A novel function of thyroid hormone receptors in promoting metastasis of human hepatoma cells via regulation of TRAIL

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ABSTRACT

Accumulating evidence has confirmed an important role of Thyroid hormone (T3) and its binding receptors (TRs) in tumor progression. However, the specific functions of TRs in carcinogenesis have yet to be clarified. Our observations have shown that TNF-related apoptosis-inducing ligand (TRAIL) is upregulated by T3 in TR-overexpressing hepatoma cell lines. Interestingly, TRAIL induces apoptosis in several cancer cell types, but also triggers non-apoptotic signals favoring tumorigenesis in apoptosis-resistant cancer cells. In our experiments, TR-overexpressing hepatoma cells treated with T3 were apoptosis-resistant, even under conditions where TRAIL was upregulated. This finding may be attributed to the simultaneous upregulation of Bcl-xL, an anti-apoptosis gene, by T3. Thus, overexpression of Bcl-xL in hepatoma cells possibly protects against apoptosis triggered by TRAIL, consequently leading to TRAIL-promoted metastasis in hepatoma cells. Notably, TRAIL was highly expressed in tumor cells of HCC patients, and this high expression was significantly correlated with the high TR expression as examined in 65 HCC tissues. Collectively, our findings indicate that TR directly binds to the TRAIL promoter to induce TRAIL expression, which acts in concert with the simultaneously activated Bcl-xL, to promote metastasis, rather than apoptosis. Our findings provide a novel mechanistic link for increased TR and TRAIL in HCC.
INTRODUCTION

The thyroid hormone, 3, 3’-5-triiodo-L-thyronine (T₃), is an important mediator that regulates the expression of genes that function in development, differentiation, cell growth and other aspects of metabolism. The actions of T₃ are mediated by nuclear thyroid hormone receptors (TRs), which are ligand-dependent transcription factors derived from two genes, TRα and TRβ. TRs comprise modular functional domains involved in ligand binding, DNA binding, homo- and hetero-dimerization with other receptors, and cooperation with other transcription factors or cofactors. Binding of ligand triggers conformation changes in TRs, in turn, stimulating the release of co-repressors and recruitment of transcriptional coactivators to enhance target gene transcription.

Accumulating evidence from recent studies supports an association between aberrant TR regulation or TR mutations and human neoplasia. However, the specific roles played by TR in tumorigenesis remain debatable. Notably, a mutant form of TR (v-erbA) with loss of ligand binding ability has been shown to cause hepatocellular carcinomas (HCC) in transgenic mice. Moreover, earlier experiments by our group and other laboratories have shown that TRα and TRβ cDNAs are truncated or mutated at high frequencies in human HCCs. A few studies to date have implicated T₃ as a potential tumor inducer in several cancer types. For example,
T₃ and TR promoted intestinal cell proliferation and intestinal tumorigenesis via cooperation with the WNT pathway and induced β-catenin and some of its targets. Additionally, hyperthyroxinemia has been reported to increase the rate of colon cancer incidence in a rat experimental model.

We used cDNA microarray to identify the genes positively regulated by T₃ in a TRα₁-overexpressing hepatoma cell line (HepG2-TRα₁), with a view to elucidating the role of T₃/TR in human hepatocellular carcinogenesis. Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL/Apo2L), a potent effector of tumorigenesis, was selected for further study.

TRAIL was cloned on the basis of sequence homology with the extracellular domain of TNF and CD95 ligand (FASL). In healthy subjects, TRAIL is expressed in most human tissues, with significantly higher levels in the immune system. Analogous to other members of the TNF family, TRAIL is expressed as a type II transmembrane protein consisting of 281 amino acids in humans. Cleavage of its C-terminal region (extracellular domain) by a cysteine protease allows the release of a soluble form of TRAIL. Both the soluble and membrane-bound forms of TRAIL are functionally active. To date, five distinct cognate receptors have been identified, including the death receptors, DR4 and DR5, decoy receptors, DcR1 and DcR2, and a soluble receptor known as osteoprotegerin (OPG). Upon binding of
TRAIL, DR4 and DR5 activate the apoptotic signal pathway. In contrast, DcR1, DcR2 and OPG lose or lack the entire cytoplasmic domain, and consequently lose the ability to transduce the apoptosis signal upon TRAIL binding.

The majority of investigations to date have focused on the ability of TRAIL to induce apoptosis in cancer cells. However, a few studies have additionally shown that TRAIL not only promotes cellular apoptosis but also triggers non-apoptotic pathways, including protein kinase C (PKC), nuclear factor kappaB (NF-κB) and mitogen-activated protein kinases (MAPK). The non-apoptotic signaling pathways stimulated by TRAIL induce genes that promote cell survival, angiogenesis and metastasis, and contribute to cancer progression.

Thus, TRAIL serves as a potential candidate for cancer therapy, but on the other hand, also promotes tumor progression. In apoptosis-resistant cancer cells, TRAIL facilitates tumor progression by promoting cell migration and invasion. Consistent with this finding, earlier studies have shown that TRAIL is abnormally expressed in several human cancers, especially high-grade stage tumors, including non-small cell lung cancer, pancreatic cancer, colorectal cancer and cholangiocarcinoma cells. However, these observations are inconsistent with the theory that TRAIL serves as a tumor killer, rather implying that the roles of TRAIL in tumorigenesis depend on whether cells are resistant to TRAIL. Bcl-xL has been
reported to suppress TRAIL-mediated apoptosis in several cell types.\textsuperscript{18,24} Experiments from the current investigation showed that T\textsubscript{3} upregulates TRAIL expression at the transcriptional level in TR-overexpressing hepatoma cells. Furthermore, the positive TRE of TRAIL was identified. Notably, T\textsubscript{3}-induced Bcl-xL expression simultaneously protected cells from TRAIL-induced cell death. Our findings suggest that TRAIL contributes to tumor progression by promoting cancer cell migration and invasion following T\textsubscript{3} treatment.
RESULTS

T$_3$ regulates *TRAIL* mRNA and protein levels in TR-overexpressing hepatoma cells

To ascertain the roles of TR in hepatocellular carcinogenesis, cDNA microarray was performed to identify the genes regulated by T$_3$ in HepG2-TR$\alpha$ cells. One of these genes, *TRAIL*, was highly stimulated after 48 h of treatment with T$_3$. Subsequently, real-time PCR was utilized with the established stable cell lines, HepG2-TR$\alpha$#1, #2, -TR$\beta$, and -Neo, verify this result. Expression levels of TR in parental and several stable cell lines are shown in Figure 1A. Following T$_3$ treatment, the *TRAIL* mRNA level was enhanced in a time-, dose-, and TR-dependent manner in various TR-overexpressing cell lines (Figure 1B).

The effect of T$_3$ on TRAIL protein expression was additionally assessed in HepG2 isogenic cell lines. After treatment with or without T$_3$ (1 or 10 nM) for 24 and 48 h, TRAIL protein levels in culture medium and total lysate were analyzed using Western blot. Notably, both cellular and secreted TRAIL protein levels were upregulated dramatically (~2- to 15-fold) by T$_3$ in HepG2-TR$\alpha$#1, #2 and -TR$\beta$ (Figures 1C, D) cell lines. In contrast, exposure of HepG2-Neo cells to T$_3$ for 24 or 48 h had no significant effects on TRAIL protein expression. In other hepatoma cell lines
expressing exogenesis or endogenous TR proteins, such as J7-TR or Huh7, T₃ enhanced the cellular and secreted TRAIL protein levels, similar to that observed in HepG2-TR cells (Figures 1C, D). Our results clearly demonstrate that T₃ increases TRAIL expression at both mRNA and protein levels in hepatoma cells in a TR-dependent manner.

**T₃ regulates TRAIL expression at the transcriptional level**

To elucidate the mechanism underlying T₃-mediated enhancement of TRAIL expression, we examined TRAIL transcript stability by suppressing new mRNA synthesis with actinomycin D (ActD; 2µg/mL) in HepG2-TRα cells. After incubation without or with 10 nM T₃ for 24 h, ActD was added, and degradation of TRAIL mRNA monitored at indicated times in these cells using real-time PCR. The half-life of TRAIL mRNA was similar in cells, regardless of the presence and absence of T₃ (Figure 2A). This result indicates that induction of TRAIL mRNA by T₃ is independent of RNA stability. To determine whether T₃ alters TRAIL transcription, we cloned the human TRAIL promoter region encompassing nucleotides from positions –3899 to +30 (promoter region 1, P1) and placed it upstream of the luciferase reporter gene in pGL3-basic vector. Several putative thyroid response elements (TREs) have been identified in this region. A series of 5’-deletion mutants of the TRAIL promoter region containing various numbers of TREs were additionally
generated (Figure 2B, P2-P7). Using these reporter constructs, the transactivation effects of TR on wild-type and mutant TRAIL promoter constructs were determined in the presence of T3. In the presence of 100 nM T3, the transactivation activity of the reporter construct containing the TRAIL promoter region –3899 to +30 (P1; four predicted TREs) was increased by approximately 4-fold in HepG2-TR cells (Figure 2B, right). Moreover, T3 treatment enhanced the transactivation activities of the –3200 to +30 (P2) and –2700 to +30 (P3) mutants up to 4- and 3.6-fold, respectively. However, the deletion mutant, –1900 to +30 (P4), lost T3-induced promoter activity (Figure 2B, right), implying that cis elements between positions -2700 and -1900 mediate the T3 effect. Two putative TREs, TRE1 and TRE2 (-2068 to -2180 and –1967 to –1977, respectively), were identified within this region. Accordingly, TRE1 and TRE2 in the TRAIL promoter region were mutated separately to determine their precise roles in T3-induced promoter activity. Mutation of TRE1 had no effect on T3-induced TRAIL promoter activity. However, the TRE2 mutation significantly reduced the response of the TRAIL promoter to T3. These results collectively suggest that TRE2, located between positions –1967 and –1977, mediates the effect of T3 on the TRAIL promoter. Further analysis of the TRE2 sequence, ATCTCTTGACCT, revealed an atypical palindromic TRE. Targeting of TRE2 by TR in vivo was confirmed using the chromatin immunoprecipitation (ChIP) assay
Binding of TR to the *Furin* promoter or *Gapdh* served as positive and negative controls, respectively.

**T3 protects TR-overexpressing hepatoma cell lines from apoptosis**

Previous studies have suggested a pro-apoptotic role of TRAIL during tumor progression. TRAIL is positively regulated by T3 in TR-expressing hepatoma cells, and it is of interest to define the physiological significance of this regulation in these cell lines. Accordingly, we examined whether T3 triggers apoptosis through induction of TRAIL in TR-overexpressing hepatoma cells. Following stimulation of HepG2-TR or J7-TR cells with T3, TRAIL expression was significantly increased. Interestingly, however, neither caspase-3 activation nor the percentage of sub-G1 population were increased upon T3 treatment (Figure 3A). Moreover, after neutralization of TRAIL activity via co-treatment with a blocking antibody (mouse anti-TRAIL monoclonal antibody, RIK-2) and T3, the percentage of apoptotic cells was not affected, compared with that in HepG2-TR or J7-TR cells co-treated with T3 and control IgG (Figure 3B). These results imply that TRAIL upregulated by T3 does not exert a significant apoptotic effect in TR-overexpressing hepatoma cells. To ascertain whether TRAIL promotes apoptotic cell death in our experimental system, HepG2, J7, HepG2-TR and J7-TR cells were treated with recombinant (r)-TRAIL. As expected, apoptosis events were induced, such as increased active caspase-3/-8 and sub-G1 populations,
following TRAIL stimulation (Figure 3C). To further confirm the correlation between T3 and TRAIL in cell death control, r-TRAIL and several chemotherapy drugs were used. Chemotherapy drugs, such as cyclohexamine (CHX), Cisplatin, and Etoposide, promote tumor cell apoptosis synergistically with TRAIL. Accordingly, the effects of co-treatment with rTRAIL and CHX, Cisplatin or Etoposide for 48 h on HepG2-TR and J7-TR cell death were examined. All the drugs enhanced TRAIL-induced cell death (Figure 3D). Surprisingly, caspase-3 activation and the percentage of sub-G1 population (hypodiploid cells) following r-TRAIL and chemotherapy drug treatments were partially reduced in T3-treated cells (Figure 3E). Our results suggest that T3 acts as an anti-apoptotic factor in TR-overexpressing hepatoma cells challenged with TRAIL or chemotherapy drugs. However, further studies are required to establish the mechanism by which T3 prevents apoptosis while activating the expression of the pro-apoptotic factor, TRAIL.

**Bcl-xL upregulation following T3 treatment protects hepatoma cells against TRAIL-induced apoptosis**

Although TRAIL promoted apoptosis in hepatoma cells, this effect was prevented by T3 in TR-overexpressing hepatoma cells, even upon significant induction of TRAIL. To define the mechanism employed by T3/TR in antagonizing the apoptotic effects of TRAIL, the target genes of TR need to be further explored. Previous
studies have implicated several genes in the apoptotic event triggered by TRAIL, including NF-κB, cellular FLICE-like inhibitory protein (cFLIP), and members of the Bcl-2 family (Bcl-2, Bcl-XL, Mcl-1, Bax, Bad and Bid). Among these factors, Bcl-xL was dramatically (~4-5-fold) induced by T3 in HepG2-TR and J7-TR cells (Figure 4A). In view of the finding that Bcl-xL desensitizes cancer cells to the apoptotic effect of TRAIL, we hypothesized that it is one of the factors mediating the anti-apoptotic effects of T3/TR. To establish the effect of Bcl-xL on TRAIL-mediated apoptosis in hepatoma cells, immunoblotting was performed. After TRAIL stimulation for 48 h in Bcl-xL overexpressing HepG2 or J7 cells, which, in turn, led to a dramatic reduction in the activation of caspase-3 and percentage of cells at the sub-G1 stage (Figure 4B, C). Furthermore, Bcl-xL overexpression enhanced the survival of cells treated with TRAIL (Figure 4D). These observations suggest that T3 simultaneously upregulates TRAIL and anti-apoptotic signals, such as Bcl-xL, which prevent the apoptotic effect of TRAIL in TR-overexpressing hepatoma cells.

**TRAIL enhances the metastatic potential of apoptosis-resistant hepatoma cells.**

Previous studies have demonstrated that in addition to inducing apoptosis, TRAIL transduces non-apoptotic signals in tumor cells resistant to apoptosis. Among the non-apoptotic signals, activation of NF-κB and p42/44 mitogen-activated protein...
kinases (MAPK) are critical for mediating cell proliferation, differentiation and migration.\textsuperscript{26} Similarly, the NF-κB response reporter (4 tandem repeat NF-κB response elements-driven, luciferase activity) was dynamically activated by TRAIL (Supplemental Figure 1A). Additionally, activated p42/44 MAPK and phosphor-ERK 1/2 were markedly elevated after TRAIL treatment (Supplemental Figure 1B). To further examine the non-apoptotic effects of TRAIL on hepatoma cells overexpressing Bcl-xL, we analyzed the expression of NF-κB and ERK target genes that promote migration and invasion in several cancer cells. The real-time PCR assay showed that members of the matrix metalloproteinase (MMP) family, such as MMP2, MMP9 and MMP7, are upregulated upon TRAIL stimulation in HepG2, J7, HepG2-Bcl-xL and J7-Bcl-xL cells (Figure 5A). Interestingly, these genes were more strongly induced in Bcl-xL-overexpressing cells resistant to TRAIL-induced apoptosis. Similar expression of these non-apoptotic target genes at the protein level was observed in adenovirus (ad)-TRAIL-infected or r-TRAIL-treated HepG2-Bcl-xL and J7-Bcl-xL cells (Figure 5B). Moreover, the gelatin zymography assay revealed increased MMP2 and MMP9 activities in the presence of TRAIL in HepG2-Bcl-xL and J7-Bcl-xL cells (Figure 5C). Taken together, these results imply that although the proapoptotic function of TRAIL is negligible, upregulation of TRAIL in hepatoma cells may facilitate cancer cell progression in other ways, such as increasing invasion.
and metastasis. To further address this issue, the transwell assay was performed to examine the metastatic abilities of parental HepG2, J7 and TRAIL-resistant Bcl-xL-overexpressing hepatoma cells in response to ad-TRAIL stimulation. After staining the cells transversed the membrane of matrigel transwell with crystal violet, Invasion of HepG2-Bcl-xL and J7-Bcl-xL cells stimulated with ad-TRAIL infection was significantly greater than that of HepG2 and J7 control cells (Figure 5D). These gain-of-function studies showed that TRAIL enhances the metastatic potential of apoptosis-resistant hepatoma cells.

**T3-promoted hepatoma cell invasion is mediated by TRAIL**

The above results suggest that upregulation of TRAIL by T3/TR in hepatoma cells triggers the invasive activity of apoptosis-resistant cells instead of inducing apoptosis. The issue of whether T3/TR influences hepatoma cell metastasis requires further examination. After transwell analysis, invasive ability was enhanced significantly upon T3 treatment. However, following co-treatment with RIK-2 and T3, the number of invasive cells was reduced (Figure 6A). In addition, RIK-2 partially suppressed the induction of MMP-2, -7 and -9 in the presence of T3 (Figure 6B). The results imply that T3-mediated upregulation of TRAIL promotes metastasis in TR-overexpressing hepatoma cells.

To ascertain whether T3/TR-induced TRAIL expression and its related effects occur
in vivo, Severe Combined Immunodeficiency (SCID) mice were injected with $1 \times 10^7$ J7-TR cells, and classified into euthyroid, hypothyroid, and hyperthyroid groups for 8 weeks before sacrifice. Mice injected with the J7-TR cells developed multiple macroscopic tumor nodules in the lung, as evident from HE staining data (Figure 6C). Although tumor formation was observed in all groups, the numbers and sizes of tumors were different. Tumor size, relative to average tumor size (per cm$^2$/lung section in mice), or the metastatic index was larger in hyperthyroid mice, compared to euthyroid or hypothyroid mice. Expression of TRAIL (observed as a dark-brown color in IHC staining) was markedly stronger in lung tumors of hyperthyroid mice, compared to that in tumors of euthyroid and hypothyroid mice (Figure 6C). The animal data clearly suggest that T$_3$ promotes invasion of TR-overexpressing hepatoma cells via TRAIL induction.

**TRAIL is upregulated in human HCC**

To determine TRAIL expression in HCC patients, we performed immunohistochemical staining (IHC) of 65 HCC biopsy samples. TRAIL was highly expressed in tumor cells, whereas relatively weak staining was observed in the adjacent non-cancerous region (Figure 7A, B). We classified TRAIL expression patterns in tumor specimens as ranging from weakly to strongly positive. In total, 29 of the 65 tumor specimens (45%) showed strong staining, 25 (38%) stained
moderately, and 11 (17%) stained weakly for TRAIL (Figure 7C). To further examine the correlation between TRAIL expression and TR in HCC tissues, consecutive tissue slides were subjected to IHC using an anti-TR antibody. About 25 specimens (38%) showed strong TR staining, 30 (46%) displayed moderate levels of TR, and 10 (16%) stained weakly (Figure 7C). Statistical analysis revealed that TR expression is positively correlated with that of TRAIL (Figure 7D; \( P=0.005 \)). These results strongly suggest that TRAIL is positively regulated by TR in HCC tumor specimens.
**Discussion**

The circulating thyroid hormone and expression of TRs in target tissues control the development and metabolic homeostasis in vertebrates. However, increasing evidence has revealed important roles in HCC. In this study, we have highlighted a novel function of T₃/TR in promoting the invasive ability of hepatoma cells, which is attributed to simultaneous regulation of TRAIL and anti-apoptotic genes, such as Bcl-xL. Induction of TRAIL by T₃ enhances hepatoma cell invasion and expression of matrix metalloproteases, including MMP2, -9 and -7. Importantly, IHC staining revealed an increase in TRAIL expression in human liver cancers, compared with adjacent normal controls. Collectively, our findings show that TR directly binds to TRE of the TRAIL promoter to induce mRNA expression, which promotes metastasis instead of apoptosis, owing to simultaneous activation of Bcl-xL. This observation provides a new mechanistic link for increased TR and TRAIL in HCC.

The roles of T₃ and TRs in tumorigenesis appear multifaceted. Results obtained with genetic knockin mouse models have revealed that TRβ mutations may lead to thyroid cancer and pituitary tumors. Moreover, the oncogenic activity of mutant TRβ is mediated by a combination of genomic and nongenomic actions to alter gene expression and signaling pathway activities. Recently, Martinez-Iglesias and co-workers (2009) also reported that TR serves as a potent suppressor of tumor
metastasis in hepatoma (SK-hep1) and breast cancer (MDA-MB-468) cells. This study suggests that TRβ1 expression in hepatoma and breast cancer cell lines that have lost TR expression may induce tumor growth retardation, partial mesenchymal-to-epithelium transition and suppression of tumor metastasis in nude mice. Moreover, the data support diverse roles of TRs at different stages of tumorigenesis. This study showed that TR-α/-/TR-β/- double knockout mice are vulnerable to skin carcinogenesis, and TRs deficiency inhibits benign tumor formation at the early stages, but promotes malignant transformation at later stages of carcinogenesis. However, the tumor suppressor role of TRs is currently a subject of controversy. Several investigators have suggested an oncogenic role of TRs in tumorigenesis. For instance, T3 induces breast cancer cell or ovarian surface epithelial cell proliferation by influencing the expression or activation of estrogen receptor, which may explain the epidemiological links between hyperthyroidism and increased risk of ovarian cancer. Kress and co-workers reported that TRα1 directly regulates several components of the Wnt pathway in an animal model, facilitating the expression of β-catenin/Tcf4 target genes and promoting cell proliferation. Interestingly, overexpression of TRα in the intestinal epithelium did not induce cancer development, but accelerated tumorigenesis in mice with a Wnt-activated Apc+/1638N genetic background. These divergent actions of TRs in
carcinogenesis may be due to differences in tissues and stages of specific tumor microenvironments.

TRAIL has been shown to induce apoptotic cell death in a wide variety of tumor cell lines in vitro, including hepatoma cells. Moreover, combination treatments with other chemotherapeutic agents (such as CHX, Etoposide or Cisplatin) sensitize tumor cells to TRAIL-induced apoptotic death. However, in our experiments, cell death did not occur. Conversely, T3 abolished apoptotic events after chemotherapeutic drug stimulation in hepatoma cells overexpressing TR, even upon high expression of TRAIL. These findings indicate that T3 acts as a survival factor in combination with TRAIL to protect hepatoma cells from apoptosis caused by chemotherapeutic drugs. The unusual non-apoptotic function of TRAIL observed following T3 induction was further explored.

Several anti-apoptotic signals or genes suppress TRAIL-induced apoptosis. Among these, Bcl-xL is of significant interest, owing to its upregulation by T3 in TR-overexpressing hepatoma cells. Previous studies have shown that overexpression of Bcl-xL reduces TRAIL-induced cell death in murine hepatitis and pancreatic ductal adenocarcinoma, while treatment with an inhibitor of Bcl-xL potently enhances TRAIL-triggered apoptosis in glioma cells. Consistent with results from pancreatic ductal adenocarcinoma studies, Bcl-xL repressed TRAIL-triggered
apoptosis in HepG2 and J7 cells. In addition to transmitting apoptotic signals, TRAIL conversely stimulates non-apoptotic signals. In human vascular endothelial cells, TRAIL enhances proliferation by activating the Akt and ERK pathways,\textsuperscript{32} and promotes metastasis of tumor cells, including human pancreatic ductal adenocarcinoma, cholangiocarcinoma cells, and colon cancer cells, that are resistant to apoptotic death induced by NFkB activation.\textsuperscript{18-20} Additionally, TRAIL induces chemotactic migration of monocytes mediated by the Rho-GTPase pathway,\textsuperscript{33} and plays an important role in immune escape of tumor cells. In colorectal cancers, effective apoptotic depletion of infiltrating CD8+ immune cells is mediated by TRAIL expression.\textsuperscript{34,35} These observations strongly suggest that TRAIL plays oncogenic roles in apoptotic-resistant cancer cells. In our study, cell invasion ability was increased significantly in HepG2-TR and J7-TR cells by T\textsubscript{3} through TRAIL activation. TRAIL enhanced HepG2-Bcl-xL and J7-Bcl-xL cell invasion \textit{in vitro}, as well as expression or activity levels of members of the matrix metalloprotease family, particularly MMP-2,-9, and -7. These proteins represent key factors in the enhancement of survival, invasion and metastasis in several malignant tumor cells.\textsuperscript{36,37} Our results conclusively demonstrate that cell invasion and metastasis are enhanced by non-apoptotic signals induced by TRAIL following T\textsubscript{3} treatment, instead of apoptosis. Based on the collective findings, we conclude that TR is a potent cancer
promoter, particularly in HCC.
Figure legends:

Figure 1. Effects of T3/TR on TRAIL mRNA and protein levels in hepatoma cell lines.

(A) Detection of TR protein in the indicated HCC cell lines and TR-overexpressing HepG2 and J7 stable cell lines. (B) HepG2-Neo HepG2-TRα1#1, HepG2-TRα1#2, HepG2-TRβ1 cell lines treated with T3-depleted medium (T3, 0 nM) or T3-supplemented medium (1 or 10 nM) for 12, 24 or 48 h. Total RNA was prepared for analyzing TRAIL expression using real-time PCR. (C) (D) HepG2 stable cell lines, J7-TR or parental Huh7 cell lines were plated in DMEM with 10% FBS for 24 h. After washing, the medium was replaced with serum-free medium with or without T3 (1 or 10 nM) for 24 and 48 h. Conditioned media (30 μg) and cell lysates (100 μg of protein) were subjected to Western blot analysis with a polyclonal antibody against TRAIL. ACTIN or Amido black-stained proteins were used as loading controls. Data are presented as means ± SEM of values from three independent experiments. Values are presented as fold activation, relative to 0 nM T3, at the indicated time-points. **p < 0.01; *p < 0.05

Figure 2. Regulation of TRAIL at the transcriptional level by T3

(A) T3 does not influence TRAIL stability at the RNA level. HepG2-TRα cells were treated with ActD for the indicated times(2 μg/ml) following T3(0 or 10
nM) stimulation for 24 h. Total mRNA was isolated and subjected to real-time PCR. Data are presented as means ± SEM of values from three independent experiments. Values are presented as percentage of RNA remaining relative to the start of ActD treatment at the indicated time-points. (B) *TRAIL* 5′-flanking regions (–3899 to +30, P1) were cloned into PGL3-basic luciferase reporter plasmid. After co-transfection with β-galactosidase (a transfection efficiency control), HepG2-TR cells were harvested following T₃ (0, 10 or 100 nM) treatment for 24 h. Luciferase activity was normalized to that of β-galactosidase. Moreover, serial deletions of the TRAIL promoter region were constructed (P2-P7) and transfected into HepG2-TR cells, and promoter activity analyzed. Data are presented as means ± SEM of values from three independent experiments performed in triplicate. (C) ChIP assays demonstrating that TR is recruited to TREs within the TRAIL promoter region. Primer sets for TRE within the TRAIL promoter region, positive control (*furin* promoter) and negative control (*gapdh*) were designed. ChIP assay data were evaluated using PCR and gel electrophoresis.

**Figure 3. T₃/TR-induced TRAIL is apoptosis-independent.**

(A) T₃ induces TRAIL activation but not apoptosis in HepG2-TR and J7-TR cells. HepG2-TR and J7-TR cells were cultured in T₃-depleted medium without or with T₃
(1 or 10 nM) treatment for 48 and 72 h. Cell lysates were subjected to Western blots with antibodies against TRAIL, activated caspase-3 and ACTIN as internal controls (upper panel). In addition, apoptotic cells following T₃ treatment for 48 h were determined using PI flow cytometry to measure the percentage of hypodiploid cells (lower panel). Data are presented as means ± SEM from experiments performed in triplicate. *P < 0.05, **P < 0.01. (B) The percentage of apoptotic cells was not affected after neutralizing TRAIL activity by co-treatment with blocking antibody (RIK-2, 10 μg/ml) and T₃ for 48 h. Data are presented as means ± SEM from experiments performed in triplicate. *P < 0.05, **P < 0.01. (C) TRAIL induces apoptotic death in hepatoma or TR-overexpressing hepatoma cells. HepG2-Neo, J7-Neo, HepG2-TR or J7-TR cells were treated with TRAIL (100 ng/ml) for 48 h. After TRAIL stimulation, activation of caspase-8 and caspase-3 were analyzed using Western blotting, and the percentage of apoptotic cells assessed with PI flow cytometry. Data are presented as means ± SEM from experiments performed in triplicate. *P < 0.05, **P < 0.01. (D) Anti-tumor effects of TRAIL, CHX, Cisplatin, and Etoposide in HepG2-TR and J7-TR cell lines. HepG2-TR and J7-TR cells were treated with or without TRAIL (100 ng/ml) in combination with vesicle, 5 μM cisplatin, 2.5 μM CHX or 20 μM Etoposide for 48 h. After stimulation, active caspase-3 was examined by Western blotting, and apoptotic cells assessed using PI
flow cytometry. (E) T3 protected HepG2-TR and J7-TR cells from TRAIL- or chemotherapy drug-induced apoptosis. HepG2-TR and J7-TR cells were treated with TRAIL or the respective chemotherapy drugs for 48 h following T3 (0 or 10 nM) stimulation for 2 days. Next, cell lysates were subjected to Western blotting with antibodies against activated caspase-3. The apoptotic effect was determined using PI flow cytometry. Data are presented as means ± SEM from experiments performed in triplicate. *P < 0.05, **P < 0.01.

**Figure 4. Bcl-XL overexpression suppresses TRAIL-induced apoptosis in hepatoma cells**

(A) Expression of related anti-apoptotic proteins in HepG2-TR and J7-TR cells following T3 treatment. HepG2-TR and J7-TR cells were cultured in the absence or presence of T3 (1 or 10 nM) for 24 and 48 h. Cell lysates (100 μg) were subjected to Western blot analysis with antibodies against Bcl-2, Bcl-xL, Mcl-1, Bad, Bid, Bax, cFLIP and ACTIN as internal controls. (B) Bcl-xL inhibits TRAIL-induced apoptosis in hepatoma cell lines. Bcl-xL was overexpressed in HepG2 and J7 cells (upper panel). After TRAIL (100 ng/ml) stimulation for 48 h, caspase-3 activation and (C) genomic DNA fragmentation were determined using Western blot analysis and PI flow cytometry. (D) Viability of HepG2-Bcl-xL, J7-Bcl-xL and the respective control cells after TRAIL treatment. The cell survival rate is presented as the relative absorbance
of Bcl-xL-overexpressing cells treated with TRAIL, compared with control cells, using the MTT assay. Data are presented as means ± SEM from experiments performed in triplicate. *P < 0.05, **P < 0.01.

**Figure 5. TRAIL increases the metastatic potential of apoptosis-resistant hepatoma cells.**

(A) Induction of MMP-2, -9 and -7 with TRAIL in HepG2, J7, HepG2-Bcl-xL or J7-Bcl-xL cells. TRAIL-treated HepG2, J7, or Bcl-xL-overexpressing lines. Total RNA was extracted and MMPs assayed with real-time PCR. Data are presented as means ± SEM from experiments performed in triplicate. Values are presented as fold activation with TRAIL treatment, relative to the vehicle control, in HepG2 or J7 cells. **p < 0.01; *p < 0.05

(B) TRAIL induces MMP protein expression. Following Ad-Trail-infection of HepG2-Bcl-xL and J7-Bcl-xL or rTRAIL stimulation of cells for 48 h, total cell lysates or conditioned medium was subjected to Western blotting for detecting protein expression of MMPs and TRAIL. ACTIN was used as an internal control.

(C) Zymography of MMP-2 and -9 in HepG2-Bcl-xL and J7-Bcl-xL cells. The conditioned media of Ad-TRAIL-infected HepG2-Bcl-xL and J7-Bcl-xL were collected and MMPs assessed as described in Materials and Methods. The positions of the proenzyme and active form of MMPs are shown.
(D) TRAIL induces apoptosis-resistant cell invasion \textit{in vitro}. After Ad-GFP or Ad-TRAIL infection of HepG2, J7, HepG2-Bcl-xL and J7-Bcl-xL, cells ($1 \times 10^5$) were added to matrigel-coated wells, as described in Materials and Methods. Promotion of invasion was quantified as fold changes using Ad-GFP-infected cells as the control. All assays were repeated at least three times. Differences were examined using the Student’s t-test. **$p < 0.01$; *$p < 0.05$.

\textbf{Figure 6. T3/TR-induced hepatoma cell metastasis is mediated via upregulation of TRAIL and MMPs.}

(A) Invasion properties of HepG2-TR and J7-TR cell lines treated without or with T3 ($10 \text{ nM}$) in the presence of control IgG ($10 \text{ μg}$) or RIK-2 ($10 \text{ μg}$). Cells ($1 \times 10^5$) were added to the matrigel-coated chamber of Transwell units and incubated for 24 h. The relative number of cells that migrated to the lower chamber was determined. (B) MMPs were induced by T3, but blocked by RIK-2. In the presence of control IgG ($10 \text{ μg}$) or RIK-2 ($10 \text{ μg}$), HepG2-TR and J7-TR cell lines were cultured in DMEM without or with T3 ($10 \text{ nM}$) for 48 h. Total RNAs were subjected to real-time PCR to analyze MMP expression. (C) T3/TR induced hepatoma cell metastasis in parallel with TRAIL induction \textit{in vivo}. SCID mice were injected with $1 \times 10^7$ J7-TR cells and divided into three groups (n=3 per group), as described in Materials and Methods. H&E staining from lungs of the indicated groups of mice was examined, and TRAIL
expression observed using IHC at 200× magnification. Scale bar, 200 μm. The metastasis index (fold density of tumor foci in the indicated groups per cm² area) in lungs is shown in the lower panel.

**Figure 7. Expression of TRAIL and TR in HCC patients.**

(A) TRAIL is upregulated in HCC patients. Sixty-five HCC biopsy samples were used for analyzing TRAIL expression. The intensities of TRAIL expression in tumors and their adjacent non-cancerous regions were scored and compared. **p < 0.01 (B) Representative image of HCC specimens, including tumor (T) and adjacent nontumor (N) cells stained with a specific anti-TRAIL antibody, are shown at 200× magnification. (C) Consecutive HCC tissue sections were stained with anti-TR (upper panel) and anti-TRAIL (lower panel) antibodies, and subjected to IHC evaluation. These samples displayed strong, moderate, and weak expression at 400× magnification in cancerous tissues. (D) Correlation of TR and TRAIL expression in HCC patients. * Statistically significant with the x² test. **p < 0.01

**Supplemental Figure 1. TRAIL induces NF-κB activation in Bcl-xL-overexpressing hepatoma cells.**

(A) Effect of TRAIL on NF-κB promoter activity. After co-transfection of the NF-κB reporter construct with β-galactosidase (a transfection efficiency control), HepG2-Bcl-xL and J7- Bcl-xL cells were harvested following TRAIL treatment for
various times. Luciferase activity was normalized to that of β-galactosidase. Values are shown as fold activation, relative to TRAIL (0 ng/ml) treatment. Data are presented as means ± SEM of values from three independent experiments performed in triplicate. **p < 0.01; *p < 0.05. (B) Effects of TRAIL on phospho-ERK1/2. Following TRAIL (100ng/ml) stimulation for the indicated times, cell lysates of HepG2-Bcl-xL and J7- Bcl-xL cells were extracted for examining phosphor-ERK1/2 expression. Total ERK 1/2 was used as an internal control.
Methods

Cell cultures

Individual hepatoma cell lines, including Huh7 and the isogenic HepG2 and J7 cell lines, were routinely cultured in Dulbecco’s Modified Eagle’s medium (DMEM) containing 10% (v/v) FBS. T3-depleted serum was prepared with AG 1-X8 resin (Bio-Rad, Hercules, CA). T3 was purchased from Sigma-Aldrich®, St. Louis, MO, USA. Cells were cultured at 37°C in a humidified atmosphere of 5% CO2 and 95% air.

Real-time PCR

Hepatoma cells were seeded in 10 cm dishes. After several reagent treatments for the indicated times, cells were harvested for RNA extraction. Total RNA was purified with TRIzol reagent, and cDNA synthesized with the Superscript II kit for RT-PCR (Life Technologies, Karlsruhe, Germany). Real-time PCR was conducted in a 15 μl reaction mixture containing 25 nM forward and reverse primers, and 1× SYBR Green reaction mix (Applied Biosystems, Carlsbad, CA, USA). The reaction was performed using the ABI PRISM 7500 sequence detection system (Applied Biosystems, Foster City, CA).

Immunoblot analysis
Total cell total lysates or supernatant were isolated and fractionated via SDS-PAGE on a 12% gel, and separated proteins transferred to a PVDF membrane and visualized by chemiluminescence with an ECL detection kit (Amersham Inc., Piscataway, NJ). The rabbit polyclonal antibody against human TRAIL was purchased from AbD Serotec (Oxford, UK). Rabbit anti-human Bcl-xL, Bcl-2, Mcl-1, Bad, Bid, Bax, active caspase-3 and cFLIP antibodies were acquired from Epitomics (Burlingame, Ca). Mouse anti-human fascin monoclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit antibodies to ERK1/2, phospho-ERK1/2 and active caspase-8 were obtained from Cell Signaling Technology (Boston, MA).

**Transient transfection and reporter assays**

To construct the *TRAIL* 5′-flanking region for promoter activity assay, fragments of the *TRAIL* promoter (−3899 to +30) and serial deletion mutants were amplified with PCR and inserted into pGL3-basic vector. HepG2-TR cells were transfected with 0.2 μg of PGL3-basic vector containing *TRAIL* promoter sequences and 0.05 μg of SVβ plasmid (Clontech, Palo Alto, CA), the β-galactosidase expression vector. At 16 h after transfection, cells receiving T3 (0, 10 or 100 nM) were incubated for an additional 24 h and lysed to measure luciferase and β-galactosidase activities. The activity of luciferase was normalized to that of β-galactosidase.

**Chromatin immunoprecipitation (ChIP) assay**
The ChIP assay was performed to determine the localization of TREs within the *TRAIL* promoter region. Proteins were cross-linked to DNA by the direct addition of 1% formaldehyde to the medium for 10 min at room temperature, as described previously\textsuperscript{38}. The mouse monoclonal antibody against TR was provided by S-Y Cheng at NIH, and anti-IgG antibody by R&D Systems, Inc. (Minneapolis, MN). The 186 bp fragment of the *TRAIL* promoter containing the predicted TRE was detected by PCR using the following primers: forward, 5′-GAGATGGAGTTAGC CGTGTTAGC-3′, and reverse, 5′-AGAGTGAGGAAAATAAGGAGGCTTA-3′. The TRE region of the *Furin* promoter was amplified with the forward primer, 5′-TACTAGCGGT TTTACGGGCG-3′, and reverse primer 5′-TCGAACAGGAGCAGAGAGCGA-3′ as the positive control. The 166 bp fragment of *Gapdh* without TRE was detected with the forward primer, 5′-CTCCAAAGACCCACTGCG-3′, and reverse primer, 5′-CCACTTGTCCTCAGGCCTAG-3′, as the negative control.

**DNA fragmentation assay**

Parental hepatoma or several stable hepatoma cell lines were treated with the indicated chemotherapeutic drugs or TRAIL following T\textsubscript{3} (0, 10 nM) treatment for 48 h. After detachment via trypsinization, cells were washed with complete cell culture medium to stop tryptic digestion and collected by centrifugation at 300 g for 5 min.
Cells were fixed in Ethanol/PBS (7:3, v/v) for 1 h at -20°C. After fixation, cells were pelleted and resuspended in PBS containing 40 mg/ml RNase A and 0.5% Triton X-100 for 1 h at 37°C. Finally, cells were pelleted again and resuspended in PBS containing 50 mg/ml propidium iodide (Sigma-Aldrich, St Louis, MO). Genomic DNA fragmentation was quantified by flow cytometric analysis of hypodiploid DNA. Data were collected and analyzed using FACScan (Becton Dickinson, Co.) equipped with CELLQuest software.

**Zymography**

Concentrated medium (80 μg) from indicated cells was diluted in 50 mM Tris-HCl (pH 7.4) in the absence of reducing agent and separated via 8% SDS–polyacrylamide gel electrophoresis in the presence of 1 mg/ml gelatin. After electrophoresis, gels were washed twice in 2.5% Triton X-100 (50 mM Tris-HCl, pH 7.4) for 15 min and incubated for 16 h at 37°C in developing buffer (10 mM CaCl₂, 150 mM NaCl, and 0.02% NaN₃ in 50 mM Tris-HCl (pH 7.5)). Gels were stained with Coomassie Blue and destained until the appearance of clear bands.

**In vitro assay of invasive activity**

The influence of T₃ on the TRAIL-mediated invasive activity of TR-overexpressing hepatoma cell lines was assessed with the Transwell rapid in vitro assay. Cell density was adjusted to 1 × 10⁵ cells/ml, and cells added to each upper chamber coated with
Matrigel (Becton-Dickinson, Franklin Lakes, NJ) in triplicate. The media used were serum-free DMEM in the upper chamber and DMEM supplemented with 20% FBS in the lower chamber. After incubation for 24 h at 37°C, the number of cells traversing the filter to the lower chamber was measured.

**Xenograft models of tumor progression and metastasis**

To investigate T₃-induced TRAIL expression *in vivo* and its physiological consequences, severe combined immunodeficiency (SCID) mice were divided into three groups, each consisting of three animals. After tail vein injection with 1×10⁷ J7-TR tumor cells, euthyroid animals were generated by treatment of drinking water with PBS for 8 weeks. To induce hypothyroidism in SCID mice, 0.02% methimazole plus 0.1% sodium perchlorate was added to drinking water after inoculation with 1×10⁷ J7-TR tumor cells. Hyperthyroid mice were generated by treatment of drinking water with T₃ (2.5 mg/l) after inoculating with 1×10⁷ J7-TR tumor cells. All animals were killed at 8 weeks after tumor inoculation, following which liver and lungs were removed. All procedures were performed in accordance with United States National Institutes of Health guidelines and the Chang-Gung Institutional Animal Care and Use Committee Guide for Care and Use of Laboratory Animals. Paraffin-embedded and formalin-fixed tissues from the lungs of SCID mice were examined using hematoxylin and eosin staining and IHC with a specific
antibody against TRAIL. Positive staining for TRAIL immunoreactivity appeared dark-brown in color.

**Human HCC specimens**

Biopsies of 65 patients with HCC diagnosed were selected for study, with informed participant consent. Samples of HCC tissues with paired adjacent non-tumor liver tissues were obtained from the Chang Gung Memorial Hospital medical research center for IHC analysis. HCC and adjacent noncancerous liver tissue samples were confirmed using H&E staining. The study protocol was approved by the Medical Ethics and Human Clinical Trial Committee at Chang-Gung Memorial Hospital.

**Immunohistochemical staining**

IHC analyses were performed with an automatic device from the Bond-max Automated Immunostainer (Vision Biosystems, Melbourne, Australia). Tissue sections were retrieved with Bond Epitope Retrieval Solution 1 (Vision BioSystems) and stained with rabbit polyclonal antibody against TRAIL and mouse monoclonal antibody against TR. The polymer detection system (Bond Polymer Refine; Vision BioSystems) was used to reduce non-specific signal noise. Subsequently, sections were treated with DAB buffer using 3,3’-diaminobenzidine as the chromogen and hematoxylin as the counterstaining reagent. In terms of TRAIL expression, 45% of the HCC specimens displayed strong staining, 38% displayed moderate staining, and
17% stained weakly. For TR expression, 38% of the tumor specimens displayed strong staining, 46% stained to a moderate extent, and 16% stained weakly. TR and TRAIL-positive tumor cells in the representative microscopic fields were independently scored by two experienced pathologists.

**Statistical analysis**

Values are expressed as means ± SEs of at least three independent experiments.

Statistical analysis was performed using Student's t-test or one-way ANOVA. **, $P < 0.01$; *, $P < 0.05$ was considered statistically significant.
Reference


