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Spheroids of adipose derived stem cells show their potential in differentiating towards the angiogenic lineage

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ABSTRACT

Introduction: Adipose derived stem cells (ASCs) are a mesenchymal stem cell population of great scientific interest due to their abundance and easiness in obtaining them from adipose tissue. Recently, several techniques for three dimensional (3D) ASCs cultivation have been developed to obtain spheroids of adipose stem cells (SASCs). It was already proved that ASCs are able to differentiate towards the endothelial lineage thus, for the first time, we investigated the ability of our 3D SASCs to differentiate endothelially and the effects of not differentiated SASC secreted factors on specific cultured cells.

Materials and methods: SASCs were differentiated with a specific medium towards endothelial lineage. Cell viability, gene and protein expression of typical endothelial markers were analysed. Moreover, tube formation, wound healing and migration assays were performed to investigate the ability in migration and angiogenic networks formation of endothelially differentiated cells. SASCs secretome were also tested.

Results: We showed the ability of SASCs to differentiate towards the endothelial lineage with an increase in cell viability of 15-fold and 8-fold at 14 and 21 days of differentiation respectively. Moreover, we showed the upregulation of VEGF-A and CD31 mRNAs of 9-fold and 1300-fold in SASCs endothelially differentiated cells, whilst protein expression was different. VEGF-A protein expression was upregulated whilst CD31 protein wasn't translated. In addition, ICAM1, VCAM1, ANGPT1, CD62E protein levels remain unchanged. SASCs were also able to organize themselves into angiogenic networks after 7 days of culturing themon ECMatrix. Secreted factors from undifferentiated 3D SASCs acted in a paracrine way on HUVECs and endothelially differentiated ASCs seeded on ECMatrix to promote angiogenic events.

Conclusions: SASCs, thanks to their multilineage differentiation potential, also possess the ability to differentiate towards endothelial lineage and to organize themselves into angiogenic networks. Moreover, they are able to promote angiogenesis through their secreted factors.

Abbreviations: ANGPT1, angiopoietin-1; ASCs, adipose derived stem cells; bFGF, basic fibroblast growth factor; CD31, cluster differentiation 31; CD62E, cluster differentiation 62 E-selectin; CM, conditioned medium; cDNA, complementary DNA; DMEM, dulbecco's modified eagle medium; EPCs, endothelial progenitor cells; EGF, epidermal growth factor; ECMatrix, extracellular matrix; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hFGF-b, human basic fibroblast growth factor – beta; HIF1α, hypoxia-inducible factor alpha; HUVECs, human umbilical vein endothelial cells; ICAM1, intercellular adhesion molecule 1; IGF-1, insulin like growth factor-1; miRNA, microRNA; MMP-2, matrix metallopeptidase 2; MMP-14, matrix metallopeptidase 14; mRNA, messenger RNA; MSCs, mesenchymal stem cells; RUNX2, runt-related transcription factor 2; SASCs, spheroids of adipose stem cells; SCM, stem cell medium; SD, standard deviation; SVF, stromal vascular fraction; 3D, three dimensional; VCAM1, vascular cell adhesion molecule 1; VCBM, vascular cell basal medium; VEGF-A, vascular endothelial growth factor A; VEGFR1, vascular endothelial growth factor receptor 1; VEGFR2, vascular endothelial growth factor receptor 2.

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1. Introduction

Mesenchymal stem cells are morphologically fibroblast-like cells (Denu et al., 2016), self-renewal cells (Yoon et al., 2014) and can be isolated from several sources such as bone marrow (Pittenger et al., 1999; Chu et al., 2020), dental pulp (Rajendran et al., 2013), adipose tissue (Mahmoudifar and Doran, 2015) and many others (Makhoul et al., 2013; Poltavtseva et al., 2014; Sahraei et al., 2022).

Recent literature studies proved that MSCs isolation from adipose tissue was advantageous if compared with bone marrow due to the lower invasiveness and higher safeness of the surgical liposuction procedures (Housman et al., 2002; Dhami and Agarwal, 2006) and the higher abundance of cells (Witkowska-Zimny and Walenko, 2011; Harasymiak-Krzyżanowska et al., 2013). It has already been proved that 2D cultures of adipose derived stem cells (ASCs), with the addition of specific culture media enriched with lineage-specific induction factors, are able to differentiate into multilineages such as chondrogenic, osteogenic, adipogenic (Kuca-Warnawin et al., 2023), cardiomyogenic, angiogenic (Shang et al., 2019), myogenic and others cell lines (Si et al., 2019).

Recently, new advances in 3D ASCs cultivation have been developed, leading to obtain spheroids of adipose stem cells (SASCs). Today, there is still no standardised technique to obtain spheroids of ASCs, but all researchers agree that 3D cultures represent better the *in vivo* condition and possess higher capacity to differentiate towards mesenchymal lineages compared to 2D-ASCs (Di Stefano et al., 2022). We showed that SASCs possess higher stemness properties than 2D ASCs (Di Stefano et al., 2021), exhibiting a typical miRNAs profile similar to the one of the induced pluripotent stem cells (iPSCs) (Di Stefano et al., 2018). It has already been proved that they have an increased differentiation potential towards chondrogenic, osteogenic and adipogenic lineages if compared to 2D ASCs (Di Stefano et al., 2015) and their ability in inducing osteogenesis in *in vivo* rabbit calvaria models. SASCs implanted with Integra at the site of injury, through the release of paracrine factors, stimulated rabbit cells to repair the bone injury and also to enhance neoangiogenesis processes (Di Stefano et al., 2020). This could due to the fact that SASCs mimic better than 2D cells what happens inside the living tissue where the cells are in three dimensions and not in two. Nowadays, only one study investigated the angiogenic differentiation potential of ASC spheroids, proving that 3D spheroids formed with a different technique showed a quick spontaneous over-expression of some osteogenic and angiogenic markers, respectively RUNX2 and CD31 compared to 2D adherent cells. Then, an increased cell differentiation was performed through the simultaneous addition of specific osteo and angio-inductive factors, finding a predominance in osteogenic differentiation (Gorkun et al., 2021).

In addition, literature shows that ASCs can play a role in tissue repair by promoting angiogenesis and neovascularisation in various pathologies such as myocardial and various types of ischemia (Gimble et al., 2007; Ma et al., 2017; Katagiri et al., 2020; Lee et al., 2020).

The aim of this work was to show the differentiation potential of SASCs towards the angiogenic lineage, not yet investigated in detail. Then, we also studied SASCs ability, or their released paracrine factors, to form tube networks and stimulate the cell proliferation.

2. Materials and methods

2.1. Cell culture

Lipoaspirates and subcutaneous adipose tissue samples were taken from healthy patients which underwent surgery in the Department of Plastic and Reconstructive Surgery at the University Hospital of Palermo, following informed consent. The samples were treated both enzymatically with collagenase (150 mg/ml, Gibco, Carlsbad, CA) and hyaluronidase (20 mg/ml, Sigma) and mechanically thanks to a thermomixer at 37 °C (30–60 min). After centrifugation at 1200 rpm for 5 min, the SVF (stromal vascular fraction) was cultured under different conditions: in suspension (3D) and in adhesion (2D).

In 3D conditions, the stem cell medium (SCM) together with growth factors as bFGF (10 ng/ml, Sigma, St. Louis, MO) and EGF (20 ng/mL, Sigma, St. Louis, MO) were added and the cells were seeded in ultralow adhesion flasks (Corning, NY). On average, the generated spheroids are composed of 30 to 100 cells. In 2D conditions, Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS was used in traditional adhesion flasks. In addition, HUVEC population was cultured in 2D adhesion conditions with Vascular Cell Basal Medium (VCBM) (MesenPRO RS, Gibco, 2337037). All the cell cultures were synchronized, by culturing them concurrently for up to 21 days. They were stored in an incubator at 37 °C and 5% CO_2 and periodically washed. In order to analyse the growth of ASCs, SASCs and HUVECs cultured in specific inductive endothelial conditions, 350.000 cells were seeded and a cell count was performed after 0, 14 and 21 days of *in vitro* differentiation.

2.2. RNA extraction and mRNA assay

RNA extraction was performed through the RNeasy Mini Kit (Qiagen). High quality RNA was quantified with Qubit 4 (Thermofisher). Then, 350 ng of RNA was reverse transcribed into cDNA using the High capacity cDNA reverse transcription kit (Applied Biosystems). Relative mRNA expression values were calculated through the Livak method from the equation $2^{-\Delta\Delta Ct}$. HIF1α (Hs00153153_m1), VEGF (Hs0090054_m1) and CD31 (Hs00169777_m1) genes were analysed on VCBM-treated cells and GAPDH (Hs02758991_g1) was used as housekeeping gene.

2.3. In vitro angiogenesis assay

After culturing ASCs, SASCs and HUVECs in VCBM for 21 days, cells were seeded on an extracellular matrix (ECMatrix™ ECM625, Sigma-Aldrich). It consists of laminin, collagen type IV, heparan sulfate proteoglycans, entactin and nidogen and several growth factors such as TGF-beta and FGF and proteolytic enzymes such as plasminogen, tPA and MMPs.

It was generated *in vitro* by mixing a solution of ECMatrix™ and cold buffer which was seeded on 96-well plates and kept in the incubator at 37 °C to solidify. After 1 h, 10,000 cells per well were added and incubated overnight. After 12 h all media were changed by adding the conditioned media from ASC and SASC cultured in their own media for 28 days. The experimental conditions for this analysis were the following: VCBM as control and conditioned medium from 3D SASCs and 2D ASCs maintained respectively in their specific media for 28 days. Then, we tested the angiogenic sprouting of the cells and also analysed the expression of angiogenic markers on cells seeded on matrix through an immunofluorescence assay. Anti-VEGF antibody (ab185238, Abcam) and anti-CD31 antibody (ab9498, Abcam) were used. In addition, Hoechst (33342, Thermofisher) was used to detect cell nuclei.

2.4. Wound healing assay

After 21 days of endothelial differentiation, the proliferation and migration of ASCs, SASCs and HUVECs were evaluated in two different assays in VCBM and experimental conditioned media. Firstly, cells were cultured into a 24-well plate until confluence and, subsequently, a scratch was performed. *I*mageJ software was used to measure the migration distance and the covered area. Secondly, 15.000 cells were seeded into the upper chamber with pore sizes of 8 μm in a 24well plate (Thermofisher) and, after 24 h, the migrated cells on the bottom of the well were counted.

2.5. Luminex investigation

By Luminex plate (BioRad), we analysed the presence of 7

endothelial analytes in SASCs, ASCs and HUVECs conditioned medium. Firstly, for each analyte, we subtracted the average value of each cell conditioned medium to the blank after that the average between the data of SASCs and ASCs samples was calculated. Finally, data were processed as intensity value in log10.

2.6. Statistical analysis

Data are expressed as mean ± standard deviation (SD) of three independent experiments conducted in triplicate. Statistical significance was observed using the One Way Anova test, followed by Bonferroni's multiple comparison test. Significance levels are indicated as p values: * $p < 0{,}001$.

3. Results

3.1. Investigation on the angiogenic differentiation potential of 3D SASCs

In order to investigate the angiogenic differentiation potential of SASCs, we performed several analyses comparing them with HUVECs and endothelially differentiated 2D ASCs, whose endothelial properties were already known. Morphological observations showed that the addition of endothelial differentiation stimuli on cell cultures led to a change in SASCs shape. In fact, the cells take on the fibroblastic-like shape characteristic of the endothelial population (Fig. 1A) if compared with the original spheroidal organization as well as mesenchymal 2D shape. The analysis of cell viability showed a different proliferation of SASCs, at the same medium condition and time period, compared to ASC and HUVEC ones. Although the latter maintained the same pattern with an increase in growth at 14 and 21 days of approximately 2.5-fold compared to baseline, SASCs showed an increase of approximately 15-fold at 14 days and 8-fold at 21 days compared to time 0. Comparison analysis amongst the three cell cultures showed that SASCs grew up approximately 6-fold more at 14 days and 3-fold more at 21 days if compared to ASCs and HUVECs (Fig. 1B). A Real-Time PCR analysis of several angiogenic mRNAs was performed on the cell cultures after 21 days of induction of endothelial differentiation in comparison to HUVEC expression. The results showed an up-regulation of VEGF-A expression levels approximately of 9-fold on SASCs and 5-fold on ASCs and CD31 up-regulation of 1300-fold on SASCs and 800-fold on ASCs. VEGFR2 and HIF1-α (data not shown) were instead reduced on SASCs (750-fold and 2-fold, respectively) and ASCs (180-fold and 1-fold, respectively) compared with HUVECs expression (Fig. 1C). We also quantified several angiogenic factors through Luminex assay, finding that VEGF-A was 6-fold up-regulated in both samples whilst VEGFR2 and CD31 were 3-fold down-regulated. In addition, ICAM1, VCAM1, ANGPT1 and CD62E (data not shown) did not change in all samples (Fig. 1D).

3.2. Investigation of cell differentiation on ECM matrix

To evaluate the ability to form angiogenic networks, SASCs previously induced to differentiate towards the endothelial lineage, were seeded on ECMatrix™. Similarly, ASCs and HUVECs were placed on the matrix as control. These tests showed different behaviours of cells. Although the specific protocol indicated the first sprouting events within 4 h followed by apoptosis phenomena, HUVECs maintained their angiogenic networks up to 24 h. In contrast, after 24 h SASCs and ASCs had not yet shown any angiogenic network and only single sprouting events were visible, so that they were maintained on ECMatrix™ for longer time. After being cultured for 7 days on matrix, both populations started to generate angiogenic networks that were maintained and observed until 14 days (Fig. 2).

3.3. Analysis of SASCs secreted factors on angiogenic networks formation

To investigate the potential stimulatory effects of secreted SASCs paracrine factors for angiogenic networks formation, the cells were

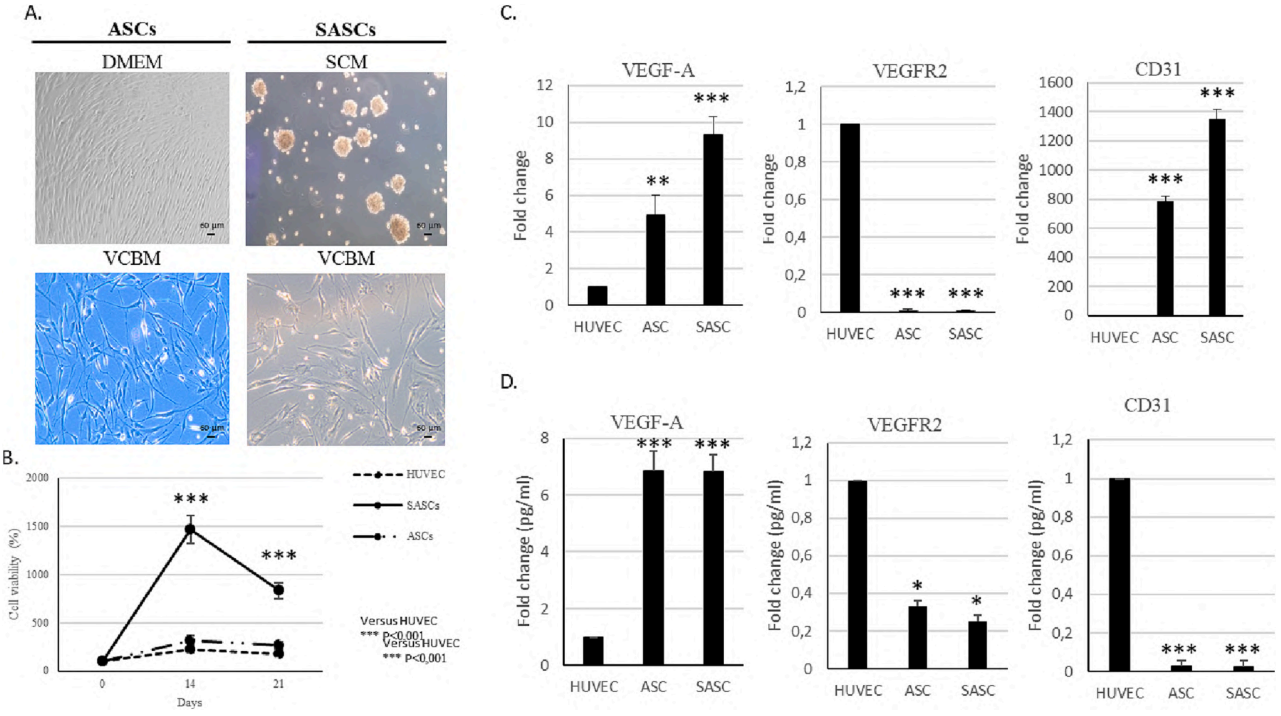

Fig. 1. Cell analysis during endothelial differentiation. A. Morphological analysis of ASCs and SASCs endothelially differentiated cells at 21 days in culture condition. On the top: cells in their own growth medium. On the bottom: cells cultured in VCBM. HUVECs and ASCs were used as control. B. Cell viability analysis of ASCs, SASCs and HUVECs in VCBM at 0, 14 and 21 days. C. Real-time PCR analysis of VEGF-A, VEGFR2 and CD31 mRNAs and D. Angiogenic Luminex assay in SASCs, ASCs and HUVECs at 21 days of endothelial differentiation.

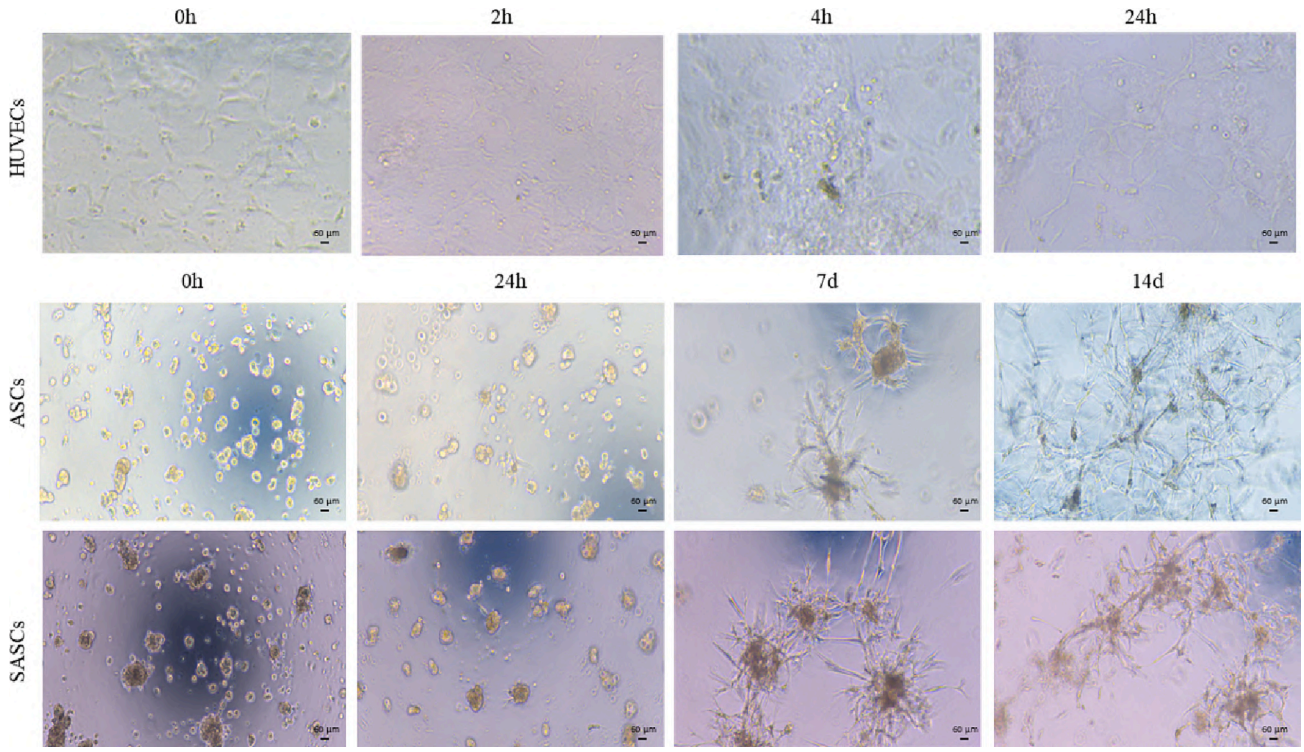

Fig. 2. Analysis of angiogenic networks on ECMatrix™. In the first lane, angiogenic network formation by HUVECs at 0 h, 2 h, 4 h and 24 h. In the second and third lanes, respectively, angiogenic network formation by ASCs and SASCs at 0 h, 24 h, 7d and 14d.

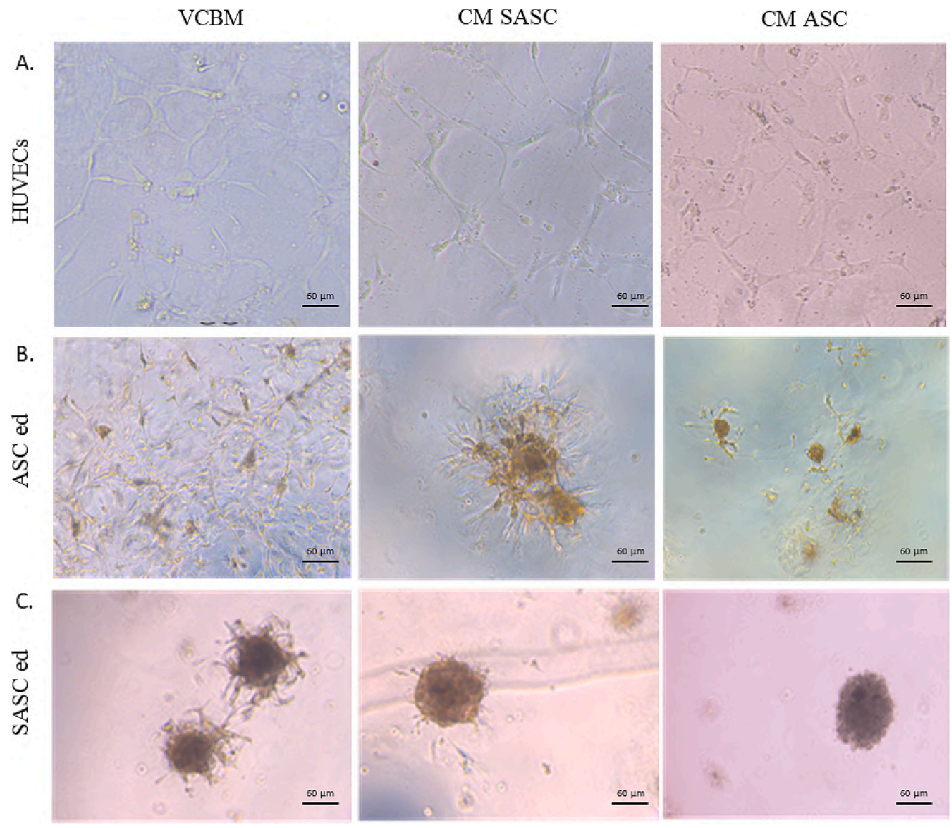

Fig. 3. In vitro assay of angiogenic networks formation on ECMatrix™. A. HUVECs in VCBM, CM SASCs and CM ASCs on ECMatrix™ at 24 h. B. ASCs endothelially differentiated cells in VCBM, CM SASCs and CM ASCs on ECMatrix™ at 7d. C. SASCs endothelially differentiated cells in VCBM, CM SASCs and CM ASCs on ECMatrix™ at 7d.

cultured in their own medium for 28 days and their secretomes were added on HUVECs, ASCs and SASCs endothelially differentiated cells seeded on ECMatrix™. The same analysis was performed with ASC secretomes, as control. The results of the assay performed on HUVECs showed the beginning of the sprouting already after the first hour, leading to the development of a more evoluted angiogenic network at 24 h, but only in the condition of SASCs secretome addition and not of ASCs' one (Fig. 3A). The analysis of angiogenic networks formation through morphological evaluation of ASCs differentiated endothelial cells showed that any sprouting event was happening at 24 h (data not shown), whilst after 7 days of SASCs secretome treatment, detectable ramifications appeared (Fig. 3B). On the contrary, the addition of ASCs CM led to very few and thin ramifications at 7 days of culture (Fig. 3B). The same treatment on SASCs endothelially differentiated cells proved that already after 24 h (data not shown), CM SASCs stimulated the beginning of sprouting phenomena, but after 7 days the cells lost this condition (Fig. 3C).

In addition, we performed immunofluorescence analysis on SASCs and ASCs endothelially differentiated cells seeded on ECMatrixTM at 7d, showing positive cells to VEGF and CD31, two typical angiogenic markers (Fig. 4).

With the aim of investigate the effect of SASCs secretome on proliferation and migration of endothelial cells, wound healing assays respectively on HUVECs, ASCs and SASCs endothelially differentiated cells were performed. The end point was fixed at 4 h for HUVEC but the others cell cultures were analysed until 72 h. The fresh conditioned media (SCM and DMEM) were used as control to analyse if their effect was due to the paracrine factors secreted by the stem cells or by the fresh medium factors. A morphological analysis revealed that CM SASC had any effect on HUVECs proliferation, in comparison to CM ASCs that stimulated a greater proliferation and migration of HUVECs after 4 h in an *in vitro* assay (Fig. 5A).

In ASC endothelially differentiated cells, no effect was visible with conditioned media but an increased wound closure was observed in SCM and DMEM conditions (Fig. 5C). The treatment of SASC endothelially differentiated cells, instead, showed an higher proliferation and migration in CM SASCs condition at 72 h (Fig. 5E). These data were quantified by ImageJ analysis (Fig. 5B,5D,5F).

In addition, to investigate if endothelially pre-differentiated cells were able to migrate, we performed a transwell migration assay. We found that respectively 20% of HUVECs in the control condition, 13% in the CM SASCs and 7% in the CM ASCs migrated. 7% of pre-differentiated SASCs migrated only in the control condition whilst in CM ASCs and SASCs both endothelially differentiated SASCs and ASCs didn't show a relevant migration (Fig. 6).

4. Discussion

Vascularization is an essential process for tissue and organ physiological functions, such as for bone and cartilage maintenance (Chiesa et al., 2020; Apelgren et al., 2021), as well as remodelling and healing of injuries. Vessels help to ensure the cell viability by supplying them with the nutrients they need to survive. In regenerative medicine field, several studies investigated the angiogenic properties of the traditional 2D ASCs for the generation of functional vascularized new tissues (Fischer et al., 2009). The capacity and role of 3D ASCs has not yet been investigated. Today, spheroids of adipose stem cells could represent a revolution in this field. In recent years, they have been extensively characterised both for their capacity to maintain stemness and for their ability to differentiate into adipocytes, chondrocytes and osteoblasts, making them suitable for the use in regeneration of bone and cartilage lesions. In particular, in an *in vivo* calvaria study with SASCs, we demonstrated that the paracrine production of specific factors has led to osteoblastic but also angiogenic regeneration. SASCs actively participated in neoangiogenesis process at the site of the lesion. Furthermore, we showed the expression of miRNAs (miR-126, miR-21, and miR-20a) and mRNAs (VEGF and HIF-1a) typically involved in angiogenesis in SASCs cultures (Di Stefano et al., 2021). From these observations, we first asked whether SASCs themselves are capable of differentiating into endothelial cells but, more importantly, what molecules they produced to stimulate vascular regeneration.

At first, we showed the capacity of SASCs to differentiate towards the endothelial lineage. Interestingly, SASCs grew up much more than HUVECs or 2D ASCs, showing a visible morphological change at 21 days of *in vitro* differentiation. Then, we confirmed the differentiation through mRNA analysis observing an increased expression of typical endothelial genes, such as VEGF-A and CD31. In addition, the hypoxia marker HIF1-α and VEGF receptor 2 (VEGFR2) gene expression were down-regulated. We hypothesized that HIF1-α lower expression could indicate that SASCs differentiated endothelial cells were not in a hypoxic condition. Moreover, several studies proved that VEGFR2 expression is down-regulated by MMP-2 and MMP-14 metalloproteases and that endothelial cells secrete high levels of MMP-2 until they create stable cell–cell contacts and tubular structures. Thus, we hypothesized that we found a VEGFR2 down-regulation probably because our differentiated cells were still at the beginning of their cell–cell contacts and tubular

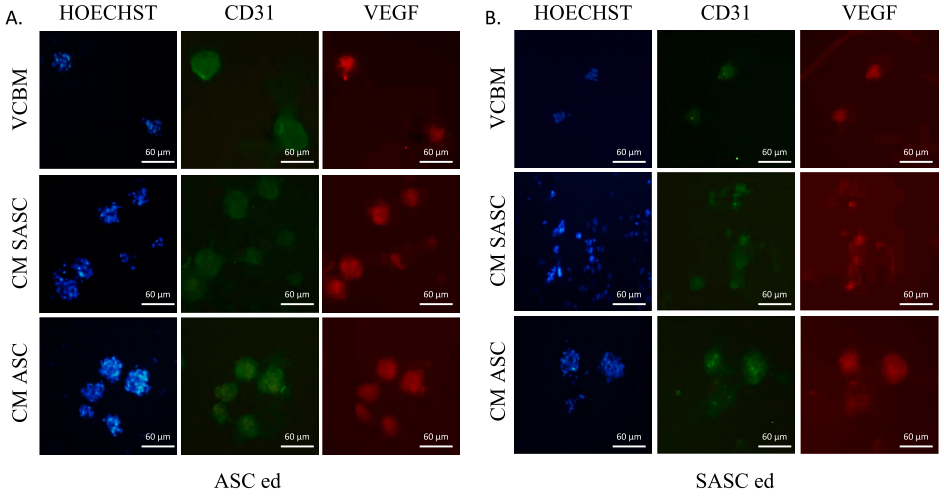

Fig. 4. Analysis of angiogenic markers on ECMatrix™. Hoechst (blue), CD31 (green) and VEGF (red) expression on A. endothelially differentiated ASCs in VCBM, CM SASCs and CM ASCs condition and on B. endothelially differentiated SASCs in VCBM, CM SASCs and CM ASCs condition. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

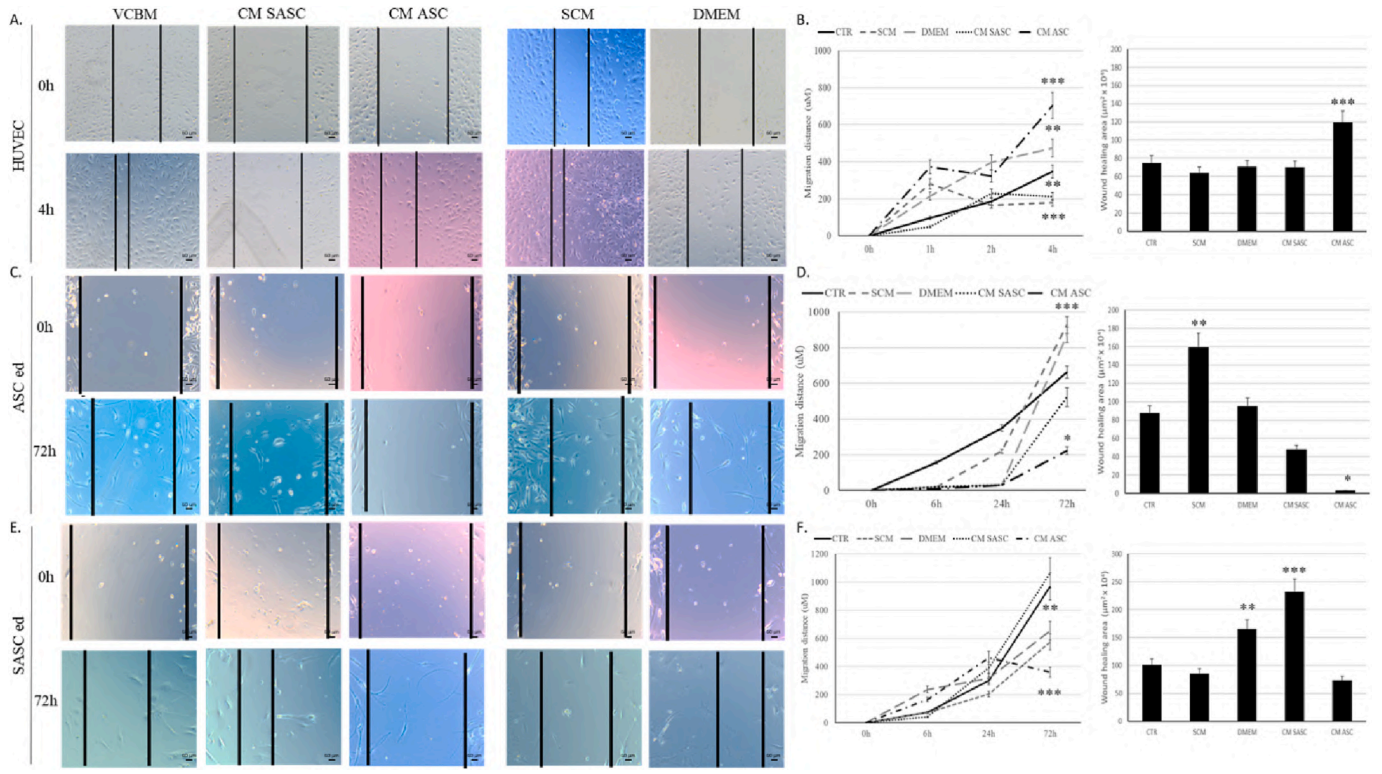

Fig. 5. Wound healing assay. A. observation of the conditioned media on HUVECs and SCM/DMEM as controls. B. Analysis of migration distance and determination of covered area at 4 h. C. Observation of the conditioned media on ASCs endothelially differentiated cells and SCM/DMEM as controls. D. Analysis of migration distance and determination of covered area at 72 h. E. Observation of the conditioned media on SASCs endothelially differentiated cells and SCM/DMEM as controls. F. Analysis of migration distance and determination of covered area at 72 h.

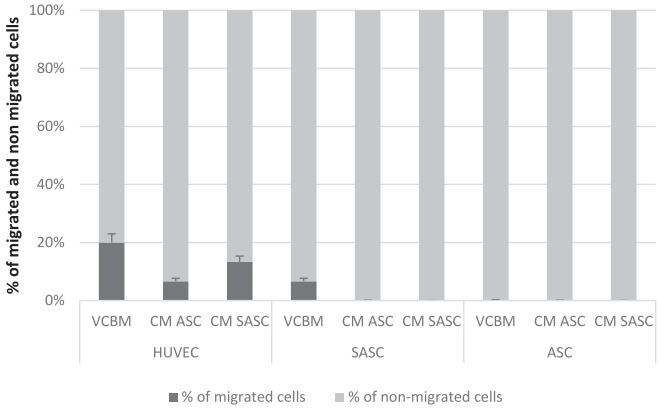

Fig. 6. Transwell migration assay. The percentage of migrated (dark grey) and non-migrated (light grey) cells after 24 h is shown.

structures formation (Rautiainen et al., 2021). VEGFR2 is not the main VEGF-A binding pathway in SASCs differentiated endothelial cells. VEGF-A ligand can bind also the less characterized VEGFR1 receptor (Weddell et al., 2018).

We also investigated the protein concentration of seven typical endothelial proteins, observing VEGF-A up-regulation and CD31 and VEGFR2 down-regulation. ICAM1, VCAM1, ANGPT1 and CD62E did not change. We hypothesized the activation of alternative post-transcriptional regulatory mechanisms that did not translate the high expression of CD31 mRNA into protein, while VEGFR2 maintains a constant trend of low gene and protein expression in both adipose cultures. These aspects find no correspondence in literature. In 2D ASCs, only a study investigated the mRNA or protein expression of VEGFR2 and CD31. In fact, Amerion et al. analysed VEGFR2 expression during the endothelial differentiation of rat ASCs, observing a constant up regulation of its mRNA for three weeks of differentiation and a lack of CD31 expression in EPCs compared to undifferentiated ASCs (Amerion et al., 2018). In addition, a study demonstrated how after 14 days of ASCs culturing in endothelial growth medium enriched with growth factors such as VEGF, hFGF-b (human basic fibroblast growth factor), EGF (epidermal growth factor) and IGF-1 (insulin like growth factor-1), ASCs expressed typical endothelial markers and presented an evident formation of angiogenic networks; ASCs plated onto Matrigel formed capillary-like structures after 24 h (Zhang et al., 2011). Similarly, pre-differentiated ASCs for 10 days with VEGF, FGF or VEGF/FGF media, showed the ability of forming tube structures during 12 h if plated on Matrigel but only in FGF and VEGF/FGF conditions (Khan et al., 2017). Moreover, co-cultures of ASCs and HUVECs respectively in a ratio 1:6 were able to organize themselves to form angiogenic networks, already after three hours (Parshyna et al., 2017). Based on these results, in order to better characterise the endothelial differentiation capacity of SASCs, we seeded cells on ECM Matrix™ showing that they formed angiogenic branches and networks already at 7 days, and that they were maintained up to 14 days.

A final important factor that we analysed in this study was the cell capacity to release factors in the culture medium. Thanks to the secreted factors, cells can exert a paracrine signalling on the other cells by conveying promoting or inhibiting messages. Many studies showed the importance of mesenchymal stem cell paracrine factors in promoting migration and proliferation of endothelial or progenitor endothelial cells (Suga et al., 2014; Kato et al., 2020; Gan et al., 2022) as well as the involvement of mesenchymal stem cell secreted factors in angiogenesis stimulation (Kuchroo et al., 2015; Maacha et al., 2020).

In this regard, we analysed the effects of SASCs released paracrine factors, defined also as secretome, on three endothelial cell lines,

through a wound healing assay. Our preliminary results showed that SASCs released factors increased the proliferation of SASCs endothelially differentiated cells, while ASCs secretome had an opposite effect, stimulating HUVECs and inhibiting ASCs endothelially differentiated cells. Data showed that this effect was probably due to the presence of stimulatory factors released by the cultured SASCs rather than by those already present in the fresh medium. Instead, the transwell migration assay showed migration ability of HUVEC treated in all media conditions and SASC endothelially differentiated cells in VCBM. ASC endothelially differentiated cells weren't able to migrate in all media conditions. In particular, the CM SASC seems to stimulate HUVECs to migrate more if compared to CM ASC, confirming a different effect of 2D and 3D cell released factors.

Further investigations on the possibility of recreate a vascularized tissue using adipose stem cell spheroids are desirable. The future perspectives will be directed to the generation of bio-mould biocompatible scaffolds where spheroids can be seeded for tissue regeneration.

CRediT authorship contribution statement

Anna Barbara Di Stefano: Conceptualization, Methodology, Validation, Formal analysis, Writing – original draft, Visualization. **Francesca Toia:** Writing – review & editing, Supervision, Funding acquisition. **Valentina Urrata:** Methodology, Validation, Investigation, Writing – original draft. **Marco Trapani:** Methodology, Investigation, Writing – original draft. **Luigi Montesano:** . **Emanuele Cammarata:** . **Francesco Moschella:** Writing – review & editing, Supervision, Funding acquisition. **Adriana Cordova:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors are unable or have chosen not to specify which data has been used.

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Valentina Urrata is PhD student of the Doctoral Course of Experimental Oncology and Surgery, Cycle XXXVI, University of Palermo (IT).

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