

**Enhanced osteogenesis in co-cultures with human mesenchymal stem cells and endothelial cells on polymeric microfiber scaffolds**

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## Abstract

In this work, human mesenchymal stem cells (hMSCs) and their osteogenically precultured derivatives were directly co-cultured with human umbilical vein endothelial cells (HUVECs) on electrospun 3D poly( $\epsilon$ -caprolactone) microfiber scaffolds in order to evaluate the co-culture's effect on the generation of osteogenic constructs. Specifically, cells were cultured on scaffolds for up to three weeks, and the cellularity, alkaline phosphatase activity (ALP), and bone-like matrix formation were assessed. Constructs with co-cultures and monocultures had almost identical cellularity after the first week, however lower cellularity was observed in co-cultures compared to monocultures during the subsequent two weeks of culture. Scaffolds with co-cultures showed significantly higher ALP activity, glycosaminoglycan and collagen production, as well as greater calcium deposition over the course of study compared to monocultures of hMSCs. Furthermore, the osteogenic outcome was equally robust in co-cultures containing osteogenically precultured and non-precultured hMSCs. The results demonstrate that the combination of MSC and HUVEC populations within a porous scaffold material under osteogenic culture conditions is an effective strategy to promote osteogenesis.

**Keywords:** Bone tissue engineering; co-culture; mesenchymal stem cells; human umbilical vein endothelial cells; 3D culture; scaffold.

## Introduction

A major challenge in current bone tissue engineering strategies is the lack of vascular ingrowth.<sup>1-</sup>  
<sup>3</sup> Native bone tissue has an abundant vascular network, without which engineered tissue must rely primarily on diffusion for oxygen and nutrient transport, which is effective only over distances of 100 to 200  $\mu\text{m}$ .<sup>4</sup> One strategy for improving the survival and osteogenesis of tissue engineered bone grafts involves the addition of endothelial cells (ECs) to cultures containing mesenchymal stem cells (MSCs).<sup>5-6</sup> MSCs are promising candidates for tissue engineering applications<sup>7-8</sup> because they have a capacity to differentiate along bone, cartilage and adipose lineages.<sup>9-12</sup> Additionally, MSCs reside in the bone marrow perivascular niche,<sup>13-14</sup> which would facilitate paracrine communication between MSCs and ECs. Studies evaluating the use of ECs in MSC or osteoblast cultures have observed the formation of microvessels in the engineered construct.<sup>15-19</sup>

Furthermore, previous studies have demonstrated that ECs are capable of enhancing the proliferation and osteogenic differentiation of MSCs.<sup>20-24</sup> Thus, co-cultures of MSCs and ECs are currently being investigated for their ability to enhance bone-formation and have shown that trophic regulation from ECs can provide necessary components for MSC osteogenic differentiation.<sup>5-6</sup> The addition of ECs to MSC or osteoblast cultures has been shown to enhance both pro-angiogenic and pro-osteogenic gene expression, stimulate alkaline phosphatase (ALP) activity, and increase mineralization.<sup>15-17, 20-23, 25-27</sup> While numerous studies have evaluated such co-cultures in two dimensions, data obtained from 3D conditions are still very limited, as studies emphasizing the osteogenic outcome have primarily been performed in monolayer<sup>19, 21, 23-24</sup> or pellet cultures<sup>20, 22, 26</sup> but not on porous scaffolds. Studies performed with co-cultures of ECs and bone-forming cells on porous scaffolds have investigated primary osteoblasts<sup>16-17, 28</sup> or an

osteoblast cancer cell line,<sup>27-28</sup> and have focused on the survival of the ECs,<sup>28</sup> angiogenic gene expression, the development of scaffold vascularization with ECs<sup>16-17, 28</sup> or the properties of the scaffold material,<sup>27</sup> but not on the osteogenic differentiation of bone forming cells (e.g., by quantifying bone-like matrix production and maturation). As such, previous co-culture studies have often utilized culture conditions favoring the angiogenic,<sup>16-17, 27-28</sup> over the osteogenic outcome.

A variety of scaffold materials have been investigated for EC and MSC co-cultures, including: poly( $\epsilon$ -caprolactone) (PCL); starch-based scaffolds with a fiber size of more than 200  $\mu\text{m}$ ,<sup>16, 17</sup> hydroxyapatite (HA) and  $\beta$ -TCP ( $\text{Ca}_3(\text{PO}_4)_2$ ) porous discs,<sup>28</sup> hydrophilic and hydrophobic titanium surfaces,<sup>27</sup> collagen mesh scaffolds,<sup>29</sup> and poly( $\epsilon$ -caprolactone) (PCL)-hydroxyapatite (HA) composite membranes.<sup>30</sup> Recently, porous microfiber mesh scaffolds made of biodegradable PCL have been developed using electrospinning.<sup>31</sup> Electrospun PCL scaffolds with 5 or 10  $\mu\text{m}$  fiber diameters used in our laboratory have been shown to successfully support MSC proliferation and osteogenic differentiation.<sup>32-33</sup>

In this study, it was hypothesized that co-cultures of human MSCs (hMSCs) and/or osteogenically precultured hMSCs and human umbilical vein endothelial cells (HUVECs) on 3D scaffolds would lead to enhanced osteogenesis over hMSCs or their derivatives alone. In order to test this hypothesis, hMSCs were co-cultured with HUVECs on electrospun PCL microfiber scaffolds under osteogenic culture conditions, and the effect on osteogenic differentiation was quantified with a variety of biochemical assays.

## Materials and Methods

### Experimental design

This study investigated a total of 8 groups, seeded onto electrospun PCL scaffolds and cultured in osteogenic medium for 7, 14, or 21 days. Three co-culture groups were investigated with the total number of cells used to seed each scaffold remaining constant ( $3.0 \times 10^5$  cells per scaffold). In the first group, hMSCs and HUVECs were cultured in a 1:1 ratio (MH). In the second group, hMSCs were precultured for 7 days in osteogenic medium before being combined at a 1:1 ratio with HUVECs (OH). In the third group, hMSCs, osteogenically precultured for 7 days, were mixed with non-precultured hMSCs and HUVECs at a 1:1:1 ratio (OMH). Additionally, five monoculture groups were investigated, which consisted of: a high density ( $3.0 \times 10^5$  cells per scaffold) of hMSCs with the same total number of cells as the co-culture (M2), a high density ( $3.0 \times 10^5$  cells per scaffold) of osteogenically pre-cultured hMSCs (O2); a low density ( $1.5 \times 10^5$  cells per scaffold) of hMSCs with the same total number of hMSCs as the co-culture groups (M1); a low density ( $1.5 \times 10^5$  cells per scaffold) of osteogenically pre-cultured hMSCs (O1); and a monoculture of HUVECs at a density of  $1.5 \times 10^5$  cells per scaffold (H).

### Human mesenchymal stem cell culture and human umbilical vein endothelial cell culture

Frozen bone marrow-derived hMSCs were kindly provided by Dr. Darwin Prockop of the Texas A&M University System Health Science Center in Temple, Texas, USA. A specification certificate provided with the cells indicated that they possessed widely accepted CD-markers, including CD90, CD105 and CD73<sup>12</sup> and were tested for the capability to differentiate towards osteogenic and adipogenic lineages until passage 4. The hMSCs were thawed and cultured for 1-2 passages in general expansion medium:  $\alpha$ -MEM without nucleosides and ribonucleosides (Gibco, Grand Island, NY) with 13% v/v fetal bovine serum (FBS, Atlanta Biologicals,

Norcross, GA), 100 units/mL penicillin, 100 µg/mL streptomycin (Gibco, Grand Island, NY) and 2-4 mM L-glutamine (Sigma, St. Louis, MO). Osteogenically pre-cultured cells were expanded for 1 passage in general media and an additional 7 days in osteogenic medium containing 10 nM dexamethasone, 10 mM β-glycerol-phosphate and 0.2 mM ascorbic acid (all from Sigma, St. Louis, MO). Cells at passage 3 were used for this study.

Frozen, pooled, primary HUVECs, (ATCC, Manassas, VA) that were certified by the vendor as von Willebrand factor positive and smooth muscle α-actin negative were expanded prior to use in our studies. The HUVECs were thawed and cultured in Vascular Cell Basal Medium containing 0.2% bovine brain extract (BBE), 5 ng/ml recombinant human epidermal growth factor (rhEGF), 10 mM L-glutamine, 0.75 units/ml heparin sulfate, 1 µg/ml hydrocortisone, 50 µg/ml ascorbic acid, 2% v/v FBS (Endothelial Cell Growth Kit-BBE) (all from ATCC, Manassas, VA) and 10 units/ml penicillin, 10 µg/ml streptomycin and 25 ng/ml amphotericin B (Gibco, Grand Island, NY). Cells at passage 4 were used for this study.

### **Scaffold fabrication and characterization**

Nonwoven poly(ε-caprolactone) (PCL) microfiber mats were fabricated using a horizontal electrospinning apparatus as previously described.<sup>31</sup> Mats were electrospun to achieve an average fiber diameter of ~10 µm using 18 wt % PCL (inherent viscosity 1.0-1.3 dl/g; Lactel, Birmingham, AL) in a solution of 5:1 v/v chloroform:methanol. The resulting PCL mats of ~1 mm thickness were stored in a desiccator until use. Five PCL microfiber mats were electrospun to generate a sufficient number of discoid scaffolds for this study (average scaffold thickness 1.05±0.05 mm). For cell seeding, discoid PCL scaffolds 8 mm in diameter were punched from the electrospun mats using a dermal biopsy punch (Miltex, Inc. York, PA), and the microfiber

morphology was assessed via scanning electron microscopy (SEM) (mean diameter  $10.6 \pm 1.6$   $\mu\text{m}$ ). The scaffolds were then sterilized by exposure to ethylene oxide (AN74i, Andersen Sterilizers, Haw River, NC) at room temperature for 14 h and aerated overnight.

### Cell seeding of scaffolds

To facilitate cell adhesion, pre-wetting of scaffolds was performed in a gradient series of ethanol (100%-25%; centrifugation for 15 min each step at 2000 rpm), followed by two rinses in phosphate buffered saline (PBS) and one rinse in general expansion medium. Scaffolds were maintained in culture medium overnight at 37 °C prior to cell seeding. Pre-wetted scaffolds were press-fitted into polymethylmethacrylate cylindrical holders specifically designed to fit the 8 mm scaffolds and localize the cell suspension above the scaffolds for the time required for cell adhesion. Cell suspensions were slowly pipetted onto the scaffolds at a density of  $1.5 \times 10^5$  cells per scaffold for monocultures of hMSCs (M1), preosteoblasts (O1) and pure HUVECs (H) or  $3.0 \times 10^5$  cells per scaffold for monocultures of hMSCs (M2), preosteoblasts (O2) and co-cultures (MH, OH, and OMH) in 200  $\mu\text{l}$  of culture medium and allowed to adhere for 4 h before general expansion medium was added to completely fill the holders. Scaffolds were transferred after 24 h into 12-well culture plates (Becton Dickinson, Franklin Lake, NJ) with 3 ml of fresh osteogenic medium. Thereafter, medium was changed twice a week for up to 21 days. Four scaffolds from each group were harvested the day after seeding to assess the initial cellularity and ALP-activity (day 1). Twelve scaffolds from each experimental cellular group were harvested after 7, 14 and 21 days of culture.

## Biochemical assays

After harvesting, the constructs were washed with PBS and frozen at  $-20\text{ }^{\circ}\text{C}$  until use for the biochemical assays. For extraction of DNA, GAG and collagen, thawed samples were digested in 500  $\mu\text{l}$  of proteinase K solution (1 mg/ml proteinase K, 0.01 mg/ml pepstatin A and 0.185 mg/ml iodoacetamide in a 50 mM tris-(hydroxymethyl-aminomethane) - 1mM ethylene-diamine-tetraacetic acid buffer, pH 7.6) in a  $56\text{ }^{\circ}\text{C}$  water bath for 16 h followed by two additional freeze-thaw cycles and 10 min sonication. For measuring of alkaline phosphatase (ALP) activity and calcium (Ca) content, scaffolds were placed into 1 ml of milliQ water after thawing, and then subjected to two additional freeze-thaw cycles followed by 10 min sonication.<sup>32</sup>

The DNA content of each cell lysate solution was quantified using the fluorometric PicoGreen assay (Invitrogen, Eugene, OR, USA) with an excitation wavelength of 490 nm and an emission wavelength of 520 nm (BioTek FLX800, Winooski, VT), as described elsewhere.<sup>34-35</sup> ALP activity was performed according to an established colorimetric protocol.<sup>35</sup> The ALP activity of samples was determined relative to a p-nitrophenol standard curve and presented as activity per scaffold. For measuring calcium content, each scaffold was removed from the aqueous cell lysates, placed into 1 ml 0.5 N acetic acid, and left on a shaker table at 200 rpm overnight to dissolve the calcium on the scaffolds. A colorimetric assay was used to quantify calcium content based on the color change that occurs when a calcium reagent (Arsenazo III, Diagnostic Chemicals, Oxford, CT) binds to free calcium.<sup>32</sup> Glycosaminoglycan (GAG) content was determined using the colorimetric dimethylmethylene blue assay.<sup>36</sup> GAG concentrations were determined relative to a chondroitin sulfate standard curve. Total collagen was extracted from scaffolds via basic hydrolysis of a 100  $\mu\text{l}$  sample solution (obtained after proteinase K digestion) with 100  $\mu\text{l}$  4N NaOH for 20 min at  $120\text{ }^{\circ}\text{C}$ . After cooling, samples were neutralized with acid,



and the resulting hydrolyzed collagen was determined by measuring hydroxyproline in a colorimetric assay as previously described.<sup>37</sup> The resulting hydroxyproline concentrations were then converted to collagen contents for each scaffold following a 1:10 ratio of hydroxyproline to collagen.<sup>32</sup> All colorimetric assays were performed on PowerWaveX340 Microplate Reader (BioTek Instruments, Winooski, VT).

## **Histology**

At each time point two scaffolds from each group were rinsed with PBS and fixed in 10% neutral buffered formalin (Fisher Scientific, Pittsburgh, PA). Cryosections of 7  $\mu\text{m}$  thickness were cut using a cryostat (Leica CM 1850 UV; Leica Biosystems Nussloch GmbH, Germany). Sections were stained with Safranin-O, von Kossa and Picrosirius red to visualize GAG, mineralized matrix and collagen fibers, respectively. Imaging was performed using a ZeissAxio Imager.Z2 microscope (equipped with Axi-oCam MRc5; Carl Zeiss MicroImaging GmbH, Germany).

## **Scanning electron microscopy**

Extracellular matrix morphology of cell-scaffold constructs was evaluated at each time point via scanning electron microscopy (SEM) (FEI Quanta 400 Environmental, Hillsboro, OR). Two scaffolds from each group were rinsed with PBS, fixed in 2.5% v/v glutaraldehyde in PBS for 30 min at room temperature and dehydrated via an alcohol gradient (50%-100%) and dried overnight in ambient conditions.

## Statistical analysis

Results are presented as mean  $\pm$  standard deviation for  $n=4$ . The differences were analyzed using one-way ANOVA followed by Tukey's post-hoc test using JMP 10 software package (SAS Institute, Cary, NC). Differences were considered significant at  $p<0.05$ .

## Results

### Scaffold cellularity

As seen in Fig. 1, an increase in scaffold cellularity over time was observed in all monoculture groups (M1, O1, M2, O2), with the monoculture containing osteogenically precultured cells (O2) displaying the highest cellularity at day 14 and 21. All co-cultures containing HUVECs (OH, OMH, MH) were seeded with the same total number of cells as the high density monocultures (M2, O2). In the co-culture groups, cell proliferation took place primarily in the first 7 days. At 14 days, DNA content in the co-cultures was significantly lower than either seeding density of the corresponding monocultures (O1 and O2 for OH and OMH; M1 and M2 for MH).

### ALP activity

ALP activity generally peaked at day 7 and then decreased over time in the case of co-cultures (OH, OMH, MH). However, ALP activity remained relatively unchanged after day 7 for monocultures of non-precultured cells (M1, M2) and decreased in the osteogenically precultured monoculture groups at day 7 or later (O1, O2) (Fig. 2). Despite lower scaffold cellularity, all co-cultures showed significantly higher total ALP activity per scaffold compared to the corresponding monocultures (O1 and O2 for OH and OMH; M1 and M2 for MH) from day 7 onward (Fig. 2). The only exception was the co-culture group with mixed precultured and non-

precultured hMSCs (OMH) and the monoculture group with osteogenically precultured cells at the higher seeding density (O2); these were not significantly different at day 21 (Fig. 2).

### **Collagen synthesis**

All groups showed increasing collagen per scaffold, as seen in Fig. 3A, as well as collagen content normalized to DNA (Fig. 3B). In the co-cultures significantly increased collagen per scaffold was observed from day 14 to day 21. The co-cultures with osteogenically precultured cells (OH, OMH) had significantly increasing normalized collagen content during the first 14 days of culture, whereas co-cultures with hMSCs (MH) showed significantly increased collagen normalized to DNA from day 14 to day 21. All co-cultures with either osteogenically precultured cells or hMSCs showed significantly higher collagen production per scaffold compared to all corresponding monocultures at day 7 of culture, as shown in Fig. 3A. The differences between groups were not significant at days 14 and 21, excluding the co-culture with hMSCs (MH), which presented a higher collagen content per scaffold in comparison to the hMSC monoculture with the lower seeding density (M1) at day 14. In contrast to total collagen content, normalized collagen content was significantly elevated for all co-culture groups compared to the corresponding monocultures at all investigated time points (Fig. 3B). Among the co-cultures, the highest collagen to DNA ratio was observed in the group with non-precultured cells (MH) at days 7 and 21.

### **GAG synthesis**

All monocultures of either osteogenically precultured or non-precultured hMSCs (O1, O2 and M1, M2, respectively) showed continuously increasing GAG contents per scaffold, as seen in Fig. 4A. Co-cultures demonstrated completely different behavior in terms of GAG production

and synthetic activity. The GAG production per scaffold remained relatively unchanged with time for all co-cultures (Fig. 4A). GAG synthetic activity of cells significantly increased only from day 7 to day 14 in all investigated co-cultures (Fig. 4B). Total GAG production per scaffold over time was the strongest in the co-culture group with osteogenically precultured cells (OH) among all tested groups, whereas synthetic GAG activity (GAG/DNA) was similar between OH and OMH groups and was the strongest in the MH group at days 7 and 21. With regard to GAG to DNA ratio, all co-cultures had significantly higher synthetic activity in comparison to corresponding monocultures.

### **Calcium deposition**

The amount of calcium deposited on scaffolds was minimal over time for all monoculture groups (Fig. 5). On the contrary, all investigated co-culture groups (OH, OMH, MH) had continuously increasing calcium deposition over time (Fig. 5). All co-cultures with either osteogenically precultured cells or hMSCs showed significantly higher calcium per scaffold compared to the corresponding monocultures at all tested time points, as shown in Fig. 5. There were no differences in calcium deposited between co-cultures with osteogenically precultured cells (OH) and hMSCs (MH) over time, but the co-culture with both cell types (OMH) showed the lowest calcium deposition per scaffold among these groups at days 7 and the highest at day 14.

### **Histology**

Histology corroborated the results of calcium, collagen and GAG biochemical assays (Fig. 6). Histological analysis showed cells and extracellular matrix (ECM) accumulated near the top surface of the scaffolds, with increasing cellular penetration and matrix distribution with time. A clear difference between all co-cultures and monocultures was observed for calcium mineralization (Fig. 6). Specifically, co-cultures showed abundant deposits of mineralized matrix

that penetrated deeper into the scaffolds with time, while monocultures displayed relatively little mineral deposition. Similarly, the co-culture groups showed greater staining for GAGs relative to the monoculture groups (Fig. 6). The co-cultures also displayed more abundant collagen-rich matrix deposition, especially on the top scaffold surface (Fig. 6).

### Scanning electron microscopy

SEM images (Supplementary Figure S1) generally showed extracellular matrix completely covering the scaffold surfaces and cells infiltrating the pores between PCL fibers, with the only exception being the HUVEC only group (H) (Supplementary Figure S2).

### Discussion

The main objective of this study was to determine whether co-culturing hMSCs with endothelial cells on PCL microfiber scaffolds could enhance osteogenesis (as determined by ALP activity and calcium deposition) and the production of bone-like extracellular matrix components (as determined by collagen and GAG matrix production). HUVECs were selected for this study because they are an established model of endothelial cells and have been shown to stimulate osteogenesis *in vitro*.<sup>20-23, 27</sup> General hMSC expansion medium with osteogenic supplements was used because of its proven ability to support osteogenesis in co-cultures of HUVECs and hMSCs,<sup>20</sup> as opposed to other media formulations that have demonstrated greater utility in maintaining and promoting angiogenesis of endothelial cells in co-culture.<sup>17, 20, 28</sup> While the absence of angiogenic growth factors in osteogenic media limits endothelial cell metabolic activity and proliferation,<sup>20</sup> it is still sufficient for manifestation of the angiogenic potential of HUVECs in co-cultures with hMSCs.<sup>15, 20, 22</sup>

From previous studies it is known that co-culturing endothelial cells with bone forming cells promotes cellular proliferation in 2D<sup>21, 23</sup> as well as in 3D.<sup>27</sup> However, since most of these findings were obtained using endothelial growth media, we evaluated the effect of 3D culture in osteogenic media. In the present study DNA content increased after 7 days of co-culture in osteogenic medium, but then decreased by 14 days. This is consistent with literature data where similar co-cultures led to a decrease in DNA content after 14 days of 2D-culture in osteogenic conditions.<sup>20</sup> Indeed, the balance between cell proliferation and differentiation during development of the osteoblastic phenotype is well established.<sup>38</sup> Consequently, the lower DNA content observed in co-cultures at 14 days relative to 7 days of culture could reflect a decrease in proliferation corresponding to osteogenic differentiation. This finding, together with the significantly increased ALP activity, as well as matrix production and maturation (collagen and GAG production and calcium deposition) observed in co-cultures at the earlier time points, supports the hypothesis that osteogenesis is enhanced in co-cultures of hMSCs and endothelial cells on microfiber scaffolds.

The osteogenic preculture conditions used for the hMSCs in this study typically result in committed osteoprogenitor cells.<sup>39</sup> In this study, it was observed that precultured and non-precultured co-cultures presented ALP activity significantly higher than corresponding monocultures. The enhancement of ALP activity in co-cultures may be mediated by p38 mitogen-activated protein kinase-dependent stabilization of mRNA for ALP.<sup>40</sup> This type of stimulation has been shown in series of studies.<sup>20-23, 25-27, 40</sup> One novel aspect of this study was that in addition to elevated ALP activity, all co-cultures were shown to have significantly higher production of the main non-mineral components of bone extracellular matrix, namely collagen and GAGs. The levels of these extracellular matrix components (as reflected by the collagen and

GAG contents of scaffolds normalized to DNA) in monocultures at each time point were always lower than those observed in co-cultures. The promotion of increased collagen matrix production by co-cultures is supported by several studies.<sup>16, 23, 25</sup> For instance, up-regulated collagen type 1 gene expression was found in co-cultures of human osteoblasts and dermal endothelial microvascular cells<sup>16</sup> and in co-cultures of HUVECs and hMSCs.<sup>23, 25</sup> However, no up-regulation in collagen expression was found in the co-culture of outgrowth endothelial cells and MSCs.<sup>15</sup> To our knowledge, no other studies have reported increased GAG production in co-cultures of MSCs and ECs in either 2D or 3D. GAGs have a variety of biological functions necessary for osteogenesis and cell-to-cell communications.<sup>41</sup> One possible reason for increased GAG production observed in co-cultures in the present study could be residual heparin sulfate, which is a component of vascular basal media commonly used for HUVEC cultivation and the media used for HUVEC culture in the present study. However no GAGs were detected in HUVEC monoculture (Fig. 4) Interestingly, it is thought that heparan sulfate proteoglycans assist in mediating the interactions between marrow stromal cells and hematopoietic cells,<sup>42-43</sup> and thus the upregulation of GAG production observed in the present study potentially could have further enhanced the effect of the co-cultures.

Consistent with the results of biochemical and matrix production in general, all co-cultures presented increased mineralized matrix deposition compared to monocultures. This result was consistent with the observation in co-cultures of enhanced ALP activity, which hydrolyzes the phosphate source for mineralization, as well as increased collagen matrix production, which provides the structural support necessary for calcium deposition.<sup>38</sup> The observed increased mineralization in co-cultures is consistent with literature data.<sup>15, 20-21</sup> Furthermore, the very low calcium deposition found in monocultures is consistent with data obtained in a recent study in

which hMSCs from several donors demonstrated minimal calcium deposition for up to 28 days of culture, while co-cultures of HUVECs and hMSCs exhibited significant mineralized matrix much earlier in culture.<sup>20</sup> This result emphasizes the role of endothelial cells as osteogenic mediators in co-culture models.

To address the question of what stage of maturity favors the promotion of osteogenesis in this study, we tested three different co-cultures including two groups with a 1:1 ratio of either osteogenically precultured or non-precultured hMSCs together with HUVECs, as well as a third group combining all three cell types (OMH). The latter group was included in order to investigate the simultaneous cross-talk between various cell types that exist together in the bone marrow niche and to investigate the possibility of decreasing the number of HUVECs needed while maintaining a suitable osteogenic outcome. MSCs reside in the perivascular niche,<sup>13-14</sup> and may be recruited to the site of bone regeneration by cues from endosteal (pre)osteoblasts as well as through signaling from the surrounding endothelium. Several studies investigating co-cultures of non-human MSCs with osteoblasts or osteoblast/osteocyte murine lines<sup>44-45</sup> have shown the potential of directing osteogenic differentiation of MSCs *in vitro* by cues from more mature cell types. However, the effects were different depending on whether terminally or partially differentiated cells were applied. Osteoblasts were shown to be more favorable for proliferation but not the differentiation of MSCs, while osteocytes significantly stimulated osteogenic outcome.<sup>45</sup> Studies have not been conducted with hMSCs co-cultured with their osteogenically committed derivative together with endothelial cells. Although in the current study there were no control groups consisting of hMSCs co-cultured with preosteoblasts without HUVECs, the data obtained were compared with other co-cultures and monocultures used (as the amount of cells were the same) and support a beneficial effect of such a co-culture. Comparing the osteogenically



precultured to non-precultured co-cultures, the present study found that the OH and OMH co-cultures resulted in higher levels of proliferation than the non-osteogenically precultured co-cultures (MH). Preculture also affected GAG and collagen matrix production and calcium content at early time points; however at later time points, the calcium content of the constructs, which is the primary marker of osteogenesis, was equal among all co-cultured populations, indicating that the preculture period did not significantly affect the co-culture construct mineralization.

The cell proportions in the co-cultures with 1:1 ratios were selected based on the literature,<sup>20, 22, 40</sup> and the current study resulted in comparable osteogenic outcomes. Additionally, for comparison of the results, several control groups with monocultures of either osteogenically precultured cells or hMSCs at lower and higher densities were included. Importantly, both seeding densities resulted in inferior osteogenic outcomes than the corresponding co-cultures. This suggests that stimulation of osteogenesis observed in co-cultures most likely does not reflect the effects of cell density; although it is evidently dependent on cell-to-cell contacts<sup>16, 25</sup> and trophic cross-talk.<sup>6</sup> In direct co-cultures, tight heterotypic cell-to-cell contacts (gap-junctions) were found between ECs and hMSCs and were thought to contribute toward activation of angiogenic and osteogenic gene expression in the cell types, respectively.<sup>16, 25</sup>

Overall, with regard to construct cellularity and ALP activity, the best outcome (indicated by the highest ALP production on a per cell basis) was achieved in the co-cultures with osteogenically precultured cells (OH, OMH), which is consistent with longer exposure to osteogenic supplements. However, diverse effects were observed in matrix production (collagen, GAGs) and maturation (calcium deposition) among the co-cultures investigated. The result that collagen and GAG synthetic activity both achieved their maximum observed value in the present study in

the co-culture with non-precultured cells (MH) was unexpected, as it indicates osteogenically precultured cells effected cell osteogenic synthetic activity in co-cultures relatively less than hMSCs. Previously it was shown that the preculture period of MSCs in osteogenic medium influences their *in vivo* bone forming potential.<sup>39</sup> Perhaps the preculture period of 7 days chosen in the present study was not appropriate to achieve positive osteogenic effects exceeding that of non-precultured hMSCs. Alternatively, it is possible that the outcome of co-cultures of HUVECs and hMSCs depends mostly on endothelial cell activity and their trophic communication with progenitor cells, which has not been characterized with respect to the stage of cell maturity. Therefore these data demonstrate that supplementation with endothelial cells promotes osteogenic differentiation of hMSCs cultured on 3D electrospun PCL microfiber scaffolds regardless of their stage of maturity.

## Conclusions

In this work, we investigated osteogenesis in polymeric microfiber scaffolds using osteogenically committed or non-committed hMSCs alone and in combined co-culture with ECs with the goal of generating engineered bone constructs. Co-cultures showed lower proliferation but more pronounced bone-like matrix formation and mineralization in comparison to hMSC monocultures. All co-cultures showed greater degrees of osteogenic differentiation compared to monocultures, which suggests the cellular cross-talk in co-cultures enhances the osteogenic potential of hMSCs in microfiber scaffolds. Based on these results, we conclude that co-cultures of ECs and hMSCs are a promising strategy to improve osteogenesis in engineered bone constructs.

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## Disclosure Statement

The authors declare no conflicts of interest.

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### Figure Captions

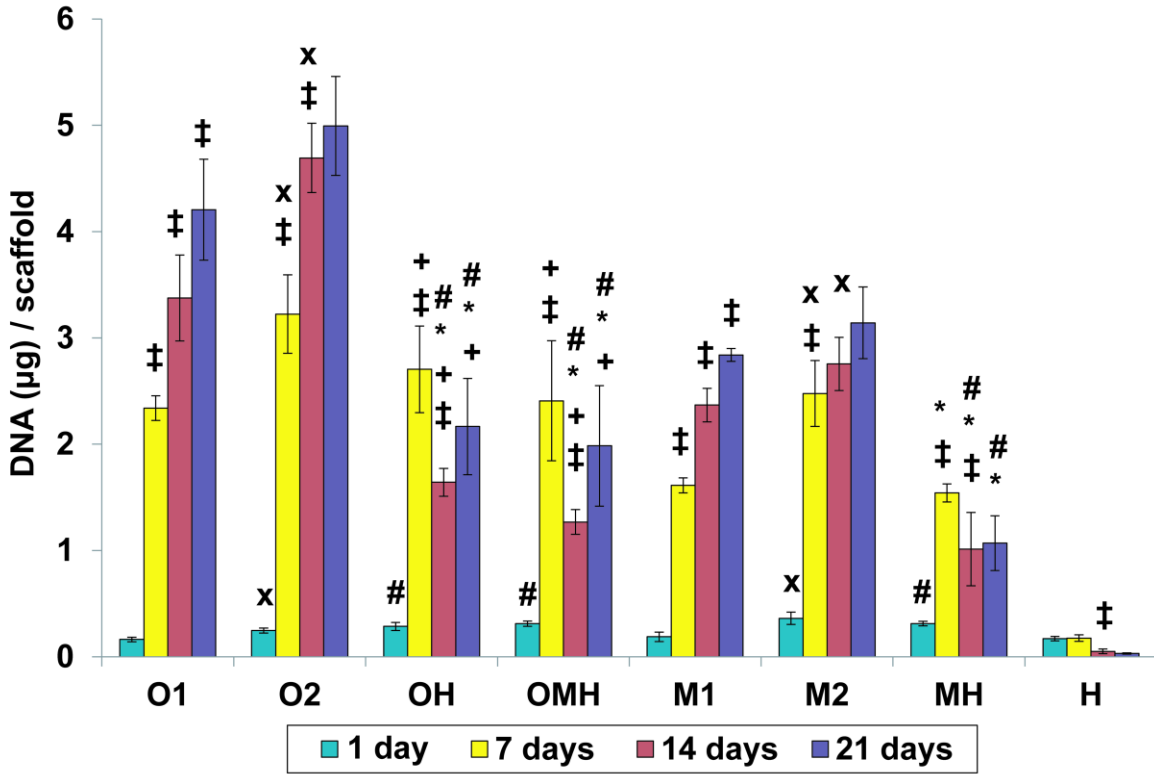


Figure 1. DNA content at 1, 7, 14, and 21 days of culture in (O1) osteogenically precultured hMSCs seeded at  $1.5 \times 10^5$  cells per scaffold, (O2) osteogenically precultured hMSCs seeded at  $3.0 \times 10^5$  cells per scaffold, (OH) 1:1 ratio of osteogenically precultured hMSCs and HUVECS seeded at  $3.0 \times 10^5$  cells per scaffold, (OMH) 1:1:1 ratio of osteogenically precultured hMSCs, hMSCs, and HUVECS seeded at  $3.0 \times 10^5$  cells per scaffold, (M1) hMSCs seeded at  $1.5 \times 10^5$  cells per scaffold, (M2) hMSCs seeded at  $3.0 \times 10^5$  cells per scaffold, (MH) 1:1 ratio of hMSCs and HUVECS seeded at  $3.0 \times 10^5$  cells per scaffold, and (H) HUVECS seeded at  $1.5 \times 10^5$  cells per scaffold. The results are presented as means  $\pm$  SD for  $n=4$ . Symbols #, \* denote significant difference of co-cultures from the corresponding monocultures (with lower and higher densities respectively), + indicates significant difference of precultured co-cultures (OH, OMH) versus co-

culture of non-precultured cells (MH), x denotes significant difference between monocultures with lower and higher seeding density, ‡ reflects significant difference to the previous time point for all groups ( $p < 0.05$ ).

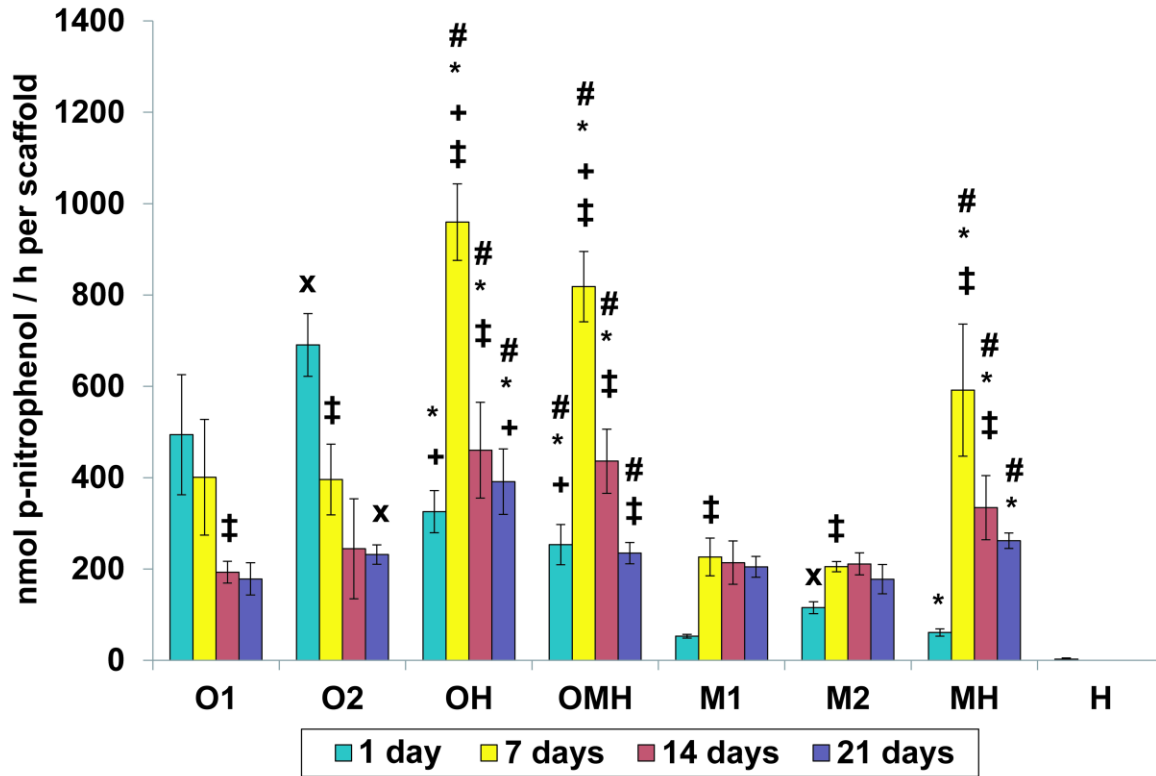


Figure 2. Alkaline phosphatase activity at 1, 7, 14, and 21 days of culture in (O1) osteogenically precultured hMSCs seeded at  $1.5 \times 10^5$  cells per scaffold, (O2) osteogenically precultured hMSCs seeded at  $3.0 \times 10^5$  cells per scaffold, (OH) 1:1 ratio of osteogenically precultured hMSCs and HUVECS seeded at  $3.0 \times 10^5$  cells per scaffold, (OMH) 1:1:1 ratio of osteogenically precultured hMSCs, hMSCs, and HUVECS seeded at  $3.0 \times 10^5$  cells per scaffold, (M1) hMSCs seeded at  $1.5 \times 10^5$  cells per scaffold, (M2) hMSCs seeded at  $3.0 \times 10^5$  cells per scaffold, (MH) 1:1 ratio of hMSCs and HUVECS seeded at  $3.0 \times 10^5$  cells per scaffold, and (H) HUVECS seeded at  $1.5 \times 10^5$  cells per scaffold. The results are presented as means  $\pm$  SD for  $n=4$ . Symbols #, \* denote significant difference of co-cultures from the corresponding monocultures (with lower and higher densities respectively), + indicates significant difference of precultured co-cultures (OH, OMH)

versus co-culture of non-precultured cells (MH), x denotes significant difference between monocultures with lower and higher seeding density, ‡ reflects significant difference to the previous time point for all groups ( $p < 0.05$ ).

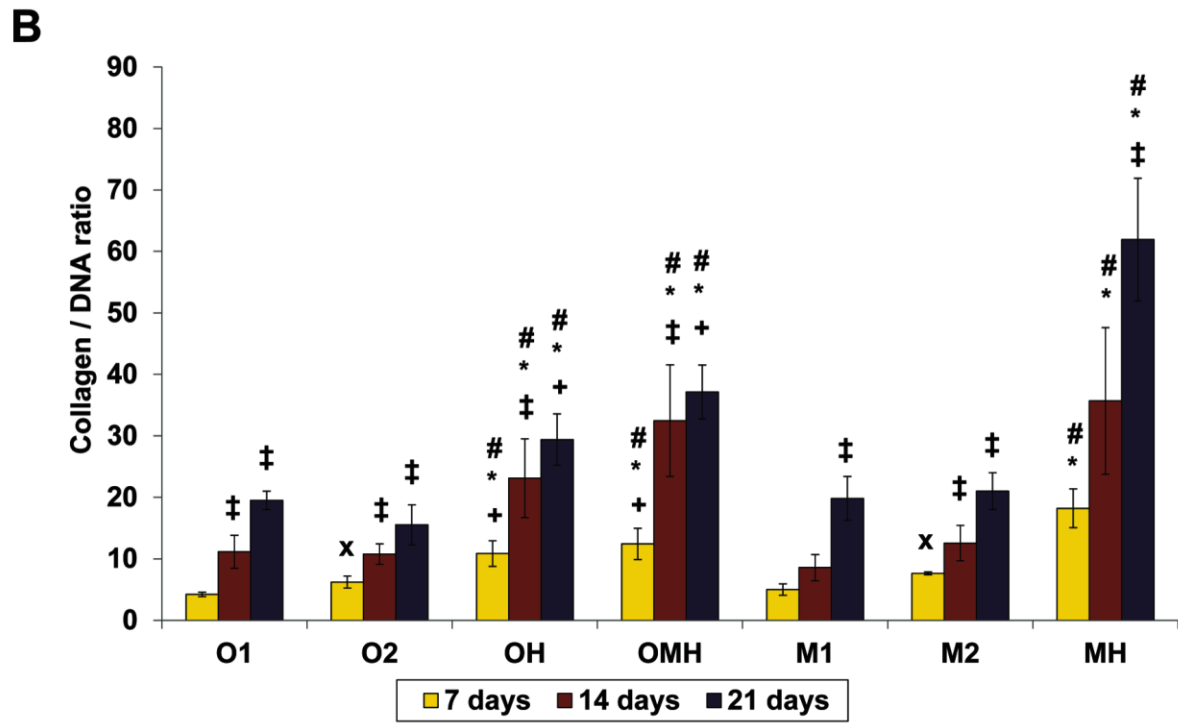
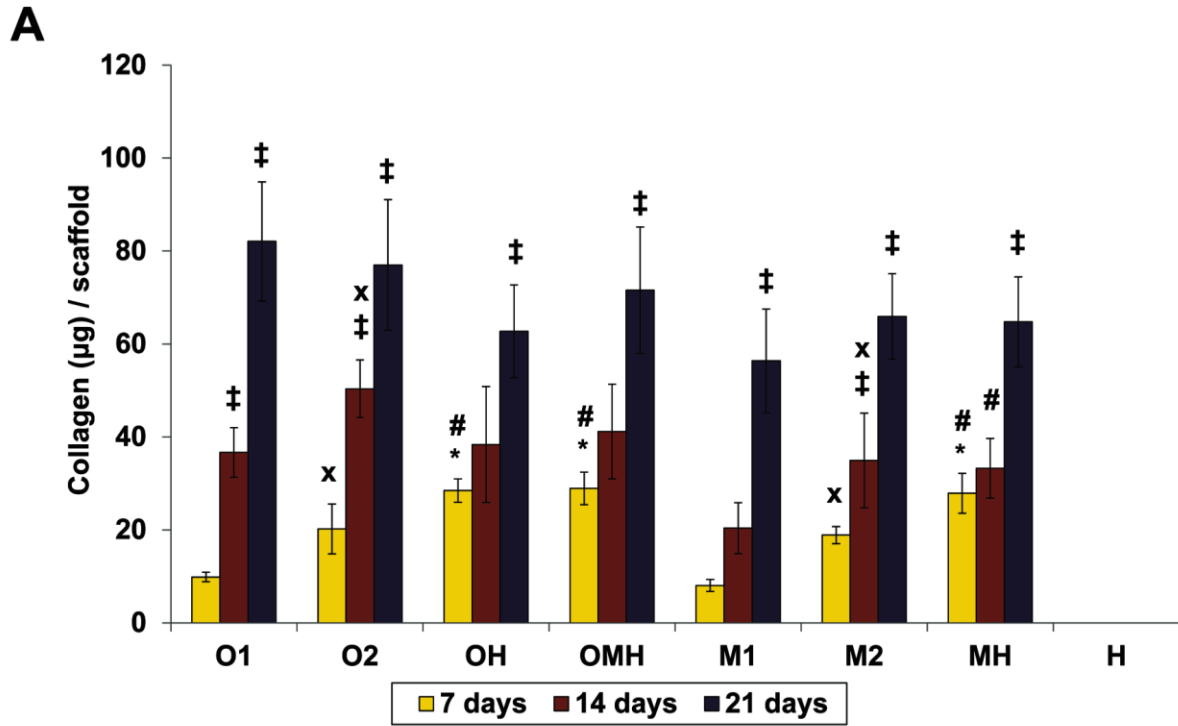


Figure 3. Collagen content (A) or normalized collagen content (B) at 7, 14, and 21 days of culture in (O1) osteogenically precultured hMSCs seeded at  $1.5 \times 10^5$  cells per scaffold, (O2) osteogenically precultured hMSCs seeded at  $3.0 \times 10^5$  cells per scaffold, (OH) 1:1 ratio of osteogenically precultured hMSCs and HUVECS seeded at  $3.0 \times 10^5$  cells per scaffold, (OMH) 1:1:1 ratio of osteogenically precultured hMSCs, hMSCs, and HUVECs seeded at  $3.0 \times 10^5$  cells per scaffold, (M1) hMSCs seeded at  $1.5 \times 10^5$  cells per scaffold, (M2) hMSCs seeded at  $3.0 \times 10^5$  cells per scaffold, (MH) 1:1 ratio of hMSCs and HUVECS seeded at  $3.0 \times 10^5$  cells per scaffold, and (H) HUVECs seeded at  $1.5 \times 10^5$  cells per scaffold. The results are presented as means $\pm$ SD for n=4. Symbols #, \* denote significant difference of co-cultures from the corresponding monocultures (with lower and higher densities respectively), + indicates significant difference of precultured co-cultures (OH, OMH) versus co-culture of non-precultured cells (MH), x denotes significant difference between monocultures with lower and higher seeding density, ‡ reflects significant difference to the previous time point for all groups ( $p < 0.05$ ).

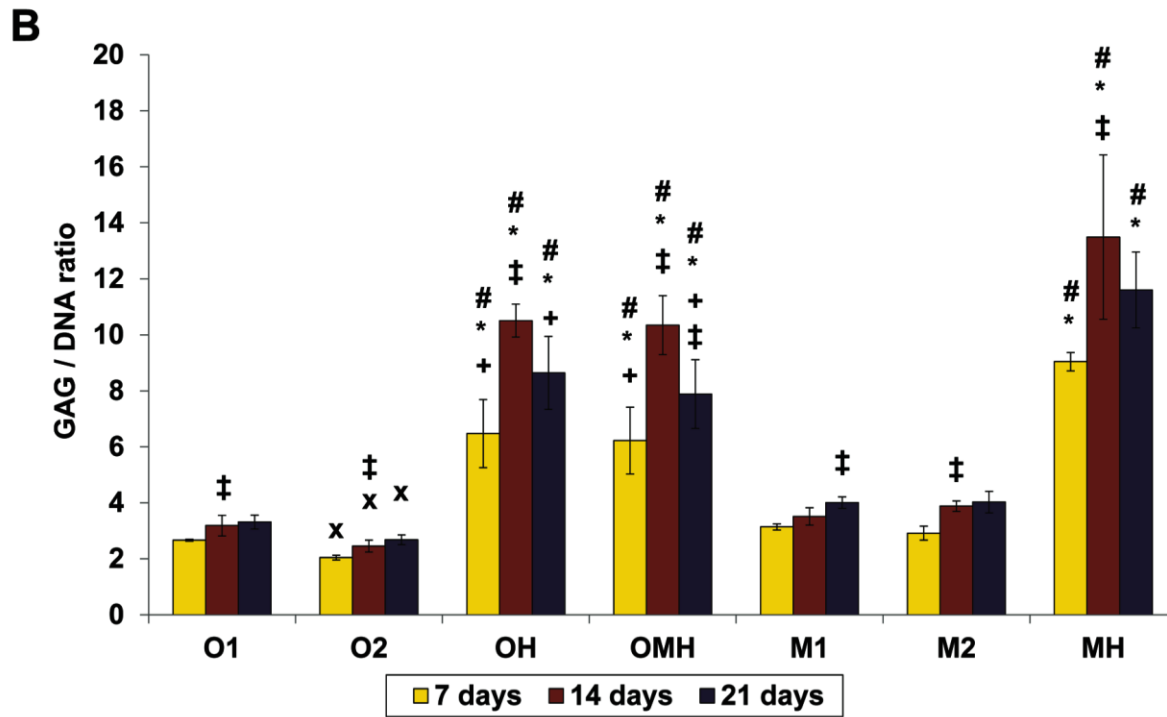
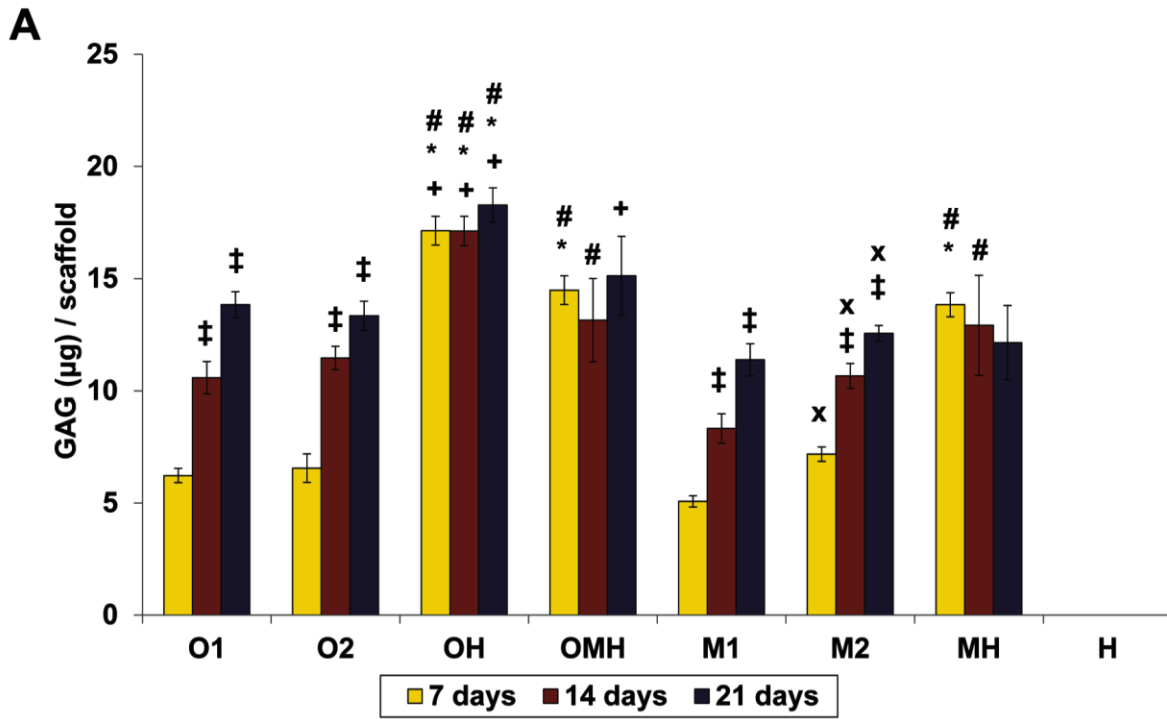




Figure 4. GAG content (A) or normalized GAG content (B) at 7, 14, and 21 days of culture in (O1) osteogenically precultured hMSCs seeded at  $1.5 \times 10^5$  cells per scaffold, (O2) osteogenically precultured hMSCs seeded at  $3.0 \times 10^5$  cells per scaffold, (OH) 1:1 ratio of osteogenically precultured hMSCs and HUVECS seeded at  $3.0 \times 10^5$  cells per scaffold, (OMH) 1:1:1 ratio of osteogenically precultured hMSCs, hMSCs, and HUVECs seeded at  $3.0 \times 10^5$  cells per scaffold, (M1) hMSCs seeded at  $1.5 \times 10^5$  cells per scaffold, (M2) hMSCs seeded at  $3.0 \times 10^5$  cells per scaffold, (MH) 1:1 ratio of hMSCs and HUVECS seeded at  $3.0 \times 10^5$  cells per scaffold, and (H) HUVECs seeded at  $1.5 \times 10^5$  cells per scaffold. The results are presented as means $\pm$ SD for n=4. Symbols #, \* denote significant difference of co-cultures from the corresponding monocultures (with lower and higher densities respectively), + indicates significant difference of precultured co-cultures (OH, OMH) versus co-culture of non-precultured cells (MH), x denotes significant difference between monocultures with lower and higher seeding density, ‡ reflects significant difference to the previous time point for all groups ( $p < 0.05$ ).

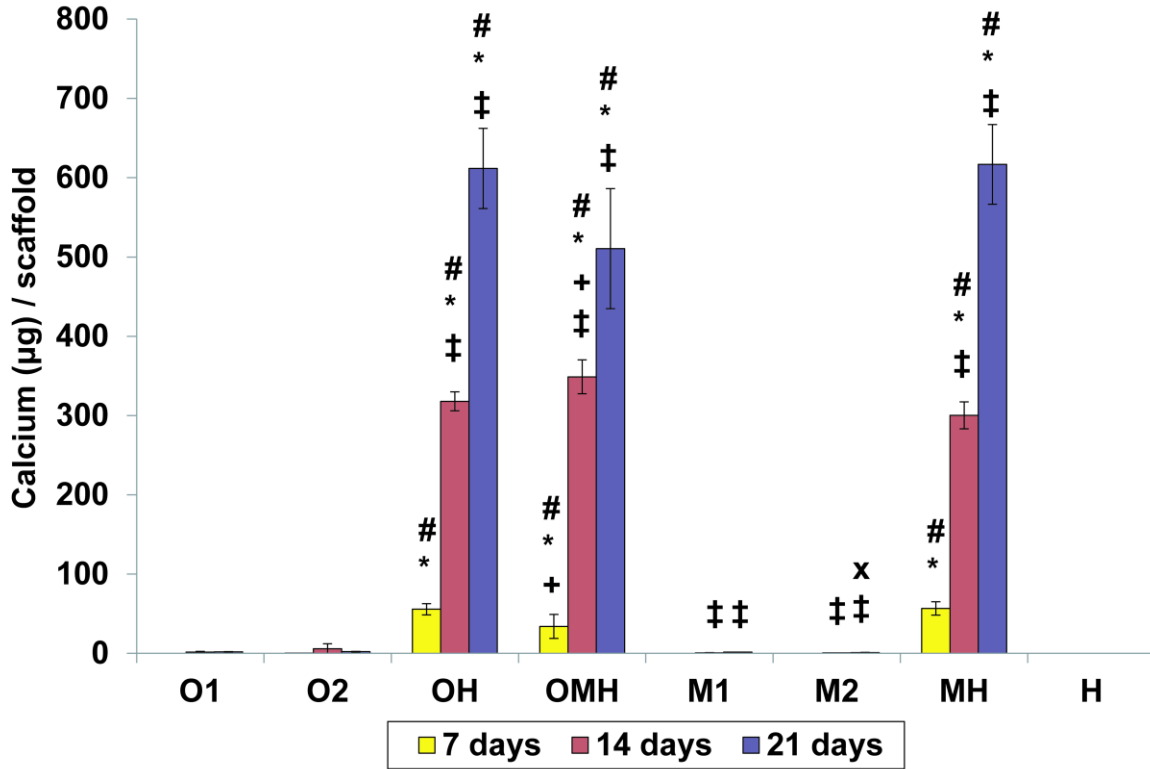


Figure 5. Calcium contents at 7, 14, and 21 days of culture in (O1) osteogenically precultured hMSCs seeded at  $1.5 \times 10^5$  cells per scaffold, (O2) osteogenically precultured hMSCs seeded at  $3.0 \times 10^5$  cells per scaffold, (OH) 1:1 ratio of osteogenically precultured hMSCs and HUVECS seeded at  $3.0 \times 10^5$  cells per scaffold, (OMH) 1:1:1 ratio of osteogenically precultured hMSCs, hMSCs, and HUVECS seeded at  $3.0 \times 10^5$  cells per scaffold, (M1) hMSCs seeded at  $1.5 \times 10^5$  cells per scaffold, (M2) hMSCs seeded at  $3.0 \times 10^5$  cells per scaffold, (MH) 1:1 ratio of hMSCs and HUVECS seeded at  $3.0 \times 10^5$  cells per scaffold, and (H) HUVECS seeded at  $1.5 \times 10^5$  cells per scaffold. The results are presented as means $\pm$ SD for n=4. Symbols #, \* denote significant difference of co-cultures from the corresponding monocultures (with lower and higher densities respectively), + indicates significant difference of precultured co-cultures (OH, OMH) versus co-culture of non-precultured cells (MH), x denotes significant difference between monocultures

with lower and higher seeding density, ‡ reflects significant difference to the previous time point for all groups ( $p < 0.05$ ).

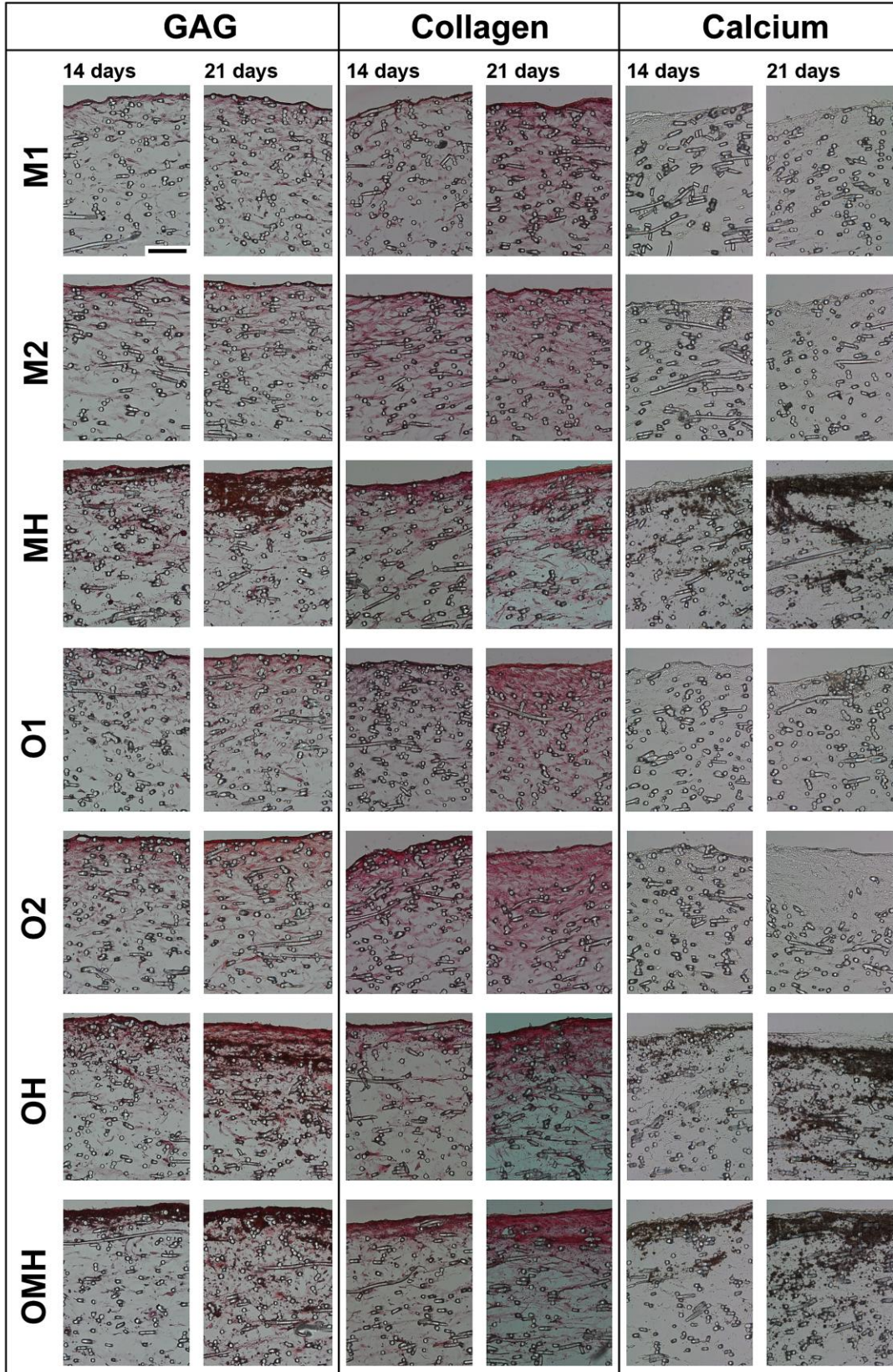
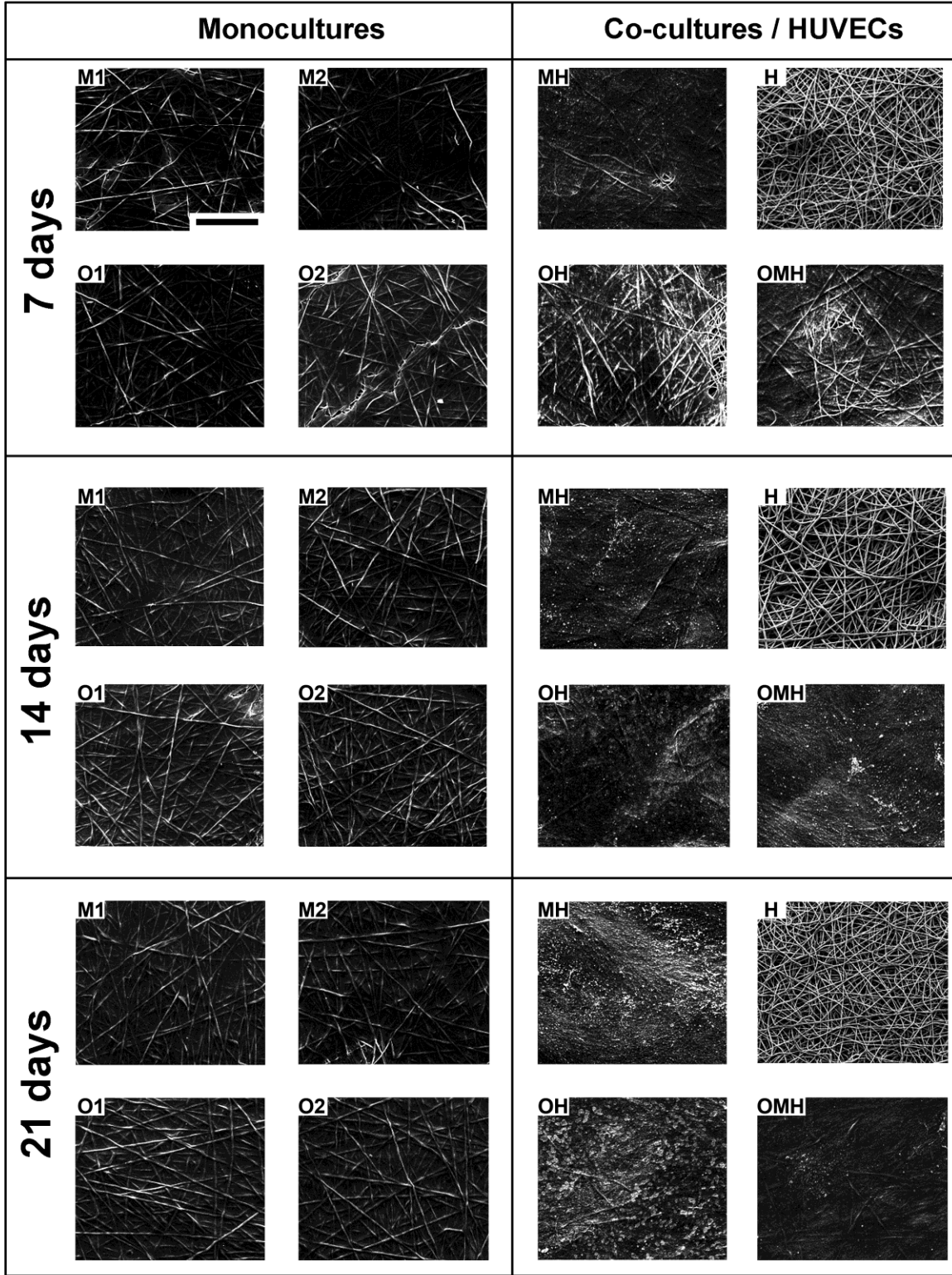


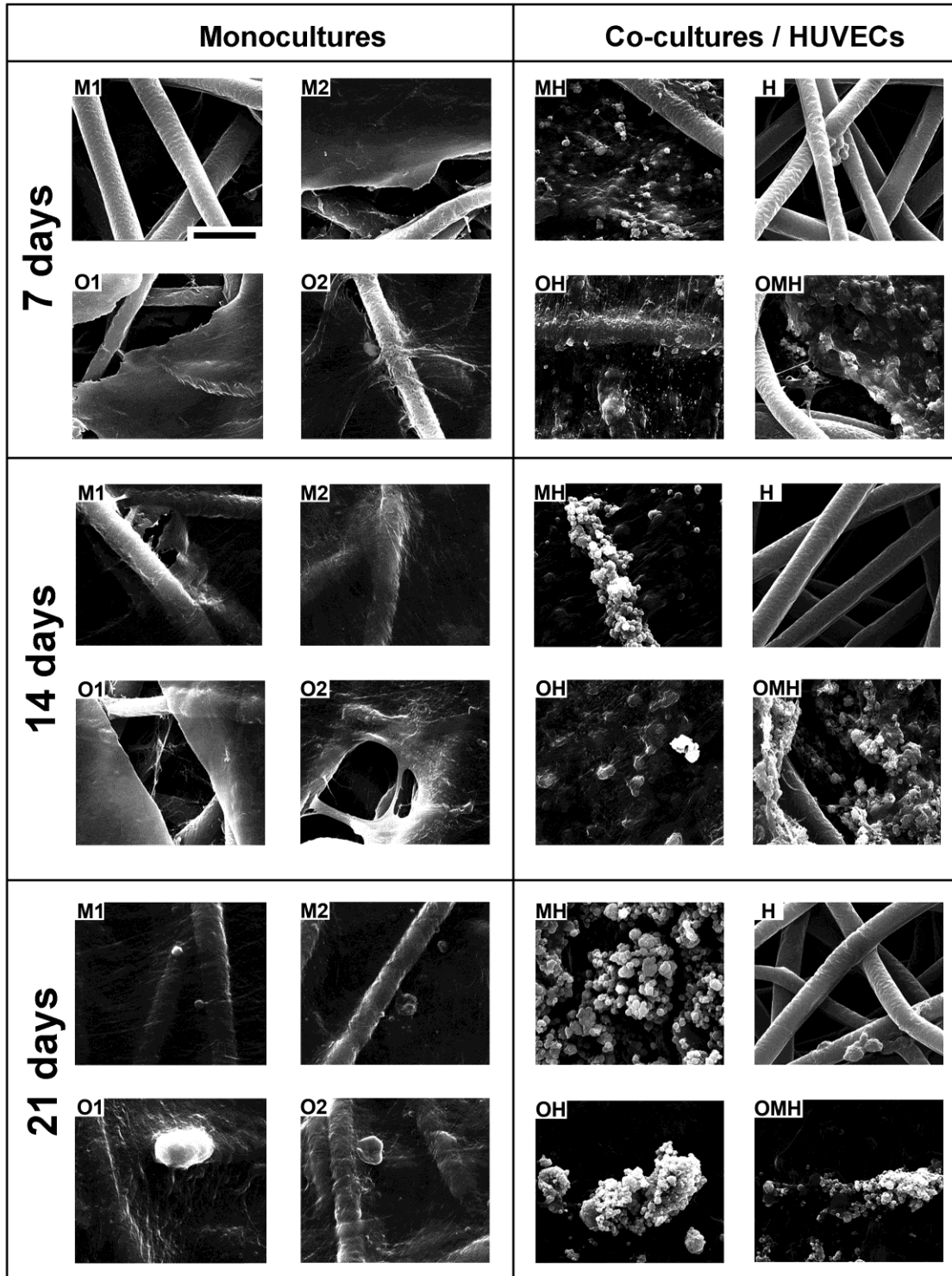
Figure 6. Bone-like matrix production, maturation, and mineralization within the PCL scaffolds with monocultures of hMSCs (M1, M2, O1, O2) and osteogenically precultured co-cultures (OH, OMH) or non-precultured co-culture hMSCs (MH) with HUVECs. Three panels display Safranin O staining for sulfated GAGs (red), PicroSirius red staining for total collagen (pink) and Von Kossa staining for calcium salts (brown). Scale bar represents 100  $\mu\text{m}$  in all images.

Supplementary Figure S1-S2. Extracellular matrix organization of scaffolds with monocultures of hMSCs (M1, M2, O1, O2) and co-cultures of hMSCs with HUVECs (MH, OH and OMH) imaged via SEM. S1 - magnification 100x, scale bar is 500  $\mu\text{m}$  and applies for all images, S2 - magnification 2000x, scale bar is 30  $\mu\text{m}$  and applies for all images. Note the absence of the matrix and the lack of any mineralization on the scaffolds with endothelial cells (H) although cells are still present on the PCL fibers.

**I**

Supplementary Figure S1. Extracellular matrix organization of scaffolds with monocultures of hMSCs (M1, M2, O1, O2) and co-cultures of hMSCs with HUVECs (MH, OH and OMH) imaged via SEM. S1 - magnification 100x, scale bar is 500  $\mu\text{m}$  and applies for all images, S2 - magnification 2000x, scale bar is 30  $\mu\text{m}$  and applies for all images. Note the absence of the matrix and the lack of any mineralization on the scaffolds with endothelial cells (H) although cells are still present on the PCL fibers.

## II





Supplementary Figure S2. Extracellular matrix organization of scaffolds with monocultures of hMSCs (M1, M2, O1, O2) and co-cultures of hMSCs with HUVECs (MH, OH and OMH) imaged via SEM. S1 - magnification 100x, scale bar is 500  $\mu\text{m}$  and applies for all images, S2 - magnification 2000x, scale bar is 30  $\mu\text{m}$  and applies for all images. Note the absence of the matrix and the lack of any mineralization on the scaffolds with endothelial cells (H) although cells are still present on the PCL fibers.