Thyroid hormone regulation of miR-21 enhances migration and invasion of hepatoma


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Abstract

Thyroid hormone (T3) signaling through the thyroid hormone receptor (TR) regulates hepatoma cell growth and pathophysiology, but the underlying mechanisms are unclear at present. Here, we have shown that the oncomir microRNA-21 (miR-21) is activated by T3 through a native thyroid hormone response element in the primary miR-21 promoter. Overexpression of miR-21 promoted hepatoma cell migration and invasion, similar to that observed with T3 stimulation in hepatoma cells. Additionally, anti-miR-21-induced suppression of cell migration was rescued by T3. The Rac-controlled regulator of invasion and metastasis, T-cell lymphoma invasion and metastasis 1 (TIAM1) was identified as a miR-21 target additionally downregulated by T3. Attenuation and overexpression of miR-21 induced upregulation and downregulation of TIAM1, respectively. TIAM1 attenuation, in turn, enhanced migration and invasion via upregulation of β-catenin, vimentin and matrix metalloproteinase-2 in hepatoma cells. Notably, correlations between TRα1, miR-21 and TIAM1 expression patterns in animal models paralleled those observed in vitro. In the clinic, we observed a positive correlation (P = 0.005) between the tumor/non-tumor ratios of TR and miR-21 expression, but a negative correlation (P = 0.019) between miR-21 and TIAM1 expression in hepatoma patients. Our findings collectively indicate that miR-21 stimulation by T3 and subsequent TIAM1 suppression promotes hepatoma cell migration and invasion.
Introduction

The thyroid hormone (T3) is an important regulator of growth, development and differentiation in vertebrates. T3 binds to thyroid hormone receptors (TR) that belong to the nuclear receptor superfamily. Human TRs are encoded by TRα and TRβ genes located on chromosomes 17 and 3, respectively. The two genes yield several polypeptides via alternative splicing and differential promoter usage. The TRα1, TRβ1 and TRβ2 genes encoding functional products have been well characterized. Accumulating evidence supports a critical role of TRs in carcinogenesis. For instance, aberrant expression of TR has been associated with human cancers (1). Earlier studies suggest that partial loss of normal TR function occurs due to reduced expression or complete loss of activity resulting in mutations and/or aberrant expression, which in turn, provides an opportunity for tumor cells to proliferate, invade, and metastasize (2).

MicroRNAs (miRNA) are small ~22-nucleotide RNA molecules that modulate gene expression via the RNA interference (RNAi) pathway. MiRNAs generally exhibit partial complementarity, and often bind within the 3’ untranslated region (3’UTR) of target mRNAs, leading to translational repression and/or degradation (3). Inappropriate expression of miRNAs is strongly associated with carcinogenesis, since the genomic aberrations observed in cancers directly reflect miRNA expression patterns. Moreover, increasing evidence shows that miRNA gene expression is dysregulated in human cancers (4, 5). Specific over- or underexpression of miRNA is correlated with particular tumor types (6, 7). To date, a number of miRNAs that appear to function as tumor suppressors or oncogenes have been identified.

A number of earlier studies have reported that T3/TR signaling plays a critical role in hepatoma (8-10). In addition, miRNAs play a fundamental role in regulating gene
expression in multicellular eukaryotes. Therefore, to clarify the functions of specific transcription factors, miRNAs can be evaluated as potential targets. In hepatoma, miRNA expression is frequently dysregulated, and specific miRNAs have been shown to regulate migration and invasion (11). It is speculated that miRNAs are critical downstream targets of TRs that participate in hepatoma cell progression. To identify the specific miRNA targets of TR, 270 miRNA expression profiles in HepG2-TRα1 cells were obtained in the presence or absence of T3 using reverse transcriptase stem-loop quantitative polymerase chain reaction (RT-qPCR) analysis. Consequently, several miRNAs up- or downregulated upon T3 stimulation were detected. We ultimately focused on miR-21, one of the T3-modulated miRNAs that is dysregulated in various cancers. Direct regulation of miR-21 by T3 was confirmed, and the mechanism underlying the effects of T3 on hepatoma cell migration further investigated.

**Materials and Methods**

**Reverse transcription-quantitative real-time PCR for miRNA and mRNA**

The RT-qPCR method for detection of mature miRNAs and mRNAs was performed as described previously (10, 12).

**Cell culture and transfection**

The human hepatoma cell lines, HepG2, Hep3B, SK-Hep1 (obtained from American Type Culture Collection) and J7 (gift from Dr. C. S Yang, National Taiwan University, Taiwan) (13) was routinely grown in Dulbecco's modified Eagle's medium.
(DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS). Cell lines have recently been authenticated using the StemElite™ ID System (Promega, Madison, WI). TRα1 and TRβ1-overexpressing cell lines were cultured, as described previously (14). The serum was depleted of T3 (Td) using an earlier procedure (15). Cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO2.

Patients and statistical methods

To determine the associations among TRα1, miR-21 and TIAM1 in hepatoma, total RNAs of tumor and adjacent non-tumor liver tissues from 40 hepatoma patients (13 females and 27 males) from the Taiwan Liver Cancer Network (TLCN) were obtained for analysis. The basic clinical characterization of the 40 patients is presented in Supplementary Table S1. The study protocol was approved by the Medical Ethics and Human Clinical Trial Committee of the Chang Gung Memorial Hospital (IRB No. 98-0798B).

Stable transfection of Hep3B cells with TIAM1 knockdown

RNA interference against TIAM1 (obtained from Academia Sinica in Taiwan), pLKO.1-shTIAM1, was used to deplete endogenous TIAM1 in Hep3B cells. Specifically, Hep3B cells were transfected with pLKO.1-shTIAM1 using Turbofect™ (Fermentas, Glen Burnie, MD). Transfected cells were incubated and selected in DMEM supplemented with 10% FBS-containing 0.3 μg/ml puromycin for at least 2 weeks until colonies could be picked.

Amplified chromatin immunoprecipitation assay
The ChIP assay was performed as described in a previous report (8). The upstream TRE region of the pri-miR-21 gene was amplified with the forward primer, 5’-AGAGGGCCGGCAAGTTTCTT-3’, starting at nucleotide -1017, and reverse primer, 5’-AATAAGGAAATGACTTATGC-3’, starting at nucleotide -958.

Immunoblot analysis

Proteins were loaded on a 6% or 10% SDS-polyacrylamide gel for electrophoresis before transfer to a PVDF membrane (PerkinElmer, Boston, MA). The membrane was blotted with antibodies specific for TR protein (C4) (16), TIAM1 (Calbiochem, Darmstadt, Germany), vimentin, β-catenin, phospho-β-catenin (Santa Cruz, Santa Cruz, CA), c-myc (GeneTex, San Antonio, TX), cyclin D1, c-Jun (Epitomics, Burlingame, CA), β-actin and GAPDH (Chemicon, Bedford, MA) for detection. Blots were incubated with horseradish peroxidase-conjugated secondary antibody, and developed using an ECL detection kit (Millipore Corp., Bedford, MA). Immunoblot assays were performed independently at least three times. The intensities of the immunoreactive bands were quantified using Image Gauge software (Fuji Film, Tokyo, Japan).

3’UTR luciferase reporter assay

Full-length TIAM1 (1950 nt) or MSH2 (272 nt) 3’UTR (Supplementary Fig. S1) was synthesized and cloned into the pMIR-REPORT vector (Applied Biosystems, Foster City, CA) containing the luciferase reporter gene (TIAM1 or MSH2 3’UTR wt). The putative miR-21 recognition sites in TIAM1 or MSH2 3’UTR were subjected to site-directed mutagenesis (TIAM1 or MSH2 3’UTR mut), and the mutated sequences validated via automated DNA sequencing. The pCDH-CMV-MCS-EF1-copGFP and
pmiRZip lentivector (SBI System Biosciences, Mountain View, CA) were used to express pre-miR-21 and antisense miR-21 small RNA, respectively. To determine the effects of miR-21 on TIAM1 3’UTR, pMIR-TIAM1-3’UTR and pre-miR-21, anti-miR-21, or negative control vector were co-transfected into HepG2 cells using TurboFect™. Similarly, HepG2 cells co-transfected with pMIR-MSH2-3’UTR and pre-miR-21 or negative control vector were examined. To determine the effects of T3 on TIAM1 3’UTR, HepG2-TRα1 cells pre-cultured in Td medium were transfected with pMIR-TIAM1-3’UTR (wt) in the presence or absence of T3. After transfection for 24 h, cells were extracted for detection of luciferase activity.

**Migration and invasion assay**

Cells (5×10⁴/200 μl) in serum-free DMEM were seeded onto the upper chambers of Transwell filter devices and DMEM containing 10% FBS added to the lower chambers, as described previously (14).

**Gelatin zymography**

An aliquot of concentrated medium (20 μg) from Hep3B-shNeo and -shTIAM1 cell cultures was loaded onto a 10% non-denaturing polyacrylamide gel containing 0.1% gelatin, as described previously (17).

**Animal model**

In Model I, male Sprague Dawley (SD) rats were subjected to thyroidectomy (Tx) at 6 weeks of age according to a previous method, with a view to examining the association between T3/TR and miR-21 in vivo (10). To investigate the correlation
between T<sub>3</sub> and miR-21 in T<sub>3</sub>-enhanced metastasis of hepatoma cells in vivo, J7-TRα1 xenografts of eu-, hypo- and hyperthyroid severe combined immunodeficiency (SCID) mice generated in a previous study by our group (18, 19) were utilized (Model II). Finally, to determine whether the negative correlation between the miR-21 and TIAM1 can be found in vivo, knockdown of miR-21 in SK-Hep1 cells was injected into SCID mice via the tail vein (Model III). All animal experiments were conducted in accordance with the National Institutes of Health Guide and Chang-Gung Institutional Animal Care and Use Committee Guide for the Care and Use of Laboratory Animals (IACUC Approval No. CGU09-011).

Statistical analysis

Phi correlation coefficients were computed to determine the correlations among the tumor/non-tumor (T/N) ratios of TRα1, miR-21 and TIAM1. Statistical analysis was performed using SPSS version 15.0 (Chicago, IL). Other statistical analysis of data was performed using Student’s t-test. Data were considered statistically significant at P<0.05. Values are presented as means ± SD of at least three independent observations.

Results

Expression profiles of miRNA in HepG2 cells treated with or without T<sub>3</sub>

Due to low expression of endogenous TRα1 in various hepatoma cell lines (Supplementary Fig. S2), a HepG2 cell line expressing high levels of TRα1 (HepG2-TRα1) was established to identify the miRNA genes potentially regulated by T<sub>3</sub>. In total, 14 miRNAs potentially regulated by T<sub>3</sub> were identified. Ten of these miRNAs
were upregulated by at least 3-fold and four miRNAs were downregulated by at least 2-fold (Supplementary Table S2). Among the 14 miRNA candidates, miR-21 has been shown to be upregulated in various cancers, including hepatoma (7), suggestive of a role as a central oncomir (20). A pilot study additionally revealed miR-21 upregulation by T₃ in HepG2-TRα₁ cells. Interestingly, T₃/TR signaling may exert both oncogenic (10) and tumor suppressor effects (21, 22). The findings that miR-21 is increased in hepatoma tissue and potentially upregulated as a target of T₃ in HepG2 cells imply that T₃ functions as an oncogene in this case.

Validation of T₃-mediated upregulation of miR-21 in HepG2-TR cells

To confirm the positive regulation of miR-21 by T₃, HepG2 cells stably expressing TRα₁ (HepG2-TRα₁) and TRβ₁ (HepG2-TRβ₁) were examined. A HepG2 cell line expressing the empty vector, Neo, was used as the control (HepG2-Neo). TR expression levels of the three stable HepG2 cell lines are shown in Fig. 1A. The observed increase in miR-21 expression after T₃ treatment in the HepG2-TR cell lines was both time- and dose-dependent (Figs. 1B and C). In contrast, no changes in miR-21 levels were evident following T₃ treatment in the HepG2-Neo cell line (Fig. 1D). Moreover, expression of the miR-21 primary transcript (pri-miR-21, accession No. AY699265, Supplementary Fig. S3) was stimulated in a time- and dose-dependent manner in HepG2-TRα₁ and -TRβ₁ cells treated with T₃ (Fig. 1E and Supplementary Fig. S4). Accordingly, we speculated that the promoter region of miR-21 possesses a thyroid hormone response element (TRE). The miR-21 gene is located within the intron of TMEM49. The TMEM49 level in T₃-treated HepG2-TRα₁ cells was thus examined to eliminate the possibility that increased miR-21 expression is attributed to T₃-regulated TMEM49. As
shown in Fig. 1F, TMEM49 mRNA expression was significantly increased by 1.5- and 1.7-fold in HepG2-TRα1 cells treated with 10 nM T3 for 48 and 72 h, while the miR-21 level was elevated approximately 4.3- and 5.9-fold under the same conditions, respectively (Fig. 1B). This difference in relative fold change between miR-21 and TMEM49 levels in HepG2-TRα1 cells (4.3-fold vs 1.5-fold and 5.9-fold vs 1.7-fold following 10 nM T3 treatment for 48 or 72 h, respectively) was significant. The differences in fold change of miR-21 (both primary and mature forms) and TMEM49 induction by T3 are indicative of direct regulation of miR-21 expression by T3, rather than co-regulation with TMEM49.

Characterization of TRE in the miR-21 gene

To determine whether the promoter region of pri-miR-21 contains TRE, we analyzed the region 3000 nucleotides upstream of the gene using Vector NTI software. Seven putative TRES were identified in the pri-miR-21 promoter region (-3000/+1) (Supplementary Fig. S5). Among these, two putative regions (TRE\textsubscript{A} and B) were predicted to possess palindromic (Pal) TRE, three (TRE\textsubscript{C,F} and G) to include inverted palindrome (Lap) TRES, and TRE\textsubscript{E} to contain direct repeat 4 (DR4) TRE. In addition, two overlapping response elements of TRE\textsubscript{D} (Lap and Pal) were predicted (Figs. 2A and B). A luciferase-based reporter plasmid containing the promoter region (-3000/-45) was constructed for the luciferase promoter assay. An approximate 3- to 5-fold increase in luciferase activity was induced by T3 (10 and 100 nM) in HepG2-TRα1 cells (Fig. 2A, fragment I), indicating a response of the promoter region to T3/TR. To further identify the putative TRE within the -3000/-45 region that responds to T3/TR, serial fragments (II to VII) were constructed for an additional promoter assay. Among these fragments,
only two containing the TRED region (fragments III and V) displayed luciferase activity under T3 treatment conditions. In addition, luciferase activity was dependent on the T3 dose (Fig. 2A). Our luciferase promoter assay results indicate that the -1109/-875 region of the pri-miR-21 promoter possesses native TRE. The ChIP assay was subsequently performed to further confirm the association of the -1109/-875 region with T3/TR in vivo. The TR and RXR binding region was narrowed down to positions -1017/-958 within this region of the pri-miR-21 promoter (Fig. 2C, right). The native TRE of furin associated with TR and RXR was used as a positive control (Fig. 2C, left), while additive IgG was employed as a negative control in the ChIP assay. In summary, our analytical results demonstrate that miR-21 is a direct TR target gene containing native TRE.

Prediction of miR-21 targets

The possible effects and mechanisms of action of T3/TR-induced miR-21 in hepatoma cells were further analyzed. Specific potential miR-21 target genes negatively regulated by T3 were selected as candidates for study. In total, 307 genes were predicted as miR-21 targets using TargetScan software (http://www.targetscan.org/). Following comparison with 3029 genes potentially downregulated by T3 in HepG2-TRα1 cells analyzed previously with cDNA microarray (data not shown), 62 were selected. Among these, 5 genes (cyclin-dependent kinase: CDK6, mismatch repair protein: MSH2, programmed cell death 4: Pdcd4, TGF-β type II receptor: TGFBR2, and T-cell lymphoma invasion and metastasis 1: TIAM1) were confirmed as miR-21 targets (23-27). Moreover, in view of the enhanced capability of hepatoma cell migration after T3 application (14), Pdcd4 and TIAM1 are reported to be associated with cell migration...
and invasion. Earlier research showed that miR-21 promotes migration and invasion through a miR-21-PDCD4-AP-1 feedback loop in hepatoma cells (28). Furthermore, a paradoxical role of TIAM1 in migration of various cancers has been documented (23, 29). Accordingly, we focused on TIAM1 for further study to validate its role in liver carcinogenesis. The issue of whether TIAM1 is also a direct target of miR-21 in hepatoma cells remains to be established. Two sequences with 7 nucleotides (AUAAGCU) of \textit{TIAM1} 3′UTR, identified as the seed region of miR-21, were predicted using TargetScan. Full-length \textit{TIAM1} 3′UTR containing two seed regions located at positions +202/+208 and +1861/+1867 were cloned into a cytomegalovirus (CMV)-driven luciferase reporter plasmid. In the presence of \textit{TIAM1} 3′UTR, miR-21 suppressed luciferase activity. This suppressive effect was diminished upon mutation of the two miR-21 binding sites in HepG2 cells (Fig. 3A, left). In addition, knockdown of endogenous miR-21 (anti-miR-21) in the presence of \textit{TIAM1} 3′UTR induced luciferase activity, which was also decreased upon mutation of the seed region of \textit{TIAM1} 3′UTR (Fig. 3A, right). To validate the effects of TIAM1 protein expression in the presence of miR-21 in hepatoma cells, Hep3B cells expressing higher levels of TIAM1 than HepG2 cells were employed for further analysis (Fig. 3B, lanes 1, 2). We observed a decrease in TIAM1 protein expression in the presence of miR-21 (Fig. 3B, lanes 4 vs. 3), and conversely, increased TIAM1 expression upon knockdown of endogenous miR-21 (using anti-miR-21) in Hep3B cells (Fig. 3B, lanes 6 vs. 5). Based on these findings, we propose that TIAM1 is a direct miR-21 target in hepatoma cells.

**Downregulation of TIAM1 by T₃/TR in Hep3B cells**

Since miR-21 is upregulated by T₃, we further examined whether \textit{TIAM1} 3′UTR
luciferase activity is affected by the thyroid hormone. As shown in Fig. 3C, a significant decrease in TIAM1 3’UTR luciferase activity was observed in HepG2-TRα1 cells in the presence of T3, compared with that in the absence of T3. Subsequently, Hep3B cells expressing high levels of endogenous TIAM1 were used to overexpress TRα1 (Hep3B-TRα1, Fig. 3D) for the purpose of validating TIAM1 regulation by T3. The dose-dependent upregulation of miR-21 in T3-treated Hep3B-TRα1 (Fig. 3E) was similar to that in HepG2-TR cells (Figs. 1B and C). T3-mediated repression of the TIAM1 protein level was observed in Hep3B-TRα1 cells (Fig. 3F, lanes 1-4). To verify that the effect of T3 on TIAM1 is mediated, at least in part, through miR-21, knockdown of endogenous miR-21 (using anti-miR-21) in Hep3B-TRα1 cells was performed. Notably, suppression of TIAM1 protein in Hep3B-TRα1 cells by T3 was rescued in cells depleted of endogenous miR-21 (Fig. 3F, lanes 6 vs. 8). The data presented in Figure 3 suggest that negative regulation of TIAM1 by T3 occurs through T3-mediated upregulation of miR-21 in hepatoma cells. MSH2, a tumor suppressor downregulated by T3/TR signaling and miR-21, was additionally examined. As shown in Supplementary Fig. S6A, the luciferase activity of full-length MSH2 3’UTR (272 nt) containing a seed site (positions +33/+39) was inhibited by miR-21, which was partially restored upon mutation of the seed region. Moreover, MSH2 expression was suppressed in the presence of T3 or upon overexpression of miR-21 (Figs. S6B and S6C, lane 2), and conversely, increased upon knockdown of miR-21 (anti-miR-21, Fig. S6C, lane 4).

Correlations among TR, miR-21 and TIAM1 in hepatoma patients

To determine whether TR, miR-21 and TIAM1 expression patterns are correlated in hepatoma, total RNA from paired tumor and adjacent non-tumor liver tissues was
extracted from a series of 40 consecutive hepatoma patients. The T/N ratios of TRα1, miR-21 and TIAM1, assessed using RT-qPCR, are presented in Supplementary Table S3. TRα1 RNA expression was higher in 87.5% (35/40) of tumor, compared to non-tumor tissues. In 35 hepatoma patients with increased TRα1 expression in tumor tissues, miR-21 was concomitantly increased in 80.0% (28/35) of the tumor regions. Additionally, TIAM1 RNA expression was decreased in 71.4% (20/28) of tumor tissues in 28 hepatoma patients displaying upregulation of TRα1 and miR-21. Phi correlation coefficients revealed a significant positive correlation between the T/N ratios of TRα1 and miR-21 (φ = 0.444, P = 0.005). Moreover, a significant negative correlation between the T/N ratios of miR-21 and TIAM1 was observed (φ = -0.370, P = 0.019).

Our analytical results imply that the correlations among TRα1, miR-21 and TIAM1 expression patterns in hepatoma patients are similar to those observed in hepatoma cells in vitro.

**T3 and miR-21 enhance Hep3B cell migration**

Stimulation of T3 promotes migration in HepG2 cells (14). Consistent with this finding, an earlier study showed that a miR-21 inhibitor suppresses migration of Huh7 cells (30). To establish the migration properties of Hep3B cells treated with T3 or displaying elevated miR-21 expression, the transwell assay was performed. Our data showed that migration of Hep3B-TRα1 cells stimulated with T3 is enhanced (Fig. 4Aa). Similarly, cell migration (Fig. 4Ab) and invasion (Fig. 4B) were promoted in miR-21-overexpressing Hep3B cells. Conversely, cell migration was inhibited in Hep3B cells depleted of endogenous miR-21 (Fig. 4Ac). Epithelial-mesenchymal transition (EMT) has been proposed as an index of enhanced metastasis in various
malignant tumor types (31). It is suggested that the EMT markers, vimentin and β-catenin, play important roles in metastasis of hepatoma (32, 33). Interestingly, increased expression of these two molecules was observed in not only Hep3B-TRα1 cells treated with T3, but also miR-21-overexpressing Hep3B cells (Fig. 4C, lanes 1-5). The downstream target proteins of β-catenin, c-myc and cyclin D1, were additionally elevated by T3 (Fig. 4C). β-Catenin was significantly upregulated (by 1.8-fold) in miR-21-overexpressing Hep3B cells, compared with the control cell line (Fig. 4C lanes 5 vs. 4), and significantly downregulated (by 60%) in the miR-21 knockdown cell line (Fig. 4C, lanes 7 vs. 6). Moreover, vimentin was downregulated upon knockdown of endogenous miR-21 (Fig. 4C, lanes 7 vs. 6). Our results collectively indicate that the effects of miR-21 and T3 on cell migration and metastasis-associated molecules (such as vimentin and β-catenin) are similar in Hep3B cells.

**TIAM1 knockdown in Hep3B cells promotes migration and invasion**

To investigate the effects of TIAM1 on cell migration and the underlying signaling pathways, we performed knockdown of TIAM1 in Hep3B or SK-Hep1 cells. The TIAM1 protein levels in four Hep3B stable cell lines are shown in Fig. 5A. The β-catenin level was significantly enhanced (by 1.6- to 1.7-fold) in the two TIAM1 knockdown Hep3B cell lines (shTIAM1; K1, K2), compared with the control cell line (shNeo; N1, N2; Fig. 5A). The phosphorylated β-catenin level was downregulated by almost 40% in the K1 and K2 cell lines. Immunoblot analysis revealed a marked increase in the expression levels of the β-catenin target proteins (c-myc, cyclin D1, and c-Jun) in the two cell lines (Fig. 5A). In addition, vimentin expression was increased in Hep3B cells depleted of TIAM1 (Fig. 5A). K1 and K2 cell lines displayed enhanced...
activity (by 1.4- to 3.6-fold) of pro-matrix metalloproteinase (MMP) 2 (72 kDa) (Fig. 5B). Moreover, migration (Figs. 5C and D) and invasion (Figs. 5E and F) abilities were increased ~3.1- to 6.0-fold and 1.5- to 2.8-fold, respectively, in these cell lines. To further verify the effect of miR-21 on metastasis in vivo, SK-Hep1 cells possessing high metastatic ability and endogenous TIAM1 expression were utilized. Expression of miR-21 was stimulated by T3 (Supplementary Fig. S7A), while TIAM1 and MSH2 levels were decreased. Moreover, c-myc and cyclin D1 were upregulated by T3 (Supplementary Fig. S7B), and β-catenin was significantly downregulated in anti-miR-21-expressing cells (Supplementary Fig. S7C). Phosphorylated β-catenin protein was upregulated in the anti-miR-21-expressing cell line. Expression levels of c-myc, cyclin D1 and vimentin were significantly decreased (Supplementary Fig. S7C).

Our data collectively indicate that miR-21 stimulation by T3 and subsequent TIAM1 suppression promote cell migration and invasion in hepatoma cells, possibly through upregulation of β-catenin, c-myc, c-jun and vimentin (Fig. 5 and Supplementary Fig. S7).

**Suppression of cell migration induced by miR-21 knockdown is rescued by T3 in Hep3B-TRα1 cells**

To confirm the finding that T3/TR signaling promotes cell migration via T3-mediated positive regulation of miR-21, Hep3B-TRα1 cells depleted of miR-21 were examined in the presence or absence of T3 using the transwell migration assay. As shown in Fig. 6, cell migration was markedly promoted in the presence of T3, compared with that in absence of T3 (Figs. 6A, I vs. II and 6B). The cell migration effect in the absence of T3 was significantly suppressed after miR-21 knockdown (Fig. 6A, I vs. III
and 6B), which was rescued following administration of T3 (Figs. 6A, III vs. IV and 6B). Moreover, promotion of cell migration by T3 was markedly repressed after miR-21 knockdown (Fig. 6A, II vs. IV and 6B). These results demonstrate that T3 rescues anti-miR-21-mediated suppression of cell migration. Furthermore, T3-induced cell migration is inhibited by anti-miR-21. Based on these findings, we propose that T3-mediated positive regulation of miR-21 promotes Hep3B cell migration.

**Correlations among TR, miR-21 and TIAM1 in vivo**

To investigate whether the effects of miR-21 in vitro can be replicated in vivo, three animal models were designed. Firstly, surgical thyroidectomy (Tx) was performed in two groups of 6-week-old male SD rats to determine the in vivo responses of miR-21 to T3 treatment. One group (Tx+T3) was injected daily with T3 for two weeks, the second group (Tx) received no T3 injections, and the third group included sham-operated controls. Rats were sacrificed at the end of the experiment (four weeks after thyroidectomy), and serum collected for T3 and TSH determination, as described previously (21). Quantitative polymerase chain reaction (Q-PCR) analysis disclosed that miR-21 expression in the Tx+T3 group was 2.8-fold that of the sham group, and conversely, 0.7-fold that of the sham group in the Tx group (Fig. 7A), indicative of a positive correlation between T3/TR and miR-21 in vivo.

Secondly, to determine whether the in vitro metastatic effect of miR-21/T3 occurs in vivo, SCID mice were injected with J7-TRα1 (a highly metastatic hepatoma cell line). Animals were subjected to hyperthyroid, euthyroid, and hypothyroid conditions after injection (18, 19). The hyperthyroid group of SCID mice injected with J7-TRα1 cells displayed higher miR-21 expression (Fig. 7B), but lower TIAM1 expression than the
 euthyroid (control) group (Fig. 7C, panels IX vs. III). Conversely, the hypothyroid group displayed decreased miR-21 (Fig. 7B) and higher TIAM1 expression (Fig. 7C, panels VI vs. III). Tumors are marked with black lines. Figures 7C, panels I, II, IV, V, VII and VIII represent hematoxylin and eosin (H&E) staining, with panels II, V and VIII depicting a higher magnification than I, IV and VII, respectively. Finally, SCID mice with miR-21-depleted SK-Hep1 (anti-miR-21) xenografts developed fewer metastatic foci in lungs, as evident from H&E staining (Fig. 7D, panel III), with a 98.2% decrease in metastatic index (tumor density per cm²) (Fig. 7E), compared with SK-Hep1-scrambled xenografts (Figs. 7D, panel I, and 7E). Moreover, a stronger dark-brown color representing TIAM1 protein was observed in the tumor areas of SK-Hep1-anti-miR-21 xenografts (Fig. 7D, panel IV, arrowhead), compared to SK-Hep1-scrambled xenografts (Fig. 7D, panel II). Analogous results were obtained with both in vivo animal and cell culture models.

Discussion

T₃/TR signaling promotes cell invasiveness involved in hepatoma progression through direct or indirect upregulation of several genes, including furin (10), methionine adenosyltransferase 1A (MAT1A) (9), plasminogen activator inhibitor-1 (PAI-1) (18), and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) (19). MiRNAs are small RNA molecules processed from endogenous precursor RNA with stem-loop structures that negatively regulate gene expression. To date, several tumor progression-related genes have been identified as specific miRNA targets (34, 35). Aberrant miRNA expression has been reported in various tumors, including liver cancer.
(36, 37). Data from the current study indicate that both T3/TR signaling and miRNAs affect tumor cell progression through TIAM1 suppression and upregulation of β-catenin, vimentin and MMP2 (Supplementary Fig. S8). Moreover, the correlations among TRα1, miR-21 and TIAM1 expression patterns in hepatoma patients appear similar to those reported in hepatoma cells.

Previous studies have reported that T3 stimulation affects the expression of specific miRNAs in mouse hepatocyte cells. T3 upregulates Gpd2, and Mup1 mRNA may be associated with decreased miR-206 expression in the presence of T3 (38). However, the molecular mechanism underlying T3-mediated regulation of miRNAs remains unclear at present. To our knowledge, this is the first study to clearly demonstrate that the miR-21 promoter region containing native TRE bound to TR and RXR is directly upregulated by T3 in hepatoma cells. Both T3/TR signaling and miR-21 stimulation promoted hepatoma cell migration. Moreover, T3 and miR-21 stimulation was associated with suppression of TIAM1 protein expression. Our experiments further confirmed that TIAM1 is a direct target gene of miR-21. Notably, suppression of TIAM1 by T3 was rescued upon knockdown of endogenous miR-21. Suppression of cell migration with anti-miR-21 was partially restored after administration of T3. Conversely, T3-enhanced cell migration was inhibited upon endogenous miR-21 knockdown. Overall, our analytical data support the promotion of hepatoma cell migration and invasion through a T3/TR-miR-21-TIAM1 pathway (Supplementary Fig. S8). In addition, the tumor suppressor gene, MSH2 (39), another miR-21 target, was downregulated through T3/TR signaling in hepatoma cells. The associations among T3/TR, miR-21 and MSH2 further confirm the pathway of T3/TR-miR-21-target in hepatoma cells. Based on the reported
function of MSH2 as a tumor suppressor, our data support a role of T3/TR as an oncogene.

Suppression of TIAM1 expression by miR-21 or knockdown of endogenous TIAM1 with RNAi led to promotion of migration and invasion of Hep3B cells. Similar results were observed with colon carcinoma cells overexpressing miR-21, whereby enhanced cell migration and invasion were inhibited upon TIAM1 overexpression (23). In addition, TIAM1 is a direct target of miR-21 in LIM 1863 colon carcinoma cells (23). TIAM1 inhibits the migratory and invasive properties of epithelial and melanoma cells via different mechanisms. In epithelial cells, TIAM1 promotes E-cadherin-mediated cell-cell adhesion and shifts the balance between invasion-promoting MMP2 and MMP9 and invasion-inhibiting tissue inhibitors of metalloproteinases (TIMP-1 and TIMP-2) to suppress cell migration and invasion ability (40). In melanoma cells, TIAM1-mediated inhibition of migration and invasion is dependent on increased Rac activity (41). In the current study, we propose that knockdown of TIAM1 promotes migratory and invasive abilities in hepatoma cells through activation of the β-catenin pathway and increase in vimentin and MMP2 levels.

Similarly, the estrogen receptor α (ERα) binds the miR-221-222 transcription start site recruiting corepressors to suppress their transcriptional activity (42). Interestingly, Zhao et al. (43) reported that ERα is negatively regulated by miR-221 and miR-222 in breast cancer. These two studies support the existence of a negative regulatory loop involving miR-221-222 and ERα (42). However, the issue of whether such a regulatory loop can be identified between miRNA and TR is yet to be established. In fact, degradation of TR induced via T3 binding has been reported previously (44). In view of
the finding that miR-27a induces a decrease in TRβ1 protein without affecting the mRNA level (45), the possibility of a miR-21-triggered decrease in TR is speculated.

Wnt/β-catenin signaling is commonly activated in hepatoma (46). Notably, hepatitis B virus X (HBX) protein is reported to activate β-catenin signaling (47). Furthermore, hepatitis C virus (HCV)-induced miR-155 expression promotes hepatocyte proliferation and tumorigenesis via activation of Wnt signaling (48). Consistently, Lan et al. (49) observed a positive correlation between activation of the β-catenin pathway and in situ expression of miR-21 in colon carcinoma cells. In the current study, we showed that β-catenin signaling is activated by miR-21, also reported to be overexpressed in hepatoma (50) and upregulated by T3/TR signaling.

In conclusion, our findings collectively illustrate that miR-21 is directly upregulated by T3 through TR binding to native TRE of the miR-21 promoter region in hepatoma cells. T3 enhances migration and invasion by targeting miR-21 to downregulate TIAM1 in hepatoma cells (Supplementary Fig. S8).

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Reference


Figure legends

Fig. 1. Upregulation of miR-21 by T3 in HepG2-TRs cells. (A) Expression of the TR protein in HepG2-Neo, -TRβ1, and -TRα1 cell lines. (B to F) Cells were incubated for 24, 48, and 72 h in the absence or presence of T3 (1, 10 nM). Expression of mature miR-21 in HepG2-TRα1 (B), HepG2-TRβ1 (C), and HepG2-Neo (D) cells using stem-loop RT-qPCR. (E) Expression of primary miR-21 in HepG2-TRα1 cells in the presence or absence of T3. (F) TMEM49 mRNA expression in HepG2-TRα1 cells in the presence or absence of T3. Values are mean ± SD from three independent experiments, each performed in duplicate. * P<0.05; ** P<0.01.

Fig. 2. The promoter region of miR-21 contains native TRE as a direct T3/TR target gene. (A) HepG2-TRα1 cells were transfected with luciferase reporter vectors with (pA3TK-Luc) or without (pGL3-Basic) a minimal thymidine kinase promoter expressing full-length and various fragments of pri-miR-21 promoter. Luciferase activity was examined after cells were incubated for 24 h in the presence or absence of T3 (10, 100 nM). The vector expressing β-galactosidase was used to normalize for transfection efficiency control. Values are mean ± SD from three independent experiments, each performed in triplicate. ** P<0.01. (B) The type of putative TREs in the pri-miR-21 promoter region presented in panel (A). (C) Binding of TRα1, together with RXR to the pri-miR-21 (right) and furin (positive control, left) promoter. Chromatin fragments were prepared from HepG2-TRα1 cells. The ChIP assay was performed using control IgG, anti-TR or anti-RXR antibody. Chromatin fragments without IgG, anti-TR or anti-RXR antibody were used as an input control. Two sets of primers for positive control TRE (furin) and pri-miR-21 TRE were employed for PCR.
and gel electrophoresis.

**Fig. 3. TIAM1 is a miR-21 target downregulated by T₃.** (A) Relative luciferase activity levels in HepG2 cells cotransfected with miR-21 (left) or anti-miR-21 (right) and *TIAM1* wild-type (wt) or mutant-type (mut) 3’UTR vector. Luciferase activity was normalized to that of β-galactosidase. Data are presented as relative fold change to pCDH or pmiRZip control vector-transfected cells (Scrambled). (B) Endogenous TIAM1 protein levels in HepG2 and Hep3B cells (left). Endogenous TIAM1 protein levels in Hep3B cells expressing miR-21 (middle) or anti-miR-21 (right). Cells expressing pCDH (middle) or pmiRZip (right) as an internal control (Scrambled). (C) Relative luciferase activity levels in HepG2-TRα1 cells transfected with *TIAM1* 3’UTR vector were incubated for 24 h in the absence or presence of T₃ (100 nM). (D) Expression of the TRα1 protein in Hep3B-Neo and -TRα1 cell lines using immunoblot assay. (E) Expression of mature miR-21 in Hep3B-TRα1 cells incubated for 24 h in the absence or presence of T₃ (10, 100 nM) using stem-loop RT-qPCR. (F) Endogenous TIAM1 protein levels in Hep3B-TRα1 cells incubated for 48 and 72 h in the absence or presence of T₃ (10 nM) (left). The protein expression of TIAM1 in Hep3B-TRα1 cells with or without miR-21 knockdown incubated for 48 h in the absence or presence of T₃ (10 nM) (right). Values are mean ± SD from three independent experiments, each performed in duplicate. *P*<0.05, **P*<0.01.

**Fig. 4. MiR-21 enhances the migration and invasion of Hep3B cells.** (A) Hep3B-TRα1 cell migration ability in the absence (Td) or presence of T₃ (T3) (a). Hep3B cells were transfected with miR-21 (b) or anti-miR-21 (c) for 72 h. Hep3B cells transfected with pCDH or pmiRZip vector (scrambled) were used as controls. The
migration ability of transfected cells was determined using the Transwell migration assay. (B) Invasive ability of Hep3B cells transfected with miR-21 or control vector (pCDH) using the matrigel-coated Transwell assay. Quantification of migratory or invasive cells is presented on the right. Data are from three independent experiments, each performed in duplicate. * \( P < 0.05 \); ** \( P < 0.01 \). (C) Expression of vimentin, \( \beta \)-catenin, c-myc, cyclin D1 and TR\( \alpha \)1 in Hep3B-TR\( \alpha \)1 cells in the absence or presence of T3 (left). Expression of vimentin and \( \beta \)-catenin in Hep3B transfected with miR-21 (middle) or anti-miR-21 (right). GAPDH is an internal control.

**Fig. 5. Knockdown of TIAM1 promotes migration and invasion of Hep3B cells.** (A) Expression of TIAM1, vimentin, \( \beta \)-catenin, phospho-\( \beta \)-catenin, c-myc, cyclin D1 and c-Jun protein in Hep3B-shNeo#1 (N1), -shNeo#2 (N2), -shTIAM1#1 (K1), and -shTIAM1#2 (K2) cell lines. (B) Expression of proMMP2 in Hep3B-shNeo and -shTIAM1 cell lines using zymograph assay. (C) Migration abilities of Hep3B-N1, -N2, -K1, and -K2 cells assessed using the Transwell migration assay. (D) Quantification of migratory cells from (C). (E) Invasion abilities of Hep3B-N1, -N2, -K1, and -K2 cells using the matrigel-coated Transwell assay. (F) Quantification of invasive cells from (E). Values are mean ± SD from three independent experiments, each performed in duplicate. * \( P < 0.05 \); ** \( P < 0.01 \).

**Fig. 6. Anti-miR-21-repressed cell migration is rescued by T3 in Hep3B-TR\( \alpha \)1 cells.** (A) Hep3B-TR\( \alpha \)1 cells were transfected with the pmiRZip (scrambled; I and II) or pmiRZip-21 (anti-miR-21; III and IV) vector, and incubated for 24 h in the absence (I and III) or presence (II and IV) of T3 for the Transwell migration assay. (B) Quantification of migratory cells from (A). Values are mean ± SD from three
independent experiments, each performed in duplicate. **$P<0.01$. 

**Fig. 7. Correlation among T₃/TR, miR-21 and TIAM1 in vivo.** (A) The expression of miR-21 from liver tissues of SD rats underwent sham (n = 4), thyroidectomies (Tx, n = 4), and application of T₃ (Tx + T₃, n = 3) was detected using real-time Q-PCR. (B) The expression of miR-21 from lung tissues of J7-TRα1 xenograft SCID mice with eu-, hypo- and hyper-thyroid (n = 3 in each group). (C) Lung tissue sections from (B) were performed H&E staining (I, IV and VII: 200×; II, V and VIII: 400×) or immunohistochemistry detected with TIAM1 (III, VI and IX: 400×). T: tumor. The black lines indicate tumor region of the 400× magnification is from the corresponding area of the 200×. (D) Lung tissue section from control SK-Hep1 xenografts (scrambled) and SK-Hep1 miR-21 knockdown (anti-miR-21) xenografts. Panels I and III displayed the H&E staining. Panels II and IV displayed the TIAM1 expression detected by immunohistochemistry. Tumor is indicated by arrowheads. (E) The metastatic index in lung tissue is shown. * $P<0.05$; ** $P<0.01$. 
Fig. 1

(A) Western blot analysis of HepG2 cells transfected with Neo, TRβ1, or TRα1.

(B) Graph showing the relative fold induction of miR-21 in HepG2-TRα1 cells treated with 0 nM, 1 nM, or 10 nM at various time points (24, 48, 72 hours).

(C) Graph showing the relative fold induction of miR-21 in HepG2-TRβ1 cells treated with 0 nM, 1 nM, or 10 nM at various time points (24, 48, 72 hours).

(D) Graph showing the relative fold induction of miR-21 in HepG2-Neo cells treated with 0 nM, 1 nM, or 10 nM at various time points (24, 48, 72 hours).

(E) Graph showing the relative fold induction of pri-miR-21 in HepG2-TRα1 cells treated with 0 nM, 1 nM, or 10 nM at various time points (24, 48, 72 hours).

(F) Graph showing the relative fold induction of TEMEM49 in HepG2-TRα1 cells treated with 0 nM, 1 nM, or 10 nM at various time points (24, 48, 72 hours).
(A) Relative Promoter Activity

(I) -3000/-45
(II) -3000/-1429
(III) -1508/-639
(IV) -738/-45
(V) -1109/-875
(VI) -916/-639
(VII) -1306/-1056

(B) Thyroid hormone response element
Pal: palindrome
Lap: inverted palindrome
DR4: direct repeat 4

(C) Furin and miR-21

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Fig. 3
**Fig 4**

(A) Migration

- **a**
  - Td vs. T3
  - Graph showing migratory cells per field.

- **b**
  - Scrambled vs. miR-21
  - Graph showing migratory cells per field.

- **c**
  - Scrambled vs. anti-miR-21
  - Graph showing migratory cells per field.

(B) Invasion

- Scrambled vs. miR-21
  - Graph showing invasive cells per field.

(C)

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Fig. 6
Thyroid hormone regulation of miR-21 enhances migration and invasion of hepatoma

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