Differentiation Capacity of Monocyte-Derived Multipotential Cells on Nanocomposite Poly(ϵ-caprolactone)-Based Thin Films

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Differentiation Capacity of Monocyte-Derived Multipotential Cells on Nanocomposite Poly(e-caprolactone)-Based Thin Films

Iro Koliakou[1,2] · Eleni Gounari[2,3] · Maria Nerantzaki[4,5] · Eleni Pavlidou[6] · Dimitrios Bikiaris[4] · Martha Kaloyianni[1] · George Koliakos[2,3]

Abstract

BACKGROUND: Monocyte-derived multipotential cells (MOMCs) include progenitors capable of differentiation into multiple cell lineages and thus represent an ideal autologous transplantable cell source for regenerative medicine. In this study, we cultured MOMCs, generated from mononuclear cells of peripheral blood, on the surface of nanocomposite thin films.

METHODS: For this purpose, nanocomposite Poly(e-caprolactone) (PCL)-based thin films containing either 2.5 wt% silica nanotubes (SiO_2ntbs) or strontium hydroxyapatite nanorods (SrHAnrds), were prepared using the spin-coating method. The induced differentiation capacity of MOMCs, towards bone and endothelium, was estimated using flow cytometry, real-time polymerase chain reaction, scanning electron microscopy and fluorescence microscopy after cells' genetic modification using the Sleeping Beauty Transposon System aiming their observation onto the scaffolds. Moreover, Wharton's Jelly Mesenchymal Stromal Cells were cultivated as a control cell line, while Human Umbilical Vein Endothelial Cells were used to strengthen and accelerate the differentiation procedure in semi-permeable culture systems. Finally, the cytotoxicity of the studied materials was checked with MTT assay.

RESULTS: The highest differentiation capacity of MOMCs was observed on PCL/SiO_2ntbs 2.5 wt% nanocomposite film, as they progressively lost their native markers and gained endothelial lineage, in both protein and transcriptional level. In addition, the presence of SrHAnrds in the PCL matrix triggered processes related to osteoblast bone formation.

CONCLUSION: To conclude, the differentiation of MOMCs was selectively guided by incorporating SiO_2ntbs or SrHAnrds into a polymeric matrix, for the first time.

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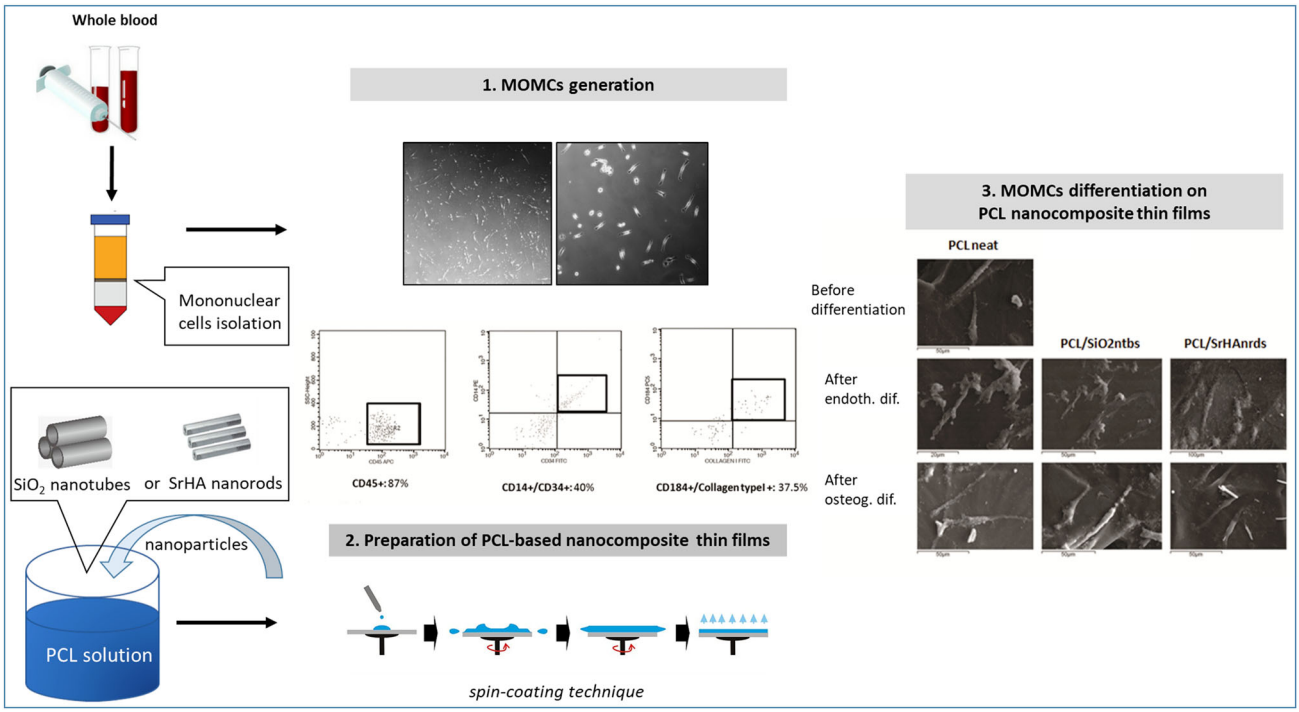
Graphical Abstract



Keywords Monocyte-derived multipotential cells · Poly(ε-caprolactone) · Silica nanotubes · Strontium hydroxyapatite nanorods

1 Introduction

As our population ages and life expectancy increases there is a great need for research in the field of regenerative medicine. Stem cell and biomaterial based therapies are being actively investigated [1–3]. The key is to find the best combination of biomaterials and cell lines in order to regenerate specific tissues [4, 5]. Both Human Bone Marrow Mesenchymal Stromal Cells (hBMSCs) and Human Wharton's Jelly Mesenchymal Stromal Cells (WJSCs) have the potential of regeneration [6, 7]. BMSCs' and WJSCs' collection has no ethical concerns, unlike embryonic stem cells (ESCs), share few embryonic features, have high cell proliferation rate and wide differentiation potential while they are hypo-immunogenic and non-tumorigenic [8, 9].

Nevertheless, both hBMSCs and WJSCs present the disadvantage of harvest. Harvest of hBMSCs involves an invasive procedure, while WJSCs need to be harvested during birth [10]. During the last years a new cell line, isolated by Seta and Kuwana called Monocyte-derived Multipotential Cells (MOMCs) has shown potential to differentiate into multiple cell lineages in a specific permissive environment and thus represent an autologous transplantable cell source for regenerative medicine [11–14]. MOMCs are a human-derived cell population with a fibroblast-like morphology characterized by a unique immunophenotype positive for CD14, CD45, CD34 and type I collagen. MOMCs have the capacity to differentiate into a variety of mesenchymal cell types including endothelial and osteogenic cells and can be easily harvested from circulating blood [15, 16].

In the present study, we investigated the differentiation capacity of MOMCs on the surface of nanocomposite thin films based on poly(ε-caprolactone) (PCL), reinforced either with 2.5 wt% silica nanotubes (SiO_2ntbs) or strontium hydroxyapatite nanorods (SrHAnrds). PCL is a biodegradable aliphatic polyester with many biomedical applications because of its biocompatibility and its modifiable properties. PCL also plays an increasingly important role in tissue engineering because it can be fashioned into porous scaffolds or carriers of cells, extracellular matrix components and bioactive agents. Since all living systems are governed by molecular behavior at nanoscale, the introduction of a nanoscopic reinforcing phase to PCL matrix offers great possibilities of obtaining improved biological properties. As a result, nanometer structural

components could be considered promising biomaterials [17–21].

Silica-based nanoparticles (NPs) have been widely used in the field of biomaterials science since 1970s, when several studies revealed the requirement of silicon in bone development [22, 23]. As silica plays an important role in skeletal tissue formation, bioactive silica has been used in order to produce bioactive glasses. When implanted into the body, bioactive glass bonds with the surrounding tissue without promoting inflammation or forming fibrous tissue [24]. The biological role of strontium has been well documented including its effects on bone cells (up-regulation of osteoblasts and down-regulation of osteoclasts). Oral administration of strontium has also been shown to increase bone density and reduce fracture risk in osteoporotic patients. Recent studies have published on other delivery methods of strontium in the form of bioactive glasses, Sr-HA and Sr-containing cements [25, 26]. The advantages of biomaterial incorporation of strontium include the local and controlled release of the Sr-ion [21]. The use of microfabrication technique in silicon and Pyrex to generate hepatic tissue and vascular tissue may be able to be combined to form thick, three-dimensional vascularized tissues. Using standard photolithography techniques, trench patterns reminiscent of branched architecture of vascular and capillary networks were etched onto silicon and Pyrex surfaces to serve as templates. With the use of microfabrication technology in tissue engineering, it now seems feasible to consider lifting endothelial cells as branched vascular networks from two-dimensional templates that may ultimately be combined with layers of parenchymal tissue, such as hepatocytes, to form three dimensional conformations of living vascularized tissue for implantation [27]. In brief, the released ionic dissolution products are capable of enhancing proliferation and differentiation of bone cells and inducing angiogenesis.

Thus, in the present study by incorporating SiO_2ntbs or SrHAnrds in the PCL matrix by applying the spin-coating method, we expect to improve the ability of PCL to selectively guide the differentiation capacity of MOMCs. WJSCs are used as a control cell line in order to compare the differentiation potential of MOMCs with WJSCs respectively. We also use Human Umbilical Vein Endothelial Cell line (HUVEC) [28] to strengthen and accelerate MOMCs differentiation to endothelium given that, in contrast to WJSCs, MOMCs are unable to be maintained in cell culture for a long time [15, 29]. The presence of HUVEC in a new semi-permeable co-culture system with MOMCs, ensures that in a limited time this easily collected category of stem cells could be exploited for autologous transplantation of a scaffold for endothelium regeneration [30, 31].

To our knowledge this is the first study on the effect of nanophase reinforcement such as SiO_2ntbs and SrHAnrds, on the capacity of MOMCs to differentiate on nanocomposite materials based on PCL. Therefore, this paper will provide practical guidance for rationally designing nanocomposite PCL films for multiple applications in regenerative medicine.

2 Materials and methods

2.1 Materials

PCL with average Mn ~ 65,000 g/mol and glass slides (plain, size 25 mm × 75 mm), were purchased from Sigma-Aldrich (St.Louis, MO, USA). Chloroform ($CHCl_3$) (99.5%), was supplied from Chem-Lab NV (Zedelgem, Belgium). Sulfuric acid (H_2SO_4) (95–98%) and hydrogen peroxide (H_2O_2) (solution 110 vol, 30%w/w) were supplied from Scharlab S.L. (Barcelona, Spain). Phosphate Buffer Saline (PBS) was used as purchased from Sigma Aldrich (St. Louis, MO, USA). DMEM high glucose w/L-glutamine w/sodium pyruvate, penicillin–streptomycin solution 100X, fetal bovine serum (FBS) and trypsin-EDTA 1x in PBS were purchased from BIOSERA (Kansas city, MO, USA), Osteogenesis medium from GIBCO-BRL (Grand Island, NY, USA) while the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) powder-MTT, hydrogen peroxide, uric acid, 3,3′,5,5′-tetramethylbenzidine (TMB) tablets, Histopaque and DMSO from Sigma Aldrich (St. Louis, MO, USA).

2.2 Cell lines

The collection of biological samples from different sources for this study approved by the Ethics Committee of Aristotle University of Thessaloniki, School of Medicine (390-9/1.7.2017) and University Hospital AXEPA of Thessaloniki (3868/24.1.2018) as described below in ethical statement section.

2.2.1 Optimized harvest, culture and characterization of MOMCs

The blood sampling of the healthy volunteer donors was performed after their endorsed consent. More precisely, 10 ml of peripheral blood were collected in anti-coagulant CPD and upon dilution in 20 ml of PBS were loaded in the half volume of Ficoll Paque. The mononuclear cell layer (Peripheral blood mononuclear cells-PBMCs) was collected upon centrifugation at 700 g for 20 min, with no brakes added. 1.5×10^6 PBMCs in DMEM complete media supplemented with 50 ng/ml SDF-1a (Millipore,

MA, USA) were plated in a 6-well plate pre-coated overnight with either fibronectin or poly-L-lysine in gelatin. The cells were incubated for 12–14 days in 37 °C with 5% CO_2 with SDF-1a enriched media changes every 2 days. Aiming the characterization of the harvested cells, flow cytometry was performed for the detection of typical surface marker expression. Briefly, upon detachment of cells with Trypsin-EDTA 1x in PBS and mild centrifugation, staining with monoclonal antibodies (mAbs) CD45, CD14, CD34, Collagen type I and CD184 (BD Biosciences, San Jose, CA USA) was performed for 15 min in the absence of light and finally analysis in BD FACS Calibur (BD Biosciences, San Jose, CA USA).

2.2.2 Harvest, culture and characterization of WJSCs

35 cm of umbilical cord were collected after the parents endorsed consent in a sterilized box. Following a normal saline wash and a mild cut up with a lancet, an overnight lysis with 4 mg/ml collagenase and 2 mg/ml dispase contained in PBS in a stirring incubator was performed. The next day, the mixture was filtered through a 70 μm Filter Unit and was subsequently centrifuged in 850 g for 10 min in RT. The pellet was re-suspended in DMEM media supplemented with 10% FBS + 1% penicillin/streptomycin (DMEM full medium) and was then plated in culture flasks for 72 h until the full cell adherence in a 37 °C incubator with 5% CO_2. Following media changes every 2–3 days and after 2–3 passages, the cells were characterized via flow cytometry for the expression of the CD90–CD105 markers and the absence of the CD45–CD34 markers.

2.3 Genetic modification of the WJSCs with venus plasmid via the Sleeping Beauty Transposon system

By applying genetic modification to WJSCs we enable the observation of our cells on scaffolds via fluorescence microscopy. The generation of a cell line capable of stable expression of green fluorescence, allow us to check the gradual differentiation of our cells throughout the culture. Between the 4–5 passage a Pt2-Venus-neo mediated nucleofection was performed. More precisely, 4×10^5 cells were mixed with 7.5 μg of plasmid DNA SB100X transposase and pT2-Venus-neo transposon expression plasmids (1:10 ratio) and were electroporated according to the manufacturer's instructions (Lonza, Basel, Switzerland). The cells were then plated in one well of a 6-well plate in the presence of DMEM full medium until reaching a 90% confluency, whereas 100 mg/ml G418 (InvivoGen, San Diego, CA, USA) was added for the selection of the genetically modified WJSCs. The cells were incubated in 37 °C with 5% CO_2 and were subjected to media changes every 2–3 days.

2.4 Isolation, characterization and culture of HUVEC

20–25 cm of umbilical cord near the placenta height, were collected after the parents endorsed consent in a sterilized box. Following a double saline wash, the umbilical cord was placed in a plastic surface with its both ends cut transversely with a lancet, in order for the umbilical vein to be visible from both sides. After a mild restrain with the use of a pincher, a wash with PBS was performed 3 times from every side of the umbilical cord in the internal side of the vein using a 5 ml syringe and 18G needle. Subsequently, the one side of the vein was wide shut and in the one a PBS solution supplemented with 25 mg collagenase was added, preheated in 37 °C for 30 min, until the complete filling of the vein cavity. After the closure of the open side, the umbilical cord was cautiously transferred in a preheated saline solution in 37 °C for 15 min. Upon the complete incubation and the opening of the one side, the content of the vein was collected following mild massages while 3 washes with PBS were performed from every side and the content was also collected. After a centrifugation at 700 g for 15 min in RT, the pellet was resuspended in Endothelial Cell Growth Medium (Lonza, Basel, Switzerland) and was subsequently plated in a T-25 culture flask and incubated in 37 °C with 5% CO_2 with media changes every 2–3 days. The cells were then characterized via flow cytometry for the expression of the CD144 surface marker.

2.5 Preparation and characterization of PCL nanocomposite thin films by spin-coating

2.5.1 Evaluation of the cytotoxicity levels SiO_2ntbs and SrHAnrds

In order to assess the cytotoxicity levels of SiO_2ntbs and SrHAnrds which were synthesized by the sol–gel method and a hydrothermal treatment, according to a process that has been described by us in detail previously [22]. Prior to cell plating, WJSCs cells were exposed to both type of NPs and the MTT assay was carried out for 24 and 72 h. As day 0 was defined as the NP's addition in the culture supernatants at three different concentrations: 10, 100 and 200 μg/ml in DMEM. For the sterilization of the solutions, 0.22 μm filter units were used. Non-NP treated cells were used as a control group in the same number as the other groups. Briefly, after the medium removal from the wells, MTT reactant was introduced in a ratio of 1:10 in DMEM culture medium and was followed by a 4 h incubation in 37 °C with 5% CO_2. Upon the removal of the MTT, 1 ml/

well of DMSO was introduced for one additional hour of incubation in the same conditions. The optical density of MTT formazan deposits was quantified by a spectrophotometer at 570 nm and 630 nm wave length (PerkinElmer, Waltham, MA, USA).

2.5.2 Preparation of PCL nanocomposite thin films by spin-coating

Nanocomposites based on PCL matrix (PCL/SiO_2ntbs 2.5 wt% and PCL/SrHAnrds 2.5 wt%) were prepared according to the following procedure. Firstly, PCL was dissolved in $CHCl_3$ to form a 10 wt% solution using magnetic stirring for 2 h at room temperature. At the same time, SrHAnrds and SiO_2ntbs, were weighted to achieve the desired final concentration (2.5 wt% based on the weight of the composite material) and dispersed separately using magnetic stirring for 2 h and ultrasonication for 20 min. In the second step, both the polymer solution and the suspensions of NPs were mixed, stirred and sonicated (15 min) to form nanocomposites with 2.5% weight fraction. The produced nanocomposite PCL films were spin-coated on glass substrates (slides), cut into 2 cm × 2 cm square-pieces. Several coating speeds were tested to achieve the desired thickness which was dependent on the used featured height. Prior to spin coating piranha solution (3:1 mixture of H_2SO_4 H_2O_2 30%.) was used to clean organic residues off substrates and to enhance the polymer adherence. For comparison purposes pure PLC films were also prepared using a similar process.

2.5.3 Chemical characterization by Fourier transform infrared spectroscopy (FTIR)

To evaluate the chemical composition of the nanocomposite samples obtained by the spin-coating process, the PCL/SiO_2ntbs 2.5 wt% and PCL/SrHAnrds 2.5 wt% nanocomposite films and their pure components: PCL (as spin-coated polymer thin film), SiO_2ntbs and SrHAnrds (in powdered form) were examined using Fourier transform infrared spectroscopy (FTIR). FTIR spectra were obtained using a Perkin-Elmer FTIR spectrometer, model Spectrum One, in absorbance mode, in the spectral region of 400–4000 cm^{-1}, using a resolution of 4 cm^{-1} and 64 co-added scans.

2.6 Differentiation towards bone and endothelium

2.6.1 Cell culture plating

Neat PCL films and the nanocomposites incorporating SiO_2ntbs or SrHAnrds, cut into pieces (10 mm × 10 mm squares) were sterilized in gradually reduced ethanol concentrations (100%–70%–50%) and after being washed three times with ddH_2O were let to air dry for 4 h under sterile conditions.

Fibrin glue was prepared after the blood sampling of a healthy volunteer donor. 15 μl of fibrin glue per film were placed in the bottom of a 24-well plate and the materials were seeded using a sterile pincher from above by applying minimal manual pressure and were let to air dry overnight under sterile conditions.

WJSCs were detached using Trypsin-EDTA 1x in PBS and were counted in a Neubauer cell counting chamber. 3×10^5 cells were re-suspended in DMEM full medium and were subsequently placed above the films of each condition. Upon air drying for 4 h in the incubator 1 ml DMEM full medium was added per well for the culture initiation. For the adherence of the MOMCs in the upper PCL surface 3 days before the completion of the culture (day 8–9), a mild detachment using Trypsin-EDTA 1x in PBS was performed and transfer without centrifugation in pre-coated with 0.1% poly-L-lysine in gelatin PCL films. 4×10^5 cells dissolved in DMEM full medium enriched with SDF-1a were plated per PCL film and were let to air dry as described above for the WJSCs. After 48 h the medium was changed for the final time towards the culture completion.

2.6.2 Induction of differentiation

2.6.2.1 Induction of differentiation towards endothelium Aiming the cell differentiation towards endothelial ones, endothelial cell growth medium was added in the WJSCs that covered the PCL film surfaces in a confluency of approximately 80% for 32 days with medium changes every 2–3 days and incubation in 37 °C with 5% CO_2. As for the attached MOMCs respectively, the induction was introduced 2 days before the completion of their 12 days generation culture and lasted 14 days with differentiation medium changes every 2–3 days in the same conditions as the WJSCs. Solely plated cells in plastic surfaces were used as a control group in the same number as the rest of the groups.

Aiming the amplification of the inducted MOMC differentiation towards endothelium, 2×10^5 HUVEC cells were counted and plated per well in the upper department of semi-permeable transwell membrane systems. 0.2 μl of endothelial cell growth medium were introduced in the start of the differentiation and remain until the first change of medium.

2.6.2.2 Induction of differentiation towards bone The induction of differentiation towards bone cells was accomplished by introducing osteogenesis medium in the culture additionally to the endothelial growth medium in

both WJSCs and MOMCs for 28 days. Cells plated in plastic surfaces were used as a control group, in the same number as the rest of the other groups.

2.7 Evaluation of differentiation

2.7.1 Fluorescent microscopy

The observation of the cells above the materials was performed in a fluorescence microscope 0, 14 and 30 days upon their attachment upon the films until the completion of the inducted differentiation for every cell line. HBO 50 mercury lamp as well as reflectors with fluorescence filter (excitation 488 nm, emission 509 nm) were used while the program for the download and editing of the photos was the Fluorescence Lite software module of AxioVision LE (Carl Zeiss, Oberkochen, German).

2.7.2 Scanning electron microscope (SEM)

All the included materials were fixed in 4% Paraformaldehyde for 20 min in RT and were subsequently let to air dry overnight. All materials and controls were covered with carbon black and observed with JEOL type SEM (JMS-840, Peabody, MA, USA), in 20 kV acceleration, electric current 45 nA and counting time 60.

2.7.3 Monitoring of the immunophenotypical differentiation potential with flow cytometry

The differentiation capacity of both WJSCs and MOMCs towards endothelial cells was estimated in the 0, 3, 14 and 31 days and 0, 3 and 14 days respectively via flow cytometry for the co-expression of the CD45/CD144 surface markers as described above.

2.7.4 Evaluation of the differentiation capacity in transcriptional level with real-time PCR

Aiming the comparison of the expression levels of genes related with self-renewal (CD34) and differentiation towards endothelial cells (CD144, VEGF) in all the includes types of films for both WJSCs and MOMCs, real-time polymerase chain reaction (PCR) with the use KAPA SYBR® FAST one step qPCR Master Mix (2X) Kit was performed. More precisely, 0, 14 and 30 days upon differentiation, the cells were detached by using 0.05% Trypsin-EDTA followed by RNA extraction procedure according to the manufacturer's instructions (Macherey–Nagel, Düren, Germany) and a subsequent RNA quantification in a NanoDrop ND-1000 UV–Vis Spectrophotometer. After a cautious primer design as well as HPLC Purification (Lab Supplies, Athens, Greece), Q-PCR for 10 ng of RNA template was performed, the results of which were analyzed by using ddCt algorithm for the analysis of the relative changes in gene expression.

2.7.5 Control of the osteogenesis induction with alizarin red staining

The successful induction of differentiation towards bone cells was verified with Alizarin Red staining. The quantification of the procedure was carried out with 10% cetylpyridinium chloride (CPC) in 10 nM sodium phosphate, pH 7.0 for 15 min in RT. The extracts were 10 times dissolved in 10% CPC and the optical density was counted in 562 nm wave length in a Perkin Elmer spectometer.

2.8 Statistical analysis

Data are presented as the mean ± SD and the Student's *t* test (unpaired, two-tailed) was used for the two-group comparisons. Differences were considered statistically significant at a value of $p \leq 0.05$.

3 Results

3.1 Cell lines

3.1.1 WJSCs and HUVEC characterization

Upon the completion of the cultures, the WJSCs displayed a typical fibroblast like-morphology before and after the genetic modification (Fig. 1A) and were additionally characterized by the co-expression of the CD90/CD105 markers as well as the absence of hematopoietic markers (CD45-/CD34-) as expected (Fig. 1B). The HUVEC cell line, respectively, displayed an endothelial cell like-morphology (Fig. 1A) while, approximately in total, expressed the endothelial marker CD144 following its successful adherence upon the plastic surface (Fig. 1B).

3.1.2 Optimization of MOMCs culture

The cultivation of MOMCs in plastic surfaces coated with poly-L-lysine in gelatin leads to the isolation of a morphologically more purified cell population in contrast to the cultures of MOMCs on fibronectin layer, where except of the MOMCs isolation, cells with different morphology such as macrophages, are also observed. The adherence of a specific cell population allow as to perform differentiation tests to specific cell subpopulations (Fig. 2A). The Poly-L-lysine/gelatin cultivated MOMCs were characterized by high expression of the CD45/CD14/CD34/CD184/Col I markers (Fig. 2B). The efficient and successful

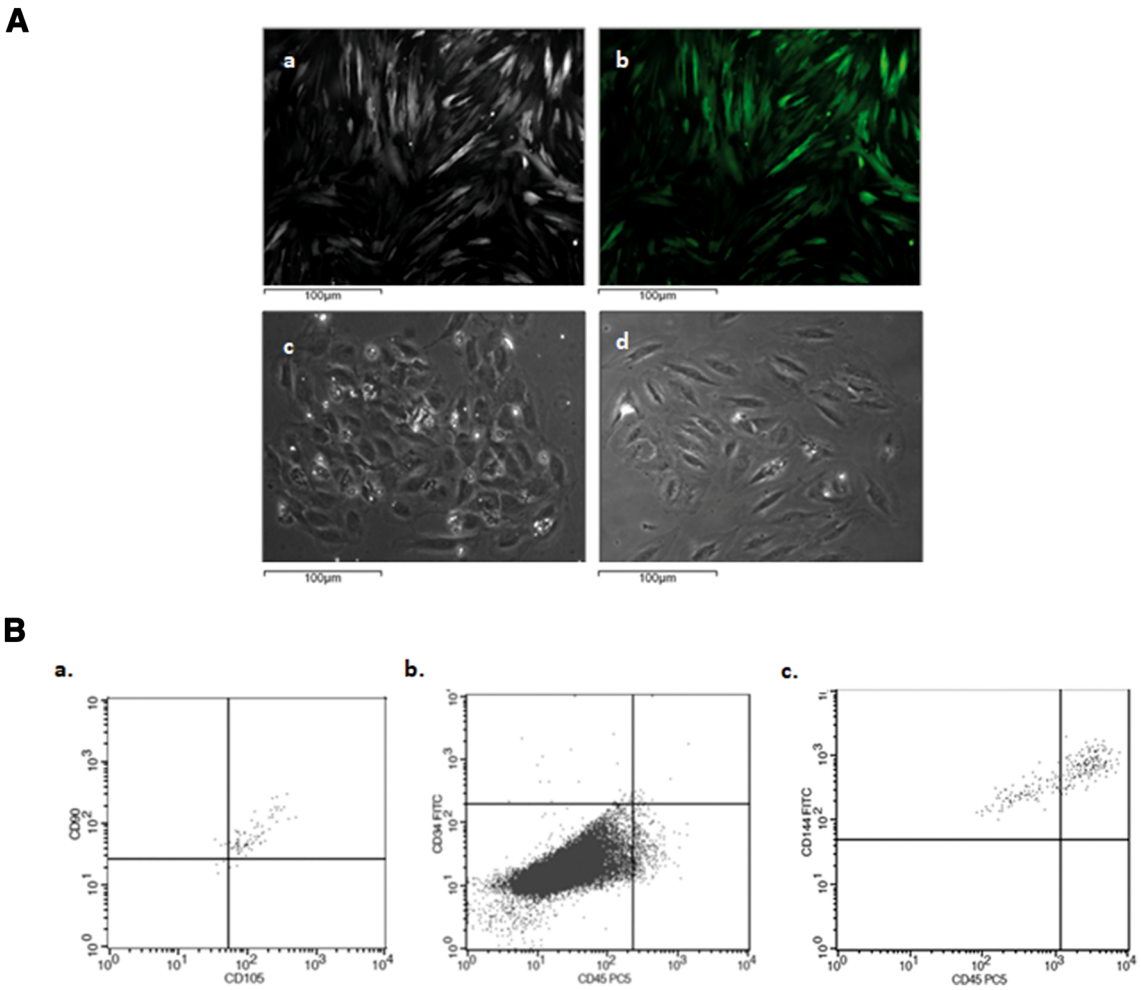


Fig. 1 **A** Morphology of genetically modified normal WJSCs (a, b) and HUVEC (c, d) in cell culture. **B** Immunophenotypic characterization of WJSCs (a, b) and HUVEC (c) via flow cytometry

MOMCs growth in the improved and novel coating medium, combined with the addition of the SDF-1a chemotactic factor, augmented the adherence capacity of the MOMCs, aiming their subsequent adherence upon the biomaterials.

3.2 *In vitro* cytotoxicity assay by MTT and chemical characterization by FTIR

3.2.1 MTT assay of SiO_2ntbs and SrHAnrds incubated with WJSCs

SiO_2ntbs and SrHAnrds displayed no significant toxicity upon their addition, even in high concentrations, in WJSCs cultures after 24 h (Fig. 3A). Even 72 h after SrHAnrds initiation in the culture and also in the maximum concentration of 200 μg/ml, NPs-mediated cytotoxicity was not observed (Fig. 3A). This rather non-cytotoxic activity of the tested NPs had therefore led us to the next step of their incorporation upon PCL films.

3.2.2 Study of chemical composition by FTIR

In this study, FTIR was used to examine the incorporation of SiO_2ntbs and SrHAnrds into the PCL biopolymer matrix by the spin-coating technique. Figure 3B shows the FTIR absorption spectra of PCL/SiO_2ntbs 2.5 wt% and PCL/SrHAnrds 2.5 wt% nanocomposite films. The FTIR spectra of pure SiO_2ntbs, SrHAnrds and PCL are also presented in Fig. 3B, for comparison purposes. In the spectrum of PCL/SiO_2ntbs 2.5 wt% the band broadening at 2942 and 2862 cm^{-1} (assigned to asymmetric and symmetric stretching of CH_2 groups of PCL) and at 1723 cm^{-1} (due to the carbonyl stretching vibration of PCL) can be seen as an is evidence of chemical interactions between the components (Fig. 3B) [23]. Additionally, while the characteristic band of the silica network (symmetric and asymmetric

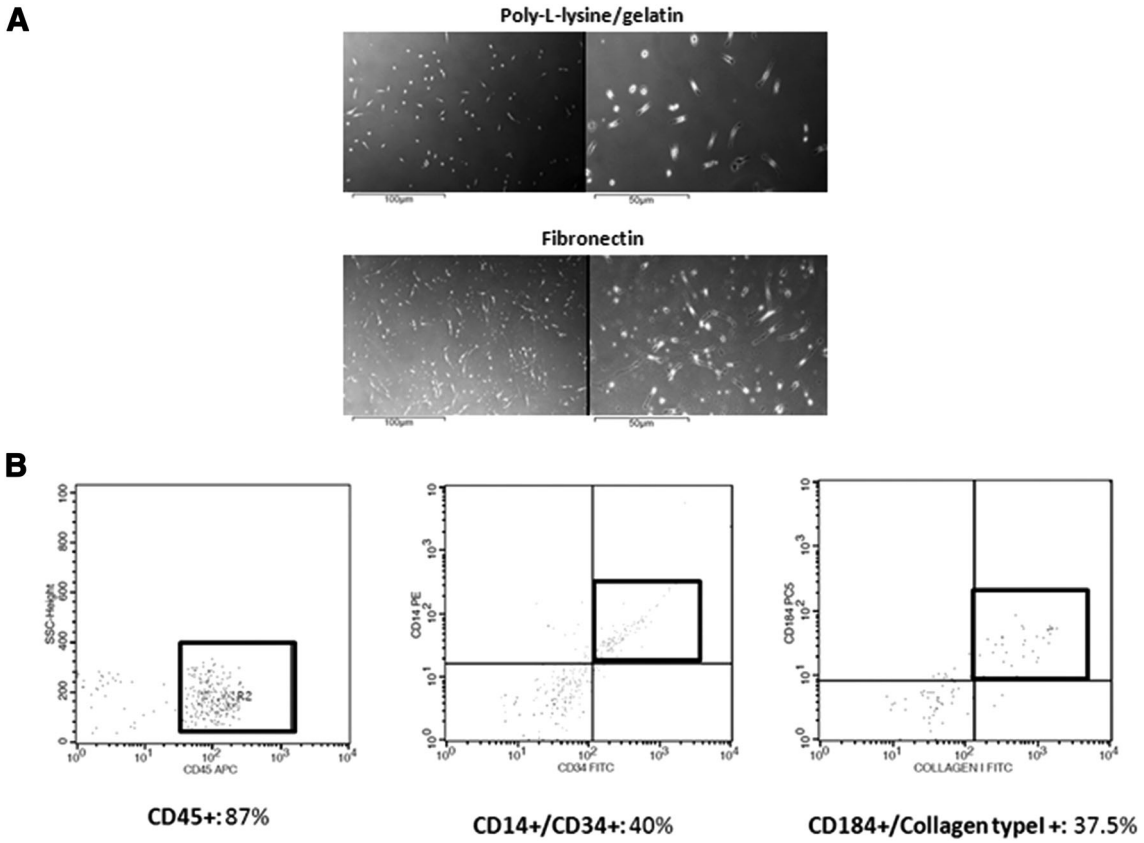


Fig. 2 **A** Comparative MOMCs culture in the presence of poly-L-lysine (upper) and fibronectin (below). **B** Immunophenotypic characterization of MOMCs after culture in the presence of poly-L-lysine and medium supplemented with SDF-1a

–Si–O–Si– stretching at 1100 cm^{-1} and 473 cm^{-1}, respectively) can't be observed in the spectrum of PCL/SiO_2ntbs 2.5 wt% sample, because PCL has multiple bands in the same spectral region, some new bands which could be attributed to O–H stretching for the silicate, arise at 3633 and 3385 cm^{-1} [24].

In Fig. 3B, the spectrum of PCL/SrHAnrds 2.5 wt% the broadening of the characteristic bands of PCL at 3000–2850 cm^{-1} and at 1723 cm^{-1} (due to asymmetric-symmetric stretching of CH_2 groups and stretching vibration of C=O group, respectively) is critical for the presumed intramolecular interaction between the nanofiller and the polymer matrix. As before, multiple overlapped peaks were detected and the characteristic absorption bands of the apatite (at 1082, 1017, 598 and 561 cm^{-1}, due to the asymmetric stretching and bending vibrations of O–P–O in PO_4^{3-} groups) can't be seen in the spectrum of the nanocomposite PCL/SrHAnrds 2.5 wt% sample [25]. Nevertheless, the successful incorporation of SrHAnrds in the polymer matrix can be confirmed by the typical OH stretching, which is present as a distinctively large band above 3300 cm^{-1}.

3.3 Evaluation of MOMCs and WJSCs differentiation

3.3.1 Differentiation towards endothelium

3.3.1.1 Morphological differentiation The differentiation of MOMCs upon the PCL films was completed 14 days after the culture initiation, due to the MOMCs distinct properties, which don't permit their time consuming culture. On the contrary, the control group WJSCs differentiated after 30 days. Aiming to accelerate the induction of differentiation, the MOMCs cultures were also boosted, apart from the differentiation medium, by the presence of HUVEC cells into semi-permeable culture systems. The morphology of the cells was characterized by gradually observed differences in their size and shape suggesting a possible differentiation towards endothelial that was finally confirmed both in transcriptional and protein level with real time PCR and flow cytometry respectively (Fig. 4). The seeding of WJSCs and MOMCs on PCL scaffolds is totally different before the induction of differentiation. This can be explained by the morphology, shape, size and cell interaction during the initial seeding. According to this observation, the following changes to the cellular

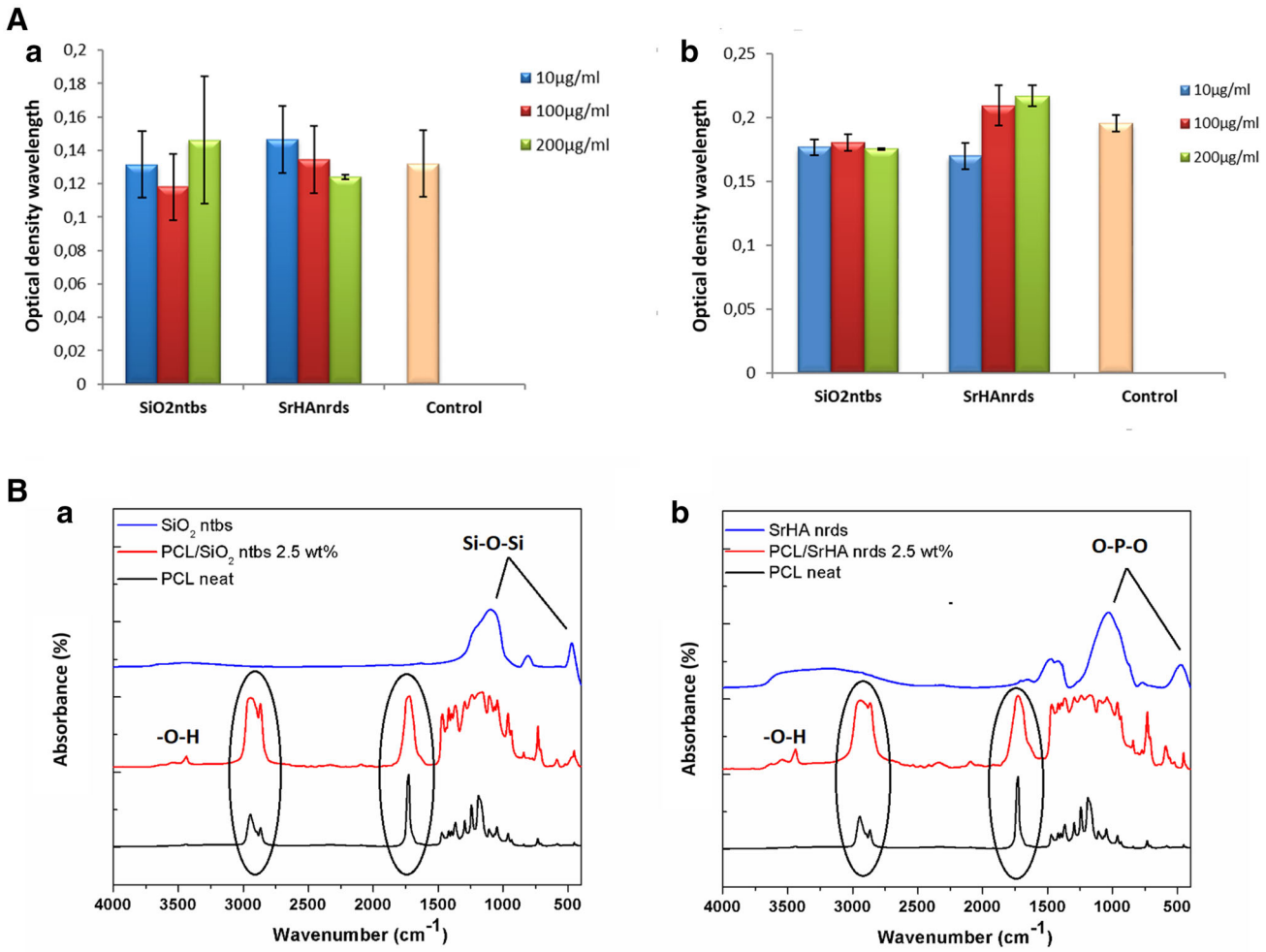


Fig. 3 **A** Testing of the NPs-mediated cytotoxicity in WJSCs cultures with MTT assay (a) 24 h and (b) 72 h after their addition in growth medium. **B** FTIR spectra of (a) PCL/SiO_2ntbs 2.5 wt% and (b), PCL/SrHAnrds 2.5 wt% in comparison to the spectra of their pure components

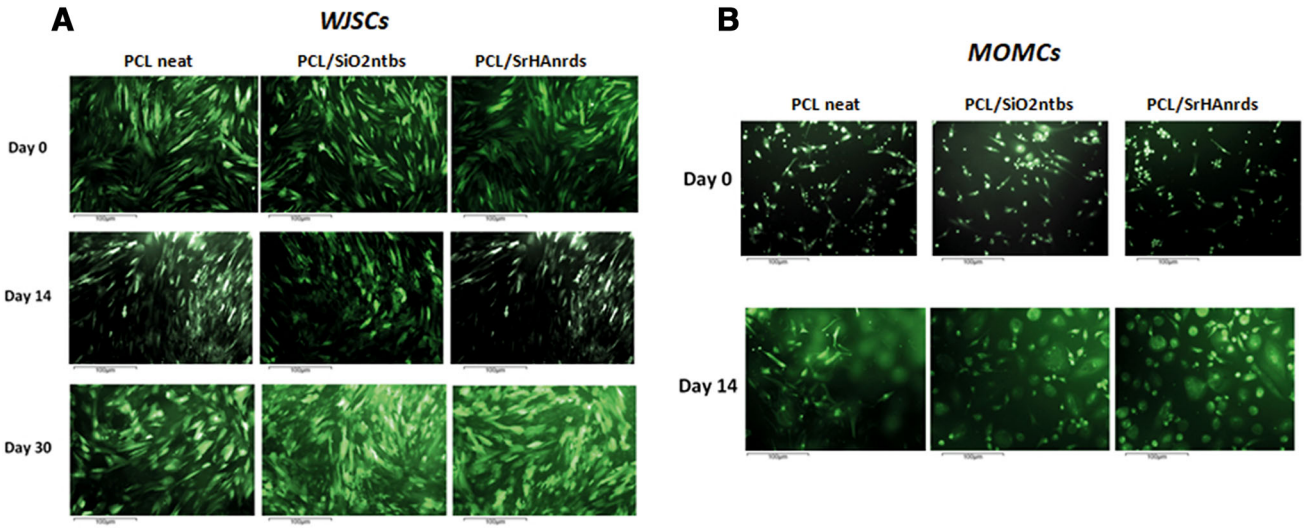


Fig. 4 Observation of morphological differentiation of **A** WJSCs and **B** MOMCs upon endothelial differentiation on different NP category PCL films

membranes as a result of induced differentiation towards endothelial cells, also lead to different final image, despite the fact that the final cell populations are characterized by similar endothelial markers. Moreover, the origin of MOMCs comparing to the WJSCs pluripotency capacity, limit us to apply different time duration for the differentiation process, something that could also affect the final cellular morphology upon the end of endothelial induction.

We present for the first time MOMCs cultures upon PCL films, before and after their induced differentiation towards endothelial cells, with SEM. Differences that depicted in cells' morphology and adherence on PCL nanocomposites with SEM, suggesting a possible differentiation that was finally confirmed with appropriate methods for the accurate quantification of endothelial induction.

3.3.2 Evaluation of differentiation in both transcriptional and protein level

The immunophenotypic characterization with flow cytometry (Fig. 5A) showed an unambiguous differentiation towards endothelial cells with a gradual increase of the respective endothelial marker CD144 expression during the culture (Fig. 5B). In both MOMCs as well as the WJSCs cultures, the final percentages of the CD144 expression were high, while the PCL/SiO_2ntbs sample displayed a statistically significant difference, proving the induced angiogenetic activity of silica. In just 14 days of triple mediated induction of differentiation, MOMCs express the endothelial marker in percentages far greater that the WJSCs ones even 30 days upon the differentiation.

In the transcriptional level, as proved by the Q-PCR upon the completion of the differentiation cultures, the CD144 expression manifests a similar path compared with the flow cytometry, while the PCL-Si films maintain the highest expression levels in both MOMCs and WJSCs (Fig. 6). The expression of the angiogenetic factor VEGFR is also high in both cell categories (Fig. 6), while the MOMCs differentiation was additionally confirmed by the gradual loss of the CD34 expression, initially characterizing the MOMCs (Fig. 6).

3.3.3 Differentiation towards bone

3.3.3.1 Morphological differentiation The morphology of the cells was characterized by gradually observed differences suggesting a possible differentiation towards bone cells for both MOMCs and WJSCs after the completion of the induced differentiation as depicted with SEM (Fig. 7).

3.3.3.2 Quantification of differentiation Following the completion of the induced differentiation towards bone cells, the evaluation was performed with the CPC method. A distinct differentiation towards bone cells was observed in both MOMCs and WJ-SCs upon the PCL/SrHAnrds 2.5 wt% films, with the MOMCs being characterized by a potential greater induction although being cultivated for just 14 days in the presence of a differentiation medium without an exogenous cellular stimulus this time (Fig. 8). It seems that for the PCL/SiO_2ntbs 2.5 wt% films the induction follows a similar differentiation capacity with the control group, presenting the beneficial role of silica in osteogenesis (Fig. 8).

4 Discussion

During the endothelial differentiation, MOMCs are progressively losing their native markers and gain endothelial lineage features in both protein as well as transcriptional level, with a statistically significant difference over the differentiated cells onto PCL/SiO_2ntbs 2.5 wt% nanocomposite films versus pristine PCL ones ($*p = 0.008$). Moreover, the nanocomposite materials, during the culture, remain non-cytotoxic, allow the growth and cell differentiation, while the impact on cell morphology confirms the successful differentiation in the presence of the appropriate stimulations.

24 h exposure of WJSCs to both SiO_2ntbs and SrHAnrds indicated no cytotoxicity. In all three concentrations, 10 μg/ml, 100 μg/ml and 200 μg/ml no significant change was observed versus the control group. In addition, after 72 h, the presence of SiO_2ntbs and SrHAnrds has no significant impact on the cell viability even in the highest given concentrations. Furthermore, MTT shows cell proliferation from day 1 to day 4.

These results indicate that both NPs present no adverse effect to WJSCs for exposure for at least 3 days. It is also worth noting that in the presence of NPs in the culture, even 3 days after the initiation, the cell proliferation rate remains in high levels and in a parallel stability in comparison with the control group. This long time period, for which the addition of NPs does not consist with toxic response, grants an extension provided either to their *in vivo* time of action or the *in vitro* induced differentiation towards the regeneration of damaged areas of the body. Several researchers have reported that using nanomaterials as 2D cell culture substrate could effectively promote the differentiation of stem cells into specific lineages, and the stiffness, surface chemistry, alignment and several other parameters of the cell culture substrate (matrix) may work together to influence the fate of stem cells [32]. Our findings provide strong evidence that the resulting nanocomposite PCL thin films not only passively interact with cells, but also serve as mechanical stimuli that trigger a series of biological alterations and modulate cell behaviors. As for

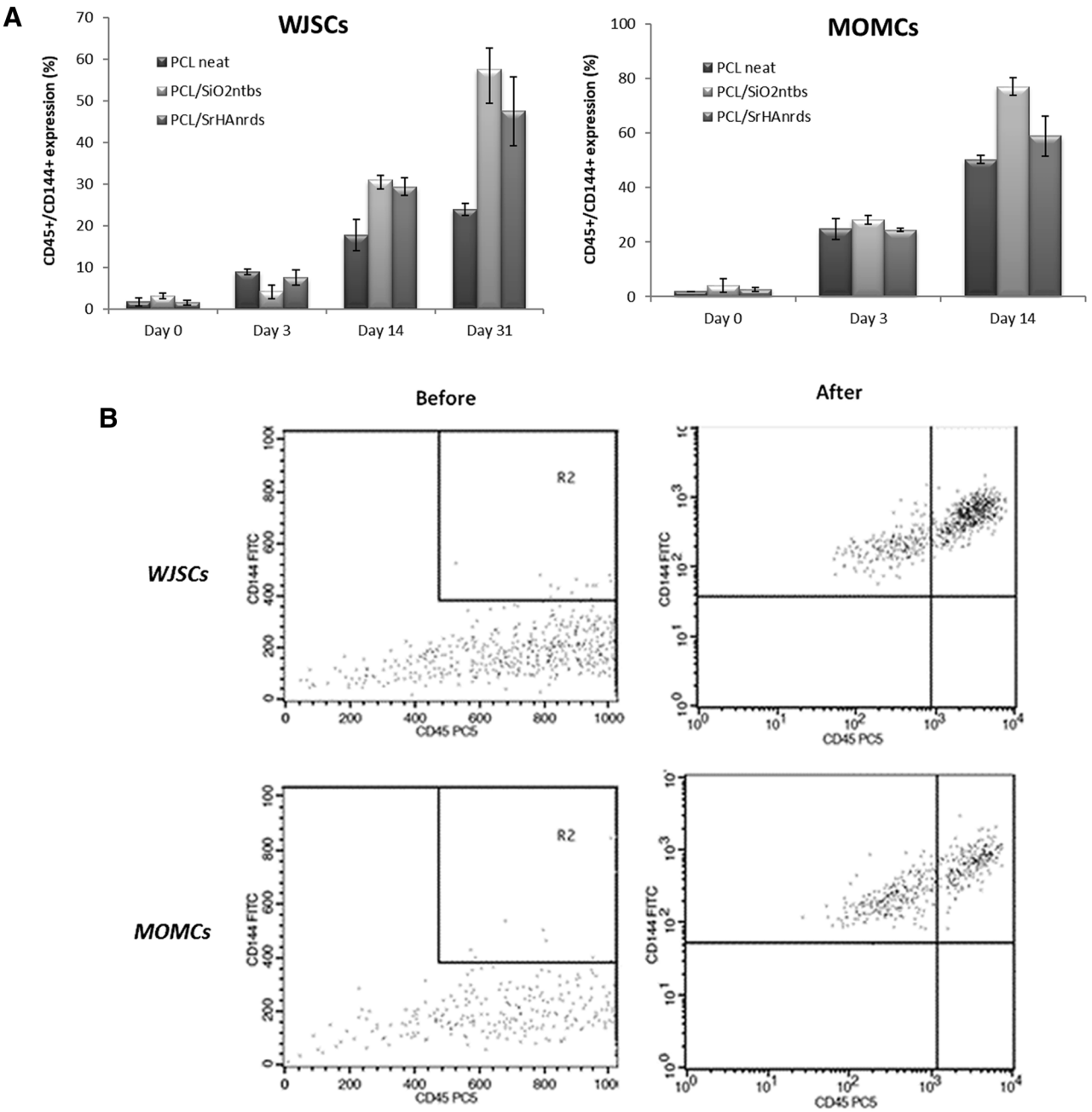


Fig. 5 **A** Immunophenotypic evaluation of the WJSCs (left) and MOMCs (right) differentiation towards endothelial cells. **B** Flow cytometry images of the CD45/CD144 marker expression before and after the completion of the WJSCs and MOMCs differentiation towards endothelium upon PCL/SiO_2ntbs films

the biological interaction and underlying mechanisms, further studies are needed to better understand how PCL matrix and the reinforcing components (SiO_2ntbs or SrHAnrds) mediate the molecular processes that are essential for regulating cell functions.

The intense regenerative potential of WJSCs renders them ideal candidates for various regenerative medicine applications [9, 26]. Their respective durability, even in the presence of high concentrations, increase the perspectives of using the two NPs for local injections in areas suffering bone damages or the accumulation of solid scaffolds enriched with either SiO_2ntbs or SrnHAnrds aiming the construction of new tissues upon a pre-constructed matrix [28].

Successful generation of MOMCs has been already confirmed in the presence of specific factors. MOMCs binding to the RGD domain of fibronectin via cell-surface α5β1 integrin [16] in combination with the interaction of

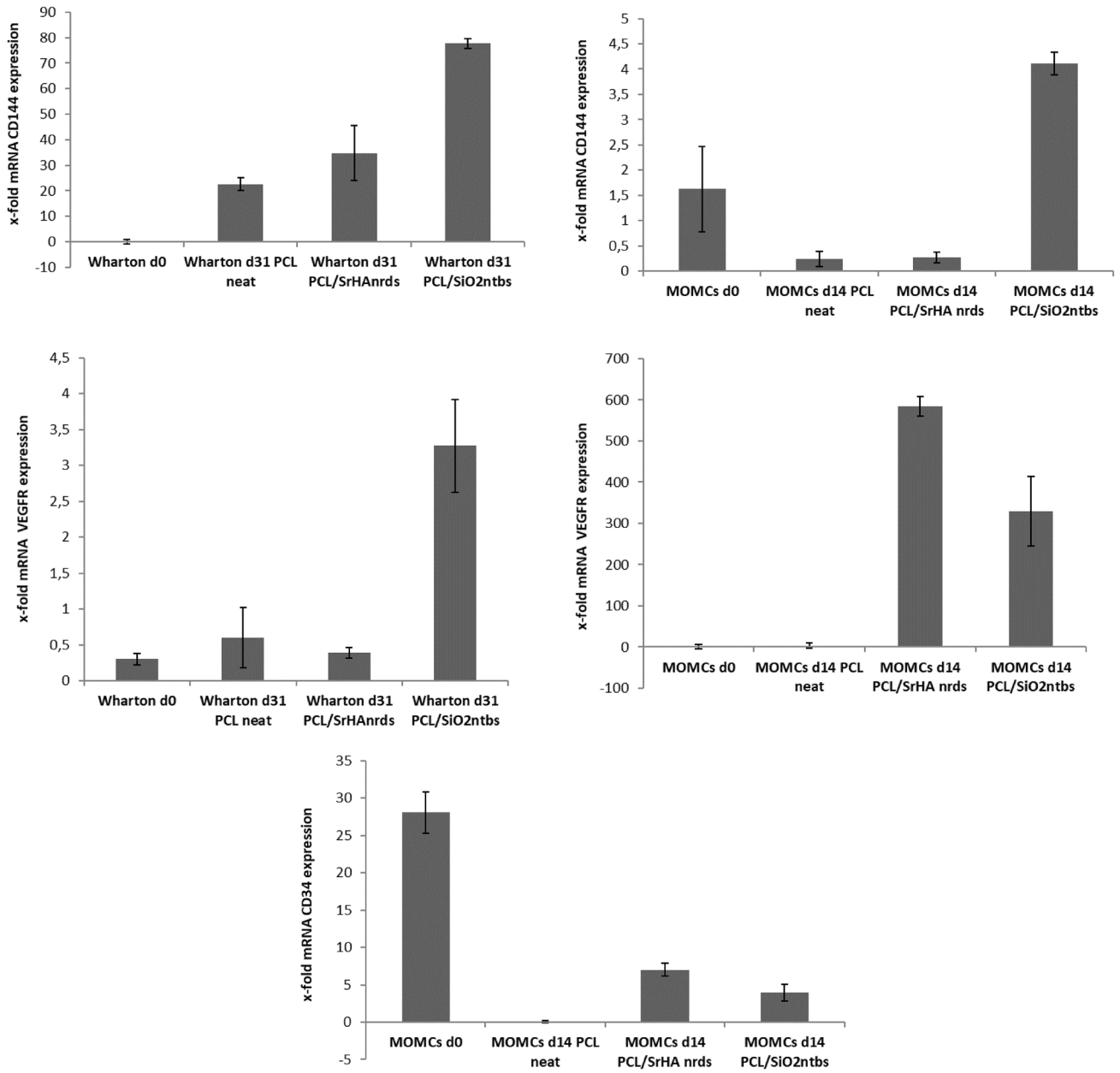


Fig. 6 Real-time PCR for the evaluation of MOMCs and WJSCs differentiation in transcriptional level

soluble SDF-1 factor with CXCR4 surface marker [15] is essential for the transformation of circulating monocytes into MOMCs, as we have proven here. This interaction throughout the culture maybe affects other cell types generation during this procedure, like macrophages, explaining larger number of observed cells compared to culture in the presence of poly-L-lysine in gelatin. For the first time, we present the successful development of MOMCs on PCL scaffolds for application to a variety of regenerative medicine applications. This study confirms the ability of MOMCs to maintain their ability to differentiate towards the desired tissues on PCL films. This element is particularly important as it makes the easily accessible MOMCs an ideal source for tissue engineering applications after their attachment to stable substrates.

Nowadays, the most promising strategy for the restore of damaged areas of the body is the stem cell therapy [29]. However, cell proliferation and differentiation resulting in tissue regeneration is difficult while cell organization to form complex tissue structure is limited without using a scaffold [3]. A three-dimensional matrix is necessary to guide and stimulate cellular activities [30]. It works as an anchor for cells that decreases the possibility of cell migration from the place of destination and allows tissue

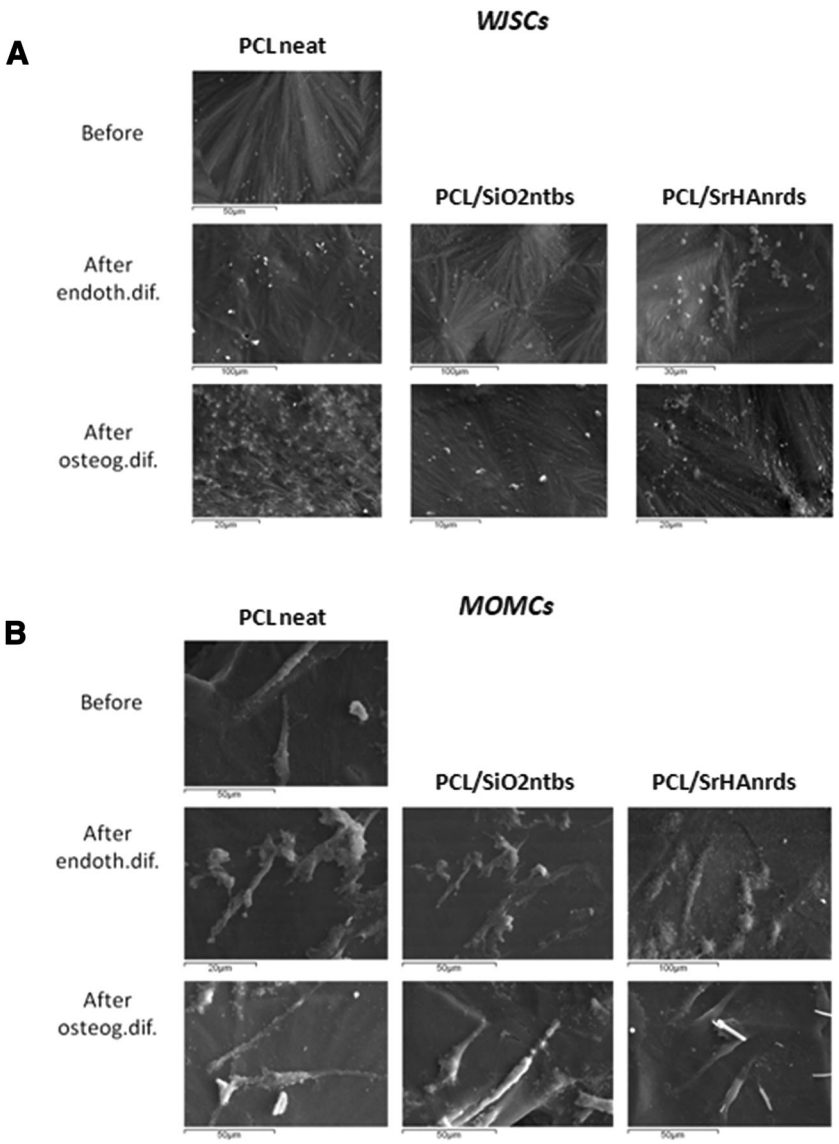


Fig. 7 **A**, **B** SEM depiction of the WJSCs and MOMCs before and after their differentiation upon PCL films

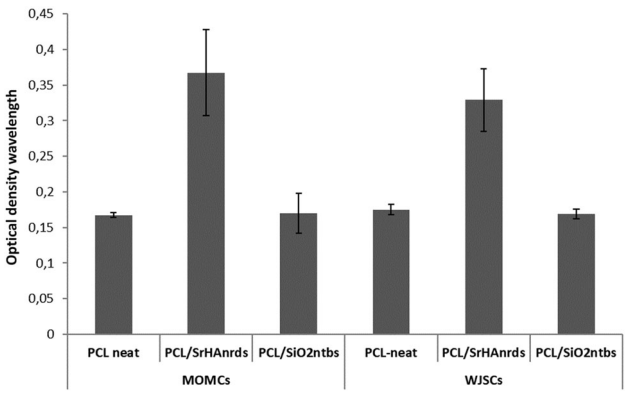


Fig. 8 Evaluation of the differentiation capacity towards bone cells with the CPC method for MOMCS as well as WJSCs

growth and differentiation *in vitro*. Despite its advantages PCL lacks bioactivity and thus the new tissue cannot bond tightly to the polymer surface. Therefore, it is important to incorporate reinforcing fillers into PCL matrix in order to result in a promising combination of bioactivity, biodegradability and strength [31]. Here we present the development of non-cytotoxic PCL nanocomposites capable of inducing targeted differentiation of adherent MOMCs into endothelium depending on the added nanofiller.

Enhancement of endothelial differentiation in the presence of HUVEC in semi-permeable culture systems contributes to the fastest differentiation of MOMCs in just 14 days compared to the WJSCs control group. Due to their previous cultivation in SDF-1a chemotactic factor for several days, MOMCs are exhausted before the initialization of differentiation procedure which requires at least 20 days without other stimulus except of the endothelial induction differentiation medium (Data not shown). By

applying the co-culture HUVEC-mediated system we facilitate and accelerate the differentiation process, especially taking into consideration that all this procedure must be performed on PCL surfaces. The HUVEC-mediated differentiation is not recommended as a way to prepare nanocomposite PCL films for endothelium regeneration. It is only an evidence that under strong stimulus MOMCs have the ability to rapidly differentiated towards the desirable tissue, as a commonly used technique to promote stem cells differentiation on a scaffold [33]. The combination of application of SiO_2ntbs and endothelial cell line in the cell culture environment accelerates the differentiation of MOMCs by ensuring the limited exhaustion of the cells in the culture. By applying the upper induction-mediated differentiation conditions we propose a novel system for fast and successful *in vitro* generation of endothelium from easily accessible MOMCs on PCL-based scaffolds.

In our work we managed to successfully isolate MOMCs from the peripheral blood of healthy volunteer donors which characterized by intense plasticity and differentiation ability towards multilineage directions. Both their successive isolation from adult tissues and their differentiation capacities make these cells ideal candidates for various forms of cellular therapies. However, cell proliferation and differentiation resulting in tissue regeneration for some diseases, is difficult without using a scaffold. Recent studies show that isolated cells are hardly able to organize themselves spontaneously to form complex tissue structures in the absence of a three dimensional matrix that guide and stimulate their activities. For this reason, we decided to use the PLC for the study of endothelial and osteogenic differentiation because it has excellent mechanical properties and slow degradation; thus it might be an optimal candidate to be used *in vivo* for tissue transplantation [34]. By incorporating SiO_2ntbs or SrHAnrds to PCL we suggest a novel way to stimulate, accelerate and facilitate the differentiation procedure during the tissue reconstruction, providing stimuli for targeted differentiation. The aim of this study was to demonstrate that nanocomposite PCL is a promising material to use as a support scaffold for the autologous reconstruction of new tissue starting from easily accessible MOMCs.

Here, we propose MOMCs as an alternative and easily accessible cell source for tissue repair applications. The developed nanocomposite PCL-based films, reinforced with SiO_2ntbs or SrHAnrds, support MOMCs attachment on their surface and promote their *in vitro* differentiation towards endothelium and bone, respectively. However, further studies should be performed to identify the underlying mechanisms of MOMCs differentiation on the surface of nanocomposite PCL-based films, in order to optimize the upper protocols, make the procedure less time-consuming and be able to propose these materials for commercial clinical use in combination with blood derivatives of patients, for applications in regenerative medicine.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement This study was approved by the Ethics Committee of Aristotle University of Thessaloniki, School of Medicine (390-9/1.7.2017) and University Hospital AXEPA of Thessaloniki (3868/24.1.2018). This research involves Human Participants after their informed consent. Peripheral blood samples were collected from healthy volunteer donors while mesenchymal stromal cells and HUVEC were isolated from Wharton's jelly after parents approval during stem cell banking in Biohellenika SA Biotechnology Company.

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