Additional Supporting Information (Materials and Methods)

The significance of TNFAIP8 in prostate cancer response to radiation and docetaxel and disease recurrence

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Antibodies, reagents and chemicals

A rabbit polyclonal antibody was custom generated against a human TNFAIP8-specific peptide, KLCDGINKMLDEENI (amino acids 174-188) as we described earlier.[6,7] The following antibodies were obtained commercially: polyclonal anti-MMP-1 (Chemicon International, Inc, Temecula, CA), polyclonal anti-MMP-2 (Chemicon International, Inc), polyclonal anti-MMP-9 (Chemicon International, Inc), polyclonal anti-MT1-MMP (MMP-14) (Chemicon International, Inc), monoclonal anti-Flk-1/VEGFR2 (Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal anti-VEGF (Santa Cruz), monoclonal anti-Karyopherin α/Rch-1 (BD Transduction Laboratories, Sparks, MD), monoclonal anti-Importin α1 (Sigma-Aldrich Co., St. Louis, MO), monoclonal anti-PARP (Santa Cruz), anti-LaminB1 (Sigma), monoclonal anti-α-Tubulin (Sigma), polyclonal anti-GAPDH (Trevigen, Gaithersburg, MD), polyclonal anti-Actin (Sigma), horseradish peroxidase-conjugated secondary antibodies anti-mouse IgG and anti-rabbit IgG (GE Healthcare, Waukesha, WI). Docetaxel (TAXOTERE) (Aventis Pharmaceuticals, Bridgewater, NJ) was purchased from the pharmacy at the Georgetown University hospital.

Androgen treatment, Northern blotting and immunoblotting

Logarithmically growing LNCaP cells were cultured in IMEM with 5% FBS for three days and medium was then switched to IMEM containing 5% charcoal stripped fetal calf serum for overnight. Stock solution (5 mM) of synthetic androgen R1881 (NEN, Boston, MA) was prepared in DMSO (Sigma). The cells were stimulated with indicated concentrations of R1881 for various times. Alternatively, LNCaP cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS (Invitrogen), followed by serum-free RPMI 1640 medium for overnight in a humidified atmosphere of 5% CO_2 and 95% air at 37ºC. The cells were stimulated with R1881 at indicated concentrations prior to harvesting for RNA and protein analyses as detailed below.

Northern blotting and Western blotting

TNFAIP8 mRNA expression was detected by Northern blot analysis using radiolabeled *TNFAIP8* cDNA as probe. In brief, androgen-treated LNCaP cells were trypsinized and total RNA was isolated with RNA-Bee reagent according to the manufacture's specifications (Tel-test, Inc, Friendswood, TX). Total RNA was electrophoresed on 1% agarose-formaldehyde gel (20 μg/lane), transferred overnight to nylon membrane (Amersharm Pharmacia Biotech, Piscataway, NJ), and fixed by UV cross-linking. Total RNA blot (20μg/lane) was sequentially hybridized to [^{32}P]-UTP labeled *TNFAIP8* cDNA coding for the open reading frame, followed by *GAPDH* cDNA probe as described earlier.[3] Autoradiographs were computer-scanned and *TNFAIP8* mRNA expression was normalized to the *GAPDH* signal in the corresponding lane.

For immunoblotting, androgen-treated LNCaP cells were washed twice with ice-cold phosphate buffered saline (PBS) and subsequently scrapped and lysed on a shaker for 30 min at 4^{o}C in lysis buffer (100 mM HEPES, pH 7.5, 1% Nonidet P-40, 150 mM sodium chloride, 10% glycerol) supplemented with protease inhibitor cocktail tablets (1 tablet per 50 ml of lysis buffer) (Roche Diagnostics, Indianapolis, IN). The whole cell lysates were collected by microcentrifugation for 20 min at 4ºC. Protein concentrations were determined using Coomassie Plus – The Better Bradford Assay Reagent according to the manufacturer's specifications (Pierce). The proteins were separated by electrophoresis on a NuPAGE 4-12% Bis-Tris gel (Invitrogen) (approximately 30 μg per lane) and transferred to PVDF membrane (Immobilon-P, Millipore, Bedford, MA). The membrane was probed with anti-TNFAIP8 antibody (1:1000 dilution), followed by detection of TNFAIP8 by enhanced chemiluminescence (Amersham Pharmacia Biotech). The blot was stripped with Re-Blot plus mild antibody stripping solution (Chemicon International, Inc.) and reprobed with anti-α-Tubulin antibody.

Formulation of liposome-entrapped TNFAIP8 antisense oligonucleotide

We have previously demonstrated that terminal base-modified oligodeoxyribonucleotides (oligos) are nuclease-sensitive and rapidly degraded *in vivo*, and cationic liposome-entrapped oligos exhibit significantly improved pharmacokinetics and bio-distribution in athymic mice.[15] Therefore, cationic liposome-entrapped terminal base-modified TNFAIP8 antisense oligo (LE-AS5) and control mismatch oligo (LE-MM) (particle size approximately 450 nm, entrapment

efficiency > 85%) were used in present *in vivo* studies. A proprietary formulation of the lyophilized lipids (dimethyldioctadecyl ammonium bromide, egg phosphatidylcholine, and cholesterol) was provided by NeoPharm, Inc. (Lake Forest, IL). The lyophilized lipids were reconstituted at room temperature with 2 mg/ml of oligo solution using oligo to lipid ratio of 1:15 (w/w) as reported earlier.[16] The mixture was vortexed vigorously for 2 min, followed by hydration at room temperature for 2 hr and sonication for 10 min. Control blank liposomes (BL) were prepared in the absence of the oligo.

Liposome-entrapped TNFAIP8 antisense oligonucleotide and radiation treatments *in vivo*

PC-3 tumor cells were injected subcutaneously in the left flank regions of 6- to 8- week old male athymic nude mice (5.0×10^6 cells/150 μl). Tumor-bearing animals (tumor size, approximately 40 mm^3) were randomized into six treatment groups (n = 5 - 8). The first day of treatment was designated as day 0. LE-AS5 and LE-AS5 + ionizing radiation (IR) groups received 10 intravenous (i.v., via tail vein) injections of 25.0 mg/kg/day of LE-AS5 over 14 days (day 0, 1, 3, 4, 6, 7, 9, 10, 12, 13). Radiation (3.8 Gy/day) was delivered to the tumors of mice in the IR alone and LE-AS5 + IR treatment groups once daily from day 5 through day 8 using a [^{137}Cs] irradiator (J.L. Shepard Mark I; 2.37 Gy/minute). In the combination group, the LE-AS5 and IR treatments were given six to eight hours apart. Control animals received i.v. dose of BL or LE-MM on the same dosing schedule as LE-AS5 or were left untreated (UT). Tumor growth was monitored twice a week. Tumor volumes were determined from caliper measurements of the three major axes (a,b,c) and calculated using abc/2, an approximation for the volume of an ellipsoid (π abc/6). Individual tumor volume was calculated as the percentage of initial (pre-treatment) tumor volume on day 0 (the first day of dosing, 100%). The mean tumor volume (percentage of initial) ± S.E. for each group was plotted. Two mice from each treatment group were sacrificed within 6-12 hr after the last treatment and tumor tissues were excised for Western blot analysis. PC-3 tumor tissues were homogenized in the lysis buffer. The lysates were clarified by centrifugation and protein concentrations were measured. The lysates were resolved by 15% SDS-PAGE, electroblotted onto the PVDF membrane. The membrane was sequentially probed with anti-TNFAIP8 antibody and anti-GAPDH polyclonal antibody. The antigen-antibody complex was detected by the ECL-PLUS reagent. The signals were scanned using a Personal Densitometer (Amersham Pharmacia Biotech), and quantified (Image-Quant software).

The TNFAIP8 protein expression was normalized against the GAPDH signal in the same lane. The study was terminated on day 29. The statistical significance of differential findings between experimental and control groups was determined by Student's t-test and considered statistically significant if two-tailed P values were <0.05.

Liposome-entrapped TNFAIP8 antisense oligonucleotide and docetaxel treatments *in vivo*

PC-3 tumor cells were injected subcutaneously in the left flank regions of 6- to 8- week old male athymic nude mice (5.0×10^6 cells/150 μl). Tumor-bearing animals (tumor size, approximately 65 mm^3) were randomized into six treatment groups (n = 6 - 8). Initial diluted solution of 10 mg/ml docetaxel was prepared according to the supplier's instructions (Aventis Pharmaceuticals Inc. Bridgewater, NJ). Subsequently, 0.4 mg/ml stock solution of docetaxel was prepared in normal saline. The first day of treatment was designated as day 0. LE-AS5 and LE-AS5 + docetaxel groups received 10 i.v. injections of 25.0 mg/kg/day of LE-AS5 over 14 days (day 0, 1, 3, 4, 6, 7, 9, 10, 12, 13). Docetaxel was given intravenously on day 2 and day 8 at 10 mg/kg/day to docetaxel alone and LE-AS5 + docetaxel groups. In the combination group, the LE-AS5 treatment was routinely scheduled at least 4 - 6 hr prior to the drug treatment. Control animals received i.v. dose of BL or LE-MM on the same dosing schedule as LE-AS5 or were left untreated (UT). Two mice from each treatment group were sacrificed within 6-12 hr after the last treatment and tumor tissues were excised for expression analysis as detailed above. Tumor growth was monitored twice a week. Individual tumor volume was calculated as the percentage of initial (pre-treatment) tumor volume on day 0 (the first day of dosing, 100%). The mean tumor volume (percentage of initial) ± S.E. for each group was plotted. The study was terminated on day 29. The statistical significance of differential findings between experimental and control groups was determined by Student's t-test and considered statistically significant if two-tailed P values were <0.05.

TNFAIP8 siRNA treatment, immunoprecipitation and immunoblotting

Logarithmically growing PC-3 cells were seeded into 100 mm dishes (1.5×10^6 cells/dish) in complete IMEM medium containing 10% FBS. Next day, medium was switched to 5 ml Opti-MEMI medium (GIBCO Invitrogen) containing indicated concentration of stealth TNFAIP8 siRNA (Invitrogen) and Lipofectamine 2000 complex prepared according to supplier's

instructions (Invitrogen). Three sets of TNFAIP8 siRNA duplex oligoribonucleotide sequences were used as follows. Si1 (5'-3'), UUA AGG UGG UGG CGA UGG AUU UGG A and UCC AAA UCC AUC GCC ACC ACC UUA A; Si2 (5'-3'), UAA AUU UCU CCA UCA AUG CUA GCU C and GAG CUA GCA UUG AUG GAG AAA UUU A; and Si3 (5'-3'), UUU AAC AGC CUG GAU AAC ACA UUC C and GGA AUG UGU UAU CCA GGC UGU UAA A. Control cells were treated with the same concentration of a Stealth RNAi Negative control duplex denoted as ScrambledSi/ScrSi (Invitrogen). At 4-6 hr post-transfection, 2.5 ml of DMEM containing 30% FBS was added to the medium. After 72 hr, the medium was removed and cells were rinsed with 1X PBS. The immunoblotting and immunoprecipitation assays were performed as detailed below.

TNFAIP8 immunoprecipitation and immunoblotting

For Western blotting, cells were lysed in ice cold Triton X lysis buffer (1% Triton X-100 (Biorad Laboratories, Inc., Hercules, CA), 0.1%SDS, 0.5% sodium deoxycholate, 10 mM NaCl) containing 1 tablet of protease inhibitor (Roche Diagnostics) per 25 ml of lysis buffer. Alternatively, cells were lysed in NP-40 lysis buffer as above. The whole cell lysate was centrifuged at 14,000 rpm in Beckman microcentrifuge for 20 min at 4^{o}C. Protein concentration in the supernatant was determined by the Pierce Protein BCA assay (Pierce). Subsequently, the sample was boiled in 20 µl of SDS sample buffer (125 mM Tris HCL, pH 6.8, 4% SDS, 20% glycerol, 0.0045% bromophenol blue) (Sigma). Thirty to fifty micrograms of protein was resolved by 4-12% NuPAGE, followed by electroblotting onto a PVDF membrane (Immobilon-P). The PVDF membrane was blocked in 1X TBS (Biorad) containing 0.1% Tween 20 (Biorad) and 5% non-fat milk (Biorad) for 1 hr. The membrane was probed with anti-TNFAIP8 antibody for overnight (1: 1000 dilution in 1X TBS-T), followed by goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (1:20,000 dilution in 1X TBS-T) for 2 hr at room temperature. Immunoreactive protein bands were detected with enhanced chemilumincescence reagent (ECL-PLUS) according to the manufacturer's instructions (Amersham Pharmacia Biotech). Blot was stripped with mild reblot buffer for 15 min and then reprobed with anti-GAPDH antibody (1: 10000 dilution).

For immunoprecipitation, cells were washed twice with ice-cold phosphate buffered saline (PBS) and subsequently scraped in RIPA Buffer (Sigma) supplemented with phosphatase

inhibitor cocktail I (Sigma), phosphatase inhibitor cocktail II (Sigma), and protease inhibitor tablet (Roche Diagnostics). The cell lysate was microcentrifuged and the supernatant was preclarified with protein A agarose beads (Roche Diagnostics). The precleared lysate (2 mg protein) was incubated with 100 µl of agarose-conjugated anti-TNFAIP8 antibody at 4°C overnight. The TNFAIP8 immune-complex was collected by centrifugation and beads were washed twice with RIPA lysis buffer, once with 0.5 M LiCl and once with100 mM Tris-HCl, pH 7.4, followed by 4-12% NuPAGE and immunoblotting with anti-TNFAIP8 antibody as above.

Subcellular fractionation

Logarithmically growing prostate cancer cells (LNCaP, PC-3 and DU-145) were collected by trypsinization and washed twice with ice-cold PBS. Buffer A (25 mM Tris-HCl, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 2 mM dithiothreitol, complete protease inhibitors (Roche)) was added (4 ml buffer A/g wet pellet) and the cell suspension was incubated for 10 min on ice. An equal volume of buffer B (buffer A containing 0.5 M sucrose) was added to the cell suspension, and the cells were homogenized by 40 strokes in a dounce homogenizer equipped with a tight-fitting pestle B. The homogenate was microcentrifuged at 600 x *g* at 4°C for 5 min. The pellet represented the initial nuclear fraction. The supernatant was microcentrifuged at 10,000 x *g* at 4°C for 30 min. The next supernatant was then centrifuged at 120,000 x *g* at 4°C for 1 hr. The resulting supernatant was used as the cytosolic fraction. The initial nuclear fraction was washed twice in buffer A, and extracted by mild agitation in 100 µl of buffer E (25 mM Tris-HCl, pH 7.5, 1.5 mM $MgCl_2$, 750 mM KCl, 2 mM dithiothreitol, complete protease inhibitors (Roche) at 4 °C for 1 hr, and microcentrifuged at 16,000 x *g* at 4°C for 1 hr. The supernant represented the nuclear fraction.

Antibody microarray and bioinformatics for analysis of TNFAIP8 interacting proteins

PC-3 cells were treated with TNFAIP8 siRNA (Si2siRNA/siRNA) or a scrambled siRNA (ScrsiRNA/MM). The knockdown of TNFAIP8 in siRNA treated cells versus ScrsiRNA treated cells was verified by immunoprecipitation and immunoblotting with anti-TNFAIP8 antibody. The TNFAIP8 immune-complexes were analyzed for differences in levels of proteins co-immunoprecipitating with TNFAIP8 in siRNA versus ScrsiRNA- treated samples using antibody microarray (Antibody Microarray 507, Clontech) according to the manufacturer's instructions as

we have detailed earlier.[20,21] Samples were labeled with Cy3, and applied on the microarray together with a mixture of cell proteins labeled with Cy5 as a "gold-standard." Each sample was incubated with a 507 feature antibody microarray, washed, and the bound proteins were assayed on a sensitive fluorescence reader. The fluorescence at each site on the antibody microarray was measured on a GenePix array reader (New Milton, New Hampshire, U.K.), and downloaded to an Excel spread sheet. Since each of the 507 microarray sites contains duplicate antibody spots, data from 4 independent technical replicates of each sample were available for averaging and were used to perform statistical tests to evaluate the statistical significance of the results. Both sample and "gold-standard" intensities were normalized to the respective median for each array. Protein intensities were corrected for spot variations by using the Cy5-labeled "gold-standard" intensity on the same spot. Since the exact same "gold-standard" was used for all samples the ratio of Cy3 to Cy5 intensities gave the ratio of sample to "gold-standard" for each individual spot. These corrected values were used to calculate the ratios as well as the P-values for the significance of the difference between the ScrsiRNA and TNFAIP8 siRNA (Si2 siRNA) levels for each protein on the array. Fold change represents the ratio of largest of MM.Si, divided by smallest. The Ingenuity Pathway Analysis of the antibody microarray data was used to identify significant functional categories, canonical pathways, and connectivity maps affected by TNFAIP8 or its knockdown.