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Original Article

Melatonin Regulates Breast Cancer Progression by the lnc010561/miR-30/FKBP3 Axis

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Melatonin has been recognized to slow breast cancer growth. The molecular mechanisms may involve long non-coding RNAs (lncRNAs). However, little is known on how melatonin affects lncRNA expression and function in breast cancer. We used microarrays to explore the expression profile of mRNAs and lncRNAs in melatonin-treated breast cancer cells. Kyoto encyclopedia of genes and genomes (KEGG) and Reactome pathways analysis were performed to identify the signaling pathways affected by altered expressed mRNAs after melatonin treatment. To explore the functions and mechanisms of the selected differentially expressed mRNA and lncRNA in breast cancer, we performed a series of experiments. We found that FK506-binding protein 3 (FKBP3) and lnc010561 were downregulated in melatonin-treated breast cancer cells. Knockdown of FKBP3 and lnc010561 inhibited breast cancer proliferation and invasion, and induced apoptosis. Also, lnc010561 and FKBP3 functioned as competing endogenous RNAs (ceRNAs) for miR-30. Our findings suggested that melatonin regulated breast cancer progression by the lnc010561/miR-30/FKBP3 axis. Melatonin may, therefore, function as an anticancer strategy for breast cancer.

INTRODUCTION

Melatonin has been reported to have anti-cancer properties, including suppressing tumor cell metabolism and inducing tumor suppressor genes, and is also involved in cancer cell motility and invasiveness.[1] Several clinical studies have demonstrated the potential use of melatonin in cancer treatment.[2] In osteosarcoma, melatonin attenuates cell invasion by suppressing CCL24 through inhibiting JNK pathway.[3] Moreover, melatonin could suppress cancer stem cells by regulating cellular prion protein/Oct4 axis[4] and could promote the sensitivity of paclitaxel in brain cancer stem cells.[5]

Breast cancer progression is related to multiple mechanisms,[6,7] and melatonin is involved in breast cancer cell proliferation,[8] apoptosis,[9] and metastasis[10] through regulating different pathways. Non-coding RNAs are important regulators involved in cancer progression.[11–13] During the past few decades, plenty of long non-coding RNAs (lncRNAs) have been involved in breast cancer development,[14] but how melatonin affects lncRNA expression and function in breast cancer remains unknown. In this study, we performed whole-genome mRNA and lncRNA expression microarrays to explore the expression profile of mRNAs and lncRNAs in melatonin-treated breast cancer cells. KEGG and Reactome pathways analyses were performed to identify the signaling pathways affected by altered expressed mRNAs after melatonin treatment. To explore the functions and mechanisms of the selected differentially expressed mRNA and lncRNA in breast cancer, we performed a series of experiments. Our results revealed FK506-binding protein 3 (FKBP3), a member of the immunophilin protein family that plays a role in immunoregulation, and lnc010561 (NONMMUT010561) were significantly downregulated in melatonin-treated breast cancer cells. Knockdown of FKBP3 and lnc010561 inhibited breast cancer cell proliferation and invasion, and induced cell apoptosis. Further experiments revealed that specifically lnc010561 and FKBP3 functioned as competing endogenous RNAs (ceRNAs) for miR-30. Our findings suggested that melatonin regulated breast cancer progression by the lnc010561/miR-30/FKBP3 axis, and melatonin may, therefore, function as an anticancer strategy in human breast cancer.

RESULTS

FKBP3 Knockdown Suppresses Breast Cancer Progression

To explore the expression profile of mRNAs in melatonin-treated breast cancer cells, we performed whole-genome mRNA expression microarrays on melatonin-treated 4T1 and 891 cells. KEGG (Figure 1A) and Reactome (Figure 1B) pathways analysis showed the signaling pathways affected by altered expressed mRNAs after melatonin treatment, including cell cycle. We observed 486 mRNAs downregulated at least 2-fold in the melatonin-treated 4T1 cells, whereas 840 mRNAs downregulated in the melatonin-treated 891 cells. Furthermore, 203 mRNAs downregulated at least 2-fold both in melatonin-treated 4T1 and 891 cells (Figure 1C). Among these 203 mRNAs, we detected FKBP3 (also known as FKBP25), a member of the FKBP family and required for

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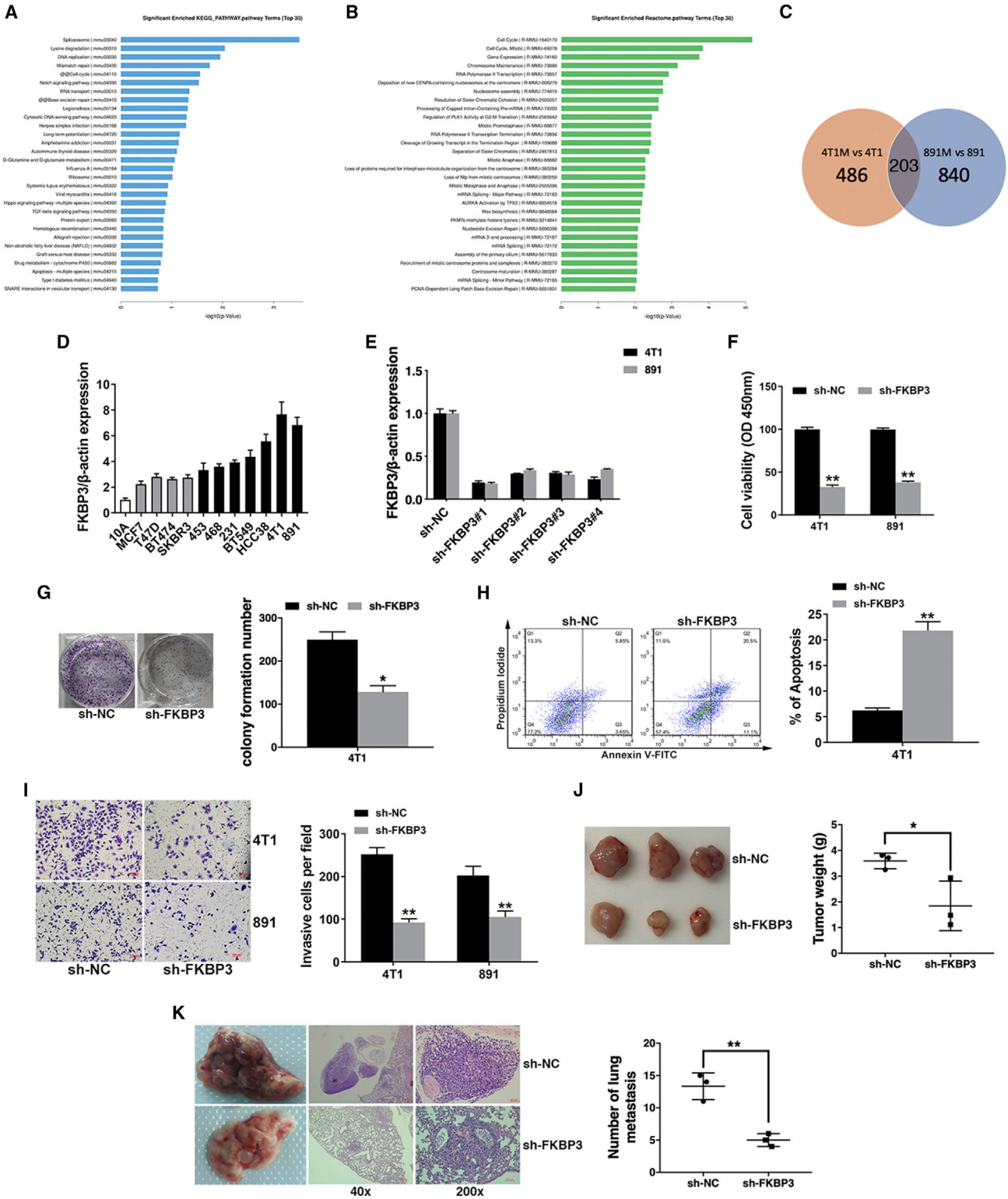

Figure 1. FKBP3 Knockdown Suppresses Breast Cancer Progression

(A) KEGG pathway analysis was performed. (B) Reactome pathway analysis was performed. (C) Venn diagrams showing the overlap mRNAs downregulated at least 2-fold in melatonin-treated 4T1 and 891 cells. (D) FKBP3 expression in breast cell lines obtained by qRT-PCR. (E) qRT-PCR showed that FKBP3 knockdown was successful using

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cell-cycle progression.[15] However, little is known about the expression and function of FKBP3 in breast cancer.

We detected the expression of FKBP3 and found it upregulated in breast cancer cell lines (Figure 1D). Thus, we used short hairpin RNA (shRNA) to knock down FKBP3 expression to explore the function of FKBP3 in breast cancer progression. Figure 1E showed that knockdown was successful using sh-FKBP3#1, which was used in the following experiments. Cell Counting Kit-8 (CCK-8) assay showed that FKBP3 knockdown significantly inhibited cell proliferation (Figure 1F). FKBP3 knockdown also reduced cell colony formation ability (Figure 1G). Apoptosis assay revealed that FKBP3 knockdown induced cell apoptosis (Figure 1H). Transwell assay revealed that FKBP3 knockdown inhibited cell invasion (Figure 1I). To further explore the function of FKBP3 *in vivo*, we established mouse xenograft models. FKBP3 knockdown significantly decreased tumor growth (Figure 1J) and lung metastasis (Figure 1K). These results demonstrate that FKBP3 knockdown suppresses breast cancer progression.

FKBP3 Is a Target Gene of miR-30

TargetScan was used to explore the potential microRNAs (miRNAs) regulating FKBP3, and miR-30 was predicted (Figure 2A). miR-30 has been reported to function as a tumor suppressor and is also involved in controlling cell-cycle progression.[16] Thus, we detected the expression of miR-30 and found it downregulated in breast cancer cell lines (Figure 2B). We performed the luciferase reporter assay to confirm the direct binding of FKBP3 and miR-30. When cells were transfected with wild-type (WT) luciferase vector, the luciferase activity decreased markedly. However, the mutant vector had no such effect (Figure 2C), and the expression of FKBP3 was downregulated by miR-30, whereas locked nucleic acid (LNA)-miR-30 upregulated FKBP3 expression (Figures 2D and 2E). These results indicate that FKBP3 is a target gene of miR-30.

CCK-8 assay showed that miR-30 inhibition reversed the suppressed cell proliferation induced by FKBP3 knockdown (Figure 2F). The reduced cell colony formation ability induced by FKBP3 knockdown was also reversed by miR-30 inhibition (Figure 2G). Apoptosis assay revealed that miR-30 inhibition reversed the increased cell apoptosis induced by FKBP3 knockdown (Figure 2H). Transwell assay revealed that cell invasion inhibited by FKBP3 knockdown was reversed by miR-30 inhibition (Figure 2I), and miR-30 inhibition reversed the decreased tumor growth (Figure 2J) and lung metastasis (Figure 2K) induced by FKBP3 knockdown. These results demonstrate that FKBP3 is a target gene of miR-30, and the function of FKBP3 is regulated by miR-30.

lnc010561 Knockdown Suppresses Breast Cancer Progression

To explore the expression profile of lncRNAs in melatonin-treated breast cancer cells, we performed lncRNA microarrays on melatonin-treated 4T1 and 891 cells. The results showed that there are 451 lncRNAs downregulated at least 2-fold in melatonin-treated 4T1 cells and 1,019 in melatonin-treated 891 cells, and 66 lncRNAs downregulated at least 2-fold in both melatonin-treated 4T1 and 891 cells (Figure 3A). Among these 66 lncRNAs, we verified the expression of the top five downregulated lncRNAs and found that NONMMUT010561 downregulated the most in melatonin-treated 4T1 and 891 cells (Figure 3B). Thus, we decided to study this lncRNA and named it lnc010561.

We used shRNA to knockdown lnc010561 expression to explore the function of lnc010561 in breast cancer progression. Figure 3C showed that knockdown was successful using sh-010561#1, which was used in the following experiments. CCK-8 assay showed that lnc010561 knockdown significantly inhibited cell proliferation (Figure 3D). lnc010561 knockdown also reduced cell colony formation ability (Figure 3E). Apoptosis assay revealed that lnc010561 knockdown induced cell apoptosis (Figure 3F). Transwell assay revealed that lnc010561 knockdown inhibited cell invasion (Figure 3G). To further explore the function of lnc010561 *in vivo*, we established mouse xenograft models. lnc010561 knockdown significantly decreased tumor growth (Figure 3H) and lung metastasis (Figure 3I). These results demonstrate that lnc010561 knockdown suppresses breast cancer progression.

lnc010561 Is a Target of miR-30

We explored the intracellular location of lnc010561 and found that lnc010561 mainly distributed in the cytoplasm (Figure 4A), and bioinformatics analysis showed complementary sequences for miR-30 in lnc010561 (Figure 4B). To confirm the binding between lnc010561 and miR-30, we conducted RNA immunoprecipitation (RIP) assay and found that miR-30 could bind to lnc010561 (Figure 4C). Then we conducted luciferase reporter assay and found that the luciferase activity decreased after transfection with WT luciferase vector and miR-30 (Figure 4D), which indicated that lnc010561 is a target of miR-30.

CCK-8 assay showed that miR-30 inhibition reversed the suppressed cell proliferation induced by lnc010561 knockdown (Figure 4E). The reduced cell colony formation ability induced by lnc010561 knockdown was also reversed by miR-30 inhibition (Figure 4F). Apoptosis assay revealed that miR-30 inhibition reversed the increased cell apoptosis induced by lnc010561 knockdown (Figure 4G). Transwell assay revealed that cell invasion inhibited by lnc010561 knockdown was reversed by miR-30 inhibition (Figure 4H), and miR-30 inhibition reversed the decreased tumor growth (Figure 4I) and lung metastasis (Figure 4J) induced by lnc010561 knockdown. These results demonstrate that

sh-FKBP3#1. (F) CCK-8 assay was performed after transfection. (G) Colony formation assay was performed (left) and quantified (right). (H) Apoptosis assay was performed (left) and quantified (right). (I) Transwell assay was performed (left) and quantified (right). (J) Representative images of xenograft tumors are shown (left), and tumor weight was quantified (right, n = 3 per group). (K) Representative images of lung metastatic nodules and H&E-stained sections are shown (left), and the number of metastatic nodules was quantified (right, n = 3 per group). *$p < 0.05$, **$p < 0.01$.

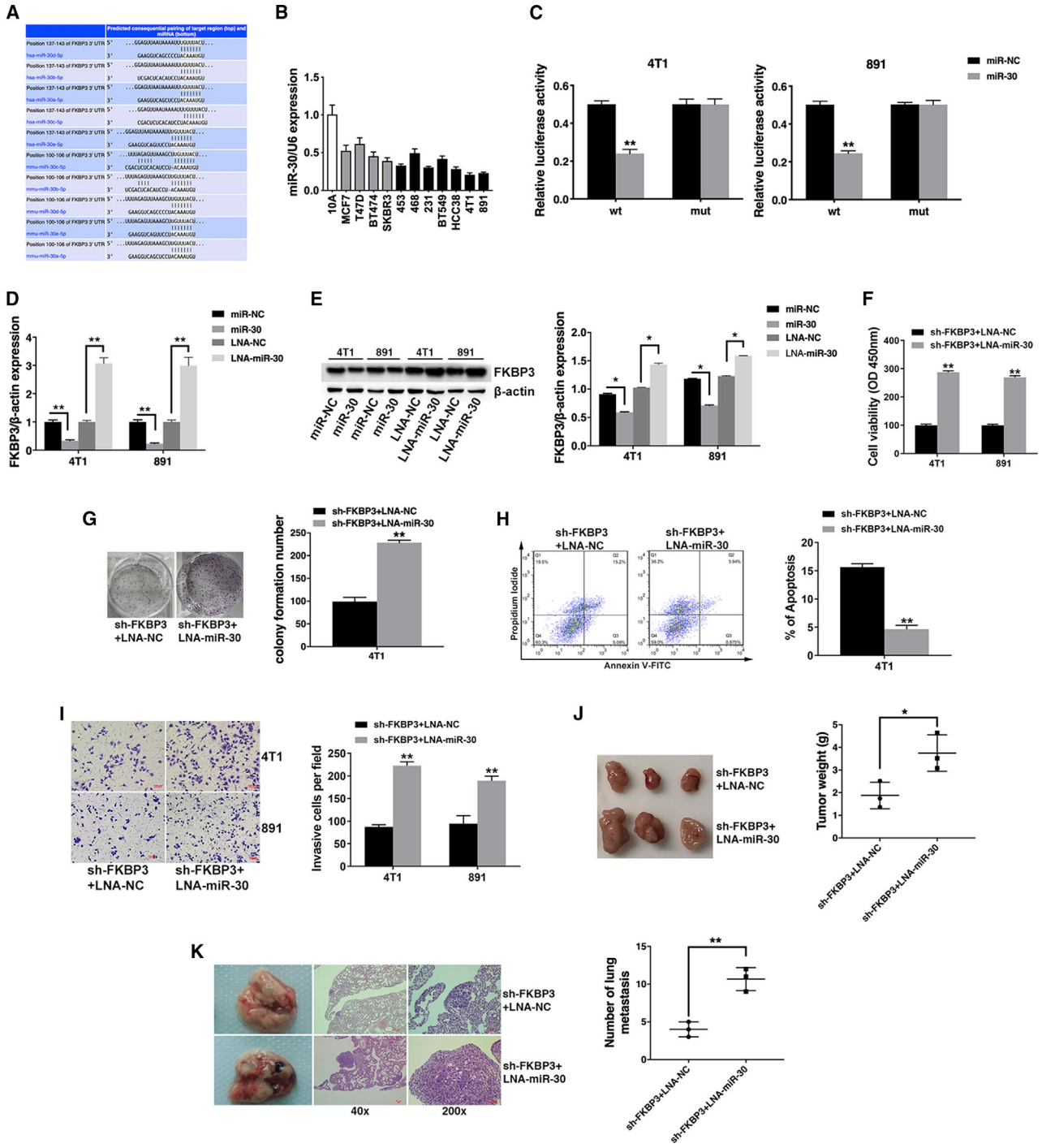


Figure 2. FKBP3 Is a Target Gene of miR-30

(A) The predicted binding sites of miR-30 within FKBP3 are shown. (B) The expression of miR-30 was detected in breast cancer cell lines. (C) Luciferase assay of cells transfected with vectors containing FKBP3 3′ UTR (WT) or mutant version (mut). (D) Expression of FKBP3 after transfection with miR-30 mimics or LNA-miR-30 determined by qRT-PCR. (E) Expression of FKBP3 after transfection with miR-30 mimics or LNA-miR-30 determined by western blotting (left) and quantified by ImageJ (right). (F) CCK-8 assay was performed after transfection. (G) Colony formation assay was performed (left) and quantified (right). (H) Apoptosis assay was performed (left) and quantified (right). (I) Transwell assay was performed (left) and quantified (right). (J) Representative images of xenograft tumors are shown (left), and tumor weight was quantified (right, n = 3 per group). (K) Representative images of lung metastatic nodules and H&E-stained sections are shown (left), and the number of metastatic nodules was quantified (right, n = 3 per group). *$p < 0.05$, **$p < 0.01$.

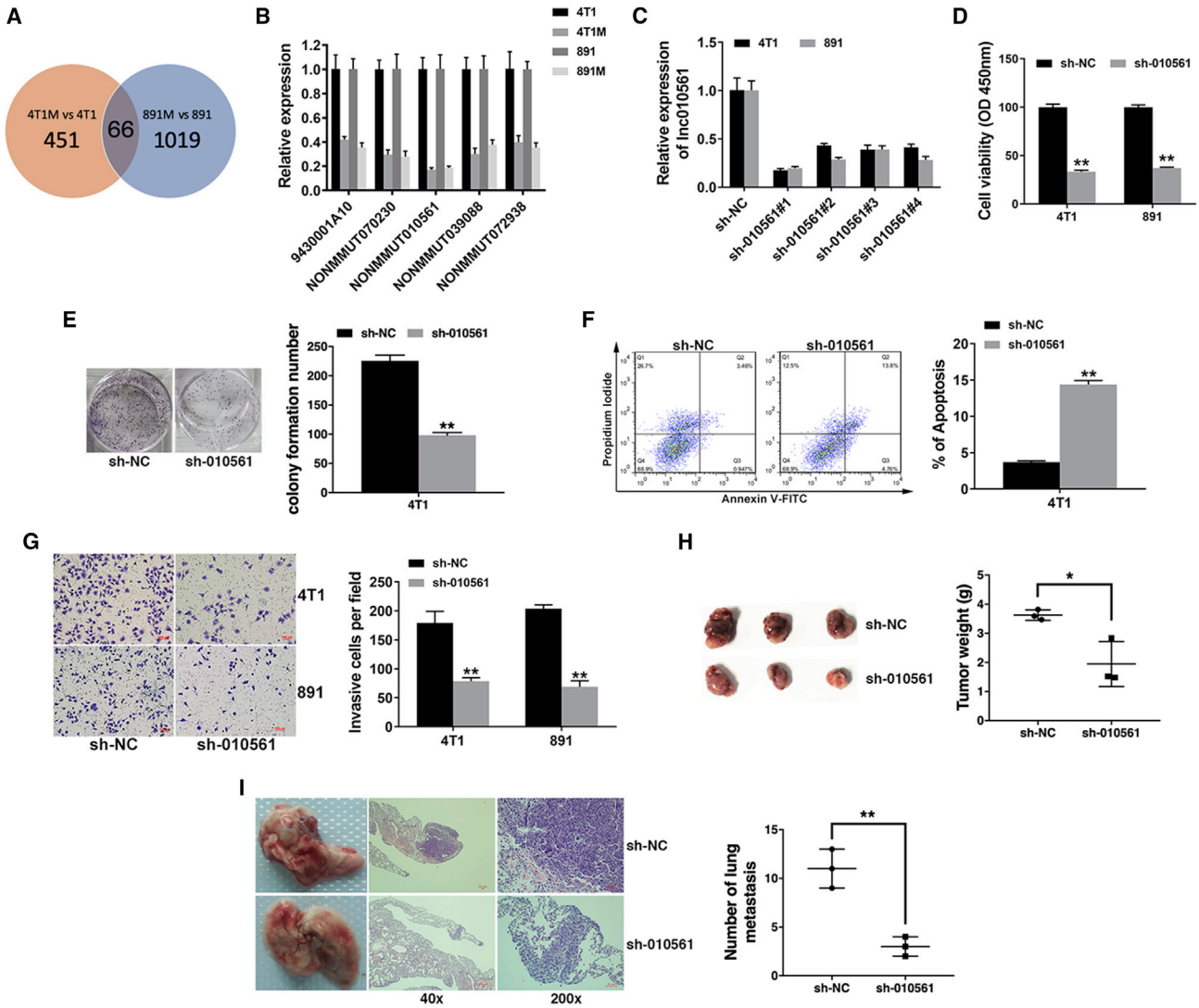


Figure 3. lnc010561 Knockdown Suppresses Breast Cancer Progression

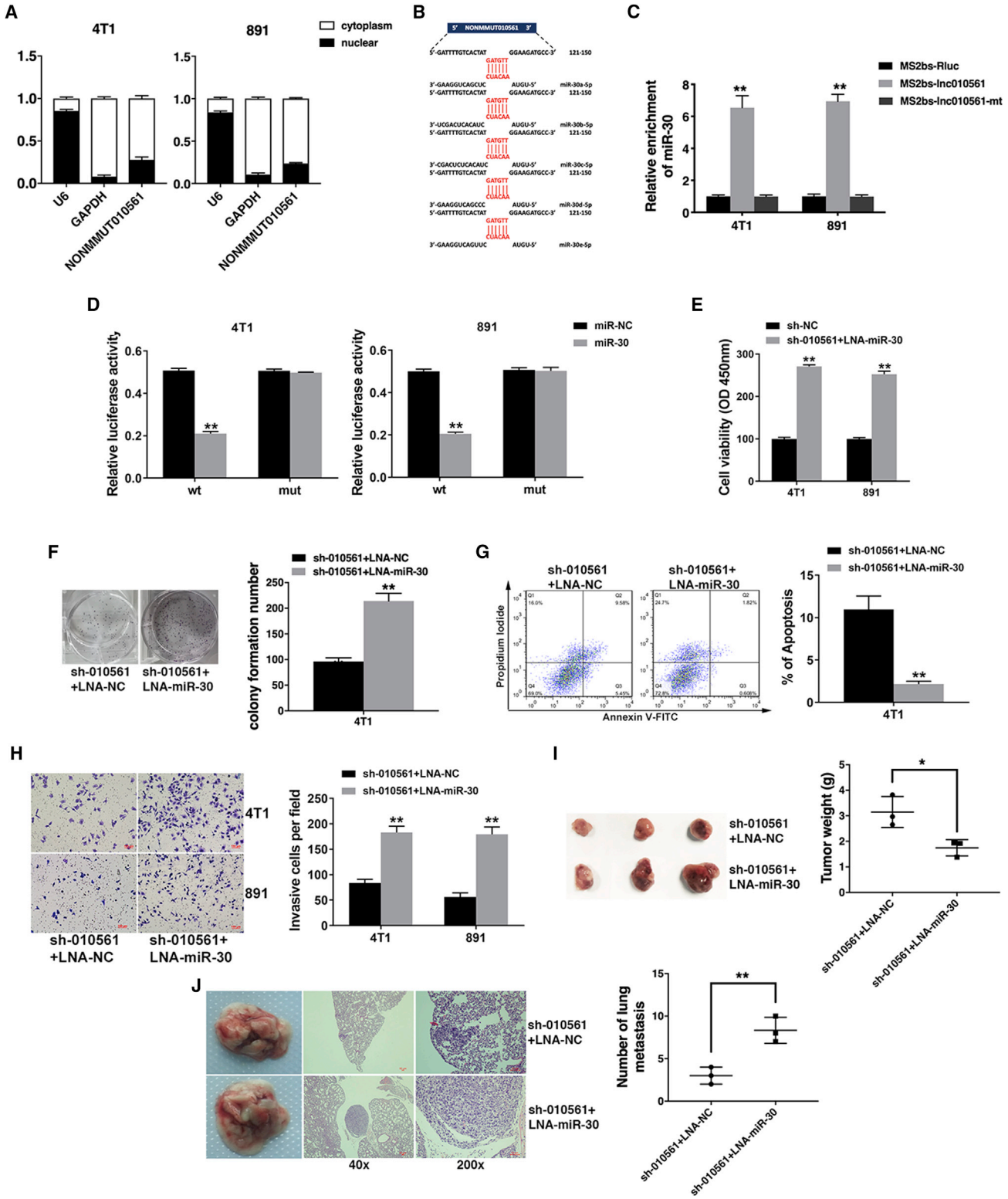
(A) Venn diagrams showing the overlap lncRNAs downregulated at least 2-fold in melatonin-treated 4T1 and 891 cells. (B) The expression of the top five overlap downregulated lncRNAs was detected by qRT-PCR. (C) qRT-PCR showed that lnc010561 knockdown was successful using sh-010561#1. (D) CCK-8 assay was performed after transfection. (E) Colony formation assay was performed (left) and quantified (right). (F) Apoptosis assay was performed (left) and quantified (right). (G) Transwell assay was performed (left) and quantified (right). (H) Representative images of xenograft tumors are shown (left), and tumor weight was quantified (right, n = 3 per group). (I) Representative images of lung metastatic nodules and H&E-stained sections are shown (left), and the number of metastatic nodules was quantified (right, n = 3 per group). *p < 0.05, **p < 0.01.

lnc010561 is a target of miR-30, and the function of lnc010561 is regulated by miR-30.

lnc010561 and FKBP3 Act as ceRNAs to Regulate miR-30

RIP assay on Ago2 showed that lnc010561, FKBP3, and miR-30 were all mainly enriched in the Ago2 group (Figure 5A). Moreover, lnc010561 knockdown decreased enrichment of lnc010561 to Ago2 but increased enrichment of FKBP3 to Ago2 (Figure 5B). In parallel, FKBP3 knockdown decreased enrichment of FKBP3 to Ago2 but increased enrichment of lnc010561 to Ago2 (Figure 5C). These results indicated that lnc010561 and FKBP3 could serve as ceRNAs and competed for binding with miRNAs.

We continued to confirm the regulation of lnc010561, FKBP3, and miR-30 and found that knockdown of lnc010561 downregulated FKBP3 expression, but LNA-miR-30 could rescue the FKBP3 expression (Figures 5D and 5E). In parallel, lnc015192 expression decreased



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after FKBP3 knockdown and increased when miR-30 was inhibited (Figure 5F). Taken together, these data reveal that lnc010561 and FKBP3 serve as ceRNAs for miR-30 to regulate breast cancer progression.

DISCUSSION

This study shows that higher melatonin levels are associated with lower risk for breast cancer,[17] and the melatonin role in breast cancer is mediated through several molecular pathways.[18] Melatonin inhibits cell proliferation and induces cell apoptosis by suppressing cyclooxygenase-2 (COX-2)/prostaglandin E2 (PGE2), p300/nuclear factor-κB (NF-κB), and phosphatidylinositol 3-kinase (PI3K)/Akt signaling and activating the Apaf-1/caspase-dependent apoptotic pathway.[19] Melatonin inhibits cell invasion by inhibiting the p38 pathway and matrix metalloproteinases 2 and 9 (MMP-2/9).[20] Melatonin promotes sensitivity of breast cancers to doxorubicin and drives tumor regression.[21] Furthermore, melatonin pretreatment could sensitize breast cancer cells to radiation.[22] However, how melatonin affects lncRNA expression and function in breast cancer remains unknown.

In this study, we performed whole-genome mRNA and lncRNA expression microarrays to explore the expression profile of mRNAs and lncRNAs in melatonin-treated breast cancer cells. KEGG and Reactome pathways analysis showed that melatonin affected cell-cycle signaling pathways. Among the mRNAs downregulated in both melatonin-treated 4T1 and 891 cells, we noticed FKBP3, a protein required for cell-cycle progression[15] and regulated by p53-mediated repression.[23] FKBP3 has been found to be upregulated in non-small cell lung cancer and could promote cell proliferation, which closely correlated with poor survival.[24] Nevertheless, little is known about the expression and function of FKBP3 in breast cancer. Here, we found that FKBP3 was upregulated in breast cancer cell lines and when knockdown of FKBP3 suppressed breast cancer proliferation and invasion and induced cell apoptosis. Further investigation found that FKBP3 was a target gene of miR-30, and the expression and function of FKBP3 were regulated by miR-30.

miR-30 has long been reported to function as a tumor suppressor.[25] miR-30 is also involved in controlling cell-cycle progression and tumor formation.[16] Overexpression of miR-30 in lungs postpones metastasis.[26] In addition, miR-30 also inhibits invasion, osteomimicry, and bone destruction.[27] In clear cell renal carcinoma, miR-30 inhibits proliferation, angiogenesis, and xenograft tumor growth.[28] In breast cancer, miR-30a is also involved in cell growth and metastasis.[29] Reduced miR-30a expression correlates with p53 inactivation, lymph node positivity, and poor prognosis.[30] Here, we found that miR-30 suppressed breast cancer progression by regulating the expression and function of FKBP3.

Non-coding RNAs are important regulators involved in cancer progression.[11–13] Among them, lncRNAs could act as tumor suppressors or oncogenes.[31] To explore the expression and function of lncRNAs in melatonin-treated breast cancer, we performed lncRNA microarrays. We found that lnc010561 was downregulated in melatonin-treated breast cancer cells. Knockdown of lnc010561 inhibited breast cancer cell proliferation and invasion, and induced cell apoptosis. Further investigation revealed that lnc010561 was a target of miR-30, and the function of lnc010561 was regulated by miR-30.

ceRNA is a mechanism by which RNAs regulate each other by competing with shared miRNAs.[32] lncRNAs could function as ceRNAs to inhibit miRNA function and promote mRNA function.[33] In this study, our experiments revealed that lnc010561 and FKBP3 functioned as ceRNAs and competed for binding with miR-30 to regulate breast cancer progression.

Conclusions

Our findings suggested that melatonin regulated breast cancer progression by the lnc010561/miR-30/FKBP3 axis, and melatonin may, therefore, function as an anticancer strategy in human breast cancer.

MATERIALS AND METHODS

Microarray and Cluster Analysis

Breast cancer cell lines 4T1 and 891 were treated with melatonin (100 nM; Sigma-Aldrich, USA) for 24 h; then RNA was extracted. Microarray analysis was conducted with an Affymetrix GeneChip Mouse Genome 430 2.0 Array and Agilent Mouse lncRNA Chip (Capitalbio Technology Corporation, China). Quantile normalization and data processing were carried out with GeneSpring software v.13.0 (Agilent). Heatmaps were generated by Cluster 3.0 software.

Cell Culture and Transfection

Breast cell lines were obtained from American Type Culture Collection (USA). Cells were cultured under standard conditions and confirmed without mycoplasma infection. DNA fingerprinting was conducted to verify cell authenticity.

Cells were transfected with Lipofectamine 2000 (Invitrogen, USA). shRNAs targeting FKBP3 and lnc010561 were synthesized by GeneCopoeia (USA). Lentiviruses expressing shRNAs were produced by Lenti-Pac HIV Expression Packaging Kit (GeneCopoeia). FKBP3 and lnc010561 knockdown cells were selected with 2 μg/mL

Figure 4. lnc010561 Is a Target of miR-30

(A) U6, GAPDH, and lnc010561 levels were detected by qRT-PCR. (B) The predicted binding sites of miR-30 within lnc010561 are shown. (C) MS2-based RIP assay confirmed the binding of miR-30 and lnc010561. (D) Luciferase assay of cells transfected with vectors containing binding sites of miR-30 within lnc010561 (WT) or mutant version (mut). (E) CCK-8 assay was performed after transfection. (F) Colony formation assay was performed (left) and quantified (right). (G) Apoptosis assay was performed (left) and quantified (right). (H) Transwell assay was performed (left) and quantified (right). (I) Representative images of xenograft tumors are shown (left), and tumor weight was quantified (right, n = 3 per group). (J) Representative images of lung metastatic nodules and H&E-stained sections are shown (left), and the number of metastatic nodules was quantified (right, n = 3 per group). *$p < 0.05$, **$p < 0.01$.

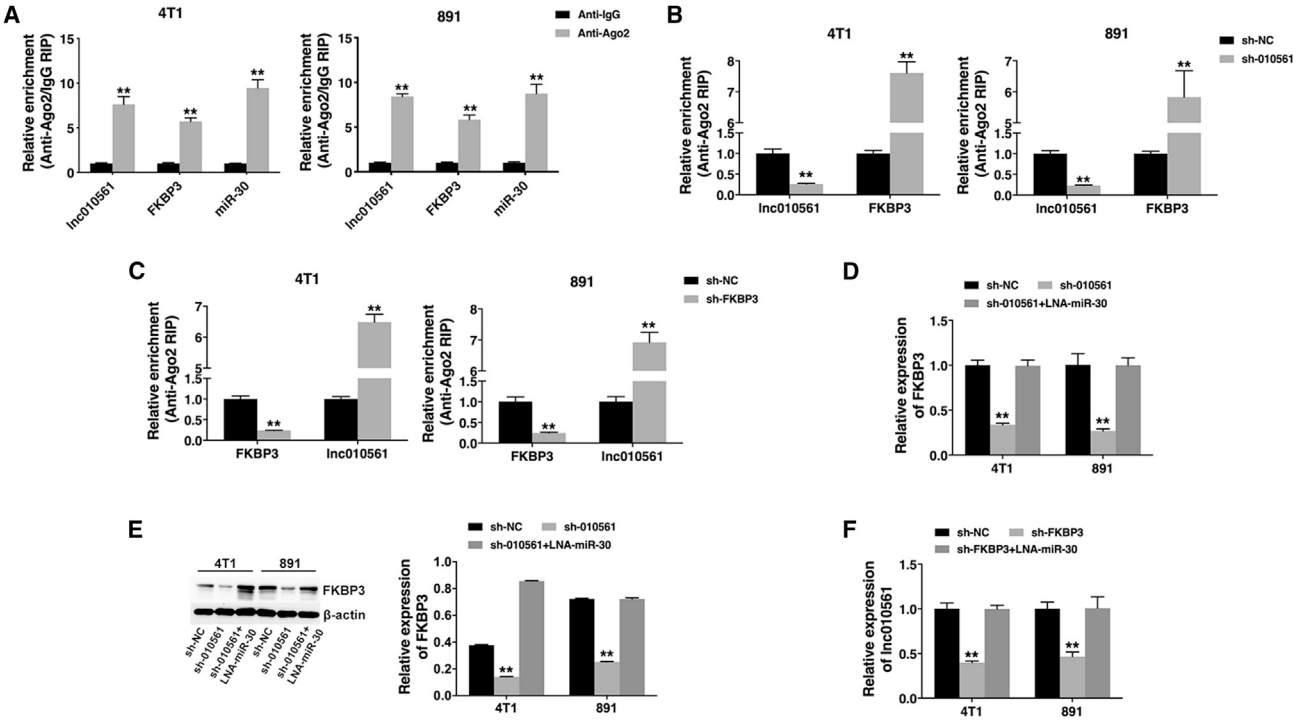

Figure 5. lnc010561 and FKBP3 Act as ceRNAs to Regulate miR-30

(A) RIP assay showed the enrichment of lnc010561, FKBP3, and miR-30 on Ago2 relative to IgG. (B) RIP assay on Ago2 after transfected with sh-010561. (C) RIP assay on Ago2 after transfected with sh-FKBP3. (D) Cells were transfected; then FKBP3 level was detected by qRT-PCR. (E) FKBP3 level was detected by western blotting (left) and quantified by ImageJ (right). (F) Cells were transfected; then lnc010561 level was detected by qRT-PCR. **$p < 0.01$.

puromycin. miR-30 mimics and inhibitors were purchased from GeneCopoeia.

Quantitative RT-PCR Analysis

Total RNA was extracted with TRIzol (Invitrogen). Cytoplasmic and nuclear RNA were extracted with PARISTM Kit (Invitrogen). PrimeScript RT Master Mix and SYBR Premix Ex Taq II (Takara, Japan) were used to conduct qRT-PCR with Bio-Rad CFX96 PCR System (USA). The primers were synthesized by Invitrogen (Table S1). For the miRNA, All in One miRNA qRT-PCR Detection Kit (GeneCopoeia) was used. Threshold cycle (CT) value was normalized to β-actin or U6 with the $2^{-\Delta\Delta Ct}$ method.

CCK-8 Assay

Cells (1×10^3) were seeded into 96-well plates, and CCK-8 solution (Dojindo Laboratories, Japan) was added 48 h after transfection. The absorbance at 450 nM was measured after incubation for 2 h at 37°C.

Colony Formation Assay

Cells (1×10^3) were seeded in six-well plates and incubated at 37°C for 2 weeks. Colonies were fixed in methanol, stained with 0.1% crystal violet, imaged, and counted.

Apoptosis Assay

Annexin V/propidium iodide staining and flow cytometry were performed with Andy Fluor 488 Annexin V/PI Kit (GeneCopoeia) according to the manufacturer's guidelines.

Transwell Assay

Transwell assays were performed with migration chambers (BD Biosciences, USA). Cells (1×10^4) were seeded, and medium with 10% FBS was added to the lower chamber as a chemoattractant. After 24 h, cells were fixed in methanol, stained with 0.1% crystal violet, imaged, and counted.

Mouse Xenograft Model

Cells (2×10^6) were subcutaneously injected into the dorsal flanks of 4-week-old female BALB/c nude mice (three mice per group). Xenografts were excised under anesthesia after 4 weeks, and tumor weights were measured.

For lung metastasis, cells (1×10^5) were injected through tail veins (three mice per group). After 8 weeks, the lungs were excised under anesthesia, and the numbers of macroscopically visible metastatic nodules were counted and validated by hematoxylin and eosin (H&E) staining.

All animal studies were approved and performed according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Sun Yat-Sen University Cancer Center.

Luciferase Reporter Assay

The FKBP3 3′ UTR or lnc010561 sequences, including the miR-30 binding sites, were inserted into the pGL3 luciferase vector (Promega, USA). Mutations in the miR-30 seed region were conducted with Fast Site-Directed Mutagenesis Kit (TIANGEN, China) and served as a mutant control. Luciferase intensity was measured by dual-luciferase reporter assay system (Promega).

Western Blot

In brief, proteins were extracted, quantified, and separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA); then incubated with 5% skim milk at room temperature for 1 h and with primary antibody of FKBP3 (1:1,000; Abcam, USA); and then incubated with horseradish peroxidase (HRP)-labeled secondary antibody (CST) and detected by chemiluminescence. Anti-β-actin antibody (1:1,000; Affinity, USA) was used as a control.

RIP Assay

Cells were transfected with MS2bs-lnc010561, MS2bs-010561-mt, or MS2bs-Rluc and MS2bp-GFP. After 48 h, RIP was performed with Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore). The RNA complexes were then purified, and the level of miR-30 was quantified. RIP assay on Ago2 was performed with anti-Ago2 antibody (Millipore), and the levels of lnc010561, FKBP3, and miR-30 were measured.

Statistical Analysis

Statistical analysis was performed with SPSS 19.0 software. Comparisons between groups were performed with t tests. Unless otherwise indicated, data are presented as the mean ± SD of three independent experiments. $p < 0.05$ was considered statistically significant.

Declarations

Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee of Sun Yat-Sen University Cancer Center Health Authority and was performed according to the ethical standards of the Declaration of Helsinki. All animal studies were approved and performed according to the guidelines of the IACUC of Sun Yat-Sen University Cancer Center.

Availability of Data and Materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2019.12.019.

AUTHOR CONTRIBUTIONS

H.T. and Xiaoming Xie designed the experiments. P.L., Xinhua Xie, and A.Y. performed the experiments. Y.K. and D.A.-G. analyzed and interpreted the data. Z.T. and L.Z. were the major contributors in writing the manuscript. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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