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ORIGINAL ARTICLE

DcR2 (TRAIL-R4) siRNA and adenovirus delivery of TRAIL (Ad5hTRAIL) break down *in vitro* tumorigenic potential of prostate carcinoma cells

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High levels of decoy receptor 2 (DcR2; TRAIL-R4) expression are correlated with TRAIL resistance in prostate cancer cells. In addition, upregulation of TRAIL death receptor (DR4 and DR5) expression, either by ionizing radiation or chemotherapy, can sensitize cancer cells to TRAIL. Considering more than half of human cancers are TRAIL resistant, modulation of surface TRAIL receptor expression appears to be an attractive treatment modality to counteract TRAIL resistance. In this study, three siRNA duplexes targeting DcR2 receptor were tested. Ad5hTRAIL infections were performed to overexpress human full-length TRAIL to induce cell death, and the *in vitro* tumorigenic potential of prostate cancer cells was assessed using colony-forming assays on soft agar. The DU145 and LNCaP prostate cancer cell lines, which express high levels of DcR2, were resistant to Ad5hTRAIL-induced death. Downregulation of surface DcR2 expression by siRNA sensitized these prostate cancer cell lines to Ad5hTRAIL. In addition, DcR2 siRNA-mediated knockdown of DcR2, followed by Ad5hTRAIL infection, dramatically reduced the *in vitro* tumorigenic potential of prostate cancer cells. Collectively, our results suggest the potential for combining receptor-specific siRNA with TRAIL in the treatment of certain cancers.
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Introduction

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a type 2 membrane protein in the TNF family[1] that can bind to five different receptors: four of these are membrane bound (DR4/TRAIL-R1, DR5/TRAIL-R2, decoy receptor 1 (DcR1)/TRAIL-R3 and DcR2/TRAIL-R4) and one is soluble (Osteoprotegrin—OPG).[2] Two of these membrane receptors, DR4 and DR5, act as apoptosis-inducing receptors, whereas DcR1, DcR2 and OPG act as antagonistic receptors since they do not signal for cell death. Interestingly, TRAIL exhibits cancer-specific apoptosis-inducing properties, and does not induce cell death in normal cells and tissues.[3,4] Decoy receptor expression on normal cells was initially hypothesized as the explanation why normal cells were resistant to TRAIL. However, the decoy receptors are also expressed by cancer cells, prompting a more rigorous investigation into the mechanism(s) controlling TRAIL resistance in cancer cells.[5,6]

TRAIL death receptor upregulation is one means to sensitize cancer cells to TRAIL. For example, chemotherapeutic agents (such as cisplatin, topotecan and gimatecan) and ionizing radiation upregulate TRAIL death receptor expression in cancer cells, such as esophageal squamous cell carcinoma[7] and androgen-independent prostate carcinoma cell lines.[8] Furthermore, human colon carcinoma cells are sensitized to TRAIL by COX-2 inhibition through DR5 clustering at the cell surface.[9] The proteasome inhibitor, MG132, also upregulates DR5 expression and sensitizes cancer cells to TRAIL-induced apoptosis.[10] Ionizing radiation-induced DR5 expression on breast cancer cells[11] and prostate cancer cells[12] has resulted in their sensitization to TRAIL-mediated apoptosis.

Modulation of intracellular antiapoptotic pathways represents another means of influencing TRAIL sensitivity. For example, nuclear factor κappa B signaling contributes substantially to the TRAIL resistance of cancer cells.[13] 17-allylamino-17-demethoxygeldanamycin inhibition of nuclear factor κappa B signaling sensitizes lung cancer cells to TRAIL.[14] Similarly, nuclear factor κappa B inhibition using a gene therapy approach

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(AdIKKβKA)[15] or curcumin[16] can sensitize hormone-refractory prostate cancer cells to TRAIL-induced apoptosis. The downregulation of the antiapoptotic proteins, such as FLICE-inhibitory protein and survivin, by various techniques (such as using the antidiabetic drug troglitazone) also sensitizes human cancer cells to TRAIL-induced apoptosis.[17] 3,3′-diindolylmethane downregulated c-FLICE-inhibitory protein expression and sensitized cancer cells to TRAIL.[18] Finally, the overexpression or the activation of proapoptotic molecules are necessary to sensitize cancer cells to TRAIL. Selenite generates a rapid superoxide burst and activates the p53 pathway, leading to Bax upregulation and mitochondrial translocation to facilitate apoptosis in prostate cancer cells.[19] The combination of 5-Aza-2′-deoxycytidine and IFN-γ-sensitized neuroblastoma and medulloblastoma cells to TRAIL-induced apoptosis through the upregulation of caspase-8.[20]

This study explored the feasibility of regulating TRAIL sensitivity by targeting TRAIL decoy receptor expression using DcR2 siRNA instead of upregulating TRAIL death receptors in cancer cells. Our results indicate that the combinatorial use of a DcR2 siRNA strategy and Ad5hTRAIL infection dramatically affect the tumorigenic potential of prostate cancer cells.

Materials and methods

Gene therapy vectors and cell lines

The recombinant adenoviral vectors Ad5hTRAIL[21] and AdCMVLacZ[22,23] were amplified as described previously.[24,25] Briefly, virus purifications were performed by CsCl banding and the adenoviral vectors were stored at −80 °C in 10 mM Tris containing 20% glycerol. The particle titers of the adenoviral stocks were determined by A_{260} measurements. Viral dose applied is given as multiplicity of infection (MOI), which refers to the virus titer as DNA particles per cell. Plaque titering on 293 cells and expression assays for encoded proteins were used to assess functionality. The human prostate tumor cell lines, DU145 and LNCaP, were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, penicillin, streptomycin, sodium pyruvate, non-essential amino acids and HEPES (hereafter referred to as complete RPMI).

Quantitative RT-PCR

An SDS 7500, ABI Prism instrument was used for quantitative transcript detection. TRIzol reagent (Life Technologies, Gaithersburg, MD) was used to isolate total RNA from the prostate cancer cell lines. Two micrograms of the total RNA was reverse transcribed using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, Cat. N8080234). DR4, DR5, DcR1 and DcR2 primer and probe sequences were reported previously by our group.[13,15,26] Ribosomal RNA (rRNA) primers and probe were purchased from PE Applied Biosystems (Cat. 4308329), and served as an internal control in the same reaction. The TaqMan PCR was administered according to the manufacturer's instructions (Applied Biosystems Cat. N8080228), and the $\Delta\Delta C_t$ technique was employed to determine the relative quantities of TRAIL receptors for each sample. A 50 µl reaction mixture was prepared for the reverse transcription step in reverse transcription buffer with 2.25 mM $MgCl_2$, 50 µM of each dNTP, 2.5 µM Random Hexamer, 0.6 U µl^{-1} RNase inhibitor and 1.25 U µl^{-1} reverse transcriptase. The thermal cycling protocols were 10 min at 25 °C, followed by 60 min at 48 °C. TaqMan Universal Master Mix was used for the TaqMan PCR reaction with 50 pmol of primer and probe mixture including 250 ng of cDNA. PCR thermal cycling conditions were: 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C.

Flow cytometry

Anti-TRAIL receptor flow cytometry set (Alexis Biochemicals, Cat. ALX-850-273-KI01) was used to detect the TRAIL receptor protein expression on the cell surface. This kit contains mAb to DR4 (clone HS101, Cat. 804-297A), DR5 (clone HS201, Cat. 804-298A), DcR1 (clone HS301, Cat. 804-344A) and DcR2 (clone HS402, Cat. 804-299A) and 5 µg ml^{-1} of the primary antibodies were utilized for each reaction. Biotinylated goat anti-mouse IgG1 (Cat. ALX-211-202) was used as a secondary antibody prior to the addition of streptavidin-PE (Cat. ANC-253-050). Flow cytometric analyses were performed using Beckman Coulter EPICS ALTRA (HyPerSort) flow cytometry instrument located at the Human Gene Therapy Unit of Akdeniz University Hospitals and Clinics. As an isotype control, purified mouse IgG1 (MOPC 31C, Cat. ANC-278-010) was utilized. Surface TRAIL expression was revealed using a TRAIL-specific mAb (ALX-804-296-C100), followed by a PE-conjugated antibody to mouse IgG1 (ALX-211-201-CO50). In this study, a FITC-conjugated human Annexin V (ALX-209-250-T100) was used to detect cells undergoing apoptosis using flow cytometry. Annexin V staining procedure was performed according to the manufacturer's protocols (Alexis Biochemicals, Lausen, Switzerland).

Live/dead cellular viability/cytotoxicity assay

Live cells were discriminated from dead using live/dead cellular viability/cytotoxicity kit from Molecular Probes (Eugene, OR). Calcein AM and Ethidium homodimer-1 (EthD-1) are the interactive reagents in this assay. Intracellular calcein esterase activity is detected by a fluorogenic substrate, Calcein AM. Active esterase present in live cells with intact membranes modifies Calcein AM to a green fluorescent compound (calcein), serving as a marker for viable cells. EthD-1, a red fluorescent nucleic acid stain, cannot penetrate unharmed cell membranes, so only damaged cells uptake the dye and stain positive.

TRAIL-R4 gene silencing using siRNA

DcR2 siRNA (sc-35185), siRNA transfection medium (sc-36868) and siRNA transfection reagent (sc-29528) were used with DU145 and LNCaP prostate cancer cells as described by the manufacturer (Santa Cruz Biotechno-

logy). The DcR2 siRNA kit is a pool of three siRNA sequences designed for TRAIL-R4 mRNA silencing (Gene Bank accession no.: NM_003840), and are as follows. Strand A: GGAUGGUCAAGGUCAGUAA; Strand B: CCCUAUCACUACCUUAUCA; Strand C: GCUUGGGAAUGGUGUGAAA. A T7-mediated siRNA synthesis was carried out as described previously.[27] The prostate cancer cells were cultured at a density of 2×10^5 cells well^{-1} after being resuspended in fresh RPMI-1640 containing 10% fetal bovine serum without antibiotics in 24-well plates. Cells were grown overnight to 40–50% confluency, after which a mixture of the transfection solutions A and B (solution A: 1.8 μl 10 μM siRNA mixed with 30 μl siRNA transfection medium; solution B: 1.8 μl siRNA transfection reagent mixed with 7.25 μl siRNA transfection medium) was prepared and added for 20 min at room temperature. The culture medium was removed, and 300 μl fresh growth medium (10% serum, without antibiotics) was added to each well before the addition of the transfection reagent complex. Cells were then incubated for 30 h at 37 °C, and then infected with either Ad5hTRAIL or AdCMVLacZ. Control conditions were uninfected/untransfected, uninfected/transfected and infected/untransfected cells. Fluorescein-conjugated control siRNA-A (sc-36869) was utilized to monitor transfection efficiency using flourescent microscopy, and greater than 95% of the cells were transfected by this procedure. As a negative control, control siRNA-A (sc-37007) containing scrambled sequences was used.

In vitro *siRNA synthesis*

DNA oligonucleotides encoding top and bottom strand target sequences were purchased from Integrated DNA Technologies (Coralville, IA). The sequences of the three oligonucleotides used to synthesize siRNA have been reported,[27] and the 3′ ends of each primer included a T7 promoter sequence for *in vitro* transcription. AmpliScribeT7 high-yield transcription kit (Epicentre Technologies, Madison, WI) was used for the *in vitro* siRNA synthesis. Briefly, 1 μg target oligo and 1 μg of T7 promoter oligo were mixed by heating at 95 °C for 5 min and then allowed to cool to 70 °C gradually. The tube was moved to a 37 °C water bath for an additional 15 min to facilitate annealing and obtain dsDNA. *In vitro* siRNA synthesis was performed according to the manufacturer's protocol. Transcription mix contained 1 × T7 AmpliScribe buffer, 7.5 mM rNTP, 10 mM DTT, T7 RNA polymerase and 1 μg of dsDNA as a template. RNase free-DNase I 1 U was then added for 15 min. Top and bottom strand RNA generated in separate reactions were annealed to obtain small interfering double-stranded RNA using the following procedure. After mixing the two transcription reactions, the sample was heated at 95 °C for 5 min and then allowed to slowly cool to 70 °C. The reaction tube was incubated at 37 °C for additional 15 min. Finally, three volumes of ethanol and 100 μl nuclease-free water with 1 mM EDTA and 0.125 M LiCl were used to precipitate the siRNA.

Transfection of TRAIL-R4 cDNA into Cos-7 cells and RNA interference in Cos-7, DU145 and LNCaP cells

Monkey kidney fibroblast cells (Cos-7) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, penicillin/streptomycin mixture. The human prostate cancer cells were maintained as described above. Lipofectamine plus reagent (Invitrogen Life Technologies, Carlsbad, CA) was used to transfect cells according to the manufacturer's protocol. A plasmid vector containing human TRAIL-R4 cDNA alone (0.3 μg), or in combination with the *in vitro*-transcribed siRNAs (0.3 μg) for TRAIL-R4 were used to transfect 2×10^5 Cos-7 cells. DU-145 and LNCaP cells were transfected as described above with *in vitro*-transcribed siRNAs (labeled as siRNA 1, 2 and 3) for TRAIL-R4, individually or in combination, to measure the silencing of the endogenous TRAIL-R4 gene expression. RNA was isolated 24 h after transfection using Trizol reagent for the subsequent quantitative RT-PCR assay as described above.

Colony-forming assay on soft agar

Culture plates were first covered with a layer of 0.5% agar in medium supplemented with 20% FBS to prevent the attachment of the cells to the plastic substratum. Transfections using DcR2 siRNA reagents were performed as described above. After the infection with various viral vectors, prostate cancer cells were trypsinized, counted, mixed with agar (final 0.35%) and seeded in 60 mm dishes. Twelve days after seeding, cells were stained with crystal violet and the number of positive cells was counted. Experiments were performed in triplicate.

Statistical analysis

Statistical analysis was performed using Prism (GraphPad Software; San Diego, CA). Error bars in all figures represent ± s.e.m. Statistical tests used for the data analysis are specifically described in the figure legends.

Results

TRAIL expression following Ad5hTRAIL infection does not overcome TRAIL resistance in DU145 and LNCaP prostate carcinoma cell lines

We have recently described differential sensitivities of prostate carcinoma cell lines to TRAIL, and high DcR2 expression correlated with TRAIL resistance.[15] In addition, high levels of DcR2 expression were not restricted only to the prostate cancer cell lines, but a similar correlation was also observed in patients with prostate carcinoma.[28] Thus, the current study was initiated to directly investigate the connection between high DcR2 expression and TRAIL resistance. To first rule out the possibility that inadequate TRAIL expression from the Ad5hTRAIL vector was the reason for the lack of any Ad5hTRAIL-mediated cytotoxicity in DU145 and LNCaP, we examined surface TRAIL levels by flow cytometry after Ad5hTRAIL infection. TRAIL was readily detected after Ad5hTRAIL infection in more than

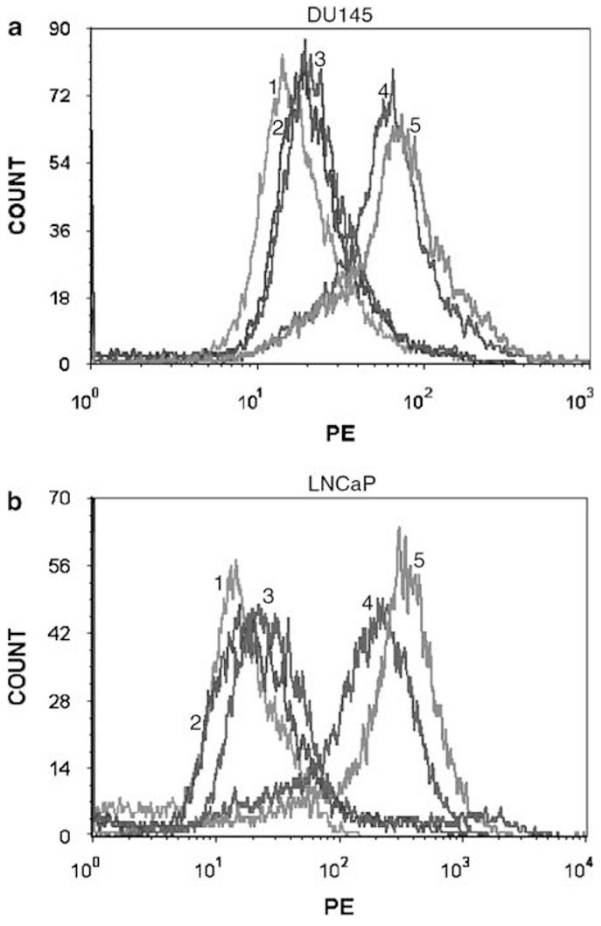


Figure 1 Adenovirus-mediated TNF-related apoptosis-inducing ligand expression in DU145 (**a**) and LNCaP (**b**). Cells (10^4) were gated for each histogram. Conditions for the infections were as follows: 1, uninfected isotype control; 2, uninfected but TRAIL antibody treated; 3, infected with AdCMVLacZ (10 000 multiplicity of infection (MOI)); 4, infected with Ad5hTRAIL (5000 MOI); 5, infected with Ad5hTRAIL (10 000 MOI). Results are representative of at least two independent experiments.

90% of the DU145 (Figure 1a) and LNCaP cell lines (Figure 1b), but not after infection with AdCMVLacZ. We can, therefore, be certain that TRAIL resistance in these cells is not due to poor TRAIL expression after Ad5hTRAIL infection, and is probably mediated by either antiapoptotic mechanisms at the cell surface or within the cell.

siRNA-mediated downregulation of DcR2 expression sensitizes DU145 and LNCaP to Ad5hTRAIL

We next examined the TRAIL receptor profile on DU145 cells by flow cytometry. Significant levels of both TRAIL death receptors, DR4 and DR5 and the decoy receptor, DcR2, were detectable on the surface, similar to that reported previously (Figure 2).[15] Because DU145 is TRAIL-resistant, siRNA-specific for DcR2 was used to determine the potential protective effect of DcR2 expression against TRAIL. Quantitative RT-PCR analysis confirmed an $80\pm6\%$ decrease in DcR2 mRNA expression following DcR2 siRNA treatment (data not shown). Consistent with that result, flow cytometry analysis revealed a $75\pm6\%$ decrease in the DcR2 protein expression on the surface (Figure 3a). No decrease in DcR2 expression was observed when siRNA-A was used in the transfection instead of the DcR2 siRNA. Importantly, DU145 cells expressing lowered DcR2 levels following DcR2 siRNA treatment demonstrated a $57\pm5\%$ decrease in viability after infection with the highest dose of Ad5hTRAIL, but not AdCMVLacZ (Figure 3b). In addition, surface TRAIL receptor expression profile in LNCaP cells was revealed by flow cytometry. Clearly, both the death receptors (DR4 and DR5) and the decoy receptors (DcR1, DcR2) were expressed on the surface as reported previously (Figure 4).[15] Similar results were observed with LNCaP, where there was an $87\pm7\%$ decrease in DcR2 mRNA expression, as demonstrated by quantitative RT-PCR, following the DcR2 siRNA approach (data not shown). Additionally, DcR2 siRNA, but not siRNA-A, transfection downregulated DcR2 surface expression by $83\pm5\%$, without affecting the expression pattern of other receptors (DR4, DR5 and DcR1; Figure 5a). There was also a $66\pm7\%$ decrease in the viability of LNCaP cells after Ad5hTRAIL infection at the highest dose, but not with AdCMVLacZ, following DcR2 siRNA treatment (Figure 5b). These results clearly show the protective effect of DcR2 on DU145 and LNCaP cells from the cytotoxic effects of TRAIL.

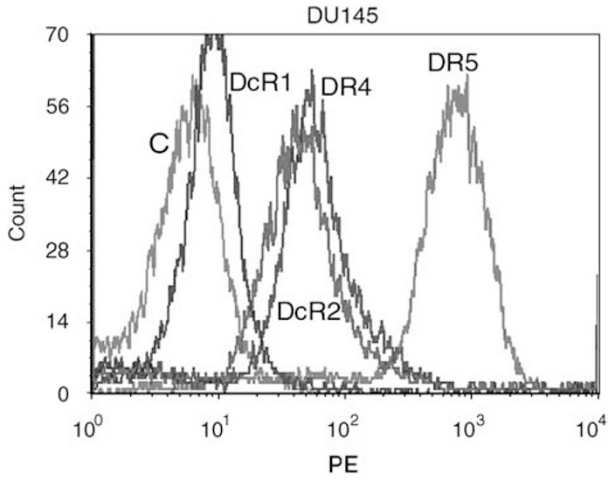


Figure 2 TNF-related apoptosis-inducing ligand receptor expression on DU145 prior to DcR2 siRNA treatment. The type of TRAIL receptors expressed is given above each peak. C = isotype control staining.

The efficacy of an individual siRNA duplex in inhibiting DcR2 expression in prostate cancer cell lines

The commercially available DcR2 siRNA kit used in Figures 3 and 5 included a combination of three different siRNA oligos. Thus, we were interested in testing the silencing effect of the *in vitro* synthesized DcR2 siRNA duplexes individually in the prostate cancer cell lines. Preliminary studies were performed on Cos-7 cells

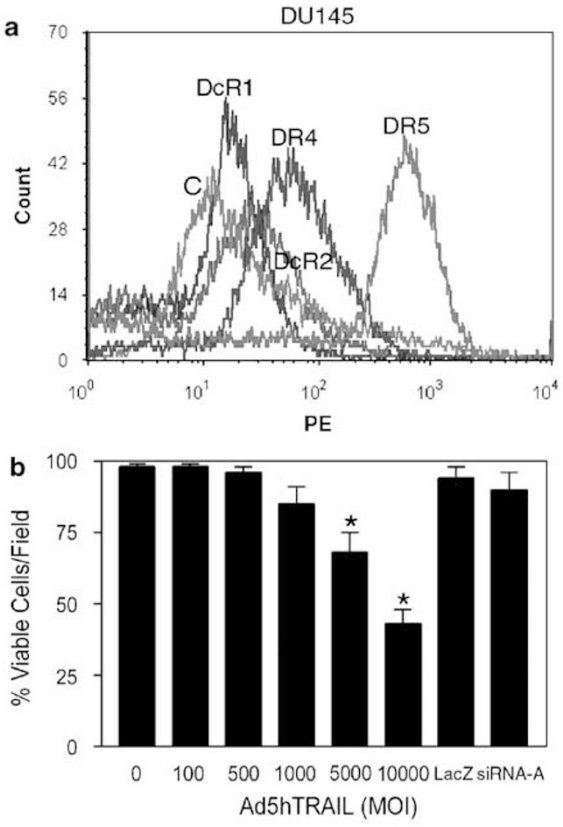


Figure 3 TNF-related apoptosis-inducing ligand receptor expression on DU145 after the DcR2 siRNA treatment (**a**). Cellular viability following siRNA transfections and Ad5hTRAIL or AdCMVLacZ infection (**b**). LacZ marked column refers to DcR2 siRNA-transfected and AdCMVLacZ-infected (10 000 multiplicity of infection (MOI)) samples in place of Ad5hTRAIL. siRNA-A labeled column depicts siRNA-A (consisting of scrambled sequence of oligonucleotides) transfected and Ad5hTRAIL (10 000 MOI) infected samples. All other columns display DcR2 siRNA-transfected Ad5hTRAIL-infected (at increasing MOIs) assay results. One-way analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test was employed to reveal the statistical significance among the groups. Results depict the mean ($\pm$s.e.m.) of six independent data points. *$P<0.01$.

transfected with a DcR2 expression vector to document the specificity of the silencing phenomenon. Quantitative RT-PCR analyses were conducted following the transfection with each of the individual siRNA duplex or with the combination of the three as described in Materials and methods. Each of the DcR2 siRNA duplexes efficiently downregulated DcR2 mRNA levels $>80\%$ when used individually (data not shown). Although 50–60% decrease in DcR2 mRNA levels was observed with the individual siRNA duplex in DU145 cells, the combination of the three resulted in 80% decrease in DcR2 mRNA (Figure 6a). No such decrease was observed with siRNA-A transfection. Moreover, DcR2 mRNA silencing was stronger (72–82% inhibition) in LNCaP cells compared to what was observed in DU145 cells. Although, the transfection combining the three siRNA duplexes was more effective ($85\pm2\%$ inhibition) on average than their

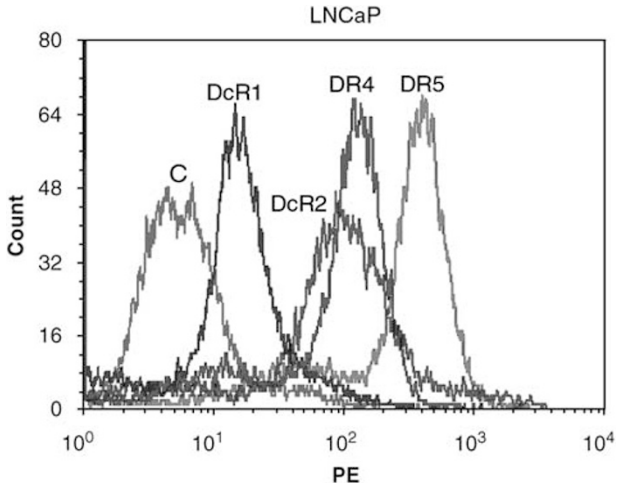


Figure 4 TNF-related apoptosis-inducing ligand receptor expression on LNCaP prior to DcR2 siRNA treatment. C = isotype control staining.

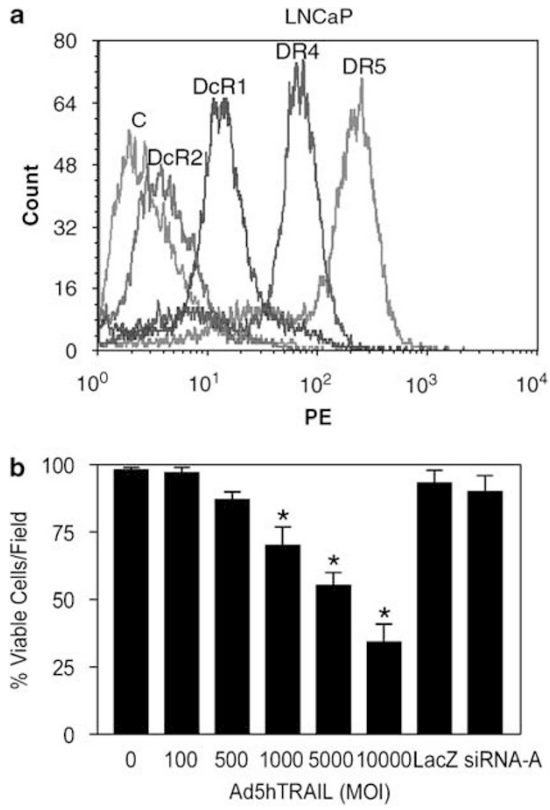


Figure 5 TNF-related apoptosis-inducing ligand receptor expression on LNCaP after DcR2 siRNA treatment (**a**). Cellular viability following siRNA transfections and Ad5hTRAIL or AdCMVLacZ infection (**b**). LNCaP cells were transfected with DcR2 siRNA prior to Ad5hTRAIL infection at increasing doses. LacZ column displays DcR2 transfections and AdCMVLacZ infection performed at 10 000 multiplicity of infection (MOI) instead of Ad5hTRAIL. siRNA-A labeled column includes siRNA-A transfection and Ad5hTRAIL infection performed at 10 000 MOI. ANOVA followed by Dunnett's Multiple Comparison Test was employed to reveal the statistical significance among the groups. Data represent the mean ($\pm$s.e.m.) of two independent assays ($n=6$). *$P<0.01$.

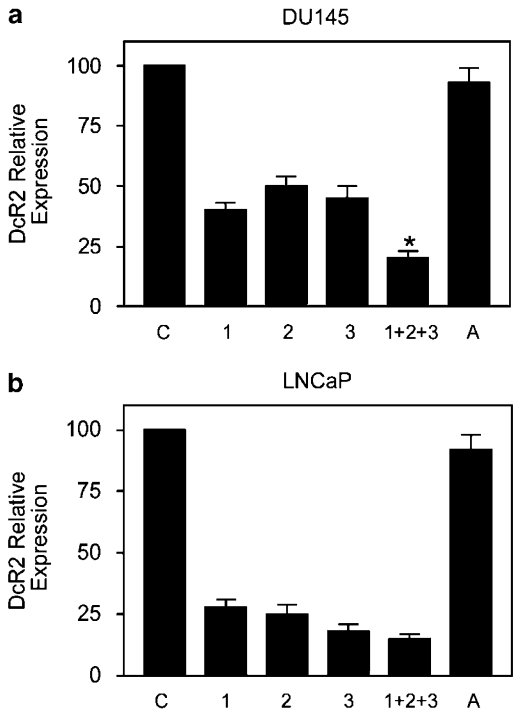


Figure 6 DcR2 expression is downregulated by *in vitro* synthesized siRNA constructs in DU145 (**a**) and LNCaP (**b**). *In vitro* synthesized siRNA duplexes were transfected at 0.3 μg of each. The combined transfection sample (1 + 2 + 3) included 0.9 μg of the siRNA duplex in total (0.3 μg of each). siRNA-A (0.9 μg) is used as a non-specific control for the transfection. DcR2 expression levels were detected by quantitative RT-PCR following the transfections. C = untransfected control samples. ANOVA followed by Bonferroni's Multiple Comparison Test was employed to distinguish the effect of individual siRNA constructs versus the combination of the three (the mixture). $^*P<0.05$.

individual administration in LNCaP cells, the difference was not statistically significant (Figure 6b).

DcR2 siRNA approach followed by Ad5hTRAIL infection results in apoptosis of prostate cancer cells

To prove that apoptosis is the mechanism of TRAIL-induced cytotoxicity under the setting of DcR2 downregulation in prostate cancer cells; Annexin V staining was performed before flow cytometry. Thus, DcR2 siRNA or siRNA-A transfections were performed prior to Ad5hTRAIL infection of DU145 cells. Apoptotic cell death was quantified by flow cytometry using an Annexin-V-FITC staining kit 40 h after transfection. Minimum cell staining with Annexin V was detected in siRNA-A-transfected and Ad5hTRAIL-infected cells (Figure 7). Conversely, DcR2 siRNA treatment followed by Ad5hTRAIL infection generated significant levels of apoptotic cell death manifested as high levels of Annexin V binding. As a result, only under the setting of DcR2 downregulation, Ad5hTRAIL infection produced apoptotic cell death in DU145 prostate cancer cells. No such effect was observed when Ad5hTRAIL infection or

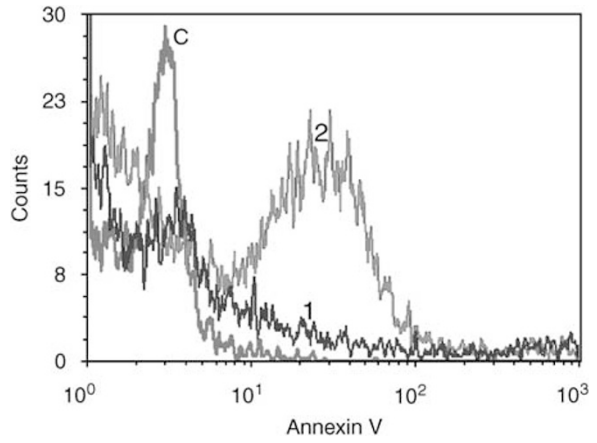


Figure 7 DcR2 siRNA treatment and Ad5hTRAIL infection results in apoptosis in DU145 prostate cancer cell line. FITC-conjugated Annexin V and Propidium Iodide (PI) stainings were employed using DU145 cell line. To accomplish this, prostate cancer cells were first transfected either with siRNA-A (depicted as 1 = negative control) or DcR2 siRNA (marked as 2) prior to infection with Ad5hTRAIL virus at an multiplicity of infection of 10 000 DNA particles per cell^{-1}. Flow cytometry analyses were performed 20 h after the infection. C = untransfected and uninfected samples. Cells (10^4) were gated for each histogram. Annexin V binding assay was repeated two times and a representative experiment is shown.

DcR2 siRNA or siRNA-A transfections were performed individually (data not shown).

In vitro *tumorigenic potential of prostate cancer cells is significantly reduced by DcR2 siRNA approach followed by Ad5hTRAIL infection*

Because of the correlation between the tumorigenic potential of cells *in vivo* and their ability to grow in an anchorage-independent manner *in vitro,* a colony-forming assay was performed on soft agar to determine whether Ad5hTRAIL and DcR2 siRNA alone or in combination would influence the tumorigenic potential of prostate cancer cell lines *in vitro*. While neither AdCMVLacZ infection nor DcR2 siRNA treatment influenced the colony-forming ability of prostate cancer cells on soft agar, Ad5hTRAIL infection alone only exhibited a mild effect. Consistent with the data presented above, the combination of DcR2 siRNA and Ad5hTRAIL infection dramatically reduced the number of colonies formed on soft agar both in DU145 (Figure 8a) and LNCaP (Figure 8b) cell lines. In addition, subtle differences in the efficacy of the combined treatments were noticed between DU145 and LNCaP cell lines, such that the efficacy of the combination treatment was greater in DU145 cells (68% reduction) compared to that of LNCaP (49% reduction).

Discussion

TRAIL induces apoptosis in cancer cells without having adverse effects on normal cells. However, many tumor

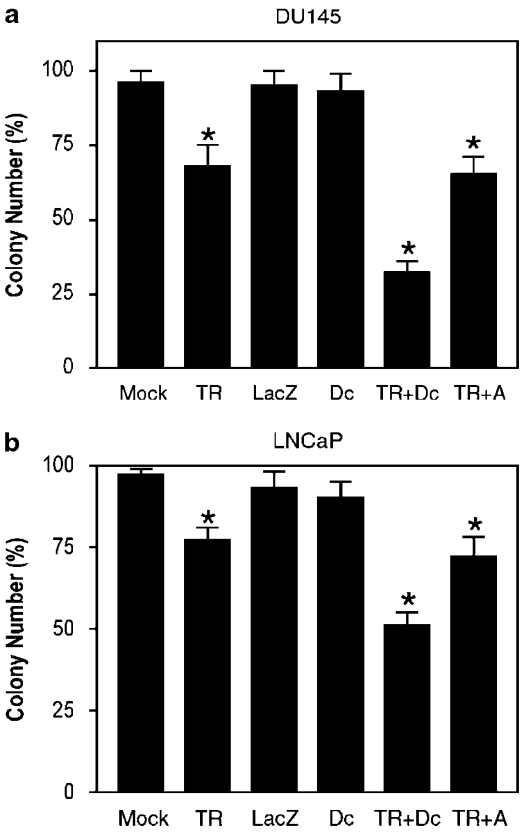


Figure 8 Ad5hTRAIL infection and DcR2 siRNA reduced *in vitro* tumorigenic potential of prostate cancer cell lines. Colony forming assays were performed as described in Materials and methods using DU145 (**a**) or LNCaP (**b**). Prostate cancer cell lines were transfected either with DcR2 siRNA (Dc) or siRNA-A (**a**). Infections were performed either with Ad5hTRAIL (TR) or AdCMVLacZ (LacZ) vectors. While TR-Dc column depicts DcR2 siRNA transfected and Ad5hTRAIL infected cells, TR-A column displays siRNA-A-transfected and Ad5hTRAIL-infected samples. Statistical significances were determined using ANOVA followed by Dunnett's Multiple Comparison Test. Statistical comparisons were made relative to mock-treated cells. Results are representative of at least three independent experiments. $^*P<0.01$.

types are also TRAIL-resistant, which may facilitate cancer progression.[29] The sensitivity to TRAIL-induced apoptosis can be modulated at different levels in apoptosis-inducing signaling cascade, starting at the receptor level. Upregulation of the TRAIL death receptors (DR4 or DR5) is a well-known strategy to sensitize cancer cells to TRAIL. For example, cardiac glycosides,[30] various histone deacetylase inhibitors,[31–34] and α-tocopheryl succinate (α-TOS) induce DR4 and DR5 expression and sensitize tumor cells derived from a variety of tissue types to TRAIL.[35] While the intracellular retention of DR4 and DR5 in TRAIL overexpressing cells can lead to TRAIL resistance in cancer cells,[36] we know little about the significance of decoy receptor expression contributing to the TRAIL resistance.[37] For example, while high levels of DR5 expression correlated with TRAIL sensitivity in advanced prostate cancer cell lines, TRAIL-resistant prostate cancer cells exhibited higher DcR2 expression compared to non-neoplastic cells.[38] This particular study demonstrated that TRAIL-induced apoptosis mainly relied on the receptor composition of prostate cancer cells, and the modulation of the surface TRAIL receptor expression might have some clinical value for advanced tumor treatment. Finally, TRAIL decoy receptors were claimed to mediate TRAIL resistance in acute myeloid leukemia cells.[39]

siRNA technology is a feasible option to selectively downregulate gene expression for various purposes, including the sensitization of cancer cells to TRAIL. For example, X-linked inhibitor of apoptosis (XIAP) regulates TRAIL sensitivity in pancreatic carcinoma cells, and the targeting of XIAP via RNA interference drastically reduced cell survival and enhanced TRAIL sensitivity in pancreatic cancer.[40] Similarly, siRNA-mediated downregulation of survivin sensitized human hepatoma cells to TRAIL-induced apoptosis.[41] Accordingly, a DcR2 siRNA approach downregulated receptor expression on surface and this resulted in the sensitization of breast cancer[26] and lung cancer[27] cells to adenoviral delivery of TRAIL. High levels of TRAIL decoy receptor expression (DcR2) were also recently correlated with TRAIL resistance in some of the prostate cancer cell lines.[15] Analysis of prostate sections suggested that not only patients with benign prostate hyperplasia but also organ-confined prostate carcinoma or advanced prostate carcinoma displayed increased DcR2 receptor expression.[28] Moreover, elevated DcR2 expression levels in prostate cancer patients were linked to high Gleason scores, PSA recurrence and decreased survival.[42] Despite these studies, the relevance of TRAIL decoy receptor expression on TRAIL resistance or tumoricidal potential has not been directly investigated for the prostate cancer cells. Thus, this study explored the possibility of sensitizing hormone refractory prostate cancer cell lines (DU145 and LNCaP) to TRAIL using siRNA to target the DcR2 receptor.

High levels of DcR2 expression correlated with TRAIL resistance in DU145 and LNCaP and DcR2-specific siRNA decreased the expression of the DcR2 receptor and sensitized both cell lines to Ad5hTRAIL. This study provides direct evidence for DcR2 expression conferring TRAIL resistance. Moreover, the combination of DcR2 siRNA and Ad5hTRAIL dramatically reduced the number of colonies formed on soft agar, indicating that such a combined approach would not only reduce the cell viability but also the tumorigenic potential of prostate cancer cells. Subtle differences in the efficacy of each treatment alone or in combination were also observed when measuring cell viability and colony-forming assays. These variations can be explained by the differences in the assay types and the duration of the experiments, as the colony formation assay requires longer incubation periods than needed to measure cellular viability. In conclusion, a DcR2 siRNA approach followed by an Ad5hTRAIL infection might be of some use to overcome potential TRAIL resistance in patients with prostate carcinoma.

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References

1 Griffith TS, Lynch DH. TRAIL: a molecule with multiple receptors and control mechanisms. *Curr Opin Immunol* 1998; **10**: 559–563.
2 Griffith TS, Rauch CT, Smolak PJ, Waugh JY, Boiani N, Lynch DH *et al*. Functional analysis of TRAIL receptors using monoclonal antibodies. *J Immunol* 1999; **162**: 2597–2605.
3 Griffith TS, Chin WA, Jackson GC, Lynch DH, Kubin MZ. Intracellular regulation of TRAIL-induced apoptosis in human melanoma cells. *J Immunol* 1998; **161**: 2833–2840.
4 Griffith TS, Fialkov JM, Scott DL, Azuhata T, Williams RD, Wall NR *et al*. Induction and regulation of tumor necrosis factor-related apoptosis-inducing ligand/Apo-2 ligand-mediated apoptosis in renal cell carcinoma. *Cancer Res* 2002; **62**: 3093–3099.
5 Held J, Schulze-Osthoff K. Potential and caveats of TRAIL in cancer therapy. *Drug Resist Updat* 2001; **4**: 243–252.
6 Sanlioglu AD, Koksal T, Baykara M, Luleci G, Karacay B, Sanlioglu S. Current progress in adenovirus mediated gene therapy for patients with prostate carcinoma. *Gene Ther Mol Biol* 2003; **7**: 113–133.
7 Kondo K, Yamasaki S, Sugie T, Teratani N, Kan T, Imamura M *et al*. Cisplatin-dependent upregulation of death receptors 4 and 5 augments induction of apoptosis by TNF-related apoptosis-inducing ligand against esophageal squamous cell carcinoma. *Int J Cancer* 2006; **118**: 230–242.
8 Perego P, Ciusani E, Gatti L, Carenini N, Corna E, Zunino F. Sensitization to gimatecan-induced apoptosis by tumor necrosis factor-related apoptosis inducing ligand in prostate carcinoma cells. *Biochem Pharmacol* 2006; **71**: 791–798.
9 Martin S, Phillips DC, Szekely-Szucs K, Elghazi L, Desmots F, Houghton JA. Cyclooxygenase-2 inhibition sensitizes human colon carcinoma cells to TRAIL-induced apoptosis through clustering of DR5 and concentrating death-inducing signaling complex components into ceramide-enriched caveolae. *Cancer Res* 2005; **65**: 11447–11458.
10 He Q, Huang Y, Sheikh MS. Proteasome inhibitor MG132 upregulates death receptor 5 and cooperates with Apo2L/TRAIL to induce apoptosis in Bax-proficient and -deficient cells. *Oncogene* 2004; **23**: 2554–2558.
11 Shankar S, Singh TR, Chen X, Thakkar H, Firnin J, Srivastava RK. The sequential treatment with ionizing radiation followed by TRAIL/Apo-2L reduces tumor growth and induces apoptosis of breast tumor xenografts in nude mice. *Int J Oncol* 2004; **24**: 1133–1140.
12 Shankar S, Singh TR, Srivastava RK. Ionizing radiation enhances the therapeutic potential of TRAIL in prostate cancer *in vitro* and *in vivo*: intracellular mechanisms. *Prostate* 2004; **61**: 35–49.
13 Karacay B, Sanlioglu S, Griffith TS, Sandler A, Bonthius DJ. Inhibition of the NF-kappaB pathway enhances TRAIL-mediated apoptosis in neuroblastoma cells. *Cancer Gene Ther* 2004; **11**: 681–690.
14 Wang X, Ju W, Renouard J, Aden J, Belinsky SA, Lin Y. 17-allylamino-17-demethoxygeldanamycin synergistically potentiates tumor necrosis factor-induced lung cancer cell death by blocking the nuclear factor-kappaB pathway. *Cancer Res* 2006; **66**: 1089–1095.
15 Sanlioglu AD, Koksal IT, Karacay B, Baykara M, Luleci G, Sanlioglu S. Adenovirus-mediated IKKbetaKA expression sensitizes prostate carcinoma cells to TRAIL-induced apoptosis. *Cancer Gene Ther* 2006; **13**: 21–31.
16 Deeb DD, Jiang H, Gao X, Divine G, Dulchavsky SA, Gautam SC. Chemosensitization of hormone-refractory prostate cancer cells by curcumin to TRAIL-induced apoptosis. *J Exp Ther Oncol* 2005; **5**: 81–91.
17 Schultze K, Bock B, Eckert A, Oevermann L, Ramacher D, Wiestler O *et al*. Troglitazone sensitizes tumor cells to TRAIL-induced apoptosis via down-regulation of FLIP and Survivin. *Apoptosis* 2006; **11**: 1503–1512.
18 Li X, Raikwar SP, Liu YH, Lee SJ, Zhang YP, Zhang S *et al*. Combination therapy of androgen-independent prostate cancer using a prostate restricted replicative adenovirus and a replication-defective adenovirus encoding human endostatin-angiostatin fusion gene. *Mol Cancer Ther* 2006; **5**: 676–684.
19 Hu H, Jiang C, Schuster T, Li GX, Daniel PT, Lu J. Inorganic selenium sensitizes prostate cancer cells to TRAIL-induced apoptosis through superoxide/p53/Bax-mediated activation of mitochondrial pathway. *Mol Cancer Ther* 2006; **5**: 1873–1882.
20 Fulda S, Debatin KM. 5-Aza-2′-deoxycytidine and IFN-gamma cooperate to sensitize for TRAIL-induced apoptosis by upregulating caspase-8. *Oncogene* 2006; **25**: 5125–5133.
21 Griffith TS, Anderson RD, Davidson BL, Williams RD, Ratliff TL. Adenoviral-mediated transfer of the TNF-related apoptosis-inducing ligand/Apo-2 ligand gene induces tumor cell apoptosis. *J Immunol* 2000; **165**: 2886–2894.
22 Sanlioglu S, Engelhardt JF. Cellular redox state alters recombinant adeno-associated virus transduction through tyrosine phosphatase pathways. *Gene Therapy* 1999; **6**: 1427–1437.
23 Sanlioglu AD, Karacay B, Benson PK, Engelhardt JF, Sanlioglu S. Novel approaches to augment adeno-associated virus type-2 endocytosis and transduction. *Virus Res* 2004; **104**: 51–59.
24 Engelhardt JF, Yang Y, Stratford-Perricaudet LD, Allen ED, Kozarsky K, Perricaudet M *et al*. Direct gene transfer of human CFTR into human bronchial epithelia of xenografts with E1-deleted adenoviruses. *Nat Genet* 1993; **4**: 27–34.
25 Doerschug K, Sanlioglu S, Flaherty DM, Wilson RL, Yarovinsky T, Monick MM *et al*. First-generation adenovirus vectors shorten survival time in a murine model of sepsis. *J Immunol* 2002; **169**: 6539–6545.
26 Sanlioglu AD, Dirice E, Aydin C, Erin N, Koksoy S, Sanlioglu S. Surface TRAIL decoy receptor-4 expression is correlated with TRAIL resistance in MCF7 breast cancer cells. *BMC Cancer* 2005; **5**: 54.
27 Aydin C, Sanlioglu AD, Karacay B, Ozbilim G, Dertsiz L, Ozbudak O *et al*. Decoy receptor-2 small interfering RNA (siRNA) strategy employing three different siRNA constructs in combination defeats adenovirus-transferred tumor necrosis factor-related apoptosis-inducing ligand resistance in lung cancer cells. *Hum Gene Ther* 2007; **18**: 39–50.
28 Sanlioglu AD, Koksal IT, Ciftcioglu A, Baykara M, Luleci G, Sanlioglu S. Differential expression of TRAIL and its receptors in benign and malignant prostate tissues. *J Urol* 2007; **177**: 359–364.

29 Malhi H, Gores GJ. TRAIL resistance results in cancer progression: a TRAIL to perdition? *Oncogene* 2006; **25**: 7333–7335.
30 Frese S, Frese-Schaper M, Andres AC, Miescher D, Zumkehr B, Schmid RA. Cardiac glycosides initiate Apo2L/TRAIL-induced apoptosis in non-small cell lung cancer cells by up-regulation of death receptors 4 and 5. *Cancer Res* 2006; **66**: 5867–5874.
31 Butler LM, Liapis V, Bouralexis S, Welldon K, Hay S, Thai le M *et al*. The histone deacetylase inhibitor, suberoylanilide hydroxamic acid, overcomes resistance of human breast cancer cells to Apo2L/TRAIL. *Int J Cancer* 2006; **119**: 944–954.
32 VanOosten RL, Moore JM, Karacay B, Griffith TS. Histone deacetylase inhibitors modulate renal cell carcinoma sensitivity to TRAIL/Apo-2L-induced apoptosis by enhancing TRAIL-R2 expression. *Cancer Biol Ther* 2005; **4**: 1104–1112.
33 Earel Jr JK, VanOosten RL, Griffith TS. Histone deacetylase inhibitors modulate the sensitivity of tumor necrosis factor-related apoptosis-inducing ligand-resistant bladder tumor cells. *Cancer Res* 2006; **66**: 499–507.
34 VanOosten RL, Earel Jr JK, Griffith TS. Enhancement of Ad5-TRAIL cytotoxicity against renal cell carcinoma with histone deacetylase inhibitors. *Cancer Gene Ther* 2006; **13**: 628–632.
35 Tomasetti M, Andera L, Alleva R, Borghi B, Neuzil J, Procopio A. Alpha-tocopheryl succinate induces DR4 and DR5 expression by a p53-dependent route: implication for sensitisation of resistant cancer cells to TRAIL apoptosis. *FEBS Lett* 2006; **580**: 1925–1931.
36 Wenger T, Mattern J, Penzel R, Gassler N, Haas TL, Sprick MR *et al*. Specific resistance upon lentiviral TRAIL transfer by intracellular retention of TRAIL receptors. *Cell Death Differ* 2006; **13**: 1740–1751.
37 Terzioglu E, Bisgin A, Sanlioglu AD, Ulker M, Yazisiz V, Tuzuner S *et al*. Concurrent gene therapy strategies effectively destroy synoviocytes of patients with rheumatoid arthritis. *Rheumatology (Oxford)* 2007; **46**: 783–789.
38 Hesry V, Piquet-Pellorce C, Travert M, Donaghy L, Jegou B, Patard JJ *et al*. Sensitivity of prostate cells to TRAIL-induced apoptosis increases with tumor progression: DR5 and caspase 8 are key players. *Prostate* 2006; **66**: 987–995.
39 Riccioni R, Pasquini L, Mariani G, Saulle E, Rossini A, Diverio D *et al*. TRAIL decoy receptors mediate resistance of acute myeloid leukemia cells to TRAIL. *Haematologica* 2005; **90**: 612–624.
40 Vogler M, Durr K, Jovanovic M, Debatin KM, Fulda S. Regulation of TRAIL-induced apoptosis by XIAP in pancreatic carcinoma cells. *Oncogene* 2007; **26**: 248–257.
41 Nakao K, Hamasaki K, Ichikawa T, Arima K, Eguchi K, Ishii N. Survivin downregulation by siRNA sensitizes human hepatoma cells to TRAIL-induced apoptosis. *Oncol Rep* 2006; **16**: 389–392.
42 Koksal IT, Sanlioglu AD, Karacay B, Griffith TS, Sanlioglu S. TRAIL-R4 decoy receptor gene expression is correlated with high Gleason scores, PSA recurrence and decreased survival in patients with prostate carcinoma. *Urol Oncol* (in press).