Biochemical Indicators of Implantation Success of Tissue-Engineered Oral Mucosa

Journal of Dental Research
2015, Vol. 94(1) 78–84
© International & American Associations
for Dental Research 2014
Reprints and permissions:
sagepub.com/journalsPermissions.nav
DOI: 10.1177/0022034514554225
jdr.sagepub.com

S. Kuo^{1*}, Y. Zhou^{2*}, H.M. Kim³, H. Kato¹, R.Y. Kim^{1,4}, G.R. Bayar¹, C.L. Marcelo⁴, R.T. Kennedy², and S.E. Feinberg^{1,4}

Abstract

Real-time (RT) determination of the health of in vitro tissue-engineered constructs prior to grafting is essential for prediction of success of the implanted tissue-engineered graft. In addition, the US Food and Drug Administration requires specific release criteria in RT prior to the release of tissue-engineered devices for human use. In principle, assessing the viability and functionality of the cellular component can be achieved by quantifying the secretion of growth factors and chemokines of tissue-engineered constructs. Ex vivo-produced oral mucosa equivalents (EVPOMEs) were fabricated under thermally stressed conditions at 43 °C for 24 h to create a functionally compromised EVPOME. We used microchannel enzyme-linked immunosorbent assay to evaluate the functionality of the cellular component, oral keratinocytes, of stressed and unstressed EVPOMEs by measuring the release of vascular endothelial growth factor (VEGF), interleukin-8 (IL-8), human β -defensin 1 (hBD-1), and tissue inhibitor of metalloproteinase 1 and 2 (TIMP-1 and -2) into the spent medium, which was collected on the same day prior to graft implantation into severe combined immunodeficiency mice. Implanted EVPOMEs' histology on the seventh postimplantation day was used to correlate outcomes of grafting to secreted amounts of IL-8, hBD-1, VEGF, TIMP-1, and TIMP-2 from corresponding EVPOMEs. Our findings showed that significantly higher levels of IL-8, hBD-1, and TIMP-2 were secreted from controls than from thermally stressed EVPOMEs. We also found a direct correlation between secreted VEGF and IL-8 and blood vessel counts of implanted EVPOMEs. We concluded that measuring the constitutive release of these factors can be used as noninvasive predictors of healthy tissue-engineered EVPOMEs in RT, prior to their implantation.

Keywords: angiogenesis, chemokines, cytokines, immunoassay, keratinocytes, transplants

Introduction

Oral mucosa can be damaged or lost after surgeries for tumor removal, trauma, or periodontal diseases. Repair of oral mucosa defects using autografts is hindered by the limited amount of healthy tissue available. To address this problem, 3-dimensional (3D) in vitro fabricated autogenous human tissue-engineered oral mucosa has been developed. In addition to the applications of clinical transplantation, 3D tissue-engineered oral mucosa can also be used as an in vitro model in the studies of cell-cell and cell-biomaterial interactions, allowing manipulations that mimic the cellular and tissue natural environment (Moharamzadeh et al. 2012).

An example of 3D tissue-engineered human oral mucosa is the ex vivo-produced oral mucosa equivalent (EVPOME) prepared using primary human oral keratinocytes (Izumi et al. 1999). The quality of EVPOMEs before grafting into

¹Department of Oral and Maxillofacial Surgery, School of Dentistry, University of Michigan, Ann Arbor, MI, USA

²Department of Chemistry, University of Michigan, Ann Arbor, MI, USA

³Center for Statistical Consultation and Research, University of Michigan, Ann Arbor, MI, USA

⁴Department of Surgery, Medical School, University of Michigan, Ann Arbor, MI, USA

*Authors contributing equally to this article.

A supplemental appendix to this article is published electronically only at <http://jdr.sagepub.com/supplemental>.

Corresponding Authors:

R.T. Kennedy, Department of Chemistry, University of Michigan, 930 N. University Ave, Ann Arbor, MI 48109-1055, USA.

Email: rtkenn@umich.edu

S.E. Feinberg, Department of Oral and Maxillofacial Surgery, University of Michigan Health System, Towsley Center Rm G1114, 1515 East Medical Center Drive, Ann Arbor, MI 48109-5222, USA.

Email: sefein@umich.edu

patients affects the success of graft take and tissue maturation of the EVPOMEs in vivo, so evaluation becomes an important issue to ensure the success of tissue-engineered construct implantation. The assessment has to be made non-invasively and in real time, as is required by the US Food and Drug Administration (FDA) for a release criterion of tissue-engineered devices for use in humans. Previously, we reported that the cellular component of the EVPOME secreted interleukin-8 (IL-8) and vascular endothelial growth factor (VEGF) that could correlate with EVPOME viability (Xu et al. 2009).

IL-8 has been found to enhance wound healing (Rennekampff et al. 2000) because of its ability to induce keratinocyte migration and proliferation (Tuschil et al. 1992; Morelli et al. 2011), while VEGF is important for vascular development (Breen 2007). Human β -defensin 1 (hBD-1) is an important antimicrobial peptide in epithelial tissues (Prado-Montes de Oca 2010) and can also stimulate the differentiation of keratinocytes (Abiko et al. 2003). Osborne and Schmid (2002) showed that tissue inhibitor of metalloproteinase 1 and 2 (TIMP-1 and -2) may support wound reepithelialization. Morelli et al. (2011) evaluated the expression of TIMP-1 and -2 during the wound healing of periodontal tissue reconstructive procedures using gingival fluid. Therefore, the concentrations of constitutively released VEGF, IL-8, hBD-1, TIMP-1, and TIMP-2 in the spent medium of EVPOME cultures may be indicators of cell viability and functionality.

The goal of this study was to examine the applicability of using IL-8, hBD-1, VEGF, TIMP-1, and TIMP-2 concentrations in EVPOME cultures as in vitro indicators of EVPOME implantation success. To reach this goal, we created a group of thermally stressed EVPOMEs that were cultured at 43 °C for 24 h to intentionally destroy a majority of the cellular layers of EVPOMEs (Winterroth et al. 2011). The viability of cellular components of control EVPOMEs was found to be higher than thermally stressed EVPOMEs (Chen et al. 2014). We hypothesized that the secretion amounts of IL-8, hBD-1, VEGF, TIMP-1, and TIMP-2 would be lower for thermally stressed than control EVPOMEs. Toward this goal, the levels of target proteins were determined by enzyme-linked immunosorbent assay (ELISA). Postimplanted EVPOMEs were evaluated for reepithelialization and neovascularization using anti-pan-keratin and anti-CD31 antibodies, respectively, as indicators of a successful grafting of the tissue-engineered devices.

Materials and Methods

Primary Human Oral Keratinocyte Cultures

All procedures of harvesting human oral mucosal tissue were approved by the University of Michigan Institutional Review Board. The method for culturing primary human oral keratinocytes was described previously (Xu et al.

2009). Briefly, primary human oral keratinocytes were enzymatically dissociated using 0.04% trypsin (Sigma-Aldrich, St. Louis, MO) from the tissue samples, and cell cultures were established in serum-free chemically defined culture medium (EpiLife and EDGS; Life Technologies, Grand Island, NY) containing 0.06 mM calcium, 25 μ g/mL gentamicin, and 0.375 μ g/mL fungizone.

ELISA

Spent medium of stressed and unstressed cultured EVPOMEs was collected on the day when EVPOMEs were implanted into mice. For microchannel ELISA, Optimser™ microfluidic ELISA plate assembly, buffers, and reagents were from Siloam Biosciences (Cincinnati, OH). IL-8 and VEGF antibody pairs were from Life Technologies (Carlsbad, CA). hBD-1 and TIMP-1 reagent kits were obtained from Peprotech (Rocky Hill, NJ). The TIMP-2 antibody pair was from R&D Systems (Minneapolis, MN). The optimized conditions and limits of detection for microchannel ELISA measurement are listed in the Appendix. Measurement was conducted according to the protocol provided by Siloam Biosciences, except for the TIMP-1 assay, whose optimal wash was 0.05% Tween-20 in phosphate buffered saline. The protein concentration was measured in triplicate based on external calibration.

EVPOMEs Manufacturing and Histology

The detailed protocol for manufacturing EVPOMEs was described previously (Izumi et al. 1999). Briefly, 200,000 oral keratinocytes/cm² were seeded on a 1-cm² acellular cadaver skin (AlloDerm™; LifeCell Corporation, Branchburg, NJ) that was presoaked in 0.05 μ g/ μ L human type IV collagen (Sigma-Aldrich). EVPOMEs were submerged in medium containing 1.2 mM Ca²⁺ for 4 d and were raised to an air-liquid phase for an additional 7 d. Thermally stressed EVPOMEs, at day 9 postseeding, were switched to 43 °C culture for 24 h and then back to 37 °C at day 10 postseeding, when all samples were replenished with fresh medium. Control EVPOMEs were maintained at 37 °C. At day 11 postseeding, 1.5 mL of spent medium from each sample was collected and frozen at –80 °C until ELISA analysis. On the same day, one-fifth of each EVPOME sample was cut and fixed in 10% formalin, embedded in paraffin, cut into 5- μ m sections, and processed by the histology core at the Dental School, University of Michigan, for hematoxylin and eosin staining. The remaining four-fifths of each EVPOME sample were grafted onto severe combined immunodeficiency (SCID) mice. Four batches of EVPOME samples were made using oral keratinocytes from 4 patients, with a total of 16 EVPOME samples including 8 control and 8 stressed samples. Each batch of EVPOME samples was manufactured using oral keratinocytes from 1 patient.

Figure 1. Box plots of the distribution of interleukin-8 (IL-8), human β -defensin 1 (hBD-1), and vascular endothelial growth factor (VEGF) (A), and tissue inhibitor of metalloproteinase 1 and 2 (TIMP-1 and TIMP-2) (B) enzyme-linked immunosorbent assay data by control and thermally stressed groups of ex vivo-produced oral mucosa equivalents. The *P* values from the test for differences in means were 0.04 for IL-8, 0.03 for hBD-1, 0.07 for VEGF, 0.15 for TIMP-1, and 0.02 for TIMP-2. Statistical analysis strategy is detailed in the Materials and Methods section.

Mice Surgery for EVPOMEs Grafting and Postimplanted EVPOME Histology

Surgical procedures in mice were detailed in a previous article (Izumi et al. 2003). Briefly, each batch of control and stressed EVPOMEs was grafted into 7- to 8-wk-old SCID mice (Charles River Laboratories, Wilmington, MA) for 1 wk. Each grafted EVPOME was overlaid with a slightly larger sterilized 0.005-inch-thickness silicon sheeting (Specialty Manufacturing, Saginaw, MI). Implanted EVPOMEs were removed from mice at 1 wk postimplantation. The samples were fixed in 10% formalin, embedded in paraffin, and cut into 5- μ m sections. Anti-pan-keratin antibody (Abcam, Cambridge, MA) with 1:250 dilutions was used to detect continuous stratification of keratinocytes and

expression of keratin to verify reepithelialization on implanted EVPOMEs (Izumi et al. 2003; Khmaladze et al. 2014). Three histology experts were blinded to experimental conditions and examined 2 slides from each sample to evaluate reepithelialization on all postimplanted EVPOMEs. The anti-CD31 antibody (Dianova, Hamburg, Germany) with 1:25 dilutions was used to detect blood vessel endothelial cells. The CD31 immunohistochemistry slides were scrambled and numbered so that they did not reflect experimental conditions. One examiner counted the blood vessels and was blinded to experimental conditions. Blood vessels that grew into the dermal equivalent (AlloDermTM) of the implanted-EVPOMEs were counted. The average number of blood vessels from 3 CD31 immunohistochemistry slides was used for each postimplanted EVPOME sample. Each slide contained 2 sections, and each section contained 1.5-mm cut strips from a whole postimplanted EVPOME sample. Six sections per sample were used to count blood vessels. One of the thermally stressed implanted EVPOMEs was found only partially covered by the silicon sheeting. As a result of that, we excluded this sample from the blood vessel count. Rat IgG2a and mouse IgG1 (Santa Cruz Biotechnology, Dallas, TX) were used for negative controls. Immunohistochemistry signals were detected using 3, 3'-diaminobenzidine chromogen for 5 min and hematoxylin as counterstaining. All animal studies were approved by the University of Michigan Committee on Use and Care of Animals.

Data Analysis

The spent medium protein level distributions were examined graphically, and summary statistics were obtained by control versus compromised EVPOMEs. The secretion levels of IL-8, hBD-1, VEGF, TIMP-1, and TIMP-2 between control and thermally stressed EVPOMEs were each compared by fitting a linear mixed-effects model in which the primary predictor was a dummy indicator for thermally stressed EVPOMEs, and statistical significance of the dummy indicator provided evidence for the differences in the concentration levels of each of IL-8, hBD-1, VEGF, TIMP-1, and TIMP-2 in EVPOME cultures between compromised and control EVPOMEs. The linear mixed-effects model included patients as random intercepts to account for potential correlation of the data from a set of control and compromised EVPOME samples prepared using oral keratinocytes of the same patient. Because the distribution of the biochemical indicators are skewed to the right, we have also used a generalized linear model with log-link function to estimate the multiplicative difference in the concentration levels of each measure between compromised and control EVPOMEs. Lastly, we assessed the correlation between the concentration levels of IL-8, hBD-1, VEGF, TIMP-1, and TIMP-2 in EVPOME cultures to the blood vessel counts. This was calculated by using the blood vessel counts

Table 1. Summary Statistics of IL-8, hBD-1, VEGF, TIMP-1, and TIMP-2 Distribution between Control ($n = 8$) and Thermally Stressed Samples ($n = 8$) at 43 °C EVPOMEs.

		Mean	SD	Median	Min, Max	Robust SE ^a	Difference (Control – Stressed)	95% CI of the Difference	P Value
IL-8	Control	16.5	12.2	9.6	4.5, 34.0	5.3	8.9	0.3, 17.4	0.04
	Stressed	7.6	5.1	6.4	0.9, 17.3	1.8			
hBD-1	Control	49.7	33.2	34.9	17.9, 105.0	10.7	29.4	3.2, 55.6	0.03
	Stressed	20.3	23.2	13.4	–2.9 ^b , 73.5	9.3			
VEGF	Control	24.1	19.6	16.6	9.9, 69.9	6.7	13.4	–1.3, 28.0	0.07
	Stressed	10.7	11.3	7.5	0.1, 37.0	4.5			
TIMP1	Control	75.9	73.5	59.9	27.6, 252.0	28.8	39.7	–14.3, 93.7	0.15
	Stressed	36.2	39.3	24.0	1.9, 127.5	16.8			
TIMP2	Control	168.4	80.3	169.6	42.5, 252.2	31.6	80.2	11.2, 149.3	0.02
	Stressed	88.1	70	63.9	27.4, 244.2	88.1			

IL-8, interleukin-8; hBD-1, human β -defensin 1; VEGF, vascular endothelial growth factor; TIMP-1, tissue inhibitor of metalloproteinase 1; TIMP-2, tissue inhibitor of metalloproteinase 2; EVPOME, ex vivo-produced oral mucosa equivalent; CI, confidence interval.

^aRobust SEs are standard errors of the mean after adjusting for potential clustering of the data within EVPOMEs generated from oral keratinocytes of the same patient. The concentration unit is pg/mL.

^bThe negative concentration was based on linear calibration and fell within ± 3 SD of the blank.

as the dependent variable. However, because the blood vessel counts were skewed to the right, we used a log-link function, and to account for potential correlation within patient, generalized estimating equation was used. Because of the small sample size of this feasibility study, statistical significance was determined at an alpha level of 0.05, even though multiple comparisons were made.

Results

ELISA Analysis of IL-8, hBD-1, VEGF, TIMP-1, and TIMP-2 from EVPOME Culture

Previously, we analyzed the secretion of IL-8 and VEGF from an EVPOME culture and found that protein secretion was highest on day 11 of culture (Xu et al. 2009). In this study, day 11 EVPOMEs were implanted into SCID mice, and the day 11 spent medium was analyzed for the constitutively secreted amounts of IL-8, VEGF, hBD-1, TIMP-1, and TIMP-2 from control and thermally stressed EVPOMEs cultures.

As shown in Figure 1 and Table 1, higher concentrations of IL-8, hBD-1, VEGF, TIMP-1, and TIMP-2 were observed in control compared with stressed samples. Specifically, the mean secretion levels of IL-8, hBD-1, and VEGF were higher in control than in stressed EVPOME samples by 8.9 pg/mL ($P = 0.04$), 29.4 pg/mL ($P = 0.03$), and 13.4 pg/mL ($P = 0.07$), respectively. In terms of relative difference, the mean secretion levels were 46% ($P = 0.10$) lower for IL-8 in thermally stressed EVPOMEs than in control EVPOMEs, 41% ($P = 0.09$) lower for hBD-1, and 45% ($P = 0.14$) lower for VEGF. No difference was found between stressed and control samples in TIMP-1 concentration (higher in control by 39.7 pg/mL; $P = 0.15$), but mean TIMP-2 concentration

was significantly higher by 80.2 pg/mL ($P = 0.02$) in control than in stressed samples. Similarly, stressed samples showed 52% reduction ($P = 0.03$) in mean TIMP-2 concentration in thermally stressed samples compared with control samples. In summary, only IL-8, hBD-1, and TIMP-2 showed a statistically significant difference in the mean concentration level; however, all biochemical indicators showed higher levels in control than in thermally stressed EVPOMEs. In addition, we evaluated the release of lactate dehydrogenase (LDH) into medium as an indicator of cell death (Kahn et al. 2010). However, LDH was at undetectable levels despite our best efforts.

Examination of Development of Implanted Control and Compromised EVPOMEs

To examine the growth and development of grafted EVPOMEs, we removed a small portion of each EVPOME before implantation for preimplantation histology and the entire corresponding implanted EVPOMEs for 1 wk postimplantation histology. Thermally stressed EVPOMEs showed obvious destruction of cellular layers in vitro before implantation (Fig. 2A, top row). Continuous epithelial differentiation and stratification of EVPOMEs were seen in both control and compromised grafted EVPOMEs at 1 wk; however, a greater stratification amount was observed in control than in compromised EVPOMEs (Fig. 2A, middle row).

One of the hallmarks of the success of grafting is the development of blood vessels within the implanted grafts that maintain the nutrient supply to the epithelial layer of the grafts. We used anti-mouse CD31 antibody to detect the development of blood vessel ingrowth into the grafted EVPOME dermal component (Fig. 2A, bottom row). Blood vessel formation between control and compromised EVPOMEs was significantly

Figure 2. Effect of thermal stress on reepithelialization and neovascularization of EVPOME. **(A)** Comparison of preimplanted ex vivo-produced oral mucosa equivalents (EVPOMEs) and reepithelialization and neovascularization of postimplanted EVPOMEs between control and thermally stressed EVPOMEs. C represents control EVPOMEs, and S represents thermally stressed EVPOMEs. The letter “A” shows the area of AlloDerm™ used as a scaffold for EVPOME manufacturing. The top row shows the hematoxylin and eosin staining of preimplanted EVPOMEs. The middle row shows the immunohistochemistry of postimplanted EVPOMEs by anti-pan-keratin antibody to detect keratinocytes and keratin structures on postimplanted EVPOMEs after 1 wk. The bottom row shows the detection of blood vessels on implanted EVPOMEs by anti-CD31 antibody. The yellow arrowheads mark the location of blood vessels, whose endothelial membranes were detected by anti-CD31 antibody. Each scale bar represents 100 µm on the top and middle rows and 50 µm on the bottom row. **(B)** Box plot shows the distribution of blood vessels on control and thermally stressed EVPOMEs implanted.

different at 1 wk postimplantation (Fig. 2B). Blood vessels perforated through the dermal equivalent of the implanted graft were obvious on control EVPOMEs; in contrast, few blood vessels were observed in the thermally stressed EVPOMEs. We found mean blood vessel counts to be significantly lower by 12.9 ($P = 0.008$) in implanted thermally-stressed EVPOMEs than those in implanted control EVPOMEs. Lastly, when blood vessel counts were modeled using various markers in spent medium, we found VEGF and IL-8 to be significant predictors of blood vessel counts in implanted EVPOMEs (Table 2). Based on the model, every 10-pg/mL increase in VEGF was associated with a 48% ($1.04^{10} - 1$) increase in the expected blood vessel count, holding IL-8 level constant, while every 10-pg/mL increase in IL-8 was associated with a 54% (0.94^{10}) reduction in the expected blood vessel count, holding VEGF level constant.

Discussion

Selecting the most robust tissue-engineered grafts to be used on patients prior to surgery is important to ensure optimal postoperative implant integration. Noninvasive and real-time determination of EVPOME viability prior to implantation is desired and required by the FDA. We employed microchannel ELISA measurements of IL-8, hBD-1, VEGF, TIMP-1, and TIMP-2 to assess the functional viability of the cellular component of grafts. The microchannel ELISAs showed a statistically significant decrease of selected protein secretion in thermally stressed EVPOMEs compared with controls. This difference correlated with histology assessments for epithelial stratification and microvessel growth into the dermal component.

Table 2. Model^a for Blood Vessel Counts in Implanted EVPOMes at 1 wk with VEGF and IL-8 in Spent Medium.

Blood Vessel Count	exp(b) ^b	SE	Z	P Value	95% CI
VEGF	1.04	0.009	4.04	<0.001	1.02, 1.05
IL-8	0.94	0.06	-3.99	<0.001	0.91, 0.97
Constant	9.11	2.32	8.65	<0.001	5.52, 15.01

EVPOME, ex vivo-produced oral mucosa equivalents; VEGF, vascular endothelial growth factor; IL-8, interleukin-8; SE, standard error; CI, confidence interval.

^aGeneralized linear model with log link and with generalizing estimating equation to account for the potential correlation of blood vessel count data of the EVPOMes from the same patient.

^bParameter estimates are exponentiated for ease of interpretation; they can be interpreted as the ratio of expected mean in blood vessel count associated with every 1-unit increase in the predictors.

Postimplanted EVPOME histology showed significantly different outcomes between control and stressed grafts. In control EVPOMes, continuous reepithelialization of the cellular layer and formation of blood vessels within the dermis was seen at 1 wk after implantation. In contrast, stressed EVPOMes showed a more disorganized epithelial layer and a decreased number of invading vessels within the dermis at 1 wk after grafting.

Wound healing is regulated by various growth factors and chemokines (Werner and Grose 2003; Barrientos et al. 2008), among which IL-8 and VEGF are secreted in large amount by keratinocytes (Xu et al. 2009). IL-8 is known to increase keratinocyte migration and proliferation and promote reepithelialization (Tuschil et al. 1992; Morelli et al. 2011). Reepithelialization observed in our postimplantation EVPOME histology might be a result of secretion of IL-8 or TIMP-1 and TIMP-2 from keratinocytes in EVPOMes. The formation of blood vessels on implanted grafts is a critical factor in determining the success of grafting because blood vessels supply oxygen, nutrients, and remove metabolites from cells and tissues. IL-8 was reported to be a factor in inducing neovascularization (Strieter et al. 1992; Li et al. 2003). VEGF is important for vascular development and ingrowth (Breen 2007). Our statistical analysis data showed a correlative relationship between blood vessel counts of implanted EVPOMes and the secreted concentration of IL-8 and VEGF. Interestingly, our statistical data showed an inverse relationship of IL-8 and blood vessel counts. Our statistical data suggested that lower concentration of IL-8 was better for neovascularization when VEGF secretion was kept constant. It is possible that there is a feedback regulatory mechanism between IL-8 and VEGF on blood vessel growth on our implanted EVPOMes or that the timing of IL-8 secretion is important in blood vessel development. Knockdown of IL-8 or VEGF on EVPOMes by shRNA can be used to examine the issue in a future study. Postimplanted control EVPOMes had higher level of neovascularization than postimplanted thermally stressed EVPOMes, which indicated a better integration into hosts for implanted control EVPOMes. Increased VEGF secretion from the intact epithelial layer on the control EVPOMes

could explain the increase in neovascularization observed at 1 wk postimplantation.

These results indicated that we have created an in vitro model system using control and thermally stressed EVPOMes, in which the cell layer was affected sufficiently to create an obvious dissimilar differentiation pattern in vivo. This model allowed us to correlate suitable growth factors/chemokines as in vitro indicators of successful implantation of EVPOMes 1 wk postgrafting. Various biochemical indicators evaluated in this study showed potential as indicators of a well-stratified EVPOME. The analyses of secretion levels of these factors can be performed by microchannel ELISA noninvasively with a turnaround time of just 2 to 3.7 h, prior to release of the grafts for surgery.

Author Contributions

S. Kuo, contributed to conception, design, and data acquisition, drafted and critically revised the manuscript; Y. Zhou, contributed to design and data acquisition, drafted and critically revised the manuscript; H.M. Kim, contributed to conception, design, and data analysis, critically revised the manuscript; H. Kato, R.Y. Kim, G.R. Bayar, contributed to design and data acquisition, critically revised the manuscript; C.L. Marcelo, contributed to conception and data interpretation, critically revised the manuscript; R.T. Kennedy, contributed to design and data interpretation, critically revised the manuscript; S.E. Feinberg, contributed to conception, design, data analysis, and interpretation, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

Acknowledgments

This work was supported by the National Institutes of Health (NIH) grant R01DE019431-01 (to S.E. Feinberg). We thank Paula Arrowsmith for EVPOME immunohistochemistry. The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

References

- Abiko Y, Nishimura M, Kusano K, Yamazaki M, Arakawa T, Takuma T, Kaku T. 2003. Upregulated expression of human

- b defensin-1 and -3 mRNA during differentiation of keratinocyte immortalized cell lines, HaCaT and PHK16-0b. *J Dermatol Sci.* 31(3):225–228.
- Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M. 2008. Growth factors and cytokines in wound healing. *Wound Rep Reg.* 16(5):585–601.
- Breen EC. 2007. VEGF in biological control. *J Cell Biochem.* 102(6):1358–1367.
- Chen LC, Lloyd WR, Kuo S, Kim HM, Marcelo CL, Feinberg SE, Mycek MA. 2014. The potential of label-free nonlinear optical molecular microscopy to non-invasively characterize the viability of engineered human tissue constructs. *Biomaterials.* 35(25):6667–6676.
- Izumi K, Feinberg SE, Terashi H, Marcelo CL. 2003. Evaluation of transplanted tissue-engineered oral mucosa equivalents in severe combined immunodeficient mice. *Tissue Eng.* 9(1):163–174.
- Izumi K, Takacs G, Terashi H, Feinberg SE. 1999. Ex vivo development of a composite human oral mucosal equivalent. *J Oral Maxillofac Surg.* 57(5):571–577.
- Kahn E, Baarine M, Pelloux S, Riedinger JM, Frouin F, Tourneur Y, Lizard G. 2010. Iron nanoparticles increase 7-ketocholesterol-induced cell death, inflammation, and oxidation on murine cardiac HL1-NB cells. *Int J Nanomed.* 5:185–195.
- Khmaladze A, Kuo S, Kim RY, Matthews RV, Marcelo CL, Feinberg SE, Morris MD. 2014. Human oral mucosa tissue-engineered constructs monitored by raman fiberoptic probe. *Tissue Eng Part C Methods.* 2014 Jul 7. [Epub ahead of print].
- Li A, Dubey S, Varney ML, Dave BJ, Singh RK. 2003. IL-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases production and regulated angiogenesis. *J Immunol.* 170(6):3369–3376.
- Moharamzadeh K, Colley H, Murdoch C, Hearnden V, Chai WL, Brook IM, Thornhill MH, Macneil S. 2012. Tissue-engineered oral mucosa. *J Dent Res.* 91(7):642–650.
- Morelli T, Neiva R, Nevins ML, McGuire MK, Scheyer ET, Oh TJ, Braun TM, Nör JE, Bates D, Giannobile WV. 2011. Angiogenic biomarkers and healing of living cellular constructs. *J Dent Res.* 90(4):456–462.
- Osborne CS, Schmid P. 2002. Epidermal-dermal interactions regulate gelatinase activity in Apligraf®, a tissue-engineered human skin equivalent. *Br J Dermatol.* 146(1):26–31.
- Prado-Montes de Oca E. 2010. Human β -defensin 1: a restless warrior against allergies, infections and cancer. *Int J Biochem Cell Biol.* 42(6):800–804.
- Rennekampff HO, Hansbrough JF, Kiessig V, Doré C, Sticherling M, Schröder JM. 2000. Bioactive interleukin-8 is expressed in wounds and enhances wound healing. *J Surg Res.* 93(1):41–54.
- Strieter RM, Kunkel SL, Elnor VM, Martonyi CL, Koch AE, Polverini PJ, Elnor SG. 1992. Interleukin-8 a corneal factor that induces neovascularization. *Am J Pathol.* 141(6):1279–1284.
- Tuschil A, Lam C, Haslberger A, Lindley I. 1992. Interleukin-8 stimulates calcium transients and promotes epidermal cell proliferation. *J Invest Dermatol.* 99(3):294–298.
- Werner S, Grose R. 2003. Regulation of wound healing by growth factors and cytokines. *Physiol Rev.* 83(3):835–870.
- Winterroth F, Lee J, Kuo S, Fowlkes JB, Feinberg SE, Hollister SJ, Hollman KW. 2011. Acoustic microscopy analyses to determine good vs. failed tissue engineered oral mucosa under normal or thermally stressed culture conditions. *Ann Biomed Eng.* 39(1):44–52.
- Xu Q, Izumi K, Tobita T, Nakanishi Y, Feinberg SE. 2009. Constitutive release of cytokines by human oral keratinocytes in an organotypic culture. *J Oral Maxillofac Surg.* 67(6):1256–1264.