

### Short Technical Report

# Fluorescence-Based Functional Assay for Wnt/ $\beta$ -Catenin Signaling Activity

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#### ABSTRACT

Aberrant activation of  $\beta$ -catenin signaling has been implicated in the development of human cancers. As a Wnt signal transducer,  $\beta$ -catenin forms a complex with the lymphocyte enhancer-binding factor/T cell factor transcription factor and activates downstream targets that promote cell proliferation. Here we developed a Wnt-dependent  $\beta$ -catenin-mediated heterologous transactivation system, which consisted of a chimeric transcription factor constructed by fusing the GAL4 DNA-binding domain with the full-length  $\beta$ -catenin, and a GAL4-responsive reporter expressing GFP. The chimeric transcription factor was highly unstable and exerted no detectable transactivating effect on the GAL4-responsive reporter. However, lithium and Wnt1 significantly stabilized this chimeric transactivator, indicating that this transactivation system is regulated by  $\beta$ -catenin in a Wnt-responsive fashion. Thus, this transactivation system could be used as a functional reporter to identify potential upstream factors that deregulate  $\beta$ -catenin signaling during tumorigenesis, as well as to screen for potential anti-cancer agents that specifically inhibit  $\beta$ -catenin signaling in human tumors.

#### INTRODUCTION

$\beta$ -catenin is an essential signal transducer of the Wnt/Wingless pathway (2,27). Wnt ligands initiate the signaling pathway by binding to the *frizzled* receptors and co-receptors low-density lipoprotein receptor-related protein (LRP)5 and LRP6, leading to phosphorylation of the *disheveled* protein. It then associates with Axin and the adenomatous polyposis coli (APC) tumor suppressor, preventing glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ) from phosphorylating  $\beta$ -catenin. Unphosphorylated  $\beta$ -catenin escapes recognition by  $\beta$ -transducin repeat-containing protein (TrCP)/proteasome complex and eventually translocates to the nucleus where it engages transcription factors lymphocyte enhancer-binding factor (LEF)/T cell factor (TCF)4 to activate the expression of downstream genes, such as c-Myc and cyclin D1 (8,18,22,25).

The involvement of  $\beta$ -catenin in tumorigenesis was first established in colorectal cancer (10,18), where  $\beta$ -catenin was found to form a complex with the APC tumor suppressor (10). The importance of  $\beta$ -catenin in tumorigenesis has been further highlighted by the discovery of oncogenic mutations of the  $\beta$ -catenin gene in colon cancers containing the wild-type APC gene (14,19). Mutant  $\beta$ -catenin protein becomes more stable because it is capable of bypassing APC-targeted degradation. Subsequently,  $\beta$ -catenin mutations have been uncovered in a variety of human tumors (17). Thus, the collective genetic data strongly suggest that deregulation of  $\beta$ -catenin signaling could be involved in the development of many human malignancies. In fact,

oncogenic  $\beta$ -catenin has been shown to induce tumor formation in transgenic animals (6,7), whereas  $\beta$ -catenin mutations have been frequently detected in tumors induced by either carcinogens or activated oncogenes (4,26). Thus, the deregulated  $\beta$ -catenin signaling pathway constitutes a logical target for anti-cancer therapy.

In this study, we sought to develop an assay system that could assess the functional status of  $\beta$ -catenin signaling. Specifically, to establish a Wnt-regulated  $\beta$ -catenin-mediated heterologous transactivation system, we constructed a chimeric transcription factor by fusing the yeast GAL4 DNA-binding domain with the full-length human  $\beta$ -catenin. The reporter expressed GFP driven by a GAL4-responsive promoter. Our data indicated that this heterologous transactivation system is regulated by  $\beta$ -catenin in a Wnt-responsive fashion. Thus, this fluorescence-based assay system can be used as a functional reporter to identify potential upstream factors that deregulate  $\beta$ -catenin signaling during tumorigenesis and to screen for potential anti-cancer agents that specifically inhibit  $\beta$ -catenin signaling.

#### MATERIALS AND METHODS

##### Cell Culture and Chemicals

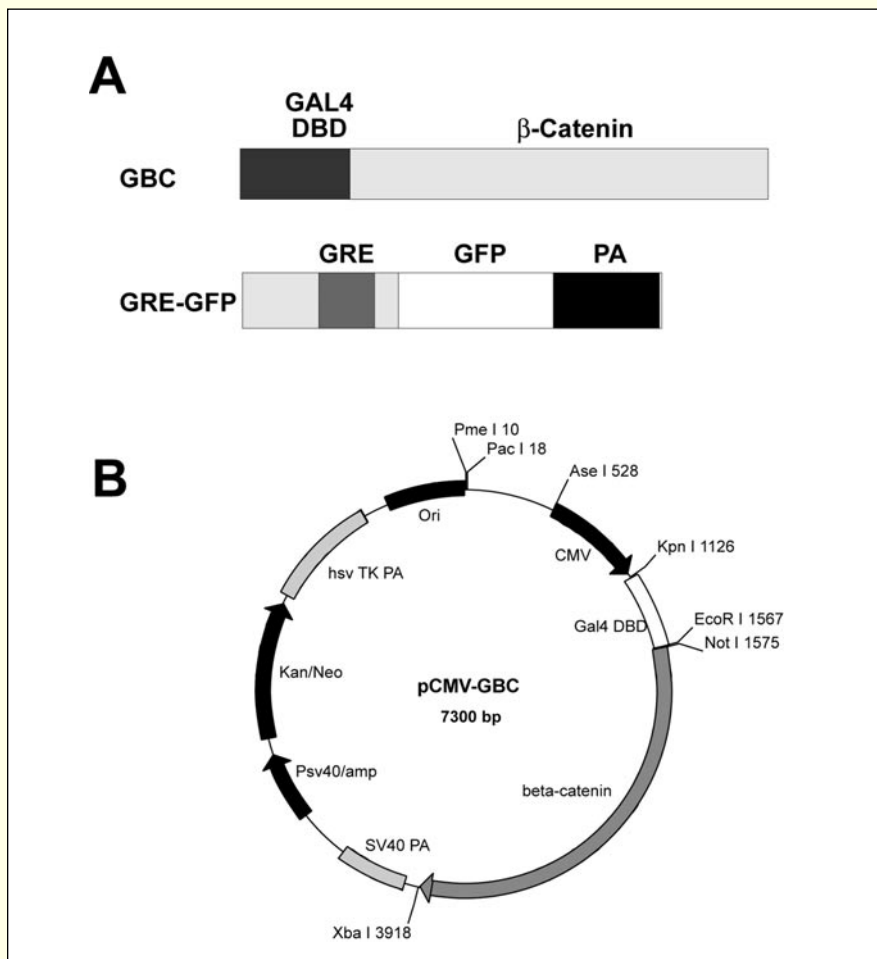
Human embryonic kidney 293 cell line, human colon cancer line HCT116, human osteosarcoma line 143B, and human chondrosarcoma line HTB-94 were obtained from the ATCC (Manassas, VA, USA). Human osteosarcoma line MNNG/HOS was kindly provided by Dr. Sabine Krueger of University of

Magdeburg, Germany (13). HTB-94 and 293 cells were maintained in complete DMEM supplemented with 10% FBS (Mediatech, Herndon, VA, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in 5% CO<sub>2</sub>. HCT116 cells were maintained in complete McCoy's 5A medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in 5% CO<sub>2</sub>. MNNG/HOS and 143B lines were maintained in complete Minimum Essential Medium Eagle supplemented with 10% FBS, 2 mM L-glutamine (Mediatech), 1× nonessential amino acids (Mediatech), 1 mM sodium pyruvate (Mediatech), 100 U/mL penicillin, and 100 µg/mL

streptomycin at 37°C in 5% CO<sub>2</sub>. Unless indicated otherwise, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Fisher Scientific (Pittsburgh, PA, USA).

### Construction of a GAL4-β-Catenin (GBC) Chimeric Transcription Factor and GAL4-Responsive GFP Reporter (GRE-GFP)

The DNA-binding domain (i.e., amino acids 1–147) of the yeast transcription factor GAL4 was amplified by PCR with the following pair of primers: 5'-CGGGGTACCACCATGGGCATGAAGCTACTGTCTTCTATC-3' and 5'-TTTGCGGCCGCGAATTC-



**Figure 1. Construction of a Wnt-regulated β-catenin-mediated heterologous transactivation system.** (A) Structural representation of the two components of the GEM system. GBC was constructed by fusing the GAL4 DNA-binding domain (GAL4 DBD, amino acids 1–147) with the full-length human β-catenin. As a reporter, GRE-GFP expressed GFP under the control of GRE. (B) Schematic representation of the expression vector for the chimeric GBC. The expression of GBC was driven by a CMV promoter. This vector also contained a kanamycin/neomycin expression cassette driven by a prokaryotic and eukaryotic hybrid promoter, Psv40/amp, which conferred kanamycin resistance in bacterial cells and geneticin resistance in mammalian cells, respectively. PA, polyadenylation signal.

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CGGCGATACAGTCAACTG-3'. The amplified fragment was subcloned into the *KpnI* and *NotI* sites of pCMV-NK3, resulting in pCMV-NK3-G4DB. To fuse the  $\beta$ -catenin in-frame with the GAL4 DNA-binding domain, the following primers were used to amplify the N-terminal portion (i.e., amino acids 1–90) of  $\beta$ -catenin: 5'-CCGGAA-TTCGGCATGGCTACTCAAGCTGATTTG-3' and 5'-GAGCTCGAGTCAT-TGCATACTGTC-3'. The amplified fragment was subcloned into the *EcoRI* and *XhoI* sites of pCMV-NK3-G4DB, followed by inserting an *XhoI/XbaI* fragment containing the rest of the  $\beta$ -catenin coding sequence (i.e., amino acids 91–781). The resultant construct was designated pCMV-GBC (Figure 1). A reporter vector, pGRE-GFP, which expressed GFP under the control of a GAL4-responsive promoter, was generously provided by Bert Vogelstein of Johns Hopkins Oncology Center (3). All PCR-amplified fragments and cloning junctions were verified by DNA sequencing.

### Establishment of 293-GEM Stable Cell Lines

Exponentially growing HEK 293 cells were co-transfected with 1  $\mu$ g pGRE-GFP plasmid DNA and 0.2  $\mu$ g pCMV-GBC plasmid per 25-cm<sup>2</sup> tissue culture flask by using LipofectAMINE™ according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). At 24 h after transfection,

cells were trypsinized and replated into 96-well cell culture plates at multiple dilutions. Stable clones were selected in the presence of G418 at a final concentration of 0.3 mg/mL for two weeks. Clones derived from single cells were grown up for further characterization.

### Western Blotting Analysis

Cleared total cell lysate was denatured by boiling the samples in Laemmli sample buffer and were loaded on to a 4%–20% gradient or 8% SDS-polyacrylamide gel (approximately 10  $\mu$ g total protein/lane). After being resolved by electrophoresis, proteins were transferred to an Immobilon™-P membrane (Millipore, Bedford, MA, USA) via electroblotting. The membrane was blocked with 5% nonfat milk in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween® 20) at room temperature for 1 h and probed with an anti- $\beta$ -catenin antibody (Transduction Laboratories, Lexington, KY, USA) or anti-HA antibody (Roche Applied Science, Indianapolis, IN, USA) for 60 min, followed by a 30-min incubation with an anti-mouse IgG secondary antibody conjugated with HRP (Pierce Chemical, Rockford, IL, USA). The presence of GBC fusion protein or HA-tagged Wnt-1 was detected by using the SuperSignal® West Pico Chemiluminescent Substrate kit (Pierce Chemical) and recorded by using a Kodak 440<sub>CF</sub> Image Station.

### Construction of a Recombinant Adenovirus Expressing Wnt1 and Preparation of Wnt1-Conditioned Medium

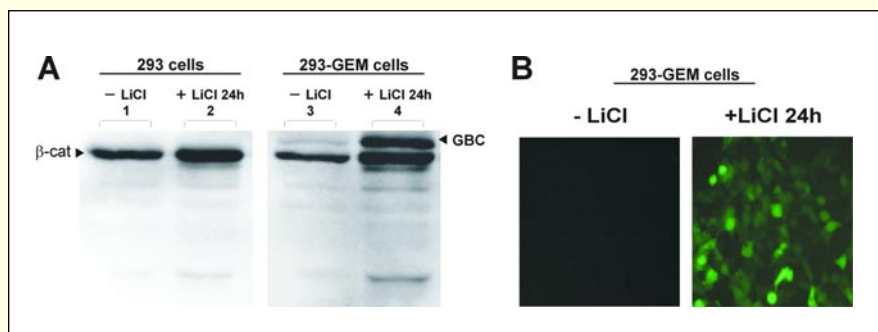
The cDNA encoding mouse Wnt1 was tagged with an HA epitope at its C-terminus and was subcloned into the pAdTrack-CMV vector, resulting in pAdTrack-Wnt1. Recombinant adenovirus expressing Wnt1 (i.e., AdWnt1) was generated as previously described (9). It is noteworthy that the AdWnt1 virus also expressed GFP, allowing easy detection of gene transduction efficiency upon infection. To prepare Wnt1-conditioned medium, the purified AdWnt1 was used to infect exponentially growing HCT116 cells in 75-cm<sup>2</sup> tissue culture flasks (MOI = 20). Adenovirus-containing medium was removed at 6 h after infection and replaced with 10 mL DMEM per 75-cm<sup>2</sup> flask. The Wnt1-conditioned medium was collected at 24 h and, after passing through 0.2- $\mu$ m filters, was kept at 4°C.

### FACS® Analysis

Exponentially growing 293-GEM cells were exposed to either Wnt1-conditioned medium or a control medium derived from AdGFP-infected cells. Cells were collected by trypsinization at 0, 24, and 48 h after stimulation and were resuspended in complete DMEM medium at approximately 5  $\times$  10<sup>5</sup> cells/mL. Cells were subjected to FACSscan™ (BD Biosciences, San Jose, CA, USA) with the signal gated to measure specific GFP-mediated fluorescence. Signal threshold was determined by using the parental 293 cells. Approximately 10 000 cells were analyzed to determine their mean fluorescence intensity.

### Luciferase Assays

Exponentially growing 293-GEM cells were seeded in 12-well cell culture plates and transfected with 1  $\mu$ g pTOP-Luc plasmid (i.e., a TCF4 responsive reporter) (8,14) per well using LipofectAMINE. At 15 h after transfection, Wnt1-conditioned medium was added to the transfected cells. Conditioned medium derived from the AdGFP-infected HCT116 cells was used as a control. At 24 h after stimula-



**Figure 2. Effect of lithium chloride on 293-GEM stable cell lines.** (A) Stabilization of chimeric GBC transcription factor by lithium. Exponentially growing parental 293 cells and 293-GEM cells were stimulated with lithium chloride (30 mM) for 0 h (lanes 1 and 3) and 24 h (lanes 2 and 4). Total cell lysate was collected and subjected to 8% SDS-PAGE. The presence of the endogenous  $\beta$ -catenin and the chimeric GBC was probed with an anti- $\beta$ -catenin antibody followed by enhanced chemiluminescence detection. (B) Lithium induction of GFP expression in 293-GEM stable cell lines. Subconfluent 293-GEM cells were stimulated with lithium chloride (30 mM) for 24 h (+LiCl 24h). 293-GEM cells without lithium stimulation served as a control (-LiCl). Expression of GFP was recorded using fluorescent microscopy.

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tion, cells were lysed and collected for assays of luciferase activity using the Luciferase Assay kit (Promega, Madison, WI, USA). Each assay condition was done in triplicate.

### RESULTS AND DISCUSSION

#### Construction of a Chimeric GBC Transcription Factor by Fusing the GAL4 DNA-Binding Domain with Full-Length $\beta$ -Catenin

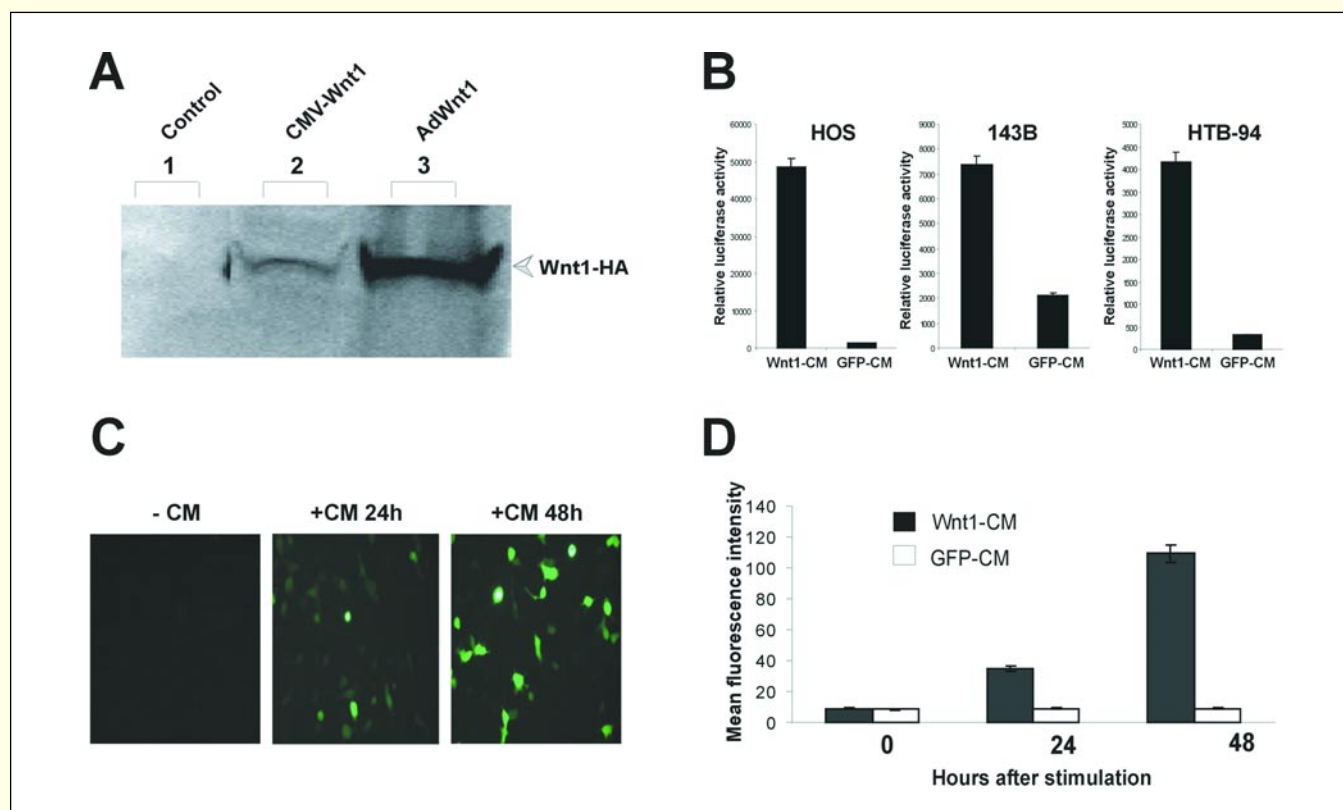
As an essential transducer of the Wnt signal,  $\beta$ -catenin exerts its biological effects by forming a complex with the LEF-1/TCF transcription factor family. In this transcription complex,  $\beta$ -catenin provides transactivating activi-

ty, and LEF-1/TCF provides sequence-specific DNA-binding activity. The transcription activity of the  $\beta$ -catenin/TCF complex is primarily affected by the stability of free  $\beta$ -catenin protein, which is negatively modulated by the tumor suppressor APC. In this study, we investigated whether  $\beta$ -catenin could regulate a heterologous transactivation system in a Wnt-dependent fashion. This heterologous transcription system consisted of two components, a chimeric transactivator and a GFP reporter. The chimeric transactivator (i.e., GBC) was engineered by fusing the DNA-binding domain (i.e., amino acids 1–147) of yeast transcription factor GAL4 with the full-length human  $\beta$ -catenin (Figure 1A). Within the resultant GBC chimeric transcription factor,

the  $\beta$ -catenin moiety conferred transactivation activity, while the sequence-specific DNA-binding ability was derived from the well-characterized yeast GAL4 DNA-binding domain. The expression of this chimeric GBC transcription factor was under the control of a CMV promoter (Figure 1B). As for the reporter plasmid GRE-GFP, the expression of GFP was under the control of a minimal promoter containing a GAL4-responsive element (Figure 1A).

#### Establishment and Characterization of Stable 293-GEM Cell Lines

To establish stable cell lines that contained both the chimeric transactivator and the reporter, we co-transfected pCMV-GBC and pGRE-GFP plasmids



**Figure 3. Effect of Wnt1-conditioned medium on 293-GEM stable cell lines.** (A) Recombinant adenovirus-mediated expression of Wnt1. Subconfluent HCT116 cells were either transfected with the pCMV-Wnt1 plasmid (lane 2) or infected with AdWnt1 (lane 3). AdGFP-infected HCT116 cells were used as a control (lane 1). At 24 h after transfection and infection, total cell lysate was collected and subjected to 4%–20% SDS-PAGE. The expression of the HA-tagged Wnt1 protein was detected with an anti-HA antibody using the enhanced chemiluminescence procedure. (B) Activation of a TCF4-responsive luciferase reporter by Wnt1-conditioned medium. Exponentially growing cells were transfected with pTOP-Luc reporter. At 15 h after transfection, cells were stimulated with either Wnt1-conditioned medium (Wnt1-CM) or a control medium derived from AdGFP-infected cells (GFP-CM) for additional 24 h. Cell lysate was collected and assayed for luciferase activity. Each assay condition was done in triplicate. (C) Induction of GFP expression in 293-GEM cells by Wnt1-conditioned medium. Subconfluent 293-GEM cells were exposed to Wnt1-conditioned medium. Expression of GFP was recorded at 24 h (+CM 24h) and 48 h (+CM 48h) after stimulation by using fluorescent microscopy. Unstimulated 293-GEM cells were used as a control (-CM). (D) FACS analysis of 293-GEM cells stimulated with Wnt1-conditioned medium. 293-GEM cells were exposed to either Wnt1-conditioned medium (Wnt1-CM) or a control medium derived from AdGFP-infected cells (GFP-CM). Cells were collected at indicated times and subjected to FACS analysis. See text for details.

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into 293 cells. 293 cells were chosen because the  $\beta$ -catenin signaling pathway is intact and the basal activity of this pathway is negligible. Stable clones derived from single cells were selected in the presence of G418 at a final concentration of 0.3 mg/mL. Resultant stable clones were designated 293-GEM lines. As shown in Figure 2A, the expression of the chimeric transactivator GBC was detectable at a very low level in a representative 293-GEM stable line (Figure 2A, lane 3). However, chimeric GBC expression was shown to significantly increase in the presence of lithium for 24 h (Figure 2A, lane 4), suggesting that the GBC fusion protein was effectively stabilized by lithium stimulation. It has been reported that lithium can mimic the Wnt signal by inhibiting the kinase activity of GSK3 $\beta$  (12,23). Accordingly, it was demonstrated that the endogenous level of  $\beta$ -catenin was shown to increase after lithium stimula-

tion (Figure 2A, lanes 2 and 4).

When the activity of GRE-GFP reporter was examined in the 293-GEM stable lines, the basal expression of GFP was not detected (Figure 2B). However, in the presence of lithium, GFP expression increased significantly in a time-dependent manner (Figure 2B). At 24 h of lithium stimulation, expression of GFP was detected in almost every cell of the 293-GEM lines, further suggesting that the GRE-GFP reporter in the 293-GEM cells could be effectively activated by lithium, a well-characterized Wnt surrogate.

### **Generation of Biologically Active Wnt1-Conditioned Medium Using a Recombinant Adenovirus Expressing Wnt1**

Although lithium was shown to activate the 293-GEM cells, it may also target other cellular processes. Thus,

we sought to test the effect of the Wnt1 protein on these cells. Wnt molecules are a large family of highly glycosylated proteins. The expression and purification of biologically active Wnt proteins from prokaryotic cells, insect cells, or mammalian cells have met with very limited success (1,11,16). Here we explored the possibility of using a recombinant adenoviral vector as a means of producing biologically active Wnt1 protein. Specifically, a cDNA fragment encoding the full-length mouse Wnt1 was subcloned into pAdTrack-CMV vector. A recombinant adenovirus (AdWnt1) was generated by using our recently developed AdEasy system (9). To facilitate the detection of exogenous Wnt1 expression, the Wnt1 coding sequence also contained an HA-tag at its C-terminus. It is noteworthy that the generated recombinant virus, AdWnt1, also expressed GFP as a tracking marker for infectivity

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and transduction efficiency. As shown in Figure 3A, AdWnt1-mediated expression of Wnt1 protein was readily detected by Western blotting in adenovirus-infected HCT116 cells. In fact, the expression level of HA-tagged Wnt1 in AdWnt1-infected cells was significantly higher (Figure 3A, lane 3) than the expression level of HA-tagged Wnt1 in the cells transfected with a conventional expression vector (Figure 3A, lane 2), suggesting that the adenoviral vector AdWnt1 exhibited much higher gene delivery efficiency.

The Wnt1-conditioned medium was prepared from AdWnt1-infected HCT116 cells and was tested for its ability to activate a TCF4-responsive luciferase reporter in three commonly used cell lines. Briefly, MNNG/HOS, 143B, and HTB-94 cells were transfected with a TCF4-responsive luciferase reporter plasmid (i.e., pTOP-Luc) for 15 h, followed by the addition of either Wnt1-conditioned medium or a control medium derived from AdGFP-infected

HCT116 cells. At 24 h after stimulation, the cell lysate was collected and subjected to luciferase activity assays. As shown in Figure 3B, the luciferase activity was significantly increased in the presence of Wnt1-conditioned medium in all three tested cell lines. Specifically, there was a 41.5-fold increase in MNNG/HOS, 3.4-fold increase in 143B, and 13.3-fold increase in HTB-94 cells. The varied magnitudes of activation may reflect differences in transfection efficiency and/or basal activity of  $\beta$ -catenin signaling in the tested cell lines. Nevertheless, our results demonstrated that the prepared Wnt1-conditioned medium was biologically active. In addition, the prepared Wnt1-conditioned medium was shown to be stable at 4°C for up to two months.

### Activation of 293-GEM Cells by Wnt1-Conditioned Medium

We next tested whether the prepared Wnt1-conditioned medium could induce GFP expression in 293-GEM cell lines. Briefly, subconfluent 293-GEM cells were exposed to Wnt1-conditioned medium for 24 and 48 h. As shown in Figure 3C, the Wnt1-conditioned-medium-induced GFP expression was readily detected at 24 h after stimulation, and a stronger GFP signal was observed at 48 hours. Furthermore, when 293-GEM cells stimulated with either Wnt1-conditioned medium or a control GFP-conditioned medium were subjected to FACS analysis, the mean fluorescence intensity was shown to have an approximately 2-fold increase at 24 h and a 12-fold increase at 48 h after Wnt1-conditioned medium stimulation, respectively (Figure 3D). Thus, our findings have demonstrated that the chimeric GBC-mediated transactivation of GRE-GFP reporter was indeed specifically regulated by the Wnt signal.

### 293-GEM Cells Can Be Used as a Functional Barometer for Wnt/ $\beta$ -Catenin Signaling

The activity of Wnt/ $\beta$ -catenin signaling is usually determined by measuring the transcriptional regulation of downstream target genes or a reporter driven by  $\beta$ -catenin/TCF-responsive promoter. In the latter case, such a re-

porter is usually driven by multiple copies of TCF-binding sites. Although this kind of reporter is easy to be constructed and generally works well in transient assays with sensitive reporter genes (e.g., luciferase), we have found that the  $\beta$ -catenin/TCF-responsive promoter is usually weak and exhibits high nonspecificity. In this study, by utilizing a chimeric transactivator between the GAL4-DNA-binding domain and the full-length human  $\beta$ -catenin, we demonstrated that this heterologous transcription system was tightly regulated by  $\beta$ -catenin in a Wnt signal dependent manner. Therefore, the reported 293-GEM system may serve as a biological barometer that measures the functional status of  $\beta$ -catenin signaling. Taken together, the development of the 293-GEM cells may have at least two important ramifications.

First, the 293-GEM cells can be used as a functional reporter to identify potential upstream factors that deregulate  $\beta$ -catenin signaling in human cancer. The transcription activity of  $\beta$ -catenin/TCF complex is primarily regulated by the stability of free  $\beta$ -catenin protein, which is negatively modulated by APC/Axin/GSK3 $\beta$  activity. Mutations of either  $\beta$ -catenin or its negative regulators (such as APC or Axin) would lead to the stabilization of  $\beta$ -catenin protein (17). In fact, oncogenic mutations of  $\beta$ -catenin have been detected in a broad range of human tumors, albeit at a low frequency (i.e., <10%) (18). Moreover, cytoplasmic and/or nuclear accumulation of  $\beta$ -catenin protein has also been frequently observed in many forms of human tumors that do not harbor  $\beta$ -catenin mutations (5,15,20,21,24), suggesting that deregulation of  $\beta$ -catenin may play an important role in human tumorigenesis. However, with the exception of colorectal cancer, the molecular mechanisms underlying  $\beta$ -catenin deregulation are not known in most human tumors. Thus, the 293-GEM cells can be used as a reporter for functional cloning studies to identify potential upstream factors that deregulate  $\beta$ -catenin signaling in human cancer.

Second, the 293-GEM cells can be used as a functional reporter to perform high-throughput screening for potential anti-cancer agents that specifically in-

hibit  $\beta$ -catenin signaling in human cancer. Given the fact that  $\beta$ -catenin signaling is frequently deregulated in human cancer, efficacious anti-cancer agents may be developed by specifically targeting this signaling pathway. In this study, we have demonstrated that the activation of the 293-GEM cells is tightly regulated by the Wnt signal. Therefore, it is conceivable that these cells can be used to screen for compounds or small peptides that antagonize the Wnt signal. Moreover, the built-in nature of the Wnt-regulated GFP expression in the 293-GEM cells would offer a high-throughput capability for drug discovery.

In summary, we have successfully demonstrated that  $\beta$ -catenin can transactivate a heterologous transcription system in a Wnt signal-dependent manner. The established 293-GEM cells can be used as a functional indicator of the Wnt/ $\beta$ -catenin signaling pathway. Thus, these cells could be utilized to dissect the molecular mechanisms underlying the deregulation of  $\beta$ -catenin signaling in human cancer and to screen for potential anti-cancer agents that specifically inhibit the  $\beta$ -catenin signaling pathway.

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