The IncRNA PDIA3P Interacts with miR-185-5p to Modulate Oral Squamous Cell Carcinoma Progression by Targeting Cyclin D2

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Long noncoding RNAs (lncRNAs) are emerging as important regulators during tumorigenesis by serving as competing endogenous RNAs (ceRNAs). In this study, the qRT-PCR results indicated that the IncRNA protein disulfide isomerase family A member 3 pseudogene 1 (PDIA3P) was overexpressed in oral squamous cell carcinoma (OSCC) and decreased the survival rate of OSCC patients. CCK-8 and clonal colony formation assays were used to detect the effects of PDIA3P on proliferation. Results revealed that silencing PDIA3P by small interfering RNA (siRNA) inhibited OSCC cell proliferation and repressed tumor growth and reduced the expression of proliferation antigen Ki-67 in vivo. Furthermore, the interaction between PDIA3P and miRNAs was then analyzed by qRT-PCR and luciferase reporter gene assay. We found that PDIA3P negatively regulated miR-185-5p in OSCC cells. Simultaneously, we found that silencing PDIA3P by siRNA suppressed proliferation via miR-185-5p in OSCC cells. Moreover, silencing PDIA3P by siRNA inhibited CCND2 protein (no influence on mRNA levels) expression via miR-185-5p in OSCC cells, and CCND2 facilitated cell proliferation of SCC4 and SCC15 cells induced by sh-PDIA3P#1. Therefore, our study demonstrated that PDIA3P may be a therapeutic target for the treatment of OSCC.

INTRODUCTION

Oral squamous cell carcinoma (OSCC), an epithelial neoplasm found in 80%–90% of head and neck cancer,1,2 is among the ten most frequent human malignancies and mostly arises in the context of alcohol abuse, smoking, and human papillomavirus (HPV) infection.3 OSCC lesions are histopathologically characterized by the presence of a different degree of keratin production, squamous differentiation, mitotic activity, invasive growth and metastasis, and nuclear pleomorphisms. No promising progress in the treatment of OSCC has been made over the past decades.4,5 Although several advances have been obtained in its treatment, OSCC prognosis still remains poor, with a 5-year survival ratio of nearly 50%.1 It has been reported that the tumorigenesis and progression for OSCC are an intricate process involved in multiple genetic and epigenetic alterations.6,7 Therefore, elucidation of the molecular mechanisms referred in the initiation and oncogenesis of OSCC is of great importance to improve treatment outcome.

Long non-coding RNAs (incRNAs) are non-protein coding transcripts longer than 200 nt in length, which are involved in multiple biological functions, such as gene transcription regulation,8 RNA processing,9 modulation of apoptosis and invasion,10 miRNA host genes,11 chromatin modification,12 and marker of cell fate,13 etc. Dysregulation of lncRNAs has occurred in multiple human diseases, including neurological disorders14 and cancers. Additionally, lncRNAs can not only function as a decoy for splicing factors or as antisense transcripts resulting in splicing malfunctioning,15,16 but also serve as competing endogenous RNAs (ceRNAs) in several tumors.17–20 Several studies have implied that lncRNAs, including HOTAIR21 and GTF2IRD2P1,22 are related to OSCC tumorigenesis. Nevertheless, the detailed clinical significance and molecular mechanisms of lncRNAs in OSCC neoplasia and development still remain largely unclear.

Protein disulfide isomerase family A member 3 pseudogene 1 (PDIA3P1, NR_002305) is a 2099-bp IncRNA that maps to chromosome 1q21.1 (https://www.ncbi.nlm.nih.gov/gene/?term=PDIA3P).

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Recently, Zhang et al.22 have reported that PDIA3P is overexpressed in OSCC patients, whereas until now, the relationship between PDIA3P expression and OSCC oncogenesis has not been explored yet. Thus, the functions and underlying molecular mechanisms for PDIA3P in OSCC still need to be further elaborated.

In the present study, we demonstrated that PDIA3P was upregulated in OSCC tissues. PDIA3P upregulated the miR-185-5p target gene CCND2 by competitively sponging miR-185-5p and then promoted OSCC cell proliferation. PDIA3P may function as a part of the ceRNA network.

RESULTS

PDIA3P Is Overexpressed in OSCCs and Suppresses the Survival Rate of OSCC Patients

We randomly collected OSCC tissues and paired noncancerous tissues from 58 patients. The RNA expression level of PDIA3P was measured by qRT-PCR and found to be increased in OSCC tissues compared with corresponding noncancerous tissues (Figure 1A). Moreover, we found that the RNA expression level of PDIA3P was upregulated in OSCC cell lines (SCC4, SCC9, SCC1, SCC25, TU183, HSU3, FADU, OECD-M1, SNU1041, and SCC15) compared with a normal human oral keratinocytes cell line NHOK (p < 0.05), and PDIA3P expression was higher in SCC4 and SCC15 cells than in other OSCC cells (Figure 1B). Hence, the following experiments were performed using SCC4 and SCC15 cells. Results also indicated that PDIA3P reduced the survival rate of OSCC patients (p < 0.05) (Figure 1C). These results demonstrated that higher PDIA3P expression was related to poorer prognosis, and overexpression of PDIA3P might be crucial for OSCC progression.

PDIA3P Promotes OSCC Cell Growth In Vivo

We also studied the impact of PDIA3P on OSCC cell proliferation. To this end, SCC4 and SCC15 cells were transfected with control short hairpin RNA (shRNA) or shRNAs against PDIA3P, i.e., sh-PDIA3P#1, sh-PDIA3P#2, and sh-PDIA3P#3, and pcDNA3.1-CT-GFP-PDIA3P. The qRT-PCR results indicated that sh-PDIA3P#1 effectively knocked down PDIA3P (Figure 2A). Thus, SCC4 and SCC15 cells were transfected with control or sh-PDIA3P#1 or control or pcDNA3.1-CT-GFP-PDIA3P. The CCK-8 results showed that silencing PDIA3P by RNAi inhibited SCC4 and SCC15 cell proliferation (p < 0.05) (Figures 2B–2E). Moreover, the clonal colony forming assay results also showed that silencing PDIA3P by RNAi inhibited SCC4 and SCC15 cell proliferation (p < 0.05) (Figures 2F and 2G). However, overexpressed PDIA3P facilitated cell growth of SCC4 and SCC15 cells (Figures 2B–2G), which clearly revealed that PDIA3P markedly promoted cell growth in OSCC cells.

PDIA3P Knockdown Suppresses OSCC Cell Growth In Vivo

To further explore whether silencing PDIA3P could be involved in affecting tumor growth in vivo, SCC4 and SCC15 cells transfected with sh-NC or sh-PDIA3P#1 were inoculated into male nude mice. Results demonstrated that the tumor size in the sh-PDIA3P#1 group was smaller compared with the sh-NC group 36 days after injection (Figures 3A and 3B). The tumor weight of the sh-PDIA3P#1 group was also significantly less than that of the sh-NC group (Figures 3C and 3D). Additionally, immunohistochemistry (IHC) analysis identified that the tumors isolated from SCC4/sh-PDIA3P#1 and SCC15/sh-PDIA3P#1 cells revealed fewer Ki-67-staining areas than those from control cells (Figure 3E). Hence, we confirmed that silencing PDIA3P could repress tumor growth in vivo.
A) Relative PDIA3P expression (ΔΔCt)

B) OD-450 Values

C) SCC4

D) SCC15

E) SCC15

F) Images of cell cultures

G) Colony formation ratio (%)
SCC4 and SCC15 cells (Figure 5E). Furthermore, we assessed the association between PDIA3P overexpression suppressed miR-185-5p expression both in SCC4 and SCC15 cells. Moreover, miR-185-5p decreased the protein expression but had no influence on the mRNA expression for CCND2 in SCC4 and SCC15 cells (Figures 6D and 6E). Additionally, our results also demonstrated the miRNA of CCND2 expression had no significant correction with the expression of miR-185-5p in OSCC samples (r = −0.1997, p = 0.1372) (Figure 6F). Further, our results also demonstrated that CCND2 was highly expressed, whereas miR-185-5p was lowly expressed in OSCC tissues (Figures 6G and 6H). We then explored the role and mechanism of miR-185-5p on OSCC cell growth. Results of Trypan blue staining revealed miR-185-5p treatment suppressed cell growth, and pcDNA3.1-CT-GFP-CCND2 (with a full 3′ UTR mRNA for CCND2) treatment facilitated cell growth in SCC4 and SCC15 cells (Figures 6I and 6J). However, when SCC4 and SCC15 cells were treated with miR-185-5p plus pcDNA3.1-CT-GFP-CCND2, the advantageous role of CCND2 on cell growth was reversed by miR-185-5p, and the growth-inhibitory effect of miR-185-5p was inversely correlated with the expression of CCND2 in SCC4 and SCC15 cells (Figures 6I and 6J). These data demonstrated that miR-185-5p suppressed cell growth by directly targeting 3′ UTR of CCND2 mRNA. Additionally, pcDNA3.1-CT-GFP-PDIA3P treatment reversed the growth inhibitory effect of sh-CCND2 in SCC4 and SCC15 cells (Figures 6I and 6J), which demonstrated that PDIA3P facilitated cell growth partially through upregulation of CCND2. Furthermore, we next explored the role of PDIA3P and miR-185-5p on the protein expression of CCND2. Results demonstrated that both miR-185-5p and sh-CCND2 treatment suppressed protein expression of CCND2, whereas both pcDNA3.1-CT-GFP-PDIA3P and pcDNA3.1-CT-GFP-CCND2 treatment significantly enhanced protein expression of CCND2 in SCC4 and SCC15 cells (Figures 6I and 6J), respectively. However, when SCC4 and SCC15 cells were treated with pcDNA3.1-CT-GFP-PDIA3P plus sh-CCND2, the beneficial role of PDIA3P on protein expression of CCND2 was suppressed by knockdown of CCND2, and the negative effect of sh-CCND2 was alleviated by over-expression of PDIA3P (Figures 6I and 6J). These findings suggest that the oncogenic roles of PDIA3P are at least partially mediated by miR-185-5p-CCND2 axis in OSCC.

**DISCUSSION**

IncRNAs can regulate protein-coding genes, transcription, and post-transcription, and play crucial roles in biological processes. Many studies have shown that IncRNAs acted as oncogenes or anti-oncogenes. This study further verified whether PDIA3P can act as an anti-oncogene or oncogene in OSCC. Although the molecular mechanism of PDIA3P in OSCC were demonstrated, the detailed mechanisms were still to be confirmed in the future work. In summary, this study firstly explored the expression, function, and mechanism of PDIA3P in OSCC tissues and cells, which will provide new ideas for the development of OSCC clinical diagnosis and treatment in the future.
Figure 3. PDIA3P Knockdown Suppresses NPC Cell Growth In Vivo
(A and B) Tumor volume in nude mice: SSC4 (A) and SCC15 (B). (C and D) Tumor weight in nude mice: SSC4 (C) and SCC15 (D). Each group contained six mice (n = 6).
(E) Representative images of Ki-67 staining. Scale bar, 100 μm. Assays were performed in triplicate. *p < 0.05. Mean ± SEM is shown. Statistical analysis was conducted using Student’s t test.
tumor-suppressive genes to affect tumorigenesis, metastasis, prognosis, or diagnosis.\textsuperscript{10,19,20} Until now, the effects and mechanisms of lncRNAs on OSCC tumorigenesis and progression are still largely unclear. In the current study, we discovered that the lncRNA PDIA3P expression was higher in OSCC tissues than in corresponding noncancerous tissues. Concurrently, PDIA3P was associated with reduced survival in OSCC patients. We also proved that PDIA3P facilitated cell proliferation in OSCC cells. We also discovered that PDIA3P was a direct target for miR-185-5p. PDIA3P exerted its oncogenic role on OSCC in large part due to its effects to act as a ceRNA for miR-185-5p and subsequently activated the CCND2 signaling pathway. Therefore, we speculated that PDIA3P may play a vital role in the development and progression of OSCC.

To our knowledge, our present work is the first one to directly investigate the association between PDIA3P expression and OSCC. Herein, we found PDIA3P was markedly higher expressed in OSCC tissues than that of their counterparts. We also found that higher expressed PDIA3P corrected with poorer OSCC prognosis, as confirmed by Kaplan-Meier analysis, suggesting that PDIA3P levels might be used as a prognostic biomarker to assist in distinguishing patients with higher risks of OSCC progression. In addition, overexpressed PDIA3P significantly promoted OSCC cell growth in vitro, whereas silencing PDIA3P repressed it. Collectively, our data imply that PDIA3P may function as an oncogene and be favorable to OSCC tumorigenesis and progression.

An increasing number of studies have shown that miRNAs played important roles in regulating biological processes, including cell proliferation, metastasis, apoptosis, angiogenesis, and inflammation, in a variety of tumors.\textsuperscript{18,25} Specifically, many studies have indicated that various miRNAs, such as miR-455-5p,\textsuperscript{25} miR-211,\textsuperscript{31} miR-375,\textsuperscript{26} miR-19a/b,\textsuperscript{27} miR-204,\textsuperscript{32} and miR-187,\textsuperscript{33} were related to the development of OSCC. lncRNAs can function as ceRNA sponges for miRNAs to regulate the degradation of miRNA targets.\textsuperscript{18–20} A handle of lncRNAs have been reported, such as GAS5,\textsuperscript{28,29} NEAT1 [1520906528], XIST,\textsuperscript{30} and CTCAT.\textsuperscript{20} In our study, we selected 7 miRNAs associated with PDIA3P, namely, miR-185-5p, miR-185-375, miR-19a/b, miR-204, miR-187, miR-330-5p, and miR-552-3p, miR-4423-3p, miR-326, and miR-135a-5p, using bioinformatic prediction and qRT-PCR verification. We further validated the RNA expression levels of miRNAs and found that miR-185-5p was lowly expressed in OSCC tissues. We then found that PDIA3P negatively regulated miR-185-5p in OSCC cells, and PDIA3P facilitated cell proliferation via miR-185-5p in OSCC cells.

Having a revelation of the essential effects of miR-185-5p on the suppression OSCC progression, we searched for the potential mechanisms that participated in its function. It has been reported that miR-185 is a novel tumor suppressor that negatively modulates the Wnt/β-catenin pathway in human colorectal cancer.\textsuperscript{30} miR-185-5p regulates cell function Kruppel-like factor 2 (KLF2) in atherosclerosis-related vascular dysfunction.\textsuperscript{35} Zhu et al.\textsuperscript{36} also found that miR-185 inhibited cell proliferation and epithelial-mesenchymal transition in hepatocellular carcinoma by targeting S62. But among all of the predicted target genes for miR-185-5p, we discovered CCND2 functioned as a critical effector of miR-185-5p. CCND2 plays an essential role in regulating progression from the G1-phase to the S-phase by forming complexes in the cytoplasm that will enter the nucleus and destroy cell-cycle suppressive retinoblastoma protein.\textsuperscript{37} A previous study has indicated that overexpression of miR-155 promotes the proliferation and invasion of oral squamous carcinoma cells by regulating BCL6/CCND2.\textsuperscript{37} We confirmed that CCND2 was highly expressed in OSCC tissues, and overexpressed CCND2 facilitated cell proliferation in OSCC cell lines, whereas silencing CCND2 expression suppressed it. Moreover, we predicted CCND2 as a direct target of miR-185-5p at its 3’ UTR miRNA by bioinformatics, which was confirmed by luciferase reporter assays. Our results also demonstrated that miR-185-5p regulated CCND2 at the post-transcription level. Additionally, our data also revealed miR-185-5p could function as a tumor suppressor on OSCC by directly targeting CCND2. Furthermore, our data also revealed that PDIA3P’s oncogenic roles are in part through sponging miR-185-5p and then activating CCND2.

In summary, our results indicated that lncRNA PDIA3P was overexpressed in OSCC and decreased the survival rate of OSCC patients. Moreover, PDIA3P facilitated cell proliferation in OSCC cells via miR-185-5p. A qRT-PCR was then used to screen differentially
expressed miRNAs associated with PDIA3P. Specifically, PDIA3P negatively regulated miR-185-5p in OSCC cells, and silencing PDIA3P by shRNA suppressed CCND2 protein expression via miR-185-5p in OSCC cells. Our study demonstrated that PDIA3P may be a therapeutic target for the treatment of OSCC.

MATERIALS AND METHODS

Ethical Statement and Tissue Collection

58 patients diagnosed as OSCC were involved in this study. All histological diagnoses were performed by the pathology department in the Department of Oncology, Wuhan Pu-Ai Hospital, Tongji Medical College, Huazhong University of Science and Technology. Normal oral mucosal tissues were obtained from non-tumor adjacent tissues or patients with reshaping of gingival tissues. Informed consent was obtained from all subjects. All experimental protocols were approved by the Ethics Committee in Wuhan University and Huazhong University of Science and Technology. None of these patients received any pre-operative chemotherapy or radiotherapy.

Cell Lines and Plasmids

Ten OSCC cell lines (SCC4, SCC9, SCC1, SCC25, TU183, HSU3, FADU, OEC-M1, SNU1041, and SCC15) and the NHOK cell lines were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Cells were routinely cultured as previously described. All cells were cultured at 37°C in a 5% CO2 atmosphere and maintained in 10% fetal bovine serum (FBS) (Kibbutz BeitHaemek, Israel) within 3 months of resuscitation from the frozen aliquots, with lower than 20 passages for each serum (FBS) (Kibbutz BeitHaemek, Israel) within 3 months of resuscitation from the frozen aliquots, with lower than 20 passages for each experiment. Plasmid pcDNA3.1-CT-GFP-CCND2 (NM_001759, with full length of 3’ UTR) and pcDNA3.1-CT-GFP-PDIA3P were prepared by us. RNAi sequences are shown in the Supplemental Information.

Cell Transfection and Stable Cell Lines

Cells were transfected with DNA plasmids using TransFast transfection reagent Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. For screening stable cell lines, 48 hr after transfection, cells were plated in the selective medium containing G418 (1,000 μg/mL, Invitrogen, UK) for the next 4 weeks or so, and the selective media were replaced every 3 days.

Western Blot Analysis

Western blot was performed using the protocol described previously. Briefly, the logarithmically growing cells were washed twice with ice-cold PBS (Hyclone) and lysed in a RIPA lysis buffer. Cells lysates were centrifuged at 12,000 g for 20 min at 4°C after sonication on ice, and the supernatant was separated. After being boiled for 5–10 min in the presence of 2-mercaptoethanol, samples containing cell proteins were separated on a 10% SDS-PAGE and transferred onto a nitrocellulose membranes, then blocked in 10% dry milk-TBST (20 mM Tris-HCL [pH 7.6], 127 mM NaCl, and 0.1% Tween 20) for 1 hr at 37°C. Following three washes in Tris-HCl, pH 7.5, with 0.1% Tween 20, the blots were incubated with 0.2 μg/mL of antibody (appropriate dilution) overnight at 4°C. Following three washes, membranes were then incubated with secondary antibody for 60 min at 37°C or 4°C overnight in TBST. Signals were visualized by enhanced chemiluminescence (ECL). The following primary antibodies were used: rabbit anti-CCND2 (Santa Cruz, CA, USA) and rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Santa Cruz, CA, USA).

qRT-PCR

RNA isolation and qRT-PCR was performed as described previously. Total RNA was extracted from cells using Trizol reagent (Sangon Biotech) according to the manufacturer’s instructions. For miRNAs and IncRNA quantification, RNA was reverse transcribed to cDNA using the PrimeScript RT reagent Kit with gDNA Eraser (Takara). Quantitative real-time PCR was performed using cDNA primers specific for miRNA or IncRNA. The gene GAPDH was used as an internal control. For miRNA quantification, reverse transcription was performed using the Mir-X miRNA First Strand Synthesis Kit (Takara). miRNA-specific 5’ primers and the rRNA 3’ primer was used during quantitative real-time PCR. The gene U6 was used as an internal control. Primer sequences are provided in Table S2. All the real-time PCR reactions were performed using Takara’s SYBR Premix Ex Taq II (TliRNaseH Plus) in Applied Biosystems 7300 Fast Real-Time PCR System. The 2⁻ΔΔCt method was used for quantification, and fold change for target genes was normalized by internal control.

Colony Formation Assay

The treated SCC4 and SCC15 cells were cultured for 14 days, and the colonies were then fixed with methanol and dyed with Giemsa dye solution before counting the colony-forming units.

Luciferase Reporter Assays

Luciferase reporter assays were conducted as previously described.

CCK-8 Assay

Cell growth was measured using the cell proliferation reagent WST-8 (Roche Biochemicals, Mannheim, Germany). After plating cells in 96-well microtiter plates (Corning Costar, Corning, NY) at
Figure 6. PDIA3P’s Oncogenic Activity Is in Part through Negative Regulation of miRNA-185-5p and Then Activation of Cyclin D2 in OSCC Cells

(A) The 3’ UTR of CCND2 harbors one miR-185-5p cognate site. (B) Relative luciferase activity of reporter plasmids carrying wild-type or mutant CCND2 3’ UTR in SCC4 and SCC15 cells co-transfected with miR-NC or miR-185-5p-5p. (C) Relative miR-185-5p expression after transfection with miR-NC and miR-185-5p. (D) Relative CCND2 mRNA expression after transfection with miR-NC and miR-185-5p. (E) Relative cyclin D2 protein expression after transfection with miR-NC and miR-185-5p. (F) The relationship between CCND2 mRNA expression and miR-185-5p expression in OSCC tissues. (G) and (H) mRNA expression of CCND2 (G) and miR-185-5p (H) in OSCC tissues. (I) and (J) Up-statistical analysis of Trypan blue staining: SCC4 (I) and SCC15 (J). Down-protein expression of cyclin D2 in vector, miR-185-5p, pcDNA3.1-CT-GFP-CCND2, miR-185-5p plus pcDNA3.1-CT-GFP-CCND2, pcDNA3.1-CT-GFP-PDIA3P, sh-CCND2, or pcDNA3.1-CT-GFP-PDIA3P plus sh-CCND2-treated SCC4 and SCC15 cells. Assays were performed in triplicate. *p < 0.05. Mean ± SEM is shown. Statistical analysis was conducted using the one-way ANOVA test.
1.0 \times 10^5/well, 10 \muL of CCK-8 was added to each well at the time of harvest, according to the manufacturer’s instructions. 1 hr after adding CCK-8, cellular viability was determined by measuring the absorbance of the converted dye at 450 nm.

**Trypan Blue Staining**

Cell viability was assessed using the Trypan blue (Lanza, Basel, Switzerland) exclusion method. The SCC4 and SCC15 cells were seeded in 24-well culture plates at a density of 3 \times 10^5 cells per well, and the cells were then transfected with Vector, miR-185-5p, pcDNA3.1-CT-GFP-CCND2, miR-185-5p plus pcDNA3.1-CT-GFP-CCND2, pcDNA3.1-CT-GFP-PDIA3P, sh-PDIA3P, or pcDNA3.1-CT-GFP-PDIA3P plus sh-PDIA3P. Each cell suspension was mixed with an equal volume of 0.4% Trypan blue solution, and the living cells were quantified using a hemocytometer. The cells were also counted using a microscope. The data are representative of three independent experiments performed on different days.

**RNA Pull-Down Assays**

PDIA3P transcripts were transcribed using T7 RNA polymerase (Ambio life) in vitro, and then by using the RNase Plus Mini Kit (QIAGEN) and treating with DNase I (QIAGEN). Puriﬁed RNAs were biotin labeled with the Biotin RNA Labeling Mix (Ambio life). Isolated total RNAs were biotinylated with the Biotin RNA Labeling Mix (Ambio life) in vitro, and then by using the RNeasy Plus Mini Kit (QIAGEN) and treating with DNase I (QIAGEN). Puriﬁed RNAs were biotin labeled with the Biotin RNA Labeling Mix (Ambio life). Positive control, negative control, and biotinylated RNAs were mixed and incubated with SCC4 and SCC15 cell lysates. Then, magnetic beads were added to each binding reaction and incubated at room temperature. Finally, the beads were washed, and the eluted proteins were detected by western blot analysis.

**Statistical Analysis**

The results were analyzed using GraphPad Prism (GraphPad, La Jolla, CA, USA) and the SPSS 23.0 software (SPSS, Chicago, IL, USA). The signiﬁcance of differences was assessed using Student’s t test or a one-way ANOVA. All data are expressed as mean ± SEM; p < 0.05 indicates a signiﬁcant difference.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes one ﬁgure and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.omtn.2017.08.015.

**AUTHOR CONTRIBUTIONS**

Participated in research design: C.S., G.L., S.L., L.Z., and D.L.; Conducted experiments: C.S., S.L., and G.L.; Contributed new reagents or analytic tools: C.S., S.L., G.L., F.G., T.B., L.Z., and Y.F.; Performed data analysis: C.S., S.L., and G.L.; Wrote or contributed to the writing of the manuscript: C.S., G.L., S.L., L.Z., and D.L.

**CONFLICTS OF INTEREST**

The authors declare no conﬂict of interest.

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