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Thyroid hormone negatively regulates tumorigenesis through suppression of BC200

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Abstract

Thyroid hormone (T_3) and its receptor (TR) are involved in cancer progression. While deregulation of long non-coding RNA (lncRNA) expression has been detected in many tumor types, the mechanisms underlying specific involvement of lncRNAs in tumorigenicity remain unclear. Experiments from the current study revealed negative regulation of BC200 expression by T_3 /TR. BC200 was highly expressed in hepatocellular carcinoma (HCC) and effective as an independent prognostic marker. BC200 promoted cell growth and tumor sphere formation, which was mediated via regulation of cell cycle-related genes and stemness markers. Moreover, BC200 protected cyclin E2 mRNA from degradation. Cell growth ability was repressed by T_3 , but partially enhanced upon BC200 overexpression. Mechanistically, BC200 directly interacted with cyclin E2 and promoted CDK2–cyclin E2 complex formation. Upregulation of cell cycle-related genes in hepatoma samples was positively correlated with BC200 expression. Our collective findings support the utility of a potential therapeutic strategy involving targeting of BC200 for the treatment of HCC.

Key Words

- thyroid hormone
- non-coding RNA
- tumor-initiating cell
- mRNA stability
- overall survival

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Introduction

Thyroid hormone (T_3) and its receptor (TR) are involved in cell growth, metabolism, autophagy and cancer progression (Wu *et al.* 2013, Chi *et al.* 2016). Two TR genes, TR α 1 and TR β 1, have been identified on chromosomes 17 and 3, respectively. The receptors function as ligand-dependent transcriptional factors through binding to specific regions known as thyroid hormone response elements (TREs) that are usually located in the promoter

region of target genes. T_3 /TR has been shown to reduce tumor formation in various cancer types, including hepatocellular carcinoma (HCC) (Chi *et al.* 2013) and breast cancer (Martinez-Iglesias *et al.* 2009), suggestive of a tumor suppressor function.

According to the tumor-initiating cell (TIC) concept, a subset of cancer cells possesses stem cell features that are indispensable for tumor formation (Plaks *et al.* 2015).

TICs are generally characterized by their capacity for self-renewal and generate progeny to create the tumor bulk. Accumulating evidence supports the involvement of TICs in perpetuation of various cancers, including liver (Zhu *et al.* 2016), breast (Nadal *et al.* 2013), prostate (Vander Griend *et al.* 2008), brain (Brescia *et al.* 2013) and colon (Jing *et al.* 2015). In liver cancers, CD133/Prominin-1, a transmembrane hematopoietic stem cell antigen, has been identified as a putative marker of TICs. Notably, CD133 promotes tumorigenic capacity in cancer stem cells through activation of PI3K/AKT/mTOR (Xia & Xu 2015) or β -catenin signaling pathways (Mak *et al.* 2012).

Long non-coding RNAs (lncRNAs) are a class of non-protein coding transcripts longer than 200 nucleotides that regulate complex cellular functions, such as cell growth, differentiation, metabolism and metastasis (Rinn & Chang 2012). Recently, dysregulation of many HCC-related lncRNAs such as lncRNA-ATB (Yuan *et al.* 2014), NEAT1 (Mang *et al.* 2017), HOTAIR (Gao *et al.* 2016) and MALAT1 (Malakar *et al.* 2017) have been identified. Brain cytoplasmic RNA 1 (BCYRN1 or BC200), hereafter referred to as BC200, is overexpressed in several tumor types, including esophageal squamous cell carcinoma (Zhao *et al.* 2016), breast (Iacoangeli *et al.* 2004) and lung cancer (Hu & Lu 2015). However, the mechanisms underlying functional impairment and specific involvement of BC200 in HCC remain to be established.

Here, we aimed to elucidate the involvement of specific deregulated lncRNAs and target genes mediated by T_3 /TR in tumor formation. Experiments from the current study revealed BC200 as a target gene downregulated by T_3 /TR. BC200 promoted cell growth and oncogenic sphere formation of hepatocarcinoma cells through upregulation of cell cycle-related genes. Furthermore, T_3 /TR signaling inhibited cell growth through suppression of BC200. Our results collectively demonstrate novel associations among T_3 /TR, BC200, cyclin E2 and cyclin-dependent kinases (CDK) 2 that are involved in regulation of the tumor formation.

Materials and methods

Cell culture

The human hepatoma cell lines, HepG2, Hep3B, SK-Hep1 (obtained from American Type Culture Collection), Huh7 (gift from Dr. T.Y Hsieh, Tri-Service General Hospital, Taiwan) (Shiu *et al.* 2013) and J7 (gift from Dr. C S Yang, National Taiwan University, Taiwan) (Chen *et al.* 2002)

were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS). Cells were grown at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The cell lines were authenticated using the Promega StemElite ID System, which is a short tandem repeat-based assay (Supplementary Fig. 1, see section on [supplementary data](#) given at the end of this article). All experiments were conducted with cells from passage numbers 5–20. Serum was depleted of T_3 , as described previously (Chen *et al.* 2008).

lncRNA profiling

Human Disease-Related lncRNA Profiler (System Biosciences, Mountain View, CA, USA) consisting of 83 lncRNAs was used. Briefly, total RNA was isolated from HepG2-TR α 1 cells treated with/without T_3 for 24 h and two paired HCC specimens. The detection of lncRNA was performed according to the manufactory's instructions. Values are expressed as log 2-transformed relative fold increase or decrease in lncRNA expression, relative to that in without T_3 treatment or adjacent non-tumorous tissues after normalization to the internal control. A positive log 2-transformed fold-change indicates higher expression in T_3 treatment and tumor specimens, whereas a negative value signifies relatively decreased expression. A Student's *t*-test was performed to identify significantly and differentially expressed lncRNAs with a fold-change ≥ 2.0 or ≤ 0.5 , $P < 0.05$.

Human hepatoma specimens

Hepatoma samples from Taiwan Liver Cancer Network (TLCN) were selected for study and subjected to qRT-PCR and western blot analyses (Chang *et al.* 2016). These analyses were carried out under informed consent. The protocol was approved by the Medical Ethics and Human Clinical Trial Committee at Chang Gung Memorial Hospital (Institutional Review Board, No: 103-4866B).

Quantitative reverse transcription-PCR (qRT-PCR)

To quantify lncRNA transcripts, total RNA was extracted from cells with TRIzol reagent kit (Life Technologies Inc.) and converted into cDNA using reverse transcriptase (Life Technologies). qRT-PCR was conducted in a total reaction volume of 15 μ L containing forward and reverse primers and 1X SYBR Green mix (Applied Biosystems). The ABI

Prism 7500 Fast Real-Time PCR system (Life Technologies) was employed for qRT-PCR analysis. The primer sequences were listed in Supplementary Table 1.

RNA *in situ* hybridization (RNA-ISH)

In situ detection of BC200 expression was performed on formalin-fixed paraffin-embedded HCC samples. BC200 anti-sense RNA probes were labeled with digoxigenin (DIG) using a DIG RNA labeling kit (Roche). Before the labeling reaction, BC200 plasmid was digested with restriction enzymes for linearization. Further, *in vitro* transcription from plasmid was carried out using SP6 RNA polymerase. After de-paraffinization and rehydration, samples were treated with proteinase K (20 µg/mL) to digest tissues before hybridization, which was conducted at 37°C for 30 min. Sections were prehybridized in buffer containing 4×SSC and 50% deionized formamide for at least 10 min at 37°C and hybridized overnight with DIG-labeled probe at 62°C in solution containing 40% deionized formamide, 10% dextran sulfate, 1× Denhardt's solution, 4× SSC, 10 mM DTT and 1 µg/mL yeast t-RNA. After stringent washing, signals were detected using anti-DIG-AP antibodies (1:800 dilution, Roche) and NBT/BCIP substrate (Roche).

Immunoblot analysis

The immunoblot procedure was performed as described previously (Wu *et al.* 2011). Antibodies specific for cyclin E1, cyclin E2 and CDK2 (Santa Cruz Biotechnology Inc.), p21 (Thermo Fisher Scientific Inc.), p27 (Sigma-Aldrich), CD133 (Miltenyi Biotec, Auburn, CA, USA), CD44, Sox2, Nanog (GeneTex, Inc., Irvine, CA, USA) and GAPDH (Merck Millipore) were used. Band intensities were calculated using Image Gauge software (Fujifilm, Tokyo, Japan). The PVDF membranes were reprobed for different antibodies after using TOOLStripping Buffer (BIOTOOLS CO., LTD. Taiwan). The signal intensities of expression of target genes were normalized to those of GAPDH.

Establishment of overexpression and knockdown stable cell lines

For ectopic expression of BC200 ncRNA, the sequences were amplified via PCR and cloned into pLKO-TRC001 vector. BC200 shRNAs (shBC200#1 and shBC200#2) were designed and cloned into pLKO-TRC001 vector. The shRNA sequences were shown below:

shBC200#1-F: 5'-CCGGGAGACCTGCCTGGGCAATATACTCGAGTATATTGCCCAGGCAGGTCTCTTTT-3'
shBC200#1-R: 5'-CTCTGGACGGACCCGTTATATGAGCTCATATAACGGGTCCGTCCAGAGAAAAATTAA-3'
shBC200#2-F: 5'-CCGGACTTCCCTCAAAGCAACAACCTCGAGGGTTGTTGCTTTGAGGGAAGTTTTT-3'
shBC200#2-R: 5'-TGAAGGGAGTTTCGTTGTTGGGAGCTCCCAACAACGAACTCCCTTCAAAAAATTAA-3'

Single shRNA plasmid and virus package plasmids (pCMV-ΔR8.91 and pMD.G) were co-transfected into 293FT, and the virus harvested after 72 h of transfection. A pool of stably infected cells was selected in medium containing puromycin.

Cell proliferation

Cells (1×10^5) were grown on 6 cm dishes. At the indicated time-points, cell growth rates were determined with trypan blue exclusion and quantitated using the LUNA Automated Cell Counter.

Soft agar assay

Stable cells (10,000/well) were seeded in 12-well plates for layer agar cultures. Cells were resuspended in 0.33% agar in DMEM containing 10% FBS, and the upper layer replaced once a week in complete medium. After 3 weeks, colonies were stained with 0.01% (w/v) crystal violet. Images of all plates were obtained under a microscope (Olympus IX71), and colony numbers scanned and counted with Image J.

RNA immunoprecipitation assay

The RNA immunoprecipitation (RIP) assay was performed as described previously (Hsieh *et al.* 2014). Antibodies for RIP assay against cyclin E2 (Santa Cruz) were used.

Cell sorting and flow cytometry

Cell sorting via flow cytometry was performed on HCC cells using PE-conjugated monoclonal mouse anti-human CD133/1 (Miltenyi Biotec). Isotype control mouse IgG1k-PE (eBioscience, San Diego, CA, USA) served as the negative control.

Sphere formation assay

For the sphere formation assay, cells (1×10^3) were plated to the ultralow attachment 6-well plate (Corning Inc., Corning, NY, USA) with DMEM/F12 medium containing

20 ng/mL basic fibroblast growth factor, 20 ng/mL epidermal growth factor and B27. Two weeks later, the whole image of each well was taken and the number of spheres larger than 100 μ m was manually counted using ImageJ software.

Animal models

Model I: Five-week-old male nude mice were subcutaneously injected with BC200-overexpressing Hep3B cells (5×10^6 , $n=3$ per group) and BC200-depleted SK-Hep1 cells (2×10^6 , $n=4$ per group) to assess the effects of the lncRNA on tumor formation ability. Tumor volumes (mm^3) were measured using the formula: $(W^2 \times L)/2$ (W , smallest diameter; L , longest diameter). All animals were killed at the indicated time-points after tumor inoculation. **Model II:** Hyper- and eu-thyroid mice ($n=8$ per group) were generated and samples preparations were described in our previous report (Chi *et al.* 2016). Briefly, the effects of T_3 repressed diethylnitrosamine (DEN)-induced liver injury were determined in C57BL/6 male mice. Mice were treated with T_3 (10 μ g/100 g body weight) before intraperitoneal injection of DEN (100 mg/kg). All groups of mice received continued T_3 after DEN injection. Animal experiments were approved by the committee and performed according to the guidelines of the Chang Gang Institutional Animal Care and Use Committee Guide for the Care and Use of Laboratory animals (CGU13-106).

Statistical analysis

Results are presented as means $\pm$ S.D. of three independent experiments. Statistical analysis was performed with SPSS version 15 software (SPSS Inc., Chicago, IL, USA) using the Mann–Whitney test for comparison of two groups and one-way ANOVA, followed by Tukey's *post hoc* test, for two or more groups. Kaplan–Meier survival curves were employed to analyze survival outcomes. Overall survival (OS) with death as an event was analyzed using the log-rank test. P values <0.05 were considered significant.

Results

BC200 is downregulated by T_3 /TR

To identify T_3 /TR-related lncRNAs that are differentially expressed in HCC, lncRNA expression profile screening was performed using the SYBR Green-based qRT-PCR array in TR-overexpressing HepG2 cell lines and HCC specimens. Candidate lncRNAs that were simultaneously

downregulated by T_3 /TR and upregulated in HCC were selected for further study, leading to the identification of BC200 (Supplementary Fig. 2A). Expression levels of BC200 decreased by 50–80% after incubation of HepG2-TR α 1 and HepG2-TR β 1 with T_3 for 24–72 h (Fig. 1A). In contrast, BC200 expression levels were only marginally regulated by T_3 in HepG2-neo cells (Fig. 1A). In other hepatoma cell lines, including J7-TR α 1 and Huh7 expressing exogenous and endogenous TR protein, T_3 suppressed BC200 expression (Fig. 1B), similar to results obtained with HepG2-TR cells. These findings indicate that T_3 /TR signaling negatively regulates BC200 expression in a dose- and time-dependent manner *in vitro*. Promoter activity analysis was further performed to determine whether BC200 is regulated by T_3 at the transcriptional level. Putative negative TREs (nTRE) in the BC200 promoter region (I–VI), based on the sequences from nTRE-thyroid-stimulating hormone (TSH) (Nakano *et al.* 2004) or nTRE-Nm23-H1 (Lin *et al.* 2000), were identified with bioinformatics tools (Supplementary Fig. 2B). In the presence of T_3 , luciferase activity was decreased by 30–40% in constructs I, II, III and IV. Repression of luciferase activity was alleviated upon deletion of region d (–305/–289) in constructs V and VI (Supplementary Fig. 2B), indicating that potential nTRE of the BC200 promoter is located within this region. TR binding to promoter fragment VI of BC200 *in vivo* was validated using the ChIP assay. Specific binding of TR to the BC200 promoter fragment was confirmed based on pulldown with the TR antibody, but not mopc21 (immunoglobulin) antibody (Supplementary Fig. 2C).

BC200 expression is upregulated in HCC

qRT-PCR and ISH were performed to validate the profiling results. Notably, BC200 expression was significantly upregulated in HCC tissues compared with benign and adjacent normal tissues (Fig. 1C). Consistently, the ISH assay revealed high expression of BC200 in HCC (Fig. 1D). Furthermore, BC200 expression in HCC was positively correlated with tumor type, tumor size, vascular invasion and pathological stage (Table 1). Kaplan–Meier survival analysis showed association of high BC200 expression with poor OS rate in HCC patients (Fig. 1E). Multivariate Cox proportional hazard regression analysis revealed that BC200 is an independent prognostic factor associated with survival (Table 2). These data clearly support a critical role of BC200 in hepatocarcinogenesis.

Figure 1

BC200 is downregulated by T_3 /TR and highly expressed in HCC. (A and B) HepG2-TR, HepG2-neo, J7-TR and HuH-7 cells were treated with T_3 for 24–72 h, and BC200 levels measured using qRT-PCR. 18S rRNA was used as the internal control. (C) qRT-PCR analysis of BC200 expression in human HCC. 18S rRNA was used as the internal control. (D) ISH analysis of BC200 expression in human HCC. (E) Kaplan–Meier analysis of overall survival based on BC200 expression in HCC specimens. Mean expression of BC200 was used as the cutoff. Overall survival was analyzed using the log-rank test.

BC200 promotes cell growth and transformation *in vitro* and *in vivo*

The precise functions of the majority of lncRNAs are still unknown. The co-expression network analysis has been used to predict function of unknown non-coding RNA since genes regulated by the specific regulator or a set of genes with the same function would be co-expressed (Lee *et al.* 2004, Gong *et al.* 2016, Shang *et al.* 2016). This type of approach may be effectively used for exploring the roles of lncRNAs in cancer progression (Lee *et al.* 2004). The Oncomine database (www.oncomine.com) was used

to systematically assess the relative BC200 expression in different cancer types. However, only one publicly available dataset contains information of BC200 was found to exhibit a significant upregulation of BC200 in lung cancer (Supplementary Fig. 2D). In our study, co-expression analysis for BC200-involved pathway was performed using Pearson correlation in a publicly available dataset (GSE32863). Pathway analysis was further carried out by Gene Set Enrichment Analysis tool after genes obtained from co-expression network analysis. The results indicate that cell cycle mitotic and DNA replication pathway are significantly enhanced and positively correlated with

Table 1 Clinicopathological correlations of BC200 in HCC specimens.

Parameters	Cases (n=240)	Mean $\pm$ s.e.	P ^a
Age (years)			
<65	148	7.971 $\pm$ 1.838	0.4403
$\geq$ 65	92	5.122 $\pm$ 0.6504	
Gender			
Male	131	4.340 $\pm$ 0.4438	0.1600
Female	109	3.721 $\pm$ 0.3843	
Cirrhosis			
No	142	5.891 $\pm$ 1.106	0.3239
Yes	98	8.310 $\pm$ 2.354	
AFP			
Low	107	5.059 $\pm$ 1.400	0.1404
Medium	49	5.485 $\pm$ 1.438	
High	84	10.1 $\pm$ 2.654	
Viral status			
NBNC	32	5.206 $\pm$ 1.452	0.9081
HBV	122	7.812 $\pm$ 1.890	
HCV	81	6.335 $\pm$ 1.859	
HBV & HCV	5	3.629 $\pm$ 1.146	
Tumor type			
Solitary	181	5.460 $\pm$ 0.9396	0.0322
Multiple	59	11.23 $\pm$ 3.715	
Tumor size			
<5 cm	137	5.172 $\pm$ 1.192	0.0343
$\geq$ 5 cm	103	9.150 $\pm$ 2.183	
Vascular invasion			
No	128	4.090 $\pm$ 0.6392	0.01
Yes	112	10.07 $\pm$ 2.351	
Pathological stage			
I	116	4.185 $\pm$ 0.6977	0.0376
II	71	7.686 $\pm$ 2.102	
III	53	11.69 $\pm$ 4.126	
Grading			
1	11	2.173 $\pm$ 0.5413	0.2778
2	166	5.898 $\pm$ 1.047	
3	63	10.28 $\pm$ 3.444	

^aMann–Whitney *U* test (for two groups) or Kruskal–Wallis test (for > two groups).

BC200 expression (Supplementary Fig. 2E). Based on the indirect pathway analysis data, we examined BC200 potential effects on cell growth via cell counting and soft agar assay in hepatoma cells. Based on indirect pathway analysis data, we examined BC200 potential effects on cell growth via cell counting and soft agar assay in hepatoma cells. Notably, BC200 showed low expression in well and moderately differentiated cell lines (HepG2, Huh7 and Hep3B) but high expression in poorly differentiated cell lines (J7, Mahlavu and SK-Hep1) (Supplementary Fig. 3A, upper panel). Interestingly, poorly differentiated cell lines exhibited a higher growth rate than well-differentiated cell lines (Supplementary Fig. 3A, lower panel), suggesting that expression of BC200 is positively correlated with cell growth ability. Stable BC200 knockdown or

overexpression in J7, SK-Hep1, Hep3B and Huh7 cell lines was subsequently established (Supplementary Fig. 3B). Cell growth and soft agar assay were suppressed in J7 and SK-Hep1 cells after depletion of endogenous BC200 (Fig. 2A and B). Conversely, increased proliferation was observed in BC200-overexpressing Hep3B and Huh7 cells (Fig. 2A and B). To confirm whether the *in vitro* phenotype can be recapitulated *in vivo*, tumor formation was examined in nude mice. To this end, stable cells were subcutaneously injected into nude mice and tumor growth rates were determined. Overexpression of BC200 significantly promoted tumor growth and tumor weights, compared with the control group (Fig. 2C). Conversely, tumor growth curves and tumor weights were markedly reduced in BC200-depleted SK-Hep1 cells, compared with the control group (Fig. 2C). Our collective findings support a tumor promoter role of BC200 in HCC, both *in vitro* and *in vivo*.

BC200 is required for self-renewal maintenance of liver cancer stem cells

Our findings suggest that BC200 is responsible for conferring growth advantages at an early stage of tumor development. As specified earlier, TICs have the capacity to self-renew and regenerate new tumors. To determine the association between TIC behavior and BC200, flow cytometry of the CD133⁺ TIC population in HCC cell lines was performed. The data revealed the presence of the CD133⁺ population in HepG2 and Huh7 cells, with expression ranging from 8 to 68% (Supplementary Fig. 4A). HepG2 and Huh7 cell lines were further sorted into CD133⁻ and CD133⁺ populations and confirmed via western blot (Supplementary Fig. 4B). BC200 was upregulated in CD133⁺ HepG2 cells (Supplementary Fig. 4C). To determine whether BC200 functions in liver TIC self-renewal, the sphere formation assay was performed. Knockdown of BC200 dramatically reduced the tumor sphere formation frequencies of CD133⁺ subsets of HepG2 and Huh7 cells, compared with sh-luc cells (Fig. 2D). To ascertain the role of T₃/TR in liver TIC self-renewal, CD133⁺ HepG2 cells were infected with Adenovirus-TR α 1 (Ad-TR α 1) or Adenovirus-TR β 1 (Ad-TR β 1) and tumor sphere formation with/without T₃ examined. T₃ clearly suppressed tumor sphere formation in CD133⁺ HepG2 cells overexpressing Ad-TR α 1 or Ad-TR β 1 relative to non-treated cells (Fig. 2E). In view of these results, we propose that BC200 is required for maintaining stemness ability in HCC.

Table 2 Univariate and multivariate Cox regression analyses for overall survival.

Variable	Univariate analysis	P	Multivariate analysis	P
	HR (95% CI)		HR (95% CI)	
BC200 (high vs low)	1.838 (1.156–2.922)	0.01	1.618 (1.011–2.589)	0.045
Age (≥ 50 vs < 50)	1.154 (0.767–1.736)	0.493	–	–
Gender (male vs female)	0.912 (0.608–1.366)	0.654	–	–
Cirrhosis (yes vs no)	1.206 (0.804–1.808)	0.366	–	–
AFP (high vs medium vs low)	1.310 (1.046–1.640)	0.019	1.080 (0.858–1.360)	0.51
Tumor size (≥ 5 vs < 5 cm)	2.717 (1.796–4.108)	< 0.001	1.705 (1.035–2.810)	0.036
Tumor type (multiple vs solitary)	2.135 (1.415–3.223)	< 0.001	1.170 (0.627–2.181)	0.622
Vascular invasion (present vs absent)	1.824 (1.519–2.191)	< 0.001	1.561 (1.124–2.166)	0.008
Pathological stage (III vs II vs I)	2.176 (1.695–2.794)	< 0.001	1.043 (0.549–1.980)	0.898
Grading (3 vs 2 vs 1)	1.147 (0.777–1.694)	0.49	–	–

CI, confidence interval; HR, hazard ratio.

BC200 is involved in T_3 /TR-mediated repression of cell growth ability

CDKs and cyclin-dependent kinase inhibitors play important roles in control of cell growth (Abbas & Dutta 2009, Malumbres 2014). Deregulation of these proteins is a hallmark of several diseases, including cancers. To assess the contributory role of BC200 in cell growth, its effects on the mRNA levels of cell cycle-related genes were examined. The p21 transcript levels were increased in SK-Hep1 cells after knockdown of BC200 expression while mRNA levels of CDK2, cyclin E1 and cyclin E2 were reduced (Fig. 3A, left panel). Conversely, overexpression of BC200 led to opposite effects (Fig. 3A, right panel). As expected, levels of the corresponding proteins were consistent with mRNA results, both *in vitro* and *in vivo* (Fig. 3B and C). In addition, BC200 depletion in CD133⁺ HepG2 cells led to reduced expression of pluripotent transcription factors (Nanog and Sox2), CD44 and cell cycle-related genes, compared with control (sh-luc) cells (Fig. 3D). To confirm the involvement of BC200 in T_3 /TR-regulated cell growth, HepG2-TR β 1 cells were transfected with BC200-expressing or control (vc) plasmid and proliferation examined in the presence or absence of T_3 . T_3 clearly repressed HepG2-TR β 1 cell growth. Interestingly, this effect was partially rescued by BC200 in the presence of T_3 (Fig. 3E). Notably, protein levels of cyclin E2 and CDK2 were clearly increased in BC200-overexpressing HepG2-TR β 1 cells treated with T_3 , compared with their T_3 -treated control counterparts (Fig. 3E, lanes 2 vs 4). Our results collectively demonstrate that T_3 /TR suppresses BC200 expression, leading to modulation of cell cycle-related genes, and ultimately, inhibition of cell proliferation.

BC200 protects cyclin E2 mRNA from degradation

To clarify the regulatory mechanism of cyclin E2, its expression was measured using qRT-PCR after treatment

with actinomycin D (ActD, a transcriptional inhibitor). Knockdown of BC200 promoted cyclin E2 mRNA degradation in SK-Hep1 cells (Fig. 4A), suggesting a protective effect of BC200 against degradation. To determine the functional sequence of BC200, we established stable expression of BC200-wt (1–200), BC200-F1 (1–119), BC200-F2 (122–200) and BC200-F3 (51–119) in BC200 knockdown cell lines. Overexpression of BC200-wt and BC200-F1 partially delayed cyclin E2 mRNA degradation, compared to the control group, while overexpression of BC200-F2 and BC200-F3 had no effect on the stability of these mRNAs (Fig. 4B). These findings suggest that a functional sequence of 1–50 nucleotides participates in regulation of cyclin E2 mRNA stability. Previously, cyclin E-CDK2 complex has been considered an essential and master regulator of progression through G1 phase of the cell cycle (Koff *et al.* 1992). To test this hypothesis, co-immunoprecipitation assay was performed in BC200 stable cell lines. We found BC200 promoted association with CDK2-cyclin E2, but not CDK2-cyclin E1 (Fig. 4C). These findings indicated that the critical role of BC200 in cyclin E2-CDK2 activity in HCC cells. Next, the physical interaction of BC200 and cyclin E2 was examined using RIP assay. The results showed a specific enrichment of BC200 (but not GAPDH mRNA) coprecipitated within the cyclin E2 (Fig. 4D), indicating BC200 associated with cyclin E2 through direct binding and enhanced CDK2–cyclin E2 complex formation. Furthermore, levels of the p27 and p21 were decreased in cyclin E2-overexpressing cells compared to control cells (Fig. 4E).

Cyclin E2 and CDK2 are highly expressed in HCC and positively correlated with BC200

T_3 protects hepatocyte from DEN-induced HCC-like liver tumors in mice model (Chi *et al.* 2016). To further

Figure 2

BC200 promotes cell growth and transformation ability. Effects of BC200 on cell growth (A) and soft agar colony formation (B). (C) Comparison of tumor volumes between hepatoma xenografts in BC200 stable cell lines. Xenografts were derived from Hep3B (5×10^6 , $n=3$ per group) and SK-Hep1 cells (2×10^6 , $n=4$ per group). Tumor volumes and weights were measured in xenografts after subcutaneous injection of cells. (D) Sphere formation assay of BC200-silenced stable cells. (E) CD133⁺ HepG2 cells treated with T₃ or left untreated were infected with adenovirus-TRα1 (Ad-TRα1) and adenovirus-TRβ1 (Ad-TRβ1), and the sphere formation assay performed.

determined T₃ represses this phenotype through BC200/cyclin E2/CDK2 cascade, the expression levels of these genes were determined in DEN/hyper- and DEN/euthyroid mice. The expression levels of BC200 and cell cycle-related genes were repressed in DEN/hyperthyroid group, compared with DEN/euthyroid group (Fig. 5A and B). Our results suggest that T₃ suppresses DEN-induced hepatocarcinogenesis through BC200/cyclin E2/CDK2 cascade. To determine whether cyclin E2 and CDK2 are associated with tumor formation, mRNA levels of these molecules were determined in HCC specimens using qRT-PCR. The markers were significantly expressed in HCC compared with the corresponding adjacent

non-tumor counterparts (Fig. 5C and Supplementary Fig. 5A), consistent with high expression in HCC reported earlier from two publicly available datasets (Supplementary Fig. 5B and C). Furthermore, both TRα1 and TRβ1 were downregulated in HCC relative to the normal control group (Supplementary Fig. 5B and C). Notably, cyclin E2 and CDK2 expression were significantly correlated with OS of HCC patients (Supplementary Fig. 6A), similar to findings from the public dataset (Supplementary Fig. 6B). Spearman's correlation coefficient analysis revealed significant positive correlation of BC200 with cyclin E2 and CDK2 (Supplementary Table 2). Considering the positive correlation between these markers in HCC

Figure 3

BC200 regulates cell cycle-related genes and is involved in T₃/TR-mediated cell growth function. (A) qRT-PCR analysis of total RNA in the indicated cell lines using 18S rRNA as a loading control to determine the mRNA expression levels of cell cycle-related genes. (B and C) Western blot analysis of cyclin E, CDK2, p21 and p27 expression in the indicated cell lines and tissues. (D) Immunoblot analysis of the expression patterns of CD44, Nanog, Sox2 and cell cycle-related genes in the indicated cell lines. GAPDH was used as the loading control. (E) Left panel: Western blot analysis of expression of cell cycle-related genes in the presence and absence of T₃. Right panel: Vector control (vc) and BC200 cell lines stably expressing in HepG2-TRβ1 were grown in 6 cm dishes, and cell growth detected in the presence and absence of T₃.

tumors, their combined influence on patient outcomes were evaluated. Patients were classified into three groups (I–III) using the median value as cutoff. Patients in group III (higher expression of both genes) showed significantly

poorer OS than those in group I (lower expression of both genes) (Fig. 5D). Our findings clearly support an important regulatory function of the BC200/cyclin E2/CDK2 axis in hepatoma (Fig. 5E).

Figure 4

BC200 promotes cyclin E2-CDK2 complex formation. (A and B) Half-life of cyclin E mRNA was calculated in the indicated cell lines after adding actinomycin D (ActD), normalizing to 18S rRNA. (C) Lysates from indicated cells were immunoprecipitated with IgG, anti-cyclin E1 and anti-cyclin E2 antibody, respectively. The immunoprecipitates were analyzed by Western blotting with an anti-CDK2 antibody. (D) RIP experiments were performed in J7 and SK-Hep1 cells and the coprecipitated RNA was subjected to qRT-PCR for BC200. GAPDH was used as negative controls. (E) Immunoblot analysis of the expression patterns of p27 and p21 in the indicated cell lines.

Discussion

Previous studies have confirmed that lncRNAs and transcriptional factors form feedback or feed-forward loops, which play critical roles in biological processes. For example, the MYC-induced long non-coding RNA (MINCR) is activated by MYC and modulates the MYC transcription network (Doose *et al.* 2015). LincRNA-p21 is upregulated by HIF1 α and essential for hypoxia-enhanced glycolysis (Yang *et al.* 2014). In view of these associations, it is proposed that deregulation of lncRNAs by transcriptional factors could cause disease or cancer progression. Therefore, transcriptional factor-mediated lncRNA regulation is a significant focus of cancer biology research. In the current study, BC200 was identified as a novel gene downregulated by T₃/TR that modulates thyroid hormone-mediated functions.

BC200 is reported to be expressed at high levels in invasive carcinoma of the breast (Iacoangeli *et al.* 2004). Earlier receiver-operating characteristics analysis of sensitivity and specificity confirmed the diagnostic power of BC200 RNA as a molecular marker of invasive breast cancer. However, the functional and clinical significance of BC200 in HCC remains to be established. Here, we showed that BC200 promotes cancer cell growth,

both *in vitro* and *in vivo* and is positively correlated with tumor type, tumor size, vascular invasion and pathological stage, supporting its utility as an independent prognostic factor associated with OS. Additionally, BC200 is known to act as a translational regulator in neurons (Iacoangeli & Tiedge 2013). Data from the current study support a novel role of BC200 in regulation of cyclin E2 mRNA stability. Functional sequences of BC200 1–50 nucleotides in length were responsible for regulating cyclin E2 mRNA stability. On the other hand, BC200 expression appeared positively correlated with the cell proliferation rate. These results were similar to previous findings (Booy *et al.* 2017). Interestingly, BC200 was upregulated in CD133⁺ HepG2 cells and stimulated stemness marker expression (Sox2 and Nanog), in turn, promoting sphere-formation capacity. Accordingly, we concluded that BC200 regulates early-stage HCC formation.

In our experiments, several genes that are positively or negatively regulated by T₃ were identified in the HepG2-TR α 1 cell line, including endoglin (Lin *et al.* 2013), pituitary tumor-transforming 1 (PTTG1) (Chen *et al.* 2008), Dickkopf 4 (DKK4) (Liao *et al.* 2012), Ubiquitin-like with PHD and ring finger domains 1 (UHRF1) (Wu *et al.* 2015) and Death-associated protein kinase 2 (DAPK2) (Chi *et al.* 2016), proposed to suppress

Figure 5

The BC200/cyclin E2/CDK2 axis is correlated with poor prognosis in HCC patients. QRT-PCR (A) and immunoblot analysis (B) of hepatic levels of BC200, cyclin E2 and CDK2 in the numbers of mice receiving DEN treatment ($n=8$ per group). The quantitative results of western blot were shown. (C) qRT-PCR analysis of cyclin E2 and CDK2 expression in 140 pairs of human HCC (T) and adjacent normal (N) tissues using 18S rRNA as the loading control. (D) Kaplan-Meier overall survival analysis curve for high- or low-risk survival groups were measured in 140 paired HCC patients. High BC200 and simultaneous high the other genes were significantly associated with poorest overall survival. *P* values were determined via the log-rank test. (E) Proposed model for the T_3 /TR/BC200/stemness marker/cell cycle-related gene pathway in regulation of tumor formation and self-renewal of hepatoma.

tumor formation. Previously, hypothyroidism was shown to be associated with significantly elevated risk for HCC (Hassan *et al.* 2009). A recent study by our group suggests that the T_3 /PTEN-induced kinase 1/Parkin pathway plays an important role in protecting hepatocytes from HBx-induced HCC (Chi *et al.* 2017). Catalano and co-workers demonstrated that T_3 reduces the tumorigenic potential of colorectal cancer-CSC to a significant extent through regulating Wnt and BMP signaling (Catalano *et al.* 2016). In contrast, the group of Wang showed that the thyroid hormone (T_4) promotes cell self-renewal in HCC cells through TR α , but not TR β (Wang *et al.* 2016). Kress and colleagues reported that TR α 1 overexpression and Wnt pathway activation enhance colorectal cancer formation (Kress *et al.* 2010). Specifically, the group demonstrated that TR α 1 promotes tumorigenesis in APC $^{+}/1638N$ mice with a Wnt-activated genetic background but TR α 1 overexpression alone is unable to trigger tumor formation. Thus, T_3 /TRs may perform a dual function as an oncogene or tumor suppressor in different genetic backgrounds.

In conclusion, we have made conceptual progress toward advancing the theory that T_3 /TR suppresses tumor growth and tumor sphere formation via reduction of BC200. Mechanistically, BC200 promotes

cyclin E2 expression via regulation of mRNA stability. The identification of a novel pathway interlinking T_3 /TR, BC200, cyclin E2 and CDK2, which regulates proliferation and tumor sphere formation of hepatoma cells, presents potential therapeutic strategies involving targeting of BC200 and associated molecules for treatment of HCC.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/ERC-18-0176>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Conception and design of the study: Yang-Hsiang Lin, Meng-Han Wu, Kwang-Huei Lin; Acquisition of data: Yang-Hsiang Lin, Meng-Han Wu, Ya-Hui Huang, Hsiang-Cheng Chi, Wen-Yu Chuang, Chia-Jung Yu, Ching-Ying Chen; Analysis and interpretation of data: Yang-Hsiang Lin, Ya-Hui Huang, Chau-Ting Yeh, Chung-Ying Tsai, I-Hsiao Chung; Drafting of the manuscript: Yang-Hsiang Lin, Meng-Han Wu, Kwang-Huei Lin; Statistical analysis: Yang-Hsiang Lin, Ya-Hui Huang, Chau-Ting Yeh; Study supervision: Kwang-Huei Lin.

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