**Signaling and Regulation**

**Noncanonical Wnt11 Inhibits Hepatocellular Carcinoma Cell Proliferation and Migration**

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**Abstract**

The canonical Wnt signaling is frequently activated due to overexpression 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 with the adjacent uninvolved liver as measured by quantitative real-time reverse transcription-PCR and Western blot analysis. In human HCC cell lines, overexpression of Wnt11 activated protein kinase C signaling. Protein kinase C antagonized the canonical signaling through phosphorylation of β-catenin and reduced T-cell factor-mediated transcriptional activity, resulting in a decrease of cell proliferation. Furthermore, ectopic expression of Wnt11 promotes RhoA/Rho kinase activation. We found that activated Rho kinase inhibited Rac1 to reduce cell motility and migration. These observations suggest a novel role for Wnt11 as a tumor suppressor during hepatocarcinogenesis because loss of expression promotes the malignant phenotype via both canonical and noncanonical Wnt signaling pathways. *Mol Cancer Res; 8(2); 254–65. ©2010 AACR.*

**Introduction**

Hepatocellular carcinoma (HCC) 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 overexpression 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 include cell proliferation, differentiation, polarity, and migration to affect the organization of the body plan and tissue patterning (5). Moreover, constitutive activation of Wnt signaling 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 and the low-density lipoprotein-related protein coreceptor 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 and survival and modify cell fate. In the absence of Wnt stimulation, β-catenin is phosphorylated within the adenomatous polyposis coli (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, Wnt5a, and Wnt11 ligands (8). Although noncanonical Wnt pathways are diverse and less well characterized, they are important in polarized cell movement and organ morphogenesis through cytoskeletal rearrangement involving the small GTPases RhoA and Rac1. In addition, the noncanonical Wnt/Ca²⁺ 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, Wnt5a, Wnt6*, and *Wnt11*) among the 19 family members were involved. Wnt3 was shown to activate the canonical pathway via binding to the Frizzled-7 receptor and lead to increased HCC cell proliferation and motility. More importantly, Wnt3 expression was upregulated in human HCC compared with 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 with 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.

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 pathologic features of these HCC patients have been presented (4). Use of these tissues was approved by the Brown University Institutional Review Board.

Quantitative Real-time Reverse Transcription-PCR
To determine the levels of Wnt11 mRNA expression, real-time reverse transcription-PCR was done as previously described (2) using the following primers: Wnt11, 5′-TTCCGTAGCTCTCTATGGAAG-3′ and 5′-AGACACGGCTCTAGTTGCA-3′; 18S rRNA, 5′-GGACACGGACAGGATGACA-3′ and 5′-ACCACCAGAATCGAGAAGA-3′. The copy number of Wnt11 mRNA was quantified by the Ct values compared with standard curves and followed by normalization to 18S rRNA. All reactions were done in triplicate.

Immunohistochemical Staining
Formalin-fixed, paraffin-embedded tumor and adjacent uninvolved peritumoral tissue sections were applied for Wnt11 protein expression by immunohistochemistry. Sections were deparaffinized, rehydrated, and subsequently incubated with anti-Wnt11 antibody (Santa Cruz Biotechnology) overnight at 4°C. Anti-rabbit IgG antibody conjugated with horseradish peroxidase (EnVision+ System, Dako) was incubated for 45 min at room temperature; immunoreactivity was detected using 0.1% 3,3′-diaminobenzidine and 0.005% H2O2 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 (In- vitrogen) 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.) according to the manufacturer’s instructions. To obtain FOCUS cells stably transfected with empty vector (F-C) or Wnt11-myc (FW11-1 and FW11-2), colonies of G418-resistant cells were selected and expanded for further characterization. For analysis of TCF transcriptional activity, we used a TOPFlash/FOPFlash reporter gene assay as previously described (4).

Control small interfering RNA (siRNA) and specific for human Wnt11 (Santa Cruz Biotechnology) were transfected into cells at a concentration of 100 nmol/L using the siPORT NeoFX transfection agent (Ambion). Knockdown efficiency of Wnt11 was examined by Western blot analysis.

Western Blot Analysis
Western blot analysis was done as previously described (2) using the following antibodies: β-catenin (BD Biosciences), phospho-β-catenin (Ser33/37/Thr41), phospho-GSK-3β (Ser9; Cell Signaling), GSK-3β, actin, axin, APC, c-Myc, and Wnt11 (H-95; Santa Cruz Biotechnology). To detect endogenous Wnt11 expression, Solution 1 for Primary Antibody (Calbiochem) and Solution for Secondary Antibody (Calbiochem) were used to increase a ratio of immunoreactivity to background and SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) was used for detection.

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

Measurement of Cytosolic Ca2+
The Ca2+ levels in cells were determined using the fluorescent fura-2 acetoxymethyl ester probe (fura-2/AM; Invitrogen). After overnight serum starvation, cells were loaded with fura-2/AM (2 μmol/L) for 45 min at 37°C in modified Hank’s solution. Fura-2/AM fluorescence was measured in a SpectraMax M5 (Molecular Devices Co.). The levels of intracellular Ca2+ were estimated by using excitation at 340 and 380 nm and by the ratio of fluorescence intensities detected at 510 nm.

PKC Activity Assay
A nonradioactive PKC kinase assay kit (Promega) was used (11). Briefly, cells were lysed and centrifuged at 100,000 × g 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.
**Results**

**Wnt11 Expression Is Downregulated in Human HCC**

To explore the Wnt11 signaling pathway in HCC, we measured expression in four different HCC cell lines using quantitative real-time reverse transcription-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 with 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 with peritumoral tissue. Downregulation of Wnt11 protein was also confirmed by immunohistochemical staining of a representative example (case 5) as shown in Fig. 1E.

**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 (13, 14) as well as hematopoietic stem cells (15). 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 of Wnt11 mRNA was in HepG2 cells. The expression of Wnt11 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). Overexpression of Wnt11-myc in FOCUS and Huh7 cells resulted in a significant reduction of TCF activity compared with control (empty vector), 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 by double immunostaining with both anti–β-catenin (red) and anti-c-Myc (green) antibodies. As shown by a representative example presented in Fig. 2B, the...
FIGURE 1. Wnt11 expression in human HCC tissue samples and cell lines. A, the level of Wnt11 mRNA was measured in four 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, 17 paired human HCCs and corresponding adjacent HCC-free tissues were analyzed for Wnt11 mRNA expression. Wnt11 was significantly downregulated in HCC tumors compared with 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. Black columns, mRNA levels in HCC tissues; white columns, mRNA levels 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. Bottom, expression of Wnt11 protein was detected by anti-Wnt11 antibody and actin was used as a loading control; top, the level of Wnt11 protein was plotted as a ratio to actin. Note that 8 of 10 samples exhibited reduced expression level in tumor (T) compared with peritumoral (pT) tissues. E, Wnt11 protein expression in human HCC tissue samples using immunohistochemical staining. Representative example (case 5) of HCC and peritumoral area was immunostained with anti-Wnt11 antibody (brown) and counterstained with hematoxylin (blue). Right, weak but clear positive signal for Wnt11 was found in the cytoplasm of hepatocytes in the peritumor liver tissue; left, the immunoreactive Wnt11 in the HCC tissue was negative. Magnification, ×100.
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 with control cells. Furthermore, the reduced level of β-catenin was associated with an increase in phospho-β-catenin. There was no change of total β-catenin 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 (16). This finding is consistent with the result displayed in Fig. 2A, indicating that overexpression 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-GSK-3β 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 used 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 because 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, 17, 18). Therefore, the effects of Wnt11 on cell proliferation and apoptosis were investigated. As shown in Fig. 2E, Wnt11-overexpressing cells (FW11-1 and FW11-2) exhibited significantly reduced growth rates compared with controls (F-C) at days 4 and 5 of culture. It is noteworthy that terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assays revealed no differences in apoptosis rates between Wnt11-overexpressing and control cells (data not shown).

**FIGURE 2.** Overexpression 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 have an inactivating β-catenin mutation. B, effect of Wnt11 on β-catenin nuclear accumulation in Huh7 cells by a double-immunofluorescence 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, overexpression of Wnt11 decreased β-catenin levels in a GSK-3β-independent manner. Overexpression 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. Bottom, the bar graphs depict the results of densitometric analysis from the Western blots. 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 (Wnt11-myc). E, reduced cell proliferation in Wnt11-overexpressing cells. Cell proliferation rate was significantly decreased in FOCUS (FW11-1 and FW11-2) compared with control cells (F-C). Top, constitutive Wnt11 overexpression was shown by Western blot analysis. *, P < 0.05 versus control cells.
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 (22) and implies that Wnt11 may influence HCC cell migration and motility. By using the wound-healing assay, it was found that Wnt11-overexpressing cells (FW11-1 and FW11-2) had a significant reduction of cell migration as compared with control (Fig. 4B). To confirm this observation, we tested cell motility using a Transwell chamber assay. Cell motility in Wnt11-overexpressing cells (FW11-1 and FW11-2) was decreased by 42% compared with 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 or control siRNA was transfected; knockdown of Wnt11 expression was verified by Western blot analysis (Fig. 4D, top). The reduction of Wnt11 expression by siRNA partially restored cell motility in Wnt11-overexpressing cells. Moreover, knockdown of
**FIGURE 4.** Effects of Wnt11 on cell migration. A, morphologic alterations in a stable transfected Wnt11-overexpressing FOCUS (FW11-1). Left, FW11-1 cells reveal a smooth leading edge with few membrane protrusions compared with control cells; right, control and FW11-1 were subjected to immunostaining for filamentous actin with rhodamine-phalloidin (red). Note that control cells are characterized by thin stress fiber at the leading front edge of cells, whereas Wnt11-overexpressing cells show many bundled stress fibers without preferred orientation and suggest a less motile phenotype. B, delayed cell migration in stable Wnt11-overexpressing clones compared with 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-overexpressing 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, cell migration in Wnt11-overexpressing cells (FW11-1 and FW11-2) was significantly reduced by 40\% compared with control (F-C); bottom, a representative cell motility assay stained with crystal violet. The number of migrated cells was decreased in FW11-1 compared with control. D, the Wnt11-mediated inhibition of cell motility was partially rescued by Wnt11-siRNA (W11-si). Top, knockdown of Wnt11 expression with siRNA was confirmed by Western blot analysis. E, knockdown of Wnt11 resulted in increased cell motility in Hep3B cells.
endogenous Wnt11 by siRNA in Hep3B cells resulted in increased cell motility (Fig. 4E).

Signaling through the noncanonical Wnt/planar cell polarity regulates cytoskeletal architecture through small GTPases, including RhoA and Rac1. Furthermore, RhoA and Rac1 are involved in the formation of stress fibers and membrane protrusions (23, 24). 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, overexpression of Wnt11 in both FOCUS and HuH7 cells increased RhoA as well as ROCK activity compared with control. In contrast, Rac1 activity was decreased in both stable (FW11-1 and FW11-2) and transient (W11-myc) Wnt11-overexpressing cells compared with controls (Fig. 5B).

Recently, it has been reported that Wnt/planar cell polarity signaling promotes RhoA activity and inhibits Rac1 in neural crest (25) and human GNS-3314 glioblastoma cells (26). Accordingly, the observation that RhoA/ROCK activation may lead to inhibition of Rac1 allowed us to test this concept in the Wnt11-overexpressing 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 (27). 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 about the 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 (13, 14). However, the mechanisms that drive Wnt11 inhibitory effects on canonical signaling seem to be tissue specific and poorly understood. Here, we show that Wnt11 activated the Ca2+/PKC pathway and down-regulated β-catenin levels in cells, resulting in inhibition of canonical signaling. Our findings agree with a report showing that PKC phosphorylates β-catenin to subse-

quently inhibit Wnt3a-mediated canonical signaling in HEK 293 cells (28).

Wnts regulate mammalian cell migration. Wnt3a has been shown to stimulate the migration of myeloma and Chinese hamster ovary cells (29, 30), whereas Wnt5a repressed migration of thyroid carcinoma cells (13). Similarly, the noncanonical Wnt11 ligand inhibits migration and motility through modulation of RhoA/ROCK and Rac1 activities in HCC cells. Cross talk between Rac and RhoA has been described in various cell types. Indeed, several reports suggest that RhoA suppresses Rac1 activity in different cell lines (31–36). In addition, Rac1 activity is increased in fibroblasts and neutrophils following treatment with Y27632, indicating that ROCK may be involved in Rac1 inactivation (34, 37). Recently, Liu et al. (38) reported that Rac1 plays a crucial role in the control of HCC cell motility and metastasis. 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 (39). In agreement with these findings, our observations reveal that Wnt11-overexpressing cells showed less lamellipodia formation (Fig. 4A), possibly through inactivation of Rac1 via ROCK.

Several studies have shown that upregulation of RhoA expression correlated with tumor progression and metastasis in HCC (40–42), whereas treatment with Y27632 suppressed cell migration and invasion (43, 44), which is in contrast with our observations. However, consistent with our results are studies that showed that Rac1 activation was associated with increased HCC cell motility and migration (38, 45). 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/planar cell polarity signaling promotes RhoA activity and activated RhoA inhibits Rac1 (25, 26). 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. ROCK 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 cross talk between RhoA and Rac1 in HCC.

We found that the expression level of Wnt11 mRNA was significantly decreased in HCC tissue compared with 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, 46), we hypothesize that loss of Wnt11 signaling activity may contribute to the development of HCC. It is noteworthy that the biological function of Wnt11 seems to directly oppose 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...
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 amounts 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-overexpressing cells, either transiently (W11-myc) or stably (FW11-1 and 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-overexpressing or control cells were treated with Y27632 followed by Rac1 activity assay. Y27632 restored the inhibition of Rac1 activity mediated by Wnt11, indicating that ROCK is responsible for the reduced Rac1 activity.

D, inhibition of cell motility was restored by ROCK inhibitors (HA1077 and Y27632), not by Bisl.

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FIGURE 6. Schematic diagram of the potential cross talk 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. Black arrows, Wnt11-mediated activation of RhoA/ROCK inhibits Rac1 activity and leads to inhibition of cell migration and motility. Both Wnt3 (4) and Wnt11 may be involved in tumorigenesis by reciprocal mechanisms. Thus, overexpression of Wnt3 or downregulation 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.

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 Wnt1 and Wnt3 function to regulate activity of canonical β-catenin signaling in liver. Because dysregulation of β-catenin signaling is important in tumor formation, further analysis of these two pathways may provide insight into HCC pathogenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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