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Long noncoding RNA NORAD promotes the progression of retinoblastoma by sponging miR-136-5p/PBX3 axis

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Abstract. – OBJECTIVE: The specific roles of long noncoding RNAs (lncRNAs) have been found in human cancers, including retinoblastoma (RB). However, the function of lncRNA-NORAD has not been reported in RB. Therefore, the regulatory mechanism of lncRNA-NORAD was investigated in the development of RB.

PATIENTS AND METHODS: The experimental tissues were collected from 24 RB patients and 6 patients with ruptured globes. The average age of all patients was 2.78 years (range, 2 months to 11 years). The mRNA and protein expression was measured by Real Time-quantitative Polymerase Chain Reaction (RT-q and Western blot analysis. The functional mechanism of NORAD was assessed by Cell Count Kit-8 (CCK-8), transwell, and Dual-luciferase reporter assays.

RESULTS: Upregulation of NORAD and downregulation of miR-136-5p were found in RB. Functionally, knockdown of NORAD or miR-136-5p overexpression restrained cell viability, invasion, and migration. In addition, NORAD acts as a ceRNA of miR-136-5p in RB. miR-136-5p was found to directly target PBX3. Furthermore, knockdown of PBX3 inhibited the progression of RB. More importantly, the NORAD/miR-136-5p axis is involved in RB progression by mediating PBX3.

CONCLUSIONS: LncRNA NORAD, serving as a ceRNA of miR-136-5p, accelerates RB progression by upregulating PBX3.

Key words:
LncRNA NORAD, Retinoblastoma, MiR-136-5p, PBX3.

Introduction

Retinoblastoma (RB) is the most common intraocular tumor in children with family genetic predisposition[1]. RB can cause visual impairment and fundus changes. Failure to treat RB in time can result in intracranial or distant metastasis[2]. At present, chemotherapy, topical therapy, surgical treatment can be used to remove RB tumors and preserve vision. However, when the risk of tumor metastasis is high and the tumor volume exceeds half of the eyeball, eyeball removal should be considered. In addition, the prognosis in patients with RB is associated with tumor size and timing of treatment. Therefore, early detection and treatment are crucial to RB patients.

Long noncoding RNA (lncRNA) is a class of RNA molecules with a transcription length of more than 200 nt, which can regulate gene expression through chromatin remodeling, transcription regulation, and post-transcriptional processing. Although lncRNA does not encode proteins, their expression is still specific in different tissues and developmental stages[4]. In the pathogenesis of RB, many lncRNAs have been found to be involved in tumorigenesis. For example, lncRNA PVT1 expression was increased in RB, while knockdown of lncRNA PVT1 suppressed RB progression by sponging miR-488-3p[5]. In contrast, downregulation of lncRNA BDNF-AS was identified in RB and overexpression of lncRNA BDNF-AS restrained RB development[6]. Now, the role of lncRNA NORAD in other cancers has caught our attention. For example, NORAD was upregulated in epithelial ovarian cancer and promoted cancer cell functions by competing with miR-155-5p[7]. Additionally, the high expression of NORAD has been reported to predict poor prognosis in bladder cancer patients and promote bladder cancer clinical progression and metastasis[8]. However, the specific role of NORAD in RB is unclear.

In addition, NORAD was predicted to have a binding site with miR-136-5p in this study. Recently, miR-136-5p has been extensively investigated in human cancers. In lung squamous cell cancer and renal cell carcinoma, downregulation and inhibitory effect of miR-136-5p have been identified[9,10]. It has been reported[11] that lncRNA CRNDE promot-

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ed malignancy of glioma by downregulating miR-136-5p. However, the regulatory mechanism of NORAD/miR-136-5p axis is unknown in RB. Previous studies[12,13] have shown that miR-136-5p plays an important role in human cancers by regulating some target genes, such as HOXC10 and RASAL2. Here, Pre-B-cell leukemia homeobox 3 (PBX3) was found to have a binding site to miR-136-5p. It has been reported that PBX3 can regulate the progression of human cancers. For example, upregulation of PBX3 was detected in gastric cancer and promoted cell proliferation[14]. Similarly, PBX3 was also found to promote cell invasion and migration in colorectal cancer by activating the MAPK/ERK pathway[15]. Moreover, PBX3 has been proposed to regulate tumorigenesis by mediating some miRNAs, such as miR-144-3p and miR-320[16,17]. However, it is unclear whether miR-136-5p plays a role in RB by regulating PBX3.

In this study, the abnormal expression and function of lncRNA NORAD were detected in RB. Additionally, the possible functional mechanisms of NORAD/miR-136-5p/PBX3 axis in RB were elucidated. Our research will provide therapeutic targets for RB patients.

Patients and Methods

Clinical Tissues

RB tissues were obtained from 24 patients with RB during enucleation in [illegible] Hospital. Six normal retinal tissues were collected from the ruptured patient's eye. These tissues were frozen in liquid nitrogen and stored at [illegible]0°C for further experiments. Informed consents were obtained from all patients. This investigation was approved by the Institutional Ethics Committee of Yantaishan Hospital. This study was conducted in accordance with the Declaration of Helsinki.

[illegible] Line and Culture

Human [illegible] pigment epithelial cells ARPE-19 and [illegible] cell line Y79 were obtained from the American [illegible] Culture Collection (ATCC; Manassas, VA, USA). These cells were incubated in Roswell Park Memorial Institute-1640 (RPMI-1640) medium containing 10% fetal bovine serum (FBS; 37 °C, 5% CO_2, Gibco, Grand Island, NY, USA).

Cell Transfection

NORAD or PBX3 complementary DNA was inserted into pcDNA3.1 expression vector. NORAD and PBX3 siRNA or miR-136-5p mimics and inhibitor were purchased from GenePharma (Shanghai, China). Next, they were transfected into Y79 cells with Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA), respectively.

RNA Isolation, Reverse Transcription and Real Time-Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from RB tissues and cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA was reversely transcribed into complementary DNA (cDNA) using the PrimeScript RT reagent kit (TaKaRa, Dalian, China). RT-qPCR assay was performed using [illegible]-time PCR Mixture assay (TaKaRa, Dalian, China) with primers. The primers used were: NORAD forward, 5'-C[illegible] GGA[illegible]G T[illegible] GCG AAG T-3' and reverse, 5'-AGA [illegible]GG TGG GCA TTT-3'; miR-136-5p forward, 5'-ACA CTC CAG CTG GGA CTC [illegible] TTG TTT T-3' and reverse, 5'-[illegible]TG CAG [illegible] CCG AGG T-3'; U6-forward: 5'-GCT TCG GCA GCA CAT ATA CTA [illegible]A T-3' and reverse, 5'-CGC TTC ACG AAT T[illegible] CGT G[illegible] AT-3'; PBX3-forward: 5'-CAA GT[illegible]C AAT GTG-3' and reverse, 5'-ATG TAG CTC AGG GAA AAG TG-3'; GAPDH forward: 5'-AGA AGG CTG GGG CTC ATT [illegible] and reverse, 5'-AGG GGC CAT CCA CAG TCT TC-3'. NORAD and miR-136-5p expression was normalized to U6, while PBX3 was normalized to GAPDH. Their expressions were quantified with the $2^{-\Delta\Delta Cq}$ method.

Cell Counting Kit-8 (CCK-8) Assay

Transfected Y79 cells (2×10^3 cells/well) were seeded in 96-well plates and incubated in RPMI-1640 medium for 24, 48, 72 or 96 h, respectively. Then, the cells were incubated with 10 µL CCK-8 reagents for 4 h. Next, we discarded the medium and added dimethyl sulfoxide. After shaking for 10 min, OD490 was detected with a microplate reader (Olympus, Tokyo, Japan).

Transwell Assay

Cell invasion was detected in the upper chamber with Matrigel. Cell migration experiment was performed without Matrigel. After 30 min, Y79 cell suspension (2×10^3 cells/well) was added to the transwell upper chamber. Next, RPMI-1640 medium (10% FBS) was added to 24-well plates in lower chamber. After 24 h, 0.1% crystal violet was applied to stain the moving cells. Observation and photographing were performed by a light microscope.

Dual-Luciferase Reporter Assay

The 3'-UTR of wild-type and mutant NORAD (wt-NORAD and mut-NORAD) or PBX3 (wt-PBX3 and mut-PBX3) were amplified and inserted into the pmiR-GLO vector (Promega, Beijing, China). Then, the above reporter plasmids and miR-136-5p mimics were transfected into Y79 cells. After 48 h, luciferase activity was measured by a Dual-Luciferase reporter assay system (Promega, Madison, WI, USA).

Statistical Analysis

Data were analyzed using SPSS 19.0 (SPSS IBM, Armonk, NY USA) or GraphPad Prism 6 (La Jolla, CA, USA) and shown as mean ± SD. Differences between the two groups were analyzed using Student's *t*-test. Comparisons between multiple groups were performed using One-way ANOVA test followed by post-hoc test (Least Significant Difference). $p<0.05$ was considered to be a significant difference.

Results

The Dysregulation of NORAD and MiR-136-5p Was Found in RB

First, the expression of NORAD and miR-136-5p was examined in RB tissues and cell lines. We found that NORAD was upregulated in RB tissues compared to normal tissues (Figure 1A). Furthermore, NORAD expression in Y79 cells was higher than in ARPE-19 cell[illegible]). In contrast, miR-136-5p was do[illegible]egulated [illegible]RB tissues compared to normal t[illegible]s (Figure [illegible]C). Similarly, miR-136-5p ex[illegible]ssion [illegible] signi[illegible]nt-ly decreased in Y79 c[illegible] compared [illegible]E-19 cells (Figure 1D). T[illegible]e res[illegible] demo[illegible]ate that the dysregulatio[illegible] and [illegible]iR-136-5p is involved in th[illegible]morig[illegible]is of [illegible].

LncRNA [illegible] Acts as [illegible]ceRNA of MiR-136-5p [illegible]RB

[illegible]ition, Star[illegible] version 2.0 (http://starb[illegible].sysu.edu.cn/) predicts that miR-136-5p has a

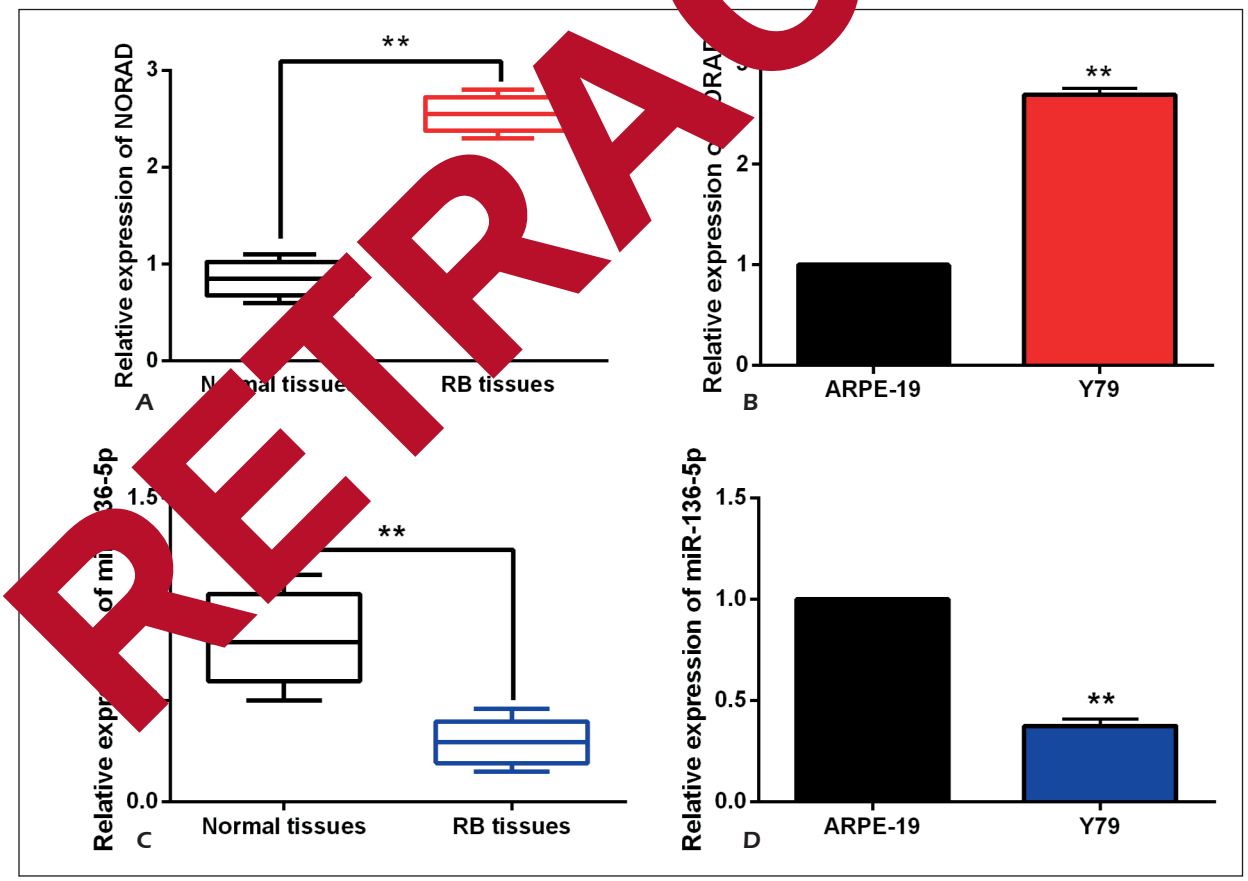

Figure 1. The dysregulations of NORAD and miR-136-5p were found in RB. **A**, NORAD expression in RB tissues. **B**, NORAD expression in Y79 and ARPE-19 cells **C**, MiR-136-5p expression in RB tissues. **D**, MiR-136-5p expression in Y79 and ARPE-19 cells. *$p< 0.01$.

binding site to NORAD (Figure 2A). Next, Dual-Luciferase reporter assay was performed in Y79 cells to further confirm their relationship. We found that miR-136-5p mimics significantly reduced the luciferase activity of wt-NORAD, but had little effect on mut-NORAD (Figure 2B). NORAD was also found to be negatively correlated with miR-136-5p expression in RB tissues (Figure 2C). Next, miR-136-5p mimics, miR-136-5p inhibitor, si-NORAD or NORAD vector was transfected into Y79 cells, respectively. RT-qPCR showed that NORAD expression was downregulated by si-NORAD and upregulated by NORAD vector (Figure 2D). Then, how NORAD regulates miR-136-5p expression was analyzed in Y79 cells. RT-qPCR showed that NORAD vector reduced miR-136-5p expression, while si-NORAD increased the expression of miR-136-5p (Figure 2D). Besides that, we found that miR-136-5p mimics enhanced its expression, while miR-136-5p inhibitor reduced its expression in Y[illegible] (Figure 2E). Additionally, miR-136-5[illegible]mimics r[illegible]ed

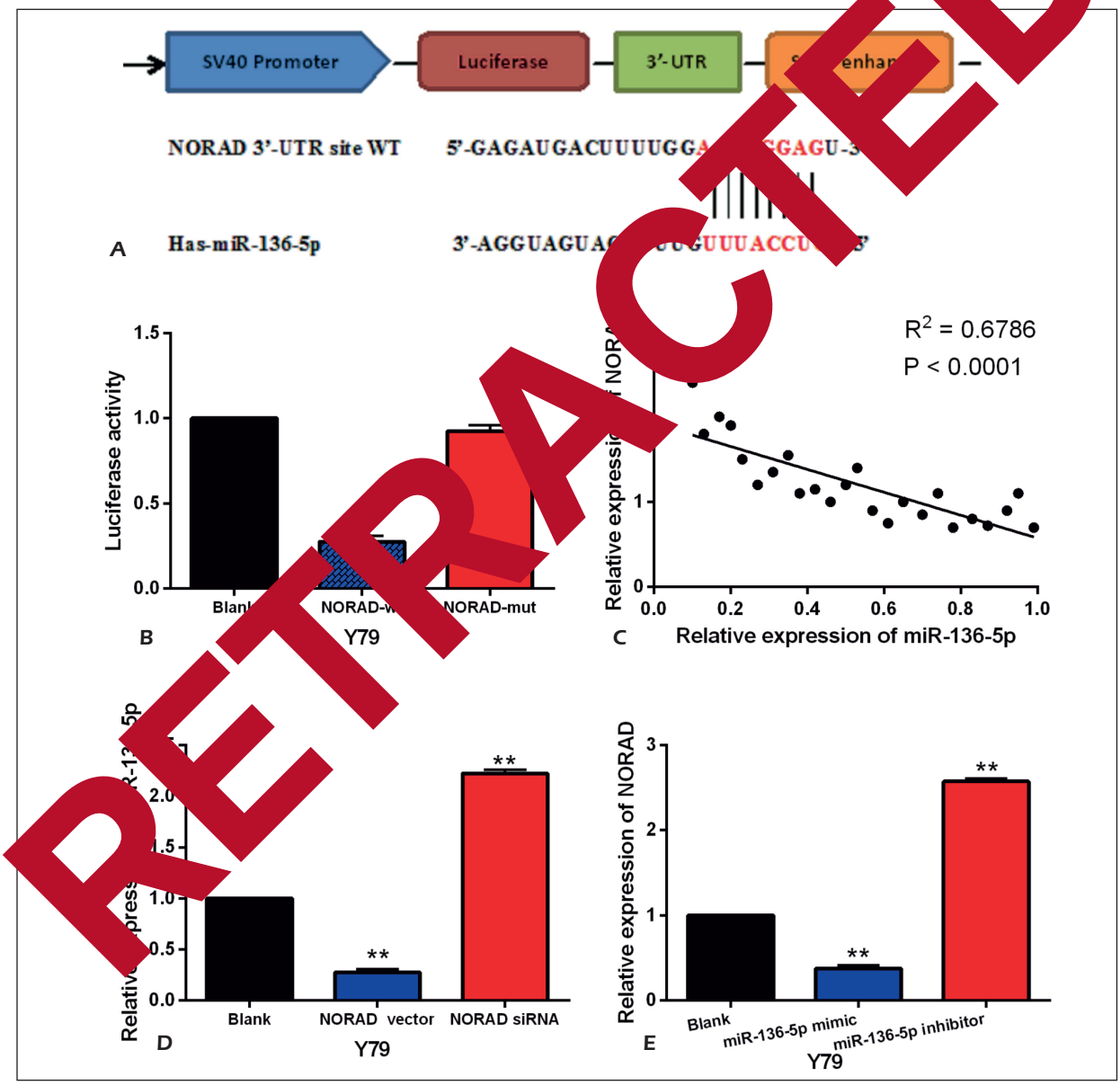

Figure 2. NORAD acts as a ceRNA of miR-136-5p in RB. **A**, The binding sites between NORAD with miR-136-5p. **B**, Luciferase reporter assay. **C**, MiR-136-5p inversely regulated NORAD expression in RB tissues. **D**, MiR-136-5p expression regulated by si-NORAD and NORAD vector in Y79 cells. **E**, NORAD expression in Y79 cells containing miR-136-5p mimics or inhibitor. $^{**}p<0.01$

NORAD expression, while miR-136-5p inhibitor enhanced NORAD expression in Y79 cells (Figure 2G). These findings reveal that NORAD acts as a ceRNA of miR-136-5p in RB.

NORAD/MiR-136-5p Axis Regulates the Progression of RB

To investigate the interaction between NORAD and miR-136-5p, miR-136-5p inhibitor was transfected into Y79 cells with si-NORAD. As expected, the decreased expression of NORAD induced by si-NORAD was restored by miR-136-5p inhibitor (Figure 3A). In addition, downregulation of NORAD restrained proliferation of Y79 cells. Downregulation of miR-136-5p abolished the inhibition of cell proliferation induced by si-NORAD in Y79 cells (Figure 3B). Knockdown of NORAD also inhibited cell migration and invasion in Y79 cells. MiR-136-5p inhibitor also restored si-NORAD mediated inhibition of cell migration and invasion (Figures 3C, 3D). Therefore, NORAD was considered to act as a tumor promoter in RB by targeting miR-136-5p.

Next, Y79 cells were transfected with NORAD vector and miR-136-5p mimics. We found that upregulation of NORAD reduced the incr[illegible] expression of miR-136-5p induced by its mi[illegible]s (Figure 3E). Functionally, cell migration, invasi[illegible] and proliferation were restrained b[illegible]R-136-5[illegible] overexpression. The reverse eff[illegible]o[illegible]RAD vector on miR-136-5p mimics [illegible]s also [illegible]ntified in Y79 cells (Figures 3F, 3G, a[illegible]H). [illegible]t imply that overexpressio[illegible] mi[illegible]5p inhibits RB progression by me[illegible]ng NOR[illegible]

PBX3 is a Dire[illegible] Target [illegible]MiR-136-5p

TargetScan [illegible]ttp://www.ta[illegible]can.org) predicts that [illegible]136-5[illegible]as a binding site to PBX3 (Figure 4A). [illegible]uci[illegible]ase r[illegible]porter assay showed that miR-136-[illegible]imic[illegible]duced the luciferase acti[illegible]y of wt-PB[illegible] had no effect on mut-PBX3 (Fi[illegible]e 4B). [illegible]rthermore, overexpression of miR-[illegible]duced PBX3 expression, whereas [illegible]nregulation of miR-136-5p promoted PBX3 [illegible]ression in Y79 cells (Figures 4C, 4D). In additi[illegible]upregulation of PBX3 was identified in RB tissues compared to normal tissues (Figure 4E). PBX3 was negatively correlated with miR-136-5p expressions in RB tissues (Figure 4F). On the contrary, NORAD was positively correlated with the expression of PBX3 in RB tissues (Figure 4G). Based on these findings, we think that PBX3 is a direct target of miR-136-5p and is positively regulated by NORAD in RB.

NORAD/MiR-136-5p Axis Regulates RB Progression by Mediating PBX3

To investigate the interaction between NORAD/miR-136-5p axis and PBX3, NORAD vector or miR-136-5p inhibitor was transfected into Y79 cells with si-PBX3. RT-qPCR showed that si-PBX3 reduced its expression in Y79 cells. But NORAD vector or miR-136-5p inhibitor restored this decrease in PBX3 expression (Fig[illegible]5A, 5B). More importantly, it was found th[illegible]n of PBX3 restrained Y79 cell pr[illegible]ration, w[illegible]as upregulation of NORAD or [illegible]nregulati[illegible]of miR-136-5p attenuated si[illegible]X3 i[illegible]d inhi[illegible]on of cell proliferation (F[illegible]e 5C). Sim[illegible]own-regulation of PBX3 [illegible]strain[illegible]ell mig[illegible]ion and invasion in Y79 [illegible]ll[illegible]r[illegible]ation [illegible]f NORAD or downregulati[illegible]f mi[illegible]-5p [illegible]ished the inhibitory eff[illegible]f si-PBX3 [illegible] cell migration, and inva[illegible](F[illegible]es 5D, 5E). Taken together, the NORAD/miR-136[illegible]axis promotes the develop-me[illegible]B by regul[illegible]PBX3 expression.

Discussion

Recently, many lncRNAs have been shown to [illegible]important roles in RB. For example, lncRNA [illegible]was upregulated in RB and acted as a RB tumor promoter[18]. In our study, upregulation of lncRNA NORAD was also found in RB. In addition, knockdown of NORAD restrained proliferation, migration and invasion of RB cells. Consistent with our results, NORAD expression was also found to increase in other malignancies, such as breast cancer and non-small cell lung cancer[19,20]. Furthermore, overexpression of NORAD has been found to promote cell invasion and migration in malignant melanoma[21]. In addition, NORAD promoted cell proliferation in human osteosarcoma by endogenously competing with miR-199a-3p[22]. The role of NORAD in other cancers is the same as our study. Here, NORAD was found to act as a molecular sponge of miR-136-5p in RB. MiR-136-5p inhibitor attenuated the inhibitory effect of NORAD downregulation in RB. Gao et al[23] reported that NORAD promoted cell proliferation and glycolysis of non-small cell lung cancer by sponging miR-136-5p, which is similar to our results.

In our research, the dysregulation of miR-136-5p was investigated in RB. It was identified that miR-136-5p was downregulated in RB, and overexpression of miR-136-5p inhibited the progression of RB. We also found that upregulation of NORAD impaired the inhibitory effect of miR-136-5p in RB.

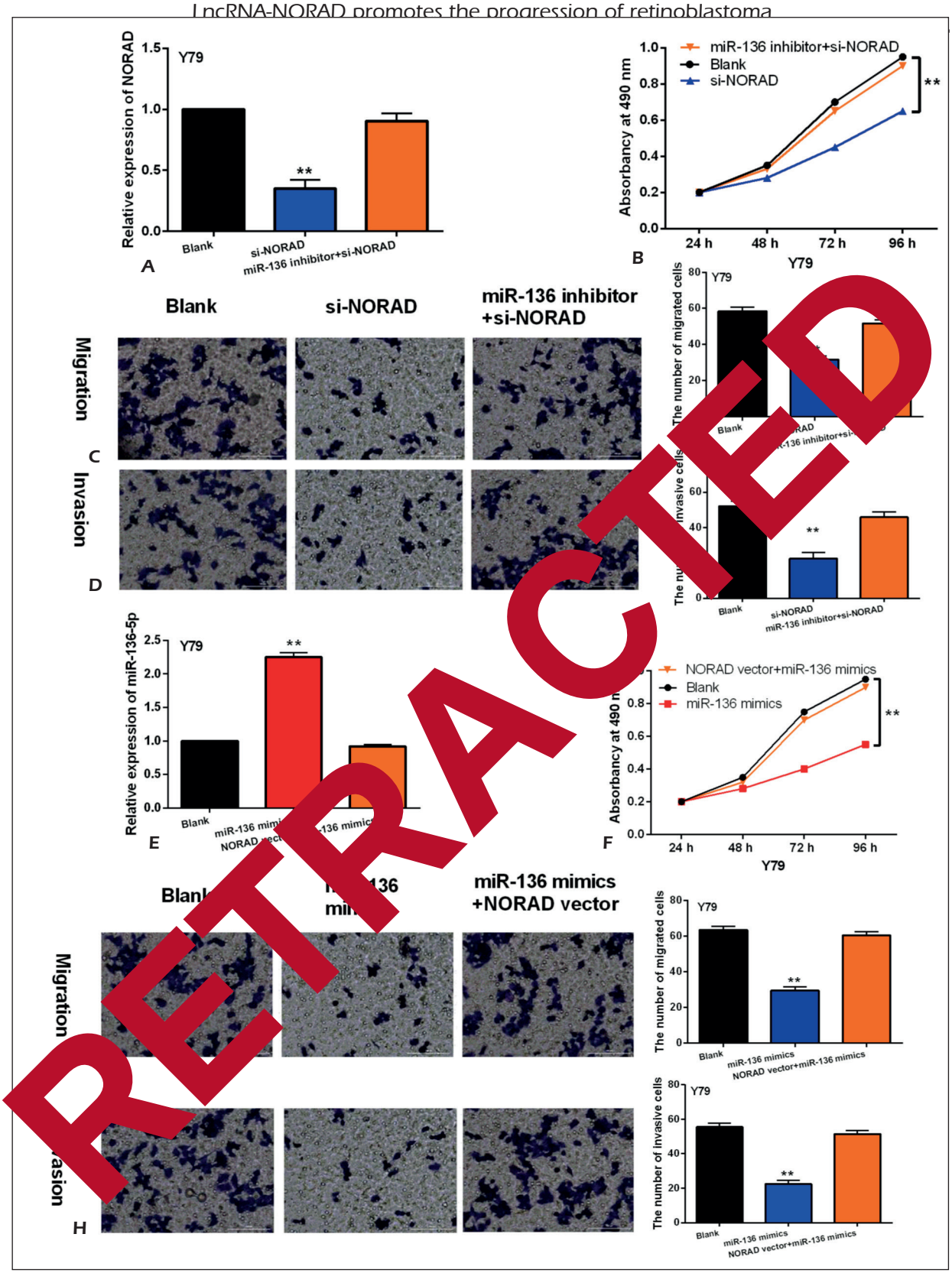

Figure 3. NORAD/miR-136-5p axis regulates the progression of RB. **A**, NORAD expression in Y79 cells with si-NORAD or si-NORAD +miR-136 inhibitor. **B-D**, Cell proliferation, migration and invasion in Y79 cells with si-NORAD or si-NORAD +miR-136 inhibitor (magnification, 200X). **E**, MiR-136-5p expression in Y79 cells with its mimics or NORAD vector+miR-136-5p mimics. **F-H**, Cell proliferation, migration and invasion in Y79 cells with miR-136-5p mimics or NORAD vector+miR-136-5p mimics (magnification, 200X). $^{**}p<0.01$

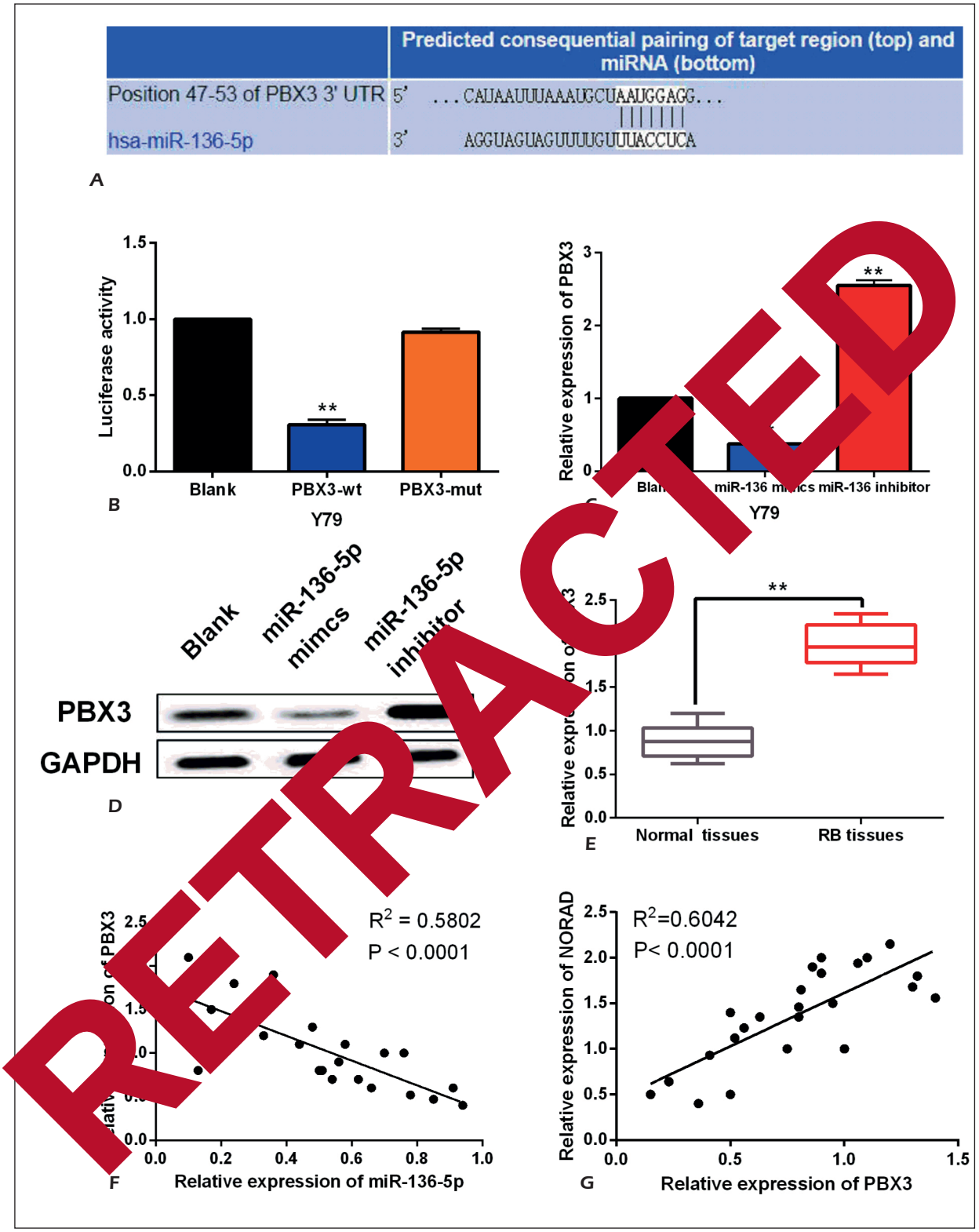

Figure 4. PBX3 is a direct target of miR-136-5p. **A**, MiR-136-5p has binding sites with PBX3. **B**, Luciferase reporter assay. **C-D**, PBX3 expression regulated by miR-136-5p mimics or inhibitor in Y79 cells. **E**, PBX3 expression in RB tissues. **F**, MiR-136-5p was negatively correlated with PBX3 in RB tissues. **G**, PBX3 was negatively correlated with NORAD in RB tissues. $^{**}p<0.01$.

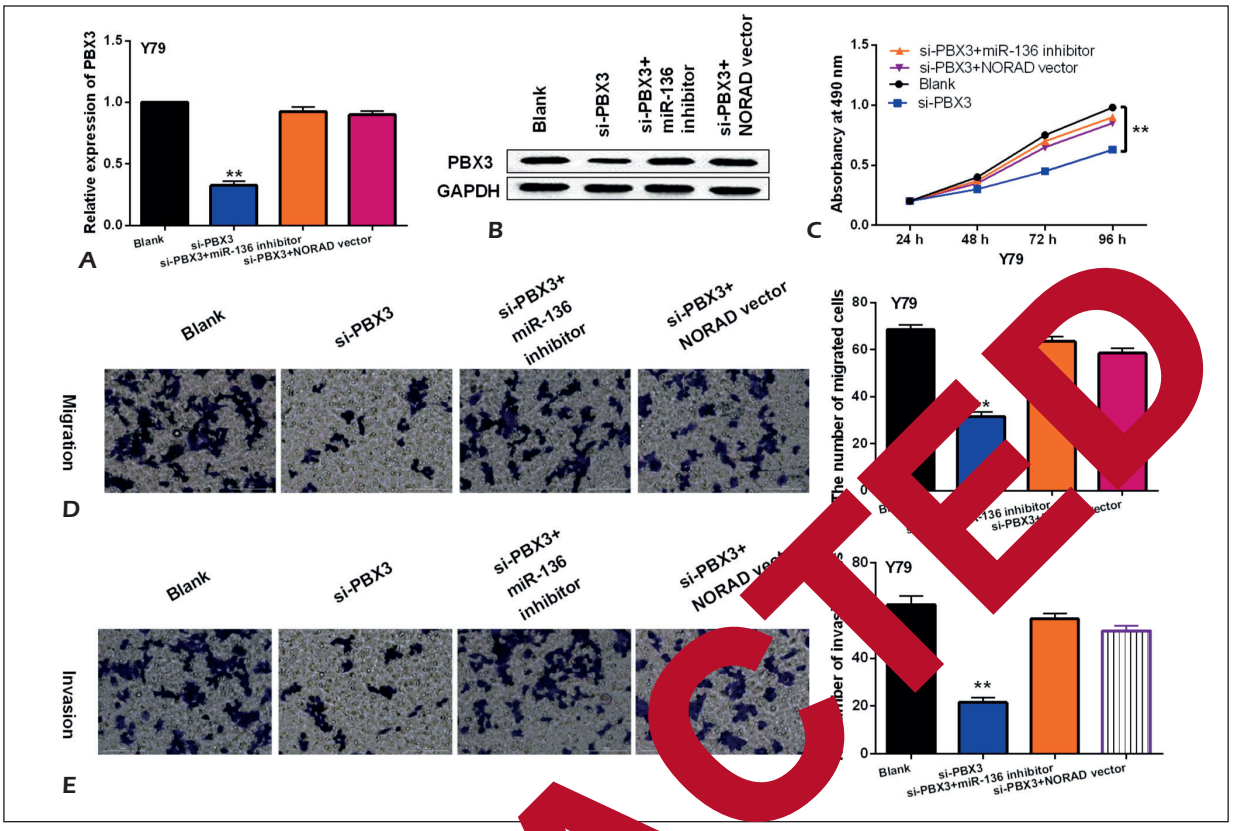

Figure 5. NORAD/miR-136-5p axis regulates RB pro[illegible]sion [illegible]ting PBX3. **A-B**, PBX3 expression in Y79 cells with si-PBX3, si-PBX3+miR-136 inhibitor or si-PBX+NORA[illegible]. **C-E**, [illegible]ell proliferation, migration and invasion in Y79 cells with si-PBX3, si-PBX3+miR-136 inhibitor [illegible]PBX+N[illegible] vector (magnification, 200X). $^{**}p<0.01$.

This indicates that NOR[illegible] and [illegible]136-5p are competitive endogen[illegible]RNAs. T[illegible] findings have not been repo[illegible]n [illegible]vious st[illegible]s. Similar to our resul[illegible] decrease[illegible]pression and inhibitory role [illegible]iR-136-5p we[illegible]lso detected in other cance[illegible]includ[illegible] gastric cancer and osteosarcoma[24,illegible]rtl[illegible]more, lncRNA CRNDE has been d[illegible]onst[illegible] to in[illegible]ase IRX5 expression and [illegible]o[illegible] the [illegible]pment of hepatocellular [illegible]inoma[illegible] medi[illegible]g miR-136-5p[26]. Here, we [illegible]fou[illegible]NORAD increased PBX3 expression competitively binding to miR-136-5p, thereby accelerating RB development.

MiR-[illegible]5p was observed to directly target PBX3 in this study. Furthermore, upregulation and carcinogenesis of PBX3 were detected in RB. Similar to our results, high expression of PBX3 was also detected in hepatocellular carcinoma and multiple myeloma[27,28]. Functionally, knockdown of PBX3 has been reported to restrain invasion, migration, and proliferation of melanoma cells[29]. The same effect of PBX3 was also found in RB. Upregulation of NORAD and downregulation of miR-136-5p can reverse the effect of PBX3 in RB, which has not been reported previously. It indicates that NORAD/miR-136-5p axis promotes the progression of RB by upregulating PBX3. Consistent with our results, it was reported that lncRNA UCA1 promoted proliferation and metastasis of lung tumor cells by regulating the miR-144/PBX3 axis[30]. These findings reveal that NORAD/miR-136-5p/PBX3 axis plays an important role in RB progression.

Conclusions

In summary, we first proved that NORAD/miR-136-5p/PBX3 axis is involved in the pathogenesis of RB. More importantly, lncRNA NORAD promotes the progression of RB by acting as a ceRNA of miR-136-5p and regulating PBX3 expression. This suggests that lncRNA NORAD is an important regulator in the progression of RB.

Conflict of Interests

The authors declare that they have no conflict of interests.

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