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Noncanonical Wnt11 inhibits hepatocellular carcinoma cell proliferation and migration

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Abstract

The canonical Wnt signaling is frequently activated due to over-expression and/or mutations in components of this pathway in hepatocellular carcinoma (HCC). However, the biological role of noncanonical Wnt-mediated signaling in HCC with respect to the signaling pathways involved and their physiologic function is unknown. Here we report the role of Wnt11, a member of the noncanonical cascade, in hepatic oncogenesis. The expression levels of Wnt11 mRNA and protein were significantly downregulated in human HCC tumors compared to the adjacent uninvolved liver as measured by quantitative real-time RT-PCR and western blot analysis. In human HCC cell lines, over-expression of Wnt11 activated PKC signaling. PKC antagonized the canonical signaling through phosphorylation of β-catenin, and reduced TCF mediated transcriptional activity resulting in a decrease of cell proliferation. Furthermore, ectopic expression of Wnt11 promotes RhoA/Rho kinase (ROCK) activation. We found that activated ROCK inhibited Rac1 to reduce cell motility and migration. These observations suggest a novel role for Wnt11 as a tumor suppressor during hepatocarcinogenesis since loss of expression promotes the malignant phenotype via both canonical and noncanonical Wnt signaling pathways.

Keywords

Wnt11; HCC; PKC; Rac1; noncanonical Wnt

Introduction

Hepatocellular carcinoma is one of the most prevalent malignant neoplasms worldwide. Although the major etiologies of HCC are now well defined and include chronic viral hepatitis B and C, toxins, drugs and metabolic liver diseases, the molecular mechanisms that contribute to tumor initiation or progression are poorly understood. There is increasing evidence that aberrantly activated Wnt signaling due to over-expression of upstream components, and/or mutations in signaling proteins of this pathway is a common early event in the molecular pathogenesis of this disease (1-4).

Wnt proteins play a significant role during normal and pathologic developmental processes that includes cell proliferation, differentiation, polarity and migration to affect the organization of the body plan and tissue patterning (5). Moreover, constitutive activation of Wnt signaling

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contributes to the development of human tumors and thus may participate in tumor progression as well as metastasis (6). Wnts transduce canonical and noncanonical signaling pathways. In the canonical Wnt cascade, these ligands bind to Frizzled receptors (FZD) and the low-density lipoprotein-related protein (LRP) co-receptor that inactivates the β-catenin destruction complex and results in stabilization of β-catenin in the cytoplasm followed by translocation into the nucleus. In this regard, β-catenin binds to T-cell factor (TCF) transcription factors to activate Wnt responsive target genes. The activated transcriptional programs direct cell proliferation, survival and modify cell fate. In the absence of Wnt stimulation, β-catenin is phosphorylated within the APC, axin, glycogen synthase kinase-3β (GSK-3β) and CK1 complex followed by proteasomal degradation (7).

Noncanonical Wnt signaling is β-catenin-independent. This cascade may be activated by Wnt4, 5a, and 11 ligands (8). Although noncanonical Wnt pathways are diverse and less well characterized, they have been shown to be important in polarized cell movement and organ morphogenesis through cytoskeletal rearrangement involving the small GTPases RhoA and Rac1. In addition, the noncanonical Wnt/Ca^{2+} pathway initiated through protein kinase C (PKC) and calcium/calmodulin-dependent protein kinase II (CaMKII) is known to antagonize canonical β-catenin signaling (9,10).

Recently, we have investigated the expression of Wnt ligands in human HCC cell lines and found that four Wnt genes (Wnt3, 5a, 6, and 11) among the 19 family members were involved. Wnt3 was shown to activate the canonical pathway via binding to the FZD7 receptor and led to increase HCC cell proliferation and motility. More important, Wnt3 expression was upregulated in human HCC compared to the adjacent peritumoral tissues (4). However, the function of noncanonical Wnt11 in HCC has not been explored.

In the current study, we analyzed the expression of Wnt11 in human HCC; mRNA and protein levels were found to be downregulated in tumors compared to uninvolved liver tissue. With respect to human HCC cell lines, we observed that Wnt11 antagonized the canonical β-catenin signaling by promoting phosphorylation of β-catenin via PKC activation; the functional consequence is reduced HCC cell proliferation. In this context, Wnt11 activated RhoA and Rho kinase (ROCK), which inhibited Rac1 activity and led to suppression of cell migration and motility. Thus, these findings suggest that Wnt11 may play a role as a tumor suppressor during hepatocarcinogenesis.

Results

Wnt11 expression is downregulated in human HCC

To explore the Wnt11 signaling pathway in HCC, we measured expression in 4 different HCC cell lines using quantitative real-time RT-PCR. As shown in Fig. 1A, the level of Wnt11 mRNA was highest in HepG2 followed by Hep3B and Huh7; however, Wnt11 was undetectable in FOCUS cells. The expression of Wnt11 was also assessed in 17 pairs of tumor and corresponding adjacent peritumoral tissue. Wnt11 mRNA expression was significantly downregulated in tumor ($p = 0.017$) compared with the adjacent uninvolved liver tissue (Fig. 1B). Eleven of 17 (65%) showed decreased expression of Wnt11 mRNA in HCC tumor compared to peritumoral tissues (Fig. 1C). We also evaluated the level of Wnt11 protein in 10 pairs of tumor and peritumoral tissue by western blot analysis. As shown in Fig. 1D, 8 of 10 (80%) exhibited downregulation of Wnt11 protein in tumor compared to peritumoral tissue. Downregulation of Wnt11 protein was also confirmed by immunohistochemical staining of a representative example (Case 5) as shown in Fig. 1E.

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Wnt11 inhibits the canonical β-catenin signaling pathway

It has been reported that noncanonical Wnt ligands may inhibit the canonical signaling cascade in thyroid and prostate adenocarcinomas (11,12), as well as hematopoietic stem cells (13). The observation that Wnt11 mRNA expression was downregulated in HCC tumors led us to examine if exogenous expression of Wnt11 modulates canonical β-catenin signaling. In this context, we chose three different HCC cell lines for analysis. FOCUS and Huh7 cell lines exhibited no or low levels of Wnt11 expression, respectively (Fig. 1A). The highest level were observed in HepG2 cells which contain a mutant β-catenin gene (14). To evaluate activation of canonical Wnt signaling in these HCC cell lines, we measured the TCF transcriptional activity using a TCF-reporter plasmid. A Wnt11-myc expressing construct was transiently transfected into FOCUS, Huh7 and HepG2 cell lines; Wnt11 protein levels were determined with an anti-c-Myc antibody (Fig. 2C). Over-expression of Wnt11-myc in FOCUS and Huh7 cells resulted in a significant reduction of TCF activity compared to control (EV) whereas there was no change as expected in HepG2 cells (Fig. 2A).

To elucidate the potential mechanisms of the Wnt11-mediated inhibition of canonical signaling, we first investigated the effect of Wnt11 on β-catenin nuclear accumulation. FOCUS and Huh7 cells were transiently transfected with Wnt11-myc or empty vector plasmids followed double-immunostaining with both anti-β-catenin (red) and c-Myc (green) antibodies. As shown by a representative example presented in Fig. 2B, the control Huh7 cells revealed high level of β-catenin accumulation in the nucleus (yellow arrows). In contrast the Wnt11-transfected Huh7 cells revealed a striking decrease of β-catenin nuclear accumulation that now localized to the plasma membrane (white arrows). The cellular level of β-catenin expression was also examined by Western blot analysis. As shown in Fig. 2C, the total β-catenin level was decreased in Wnt11-overexpressing FOCUS and Huh7 as compared to control cells. Furthermore, the reduced level of β-catenin was associated with an increase in phospho-β-catenin. There was no change of total- and phospho-β-catenin levels in HepG2 cells due to the presence of a truncated form of β-catenin which does not undergo proteasomal degradation (14). This finding is consistent with the result displayed in Fig. 2A indicating that over-expression of Wnt11 in HepG2 cells had no effect on TCF transcriptional activity. We evaluated if Wnt11 expression influenced the activity of GSK-3β by Western blot analysis. However, the levels of phospho- and total GSK-3β were not influenced by Wnt11. Therefore, it is unlikely that GSK-3β was responsible for the enhanced phosphorylation of β-catenin induced by Wnt11. To further confirm whether GSK-3β was a participant in Wnt11 signaling, we utilized LiCl as a specific inhibitor of GSK-3β. LiCl treatment substantially increased TCF reporter activity in control cells; however, Wnt11 expression still inhibited TCF reporter activity by 80% in FOCUS cells (Fig. 2D). We are led to believe that Wnt11 may inhibit the canonical β-catenin signaling via GSK-3β-independent pathways since ectopic expression of Wnt11 may regulate TCF transcriptional activity via β-catenin phosphorylation. Moreover there was no effect on expression levels of other components in the β-catenin destruction complex such as axin and APC (data not shown).

Previous reports have shown that reduced levels of β-catenin are directly associated with inhibition of human HCC cell proliferation and survival (4,15,16). Therefore, the effects of Wnt11 on cell proliferation and apoptosis were investigated. As shown in Fig. 2E, Wnt11 over-expressing cells (FW11-1 and FW11-2) exhibited significantly reduced growth rates compared to controls (F-C) at days 4 and 5 of culture. It is noteworthy that TUNEL assays revealed no differences in apoptosis rates between Wnt11 over-expressing and control cells (data not shown).

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Wnt11 activates PKC Signaling

It has been previously reported that the noncanonical Wnt/Ca^{2+} pathway inhibits the canonical β-catenin cascade (17,18). Therefore, we determined if Wnt11 altered intracellular Ca^{2+} levels. As shown in Fig. 3A, the Ca^{2+} signal intensity was increased in two different Wnt11 stable transfected FOCUS clones compared to control cells. Given the fact that Wnt11 increased cytosolic free Ca^{2+}, it became of interest to test for activation of PKC and CaMKII calcium-dependent kinases. The PKC activity was increased following over-expression of Wnt11 in FOCUS (19%) and Huh7 (28%) compared to control (Fig. 3B). It is known that PKC translocates from the cytoplasm to the plasma membrane following activation (19). As shown in Fig. 3C, immunofluorescent studies revealed translocation of PKC to the plasma membrane in Wnt11 overexpressing (FW11-1), but not in control cells (F-C). In addition, suppression of endogenous Wnt11 expression by siRNA induced decreased PKC activity in Hep3B cells (Fig. 3D), suggesting that Wnt11 influenced on activation of PKC. In contrast, Wnt11 had no effect on CaMKII activity as assessed by Western blot analysis. Indeed, the protein levels of CaMKII and phosphorylated CaMKII (active form) were unchanged compared to controls (data not shown).

To further define if PKC activity was responsible for antagonism of the canonical Wnt signaling pathway, BisI (PKC) and Y27632 (Rho kinase; ROCK) inhibitors were employed. As shown in Fig. 3E, treatment with BisI but not Y27632 restored the reduced TCF activity mediated by Wnt11. This experiment demonstrated that PKC was involved in reduced canonical Wnt signaling as mediated through Wnt11. Taken together, Wnt11 appears to antagonize the canonical β-catenin pathway via activation of PKC, and subsequently leads to inhibition of cell growth (Fig. 2D).

Wnt11 inhibits HCC cell migration

Following stable transfection of Wnt11 into FOCUS cells, substantial changes in morphology were observed. Control cells appeared flattened and irregular with many lamellipodia at the leading edge consistent with a migratory cell phenotype. In contrast, Wnt11-overexpressing cells (FW11-1) exhibited a smooth leading edge with few lamellipodia (Fig. 4A, left). Therefore, the possible role of Wnt11 on reorganization of the actin cytoskeleton was examined by rhodamine-conjugated phalloidin staining. FOCUS cells over-expressing Wnt11 revealed an extensive rearrangement of stress fibers, whereas phalloidin staining in control cells showed far less stress fiber formation and these structures were localized primarily to the leading edge of the cells (F-C) (Fig. 4A, right).

The rearrangement of cytoskeletal elements is known to play an important role in cell migration (20), and implies that Wnt11 may influence HCC cell migration and motility. By using the Wound-healing assay, it was found that Wnt11 over-expressing cells had a significant reduction of cell migration as compared to control (Fig. 4B). To confirm this observation, we tested cell motility using a transwell chamber assay. Cell motility in Wnt11 over-expressing cells (FW11-1, FW11-2) was decreased by 42% compared to control (Fig. 4C). To further examine the effect of Wnt11 on cell motility, we determined if knockdown of Wnt11 expression using siRNA would restore the decreased cell motility as mediated by Wnt11. A siRNA specific for Wnt11 (W11-si) or control siRNA (C-si) was transfected; knockdown of Wnt11 expression was verified by Western blot analysis (Fig. 4D, upper panel). The reduction of Wnt11 expression by siRNA partially restored cell motility in Wnt11 over-expressing cells. Moreover, knockdown of endogenous Wnt11 by siRNA in Hep3B cells resulted in increased cell motility (Fig. 4E).

Signaling through the noncanonical Wnt/planar cell polarity (PCP) regulates cytoskeletal architecture through small GTPases including RhoA and Rac1. Furthermore, RhoA and Rac1

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are known to be involved in the formation of stress fibers and membrane protrusions (21,22). Therefore, we explored the idea that inhibition of HCC cell migration and motility as mediated by Wnt11 may be directed through RhoA and/or Rac1 activation. As shown in Fig. 5A, over-expression of Wnt11 in both FOCUS and Huh7 cells increased RhoA as well as ROCK activity compared to control. In contrast, Rac1 activity was decreased in both stable (FW11-1, FW11-2) and transient (W11-myc) Wnt11 over-expressing cells compared to controls (Fig. 5B).

Recently, it has been reported that Wnt/PCP signaling promotes RhoA activity and inhibits Rac1 in neural crest (23) and human GNS-3314 glioblastoma cells (24). Accordingly, the observation that RhoA/ROCK activation may lead to inhibition of Rac1 allowed us to test this concept in the Wnt11 over-expressing HCC cells. As shown in Fig. 5C, reduced Rac1 activity was abolished using the ROCK inhibitor Y27632, suggesting that ROCK is an upstream effector of Rac1. Y27632 has previously been found to suppress HCC cell migration and invasion (25). Thus, it was determined if ROCK was involved in alteration of cell motility as mediated by Wnt11. It is noteworthy that ROCK inhibitors Y27632 and HA1077 restored motility, whereas BisI, a PKC inhibitor did not (Fig. 5D). Taken together, these studies suggest that Wnt11 activates RhoA/ROCK, and the activated ROCK subsequently inhibits Rac1 that contributes to decreased cell migration and motility.

Discussion

The canonical β-catenin signaling pathway plays a major role in the pathogenesis of HCC. However, there is no information regarding function of the noncanonical cascade. Recently, we reported that the Wnt11 ligand is expressed in HCC cell lines (4). In the present study, Wnt11-mediated signaling was assessed in the context of how it may contribute to the development of HCC. Evidence was provided that Wnt11 may function by antagonizing the canonical β-catenin cascade through activation of PKC, which subsequently leads to inhibition of cell growth. In addition, Wnt11 inhibited cell migration through modulation of RhoA/ROCK and Rac1 activity.

Recent studies suggest that noncanonical Wnt signaling alters the canonical β-catenin cascade in different tumor types (11,12). However, the mechanisms that drive Wnt11 inhibitory effects on canonical signaling appear to be tissue specific and poorly understood. Here we show that Wnt11 activated the Ca^{2+}/PKC pathway and downregulated β-catenin levels in cells, resulting in inhibition of canonical signaling. Our findings are in agreement with a report demonstrating that PKC phosphoylates β-catenin to subsequently inhibit Wnt3a mediated canonical signaling in HEK 293 cells (26).

Wnts regulate mammalian cell migration. Wnt3a has been shown to stimulate the migration of myeloma and Chinese hamster ovary cells (27,28), whereas Wnt5a repressed migration of thyroid carcinoma cells (11). Similarly, the noncanonical Wnt11 ligand inhibits migration and motility through modulation of RhoA/ROCK and Rac1 activities in HCC cells. Crosstalk between Rac and RhoA has been described in various cell types. Indeed, several reports suggest that RhoA suppresses Rac1 activity in different cell lines (29-34). In addition, Rac1 activity is increased in fibroblasts and neutrophils following treatment with Y27632, indicating that ROCK may be involved in Rac1 inactivation (32,35). Recently, Liu et al. reported that Rac1 plays a crucial role in the control of HCC cell motility and metastasis (36). Moreover, it has been observed that reduced Rac activity inhibits lamellipodia formation required for a migratory cell phenotype, whereas Y27632 treatment increased lamellipodia through Rac activation (37). In agreement with these finding, our observations reveal that Wnt11 over-expressing cells showed less lamellipodia formation (Fig. 4A), possibly through inactivation of Rac1 via ROCK.

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Several studies have shown that upregulation of RhoA expression correlated with tumor progression and metastasis in HCC (38-40), whereas treatment with Y27632 suppressed cell migration and invasion (41,42) which is in contrast our observations. However, consistent with our results, are studies that demonstrated Rac1 activation was associated with increased HCC cell motility and migration (36,43). Previous studies have not explored dual activation of RhoA and Rac1 in HCC in the context of noncanonical Wnt11 signaling. It is important to note that in other cell types, Wnt/PCP signaling promotes RhoA activity and activated RhoA inhibits Rac1 (23,24). In agreement with these reports, our experiments reveal that Wnt11 activates RhoA and ROCK; Rac1 activity was inhibited by ROCK and results in suppression of HCC cell migration and motility. Hepatocellular carcinoma is characterized by a migratory/invasive cell phenotype, due, in part, to Rac1 activation. Our results suggest that loss of Wnt11 noncanonical signaling may contribute to the activation of Rac1. It will be of interest to further examine crosstalk between RhoA and Rac1 in HCC.

We found the expression level of Wnt11 mRNA was significantly decreased in HCC tissue compared to the adjacent uninvolved liver. Given the observation of canonical pathway antagonism by Wnt11 as revealed in the present study and the well-established link between aberrant activation of canonical signaling and cancer development (5,44), we hypothesize that loss of Wnt11 signaling activity may contribute to the development of HCC. It is noteworthy that the biological function of Wnt11 appears to directly opposes the activity of Wnt3 which is known to stimulate cell proliferation and motility (4). As shown by the scheme presented in Fig. 6, both Wnt3 and Wnt11 may be involved in tumorigenesis through different but reciprocal mechanisms. In this context, a balance of expression between Wnt3/canonical and Wnt11/noncanonical signaling may be important for homeostatic regulation of hepatocyte cell growth and migration in the normal liver. Cellular alterations in this balance could contribute to the development of HCC. In support of this idea, recent studies describe mutual antagonism of canonical β-catenin signaling by components of the noncanonical cascade during embryonic development (17,45,46). Furthermore, when the ratio of Wnt3 to Wnt11 expression was calculated on the same HCC samples to evaluate the balance between the two ligands, a value of 17.2 was found for tumor compared to 1.7 in adjacent uninvolved liver tissue (ref. 4 and Fig. 1B). This finding is consistent with previous observations demonstrating activation of canonical β-catenin signaling due to over-expression of Wnt3 and Frizzled-7 in HCC (4). The canonical signaling pathway would be further amplified by downregulation of an inhibitory Wnt11 mediated signal to further promote a proliferative, migratory and invasive cell phenotype.

In summary, our study suggests a significant role for loss of Wnt11 mediated signaling during hepatocarcinogenesis. Wnt11 influences both the canonical and noncanonical signaling pathways in HCC and participates in the negative regulation of cell proliferation and migration (Fig. 6). It is likely that Wnt11 and Wnt3 function to regulate activity of canonical β-catenin signaling in liver. Since dysregulation of β-catenin signaling has been shown to be important in tumor formation, further analysis of these two pathways may provide insight into HCC pathogenesis.

Materials and Methods

Human HCC tissues

Seventeen pairs of HCC and matched peritumoral liver tissue were obtained from South Korean patients who underwent surgical resection for diagnosis and therapy. Clinical and pathological features of these HCC patients has been presented (4). Use of these tissues was approved by the Brown University Institutional Review Board.

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Quantitative real-time RT-PCR

To determine the levels of Wnt11 mRNA expression, real-time RT-PCR was performed as previously described (2) using the following primers: Wnt11, 5′-TTCCGATGCTCCTATGAAGG-3′ and 5′-AGACACCCCATGGCACTTAC-3′; 18S rRNA, 5′-GGACACGGACAGGATTGACA-3′ and 5′-ACCCACGGAATCGAGAAAGA-3′. The copy number of Wnt11 mRNA was quantified by the Ct values compared to standard curves and followed by normalization to 18S rRNA. All reactions were performed in triplicate.

Immunhistochemical staining

Formalin-fixed, paraffin-embedded tumor and adjacent uninvolved peritumoral tissue sections were applied for Wnt11 protein expression by immunohistochemistry. Sections were deparafinized, rehydrated, and subsequently incubated with anti-Wnt11 antibody (sc-50360; Santa Cruz Biotechnology, CA) overnight at 4°C. Anti-rabbit IgG antibody conjugated with horseradish peroxidase-labeled polymer (EnVision+ System; Dako) was incubated for 45 min at room temperature; immunoreactivity was detected using 0.1% 3-3″-diamino-benzidine-tetrahydrochloride and 0.005% H_2O_2 in 0.1 mol/L Tris-HCl buffer (pH 7.4). Sections were counterstained with hematoxylin (Zymed) and examined under a light microscopy.

Cell culture and transfection studies

Human HCC cell lines (FOCUS, Huh7, Hep3B, and HepG2) were propagated in DMEM with 10% fetal bovine serum. The human Wnt11 cDNA (Wnt11-myc) was cloned from the HepG2 cells using the TA cloning kit (Invitrogen™, Carlsbad, CA) and subcloned into the pcDNA3.1/myc-His B vector (Invitrogen™). The Wnt11-myc or empty vector plasmid was transfected into HCC cells using a LT1 transfection reagent (Mirus Bio Co., Madison, WI), according to the manufacturer's instructions. To obtain FOCUS cells stably transfected with empty vector (F-C) or Wnt11-myc (FW11-1, FW11-2), colonies of G418-resistant cells were selected and expanded for further characterization. For analysis of TCF transcriptional activity, we employed a TOPFlash/FOPFlash reporter gene assay as previously described (4).

Control small interfering RNA (siRNA) and specific for human Wnt11 (Santa Cruz, Santa Cruz, CA) were transfected into cells at a concentration of 100 nM using the siPORT™ NeoFX™ transfection agent (Ambion, Austin, TX). Knockdown efficiency of Wnt11 was examined by Western blot analysis.

Western blot analysis

Western blot analysis was performed as previously described (2) using the following antibodies; β-catenin (BD Biosciences, San Diego, CA), phospho-β-catenin (Ser33/37/Thr41), phospho-GSK-3β (Ser9) (Cell Signaling, Beverly, MA), GSK-3β, actin, Axin, APC, c-Myc, and Wnt11 (H-95) (Santa Cruz). To detect endogenous Wnt11 expression, Solution 1 for Primary antibody (Calbiochem) and Solution for Secondary antibody (Calbiochem) were utilized to increase a ratio of immunoreactivity to background and SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, IL) was used for detection.

Immunofluorescent staining

Cells were incubated with β-catenin and c-Myc antibodies followed by FITC- or Texas red-conjugated secondary antibodies (Vector Lab., Burlingame, CA). For PKC immunostaining, anti-PKC (Cell Signaling) and Alexa594- or FITC-conjugated secondary antibodies were used. Immunostaining was examined under an Olympus IX70 fluorescence or a Zeiss 410 confocal microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY).

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Measurement of cytosolic Ca^{2+}

The Ca^{2+} levels in cells were determined using the fluorescent fura-2 acetaxymethyl ester probe (fura-2/AM) (Invitrogen™). After overnight serum-starvation, cells were loaded with fura-2/AM (2 uM) for 45 min at 37°C in modified Hank's solution. Fura-2/AM fluorescence was measured in a SpectroMax M5 (Molecular Devices Co, Sunnyvale, CA). The levels of intracellular Ca^{2+} were estimated by using excitation at 340 nm and 380 nm and by the ratio of fluorescence intensities detected at 510 nm.

PKC activity assay

A non-radioactive PKC kinase assay kit (Promega, Madison, WI) was used (47). Briefly, cells were lysed and centrifuged at 100,000g for 1 h, and the PKC activity was assessed using equal amount of protein. Reactions were processed at 30°C for 30 min and the phosphorylated peptide substrate was analyzed by agarose gel electrophoresis.

RhoA and Rac1 activity assay

The activities of RhoA and Rac1 were analyzed with EZ-Detect Rho and Rac1 Activation Kit (Pierce biotechnology, Rockford, IL), respectively. These assays utilize GST-fusion proteins that specifically bind to the active forms of RhoA or Rac1. Cells were rapidly lysed on ice and whole cell lysates were incubated with either GST-human Rhotekin (for active RhoA) or GST-human p21-activated kinase1 (PAK1; for active Rac1) for 1 h at 4°C. The resin was washed and GTP-bound RhoA or Rac1 was collected for Western blot analysis with anti-RhoA or Rac1 antibodies. Whole cell lysates were also subjected to immunoblot analysis as a control.

Kinase inhibitors

To block kinase activity, bisindolylmaleimide I (BisI, PKC inhibitor), LiCl (GSK-3β inhibitor), and Y27632 and HA1077 (ROCK inhibitors) was used. Cells were treated with these agents for 1 or 24 h, and subjected to further experiments. All kinase inhibitors were obtained from Calbiochem.

Cell proliferation assay

We used the CellTiter 96 AQeous Non-Radioactive Cell Proliferation Assay (Promega). Briefly, cells (1×10^4) were seeded into 24-well plates and cell proliferation was measured with a combined MTS/PMS solution for 5 d. Results were presented as the average absorbance of 3 wells per experiment.

Wound healing and transwell cell motility assays

Cell migration was assessed using a scratch wound assay (4). A scratch was made using a sterile micropipette tip and cells were washed to remove floating cells and debris. The wound closure was photographed at the indicated time points from same area of the wound. Results were derived from three independent wells.

Cell motility was evaluated using a luminescence-based assay as described previously with some modifications (48). In brief, cells (5×10^4) were seeded into the upper chamber of 12-well plate separated by a Transparent PET Membrane (BD Biosciences). Migrated cells were harvested with a cotton swab and luminescence was measured after incubation with ATPLite substrate (PerkinElmer). The results are expressed as a percent of migrated to total number of cells and the experiments were performed in triplicate. For microscopic visualization, the migrated cells were fixed with 4% paraformaldehyde for 10 min followed by staining with 4% crystal violet for 30 min at 20 °C. The cells were visualized at ×40 magnification.

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Statistical analysis

All analyses were performed with R's software (R Foundation for Statistical Computing, Austria). The results were reported as mean ± SD.

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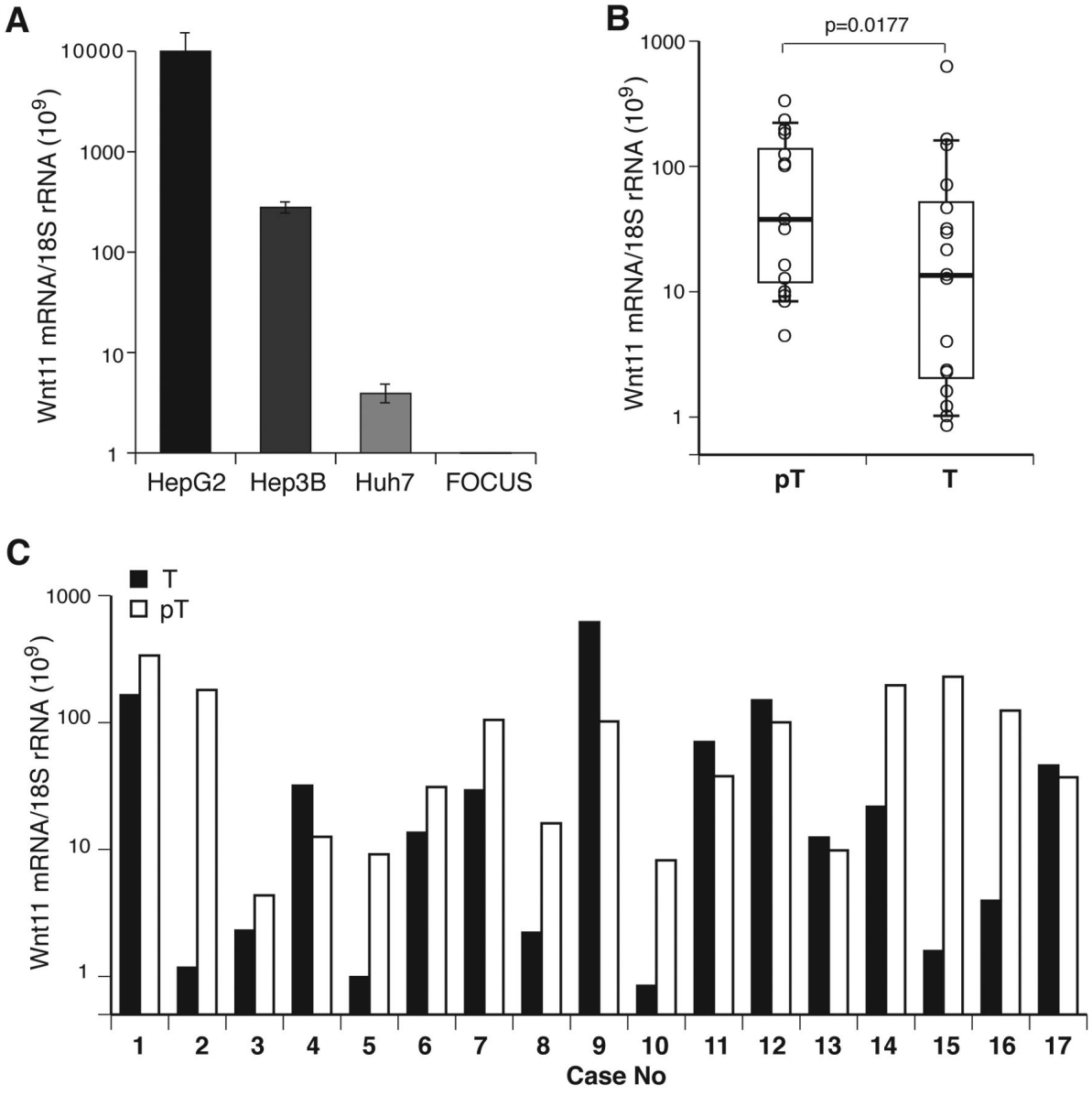

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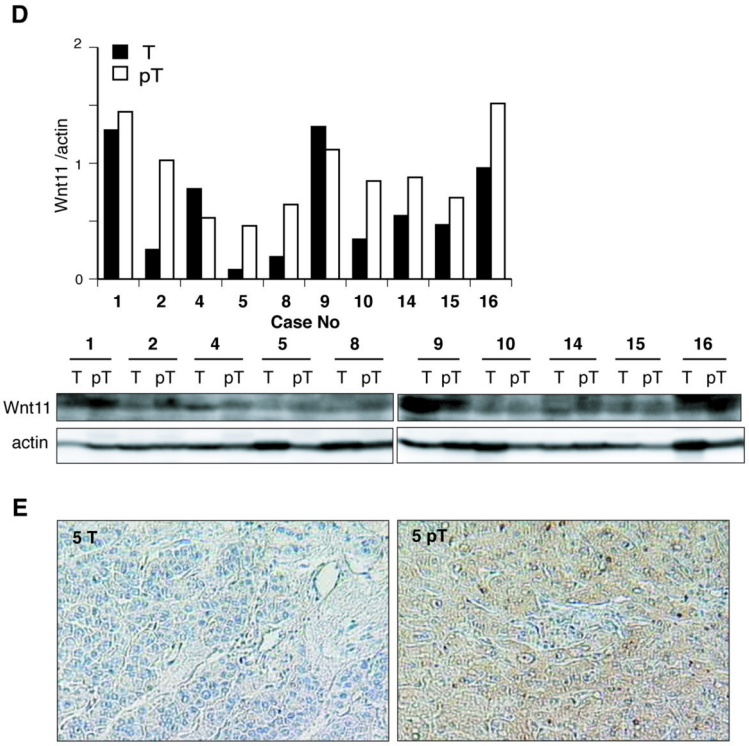

Figure 1.

Wnt11 expression in human HCC tissue samples and cell lines. *A*, The level of Wnt11 mRNA was measured in 4 different HCC cell lines (HepG2, Hep3B, Huh7, and FOCUS) and plotted as copy numbers. No detectable expression of Wnt11 mRNA was found in FOCUS cells. *B*, Seventeen paired human HCC and corresponding adjacent HCC-free tissue were analyzed for Wnt11 mRNA expression. Wnt11 was significantly downregulated in HCC tumors compared to the adjacent peritumoral liver tissue. Horizontal bars indicate the mean values within each group of samples. Statistical comparisons were made using paired t-tests (p=0.0177). *C*, The level of Wnt11 mRNA plotted as a bar graph. The black bars represent the mRNA levels in HCC tissues, and the white bars in corresponding peritumoral areas. Within the paired samples, 11 of 17 (65%) showed decreased expression of Wnt11 mRNA in tumors compared with corresponding peritumoral tissues. *D*, Western blot analysis of Wnt11 expression in human HCC. Expression of Wnt11 protein was detected by anti-Wnt11 antibody and actin was used as a loading control (bottom panel). The level of Wnt11 protein was plotted as a ratio to actin (top panel). Note that 8 of 10 samples exhibited reduced expression level in tumor (T) compared to peritumoral (pT) tissues. *E*, Wnt11 protein expression in human HCC tissue samples using immunohistochemical statining. Representative example (case #5) of HCC and pertitumoral area was immunostained with anti-Wnt11 antibody (brown color) and counterstained with hematoxylin (blue color). Weak but clear positive signal for Wnt11 was found in the cytoplasm of hepatocytes in the peritumor liver tissue (right). In contrast, the immunoreactive Wnt11 in the HCC tissue was negative (left). (Magnification, × 100)

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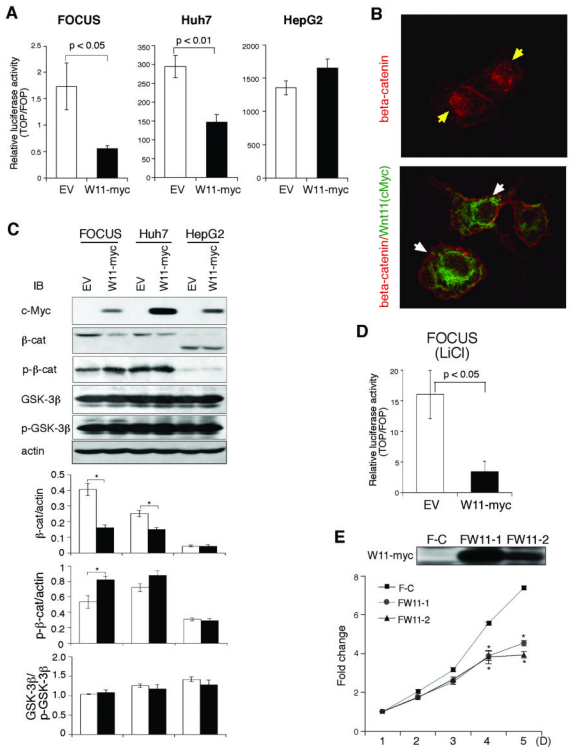

Figure 2.

Over-expression of Wnt11 inhibits canonical Wnt signaling. *A*, Effects of Wnt11 on TCF transcriptional activity. The TCF activity was significantly decreased in FOCUS and Huh7 cells by 68% and 50%, respectively, but not in HepG2 cells which has an inactivating β-catenin mutation. *B*, Effect of Wnt11 on β-catenin nuclear accumulation in Huh7 cells by a double-immunofluoescence staining. Nuclear accumulation of β-catenin was reduced in Wnt11-expressed cells (bottom, white arrows), whereas control cells revealed high level of β-catenin accumulation in the nucleus (top, yellow arrows). *C*, Over-expression of Wnt11 decreased β-catenin levels in the manner of GSK-3β-independent. Over-expression of Wnt11 reduced total β-catenin levels with a concomitant increase in phospho-β-catenin; however, there was no difference between total GSK-3β and phospho-GSK-3β level. The bar graphs depict the results of densitometric analysis from the Western blots (bottom). The results are reported as the mean of three independent experiments. *: $p < 0.05$ *D*, Effect of GSK-3β inhibitor (LiCl) on β-catenin-dependent TCF activity in Wnt11 overexpressing cells. LiCl treatment did not rescue the reduced TCF activity observed in Wnt11 overexpressing FOCUS cells (W11-myc). *E*, Reduced cell proliferation in Wnt11 over-expressing cells. Cell proliferation rate was significantly decreased in FOCUS (FW11-1 and FW11-2) compared to control cells (F-C). Constitutive Wnt11 over-expression was demonstrated by Western blot analysis (top panel). *: $p < 0.05$ versus control cells

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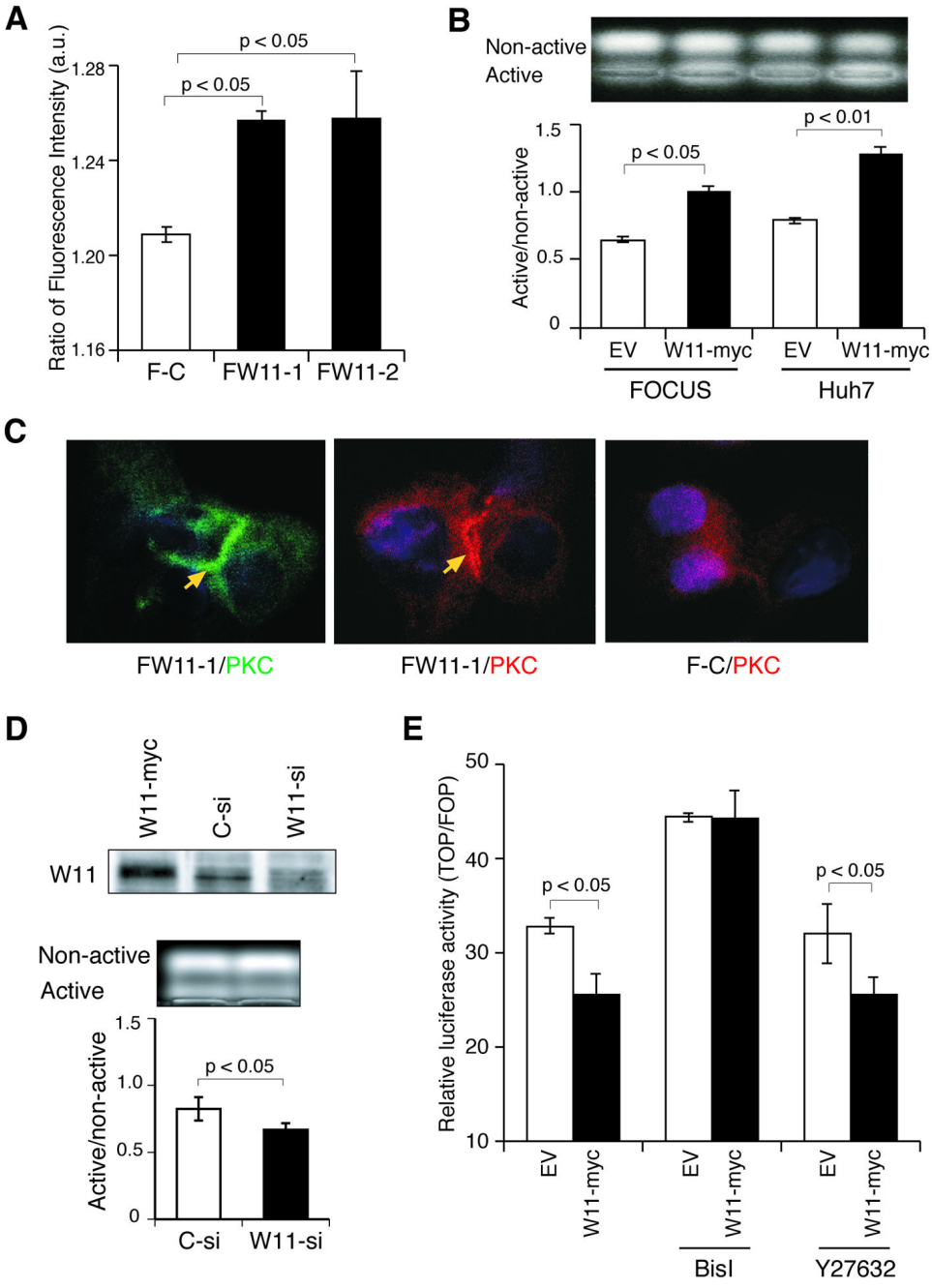

Figure 3.

Wnt11 expression activated PKC signaling. *A*, Effect of Wnt11 on intracellular Ca^{2+}, which was significantly increased in Wnt11 over-expressing (FW11-1, FW11-2) compare to control (F-C) cells. *B*, Effect of Wnt11 on PKC activity. Bar graph represents relative optical densities that reflect enzymatic activities. PKC activities are increased by 19% in FOCUS and 28% in Huh7 cells. *C*, Effect of Wnt11 on cellular distribution of PKC in FOCUS cells. Yellow arrows indicate translocation of activated PKC to the plasma membrane as visualized by FITC (green)- and Alexa594 (red)- conjugated secondary antibodies. *D*, Knockdown of Wnt11 expression with siRNA resulted in decreased PKC activity in Hep3B cells. (Top panel) Immunoblot analysis to verify knockdown of endogenous Wnt11 protein expression. Control siRNA (C-si)

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or Wnt11-siRNA (W11-si) was transfected into Hep3B cells. Ectopic expression of Wnt11-myc (W11-myc) was used as a positive control. Samples were immunoblotted with anti-Wnt11 antibody to detect endogenous Wnt11 protein. (Bottom panel) PKC activity was decreased in Wnt11-siRNA transfected Hep3B cells. *E*, The effect of PKC (BisI) and ROCK (Y27632) inhibitors on TCF activity measured after 24 h incubation with BisI or Y27632. BisI rescued the inhibition of TCF activity as mediated by Wnt11. In contrast, Y27632 had no effect.

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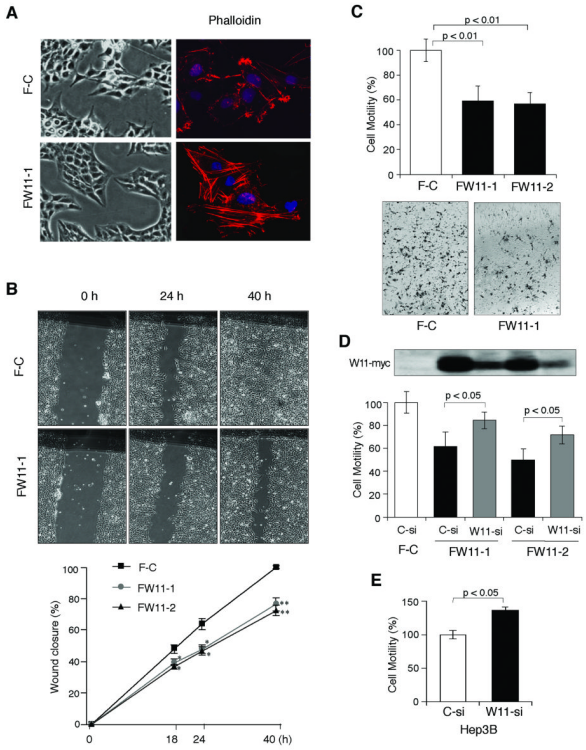

Figure 4.

Effects of Wnt11 on cell migration. *A*, Morphological alterations in a stable transfected Wnt11 over-expressing FOCUS (FW11-1). FW11-1 cells reveal a smooth leading edge with few membrane protrusions compared to control cells (left panel). Control and FW11-1 were subjected to immunostaining for F-actin with rhodamine-phalloidin (red, right panel). Note that control cells are characterized by thin stress fiber at the leading front edge of cells, whereas Wnt11 over-expressing cells show many bundled stress fiber without preferred orientation and suggest a less motile phenotype. *B*, Delayed cell migration in stable Wnt11 over-expressing clones compared to control cells using a wound-healing assay. The cells were photographed at the identical location at the time indicated. At 40 h, most of the wound was closed with migrating cells in the controls (F-C), whereas it remained open in Wnt11 over-expressing cells (FW11-1). Graph of the wound closure plotted against time. * $p < 0.05$, ** $p < 0.001$ versus control. *C*, Wnt11 inhibited cell motility using a Transwell chamber assay. (Top panel) Cell migration in Wnt11 over-expressing cells (FW11-1, FW11-2) was significantly reduced by 40 % compared to control (F-C). (Bottom panel) A representative cell motility assay stained with crystal violet. Number of migrated cells was decreased in FW11-1 compared to control. *D*, The Wnt11-mediated inhibition of cell motility was partially rescued by Wnt11 siRNA (W11-si). Knockdown of Wnt11 expression with siRNA was confirmed by Western blot analysis (top panel). *E*. Knockdown of Wnt11 resulted in increased cell motility in Hep3B cells.

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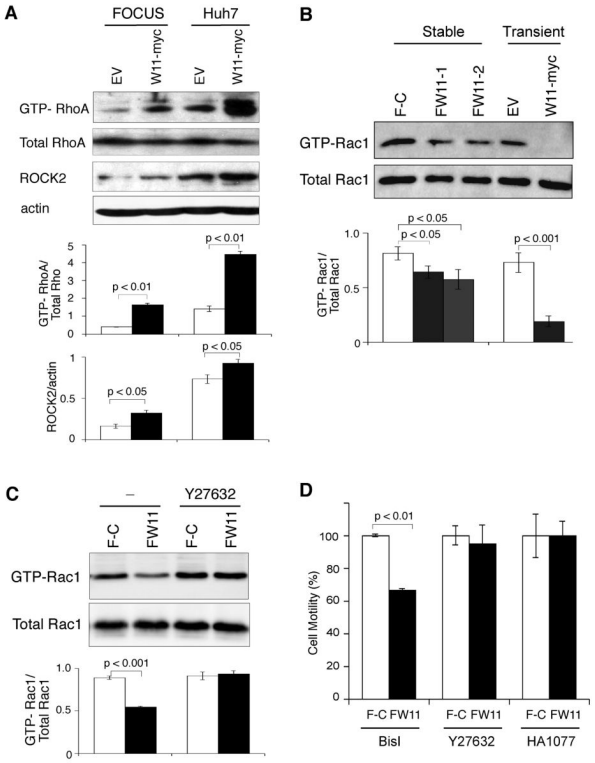

Figure 5.

Wnt11 decreased FOCUS cell motility through the RhoA/ROCK and Rac1 pathway. *A*, Wnt11 induced activation of RhoA/ROCK in FOCUS and Huh7 cells. Whole cell lysates were used to pull-down the active form of RhoA (GTP-RhoA) with GST-human rhotekin and followed by Western blot analysis. Equal amount of total cell lysates serve as controls (total RhoA). The bar graph shows the ratio of active RhoA to total RhoA, and ROCK2 to actin. *B*, Wnt11 decreased Rac1 activity in FOCUS cells. Wnt11 over-expresssing cells, either transiently (W11-myc) or stably (FW11-1, FW11-2) exhibited reduced Rac1 activity assessed by pull-down assay with GST-human Pak1 to detect active Rac1 (GTP-Rac1) using Western blot analysis. Aliquots of the respective lysates serve as controls for analyzing total amount of Rac1 protein (total Rac1). *C*, ROCK inhibitor (Y27632) rescued the decreased Rac1 activity mediated by Wnt11. Wnt11 over-expressing or control cells were treated with Y27632, followed by Rac1 activity assay. Y27632 restored the inhibition of Rac1 activity medicated by Wnt11, indicating that ROCK is responsible for the reduced Rac1 activity. *D*, Inhibition of cell motility was restored by ROCK inhibitors (HA1077, Y27632), not by BisI.

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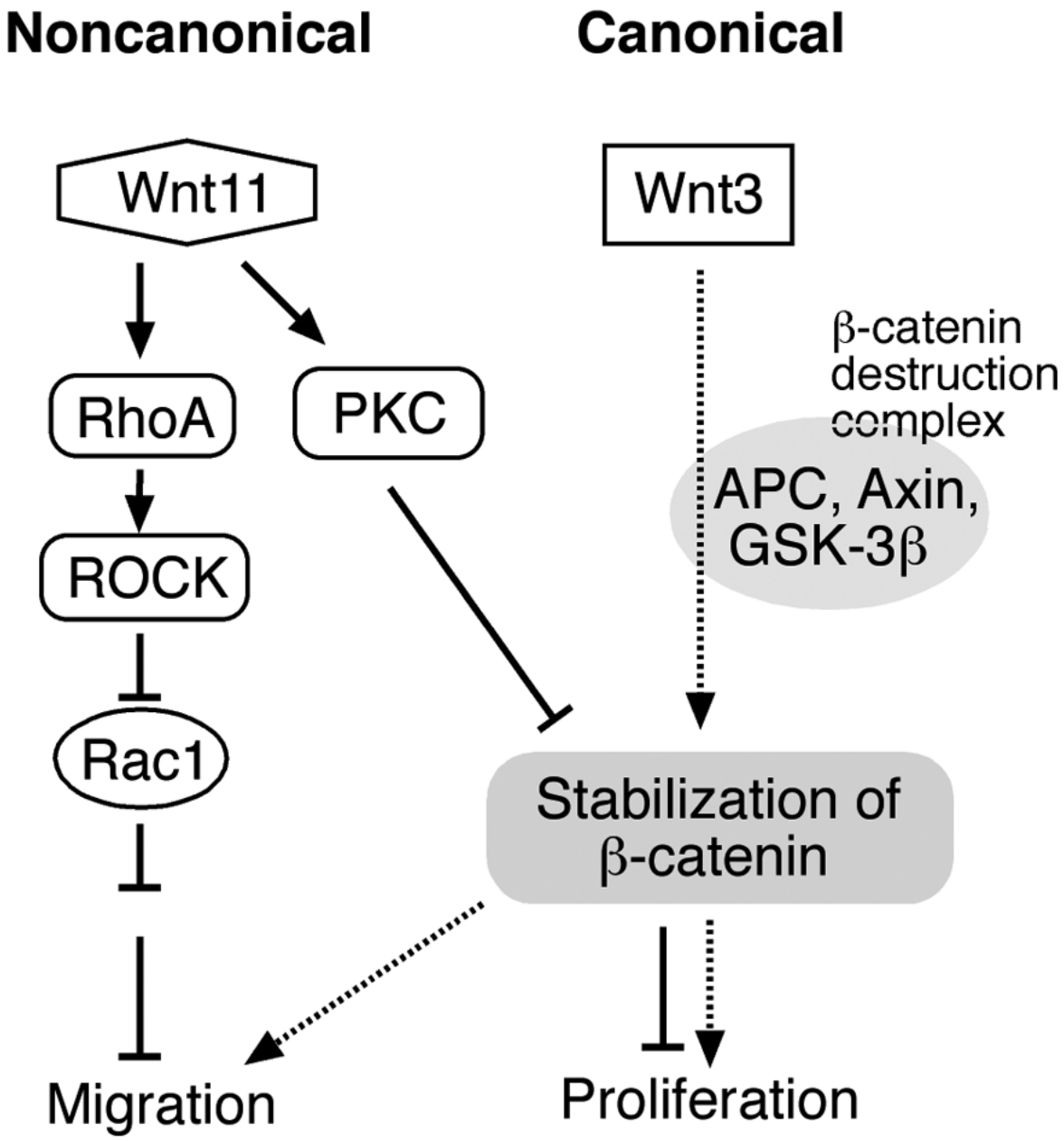

Figure 6.

Schematic diagram of the potential crosstalk between canonical and noncanonical pathways as mediated by Wnt11 in HCC cells. Activation of the canonical Wnt signaling induces cell proliferation and migration through β-catenin stabilization and translocation to the nucleus where it activates Wnt responsive target genes (dotted arrows). The activation of PKC by Wnt11 triggers β-catenin phosphorylation and results in inhibition of cell proliferation. Wnt11-mediated activation of RhoA/ROCK inhibits Rac1 activity, and leads to inhibition of cell migration and motility (black arrows). Both Wnt3 (4) and Wnt11 may be involved in tumorigenesis by reciprocal mechanisms. Thus, over-expression of Wnt3 or down-regulation of Wnt11 or both may activate canonical β-catenin signaling and contribute to a highly motile, invasive and proliferative HCC phenotype. Therefore, a balance between Wnt3/canonical and

Wnt11/noncanonical signaling may be important for homeostatic regulation of β-catenin signaling in liver and alterations in this balance may contribute to hepatic oncogenesis.

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