Original Paper

Lnc-SNHG1 Activates the TGFBR2/SMAD3 and RAB11A/Wnt/ β -Catenin Pathway by Sponging MiR-302/372/373/520 in Invasive Pituitary Tumors

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Key Words

Lnc-SNHG1 • TGFBR2 • RAB11A • Wnt/ β -catenin • SMAD3 • Pituitary tumor

Abstract

Background/Aims: Long noncoding RNAs (lncRNAs) are critical regulators in various diseases including human cancer and function as competing endogenous RNAs (ceRNAs) to regulate microRNAs (miRNAs). **Methods:** Quantitative real-time PCR (qRT-PCR) was used to analyze the expression of lnc-SNHG1 and miR-302/372/373/520 in pituitary tumor tissues and cell lines. Cell proliferation was investigated using MTT and cell count assays. The mechanisms by which lnc-SNHG1 affects pituitary tumor progression were investigated using Western blot assays, cell migration assays, immunohistochemistry, immunofluorescence, luciferase reporter assay, tumor xenografts, and flow cytometry. **Results:** We found that lnc-SNHG1 was overexpressed in invasive pituitary tumor tissues and cell lines. Ectopic expression of lnc-SNHG1 promoted cell proliferation, migration, and invasion, as well as the epithelial-mesenchymal transition (EMT), by affecting the cell cycle and cell apoptosis *in vitro* and tumor growth *in vivo*. Further study indicated that overexpression of lnc-SNHG1 markedly inhibited the expression of miR-302/372/373/520 (miRNA-pool) which is down-regulated in invasive pituitary tumor cells. Moreover, overexpression of lnc-SNHG1 significantly promoted the expression of TGFBR2 and RAB11A, the direct targets of miR-302/372/373/520. Finally, lnc-SNHG1 activates the TGFBR2/SMAD3 and RAB11A/Wnt/ β -catenin pathways in pituitary tumor cells via sponging miR-302/372/373/520. **Conclusions:** Our data suggest that lnc-SNHG1 promotes the progression of pituitary tumors and is a potential therapeutic target for invasive pituitary tumor.

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Published by S. Karger AG, Basel

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Introduction

Long noncoding RNAs (lncRNAs) are important new members of the noncoding RNA family, which comprises RNAs longer than 200 bases that do not encode proteins [1]. To date, many lncRNAs have been found to play important roles in the genesis and progression of tumors [2]. For example, altered levels of lncRNAs have been observed in squamous cell cancers [3], gastric cancer [4], gallbladder cancer [5], hepatocellular carcinoma [6], colorectal cancer [7], and pancreatic ductal adenocarcinoma [8], indicating that aberrant expression of certain lncRNAs contributes to carcinogenesis, including pituitary tumors [9].

Invasive pituitary tumors are aggressive and result in high mortality because medical and radiation therapies are either only partially effective or are completely ineffective [10, 11]. Moreover, some pituitary tumors can invade areas outside of the central nervous system such as the intracranial region, which can lead to serious health problems, even death [12]. Therefore, understanding the molecular pathogenesis of the invasive pituitary tumors and discovering novel therapeutic targets would facilitate early detection and improve patient survivals.

Previous studies have shown lnc-SNHG1 is overexpressed in a variety of human cancers, including colorectal carcinoma [13], gastric carcinoma [14], esophageal cancer [15], esophageal cancer [16], and hepatocellular carcinoma [17], and enhances tumor progression through various signaling pathways. However, the important roles and potential functions of lnc-SNHG1 remain unclear in pituitary tumors.

Here, we first investigated the expression level of lnc-SNHG1 in pituitary tumor samples and demonstrated up-regulation of lnc-SNHG1 in invasive pituitary tumor tissues and cell lines. Functional analysis showed that ectopic expression of lnc-SNHG1 promoted cell proliferation by accelerating the cell cycle and inhibiting cell apoptosis both *in vitro* and *in vivo*. In addition, transwell migration analysis revealed that the inhibitory effect of lnc-SNHG1 down-regulation on cell migration and cell invasion occurred by inhibition of EMT in GH1 and RC-4B/C cells. Further, we identified miR-302/372/373/520 (miRNA-pool) as the targets for lnc-SNHG1 using RNA pull-down. In addition, we suggest that RAB11A and TGFBR2 act as targets of this miRNA-pool and play an oncogenic role in pituitary tumor progression by activating the SMAD3 and Wnt/PCP signaling pathways, respectively. Taken together, our results suggest that lnc-SNHG1/miRNA-pool/RAB11A and TGFBR2 axis could be a valuable target for developing therapies against human pituitary tumors.

Materials and Methods

Tissue Samples and Ethics Statement

Human tissue specimens (n=58) were obtained from pituitary tumor patients who provided informed consent, including 48 cases of invasive and 10 cases of non-invasive pituitary tumor, at the China-Japan Friendship Hospital, Jilin University, in Changchun, Jilin, China. No patients had undergone radiotherapy, chemotherapy, or hormone therapy before surgery. Tissue samples were immediately frozen in liquid nitrogen at the time of surgery and stored at -80 °C until RNA extraction. Informed consent for this study was obtained from all patients, and this study was approved by the Research Ethics Committee of Jilin University.

Cell Culture and Cell Transfection

GH1 and RC-4B/C cell lines were purchased from the ATCC cell bank (American Type Culture Collection, Manassas, VA, USA). GH1 was routinely cultured in ATCC-formulated F-12K medium (Invitrogen, Carlsbad, CA, USA) containing 2.5% fetal bovine serum (Gibco, USA), 15% horse serum (Gibco) and was then grown in an incubator at 37 °C with 5% CO₂. RC-4B/C was routinely cultured in Dulbecco's Modified Eagle's medium (Invitrogen) with 4 mM L-glutamine (Sigma-Aldrich Corp., USA) supplemented with 0.01 mM nonessential amino acids (Sigma-Aldrich Corp.), 15 mM HEPES (Sigma-Aldrich Corp.), 0.2 mg/ml bovine serum albumin (Sigma-Aldrich Corp.), 2.5 ng/ml epidermal growth factor (Sigma-Aldrich Corp.) and 10% fetal bovine serum (Gibco). Cells were transfected with various constructs using Lipofectamine® 2000 (Invitrogen).

RNA extraction and RT-PCR assay

Total RNAs from human pituitary tumor tissues and pituitary tumor cells were extracted using a TRIzol® Reagent kit (Invitrogen) according to the manufacturer's protocol. First-strand cDNA was reverse transcribed from total RNA using the TIANScript II cDNA kit (Tiangen, Beijing, China). Real-time PCR (RT-PCR) was performed on an ABI7500 quantitative PCR instrument (Applied Biosystems) with reactions prepared using the SYBR® Green Master Mix kit (Invitrogen, Carlsbad, CA, USA). All reactions, including no template controls, were run in triplicate. The relative fold-changes in transcript levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The sequences of primers used are shown in Table 1.

Plasmid construction

The overexpression plasmids lnc-SNHG1, pri-miR-302, pri-miR-372, pri-miR-373 and pri-miR-520 were constructed. The primers used to amplify inserts by PCR are shown in Table 1. The shRNA sequence for SNHG1 and the sequences for 3'UTR and 3'UTR mutants of TGFBR2 and RAB11A are also shown in Table 1.

MTT assay

Cells were transiently transfected with the indicated plasmids for 24 h and then seeded into 96-well plates at a density of 6×10^3 cells/well in 120 μ l cell culture medium. At 24 h, 48 h, or 72 h after transfection, 12 μ l MTT buffer was added to each well, and then the cells were incubated at 37 °C for another 6 h. The medium was then removed, and the precipitated formazan was dissolved in 100 μ l DMSO, and absorbance of the solution was measured at 570 nm.

Colony formation assay

For the colony formation assay, GH1 and RC-4B/C cells were counted at 24 h after transfection and seeded into 24-well plates at 100 cells/well. The culture medium was replaced every 3 days. After approximately two weeks, colonies were washed with 1x phosphate-buffered saline (PBS), stained with common crystal violet dye, and colonies containing >50 cells were counted.

Transwell migration and invasion assays

Migration and invasion assays were performed using Transwell® chamber inserts. For transwell migration assay, 4×10^4 GH1 or RC-4B/C cells were seeded into the Transwell chamber. For invasion assay, 4×10^4 GH1 or RC-4B/C cells were placed on the upper chamber of each insert, which was coated with 10 μ g Matrigel® (2 mg/ml). The bottom of the insert was incubated in 700 μ l medium containing 20% FBS, and the chamber is then maintained in 5% CO₂ at 37 °C for 48 h. Subsequently, the cells that had migrated were fixed, stained, and counted.

Tumor xenograft model

In short, subcutaneous tumor xenografting was performed by injecting the right flank of nude mice with 1×10^7 cells that had been transfected with the indicated plasmids. After 4 weeks, the mice were sacrificed, and

Table 1. Primers used for reverse transcription and PCR in this study

Gene	Sequence (5'-3')
lnc-SNHG1	qPCR-F: 5'-GGAAGTTACAGGTC-3' qPCR-R: 5'-TGGCTCCCAAGTGTCTTA-3' RT-F: 5'-GTCGTATCCAGTGCAGGGTCCGA GGTGCACCTGGATACGACTCACCA-3'
miR-302	qPCR-F: 5'-TGCCTGAAGTCTTCCATGTT-3' RT-F: 5'-GTCGTATCCAGTGCAGGGTCCGA GGTGCACCTGGATACGACTCACCA-3'
miR-372	qPCR-F: 5'-TGCCTGAAGTCTTCCATGTT-3' RT-F: 5'-GTCGTATCCAGTGCAGGGTCCGA GGTGCACCTGGATACGACTCACCA-3'
miR-373	qPCR-F: 5'-TGCCTGAAGTCTTCCATGTT-3' RT-F: 5'-GTCGTATCCAGTGCAGGGTCCGA GGTGCACCTGGATACGACTCACCA-3'
miR-520	qPCR-F: 5'-TGCCTGAAGTCTTCCATGTT-3' RT-F: 5'-GTCGTATCCAGTGCAGGGTCCGA GGTGCACCTGGATACGACTCACCA-3'
U6	qPCR-F: 5'-TGCCTGAAGTCTTCCATGTT-3' RT-F: 5'-GTCGTATCCAGTGCAGGGTCCGA GGTGCACCTGGATACGACTCACCA-3'
U6	qPCR-F: 5'-TGCCTGAAGTCTTCCATGTT-3' RT-F: 5'-GTCGTATCCAGTGCAGGGTCCGA GGTGCACCTGGATACGACTCACCA-3'
miRNAs	qPCR-F: 5'-TGCCTGAAGTCTTCCATGTT-3' RT-F: 5'-GTCGTATCCAGTGCAGGGTCCGA GGTGCACCTGGATACGACTCACCA-3'
β -actin	qPCR-F: 5'-TGCCTGAAGTCTTCCATGTT-3' RT-F: 5'-GTCGTATCCAGTGCAGGGTCCGA GGTGCACCTGGATACGACTCACCA-3'
Oligo dT	qPCR-F: 5'-TGCCTGAAGTCTTCCATGTT-3' RT-F: 5'-GTCGTATCCAGTGCAGGGTCCGA GGTGCACCTGGATACGACTCACCA-3'
TGFBR2	qPCR-F: 5'-TGCCTGAAGTCTTCCATGTT-3' RT-F: 5'-GTCGTATCCAGTGCAGGGTCCGA GGTGCACCTGGATACGACTCACCA-3'
RAB11A	qPCR-F: 5'-TGCCTGAAGTCTTCCATGTT-3' RT-F: 5'-GTCGTATCCAGTGCAGGGTCCGA GGTGCACCTGGATACGACTCACCA-3'
lnc-SNHG1	F: 5'-GGGGTACCGTCTTCTATTTCTACTGCTCGT-3' R: 5'-CGGGATCCATGTAACAACATTTTATTTTCATC-3'
miR-302	F: 5'-CGGGGTACCGTCTTCTATTTCTACTGCTCGT-3' R: 5'-CCGCTCGAGCCTGTAGCCAAGAAGCTG-3'
miR-372	F: 5'-CGGGGTACCGTCTTCTATTTCTACTGCTCGT-3' R: 5'-CCGCTCGAGCCTGTAGCCAAGAAGCTG-3'
miR-373	F: 5'-CGGGGTACCGTCTTCTATTTCTACTGCTCGT-3' R: 5'-CCGCTCGAGCCTGTAGCCAAGAAGCTG-3'
miR-520	F: 5'-CGGGGTACCGTCTTCTATTTCTACTGCTCGT-3' R: 5'-CCGCTCGAGCCTGTAGCCAAGAAGCTG-3'
TGFBR2	3'UTR-F: 5'-GATCCTAGGATAAGCTGTGTAGCACTTCAAGCTTG-3' 3'UTR-R: 5'-AATTCAGCTTGAAGTGTCTAAGCACTTATCTAG-3'
TGFBR2	3'UTR-m-F: 5'-GATCCTAGGATAAGCTGTGTAGCACTTCAAGCTTG-3' 3'UTR-m-R: 5'-AATTCAGCTTGAAGTGTCTAAGCACTTATCTAG-3'
RAB11A	3'UTR-F: 5'-GATCCCGTGGATAATATTAAAGCACTTAAAGCTTG-3' 3'UTR-R: 5'-AATTCAGCTTGAAGTGTCTAAGCACTTATCTAG-3'
RAB11A	3'UTR-m-F: 5'-GATCCCGTGGATAATATTAAAGCACTTAAAGCTTG-3' 3'UTR-m-R: 5'-AATTCAGCTTGAAGTGTCTAAGCACTTATCTAG-3'

pituitary tumor volumes and weights were measured. All animal experiments were performed with the approval of Jilin University.

Flow-cytometric cell cycle and apoptosis analyses

At 48 h after transfection, transfected GH1 or RC-4B/C cells were harvested by trypsinization and resuspended in cold PBS for analysis. For cell cycle analysis, cells were stained with propidium iodide (PI) according to the manufacturer's protocol. The rate of cell apoptosis was determined using an Annexin V-FITC/PI Apoptosis Detection kit (Nanjing Kaiji Biotechnology Development Co., Ltd., Nanjing, China) according to manufacturer's protocol.

Western blot assay

Cell extracts were washed with 1x PBS buffer, prepared with RIPA buffer supplemented with cocktail, and protein concentrations were quantified using the BCA Protein Assay kit (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's protocols. Equal amount of protein were separated by 10% SDS-PAGE and subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were then blocked with 5% non-fat milk in Tris-buffered saline with Tween®20 (TBST) for about 2 h, followed by incubation with primary antibodies against GAPDH (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), caspase 3 (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA), PARP (Santa Cruz Biotechnology, Inc.), E-cadherin (Wanlei Biotechnology, Shanghai, China), Vimentin (Wanlei Biotechnology, Shanghai, China), ICAM1 (Wanlei Biotechnology, Shanghai, China), TGFB2 (Proteintech, Wuhan, China), RAB11A (Proteintech, Wuhan, China), SMAD3 (Cell Signaling Technology, Danvers, MA), pSMAD3 (Cell Signaling Technology, CA, USA), β-catenin (Cell Signaling Technology, CA, USA), c-myc (Cell Signaling Technology, CA, USA), and cyclin D1 (Cell Signaling Technology, CA, USA) overnight at 4°C. After washing with TBST, the blots were incubated with horseradish peroxidase (HRP) conjugated secondary antibody (Cell Signaling Technology, CA, USA) at 37°C for 1 h. Then, the proteins of interest were visualized using enhanced chemiluminescence (ECL) (Wanlei Biotechnology, Shanghai, China) and densitometric analysis was performed using Gene Analyzer system (Beijing Liuyi Instrument Factory, Beijing, China).

Statistical analysis

Data are presented as the mean ± standard deviation (SD) from at least three independent experiments. Statistical analyses were performed using Student's t-tests, for which $p < 0.05$ indicated a statistically significant difference.

Results

Up-regulated lnc-SNHG1 promotes cell proliferation by accelerating the cell cycle and inhibiting apoptosis in GH1 and RC-4B/C cells

We assessed the expression of lnc-SNHG1 using qRT-PCR assays of RNAs extracted from pituitary tumor tissues and cells. As shown in Fig. 1A, the expression of lnc-SNHG1 was up-regulated in invasive pituitary tumor tissues compared with non-invasive pituitary tumor tissues (Fig. 1A). In addition, the expression level of lnc-SNHG1 was also up-regulated in pituitary tumor cells (Fig. 1B). We also detected the expression levels of lnc-SNHG1 used immunohistochemical *in situ* and the results showed that lnc-SNHG1 was up-regulated in tumor tissues (Fig. 1C). To confirm whether lnc-SNHG1 affects pituitary tumorigenesis, constructs encoding pcDNA3, lnc-SNHG1, pSilencer, or shR-SNHG1 were transfected into GH1 and RC-4B/C cells, and the RNAs encoded by each of these plasmids were effectively expressed (Fig. 1D). We then performed MTT and colony formation assays to assess cell proliferation. MTT assays revealed that overexpression of lnc-SNHG1 promoted GH1 and RC-4B/C cell viability and knockdown of lnc-SNHG1 inhibited GH1 and RC-4B/C cell viability (Fig. 1E and F). Further, colony formation assays also indicated that overexpression of lnc-SNHG1 promoted the proliferation of GH1 and RC-4B/C cells and knockdown of lnc-SNHG1 inhibited the proliferation of GH1 and RC-4B/C cells at 24, 48, and 72 h (Fig. 1G). We analyzed the cell cycle

Fig. 1. Lnc-SNHG1 promotes cell proliferation by accelerating the cell cycle and inhibiting apoptosis. (A) qRT-PCR assays indicate Lnc-SNHG1 levels in the indicated tumor tissues. (B) qRT-PCR assays show Lnc-SNHG1 levels in the indicated cell types. (C) Lnc-SNHG1 levels were detected by hybridization in situ. (D) The efficiency of overexpression and knockdown of Lnc-SNHG1 by each transfected plasmid was determined using qRT-PCR assays. (E, F) The effect of Lnc-SNHG1 on cell viability was determined using MTT assays. (G) Relative colony formation rates of GH1 and RC-4B/C cells under indicated treatments were determined using colony formation assays. (H, I) Flow cytometric cell cycle assays showed that Lnc-SNHG1 increased the proliferation index of GH1 and RC-4B/C cells. (J) Flow cytometric cell apoptosis assays showed that Lnc-SNHG1 overexpression inhibited apoptosis in GH1 and RC-4B/C cells. (K) Hoechst 33258/PI staining assays showed that Lnc-SNHG1 overexpression inhibited apoptosis in GH1 and RC-4B/C cells. (L) Western blot assays show cleaved caspase 3 and PARP protein quantities after transfection with Lnc-SNHG1 or shR-SNHG1 compare with the non-transfected control group in GH1 and RC-4B/C cells. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

distributions of GH1 and RC-4B/C cells using flow cytometry with propidium iodide staining. Compared with control cells, ectopically expressing Lnc-SNHG1 exhibited an accelerated cell cycle and knockdown of Lnc-SNHG1 caused cell cycle arrest in the G0/G1 phase 48 h after transfection (Fig. 1G and I). Flow cytometric apoptosis assays showed that Lnc-SNHG1 overexpression reduced cell apoptosis and SNHG1 knockdown promoted apoptosis in GH1 and RC-4B/C cells (Fig. 1J). In addition, Hoechst 33258/PI staining assay showed that Lnc-SNHG1 overexpression inhibited apoptosis compared to non-transfected controls (Fig. 1K). Furthermore, the noted effect of Lnc-SNHG1 on apoptosis was also confirmed because Lnc-SNHG1 overexpression induced activating cleavage of PARP and caspase3 was induced in GH1 and RC-4B/C cells that overexpressed Lnc-SNHG1 (Fig. 1L).

Up-regulated Lnc-SNHG1 facilitates tumor growth in vivo and promotes cell migration, invasion, and EMT

To investigate the effects of Lnc-SNHG1 on the growth of pituitary tumors *in vivo*, GH1 cells were transfected with pcDNA3, Lnc-SNHG1, pSilencer, or shR-SNHG1 and were subcutaneously injected into nude mice. The average weights of tumors derived from Lnc-SNHG1-transfected GH1 and RC-4B/C cells were significantly greater than those of control groups (Fig. 2A). Our results showed that tumors from the Lnc-SNHG1 transfected GH1 and RC-4B/C cells grew faster than those from the control non-transfected group during the entire tumor growth period and that the Lnc-SNHG1 knockdown inhibited tumor growth (Fig. 2B and C). Further, Ki67 was detected by IHC that revealed significantly increased Ki67 expression in tumors of the Lnc-SNHG1-treated group compared with the control non-

Fig. 2. lnc-SNHG1 facilitates tumor growth and promotes cell migration, invasion, and EMT. (A, B, C) Tumor growth rate significantly increased after treatment with lenti-SNHG1 in GH1 and RC-4B/C cells. (D) Ki67 expression in the indicated tissues as shown by IHC staining. (E, F) Transwell migration and invasion assays show lnc-SNHG1 overexpression promoted cell migration and invasion. (G) qRT-PCR assay indicating mRNA levels of ICAM-1, Vimentin, and E-cadherin after transfection with the indicated plasmids in GH1 and RC-4B/C cells. (H) Western blot assay indicating protein levels of ICAM-1, Vimentin, and E-cadherin after transfection with the indicated plasmids in GH1 and RC-4B/C cells. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

transfected group (Fig. 2D). Transwell migration and invasion assays revealed that ectopic expression of lnc-SNHG1 drastically enhanced GH1 and RC-4B/C cell migration and invasion and that knockdown of lnc-SNHG1 dramatically decreased GH1 and RC-4B/C cell migration and invasion (Fig. 2E and F). To further investigate the regulation of EMT by lnc-SNHG1, the expression of the EMT markers E-cadherin, Vimentin, and ICAM-1 was analyzed by qRT-PCR and western blot assay. Results indicated that Vimentin and ICAM-1 expression was significantly increased by lnc-SNHG1 overexpression but attenuated by SNHG1 knockdown as compared to control non-transfected cells, and that the expression of E-cadherin was attenuated by lnc-SNHG1 overexpression but increased by SNHG1 knockdown in GH1 and RC-4B/C cells (Fig. 2G and H).

lnc-SNHG1 interacts with miR-302/372/373/520 and inhibits their activity

To explore the potential mechanisms underlying the association between lnc-SNHG1 and miR-302/372/373/520, we firstly identified whether miR-302/372/373/520 could interact with lnc-SNHG1 via complementary nucleotide sequences. Using blast prediction at the RegRNA 2.0 database (<http://regRNA2.mbc.nctu.edu.tw/>), we found that lnc-SNHG1 carries potential miR-302/372/373/520 targeting sites (Fig. 3A and B). Next, we sought to examine whether lnc-SNHG1 could influence miR-302/372/373/520 levels in pituitary tumors. We found that miR-302/372/373/520 was significantly down-regulated in GH1 and RC-4B/C cells transfected with lnc-SNHG1 compared with the control non-transfected cells (Fig. 3C and D). In addition, we found that overexpression of miR-302/372/373/520 markedly inhibited the expression of lnc-SNHG1 in GH1 and RC-4B/C cells (Fig. 3E and F). Subsequently, we examined the expression of miR-302/372/373/520 in GH1 and RC-4B/C cells and in pituitary tumor tissues using qRT-PCR. We found that miR-302/372/373/520 was distinctly down-regulated in GH1 and RC-4B/C cells and in the invasive pituitary tumor tissues (Fig. 3G and H). We then performed Pearson correlation analysis to evaluate the relationship between lnc-SNHG1 and miR-302/372/373/520 levels. Interestingly, our data suggest that lnc-SNHG1 levels were significantly negatively correlated with miR-302/372/373/520 levels

Fig. 3. Lnc-SNHG1 interacts with miR-302/372/373/520 and inhibits their activity. (A) Analysis using RegRNA revealed the potential interactions between Lnc-SNHG1 and each miRNA analyzed here. (B) MiRbase showed that these miRNAs share similar seed sequences. (C, D) Relative levels of miRNAs in GH1 and RC-4B/C cells transfected with the indicated plasmids, as determined by qRT-PCR assays. (E, F) Relative Lnc-SNHG1 levels in GH1 and RC-4B/C cells transfected with the indicated plasmids as shown by qRT-PCR assays. (G) Relative miRNA levels in the indicated cells by RT-qPCR assay. (H) Relative miRNA levels in tissues as revealed by qRT-PCR assays. (I) Correlations between Lnc-SNHG1 and miRNA levels in tissues was shown. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

in pituitary tumors (Fig. 3I). Together, our results demonstrate that Lnc-SNHG1 levels are abnormally up-regulated in pituitary tumors and are negatively associated with miR-302/372/373/520 levels.

miR-302/372/373/520 directly target *TGFBR2* and *RAB11A*

By using the online database Targetscan (http://www.targetscan.org/vert_71/) to search for miRNA targets, we noted that *TGFBR2* and *RAB11A* are potential targets of miR-302/372/373/520 (Fig. 4A and B). We then examined whether miR-302/372/373/520 modulate the expression of *TGFBR2* or *RAB11A*. To verify the putative binding site(s) for miR-302/372/373/520 in the 3'UTR of the *TGFBR2* and *RAB11A* genes, we constructed two reporter vectors containing either wild-type *TGFBR2* and *RAB11A* 3'UTRs or mutant *TGFBR2* and *RAB11A* 3'UTRs (Fig. 4C). As shown in Fig. 4D and E, the relative EGFP activity of the *TGFBR2* and *RAB11A* 3'UTR WT vectors was inhibited by miR-302/372/373/520 overexpression in GH1 and RC-4B/C cells, while the EGFP activity of the *TGFBR2* and *RAB11A* 3'UTR mut vectors was not inhibited (Fig. 4F and G). To further confirm the regulation of *TGFBR2* and *RAB11A* by miR-302/372/373/520, qRT-PCR and western blot assays were performed. Our results illustrated that both mRNA and protein levels of *TGFBR2* and *RAB11A* were significantly decreased in GH1 and RC-4B/C cells transfected with pri-miR-302/372/373/520 (Fig. 4H, I, and J). These results demonstrated that *TGFBR2* and *RAB11A* could be directly targeted by miR-302/372/373/520 in GH1 and RC-4B/C cells.

miR-302/372/373/520 inhibits cell proliferation, cell migration and EMT process

The roles of miR-302/372/373/520 in pituitary tumor cells were evaluated by transfection of miR-302/372/373/520 overexpression plasmids into GH1 and RC-4B/C cells (Fig. 5A). An MTT assay indicated that transfection with pri-miR-302/372/373/520

Fig. 4. miRNA-pool (miR-302/372/373/520) can directly target TGFBR2 and RAB11A. (A, B) The predicted miRNA-pool binding sites in TGFBR2 and RAB11A mRNA were revealed using Targetscan. (C) The mutated 3'UTRs of TGFBR2 and RAB11A mRNA are shown. (D, E) GH1 and RC-4B/C cells were co-transfected with either pcDNA3/EGFP-TGFBR2 3'UTR or pcDNA3/EGFP-RAB11A 3'UTR and pri-miRNAs. EGFP intensity was determined spectrophotometrically, and the value of emission at OD488 from transfection with pcDNA3 was set to one. (F, G) GH1 and RC-4B/C cells were co-transfected with either pcDNA3/EGFP-TGFBR2 3'UTR mut or pcDNA3/EGFP-RAB11A 3'UTR mut and pri-miRNAs. EGFP intensity was determined spectrophotometrically, and the emission value at OD488 from transfection with pcDNA3 was set to one. (H, I) TGFBR2 and RAB11A mRNA levels in GH1 and RC-4B/C cells transfected with pri-miRNAs were measured using qRT-PCR assays. (J) TGFBR2 and RAB11A protein level in GH1 and RC-4B/C cells transfected with pri-miRNAs were determined by western blot assays. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

significantly inhibited the proliferation of GH1 and RC-4B/C cells (Fig. 5B). In addition, up-regulation of miR-302/372/373/520 weakened the relative colony formation rate of GH1 and RC-4B/C cells (Fig. 5C). miR-302/372/373/520 significantly inhibited the proliferation of GH1 and RC-4B/C cells. We hypothesized that miR-302/372/373/520 might regulate the cell cycle in pituitary tumor cells. The addition of pri-miR-302/372/373/520 blocked the cell cycles of GH1 and RC-4B/C cells, as shown by flow cytometric cell cycle analysis (Fig. 5D). Further, the cell proliferation index of GH1 and RC-4B/C cells treated with pri-miR-302/372/373/520 markedly decreased (Fig. 5E). To investigate whether apoptosis took part in the negative effect of miR-302/372/373/520 on cell proliferation, the relative apoptosis rate of GH1 and RC-4B/C cells were analyzed by flow cytometric apoptosis assays. Figure 5F shows that the relative apoptotic rate of GH1 and RC-4B/C cells was significantly higher in the miR-302/372/373/520-treated group than in the control groups. In addition, Hoechst 33258/PI staining assays showed that overexpression of miR-302/372/373/520 promoted apoptosis compared to the control group (Fig. 5G). Further, transwell migration assays showed that cell migration decreased upon treatment of GH1 and RC-4B/C cells with pri-miR-302/372/373/520 (Fig. 5H). Finally, we found that the levels of the mesenchymal markers Vimentin and ICAM-1 became significantly decreased after overexpression of miR-302/372/373/520. In contrast, the level of the epithelial marker E-cadherin increased in cells overexpressing miR-302/372/373/520 compared with the control group (Fig. 5I).

Lnc-SNHG1 activates the Wnt/ β -catenin and TGFBR2/SMAD3 pathway by inhibiting the miR-302/372/373/520

qRT-PCR and western blot assays were used to explore whether TGFBR2 and RAB11A are regulated by Lnc-SNHG1. As shown in Fig. 6A and B, overexpression of Lnc-SNHG1

Fig. 5. miRNA-pool (miR-302/372/373/520) inhibits cell proliferation, cell migrations and EMT. (A) qRT-PCR assays revealed relative miRNA levels in GH1 and RC-4B/C cells transfected with the indicated plasmids. (B) The effect of miRNA-pool on cell viability was determined using MTT assays. (C) Relative colony formation rates of GH1 and RC-4B/C cells with indicated treatments were determined by colony formation assays. (D, E) Flow cytometric cell cycle assays showed that miRNA-pool decreased the proliferation index of GH1 and RC-4B/C cells. (F) Flow cytometric apoptosis assays showed that miRNA-pool overexpression promoted the apoptosis of GH1 and RC-4B/C cells. (G) Hoechst 33258/PI staining assays showed that miRNA-pool overexpression promoted inhibited the apoptosis of GH1 and RC-4B/C cells. (H) Transwell migration assays showed that miRNA pool overexpression promoted cell migration. (I) Western blot assays showed the protein levels of ICAM-1, Vimentin, and E-cadherin in GH1 and RC-4B/C cells after transfection with pri-miRNAs. **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

increased both mRNA and protein levels of TGFBR2 and RAB11A, while inhibition of lnc-SNHG1 reduced the mRNA and protein levels of TGFBR2 and RAB11A (Fig. 6A and B). TGFBR2 and RAB11A reportedly activate the SMAD3 and Wnt/ β -catenin signaling pathways, respectively, driving tumorigenesis [18, 19]. Considering the regulatory effect of lnc-SNHG1 on TGFBR2 and RAB11A expression, we attempted to further elucidate the role of lnc-SNHG1 in the SMAD3 and Wnt/ β -catenin signaling pathway. Our results showed that overexpression of lnc-SNHG1 significantly increased pSMAD3 and nSMAD3 protein levels, but co-transfection with lnc-SNHG1 and miR-302/372/373/520 (miRNA-pool) partially rescued it in GH1 and RC-4B/C cells (Fig. 6C). Additionally, we examined the distribution of SMAD3 protein by immunofluorescence assay in GH1 cells and found that overexpression of lnc-SNHG1 significantly increased the nuclear distribution of SMAD3 but co-transfection with lnc-SNHG1 and miRNAs pool partly rescued it (Fig. 6D). Western blot assays were also used to detect downstream effectors of the Wnt signaling pathway in cells transfected with the indicated plasmids. As shown in Fig. 6E, the expression of A- β -catenin, c-myc, and cyclin D1 were increased upon lnc-SNHG1 overexpression (Fig. 6E). IF assays showed that overexpression of lnc-SNHG1 significantly increased the nuclear distribution of β -catenin, but co-transfection with lnc-SNHG1 and miRNA pool partly rescued it in GH1 cells (Fig. 6F). Finally, TOP/FOP luciferase reporter assays were also performed and showed that lnc-SNHG1 overexpression increased the TOP/FOP flash ratio, but co-transfection with lnc-SNHG1 and miRNA-pool partly rescued it in GH1 and RC-4B/C cells (Fig. 6G).

Fig. 6. Lnc-SNHG1 activates the Wnt/ β -catenin and TGFBR2/SMAD3 pathways by inhibiting the miR-302/372/373/520. (A) qRT-PCR assays indicate TGFBR2 and RAB11A mRNA levels transfected with the indicated plasmids. (B) Western blot assays show protein levels of TGFBR2 and RAB11A transfected with the indicated plasmids. (C) Western blot assays showed pSMAD3, nSMAD3 and SMAD3 protein levels in GH1 cells transfected with the indicated plasmids. (D) IF assays reveal the distribution of SMAD3 in GH1 cells transfected with the indicated plasmids. Original magnification are 1000 $\times$. (E) Western blot assays indicate that the protein levels of A- β -catenin, c-myc, and cyclin D1 in GH1 cells transfected with the indicated plasmids. (F) IF assays reveal the distribution of β -catenin in GH1 cells transfected with the indicated plasmids. Original magnification are 1000 $\times$. (G) TOP/FOP luciferase reporter assay was performed to detect β -catenin activity. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Discussion

Increasing evidence suggests that lncRNAs are involved in carcinogenesis and the progression of invasive pituitary tumors [20]. For example, the expression of lnc-MEG3 gradually decreased while the expression of lnc-HOTAIR gradually increased in invasive pituitary adenomas, indicating that lnc-MEG3 and lnc-HOTAIR are potential diagnostic markers of invasive non-invasive pituitary adenoma [21]. Recently, Liu et al. reported that up-regulation of lnc-SNHG1 enhances cell proliferation, migration, and invasion in cervical cancer [22]. Furthermore, Liu et al. reported that lnc-SNHG1 promotes tumorigenesis and cancer progression by regulating transcription of both proximal and distal genes in multiple types of cancer [23]. However, the roles of lnc-SNHG1 in human pituitary tumor have not yet been elucidated. We found that lnc-SNHG1 is up-regulated in invasive pituitary tumor tissues and cells. The up-regulation of lnc-SNHG1 promotes cell proliferation, cell migration, cell invasion, and EMT by accelerating the cell cycle and inhibiting apoptosis *in vitro*, and also facilitates tumor growth by increasing Ki67 expression *in vivo*.

In recent years, many studies have reported that lncRNAs could function as a competing endogenous RNAs (ceRNAs) to regulate the functions of microRNAs (miRNAs) in human disease processes, included many cancers [24, 25]. Cui et al. reported that miR-101-3p could act as a target of lnc-SNHG1 in non-small cell lung cancer (NSCLC) and that the inhibition of NSCLC progression induced by lnc-SNHG1 knockdown required the activity of miR-101-3p [26]. In the present study, we identified miR-302/372/373/520 as the inhibitory targets of lnc-SNHG1 by nucleotide sequence complementarity analysis and functional assays. In addition, Pearson correlation analysis showed a remarkably negative correlation between lnc-SNHG1 expression and miR-302/372/373/520 expression in the invasive pituitary tumor tissues. Furthermore, we found that miR-302/372/373/520 overexpression could inhibit cell proliferation by blocking the cell cycle and promoting apoptosis and could inhibit

the EMT by weakening cell migration in GH1 and RC-4B/C cells.

Wnt/ β -catenin signaling is known to regulate a broad range of cellular processes, such as cell proliferation, cell invasion, and cell differentiation by regulating functions of the multi-functional β -catenin protein, a crucial signaling molecule in the Wnt/ β -catenin pathway [27]. Yu et al. have reported that RAB11A overexpression could enhance β -catenin/T-cell factor (TCF) transcriptional activity with a

corresponding change in the expression of Wnt target genes including cyclin D1, cyclin E, MMP7, and c-myc in pancreatic cancer [18]. In the present study, we demonstrated that RAB11A could act as a target of miR-302/372/373/520 and found that lnc-SNHG1 played an oncogenic role in pituitary tumor progression by activating the RAB11A/Wnt/ β -catenin signaling pathway by regulating the expression of A- β -catenin, cyclin D1. TGF- β /SMAD3 signaling is a major contributor to the development of many diseases, including renal fibrosis [28], hepatic fibrosis [29], glioblastoma [30], and pulmonary fibrosis [31]. Here, we have demonstrated that lnc-SNHG1 could regulate the TGFBR2/SMAD3 pathway by competitively inactivating miR-302/372/373/520 in GH1 and RC-4B/C cells. Furthermore, we showed that miR-302/372/373/520 can directly target the 3'UTR of TGFBR2 mRNA.

Taken together, our results reveal an oncogenic function of lnc-SNHG1 in invasive pituitary tumors. Overexpression of lnc-SNHG1 up-regulated TGFBR2 expression and thus increased the activity of the TGF/SMAD3 signaling pathway. Overexpression of lnc-SNHG1 also up-regulated RAB11A expression and activated the Wnt/ β -catenin pathway (Fig. 7A and B). Further, lnc-SNHG1 interacted with miR-302/372/373/520 (miRNA-pool) and inhibited their activity. These findings will help us better understand the molecular mechanisms of the carcinogenesis of invasive pituitary tumors and might have potential diagnostic and therapeutic value in the future.

Acknowledgements

This project was supported by Jilin Scientific and Technological Development Program (No. 2017022309).

Disclosure Statement

The authors declare to have no conflict of interests.

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Fig. 7. Model of regulation mediated by lnc-SNHG1. A) lnc-SNHG1 interacts with miR-302/372/373/520 and p-SMAD3. B) lnc-SNHG1 activates the SMAD3 and Wnt pathways by regulating the miRNA-pool and their target genes.

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