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Original Article

miR-132 and miR-212 cluster function as a tumor suppressor in thyroid cancer cells by CSDE1 mediated post-transcriptional program

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Abstract: microRNAs (miRNAs) are small non-coding RNA molecules which have been reported to be associated with the development of cancers. However, the role of miRNAs in thyroid cancer remains unclear. Here, we identified that miR-132/212 cluster as tumor suppressor in thyroid cancer. Overexpression or knockdown of miR-132/212 in thyroid cancer cells resulted in inhibited or enhanced proliferation. Furthermore, *CSDE1* was identified as the direct and functional target of miR-132/212. Knockdown of *CSDE1* expression upregulated PTEN expression and inhibits AKT activation. Suppressed proliferation was also observed in *CSDE1* inhibition cells. Moreover, overexpression of *CSDE1* reversed miR-132/212 mediated proliferation suppression. In summary, our findings highlight the importance of miR-132/212 as tumor suppressor in thyroid cancer by directly targeting *CSDE1*.

Keywords: CSDE1, miR-132, miR-212, proliferation, thyroid cancer

Introduction

Thyroid cancer is the most common malignant tumor of endocrine organs [1]. The worldwide incidence of thyroid cancer has been going up steadily and has almost tripled over the past 3 decades in the US and all over the world [2]. The estimated new cancer cases of the disease are 64,300 and accounts for approximately 3% of all new diagnosed cases in the US [3]. With the development and application of next generation sequencing (NGS) technology, the profile of thyroid cancer genetic change has been discovered. Just like other cancers, thyroid cancer initiate and progression occurs through accumulation of multiple genetic and epigenetic alterations. The most important changes are MAPK and PI3K-AKT pathway [4].

MicroRNAs (miRNAs) are small non-coding RNAs which function in RNA silencing and post-transcriptional regulation of gene expression by binding 3' untranslated region (3'UTR) of mRNAs. Dysregulated expression of oncomiRs and tumor suppressor miRNAs has been found to participate the process of tumor progression [5]. Many miRNAs have been found to be deregulated in thyroid cancer, such as miR-146b, miR-221 and miR-222 in papillary carcinomas, miR-197, miR-346 and miR-155 in follicular carcinomas and miR-30d, miR-125b, miR26a and miR-30a-5p in anaplastic carcinomas [1]. However, our understanding of miRNAs in thyroid cancer is just at beginning.

miR-132 and miR-212 are tandem miRNAs sharing close sequences highly conserved. Deregulation of miR-132/212 cluster is associated with Alzheimer's disease and tauopathies [6]. In malignant disease, miR-132/212 cluster exhibits controversial biological functions in different genetic context. miR-132/212 promotes gastric and pancreatic cancer cells proliferation and tumor growth, while in lung and ovarian cancer, miR-132/212 suppresses tumor cells migration and invasion [7-11].

Cold shock domain containing E1 (CSDE1), also known as upstream-of-N-Ras (UNR), is a conserved RBP containing five cold-shock domains (CSDs) that bind single-stranded RNA. Recent study indicates CSDE1 drives a post-transcrip-

tional program to promote melanoma invasion and metastasis [12]. However, the role of CSDE1 in thyroid cancer has not been characterized.

In the current study, we explored the functional role of miR-132/212 cluster in thyroid cancer cells and established a link between miR-132/212 and CSDE1 mediated PI3K-AKT pathway activation in the disease.

Materials and methods

Cell culture, transfection and reagents

Thyroid cancer cell lines were obtained from Chinese Academy of Sciences and maintained according to their recommendations. Transient transfection was performed using the Lipofectamine 2000 (Invitrogen). Antisense oligonucleotides were purchased from Sigma Aldrich. ASO-miR132 (5'-AGUAACAAUCGAAAGCCACGGU-3'), ASO-miR212 (5'-AGUAAGCAGUCUAGAGCCAAGGU-3'), ASO-NC (5'-CAGUACUUUUGUGUAGUACAA-3').

miRNA target prediction

miRNA target analysis of miR-132 and miR-212 was applied using the miRanda, TargetScan and PicTar algorithms. The functions of predicated targets were taken into consideration.

Lentivirus infection

Lentivirus of pGLV-miR132, pGLV-miR212 and pGLV-vector was obtained from Shanghai Genepharma Co., Ltd. Lentivirus of pLKO.1 CSDE1-1, pLKO.1 CSDE1-1 and pLKO.1 scramble was obtained from Shanghai Genepharma Co., Ltd. The targets of pLKO.1 CSDE1-1 or pLKO.1 CSDE1-1 are 5'-CACTAATGAAGCCCGAGAAAT-3' and 5'-CTGTAAGTGCTCGCAACATTA-3', respectively. Virus supernatant was incubated on target cells for 12 hours with 5 μg/ml polybrene, following the manufacturer's instructions. Infected cells were selected in puromycin, as optimized for each cell line.

Luciferase reporter assay

Cells of 80% confluence in 24-well plates were transfected using Lipofectamine 2000 reagent (Invitrogen). Firefly luciferase reporter gene construct (200 ng) with wildtype or mutant 3'-UTR of CSDE1 and 1 ng of the pRL-SV40 Renilla luciferase construct (for normalization) were cotransfected per well. Cell extracts were prepared 24-48 h after transfection, and the luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega).

CCK8 assay

Thyroid cancer cells (3,000 cells/well) were placed in 96-well plates. At 24 h following treatment, the cells were continually cultured for 24-72 h. At Day 0, 1, 2, 3, 4 and 5, 10 μl of CCK8 reagent was added to each well. The cells were incubated at 37°C for another 2 h, after shaking for 20 min, the absorbance was detected at 450 nm on a μ Quant Universal Microplate Spectrophotometer (Bio-Tek Instruments, Winooski, USA).

Colony formation assay

Thyroid cancer cells infected or transfected with indicated plasmid or lentivirus were trypsinized and 2000 cells were placed in a 6-well plate. The cells then were cultured for 10-14 days with medium replaced every 2 days. Cells were stained for 10 minutes with 0.05% crystal violet. Cells in five random fields of view at ×100 magnifications were counted and expressed as the average number of cells per field of view. All assays were performed in triplicate.

BrdU incorporation assay

BrdU incorporation assay were performed as an indicator of proliferation. Cell Proliferation ELISA Kit (Roche) was applied following the manufacture's instruction. All assays were performed in triplicate.

mRNA extraction and RT-qPCR assay

For total RNA extraction, samples were processed using the RNeasy Mini Kit (Qiagen) according to manufacturer's procedure. 1 μg of RNA was reverse-transcribed using M-MLV Reverse Transcriptase and oligo-dT primers (Invitrogen). miRNAs were reverse transcribed to generate cDNA using stem-loop reverse transcriptase (RT) primers. Target genes and controls were analyzed by qRT-PCR using SYBR® Select Master Mix (Life Technologies). GAPHD and U6 snRNA were used as endogenous control for mRNA and miRNA; fold changes were calculated using the ΔΔ Ct method in Microsoft Excel. Primer sequences were CSDE1, TTTG-

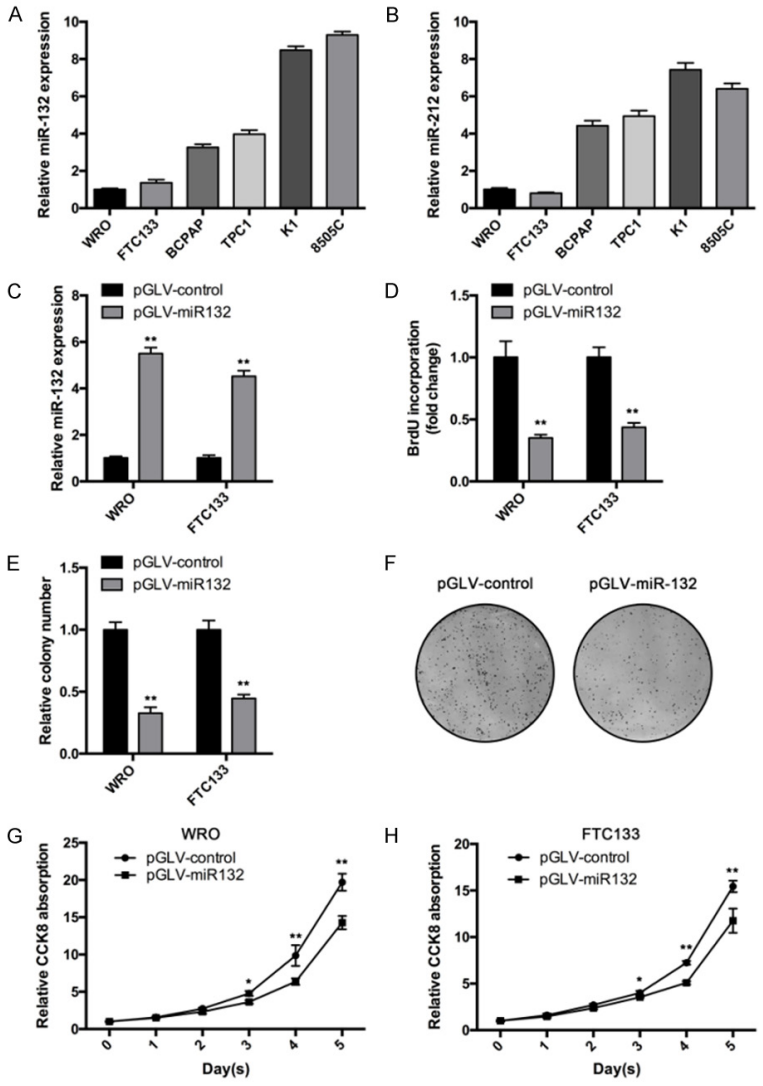

Figure 1. miR-132 suppresses thyroid cancer cells proliferation in vitro. A and B. miR-132 and miR-212 expression in six thyroid cancer cell lines was determined by qRT-PCR assay. C. WRO and FTC133 cells were infected with expressing or control lentivirus. Then expression level of miR-132 was determined by RT-qPCR assay. D. WRO and FTC133 cells overexpressing miR-132 or control cells were subjected to BrdU incorporation assay to determine the cell proliferation. E and F. WRO and FTC133 cells overexpressing miR-132 or control cells were subjected to colony formation assay. F. Representative graphs of WRO cells in colony formation assay. G and H. WRO and FTC133 cells were infected miR-132 expressing or control lentivirus. Then cell proliferation curve was determined using CCK8 reagent. Bars, mean of each group ± SD (n = 3). *, $P < 0.05$, **, $P < 0.01$.

ATCCAAACCTTCTCCACAA and CAATAACCCCAGTTTCACGCA, GAPDH, GGAGCGAGATCCCTCCAAAAT and GGCTGTTGTCATACTTCTCATGG, miR-132, ACCGTGGCTTTCGATTG and GGTCCAGTTTTTTTTTTTTTTTAGTAAC, miR212, GCAGACCTTGGCTCTAGAC and TCCAGTTTTTTTTTTTTTTAGTAAGCA, U6, GTCGTATCCAGTGCAGGGTCCGAGGTATT and CGCACTGGATACGACAAAATATGGAAC.

Western blot assay

Whole-cell extracts were obtained by lysis of cells in an appropriate volume of ice-cold radioimmunoprecipitation assay (RIPA) buffer. Nuclear protein was extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) following the manufacturer's instructions. Cell lysates were separated on 10% SDS denatured polyacrylamide gel electrophoresis (PAGE) gels, transferred to nitrocellulose membranes and blocked in phosphate-buffered saline/Tween-20 containing 5% nonfat milk. Membranes were probed with dilutions of primary antibodies followed by incubation with HRP-conjugated secondary antibodies. After extensive washing, proteins were visualized by enhanced chemiluminescence and exposure to film (Fujifilm, Tokyo, Japan). Anti-CSDE1 antibody was purchased from Abcam, anti-PTEN, EKR1/2, p-ERK1/2 and anti-p-AKT antibodies were from Cell signaling, anti-β-actin antibody was from Sigma Aldrich.

Statistical analysis

Experimental results are expressed as mean ± standard deviation (SD). Statistically significant differences between groups were determined using a two-tailed unpaired Student's t-test; $P < 0.05$ was considered significant.

Results

miR-132 suppresses thyroid cancer cells proliferation in vitro

To investigate miR-132/212 cluster's role in thyroid cancer, we first examined their expression in six thyroid cancer cell lines. As shown in **Figure 1A**, miR-132 showed high expression

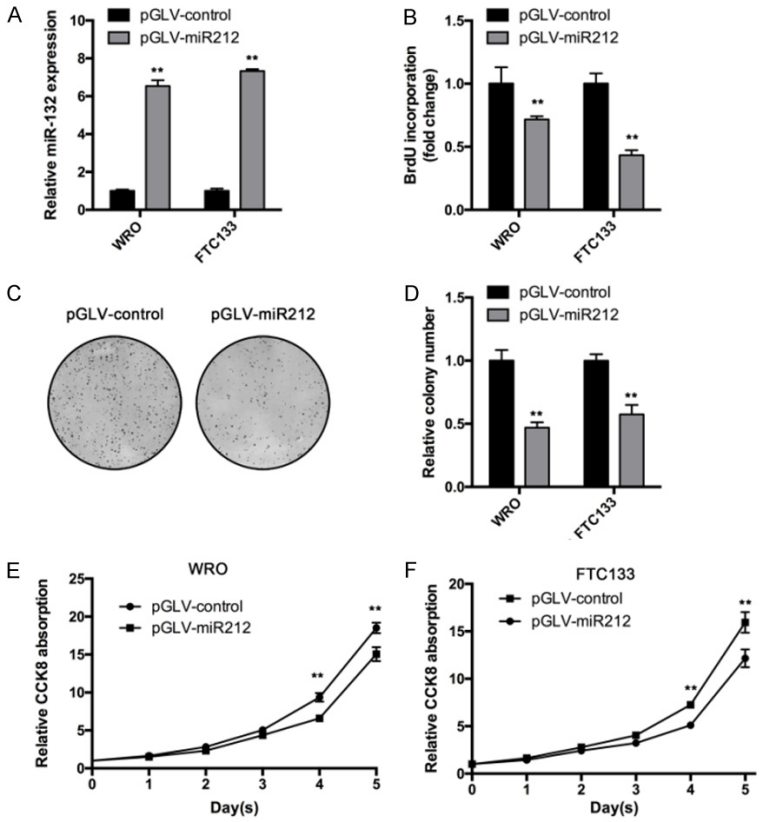

Figure 2. miR-212 suppresses thyroid cancer cells proliferation in vitro. A. WRO and FTC133 cells was infected with miR-212 expressing or control lentivirus. Then expression level of miR-212 was determined by RT-qPCR assay. B. WRO and FTC133 cells overexpressing miR-212 or control cells were subjected to BrdU incorporation assay to determine the cell proliferation. C and D. WRO and FTC133 cells overexpressing miR-212 was subjected to colony formation assay. D. Representative graphs of WRO cells in colony formation assay. E and F. WRO and FTC133 cells were infected with lentivirus expressing miR-212. Then cell proliferation curve was determined using CCK8 reagent. Bars, mean of each group ± SD (n = 3). **, $P < 0.01$.

level in K1 and 8505C cells, and low expression level in WRO and FTC133. Interestingly, miR-212 showed similar expression pattern in these cell lines (**Figure 1B**). These results indicate miR-132 and miR-212 could share similar effector. Then we chose WRO and FTC133 cells for further investigation because of their low level of miR-132 expression. Using lentivirus vector, we stably overexpressed miR-132 in WRO and FTC133 cells. qRT-PCR assay was performed to confirmed the expression (**Figure 1C**). Then we used thymidine analog BrdU (5-bromo-2' deoxyuridine) measure cell proliferation. miR-132 significantly reduced BrdU incorporation of WRO cells (**Figure 1D**). Similar results were obtained from FTC133 cells. miR-132 reduced BrdU incorporation in FTC133 cells (**Figure 1D**). In consistent with these data, miR-132 overexpressing cells produced less colonies compared with control cells (**Figure 1E** and **1F**). Cell Counting Kit-8 (CCK-8) assay is a sensitive colorimetric assay for the determination of cell viability in cell proliferation and cytotoxicity assays. CCK8 assay showed that miR-132 overexpressing cells proliferated slower than control cells (**Figure 1G** and **1H**). Taken together, these results indicate that miR-132 inhibits cell proliferation in thyroid cancer.

miR-212 suppresses thyroid cancer cells proliferation in vitro

To further investigate whether miR-212 has similar effects in thyroid cancer, we enhanced miR-212 expression in WRO and FTC133 cells by lentivirus. Overexpression efficiency was determined by qRT-PCR assay (**Figure 2A**). Then we performed BrdU assay. In agreement with miR-132, miR-212 suppressed BrdU incorporation in WRO and FTC133 cells (**Figure 2B**). In addition, results from colony formation assay showed that miR-212 reduced colony numbers of WRO and FTC133 cells (**Figure 2C**). Cell proliferation was also impacted by miR-212 overexpression. WRO and FTC133 cells infected with miR-212 expressing lentivirus showed suppressed CCK8 absorption at Day 4 and 5. Taken together, these results indicate that miR-212 showed similar biological function of miR-132 that inhibits thyroid cancer proliferation.

Inhibition of miR-132/212 promotes thyroid cancer cell proliferation in vitro

To validate the miR-132/212's role in thyroid cancer cells, we inhibited their expression in miR-132/212 high expression cells, K1 and 8505C. RT-qPCR assay was performed to confirm the downregulation (**Figure 3A** and **3B**). In consistent with previous results, miR-132 inhi-

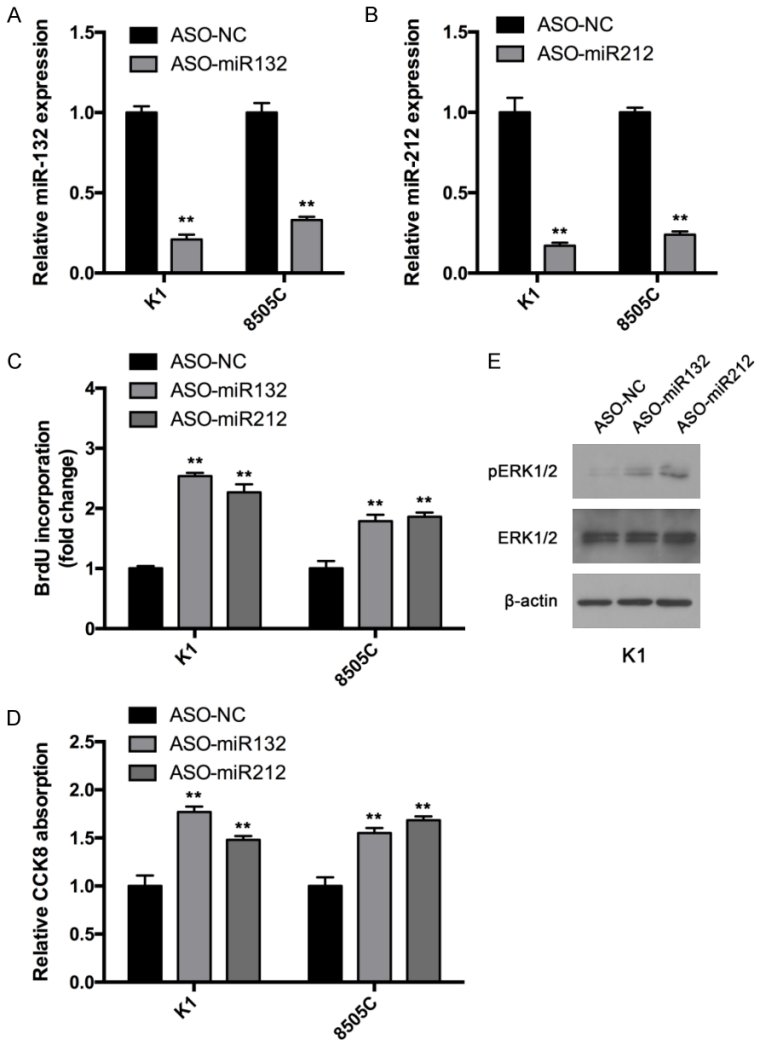

Figure 3. Inhibition of miR-132/212 promotes thyroid cancer cell proliferation in vitro. A. K1 and 8505c cells were transfected with ASO-miR132 or ASO-NC. Then miR-132 expression was determined by RT-qPCR assay. B. K1 and 8505c cells were transfected with ASO-miR212 or ASO-NC. Then miR-132 expression was determined by RT-qPCR assay. C. K1 and 8505c cells were transfected with ASO-miR132/212 or ASO-NC. 48 hours later, BrdU incorporation was determined. D. K1 and 8505c cells were transfected with ASO-miR132/212 or ASO-NC. 48 hours later, CCK8 absorption was determined. E. p-ERK1/2 and total ERK1/2 expression was determined by western blot assay. Bars, mean of each group ± SD (n = 3). **, $P < 0.01$.

bition significantly enhanced BrdU incorporation in K1 cells. Similar results were obtained from 8505C cells (**Figure 3C**). miR-212 inhibition also promoted BrdU incorporation in both K1 and 8505C cells (**Figure 3C**). In addition, CCK8 assay showed that downregulation of miR-132 in K1 and 8505C cells increased CCK8 absorption compared with control (**Figure 3D**). Inhibition of miR-212 obtained similar results (**Figure 3D**). Furthermore, we also examined ERK activation. Result showed that inhibition of miR-132 and miR-212 enhanced ERK phosphorylation in K1 cells (**Figure 3E**). Taken together, these results suggest miR-132/212 as tumor suppressive miRNAs in thyroid cancer.

miR-132/212 co-target the 3'-UTR of CSDE1 mRNA and downregulates CSDE1 expression in thyroid cancer cells

To investigate the molecular mechanism by which miR-132/212 exerts their functions, we used three algorithms to predict potential targets of miR-132/212. Interestingly, analysis results showed that 3'-UTR of CSDE1 contains the both miR-132 and miR-212 complementary sequence (**Figure 4A**). We constructed luciferase reporter vector with either wildtype or mutated 3'UTR of CSDE1 mRNA (**Figure 4A**). We then transfected wildtype 3'UTR of CSDE1 mRNA reporter vector in WRO-pGLV-miR132/212 or control cells. Luciferase activity was reduced in WRO-pGLV-miR132 cells or WRO-pGLV-miR212 cells (**Figure 4B**). Similar results were obtained from FTC133 cells. Luciferase activity was suppressed in FTC133-pGLV-miR132 or FTC133-pGLV-miR212 cells (**Figure 4B**). Then we cotransfected ASO-NC or ASO-miR-132/212 with wildtype 3'UTR of CSDE1 mRNA reporter vector in K1 cells. Results showed that luciferase activity was significantly lower in cell transfected with ASO-miR132/212 (**Figure 4B**). In addition, luciferase activity was also decreased in ASO-8505C cells transfected with ASO-miR132/212 (**Figure 4B**). Furthermore, RT-qPCR assay showed that miR132 or miR-212 overexpression suppressed CSDE1 mRNA

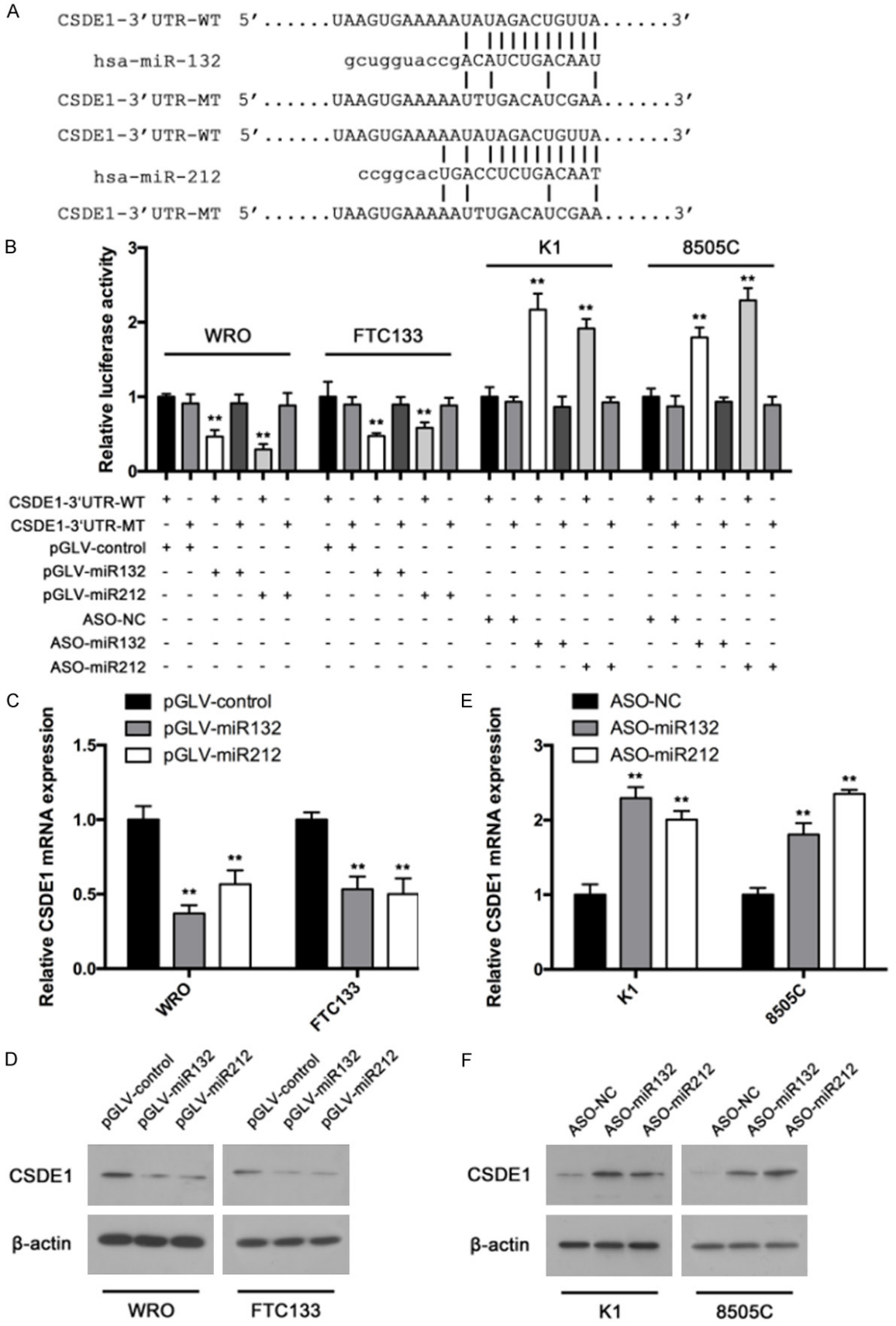
A
CSDE1-3'UTR-WT 5'.....UAAGUGAAAAAUAUAGACUGUUA.....3'
hsa-miR-132 gcugguaccgACAUCUGACAAU
CSDE1-3'UTR-MT 5'.....UAAGUGAAAAAUTUGACAUCGAA.....3'
CSDE1-3'UTR-WT 5'.....UAAGUGAAAAAUAUAGACUGUUA.....3'
hsa-miR-212 ccggcacUGACCUCUGACAAT
CSDE1-3'UTR-MT 5'.....UAAGUGAAAAAUTUGACAUCGAA.....3'
B
Relative luciferase activity
WRO
FTC133
K1
8505C
CSDE1-3'UTR-WT
CSDE1-3'UTR-MT
pGLV-control
pGLV-miR132
pGLV-miR212
ASO-NC
ASO-miR132
ASO-miR212
C
Relative CSDE1 mRNA expression
pGLV-control
pGLV-miR132
pGLV-miR212
WRO
FTC133
E
Relative CSDE1 mRNA expression
ASO-NC
ASO-miR132
ASO-miR212
K1
8505C
D
pGLV-control
pGLV-miR132
pGLV-miR212
CSDE1
β-actin
WRO
FTC133
F
ASO-NC
ASO-miR132
ASO-miR212
CSDE1
β-actin
K1
8505C

Figure 4. miR-132/212 co-target the 3'-UTR of *CSDE1* mRNA and downregulates *CSDE1* expression in thyroid cancer cells. A. The sequences of the predicted miR-132/212 binding site and the CSDE1 3' -UTR segments containing the wildtype or mutant binding site are shown. B. Relative luciferase activity was determined after 3'-UTR reporter plasmids were co-transfected indicated plasmid. C and D. WRO and FTC133 cells were infected with miR-132/212 expressing or control lentivirus. Then CSDE1 expression was determined by RT-qPCR and western blot assay. E and F. K1 and 8505C cells were transfected with ASO-miR-132/212 expressing lentivirus. Then CSDE1 expression was determined by RT-qPCR and western blot assay. Bars, mean of each group ± SD (n = 3). **, $P < 0.01$.

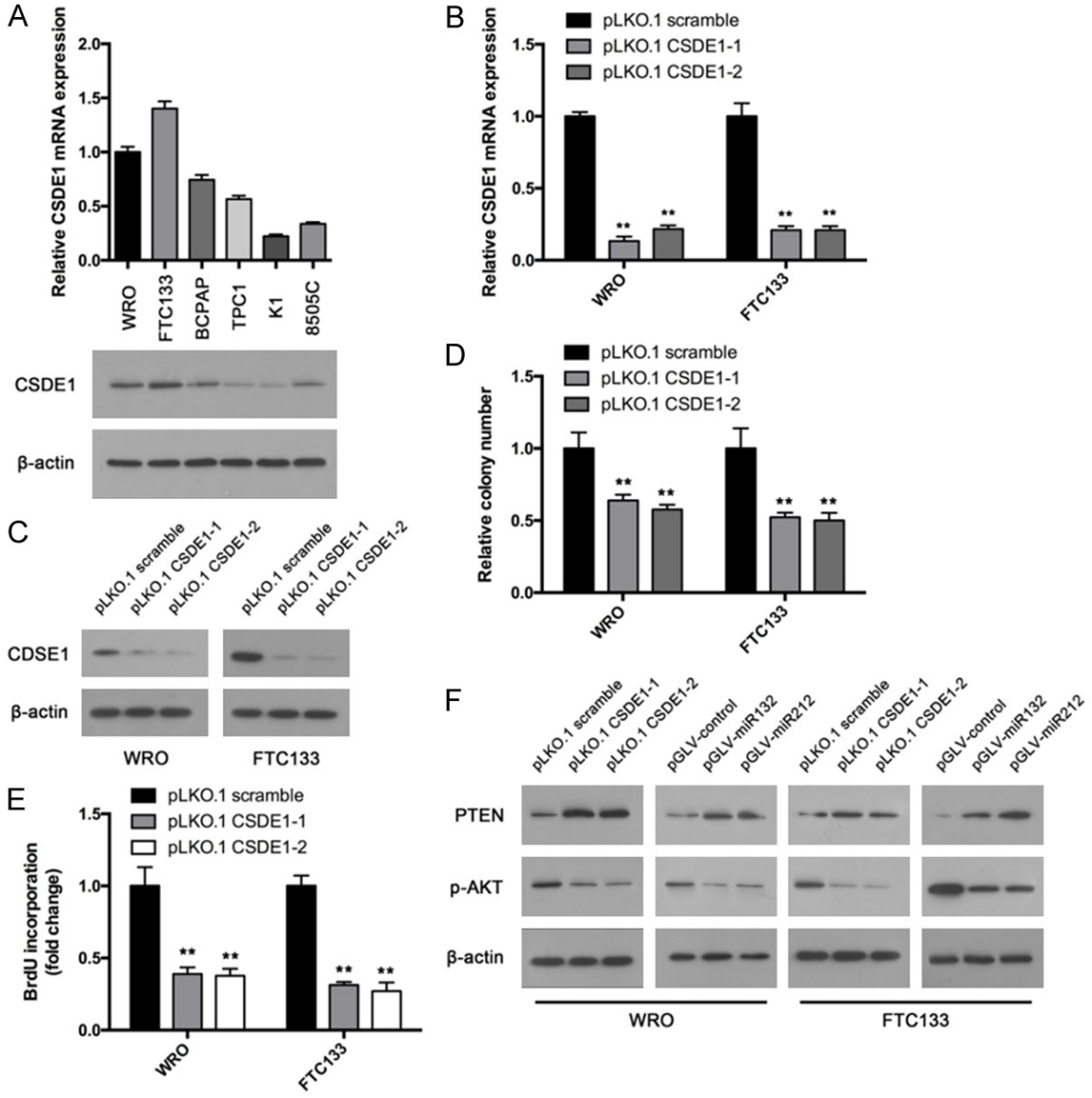

Figure 5. miR-132/212 and CSDE1 promotes proliferation of thyroid cancer cells by activating AKT. A. CSDE1 expression in six thyroid cancer cell lines was determined by qRT-PCR assay and western blot assay. B and C. WRO and FTC133 cells was infected with CSDE1 targeting shRNA or scramble control lentivirus. Then expression level of CSDE1 was determined by RT-qPCR and western blot assay. D. WRO and FTC133 cells with CSDE1 knockdown or control were subjected to colony formation assay. E. WRO and FTC133 cells with CSDE1 knockdown or control were subjected to BrdU incorporation assay to determine the cell proliferation. F. PTEN and p-AKT expression in indicated cells was determined by western blot assay. Bars, mean of each group ± SD (n = 3). **, $P < 0.01$.

expression in both WRO and FTC133 cells (**Figure 4C**). Inhibition of miR-132 or miR-212 could upregulate CSDE1 mRNA in K1 and 8505C cells (**Figure 4E**). In addition, we confirmed these alterations by western blot assay (**Figure 4D** and **4F**).

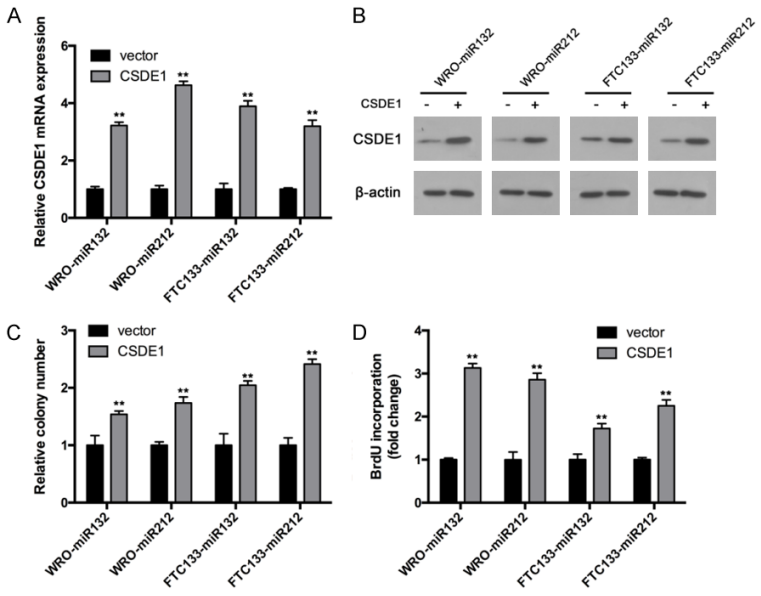

Figure 6. CSDE1 overexpression reversed miR-132/212 induced cell proliferation suppression. (A) and (B). miR-132 or miR-212 overexpression cells was transfected with CSDE1 overexpression vector or control. CSDE1 expression was determined by RT-qPCR and western blot assay. Then colony formation (C) and BrdU incorporation (D) assay was performed. Bars, mean of each group ± SD (n = 3). **, $P < 0.01$.

miR-132/212 and CSDE1 promotes proliferation of thyroid cancer cells by activating AKT

CSDE1 is a RNA binding proteins (RBPs). However, its role in thyroid cancer has not been reported. To explore the functions of CSDE1 in thyroid cancer, we first examined its expression in thyroid cancer cell lines. Results showed that CSDE1 high expresses in WRO and FTC133 cells and low expresses in K1 and 8505C cells (**Figure 5A**). Interestingly, these results negatively correlate with miR-132/212 expression (**Figure 1A**), which indicates the link between miR-132/212 and CSDE1. Then we knockdowned CSDE1 expression in WRO and FTC133 cells with two different shRNA. RT-qPCR and western blot assay confirmed the downregulation (**Figure 5B** and **5C**). Colony formation was performed. Both two shRNA could decrease the colony number of WRO and FTC133 cells (**Figure 5D**). BrdU incorporation assay provides similar results. CSDE1 downregulation suppressed BrdU incorporation in WRO and FTC133 cells (**Figure 5E**). Previous study indicates that CSDE1 suppress PTEN expression using a post-transcriptional program. We examined PTEN expression in CSDE1 knockdown and control cells. Upregulation of PTEN after CSDE1 suppression was observed (**Figure 5F**). Furthermore, CSDE1 downregulation inhibited AKT activation in WRO and FTC133 cells (**Figure 5F**). Because miR-132/212 targets CSDE1, we wondered if miR-132/212 could also suppress AKT activation. Results showed that overexpression of miR-132/212 upregulated PTEN expression and inhibited AKT activation in WRO and FTC133 cells (**Figure 5F**).

CSDE1 overexpression reversed miR-132/212 induced cell proliferation suppression

Previous results indicate that miR-132/212 may exert their tumor suppressive effects by targeting CSDE1. To validate this hypothesis, we overexpressed CSDE1 in WRO-miR-132/212 and FTC133-miR-132/212 cells. RT-qPCR and western blot assay confirmed the upregulation (**Figure 6A** and **6B**). Colony formation assay showed that CSDE1 overexpression cells reversed miR-132/212 induced colony number decrease (**Figure 6C**). Furthermore, CSDE1 promoted BrdU incorporation in miR-132/212 overexpression cells (**Figure 6D**).

Discussion

microRNAs are critical regulators of gene expression. Dysregulation of microRNAs has been found in a variety of malignant diseases. In current study, we established a link between miR-132/212 cluster and CSDE1 mediated post-transcriptional program in thyroid cancer cells.

Although several reports indicate miR-132/212 cluster have critical roles in cancer progression, the results are controversial. Furthermore, little is known about the specific function of the cluster and mechanism behind it in thyroid cancer. Our results indicate a tumor suppressive role of miR-132/212 cluster. Colony formation assay showed that both miR-132 and miR-212 decreased colony numbers of WRO and FTC133 cells. BrdU incorporation was also suppressed by enhance overexpression of miR-132/212. In consistent with these results, inhibition of the

expression of both miRNAs promoted K1 and 8505c cells growth.

RNA binding proteins (RBPs) are proteins that bind to the double or single stranded RNA in cells and participate in forming ribonucleoprotein complexes. Recent studies indicate RBPs participate in tumorigenesis and progression [13]. CSDE1 is a conserved RBP which regulates mRNA translation and stability [14]. Recent studies indicate the oncogenic role of CSDE1 in melanoma [12]. In the current study, we confirmed oncogenic role of CSDE1 in thyroid cancer cells. CSDE1 suppresses proliferation of thyroid cancer cells. In consistent with previous study, CSDE1 negatively regulated PTEN expression and AKT activation in WRO and FTC133 cells.

In addition, we found that miR-132/212 target CSDE1 in thyroid cancer cells. CSDE1 3'-UTR segments contains both miR-132 and miR-212 complementary sequence. Overexpression of either miR-132 or miR-212 could downregulate CSDE1 expression. Inhibition of miR-132/212 expression could upregulate CSDE1. Furthermore, manipulating miR-132/212 expression could alter luciferase activity of CSDE1 3'UTR reporter. Forced expression of CSDE1 in miR-132/212 overexpression cells could reverse miR-132/212 induced tumor suppressive effects.

In summary, our study establishes a link between dysregulation expression of miR-132/212 cluster and CSDE1 mediated post-transcriptional program in thyroid cancer cells. PTEN and AKT activation are also regulated by the cluster. These findings provide us a new mechanism by which thyroid cancer progress and a potential therapeutic target.

Acknowledgements

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Disclosure of conflict of interest

None.

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