

Oncogenic Role of Long Noncoding RNAMALAT1 in Thyroid Cancer Progression through Regulation of the miR-204/IGF2BP2/m6A-MYC Signaling

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Accumulating studies highlight the role of long noncoding RNAs (lncRNAs)/microRNAs (miRNAs)/messenger RNAs (mRNAs) as important regulatory networks in various human cancers, including thyroid cancer (TC). This study aimed to investigate a novel regulatory network dependent on lncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) in relation to TC development. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) and western blot were initially employed to detect the expression of MALAT1, insulin-like growth factor 2 mRNA binding protein 2 (IGF2BP2), and myelocytomatosis (MYC) in TC cells. Interactions among MALAT1, miR-204, and IGF2BP2 were then identified *in vitro*. The biological processes of proliferation, migration, invasion, and apoptosis were evaluated *in vitro* via gain- and loss-of-function experiments, followed by *in vivo* validation using xenograft mice. Our data indicated that MALAT1 and IGF2BP2 were highly expressed, while miR-204 was poorly expressed in TC. IGF2BP2 was verified as a target of miR-204. MALAT1 was found to upregulate IGF2BP2 and enhance MYC expression via m6A modification recognition by competitively binding to miR-204, conferring a stimulatory effect on proliferation, migration, and invasion of TC cells, which was accompanied by weakened tumor growth and cell apoptosis. Altogether, the central findings of our study suggest that MALAT1 contributes to TC progression through the upregulation of IGF2BP2 by binding to miR-204.

INTRODUCTION

Thyroid cancer (TC) remains a notable endocrine malignancy that accounts for approximately 3% of all malignancies globally.¹ The morbidity and mortality rates of TC have exhibited drastic increases in past decades, making TC the 4th leading cause of cancer on a global scale.² TC occurrence has been attributed to a wide array of factors, including genetic factors, environmental exposure, and epigenetic alteration.³ Despite commendable advancements in terms of detection and surgical management techniques, including surgical resection, radiotherapy and hormone treatment,^{4,5} the cure rate for TC remains alarmingly low, highlighting the importance of devel-

oping more efficacious therapies. In order to identify more efficacious TC treatment approaches, an improved understanding of the underlying mechanisms associated with TC progression and pathogenesis is crucial.

Emerging evidence has emphasized the critical roles played by long noncoding RNAs (lncRNAs) in the regulation of TC initiation and progression.⁶ For instance, the oncogenic property of lncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) has been implicated in papillary TC and may even possess the potential to function as a diagnostic marker for TC.⁷ Upregulated lncRNA MALAT1 levels are also known to exacerbate the progression of anaplastic TC by means of regulating microRNA (miR)-200a-3p.⁸ Similarly, existing literature has indicated that lncRNA MALAT1 competitively binds to miR-204 and regulates its expression in lung adenocarcinoma.⁹ Notably, the expression of miR-204 has been linked with various diseases. In addition, the overexpression of miR-204 has been reported to repress breast cancer cell proliferation and metastasis properties through its inhibitory role on phosphorylated phosphatidylinositol 3-kinase and phosphorylated protein kinase B expression.¹⁰ Moreover, miR-204 has been reported to play a tumor suppressor role illustrated by its ability to force the sensitivity of ovarian cancer cells to chemotherapy.¹¹ Furthermore, augmented expression of miR-204 has been demonstrated to restrain the proliferation and invasion capacities of papillary TC cells.¹² Meanwhile, insulin-like growth factor 2 mRNA binding protein 2 (IGF2BP2) has been identified as a target of miR-204 that negatively regulates IGF2BP2 expression in clear cell renal cell carcinoma cells.¹³ IGF2BP2 is detected to be significantly overexpressed in cancer tissues, and correlates to short overall survival and disease-free survival of patients suffering from papillary TC.¹⁴ Intriguingly, we conducted

Received 25 February 2020; accepted 21 September 2020;
<https://doi.org/10.1016/j.omtn.2020.09.023>

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microarray analysis and found that IGF2BP2 expression is significantly upregulated in the TC tumor samples from the Gene Expression Omnibus (GEO): GSE97001 dataset in the GEO database and The Cancer Genome Atlas (TCGA). Elevated levels of IGF2BP2 were inversely proportional to the survival rate of patients with TC. Therefore, we hypothesized that the miR-204-dependent IGF2BP2 axis may be involved in the modulation of lncRNA MALAT1 on TC development. In addition, as previously reported, IGF2BP2 possesses the ability to recognize modifications in the RNA N6-methyladenosine (m6A), which is a regulatory mechanism for its gene expression in the myelocytomatosis (MYC) gene and subsequently elevates the MYC expression.^{15,16} MYC has been reported to also initiate the progression of human anaplastic TC,¹⁷ highlighting the possible involvement of IGF2BP2 in the initiation of TC. In accordance with the aforementioned exploration of literature, the current study aimed to elucidate the specific mechanism of the lncRNA MALAT1/miR-204/IGF2BP2/m6A-MYC axis in TC.

RESULTS

miR-204 Targets and Downregulates IGF2BP2 Expression in TC

Previous research has documented significantly decreased levels of miR-204 in TC, highlighting its potential as a TC marker;¹⁸ however, the underlying mechanism by which miR-204 influences TC remains unclear. Hence we initially predicted the downstream target genes of miR-204 using mirDIP (<http://ophid.utoronto.ca/mirDIP/index.jsp#r>), TargetScan (http://www.targetscan.org/vert_71/), starBase (<http://starbase.sysu.edu.cn/>), and RNA22 (<https://cm.jefferson.edu/rna22/Precomputed/>) databases. In addition, using the GEO database (<https://www.ncbi.nlm.nih.gov/geo/>), the TC expression dataset GEO: GSE97001 was obtained, and the gene expressions retrieved from the dataset were subjected to differential analysis with $|\log\text{Fold-Change}| > 1$ and $p < 0.05$ serving as the criteria. The upregulated genes in tumors following differential analysis were overlapped with the predicted targets of miR-204 (Figure 1A), revealing a total of 61 potential target genes. Among these genes, IGF2BP2 expression was found to be markedly upregulated in the tumor samples from the GEO: GSE97001 dataset (Figure 1B). Moreover, analysis from TCGA also indicated that IGF2BP2 was highly expressed in TC samples (Figure 1C), while elevated levels of IGF2BP2 were inversely proportional to the survival rate of patients succumbing to TC (Figure 1D). Based on these findings, we asserted the notion that miR-204 possesses the ability to target and inhibit IGF2BP2 in TC. In order to verify this, we detected the miR-204 and IGF2BP2 expression in TC cells using quantitative reverse transcription polymerase chain reaction (qRT-PCR). The results (Figure 1E) provided data indicating that miR-204 expression in BCPAP, TPC-1, and SW579 cell lines was decreased, whereas IGF2BP2 was elevated when compared with the normal thyroid cell line Nthy-ori 3-1. Notably, the SW579 cell line exhibited the most dramatic changes in terms of miR-204 and IGF2BP2 expressions, and therefore was selected as the study subject. Figure 1F illustrates that the luciferase activity of IGF2BP2-3' untranslated region (UTR)-wild-type (WT) was diminished in cells transfected with miR-204 mimic, whereas no such effect was detected in the IGF2BP2-3' UTR mutant (mut), indicating that miR-204 could

target IGF2BP2 by binding to its 3' UTR. Additionally, qRT-PCR and western blot analysis results revealed evidence of elevated miR-204 and a decline in IGF2BP2 levels in cells transfected with miR-204 mimic, while IGF2BP2 level significantly increased in cells transfected with miR-204 inhibitor (Figures 1G and 1H). Taken together, the aforementioned findings supported the notion that miR-204 targeted IGF2BP2 and curtailed its expression in TC.

miR-204 Suppresses Proliferation, Invasion, and Migration in TC Cells while Augmenting Their Apoptosis by IGF2BP2 Inhibition

After uncovering that miR-204-targeted IGF2BP2 in TC cells, we subsequently set out to ascertain whether miR-204 regulated the biological functions of TC cells via IGF2BP2. SW579 cells were treated with miR-204 mimic or IGF2BP2 overexpression plasmid (oe-IGF2BP2). The results of western blot analysis provided data suggesting that the expression of IGF2BP2 was diminished after the cells had been treated with miR-204 mimic, whereas this effect was reversed following treatment with miR-204 mimic + oe-IGF2BP2 (Figure 2A). Meanwhile, the application of Cell Counting Kit-8 (CCK-8) assay demonstrated that SW579 cell proliferation was diminished as a result of miR-204 mimic treatment, whereas enhanced cell proliferation was noted after IGF2BP2 overexpression. Moreover, transfection with miR-204 mimic + oe-IGF2BP2 brought about an increase in SW579 cell proliferation relative to miR-204 mimic + oe-negative control (NC) transfection (Figure 2B). Transwell assay revealed that cell migration and invasion were reduced in the presence of miR-204 mimic, whereas overexpression of IGF2BP2 led to the opposite results. Meanwhile, simultaneous overexpression of miR-204 and IGF2BP2 induced an enhancement in cell migration and invasion (Figure 2C). As depicted in Figure 2D, flow cytometry revealed that cell apoptosis was markedly elevated following miR-204 mimic transfection, which was abolished upon transfection with miR-204 mimic + oe-IGF2BP2. Similarly, a diminished rate of cell apoptosis was detected in IGF2BP2-overexpressing SW579 cells. Taken together, the aforementioned results suggested that miR-204 could impede the proliferation, migration, and invasion potentials and potentiate apoptosis of TC cells via IGF2BP2 inhibition.

lncRNA MALAT1 Elevates the IGF2BP2 Expression by Binding to miR-204 in TC Cells

Then we intended to investigate the underlying upstream mechanism by which miR-204 affects TC. Following literature retrieval, we found that miR-204 is regulated by MALAT1, and that MALAT1 can positively regulate the expression of Smad4 by sponging miR-204, and thus promote the osteogenic differentiation of valve interstitial cells.¹⁹ In addition, MALAT1 upregulates the expression of SLUG, a target gene of miR-204, by competitively binding to miR-204 both *in vitro* and *in vivo*, which promotes epithelial-mesenchymal transition (EMT) and metastasis of lung adenocarcinoma cells.⁹ In the current study, the starBase database (<http://starbase.sysu.edu.cn/>) predicted that the lncRNA MALAT1 could bind to miR-204 and consequently regulate its expression (Figure 3C). Subsequent qRT-PCR results indicated that lncRNA MALAT1 expression in SW579 cell lines was notably higher compared with that in the Nthy-ori 3-1 cell line

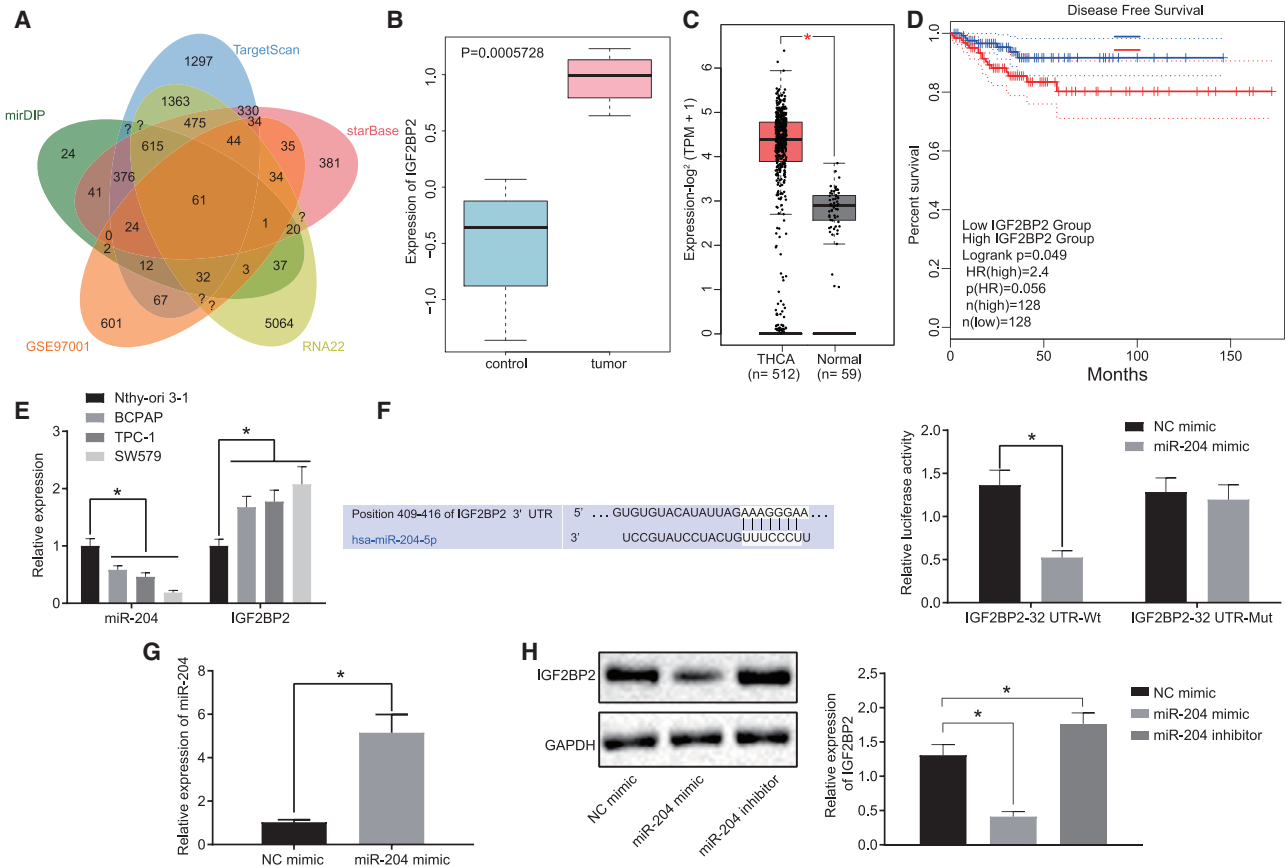


Figure 1. IGF2BP2 Is a Target Gene of miR-204 in TC

(A) The intersection of the predicted downstream targets of miR-204 and differentially expressed genes in the GEO: GSE97001 dataset. The five ovals in the figure respectively represent the predicted results and differential analysis results, and the middle part represents the intersection of five groups of data. (B) The differential expression of IGF2BP2 gene in the GEO: GSE97001 dataset. The abscissa represents the sample number, and the ordinate represents the differentially expressed gene. Blue boxplot indicates control samples, and red boxplot indicates tumor samples. (C) Differential expression of IGF2BP2 in the TCGA database; red boxplot represents tumor samples, and gray boxplot represents normal samples ($p < 0.01$). (D) Disease-free survival analysis of patients with high (blue line) or low (red line) IGF2BP2 expression in TC. The dotted line indicates 95% confidence interval. (E) The expression patterns of miR-204 and IGF2BP2 in TC cells determined by qRT-PCR, normalized to U6 and GAPDH, respectively. (F) The binding of miR-204 to IGF2BP2 3' UTR predicted online and confirmed by dual-luciferase reporter gene assay. (G) Transfection efficiency detected by qRT-PCR. (H) Western blot analysis of IGF2BP2 protein levels in cells following miR-204 mimic/inhibitor transfection, normalized to GAPDH. Data (mean \pm standard deviation) between two groups were compared with unpaired t test. The experiment was repeated three times independently. $*p < 0.05$. THCA, thyroid cancer.

(normal thyroid cell line) (Figure 3A), corresponding to lower miR-204 expression in the SW579 cell line relative to the Nthy-ori 3-1 cell line. The results of cellular localization of lncRNA MALAT1 detected by fractionation of nuclear/cytoplasmic RNA assay are depicted in Figure 3B, which suggested that lncRNA MALAT1 was expressed both in the nucleus and the cytoplasm. In addition, luciferase activity was observed to be reduced in cells co-transfected with miR-204 mimic and pGL3-MALAT1 ($p < 0.05$), but unaltered in cells co-transfected by miR-204 mimic and pGL3-MALAT1-mut ($p > 0.05$; Figure 3C). Meanwhile, RNA binding protein immunoprecipitation (RIP) assay results indicated that lncRNA MALAT1 and miR-204 expressions were both considerably higher following Argonaute2 (Ago2) treatment relative to immunoglobulin G (IgG) treatment ($p < 0.05$; Figure 3D). Next, the influence of lncRNA MALAT1 on

the expressions of miR-204 and IGF2BP2 was investigated following cell transfection. qRT-PCR and western blot analysis results illustrated upregulated miR-204 and downregulated IGF2BP2 levels in lncRNA MALAT1-silenced cells, which was abrogated by treatment with short hairpin RNA (sh)-MALAT1 + miR-204 inhibitor (Figures 3E and 3F). The obtained results above suggested that lncRNA MALAT1 could upregulate IGF2BP2 by binding to miR-204 in TC cells.

IGF2BP2 Elevates MYC Expression by Recognizing the m6A Modification of MYC in TC Cells

After the upstream mechanism, we shifted our focus to investigating the downstream regulatory mechanism of IGF2BP2 in TC. The results from western blot analysis demonstrated that compared with the normal thyroid cell line Nthy-ori 3-1, MYC protein levels were

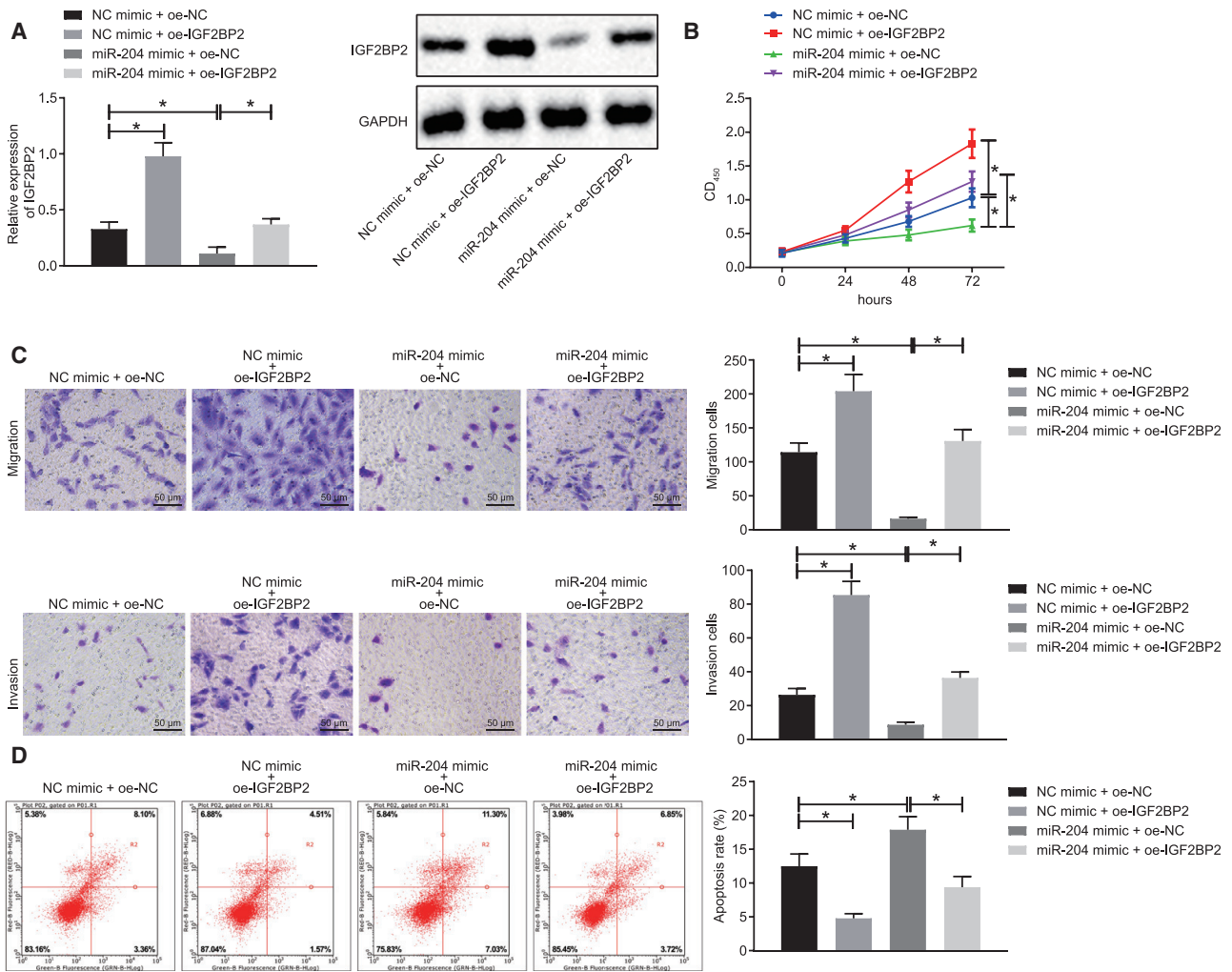


Figure 2. miR-204 Restraints Proliferation, Migration, and Invasion of TC Cells but Induces Cell Apoptosis through Repressing IGF2BP2

SW579 cells were treated with NC mimic + oe-NC, miR-204 mimic + oe-NC, NC mimic + oe-IGF2BP2, or miR-204 mimic + oe-IGF2BP2. (A) Western blot analysis of IGF2BP2 protein levels in cells, normalized to GAPDH. (B) Cell proliferation assessed by CCK-8 assay. (C) Cell migration and invasion measured by Transwell assay (original magnification $\times 200$). (D) Cell apoptosis measured by flow cytometry. Data (mean \pm standard deviation) between two groups were compared using unpaired t test, while those among multiple groups were compared with one-way ANOVA, followed by Tukey's post hoc test. The experiment was repeated three times independently. * $p < 0.05$.

higher in the TC cell line SW579 (Figure 4A). METTL14 represents a m6A modification enzyme of MYC. In order to diminish the m6A modification level of MYC in TC cells, METTL14 was silenced in the SW579 cells, with Figure 4B illustrating reduced METTL14 protein levels in METTL14-silenced SW579 cells. In addition, our data revealed that METTL14 silencing triggered a decrease in the m6A modification of MYC and m6A expression in cells, which was detected using Me-RIP and immunoprecipitation (IP) assays (Figure 4C). Meanwhile, western blot analysis demonstrated that sh-METTL14-treated SW579 cells exhibited diminished MYC protein levels (Figure 4D). RIP assay subsequently demonstrated that IGF2BP2 bound to the enriched mRNA m6A of MYC and when METTL14 was silenced, mRNA m6A of MYC binding to IGF2BP2 was diminished (Figure 4E), indicating that IGF2BP2 recognized

the m6A modification of MYC. In addition, results from western blot analysis highlighted a reduction in IGF2BP2 and MYC protein expression in sh-IGF2BP2-transfected SW579 cells (Figure 4F). Altogether, the aforementioned findings indicated that IGF2BP2 could upregulate MYC by recognizing the m6A modification of MYC in TC cells.

IncrNA MALAT1 Silencing Curbs Proliferation, Invasion, and Migration in TC Cells while Potentiating Their Apoptosis via the miR-204/IGF2BP2/m6A-MYC Axis

We subsequently set out to explore the influence of lncRNA MALAT1 on TC cells via regulation of the miR-204/IGF2BP2/m6A-MYC axis; SW579 cells were treated with sh-NC + NC inhibitor, sh-MALAT1 + NC inhibitor, or sh-MALAT1 + miR-204 inhibitor. As illustrated in

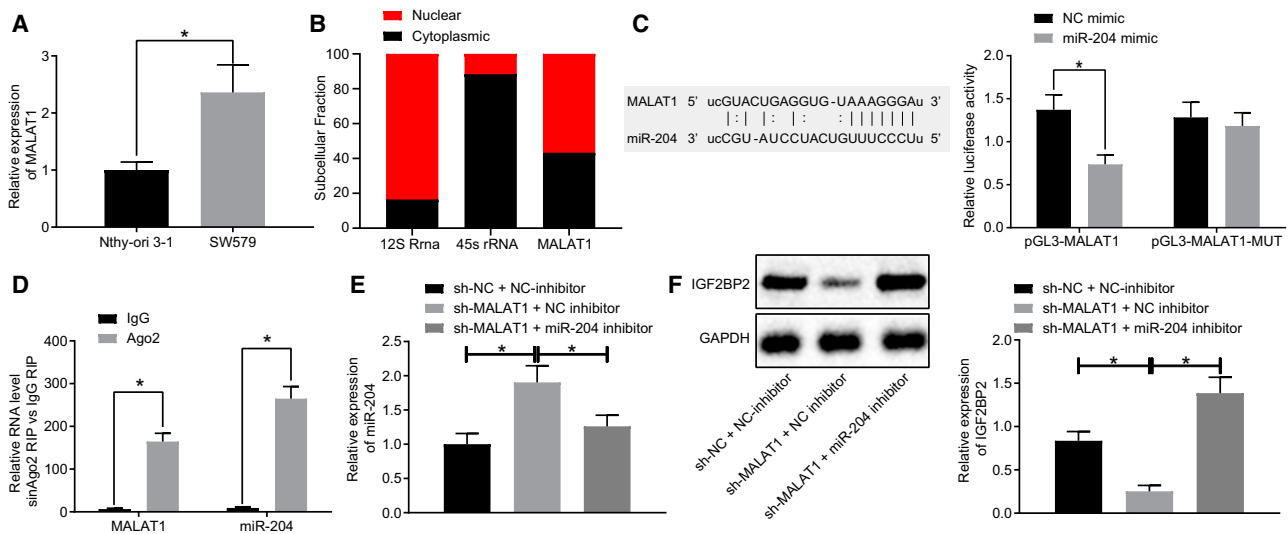


Figure 3. IncRNA MALAT1 Binds to miR-204, Leading to Upregulated IGF2BP2 Expression in TC Cells

(A) IncRNA MALAT1 expression patterns in TC cells determined by qRT-PCR, normalized to GAPDH. (B) Cellular localization of IncRNA MALAT1 detected by fractionation of nuclear/cytoplasmic RNA. (C) The binding of miR-204 to IncRNA MALAT1 confirmed by dual-luciferase reporter gene assay. (D) The enrichment of miR-204 and IncRNA MALAT1 in Ago2 and IgG detected by RIP assay. (E) Expression of miR-204 and IncRNA MALAT1 in TC cells after inhibition of miR-204 and IncRNA MALAT1 determined by qRT-PCR, normalized to U6. (F) Western blot analysis of IGF2BP2 protein levels in TC cells after inhibition of miR-204 and IncRNA MALAT1, normalized to GAPDH. Data (mean \pm standard deviation) between two groups were compared using unpaired t test, while those among multiple groups were compared with one-way ANOVA, followed by Tukey's post hoc test. The experiment was repeated three times independently. * $p < 0.05$.

Figure 5A, RIP assay illustrated that MYC mRNA binding to IGF2BP2 was reduced as a result of IncRNA MALAT1 silencing, which was normalized in cells transfected with sh-MALAT1 + miR-204 inhibitor. Moreover, western blot analysis revealed a downward trend in MYC protein levels in IncRNA MALAT1-silenced SW579 cells, whereas an opposite trend was identified following sh-MALAT1 + miR-204 inhibitor treatment (Figure 5B). Altogether, the above results suggested that IncRNA MALAT1 silencing resulted in downregulation of miR-204-dependent IGF2BP2 to inhibit the m6A/MYC expression in TC cells.

Figures 5C–5E depict the cellular function assessment results, which suggested that the proliferation, invasion, and migration were all markedly reduced, whereas cell apoptosis was enhanced in IncRNA MALAT1-silenced cells, which was neutralized by transfection with miR-204 at the same time. Taken together, IncRNA MALAT1 silencing could potentially possess the ability to curtail TC cell proliferation, invasion, and migration abilities, and trigger cell apoptosis via the miR-204/IGF2BP2/m6A-MYC axis.

Silencing IncRNA MALAT1 Retards TC Tumor Growth via IGF2BP2 Suppression *In Vivo*

Finally, we explored the effects associated with IncRNA MALAT1 and IGF2BP2 on transplanted tumors in nude mice *in vivo*. As exhibited in Figures 6A–6C, the silencing of IncRNA MALAT1 was accompanied by reductions in the tumor volume and weight of mice, which was normalized in mice treated with sh-MALAT1 + oe-IGF2BP2.

In addition, the qRT-PCR results revealed a decline in the expressions of IncRNA MALAT1, IGF2BP2, and MYC, and an elevation in the miR-204 expression in mice following IncRNA MALAT1 silencing. Moreover, immunohistochemical staining showed that the expression of MYC was diminished in response to sh-MALAT1 treatment alone, whereas heightened levels were identified following treatment with sh-MALAT1 + oe-IGF2BP2 (Figure 6D). TUNEL staining further revealed that cell apoptosis was promoted in the presence of sh-MALAT1 + oe-NC, the effects of which were abrogated by oe-IGF2BP2 delivery (Figure 6E). However, the expression of IGF2BP2 and MYC was enhanced, whereas that of IncRNA MALAT1 and miR-204 remained unchanged in mice transplanted with cells transfected with sh-MALAT1 + oe-IGF2BP2 compared with those in mice transplanted with sh-MALAT1 + oe-NC cells (Figure 6F). Altogether, the results obtained suggested that depletion of IncRNA MALAT1 could inhibit the MYC expression and consequently inhibit the growth of tumors in mice via miR-204 upregulation.

Effect on Migration and Invasion Is Not Affected by Increased Apoptosis on the Expression of miR-204

The TC cells were treated with 50 μ M Z-VAD-FMK for 24 h for Transwell assay in order to detect cell migration and invasion. Our obtained results revealed that miR-204 mimic induced suppression of the migration and invasion of TC cells, whereas no significant difference was observed in relation to the migration and invasion of TC cells following the treatment of miR-204 mimic + Z-VAD-FMK (apoptosis inhibitor) relative to the cells following miR-204 mimic

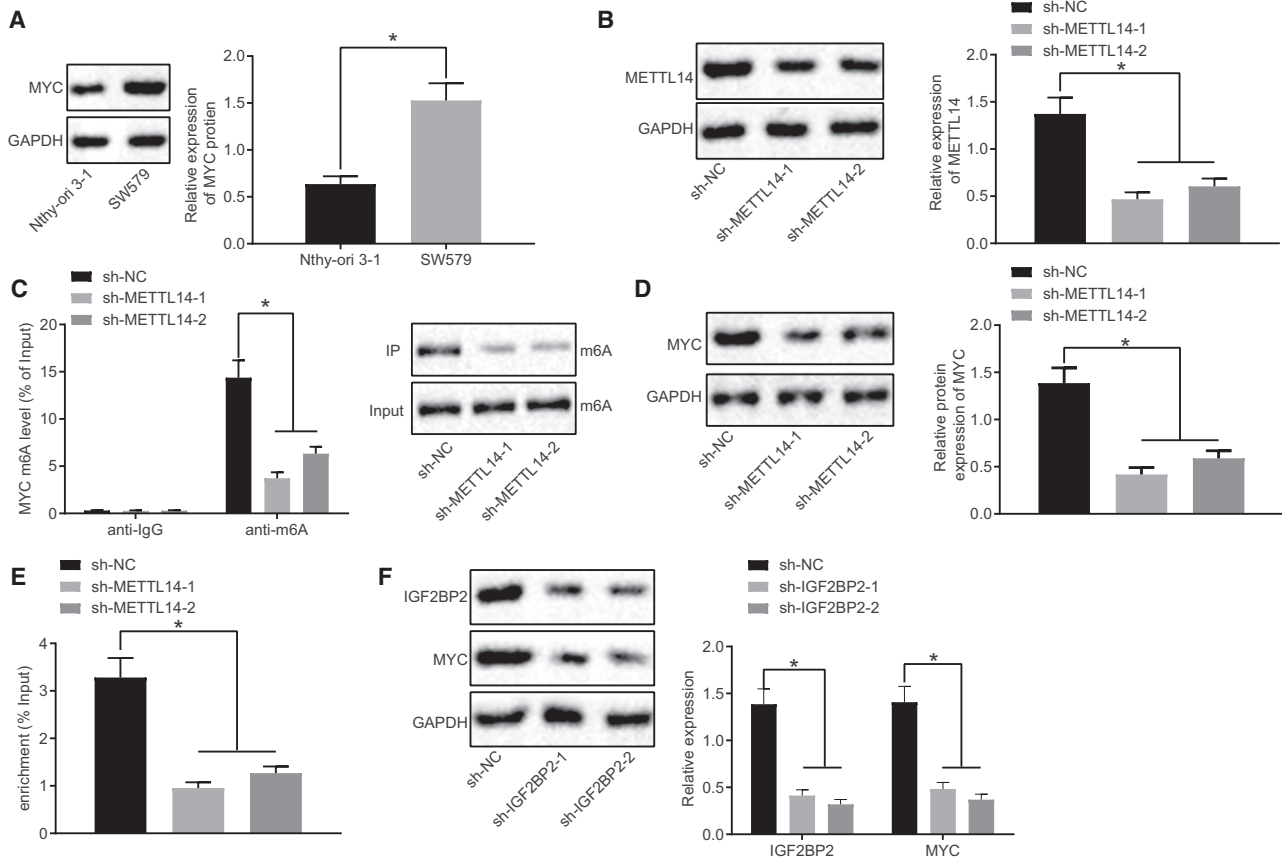


Figure 4. IGF2BP2 Promotes MYC Expression by Recognizing the m6A Modification of MYC in TC Cells

(A) Western blot analysis of MYC protein levels in Nthy-ori 3-1 and SW579 cells, normalized to GAPDH. (B) The silencing efficiency determined by western blot analysis. (C) m6A modification levels of MYC and m6A expression in cells after METTL14 silencing measured by Me-RIP and IP assays. (D) Western blot analysis of MYC protein levels in METTL14-silenced cells, normalized to GAPDH. (E) Binding of IGF2BP2 to MYC assessed by RIP assay. (F) Western blot analysis of MYC and IGF2BP2 protein levels in IGF2BP2-silenced cells, normalized to GAPDH. Data (mean \pm standard deviation) were compared with unpaired t test. The experiment was repeated three times independently. * $p < 0.05$.

treatment alone (Figure S1). Taken together, these findings suggested that the effect of miR-204 on TC migration and invasion was not dependent on apoptosis.

DISCUSSION

Accumulating evidence has continued to implicate lncRNA MALAT1 in the clinicopathological features of a wide array of human cancers, and even highlight its potential as a prognostic marker.²⁰ However, the molecular mechanisms by which the lncRNA MALAT1 influences TC remain largely unknown. Thus, we designed the current study to analyze the potential role of lncRNA MALAT1 in TC together with the aim of elucidating the underlying molecular mechanisms. Our findings unveiled that lncRNA MALAT1 led to an increase in the expression of IGF2BP2 by binding to miR-204, and IGF2BP2 could recognize the m6A modification of MYC to elevate MYC expression, which ultimately accelerated the proliferation, invasion, and migration abilities of TC cells and impeding cell apoptosis.

Initially, we identified that miR-204 was poorly expressed, whereas IGF2BP2 and lncRNA MALAT1 were highly expressed in TCs. Consistently, lncRNA MALAT1 has been previously noted to be abundantly expressed in papillary TC tissues when compared with corresponding noncancerous tissues.⁷ Similarly, a previous study highlighted that miR-204 was drastically downregulated in papillary TC tissues and went on to conclude that it holds the potential to function as a diagnostic biomarker for papillary TC.¹⁸ Another study also demonstrated that miR-204 levels were diminished in TC specimens and cell lines.²¹ Interestingly, based on bioinformatics analysis, we identified that miR-204 targets IGF2BP2, which is highly expressed in TC tumors, that has an inverse association with the survival rate of TC patients. The oncogenic property of IGF2BP2 has been well documented in various types of solid cancers. For instance, augmented levels of IGF2BP2 have been detected in acute myelocytic leukemia primary cells.²² Moreover, existing literature has indicated that IGF2BP2 is often amplified in pancreatic cancer tissues versus normal pancreatic tissues and associated with poor PC survival

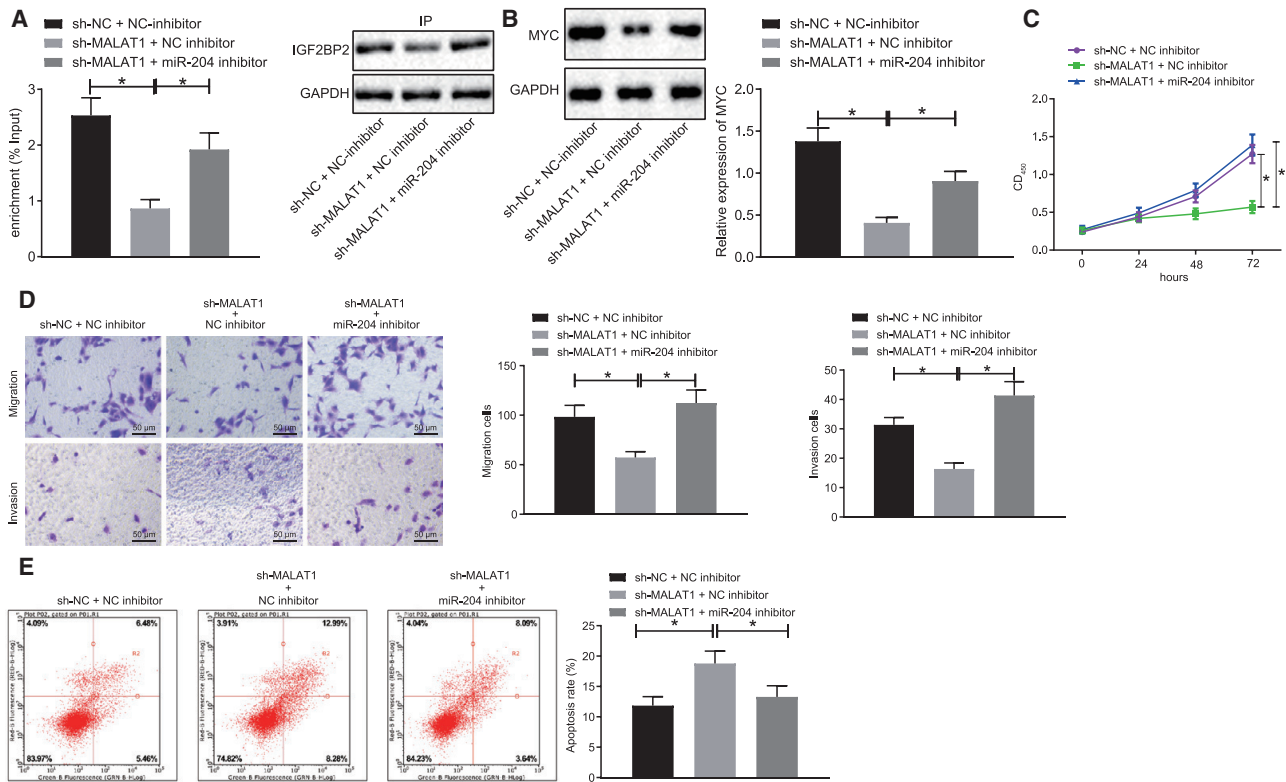


Figure 5. Silencing of lncRNA MALAT1 Suppresses Proliferation, Migration, and Invasion and Promotes Apoptosis in TC Cells via the miR-204/IGF2BP2/m6A-MYC Axis

SW579 cells were treated with sh-NC + NC inhibitor, sh-MALAT1 + NC inhibitor, or sh-MALAT1 + miR-204 inhibitor. (A) Relationship between lncRNA MALAT1 and MYC detected using RIP assay. (B) Western blot analysis of MYC protein levels in cells, normalized to GAPDH. (C) The proliferation of cells assessed by CCK-8 assay. (D) Cell migration and invasion measured by Transwell assay (original magnification $\times 200$). (E) Cell apoptosis measured by flow cytometry. Data (mean \pm standard deviation) among multiple groups were compared with one-way ANOVA, followed by Tukey's post hoc test. The experiment was repeated three times independently. * $p < 0.05$.

rates.²³ Nevertheless, our knowledge of IGF2BP2 functioning in TC is still very primitive. Findings from TC cells revealed that miR-204 expression was upregulated in TC cells, whereas IGF2BP2 expression was downregulated. Then, dual-luciferase reporter gene assay was employed in our study and verified that IGF2BP2 serves as a target of miR-204 and is negatively regulated by miR-204 in TC cells. Intriguingly, evidence has confirmed IGF2BP2 as a target regulated by miR-204-5p in clear cell renal cell carcinoma cells.¹³ Additionally, IGF2BP2 is a target gene of miR-204-3p in glioma, which was largely consistent with the observations of the current study.²⁴ Furthermore, our results revealed that IGF2BP2 elevated the MYC expression by recognizing the m6A modification of MYC in TC cells, which was supported by previous research conducted by Huang et al.¹⁶ Meanwhile, the overexpression of MYC has been shown to promote the occurrence of TC, and thus targeting MYC may well prove to be a fruitful preventive therapeutic strategy from an anaplastic TC perspective,¹⁷ highlighting the possible key regulatory role of IGF2BP2 in the occurrence of TC.

Further experimentation in the current study revealed that lncRNA MALAT1 bound to miR-204 to dramatically elevate TC cell prolifer-

ation, invasion, and migration abilities, and reduce cell apoptosis *in vitro* by upregulating IGF2BP2. This was consistent with the observations of a previous study that highlighted an interaction between lncRNA-miRNA-mRNA in TC, whereby lncRNAs were observed to regulate mRNA expressions by means of interacting with miRNAs.²⁵ For instance, lncRNA UCA1 is known to promote colony formation in addition to cell proliferation in papillary TC via miR-204-mediated bromodomain containing 4 activation *in vitro*, while stimulating papillary TC tumor growth *in vivo*.²⁶ Meanwhile, a previous study found that overexpression of miR-204-5p would suppress papillary TC cell proliferation and induce cell apoptosis by inhibiting the IGF2BP5 expression.²⁷ High levels of miR-204 expression have been reported to repress the proliferation and invasion abilities of retinoblastoma cells.²⁸ Likewise, a previous functional assay presented evidence indicating that IGF2BP2 downregulation caused by miR-592 led to a drastic cell-cycle blockade and a decline in glioma cell proliferation, invasion, and migration *in vitro*.²⁹ Alternatively, the oncogenic role of lncRNA MALAT1 in lung adenocarcinoma has been previously shown via the upregulation of SLUG and competitive "sponging" of miR-204.⁹ Accumulating studies have also indicated that lncRNA MALAT1 promotes proliferation, invasion, and

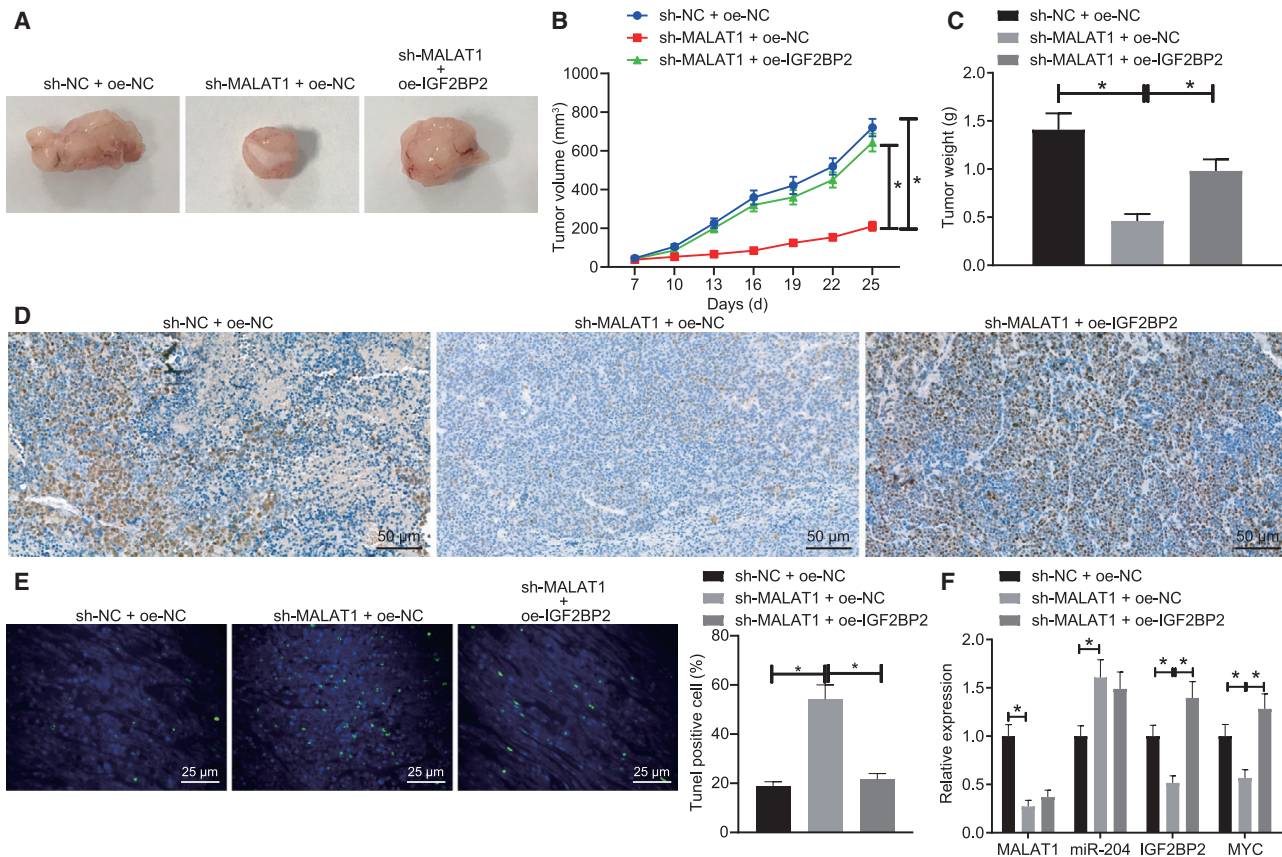


Figure 6. Silencing of lncRNA MALAT1 Inhibits MYC Expression and TC Tumor Growth *In Vivo* by Downregulating IGF2BP2

SW579 cells were treated with sh-NC + oe-NC, sh-MALAT1 + oe-NC, or sh-MALAT1 + oe-IGF2BP2 and then transplanted into nude mice. (A–C) The representative images of xenograft tumors and quantitative analysis of tumor weight and volume. (D) Expression patterns of MYC in tumor sections identified by immunohistochemical staining (original magnification 200 \times). (E) Cell apoptosis in tumor tissues detected by TUNEL assay (original magnification 200 \times). (F) Expression patterns of miR-204, lncRNA MALAT1, IGF2BP2, and MYC in mice detected by qRT-PCR and normalized to U6 and GAPDH, respectively. Data (mean \pm standard deviation) among multiple groups were compared with one-way ANOVA, followed by Tukey's post hoc test. $n = 6$. * $p < 0.05$.

angiogenesis in TC cells, which is in line with our findings.^{30,31} All of the aforementioned evidence points toward the conclusion that lncRNA MALAT1 might promote the expression of IGF2BP2 by downregulating miR-204. In addition, MYC has been highlighted to regulate the apoptosis of coelomocytes by directly targeting Bax expression in sea cucumber *Apostichopus japonicus*.³² In our study, MYC expression was detected to be regulated by the lncRNA MALAT1/miR-204 signaling. Therefore, we speculated that overexpression of miR-204 promoting cell apoptosis may be closely correlated to MYC-dependent cell apoptosis.

Taken together, the central findings of the current study suggest that lncRNA MALAT1 competitively binds to miR-204 and releases IGF2BP2, which induces m6A of MYC and promotes TC cell proliferation, invasion, migration, and anti-apoptosis. These findings provide insight into potential intervention strategies for TC in the future. However, there were certain limitations faced during the current study, including a limited sample size that prevented us from further validating the great significance of the lncRNA MALAT1/miR-204/

IGF2BP2/m6A-MYC axis in the progression of TC. Consequently, a further in-depth investigation into the specific mechanisms underlying the lncRNA MALAT1/miR-204/IGF2BP2/m6A-MYC axis will be conducted in our future studies to facilitate therapeutic development against TC.

MATERIALS AND METHODS

Ethics Statement

The study protocols were approved by the Institutional Animal Care and Use Committee of the Second Affiliated Hospital of Zhejiang University School of Medicine. All animal experimentation was performed in strict adherence with the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. Extensive efforts were made to minimize the pain, suffering, and discomfort of animals used during our study.

Cell Treatment

TC cell lines BCPAP (BNCC338685), TPC-1 (BNCC337912), SW579 (BNCC100182), and normal thyroid cell line Nthy-ori 3-1

Table 1. Primer Sequences for qRT-PCR

Target	Sequence
miR-204	F: 5'-GCTTCAGCAAAGACAACGAG-3'
	R: 5'-GTGTAATGCAGGACCACAGC-3'
U6	F: 5'-GCTTCGGCAGCACATACTAAAAT-3'
	R: 5'-CGCTTCACGAATTTGCGTGCAT-3'
MALAT1	F: 5'-GAATTGCGTCATTTAAAGCCTAGTT-3'
	R: 5'-GTTTCATCCTACCACTCCCAATTAAT-3'
MYC-m6A	F: 5'-GCATACATCTGTCCGTCCA-3'
	R: 5'-GTCGTTTCCGCAACAAGTCCC-3'
GAPDH	F: 5'-GCACCGTCAAGGCTGAGAAC-3'
	R: 5'-TGGTGAAGACGCCAGTGA-3'

F, forward; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MALAT1, metastasis-associated lung adenocarcinoma transcript 1; miR, microRNA; MYC-m6A, myelocytomatosis-RNA N6-methyladenosine; qRT-PCR, quantitative reverse transcription polymerase chain reaction; R, reverse.

(BNCC340487) were provided by the Beijing Beina Chuanglian Biotechnology Research Institute (Beijing, China). Following resuscitation, the aforementioned cells were cultured in Roswell Park Memorial Institute 1640 medium containing 10% fetal bovine serum (FBS) (GIBCO, Carlsbad, CA, USA) in a 5% CO₂ incubator at 37°C with saturated humidity (Thermo Fisher Scientific, Rockford, IL, USA). Upon reaching 90% confluence, the cells were subjected to 0.25% trypsin treatment and sub-cultured at a ratio of 1:3.

The cells were subsequently transfected with miR-204 mimic, miR-204 mimic + oe-IGF2BP2, sh-MALAT1 + miR-204 inhibitor, sh-METTL14 and sh-IGF2BP2, as well as their corresponding NCs (NC mimic, NC mimic + oe-NC, miR-204 mimic + oe-NC, NC mimic + oe-IGF2BP2, sh-NC + NC inhibitor, sh-MALAT1 + NC inhibitor, and sh-NC), respectively. The overexpression plasmid pCMV6-AC-GFP was obtained from Fenghui Biotechnology (FH1215; Hunan, China) with the silencing plasmid pGPU6/Neo procured from Shanghai GenePharma (Shanghai, China). Transfection was performed using a Lipofectamine 2000 kit (11668019; Invitrogen, Carlsbad, CA, USA). The medium was renewed with Dulbecco's modified Eagle's medium containing 10% FBS and penicillin/streptomycin after 6 h of transfection. After 48 h of additional culture, the cells were collected, and the transfection efficiency was identified and recorded.

RNA Extraction and Quantification

Total RNA content was extracted from the cells with the use of TRIzol reagents (Invitrogen). A NanoDrop2000 micro UV spectrophotometer (1011U, NanoDrop 2000 spectrophotometer; Thermo Scientific) was employed for the detection of total concentration and purity of the extracted RNA. The collected RNA was subsequently reversely transcribed into complementary DNA (cDNA) using TaqMan MicroRNA Reverse Transcription Kits (4427975; Applied Bio-

systems, Foster City, CA, USA)/PrimeScript RT reagent Kit (RR047A; Takara, Japan). The Takara company was sanctioned to design and synthesize the primers for miR-204 and lncRNA MALAT1 (Table 1). qRT-PCR was then performed using an ABI 7500 instrument (Applied Biosystems). U6 was regarded as an internal reference for miR-204, while glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was considered the internal reference for the remaining genes. The fold changes were calculated by means of relative quantification (the 2^{-ΔΔCt} method).

Western Blot Analysis

Total protein content was extracted from the cells using radioimmunoprecipitation assay lysis buffer (P0013C; Beyotime Biotechnology, Shanghai, China) containing phenylmethylsulfonyl fluoride and quantified with the use with bicinchoninic acid protein assay kits. Next, 50 μg protein was subjected to separation by means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes using the wet-transfer method. The membrane was then blocked with 5% skim milk at ambient temperature for 1 h and overnight incubation at a temperature of 4°C with primary rabbit antibodies to IGF2BP2 (1:1,500, ab151463), MYC (1:1,000, ab39688), METTL14 (1:1,500, ab98166), and GAPDH (1:2,500, ab9485). The aforementioned antibodies were procured from Abcam (Cambridge, UK). Next, the membrane was re-probed with the horseradish peroxidase (HRP)-conjugated secondary goat anti-rabbit antibody to IgG (1:2,000, ab97051; Abcam) for 1 h. The immunocomplexes on the membrane were subsequently visualized by means of enhanced chemiluminescence kits (BB-3501; Amersham, Little Chalfont, UK) and photographed using the Bio-Rad image analysis system (Bio-Rad Laboratories, Hercules, CA, USA). Finally, band intensities were subjected to quantification using Quantity One v.4.6.2 software. The ratio of the gray value of the target band to GAPDH was considered to be reflective of the relative protein expression.

Dual-Luciferase Reporter Gene Assay

IGF2BP2-3' UTR and lncRNA MALAT1 cDNA fragments with the miR-204 binding site were inserted into the pGL3 plasmids. More specifically, IGF2BP2-3' UTR-mut and lncRNA MALAT1-mut fragments were constructed by site-directed mutagenesis and subsequently inserted into pGL3 plasmids with the insertion confirmed by sequencing. Lipofectamine 2000 kits were employed to co-transfect recombinant vectors of pGL3-MALAT1, pGL3-MALAT1-mut, pGL3-IGF2BP2-3' UTR, and pGL3-IGF2BP2-3' UTR-mut with miR-204 mimic or NC-mimic into HEK293T cells, respectively. Following a 48-h period of transfection, the cells were harvested and lysed. The relative light unit (RLU) was detected using luciferase assay detection kits (K801-200; BioVision, Milpitas, CA, USA) with Renilla luciferase activity serving as the internal reference. Luciferase reporter gene detection was performed using the dual-luciferase reporter gene analysis system (Promega, Madison, WI, USA). The relative luciferase activity was expressed as the ratio of the RLU value of Firefly luciferase to the RLU value of Renilla luciferase.

Fractionation of Nuclear/Cytoplasmic RNA

Fractionation of the nuclear/cytoplasmic RNA was performed with the help of the PARISTM Kit Protein and RNA Isolation System (Invitrogen). In brief, TC cells were trypsinized, which was then halted following the addition of 2 mL medium. Next, the samples were harvested and subjected to centrifugation at 4°C and 500 × *g* for 5 min, followed by phosphate-buffered saline (PBS) rinsing of the sediment with the supernatant discarded. Next, 500 μL of cell fractionation buffer was added to the samples, subjected to gentle mechanical dissociation, and allowed to stand on ice for 5–10 min, followed by 5-min centrifugation at 4°C. The supernatant (cytoplasm) was subsequently transferred to a new 2-mL sterile enzyme-free tube for an additional 5-min period of centrifugation at 4°C. Subsequently, 500 μL of cell fractionation buffer was supplemented to the precipitate (nucleus), with 500 μL of 2 × lysis/binding solution then added. The supernatant was removed after gentle trituration and mixture reaction on ice. Then, precooled 500 μL of cell disruption buffer was added and mixed with a strong vortex. Next, 500 μL absolute ethanol was added, gently triturated, and mixed well, after which an adsorption column was placed onto the collection tube with 700 μL of reaction solution added each time, followed by 30 s of centrifugation at 12,000 × *g*. After removal of the liquid in the collection tube, the columns were supplemented with 700 μL of wash solution I and centrifuged at 12,000 × *g* for 30 s, after which the liquid in the collection tube was withdrawn. The columns were subsequently supplemented with 500 μL wash solution 2/3 and centrifuged at 12,000 × *g* for 30 s, with the liquid in the collection tube discarded. The columns were then centrifuged at maximum rotation speed for 1 min. The adsorption columns were finally loaded with new collection tubes, eluted by the addition of 40 μL elution solution (water bathed to 95°C ahead), and centrifuged at 12,000 × *g* for 30 s. An additional 10 μL of elution solution was added for elution. Lastly, the expression patterns of lncRNA MALAT1 were detected by means of qRT-PCR, with 45S rRNA serving as the internal reference in the nucleus and 12S rRNA as the internal reference in the cytoplasm.

RIP

The cells were lysed on ice for 5 min with equal volumes of RIP lysis (P0013B; Beyotime), followed by 10-min centrifugation at 14,000 rpm at 4°C, with the supernatant obtained. The binding of lncRNA MALAT1 and miR-204 to Ago2 protein and the binding of MYC mRNA to the IGF2BP2 protein were detected using RIP kits (Millipore, Billerica, MA, USA). In brief, 50 μL magnetic beads of each co-precipitation reaction system was re-suspended in 100 μL RIP Wash Buffer, followed by 6 h of incubation at 4°C and addition of 5 μg rabbit monoclonal antibody to Ago2 (ab186733, 1:50; Abcam) or mouse monoclonal antibody to IGF2BP2 (ab128175, 1:50; Abcam) for binding. The magnetic beads-antibody complex was then re-suspended in 900 μL RIP Wash Buffer followed by overnight incubation with 100 μL cell extract at 4°C. The samples were then placed onto a magnetic pedestal to collect the bead-protein complex. The samples were subsequently detached using protease K, after which the RNA content was extracted for qRT-PCR detection. The rabbit antibody against IgG (ab172730, 1:100; Abcam) was regarded as the NC.

Me-RIP

Total RNA content was isolated from TC cells using the TRIzol method, with the mRNA in the extracted RNA separated and purified using PolyAtract mRNA Isolation Systems (A-Z5300; A&D Technology Corporation, Beijing, China). Antibody against m6A (1:500, ab151230; Abcam) or IgG (ab109489, 1:100; Abcam) was added to the IP buffer (20 mM Tris [pH 7.5], 140 mM NaCl, 1% Nonidet P-40, and 2 mM ethylenediaminetetraacetic acid [EDTA]) and incubated with protein A/G magnetic beads for 1 h for binding. The purified mRNA, as well as the magnetic bead-antibody complex, was then added to the IP buffer with ribonuclease inhibitor and protease inhibitor for overnight incubation at 4°C. The expression of m6A was detected by IP assay. The RNA was eluted with elution buffer, extracted using the phenol-chloroform method, purified, and then analyzed with qRT-PCR. The primer sequences applied in the experimentation are illustrated in Table 1.

CCK-8 Assay

CCK-8 kits (CK04; Dojindo Laboratories, Tokyo, Japan) were employed to measure TC cell proliferation. In short, the cells at the logarithmic phase of growth were inoculated in 96-well plates (at a density of 1 × 10⁴ cells/well) and pre-cultured for 24 h. At 0, 24, 48, and 72 h posttransfection, CCK-8 solution (10 μL) was added to each well for a 3-h period of incubation at 37°C. The optical density values at 450 nm were subsequently determined using a microplate reader. The value was indicative of the number of proliferative cells in the medium. Finally, a cell growth curve was plotted based on the values collected.

Transwell Assay

Matrigel (YB356234; Shanghai Yubo Biotechnology, Shanghai, China) stored at –80°C in a refrigerator was taken out and melted overnight to a temperature of 4°C. Next, 200 μL of serum-free medium was supplemented with 200 μL of Matrigel at 4°C and mixed in an even manner in order to dilute the matrix. Next, 50 μL of Matrigel postdilution was supplemented to the apical Transwell chamber and incubated for 2–3 h until solidification. The TC cells were then detached, counted, and prepared as a cell suspension with serum-free medium. Afterward, 200 μL of cell suspension was supplemented into the apical chamber of each well, and 800 μL conditioned medium containing 20% FBS was subsequently added to the basolateral chamber. Next, 50 μm apoptosis inhibitor, Z-VAD-FMK (#S7023; Selleck Chemicals, Houston, TX, USA), and DMSO (NC) were respectively added and cultured with TC cells for 24 h. The Transwell plate was then subjected to incubation at 37°C for 20–24 h and 10 min of rinsing with formaldehyde. The samples were then stained with 0.1% crystal violet and allowed to stand at ambient temperature for 30 min. The cells on the upper surface of the chamber were wiped off with cotton balls. Finally, the remaining cells were observed, photographed, and counted under an inverted microscope. In regard to the Transwell migration experiment, no Matrigel was required, and the incubation time was 16 h. A minimum of four fields were randomly selected, and cells in the selected fields were counted under a microscope.³³

Flow Cytometry

After 48 h of transfection, the TC cells were detached with 0.25% trypsin containing no EDTA (YB15050057; Shanghai Yubo Biotechnology) and collected into flow tubes. Next, centrifugation was performed, after which the supernatant was removed. The cells were then subjected to another round of centrifugation with the supernatant discarded. As per the instructions of the Annexin V-fluorescein isothiocyanate (FITC) cell apoptosis detection kit (K201-100; Bio-Vision, Milpitas, CA, USA), Annexin V/propidium iodide (PI) dye solution was prepared with an Annexin V-FITC, PI, and N-2-hydroxyethylpiperazine-N-ethane-sulphonic acid (HEPES) buffer solution at a ratio of 1:2:50. Every 100 μ L of dye solution was applied in order to re-suspend 1×10^6 cells, which was then evenly mixed by shaking. After a 15-min period of incubation at ambient temperature, 1 mL HEPES buffer (PB180325; Procell, Wuhan, Hubei, China) was added to the cells. Afterward, cell apoptosis was determined after fluorescence was initiated by excitation at 488 nm (FITC) and was measured by emission filters at 525 nm (FITC) and 620 nm (PI).

TC Xenografts in Nude Mice

A total of 18 female BALB/c nude mice (aged 4–5 weeks old, weighing 15–18 g) were obtained from Shanghai SLAC Laboratory Animal (Shanghai, China). The mice were subsequently randomized into three groups, with six mice in each group. The TC SW579 cell lines stably transfected with sh-NC + oe-NC, sh-MALAT1 + oe-NC, or sh-MALAT1 + oe-IGF2BP2 were subcutaneously injected into the nude mice to generate SW579 cells in xenograft mouse models. In brief, SW579 cells at the logarithmic stage of growth were prepared into a cell suspension (a concentration of about 1×10^7 cells/mL). The prepared cell suspension was then injected into the skin of the left armpit of the nude mice using a 1-mL syringe. Tumor growth was observed and recorded on the 7th, 14th, 21st, 28th, and 35th days after inoculation. On the 36th day after inoculation, the mice were euthanized by means of cervical dislocation, after which the tumors were extracted. Finally, the tumors were weighed followed by qRT-PCR analysis.

Immunohistochemical Staining

Xenograft tumors were resected, fixed with 4% paraformaldehyde, paraffin embedded, sliced into 5- μ m sections, and dewaxed. Following streptavidin-peroxidase (SP) treatment, antigen retrieval was performed using microwave heating until boiling. The heating was held for 5 min, after which it was repeated again. After cooling to ambient temperature, the sections were blocked with normal goat serum. The known positive TC sections were employed as the positive control, whereas IgG was considered as the NC in lieu of the primary antibody. Immunohistochemical staining was conducted using Histostain SP-9000 immunohistochemical staining (Invitrogen). The sections were then probed with primary antibody to MYC (1:1,000, ab39688; Abcam) at 4°C overnight and re-probed with the secondary antibody of goat anti-rabbit antibody to IgG (1:10,000, ab6721; Abcam) at 37°C for 30 min, followed by incubation with HRP-labeled working solution. The coloration in the sections was visualized by 3,3'-N-diaminobenzidine for 5–10 min. The staining

period was controlled microscopically. After counterstaining with hematoxylin for 1 min, the sections were mounted with gum and photographed.

Terminal Deoxynucleotidyl Transferase (TdT)-Mediated 2'-Deoxyuridine 5'-Triphosphate (dUTP) Nick End Labeling (TUNEL) Staining

Cell apoptosis in paraffin-embedded xenograft tumor sections was detected as per the instructions of the manual of the commercially purchased TUNEL kits (Boster Biological Technology, Wuhan, Hubei, China). In brief, after dewaxing and dehydration, the coverslip was treated with Proteinase K (Boster) at room temperature for 20 min and incubated with TUNEL reaction solution at 37°C for 60 min. Cell apoptosis was observed under a fluorescence microscope (BX-60; Olympus, Tokyo, Japan) (excitation wavelength: 450–500 nm, emission wavelength: 515–565 nm). NC was set by incubating with labeling solution in the absence of terminal transferase for replacement of TUNEL reaction solution, whereas the positive control was set by incubating with Bovine pancreas DNase I (0.01 mg/mL; Boster). The number of apoptotic cells was determined using Image-Pro Plus 6.0 software.

Statistical Analysis

All experimental data were analyzed using the SPSS 19.0 software (IBM, Armonk, NY, USA). Measurement data were expressed as mean \pm standard deviation. Data conforming to normal distribution and homogeneity of variance were compared with independent sample t test between two groups and one-way analysis of variance (-ANOVA) among multiple groups, followed by the application of a Tukey post hoc test. A p value <0.05 was considered to be indicative of statistical significance.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.omtn.2020.09.023>.

AUTHOR CONTRIBUTIONS

M.Y. designed the experiments. S.D. collated the data. H.H. and T.Z. carried out data analyses and conducted the experiments. S.D. and M.S. contributed to drafting the manuscript. All authors have read and approved the final submitted manuscript.

CONFLICTS OF INTEREST

The authors declare no competing interests.

ACKNOWLEDGMENTS

We acknowledge and appreciate our colleagues for their valuable efforts and comments on this paper.

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Supplemental Information

**Oncogenic Role of Long Noncoding RNAMALAT1 in
Thyroid Cancer Progression through Regulation
of the miR-204/IGF2BP2/m6A-MYC Signaling**

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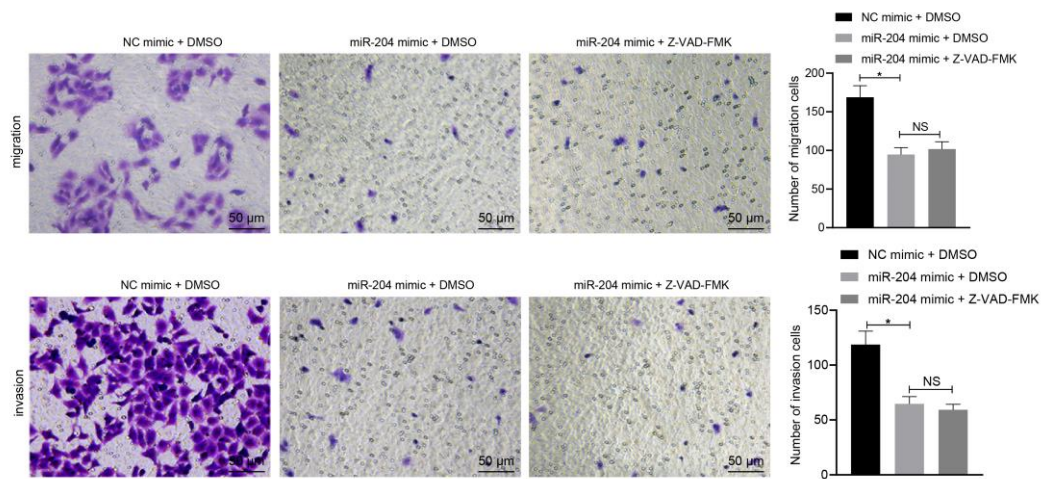


Figure S1 Apoptosis is not involved in the action of miR-204 on TC cell migration and invasion. Cell migration and invasion measured by Transwell assay ($\times 200$). DMSO and Z-VAD-FMK ($50 \mu\text{m}$) was added and cultured with TC cells for 24 h. Data (mean \pm standard deviation) were compared with unpaired *t* test. The experiment was repeated 3 times independently. * $p < 0.05$.