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The effects of Cytosporone-B, a novel anti-fibrotic agent, on vocal fold fibroblasts

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Abstract

Objectives/Hypothesis—Our laboratory recently described NR4A1 as an endogenous inhibitor of TGF- β -induced vocal fold (VF) fibrosis. Our prior report described the temporal expression of NR4A1 during VF healing *in vivo* and the effects of NR4A1 knockdown on fibroplastic cell activities *in vitro*. Based on these findings, we hypothesized that Cytosporone-B (Csn-B), a NR4A1 agonist, may hold significant therapeutic potential.

Study Design—In vitro.

Methods—Human VF fibroblasts were exposed to TGF- β 1+/-Csn-B. Expression of genes related to fibrosis were quantified. In addition, contraction was assayed as a surrogate for the fibrotic phenotype in our cell line.

Results—TGF- β 1 stimulated COL1A1 and ACTA2, as expected. Csn-B significantly downregulated TGF- β 1-mediated upregulation of these genes ($p=0.009$, $p=0.03$, respectively). Csn-B had no effect on genes related to TGF- β /Smad signaling. Csn-B also decreased the TGF- β 1-mediated contractile phenotype in our cells ($p=0.004$).

Conclusions—NR4A1 is an endogenous inhibitor of fibrosis in the vocal folds and Csn-B, as an NR4A1 agonist, may evolve as an ideal, therapeutic candidate for this challenging condition.

Level of Evidence—N/A

Keywords

Vocal fold; voice; fibrosis; NR4A1; nuclear receptor; transforming growth factor- β

INTRODUCTION

Vocal fold fibrosis remains one of the most intractable and challenging disorders in laryngology. The fibrotic tissue phenotype is characterized by disruption of the layered

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structure of vocal fold lamina propria in which hyaluronic acid (HA) and elastin are decreased and replaced by excessive collagen bundle accumulation.¹ This altered structure often results in increased tissue stiffness and loss of viscoelasticity. Furthermore, once scar is mature, it is increasingly difficult to recover functional mucosal wave propagation due to the structural aberrations associated with replacement of the innate hyaluronic acid-rich tissue with collagen bundles. Several treatment approaches have evolved to clinical utility including injectable growth factors like hepatocyte growth factor (HGF) which appears to hold significant anti-fibrotic potency and favorable data have emerged from clinical trials.² However, these favorable effects are not uniform and the development of targeted therapeutic approaches remains a priority for this challenging clinical population.

One of the primary mechanisms underlying the development of fibrosis is TGF- β dysregulation following injury; many fibrotic conditions are characterized by sustained and exuberant TGF- β signaling resulting in altered extracellular matrix metabolism and fibroblast-myofibroblast differentiation.³⁻⁵ Global suppression of TGF- β signaling is not ideal as TGF- β has a wide range of biological functions and is critical for maintenance of homeostatic cell activities. In this regard, RNA-based therapies have recently evolved focusing on targeting specific signaling proteins, Smads, in the TGF- β cascade. Upon stimulation, Smad2/Smad3 heterodimerize and translocate to nucleus to regulate the transcription,⁶ and specifically, Smad3 is thought to be a master regulator of fibrosis. Smad7, however, is a competitive inhibitor of SMAD activation acting as an innate anti-fibrotic agent.⁷ Our laboratory recently described the antifibrotic potential for limiting SMAD3 expression in human vocal fold fibroblasts (VFFs),^{7,8} and more recently, *in vivo*.⁹ As an alternative, or potentially complimentary to this work, nuclear receptor 4A1 (NR4A1, also known as NUR77, TR3, NGFI-B, or NAK-I) was recently reported to localize downstream of TGF- β /Smad signaling and may be particularly relevant to the development of fibrosis, or globally, wound healing in general.¹⁰ NR4A1 was initially discovered as a rapid induction receptor induced by multiple stimuli in tissues, cells, and organs. It acts as a cofactor along with other integral cofactors of specificity protein 1 (Sp1) transcription factor.^{11,12} NR4A1 is implicated in wide variety of physiological conditions including metabolic, cardiovascular, neurogenic diseases, arthritis, inflammatory and immunological conditions as well as apoptosis of cells which is applied for establishing novel therapies for cancer treatment.

Palumbo-Zerr et al recently reported the regulatory effects of NR4A1 by inhibiting the profibrotic effects of TGF- β employing murine skin, lung, and liver fibrosis models.¹⁰ In addition, cytosporone-B (Csn-B), a fungal metabolite, was identified as an agonist binding to the ligand-binding domain of NR4A1.¹³ Our group recently described enhanced fibrotic features in human vocal fold fibroblasts with NR4A1 knockdown; specifically, upregulation of extracellular matrix associated with tissue fibrosis, increased myofibroblast differentiation, and collagen gel contraction.¹⁴ Conversely, we hypothesized that Csn-B would enhance the anti-fibrotic features of NR4A1 under TGF- β 1-stimulation in human VFFs; affirmative findings could provide additional foundational justification to further investigate the therapeutic potential of Csn-B for vocal fold fibrosis.

MATERIALS AND METHODS

Cell culture

An immortalized human vocal fold fibroblast cell line created in our laboratory was employed for all *in vitro* experimentation. This cell line, referred to as HVOX, has been shown to be stable through multiple population doublings;¹⁵ cells in passages 20–30 were used. Cells were cultured on 6-well plates in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic at 37°C under standard cell culture conditions. Following 12 hour serum starvation, HVOX were treated with serum-free DMEM +/- TGF- β 1 (10ng/mL). Csn-B (Sigma-Aldrich, St. Louis, MO) in DMSO was concurrently added to the medium at a final concentration of 1 μ M. Recombinant human TGF- β 1 (Life Technologies, Grand Island, NY) was employed for cell stimulation.

Quantitative polymerase chain reaction

Cells were harvested at 6 or 24 hours. Total RNA was extracted via the RNeasy Mini Kit (Qiagen, Valencia, CA). RNA was reverse transcribed with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative polymerase chain reaction (qRT-PCR) employed the TaqMan Gene Expression kit (Life Technologies, Waltham, MA) and StepOne Plus (Applied Biosystems). The following Taqman primer probes were employed: *NR4A1* (Hs00374226_m1), *SMAD2* (Hs00183425_m1), *SMAD3* (Hs00969210_m1), *SMAD7* (Hs00998193_m1), *TGF- β 1* (Hs00998133_m1), *COL1A1* (Hs00164004_m1), *ACTA2* (Hs00426835_g1), *SERPINE1* (Hs00167155_m1), and *GAPDH* (Hs02758991_g1). A description of these gene targets is provided in Table 1. The Ct method was employed with *GAPDH* as the housekeeping gene.

Collagen gel contraction

Gel contraction assays were performed as previously described by our laboratory.⁸ Briefly, collagen gels were created using PureCol (Advanced BioMatrix, San Diego, CA) as previously reported¹⁶ using 0.5-inch Teflon rings. HVOX were gently pipetted onto gels at a concentration of 6.0×10^4 cells in 200 μ L complete media and incubated for 2 hours. The gels were then submerged in each media condition supplemented with 1% FBS. Gels were imaged at 0 and 12 hours using a digital 8-megapixel camera (Apple, Inc. Cupertino, CA). Contraction was quantified by measuring the gel area in pixels using ImageJ (National Institutes of Health). Percent contraction was then calculated via initial area–final area/initial area (%).

Statistical Analyses

Analyses were performed using StatView 5.0 (SAS Institute, Berkeley, CA). For all analyses, the dependent variable of interest was subjected to a one way analysis of variance. If the main effect was significant at $p < 0.05$, post hoc comparisons were performed via the Scheffé method. Significant differences were displayed. Statistical significance was defined as $p < 0.05$; data are expressed as means $\pm$ standard deviation (SD).

RESULTS

Csn-B altered TGF- β 1-mediated transcriptional events

Six hours of Csn-B exposure had no effect on *SMAD2*, *SMAD3*, or *SMAD7*. TGF- β 1 appeared to upregulate Csn-B, but this difference did not reach significant (Figure 1A–1D). At 24 hours, *COL1A1* and *ACTA2* were significantly downregulated in Csn-B treated cells under TGF- β stimulation ($p=0.009$, $p=0.03$, respectively; Figure 2A, 2B), whereas *SERPINE1* was not responsive to Csn-B (Figure 2C).

TGF- β -mediated collagen gel contraction was reduced by Csn-B

As shown in Figure 3A, stimulatory effects of TGF- β 1 on gel contraction were observed after 12 hours, and this response was reduced by Csn-B. Image analysis confirmed significant differences between Csn-B treatment compared to TGF- β 1 stimulation ($p=0.004$; Figure 3B).

DISCUSSION

Given the profound impact of vocal fold fibrosis, we sought to investigate the regulatory effects of Csn-B as a stimulator of NR4A1 with the ultimate goal of developing a novel therapeutic to eventually progress to clinical trials. NR4A1 is classified as an adopted orphan receptor,¹⁷ and specifically, NR4A1 was recently implicated in carcinogenesis and may evolve as a novel target for anticancer pharmaceuticals.¹⁸ Zhan et al initially identified Csn-B as a specific agonist of NR4A1 to enhance transcriptional activity and regulated glucogenesis and apoptosis in cancer which may represent potential targets for both hypoglycemia and some cancers. More recently, Palumbo-Zerr et al addressed fibrotic conditions in multiple animal models by administering Csn-B systemically and achieved favorable cellular and histological findings. Vocal fold fibrosis is an ideal candidate for applying this promising compound given the anatomical location which permits direct injection to avoid systemic morbidity.

We hypothesized that Csn-B had potential therapeutic, anti-fibrotic effects on vocal fold fibroblasts, and performed preliminary experiments employing fibrosis-related gene analyses and collagen gel contraction assays. *COL1A1* and *ACTA2* was significantly downregulated with Csn-B treatment in TGF- β stimulated fibroblasts. These two genes encoding collagen type I and myofibroblast activation, respectively, both of which are strong indicators of tissue fibroplasia. Conversely, *SMAD2*, *SMAD3*, and *SMAD7* expression did not change in response to Csn-B. Our group previously reported siRNA for NR4A1 enhanced pro-fibrotic features of TGF- β 1-stimulated cells with no significant changes in *SMAD2* or *SMAD3* expression.¹⁴ We observed the tendency towards upregulation of endogenous TGF- β 1 induced by Csn-B. Interestingly, no previous data describes a relationship between endogenous TGF- β 1 and Csn-B, but presumably, this result could be derived from feedback of cellular anti-fibrotic signaling induced by Csn-B treatment. Further investigation is warranted to verify this finding. Cumulatively, Smad signaling appeared to not respond to either stimulation or suppression of NR4A1, which is a bit counterintuitive as Smad3 is considered a master regulator of various fibrotic conditions including vocal fold fibroplasia.

^{7,19} This independence between Smad signaling and NR4A1 could be viewed as favorable; certainly, further investigation is warranted. With regard to contraction, Csn-B significantly reduced TGF- β 1 mediated gel contraction. These assays are thought to reflect the physiological condition during wound healing and the fibrotic properties of stimulated cells. These data further confirm the antifibrotic features described in the gene analyses, and are compatible with our previous reports regarding accelerated contraction with siRNA for NR4A1.¹⁴

The current study is not without limitation; as with all *in vitro* investigation, limited sample size, a single cell line, and the lack of *in vivo* confirmation of relevant phenomena limit the overall applicability of these data. In addition, this investigation is far from comprehensive and included limited dependent variables related to fibrosis. Regardless, the data provide cursory insight regarding the potential therapeutic effects of Csn-B for vocal fold fibrosis. In this regard, Csn-B is not yet approved for clinical use and further investigation regarding the regulatory effects of this compound is warranted, with the ultimate goal of administration for this challenging disorder.

CONCLUSIONS

Csn-B is the specific stimulator of NR4A1. Csn-B decreased fibrosis-related gene expression and reduced collagen contraction in TGF- β 1-stimulated human vocal fold fibroblasts. These preliminary data are the first to describe the anti-fibrotic potential of Csn-B for vocal fold fibrosis; these findings warrant further investigation to elucidate the possibility of Csn-B as a novel therapeutic agent.

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Figure 1. Effects of NR4A1 agonist, Csn-B, on genes related to TGF- β signaling. (A–D) *SMAD* family and endogenous *Tgf- β 1* mRNA in TGF- β 1-stimulated HVOX were upregulated in response to Csn-B (n = 4 for all; *p<0.05).

Figure 2.

Effects of NR4A1 agonist, Csn-B, on genes related to extracellular matrix metabolism. (A) *COL1A1* and (B) *ACTA2* were downregulated in response to Csn-B (n = 4 for all; *p<0.05).

Figure 3.

Effects of Csn-B on three-dimensional collagen gel contraction. (A) Representative gels under each culture condition immediately following cell seeding and after 12 hours under each treatment condition. All images were cropped along the periphery of the well. (B) The stimulatory effects of TGF-β1 on contraction were significantly reduced in response to Csn-B (n=4 for all, * $p<0.05$).

Table 1

Description of gene targets included, their symbol, and a cursory description of their role in the development and maintenance of fibrosis.

Gene Target	Symbol	Function
Nuclear Receptor Subfamily 4 Group A Member 1	NR4A1	Nuclear transcription factor; endogenous inhibitor of TGF- β
Mothers against decapentaplegic homolog 2	SMAD2	TGF- β signaling protein
Mothers against decapentaplegic homolog 3	SMAD3	TGF- β signaling protein
Mothers against decapentaplegic homolog 7	SMAD7	TGF- β signaling protein-competitive inhibitor of Smad2/3
Transforming Growth Factor- β	TGF- β 1	Regulator of multiple cell activities associated with tissue fibrosis
Type I Collagen	COL1A1	Primary extracellular component of fibrosis
Smooth muscle alpha (α)-2 actin	ACTA2	Indicator of cellular contractile features associated with fibrosis
Serpin Family E Member 1	SERPINE1	Inhibitor of fibrinolysis