Title

An in vitro scaffold-free epithelial–fibroblast coculture model for the larynx

Journal

The Laryngoscope, 127(6)

ISSN

0023-852X

Authors

Walimbe, Tanaya
Panitch, Alyssa
Sivasankar, M Preeti

Publication Date

2017-06-01

DOI

10.1002/lary.26388

Peer reviewed

HHS Public Access

Author manuscript

Laryngoscope. Author manuscript; available in PMC 2018 June 01.

Published in final edited form as:

Laryngoscope. 2017 June ; 127(6): E185–E192. doi:10.1002/lary.26388.

An in-vitro scaffold-free epithelial-fibroblast coculture model for the larynx

Tanaya Walimbe, B.S.[1], **Alyssa Panitch, Ph.D.**[1], and **M. Preeti Sivasankar, Ph.D., CCC-SLP**[1,2]

[1]Weldon School of Biomedical Engineering, Purdue University, West Lafayette, IN

[2]Department of Speech, Language, and Hearing Sciences, Purdue University, West Lafayette, IN

Abstract

Objective—Physiologically relevant, well-characterized *in vitro* vocal fold coculture models are needed to test the effects of various challenges and therapeutics on vocal fold physiology. We characterize a healthy state coculture model, created by using bronchial/tracheal epithelial cells and immortalized vocal fold fibroblasts. We also demonstrate that this model can be induced into a fibroplastic state to overexpress stress fibers using TGFβ1.

Method—Cell metabolic activity of immortalized human vocal fold fibroblasts incubated in different media combinations were confirmed with MTT assay. Fibroblasts were grown to confluence and primary bronchial/tracheal epithelial cells suspended in coculture media were seeded directly over the base layer of the fibroblasts. Cells were treated with TGFβ1 to induce myofibroblast formation. Cell shape and position was confirmed by live cell tracking, fibrosis was confirmed by probing for α smooth muscle actin (α-SMA) and phenotype was confirmed by immunostaining for vimentin and E-cadherin.

Results—Fibroblasts retain metabolic activity in coculture epithelial media. Live cell imaging revealed a layer of epithelial cells atop fibroblasts. α-SMA expression was enhanced in TGFβ1 treated cells, confirming that both cell types maintained a healthy phenotype in coculture, and can be induced into overexpressing stress fibers. Vimentin and E-cadherin immunostaining show that cells retain phenotype in coculture.

Conclusion—These data lay effective groundwork for a functional coculture model that retains the reproducibility necessary to serve as a viable diagnostic and therapeutic screening platform.

Level of Evidence—NA

Keywords

Coculture; in vitro model; larynx; fibroblast; epithelial

Correspondence may be addressed to: Preeti M. Sivasankar, Ph.D., CCC-SLP, Department of Speech, Language, and Hearing Sciences, Purdue University, West Lafayette, IN 47907, msivasankar@purdue.edu, Phone: (765) 496 6628, Fax: (765) 494 0771.

Conflict of Interest: None

This manuscript will be presented at the 96th annual American Bronchoesophagological Asociation (ABEA) meeting held in conjunction with Combined Otolaryngology Spring Meetings (COSM) in Chicago, IL, USA on May 18th–19th 2016.

Introduction

In healthy vocal folds, the epithelium serves as a first line of defense, acting as a selective biochemical barrier.[1–3] Underlying fibroblasts in the lamina propria synthesize most of the extracellular matrix (ECM) in response to autocrine and paracrine signals from the surrounding environment.[4,5] Homeostasis is thus maintained by complex interactions between various cell types and the ECM.[2,4,6,7] Scarring, the most common cause of vocal deficiencies[8–11] disrupts homeostasis by causing a cascade of events involving cell signaling, wound healing, and matrix reorganization.[11,12]

Despite advances in understanding the pathophysiology, effective treatments for scarring remain elusive. While matrix reorganization does take place in the lamina propria, the epithelium is also disrupted upon injury and undergoes restoration.[4] Much research about scarring has focused on lamina propria reorganization using fibroblasts alone[13–19] or stem cells,[20–26] and there is emerging evidence on the response of epithelial cells to injury.[6,26] The lack of an epithelial cell line for the vocal folds, poor proliferative capacity of primary cells, limited viability of ex-vivo tissue, and difficulties associated with handling and cost of animal models have limited investigations into the interactions between the two cell types.

It is widely accepted that cellular and molecular signaling between the epithelial cells and fibroblasts is one of the many factors involved in maintaining homeostasis *in vivo*,[6,27] but the underlying mechanisms remain largely unexplored. Fibroblasts provide an incomplete picture of wound healing response *in vivo*, since epithelial restoration and signaling events are also likely to play an important role in mediating wound-healing response. While stem cells have advantages such as tunability and increased control of differentiation, there are no models sourced from primary human cells to compare stem cell based approaches to. In order to explore the combination of these epithelial-mesenchymal interactions, our goal was to develop a reproducible, high throughput, physiologically relevant, *in vitro* bench top model, which takes into account direct interactions between epithelial cells and fibroblasts.

Direct signaling events between multiple cell types have been shown to be crucial in growth, migration, and differentiation of cells.[27–32] Direct contact between cell types can be facilitated in a scaffold-based[18,24,26,33] or scaffold-free[27,28,31] platform. Cells respond differently to the material properties of the scaffold.[34] A scaffold-free model enables matrix formation guided by cells themselves based on contact, independent of signaling cues from the scaffold. To investigate whether epithelial-fibroblast interaction, affects cell morphology and phenotype *in vitro*, we established a direct contact, scaffold free cell culture model. We also investigated whether our co-culture model could be induced into an *in vitro* fibroplastic state by the use of TGF-β1. TGF-β1 is a potent cytokine responsible for inducing a wide range of functions such as tissue repair and homeostasis, inflammatory responses, extracellular matrix production and cell proliferation and differentiation.[35] Of particular interest is its ability to induce continuous stress fiber formation and myofibroblast differentiation in fibroblasts, which leads to excess collagen deposition;[5,17,36] thus recapitulating an important aspect of fibrotic phenotype *in vitro*. To the best of our knowledge, such an *in vitro* platform to study pathophysiology of TGF-β1 mediated fibrosis and screen for therapeutics does not exist. Based on the assumption that primary cells from

the airway are exposed to a similar environment as the larynx and to overcome the shortcoming of limited availability of vocal fold epithelial cells, we chose airway, cuboidal bronchial/tracheal epithelial cells for coculture. This simple coculture model can provide a more physiologically relevant diagnostic and therapeutic platform to carry out more detailed *in vitro* vocal fold research.

Materials and Methods

Cell culture

Immortalized Vocal Fold Fibroblasts—The immortalized vocal fold fibroblast (I-hVFF) cell line[37] was obtained from Dr. Susan Thibeault at the University of Wisconsin, Madison. The fibroblast culture was maintained in 25 cm^2 tissue-culture (TC)-treated flasks in a 5% CO_2 and 37°C environment in Dulbecco's modified eagle medium (DMEM, Sigma-Aldrich) supplemented with 10% Fetal Bovine Serum (Corning, Corning, NY), 1% Penicillin/Streptomycin (Invitrogen, Carlsbad, CA), 1% MEM non-essential amino acid solution (Sigma-Aldrich, St. Louis, MO) and 200 μg/ml of Geneticin (G418, Teknova, Hollister, CA). Cells from passage 8–12 were used throughout the experiments. All experiments were performed on cell culture treated Ibidi μ-slide Angiogenesis (Ibidi USA Inc., Madison, WI) at an initial seeding density of 3–4 × 10^4 cells/cm^2 unless otherwise mentioned.

Primary Bronchial/Tracheal Epithelial Cells (ECs)—Primary bronchial/tracheal epithelial cells were purchased from ATCC (Manassas, VA; Catalog # PCS-300-010) and grown in complete airway epithelial cell serum free basal medium (ATCC-PCS-300-030) supplemented with bronchial/tracheal cell growth kit (ATCC-PCS-300-040) and gentamicin/amphotericin B solution (ATCC-PCS-999-025). The cells were maintained in 25 cm^2 TC-treated flasks in a 5% CO_2 and 37°C humidified environment. Cells from passage 2–7 were used throughout the experiments at an initial seeding density of 6–7×10^4 cells/cm^2 unless otherwise mentioned.

MTT assay for I-hVFF metabolic activity in coculture media

The effect of different media combinations and cell densities on I-hVFF metabolic activity was tested by using the Vybrant® MTT Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR). The purpose of the assay was to ensure that cells retained metabolic activity at confluence. Cells were seeded in 96 well plates at densities of either 25,000 cells/cm^2 or 37,500 cells/cm^2; and incubated in 4 respective media formulations consisting of: 1) complete fibroblast growth media (control media), 2) complete epithelial growth media, 3) modified media which consisted of complete epithelial growth media with non essential MEM solution and 4) phenol red free complete epithelial growth media. After 48 hours of incubation, 100μL media and MTT solution was added to each well and incubated for 4 hours. 100uL of SDS-HCl solution was then added to each well, mixed thoroughly and incubated for 4 hours to dissolve the formazan formed. Absorbance was read at 570nm on a Spectramax M5 microplate reader (Molecular Devices, Sunnyvale, CA) and was directly co-related with cell viability.

Coculture model

Healthy coculture model—I-hVFFs were seeded and grown for 48 hours to confluence in complete fibroblast growth medium with a media change after 24 hours. ECs were then directly seeded on top of the confluent layer of I-hVFFs, in a suspension of complete epithelial cell media with 2% FBS, which was chosen as the coculture media. After 18–24 hours, the ECs form a layer on top of the I-hVFFs, and are ready to be used as a coculture model (Figure 1).

TGF-β1 coculture model—TGF--β1 was used to induce stress fiber formation in cells. I-hVFFs were seeded and grown for 48 hours in complete fibroblast growth medium with a media change after 24h. After 48 hours, ECs were seeded directly on top of the I-hVFFs in complete epithelial media containing 10 ng/mL TGFβ1. 18–24 hours after seeding, the ECs formed a layer on top of the I-hVFFs (Figure 1).

Live Cell Imaging In Co-Culture

For live cell tracking, I-hVFFs were incubated with 10 μM Cell Tracker™ Orange CMRA (Molecular Probes) for 45mins and washed 3 times with 1X D-PBS. Cells were then trypsinized and seeded at a density of 3×10^4 cells/cm^2. After 48 hours, ECs were labeled with 10 μM Cell Tracker ™ Green CMFDA (Molecular Probes) for 45 minutes, trypsinized and seeded directly on top of the I-hVFFs. Cultures were visualized using a Leica DMI6000 B inverted microscope with an EL6000 external light source (Leica Microsystems, Wetzler, Germany) 24 hours after seeding ECs using a ×10objective. A CooolSNAP HQ^2 camera and Leica application suite (LAS) AF6000 software (Leica Microsystems) were utilized for image acquisition. All images were taken using identical settings for exposure time. Images were edited using ImageJ (National Institutes of Health, Bethesda, MD).

Immunofluorescence studies to assess phenotype

24 hours after seeding ECs in epithelial media containing 2% serum, cultures were fixed in 3% paraformaldehyde for 30 min, permeabilized using 0.5% Triton X-100 (Sigma) in PBS for 30 min, and blocked in 5% milk for 2 hours. Cultures were immunostained for α-smooth muscle actin (αSMA), vimentin and E-cadherin sequentially. To identify stress fibers, cultures were incubated with mouse anti-human αSMA (Sigma; 1:200 dilution) overnight at 4°C followed by incubation with goat anti-mouse AlexaFluor® 488 (Invitrogen, 1:400 dilution) for 2 hours. To identify I-hVFFs in culture, we used Vimentin, a mesenchymal cell marker. Cultures were incubated overnight at 4°C with rabbit anti-human vimentin (Cell Signaling Technology, 1:100 dilution), followed by incubation with goat anti-rabbit AlexaFluor® 555 (Cell Signaling Technology, 1:400 dilution) for 2 hours. To identify ECs in culture, we used E-cadherin, an adherens junction protein. Cultures were incubated overnight at 4°C with rabbit anti-human E-cadherin (Cell Signaling Technology, 1:50 dilution), followed by incubation with goat anti-rabbit AlexaFluor® 555 (Cell Signaling Technology, 1:400 dilution) for 2 hours. Primary antibody was eliminated from the negative control to confirm specificity of secondary antibodies. Samples were washed 3 times to ensure removal of previous antibody. The samples were counterstained with 4′, 6 -

Diamidino-2-phenylindole (DAPI) for nuclear staining and were visualized using the Leica DMI6000 B inverted microscope at a ×10 objective.

Statistical Analysis

Statistical Analysis was completed using Minitab 17 (State College, PA). The data for the MTT assay are represented as mean with error bars corresponding to standard deviation. Single factor analysis of variance (ANOVA) and Tukey's significant difference post hoc tests were performed to look for differences in both, cell seeding density and different media formulations, and a p value at or below 0.05 was considered statistically significant.

Results

MTT Assay for I-hVFF Metabolic Activity

Data comparing different I-hVFF cell seeding densities and different media combinations is shown in Figure 2. A statistically significant difference in I-hVFF metabolic activity was seen between seeding densities of 37,500 cells/cm^2 and 25,000 cells/cm^2 ($p < 0.05$). To achieve a monolayer of I-hVFFs, cell seeding density of 37,500 cells/cm^2 was selected for all future experiments. I-hVFF viability was highest for control media ($p < 0.05$), but was preserved in epithelial media, which was used as the media for subsequent studies.

Live Cell Coculture Visualization

Fibroblasts and epithelial cells retained viability, as seen from live cell tracker imaging. Fibroblasts displayed an elongated, spindle-shaped morphology and were spread out, and the epithelial cells retained cobblestone-like morphology, and formed a layer on top of the fibroblasts (Figure 3).

α-SMA Expression

α-SMA, a well-known marker of myofibroblastic phenotype, was observed in all cells types (Figure 4). Induction with TGF-β1 increased stress fiber formation in both, fibroblasts and coculture models, indicating myofibroblast differentiation.

E-cadherin Expression

E-cadherin, a common epithelial adherens junction marker, was retained in epithelial cells in monoculture and coculture. E-cadherin expression was maintained after addition of TGF-β1 (Figure 5).

Vimentin Expression

Vimentin, a common mesenchymal cell marker, was expressed by fibroblasts in monoculture and coculture, with and without the addition of TGF-β1 (Figure 6). Mild vimentin staining was observed in epithelial cells.

Discussion

Here we characterize a scaffold-free, direct contact epithelial-fibroblast coculture model for the larynx. We demonstrate that both epithelial cells and fibroblasts retain their morphology

in coculture, and express cell-specific proteins. We also show that the addition of TGFβ1 induces stress-fiber formation. Activation of myofibroblasts, which express α-SMA, is a characteristic marker of fibrosis.[38] Fibroblasts were grown to confluence for 48 hours to facilitate matrix formation before epithelial cell seeding. This coculture model lays the groundwork for developing a reproducible test bed for high throughput, controlled screening of therapeutics for laryngeal pathologies in the future.

The role of vocal fold epithelium in wound healing response has been gaining increased attention.[6] Vocal fold epithelial cells play a pivotal role as a physical and biochemical barrier.[1–3] Little is known about the impact of epithelial-fibroblast signaling in the larynx, precluding advances in disease diagnosis and new candidate drug screenings. *In vitro* models provide the advantage of a controlled environment for simulating disease states and screening therapeutics, and are capable of significantly decreasing the cost and amount of time required to screen therapeutics. An *in vitro* direct contact coculture model between primary bronchial/tracheal epithelial cells and vocal fold fibroblasts, two predominant cell types, can enumerate some of the signal transduction and cell-cell interactions *in vivo*.

Current bench top models of the vocal folds use polymeric scaffolds made of collagen, decellularized ECM, hyaluronan or fibrin scaffolds by themselves, or in combination with stem cells and fibroblasts.[24,26,33,39] These models have focused on engineering a functional, multi-layered mucosa but not on establishing a test bed for therapeutics. Our model offers information about cell interactions in fibrotic state while retaining the tunability and reproducibility necessary to serve as a viable diagnostic and therapeutic test platform.

There are some potential limitations to this study. Cell phenotype was maintained in coculture for 24 hours. Future studies will include testing therapeutics in this model. The media for the coculture experiments was epithelial growth media. We chose to use this media because primary epithelial cells are more susceptible to phenotypic changes than immortalized fibroblasts. This might explain why some epithelial cells in monoculture stained for vimentin. Follow-up studies will probe for epithelial-mesenchymal transition using the coculture model. This question is of interest because epithelial-mesenchymal transition, characterized by the reduction in expression of epithelial adhesion proteins like E-cadherin, and increase in expression of mesenchymal markers such as vimentin and α-SMA, has been associated with multiple diseases such as cancers[40–42] and fibrosis[43,44] in multiple tissues. The addition of 2% FBS was further shown to preserve epithelial and fibroblast phenotype and hence was used for immunofluorescence studies. Overall, the epithelial cells and fibroblasts survived in coculture and could be induced into a fibrotic model with TGF-β1. While mild α-SMA expression was observed in the control group, significantly higher expression was seen after treating with TGF-β1, suggesting fibrosis. This finding corroborates with published reports.[38] Finally, The use of a non-vocal fold source of epithelial cells is also a limitation. However, cuboidal epithelial cells, which line the airway, are exposed to a similar environment as vocal fold epithelia, and can provide important insights into epithelial-mesenchymal interactions.

Conclusion

In summary, we characterized an epithelial-fibroblast coculture. We demonstrated that epithelial cells expressed E-cadherin and that fibroblasts expressed vimentin in coculture at 24 hours. We further showed that the addition of TGF-β1 could induce the model into overexpressing stress fibers. Although, an *in vitro* test bench model has its limitations of being contrived, it provides an important platform for high throughput and targeted screening of a variety of therapeutics before translations into animal models.

Acknowledgments

Funding: Funding was provided from R01DC011759 (National Institutes of Health/National Institute on Deafness and other Communication Disorders).

The authors thank Dr. Sarah Calve for providing access to her microscope facility. The Purdue Speech Language and Hearing Sciences and Weldon School of Biomedical Engineering supported this work. The study was funded by R01DC011759 (National Institutes of Health/National Institute on Deafness and other Communication Disorders).

References

1. Levendoski EE, Leydon C, Thibeault SL. Vocal Fold Epithelial Barrier in Health and Injury A Research Review. Journal of speech, language, and hearing research : JSLHR. 2014; 57(5):1679–1691.
2. Erickson-Levendoski E, Sivasankar MP. Role for Ion Transport in Porcine Vocal Fold Epithelial Defense to Acid Challenge. Otolaryngology -- Head and Neck Surgery. 2012; 146(2):272–278. [PubMed: 22086905]
3. Sivasankar M, Erickson E, Rosenblatt M, Branski RC. Hypertonic challenge to porcine vocal folds: effects on epithelial barrier function. Otolaryngology--head and neck surgery : official journal of American Academy of Otolaryngology-Head and Neck Surgery. 2010; 142(1):79–84. [PubMed: 20096227]
4. Palencia L, Das A, Palecek SP, Thibeault SL, Leydon C. Epidermal growth factor mediated healing in stem cell-derived vocal fold mucosa. The Journal of surgical research. 2015; 197(1):32–38. [PubMed: 25818979]
5. Hiwatashi N, Bing R, Kraja I, Branski RC. Mesenchymal stem cells have antifibrotic effects on transforming growth factor-β1-stimulated vocal fold fibroblasts. The Laryngoscope. 2016
6. Leydon C, Imaizumi M, Bartlett RS, Wang SF, Thibeault SL. Epithelial cells are active participants in vocal fold wound healing: an in vivo animal model of injury. PloS one. 2014; 9(12):e115389. [PubMed: 25514022]
7. Gray SD. Cellular physiology of the vocal folds. Otolaryngologic clinics of North America. 2000; 33(4):679–698. [PubMed: 10918654]
8. Friedrich G, Dikkers FG, Arens C, et al. Vocal fold scars: current concepts and future directions. Consensus report of the phonosurgery committee of the European laryngological society. Eur Arch Otorhinolaryngol. 2013; 270(9):2491–2507. [PubMed: 23605306]
9. Kutty JK, Webb K. Tissue engineering therapies for the vocal fold lamina propria. Tissue engineering. Part B, Reviews. 2009; 15(3):249–262. [PubMed: 19338432]
10. Heris HK, Miri AK, Ghattamaneni NR, et al. Microstructural and mechanical characterization of scarred vocal folds. Journal of biomechanics. 2015; 48(4):708–711. [PubMed: 25648495]
11. Hirano S, Minamiguchi S, Yamashita M, Ohno T, Kanemaru S-i, Kitamura M. Histologic Characterization of Human Scarred Vocal Folds. Journal of Voice. 2009; 23(4):399–407. [PubMed: 18395421]
12. Lim X, Tateya I, Tateya T, Muñoz-Del-Río A, Bless DM. Immediate Inflammatory Response and Scar Formation in Wounded Vocal Folds. Annals of Otology, Rhinology & Laryngology. 2006; 115(12):921–929.

13. Chhetri DK, Head C, Revazova E, Hart S, Bhuta S, Berke GS. Lamina propria replacement therapy with cultured autologous fibroblasts for vocal fold scars. Otolaryngology--head and neck surgery : official journal of American Academy of Otolaryngology-Head and Neck Surgery. 2004; 131(6): 864–870. [PubMed: 15577782]
14. Hahn MS, Teply BA, Stevens MM, Zeitels SM, Langer R. Collagen composite hydrogels for vocal fold lamina propria restoration. Biomaterials. 2006; 27(7):1104–1109. [PubMed: 16154633]
15. Hiwatashi N, Hirano S, Mizuta M, et al. Biocompatibility and efficacy of collagen/gelatin sponge scaffold with sustained release of basic fibroblast growth factor on vocal fold fibroblasts in 3-dimensional culture. The Annals of otology, rhinology, and laryngology. 2015; 124(2):116–125.
16. Chen X, Thibeault SL. Biocompatibility of a synthetic extracellular matrix on immortalized vocal fold fibroblasts in 3-D culture. Acta biomaterialia. 2010; 6(8):2940–2948. [PubMed: 20109588]
17. Chen X, Thibeault SL. Response of Fibroblasts to Transforming Growth Factor-β1 on Two-Dimensional and in Three-Dimensional Hyaluronan Hydrogels. Tissue Engineering. Part A. 2012; 18(23–24):2528–2538. [PubMed: 22734649]
18. Farran AJ, Teller SS, Jha AK, et al. Effects of matrix composition, microstructure, and viscoelasticity on the behaviors of vocal fold fibroblasts cultured in three-dimensional hydrogel networks. Tissue Engineering. Part A. 2010; 16(4):1247–1261. [PubMed: 20064012]
19. King SN, Chen F, Jette ME, Thibeault SL. Vocal fold fibroblasts immunoregulate activated macrophage phenotype. Cytokine. 2013; 61(1):228–236. [PubMed: 23123198]
20. Chen X, Thibeault SL. Cell-cell interaction between vocal fold fibroblasts and bone marrow mesenchymal stromal cells in three-dimensional hyaluronan hydrogel. J Tissue Eng Regen Med. 2013
21. Choi JW, Park JK, Chang JW, et al. Small intestine submucosa and mesenchymal stem cells composite gel for scarless vocal fold regeneration. Biomaterials. 2014; 35(18):4911–4918. [PubMed: 24679493]
22. Farran AJE, Teller SS, Jia F, Rodney JC, Duncan RL, Jia X. Design and Characterization of a Dynamic Vibrational Culture System. Journal of tissue engineering and regenerative medicine. 2013; 7(3):213–225. [PubMed: 22095782]
23. Fishman JM, Long J, Gugatschka M, et al. Stem cell approaches for vocal fold regeneration. The Laryngoscope. 2016 n/a–n/a.
24. Imaizumi M, Sato Y, Yang DT, Thibeault SL. In vitro epithelial differentiation of human induced pluripotent stem cells for vocal fold tissue engineering. The Annals of otology, rhinology, and laryngology. 2013; 122(12):737–747.
25. Johnson BQ, Fox R, Chen X, Thibeault S. Tissue regeneration of the vocal fold using bone marrow mesenchymal stem cells and synthetic extracellular matrix injections in rats. Laryngoscope. 2010; 120(3):537–545. [PubMed: 20131370]
26. Leydon C, Selekman JA, Palecek S, Thibeault SL. Human Embryonic Stem Cell-Derived Epithelial Cells in a Novel In Vitro Model of Vocal Mucosa. Tissue Engineering Part A. 2013 130626063917000.
27. Suzuki S, Sato M, Senoo H, Ishikawa K. Direct cell-cell interaction enhances pro-MMP-2 production and activation in co-culture of laryngeal cancer cells and fibroblasts: involvement of EMMPRIN and MT1-MMP. Experimental cell research. 2004; 293(2):259–266. [PubMed: 14729463]
28. Scott RA, Ramaswamy AK, Park K, Panitch A. Decorin mimic promotes endothelial cell health in endothelial monolayers and endothelial–smooth muscle co-cultures. Journal of Tissue Engineering and Regenerative Medicine. 2015 n/a–n/a.
29. Lombardo Bedran TB, Palomari Spolidorio D, Grenier D. Green tea polyphenol epigallocatechin-3-gallate and cranberry proanthocyanidins act in synergy with cathelicidin (LL-37) to reduce the LPS-induced inflammatory response in a three-dimensional co-culture model of gingival epithelial cells and fibroblasts. Archives of Oral Biology. 2015; 60(6):845–853. [PubMed: 25791329]
30. Xu W, Hu R, Fan E, Han D. Adipose-Derived Mesenchymal Stem Cells in Collagen—Hyaluronic Acid Gel Composite Scaffolds for Vocal Fold Regeneration. Annals of Otology, Rhinology & Laryngology. 2011; 120(2):123–130.

31. Chaterji S, Park K, Panitch A. Scaffold-free in vitro arterial mimetics: the importance of smooth muscle-endothelium contact. Tissue Engineering. Part A. 2010; 16(6):1901–1912. [PubMed: 20088699]
32. Papazian D, Wagtmann VR, Hansen S, Wurtzen PA. Direct contact between dendritic cells and bronchial epithelial cells inhibits T cell recall responses towards mite and pollen allergen extracts in vitro. Clinical and experimental immunology. 2015
33. Long JL, Zuk P, Berke GS, Chhetri DK. Epithelial differentiation of adipose-derived stem cells for laryngeal tissue engineering. Laryngoscope. 2010; 120(1):125–131. [PubMed: 19856398]
34. Liao H, Munoz-Pinto D, Qu X, Hou Y, Grunlan MA, Hahn MS. Influence of hydrogel mechanical properties and mesh size on vocal fold fibroblast extracellular matrix production and phenotype. Acta biomaterialia. 2008; 4(5):1161–1171. [PubMed: 18515199]
35. Biernacka A, Dobaczewski M, Frangogiannis NG. TGF-β signaling in fibrosis. Growth factors (Chur, Switzerland). 2011; 29(5):196–202.
36. Vyas B, Ishikawa K, Duflo S, Chen X, Thibeault SL. Inhibitory effects of hepatocyte growth factor and interleukin-6 on transforming growth factor-beta1 mediated vocal fold fibroblast-myofibroblast differentiation. The Annals of otology, rhinology, and laryngology. 2010; 119(5): 350–357.
37. Chen X, Thibeault SL. Novel isolation and biochemical characterization of immortalized fibroblasts for tissue engineering vocal fold lamina propria. Tissue engineering. Part C, Methods. 2009; 15(2):201–212. [PubMed: 19108681]
38. Branco A, Bartley SM, King SN, Jette ME, Thibeault SL. Vocal fold myofibroblast profile of scarring. Laryngoscope. 2016; 126(3):E110–117. [PubMed: 26344050]
39. Choi JS, Lee S, Kim da Y, Kim YM, Kim MS, Lim JY. Functional remodeling after vocal fold injury by small intestinal submucosa gel containing hepatocyte growth factor. Biomaterials. 2015; 40:98–106. [PubMed: 25433606]
40. Vu T, Jin L, Datta PK. Effect of Cigarette Smoking on Epithelial to Mesenchymal Transition (EMT) in Lung Cancer. Journal of clinical medicine. 2016; 5(4)
41. Davis FM, Stewart TA, Thompson EW, Monteith GR. Targeting EMT in cancer: opportunities for pharmacological intervention. Trends in pharmacological sciences. 2014; 35(9):479–488. [PubMed: 25042456]
42. Ginnebaugh KR, Ahmad A, Sarkar FH. The therapeutic potential of targeting the epithelial-mesenchymal transition in cancer. Expert opinion on therapeutic targets. 2014; 18(7):731–745. [PubMed: 24758643]
43. Chen T, Nie H, Gao X, et al. Epithelial-mesenchymal transition involved in pulmonary fibrosis induced by multi-walled carbon nanotubes via TGF-beta/Smad signaling pathway. Toxicology letters. 2014; 226(2):150–162. [PubMed: 24530353]
44. Li M, Luan F, Zhao Y, et al. Epithelial-mesenchymal transition: An emerging target in tissue fibrosis. Experimental biology and medicine (Maywood, NJ). 2016; 241(1):1–13.

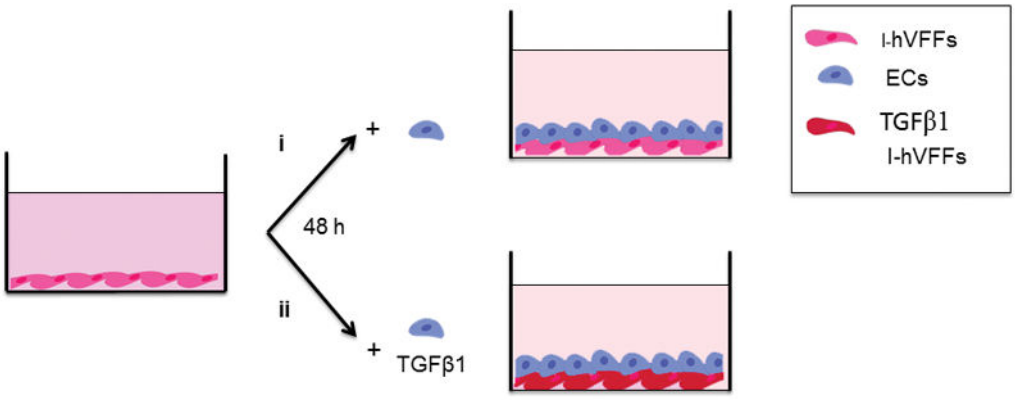

Figure 1.

Visualization of experimental design. I-hVFFs were seeded at 3–4 × 10^4 cells/cm^2 and allowed to grow to confluence for 48 hours to enable extracellular matrix formation. After 48 h, ECs were seeded directly on top at a seeding density of 6–7 × 10^4 cells/cm^2, either in (i) complete epithelial media to create a healthy coculture or in (ii) complete epithelial media supplemented with 10 ng/ml TGFβ1 to induce into a fibrotic coculture model

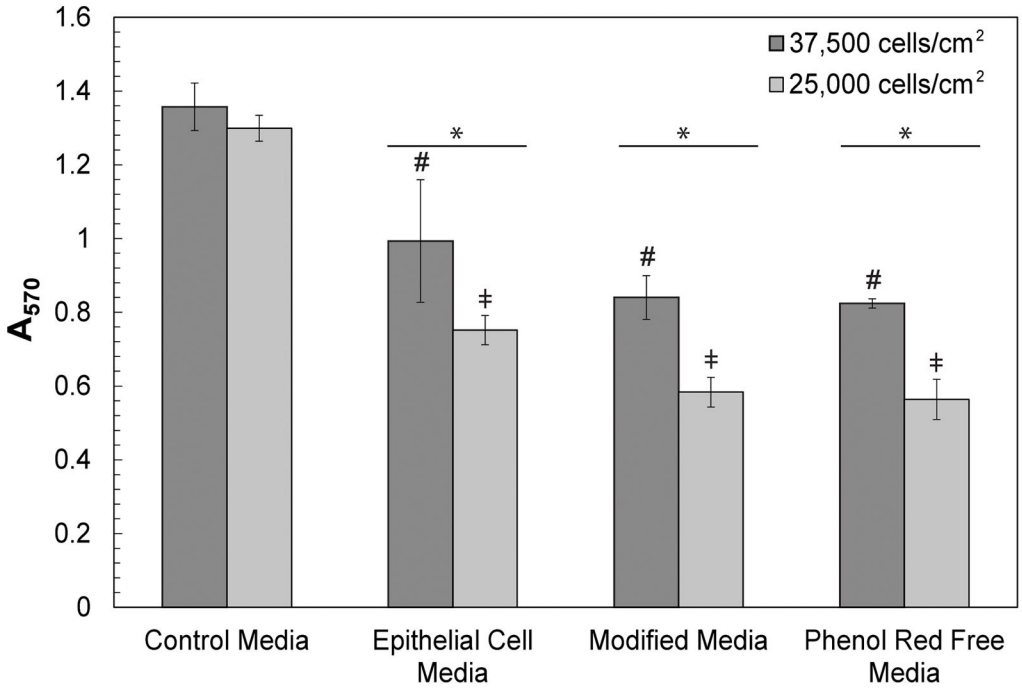

Figure 2.

MTT assay on I-hVFFs for metabolic activity. I-hVFFs performed better in epithelial media at a seeding density of 37,500 cells/cm^2 and was hence, chosen for future experiments. As metabolic activity was retained in all medias, epithelial media was chosen as coculture media to ensure that the primary ECs maintain phenotype

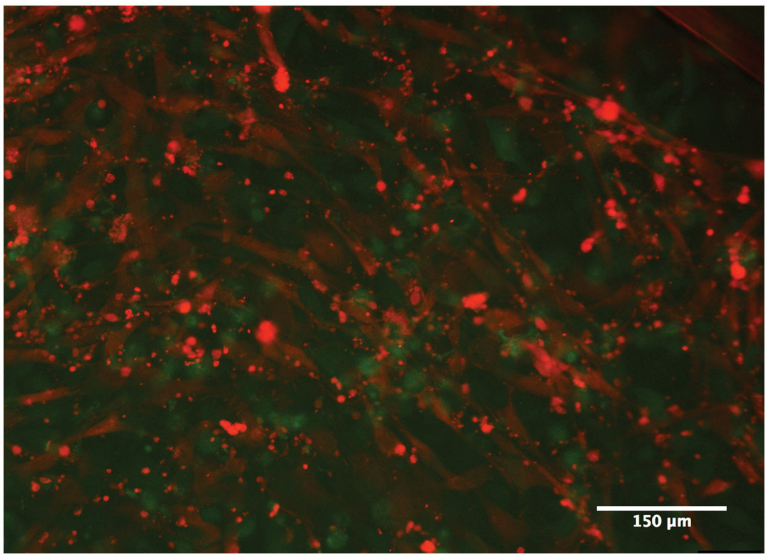

Figure 3.

Coculture visualization using live Cell Tracker®. I-hVFFs were tracked with Cell Tracker® Orange CMRA, and ECs were tracked with Cell Tracker® Green CMFDA. Both cells maintain morphology in coculture. Imaged after 24 h of coculture. Scale bar = 150 µm

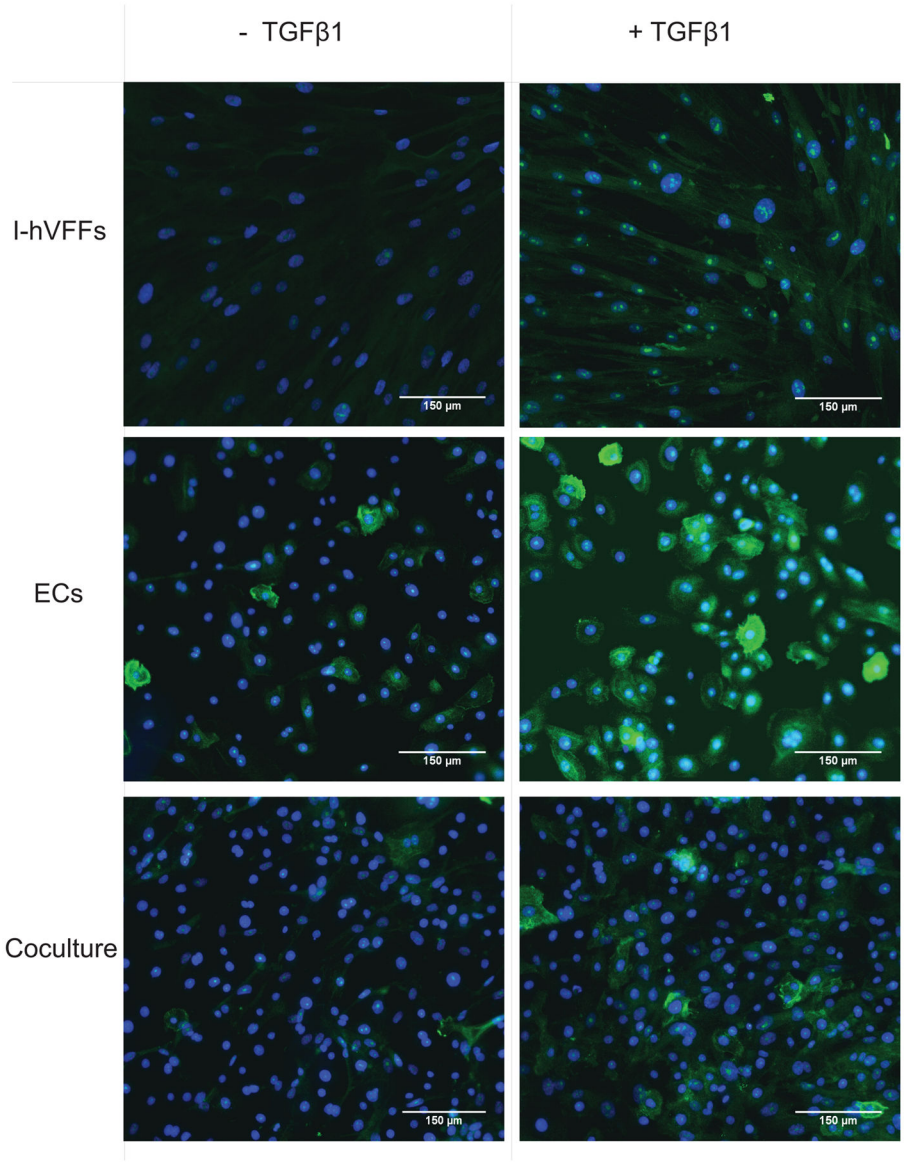

Figure 4.

Representative images of α-SMA expression in monocultures of I-hVFFs and ECs, and in coculture, with and without addition of TGFβ1. Increased expression of α-SMA was seen after induction with TGFβ1, suggesting a fibrotic phenotype. Scale bars = 150 μm

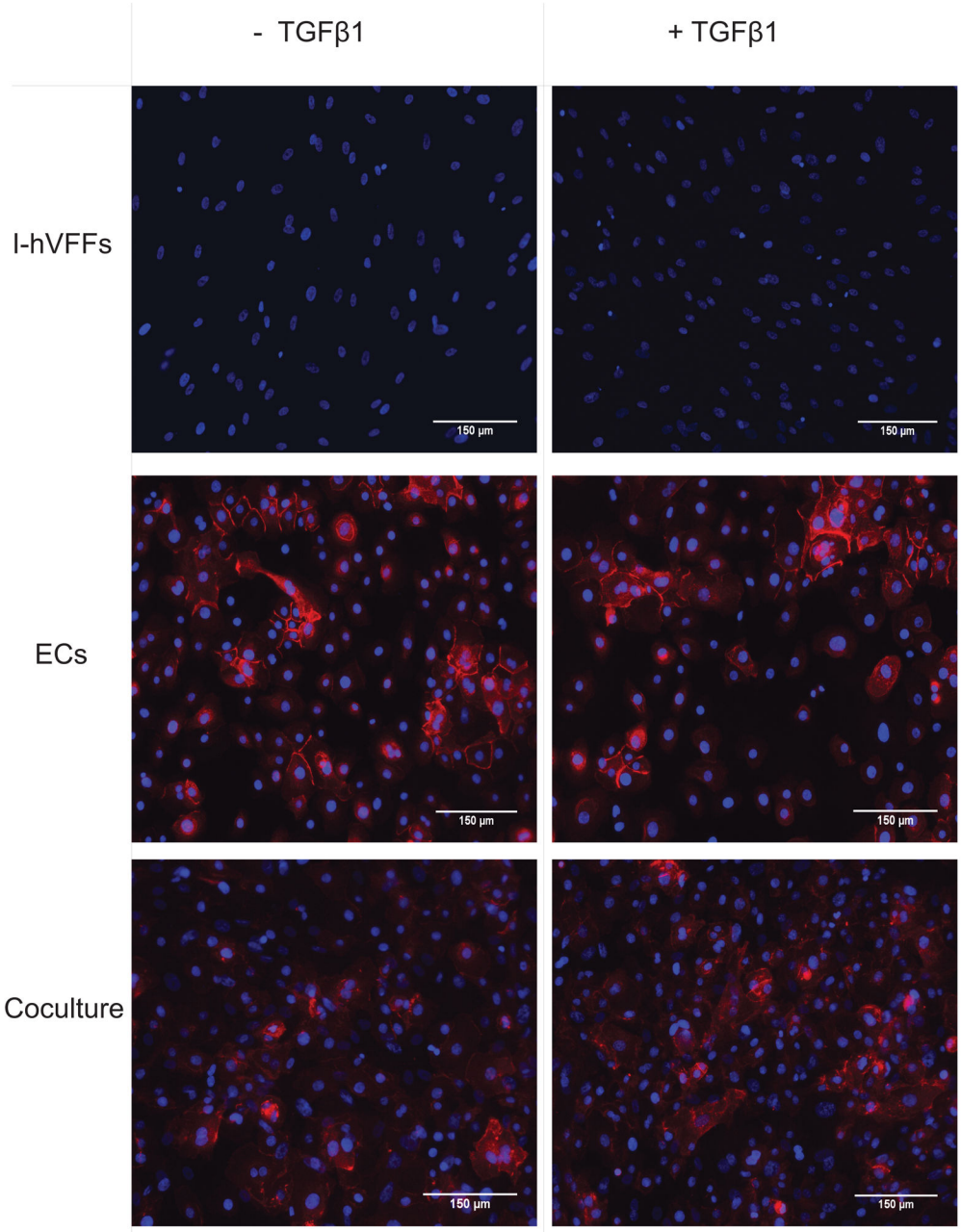

Figure 5.
Representative images of E-cadherin expression seen in monocultures of ECs and in coculture. Addition of TGFβ1 did not seem to change expression levels. Scale bars = 150 μm

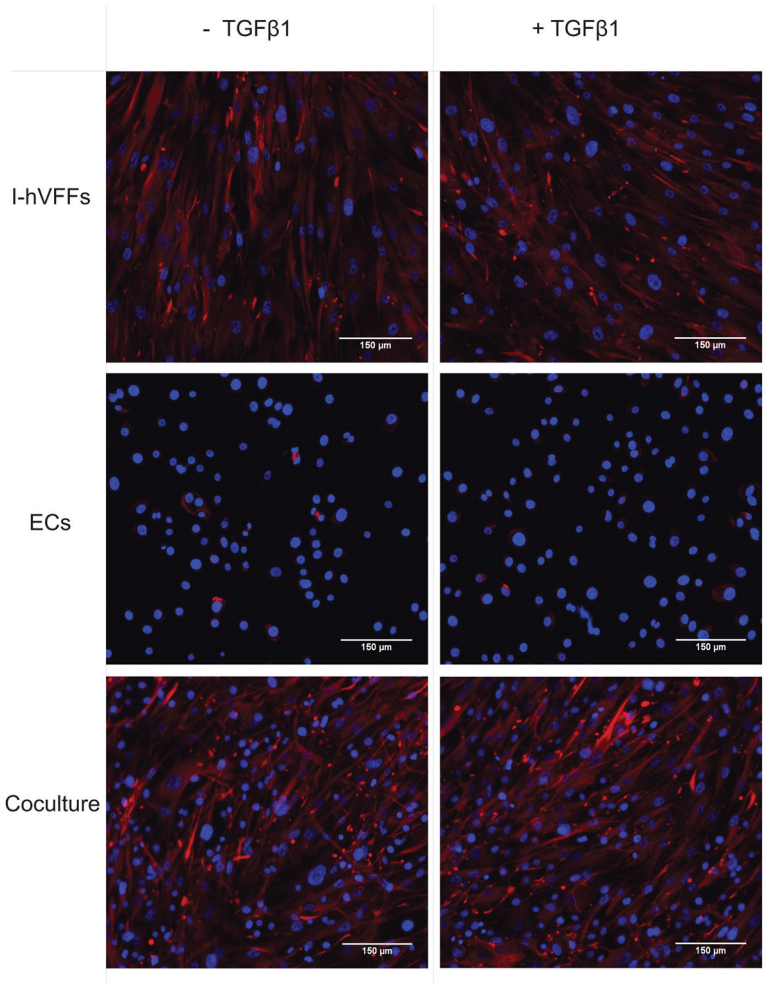

Figure 6.
Representative images of Vimentin expression seen in monocultures of I-hVFFs and in coculture, with and without addition of TGFβ1. Scale bars = 150 μm