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Original Article

NCK1 antisense RNA 1 (NCK1-AS1) exerts pro-fibrosis property in oral mucosa through modulation of miR-137/NCK1 axis

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KEYWORDS
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MicroRNA-137;
NCK1

Abstract *Background/Purpose:* Oral submucous fibrosis (OSF) is a premalignant condition of the oral cavity, and its pathogenesis remains largely unknown. A multitude of non-coding RNAs are aberrantly expressed in OSF, and their implication for the development of OSF is a matter meriting investigation.
Materials and methods: The functional role of long non-coding RNA NCK1-AS1 in myofibroblast activation of fibrotic buccal mucosal fibroblasts (fBMFs) derived from OSF tissues was assessed. Wound healing, collagen gel contraction and transwell migration assays have been employed to assess the myofibroblast activities. In addition, a luciferase-based reporter assay was used to illustrate the potential mechanism underlying the regulation of NCK1-AS1 in myofibroblast activation.
Results: Silencing of NCK1-AS1 markedly downregulated myofibroblast activation and the expression of fibrosis markers in fBMFs. Besides, we demonstrated that NCK1-AS1 directly interacted with microRNA-137 (miR-137) and was negatively correlated with it. Moreover, we found that NCK1 was a target of miR-137 and positively related to NCK1-AS1. Our results

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demonstrated that NCK1-AS1 may regulate myofibroblast activation by suppressing miR-137 and upregulating NCK1.
Conclusion: We showed that NCK1-AS1 acted as a sponge of miR-137 and titrated the suppressive effect of miR-137 on NCK1 to modulate myofibroblast activation in OSF condition.
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Introduction

Oral submucous fibrosis (OSF) is a gradually developing fibrosis condition that may eventually become cancerous. It has been indicated that this oral potentially malignant disorder has a 7%—30 % risk of progressing to oral squamous cell carcinoma (OSCC), and the development of OSF involves various biological processes, such as a dysregulation of collagen metabolism, epithelial—mesenchymal transition (EMT), and persistent myofibroblast transdifferentiation.[1] Among various mediators, non-coding RNAs have attracted wide attention in the field of fibrosis treatment as therapeutic targets. Non-coding RNAs are RNA molecules that do not undergo the translation process of protein synthesis, and can be divided into infrastructural (e.g. small nucleolar RNAs) and regulatory non-coding RNAs (e.g. microRNAs and long non-coding RNAs). In fact, several studies have revealed the significance of microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) in the changes of gene regulatory networks that were associated with the accumulation of collagen,[2] EMT,[3] and myofibroblast activation in OSF.[4] One of the key areas of current research interest is the competitive endogenous RNA (ceRNA) hypothesis. It is suggested that these lncRNAs interact with miRNAs via miRNA-binding sites, the miRNA response elements (MREs), and then titrate the effects of miRNAs on their target genes.[5]

Noncatalytic region of tyrosine kinase adaptor protein 1 antisense 1 (NCK1-AS1), also known as NCK1 divergent transcript or SLC35G2-AS1, is a 1073-bp lncRNA located on chromosome 3q22.3.[6] NCK1-AS1 is primarily transcribed in the lymph nodes, spleen, and 25 other tissues. Recently, notable overexpression of NCK1-AS1 has been observed in various tumors, such as cervical cancer,[7] glioma,[8] and esophageal squamous cell carcinoma (ESCC).[9] Besides, the expression level of plasma NCK1-AS1 was upregulated in early-stage OSCC patients and overexpression of NCK1-AS1 promoted cell migration and invasion of OSCC cells.[10] Moreover, our recent work has revealed that the expression of NCK1-AS1 affected the myofibroblast activities of fibrotic buccal mucosal fibroblasts (fBMFs).[11] Given that NCK1-AS1 is implicated in numerous malignant conditions (e.g. OSCC) and myofibroblast activation, whether its dysregulation contributes to the development of precancerous OSF is a topic worthy of investigation.

Herein, we assessed the functional role of NCK1-AS1 in the myofibroblast activities of fBMFs derived from OSF tissues and identified the putative interacting factor of NCK1-AS1 that mediated its property in order to elucidate the molecular basis that underlies the regulatory role of NCK1-AS1 in the development of OSF.

Materials and methods

Cell culture for fibrotic buccal mucosa fibroblasts

Buccal mucosa tissues were obtained from OSF patients and healthy individuals. This is followed by primary cell isolation and culture methods, as described in this reference.[2] The isolated buccal mucosa tissues from surgery were then immediately transported to the laboratory in Hanks' Balanced Salt Solution at 4 °C for further processing. Following trypsinization, the tissues were cultured in DMEM medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10 % fetal bovine serum (FBS) (Thermo Fisher Scientific), and 1 % penicillin-streptomycin cocktail (Thermo Fisher Scientific) and plated into 25-T flasks for 14 days. Spindle-shaped cells that migrated from the tissue explants were identified as buccal fibroblasts. These cells were continuously propagated and used for subsequent experiments between the 3rd and 8th passages. To ensure the integrity of the cell cultures, mycoplasma contamination was checked using short tandem repeat DNA profiling.

Quantitative reverse transcription polymerase chain reaction analysis

RNA extraction and quality control were performed using established protocols.[12] The isolated RNA was then converted into complementary DNA (cDNA) using the Superscript III enzyme. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was carried out on an ABI StepOne™ Real-Time PCR System (Thermo Fisher Scientific) to quantify the expression of NCK-AS1. The primer sequences utilized were as previously described.[11] The expression levels of NCK-AS1 were normalized to GAPDH and calculated using the 2-ΔCt method.[11]

Collagen gel contraction assay

Cells were suspended in a collagen gel solution from Sigma—Aldrich (St. Louis, MO, USA), and seeded into a 24-well plate at 37 °C for 2 h. After gel polymerization, the cultures were incubated in media of 0.5 mL for 48 h. The change in collagen gel size, or contraction index, was measured using ImageJ software. The detail was described in the previous study.[13]

Transwell migration assays

This assay was executed similarly to our previous study.[14] Cells of 1×10^5 were seeded into the top chamber of a transwell (Corning, Acton, MA, USA), and a chemoattractant was added to the bottom chamber. After a 24-h incubation, migrated cells were stained with crystal violet and the numbers of cells in five randomly selected areas were counted.

Western blot analysis

In accordance to our previous study, 20 μg of total protein from whole-cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (Merck, Darmstadt, Germany).[4] Non-specific binding sites were blocked with 5 % bovine serum albumin (BSA) (Merck) before incubating the membrane with primary antibodies at 4 °C overnight. Subsequently, the membrane was exposed to HRP-conjugated secondary antibodies at room temperature for 1 h. The signals formed by antibody binding were envisioned using enhanced chemiluminescence (ECL) substrate (Merck) and taken using the LAS-1000 plus analyzer (GE Healthcare, Piscataway, NJ, USA). GAPDH was used as an internal reference for normalization purposes. All antibodies utilized in this study were bought from Cell Signaling Technology (Danvers, MA, USA).

Lentivirus production and NCK-AS1 knockdown

As described in our previous study, the pLV-RNAi vector (Biosettia Inc., San Diego, CA, USA) was utilized for gene knockdown.[15] Short-hairpin RNA (shRNA) sequences targeting NCK-AS1 were synthesized, cloned into the pLV-RNAi vector as per the manufacturer's instructions, and used to generate lentiviral expression vectors. A pLV-vector targeting luciferase served as the negative control (Sh-Luc). Lentivirus was produced by co-transfecting 293T cells (American Type Culture Collection, VA, USA) with a plasmid DNA mixture containing the lentivector and helper plasmids (VSVG and Gag-Pol), using Lipofectamine 2000 (Invitrogen, Waltham, MA, USA).

miR-137 overexpression

miR-137 mimics or a scramble sequence negative control (miR-Scr.) were transfected into cells using Lipofectamine 2000 to achieve miR-137 overexpression.[16] All oligonucleotides were obtained from Applied Biosystems.

Dual-luciferase reporter assay

The 3′UTR sequence was cloned into the pMIR-REPORT vector (Life Technologies, NY, USA), generating a wild-type reporter construct (Wt). A mutant reporter (Mut) was then created by mutating the predicted miR-137 binding site within the Wt construct. Cells were co-transfected with either the Wt or Mut reporter and miR-137 mimics or a scramble control (miR-Scr) using Lipofectamine 2000. Luciferase activity, normalized to transfection efficiency, served as a measure of reporter activity.[16]

Statistical analysis

Data were analyzed using Statistical Package of Social Sciences (SPSS). Results are expressed as mean ± SD. Statistical significance was determined by Student's t-test, one-way ANOVA with Tukey's post hoc test, or other appropriate tests. $P < 0.05$ was considered significant.[4]

Results

First, we examined the effect of NCK1-AS1 on myofibroblast activation by silencing of NCK1-AS1 in two fBMFs derived from OSF tissues (Fig. 1A). During tissue repair, the activated smooth muscle α-actin (α-SMA)-expressing myofibroblasts migrate to the injured site to synthesize extracellular matrix (ECM) components and generate high contractile forces for wound retraction. Hence, measurement of the decrease in size of a gel matrix can indicate the contractile forces generated by myofibroblasts propagate throughout the collagen matrix. As shown in Fig. 1B, interruption of NCK1-AS1 markedly inhibited the wound healing capacity of fBMFs. Besides, suppression of NCK1-AS1 reduced collagen gel contractility of fBMFs (Fig. 1C) and the expression of fibrosis markers, including alpha smooth muscle actin (α-SMA) and collagen type I alpha 1 chain (COL1A1) (Fig. 1D).

Various studies have shown that NCK1-AS1 exerted its capacities in tumorigenesis and drug resistance in ovarian cancer, glioma, osteosarcoma, gastric cancer, non-small cell lung cancer and laryngeal squamous cell carcinoma via targeting miR-137.[17–21] To assess the direct interaction of miR-137 and NCK-AS1, a luciferase reporter assay was conducted and the binding site between NCK1-AS1 and miR-137 were displayed in Fig. 2A. We observed that the luciferase activity of wild-type (wt)-NCK1-AS was reduced by miR-137 overexpression in fBMFs, while that of mutant (mut)-NCK1-AS1 was unaffected by miR-137 mimics (Fig. 2B). In line with these results, we also found that there was an inverse correlation between NCK-AS1 and miR-137 (Fig. 2C). These findings suggested that miR-137 was negatively regulated by NCK1-AS1.

Furthermore, a couple of studies have shown that non-catalytic region of tyrosine kinase adaptor protein 1 (NCK1) was a downstream target of miR-137 that mediated the oncogenic role of NCK1-AS1.[17,21] It has been shown that the adaptor protein NCK1 interacted with protein tyrosine phosphatase 1B (PTP1B)[22] and played a role in the regulation of the PI3K/Akt pathway, which was implicated in the development of OSF and OSCC.[23,24] To examine whether NCK1-AS1 affected myofibroblast activation via binding with miR-137 to upregulate NCK1, we explored the binding capacity of miR-137 and NCK1 by performing a luciferase reporter assay. The sequences of wild-type (wt)-NCK1 and mutated (mut)-NCK1 were inserted into the luciferase reporter vector followed by co-transfection with miR-137 mimic (Fig. 3A). We showed that miR-137 mimics led to an obvious decline in the luciferase activity of wt-NCK1, but failed to change the relative luciferase activity of mut-NCK1 (Fig. 3B).

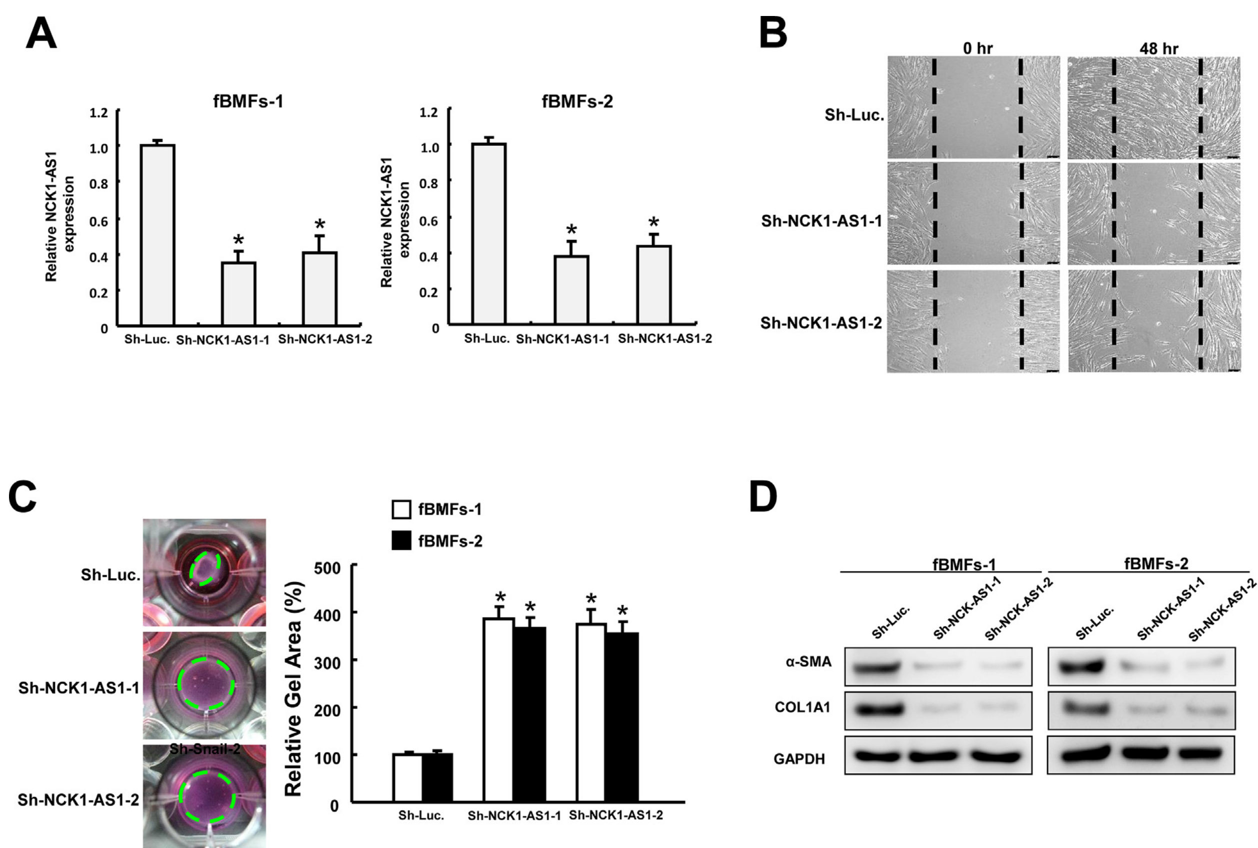

Figure 1 Silencing of NCK1-AS1 diminishes myofibroblast features of fibrotic buccal mucosal fibroblasts (fBMFs). (A) The expression of NCK1-AS1 in two fBMFs derived from OSF tissues was silenced by Sh-NCK1-AS1-1 and Sh-NCK1-AS1-2. (B) Wound healing and (C) collagen gel contraction in fBMFs with Sh-NCK1-AS1-1 and Sh-NCK1-AS1-2. (D) The expression levels of fibrosis markers, including alpha smooth muscle actin (α-SMA) and collagen type I alpha 1 chain (COL1A1), in fBMFs with Sh-NCK1-AS1-1 and Sh-NCK1-AS1-2. * $P < 0.05$ compared to Sh-Luc.

As expected, there was a positive correlation between NCK1-AS1 and NCK1 (Fig. 3C). Moreover, we showed that silencing of NCK1-AS1 resulted in downregulation of NCK1, whereas inhibition of miR-137 blocked it (Fig. 3D). Most importantly, we demonstrated that the reduced myofibroblast activities, including collagen gel contractility (Fig. 4A) and transwell migration capacity (Fig. 4B), in fBMFs with silenced NCK1-AS1 were rebooted in the presence of miR-137 inhibitor. Taken together, these results demonstrated that NCK1-AS1 regulated myofibroblast activation through serving as a sponge of miR-137 to antagonize the inhibitory impact of miR-137 on NCK1.

Discussion

The ceRNA hypothesis was proposed in 2011 and has been proven to be an important mechanism underlying the development of various diseases, including OSF.[5] For instance, lncRNA MetaLnc9,[25] FENDRR,[2] and XIST[4] all have been found to be ceRNAs for miR-143, miR-214 and let-7i, respectively, and contributed to oxidative stress and myofibroblast transdifferentiation. In the present study, we demonstrated that NCK1-AS1 can function as a ceRNA of miR-137 to affect the expression of NCK1 and myofibroblast activities. NCK1-AS1 was aberrantly upregulated in tissues of multiple types of cancers,[8,9] including head and neck squamous cell carcinoma.[21] Furthermore, the plasma NCK1-AS1 was elevated in early-stage OSCC patients, which may lead to higher cell migration and invasion capacities of OSCC cells.[10] Our results showed that NCK1-AS1 not only played an oncogenic role in OSCC, but also possessed fibrosis features during the development of precancerous OSF. Activation of TGF-β1 has long been considered as a crucial event for the pathogenesis of the areca nut-associated OSF,[26,27] and our previous work has revealed the implication of NCK1-AS1 in OSF may be mediated by regulation of TGF-β1 signaling. It has been shown that forced upregulation of NCK1-AS1 resulted in an elevation of TGF-β1 production in ESCC[9] and prostate cancer cells,[28] and here we demonstrated that NCK1-AS1 may play a part in persistent myofibroblast activation through restricting the inhibitory effects of miR-137 on NCK1.

One of the previous studies has shown that miR-137 was downregulated in a panel of 18 OSCC cell lines, and acted as a tumor suppressor miRNA in OSCC silenced by tumor-specific DNA hypermethylation that is located in CpG islands.[29] Moreover, it was reported that the promoter methylation status of miR-137 was associated with overall survival of OSCC.[30] In addition, the malignancy prediction potential from miR-137 promoter methylation status was

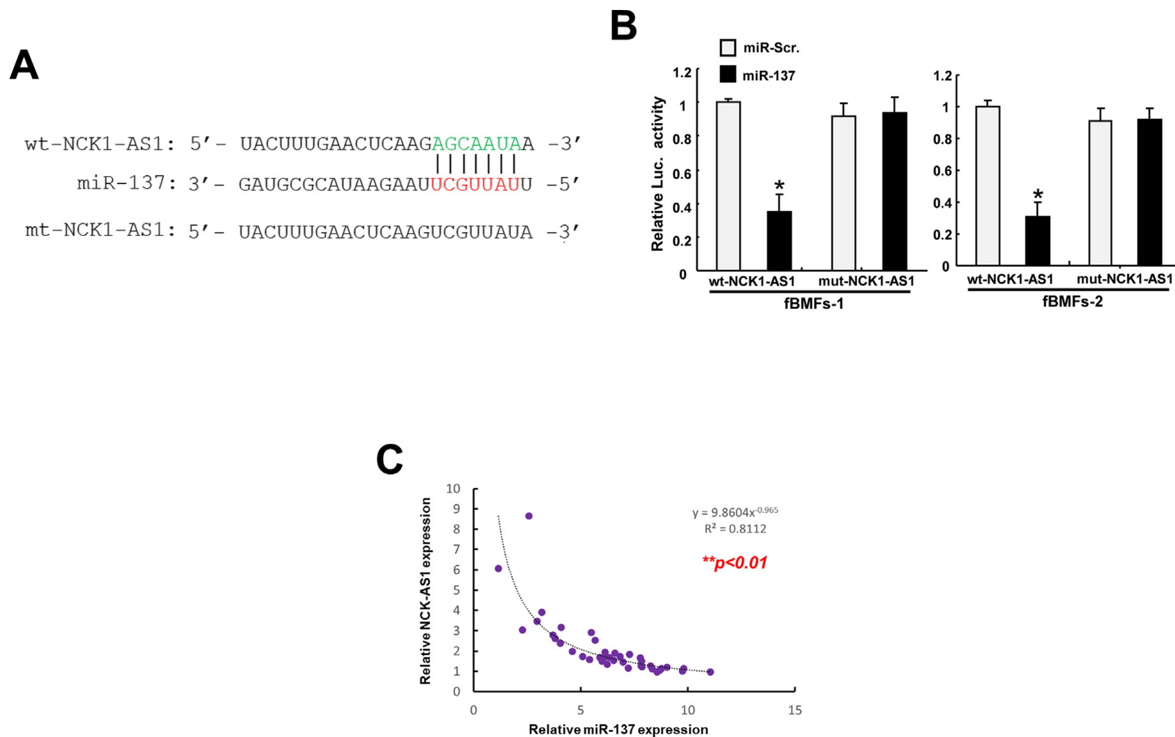

Figure 2 NCK1-AS1 directly binds with miR-137 and negatively regulates it. (A) The predicted binding site between miR-137 and NCK1-AS1 was illustrated. (B) The luciferase reporter assay was designed to confirm the binding relation between miR-137 and NCK1-AS1. (C) An inverse relationship between miR-137 and NCK1-AS1 was observed. * $P < 0.05$ compared to miR-Scramble (miR-Scr.) control.

examined and aberrant promoter methylation of miR-137 may represent an early alteration during the malignancy for patients with oral lichen planus (another potentially malignant lesion).[31] Besides, ectopic expression of miR-137 has been shown to increase the expression of the epithelial biomarker, E-cadherin, and downregulate the mesenchymal biomarker, N-cadherin, vimentin and Snail expression, suggesting that miR-137 may reduce the process of epithelial—mesenchymal transition (EMT).[32] Even though the epigenetic silencing of miR-137 may play a pivotal role during oral carcinogenesis, little is known about the significance of miR-137 in precancerous OSF. It has been revealed that suppression of miR-137-3p further increased cell proliferation of the TGF-β-stimulated mouse hepatic stellate cells and increased the expression of ECM components.[33] In accordance with this finding, we also showed that inhibition of miR-137 promoted myofibroblast activation in fBMFs with sh-NCK1-AS1. Several studies have shown that NCK1-AS1 exerted its capacities in tumorigenesis through regulating miR-137,[17-21] and our results demonstrated that NCK1-AS1 exerted its fibrosis property via sponging miR-137 as well. Additionally, we showed that NCK1 was a putative target of miR-137, which mediated the regulatory effects of NCK1-AS1 on myofibroblast activation.

NCK1, also known as NCK adaptor protein 1, has been found to be composed of one Src homology 2 (SH2)-domain and three SH3 domains[34] and implicate in transducing signals from receptor tyrosine kinases to a variety of downstream recipients that link cell surface receptors with the actin cytoskeleton.[35] NCK consists of two closely related members, NCK1 (Nckα) and NCK2 (Nckβ), that exhibit 68 % amino acid identity with each other.[34] It has been shown that NCK1/2-deficient podocytes displayed defects in actin organization and cell adhesion.[36] Also, the absence of NCK1 disrupted adipogenesis by altering the preadipocyte phenotype to a less committed state, characterized by decreased expression of preadipocyte lineage markers, increased cell proliferation, and heightened collagen production.[37] Currently, there was a limited studies showing the effect of NCK1 on fibrosis and our results revealed that NCK1 may participate in the regulation of NCK1-AS1 on myofibroblast activities. As abovementioned, NCK1 was a direct target of miR-137 that mediated the tumor-promoting role of NCK1-AS1.[17,21] Besides, it was also associated with the regulation of the PI3K/Akt signaling, which was crucial in the development of OSF[23] and OSCC.[24] As such, it is appealing to investigate whether NCK1-AS1 contributes to oral fibrogenesis through the PI3K/Akt signaling via modulation of the miR-137/NCK1 axis in the future. Also, further investigation is warranted to explore the molecular mechanisms by which the increase of NCK1 affects the development of OSF.

In summary, our results showed that silencing of NCK1-AS1 mitigated myofibroblast activation of fBMFs. We demonstrated that NCK1-AS1 may contribute to persistent myofibroblast activities by upregulating NCK1 via interaction with miR-137. Of note, this experiment used only *in vitro* models and it cannot fully replicate the *in vivo* environment. It is necessary to use animal models or patient samples for further validation. On the whole, our data demonstrated that therapeutic approaches to attenuate the expression of NCK1-AS1 in fBMFs may serve as a promising direction for OSF treatment.

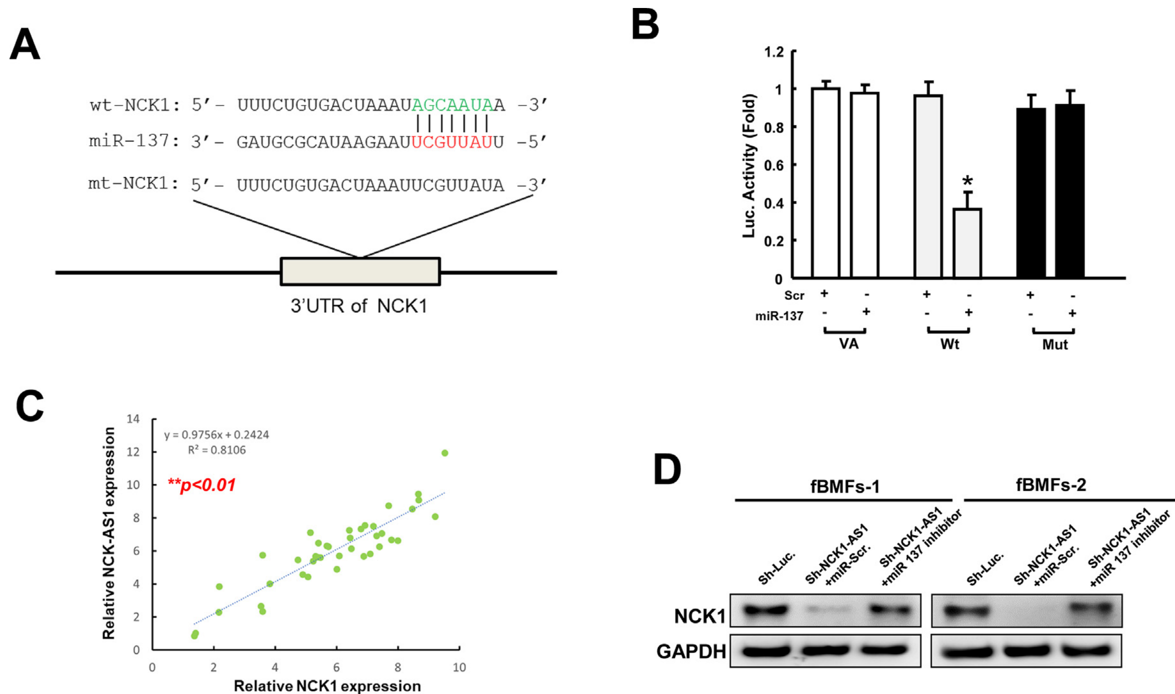

Figure 3 NCK1 is a putative target of miR-137 and positively correlated with NCK1-AS1. (A) The predicted binding sites between NCK1 and miR-137 was indicated. (B) The luciferase reporter assay showed the activity within miR-137 and NCK1 wild type (wt-NCK1) or mutant (mt-NCK1). (C) The expression of NCK1 was positively related to NCK1-AS1. (D) The expression of NCK1 in fBMFs with Sh-NCK1-AS1+ miR-Scr. or Sh-NCK1-AS1+ miR-137 inhibitor. * $P < 0.05$ compared to the control group.

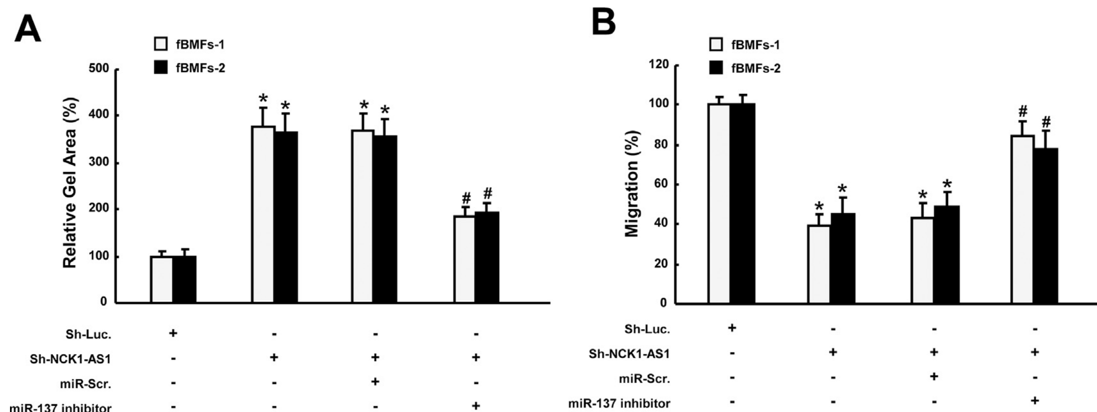

Figure 4 Downregulation of miR-137 is required for the downregulation of myofibroblast activities in fBMFs with sh-NCK1-AS1. (A) Collagen gel contraction and (B) transwell migration assays were applied to examine whether the effect of silencing of NCK1-AS1 on myofibroblast activities was reversed by miR-1137 inhibitor. * $P < 0.05$ compared to the control group. #$P < 0.05$ compared to Sh-NCK1-AS1 group.

Declaration of competing interest

All authors have no conflicts of interest relevant to this article.

Acknowledgments

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