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The effect of human osteoblasts on proliferation and neo-vessel formation of human umbilical vein endothelial cells in a long-term 3D co-culture on polyurethane scaffolds☆

Alexander Hofmann [a,*], Ulrike Ritz [a], Sophie Verrier [b], David Eglin [b], Mauro Alini [b], Sabine Fuchs [c], C. James Kirkpatrick [c], Pol Maria Rommens [a]

[a] Department of Trauma Surgery, Johannes Gutenberg University School of Medicine, Langenbeckstrasse 1, D-55101 Mainz, Germany
[b] AO-Research Institute, Davos, Switzerland
[c] Institute of Pathology, Johannes Gutenberg University School of Medicine, Germany

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ABSTRACT

Angiogenesis is a key element in early wound healing and is considered important for tissue regeneration and for directing inflammatory cells to the wound site. The improvement of vascularization by implementation of endothelial cells or angiogenic growth factors may represent a key solution for engineering bone constructs of large size. In this study, we describe a long-term culture environment that supports the survival, proliferation, and *in vitro* vasculogenesis of human umbilical vein endothelial cells (HUVEC). This condition can be achieved in a co-culture model of HUVEC and primary human osteoblasts (hOB) employing polyurethane scaffolds and platelet-rich plasma in a static microenvironment. We clearly show that hOB support cell proliferation and spontaneous formation of multiple tube-like structures by HUVEC that were positive for the endothelial cell markers CD31 and vWF. In contrast, in a monoculture, most HUVEC neither proliferated nor formed any apparent vessel-like structures. Immunohistochemistry and quantitative PCR analyses of gene expression revealed that cell differentiation of hOB and HUVEC was stable in long-term co-culture. The three-dimensional, FCS-free co-culture system could provide a new basis for the development of complex tissue engineered constructs with a high regeneration and vascularization capacity.

1. Introduction

Current progress in tissue engineering research has revealed tremendous potential and new perspectives for the treatment of bone defects and fracture non-union. Reintroduction of *in vitro* expanded osteoblasts, bone marrow stromal cells, or mesenchymal stem cells in a state that guarantees their differentiation into functional bone matrix-producing cells is now widely considered a potential alternative to autologous bone grafting with a most promising future.

In a clinical study, Schimming et al. demonstrated that tissue engineered bone constructs for maxillary sinus augmentation, which is considered too small for most orthopedic and trauma surgery purposes, was not successful in many cases due to insufficient oxygen supply [1]. Other authors have suggested that the limited diffusion of oxygen and nutrients in a static cell culture environment as well as after *in vivo* implantation of tissue engineered bone may constrain cell growth and bone healing [2,3]. This problem may be amplified in cases of long bone defects since extraosseous blood supply is often significantly impaired in fracture non-unions, and medullary vasculature is not sufficient to adequately supply the defect site [4,5]. Therefore, the induction of mechanisms to improve angiogenic processes in tissue engineered bone constructs is now a major focus of research in this field [6–13].

The processes of angiogenesis and osteogenesis have been thought to be dependent on a closed interaction between endothelial cells and osteoblasts [6,14,15]. Meury et al. showed that osteoblastic differentiation of bone marrow stromal cells is controlled by endothelial cells by interfering with osterix (Osx) expression [16]. Furthermore, Villars et al. and Wang et al. demonstrated that endothelial cell differentiation and the crosstalk between endothelial cells and osteoblasts not only involve diffusible factors expressed by osteoblasts (e.g. VEGF) but are also influenced by cell membrane proteins and gap junctions in two-dimensional co-cultures [14,17]. Although these studies provide new insights into the relationship between the two cell types, knowledge about their interaction in a more complex situation involving different biomaterials is limited.

☆ This work was performed at the Department of Trauma Surgery, Johannes Gutenberg University School of Medicine, Germany.

* Corresponding author. Tel.: +49 6131 17 2359; fax: +49 6131 17 6612.
E-mail address: hofmann.trauma-surgery@gmx.net (A. Hofmann).

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In this study, we focused on the process of cell organization and neo-vessel formation in endothelial cell cultures on polyurethane scaffolds. In particular, we addressed the question of how the parameters of endothelial cell proliferation and differentiation would be influenced by differentiated human osteoblasts in a sophisticated three-dimensional long-term culture environment that is relevant for bone tissue engineering. This study should provide a new basis for the future development and biological improvement of complex bioengineered bone constructs.

2. Materials and methods

2.1. Polyurethane synthesis and scaffold preparation

A biodegradable polyurethane was synthesized using hexamethylene-1,6-diisocyanate, poly(ε-caprolactone) diol ($MW = 530\ g\ mol^{-1}$) and 1,4,3,6-dianhydro-D-sorbitol, and the scaffold was prepared by a salt leaching-phase inverse process previously described [18]. The polyurethane sponge was analyzed by the buoyancy method, computer-assisted microtomography and scanning electron microscopy presented a porosity of 85%, an interconnectivity of $99 \pm 39\ \mu m$, a macro- and a micro-average pore size equal to $216 \pm 112\ \mu m$ and $10 \pm 5\ \mu m$, respectively. The resorbable polyurethane scaffolds, which were sterilized by an ethylene oxide process, did not degrade significantly during the time of the study (no weight loss and no change of geometry).

2.2. Culture of human umbilical vein endothelial cells (HUVEC)

Primary cultures of HUVEC(-B) were purchased from PromoCell GmbH (Heidelberg, Germany), cultured in ECGM (Endothelial Cell Growth Medium, PromoCell, Heidelberg, Germany), supplemented with the provided kit, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate, in a humidified atmosphere (5% CO_2, 37 °C), with the media changed twice a week. Fifth passage cells were used for cell phenotype evaluation and for experiments.

2.3. Culture of human osteoblasts (hOB)

Endosteal cancellous bone fragments were taken from six patients undergoing orthopedic and trauma surgery. The bone fragments used for the study were considered surgical waste and would otherwise have been discarded. Informed consent was obtained from all patients and the local ethical committee approved the investigations. The patients did not suffer from any conditions known to affect skeletal healing. Osteoblast cultures were prepared using an established outgrowth culture protocol as previously described [19,20]. Bone pieces were vigorously rinsed in phosphate-buffered saline (PBS) and digested with collagenase type IV (Sigma–Aldrich Co.) for 45 min at 37 °C. After washing in PBS, in order to remove blood and fat residues, bone specimens were placed in 6-well plates (Becton-Dickinson, Heidelberg) and cultured in DMEM/F12 (Biochrom, Germany), supplemented with 10% heat-inactivated fetal calf serum (PAA Lab, Austria), 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, 100 μg/ml ascorbate, 50 μg/ml glycerolphosphate, and 10^{-8} M dexamethasone, in a humidified atmosphere (5% CO_2, 37 °C). The media were changed twice a week. Third passage cells were used for the evaluation of the osteoblastic phenotype and for experiments.

2.4. Cell phenotype of HUVEC and hOB

The phenotype of HUVEC was verified using RT-PCR (Table 1) and flow cytometry for the endothelial cell marker CD31 (PECAM-1, Miltenyi Biotec, Bergisch Gladbach, Germany). Subconfluent cultures of hOB were used for the assessment of the osteoblastic cell phenotype in each culture. Expression of the osteoblast markers osteonectin (ON) and osteopontin (OP) were examined by RT-PCR (Table 1). The expression of alkaline phosphatase (ALP) was visualized using 5-bromo-4-chloro-3-indolyl-phosphate/nitro-blue-tetrazolium as the substrate (Sigma–Aldrich Co.). Mineralization of the extracellular matrix was detected using Alizarin Red-S (Sigma–Aldrich) after culturing the cells for four weeks. The mouse fibroblast cell line L-929 was used as a negative control for all staining procedures.

2.5. Preparation of platelet-rich plasma (PRP) and platelet-released growth factors (PRGF)

PRP was prepared from blood aspirates collected in CPDA-tubes (Sarstedt, Nümbrecht) and centrifuged at $200 \times g$ for 30 min at room temperature. The plasma supernatants were pooled, transferred into a new 1 ml Falcon-tube, and centrifuged at $2000 \times g$ for 5 min at room temperature to obtain a platelet pellet. The pellet was resuspended in 1/10th of the initial blood volume with plasma supernatant. The resulting solution was called PRP. PRGF was produced by resuspending the platelet pellet with PBS in order to avoid spontaneous gelling (same volume). PRGF was activated prior to addition to cell culture medium by five freeze and thaw cycles.

2.6. Three-dimensional co-cultures of HUVEC/hOB on polyurethane scaffolds

Cylindrical biodegradable polyurethane scaffolds (diameter 8 mm, height 4 mm) were completely degassed in vacuum and pre-incubated in Iscove's-medium for 24 h (Biochrom AG, Berlin, Germany) prior to cell seeding. Cells were trypsinized, washed several times in PBS, and resuspended in PRP for a few minutes. Three different groups were used: (A) monoculture of HUVEC (1.4 million cells/scaffold), (B) monoculture of hOB (1.4 million cells/scaffold), and (C) co-culture HUVEC/hOB (0.7 million HUVEC + 0.7 million hOB/scaffold). Scaffolds were compressed manually using tweezers and cell suspensions were infused into the scaffold by slowly relaxing the scaffold and pipetting the cell suspension onto the top of the scaffold. Polymerization of PRP was induced using 5 U thrombin in order to retain cell suspensions within the scaffolds. Scaffolds were cultured for two and six weeks in Iscove's medium (FCS free, +10% PRGF) under static conditions. The media was changed every two days.

2.7. Immunofluorescence

In order to obtain additional information about cellular organization of the co-cultures during the first two weeks of incubation, three different cultures of hOB were labeled with a cell tracker green fluorochrome (Molecular Probes, Leiden, NL) to differentiate between the different cell types in the co-cultures. Mono- and co-culture were investigated 14 days after seeding using immunofluorescence for the endothelial cell marker CD31 (PECAM-1). After washing with PBS, cells were fixed with paraformaldehyde, permeabilized using Triton-X, washed with PBS/bovine serum albumin (BSA), and incubated with the primary antibody mouse-anti-human CD31 (M0823 DaKo, diluted 1:50) in 1% PBS/BSA for 40 min at room temperature. Cells were washed and labeled with the corresponding antibody Alexa Fluor®-546 conjugated anti-mouse (Molecular Probes Inc.) diluted 1:1000 in 1% BSA/PBS for 40 min at room temperature. Cell nuclei were counterstained using Hoechst 33258 (Sigma–Aldrich Co.). Finally, scaffolds were mounted with Gelmount (M01, Biomeda, Foster City, CA) and examined using a Leica TCS-NT confocal laser scanning microscope (Leica, Bensheim, Germany).

2.8. Histological and immunohistological evaluation

The evaluation of cell morphology and organization within the scaffolds in a long-term environment was performed using histological and immunohistological staining. After six weeks in culture, scaffolds were fixed using neutral-buffered formalin and mounted in paraffin. Five-micron sections were processed for the histological evaluation. A standard staining with hematoxylin–eosin and Nuclear fast Red was performed for analysis of cell integrity and morphology. Immunohistochemical evaluation was performed using specific antibodies for osteonectin (monoclonal mouse-anti-human-ON, Zymed Laboratories Inc., San Francisco, USA),

Table 1
Primer sequences used for qPCR

Gene symbol	GenBank ID	Primer sequences	Annealing Temp., °C	Product size, bp
GAPDH	NM_002046	5′-CGTCTTCACCACCATGGAGA 3′-CGGCCATCACGCCAGTTT	58	300
CD31	NM_000442	5′-GAGTCCTGCTGACCCTTCTG 3′-CACTCCTTCCACCAACACCT	52	350
Collagen type I	NM_000088	5′-CCAAAGGATCTCCTGGTGAA 3′-GGAAACCTCTCTCGCCTCTT	52	490
Osteonectin	NM_003118	5′-TGCCTGATGAGACAGAGGTG 3′-AAGTGGCAGGAAGAGTCGAA	52	304
Osteopontin	NM_001040058	5′-CATCACCTGTGCCATACCAG 3′-GGGGACAACTGGAGTGAAAA	52	418
vWF	NM_00552	5′-ATGATTCCTGCCAGATTTGC 3′-AGACTCTTTGGTCCCCCTGT	52	328

osteopontin (monoclonal mouse-anti-human-OP, R&D Systems Inc., Wiesbaden, Germany), vWF (monoclonal mouse-anti-human-vWF, Abcam, Cambridgeshire, UK), and CD31 (monoclonal mouse-anti-human CD31, DAKO, Denmark). Cell nuclei were counterstained with Mayer's hemalun.

2.9. Quantitative RT-PCR

Total RNA was extracted and purified using Trizol (Invitrogen, Karlsruhe, Germany) and RNeasy Micro Kit (Qiagen GmbH, Hilden, Germany). RNA integrity was evaluated by electrophoresis. Quantitative real-time PCR were performed in order to evaluate the stability of the hOB and HUVEC phenotypes with regard to various differentiation markers using 7300 ABI Sequence Detection System (Applied Biosystems). Validated QuantiTech Primer Assay Kits were used for the following genes: ALP (QT00012957), collagen type I (QT00037793), collagen type IV (QT00005250), CD31 (QT00081172), osteopontin (QT01008798), von-Willebrand-factor (QT00051975), Cbfa1/Runx2 (QT00020517), and 18S RNA housekeeping gene as the control (QT00199367). Real-time PCR reactions were carried out using 100 ng cDNA samples, 1XQuantiTech primers, and 1XQuantiTech SYBR-Green PCR master mix in a total volume of 30 μl. The reactions were performed using the following thermal profile: 15 min at 95 °C (PCR initial activation step) and 40 cycles of 15 s at 94 °C, 30 s at 55 °C, and 35 s at 72 °C. This was followed by a dissociation step to confirm specificity of the reaction. The data were analyzed with SDS 2.1 software (Applied Biosystems) and quantified using the $2^{-\Delta Ct}$ method. Measurement values were indicated as fold-expression (10,000×) using the housekeeping gene 18S for normalization.

2.10. Statistical analysis

Values were presented as means ± SD. All experiments were independently repeated three times with different cell preparations and all measurements were done at least in triplicate. Comparisons between groups were performed using the non-parametric Mann–Whitney *U*-Test. Differences with a $p < 0.016$ ($0.05/n$ of comparisons) were considered statistically significant. All statistical analyses were performed using SPSS-10.07-software.

3. Results

3.1. Cell phenotype

In order to examine the interaction of HUVEC with human osteoblasts, both cell types were tested with regard to their specific cell phenotype. Cultures of HUVEC showed typical cobblestone-like morphology and grew in a typical monolayer fashion (Fig. 1A). Flow cytometry revealed that 80–90% of HUVEC used for the experiments were positive for CD31 (Fig. 1B and D). After hOB reached confluence, 80–90% of the cells were positive for ALP, whereas ALP-activity was negligible in HUVEC (Fig. 1C). Accordingly, after four weeks in culture with osteogenic culture supplements, Alizarin Red-S-positive bone nodule formation was present in hOB-cultures, whereas HUVEC did not mineralize extracellular matrix (Fig. 1C). Furthermore, microscopic observation of HUVEC cultures revealed disintegration of the monolayer and decreased cell numbers after four weeks (data not shown). Analysis of gene expression using RT-PCR revealed specific expression of the endothelial cell markers CD31 and vWF as well as the osteoblast differentiation markers collagen type I and osteopontin (Fig. 1D). As expected, expression of osteonectin was evident in both cell culture types. Therefore, ON was excluded from gene expression assays in the co-cultures.

3.2. Immunofluorescence

In a pilot experiment using mono- and co-culture of HUVEC and hOB, we found that after a culture period of 14 days most cells were present in the outer part of the scaffold (data not shown). In order to get a better overview about the cell organization in this stadium of the co-cultures, we used immunofluorescent tracking of the cells and fluorescent laser microscopy for the examination. Monocultures of hOB revealed stable dye incorporation of the cell tracker green in the cell membrane and presented typical morphology of fibroblastic cells (Fig. 2A). Monocultures of HUVEC presented more flattened, cobblestone-like morphology and positive PECAM-1-immunostaining of the cell contacts (Fig. 2B). In both monocultures, cells attached to the polyurethane scaffold and proliferated along the predetermined structure. In co-cultures, both cell types were present in close contact to each other (Fig. 3C). Interestingly, the number of hOB in the co-culture was visibly lower compared with the monoculture. HUVEC that associated with hOB appeared more elongated and organized in individual cell foci. Tube-like structures could not be detected in this culture period by examination of the outer part of the scaffolds.

3.3. hOB-monocultures

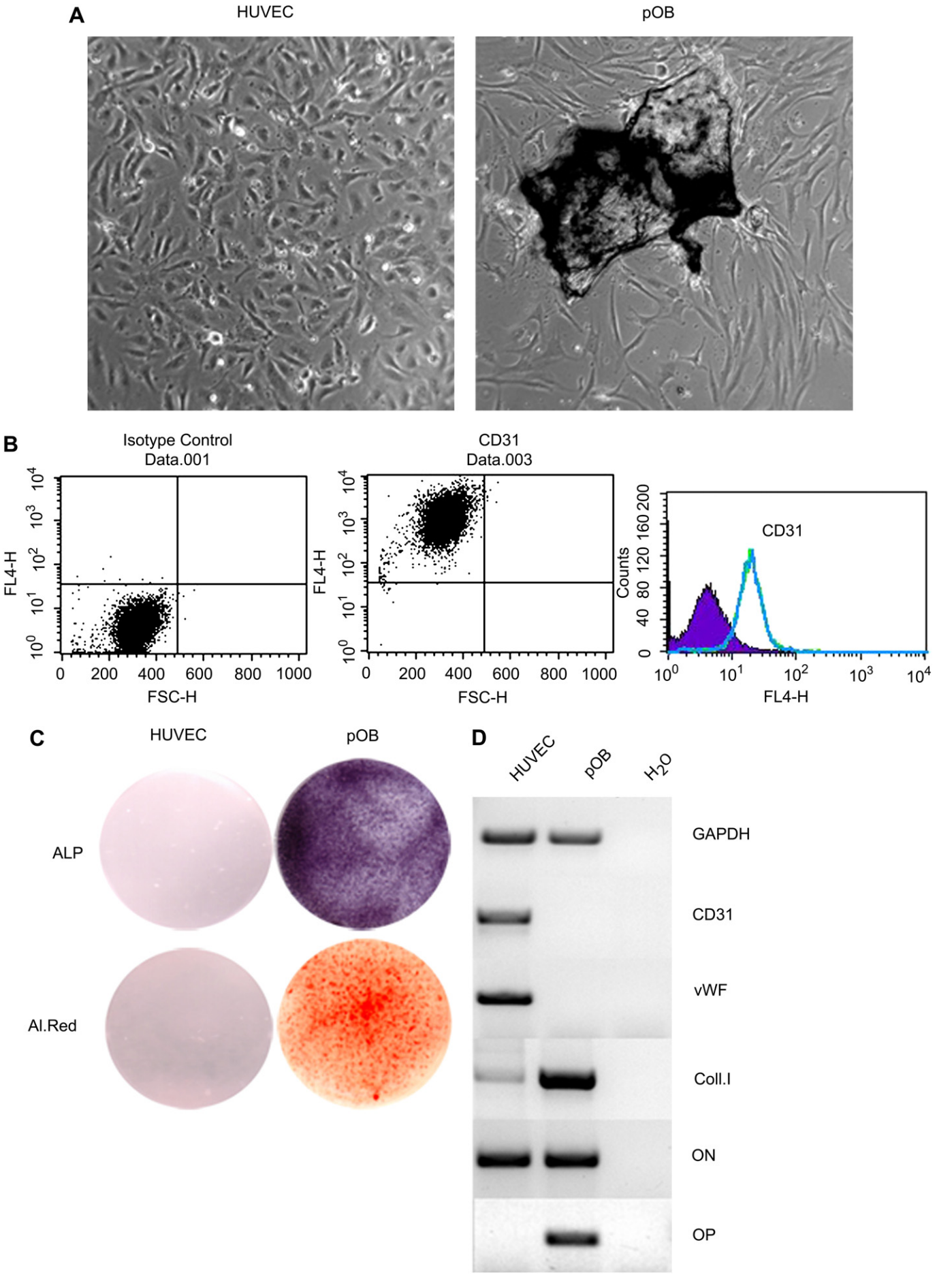
Histological evaluation of hOB-monocultures after a long-term culture period revealed that these cells were able to form two morphologically distinct cell populations on polyurethane scaffolds. One part of the cells presented cuboid-like morphology in mono- and multilayer, which is associated with the predetermined scaffold surface (Fig. 3A and B). These strongly OP- and ON-positive cells (Fig. 4) were present only in the outer part of the scaffolds (app. 500 μm). They were evocative of a kind of newly formed osteoid; however, the amount of extracellular matrix was not comparable to *in vivo* bone formation. Analysis of consecutive histological section series revealed that morphological structures formed by these cells contained several "empty" cavities (‡ in Fig. 3A and B), which could not be related to previously described neo-vessel formation and tube-like structures observed for endothelial cells (see below). Another part of the cells was present in all scaffold segments. These cells were much smaller and grew within the scaffold pores without any visible association with the scaffold surface (arrows in Fig. 3A and B). This second type of cells showed much weaker immunostaining for OP and ON (Fig. 5).

3.4. HUVEC-monocultures

A significant number of HUVECs did not survive the long-term culture period of six weeks in a monoculture (Fig. 3C and D). The outer cell surface on the polyurethane scaffold showed complete disintegration of cellular structures and could only be detected as an amorphous layer containing residues of cell nuclei (Fig. 3D). Only a few intact cells could be detected in association with the scaffold surface (Fig. 3C). However, no cells were detectable in the deeper parts of the scaffold. For further analysis of gene expression, small amounts of RNA could be isolated from only two monocultures of HUVEC.

3.5. hOB-HUVEC-co-cultures

Co-cultures of primary human osteoblasts and human umbilical vein endothelial cells were characterized by impressive formation of multiple sprouts and tube-like structures (arrows in Fig. 3E–J; Fig. 4). In contrast to cavities formed by hOB, these lumens were branched, interconnected, and of much smaller diameter (Fig. 5A–D). The lumens were formed predominantly of a single elongated cell layer (Fig. 5C–D). The outer part of the scaffold contained a thick multilayer of CD31+/vWF+-cells with multiple apertures that were connected to the tube-like structures penetrating the scaffold (Figs. 3E–G and 4, vWF). Tube-like structures could be identified both in longitudinal- and cross-section (Fig. 5A–D). The tubes did not show close association with the scaffold surface, but they were predominantly localized in the center of the pores and were surrounded by mesenchymal cells and "stroma-like" extracellular matrix. Some elongated small diameter-tubes were also present in the center of the scaffold (Fig. 4, CD31). The tube-like structures were strongly positive for the endothelial cell markers CD31 and vWF (Fig. 4). According to the results observed via immunofluorescence microscopy, the number of OP- and ON-positive cells was lower than values observed with the monocultures. The highest


A
HUVEC
pOB
B
Isotype Control
Data.001
CD31
Data.003
FL4-H
FSC-H
Counts
CD31
C
HUVEC
pOB
ALP
Al.Red
D
HUVEC
pOB
H2O
GAPDH
CD31
vWF
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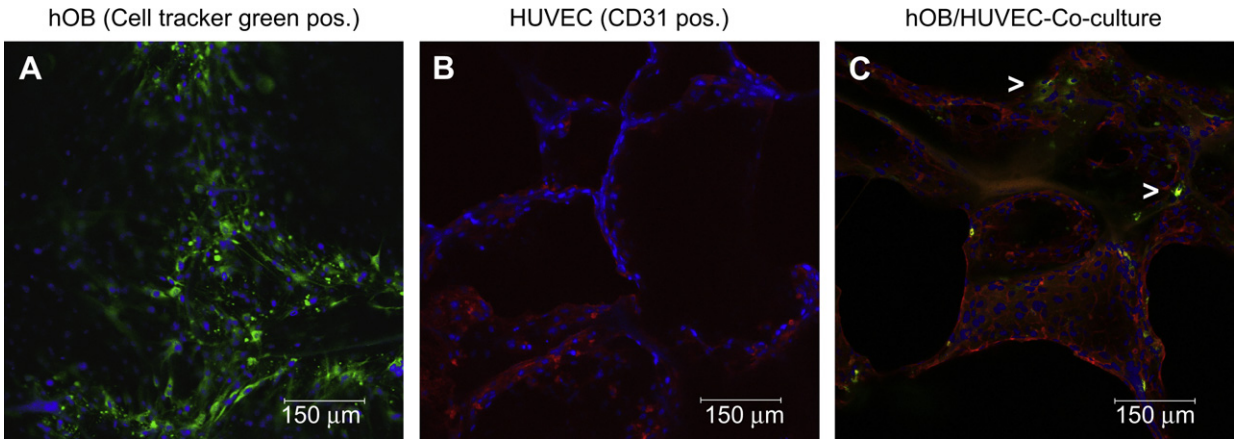

Fig. 2. Merged confocal laser microscopy images of cellular organization at day 14. Cell nuclei were stained with Hoechst 33258 (blue dye). hOB (stained with cell tracker green, A and C) were found in close contact to the HUVEC (C, white arrows), which were stained with a specific antibody against CD31 (B and C, red dye).

positive staining for these two markers was detected in small "stroma"-like cells (Fig. 4). Interestingly, some detected tube-like structures contained cellular debris and residues of cell nuclei (Figs. 3I–J, 4 and 5).

3.6. Quantitative RT-PCR

Quantitative RT-PCR was performed in order to analyze expression of diverse differentiation markers using independent methods. Expression of osteoblast-specific transcription factor Cbfa1/Runx2, as well as the expression of ALP and collagen type I was not influenced by HUVEC (Fig. 6). However, the gene expression of the extracellular matrix protein osteopontin was significantly inhibited in co-cultures with HUVEC ($p = 0.001$). Due to significantly reduced cell numbers in HUVEC-monocultures, small amounts of RNA could be isolated only in two replicates. However, to evaluate gene expression changes these two samples were pooled for RT-PCR analysis. The results revealed that expression of the endothelial differentiation markers CD31 and vWF was unchanged in hOB/HUVEC-co-cultures with hOB after normalization of the gene expression level to the housekeeping gene 18S. According to the histological results, which revealed tube-like formation only in co-cultures, expression of collagen type IV was significantly induced in this group.

4. Discussion

Fracture healing is a complex process that requires activation, migration, and differentiation of various cells that are capable of extracellular matrix synthesis and formation of different morphological structures. Thereby, the process of angiogenesis is a key element in early wound and fracture healing and is considered one of the most important requirements for sufficient oxygen and nutrient supply as well as for directing inflammatory cells to the wound site. Therefore, the improvement of vascularization in tissue engineered constructs by implementation of endothelial cells or angiogenic growth factors may be a key solution for engineering bone constructs of large dimensions. In this study, we describe a long-term culture environment that supports the survival, proliferation, and *in vitro* vasculogenesis of endothelial cells employing polyurethane scaffolds, platelet-rich plasma, and primary human osteoblasts. We clearly show that hOB and HUVEC were capable of spontaneous tissue-like organization and formation of multiple tube-like structures that were positive for the endothelial cell markers CD31 and vWF. Immunohistochemistry and qPCR analysis of gene expression revealed stable osteoblastic and endothelial gene expression profiles.

Degradable and highly porous polyesterurethane (polyurethane)-foams were introduced as potential biomaterials for bone and cartilage repair in the late 1990's, [21–23]. This polymer exhibits some specific advantages since it can be easily handled and shaped. Previous studies have reported that polyurethane supports cell adhesion and proliferation of human osteoblasts and other cell types [17,21,24–26]. Polyurethanes have also been used for replacement of vascular structures since it promotes endothelial cell growth and formation of a neo-intima [27–29]. The highly porous interconnected polyurethane foams used in this study did not show any apparent adverse effects on cell growth. In this study, histological evaluation revealed that cells found on the surface and inside the foams did not exhibit significantly different cell morphology.

Recent studies reported only a primitive *in vitro* organization of HUVEC in short-term 3D co-cultures with osteoprogenitors and osteoblasts [6,30]. The authors suggested that additional factors might be necessary to induce the maturation of the prevascular networks because lumen formation was enhanced *in vivo* after subcutaneous implantation. Our results indicate that growth factors released from PRP are likely to provide additional factors for growth and maturation of HUVEC. Different studies demonstrated that PRP promotes homing, adhesion, cell proliferation, and migration of endothelial cells [31–34], the proliferation and differentiation of osteoblasts [35,36], and the osteoblastic differentiation of bone marrow mesenchymal stem cells [37] due to high concentrations of different osteogenic and endothelial growth factors [38,39]. Therefore, PRP can be used for embedding cells within the scaffold and as a source of growth factors important for both cell types. This three-dimensional, FCS-free co-culture system could provide a new basis for the development of complex tissue engineered constructs with a high regeneration and vascularization capacity.

Previous studies provided evidence that different osteoblast cell types may have different effects on morphological organization of endothelial cells; and, furthermore, different endothelial cell types may possess different morphological organizations in response to

Fig. 1. Cell phenotype of HUVEC and hOB used in the study. (A) Monocultures of HUVEC (left) were characterized by typical cobblestone-like morphology whereas outgrowth cultures of primary human osteoblasts (right) represented an elongated, spindle-like, fibroblastic phenotype. (B) Flow cytometric analysis of HUVEC cultures for the endothelial cell marker CD31 (PECAM-1) revealed that 80–90% of the cells used in this study were positive for this marker. (C) Four-week cultures of hOB were strongly positive for the marker ALP (blue dye) and for bone nodule formation (calcium depositions stained red). HUVEC were entirely negative for this marker and nodule formation. (D) RT-PCR analysis of gene expression in HUVEC and hOB-monocultures. Gene expression of CD31, vWF, Coll1a, and OP clearly distinguished the two different cell phenotypes. Expression of osteonectin was apparent in both cell types.

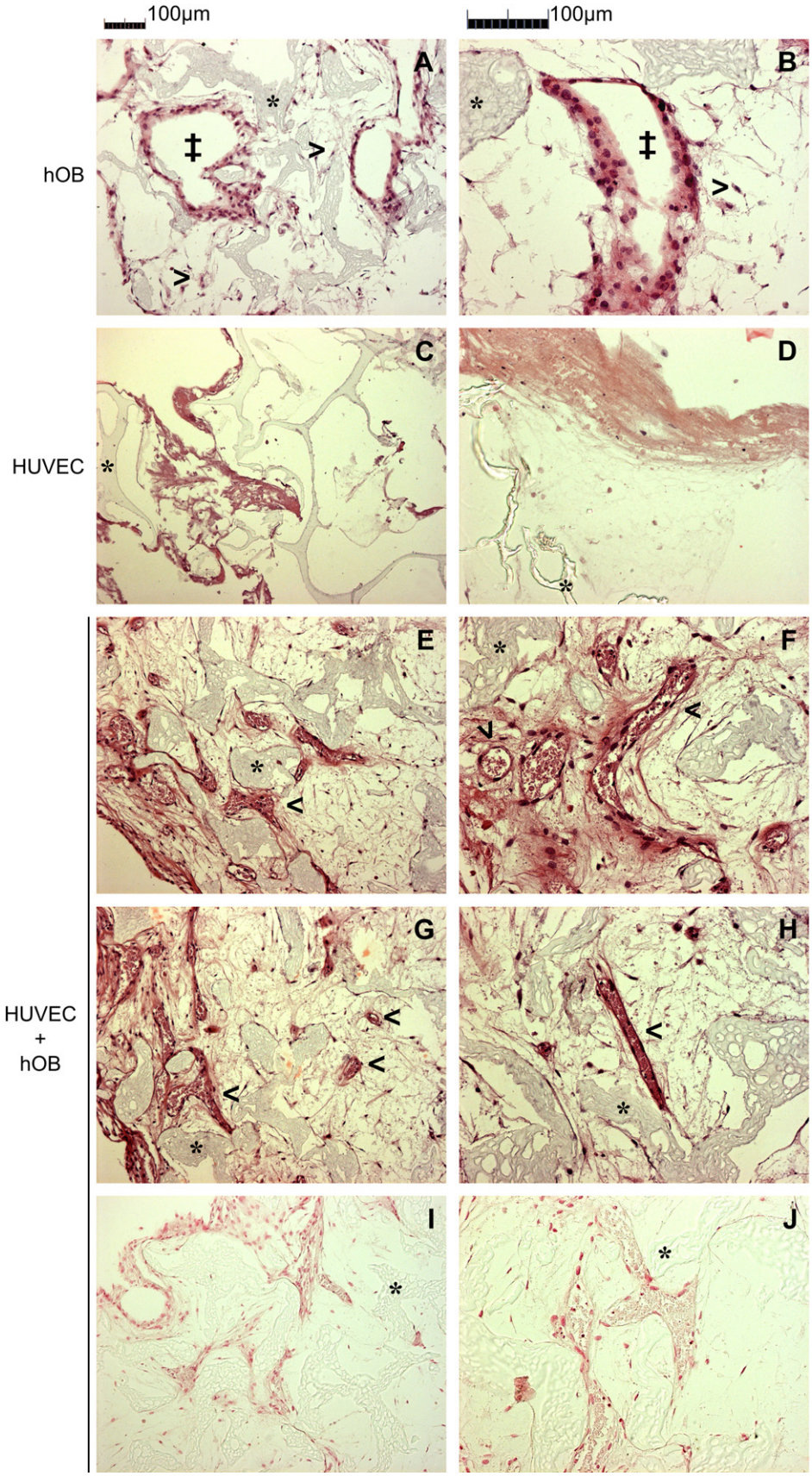

Fig. 3. Histological section of mono- (A–D) and co-cultures (E–H) were stained with hematoxylin/eosin. (A) and (B) Empty cavities in hOB-monocultures indicated with ‡ were surrounded by cuboid-like cells that were associated with the scaffold surface. Tube-like formation was apparent only in co-cultures. The tubes were localized within the scaffold pores and were surrounded by small spindle-like cells and extracellular matrix. The opaque glassy structure of polyurethane is indicated with asterisks. The Nuclear fast Red staining (I–J) showed that cellular debris included in the lumens of the neo-vessels contained residues of cell nuclei.

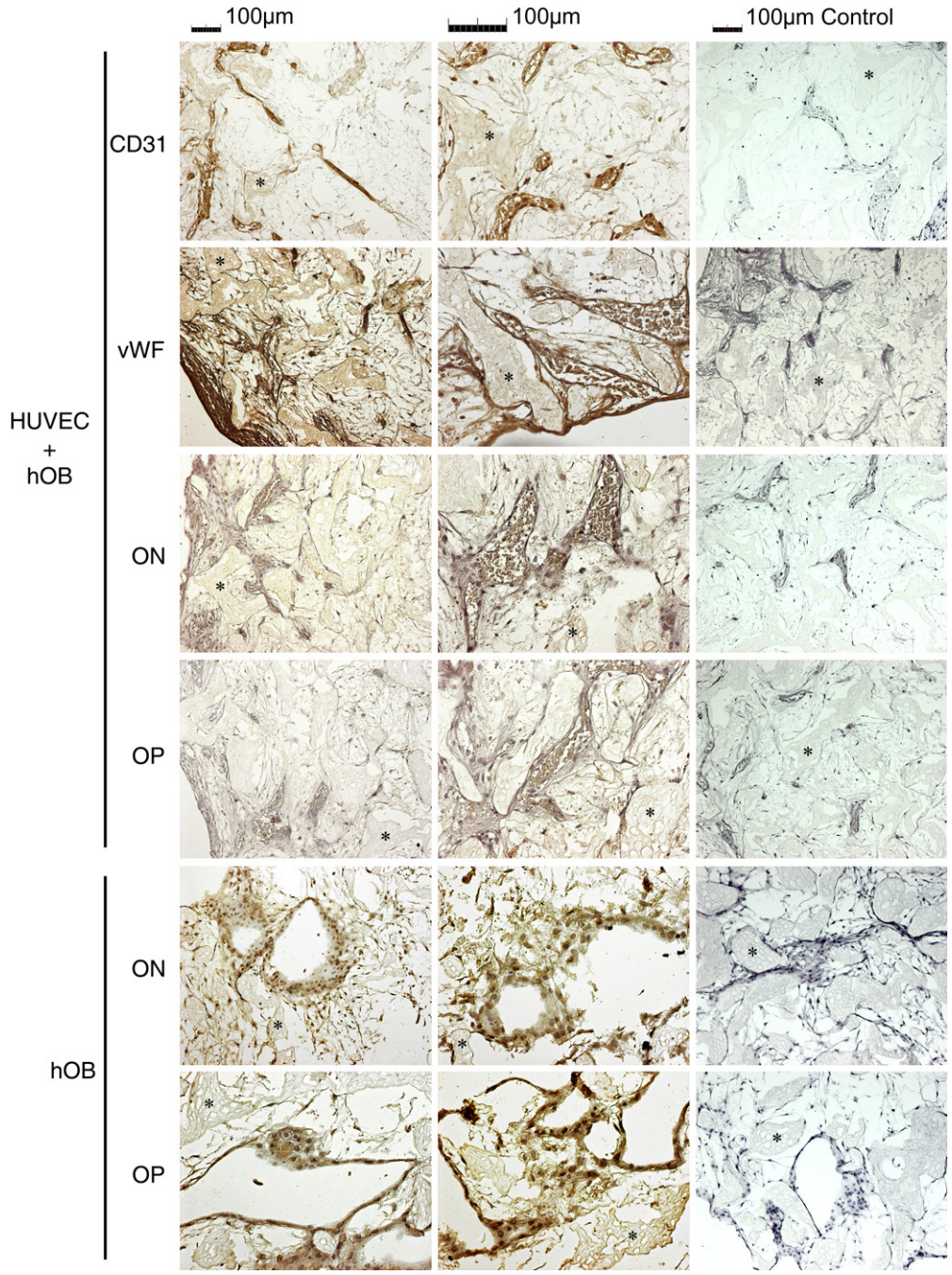

Fig. 4. Immunohistological detection of endothelial and osteoblast cell markers. The neo-vessels were strongly positive for CD31 and vWF. Both cuboid-like and small spindle-like cells in monocultures of hOB were positive for ON and OP, providing evidence that human osteoblasts are capable of possessing different morphological phenotypes *in vitro*. Negative controls were stained using the same protocol but without the primary antibody and counterstained with Mayer's hemalun. Extracellular matrix proteins ON and OP were also localized in the surrounding matrix fibers. Immunostaining for ON and OP was weakly positive in all co-cultures. The ON- and OP-positive staining of the cellular debris within the lumens was considered as non-specific. The opaque glassy structure of polyurethane is indicated with asterisks.

osteoblasts [6,7]. Outgrowth endothelial cells, which were first described by Lin et al. [40], are capable of forming an interconnected network of cord-like structures reminiscent of a pre-vascular-like formation after one week of incubation when co-cultured with the osteosarcoma cell line MG63 [6]. In a short-term co-culture, HUVEC retain their cobblestone-like morphology and remain organized in individual, non-interconnected cell foci, but they show an opening of intercellular contacts, which is considered as the very beginning of endothelial sprouting. Wenger et al. [41] reported that the ability of HUVEC to form tube-like structures is inhibited in HUVEC/hOB-spheroids seeded onto collagen matrices despite angiogenic stimulation with VEGF and bFGF. However, the same authors reported that this effect could not be reproduced in co-cultures of hOB and endothelial progenitor cells [42]. With regard to tissue engineering approaches, the results of these studies clearly demonstrate that the phenotype of endothelial cells used, as well as the seeding order (direct or non-direct contact), may cause different effects on proliferation and differentiation of both cell types. Our results in a long-term co-culture revealed that primary hOB support the survival, proliferation, and neo-vessel formation of HUVEC when seeded simultaneously onto polyurethane scaffolds. In contrast, HUVEC neither proliferated nor formed any apparent

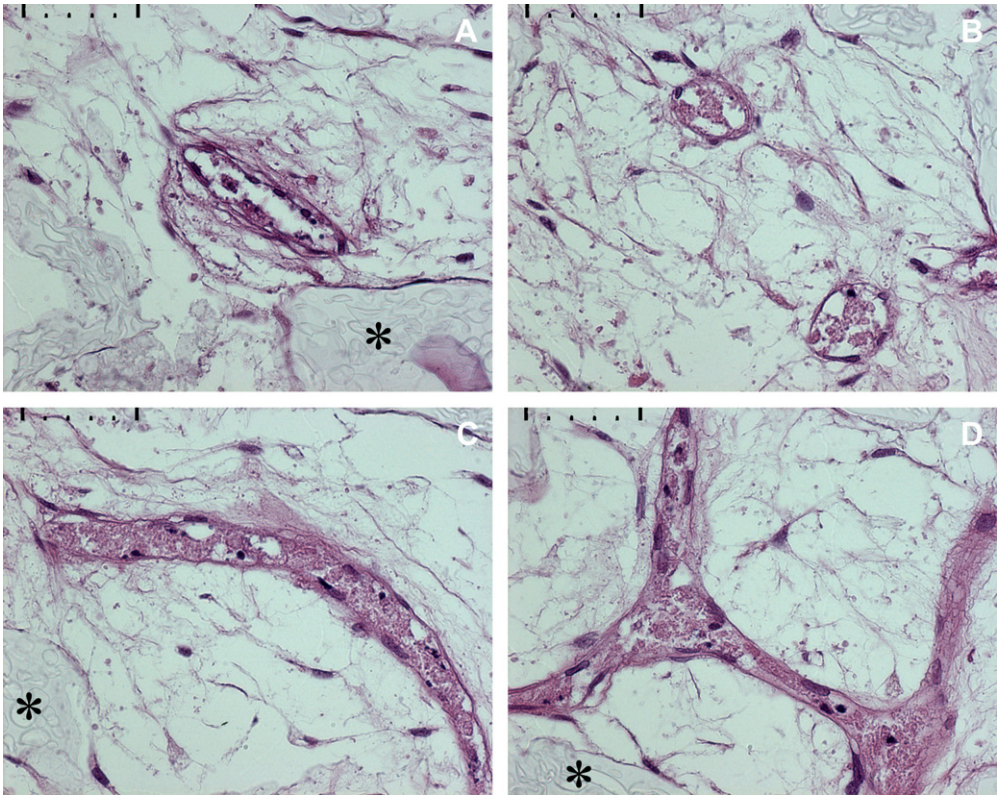

Fig. 5. High magnification of cross- (A and B) and longitudinal (C and D) HE-sections of HUVEC/hOB-co-cultures providing evidence of neo-vessel-like organization of HUVEC. Formation of tube-like structures and lumens were only apparent in co-cultures. The lumens contained cellular debris and residues of cell nuclei. This prevascular network was highly interconnected (D). The shape of the tubes was not predetermined by the scaffold (*). Note the "tissue-like" formation of the surrounding spindle-like cells and extracellular matrix fibers reminiscent of a stroma that was in close relationship to the tubes. Scale bar: 50 μm.

tube-like structures in a monoculture under the same culture conditions. Interestingly, very few cells identified histologically in HUVEC-monocultures expressed CD31- and vWF-mRNA. Their cellular expression levels were comparable in both groups even after normalization to levels of 18S-mRNA. In contrast, the expression of type IV collagen, which is one of the basal membrane constituents synthesized by endothelial cells, was significantly induced in co-cultures correlating with the detected tube-like structures (Fig. 6). Since cell ratios are difficult to estimate accurately in co-cultures, additional correction for the number of each cell type could not be performed. Therefore, gene expression of all these markers should be appraised much higher in co-cultures because the expression level of 18S in HUVECs is only a part of the total expression level in the co-culture. However, our results confirm the study of Unger et al. [7], who reported that the life span of human dermal microvascular endothelial cells was significantly enhanced in a co-culture with hOB being present for up to at least 42 days, compared to the monoculture where cells began to die rapidly after one week without passage.

Several *in vitro* studies have shown that soluble factors expressed by endothelial cells regulate the function of osteoblast cells [43–46]. As such, the vascular endothelial growth factor (VEGF) expressed by osteoblasts seems to be the most important signal molecule with significant effects on both cell types [47–51]. Cytokines expressed by endothelial cells (e.g. endothelin-1) have been reported to induce osteoblast proliferation and differentiation [52]. Although most authors found concordantly that endothelial cells have a positive effect on osteoblast proliferation [43,44,46], their influence on osteoblast differentiation has been discussed controversially [16,46,53]. Meury et al. [16] demonstrated that HUVEC inhibited osteoblastic differentiation of human bone marrow stromal cells by interfering with OSX-expression. Jones et al. [46] also showed that expression of ALP and osteocalcin, as well as the number of bone nodules in rat osteoblast cultures, were reduced by exposure to media conditioned by microvessel cells. Contrary to these results, Sun et al. [54] and Guillotin et al. [53] demonstrated that endothelial cells are capable of inducing osteoblast differentiation of rat and human osteoprogenitor cells *in vitro* as well as osteogenesis *in vivo*. When comparing different experimental studies, it is most important to notice that different effects can be observed in direct and non-direct co-cultures of endothelial and osteoblast cells, and that gap-junctional communication between both cell types is required for the regulation of the cell response [14].

In the present work, we found distinct morphological differences between mono- and co-culture of hOB. The osteoblasts with cuboid-like cell morphology that were strongly positive for the markers OP and ON in a hOB-monoculture were absent in co-cultures. Of course, it is possible that these cells were included in the thick outer cell layer, but lost the strong positive immunostaining for OP and ON (Fig. 5). Small mesenchymal cells that were present in hOB-monocultures could be identified in all co-cultures. Interestingly, with respect to the limitation of qPCR for estimation of gene expression in co-cultures, expression of Cbfa1/Runx2, ALP, and collagen type I was unchanged, indicating that the early stage of osteoblastic differentiation was not influenced significantly by HUVEC. However, according to our OP-immunostaining results, gene expression of OP was reduced significantly in hOB/HUVEC-co-cultures. This effect may be explained by the fact that later stages of osteoblast differentiation may be influenced by endothelial cells. Nevertheless, according to our qPCR results, we concluded that human umbilical vein endothelial cells are unlikely to completely inhibit the expression of the osteoblast cell differentiation markers Cbfa1/Runx2, collagen type 1, and ALP in human osteoblasts. Further evaluation

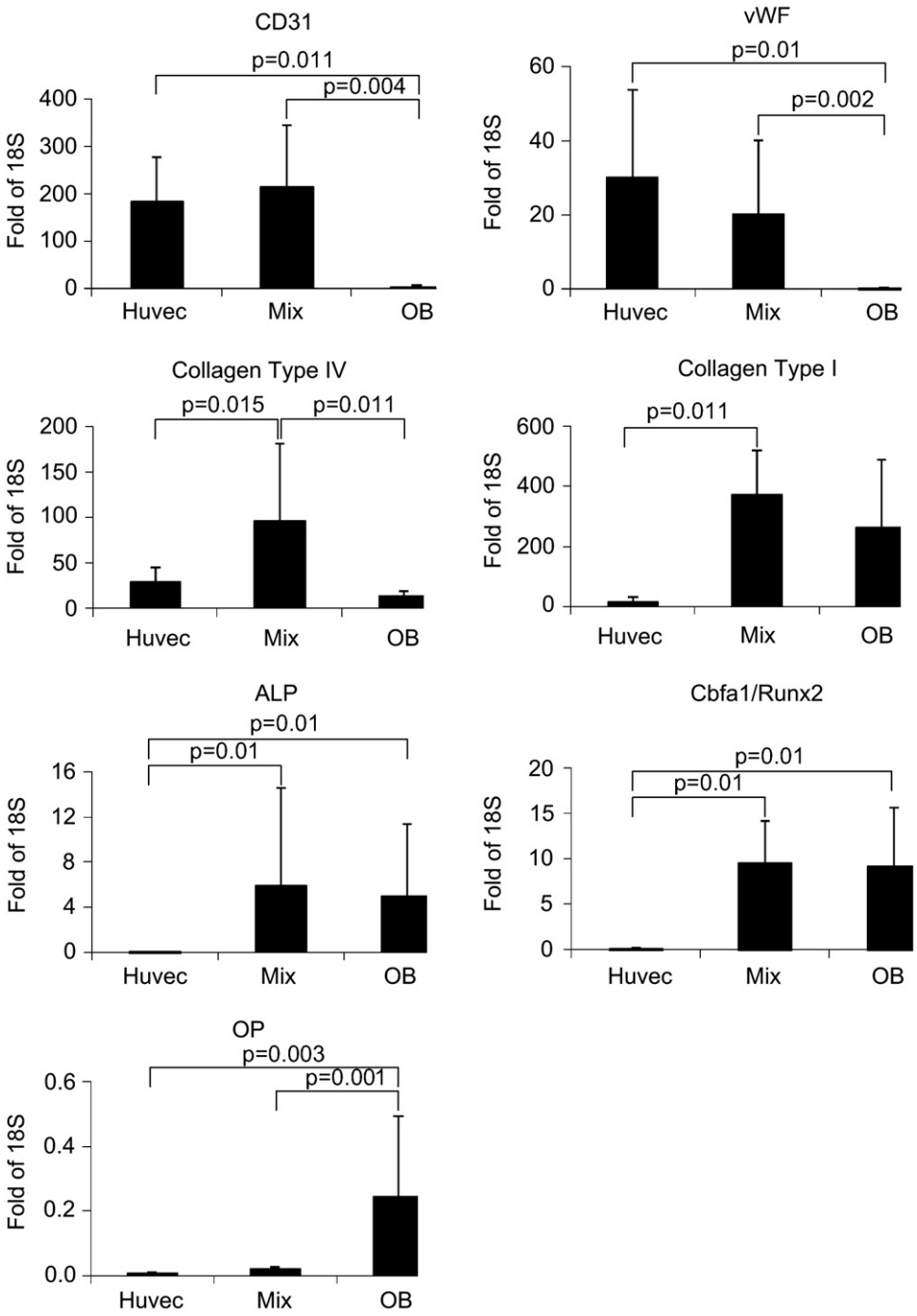

Fig. 6. Quantitative PCR analysis of marker gene expression. Comparisons between the groups were performed using the non-parametric Mann–Whitney *U*-test. $2^{-\Delta Ct}$: fold-expression normalized to 18S-mRNA expression.

is required in order to address the hypothesis that cell proliferation and later differentiation stages of hOB may by altered by HUVEC, and how hOB/HUVEC-co-cultures would develop under dynamic culture conditions. In addition, future research should address the question of whether implementation of endothelial cells in a pre-seeding step would be beneficial for healing of tissue engineered constructs *in vivo*.

5. Conclusion

In this study, we clearly demonstrate that human osteoblasts support cell proliferation, differentiation, and neo-vessel formation of human umbilical vein endothelial cells cultured on polyurethane scaffolds. In contrast, in a long-term monoculture, HUVEC do neither proliferate nor form any vessel-like structures. This three-dimensional, co-culture system could provide a new basis for the development of complex tissue engineered constructs with a high regeneration and vascularization capacity.

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