

PLATELET-RICH FIBRIN SECRETOME INDUCES THREE DIMENSIONAL ANGIOGENIC ACTIVATION *IN VITRO*

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Abstract

Different tissue engineering techniques are used to support rapid vascularisation. A novel technique is the use of platelet-rich fibrin (PRF), an autologous source of growth factors. This study was the first to investigate the influence of PRF matrices, isolated following different centrifugation protocols, on human dermal vascular endothelial cells (ECs) in mono-culture and co-culture with human primary fibroblasts (HFs) as an *in vitro* model for tissue regeneration. Focus was placed on vascular structure formation and growth factor release. HFs and ECs were cultivated with PRF prepared using a high (710 ×g) or low (44 ×g) relative centrifugation force (RCF) over 14 d. Immunofluorescence staining and immunohistochemistry were used to evaluate the microvascular formation. Cell culture supernatants were collected for evaluation of growth factor release. The results showed a PRF-mediated effect on the induction of angiogenesis in ECs. Microvessel-like structure formation was promoted when ECs were combined with low-RCF PRF as compared to high-RCF PRF or control group. The percentage of vascular lumen area was significantly higher in low-RCF PRF, especially at day 7, which coincided with statistically significantly higher growth factor [vascular endothelial factor (VEGF), transforming growth factor β 1 (TGF- β 1) and platelet derived growth factor (PDGF)] concentration measured in low-RCF PRF as compared to high-RCF PRF or control group. In conclusion, reducing the RCF according to the low-speed centrifugation concept (LSCC) resulted in increased growth factor release and angiogenic structure formation with EC mono-culture, suggesting that PRF may be a highly beneficial therapeutic tool for tissue engineering applications.

Keywords: Platelet-rich fibrin, low speed centrifugation concept, angiogenesis, secretome.

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Introduction

Rapid vascularisation of engineered tissues is necessary for regeneration of tissue defects, caused by trauma or tumour resection, since the vasculature supplies cells with nutrients and oxygen (Auger *et al.*, 2013; Liu *et al.*, 2017). For this reason, one of the main goals in the fields of tissue engineering and regenerative medicine is the generation of tissues that can be used in place of autologous tissues. For optimal integration, engineered tissues need to possess several features; the inability to achieve sufficient vascularisation is one of the key limitations hindering the translation of regenerative

technologies from bench to bedside. Different tissue engineering approaches, such as the use of skin and bone substitute materials, have been developed to support bone and soft tissue regeneration. Therefore, various concepts have been implemented to obtain tissue-like constructs, *i.e.* pre-cultured biomaterials containing living cells to facilitate tissue vascularisation. In previous studies, “biologisation” of acellular biomaterials prior to their implantation *in vivo* has improved their integration in the application region (Al-Maawi *et al.*, 2017; Barbeck *et al.*, 2015). In this context, biomaterials that are pre-cultured using human osteoblasts and endothelial cells undergo significantly higher vascularisation

in vivo as compared to pure biomaterials (Fuchs *et al.*, 2009; Ghanaati *et al.*, 2011; Unger *et al.*, 2010). Barbeck *et al.* (2016) demonstrated the positive effect of monocytes isolated from the peripheral blood on vascularisation. The isolated monocytes were cultured on a bone substitute material prior to its subcutaneous implantation *in vivo*. The addition of peripheral blood monocytes to the biomaterial results in a higher rate of vascularisation when compared to the untreated biomaterial. In addition, peripheral blood neutrophils have a significantly positive effect on angiogenesis and osteogenesis *in vitro* (Herath *et al.*, 2018). Nevertheless, these strategies require cell isolation and, in some cases, cell growth and expansion for 3-4 weeks prior to biomaterial implantation, a protocol that currently hinders translation to the clinics.

In the context of a clinically applicable method, platelet-rich fibrin (PRF) has been introduced as a beneficial tool to enhance tissue regeneration (Choukroun *et al.*, 2006a; Choukroun *et al.*, 2006b; Choukroun, 2001; Dohan *et al.*, 2006a; Dohan *et al.*, 2006b; Dohan *et al.*, 2006c; Ghanaati *et al.*, 2014). This blood concentration-system involves centrifugation of the patient's own peripheral blood in a single step. According to the protocol implemented, it is possible to obtain solid as well as injectable liquid PRF matrices without the requirement of additives such as anticoagulants (Choukroun and Ghanaati, 2018). The resulting PRF contains fibrin, platelets, leukocytes and plasma proteins. Furthermore, Ghanaati *et al.* (2014) showed that it could function as a tailored drug delivery system. Based on the low-speed centrifugation concept (LSCC), Choukroun and Ghanaati (2018) reported the influence of systematically altering the applied relative centrifugal force (RCF) on cell number and growth factor release within the injectable PRF. The number of leukocytes, neutrophils, monocytes, lymphocytes and platelets is significantly higher in PRF matrices prepared using low RCF as compared to those prepared using high RCF. Additionally, the total growth factor release of platelet-derived growth factor (PDGF)-BB, transforming growth factor β 1 (TGF- β 1), endothelial growth factor (EGF), vascular endothelial factor (VEGF) and matrix metalloproteinase 9 (MMP-9) is significantly higher in the low RCF group than that in the high RCF group (Wend *et al.*, 2017).

Relevant *in vitro* models include the use of human primary fibroblasts (HFs) and endothelial cells (ECs) embedded in three-dimensional (3D) substrates (DeCicco-Skinner *et al.*, 2014; Liu *et al.*, 2017). A variety of endothelial culture media are reported (Price, 2017) to be enriched with varying concentrations of serum and synthetic growth factors, such as VEGF, fibroblast growth factor (FGF), insulin growth factor (IGF) and EGF. Although the methods mentioned previously provide conditions for the formation of vascular structures, the synthetic combination of growth factors (GFs) in a culture medium lacks the complexity and repertoire of proteins secreted

naturally (secretome) into the extracellular space (Veronesi *et al.*, 2017). The role of the secretome, *i.e.* all the factors released by the cells within the physiological environment, in tissue regeneration is gaining interest as the best method to recapitulate the broad beneficial effects of paracrine mechanisms. The secretome includes trophic factors (chemokines, cytokines, GFs, hormones and lipid mediators) with the capacity to affect receptor-mediated survival pathways, differentiation, pro- and anti-inflammatory cell behaviour and wound healing (Burdon *et al.*, 2011; Mukherjee and Mani, 2013). Considering PRF as a natural source of growth factors, Dohle *et al.* (2017) demonstrated the effectiveness of injectable PRF for inducing vascular formation and osteogenic differentiation *in vitro*. In this context, co-cultures of outgrowth endothelial cells (OECs) and primary osteoblasts incubated in injectable PRF clots produce significantly more microvessel-like structures as compared with co-cultures on cell culture plastic. These results suggest a pro-angiogenic property of injectable PRF in the *in vitro* co-culture model.

The purpose of the present study was to investigate the influence of PRF-matrices prepared using high (710 $\times g$) or low (44 $\times g$) RCF on human dermal vascular endothelial cells (HDMECs) in monoculture and in co-culture with HFs. The final aim was to implement an *in vitro* model system for soft tissue regeneration in which vascular structure formation and growth factor secretion could be studied. Reducing the time of cell culturing to that required to induce the process of angiogenesis would facilitate the translation to the clinical environment of numerous cell-based tissue engineering techniques. Furthermore, this study may provide basic evidence that PRF could serve as "biologisation" tool of acellular biomaterials as a clinically applicable concept.

Materials and Methods

Isolation and cultivation of primary cells

HFs and HDMECs were isolated from excess-tissue samples of patients undergoing cleft lip reconstruction, according to a standard protocol (Unger *et al.*, 2002). The surgeries occurred in the plastic and maxillofacial surgery service of the University Hospital of Frankfurt, Germany. The acquisition and application of all cells that were used in the study were in accordance with the principle of informed consent and approved by the responsible Ethics Commission of the state of Hessen, Germany. ECs were cultivated at 37 °C and 5 % CO₂ in 75 cm² flasks with Endothelial Cell Growth Basal Medium-2 (EBMTM-2; Lonza), supplemented with EGMTM-2 Endothelial Cell Growth Medium-2 BulletKitTM (Lonza), 1 % penicillin/streptomycin (Sigma-Aldrich) and 4 % foetal bovine serum (Biocrom). HFs were grown and cultivated in 75 cm² flasks using Dulbecco's modified minimum essential medium (DMEM) medium (Biocrom), supplemented with

Table 1. Different PRF protocols generated by different RCFs. Based on the LSCC, high (710 ×g) and low-RCF (44 ×g) centrifugation protocols were selected for comparison in the study.

PRF protocol	Rotation (rpm)	Time (min)	g-force (RCF)	Tube material	Radius (mm)	Centrifuge
PRF low	600	8	44	plastic	110	Duo centrifuge
PRF high	2,400	8	710	plastic	110	Duo centrifuge

1 % penicillin/streptomycin (Sigma-Aldrich) and 4 % foetal bovine serum (Biochrom). For the study, ECs were trypsinised and expanded until passage 4 and HFs until passage 7.

PRF-LSCC

PRF was obtained from 3 healthy donors and each donor was used independently. Blood was collected from the antecubital vein using a vacuum blood-collection butterfly. The blood was immediately transferred into 10 mL i-PRF[®] tubes (Process for PRF, Nice, France) and centrifuged in a Duo[®] centrifuge (Process for PRF). Based on the LSCC, a high (710 ×g) and a low-RCF (44 ×g) centrifugation protocol were selected for comparison (Table 1). After centrifugation, 2 layers could be observed: the red blood cells at the bottom and the injectable PRF at the top, containing fibrin, platelets, leukocytes and plasma proteins. Although the i-PRF[®] tubes do not contain anti-coagulants, PRF did not clot immediately, but after 10 to 15 min following centrifugation and depending on the donor. The layers of the generated injectable PRF which were still liquid after centrifugation were immediately aspirated from all tubes using a syringe. Low- and high-RCF PRF were homogenised separately for each donor in 15 mL tubes. The resulting homogenised PRF was used for cell culture experiments as described in the following section. As control group, 1 mL of high- and low-RCF PRF, respectively, was cultivated alone with 1 mL of EBMTM-2.

Cell culture experiments

Primary cells were isolated from excess tissue of infant patients undergoing cleft lip reconstruction and PRF was generated from 3 additional human donors. The cell culture experiments were conducted in triplicate in 24-well plates. In the experimental groups, a total of 1 mL of high-RCF or low-RCF PRF was deposited in each well for 20 min until clotted; in the control group, 1 mL of collagen type I from rat tail (Sigma-Aldrich) was deposited in the wells. On top of the collagen and the clotted PRF, 20,000 cells per well (HFs or ECs) were seeded for mono-culture; for co-culture, 10,000 HFs and ECs, respectively, were seeded per well. Cells were resuspended in 1 mL of EBMTM-2 enriched with EGMTM-2 kit supplements. The individual experimental groups are indicated in Table 2. After 7 or 14 d of cultivation, cells were fixed in formaldehyde for immunofluorescence staining and

immunohistochemistry. Cell culture supernatants were collected after 4, 7 and 14 d and replaced with fresh medium. The collected supernatants were assayed for growth factor concentration by enzyme-linked immunosorbent assay (ELISA).

Protein quantification by ELISA

Quantification of growth factors within the cell culture supernatants and from PRF cultivated alone was performed using quantitative sandwich ELISA. Briefly, the release of TGF-β1, VEGF and PDGF-BB was quantified using the DuoSet ELISA kit (R&D Systems) following the manufacturer's instructions. The absorbance assay was conducted using a microplate reader (Infinite M200, Tecan, Grödig, Austria) set to a wavelength of 450 nm with a reference reading at 570 nm. The output data were analysed using a 4-parameter logistic curve fit and presented graphically using Graph-Pad Prism version 6.0 (GraphPad Software).

Immunofluorescence staining and immunohistochemistry

For immunofluorescence staining, cell culture wells were fixed in formaldehyde 4 % for 10 min and cells were washed three times with phosphate-buffered saline (PBS). Cells were permeabilised for 5 min using 0.1 % Triton-X in PBS, followed by incubation with mouse anti-human CD-31 antibody (1 : 40, Dako, M0823) diluted in 1 % bovine serum albumin (BSA) in PBS for 45 min at room temperature. After washing with PBS, cells were incubated with anti-mouse antibody Alexa Fluor[®] 488 secondary antibody (Life Technologies) diluted 1 : 1000 in 1 % BSA/PBS for 45 min at room temperature while avoiding light exposure. Additionally, counterstaining of cell nuclei was performed with 4',6-diamidino-2-phenylindole (DAPI) (1 : 125) and cells were mounted with Fluoroshield (ImmunoBioScience Corp., Mukilteo, WA, USA). Visualisation was carried out using a confocal laser scanning microscope (Leica TCS-NT) and the z-stacks obtained were used for 3D visualisation using the open source software Image J (Java 1.8.0_112) (Rueden *et al.*, 2017). For immunohistochemistry, samples were fixed in 4 % buffered formalin (Roti-Histofix 4 % acid-free pH 7, Carl-Roth), dehydrated using a tissue processor (Leica TP1020), embedded in paraffin wax, sectioned using a microtome (Leica RM2255) and stained for CD31. Tissue sections were rehydrated in a

Table 2. Experimental groups. High-RCF or low-RCF PRF were combined with 20,000 cells per well (HFes or ECs) for mono-culture experiments and 10,000 HFes and ECs, respectively, for co-culture experiments. Cells were resuspended in 1 mL of EBM™-2 enriched with EGM™-2 kit supplements.

		Cell culture medium	Collagen	PRF high	PRF low	ECs	HFes
Control	PRF high RCF	1 mL	-	1 mL	1 mL	-	-
	PRF low RCF	1 mL	-	1 mL	1 mL	-	-
	ECs	1 mL	1 mL	-	-	-	20,000
	HFes	1 mL	1 mL	-	-	20,000	-
	Co-culture	1 mL	1 mL	-	-	10,000	10,000
PRF high	ECs + PRF high	1 mL	-	1 mL	-	-	20,000
	HFes + PRF high	1 mL	-	1 mL	-	20,000	-
	HFes + ECs + PRF high	1 mL	-	1 mL	-	10,000	10,000
PRF low	ECs + PRF low	1 mL	-	-	1 mL	-	20,000
	HFes + PRF low	1 mL	-	-	1 mL	20,000	-
	HFes+ ECs + PRF low	1 mL	-	-	1 mL	10,000	10,000

descending ethanol series before commencing the immunostaining protocol. The antigen was retrieved using the heat-induced epitope retrieval (HIER) method (Udeabor *et al.*, 2017) with citrate pH 6 buffer at 95 °C for 20 min. Samples were rinsed with buffer solution [tris-buffered saline (TBS) with Tween 20 in a ratio of 20 : 1] before staining with monoclonal mouse anti-human CD31 antibody (1 : 120; Dako, M0823) by Autostainer (Lab Vision Autostainer 360, ThermoFisher Scientific). An anti-mouse secondary antibody was used (HRP Ultravision Quanto Detection System, ThermoFisher Scientific) and chromogenic visualisation was achieved using AEC peroxidase (Dako). Histological examination was performed using a light microscope (Nikon Eclipse 80i) and images were taken with a connected Nikon DS-Fi1/digital camera and a Nikon digital sight unit DS-L2.

Measurements of vascular structures

Immunohistochemical staining analyses were conducted using an established protocol (Dohle *et al.*, 2017). A 20× magnification scan of each sample was obtained using a light microscope (Nikon Eclipse 80i®) equipped with an automatic scanning table (ProScan III, Prior, Rockland, MA, USA), a Nikon DS-Fi1 digital camera and a Nikon Digital sight DS-L2 unit. The histochemically stained structures (CD31) were manually selected using the area tool of the “annotations and measurements” function

of the NIS Elements software (Nikon). The percent vascularisation was calculated as the total area of the vessel/lumen structures divided by the total area of the image. For image quantification, three images for each experimental group were analysed.

Statistical analyses

Results were calculated as mean ± standard deviation (SD) and evaluated for significant differences at each time point with one-way analysis of variance (ANOVA) using GraphPad Prism6.0 software (GraphPad Software Inc.). Differences were considered statistically significant for * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. The compiled data were plotted with GraphPad Prism 6.0 software.

Results

Effects of low- and high-RCF PRF on angiogenic structure formation

Cell/PRF-complexes (ECs + PRF and ECs + HFes + PRF using low-RCF and high-RCF PRF), as well as the control groups without addition of PRF, were stained for immunofluorescence detection of the EC-specific marker CD31 to identify ECs (Fig. 1,2). Additionally, cell/PRF-complexes were stained for immunohistochemical detection of CD31 to visualise vessel-like luminal structures (Fig. 3). Qualitative

evaluation of CD31 immunofluorescence in the control group without PRF (ECs) revealed a typical cobblestone-like morphology of ECs in mono-culture, which were organised into a monolayer after 7 and 14 d of cultivation (Fig. 1a,g). ECs in control co-cultures consisting of ECs and HFs without addition of PRF were organised into EC aggregates after 7 d of cultivation (Fig. 1d) and started to form few angiogenic structures after 14 d of co-cultivation (Fig. 1j). In contrast, combining ECs in mono-culture with PRF generally resulted in a distinct angiogenic activation of ECs, suggested by the formation of many microvessel-like structures (Fig. 1b,c,h,i) after 7 and 14 d, compared to the control groups without the addition of PRF. This PRF-mediated effect on the

induction of angiogenesis was even more clear when low-RCF PRF was applied (Fig. 1c,i). This difference in the angiogenic activation between the two PRF preparations was distinct after both cultivation time points. When ECs were combined with low-RCF PRF, ECs formed a very complex network of microvessel-like structures with larger lumena invading the PRF matrix (Fig. 2). Although the formation of single microvessel-like structures was detected when EC/HF co-cultures were combined with PRF, the significant pro-angiogenic effect of PRF on ECs in mono-culture was not observed for the co-cultures (Fig. 1e,f,k,l). The dependence of angiogenic structure formation in ECs on the RCF was also confirmed by immunohistochemistry (Fig.

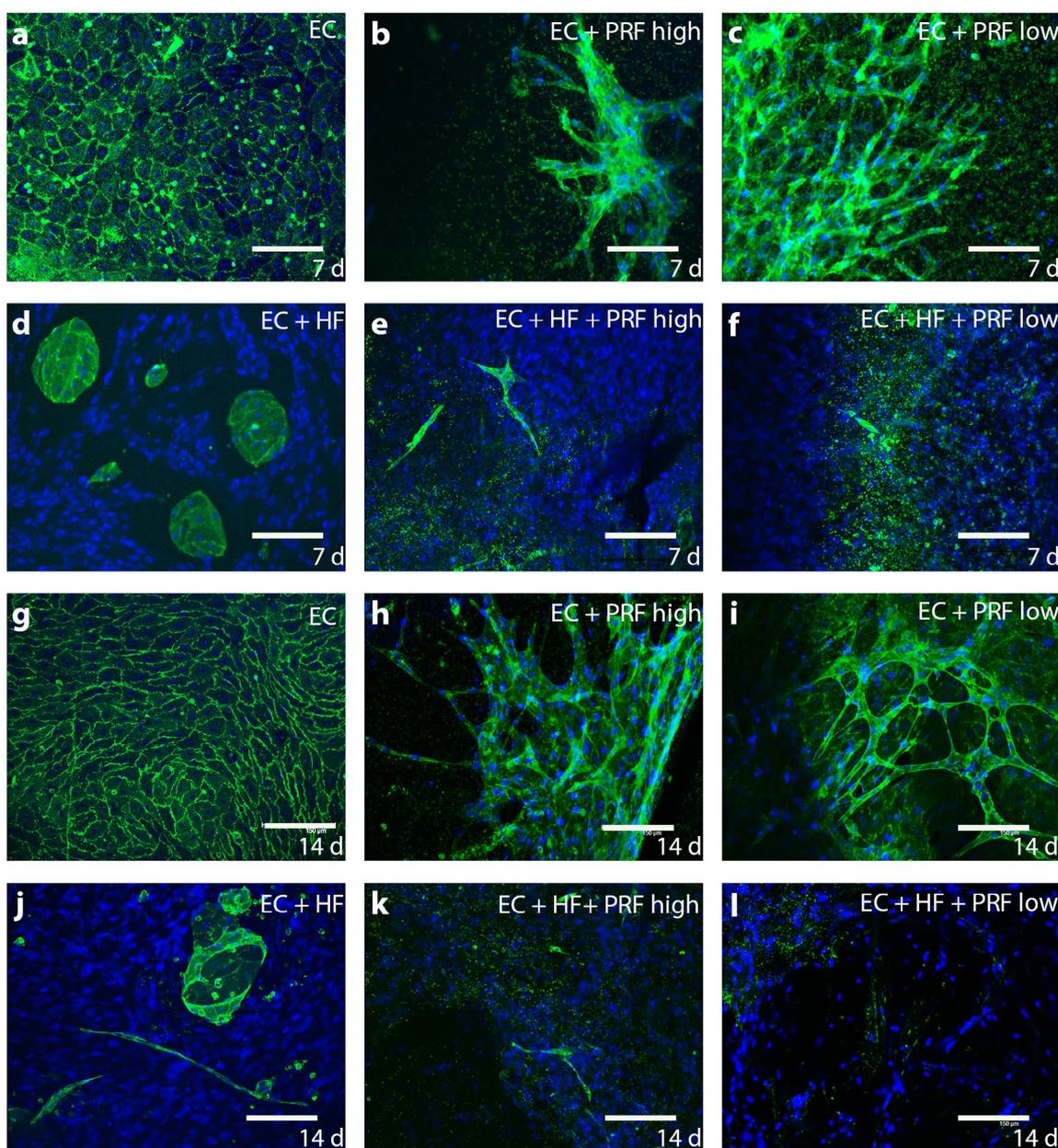


Fig. 1. Lumen and microvessel-like structure formation of ECs in mono-culture and ECs and HFs in co-culture mixed with PRF prepared using the LSCC. Cells were cultivated with a (b,e,h,k) high or (c/f/i/l) low RCF PRF as experimental groups and (a/d/g/j) without PRF as control. Whole cell complexes were stained by immunofluorescence for CD31 (green) and DAPI (blue) after (a-f) 7 and (g-l) 14 d of cultivation. Scale bars: 150 μ m.

3). CD31 immunostaining of low- and high-RCF PRF complexes with EC and EC/HF co-culture showed a significantly larger amount of CD31-positive luminal structure formation in EC monoculture in which low-RCF PRF had been applied as compared to high-RCF PRF after 7 or 14 d (Fig. 3). Quantitative evaluation of the total amount of vessel lumina (% of total area) indicated a statistically larger number of vascular lumina in EC + PRF low-RCF complexes on day 7 and 14 as compared to EC + PRF high-RCF complexes (Fig. 3i). When compared with the immunofluorescence staining of cell/PRF complexes depicted in Fig. 1, co-culture/PRF complexes generally formed vascular luminal structures to a lesser extent on day 7 and 14, although a pro-angiogenic effect of low-RCF PRF was observed for co-culture experiments after 14 d of cultivation (Fig. 3j).

PRF-mediated effect on protein release in monocultures and co-cultures

To gain insight into the molecular basis of the pro-angiogenic effect of PRF generated by LSCC, the release of VEGF, PDGF-BB and TGF- β 1 was measured in all experimental groups after 4, 7 and 14 d of cultivation (Fig. 4-6). Data compared the effect of PRF on the individual cell types by time points and, more specifically, on ECs and HFs in mono-culture and co-culture relatively to the control (low-RCF PRF without cells) (Fig. 4-6a,b,c). Absolute growth factor concentrations at all time points for the different experimental groups are shown to evaluate growth factor secretion from 4 to 14 d (Fig. 6d,e,f).

VEGF

On day 4, the highest VEGF concentration was observed in supernatants of HF/low-RCF PRF clots, but this was not statistically significant when

compared to the HF + PRF high-RCF and to the HF control group without PRF (Fig. 4a). Although there seemed to be more VEGF in EC supernatants as well as in co-culture supernatants when cells were embedded in low-RCF PRF, compared to the VEGF concentration in supernatants of cells embedded in high-RCF PRF, this result was not statistically significant (Fig. 4a). On day 7, there was an apparent, albeit not statistically significant, increase in VEGF concentration of HFs cultivated with low- or high-RCF PRF as compared to control HFs without PRF (Fig. 4b). In general, there were no distinct differences in VEGF concentration between the supernatants of all other experimental groups after 7 d of cultivation (Fig. 4b). After 14 d of cultivation, VEGF content was generally low in supernatants of all tested experimental groups as compared to control HF mono-culture and control low-RCF PRF without additional cells (Fig. 4c). On day 14, VEGF concentration was significantly higher in supernatants of low-RCF PRF as compared to high-RCF PRF. The effect of PRF on HFs was clear when the absolute VEGF concentration was evaluated by time point and experimental group (Fig. 4d). VEGF concentration increased in cell culture supernatants when HFs were embedded in PRF with low-RCF and high-RCF PRF from 4 up to 7 d as compared to control HFs without PRF cultivation. No increase in VEGF protein secretion was measured for ECs in mono-culture or co-culture (Fig. 4e,f).

PDGF-BB

After the first time point (4 d), the concentration of PDGF-BB was significantly higher in the supernatants of HF mono-culture, EC mono-culture and co-cultures of ECs and HFs when the cells were combined with low-RCF PRF as compared to the mono-cultures

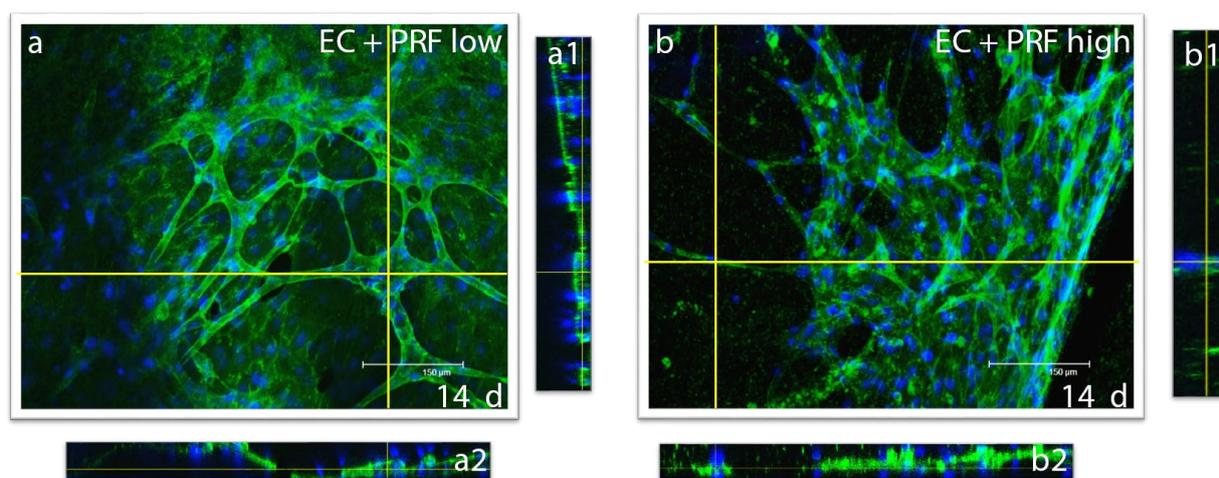


Fig. 2. Immunofluorescence staining of PRF/cell complex after 14 d of cultivation. Representative images of 3D projections using z-stacks obtained from the confocal microscopy with orthogonal views using the open source software Image J. Qualitative evaluation of lumen size formation by ECs cultivated with (a) PRF low-RCF as compared to (b) PRF high-RCF. The intersection of the yellow lines indicates the centre of the lumen displayed in the YZ (Fig. a1-b1) and XZ (Fig. a2-b2) planes. A more complex network of vessel-like structures with bigger lumen size can be observed in the EC + PRF-low as compared to the EC + PRF-high RCF. Scale bars: 150 μ m. **a** and **b** correspond to Fig. 1i and 1h, respectively.

combined with high-RCF PRF or without PRF (Fig. 5a). Similar results were also observed after 7 d (Fig. 5b). PDGF concentration was increased in HF/low-RCF PRF complexes and in EC/low-RCF PRF complexes after 7 d as compared to the other experimental groups, although statistical significance was found only for low-RCF PRF on EC mono-culture (Fig. 5b). The effect of low-RCF PRF became even clearer at the third time point (Fig. 5c). After 14 d of cultivation, the concentration of PDGF in supernatants of EC mono-culture increased tremendously in a PRF-dependent manner as compared to control mono-culture and to all other experimental groups. Supernatants of EC/low-RCF PRF complexes contained ~13 times more PDGF than the corresponding control group without PRF, a result that was statistically significant (Fig. 5c). Additionally, PDGF content in supernatants of ECs combined with high-RCF PRF also increased significantly when compared to control EC mono-cultures. The difference in absolute PDGF concentration between EC supernatants with or without PRF also supported these data (Fig. 5e). The highest PDGF concentration was observed with ECs combined with low-RCF PRF

for 14 d, whereas this effect was not observed when HF mono-culture or in co-culture with ECs were combined with PRF (Fig. 5d,f).

TGF- β 1

Low-RCF PRF clots without additional external cells released more TGF- β 1 into the surrounding medium than high-RCF PRF clots at all three time points, with statistical significance after 4 and 7 d of cultivation (Fig. 6a,b,c). After 4 and 7 d, TGF- β 1 concentration in cell culture supernatants was significantly higher than in supernatants of HFs and ECs in mono-culture and co-culture when the cells were mixed with low-RCF PRF as compared to control cells without PRF and cells embedded in high-RCF PRF (Fig. 6a,b). After 14 d, there were no distinct differences between the different experimental groups in terms of detectable TGF- β 1 (Fig. 6c). Although TGF- β 1 concentration in the cell culture supernatants of ECs, HFs and co-cultures was very high with low-RCF PRF at 4 d, it gradually decreased to a level comparable to that in supernatants of high RCF PRF clots with mono-cultures and supernatants of cells without PRF after 14 d of cultivation (Fig. 6d,e,f).

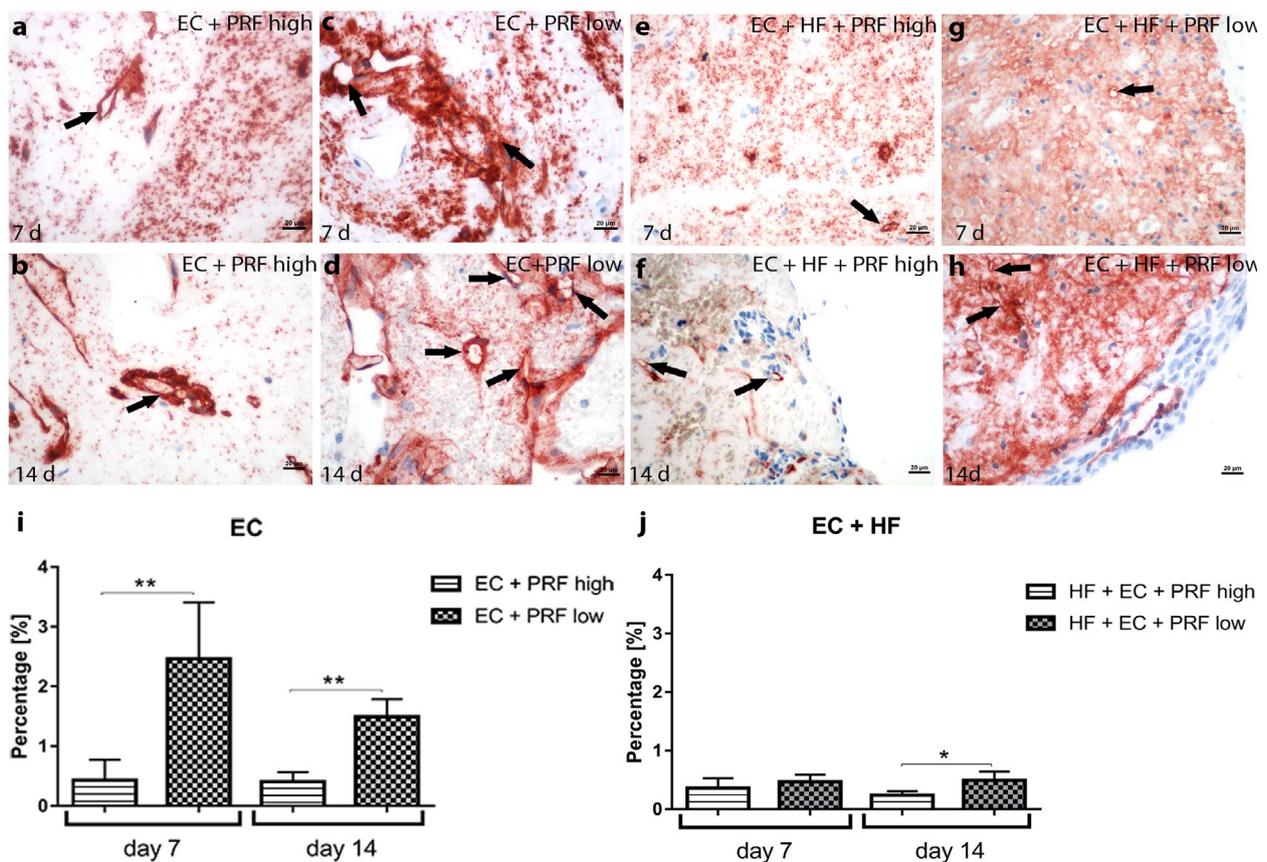


Fig. 3. Lumen and microvessel-like structure formation of ECs in mono-culture and ECs and HFs in co-cultures mixed with PRF prepared using the LSCC. Cells were cultivated with a (a,b,e,f) high- or (c,d,g,h) low-RCF PRF. Histological sections of PRF-cell complexes were stained immunohistochemically for CD31 after 7 and 14 d of cultivation. Black arrows highlight positively stained lumen structures formation. (a-h) Scale bars: 20 μ m. (i,j) The percentage (%) of vascularisation was calculated from the total vessel/lumen area and the total area of the images. Data error bars are calculated as SD of the mean. Statistical significance was evaluated by means of ANOVA. Differences were considered statistically significant for * $p < 0.05$ and ** $p < 0.01$.

Discussion

The use of PRF as a promising material for tissue engineering has recently gained attention among dentists in the field of regenerative medicine since PRF seems to promote a material-induced physiological tissue reaction in defective areas that leads to better wound healing and tissue repair (Miron *et al.*, 2017). PRF is a blood concentrate containing inflammatory cells and growth factors embedded in a fibrin network, which can be easily obtained from a patient's own peripheral blood using a one-step centrifugation process without addition of anticoagulants (Choukroun, 2001; Ghanaati *et al.*, 2014). With regard to this, the cellular and structural composition of PRF can be modified through the application of different RCFs and centrifugation times (Choukroun and Ghanaati, 2018; El Bagdadi *et al.*, 2017). Implementation of the LSCC resulted in an increased concentration of inflammatory cells and growth factors released from injectable and solid PRF matrices as a consequence of a reduction

in the RCF (El Bagdadi *et al.*, 2017; Wend *et al.*, 2017). Wend *et al.* (2017) showed that decreasing the RCF leads to a larger number of leukocytes, lymphocytes, neutrophils, monocytes and platelets in the resulting injectable PRF when compared to PRF generated at higher RCFs. Therefore, the amount of growth factor and cytokine release in PRF matrices increases with decreasing RCF forces. The present study investigated, for the first time, two different PRF matrices produced at low- (44 \times g) and high- (710 \times g) RCF and their influence on the formation of angiogenic structures and growth factor release, using HDMECs in mono-culture and in co-culture with HFs as an *in vitro* model system for soft tissue regeneration. In general, the present comparative study revealed a distinct PRF-dependent, pro-angiogenic effect on ECs in mono-culture, as demonstrated by an increase in microvessel-like structures when ECs were combined with both types of PRF as compared to the appropriate control groups without PRF. Interestingly, this pro-angiogenic effect of PRF on ECs was significantly larger when low-

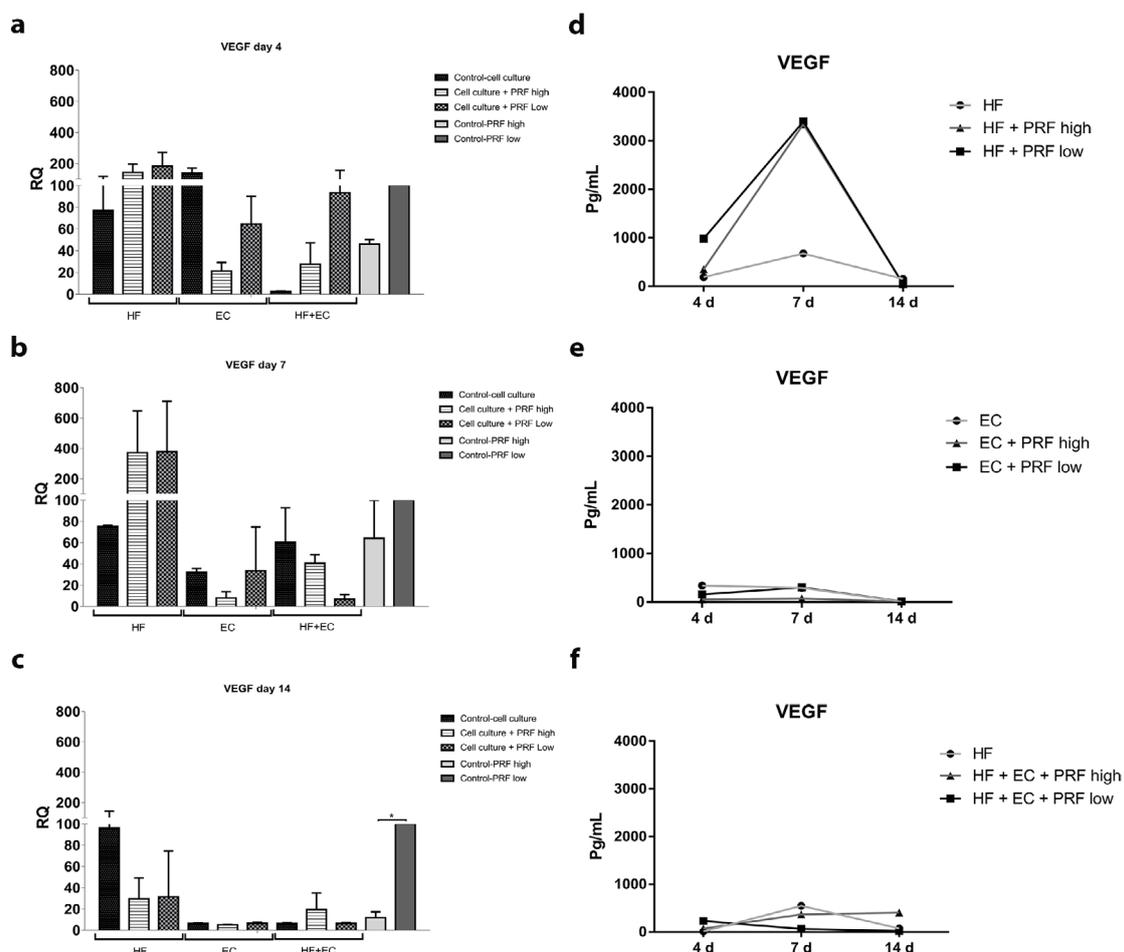


Fig. 4. Quantification of VEGF content within the cell culture supernatants released from cell/PRF-complexes by ELISA. Mono- and co-cultures were cultivated with a high or low RCF PRF prepared using the LSCC; control group was cultivated without PRF. (a-c) Results are relatively quantified (RQ) to the control PRF low-RCF matrix without additional cells. (d-f) Absolute concentrations at 4, 7 and 14 d. Data error bars are calculated as SD of the mean. Statistical significance was evaluated by means of ANOVA. Differences were considered statistically significant for * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

RCF PRF was employed as compared to high-RCF PRF. Analysis of growth factor production in PRF produced at different RCF confirmed previous results (Choukroun and Ghanaati, 2018; El Bagdadi *et al.*, 2017; Wend *et al.*, 2017). A larger release of TGF- β 1 and PDGF-BB in low-RCF PRF and in low-RCF PRF combined with cells was observed, indirectly suggesting that larger cell numbers were generated in the low PRF/cell complexes by the LSCC. Considering that reducing the applied RCF in low-RCF PRF results in a higher concentration of leukocytes and their subtypes, especially neutrophils (Ghanaati *et al.* 2014), the observed pro-angiogenic effect may be related to the presence of a large number of neutrophils and their released neutrophil extracellular traps (NETs). This is supported by an *in vitro* study showing the positive influence of neutrophils on angiogenesis and osteogenesis *in vitro* (Herath *et al.*, 2018). In addition, during tissue repair, activated platelets accumulate

in the wound area and form a fibrin clot that closes the wound and secretes growth factors – such as PDGF or TGF- β 1 – which are, therefore, key players in the activation of immune cells, wound healing and tissue regeneration (Gurtner *et al.*, 2008; Martin, 1997). In particular, the interaction of platelets with leukocytes, such as monocytes, macrophages and neutrophils, is a requisite for normal wound healing and tissue regeneration. Activated immune cells, such as monocytes, release different growth factors, *e.g.* PDGF-BB and TGF- β 1, that attract fibroblasts to sites of injury where they produce an extracellular matrix within the wound tissue (Eming *et al.*, 2007; Werner and Grose, 2003). Additionally, TGF- β 1, PDGF-BB and VEGF in concert are essential for the stabilisation of vascular structures (Beck and D'Amore, 1997). The larger release of PDGF-BB and TGF- β 1 in the low-RCF PRF/cell complexes as compared to the high-RCF PRF/cell complexes resembled the initiation of wound

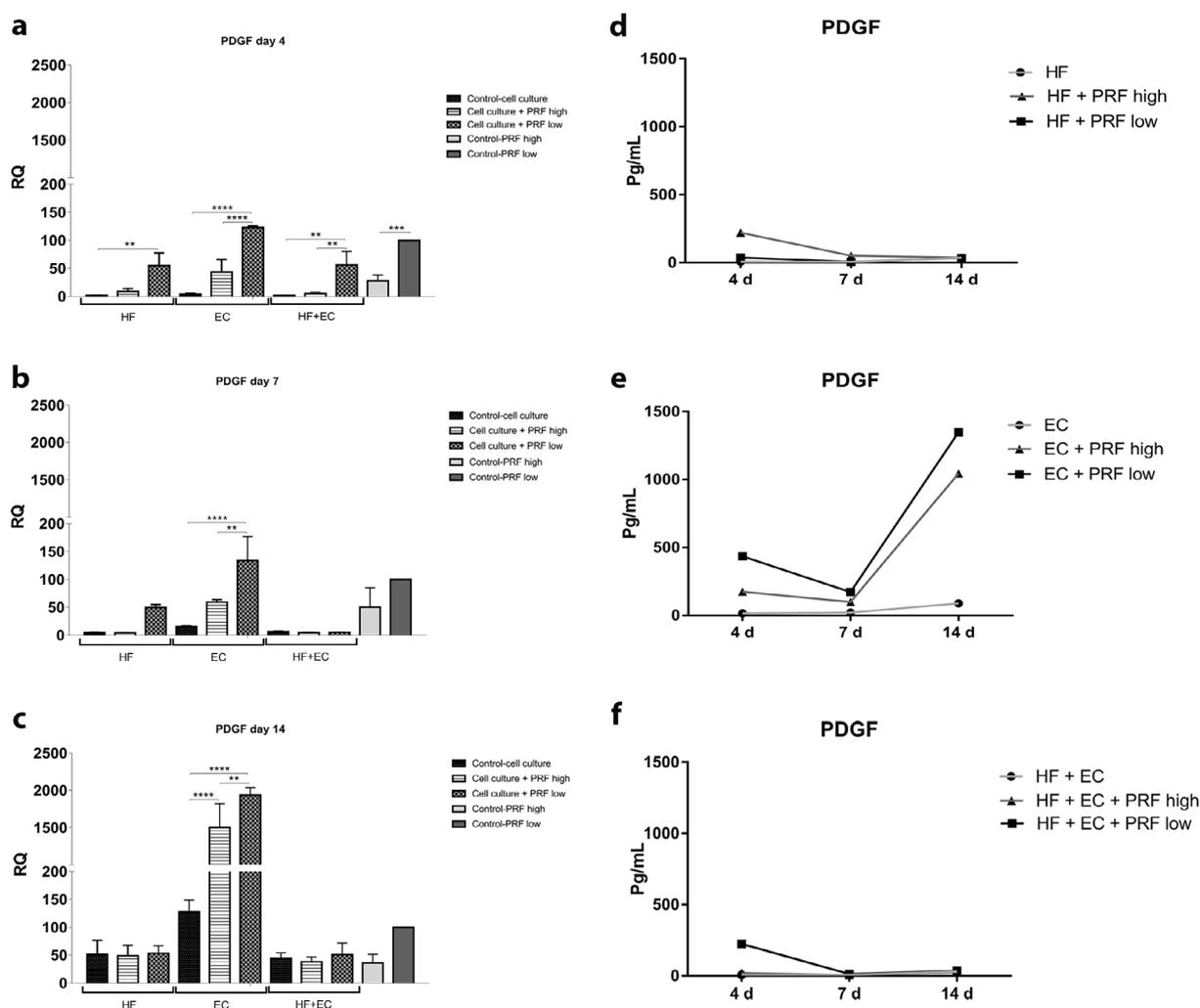


Fig. 5. Quantification of PDGF-BB content within the cell culture supernatants released from cell/PRF-complexes by ELISA. Mono- and co-cultures were cultivated with a high- or low-RCF PRF prepared using the LSCC; control group was cultivated without PRF. (a-c) Results are relatively quantified (RQ) to the control PRF low-RCF matrix without additional cells. (d-f) Absolute concentrations at 4, 7 and 14 d. Data error bars are calculated as SD of the mean. Statistical significance was evaluated by means of ANOVA. Differences were considered statistically significant for * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

healing mediated by the addition of PRF produced by the LSCC. Furthermore, activated leukocytes secrete VEGF within the wound area, which finally leads to the induction of blood vessel formation, underscoring the close interaction of the different cell types during wound healing processes and angiogenesis (Jenne *et al.*, 2013; Nurden, 2011).

The results obtained could provide an idea about the potential origin of the growth factor and their pattern of consumption or accumulation in the cell culture supernatant, leading to vascular formation. The significant pro-angiogenic effect of low-RCF PRF on ECs may be attributed to higher VEGF expression, since the release of VEGF was observed to be larger in low-RCF PRF as compared to high-RCF PRF, leading to the assumption that this difference in VEGF expression was already triggering the ECs to form microvessel-like structures. Given

that there was almost no detectable VEGF in the EC/PRF groups after 14 d of cultivation, it is reasonable to assume that during cultivation with PRF, ECs adsorbed the free VEGF released from PRF into the supernatants by VEGF binding to its receptor, VEGF receptor 2 (VEGFR2). This led to their angiogenic activation, which finally resulted in microvessel-like structure formation. Blocking of soluble VEGF using conditioned medium from soluble VEGFR2-producing cells results in VEGF ligand inhibition, reduction of free VEGF in cell culture supernatants and inhibition of angiogenesis in a triple-culture model system for bone tissue engineering (Dohle *et al.*, 2014). In contrast, the lower observed concentrations of growth factors and angiogenic structure formation in the PRF/co-culture complexes as compared to the mono-culture might be associated with an excessive supply of growth factors,

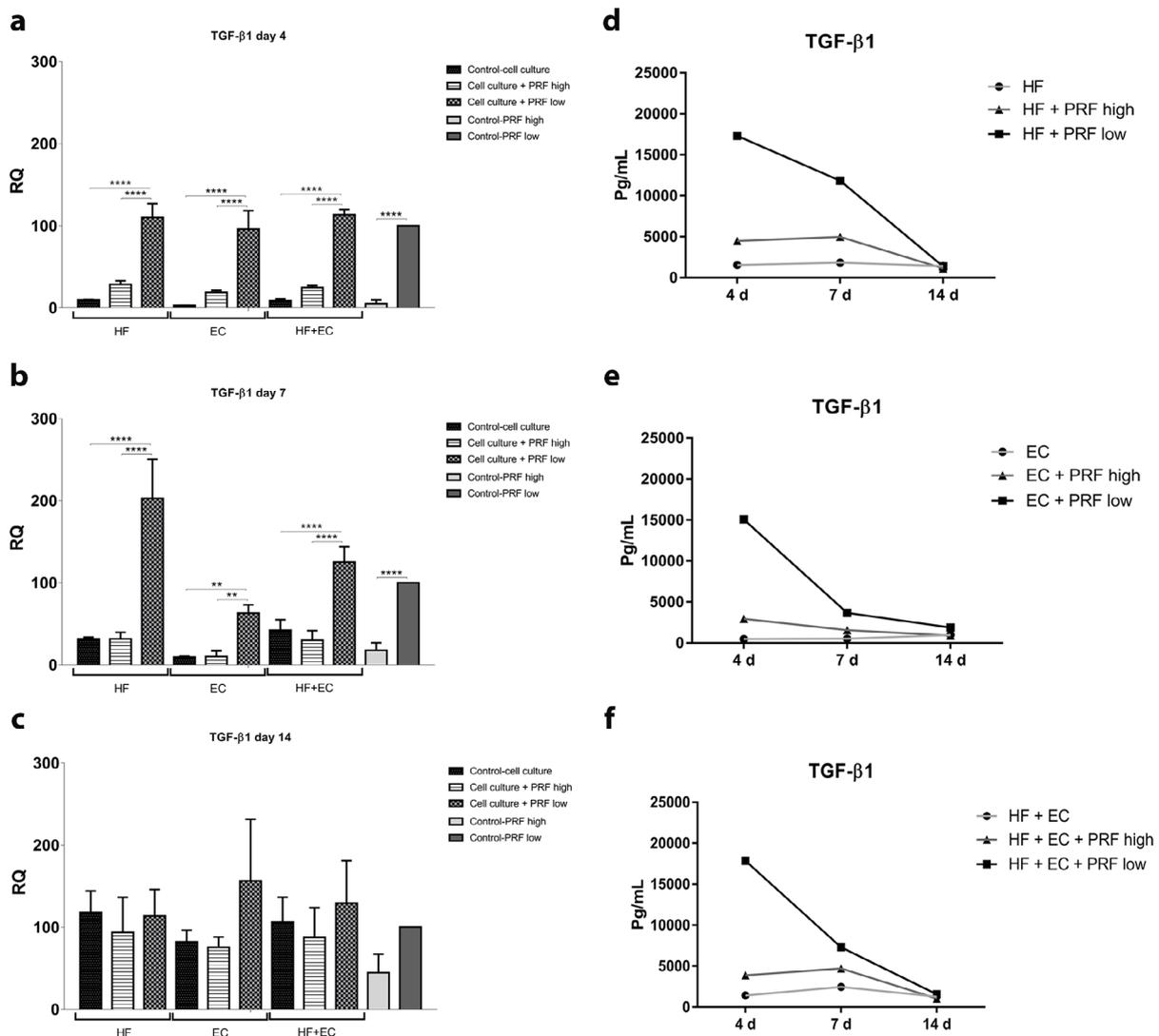


Fig. 6. Quantification of TGF-β1 content within the cell culture supernatants released from cell/PRF-complexes by ELISA. Mono- and co-cultures were cultivated with a high- or low-RCF PRF prepared using the LSCC; control group was cultivated without PRF. (a-c) Results are relatively quantified (RQ) to the control PRF low-RCF matrix without additional cells. (d-f) Absolute concentrations at 4, 7 and 14 d. Data error bars are calculated as SD of the mean. Statistical significance was evaluated by means of ANOVA. Differences were considered statistically significant for * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

i.e. VEGF and TGF- β 1, deriving from PRF itself and additionally released by HFs. In general, highest VEGF and TGF- β 1 concentrations could be found in HF/PRF-complexes after 4 and 7 d. Growth factor concentrations in supernatants of co-culture/PRF complexes were comparatively lower, supposedly being consumed by ECs in these complexes. Both VEGF and TGF- β 1 might induce angiogenesis but have opposite effects on ECs, since the crosstalk between these growth factors also controls ECs apoptosis (Hicklin and Ellis, 2005). VEGF protects ECs from apoptosis whereas TGF- β 1 induces apoptosis, demonstrating the necessity for a precise regulation of their expression and interaction (Ferrari *et al.*, 2006; Ferrari *et al.*, 2009). In addition, TGF- β 1 downregulates VEGFR2 expression, therefore enabling abolishment of angiogenic structure formation mediated through VEGF (Mandriota *et al.*, 1996) and, possibly, limiting VEGF effect in the co-culture/PRF-complexes.

Another explanation for the PRF-mediated pro-angiogenic effect in this *in vitro* model relates to the fibrin structure of PRF itself, which serves as a 3D matrix. In vasculogenesis models, the 3D matrix is a *sine qua non* condition for the formation of 3D vascular structures *in vitro* (Vailhe *et al.*, 2001). In terms of cell-based vascularisation strategies, the rapid initiation of neovascularisation *in vivo* can only be achieved by co-implantation of pro-angiogenic matrices such as Matrigel® (Fuchs *et al.*, 2009). In previous *in vitro* 3D culture models, the modification of the mechanical properties (*i.e.* Young's modulus, stress/strain) of synthetic manufactured extracellular matrices (S-ECM) originating from collagen, hyaluronic acid or fibrin hydrogel has shown an effect on cellular physiological processes (Nebuloni *et al.*, 2016; Soofi *et al.*, 2009). Based on previous studies, it is arguable that the structure of PRF could be modified by the RCF (Kubesch *et al.* 2018). In this context, more vascularisation could be expected *in vivo* in nude mice, depending on the porous structure of the implanted PRF. The changes in fibrin structure gained from different RCFs could be pulled together with a larger number of angiogenic structures in the more porous low-RCF PRF matrix as compared to the high-RCF PRF and control group. The effect of the fibrin structure of PRF on the angiogenic arrangement of ECs is documented *in vivo* (Kubesch *et al.* 2018). The tissue reaction in subcutaneous implantation model of severe combined immunodeficient (SCID) mice clearly differs from the present *in vivo* results, likely due to differences in the implanted fibrin scaffold composition and porous structure of the implanted PRF matrix. According to Kubesch *et al.* (2018), the implanted low-RCF PRF has a more porous structure and results in a higher vascularisation rate as compared to implantation of high-RCF PRF. In general, a more porous and softer matrix appears to facilitate angiogenesis. Therefore, fine-tuning the mechanical properties of PRF matrices for *in vitro* cell tissue engineering and for clinical practices (*i.e.* plug

PRF, pressed or unpressed PRF matrices) to resemble the cell microenvironment should be a topic of further studies.

The aim of the present study was to compare the distinct proangiogenic effect of low- and high-RCF PRF with regard to EC angiogenic activation. The applied methods contributed to refute that angiogenesis was not being facilitated through co-cultivation itself and permitted the observations that angiogenic structures were, in fact, induced by PRF. A limitation of the study may be the absence of a control group using a S-ECM. In this regard, further experimental scenarios with different S-ECM as control groups are currently being conducted to determine if the proangiogenic effect of PRF is mediated by cell content, matrix structure and/or its secretome. Additionally, phenotypic characterisation of ECs and HFs during cell culture was not performed. This led to the question whether a high growth factor concentration is a possible risk factor if PRF is applied clinically, considering the observed proangiogenic effects. To the authors' knowledge, there is no information in the literature regarding side effects or tumorigenesis induced by PRF. In a recently published systematic review involving 73 clinical studies, no reports were found of any side effects or tumorigenesis after PRF application for the treatment of different pathologies (Ghanaati *et al.*, 2018b). Furthermore, long-term clinical follow-up studies can be found in the literature describing no negative effects of PRF application up to 72 months (Simonpieri *et al.*, 2011). Additionally, preliminary results of temporomandibular joint (TMJ) infiltrations with i-PRF for treatment of a pathological state known as "internal derangement" and localised TMJ pain were reported by Albilgia *et al.* (2018). 37 patients participated in the study with an observation period of 12 months. During the study, the patients reported reduction of pain and improvement of function. No side effects were observed and the patients are, currently, still under observation (Albilgia *et al.*, 2018). In an additional clinical case, Ghanaati *et al.* (2018a) reported the use of i-PRF as a filling skin material for treating hyaluronic-acid-related complications, with a follow up of 24 months. In this case, PRF injections promoted wound healing and minimised scar formation with no clinical signs of tumorigenesis induction (Ghanaati *et al.*, 2018a). In a further clinical report, a patient affected by squamous cell carcinoma in the anterior floor of the mouth was subjected to a tumour resection including an anterior block resection of the mandible. After 16 months of tumour therapy, the patient underwent reconstruction with an individualised 3D titanium mesh and the combination of PRF with a xenogeneic bone substitute biomaterial (Bios-Oss®, Geistlich Biomaterials, Wolhusen, Switzerland). After a follow up of 12 months, the patient was rehabilitated with dental implants and prosthetics. The histological analyses of bone biopsies showed new bone

formation and a well vascularised connective tissue. It is noteworthy that after 28 months of follow up there was no evidence of tumour recurrence (Lorenz *et al.*, 2018).

Based on previous studies that evaluated single components of the peripheral blood [*i.e.* platelets (Nurden 2011), neutrophils (Herath *et al.*, 2018) and monocytes (Barbeck *et al.*, 2016)] to understand their individual role in tissue engineering, the present study proposed a complex system containing cells and proteins existing within the peripheral blood and aimed at understanding their effect at different concentrations, using high- and low-RCF to vary their amounts. The important role of the secretome for tissue regeneration has come into focus, as it was found to contain the necessary chemokines, cytokines and growth factors that positively affect wound healing (Burdon *et al.*, 2011; Gaetani *et al.*, 2018). Therefore, PRF might be a useful therapeutic “biologisation” instrument of acellular biomaterials to increase their regenerative potential by providing additional secretion of growth factors with increased pro-angiogenic activity. Reducing the RCF of PRF had a significant influence on growth factor secretion and induction of angiogenesis in an *in vitro* model system for soft tissue engineering in which immune cells, growth factors and the structure of the fibrin matrix seemed to act together to create an appropriate microenvironment that might improve wound healing and vascularisation.

Conclusion

The present study evaluated the effect of PRF produced either with a high- (710 ×g) or low- (44 ×g) RCF according to the LSCC on human vascular ECs and HFs in mono-culture and in co-culture. PRF-mediated effects on the growth factor performance and the pro-angiogenic effect of PRF on this *in vitro* model system were analysed. PRF generated with low-RCF and combined with ECs resulted in a significantly larger release of growth factors and a significantly larger number of microvessel-like structures as compared to high-RCF PRF and control groups. In conclusion, PRF is a beneficial tool for increasing growth factor release and angiogenesis for *in vitro* cell tissue-engineering applications. Using low-RCF PRF, most likely meeting good clinical practice guidelines, it might be possible to facilitate a material-induced physiological tissue reaction in defective areas, leading to better wound healing and tissue repair.

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Discussion with Reviewers

Markus Ioibl: Do the authors think similar proangiogenic effects in the EC mono-culture could be obtained by adding recombinant VEGF in the same concentration as measured in the study?

Authors: The proangiogenic effect was, indeed, partly mediated through an increased VEGF expression in the EC-PRF complexes. This was demonstrated during the study and it might lead to the assumption that simply adding recombinant VEGF instead of using PRF could also induce microvessel-like structure formation in EC mono-culture. Nevertheless, to form angiogenic structures, EC mono-culture essentially need a stabilising matrix: either a matrix that is produced by another cell type, e.g. osteoblasts or fibroblasts (Dohle *et al.*, 2010; Dohle *et al.*, 2011; Fuchs *et al.*, 2009), or matrices such as Matrigel® or fibringels that are generally used in different angiogenesis arrays (Fuchs *et al.*, 2009; Stahl *et al.*, 2004).

Markus Ioibl: Do the authors consider the effect of PRF as a collective effect of different bioactive molecules or could they name one key player for the promotion of vascularisation?

Authors: We consider the effect of PRF as a collective effect of different bioactive molecules in combination with the fibrin structure, regarding PRF as a unity to study PRF-mediated effects in its entirety. Further studies are currently ongoing separating PRF into its components and analysing their effects individually.

Elena Della Bella: PRF has a pro-angiogenic effect on EC monocultures, but this is not so evident when ECs are co-cultivated with HFs. How could you explain this difference? This should be investigated more in details in the eventuality of a clinical translation of the results.

Authors: The difference in PRF-mediated proangiogenic effect on EC mono-cultures as compared to co-cultures with regard to angiogenic structure formation might be explained by an excessive supply of growth factors, *i.e.* VEGF and TGF- β 1, deriving from PRF itself but also additionally released by the HFs. The highest VEGF and TGF- β 1 concentrations can be found in HF/PRF-complexes after 4 and 7 d. Growth factor concentrations in supernatants of co-culture/PRF-complexes were comparatively lower, supposedly because the growth factors might be consumed by ECs in these complexes. VEGF and TGF- β 1 are able to induce angiogenic structure formation but might also have opposing effects on ECs since the crosstalk between

these growth factors also controls EC apoptosis (Hicklin and Ellis, 2005). In general, a co-culture in combination with another factor, such as PRF, is a highly complex system which is similar to the *in vivo* situation. Therefore, it is usually very difficult to determine exactly which cell type is doing what and how, because the different cell types act together as a unique system and each cell type is influencing the other through paracrine or direct communication,

including complicated feedback mechanisms. Nevertheless, we are planning a further study to explain the differences in the angiogenic activation, analysing cell communication and occurring mechanisms in PRF/cell-complexes.

Editor's note: The Scientific Editor responsible for this paper was Mauro Alini.