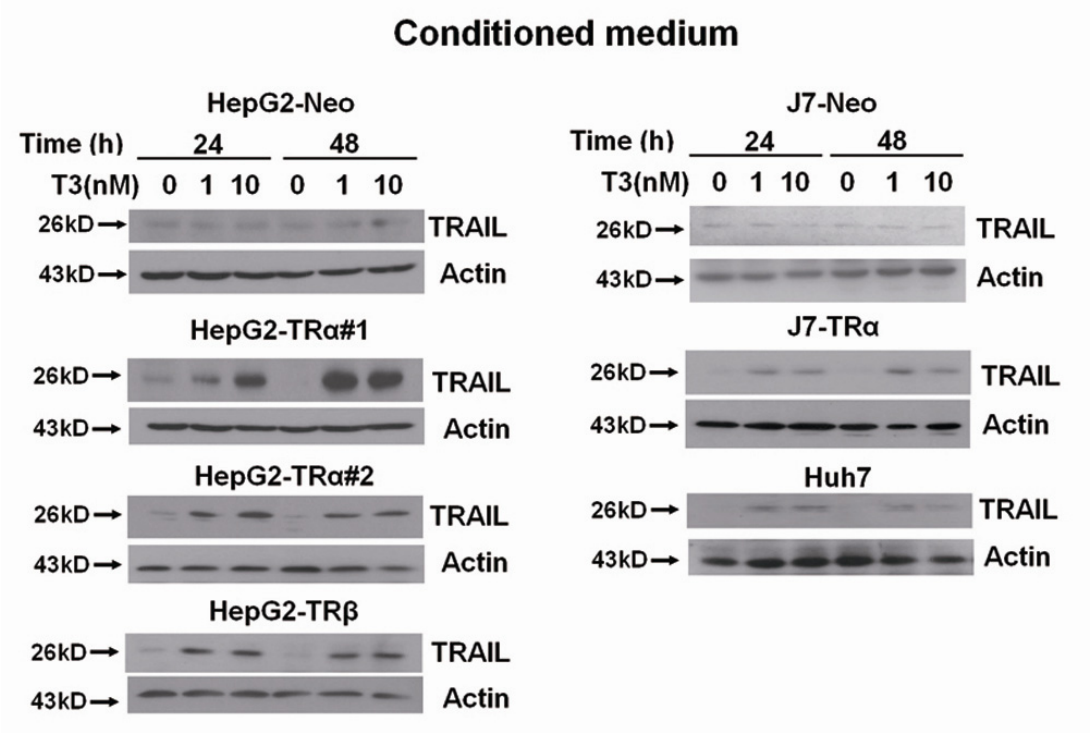
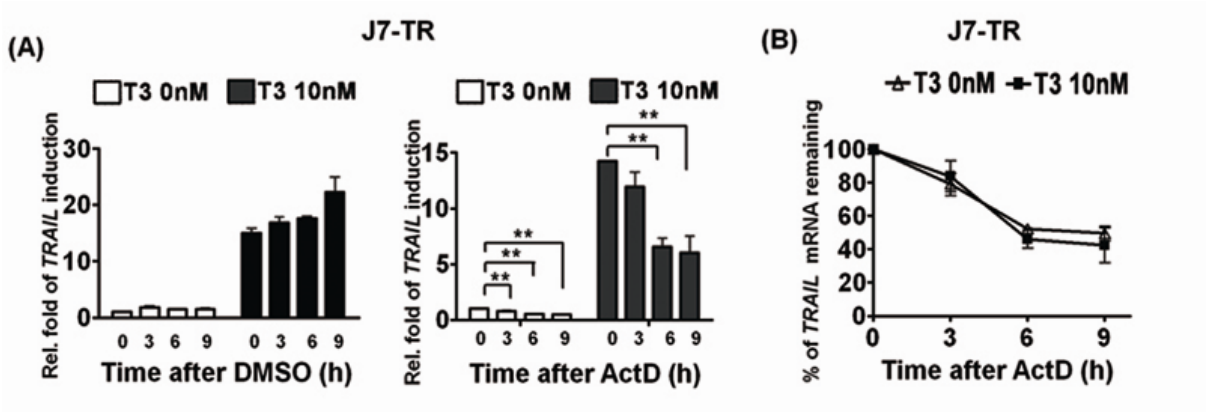
Supplemental materials



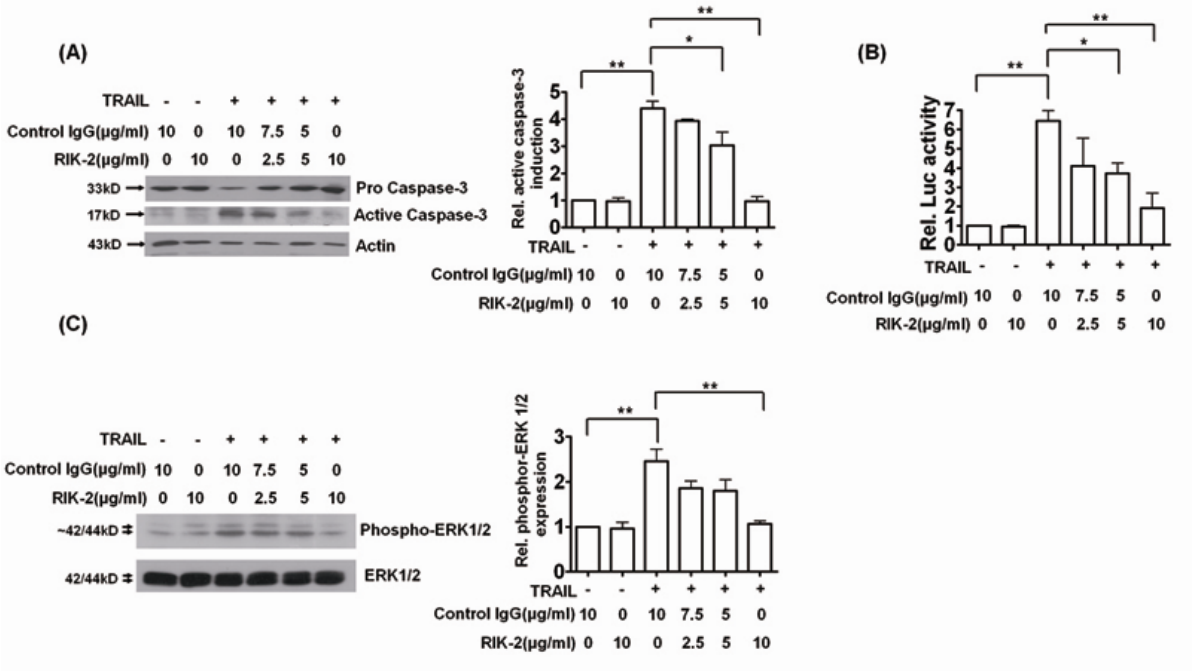
Supplemental Figure 1. Effects of T_3/TR on secreted TRAIL protein levels in hepatoma cell lines.

TRAIL protein levels in conditioned medium (30 μg total protein) of parental and stable HCC cell lines kept in serum-free medium with or without T_3 (1 or 10 nM) for 24 and 48 hr were analyzed with Western blot. To ensure the depletion of T_3 in the cells, they were kept in Td medium for 24 hr before replaced by serum-free medium. The signals of Actin were used as loading controls.



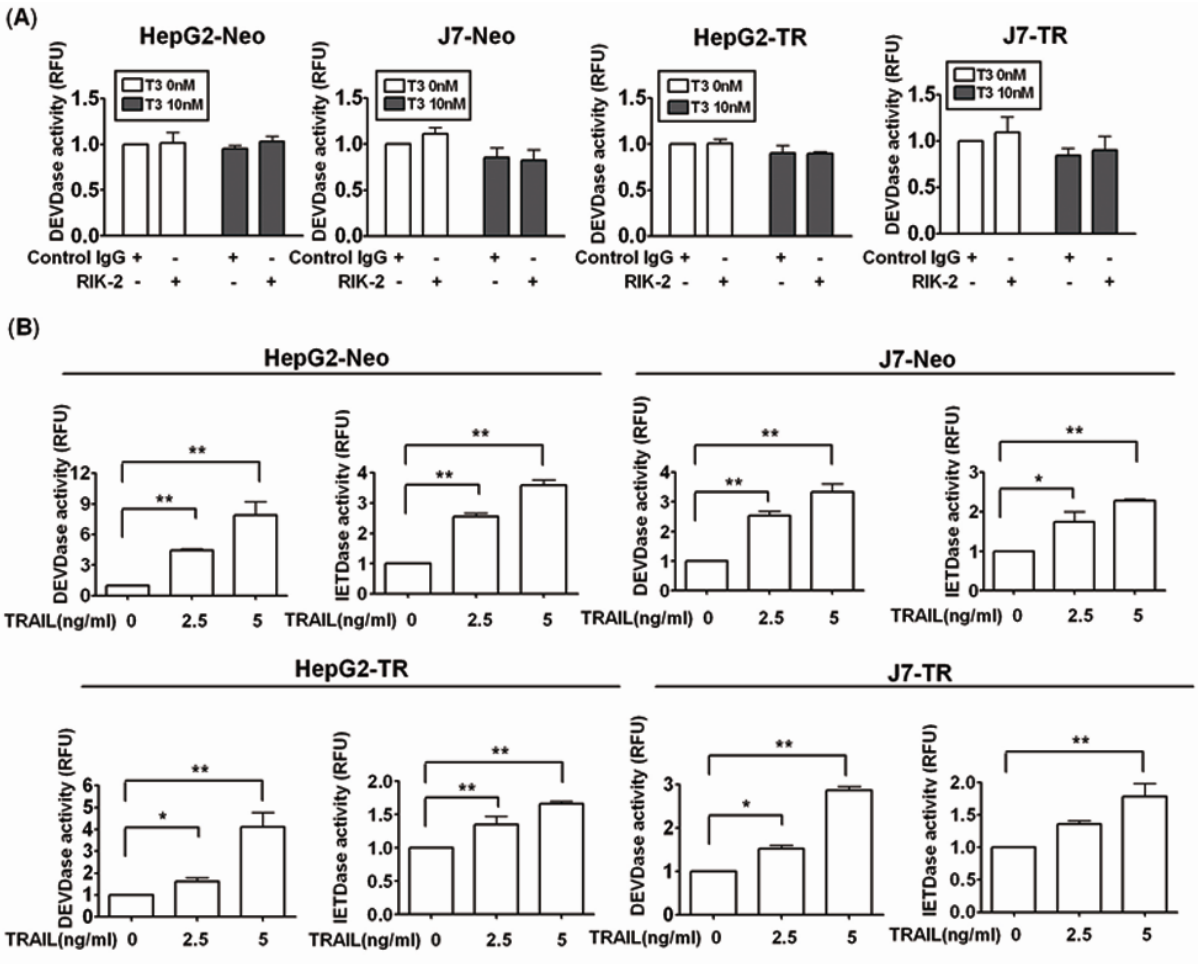
Supplemental Figure 2. T_3 does not influence TRAIL stability at the RNA level.

(A) J7-TRα cells were treated with Vehicle or ActD for the indicated times (2 μg/mL) following T_3 (0 or 10 nM) stimulation for 24 h. Total mRNA was isolated and subjected to real-time PCR. The fold induction of *TRAIL* relative to the start of treatment in the absence of T3 treatment was shown (Left and medina panel). (B) Furthermore, these values are also presented as percentage of RNA remaining relative to the start of ActD treatment at the indicated time-points. Data are presented as means ± SEM of values from three independent experiments.



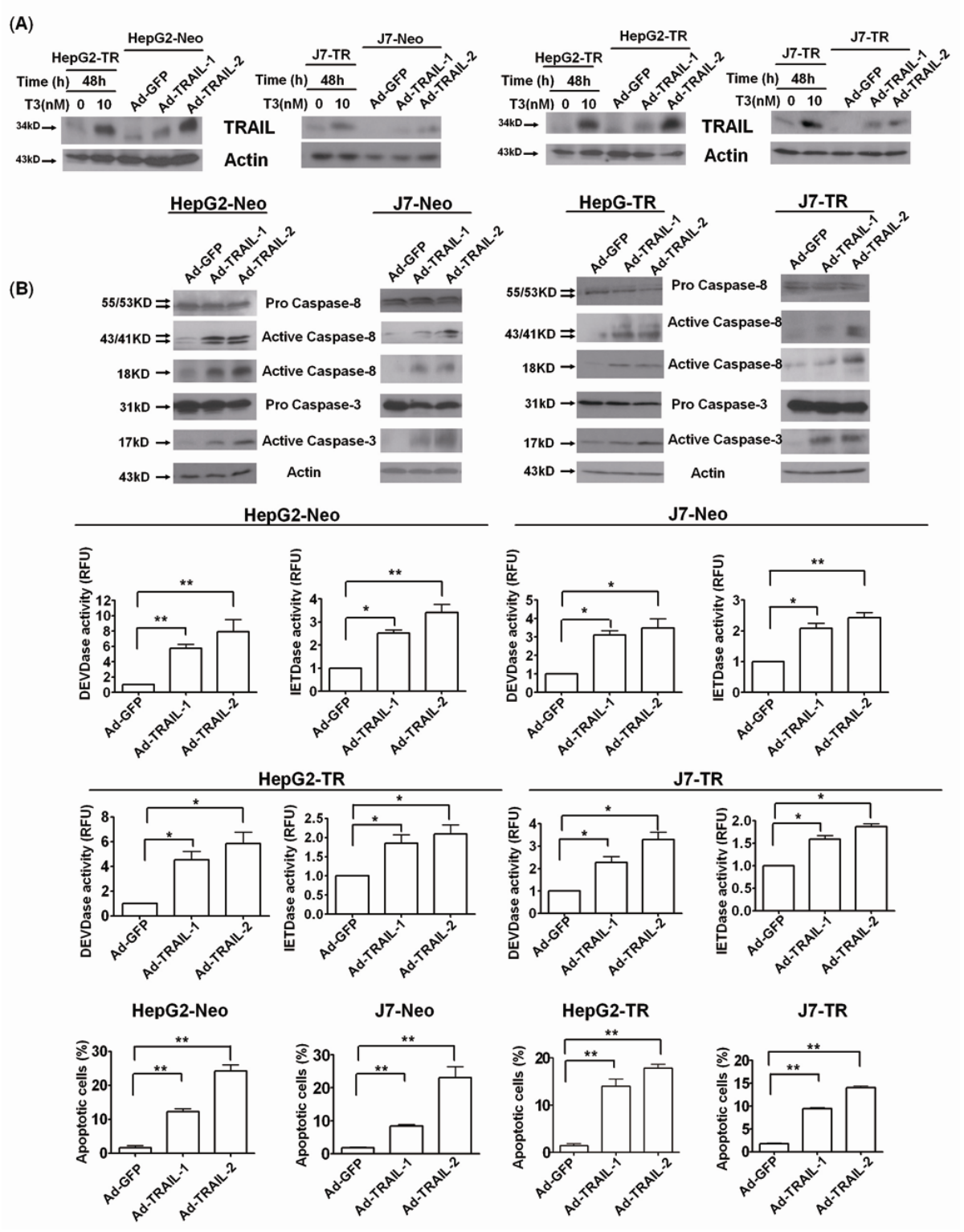
Supplemental Figure 3. RIK-2 efficiently suppresses TRAIL activity

(A) RIK-2 suppressed TRAIL induced Caspase-3 activation. r-TRAIL was pre-incubated with several concentration of RIK-2 (r-TRAIL plus RIK-2) for 2hr. After treating HepG2 cells with r-TRAIL or without RIK-2 for 24h, cell lysates were extracted for detecting the Caspase-3 activation by Western blot. Actin was used as an internal control. The intensities of Active Caspase-3 band was quantified and normalized to that of Actin. Values are shown as fold induction relative to the cells treated with control IgG (10μg/mL). (B) NF-κB reporter construct was co-transfection with SVβ vector (expressing β-galactosidase for transfection efficiency control) into HepG2 cells. After transfection, cells were treated with r-TRAIL with/without RIK-2 for 8hr before harvested. Luciferase activity was normalized to that of β-galactosidase and shown as fold activations relative to that of vehicle treatment. (C) Following r-TRAIL plus RIK-2 stimulation for 1 hour, the cell lysates of HepG2 cells were extracted for detecting the expression of phospho-ERK 1/2 by Western blot. The signals of total ERK 1/2 acts as internal control. The intensities of phospho-ERK 1/2 bands were quantified and normalized to that of ERK 1/2. Values are shown as fold induction relative to the cells treated with control IgG (10μg/mL). Value is presented as means ± SEM from experiments performed in triplicate and the difference between treatments were analyzed with one-way ANOVA. *and **: $p < 0.05$ and $p < 0.01$, respectively.



Supplemental Figure 4. r-TRAIL enhanced Caspase-3 and -8 activities.

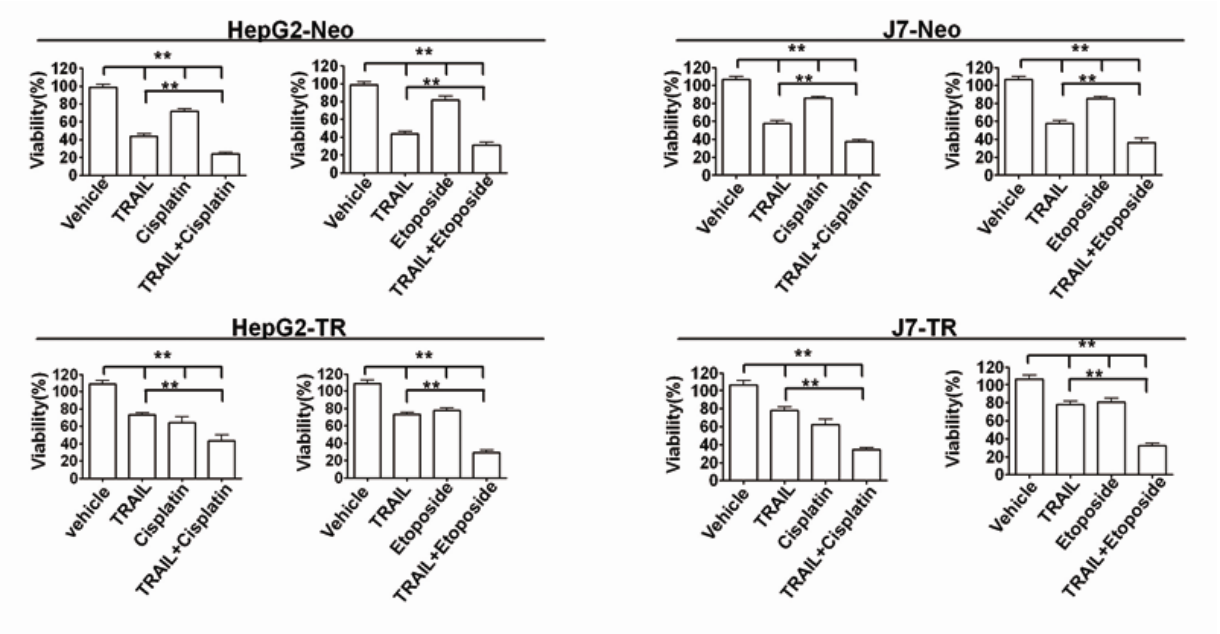
(A) Neither control IgG nor TRAIL blocking antibody (RIK-2, 10μg/mL) co-treatment with T3 for 48 h induced Caspase-3 activity in control or TR overexpressing hepatoma cell lines (B) TRAIL induces apoptosis in control or TR-overexpressing hepatoma cells. HepG2-Neo, J7-Neo, HepG2-TR or J7-TR cells were treated with r-TRAIL (2.5 or 5ng/mL) for 48 h. After TRAIL stimulation, activation of Caspase-3 and Caspase-8 were analyzed DEVDase.and IETDase activity assay



Supplemental Figure 5. Ad-TRAIL causes apoptosis in control or TR-overexpressing hepatoma cells.

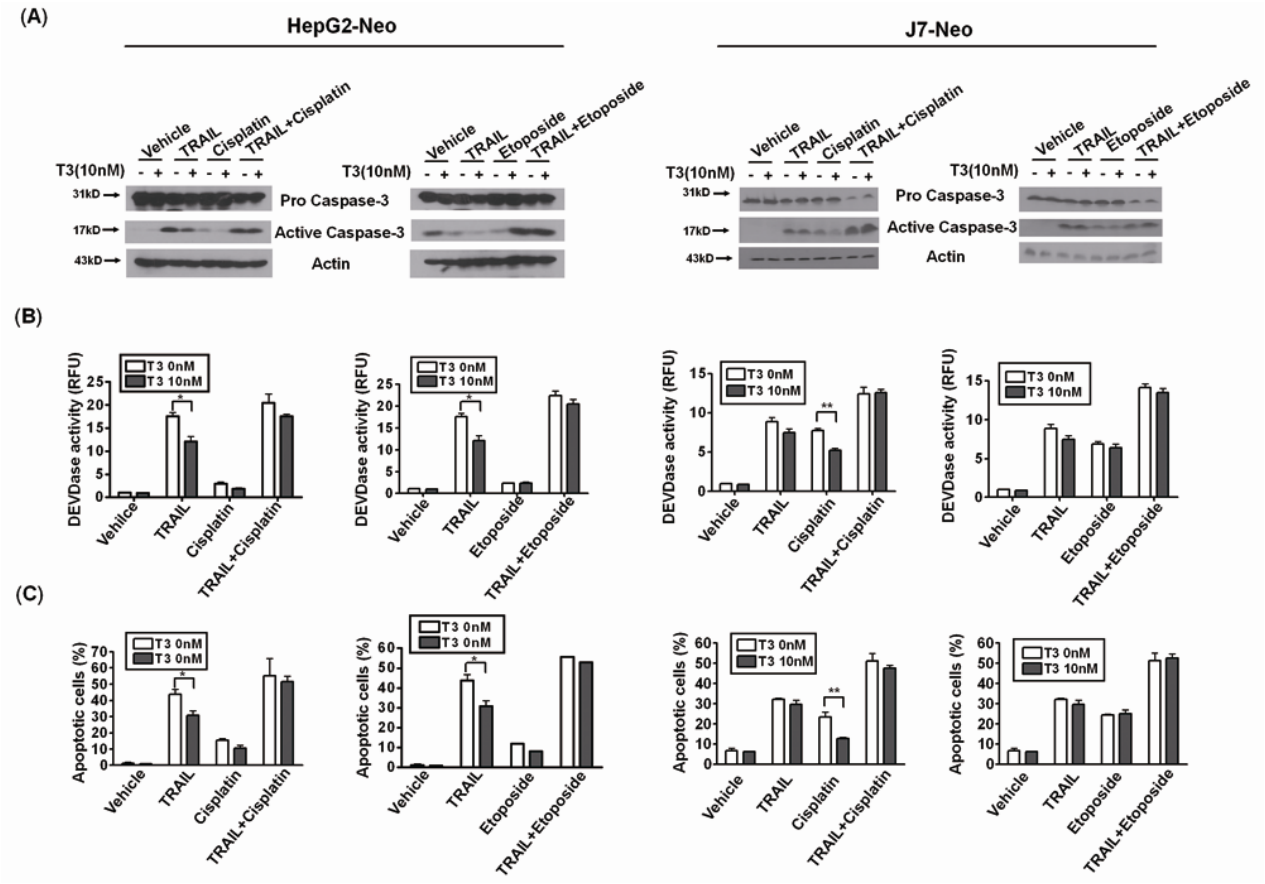
(A) HepG2-Neo, J7-Neo, HepG2-TR or J7-TR cells were infected with TRAIL-expressing adenovirus (Ad-TRAIL) and their TRAIL protein expression level was examined using Western blot. The signals of Actin act as an internal controls. (B)

After Ad-TRAIL transduction, activation of Caspase-8 and Caspase-3 were analyzed using western blotting (upper panel) and DEVDase.and IETDase activity assay (middle panel). The percentage of apoptotic cells assessed with PI-stained flowcytometry (lower panel). Data are presented as means ± SEM from experiments performed in triplicate and the difference between treatments were analyzed with one-way ANOVA.. *and **: $p < 0.05$ and $p < 0.01$ respectively.



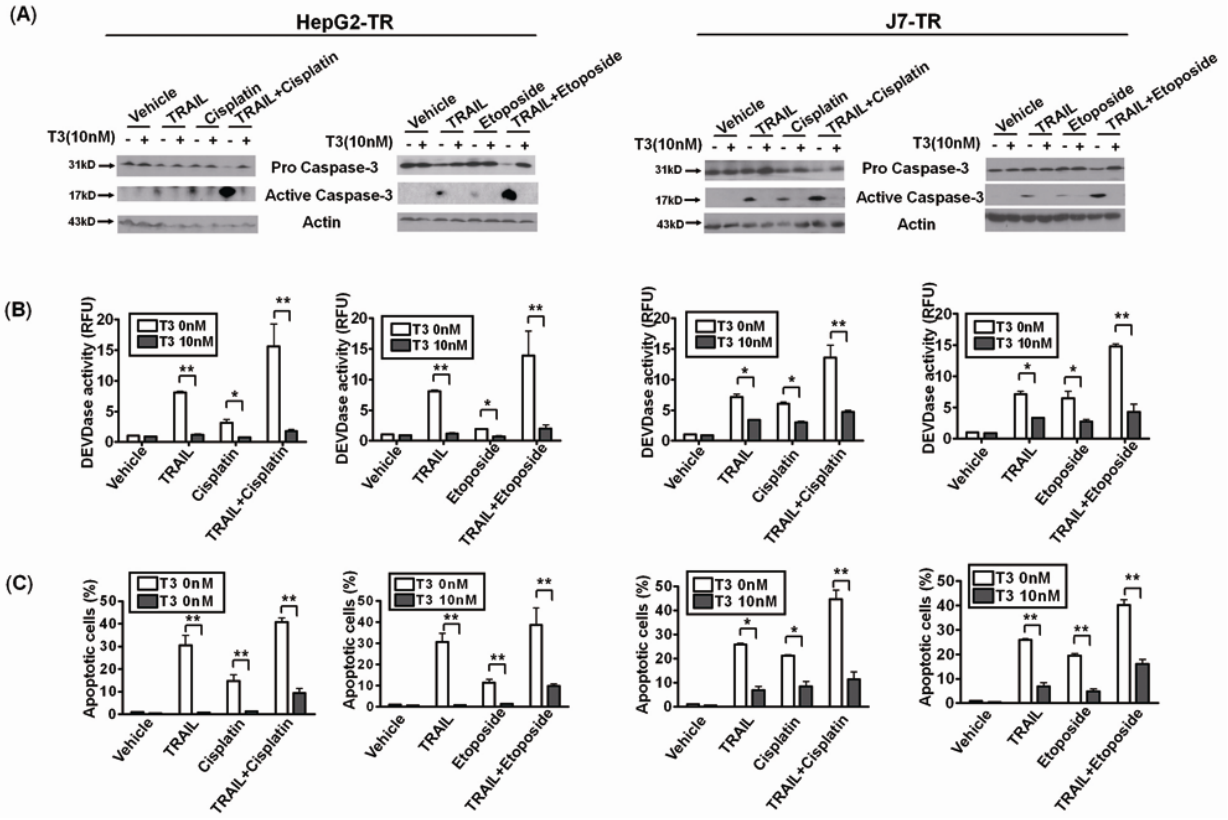
Supplemental Figure 6. Cisplatin and Etoposide synergistically enhanced TRAIL-induced cell death

HepG2-Neo, J7-Neo, HepG2-TR and J7-TR cells were treated with or without r-TRAIL (100 ng/mL) in combination with vesicle 5 μM Cisplatin, or 40 μM Etoposide for 24h. Then, cell viability was measured by MTT assay and presented as relative absorbance compared with vehicle treated control cells. Data are presented as means ± SEM from experiments performed in triplicate and the difference between treatments were analyzed with one-way ANOVA.. *and **: $p < 0.05$ and $p < 0.01$ respectively.



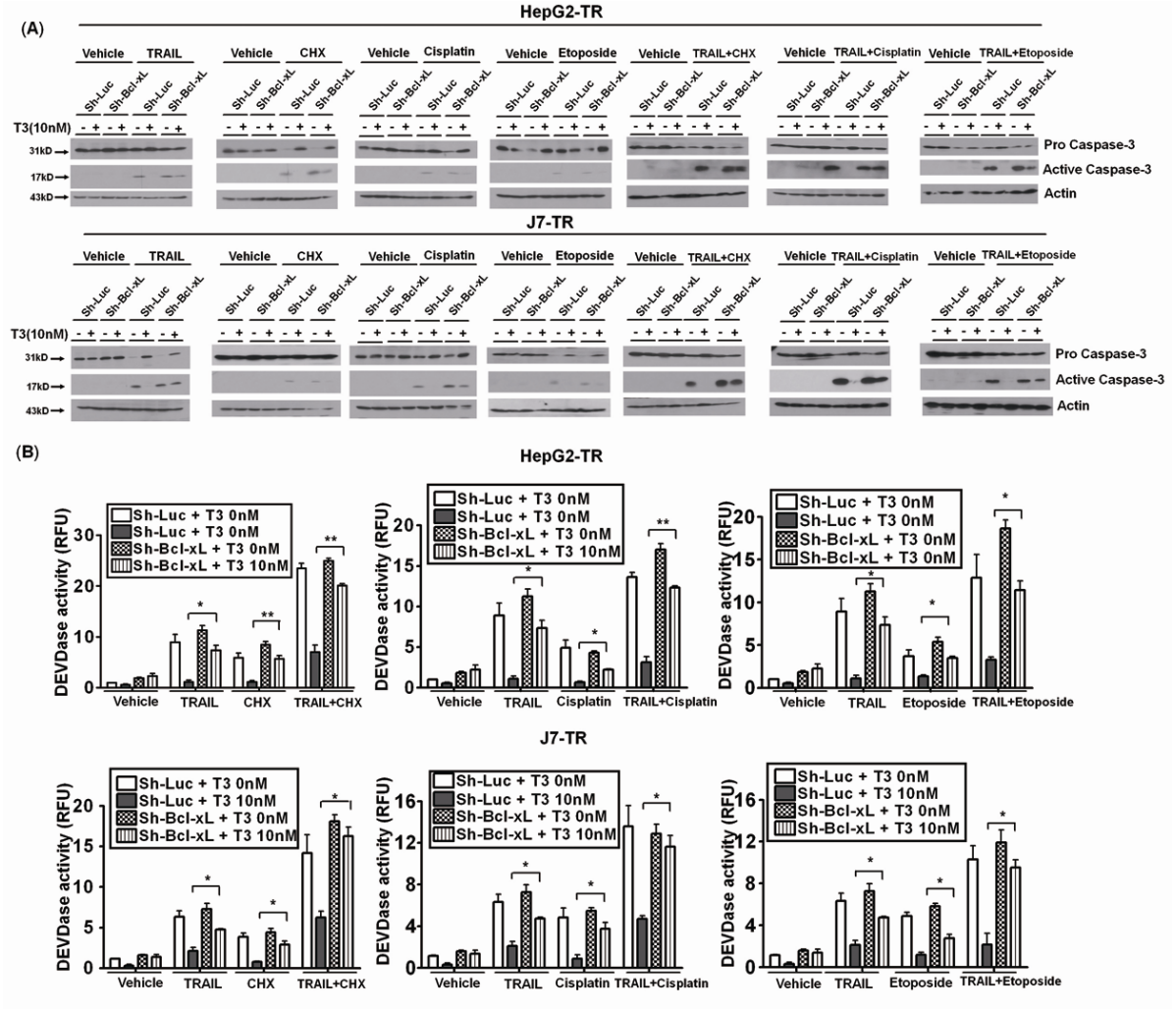
Supplemental Figure 7. T_3 marginally suppressed TRAIL, Cisplatin or Etoposide induced apoptosis in control hepatoma cells.

T_3 only slightly protected control cells from r-TRAIL and therapeutical drugs caused apoptosis. HepG2-Neo and J7-Neo cells were treated with r-TRAIL, therapeutical drugs , or their combination for 24 h after in T_3 (0 or 10 nM) stimulation for 2 days. Then, activation of Caspase-3 was analyzed using (A) Western blotting and (B) DEVDase activity assay. (C) The percentage of apoptotic cells assessed with PI-stained flowcytometry. Data are presented as means ± SEM from experiments performed in triplicate and the difference between treatments were analyzed with one-way ANOVA. *and **: $p < 0.05$ and $p < 0.01$ respectively.



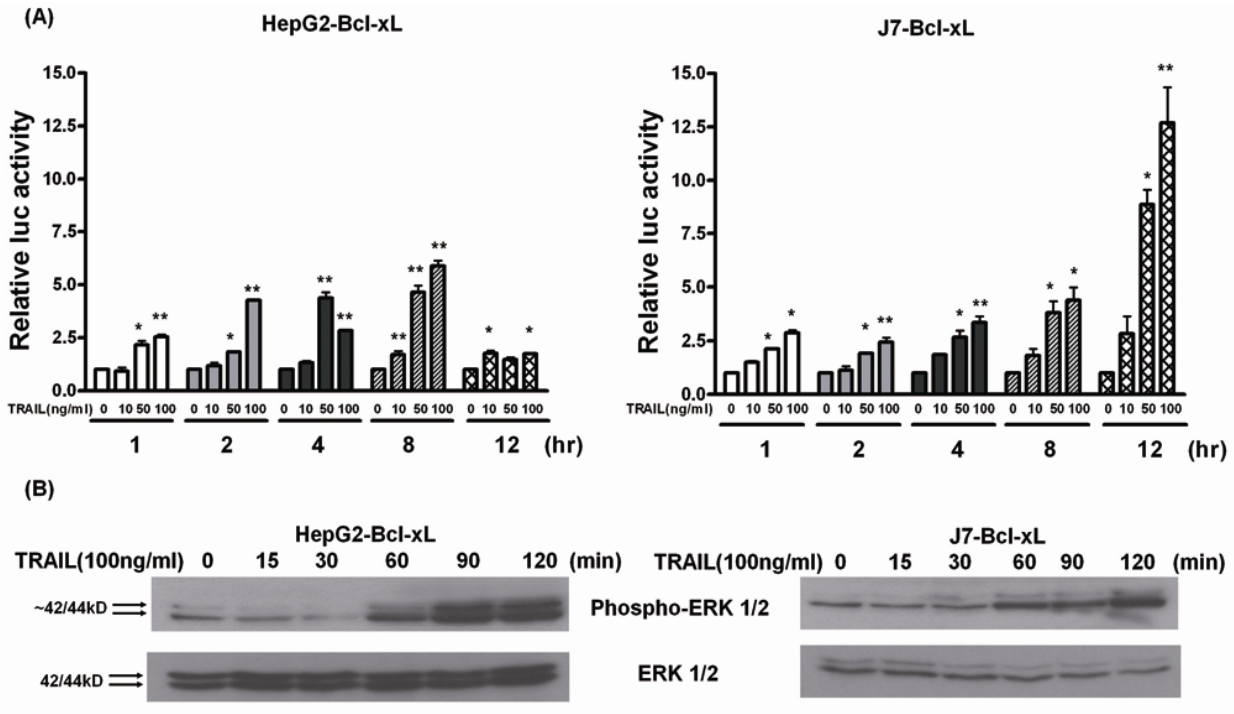
Supplemental Figure 8. T_3/TR suppresses r-TRAIL and chemotherapeutic drugs -induced apoptosis.

T_3 significantly protected TR-overexpressing cells, from r-TRAIL and therapeutical drugs apoptosis. HepG2-TR and J7-TR cells were treated with r-TRAIL, therapeutical drugs , or their combination for 24 h after in T_3 (0 or 10 nM) stimulation for 2 days. Then, activation of Caspase-3 was analyzed using (A) Western blotting and (B) DEVDase activity assay. (C) The percentage of apoptotic cells assessed with PI-stained flowcytometry. Data are presented as means ± SEM from experiments performed in triplicate and the difference between treatments were analyzed with one-way ANOVA.. *and **: $p < 0.05$ and $p < 0.01$ respectively.



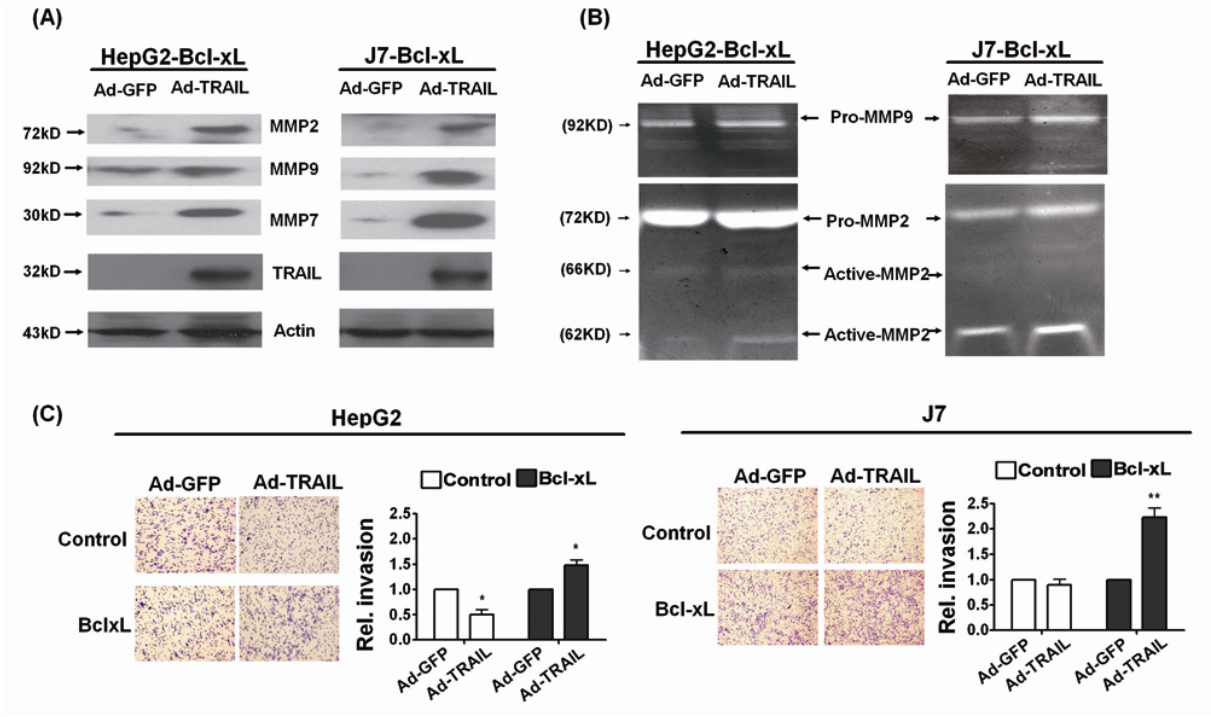
Supplemental Figure 9. Bcl-xL mediated the apoptotic protection effect of T3/TR in hepatoma cells

HepG2-TR-shLuc, J7-TR-shLuc, HepG2-TR-shBcl-xL and J7-TR-shBcl-xL cells were treated with apoptosis-inducing agents for 24 h after in T_3 (0 or 10 nM) stimulation for 2 days. Then, Caspase-3 activity was analyzed with (A) Western blot and (B) DEVDase activity assay. Data are presented as means ± SEM from experiments performed in triplicate and the difference between treatments were analyzed with one-way ANOVA. *and **: $p < 0.05$ and $p < 0.01$ respectively.



Supplemental Figure 10. TRAIL induces NF-κB activation in Bcl-xL-overexpressing hepatoma cells.

(A) Effect of r-TRAIL on NF-κB promoter activity. NF-κB reporter construct was co-transfection with SVβ vector (expressing β-galactosidase for transfection efficiency control) into HepG2-Bcl-xL and J7- Bcl-xL cells cells. After transfection, cells were treated with r-TRAIL for 1-12 hr before harvested. Luciferase activity was normalized to that of β-galactosidase and shown as fold activations relative to that of vehicle treatment at each time point. Data are presented as means ± SEM from experiments performed in triplicate and the difference between treatments were analyzed with one-way ANOVA. *and **: $p < 0.05$ and $p < 0.01$ respectively. (B) Effects of r-TRAIL on phospho-ERK1/2. Following r-TRAIL (100 ng/mL) stimulation for the indicated times, cell lysates (100 μg) of HepG2-Bcl-xL and J7-Bcl-xL cells were extracted for examining phosphor-ERK1/2 expression with Western blot. Total ERK 1/2 was used as an internal control.

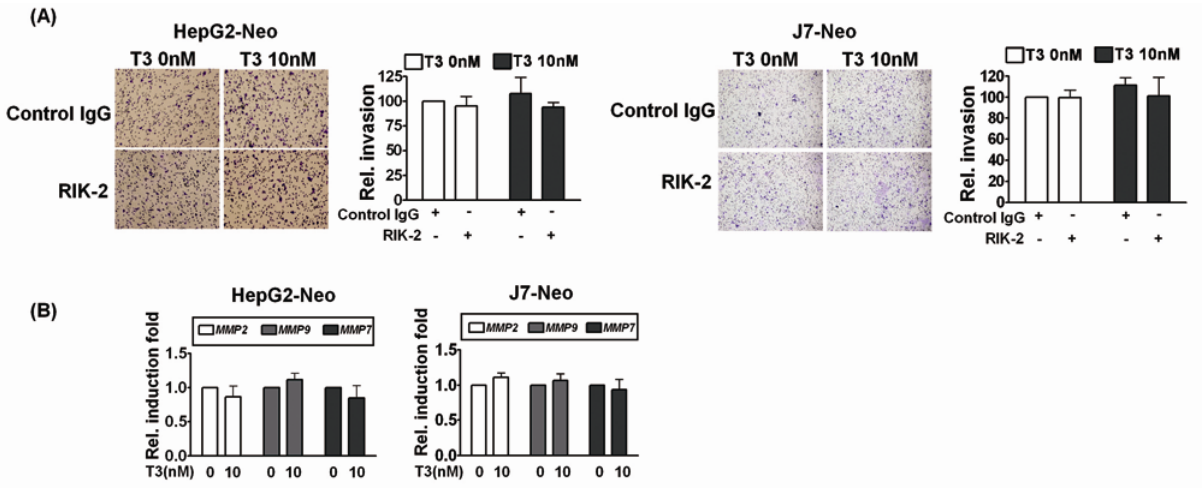


Supplemental Figure 11. Invasive ability of apoptosis-resistant hepatoma cells was enhanced by Ad-TRAIL transduction

(A) TRAIL induces MMPs protein expression. Following Ad-TRAIL infection for 48 h, total cell lysates or conditioned medium from HepG2-Bcl-xL and J7-Bcl-xL was subjected to Western blot analysis for detecting protein expression of MMPs and TRAIL. The signals of Actin were used as internal controls.

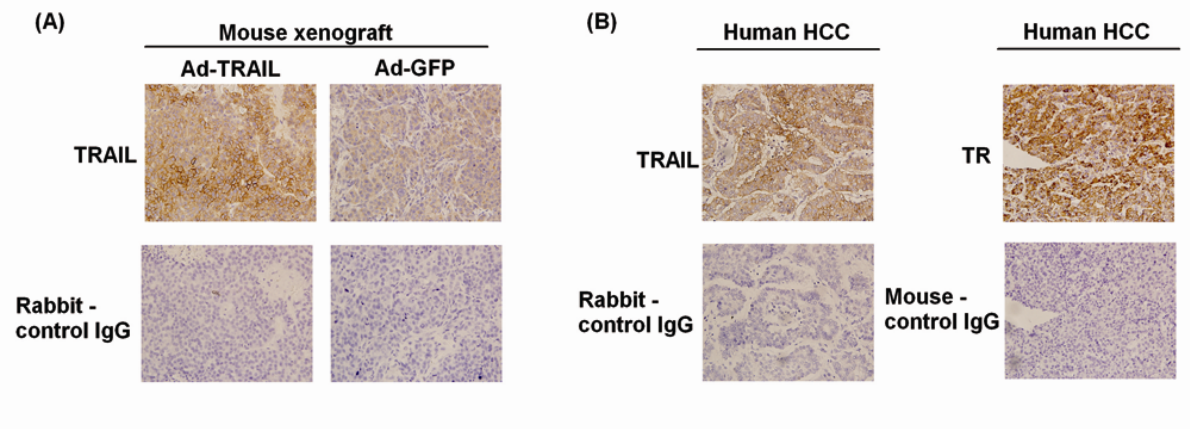
(B) Zymography assays of MMP-2 and -9 in HepG2-Bcl-xL and J7-Bcl-xL cells. The conditioned media from Ad-TRAIL-infected HepG2-Bcl-xL and J7-Bcl-xL cells were collected and MMPs activity was assessed with zymography assay. The positions of the proenzyme and active form of MMPs are shown.

(C) TRAIL induces apoptosis-resistant cell invasion *in vitro*. After Ad-GFP or Ad-TRAIL infection, control HepG2 and J7,or Bcl-xL overexpressing HepG2 and J7 cells (1×10^5) were added to matrigel-coated wells of Transwell units for 24 h, The relative number of cells that migrated to the lower chamber was stained and determined. Promotion of invasion was quantified as fold changes relative to that of Ad-GFP-infected cells. All assays were repeated at least three times and differences among treatments were examined using the Student's t-test. *and **: $p<0.05$ and $p<0.01$ respectively



Supplemental Figure 12. T_3 can not induce hepatoma cell metastasis in control hepatoma cell lines.

(A) Invasion properties of HepG2-Neo and J7-Neo cell lines treated without or with T_3 (10 nM) in the presence of control IgG (10 μg/mL) or RIK-2 (10 μg/mL) were determined with transwell assay. Cells (1×10^5) 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. Promotion of invasion was quantified as fold changes as compared with that of cells treated with control IgG in the absence of T_3. All assays were repeated at least three times and differences among treatments were examined using one-way ANOVA. $p<0.05$ and $p<0.01$, respectively. (B) HepG2-Neo and J7-Neo cells were cultured in DMEM without or with T_3 (10 nM) for 48 hr. Total RNAs were subjected to real-time PCR analysis for determining *MMP*s expression. The expression level of *MMP*s in cells without T_3 medium was set as 1 fold. All assays were repeated at least three times and differences among treatments were examined using Student's *t*-test. $p<0.05$ and $p<0.01$ respectively



Supplemental Figure 13. Detecting TRAIL and TR expression in mouse xenograft of hepatoma cells or in human HCCs by IHC analysis

(A) TRAIL antibody detected strong signal in TRAIL overexpressing, but not in GFP overexpressing, J7-Bcl-xL xenografts. control rabbit IgG or rabbit anti-TRAIL antibody were used to detect xenografts of either GFP or TRAIL overexpressing J7-Bcl-xL cells grown in the liver of SCID mice using IHC analysis. (B) Rabbit control IgG and TRAIL antibodies, or mouse control IgG and TR antibodies were used to detect TRAIL and TR expression in human HCC tissues. The representative images from the same tumor were shown here. Scale bar: 200 μm.