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Electronic Supplementary Material

TGF-β1 mediates the radiation response of prostate cancer

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Supplementary Methods

Animal tumor model and Radiation

In the ectopic tumor implantation model, TRAMP-C1 and TRAMP-HR transfectants (1×10^6 cells per implantation, five animals per group) were subcutaneously implanted into the dorsal gluteal region. In the orthotopic tumor implantation model, TRAMP-C1 and TRAMP-HR transfectants (1×10^6 cells per implantation, five animals per group) were intra-operatively implanted into the lateral region of the prostate gland. The extent of orthotopic tumor invasion was measured 2 weeks after implantation or the indicated time points. The effect of TGF-β1 on Tregs accumulation was also investigated *in vivo*. For the treated group, intraperitoneal injection of TGF-β1 (10 μg/kg, two times per week) or neutralizing antibody against TGF-β1 (10 mg/kg, three times per week) was started 1 day before tumor implantation and continued for 2 weeks. To investigate the effect of Tregs on tumor regrowth following irradiation, neutralizing antibody against CD25 (100 mg/kg, 2 times per week) was intraperitoneally injected from 1 day before tumor implantation or local irradiation. When indicated, tumor-bearing mice were imaged at the Molecular Imaging Center of our Hospital. To determine the radiosensitivity *in vivo*, local irradiation (10 Gy) was performed when the ectopic tumor volume reached 0.5 cm^3, and tumor size was measured every 3 days thereafter. For local irradiation, mice were anesthetized and restrained to irradiate a field including the ectopic tumor with at least 5-mm

margins and a 1.5 cm bolus on the surface using a linear accelerator (Varian Medical System). Control mice were subjected to sham irradiation. Relative tumor volume normalized to tumor size at the time of irradiation determined the curves of tumors in mice exposed to radiation.

PET imaging

Tumor bearing mice were imaged at the Molecular Imaging Center of our Hospital. Serial PET scans were performed for experimental mice at indicated times after tumor implantation. Animals fasted overnight under the standard condition of light. Warming was started 30 minutes before FDG-radiotracer injection and continued throughout the uptake and imaging period under a tungsten lamp. The scanning room temperature was controlled at 24°C at all time. Under temporary isoflurane anesthesia, FDG-radiotracer was administered intravenously, and a dynamic frame acquisition was initiated simultaneously. The total acquisition time was 120 minutes.

Immunohistochemical staining and immunofluorescence for tissue specimens

Formalin-fixed, paraffin-embedded tissues were cut into 5-μm sections, mounted on slides, de-paraffinized with xylene, and dehydrated using a graded ethanol series. Antibodies specific for TGF-β1, p-H2AX, ki-67, vascular endothelial growth factor (VEGF), and active caspase 3 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), Research & Diagnostics Systems, Inc. (Minneapolis, MN USA), Cell Signaling (Danvers, MA), and Chemicon (Temecula, CA). Sections were incubated overnight at 4°C with antibodies against target proteins. After three PBS washes, the sections were incubated for 10 min with a biotinylated secondary antibody, stained with peroxidase-avidin, and washed with PBS. AEC (3-amino-9-ethylcarbazole) solution was added and the sections were counterstained with hematoxylin. Ki-67 expression is usually estimated as the percentage of tumor cells positively stained by the antibody, with nuclear staining being the criterion of positivity. Thus, Ki-67 reactivity, defined as percent tumor cells staining positive as measured by immunohistochemical (IHC) staining, is a specific nuclear marker for cell proliferation. IHC data were analyzed using Image Pro Plus 6.3 (IPP). Frozen tissue specimens were cut into 5–8-μm cryostat

sections, warmed to room temperature, fixed for 10 min in cold acetone (-20°C), and incubated for 20 min with PBS containing 10% goat serum. They were then incubated overnight at 4°C with antibodies against TGF-β1, CD4, E-cadherin, CD31, and Foxp3+, washed three times with PBS, and incubated for 1 h with fluorescein- or Texas red-conjugated secondary antibodies. The quantification for E-cadherin expression is to calculate the value of the cell number positive for E-cadherin immunofluorescence divided by the total cell number for each condition. The quantification for CD31 expression is to calculate the area of positive for CD31 immunofluorescence divided by the total DAPI positive area for each condition.

Enzyme-linked immunosorbent assay (ELISA) for TGF-β1 levels in vitro and in vivo

Levels of TGF-β1 in cellular supernatants and murine serum samples were analyzed by ELISA using the Human or Mouse TGF-β1 Quantikine ELISA Kit (R&D Systems). To measure TGF-β1 levels in cellular supertnants, cells were cultured with 1 ml of serum-free medium for 24 h in six-well plates. The medium was collected and clarified by centrifugation at 3000 g. For circulating TGF-β1 levels *in vivo*, blood samples were drawn from mouse hearts for assay and sera were kept frozen and then thawed shortly before determination of TGF-β1 levels.

Immunoblot analysis

For western blotting of whole-cell extracts, cells were treated with lysis buffer (Calbiochem, La Jolla, CA). An NE-PER kit (Pierce, Rockford, IL) was used to separate nuclear and cytoplasmic proteins. Equal amounts of protein were loaded onto SDS-PAGE gels. After electrophoresis, the proteins were transferred to nitrocellulose membranes. Antibodies specific for TGF-β1, Bcl-2, Bax, cleaved caspase 3, p-H2AX, p-ATM, and p53 were obtained from Santa Cruz Biotechnology, Inc., R&D Systems, Inc. (Minneapolis, MN), and Cell Signaling (Danvers, MA). Each membrane was re-probed with an antibody specific for r-tubulin or nuclear lamin to normalize protein loading.

Supplementary Figure Legends

Supplementary Figure 1

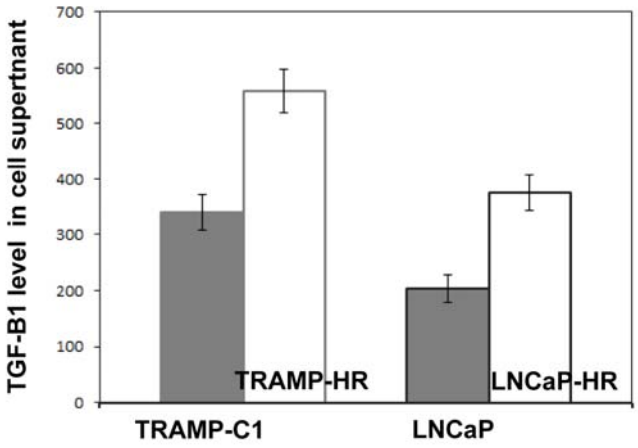

Figure 1 Levels of TGF-β1 in prostate cancer. The level of TGF-β1 in cell supernatant was examined by ELISA analysis.

Supplementary Figure 2

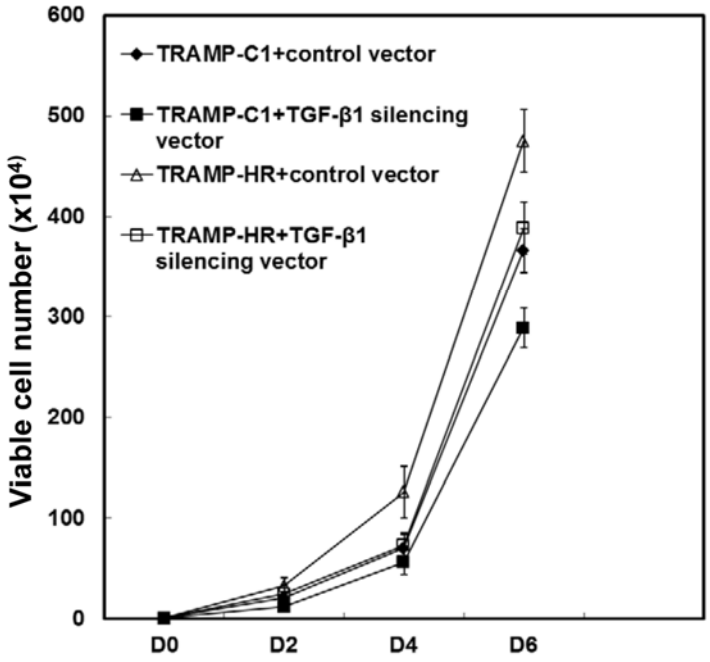

Figure 2 Effects of TGF-β1 on tumor growth in vitro. The same number of cells (10^4) were plated in each plate on day 0 and allowed to grow in their respective cultures. We counted the number of viable cells after incubation or 2, 4, and 6 days. The Y-axis represents the viable cell number. Point means of three separate experiments, bars SD; *P<0.05.

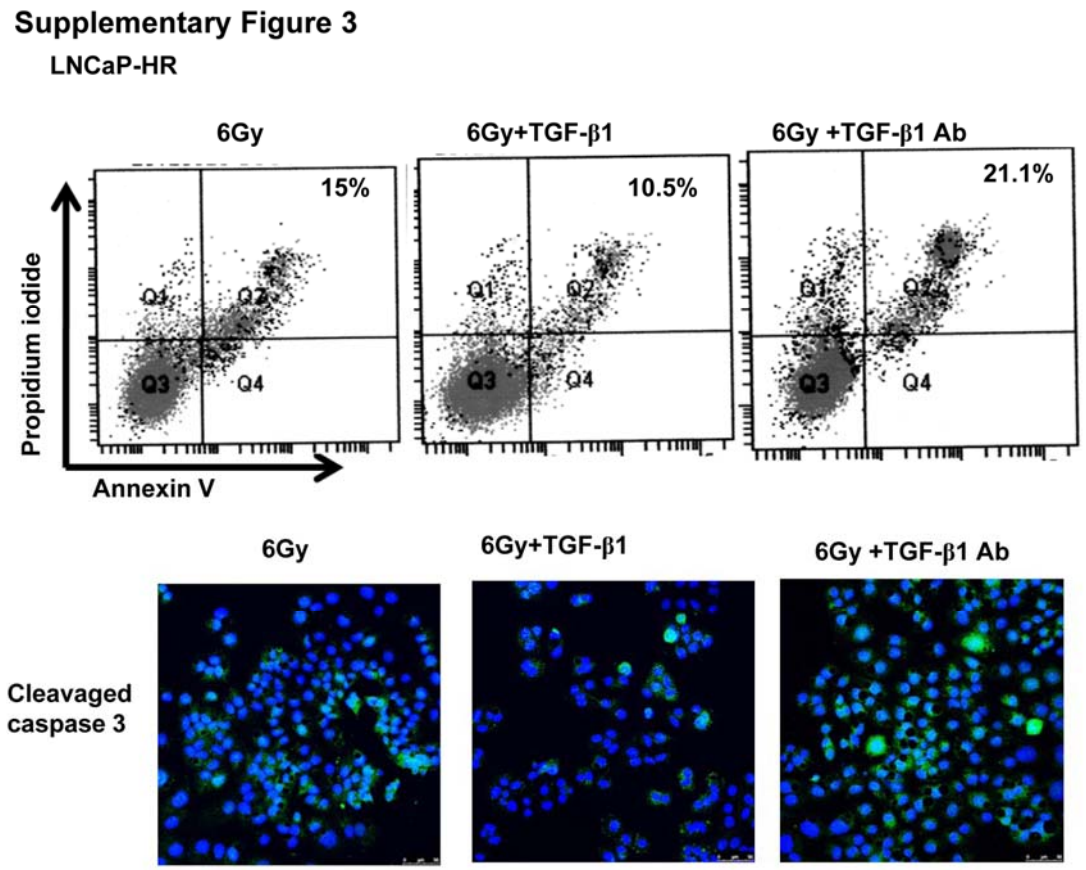

Figure 3 Effects of TGF-β1 on radiation responses *of human prostate cancer cells in vitro.* The *in vitro* effects of TGF-β1 on apoptosis and cell death, as evaluated by immunofluoresence staining with cleavaged caspase 3 and FACS with Annexin V-PI staining in irradiated cells.

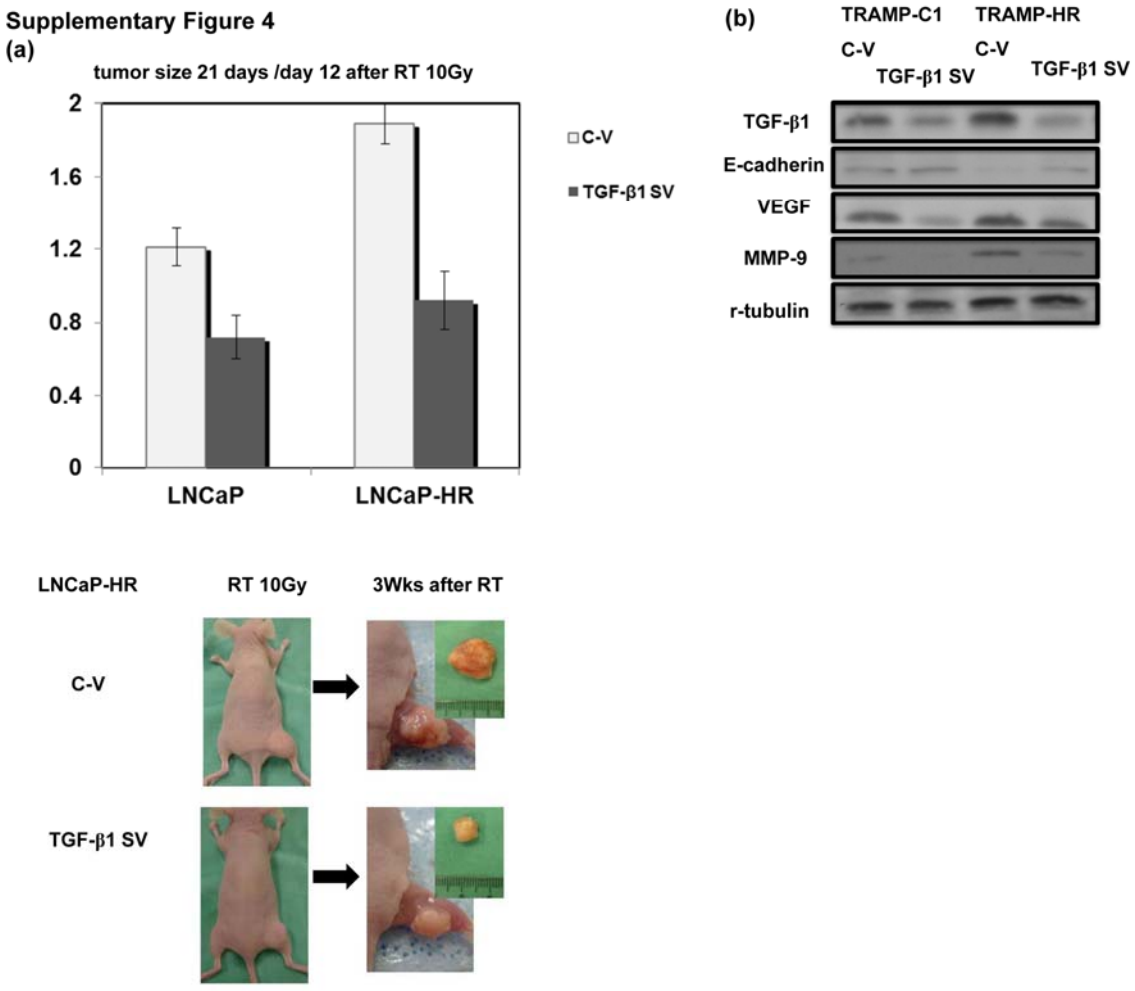

Figure 4 Effects of TGF-β1 on radiation responses *of human prostate cancer cells in vivo.* (a) The effect of TGF-β1 silencing vectors on tumor regrowth after irradiation are demonstrated by the relative tumor size at the indicated times normalized to the tumor size 12 days after irradiation, and (b) Western Blotting analysis for the expressions of TGF-β1, MMP-9, VEGF and E-cadherin