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Wnt11 Signaling Promotes Proliferation, Transformation, and Migration of IEC6 Intestinal Epithelial Cells*

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Abstract

Wnts are morphogens with well recognized functions during embryogenesis. Aberrant Wnt signaling has been demonstrated to be important in colorectal carcinogenesis. However, the role of Wnt in regulating normal intestinal epithelial cell proliferation is not well established. Here we determine that Wnt11 is expressed throughout the mouse intestinal tract including the epithelial cells. Conditioned media from Wnt11-secreting cells stimulated proliferation and migration of IEC6 intestinal epithelial cells. Co-culture of Wnt11-secreting cells with IEC6 cells resulted in morphological transformation of the latter as evidenced by the formation of foci, a condition also accomplished by stable transfection of IEC6 with a Wnt11-expressing construct. Treatment of IEC6 cells with Wnt11 conditioned media failed to induce nuclear translocation of β-catenin but led to increased activities of protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase II. Inhibition of protein kinase C resulted in a decreased ability of Wnt11 to induce foci formation in IEC6 cells. Finally, E-cadherin was redistributed in Wnt11-treated IEC6 cells, resulting in diminished E-cadherin-mediated cell-cell contact. We conclude that Wnt11 stimulates proliferation, migration, cytoskeletal rearrangement, and contact-independent growth of IEC6 cells by a β-catenin-independent mechanism. These findings may help understand the molecular mechanisms that regulate proliferation and migration of intestinal epithelial cells.

Wnt signaling pathways have established importance in determining tissue development, differentiation, cell fate, and embryonic patterning (reviewed in Refs. 1–3). Mutations in the Wnt signaling pathways are involved in ~85% of sporadic cases of colorectal cancer (4,5). In colorectal cancer, inactivating mutations in the genes encoding adenomatous polyposis coli (6,7), axin/conductin (8,9), or activating mutations of β-catenin (10,11) result in β-catenin stabilization, translocation into the nucleus, and the activation of the TCF[1]/lymphoid-enhancing factor family of transcription factors (12). The importance of this pathway in the

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[1]The abbreviations used are: TCF, T-cell factor; CamKII, Ca^{2+}/calmodulin-dependent protein kinase II; CM, conditioned media; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; PKC, protein kinase C; FACS, fluorescence-activated cell sorting; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; FITC, fluorescein isothiocyanate.

maintenance of intestinal cell proliferation in crypt cells has been demonstrated by gene knockout studies of *Tcf-4*, which results in loss of the stem cell compartment (13). Tumors are thought to originate from these crypt cells although progression into malignancy requires additional transforming signals that further enhance growth, invasiveness, and differentiation (3).

Wnts, with at least 19 identified homologues in humans (reviewed in Refs. 14–18), are present in most adult tissues. The analysis of Wnt expression in the gastrointestinal tract has been performed largely in the context of embryonic development (19–22) and by analysis of expression levels in cancerous intestinal tissues or cancer cell lines (3,23,24). From these studies, several Wnts have been identified as potential signaling molecules in intestinal cancers including Wnts 2, 4, 5a, 6, 7a, and 11. In contrast, there is relatively sparse literature that examines the expression of Wnts in the normal adult intestinal tract (24). However, the significance of the Wnt signaling pathway is illustrated by recent studies (25,26) showing that over-expression of a Wnt inhibitor, Dickkopf1 (DKK1), can inhibit proliferation of the intestinal epithelial cells in mice.

Although the classical ("canonical") mechanism by which Wnt exerts its action depends on the nuclear localization of β-catenin and subsequent transcriptional activation of target genes (17,27–29), increasing evidence indicates that β-catenin-independent ("noncanonical") mechanisms are also important in signal transduction initiated by Wnt (30–34). For example, β-catenin-independent Wnt signal transduction has been shown to involve the activation of Ca^{2+}/calmodulin-independent kinase II (CamKII) and protein kinase C (PKC) in a manner that is modulated by intracellular concentrations of Ca^{2+} (33,35–37). Evidence also indicates that activators of the Wnt/Ca^{2+} pathway can antagonize the β-catenin-dependent Wnt pathway (38–40), suggesting a possible mechanism by which cell proliferation can be modulated. This antagonism might explain why inactivating mutations in Wnts that function through alternative pathways are also associated with a transformed phenotype (41). The co-existence of multiple Wnts that function via multiple pathways in the intestine suggests a complex interplay and perhaps sub-compartmentalization of effects of Wnt signaling that has not thoroughly been investigated to date.

To characterize further the function of the Wnt family of proteins in regulating intestinal epithelial cell proliferation, we aimed at establishing the identity and location of Wnts expressed in the mouse intestinal tract and at characterizing such Wnts with regard to intestinal epithelial cell proliferation. We show that Wnt11 is one protein in this family that has a relatively compartmentalized pattern of expression in the gut, and that both exogenous treatment of intestinal epithelial cells IEC6 with Wnt11 and overexpression of Wnt11 in IEC6 result in accelerated proliferation, migration, and transformation in a manner that is dependent on PKC and/or CamKII but independent of β-catenin. These findings underscore the importance of noncanonical signaling pathways of Wnt in modulating proliferation of intestinal epithelial cells.

EXPERIMENTAL PROCEDURES

Cell Lines

The nontransformed rat small intestinal epithelial cell line, IEC6 (42,43), was purchased from American Type Culture Collection (ATCC). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM), 5% fetal bovine serum (FBS), and 0.1 units/ml insulin. C57MG-Wnt1, provided by A. Levine (see Ref. 44), and Caco2-BBE cells, provided by D. Merlin (see Ref. 45), were both cultured in DMEM and 10% FBS. All media were supplemented with 100 units/ml penicillin and 100 mg/ml streptomycin. A Wnt11-transfected quail cell line, W11ox, and its parental cell line, QCE6 (46), were cultured on fibronectin-coated plates (1 μg/cm^3

fibronectin in phosphate-buffered saline (PBS)) in media consisting of minimum essential medium with 292 mg/liter glutamine, 10% tryptose phosphate broth, 2% heat-inactivated chicken serum, 10% iron-supplemented calf serum, and 200 μg/ml G418 for W11ox.

Generation of Conditioned Media

The media (control media) used to generate conditioned media (CM) were minimum essential medium supplemented with insulin/transferrin/selenium, 10% tryptose phosphate broth, 4 g/liter Albumax, and 20 μg/liter dexamethasone. Conditioned media were generated by culturing W11ox cells to 60% confluency, washed once with control media, and maintained in control media for an 3 additional days. The conditioned media were harvested, centrifuged to remove debris, and stored at −80 °C. Conditioned media were depleted of Wnt11 protein by incubation with 4 μg/ml rabbit polyclonal anti-mouse Wnt11 antibody at 4 °C overnight.

Generation of Wnt11 Transfectants of IEC6 Cells

A full-length cDNA encoding the mouse *Wnt11* was obtained by PCR amplification based on the published sequence and cloned into a TA cloning vector, pCR®-II (Invitrogen). The cDNA was then subcloned into the EcoRI restriction sites of the expression vector, pcDNA3 (Invitrogen), and sequenced for orientation and fidelity. IEC6 cells were transfected by electroporation (300 V, 1 millifarad) with pcDNA3-Wnt11 or the pCDNA3 vector control. Transfectants were selected in 1 mg/ml G418. Independent stable populations were tested for their ability to form foci in soft agar by plating 10^4 cells in 2.5% agar culture medium with antibiotic selection on plates with a 5% agar base. Cells were cultured for 3 weeks, and visible foci were selected and clonally expanded for further analysis (IEC6/W11 cells). IEC6 cells stably transfected with the pcDNA3 vector were selected with G418 and pooled as controls (IEC6/V cells).

Co-culture Experiments

5×10^5 IEC6 cells were co-cultured in 10-cm^2 dishes with 10^2 W11ox cells in antibiotic-free W11ox culture media. Media were changed every 4 days, and cells were monitored for foci after 3 weeks. Co-culture experiments were performed in transwell plates with 6-well polyester inserts (Corning Glass) with 0.4-μm membrane pore sizes. 10^5 W11ox cells were plated in the wells as a feeder layer, and 10^5 IEC6 cells were plated in the inserts and both grown in G418-free W11ox media. Media were changed once a week, and monolayers were observed after 3 weeks of culture. Foci images were captured on a Coolpix MDC Lens (Nikon). Foci sizes were measured after staining transwell membranes with 0.1% methylene blue following fixation in 75% ethanol.

Differentiation Model Using Caco2-BBE Cells

Caco2-BBE cells were cultured to confluency in growth media and then allowed to continue growing in control CM, W11 CM, or normal growth media. Cells were harvested at 0, 3, 6, 12, and 18 days post-confluency for standard fluorescence-activated cell sorting (FACS) analysis to determine the cell cycle profiles. Harvested cells were trypsinized, pelleted, washed twice in PBS, fixed in ethanol prior to staining with propidium iodide, and analyzed using FACS techniques (47). The percentages of cells in G_1 and S phases were compared among different treatments.

Reverse Transcription-PCR

Total RNA extracted from multiple tissues of wild type C57BL/6 mice was prepared using Trizol reagent (Invitrogen). Colon and small intestine RNA was reverse-transcribed using oligo (dT) and Superscript II (Invitrogen). Degenerate primers for *Wnt* genes spanning the conserved 5′ and 3′ sequences of QECKCH and FHWCC (48) were used to amplify all *Wnt* species from

the cDNA pool by PCR, with the expected product size ranging between 400 and 550 bp. Specific primers for amplifying Wnt1, Wnt3a, Wnt4, Wnt5a, and Wnt11 (Table I), based on representation in embryonic tissues (20), were also used to amplify *Wnts* from the cDNA pool. Primers were purchased from Integrated DNA Technologies, Inc. Amplified products were separated on 1% agarose gels. PCR products were cloned using the TOPO-TA kit for sequencing (Invitrogen) and submitted for sequence analysis (Emory DNA Core Facility).

Northern Blot Analysis

Total RNA from intestinal and nonintestinal tissue was separated by denaturing agarose gel electrophoresis and transferred onto Nytran membrane (Hybond^{TM}; Amersham Biosciences) for Northern blot analysis. Complementary cDNA probes encoding Wnt11 and β-actin were labeled with [α-^{32}P]dATP by using random priming kits (Stratagene) and purified with MicroBio® spin P-30 chromatography columns (Bio-Rad). Blots were pre-hybridized in QuikHyb® hybridization reagent (Stratagene) at 68 °C for 20 min, hybridized with labeled probe for 1 h, washed in 2× SSC and 0.1% SDS twice for 15 min at room temperature, and washed in 0.1× SSC and 0.1% SDS at 68 °C for 30 min before autoradiography.

Proliferation Assays

IEC6 cells at 70–80% confluency were trypsinized and plated in 96-well plates at a concentration of 5×10^3 cells per well and allowed to attach for 4 h in IEC6 media. Media were then replaced with IEC6 growth media containing 5% FBS, control serum-free media (control CM), Wnt11 conditioned media from W11ox (W11 CM), or depleted W11 CM. At the specified time points, cells were assayed for proliferation by incubation for 2 h with an MTS-based reagent (Promega/Roche Applied Science), a colorimetric method for the determination of viable cells in proliferation. MTS is bioreduced by cells into a formazan that is soluble in tissue culture medium and can be measured directly on a multiplate reader (Molecular Devices) at 490 nm.

Western Blot Analysis, PKC, and CamKII Assays

Protein was extracted from cultured cells by incubation in 1× lysis buffer (Cell Signaling) consisting of 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM sodium vanadate, and 1 μg/ml leupeptin supplemented with a protease inhibitor mixture (Roche Applied Science), vortexed, and followed by centrifugation at 2,000 × *g* for 20 min. Protein lysates were quantified by Bradford assays (Bio-Rad). Western blot analysis was performed by standard protocols using a rabbit polyclonal antibody generated against a mouse Wnt11 peptide, W11259 (49). The blots were incubated with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology), and signals were detected by enhanced chemiluminescence (Amersham Biosciences). Protein lysates were also used in PKC and CamKII activity assays using the PepTag® nonradioactive detection of protein kinase C assay kit (Promega; V5330) or radioactive protein kinase C and CamKII assay kits (Upstate Biotechnology, Inc., catalog numbers 17–139 and 17–135, respectively). An active and purified PKC enzyme was used as a positive control, and water or lysis buffer was used as a negative control. In certain experiments, calphostin C (Calbiochem) was included to inhibit PKC at a concentration of 100 nM in Me_2SO.

Migration Assays

IEC6 cells grown to confluency were scratched with a 0.5-mm pipette tip to cause wounding. Images were taken of all cultures immediately following wounding, and the location was referenced by indelible ink. Media were replaced with 5% serum media, control CM, W11 CM, or depleted W11 CM. Images of the plates at the referenced locations were captured at

intervals of 6 and 24 h. The wound gap was measured, and the percent wound repair was determined for each time point. All treatments were assessed in triplicate.

Luciferase Assays

IEC6 cells were harvested from a 70–80% sub-confluent culture and resuspended in serum- and antibiotic-free DMEM at a concentration of 1.25×10^7 cells/ml. Transfection was performed by electroporation (300 V, 1 millifarad) of 10^7 cells with 15 μg of TCF3WT-pGLS-luciferase (Clontech) and co-transfected with 0.5 μg of cytomegalovirus-*Renilla* luciferase and pEGFP-EC3 (Clontech) constructs as transfection controls. Fifty μl of the transfected cells were plated in 6-well plates overnight in DMEM supplemented with 10% FBS. Test media were then added, and cells were incubated for an additional 24 h prior to harvesting and assaying for luciferase activity using the Dual Luciferase Reporter Assay system (Promega) per the manufacturer's protocol.

Immunofluorescence and Immunohistochemistry

Immunofluorescence was performed on cells cultured on coverslips to 50–80% confluency and treated with 5% serum media, control CM, Wnt11, or Wnt1 conditioned media for 8 h. The cultures were washed twice in PBS, incubated in methanol at −20 °C for 15 min, and washed twice in cold PBS. Cells were blocked for 1 h in 0.5% bovine serum albumin at 4 °C. Cells were then incubated for 1 h with the following primary antibodies (Santa Cruz Biotechnology) at a 1:100 dilution, β-catenin, E-cadherin, and LI-cadherin, and then washed three times for 5 min in PBS followed by a 1-h incubation in the secondary antibodies anti-mouse IgG FITC 1:300 and anti-goat FITC 1:160 (Sigma). The nuclear stain To-Pro3 (Molecular Probes) was added to the final PBS wash (200 ng/ml); the coverslips were rinsed once in double-distilled H_2O and mounted onto coverslips with mounting media (Sigma) for observation under a fluorescent microscope (Nikon eclipse TS-100 and/or LSM 510 Zeiss Confocal Microscope). A rabbit polyclonal antibody against a mouse Wnt11 peptide (49) was used for immunohistochemistry. Paraffin-embedded sections from the colon and jejunum of two C57BL/6 mice were incubated in a 1:1000 dilution of the primary antibody followed by immunodetection by standard techniques. A 2-h incubation with 5× blocking peptide was performed prior to immunohistochemistry as a control.

RESULTS

Identification and Localization of Wnt11 in Mouse Tissues

In order to identify Wnt molecules that are expressed in normal adult mouse gastrointestinal tract, C57BL/6 mouse colon and small intestine were harvested and RNA extracted. Following reverse transcription, cDNAs were amplified using primers specific for Wnts shown previously to be present in embryonic intestine or associated with intestinal cancers. By using these sequence-specific primers, only Wnt11 was amplified, whereas the remaining primers against Wnt 1, 3a, 4, and 5a did not yield any products. As a further screen for intestinal *Wnt* expression, reverse-transcribed cDNA was PCR-amplified using well characterized degenerate primers against the conserved regions of known Wnts (48). However, this approach did not identify any other Wnt genes as candidates for intestinal tract expression. Thus, we focused subsequent experiments on Wnt11.

We first determined the tissue distribution of *Wnt11* transcripts in a panel of RNA extracted from various adult mouse tissues. As shown in Fig. 1 by Northern blot analysis, *Wnt11* transcripts were present in the distal and proximal colon and small intestine as well as a variety of other tissues including the liver, kidney, spleen, heart, brain, and whole embryo. These data are consistent with reports of function of Wnt11 in kidney (50) and heart morphogenesis (46,

51). The presence of *Wnt11* transcript in both small and large intestines suggests that it may serve an equally important function in the intestinal tract.

Cellular Localization of Wnt11 in the Intestinal Tract

To localize further the specific cell types in the intestinal tract that express *Wnt11*, we performed immunohistochemical studies of the adult mouse jejunum and colon using a polyclonal antibody generated against a mouse Wnt11 peptide (49). The specificity of the antibody was demonstrated by the lack of signals in sections that were blocked with excessive amounts of the peptide antigen (Fig. 2*B*, *D*, and *F*). As seen in Fig. 2*A*, *C*, and *E*, the smooth muscle in both muscularis propria and muscularis mucosa in the jejunum, distal colon, and proximal colon, respectively, stained strongly. Staining was also observed in cells of the lamina propria but not in the submucosa. Finally, epithelial cells lining both the crypt region as well as the villus and surface epithelium of the small and large intestine, respectively, stained positively. The presence of Wnt11 in the intestinal epithelium prompted us to investigate further the biological function of Wnt11 in intestinal epithelial cells.

The Effect of Wnt11 on Intestinal Epithelial Differentiation

We first examined the effect of Wnt11 on cell differentiation because of its presence in the differentiated epithelial cells within the intestinal epithelium. This was accomplished by studying the effect of Wnt11 on a well established culture model of intestinal epithelial differentiation, Caco2-BBE (45,52,53). These cells spontaneously undergo enterocyte differentiation upon confluency-mediated cell cycle arrest. Caco2-BBE cells were treated with conditioned media harvested from W11ox cells or control conditioned media that were not exposed to any cells. As seen in Fig. 3, post-confluent Caco2-BBE cells treated with control conditioned media over a 3-week period exhibited sustained cell cycle arrest as demonstrated by the persistent decrease in the population of cells in the S phase (Fig. 3*A*) and the persistent increase in the population of cells in the G_1 phase (Fig. 3*B*) of the cell cycle. In contrast, Caco2-BBE cells treated with conditioned media from Wnt11-secreting QCE6 cells had an unexpected increase in the population of cells in the S phase of the cell cycle, particularly toward the late stage of treatment (Fig. 3*A*). The G_1 population of cells was concomitantly decreased in this period (Fig. 3*B*). At 3 weeks post-confluency, the activity of alkaline phosphatase, a marker of differentiation, was significantly lower in cells treated with Wnt11 CM than that treated with control media (results not shown). These findings suggest that Wnt11 is not able to induce differentiation in the Caco2-BBE culture model despite its presence in the differentiated epithelial cells of the villus tip and surface epithelium of the small and large intestine, respectively, as revealed by immunohistochemical examination (Fig. 2).

The Effects of Wnt11 on Proliferation of Intestinal Epithelial Cells

The inability of Wnt11 conditioned media to induce differentiation and its ability to increase the percentage of cells in the S phase of the cell cycle, despite a post-confluent stage of Caco2-BBE cells (Fig. 3), led us to investigate whether Wnt11 may stimulate cell proliferation. We chose the nontransformed rat intestinal epithelial cell line, IEC6 (42,43), as a model to study the effect of Wnt11 on cell proliferation. As Wnts can exert both autocrine and paracrine effects when secreted into the extracellular matrix (54), we first examined a system in which exogenously produced Wnt11 was used to treat IEC6 cells. As seen in Fig. 4*B*, conditioned media harvested from a Wnt11-transfected quail cell line, W11ox (46), contained appreciable amounts of Wnt11 (*lane 2*).

The results in Fig. 4*A* show that when maintained in media supplemented with serum, IEC6 cells exhibited a typical exponential pattern of growth during the 4 days in culture. Similarly, Wnt11 CM was able to stimulate growth of the cells, albeit not as robustly as that in serum-containing media (Fig. 4*A*). However, control serum-free conditioned media that did not

contain Wnt11 (Fig. 4*B*, *lane 1*) failed to increase the number of cells over a period of 4 days (Fig. 4*A*). These results indicate that exogenously added Wnt11 has a pro-proliferative effect on IEC6 cells.

To determine whether the effect of Wnt11 conditioned media on proliferation of IEC6 cells was indeed due to Wnt11, we performed the proliferation experiments using Wnt11 conditioned media that had first been depleted of Wnt11 using an anti-quail Wnt11 antibody (46). As shown in Fig. 5*A*, depletion of Wnt11 significantly reduced the ability of Wnt11 conditioned media to stimulate proliferation of IEC6 cells after 3 days of treatment (Fig. 5*A*, compare *W11 CM* and *W11 depl.*; $p < 0.001$). Moreover, we showed that Wnt11 may function in an autocrine fashion as stably transfected clones of IEC6 cells with Wnt11 (IEC6/W11), but not those transfected with vector alone (IEC6/Vector), were able to proliferate when maintained in a serum-free media (Fig. 5*B*). Combining the results of Figs. 4 and 5, we conclude that IEC6 cells can be stimulated to proliferate when treated with either exogenous or endogenous Wnt11.

Long Term Wnt11 Stimulation Leads to Transformation of IEC6 Cells

To investigate the long term effect of Wnt11 on IEC6 cells, we directly co-cultured W11ox and IEC6 cells. Prominent foci were observed following 21 days of co-culture (Fig. 6*B*). These foci were masses of cells that appeared as raised areas (Fig. 6*B*) on the otherwise evenly distributed and flattened monolayer of IEC6 cells when cultured alone (Fig. 6*A*). To address the potential concern that the foci were of indeterminate origin (from IEC6 or W11ox) and to better mimic a physiologic model of subepithelial cells underlying crypt epithelial cells as sources of Wnt11, we conducted co-culture experiments using transwells to separate IEC6 and W11ox cells. As seen in Fig. 6*C*, IEC6 cells maintained a monolayer configuration when grown in the absence of any feeder layer. In contrast, foci were formed in IEC6 cells when W11ox cells were used in the feeder layer (Fig. 6*D*). These findings confirm that Wnt11 does have a long term growth-sustaining effect and is capable of transforming IEC6 cells as evidenced by the contact-independent cell aggregates formed in the cultures.

As an additional and independent means to demonstrate the transforming effect of Wnt11 on IEC6 cells, we cultured clonally derived stable transfectants of IEC6 cells with Wnt11 (IEC6/W11) in soft agar and monitored for anchorage-independent formation of foci. As seen in Fig. 6*E*, each of the six clones of IEC6/W11 selected was able to form foci following 21 days of growth in soft agar. In contrast, neither the nontransformed parental IEC6 cells nor IEC6 cells stably transfected with the pcDNA3 vector (IEC6/V) formed any foci. Combining the results of the three different assays, it is clear that long term exposure of IEC6 cells to Wnt11 leads to a transformed phenotype.

Analysis of Wnt11 Signaling Pathways in IEC6 Cells

Transformation by Wnt is a phenotype that is commonly associated with the classical (canonical) signaling pathway that is mediated by β-catenin. However, Wnt11 has been reported to function via a β-catenin-independent pathway in other systems (36,51,55–58). This apparent contradiction prompted us to examine the signaling mechanism of Wnt11 in intestinal cells by using the IEC6 cell line. The crucial activation step in the canonical Wnt/β-catenin pathway is the translocation of β-cate-nin into the nucleus of the cell upon Wnt treatment (17,27–29). We therefore performed immunofluorescence studies of β-catenin in IEC6 cells after short term (8 h) treatment with Wnt11. As seen in Fig. 7, treatment of IEC6 cells with Wnt11 conditioned media failed to result in the translocation of β-catenin into the nucleus (Fig. 7, *G–I*), in a manner similar to those treated with either serum (Fig. 7, *A–C*) or serum-free media (Fig. 7, *D–F*). In contrast, treatment of IEC6 cells with conditioned media obtained from C57MGWnt1 cells for 8 h resulted in nuclear translocation of β-catenin (Fig. 7, *J–L*).

To validate these results further, a luciferase reporter construct under the control of a β-catenin-activated minimal c-*fos* promoter containing upstream TCF3-response elements (59,60) was used to assess the ability of Wnt to activate β-catenin-dependent transcription. As seen in Fig. 7*M*, luciferase activity in transfected cells was only stimulated after treatment with conditioned media from C57MGWnt1 but not W11ox. In fact, Wnt11 stimulation may actually repress the promoter activity as demonstrated by the decreased luciferase activity compared with the control. These findings indicate that the effect of Wnt11 on proliferation and transformation of IEC6 cells is mediated by a β-catenin-independent mechanism.

Wnt11 Stimulates PKC and CamKII Activities in IEC6 Cells

Previous studies indicate that signaling by Wnt11 can be mediated by PKC, CamKII, or JNK (36,38,51,55–58,61). We measured PKC and CamKII activities in IEC6 cells following Wnt11 treatment at various time points. Fig. 8*A* shows that PKC activity in IEC6 cells increased steadily until 8 h after Wnt11 treatment and remained above base-line level at 24 h following treatment. Calcium-dependent CamKII activity was also significantly elevated between 30 and 120 min post-treatment with Wnt11 conditioned media when compared with those treated with control media (Fig. 8*B*). These results indicate that Wnt11 stimulates both PKC and CamKII activities in IEC6 cells. Similarly, the steady-state PKC and CamKII activities were both higher in stable transfectants of IEC6 by Wnt11 when compared with vector-transfected cells (Fig. 9*A* and *B*, respectively).

To demonstrate further that PKC is a mediator of the biological activity of Wnt11 in stimulating proliferation of IEC6 cells, we investigated the effect of a PKC-specific inhibitor, calphostin C, on the transforming activity of Wnt11 using the focus formation assay in co-cultured transwells. Foci number and size were used to quantify the effect of PKC inhibition on transformation. As shown in Fig. 10*A*, the number of foci formed in the presence of calphostin C was significantly reduced when compared with the co-cultured IEC6 cells treated with the vehicle, Me_2SO. This was accompanied by a significant difference in the morphology and size of the foci as demonstrated in Fig. 10*B*. These results suggest that PKC is involved in mediating the pro-proliferative and transforming effect of Wnt11 on IEC6 cells.

Wnt11 Induces the Internalization of E-cadherin in IEC6 Cells

PKC activation has been reported to disrupt cell contacts of intestinal cells concomitant with the internalization of E-cadherin (62). We therefore examined the distribution of E-cadherin in IEC6 cells following treatment with Wnt11 conditioned media. To explore changes of other cytoskeleton-associated proteins, we examined the distribution of LI-cadherin, which is expressed exclusively in the liver and intestine (63). Fig. 11 demonstrates that Wnt11 conditioned media causes a redistribution of E-cadherin in IEC6 cells (Fig. 11*B*) in contrast to cells treated with control media (Fig. 11*A*). Twelve hours of treatment with Wnt11 conditioned media results in the internalization of E-cadherin from the membrane to close association with the nucleus with radiating extensions into the cytoplasm (Fig. 11*B*). Cells treated with control media show the expected periplasmic distribution underlying the cell membrane to form a "chicken wire" pattern (Fig. 11*A*). Wnt11 treatment does not alter the periplasmic distribution of L1-cadherin (Fig. 11*C* and *D*), indicating the specificity of its effect on E-cadherin.

Wnt11 Enhances the Migration of IEC6 Cells

The activation of PKC has been associated not only with cytoskeletal rearrangements but also with increased motility of various cell types (64,65). To determine whether redistribution of E-cadherin and the apparent loss of E-cadherin-mediated cell-cell contacts affected cell movement or migration, we performed a scratch assay (Fig. 12). The movement of cells across a wounded area of the cell monolayer indicates the migration of cells in a process independent of proliferation (66). As shown in Fig. 12, cells cultured under normal serum-containing

conditions migrated rapidly and completely covered the wound in 24 h. The ability of the cells to migrate was significantly impaired when they were treated with serum-free control conditioned media. In contrast, the addition of Wnt11 conditioned media enhanced the migration of cells as compared with those treated with control media. The specificity of the treatment was demonstrated by the reduction in migration of cells treated with Wnt11 conditioned media that had been depleted of Wnt11 (Fig. 12).

DISCUSSION

Differentiated intestinal epithelial cells originate from the dividing stem cell compartment of the crypt that migrate upward to the villus tip or downward to the crypt base (67–72). The importance of the Wnt/β-catenin pathway in regulating intestinal epithelial cell proliferation has been described in recent studies (2,3,22,26). In this pathway, Wnt signaling inhibits β-catenin degradation, resulting in an accumulation of β-catenin and subsequent translocation into the nucleus. In the nucleus, β-catenin activates TCF/lymphoid-enhancing factor-responsive genes, which include important growth-promoting genes such as c-*myc* and cyclin D1 (73–75). Notably, β-catenin is localized to the nucleus of proliferating intestinal crypt cells (76), and gene disruption studies have demonstrated the absolute requirement of TCF4 in crypt cell maintenance (77). However, despite evidence showing the presence of this signaling pathway, no specific Wnts have been directly implicated in the regulation of crypt cell proliferation. The source and the nature of the Wnt signal in the intestinal crypt and whether a concentration gradient of Wnt secretion exists in the intestinal crypt are still largely undetermined.

Here we show that Wnt11 mRNA is present in various mouse tissues such as the heart and kidney as reported previously (50,78). The distribution of Wnt11 in these tissues corresponds well with its functions in the morphogenesis of cardiac myocytes (46) and differentiation of cardiac conduction cells (78) and kidney ureter (50,79). In the developing gastrointestinal tract, Wnt11 is localized in the endodermal layer of the esophagus and colon as well as in the mesenchymal layer of the stomach (20). A recent study (21) also confirmed the localization of Wnt11 to the developing chick hindgut with a concentration in the regions of ceca and cloaca. Our study shows that *Wnt11* transcripts in the intestinal tract persist into the adult mouse and are present in both the small and large intestine (Fig. 1).

In situ hybridization studies have demonstrated the presence of Wnt5a in the colonic mucosa with a concentration at the base of the crypt epithelium (24). Experiments in *Xenopus* embryos also indicate that Wnt5a and Wnt11 are members of a single functional class (80). The result of our study is consistent with those of published work and suggests that Wnt5a and Wnt11 may have overlapping or complementary functions in the intestinal epithelium. Moreover, our study is the first to provide a description of the different cell types in the adult mouse intestinal tract that contain Wnt11. In addition to the intestinal epithelial cells, we observed abundant staining for Wnt11 in the smooth muscle cells including the muscularis propria and muscularis mucosa. Wnt11 is also noted in cells in the lamina propria. As both muscularis mucosa and lamina propria are adjacent to the epithelium, it is possible that they are the sources of secreted Wnt11, which may regulate important biological processes in the intestinal epithelium.

The relative uniform distribution of Wnt11 between the differentiated villus and surface epithelium of the small and large intestine, respectively, and the proliferating crypt epithelium suggests that Wnt11 may regulate either proliferation or differentiation of the epithelial cells. The ability of Wnt11 to stimulate a proliferative response in Caco2-BBE cells that have undergone confluency-mediated cell cycle arrest as shown in Fig. 3 suggests that Wnt11 is involved in induction of proliferation rather than differentiation of epithelial cells. Indeed, Wnt11 is able to enhance proliferation of IEC6 cells and lead to foci formation upon prolonged

treatment. These effects are accomplished by treatment of conditioned media containing Wnt11, direct and indirect co-culturing between Wnt11-secreting cells and IEC6 cells, and stable transfection of IEC6 cells with a Wnt11-expressing plasmid. The specificity of the effects was also demonstrated by the ability of a Wnt11 antibody to block the pro-proliferative effect of Wnt11. The results of these experiments demonstrate that Wnt11 works in both an autocrine (endogenous expression by stable transfection) and paracrine (exogenous treatment by conditioned media or co-culture) fashion to stimulate intestinal epithelial cell proliferation.

How does Wnt11 exert its effects in stimulating proliferation of IEC6 cells? We show here that Wnt11 does not function through the β-catenin pathway (Fig. 7). Instead, we show that Wnt11 signaling in IEC6 cells involves both PKC and CamKII and that their activation occurs in both exogenously treated cells (Fig. 8) and endogenously transfected cells (Fig. 9). Moreover, inhibition of PKC reduces the ability of co-cultured Wnt11-secreting cells to induce the formation of foci in IEC6 cells (Fig. 10). The role of the PKC- and CamKII-dependent Wnt pathways has not been documented previously in the intestine, although both pathways have been shown to be important in mediating the effect of certain Wnts, including Wnt5a and -11, during cell fate determination in *Xenopus* embryos (36). These two pathways have collectively been named the Wnt/Ca^{2+} pathway (33). It is of interest to note that the Wnt/Ca^{2+} pathway can block the Wnt/β-catenin pathway during *Xenopus* gastrulation and the two pathways antagonistically regulate the convergent extension movements in *Xenopus* (38). The results of this study showing that Wnt11 suppresses TCF3-driven transcriptional regulation in IEC6 cells (Fig. 7*M*) are consistent with the previously observed cross-talk between the two distinct Wnt signaling cascades (38).

In addition to stimulating proliferation of IEC6 cells, results of our study indicate that Wnt11 induces internalization of E-cadherin (Fig. 11), a membrane-associated protein that mediates intercellular adhesion in intestinal epithelial cells (81). It is of interest to note that previous studies have shown that cytoskeletal rearrangement can stimulate expression of *Wnt5a* (82), but the reverse has not been established. We also show that Wnt11 causes increased cell migration as demonstrated by a wound-healing scratch assay (Fig. 12). Although we did not document a direct connection between rearrangement of E-cadherin and increased migration caused by Wnt11, previous studies using transgenic mice have highlighted the importance of E-cadherin in controlling epithelial cell migration along the crypt-villus axis of the small intestine (83–85). The ability of Wnt11 in stimulating migration of IEC6 cells is reminiscent of the recent finding that expression of *Wnt5a* is increased in metastatic human melanoma cells and that this increase directly correlates with increased PKC activity and motility of the tumor cells (65). Combining the results of published studies and the present study, it becomes apparent that several Wnts in the Wnt/Ca^{2+} class, including Wnt11 and Wnt5a, can regulate a multitude of biological processes in the intestinal epithelial cells such as proliferation and migration.

A role for Wnt11 in the promotion of tumorigenesis has been implied as Wnt11 is up-regulated in several cancers, including primary colorectal cancer (86). Similar to our study, Wnt11 has been shown to induce transformation of mammary epithelial cells (49). Related studies have also shown that PKC activation and increased motility due to E-cadherin rearrangement are associated with increased aggressiveness (65,87,88). Moreover, overexpression of PKC in the MCF-7 breast cancer cells enables anchorage-independent growth and metastasis in nude mice (89) and reduces E-cadherin expression, which also correlates with increased invasion and metastasis (90,91). These studies may provide some insight into how Wnt11 could act as a tumor promoter. Additional studies on the expression of *Wnt11* in human cancers including colorectal cancer and the consequence of altered expression of *Wnt11* may have on the phenotype of tumors would require further investigation.

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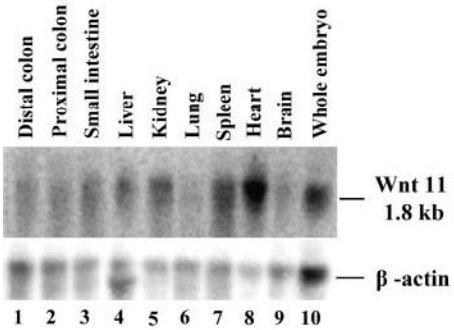

Fig. 1. Tissue distribution of the *Wnt11* transcript in mouse tissues

Twenty μg of total RNA from each of the indicated mouse tissues was analyzed by Northern blotting against a Wnt11 (*upper panel*) or β-actin cDNA probe. The size of the *Wnt11* transcript is indicated.

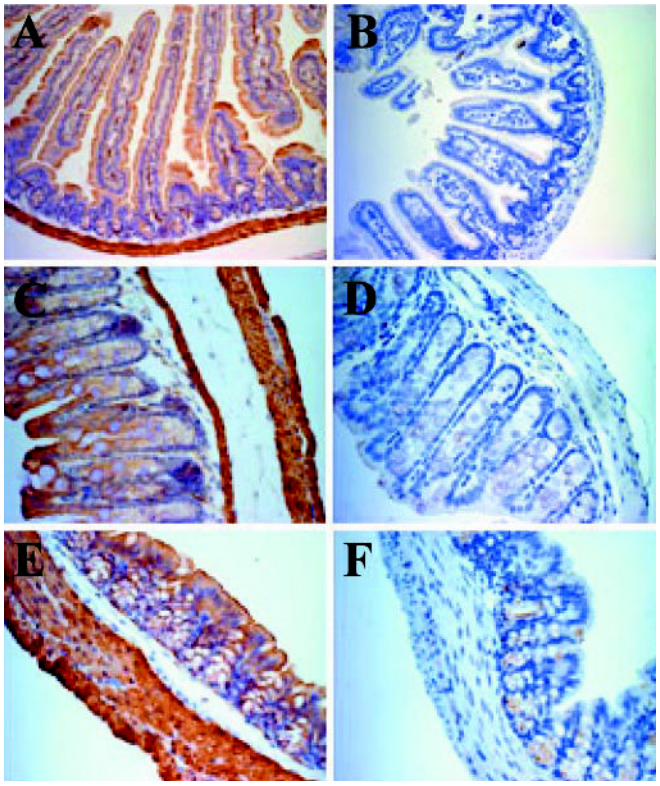

Fig. 2. Immunohistochemical localization of Wnt11 in adult mouse jejunum and colon

Immunohistochemical staining for Wnt11 using a rabbit polyclonal anti-mouse Wnt11 peptide antibody was performed on sections of adult mouse jejunum (*A* and *B*), proximal colon (*C* and *D*), and distal colon (*E* and *F*). *B*, *D*, and *F*, excessive amounts of the peptide antigen were used to block the antibody.

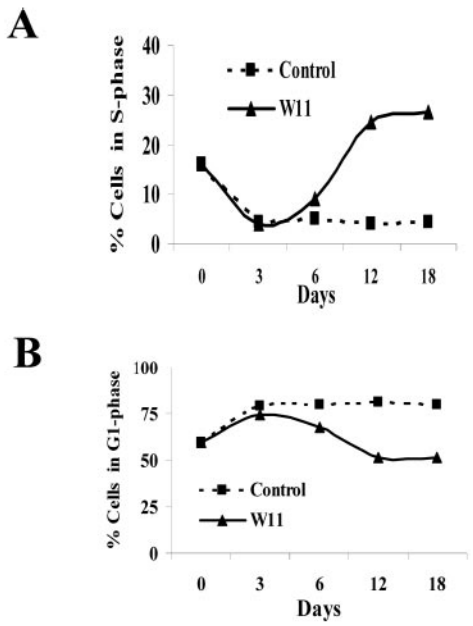

Fig. 3. Effect of Wnt11 on cell cycle-dependent differentiation of Caco2-BBE cells

Caco2-BBE cells were cultured to confluency and treated with either conditioned media harvested from Wnt11-secreting QCE6 cells (W11) or control media that were not exposed to any cells for a period of 18 days post-confluency. Cell cycle profiles were determined by FACS analysis, and the percentages of cells in the S and G_1 phase of the cell cycle over time are shown in *A* and *B*, respectively.

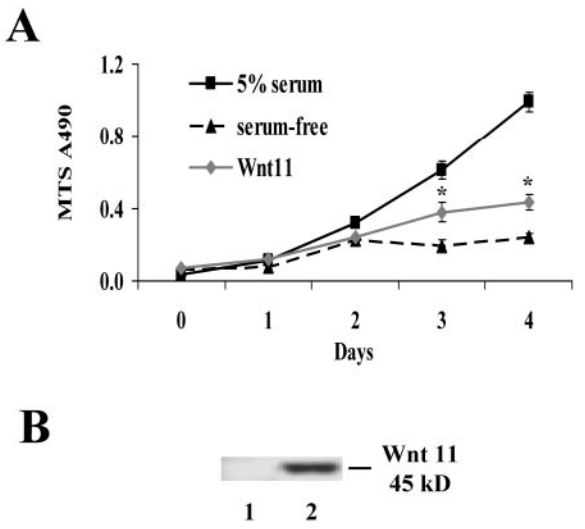

Fig. 4. Effect of Wnt11 on proliferation of IEC6 cells

A, proliferation of IEC6 cells treated with 5% serum, control serum-free media, and Wnt11 conditioned media was measured over 4 days using the MTS assays and presented as absorbance at A_{490} (*A490*); $n = 4$ for each time point. *Vertical bars* are standard deviations. *, $p < 0.001$ when compared with control serum-free, media-treated cells. *B*, presence of Wnt11 in conditioned media. Western blot analysis of Wnt11 protein was conducted on control serum-free media (*lane 1*) and conditioned media from Wnt11-secreing cell line W11ox (*lane 2*). The 45-kDa Wnt11 protein band is present only in the conditioned media from the W11ox cell line.

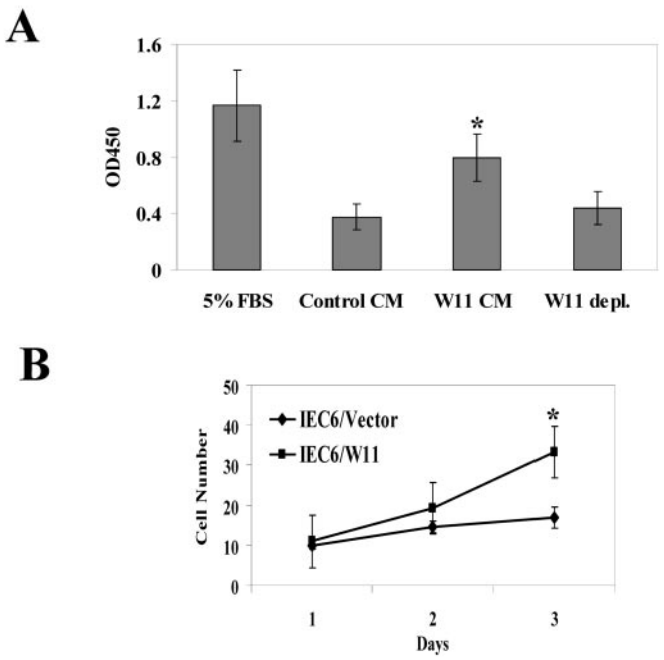

Fig. 5. Effect of Wnt11 depletion and stable transfection on proliferation of IEC6 cells

A, IEC6 cells were cultured for proliferation assays in the presence of 5% FBS, control CM, W11 CM, or W11 CM-depleted (*W11 depl*) for Wnt11 over a 3-day period. Proliferation was measured by MTS assay readings at A_{490}; $n = 6$. *Vertical bars* are standard deviations. *, $p < 0.001$ when compared with Wnt11-depleted conditioned media. *B*, IEC6 cells were stably transfected with a Wnt11-expressing vector or vector alone and clonally selected. A single clone was selected and cultured in serum-free media over 3 days and examined for proliferation; $n = 3$ for each time point. *Vertical bars* indicate standard deviations. *, $p < 0.05$ when compared with IEC/Vector cells.

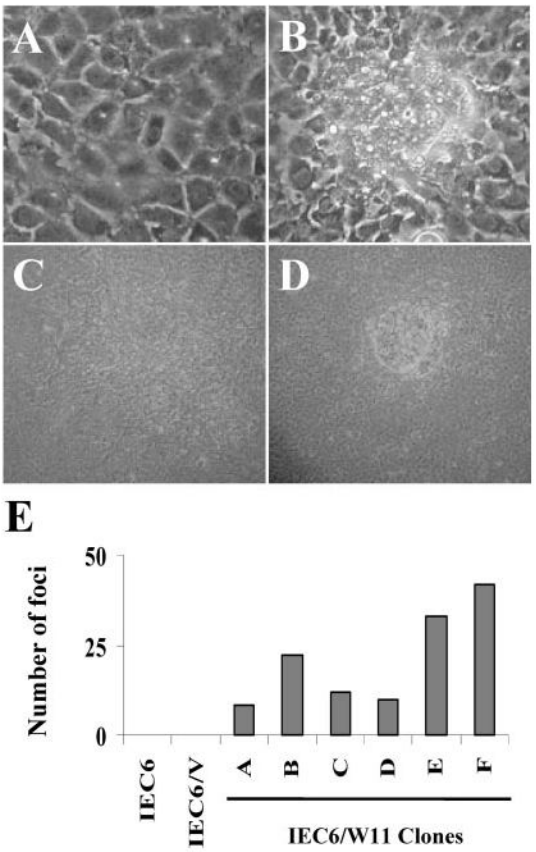

Fig. 6. Effect of long term exposure of IEC6 cells to Wnt11

Foci formation was measured following direct or transwell co-culture of IEC6 cells with W11ox cells. 5×10^5 IEC6 cells were cultured alone (*A*) or with 100 W11ox cells (*B*) for 21 days. IEC6 cells were also grown in transwell insert with no feeder layer (*C*) or with 10^5 W11ox feeder cells (*D*) for 14 days. *E*, transfected IEC6 cells selected in G418 followed by growth on soft agar demonstrate foci formation only in six independent clones of pCDNA3-Wnt11-transfected cells (IEC6/W11) but not with pcDNA3 vector alone (IEC6/V) or untransfected IEC6 cells.

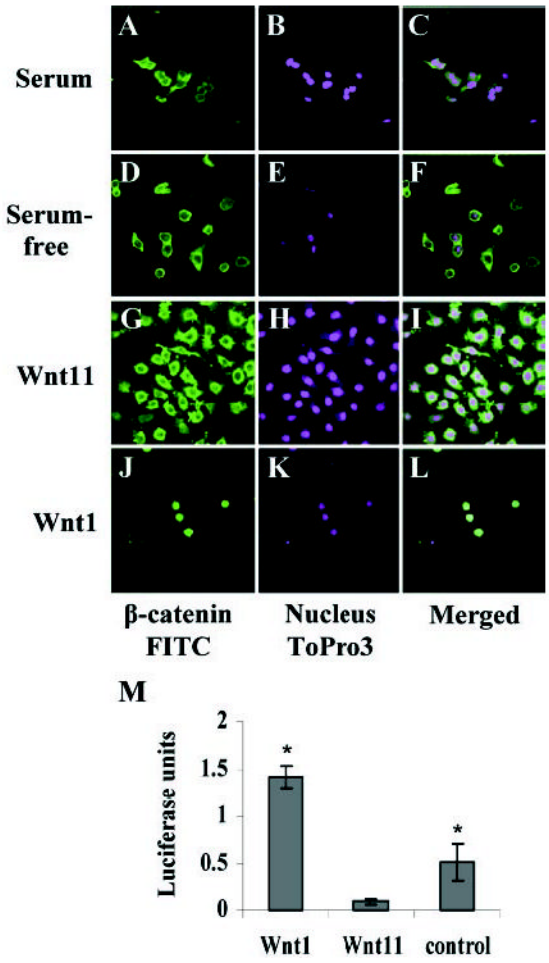

Fig. 7. Signaling mechanism of Wnt11 in IEC6 cells

Nuclear localization of β-catenin in IEC6 cells was assessed by indirect immunofluorescence after 8 h of treatment with 5% FBS (*A*), serum-free control media (*D*), Wnt11 conditioned media (*G*), or Wnt1 conditioned media (*J*). *B*, *E*, *H*, and *K* show nuclear stain with ToPro3; *C*, *F*, *I*, and *L* are merged images. β-Catenin was visualized by the green FITC stain, and the nucleus was labeled *red*. *M*, effect of Wnt11 and Wnt1 on the activity of a TCF3-luciferase promoter construct in IEC6 cells. Fifteen μg of TCF3-luciferase reporter were transiently transfected with 0.5 μg *Renilla*-luciferase and 5 μg of GFP-E3 plasmids as internal controls for transfection efficiency. Cells were treated for 24 h with Wnt1, Wnt11, or control conditioned media. Luciferase activity is represented as the ratio of luciferase activity to *Renilla* luciferase; $n = 3$. *Vertical bars* are standard deviation. *, $p < 0.05$ compared with Wnt11 conditioned media-treated cells.

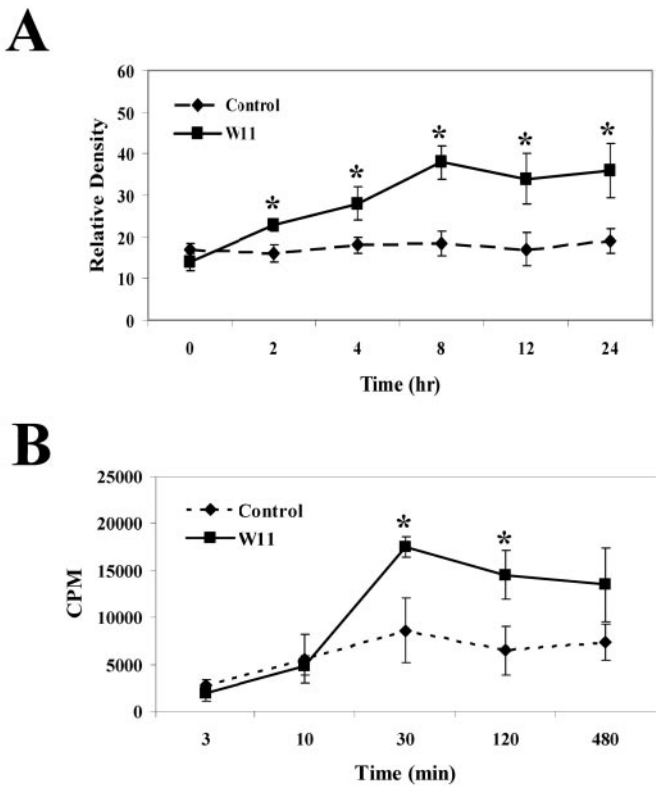

Fig. 8. Effect of Wnt11 on PKC and CamKII activities in IEC6 cells

Enzymatic assays for PKC (*A*) and CamKII (*B*) were conducted in IEC6 cells treated with Wnt11 conditioned media or control media for the periods indicated. The PKC assay was performed using a nonradioactive, colorimetric kit (Promega). Shown are the relative optical densities that reflect enzymatic activities. The CamKII assay was conducted by using a radioactive assay kit (Upstate Biotechnology, Inc.) that expresses enzymatic activity in counts/min; $n = 3$ for each time point. *Vertical bars* are standard deviations. *, $p < 0.05$ when compared with cells treated with control media for the same time.

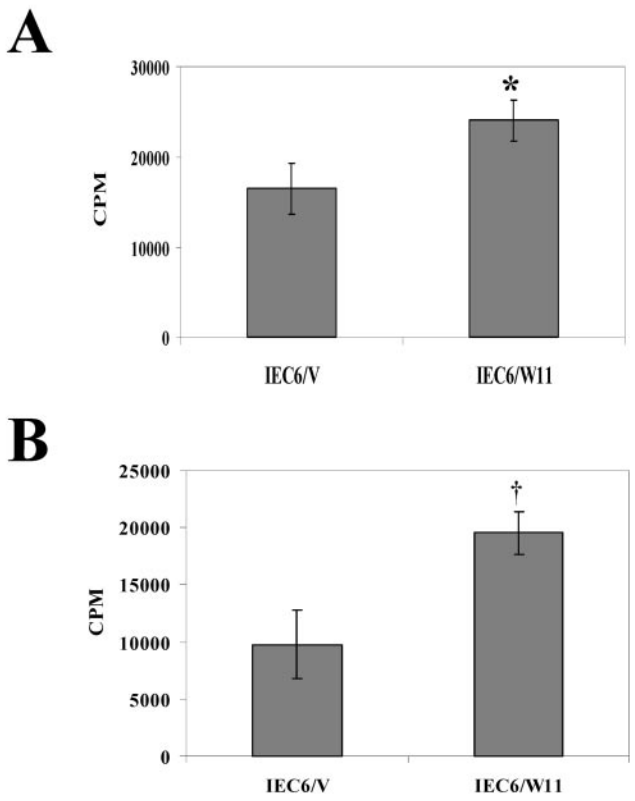

Fig. 9. PKC and CamKII activities in Wnt11-transfected IEC6 cells

PKC (*A*) and CamKII (*B*) activities were conducted in stably transfected IEC6 cells with Wnt11 (*IEC6/W11*) or vector (*IEC6/V*) by using the radioactive PKC and CamKII assay kit from Upstate Biotechnology, Inc., respectively; $n = 4$. *Vertical bars* are standard deviations. *, $p < 0.05$; †, $p < 0.005$ when compared with vector-transfected cells in *A* and *B*, respectively.

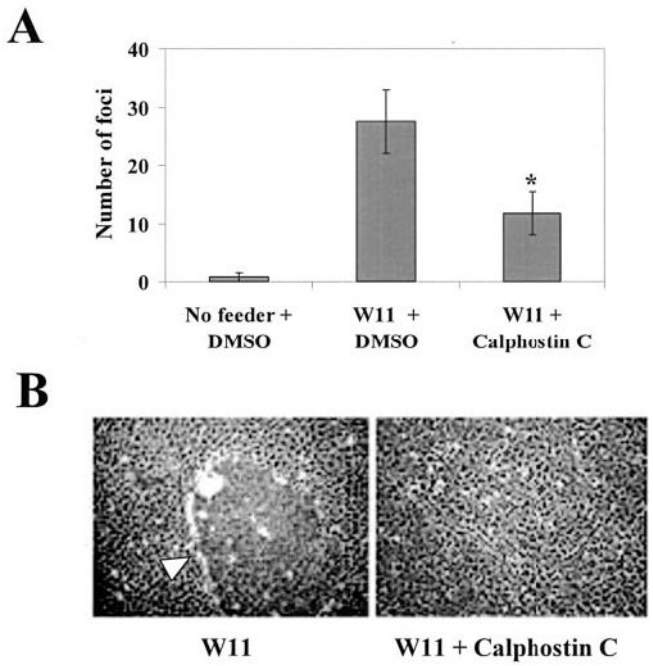

Fig. 10. Effect of PKC inhibition on the ability of Wnt11 to induce foci formation in IEC6 cells

A, focus formation assays were conducted as described under "Experimental Procedures" using IEC6 cells grown on transwell inserts in the absence of any feeder cells, W11ox or W11ox, with 100 nM calphostin C. Me_2SO (*DMSO*) was used when calphostin C was not included. Media were replaced every other day and the number of foci counted after 3 weeks; $n = 3$. *Vertical bars* are standard deviations. *, $p < 0.005$ when compared with the W11ox + Me_2SO group. *B* is an example of the morphological differences in foci formed in IEC6 cells co-cultured with W11ox and treated or not with calphostin C. The *arrowhead* points to the margin of a large focus.

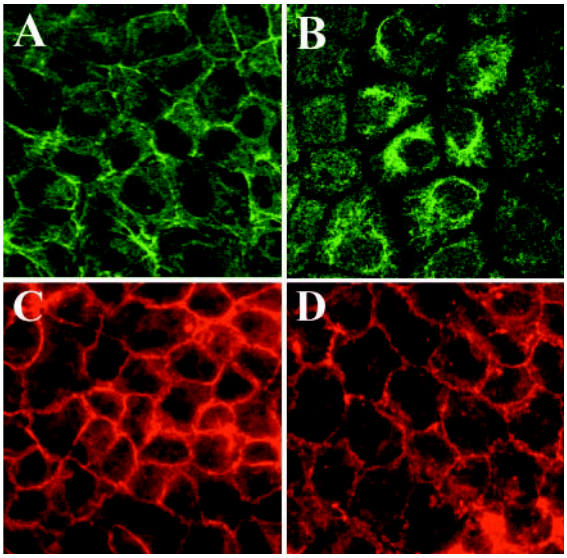

Fig. 11. Effect of Wnt11 on cellular distribution of E- and LI-cadherin in IEC6 cells

IEC6 cells plated on coverslips in 24-well plates were treated with either control serum-free media (*A* and *C*) or Wnt11 conditioned media (*B* and *D*) for 12 (E-cadherin; *green*) or 24 h (LI-cadherin; *red*) and processed for immunocytochemical detection using the appropriate antibodies. Confocal microscopy (×100) was used to capture images.

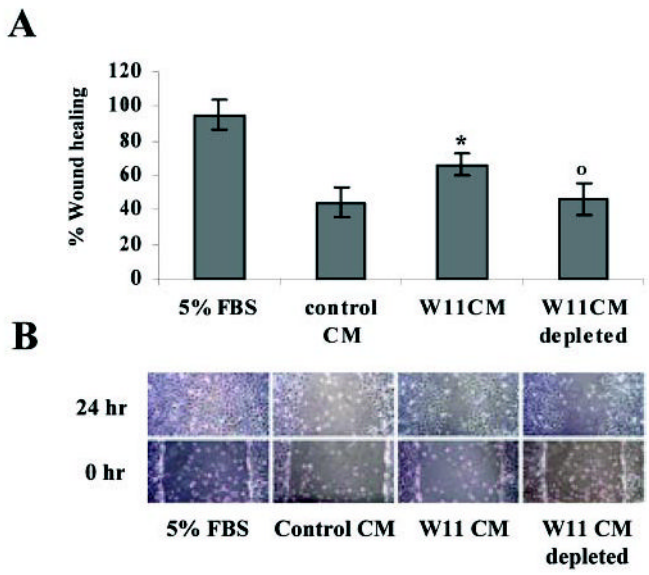

Fig. 12. Effect of Wnt11 on migration of IEC6 cells

Confluent IEC6 cells were wounded by scratching with a pipette tip. The plates were photographed immediately following wounding. Culture media were replaced with normal media containing 5% FBS control serum-free media, Wnt11 conditioned media, or Wnt11 conditioned media that had been depleted of Wnt11 with an anti-Wnt11 antibody (*A*). The plates were photographed at the identical location of the initial image at 24 h (*B*). The % wound healing is calculated based on the sealing of wound in 24 h; $n = 3$. *Bars* are standard errors. *, $p < 0.05$ when compared with control conditioned media-treated cells. ○, $p < 0.05$ when compared with Wnt11 conditioned media-treated cells.

Table I

Sequences of primers used to amplify Wnt sequences

Product	Primer sequence
Wnt degenerate	Reverse, AAAATCTAGARCARCACCARTGRAA
	Forward, GGGGAATTCCARGARTGYAARTGYCAT
Wnt1	Reverse, GCACGTCAACGCCGTCGACTGC
	Forward, CGGTTCTCACGTTTACGGTGCC
Wnt3a	Reverse, GGTCACCACCCACCTATATCG
	Forward, GTGGGTATCTTGTCTTGTCTGAG
Wnt4	Reverse, GCCGGTCGAGGTGAACGCG
	Forward, GCACGACCGCACGACAGGG
Wnt5a	Reverse, CTGTATCGTCGTGGTCACTTTGAACG
	Forward, GTGCATGCGCCACTCGCGTCGA
Wnt11	Reverse, CTTGCAGACGTAGCGCTCCA
	Forward, TCCAACGGAAGCGACAGCTG