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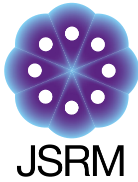

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Original Article

The construction of a microenvironment with the vascular network by co-culturing fibroblasts and endothelial cells

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ABSTRACT

Introduction: Extracellular matrix (ECM) synthesis and deposition in fibroblasts, and vascularization via endothelial cells are essential for successful tissue regeneration. Fibroblasts can produce both ECM, physical support for maintaining homeostasis, and bioactive molecules, such as growth factors and cytokines. Endothelial cells can secrete growth factors and form vascular networks that enable the supply of nutrients and oxygen and remove metabolic products.
Methods: In this study, we focused on combining Human Periodontal Ligament Fibroblasts (HPLF) and Human Umbilical Vein Endothelial Cells (HUVEC) for tissue regeneration in clinical applications.
Results: The fibroblastic and angiogenic phenotypes were promoted in co-culture with HPLF and HUVEC at a ratio of 1:1 compared to HPLF or HUVEC mono-culture. The gene expression of ECM components and angiogenesis-related factors was also enhanced by HPLF/HUVEC co-culture. Despite an apparent increase in the expression of angiogenic factors, the levels of secreted growth factors decreased under co-culture conditions. These data suggest that ECM constructed by HPLF and HUVEC would act as a storage site for growth factors, which can later be released. Our results showed that cell-to-cell interactions between HPLF and HUVEC enhanced collagen synthesis and endothelial network formation, leading to the creation of highly vascularized constructs for periodontal tissue regeneration.
Conclusion: Successful periodontal tissue regeneration requires microenvironmental reconstruction and vascularization, which can be achieved using a co-culture system. In the present study, we found that fibroblastic and angiogenic phenotypes were enhanced by the co-culture of HPLF and HUVEC. The optimal culture conditions (1:1) could potentially accelerate tissue engineering, including ECM synthesis and EC tube formation, and these approaches can improve therapeutic efficacy after transplantation.
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1. Introduction

The periodontal tissue is a functional unit that integrates several tissues, including the cementum, periodontal ligament (PDL), and alveolar bone. Chronic periodontal diseases destroy the periodontal tissue, leading to tooth loss [1–3]. Periodontitis is initiated by bacterial infection and progresses with the infiltration of neutrophils and macrophages, activation of osteoclasts, and bone resorption [4]. Damaged tissues have a low capacity for self-renewal, which makes it difficult to reconstruct structural and functional tissues. Flap surgery and guided tissue regeneration (GTR) [5] are widely used treatments, although they focus on bone formation. Current treatment methods have difficulty completely repairing damaged periodontal tissues [6,7].

The PDL connects the cementum to the alveolar bone by Sharpey's fibers, bundles of type I collagen, 0.15 is 0.38 mm thick. Their main functions are to transmit and ab-sorb mechanical stress and supply nutrients to the surrounding tissues [8]. PDL is a connective tissue, similar to tendons and other ligaments [9]. In regenerated PDL, collagen fibers should ideally be highly organized, inserted vertically into the regenerated cementum and newly-formed bone, and tightly adhered [10]. Fibroblasts account for approximately 50–60 % of all PDL cells [11]. PDL includes macrophages, lymphocytes, and endothelial cells (ECs) that form the inner walls of blood vessels [12].

Tissue engineering attempts to introduce a vascular network to improve cell viability after transplantation. Growth of newly-formed blood vessels in transplanted tissue structures is a highly dynamic process. The first step is the activation of host microvasculature at the

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graft site by Vascular Endothelial Growth Factor (VEGF) and Fibroblast Growth Factor 2 (FGF2) [13]. Spontaneous repair is a multistep process involving multiple cytokines in the appropriate extracellular matrix (ECM) microenvironment. The release of cytokines at appropriate times is important for tissue regeneration. FGF-2 binds with high affinity to heparan sulfate proteoglycans located on the surface of most cells and within the ECM. VEGF also bounds the cell surface or ECM [14,15]. FGF2, a member of the FGF family, plays multifaceted roles in cellular and metabolic homeostasis [16]. FGF2 is an essential growth factor released from fibroblasts as a cytokine that functions as a potent inducer of angiogenesis [17], proliferation [18], and migration [19] during wound healing. On the other hand, VEGF has attracted attention as a potent factor in inducing angiogenesis, inducing migration of angioblasts to blood islands, and differentiation into ECs [20–22]. VEGF also inhibits apoptosis in ECs [21–25]. FGF2 stimulates VEGF expression in ECs, and FGF2 and VEGF have synergistic angiogenic effects [21,24,26]. In this study, human periodontal ligament fibroblasts (HPLF) and human umbilical vein endothelial cells (HUVEC) were co-cultured at different ratios on 2-D culture environment to determine the optimal culture conditions for ECM production and angiogenesis, which are important processes in wound healing. In this model, the co-culture of HPLF and HUVEC at a ratio of 1:1 enhanced gene expression and stored growth factors related to wound healing. This approach allowed us to create periodontal ligament-like tissue with a vascular network *in vitro*.

2. Materials and methods

2.1. Materials

Chemicals and reagents were purchased from the following manufacturers.

A saturated picric acid solution, Sirius Red, Alizarin Red S, 28 % ammonia water, and 4 % paraformaldehyde phosphate buffer were purchased from Wako Pure Chemical Industries (Osaka, Japan). Sodium dihydrogen phosphate, potassium dihydrogen phosphate, sodium chloride, potassium chloride, sodium bicarbonate, 99 % 2-mercaptoethanol, 0.01 M hydrochloric acid, 0.1 M sodium hydroxide, and DAPI (4′,6-diamidino-2-phenylindole) were purchased from Dojindo (Kumamoto, Japan). ECGM2 (Endothelial Cell Growth Medium 2), PrimeScript RT Master Mix, and TB Green® Fast qPCR Master Mix were purchased from TaKaRa Bio (Shiga, Japan). DMEM (Dulbecco's Modified Eagle Medium), Alexa Fluor™ 594 goat anti-mouse IgG1 (γ1), and Alexa Fluor™ 488 goat anti-rabbit IgG (H + L) were purchased from Thermo Fisher Scientific (MA, USA). The Monoclonal Mouse Anti-Human CD31 antibody was purchased from Dako (Glostrup, Denmark). FastGene™ RNA Basic Kit was purchased from Nippon Genetics (Tokyo, Japan). The Human VEGF ELISA kit was purchased from R&D Systems (Minneapolis, MN, USA). Human FGF2 ELISA kit was purchased from RayBiotech (GA, USA). Human periodontal ligament fibroblasts (HPLF) were purchased from ScienCell Research Laboratories (CA, USA). Human umbilical vein endothelial cells (HUVEC) were purchased from Takara Bio (Shiga, Japan).

2.2. Cell culture

In this study, human periodontal ligament fibroblasts (HPLF), the major constituent cells of the periodontal ligament, and human umbilical vein endothelial cells (HUVEC), the constituent cells of blood vessels, were used. HPLF were cultured in DMEM supplemented with 10 % heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. HUVEC were cultured in ECGM2. HPLF and HUVEC were grown on each well of 24-well plate in a humidified atmosphere containing 5 % CO_2 at 37 °C. In this study, all experiments were conducted using HPLF at passages 3–5, and HUVEC at passages 1–2.

2.3. Co-culture of HPLF and HUVEC

HPLF and HUVEC were grown in an equivalent mixture of DMEM and ECGM2.

HPLF and HUVEC were suspended at a ratio of 4:1, and 1:1 with an equivalent mixture of DMEM and ECGM2. The suspended cells (8.0×10^4 or 2.0×10^4 cells, and 5.0×10^4 or 5.0×10^4 cells) were seeded on each well of a 24-well plate. As controls, HPLF and HUVEC were seeded in an equivalent mixture of DMEM and ECGM2 at the same density. The culture medium was refreshed every two days, and maintained for a maximum of 14 days.

2.4. Sirius Red staining

Sirius Red staining was conducted to investigate the effect of the co-culture of HPLF and HUVEC on collagen synthesis. HPLF and HUVEC were seeded on each well of 24-well plate at a total density of 5×10^4 cells/cm^2 and cultured in an equivalent mixture of DMEM and ECGM2. After 7, 10, and 14 days of culture, Sirius Red staining was performed at 25 °C. Briefly, cells were fixed with 4 % paraformaldehyde/PBS for 15 min. The cells were washed with ultrapure water and air-dried for 15 min. The cells were stained with 0.1 % Sirius Red in Saturated Picric Acid Solution for 1 h. After washing with ultrapure water, cells were treated with 0.1 M hydrochloric acid for 15 min. Samples were examined under a microscope after air-drying for 15 min. Samples were treated with 0.01 M sodium hydroxide solution for 30 min for extraction of collagen. The absorbance of the extracted solution was measured at 550 nm using a spectrophotometer. 0.01 M sodium hydroxide solution was used as a control.

2.5. Immunofluorescence staining and quantification of cellular junctions

Immunofluorescence staining was conducted to investigate the tube-forming ability of the co-culture of HPLF and HUVEC, immunofluorescence staining was conducted. HPLF and HUVEC were seeded on each well of 24-well plate at a total density of 5×10^4 cells/cm^2 and cultured in an equivalent mixture of DMEM and ECGM2. Cell cultures were grown for 14 days and immunofluorescence staining was performed as described below. Cells were fixed with 4 % paraformaldehyde in PBS for 15 min at 25 °C. After washing with PBS, the cells were permeabilized with 0.1 % Triton X-100 in PBS for 15 min at 25 °C. After rinsing with PBS, cells were treated overnight with primary antibodies at 4 °C. Monoclonal Mouse Anti-Human CD31 antibody (Dako, 1:250) and Polyclonal Rabbit Anti-Human Collagen type-I antibody (Abcam, 1:500) were used as primary antibodies. The cells were again washed with PBS, and then stained with secondary antibodies for 1 h in the dark at 25 °C. Alexa Fluor™ 594 goat anti-mouse IgG1 (γ1) (Thermo Fisher Scientific, 1:500) and Alexa Fluor™ 488 goat anti-rabbit IgG (H + L) (Thermo Fisher Scientific, 1:500) were used as secondary antibodies. After rinsing with PBS, the cells were examined under a fluorescence microscope (BZ-X710, Keyence, Osaka, Japan). The number of cellular junctions in three or more directions, vascular length, and lumen-forming area in immunofluorescence-stained images of CD31 was counted using the Image J image analysis software. The average value from 3 fields in each experiment was used for the statistical analysis.

2.6. Quantitative Real-Time PCR (qRT-PCR)

The expression of marker genes, such as *Col1A1*, *Mkx*, *FGF2*, and *VEGF*, was examined using qRT-PCR. HPLF and HUVEC were seeded

on each well of 24-well plate at a total density of 5×10^4 cells/cm^2 and cultured in an equivalent mixture of DMEM and ECGM2. The culture medium was refreshed every two days, and the culture was maintained for a maximum of 14 days. Total RNA was extracted using a FastGene™ RNA Basic Kit. Single-stranded complementary DNA was synthesized using the Transcriptor First-Strand cDNA synthesis Kit according to the manufacturer's protocol. Primers used in these experiments are listed in Table 1. The quantitative-PCR analysis was conducted on a StepOne® Real-Time PCR System (Thermo Fisher Scientific, MA, USA) using SYBR® Green Reagents. The amounts of mRNA were calculated as relative quantities compared to GAPDH and analyzed with the $2^{-\Delta\Delta Ct}$ method.

2.7. ELISA (Enzyme-Linked Immuno Sorbent Assay)

The culture supernatant was collected to investigate the concentration of FGF2 and VEGF in the culture supernatant under each culture condition. The total cell density was seeded at 5×10^4 cells/cm^2 on 24-well plate and cultured in an equal -volume mixture of DMEM and ECGM2. Culture conditions were set at ratios of 4:1 and 1:1 of HPLF to HUVEC. As a control, HPLF and HUVEC were seeded under identical conditions. The culture medium was changed every two days for a maximum of 14 days. On days 7 and 14, the medium was switched to a serum-free DMEM/ECGM2 mixture in equal volumes, and the cells were cultured for two days. The supernatant was collected in 1.5 mL tubes and centrifuged (8000 rpm, for 5 min). The samples were filter-sterilized, collected in 1.5 mL tubes, and stored at −80 °C. The concentrations of FGF2 and VEGF were measured using the Human bFGF ELISA Kit (RayBio®, ELH-bFGF-1) and the Human VEGF Quantikine® ELISA Kit (R&D Systems, DVE00) according to the manufacturer's protocol.

2.8. Statistical analysis

Data were statistically analyzed for determination to determine the mean and standard deviation (S.D.) of the mean. Significant differences were determined using GraphPad Prism software (version 10; GraphPad, San Diego, CA, USA). Student's t-test was used to determine statistical differences between two groups. Two-way ANOVA followed by Bonferroni multiple comparison post hoc tests was used to compare the levels of different experimental groups. A probability value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Co-culture of HPLF and HUVEC enhanced collagen synthesis

Collagen is a major component of ECM and is necessary for the migration of endothelial cells (ECs) to construct a vascular network. The formation of collagen scaffolds is important for initial cell adhesion during tissue regeneration [27]. The amount of collagen was measured under each culture condition to investigate the effect of the co-culture of HPLF and HUVEC on collagen synthesis (Fig. 1). Appropriate co-culture conditions allow for the construction of a native tissue microenvironment. Our preliminary study showed that collagen synthesis was not correlated with the proportion of HUVEC (HPLF/HUVEC ratios: 1:1, 1:2, and 1:4). However, highly vascularized networks could be seen in 1:1 HPLF/HUVEC co-culture condition among them (Fig. S1). Therefore, HPLF was cultured with HUVEC in different ratios (HPLF/HUVEC ratios:4:1 and 1:1) under 2-D culture conditions. A monoculture of HPLF and HUVEC was used as a control. Collagen synthesis in HPLF mono-culture and HPLF/HUVEC co-culture significantly increased during the culture period, especially on day 14. For the monolayer culture of HUVEC, the level of collagen synthesis was the lowest at all times. No significant changes were observed in HPLF mono-culture and both HPLF/HUVEC co-culture (4:1 and 1:1) during culture period. However, We observed a tendency for collagen production to increase as the percentage of HUVECs increased. These results indicate that the co-culture of HPLF and an optimal amount of HUVEC could enhance collagen synthesis in HPLF.

Table 1
Primers used in this study.

Gene	Accession #	Sequence	
GAPDH	NM_001256799.3	Forward	GTCTCCTCTGACTTCAACAGCG
		Reverse	ACCACCCTGTTGCTGTAGCCAA
Col1A1	NM_000088.4	Forward	GGGATTCCCTGGACCTAAAG
		Reverse	TCCCTGAGCTCCAGCCTCTCC
Mkx	NM_001242702.	Forward	TTACAAGCACCGTGACAACC
		Reverse	AAGCCGACGTCTTGCATTAG
FGF2	NM_001361665.2	Forward	GAGCGACCCTCACATCAA
		Reverse	CGTTTCAGTGCCACATACC
VEGF	NM_001025366.3	Forward	CGCAGCTACTGCCATCCAAT
		Reverse	GTGAGGTTTGATCCGCATAATCT

3.2. Effects of HPLF/HUVEC co-culture on tube formation ability

Blood vessels supply oxygen and nutrients to the surrounding tissues and remove waste products. The first step in angiogenesis proceeds by binding growth factors to receptors on ECs, thereby activating an intracellular transduction cascade. To explore the effect on the tube formation ability by the HPLF/HUVEC co-culture, HPLF and HUVEC were seeded on each well of 24-well plate at a total cell number of 5×10^4 cells/cm^2 at ratios of 4:1 and 1:1 on 24-well plate. HPLF and HUVEC mono-cultures were used as controls. Immunofluorescent staining for CD31 and type I collagen was performed (Fig. 2). CD31 was localized on cell periphery under both HUVEC mono-culture and co-culture conditions. Type I collagen was observed under all conditions except in the HUVEC mono-culture. EC tube formation was observed under both co-culture conditions (4:1 and 1:1). Under co-culture conditions, the localization of the CD31-expressing endothelial cells was consistent with that of type I collagen. These results suggest that ECs aligned and formed tube-like structures using collagen synthesized by HPLF as a scaffold. Next, the number of junctional complexes under co-culture conditions was quantified using the ImageJ software (Fig. 3). The number of junctions increased significantly with an increment in the proportion of HUVECs, although the vascular length and network area did not change. These data revealed that the optimal co-culture condition (1:1) accelerated collagen synthesis (Fig. 1) and angiogenic phenotypes (Fig. 2) via cell-to-cell interactions.

3.3. Changes in the ratio of HPLF and HUVEC under co-culture conditions

The proliferation of each cell under co-culture conditions was determined by counting the total cell number and measuring the proportion of each cell by CellDrop™ (DeNovix®, USA). GFP-labeled HPLF (GFP-HPLF) and HUVEC were seeded at a total cell density of 5×10^4 cells/cm^2 at co-culture ratios of 4:1 and 1:1 and cultured for 1, 3, 5, and 7 days (Fig. 4). The proportion of GFP-HPLF increased as the culture period progressed, whereas that of HUVEC decreased. After 3 days of culture, we observed a dramatic reduction in the ratio of HUVEC at a co-culture ratio of 4:1 (6 % of HUVEC on day 3). However, there was a modest decline in the ratio of HUVEC at a co-culture ratio of 1:1. Under co-culture condition, the cell number of HUVEC did not change a lot, though that of HPLF increased significantly. The presence

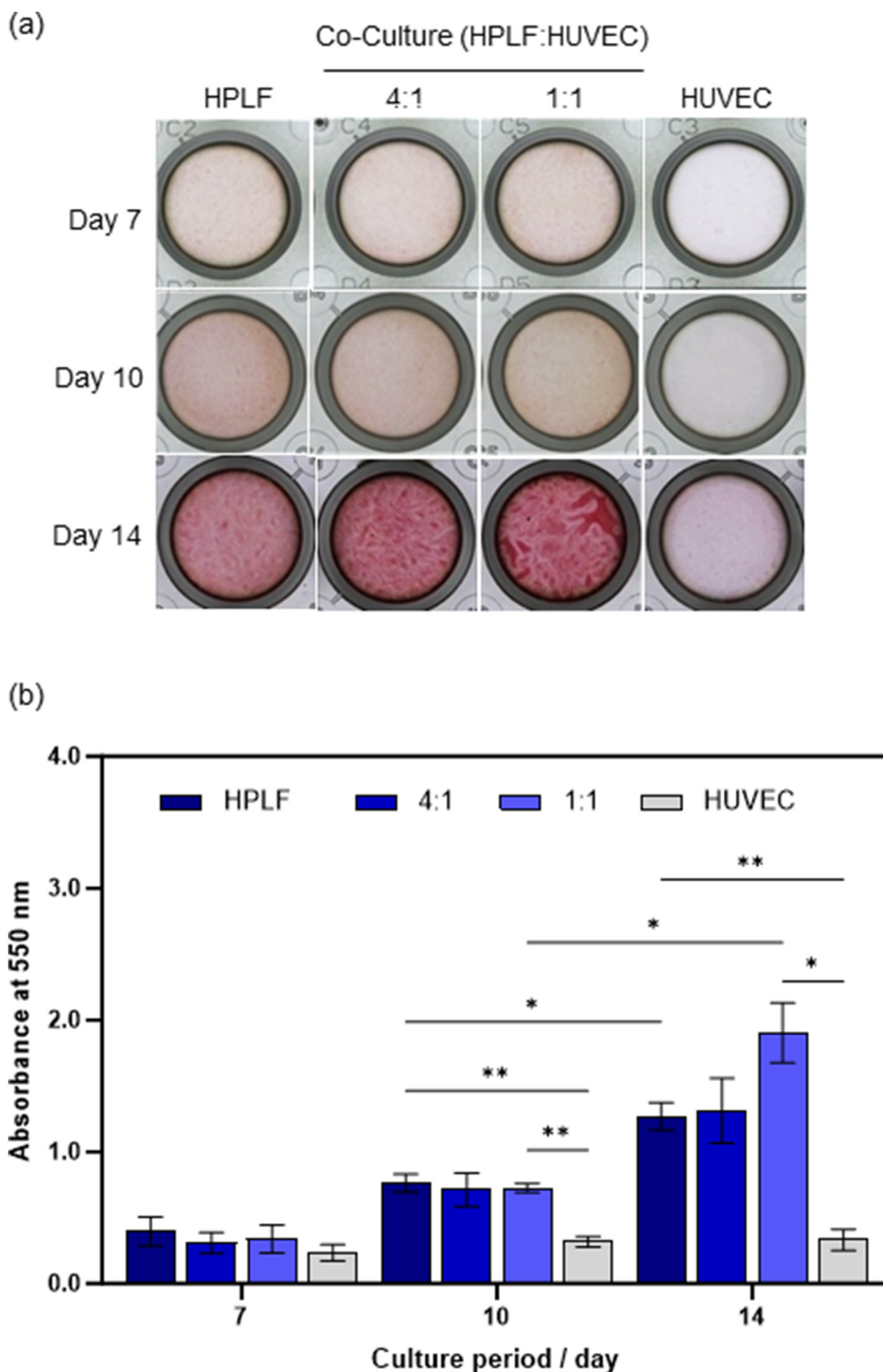

Fig. 1. Comparison of collagen synthesis under different culture conditions. (a) Images of Sirius Red staining, (b) Quantitative data of collagen synthesis. HPLF and HUVEC were seeded on each well of 24-well plate at a total cell density of 5×10^4 cells/cm^2 and cultured for 7, 10, and 14 days. Data represent as means ± S.D. of three independent experiments. *, $p < 0.05$; and **, $p < 0.01$ for between-group comparisons.

of a certain amount of HUVEC led to increased cell-to-cell interactions, resulting the enhancement of collagen synthesis and EC tube formation at a co-culture ratio of 1:1.

3.4. *Gene expression profile of mono- and co-culture system*

Quantitative polymerase chain reaction (qRT-PCR) analysis was performed to assess the specific gene expression under mono- and coculture conditions. We analyzed *Col1A1*, the major collagen in HPLF (Fig. 5a), *Mkx*, a tendon-specific transcription factor (Fig. 5b), *FGF2* and *VEGF*, angiogenesis-related genes (Fig. 5c and d). *Col1A1* expression in HPLF monoculture decreased significantly with the passage of the culture period. In HUVEC monoculture, a very small amount of *Col1a1* was not detectable. In contrast, the expression level of *Col1A1* under co-culture conditions was higher than that under mono-culture after 10 days. These data indicate that the co-culture of HPLF and HUVEC increased the expression levels of *Col1A1*. Interactions between HPLF and HUVEC regulate gene

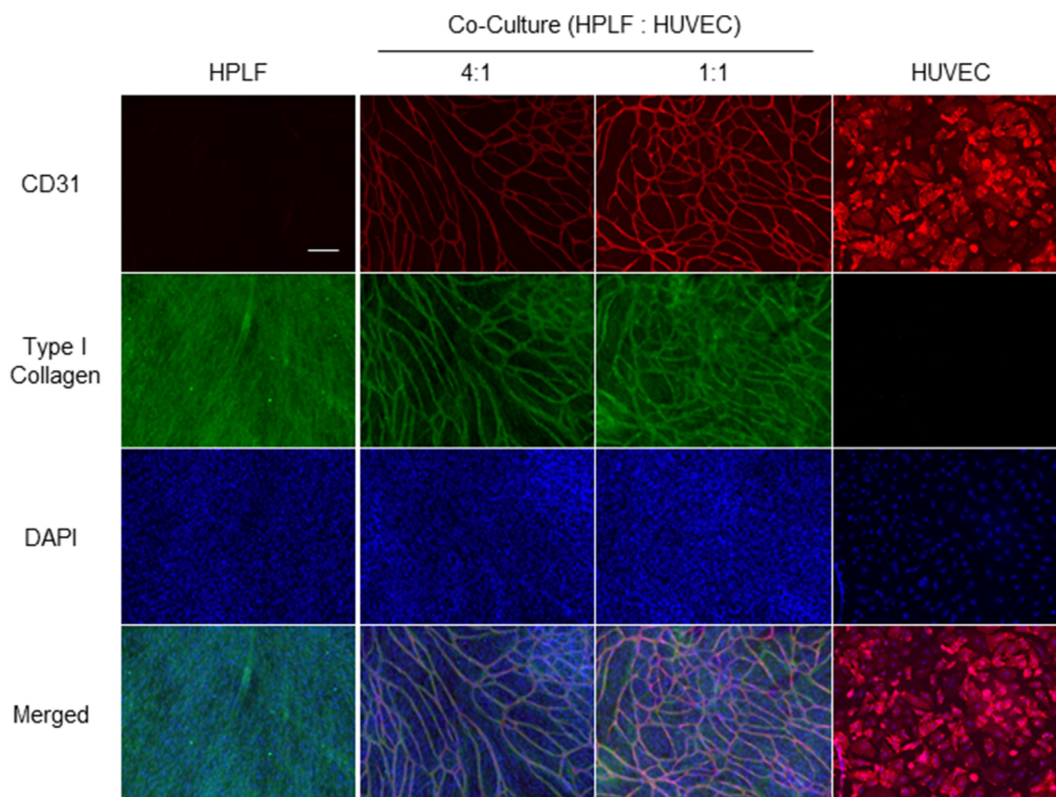

Fig. 2. Immunofluorescence analysis of CD31 and type I collagen under mono- and co-culture conditions. HPLF, HUVEC, and HPLF and HUVEC cocultures were seeded at a total cell number of 5.0×10^4 cells/cm^2. After 14 days culture, cells were fixed and stained with anti-CD31 for CD31 (red), anti-type I collagen for type I collagen (green), and DAPI for nuclei (blue). They were viewed with a fluorescence microscope 10 × magnifications (scale bar: 100 μm).

expression. A comparison of different co-culture conditions (4:1 vs. 1:1) showed that a higher number of HUVEC cells could enhance the *Co11A1* gene expression at day 14. *Mkx*, a tendon-specific transcription factor, was assessed for membranous properties. The expression levels of *Mkx* in both co-culture conditions were lower than those in the HPLF mono-culture on day 7, contributing to the number of HPLF. However, no significant changes were observed in any of the other groups. Our data suggested that the co-culture of HPLF and HUVEC did not affect the expression of *Mkx* in long-term culture.

The expression levels of *FGF2* and *VEGF* were examined to evaluate angiogenesis. In HPLF monocultures, the expression of *FGF2* significantly decreased with the passage of culture time (Fig. 5c). On the other hand, *FGF2* expression in HUVEC mono-culture increased as the culture period passed, though the level was lower than HPLF. Under co-culture conditions, the expression level of *FGF2* at 1:1 condition was the highest among all samples over time in culture. The level of *FGF2* at 1:1 condition decreased with the culture period under co-culture conditions though it remained higher than in the other samples. The expression of *VEGF* in HPLF monocultures also decreased over time in culture (Fig. 5d). *VEGF* expression in HUVEC was much lower than that in HPLF. Under the co-culture condition, the expression level of *VEGF* in the 1:1 condition on day 7 was the highest among all the samples. These results showed that the co-culture of HPLF and HUVEC enhanced the expression of *VEGF*. The increase in the ratio of HUVEC may be correlated with the *FGF*2 derived from HPLF.

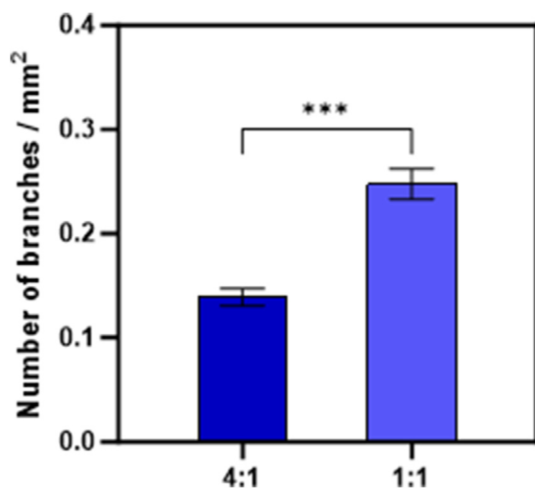

Fig. 3. Quantification of the number of junctions under co-culture condition. HPLF and HUVEC were seeded at a total cell count of 5.0×10^4 cells/cm^2 and cultured for 14 days. The tube-like structure was assessed by immunofluorescence staining. The number of junctions was determined by ImageJ. Data represent as means ± S.D. of three independent experiments. ***, $p < 0.01$ compared with co-culture (4:1).

3.5. Effect of co-culture of HPLF and HUVEC on the secretion of growth factors

Cell-to-cell interactions regulate various changes in co-culture. We focused on indirect cell-to-cell interactions and examined the cytokines that stimulate angiogenesis, such as FGF2 (Fig. 6) and VEGF (Fig. 7). In the monoculture of HUVEC, FGF2 was secreted the most compared to other conditions. No apparent differences were observed between the HPLF mono-culture and the co-culture. A comparison with between days 7 and 14 under co-culture

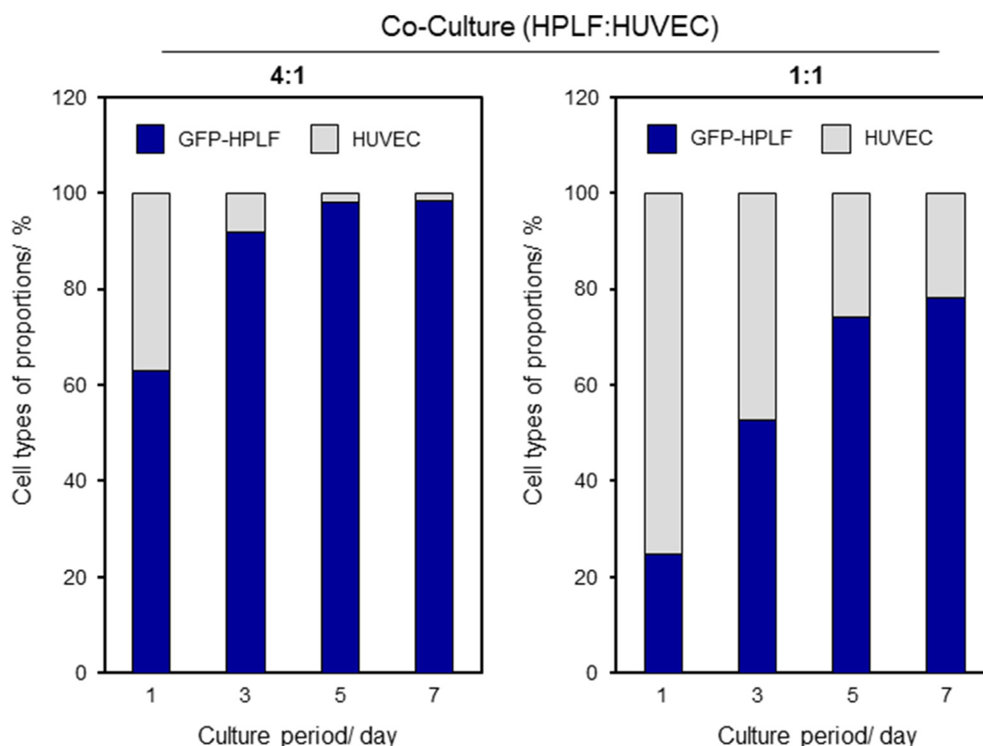

Fig. 4. Changes in the proportions of HPLF and HUVEC under co-culture conditions. GFP-labeled HPLF (GFP-HPLF) and HUVEC were seeded on 24-well plate at a total cell density of 5×10^4 cells/cm^2 at co-culture ratios of 4:1 and 1:1 and cultured for 1, 3, 5, and 7 days. The proportion of GFP-HPLF and HUVEC in the co-culture was determined by cell counter. Data are shown as the average of three different samples.

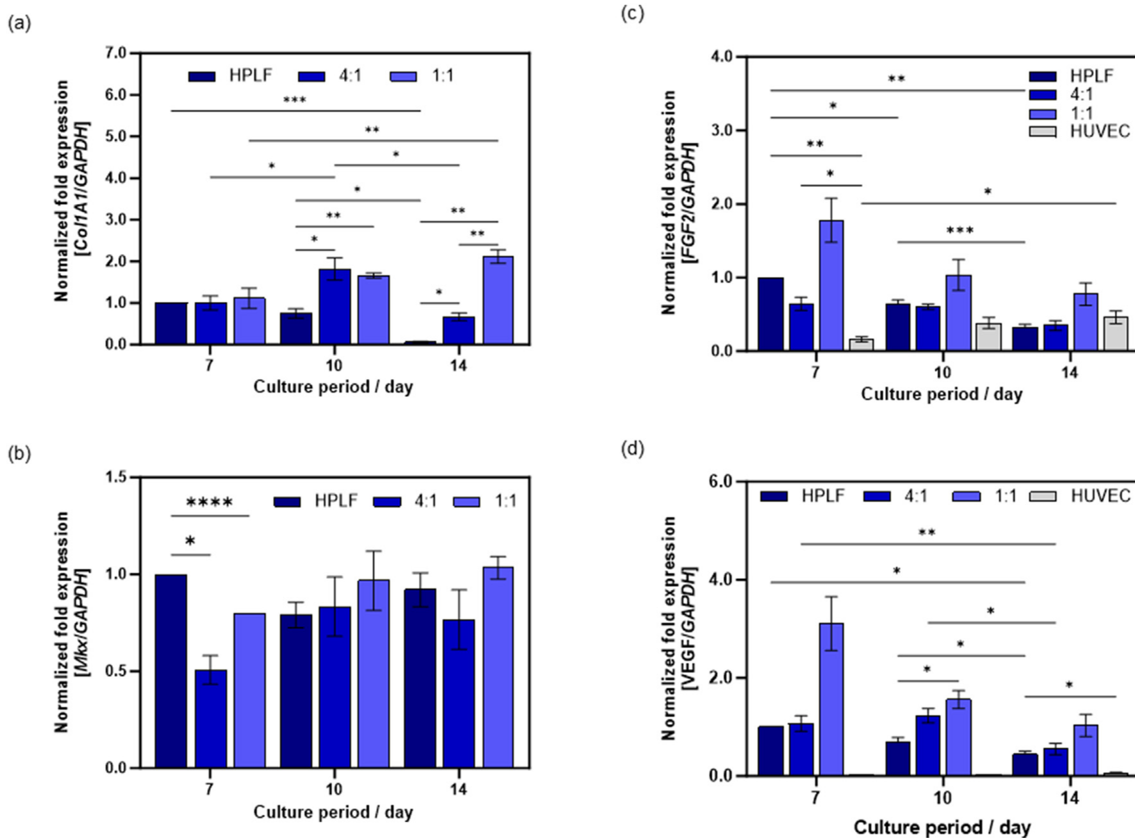

Fig. 5. Gene expression profile of mono- and co-culture system. Relative expression of *Col1A1* (a), *Mkx* (b), *FGF2* (c), and *VEGF* (d). HPLF, HUVEC, and HPLF and HUVEC were seeded at a total cell count of 5.0×10^4 cells/cm^2 and cultured for 7, 10 and 14 days. After extraction of total RNA, synthetic cDNA was prepared and qRT-PCR was performed. The amounts of mRNA were calculated as relative quantities compared to GAPDH and analyzed with the $2^{-\Delta\Delta}$Ct method. Data represent as means ± S.D. of three independent experiments. *, $p < 0.05$; **, $p < 0.001$, ***, $p < 0.001$; and ****, $p < 0.0001$ for between-group comparisons.

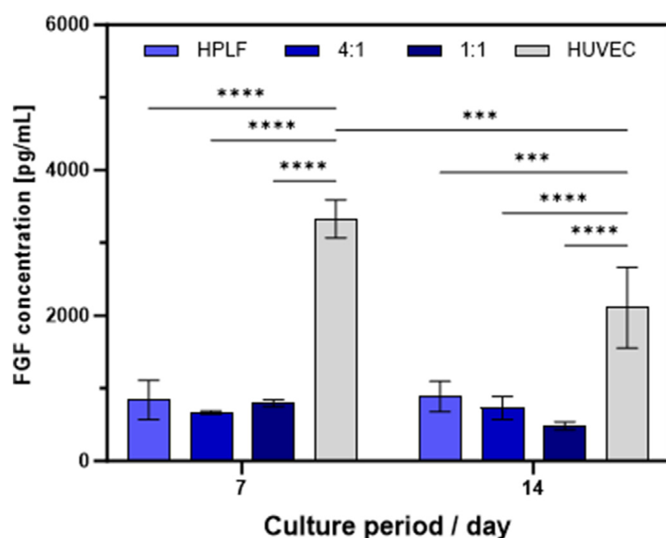

Fig. 6. Comparison of FGF2 secretion between mono- and co-culture. HPLF, HUVEC, HPLF and HUVEC were seeded at a total cell density of 5.0 × 10^4 cells/cm^2 and cultured in an equal volume mixture of DMEM and ECGM2 for 7 and 14 days. The medium was changed to serum-free DMEM medium, and the cultures were cultured for 2 more days. The medium supernatant was collected and the FGF2 concentration was measured using ELISA. Data represent as means ± S.D. of three independent experiments. ***, $p < 0.001$ and ****, $p < 0.0001$ for between-group comparisons.

conditions (1:1) showed a slight reduction in FGF2 secretion (day 7; 796.9 ± 47.23 pg/mL, day 14; 486.03 ± 54.53 pg/mL). Our data imply that, under co-culture conditions, FGF2 would bind with heparan sulfate proteoglycans located on the surface of most cells and within the ECM. FGF2 released from ECM may stimulate each cell type, contributing to collagen synthesis and EC tube formation.

High concentrations of VEGF were secreted from HPLF mono-culture on day 7 (Fig. 7). In contrast, VEGF was undetectable in HUVEC monocultures during the culture period. Between the HPLF mono-culture and co-culture conditions on day 7, the level of VEGF was significantly lower in the 1:1 co-culture than in the HPLF mono-culture (HPLF; 2171 ± 556 pg/mL, 1:1; 1236 ± 31 pg/mL). On day 14, the concentration of VEGF decreased in co-culture of 4:1 (496 ± 81.8 pg/mL) and 1:1 (496 ± 69.8 pg/mL) compared with the mono-culture of HPLF (681 ± 36.7 pg/mL). These results indicate that VEGF secreted from HPLF was also bounds the cell surface or ECM, contributing to collagen synthesis and EC tube formation.

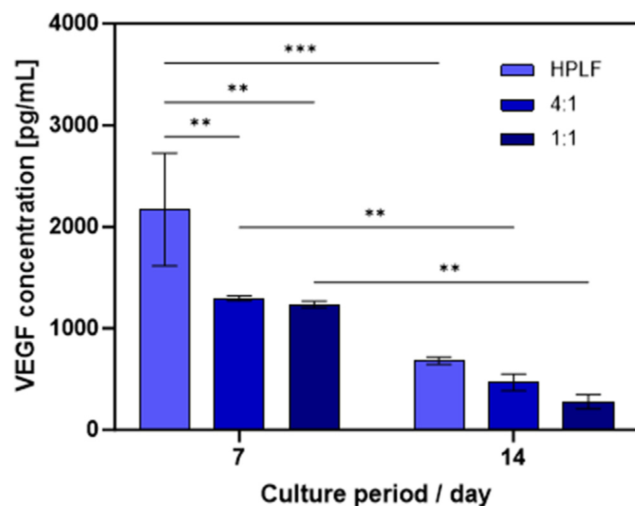

Fig. 7. Comparison of VEGF secretion between mono- and co-culture. HPLF, HUVEC, HPLF and HUVEC were seeded on 24-well plate at a total cell density of 5.0 × 10^4 cells/cm^2 and cultured in an equal volume mixture of DMEM and ECGM2 for 7 and 14 days. The medium was changed to serum-free DMEM medium, and the cultures were cultured for 2 more days. The medium supernatant was collected and the VEGF concentration was measured using ELISA. Data represent as means ± S.D. of three independent experiments. **, $p < 0.001$, and ***, $p < 0.001$ for between-group comparisons.

4. Discussion

Periodontal tissue is composed of multiple tissues, damaged by periodontal disease making it difficult to reconstruct normal tissue [1–4]. Wound healing is a complex integrated biological process involving ECM molecules, cytokines, proliferation and migration of multiple cells, ECM deposition, and reorganization of the blood transport system [28]. The ECM is a highly dynamic non-cellular network in all tissues that plays an important role in homeostasis [29]. It is essential not only for maintaining tissue homeostasis, but also as a signaling hub that transduces cascades for cellular function. The ECM also serves as a reservoir for growth factors released after injury to modulate tissue behaviour and activate repair programs [30].

In this study, we first examined the effect of co-culture HPLF with HUVEC on collagen synthesis, which is the main component of the ECM. According to recent study, collagen in the PDL is mainly composed of type I α1 (33.77 %), type III α1 (10.35 %), and type I α2 (6.69 %), and collagen in HUVEC is type IV, a component of the basement membrane [31]. In our results, collagen synthesis in the co-culture was comparable to that in the HPLF mono-culture on days 7 and 10, while a significant increase was seen on day 14 only in the 1:1 co-culture (Fig. 1). These results suggest that an optimal amount of HUVEC that can interact with HPLF promotes collagen synthesis in HPLF. Immunofluorescent analysis also showed that the amount of collagen I was higher at a co-culture ratio of 1:1 than that at 4:1 condition (Fig. 2). Furthermore, we could see numerous collagen fibers and depositions within the tube network (asterisks in Fig. S2). These data suggest that collagen functioned as a scaffold for tube formation. In the co-culture of PDL and ECs, cell-to-cell interactions stimulate EC tube formation [32]. These data imply that the enhanced collagen synthesis in the co-culture contributes to the construction of endothelial network structure. qRT-PCR analyses showed that *Col1A1* gene expression significantly decreased with the passage of culture days in the HPLF monoculture. In contrast, *Col1A1* expression under co-culture conditions was higher than that in the mono-culture after 10 days of culture. *Col1A1* gene expression in the 1:1 co-culture was also higher than that in the 4:1 co-culture, with a significant increase in collagen synthesis and the number of junctions on day 14. These results are consistent with those of previous reports showing that *Col1A1* promotes the formation of luminal structures in vascular ECs [33].

Next, we examined the effect of the co-culture of HPLF and HUVEC on angiogenesis. Immunofluorescent analysis showed the EC alignment and tube formation (Fig. 2) under co-culture conditions. In addition, type I collagen was localized over the entire field of view in HPLF monocultures. On the other hand, collagen I colocalized with CD31 in HUVEC under the co-culture condition. These data suggest that collagen in HPLF functions like a scaffold for migration and growth of ECs, resulted in the formation of EC tube networks (Fig. S2). In this study, the co-culture of HPLF and HUVEC induced EC tube formation through the intercellular action of each cell. In particular, under 1:1 co-culture conditions, angiogenesis-related factors, including FGF2 and VEGF, may function as transduction molecules between both cells. FGF2 and VEGF are key mediators of angiogenesis [34], and numerous studies have reported on these factors. FGF2 promotes the proliferation of ECs and

repair of damaged vessels [35,36]. During *in vivo* angiogenesis, VEGF induces lumen formation in ECs, whereas Notch signaling converts adjacent cells into stem cells and induces VEGF receptor expression [37–39].

The levels of FGF2 and VEGF were analyzed by qRT-PCR (Fig. 5) and ELISA (Figs. 6 and 7). Our findings indicate that under co-culture conditions, FGF2 secreted from HPLF and HUVEC may be used by their respective cells and stored in ECM, contributing to collagen synthesis and EC tube formation. This phenomenon can preferentially occur in a larger number of HUVECs, contributing to tissue reconstruction. According to a previous report, FGF2 administration to HUVEC promotes cell proliferation [40,41] and migration [42,43]. These data suggest that the degree of decline in the FGF2 concentration under co-culture conditions is dependent on the number of HUVEC. Additionally, the decrease of FGF2 would derive from binding with the cell surface or ECM (Fig. 6). The expression of *VEGF* in HPLF monocultures also significantly decreased over time in culture. *VEGF* expression in HUVEC was much lower than that in HPLF. Under the co-culture condition, the expression level of *VEGF* in the 1:1 condition on day 7 was the highest among all the samples. These results indicate that the co-culture of HPLF and HUVEC enhanced the expression of *VEGF*. The increase in the ratio of HUVEC was correlated with FGF2 derived from HPLF. Our data suggest that VEGF released from ECM, which can serve as a storage place, would proceed tube formation depending on the number of cells in HUVEC with VEGER. A previous study demonstrated that HUVECs express *VEGFR* and that lumen formation proceeds by using VEGF, which is dependent on the concentration of VEGF [44]. In this study, the concentration of VEGF was also lower in the 1:1 co-culture, in which tube formation progressed significantly. These data suggested that VEGF was also stored in ECM, resulting in stabilizing the capillary-like formations.

Synergistic effects of FGF2 and VEGF have been shown in human cord blood mononuclear cells transfected with a plasmid expressing FGF2 and VEGF; cell proliferation was enhanced, and the process of reconstructing vascular structures occurred at an earlier stage [45]. Other studies have shown that the co-treatment of MPDL22 cells with FGF2 and VEGF significantly enhanced cell migration. When MPDL22 cells were co-cultured with bEnd5 cells, a mouse endothelial cell line, the production level of VEGF was increased, and lumen formation was induced [32]. In the present study, the co-culture of HPLF and HUVEC showed significantly higher expression levels of *VEGF* on day 7 compared to the HPLF mono-culture (Fig. 5c). Secreted FGF2 was most highly expressed in HUVEC alone, whereas VEGF was highly expressed in the HPLF monocultures. Therefore, under the 1:1 co-culture conditions, the growth factors produced by HPLF and HUVEC interacted with each other and showed synergistic effects, contributing to the significant increase in *VEGF* gene expression.

Our data lead to the following processes for constructing a microenvironment with vascular network.

i) The viability of HUVEC was enhanced by VEGF secreted from HPLF.
ii) FGF2 secreted by activated HUVECs accelerates collagen production in HPLF.
iii) VEGF secreted from HPLF aligned and formed a tube-like structure on the collagen scaffold.
iv) The ECM constructed by fibroblasts and ECs acts as a storage place of growth factors.

This system can be applied to various regenerative tissues by selecting appropriate fibroblasts, and the optimal conditions for each tissue should be determined.

5. Conclusions

Successful periodontal tissue regeneration requires microenvironmental reconstruction and vascularization, which can be achieved using a co-culture system. The ECM plays an important role in cell-to-cell communication via paracrine signaling, which entraps and stores bioactive molecules secreted from the cells. Additionally, vascular networks support tissue regeneration by supplying essential substances, such as oxygen, nutrients, and cytokines. In the present study, we found that fibroblastic and angiogenic phenotypes were enhanced by the co-culture of HPLF and HUVEC, especially at a co-culture ratio of 1:1. The presence of HPLF-stimulated in HUVEC led to increased EC tube formation. On the other hand, HUVEC stimulated HPLF, which in turn increased cell proliferation and ECM production. Taken together, optimal culture conditions (1:1) could potentially accelerate tissue engineering, including collagen synthesis and EC tube formation, and these approaches can improve therapeutic efficacy after transplantation.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2023.12.004.

References

[1] Chapple IL. Periodontal disease diagnosis: current status and future developments. J Dent 1997;25:3–15.
[2] Van Dyke TE. The management of inflammation in periodontal disease. J Periodontol 2008;79:1601–8.
[3] Papapanou PN, Sanz M, Buduneli N, Dietrich T, Feres M, Fine DH, et al. Periodontitis: consensus report of workgroup 2 of the 2017 world workshop on the classification of periodontal and peri-implant diseases and conditions. J Periodontol 2018;89:S173–82.
[4] Feng Z, Weinberg A. Role of bacteria in health and disease of periodontal tissues. Periodontology 2000 2006;40:50–76.
[5] Ramseier CA, Rasperini G, Batia S, Giannobile WV. Advanced reconstructive technologies for periodontal tissue repair. Periodontology 2000 2012;59:185–202.
[6] Cardoso EM, Reis C, Manzanares-Céspedes MC. Chronic periodontitis, inflammatory cytokines, and interrelationship with other chronic diseases. Postgrad Med 2018;130:98–104.
[7] Bosshardt DD, Sculean A. Does periodontal tissue regeneration really work? Periodontology 2000 2009;51:208–19.
[8] Storey E. The nature of tooth movement. Am J Orthod 1973;63:292–314.
[9] Nanci A, Bosshardt DD. Structure of periodontal tissues in health and disease. Periodontology 2000 2006;40:11–28.
[10] Zhu W, Zhang Q, Zhang Y, Cen L, Wang J. PDL regeneration via cell homing in delayed replantation of avulsed teeth. J Transl Med 2015;13:357.
[11] McCulloch CA, Bordin S. Role of fibroblast subpopulations in periodontal physiology and pathology. J Periodontal Res 1991;26:144–54.
[12] Naveh GR, Lev-Tov Chattah N, Zaslansky P, Shahar R, Weiner S. Tooth-PDL-bone complex: response to compressive loads encountered during mastication - a review. Arch Oral Biol 2012;57:1575–84.
[13] Carmeliet P, Jain RK. Molecular mechanisms and clinical applications of angiogenesis. Nature 2011;473:298–307.
[14] Neve A, Cantatore FP, Maruotti N, Corrado A, Ribatti D. Extracellular matrix modulates angiogenesis in physiological and pathological conditions. BioMed Res Int 2014;2014:756078.
[15] Wilgus TA. Growth factor-extracellular matrix interactions regulate wound repair. Adv Wound Care 2012;1:249–54.
[16] Li X. The FGF metabolic axis. Front Med 2019;13:511–30.
[17] Presta M, Foglio E, Churruca Schuind A, Ronca R. Long pentraxin-3 modulates the angiogenic activity of fibroblast growth factor-2. Front Immunol 2018;9:2327.

[18] Sahni A, Francis CW. Stimulation of endothelial cell proliferation by FGF-2 in the presence of fibrinogen requires alphavbeta3. Blood 2004;104:3635–41.
[19] Schmidt A, Ladage D, Schinköthe T, Klausmann U, Ulrichs C, Klinz FJ, et al. Basic fibroblast growth factor controls migration in human mesenchymal stem cells. Stem Cell 2006;24:1750–8.
[20] Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. Endocr Rev 2004;25:581–611.
[21] Takahashi H, Shibuya M. The vascular endothelial growth factor (VEGF)/VEGF receptor system and its role under physiological and pathological conditions. Clin Sci (Lond) 2005;109:227–41.
[22] Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z. Vascular endothelial growth factor (VEGF) and its receptors. Faseb J 1999;13:9–22.
[23] Heloterä H, Alitalo K. The VEGF family, the inside story. Cell 2007;130:591–2.
[24] Ucuzian AA, Gassman AA, East AT, Greisler HP. Molecular mediators of angiogenesis. J Burn Care Res 2010;31:158–75.
[25] Onimaru M, Yonemitsu Y. Angiogenic and lymphangiogenic cascades in the tumor microenvironment. Front Biosci (Schol Ed) 2011;3:216–25.
[26] Ley CD, Olsen MW, Lund EL, Kristjansen PE. Angiogenic synergy of bFGF and VEGF is antagonized by Angiopoietin-2 in a modified in vivo Matrigel assay. Microvasc Res 2004;68:161–8.
[27] Minor AJ, Coulombe KLK. Engineering a collagen matrix for cell-instructive regenerative angiogenesis. J Biomed Mater Res B Appl Biomater 2020;108: 2407–16.
[28] Eming SA, Martin P, Tomic-Canic M. Wound repair and regeneration: mechanisms, signaling, and translation. Sci Transl Med 2014;6:265sr6.
[29] Bonnans C, Chou J, Werb Z. Remodelling the extracellular matrix in development and disease. Nat Rev Mol Cell Biol 2014;15:786–801.
[30] Hynes RO. The extracellular matrix: not just pretty fibrils. Science 2009;326: 1216–9.
[31] Hosiriluck N, Kashio H, Takada A, Mizuguchi I, Arakawa T. The profiling and analysis of gene expression in human periodontal ligament tissue and fibroblasts. Clin Exp Dent Res 2022;8:658–72.
[32] Yanagita M, Kojima Y, Kubota M, Mori K, Yamashita M, Yamada S, et al. Cooperative effects of FGF-2 and VEGF-A in periodontal ligament cells. J Dent Res 2014;93:89–95.
[33] Strömblad S, Cheresh DA. Cell adhesion and angiogenesis. Trends Cell Biol 1996;6:462–8.
[34] Seo H-R, Jeong HE, Joo HJ, Choi S-C, Park C-Y, Kim J-H, et al. Intrinsic FGF2 and FGF5 promotes angiogenesis of human aortic endothelial cells in 3D microfluidic angiogenesis system. Sci Rep 2016. https://doi.org/10.1038/srep28832.
[35] Przybylski M. A review of the current research on the role of bFGF and VEGF in angiogenesis. J Wound Care 2009;18:516–9.
[36] Lee JG, Kay EP. FGF-2-Induced wound healing in corneal endothelial cells requires Cdc 42 activation and Rho inactivation through the phosphatidylinositol 3-kinase pathway. Invest Ophthalmol Vis Sci 2006;47:1376–86.
[37] Gerhardt H. VEGF and endothelial guidance in angiogenic sprouting. Organogenesis 2008;4:241–6.
[38] Hellström M, Phng L-K, Hofmann JJ, Wallgard E, Coultas L, Lindblom P, et al. Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. Nature 2007;445:776–80.
[39] Potente M, Gerhardt H, Carmeliet P. Basic and therapeutic aspects of angiogenesis. Cell 2011;146:873–87.
[40] Klint P, Kanda S, Kloog Y, Claesson-Welsh L. Contribution of Src and Ras pathways in FGF-2 induced endothelial cell differentiation. Oncogene 1999;18:3354–64.
[41] Kanda S, Hodgkin MN, Woodfield RJ, Wakelam MJ, Thomas G, Claesson-Welsh L. Phosphatidylinositol 3'-kinase-independent p70 S6 kinase activation by fibroblast growth factor receptor-1 is important for proliferation but not differentiation of endothelial cells. J Biol Chem 1997;272:23347–53.
[42] Kanda S, Lerner EC, Tsuda S, Shono T, Kanetake H, Smithgall TE. The non-receptor protein-tyrosine kinase c-Fes is involved in fibroblast growth factor-2-induced chemotaxis of murine brain capillary endothelial cells. J Biol Chem 2000;275:10105–11.
[43] Tanaka K, Abe M, Sato Y. Roles of extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinase in the signal transduction of basic fibroblast growth factor in endothelial cells during angiogenesis. Jpn J Cancer Res 1999;90:647–54.
[44] Zhang Z, Shuai Y, Zhou F, Yin J, Hu J, Guo S, et al. PDLSCs regulate angiogenesis of periodontal ligaments via VEGF transferred by exosomes in periodontitis. Int J Med Sci 2020;17:558–67.
[45] Salafutdinov II, Gazizov IM, Gatina DK, Mullin RI, Bogov AA, Islamov RR, et al. Influence of recombinant codon-optimized plasmid DNA encoding VEGF and FGF2 on Co-induction of angiogenesis. Cells 2021;10:432.