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ORIGINAL ARTICLE

Cultivation of EPC and co-cultivation with MSC on β-TCP granules in vitro is feasible without fibronectin coating but influenced by scaffolds' design

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Abstract

Introduction Meanwhile, the osteoconductive properties of frequently used synthetic bone grafts can be improved by the use of osteoinductive cells and growth factors. Nevertheless, the cultivation of endothelial progenitor cells (EPC) seems to be difficult and requires a pre-conditioning of the scaffolds with fibronectin. Additionally, the influence of the scaffolds' design on cell cultivation is not fully elucidated.

Methods As scaffold, a commercially available β-tricalcium phosphate was used. 5×10^5 EPC, or 5×10^5 MSC or a combination of each 2.5×10^5 cells was seeded onto the granules. We investigated seeding efficiency, cell morphology, cell metabolism, adherence, apoptosis and gene expression of EPC and MSC in this in vitro study on days 2, 6 and 10.

Results Total number of adherent cells was higher on the β-TCP without fibronectin coating. The number of cells in all approaches significantly declined when a solid β-TCP was used. Metabolic activity of MSC was comparable throughout the scaffolds and increased until day 10. Additionally, the amount of supernatants VEGF was higher for MSC than for EPC.

Discussion Our results demonstrate that a coating of the scaffold for successful cultivation of EPC in vitro is not necessary. Furthermore, our study showed that structural differences of the scaffolds significantly influenced cell adherence and metabolic activity. Thereby, the influence on EPC seems to be higher than on MSC.

Keywords Scaffold · Stem cell · Tricalcium phosphate · Fibronectin

Abbreviations

β-TCP/β-TCP-FN	Beta-tricalcium phosphate/-fibronectin
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CS	ChronOS strip
DMEM	Dulbecco's Modified Eagle Medium
DNA	Desoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EPC	Endothelial progenitor cell
FCS	Fetal calf serum
MTT	Methyl-thiazolyl-tetrazolium
MSC	Mesenchymal stem cell
PBS	Phosphate-buffered solution
(RT-) PCR	(Real-time) polymerase chain reaction
RNA	Ribonucleic acid
SEM	Standard error of mean
SEM	Scanning electron microscopy
VEGF	Vasoendothelial growth factor
vWF	Von Willebrand factor

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Introduction

Under normal conditions, bone healing leads to stable bony conditions and with completion of the remodeling the bone is scare free without deviation of the axis. However, in large bone defects, after tumors or infections, the physiological bone regeneration can fail. In these cases, orthopedic surgeons can use different strategies to improve the complex physiological process including a stable fixation as well as the usage of adjuncts such as osteoconductive scaffolds, growth factors or osteogenic cells [1, 2]. Despite the numerous possibilities in reconstructive surgery, autologous bone grafting remains

the gold standard. Bone usually is taken from the patients' iliac crest. This procedure is, however, fraught with donor site morbidity like postoperative pain and limited amount of bone material [3, 4].

Bone tissue engineering probably provides a suitable alternative to autologous bone. Giannoudis et al. summarized the special preconditions for proper bone tissue engineering. They proposed a diamond concept incorporating mechanical environment, growth factors, regenerative cells, scaffolds and vascularization [5, 6].

It has been proved in a variety of bone healing models that marrow stromal cells (MSC), usually provided on a scaffold, promote bone healing probably due to osteogenic differentiation [7, 8].

However, the size of the bone defect may limit the ingrowth of bone-forming cells, and the lack of vessels does not provide a sufficient nutritional support for the bone graft. Early vascularization of the scaffold in the bone defect may be necessary for ingrowth of osteogenic reparative cells to regenerate bone in vivo. This can be achieved by endothelial progenitor cells (EPC). We demonstrated in own previous work that the simultaneous cultivation of both cell types on different scaffolds is feasible [9]. Furthermore, the combined application of both cell types on a β-TCP scaffold showed improved vascularization and bone healing in critical size defects in rats [10].

Depending on the shape of the bone defect other form factors of the scaffold than granules might though be more feasible. The granular β-TCP scaffold used in our previous work is also available as a flexible composite scaffold consisting of a resorbable polymer poly(lactide-co-ε-caprolactone) carrier and β-TCP granules.

So far it has been assumed that a coating of surfaces with fibronectin is crucial to promote adhesion and to maintain endothelial differentiation of EPC. Thus, fibronectin-coated β-TCP scaffolds were applied in our previous preclinical studies [9–12].

However, the necessity of a fibronectin coating adds further complexity to scaffold development, storage and application in clinical routine.

Therefore, we examined whether fibronectin coating of a granular β-TCP scaffold is necessary for the initial adherence, survival and function of EPC in single culture and coculture with MSC.

We additionally characterized EPC and MSC seeded on a composite scaffold based on the same β-TCP and a flexible poly(lactide-co-ε-caprolactone) carrier to elucidate the influence of different form factors on the cytocompatibility.

Methods

Ethics

MSC were isolated from bone marrow samples taken by fine needle puncture of the iliac crest. EPC were isolated from buffy coat. Both were provided by the Red Cross blood donor service Baden-Wuerttemberg–Hessen. The use of anonymized bone marrow, respectively, buffy coat for research purposes was approved by the local ethics committee (Project No. 329/10) and all donors signed informed consent.

Isolation of MSC

Bone marrow was obtained from five individual donors. Each bone marrow was diluted 1:5 with PBS (without calcium and magnesium, PBS −/−) and layered 1:1 on a Ficoll density gradient (1.077 g/ml, 1100 g, 30 min, Biochrom, Berlin, Germany). Mononuclear cells were then collected from the interphase and washed twice (PBS −/−, 900 g, 10 min) before they were resuspended in medium (MesenCult + Supplements, CellSystems, St. Katharinen, Germany). Mononuclear cells were seeded in a density of 4×10^6 cells/cm^2 and were cultured over at least three weeks. Medium was exchanged three times a week. Cells were passaged if the culture reached 80% confluence. MSC of the third to the fifth culture passage were used for the experiments. MSC were prestained with the cell tracker carboxyfluorescein diacetate succinimidyl ester (CFSE) prior to seeding on to the scaffolds, thereby following the instructions of the manufacturer (Molecular probes, Thermo-Fisher, Schwerte, Germany). In brief, 1×10^6 MSC were incubated in 5 mL prewarmed PBS. The CFSE solution was added in a final concentration of 10 mM and incubated at 37 C° for 10 min. Then, the staining was quenched by the addition of 25 mL of ice-cold DMEM + 10% FCS to the cells. Cells were incubated for further 5 min on ice followed by two washes at 300 g for 10 min using DMEM + 10% FCS. The cells were resuspended in medium and the seeding procedure was performed as described later.

Isolation of endothelial progenitor cells (EPC)

EPC were isolated from buffy coats obtained from five individual donors. The isolation was performed by density gradient (Ficoll, 1.077 g/ml, 800 g, 20 min, Biochrom, Berlin, Germany). Mononuclear cells were then collected from the interphase, were washed twice with PBS−/− and seeded into fibronectin-coated culture flasks (75 cm^2) in a density of 2×10^6 cells/cm^2 in Endothelial

Basal Medium—2 + EGM-2—Single Quots, (Lonza, Heidelberg, Germany) at 37 °C, 5% CO_2. Non-adherent cells were removed during medium exchange after 72 h. Cells were removed from culture flask by *Accutase* treatment (*Accutase*, PAA Laboratories, Linz, Austria) and used for experiments after two further days of cultivation. A parallel preparation was performed to evaluate the percentage of endothelial cell-like differentiated cells. Cells were incubated with 2.4 µg/mL 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein (DiL, CellSystems, St. Katharinen, Germany) in EBM supplemented with 20% FCS for 1 h. Cells were then fixed with 2% paraformaldehyde for 10 min, and after washing with PBS +/+ they were incubated with FITC-labeled *Ulex europaeus* agglutinin-1 [10 µg/mL] (lectin, Sigma, Deisenhofen, Germany) for 1 h. Cells presenting double-positive fluorescence were considered to be EPC [13, 14]. Only preparations with a percentage of endothelial-like differentiated cells of greater than 80% were used.

Scaffolds

ChronOS granules (diameter 1.4–2.8 mm, DePuy Synthes, West Chester, USA), a β-tricalcium phosphate, as well as the composite material *ChronOS Strip* (CS, DePuy Synthes) consisting of a poly(lactide co-ε-caprolactone) carrier engaged with β-TCP granules were used. The β-TCP granules offer a porosity of 60%, interconnecting pores with a size of 100–500 µm, low mechanical stability and moderate biodegradability.

For the experiments, *ChronOs Strip* was cut into pieces of 5 × 5 mm under sterile conditions.

Fibronectin coating of β-TCP granules

Sterile β-TCP granules were incubated for 30 min in a fibronectin solution (10 mg/ml, Sigma, Deisenhofen, Germany) in PBS−/−. Then the supernatant was removed and replaced by PBS−/. The granules were immediately placed as a dense single layer in a 24-well plate (Nunc, Wiesbaden, Germany) using sterile forceps followed by the subsequent cell seeding procedure. Fibronectin-coated β-TCP is referred to as β-TCP-FN in the following.

Seeding of cells

Sterile β-TCP, β-TCP-FN granules, respectively, CS were placed as dense monolayer to individual wells (4 cm^2) of 12-well plates (Nunc, Wiesbaden, Germany). Subsequently, EPC, MSC, respectively, a 1:1 mixture of both cells were seeded in a density of 1.25×10^5 cells/cm^2 scaffold monolayer (5×10^5 cells/well) in a volume of 100 µL medium (500 µL/well) as described in [9, 15–17] and incubated for 10 min at 37 °C. After that the medium-containing non-adherent cells was recovered and dripped once again over the respective scaffolds and incubated for further 10 min at 37 °C. The last step was repeated one more time.

Then the granules were transferred into a new well plate and supplemented with culture medium. This medium consisted of 2/3 Mesencult + supplements + 1/3 EBM-2 + EGM2 supplements as described in [9]. This mixture maintained endothelial characteristics of EPC as well as osteogenic gene expression of MSC as shown by RT-PCR [9, 15, 18]. The initially used wells were treated with *Accutase* to recover all cells that did not adhere to the test scaffolds to determine the cell seeding efficacy.

The subsequent experiments (cell adhesion, metabolic activity, gene expression, VEGF synthesis) were performed on days 2, 6 and 10 after cell seeding [9, 15, 18].

Cell adhesion

The relative number of cells was determined using a fluorescence microscope (AxioObserver, Carl Zeiss, Gottingen, Germany). Adherent MSC were identified via their CFSE prestaining and EPC were visualized by DiL staining. Granules seeded with EPC and MSC were incubated with 1 µl DiLacLDL per 100 µl nutrient solution and incubated for 1 h at 37°C/5% CO_2. Subsequently, cells were washed twice with PBS, fixed with 2% formalin in PBS (10 min), followed by three washes with PBS−/−. Nuclei of the adherent cells were stained by subsequent incubation with DAPI (4′,6-diamidino-2-phenylindole, Sigma-Aldrich, 10 µg/mL, 10 min) followed by four washes with PBS−/−. The number of adherent EPC and MSC cells was counted on ten randomly chosen high power fields at 100-fold magnification. CSFE-positive cells with DAPI-stained nucleus were judged as MSC, DiL-positive cells with a DAPI-stained nucleus were counted as EPC.

Metabolic activity

On days 2, 6 and 10 after seeding of the cells, the metabolic activity was measured using the methyl-thiazolyl-tetrazolium (MTT) test (Roche Diagnostics, Mannheim, Germany). Each three β-TCP granules were transferred into a fresh 96-well plate, respectively, one piece of composite material into a well of a 48-well plate to prevent false-positive results caused by cells adhering to the bottom of the well. 90 µL of medium and 10 µL of MTT labeling reagent were added to each well and cells were incubated for an additional 4 h. Next, the cells were incubated overnight with a solubilization solution. The supernatant was collected and transferred to another 96-well plate. The absorbance at 570 nm was then measured with an ELISA reader (Ceres UV900c, BioTek Instruments, Winooski, VT, USA). To determine

the metabolic equivalent, increasing numbers (1000, 2500, 5000, and 10,000) of MSC and EPC were seeded directly in 96-well plates and assessed separately.

Secretion of vascular endothelial growth factor (VEGF)

The secretion of VEGF into the nutrient solution as a functional parameter of EPC and MSC was determined on days 2, 6, and 10. The supernatants were kept at – 80 °C, later measurement was performed using a VEGF-A-ELISA (R&D Systems, Wiesbaden, Germany) following the manufacturer's instruction. Values were corrected for the VEGF content of the medium.

Apoptosis

Apoptotic activity of EPC on the scaffolds was measured 24 h after seeding by means of Caspase 3/7 activity assessment (Caspase 3&7 Flica Kit, Immunochemistry Technologies LCC), thereby following the manufacturer's instructions. The assay is based on the binding of the fluorescent inhibitor probe FAM-DEVD-FMK to active caspase 3 and 7 enzymes which can be detected by green fluorescence. Non-apoptotic and apoptotic EPC per high power field (100-fold magnification) were subsequently assessed using fluorescence microscopy. Three high power fields per experiment ($n=5$) were counted and the mean value for each experiment was calculated. Those mean values were used for the subsequently following statistical analysis.

Endothelial and osteogenic gene activity

In brief, total RNA was isolated using the RNeasy system (Qiagen, Hilden, Germany) following the manufacturer's instructions with the following exception. Approximately 50 µL granules, respectively, one piece of the composite scaffold (5 × 5 mm) that had been sown with progenitor cells was incubated in RLT buffer for 3 min, the mixture was gently vortexed and the supernatant was subjected to the RNA isolation procedure. The quality and quantity of RNA was determined using the NanoDrop ND-1000 device (Nanodrop technologies, Wilmington, Delaware, USA). Contaminating genomic DNA was removed by digestion with the RNase-free DNase Kit following the manufacturer's protocol (Qiagen). Each 250 ng of RNA was reversely transcribed using an Affinity script QPCR cDNA synthesis kit (Stratagene, La Jolla, CA, USA), following the manufacturer's instructions. Real-time PCR was performed on a Stratagene MX3005P QPCR system (Stratagene, La Jolla, CA, USA). PCR was performed using the primer assays for human collagen-1 (COL1A, NM 000088, catalog number PPH01299F), alkaline phosphatase (ALPL, NM 000478, catalog number PAHS-026), core-binding factor-1 (cbfa-1 also known as Runt-related transcription factor 2, RUNX2, NM 004348.3, catalog number PPH01897B), osteocalcin (BGLAP, bone gamma-carboxyglutamate (gla) protein, NM 199173.3, catalog number PPH01898A), vascular endothelial growth factor (VEGF, NM 003376.4, catalog number PPH00251B), and von Willebrand factor (vWF, NM 000552.3, catalog number PPH02567E). As reference gene, the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH, NM 002046.3, catalog number PPH00150E) was measured. All primer assays were purchased from Qiagen. A melting curve analysis was applied to ensure the specificity of the PCR reaction. Relative quantification of the mRNA levels of the target genes was determined using the comparative CT (threshold cycle values) method ($2^{-\Delta CT}$ method). The results are presented as fold change to GAPDH gene expression. Assessment of gene expression was performed on days 2, 6 and 10 after cell seeding.

Analysis of adhering cells using scanning electron microscopy (SEM)

2 , 6 and 10 days after cell seeding as described before, untreated and treated scaffolds were fixed with glutaraldehyde (2%) for 30 min and subsequently dehydrated by ascending grades of alcohol (25, 50, 75, 96, 100% ethanol) for 15 min per step. Scaffolds were then incubated in 1,1,1,3,3,3-hexamethyldisilazane (Merck Schuchardt, Hohenbrunn, Germany) overnight and drained. Afterwards, the samples were sputtered with gold (5 × 60 s, Agar Sputter Coater; Agar Scientific Ltd., Stansted, United Kingdom) and analyzed using a Hitachi FE-SEM S4500 (Hitachi, Dusseldorf, Germany) with a voltage of 5 kV. The images were digitally recorded using the Digital Image Processing System 2.6 (Point Electronic, Halle, Germany).

Statistical analysis

Results are presented as mean values and standard error of mean (SEM). Kruskal–Wallis test with Dunn's post hoc test for multiplicity was used for comparisons between the groups and for the analysis of changes during the follow-up period (day 2 vs day 6 and day 10). A p value < 0.05 indicates statistical significance.

Results

Seeding efficacy is independent from scaffold and cell type

Initial adherence for EPC on the three specimens, β-TCP, β-TCP-FN and composite scaffold was comparable. 88 ± 3%

adherence could be shown for EPC on β-TCP, whereas 91 ± 3% were adherent on β-TCP-FN, 89 ± 2% EPC were adherent to the composite scaffold. Similar results were observed for MSC (β-TCP: 81 ± 5%, β-TCP-FN 92 ± 3%, composite scaffold 89 ± 4%). No statistical differences were found between the groups.

SEM also proved the presence of EPC and MSC on the scaffolds on day 2 and 6 after seeding. EPC on day 2 showed a fusiform phenotype with few pseudopodia. The morphology of EPC was similar on β-TCP and β-TCP-FN, whereas also rounded and shrunken EPC were frequently seen on the composite scaffold. EPC's morphology changed significantly from day 2 to day 6 on β-TCP and β-TCP-FN scaffolds in comparable manner, the cells appeared more flattened and branched. EPC on the composite scaffolds appeared rather necrotic, demonstrating either a thin and elongated or a rounded and shriveled phenotype (Fig. 1a).

MSC appeared as large flattened cells on all scaffolds tested and no significant changes in phenotype were observed between day 2 and day 6 (Fig. 1b).

Cell adhesion enhanced on β-TCP

Relative numbers of stained EPC and MSC on the scaffolds were analyzed by mean of fluorescence microscopy. Additionally, a metabolic equivalent number of EPC and MSC was assessed by MTT assay.

The relative number of EPC was significantly higher on β-TCP and β-TCP-FN compared to the composite scaffold throughout the whole observation period ($p < 0.05$). Furthermore, the number of adherent EPC was significantly higher on β-TCP on day 10 after seeding compared to the number of cells found on β-TCP-FN ($p < 0.05$). The increase of the relative EPC number on β-TCP between day 2 and day 10 was not significant ($p = 0.1$, Fig. 2a).

The relative number of adhering MSC was significantly increased on uncoated β-TCP compared to β-TCP-FN on day 2 and day 6 ($p < 0.05$) and significantly higher compared to the composite scaffold during the whole observation period ($p < 0.05$, Fig. 2b).

Furthermore, we observed a shift of the MSC/EPC ratio in favor to MSC during the whole observation period on β-TCP-FN and the composite scaffold under co-culture conditions ($p < 0.05$, Fig. 2c).

The metabolic activity is closely related to the absolute cell number. Similar to the relative cell numbers assessed by fluorescence microscopy, we observed significantly increased metabolic activity of EPC seeded on β-TCP in comparison to EPC seeded on β-TCP-FN, respectively, on the composite scaffold on day 2 and day 6 after cell seeding. Metabolic activity of MSC was similar on all scaffolds, a not significant increase of activity was noted for all scaffolds on day 10 (Fig. 2d, e).

EPC's apoptosis is not correlated to scaffold

We investigated whether the decrease of the EPC number on the composite scaffold is due to increased apoptotic activity. Therefore, the activity of caspase-3 and -7 was analyzed on day 1 after seeding. The percentage of EPC demonstrating

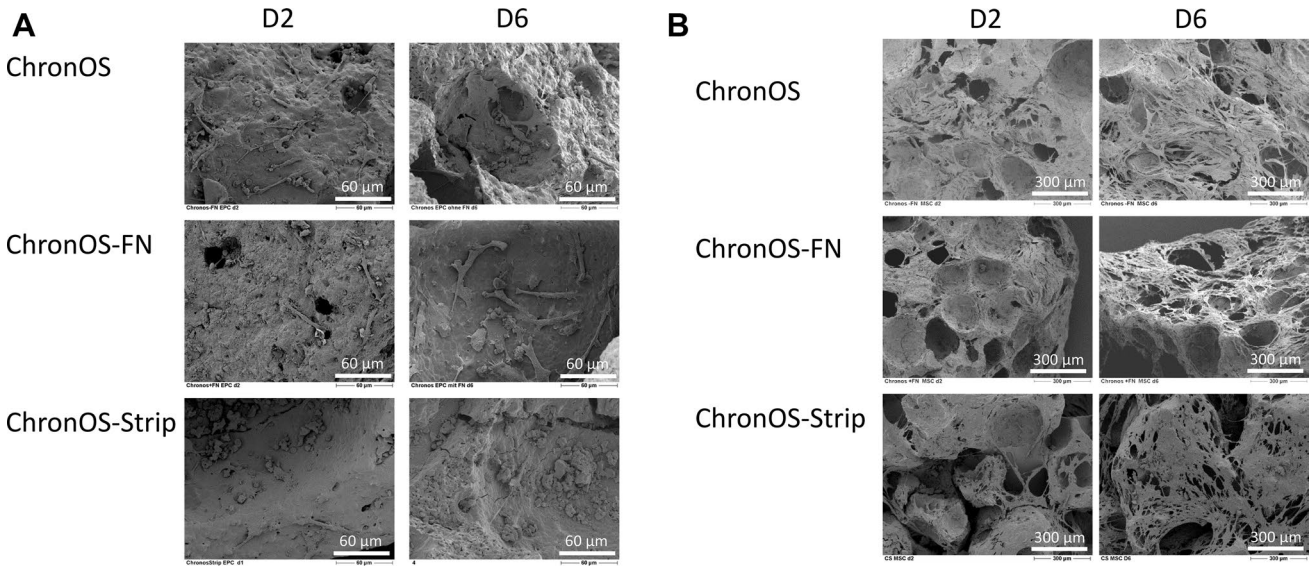

Fig. 1 **a** SEM images: EPC on β-TCP (top), β-TCP-FN (middle) and composite (bottom) on days 2 and 6 after sowing. EPC on ChronOS Strip show a strongly impaired phenotype. On day 10 after sowing, EPC which appeared vital were not detectable on any of the test materials. Scale bar represents 60 µm. **b** SEM images showing MSC on β-TCP (top), β-TCP-FN (middle) and composite (bottom) on days 2 and 6 after sowing

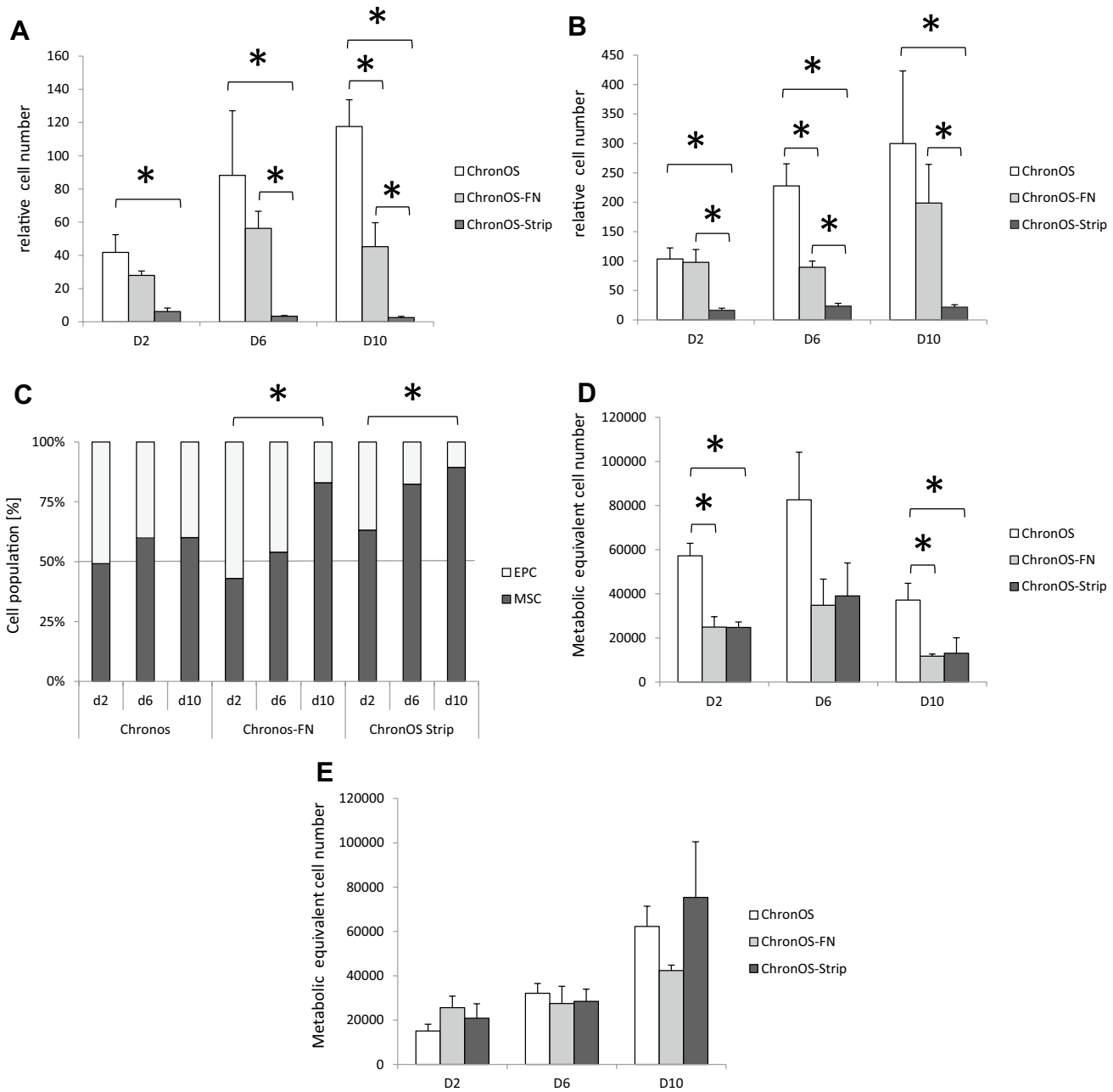

Fig. 2 **a** Relative number of adherent EPC on β-TCP (white), β-TCP-FN (gray) and composite (black) over time. Detection of the EPC was by means of DiL staining and fluorescence microscopy. For each EPC preparation, five randomized high power fields were counted at 100x magnification, $*p < 0.05$. **b** Relative number of adhering MSC to β-TCP, β-TCP-FN and composite over time. Detection of the MSC was achieved by staining the cell nuclei with DAPI and fluorescence microscopy, $*p < 0.05$. **c** Cells in co-culture. Showing the ratio of EPC/MSC on β-TCP (left), β-TCP-FN (middle) and composite (right), $*p < 0.05$. **d** Significantly increased metabolic activity of EPC on β-TCP (white), β-TCP-FN (gray) and composite (black) over the time. Values are given as equivalent cell number. The metabolic activity was determined by the MTT test, $*p < 0.05$. **e** Metabolic activity of MSC on β-TCP (white), β-TCP-FN (gray) and composite (black) over time. Values are given as "equivalent cell count MSC" In this experiment, only MSC were used. $N = 5$ MSC preparations, $*p < 0.05$

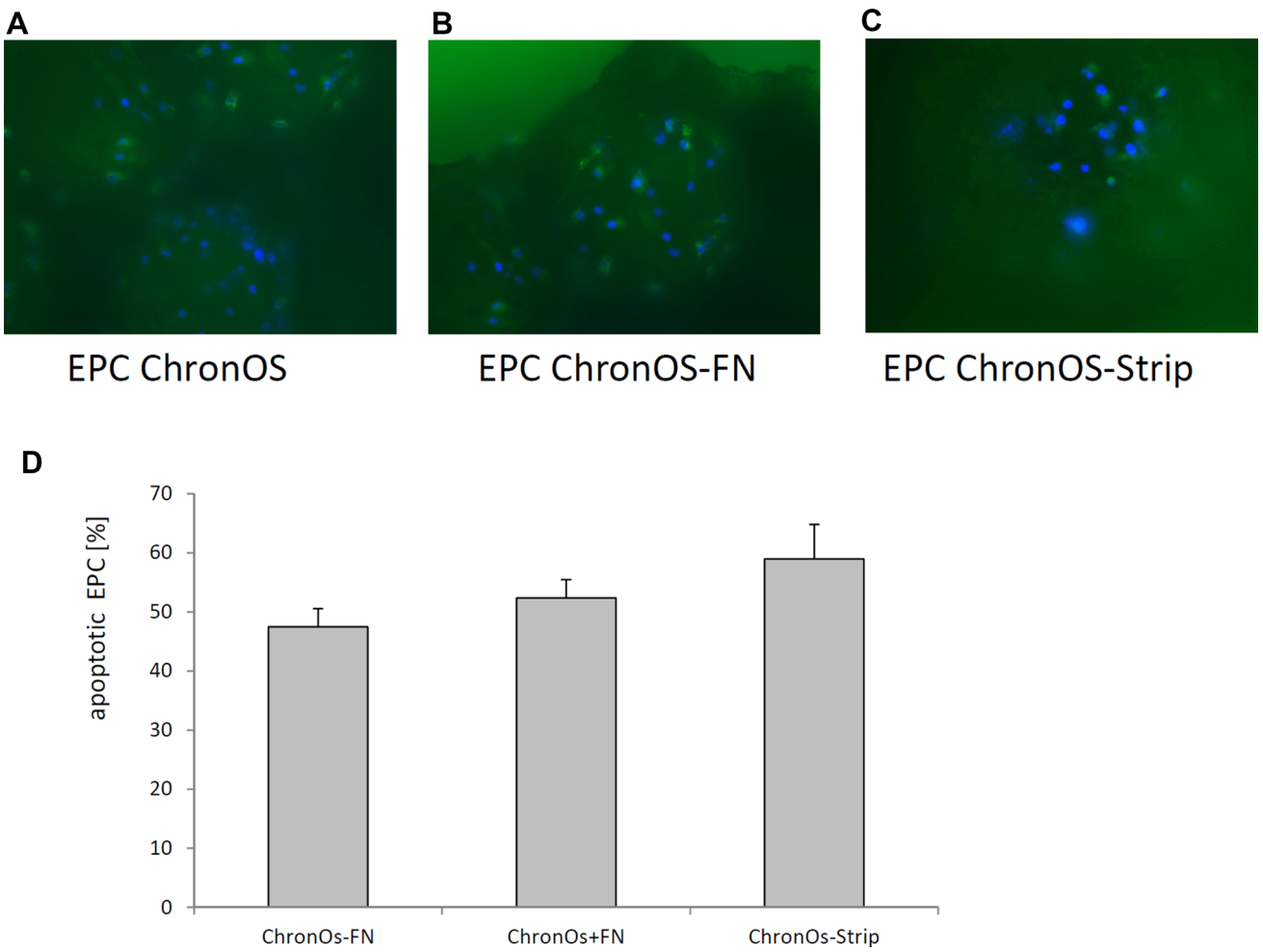

Fig. 3 Fraction of apoptotic EPC depending on the scaffold. Apoptosis was detected via FLICA staining. Active caspase-3/7 leads to intracellular accumulation of the fluorescent substrate. Cells in which apoptotic processes occur carry a green fluorescence. Nuclei were counterstained with DAPI. Scale bar represents 100 µm

apoptotic activity did not differ significantly between the scaffolds (Fig. 3a–d).

Cell differentiation is impaired on the composite scaffold

VEGF mainly origins from MSC

The VEGF concentrations were assessed in supernatants of EPC, MSC and co-culture of both cell types. EPC as well as MSC-released VEGF. We were able to demonstrate that MSC produce significantly more VEGF on all scaffolds. The amount of VEGF released by MSC was approximately tenfold higher compared to EPC. Accordingly, the amount of VEGF release in cocultures was approximately 50% reduced in comparison to MSC cultures.

The amount of VEGF released by EPC, respectively, MSC were independent of the scaffold used. Additionally, there was no significant change of VEGF release during the observation period (Fig. 4a–c).

Co-cultivation leads to increase of osteocalcin expression

Gene expression was measured on days 2, 6 and 10 after cell seeding. The gene expression of vWF was measured as prototypic endothelial marker in EPC, gene expression of collagen-1α, osteocalcin, RUNX2 and osteonectin was measured to determine osteogenic activity of MSC. VEGF, expressed in both cell types, was also assessed.

The gene expression of vWF was not significantly altered by the different scaffolds and remained approximately constant during the whole observation period in EPC single culture and under EPC/MSC co-culture conditions. The gene expression of collagen-1α remained constant in MSC over the whole observation period regardless of the scaffold used for cultivation. However, a significant

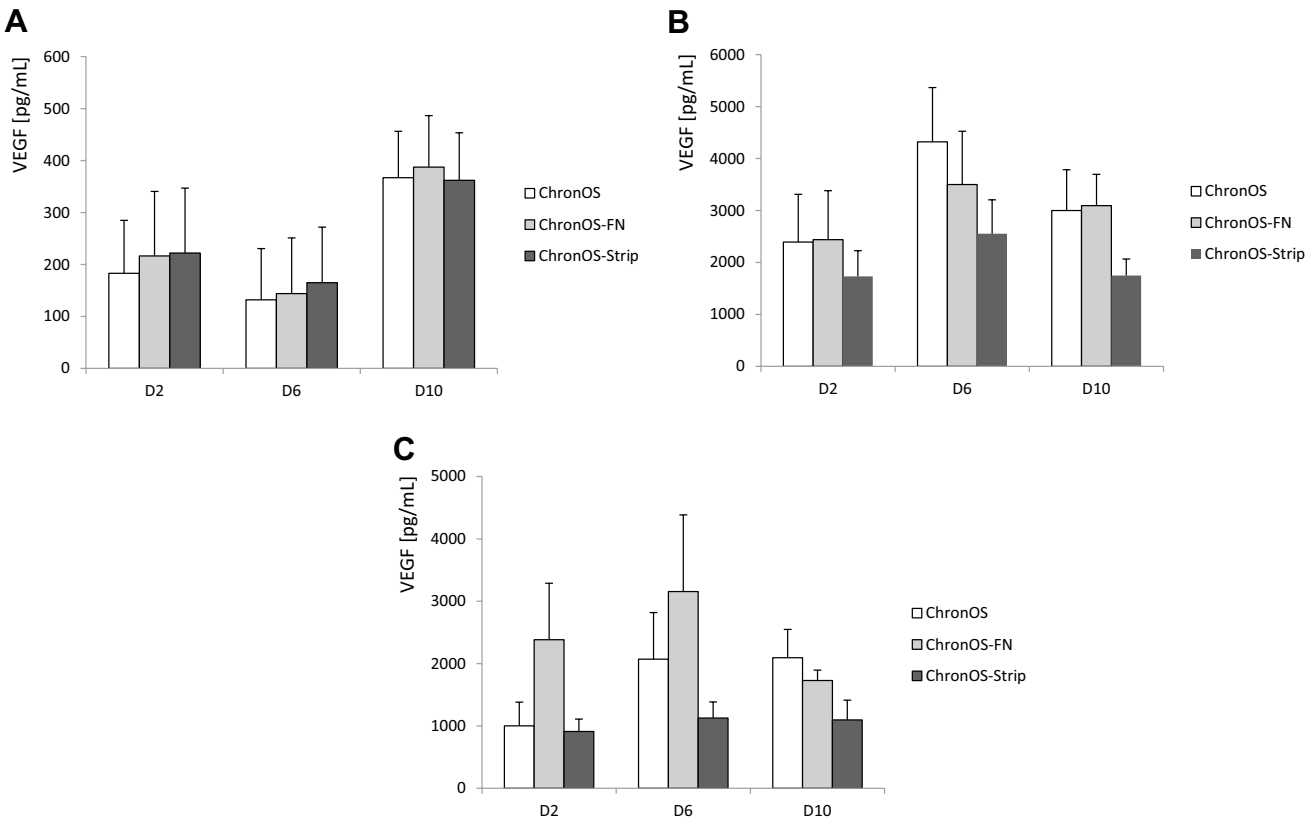

Fig. 4 **a** VEGF secretion of EPC to β-TCP (white), β-TCP-FN (gray) and composite (black) on the measurement dates. Values are given as pg / mL, $*p < 0.05$. **b** VEGF secretion of MSC sowed to the different scaffolds. Values are given as pg/ml, $*p < 0.05$. **c** Secretion of the MSC/EPC coculture on the different days, depending on the scaffold. Values are given as pg/ml, $*p < 0.05$

Table 1 Expression of collagen 1 α (COL1A) of MSC (a) and coculture (b) sowed on the three tested scaffolds

	COL1A D2	COL1A D6	COL1A D10
(a) MSC			
β-TCP	988 ± 265	636 ± 367	1341 ± 703
β-TCP-FN	742 ± 152	2798 ± 1588	620 ± 303
Composite	1056 ± 500	719 ± 427	240 ± 146
(b) MSC/EPC			
β-TCP	1198 ± 903	1110 ± 493	184 ± 72
β-TCP-FN	631 ± 317	985 ± 146	143 ± 99
Composite	142 ± 55	4168 ± 4071* vs D10	69 ± 16

Values are given as standard error of mean and are presented as a multiple of the GAPDH gene expression, $*p < 0.05$

Table 2 Expression of the housekeeping gene osteocalcin (BGLAP) of MSC (a) and the MSC/EPC coculture (b)

	BGLAP D2	BGLAP D6	BGLAP D10
(a) MSC			
β-TCP	0.44 ± 0.18	0.05 ± 0.02	0.7 ± 0.3
β-TCP-FN	1.1 ± 0.9	0.07 ± 0.02	0.3 ± 0.14
Composite	1.7 ± 1.2	0.08 ± 0.03	0.27 ± 0.14
(b) MSC/EPC			
ChronOS	2.20 ± 1.95	0.16 ± 0.10	4.45 ± 4.12* vs D6
ChronOS-FN	1.26 ± 0.99	0.23 ± 0.07	0.45 ± 0.39
ChronOS Strip	0.23 ± 0.14	0.43 ± 0.29* vs D2	1.04 ± 0.82

Values are given as standard error of mean for all three test days on the different scaffolds, $*p < 0.05$

decrease of collagen-1α gene expression was noted on the composite scaffold under co-culture conditions (Table 1). The gene expression of osteocalcin also did not differ between the scaffolds, though a significant increase was noted under co-culture conditions between day 6 and day 10 on β-TCP and a significant decline between day 2 and day 6 on the composite scaffold (Table 2). No significant differences could be shown regarding RUNX2 (Table 3) and osteonectin (Table 4) but expression was lowest on composite scaffold on day 6 and 10.

VEGF gene expression in EPC and MSC also did not differ in dependency of the scaffold used at any point in time. Moreover, VEGF gene expression in MSC was

Table 3 Expression of RUNX2 in MSC (a) and in the MSC/EPC coculture (b)

	RUNX2 D2	RUNX2 D6	RUNX2 D10
(a) MSC			
β-TCP	0.24 ± 0.09	0.55 ± 0.31	0.99 ± 0.62
β-TCP-FN	0.21 ± 0.49	1.02 ± 0.60	0.21 ± 0.06
Composite	0.59 ± 0.34	0.18 ± 0.11	0.18 ± 0.06
(b) MSC/EPC			
β-TCP	0.73 ± 0.40	0.35 ± 0.27	1.45 ± 1.24
β-TCP-FN	0.28 ± 0.16	0.68 ± 0.36	0.21 ± 0.07
Composite	0.08 ± 0.04	0.09 ± 0.03	0.31 ± 0.22

Values are given as mean values and standard error of mean for all three test days on the different scaffolds, $*p < 0.05$

Table 4 Expression of osteonectin in MSC (a) and in the MSC/EPC coculture (b)

	Osteonectin D2	Osteonectin D6	Osteonectin D10
(a) MSC			
β-TCP	156 ± 33	302 ± 179	182 ± 144
β-TCP-FN	142 ± 37	526 ± 268	108 ± 69
Composite	333 ± 258	151 ± 114	52 ± 28
(b) MSC/EPC			
β-TCP	318 ± 289	247 ± 115	38.7 ± 11.7
β-TCP-FN	175 ± 111	207 ± 41	22.9 ± 15.8
Composite	50.1 ± 19.6	42.8 ± 28.3	15.8 ± 6.6

Values are given as standard error of mean for all three test days on the different scaffolds, $*p < 0.05$

Table 5 Expression of von Willebrand factor (vWF) in EPC (a) and MSC/EPC cocultures (b)

	vWF D2	vWF D6	vWF D10
(a) EPC			
β-TCP	2.12 ± 1.27	1.76 ± 0.51	2.86 ± 1.42
β-TCP-FN	1.13 ± 0.31	4.01 ± 2.64	7.66 ± 4.79
Composite	1.28 ± 0.94	2.55 ± 0.67	7.36 ± 5.53
(b) MSC/EPC			
β-TCP	0.48 ± 0.44	0.31 ± 0.12	0.99 ± 0.58
β-TCP-FN	0.54 ± 0.29	0.66 ± 0.31	7.98 ± 5.42
Composite	0.35 ± 0.23	0.19 ± 0.11	1.15 ± 0.93

Cultured on β-TCP, β-TCP-FN and composite on days 2, 6, 10 after sowing. Values are gives as standard error of mean and are presented as a multiple of the GAPDH gene expression

mostly significantly higher compared to EPC. A significant decrease over the time (day 6 vs day 10) was seen under co-culture conditions on β-TCP-FN (Tables 5, 6).

Table 6 Expression of vasoendothelial growth factor (VEGF) in EPC (a) and MSC (b)

	VEGF D2	VEGF D6	VEGF D10
(a) EPC			
β-TCP	0.03 ± 0.11	0.003 ± 0.002	0.01 ± 0.006
β-TCP-FN	0.04 ± 0.01	0.11 ± 0.06	0.02 ± 0.008
Composite	0.02 ± 0.01	0.02 ± 0.01	n.a
(b) MSC			
β-TCP	0.24 ± 0.09	1.41 ± 1.04	0.23 ± 0.21
β-TCP-FN	0.63 ± 0.49	3.13 ± 1.91	0.16 ± 0.11
Composite	2.22 ± 1.89	0.85 ± 0.82	0.16 ± 0.12

Cultured on the three different scaffolds as given below. Values are given as standard error of mean and are presented as a multiple of the GAPDH gene expression

Discussion

The aim of the present study was to evaluate, if a fibronectin pre-coating of a granular β-TCP is needed to ensure adhesion, survival and function of EPC and MSC seeded to the scaffold. Furthermore, we evaluated the cytocompatibility of a composite scaffold based on the same β-TCP preparation to human EPC and MSC. We provided evidence that a fibronectin coating is not mandatory for the adhesion, survival and function of EPC and MSC seeded on the granular β-TCP scaffold. Moreover, we found evidence that the survival and the function of both cell types seeded to the composite scaffold were at least partly impaired in comparison to the function of cells seeded to granular β-TCP.

The application of biomaterials in bone healing should be safe and surgically as easy as possible. Numerous products are already in commercial use, however, with oftentimes limited clinical acceptance and results [19–21]. Therefore, despite its various limitations such as donor site morbidity and limited supply, autologous bone grafting remains the golden standard.

Thus, further research is necessary to improve the effectivity of biomaterials for bone healing purposes. Giannoudis et al. summarized the needs for bone healing supported by tissue engineering strategies in a diamond concept. The diamond concept combines biological aspects such as growth factors and reparative cells with chemical, respectively, mechanical needs to achieve proper bone healing [5].

The evaluation of beneficial characteristics to ensure a high cytocompatibility and function of reparative cells is therefore an emerging field of research. Our previous work demonstrated that the same granular β-TCP used in the present study holds an excellent cytocompatibility for various cell types in comparison to other synthetic and natural biomaterials including high initial adherence, increased rate of

cell survival and partly osteoinductive properties as measured by RT-PCR [9, 15, 16, 22]. The high cytocompatibility of that β-TCP scaffold is probably due to its surface characteristics which play a crucial role for cell attachment and activity [23, 24].

Role of fibronectin pre-coating

Granular β-TCP precoated with fibronectin was used as scaffold in our previous studies [9, 16, 17]. The use of a fibronectin precoating is based on the widely accepted assumption that this extracellular matrix protein improves adherence and differentiation of EPC [11, 13, 16]. In contrast, we were able to demonstrate that waiving of fibronectin pre-coating of the β-TCP scaffold did not affect the number of adherent EPC, their metabolic activity or expression of endothelial genes. Reasons for that surprising finding might be based on the protocol used for generation of EPC from buffy coat. EPC were pre-differentiated on fibronectin-coated plastic ware over 5 days before they were used for experiments. Thus, EPC used in the present work can be ascertained as early or myeloid EPC [13, 25]. It has been reported that myeloid EPC acquire additional adhesion receptors such as CD54 and CD106 during differentiation process [26]. Hence, one might argue that the receptor configuration of differentiated EPC is sufficient to ensure binding to an uncoated β-TCP scaffold. Moreover, the pre-differentiation and the cultivation in a medium comprising one-third (v/v) endothelial basal medium-2 (EBM-2) probably leads to a maintenance of the endothelial differentiation state as indicated by similar expression of endothelial marker genes as described in [9]. Thus, a further coating of the scaffold with fibronectin might not be necessary to maintain the endothelial differentiation of the EPC seeded to the scaffold.

Evidence is found that fibronectin promotes the osteogenic differentiation as demonstrated by several research groups. Fibronectin was 'the only substrate to promote calcium deposition in standard culture medium at day 21' as demonstrated by [27]. Furthermore, 'high ALP gene (ALPL) expression and associated enhancement of mineralization were observed on collagen type I, fibronectin- and vitronectin-treated plates' as reported by [28]. The differences to our findings might be due to the much longer cultivation period of 21 days compared to 10 days in the present study as well as unequal materials used for cultivation compared to β-TCP scaffold in this work.

A fibronectin pre-coating of scaffolds is a costly and time-consuming process. Since the waiver of a fibronectin pre-coating did not lead to an impairment of cell adherence, survival and differentiation, we propagate that a fibronectin pre-coating of a β-TCP scaffold can be omitted.

VEGF synthesis

Early vascularization is crucial in bone healing, whereas inadequate vessel formation can lead to delayed union [29]. Early EPC can induce new vessel formation in two different ways. Early EPC incorporate to a small percentage into newly formed blood vessels [17], but generally it is assumed that their effect on revascularization is predominantly based on the secretion of proangiogenic factors such as VEGF, and thus, attraction of further endothelial cells [30, 31]. Interestingly, we observed a much higher VEGF release by MSC in comparison to EPC in the present study. The production of VEGF is not limited to endothelial cells and it has been shown that many cell types were able to synthesize VEGF under hypoxic conditions [32, 33].

In terms of MSC, it has been reported that 'stress, in the form of hypoxia or TNF, activates MSC to release VEGF by STAT3 and p38 MAPK-dependent mechanisms'. Furthermore, the amount of VEGF produced after hypoxia was within the scale that we observed in our study [34]. It can be assumed that VEGF release is triggered by local hypoxia. Local hypoxia occurred presumably in areas of low perfusion due to dense granule packing and/or within pores. The VEGF synthesis mediated by MSC and EPC seeded on the β-TCP scaffold can also be observed *in vivo* as demonstrated in own previous work. VEGF was abundant in bone defects filled with β-TCP-FN seeded with EPC, MSC or a combination of both cell types in comparison to defects filled with β-TCP without cells or autologous bone graft. Furthermore, a correlation between the released VEGF and the blood vessel density within the defect was seen [17].

Cellular activity in single and co-culture conditions and on the composite scaffold

Previously, we observed a synergistic effect of co-transplanted EPC and MSC into a critical size defect of athymic rats with regard to the bone healing response. The bone healing was much improved in comparison to animals that received either EPC or MSC [16].

This effect was also described by [35] using a mice model. Hence, the function of co-cultivated EPC and MSC on the different scaffolds was also analyzed. A marked shift of the ratio MSC to EPC towards MSC was noted throughout the observation period, if both cell types were co-cultured on the biomaterials. This change is probably due to the divergent proliferation characteristics of EPC and MSC. Evidence is reported that early EPC demonstrate a rather weak proliferative activity [36], whereas MSC were characterized by a high proliferation rate.

The co-cultivation of EPC and MSC on the scaffolds in the present study leads neither to significantly increased

mRNA synthesis nor to significantly increased VEGF release, hence, costimulatory effects were not seen during the observation period. The significantly reduced VEGF release under co-culture conditions compared to MSC cultured alone might be due to the high discrepancy in VEGF synthesis of EPC and MSC seen and the reduced number of MSC in co-culture experiments.

Hence, the results presented here do not provide an explanation for the significantly increased bone healing response induced by concomitantly transplanted EPC and MSC.

Using the more solid composite scaffold, we observed reduced cell adherence as well as metabolic activity of MSC and EPC compared to uncoated, respectively, fibronectin pre-coated granular β-TCP. Actually, the granular β-TCP as well as the composite scaffold provides pore sizes that were suitable for osteoconduction and the ingrowth of newly formed blood vessels [16, 37]. But in contrast to granular β-TCP, the composite scaffold in its commercial form (strip, 1 cm wide) is characterized by increased diffusion distances leading to a lack of nutrition and oxygen which both result in a reduction of cellular activity and cell number.

This study has some limitations: First of all, this is an in vitro study and the results of these experiments have to be confirmed in vivo. Observation period was limited to ten days. In this period, culture medium was changed three times. Maybe different results could be shown if a continuous flow chamber is used which lead to a better nutritional supply.

Conclusion

We were able to demonstrate that a preconditioning of a β-TCP scaffold with fibronectin is not necessary for successful cultivation of EPC. Furthermore, the number and activity of MSC seeded on β-TCP is not influenced by a fibronectin pre-coating. Moreover, we found evidence that the size of the scaffold is a critical factor for the survival and activity of adherent cells. Cultivation of EPC, as well as MSC seems to be more effective on granules than on a more solid composite scaffold based on β-TCP layered onto a poly(lactide-co-ε-caprolactone) carrier. However, it must be clearly stated that the presented results are limited to in vitro conditions, and thus further experiments were obligatory to confirm our results in vivo.

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Compliance with ethical standards

Conflict of interest Philipp Störmann, Juliane Kupsch, Kerstin Kontradowitz, Maximilian Leiblein, René Verboket, Caroline Seebach, Ingo Marzi, Dirk Henrich and Christoph Nau hereby declare that they have no conflicts of interest to disclose.

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