



LncRNA FOXD2-AS1 Functions as a Competing Endogenous RNA to Regulate TERT Expression by Sponging miR-7-5p in Thyroid Cancer

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Long non-coding RNA FOXD2 Adjacent Opposite Strand RNA 1 (FOXD2-AS1) has been widely reported to be implicated in the progression and recurrence of several cancers. The clinical significance and functional role of FOXD2-AS1 in thyroid carcinoma remain unknown. FOXD2-AS1 expression was evaluated by analyzing thyroid cancer RNA sequencing dataset from The Cancer Genome Atlas (TCGA). *In vitro* and *in vivo* assays were performed to assess the biological roles of FOXD2-AS1 in thyroid cancer cells. Western blot, luciferase, immunoprecipitation (IP), and RNA immunoprecipitation (RIP) assays were used to identify the underlying miRNA and mRNA target mediating the biological roles of FOXD2-AS1 in thyroid cancer cells. FOXD2-AS1 was upregulated in thyroid carcinoma tissues and cells. High expression of FOXD2-AS1 significantly correlated with clinical stage, recurrence of thyroid carcinoma. Silencing FOXD2-AS1 inhibited cancer stem cell-like phenotypes and attenuates the anoikis resistance *in vitro*. Downregulating FOXD2-AS1 represses the tumorigenesis of thyroid carcinoma cells *in vivo*. FOXD2-AS1 acts as a competitive endogenous RNA (ceRNA) for miR-7-5p, up-regulating the expression of telomerase reverse transcriptase (TERT), which further promotes the cancer stem cells features and anoikis resistance in thyroid cancer cells. Our findings indicate that FOXD2-AS1 functions as an oncogenic regulator in the development of thyroid cancer, contributing to early recurrence of thyroid cancer.

Keywords: FOXD2-AS1, TERT, cancer stem cells, recurrence, thyroid cancer

INTRODUCTION

Thyroid cancer is the most frequent endocrine malignancy with an increasing incidence (1, 2). Among histological subtypes, papillary thyroid carcinoma (PTC) accounts for 90% of cases (3). PTCs have a favorable 5-year survival rate (over 95%) (4), however, about 5–10% PTC patients present recurrence after therapy (4, 5).

Long non-coding RNAs (lncRNAs) are newly identified class of non-coding RNA, longer than 200 nucleotides (6, 7). lncRNAs have been demonstrated to be involved in multiple biological

processes, including transcription or post-transcription, epigenetic modification and mRNA processing (6, 7). Recently, accumulating studies have reported that lncRNAs play important role in the development, progression and metastasis of various types of cancer (8–11). However, literatures regarding the effects of lncRNAs on PTC are relatively scanty. The lncRNA, FOXD2 Adjacent Opposite Strand RNA 1 (FOXD2-AS1), has been reported to function as an oncogenic lncRNA in several human cancer types. For example, FOXD2-AS1 was found to be overexpressed in bladder cancer tissues, which further promoted bladder cancer progression and recurrence through forming a positive feedback loop with Akt and E2F1 (12); in addition, Xu et al. have reported that overexpression of FOXD2-AS1 contributed to carcinogenesis of gastric cancer, and predicted poor prognosis in gastric cancer patients (13). Furthermore, Lu's study has shown that FOXD-AS1 expression was significantly associated with overall survival in thyroid cancer patients (14). However, to date, the functional role of FOXD2-AS1 in the progression of thyroid cancer is unclear.

lncRNAs can function as a competing endogenous RNAs (ceRNAs, or a molecular sponge) to modulate microRNAs (miRNAs) expression, and focus on the miRNA-mediated lncRNA/mRNA crosstalk (15). miRNAs are a class of endogenous, small non-coding RNAs containing about 22 nucleotides that are involved in regulation of downstream target genes expression at a posttranscriptional level via binding with specific sequences in the 3' untranslated region (3'UTR) of downstream target genes (16). Aberrant expression of miRNAs has been demonstrated to be involved in the tumorigenesis and metastasis of cancers (17–21). Studies have shown that miR-7-5p

is downregulated in PTC (22–24). The aim of the current report is to evaluate the FOXD2-AS1 expression in thyroid cancer.

In the current study, we found that FOXD2-AS1 was upregulated in thyroid cancer tissues, and high levels of FOXD2-AS1 were positively associated with poor recurrence-free survival in thyroid cancer patients via analyzing thyroid cancer RNA sequencing dataset from The Cancer Genome Atlas (TCGA). Loss of function experiments revealed that silencing FOXD2-AS1 repressed CSCs characteristics and anoikis resistance in thyroid cancer cells *in vitro*, and tumorigenesis *in vivo*. Furthermore, mechanistic investigations revealed that FOXD2-AS1 functioned as a ceRNA by sponging miR-7-5p, which further upregulated telomerase reverse transcriptase (TERT) expression in thyroid cancer cells, finally contributing to the early recurrence of thyroid cancer. Therefore, our findings offer new insights into the molecular function of FOXD2-AS1 in thyroid cancer, supporting the notion that FOXD2-AS1 may be used as an early recurrent factor in thyroid cancer patients.

MATERIALS AND METHODS

Cell Lines and Cell Culture

Normal primary thyroid follicular epithelial cells (PTFE) and thyroid duct cell carcinoma cells TT was purchased from Procell (Procell Life Science and Technology Co., Ltd., Wuhan, China). Thyroid cancer cell lines, including PTC cell lines (B-CPAP and BHT101), and anaplastic thyroid cancer (ATC) cell lines (CAL-62 and 8305C) were obtained from Cell Bank of Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). PTFE were cultured in CM-H023 medium (Procell, China), and thyroid cancer cell lines were cultured in RPMI-1640 medium (Life Technologies, Carlsbad, CA, US) supplemented with penicillin G (100 U/ml), streptomycin (100 mg/ml) and 10% fetal bovine serum (FBS, Life Technologies). All cell lines were cultured at 37°C in a humidified atmosphere with 5% CO₂.

RNA Extraction, Reverse Transcription, and Real-Time PCR

RNA from tissues and cells was extracted (TRIzol, Life Technologies) according to the manufacturer's instructions.

TABLE 1 | A list of primers used in the reactions for real-time PCR.

Gene	Sequence (5'-3')
FOXD2-AS1-up	ACTGGCTTGAAGCGGAGTTTG
FOXD2-AS1-dn	TTAGAGAAATCTGCGGGCGTAG
GAPDH-up	TCCTCTGACTTCAACAGCGACAC
GAPDH-dn	CACCTGTTGCTGTAGCCAAATTC
NANOG-up	TCCAACATCCTGAACCTCAGCTA
NANOG-dn	AGTCGGGTTACACAGGCATC
SOX2-up	GTGAGCGCCTGCAGTACAA
SOX2-dn	GCGAGTAGGACATGCTGTAGGTG
OCT4-up	TGAAGCTGGAGAAGGAGAAGCTG
OCT4-dn	GCAGATGGTTCGTTGGCTGA
BMI-1-up	TCGTTGTTTCGATGCATTCT
BMI-1-dn	CTTTCATTGTCTTTCCGCC
ABCG2-up	ATGAACACACATGTGCAACCATC
ABCG2-dn	CACAGAAACACAACACTTGGCTGTA
ALDH1A1-up	ACAGTGGTTGTCAAACCAGCAGAG
ALDH1A1-dn	TGTAGGCCCATAAACCAGGAACAATA
KLF4-up	CCCCGTGTGTTTACGGTAGT
KLF4-dn	GAGTCCCATCTCAAGGCAC
TERT-up	TCACGGAGACCAGTTCACAA
TERT-dn	TTCAAGTGCTGTCTGATTCCAAT

TABLE 2 | A list of primers used in the reactions for clone PCR.

Gene	Sequence (5'-3')
shFOXD2-AS1-1#-up	CCGGCAGCGATTATGCGGATCTAATCTCGAG ATTAGA TCCGCATAATCGCTGTTTTTG
shFOXD2-AS1-1#-dn	AATTCAAAAACAGCGATTATGCGGATCTAA TCTCGAG ATTAGATCCGCATAATCGCTG
shFOXD2-AS1-2#-up	CCGGGGGCAAGTTCGAGAGTGAATCTCGAG ATTCA CTCTCGAACTTTGCCCTTTTTG
shFOXD2-AS1-2#-dn	AATTCAAAAAGGGCAAGTTCGAGAGTGAATC TCG AGATTCACCTCGAACTTTGCC
sh-Scramble-up	CCGGTTCCTCCGAACGTGTCCAGTCTCGAGAC GTGAC ACGTTCGGAGAATTTTTG
sh-Scramble-dn	AATTCAAAAATTCTCCGAACGTGTCCAGTCTC TCGAGA CGTGACACGTTCCGGAGAA

Messenger RNA (mRNA), lncRNA and miRNA were reverse transcribed from the total RNA using the Revert Aid First Strand cDNA Synthesis Kit (Thermo, USA) according to the manufacturer's protocol. Complementary DNA (cDNA) was amplified and quantified on ABI 7500HT system (Applied Biosystems, Foster City, CA, USA) using SYBR Green I (Applied Biosystems). The primers used in the reactions are listed in **Table 1**. Real-time PCR was performed according to a standard method, as described previously (25). Primers for miR-7-5p and miR-7-1-3p were synthesized and purified by RiboBio (Guangzhou, China). U6 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as endogenous controls for miRNA or mRNA, respectively. Relative fold expressions were calculated with the comparative threshold cycle method according to a previous study (26).

Plasmid, miRNA Inhibitor, and Transfection

Human FOXD2-AS1 cDNA (Vigene Biosciences, Shandong, China) was cloned into the pcDNA3.1(+) plasmid. Knockdown of endogenous FOXD2-AS1 was performed by cloning two short

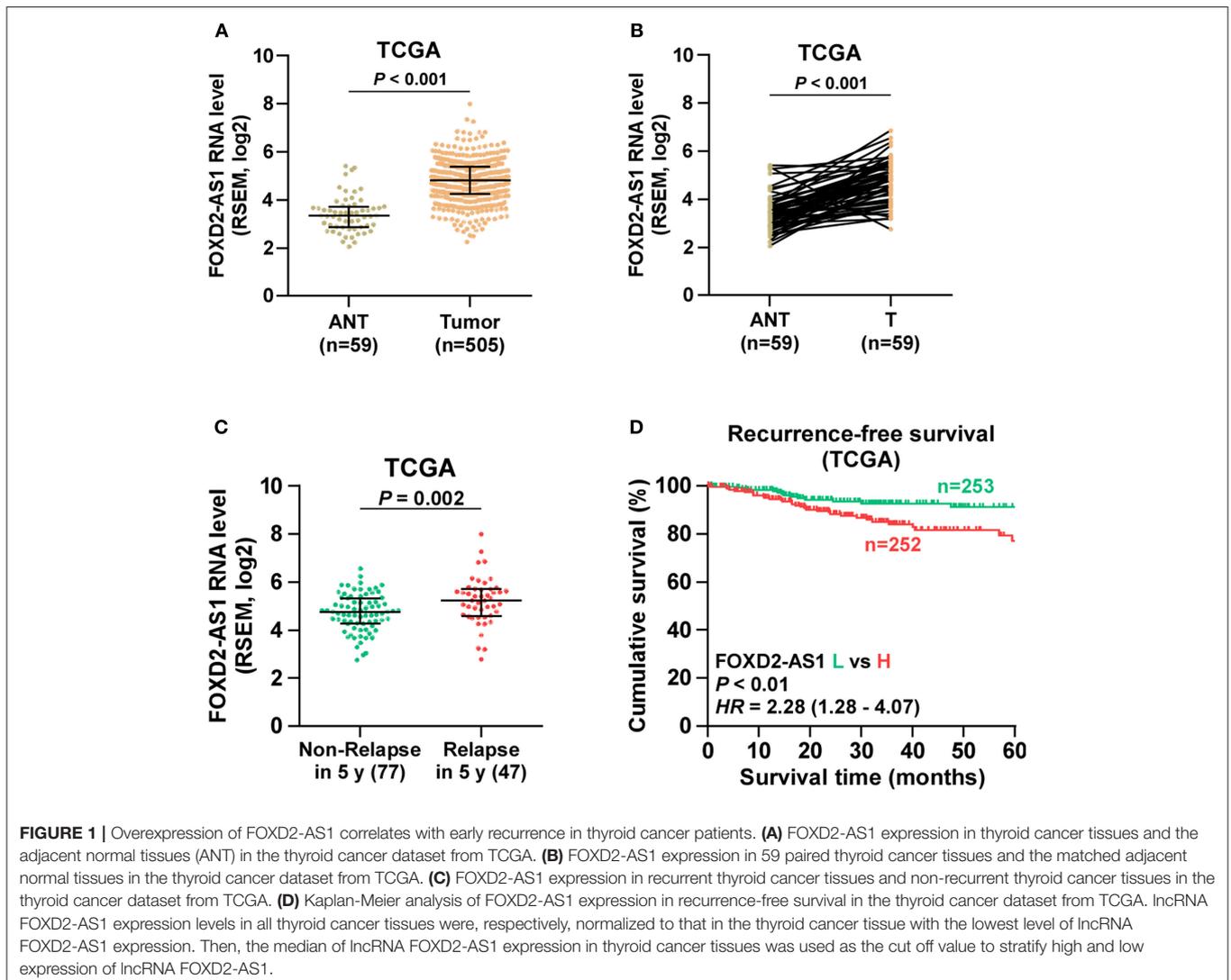
hairpin RNA (shRNA) oligonucleotides into the GV493 vector (GenChem, Shanghai, China). The sequences of the two separate shRNA fragments and scramble are listed in **Table 2**. The miR-7-5p inhibitor and the negative control (RiboBio, China) were achieved. Transfection of siRNAs and plasmids was performed as previously described (27).

Western Blotting Analysis

Western blot was performed according to a standard method (28). Antibodies against cytochrome C, ABCG2, SOX2, NANOG, BMI-1, and LSD1 (Cell Signaling Technology, Cambridge, USA) and TERT (Invitrogen, California, USA) were obtained. The membranes were re-probed with an anti- α -tubulin antibody (Cell Signaling Technology) as the loading control.

Anchorage-Independent Growth Assay

Five-hundred cells were suspended in 2 ml of complete medium containing 0.3% agar (Sigma, Burlington, USA). This experiment was performed according to standards (29) and three times independently for each cell line.



Flow Cytometric Analysis

Flow cytometric analysis (keyGen BioTECH) was performed as standards (30). The cell's inner mitochondrial membrane potential ($\Delta\psi_m$) was detected by flow cytometric analysis using MitoScreen JC-1 staining kit (keyGen BioTECH). Briefly, cells were dissociated with trypsin and resuspended at 1×10^6 cells/ml in Assay Buffer, and then incubated at 37°C for 15 min with $10 \mu\text{l/ml}$ JC-1. Before analyzed by the flow cytometer, cells were washed twice by Assay Buffer. Flow cytometric data were analyzed using FlowJo v10 software (TreeStar Inc., USA).

Caspase-9 or Caspase-3 Activity Assays

The activity of caspase-9 or caspase-3 was analyzed by spectrophotometry (Keygen, China) (31). Briefly, 5×10^6 cells or 100 mg fresh tumor tissues were washed with cold phosphate-buffered saline (PBS) and resuspended in Lysis Buffer and incubated in ice for 30 min. Mixed the $50 \mu\text{l}$ cell suspension, $50 \mu\text{l}$ Reaction Buffer, and $5 \mu\text{l}$ Caspase-3/-9 substrate, and then incubated at 37°C for 4 h. The absorbance was measured at 405 nm, and bicinchoninic acid (BCA) protein quantitative analysis was used as the reference.

Side Population Analysis

The cell suspensions were labeled (Hoechst 33342, Molecular probes – #H-3570) and dye for side population analysis. Cells were resuspended at 1×10^6 pre-warmed (OptiMEM, Gibco, USA) containing 2% fetal bovine serum (FBS) (Gibco, USA) at a density of $10^6/\text{mL}$. Hoechst 33342 dye was added at a final concentration of 5 mg/mL in the presence or absence of verapamil (50 mmol/L; Sigma) and the cells were incubated at 37°C for 90 min with intermittent shaking. At the end of the incubation, the cells were washed with OptiMem containing 2% FBS and centrifuged down at 4°C , and resuspended in ice-cold OptiMem containing 2% FBS and 10 mmol/L HEPES. Propidium iodide (Sigma, USA) at a final concentration of 2 mg/mL was added to the cells to gate viable cells. The cells were filtered through a 40- μm cell strainer to obtain single cell suspension before sorting. Analysis and sorting were done (FACS AriaI, Becton Dickinson). The Hoechst 33342 dye was excited at 355 nm and its dual-wavelength emission at blue and red region was plotted to get the side population (SP) scatter.

Spheroid Formation Assay

Cells (500 cells/well) were seeded into 6-well plates (Ultra Low Cluster, Corning) and cultured (32). After 10–12 days, the number of cell spheroids (tight, spherical, non-adherent masses $>50 \mu\text{m}$ in diameter) were counted, and images of the spheroids were scored under an inverse microscope (spheroids formation efficiency = colonies/input cells $\times 100\%$).

RNA Immunoprecipitation Assay

Cells were co-transfected with pIRESneo-FLAG/HA-Ago2 (#10822; Addgene Inc., USA), followed by HA-Ago2 immunoprecipitation using HA-antibody (33). Real-time PCR analysis of the immunoprecipitation (IP) material was used to test the association of the miR-7-5p with the RNA-induced silencing complex (RISC).

RNA Pull-Down Assay

Details of the RNA pull-down experiment were obtained (34). The proteins in the pull-down products were then examined by western blot technique.

Tumor Xenografts

To study the effect of FDX2-AS1 on the tumorigenesis of thyroid cancer cells, the 6-week-old BALB/c-nu mice were randomly divided into four groups ($n = 6$ per group). Cells (5×10^6 , 1×10^6 , 5×10^5 , and 1×10^5) were inoculated subcutaneously together with Matrigel (final concentration of 25%) into the inguinal folds of the nude mice, respectively. Tumor volume was determined using an external caliper and calculated using the equation $(L \times W^2)/2$. On day 38, tumors were excised, weighed and stored in liquid nitrogen tanks. All the animal experimental procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee. The protocol was approved by the Animal Ethics Committee of the China-Japan Union Hospital of Jilin University.

Statistical Analysis

All values are presented as means \pm standard deviation (SD). Significant differences were determined using GraphPad 5.0

TABLE 3 | The relationship between FDX2-AS1 expression level and clinical pathological characteristics in 505 patients with thyroid carcinoma (from TCGA).

Parameters	Number of cases	FOXD2-AS1 expression		P-values
		Low	High	
HISTOLOGIC				
PTC	495	248	247	0.995
ATC	10	5	5	
GENDER				
Male	136	64	72	0.407
Female	369	189	180	
AGE				
>55	338	180	158	0.044*
≤55	167	73	94	
T CLASSIFICATION				
T1-T2	309	173	136	0.001*
T3-T4	194	80	114	
N CLASSIFICATION				
N0	230	132	98	< 0.001*
N1	225	88	137	
M CLASSIFICATION				
M0	282	135	147	0.18
M1	9	2	7	
STAGE				
I	224	116	108	0.004*
II-IV	64	20	44	
RECURRENCE STATUS				
No (>5 years)	79	46	33	0.003*
Yes (≤5 years)	46	14	32	

*PTC, papillary thyroid carcinoma; ATC, anaplastic thyroid carcinoma; NA, Not available.

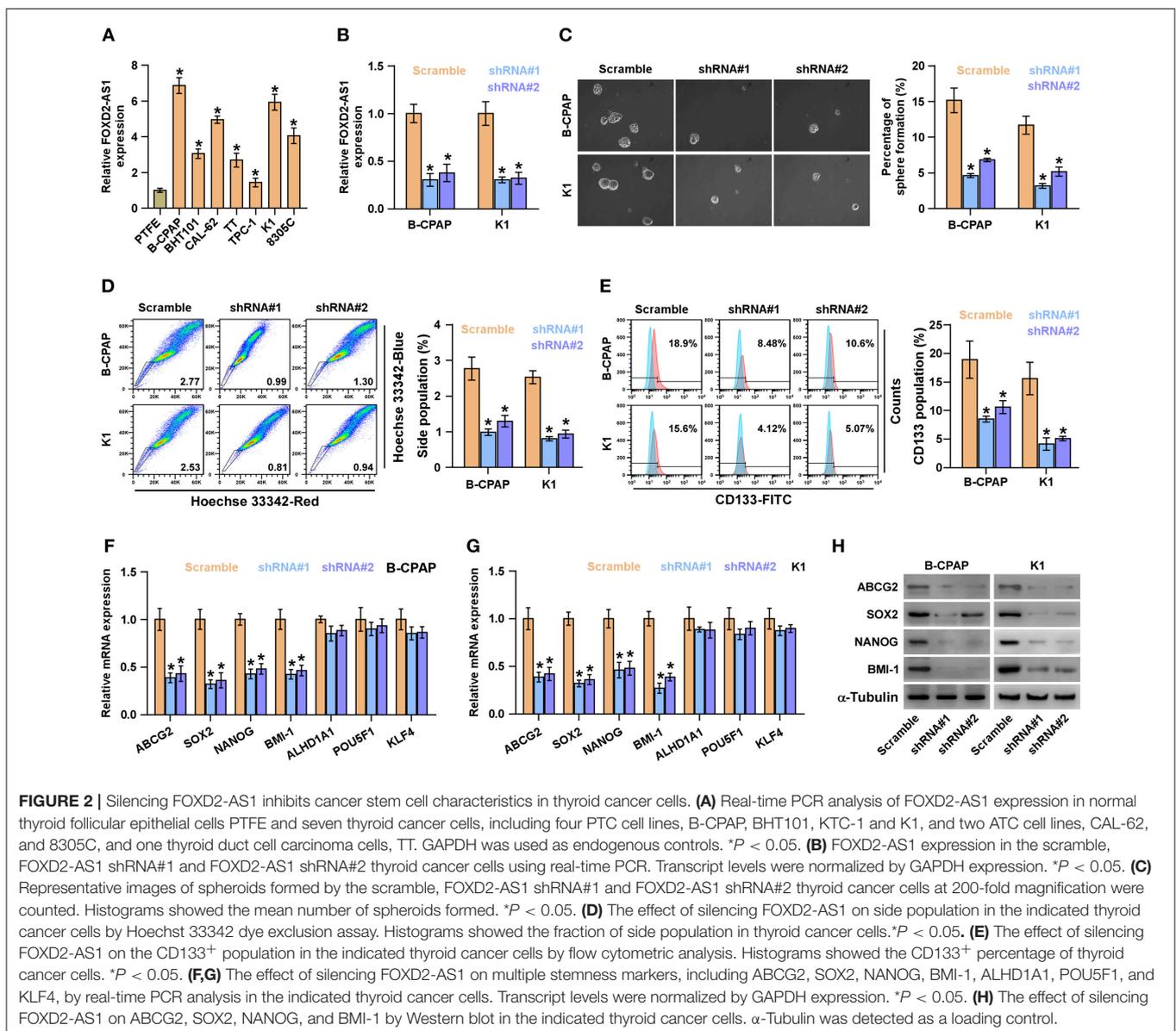
software (USA). Student's *t*-test was used to determine statistical differences between the two groups. One-way ANOVA was used to determine statistical differences between multiple testing. The chi-square test was used to analyze the relationship between FOXD2-AS1 expression and clinicopathological characteristics. Survival curves were plotted using the Kaplan Meier method and compared by log-rank test. $P < 0.05$ was considered significant. Experiments were repeated three times.

RESULTS

FOXD2-AS1 Is Up-Regulated in Thyroid Cancer Tissues

Through analyzing RNA sequencing dataset of thyroid cancer from TCGA, we found that expression level of FOXD2-AS1 was

increased in thyroid cancer tissues compared with the adjacent normal tissues (ANT) (**Figure 1A**). Furthermore, upregulation of FOXD2-AS1 in 59 paired thyroid cancer tissues was demonstrated compared with the matched ANT in the majority of thyroid cancer tissues (**Figure 1B**). Overexpression of FOXD2-AS1 was found to significantly correlate with age, T classification, N classification, clinical stage, and recurrence status in thyroid cancer patients via analyzing clinical dataset of thyroid cancer patients from TCGA (**Table 3**). Importantly, analysis result of TCGA showed that FOXD2-AS1 expression was significantly elevated in recurrent thyroid cancer tissues compared with those in non-recurrent thyroid cancer tissues (**Figure 1C**), and high expression of FOXD2-AS1 predicted poor recurrence-free survivals (**Figure 1D**). Therefore, these results suggest that overexpression of FOXD2-AS1 may be implicated in the early recurrence of thyroid cancer.



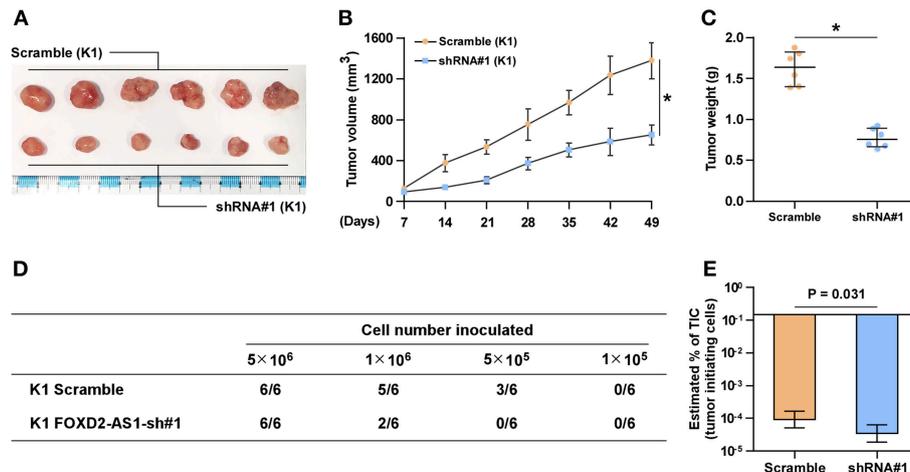


FIGURE 3 | Downregulation of FOXD2-AS1 inhibits the CSCs characteristics *in vivo*. **(A)** The tumors formed by the scramble and FOXD2-AS1 shRNA#1 K1 cell were shown ($n = 6$, each group). **(B)** The effect of silencing FOXD2-AS1 on the tumor volumes in the indicated mice groups from the fifth day at 7 days interval after inoculation of 5×10^6 cells. Data presented are the mean \pm s.d. **(C)** The effect of silencing FOXD2-AS1 on the tumor weights in the indicated mice groups after inoculation of 5×10^6 cells. $^*P < 0.05$. **(D)** The formation number of tumors generated by different amounts of K1 cells in the indicated mice groups. **(E)** The estimated percentage of tumor-initiating cells required to generate tumors in the indicated mice groups.

Silencing FOXD2-AS1 Attenuates CSCs Characteristics *in vitro* and *in vivo*

Existence of CSCs has been reported to contribute to the recurrence of thyroid cancer (35). Therefore, the effects of FOXD2-AS1 on the CSCs phenotypes of thyroid cancer cells were further investigated. The expression levels of FOXD2-AS1 in normal thyroid follicular epithelial cells PTFE and seven thyroid cancer cells were first examined, the results showed that FOXD2-AS1 level was differentially upregulated in thyroid cancer cells compared with that in PTFE cells (Figure 2A). As B-CPAP and K1 cells expressed the highest levels of FOXD2-AS1, thus we further knocked down FOXD2-AS1 expression in B-CPAP and K1 cells by retrovirus infection (Figure 2B). Spheroids formation assay was performed and the results showed that silencing FOXD2-AS1 inhibited spheroids formation ability in thyroid cancer cells (Figure 2C). Moreover, downregulating FOXD2-AS1 decreased the fraction of side population (SP) cells and CD133⁺ population of thyroid cancer cells by flow cytometry (Figures 2D,E). RT-PCR analysis showed that silencing FOXD2-AS1 repressed the expression of ABCG2, SOX2, NANOG, and BMI-1, but not of ALHD1A1, POU5F1, and KLF4 expression (Figures 2F,G), which was further validated by western blot (Figure 2H).

The effect of FOXD2-AS1 on the tumorigenesis of thyroid cancer cells was further investigated *in vivo*. The results of animal experiments revealed that silencing FOXD2-AS1 reduced the volume and weight of tumors compared with those in the scramble group after implantation of 5×10^6 cells (Figures 3A–C). Of note, after inoculation of 5×10^5 scramble cells, the tumors were only detected in the scramble group compared with those in FOXD2-AS1-silencing group (Figure 3D). Furthermore, silencing FOXD2-AS1 increased the number of tumor initiating cells (TIC) required to develop

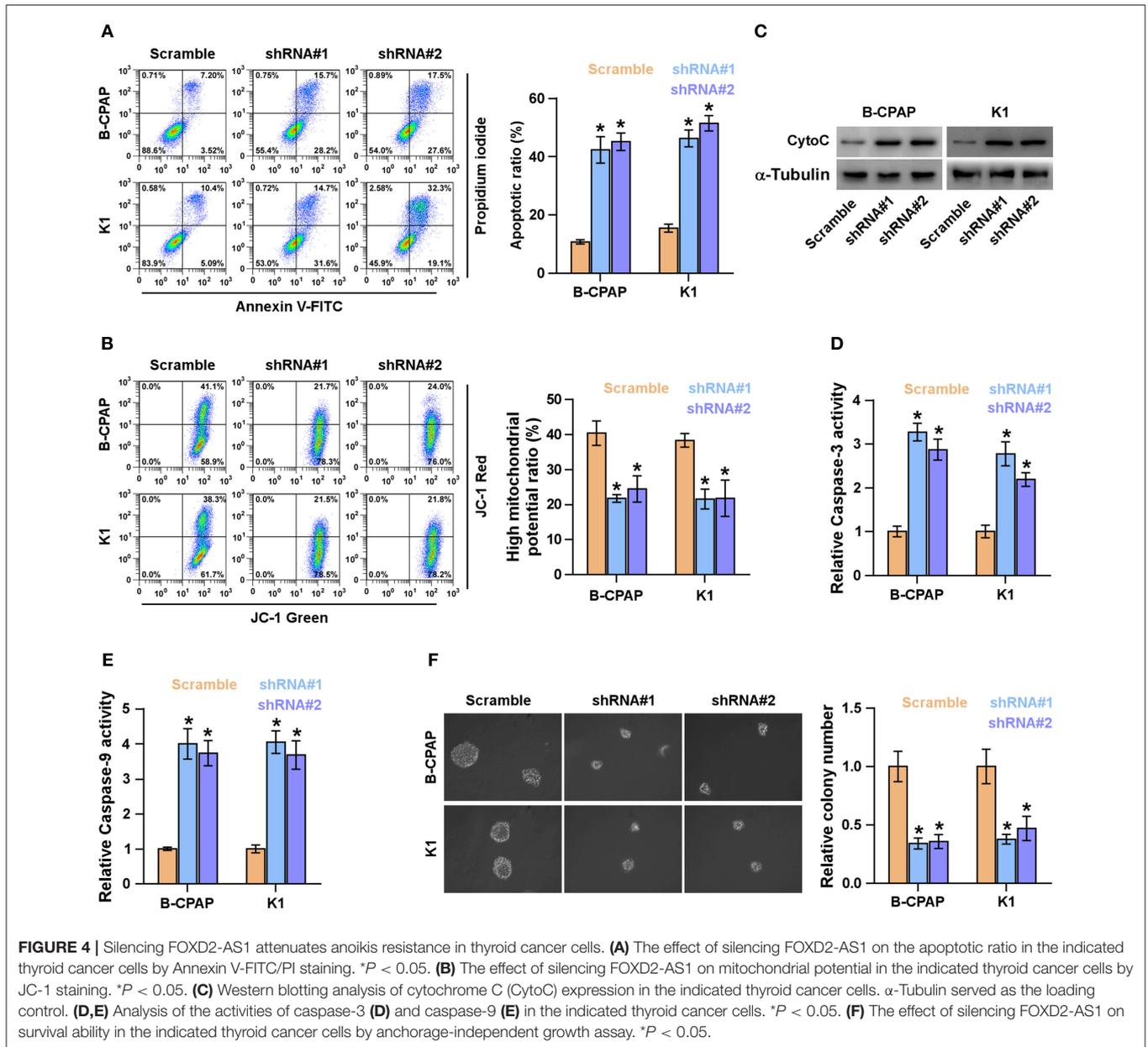
tumor in mice compared with that in the scramble mice group (Figure 3E). Taken together, these results demonstrate that silencing FOXD2-AS1 inhibits CSCs characteristics *in vitro* and *in vivo*.

Silencing FOXD2-AS1 Attenuates Survival and Anoikis Resistance in Thyroid Cancer Cells

Anoikis resistance ability has been identified to be a major hallmark of TICs (36, 37). Therefore, the effects of FOXD2-AS1 on anoikis resistance of thyroid cancer cells were further examined. As shown in Figure 4A, silencing FOXD2-AS1 increased the apoptosis rate of thyroid cancer cells. In addition, silencing FOXD2-AS1 reduced the mitochondrial potential of thyroid cancer cells via mitochondrial membrane potential assay (Figure 4B). The effect of FOXD2-AS1 on the expression of cytochrome C (CytoC) and the caspase-3 and -9 activity were further examined, and we found that silencing FOXD2-AS1 enhanced the expression of CytoC and the activity of caspase-3 or -9 in thyroid cancer cells (Figures 4C–E). Anchorage-independent growth assay showed that silencing FOXD2-AS1 repressed the survival ability of thyroid cancer cells (Figure 4F). Collectively, these results indicate that silencing FOXD2-AS1 reduces anoikis resistance and survival in thyroid cancer cells.

Silencing FOXD2-AS1 Down-Regulates TERT at the Post-transcriptional Level

Several studies have demonstrated that overexpression of TERT is not only involved the development and aggressive behaviors of thyroid cancer cells (38), but also predicts early recurrence in thyroid cancer patients (39). Furthermore, promoter mutation of TERT has been identified to be significantly correlated with aggressiveness and recurrence in thyroid cancer in our

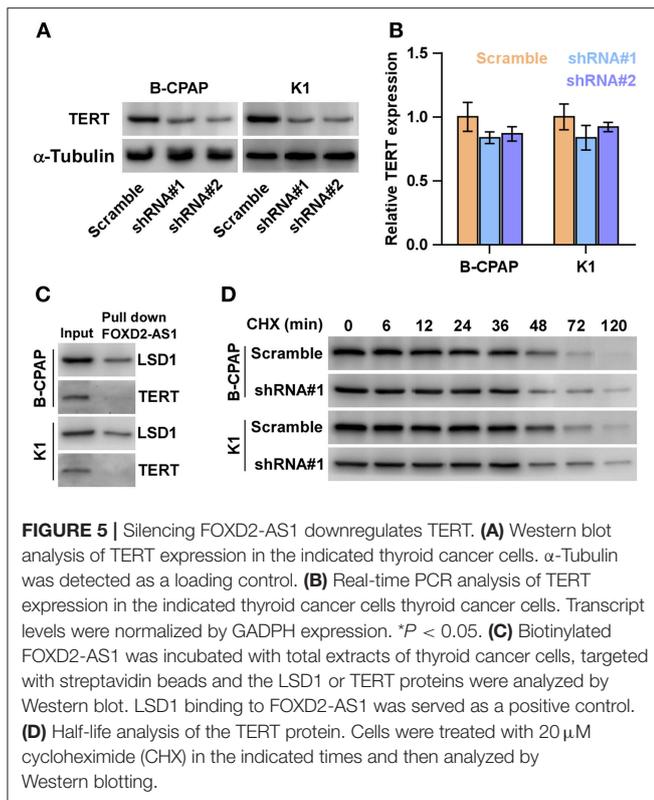


previous studies (39–41). Therefore, we posited that FOXD2-AS1 promotes the recurrence of thyroid cancer via regulating TERT expression. As expected, TERT expression was repressed by downregulation of FOXD2-AS1 in thyroid cancer cells (Figure 5A). Strikingly, the mRNA level of TERT was not affected by FOXD2-AS1 (Figure 5B), suggesting that FOXD2-AS1 post-transcriptionally regulated TERT expression. A major role of lncRNAs in modulating downstream factors is as RNA-binding proteins (34, 41) such as LSD1 (42). The interactions between FOXD2-AS1 and LSD1 was confirmed by RNA pull-down assay, but FOXD2-AS1 had no interaction with TERT (Figure 5C). The half-life period of TERT was not affected by FOXD2-AS1 in thyroid cancer cells (Figure 5D). Thus, these findings indicate that some unknown post-transcriptional regulatory mechanism

may be implicated in the regulatory role of FOXD2-AS1 in TERT in thyroid cancer cells.

FOXD2-AS1 Functions as a ceRNA to Sponge miR-7-5p

Using the publicly available algorithm TargetScan, we found that miR-7-5p that has been reported to act as a tumor-suppressive miRNA in thyroid cancer (42, 43) had 4 miRNA recognition sequences on FOXD2-AS1, suggesting that miR-7-5p was a potential target of FOXD2-AS1 (Figure 6A). RT-PCR analysis showed that silencing FOXD2-AS1 increased miR-7-5p, but not miR-7-1-3p, expression in thyroid cancer cells (Figure 6B). The interaction of FOXD2-AS1 with miR-7-5p was further analyzed by RNA immunoprecipitation (RIP) experiment, and



the results showed that FOXD2-AS1 was enriched in Ago2-containing miR-7-5p immunoprecipitate compared with control immunoglobulin G (IgG) or miR-7-1-3p immunoprecipitates (Figures 6C,D). Moreover, RIP results indicated that mutation of any four recognition site of miR-7-5p on FOXD2-AS1 differentially reduced the binding of miR-7-5p with FOXD2-AS1 compared with wild-type FOXD2-AS1 in thyroid cancer cells (Figures 6E,F). Therefore, these findings indicate that FOXD2-AS1 may act as a ceRNA to regulate miR-7-5p expression in thyroid cancer cells.

TERT Is a Target of miR-7-5p

Interestingly, TERT was found to be a potential target of miR-7-5p (Figure 6G). Silencing FOXD2-AS1 dramatically reduced the TERT expression (Figure 6H). Importantly, inhibition of miR-7-5p increased the TERT expression in FOXD2-AS1-silenced thyroid cancer cells (Figure 6H). Thus, our results demonstrate that FOXD2-AS1 functions as a ceRNA sponge to disrupt the inhibitory effect of miR-7-5p on TERT, finally upregulating TERT expression in thyroid cancer cells.

The Effects of FOXD2-AS1 on CSCs Features of Thyroid Cancer Cells Depend on miR-7-5p

We further investigated whether FOXD2-AS1 has an effect on CSCs phenotype of thyroid cancer cells via sponging miR-7-5p, and found that inhibition of miR-7-5p enhanced the sphere formation ability, SP fraction and CD133⁺ population

repressed by downregulation of FOXD2-AS1 (Figures 6I–K). Therefore, our results indicate that FOXD2-AS1 promotes CSCs features by competitively binding miR-7-5p in thyroid cancer cells.

Correlation of FOXD2-AS1 With miR-7-5p and TERT

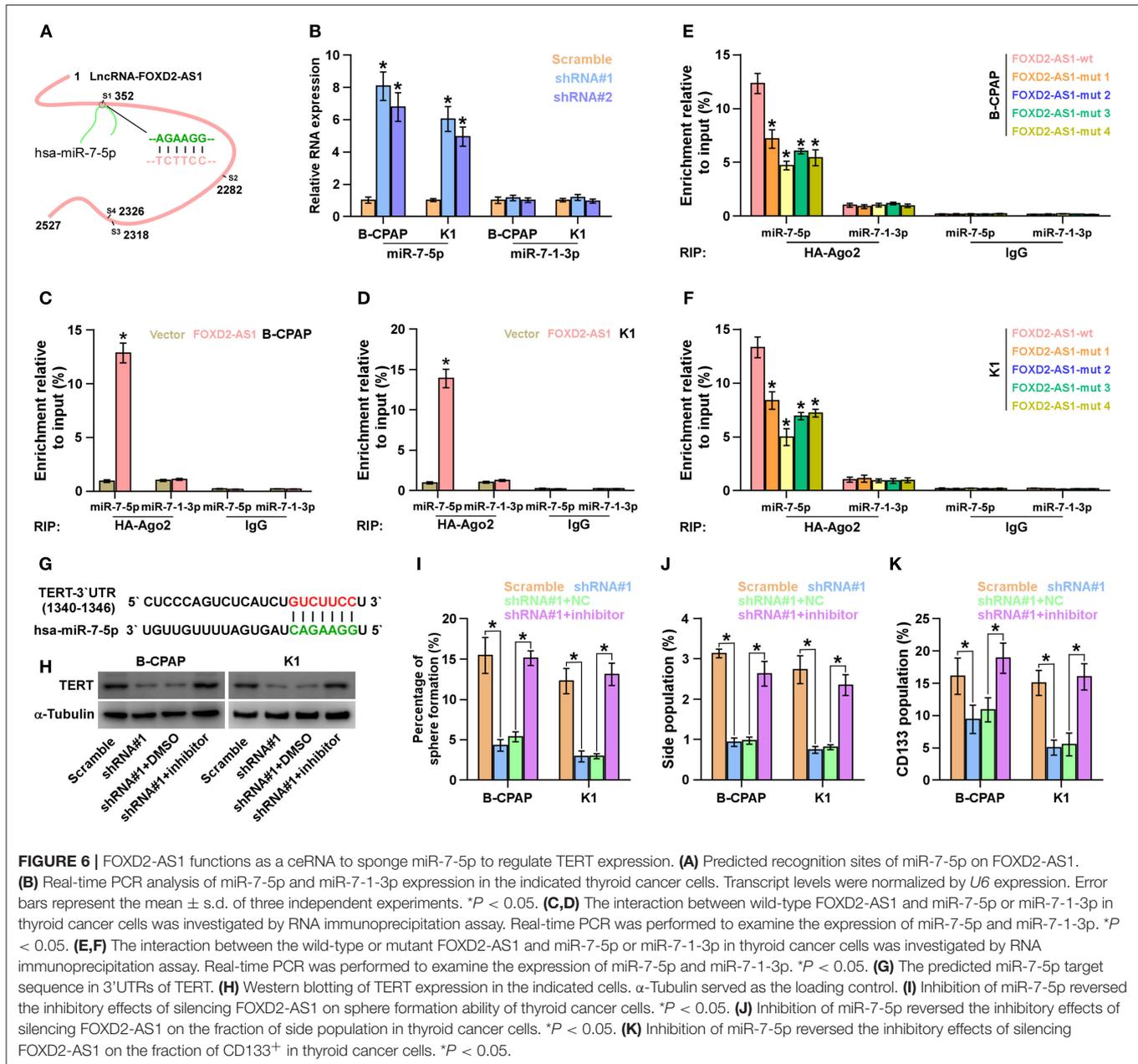
The clinical association of FOXD2-AS1 with miR-7-5p and TERT were further investigated in the tumors tissues of mice formed by 5×10^6 FOXD2-AS1-silencing cells and the scramble cells. RT-PCR was performed to measure the expression of FOXD2-AS1 and miR-7-5p in the tumor tissues, and the results showed that FOXD2-AS1 expression levels were decreased in the tumor tissues from FOXD2-AS1-silencing mice group compared with those from the scramble mice group; conversely, miR-7-5p expression levels were elevated (Figures 7A,B). Furthermore, TERT expression was reduced in the tumor tissues from FOXD2-AS1-silencing mice group compared with those from the scramble mice group (Figure 7C). In addition, we further analyzed the correlation between FOXD2-AS1, miR-7-5p, and TERT expression in clinical samples from TCGA, and found that FOXD2-AS1 expression levels significantly and positively correlated with TERT expression (Figure 7D), and was negatively associated with miR-7-5p in thyroid cancer tissues (Figure 7E). Collectively, our results demonstrate that TERT expression is negatively associated with miR-7-5p expression and positively correlated with FOXD2-AS1 expression.

DISCUSSION

The clinical significance and biological function of FOXD2-AS1 in thyroid cancer are under-reported unknown. In the current study, we found that FOXD2-AS1 was upregulated in thyroid cancer tissues, and high expression of FOXD2-AS1 predicted recurrence in thyroid cancer patients. Silencing FOXD2-AS1 abrogated the CSCs-like phenotypes of thyroid cancer cells *in vitro* and the tumorigenesis of thyroid cancer cells *in vivo*. Therefore, our findings suggest the oncogenic role of FOXD2-AS1 in thyroid cancer.

The underlying mechanisms by which lncRNAs promote the tumorigenesis and metastasis of cancer involves transcription or post-transcription, epigenetic modification and mRNA processing (6, 7). It is now increasingly acknowledged that lncRNAs regulate development and progression via sponging an array of downstream miRNAs. Indeed, FOXD2-AS1 has been extensively reported to sponge a mass of miRNAs in a variety of cancers. In bladder cancer, FOXD2-AS1 was found to be overexpressed in bladder cancer tissues, which further promoted bladder cancer progression and recurrence through forming a positive feedback loop with Akt and E2F1 (12). In gastric cancer, FOXD2-AS1 acted as a molecular sponge of miR-136 to promote cancer overexpression of FOXD2-AS1 contributed to carcinogenesis of gastric cancer and predicted poor prognosis in gastric cancer patients (13).

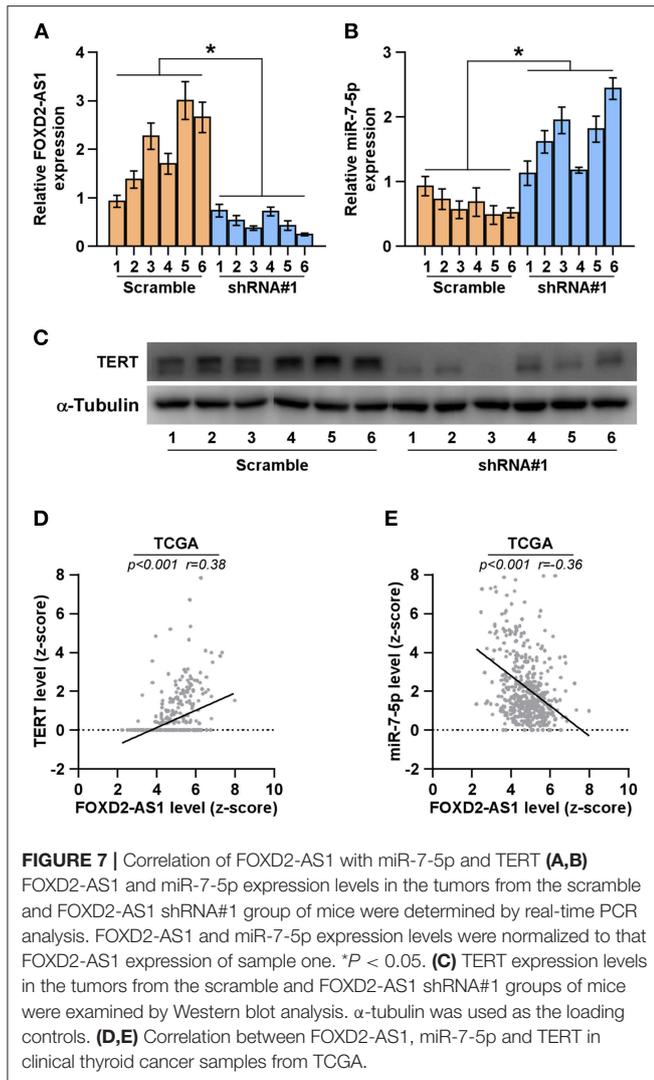
Our results found that silencing FOXD2-AS1 reduced the protein, but not the mRNA, expression levels of TERT in



thyroid cancer cells, suggesting that FOXD2-AS1 regulates TERT expression at post-transcriptional level. Telomerase reverse transcriptase (TERT, or hTERT) is a catalytic subunit of the telomerase enzyme, comprising the most important unit of the telomerase complex (44), and its aberrant expression has been widely reported in a variety of cancer types (45, 46). Of note, studies have reported that overexpression of TERT not only promotes the development and aggressive behaviors of thyroid cancer cells (38), but also predicts early recurrence in thyroid cancer patients (39). Our previous studies have widely demonstrated that the promoter mutation of TERT was significantly correlated with aggressiveness and recurrence in thyroid cancer (39–41). Bioinformatics analysis revealed the four

recognition sequences of miR-7-5p on FOXD2-AS1. RT-PCR showed that silencing FOXD2-AS1 enhanced the expression of miR-7-5p in thyroid cancer cells. Downregulating miR-7-5p abolished the inhibitory effects of FOXD2-AS1 down-expression on the CSC phenotypes of thyroid cancer cells. In addition, FOXD2-AS1 acted as an endogenous sponge or decoy for miR-7-5p, which further relieved the inhibitory effects of miR-7-5p on TERT, leading to the recurrence of thyroid cancer patients. Therefore, our results unravel a novel mechanism underlying the oncogenic role of FOXD2-AS1 in thyroid cancer.

It has been extensively reported that lncRNAs can serve as a prognostic marker in thyroid cancer. Li et al. have found



four-lncRNA signature complex as an independent prognostic predictor in PTC patients by genome-wide analysis of lncRNA expression profiles in a large cohort of PTC patients from TCGA

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(47). Consistently, a study from Luo et al. have revealed that lncRNAs, including AC079630.2, CRNDE, and CTD-2171N6.1, were closely related to the progression and survival of PTC, suggesting that this lncRNA may serve as a potential biomarker to predict the survival of PTC patient (48).

Lu's study has shown that FOXD2-AS1 expression was significantly associated with overall survival in thyroid cancer patients (14). In this study, we identified that lncRNA FOXD2-AS1 expression was elevated in thyroid cancer tissues, particularly in recurrent thyroid cancer tissues. Importantly, Kaplan-Meier survival analysis showed that thyroid cancer patients with high expression of FOXD2-AS1 displayed early recurrence compared with those with low expression of FOXD2-AS1.

In summary, our study demonstrates that FOXD2-AS1 functions as a competing endogenous RNA to upregulate TERT expression by sponging miR-7-5p in thyroid cancer. Therefore, our results provide new insights into the mechanism that clarifies the function of FOXD2-AS1 in thyroid cancer, supporting the idea that FOXD2-AS1 can serve as a novel recurrent marker or a potential target in thyroid cancer.

ETHICS STATEMENT

All the animal experimental procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee. The protocol was approved by the Animal Ethics Committee of the China-Japan Union Hospital of Jilin University.

AUTHOR CONTRIBUTIONS

XL and HS designed the study. XL, QF, SL, NL, FL, CL, and CS performed the experiments and acquired the data. XL, QF, SL, and NL analyzed and interpreted the results. XL, GD, and HS wrote the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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