Implementing Efficient Peptoid-Mediated Delivery of RNA-Based Therapeutics to the Vocal Folds

Shigeyuki Mukudai, MD, PhD; Iv Kraja, BS; Renjie Bing, MD; Danielle M. Nalband, PhD; Mallika Tatikola, BS; Nao Hiwatashi, MD, PhD; Kent Kirshenbaum, PhD; Ryan C. Branski, PhD

Objective: We hypothesize that Smad3 is a master regulator of fibrosis in the vocal folds (VFs) and RNA-based therapeutics targeting Smad3 hold therapeutic promise. Delivery remains challenging. We previously described a novel synthetic peptoid oligomer, lipitoid L0, complexed with siRNA to improve stability and cellular uptake. An advantage of these peptoids, however, is tremendous structural and chemical malleability to optimize transfection efficiency. Modifications of L0 were assayed to optimize siRNA-mediated alteration of gene expression.

Methods: In vitro, Smad3 knockdown by various lipitoid variants was evaluated via quantitative real-time polymerase chain reaction in human VF fibroblasts. Cytotoxicity was quantified via colorimetric assays. In vivo, a rabbit model of VF injury was employed to evaluate the temporal dynamics of Smad3 knockdown following injection of the L0-siRNA complex.

Results: In vitro, similar reductions in Smad3 expression were established by all lipitoid variants, with one exception. Sequence variants also exhibited similar nontoxic characteristics; no statistically significant differences in cell proliferation were observed. In vivo, Smad3 expression was significantly reduced in injured VFs following injection of L0-complexed Smad3 siRNA at 1 day postinjection. Qualitative suppression of Smad3 expression persisted to 3 days following injury, but did not achieve statistical significance.

Conclusions: In spite of the chemical diversity of these peptoid transfection reagents, the sequence variants generally provided consistently efficient reductions in Smad3 expression. L0 yielded effective, yet temporally limited knockdown of Smad3 in vivo. Peptoids may provide a versatile platform for the discovery of siRNA delivery vehicles optimized for clinical application.

Key Words: Voice, vocal fold, fibrosis, siRNA, lipitoid, Smad3.

Level of Evidence: NA

INTRODUCTION

Vocal fold (VF) scar continues to pose a significant clinical challenge. Scar and the associated tissue stiffness contribute to decreased vibratory pliability of the VF mucosa often resulting in altered phonatory physiology and voice quality.1-3 Following injury, transforming growth factor beta (TGF-β)/Smad signaling plays an important role4 and the actions of TGF-β1 are mediated via a unique signaling pathway providing ample targets for intervention. Upon TGF-β1 binding to the cell surface receptor, the Smad family of proteins is phosphorylated, heterodimerize, and translocate to the nucleus to regulate transcription. Activated Smads have been implicated in a variety of fibrotic processes, suggesting that Smad activation plays a central role in both collagen production and accumulation.5

Our laboratory hypothesized that Smad3 is a principal mediator of the fibrotic phenotype in the VFs and we further posited that alteration of Smad3 expression through short interfering (si)RNA holds therapeutic promise. Nevertheless, delivery of oligonucleotides for siRNA-mediated therapies remains challenging largely due to the proteolytic tissue environment.6 To address this issue, our laboratory employed a synthetic peptidomimetic oligomer, lipitoid L0, complexed with siRNA to improve efficiency of RNA-based therapeutics and we recently described in vivo knockdown of Smad3 employing L0.7 These peptoid transfection reagents, however, afford substantive chemical diversity to optimize transfection efficacy. L0 was chosen as an optimal sequence due to its exceptional performance during in vitro DNA transfection trials in HT1080 cells, as well as its ability to protect nucleic acid cargo from hydrolytic cleavage.8

Although L0 performed well both in vitro and in vivo, we sought to exploit the inherent flexibility of these peptoids to ensure optimal efficiency. We hypothesized that lipitoids incorporating both alkyl and aryl side chain groups may provide transfection efficiency and form stronger nanoparticle complexes with genetic cargo, protecting it from hydrolysis. Based on this hypothesis, we designed additional lipitoid variants L1-L8 (Figs. 1 and 2; “lipitoid” and L0 are used interchangeably); these figures illustrate the
inherent flexibility associated with lipitoid structure. We then screened these variants with regard to knockdown efficiency and cytotoxicity at 24 hours, acknowledging that additional time points may be indicated for more comprehensive evaluation. We then examined the temporal profile of Smad3 knockdown in vivo. Cumulatively, these data provide a logical progression of work from our laboratory, and more importantly, serve as a platform for the continued evolution of RNA-based therapeutics into the clinical milieu.

MATERIALS AND METHODS

In Vitro Experimentation

CELL MODEL. All in vitro experiments employed an immortalized human VF fibroblast cell line created in our laboratory referred to as HVOX.5 Cells were grown in Dulbecco’s modified eagle medium (DMEM; Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Life Technologies) and 1% antibiotic/antimycotic (Life Technologies).

L0–L8 SYNTHESIS. All lipitoids were synthesized according to the modified solid phase synthesis protocol previously described by the Zuckermann group.10 Conjugation of the lipid to the peptoid was conducted in glass scintillation vials. After assessing the lipitoid product purities by analytical reversed phase high performance liquid chromatography (RP-HPLC), they were either used crude or purified by preparatory RP-HPLC. Synthesis of peptoid oligomers was initiated on Rink Amide resin (Nova Biochem, 100–200 mesh, loading-0.74 mmol/g). Bromoacetic acid (97%) was supplied by Sigma-Aldrich. Triisopropylsilane, and 2.5% water (4 mL) at room temperature for 10 minutes. The solution was removed under reduced pressure and the crude lipitoids were resuspended in acetonitrile/water, from the resin using a cocktail containing 95% TFA, 2.5% chloromethane (5 x 1 mL). The lipitoid products were then cleaved primary amine (1 M in DMF) for 30 minutes with agitation at room temperature. The resin was then washed with 5x1mL DMF (5 x 1 mL). This two-step bromoacetylation/displacement iterative process was repeated until the desired oligomer chain length and monomer sequence composition was achieved. Prior to lipid addition, the peptoid sequence was bromoacetylated once more and the resin washed and transferred to a glass scintillation vial. A 0.2 M solution of lipid, either 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine, or 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine, was prepared in 15% methanol/chlorobenzene and to it was added 0.95 eq. of 11 M KOH. The solution was centrifuged, and the supernatant removed and added to the scintillation vial containing the resin. Resins were exposed to the lipid solution for approximately 16 hours at 37-C with agitation. The resins were then washed with 15% methanol/chlorobenzene, DMF (5 x 1 mL), and finally dichloromethane (5 x 1 mL). The lipitoid products were then cleaved from the resin using a cocktail containing 95% TFA, 2.5% trisopropylsilane, and 2.5% water (4 mL) at room temperature for 10 minutes. The solution was removed under reduced pressure and the crude lipitoids were resuspended in acetonitrile/water, frozen, and lyophilized. Once thoroughly dried, crude lipitoids were stored at 4°C until characterization and purification.

CHARACTERIZATION AND PURIFICATION OF L0 VARIANTS. Lyophilized powders were suspended in a solution of aqueous acetonitrile then analyzed by matrix assisted laser desorption/ionization - time of flight mass spectrometry (MALDI-TOF/TOF) and analytical RP-HPLC. Mass spectrometry was performed on an Agilent 1100 series LC/MS VL MS spectrometer or a Bruker Maldi-TOP TOF UltraflteXtreme MS spectrometer.

Laryngoscope Investigative Otolaryngology: December 2019 Mukudai et al.: Optimized RNA-Based Vocal Fold Therapeutics

Fig. 1. Chemical structure of the L0 variants, L1–L8, depicted to indicate their trimeric repeat sequence motifs.

Fig. 2. First and second generation lipitoid sequence identities (A); and representation of the peptoid monomers used in the lipitoid sequences. L0 is also notated as “lipitoid”, as this is the original nomenclature (B). Nmpe: N-(2-(p-methoxyphenyl)glycine; Nae: N-(aminoethyl)glycine; Nia: N-(isoamyl)glycine, and Npn: N-(pentyl)glycine. DMPE = 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine; DPPE = 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine; POPE = 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine.
using a saturated solution of α-Cyano-4-hydroxycinnamic acid prepared in 70% ACN(aq) with 0.1% TFA. Purity was assessed by reverse-phase HPLC (DeltaPak analytical C18 column, Waters, 5 μm, 300 Angstroms, 3.9 × 150 mm) on an Agilent 1200 Infinity LC system. L1–L8 were detected at 220 nm. Crude L1, L2, and L3 were analyzed over a linear gradient linear gradient of 30–95% aqueous ACN with 0.1% TFA over 40 minutes at a flow rate of 0.5 mL/min. Crude L4–L8 were analyzed over a linear gradient of 50–95% aqueous ACN with 0.1% TFA over 60 minutes at a flow rate of 0.5 mL/min. Crude L1, L2, L4, and L8 were obtained at >90% purity and L3 at >80%. These lyophilized powders were stored at 4°C until in vitro testing. L5–L7 were purified to >95% by RP-HPLC using a preparatory C18 column. HPLC was performed on a Waters 2489 instrument using a Phenomenex Jupiter C18 column (Phenomenex, 15 μm, 300Angstroms, 10 × 250 mm) and detected at 220 nm. L6 and L7 were purified over linear gradient of 30–95% aqueous ACN with 0.1% TFA over 40 minutes at a flow rate of 5 mL/min; L5 necessitated a linear gradient of 15–95% aqueous ACN with 0.1% TFA over 60 minutes at a flow rate of 5 mL/min. Fractions were consolidated, frozen, and lyophilized. Once dried, purified lipitoids were stored at 4°C until analysis and in vitro testing.

**TRANSFECTION.** HVOX were grown to 80% confluence. A mixture of 5 μL siRNA (5 μM) and 5 μL of lipotid (1.07 mg/mL) were combined in 500 μL of reduced serum media (Opti-MEM; Life Technologies) and incubated at room temperature for 20 minutes. A nonsense siRNA sequence was employed as a control condition. After incubation, the mixture was then added to cells in a six-well plate along with 1.5 mL of DMEM (Life Technologies). Antibiotic/antimycotic additives were omitted during transfection. Cells were treated with the transfection media for 24 hours, and then harvested for RNA extraction.

**RNA EXTRACTION.** RNA was extracted from harvested cells with the Qiagen RNeasy Kit (Qiagen, Valencia, CA) using the manufacturer's protocols. A total volume of 30 μL of RNA was extracted from each sample. RNA was then quantified using the NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, DE) following standard protocols.

**QUANTITATIVE POLYMERASE CHAIN REACTION.** Taqman RNA-to-Ct 1-Step kit (Applied Biosystems, Grand Island, NY) was used to perform quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) from harvested RNA under the manufacturer's protocols. cDNA obtained from reverse transcription was then used to perform quantitative RT-PCR using the Via7 Real-Time PCR System (Applied Biosystems). Sequences for Smad3 and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained for Taqman gene expression assays (Applied Biosystems). Amplification data were obtained using the comparative ΔΔCt method.

**CELL PROLIFERATION ASSAY.** HVOX were treated with 5.0 μg/mL of each lipotid variant for 24 hours. After treatment, the medium was changed and the cells were then treated with 20 μL of CellTiter 96 Aqueous One (Promega, Fitchburg, WI) for 2 hours. Absorption at 490 nm was quantified to determine cell viability.

**In Vivo Experimentation**

**VF INJURY.** All procedures were approved by the Institutional Animal Care and Use Committee at the New York University School of Medicine. Fifteen 2–3 kg New Zealand White rabbits were randomly assigned to each treatment group; L0 + nonsense siRNA and L0 + Smad3 siRNA; an additional five rabbits were included as control/no treatment (n = 15 per treatment group, 5 control; 35 total animals). Each group was further categorized according to time of sacrifice: 1, 2, and 3 days after injection (n = 5 each). All animals received intravenous injection of ketamine (70 mg/kg) and xylazine (2 mg/kg) for induction of anesthesia. Inspired isoflurane was added for anesthetic maintenance. The animals were placed supine on an operating table and the larynx was visualized via a slotted laryngoscope (size 1; Karl Storz, Flanders, NJ). Under the guidance of 2.7 mm, 0° or 30 telescope (Karl Storz), the VFVs were injured unilaterally by puncturing the lamina propria from the thyroarytenoid muscle via insertion of a 22-gauge needle at the lateral portion of right VF, followed by removal of the lamina propria with microscissors and microforceps. This model and the surgical technique have been described previously by our group.

**TRANSFESSION SOLUTIONS.** Ten microliters of 5 μM Smad3 siRNA were complexed with 10 μL of 210 μM lipotid diluted in deionized water/1x phosphate buffered saline (PBS) buffer by adding the siRNA to the lipotid solution. The final injected lipotid concentration was determined via 1.07 mg/mL (initial lipotid concentration in H2O) × 5 μL = 5.35 μg in 20 μL of 1x PBS. This solution was allowed to stand at room temperature for 20 minutes to optimize nanoparticle dimension, as previously described and based on our previous findings.11 In addition, siRNA-lipotid complexes were formed at an approximate lipotid to siRNA molar ratio of 42:1, establishing a positive/negative charge ratio of 3:1, based on work from the Zuckerman Laboratory.12 Nonsense siRNA solution was prepared under identical conditions.

**VF INJECTION.** Seven days after injury, animals underwent anesthesia and laryngeal visualization as described above. This time point was selected to allow for re-epithelialization of the injury site to provide a "pocket" for the injectate to reside. Previous attempts to inject into the acutely injured VF lead to decreased precision and loss of injectate at the injury site. The intralaryngeal approach was then employed to inject 20 μL of 0.1 mg/kg administered dose of nonsense siRNA or Smad3 siRNA complexed with L0 into the subepithelial layer of the scarred right lamina propria using a microinjection system (Hamilton Company, Reno, NV) with a 27-gauge needle. The location of the injection site was confirmed visually by distention of the VF mucosa. At 1, 2, or 3 days following injection, the animals were sacrificed and the larynges were harvested and immersed in RNALater RNA Stabilization Regent (Quagen, Inc.) and stored at −80°C until analysis.

**QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION.** The bilateral VFVs were dissected under magnification. mRNA extraction, quantification of mRNA, and RT-PCR were performed as previously described.7 Expression was presented as fold change relative to the uninjured VF. In addition, the uninjured left VF served as an internal control.

**STATISTICAL ANALYSES.** In vitro experimentation was performed in triplicate. The dependent variable of interest was subjected to a one-way analysis of variance. If the main effect was significant, post hoc comparisons were performed via the Scheffé method. For the in vivo study, a two-way analysis of variance (ANOVA) followed by the Scheffé post hoc test was employed to investigate differences in gene expression at each time point. When interactions were present between treatment and time point, a one-way factorial ANOVA followed by the Scheffé post hoc test were performed. Statistical significance was defined as P < .05.
RESULTS

In Vitro

SMAD3 EXPRESSION DECREASED WITH ALL L0 VARIANTS. All lipitoid samples resulted in statistically significant knockdown of Smad3 relative to control (Fig. 3A). Gene expression in the negative control cells (N/S), transfected with random sequences of siRNA, was not altered. No significant changes in Smad3 expression were observed across L0 variants with the exception of L6. Specifically, L6 yielded 0.673-fold change relative to control, which was the least effective knockdown among the L0 variants. L3 yielded the greatest Smad3 knockdown at 0.143-fold change relative to control, but this difference was not significant when compared to L0.

L0 VARIATION HAD NO EFFECT ON CELL PROLIFERATION. Cell proliferation during transfection with L0–L8 was compared to a control and a negative control in FBS-free media (Fig. 3B). A 0.653-fold change was observed between the negative control condition and untreated cells. All L0 variants maintained proliferation levels above the negative control conditions. No statistically significant differences were observed between L0 variants. Specifically, variants L1–L8 did not differ significantly from L0. L0 resulted in a 0.871-fold change compared to untransfected cells. Other variations of L0 resulted in a fold change between the lowest (L6) 0.784 and the highest (L1) 0.981 compared to the same control group.

In Vivo

L0-COMPLEXED SIRNA SUPPRESSED SMAD3 GENE EXPRESSION ACUTELY FOLLOWING INJECTION, BUT THIS EFFECT WAS NOT SUSTAINED. Smad3 mRNA expression increased significantly 1, 2, and 3 days following injury (Fig. 4). Although Smad3 mRNA expression decreased in response to L0 + Smad3 siRNA injection, qualitative suppression of Smad3 expression was observed in the injured VFs at 2 and 3 days following L0 + Smad3 siRNA injection relative to the control. This difference did not achieve significance (P = .289 and .782, respectively).

DISCUSSION

VF scar poses a significant clinical challenge and optimal treatments for this highly morbid condition have not yet been established. Collectively, many laboratories have provided valuable insight regarding the processes underlying the development of this challenging tissue phenotype. Our laboratory has acquired substantive data suggesting a regulatory role for Smad3 in the development of VF fibrosis and hypothesized that targeting TGF-β signaling, and more precisely, Smad signaling were likely to yield encouraging clinical outcomes. In that regard, localized delivery of RNA-based therapies likely hold great promise. Delivery of adequate concentrations to the target organ is challenging as the inherent instability of siRNA leads to rapid degradation.

Fig. 3. (A) Twenty-four hour treatment with all lipitoid samples (L0–L8) resulted in statistical significant knockdown of Smad3 in vitro. The negative control cells (N/S), which were transfected with random sequences of siRNA, did not result in significant change from an unaffected control group, and (B) maintained cell proliferation levels above that of the negative control sample containing no FBS 24 hours following transfection. No statistically significant differences were observed between L0 variants (*P < .05 vs. control; #P < .05 vs. other peptoids). FBS = fetal bovine serum; N/S = nonsense siRNA.

Fig. 4. Expression of Smad3 mRNA at 1, 2, and 3 days after injection of L0-siRNA complex (n = 5). Smad3 expression was standardized to glyceraldehyde-3-phosphate dehydrogenase and presented as fold change relative to the uninjured vocal fold as a control (mean ± SEM; *P < .05). N/S = nonsense siRNA.
In addition, negatively charged siRNA typically cannot cross the cell membrane via passive diffusion.16 Therapeutic delivery of siRNA requires optimization of delivery mechanisms to protect siRNA from degradation,17 deliver siRNA to target cells and avoid off-target effects, facilitate cellular uptake and endosomal escape, and finally, release siRNA intracellularly so it will be accessible to the cell machinery.

Our laboratory recently described both in vivo and in vitro utility of a novel synthetic oligomer, lipoid L0, complexed with siRNA to improve stability and cellular uptake with the goal of increased efficiency of RNA-based therapeutics. We were the first group to show preclinical data regarding the efficacy of RNA-based therapeutics to locally alter transcription in the VFs. This initial study, however, only quantified Smad3 mRNA expression 24 hours following treatment and more long-term time points are necessary to provide a platform for clinical indication. Furthermore, our synthetic oligomers are highly amenable to modifications in the structural and chemical properties (Figs. 1 and 2). In the current study, we sought to perform preliminary screening of these peptoids with regard to regulation of transcription and cytotoxicity at limited time points to ensure our approach to in vivo therapeutics was sound. Certainly, this screening was not comprehensive, and ideally, could include multiple additional dependent variables across numerous time points. In spite of substantive chemical changes across the nine different structures included in the current study, the effect on Smad3 knockdown was essentially unchanged in vitro, with the exception of L6. L3 yielded the greatest Smad3 knockdown relative to control, but post hoc statistical analyses failed to achieve significance when compared to L0. In addition, across all L0 variants, cell proliferation was not significantly affected. Cumulatively, these data, although not particularly innovative, are critical to the progression of this line of investigation, and ultimately, the progression to clinical experimentation.

Our group previously reported significant Smad3 upregulation acutely following injury,13 we also recently published the first in vivo application of RNA-based therapeutics to locally alter the VF gene environment.14 In the current study, qualitative suppression of Smad3 expression persisted 2 days following treatment. This difference, however, did not achieve statistical significance. We previously reported Smad3 suppression for up to 96 hours in vitro;15,16 this discrepancy likely speaks to the inherent complexity of the in vivo environment. Regardless, these data are quite encouraging and provide some temporal insight into the utility of RNA-based therapeutics. Ultimately, multiple treatments may be required for optimized efficacy. These issues certainly warrant further investigation with the ultimate goal of transitioning to clinical application.

Enthusiasm regarding these findings should be somewhat tempered; the current study is certainly not without limitations that warrant further discussion. First, the scope of the work is relatively narrow. The primary dependent variable was Smad3 mRNA expression. Clearly, the inherent tissue response to injury is complex invoking multiple cell types and soluble mediators. TGF-β is a known mediator of fibrosis and was not queried. With that being said, our laboratory previously described the downstream effects of Smad3 knockdown and found TGF-β expression to be largely unaffected. However, expression of TGF-β-induced profibrotic compounds such CTGF, ACTA2, and SNAI1 was reduced in response to Smad3 knockdown.14 In addition, given that our therapeutic strategy targets transcriptional events, we singularly assayed mRNA expression. Clearly, future investigation warrants additional quantitative analyses including translational assays, and more importantly, histological and biomechanical analyses to elucidate the functional implications for Smad3 knockdown in the injured VF. In addition, the in vitro work was certainly not comprehensive, and experimentation regarding peptoid variability should be considered a screening of variants. This screening confirmed that our approach with L0 is likely appropriate.

CONCLUSION

Lipoid L0 transfection reagent successfully mediated temporally limited knockdown of Smad3 in vivo following VF injury. Variations within these versatile oligomers facilitate the development of transfection reagents optimized to address a range of therapeutic modalities. A set of sequence variants were synthesized and screened; in the current study, the original transfection reagent design exhibited significant promise and is a likely candidate for transition to clinical utility.

BIBLIOGRAPHY

1. Hirano S, Minamiguchi S, Yamashita M, Ohno T, Kanemaru S, Kitamura M. Histologic characterization of human scarred vocal folds. J Voice 2009;23: 409–417.
2. Hirano S. Current treatment of vocal fold scarring. Curr Opin Otolaryngol Head Neck Surg 2005;13:143–147.
3. Benninger MS, Alessi D, Archer S, et al. Vocal fold scarring: current concepts and management. Otolaryngol Head Neck Surg 1996;115:474–482.
4. O’Kane S, Ferguson MW. Transforming growth factor beta s and wound healing. Int J Biochem Cell Biol 1997;29:63–78.
5. Branski RC, Bing R, Kraja I, Amin MR. The role of Smad3 in the fibrotic phenotype in human vocal fold fibroblasts. Laryngoscope 2016;126: 1151–1156.
6. Burnett JC, Rossi JJ. RNA-based therapeutics: current progress and future prospects. Chem Biol 2012;19:69–71.
7. Hiwatashi N, Kraja I, Benedict PA, et al. Nanoparticle delivery of RNA-based therapeutics to alter the vocal fold tissue response to injury. Laryngoscope 2017;128:E178–E183.
8. Huang CY, Uno T, Murphy JE, et al. Lipoiditoids—novel cationic lipids for cellular delivery of plasmid DNA in vitro. Chem Biol 1998;5:345–354.
9. Branski RC, Barbieri SS, Wexelser BB, et al. The effects of transforming growth factor-beta s on human vocal fold fibroblasts. Ann Otol Rhino Laryngol 2009;118:218–226.
10. Murphy JE, Uno T, Hamer JD, Cohen FE, Dwariki V, Zuckerman RN. A combinatorial approach to the discovery of efficient cationic peptidic reagents for gene delivery. Proc Natl Acad Sci U S A 1998;95:1517–1522.
11. Hiwatashi N, Kraja I, Benedict PA, et al. Nanoparticle delivery of RNA-based therapeutics to alter the vocal fold tissue response to injury. Laryngoscope 2016;126:E178–E183.
12. Kocza YU, Kirshenbaum K, Zackmann NR. Nanometer-scale siRNA car- riers incorporating peptidomeric oligomers: physical characterization and biological activity. Int J Nanomed 2014;9:2271–2285.
13. Hiwatashi N, Benedict PA, Dion GR, et al. Smad3 expression and regula- tion of fibroplasia in vocal fold injury. Laryngoscope 2017;127:E308–E316.
14. Paul BC, Rafii BY, Gandonu S, et al. Smad3: an emerging target for vocal fold fibrosis. Laryngoscope 2014;124:2327–2331.
15. Whitehead KA, Langer R, Anderson DG. Knockdowning barriers: advances in siRNA delivery. Nat Rev Drug Discov 2009;8:129–138.
16. Lu JJ, Langer R, Chen L. A novel mechanism is involved in cationic lipid- mediated functional siRNA delivery. Mol Pharm 2009;6:763–771.
17. Larson SD, Jackson LN, Chen LA, Rychahou PG, Evers BM. Effectiveness of siRNA uptake in target tissues by various delivery methods. Surgery 2007;142:262–269.
18. Kraja I, Bing R, Hiwatashi N, et al. Preliminary study of a novel transfection modality for in vivo siRNA delivery to vocal fold fibroblasts. Laryngoscope 2017;127:E291–E297.