Published in final edited form as:

Laryngoscope. 2019 May ; 129(5): E187–E193. doi:10.1002/lary.27570.

Phosphorylation of the glucocorticoid receptor alters Smad signaling in vocal fold fibroblasts

Shigeyuki Mukudai, M.D.,Ph.D.¹, Nao Hiwatashi, M.D.,Ph.D.¹, Renjie Bing, M.D.¹, Michael Garabedian, Ph.D.², and Ryan C. Branski, Ph.D.¹

¹NYU Voice Center, Department of Otolaryngology-Head and Neck Surgery, New York University School of Medicine, New York, New York

²Department of Microbiology, New York University School of Medicine, New York, New York

Abstract

Objectives/Hypothesis.—Direct glucocorticoid (GC) injection for vocal fold (VF) scarring has evolved as a therapeutic strategy, but the mechanisms underlying the anti-fibrotic effects remain unclear. GCs act via the glucocorticoid receptor (GR), which is phosphorylated at multiple serine residues in a hormone-dependent manner to affect bioactivity. We hypothesize that GCs regulate Smad signaling via GR phosphorylation in vocal fold fibroblasts (VFFs).

Study Design.—In vitro

Methods.—Human VFFs were treated with dexamethasone (DM; 10^{-5} – 10^{-7} M) +/- Transforming Growth Factor (TGF)- β 1 (10ng/ml). RU486 (10^{-6} M) was employed to isolate the regulatory effects of GR. Total GR, Ser²¹¹, and Ser²⁰³ phosphorylation was examined via SDS-PAGE and immunocytochemistry. Quantitative polymerase chain reaction was employed to determine GR-mediated effects of DM on genes related to fibrosis.

Results.—Total GR and Ser²¹¹ phosphorylation was observed predominantly in the nucleus 1 hour after DM administration. DM decreased total GR expression, but Ser²⁰³ and Ser²¹¹ phosphorylation increased. RU486 limited the effects of DM. *SMAD3* and *SMAD7* mRNA expression significantly decreased 4 hours after DM administration ($p<0.05$); this response was negated by RU486. *COL1A1* remained unchanged and *ACTA2* significantly increased following 24 hours of DM treatment ($p<0.05$).

Conclusions.—DM regulated TGF- β 1 signaling via altered *SMAD3* and *SMAD7* expression. This response was associated with altered GR phosphorylation. These findings provide insight into the mechanisms of steroidal effects on vocal fold repair; ultimately, we seek to enhance therapeutic strategies for these challenging patients.

Level of Evidence: N/A

Address all correspondence to: Ryan C. Branski, Ph.D., NYU Voice Center, Department of Otolaryngology-Head and Neck Surgery, New York University School of Medicine, 345 East 37th Street, Suite 306, New York, NY 10016, Phone: 646.754.1207, Fax: 646.754.1222, ryan.branski@nyumc.org.

The authors have no financial disclosures or conflicts of interest.

Keywords

vocal fold; voice; glucocorticoid; glucocorticoid receptor; phosphorylation; transforming growth factor- β

INTRODUCTION

Vocal fold (VF) scarring continues to pose a significant clinical challenge and is often associated with repeated phonotraumatic or inflammatory events. Scar and the associated tissue stiffness result in decreased vibratory pliability of the vocal fold mucosa and often underlies aberrant voice quality and resultant limitations in communicative effectiveness.¹⁻³ Optimal treatment and strategies for prevention of VF scarring have not yet been established; however, direct glucocorticoid (GC) injection to focal regions of VF fibrosis has evolved as an increasingly common therapeutic option. Observational data suggest that these injections hold some degree of therapeutic potential,⁴ and intraoperative GCs are commonly employed under the assumption that they prevent scar formation following laryngeal surgery.⁵ However, the mechanism(s) underlying the anti-fibrotic effects of GCs remain unclear, and GCs are a large class of compounds with diverse pharmacokinetic properties. Clinically, no clear guidelines exist regarding GC use for VF scarring.^{6,7} The acquisition of pre-clinical, mechanistic insight into factors relevant to optimal therapeutic outcomes is critical to ultimately developing novel, targeted treatments for this challenging patient population.

In that regard, fibroblasts play a critical role in regulating extracellular matrix (ECM) turnover under normal conditions, and following injury, fibroblasts differentiate into myofibroblasts. This altered phenotype is associated with both increased metabolic and contractile properties. Excessive myofibroblast activity, has been shown to mediate the development of fibrosis across tissue types including the vocal folds.⁸ The stimuli for this differentiation is thought to be Transforming Growth Factor (TGF)- β . The actions of TGF- β 1 are mediated via a unique signaling pathway. Briefly, receptor-activated SMAD2 and SMAD3 are phosphorylated via TGF- β 1 binding to the receptor and heterodimerizing with SMAD4. This complex then translocates to the nucleus to regulate transcription. Conversely, SMAD7 is a competitive inhibitor of SMAD activation, and therefore is thought to be antifibrotic. Activated Smads have been implicated in a variety of fibrotic processes, suggesting that Smad activation plays a central role in fibrosis.⁹ Ideally, therapies for vocal fold fibrosis should likely alter the fibroblast phenotype and emerging data suggest that GCs attenuate TGF- β /Smad signaling in human fetal lung fibroblasts.^{10,11} Fetal lung fibroblasts are likely a unique niche; we sought to investigate the effects of GCs on vocal fold fibroblasts (VFFs).

Emerging evidence suggests that fibroblasts are target cells for GCs and express the glucocorticoid receptor (GR).¹²⁻¹⁴ Our laboratory recently immunolocalized the GR in the vocal fold mucosa and GCs altered VFF cell growth, the expression of ECM genes, and collagen secretion.^{12,14} GCs pass through the cell membrane and form complexes by binding with nuclear receptors. The resulting complex binds to DNA of specific genes and

regulates transcription. Although ligand binding is essential for activation of GR, the receptor is also subject to post-translational modification through phosphorylation.¹⁵ GR is phosphorylated in the absence of hormones, and additional phosphorylation events may occur following agonist binding. Three major serine sites are phosphorylated within the N-terminal region of the receptor and are involved in transcriptional regulation (Ser²⁰³, Ser²¹¹, and Ser²²⁶). The specific GR phosphorylation site may determine target gene activity, cofactor interaction, strength and duration of receptor signaling, and/or receptor stability. For example, phosphorylation at Ser²¹¹ has been associated with nuclear translocation and transcriptional activation of GR after hormone treatment. Phosphorylation at Ser²⁰³ has been shown to be a determinant of ligand-dependent down-regulation of GR.^{16,17} [ENREF 17](#) Novel phosphorylation-specific antibodies to these phosphoserine sites were recently developed to allow for analysis of GR phospho-isoform expression and localization with increased fidelity.^{16,17} We hypothesized that GCs regulate Smad signaling via GR phosphorylation in VFFs. Therefore, we sought to quantify the effects of dexamethasone (DM) on Ser²¹¹ and Ser²⁰³ phosphorylation and regulation of TGF- β 1 signaling.

MATERIALS AND METHODS

Cell culture.

An immortalized human vocal fold fibroblast cell line created in our laboratory was employed for all experimentation. This cell line, referred to as HVOX, has been shown to be stable through multiple population doublings; cells in passages 20–30 were used. Cells were cultured on plates in phenol red-free Dulbecco's Modified Eagle's medium (DMEM) containing 10% charcoal-dextran-treated fetal bovine serum (FBS) and 1% antibiotic/antimycotic (Life Technologies, Grand Island, NY) at 37°C under standard cell culture conditions.

Immunocytochemistry for GR.

HVOX were seeded on chamber slides for two days and examined after one hour of incubation at 37°C in the presence of 10⁻⁷M DM (Sigma-Aldrich, St. Louis, MO) or control (equal volume of dimethyl sulfoxide, DMSO). All cells were fixed with 4% paraformaldehyde at 37°C for 10 minutes, washed with PBS, and blocked for one hour at room temperature with PBS solution containing 0.3% Triton-X and 5% bovine serum albumin. Cells were then incubated with the following antibodies for 24 hours at 4°C: rabbit anti-total GR (1:100; Cell Signaling, Danvers, MA), rabbit anti-hGR-S203P (1:500) and rabbit anti-hGR-S211P (1:1000). Alexa-Fluor 488 goat anti-rabbit IgG (1:500; Invitrogen, Carlsbad, CA) were used as secondary antibody. Stained sections were visualized, and images were captured using a Nikon Eclipse Ni-U fluorescence microscope (Nikon Inc., Tokyo, Japan).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Following 12 hours of serum starvation, HVOX were treated with serum/phenol red-free DMEM +/- DM (10⁻⁷ M) +/- TGF- β 1 (10 ng/ml) +/- RU486 (10⁻⁶ M, Sigma-Aldrich) and harvested at one hour, or with serum/phenol red-free DMEM + DM (10⁻⁷ M) and harvested at 1, 4, 24 or 48 hours. Recombinant human TGF- β 1 was employed for experimentation.

RU486, a GR antagonist, was employed to isolate the regulatory effects of GR. After treatment, the chamber slide inserts were removed and the plates were washed twice with cold PBS. HOVX were harvested via cell scraping and total cellular protein was extracted using the Mammalian Protein Extraction Reagent (Thermo Scientific, Waltham, MA), supplemented with Halt Protease Inhibitor Cocktail (Thermo Scientific), 0.5M EDTA Solution 100x (Thermo Scientific), Calyculin A (Cell Signaling), and 2-mercaptoethanol (Life Technologies). Total protein was quantified via the Pierce 660nm Protein Assay (Thermo Scientific). Each protein lysate was loaded on 8% sodium dodecyl sulfate (SDS)-polyacrylamide gels. Protein was then transferred to PVDF membranes (Invitrogen) and blocked with I-Block (Applied Biosystems, Foster City, CA) overnight at 4°C. Membranes were incubated with a primary antibody against rabbit anti-total GR (1:1000; Cell Signaling) for 1 hour, rabbit anti-hGR-S203P (1:3000) for 3 hours, rabbit anti-hGR-S211P (1:1000) overnight, or β -actin (1:5000; Cell Signaling) for 1 hour at 4°C followed by 1 hour incubation with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling). Blots were visualized using chemiluminescence detection.

Quantitative real-time polymerase chain reaction.

Following 12 hours of serum starvation, cells were treated with serum/phenol red-free DMEM +/- DM (10^{-5} , 10^{-6} , 10^{-7} M, Sigma-Aldrich) +/- TGF- β 1 (10 ng/ml, Life Technologies) +/- RU486 (10^{-6} M, Sigma-Aldrich) and harvested at 4 and 24 hours. Total RNA was extracted via the RNeasy Mini Kit (Qiagen, Valencia, CA) and reverse transcribed with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The TaqMan Gene Expression kit (Life Technologies) and StepOne Plus (Applied Biosystems) were employed for quantitative analyses. Taqman primer probes for *SMAD3* (Hs00969210_m1), *SMAD7* (Hs00998193_m1), *COL1A1* (Hs00164004_m1), *ACTA2* (Hs00426835_g1) and *GAPDH* (Hs02758991_g1) were employed. The Ct method was employed with *GAPDH* as the housekeeping gene for the determination of relative expression levels.

Immunocytochemistry for ACTA2.

HOVX were seeded on chamber slides for two days. Following 12 hours of serum starvation, cells were treated with serum/phenol red-free DMEM +/- DM (10^{-7} M, Sigma-Aldrich) +/- TGF- β 1 (10 ng/ml, Life Technologies) and harvested at 48 hours. All cells were fixed with 4% paraformaldehyde at 37°C for 10 minutes, washed with PBS, and blocked for one hour at room temperature with PBS solution containing 0.3% Triton-X and 5% bovine serum albumin. The slides were incubated at 4°C overnight with primary mouse monoclonal antibodies against ACTA2 (1:100; Sigma-Aldrich) and then the corresponding Alexa-Fluor 555 goat anti-mouse immunoglobulin G (1:500; Invitrogen) secondary antibody. Nuclei were counterstained with DAPI. Digital images were captured, and both the total cell count and ACTA2 positive cells were counted in 10 randomly chosen, 3.0mm^2 fields for each condition under 10 $\times$ magnification.¹⁸

Statistical Analyses.

Data were expressed as means $\pm$ SEM and analyzed via one-way analysis of variance (ANOVA). If a main effect was observed, post hoc Tukey tests for data with normal distribution and Kruskal-Wallis and Mann-Whitney U tests for data with non-normal

distribution were performed using IBM SPSS Statistics (v23) for Windows. $p < 0.05$ was considered significant.

RESULTS

Subcellular distribution of endogenous Ser²⁰³ and Ser²¹¹ phosphorylated forms of GR.

In the absence of DM, total GR and P-211 immunoreactivity was detected in the nucleus and cytoplasm of HVOX. Following DM exposure, localization shifted predominantly to the nucleus (Figure 1A, 1B). P-203 was localized predominantly in the cytoplasm in the absence and presence of DM (Figure 1C).

Effects of DM, RU486 and TGF- β 1 on GR phosphorylation.

As shown in Figure 2, whereas DM (10^{-7} M) decreased total GR expression, Ser²⁰³ and Ser²¹¹ phosphorylation increased. RU486 (10^{-6} M) limited the effects of DM. TGF- β 1 (10 ng/ml) did not alter total GR expression or phosphorylation of Ser²⁰³ and Ser²¹¹.

DM downregulated total GR.

DM (10^{-7} M) reduced total GR expression in a time-dependent manner (Figure 3).

DM altered gene expression of *SMAD3*, *SMAD7*, *COL1A1* and *ACTA2*.

SMAD3 and *SMAD7* significantly decreased 4 hours after DM exposure (Figure 4A and 4B). *COL1A1* remained unchanged and *ACTA2* significantly increased following 24 hours of DM treatment (Figure 4C and 4D). No concentration effect for DM was noted. RU486 reduced DM-induced decrease in *SMAD3* and *SMAD7* mRNA expression (Figure 5A and 5B). RU486 reduced DM-induced increase in *ACTA2* mRNA expression (Figure 5C).

DM altered TGF- β 1-mediated transcriptional events.

TGF- β 1 significantly increased *SMAD7*, *COL1A1* and *ACTA2* mRNA expression (Figure 6B, 6C, and 6D). The combination of DM and TGF- β 1 significantly decreased *SMAD3* and *SMAD7* (Figure 6A and 6B) compared to TGF- β 1 alone at 4 hours. *COL1A1* was unchanged (Figure 6C) and *ACTA2* significantly increased compared to TGF- β 1 alone (Figure 6D) following 24 hours of DM treatment.

DM enhanced myofibroblast differentiation.

TGF- β 1 altered both cell size and the appearance of stress fibers qualitatively. Increased *ACTA2* expression was observed 48 hours after DM exposure (Figure 7A); the ratio of *ACTA2*-positive cells significantly increased 48 hours after DM exposure (Figure 7B).

DISCUSSION

Effective therapeutics and preventative strategies for vocal fold scar remain elusive. We hypothesize that enhanced understanding of key biochemical triggers underlying the sequence of events leading to the development of the aberrant, fibrotic phenotype is likely to elucidate novel targets for intervention. Glucocorticoids have historically been thought to be powerful immune modulators with ubiquitous use across inflammatory processes. The utility

of GCs in more chronic fibroplastic processes is less clear. However, emerging clinical data suggest some degree of efficacy of direct steroid injection into vocal fold scar.⁴ We sought to investigate potential interactions between glucocorticoids and TGF- β , a mediator of fibrosis across tissues including the vocal folds.

In the current study, DM regulated GR phosphorylation and TGF- β 1 signaling in human VFFs. To the best of our knowledge, these data are the first to describe immunoreactivity and changes in specific GR residues (203 and 211) associated with GC exposure in VFFs. Moreover, DM decreased *SMAD3* mRNA expression consistent with its antifibrotic potential. However, DM also increased TGF- β 1-induced fibroblast-myofibroblast differentiation which is inconsistent with the concept of glucocorticoids holding significant anti-fibrotic potential.

The distribution of total GR in HVOX indicated that ligand binding led to near complete nuclear localization of the receptor; consistent with our previous reports.^{12,17} DM acted on human VFFs directly via GR. Similarly, the distribution of P-203 and P-211 was also consistent with our previous report,¹⁷ suggesting that differentially phosphorylated receptor residues were located in unique subcellular compartments, likely modulating distinct aspects of receptor function. DM downregulated total GR over time and increased Ser²⁰³ and Ser²¹¹ phosphorylation in human VFFs. These effects were extinguished with RU486, a GR antagonist. These findings are largely consistent with established dogma. However, we conceptualized a model of GR modulation via phosphorylation in the absence of DM. Although DM stimulated GR phosphorylation primarily at Ser²⁰³, a population of unphosphorylated receptor molecules may persist. With time, Ser²⁰³ underwent *dephosphorylation* such that the ratio of phosphorylated Ser²¹¹ to phosphorylated Ser²⁰³ increased. Differential modification of GR by phosphorylation likely induces a distinct conformation and influences GR association with additional coregulatory proteins to ultimately modulate GR transactivation, stability, and subcellular location.¹⁷ The current data are consistent with this hypothesis regarding GR-dependent phosphorylation. Conversely, phosphorylation was not affected by TGF- β 1, consistent with previous report.¹⁹

Dexamethasone significantly decreased *SMAD3* and *SMAD7*, and increased *ACTA2* mRNA in our cell line. Previous reports suggested that GR inhibited TGF- β signaling by directly targeting the transcriptional activation function of SMAD3,¹⁹ consistent with our data. Our laboratory and others identified SMAD3 as a principle mediator of fibrotic VF wound healing due to its role in TGF- β 1 signaling, which in turn regulates numerous fibroblast activities including migration, proliferation, and production of extracellular matrix.^{9,20,21,22,23} Clearly, these data suggest that DM has anti-fibrotic effects. However, SMAD7, a homolog and competitive inhibitor of SMAD3, was also downregulated. SMAD7 downregulation in response to DM was both contradictory to our hypothesis and conceptually interesting. One possible explanation is that this response may be a component of a feedback loop for SMAD3 downregulation.

Collectively, these responses were negated by RU486, confirming GR dependence. Interestingly, GCs were previously shown to have no effect on SMAD expression in human fetal lung fibroblasts, mouse and rat osteoblastic cells, and human trabecular meshwork cells

and tissues^{10,11,24,25} suggesting some geographic specificity. In addition, no concentration effect evolved with regard to DM exposure on our cells. Unfortunately, these data provide little insight regarding relevant concentrations for clinical use and this issue warrants further investigation. DM did not decrease *Col1A1* mRNA in human VFFs which is contradictory to our previous work regarding the effects DM on collagen secretion.¹⁴ This discrepancy, however, is actually consistent with data from other cells.²⁶ The mechanism was thought to be lysosome-mediated autophagy induced degradation of COL1A1 protein abundance by cortisol. These phenomena warrant further investigation.

In addition, DM increased *ACTA2* mRNA and the ratio of ACTA2 positive cells in our cell line, suggesting DM synergized with TGF- β 1 to induce fibroblast-myofibroblast differentiation consistent with data from human fetal lung fibroblasts.²⁷ These data are wholly contrary to our overarching hypothesis, and may underlie suboptimal outcomes with DM treatment in the clinical milieu. For example, no differences were reported between steroid-injected and control groups after vocal fold surgery.²⁸ Furthermore, prior data did not support the use of DM to prevent pulmonary fibrosis and the authors speculated that DM actually enhanced the fibrotic response.^{27,29} GCs also adversely affected patient prognosis in idiopathic pulmonary fibrosis.^{30–34} In addition, with regard to mechanism, previous work suggested that prednisolone suppressed lung inflammatory edema, but failed to suppress pulmonary fibrosis.³⁴ These results suggest that GCs may be effectively limit the acute inflammatory response following vocal fold injury, but less ideal for more chronic vocal fold fibrosis. Clearly, the timing of GC administration may be of critical importance in order effectuate therapeutic efficacy; further investigation in this regard is warranted. Furthermore, the current study is not without limitation. As noted previously, GCs are a vast category of compounds with diverse pharmacokinetic profiles. The current study included only DM as it appears to hold the highest therapeutic (e.g., half-life, anti-inflammatory potential, etc.). It is unclear if these effects are consistent across GCs. With the evolution GC use in laryngology, further investigation is fundamental to optimal therapeutics.

CONCLUSION

DM regulated TGF- β 1 signaling via altered *SMAD3* and *SMAD7* expression. This response was associated with altered GR phosphorylation. These findings provide preliminary insight regarding the mechanisms of steroidal effects on vocal fold injury, with the goal of enhanced therapeutic strategies for these challenging patients.

Acknowledgments

Funding for this work was provided by the National Institutes of Health/National Institute on Deafness of and Other Communication Disorders (RO1 DC013277 and DC DC017397, PI-Branski)

REFERENCES

1. Hirano S, Minamiguchi S, Yamashita M, Ohno T, Kanemaru S, Kitamura M. Histologic characterization of human scarred vocal folds. *Journal of voice : official journal of the Voice Foundation* 2009; 23:399–407. [PubMed: 18395421]
2. Hirano S Current treatment of vocal fold scarring. *Current opinion in otolaryngology & head and neck surgery* 2005; 13:143–147. [PubMed: 15908810]

3. Benninger MS, Alessi D, Archer S et al. Vocal fold scarring: current concepts and management. *Otolaryngology--head and neck surgery : official journal of American Academy of Otolaryngology-Head and Neck Surgery* 1996; 115:474–482. [PubMed: 8903451]
4. Mortensen M, Woo P. Office steroid injections of the larynx. *The Laryngoscope* 2006; 116:1735–1739. [PubMed: 17003727]
5. Campagnolo AM, Tsuji DH, Sennes LU, Imamura R. Steroid injection in chronic inflammatory vocal fold disorders, literature review. *Brazilian journal of otorhinolaryngology* 2008; 74:926–932. [PubMed: 19582351]
6. Govil N, Rafii BY, Paul BC, Ruiz R, Amin MR, Branski RC. Glucocorticoids for vocal fold disease: a survey of otolaryngologists. *Journal of voice : official journal of the Voice Foundation* 2014; 28:82–87. [PubMed: 24050821]
7. Rafii B, Sridharan S, Taliencio Set al. Glucocorticoids in laryngology: a review. *The Laryngoscope* 2014; 124:1668–1673. [PubMed: 24474440]
8. Darby IA, Laverdet B, Bonte F, Desmouliere A. Fibroblasts and myofibroblasts in wound healing. *Clinical, cosmetic and investigational dermatology* 2014; 7:301–311.
9. Branski RC, Bing R, Kraja I, Amin MR. The role of Smad3 in the fibrotic phenotype in human vocal fold fibroblasts. *The Laryngoscope* 2016; 126:1151–1156. [PubMed: 26422444]
10. Wen FQ, Kohyama T, Skold CM et al. Glucocorticoids modulate TGF-beta production. *Inflammation* 2002; 26:279–290. [PubMed: 12546137]
11. Wen FQ, Kohyama T, Skold CM et al. Glucocorticoids modulate TGF-beta production by human fetal lung fibroblasts. *Inflammation* 2003; 27:9–19. [PubMed: 12772773]
12. Mukudai S, Matsuda KI, Nishio T et al. Differential responses to steroid hormones in fibroblasts from the vocal fold, trachea, and esophagus. *Endocrinology* 2015; 156:1000–1009. [PubMed: 25514085]
13. Gadson PF, Russell JD, Russell SB. Glucocorticoid receptors in human fibroblasts derived from normal dermis and keloid tissue. *The Journal of biological chemistry* 1984; 259:11236–11241. [PubMed: 6381497]
14. Zhou H, Sivasankar M, Kraus DH, Sandulache VC, Amin M, Branski RC. Glucocorticoids regulate extracellular matrix metabolism in human vocal fold fibroblasts. *Laryngoscope* 2011; 121:1915–1919. [PubMed: 22024844]
15. Bodwell JE, Webster JC, Jewell CM, Cidlowski JA, Hu JM, Munck A. Glucocorticoid receptor phosphorylation: overview, function and cell cycle-dependence. *The Journal of steroid biochemistry and molecular biology* 1998; 65:91–99. [PubMed: 9699861]
16. Lee MJ, Wang Z, Yee H et al. Expression and regulation of glucocorticoid receptor in human placental villous fibroblasts. *Endocrinology* 2005; 146:4619–4626. [PubMed: 16055431]
17. Wang Z, Frederick J, Garabedian MJ. Deciphering the phosphorylation “code” of the glucocorticoid receptor in vivo. *The Journal of biological chemistry* 2002; 277:26573–26580. [PubMed: 12000743]
18. Hiwatashi N, Bing R, Kraja I, Branski RC. NR4A1 is an endogenous inhibitor of vocal fold fibrosis. *The Laryngoscope* 2017.
19. Song CZ, Tian X, Gelehrter TD. Glucocorticoid receptor inhibits transforming growth factor-beta signaling by directly targeting the transcriptional activation function of Smad3. *Proceedings of the National Academy of Sciences of the United States of America* 1999; 96:11776–11781. [PubMed: 10518526]
20. Branski RC, Barbieri SS, Weksler BB et al. Effects of transforming growth factor-beta1 on human vocal fold fibroblasts. *The Annals of otology, rhinology, and laryngology* 2009; 118:218–226.
21. Lim X, Tateya I, Tateya T, Munoz-Del-Rio A, Bless DM. Immediate inflammatory response and scar formation in wounded vocal folds. *The Annals of otology, rhinology, and laryngology* 2006; 115:921–929.
22. Leask A, Abraham DJ. TGF-beta signaling and the fibrotic response. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 2004; 18:816–827. [PubMed: 15117886]
23. Border WA, Noble NA. Transforming growth factor beta in tissue fibrosis. *N Engl J Med* 1994; 331:1286–1292. [PubMed: 7935686]

24. Iu MF, Kaji H, Sowa H, Naito J, Sugimoto T, Chihara K. Dexamethasone suppresses Smad3 pathway in osteoblastic cells. *The Journal of endocrinology* 2005; 185:131–138. [PubMed: 15817834]
25. Kasetti RB, Maddineni P, Patel PD, Searby C, Sheffield VC, Zode GS. Transforming growth factor beta2 (TGFbeta2) signaling plays a key role in glucocorticoid-induced ocular hypertension. *The Journal of biological chemistry* 2018; 293:9854–9868. [PubMed: 29743238]
26. Mi Y, Wang W, Zhang C et al. Autophagic Degradation of Collagen 1A1 by Cortisol in Human Amnion Fibroblasts. *Endocrinology* 2017; 158:1005–1014. [PubMed: 28323983]
27. Gu L, Zhu YJ, Guo ZJ, Xu XX, Xu WB. Effect of IFN-gamma and dexamethasone on TGF-beta1-induced human fetal lung fibroblast-myofibroblast differentiation. *Acta pharmacologica Sinica* 2004; 25:1479–1488. [PubMed: 15525471]
28. Coleman JR, Smith S Jr., Reinisch Let al. Histomorphometric and laryngeal videostroboscopic analysis of the effects of corticosteroids on microflap healing in the dog larynx. *The Annals of otology, rhinology, and laryngology* 1999; 108:119–127.
29. Brenner RE, Felger D, Winter C, Christiansen A, Hofmann D, Bartmann P. Effects of dexamethasone on proliferation, chemotaxis, collagen I, and fibronectin-metabolism of human fetal lung fibroblasts. *Pediatric pulmonology* 2001; 32:1–7. [PubMed: 11416869]
30. Raghu G, Rochwerf B, Zhang Y et al. An Official ATS/ERS/JRS/ALAT Clinical Practice Guideline: Treatment of Idiopathic Pulmonary Fibrosis. An Update of the 2011 Clinical Practice Guideline. *American journal of respiratory and critical care medicine* 2015; 192:e3–19. [PubMed: 26177183]
31. Turner-Warwick M, Burrows B, Johnson A. Cryptogenic fibrosing alveolitis: response to corticosteroid treatment and its effect on survival. *Thorax* 1980; 35:593–599. [PubMed: 7444826]
32. American Thoracic Society. Idiopathic pulmonary fibrosis: diagnosis and treatment. International consensus statement. American Thoracic Society (ATS), and the European Respiratory Society (ERS). *American journal of respiratory and critical care medicine* 2000; 161:646–664. [PubMed: 10673212]
33. Collard HR, King TE, Jr. Demystifying idiopathic interstitial pneumonia. *Arch Intern Med* 2003; 163:17–29. [PubMed: 12523913]
34. Oku H, Shimizu T, Kawabata T et al. Antifibrotic action of pirfenidone and prednisolone: different effects on pulmonary cytokines and growth factors in bleomycin-induced murine pulmonary fibrosis. *European journal of pharmacology* 2008; 590:400–408. [PubMed: 18598692]

Figure 1.

Subcellular distribution of endogenous Ser²¹¹ and Ser²⁰³ phosphorylated forms of GR in human vocal fold fibroblasts. (A) Total GR immunostaining in the absence (left) or presence (right) of 10^{-7} M dexamethasone (DM). (B) GR phospho-Ser²⁰³ (P-211) immunostaining in the absence (left) or presence (right) of 10^{-7} M DM. (C) GR phospho-Ser²¹¹ (P-203) immunostaining in the absence (left) or presence (right) of 10^{-7} M DM. Scale bar: 50 μ m.

Figure 2.

Effects of dexamethasone (DM), RU486 and transforming growth factor (TGF)-β1 on GR phosphorylation. Human vocal fibroblasts were treated with dimethyl sulfoxide (DMSO), 10^{-7} M DM, 10^{-6} M RU486, or 10^{-7} M DM + 10^{-6} M RU486, TGF-β1 (10 ng/ml) for 1 hour, and whole cell extracts were prepared. Equal amounts of protein from each treatment were analyzed by immunoblotting with anti-total GR antibody, anti-phospho-Ser²⁰³ (P-203) or anti-phospho-Ser²¹¹ (P-211) antibodies.

Figure 3.

Dexamethasone (DM)-dependent down regulation of total GR in human vocal fold fibroblasts (HVOX). HVOX were treated with dimethyl sulfoxide (DMSO) or 10^{-7} M DM for the indicated time. Whole-cell lysates were prepared, normalized, and analyzed by immunoblotting with antibodies to total GR (top) or β -actin (bottom) as a loading control.

Figure 4.

DM (10^{-5} , 10^{-6} , 10^{-7} M) administration altered gene expressions of *SMAD3* (A), *SMAD7* (B), *COL1A1* (C) and *ACTA2* (D). n=5 for all; *p<0.05 versus control.

Figure 5.

RU486 blocked the effects of dexamethasone (DM) in *SMAD3* (A), *SMAD7* (B) and *ACTA2* (C) mRNA expression. n=5 for all; *p<0.05 versus control; #p<0.05 versus DM.

Figure 6.

SMAD3 (A), *SMAD7* (B), *COL1A1* (C) and *ACTA2* (D) mRNA levels were examined by quantitative real-time PCR 4 or 24 hours after transforming growth factor (TGF)- β 1 +/- dexamethasone (DM). n=5 for all; *p<0.05 versus control; Φ p<0.05 versus TGF- β 1.

Figure 7.

Effects of dexamethasone (DM) on ACTA2 expression. (A) Representative immunofluorescent images of human vocal fold fibroblasts under experimental conditions. Blue, DAPI and red, ACTA2. Scale bar: 100 μ m. (B) Ratio of ACTA2-positive cells was calculated from 10 randomly chosen fields of four samples in each group. The ratio of ACTA2-positive cells under transforming growth factor (TGF)- β 1 stimulation was significantly increased 48 hours after DM administration. N=5; *p<0.05 versus control; #p<0.05 versus DM; Φp<0.05 versus TGF- β 1. DAPI = 4', 6-Diamidino-2-phenylindole dihydrochloride.