L1 Peptide–Conjugated Fibrin Hydrogels Promote Salivary Gland Regeneration

Appendix

MATERIALS AND METHODS

Materials

Lyophilized fibrinogen from human plasma was purchased from EMD Millipore (Billerica, MA). Spectra/Por 7 dialysis membrane (MWCO = 3.5 kDa) was purchased from Spectrum Laboratories (Rancho Dominguez, CA). A Whatman syringe filter (0.8 μm) was purchased from GE Healthcare Life Sciences (Pittsburgh, PA). A Millex syringe filter (0.22 μm) was purchased from Merck Millipore (Billerica, MA). Paraformaldehyde (PFA) was purchased from Baker (Phillipsburg, NJ). Insulin-transferrin-sodium selenite media supplement, retinoic acid, hydrocortisone, gentamicin, epidermal growth factor (EGF) from murine submaxillary gland, $_{DL}$-dithiothreitol (DTT) and ε-aminocaproic acid (εACA) were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's modified eagle medium/nutrient mixture F-12 (1:1) (DMEM/F12 (1:1)), fetal bovine serum (FBS), glutamine, Lab-Tek chambered coverglass (8-well), Alexa Fluor 568 conjugated phalloidin, sulfosuccinimidyl 6-(3'-(2-pyridyldithio)propionamido)hexanoate (Sulfo-LC-SPDP) and DyLight 680 NHS-ester were purchased from Thermo Fisher Scientific (Newington, NH). Mouse anti-E-cadherin antibody was purchased from BD Biosciences (San Jose, CA). TO-PRO-3 iodide, rabbit anti-zonula occludens-1 (ZO-1) antibody, Alexa Fluor 488 conjugated anti-rabbit IgG secondary antibody and Alexa Fluor 568 conjugated anti-mouse IgG secondary antibody were purchased from Invitrogen (Carlsband, CA). Rabbit anti-aquaporin 5 (AQP5), rabbit anti-TMEM-16A, rabbit anti-Ki67, and Picro Sirius Red Stain Kit were

purchased from Abcam (Cambridge, MA). Mouse Na^+/K^+-ATPase α antibody was purchased Santa Cruz Biotechnology (Santa Cruz, CA). Masson's Trichrome Stain kit was purchased from Polysciences (Warrington, PA). Peptides were synthesized by University of Utah DNA/Peptide synthesis core facility.

Synthesis of L1 Peptides and L1 peptide-conjugated fibrinogen

The overall synthesis scheme of peptides and peptide-conjugated fibrinogen was the same as previously reported (Nam et al. 2016). Briefly, two biologically active L1 peptides (YIGSR and A99) containing a cysteine and two glycine residues at the N-terminus were designed for site-specific modification. Then, peptides were prepared by Fmoc solid-phase peptide synthesis. The peptide sequences were as follows: A99 (CGGALRGDN-amide), YIGSR (CGGADPGYIGSRGAA-amide). In order to create a thiol-reactive fibrinogen, sulfo-LC-SPDP was used as a cross-linker. The primary amine groups of fibrinogen were reacted with the cross-linker. Then, the activated fibrinogen was conjugated with cysteine-ended L1 peptides (Appendix Fig. 1). The final product was purified, lyophilized, and then stored at -80 ºC.

DyLight 680 conjugated fibrinogen

To monitor fibrin hydrogels stability *in vivo*, fluorescently labeled fibrinogen was also produced. Briefly, lyophilized fibrinogen (51.28 mg) was dissolved in 0.05 M sodium borate buffer at pH 8.5 (10 mg/mL). One hundred microliters of DyLight 680 solution (10 mg/mL, DMF) was added to the fibrinogen solution and incubated for 1 h at room temperature. Non-

reacted reagent was removed from fibrinogen solution by dialysis (MWCO = 3.5 kDa). The concentration of dye labeled fibrinogen was calculated using the following equation:

$$Dye\ labeled\ fibrinogen\ (mg/mL) = \frac{A_{280} - (A_{684} \times 0.128)}{\varepsilon_{Fib}} \times DilutionFactor\ (eq.1)$$

where $\varepsilon_{Fib,}$ the extinction coefficient at 280 nm for human fibrinogen, is 1.51 mL mg^{-1} cm^{-1}.

The degree of labeling was calculated using the following equation:

$$Moles\ dye\ per\ mole\ protein = \frac{A_{684}\ of the\ labeled\ protein \times DilutionFactor}{\varepsilon_{Fluor} \times protein\ concentration\ (M)}\ (eq.2)$$

where $\varepsilon_{Fluor,}$ the extinction coefficient at 684 nm for DyLight 680 Dye, is 140,000 ($M^{-1}cm^{-1}$).

Fibrin hydrogels preparation

For *in vitro* experiments, FH was fabricated by dissolving fibrinogen (2.5 mg/mL) and plasma-derived bovine thrombin (2.5 U/mL) in Tris-buffered saline (TBS) with $CaCl_2$ (2.5 mM) and εACA (2 mg/mL). For L_{1p}-FH, YIGSR-conjugated fibrinogen (1.25 mg/mL) and A99-conjugated fibrinogen (1.25 mg/mL) were used as monomers instead of fibrinogen (2.5 mg/mL). Then, one hundred microliters of hydrogel mixture were applied per well in eight-well chambers and allowed to solidify at 37°C in a humidified incubator. For *in vivo* experiments, L_{1p}-FH^{680} (DyLight 680 labeled L_{1p}-FH) was generated by dissolving YIGSR-conjugated fibrinogen (1.00 mg/mL) and A99-conjugated fibrinogen (1.00 mg/mL), DyLight 680 labeled fibrinogen (0.5 mg/mL) and plasma-derived bovine thrombin (2.5 U/mL) in TBS with $CaCl_2$ (2.5 mM) and εACA (2 mg/mL). For the control group, FH^{680} (DyLight 680 labeled FH) was fabricated by mixing fibrinogen (2 mg/mL), DyLight 680 labeled fibrinogen (0.5 mg/mL) and plasma-derived bovine thrombin (2.5 U/mL) in TBS with $CaCl_2$ (2.5 mM) and εACA (2 mg/mL). Then, twenty microliters of fibrin hydrogel mixture were added at the wound site.

	Volume used	Hydrogels	Composition (picomole)			
			Fibrinogen	**DyLight 680**	**YIGSR**	**A99**
In vitro	100 μL	FH	733.1	-	-	-
	100 μL	L_{1p}-FH	716.7	-	2141.0	2159.1
In vivo	20 μL	FH^{680}	146.3	144.9	-	-
	20 μL	L_{1p}-FH^{680}	143.6	144.9	342.6	345.5

Appendix Table 1. Composition of fibrin hydrogels

Preparation of mouse submandibular gland cell clusters

Female C57BL/6 mice at 5-7 weeks of age were anesthetized via intraperitoneal (IP) injection with 80-100 mg/kg Ketamine and 5 mg/kg Xylazine. Mice were euthanized by abdominal exsanguination and submandibular glands were removed, processed and plated as described below. All animal usage, anesthesia, and surgery were conducted with the approval of the University of Utah Institutional Animal Care and Use Committee, in accordance with their strict guidelines.

Freshly dispersed cell clusters from C57BL/6 mSMGs were prepared as follows: mSMGs were minced and then placed in a 35 mL GentleMACS C Tube (Miltenyi Biotec, Auburn, CA) containing dispersion medium [6.5% (v/v) tumor dissociation enzyme mix (Miltentyi Biotec) in DMEM]. Following manufacturer's instruction, the tissue was enzymatically and mechanically digested using alternating steps of dissociation with the GentleMACS Dissociator (Miltenyi Biotec) and 20 min incubation at 37°C in a shaking water bath. After three dissociation steps and two incubations, the cells were centrifuged at 150 × g for five min at 37°C and the dispersion medium was removed. Cells were then resuspended in 10 mL complete medium [DMEM/Ham's F-12 (1:1) containing 2.5% (v/v) FBS along with the following supplements: 2 nM

triiodothyronine, 0.1 μM retinoic acid, 0.4 μg/ml hydrocortisone, 80 ng/ml EGF, 5 ng/ml sodium selenite, 5 mM glutamine, 5 μg/ml insulin, 5 μg/ml transferrin] and passed sequentially through 100 μm, 70 μm and 40 μm cell strainers. Cells were then centrifuged at 150 ×g for five min at 37°C and resuspended once more in complete medium (described above). Cells were counted using a hemocytometer and plated as described below.

Plating of mSMG cell clusters on FH or L_{1p}-FH

One hundred microliters of FH or L_{1p}-FH were pipetted into wells of 8-well chambered coverglass slides and allowed to set for 24 h at 37°C. Then, freshly dissociated mSMG (described above) cell clusters were plated at a density of approximately 20-30,000 cell clusters per well and allowed to attach for 24 h. The following day, cells were washed once to remove dead and suspended cells, and medium (described above) was replaced every other day prior to fixation, bright field and confocal analysis.

Counting of mSMG cell clusters on FH or L_{1p}-FH

After six days of growth, the mSMG cell clusters were counted via bright field imaging. Clusters were counted prior to fixation so as to capture any clusters that might wash away during fixing procedures. Using an EVOS XL Core (Life Technologies, Grand Island, NY) microscope, two images were captured per well (top left and bottom right) using a 4× objective. Round sphere-like cell clusters were counted and divided by the area imaged to determine the number of cell clusters/mm^2 (Fig. 1). Only round clusters possessing clearly delineated smooth dark edges were counted, so as to exclude non-sphere-like clusters or clumps.

Surgical procedure

To demonstrate the ability of L_{1p}-FH^{680} to form new and functional tissue, we designed an animal model of surgically wounded mSMG. For pain control animals were given Carprofen (5 mg/kg) orally 1 dose prior to surgery which continued daily as needed for pain. In addition, mice received a local anesthetic (Marcaine) at the surgical site at the time of surgery. For the surgical procedure, mice were anesthetized with 3% isoflurane with an oxygen flow rate set at approximately 2.0 L/min. Then, a skin incision of approximately 1 cm in length was made along the anterior surface of the neck (Fig. 2A). Then, both mSMG were exposed and a circular surgical wound was created using a 3 mm diameter biopsy punch (Fig. 2B). To determine the effects of L_{1p}-FH^{680} on formation of new glandular tissue, the wound in one mSMG was filled either with L_{1p}-FH^{680} or FH^{680} while the contralateral gland was left with no scaffold (Fig. 2C). Finally, the skin incision was sutured (Fig. 2D) and post-surgical studies at days 3 and 8 were performed. The number of animals used in this experiments and the times studied are summarized in Appendix Table 2.

	None	FH^{680}	L_{1p}-FH^{680}
3 day	5	5	5
8 day	5	5	5

Appendix Table 2. Number of mice used in this study

Monitoring of Scaffold Stability

Fluorescence signal intensity of DyLight 680 labeled hydrogel was measured *in vivo* using the Xenogen IVIS 100 Bioluminescent Imager (University of Utah, Center for Quantitative Cancer Imaging) at post-surgery days 3 and 8 (Fig. 2E, 2F). Briefly, mice were anesthetized as described above and fluorescent images were acquired with filter set with excitation/emission filters at

692/712 nm. The fluorescence values were then corroborated with dissected glands using a Bio-Rad Chemi-Doc^{TM} MP imaging system (Bio-Rad, Hercules, CA) (Fig. 2G, H).

Hematoxylin and eosin staining

Gland sections from each group were deparaffinized with xylene and rehydrated with serial ethanol solutions (100%, 70%, and 50%) and distilled water. The rehydrated sections were stained with Harris Hematoxylin for 6 min. Then, hematoxylin stained sections were washed with distilled water for 2 min, 0.5% lithium carbonate (Li_2CO_3 (w/v)) for 1 min, and distilled water for 1 min. Slides were then washed with 95% ethanol for 1 min, followed by a 1 min incubation with Eosin and washed with 95% ethanol for 1 min. Finally, hematoxylin and eosin stained gland sections were washed three times with 100% ethanol, cleared in xylene, and mounted with a xylene-based mounting medium. The samples were examined using a Leica DMI6000B inverted microscope (Leica Microsystems, Wetzlar, Germany).

Masson's trichrome staining

The rehydrated sections were re-fixed in Bouin's solution at 60°C for 1 h. To remove yellow color from sections, re-fixed sections were rinsed in running tap water for 10 min. Then, sections were washed with distilled water for 5 min. For nuclei staining, sections were stained with Weigert's iron hematoxylin solution for 10 min, rinsed with running warm tap water for 10 min, and washed with distilled water for 5 min. For cytoplasm staining, sections were incubated with Biebrich scarlet acid fuchsine solution for 5 min, and washed three times with distilled water for 2 min. For collagen staining, sections were incubated in phosphotungstic/phosphomolybdic acid for 15 min, transferred directly to aniline blue solution, stained for 5 min, and washed three times

with distilled water for 2 min. Then, stained sections were differentiated in 1% acetic acid solution for 1 min, washed two times with distilled water for 2 min. Finally, sections were dehydrated with serial ethanol solutions (95% and 100%), cleared in xylene, and mounted with a xylene-based mounting medium. The samples were examined using a Leica DMI6000B.

Picrosirius red staining

The rehydrated sections were stained with picrosirius red solution for 1 h, and washed three times with acetic acid solution (0.5%). Then, stained sections were rinsed three times in absolute ethanol, cleared in xylene, and mounted with a xylene-based mounting medium. The samples were examined using a Leica DMI6000B.

Confocal microscopy

In vitro: mSMG cell clusters were fixed in 4% paraformaldehyde for 20 min at room temperature, incubated with 0.1% Triton X-100 in phosphate buffered saline (PBS) for 5 min and washed with PBS. Cells were then incubated with 5% goat serum, containing 10 μM digitonin for 2 h at room temperature and washed three times with PBS. Spheres were stained for 20 min with Alexa Fluor 568 conjugated phalloidin to stain for F-actin (1:400 dilution in PBS; Sigma) and counter-stained with TO-PRO-3 iodide at room temperature for 15 min at 1:1000 dilution and washed 3 times with PBS, for 5 min each. Finally, specimens were analyzed using a confocal Zeiss LSM 700 microscope using a 20× objective. A total depth of 20 μm was acquired for each sample, and total projection was visualized in the xy planes.

In vivo: mSMG tissue sections were immersed in 10% neutral formalin at room temperature for at least 24 h, dehydrated in serial ethanol solutions (50%, 70%, and 100% for 2 h each),

embedded in paraffin wax and cut into 7 μm sections. Sections from each group were deparaffinized with xylene and rehydrated with serial ethanol solutions (100%, 70%, and 50%). Sections were rinsed with distilled water three times, and then incubated in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) at 95 °C for 30 min. Then, sections were washed with distilled water and permeabilized with 0.1% Triton X-100/PBS at room temperature for 45 min, blocked in 5% goat serum in PBS for 1 h at room temperature, and incubated at 4 °C with the following combinations of primary antibodies in 5% goat serum overnight as follows: rabbit anti-Ki67 (1:200 dilution), rabbit anti-ZO-1 (1:100 dilution), mouse anti-E-cadherin (1:100 dilutions), rabbit anti-TMEM-16A (1:100 dilutions), mouse anti-cytokeratin 7 (K7, 1:500 dilution), rabbit anti-aquaporin 5 (AQP5, 1:200 dilution) and mouse anti-Na^+/K^+-ATPase α antibody (1:200 dilution). Tissue sections were washed three times for 5 min with PBS and incubated for 1 h with Alexa Fluor 488 conjugated anti-rabbit secondary antibody (1:500 dilution) and Alexa Fluor 568 conjugated anti-mouse IgG secondary antibody (1:500 dilution) in 5% goat serum at room temperature. Sections were then washed three times with PBS, for 5 min each, counter-stained with TO-PRO-3 iodide at room temperature for 15 min (1:1000 dilution) and washed 3 times with PBS, for 5 min each. Finally, specimens were analyzed using a confocal Zeiss LSM 700 microscope using a 10× or 20× objective. A total depth of 7 μm was acquired for each sample, and total projection was visualized in the xy planes.

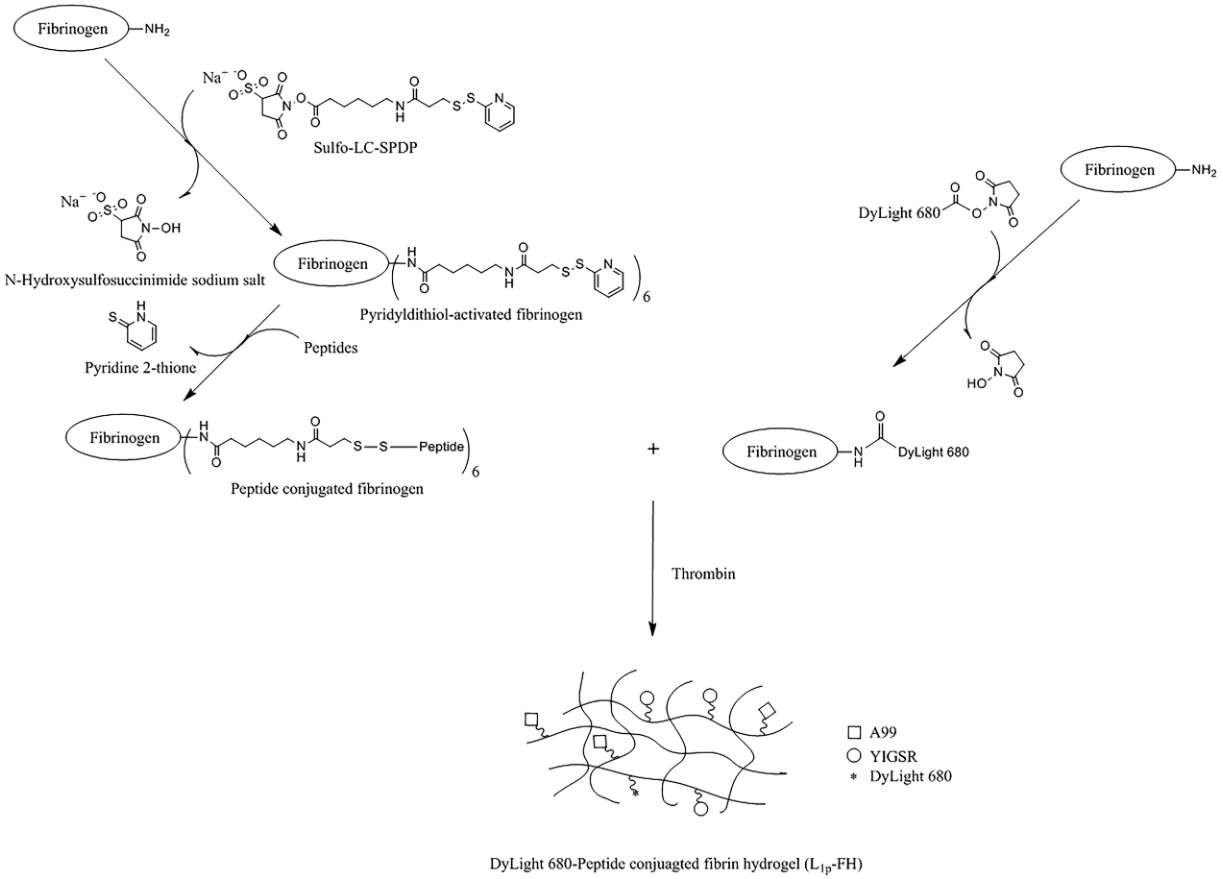
Ki67 positive cells quantification

For this study, we used five tissue sections per group. Confocal images of samples were captured at 20× magnification using a confocal Zeiss LSM 700. The percentage of Ki67 positive cells was calculated using ImageJ software. All statistical analyses were performed with

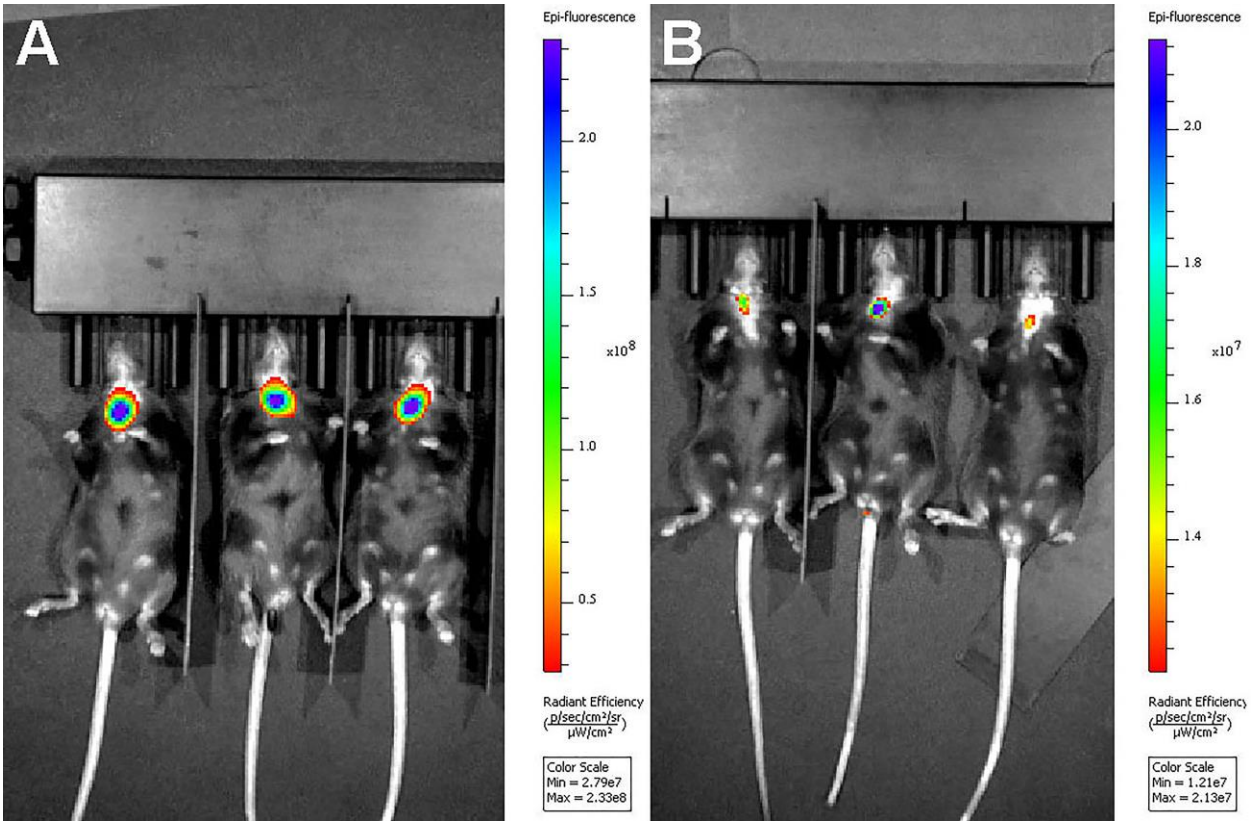
GraphPad Prism 6. Data were analyzed by one-way analysis of variance (ANOVA) followed by pairwise post hoc Tukey's t-tests where $p < 0.001$ represents significant differences between experimental groups.

APPENDIX REFERENCE

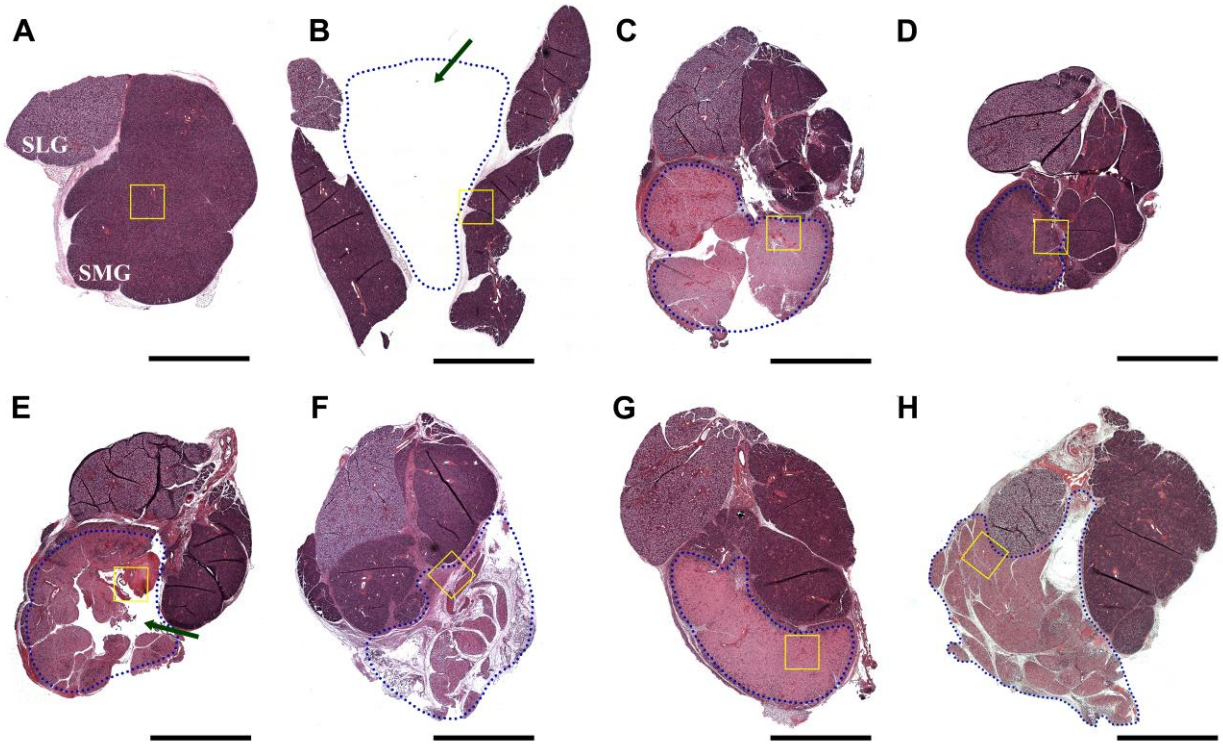
Nam K, Jones JP, Lei P, Andreadis ST, Baker OJ. 2016. Laminin-111 peptides conjugated to fibrin hydrogels promote formation of lumen containing parotid gland cell clusters. Biomacromolecules. 17(6):2293-2301.



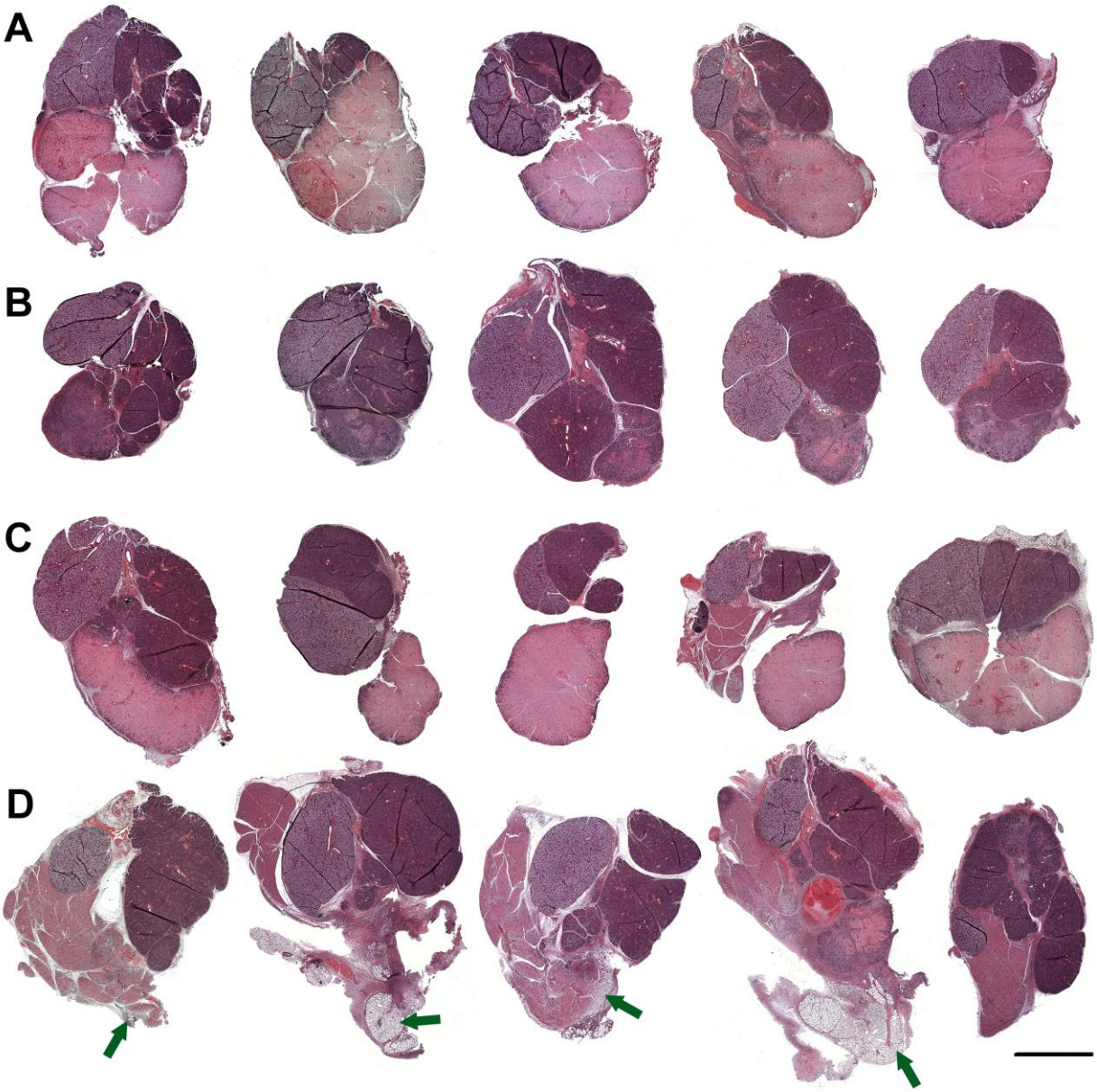
Appendix Fig. 1. The synthesis scheme and structure of L1 peptide and DyLight 680 conjugated fibrin hydrogel (L_{1p}-FH^{680}).



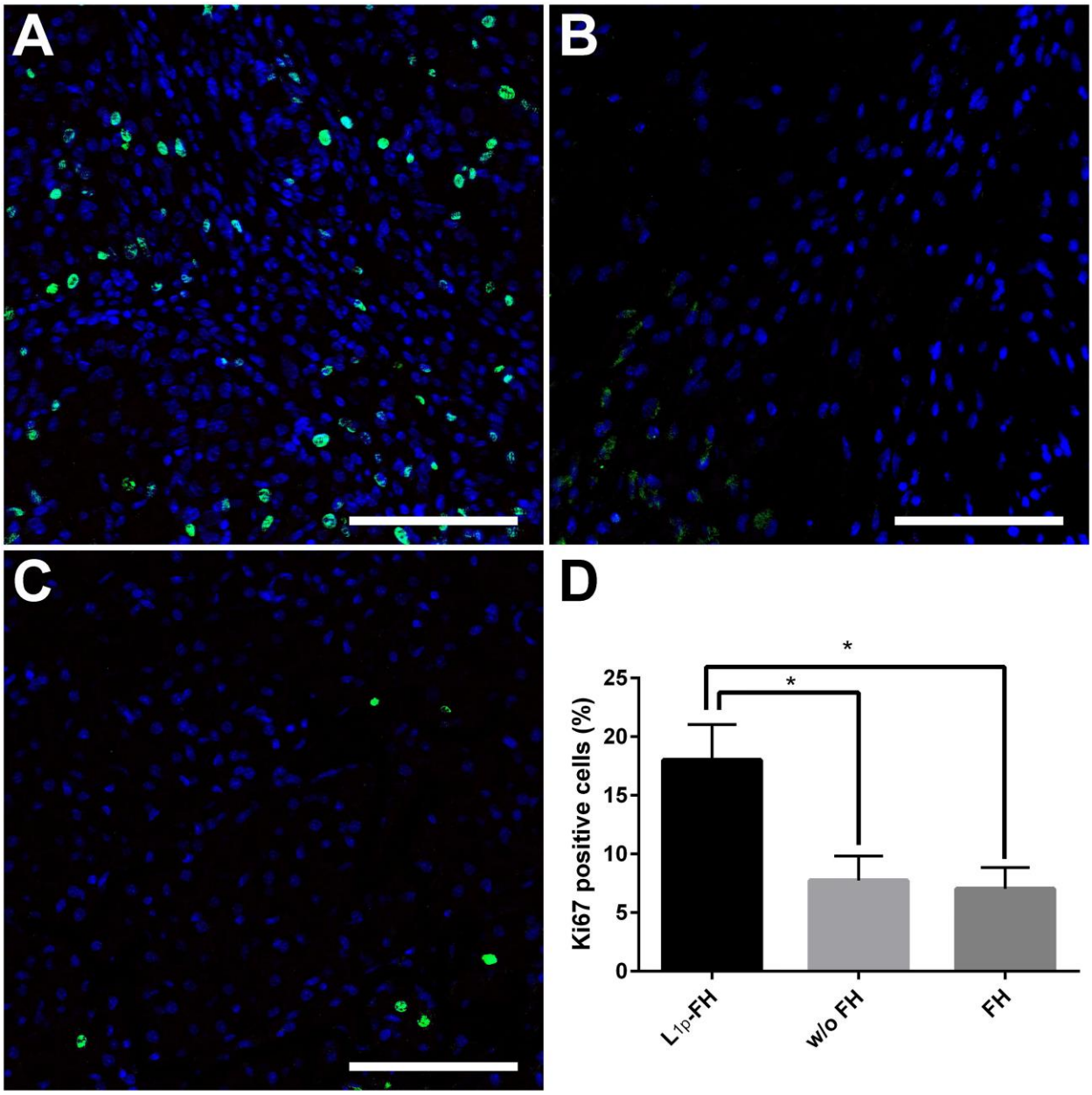
Appendix Fig. 2. The fluorescence intensity of the hydrogels on day 3 (A) and day 8 (B) post-surgery.



Appendix Fig. 3. Hematoxylin and eosin (H&E) stained histological sections of non-wounded (A: day 0), wounded mSMG without scaffold (B: day 0), wounded mSMG with L_{1p}-FH^{680} (C: day3, D: day 8), wounded mSMG without scaffold (E: day3, F: day 8), and wounded mSMG with FH^{680} (G: day3, H: day 8). Blue dotted areas are wounded areas. Scale bars represent 2 mm. SMG means submandibular gland and SLG means sublingual gland. Representative image from a total of n = 5 mice per group.



Appendix Fig. 4. Hematoxylin and eosin-stained histological sections of wounded mSMG with L_{1p}-FH^{680} (A; day 3, B; day 8) and FH^{680} (C; day 3, D; day 8). Tiled images generated by Leica DMI6000B system. Magnification 10×, bar = 2 mm.



Appendix Fig. 5. Ki67 stained sections of wounded mSMG with L_{1p}-FH^{680} (A), wounded mSMG without scaffold (B), and wounded mSMG with FH^{680} (C). (D) The percentage of Ki67 positive cells (green) in wounded area was calculated. Data represent the mean ± SD from five specimens per group where asterisks indicate significant differences between experimental groups by an analysis of variance (ANOVA; $*p < 0.001$). Scale bars represent 100 μm.