A Mammalian Two-Hybrid System for Adenomatous Polyposis Coli-Mutated Colon Cancer Therapeutics

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Abstract
Colon cancer cells frequently lose expression of the tumor suppressor adenomatous polyposis coli (APC). As result, β-catenin accumulates and activates transcription of Tcf-responsive genes. Here we describe a novel mammalian two-hybrid system that selectively kills APC-mutated cells. This system consists of GAL4/β-catenin, VP16/Tcf4, and a gene that is transcribed when GAL4 and VP16 associate. In APC-mutated human colon cancer cells, such as SW480, GAL4/β-catenin accumulates, and in the presence of VP16/Tcf4, induces high levels of expression of the reporter gene. Expression of wild-type APC reduced GAL4/β-catenin and intact β-catenin levels and inhibited reporter gene expression. In colon cancer cells such as SW480 that have wild-type APC, GAL4/β-catenin was degraded, and expression levels of the output gene were low. Replacement of the reporter gene with a suicide gene resulted in selective killing of SW480 cells. This system may be applicable for broader use of gene therapy by targeting diseases that involve protein degradation.

Introduction
Mutations in the APC tumor suppressor gene occur in familial adenomatous polyposis patients (1, 2), and in sporadic colorectal cancer (3). APC is necessary for complex formation between GSK-3β and β-catenin (4) and degradation of cytoplasmic β-catenin via the ubiquitin-proteasome pathway (5, 6). Functional loss of APC by genetic mutation in colorectal cancer, therefore, causes accumulation of β-catenin (7). The fact that colorectal cancer cells that retain wild-type APC have mutations in the GSK-3β phosphorylation site of β-catenin, which also stabilizes β-catenin protein in the cells (8), implies that stabilization of β-catenin is one of the major consequences of APC loss. Moreover, in mouse models, both APC-mutant mice (9) and β-catenin-mutated mice (10) form intestinal polyps. In humans, mutations in APC are observed at early stages of colon cancer development even before ras or p53 mutation (11).

β-Catenin that accumulates in colorectal cancer cells forms complexes with transcription factor Tcf4 (12, 13) and facilitates expression of Tcf/lef-1-dependent gene expression such as cyclin D1 (14), c-myc (15), and peroxisome proliferator-activated receptor δ (16).

The yeast two-hybrid system has been used to study protein-protein interactions or to identify interacting partners for many proteins (17). This system consists of basically three components. The first component of Tcf/lef-1-dependent gene expression such as cyclin D1, which also stabilizes β-catenin via the ubiquitin-proteasome pathway, induces high levels of expression of the reporter gene. Expression of wild-type APC reduces GAL4/β-catenin and intact β-catenin levels and inhibits reporter gene expression. In colon cancer cells such as SW480 that have wild-type APC, GAL4/β-catenin was degraded, and expression levels of the output gene were low. Replacement of the reporter gene with a suicide gene resulted in selective killing of SW480 cells. This system may be applicable for broader use of gene therapy by targeting diseases that involve protein degradation.

Materials and Methods
Cells. Human cancer cell lines were obtained from The American Type Culture Collection (Rockville, MD). Human colon cancer cell lines SW480 and SW48 were maintained in L-15 medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% heat-inactivated FBS (Life Technologies, Inc.) in a 0.8% CO₂ incubator at 37°C. The human osteosarcoma cell line U2-OS and embryonic kidney cell line 293 were maintained in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% FBS in a 5% CO₂ incubator at 37°C.

Plasmids. The pBIND, pBIND/Id, pACT, and pG5Luc plasmids were purchased from Promega Corp. (Madison, WI). pp53-EGFP was purchased from Clontech (Palo Alto, CA). The GAL4 fusion β-catenin expression plasmid, pBIND/β-catenin, and VP16 fusion Tcf4 expression plasmids, pACT/ Tcf4, were prepared as follows. The human β-catenin cDNA with BamHI linker was prepared with PCR and cloned into BamHI site of pBEND. The human Tcf4 cDNA with BamHI linker was prepared with PCR and cloned into BamHI site of pBEND. The pcDNA3/pp53-EGFP plasmid was purchased from Promega Corp. (Madison, WI). The p53-Luc, pAP-1-Luc, and pCRE-Luc plasmids were purchased from Stratagene (La Jolla, CA). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: APC, adenomatous polyposis coli; EGFP, enhanced green fluorescent protein.
transfection efficiency was normalized by Renilla luciferase activity, which was simultaneously expressed from the pBIND plasmids.

**Fluorescence-activated Cell Sorter Analysis.** The pG5/p53-EGFP or pG5/EGFP plasmid was cotransfected with pBIND/WT-β-catenin and pACT/FL-Tcf4 plasmids to SW480 cells. After 48–72 h incubation, cells were trypsinized and washed twice with PBS. Cells were suspended in PBS containing 10 μM propidium iodide and analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Cell viability profile of EGFP-positive cells was analyzed by the uptake of propidium iodide.

**Results**

**Functional Expression of Two-Hybrid Proteins in a Colon Cancer Cell Line** We constructed four fusion protein expression plasmids, pBIND/WT-β-catenin, pBIND/MT-β-catenin, pACT/FL-Tcf4, and pACT/DN-Tcf4, which express GAL4/WT-β-catenin, GAL4/MT-β-catenin, VP16/FL-Tcf4, and VP16/DN-Tcf4, respectively (Fig. 2A). GAL4/WT-β-catenin is a fusion protein that consists of the DNA binding domain of yeast GAL4 protein and full-length human β-catenin. GAL4/MT-β-catenin has a truncated form of β-catenin, which lacks the GSK-3β recognition site. VP16/FL-Tcf4 is a fusion protein that consists of the transcription activation domain of herpes simplex virus VP16 and full-length human Tcf4. VP16/DN-Tcf4 has a truncated form of Tcf4, which lacks the β-catenin binding site. We transfected pBIND/WT-β-catenin, pBIND/MT-β-catenin, pACT/FL-Tcf4, or pACT/DN-Tcf4 to SW480 cells and detected those fusion protein expression levels using immunoblotting. Expressed GAL4/β-catenin fusion proteins were recognized by both anti-GAL4 and anti-β-catenin antibodies, as well as VP16/Tcf4 proteins, which were recognized by both anti-VP16 and anti-Tcf4 antibodies (Fig. 2B). The cotransfection of pBIND/WT-β-catenin, pACT/FL-Tcf4, and pG5/Luc led to an expression of the output gene, firefly luciferase (Fig. 2C). This output gene expression level was strictly controlled by the expression of the GAL4/β-catenin and VP16/FL-Tcf4 combination, because the replacement of pBIND/WT-β-catenin plasmid to pBIND/Id or pACT/FL-Tcf4 plasmid to pACT/DN-Tcf4 failed to increase firefly luciferase activity.

**Regulation of β-Catenin/Tcf4 Two-Hybrid System by APC.** We next examined whether the GAL4/WT-β-catenin protein was degradable in an APC-dependent manner. Wild-type APC expression plasmid was cotransfected with pBIND/WT or MT-β-catenin, and pACT/FL-Tcf4 and pG5/Luc activity was measured. The addition of APC in the pBIND/WT-β-catenin system resulted in a drastic decrease of firefly luciferase activity, whereas pBIND/MT-β-catenin system was not affected by APC expression (Fig. 3A). Next, the protein levels of GAL4/WT-β-catenin and myc-tagged WT-β-catenin protein levels were reduced, whereas neither GAL4/MT-β-catenin nor myc-tagged MT-β-catenin protein levels were changed (Fig. 3B). Neither VP16/Tcf4 nor intact Tcf4 protein levels were affected by APC expression.

**Regulation of the β-Catenin/Tcf4 Two-Hybrid System in Various Cell Types.** To confirm these results, we performed the two-hybrid assay using human osteosarcoma U-2OS and human embryonic kidney 293 cells, which express wild-type APC. We examined the expression levels of cytoplasmic β-catenin and APC first (Fig. 4A). Both U-2OS and 293 showed low levels of cytoplasmic β-catenin. These two cell lines also showed an APC band at the...
expected molecular size. These results indicated that the degradation mechanism of \( \beta \)-catenin was intact in these cell lines. SW480, in which APC is mutated, did not show an APC band at the expected size. SW48, in which \( \beta \)-catenin is mutated, exhibited high levels of \( \beta \)-catenin despite the presence of wild-type APC. Fig. 4B shows the expression levels of GAL4/WT-\( \beta \)-catenin and VP16/Tcf4 in SW480 Cells. Cells were transfected with pBIND/\( \beta \)-catenin or pACT/Tcf4 plasmid and cultured for 24 h. Then expression levels of fusion proteins were detected by Western blot. C, specificity of the two-hybrid system. SW480 cells were transfected with various combinations of GAL4 fusion protein, VP16 fusion protein, and pG5/luc plasmids and cultured for 24 h. The luciferase activity of each sample was measured by a luminometer. WT, wild type; MT, mutated; FL, full length; DN, dominant negative.

**Application of the Mammalian Two-Hybrid System to in Vitro Gene Therapy.** We next switched the output gene from luciferase to p53-EGFP. We expressed p53-EGFP in SW480 cells in the presence of a p53-responsive element-driven luciferase plasmid to examine whether the p53-EGFP fusion protein was functionally equivalent to wild-type p53. Transfected p53-EGFP selectively activated the p53-responsive element-driven luciferase, whereas negative control lacZ did not activate p53-responsive element-driven luciferase (Fig. 5A). p53-EGFP expression was observed as a green light under the fluorescence microscope (data not shown) and was also recognized as a Mr 80,000 protein by anti-p53 antibody (Fig. 5B). The viability of those p53-EGFP-expressing cells was analyzed by flow cytometry. The two-hybrid plasmid-transfected cells were harvested after 48 or 72 h incubation. About 20% of the pG5/p53-EGFP plasmid-transfected cells survived, whereas \( \sim \)80% of the pG5/EGFP-transfected cells survived (Fig. 5C). In this experiment, pG5/EGFP-transfected cells showed a higher intensity of GFP light than pG5/p53-EGFP-transfected cells (data not shown).

**Discussion**

Gene therapy offers the promise of selective tumor cell killing based on molecular defects that cause cancer. Many technical issues must be resolved before the full potential of gene therapy is realized, but there is reason to believe that this will be a successful therapeutic approach in the future (19).
In this report, we describe a novel approach that could be applied to kill tumor cells selectively. This approach targets cancer cells that are caused by a specific protein-degradation disorder and uses the mammalian two-hybrid system to control suicide gene expression. Accumulation of β-catenin in the cytoplasm occurs in most colon cancer cells, and ~80% of those are caused by dysfunctional APC. Our system takes advantage of the fact that β-catenin is not degraded efficiently in APC-mutated cells, but the principle could be used in other disorders that involve abnormal protein degradation. This system can also be used to identify signaling pathways that regulate association of β-catenin and Tcf as well as forming the basis of a screen for inhibitors of this interaction. Indeed, the mammalian two-hybrid system has general utility for monitoring protein-protein interactions in cells and manipulating these interactions for therapeutic purposes.

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References


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