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# Adipose stem cell-derived osteoblasts sustain the functionality of endothelial progenitors from the mononuclear fraction of umbilical cord blood

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ABSTRACT

Vascularization is the most pressing issue in tissue engineering (TE) since ensuring that engineered constructs are adequately perfused after in vivo transplantation is essential for the construct's survival. The combination of endothelial cells with current TE strategies seems the most promising approach but doubts persist as to which type of endothelial cells to use. Umbilical cord blood (UCB) cells have been suggested as a possible source of endothelial progenitors. Osteoblasts obtained from human adipose-derived stem cells (hASCs) were co-cultured with the mononuclear fraction of human UCB for 7 and 21 days on carrageenan membranes. The expression of vWF and CD31, and the DiI-AcLDL uptake ability allowed detection of the presence of endothelial and monocytic lineages cells in the co-culture for all culture times. In addition, the molecular expression of CD31 and VE-cadherin increased after 21 days of co-culture. The functionality of the system was assessed after transplantation in nude mice. Although an inflammatory response developed, blood vessels with cells positive for human CD31 were detected around the membranes. Furthermore, the number of blood vessels in the vicinity of the implants increased when cells from the mononuclear fraction of UCB were present in the transplants compared to transplants with only hASC-derived osteoblasts. These results show how endothelial progenitors present in the mononuclear fraction of UCB can be sustained by hASC-derived osteoblast co-culture and contribute to angiogenesis even in an in vivo setting of inflammatory response.



## 1. Introduction

It is well established that vascularization is a critical issue when engineering a tissue [1,2]. Improper inosculation and blood perfusion after transplantation can result in the failure of an implant especially if its thickness prevents suitable oxygen and nutrient diffusion to the transplanted cells. This is a pressing issue in bone tissue engineering (TE) where typically a 3-D scaffold is seeded with osteogenic or osteoprogenitor cells, and transplanted in vivo for subsequent bone formation [1,2]. The current limited clinical potential of this approach is directly related to deficient vascularization in vivo, which results in necrosis in the bulk of the scaffold [1–5]. Therefore, several strategies are being employed to try to circumvent this important challenge, one of which comprises the use of endothelial cells to accelerate inosculation and the implant's vascularization after transplantation [6].

Endothelial progenitor cells (EPCs) are one type of endothelial cells that has recently attracted attention. These cells were first isolated from the mononuclear fraction of human peripheral blood by Asahara et al. [7] but have, since then, also been proven to exist in the bone marrow [8] and in umbilical cord blood (UCB) [9]. Studies have shown that EPC transplantation can increase angiogenesis and vessel formation in different ischemia models [10–13]. UCB is considered the best source of EPCs since it contains 10 times more CD34+ cells, one of the markers used to isolate EPCs, than peripheral blood [14]. Furthermore, other blood mononuclear cells, such as monocytes [15,16] and lymphocytes [17], have been shown to play a positive and essential role in the in vitro formation of EPC colonies, pointing to a potential advantage of not specifically isolating $CD34^+$ cells or other subpopulations to obtain EPCs.

The intimate relationship between bone tissue and blood vessels is well studied. Bone formation is always accompanied by blood vessel development both in embryogenesis and postnatal growth [18]. Bone multicellular units (BMUs), responsible for bone remodeling, are always adjacent to a blood vessel that grows as the BMU advances [19,20]. At the cellular level, endothelial cells have been proven to modulate the activity of osteoblasts and osteoblast progenitors by direct and indirect interactions [21–28]. On the

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other hand, osteoblasts are capable of controlling angiogenesis through the production of factors such as vascular endothelial growth factor (VEGF) [29–31], which is in turn upregulated when osteoblasts are in contact with endothelial cells [6]. More importantly, osteoblasts have been proven to support the maintenance of cells such as hematopoietic stem cells in several in vitro models [32,33] by direct contact [32].

Carrageenans (CRGs) are a class of galactan polysaccharides that behave as extracellular matrix materials, like agars, in specific species of red seaweeds, including *Gigartina*, *Chondrus* and *Eucheuma* [34–36]. These galactan polysaccharides have been used in many industrial and some pharmaceutical applications [35,37] and have been recently proposed for biomedical applications [38–40]. The CRGs are grouped into several sub-types according to their sulfation patterns and distribution of 3,6-anhydro-D-galactose residues [34,35]. In the present work, a kappa–iota hybrid CRG derived from the seaweed *Chondrus crispus* was used. This hybrid CRG presents several processing advantages, including a higher degree of flexibility, when compared to kappa CRG, and a higher robustness when compared to pure iota CRG [41,42]. Being a naturally occurring hybrid polymer, it behaves as a monocomponent system and not like a blend, concentrating in a single molecule characteristics of both kappa and iota types.

Therefore, the aim of this study was to define a co-culture system, using CRG membranes as carriers, composed by hASC-derived osteoblasts and cells from the mononuclear fraction of UCB by taking advantage of the osteoblast capacity to generate angiogenic conditions. The capacity of hASC-derived osteoblasts cultured on CRG membranes to support the in vitro survival of human endothelial progenitors present in the mononuclear fraction of UCB was addressed. The developed systems were then transplanted into nude mice in order to elucidate their in vivo functionality and therefore to allow us to consider the potential use of the mononuclear cells from UCB, without further selection, as a source of endothelial progenitors supported by co-culture with osteoblasts.

## 2. Materials and methods

### 2.1. Carrageenan membrane production

CRG-type iota–kappa (hybrid) extracted from the red algae *C. crispus* by CEAMSA Company (Spain) was used to produce the membranes. CRG was dissolved in distilled water to a final concentration of 2% w/v. While hot, 25–35 ml of the solutions were placed in square Petri plates and left to dry at room temperature for 48 h. To preserve their structural features while cutting, the formed membranes were placed in a low-hydrating solution of 17% (v/v) ethanol plus 3% (w/v) KCl (Sigma, USA) and cut into small discs of 1.1 cm diameter and 1 mm thickness. The CRG membranes were crosslinked in a water solution of 50% (v/v) 2-propanol (Fluka, Czech Republic), and 0.32 M KOH (Fluka) and 4.63 mM of epichlorohydrin (ECH, Aldrich, USA) for 24 h, at 37 °C under 100 rpm agitation. The samples were then washed twice with a 17% ethanol (v/v) and 0.1 M of NaOH (Panreac, Spain) solution, aiming at blocking the partially reacted ECH and twice with water at 50 °C for 1 h. Each group of CRG membranes used on each assay was then sterilized overnight with 70% ethanol. Before cellular assays, the membranes were maintained in sterile phosphate-buffered saline (PBS, Sigma) with 3% (w/v) KCl and washed twice in PBS immediately before cell seeding.

### 2.2. Cell isolation

UCB and adipose tissue were obtained under cooperation agreements established with 3B's Research Group and after approval of the respective Ethical Committees. All samples were obtained with the agreement of the patients and under total anonymity.

#### 2.2.1. Cord blood mononuclear cells (CBMNCs)

Cord blood was collected during planned caesareans in the Hospital de São Marcos, Braga, Portugal. Cord was clamped in the proximity of the abdomen of the newborn and cut. Cord blood was collected from the vein, before placenta removal, directly to the collection bag containing CPDA I (Fenway Europe Sprl, Belgium) as anticoagulant. Transport was carried out under temperature-controlled conditions and samples were processed within 2 h after collection. The blood was then diluted 2:1 in PBS (Sigma) and placed over Histopaque 1077 (Sigma) for density gradient centrifugation. For this, 5 ml of Histopaque at room temperature was placed in a 15 ml conical falcon tube and 3 ml of the cell suspension was carefully layered over it. The tubes were then centrifuged at 400*g* for 30 min at room temperature. Cells in the interface between plasma and Histopaque were recovered using a Pasteur pipette, washed three times in PBS (Sigma), counted and used for establishing the co-culture.

#### 2.2.2. Human adipose derived stem cells (hASCs)

Discarded subcutaneous adipose tissue from liposuction procedures was collected to sterile containers with 100 ml of PBS with 10% antibiotics (Invitrogen, USA) at the Hospital da Prelada, Porto, Portugal. Lipoaspirates were washed with PBS, in order to discard the majority of blood, and then digested with 0.05% collagenase II (Sigma) at 37 °C for 45 min. Following that, the digested tissue was passed through a strainer and centrifuged at 1000*g* for 10 min at 4 °C. The obtained pellet was resuspended in PBS and centrifuged again at 800*g* for another 10 min at 4 °C. The resulting pellet was then resuspended in minimum essential medium alpha-modification (alpha-MEM, Invitrogen), supplemented with 10% FBS (Gibco, USA) and 1% antibiotic–antimycotic (Gibco), filtered with a 100 μm pore size cell strainer (BD Biosciences, USA) and plated in tissue culture polystyrene flasks, using the culture medium described. After 24 h, colonies of spindle-shaped adhered cells started to appear.

### 2.3. Flow cytometry

After isolation, CBMNCs were screened by flow cytometry for the following markers: CD14-PE, CD45-FITC, CD31 APC, CD34-PE and CD73-PE (all from BD Pharmingen, USA), and CD105-FITC and CD90-APC (eBiosciences, USA). Cells were resuspended in cold PBS with 2% (w/v) bovine serum albumin (BSA) (Sigma) and 100 μl of cell suspension, with $2 \times 10^5$ cells, was incubated with the antibodies at the concentration advised by the manufacturer. After incubation for 20 min at room temperature in the dark, cells were washed with PBS/BSA, resuspended in PBS with 1% formaldehyde (Sigma) and analyzed in a BD FACScalibur flow cytometer (BD Biosciences). Cells of interest were gated in a forward vs. side scatter dot plot with a linear scale. Isotype controls were made to discern non-specific from specific staining. A minimum of 10,000 gated events were acquired and displayed in dot plots created using the Cyflogic software (version 1.2.1, CyFlo Ltd., Finland).

### 2.4. Co-culture set-up

The isolated hASCs at passage 3 were cultured in alpha-MEM supplemented with osteogenic factors, $10^{-8}$ M dexamethasone (Sigma), 10 mM beta-glycerophosphate (Sigma) and 50 μg ml$^{-1}$ ascorbic acid (Sigma) for 3 weeks in order to induce differentiation into the osteogenic lineage. After differentiation (Supplemental Fig. 1), cells were seeded on the membranes at a concentration of $82 \times 10^3$ cells cm$^{-2}$ that, according to our previous experience

with mesenchymal stem cells (MSCs) [43], would allow the cultures to reach confluence after 5 days. Cells were cultured in M199 medium (Sigma), commonly used to culture primary endothelial cells, supplemented with 10% FBS, 1% antibiotic–antimycotic, osteogenic factors, 50 μg ml$^{-1}$ endothelial cell growth supplement (ECGS, BD Biosciences) and 50 μg ml$^{-1}$ heparin (Sigma). After this time, the mononuclear cells isolated from cord blood were seeded on the membranes at a concentration 10-fold higher than that of hASCs, $82 \times 10^4$ cells cm$^{-2}$, given the reduced percentage of endothelial progenitors in the total cell number [44] (and cultured for a further 7 and 21 days in M199 medium supplemented with 10% FBS, 1% antibiotic–antimycotic, osteogenic factors, 50 μg ml$^{-1}$ ECGS and 50 μg ml$^{-1}$ heparin). Monocultures of hASC-derived osteoblasts in the membranes were used as controls.

### 2.5. DiI-AcLDL uptake assay

After each time point, co-cultures were incubated with 10 μg ml$^{-1}$ of the complex DiI-AcLDL (Invitrogen) for 4 h, at 37 °C and 5% $CO_2$. Cells were then washed with warm medium and fixed with 3.7% (v/v) buffered formalin (Sigma) for 20 min at room temperature. Cells were then counterstained with DAPI (Invitrogen) and visualized using an Axioplan Imager Z1 fluorescence microscope (Zeiss, Germany).

### 2.6. Immunocytochemistry

After each culture time point, the samples were fixed in a 3.7% (v/v) buffered formalin solution for 30 min at room temperature. After fixation, cells were washed three times in PBS and used immediately for immunocytochemistry. Non-specific binding was blocked by incubating the membranes with a 3% (w/v) BSA (Sigma) solution in PBS (Sigma) for 30 min. Cells were then incubated with the primary antibody diluted in 1% BSA/PBS for 1 h at room temperature. Mouse anti-human CD31 (1:30, Dako, Denmark) and mouse anti-human vWF (1:100, Dako) primary antibodies were used. Cells on the membranes were then washed three times with PBS (Sigma) and incubated for 1 h at room temperature with an appropriate AlexaFluor (Invitrogen) secondary antibody diluted 1:500. After this time cells were washed in PBS, counterstained with DAPI, and analyzed in an Axioplan Imager Z1 fluorescence microscope (Zeiss).

### 2.7. Quantitative real-time RT-PCR

Messenger RNA (mRNA) of cells was extracted, after each in vitro time-point, using Tri-reagent (Sigma) according to the manufacturer's instructions. 800 μl of the reagent was added per $1 \times 10^6$ cells and samples were homogenized by vigorous pipetting. Following 5 min incubation, 160 μl of chloroform (Sigma) was added to each sample, which were then incubated for 15 min at 4 °C, and then centrifuged at 13,000 rpm and 4 °C for 15 min. After centrifugation the aqueous part was collected and an equal volume of isopropanol (Sigma) was added. Following an overnight incubation at −20 °C, samples were centrifuged at 9000 rpm and 4 °C for 10 min. Supernatants were discarded and pellets were washed in ethanol, centrifuged at 9000 rpm and 4 °C for 5 min and resuspended in 12 μl of RNase/DNase-free water (Gibco, UK). RNA quantity and purity were assessed with a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA). Samples with a 260/280 ratio between 1.6 and 2.0 were used for cDNA synthesis. The cDNA synthesis was performed using iScript cDNA synthesis Kit (BioRad, USA) and the thermoblock of the MiniOpticon Real-Time PCR Detection System (BioRad). An initial amount of 2 μg of mRNA was used in a total volume of 20 μl of RNase/DNase-free water.

Osteocalcin and osteopontin transcripts were quantified in the cDNA samples using a quantitative real-time PCR reaction. For each sample GAPDH was used as the housekeeping gene. The primers were designed using Primer 3 software (version 0.4.0) and synthesized by MWG Biotech (Germany) as follows: human GAPDH forward 5′ ACA GTC AGC CGC ATC TTC TT3′ and reverse 5′ GAC AAG CTT CCC GTT CTC AG 3′; human osteocalcin forward 5′ GTG CAG AGT CCA GCA AAG GT 3′ and reverse 5′TCC CAG CCA TTG ATA CAG GT 3′: human osteopontin forward 5′CCC ACA GAC CCT TCC AAG TA 3′ and reverse 5′ GGG GAC AAC TGG AGT GAA AA 3′; human collagen I forward 5′ AGC CAG CAG ATC GAG AAC AT 3′ and reverse 5′ ACA CAG GTC TCA CCG GTT TC 3′; human VE-cadherin forward 5′ CAC AGT GCT GGC CAT GGA 3′ and reverse 5′ GTC CTG CGG ATG GAG TAT CC 3′; human CD31 forward 5′ AAG GCC AGA TGC ACA TCC 3′ and reverse 5′ TTC TAC CCA ACA TTA ACT TAG CAG G 3′; human vWF forward 5′ CCC TGG GTT ACA AGG AAG AAA AT 3′ and reverse 5′ AGT GTC ATG ATC TGT CCT CCT CTT AG 3′. A concentration of 200 nM of primer was used in a final volume of 25 μl of sample. The real-time PCR reaction was done using the iQ Syber Green SuperMix (BioRad), following manufacturer's instructions, in a MiniOpticon Real-Time PCR Detection System (BioRad).

The relative quantification of gene expression was performed using the $2^{-\Delta\Delta CT}$ method (Perkin Elmer User Bulletin No. 2). All values were first normalized against GAPDH values and the results of the expression of the osteogenic genes in the co-cultures were normalized using osteoblast monocultures prior to establishing the co-cultures while the results of the expression of endothelial genes were normalized using UCB mononuclear cells after isolation and prior to co-culture.

### 2.8. In vivo assay

All in vivo assays were performed after consent from the internal Animal Experimentation Ethical Committee.

Three animals were used per condition, per culture time point and per implantation time point for a total of 24 animals. Four week old female Balb/C nude mice with an average weight of 20 g (Charles River Laboratories Inc., USA) were anesthetized with a mixture of ketamine (1.2 mg/mouse s.c., Imalgene® 1000, Merial, Lyon, France) and medetomidine (20 μg/mouse s.c., Domitor®, Orion Corp., Finland) prepared in physiological serum. After the confirmation of analgesia/anesthesia, one incision was performed, reaching a maximum of 1.5 cm, in the intrascapular region. Two craniolateral oriented pockets were created by blunt dissection and the implants were subcutaneously inserted. Two CRG membranes with the same experimental condition were implanted in each animal. The incisions were sutured and the mice transferred to heated recovery compartments. When the recovery from analgesia/anesthesia was confirmed, they were returned to their respective cages and kept under food and drink ad libitum. After 7 and 21 days after implantation, animals were killed with an intracardiac overdose of anesthesia for implant retrieval, and processed for characterization.

### 2.9. Histology

Standard hematoxylin and eosin (H&E) staining was used to make a general morphological assessment of the sectioned tissues. Briefly, sections were deparaffinized in xylene (Thermo Scientific, USA) and hydrated by incubation with increasingly diluted ethanol solutions. Sections were then washed in water and incubated with hematoxylin (Sigma) for 5 min. After this, sections were washed under running tap water for 10 min and finally double-distilled $H_2O$. After incubation with eosin (Sigma) for 1 min, sections were

dehydrated with ethanol, washed with xylene and mounted with Histofluid (Marienfeld, Germany).

### 2.10. Immunohistochemistry

After deparaffinization, sections were incubated with antigen retrieval solution (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) and heated in a microwave for 2 min at maximum power. After cooling, sections were incubated with 3% $H_2O_2$ for 5 min to inhibit non-specific peroxidase activity and washed with PBS. The next step was to block non-specific protein interactions by incubating with a 3% (w/v) BSA solution for 30 min. Sections were then washed in PBS-tween 20 (PBS-T, Sigma) and incubated with the mouse anti-human CD31 (1:30, Dako) primary antibody overnight at 4 °C. Sections were then washed in PBS-T and incubated for 45 min at room temperature with a biotinylated secondary antibody (Vector Labs, UK). After washing, cells were incubated first with Streptavidin-HRP (Vector Labs) and then with 3,3′-diaminobenzidine (DAB) chromogenic substrate (Vector Labs). Cells were then observed in an in an Axioplan Imager Z1 microscope (Zeiss).

### 2.11. Blood vessel quantification

After H&E staining, sections were observed in an Axioplan Imager Z1 microscope and analyzed for blood vessels using Axiovision software (Zeiss). The number of perfused blood vessels was counted in the periphery of the implants within a 200 μm distance (Supplemental Fig. 2). Three different sections with two cuts per sample (n = 6) were used in this procedure.

### 2.12. Statistical analysis

In vitro data was obtained from four independent experiments with three replicates for each condition and averaged. Standard deviation is reported as a measure of sample deviation. Statistical analysis of blood vessel quantification was performed using one-way ANOVA test with the Tukey–Kramer method as a post-hoc pairwise comparison test. The statistical analysis of the quantitative real-time RT-PCR results was performed using the Student's *t*-test for n = 4. Values were considered statistically significant for $p < 0.05$.

## 3. Results

### 3.1. CBMNC phenotype

The expression profile of CD14, CD45, CD34, CD31, CD90, CD105 and CD73 markers in the isolated human CBMNCs was determined by flow cytometry (Fig. 1). The isolated cells expressed the hematopoietic marker CD34 (1.8%), the monocytic marker CD14 (4.94%), the CD90 (6.27%) and the CD105 (7.8%) surface proteins. In addition, 13.8% of the population was positive for the expression of CD73, while 4.9% expressed the endothelial marker CD31. As expected, almost all of the mononuclear fraction (close to 93%) was positive for CD45 (not shown).

### 3.2. Co-culture characterization

The uptake of the DiI-AcLDL was assessed after 7 and 21 days of in vitro co-culture. Cells with internalized DiI-AcLDL were observed throughout the surface of the membranes and without any specific organization (Fig. 2). The morphology and distribution of cells did not change throughout the time of co-culture. A similar cellular distribution was observed when cells in the co-culture were stained for CD31 and vWF markers (Fig. 2). As for the DiI-AcLDL, the pattern of expression of CD31 and vWF was similar for all the tested co-culture times.

The quantification of the transcripts for the genes of interest was also performed after 7 and 21 days of co-culture (Fig. 3). The expression level of osteogenic genes, osteopontin, osteocalcin and collagen I, did not change in relation with monocultures for the same time period. On the contrary, the expression of vWF, VE-cadherin and CD31 genes significantly increased when compared to monocultures for the same time period. The variation of the expression of vWF for 7 days of co-culture was of 2.8-fold, while for 21 days of co-culture the fold change was of 2.7. In the case of the VE-cadherin gene, for 7 days of co-culture the expression varied 3.0-fold while for 21 days of co-cultures there was a change of 13.5-fold. Regarding the CD31 gene expression a positive

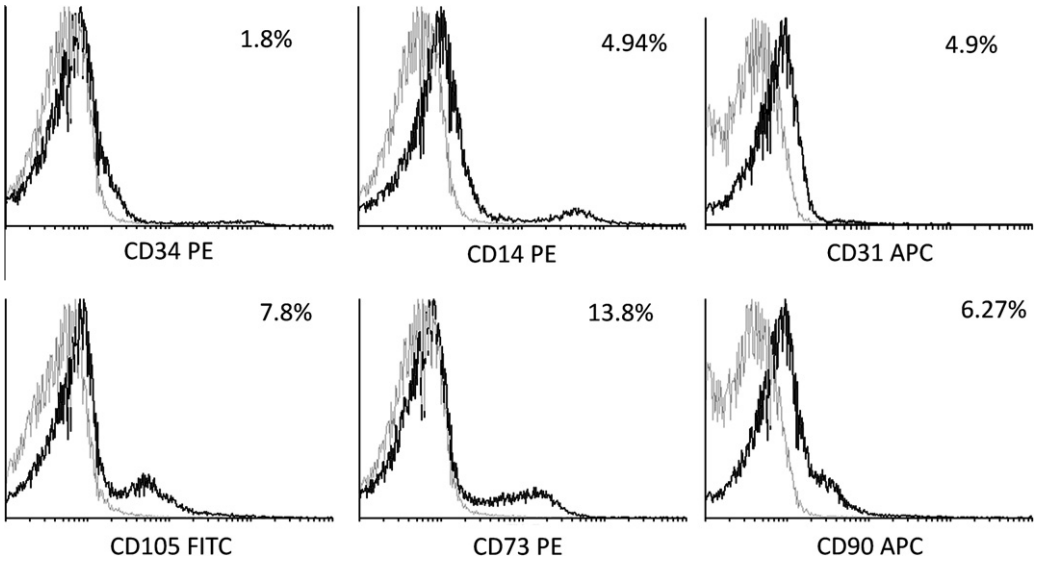


**Fig. 1.** Flow cytometry analysis of CBMNCs immediately after isolation and prior to seeding. Cells were gated to exclude dead cells, platelets and debris of erythrocytes. The black lines represent stained cells; grey lines are the controls. The percentage of positive cells for each marker is indicated in the graph.

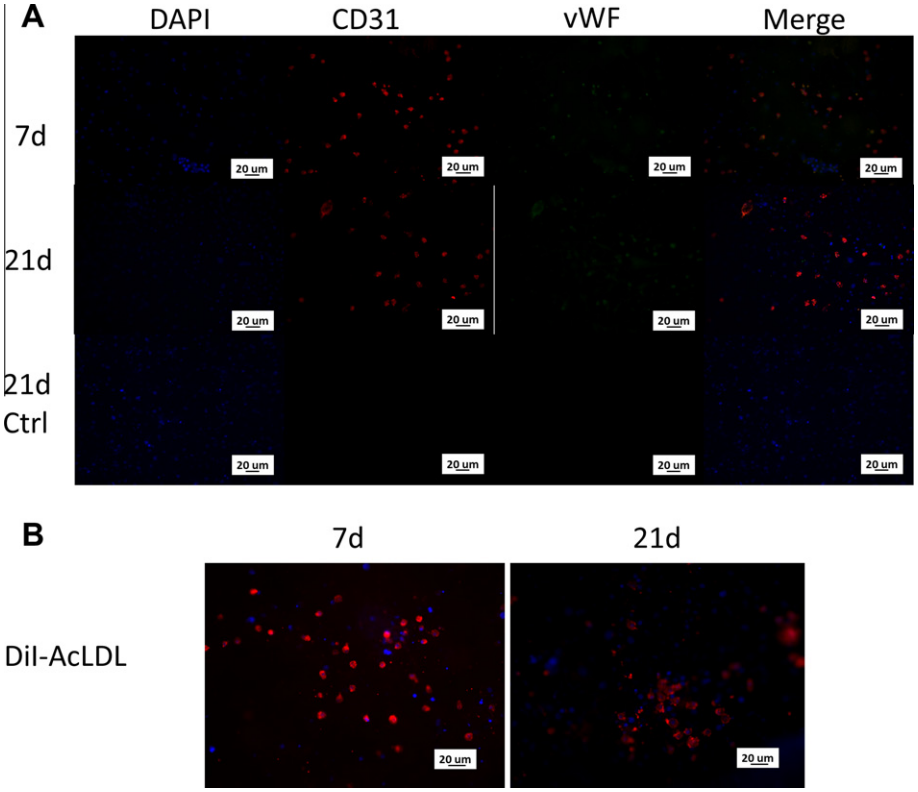


**Fig. 2.** Co-cultures of osteoblasts and CBMNCs on the carrageenan membranes after 7 and 21 days. (A) Endothelial cell markers, CD31 (red) and vWF (green) were detected by immunocytochemistry. ASC-derived osteoblast controls are shown (Ctrl). (B) DiI-AcLDL (red) staining identifies cells from the phagocytic and endothelial lineages. Nuclei were stained with DAPI (blue).

variation of 3.3-fold was detected after 7 days of co-culture, while for 21 days of co-culture, the variation was of 9.3-fold.

### 3.3. In vivo performance

The nature of the host reaction was analyzed based on the histological and immunohistological characterization of the tissues surrounding the different implants recovered (Fig. 4). The histological processing altered the morphology of the membranes and their localization in the tissue. More specifically, the size of the constructs decreased after treatment for histological analysis. Therefore, we have marked the tissue–construct interface with black dashes. The histological evaluation of the obtained sections showed no particular differences between the co-cultured (Fig. 4A–H) and monocultured (Fig. 4I–L) CRG membranes for 7 and for 21 days in vitro, for each implantation period. A significant inflammatory infiltrate surrounded the membranes at day 7 of implantation of both systems co-cultured in vitro for 7 (Fig. 4A) and 21 (Fig. 4C) days.

This infiltrate was characterized by the presence of some polymorphonuclear neutrophils, of a significant number of macrophages, either resident or recruited from circulation, and some foreign body giant cells (FBGCs) and lymphocytes (Fig. 4E and G). The inflammatory infiltrate around the membranes co-cultured in vitro for 21 days was surrounded by vascularized adipose tissue (Fig. 4C and G).

After 21 days of implantation of the membranes, the extension of the inflammatory infiltrate clearly increased (Fig. 4B, D, J and L) as compared with the early implantation time period. The presence of many mononuclear cells, similarly to the early time period (Fig. 4F and H), was observed. Moreover, macrophages fused together, forming FBGCs, lymphocytes maintained their apparent activation status, and some plasma cells were identified.

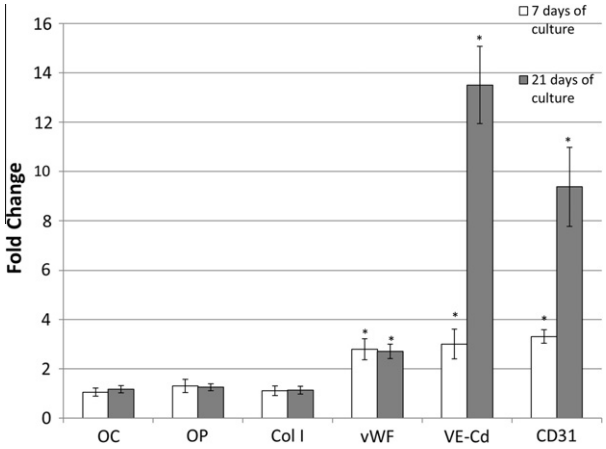


**Fig. 3.** Quantitative real-time RT-PCR of osteopontin (OP), osteocalcin (OC), collagen I (COL I), vWF, VE-cadherin (VE-Cd) and CD31 transcripts in co-cultures of osteoblasts and CBMNCs on carrageenan membranes after 7 and 21 days. $^{*}p < 0.001$ in relation to the monoculture controls.

Perfused blood vessels around the implants with the co-cultures were found to possess cells positive for human CD31 marker (Fig. 5A–D) while this was not the case of the implants with the monocultures of hASC-derived osteoblasts (Fig. 5E–H).

Blood vessels were quantified in the periphery of the implants within a 200 μm distance for all the conditions, with and without UCB MNC (Fig. 6). The number of vessels did not significantly vary with the in vitro culture and implantation times. However, the number of blood vessels was always greater when MNCs from cord blood were present. This difference was statistically significant when comparing the co-culture conditions and their respective monoculture control.

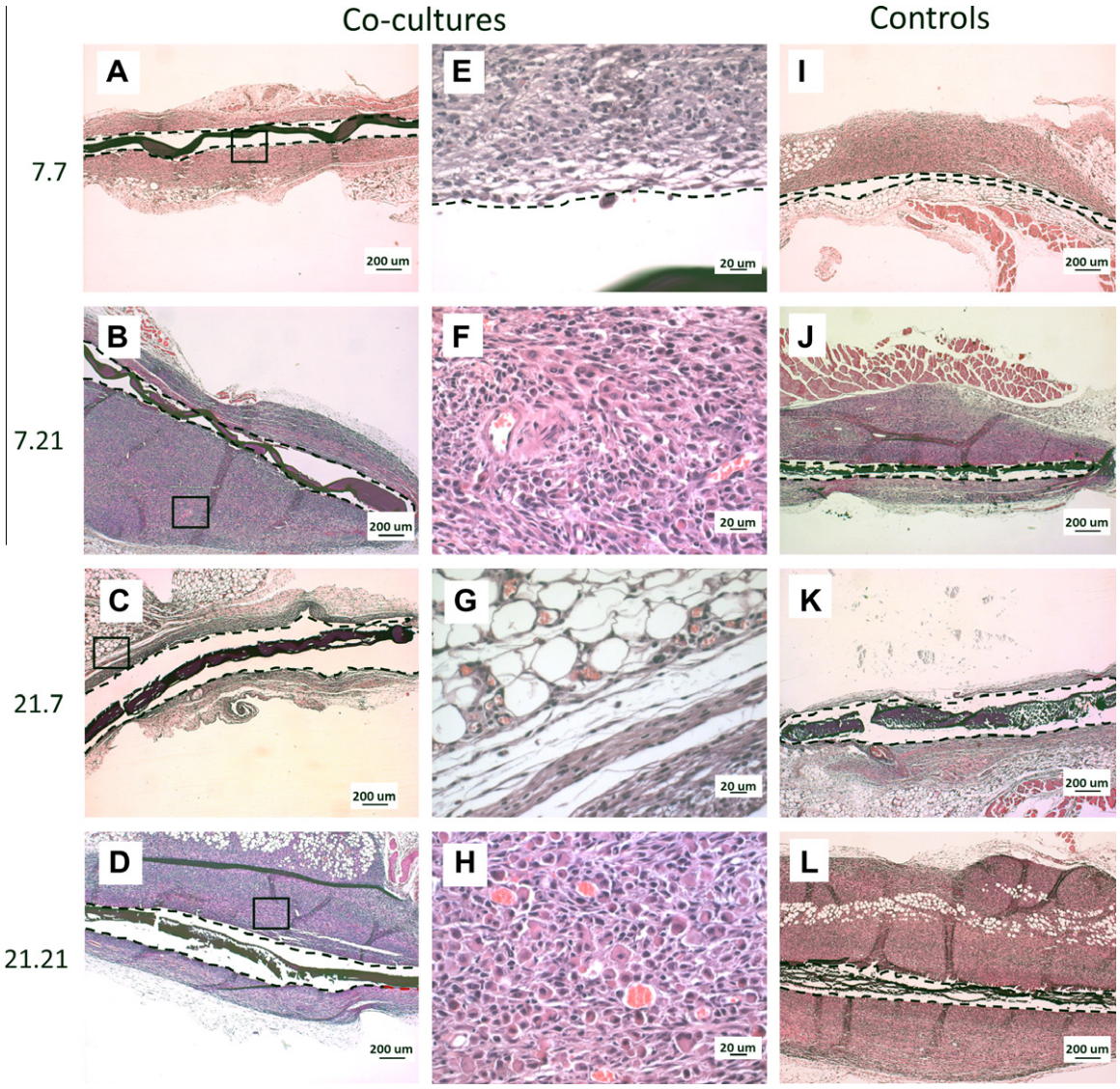


**Fig. 4.** Histological characterization of carrageenan membranes with co-cultured hASCs-derived osteoblasts and CBMNCs (co-cultures) (A–H) and monocultures of hASCs-derived osteoblasts (controls) (I–L) in vitro for 7 (A, B, E, F, I, J) and 21 (C, D, G, H, K, L) days, and after 7 (A, C, E, G, I, K) and 21 (B, D, F, H, J, L) days of implantation. Implant sections were stained with hematoxylin–eosin. The black dashes mark the construct–tissue interface before histological processing. (E)–(H) represent the zoomed areas indicated in (A)–(D).

## 4. Discussion

UCB has been shown to contain endothelial progenitors that can be used to promote the vascularization of bone tissue engineered constructs [45]. Furthermore, current cord blood cryopreservation techniques allow the preservation of the potential of endothelial progenitors to be isolated from this source [46,47]. Reports showing the positive influence of diverse populations of the UCB mononuclear fraction in the behavior of EPCs have also reinforced the potential of this cell source [17,48]. Mononuclear fractions of peripheral blood depleted of $CD14^+$ monocytes lose the ability to form colony forming unit-endothelial cells (CFU-ECs) [48]. In fact, $CD14^+$ cells have been suggested to be pro-angiogenic [17] and capable of differentiating into endothelial cells [16]. Furthermore, T cells were proven to be essential to the CFU-EC formation by $CD14^+$ cells [17]. Using MNCs from cord blood for vascularization purposes could then be an advantageous alternative to the typically used isolation of its specific sub-populations [7].

Several co-culture constructs of EPCs with MSCs/osteoblasts have been described for bone TE [45,49,50]. Although most of them successfully lead to increased vascularization of their constructs because of EPCs, the isolation and culture of these cells is a long and complex process. This work therefore proposes the use of the complete mononuclear fraction of UCB in order to be able to define a co-culture system carrying functional endothelial progenitors.

The present work allowed verified that osteoblasts obtained from adipose-derived stem cells support the survival of endothelial progenitors present in the mononuclear fraction of UCB. Taichman and colleagues [33] previously demonstrated how osteoblasts can support the maintenance of hematopoietic progenitors in vitro. Later Jung et al. [32] proved that this supportive effect of osteoblasts required direct cell–cell contact. We hypothesized that osteoblasts can have a similar action on the endothelial progenitors present in the mononuclear fraction of the UCB. Throughout the culture times, the monolayers of osteoblasts supported the culture of $CD31^+$ and $vWF^+$ cells and of cells capable of uptaking DiI-AcLDL. These properties are commonly used to define EPCs; however, blood monocytes have been shown to present these markers under angiogenic conditions [48,51]. In addition, overexpression of the

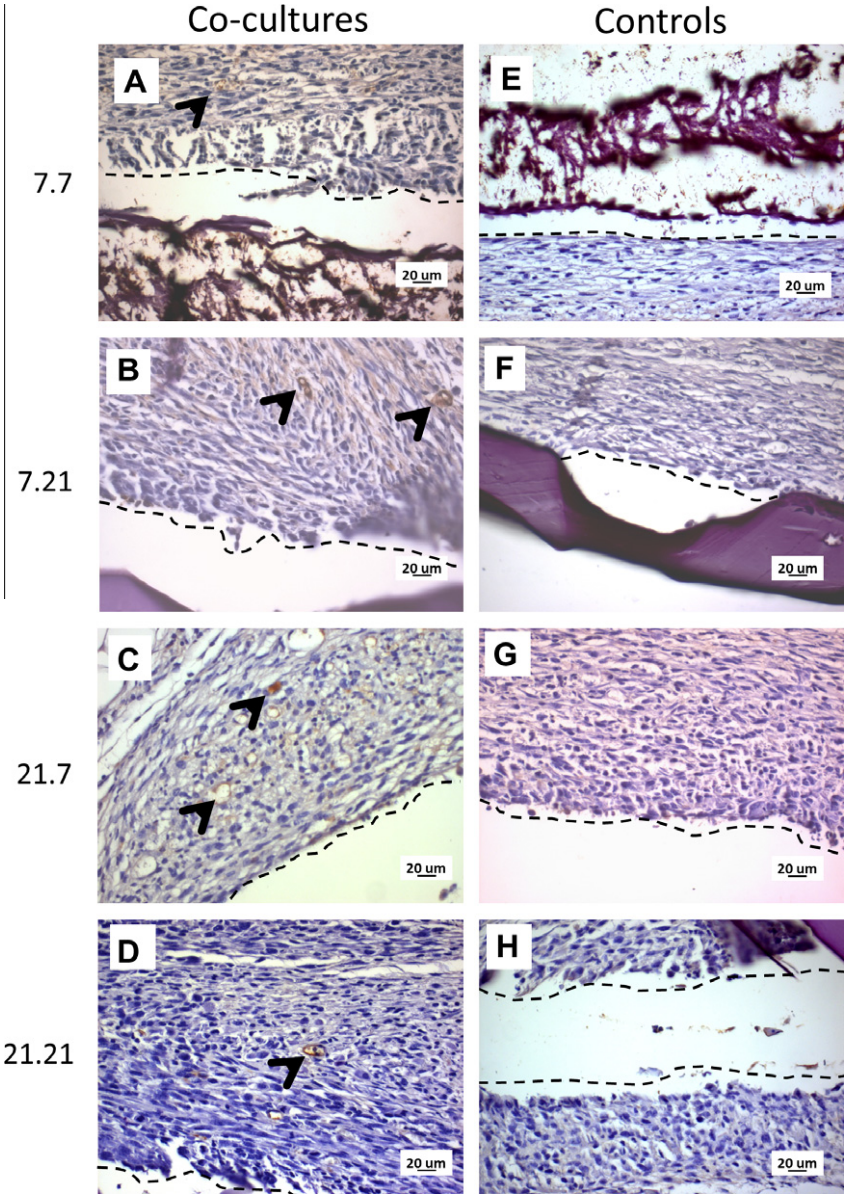


**Fig. 5.** Immunohistological characterization of carrageenan membranes with co-cultured hASCs-derived osteoblasts and CBMNCs (co-cultures) (A–D) and monocultures of hASCs-derived osteoblasts (controls) (E–H) in vitro for 7 (A, B, E, F) and 21 (C, D, G, H) days, and after 7 (A, C, E, G) and 21 (B, D, F, H) days of implantation. Immunostaining was performed to localize human CD31 marker around the implant. Sections were counterstained with hematoxylin. The black dashes mark the construct–tissue interface before histological processing.

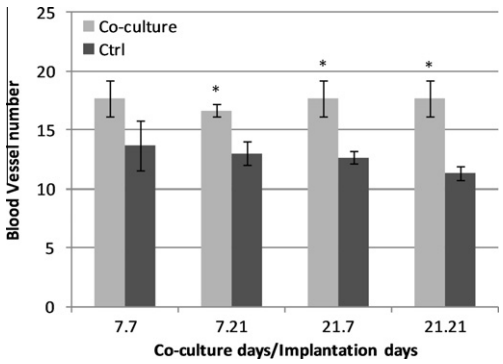


**Fig. 6.** Quantification of blood vessels within 200 μm around the surface of the implanted membranes. Implanted carrageenan membranes with co-cultures (co-culture) and with osteoblast monocultures (Ctrl) were compared. *Statistical significance at $p \leqslant 0.05$.

endothelial genes vWF, CD31 and VE-cadherin was detected in comparison with monocultures of MNCs under the same culture conditions and was more significant in the case of CD31 and VE-cadherin genes after 21 days of co-culture. This result for VE-cadherin is particularly remarkable as its expression is strongly linked to the maintenance of the integrity of new blood vessels [52]. Although striking, this promotion of the expression of endothelial genes is not unexpected. In fact osteoblasts produce factors, such as VEGF [31], that are capable of inducing the endothelial phenotype from progenitors [7]. Furthermore, the ECM deposited by osteoblasts is composed of fibronectin which may support the adherence of endothelial progenitors from the CBMNCs [53]. Fuchs and colleagues [50] have in fact demonstrated that outgrowth endothelial cells, a type of EPC, have increased endothelial activity when co-cultured with osteoblasts.

Unlike the endothelial lineage-related genes, the expression of the osteogenic genes osteopontin, osteocalcin and collagen I

remained unchanged, which is not unexpected as the osteoblastic supporting cells were fully differentiated at the time of the co-culture set-up. Co-culture systems established with MSCs or other osteoblast progenitors instead of osteoblasts are probably more likely to be influenced by the other cell types. This was demonstrated by Fedorovich and colleagues [54] in a co-culture model where EPCs promote the osteogenic differentiation of MSCs, while these promote the proliferation of the EPCs. In our strategy, completely differentiated cells were the ones we wanted to test.

CRGs are natural sulfated polysaccharides are a widely used in the food industry [55]. Recently, kappa-CRGs have been proposed for regenerative medicine purposes [38–40], presumably due to their favorable mechanical properties and anticoagulant effect [56]. We hypothesized that membranes produced using a kappa–iota hybrid type of this natural polymer could act as carriers to test the functionality of the developed co-cultures in vivo. To our surprise, a very strong and progressive inflammatory reaction was detected after implantation of the CRG membranes. Furthermore, the amount and nature of the inflammatory infiltrate did not vary with the implant type (CRG membranes only withhASC-derived osteoblasts or CRG membranes with hASC-derived osteoblasts co-cultured with CBMNCs), which seems to indicate that the cellular component did not significantly influence the inflammatory reaction. Solutions of CRG from kappa, iota and lambda types have been used to induce acute inflammation in animal models [57–59]. Moreover, as Tobacman thoroughly reviewed [55], the ingestion of CRG, used in the food industry, is associated with the onset of several lesions in the gastrointestinal tract in several animal models. Its use as a food additive is in fact very controversial due to health concerns [55,60,61]. Additionally, an acute inflammatory response, characterized by an abundant neutrophilic exudate and high levels of c-reactive protein, was reported after implanting gelatin/kappa-CRG (55%/45% w/w) sponges. The intensity of the reaction was nevertheless milder when the weight percentage of CRG was lowered to 9% suggesting a dose-dependent inflammatory effect of this polysaccharide. A similar acute inflammatory response that progressed to chronic inflammation was observed after implanting our membranes made of hybrid CRG. Although these results impair their potential use for TE or regenerative medicine purposes, the functionality of the defined co-culture system was nevertheless demonstrated.

A link between chronic inflammation and angiogenesis is well described in the literature [62–64] as there are many factors secreted by inflammatory cells that can act on EPCs [65,66]. Furthermore, differentiation of EPCs and their contribution to angiogenesis in pathological situations has also been observed experimentally [8,67]. Although the animal model used in the present work lacks an adaptive immune response, it is highly capable of presenting a strong inflammatory response. Human $CD31^+$ cells were found forming the wall of perfused blood vessels in the vicinity of the implant for all the times of culture. This suggests that an endothelial progenitor population present in the co-cultures was able to respond to the stimuli present at the implantation site and to migrate out of the membranes and differentiate, and to contribute to angiogenesis in the inflammatory setting. These results also demonstrate the usefulness of the mononuclear fraction of UCB as a source of endothelial cells or endothelial progenitors without previous selection. In addition to the proven active participation of transplanted CBMNCs in new blood vessels formed, their impact over the number of vessels formed around the implants was also demonstrated. A significantly higher number of vessels formed around the implants containing the CBMNCs than around the implants only with hASC-derived osteoblasts.

Overall, this work suggests that hASC-derived osteoblasts can support the maintenance of endothelial progenitors present in CBMNCs. Furthermore, the latter can be used to increase vascularization in vivo in an inflammatory setting. This can potentially be very interesting for future bone TE approaches although more studies need to be made, especially using biomaterial carriers/supports capable of eliciting host reactions with different degrees of severity.

## 5. Conclusions

The current work showed that hASC-derived osteoblasts sustain in vitro the survival of endothelial progenitors present in the mononuclear fraction of UCB in a direct co-culture system established using CRG membranes as supports. Although it is proven that the produced CRG membranes are not suitable for biomedical applications, the functionality of the cells from UCB co-cultured with osteoblasts was demonstrated in an in vivo inflammatory setting. The presence of human $CD31^+$ cells in blood vessels around the implant, as well as the increased number of blood vessels when the mononuclear fraction of UCB was used, confirms the existence of a sub-population of that fraction that can contribute to angiogenesis. This is an indication of the potential use of the mononuclear cells from the UCB, without further selection, as a source of endothelial progenitors supported by co-culture with osteoblasts.

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## Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figs. 2, 4 and 5, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at http://dx.doi.org/10.1016/j.actbio.2012.09.013.

## Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2012.09.013.

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