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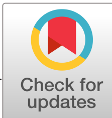

RESEARCH ARTICLE

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Bovine pericardium membrane as new tool for mesenchymal stem cells commitment

Letizia Ferroni[1,2] | Chiara Gardin[1,2] | Gloria Bellin[1,2] | Vincenzo Vindigni[3] | Carmen Mortellaro[4] | Barbara Zavan[1,2]

[1] GVM Care & Research, Maria Cecilia Hospital, Cotignola, Italy

[2] Department of Medical Sciences, University of Ferrara, Ferrara, Italy

[3] Plastic Clinic Surgery, University of Padova, Padova, Italy

[4] Department of Health Sciences, "A. Avogadro" University of Eastern Piedmont, Novara, Italy

Correspondence
Barbara Zavan, GVM Care & Research, Maria Cecilia Hospital, Cotignola 48033 (RA), Italy.
Email: zvnbbr@unife.it

Abstract

Acellular matrices are widespread biomaterials used in surgical practice as tissue reinforcement and anatomical support to favor tissue regeneration. It is clear that a fundamental role in the regeneration of tissue is played by cell–material interaction. In this work, the interaction between a bovine pericardium membrane and human adult stem cells was investigated by microscopy analysis and gene expression analysis. Parallel cell cultures were prepared on the pericardium membrane or tissue culture plate. They were incubated in basal growth medium or in adipogenic differentiation medium to perform experiments on the seventh and the 14th day of culture. Results demonstrated that the membrane allows cell viability, adhesion, and proliferation of human stem cells. During adipogenic commitment on the membrane, the accumulation of cytoplasmatic lipid droplets and the expression of adipogenic gene PPARG, CEBPA, GLUT4, FABP4, and ADIPOQ were detected. Concurrently, a downregulation of mesenchymal stem cell gene CD29, CD90, and CD105 was detected. In basal medium, the adipogenic gene expression was upregulated, whereas the mesenchymal markers were indifferently expressed. These findings suggest that the bovine pericardium membrane is a biocompatible matrix and that their rough surface allows cell adhesion, spreading, and proliferation. The surface morphology activates mechanochemical signals that stimulate the adipogenic commitment of stem cells in basal medium and potentiate their commitment in adipogenic differentiation medium.

KEYWORDS

acellular matrix, adipogenic commitment, bovine pericardium membrane, stem cells, tissue regeneration

1 | INTRODUCTION

Regenerative medicine is the innovative field that, working with tissue engineering, is focus on the research and development of novel strategies directs to find solution in tissue loss problems. One of the main strategies used in tissue engineering is the development of biomaterials, with similar geometry to the extracellular matrix (ECM) of tissue that need to be replaced, providing environmental cues promoting migration and commitment of cells involved in tissue regeneration (Spear, Seruya, Clemens, Teitelbaum, & Nahabedian, 2011; Gardin et al., 2015; Casadei et al., 2012). During the design of novel materials, the researchers focus their attention mainly on chemical or physical mechanisms by which the extracellular environment influences the cells, with the aim to develop scaffolds able to drive cellular response, most of all when they are involved on long-term tissue support. Cell–material interactions are particularly important because the initial contact between cells and the biomaterial can define the success of substitute integration. As part of the

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tissue microenvironment presented to the cells, the choice of an optimal biomaterial and its surface morphology are central for the adhesion, spreading, migration, proliferation and differentiation of cells, protein adsorption, and, finally, for the formation of new tissue. In fact, the biomaterial must provide the physical support at the moment of implantation in order to assist tissue function while new ECM is being deposited (Bressan, Carraro, et al., 2013; Figallo, Flaibani, Zavan, Abatangelo, & Elvassore, 2007; Banik, Riley, Platt, & Brown, 2016). Different types of materials have been developed in order to support tissue regeneration, starting from the completely synthetic one to the natural one. In this last group, acellular matrices have gained increasing use in several clinical application such as on expander-based breast reconstruction (Vardanian et al., 2011, Buck, Heyer, Wayne, Yeldandi, & Kim, 2009). Breast cancer is the most common cancer in women. About 70% of women who undergo a mastectomy will proceed to have an alloplastic reconstruction. Several technical variations exist for postmastectomy breast reconstruction and can be categorized into autologous versus alloplastic, immediate versus delayed, and single versus two staged (Chun et al., 2010; de Blacam et al., 2012). Acellular dermal matrices are the most applied since the 1990s, are immunologically inert, and act as biological scaffolds for reepithelialization, neovascularization, and fibroblast infiltration. Moreover, they facilitate immediate reconstruction, improving implant positioning via better definition of the inframammary and lateral mammary folds, shorting expansion times in tissue-expander reconstructions. They improve capsular contracture rates and mask implant rippling providing an additional layer between the prosthesis and overlying mastectomy skin and also reduce rates of implant/expander migration reducing discomfort during postoperative expansion. However, there are concerns regarding potential increased risks of infection, inflammatory reaction, seroma, masking tumor recurrence, and significant costs (Jansen & Macadam, 2011; Parikh, Pappas-Politis, & Smith, 2012). In this perspective, recent research has focused its attention on a new acellular scaffold made of bovine pericardium able to regenerate tissues optimally (Semprini, Cattin, De Biasio, Cedolini, & Parodi, 2012; Gubitosi et al., 2014). The acellular bovine pericardium membrane (BPM) is characterized by high strength, allows a minimal percentage of infection, is relatively low cost, and can be used directly without a preliminary preparation (Limpert, Desai, Kumpf, Fallucco, & Aridge, 2009). For regenerative medicine purposes, the BPM has been successfully used in cardiovascular field. In fact, BPM is one of the preferred biological materials in cardiovascular and thoracic surgical procedures thanks to its handling and a lower risk of infection and thrombosis compared with synthetic patching materials (Lauterio et al., 2017). It has been effectively used for the development of surgical bioprosthetic heart valves and transcatheter aortic valves (Caballero, Sulejmani, Martin, Pham, & Sun, 2017). Moreover, patches of BPM have been increasingly used in vessel reconstruction such as carotid (Olsen, Mcquinn, & Feliciano, 2016) and aorta (Umashankar, Sabareeswaran, & Shenoy, 2017). In addition, BPM has been used for major venous reconstruction in oncological liver (Jara et al., 2015) and pancreatic surgery (Pulitanó et al., 2013). Furthermore, BPM has been tested as guided bone regeneration membrane to facilitate bone regeneration in critical-sized osseous defects, as well as healing of bone defects around dental implants (Sterio, Katancik, Blanchard, Xenoudi, & Mealey, 2013; Gardin et al., 2015). In the present work, the interactions between BPM and human adipose-derived stem cells (ADSCs) have been studied. Mesenchymal stem cells isolated from adipose tissue have been chosen due to their easy accessibility, known plasticity, and differentiation capability (Zavan et al., 2007; Gardin et al., 2012; Ferroni et al., 2013). The multilineage capacity of ADSCs offers the potential to repair, maintain, or enhance various tissues. In fact, ADSCs are able to differentiate into cells of the mesodermal lineage, including osteoblasts (Paduano, Marrelli, Amantea, et al., 2017), chondrocytes (Pak, Lee, Kartolo, & Lee, 2016), or adipocytes (Casadei et al., 2012). In addition, such cells can produce chemokines that are useful to facilitate the homing of endogenous stem cells in the site of defect. Secreting trophic factors, ADSCs promote proliferation and differentiation of local progenitor cells, as well as the activation of regenerative and reparative processes (Paduano, Marrelli, Amantea, et al., 2017). Moreover, ADSCs induce or increase neovascularization and have immunomodulatory and antioxidant properties (Atashi, Modarressi, & Pepper, 2015).

In light of these considerations, we have performed in vitro experiments to test BPM as a scaffold for the regeneration of adipose tissue in order to identify a biomaterial useful for breast reconstruction. To achieve this aim, we have seeded ADSCs on BPM to test cell adhesion, spreading, morphology, proliferation, and adipogenic commitment.

2 | MATERIALS AND METHODS

2.1 | Cell isolation and culture

Human adult stem cells were isolated from human abdominal fat of healthy donors (age: 21–36) undergoing cosmetic surgery procedures, following the guidelines of the University of Padova's Plastic Surgery Clinic. As already described elsewhere (Ferroni et al., 2018; Bressan et al., 2015), the adipose tissues were digested with 0.075% collagenase Type II (Sigma-Aldrich, Saint Louis, Missouri, USA) in Hanks' balanced salts solution (Euroclone, Milano, Italy) for 2 hr at room temperature. Cells from the stromal–vascular fraction were rinsed with phosphate-buffered saline (EuroClone). Red blood cells were removed by a step in red blood cells lysis buffer (Sigma-Aldrich) run for 10 min at room temperature. The resulting viable human ADSCs were seeded in basal medium (BM) consisting of Dulbecco's modified Eagle's medium (Euroclone) supplemented with 10% fetal bovine serum (EuroClone), and 1% penicillin/streptomycin (EuroClone). ADSCs were maintained at 37°C and 5% CO_2, and culture medium was changed twice a week. Cells were characterized by fluorescence-activated cell sorting analysis as it has been done by Gardin et al. (2018).

2.2 | Biomaterial and three-dimensional culture

A sterile natural membrane deriving from bovine pericardium has been employed to perform three-dimensional (3D) cultures. The membrane is a non-cross-linked acellular collagen matrix that has retained 92% of native Type I collagen (Tutomesh®, Tutogen Medical GmbH, Germany). Membrane squares (1 × 1 cm) were positioned on the bottom of 24-well plates with the rough side facing upwards, and 5×10^4 human viable cells have been seeded on each of them. The viable cells were counted by the trypan blue exclusion test (Sivolella et al., 2015). Briefly, ADSCs were detached with a solution of 0.25% trypsin and 0.02% EDTA (EuroClone) and then pelleted. The cell was suspended in 1 ml of BM; 20 μl of cell suspension was added to 80 μl of trypan blue (EuroClone), and cells were counted using a Burker's chamber (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany). After seeding, 3D cultures have been maintained in BM or in adipose differentiation medium (ADM) consisting in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 10 μg/ml insulin (Sigma-Aldrich), 0.5 mM isobutylmethylxanthine (Sigma-Aldrich), 0.1 mM indomethacin (Sigma-Aldrich), and 1 μM dexamethasone (Sigma-Aldrich); 5×10^4 ADSCs seeded onto 24-well tissue culture plate (TCP) represented the control condition. All cultures have been incubated at 37°C and 5% CO_2 up to 14 days, and culture medium was changed twice a week (Ferroni et al., 2016).

2.3 | Cell viability assay

The viability of ADSCs by the methyl thiazolyl-tetrazolium (MTT) assay was detected (Gardin et al., 2014; Bressan et al., 2013). The test is based on mitochondria viability, that is, only functional mitochondria can oxidize an MTT solution, giving a typical blue-violet product. After harvesting the culture medium, the cell cultures were incubated for 3 hr at 37°C in 1 ml of 0.5 mg/ml MTT solution prepared in phosphate-buffered saline solution. After removal of the MTT solution, 0.5 ml of 10% dimethyl sulfoxide in isopropanol was added for 30 min at 37°C. For each sample, absorbance values at 570 nm in duplicate on 200 μl aliquots were recorded using a multilabel plate reader (Victor 3 Perkin Elmer). MTT solution was added to the BPM without cells to perform the blank. All samples were examined after 7 and 14 days of culture.

2.4 | Morphology analyses

The adhesion and proliferation of ADSCs onto BPM by scanning electron microscopy (SEM) and hematoxylin and eosin (H&E) staining have been observed. SEM samples were fixed with 2.5% glutaraldehyde (Sigma-Aldrich) in 0.1 M of cacodylate buffer (Sigma-Aldrich), dehydrated in ethanol, critical point dried, and then gold-palladium coated (Brunello et al., 2018). All images were obtained using a JEOL 6360LV SEM microscope (JEOL, Tokyo, Japan) at the Centro di Analisi e Servizi Per la Certificazione (CEASC, University of Padova, Padova, Italy). To perform H&E staining, 3D cultures were fixed in 4% formaldehyde pH 7 (Kaltek, Padova, Italy) and then embedded in paraffin (Plastiwax, Kaltek); 5-μm-thick sections were stained with the nuclear dye, hematoxylin (Sigma-Aldrich), and the counterstain, eosin (Sigma-Aldrich; Ferroni et al., 2015).

2.5 | Oil Red O staining and quantification

An Oil Red O (ORO; Sigma-Aldrich) stock solution was made dissolving the powder in isopropanol at the concentration of 3.5 mg/ml, and then an ORO working solution was prepared adding three parts of ORO stock solution to two parts of distilled water. Cells were stained with 0.5 ml of fresh ORO working solution for 15 min at room temperature. After four washes with distilled water, phase-contrast images were taken, and ORO staining was extracted with 0.25 ml 100% isopropanol. For each sample, OD values at 490 nm were measured by a multilabel plate reader (Victor 3 Perkin Elmer).

2.6 | Real-time polymerase chain reaction

Total RNA was extracted with Total RNA Purification Plus Kit (Norgen Biotek Corporation, Ontario, Canada), according to the manufacture procedures; 500 ng of total RNA of each sample was reverse transcribed with SensiFAST™ cDNA Synthesis Kit (Bioline GmbH, Germany) in the LifePro Thermal Cycler (Bioer Technology, China) following the manufacture conditions: annealing at 25°C for 10 min, reverse transcription at 42°C for 45 min, and inactivation at 85°C for 5 min. The resultant cDNA samples were stored at −20°C until the next use. Real-time polymerase chain reaction was performed using SensiFAST™ SYBR No-ROX mix (Bioline GmbH) with 400 nM of primers on a Rotor-Gene 3000 (Corbett Research, Sydney, Australia). Thermal cycling conditions were as follows: denaturation at 95°C for 2 min; followed by 40 cycles of denaturation at 95°C for 5 s; annealing at 60°C for 10 s; and elongation at 72°C for 20 s. Data analysis was performed using the widely adopted 2ΔΔCt method (Pfaffl, 2001). Ct values of target genes were normalized to that of housekeeping gene (TFRC: transferrin receptor 1). Results were reported as fold regulation of target genes in 3D culture compared with ADSCs on TCP. Human primers were selected by Primer 3 software (Table 1).

2.7 | Statistical analyses

One-way analysis of variance for data analyses was used. The repeated measures analysis of variance with a post hoc analysis was performed using Bonferroni's correction for multiple comparisons, and *t* tests were used to ascertain significant differences ($p < .05$). Repeatability was calculated as the standard deviation of the difference between measurements. All testing was performed in SPSS 16.0 software (SPSS Inc., Chicago, IL, USA; license of the University of Padova, Padova, Italy).

TABLE 1 Human primer sequences

Gene	Sequences (5′-3′)	Amplicon
ADIPOQ	GTTGTGTGCCTGTTTCTGACC GCATCTATCATCCACTCTCCTATTTCTG	153
CD29	TGCAACAGCTCTCACCTACG GTGAAACAAGATGGGCAACTCA	100
CD90	CAGCATTCTCAGCCACAACC CCTCATCCTTTACCTCCTTCTCCA	158
CD105	GGTGACGGTGAAGGTGGAA TCTGCATGTTGTGGTTGGC	113
CEBPA	GGACTTGGTGCGTCTAAGATGAG GCATTGGAGCGGTGAGTTTG	147
FABP4	TGACCTGGACTGAAGTTCGC AAGCACAATGAATACATCATTACATCACC	193
GLUT4	CCAGTATGTTGCGGAGGCTA TCAAGTTCTGTGCTGGGTTTCA	189
PPARG	CAGGAGATCACAGAGTATGCCAA TCCCTTGTCATGAAGCCTTGG	173
TFRC	TGTTTGTCATAGGGCAGTTGGAA ACACCCGAACCAGGAATCTC	222

Abbreviations: ADIPOQ, adiponectin; CD29, integrin subunit beta 1; CD90, Thy-1 cell surface antigen; CD105, endoglin; CEBPA, CCAAT enhancer binding protein alpha; FABP4, fatty acid binding protein 4; GLUT4, solute carrier family 2 (facilitated glucose transporter) member 4; PPARG, peroxisome proliferator activated receptor gamma; TFRC, transferrin receptor 1.

3 | RESULTS

3.1 | Cell seeding and 3D culture in BM

Human ADSCs were isolated from abdominal adipose tissue of healthy donors and then amplified in BM up to passage 3. After harvesting with trypsin, 5×10^4 adult stem cells were seeded on the rough surface of BPM (1 × 1 cm square) or directly on TCP (control condition). Cell survival, adhesion, and proliferation have been analyzed by means of SEM and H&E staining, and MTT assay after 7 and 14 days of culture (Figure 1). On the seventh day, ADSCs seeded on the membrane appeared large, flattened and with a spindle-shaped morphology (Figure 1a). The cell adhesion on the membrane was also visible by H&E staining that confirmed the presence of a continuous layer of cells on the membrane surface (Figure 1b). However, the MTT assay showed lower cell growth compared with the monolayer culture of about 20% (Figure 1e). On the 14th day, the SEM acquisition showed an increase in cell number. They were not arranged neatly next to each other but appear slightly overlapping and with a cuboidal shape (Figure 1c). Figure 1e shows a cross section of the 3D culture stained with H&E after 14 days of culture: The proliferation has led the ADSCs to occupy the entire surface while the membrane appeared more lax than at 7 days. Even at 14 days, the MTT assay showed lower cell growth compared with monolayer culture (Figure 1c). However, by comparing cell growth on the membrane over time, a significant increase ($p < .001$) on 14th day compared with seventh day was observed.

3.2 | Adipogenic commitment of 3D culture

The interactions of ADSCs with the BPM during adipogenic differentiation have been tested. In particular, 5×10^4 ADSCs seeded on BPM or TCP (control condition) in ADM have been incubated up to 14 days. After 7 days of incubation in ADM, the SEM analysis showed less ADSCs adherent to the membrane than those grown in BM. The cells showed a cuboidal morphology and clung to the membrane by filipods (Figure 2a). H&E staining also demonstrated the presence of cells on the membrane surface (Figure 2b). The MTT assay showed that cell

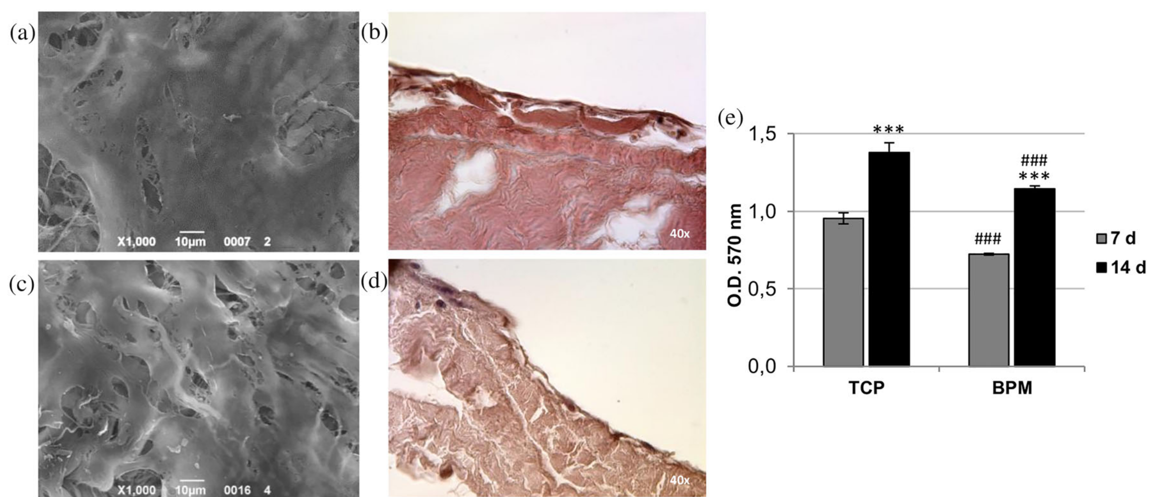

FIGURE 1 Human adipose-derived stem cells adhesion and proliferation on bovine pericardium membrane in basal medium. (a) Scanning electron microscopy after 7 days of culture, (b) hematoxylin and eosin after 7 days of culture, (c) scanning electron microscopy after 14 days of culture, (d) hematoxylin and eosin of adipose-derived stem cells after 14 days, and (e) MTT assay of tissue culture plate and bovine pericardium membrane after 7 and 14 days; all values are presented as averages of four measures ± standard deviation (7 vs. 14 days: $^{***}p < .001$; tissue culture plate vs. bovine pericardium membrane: $^{###}p < .001$) [Colour figure can be viewed at wileyonlinelibrary.com]

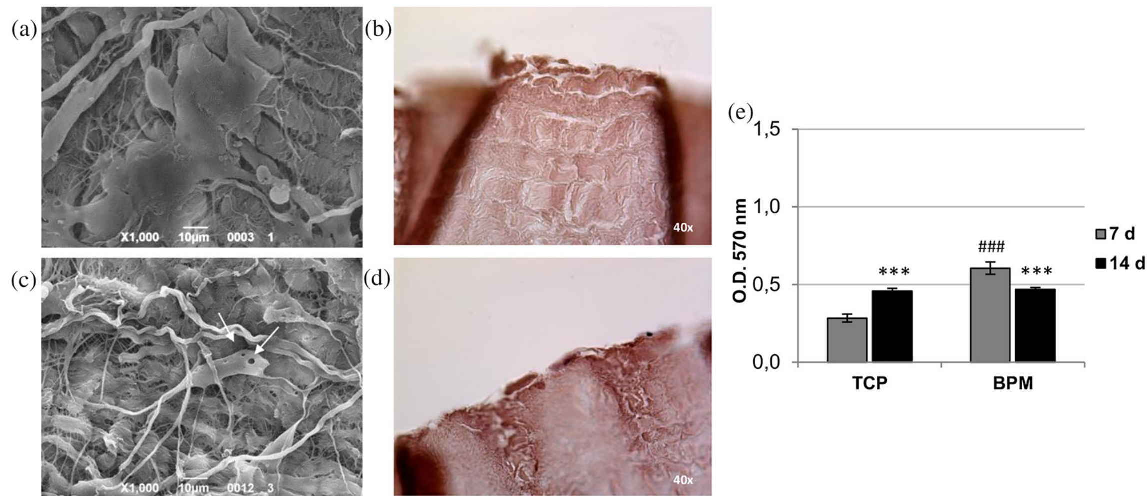

FIGURE 2 Human adipose-derived stem cells adhesion and proliferation on bovine pericardium membrane (BPM) in adipose differentiation medium. (a) Scanning electron microscopy after 7 days of culture, (b) hematoxylin and eosin after 7 days of culture, (c) scanning electron microscopy after 14 days of culture, (d) hematoxylin and eosin of adipose-derived stem cells after 14 days, and (e) MTT assay of tissue culture plate and bovine pericardium membrane after 7 and 14 days; all values are presented as averages of four measures ± standard deviation (7 vs. 14 days: $^{***}p$ < .001; tissue culture plate vs. bovine pericardium membrane: $^{###}p$ < .001) [Colour figure can be viewed at wileyonlinelibrary.com]

viability was significantly (p < .001) higher on the membrane compared with TCP (Figure 2e). On the 14th day, the cells appeared anchored to the membrane with numerous filipods and showed intracellular lipid drops (white arrows, Figure 2c). H&E staining at 14 days is in agreement with the SEM observations (Figure 2d). The MTT assay on the 14th day showed less cell viability than on the seventh day, but equal to cell viability on TCP (Figure 2e).

The presence of cytoplasmic lipid droplets is an indicator of the adipose phenotype induction, and the ORO dye is commonly used to stain them. ORO staining on cells cultured on BPM or on TCP and incubated for 7 or 14 days in ADM or in BM were performed (Figure 3). ORO staining of the 3D cultures in ADM (Figure 3a) revealed a progressive accumulation of intracellular lipid drops over time: a significant increase (p < .01) in bound dye quantification was recorded after 14 days of culture in ADM (Figure 3b). On the contrary, incubation of 3D cultures in BM did not determine the appearance of intercellular lipid drops (Figure 3c), and the quantification of the bound dye does not show significant variations between 7 and 14 days (Figure 3d). Control cells seeded on TCP showed the same behavior: In ADM, they presented a strong affinity for the lipid dye (Figure 3 e), recording a significant (p < .001) increase in bound dye quantification after 14 days of incubation (Figure 3f). Conversely, in BM, the lipid accumulations have not been detected (Figure 3g,h).

To access adipogenic commitment, we analyzed also the expression profile of genes involved in adipogenic differentiation, such as adiponectin (ADIPOQ), CCAAT enhancer binding protein alpha (CEBPA), fatty acid binding protein 4 (FABP4), solute carrier family 2 (facilitated glucose transporter) member 4 (GLUT4), and peroxisome proliferator activated receptor gamma (PPARG) by real-time polymerase chain reaction (Figure 4). The nuclear receptor transcription factor PPARG is the master regulator that control adipogenic differentiation. It cooperates with the transcription factor CEBPA during terminal differentiation by inducing cell growth arrest, and transcriptionally regulating the expression of adipocyte specific genes FABP4, GLUT4, and ADIPOQ (Chiarella et al., 2018). After 7 days of incubation in ADM, the expression of adipogenic genes was significantly higher on the membrane compared with TCP (Figure 4a). On the contrary, on the 14th day, significant differences between 3D culture and monolayer culture were not recorded (Figure 4b). Surprisingly in BM, the expression of all adipogenic markers was significant greater on the membrane compared with TCP both at 7 (Figure 4c) and at 14 days (Figure 4d).

Concurrently, the gene expression of mesenchymal stem cell markers, such as integrin subunit beta 1 (CD29), Thy-1 cell surface antigen (CD90), and endoglin (CD105) was analyzed (Figure 5). Adult stem cells seeded on the membrane and incubated in ADM showed a significant downregulation in the gene expression of the analyzed markers, both on the seventh (Figure 5a) and 14th days (Figure 5b). Even on TCP, these genes have undergone a significant downregulation when incubated in ADM, albeit to a lesser extent (Figure 5c,d). By comparing the two culture surfaces in the same culture condition, all the genes were indifferently expressed in BM both at 7 and at 14 days (Figure 5e,f, respectively), and the same profile occurred after 7 days in ADM (Figure 5g). Instead, a significant downregulation in CD29 and CD105 gene expression was recorded on the membrane after 14 days in ADM (Figure 5h).

4 | DISCUSSION

The use of acellular matrix is widespread in surgical practice, especially when autograft or acellular dermal allograft are not practicable. Acellular matrices are commonly used for cervical, breast, and abdominal wall reconstruction, where they work as tissue reinforcement and

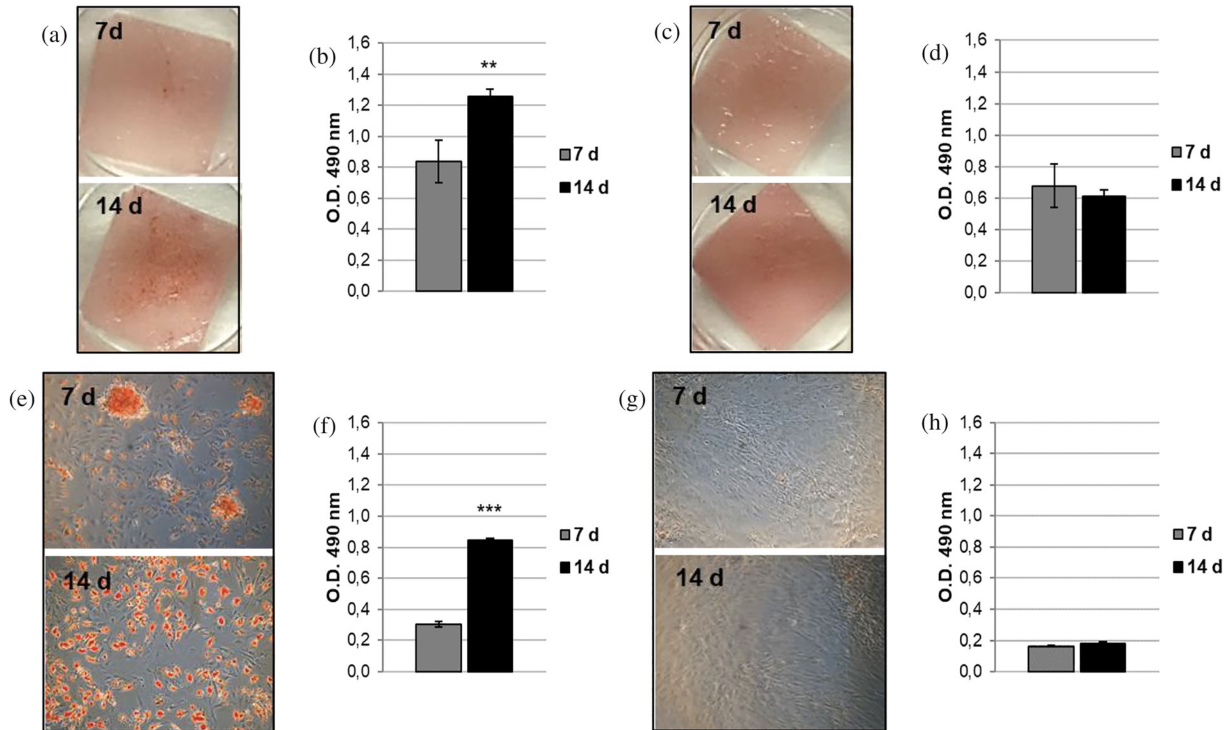

FIGURE 3 Oil Red O staining and quantification on bovine pericardium membrane or on tissue culture plate after 7 and 14 days. (a) Staining on membrane in adipose differentiation medium, (b) quantification on membrane in adipose differentiation medium, (c) staining on membrane in basal medium, (d) quantification on membrane in basal medium, (e) staining on tissue culture plate in adipose differentiation medium, (f) quantification on tissue culture plate in adipose differentiation medium, (g) staining on tissue culture plate in basal medium, and (h) quantification on tissue culture plate in basal medium. All values are presented as averages of three measures ± standard deviation ($^{***}p$ < .001) [Colour figure can be viewed at wileyonlinelibrary.com]

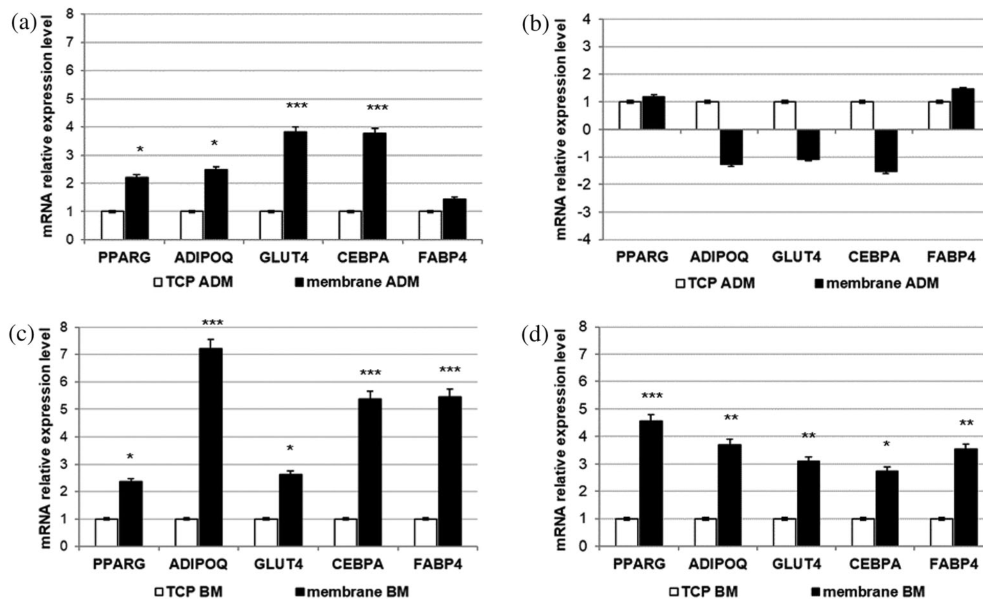

FIGURE 4 Gene expression profile of adipogenic markers (a) in adipose differentiation medium on the seventh day, (b) in adipose differentiation medium on the 14th day, (c) in basal medium on the seventh day, and (d) in basal medium on the 14th day. Data are reported as fold regulation of target genes in bovine pericardium membrane compared with tissue culture plate ($^{*}p$ < .05, $^{**}p$ < .01, and $^{***}p$ < .001). ADIPOQ, adiponectin; CEBPA, CCAAT enhancer binding protein alpha; FABP4, fatty acid binding protein 4; GLUT4, solute carrier family 2 (facilitated glucose transporter) member 4; PPARG, peroxisome proliferator activated receptor gamma

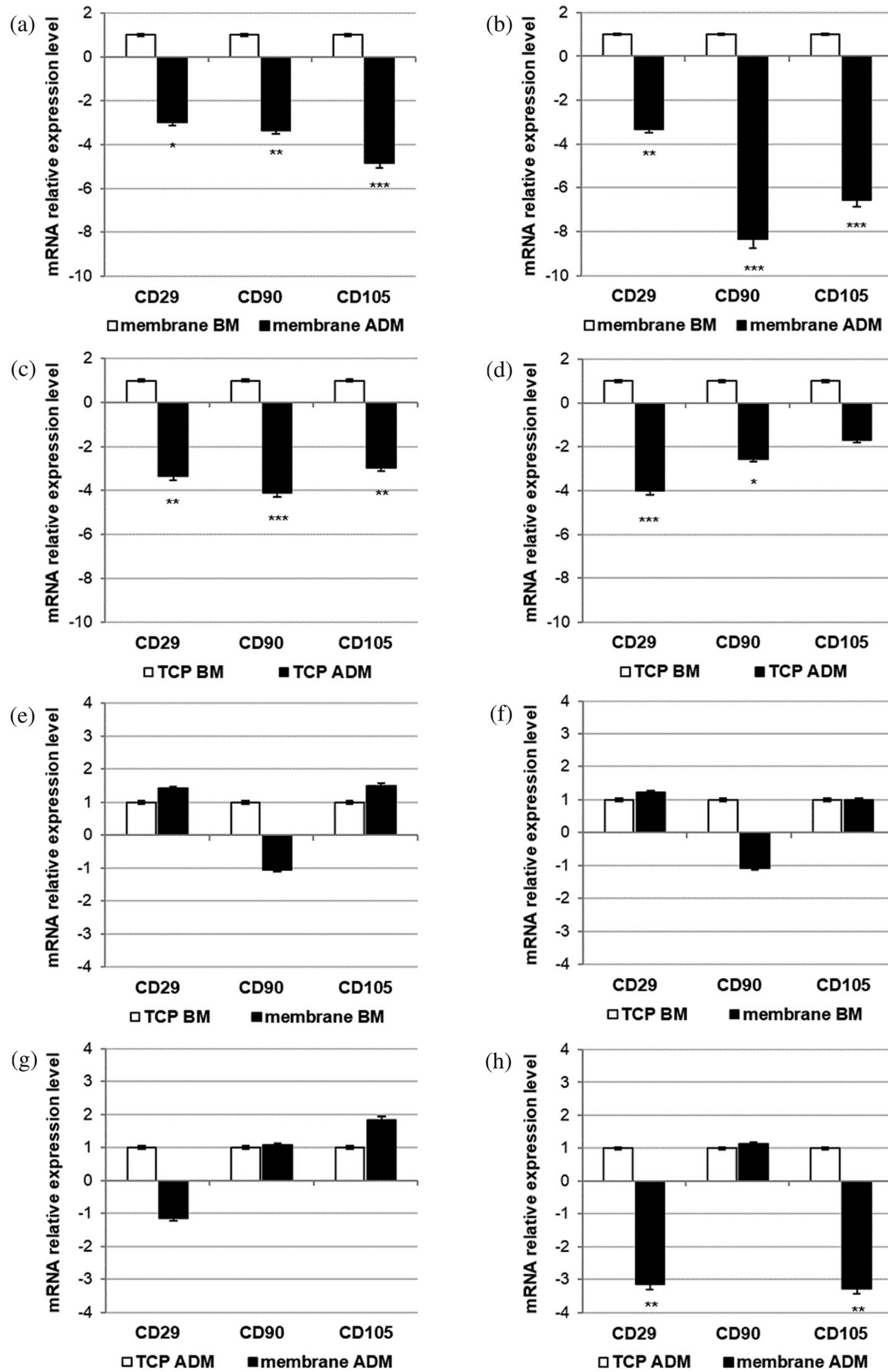

FIGURE 5 Gene expression profile of mesenchymal stem cell markers (a) on bovine pericardium membrane at 7 days, (b) on bovine pericardium membrane at 14 days, (c) on tissue culture plate at 7 days, (d) on tissue culture plate at 14 days; data are reported as fold regulation of target genes in adipose differentiation medium compared with basal medium (e) in basal medium after 7 days, (f) in basal medium after 14 days, (g) in adipose differentiation medium after 7 days, (h) in adipose differentiation medium after 14 days. Data are reported as fold regulation of target genes on membrane compared with tissue culture plate ($^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$). CD29, integrin subunit beta 1; CD90, Thy-1 cell surface antigen; CD105, endoglin

anatomical support. Among the acellular membranes of natural origin, the acellular bovine pericardium is relatively low cost, high strength, and has a minimal infection rate. Therefore, it does not require any preparation time and can be used directly out of the packaging (Limpert et al., 2009). The purpose of this work was to study the interaction between a BPM and human mesenchymal stem cells by in vitro experiments. The morphology, adhesion, proliferation, and cytophysiology of ADSCs were evaluated after incubation both in BM and in ADM. The microscopy analysis revealed that the rough side of BPM allows adhesion, spreading, and proliferation of human stem

cells. Indeed, cells were able to adhere to matrix fibers both in poor and in high confluence, as demonstrated by SEM and H&E staining of the 3D culture incubated in BM. Despite during adipogenesis cell morphology convert from a fibroblastic to a more spherical shape, microscopy analysis demonstrated that cells remained anchored to the membrane until the end of the adipogenic differentiation process. As previously demonstrated, the development of the adipose phenotype involves a reduction in cell proliferation due to the activation of the transcription factors PPARG and CEBPA (Ferroni et al., 2018). In agreement, MTT assay showed a reduction in cell viability of cells cultured on the membrane during the adipogenic differentiation, although it was higher compared with the one on TCP. This was probably due to the roughness of matrix surface that allows a better anchoring of spherical cells compared with TCP. Furthermore, the membrane surface promoted the accumulation of cytoplasmic lipid droplets, as demonstrate by the great amount of bound ORO dye recorded during the adipogenic differentiation, mostly on 14th day. The genes expression analysis of the transcription factors PPARG and CEBPA and of genes regulated by them FABP4, GLUT4, and ADIPOQ showed an expression synchronization with the development of the adipose phenotype. By comparing gene expression in 3D culture with that in monolayer culture, the cited genes showed high mRNA levels on the membrane after 7 days and equal levels after 14 days. In the meantime, the appearance of cytoplasmic lipid drops was minimal on the seventh day and maximum after 14 days. These observations suggest that the 3D culture favors the early expression of adipogenic genes and supports high level of gene expression until the development of adipose phenotype. Surprisingly, the membrane supported the expression of adipocyte markers not only when cells were grown in ADM but also in BM. By comparing the gene expression on the two surfaces in the BM, the expression of the adipogenic markers in 3D culture was higher than that in monolayer culture both at 7 and at 14 days.

Moreover, the expression of the genes CD29, CD90, and CD105, related to mesenchymal phenotype, has undergone a larger downregulation in cells cultured in ADM, especially if grown on BMP. On the 14th day, when the expression of adipogenic markers and the accumulation of lipid drops are maximum on BMP, a large downregulation in the expression of CD29 and CD105 was also observed. Instead, by comparing the two surfaces in the BM, the mesenchymal markers were indifferently expressed, both at 7 and at 14 days. The ADSCs are a nonhomogeneous population expressing several surface markers including CD29, CD44, CD63, CD73, CD90, and CD105; meanwhile, they are negative for hematopoietic antigens such as CD14, CD31, and CD45 (Paduano, Marrelli, Palmieri, & Tatullo, 2016). After culturing and passaging, the expression of some surface markers can change; in particular, the expression level of CD29, CD90, and CD105 increases with passaging due to the selection of a cell population with more homogenous cell surface markers (Tsuji, Rubin, & Marra, 2014). In our experimental setting, the observed downregulation of these genes could be related to the selection of a population of cells with adipocyte phenotype.

These findings suggest that the surface morphology of BPM influences the expression of adipocyte genes, probably through a mechanic manner, but the development of the adipose phenotype needs stimuli from the external microenvironment, such as the factors present in the ADM. This is in agreement with what has been shown in other studies in which adult stem cells of different sources were conditioned by the interaction with the ECM. In fact, cell–matrix interactions induce signaling essential for cell behavior, making ECM composition a crucial factor in stem cell proliferation and lineage-specific differentiation (Paduano, Marrelli, Alom, et al., 2017). For example, matrix derived from decellularized skeletal muscles promote myogenesis when transplanted in animal models and have the potential to reconstruct skeletal muscle tissue (Aulino et al., 2015). Hydrogels derived from decellularized and demineralized bovine bone ECM possess distinct mechanical and biological properties able to stimulate osteogenic differentiation of human stem cells (Paduano, Marrelli, Alom, et al., 2017). Furthermore, the commitment of stem cells depends on the rigidity of the matrix: the most rigid substrates promote osteogenic differentiation, on the contrary, the less rigid the adipocyte (Ivanovska et al., 2017).

In conclusion, the interactions of human mesenchymal stem cells with an acellular membrane of bovine pericardium have been investigated. In vitro experiments have demonstrated the biocompatibility of the substrate: The rough surface of BPM allows the adhesion, spreading, and proliferation of ADSCs. Furthermore, BPM is a mechanochemical signaling activator that regulates the fate of ADSCs. In fact, the membrane stimulated the adipogenic commitment of ADSCs in BM and potentiated their commitment in adipogenic differentiation medium. In light of these observations, the BPM could be used for regenerative medicine purposes when adipogenic induction is needed to replace adipose tissue, such as after breast cancer resection or even plastic transplantation.

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CONFLICT OF INTEREST

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ORCID

Barbara Zavan https://orcid.org/0000-0002-4779-4456

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