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ORIGINAL ARTICLE

The impact of intercellular communication for the generation of complex multicellular prevascularized tissue equivalents

Martin Heller[1,2] | Heide-Katharina Bauer[1,2] | Roxana Schwab[1,2] | Sebastian Blatt[2,3] | Katharina Peters[1,2] | Sandra Nezi-Cahn[1,2] | Ronald E. Unger[2,4] | Annette Hasenburg[1] | Walburgis Brenner[1,2]

[1]Department of Gynecology, University Medical Center of the Johannes Gutenberg University Mainz, Mainz, Germany

[2]BiomaTiCS—Biomaterials, Tissues and Cells in Science, University Medical Center of the Johannes Gutenberg University Mainz, Mainz, Germany

[3]Department of Maxillofacial Surgery, University Medical Center of the Johannes Gutenberg University Mainz, Mainz, Germany

[4]Institute for Pathology, University Medical Center of the Johannes Gutenberg University Mainz, Mainz, Germany

Correspondence
Walburgis Brenner, Department of Gynecology, University Medical Center of the Johannes Gutenberg University Mainz, Langenbeckstr. 1, 55131 Mainz, Germany.
Email: brenner@uni-mainz.de

Abstract

In reconstructive surgery the use of prevascularized soft tissue equivalents is a promising approach for wound coverage of defects after tumor resection or trauma. However, in previous studies to generate soft tissue equivalents on collagen membranes, microcapillaries were restricted to superficial areas. In this study, to understand which factors were involved in the formation of these microcapillaries, the levels of the angiogenic factors vascular endothelial growth factor (VEGF), Interleukin-8 (IL-8), and basic fibroblast growth factor (bFGF) in the supernatants of the tissue equivalents were examined at various time points and conditions. Additionally, the influence of these factors on viability, proliferation, migration, and tube formation in monocultures compared to cocultures of fibroblast and endothelial cells was examined. The results showed that VEGF production was decreased in cocultures compared to fibroblast monocultures and the lowest VEGF levels were observed in endothelial cell monocultures. Additionally, the highest levels of IL-8 were observed in cocultures compared to monocultures. Similar results were observed for bFGF with lowest levels seen within the first 24 hr and highest levels in cocultures. VEGF and IL-8 were shown to promote endothelial cell viability, proliferation and migration and angiogenic parameters such as tube density, total tube length, and number of tube branches. Addition of VEGF and IL-8 to cocultures resulted in accelerated and denser formation of capillary-like structures. The results indicate that VEGF, IL-8, and bFGF strongly influence cellular behavior of endothelial cells and this information should be useful in promoting the formation of microcapillary-like structures in complex tissue equivalents.

KEYWORDS

angiogenic growth factors, coculture, HDMEC, intercellular communication, microvascular endothelial cells

Martin Heller and Heide-Katharina Bauer contributed equally to this study.

1 | INTRODUCTION

Current studies indicate that tissue engineering is a promising approach in reconstructive surgery to generate complete and functional tissues and organs. In regenerative medicine applications, autologous tissue-equivalents can be used for repairing large wounds after tumor resection or for the reconstruction of inherent or acquired tissue defects. In gynecological, urological, head and neck regions, such defects are common and would benefit from using tissue-equivalents. Gynecological applications such as atresia and aplasia or after tumor resections, trauma or sex change surgeries would benefit from such surgical reconstructions (Callens et al., 2014; Kimberley, Hutson, Southwell, & Grover, 2012; Sadri-Ardekani & Atala, 2015). Similar benefits would be seen in patients with urethral defects such as hypospadias and epispadias due to genetical defects, strictures as a result of trauma or tumor resections or patients undergoing reconstructive surgery for a cleft lip or palate (Baskin & Ebbers, 2006; Fillmore & Rieck, 2014; Hampson, McAninch, & Breyer, 2014; Mossey et al., 2011; Raya-Rivera et al., 2011).

For all of the above applications, buccal mucosa tissue is the most suitable donor tissue and represents the gold standard in most of these reconstructive applications (Chiapasco, Colletti, Romeo, Zaniboni, & Brusati, 2008; De Bree et al., 2008; de Trey & Morrison, 2013; Dhillon, Mohan, Raju, & Lakhanpal, 2013; Khan, Zaheer, & Gupta, 2013; Tompkins, Vaughn, Shaikh, Stocks, & Thompson, 2015). However, disadvantages of autologous buccal mucosa are the limited access and quantity and make the wound coverage of large areas nearly impossible. Furthermore, the use of autologous tissue is often associated with severe complications at the initial surgical area such as pain, scar formation, strictures, and paresthesia (Almela, Brook, & Moharamzadeh, 2016; Heller et al., 2016; Markiewicz, Margarone, Barbagli, & Scannapieco, 2007; Moharamzadeh, Brook, van Noort, Scutt, & Thornhill, 2007; Mungadi & Ugboko, 2009). Since the success of an operative reconstruction is mainly determined by the size of adequate donor tissue for the tissue transfer (Tompkins et al., 2015), one of the major problems is the limited quantity of applicable donor tissue.

One solution to the problems associated with autologous transplants is the creation of artificial tissue equivalents in vitro using tissue engineering strategies. Recent studies have shown that a "full thickness mucosa" could be constructed using keratinocytes alone or with a coculture of keratinocytes and fibroblasts cultivated on dermal matrices (Izumi, Feinberg, Iida, & Yoshizawa, 2003; Lauer & Schimming, 2001; Moharamzadeh et al., 2007). However, one of the major problems with such artificial tissue equivalents is a delayed or missing blood vessel supply after transplantation leading to insufficient in-growth and necrosis, which can finally result in the loss of the transplant (Black, Berthod, L'heureux, Germain, & Auger, 1998; Boyce, 1996; Tremblay, Hudon, Berthod, Germain, & Auger, 2005).

In order to overcome this problem, we have previously shown that it is possible to generate a prevascularized buccal mucosa equivalent as either a co- or triculture of buccal epithelial cells, fibroblasts and microvascular endothelial cells from the preputium based on a collagen matrix (Bio-Gide®, Geistlich, Wolhusen, Switzerland) (Heller et al., 2016). After transplantation into the neck area of nude mice, the preformed vasculature in our tissue engineered transplant demonstrated anastomosis to the host vasculature. However, despite these promising advances, the formation of the capillary-like structures inside the tissue equivalent was restricted to superficial areas only of the collagen matrix. We suspected that this was due to the absence of biochemotactical signals from angiogenic factors and this prevented the migration of endothelial cells into the deeper areas of the tissue equivalent. The formation of capillary-like structures throughout the entire tissue equivalent is regarded as a crucial step in providing a rapid blood supply to the cells in the tissue equivalent after transplantation.

The events of blood vessel formation are based on the biological mechanisms of angiogenesis and vasculogenesis and are strictly regulated and controlled by the complex interactions of growth factors, cytokines and transcription factors. There are various angiogenic factors involved in this complex inter- and intracellular interactions such as VEGF (vascular endothelial growth factor), bFGF (basic fibroblast growth factor), IL-8 (Interleukin-8), TGF (transforming growth factor), TNF-α (tumor necrosis factor), Angiopoietin and HIF (hypoxia-inducible factor 1) (Carmeliet & Jain, 2011; Karamysheva, 2008; Unger, Dohle, & Kirkpatrick, 2015). In angiogenesis and prevascularization, VEGF is regarded as one of the most important factors (Adair & Montani, 2010a; Chung & Ferrara, 2011). In healthy tissue under normoxic conditions, the formation and stabilization of the vasculature is regulated by autocrine mechanisms, in which, among other factors, low concentrations of VEGF-A and bFGF are involved (Augustin, Koh, Thurston, & Alitalo, 2009; Carmeliet & Jain, 2011; Domigan et al., 2015; Koch, Tugues, Li, Gualandi, & Claesson-Welsh, 2011; Lee et al., 2007; Potente, Gerhardt, & Carmeliet, 2011). IL-8, which enhances endothelial survival and proliferation directly and is involved in persistent angiogenesis during inflammatory processes is another essential factor (Koch et al., 1992; Li, Dubey, Varney, Dave, & Singh, 2003). However, it is not clearly understood in what way these factors are involved in the prevascularization process in an in vitro generated tissue equivalent. In order to understand the complex intercellular interactions within the tissue equivalent, the aim of this study was to examine the occurrence of the angiogenic factors VEGF, IL-8 and bFGF and their impact on microcapillary formation in complex cocultures.

2 | MATERIALS AND METHODS

2.1 | Cell isolation and cell culture

Primary fibroblasts were isolated from human gingiva and primary microvascular endothelial cells were isolated from human juvenile foreskin (Heller, Frerick-Ochs, Stein, Thüroff, & Brenner, 2015; Squier & Brogden, 2011), obtained from patients who underwent surgery at the Department of Maxillofacial Surgery or the Department of Urology, University Medical Center Mainz, Germany, respectively.

The study was performed in agreement with the Declaration of Helsinki and approved by local ethics committee (Landesärztekammer Rheinland-Pfalz, Mainz, Germany: 837.439.05). Informed consent was obtained from each patient. For the experiments, fibroblasts were used up to passage 8, endothelial cells up to passage 4.

Fibroblast isolation procedure: In brief, for fibroblast isolation the connective tissue of buccal mucosa was cut into small fragments (1×1 mm^2), transferred and cultivated in 25 cm^2 cell culture flasks using DMEM medium with 10% FCS, 100 U/100 μg/ml Penicillin/Streptomycin and 2.5 μg/ml Fungizone (Gibco, Darmstadt, Germany). After 1 week of cultivation, cell medium was changed at least every 3 days.

Microvascular endothelial cell isolation: Microvascular endothelial cells (human dermal microvascular endothelial cells, HDMEC) were isolated from human juvenile foreskin as described by Peters and coworkers (Peters et al., 2002), and cultured in PC-Medium (Customer Formulation Medium, Promo Cell, Heidelberg, Germany) supplemented with 15% FCS, 10 μg/ml Na-heparin, 0.2 ng/ml bFGF, 100 U/100 μg/ml Penicillin/Streptomycin and 2.5 μg/ml Fungizone. All cells were cultivated at 37°C, 5% CO_2, and 95% relative humidity.

2.2 | Characterization of the isolated cells

For cell identification, immunohistological staining using cytospin preparations was performed. In brief, cells were centrifuged and immobilized on microscope slides (maximum speed, 10 min) and air dried. The slides were then fixed in 100% ethanol for direct staining. After washing in aqua dest. and blocking the endogenous peroxidase using *Peroxidase blocking solution* (Dako, Hamburg, Germany), the samples were incubated with the primary antibody for 1 hr. For the identification of endothelial cells, CD31 was detected using Anti-Human CD31, #M0823, Clone JC70A, Dako Mouse monoclonal (1:100) (Dako, Hamburg, Germany). The expression of α-SMA on fibroblasts was identified using anti-human alpha smooth muscle actin, #ab5694, abcam Rabbit polyclonal (1:50) (Abcam, UK). To detect the bound antibodies a DAB based kit system (Universal LSAB™2 Kit/HRP, Rabbit/Mouse) (Dako, Hamburg, Germany) was used. Negative controls were performed by omitting the primary antibody. Finally, slides were counterstained by hemalaun and evaluated using a light microscope.

2.3 | Cultivation of the mono- and coculture on a collagen membrane

Mono- and cocultures were prepared using 2×10^5 fibroblasts and 4×10^5 endothelial cells. The cell cultures were generated by placing the collagen membrane Bio-Gide® (Geistlich, Wolhusen, Swiztzerland, punch of 6 mm diameter, 28.3 mm^2) into cell culture inserts for 24 well plates (Netwell Insert No. 3477 from Corning Costar Corporation, Amsterdam, Netherlands). After equilibration of the collagen membranes using standard culture medium, the cells were seeded on the porous side of the membranes.

For analyzing the influence of the seeding order on microcapillary formation in cocultures, different seeding orders of the specific cell types were investigated. First, 4×10^5 endothelial cells were seeded onto the porous side on the membrane and after 24 hr 2×10^5 fibroblasts were added to the membrane. In a second approach, 2×10^5 fibroblasts were seeded 24 hr before 4×10^5 endothelial cells were added to the membrane. Endothelial cell culture medium was used and changed every 2–3 days.

2.4 | Collection of cell culture supernatant

For analyzing the cytokine levels observed in the supernatant by the first cell-type during the time period before seeding with the respective second cell type (24 hr), the culture medium was replaced with serum-free medium 24 hr before collecting the supernatant of monocultures after 2, 4, 6, 8, 12, and 24 hr. For comparison, in cocultures, both cell types were seeded onto the collagen membrane at the same time and the supernatants where collected similarly.

In addition, for the analyses of long-term culture of the cells, supernatants from cocultures (first fibroblasts then endothelial cells) were collected after 3, 7, 11, 15, 19, and 21 days, in each case after serum-free culture for 24 hr prior to collecting the supernatant as described above. Collected supernatants were stored at −20°C until use.

2.5 | Visualization of the mono- and coculture on a collagen membrane

For visualization of endothelial cells on the collagen membrane, confocal laser scanning microscopy (CLSM) was used. Endothelial cells where visualized by CD31 staining. In brief, collagen membranes were fixed in 3.7% paraformaldehyde for 15 min and then treated with 0.2% Triton X 100 for 10 min. The primary mouse-anti-human-CD31 antibody (1:50 in 1% BSA), clone JC70A (Dako, Hamburg, Germany) was incubated for 1 hr. The secondary anti-mouse antibody "alexa 488" (Invitrogen, Gibco Life Technologies GmbH, Darmstadt, Germany) was used with a dilution of 1:1000 in 1% BSA for 1 hr in the dark. After a final washing step, samples were analyzed with the TCS SP2 CLSM from Leica, Wetzlar, Germany.

2.6 | Analyses of the cytokine expression

For analyses of the intercellular communication, the concentration of VEGF, IL-8, and bFGF in the collected supernatants were quantified by using ELISA kits from R&D Systems (Minneapolis, USA) according to the manufacturer's specifications. Samples were analyzed at a wavelength of 450 nm and a wavelength correction of 560 nm. Quantification was performed using standard series of at least five values

and a linear regression. In each experiment triplicate measurements and three independent experiments using cells from three different donors were performed. Statistical analyses were performed using mean values and *SD*s and the software SPSS.

2.7 | Cell viability assay

In order to investigate the influence of the angiogenic factors VEGF, IL-8, and bFGF on the viability of endothelial cell culture MTT assays were performed as described (Breuksch et al., 2017). For the experiments, wells of a 96 well plate were coated with 60 µl of a 0.2% gelatin solution and 5×10^3 endothelial cells per well were seeded in 100 µl culture medium. After 24 hr, cell culture medium was replaced with serum-free medium and incubated for an additional 24 hr. Then angiogenic factors (Sigma–Aldrich Chemie GmbH, Taufkirchen, Germany) were added to the medium using following concentrations: VEGF: 5, 10, 25, 50, and 100 ng/ml; IL-8:5, 25, 50, 100, and 500 ng/ml; bFGF: 1, 2, 3, 4, and 5 µg/ml. After 24 hr of incubation the MTT assay was performed. In brief, 20 µl of 0.5% MTT solution per well was added and incubated at 37°C for 3 hr on a stirrer. Then supernatants were discarded and wells were washed with DPBS. Solubilization of tetrazolium-precipitate was performed adding isopropanol for 15 min on a stirrer at 500 rpm. 100 µl of supernatant was then transferred to a fresh 96-well plate and the absorbance was measured at 570 nm using an Anthos 2010 spectrophotometric microplate reader (Anthos Labtec Instruments, Cambridge, GB). The viability rate was calculated as percentage of the untreated cells. The assays were performed in quadruplicate for each time point and with three independent experiments using cells from three different donors. Statistical analyses were performed using mean values and *SD*s and the software SPSS.

2.8 | Proliferation assay

To analyze the influence of the angiogenic factors VEGF, IL-8 and bFGF on endothelial cells proliferation, BrdU assays were performed (*Cell Proliferation ELISA BrdU* Kit, Roche) (Haber et al., 2015). Cells were seeded as described for the MTT assay and pretreated with FCS-free medium for 24 hr. Ten µl/well BrdU-Labeling-Solution (1:100 in DPBS) was added to the cells and incubated for 2 hr at 37°C und 5% CO_2. Afterwards supernatants were discarded and 200 ml/well FixDenat-solution was added and incubated for additional 30 min at room temperature. After discarding supernatants, BrdU-antibody-solution (Anti-BrdU-POD 1:100 in *Antibody Dilution Solution*) was added and incubated for 60 min at room temperature. After washing, staining was allowed to develop for 15 min using 100 µl/well substrate solution at room temperature. The reaction was stopped with 25 µl/well 1 M H_2SO_4. Samples were analyzed at a wavelength of 450 nm and a wavelength correction of 650 nm. All BrdU measurements were performed in triplicate in three independent experiments using cells from three different donors. Statistical analyses were performed using mean values and *SD*s and the software SPSS.

2.9 | Migration assay in a Boyden chamber

For analyzing the chemotactic effect of VEGF, IL-8, and bFGF on endothelial cells, a modified Boyden chemotaxis chamber was used (Neuroprobe, Gaithersburg), as described previously (Haber et al., 2015). The chamber consists of an upper and a lower department separated by a porous polycarbonate membrane with 8 µm pore diameter (Neuro Probe, Gaithersburg, USA). Before analysis, endothelial cells were cultivated in serum-free culture medium for 24 hr. The wells of the lower part of the chamber were filled with 29 µl (according to the manufacturer's instructions) of VEGF (5, 10, 25, 50, and 100 ng/ml), IL-8 (5, 25, 50, 100, and 500 ng/ml) or bFGF (1, 2, 3, 4, and 5 µg/ml) in serum-free medium. The wells were covered by the polycarbonate membrane after having been equilibrated in DPBS for 2 min. The wells of the upper part were filled with 50 µl of cell suspension (6×10^5 cells/ml in serum-free medium). The chamber was then incubated for 16 hr in a moistened atmosphere with 5% CO_2 at 37°C. Afterwards, cells were removed from the upper membrane side of the transwell by washing in buffer solution and by mechanical detachment using a rubber scraper. Migrated cells on the lower side of the membrane were then fixed in methanol for 1 min and dyed with hemacolor (Merck, Darmstadt, Germany). The dyed membrane was then transferred onto a microscope slide and covered with immersion oil. The cells were counted in an area of 2.5 mm^2 on the porous membrane. For each concentration, experiments were performed in quadruplicate and repeated with three independent experiments by using cells from three different donors. Statistical analyses were performed using mean values and *SD*s and the software SPSS.

2.10 | Tube formation assay

In order to analyze the effect of the angiogenic factors on angiogenesis, a tube formation assay with endothelial cells was performed by using *Angiogenesis µ-Slides* (Ibidi GmbH, Martinsried, Germany). In brief, in the inner wells of the µ-slides 15 µl of Matrigel was added and incubated for 1 hr at 4°C. For polymerization of Matrigel, µ-slides were incubated over night at 37°C. In each well endothelial cells with a concentration of 1×10^4 cells/50 µl in serum-free medium were seeded. After 1 hr of cell adhesion, angiogenic factors (Sigma–Aldrich Chemie GmbH, Taufkirchen, Germany) were added to the medium using following concentrations: VEGF: 5, 10, 25, 50, and 100 ng/ml; IL-8:5, 25, 50, 100, and 500 ng/ml; bFGF: 1, 2, 3, 4, and 5 µg/ml. As negative control serum-free medium was used. After 6 hr of incubation at 37°C cells were stained with Calcein-AM (Thermo-Fisher, Waltham, USA) (20 µg/ml). Fluorescence microscopy was performed using a standard FITC filter at a wavelength of 494 nm. Micrographs were analyzed with respect to different parameters of tube formation such as tube length, density, and number of branches (Wimasis GmbH, München, Germany). Each analysis was performed in triplicate

in three independent experiments by using cells from three different donors. Data was analyzed using mean values and *SD*.

2.11 | Effect of the angiogenic factors on the formation of microcapillaries in the mucosa equivalent

In order to analyze the effect of the angiogenic factors VEGF and IL-8 on the microcapillary formation inside the mucosal tissue equivalent, cocultures of endothelial cells and fibroblasts on the collagen membranes Bio-Gide® (Geistlich) were prepared as described above and treated by VEGF (100 ng/ml), IL-8 (500 ng/ml), a combination of VEGF and IL-8 in the same concentrations or standard endothelial cells medium as control.

The microcapillary formation was evaluated after 7, 14, and 21 days cultivation via CLSM as described above. Cell culture medium was changed every 2–3 days. The experiment was performed threefold by using cells from three donors.

3 | RESULTS

3.1 | Fibroblast and endothelial cell characterization

Isolated primary fibroblasts and endothelial cells were characterized using immunohistology for the presence of CD31 staining on endothelial cells and α-SMA on fibroblasts. Cells were greater than 99% positive for the respective markers (data not shown).

3.2 | Influence of the seeding order on the microcapillary formation inside the tissue equivalent

In order to evaluate the influence of the cells used in this study on each other and to investigate the influence of the cultivation conditions on the formation of microcapillary-like structures, the two cell types were seeded in different orders using two different approaches. Seeding endothelial cells 24 hr before adding fibroblasts led to no distinct formation of capillary-like structures after 7 and 14 days. After 21 days capillary-like structures were observed, although endothelial cells appeared flat and not terminally differentiated (Figure 1a). In contrast, by seeding fibroblasts 24 hr prior to the addition of endothelial cells, microcapillaries were already seen after 7 days. After 14 and 21 days, vascular structures appeared well defined and endothelial cells showed a distinctly differentiated cell morphology (Figure 1b).

3.3 | Expression of VEGF, IL8 and bFGF by fibroblasts and endothelial cells cultured for 24 hr on the collagen membrane

The short-term levels of VEGF in monoculture of endothelial cells or fibroblasts were analyzed and compared to cell cocultures after 24 hr. Within the first 24 hr the highest VEGF levels were observed in fibroblast monocultures. The presence of VEGF in endothelial cell monocultures was lower than the levels observed in cocultures. The VEGF levels in coculture increased gradually from 1,180 pg/ml after 2 hr to its maximum of 1,800 pg/ml after 8 hr. After 4 hr the VEGF level in fibroblast monocultures was significantly increased compared to the cocultures (p = .043). After 12 and 24 hr the levels decreased again to 1,440 pg/ml. The VEGF expressed in the cocultures was distinctly lower than the sum of both monocultures at all-time points examined (Figure 1c). It appears that the interaction of fibroblasts and endothelial cells in cocultures leads to a suppressed growth factor expression.

Within the first 24 hr of cultivation, the IL-8 levels observed in the coculture exhibited consistently higher values compared to the levels observed in monocultures of endothelial cells or fibroblasts, respectively. A gradual and significant increase from 1,520 pg/ml after 2 hr to 3,030 pg/ml after 24 hr could be observed in cocultures (p = .008). Monocultures of fibroblast showed lower IL-8 levels than cocultures but a significant increase after 24 hr compared to 2 hr (p = .008). However, in endothelial cell monocultures low levels were observed. During the first 8 hr of cultivation, the sum of the IL-8 amounts from both monocultures was equal to the level observed in the coculture. After 6 (p = .037), 12 (p = .047) and 24 (p = .012) hr the IL-8 level in endothelial cell monocultures was significantly lower compared to the monocultures of fibroblasts. Interestingly, after 12 and 24 hr, the levels in cocultures decreased compared to the sum of the levels in the monocultures (Figure 1d). These findings indicate a suppressing effect of the cellular interaction within the coculture after 12 and 24 hr.

The levels of bFGF within the first 24 hr were highest in cocultures at every time point compared to both monocultures except for 4 and 24 hr. After 2 hr a significant higher bFGF level was observed in cocultures compared to the level found in endothelial cell monocultures (p = .018). 600 pg/ml were observed after 2 hr, this increased to a maximum of 1,335 pg/ml after 8 hr and decreased again to 495 pg/ml after 24 hr. Similar to the VEGF levels, lower levels of bFGF could be observed, when compared to the sum of the monoculture levels at all of time points examined (Figure 1e).

3.4 | Long-term levels of VEGF, IL8, and bFGF by fibroblasts and endothelial cells cultured on collagen membranes

After seeding fibroblasts 24 hr prior to endothelial cells on the collagen membrane, the long-term levels of cytokines up to 21 days were measured in monocultures of both cell types as well as in cocultures. During the 21 days of the incubation period, the VEGF levels in the cocultures increased from 675 pg/ml after 3 days up to 1,440 pg/ml after 19 days. At day 21, the VEGF concentration decreased again to 1200 pg/ml. In the case of fibroblast monocultures, a significant higher level of VEGF could be observed after 19 (p = .035) and

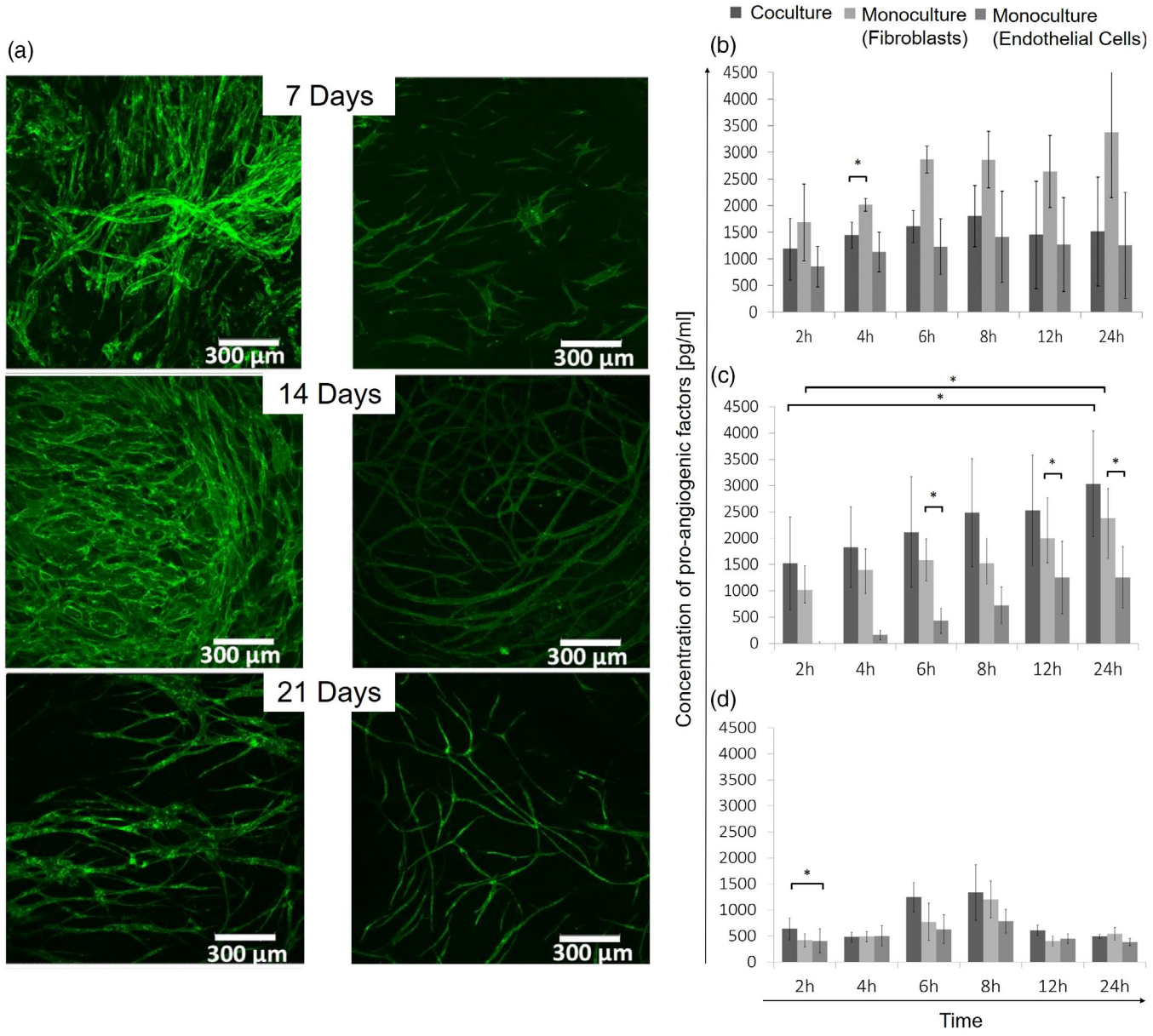

FIGURE 1 (a, b) Images of capillary structures in cultures of fibroblasts and endothelial cells. Fibroblasts and endothelial cells were seeded onto collagen membranes in different orders and cultivated for 7, 14, and 21 days after which cells with stained with endothelial cell-specific CD31 and examined in a confocal microscope. (a) Endothelial cells were seeded initially followed 24 hr later by the addition of fibroblasts, (b) fibroblast cells were seeded initially followed 24 hr later by the addition of endothelial cells. Seeding fibroblasts 24 hr prior to the addition of endothelial cells (a) led to a more distinct formation of capillary-like structures than seeding endothelial cells first followed by fibroblasts (b). Levels of angiogenic factors VEGF (c), IL-8 (d), and bFGF (e) in mono- and cocultures of fibroblasts and endothelial cells on collagen membranes up to 24 hr after cultivation begin. Supernatants were collected after 2, 4, 6, 8, 12, and 24 hr. The angiogenic factors were then quantified using ELISA. Presented are the mean values and standard errors of three independent ELISA measurements. Statistical analyses were done by comparing each value (1) to the values of the earliest time point (2 hr), (2) monocultures to cocultures, and (3) monocultures to each other using a Student's *t* test (* for *p*-value <.05 is defined as significant)

21 (p = .029) days, for endothelial cell monocultures after 21 (p = .019) days when compared to the level after 3 days.

In contrast to the first 24 hr, when the VEGF levels were highest in the fibroblast monocultures, the VEGF levels in cocultures were consistently higher compared to the monocultures up to 21 days. During this time period, distinctly lower levels could be observed in endothelial cells monocultures compared to monocultures of fibroblasts or cocultures of fibroblasts and endothelial cells. In the long-term results, the sum of the individual monoculture VEGF levels was as high as the levels in cocultures, indicating no distinct effect of the cellular interactions within the cocultures on the levels of VEGF (Figure 2a).

In the case of IL-8, the level was highest in the coculture after 3 and 7 days compared to the monocultures of fibroblasts and endothelial cells with 755 pg/ml and 970 pg/ml, respectively. At day

11, levels of IL-8 in fibroblast monocultures were equal to those in coculture and afterwards, the levels of IL-8 in fibroblast monocultures were consistently higher compared to those in the cocultures. After 15 days a significant higher IL-8 level could be observed in endothelial cell monocultures compared to the value measured at day 3 (p = .013). After 21 days, the IL-8 level in cocultures was 1,100 pg/ml compared to 1,400 pg/ml in fibroblast monocultures (Figure 2b). Beginning with day 11, the sum of the IL-8 levels in both monocultures was higher than that of the cocultures, demonstrating suppressing effects of the interacting cells within the cocultures.

During the 21 days incubation period, the bFGF level remained constant between 90 and 100 pg/ml in coculture and, except for day 11, at all investigation time points, was lower than in fibroblast monocultures, which had comparably increased levels between 130 and 180 pg/ml and a significant increased bFGF level after 21 days (p = .046). Between day 3 and day 11, cocultures had less bFGF than endothelial cell monocultures. As observed in the short-term incubations, decreased levels for bFGF were observed in cocultures compared to the sum of the monoculture levels at all-time points (Figure 2c). This indicates suppressing effects on bFGF expression most likely due to the cellular interactions within the coculture.

3.5 | Viability of endothelial cell culture after cytokine treatment

In order to investigate the impact of the pro-angiogenic factors VEGF, IL-8, and bFGF on total cell viability of endothelial cells, MTT assays

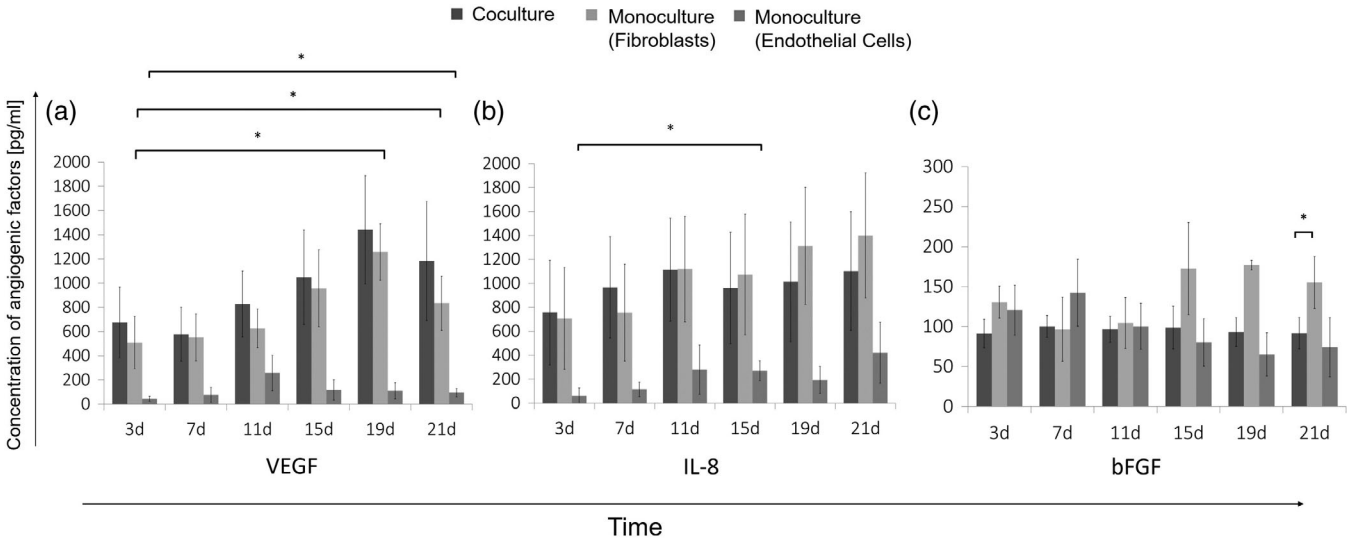

FIGURE 2 Levels of angiogenic factors VEGF (a), IL-8 (b), and bFGF (c) in mono- and cocultures of fibroblasts and endothelial cells on collagen membranes at various time points up to 21 days of cultivation. The cultivation medium was replaced with serum-free medium 24 hr prior to collecting the supernatants after which medium with serum was added again. Supernatants were collected at various time points and the presence of the angiogenic factors were quantified using ELISA. Quantification was performed using mean values and standard errors of five independent ELISA measurements. Statistical analyses were done by comparing each value (1) to the values of the earliest time point (3 days), (2) monocultures to cocultures and (3) monocultures to each other using a Student's t test (* for p-value <.05 is defined as significant)

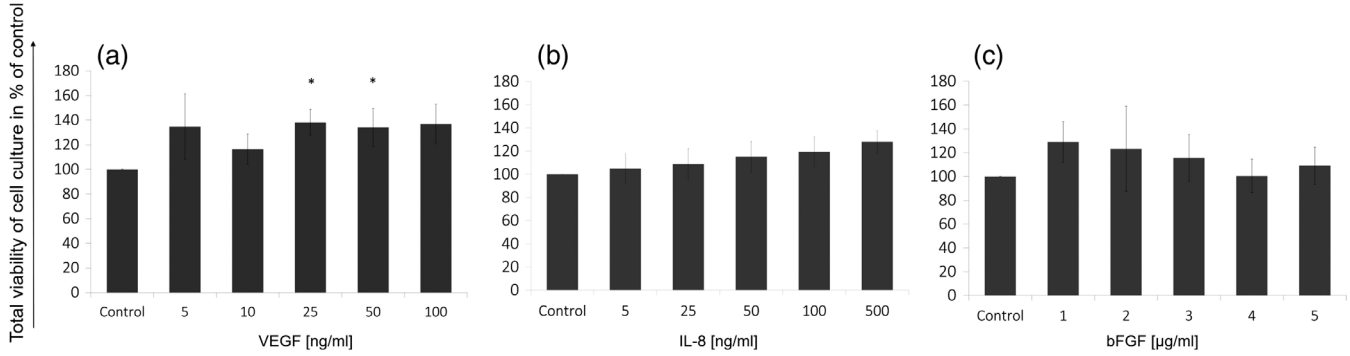

FIGURE 3 Total viability of endothelial cells determined by the MTT assay after exposure to various concentrations of VEGF (a), IL-8 (b), and bFGF (c) for 24 hr. Cells without treatment were used as control and were set to 100% viability. The effect of the angiogenic factors on the cell viability is shown using mean values and standard deviation from four independent experiments. Statistical analyses were done by comparing each value to the control using a Student's t test (* for p-value <.05 is defined as significant)

were performed after treating cells with the different concentrations of the used growth factors. Exposure of cells to VEGF resulted in higher MTT levels compared to untreated endothelial cells indicating a stimulation of the proliferation of the cells. Concentrations of 25 ng/ml (p = .008) and 50 ng/ml (p = .032) led to a similar significant increase up to 40% higher compared to control (Figure 3a). Treating endothelial cells with IL-8 led to an enhancement of total viability in a concentration dependent manner. At a concentration of 500 ng/ml the levels of MTT were 130% higher compared to the control (Figure 3b). Different concentrations of bFGF led to only a slight increase in MTT levels compared to the control. The strongest effect was observed at a concentration of 1 μg/ml with an increase of 30% compared to the control. Higher concentrations of bFGF appeared to have no effect on the cell culture viability compared to the control level (Figure 3c).

3.6 | Cell proliferation of endothelial cells after cytokine treatment

Endothelial cell proliferation increased in correlation to the concentration of all investigated pro-angiogenic factors. VEGF significantly increased proliferation at all concentrations tested. A concentration of 5 ng/ml VEGF increased the proliferation by 64% (p = .002) with a maximum of 84% enhancement with concentrations of 50 ng/ml VEGF (p = .044). 100 ng/ml led to an increase of 75% (p = .018) compared to control (Figure 4a).

Compared to the control, IL-8 led to increased proliferation at concentrations from 5 ng/ml to 500 ng/ml with an increase of 26% and 77%, respectively. A significant enhancement of proliferation was observed at concentrations of 50 ng/ml (p = .006) and 100 ng/ml (p = .006) with an increase of 44% and 58%, respectively (Figure 4b).

Treatment of endothelial cells with bFGF led to enhanced proliferation from 1 μg/ml to 4 μg/ml with a significant enhancement at concentrations of 3 μg/ml with 68% (p = .044) and 4 μg/ml with 58% (p = .008) compared to control. The enhancing effect on proliferation increased gradually with a peak at concentration levels of 3 μg/ml, followed by a decrease at higher concentrations levels with the lowest effect at 5 μg/ml with 4% enhancement compared to the control (Figure 4c).

3.7 | Cell migration of endothelial cells after cytokine treatment

In order to analyze the influence of angiogenic factors on endothelial cell migration, we performed a Boyden chamber migration assay by using the cytokines as chemotactical attractants. Migration by chemotactical attraction using VEGF increased gradually in with increasing concentrations. Treatment with VEGF at a concentration of 5 ng/ml we observed no significant increase in migration compared to the control. At concentration levels higher than 10 ng/ml (p = .007) VEGF we noticed a significant increase in migration up to sevenfold at concentration levels above 25 ng/ml (25 ng/ml: p = .004; 50 ng/ml: p = .018; 100 ng/ml: p < .001) (Figure 5a).

A similar result could be observed when IL-8 was applied to the cells. Endothelial cell migration was significantly enhanced at all concentrations with a total increase of 13-fold (p = .010) at 5 ng/ml up to a maximum of over 30-fold at 500 ng/ml (p = .0004).

Compared to VEGF and IL-8, bFGF showed the lowest impact on the chemotactical migration of endothelial cells. Nevertheless, cell migration was significantly increased by bFGF at all concentrations examined. With concentrations of 1 μg/ml (p = .024) to 3 μg/ml (p = .006) a 2.2 to threefold increase in cell migration was observed, respectively. At higher bFGF concentrations, 4 μg/ml (p = .002) and 5 μg/ml (p = .019), cell migration activity decreased again but exhibited elevated levels compared to the control (Figure 5c).

3.8 | Tube formation of endothelial cells after cytokine treatment

The angiogenic effect of VEGF, IL-8, and bFGF on the parameters tube density, total tube length and number of branches was analyzed using a tube formation assay. Cells treated with VEGF exhibited an

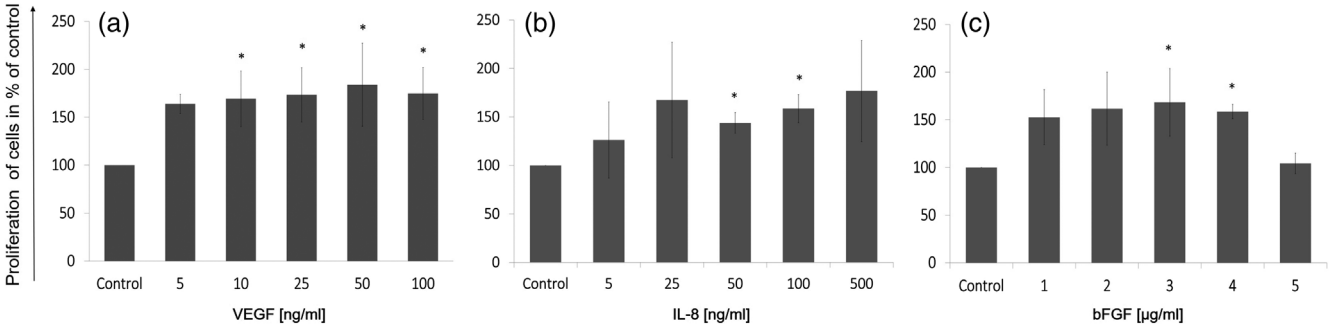

FIGURE 4 Proliferation of endothelial cells after treatment with different concentrations of angiogenic factors VEGF (a), IL-8 (b), and bFGF (c) for 24 hr, determined via BrdU incorporation. Untreated cells were used as control and were used as 100% proliferation. The effect of the angiogenic factors on cell proliferation was determined by using mean values and standard deviation from four independent experiments. Statistical analyses were carried out for each value compared to the control using a Student's t test (* for p-value <.05 is significant)

increase of all angiogenic parameters. The tube density was highest at concentrations of 5 ng/ml and 50 ng/ml VEGF and led to a 3.7 and 4.5-fold increase compared to the control, respectively. A homogeneous effect of increasing VEGF between 5 ng/ml and 50 ng/ml on total tube length could be observed with up to a 2.3-fold total increase compared to the control. At a concentration of 100 ng/ml VEGF, less total tube length was observed compared to the 5 and 50 ng/ml concentrations but still exhibited a 1.7-fold increase compared to the control. Addition of VEGF exhibited the greatest effect on the number of tube branches. At concentrations of 5 ng/ml and 50 ng/ml, a 5.4-fold to 5.5-fold increase in branches was observed, whereas at a concentration of 100 ng/ml, a lower increase compared to the control with a 3.8-fold increase was observed (Figure 6a).

A similar effect on all investigated angiogenic parameters was observed after exposure of the cells to concentrations between 1 ng/ml and 100 ng/ml IL-8. Addition of increasing IL-8 concentrations led to a gradual increase in tube density ranging from 3.4 to 3.8-fold, whereas the total length of tubes increased up to twofold compared to control. The number of branches also increased 4.9-fold to 5.5-fold from low to high concentrations. Interestingly, at the highest IL-8 concentration, 500 ng/ml, only very small changes were observed in the various angiogenic parameters (Figure 6b).

Addition of bFGF resulted in only very slight changes in the angiogenic parameters compared to VEGF and IL-8. After treating endothelial cells with bFGF, only a 2.3 to 3.3-fold increase in the tube density could be observed, whereas total tube length showed a maximum 1.8-fold increase compared to control. The greatest effect of bFGF was observed in the total number of branches. At concentration of 1 μg/ml, a 3.9-fold increase in the number of branches was observed. At concentrations from 2 μg/ml to 5 μg/ml, a 2.7-fold to 3.5-fold increase in number of branches was observed (Figure 6c).

3.9 | Formation of capillary-like structures in the collagen membrane after treatment with VEGF or IL-8

Since the addition of VEGF and IL-8 increased endothelial cell migration and angiogenic parameters in the tube formation assay on cell culture plastic, the effects of the addition of VEGF and IL-8 on the formation of capillary-like structures in coculture of endothelial cells and fibroblasts on a collagen membrane were examined. Treating cells with 100 ng/ml VEGF or 500 ng/ml IL-8 alone resulted in the formation of microcapillary-like structures in 14 days. With increased

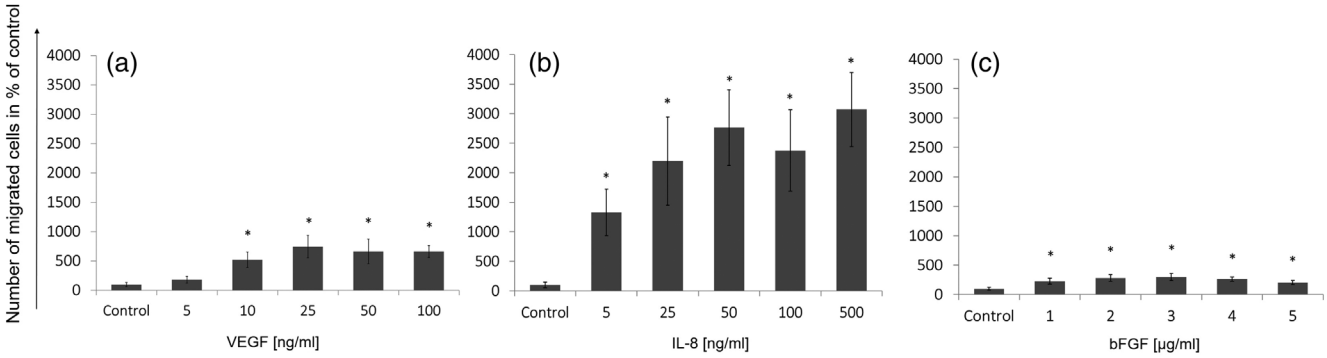

FIGURE 5 Endothelial cell migration toward VEGF (a, *n* = 5), IL-8 (b, *n* = 3) and bFGF (c, *n* = 3) at different concentrations over 16 hr, using a Boyden chamber migration assay. As control the migration of cells toward serum-free medium was analyzed. The effect of the angiogenic factors on the cell migration was presented by using mean values and standard deviation of independent experiments. Statistical analyses for each value were determined and compared to the control using a Student's *t* test (* for *p*-value <.05 is significant)

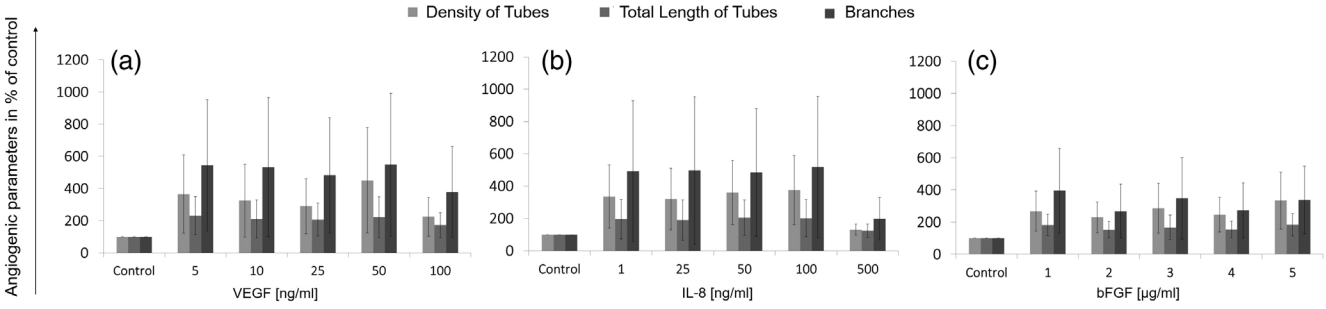

FIGURE 6 Effect of the angiogenic factors VEGF (a), IL-8 (b), and bFGF (c) in different concentrations on tube formation by endothelial cells. Density of tubes, total length of tubes and number of branches were examined. Tube formation of untreated endothelial cells was used as control. Quantification was performed using mean values and standard errors of at least four independent measurements

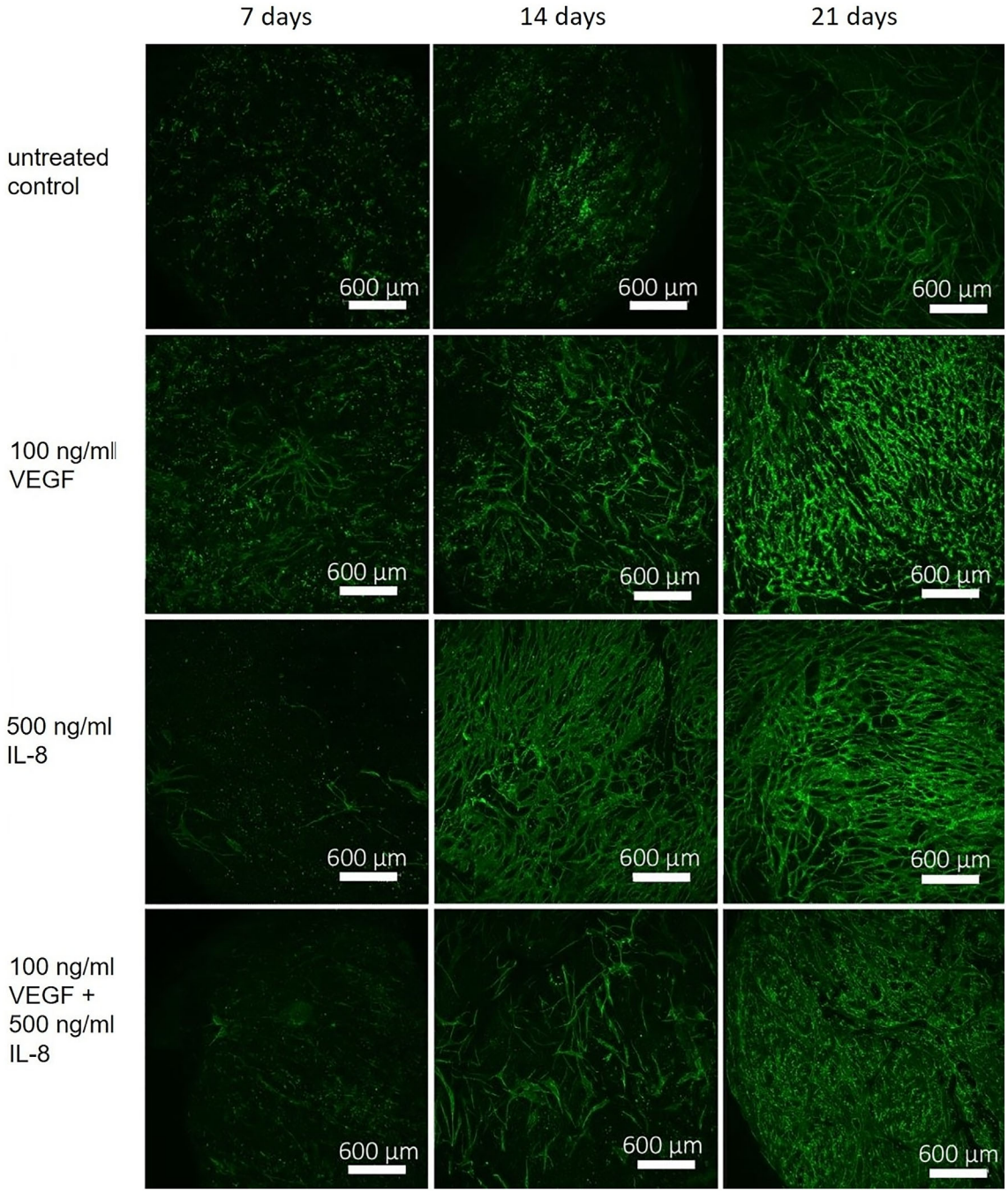

FIGURE 7 Images of cocultures of cells stained with CD31 to identify endothelial cells. Effects of VEGF and/or IL-8 on the formation of capillary-like structures within the buccal mucosa equivalent. Cocultures of endothelial cells and buccal mucosa fibroblast were cultivated on a collagen membrane for 7, 14, and 21 days. Samples were treated with 100 ng/ml VEGF, 500 ng/ml IL-8 or combination of 100 ng/ml VEGF + 500 ng/ml IL-8. Untreated samples were used as control

cultivation time (at 21 days) in the presence of VEGF, less microcapillary structures were observed and a broader and undifferentiated cell morphology was seen. Cells exposed to IL-8 continuously formed capillary-like structures up to day 21. Interestingly, when VEGF and IL-8 were added together, no microcapillary-like structures were observed after 7 or 21 days of cultivation. However, capillary-like structures were observed on day 14 in cultures with a combination of VEGF and IL-8 (Figure 7).

4 | DISCUSSION

The generation of prevascularized buccal mucosa equivalents is a promising approach in reconstructive surgery. In earlier work, our group demonstrated the successful generation and cultivation of a complex tissue equivalent in a triculture of buccal epithelial cells and fibroblasts combined with endothelial cells on a collagen membrane (Heller et al., 2016). However, in this model, the formation of

capillary-like structures was only observed in peripheral areas of the collagen membrane. We surmised that endothelial cell migration into deeper regions of the matrix was prevented due to the absence of intercellular interactions.

In this study, we demonstrated that cultivation conditions such as the seeding order can have stimulating or suppressing effects on cellular behaviors such as migration, proliferation or differentiation and that this has an effect on the formation of capillary-like structures. Seeding fibroblasts prior to endothelial cells led to an enhancement of the formation of capillary-like structures, suggesting that earlier seeding of fibroblasts might facilitate endothelial cell migration as well as capillary formation. Fibroblasts begin to migrate into three-dimensional collagen matrices after an initial lag-period of 8–16 hr (Grinnell, Rocha, Iucu, Rhee, & Jiang, 2006). After the initial migration period fibroblasts begin to secret soluble molecules such as VEGF, IL-8, and bFGF that act chemotactically on endothelial cells (Velazquez, Snyder, Liu, Fairman, & Herlyn, 2002). In the present study, an expression of VEGF, IL-8, and bFGF by fibroblasts was observed during the first 24 hr of cultivation, when seeded in monoculture on collagen membranes. The higher levels of cytokines in monocultures of fibroblasts for 24 hr prior to the addition of endothelial cells may be responsible for the higher rates of migration and angiogenic phenotypes observed by endothelial cells. Surprisingly, the expression patterns of VEGF, IL-8, and bFGF were distinctly decreased in the cocultures compared to the respective monocultures. Both VEGF and bFGF exhibited decreased levels at all-time points in the first 24 hr, whereas IL-8 expression began to decrease after 12 hr and continued after 24 hr in the cocultures. Due to the comparably low levels of the angiogenic factors in endothelial cell monocultures and the lower total levels in cocultures, it appears that fibroblasts play a crucial role in cellular communication. In cocultures, the endothelial cells may induce a suppression of the expression of the angiogenic factors produced by fibroblasts. Interestingly, after continued incubation times up to 21 days, the VEGF levels increased in the cocultures. However, IL-8 levels remained suppressed in cocultures compared to monocultures over the entire cultivation period up to 21 days. These results can be interpreted as expression changes during angiogenic processes in which IL-8 seems to become more important for differentiation over time.

After the initial 24 hr a decreased level of VEGF was observed in cocultures after which the levels increased and were similar to those observed in monocultures and remained so for the duration of the cultivation period of 21 days. The low levels may promote an initial cell migration and as time progresses and VEGF increases again, VEGF suppresses migration and induces differentiation. Since low levels of IL-8 levels were observed during the entire coculture period it is possible that low concentrations are necessary for the formation of capillary-like structures. Others have shown that IL-8 has angiogenic effects and that it induces proliferation, survival and migration of endothelial cells correlated to angiogenesis (Heidemann et al., 2003; Li et al., 2005). Endothelial cell proliferation and viability is a prerequisite for angiogenesis (Adair & Montani, 2010b; Gerhardt et al., 2003). In order to investigate the impact of angiogenic factors produced in the coculture model described in this study, the influence of VEGF, IL-8, and bFGF on the proliferation and viability of the different cell types was examined. Addition of these factors resulted in enhanced viability and proliferation of cells in a concentration-dependent manner. This indicated that VEGF and IL-8 and to a lesser extent bFGF have a significant effect on the formation of capillary-like structures in vitro in the tissue-engineered mucosa equivalent described in the present study.

Endothelial cell proliferation and migration are essential for tubulogenesis and are involved in many important processes during angiogenesis (Arima et al., 2011; Costa et al., 2016). A migration of endothelial cells into a scaffold would be absolutely essential in order for the formation of a three-dimensional network of capillary-like structures throughout a tissue equivalent to take place. A Boyden Chamber Migration Assay was used to examine the effects of the angiogenic factors VEGF, IL-8 and bFGF on monocultures of cells used in this study. VEGF and IL-8 demonstrated an enhanced chemotactical migration of endothelial cells whereas bFGF showed little effect on migration. VEGF and IL-8 have been shown to have a pro-migratory potential on cells. VEGF acts via the focal adhesion kinase (FAK) pathway, triggering migration by a number of different mechanisms (Cary, Han, Polte, Hanks, & Guan, 1998; Eliceiri et al., 2002; Romer, McLean, Turner, & Burridge, 1994; Zhao & Guan, 2011). Effects on migration by IL-8 have been shown to be due to activation of PLC and ERK, resulting in enhanced migration (Heidemann et al., 2003). In the present study, decreased levels of VEGF and IL-8 expression were observed in the initial phase of cocultivation. However, VEGF and IL-8 added to cultures stimulated migration of cells. This indicated that an interaction of fibroblasts and endothelial cells, where lower levels of VEGF and IL-8 were observed in the initial coculture period, prevented migration and induced differentiation and the formation of capillary-like structures by endothelial cells.

Similar to the migration assay, capillary formation was enhanced by the addition of VEGF, IL-8 and bFGF. Capillary formation was lower when VEGF and IL-8 exceeded certain thresholds. Capillary formation decreased at concentrations above 50 and 100 ng/ml for VEGF and IL-8, respectively. This was not observed for bFGF. An excess of VEGF and IL-8 in cocultures (>50 and 100 ng/ml, respectively), may result in angiogenic inhibition and thus alter the biological effects of how the angiogenic factors are utilized by the cells. In the case of bFGF, it can be assumed that the critical threshold, in which the described silencing effect occurred, was not reached at the concentrations utilized in the present studies.

The angiogenic factors examined in this study interact with different receptor molecules, activating intracellular signaling pathways that are directly or indirectly associated with angiogenesis. VEGFR2, is the primary VEGF-receptor and is activated by VEGF-A, stimulating many of the processes leading to angiogenesis (Koch et al., 2011). Capillary formation by endothelial cells is part of the dynamic processes in which spreading, prolongation and branching take place during angiogenesis (Arima et al., 2011). The activation of VEGFR2 leads to a dimerization and autophosphorylation of the receptor (Simons,

Gordon, & Claesson-Welsh, 2016), generating binding sites for the recruitment of adaptor molecules and kinases. Over these binding sites, intracellular signaling pathways are initiated such as the PI3K-AKT-pathway that regulates viability, proliferation and migration (Graupera & Potente, 2013; Karali et al., 2014). During VEGFR2 activation, PI3K is activated by kinases of the sarcoma family (SFKs) in combination with receptor tyrosine kinases (RTK) AXL or VE-cadherin (Carmeliet et al., 1999; Ruan & Kazlauskas, 2012). These processes lead to a complex phosphorylation cascade in which the second messenger molecule PIP3 (Phosphatidyl-inositol-[3,4,5]-triphosphate) is involved and acts as activator of other kinases such as PDK (Phosphoinositide-dependent kinase)-1, MAP-kinase (mitogen-activated protein kinase), ERK 5 (Extracellular signal Regulated Kinase), or mTORC-2 (mechanistic target of rapamycin complex) (Graupera & Potente, 2013; Koch et al., 2011; Roberts, Holmes, Muller, Cross, & Cross, 2010). PI3K-independent processes are based on the autophosphorylation activity of VEGFR2 that activates PLC (phospholipase C) and PKC (protein kinase C) leading over activation of ERK 1/2 to an enhancement of proliferation (Wong & Jin, 2005). The signal molecule AKT acts as mediator in the phosphorylation cascade and promotes endothelial cell survival and proliferation by inhibition of pro-apoptotic molecules and stimulation of anti-apoptotic molecule expression (Gerber et al., 1998; Koch et al., 2011).

IL-8 is part of the CXC-chemokine family acting on the G-protein associated receptors CXCR1 and CXCR2 (Heidemann et al., 2003; Waugh & Wilson, 2008). Similar to the VEGF signaling pathway, IL-8 also acts by activating PI3K-AKT, which regulates pro- and anti-apoptotic genes, leading to enhanced viability and proliferation (Augustin et al., 2009; Koch et al., 2011; Li et al., 2005; Luppi, Longo, De Boer, Rabe, & Hiemstra, 2007; Waugh & Wilson, 2008). We found that the combination of VEGF and IL-8 resulted in a lower capillary-like structure formation than when added individually. This effect may be due to both cytokines partly activating the same signaling pathways, so that an additive effect can be excluded. When a strong activation of a signaling pathway by different triggers occurs, regulatory effects may be regulated at a higher rate (Brandman & Meyer, 2008). This may explain the lower amounts of capillary formation on the collagen membrane used in the present study observed when both VEGF and IL-8 were added together.

In contrast to VEGF and IL-8, the effects of bFGF on capillary formation were nearly insignificant on the various parameters investigated. Although bFGF has been shown to be a primary factor in angiogenesis (Granato et al., 2004), due to the absence of major effects in the present tissue equivalent model, studies were focused on VEGF and IL-8 and their effects on capillary formation. The factor bFGF acts by activating FGFR1, but bFGF also interacts with the PDGFR (platelet-derived-growth factor receptor) (Guo et al., 2012). Similar to VEGF and IL-8, bFGF has been shown to activate PI3K-AKT and ERK and these factors have been shown to activate cellular parameters such as viability, proliferation, and migration. In the present study, it is likely that activation of the VEGF and IL-8 signaling pathways interacted with the signal transduction of bFGF, leading to lower bFGF levels.

Cellular communication can be influenced by many factors. In addition to direct cellular interactions there are many external factors, such as collagen-cell interactions, that have to be considered as crucial influencing factors for cellular behavior. It is well known that collagen can have an impact on morphological characteristics (Grinnell, 2003; Iordan, Duperray, Gérard, Grichine, & Verdier, 2010; Jiang & Grinnell, 2005) and that angiogenic properties of fibroblasts and endothelial cells and their cellular expression of VEGF, IL-8 and bFGF can be enhanced by collagen (Kahn et al., 2000; Newman, Nakatsu, Chou, Gershon, & Hughes, 2011; Pinney, Liu, Sheeman, & Mansbridge, 2000). Collagen-cell-interactions are mainly mediated by integrin receptor interactions (Barczyk, Carracedo, & Gullberg, 2010; White, Puranen, Johnson, & Heino, 2004). Integrins initiate signal transduction pathways such as PI3K-AKT-, MAP-Kinase-, and ERK-signaling (Chen, Kinch, Lin, Burridge, & Juliano, 1994; Legate, Wickström, & Fässler, 2009; Li, Li, Sun, Lin, & Zhou, 2016) and control the expression of cytokines and growth factors such as VEGF, IL-8, and bFGF (Clarke et al., 2012; Dai et al., 2009; Li et al., 2016; Yang & Rizzo, 2013). It was found that VEGF levels in brain endothelial cells can be induced over the ERK-signaling pathway by activation of $\alpha 5\beta 1$-integrins, inhibition of αv-integrin results in a reduced VEGF level (Clarke et al., 2012; Franco, Roswall, Cortez, Hanahan, & Pietras, 2011; Montenegro et al., 2012). Based on this, the downstream signaling pathway NF-κB and its participation to the expression of VEGF and IL-8 could be demonstrated (Franco et al., 2011; Hoffmann, Dittrich-Breiholz, Holtmann, & Kracht, 2002; Mukaida, Okamoto, Ishikawa, & Matsushima, 1994; Scatena et al., 1998). Interestingly, an opposite effect was observed in fibroblasts. Inhibition of $\alpha v\beta 3$-integrins resulted in enhanced VEGF expression (Montenegro et al., 2012). However, since the cultivation of fibroblasts on collagen matrices activated the NF-κB-pathway (Xu, Zutter, Santoro, & Clark, 1998), the stimulation of other members of the integrin family might be a possible explanation for these opposing effects. In addition to the influence on growth factor expression patterns, collagen stimulates cells to secret matrix metalloproteases (MMP) which are involved in remodeling processes. It was shown that these remodeling processes are necessary for invasion of endothelial cells into surrounding tissues and resulting in vascularization (Seandel, Noack-Kunnmann, Zhu, Aimes, & Quigley, 2001). With respect to the capillary formation in the mucosa equivalent, remodeling processes may be a crucial aspect for restructuring the collagen membrane by the fibroblasts.

5 | CONCLUSION

In this study, we showed that in a complex 3D-coculture of fibroblasts and microvascular endothelial cells growing on a collagen membrane, the formation of microvascular structures is dependent on the levels of the Vascular Endothelial Growth Factor (VEGF), Interleukin-8 (IL-8) and to a lesser extent of basic fibroblast growth factor (bFGF) produced by fibroblasts. Addition of VEGF and IL-8 to the coculture model increases the formation of capillary-like structures and resulted

in a complete network of microcapillary formation throughout the tissue-engineered mucosa equivalents. Applying this methodology to the generation of tissue-engineered constructs in vitro should lead to a better soft tissue integration and survival after implantation.

REFERENCES

Adair, T. H., & Montani, J. P. (2010b). Angiogenesis. In colloquium series on integrated systems physiology: From molecule to function. *Morgan & Claypool Life Sciences.*, *2*, 1–84.

Adair, T. H., & Montani, J.-P. (2010a). Angiogenesis. *Colloquium Series on Integrated Systems Physiology: From Molecule to Function*, *2*, 1–84.

Almela, T., Brook, I. M., & Moharamzadeh, K. (2016). Development of three-dimensional tissue engineered bone-oral mucosal composite models. *Journal of Materials Science. Materials in Medicine*, *27*, 65.

Arima, S., Nishiyama, K., Ko, T., Arima, Y., Hakozaki, Y., Sugihara, K., … Kurihara, H. (2011). Angiogenic morphogenesis driven by dynamic and heterogeneous collective endothelial cell movement. *Development*, *138*, 4763–4776.

Augustin, H. G., Koh, G. Y., Thurston, G., & Alitalo, K. (2009). Control of vascular morphogenesis and homeostasis through the angiopoietin-tie system. *Nature Reviews. Molecular Cell Biology*, *10*, 165–177.

Barczyk, M., Carracedo, S., & Gullberg, D. (2010). Integrins. *Cell and Tissue Research*, *39*, 269–280.

Baskin, L. S., & Ebbers, M. B. (2006). Hypospadias: Anatomy, etiology, and technique. *Journal of Pediatric Surgery*, *41*, 463–472.

Black, A. F., Berthod, F., L'heureux, N., Germain, L., & Auger, F. A. (1998). In vitro reconstruction of a human capillary-like network in a tissue-engineered skin equivalent. *The FASEB Journal*, *12*, 1331–1340.

Boyce, S. (1996). Cultured skin substitutes: A review. *Tissue Engineering*, *2*, 255–266.

Brandman, O., & Meyer, T. (2008). Feedback loops shape cellular signals in space and time. *Science*, *322*, 390–395.

Breuksch, I., Prosinger, F., Baehr, F., Engelhardt, F. P., Bauer, H. K., Thüroff, J. W., … Brenner, W. (2017). Integrin α5 triggers the metastatic potential in renal cell carcinoma. *Oncotarget*, *8*, 107530–107542.

Callens, N., De Cuypere, G., De Sutter, P., Monstrey, S., Weyers, S., Hoebeke, P., & Cools, M. (2014). An update on surgical and non-surgical treatments for vaginal hypoplasia. *Human Reproduction Update*, *20*, 775–801.

Carmeliet, P., & Jain, R. K. (2011). Molecular mechanisms and clinical applications of angiogenesis. *Nature*, *473*, 298–307.

Carmeliet, P., Lampugnani, M. G., Moons, L., Breviario, F., Compernolle, V., Bono, F., … Dejana, E. (1999). Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. *Cell*, *98*, 147–157.

Cary, L. A., Han, D. C., Polte, T. R., Hanks, S. K., & Guan, J. L. (1998). Identification of p130Cas as a mediator of focal adhesion kinase-promoted cell migration. *The Journal of Cell Biology*, *140*, 211–221.

Chen, Q., Kinch, M. S., Lin, T. H., Burridge, K., & Juliano, R. L. (1994). Integrin-mediated cell adhesion activates mitogen-activated protein kinases. *The Journal of Biological Chemistry*, *269*, 26602–26605.

Chiapasco, M., Colletti, G., Romeo, E., Zaniboni, M., & Brusati, R. (2008). Long-term results of mandibular reconstruction with autogenous bone grafts and oral implants after tumor resection. *Clinical Oral Implants Research*, *19*, 1074–1080.

Chung, A. S., & Ferrara, N. (2011). Developmental and pathological angiogenesis. *Annual Review of Cell and Developmental Biology*, *27*, 563–584.

Clarke, D. N., Al Ahmad, A., Lee, B., Parham, C., Auckland, L., Fertala, A., … Bix, G. J. (2012). Perlecan domain V induces VEGf secretion in brain endothelial cells through integrin α5β1 and ERK-dependent signaling pathways. *PLoS One*, *7*, e45257.

Costa, G., Harrington, K. I., Lovegrove, H. E., Page, D. J., Chakravartula, S., Bentley, K., & Herbert, S. P. (2016). Asymmetric division coordinates collective cell migration in angiogenesis. *Nature Cell Biology*, *18*, 1292–1301.

Dai, J., Peng, L., Fan, K., Wang, H., Wei, R., Ji, G., … Guo, Y. (2009). Osteopontin induces angiogenesis through activation of PI3K/AKT and ERK1/2 in endothelial cells. *Oncogene*, *28*, 3412–3422.

De Bree, R., Rinaldo, A., Genden, E. M., Suarez, C., Rodrigo, J. P., Fagan, J. J., … Leemans, C. R. (2008). Modern reconstruction techniques for oral and pharyngeal defects after tumor resection. *European Archives of Oto-rhino-laryngology: Official Journal of the European Federation of Oto-Rhino-Laryngological Societies (EUFOS): affiliated with the German Society for Oto-Rhino-Laryngology—Head and Neck Surgery*, *265*, 1–9.

de Trey, L. A., & Morrison, G. A. (2013). Buccal mucosa graft for laryngotracheal reconstruction in severe laryngeal stenosis. *International Journal of Pediatric Otorhinolaryngology*, *77*, 1643–1646.

Dhillon, M., Mohan, R. S., Raju, S. M., & Lakhanpal, M. (2013). Ackerman's tumour of buccal mucosa in a leprosy patient. *Leprosy Review*, *84*, 151–157.

Domigan, C. K., Warren, C. M., Antanesian, V., Happel, K., Ziyad, S., Lee, S., … Iruela-Arispe, M. L. (2015). Autocrine VEGF maintains endothelial survival through regulation of metabolism and autophagy. *Journal of Cell Science*, *128*, 2236–2248.

Eliceiri, B. P., Puente, X. S., Hood, J. D., Stupack, D. G., Schlaepfer, D. D., Huang, X. Z., … Cheresh, D. A. (2002). Src-mediated coupling of focal adhesion kinase to integrin alpha(v)beta5 in vascular endothelial growth factor signaling. *The Journal of Cell Biology*, *157*, 149–160.

Fillmore, W. J., & Rieck, K. L. (2014). Buccal mucosa grafting for male urethroplasty: Long-term follow up of patients' experience with a team approach and primary closure. *Journal of Oral and Maxillofacial Surgery, Medicine, and Pathology*, *26*, 437–442.

Franco, M., Roswall, P., Cortez, E., Hanahan, D., & Pietras, K. (2011). Pericytes promote endothelial cell survival through induction of autocrine VEGF-A signaling and Bcl-w expression. *Blood*, *118*, 2906–2917.

Gerber, H. P., McMurtrey, A., Kowalski, J., Yan, M., Keyt, B. A., Dixit, V., & Ferrara, N. (1998). Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3′-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. *The Journal of Biological Chemistry*, *273*, 30336–30343.

Gerhardt, H., Golding, M., Fruttiger, M., Ruhrberg, C., Lundkvist, A., Abramsson, A., … Betsholtz, C. (2003). VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *The Journal of Cell Biology*, *161*, 1163–1177.

Granato, A. M., Nanni, O., Falcini, F., Folli, S., Mosconi, G., De Paola, F., … Volpi, A. (2004). Basic fibroblast growth factor and vascular endothelial growth factor serum levels in breast cancer patients and healthy women: Useful as diagnostic tools? *Breast Cancer Research: BCR*, *6*, R38–R45.

Graupera, M., & Potente, M. (2013). Regulation of angiogenesis by PI3K signaling networks. *Experimental Cell Research*, *319*, 1348–1355.

Grinnell, F. (2003). Fibroblast biology in three-dimensional collagen matrices. *Trends in Cell Biology*, *13*, 264–269.

Grinnell, F., Rocha, L. B., Iucu, C., Rhee, S., & Jiang, H. (2006). Nested collagen matrices: A new model to study migration of human fibroblast populations in three dimensions. *Experimental Cell Research*, *312*, 86–94.

Guo, S., Yu, L., Cheng, Y., Li, C., Zhang, J., An, J., … Li, Z. (2012). PDGFRβ triggered by bFGF promotes the proliferation and migration of endothelial progenitor cells via p-ERK signalling. *Cell Biology International*, *36*, 945–950.

Haber, T., Jöckel, E., Roos, F. C., Junker, K., Prawitt, D., Hampel, C., … Network, G. R. C. T. (2015). Bone meta stasis in renal cell carcinoma is preprogrammed in the primary tumor and caused by AKT and integrin α 5 signaling. *The Journal of Urology*, *194*, 539–546.

Hampson, L. A., McAninch, J. W., & Breyer, B. N. (2014). Male urethral strictures and their management. *Nature Reviews. Urology*, *11*, 43–50.

Heidemann, J., Ogawa, H., Dwinell, M. B., Rafiee, P., Maaser, C., Gockel, H. R., … Binion, D. G. (2003). Angiogenic effects of interleukin 8 (CXCL8) in human intestinal microvascular endothelial cells are mediated by CXCR2. *The Journal of Biological Chemistry*, *278*, 8508–8515.

Heller, M., Frerick-Ochs, E., Stein, R., Thüroff, J. W., & Brenner, W. (2015). Prävaskularisierung eines In-vitro-Mundschleimhautäquivalents zur Regeneration der Urethra. *Der Urologe*, *54*, 1105–1107.

Heller, M., Frerick-Ochs, E. V., Bauer, H.-K., Schiegnitz, E., Flesch, D., Brieger, J., … Brenner, W. (2016). Tissue engineered pre-vascularized buccal mucosa equivalents utilizing a primary triculture of epithelial cells, endothelial cells and fibroblasts. *Biomaterials*, *77*, 207–215.

Hoffmann, E., Dittrich-Breiholz, O., Holtmann, H., & Kracht, M. (2002). Multiple control of interleukin-8 gene expression. *Journal of Leukocyte Biology*, *72*(5), 847–855.

Iordan, A., Duperray, A., Gérard, A., Grichine, A., & Verdier, C. (2010). Breakdown of cell-collagen networks through collagen remodeling. *Biorheology*, *47*, 277–295.

Izumi, K., Feinberg, S. E., Iida, A., & Yoshizawa, M. (2003). Intraoral grafting of an ex vivo produced oral mucosa equivalent: A preliminary report. *International Journal of Oral and Maxillofacial Surgery*, *32*, 188–197.

Jiang, H., & Grinnell, F. (2005). Cell-matrix entanglement and mechanical anchorage of fibroblasts in threedimensional collagen matrices. *Molecular Biology of the Cell*, *16*, 5070–5076.

Kahn, J., Mehraban, F., Ingle, G., Xin, X., Bryant, J. E., Vehar, G., … Geritsen, M. E. (2000). Gene expression profiling in an in vitro model of angiogenesis. *The American Journal of Pathology*, *56*, 1887–1900.

Karali, E., Bellou, S., Stellas, D., Klinakis, A., Murphy, C., & Fotsis, T. (2014). VEGF signals through ATF6 and PERK to promote endothelial cell survival and angiogenesis in the absence of ER stress. *Molecular Cell*, *54*, 559–572.

Karamysheva, A. F. (2008). Mechanisms of angiogenesis. *Biochemistry Moscow*, *73*, 751–762.

Khan, S., Zaheer, S., & Gupta, N. D. (2013). Oral psoriasis: A diagnostic dilemma. *European Journal of General Dentistry*, *2*, 67.

Kimberley, N., Hutson, J. M., Southwell, B. R., & Grover, S. R. (2012). Vaginal agenesis, the hymen, and associated anomalies. *Journal of Pediatric and Adolescent Gynecology*, *25*, 54–58.

Koch, A. E., Polverini, P. J., Kunkel, S. L., Harlow, L. A., DiPietro, L. A., Elner, V. M., … Strieter, R. M. (1992). Interleukin-8 as a macrophage-derived mediator of angiogenesis. *Science*, *258*, 1798–1801.

Koch, S., Tugues, S., Li, X., Gualandi, L., & Claesson-Welsh, L. (2011). Signal transduction by vascular endothelial growth factor receptors. *The Biochemical Journal*, *437*, 169–183.

Lauer, G., & Schimming, R. (2001). Tissue-engineered mucosa graft for reconstruction of the intraoral lining after freeing of the tongue: A clinical and immunohistologic study. *Journal of Oral and Maxillofacial Surgery*, *59*, 169–177.

Lee, S., Chen, T. T., Barber, C. L., Jordan, M. C., Murdock, J., Desai, S., … Iruela-Arispe, M. L. (2007). Autocrine VEGF signaling is required for vascular homeostasis. *Cell*, *130*, 691–703.

Legate, K. R., Wickström, S. A., & Fässler, R. (2009). Genetic and cell biological analysis of integrin outside-in signaling. *Genes & Development*, *23*, 397–418.

Li, A., Dubey, S., Varney, M. L., Dave, B. J., & Singh, R. K. (2003). IL-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases production and regulated angiogenesis. *The Journal of Immunology*, *170*, 3369–3376.

Li, A., Varney, M. L., Valasek, J., Godfrey, M., Dave, B. J., & Singh, R. K. (2005). Autocrine role of interleukin-8 in induction of endothelial cell proliferation, survival, migration and MMP-2 production and angiogenesis. *Angiogenesis*, *8*, 63–71.

Li, G., Li, Y.-Y., Sun, J.-E., Lin, W. H., & Zhou, R. X. (2016). ILK–PI3K/AKT pathway participates in cutaneous wound contraction by regulating fibroblast migration and differentiation to myofibroblast. *Laboratory Investigation*, *96*, 741–751.

Luppi, F., Longo, A. M., De Boer, W. I., Rabe, K. F., & Hiemstra, P. S. (2007). Interleukin-8 stimulates cell proliferation in non-small cell lung cancer through epidermal growth factor receptor transactivation. *Lung Cancer*, *56*, 25–33.

Markiewicz, M. R., Margarone, J. E., Barbagli, G., & Scannapieco, F. A. (2007). Oral mucosa harvest: An overview of anatomic and biologic considerations. *EAU-EBU Update Series*, *5*, 179–187.

Moharamzadeh, K., Brook, I. M., van Noort, R., Scutt, A. M., & Thornhill, M. H. (2007). Tissue-engineered oral mucosa: A review of the scientific literature. *Journal of Dental Research*, *86*, 115–124.

Montenegro, C. F., Salla-Pontes, C. L., Ribeiro, J. U., Machado, A. Z., Ramos, R. F., Figueiredo, C. C., … Selistre-de-Araujo, H. S. (2012). Blocking αvβ3 integrin by a recombinant RGD disintegrin impairs VEGF signaling in endothelial cells. *Biochimie*, *94*, 1812–1820.

Mossey, P. A., Shaw, W. C., Munger, R. G., Murray, J. C., Murthy, J., & Little, J. (2011). Global oral health inequalities: Challenges in the prevention and management of orofacial clefts and potential solutions. *Advances in Dental Research*, *23*, 247–258.

Mukaida, N., Okamoto, S. I., Ishikawa, Y., & Matsushima, K. (1994). Molecular mechanism of interleukin-8 gene expression. *Journal of Leukocyte Biology*, *56*, 554–558.

Mungadi, I. A., & Ugboko, V. I. (2009). Oral mucosa grafts for urethral reconstruction. *Annals of African Medicine*, *8*, 203–209.

Newman, A. C., Nakatsu, M. N., Chou, W., Gershon, P. D., & Hughes, C. C. (2011). The requirement for fibroblasts in angiogenesis: Fibroblast-derived matrix proteins are essential for endothelial cell lumen formation. *Molecular Biology of the Cell*, *22*, 3791–3800.

Peters, K., Schmidt, H., Unger, R. E., Otto, M., Kamp, G., & Kirkpatrick, C. J. (2002). Software-supported image quantification of angiogenesis in an in vitro culture system: Application to studies of biocompatibility. *Biomaterials*, *23*, 3413–3419.

Pinney, E., Liu, K., Sheeman, B., & Mansbridge, J. (2000). Human three-dimensional fibroblast cultures express angiogenic activity. *Journal of Cellular Physiology*, *183*, 74–82.

Potente, M., Gerhardt, H., & Carmeliet, P. (2011). Basic and therapeutic aspects of angiogenesis. *Cell*, *146*, 873–887.

Raya-Rivera, A., Esquiliano, D. R., Yoo, J. J., Lopez-Bayghen, E., Soker, S., & Atala, A. (2011). Tissue-engineered autologous urethras for patients who need reconstruction: An observational study. *Lancet (London, England)*, *377*, 1175–1182.

Roberts, O. L., Holmes, K., Muller, J., Cross, D. A., & Cross, M. J. (2010). ERK5 is required for VEGF-mediated survival and tubular morphogenesis of primary human microvascular endothelial cells. *Journal of Cell Science*, *123*, 3413.

Romer, L. H., McLean, N., Turner, C. E., & Burridge, K. (1994). Tyrosine kinase activity, cytoskeletal organization, and motility in human vascular endothelial cells. *Molecular Biology of the Cell*, *5*, 349–361.

Ruan, G.-X., & Kazlauskas, A. (2012). Axl is essential for VEGF-A-dependent activation of PI3K/Akt. *The EMBO Journal*, *31*, 1692–1703.

Sadri-Ardekani, H., & Atala, A. (2015). Regenerative medicine for the treatment of reproductive system disorders: Current and potential options. *Advanced Drug Delivery Reviews*, *82–83*, 145–152.

Scatena, M., Almeida, M., Chaisson, M. L., Fausto, N., Nicosia, R. F., & Giachelli, C. M. (1998). NF-κB mediates αvβ3 integrin-induced endothelial cell survival. *The Journal of Cell Biology*, *141*, 1083–1093.

Seandel, M., Noack-Kunnmann, K., Zhu, D., Aimes, R. T., & Quigley, J. P. (2001). Growth factor-induced angiogenesis in vivo requires specific cleavage of fibrillar type I collagen. *Blood*, *97*(8), 2323–2332.

Simons, M., Gordon, E., & Claesson-Welsh, L. (2016). Mechanisms and regulation of endothelial VEGF receptor signalling. *Nature Reviews. Molecular Cell Biology*, *17*, 611–625.

Squier, C. A., & Brogden, K. A. (2011). *Human oral mucosa: Development, structure, and function*. Chichester, West Sussex, UK: Wiley-Blackwell.

Tompkins, J. J., Vaughn, C. A., Shaikh, F. A., Stocks, R. M., & Thompson, J. W. (2015). Palatopharyngoplasty with bilateral buccal mucosal graft repair to alleviate oropharyngeal stenosis. *International Journal of Pediatric Otorhinolaryngology*, *79*, 1579–1581.

Tremblay, P.-L., Hudon, V., Berthod, F., Germain, L., & Auger, F. A. (2005). Inosculation of tissue-engineered capillaries with the host's

vasculature in a reconstructed skin transplanted on mice. *American Journal of Transplantation*, *5*, 1002–1010.

Unger, R. E., Dohle, E., & Kirkpatrick, C. J. (2015). Improving vascularization of engineered bone through the generation of pro-angiogenic effects in co-culture systems. *Advanced Drug Delivery Reviews*, *94*, 116–125.

Velazquez, O. C., Snyder, R., Liu, Z.-J., Fairman, R. M., & Herlyn, M. (2002). Fibroblast-dependent differentiation of human microvascular endothelial cells into capillary-like 3-dimensional networks. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, *16*, 1316–1318.

Waugh, D. J. J., & Wilson, C. (2008). The interleukin-8 pathway in cancer. *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research*, *14*, 6735–6741.

White, D. J., Puranen, S., Johnson, M. S., & Heino, J. (2004). The collagen receptor subfamily of the integrins. *The International Journal of Biochemistry & Cell Biology*, *36*, 1405–1410.

Wong, C., & Jin, Z.-G. (2005). Protein kinase C-dependent protein kinase D activation modulates ERK signal pathway and endothelial cell proliferation by vascular endothelial growth factor. *The Journal of Biological Chemistry*, *280*, 33262–33269.

Xu, J., Zutter, M. M., Santoro, S. A., & Clark, R. A. (1998). A three-dimensional collagen lattice activates NF-κB in human fibroblasts: Role in integrin α 2 gene expression and tissue remodeling. *The Journal of Cell Biology*, *140*, 709–719.

Yang, B., & Rizzo, V. (2013). Shear stress activates eNOS at the endothelial apical surface through β1 containing integrins and caveolae. *Cellular and Molecular Bioengineering*, *6*, 346–354.

Zhao, X., & Guan, J.-L. (2011). Focal adhesion kinase and its signaling pathways in cell migration and angiogenesis. *Advanced Drug Delivery Reviews*, *63*, 610–615.

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