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## The biomimetic surface topography of *Rubus fruticosus* leaves stimulate the induction of osteogenic differentiation of rBMSCs

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### Abstract

The interaction between cells and biomaterials is essential for the success of biomedical applications in which the implantation of biomaterials in the human body is necessary. It has been demonstrated that material's chemical, mechanical, and structural properties can influence cell behaviour. The surface topography of biomaterials is a physical property that can have a major role in mediating cell–material interactions. This interaction can lead to different cell responses regarding cell motility, proliferation, migration, and even differentiation. The combination of biomaterials with mesenchymal stem cells (MSCs) for bone regeneration is a promising strategy to avoid the need for autologous transplant of bone. Surface topography was also associated with the capacity to control MSCs differentiation. Most of the topographies studied so far involve machine-generated surface topographies. Herein, our strategy differentiates from the above mentioned since we selected natural surface topographies that can modulate cell functions for regenerative medicine strategies. *Rubus fruticosus* leaf was the selected topography to be replicated in polycaprolactone (PCL) membranes through polydimethylsiloxane moulding and using soft lithography. Afterwards, rat bone marrow stem cells (rBMSCs) were seeded at the surface of the imprinted PCL membranes to characterize the bioactive potential of our biomimetic surface topography to drive rBMSCs differentiation into the osteogenic lineage. The selected surface topography in combination with the osteogenic inductive medium reveals having a synergistic effect promoting osteogenic differentiation.

## 1. Introduction

Mesenchymal stem cells (MSCs) have been proposed to improve advanced therapies applications. MSCs show differential growth rate, proliferation, morphology, and differentiation potential depending on their source of origin and biophysical cues used during its culture [1–3]. These cells have been isolated from different species, such as human, mouse and rat and from different sources such as bone marrow, adipose tissue, placenta or cord blood [1, 4, 5]. Moreover, MSCs present multilineage differentiation potential, such as the differentiation into osteogenic lineage,

and low immunogenic reaction by allogeneic hosts that makes them a promising cell source for bone cell-therapy approaches [6, 7]. Usually, the differentiation of MSCs *in vitro* is promoted using a set of media formulations that contain several components to stimulate the differentiation of MSCs into desired lineages. However, the use of these formulations can result on off-target effects [8]. The stimulation of MSCs undergo osteogenic differentiation usually resorts to a cocktail of dexamethasone, ascorbic acid and glycerophosphate or by the use of high doses of soluble factors, such as bone morphogenetic proteins [9]. *In vivo* the extracellular matrix (ECM) is dynamic

and provides both physical and functional cues that cells perceive and respond to through the process of mechanotransduction [10]. Traditional approaches to induce e.g. osteogenesis of MSCs, which are typically carried out using standard tissue-culture plates, do not recapitulate any physical properties of ECM.

Bone regenerative medicine is an important area of research since this tissue is among the most commonly transplanted. Indeed, it is estimated that 2.2 million bone transplants are performed annually worldwide [11]. Ideally, to promote successful bone regeneration, autologous bone grafts are preferred. However, the availability of donor tissue for autologous grafting is limited and there is significant patient morbidity at the donor site [12, 13]. Subsequently, other strategies for bone replacement are urgently required, and herein regenerative medicine can provide excellent alternatives using biocompatible and biomimetic materials [14, 15]. The use of suitable biomaterials to promote bone formation and integration requires a positive interaction with cells. These biomaterials should promote a functional cell–material interaction to become appropriate substitutes for damaged bone avoiding the use of autologous bone grafts [16]. Nevertheless, critical limitations are still associated with current engineered bone grafts, namely the insufficient induction of osteogenesis by the implanted scaffolds usually caused by the loss of osteogenic capacity of the cells [15].

Currently approaches in bone regenerative medicine are based combining the potential of MSCs with biomaterials. The interaction of these cells with the selected material is preponderant in the success of the strategy, where both biochemical and physical properties of the biomaterial play a key role for its success [17]. Regarding the physical properties of the biomaterials, surface topography came up as a first interaction of the interface cells–material. The topography of biomaterials plays an important role in regulating stem cell fate. The substrate's topography has been reported to be able to regulate the differentiation of stem cells in different lineages. Notable studies report on the induction of differentiation of MSCs on osteogenic [16, 18, 19]. Briefly, MSCs start to form lamellipodia and filopodia selecting the ideal way to adhere to the substrate. During the adhesion process, focal adhesions are established. Through mechanotransduction processes, the formation of focal adhesions can regulate several signalling pathways induced by cytoskeleton remodelling. Many different signalling pathways can be activated which can be directly related to the topography-induced gene expression [20]. The specific topographic features sensed by focal adhesions lead to cell differentiation due to the activation of those signalling pathways that will result in the induction of the differentiation

into a certain lineage. FAK [21], ERK/MAPK [22], Rho-ROCK [23, 24], or Wnt [25–27] signalling pathways are some examples of the signalling pathways that reveals to be preponderant in determining MSCs topography-induced osteogenic differentiation.

A greatest challenge in regenerative medicine it is to tune the properties of biomaterials to mimic the natural properties of native extracellular matrix. Regarding to the topographical surface patterns to direct differentiation of MSCs, the majority of topographical designs studied to date have been machine generated. Grooves, ridges, pits and pillars have been the most studied topographical cues that have been studied [28]. Several studies over a decade ago have demonstrated that a regular nanotopographical arrangement with slight offset can increase focal adhesion formation and modulate the osteogenic differentiation. Using nature as a template, the use of biomimetic approaches might offer additional advantages over the topographical designed approaches and provide more efficient surface patterns for regenerative medicine applications [29]. In the literature it is possible to find studies that report on the use of biomimetic surface topographies to study their influence on osteogenic differentiation. As example, negative replicas of the surface of rose petals, parsley leaf, and daisy petals were used to determine the biological performance of human adipose-derived stem cells (ADSCs) on top of these substrates and their potential to drive osteogenic differentiation of those cells [30]. The oyster shell (*Pinctada maxima*) surface topography was also tested to evaluate the effect of both surface topographies on human MSCs on top of its different surface topographies (prism and nacre) revealing to have different capacities to drive osteogenic differentiation [29, 31, 32].

Herein, we hypothesize that a biomimetic surface topography of *Rubus fruticosus* leaf could be used to induce the osteogenic differentiation of rat bone marrow stem cells (rBMSCs). *R. fruticosus* leaf presents a hierarchical surface topography whose main motifs are in a scale that is reported to have an impact on cellular behaviour ranging from nano- to micrometre scale. Previously, we already reported the replication of this surface on polycaprolactone (PCL) spin casting membranes and its cytocompatibility [33]. In this work we aim evaluate their potential to promote the osteogenic differentiation of rBMSCs. *In vitro* assessment was performed by culturing rBMSCs cells on top of the membranes evaluating morphology and expression of the most remarkable osteogenic markers. The osteogenic differentiation was evaluated with and without biochemical stimulation aiming to assess not only the effect of the topography by itself, but also a possible synergistic effect established by the use of this biomimetic topography and biochemical stimulation.

## 2. Experimental section

### 2.1. Fabrication of spin casting PCL membranes

The production of the membranes was performed in accordance with our previous study [33]. Briefly, spin casting membranes were fabricated from 1 ml of a solution of 20% of PCL (PCL-Mn 70 000–90 000 by GPC, Sigma, 440 744) homogenized in dichloromethane (Dichloromethane for HPLC,  $\geq 99.8\%$ , contains amylene as a stabilizer, Laborspirit). Then, using a spin coater (Laurell technologies) with the spinning settings were: 5 min at a velocity of 1500 rpm, an acceleration of 9300 rpm s<sup>-1</sup>, the solution was dispersed in a glass petri dish.

### 2.2. Production of polydimethylsiloxane (PDMS) stamps and imprinting of its surface on spin casting PCL membranes

The production of the PDMS stamps was performed in accordance with our previous study [33]. In summary, first we produced the negative replica of *R. fruticosus* leaves surface with PDMS (SYLGARD 184 Silicone elastomer kit, SCANSCI, DCE-1673 921) (figure 1-I). Then, spin casted PCL membranes was placed in contact with the PDMS negative replica of the leaf and through the use of Nanoimprinter (Obducat technologies, Model Eitre 3, Serial number: 003-5095) the imprinting was performed (figure 1-II). The imprinting is initiated inducing under a pressure of 3 bar for 30 s with increasing the temperature up to 60 °C. Then, the pressure is raised to 5 bar for 1200 s. In the end, the pressure is released, and the set is kept in contact while waiting for it reaches the room temperature.

### 2.3. Surface modification of the membranes

To perform the modification of the membranes surface with amine groups it was performed an aminolysis reaction. For that, PCL membranes were submerged in hexamethylenediamine (HMD)/2-propanol (CHROMASOLV™ for HPLC) at 10% wt/v (pH was adjusted to 7) (Sigma-Aldrich) overnight at room temperature. Then, PCL membranes were carefully washed three times with distilled water to eliminate the unreacted HMD [34].

## 3. Biological activity

### 3.1. rBMSCs isolation and culture

rBMSCs were obtained from tibiae and femurs of three rats of each strain as previously described by our group [35]. The experimental protocol involving animal experimentation was approved by Direção Geral da Alimentação e Veterinária (national) (DGAV023875) and followed the European Community standards (European Union Directive 2010/63/EU). Briefly, using sharp scissors the ends of the tibia and femur were cut and the bone marrows flushed out three times with  $\alpha$ -minimum

essential medium (MEM) culture medium, supplemented with 10% (V/V) foetal bovine serum (FBS), 500 units ml<sup>-1</sup> of penicillin and 500  $\mu$ g ml<sup>-1</sup> of streptomycin. Cell suspensions from each rat were seeded onto 150 cm<sup>2</sup> culture flasks (TPP Techno Plastic Products AG) and incubated at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere. After 3 d, all non-adherent cells were removed and after washing with phosphate-buffered saline (PBS), fresh culture  $\alpha$ -MEM medium supplemented with 10% (V/V) FBS, 100 units ml<sup>-1</sup> of penicillin and 100  $\mu$ g ml<sup>-1</sup> of streptomycin was added for further rBMSCs (adherent cells) growth. The medium was changed every 2–3 d and after reaching  $\approx 70\%$  confluence, adherent cells were detached using TrypLE™ Express Enzyme (1X), phenol red (ThermoFisher) All experiments were performed with cells at passage  $\leq 4$  to minimize the morphological, phenotypic, and genetic alterations that can result from an extensive *in vitro* passage. These rBMSCs were characterized by specific cell-surface markers of MSCs assessing their ability for differentiation potential into osteogenic, chondrogenic and adipogenic lineage when cultured on tissue culture polystyrenes (TCPs). For that, we use a rat MSC Functional Identification Kit (R&D Systems, Inc., SC020), according to manufacturer's instructions (supplementary material).

Herein, rBMSCs were cultured in  $\alpha$ -MEM culture medium supplemented with 2.2 g l<sup>-1</sup> sodium bicarbonate, 10% FBS, and 1% penicillin–streptomycin. Preceding the seeding of the cells on top of PCL membranes, square-shaped membranes (11.5 × 11.5 mm) were sterilized by soaking them in 70% ethanol and let them become dry in a sterile laminar flow chamber. Then, they were exposed to UV light for 30 min on both sides. Non-adherent 24 well-plates were used to perform the studies where 50 000 rBMSCs cells were seeded on the top of each membrane. TCP disks of 13 mm were used as cell culture control. In parallel, cells were cultured in the same conditions but using the osteogenic inductive medium:  $\alpha$ -MEM culture medium supplemented with 2.2 g l<sup>-1</sup> sodium bicarbonate, 10% FBS, and 1% penicillin–streptomycin, 10 mM  $\beta$ -glycerophosphate, 10 mM dexamethasone, 200  $\mu$ M ascorbic acid. The samples were analysed in triplicate in three independent assays ( $n = 3$ ).

### 3.2. RNA isolation and real-time quantitative polymerase chain reaction (RT-PCR)

At 7, 14 and 21 d of culture, the collected samples were washed with PBS, immersed in Tri reagent® (Life Science, USA), and stored at  $-80$  °C until further use. Total RNA extraction was performed using Tri reagent® method according to the manufacturer's directions. The concentration and purity of the extracted RNA was determined using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc. USA). RNA was reverse transcribed into cDNA using the qScript cDNA synthesis kit (Quanta Biosciences,

**Table 1.** Primer sequences used for RT-PCR procedures<sup>a</sup>.

|                          | Gene               | Forward (5'-3')          | Reverse (5'-3')         |
|--------------------------|--------------------|--------------------------|-------------------------|
| Osteogenic markers (rat) | <i>GAPDH</i>       | CAACTCCCTCAAGATTGTCAGCAA | GGCATGGACTGTGGTCATGA    |
|                          | <i>Runx2</i>       | CAGTTCCCAGGCATTTCATC     | CAGCGTCAACACCATCATTC    |
|                          | <i>Osterix</i>     | CACTGGCTCCTGGTTCTCTC     | CCACTCCTCCTCTTCGTGAG    |
|                          | <i>COL I</i>       | GCAAGGTGTTGTGCGATGACG    | GGGAGACCACGAGGACCAGAG   |
|                          | <i>Osteopontin</i> | ATCTCACCATTCCGATGAATCT   | TCAGTCCATAAGCCAAGCTATCA |
|                          | <i>Osteocalcin</i> | CATCTATGGCACCACCGTTT     | AGAGAGAGGGAACAGGGAGG    |
|                          | <i>BSP</i>         | TGTCCTTCTGAACGGGTTC      | CTTCCCCATACTCAACCGTG    |

<sup>a</sup> *GAPDH* = glyceraldehyde 3-phosphate dehydrogenase; *Runx 2* = Runt-related transcription factor 2; *COL I* = collagen type I; *BSP* = bone sialoprotein.

VWR, USA), according to the manufacturer instructions. Subsequently, the obtained cDNA was used as a template for the amplification of the target genes shown in table 1, according to manufacturer's instructions of the PerfeCta™ SYBR® Green system (Quanta Biosciences, VWR, USA). The qPCR reactions were carried out in a Mastercycler® ep Gradient S realplex® thermocycler (Eppendorf; Germany) [36].

Glyceraldehyde-3-phosphate-dehydrogenase gene (*GAPDH*) was used as reference gene, and the expression of all target genes was normalized to the expression of this housekeeping gene for the same sample. The gene expression quantification was performed according to the Livak method ( $2^{-\Delta\Delta CT}$  method), considering the TCP basal medium condition (negative control) as calibrator.

### 3.3. Immunocytochemistry

Immunocytochemistry was performed after 21 d of culture to evaluate if the cells cultured in PCL membranes. Briefly, cells were fixed in 10% neutral buffered formalin (ThermoFisher) for 4 h at 4 °C and then washed in PBS.

Immunocytochemistry was using a mouse monoclonal antibody against osteocalcin (clone OC4-30, Abcam Ltd; dilution 1:100), a mouse monoclonal antibody against Runx2 (clone AS110, MERCK; dilution 1:100), a rabbit polyclonal antibody against bone sialoprotein II (AB1854, MERCK; dilution 1:100). Briefly, the cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min. To block non-specific binding, the samples were incubated with 3% bovine serum albumin (BSA) in PBS, for 30 min at room temperature. After blocking unspecific binding, primary antibodies were incubated overnight at 4 °C. The next day, cells were washed with PBS and incubated with the secondary antibody Alexa Fluor 488 (against mouse or rabbit) (Alfagene; 1:500 diluted in 1% BSA/PBS) at room temperature in the dark for 1 h. After several rinses in PBS, phalloidin-tetramethylrhodamine B isothiocyanate was incubated (Sigma-Aldrich, diluted 1:1000) for 30 min in the dark.

The PCL membranes were analysed under a fluorescence microscope (Zeiss, Axio Imager Z1m), the images were recorded digitally and further processed using Zen Lite Software (Zeiss) [37].

### 3.4. Scanning electron microscopy (SEM)

SEM micrographs were taken to analyse the morphology of cells in the top of the membranes. Briefly, after each defined time point, rBMSCs cultured on top of the different substrates were fixed with 10% formalin and stored at 4 °C. To perform the SEM analysis, dehydration was performed using a gradient of increasing concentrations of ethanol (10%, 20%, 40%, 60%, 80%, 90%, 95%, and 100%). Then, the samples were sputter-coated with gold (Fisons Instruments, model SC502; England) for 2 min at 15 mA. Microphotographs were recorded at 5 kV with magnifications of 20× and 150× using a SEM (Leica Cambridge, model S360; England) [38].

### 3.5. Calcium detection by alizarin red staining

Mineralization of the extracellular matrix was assessed with samples from the 21st day of culture. Following fixation in 10% formalin solution (Sigma-Aldrich, Germany) overnight, the samples were washed five times in distilled water before staining with 2% Alizarin Red (Merck, Germany) for 30 min. Alizarin red was removed from the samples with distilled water. The stained samples were then air dried and observed under an optical microscope (BX61, Olympus Corporation, Germany), and images captured under the same settings [16].

### 3.6. Statistical analysis

The statistical analysis was performed using the SPSS statistic software (release 24.0.0.0 for Mac). First, Shapiro–Wilk test was used to ascertain the data normality and Levene test for the homogeneity of variances. Observing this, the normality and variance homogeneity were rejected; nonparametric tests were used (Kruskal–Wallis test followed by Tukey's Honest Significant Difference test). The confidence interval used was 99% and  $p \leq 0.01$  were regarded as statistically significant. The statistical significance was recorded as the  $p$ -value \* $p < 0.01$ ; \*\* $p < 0.001$ ;

\*\*\* $p < 0.0001$ . Three samples from three independent assays were used.  $p < 0.01$ ; \*\* $p < 0.001$ ; \*\*\* $p < 0.0001$ .

## 4. Results

In this work, the bottom surface of a leaf from *R. fruticosus* was replicated according to the procedure firstly reported by our group [33]. A schematic representation of the process from the replication of the surface topography on PDMS, passing through the imprinting on PCL spin casting membranes and the surface chemistry modification it is represented on figures 1(I), (II) and (III) respectively. We aimed to test the potential of these biomimetic patterned PCL membranes to promote the rBMSCs differentiation into osteogenic lineage. The production of biomimetic PCL membranes (BpM) and bare PCL membranes (NpM) as well as its characterization was previously described by us [33]. In the figures 1(IV) it is shown surface patterned of both biomimetic patterned PCL membranes (figures 1-IV(A) and (B)) and bare PCL membranes (figure 1-IV(C)). On the higher magnification image (figure 1-IV(B)), it is possible to see that the replication technique was achieved with high detail, being able to replicate many topographical features such as the structural fibre-like component of the veins and the stomata structures.

In the conception of the present study, we intended to evaluate if the selected topography it is able to induce the osteogenic differentiation of rBMSCs without any biochemical stimulation. To isolate the effect of the topography we used two different controls: the TCPs, that represent the typically used cell culture substrate, and NpM, that are flat PCL membranes that can give us information about the influence of the surface chemistry of the selected polymeric membranes on rBMSCs.

Figure 2 presents the SEM analysis of the rBMSCs cultured on top of the different substrates and under both basal and osteogenic inductive medium. SEM micrographs show that rBMSCs are able to populate almost all the available surface area after 21 d of culture under basal medium conditions with a formation of a monolayer in all analysed substrates (figures 2(A)–(C)). Under the osteogenic inductive medium, there is no cell growth sufficient to form a monolayer when rBMSCs are cultured on NpM (figure 2(E)). The cells seem not able to cover the whole surface, although we observe that these cells are able to populate all the surfaces on TCPs and BpM.

Since we intend to evaluate the potential of the selected surface topography to induce osteogenesis, we evaluate the expression of Runx2, osteocalcin and bone sialoprotein (BSP) proteins, that are osteogenic markers, by immunocytochemistry (figure 3). Regarding the expression of Runx2 (green staining),

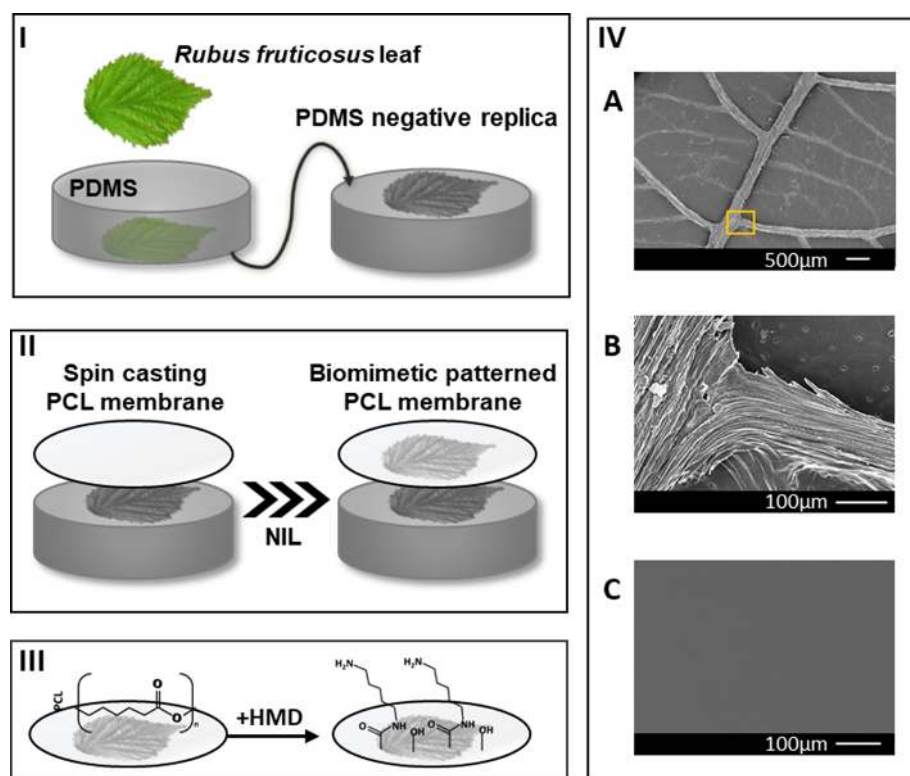
we observe that it is expressed by rBMSCs on all analysed substrates after 21 d of culture. Osteocalcin expression (green staining) it is more notorious on cells cultured under osteogenic inductive medium, however, under basal medium, is more notorious when they are cultured on BpM than when they are cultured on NpM. The immunodetection of BSP (green staining) reveals that the expression of this protein is more notorious when the studied cells are cultured on BpM when compared with rBMSCs cultured on NpM in the condition where cells were cultured under the basal medium, although, under osteogenic inductive medium, rBMSCs cultured on both NpM and BpM present BSP expression.

To evaluate if rBMSCs were induced to differentiate into osteogenic lineage, we performed the analysis of the expression of osteogenic-related genes in all tested conditions. As such, the analysis of the expression of BSP Runx-2 (Runt-related transcription factor 2), collagen type I, osteix, osteopontin and osteocalcin by rBMSCs revealed that, as expected, cells cultured under the osteogenic induction medium presented a higher expression of the analysed genes. Moreover, rBMSCs cultured on top of BpMs with basal medium express higher levels of osteogenic-related genes, with a higher significance on day 21 of culture, when compared with rBMSCs cultured on top of both NpMs and TCPs. Furthermore, the analysis of the results for rBMSCs cultured under the osteogenic medium reveals that, the expression of osteogenic-related genes is higher when they are cultured on top of PCL membranes with the selected surface topography (figure 4).

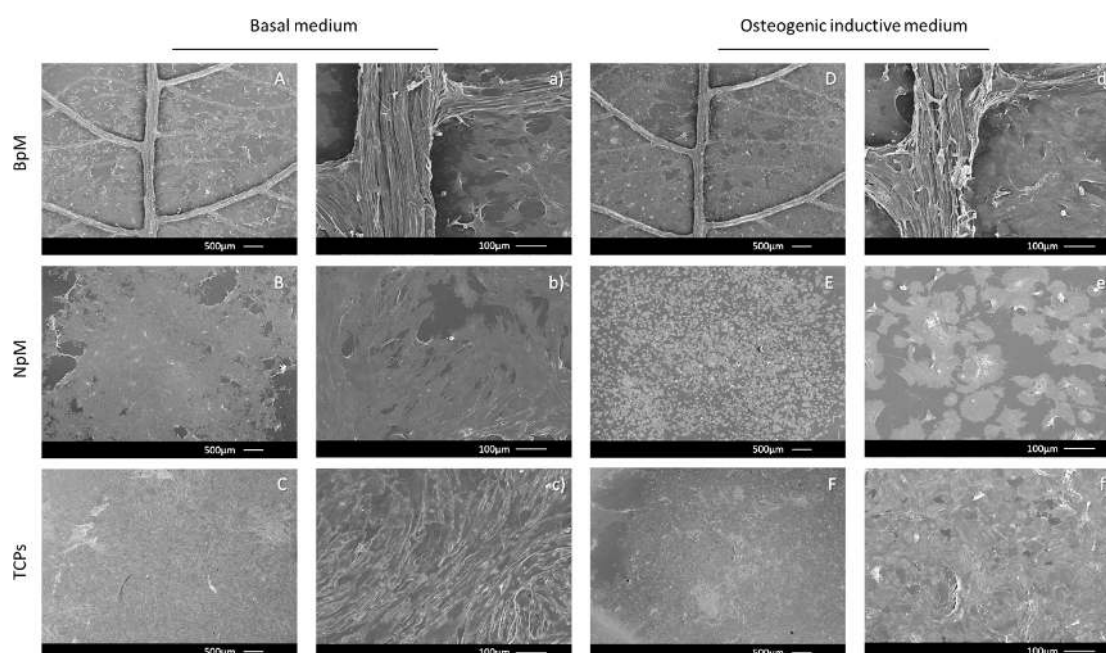
To assess the mineralization of the extracellular matrix on the last stage of osteogenesis, the samples of rBMSCs cultured on top of different studied substrates and under both basal and osteogenic inductive medium were stained with alizarin red after 21 d in culture (figure 5). The basal medium condition with the cells cultured on TCPs and on NpM surfaces did not present a relevant degree of mineralization (figures 5(A) and (B), respectively). It is noticeable that the condition where rBMSCs were cultured on top of BpM under the basal medium presented calcium deposition that is observed by the presence of red staining on the figure 5(C). As expected, all samples cultured under osteogenic inductive medium presented mineralization (figures 5(D)–(F)). The condition where rBMSCs were cultured on top of BpM presents a higher degree of mineralization (figure 5(F)) that is observed by the presence of more calcium deposits staining in red.

## 5. Discussion

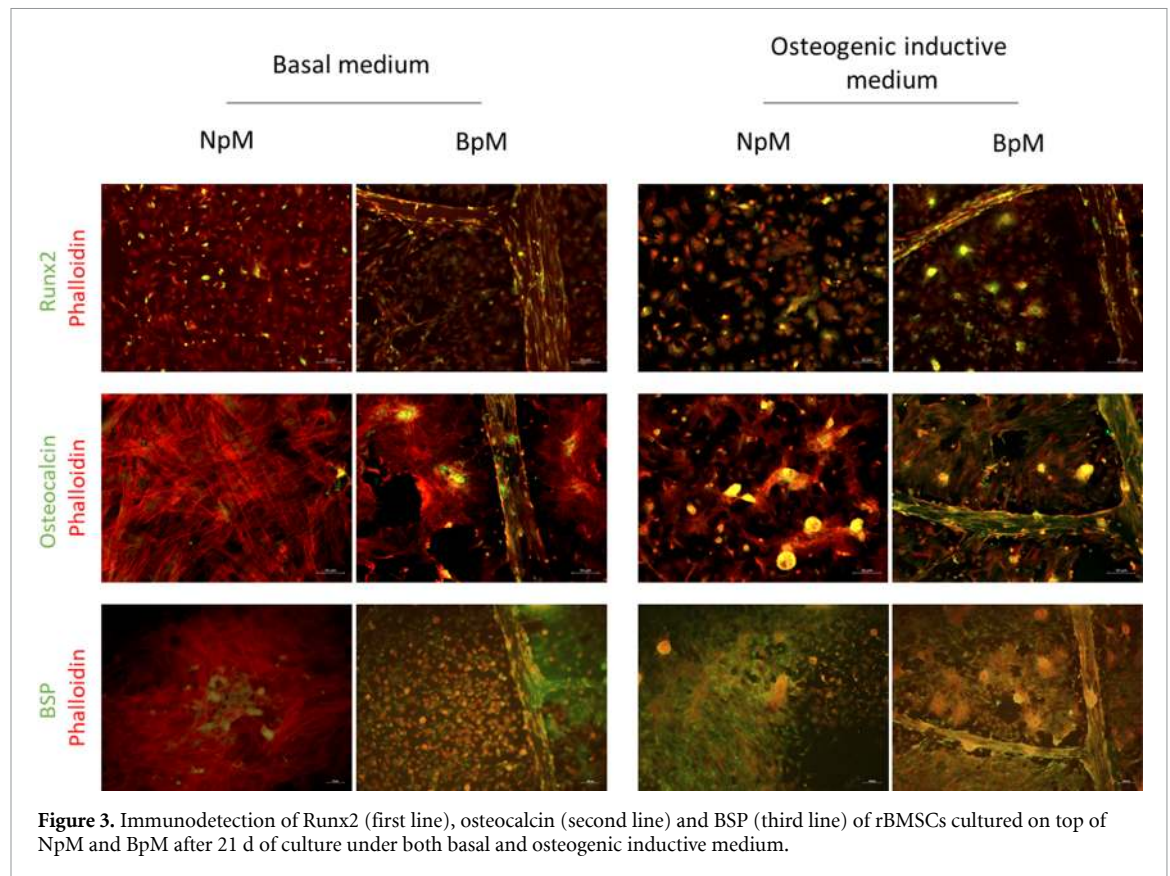
The substrate's surface topography has been described to have a deep influence regarding the cell motility, proliferation, and differentiation. Regarding



**Figure 1.** (I–III) Schematic representation of the procedure to obtain PCL membranes. (I) Rapid molding fabrication technique to obtain a PDMS negative replica of the surface topography of *Rubus fruticosus*; (II) soft lithography technique to perform the imprinting of the PDMS surface topography on Spin casting PCL membranes. (III) Aminolysis reaction on the surface of imprinted PCL membranes performed with HMD. (IV) SEM micrographs of the surface of PCL membranes: (A) BpM; (B) higher magnification of the BpM region represented in (A) with the yellow square. (C) NpM.



**Figure 2.** SEM micrographs of rBMSCs cells cultured on different substrates and under both basal and osteogenic inductive medium. (A)–(C) rBMSCs cultured under basal medium on top of BpM (A), NpM (B), TPCs (C). (D)–(F) rBMSCs cultured under osteogenic inductive medium on top of BpM (D), NpM (E), TPCs (F). The images (a)–(f) are a higher magnification image of the correspondent (A)–(F) image. Scale bar: 500 μm (A)–(F) and 100 μm (a)–(f).

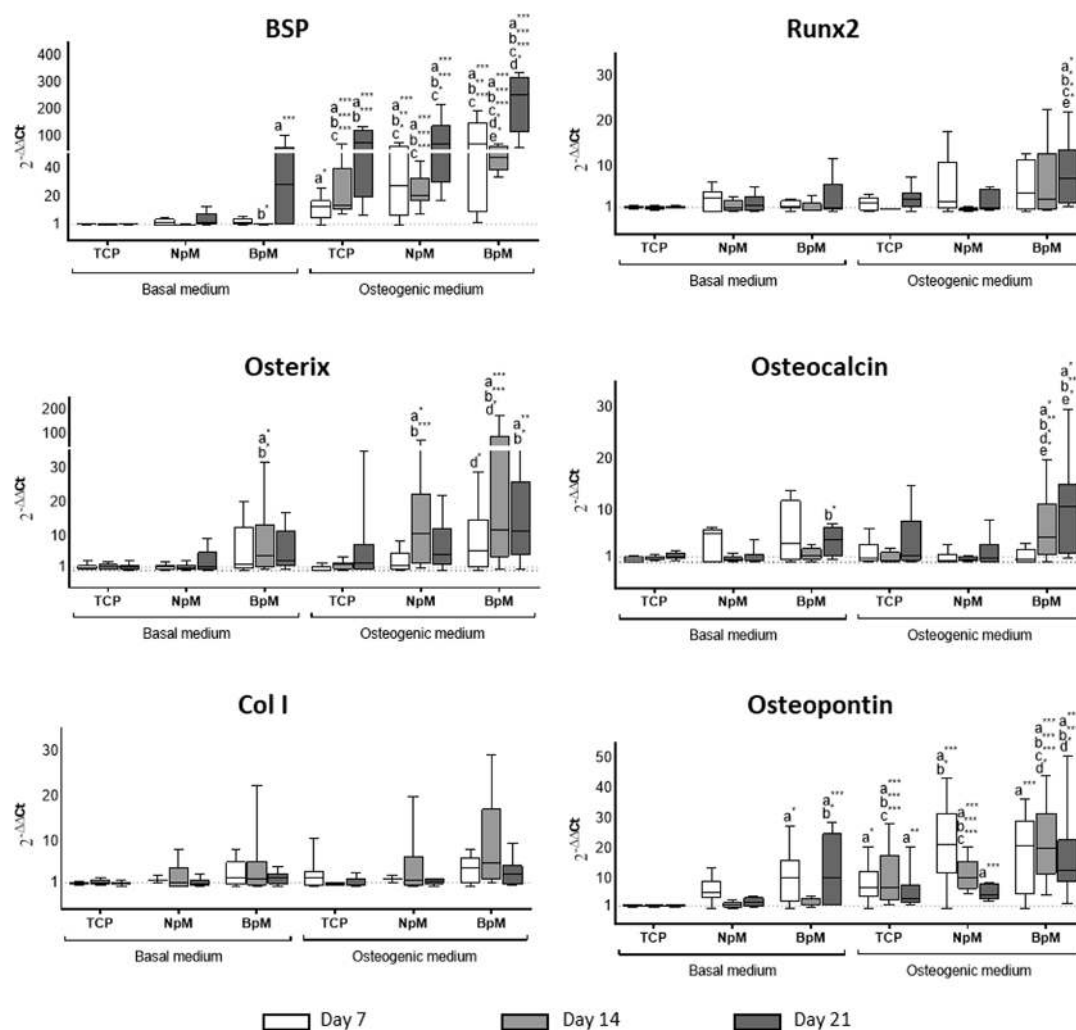


the MSCs differentiation, there are several substrate topographies that have been studied to induce MSCs differentiation, in particular into the osteogenic lineage. In fact, most of the topographies studied so far include artificially generated designs that are typically built with the combination of different size-scale artificial motifs such as grids, lines, pillars, or other geometric structures. In this work, we intend to demonstrate that biomimetic surfaces with a relevant size-scale topography can be a suitable alternative to be used in bone regenerative medicine strategies.

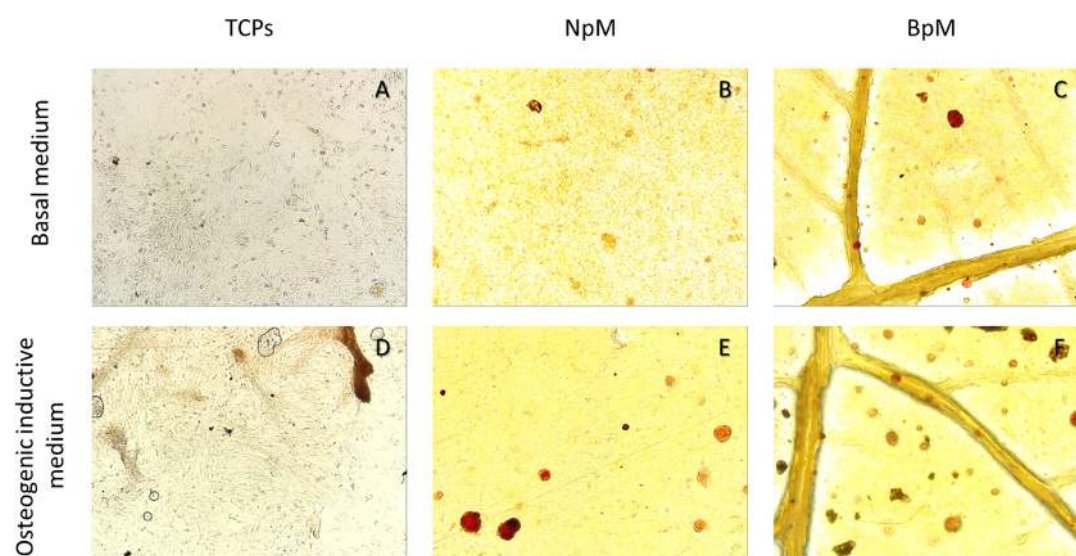
We selected the surface topography of *R. fruticosus* whose replication on PCL membranes was previously described and successfully achieved by us (figure 1) [33]. We aimed to evaluate if the selected topography is able to induce the osteogenic differentiation of rBMSCs without any other biochemical stimulation.

Several genes are associated and can be used as osteogenic markers. Herein we studied the expression of BSP, Runx2, Osterix, Osteocalcin, Collagen type 1 and Osteopontin. Their selection was based on different roles that they represent in bone morphogenesis and mineralization. BSP is a component of the extracellular matrix of hard tissues such as bone [39, 40]. Runx2 [41, 42] and osterix [43–45] are transcription factors that are associated with the osteoblast differentiation and bone morphogenesis. Osteocalcin is a calcium binding protein that plays

a key role in bone mineralization [46]. Intracellular Osteopontin is responsible for both regulation of cytoskeleton dynamics and gene expression, and extracellular is responsible for attachment, migration and bone remodelling [47, 48]. Collagen type 1 is a major component of bone extracellular matrix [49]. Our results suggest that the selected polymer reveals not to have an significant influence on the expression of osteogenic markers by rBMSCs. However, the selected topography reveals to promote the osteogenic differentiation of rBMSCs without any other biochemical stimulation. Moreover, we intend to evaluate if the effect of the substrate topography can, somehow, be conjugated with the effect of the typically used osteogenic induction medium. Again, the results of the expression of osteogenic markers of cells cultured on top of the three different selected substrates reveals that there is no statistically significant influence of the surface chemistry of PCL on rBMSCs differentiation when we compare the conditions of TCPs and NpM using osteogenic inductive medium. However, the expression of osteogenic markers of rBMSCs cultured on top of BpM is higher when compared with the expression by these cells cultured on flat surfaces with osteogenic inductive medium and higher of the expression by these cells cultured on BpM without any biochemical stimulation. These results reveal that the selected topography and the use of osteogenic inductive medium have a



**Figure 4.** Relative expression of osteogenic markers by rBMSCs. The expression was normalized against the GAPDH gene and the quantification was performed according to the Livak method, considering the control condition (TCP-basal medium) as calibrator. Data were analysed by the Kruskal–Wallis test, followed by the Tukey's honestly significant difference (HSD) test ( $p < 0.01$ ): a denotes significant differences compared to TCP-basal medium, b denotes significant differences compared to NpM-basal medium, c denotes significant differences compared to BpM-basal medium, d denotes significant differences compared to TCP-osteogenic medium and e denotes significant differences compared to NpM-osteogenic medium; \* $p < 0.01$ ; \*\* $p < 0.001$ ; \*\*\* $p < 0.0001$ .



**Figure 5.** Alizarin red staining of rBMSCs on TCPs, NpM and BpM as a result of a mineralization pattern in bone cells matrix after 21 d of cell culture with both basal and osteogenic inductive medium.

synergistic effect on rBMSCs on their differentiation into osteogenic lineage.

The mineralization is an essential step regarding the formation of hard tissues such as bone. It is the mineralization of extracellular matrix that provides rigidity to the tissues that makes them able to support the body mass or to protect the internal soft organs [50–52]. Our results showed that, the mineralization of ECM by rBMSCs occurs when these cells are cultured under the osteogenic inductive medium in all analysed substrates. Nevertheless, when we cultured rBMSCs under basal medium, we observe a significant mineralization degree when these cells are cultured on top of BpM in contrast to what it is observed to the conditions where these cells are cultured on top of TCPs and NpM (figure 5).

Overall, we observe that, PCL membranes with the biomimetic surface of *R. fruticosus* leaf are able to induce osteogenic differentiation of rBMSCs without any biochemical stimulation. Nevertheless, the combination of both physical cues and biochemical stimulation represents a synergistic effect regarding rBMSCs osteogenic differentiation. Furthermore, taking into account these findings, follow up studies can be performed to understand the mechanism that underlies the promotion of rBMSCs differentiation by the physical stimuli provided by the surface topography of the *R. fruticosus* leaf. From the adhesion of cells to the substrate, passing through the cytoskeleton remodelling and the activation of cascades that regulate the gene expression of osteogenic markers, FAK [21], ERK/MAPK [22, 53], Rho-ROCK [23, 24, 54], or Wnt [25–27, 55] signalling pathways are reported to be preponderant in determining MSCs topography-induced differentiation. The study of those signalling pathways may be able to identify the mechanisms that are responsible for the osteogenic differentiation of rBMSCs that results from the physical stimuli provided by the surface topography of the *R. fruticosus* leaf.

Previous works reports on the use of negative replicas of the surface of Rose petals, parsley leaf, and daisy petals imprinted on substrates for ADSCs culture. Differences in ADSCs cytoskeleton arrangement were observed comparing those cells cultured on different substrates studied. Moreover, the hydroxyapatite substrates imprinted with the negative replica of the surface of parsley leaf and daisy petals reveal to promote a higher expression of osteogenic associated genes (Collagen I, Runx2, ALP, and Osteopontin) than a flat surface based on the same material. By contrast, hydroxyapatite substrates imprinted with the negative replica of the surface of rose petals reveal to be responsible for a decrease in the expression of those genes when compared to the other analysed substrates [30]. As such, besides *R. fruticosus* leaves surface topography, other plant-derived biomimetic surface topographies can present a potential to drive osteogenic differentiation of MSCs.

## 6. Conclusions

Engineered bone grafts face critical limitations that are associated with the insufficient induction of osteogenesis of by the implanted scaffolds usually caused by the loss of osteogenic capacity of the cells. The developed biomimetic patterned PCL membranes that were able to induce osteogenic differentiation of rBMSCs. The *R. fruticosus* leaf's surface reveals to promote osteogenic differentiation of rBMSCs without any biochemical stimulation. The selected surface topography in combination with the osteogenic inductive medium reveals to have a synergistic effect promoting the expression of higher levels of osteogenic markers. These results give positive insights about the conception of new biomaterial-based platforms with natural topographic cues that are able to potentiate the osteogenesis of MSCs.

## Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).

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## Conflicts of interest

The authors declare no conflicts of interest.

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