

# Wnt-1 but not Epidermal Growth Factor Induces $\beta$ -Catenin/T-Cell Factor-dependent Transcription in Esophageal Cancer Cells<sup>1</sup>

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## ABSTRACT

$\beta$ -Catenin plays an important role in signal transduction pathways that regulate cellular differentiation and proliferation. The increased concentration of this protein in the cytoplasm favors its binding to the T-cell factor (TCF) family of DNA-binding proteins, and it subsequently translocates to the nucleus, where it induces transcription of specific genes. We explored mechanisms that lead to activation of  $\beta$ -catenin/TCF-dependent transcription in esophageal squamous cell carcinoma (ESCC) independent of adenomatous polyposis coli and  $\beta$ -catenin mutation. Electrophoresis mobility shift assay demonstrated that TCF4 and  $\beta$ -catenin form a complex and have DNA binding activity. However, there was no constitutive activation of  $\beta$ -catenin/TCF-dependent transcription. Coculture experiments demonstrated that Wnt-1, but not Wnt-5A and Wnt-7A, activated the TCF reporter gene. Additionally, when cultured with Wnt-1-conditioned media, ESCC cell lines showed an accumulation of  $\beta$ -catenin in the cytoplasm. Although both Wnt and epidermal growth factor inactivate glycogen synthase kinase 3 $\beta$ , activation of epidermal growth factor receptor did not stabilize  $\beta$ -catenin. A comparison of extracellular stimuli suggests that specific Wnt family members stabilize  $\beta$ -catenin with resulting activation of TCF-dependent transcription in ESCC.

## INTRODUCTION

Human esophageal cancer is an aggressive tumor with a poor prognosis (1). Recently, accumulation of nuclear and cytoplasmic  $\beta$ -catenin has been observed in ESCC<sup>3</sup> (2, 3). It is involved in Wnt-Frizzled pathways that regulate cellular differentiation and proliferation (4, 5). In the absence of Wnt signals, cytoplasmic  $\beta$ -catenin is low because the protein is targeted for degradation by a multiprotein complex, which includes the APC protein, GSK3 $\beta$ , and conductin or axin. Mutations in the APC or  $\beta$ -catenin genes that interfere with  $\beta$ -catenin degradation cause accumulation of  $\beta$ -catenin protein (6–10). The increased concentration of this protein in the cytoplasm favors its binding to the TCF/LEF family of DNA-binding proteins, and it subsequently translocates to the nucleus, where it induces transcription of specific genes stimulating cell growth. Recently, *cyclin D1* (11, 12), *c-myc* (13, 14), *cyclooxygenase-2* (15), and *multidrug resistance 1* (16) have been identified as target genes of  $\beta$ -catenin. This mechanism has been proposed as an important step in colorectal carcinogenesis. Furthermore, *metalloproteinase matrilysin* (17) and *peroxisome proliferator-activated receptor  $\delta$*  (18) have been also identified as target genes of the  $\beta$ -catenin/TCF complex, and

these genes play an important role in the development and formation of some neoplasias (17, 19).

Oncogene and tumor suppressor gene alterations in ESCCs are observed frequently (20, 21). Among these alterations are overexpression of cyclin D1 and EGFR and mutation of p53 and p16. Although accumulation of nuclear and cytoplasmic  $\beta$ -catenin has been observed in ESCC, APC (3, 22) and  $\beta$ -catenin (3) mutations have not been found in ESCC. Therefore, another mechanism for cytoplasmic  $\beta$ -catenin accumulation might exist in ESCC.

GSK3 $\beta$  is a key enzyme that phosphorylates  $\beta$ -catenin at NH<sub>2</sub>-terminal serine threonine residues. GSK3 $\beta$  has also been shown to phosphorylate cyclin D1 on threonine 286 and lead to its ubiquitination and subsequent proteolysis (23). GSK3 inactivation is mediated by at least two pathways: (a) activated peptide growth factor receptor, including EGF, platelet-derived growth factor, and insulin, leads to activation of Akt, a protein kinase that phosphorylates and inactivates GSK3 $\beta$ ; and (b) activation of the Wnt pathway leads to inhibition of GSK3 $\beta$  through an unknown mechanism.

The Wnt signaling pathway is conserved in various organisms from *Caenorhabditis elegans* to mammals and plays important roles in development (24), cell proliferation (25), and cell movement (24, 25). Wnt signaling stabilizes cytoplasmic  $\beta$ -catenin. More recently, the Frizzled family of seven transmembrane proteins has been shown to act as a receptor for Wnt proteins and therefore may be involved in cell migration patterns. In particular, FZE3, which is a member of the human Frizzled gene family, was found to be specifically expressed in ESCC (26). However, whereas Wnt is a candidate for the regulation of cytoplasmic  $\beta$ -catenin, little is known with regard to the molecular relationship between Wnt and  $\beta$ -catenin in ESCC. There have been no studies to date that examine how Wnt family members regulate  $\beta$ -catenin in ESCC or gastrointestinal cancers.

In ESCC with EGFR overexpression, we have previously shown that EGF activates Akt (27) and the cyclin D1 promoter (28). Thus, in addition to Wnt-mediated effects, it is possible that there may be interplay between the EGFR signaling pathway and the Wnt signaling pathway through inhibition of GSK3 $\beta$ .

To examine the potential role of Wnt in the development and progression of ESCC, we show that Wnt-1 leads to accumulation of  $\beta$ -catenin in the cytoplasm, with resulting activation of  $\beta$ -catenin/TCF-dependent gene transcription. By contrast, activation of the EGFR pathway does not lead to  $\beta$ -catenin stabilization and accumulation.

## MATERIALS AND METHODS

**Plasmids.** pGL3-OT and pGL3-OF (gifts of Dr. Bert Vogelstein) are luciferase reporter constructs containing wild-type and mutated trimeric TCF binding sites, respectively (4). pCIneo- $\beta$ -catenin XL and pCIneo- $\beta$ -catenin  $\Delta$ 45 are expression vector constructs for wild-type and constitutively active mutant forms of  $\beta$ -catenin, respectively (gifts of Dr. Bert Vogelstein; Ref. 29). pCS2MT-axin GID and pCS2MT-axin GID L/P are constructs that express wild-type and mutated GIDs of *Xenopus laevis* axin, respectively (30). Mouse Wnt-1, Wnt-5A, and Wnt-7A cDNA were cloned into pCS2MT for constitutive ectopic expression.

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<sup>3</sup> The abbreviations used are: ESCC, esophageal squamous cell carcinoma; APC, adenomatous polyposis coli; GSK, glycogen synthase kinase; TCF, T-cell factor; LEF, lymphoid enhancer-binding factor; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EMSA, electrophoresis mobility shift assay; GID, glycogen synthase kinase 3 $\beta$  interaction domain; IP, immunoprecipitation.

**Cell Lines.** Human ESCC cell lines (TE1, TE3, TE5, TE8, TE10, TE11, TE12, and TE15) and human hepatoblastoma HepG2 cells, embryonic kidney 293 cells, and the 293 cell stable transfectants expressing Wnt-1, Wnt-5A, and Wnt-7A were cultured under standard conditions, namely, at 37°C under 5% CO<sub>2</sub> in DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% FCS (Sigma Chemical Co., St. Louis, MO), 100 units/ml penicillin, and 100 µg/ml streptomycin (Life Technologies, Inc.). The 293 stable transfectants were generated by transfecting Wnt expression vectors using FuGENE 6 (Roche Diagnostics Corp., Berkeley, CA) according to the manufacturer's instructions, followed by G418 selections. Transfected cells were maintained as pools.

**Transient Transfection and Luciferase Assays.** We performed transfections using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer's instructions. At 24 h before transfection,  $2.5 \times 10^5$  cells were seeded in 12-well plates. To assay the ability of Wnt to activate TCF/LEF transcription, TE series cell lines were transfected with 0.25 µg of the pGL3-OT or pGL3-OF reporter plasmid. At 36 h after transfection, cells were harvested for luciferase activity. Luciferase assays were performed by using the Luciferase Assay System (Promega, Madison, WI) in the Microtiter Plate Luminometer (DYN-EX Technology, Chantilly, VA). The luciferase activity was normalized by cotransfected β-galactosidase. The mean of luciferase/β-galactosidase activities with pGL3-OT was represented as a percentage of that obtained with pGL3-OF (100%) transfected in parallel. Transfections were carried out at least three times, and variation between experiments was not greater than 15%.

**Antibodies, Western Blot Assays, and IP.** Antibodies against TCF1 and TCF4 were obtained from Upstate Biotechnology (Lake Placid, NY), and mouse monoclonal antibodies against β-catenin and against E-cadherin were obtained from Transduction Laboratories (Lexington, KY). The antibody against APC (Ab-6) was obtained from Oncogene Research Products (Boston, MA), and an antibody against axin was developed by Dr. Peter S. Klein. A mouse monoclonal antibody against GSK3β was obtained from BD Transduction Laboratories (San Diego, CA). A rabbit polyclonal antibody against phospho-GSK3β (Ser9) was obtained from Cell Signaling (Beverly, MA). A mouse monoclonal antibody against β-actin was obtained from Sigma Chemical Co. Secondary antimouse and antirabbit horseradish peroxidase-conjugated antibodies were obtained from Amersham Pharmacia Biotech (Piscataway, NJ).

Whole-cell extracts were prepared with lysis buffer [20 mM Tris (pH 7.2), 150 mM NaCl, 10% glycerol, 1% NP40, 10 mM sodium fluoride, 30 mM sodium PP<sub>i</sub>, 1 mM EDTA, 1 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride] containing a protease inhibitor mixture tablet (Roche Molecular Biochemicals, Indianapolis, IN). Nuclear extract preparations from cultured cells were performed essentially as described previously (31). Lysates were precleared, and protein concentration was determined by the BCA protein assay (Pierce, Rockford, IL). For electrophoresis, lysates containing 10 µg of protein were supplemented with SDS loading buffer and separated on 10% Tris-glycine gels (PAGE; BioWhittaker Molecular Applications, Rockland, ME). Proteins were transferred to a polyvinylidene fluoride membrane (Immobilon P; Millipore, Bedford, MA) by electroblotting. The blots were incubated in Tris-buffered saline containing 0.1% Tween 20 and 5% nonfat dry milk (Bio-Rad Laboratories, Hercules, CA) during the blocking step and in 0.1% Tween 20 and 5% nonfat dry milk during the antibody incubation steps. The anti-TCF1, anti-TCF4, anti-β-catenin, anti-GSK3β, and anti-phospho-GSK3β antibodies were used at a 1:1000 dilution. Anti-β-actin antibody was used at a 1:5000 dilution. The horseradish peroxidase-conjugated goat antimouse or antirabbit immunoglobulin (Pierce) was used at a 1:2000 dilution. Antibody complexes were detected by enhanced chemiluminescence (ECL pulse; Amersham Life Science, Arlington Heights, IL) and exposure to X-OMAT film (Kodak, Rochester, NY).

For IP, cells were washed three times with ice-cold PBS and then lysed in IP buffer [100 mM NaCl, 50 mM Tris-HCl (pH 7.5), and 0.5% NP40] with complete protease inhibitor mixture. Anti-APC antibody or anti-axin antibody was added to lysates for 1 h at 4°C, and then protein A beads (Santa Cruz Biotechnology, Santa Cruz, CA) were added for 1 h. Immunoprecipitates were washed three times with IP buffer.

**Cytoplasmic Fraction of β-Catenin.** Cytoplasmic extracts from cultured cells were prepared essentially as described previously (32). To collect conditioned media, 293-Wnt-1 cells were grown to 80% confluence. Cells were

washed with PBS and maintained in regular DMEM for 24 h before harvesting the medium. The conditioned media were subjected to centrifugation at 1,000 rpm for 5 min at 4°C and stored at -80°C until use. To prepare cytoplasmic fractions, cells were washed and collected in ice-cold PBS. Cell pellets were resuspended in ice-cold hypotonic buffer [25 mM Tris (pH 7.5), 1 mM EDTA, 25 mM NaF, and 1 mM DTT] with complete protease inhibitor mixture (Roche Molecular Biochemicals). Cells were lysed after incubation on ice for 10 min. The lysates were subjected to ultracentrifugation at  $100,000 \times g$  for 30 min at 4°C, and the supernatant was collected.

**EMSA.** Nuclear extracts from cultured cells were prepared essentially as described previously (31). To make the <sup>32</sup>P-labeled probe, 5 pmol of a double-stranded oligonucleotide, 5'-CCCTTTGATCTTACC-3', were radiolabeled by the kinase reaction in a buffer consisting of 70 mM Tris-HCl (pH 7.6), 10 mM MgCl<sub>2</sub>, 5 mM DTT, 50 pmol of [γ-<sup>32</sup>P]ATP (3,000 Ci/mmol; New England Nuclear Life Science Products, Boston, MA), and 40 units of T4 polynucleotide kinase (New England Biolabs, Beverly, MA) and purified with a Microspin G-25 column (Amersham Pharmacia Biotech). EMSAs were carried out by incubating 5 µg of nuclear extract with 10 fmol of the <sup>32</sup>P-labeled oligonucleotide DNA probe (60,000 cpm) in a 20-µl binding reaction containing 10 mM HEPES (pH 7.9), 60 mM KCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, and 0.1 mg poly(dA-dT)/poly(dA-dT) (Amersham Pharmacia Biotech). After incubation at room temperature for 15 min, the samples were loaded on to a 4% 1× Tris glycine gel and electrophoresed at 10 V/cm for 1.5 h. For competition experiments, the nuclear extract was preincubated with 100-fold molar excess of unlabeled oligonucleotides before the addition of the <sup>32</sup>P-labeled oligonucleotide DNA probe [wild-type TCF, 5'-CCCTTTGATCTTACC-3'; mutant TCF, 5'-CCCTTTGGCCTTACC-3' (described in Ref. 4)]. Immune supershift assays were performed using 0.25 µg of anti-β-catenin, anti-E-cadherin, and anti-TCF4 antibodies, and the antibodies were preincubated with the nuclear extract at room temperature for 10 min before the addition of the <sup>32</sup>P-labeled oligonucleotide probe, as described previously (31).

## RESULTS

### TCF-dependent Transcription Is Inactive in ESCC Cell Lines.

To determine TCF-dependent transcriptional activity, we transfected pGL3-OT and pGL3-OF luciferase reporter constructs in a series of ESCC cell lines. None of the ESCC cell lines tested showed any appreciable TCF-dependent transcriptional activity (Fig. 1a). The lack of constitutive activation of TCF-dependent transcription in ESCC cell lines suggested that these cell lines are likely to carry intact genes for β-catenin, APC, or axin, whose mutations are frequently observed in certain subsets of cancers with activated β-catenin-dependent TCF-dependent transcription. Indeed, we did not find any APC or β-catenin mutation in any of the ESCC cell lines.<sup>4</sup>

To exclude the possibility that ESCC cell lines lack the necessary transcriptional machinery, we ectopically expressed several genes known to potentially activate β-catenin-dependent TCF-dependent transcription. As shown in Fig. 1, b and c, transfected mutant β-catenin as well as an axin fragment harboring GID (axin-GID), the latter of which is a potent GSK3β inhibitor, both robustly activated pGL3-OT in TE8 cells. Similar observations were made in other cell lines, with the curious exception of TE12 cells, in which TCF-dependent transcription was not activated by highly expressed mutant β-catenin or axin-GID (data not shown).

Western blotting showed that all examined ESCC cell lines express TCF4. Of the ESCC cell lines, only TE8 and TE1 express TCF1 (Fig. 2). In addition, EMSAs demonstrated that nuclear extracts from TE8 cells (Fig. 3) and other ESCC cell lines (data not shown) contain TCF-specific DNA binding activities with TCF4 and barely detectable β-catenin as a part of the DNA-TCF4 complex (Fig. 3).

<sup>4</sup> Unpublished observations.

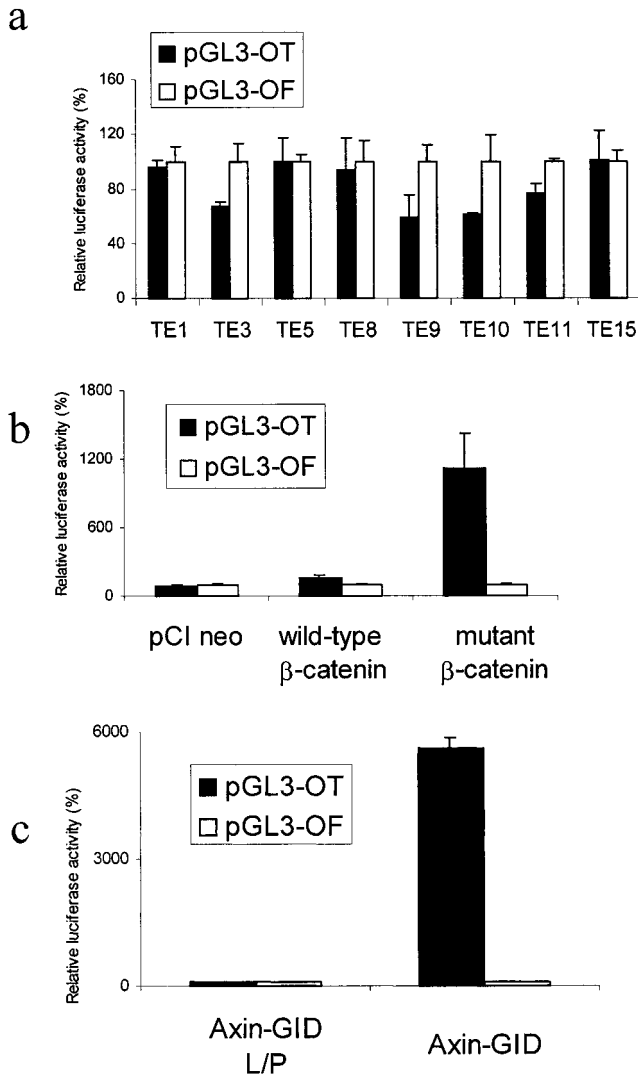


Fig. 1. TCF-dependent transcriptional activity in ESCC cell lines. *a*, ESCC cell lines were transiently transfected with luciferase reporter constructs. The ratio of reporter luciferase activity: $\beta$ -galactosidase activity is indicated.  $\beta$ -Galactosidase activities were determined by standard methods as a control for transfection efficiency. The results of all experiments are expressed as mean  $\pm$  SD (experiments were performed in triplicate, and at least four independent experiments were performed). *b*, Effects of  $\beta$ -catenin stabilization on TCF-dependent transcriptional activity in TE8 cells are shown. Empty vector (pCI neo), wild-type  $\beta$ -catenin, or mutant  $\beta$ -catenin was cotransfected with luciferase reporter constructs. pCI neo served as transfection control. Transfection of mutant  $\beta$ -catenin activated the TCF/LEF reporter gene as compared with wild-type counterparts. *c*, Effects of axin on TCF-dependent transcriptional activity in TE8 cells are shown. Axin-GID (inhibitor of GSK3 $\beta$ ) or axin-GID L/P (no effect on GSK3 $\beta$ ) was cotransfected with luciferase reporter constructs. Transfection of axin-GID activated the TCF/LEF reporter gene as compared with mutant axin-GID counterparts.

**Regulation of TCF-dependent Transcriptional Activity by Wnt-1 in ESCC Cell Lines.** To investigate the possible activation of the Wnt signaling pathway in ESCC cell lines, we tested the effect of Wnt-1, Wnt-5A, and Wnt-7A on TCF-dependent transcriptional activity. When pGL3-OT/pGL3-OF reporters were transfected in ESCC cell lines followed by cocultivation with 293 cells that stably express Wnt-1, all cell lines except TE12 demonstrated TCF-dependent transcriptional activity (Fig. 4a). Transcriptional activation was not observed when the transfected cells were cocultured with only parental 293 cells (Fig. 4a). Because TCF-dependent transcriptional activity was not observed in coculture with 293 cells, Wnt family growth factors are unlikely to be expressed in the parental cells. Therefore, the activated TCF-dependent transcription in ESCC cell lines is attributable to the expressed Wnt-1 in the medium. Consistent with this

idea, TCF-dependent transcription in TE8 cells was activated as a function of the cell number of the cocultured stable transfectants (Fig. 4b). Similarly, TE1 and TE3 cells were variably activated depending on the cell number of the cocultured stable transfectants (data not shown). 293-Wnt-1-conditioned medium also activated TCF-dependent transcription in ESCC cell lines including TE8 cells (data not shown), although the activation was less prominent than that observed in coculture experiments. When TE8 cells were cocultured with 293 cells expressing Wnt-7A or Wnt-5A, TCF-dependent transcription was not activated (Fig. 4b). Likewise, TE1 and TE3 cells failed to exhibit TCF-dependent transcriptional activities when cocultured with Wnt-7A- or Wnt-5A-expressing cells. To confirm the specificity of Wnt-1, TE series cell lines were cocultivated with Wnt-expressing 293 cells. All ESCC cell lines showed Wnt-1-specific TCF-dependent transcriptional activation (data not shown).

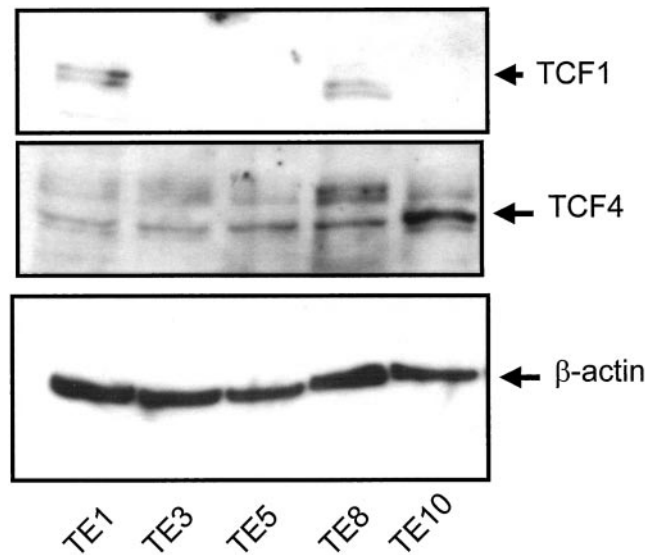


Fig. 2. Expression of TCF1 and TCF4 proteins in ESCC cell lines. Western blotting with the anti-TCF1 antibody (top panel) and the anti-TCF4 antibody (middle panel) was performed in ESCC as described in "Materials and Methods." Both proteins recognized a  $M_r$  60,000 band (arrow). The same membrane was reprobbed with an anti- $\beta$ -catenin antibody as a loading control (bottom panel).

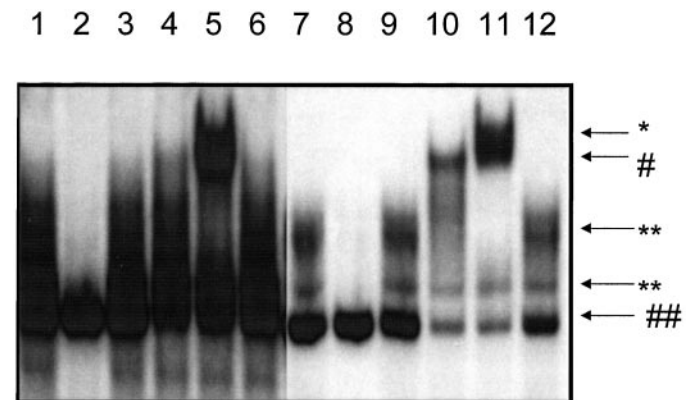
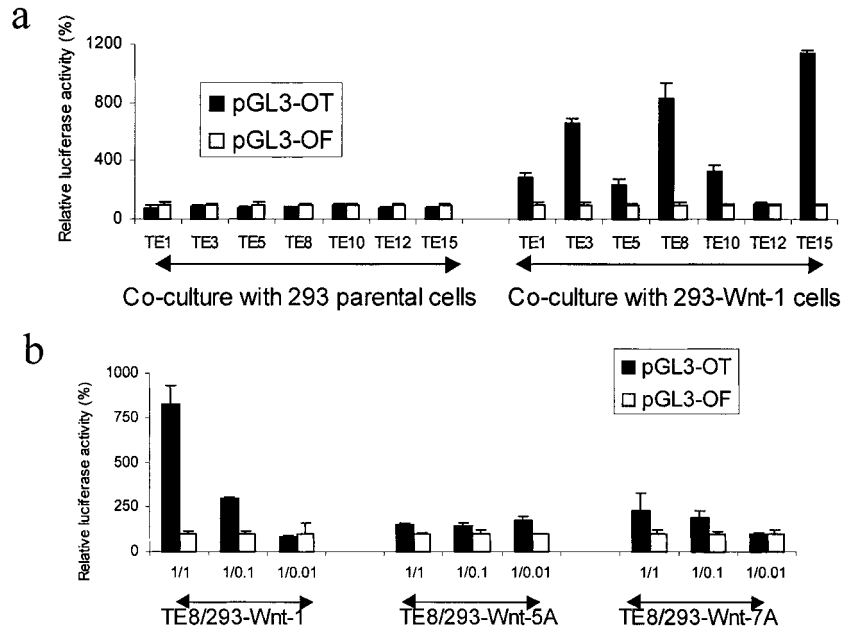


Fig. 3.  $\beta$ -Catenin binding to the TCF promoter in TE8 cells. EMSA with the anti- $\beta$ -catenin antibody was performed in TE8 and HepG2 cells (positive control for  $\beta$ -catenin and TCF4) as described in "Materials and Methods." The arrow indicates the supershifted  $\beta$ -catenin-TCF complex. Each lane was loaded as follows: Lanes 1–6, TE8 cell nuclear extracts; Lanes 7–12, HepG2 cell nuclear extracts; Lanes 1 and 7, nuclear extracts only. Lanes 2 and 8,  $\times 100$  wild-type competitor; Lanes 3 and 9,  $\times 100$  mutant competitor; Lanes 4 and 10,  $\beta$ -catenin antibody; Lanes 5 and 11, TCF4 antibody; and Lanes 6 and 12, E-cadherin antibody. \*, TCF4 supershift; \*\*, TCF; #, TCF/ $\beta$ -catenin supershift; ##, nonspecific band.

Fig. 4. Effects of Wnt family members on TCF-dependent transcriptional activity in ESCC cell lines. *a*, ESCC cells were cocultured with Wnt-1 stably transfected 293 cells or parental 293 cells and transiently transfected with luciferase reporter constructs. The ratio of reporter luciferase activity: $\beta$ -galactosidase activity is indicated. The results of all experiments are expressed as mean  $\pm$  SD (experiments were performed in triplicate, and at least four independent experiments were performed). *b*, TE8 cells were cocultured with 293-Wnt-1, 293-Wnt-5A, or 293-Wnt-7A and transfected with luciferase reporter constructs. The cell number ratio between TE8 and 293-Wnt-1, 293-Wnt-5A, or 293-Wnt-7A cells was 1:1, 1:0.1, or 1:0.01, respectively. The ratio of reporter luciferase activity: $\beta$ -galactosidase activity is indicated. The results of all experiments are expressed as mean  $\pm$  SD (experiments were performed in triplicate, and at least four independent experiments were performed).



**Wnt-1 Stabilizes  $\beta$ -Catenin in ESCC Cell Lines.** Because Wnt-1 activated TCF-dependent transcription in ESCC cell lines, we next determined whether Wnt-1 leads to cytoplasmic accumulation of  $\beta$ -catenin in these cells. When cells were incubated with 293-Wnt-1-conditioned medium, the amount of cytoplasmic  $\beta$ -catenin increased in TE8 cells (Fig. 5). Accumulation of  $\beta$ -catenin in the cytoplasm was observed at 1.5 h and sustained until 4 h.

The conditioned media successfully activated pGL3-OT reporter in ESCC cell lines, including TE8. It was also used to demonstrate increased cytoplasmic accumulation of  $\beta$ -catenin in TE1 cells (data not shown). However, the Wnt-conditioned media did not induce detectable TCF-specific DNA binding activity in TE1 and TE8 cells (data not shown).

**Differential Regulation of TCF-dependent Transcriptional Activity by Wnt and EGFR Pathways.** To explore the modulation of TCF-dependent transcriptional regulation by EGFR, an important pathway in the pathogenesis of ESCC, we tested whether this pathway

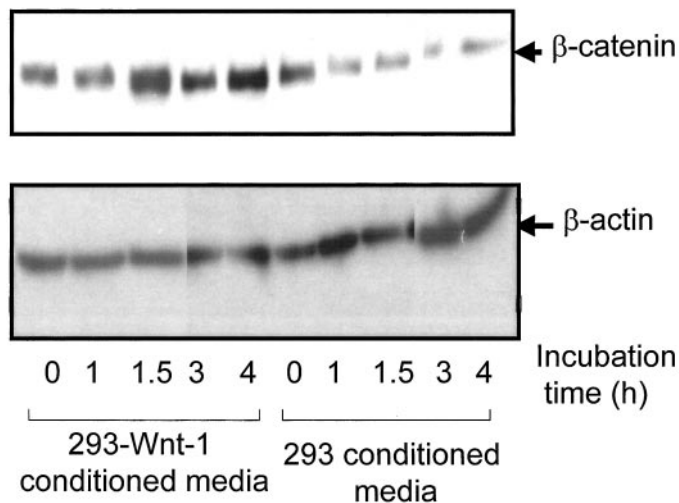


Fig. 5. Wnt-1 leads to an accumulation of cytoplasmic  $\beta$ -catenin in TE8 cells. TE8 cells were cultured using 293-conditioned media or 293-Wnt-1-conditioned media. Accumulation of  $\beta$ -catenin in the cytoplasm is observed after 1.5 h with 293-Wnt-1-conditioned media and is sustained for up to 4 h.  $\beta$ -Actin served as a loading control.

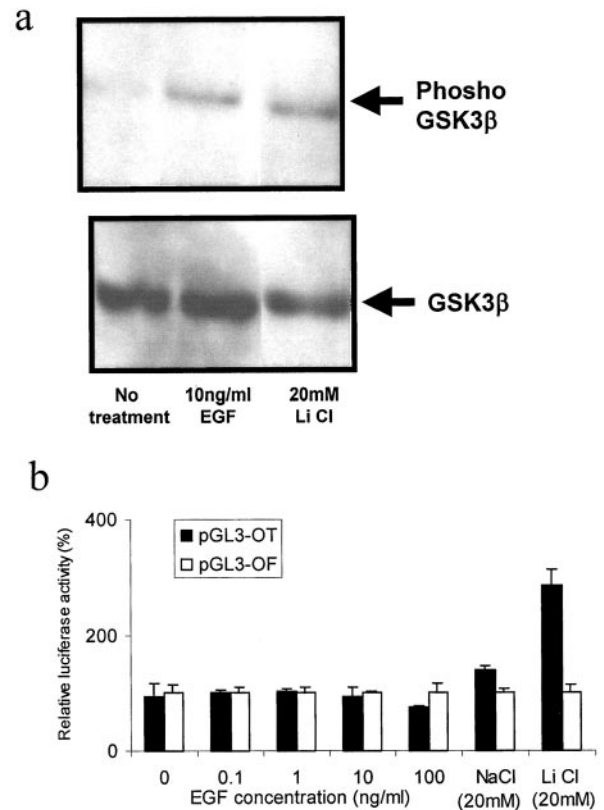


Fig. 6. Effects of EGF or lithium chloride on phosphorylation of GSK3 $\beta$  Ser-9 and TCF-dependent transcriptional activity in TE8 cells. *a*, TE8 cells were cultured with media containing 10 ng/ml EGF or 20 mM lithium chloride (control). GSK3 $\beta$  was phosphorylated by both stimuli. Total GSK3 $\beta$  served as a loading control. *b*, TE8 cells were incubated with media using various EGF concentrations (20 mM lithium chloride or NaCl). EGF did not activate TCF-dependent transcription; however, lithium chloride activated TCF-dependent transcription.

leads to inactivation of GSK3 $\beta$  in ESCC cells. When TE8 cells were incubated in 10 ng/ml EGF or 20 mM lithium chloride, a specific GSK3 $\beta$  inhibitor, GSK3 $\beta$  was phosphorylated at the serine 9 residue (Fig. 6a). However, EGF did not activate TCF-dependent transcrip-

tional activity in TE8 cells (Fig. 6b), whereas lithium chloride did. Furthermore, no synergistic effect of EGF and Wnt on TCF-dependent transcription was observed in TE8 cells (data not shown). We then examined the effects of EGF on free GSK3 $\beta$  or complexed GSK3 $\beta$  (immunoprecipitated with APC or axin antibody). EGF can phosphorylate free GSK3 $\beta$  (data not shown); however, phosphorylated GSK3 $\beta$  is not detectable in the immunoprecipitated fraction (data not shown).

## DISCUSSION

We report the activation of TCF promoter luciferase reporter gene by external Wnt stimuli in ESCC cells. We demonstrate the accumulation of  $\beta$ -catenin in the cytoplasm by incubation with Wnt-1-conditioned media in ESCC cell lines. In addition, Wnt-1 activates the TCF promoter luciferase reporter gene in a dose-dependent fashion. These findings indicate that Wnt-1 can regulate TCF-dependent transcriptional activity through  $\beta$ -catenin in ESCC cells. To our knowledge, this is the first report of functional TCF activation by Wnt-1 in ESCC cells.

Wnt-1 was identified as a preferred integration site for mouse mammary tumor virus in breast adenocarcinoma (33). In addition, Wnt-1 is able to transform cultured mammary epithelial cell lines (33), and transgenic mice expressing Wnt-1 in the mammary gland develop mammary epithelia hyperplasia and adenocarcinoma (34).

The Wnt ligands are functionally divided into two classes: (a) the transforming class; and (b) the nontransforming class (35). The transforming class of Wnt ligands triggers a signaling cascade referred to as the canonical Wnt pathway. This involves the cytoplasmic proteins dishevelled, GSK3 $\beta$ , APC, and axin. This signaling pathway stabilizes  $\beta$ -catenin and increases free intracellular pools of  $\beta$ -catenin through inactivation of GSK3 $\beta$  (6). GSK3 $\beta$  is also inhibited by EGF (36), and EGFR is frequently overexpressed in ESCCs (21). Indeed, the cooperation of Wnt-1 and mitogen-activated protein/extracellular signal-regulated kinase (MEK) 1, a downstream target of the EGFR signaling pathway, can promote cyclin D1 accumulation and cellular transformation (37). A recent report showed that Wnt-1-mediated signaling inhibits apoptosis and that the inhibition is independent of the c-Jun-NH<sub>2</sub>-terminal kinase and Akt pathways (38). We demonstrated that the EGFR pathway did not activate TCF-dependent transcription, possibly because it may only phosphorylate free GSK3 $\beta$ , which does not interact with  $\beta$ -catenin (39, 40). In contrast, activation of the Wnt pathway stabilizes cytoplasmic  $\beta$ -catenin and enhances TCF-dependent transcription. Because the phosphorylation of GSK3 $\beta$  by EGF does not activate TCF-dependent transcription, the inhibition of GSK3 $\beta$  by the Wnt pathway may arise through a different mechanism. For example, Wnt signaling may cause the axin/GSK3 $\beta$ / $\beta$ -catenin complex to at least partially disassemble, as has been observed in cultured cells (39, 40). These results indicate that cells can selectively regulate multiple inputs on GSK3 $\beta$  to elicit substrate-specific outputs.

Although mutations in downstream components of Wnt signaling have been associated with a variety of human cancers, mutations in the Wnt gene have not been found in human cancers (25, 38). Furthermore, although accumulation of  $\beta$ -catenin is observed in ESCC, mutations of APC or  $\beta$ -catenin have not been found in ESCC. It has been observed that cytoplasmic  $\beta$ -catenin is more prominent at the invading front of a cancer nest (2). It is possible that this may be attributable to Wnt signaling from mesenchymal or stromal cells contiguous to the tumor tissue.

Several Wnt family members are expressed in normal human adult tissues (41). There is currently little quantitative information regarding the affinity and specificity of interactions among individual mem-

bers of these protein families (42). A long-standing difficulty in studying the action of mammalian Wnt genes has been the inability to isolate their products in a soluble form that demonstrates functional activity (43). Secreted Wnt is known to have a paracrine effect (44). In addition, Wnt requires association with extracellular matrix components such as proteoglycan for its stable activity (45). This may account for the undetectable nuclear  $\beta$ -catenin-TCF-DNA complex in ESCC cells by EMSA with conditioned media. In the present study, we have shown for the first time that Wnt-1-conditioned medium induces the accumulation of  $\beta$ -catenin in ESCC cells. Our results indicate that Wnt-1 protein can act on target cells as a soluble secreted factor.

The stabilized free  $\beta$ -catenin has been found to bind to high mobility group box-containing transcription factors including LEF (7) and TCF (4). It has been demonstrated that TCF4 is expressed in adult tissues other than the intestinal epithelium; therefore, dysregulation of TCF4/ $\beta$ -catenin signaling activity is potentially involved in the onset or progression of other forms of human cancer (46). Because all examined ESCC cell lines expressed TCF4, this does not appear to be a rate-limiting factor. Whereas expression of TCF1 (47) may be important in regulating the availability of TCF4- $\beta$ -catenin complexes to bind DNA, this does not appear to play a role in the cell lines tested.

The role of Wnt-1 or another Wnt that signals through  $\beta$ -catenin in esophageal carcinogenesis is underscored by its specificity in regulating TCF-dependent transcription and may be broadly applicable to squamous and gastrointestinal carcinogenesis, which requires further testing.

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