Positive regulation of *spondin 2* by thyroid hormone is associated with cell migration and invasion

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Abstract

The thyroid hormone 3,3',5-triiodo-L-thyronine (T₃) regulates growth, development, and differentiation processes in animals. These activities are mediated by the nuclear thyroid hormone receptors (TRs). Microarray analyses were performed previously to study the mechanism of regulation triggered by T₃ treatment in hepatoma cell lines. The results showed that *spondin 2* was regulated positively by T₃. However, the underlying mechanism and the physiological role of T₃ in the regulation of *spondin 2* are not clear. To verify the microarray results, *spondin 2* was further investigated using semi-quantitative reverse transcription-PCR and western blotting. After 48 h of T₃ treatment in the HepG2–TR α 1#1 cell line, *spondin 2* mRNA and protein levels increased by 3.9- to 5.7-fold. Similar results were observed in thyroidectomized rats. To localize the regulatory region in *spondin 2*, we performed serial deletions of the promoter and chromatin immunoprecipitation assays. The T₃ response element on the *spondin 2* promoter was localized in the –1104/–1034 or –984/–925 regions. To explore the effect of *spondin 2* on cellular function, *spondin 2* knockdown cell lines were established from Huh7 cells. Knockdown cells had higher migration ability and invasiveness compared with control cells. Conversely, *spondin 2* overexpression in J7 cells led to lower migration ability and invasiveness compared with control cells. Furthermore, this study demonstrated that *spondin 2* overexpression in some types of hepatocellular carcinomas is TR dependent. Together, these experimental findings suggest that *spondin 2*, which is regulated by T₃, has an important role in cell invasion, cell migration, and tumor progression.

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Introduction

The actions of thyroid hormones, which regulate growth, development, and differentiation, are mediated by nuclear thyroid hormone receptors (TRs; Yen 2001). TRs are ligand-dependent transcription factors that comprise modular functional domains that mediate hormone binding (ligands), DNA binding, receptor homo- and heterodimerization, and interaction with other transcription factors and cofactors (Lazar 1993, Cheng 2000). A lack of 3,3',5-triiodo-L-thyronine (T₃) hormones in early human development reduces growth and may cause mental retardation (Yen 2001,

Forrest *et al.* 2002). TRs are derived from two genes, TR α and TR β , which are located on chromosomes 17 and 3, respectively (Cheng 2000, Yen 2001, Huang *et al.* 2006, Tai *et al.* 2007). Each of these genes expresses two receptor isoforms, 1 and 2, because of alternate splicing of their primary transcripts or of different promoter usage (Cheng 2000, Yen 2001, Chen *et al.* 2008a,b). The principal function of TRs (as transcription factors) is the regulation of the expression of target genes by binding directly to specific DNA elements termed thyroid hormone response elements (TREs) located in the promoter region of these genes.

The ability to bind specific sequences in target genes is crucial for TR function (Huang et al. 2008). In the absence of the T_3 ligand, TRs suppress the expression of target genes, which is a phenomenon known as transcriptional silencing. This process is believed to be mediated by interaction, via the ligand-binding domain, between the receptor and transcriptional corepressors, such as the silencing mediator of retinoic acid and TR (Koenig 1998). Ligand binding induces dissociation of TRs from corepressors, causing the recruitment of transcriptional coactivators, such as the steroid receptor coactivator (SRC), and subsequent induction of the expression of the target gene (Weiss & Ramos 2004).

Spondin 2 (also called mindin) is a member of the mindin–F-spondin family of extracellular matrix proteins. This family includes rat F-spondin and mindin, zebrafish mindin 1 and mindin 2, and *Drosophila melanogaster* M-spondin (He et al. 2004). All members of the mindin–F-spondin family share three domains: FS1 (for F-spondin), FS2, and thrombospondin type 1 repeats. Spondin 2 functions as a pattern recognition molecule for microbial pathogens. Spondin 2-deficient macrophages exhibit defective responses to a broad spectrum of microbial stimuli (Jia et al. 2005). The precise mechanism of T_3 -mediated spondin 2 regulation has never been reported and is the focus of this study.

Here, we demonstrated that T_3 up-regulated spondin 2 expression in HepG2–TR α 1 and –TR β 1 cells. In addition, the effect of T_3 on spondin 2 expression did not require *de novo* synthesis of cellular proteins. A similar regulation was observed *in vivo*. This study also demonstrated that TR proteins bind directly to spondin 2 5′-flanking regions. Finally, our experimental data suggest that alteration of the expression levels of spondin 2 affected cell invasiveness and migration.

Materials and methods

Cell culture

The human hepatoma cell lines Huh7, J7, HepG2, and HepG2 sublines were routinely grown in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS). Both TR α 1- and β 1-overexpressing cell lines were described previously (Chen et al. 2008b). Here, we used the TR-overexpressing cell lines HepG2–TR α 1 and HepG2–TR β 1, and the control cell line HepG2–Neo. Serum was depleted of T_3 (Td), as described previously (Samuels et al. 1979). Cells were cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO₂.

Immunoblotting

Total cell lysates were resolved using 10% SDS-PAGE. The separated proteins were then transferred onto a nitrocellulose membrane (Amersham), as described previously (Huang et al. 2006).

Cloning of *spondin 2* promoter fragments and assay of their activity

Fragments of the *spondin 2* promoter (–2555/–80) were inserted into the pGL3-Luc (Promega) or pA3TK-Luc vector. Serial deletion mutants of the promoter were amplified by PCR. Promoter construct sequences were confirmed via automatic DNA sequencing. To determine the transactivation activity of the TREs on the *spondin 2* promoter, HepG2–TR α 1#1 cells (5×10^5 cells/24-well dish) were transfected with 0.2 μ g of pGL3-Luc vector containing *spondin 2* promoter sequences and 0.05 μ g of the pSV β plasmid (which is a β -galactosidase expression vector; Clontech) using the Lipofectamine reagent. Twenty-four hours after transfection, some cells were received 10 nM T_3 . These and untransfected cells were incubated for an additional 24 h, and were then lysed to measure luciferase and β -galactosidase activities (Sambrook & Russell 2001). The activity of luciferase was normalized to the β -galactosidase activity.

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) assays were performed to determine the interaction of TR with the TREs on the *spondin* promoter region. HepG2–TR α 1 cells were treated with or without 10 nM T_3 for 24 h, harvested, and cross-linked for 10 min using 1% formaldehyde. The reaction was terminated by the addition of 0.125 M glycine. Cells were washed four times in ice-cold PBS, resuspended in RIPA lysis buffer (0.1% SDS, 0.1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris (pH 8.0), and 5 mM EDTA) in the presence of protease inhibitors (1 mM each of phenylmethanesulfonyl fluoride, aprotinin, and leupeptin), and sonicated to shear chromatin using a Misonix Sonicator 3000 Homogenizer (Mandel Scientific Company Inc., Guelph, Ontario, Canada). The sonicated DNA fragments were in the range of 100–1000 bp. Samples were precleared with 60 μ l protein A/G-agarose (Sigma Chemicals) for 30 min at 4 °C. Complexes were immunoprecipitated with 2 μ g anti-TR antibody (which were a gift from S-Y Cheng, National Cancer Institute), anti-RXR β antibody (Santa Cruz, Santa Cruz, CA, USA), or anti-IgG antibody (R&D Systems, Inc., Minneapolis, MN, USA).

The 110-bp fragment of the *spondin 2* promoter containing the predicted TRE-binding sites was detected by PCR using the following primers: forward 5'-TGCCCTCTGGCCACAGTGCAG-3', reverse 5'-TCCCGGCGCAGGCATCCCTGG-3'; or forward 5'-GCCAGGAGCTTCCCCAGCCAC-3', reverse 5'-GAAATTAGAGCCAGCG-3'.

Establishing spondin 2 knockdown or over-expressing stable hepatocellular carcinoma cell lines

shRNA clones targeting *spondin 2* were purchased from the National RNAi Core Facility (Institute of Molecular Biology, Academia Sinica, Taiwan). Transfection of shRNA to target the endogenous *spondin 2* gene in Huh7 cells was performed using the Lipofectamine reagent (Invitrogen). After 24 h of incubation, cells were transferred to medium containing puromycin for selection. After 2 weeks of selection, specific repression of the targeted gene was confirmed by western blot analysis. Alternatively, the *spondin 2* cDNA was cloned and stably expressed in J7 cells.

Animals

In accordance with techniques described elsewhere, thyroidectomy (Tx) was performed on two groups of 6-week-old male Sprague–Dawley (SD) rats ($n=10/\text{group}$) weighing 130–150 g, and sham operations were performed on one group of 6-week-old male rats ($n=10$; Chen *et al.* 2008a). All animal experiments in this study complied with National Institutes of Health (NIH) guidelines and the Chang-Gung Institutional Animal Care and Use Committee Guide for Care and Use of Laboratory Animals.

Semi-quantitative reverse transcription-PCR

Total RNA was extracted from cells using TRIzol, as described previously (Tai *et al.* 2007). Subsequently, cDNA was synthesized using the Superscript II kit for reverse transcription (RT)-PCR (Life Technologies), as described previously (Tai *et al.* 2007). Semi-quantitative RT-PCR was conducted in a 10 μl reaction mixture containing 100 ng forward and reverse primers, reaction mix (Applied Biosystems, Foster City, CA, USA), and varying concentrations of templates, as described (Tai *et al.* 2007). The exponential amplification conditions were carefully controlled to obtain data in a linear range of amplification. The human *spondin 2* PCR sequences used were as follows: forward, 5'-CCAAATACAGCATCACCTTCAC-3', reverse, 5'-GACGCACTCAGCCTCTTCTT-3'. The rat *spondin 2* PCR primers used were as follows:

forward, 5'-TGACAACTCATTCTGGGATGA-3', reverse, 5'-GCCTCGCTGGTATTTTCAATCT-3'. Real-time quantitative RT-PCR (qRT-PCR) was performed, as described previously (Chen *et al.* 2008b).

Zymography assay for matrix metalloproteinase-2 and -9

Cells (4×10^6) were plated in DMEM medium containing 10% FBS. After 24-h incubation, cells were washed and incubation was continued in serum-free medium supplemented with T_3 . The medium was collected 24 h later and was concentrated to roughly 500 ng/ μl using an Amicon ultra-4 membrane (Millipore, Bellerica, MA, USA). Forty micrograms of concentrated medium were diluted in 50 mM Tris–HCl, pH 8.0, without a reducing agent and separated using 10% SDS-PAGE in the presence of 1 mg/ml gelatin. After electrophoresis, gels were washed in 2.5% Triton X-100 for 30 min and incubated for 16 h at 37 °C in 40 mM Tris–HCl, pH 8.0, 0.01% NaN_3 , and 10 mM CaCl_2 . Gels were stained with Coomassie brilliant blue R-250 and destained in 5% methanol and 7.5% acetic acid until clear bands appeared.

In vitro assay of invasive activity

The influence of T_3 on the *spondin 2*-mediated invasive activity of HepG2–TR α 1 or HepG2–*spondin 2* cell lines was assessed using a Transwell rapid *in vitro* assay, as described previously (Lin *et al.* 2000). Briefly, cell density was adjusted to 5×10^5 cells/ml, and 200 μl of this suspension was added to each well coated with Matrigel (Becton-Dickinson, Franklin Lakes, NJ, USA) in triplicate. The medium in the upper chamber was serum-free DMEM, and the medium in the lower chamber was supplemented with 10% FBS. After incubation for 20 h at 37 °C, we determined the number of viable cells that had traversed the filter to the lower chamber.

Hepatocellular carcinoma specimens

One hundred and twelve patients with hepatocellular carcinoma (HCC) diagnosed between 2000 and 2003 were consecutively selected for this study, after written informed consent was obtained. The study protocol was approved by the Medical Ethics and Human Clinical Trial Committee of the Chang-Gung Memorial Hospital.

Immunohistochemistry

Formalin-fixed and paraffin-embedded liver tissue samples were examined by immunohistochemistry with a polyclonal antibody to *spondin 2* (R&D),

using the avidin–biotin complex method, as described previously (Hsu *et al.* 1981). Positive staining corresponded to cancerous cells with dark-brown *spodin 2* immunoreactivity.

Statistical analysis

Values are expressed as means $\pm$ S.E.M. of at least three observations. The statistical analysis of data was carried out using Student's *t*-test. $P < 0.05$ was considered significant.

Results

Effect of T_3 treatment on *spodin 2* gene expression at both the protein and mRNA levels

The five HCC cell lines used in this study were HepG2–TR α 1#1, HepG2–TR α 1#2, HepG2–TR β 1, Huh7, and HepG2–Neo. The TR protein was overexpressed in these cells at ~ 6.4 -, 3.8-, 4.2-, and

2.1-fold respectively compared with the HepG2–Neo control cell line (Fig. 1A). The effect of TR over-expression on *spodin 2* protein expression was assessed after incubation of HepG2 isogenic and Huh7 cell lines in medium containing various levels of T_3 at different time points (Fig. 1B). The cytosolic and secreted *spodin 2* had an apparent molecular weight of ~ 42 kDa in reducing conditions. In HepG2–TR α 1#1 cells, *spodin 2* protein levels were increased 2.3-fold after treatment with 10 nM T_3 for 24 h. The expression continued to increase with incubation time (3.5-fold at 48 h and 5.7-fold at 72 h). Surprisingly, the increase in the concentration of T_3 from 1 to 10 nM did not enhance significantly the induced production of *spodin 2* in TR α 1#1 and #2 cells at 24–48 h (Fig. 1B). In HepG2–TR β 1 cells, the upregulation of *spodin 2* by T_3 was about two- and six-fold higher after incubation with 10 nM T_3 for 24 and 48 h respectively (Fig. 1B). Moreover, exposure of control HepG2–Neo cells to 10 nM T_3 for 48 h did not

Figure 1 Activation of *spodin 2* by T_3 in HCC cell lines at the protein and mRNA level. (A) Expression of TR protein in HepG2–TR α 1#1, –TR α 1#2, –TR β 1, –Neo, and Huh7 cell lines. (B) Cells were treated with or without 1–10 nM T_3 for 12–48 h. Cell lysates (50 μ g) were then subjected to immunoblot analysis using polyclonal antibodies against *spodin 2*. The position of the 42-kDa *spodin 2* protein is indicated in the five HCC cell lines. (C) Expression of *spodin 2* in Huh7 cells. (D) Secreted *spodin 2* was detected in HepG2–TR α 1#1 and Huh7 cells. (E) *Spodin 2* mRNA expression was analyzed by northern blot in the HepG2–TR α 1#1 cell line. (F) Total RNA was isolated and *spodin 2* expression was analyzed by RT-PCR, as described in the ‘Materials and methods’ section.

affect *spodin 2* protein expression significantly (Fig. 1B). Similar results were observed in the HCC cell line Huh7 (Fig. 1C), which expressed detectable endogenous TR protein (Fig. 1A). The T₃ (1–10 nM)-induced upregulation of *spodin 2* was roughly 1.5- to 3-fold higher in this cell line at 12–48 h (Fig. 1C). As *spodin 2* is a secreted protein, we then examined supernatants using immunoblot analysis. We found that the levels of *spodin 2* in the supernatants were also induced by T₃ treatment and were about six- to seven-fold or 1.8- to 2.5-fold higher in the HepG2–TRα1#1 or Huh7 cell lines respectively at various time points (Fig. 1D). Secreted *spodin 2* was not induced significantly in HepG2–Neo cells (Fig. 1D).

Northern blot or RT-PCR analyses were employed to assess the presence of a similar effect of T₃ on

spodin 2 mRNA expression. A 1.9-kb transcript was detected in all three TR-overexpressing cell lines. Representative results are shown in Fig. 1E. The fold increase in the T₃-mediated expression of *spodin 2* mRNA was also assessed by semi-quantitative RT-PCR analysis (Fig. 1F). *Spodin 2* mRNA levels were increased by ~2.0-fold after treatment of HepG2–TRα1#1 cells with 1 nM T₃ for 12 h. The expression continued to increase with incubation time (3.9-fold at 12 h, 4.1-fold at 24 h, and 3.5-fold at 48 h) in the presence of 10 nM T₃. A similar induction pattern was observed in HepG2–TRα1#2 cells. In HepG2–TRβ1 cells, the T₃-mediated upregulation of *spodin 2* mRNA was about 2.8- to 3.2-fold higher after T₃ treatment (10 nM) for 48 h (Fig. 1F). Moreover, exposure of control HepG2–Neo cells to

Figure 2 T₃-dependent activation of the *spodin 2* promoter by TR and direct binding of TR proteins to the TREs spanning the –1104/–1034 and –984/–925 fragments of the *spodin 2* promoter. (A) HepG2–TRα1 cells were transfected with a luciferase reporter plasmid driven by the *spodin 2* 5'-flanking region (–2555 to –80, which contained three putative TREs (a, b, and c)) with or without a minimal thymidine kinase promoter (pA3TK-Luc), as well as with vectors expressing TRα and β-galactosidase (which was used as a transfection efficiency control). Cells were then incubated for 24 h in the presence or absence of T₃ (10 nM) before harvesting to determine luciferase activity. The activity of luciferase was normalized to the activity of β-galactosidase. Various deletion mutants of the *spodin 2* 5'-flanking region were also generated based on the pA3TK-Luc vector and were transfected. The promoter region contained in these mutants is shown. Data are means ± s.e.m. of values from three independent experiments, each performed in triplicate. (B) Sequence and location of two putative wild-type and mutant TREs. (C) ChIP assays demonstrated that TR, together with RXR, was recruited to the *spodin 2* 5'-flanking region. Two sets of primers for *spodin 2* TRE, positive control TRE (*furin*) and the negative control (*GAPDH*), were prepared. ChIP assay results were evaluated by PCR and gel electrophoresis. Representative results are shown. All ChIP assays were repeated at least three times.

10 nM T_3 for 48 h did not affect *spondin 2* expression significantly (Fig. 1F). A similar but slightly weaker induction was observed in Huh7 cells (Fig. 1F), which expressed detectable endogenous TR proteins (Fig. 1A). The T_3 (1–10 nM)-induced *spondin 2* mRNA expression was roughly 1.2- to 1.8-fold higher in this cell line at 12–48 h (Fig. 1F). These results imply that the effect of T_3 on *spondin 2* protein expression was mediated, at least in part, at the transcriptional level.

T_3 -induced *spondin 2* expression at the transcriptional level

Promoter activity assays were used to determine whether regulation of *spondin 2* gene expression by T_3 occurred at the transcriptional level. The *spondin 2* 5'-flanking region encompassing nucleotides –2555/–80 (Fig. 2A, construct A2) was cloned and placed upstream of the luciferase reporter gene in pGL3-Luc (construct A1) or pA3TK-Luc (construct A3). Various deletion mutants were also prepared to identify potential TREs. We used these reporter constructs to determine the effect of TR transactivation

via T_3 on the *spondin 2* 5'-flanking region. Figure 2 illustrates the transactivational activity of the –2555/–80 region (construct A2; +1 corresponds to the transcriptional initiation site): the luciferase activity of this construct was increased 1.92-fold in the presence of T_3 in HepG2-TR α 1 cells. The pGL3-Luc (A1) or pA3TK-Luc (A3) vectors were used as controls. The truncation of the –2555/–80 (A2) fragment to yield the –2555/–1390 (A4), –1625/–650 (A5), and –798/–80 (A6) constructs revealed that the T_3 -induced activation was about 2.91-fold higher when cells were transfected with the A5 construct. Conversely, the activity of constructs A4 and A6 was not induced by T_3 (Fig. 2A). Similarly, the truncation of construct A5 to yield the –1625/–1205 (A7), –1254/–905 (A8), and –935/–755 (A11) fragments revealed that the T_3 -induced activation was about 2.38-fold higher in cells transfected with the A8 construct. There were two putative TREs (TREb and TREc) in construct A8. This fragment (A8) was further truncated to yield the –1104/–1034 (A9, TREb) and –984/–925 (A10, TREc) fragments, both of which exhibited 2.80- to 2.88-fold higher T_3 -induced activity (Fig. 2A).

Figure 3 Induction of *spondin 2* expression by thyroid hormones in rat liver. (A) The expression of *spondin 2* protein in the liver of sham, Tx, or Tx + T_3 male SD rats was assessed by western blot analysis. The intensity of the bands corresponding to *spondin 2* and actin on the blots was quantified. (B) The expression of *spondin 2* mRNA in the liver of sham, Tx, and Tx + T_3 male SD rats was determined by qRT-PCR and was analyzed by agarose gel. Values (means $\pm$ S.E.M.) are shown as fold induction of the control (Tx) group, and the significance of differences was examined using Student's *t*-test. Data are means $\pm$ S.E.M. of values from two independent experiments ($n=10$ rats per group). ** $P<0.01$.

Mutation of these two TREs to yield constructs –1104/–1034 (A12) and –984/–925 (A13) led to a decrease in the T₃-induced activity. Together, these experimental results suggest that T₃ induced *spodin 2* gene expression at the transcriptional level, and demonstrate clearly that the sequences located between positions –1104/–1034 (A9) and –984/–925 (A10) confer T₃ responsiveness to the *spodin 2* gene. Furthermore, the sequences –1077/–1066 (AGGC-CATGCCAG) and –950/–939 (AGGTCACAC-CCT) comprised an atypical palindromic TRE (Fig. 2B). The above sequences are homologous to the reported TRE consensus palindromic sequence (AGGTCATGACCT). Furthermore, the similarity of the two TREs between humans and rodents was 58.3% (seven out of 12 nucleotides were identical; Fig. 2C).

Analysis of the binding of TR to the –1104/–1034 or –984/–925 fragments of the *spodin 2* promoter using a ChIP assay

We employed ChIP assays to examine whether endogenous TR was recruited to the *spodin 2* gene promoter in living cells. As shown in Fig. 2C, the ChIP assay demonstrated that both TR and RXR- β were clearly recruited to the two TRE-binding sites (b and c; Fig. 2C, lanes 11, 12, 15, and 16), whereas control IgG showed only background levels (Fig. 2C, lanes 10 and 14). In contrast, a set of primers for the negative control (Fig. 2C, lanes 1–4, human glyceraldehyde 3-phosphate dehydrogenase gene) did not yield any detectable bands. However, the positive control (Fig. 2C, lanes 5–8, human *furin* gene, which contains a functional TRE) (Chen *et al.* 2008a) yielded

Figure 4 Knockdown of *spodin 2*-promoted hepatoma cell migration and invasion. (A) *Spondin 2* protein expression levels in two siRNA knockdown Huh7 cell lines (shSP2 #9 and #16) or EGFP knockdown control cells (shEGFP #6 and #8). Huh7 cells (4×10^6) were plated in DMEM medium containing 10% FBS. After 24-h incubation and washing, the medium was replaced with serum-free medium. The medium was then collected for MMP detection. Preparation and zymography were performed as described in the 'Materials and methods' section; the position of the proenzyme and active form of MMPs is shown on the right. (B) Migration or (C) invasion properties of two *spodin 2* knockdown cell lines or two control cell lines. The cell lines were added to the upper chamber of Transwell units and incubated for 24 h. The number of cells that traversed the filter to the lower chamber was then determined and expressed as the total number of cells to provide an index of invasive activity. (D) mRNA expression levels of invasion-related genes, as determined by qRT-PCR. Data are means $\pm$ S.E.M. of values from three independent experiments. The numbers above some columns indicate the fold induction in expression compared with that of control cells. All assays were repeated at least three times. The significance of differences was examined using Student's *t*-test.

detectable bands (lanes 7 and 8). The results of the ChIP assays supported the finding that activated TR induces *spondin 2* gene expression in HepG2–TR cells.

In vivo expression of spondin 2 protein induced by T_3

To determine the *in vivo* response of the *spondin 2* gene to T_3 treatment, two groups of 6-week-old male SD rats ($n=10$ in each group) were thyroidectomized. Subsequently, the first group was injected with T_3 daily for 2 weeks (Tx + T_3), and the second group (Tx, used as the control) received no T_3 injections. Hypo-, hyper-, and euthyroid rats have been established successfully, as described previously (Huang et al. 2006). Western blot analysis demonstrated that liver spondin 2 protein levels were elevated by 9.2-fold in the T_3 -treated group (Tx + T_3) compared with the Tx group (Fig. 3A). Furthermore, spondin 2 mRNA levels in the Tx + T_3 group were 16.5-fold higher than in the Tx group (Fig. 3B). The spondin 2 protein and mRNA levels in sham-operated rats were 6.1- and 8.4-fold higher respectively compared with those detected in Tx rats (Fig. 3).

The function of spondin 2 was associated with cell migration and invasion *in vitro*

The function of spondin 2 was determined via manipulation of its expression levels in Huh7 cells, which express high levels of the endogenous spondin 2 protein (Fig. 4A), or in J7 cells that do not express detectable endogenous spondin 2 protein (J7-V, Fig. 5A). Matrix metalloproteinases (MMPs) are important zinc- and calcium-dependent proteinases that degrade extracellular matrix components and numerous other proteins (Sternlicht & Werb 2001). Knockdown of spondin 2 increased the expression of pro-MMP-2 (72 kDa) by about 2.5- to 2.7-fold in Huh7 cells (Fig. 4A). The migration ability of spondin 2 knockdown Huh7 cells (shSP2 #9 and #16) was enhanced significantly ($P<0.01$) by about 4.1- or 6.0-fold compared with the migration ability of shEGFP (#6 and #8) control cells (Fig. 4B). Images of cell density were shown for two control cell lines (shEGFP #6 and #8) and two knockdown cell lines (shSP2 #9 and #16; Fig. 4B). Consequently, the invasiveness of Huh7–shSP2 #9 and #16 cells was increased by ~4.5- to 4.9-fold, as assessed using the Transwell assay (Fig. 4C).

Figure 5 Overexpression of spondin 2-repressed hepatoma cell migration and invasion. (A) Spondin 2 protein expression levels in three J7 cells overexpressing spondin 2 (clones 12, 15, and 19) or in J7-V control cells. J7 cells (5×10^5) were plated in DMEM medium containing 10% FBS. (B) Migration or (C) invasion properties of three spondin 2-overexpressing cell lines or of a control cell line. The cell lines were added to the upper chamber of Transwell units and incubated for 24 h, as described in Fig. 4. (D) mRNA expression levels of invasion-related genes, as determined by qRT-PCR. Data are means $\pm$ s.e.m. of values from three independent experiments. All assays were repeated at least three times. The significance of differences was examined using Student's *t*-test. ** $P<0.01$; * $P<0.05$.

The expression of the adhesion molecule E-cadherin is inversely correlated with the aggressive phenotype of numerous epithelial cancers (Gervais *et al.* 2007), whereas fibronectin (FN) serves as a general cell adhesion molecule by anchoring cells to collagen or proteoglycan substrates (Potts & Campbell 1994). The expression of these two proteins was reduced in the two shSP2 cells compared with the control cells (shEGFP; Fig. 4D). However, invasion-associated genes, such as *snail* (Olmeda *et al.* 2007) or *vimentin* (Singh *et al.* 2003), were highly induced in the two spondin 2 knockdown cell lines (hSP2 #9 and #16, Fig. 4D).

Furthermore, we determined the migration and invasion ability of J7 cells overexpressing spondin 2. Three spondin 2-overexpressing sublines (clones 12, 15, and 19; cytosolic and secreted, Fig. 5A) were established. The migration (Fig. 5B) or invasion (Fig. 5C) ability were decreased significantly ($P < 0.01$) in spondin 2-overexpressing cells compared with control cells (J7-V, Fig. 5A). Images of cell density were shown

for one control cell line and three overexpressing cell lines (Fig. 5B and C). The expression of *E-cadherin* and *FN* was increased in the three spondin 2-overexpressing cells (Fig. 5D). However, the expression of *snail* and *vimentin* was significantly reduced in the three spondin 2-overexpressing cells (Fig. 5D).

Spondin 2 was up-regulated in human HCC

Expression of spondin 2 protein was detected in most of the HCC tissues analyzed, as assessed by western blotting. Approximately 53.6% (60/112) of the cancer tissues displayed up-regulated spondin 2 expression compared with that detected in matched noncancerous adjacent tissues (Fig. 6A). The results from 14 representative paired HCC specimens, which are shown in Fig. 6A, showed an increase in TR protein expression and the concomitant increase in spondin 2 protein expression in HCC tissues. To investigate the expression levels and

Figure 6 Overexpression of spondin 2 in human HCC. (A) The spondin 2 and TR proteins were overexpressed in the 14 representative tumor tissues (T) and were compared with matched noncancerous adjacent tissues (N) using western blot analysis. Equal loading was confirmed by actin immunodetection. The figure shows a compilation of separate blots, which were pieced together. (B) Panels IN, IIN, and IIIN depict noncancerous adjacent tissues, and panels IT, IIT, and IIIT depict hepatic cancerous tissues. Positive staining of spondin 2 is indicated by a dark-brown color. Spondin 2 expression was detected mainly in cancerous cells. N, normal; T, tumor.

location of *spondin 2* in tissues, we performed immunohistochemical staining in liver cancerous and matched noncancerous tissues. Figure 6B shows three pairs of representative cases (I, II, and III). Cancerous tissues (IT, IIT, and IIIT) and their noncancerous counterparts (IN, IIN, and IIIN) were used. The dark-brown immunostaining was most prevalent in cancerous cells, whereas low-level signal was observed in noncancerous cells. However, the intensity of the staining in cancerous cells did not correlate with pathological stages (when comparing early and advanced disease stages).

Discussion

This study characterized *spondin 2*, which was previously identified in a cDNA microarray screening as a T_3 -responsive gene in HepG2-TR α 1 cells. The regulation of *spondin 2* by T_3 and the significance of this regulation in HCC were not reported previously. Our results indicate that the T_3 -mediated regulation of *spondin 2* occurs via a direct interaction and is TRE dependent. The promoter activity analysis demonstrated that two TRE sites at -1104/-1034 and -984/-925 upstream of human *spondin 2* participated in T_3 -induced changes in *spondin 2* gene transcription. Furthermore, alteration of *spondin 2* expression influenced cell migration and invasion activity. The observation of a similar regulatory process in thyroidectomized rat specimens was consistent with these findings. Notably, the expression of *spondin 2* was positively correlated with the expression of TR protein in HCCs.

Luo et al. (2006) reported that several genes, including glypican 3, *spondin 2*, and osteopontin, are overexpressed in HCC versus adjacent tissues. *Spondin 2* has also been shown to be expressed differentially in primary lung cancers (Manda et al. 1999). In addition, Simon et al. (2007) reported that B7-H4, *spondin 2*, and DcR3 are potential ovarian cancer markers that may improve early detection of cancer. The human *spondin 2* homolog, *spondin 2*/RG-1, is a prostate-associated protein, and its expression is maintained in primary prostate tumors and in metastases to lymph nodes and bone tissues (Parry et al. 2005). In this study, we reported that the *spondin 2* protein was detected in most of the HCCs analyzed. However, knockdown of *spondin 2* increased the invasive ability of cells. Conversely, the migration and invasion ability were decreased significantly in *spondin 2*-overexpressing cells. Nevertheless, the migration and invasion ability were not necessarily increased in tumor cells. The size and number of tumors are important predictive factors of vascular invasion.

In addition, the incidence of vascular invasion increases with increasing tumor size (Pawlik et al. 2005). Tumors with a lower invasive ability are usually associated with better prognosis (Shi et al. 2001). Several factors, such as the urokinase plasminogen activator receptor (Laufs et al. 2006) and Nm23 (Xiao et al. 1998), are reportedly involved in tumor invasion.

The transcription factor snail has been proposed as an important mediator of tumor invasion because of its role in the downregulation of E-cadherin, and in the induction of epithelial-mesenchymal transitions (Battle et al. 2000, Cano et al. 2000, Olmeda et al. 2007). Expression of snail in pancreatic cancer promotes metastasis and chemoresistance (Yin et al. 2007), and is associated with lower overall survival in ovarian cancer patients (Bleichschmidt et al. 2008). Overexpression of vimentin is significantly associated with HCC (Zhao et al. 2008), and contributes to prostate cancer invasion and metastasis via Src regulation (Wei et al. 2008). Furthermore, the expression of E-cadherin is inversely associated with the expression of vimentin (Hu et al. 2004). A synthetic peptide from FN has been shown to inhibit experimental metastasis of murine melanoma cells (Humphries et al. 1986). A polymeric form of FN has antimetastatic effects against multiple tumor types. Treatment with this polymeric form of FN blocks cell spreading and migration *in vitro*, which suggests a possible mechanism for its antimetastatic effect (Pasqualini et al. 1996). Consequently, manipulation of *spondin 2* expression altered the migration and invasion capacity of two HCC cell lines.

The role of TRs in HCC is controversial. Several studies have proposed that TRs may function as tumor suppressors (Barlow et al. 1994, Yap et al. 1996, Bhat et al. 1997, Barrera-Hernandez et al. 1998, Ying et al. 2003, Garcia-Silva & Aranda 2004, Kato et al. 2004, Joseph et al. 2007, Rocha et al. 2007). These studies suggest that a partial loss of normal TR function caused by a decrease in the expression or complete loss of normal TR activity because of mutation and/or aberrant expression provides an opportunity for tumors to proliferate, metastasize, and invade other tissues. However, increasing evidence supports the notion that wild-type TR could play an enhanced role in carcinogenesis (Davis et al. 2006, Plateroti et al. 2006, Rae et al. 2007, Hall et al. 2008, Tsui et al. 2008). These observations suggest that TR plays a dual role in human carcinogenesis. As TR functions are complex and tissue- and time-specific, aberrant expression of the various TR isoforms has different effects and is associated with different types of tumor or stages of

tumor development. Notably, the effect of TR on hepatocarcinogenesis depends on the quantitative aspect (expression level) or quality (mutation) of the alterations in the TR genes in different stages of HCC. Further studies are required to elucidate the clinicopathological significance of TR.

In conclusion, this investigation demonstrated that overexpression of spondin 2 in some types of HCCs was TR dependent and may play a crucial role in the development of HCC (Chen *et al.* 2008a,b). In addition, ligand-activated TR directly transactivated *spondin 2* gene transcription via critical TR-binding sites located within the 5'-flanking region of the gene. Moreover, alteration of spondin 2 expression via overexpression or underexpression of the protein caused changes in cell migration and invasion. Clearly, an important role can be postulated for TR in liver carcinogenesis.

Declaration of interest

The authors declare that there are no conflicts of interest that would prejudice the impartiality of this scientific work.

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