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VEGF-C-driven lymphatic survival signaling promotes periodontal repair

Lymphatic remodeling facilitates periodontal repair via VEGF-C-mediated survival signaling

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Abstract

Lymphatic vessels play a pivotal role in tissue homeostasis and repair, yet their function in periodontal healing remains poorly defined. In this study, we investigated the spatial dynamics and functional significance of lymphatics during periodontal wound repair using a mouse model of ligature-induced periodontitis. By combining Prox1-tdTomato transgenic reporter mice with advanced tissue clearing and light sheet microscopy, we generated high-resolution three-dimensional images of gingival lymphatic networks under both physiological and repair conditions. Our observations revealed that lymphatic vessels undergo active and spatially organized reassembly during the healing phase. Notably, we found a region-specific increase in lymphatic branching and density in the marginal gingiva, suggesting localized lymphangiogenesis. Time-course analysis confirmed that this lymphatic remodeling peaks during the granulation tissue formation phase.

We further identified epithelial upregulation of *Vegfc* during the repair phase and demonstrated that local administration of VEGF-C C156S, a VEGFR-3-specific agonist, enhanced lymphatic vessel function and alveolar bone repair. To explore the underlying molecular mechanisms, we successfully established, for the first time, primary cultures of gingiva-derived lymphatic endothelial cells (LECs). Comparative transcriptomic analysis revealed distinct signatures of gingival LECs compared to lymph node or dermal LECs. These cells maintained typical LEC characteristics *in vitro* and responded robustly to VEGF-C stimulation. RNA sequencing and functional apoptosis assays revealed significant modulation of apoptosis-related genes, including downregulation of *Elf3* and upregulation of *Siah1b*, suggesting a survival-promoting role for VEGF-C signaling in LECs.

Collectively, our findings highlight a previously underappreciated regenerative role of lymphatic vessels in the periodontium and establish lymphangiogenic activation as a potential therapeutic strategy for enhancing periodontal repair. The successful culture of primary gingival LECs further provides a novel platform for mechanistic studies in oral lymphatic biology.

Introduction

The vascular system in vertebrates consists of two distinct yet complementary networks: blood vessels and lymphatic vessels. While blood vessels primarily supply oxygen and nutrients, lymphatic vessels regulate interstitial fluid drainage, immune cell trafficking, and tissue homeostasis(Alitalo 2011; Oliver et al. 2020). Lymphatic vessels comprise endothelial cells expressing Prox1, Lyve-1, and podoplanin. Their neogenesis is regulated by the VEGF-C signaling pathway via VEGFR-3 on lymphatic endothelial cells. Loss or dysfunction of this pathway causes tissue edema(Karkkainen et al. 2000; Butler et al. 2007).

Recent studies show lymphatic vessels aid tissue repair in the heart, skin, and other organs(Gur-Cohen et al. 2019; Liu et al. 2020; Biswas et al. 2023). In periodontal tissues, the presence of lymphatics has been documented histologically(Ushijima et al. 2008), yet their organization and contribution to wound healing remain unclear. While wound healing is a well-orchestrated biological process that restores tissue integrity, wound repair can be enhanced by various therapeutic approaches aiming to restore both structure and function. Understanding the repair roles of lymphatics in periodontal tissues may open new avenues for therapeutic intervention.

Periodontal wound healing is a complex process involving hemostasis, inflammation, granulation tissue formation, and remodeling. While the role of blood angiogenesis in supplying oxygen and nutrients is well established, the contribution of lymphatic vessels—specifically their timing and function—remains less understood. We hypothesize that lymphatic vessels are crucial during the transition from inflammation to tissue formation by clearing inflammatory filtrate and facilitating immune resolution.

Although lymphatic vessels have been implicated in inflammatory periodontal conditions(Mkonyi et al. 2010; Raica et al. 2015; Papadakou et al. 2017) and are known to expand in response to *Porphyromonas gingivalis* infection(Mkonyi et al. 2012), prior research has been limited to static, cross-sectional observations. While serial sectioning and reconstruction have been attempted, these methods are fraught with challenges, including tissue distortion, alignment artifacts, and the sheer labor required, often failing to capture the true, continuous three-dimensional architecture of the vascular network. This hurdle has limited understanding of how these networks respond to injury and repair. Therefore, a key objective of this study was to overcome this limitation by applying advanced tissue clearing and light-sheet microscopy, enabling an unbiased, holistic view of the lymphatic vasculature throughout the entire gingival tissue.

Furthermore, while lymphangiogenesis has been shown to facilitate tissue repair in other systems(Huggenberger et al. 2010; Liu et al. 2020), it is unclear whether similar mechanisms operate in the periodontium. The functional impact of modulating lymphatic growth during

healing by enhancing lymphatic vessels has not been systematically investigated. This study aimed to elucidate the dynamic role of lymphatic vessels in periodontal wound healing by employing both *in vivo* and *in vitro* methodologies. We examined the effects of local delivery of VEGF-C (C156S), a lymphangiogenic growth factor, on tissue repair. In parallel, primary gingival lymphatic endothelial cells were also isolated to investigate VEGF-C-induced molecular responses. Through this multifaceted approach, we aimed to provide mechanistic insights into the repair functions of lymphatics in periodontal tissues and explore their potential as therapeutic targets.

Materials and Methods

Animals and Ethical Approval

All animal procedures were approved by the Animal Experimentation Committee of the University of Osaka Graduate School of Dentistry (Approval Nos. 31-001-0, R-03-008-0). The following mouse lines were used: Prox1-tdTomato (JAX #018128) and Tie2-GFP (JAX #003658). These Prox1-tdTomato mice were selected because they provide robust and specific labeling of lymphatic cytoplasm. This genetic labeling is essential for high-resolution 3D imaging in dense, collagen-rich gingival tissue, where antibody penetration can be inconsistent. Wild-type C57BL/6J mice (Japan SLC) served as **control** for GFP and tdTomato signal. Mice of both sexes aged 6–10 weeks were used. All mice were housed under SPF conditions with a 12-hour light/dark cycle and ad libitum food and water. This study is reported in accordance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines. Humane endpoints were established based on signs of severe distress, with daily monitoring. No animals met these criteria for removal from the study during the experimental period.

Ligature-Induced Periodontitis Model

Periodontitis was induced by ligating the upper left second molar using a 5-0 silk suture under general anesthesia (Abe and Hajishengallis 2013; Marchesan et al. 2018). Anesthesia was achieved via intraperitoneal injection of a three-drug cocktail consisting of medetomidine (0.75 mg/kg), midazolam (4 mg/kg), and butorphanol (5 mg/kg). Antisedan (0.75 mg/kg) aided for recovery. The ligature was maintained for 7 days and then removed to initiate the healing phase (Day 0). **We used a split-mouth injury model, in which the ligature was placed on the upper left second molar, and the contralateral hemimaxilla served as an unligated internal control.** For all in vivo outcome measures, one hemijaw was treated as a single sample, and each group included a total of n=6–8 hemijaw samples. No unexpected or study-unrelated adverse events were observed in the animals during the experimental period.

VEGF-C (C156S) Administration

Recombinant human VEGF-C C156S (R&D Systems, #752-VC-025) was used to selectively activate lymphangiogenesis (Mäkinen et al. 2001). Immediately after ligature removal, a total of 5 µL of VEGF-C C156S solution (250 µg/mL; resulting in 1.25 µg per site and 2.5 µg per mouse) was locally injected into the palatal gingiva at both the mesial and distal aspects of the upper second molar using a microinjector (Hamilton, Model 65 RN). Controls received vehicle solution (0.1% BSA in PBS). **VEGF-C C156S or vehicle was assigned at the animal level, and both hemijaws of each mouse received the same injection.** The detailed injection procedure is demonstrated in Supplemental Movie 1. The dosage was selected based on previous studies demonstrating efficacy in skin wound healing (Gur-Cohen et al. 2019). Mice

were sacrificed on day 3, 7, and 10 post-injection for further analyses. Animals were not randomly allocated to the control and treatment groups.

Tissue Clearing and 3D Imaging

Following 4% paraformaldehyde perfusion fixation, maxillary tissues were dissected and decalcified in EDTA solution for 3 days. Samples underwent CUBIC tissue clearing using CUBIC-L and CUBIC-R reagents(Susaki et al. 2014; Matsumoto et al. 2019) with intermediate PBS washes. Nuclear staining was performed using CUBIC-HV1 with propidium iodide or DAPI for 3 days. Cleared tissues were imaged using the Lightsheet 7 microscope (Zeiss) and reconstructed with Drishti v2.7 and Amira 6.7.

Histological Analysis

14 µm frozen sections were stained with DAPI, and visualized using Leica TCS SP8 or Nikon ECLIPSE Ti microscopes. RNAscope was performed for *in situ* detection of *Vegfc* mRNA, as previously described(Iwayama et al. 2022).

Supplemental Methods

Detailed protocols for Isolation and Culture of Gingival Lymphatic Endothelial Cells, RNA-Sequencing and Ontology Analysis, the Lymphatic Drainage Assay, Histological Time-course Analysis, Apoptosis Assays (Caspase-3/7 and Annexin V), Simple Western protein assay, and Comparative RNA-seq Analysis are provided in the Supplemental File.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 10.5. Sample sizes were determined based on previous studies. For in vivo analyses, n refers to the number of hemijaw samples per group (typically n=6–8). The assumption of homogeneity of variances was assessed using Bartlett's test. As the assumption was not met, Welch's ANOVA was performed, followed by Dunnett's T3 multiple comparisons tests. Differences were considered statistically significant at $p < 0.05$.

Results

Histological Distribution and Remodeling of Lymphatic Vessels in the Periodontium

To investigate the behavior of lymphatic vessels during periodontal injury and repair, we examined their spatial distribution using Prox1-tdTomato and Tie2-GFP; Prox1-tdTomato mice. In healthy gingiva, Prox1-positive lymphatic vessels were predominantly observed within the subepithelial connective tissue, forming a fine, branched network that paralleled the distribution of Tie2-positive blood vessels (Fig. 1A). These lymphatics appeared continuous and well-organized, suggesting a stable and mature vascular architecture under homeostatic conditions. Following ligature-induced periodontal injury, we observed disruption of this network together with alveolar bone loss (Fig. 1B). Upon ligature removal (Day 0), Prox1-positive signals were significantly diminished in the interdental gingiva, and lymphatic structures appeared fragmented and discontinuous. This regression was evident near the marginal gingiva, indicating that acute inflammation and tissue breakdown severely impair the local lymphatic architecture.

To stage the course of tissue repair and guide subsequent analyses, we performed H&E staining at Days 0, 3, 7, and 9 (Supplemental Fig. S1). Day 0 (immediately after ligature removal) showed intense inflammatory cell infiltration and collagen degradation. By Day 3, granulation tissue formation was evident, consistent with active vascular remodeling. By Day 7, inflammation had subsided and tissue architecture was largely restored.

At the later repair stage defined by our H&E time course (Day 7; Supplemental Fig. S2), lymphatic vessels had reemerged throughout the previously damaged areas as alveolar bone levels approached baseline (Fig. 1C). The repaired vessels displayed a complex, interconnected morphology that resembled the original network in healthy tissues. Moreover, blood vessels appeared to reorganize in a parallel manner, often running alongside the newly formed lymphatics. These findings suggest lymphatic vessels are not static but respond to tissue injury through regression and remodeling during healing. The synchronized reorganization of lymphatics and blood vessels also implies potential coordination in their roles during tissue repair.

To complement these temporal observations, we further distinguished blood and lymphatic vasculature using double staining for isolectin GS-IB4 and injected dextran. While blood vessels (GS-IB4⁺) formed a dense capillary network, dextran distribution delineated distinct interstitial drainage pathways in the healing gingiva (Fig. 1D).

3D Visualization of Lymphatic Architecture Using Tissue Clearing

Guided by our histology time course, we focused on Day 3 post-ligature removal, corresponding to the granulation phase with active vascular remodeling and used CUBIC-

based tissue clearing and light-sheet fluorescence microscopy to obtain a three-dimensional view of lymphatic remodeling in the periodontium (Supplemental Fig. S2). This whole-mount approach preserves tissue architecture and vessel continuity without the need for sectioning, preserving spatial relationships between vessels and surrounding tissues. Compared to conventional histology, this method allowed us to appreciate the continuity and branching complexity of the lymphatic network across multiple planes. Accordingly, we analyzed gingival tissues at Day 3 post-ligature removal using light sheet microscopy of cleared samples (Fig. 2A). Notably, we observed striking regional differences in lymphatic vessel density and morphology between the ligated and unligated sides (Fig. 2A). In unligated healthy gingival tissues, Prox1-positive lymphatic vessels exhibited an organized spatial distribution extending from the marginal gingiva toward the periodontal ligament (Fig. 2B, left). The lymphatic network was positioned predominantly in the subepithelial and submucosal layers, forming long, continuous paths with minimal fragmentation, supporting the idea of an established drainage system under physiological conditions (Fig. 2B). In contrast, ligature-induced injury resulted in marked alterations of the lymphatic network (Fig. 2B, right). At day 3 post-ligature removal, cleared tissues displayed dense, newly formed lymphatic structures within the granulation tissue occupying the interdental region. These vessels had tortuous paths, frequent branching, and irregular calibers, suggesting active lymphangiogenesis. The high-resolution imaging confirmed that periodontal lymphatics undergo dynamic structural changes and spatial reorganization during wound healing (Fig. 2B). This dramatic, spatially organized lymphangiogenesis during the repair phase prompted us to investigate the underlying molecular drivers.

Local Vegfc Expression and Therapeutic Effect of VEGF-C C156S

To explore the molecular signals driving lymphatic remodeling, we investigated the expression of vascular endothelial growth factor C (Vegfc), a key lymphangiogenic factor. RNAscope *in situ* hybridization was performed on gingival tissues at day 3 post-ligature removal (Fig. 3A). In uninjured gingiva, Vegfc expression was sparse and localized predominantly to the basal epithelial layer. However, in the ligated gingiva undergoing repair, Vegfc signals were markedly upregulated and expanded across multiple epithelial layers, particularly near the wound margin. These findings suggest that epithelial cells are a major source of Vegfc during the healing process, potentially contributing to local lymphangiogenesis. Given the upregulation of epithelial Vegfc, we next sought to determine whether augmenting this signaling pathway could enhance periodontal repair. To assess the functional significance of this endogenous signal, we administered recombinant VEGF-C C156S—a mutant form of VEGF-C with selective VEGFR-3 activation—directly into the

gingival tissue upon ligature removal. This approach amplified lymphatic-specific signaling without substantially affecting blood vasculature via VEGFR-2. Three days after injection, micro-CT analysis revealed that mice treated with VEGF-C C156S exhibited visibly preserved alveolar bone morphology compared to PBS-treated controls (Fig. 3B). Quantitative analysis of CEJ–ABC distances confirmed that VEGF-C C156S significantly reduced the CEJ-ABC distance compared to the PBS vehicle control (Fig. 3C, mean difference = 0.32 mm, 95% CI [0.08 to 0.58], adjusted $p = 0.013$; Dunnett's T3 test). In contrast, no significant difference was observed between the C156S and PBS groups under control (unligated) conditions ($p = 0.90$). Furthermore, analysis of bone quality parameters revealed significantly higher BV/TV and Tb.Sp. in the VEGF-C treated group compared to controls (Fig. 3D), indicating improved bone quality. The effect was consistent across multiple measurement sites, indicating a robust biological response.

To determine if the structural increase in lymphatics translated to functional recovery, we performed a dye drainage assay. Quantitative analysis at Day 3 demonstrated that VEGF-C treatment significantly accelerated drainage compared to the vehicle group (Fig. 3E). By Day 10, drainage function had returned to comparable baseline levels in both groups. This demonstrates that VEGF-C accelerates the functional restoration of the lymphatic drainage system during the acute healing phase.

Functional Properties of Gingival LECs

To understand the cellular and molecular mechanisms by which VEGF-C C156S exerts this protective effect, we developed a novel *in vitro* model by isolating and culturing primary lymphatic endothelial cells (LECs) directly from the murine gingiva of Prox1-tdTomato mice. We report the first successful establishment and characterization of primary lymphatic endothelial cell cultures derived from murine gingiva. Flow cytometry–based cell sorting enabled the selection of tdTomato⁺ viable LECs, which were subsequently expanded under defined culture conditions (Fig. 4A). The cells retained expression of key lymphatic markers such as Lyve-1 (Fig. 4B) and exhibited tube-forming ability in three-dimensional Matrigel cultures (Fig. 4C). To further characterize the identity of these primary cells, we compared their transcriptomic profile with established lymph node and dermal LEC datasets. PCA demonstrated that gingival LECs cluster separately from lymph node and dermal LECs, indicating a unique tissue-specific signature (Fig. 4D). Heatmap analysis revealed that while all populations expressed core LEC markers and extracellular matrix, they exhibited distinct expression patterns. Notably, YAP gene was highly enriched in gingival LECs compared to other populations (Fig. 4E).

VEGF-C-driven Survival Signaling of Gingival LECs

To evaluate transcriptional responses to lymphangiogenic stimulation, LECs were cultured under serum-starved conditions and treated with VEGF-C C156S for 24 hours, followed by bulk RNA sequencing (Fig. 5A). This 24-hour time point was chosen to capture early transcriptional changes that initiate cellular responses, preceding the phenotypic changes observed at Day 3 *in vivo*. Analysis of differentially expressed genes ($q < 0.1$) revealed that VEGF-C stimulation modulated several apoptosis- and survival-related genes (Fig. 5B). Notably, the anti-apoptotic gene *Siah1b* was upregulated, while *Elf3*, a transcription factor associated with apoptosis induction, was downregulated (Supplemental Table S1 and S2). These results suggest that VEGF-C not only promotes vessel formation structurally, but also enhances LEC viability through transcriptional regulation. Gene Ontology enrichment analysis identified significant clustering of terms related to apoptosis regulation, cell survival, and stress response pathways (Fig. 5C).

To validate the RNA-seq findings functionally, we assessed apoptosis in primary LECs. VEGF-C C156S treatment significantly reduced Caspase-3/7 activity and the proportion of Annexin V-positive cells compared to vehicle control (Fig. 5D, E). Furthermore, Simple Western analysis confirmed the upregulation of *Siah1b* protein and downregulation of *Elf3* protein upon VEGF-C stimulation (Fig. 5F). These results provide robust evidence that VEGF-C signaling promotes LEC survival by suppressing apoptotic pathways.

Discussion

This study provides novel evidence that lymphatic vessels are dynamic and active participants in periodontal tissue repair, a process driven by VEGF-C-mediated survival signaling that promotes their restoration after injury. Our findings position lymphatic remodeling not as a secondary event, but as an integral component of the periodontal repair process, challenging previous perspectives.

The observed regression and subsequent reassembly of lymphatic vessels parallel phenomena reported in cardiac and cutaneous tissues following injury (Gur-Cohen et al. 2019; Liu et al. 2020). The spatial association of newly formed lymphatics with granulation tissue and blood vessels suggests a coordinated neovascularization process. Our study is the first to demonstrate the temporal and functional restoration of periodontal lymphatics. The drainage assay (Fig. 3E) provides physiological evidence that the newly formed vessels are functional and contribute to fluid clearance. This aligns with the concept that efficient drainage is a prerequisite for resolving inflammation and initiating tissue repair. Together, these findings provide molecular and morphological support for a role of VEGF-C signaling in periodontal repair, potentially by promoting organized lymphangiogenesis in injured tissues.

Although lymphangiogenesis in periodontitis has been previously noted in inflammatory context (Mkonyi et al. 2012; Raica et al. 2015), this study provides the first three-dimensional visualization of lymphatic remodeling during periodontal wound healing. These findings emphasize the functional importance of lymphatics as active participants in tissue repair (Hu et al. 2024). Importantly, this study also presents the first successful isolation and functional characterization of gingiva-derived primary LECs, providing a novel tool for future mechanistic studies in periodontal lymphangiogenesis. The comparison with other LEC types (Fig. 4D-E) highlights the unique nature of gingival lymphatics, potentially adapted to the high-mechanical stress environment of mastication.

Our results indicate that VEGF-C not only promotes lymphatic vessel formation but also confers repair effects on periodontal structures. The selective use of VEGF-C C156S, which activates VEGFR-3 without significant VEGFR-2 involvement, allowed us to assess lymphatic-specific effects (Saaristo et al. 2002; Huggenberger et al. 2010). RNA-seq data showed VEGF-C C156S modulated LEC survival through transcriptional regulation of apoptosis-related pathways. This suggests a dual role for VEGF-C in structural and cellular maintenance of lymphatics. Therapeutically, targeting lymphangiogenesis may offer a strategy for periodontal repair distinct from conventional approaches.

Mechanistically, we identified a Siah1b-ElI3 survival axis. Our data suggests that VEGF-C promotes gingival LEC survival in the stressed wound environment, in part by downregulating the pro-apoptotic factor ElI3, which may support timely formation of a functional drainage

network. Because VEGF-C and VEGFR3 effects on LEC viability are context dependent, ranging from pro-survival signaling under growth factor deprivation(Hossain et al. 2024) to sensitization to oxidative stress-induced apoptosis(Mäkinen et al. 2001), we interpret our findings as a tissue- and environment-specific pro-survival response. Overall, enhanced LEC resilience may improve clearance of inflammatory mediators and microbial products, limiting destructive inflammation and facilitating periodontal repair.

Our findings also support the growing body of evidence suggesting that lymphatic vessels play a key role in immunoregulation and regeneration in various tissues(Goto et al. 2022; Niec et al. 2022; Biswas et al. 2023). Aging and systemic diseases such as diabetes are known to impair lymphatic function(Zolla et al. 2015; Shang et al. 2019), which may, in turn, influence periodontal tissue regeneration. In tendon tissue, targeting local lymphatics via the FGFR3-BMPR1a pathway has been reported to ameliorate heterotopic ossification, suggesting that lymphatic-associated mechanisms can influence bone formation processes(Zhang et al. 2021). These findings support the broader concept that the lymphatic system can be a therapeutic target in regenerative medicine(Stritt et al. 2021).

Although periodontitis has been linked to various systemic diseases, the exact mechanisms are not fully understood. Recent studies suggest that periodontitis can exacerbate intestinal inflammation *in vivo*(Kitamoto et al. 2020). Proposed pathways include the ingestion and gut translocation of pathogenic oral bacteria, which then cause intestinal inflammation. Another pathway involves lymphocytes that accumulate in the submandibular lymph nodes and subsequently home to inflamed intestinal lesions(Kitamoto and Kamada 2022). In this study, we described the dense lymphatic network of the gingiva. This network may provide a conduit for lymphocyte trafficking to distant tissues. Further study of lymphatic function in periodontal tissues and its interaction with lymphocytes may provide insights into how periodontitis contributes to systemic disease.

This study has several limitations. First, consistent with recent work highlighting fine-tuning of VEGFR3 signaling in LECs(Kuonqui et al. 2025), the mechanisms by which VEGF-C modulates VEGFR3 downstream signaling in gingival LECs remain incompletely defined. Further *in vitro* studies using gingival LECs and pathway-specific genetic models targeting VEGF-C/VEGFR3 signaling are needed. Second, the interactions between lymphatics and other stromal or immune cells(Yeo and Angeli 2017; Koning and Mebius 2020; Wei et al. 2023) in the periodontal niche remain unexplored. Further studies are needed to determine whether lymphatics serve merely as structural components or also as instructive elements in the repair microenvironment(Frye et al. 2018).

In summary, this study offers novel insights into the role of lymphatic vessels in periodontal wound healing. Using transgenic reporter mice, tissue clearing, and RNA sequencing, we

demonstrated that lymphatics are actively remodeled following periodontal injury and contribute to tissue repair. Local expression of *Vegfc* was upregulated during the healing phase, and exogenous administration of VEGF-C C156S significantly enhanced periodontal tissue repair, supporting the therapeutic potential of lymphangiogenic stimulation. *In vitro* analyses further revealed that VEGF-C promotes survival of gingival lymphatic endothelial cells through regulation of apoptosis-related genes. Modulating lymphangiogenesis may enhance regenerative outcomes in periodontitis and related conditions. Future studies should elucidate molecular crosstalk between lymphatics and immune or stromal cells in the periodontal niche, and evaluate lymphatic-targeted therapies in translational and clinical settings.

Authors' Contributions

T. Iwayama contributed to the conception and design of the study, data acquisition, analysis, and interpretation, and drafted and critically revised the manuscript. S. Matsumoto contributed to the study design, data acquisition, and interpretation, performed all statistical analyses, and drafted and critically revised the manuscript. S. Murakami and M. Takedachi contributed to the conception and design of the study and critically revised the manuscript. P. Bhongsatiern, Y. Yoshida, Y. Koketsu, E. Tsuboi, S. Takashiba, and Y. Sato contributed to data acquisition and interpretation and critically revised the manuscript. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work.

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Declaration of Conflicting Interests

Y. Sato is an employee of Carl Zeiss Co., Ltd.

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Data availability

The study protocol was not pre-registered in a publicly available repository before the start of the study. The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Figure legends

Figure 1. Histological distribution of lymphatic and blood vessels in the gingiva.

(A) Confocal images of Tie2-GFP; Prox1-tdTomato mice showing co-localization of lymphatic vessels (magenta) and blood vessels (green) in the gingival connective tissue beneath the oral epithelium. (B-C) Fluorescence microscopy of the interdental gingiva reveals a transient loss of Prox1-tdTomato⁺ lymphatic vessels immediately after ligature removal (B) and their reappearance 7 days after ligature removal (C). Each panel is composed of a low-magnification overview (left) and a corresponding high-magnification image (right). The white dashed square in the overview denotes the area shown at higher magnification. White arrowheads mark anatomical reference points: the upper arrowhead indicates the cemento-enamel junction (CEJ), and the lower arrowhead marks the height of the alveolar bone crest (ABC); therefore, a larger vertical distance between the two arrowheads reflects a greater degree of alveolar bone resorption. Arrows indicate lymphatic vessels (magenta) and blood vessels (green). E, epithelium; CT, connective tissue; B, bone; G, gingiva. Scale bars: 100 μ m. (A-C) (D) Visualization of vascular networks using GS-IB4 (red, epithelium and underneath vessels) and functional perfusion/drainage paths visualized by Dextran injection (cyan). Representative image from multiple sections prepared and examined from at least three mice.

Figure 2. 3D Light-Sheet Microscopy of Cleared Gingival Tissue

(A) Light sheet microscopy image of CUBIC-cleared maxillae from Day 3 post-ligature removal Prox1-tdTomato mice. Increased Prox1⁺ lymphatic vessel density and branching observed in the marginal gingiva of the ligated side compared to the unligated control. Scale bars: 1 mm. (B) Reconstructed 3D images demonstrate continuous lymphatic networks (green arrows) of the ligated side compared to the unligated control. Scale bars: 100 μ m.

Figure 3. VEGF-C enhances periodontal healing

(A) RNAscope *in situ* hybridization showing increased *Vegfc* mRNA expression (magenta) in the gingival epithelium on the ligated side compared to the contralateral side. Nuclei counterstained with DAPI (blue). Scale bars: 100 μ m. E: epithelium. (B) 3D micro-CT images of maxillary alveolar bone in control (PBS) and VEGF-C-treated mice. Representative images from 6–7 samples per group. (C) Quantification of CEJ–ABC distances using 3-point and 8-point methods from 6–7 independent samples per group. * $p < 0.01$ (Dunnett's T3 test). (D) Quantitative analysis of bone quality parameters (BV/TV, Tb.Th, Tb.Sp) assessed by micro-CT. ** $p < 0.01$ (Šídák's multiple comparisons test). (E) Lymphatic drainage assay. Representative images of dye clearance and quantification of remaining dye intensity (% dye

area) at Day 3 and Day 10. **** $p < 0.0001$ (Šídák's multiple comparisons test).

Figure 4. Isolation and characterization of gingival lymphatic endothelial cells

(A) Representative FACS plots primary LEC isolation. tdTomato⁺ cells were sorted from a single cell suspension of Prox1-tdTomato gingiva for *in vitro* primary cell culture. (B) LEC marker expression. Immunocytochemistry of sorted Prox1-tdTomato⁺ cells confirmed the expression of Lyve-1. (C) *In vitro* tube formation assay. 3D Matrigel cultures of established gingival lymphatic endothelial cells showed tube-like structures. (A-C) Representative plots/images from at least three independent experiments. (D) Comparative transcriptomic signature of gingival LECs. PCA plot showing distinct clustering of Gingival LECs compared to Lymph Node and Dermal LECs. (E) Heatmaps of LEC markers, ECM interaction genes, and YAP target genes.

Figure 5. Transcriptomic response of LECs to VEGF-C stimulation.

(A) RNA-seq experimental workflow. After a 2-day starvation, LECs were treated with VEGF-C or vehicle for 1 day. (B) MA plot showing differential gene expression between VEGF-C vs. vehicle. The x-axis shows the mean log₂ read count across the two groups, and the y-axis shows the log₂ fold-change. Significantly up- or downregulated genes are highlighted in magenta ($q < 0.10$), while non-significant genes are shown in black. (C) Gene Ontology enrichment analysis showing apoptosis-related pathways. (D) Annexin V/PI Flow Cytometry analysis. ** $p < 0.01$ (unpaired t-test, $n=6$ wells). (E) Caspase-3/7 activity assay. ** $p < 0.01$ (Šídák's multiple comparisons test, $n=3$ wells). (F) Simple Western protein analysis of Siah1 and E11 protein levels.

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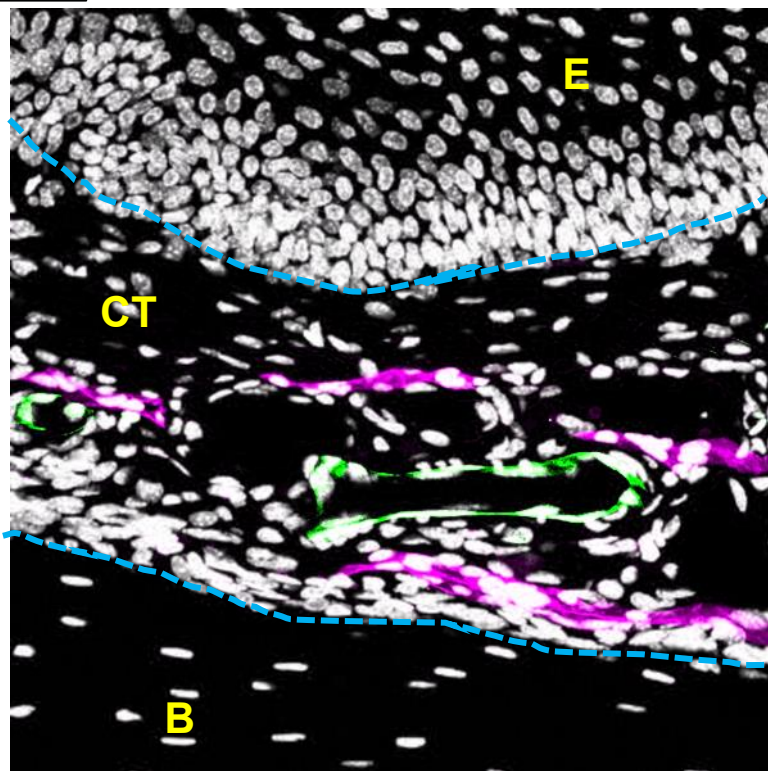
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A Healthy DAPI *Prox1-tdTomato* *Tie2-GFP*

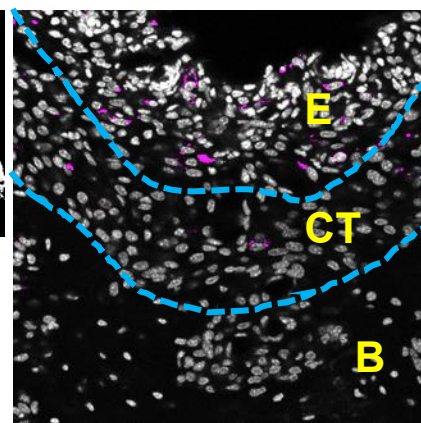
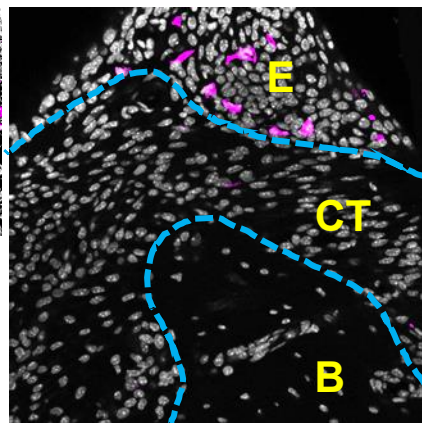
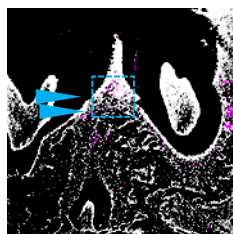


B Day 0

Control

Ligature (injury)

DAPI *Prox1-tdTomato*

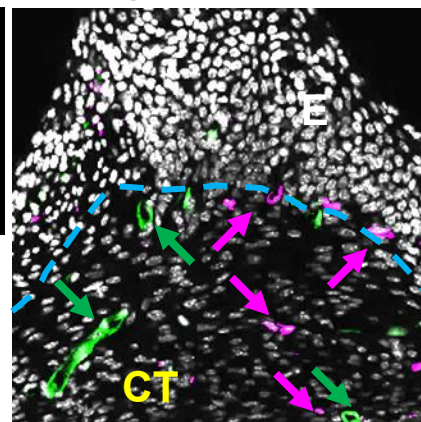
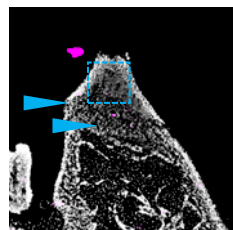
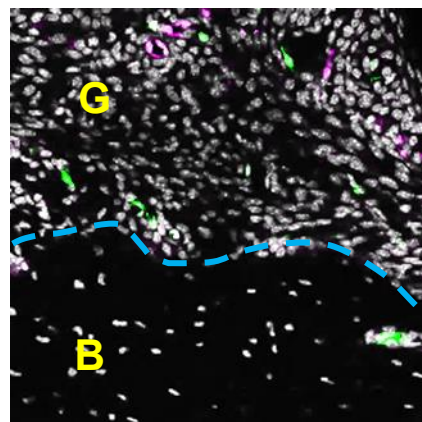
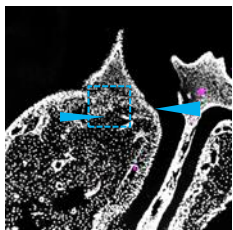


C Day 7

Control

Ligature (repair)

DAPI *Prox1-tdTomato* *Tie2-GFP*



D

Fig.1 cont'd

DAPI GS-IB4 Dextran

GS-IB4

Dextran

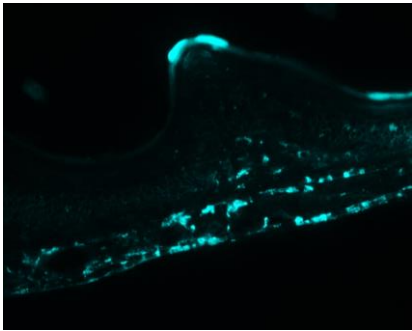
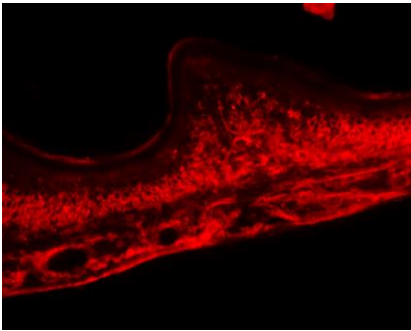
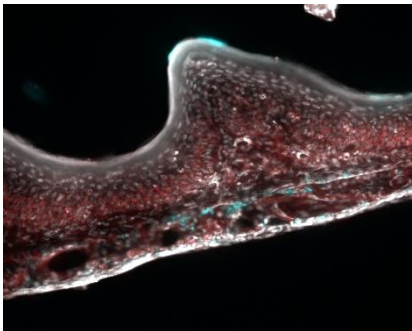


Fig.2

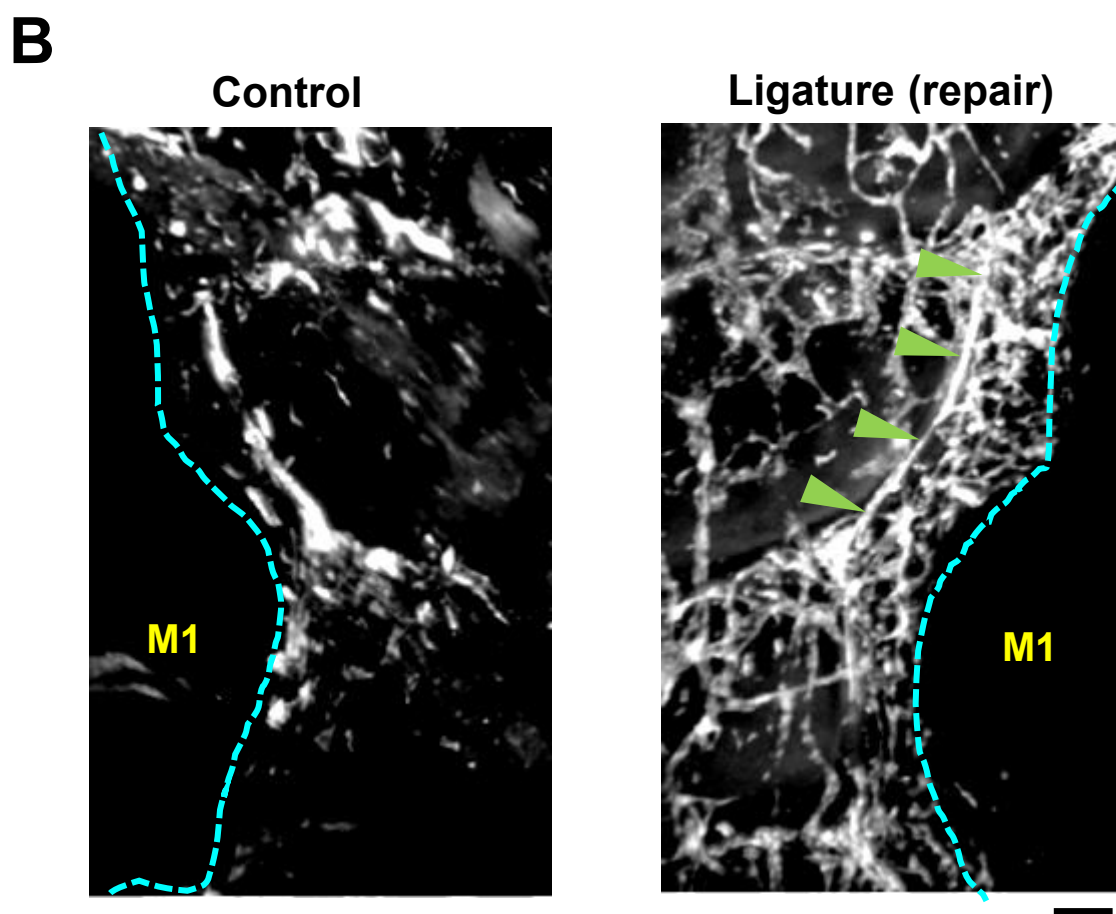
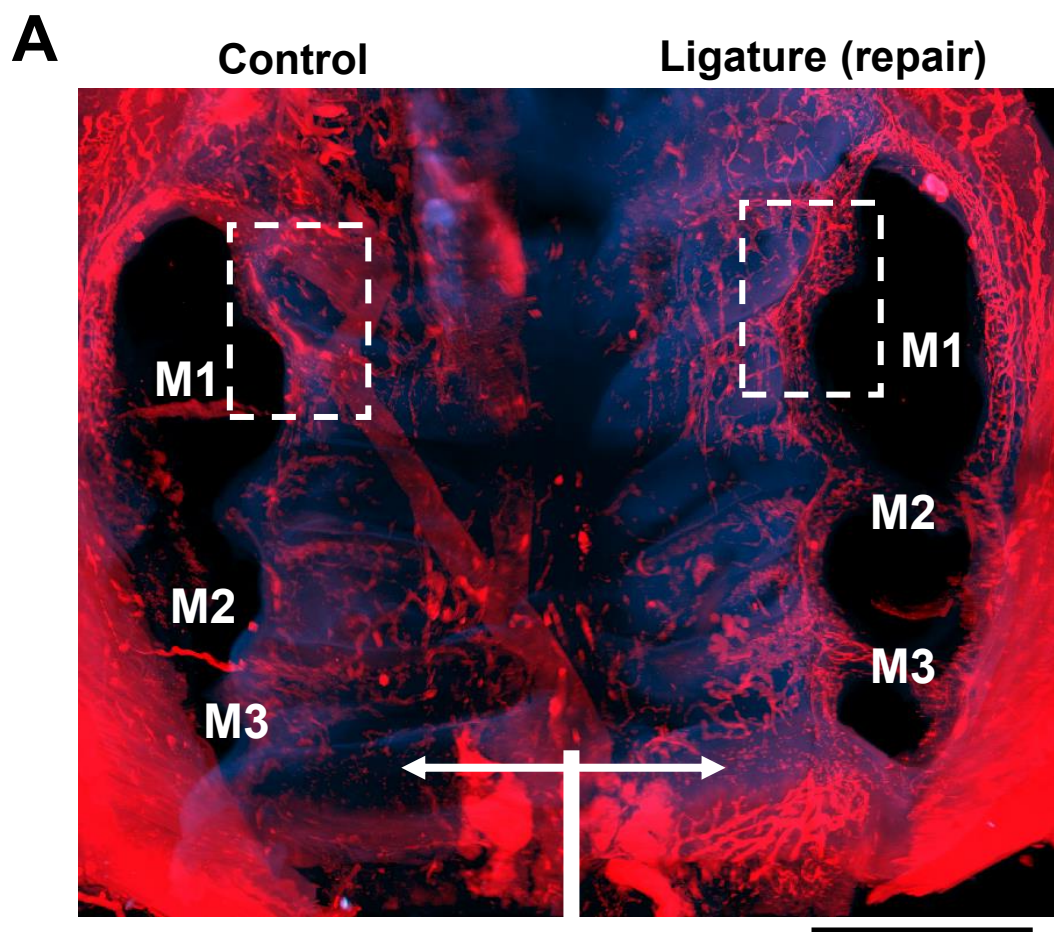
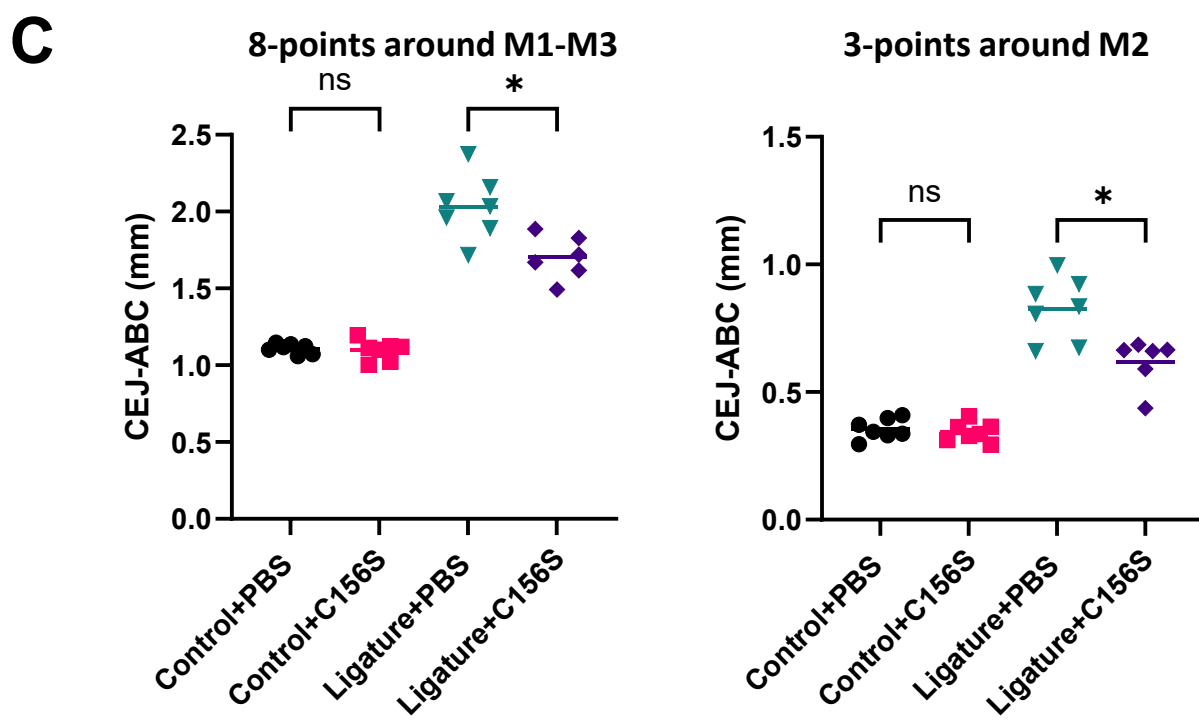
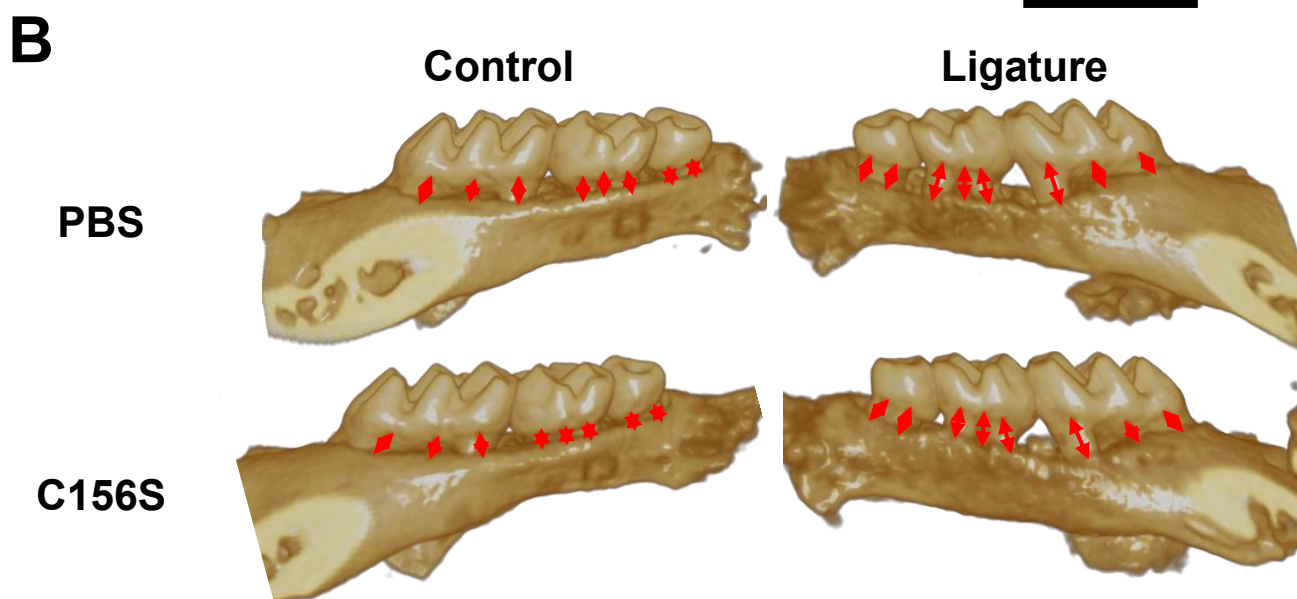
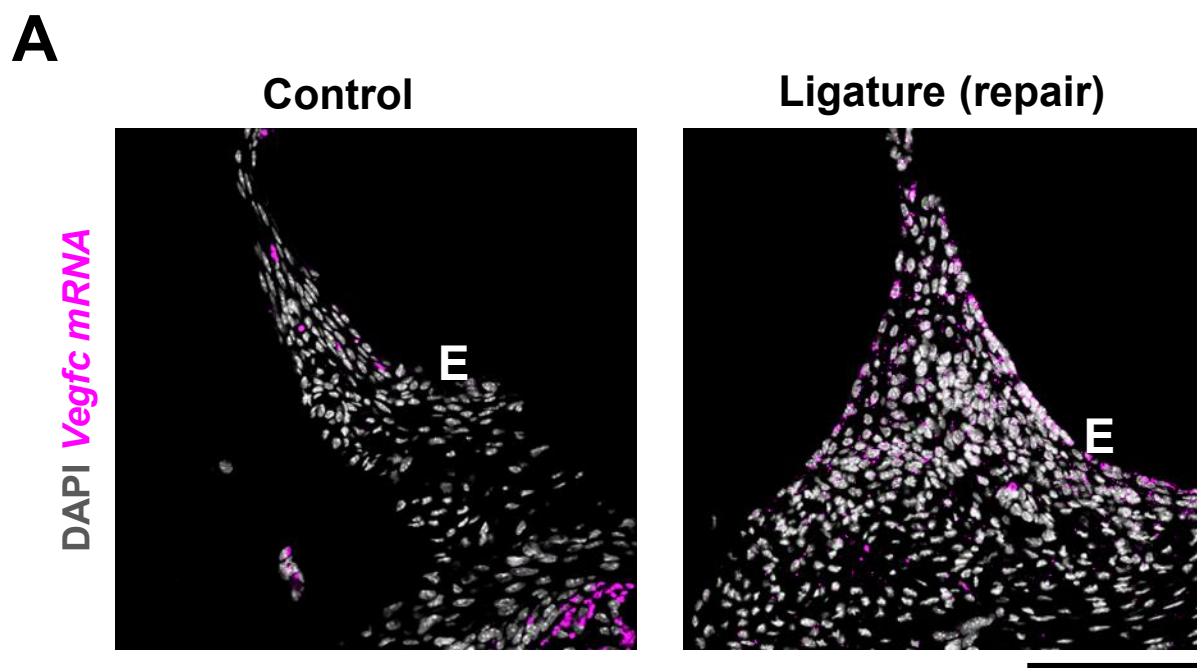
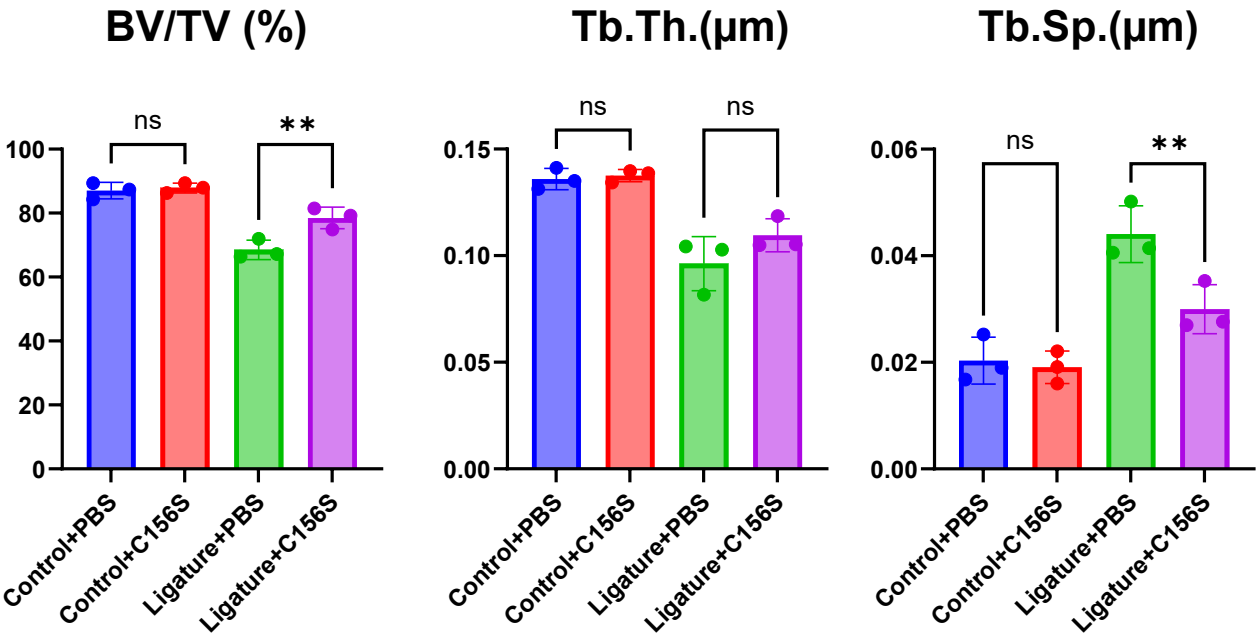


Fig.3



D



E

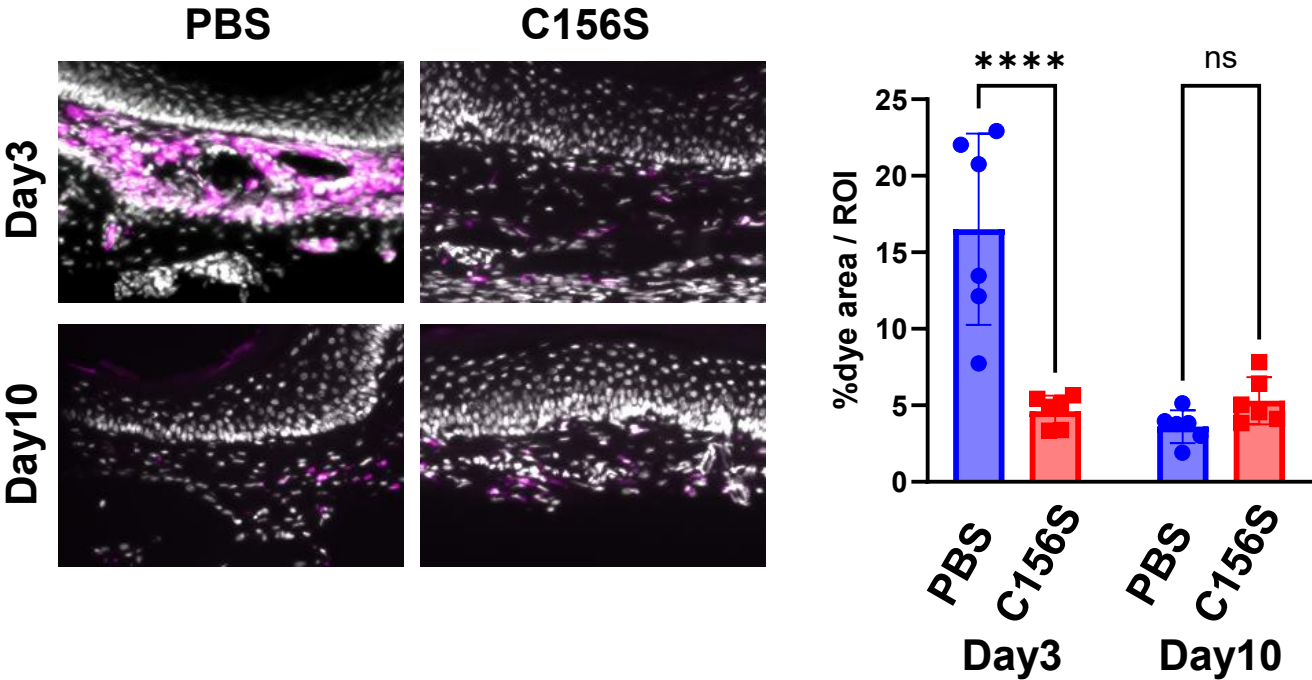
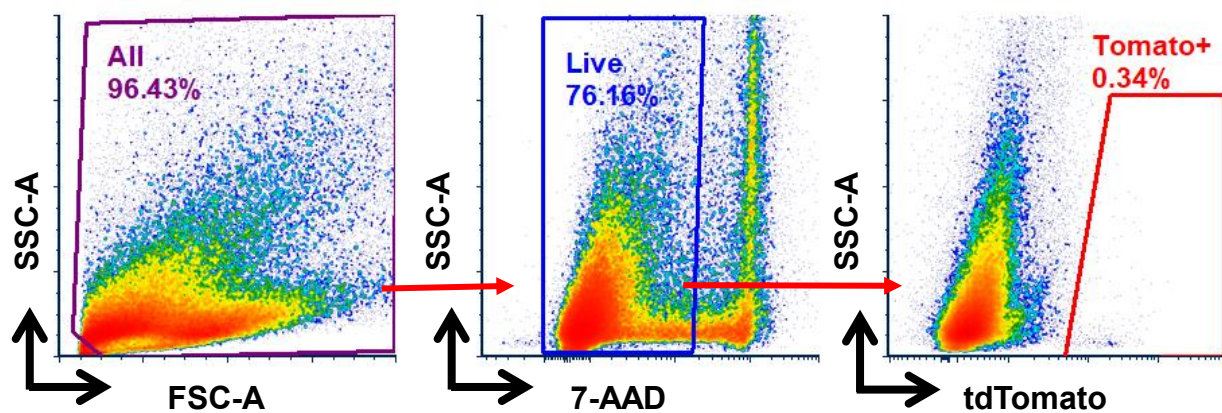
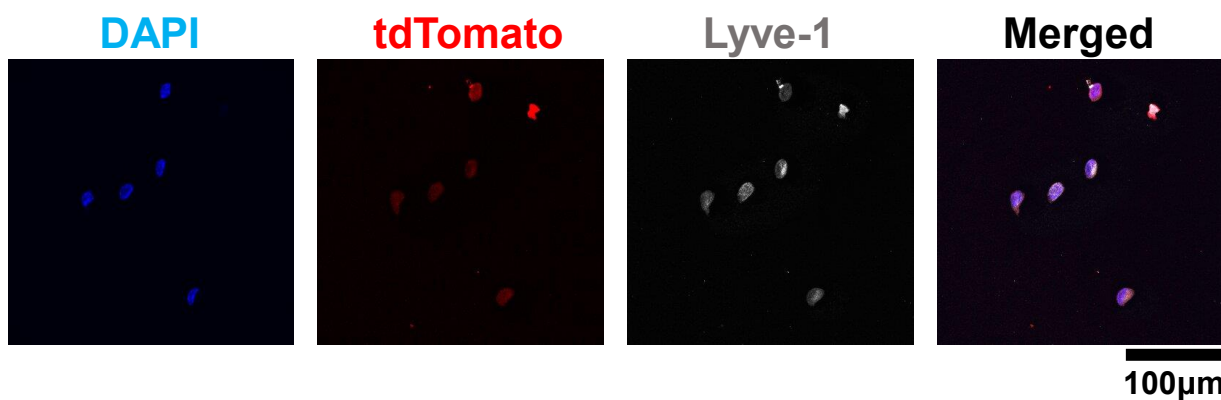
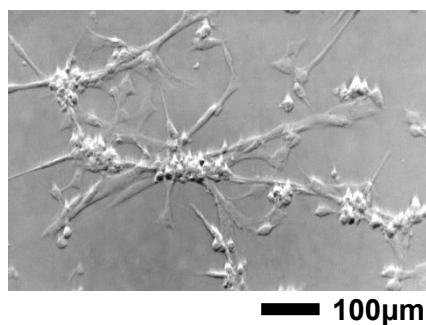
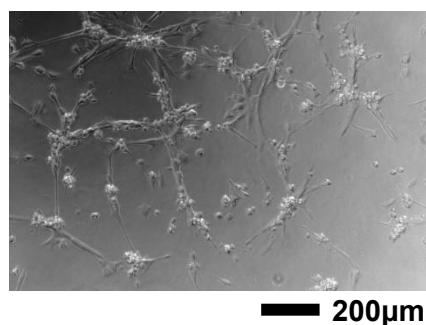
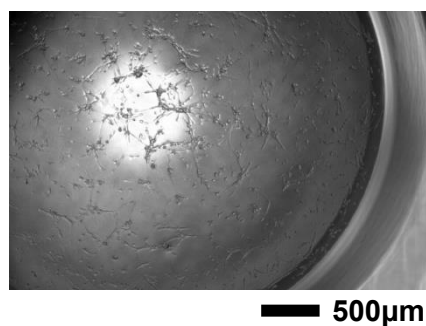
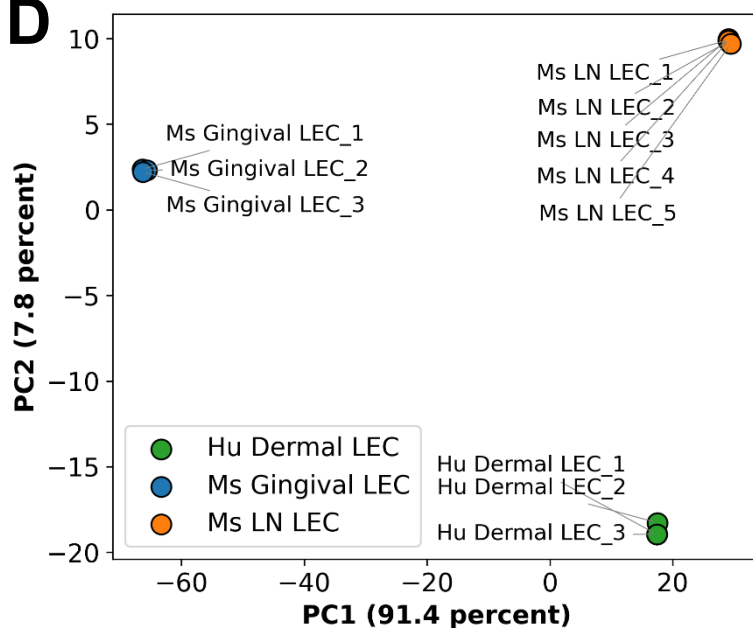


Fig.4

A**B****C****D****E**