Supplementary Materials and Methods

Telomerase activity assay (TRAP assay)

Telomerase activity was performed using TeloTAGGG telomerase PCR ELISA kit (Roche, Indianapolis) based on the "Materials and Methods" described previously. On 5 days after infection with adenoviruses at 30 MOI, all cells were harvested and extracted from cell pellets. Cell extracts were incubated with biotinylated telomerase substrate oligonucleotide (PI-TS) at 25 °C for 30 min. The extended products were amplified by PCR with primers of P1-TS and P2 under the following cycling conditions: 10 min at 95 °C for preheating, 30 cycles of 30 s at 94 °C, 30 s at 60 °C, 30 s at 72 °C and 10 min at 72 °C. PCR products were denatured and hybridized to telomeric repeat sequences-specific probe labeled with digoxigenin. TRAP products which were detected by anti-digoxigenin antibody conjugated to horseradish peroxidase (HRP) on streptavidin-coated microplate immobilized with PCR amplified products, was measured by spectrophotometer at 450 nm and 680 nm.

FACS analysis

In order to assess the infectivity of adenovirus to cancer cells, the Hep3B cells were harvested under indicated conditions, fixed in ice-cold 70% ethanol for 1 hour, and their green fluorescence analyzed using FACScaliber (BD Biosciences, San Jose, CA).

PCR analysis

The adenoviral vector copy number and endogenous hTERT concentration in Hep3B cells and was quantified by quantitative PCR (qPCR) using SYBR Tag (TaKaRa BIO INC, Japan) and ABI PRISM 7900HT (Applied biosystems, Foster City, CA). The qPCR was performed using 50 ng genomic DNA under following condition: 40 cycles at 95℃ for 30 s, and 60℃

for 1 min. The standard curve was determined for an adenovirus particle range of 10^1 to 10^9. The sequence of primers used for the hTERT amplification in Ad-CRT-infected cells were 5'-GGGAAGAGTGCTGGACAA-3' and 5'-GGATGAAGCGGAGTCTGGA-3' and performed under conditions as 94 °C for 30 s, 55 °C for 40 s and 72 °C for 1 min. For detection of hTERT mRNA, RT-PCR was performed with primers (forward 5'-TGT ACT TTG TCA AGG TGG ATG TG-3' and reverse 5'-GTA CGG CTG GAG CTC TGT CAA-3') as following conditions; 94 °C for 10 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30s.

The production of trans-spliced molecules (TSM) by Ad-PRT-E was investigated by nested RT-PCR. Total RNA extracted with TRIzol reagent (Invitrogen, CA) was obtained from cells or tissues infected with Mock and Ad-PRT-E. cDNA synthesis was processed using Superscript III kit (Invitrogen, CA) with primer for HSVtk (5'- GGG ATG GCG GTC GAA GAT GAG GG – 3') or oligo dT. The first PCR was performed with a 5' primer specific for junction area of trans-splicing reaction (5'- GGG GAA TTC AGC GCT GCG TCC TGC T-3') and with a 3' primer for the HSVtk (5'- GTT ATC TGG GCG CTT GTC AA-3') under the following cycling conditions: 10 min at 95 °C for preheating, 30 cycles of 30 s at 95 °C, 30 s at 60 °C, 30 s at 72 °C and 10 min at 72 °C. The second PCR was done with primer for inward sequence of HSVtk (forward 5'- CGT CCT GCT AAA GTT GGC CGC – 3' and reverse 5'- GCA GTT GCG TGG TGG TGG TT -3') as following conditions; 94 °C for 10 min, followed by 30 cycles of 94 °C for 30 s, 57 °C for 30 s and 72 °C for 30s. To confirm accuracy of trans-spliced molecule, the amplified PCR product was eluted and sequenced (COSMO Inc. Seoul).

Supplementary Fig. S1

Supplementary Fig. S2

Supplementary Fig. S3

Supplementary Fig. S4

Supplementary Fig. S5

Supplementary Fig. S6

Supplementary Fig. S7

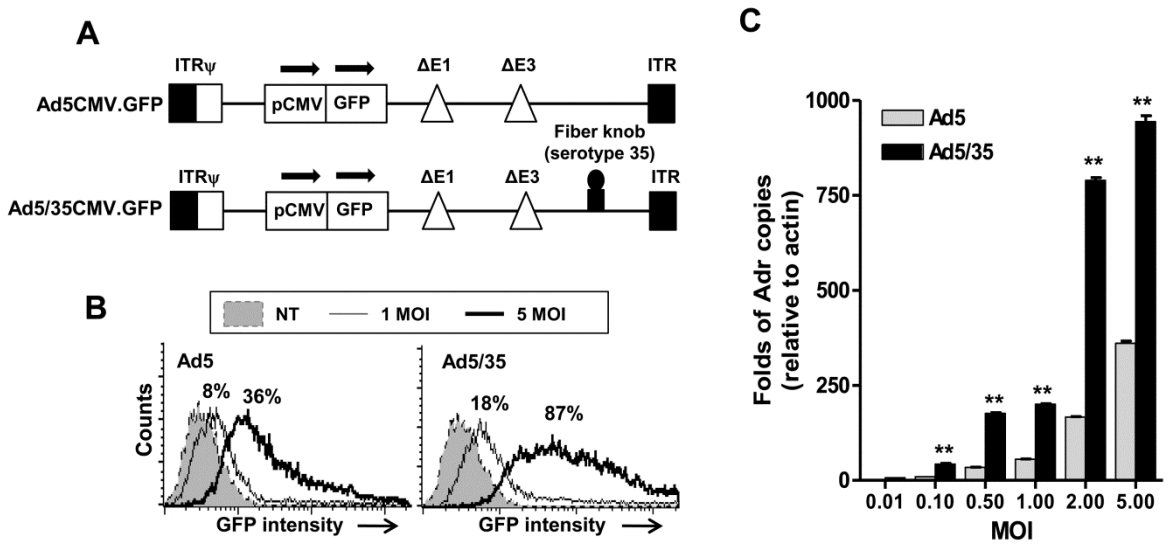

Fig. S1. Construction of chimeric adenovirus 5/35 for better transduction. (A) Structures of GFP-encoded adenoviruses engineered with chimerization of serotypes 5 and 35. **(B)** Enhanced-transduction efficiency of Ad5/35 was compared with Ad5. Hep3B cells were infected with Ad5-GFP or Ad5/35-GFP at 1 and 5 MOIs. After 48 hrs, the GFP-positive cells were analyzed by flow cytometry (histogram). **(C)** Adenovirus genomes in the Hep3B cells were calculated by real time PCR, 48 hrs following infection with the indicated MOIs as described in the Materials and Methods. Values are shown as folds of adenoviral copy numbers normalized to genomic DNA with β-actin primers. Error bars indicate the mean values ± SEM for the triplicate assays. (** $p < 0.01$)

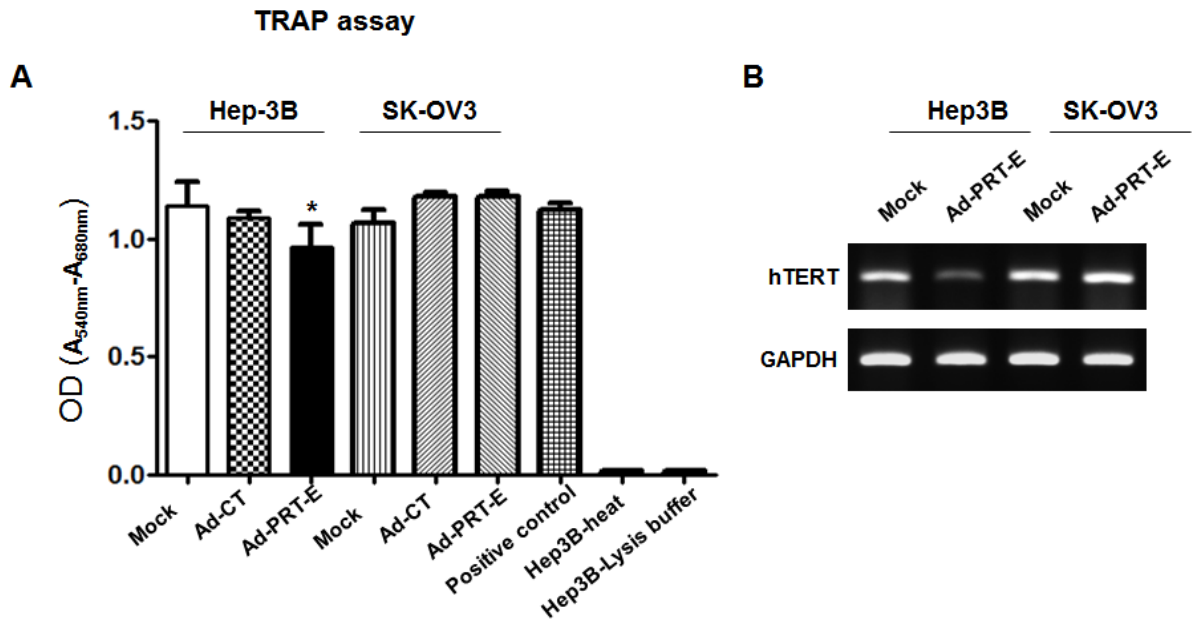

Fig. S2. HCC-specific telomerase activity of Ad-PRT-E. (A) Telomerase activity was determined by TRAP assay using TeloTAGGG telomerase PCR ELISA kit as described in "Supplmentary Materials and Methods". Error bars indicate the mean values ± SEM for triplicate assays. (* $p < 0.05$) **(B)** The decrease of hTERT mRNA by Ad-PRT-E infection determined with RT-PCR analysis. PCR procedures are described in "Supplementary Materials and Methods"

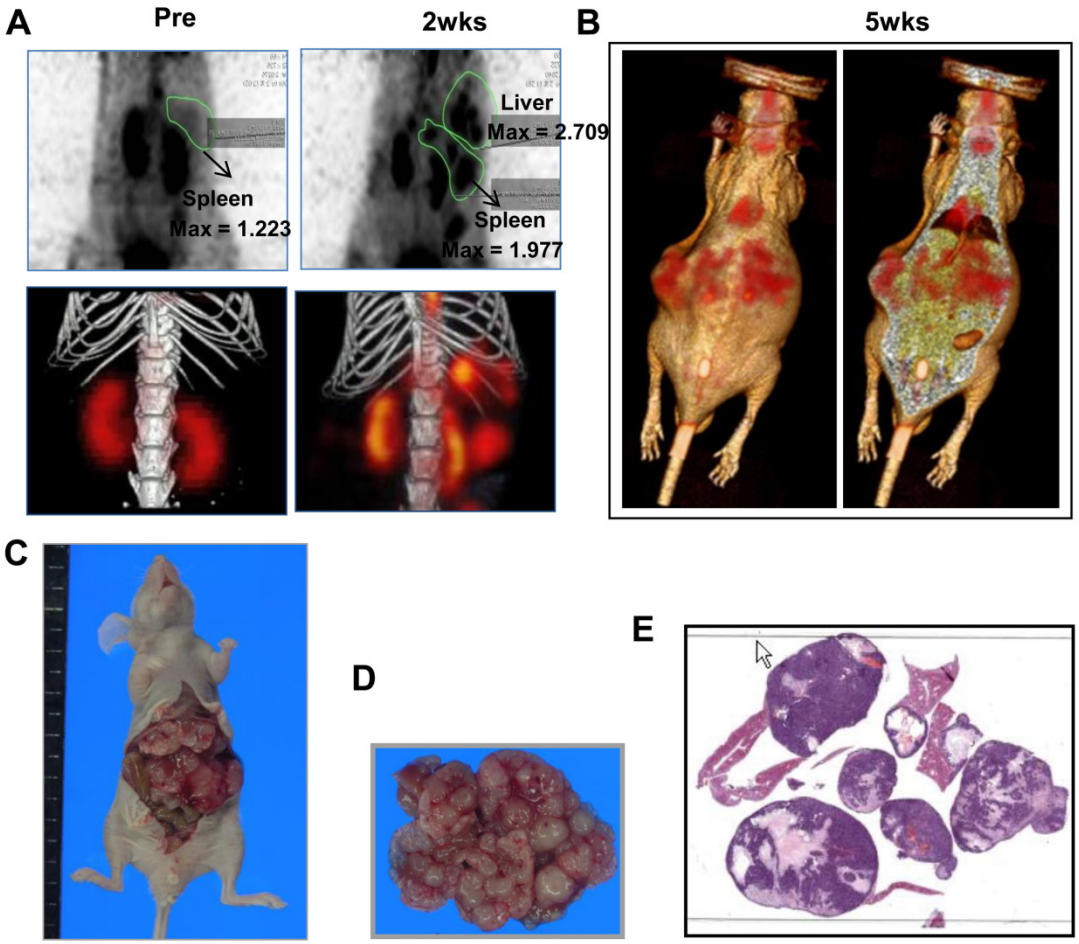

Fig. S3. Establishment of intrahepatic multifocal HCC mouse model using in vivo monitoring with animal PET/CT during 5 weeks. **(A)** Representative ^{18}F-FDG PET/CT fusion imaging of intrahepatic multifocal HCC mouse model at 0 (pre), 2 weeks. PET/CT scanning was performed before and after splenic subcapsular injection of Hep3B cells ($3x10^6$ in 100ul of PBS) every week and estimated ^{18}F-FDG uptake (SUVmax) of spleen and liver. **(B)** PET/CT fusion images at 5 weeks after tumor inoculation. **(C-E)** images of tumor mass by an autopsy (C-D) and H&E staining (E) of tumor-bearing liver at 5 weeks after tumor cell injection. Opened abdomen reveals enlarged liver lobes almost replaced by HCC masses (C-D), corresponding with microscopic features (blue masses: HCC) (E).

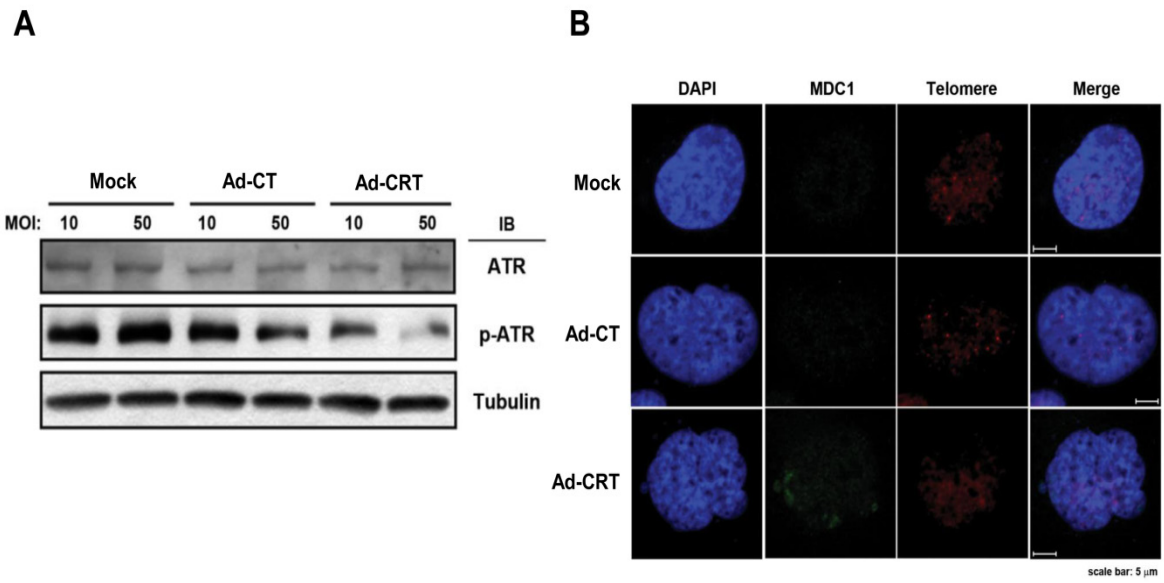

Fig. S4. Effect of Rz-mediated hTERT depletion on MDC1 and ATR phosphorylation in Ad-CRT-infected cells. (A) ATR phosphorylation was reduced in Ad-CRT-infected cells compared with Ad-CT-infected cells. **(B)** MDC1 foci were detected in Ad-CRT-infected cells, but not in Mock- or Ad-CT-infected cells. All staining was performed at 4 days after infection. MOI, multiplicity of infection.

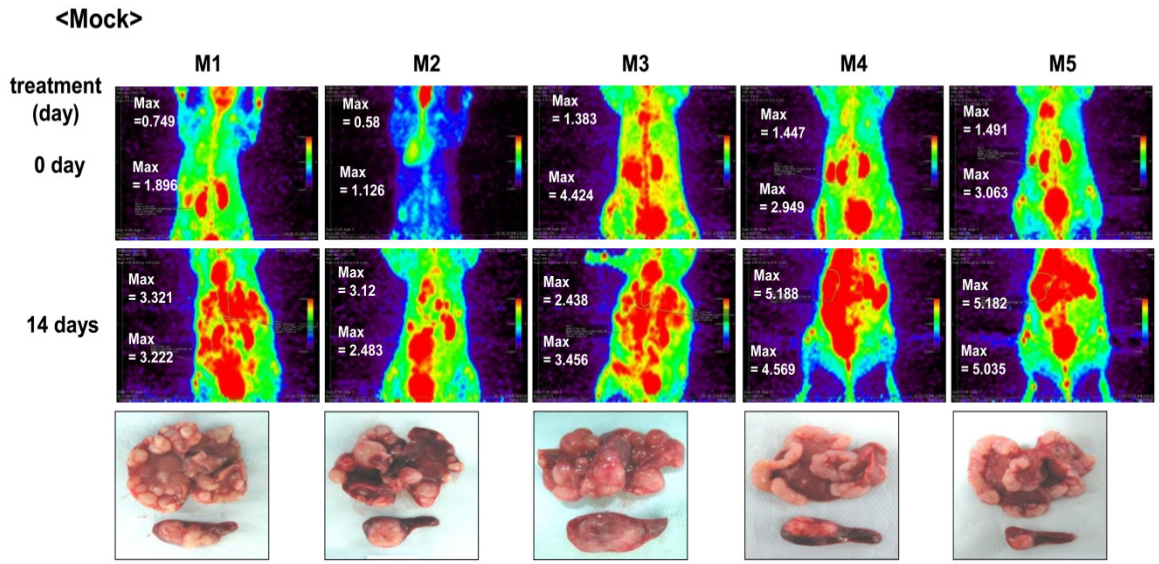

Fig. S5. *in vivo* monitoring of therapeutic effect using microPET in Mock-treated group. Individual ^{18}F-FDG PET imaging and SUVmax of Mock (M1~M5, n=5) in Fig. 6A-B. "Treatment 0 day" means the time of 2wks after intrasplenic injection of tumor cells. Max in upper area of each square box = SUVmax of liver, Max in lower area = SUVmax of spleen.

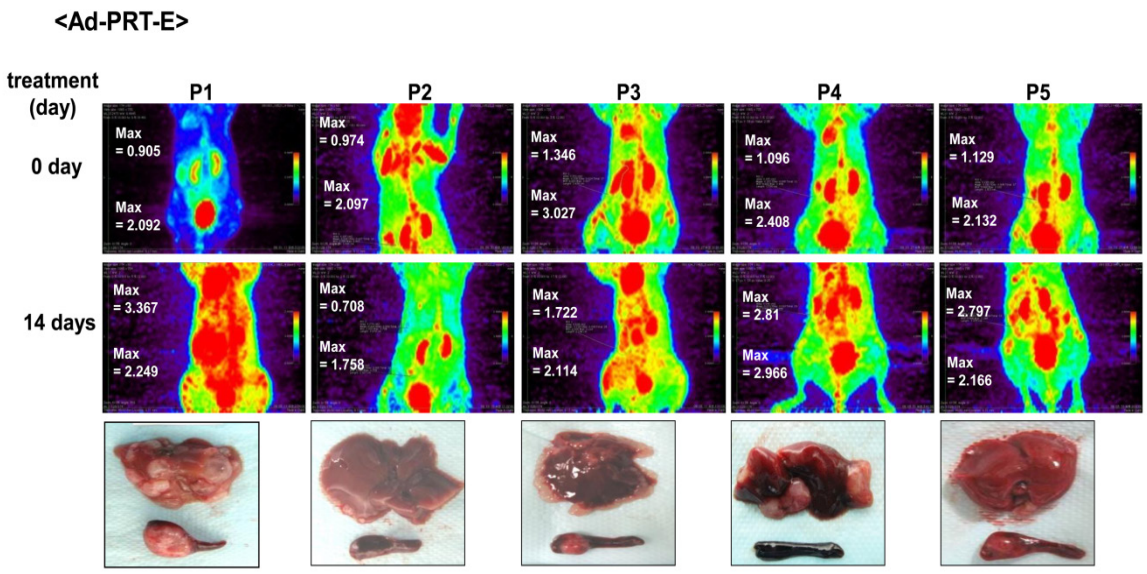

Fig. S6. *in vivo* monitoring of therapeutic effect using microPET in Ad-PRT-E-treated group. Individual ^{18}F-FDG PET imaging and SUVmax of Ad-PRT-E (P1~P5, n=5) in Fig. 6A-B. "Treatment 0 day" means the time of 2wks after intrasplenic injection of tumor cells. Max in upper area of each square box = SUVmax of liver, Max in lower area = SUVmax of spleen.

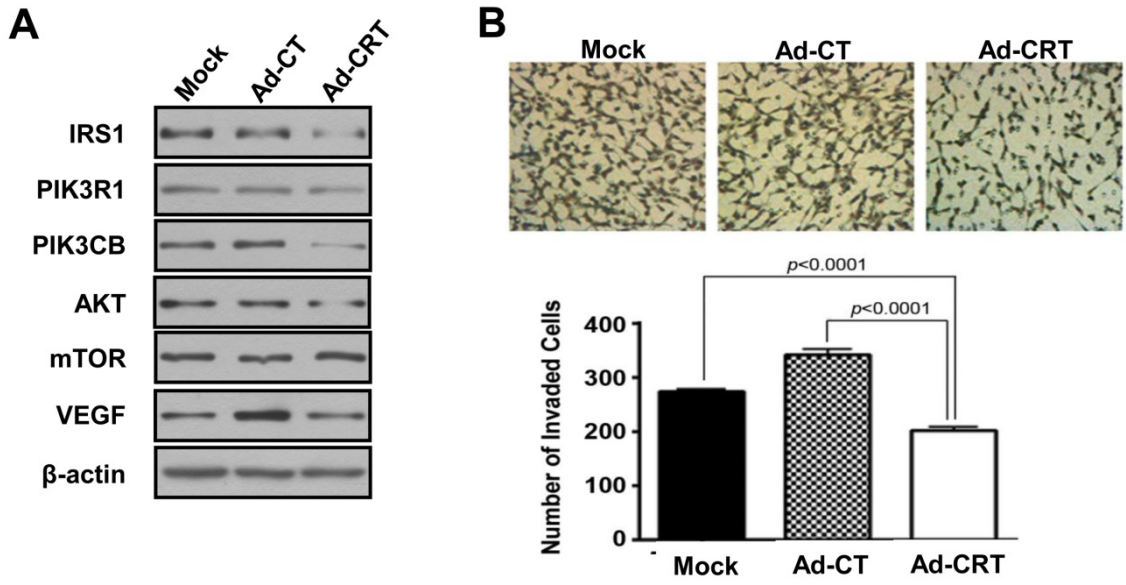

Fig. S7. Downregulation in PI3K/Akt pathway and decreased invasiveness by Rz-mediated hTERT depletion. (A) Western blots of representative molecular expression after Mock, Ad-CT, and Ad-CRT infections. **(B)** Fewer cells invaded the Matrigel in the Ad-CRT-infected Hep3B cells than in the Ad-CT-infected cells. The number of invaded cells was counted under phase contrast microscope in five random fields.