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Proteomics Clinical Applications

DATASET BRIEF

Descriptive proteomics of paired human vocal fold and buccal mucosa tissue

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Abstract

The vast majority of voice disorders is associated with changes of the unique, but delicate, human vocal fold mucosa. The ability to develop new effective treatment methods is significantly limited by the physical inaccessibility and the extremely rare occasions under which healthy tissue biopsies can be obtained. Therefore, the interest in laryngological research has shifted to human oral (buccal) mucosa, a similar and more easily available tissue. The harvesting process is less invasive and accompanied with faster healing and less scarring, compared to vocal fold mucosa. Here we report a descriptive proteomic comparison of paired human buccal and vocal fold mucosa by high-resolution mass spectrometry (CID-MS/MS). Our study identified a total of 1575 proteins detected within both tissues that are highly consistent in several crucial biological processes, cellular components, and molecular functions. Hence, our proteomic analysis will provide a fundamental resource for the laryngological research community.

KEYWORDS
descriptive proteomics, human tissue, oral buccal mucosa, vocal folds

Voice disorders are the most common communication disorder, with up to 30% of the general population developing voice disorders during their lifetime due to stimuli such as vocal overuse, air pollution or cigarette smoking [1–3]. The usual clinical symptoms involve a breathy and hoarse (dysphonic) voice, and loss of vocal control. Voice disorders may have severe consequences for individuals, especially in occupations with a high vocal demand.

Depending on the pathology and the severity of the disorder, as well as the patient's individual needs and motivation, the main goal of any current treatment is to improve vocal fold (VF) vibration by restoring the biomechanical tissue function. VF represent a unique multilayered structure, namely the epithelium, the *lamina propria* (LP), and the underlying thyroarytenoid muscle, housed in the laryngeal skeleton. Phonatory tissue function (i.e., vibration) is mainly determined by the physiological biomechanical properties of the LP, where the composition and distribution of various types of cellular components and extracellular matrix (ECM) molecules, including fibrous proteins, interstitial glycoproteins, proteoglycans, and glycosaminoglycans is essential [4, 5]. Besides the epithelium, VF fibroblasts (VFF) within

Abbreviations: VF, vocal folds; BM, buccal mucosa; LP, lamina propria; VFF, vocal fold fibroblasts; ECM, extracellular matrix; hESC, human embryonic stem cell-derived epithelial cells; TCEP, Tris(2-carbocylethyl)phosphine hydrochloride; CAA, chloracetamide; TFE, 2,2,2-Trifluoroethanol; PSM, peptide-spectra-matches; BP, biological process; CC, cellular component; MF, molecular function

the LP synthesize most of the ECM in response to stimuli from the surrounding environment, and therefore play a significant role in VF biology. To date, the ability to develop new effective treatment methods is significantly limited by the physical inaccessibility of the VF in humans, and the extremely rare occurrence of obtaining healthy VF mucosa biopsies, other than from deceased donors, due to ethical constraints [6, 7]. For practical reasons, most research in the field of laryngology has focused on a single cell type of the VF—mainly fibroblasts. Little is known about the underlying mechanisms of direct signaling events in growth, migration, and differentiation between multiple cell types located in the VF mucosa, such as macrophages, endothelial cells, stem cells or epithelial cells [8]. To overcome these shortcomings, in vitro tissue engineering and multi-cellular applications have been widely established in basic VF research, including approaches of regenerative medicine. The latest developments of research moved from two-dimensional to three-dimensional in vitro models of epithelial cell and fibroblast cocultures. Due to the absence of human VF epithelial cell lines, cells of human embryonic stem cell-derived epithelial cells (hESC) [6, 9] or oral mucosal epithelial cells [10] were used as alternative. Recently, Chen et al. published the establishment of immortalized human VF epithelial cell lines [11]. However, these are still not widely available for research purposes.

The human mucosa serves as the physiological barrier throughout the human body, most notably in the genitourinary, gastrointestinal, and the respiratory systems. Its structure and function differs slightly, depending on location and anatomy [12]. The buccal mucosa (BM) is of specific interest for laryngological research, as the tissue harvesting process is less invasive than of VF mucosa and is accompanied with faster healing and less scarring [10, 12, 13]. Furthermore, its characteristics as a non-keratinized squamous tissue, which is comparable to VF epithelium, makes it an interesting candidate for in vitro applications. Due to its biocompatibility to the various recipient tissues, it found widespread use in vaginal, urethral, conjunctival, orbital, and pharyngeal reconstruction [12, 14–17].

This dataset brief reports a descriptive study of full proteomic data sets of paired human tissue samples from BM and VF, indicating that cellular components of the BM can be attractive candidates for laryngological in vitro research, as tissues from healthy human VF cannot be obtained on a routine basis.

Paired native tissue samples from VF and BM from three true biological replicates ($n = 3$, donor demographic see Table S1) were collected during autopsy, within 6 h post mortem of patients who had previously not been intubated (Diagnostic and Research Institute of Pathology, Medical University Graz). Procedures were approved by the local ethics committee (approval no. 29–036 ex 16/17). Tissue samples were collected in decontamination medium (DM), consisting of DMEM supplemented with 1% Antibiotic-Antimycotic solution and 100 μg/mL Normocin. Snap-frozen tissue samples were homogenized with steel beads (Sigma, Austria) using MagNAlyser (Roche, Switzerland) for 3 min in 600 μL of 100 mM Tris-HCL (1% SDS, 10 mM TCEP, 40 mM CAA). After removal of the beads, homogenates were reduced, alkylated for 10 min at 95°C, and centrifuged at 15,000 × g for 7 min at 4°C. The supernatants were collected for proteomic analysis. Protein concentration was determined using the BCA-RAC assay (Thermo Fisher Scientific, USA) according to the manufacturert's instructions.

For LC-MS/MS analysis, 100 μg of solubilized protein were precipitated with four volumes of acetone overnight and obtained protein pellets were re-solubilized in TFE buffer (25% TFE, 100 mM Tris HCl pH 8.5), then diluted with 50 mM ammonium bicarbonate (to reach 10% TFE). Diluted samples were predigested with rLysC (Promega, USA; enzyme/protein 1:100) for 4 h at 37°C and digested overnight with trypsin (Promega, enzyme/protein 1:50). Peptides were desalted using SBD-RPS tips. 500 ng per sample (re-dissolved in 2% acetonitrile/0.1% formic acid in water) was subjected to LC-MS/MS analysis. Protein digests were separated by nano-HPLC (Dionex Ultimate 3000, Thermo Fisher Scientific) equipped with an Aurora (Ionoptics, Australia) nanocolumn (C18, 1 μm, 250 × 0.075 mm) at a flow rate of 300 nL/min at 50°C using the following gradient (solvent A: 0.1% formic acid in water, solvent B: acetonitrile containing 0.1% formic acid): 0–18 min: 2% B, 18–100 min: 2%–25% B; 100–107 min: 25%–35% B; 107–108 min: 35%–95% B; 108–118 min: 95% B; 118-118 min: 95%–2% B; 118–133 min: 2% B. The maXis II ETD mass spectrometer (Bruker Daltonics, Germany) was operated with the captive source in positive mode with the following settings: mass range: 200–2000 m/z, 2 Hz, capillary 16,000 V, dry gas flow 3 L/min with 150°C, nanoBooster 0.2 bar, precursor acquisition control top 20 (collision induced dissociation (CID).

The LC-MS/MS data were analyzed by MaxQuant by searching the public SwissProt human database (11393515 residues, 20467 sequences) and common contaminants. Carbamidomethylation on cystein and oxidation on methionine were set as a fixed and as a variable modification, respectively. Detailed search criteria were used as follows: trypsin, max. missed cleavage sites: 2; search mode: MS/MS ion search with decoy database search included; precursor mass tolerance ± 0.0006 Da; product mass tolerance ± 80 ppm; acceptance parameters for identification: 1% PSM FDR; 1% protein FDR. Additionally, a label free quantification (LFQ) was performed using MaxQuant [18] requiring a minimum of two ratio counts of quantified razor and unique peptides. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [19] partner repository and can be retrieved via the dataset identifier PXD025519.

We collected 3192 proteins; 147,216 high-resolution CID-MS/MS counts in total and accepted 427,115 peptide-spectrum matches after the database search. After removing reverse hits, potential contaminants, and proteins identified only by site; 20,632 unique peptides corresponding to 3094 proteins (Table S2) were identified with a FDR < 1% using Perseus version 1.6.6.0. Missing value imputation was performed based on the normal distribution (0.3 of width, 1.4 of down-shift). Filtering for peptide counts (razor+unique peptides) > 1, and 2 valid values identified a total of 1717 proteins (1397 proteins on average, SD = 53.8) with robust abundance similar across all analyzed samples (Figure 1A). Of these, 1575 (91.7%, Figure 1C, Table S3) were detected in both VF and BM - showing a high degree of overlap

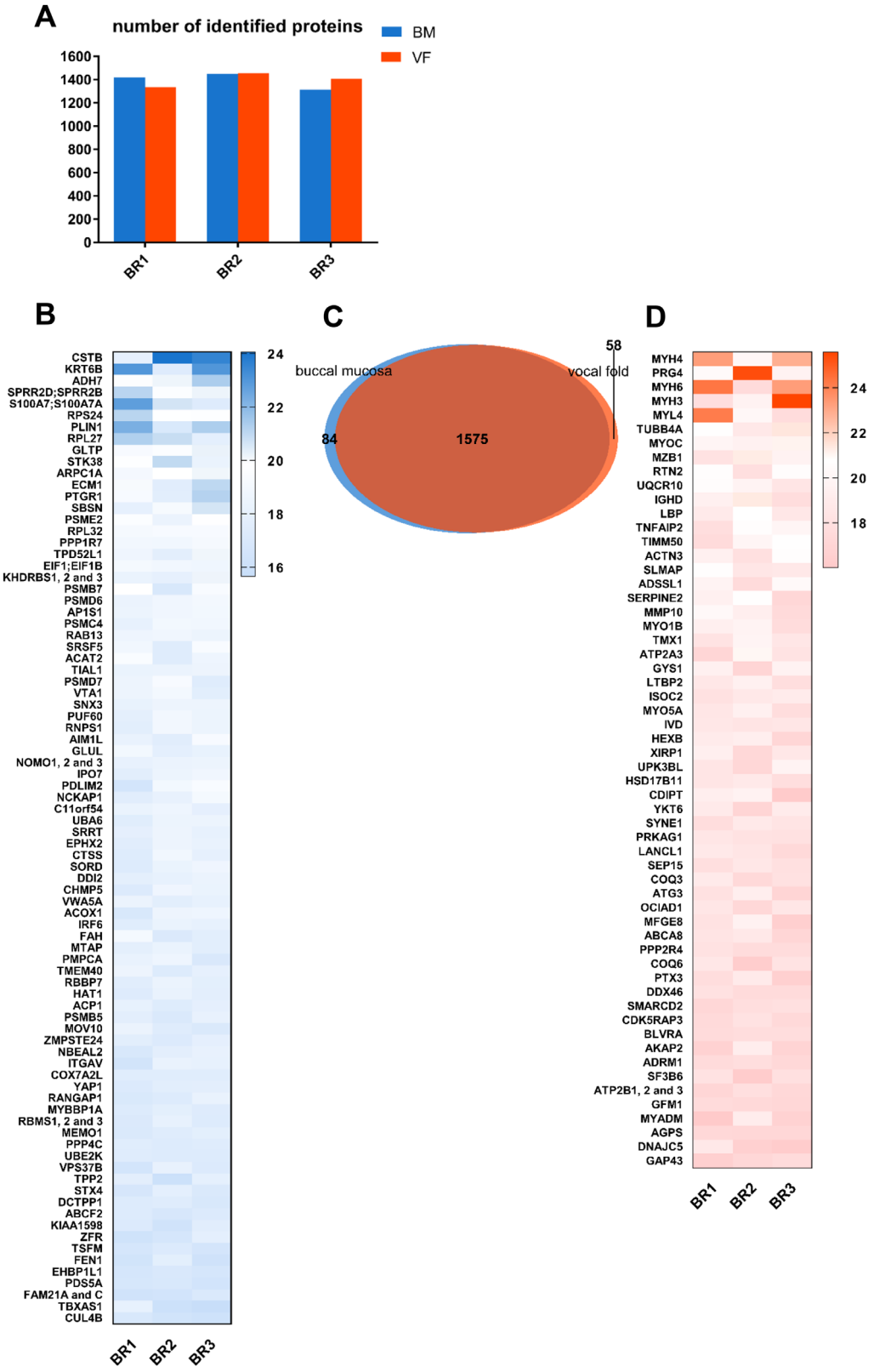

FIGURE 1 Proteomic profiling of paired tissue samples (vocal fold, buccal mucosa) of three donors. Number of unique and robustly detected proteins across distinct samples (A). Proteomic heatmap showing proteins detected solely in BM tissue samples (B). Venn diagram depicting the number of proteins detected solely in VF tissue samples, solely in BM tissue samples, and in both tissue types (C). Proteomic heatmap showing proteins detected solely in VF tissue samples (D). Proteins are specified by gene symbols and sorted in a decreasing manner according to their mean LFQ intensity values (B, D)

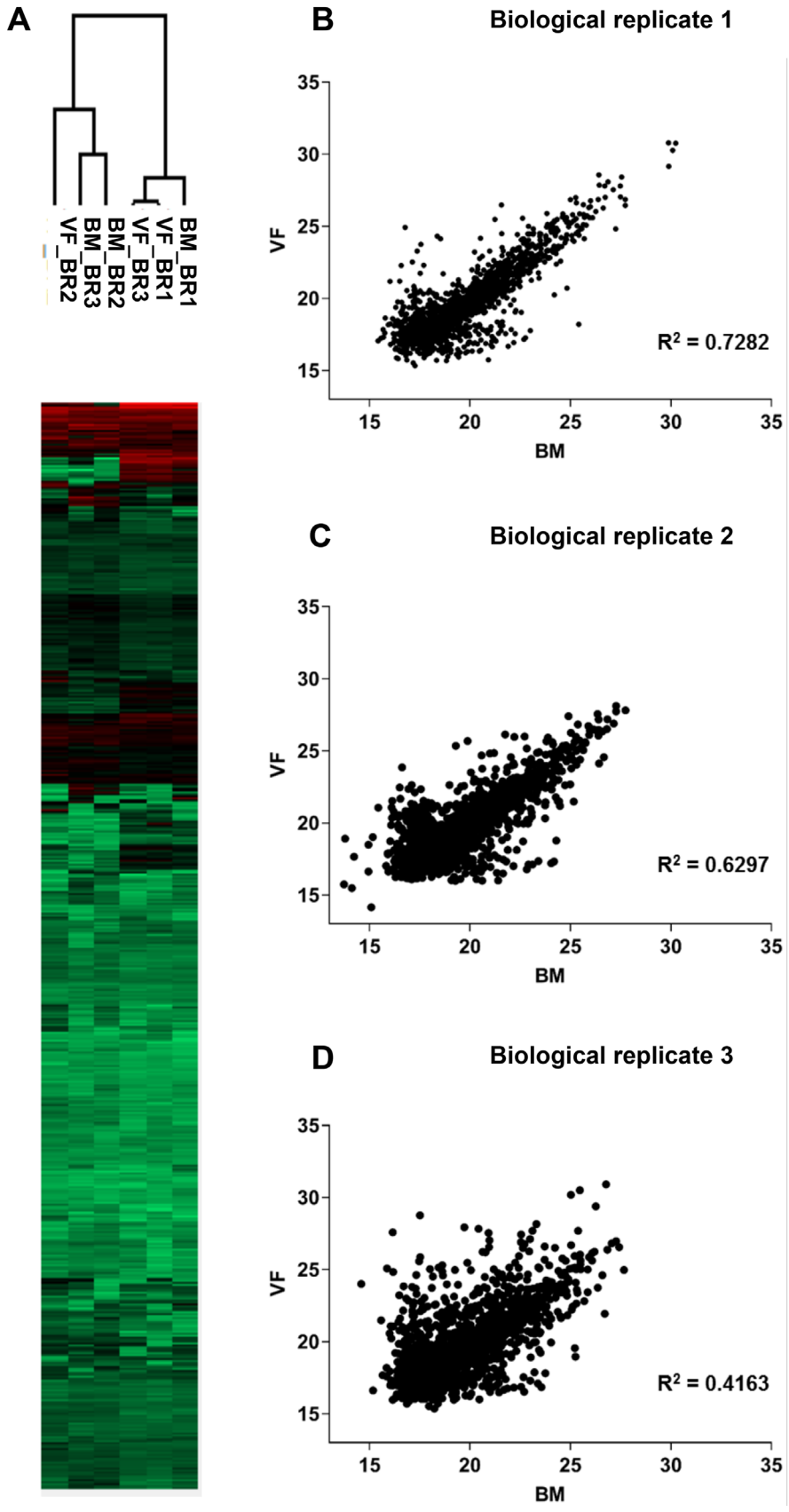

FIGURE 2 Proteomic profiling of paired tissue samples (vocal fold, buccal mucosa) of three donors. Heat map with hierarchical clustering showing log2 transformed LFQ intensity values for proteins detected in both tissue types for biological replicates (A). Statistical analysis was performed using a paired *t*-test (original FDR method of Benjamini and Hochberg, Q = 1%). Scatter plots showing log2 transformed LFQ intensity values for proteins detected in both tissue types for biological replicates (B, C, and D)

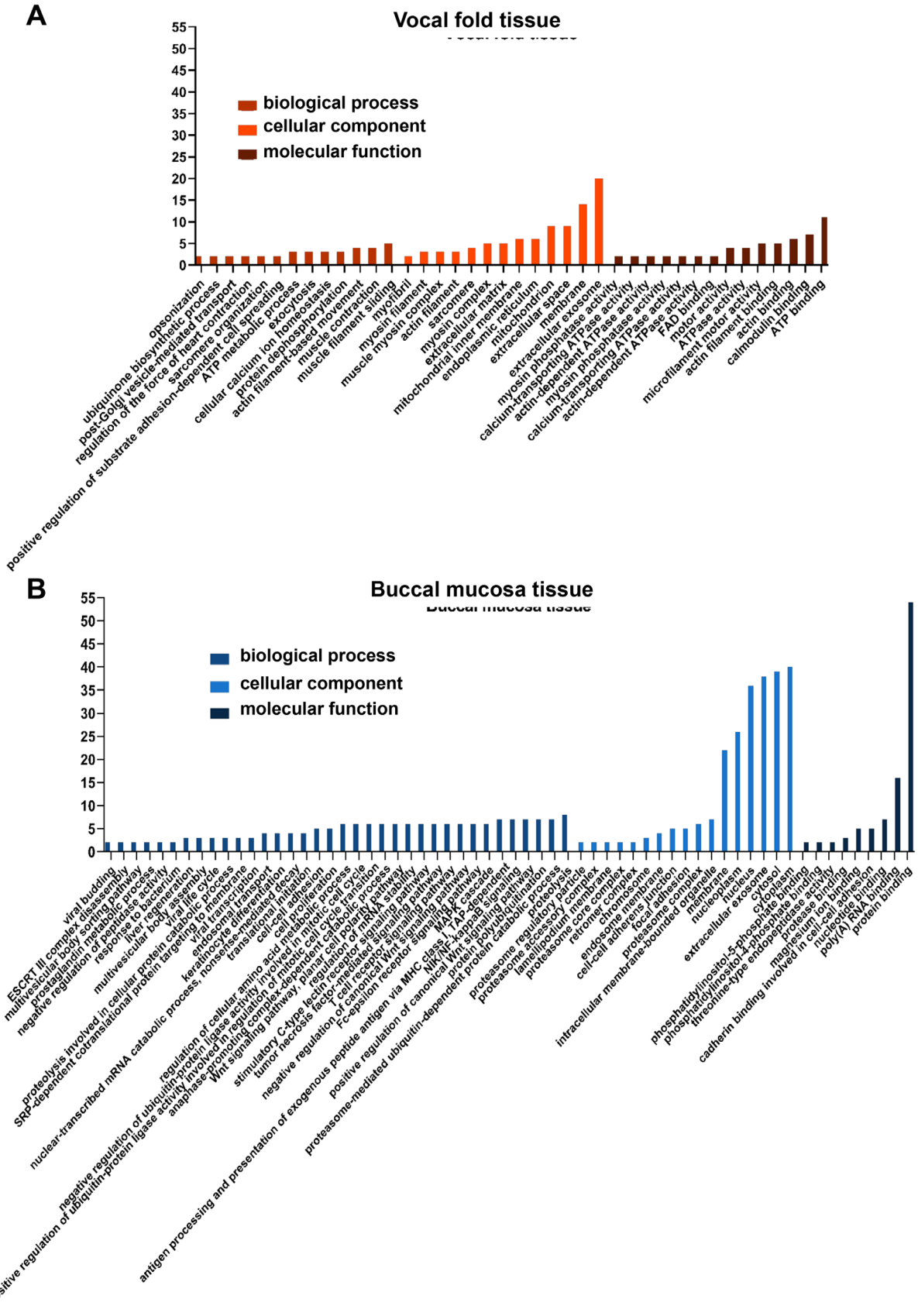

FIGURE 3 GO enrichment analysis data of paired tissue samples (vocal fold, buccal mucosa) of three donors. Major categories of proteins detected solely in VF tissue samples (A) or solely in BM tissue samples (B)

between the tissues, while 84 (4.9%, Figure 1B, Table S4) were identified only in BM, and 58 (3.4%, Figure 1D, Table S5) were found only in VF.

To test for further congruencies in the protein repertoire of both tissue types, a paired *t*-test (original FDR method of Benjamini and Hochberg, Q = 1%) of log2 transformed LFQ intensity values was conducted. No significant difference in protein expression could be identified, indicating an overall comparable protein expression level between BM and VF (Figure 2A-D).

Classification according to their roles within biological process (BP), cellular component (CC), and molecular function (MF) categories for the protein repertoire detected in both tissue types (Figure S1, BM: Table S6, VF: Table S7) was performed with GO enrichment analysis using the DAVID bioinformatics tool (http://david.ncifcrf.gov/) [20]. Collectively, these data confirmed consistent similarities for all categories between the different tissue types. GO enrichment analysis of proteins that were detected only in VF tissue (Figure 3A, Table S8) showed they are mostly muscle-related. Proteins found only in BM tissue (Figure 3B, Table S9) are associated with extracellular exosome and proteasome. Interestingly, these were shown to promote wound healing [21] and regeneration [22].

Despite the limited number of biological replicates and a lack of a reference mucosa sample, which are the limitations of our study, we provide new insights into the comparable protein content of BM and VF tissues in humans. Additional comparative analyses are required to substantiate these findings; however, protein expression similarity between BM and VF, combined with the faster healing and reduced scarring properties of BM compared to VF, could be of further interest for the development of in vitro strategies using the human BM in laryngological research. The ability to isolate a higher number of cells could favor the establishment of multi-cellular in vitro models for studying the complex processes (e.g., wound healing, fibrosis, or barrier and integrity function) in response to external stimuli in experimental treatments. This may significantly enhance current knowledge in the context of physiological and pathophysiological mechanisms in human VF.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in PRIDE at https://www.ebi.ac.uk/pride, reference number PXD025519.

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REFERENCES

1. Roy, N., Merrill, R. M., Gray, S. D., & Smith, E. M. (2005). Voice disorders in the general population: Prevalence, risk factors, and occupational impact. *The Laryngoscope*, *115*(11), 1988–1995.
2. Ramig, L. O., & Verdolini, K. (1998). Treatment efficacy: Voice disorders. *Journal of Speech, Language, and Hearing Research: JSLHR*, *41*(1), S101-116.
3. Benninger, M. S., Holy, C. E., Bryson, P. C., & Milstein, C. F. (2017). Prevalence and occupation of patients presenting with dysphonia in the united states. *Journal of Voice: Official Journal of the Voice Foundation*, *31*(5), 594–600.
4. Miri, A. K., Li, N. Y. K., Avazmohammadi, R., Thibeault, S. L., Mongrain, R., & Mongeau, L. (2015). Study of extracellular matrix in vocal fold biomechanics using a two-phase model. *Biomechanics and Modeling in Mechanobiology*, *14*(1), 49–57.
5. Walimbe, T., Panitch, A., & Sivasankar, P. M. (2017). A review of hyaluronic acid and hyaluronic acid-based hydrogels for vocal fold tissue engineering. *Journal of Voice: Official Journal of the Voice Foundation*, *31*(4), 416–423.
6. Palencia, L., Das, A., Palecek, S. P., Thibeault, S. L., & Leydon, C. (2015). Epidermal growth factor mediated healing in stem cell-derived vocal fold mucosa. *The Journal of Surgical Research*, *197*(1), 32–38.
7. Chen, X., & Thibeault, S. L. (2009). Novel isolation and biochemical characterization of immortalized fibroblasts for tissue engineering vocal fold lamina propria. *Tissue Engineering. Part C, Methods*, *15*(2), 201–212.
8. Gugatschka, M., Kojima, T., Ohno, S., Kanemaru, S.-I., & Hirano, S. (2011). Recruitment patterns of side population cells during wound healing in rat vocal folds. *The Laryngoscope*, *121*(8), 1662–1667.
9. Leydon, C., Selekman, J. A., Palecek, S., & Thibeault, S. L. (2013). Human embryonic stem cell-derived epithelial cells in a novel in vitro model of vocal mucosa. *Tissue Engineering. Part A*, *19*(19-20), 2233–2241.
10. Fukahori, M., Chitose, S.-I., Sato, K., Sueyoshi, S., Kurita, T., Umeno, H., Yamakawa, R. (2016). Regeneration of vocal fold mucosa using tissue-engineered structures with oral mucosal cells. *PLoS One*, *11*(1), e0146151.
11. Chen, X., Lungova, V., Zhang, H., Mohanty, C., Kendziorski, C., & Thibeault, S. L. (2021). Novel immortalized human vocal fold epithelial cell line: In vitro tool for mucosal biology. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, *35*(2), e21243.
12. Evans, E. W. (2017). Treating scars on the oral mucosa. *Facial Plastic Surgery Clinics of North America*, *25*(1), 89–97.
13. Guzmán-Uribe, D., Alvarado-Estrada, K. N., Pierdant-Pérez, M., Torres-Álvarez, B., Sánchez-Aguilar, J. M., & Rosales-Ibáñez, R. (2017). Oral mucosa: An alternative epidermic cell source to develop autologous dermal-epidermal substitutes from diabetic subjects. *Journal of Applied Oral Science: Revista FOB*, *25*(2), 186–195.
14. Grixti, A., & Malhotra, R. (2018). Oral mucosa grafting in periorbital reconstruction. *Orbit (Amsterdam, Netherlands)*, *37*(6), 411–428.
15. Goyal, A., Singh, M. V. A., & Dickson, A. P. (2010). Oral mucosa graft for repair of hypospadias: outcomes at puberty. *The Journal of Urology*, *184*(6), 2504–2508.
16. Mungadi, I. A., & Ugboko, V. I. (2009). Oral mucosa grafts for urethral reconstruction. *Annals of African Medicine*, *8*(4), 203–209.
17. Grimsby, G. M., & Baker, L. A. (2014). The use of autologous buccal mucosa grafts in vaginal reconstruction. *Current Urology Reports*, *15*(8), 428.
18. Cox, J., Hein, M. Y., Luber, C. A., Paron, I., Nagaraj, N., & Mann, M. (2014). Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. *Molecular & cellular proteomics: MCP*, *13*(9), 2513–2526.
19. Deutsch, E. W., Bandeira, N., Sharma, V., Perez-Riverol, Y., Carver, J. J., Kundu, D. J., Vizcaíno, J. A. (2020). The ProteomeXchange consortium

in 2020: Enabling 'big data' approaches in proteomics. *Nucleic Acids Research*, *48*(D1), D1145-D1152.
20. Huang, D. W., Sherman, B. T., & Lempicki, R. A. (2009). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature Protocols*, *4*(1), 44–57.
21. Sjöqvist, S., Ishikawa, T., Shimura, D., Kasai, Y., Imafuku, A., Bou-Ghannam, S., Kanai, N. (2019). Exosomes derived from clinical-grade oral mucosal epithelial cell sheets promote wound healing. *Journal of Extracellular Vesicles*, *8*(1), 1565264.
22. Bardag-Gorce, F., Hoft, R., Meepe, I., Garcia, J., Tiger, K., Wood, A., Niihara, Y. (2017). Proteasomes in corneal epithelial cells and cultured autologous oral mucosal epithelial cell sheet (CAOMECS) graft used for the ocular surface regeneration. *The Ocular Surface*, *15*(4), 749–758.

SUPPORTING INFORMATION

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