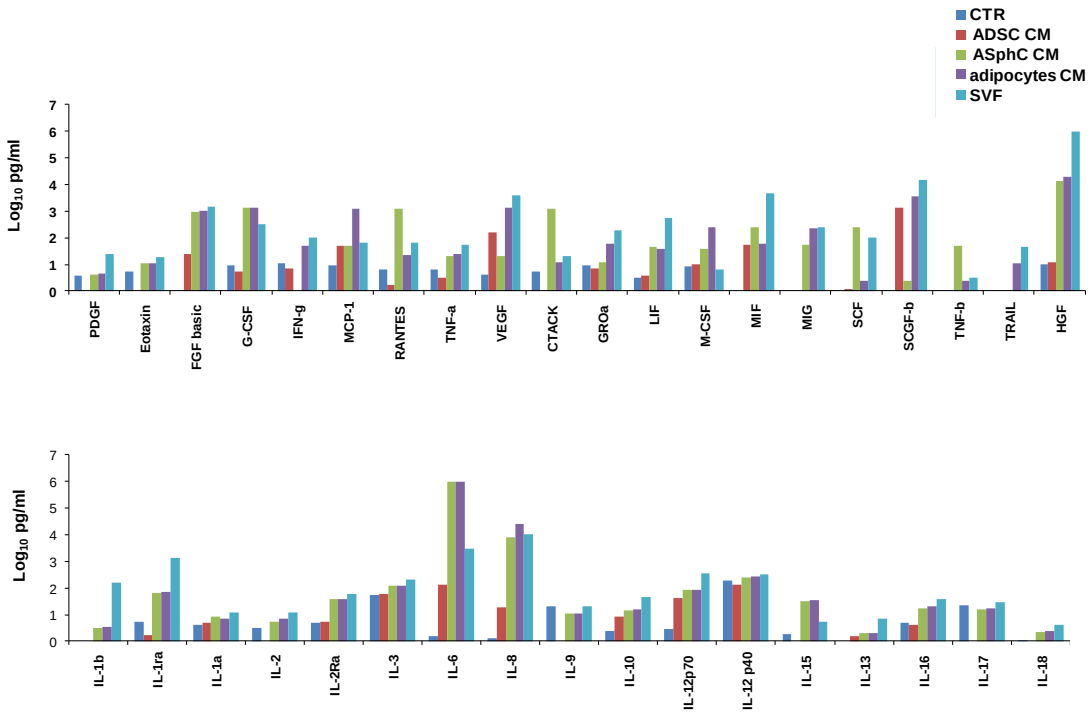


Figure 2. ASphCs and mature adipocytes compared to undigested adipose tissue (SVF) possess a comparable inflammatory,proliferative and angiogenesis Adipocytokines pattern.

Paracrine factors secreted by adipose-mesenchymal stem cells cultures, mature adipocytes and stromal vascular fraction (SVF). Data are mean ± SD of four independent experiments using cells from different patients. Whiskers: Vmin, Vmax (log10)

Adipose mesenchymal stem cell and mature adipocytes accelerate breast cancer growth by adipocytokines production.

To test the breast cancer cell viability and proliferation rate we have tested the viability assay using CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) following the manufacturer's instructions. This proliferation assay is a colorimetric method to determine the number of viable cells in proliferation. The experiments performed have shown that the luminal BCSC treated with the different adipose conditioned media (the ASphCs and mature adipocytes derived cultured cells) acquired a higher cell viability and increase the number of viable cells for the increased activity of mitochondrial dehydrogenases which leads to the increase of the amount of dye formazan formed. The assay further highlighted that the proliferative conditioned medium effect is appreciable just in the 24/48 hours, whereas it is not significant in the 72h after exposure. (Fig 3 A-B). To further confirm our results, we have performed a PKH26 dye (Sigma-Aldrich) proliferation assay and a soft agar colony forming assay. The luminal and basal BCSCs were plated in presence of ASphCs and adipocytes derived cultured cells media for 72h. Both assays have highlighted how the adipose conditioned media induce greater proliferation rate just in luminal BCSCs, instead basal ones do not undergo the effect of adipose conditioning. (Fig 3 C-D)

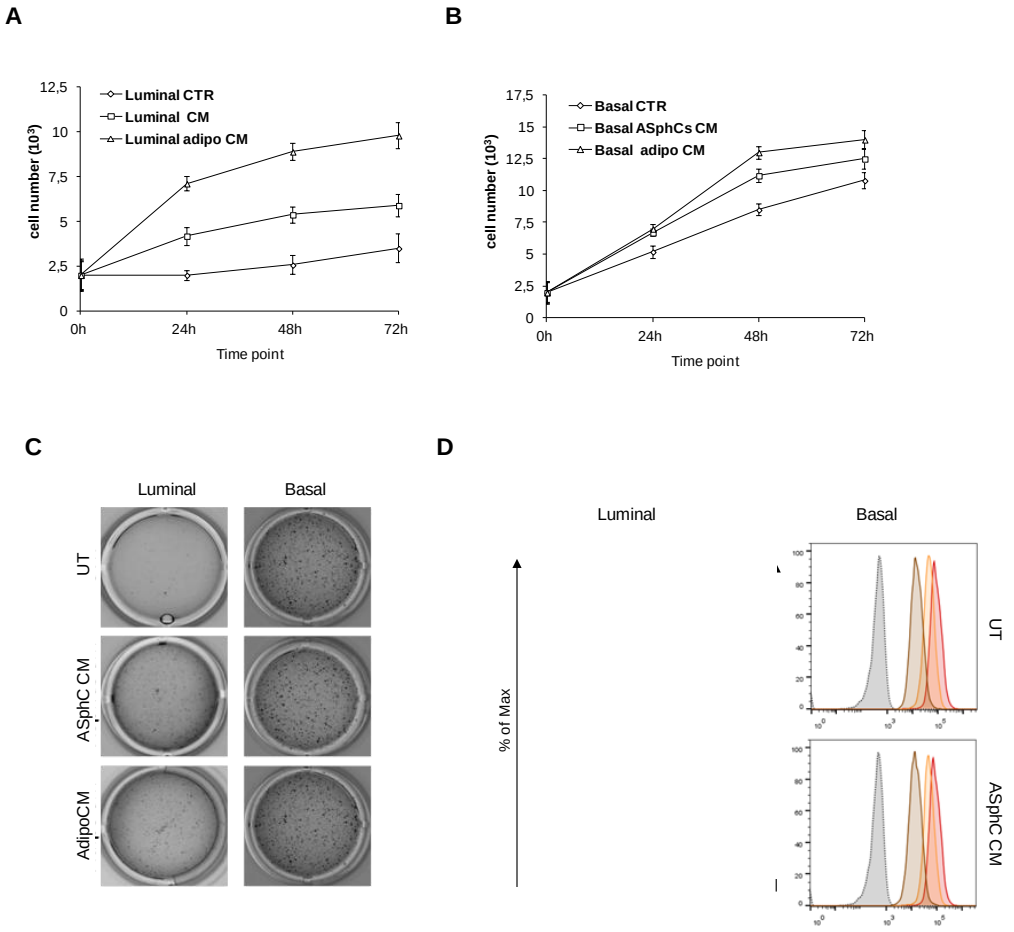
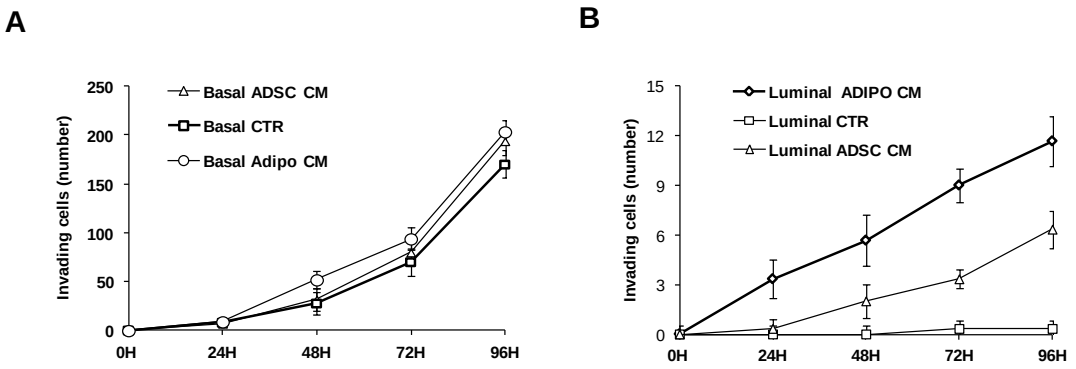


Figure 3. Luminal BCSCs treated with Adipose conditioned medium increase their proliferation rate and enhance their colonies form ability

(A-B) MTS proliferation assay performed in luminal and Basal BCSCs exposed to adipose conditioned media. **(C)** Soft agar colony forming assay of BCSCs tretated as in **(A). (D)** Representative PKH26 flow cytometry analysis of BCSCs labelled with PKH-26 at day 0 and after 24h and 48h.

Paracrine factors from adipose mesenchiymal stem cells enhance metastatic capacity in breast cancer stem cells

To evaluate the acquisition of a metastic capacity by BCSCs treated with the above cited adipose conditioned media, we have performed an invasion assay by using polycarbonate membrane filters with a diameter of 6.5 mm and pores with the size of 8µ. In the upper chamber were plated our BCSCs pretreated with conditioned media, and DMEM medium comprising 10% human AB serum added as chemioattrattante in the lower chamber. We have observed that luminal BCSCs samples acquired a invasive phenotype. (Fig 4 A-B). Further, we investigate the BCSCs migratory ability through a scratch assay. We observed the BCSCs migration to 48h. Both luminal and basal BCSC showed increased migratory capability when exposed to conditioned medium from both ASphCs and adipocytes (Figure 4 C-D)



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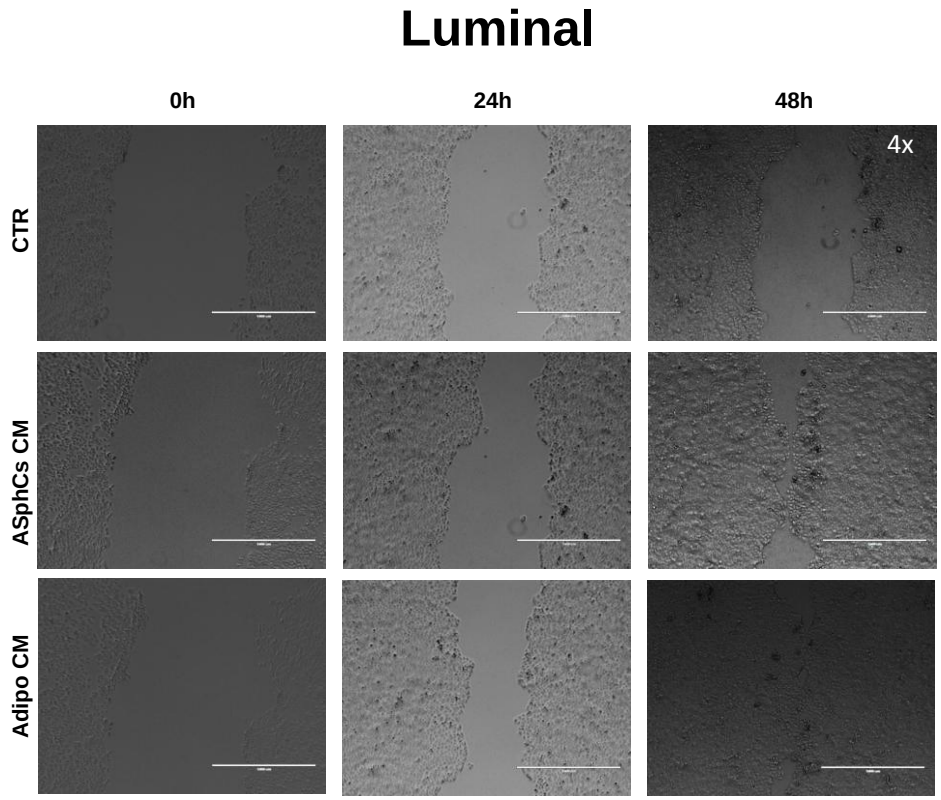
Luminal
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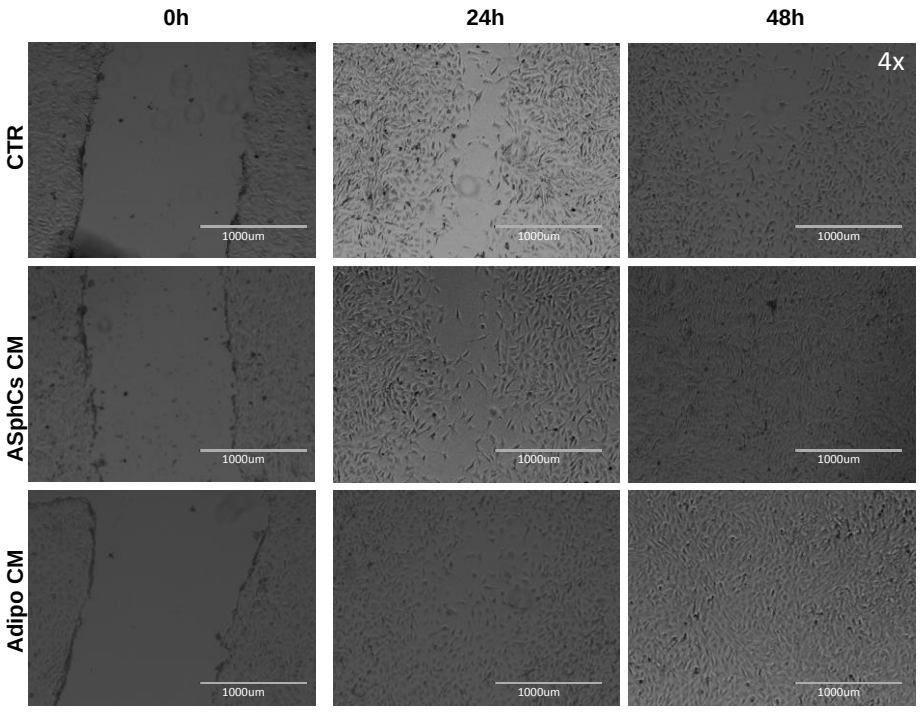
Basal
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Figure 4. Adipose conditioned luminal BCSCs acquire a metastatic potential and improve migratory ability.

(A)(B) Invasion assay of BCSCs treated with adipose conditioned media(C-D) BCSCs migration analysis in the luminal cell subset (C) and in basal cell subset (D).

Adipose conditioned medium promote an epithelial-mesenchymal transition in luminal breast cancer stem cells

In order to assess a potential gene expression profile changes in our breast cancer samples due to the presence in the culture of the adipocyte conditioned medium we have performed the human cancer pathway with RT^2 profiler PCR array. The gene expression analysis highlighted that both luminal and basal BCSCs when exposed to conditioned medium of ASphCs and adipocytes changed the expression of several important genes involved in the acquisition of a metastatic phenotype. In particulary the luminal BCSCs showed an overexpression of MIKI67 and CDC20 (cell cycle), CCL2, FGF2, KDR and ANGPT1 (angiogenesis) MAP2K and SOD1 (senescence), whike in basal ones showed an overexpression of FGF2(angiogenesis)IGFBP3 ang SERPINB2 (senescence), SNAI2 (EMT), DDT1 (DNA damage & repair) HMOX(hypoxia signaling). Different results were obtained when BCSCs were treated with adipocytes conditioned medium. A lot of genes involved in metastatic processes, proliferative and DNA damage repair angiogenic were overexpressed. In particular luminal BCSC showed an overexpression of CK14, SNAI2, SOX10, GSC and FOXC2 (EMT); KDR,VEGFC, ANGPT1 and FGF2 (angiogenesis); MIKI67, CDC20 and STMN1 (cell cycle), ERCC5 (DNA damage & repair), SERPINB2 and SOD1 (senescence).(Fig 5 A-D). The phenotypic swtich of luminal BCSCs toward a basal behaviour is confirmed by immunofluorescence analysis. (Fig 5 E)

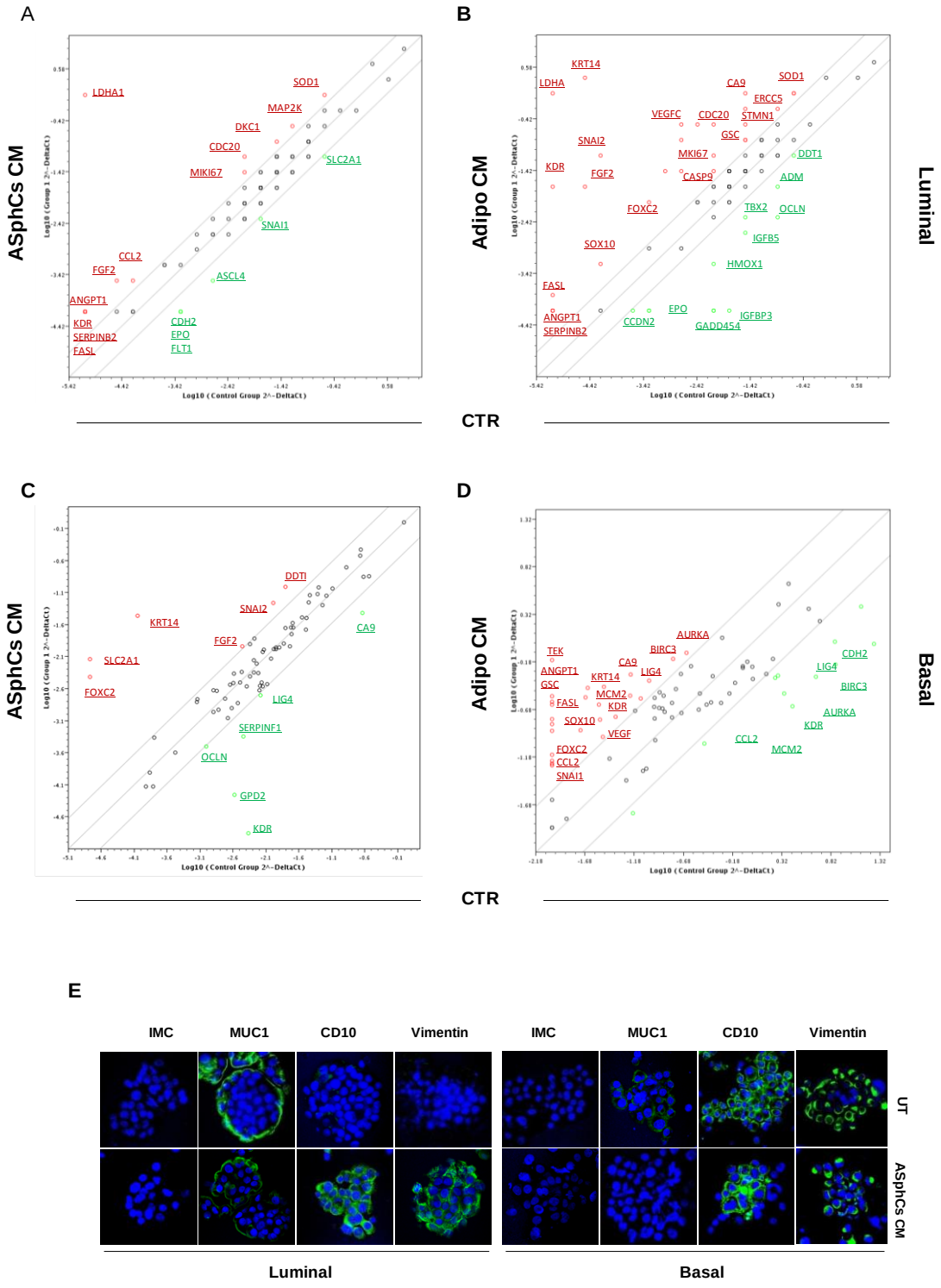

Figure 5. Exposure to the adipose conditioned medium adipose induces a switch genotype and phenotype from luminal to basal in BCSC

(A)(B) Scatter plot of human cancer pathway in luminal BCSCs treated with ASphCs medium in (A) and with mature adipocyte conditioned medium in (B) **(C)(D)** Scatter plot of human cancer pathway in basal BCSCs treated with ASphCs medium in (A) and with mature adipocyte conditioned medium in (B). **(E)** Representative confocal microscopy images of immunofluorescence (IF) analysis of Cytokeratins CD10, MUC1 and Vimentin performed in luminal and Basal BCSCs.

Conclusion and future experiments

Several scientific papers said that the use of autologous filler in lipotransfer techniques not represent for the mastectomized patients a real risk for cancer recurrence, and therefore this surgical techniques have been considered safe or very low risk, but about 1987 the American society of plastic and reconstrutive surgeons began to raise doubts about the safecty of these. In fact, recent other studies have investigated a possible long-term risk in the use of autologous fillers, and highlighted a potential risk for those subjects in which it was removed breast cancer. Hence, the elusive role of autologous filler in breast tumor development causes the safety concerns their clinical utilization. Therefore, starting from this uncertainty about the precise role of adipose tissue on tumor growth, this study, according to the many data in the literature concerning the close relation between the microenvironment and the relative tumor behavior, assumes that the autologous adipose tissue lipotransfer may influence a possible cancer relapse. Thus this work aims to shed light on all the possible mechanisms behind this harmful interaction. Furthermore, referring to some data in the literature which identify the mature adipocytes and not the pre adipocytes as the real responsible for the tumor return or its higher progression, we want to investigate the role of a adipose stem cells population, obtained by lipoaspirate samples digestion , identified as the real adipose stem cells, able to grow in non-adherence conditions in the form of spheroids, in a quiescent cell cycle state and that possess a great multidifferentation ability. This specific adipose stem cells population, appropriately characterized and analyzed by comparing with the adipose stem cells already described in the literature, were used for the co-culture studies together with breast cancere stem cells, these last considered the real makers of cancer chemotherapy resistance and minimal residual disease. Co cultures experiments have been preceded by a deep analysis of all the cytokines, adipokines and paracrine factors secreted into cell adipose culture medium. The conditioned medium analysis showed that adipose stem cultures secrete a cytokines and paracrine factors pattern decisively pro inflammatory, angiogenic, proliferative and migratory, although with a adipokines concentration less concentrated than the ones secretd by mature adipocyte cell populations and than the undigested adipose tissue which is normally used as autologous fillers surgery. Thus breast cancer stem cells were cultured in the presence of the adipose stem cells conditioned medium and also in presence of mature adipocytes conditioned medium for a specific time. BCSCs treated with adipose conditioned media have highlighted the acquisition of a more aggressive phenotype. Luminal BCSCs are especially responsive to treatment with different adipose conditioned media, infact they acquire a much higher proliferative rate and showed a invasive capacity not proper of luminal phenotype, moreover at the genotype and phenotype level they seem undergo a partial switch from luminal to basal phenotype. Therefore exposure of BCSC to adipose stem cells and adipose cells mature secretome seems to be so critical and highly detrimental, as documented in the recent scientific papers. Moreover, even if the adipose stem cells conditioned medium contribution seem to be less than conditioned medium released by mature adipocytes,

in our opinion it does not seem safe the use adipose autologous fillers enriched of adipose stem cell populations or with pre adipocytes. According to our study, in fact, the microenvironment that is generated into the operation site after lipotransfer, is a dangerous proinflammatory, proliferative and angiogenic microenvironment, which could stimulates the awakening of the few cancer stem cells remained after cancer mass surgical removing . So at the moment the best possible advice to overcome the mutilation trauma, due to demolitive surgery, is the use of others fillers such as the alloplastic fillers, some scaffolds or any more inert implants than autologous fillers.

As future prospects it could be the evaluation through the same coculture system the same data obtained from in vitro experiments also through in vivo model experiments. The BCSC tumorigenicity will be determined via subcutaneous injection BCSCs, conditioned or not treated with adipose medium, into the fat pad of 5 week-old NOD/SCID mice. After that we would investigate the possibility to ingegnerize the autologous filler with some ectopic factor, that could regulate the secretion or the prolifrative action possessed by the adipose stem cells.

Materials and Methods

Tissue collection and cell culture

Fresh tissue acquired from mastectomies of 8 patients (age 40–89) were collected at the University of Palermo and Fondazione IRCCS INT of Milan, in accordance with ethical standards. Breast tumor cells were purified from fresh tissue via enzymatic digestion as previously described [48]. Thereafter, single cell suspensions were plated in ultra-low attachment flasks (Corning) at a density of 1×10^5/ml and grown in a medium supplemented with bFGF (10 ng/ml, Sigma) and EGF (20 ng/ml, Sigma). To induce differentiation, cells were cultured in adherent conditions in Ham's/F-12 medium (Euroclone), supplemented with 5% fetal bovine serum (FBS), insulin (25µg/ml, Sigma) and hydrocortisone (1mg/ml, Sigma). MCF7 cells were purchased from ATCC and cultured in DMEM supplemented with 10% fetal bovine serum (Sigma). Adipose tissue was extracted from a lipoaspirate and subcutaneous breast tissue biopsy of 50 patients (16 males; 34 females) in compliance with our Department's policy and following patient's written consent on adipose tissue harvest and its use for research purposes. Patients ranged from 20 to 65 years of age and all selected donors were healthy and without a prior history of malignancy. Tissues were obtained either from subcutaneous adipose tissue or during liposuction of the abdominal or inner thigh regions using Coleman's cannulas, following infiltration with Klein's solution (NaCl 0.9%; lidocaine 2%; epinephrine 1:1000; $NaHCO_3$ 8.45%). Approximately, 20 cc of lipoaspirate and 0.05±0.02 gr tissue biopsies were collected from each patient and directly transferred to tissue culture tubes for enzymatic digestion and stem cell purification. Breast-derived Adipose tissue and lipoaspirate samples were digested with collagenase (1.5 mg/ml, GIBCO) and hyaluronidase (20 mg/ml, Sigma) through gentle agitation for 30 minutes at 37°C. The digested sample was centrifuged at 1200 rpm for 5 min and the recovered cells (Freshly) were plated with serum-free stem cell-specific media as previously described [59]. Cells were plated in stem cell medium in presence of bFGF (10 ng/ml, Sigma) and EGF (20 ng/ml, Sigma), in ultra-low adhesion tissue culture flasks (Corning) and placed at 37°C in a 5% CO_2 humidified incubator. In these conditions, cells grew as floating spheroids (ASphCs). Part of the isolated cells were plated in adherent conditions (DMEM+FBS 10%) and were referred as primary culture (Primary). Conversely, we termed ADSCs the commercially available STEMPRO® Human Adipose-Derived Stem Cells, plated as recommended by the manufacturer (Invitrogen). To evaluate ASphC multilineage differentiation capacity, $5x10^3$ single cells were diluted 1:4 in a cold Matrigel solution (growth factor reduced BD). This solution (50 µl/well) was dispensed into pre-warmed 24-wells plates and let polymerize for 30 minutes at 37°C. Finally, the wells were filled using 700 µl/well of basal culture medium.

Osteogenic, Adipogenenic and Chondrogenidifferentiation

Briefly, ASphCs and ADSCs were trypsinized 1 min at 37°C and plated into 24-well cell culture plates (50.000 cells/well). Cells were allowed to adhere and cultured in the STEMPRO® Osteogenesis Differentiation Kit, STEMPRO® Chondrogenesis Differentiation Kit (Invitrogen (Invitrogen) and STEMPRO® Adipogenesis Differentiation Kit (Invitrogen) up to 28 days. Cell viability, adhesion and differentiation was assessed by daily observation using an optical microscopy. Osteogenic differentiation was assessed by performing a staining for alkaline phosphatase activity (ALP) (BCIP/NBT alkaline phosphatase substrate kit, Vector Laboratories), osteopontin (OPN) (polyclonal rabbit, Sigma-Aldrich) or von Kossa for calcium deposition (Polysciences Inc.). Chondrogenic differentiation was assessed by alcian blue. Briefly, the sample was fixed in 2% PFA for 30 min at 37°C, washed in PBS and incubated with alcian blue for 30 minutes. Cells were then counterstained with nuclear fast red for 5 minutes. Adipogenic phenotype change assessed by adipored assay (Lonza) for 10 min at room temperature. Nuclei were counterstained with Toto3 and the staining was observed using a confocal microscope.

Flow cytometry

Adipose stem cells were stained with conjugated antibodies against CD44-FITC (G44-26, mouse IGg2bk, BD), CD271-ALEXA FLUOR 647 (C40-145, mouse IgG1k, BD), CD90-PE (5E10, mouse IgG1k, BD), CD45-FITC (5B1, mouse IgG2a, Miltenyi), CD19-ALEXA FLUOR 488 (HIB19, mouse IgG1k) or with purified primary, CD29 (MAR4, IgG1k, BD), CD73 (AD2, mouse IgG1k, BD), CD9 (ML13, mouse IgG1k, BD). Then, cells were labeled with goat anti-mouse IgG FITC secondary antibody (Invitrogen). BCSCs were exposed to primary antibodies CD44 (BU75, Ancell), CD24 (ML5, R&D System), CD10 (FR4D11, Santa Cruz Biotechnology), CD49f (GoH3 Miltenyi Biotec), EpCAM (AF960, R&D System) or corresponding isotype controls, rinsed and labeled with secondary antibodies. BCSCs were stained with CD49f and CD24 and successively sorted via flow cytometry using an FACSAria cell sorter (BD Biosciences). The analysis of ALDH1 activity was performed using the ALDEFLUOR kit (StemCell Technologies). For cell cycle analysis, BCSCs were fixed in 70% ethanol and incubated with 50 μg/mL propidium iodide (Sigma-Aldrich), 3.8 mmol/L sodium citrate (Sigma) and 10 μg/mL RNase (Sigma). Specific corresponding isotype matched antibodies were used as negative controls. Samples were acquired using a FACS ARIA (BD Biosciences) flow cytometer. All data were analyzed using FlowJo software (Tree Star).

Gene expression

Total RNA was extracted by using a RNeasy Mini Kit (Quiagen) and 1 μg of each sample was retro-transcribed into cDNA using a high-Capacity cDNA Reverse Transcription Kit as recommended by manufacturer (Applied Biosystems). Expression of mesenchymal stem cell genes and expression of human cancer pathway

was performed through RT^2 profiler PCR array (PAHS-082ZR and PAHS-033Z Quiagen), according to manufacturer's instructions. Arrays were performed for ASphCs, ADSCs and their differentiate cells, and for luminal and basal BCSCs. At least 2 replicates were run for each sample.).

Cell cycle and proliferation assay

Cell cycle analysis was performed on dissociated cells by staining with 50 µg/ml propidium iodide (Sigma-Aldrich) dissolved in buffer 0.1% sodium citrate (Sigma-Aldrich), 0.1% Triton X-100, 10 µg/ml RNAse (Sigma-Aldrich) for 1h on ice. Samples were acquired through a FACS Calibur flow cytometer (BD Biosciences). The proliferation assay was evaluated by using PKH26 dye (Sigma-Aldrich). $20x10^4$ of dissociated cells were stained for 1 h at 37°C with PKH26 according to manufacturer's instructions, then washed extensively with PBS and cultured for 14 additional days. PKH26 red fluorescence was analyzed by FACS Aria flow cytometer. All data were analyzed using FlowJo software. Evaluation of ASphC, ADSC, luminal and basal BCSCs proliferation was assessed using CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) according to the manufacturer's instruction.

Immunofluorescence

BCSC were cytospun at low speed on polylysine-coated glass slides BCSCs were fixed with 2% paraformaldehyde for 30 minutes at 37°C and permeabilized in 0.1% Triton X-100 (Bio-Rad Laboratories, Richmond, CA, http://www.bio-rad.com) and incubated O.N. at 4°C with the following primary antibodies: ALDH1 (44, BD), CK5 (XM26, Novocastra), CK14 (LL002, Novocastra), CK8-18 (CD10, Novocastra), MUC1 (BD Pharmigen), VIMENTIN (R28, Cell Signaling), ER (6F11, Novocastra). After two washes in PBS, cells were incubated with Alexa Fluor-conjugated secondary antibodies(Invitrogen) for 30 minutes at room temperature in the dark. Nuclei were counterstained with Toto-3 (Invitrogen). Slides were analyzed on a FV1000 confocal microscope (Olympus, Tokyo, Japan, http://www.olympus-global.com) equipped with x60 and x40 oil immersion objectives.

Luminex

Cytokines Quantification Quantification production was assessed by using multiplex Bio-Plex Pro Assays (Bio-Rad; Human Cancer Biomarker Panel 1-4 No. 171-AC500M; Human Cytokine SDF-1a Set No. 171-B6019M; TGF-b 3-plex Assay No. 171-W4001M). Raw data (mean fluorescent intensity) from all kits were analyzed by Bio-Plex Software (Bio-Rad).

Migration and Invasion assay

Transduced and control BCSCs were seeded into six-well plates at a density of $1x10^6$ cells/well and cultured to achieve a confluent cell layer in culture medium. The day

after, the growth medium was switched to serum-free medium for 16 h, after which a sterile 200 µl pipette tip was used to straight scratch a constant-diameter stripe in the confluent cell monolayer. The wells were then washed with 1 ml of PBS to remove detached cells and debris and the medium was replaced using a fresh culture medium. Wound healing was visualized by following the migrating cells in the gap during a 24-h post-scratch period using the EVOS™ fl Digital Inverted Fluorescence Microscope with a 10X objective.

Cells tested for invasive potential ($2x10^3$) were plated into Matrigel-coated (BD) transwells of 8 µm pore size (Corning), into a 24 well plate. DMEM supplemented with 10% human serum (600 µl/well) was used as chemo-attractant in the lower chamber of the transwell. Migration was observed and counted microscopically for 72 hrs.

Statistical Analysis

Data are explicited as mean ± standard deviation (SD). Statistical significance was calculated by applying Student's t-test. Significance levels were indicated as p values.

* indicates $P<0.05$, ** indicate $P<0.01$ and *** indicate $P<0.001$

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Chapter| 5

A preliminary study

The miRNAs contribution in the adipocyte maturation

Introduction

MiRNA

miRNAs discovery, which occurred only in 1993 by the group of Dr. Victor Ambros, is one of the most revolutionary scientific discoveries for the cell biology and medicine. MiRNAs reppresent a class of small endogenous single stranded non-coding RNA molecules on the size of about 18-25 nucleotides deputies to a negative gene expression regulation after transcription by binding to specific mRNAs 3'UTR and mediating its degradation or translational repression [1] (Figure 1)

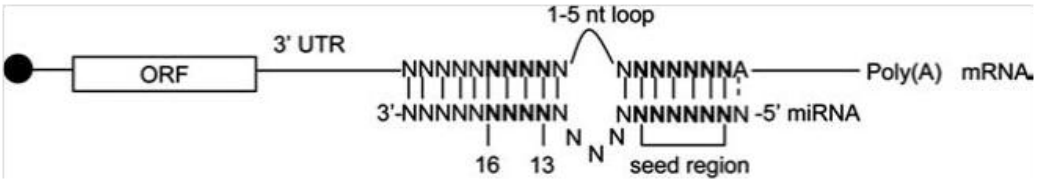

Figure.1: Graphical representation of the hybridization site of a mRNA target

Experimental evidence indicate that miRNAs play a key role in the control of various biological activities such as embryonic development, cell proliferation, metabolic homeostasis and apoptosis [2]. Computational analyzes indicate that genes coding for mRNA represent approximately 1% of the genome of different species. Along with Small interfering RNA (siRNA), Piwi RNA (piRNA), Trans-acting siRNA (tasiRNA), Small-scan RNAs (scnRNA) and repeat-associated siRNAs (rasiRNA) are part of the class of small RNA. [3] Since the RNA interference discovery have been made many efforts in trying to characterize these endogenous molecules and which have led to the identification of hundreds of miRNAs in different model organisms [4]. To date it has been identified about 1,000 different genes coding for miRNAs. [3] The miRNAs genes are distributed on all human chromosomes except the Y chromosome and in 50% of cases are organized in clusters and involving a polycistronic primary transcription [5]. Initially it was believed that most of these genes were located in an intergenic region [6-7], but recent studies have shown that genes coding for miRNAs are present in transcriptionally active regions [8], and in

particular most of these are found in intron sense, introns coding for proteins and in low percentage in exonic regions. For these reasons miRNAs are classified in intronic miRNAs protein-coding, non-coding intronic miRNAs and esonic non-coding miRNAs [3]

miRNA Biogenesis and Processing

The mature miRNA precursors come from the largest genome transcribed by RNA polymerase II as a primary miRNA variable size of approximately 100 nucleotides and as others have transcribed the hood and is polyadenilato [9-10]. This is followed by the maturation of the pri-miRNA processing through two passages in which they are involved two ribonuclease Class III: Drosha and Dicer. Ribonuclease Drosha together with its partner protein Dgcr8 processes the pri-me-nuclear RNA in a precursor molecule pre-mi-RNA of about 70 nucleotides [11-15]. This is exported from the nucleus to the cytoplasm by the action of Esportina5 that recognizes specifically the structure of pre-miRNA [16-18]. Once in the cytoplasm, the pre-miRNA is processed by the second enzyme Dicer ribonucleotidico together with the protein TRBP (Trans activator RNA BindingProtein) in the form duplex of about 21 nucleotides, where one strand is linked selectively to the enzyme complex RISC silencing. Recently it has been proposed in Caenorhabtis elegans and Drosophila melanogaster an alternative pathway independent of Drosha where a small hairpin intronic undergoes splicing in the form of pre-miRNA defined mintroni able to enter the miRNA biogenesis without the mediation of action processativa Drosha. RISC recognizes the miRNA target thanks to miRNA that has " loaded ", thanks to a partial complementarity between miRNA and mRNA target. The binding sites for miRNAs thus far described are located at the level of the 3'UTR (although recent studies have demonstrated the presence of binding sites for miRNAs also in the coding regions). The interaction requires the formation of a double-stranded structure between bases from second to eighth in the miRNA region called " seed ", and the target mRNA. To date it has been proposed several mechanisms of action for miRNAs, among the mechanisms there is the most accepted model cleavege mRNA and that of the translational repression [19-20] (Figureure 3). Other proposed mechanisms of repression include: inhibition of ribosome binding to the mRNA target, the kidnapping and deadenilazione messenger cytoplasmic bodies defined in P-bodies (Eulalio et al. 2008).

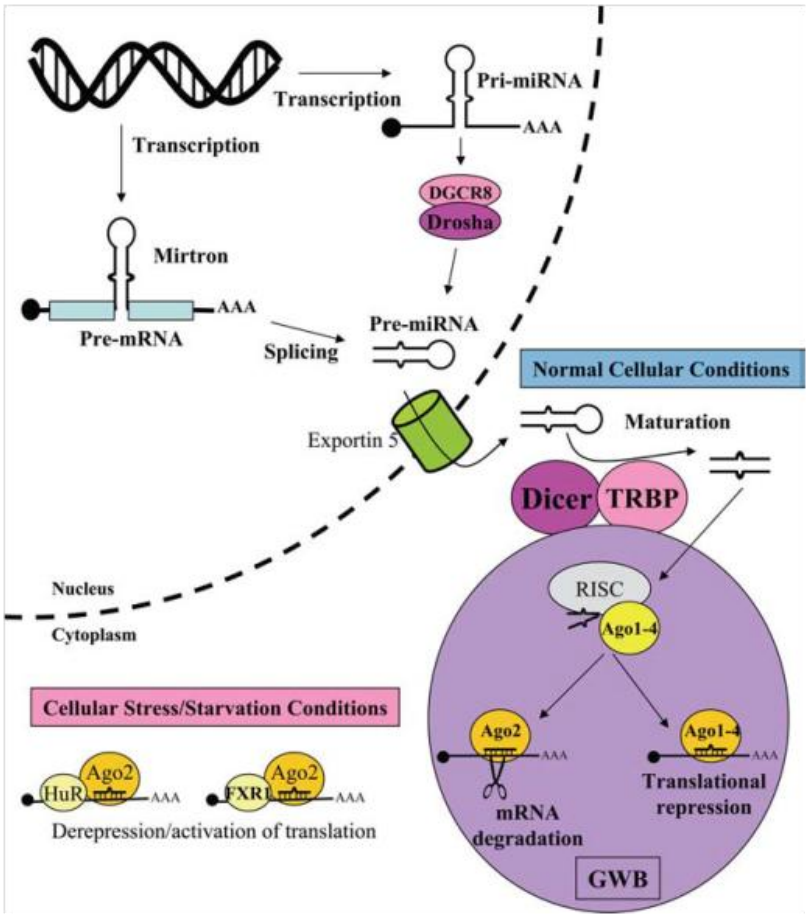

Figure.3: Biogenesis and function of miRNAs (Kaleb M.,Ann.N.Y. Accad.Sci,2008).

Mirnas and cancer

In recent years, the list of oncogenes or tumor suppressor genes considered was revised, expanding the latter to the family of miRNAs [21-22]. Depending on the capabilities we distinguish the oncomiR, which are located in regions amplified and over expressed in the tumor, and the tumor suppressor miRNAs, down regulated in tumors [23-24]. A study published in PNAS in 2004 showed that about 50% of human miRNAs are located in genomic regions frequently rearranged tumors: fragile sites, delete regions (minimal region of loss of heterozigosity, LOH) or amplified (minimal amplicons) and regions common break-point, providing further evidence of their role in the pathogenesis of cancer [25-26]. (Figureura 4)

Examples oncomiR are miR-155, amplified in many hematological malignancies such as Burkitt's lymphoma, lung cancer and breast cancer. The miR-17-92 cluster that includes 6 genes for miRNAs overexpressed in solid tumors and lymphoma diffuse large B-cell Among the tumor suppressor miRNAs were characterized miR-15a and miR-16-1, deleti in chronic lymphocytic leukemia , that target the

antiapoptotic protein BCL2, and the let-7 family, that represses the expression of the oncogene RAS. Other experimental evidence have also shown that the expression of protein processing in the biogenesis of miRNAs such as Dicer, RISC, Argonaut are respectively iporegolati in cases of lung cancer, Wilms tumors (kidney) and neuroendocrine tumors. The list of miRNAs involved in the tumorigenic processes is increasing in frequency. On the basis of the characteristics described above, the miRNA can therefore be considered good target for anti-tumor therapy. In fact the introduction of the miRNA or dell'antagomiR in tumors in which this has been lost or is overexpressed can lead to the arrest of the cell cycle and / or apoptosis.

Chromosome	Location (defining markers)	Size, Mb	miR	Hystotype	Known OG/TS
3p21.1–21.2-D	ARP-DRR1	7	*let-7g/miR-135–1*	Lung, breast cancer	—
3p21.3(AP20)-D	GOLGA4-VILL	0.75	*miR-26a*	Epithelial cancer	—
3p23–21.31(MDR2)-D	D3S1768-D3S1767	12.32	*miR-26a; miR-138–1*	Nasopharyngeal cancer	—
5q32-D	ADRB2-ATX1	2.92	*miR-145/miR-143*	Myelodysplastic syndrome	—
9q22.3-D	D9S280-D9S1809	1.46	*miR-24–1/mir-27b/miR-23b; let-7a-1/let-7f-1/let-7d*	Urothelial cancer	PTC, FANCC
9q33-D	D9S1826-D9S158	0.4	*miR-123*	NSCLC	—
11q23-q24-D	D11S927-D11S1347	1.994	*miR-34a-1/miR-34a-2*	Breast, lung cancer	PPP2R1B
11q23-q24-D	D11S1345-D11S1328	1.725	*miR-125b-1/let-7a-2/miR-100*	Breast, lung, ovary, cervix cancer	—
13q14.3-D	D13S272-D13S25	0.54	*miR-15a/miR-16a*	B-CLL	—
13q32–33-A	stSG15303-stSG31624	7.15	*miR-17/miR-18/miR-19a/miR-20/ miR-19b-1/miR-92–1*	Follicular lymphoma	—
17p13.3-D	D17S1866-D17S1574	1.899	*miR-22; miR-132; miR-212*	HCC	—
17p13.3-D	ENO3-TP53	2.275	*miR-195*	Lung cancer	TP53
17q22-t(8;17)	miR-142s/c-MYC		*miR-142s; miR-142as*	Prolymphocytic leukemia	c-MYC
17q23-A	CLTC-PPM1D	0.97	*miR-21*	Neuroblastoma	—
20q13-A	FLJ33887-ZNF217	0.55	*miR-297–3*	Colon cancer	—
21q11.1-D	D21S1911-ANA	2.84	*miR-99a/let-7c/miR-125b*	Lung cancer	—

Figure.4: List of some oncomiRNA and tumor suppressor miRNAs and related cancers in which involved (G. Calin et al. PNAS, 2004).

microRNAs in the Regulation of Adipogenesis

Several studies have reported a screening miRNA changes during adipogenic differentiation of human multipotent mesenchymal stem cells, and many of these studies demonstrated miRNAs are very important players in human MSCs adipogenic lineage commitment. [28-33]. In recent years, there has been a rapidly growing interest in the role of miRNAs in fat cell development and obesity [34-35]. Studies have showed miRNA expression in pre-adipocytes is altered during adipose tissue development and in obesity, therefore a better understanding of the pathways controlling adipogenesis is needed. Fact understanding the role miRNAs play in the proliferation and differentiation of adipocytes during fat cell development could provide new therapeutic targets. There are miRNAs can accelerate adipocyte differentiation, such as miR-143, that was the first miRNA associated with regulation of adipocyte differentiation, its expression increases in differentiating adipocytes, and antisense oligonucleotides against miR-143 inhibit human-cultured adipocyte differentiation downregulate PPARγ and GLUT-4. Several other miRNAs have been identified which can accelerate adipocyte differentiation (including miR-103, miR-107 and miR-143) are induced during adipogenesis, which may play a role in accelerating adipocyte differentiation, and then be downregulated in the obese state. One study demonstrated ERK5 (Extracellularsignal- regulated kinase 5) is targeted by

miR-143 in human pre-adipocytes [29]. The role of ERK5 in adipocyte differentiation is not clear, although it has been suggested ERK5 suppression may be involved in fine-tuning the MAPK (mitogen activated protein kinase) pathway to maintain the differentiated state. miR-103 is reported to be upregulated during differentiation of human pre-adipocytes, and its overexpression in the presence of adipogenic stimuli, adipogenesis accelerates, as shown by increased triglyceride accumulation and adipogenic gene expression. [36-41]

Another adipogenic miRNA to emerge recently is miR-210, which is a key transcription factor modulating components of WNT signaling. Overexpression of miR-210 is reported to stimulate adipocyte hypertrophy and lipid droplet formation. The upregulation of miR-210 during adipogenesis is in concordance with a suppression of genes encoding proteins in the WNT signaling pathway [42]. Any others miRNAs, such as miR-221, miR-125b, miR-34a and miR-100, were upregulated in fat depots from obese subjects and downregulated during adipocyte differentiation. miR-34a was found to be positively upregulated during adipogenesis and associated positively with BMI (Ortega et al. 2010). Others miRNAs CAN suppress adipocyte differentiation.Some studies have focused on miRNAs which appear to act as negative regulators of adipocyte differentiation. miR-27a overexpression in pre-adipocytes suppresses PPARγ expression and adipocyte differentiation [43]. Another family member, miR-27b is also downregulated during adipocyte differentiation. MiR-27b can also bind to the 3'UTR of PPARγ and repress PPARγ protein levels [31]. Interestingly,in mature adipocytes from obese mice lower miR-27a expression has been found compared to lean mice, indicating miR-27a downregulation may be necessary for adipocyte hypertrophy [44]. These studies suggest the miR-27 family could be a useful anti-adipogenic target. Potentially miR-27a mimics could be used to regulate pre-adipocyte proliferation. Another study reported miR-448 is a potential inhibitor of adipogenesis. miR-448 is encoded within the intron of HTR2C, a serotonin receptor which is upregulated during adipocyte differentiation. Kruppel-like factor 5 (KLF5) contains a putative miR-448 binding site. Overexpression of miR-448 in pre-adipocytes suppresses KLF5, triglyceride accumulation and adipogenic gene expression thus suggesting miR-448 is a negative regulator of adipocyte differentiation [45]. Inhibition of miR-15a appears to reduce preadipocyte size while promoting adipocyte proliferation. In preadipocytes miR-15a has been shown to target DLK1 at mRNA and protein level. Inhibition of miR-15a in pre-adipocytes resulted in a decrease in cell size along with an increase in cell number [46]. Furthermore, miR-222 and miR-221 are decreased during adipogenesis but upregulated in obese adipocytes, contrary, miR-185 was upregulated in mature adipocytes while downregulated in obese men.

In summary, to date studies have identified several candidate miRNAs which can accelerate or inhibit preadipocyte differentiation . These miRNAs may provide promising candidates to design anti-obesity drugs to control fat cell development. However, it remains important to examine whether miRNAs which regulate adipocyte differentiation *in-vitro* are dysregulated in human obesity *in-vivo*.

Aims of work

Tthis study is would to identify through a depth screening of many of the best known miRNA described by the scientific literature, which of them are overexpressed in adipose stem cells and in mature adipocytes. In fact many of miRNA present in our analysis have been widely described as master regulators of many fundamental biological processes such as senescence, proliferation the severance and therefore may be involved in disease processes such as cancer and obesity. Moreover it is also made extensive use of autologous adipose tissue as filler in aesthetic surgery applications and regenerative medicine, in order to reshape anatomical defects or exploiting the great capacity multi differentiation proper of mesenchymal stem cells which results in improvement of tissue regeneration. Several scientific works have investigated about a possible long-term risk in the use of autologous fillers, and these papers highlighted a tumoral recurrence potential risk for those subjects in which it was removed a previous tumoral mass. Hence, the elusive role of adipose autologous filler in causes the safety concerns their clinical utilization also in other surgical applications. Therefore, according to the many data in the literature concerning the close relation between the adipose microenvironment and many pathologies, and furthermore, referring to some data in the literature which identify the mature adipocytes and not the pre adipocytes as the real responsible for the enhancing tumor progression and obesity this work aims to shed light on all the possible mechanisms behind this harmful interaction. In partiulary we want to investigate if there are some miRNAs overexpression in a one specific adipose stem cells population, obtained by lipoaspirate samples digestion , identified as the real adipose stem cells, that are able to grow in non-adherence conditions in the form of spheroids, in a quiescent cell cycle state and that possess a great multidifferentation ability, and also in the mature adipocytes derived from the above citated adipose stem population, which could be manipulated to achieve an improvement in all those clinical applications currently in use. To this end, we are looking for oncomiRNA, miRNA tumor suppressor, miRNA involved in osteogenic maturation, and of those miRNA regulating adipocyte maturation.

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Results

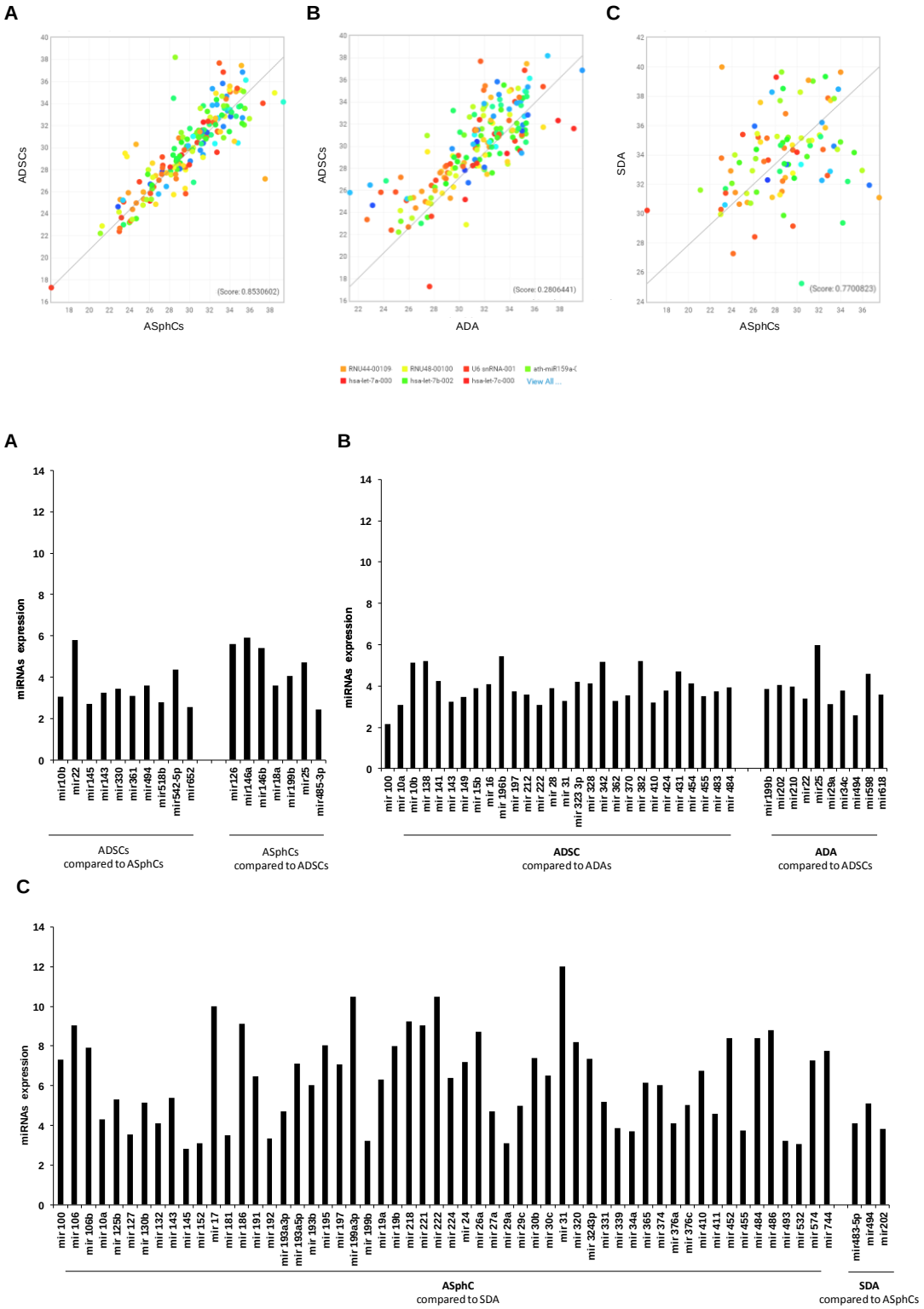

Figure 6 . Evaluation of miRNAs expression in the primary and adipose derived cultures.

(A) ASphCs human micro RNA expression comparerd to ADSC. **(B)** ADSC human micro RNA expression compared to ADSC-Derived Adipocytes (ADA). **(C)** ASphCs human micro RNA expression compared to ASphC-Derived Adipocytes (SDA).

Figure 5 Scatter plot of human MicroRNA ecpressed in inASphC, ADSC and marure adipocytes derived from ADSC (ADA) and ASphCs (SDA).

(A) Scatter plot of ASphCs micro RNA expression comparerd to ADSC. **(B)** Scatter plot of ADSC compared to ADSC-Derived Adipocytes (ADA). **(C)** Scatter plot of ASphCs compared to ASphC-Derived Adipocytes (SDA).

Materials and Methods

Adipose tissue samples and cell culture

Adipose tissue was extracted from a lipoaspirate and subcutaneous breast tissue biopsy in compliance with our Department's policy and following patient's written consent on adipose tissue harvest and its use for research purposes. Tissues were obtained either from subcutaneous adipose tissue or during liposuction of the abdominal or inner thigh regions. Breast-derived Adipose tissue and lipoaspirate samples were digested with collagenase (1.5 mg/ml, GIBCO) and hyaluronidase (20 mg/ml, Sigma) through gentle agitation for 30 minutes at 37°C. The digested sample was centrifuged at 1200 rpm for 5 min and the recovered cells (Freshly) were plated with serum-free stem cell-specific media as previously described [47]. Cells were plated in stem cell medium in presence of bFGF (10 ng/ml, Sigma) and EGF (20 ng/ml, Sigma), in ultra-low adhesion tissue culture flasks (Corning) and placed at 37°C in a 5% CO_2 humidified incubator. In these conditions, cells grew as floating spheroids (ASphCs). Conversely, we termed ADSCs the commercially available STEMPRO® Human Adipose-Derived Stem Cells, plated as recommended by the manufacturer (Invitrogen).

MIRNA Gene expression

Total RNA was extracted using a TRIzol® Reagent solution (Ambion) following manufacturer's instructions) and miRNA expression analysis was performed by Megaplex pools protocol specific for a set of 384 microRNAs (pool A) as recommended by manufacturer (Applied Biosystems). The relative quantification of microRNA expression was calculated using the equivalent Ct values where the original CT values are projected to 100% target efficiency, all the experiments were normalized using global normalization method.[27] microRNA asrays were performed for ASphCs, ADSCs and their differentiate cells. At least 2 replicates were run for each sample. miRNAs with 3 fold changes were considered for analysis.

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Chapter| 6

By promoting cell differentiation, miR-100 sensitizes basal-like breast cancer stem cells to hormonal therapy

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Keywords: Breast cancer, basal-like, differentiation, miR-100

Received: *September 30, 2014* ***Accepted***: *December 10, 2014* ***Published***: *December 11, 2014*

ABSTRACT

Basal-like breast cancer is an aggressive tumor subtype with a poor response to conventional therapies. Tumor formation and relapse are sustained by a cell subset of Breast Cancer Stem Cells (BrCSCs). Here we show that miR-100 inhibits maintenance and expansion of BrCSCs in basal-like cancer through Polo-like kinase1 (Plk1) down-regulation. Moreover, miR-100 favors BrCSC differentiation, converting a basal like phenotype into luminal. It induces the expression of a functional estrogen receptor (ER) and renders basal-like BrCSCs responsive to hormonal therapy. The key role played by miR-100 in breast cancer free-survival is confirmed by the analysis of a cohort of patients' tumors, which shows that low expression of miR-100 is a negative prognostic factor and is associated with gene signatures of high grade undifferentiated tumors. Our findings indicate a new possible therapeutic strategy, which could make aggressive breast cancers responsive to standard treatments.

INTRODUCTION

The onset and progression of malignant tumors depend on a small pool of tumor cells with biological properties similar to those of normal adult stem cells. In accordance to this cancer stem cell hypothesis, tumors are organized in a hierarchical manner and are characterized by cells that exhibit the ability to self-renew as well as to give rise to differentiated cells. The CSCs represent the apex of this hierarchy and appear to be the phenotypic and functional equivalents of normal stem cells harboring oncogenic mutations [1]. CSCs have been isolated in most human solid tumor types, suggesting their central role in tumor development, progression and recurrence [2]. The presence of a CSC pool is associated with aggressiveness and a negative prognosis in breast cancer patients. CSCs are thought to possess intrinsic resistance to current conventional therapies as compared to the bulk tumor cell population and it has been proposed that tumor recurrence is driven by this subpopulation of CSCs [3-5].

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression at a post-transcriptional level, thus monitoring several biological processes. Their deregulated expression contributes to cancer development and progression and can influence both the response to therapy [6] and the development of drug resistance [7, 8].

Recently, miRNAs have also emerged as critical players in the maintenance of pluripotency, control of self-renewal and cell fate [9]. Restricted miRNA patterns are expressed only in Embryonic Stem Cells (ESCs) [10, 11] and specific miRNAs regulate and are regulated by key stem cell genes [12, 13]. The importance of the miRNA pathway in the biology of stem cells has been confirmed in Dicer-1 knock-out mice, where the loss of Dicer-1 results in the depletion of the stem cell population in embryos [14]. Moreover, Dicer-1 deficient murine ESCs fail to differentiate [15]. The majority of miRNAs that are important in ESC biology are also involved in oncogenesis. This fuels the hypothesis that miRNAs could be determinant in cell stemness both in normal and in cancer stem cells [16, 17]. In line with this hypothesis, recent data provide evidence that miRNAs might connect stemness and metastasis. Indeed, some miRNAs specifically expressed in ESCs can be inopportunely expressed in cancer cells, promoting epithelial mesenchymal transition (EMT) [18, 19] and metastasis [20].

The miR-100 family of microRNAs is composed of three members, miR-100, miR-99a and miR-99b. Comparative studies indicate that miR-100 is the oldest known animal microRNA [21] and is widely expressed in vertebrates [22]. Recent data demonstrated that miR-100 is under-expressed in human ESCs compared to differentiated cells [23] and is required for proper differentiation of mouse ESCs [24]. The role of miR-100 in cancer is quite contradictory, since it can behave either as an oncogene or as a tumor suppressor gene, depending on the tumor type [25-27].

The present work shows that miR-100 plays a pivotal role in regulating the transition between stemness and differentiation of Breast Cancer Stem Cells (BrCSCs). The ectopic expression of miR-100 in CSCs isolated from breast cancer specimens impaired their self-renewal and tumor-initiating ability. Notably, miR-100 induced luminal differentiation in basal-like BrCSCs and rendered them sensitive to endocrine therapies, such as tamoxifen and fulvestrant.

RESULTS

MiR-100 down-regulation induces a mammosphere-like phenotype in breast cancer cells

Expression profiling studies showed that miR-100 is deregulated in various types of cancers [25-27]. Here, attention was focused on human breast cancer, where the biological role of miR-100 in tumor onset and progression remains elusive. The aim was to modulate miR-100 expression *in vitro* in breast cancer cells and study the biological consequences. The breast cancer cell line MCF7 was transiently transfected in the absence of serum, either with a miR-100 specific antagomir or a control antagomir. MiR-100 antagomir transfected cells acquired a mammosphere-like phenotype. These mammospheres retained the ability to differentiate when cultured in the presence of serum, acquiring an adherent shape (Fig. 1A). In order to ensure that antagomir-induced mammospheres showed stem cell characteristics, we analyzed the expression of the stem cell transcription factors Nanog, Oct4 and Sox2. As shown in Fig. 1B, miR-100 depleted cells expressed higher levels of the three transcription factors, compared to cells transfected with the control antagomir and to mammospheres obtained from MCF7 cells cultured in standard stem cell conditions. A wider gene expression analysis revealed that miR-100 knockdown led to a global gene reprogramming that could be responsible for the acquisition of the stem-like phenotype (Fig. 1C). Also employed was a complementary approach, evaluating miR-100 expression in mammospheres generated from breast cancer cell lines cultured in standard stem cell conditions. Consistently, the expression of the miRNA was lower in mammospheres than in the original adherent cells (Supplementary Fig. 1A, B).

Analysis of miR-100 expression in Breast Cancer Stem Cells

The level of miR-100 expression might be critical in maintaining stemness and in determining the transition from a stem to a differentiated status in cancer cells. When miR-100 expression was analyzed in a panel of CSCs isolated from basal-like and luminal breast cancer specimens (Supplementary Table 1), lower average levels of miR-100 were found in the CSCs derived from basal-like tumors (Fig. 2A). BrCSCs derived from patient 5 (P5), classified as basal-like subtype and expressing the lowest level of miR-100, were selected for further experiments. These cells displayed low levels also of the other two members of the miR-100 family, namely miR-99a and miR-99b (Supplementary Fig. 2A). The expression of the miRNAs in P5 BrCSCs was evaluated upon growth in conditions which favored differentiation. As shown in Fig. 2B and Supplementary Fig. 2B, the level of the miRNAs promptly increased upon differentiation.

MiR-100 impairs self-renewing and tumor-initiating ability of BrCSCs

In order to investigate whether miR-100 could interfere with the stem properties, an exploration of the self-renewing ability of tumor-derived P5 BrCSCs expressing stable miR-100 upon lentiviral transduction (data not shown) was undertaken. BrCSCs infected with a short hairpin scramble encoding lentivirus were

used as a control. Exogenous expression of miR-100 severely impaired the clonogenic activity of BrCSCs in *in vitro* limiting dilution assay (Fig. 3A) and in the soft agar assay (Fig. 3B). Similar results were observed in the subpopulation of BrCSCs obtained by sorting the bulk population for the expression of the breast cancer stem cell markers CD49f and CD24 [28, 29] (Supplementary Fig. 3A, B). The effect of miR-100 on BrCSC proliferation was evaluated via a cell cycle analysis. These data showed a reduced G2 phase and an enlarged sub-G1 population in miRNA transduced BrCSCs as compared to corresponding controls (Fig. 3C). Consistently, an increased apoptotic rate was revealed by an enhanced caspase3/7 activity (Supplementary Fig. 3C). Labeling of BrCSCs with the lipophilic fluorescent dye PKH-26 was used to further investigate the effect of miR-100 on self-renewal. PKH-26 is retained by quiescent stem cells whereas it is gradually lost by proliferating progenitor cells [30]. MiR-100 expression reduced the percentage of PKH-26^{high} cells (Fig. 3D, E), leading to the depletion of the BrCSC proliferating pool.

CSCs are defined as those cells able to originate the tumor and recapitulate the heterogeneity of the original tumor mass when implanted in immunocompromised mice. This inherent tumor-initiating capacity of CSCs is believed to be responsible for tumor relapse in patients. To address whether miR-100 could affect tumorigenic potential, miR-100 or scramble transduced BrCSCs were allowed to orthotopically grow in the mouse mammary gland of NOD/SCID mice. Interestingly, ectopic expression of miR-100 completely suppressed tumor growth (Fig. 4A). Histological examination of the fat pads showed that only a few breast cancer cells expressing the proliferation marker Ki67 and the stem cell marker Aldehyde Dehydrogenase 1 (ALDH1) were present in the residual tumor xenografts (Fig. 4B). Similar results (Supplementary Fig. 4) were obtained in an additional patient-derived basal-like BrCSC model (P8,

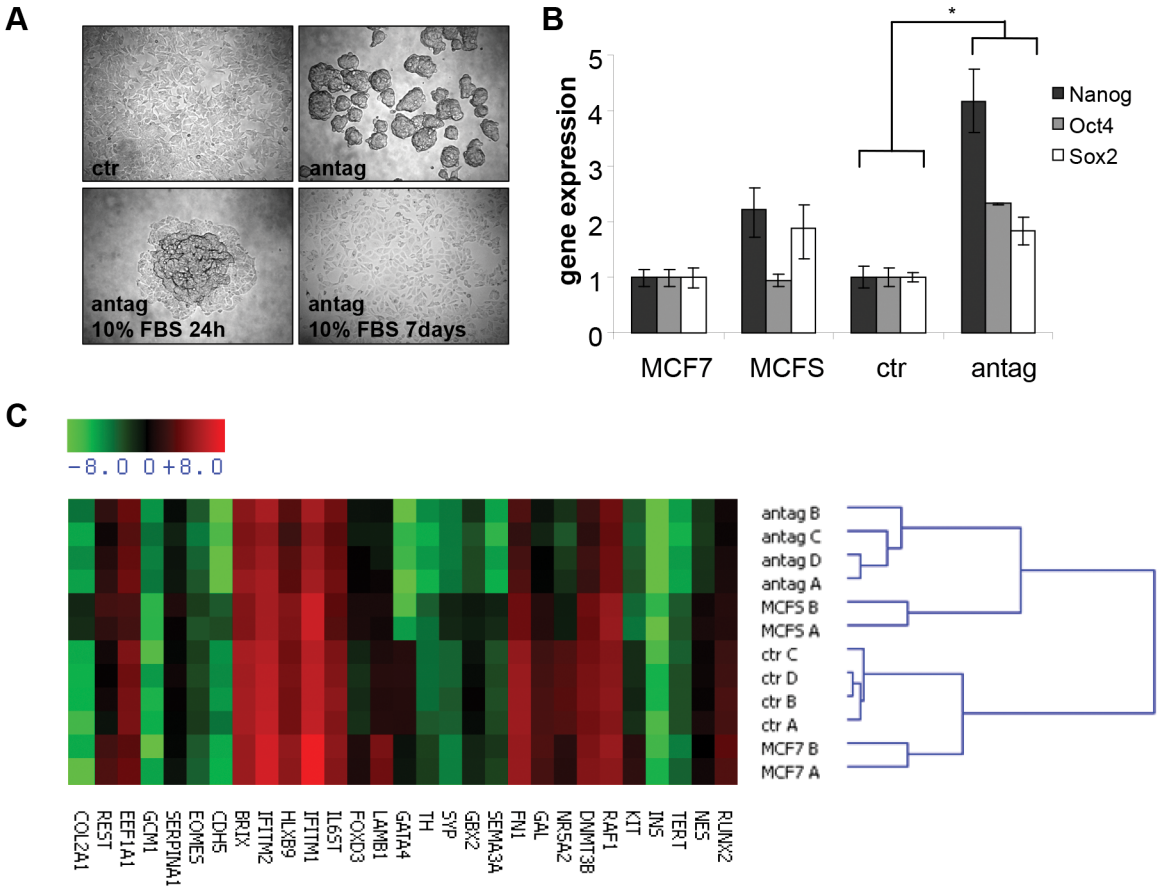

Figure 1: MiR-100 inhibition induces a stem-like phenotype in breast cancer cells. A, phase contrast images of MCF7 cells transiently transfected with a control (ctr) or a miR-100 specific antagomir (antag). Following miR-100 antagomir transfection, obtained mammospheres retained the ability to differentiate when cultured in DMEM 10% Foetal Bovine Serum (antag 10%FBS 24h; antag 10%FBS 7 days). Magnification 4x. B, stem cell transcription factors expression in control and antagomir transfected cells, analyzed by quantitative RT-PCR. Data are average ± SD of biological replicates. MCF7 cells and mammospheres obtained from MCF7 cells upon growth in stem cell conditions (MCFS) were used as controls. * P< 0.05. C, stemness and pluripotency gene expression profiling of the cells described in (B) performed using TaqMan gene expression arrays. Gene expression is reported as -ΔCT (CT gene – CT GAPDH) median-centered. A, B, C and D indicate biological replicates.

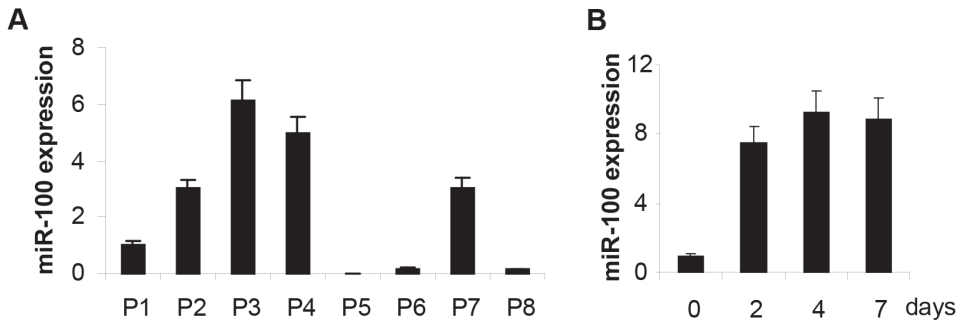

Figure 2: MiR-100 expression increases upon basal-like Breast Cancer Stem Cell (BrCSC) differentiation. A, miR-100 expression in BrCSCs derived from human breast tumors evaluated by TaqMan RT-PCR. MiR-100 expression is reported as fold changes compared to P1. P1-P4: luminal; P5-P8: basal-like. B, miR-100 expression in basal-like BrCSCs (P5) before and after growth in differentiation condition, at the indicated times. Data are representative of two independent experiments.

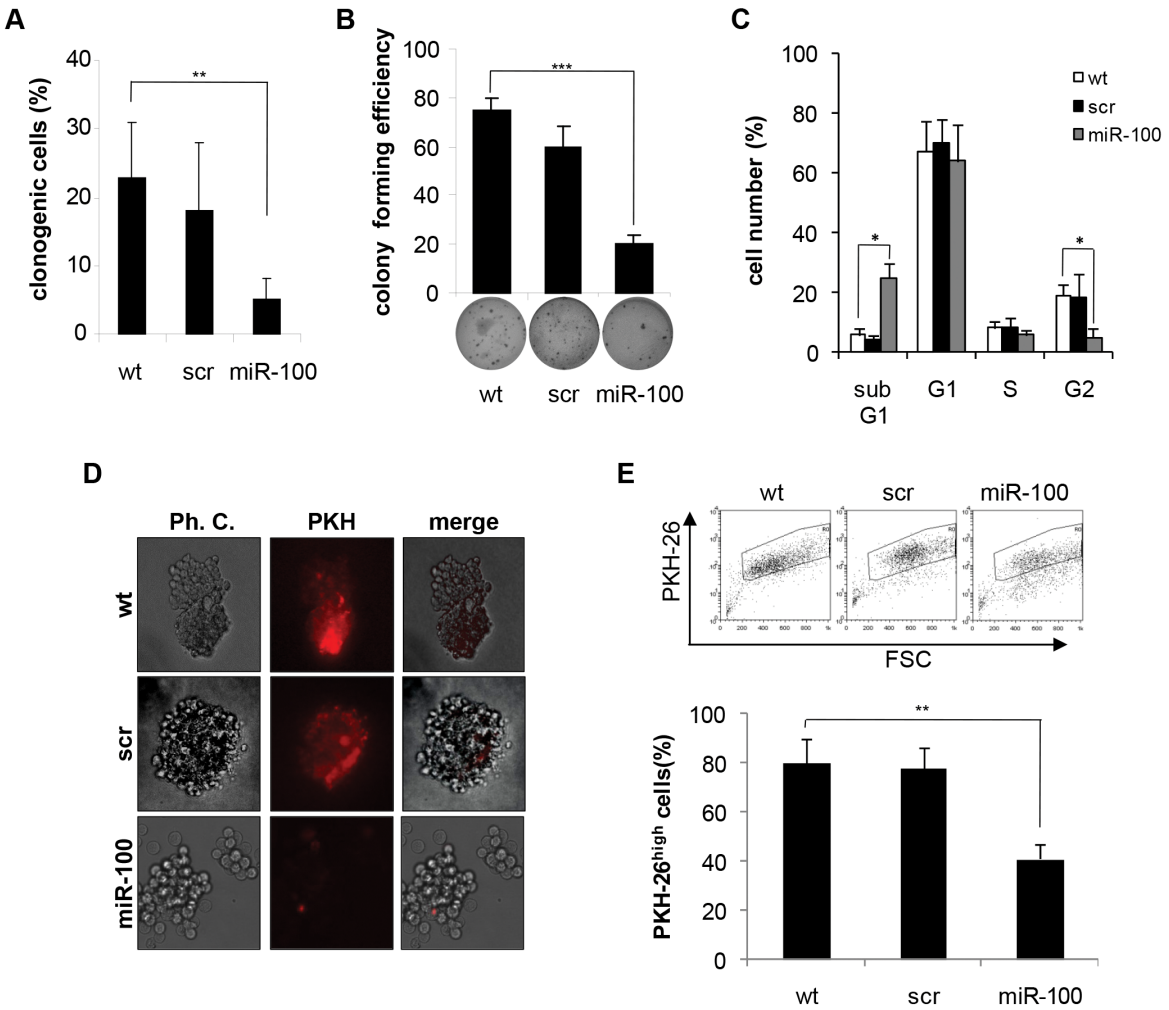

Figure 3: Ectopic expression of miR-100 in BrCSCs impairs self-renewal. A, percentage of clonogenicity in BrCSCs (P5) wild type (wt) and stably expressing either a control scramble (scr) or miR-100. Data are average + SD of 3 independent experiments. ** P<0.01. B, colony forming efficiency of BrCSCs transduced as in (A), assessed by soft agar assay (bottom); histogram shows the quantitative analysis. Data are average + SD of 3 independent experiments. *** P<0.001. C, cell cycle analysis of wt, scramble or miR-100 stably expressing BrCSCs determined by propidium iodide staining. * P<0.05. D, representative phase contrast and fluorescence microscopy analysis of wt, scramble or miR-100 transduced BrCSCs labelled with PKH-26 and cultured in soft agar up to 40 days. E, flow cytometry analysis and quantification of PKH-26 in cells transduced as in (D), after 14 days of culture. The experiments were performed in triplicates. ** P<0.01.

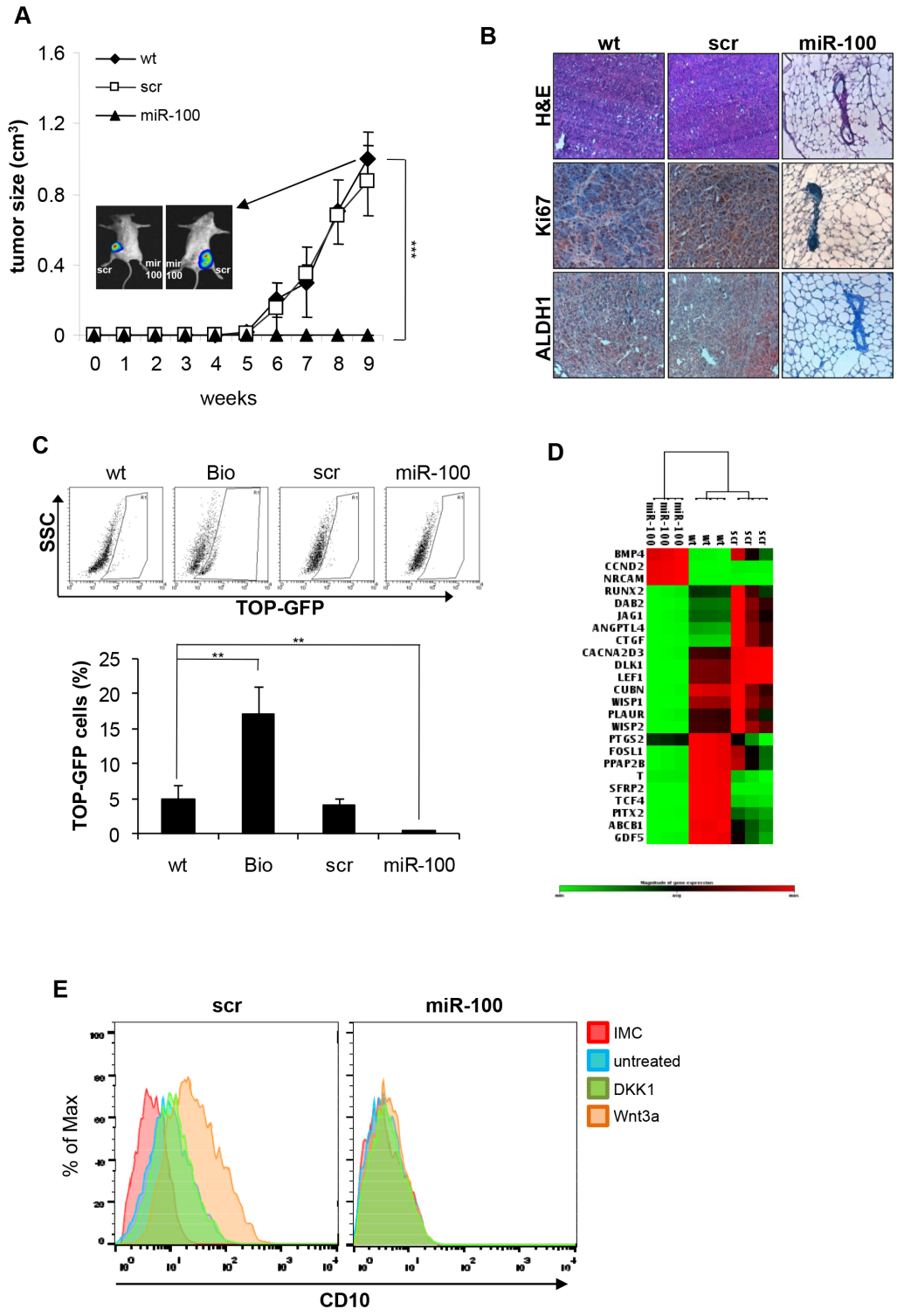

Figure 4: MiR-100 affects tumor-initiating ability and inhibits Wnt/β-catenin signaling pathway in BrCSCs. A, *in vivo* growth of BrCSCs expressed as volume of orthotopic tumors generated by fat-pad injection of either wt, scramble or miR-100 expressing BrCSCs. Insert: dorsal and ventral whole body *in vivo* imaging analysis of orthotopic tumor growth. Data are average ± SD of experimental groups containing 6 mice. *** P<0.001. B, representative Hematoxylin/Eosin (H&E) and immunohistochemical stainings (Ki67, ALDH1) of tumors originated from wt, scramble and miR-100 BrCSCs. Magnification 20x. C, percentage of TOP-GFP positive cells in wt, scramble and miR-100 transduced BrCSCs assessed by flow cytometry. As a positive control to monitor β-catenin activity, TOP-GFP reporter lentivirus-transduced BrCSCs were treated with Bio (Bio), an inhibitor of GSK-3α/β. D, heat-map showing hierarchical clustering of genes in cells transduced as in (A). Gene expression was assessed by using a Wnt target array. E, Percentage of CD10 Max fluorescence intensity in scramble or miR-100 expressing BrCSCs, untreated or stimulated either with Wnt3a or DKK1 assessed by flow cytometry.

Supplementary Table 1) displaying low levels of miRNAs of the miR-100 family (Fig 2A and Supplementary Fig. 2). These data indicate that miR-100 expression leads to the loss of CSC properties such as self-renewal and tumor-initiating ability.

MiR-100 inhibits the Wnt signaling pathway and downregulates Polo-like kinase1 (Plk1)

The Wnt/-βcatenin pathway is among the main signalling pathways involved in cancer stem cell maintenance. Tumor-initiating cells show a constitutive activation of this pathway, which can be evaluated by the LEF-1/TCF dGFP reporter [31]. In order to investigate whether the Wnt pathway contributes to miR-100 pro-differentiative program, BrCSCs wild type, scramble and miR-100 were transduced with the reporter and analyzed by flow cytometry. MiR-100 expressing BrCSCs displayed a significant reduction of β-catenin activity (Fig. 4C). Through gene array analysis, it was observed that miR-100 leads to inhibition of the Wnt signaling pathway and to downregulation of β-catenin target genes (such as WISP1/2, DLK1, TCF4 and SFRP2) that control the

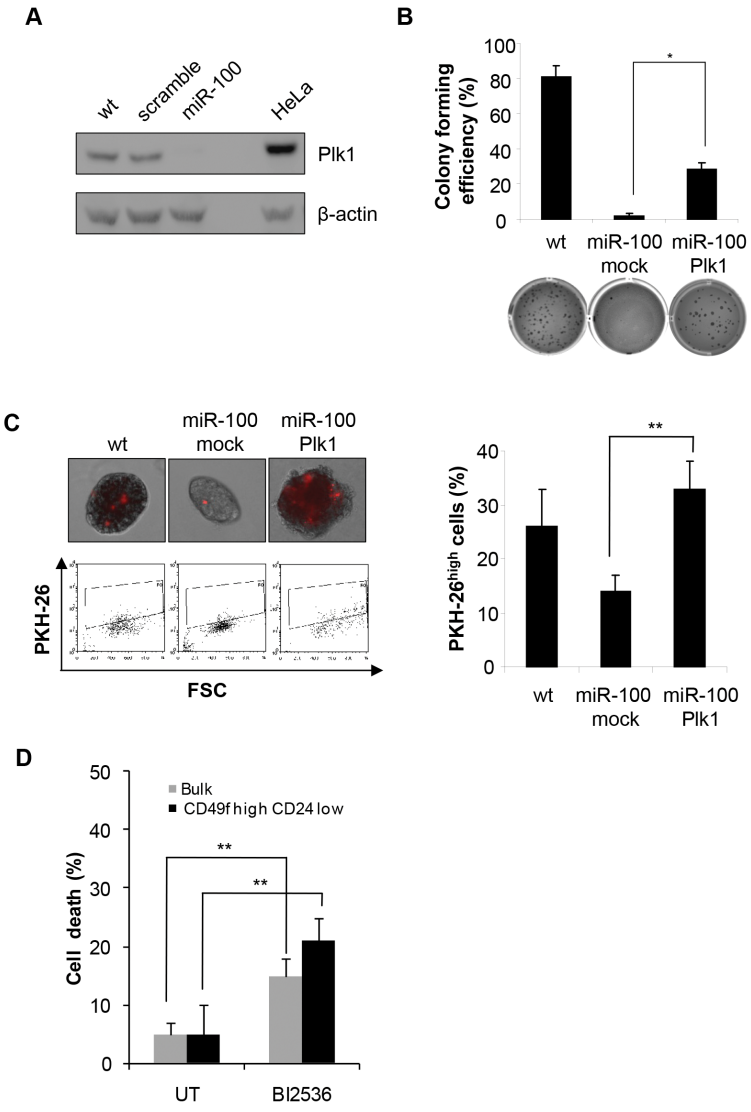

Figure 5: MiR-100 impairs CSC properties by down-regulating Plk1. A, Western blot analysis of Plk1 expression in wt, scramble or miR-100 transduced BrCSCs; a total protein lysate of HeLa cells was used as a positive control. B, colony forming efficiency of wt and miR-100 expressing BrCSCs transduced with either an empty vector (mock) or Plk1, assessed by soft agar assay; histogram shows the quantitative analysis. Data are average + SD of 3 independent experiments. C, representative fluorescence microscopy images of BrCSCs transduced as in (B) and labelled with PKH-26 (upper left). Flow cytometry analysis of PKH-26 in cells transduced as in (B) after 14 days of culture (bottom left) and the corresponding quantification (right). D, Analysis of BrCSC mortality in bulk and $CD49f^{high}/CD24^{low}$ sorted BrCSCs upon treatment with the Plk1 inhibitor BI2536 (10nM) for 72 hours. The experiments were performed in triplicates. UT: untreated.

balance between stemness and differentiation (Fig. 4D and Supplementary Fig. 5). On the contrary, BMP4, which promotes terminal differentiation of CSCs [32], was upregulated (Fig. 4D and Supplementary Fig. 5). To evaluate if the Wnt pathway is epistatic to miR-100 in controlling breast cancer stemness, we stimulated scramble and miR-100 transduced BrCSCs and evaluated the expression of the stem cell marker CD10. As shown in Figure 4E, in control cells Wnt3a stimulation increased the expression of CD10, whereas this effect was no longer visible in cells expressing miR-100. Altogether, these data infer that miR-100 expression interferes with CSC maintenance, acting downstream to the Wnt pathway, and triggers the activation of a differentiation program.

In an attempt to untangle the molecular mechanisms underlying miR-100 induced phenotype, the expression of Plk1, a known miR-100 target gene recently shown to be involved in the regulation of stem cell proliferation

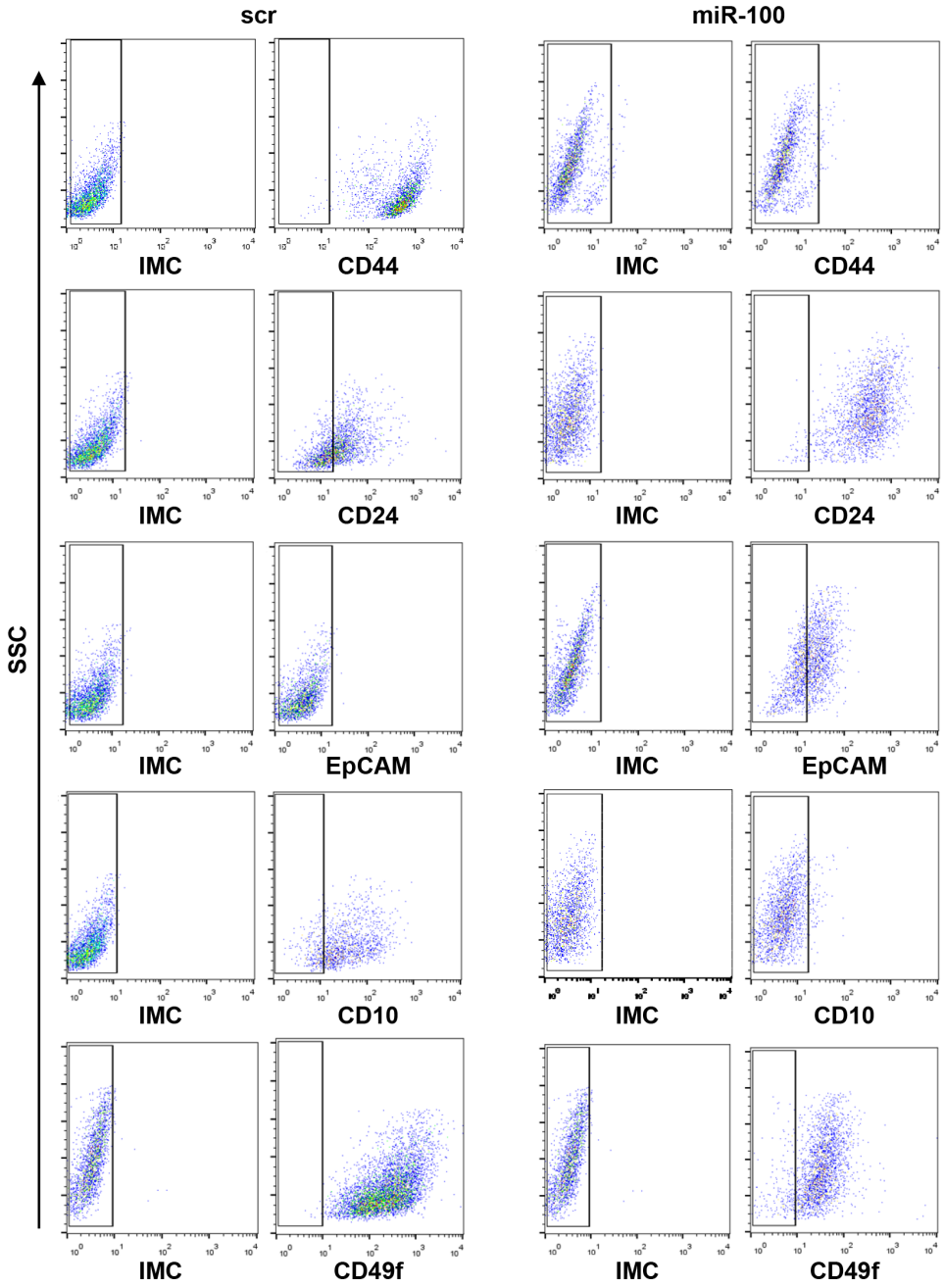

Figure 6: Ectopic expression of miR-100 reduces stem cell markers and induces markers of differentiation. Flow cytometry analysis of CD44, CD24, CD10, CD49f and EpCAM expression in BRCSCs (P5) scramble and stably expressing miR-100. IMC: Isotype Matched Control.

and differentiation [33, 34], was analyzed. MiR-100 transduced BrCSCs displayed a significant reduction of Plk1 protein (Fig. 5A). Rescue experiments were performed by re-introducing Plk1 in miR-100 expressing BrCSCs and evaluating their self-renewing ability. Upon Plk1 expression, colony forming efficiency was partially recovered (Fig. 5B), while self-renewal was restored at a level comparable to wild type BrCSCs (Fig. 5C). Consistently, the Plk1 inhibitor BI2536 impaired viability both in the bulk population of BrCSCs and in the CD49f^{high}/CD24^{low} sorted cells (Fig. 5D). These results indicate that Plk1 plays a key role in mediating miR-100 induced phenotype.

SMARCA5 and SMARCD1, two miR-100 targets belonging to the SWI/SNF protein family, have recently been shown to participate in differentiation of embryonal [24, 35] and cancer stem cells [36]. When we analyzed their expression, we found that these two proteins were significantly down-regulated in miR-100 transduced BrCSCs compared to controls (Supplementary Fig. 6A, B), suggesting that SMARC, together with Plk1, reduction could contribute to miR-100 dependent differentiation.

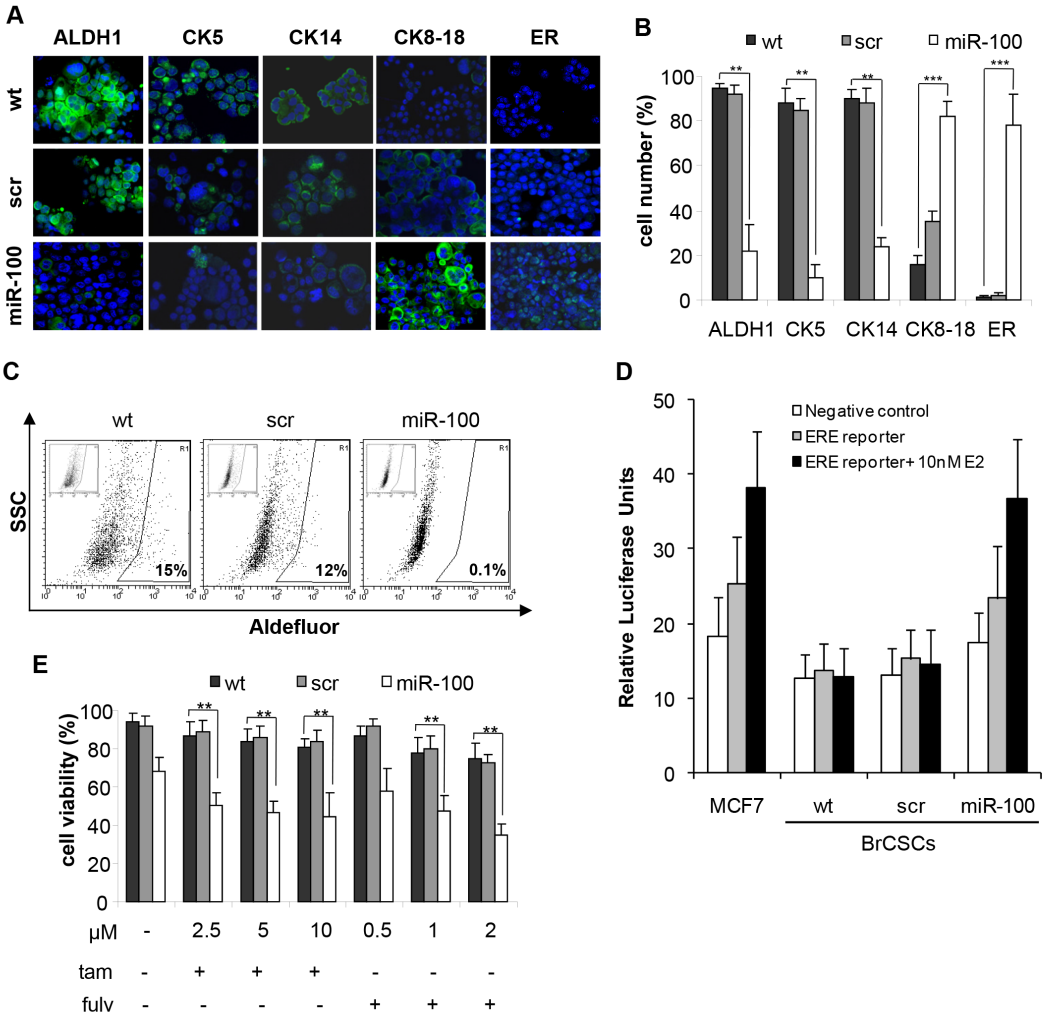

Figure 7: Ectopic expression of miR-100 reduces stem cell markers, promotes luminal differentiation and renders basal-like BrCSCs responsive to endocrine therapy. A, representative confocal microscopy images of immunofluorescence (IF) analysis of ALDH1, Cytokeratins (CK5, CK14, CK8-18) and estrogen receptor (ER) performed in BrCSCs (P5) wt and stably expressing either a control scramble or miR-100. Nuclei were counterstained by Toto-3 (blue). Magnification 40x. B, quantification of the IF staining shown in (A), performed in three independent replicates. ** P<0.01 *** P<0.001. C, representative FACS analysis of Aldefluor assay performed in wt, scramble and miR-100 BrCSCs. Cells were exposed to Aldefluor substrate (BAAA); cells treated with the specific inhibitor of ALDH1 (DEAB) are shown in the insert panels and were used to define the population with low and high (gated region) ALDH1 activity. D, representative analysis of ER-dependent transcriptional activity. The assay was performed in wt, scramble and miR-100 BrCSCs, non transfected (negative control) or transfected (ERE-reporter) with a construct where an Estrogen Responsive Element (ERE) containing promoter drives luciferase expression. Luciferase activity was evaluated in the absence or in the presence of 10nM 17-β-estradiol (E2). MCF7 cells were used as positive control of response to estradiol. E, Analysis of BrCSC viability upon treatment with tamoxifen (tam) and fulvestrant (fulv) at the indicated doses. The experiments were performed in triplicates. ** P<0.01.

MiR-100 promotes luminal differentiation and renders basal-like BrCSCs responsive to hormonal therapy

To further validate the role of miR-100 in controlling stemness and differentiation of breast cancer cells, we evaluated either by flow cytometry or immunofluorescence (IF) the expression of putative stem/progenitor and differentiation markers upon ectopic expression of the miRNA. FACS analysis showed that stem cell markers, such as CD44, CD10 and CD49f, were drastically reduced, while the differentiation markers CD24 and EpCAM increased (Fig. 6). We also assessed the expression of additional mammary stem/progenitor markers by immunofluorescence analysis. Early progenitor/stemness markers such as ALDH1, Cytokeratin 5 and myoepithelial Cytokeratin 14 were reduced in miR-100 transduced BrCSCs; conversely, the luminal epithelial markers Cytokeratin 8-18 and ER were *de novo* expressed (Fig. 7A, B and Supplementary Fig. 7A, B). Expression of ER upon miR-100 transduction was confirmed by FACS analysis as well (Supplementary Fig. 8A). Using the Aldefluor assay, we found that ALDH1 activity was also greatly reduced (Fig. 7C and Supplementary Fig. 7C). Furthermore, the luminal differentiation promoted by miR-100 was observed in $CD49f^{high}/CD24^{low}$ sorted BrCSCs as well (Supplementary Fig. 8B), confirming that miR-100 not only interferes with stemness maintenance, but also converts the breast cancer phenotype from basal to luminal-like.

Then, we wondered whether the ER pathway was functional in miR-100 expressing BrCSCs. Indeed, cells transfected with an ERE luciferase reporter displayed an increased luciferase activity in the presence of miR-100 compared to control cells (Figure 7D). Finally, we investigated whether miR-100 expression could sensitize basal-like unresponsive BrCSCs to ER inhibitors. Viability of miR-100 transduced P5 and P8 BrCSCs was significantly affected by tamoxifen and fulvestrant, at concentrations comparable to those used as optimal dose regimen for the treatment of hormone receptor positive breast cancers (Fig. 7E and Supplementary Fig. 7D). These findings indicate that the differentiation program activated by miR-100 is able to induce ER expression and to sensitize basal-like BrCSCs to endocrine therapies.

Low miR-100 expression predicts poor prognosis in breast cancer patients

To understand the clinical relevance of miR-100 as a possible prognostic factor, we examined its expression in 123 breast tumor specimens. Patients underwent radical local-regional therapy for resectable node-negative breast cancer and received no further adjuvant treatments until relapse (Supplementary Table 2). Patients were categorized according to tertiles of miR-100 expression. At a median follow-up of 60 months, low miR-100 expression was associated with worse distant metastasis-free survival in the whole population and in the subgroup with ER-positive tumors (Fig. 8A). To confirm the prognostic value of miR-100, a univariate Kaplan-Meier analysis was conducted on two validation sets of breast cancer patients who underwent surgery and received adjuvant treatments (GEO dataset superSeries GSE22220 and Supplementary Table 3). MiR-100 expression could stratify patients according to different prognosis (Supplementary Fig. 9) in these case series, as well. Moreover, as highlighted by Gene Set Enrichment Analysis (GSEA), miR-100 positively correlated with genes expressed in luminal tumors. On the other hand, low levels of the miRNA were associated with gene signatures of high-grade, poorly differentiated cancers (Fig. 8B). This confirms that low miR-100 expression is related to a more undifferentiated phenotype.

DISCUSSION

Studies performed over the past years have strengthened the hypothesis that breast tumors originate from mammary stem cells, as a consequence of dysregulation in the usually tightly controlled process of self-renewal. Despite the fact that these CSCs represent a small percentage of the tumor mass, growing evidence points to them as being the cells responsible for the life-threatening terminal evolution of the disease. They are resistant to conventional therapies and can escape anti-cancer treatments, giving rise to relapse in patients [3, 4, 37]. Therefore, untangling the molecular mechanisms underlying CSC maintenance becomes a priority for the development of new cancer therapies able to eradicate the disease.

It is known that miRNAs contribute in sustaining stemness of embryonic stem cells, since ESC maintenance is hampered by deficiency in the miRNA processing [14]. At the same time, miRNAs are essential regulators of ESC differentiation, which is associated with changes in the expression of specific miRNA patterns [38]. An important role of miRNAs in controlling self-renewal and differentiation of cancer stem cells has also recently been described [15, 39, 40].

Breast cancer is a heterogeneous disease which includes distinct types of tumors characterized by different histological origins, molecular features and prognosis [41]. Luminal tumors are characterized at the molecular level by the expression of ER and PR receptors. ER and PR signaling sustains tumor growth and thus, therapies interfering with ER activation are currently the gold standard for the treatment of this type of cancer. Basal-like tumors are defined by their lack of ER, PR and HER2 expression in about 75% of cases. These cancers are poorly differentiated and are loaded with CSCs, a feature

that is associated with a poor clinical outcome [28, 42]. The absence of the specific molecular targets in basal-like breast cancers renders ineffective the targeted therapies that significantly improve prognosis for hormone receptor-positive and HER2-overexpressing breast cancers. Therefore, a possible therapeutic strategy to treat basal-like tumors is to induce CSC differentiation and to allow the expression of genes, such as ER, to be used as targets. The strategy of using drugs that force malignant cells to terminally differentiate has been known for the past thirty years as "differentiation therapy" and is strictly connected to the concept of tissue-selective therapy (43). Such an approach drastically reduces side effects in patients since it avoids indiscriminately killing proliferating cells and instead concentrates its efficacy on cancer cells in a tissue specific manner, taking advantage of differentiation molecules that are specifically expressed in the selected tissue. This therapy has its successful paradigm in the treatment of acute promyelocytic leukemia (APL) with all-trans retinoic acid [44]. However, despite the promising results obtained in hematological malignancies, the application of differentiation therapy in solid tumors has been hampered due to inadequate knowledge of the mechanisms governing cell differentiation. The

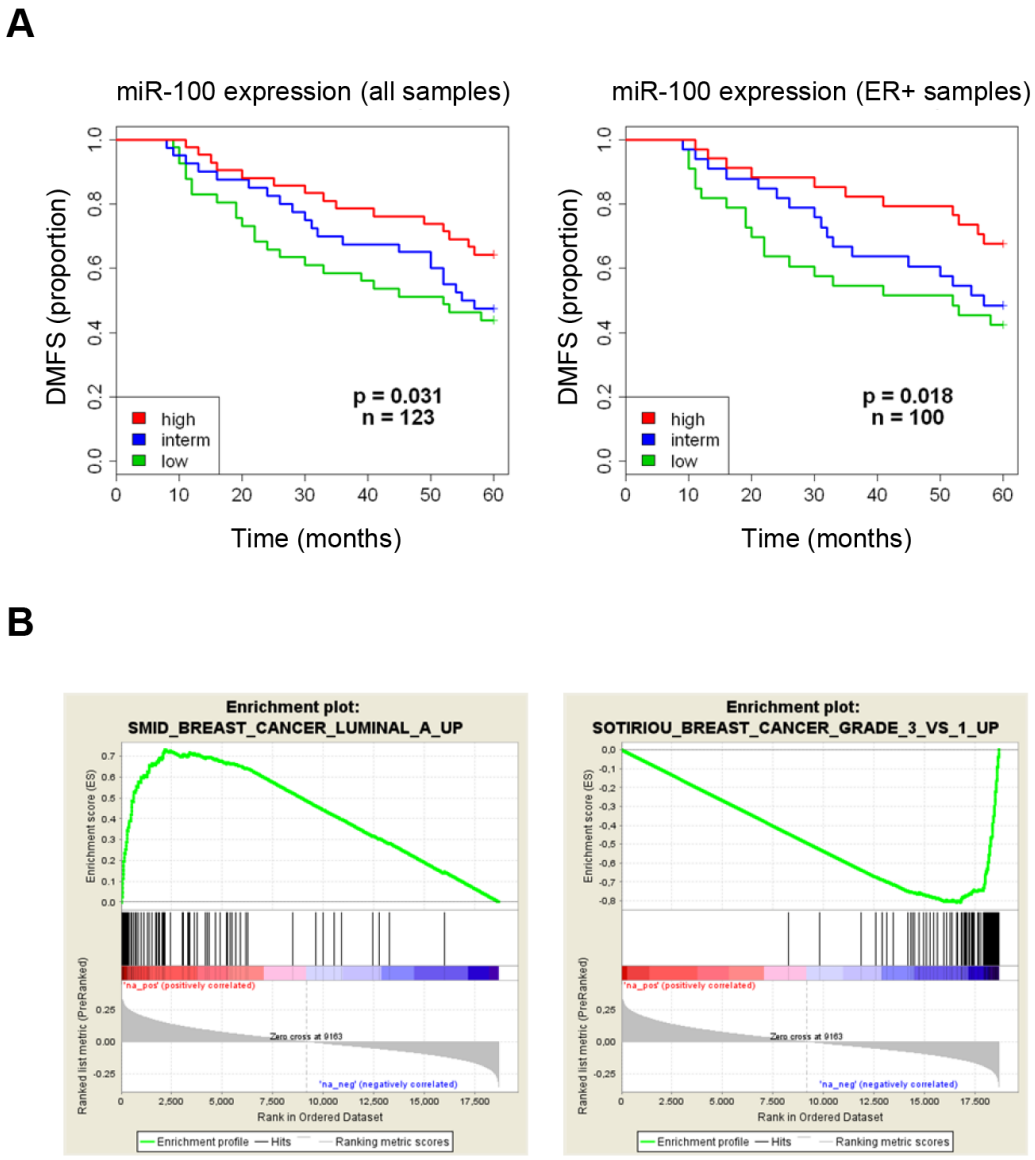

Figure 8: Low expression of miR-100 correlates with poor prognosis and high grade tumor signatures in breast cancer patients. A, Kaplan-Meier curves associated to miR-100 expression in a cohort of 123 breast cancer patients (for characteristics of patients see Supplementary Table 2). Left panel: all patients; right panel: estrogen receptor-positive patients. B, miR-100 expression was correlated with gene expression data and gene set enrichment analysis was performed on ranked genes. Two of the top positively (left) or negatively (right) correlated gene sets are reported.

current findings uncover miR-100 as a key player in the complex scenario of the differentiation process. They show that miR-100 is critical in controlling stemness and differentiation of patient-derived basal-like breast CSCs. MiR-100 interferes with the CSC properties, impairing self-renewal and blocking tumor-initiating ability.

Our results also suggest that miR-100 induces a pro-differentiative program which involves the Wnt/β-catenin pathway. Activation of β-catenin and expression of Wnt target genes are involved in the balance between stemness and differentiation. Indeed, it was found that Wnt pathway activation was reduced in cells expressing miR-100 and Wnt3a treatment was unable to promote the expression of stem cell markers. Moreover, the down-regulation of Plk1, a serine/threonine kinase target of miR-100 that controls cell cycle progression, was observed. Recently, Plk1 has been implicated in the regulation of stem cell maintenance and proliferation, as inhibiting Plk1 activity impaired growth and induced apoptosis of neurospheres [34] and of colon cancer initiating cells [33]. Here it was shown that Plk1 is required to sustain expansion of BrCSCs, as miR-100 mediated down-regulation of Plk1impairs CSC properties and depletes the CSC pool.

Furthermore data from this research provide evidence that the exogenous expression of this miRNA not only hampers the replication potential of basal-like BrCSCs, but also induces their differentiation toward a luminal phenotype. In this way, miR-100 allows the conversion of an aggressive molecular subtype of cancer into a subtype with a better prognosis, for which effective treatments are available. Indeed, miR-100 promoted the expression of the estrogen receptor and rendered basal-like cells responsive to 17-β-estradiol and to the anti-proliferative activity of tamoxifen and fulvestrant. Several miRNAs (such as miR-206, miR-221/222, miR-22, miR-17/92 and miR-145) inhibit ER expression and sustain resistance to hormonal therapies [45-47]. However, miRNAs that are able to induce *de novo* expression of this target molecule and responsiveness to endocrine treatment in triple negative breast cancer cells have never been previously described.

The results obtained in this pre-clinical system are consistent with the analysis performed on breast tumor specimens. High levels of miR-100 are associated with luminal gene signatures, while undifferentiated high grade tumors correlate with lower miRNA expression. This is also in line with recent findings that demonstrate the presence of a higher CSC content in G3 poorly differentiated tumors [30]. This could explain the poor outcome and the tendency to relapse [28]. Moreover, the analysis of breast tumor specimens from two validation data sets, which include patients who received adjuvant therapy, revealed that low expression of miR-100 correlates with a negative prognosis in ER-positive patients. This raises the question as to whether this miRNA might be a predictor of hormonal therapy response. Further studies on appropriate cohorts of patients are warranted to address this hypothesis.

Overall, these findings have relevant clinical implications, as they suggest the possibility of targeting basal-like CSCs using a pro-differentiative therapy approach, by promoting miR-100 expression. Moreover, they propose miR-100 as a response predictor to endocrine therapy and offer a therapeutic perspective in treating an aggressive tumor type, for which there are no effective therapeutic options available at this time.

METHODS

Tissue collection and cell culture

Fresh tissue acquired from mastectomies of 8 patients (age 40–89) were collected at the University of Palermo and Fondazione IRCCS INT of Milan, in accordance with ethical standards. Breast tumor cells were purified from fresh tissue via enzymatic digestion as previously described [48]. Thereafter, single cell suspensions were plated in ultra-low attachment flasks (Corning) at a density of 1×10^5/ml and grown in a medium supplemented with bFGF (10 ng/ml, Sigma) and EGF (20 ng/ml, Sigma). To induce differentiation, cells were cultured in adherent conditions in Ham's/F-12 medium (Euroclone), supplemented with 5% fetal bovine serum (FBS), insulin (25μg/ml, Sigma) and hydrocortisone (1mg/ml, Sigma). MCF7 cells were purchased from ATCC and cultured in DMEM supplemented with 10% fetal bovine serum (Sigma).

Cell transfection, lentiviral constructs and cell transduction

MCF7 cells were transiently transfected with a miR-100 specific antagomir (AM10188, Ambion) in serum free medium using Lipofectamine 2000 (Invitrogen). MiR-100 stable expression was obtained by transducing cells with a lentivirus expressing miR-100 as previously described [26]. The lentiviral short hairpin scramble (pLKO.1) used as a control was purchased from Sigma. For the *in vivo* imaging, gene transfer was performed using a TWEEN lentiviral vector containing luciferase (LUC) and green fluorescent protein (GFP) as reporter genes. For β-catenin activity assay, BRCSCs were transduced with lentiviral-TOP-dGFP-reporter (Addgene) that consists of a LEF-1/TCF-responsive promoter upstream d2-eGFP. Transfection of packaging cell line HEK-293T was performed using FuGENE Reagent (Roche) and following the manufacturer's instructions. BRCSCs were dissociated into single cells, then infected with 100 ng virus/10^5 cells.

Biological assays

For the clonogenic assay, cells were plated on a ultra-low adhesion 96-well plate at a concentration of a single cell per well and observed for 21 days. Wells containing either none or more than one cell were excluded from the analysis. For soft agar assay, 0.4% Seaplaque soft agar (Lonza) was diluted with stem cell medium and was covered by a second 0.3% soft agar layer in which BrCSCs were embedded. After 21 days, colonies were stained with 0.005% crystal violet (Sigma) for 1 hour at 37°C. For anti-estrogen treatment, cells were treated with 2.5-10 μM tamoxifen (Selleckchem) and 0.5-2 μM fulvestrant (Selleckchem) for 24-48 hours. Cell viability was assessed by means of a cell Titer Aqueous Assay Kit (Promega) following the manufacturer's instructions. Alternatively, cell death was evaluated by orange acridine/ethidium bromide staining as previously described [48] and using Cell Titer-Glo luminescent cell viability assay kit (Promega). For the assessment of apoptosis, $3x10^3$ cells/well were seeded in ultra-low adhesion 96-well plate in stem cell medium and activation of caspases 3 and 7 was evaluated after 72hours using the Caspase-Glo 3/7 Assay (Promega). The proliferative rate of stem cells was analyzed using the PKH-26 assay (2×10^{-6} M, Sigma) according to manufacturer's instructions. Samples were analyzed by FACSCalibur (BD Biosciences). For β-catenin activity experiments, cells transduced with the indicated lentiviral constructs were analyzed by FACS. As a positive control, cells were treated with BIO (1uM, Calbiochem) for 24 hours in order to inhibit GSK-3α/β. For viability assay, single cells suspensions were treated with the Plk1 inhibitor BI2536 (10nM, Selleckchem) for 72 hours.

In order to evaluate changes of CD10 expression upon Wnt pathway modulation, scramble or miR-100 BrCSCs were treated either with Wnt3a (300ng /ml) every 6 hours for 24 hours or DKK1 (200ng/ml) for 24 hours and then analyzed by flow cytometry.

FACS analysis and Cell sorting

BrCSCs were exposed to primary antibodies CD44 (BU75, Ancell), CD24 (ML5, R&D System), CD10 (FR4D11, Santa Cruz Biotechnology), CD49f (GoH3 Miltenyi Biotec), EpCAM (AF960, R&D System) or corresponding isotype controls, rinsed and labeled with secondary antibodies. BrCSCs were stained with CD49f and CD24 and successively sorted via flow cytometry using an FACSAria cell sorter (BD Biosciences). The analysis of ALDH1 activity was performed using the ALDEFLUOR kit (StemCell Technologies). For cell cycle analysis, BrCSCs were fixed in 70% ethanol and incubated with 50 μg/mL propidium iodide (Sigma-Aldrich), 3.8 mmol/L sodium citrate (Sigma) and 10 μg/mL RNase (Sigma). Samples were analyzed by FACSCalibur and CellQuest Software (BD Biosciences).

Quantitative analysis of microRNA and gene expression

Total RNA was extracted using TRI Reagent solution (Ambion) following manufacturer's instructions. Analysis of miR-100, miR-99a and miR-99b was performed starting from equal amounts of total RNA/sample (10ng) using the specific Taqman microRNA assay kits (Applied Biosystems). MiRNA expression was calculated as fold change using the delta-delta CT method and RNU48 as endogenous control.

Gene expression was evaluated by quantitative real-time PCR using the EXPRESS SYBR green (Invitrogen). Retrotranscription was performed using the High Capacity Retrotranscription Kit (Applied Biosystems) starting from 500ng of total RNA.

Expression profiling of pluripotency genes was performed starting from 1.5ug of total RNA using TaqMan Human Stem Cell Pluripotency Arrays (Applied Biosystems), according to manufacturer's instructions. Gene expression was calculated as ΔCT (CT gene - CT GAPDH). The differentially expressed genes were statistically analyzed using t-test and genes with a $P<0.05$ were included in the heat-map. Expression was reported as -ΔCT normalized to the median. The heat-map was obtained by Gedas software [49].

Expression of Wnt target genes was performed through RT^2 profiler PCR array (PAHS-243, Qiagen), according to manufacturers' instructions. Arrays were performed independently for BrCSCs wt, scamble and miR-100 and at least 3 technical replicates were run for each sample. Cycle threshold values were normalized using the average of 5 housekeeping genes on the same array (B2M, HPRT1, RPL13A, GAPDH and actin B). The comparative cycle threshold method was used to calculate the relative quantification of gene expression.

Animal models

Orthotopic xenografts were obtained by injecting 10^5 BrCSCs P5 or P8 in the murine mammary gland of three week-old female NOD/SCID mice. *In vivo* imaging was performed by a Biospace instrument upon i.p. injection of Luciferin (150 mg/kg, Promega).

Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded sections and labelled with Ki67 (MIB-1, Dako) and ALDH1 (44, BD Biosciences). Immunocomplexes were revealed by peroxidase labeled streptavidin following

manufacturer's instructions (LSAB2, Dako). Stainings were revealed using AEC (Dako) and counterstained with aqueous hematoxylin.

Immunofluorescence

BrCSCs were fixed with 2% paraformaldehyde for 30 minutes at 37°C and incubated O.N. at 4°C with the following primary antibodies: ALDH1 (44, BD), CK5 (XM26, Novocastra), CK14 (LL002, Novocastra), CK8-18 (CD10, Novocastra), MUC1 (BD Pharmigen), VIMENTIN (R28, Cell Signaling), ER (6F11, Novocastra). Thereafter, cells were labeled with secondary antibodies (Invitrogen). Nuclei were counterstained with Toto-3 (Invitrogen).

Western blot

Cell pellets were lysed in buffer (TPER, Pierce; 300 mM NaCl; 1 mM orthovanadate; 200 mM PEFABLOC, Roche; 5 μg/ml Aprotinin, 5 μg/ml Pepstatin A, 5 μg/ml Leupeptin, Sigma). Lysates (30 μg/lane) were fractioned with SDS-PAGE and blotted to PVDF. Membranes were blocked with no-fat dry milk in TBS 0.05% Tween20 and incubated overnight with a specific antibody for Plk1 (208G4, Cell Signaling), SMARCD1 (AB81621, Abcam), SMARCA5 (MAB120, Millipore) and β-actin (JLA20, Calbiochem). Densitometric analysis was performed by UVP.

ERE-reporter assay

Cells were transfected with 1μg mixture of an inducible ERE-responsive firefly luciferase construct (kindly provided by Dr. De Bortoli, University of Torino) and a constitutively expressing Renilla luciferase construct (40:1) (Cignal Reporter Assay, Qiagen) using Lipofectamine 3000 reagent (Life Technologies) according to the manufacturer's instructions. Cells were seeded into ultra-low attachment 24 wells plates (Corning) at a density of 100,000 cells per well. After 48 hours from transfection, cells were treated for 6 hours with either vehicle or 10 nM 17-β-Estradiol (E2758, Sigma) and the reporter activity was measured by luminescence. Values were normalized to Renilla luciferase activity; data are presented as relative luciferase values.

Case series at INT

The case series collected at INT in Milan consisted of 123 patients with primary invasive breast cancer and negative lymph nodes. They were subjected to radical and/or conservative surgery, plus radiotherapy. These patients, recruited from 1990 to 1998, were identified among those who developed distant metastasis within 5 years of treatment (59 patients, disease free survival range: 8-58 months) and those who were disease-free for more than 60 months (64 patients, disease free survival range: 60-185 months). The two subsets of patients were comparable in age, tumor size, histotype, ER and HER2 status. Each patient wrote an informed consent, which authorized the use of material for research purposes. The study was approved by the Independent Ethics Committee and the Institutional Review Board.

miRNA and gene expression analysis in breast cancer patients

Global gene and miRNA expression data were obtained using the HumanRef-6_v3 and Human miRNA_V2 Illumina BeadChips, respectively. Raw data were generated using the Illumina BeadStudio 3.8 software and processed using the Bioconductor *lumi* package. After quality control, the Robust Spline Normalization was applied. For gene signature expression analysis, all genes were correlated with miR-100 expression and gene sets from the C2 collection of the MSigDB were tested for their enrichment among positively or negatively correlated genes using GSEA (http://www.broadinstitute.org/gsea).

Statistical analysis

Data were expressed as average ± standard deviation. Statistical significance was determined by the t-test or by Analysis of Variance (one-way or two-way) with Bonferroni post-test. P values <0.05 were considered significant (* $P<0.05$; ** $P<0.01$; *** $P<0.001$).

ACKNOWLEDGMENTS

This work is dedicated to the memory of Dr. Raffaella Villa. The authors thank Dr. P.M. Hwang for the Plk1 expression construct, Livio Trusolino for helpful discussions, Barbara Martinoglio, Roberta Porporato and Raffaella Albano for technical assistance and Francesca Natale and Tatiana Terranova for editing the manuscript. This work was supported by the Associazione Italiana Ricerca sul Cancro (AIRC, Grant IG-11819, IG 5984 and AIRC 5x1000 to SG, Grant IG-10104 to DT, Grant IG 14415 to MT, Grant IG 12819 to GS and Grant IG-10611 and Special Programme "Innovative tools for cancer risk assessment and early diagnosis" 5x1000, grant number 12162 to MGD). AP is a fellow of AIRC 5x1000; RC and LRM are fellows of PON 2464 and 0829 respectively; DC is a fellow of the Regione Piemonte. AG is a PhD student at University of Palermo in International Immunopharmacology.

Conflicts of interest

The authors declare no competing financial interests.

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By promoting cell differentiation, miR-100 sensitizes basal-like breast cancer stem cells to hormonal therapy

Supplementary Material

MATERIALS AND METHODS

Validation case series (cohort II)

58 frozen primary invasive ductal breast cancer specimens, obtained from patients who underwent primary surgical treatment between 1988 and 2001 at a median age of 54 years (25-82), were selected from the Tumor Bank of the Department of Obstetrics and Gynecology, University of Turin. Eligibility criteria were the following: diagnosis of invasive breast cancer, all T and N stage, no distant metastasis at diagnosis, complete clinical-pathological data and updated follow up for at least 72 months and up to 100 months. All patients were treated with radical modified mastectomy or quadrantectomy and axillary dissection plus breast irradiation. High-risk node- negative and node-positive patients received adjuvant treatments (generally 6 cycles of CMF, 600 mg/m2 cyclophosphamide, 40 mg/m2 Metotrexate, 600 mg/m2 5- Fluorouracil) and/or 20 mg tamoxifen daily for 5 years in ER+ cases. ER and PR status was determined by immunohistochemical stainings, patient stage distribution was assessed as prescribed by the UICC clinical staging guidelines and tumor grading was performed according to Elston and Ellis.

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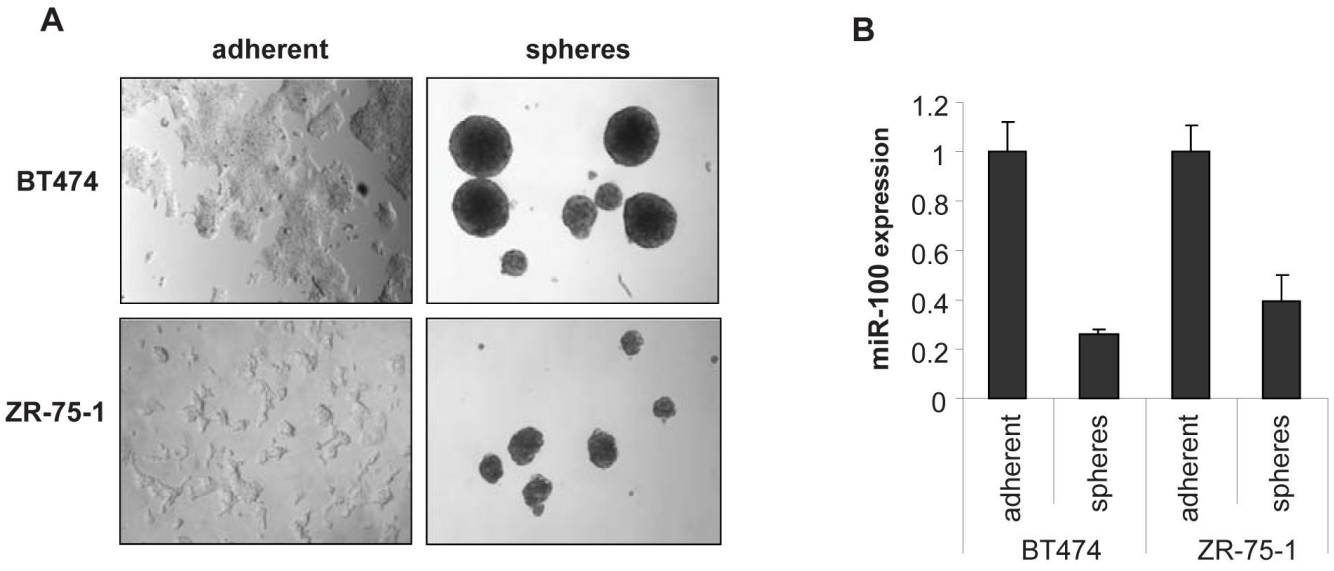
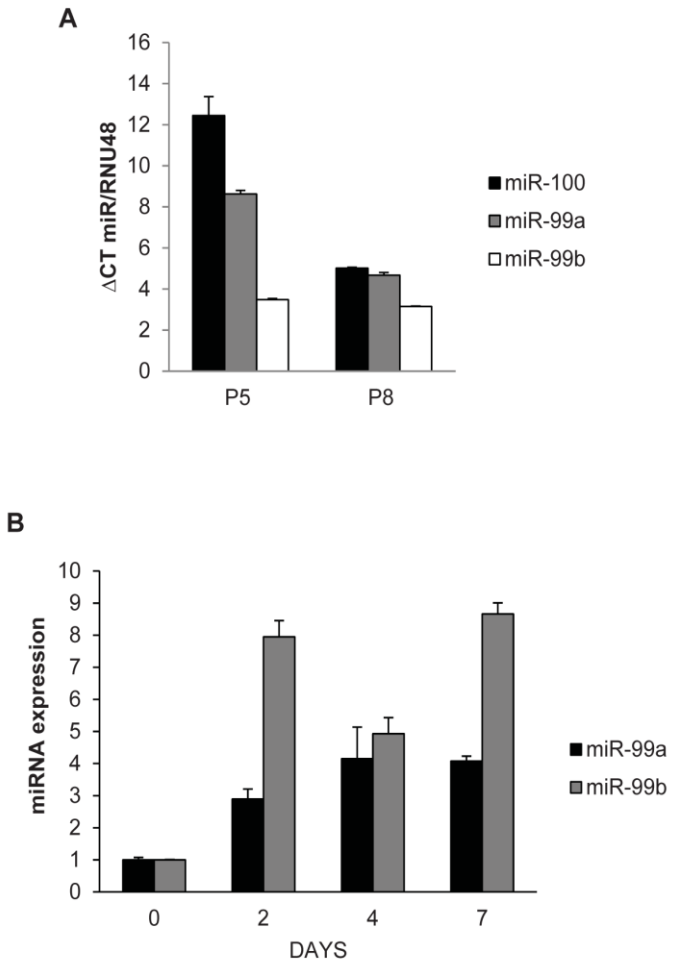
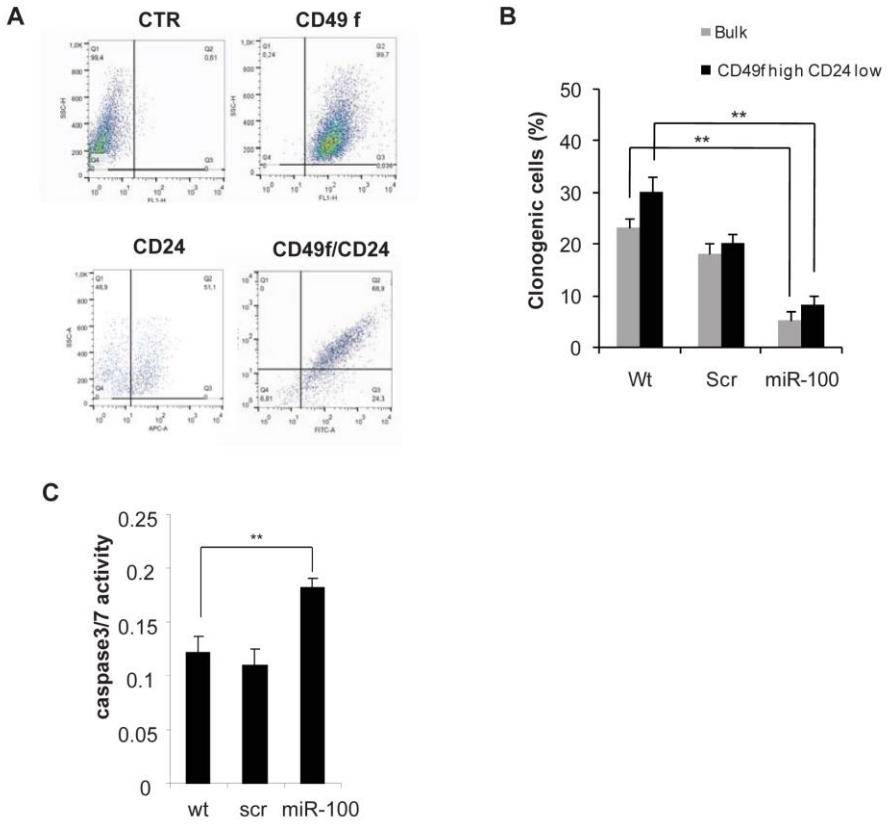


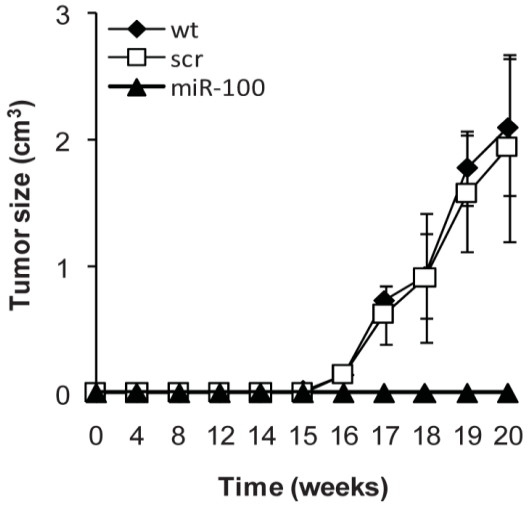
Supplementary Figure 1: MiR-100 expression is lower in mammospheres than in differentiated cells. **A,** phase contrast images of the breast cancer cell lines BT474 and ZR-75-1 grown in adherent conditions (adherent) or cultured as spheres in stem cell conditions (spheres). Magnification 4x. **B,** evaluation of miR-100 expression by TaqMan RT-PCR in the cells shown in (A), reported as fold changes compared to adherent cells. Results are representative of 2 independent experiments.



Supplementary Figure 2: MiR-100 family member expression in BrCSCs. A, miR-100, miR-99a and miR-99b expression in P5 and P8 BrCSCs evaluated by TaqMan RT-PCR and reported as ΔCT versus RNU48 endogenous control. **B,** miR-99a and miR-99b expression in P5 BrCSCs before and after growth in differentiation condition at the indicated times. Data are reported as fold change compared to undifferentiated cells (0 days).



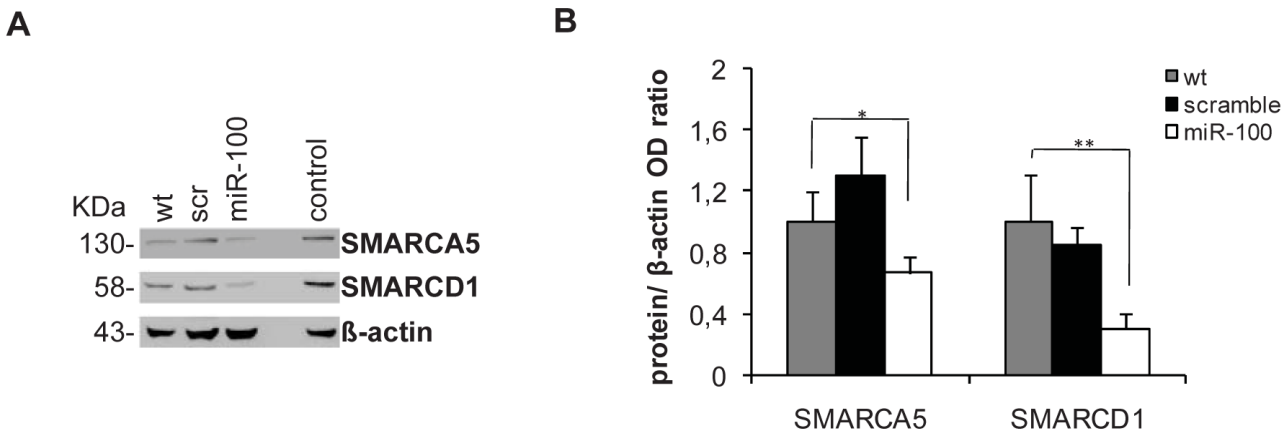
Supplementary Figure 3: MiR-100 affects BrCSC maintenance. A, representative FACS analysis and cell sorting using the CD49f and CD24 markers performed in BrCSCs. **B,** percentage of clonogenicity in bulk and $CD49f^{high}/CD24^{low}$ sorted BrCSCs wild type (wt) and stably expressing either a control scramble (scr) or miR-100. Data are average $\pm$ SD of 3 independent experiments. **C,** analysis of caspase 3/7 activation performed using the Caspase-Glo 3/7 Assay in BrCSCs transduced as in (B). **P<0.01.



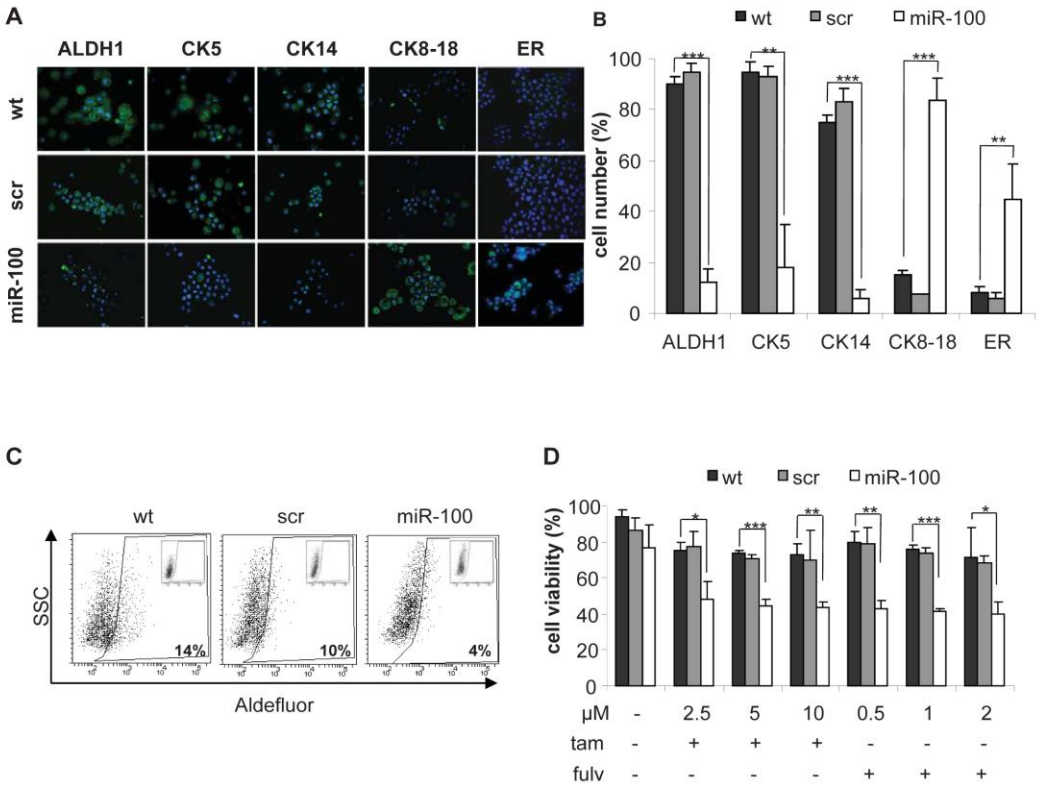
Supplementary Figure 4: MiR-100 affects tumor-initiating ability of BrCSCs. *In vivo* growth of P8 BrCSCs expressed as volume of orthotopic tumors generated by fat-pad injection of either wt, scramble or miR-100 expressing cells. Data are average ± SD of experimental groups containing 6 mice.

Gene name	Fold change	Biological function
ABCB1	-6,1688	ATP-binding cassette, sub-family B
ANGPTL4	-3,0209	growth factrs
CACNA2D3	-3,5105	Calcium Binding and Signaling
CTGF	-2,2217	adhesion
CUBN	-7,119	Calcium Binding and Signaling
DAB2	-3,142	Development & Differentiation:
DLK1	-44,1702	Development & Differentiation:
FOSL1	-3,3211	TF
GDF5	-4,0888	a member of the bone morphogenetic protein (BMP) family
JAG1	-2,5403	notch
LEF1	-19,7667	WNT Signaling
PITX2	-14,74	TF
PLAUR	-2,7734	proteolisis
PPAP2B	-3,0987	calcium signalling
PTGS2	-2,146	calcium signalling
RUNX2	-2,2064	TF
SFRP2	-23,6702	WNT Signaling
T	-6,7975	TF
TCF4	-29,9607	WNT Signaling
WISP1	-42,9623	WNT Signaling
WISP2	-3385,1405	WNT Signaling
CCND2	285,6953	Development & Differentiation:
NRCAM	24,2235	Development & Differentiation:
BMP4	5	Development & Differentiation:

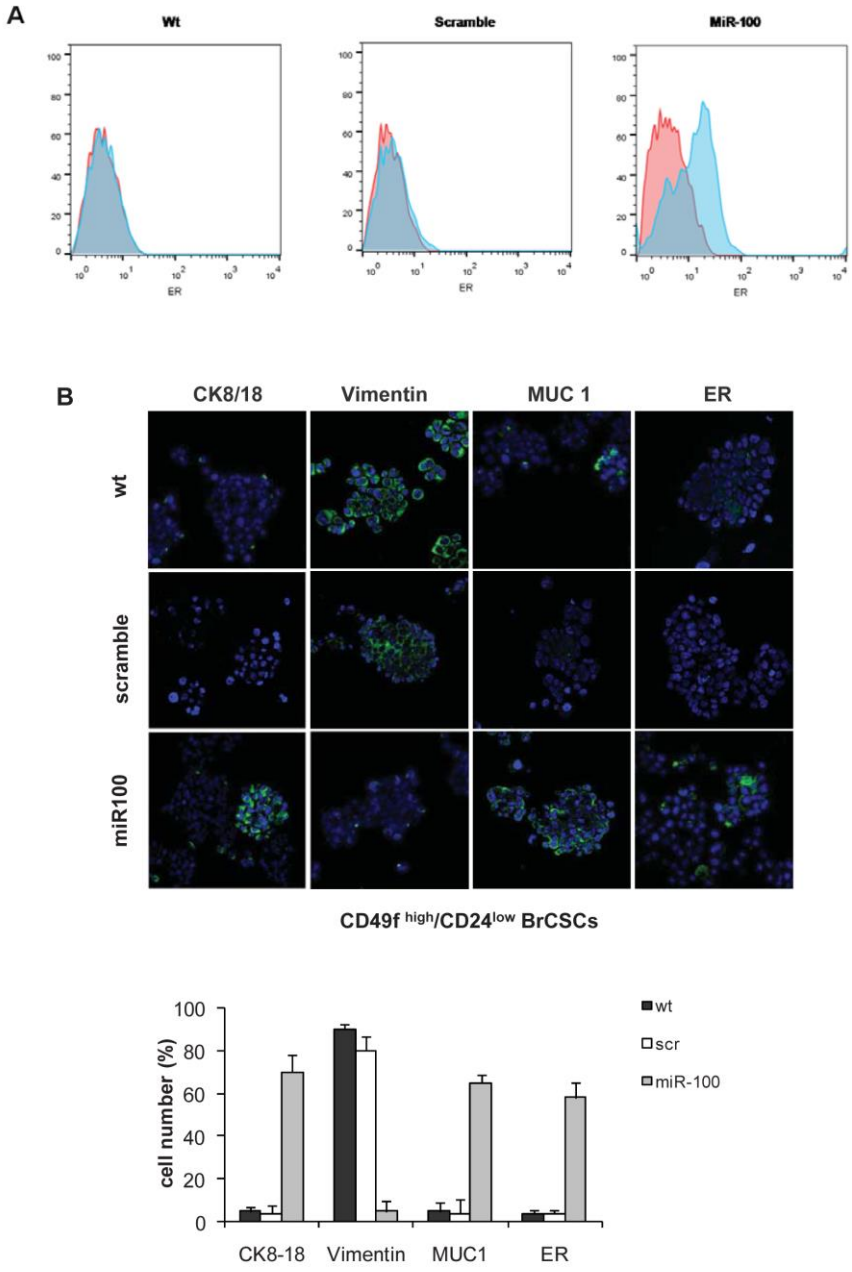
Supplementary Figure 5: MiR-100 inhibits the Wnt/β-catenin signaling pathway. Wnt target gene expression profiling of wild type and miR-100 expressing BrCSCs. In the table, gene expression levels are reported as fold changes between miR-100 expressing and wild type cells. Down-regulated genes are in green, up-regulated genes in red.



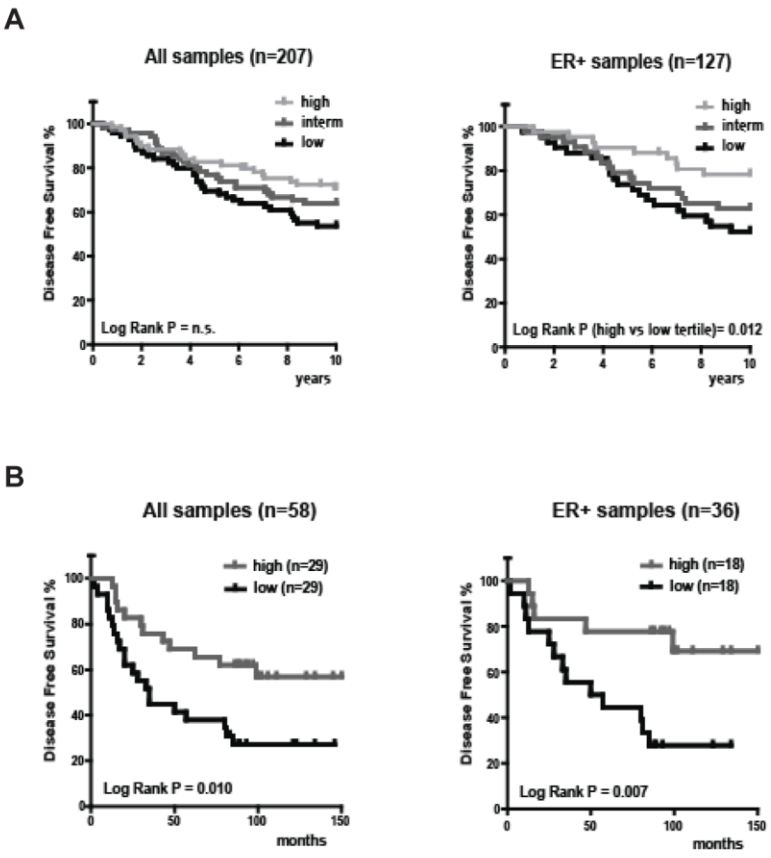
Supplementary Figure 6: MiR-100 downregulates SMARC proteins in BrCSCs. A, Immunoblotting analysis of SMARCA5 and SMARCD1 in wt, scramble or miR-100 expressing BrCSCs; control: total lysate of HeLa cells. **B**, Densitometric quantification of protein expression in BrCSCs transduced as in A. Data represent the mean ± SD of 2 independent experiments. *P<0.05, **P<0.01.



Supplementary Figure 7: Ectopic expression of miR-100 reduces stem cell markers and promotes luminal differentiation in basal-like BrCSCs. A, confocal microscopy analysis of ALDH1, Cytokeratins (CK5, CK14, CK8-18) and ER in patient-derived basal-like BrCSCs (P8), wild type (wt) or stably expressing either a control scramble or miR-100. Nuclei were counterstained by Toto-3 (blue). Magnification 40x. **B,** quantification of the IF staining shown in (A). **C,** flow citometry analysis of Aldefluor assay performed in P8 BrCSCs transduced as in (A). Cells were exposed to Aldefluor substrate (BAAA); cells treated with the specific inhibitor of ALDH1 (DEAB) are shown in the insert panel and were used to define the population with low and high (gated region) ALDH1 activity. **D,** analysis of P8 BrCSC viability upon treatment with tamoxifen (tam) or fulvestrant (fulv) at the indicated doses. *P<0.05, **P<0.01, ***P<0.001.



Supplementary Figure 8: Ectopic expression of miR-100 promotes luminal differentiation in CD49f^{high}/CD24^{low} BrCSCs. A, FACS analysis of ER expression in bulk BrCSCs wild type (wt) and stably expressing either a control scramble (scr) or miR-100. **B,** representative confocal microscopy images of IF analysis of CK8-18, Vimentin, MUC1 and ER performed in CD49f^{high}/CD24^{low} BrCSCs wt and stably expressing miR-100. Nuclei were counterstained by Toto-3 (blue). **C**, quantification of the IF staining shown in (B).



Supplementary Figure 9: Low expression of miR-100 correlates with poor prognosis in two validation cohorts of breast cancer patients. A, Kaplan-Meier curves associated with miR-100 expression in the GEO dataset superSeries GSE22220 (1). Left panel: all patients; right panel: estrogen receptor-positive patients. **B,** Kaplan-Meier curves associated with miR-100 expression in a cohort of 58 patients (for patients' characteristics see Supplementary Table 3). Left panel: all patients; right panel: estrogen receptor-positive patients.

Supplementary Table 1 Case description, tumorigenic capability and $CD44^+/CD24^-$ analysis

patient	age	Tumor type	grade	ER	PR	HER2	Ki67	Sphere formation	Xenograft	$CD44^+/CD24^-$
P1	55	IDC	G2	90%	60%	+++	>10%	yes	yes	45%
P2	69	IDC	G2	90%	60%	+	25%	yes	yes	35%
P3	74	IDC	G2	80%	80%	+++	>10%	yes	yes	15%
P4	86	ILC	G2	80%	80%	+++	<10%	yes	yes	65%
P5	85	ILC	G2	-	-	+	>10%	yes	yes	89%
P6	64	IDC	G3	-	-	+++	80%	yes	yes	75%
P7	41	IDC	G3	-	-	-	>30%	yes	no	51%
P8	51	ILC	G2	-	-	+	10-30%	yes	yes	76%

IDC: Infiltrating Ductal Carcinoma, ILC: Infiltrating Lobular Carcinoma

Supplementary Table 2 Characteristics of patients (cohort I)

	Developing distant metastases	Not developing distant metastases
Age (years)		
Range	30-85	37-82
Median	58	52.5
≤ 50	18	27
> 50	41	37
Size (cm)		
Range	0.7-9	0.6-4.5
Median	2.2	2
≤ 2	26	38
> 2	32	26
NA	1	0
Histotype		
CDI	48	49
CDI+CLI	2	5
CLI	9	3
Other	0	7
Disease Free Survival (months)		
Range	8-58	60-185
Median	28	116.5
ER		
positive	47	53
negative	12	11
HER2		
HER2 positive	9	9
HER2 negative	50	55

Supplementary Table 3 Characteristics of patients (cohort II)

	Developing distant metastases	**Not developing distant metastases**
Age (years)		
Range	25-79	32-76
Median	51.5	57
≤ 50	14	5
> 50	16	19
NA	3	1
Size (cm)		
Range	1.4-8	1-6
Median	3	2.75
≤ 2	6	5
> 2	25	15
NA	2	5
Histotype		
CDI	33	25
Disease Free Survival (months)		
Range	2-99	83-174
Median	25	102
Lymphnode status		
Positive	25	12
negative	8	13
Grade		
G1	1	0
G2	18	10
G3	13	12
NA	1	3
ER		
positive	18	18
negative	15	7
PR		
positive	16	12
negative	14	13
HER2*		
HER2 positive	6	5
HER2 negative	27	20

* assessed by Real-time analysis

Conclusions |

Conclusions

Regenerative medicine is a combination of clinical and researching interdisciplinary field aimed to repairing and regenerating tissues or organs compromises rather than replace them. (Fortier RA 2005). The potential of regenerative medicine are huge and therefore it is essential and very important keep to invest in this branch so fruitful and capable to find new prospect cures for many diseases. Exploiting regenerative medicine could overcome the lack of organs for transplantation. To date there are two main strands of therapeutic applications: The first is represented by the regeneration of solid tissues with the use of stem cells in order to reconstruct in the laboratory a piece of tissue, subsequently implanted in patients with a severe congenital malformation (Shahar M 2003); the second area concerns the cell therapy of diseases which have not a genetic origin and through with the use of well-characterized cell populations, subjected to particular treatments, such as the selection cell, in vitro expansion, the generation of clones anti-infective and anti-neoplastic (Piscaglia AC2008). This goal can be achieved by identifying a capable cell source for the regenerating damaged tissue and building a microenvironment more suitable to house and instruct cells. A source cell can be considered ideal when it is available, expandable in vitro, multipotent and able to regenerate a functional mature tissue *in vivo* without neoplastic transformation risk. Stem cells are those which are closest to this model. They found in all organisms, are undifferentiated cells capable of self-renewal and that playing a central role in homeostasis, rise new different mature progenies in order to increase tissue mass during growth or or to replace tissues due to aging or damage.Stem cells possess a hierarchical organization: from totipotent zygote, the pluripotency of embryonic stem cells (ESCs), to multipotency of adult stem cells. One promising adult stem type for regenerative medicine is the mesenchymal stem cells. Thanks to their particular characteristics MSCs are now considered as the most promising candidate for regenerative medicine and tissue engineering. The main characteristics of MSCs were first described in bone marrow tissue, but over the last 15 years, however, the attention was focused on finding an abundant valuable alternative multi-potent stem cells MSCs source that could be isolated from other tissues. Within the white adipose tissue, there is a presence of mesenchymal stem cells that are associated with potent intrinsic peculiarities. Adipose tissue mesenchymal stem cells can differentiate into a variety of cell lines and for these reasons the adipose tissue is considered an ideal MSCs source in association with therapeutic needs and in particular for the repair of defects in the regenerative medicine. Spheroids derived from human adipose tissue “ASphCs” are enriched with mesenchymal stem cells, endowed with the capability of multilineage differentiation and repairing bone injury. ASphCs showed intrinsic stem cell properties, infact despite they are quiescent, once they are forced to differentiate, they up-regulate the specific gene regulators that govern the maturation in the three different mesenchymal lineages in a more significant and higher than the commercial Adipose Derived Stem Cells, which are in our opinion non-clonogenic progenitors,

poorly able to form floating spheroids and to sustain in vitro serial passages. So it is likely that ASphCs and ADSCs may reside at different levels of the stemness hierarchy. Moreover mesenchymal stem cells have been widely used in in vivo experimental models related to the treatment of bone diseases. In this study, we have seen that Adipose stem cells were able to directly participate in bone tissue regeneration but supports that only spheroids derived from human adipose tissue "ASphCs" that expresses high levels CD271, possesses an higher in vivo multipotency, harbours the capacity to repair bone injury and could be exploited in regenerative medicine. **(Chapter 1).**

Breast carcinoma represents the most common neoplasia in women responsible for over 400,000 deaths every year. The individual risk is indeed influenced by many different factors such as family history, age, lifestyle and environment. The breast carcinomas classification for many decades was done on the histologic type and grade basis, The most common breast cancer type is the epithelial lesion, and the most frequent histotypes are ductal carcinoma and lobular that. The two histotypes may be "in situ", that is non-invasive, or "infiltrating". When cancer cells beyond the surface epithelium and spread within the underlying tissues through the lymph vessels and blood we talk about invasive tumor and the most common is ductal histology Together with the histological characterization, there is a sub molecular classification on the basis of gene expression and identified five subtypes of breast cancer: luminal A, luminal B, HER2-enriched, basal-like and claudin-low. In recent years the views of the scientific community on cancer biology has drastically changed. The Nowell stochastic model, not having an good explanation for the failure of current cancer therapies designed to kill proliferating cells, has been replaced by a different model of carcinogenesis " hierarchical " according to which a tumor would originate from a small portion of cells called " cancer stem cells ". On the basis of this model the tumor mass it is composed by a numerous proliferating cells and a small stem cells population responsible of neoplastic growth and progression in the long time. CSC model, moreover justifies how a small fraction cell is necessary and sufficient for the recapitulation of the tumor. The main problem in CSC research is the identification and characterization of this stem cell subset. The stem cell markers identification is an open scientific debate. Some markers currently used to enrich the population of cancer stem cells are CD133 for glioblastoma, colon, lung and prostate; and CD44, CD24 for the pancreas, ovary, head and breast. For this reason the molecular targeting of such highly tumorigenic cells becomes fundamental to improve the efficacy of current anti-cancer strategies. CSC state is increasingly being seen as a flexible, infact the tumor present a i heterogeneity (ITH) that may contribute to some of the difficulties in validating biomarkers for clinical use, despite the continued discovery of potential novel biomarkers. CSCs are indeed used nowadays as a marker of tumour aggressiveness and drug resistance, the CSC number and phenotype is also used by clinicians to decide the best treatment to apply. [23]. Al-Hajj and colleagues in 2002 identified by flow cytometric sorting the BCSCs " cells initiators breast cancer" based on the expression of surface antigens such as CD44, CD24, ESA, and others

additional markers were used for the characterization of these cells as CD133, ALDH1 (Ginestier and colleagues in 2007) For which cells CD44 + CD24- and ALDH 1, possess tumorigenic capacity, being able to generate tumor in immunocompromised mice since the inoculation of 20 cells. In the same study it was also shown that cells CD44 +, CD24- and ESA + would generate cells CD44 + and CD24- additional, confirming the theory of asymmetric cell division, maintaining the characteristics of self-renewal and the generation of a population heterogeneous cell also not tumorigenic that constitute the so-called tumor mass. Recent work by Pitch and colleagues of 2010 Another method for the isolation and purification of stem cell component from mammosfere cancer, used fluorescent dye PKH-26 FACS analysis (Fluorescence Activated Cell Sorter). In accordance with the fluorescence intensity it is then possible to select a population PKH-26 $^{+}$ " stem-like " (high intensity of dye) and a population PKH-26 $^{-}$ " dividing cells " (low intensity of dye). According to this profile is therefore possible to predict the biological and molecular characteristics of different mammary carcinoma: poorly differentiated or well differentiated. (**Chapter 2)**

Mastectomy is often the only possible therapy against breast cancer. and and often the surgeons to overcome the trauma of surgical mutilation suggest patients lipofilling to the implant prosthesis. This surgical techniques has been considered safe or very low risk untill the American society of plastic and recostrutive surgeons began to raise doubts about the safecty of these. The close correlation between obesity and cancer disease, highlighted in the past 20 years, it has raised concerns about a possible detrimental interplay. It is known that obesity is associated with a generalized inflammation, an elevated inflammatory cytokines expression and macrophages recruitment and endothelial dysfunction . Moreover studies on inflammatory cells infiltrating the adipose tissue, highlight that these cells generate a specific environment that perpetuates the inflammation within tissues and activate adipocytes to produce others inflammatory mediators and adipocytokines. Thus it is well established the strong correlation between the tumor and its microenvironment we suppose that latter plays a critical role in the development and metastasis cancers and that lipotransfer could represents a real potential risk for all mastectomy patients that undergo to lipofilling. In particular we think that the action of the medium released from adipose auotolo filler implanted within the lipotransfer site can induce an awakening of breast cancer stem cells still present and undetectable . Our study investigates on a specific adipose stem population, adipose cell spheres, isolated from lipoaspirati samples, considered as the true adipose stem cells, present in a quiescent state but with a great multi differentiation potential and which we think could be a viable alternative to traditional autologous lipofiller. Then through co cultures experiments between breast cancer stem cells and adipose stem cells or mature adipocytes, we investigated the effect that microenvironment generated from adipose tissue stem cell act on cancer of the breast. The results obtained say that the microenvironment generated by adipose cells spheres, although in a less concentrated than mature adipocyte and undigested adipose tissue, is pro-inflammatory,

angiogenic and proliferative. Breast cancer stem cells exposed to adipose conditioned medium increases the proliferative rate, invasive and migratory capability. In addition, breast cancer stem cell treated with adipose conditioned medium switch from a luminal to basal phenotype. Finally, our best advice for all breast carcinoma woman patients that are forced to demolitive surgery, is the use of others fillers such as the alloplastic fillers, some scaffolds or any other implants that could be less inert than autologous fillers. **(Chapter 3).**

Through a detailed preliminary study we looked for some regulatory mechanism to be exploited for the modulation of a specific stem cell population, such as that of adipose tissue

Aknowledgments|

The thanks go to all those people who contributed to my scientific and human personal growth during this long, intense and difficult course of study. Obviously I want to thank the PhD coordinator , professor Dieli, that with his constant availability support every possbile doubt. I want to thank all my adventure companions that I can hardly define simple colleagues, due to everything that we shared in these years together, and that despite the misunderstandings I have always been close, especially in complicated moments. I want to thank everyone individually, especially those who managed to not give up and do not leave in the hardest moments. I name them all because they deserve: Francesco Calo, my polyvalent friend which nothing seems impossible to do; Alessandro Gorgone, which you can always get on well and that did not ever miss a precious help in times of need;Giovanni Tomaselli the fused technician one to ask everything; Marco Bonanno, my fraternal bear friend emigrated to Africa, Simone franco, my friend "that skilled" with which you are having fun but with him you work hard and that in these last few decisive moments of study has led and guided me; my companion in misfortune Manuela Scavo with whom I had the pleasure to travel along this path of my doctorate, my acquired sisters Miriam Gaggianesi Tiziana "titi" apuzzo, the mice protector Antonella Toni Benfante from which there is always something to learn and laugh about; the sterile room lady Meri colorito that has always supported me and who never misses a kind word; Annalisa nicotra "non te la fidi" among the newcomers but immediately pleasant protagonist of the laboratory dynamics; Laura "Lauretta" Mangiapane leader of the colon cells and Alice Turbo "A Picciridda" Mistress of the breast, the young and talented newcomer Aurora Chinnici and Debora Torregrossa new handyman lab. I thank especially "Mom" Matilde Todaro that in any difficult times she has always protected and defended, and last but most important, "il mio professore" and mentor Giorgio Stassi to whom I owe so much or perhaps everything, He straightened me and made me stronger with the stick and the carrot and i always be grateful for everything good he did for me.

Of course a special thanks go to my beautiful family and to my precious and sweet Lucia that in all these years, have endured and shared all outbursts during my difficult times and that shared the successes like them.

Publications|

1. **By promoting cell differentiation, miR-100 sensitizes basal-like breast cancer stem cells to hormonal therapy.**
   Petrelli A, Carollo R, Cargnelutti M, Iovino F, Callari M, Cimino D, Todaro M, Mangiapane LR, Giammona A, Cordova A, Montemurro F, Taverna D, Daidone MG, Stassi G, Giordano S.
   Oncotarget. 2015 Feb 10;6(4):2315-30

2. **Identification and Expansion of Adipose Stem Cells with Enhanced Bone Regeneration Properties.**
   A.B. Di Stefano, PhD [1*], A. A. Leto Barone, MD [2*], A. Giammona, MSc [1*], T. Apuzzo, PhD [1], P. Moschella, MD [2], S. Di Franco, PhD [1], G. Giunta, MD [2], M. Carmisciano MD [2], C. Eleuteri PhD [1], M. Todaro MD [1,3], F. Dieli MD [3], A. Cordova MD [2], G. Stassi MD [1#] and F. Moschella MD [2]
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   J Regen Med 2015, 4:2 (http://dx.doi.org/10.4172/2325-9620.1000124)

- **ORAL PRESENTATION** during the Congress in the Parallel Sessions: ABCD Congress Bologna, Italy, 17-19 September 2015
  Topic: Stem cells, development and regenerative medicine
  Title: *Identification and expansion of adipose stem cells with enhanced bone regeneration properties.*

Human adipose sphere-derived stem cells increase the mesenchymal potential: therapeutical implication.
A.B. Di Stefano1, A. Giammona1, A. Leto Barone, G. Giunta, P. Moschella, M. Todaro1, F. Dieli, A. Cordova, G. Stassi and F. Moschella
12th Annual Meeting of the International Federation for Adipose Therapeutics and Science to be held in Amsterdam, on November 13-16, 2014

Aggiungere gli abstract dell'il4, dei chirughi plastici, di simone.

CURRICULUM VITAE

Personal information

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Education and training

Date	01/01/2013 to present
Organization providing education	**International PhD Program in Immunopharmacology (XXVI Ciclo)** University of Palermo, Italy
Place of traineeship	01/01/2013 to present
Research experience	**PhD Student at University of Palermo, Italy** Laboratory of Cellular and Molecular Patho physiology University Hospital "Paolo Giaccone", Italy Lab Head: Prof. Giorgio Stassi Project: Adipose stem cells (ASCs) in lipofilling procedure and tumoral relapse after surgical treatment. The aim of my research was the evaluation through the co culture system, both in vitro and in vivo, the correlation between Breast cancer stem cells and ASCs.
Date	19/10/2012
Title of qualification awarded	**Master's degree in Medical Biotechnology and Molecular Medicine**
Organization providing education	University of Palermo, Italy
Final degree mark	106/110
Place of traineeship	01/07/2011-19/10/ 2012
Research experience	Graduate Student at Laboratory of Cellular and Molecular Pathophysiology. University Hospital "Paolo Giaccone", Italy Lab Head: Prof. Giorgio Stassi As a graduate student I acquired skills to perform both in vitro and in vivo assays on Cancer Stem Cells and to formulate hypotheses, analyze and discuss data and organize future plans. My degree thesis was about the MiR-100 renders basal-like breast cancer stem cells responsive to hormonal therapy by inducing luminal differentiation.

Date	20/07/2010
Title of qualification awarded	**Bachelor's degree in Medical Biotechnology**
Organization providing education	University of Palermo, Italy
Final degree mark	98/110
Place of traineeship	01/11/2009 to 01/03/2010
Research experience	Under graduate student at Laboratory Biotecnologie in Virologia e nel rischio biologico. In the Dipartimento di Scienze per la Promozione della Salute G. D'Alessandro - Sezione di Microbiologia. University Hospital "Paolo Giaccone", Italy. Lab Head: Prof. Rosa Di Stefano During my undergraduate training i investigated the HBV molecular mechanisms using develop the occult infection.
Date	21 July 2000
Title of qualification awarded	**Scientific High School Diploma**
Organization providing education	Scientific High School "Giuseppe Garibaldi", Palermo, Italy
Technical skills and competences	Isolation, culture and characterization of Cancer Stem Cells and Circulating Tumor Cells from fresh specimens; *In vitro* assays (MTT assay, Invasion assay, Sphere forming assays, Colony formation assay); Flow Cytometry; Virions production using packaging cell lines; Cell transduction using lentivirus; Immunofluorescence; Immunohistochemistry; Molecular biology (general expertise including DNA and RNA extraction; PCR; RT-PCR; Real Time PCR); SDS page; Western blot analysis; Bacterial transformation; *In vivo* assays on immunocompromised mice: Subcutaneous/IP injections; Orthotopic breast surgery; Collection of blood from heart; Bioimaging analysis. Excellent ability to work in a cross-functional team and in a multicultural environment. Critical thinking and advanced research planning skills.

Membership

ABCD Junior member

ABCD Congress: Bologna, Italy, 17-19 September 2015

ORAL PRESENTATION during the Congress in the Parallel Sessions:

Topic: Stem cells, development and regenerative medicine

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Papers and Abstract

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- ***Identification and Expansion of Adipose Stem Cells with Enhanced Bone Regeneration Properties.***

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J Regen Med 2015, 4:2 (http://dx.doi.org/10.4172/2325-9620.1000124)

- ***Adiposphere-enriched dermal regenerative matrix (Integra®) promotes bone growth of calvaria defects in a xenogeneic model***

Luigi Montesano M.D.* [1], Barbara Di Stefano Ph.D.* [2]; Alessandro Giammona[2], Giovanni Cassata DVM[3], Cesare Gagliardo MD[4], Beatrice Belmonte MD[5], Ada Maria Florena MD[5], Paola Moschella MD[1], Adriana Cordova M.D. [1], Francesco Moschella[1]M.D. & Angelo A. Leto Barone, M.D.

EuRePS (European Residents in Plastic Surgery) Meeting

,Favignana, 18 to 21 of June 2015.

- ***Human adipose sphere-derived stem cells increase the mesenchymal potential: therapeutical implication.***

A.B. Di Stefano1, A. Giammona1, A. Leto Barone, G. Giunta, P. Moschella, M. Todaro1, F. Dieli, A. Cordova, G. Stassi and F. Moschella

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