Effective Treatment of Tumors with Strong β-Catenin/T-Cell Factor Activity by Transcriptionally Targeted Oncolytic Herpes Simplex Virus Vector

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Abstract

The Wnt/β-catenin/T-cell factor (Tcf) pathway is aberrantly up-regulated in the majority of colorectal cancers (CRC) and hepatoblastomas due to either an *APC* or *β-catenin* gene mutation. We constructed synthetic promoters, T and TE, which contain tandem repeats of a Tcf responsive element without and with the human 4F2 gene intronic enhancer, respectively. Although the T and TE promoters showed higher transcriptional activity than a control promoter in all CRC and hepatoblastoma cell lines tested, with low activities in most other tumor cell lines, the level of transcription varied considerably among the CRC and hepatoblastoma cell lines. In some CRC cell lines, the TE promoter displayed higher levels of transcription than even the human CMV_{IE} promoter. In those CRC cells, the *APC* gene mutations were located within a small segment between the first and second 20-amino-acid repeats in the mutation cluster region of the APC protein. We created a transcriptionally targeted oncolytic herpes simplex virus vector (bM24-TE) in which replication is driven by the TE promoter. This vector efficiently and specifically replicated in and killed tumor cells with strong β-catenin/Tcf signaling. Intratumoral injection of bM24-TE significantly reduced the growth of highly β-catenin active SW480 CRC tumors and induced a complete response in half of them, whereas it had no effect on the growth of β-catenin-inactive A549 tumors. Our results suggest that a transcriptionally regulated oncolytic herpes vector targeting β-catenin/Tcf signal is very efficacious against CRC tumors carrying an *APC* gene mutation between the first and second 20-amino-acid repeats. (Cancer Res 2006; 66(20): 10127-35)

Introduction

Recent studies on molecular carcinogenesis have highlighted the deregulation of the Wnt/β-catenin/T-cell factor (Tcf) signaling pathway as a crucial oncogenic event in a subset of tumors including colorectal cancer (CRC) and hepatoblastoma (1). In vertebrate embryos, this pathway plays important roles in the development of the neural crest, heart, and gastrointestinal tract (2–4). Although the Wnt pathway seems to have several different downstream effectors (5), many researchers thus far have focused on the canonical Wnt pathway involving β-catenin and Tcf as its key effectors. β-Catenin, which was originally reported as a protein associated with the adhesion molecules, cadherins, mediates the signal by translocating to the nucleus and acting as a transcriptional activator in complex with DNA binding protein Tcf. In normal cells, β-catenin activity is tightly and dynamically regulated by a large multiprotein complex, the so-called "β-catenin destruction complex," containing β-catenin and glycogen synthase kinase 3β (6). When the Wnt signal is off, glycogen synthase kinase 3β in this complex phosphorylates β-catenin, which triggers its subsequent ubiquitin-dependent degradation, leading to the shut-off of the signal (7, 8).

In CRC cells, β-catenin/Tcf signaling is activated mainly by mutations in the tumor suppressor gene *APC* (9, 10). The APC protein is a critical component of the β-catenin destruction complex, directly associating with β-catenin and another component, Axin (11). The majority (>60%) of somatic mutations in the *APC* gene occur within a segment called the mutation cluster region (codons 1,250-1,500) and almost all of the mutations result in truncated APC protein. Close to the mutation cluster region, there are several functional domains that directly or indirectly affect β-catenin down-regulation, including the seven 20-amino-acid repeats, three 15-amino-acid repeats, three SAMP repeats (Axin binding domains), and a centrally located nuclear exporting signal (Fig. 1*C*). *APC* mutations occurring in the mutation cluster region produce abnormal APC proteins that retain only the three 15-amino-acid repeats and the first one or two 20-amino-acid repeats while losing the other domains and failing to effectively down-regulate β-catenin (12). Indeed, >70% of sporadic CRCs contain *APC* gene mutations, which result in inefficient β-catenin degradation and up-regulation of β-catenin-mediated transcription (13). Furthermore, in half of the remaining CRCs without *APC* gene mutations, activating mutations of β-catenin are found, which make it resistant to phosphorylation and cause its abnormal accumulation, indicating the importance of β-catenin/Tcf up-regulation in CRC carcinogenesis (14). In hepatoblastomas, ~50% to 90% of cases have been reported to carry activating mutations in *β-catenin*, whereas mutation in *APC* is rare (15–17).

Although important for embryonic development, the Wnt/β-catenin/Tcf pathway is inactive in most adult tissues, except in some stem cells (18). Therefore, the aberrant activation of this signal is an ideal target for gene therapy against CRCs and hepatoblastomas. Several researchers have already reported the application of β-catenin/Tcf responsive promoters for tumor-specific suicide gene expression or replication-conditional adenovirus generation (19–21). We previously generated transcriptionally targeted oncolytic herpes simplex virus (HSV) vectors using the albumin promoter/enhancer, which specifically replicated in and killed albumin-producing hepatoma cells (22). Tumor-selective replication of the oncolytic virus was achieved by putting the

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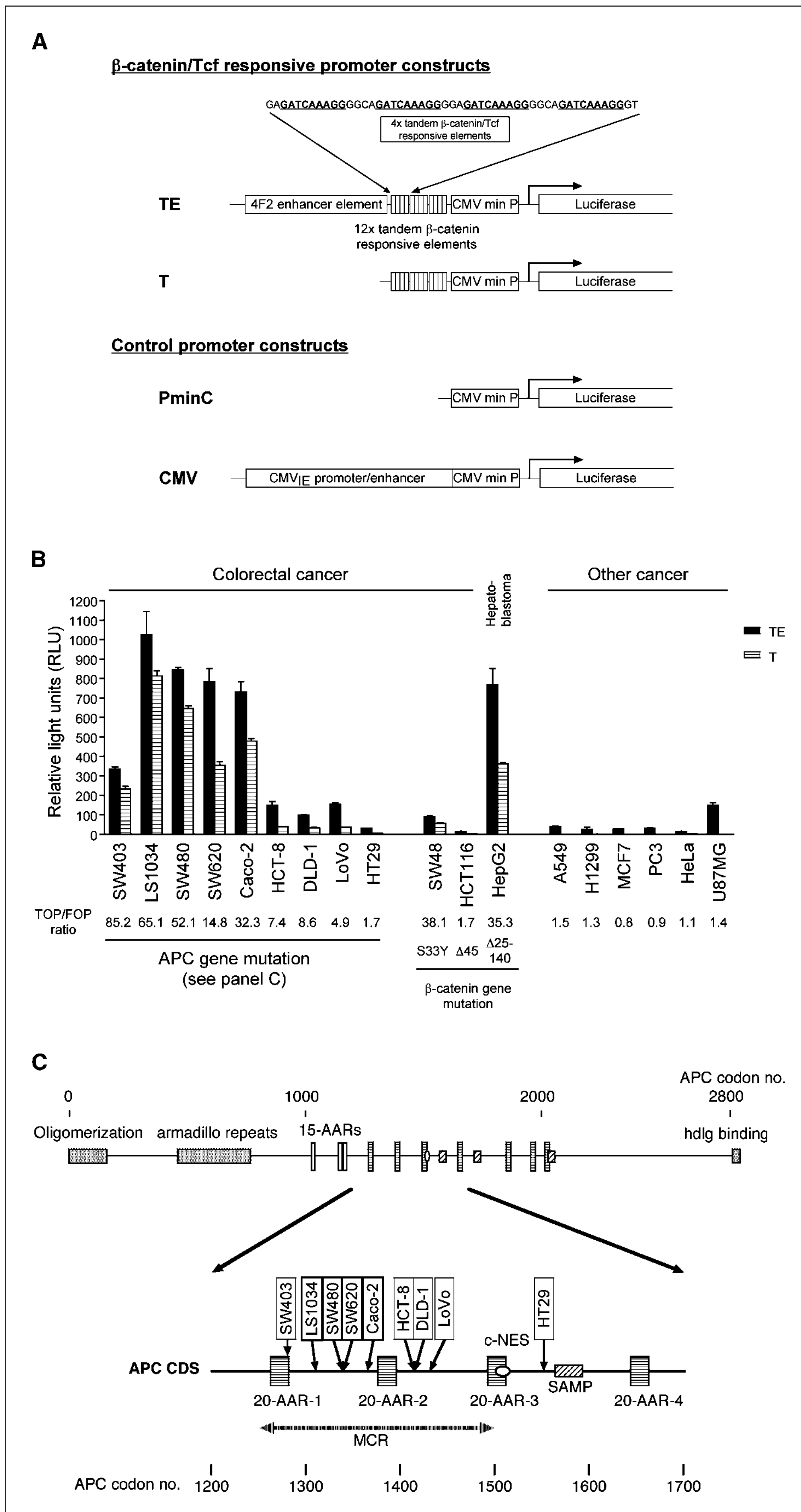

Figure 1. Assessment of β-catenin/Tcf–dependent transcriptional activity in different cell lines. *A,* construction of luciferase reporter plasmids carrying β-catenin/Tcf responsive promoters and negative-control and positive-control promoters. T promoter consists of 12 tandem repeats of a β-catenin/Tcf responsive element (*top, underlined*) followed by the human CMV_{IE} minimal promoter. TE promoter contains an enhancer element derived from the first intron of the human 4F2 heavy-chain gene inserted upstream of the T promoter. Two other control promoter constructs were also generated: PminC, carrying the human CMV_{IE} minimal promoter only, and CMV, carrying the entire human CMV_{IE} enhancer/promoter. *B,* analysis of transcriptional activities of the T and TE promoters in different human tumor cell lines with and without activated β-catenin/Tcf pathway. In the left part of the graph, CRC cell lines with *APC* gene mutation(s) are shown. These cell lines are aligned from left to right in the order of the position of the *APC* gene mutation (*C*; refs. 29, 30). In the middle part are CRC and hepatoblastoma cell lines harboring a *β-catenin* gene mutation (28, 30). Descriptions of the mutations are shown at the bottom. These three cell lines do not have an *APC* gene mutation. In the right part of the graph are other cancer cell lines, including lung adenocarcinoma (A549 and H1299), mammary adenocarcinoma (MCF7), prostate adenocarcinoma (PC3), cervical carcinoma (HeLa), and glioblastoma (U87MG). Luciferase activities were standardized for transfection efficiency using *Renilla* luciferase activity from cotransfected pRL-TK vector (Promega). *Columns,* mean from triplicate wells; *bars,* SD. In the text, the degree of transactivation is referred to as "strong" when the relative luciferase activity was >500 RLU, as "moderate" when between 100 and 500 RLU, as "low" when between 3 and 100 RLU, and as "very low" when ≤3 RLU. Ratios between the luciferase activities from TOPFLASH and FOPFLASH reporter (TOP/FOP ratio) are shown below the graph. TOP/FOP ratio has been widely used to measure β-catenin/Tcf signaling activity excluding the effect of the minimal promoter activity. *C, top,* schematic presentation of the APC protein and its domains. The central one third of APC contains several functional domains related to the down-regulation of β-catenin, including 15-amino-acid repeats (*15-AARs*; *open boxes*), 20-amino-acid repeats (*20-AAR*; *horizontally striped boxes*), SAMP repeats (*diagonally striped boxes*), and centrally located nuclear exporting signal (*c-NES*; *open ellipse*). *Bottom,* positions of the *APC* gene mutations found in CRC cell lines. Some CRC cell lines contain two *APC* mutations (SW403, LoVo, and HT29; ref. 30). As the oncogenic property of APC is basically considered to derive from the loss of its function, the positions of the *APC* mutation in these cell lines referred in the text and figure are the those of the longer APC truncation. In the other six cell lines with *APC* mutations, the wild-type allele is lost and only mutant APC is expressed (29, 30). The mutation cluster region (*MCR*), where ~60% of somatic mutations in CRCs localize, falls between the first and third 20-amino-acid repeats.

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essential HSV infected cell protein 4 (*ICP4*) gene under the control of a tumor- or tissue-specific promoter (23, 24). Because HSV vectors have a wide tissue range and have been shown to be effective against various types of tumors including brain, breast, colon, liver, and prostate (22, 25, 26), we reasoned that an HSV vector whose replication is driven by β-catenin/Tcf signaling should be an effective agent against CRCs and hepatoblastomas or other tumors with an activated Wnt pathway.

In this study, we constructed new synthetic β-catenin/Tcf responsive promoters and used them to generate transcriptionally targeted HSV vectors, which selectively replicated in tumor cells with highly activated β-catenin/Tcf signaling and induced complete remission in an *in vivo* tumor xenograft model. Interestingly, the *APC* mutations of the CRC cell lines that exhibited strong transcriptional activation from the synthetic promoter clustered in a small segment within the mutation cluster region. Our results illustrate the promise of oncolytic viral therapy targeting the Wnt/β-catenin/Tcf pathway and provide insight into the regulation of β-catenin/Tcf signaling exerted by some mutant forms of APC in CRC.

Materials and Methods

Cells and viruses. Vero, SW480, SW620, SW403, LS1034, Caco-2, DLD-1, HCT116, SW48, and U87MG cells were obtained from American Type Culture Collection (Manassas, VA) and human umbilical endothelial cells (HUVEC) from Cambrex (East Lutherford, NJ). HeLa, LoVo, HT29, HepG2, H1299, PC-3, MCF7, and 293T cells were a generous gift from M. Kaneki (Massachusetts General Hospital, Boston, MA), HCT8 from Y. Fong (Memorial Sloan-Kettering Cancer Center, NY), and A549 and MRC5 from W. Kallas (Massachusetts General Hospital). E5 cells are transformed Vero cells that express complementing levels of wild-type ICP4 on herpes simplex virus-1 (HSV-1) infection and were kindly provided by N. DeLuca (University of Pittsburgh School of Medicine, Pittsburgh, PA; ref. 27). Vero and E5 cells were grown in DMEM supplemented with 10% calf serum and HUVEC in EGM-2 (Cambrex). All other cell lines were grown in DMEM supplemented with 10% FCS. Germ-line mutations in the CRC and hepatoblastoma cell lines have been reported elsewhere (28–30). An ICP4-deletion HSV-1 mutant, d120, was provided by N. DeLuca, and an ICP6-inactivated HSV-1 mutant, *hr*R3, was provided by S. Weller (University of Connecticut Health Center, Farmington, CT; refs. 27, 31).

Plasmid and recombinant virus construction and siRNA synthesis. Oligomers coding four tandem repeats of a Tcf responsive element (4TRE) were synthesized (forward strand, 5′-CGCTAGCGAGATCAAAGGGGCA-GATCAAAGGGGAGATCAAAGGGGCAGATCAAAGGGT-3′; reverse strand, 5′-CTAGACCCTTTGATCTGCCCCTTTGATCTCCCCTTTGATCTGCCCC-TTTGATCTCGCTAGCGGTAC-3′), hybridized, and cloned into a plasmid vector. For the construction of the minimal CMV promoter sequence (−51 to +69; ref. 32), two oligomers (forward strand, 5′-CCCGCTAGC-TAGGCGTGTACGGTGGGAGGCCTATATAAGCAGAGCTCGTTTAGT-GAACCGTCAGATCGCCTGGAGACGCCATCC-3′; reverse strand, 5′-CCCGTCGACAGGCTGGATCGGTCCCGGTGTCTTCTATGGAGGT-CAAAACAGCGTGGATGGCGTCTCCAGGCGATCTGACGGTTC-3′) were hybridized and filled in with LA-Taq DNA polymerase (Takara Bio, Otsu, Japan) using a thermal cycler (MJ Research, Waltham, MA). The resultant 138-bp fragment (PminC) was cloned into pVP22/myc-His2-TOPO vector (Invitrogen, Carlsbad, CA). The T promoter was generated by inserting three 4TRE fragments in tandem upstream of the PminC sequence. The transcriptional enhancer element from the first intron of the human 4F2 heavy chain was amplified via PCR from SW480 genomic DNA with a forward primer (5′-CCTGCAGGTTCCAGGGCCACGGCGCGGGCAA-3′) and a reverse primer (5′-CTTAAGTAATCGAGACGCCCCTTCAGAC-3′) using LA-PCR kit (Takara Bio; ref. 33). The resultant 512-bp enhancer element (4F2EE) was cloned into pCR2-TOPO TA cloning vector (Invitrogen). The TE promoter was generated by inserting the 4F2EE fragment upstream of the T promoter. The T, TE, and PminC promoter fragments were cloned into pGL3-Basic vector (Promega, Madison, WI) to create pGL3-T12, pGL3-T12E, and pGL3-PminC, respectively (Fig. 1*A*). The human CMV_{IE} enhancer/promoter obtained from pVP22/myc-His2 vector (Invitrogen) was cloned into pGL3-Basic to create pGL3-CMV2. Tcf reporter plasmids, TOPFLASH and FOPFLASH, were purchased from Upstate (Lake Placid, NY). TOPFLASH contains two sets (with the second set in the reverse orientation) of three copies of the Tcf binding site followed by the minimal HSV-*tk* promoter and luciferase reporter coding sequence, and FOPFLASH contains mutated Tcf binding sites. It should be noted that these TOPFLASH/FOPFLASH vectors are different from pTOPFLASH/pFOPFLASH vectors used in previous reports (10) in that the latter contain the minimal c-*fos* promoter instead of the HSV-*tk*. Mutant β-catenin expression plasmids (pMKITneo-b-catenin-S33Y and pMKITneo-b-catenin-S37A) and pMKITneo empty vector were kindly provided by T. Akiyama (University of Tokyo, Tokyo, Japan). All of the synthetic and PCR-amplified fragments cloned into plasmid vectors were sequenced and verified.

The T and TE promoter fragments were inserted into pFLS-ICP4 shuttle vector[1] to create pFLS-12TRE and pFLS-12TRE-E. The recombinant viruses bM24-T and bM24-TE were generated using the Flip-Flop HSV-BAC system[1] with these shuttle vectors and clone purified by limiting dilution. The construction of bM24-null will be reported elsewhere.[1] Viruses were amplified on E5 cells from low-multiplicity infections. The structure of recombinant viruses was confirmed by *Hin*dIII restriction analysis of viral genomic DNA (data not shown).

For down-regulation of β-catenin, we used β-catenin-specific and control siRNA previously described (34): β-catenin, 5′-AGCUGAUAUUGAUGGA-CAGdTdT-3′ and 5′-CUGUCCAUCAAUAUCAGCUdTdT-3′; control, 5′-UUCUCCGAACGUGUCACGUdTdT-3′ and 5′-ACGUGACACGUUCGGA-GAAdTdT-3′. The siRNA duplexes were synthesized by Invitrogen.

Luciferase reporter gene assay. Cells (1×10^5) were plated in a 24-well plate 1 day before transfection and transfected with 100 ng of firefly luciferase reporter plasmid and 10 ng of internal control plasmid pRL-TK (Promega) using Lipofectamine 2000 (Invitrogen) following the protocol of the manufacturer. Twenty-four hours after transfection, cells were lysed and analyzed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega).

Immunoblot analysis. Cells (2×10^5) were plated in a 12-well plate 1 day before infection, and infected with the designated virus at a multiplicity of infection (MOI) of 2. At 10 or 16 hours after infection, the cells were lysed with 1× SDS sample buffer. Proteins obtained from $\sim 3 \times 10^4$ infected cells were analyzed by immunoblot. For the detection of ICP4, mouse anti-HSV-ICP4 monoclonal antibody (US Biological, Swampscott, MA) was used, and for the detection of actin, rabbit anti-actin polyclonal antibody (Sigma-Aldrich, St. Louis, MO). The blot was probed with either horseradish peroxidase–linked antimouse or antirabbit immunoglobulin G antibody (Amersham Biosciences, Piscataway, NJ), and chemiluminescent detection was done with the enhanced chemiluminescence Western blotting detection reagents (Amersham Biosciences).

Virus titration. Monolayer cultures of Vero cells (for *hr*R3) or E5 cells (for ICP4 mutant viruses) grown in a six-well plate were infected with serial dilutions of virus as indicated. After removal of virus inoculum, the cells were incubated in DMEM supplemented with 1% inactivated FCS and 0.1% pooled human immunoglobulin (BayGam; Bayer Corp., Elkhart, IN) at 37°C for 3 to 4 days until plaques were visible. The cells were stained by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside histochemistry (22) and β-galactosidase-positive plaques were counted.

Viral replication assay. Cells were seeded in a 12-well plate 1 day before infection and infected with the designated virus at an MOI of 1.5 or 3. At the times indicated, virus was harvested from the wells and titers were determined on Vero cells (for *hr*R3) or on E5 cells (for other recombinant

[1] T. Kuroda, R.L. Martuza, T. Todo, S.D. Rabkin, Flip-flop HSV-BAC: Bacterial artificial chromosome-based system for rapid generation of recombinant herpes simplex virus vectors using two independent site-specific recombinases. BMC Biotechnology 2006, in press.

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viruses). For the analysis of virus replication in 293T cells expressing mutant β-catenin, cells were first transfected with 200 ng of mutant β-catenin plasmid or empty vector using Lipofectamine 2000 and, 24 hours later, superinfected with bM24-TE or *hr*R3 at an MOI of 2. For the analysis of virus replication in SW480 cells after suppression of β-catenin expression by siRNA, cells were transfected with either 40 pmol of β-catenin-specific or control siRNA duplex or water only using Lipofectemine 2000 and, 48 hours later, superinfected with bM24-TE or *hr*R3 at an MOI of 1.5.

Viral cytotoxicity assay. Cells (5×10^5) were plated in a six-well plate 1 day before infection and infected with the designated viruses at an MOI of 0.05. Four days after infection, cells were harvested by trypsinization and viable cell counts were determined using a NucleoCounter (New Brunswick Scientific, Edison, NJ) following the protocol of the manufacturer.

***In vivo* treatment model.** Subcutaneous tumors were established by injecting 1×10^7 SW480 or A549 cells in the flanks of 6- to 8-week-old female BALB/c *nu/nu* mice. When tumor volumes reached 40 to 120 mm^3 (10-14 days after implantation), mice were stratified by tumor volume and then randomly assigned to treatment groups (n = 8 per group). Virus [bM24-TE or *hr*R3; 3×10^6 plaque-forming units (pfu)] or virus suspension buffer (PBS with 10% glycerol) was injected intratumorally (20 μL per injection) on days 0, 3, 5, and 7 (SW480) or days 0, 2, 6, and 8 (A549). Tumor sizes were measured weekly by external caliper and the volume calculated as $L \times W \times D$, where L is the length, W is the width, and D is the depth of the tumor.

Statistical analysis. Statistical analyses were done with Student's t test using GraphPad Prism 4 software (GraphPad Software, Inc., San Diego, CA). $P > 0.05$ was considered not significant.

Results

Construction of synthetic β-catenin/Tcf responsive promoters. Based on a previous report showing that 10 tandem repeats of Tcf-responsive elements (TRE) are necessary and sufficient to obtain specific and maximal transactivation by β-catenin/Tcf signaling (35), we generated two synthetic promoter constructs to achieve strong and specific transcriptional activation by the β-catenin/Tcf signal: (*i*) "T" promoter consisting of 12 tandem repeats of TRE followed by the minimal human CMV_{IE} promoter, and (*ii*) "TE" promoter with the intronic enhancer element derived from the 4F2 heavy-chain gene inserted upstream of the T promoter to achieve stronger transcription. A control promoter construct, "PminC," containing only the minimal CMV promoter and another control containing the human CMV_{IE} promoter/enhancer were also generated (Fig. 1*A*).

The T and TE promoters show strong transcriptional activities in a subset of colorectal and hepatocellular carcinoma cell lines. Transcriptional activities of these promoters were measured by luciferase assay in various tumor cell lines using *Renilla* luciferase driven by the HSV-*tk* promoter as internal control. As shown in Fig. 1*B*, the T and TE promoters showed strong activities in four CRC cell lines (LS1034, SW480, SW620, and Caco-2) and one hepatoblastoma cell line (HepG2). Transcription from the TE promoter was even higher than that from the CMV_{IE} promoter in the four CRC cell lines (Supplementary Table S1). Interestingly, the *APC* gene mutations of these cell lines clustered between the first and second 20-amino-acid repeats (Fig. 1*C*). The transcriptional activities of the T and TE promoters in the CRC cells carrying *APC* mutations between the second and third 20-amino-acid repeats (HCT-8, DLD-1, and LoVo) were 5- to 10-fold lower, and that in HT29 carrying an *APC* mutation between the third and fourth 20-amino-acid repeats a further 5-fold lower. SW403 containing an *APC* mutation within the first 20-amino-acid repeat showed a moderate TE promoter activity. The tumor cell lines expressing different β-catenin mutants and full-length APC exhibited variable transcriptional activities ranging from low (HCT116) to high (HepG2).

TOPFLASH and FOPFLASH vectors are a set of Tcf-reporter plasmids, and the TOPFLASH/FOPFLASH luciferase ratio has been widely used for the measurement of β-catenin/Tcf signaling activity (10). Whereas luciferase assay results using the TOPFLASH reporter alone agreed well with the T promoter results (Supplementary Fig. S1), the TOPFLASH/FOPFLASH ratio assay results (TOP/FOP ratio) showed some discrepancy (Fig. 1*B*). In all CRC and hepatoblastoma cell lines, transcription from the T promoter was at least 10-fold higher than that from the PminC promoter, reflecting activated β-catenin/Tcf signaling (Supplementary Table S1). In cancer cell lines other than CRC and hepatoblastoma, the T promoter exhibited very low transcriptional activity similar to the PminC promoter, whereas the TE promoter activities were low except in U87MG, where moderate TE promoter activity was observed presumably due to higher 4F2EE activity in the cells.

Recombinant viruses bM24-T and bM24-TE induce ICP4 expression only in cell lines with highly activated β-catenin/Tcf signaling. Recombinant viruses containing an ICP4 expression cassette driven by the T and TE promoters, designated as bM24-T and bM24-TE, respectively, were generated using a bacterial-artificial-chromosome (BAC)–based recombinant HSV cloning system (the Flip-Flop HSV-BAC system[1]; Fig. 2*A*). The bM24-null vector,[1] which carries the ICP4 coding sequence without a promoter, was used as an inactive promoter control, and *hr*R3, an ICP6 *lacZ* insertion mutant, which retains two copies of the wild-type *ICP4* gene, as an active promoter control.

Induction of ICP4 protein in different cells infected with these viruses was examined by Western blot. As shown in Fig. 2*B*, both bM24-T and bM24-TE efficiently induced ICP4 in SW480 cells, whereas ICP4 induction in HepG2 by bM24-T was much lower than that by bM24-TE. In LS1034 cells, in which both the T and TE promoters exhibited strong transcriptional activity, the level of ICP4 induction was unexpectedly low compared with SW480 and HepG2, and so was the induction by *hr*R3. ICP4 expression was undetectable by immunoblot in CRC cell lines LoVo and HT29 and non-CRC cell line A549. The inactive promoter control virus bM24-null induced no detectable ICP4 in all cell lines. These results suggest that strong promoter activity is necessary, but not sufficient, for efficient induction of ICP4; some other cellular factor(s) may also affect the level of ICP4.

Recombinant viruses bM24-T and bM24-TE selectively replicate in and kill tumor cells with highly activated β-catenin/Tcf signaling. Replication of bM24-T and bM24-TE viruses was examined by virus burst assay on different tumor cell lines. Both viruses replicated well on SW480 cells, and at 20 hours postinfection, virus yield had plateaued (Fig. 3*A*). In three other cell lines with highly activated β-catenin/Tcf signaling (HepG2, Caco-2, and SW620), bM24-TE replicated well and significantly better than bM24-T (Fig. 3*B*). The replication of both viruses in LS1034 was not efficient, as would be expected from the poor ICP4 induction (Fig. 2*B*). It has been reported that a transcriptionally targeted HSV vector did not replicate well on cells that exhibited a longer population doubling time (23); therefore, we compared the population doubling time of LS1034 with those of other cell lines (Supplementary Fig. S2) and found that it was ∼50% longer than for SW480 or HepG2, which might have contributed to the poor growth of bM24-TE in LS1034. bM24-T and bM24-TE did not replicate efficiently in two CRC cell

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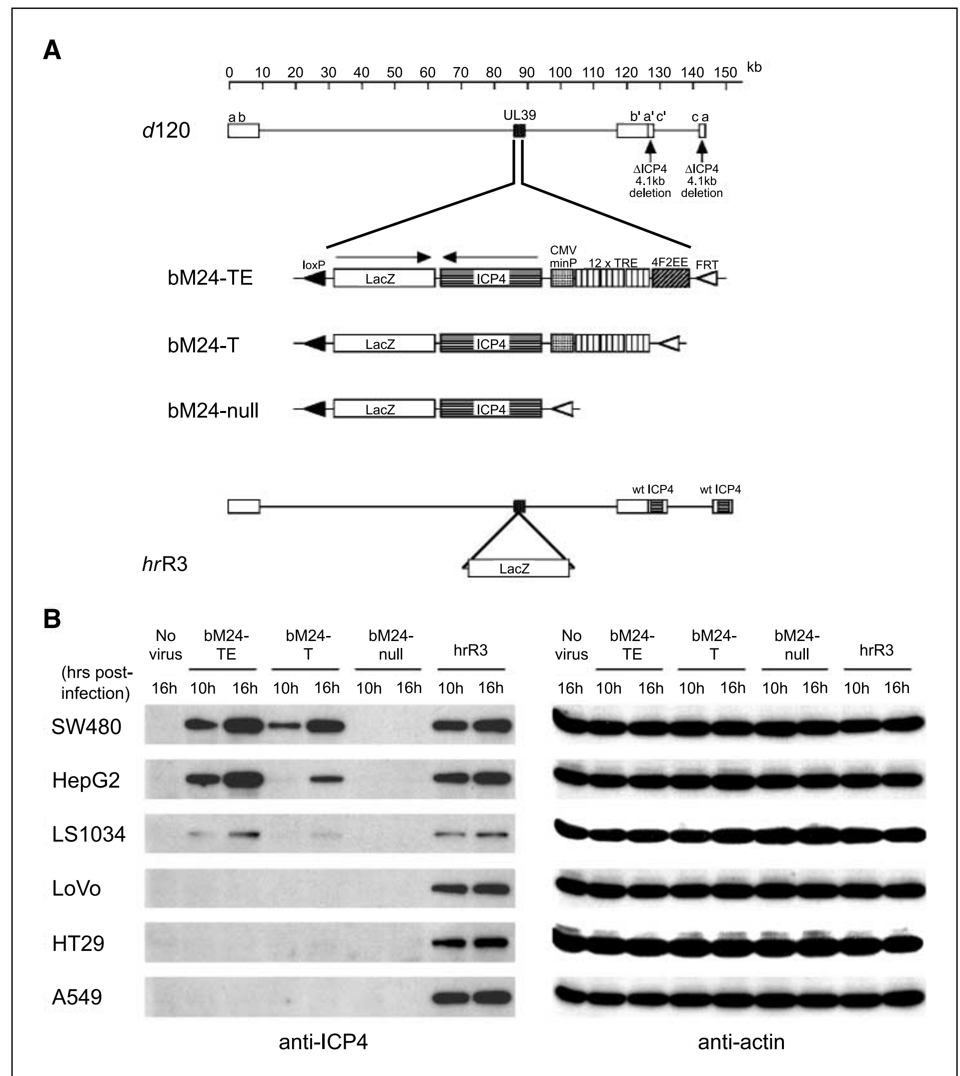

Figure 2. *A,* schematic presentation of recombinant HSV vectors used in this study. d120 is an ICP4-deletion mutant HSV and the parental strain of the transcriptionally targeted vectors. Three transcriptionally targeted vectors were generated using the Flip-Flop HSV-BAC system, by which an ICP4 expression cassette and the *LacZ* marker gene were inserted into the *UL39* gene locus of the parental virus. *UL39* encodes ICP6, the large subunit of the viral ribonucleotide reductase. bM24-T and bM24-TE carry an ICP4 expression cassette driven by the T and TE promoters, respectively. bM24-null is an inactive promoter control virus carrying a promoterless ICP4 cassette.[1] loxP (*closed triangle*) and FRT (*open triangle*) are site-specific recombinase recognition sites used for the construction of viruses in the Flip-Flop HSV-BAC system. *hr*R3 is a mutant HSV strain in which the ICP6 gene is inactivated by the insertion of the *LacZ* marker gene and carries diploid copies of the wild-type *ICP4* gene. *B,* immunoblot analysis of ICP4 protein expression in different cell lines infected with the recombinant viruses. *Left,* ICP4 protein was only detected in cells exhibiting high levels of β-catenin/Tcf signaling activity (SW480, HepG2, and LS1034) after infection with bM24-T or bM24-TE or in all cells after infection with *hr*R3. *Right,* equal loading of proteins was confirmed by probing the same filter with anti-actin antibody.

lines (LoVo and HT29) or other cancer cell lines, which had moderate to low β-catenin/Tcf activity (Fig. 3*B*). The inactive-promoter control virus bM24-null replicated in none of the cancer cell lines whereas *hr*R3 replicated efficiently in all of these cells, and all four viruses replicated very well in ICP4-complementing E5 cells (Fig. 3*B*). In normal fibroblasts (MRC5) and HUVEC, the yields of both bM24-TE and bM24-T were >2.5 log lower than that of *hr*R3 (Fig. 3*C*).

Cytotoxicity of the recombinant viruses was examined by infecting tumor cells with a low MOI of virus (0.05). As shown in Fig. 4, both bM24-T and bM24-TE effectively killed SW480 and HepG2 cells, with bM24-TE significantly more efficient than bM24-T. As a nonactive β-catenin/Tcf control, human U87MG glioblastoma cells were used because they are very susceptible to *hr*R3 (36). U87MG cells were not killed by either bM24-T or bM24-TE. Taken together, bM24-TE virus showed greater replication and cytotoxicity in highly β-catenin/Tcf–active tumor cells compared with bM24-T while retaining specificity. We therefore chose bM24-TE for further experiments.

Forced activation or suppression of β-catenin/Tcf signaling modulates bM24-TE virus replication. To test if replication of bM24-TE is actually regulated by β-catenin/Tcf activity in infected cells, we exogenously manipulated the signaling activity and examined changes in virus yield. Up-regulation of β-catenin/Tcf signaling was achieved by transiently transfecting constitutively active forms of β-catenin to 293T cells, in which the β-catenin/Tcf signaling pathway was inactive (TOP/FOP ratio, 0.97; refs. 9, 37; Fig. 5*A*). In mutant β-catenin–transfected cells, bM24-TE replicated 1 log more efficiently than in control transfected cells, whereas *hr*R3 replicated equally in both cells (Fig. 5*B*). Down-regulation of β-catenin/Tcf signaling was achieved by transfecting SW480 cells with siRNA oligonucleotides targeted to β-catenin (ref. 34; Fig. 5*C*). bM24-TE replicated 1 log less efficiently when β-catenin/Tcf siRNA was transfected, whereas *hr*R3 replication was not affected (Fig. 5*D*). These data show that the growth of bM24-TE is dependent on the activity of β-catenin/Tcf signaling in the infected cells.

bM24-TE shows antitumor efficacy in highly β-catenin/Tcf–active SW480 tumor xenografts but not in β-catenin/Tcf–inactive A549 tumors. *In vivo* antitumor efficacy of bM24-TE was examined using a human tumor xenograft model in athymic mice. Subcutaneous tumors were established with highly β-catenin/Tcf–active SW480 or β-catenin/Tcf–inactive A549 cells, which were subsequently treated with intratumorally injected

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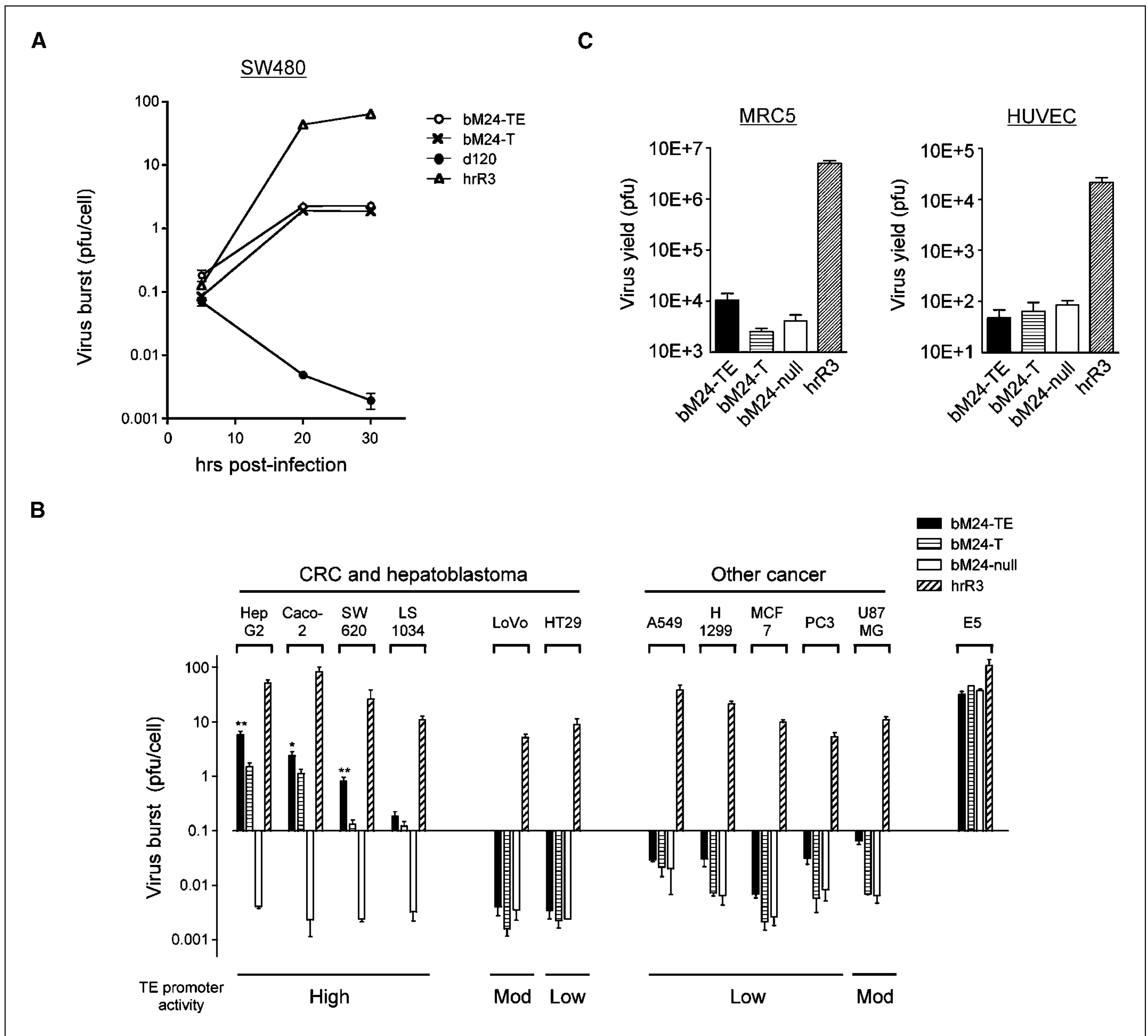

Figure 3. *A,* single-step growth experiment of recombinant viruses on SW480 cells that exhibit strong β-catenin/Tcf signaling activity. SW480 cells plated in 12-well plates were infected with indicated viruses at an MOI of 3 and progeny viruses harvested 5, 20, and 30 hours after infection and titered. *Points,* mean from triplicate wells; *bars,* SD. The virus yields of bM24-TE and bM24-T at 20 and 30 hours after infection were not significantly different. *B,* virus burst assay of the recombinant viruses on different cell lines. Cells were infected with indicated viruses at an MOI of 1.5 and progeny viruses were harvested 24 hours after infection and titered. CRC and hepatoblastoma cell lines with a mutated *APC* or *β-catenin* gene are shown on the left side of the graph, and other cancer cell lines on the right side. *hr*R3, which carries the wild-type *ICP4* gene, grew efficiently in all these cell lines and bM24-null carrying a promoterless *ICP4* gene did not grow in any of the cancer cell lines. In ICP4-complementing E5 cells, all the recombinant viruses grew very well. *Columns,* mean from triplicate wells; *bars,* SD. *, $P < 0.05$; **, $P < 0.01$, versus bM24-T. *Mod,* moderate. *C,* replication of recombinant viruses on normal MRC5 fibroblasts and HUVEC. Cells were plated in 12-well plates (MRC5, 6.4×10^4 cells; HUVEC, 5×10^4 cells) and infected with indicated viruses at an MOI of 1.5. Progeny viruses were harvested 24 hours after infection and titered. *Columns,* mean from triplicate wells; *bars,* SD.

bM24-TE or *hr*R3. Four intratumoral inoculations of 3×10^6 pfu of bM24-TE not only suppressed tumor growth and reduced the tumor volume in all animals (Fig. 6*A*) but also induced a complete response in four of the eight tumors. *In vivo* specificity is shown in that the same dose of bM24-TE failed to suppress A549 tumor growth (Fig. 6*B*). As expected, the nonspecific vector *hr*R3 was effective in both tumors, inducing a complete response in eight of eight SW480 and seven of eight A549 tumors.

Discussion

The aberrant up-regulation of the Wnt/β-catenin/Tcf pathway in CRCs and hepatoblastomas is an attractive target for tumor-specific therapy. Synthetic β-catenin/Tcf responsive promoter constructs have already been used for the targeted-expression of apoptosis and suicide genes and for the generation of transcriptionally targeted adenovirus and parvovirus vectors (19–21, 35, 38). Although cytotoxicities induced by these vectors were

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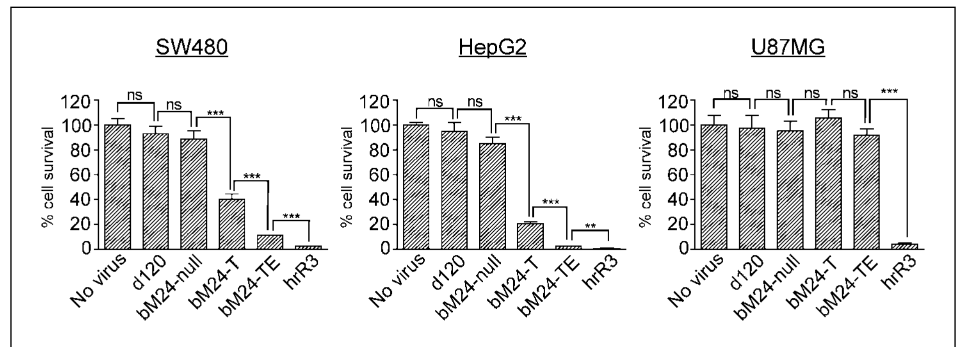

Figure 4. Cytotoxicity assay of recombinant viruses on cells with an active (SW480 and HepG2) or inactive (U87MG) β-catenin/Tcf pathway. Cells were plated in six-well plates and infected with indicated viruses at an MOI of 0.05. Five days after infection, cells were harvested by trypsinization and live cell counts were determined. *Columns,* mean from triplicate wells; *bars,* SD. **, $P < 0.01$; ***, $P < 0.001$.

specific to tumors with an activated β-catenin/Tcf pathway, the effectiveness of the treatments varied considerably due to variation in the degree of β-catenin/Tcf signal activation among these tumors (39). Thus, assessment of the magnitude of its activity in tumor cells is vital when we use this pathway for targeting in gene therapy.

For the analysis of β-catenin/Tcf signal activation, the TOPFLASH/FOPFLASH luciferase activity ratio has been the most commonly used (10). However, it does not necessarily reflect the actual level of transcription induced by β-catenin/Tcf responsive promoters. Therefore, we used luciferase activities normalized for transfection efficiency to estimate the transcriptional activities of the T and TE promoters in individual cell lines. Although these assays are prone to biases caused by variances in the activity of the cotransfected HSV-*tk* or other control promoters in each cell line, our luciferase assay results largely agreed with the results of ICP4 immunoblot and virus replication assay.

The T and TE promoters exhibited strong transcriptional activity in CRC cells carrying *APC* gene mutations between the first and second 20-amino-acid repeats (Fig. 1), indicating highly activated β-catenin/Tcf signaling in these cells. Previously, Rosin-Arbesfeld et al. (40) reported a rough inverse correlation between TOPFLASH activity and numbers of 20-amino-acid repeat retained in the truncations, which largely agrees with our present observations. These results suggest that truncated APC proteins that retain two 20-amino-acid repeats still have some activity to down-regulate

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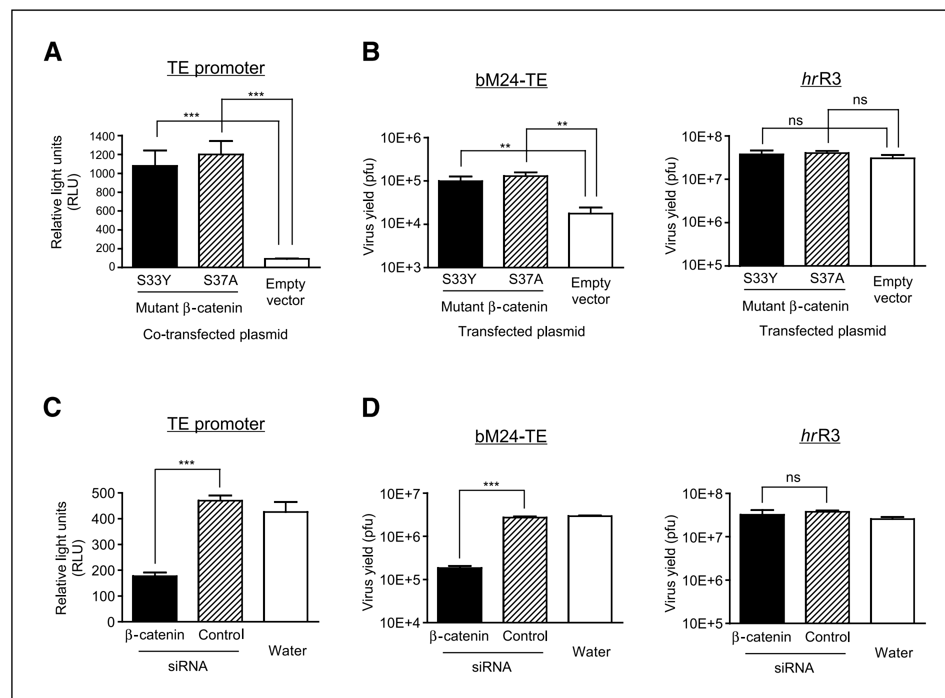

Figure 5. Forced activation or suppression of β-catenin/Tcf–dependent transcription modulates bM24-TE virus replication. *Columns,* mean from triplicate wells; *bars,* SD. **, $P < 0.01$; ***, $P < 0.001$. *A,* up-regulation of TE promoter activity in 293T cells cotransfected with constitutively active mutant β-catenin. 293T cells were cotransfected with reporter plasmids (100 ng of pGL3-T12E and 10 ng of pRL-TK) along with 100 ng of mutant β-catenin expression plasmid or empty vector. Twenty-four hours after transfection, cells were lysed and analyzed for luciferase activity. TE promoter activity was increased >10-fold by expression of mutant β-catenin compared with control. *B,* increase in bM24-TE virus yield from 293T cells transfected with mutant β-catenin. 293T cells (5×10^5 at the time of virus infection) were transfected with mutant β-catenin or empty vector (200 ng) and, 24 hours later, superinfected with bM24-TE or *hr*R3 virus at an MOI of 2. Progeny viruses were harvested 24 hours after superinfection and titered. *C,* suppression of TE promoter activity in SW480 cells transfected with β-catenin-specific siRNA. SW480 cells were first transfected with 20 pmol of siRNA duplex or water only and, 48 hours later, supertransfected with reporter plasmids (100 ng of pGL3-T12E and 10 ng of pRL-TK) using Lipofectamine 2000. Luciferase assay was done 24 hours after supertransfection. *D,* reduction in bM24-TE virus yield from SW480 cells transfected with β-catenin-specific siRNA. SW480 cells (6×10^5 at the time of virus infection) were transfected with siRNA (40 pmol) or water and, 48 hours later, superinfected with bM24-TE or *hr*R3 virus at an MOI of 1.5. Progeny viruses were harvested 24 hours after superinfection and titered.

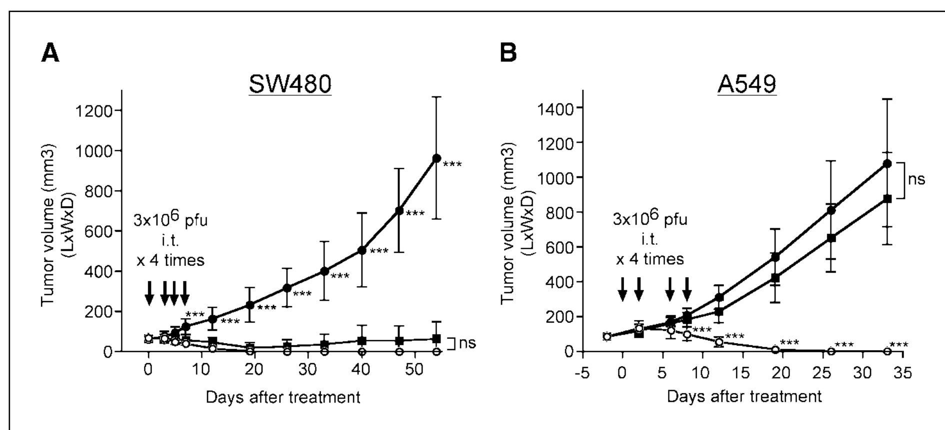

Figure 6. Treatment of human tumor xenografts in nude mice by intratumoral injection of bM24-TE or *hr*R3. *A,* intratumorally injected bM24-TE effectively reduced subcutaneous SW480 tumor mass. Subcutaneous SW480 tumors were established in the flanks of nude mice (eight mice per group), and when tumors reached ~5 mm in diameter, bM24-TE (■), *hr*R3 (○), or virus suspension buffer (●) was injected intratumorally four times on days 0, 3, 5, and 7. ***, $P < 0.001$, versus bM24-TE and *hr*R3. *Arrows,* injection of viruses. *B,* intratumorally injected bM24-TE failed to suppress subcutaneous A549 tumor growth. Subcutaneous A549 tumors were established and treated in the same manner as SW480, except that virus injection was done on days 0, 2, 6, and 8. ***, $P < 0.001$, versus bM24-TE and suspension buffer.

β-catenin compared with those retaining only one 20-amino-acid repeat. Recently, it was reported that β-catenin ubiquitination occurs in DLD-1 and HT29 cells, but not in SW480 cells (41), further supporting a difference between APCs with one or two 20-amino-acid repeats.

It is not clear why LS1034, which exhibited strong transcriptional activity from the TE promoter, did not support bM24-TE virus replication. It has been reported that a transcriptionally targeted HSV vector did not replicate well on cells with a longer population doubling time (23), which we found was longer in LS1034 than other cell lines (Supplementary Fig. S2). However, other cellular factors in LS1034 may also contribute to the low efficiency of ICP4 expression and bM24-TE viral replication in the cells.

Unfortunately, bM24-TE virus did not replicate efficiently in CRC cell lines containing an *APC* mutation after the second 20-amino-acid repeat (LoVo and HT29) due to the lower transcriptional activation of the TE promoter in these cell lines. Therefore, assessment of the nature of *APC* mutation and the level of β-catenin/Tcf signal activation may be important in clinical application. If measurement of β-catenin/Tcf signaling in the tumor cells obtained from patients proves difficult, an alternative approach would be to infect biopsy samples with virus carrying a marker gene driven by the TE promoter *ex vivo* and to measure the level of marker gene expression (42). Even if strong transcriptional activity is obtained, however, bM24-TE could not replicate efficiently in LS1034 cells. Therefore, perhaps a more straightforward method to assess the susceptibility of the tumor to bM24-TE would be to measure virus growth by infecting the CRC biopsy sample with bM24-TE.

The previously reported HSV vectors carrying a transcriptionally targeted *ICP4* gene had an inactivated HSV-*tk* gene, which compromised their suitability for clinical use (23, 24). In contrast, it should be noted that bM24-TE carries the intact HSV-*tk* gene and shows very good sensitivity to acyclovir (data not shown), as would be expected from an ICP6 mutant (25, 43). This is an important feature when we consider application of the vector in humans, so that viral replication can be terminated if undesired viral growth should occur in a patient.

In summary, four CRC cell lines carrying an *APC* mutation between the first and second 20-amino-acid repeats and one hepatoblastoma cell line carrying a mutant β-catenin showed strong transcriptional activation from the synthetic TE promoter. bM24-TE vector specifically replicated in and killed these tumor cells except LS1034 and induced a complete response in half of the SW480 xenografts in an *in vivo* tumor model. Because this vector retains very good acyclovir sensitivity, it should be further studied and considered for a clinical trial in patients with CRC or hepatoblastoma exhibiting strong β-catenin/Tcf activity.

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