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Mycoplasma affects baseline gene expression and the response to glucocorticoids in vocal fold fibroblasts

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Abstract

Introduction. *In vitro* experimentation is intentionally contrived to isolate specific phenomena in the context of profound biological complexity. Mycoplasmas in the upper airway likely contribute to this complexity and play a largely unknown role in both health and disease. Similarly, the presence and role of mycoplasma in *in vitro* investigation are largely unknown.

Hypothesis. We hypothesize mycoplasma in human vocal fold fibroblasts (VFF) will affect both basal gene-expression patterns as well as the cell response to exogenous stimuli.

Aim. We sought to determine mycoplasma presence across vocal fold fibroblast cultures, basal transcriptional changes as a function of mycoplasma, and responsiveness to exogenous glucocorticoids in mycoplasma-positive and -negative VFF.

Methodology. PCR-based mycoplasma detection was performed in an immortalized human VFF line as well as rat and rabbit primary VFF cultures and extracted rat laryngeal tissue. RNA sequencing was performed in mycoplasma-positive and -negative human cells at baseline and in response to dexamethasone.

Results. Mycoplasma was identified in the human cell line as well as primary culture from rabbits. Mycoplasma was not detected in tissue or primary culture from rat vocal folds. Basal mRNA expression in human VFF differed significantly following mycoplasma treatment. In addition, differential responses to dexamethasone were observed across multiple pathways as a function of mycoplasma presence in these cells. Pathways including apoptosis, DNA damage repair, and G1 to S cell cycle signalling were significantly enriched in mycoplasma-positive cells.

Conclusion. Variability of mycoplasma presence across culture conditions and differential responses to exogenous stimuli as a function of mycoplasma presence are potentially problematic for the translation of *in vitro* experimentation in the upper aerodigestive tract. It remains unclear if these findings represent contamination or the baseline state of this specialized tissue.

INTRODUCTION

Mycoplasmas are small, pleomorphic bacteria that lack a cell wall [1]. Although several strains are associated with serious sequelae, including mycoplasma pneumonia [2, 3], numerous non- or mildly pathogenic mycoplasma species are found throughout the body. Commensalism of mycoplasmas is primarily detected in organs exposed to the outside environment where the inherent immune response can be circumvented to permit mycoplasma entry [4–6]. The contribution of these bacteria to both homeostasis and disease states is likely significant, yet for many organs and cell types, this effect remains largely unknown. The location of the vocal folds increases the likelihood of mycoplasma colonization; the role of these bacteria in vocal-fold tissue health and disease is unknown. This issue is clinically relevant to tissue health and disease, but also particularly confounding for *in vitro* investigation and meaningful translation of laboratory findings to the clinical milieu.

Pragmatically, mycoplasma colonization, particularly in the upper aerodigestive tract, is likely to perpetuate to cell

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Abbreviations: DMEM, Dulbecco's Modified Eagle Medium; DNA, deoxyribonucleic acid; FBS, fetal bovin serum; NF, nuclear factor; PCR, polymerase chain reaction; RNA, ribonucleic acid; TGF, transforming growth factor; TNF, tumor necrosis factor; VFF, vocal fold fibroblast.

A supplementary figure is available with the online version of this article.

cultures derived from these tissues. *Mycoplasma orale* and *Mycoplasma salivarium* [7] have been identified in the upper airway and likely persist in primary cell culture. To date, the presence and/or impact of mycoplasma on vocal fold-derived cell culture is unknown. A recent review of the last 50 peer-reviewed publications describing *in vitro* investigation employing fibroblasts derived from the vocal folds, including work from our laboratory, revealed no specific mention of mycoplasma detection or treatment. Only one mention of mycoplasma was noted in the recent literature. The autologous fibroblast transplantation trial by Chhetri *et al*. reported cultures to be mycoplasma free; methodological information regarding detection and/or treatment was not provided [8]. Importantly, this human trial involved injection of fibroblasts harvested from post-auricular skin into the vocal folds, and did not include cells obtained or cultured from the airway.

Mycoplasma-free cells may limit translation of *in vitro* findings to tissues with a high likelihood of mycoplasma presence [9, 10]. Mycoplasmas are challenging to detect via traditional optical microscopy due to their small size [11]. Approximately 7% of the genes in the 1000 Genome Project [12] and 11% of 9395 surveyed rodent and primate samples from the NCBI Sequence Read Archive were mycoplasma positive [13]. In contrast, mycoplasma-free cells are commonly employed in the laboratory to control for extraneous factors related to *in vitro* cell dynamics [11, 14]. Protocols to prevent mycoplasma contamination and management [15], including antibiotics, are variably effective [6]. Antibiotics may not eradicate mycoplasma; following repeated antibiotic treatment, weak positive contamination of mycoplasma often persists in culture [16].

The vocal folds, situated at the junction of the upper and lower airways, are, arguably, the most bioactive tissues in the body. Compromised structural integrity of these specialized organs can result in substantive disability, and downstream, profound economic burden estimated at nearly $13 billion per year [17]. Fibroblasts are key to extracellular matrix metabolism and *in vitro* investigation of tissue-specific fibroblasts from the vocal folds are fundamental to our understanding of this specialized connective tissue, and more importantly, the development of novel therapeutics. Relevant to the current investigation, glucocorticoids are ubiquitously employed in the upper airway and are known regulators of multiple processes in VFF fibroblasts [18, 19]. As such, we hypothesized that, at baseline, fibroblasts from the vocal folds will be colonized with mycoplasma, and furthermore, this colonization will significantly alter both baseline genetic profiles as well as the inherent response to exogenous glucocorticoids. These data provide a foundation for future investigation regarding the role of mycoplasma in both healthy and pathologic states with a particular eye towards optimization of cell culture conditions to maximize translation to native tissue.

METHODS

Cell culture

An immortalized human vocal fold fibroblast cell line created in our laboratory, referred to as HVOX, was employed as well as primary vocal-fold fibroblast cultures from both Sprague-Dawley rats and New Zealand White rabbits, with the approval of the Institutional Animal Use and Care Committee at New York University School of Medicine. All cells were maintained in standard cell-culture conditions (37 °C, 5% CO_2) in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) (ThermoFisher Scientific, Waltham, MA, USA) and 1% antibiotic/antimycotic (Life Technologies, Long Island, NY, USA). Cells treated with 5 ng/ml dexamethasone (DM; Sigma Aldrich, Massachusetts, MA, USA) for RNASeq analyses were serum-starved overnight prior to treatment.

Laryngeal tissue analysis

A Fisher/Brown Norway rat, was sacrificed via CO_2 asphyxiation and the vocal-fold mucosa was dissected from the underlying muscle. DNA was isolated using the PureLink Genomic DNA Mini Kit (ThermoFisher Scientific).

Mycoplasma detection

The Venor GeM Mycoplasma Kit (Millipore Sigma, St. Louis, MO, USA) was employed with JumpStart Taq DNA Polymerase (Millipore Sigma) to detect mycoplasma. Following PCR, qualitative data were obtained via agarose gel. As outlined by the manufacturer, a band at 267 bp indicates mycoplasma presence, a band at 191 bp indicates no mycoplasma, and bands at both 267 and 191 bp indicate weak positive mycoplasma presence.

Mycoplasma removal

HVOX were treated with Plasmocin (InvivoGen) to remove mycoplasma and other cell wall-less bacteria in cell cultures, according to the manufacturer protocol. The detection assay was repeated after treatment to confirm efficient removal of mycoplasma.

RNA sequencing

HVOX were harvested after dexamethasone treatment for 4 h. Using the RNAeasy Mini Kit, total RNA was isolated and RNA-sequencing libraries were created with Illumina TruSeq stranded mRNA kit (Illumina, San Diego, CA, USA), using ten cycles of PCR amplification, beginning at 500 ng of total RNA (DNAse I-digested). Libraries were amplified and purified using AMPure beads (ThermoFisher Scientific), measured by Qubit (ThermoFisher Scientific) and visualized with an Agilent Bioanalyzer (Agilent, Santa Clara, CA, USA). Amplified libraries were indexed and then pooled equimolarly. Pooled libraries were run on an Illumina HiSeq 4000, 50 nucleotides in length, using three lanes. Sequencing was performed at the NYU Grossman School of Medicine sequencing core. Representative images are presented; complete gels are included as Supplemental Figures.

Bioinformatic analysis

Data obtained through RNA sequencing were analysed using ROSALIND (version 2.9–3.0; OnRamp Bioinformatics

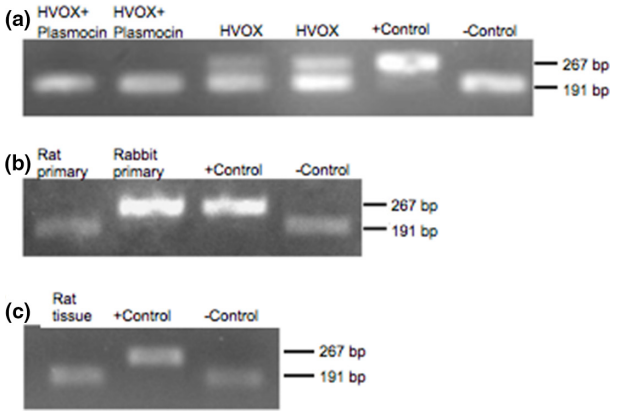

Fig. 1. Mycoplasma detection in mycoplasma-negative and mycoplasma-positive HVOX cells, rat primary vocal-fold fibroblasts, rabbit primary vocal-fold fibroblast cultures, and extracted rat laryngeal mucosa tissue. Mycoplasma detection was performed using a PCR-based detection kit and run on a 1.5% agarose gel. A band at ~267 bp represents mycoplasma positivity and a band at 191 bp indicates a lack of mycoplasma. Bands at both 267 and 191 bp indicate weak mycoplasma presence. (a) Representative gel of HVOX before and after mycoplasma treatment as well as positive and negative controls; (b) Representative gel of rat and rabbit primary vocal-fold fibroblasts as well as positive and negative controls; (c) Representative gel of DNA extracted from non-cultured rat laryngeal tissue as well as positive and negative controls.

Genomics Research Platform, OnRamp Bioinformatics, San Diego, CA, USA). Quality scores were calculated using the FastQC tool, with human genome build hg19 as a reference genome. HTseq was used to quantify individual sample reads, and DEseq2 was used to normalize reads by relative long expression and to calculate fold changes and *P*-values. Comparisons of most enriched genes (determined by analysing the log2-fold change) between each condition (mycoplasma positive/negative, dexamethasone treated/untreated) were created Wikipathways via HOMER within ROSALIND was used to determine significant genes and pathways for each comparison, a *P*-value of <0.05 was used to detect significance.

RESULTS

Mycoplasma was detected in human and rabbit vocal-fold fibroblasts

As shown in Fig. 1a, mycoplasma was detected in the human vocal-fold fibroblast cell line (HVOX); two bands were observed indicative of *weak* mycoplasma presence. Mycoplasma was also detected in rabbit primary culture vocal-fold fibroblasts (Fig. 1b). Mycoplasma was not detected in rat vocal-fold fibroblasts. We then ran the detection assay on fresh rat laryngeal tissue; no mycoplasma was detected (Fig. 1c).

Mycoplasma-altered basal gene expression in cultured vocal fold fibroblasts.

As shown in Fig. 1a, we confirmed complete removal of mycoplasma from HVOX culture following Plasmocin treatment. Baseline RNASeq analysis was then performed on mycoplasma-positive and -negative HVOX and 25074 genes were analysed by ROSALIND for expression comparisons. Nearly 19% (4732 genes) were differentially expressed between mycoplasma-positive and mycoplasma-negative HVOX cells; 20342 genes (81.13%) were expressed consistently across the two groups. The spread of significantly altered genes between mycoplasma-positive HVOX cells and mycoplasma-negative HVOX cells was significant (Fig. 2). Significant upregulation of multiple genes was observed in mycoplasma-positive cells, including genes related to apoptosis, DNA damage repair and cell cycle. Analysis of enriched pathways (Table 1) confirmed more significant genes were upregulated than downregulated. For the top two most significantly altered pathways, type II interferon signalling (IFNG) and photodynamic therapy-induced NF-κB survival signalling, no genes were downregulated.

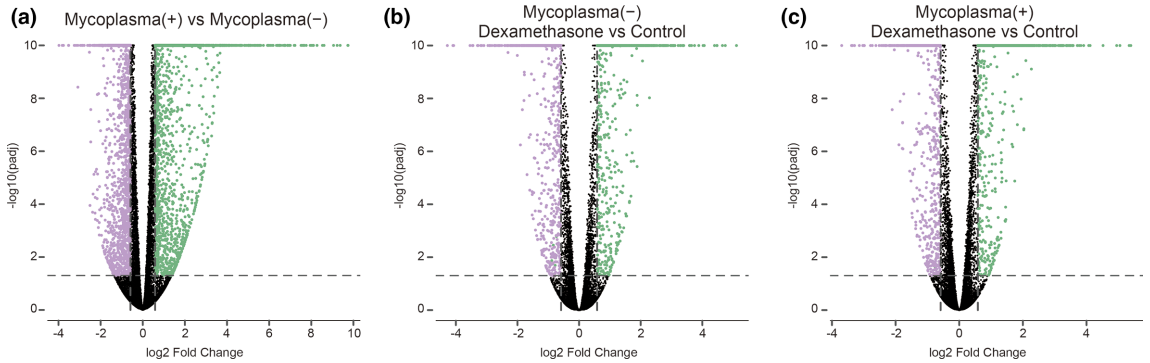

Fig. 2. Volcano plot of differentially regulated genes in the presence of dexamethasone in human vocal fold fibroblasts+/-mycoplasma. RNASeq analyses were performed to determine the relationship between mycoplasma and dexamethasone on transcriptional regulation. Comparisons between mycoplasma-positive and -negative cells (a) as well as DM and control in mycoplasma-negative cells (b) and DM and control in mycoplasma-positive cells (c). Genes with the largest absolute *P*-adjusted value were the most significantly upregulated or downregulated when the two groups were compared, using an alpha value of 0.05.

Table 1. Differentially enriched pathways between mycoplasma-negative and -positive vocal-fold fibroblasts. ROSALIND was used to analyse enriched genes in both conditions and significant pathways were determined using Wikipathways

Term	p-Adj	No. of genes in term	No. of genes in target	Upregulated	Downregulated
Type II interferon signalling (IFNG)	5.60E-06	37	26	26	0
Photodynamic therapy-induced NF-kB survival signalling	4.20E-05	35	24	24	0
Apoptosis	0.00012	86	44	39	5
DNA damage repair	0.00022	69	37	34	3
Cell cycle	0.00032	105	50	46	4
Retinoblastoma (RB) in cancer	0.00222	88	42	40	2
G1 to S cell-cycle control	0.00479	68	34	31	3
Photodynamic therapy-induced unfolded protein response	0.01298	29	18	17	1
RIG-I-like receptor signalling	0.01512	61	30	25	5
DNA replication	0.01866	42	23	22	1
TLR4 signalling and tolerance	0.02443	30	18	17	1
Gastric Cancer Network 1	0.03145	30	17	16	1
Photodynamic therapy-induced AP-1 survival signalling	0.03145	51	26	23	3
Apoptosis-related network due to altered Notch3 in ovarian cancer	0.03549	54	27	22	5
Apoptosis modulation and signalling	0.03807	94	41	34	7
Spinal cord injury	0.04972	120	49	37	12
Oncostatin M signalling pathway	0.04974	66	31	27	4

Mycoplasma-altered vocal fold fibroblast reactivity to dexamethasone

In mycoplasma-positive HVOX cells treated with dexamethasone (Fig. 2b), 1468 genes (5.85%) were differentially expressed and 23606 genes (94.15%) were expressed consistently. Genes with the largest log2-fold change were upregulated. In mycoplasma-negative HVOX cells treated with dexamethasone (Fig. 2c), 1527 genes (6.09% of total genes analysed) were differentially expressed and 23547 genes (93.91%) were expressed consistently. With dexamethasone treatment, 3580 genes (14.277%) were differentially expressed between mycoplasma-positive and -negative cells. The spread of modulated genes in this comparison was essentially equal between upregulation and downregulation. In both mycoplasma-positive and -negative cells treated with dexamethasone, adipogenesis and apoptosis pathways were significantly altered (Tables 2 and 3); however, in mycoplasma-positive cells, significantly more genes associated with apoptosis were downregulated. Fig. 3 provides a heatmap of RNA-seq data segregated by similarity; mycoplasma-positive cells were more similar to other mycoplasma-positive cells, regardless of treatment with dexamethasone.

DISCUSSION

The vocal folds pose unique therapeutic challenges given their anatomic position, importance in multiple biological functions, and unique structure. These challenges are exacerbated by the potential for mycoplasma to contribute to both tissue health and pathological conditions. To date, the presence and/or role of mycoplasma has been largely ignored. *In vitro*, it is unclear if mycoplasma-negative cells hold biological relevance, given the likelihood of colonization of the upper airway. This issue is particularly problematic if mycoplasma presence alters both basal gene-expression profiles as well as the inherent transcriptional response to exogenous stimuli. The current work sought to provide foundational insight into these issues. Admittedly, this work is not comprehensive and likely poses more questions than answers. Mycoplasma presence in human- and rabbit-derived vocal-fold fibroblasts likely suggests that, for multiple species, some level of mycoplasma colonization in the upper airway is possible. The lack of mycoplasma in rat primary culture cells may be explained by the recent improvement in managing infections in laboratory rodents; in a survey of 100 institutions in western Europe, only 3.6% of rats were infected with *M. pulmonis*. It is also possible that mycoplasma in rats is less common than in other species [20]. Furthermore, the percentage of laboratory rats

Table 2. Differentially enriched pathways between dexamethasone treated, mycoplasma-positive cells and control cells. ROSALIND was used to analyse enriched genes in both conditions and significant pathways were determined using Wikipathways

Term	p-Adj	No. of genes in term	No. of genes in target	Upregulated	Downregulated
Transcription factor regulation in adipogenesis	1.06E-03	22	11	6	5
Photogynamic therapy-induced NF-kB survival signalling	0.00136	35	14	0	14
Apoptosis-related network due to altered Notch3 in ovarian cancer	0.00576	54	17	4	13
Adipogenesis	0.00663	132	30	16	14
Lung fibrosis	0.01419	63	18	7	11
TNF related weak inducer of apoptosis (TWEAK) signalling pathway	0.0152	42	14	2	12
Prostaglandin synthesis and regulation	0.03657	30	11	5	6
Apoptosis	0.04237	86	21	5	16

with mycoplasma has declined over time, further suggesting rat mycoplasma contamination in the laboratory is rare [21]. To validate our findings in rat-derived cell culture, we ran subsequent detection on fresh rat tissue, which was also free of mycoplasma.

The functional/biological implications of mycoplasma in our human vocal-fold fibroblast cell line were then queried via RNASeq analyses. Substantive and potentially meaningful differences in gene-expression patterns were observed between mycoplasma-positive and -negative fibroblasts. This finding is not particularly surprising, but is a potential source of concern and caution with regard to the translational value of *in vitro* investigation. Mycoplasma was associated with enrichment of genes critical to DNA replication, DNA damage repair, and cell-cycle progression and signalling. In addition, significant alterations were observed in pathways related to inflammation (TLR4 signalling and tolerance, type II interferon signalling, RIG-I-like receptor signalling, etc.). Specifically, 17 differentially enriched pathways were observed between mycoplasma-positive and -negative cells. The enrichment of pathways, including DNA damage repair, DNA replication, and the cell cycle could demonstrate cell growth in response to mycoplasma via pathways related to replication. G1 to S phase cell-cycle signalling, another pathway enriched in the presence of mycoplasma, is a response to a checkpoint to a negative feedback loop to activate DNA replication and repress transcription. DNA-damage response is also activated in response to this checkpoint, as the cell cycle is disrupted in the context of sequencing errors. However, enrichment of the pathway controlling signalling for this stage could be a sign of disruption of the cell cycle, which is often associated with oncogenesis [22].

We then sought to determine the implications of mycoplasma on the cell response to exogenous stimuli. In this case, we selected dexamethasone. Inhaled, injected and systemic glucocorticoids are universally employed to treat a myriad of upper airway conditions [23]. Dexamethasone decreased the number of differentially expressed genes in our fibroblast line, in both mycoplasma-positive and -negative cells. Fewer genes were differentially expressed in the

Table 3. Differentially enriched pathways between dexamethasone treated, mycoplasma-free cells and control cells. ROSALIND was used to analyse enriched genes in both conditions and significant pathways were determined using Wikipathways

Term	p-Adj	No. of genes in term	No. of genes in target	Upregulated	Downregulated
Adipogenesis	5.10E-05	132	34	21	13
TGF-beta receptor signalling	0.00304	58	18	10	8
Oncostatin M signalling pathway	0.00577	66	19	13	6
Ectoderm differentiation	0.00852	144	31	15	16
Brain-derived neurotropic factor (BDNF) signalling pathway	0.01133	144	31	18	13
IL-2 signalling pathway	0.01198	42	14	8	6
EGF/EGFR signalling pathway	0.02332	164	33	27	6
Apoptosis	0.03102	86	21	12	9

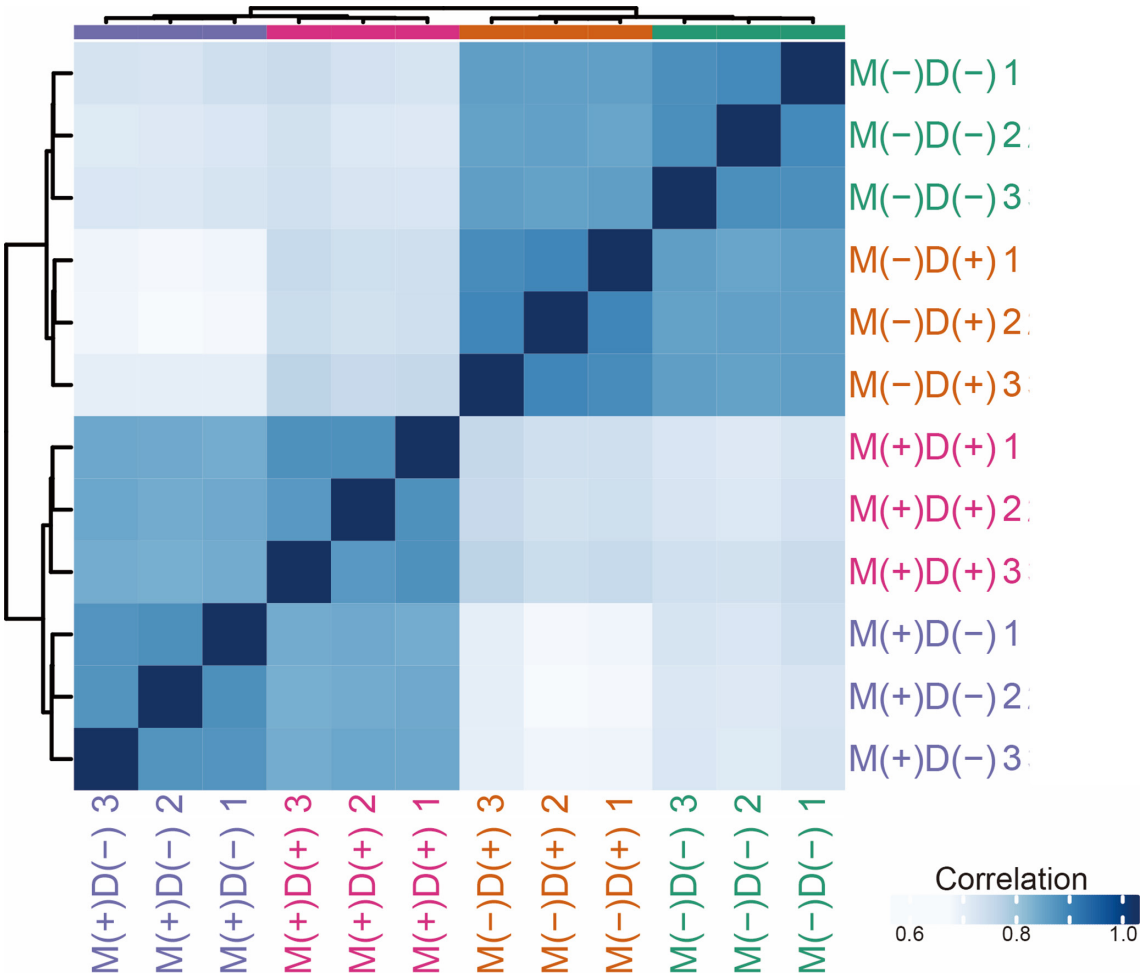

Fig. 3. Correlation heatmap of mycoplasma-positive and -negative cells treated with dexamethasone. A heatmap was generated using RNAseq data of mycoplasma-negative [M(−)] and -positive cells [M(+)], control [D(−)] or treated with dexamethasone [D(+)], uploaded to the ROSALIND bioinformatics platform.

mycoplasma-positive dexamethasone treatment group. Basal gene expression in mycoplasma-positive cells may be modulated by treatment with dexamethasone, demonstrated by the decreased percentage of differentially expressed genes in dexamethasone-treated comparisons. Dexamethasone treatment in mycoplasma-positive cells resulted in the greatest percentage of consistently expressed genes across groups, potentially suggesting many differentially expressed genes and pathways, but these two groups may be the most similar to each other. Mycoplasma-negative and -positive untreated cells had the fewest consistently expressed genes, implying that these groups may be the least similar at baseline.

In response to dexamethasone, RNA-sequencing indicated a reduced number of significantly enriched pathways. This response was anticipated given the known role of glucocorticoids on fibroblasts. Across mycoplasma-positive and -negative cells treated with dexamethasone, many overlapping pathways were enriched, including apoptosis. Apoptosis facilitates cell turnover, hypothesized to be critical for optimal wound healing [24]. In addition, photodynamic therapy-induced NF-κB survival signalling, apoptosis-related network due to altered Notch3 in ovarian cancer, and TNF- related weak induced of apoptosis (TWEAK) signalling, were also differentially expressed. Of particular interest to the vocal folds, the lung fibrosis pathway was significantly enriched only in mycoplasma-positive cells. Although these fibroblasts were derived from the vocal folds and not the lungs, this pathway may indicate enrichment of pro-fibrotic pathways. Vocal-fold fibrosis/scarring is one of the most common causes of voice-related disability [25] and these data may suggest that interactions between mycoplasma and glucocorticoids may impact the therapeutic efficacy of GCs for vocal-fold fibrosis. Conversely, in mycoplasma-negative cells treated with DM, the transforming growth factor (TGF)-β receptor signalling pathway was significantly enriched. This pathway, however, was not similarly modulated in mycoplasma-positive HVOX cells. Consistent with other tissues, TGF-β has been shown to be a master regulator of vocal-fold fibrosis [26]. Collectively,

these conflicting differential expression patterns facilitate a substantive disconnect between *in vitro* investigation and clinical translation.

CONCLUSION

Mycoplasma was detected in human- and rabbit-derived vocal-fold fibroblasts, but not in rat-derived cells. Multi-gene analysis confirmed mycoplasma-altered basal mRNA expression across multiple genes and pathways in human fibroblasts. In addition, human fibroblasts responded differently to exogenous glucocorticoids in the presence of mycoplasma. The implications for these findings are significant given that it is likely the upper aerodigestive tract of various species is colonized by mycoplasma. Additional investigation is needed to ensure appropriate *in vitro* models are employed to investigate relevant biological phenomena. The role of these bacteria in both healthy and pathologic vocal-fold disease states is of critical importance.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
All experiments were approved by the NYU Grossman School of Medicine IACUC.

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