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Functional roles of tumor necrosis factor-related apoptosis-inducing ligand–DR5 interaction in B16F10 cells by activating the nuclear factor-κB pathway to induce metastatic potential

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Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has been recognized as a promising target for cancer therapy because it can induce apoptotic cell death in tumor cells but not normal cells. Although TRAIL shows specific tumoricidal activity, resistance to TRAIL-induced apoptosis in some tumor cells has been considered a clinical obstacle of its application. It has been shown that TRAIL provides inflammatory signals that may contribute to the TRAIL-resistance of cancer cells; however, it is not known whether TRAIL itself is involved in malignant cancer cell behavior. In the present study, we examined the functional role of TRAIL in B16F10 mouse melanoma cells, which are totally insensitive to TRAIL-induced apoptosis. By establishing B16F10 cells stably expressing the nuclear factor-κB (NFκB)-luciferase reporter gene, we found that TRAIL can activate NFκB through its death receptor DR5 in B16F10 cells. Furthermore, TRAIL–DR5 interaction not only promoted malignant behaviors of B16F10 cells, such as cell proliferation and MMP-9 production, but also induced lung metastasis of B16F10 cells *in vivo*. These findings may imply a contrary role for the TRAIL–DR5 pathway in the inflammatory tumor microenvironment, in its ability to induce the metastatic potential of B16F10 melanoma cells instead of inducing apoptosis. (*Cancer Sci* 2013; 104: 558–562)

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), also known as Apo2 ligand, is a type II transmembrane protein belonging to the TNF family[1,2] of cytokines that play important roles in inflammation and immunity.[3–6] It has been recognized as a promising target for cancer therapy, because TRAIL can induce apoptotic cell death in a variety of tumor cells but not in most normal cells.[7–9] Some studies have shown that this ligand has the potential to suppress the metastatic ability of cancer cells.[10,11] So far, two cell death-inducing receptors (TRAIL-R1/DR4, TRAIL-R2/DR5) and two non-cell death-inducing receptors (TRAIL-R3/DcR1, TRAIL-R4/DcR2) have been identified for TRAIL in humans; the latter two of these may act as decoys.[5–7,12,13] In mice, only one death-inducing receptor homologous to human DR5 (mTRAIL-R2/mDR5), and two potential decoy receptors have been identified.[4,14] These death receptors signal apoptosis through a Fas-associated death domain and the caspase-8-dependent pathway.[4,6,7,13,15,16] Moreover, the cytoplasmic regions of DR5 and mDR5 contain potential TNF receptor-associated factor (TRAF)-binding motifs, which may be responsible for NFκB and MAPK activation by this receptor.[13,15–18]

Although TRAIL has shown specific tumoricidal activity, some cancer cells are totally insensitive to TRAIL-induced apoptosis and such resistance may account for a clinical obstacle. Some studies have shown that the resistance to TRAIL-induced apoptosis is caused by lower expression levels of functional TRAIL receptors.[19–22] The B16F10 murine melanoma cell line is known to show resistance in spite of the high expression of mDR5 on the cell surface.[23,24] Therefore, the effects of TRAIL on B16F10 cells have not been comprehensively explored.

As a critical transcription factor for inflammation, NFκB regulates the expression of pro-inflammatory genes associated with invasion, angiogenesis, and metastasis.[25,26] Some reports indicated that the activation of NFκB maintains resistance to TRAIL-induced apoptosis.[19,27–29] However, it is not known whether the TRAIL pathway is involved in cancer cell behavior by providing inflammatory signals.

In the present study, we investigated the role of NFκB-mediated inflammatory signals in cancer progression, particularly through the TRAIL–DR5 receptor pathway in B16F10 melanoma cells. We found that TRAIL activated the NFκB pathway through DR5 in B16F10 cells and induced a tumor-promoting effect with MMP-9 production, proliferation ability *in vitro*, and also induced lung metastasis potential *in vivo*, instead of inducing apoptosis.

Materials and Methods

Reagents. Tumor necrosis factor-α was purchased from Peprotech (Rocky Hill, NJ, USA). The pGL4.32 (luc2P/NF-kappaB-RE/Hygro) vector and D-luciferin were obtained from Promega (Madison, WI, USA). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA, USA). Hygromycin B was obtained from Nacalai Tesque (Kyoto, Japan). Anti-TRAIL (N2B2) and anti-DR5 (MD5-1) antibodies were purchased from Biolegend (San Diego, CA, USA).

Cells. Mouse melanoma B16F10 cells were maintained in DMEM and Ham's F12 medium containing 10% bovine serum (Nissui, Tokyo, Japan). Mouse B lymphoma 2PK3 cells and 2PK3 expressing mouse TRAIL (TRAIL-2PK3) cells were cultured in RPMI-1640 (Nissui) containing 0.03% L-glutamine, 0.01 M HEPES, 0.2% $NaHCO_3$, and 10% bovine serum. To establish NFκB-mediated luciferase gene expressing B16F10 cells (B16F10 NFκB), B16F10 cells (5×10^5/well) were seeded in a 6-well plate and pGL4.32 vector was transfected using Lipofectamine 2000. The cells were selected with Hygromycin B (200 μg/mL) and cloned by limiting dilution.

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To evaluate the response of NFκB *in vitro*, B16F10 NFκB transfectants or B16F10 CMV control cells (1×10^5/well) were cultured in a 96-well plate and treated with TNF-α (0.1–100 ng/mL). After incubation for 6 h, luciferase activity was measured with a multiplate reader (2030 ARVO X; Perkin Elmer Life Sciences, Boston, MA, USA).

Analysis of NFκB activation with TRAIL. The B16F10 NFκB cells (1.25×10^5/well) were cocultured with either TRAIL-expressing 2PK3 (TRAIL-2PK3) cells or control 2PK3 cells (1.25×10^5, 6.25×10^4, 1.25×10^4/well) in a 96-well plate. After 6 h incubation, luciferase activity was measured with a multiplate reader. To evaluate the specificity of TRAIL on NFκB activation in B16F10 cells, TRAIL-2PK3 (1×10^5/well) cells were pretreated with anti-TRAIL mAb (clone N2B2, 10 μg/mL) at 37°C for 1 h then cocultured with B16F10 NFκB cells at 37°C for 6 h. After the incubation, luciferase activity was measured with a multiplate reader.

Cell proliferation assay. The B16F10 CMV cells (5×10^3/well) were cocultured with TRAIL-2PK3 or control 2PK3 cells (1×10^4, 5×10^3, 2.5×10^3/well) in a 96-well plate for 48 h at 37°C. After the incubation, luciferase activity was measured with a multiplate reader.

Gelatin zymography. The B16F10 NFκB cells (5×10^5/well) were cultured with serum-free medium in a 24-well plate then treated with TNF-α (50 ng/mL) or cocultured with TRAIL-2PK3 (5×10^5/well) for another 48 h. After the incubation, cell-free supernatants were collected and mixed with sample buffer containing 2% SDS (without 2-mercaptoethanol) and incubated at 37°C for 20 min. Comparative gelatin zymography was carried out on 10% SDS-PAGE with 0.1% gelatin. Samples were electrophoresed at 10 mA for 4–5 h at 4°C. Gels were washed with buffer containing 2.5% Triton X-100 and 0.01 M Tris-HCl for 2 h at 4°C and washed with 0.01 M Tris-HCl for 40 min at room temperature. Gels were incubated in the buffer containing 0.05 M Tris-HCl, 0.5 mM $CaCl_2$, and 1 μM $ZnCl_2$ for 48 h at 37°C. After the incubation, gels were stained with Coomassie Brilliant Blue for 6 h and destained with 5% acetic acid and 10% methanol. The bands were quantified using ImageQuant LAS 4010 (GE Healthcare Japan, Tokyo, Japan).

Experimental lung metastasis model. Inbred wild-type C57BL/6 mice were purchased from Japan SLC (Tokyo, Japan). All experiments were carried out according to the guidelines of the Care and Use of Laboratory Animals of the University of Toyama (Toyama, Japan). The B16F10 CMV cells were inoculated i.v. (3×10^5) with or without pretreatment with anti-DR5 mAb (30 min, 4°C). Mice were injected with D-luciferin (150 mg/kg i.p.; Promega) 4 days after the tumor inoculation, then the lungs were removed 20 min after the D-luciferin injection to measure luminescence using an *in vivo* imaging system (IVIS Lumina II; Caliper Life Sciences, Hopkinton, MA, USA). The data was presented as the mean luminescence ± SEM.

Statistical analysis. Data were analyzed for statistical significance using Student's *t*-test. *P*-values <0.05 were considered significant.

Results

Establishment of NFκB-mediated luciferase gene stably expressing B16F10 cells. In order to determine whether TRAIL–DR5 interaction may have biological roles in B16F10 metastatic melanoma cells through NFκB-mediated inflammatory signals, we established luciferase gene-expressing B16F10 cells under an NFκB reporter (B16F10 NFκB cells). We first characterized the association between cell numbers and luciferase activity of B16F10 NFκB cells or control B16F10 CMV cells under stable cell culture conditions. There was a strong correlation between luciferase activity and cell number not only in B16F10 CMV cells (Fig. S1A,B) but also in B16F10 NFκB cells (Fig. S1C,D). These results clearly indicated that the luminescence represents cell number or viability without any stimulation in those reporter cells.

We further examined the response of B16F10 NFκB cells to TNF-α, known to be a typical inflammatory cytokine to activate the NFκB pathway. As shown in Figure 1, TNF-α induced luciferase activity in a dose-dependent manner (Fig. 1a) and

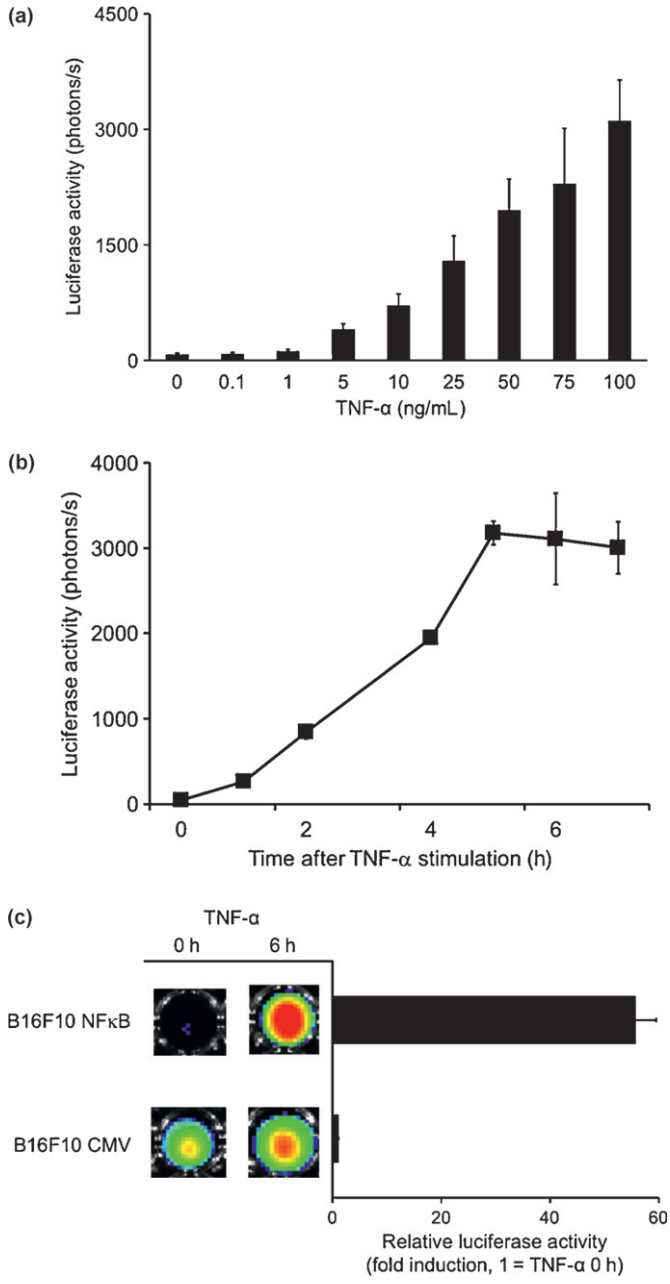

Fig. 1. Nuclear factor-κB (NFκB) activation after tumor necrosis factor-α (TNF-α) treatment in B16F10 mouse melanoma cells. (a) B16F10 NFκB cells were incubated with indicated concentrations of TNF-α for 6 h and the luminescence was measured. (b) B16F10 NFκB cells were treated with TNF-α (100 ng/mL) and the luminescence was measured at the indicated time after the TNF-α stimulation. (c) B16F10 NFκB and B16F10 CMV cells were stimulated TNF-α (100 ng/mL) for 6 h. The luminescence was measured at 0 h and 6 h after TNF-α stimulation. Error bars represent SEM.

appeared to have a peak response at 6–8 h after the TNF-α stimulation (Fig. 1b). Such induction of luciferase activity in response to TNF-α was specific for B16F10 cells expressing NFκB reporter, because B16F10 CMV cells did not show any response in its luciferase activity after TNF-α stimulation (Fig. 1c). These results indicate that B16F10 NFκB cells but not B16F10 CMV cells induce their luminescence in response to inflammatory stimulation through the NFκB pathway.

Interaction between TRAIL and DR5 activates NFκB in B16F10 cells. It is known that highly metastatic B16F10 melanoma cells are resistant to TRAIL-induced apoptosis despite their expression of DR5 receptor (Fig. S1E,F). To investigate whether TRAIL–DR5 interaction activates the inflammatory signaling pathway in B16F10 cells, we tested B16F10 NFκB cells to monitor NFκB activation in response to TRAIL stimulation. After coculture with TRAIL-2PK3 transfectants, B16F10 NFκB cells showed increased luminescence, but not with control 2PK3 cells (Fig. 2a). The reporter activity was associated with the amount of TRAIL availability within the coculture (Fig. 2a). Importantly, such induction of reporter activity was diminished in the presence of anti-TRAIL mAb (Fig. 2b). Furthermore, the activation of TRAIL receptor by agonistic anti-DR5 mAb also activated NFκB reporter in a dose-dependent manner (Fig. S2). Collectively, these results indicate that TRAIL–DR5 interaction activates the NFκB pathway in B16F10 cells.

Interaction between TRAIL and DR5 functionally activates B16F10 cells. We next examined whether TRAIL shows any functional roles in B16F10 cells in association with NFκB activation. In concert with NFκB activation, the proliferation rate of B16F10 cells was increased after 48 h of coculture with TRAIL-2PK3 cells but not with control 2PK3 cells (Fig. 3a). In addition to its activity in promoting proliferation, TRAIL–DR5 interaction also increased the production of MMP-9 from B16F10 cells, which is known to be a typical target molecule for NFκB activation. As shown in Figure 3(B), the activity of MMP-9 in the cell culture supernatant of TRAIL-stimulated B16F10 cells was higher than that of the control, and the induction of MMP-9 by TRAIL stimulation was comparable to TNF-α. These results clearly indicate that TRAIL–DR5 functionally activates B16F10 cells to facilitate their proliferation and MMP-9 production.

Finally, we examined the physiological significance of TRAIL–DR5 interactions in cancer metastasis. In an experimental lung metastasis model of B16F10 melanoma cells, we found that the activation of TRAIL receptor by pretreatment with agonistic anti-DR5 mAb enhanced the metastatic colonization of B16F10 cells (Fig. 4). Together with the functional role of TRAIL engagement in B16F10 cells *in vitro*, these results strongly imply that TRAIL–DR5 interactions have a physiological potential to enhance metastasis of B16F10 melanoma cells rather than to induce apoptosis of the cells expressing these receptors.

Discussion

Tumor necrosis factor-related apoptosis-inducing ligand is associated with one of the important effector pathways in the tumor immune surveillance, and the TRAIL signal has been known to induce the suppression of tumor metastasis by inducing apoptosis of malignantly transformed cells.[4,10,12,30] In contrast, it is also suggested that TRAIL may be involved in

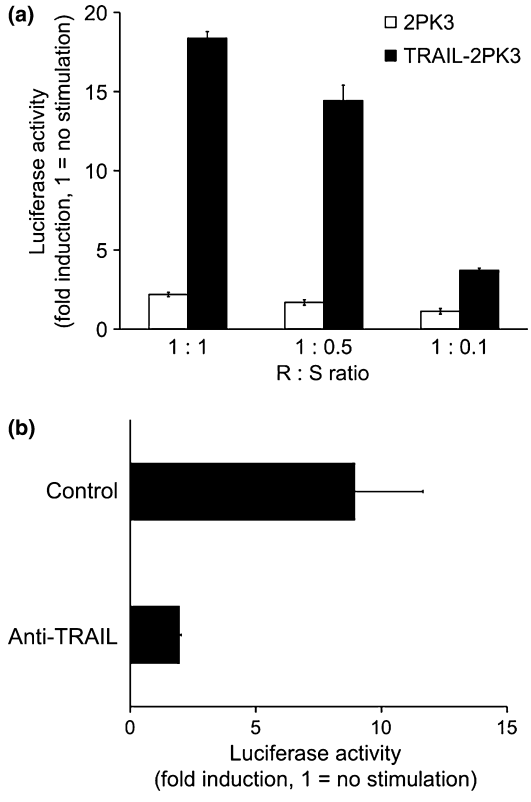

Fig. 2. Nuclear factor-κB (NFκB) activation through tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)–DR5 interaction in B16F10 mouse melanoma cells. (a) B16F10 NFκB cells were cocultured with TRAIL-2PK3 or 2PK3 at indicated responder (R):stimulator (S) ratios (B16F10 NFκB : TRAIL-2PK3 or 2PK3). After 6 h incubation, the luminescence was measured. (b) B16F10 NFκB cells were cocultured with TRAIL-2PK3 (at R:S 1:1) and N2B2 (10 μg/mL). After 6 h incubation, the luminescence was measured. Error bars represent SEM.

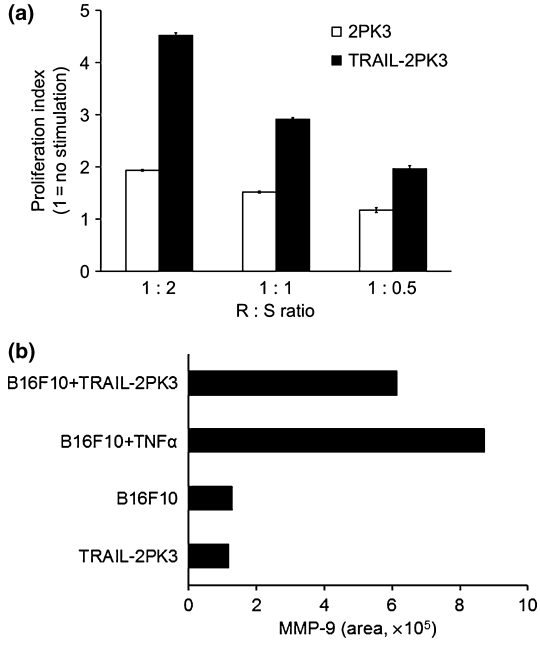

Fig. 3. Functional roles of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)–DR5 pathway in B16F10 mouse melanoma cells. (a) B16F10 CMV cells were cocultured with TRAIL-2PK3 or control 2PK3 cells at responder (R):stimulator (S) ratio of 1:1 for 48 h and luminescence was measured. Error bars represent SEM. (b) B16F10 cells were stimulated with tumor necrosis factor-α (TNF-α; 50 ng/mL) or cocultured with TRAIL-2PK3 (at R:S 1:1) for 48 h, and the cell-free supernatant was collected. Gelatin zymography was used to determine MMP-9 production and the band intensity was measured.

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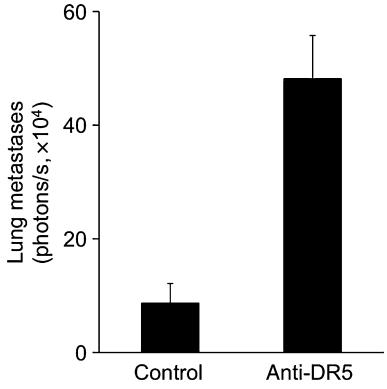

Fig. 4. Stimulation of tumor necrosis factor-related apoptosis-inducing ligand receptor enhances experimental metastasis of B16F10 mouse melanoma cells. B16F10 CMV cells were inoculated i.v. with or without pretreatment with anti-DR5 mAb. Lungs were removed 4 days after tumor inoculation to measure luminescence for determining lung metastasis. Data presented as the mean luminescence ± SEM.

cancer cell activation by providing inflammatory signals similar to other TNF superfamily members.[3,13,15–18] Previous studies have indicated that NFκB activation can be critical for acquiring resistance to TRAIL-induced apoptosis in some tumor cells.[19,27,28] In the present study, we showed the contribution of TRAIL–DR5 interaction to the activation of the NFκB pathway in B16F10 mouse melanoma cells, which is resistant to the TRAIL-induced apoptosis pathway. The TRAIL–DR5 interaction also plays a functional role in B16F10 cells by inducing their proliferation, MMP-9 production, and acquisition of metastatic potential *in vivo*.

It has been shown that cancer cells can evade TRAIL-induced apoptosis or acquire TRAIL resistance through several different mechanisms.[6,7,12,15] One particular mechanism can be the lower expression of death receptors for TRAIL, such as DR4 and DR5.[19–22] Furthermore, the intrinsic activation of anti-apoptotic machinery was also shown to be involved in acquiring TRAIL resistance in cancer cells.[12,31–33] It is also suggested that NFκB can be a key regulator for the expression of pro-inflammatory genes, including those for cancer cell proliferation and survival.[25,26] Despite the significant expression of DR5 on their cell surface,[24] murine B16F10 melanoma cells were known to be resistant to TRAIL inducing apoptosis.[23] Our present results clearly show that TRAIL stimulation in B16F10 cells activates NFκB and further promotes their cellular functions, including MMP-9 production and proliferation, which might contribute to cancer progression and metastasis. We did not find significant differences in B16F10 proliferation after a relatively short time (24 h) of coculture with TRAIL-2PK3 cells (data not shown), therefore, TRAIL–DR5 interaction may require more persistent interaction with its receptor in promoting B16F10 cell proliferation compared to its induction of apoptosis, which is generally seen 8–16 h after TRAIL ligation. Considering the TRAIL receptor ligation activates the NFκB pathway through interaction with the TNF receptor 1-associated death domain adaptor protein to recruit receptor-interacting protein kinase and TRAF2,[5,13,15,17] similar mechanisms might underlie the TRAIL-induced activation of NFκB in B16F10 cells to induce such cellular functions. Further study will be required to determine which signaling pathway is involved in the functional activation of B16F10 cells in response to TRAIL. Known to specifically express on host immune cells such as natural killer cells, dendritic cells, and activated T cells, TRAIL plays an important role in anti-tumor immune responses.[23,34–37] In contrast, we have shown that B16F10 melanoma cells may use TRAIL–DR5 interaction to promote their metastatic potential. Consistent with our current finding, it has been reported that TRAIL enhanced survival and/or proliferation in TRAIL-resistant primary leukemia cells in an NFκB-dependent manner.[38] Interestingly, it has also been reported that the NFκB pathway plays a role in the induction and maintenance of epithelial–mesenchymal transition,[26,39,40] considered to be an important process of tumor invasion and metastasis spread. Furthermore, the metastasis of TRAIL-resistant human pancreatic ductal carcinoma was promoted by TRAIL in a xenograft model.[41] Additional study is clearly required to determine whether endogenous TRAIL could be involved in the malignant progression of B16 melanoma cells; however, our current findings support a contrary role of the TRAIL–DR5 pathway in the inflammatory tumor microenvironment, in inducing the metastatic potential of cancer cells rather than inducing apoptosis in B16 melanoma cells. Considering several clinical trials of agonistic human TRAIL receptor antibodies have been undertaken to test their efficacy in cancer patients,[42–46] it would be very important to characterize patients' cancer cell types in their response to TRAIL-induced apoptosis. Collectively, our present findings propose a pro-tumor role of TRAIL–DR5 interaction in murine B16F10 melanoma cells by enhancing metastatic potential. Thus, a careful approach is required in the clinical application of the TRAIL pathway in cancer treatment, especially in TRAIL apoptosis-resistant cancer cell types.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Establishment of B16F10 CMV and B16F10 nuclear factor-κB (NFκB) mouse melanoma cells.

Fig. S2. Nuclear factor-κB (NFκB) activation by DR5 stimulation in B16F10 mouse melanoma cells.

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