The Wnt5a/Ror2 noncanonical signaling pathway inhibits canonical Wnt signaling in K562 cells

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Abstract. Wnt5a has been shown to be involved in cancer progression in a variety of tumor types, and regulates multiple intracellular signaling cascades; it is a representative ligand that activates a noncanonical Wnt signaling pathway. The mechanism governing how Wnt5a determines the specificity of these pathways and the relationship with tumorigenesis is still unknown. In this study, we aimed to clarify the tumor suppressor role of Wnt5a in leukemogenesis. In particular, we focused on Ror2 functioning as a Wnt5a receptor to mediate noncanonical Wnt signaling, which inhibits canonical Wnt signaling in K562 cells. We found that up-regulation of Wnt5a expression increased Ror2 expression in K562 cells and Wnt5a and Ror2 were co-expressed in the cytoplasm. Also, Wnt5a induced the intrnalization of Ror2. Co-immunoprecipitation experiments were performed to determine whether Ror2 binds to Wnt5a, and inhibits Wnt5a binding with Frizzled4 and LRP5 in Wnt5a treated K562 cells. Wnt5a had no effect on total ß-catenin expression levels, but regulated tyrosine phosphorylation of ß-catenin and translocation of ß-catenin from the cytoplasm to the nucleus. Furthermore, expression of Wnt5a was associated with suppression of ß-catenin/TCF-dependent transcriptional activity and down-regulated the expression of cyclin D1, a downstream target gene of the canonical Wnt signaling pathway. We hypothesize that Wnt5a plays the role of a tumor suppressor in leukemogenesis through the Wnt5a/Ror2 noncanonical signaling pathway that inhibits Wnt canonical signaling.

Introduction

Wnts are secreted glycoproteins that control vital biological processes including development and cancer progression (1). Wnt signaling has traditionally been classified as canonical (Wnt/ß-catenin) or noncanonical and at least 19 Wnt members have been shown to be present in mammals to date (2). The members exhibit unique expression patterns and have distinct functions in development and tumorigenesis. The majority of Wnt signaling has been attributed to the activation of Frz receptors, but an alternative possibility is that Wnts carry out their diverse roles via different receptors.

Wnt5a, a member of the Wnt family suggested to have a role in hydrophobic cell-cell interactions, is predominantly characterized as a noncanonical Wnt ligand that activates intracellular signaling via distinct receptors or co-receptors (3). One such receptor is Ror2, an orphan tyrosine kinase possessing an extracellular cysteine-rich Wnt binding domain. Ror2 is a transmembrane protein with tyrosine kinase activities that plays a crucial role during developmental morphogenesis (4,5). Previous studies concerning the interactions between Ror2 and Wnt signaling have indicated that Ror2 has multiple functions depending on the cellular context (6). However, the interaction between Ror2 and Wnt5a to mediate noncanonical Wnt signaling pathway has received great attention in recent years (5-8). Ror2 has been shown to be a receptor for Wnt5a, inducing a noncanonical cascade involving activation of c-Jun-N-terminal kinase (JNK) and inhibition of the canonical signaling (6). In addition, developmental phenotypes exhibiting an absence of Ror2 and Wnt5a lead to dwarfism, shortened limbs, facial abnormalities, ventricular septal defects in the heart, and abnormalities in lung development (5).

Wnt5a has been reported to act as a tumor suppressor in breast, colon and thyroid carcinomas (9-11). Wnt5a methylation has been associated with decreased Wnt5a mRNA expression and Wnt5a hypermethylation correlated with up-regulation of cyclin D1 expression (12). In particular, Wnt5a hemizygous mice develop myeloid leukemia and B-cell lymphomas with loss of Wnt5a function (13). Previously, our laboratory indicated that Wnt5a expression was down-regulated in myeloid leukemia (14). Exogenous Wnt5a can inhibit growth and induce differentiation of K562 cells (15). In contrast, it has been suggested that Wnt5a has oncogenic properties because it promotes progression in lung and prostate cancers and in malignant melanoma (8,16). There are conflicting data in the literature regarding the mechanism of the complex signaling through distinct receptors (17).

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Therefore, the function of Wnt5a in human cancers is controversial.

ß-catenin is a multifunctional protein that acts as a transcriptional co-activator mediating transduction of the canonical Wnt signaling pathway. In the absence of Wnt, an intact degradation complex consisting of glycogen synthase kinase-3ß (GSK-3ß), adenomatous polyposis coli (APC) and Axin is required for the degradation of intracellular ß-catenin. In the presence of Wnt, stabilization and accumulation of ß-catenin in the cytosol, results in subsequent translocation to the nucleus. There it binds to T cell factor 4/lymphocyte enhancer factor and activates TCF target genes (18) including c-myc (19), cyclin D1 (20), peroxisome proliferator activated receptor, cyclooxygenase-2, NOTUM (21), GS (22), TBX3 (23), Axin2 (24), LECT2 (25) and EpCAM (26). Moreover, phosphorylation of ß-catenin regulates the intracellular expression, nuclear localization and transcription of target genes.

In this study, we demonstrated that the presence of Wnt5a resulted in Ror2 localization in the nuclear invagination cytoplasmic area, and that expression levels of Ror2 were correlated with Wnt5a expression levels. Ror2 binds to Wnt5a and inhibits Wnt5a interaction with the Frizzled4 and LRP5 receptors. In addition, we confirmed that Wnt5a regulated the phosphorylation of ß-catenin, affected the translocation of ß-catenin from the cytoplasm to the nucleus. We report that Wnt5a activates the Ror2-mediated noncanonical Wnt pathway and inhibits the nuclear localization of ß-catenin rather than affecting the total amount of ß-catenin expressed in K562 cells. We further provide evidence that Wnt5a decreases ß-catenin transcriptional activity and suppresses expression of the downstream target gene. These results suggest that Wnt5a serves as an antagonist to the canonical Wnt signaling pathway via Wnt5a/Ror2 non-canonical signaling and plays the role of a tumor suppressor in human leukemia.

Materials and methods

Cell culture and treatments. The human erythroleukemia cell line, K562, was cultured in RPMI-1640. All media were supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, and 100 units/ml streptomycin. Cell cultures were incubated at 37˚C in 5% CO_2/95% air. The K562 cells that stably over-expressed the Wnt5a protein were constructed successfully in our laboratory and were compared with K562-vector cells. In addition, K562 cells were infected with Wnt3a, Wnt5a or GFP adenovirus for 48 h.

Immunofluorescence. K562 cells were washed with phosphate-buffered saline (PBS). Cells were permeabilized using 0.1% Triton X-100 in PBS for 10 min, washed and blocked with blocking buffer for 1 h, and incubated at 4˚C overnight. Primary antibodies used include: Ror2 (Santa Cruz Biotechnology), Wnt5a (Santa Cruz Biotechnology) and ß-catenin (Abcam, Inc.). The cells were washed with PBS for 1 h and probed with FITC and Cy3 secondary antibodies (Beyotime Institute of Biotechnology). They were then washed, examined under fluorescence and imaged using a Zeiss LSM 510 META confocal microscope.

Immune-electron microscopy. K562 cells were incubated overnight at 4˚C with anti-Ror2 antibody (Cell Signaling) diluted 1:50 with PBS. After washing with PBS, the cells were incubated for 1 h with HRP-labeled antibody, washed again with PBS, and incubated for 10 min in PBS containing 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB). They were immersed in 1% osmium tetroxide for 2 h, acetone dehydrated, embedded and sectioned. After uranium and lead staining, the cells were examined using a TECNAI 10 electron microscope (Philips).

Co-immunoprecipitation and Western blotting. Cell lysates were prepared according to immunoprecipitation procedures with a Wnt5a antibody using a standard overnight immuno-precipitation. The precipitated complexes were separated by 8 or 10% SDS-PAGE electrophoresis and Western blot analysis was performed. The Western blots were incubated with Ror2, Frizzled4, or LRP5 antibodies (Santa Cruz Biotechnology). Whole cell lysates were obtained by incubating cells with 100 μl of lysis buffer per 20 μg cells on ice for 30 min. Cellular debris was removed by centrifugation. Nuclear and cytoplasmic extracts were separated using a nuclear and cytoplasmic extraction kit as described by the manufacturer (Beyotime Institute of Biotechnology). Protein concentrations were determined using the BCA assay (Beyotime Institute of Biotechnology). Equivalent amounts of protein were resolved by SDS-PAGE electrophoresis. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes, which were blocked in 5% BSA and incubated with the relevant antibody. Western blotting was carried out using the chemiluminescence detection system (Bio-Rad). Antibodies to the following proteins were used for this study: ß-catenin, phosphorylated ß-catenin (Cell Signaling), Wnt5a, Ror2, cyclin D1 and ß-actin (Santa Cruz Biotechnology).

Transient transfection with dual luciferase assays. Cells were infected with adenovirus TOPflash or FOPflash. FOPflash has mutated copies of the TCF/LEF sites and is used as a control for measuring nonspecific activation of the reporter. After infection for 10 h to normalize transfection efficiency in reporter assays, cells were co-transfected with 0.2 mg of the internal control reporter *Renilla reniformis* luciferase driven under the TK promoter (pRL-TK; Promega). Thirty hours after infection with adenovirus TOPflash or FOPflash, a luciferase assay was performed using the Dual Luciferase Assay System kit, in accordance with the manufacturer's protocols (Promega).

Statistical analysis. Results are expressed as mean ± SE. Statistical analysis was carried out using analysis of variance. A P-value <0.01 was considered significant.

Results

Increasing Wnt5a is positively correlated with Ror2 expression in K562 cells. We focused on the function of Ror2 as a Wnt5a receptor and explored the relationship between Wnt5a and Ror2 in leukemia. Furthermore, we assessed whether Wnt5a inhibits the canonical Wnt signaling pathway

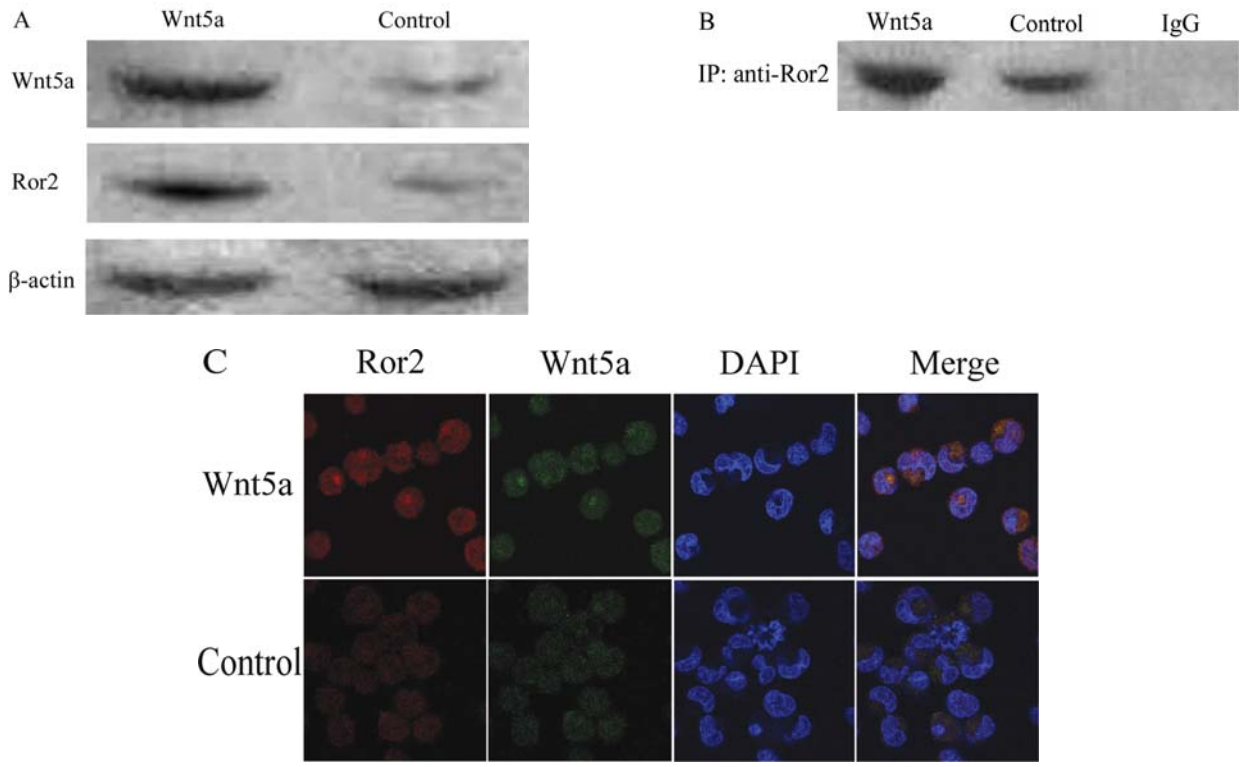


Figure 1. Ror2 expression correlates with levels of Wnt5a expression in K562 cells. (A) There is strong positive expression of Wnt5a and Ror2 in the Wnt5a-treated groups, and a weak expression in the control groups. (B) Co-immunoprecipitation experiments of Wnt5a proteins and Ror2. The lysates were immunoprecipitated with Wnt5a antibody. The Western blot was incubated with Ror2 antibody to detect binding between Wnt5a and Ror2 proteins. (C) Ror2 and Wnt5a co-expression in K562 cells. The expression level of Ror2 in the Wnt5a-treated cells was significantly higher than in control cells. Furthermore, Wnt5a and Ror2 co-localized in foci of the nuclear invagination area in the Wnt5a groups.

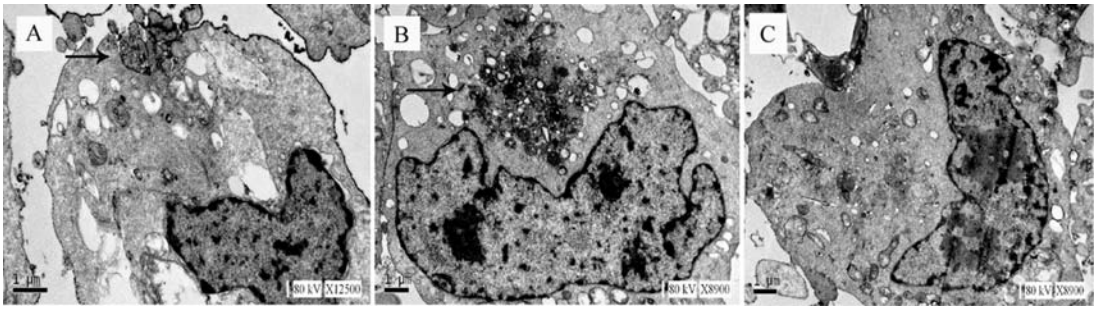
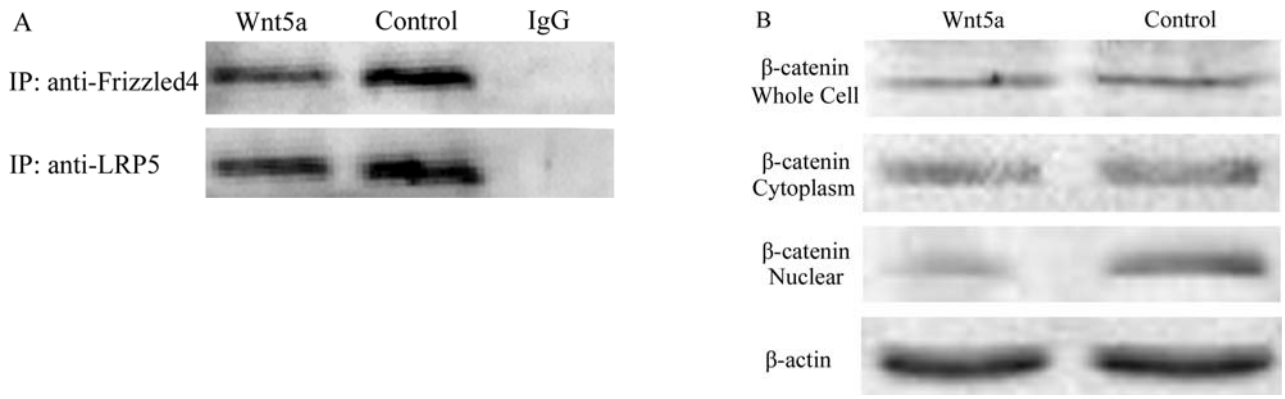


Figure 2. Immune-electron microscopy of Ror2 localization in K562 cells. (A) Wnt5a-treated groups had immunoreactive particulate matter on the plasmalemma (arrow), which was internalized into the cytoplasm. (B) The Ror2 receptor localized in the nuclear invagination cytoplasmic area in the Wnt5a-treated group (arrow). (C) Control cells exhibited no immunoreactivity.

via the Wnt5a/Ror2 noncanonical signaling and whether it plays a role of a tumor suppressor in human leukemia. The K562 cells that stably over-expressed Wnt5a protein were constructed successfully in our laboratory and were compared with K562-vector cells (27) (Fig. 1A). Western blotting was utilized to examine the protein expression of Ror2 in K562 cells. There was a weak positive expression of Ror2 in control cells which dramatically increased when Wnt5a was over-expressed (Fig. 1A). Therefore, we investigated if Wnt5a plays a role in the expression of Ror2.

Co-immunoprecipitation experiments will provide the foundation for subsequent studies analyzing Wnt5a signaling via Ror2 in K562 cells. Here we show that Wnt5a can co-immunoprecipitate with Ror2 when the former is over-expressed in K562 cells, and it displays reduced binding to the Ror2 in control cells (Fig. 1B). This implies that a specific Wnt5a/Ror2 interaction mediates the Wnt5a activity. In addition, the expression of Ror2 and Wnt5a was determined using anti-Ror2 and anti-Wnt5a antibodies. Immunofluorescence demonstrated weak Wnt5a and Ror2 staining dispersed throughout the cytoplasm in control cells. In the presence of Wnt5a, cells expressed Ror2 and Wnt5a and intense staining was detected in nuclear invagination area foci in some cells (Fig. 1C). The presence of Wnt5a resulted in increased intensity of Ror2 staining, and Wnt5a and Ror2 proteins have overlapping expression, implying



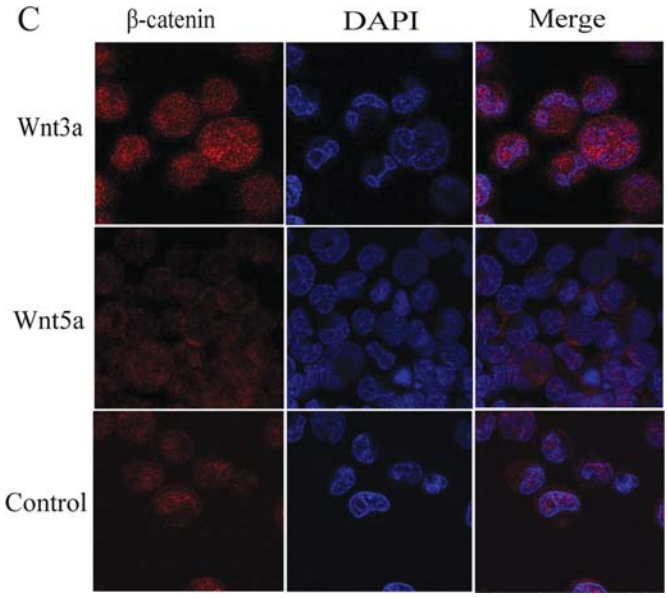


Figure 3. Wnt5a reduced binding to Frizzled4 and LRP5 receptors and affected the expression and localization of ß-catenin in the nuclear of K562 cells. (A) Co-immunoprecipitation demonstrated that Wnt5a proteins reduced binding to Frizzled4 and LRP5 in over-expressed K562 cells compared with control cells. (B) Western blot analysis of ß-catenin levels in whole cells and nuclear and cytoplasmic extracts of K562 cells. These results show that Wnt5a has no effect on total and cytoplasmic ß-catenin expression, but can decrease expression of ß-catenin in the nucleus. (C) Wnt5a affects the nuclear localization of ß-catenin. Significant differences in ß-catenin signal intensity and localization were observed when cells were infected with Wnt3a or Wnt5a adenovirus for 48 h. Wnt5a-treated groups display a staining pattern in the membrane and cytoplasm. In contrast, Wnt3a staining was more intense than in other groups, with ß-catenin staining observed in the cytoplasm and nucleus.

that Wnt5a affects the levels of Ror2. We suggest that these results could be due to internalization of Ror2 upon ligand binding, explaining why cells that express Wnt5a also demonstrate Ror2 localization in the nuclear invagination area.

Immune-electron microscopy of Ror2 expression. The electron micrograph shows Wnt5a-treated groups with immunoreactive particulate matter on the membrane and internalized into the cytoplasm of K562 cells (Fig. 2A). As shown, Ror2 immunoreactivity was present in the nuclear invagination cytoplasmic area of the Wnt5a treated groups (Fig. 2B). Cytoplasmic organelles such as lysosomes are used to digest macromolecules from phagocytosis, autophagy and endocytosis. Receptor proteins are recycled from the cell surface, and this requires internalization through ligand-bound receptors. Consequently, the presence of Wnt5a resulted in Ror2 being localized in the nuclear invagination cytoplasmic area, supporting the theory that Ror2 is internalized into the cytoplasm (8). The Wnt5a-mediated Ror2 receptor internalization is important for activation of the noncanonical Wnt signaling in K562 cells.

Wnt5a reduces binding to the Frizzled4 and LRP5 receptors and influences the nuclear localization of ß-catenin. Canonical Wnt signaling involves the binding of Wnt ligands to the cell surface receptor Frizzled4 and to the co-receptors LRP5/6. We tested the hypothesis that Wnt5a activates Ror2-mediated noncanonical signaling resulting in the inhibition of the canonical Wnt pathway in K562 cells. Co-immunoprecipitation demonstrated that Wnt5a reduced binding to Frizzled4 and LRP5 receptors in over-expressed K562 cells compared to control cells (Fig. 3A). In addition, the effect of Wnt5a on ß-catenin expression in K562 cells was examined and there was no change in the total levels of ß-catenin (Fig. 3B). Western blotting for ß-catenin was performed on nuclear fractions of K562 cells with or without Wnt5a stimulation. It should be noted that Wnt5a markedly decreased ß-catenin levels in the nucleus but not in the cytoplasm (Fig. 3B). Immunofluorescent confocal microscopy confirmed the accumulation of endogenous ß-catenin in response to Wnt3a stimulation with nuclear levels of ß-catenin being higher than in the Wnt5a-treated groups (Fig. 3C). Wnt3a activity is required for nuclear localization of ß-catenin in response to the canonical Wnt pathway. Therefore, we demonstrate that Wnt5a negatively regulates ß-catenin nuclear levels and its localization activates noncanonical Wnt pathway signaling by antagonizing the canonical Wnt signaling pathway.

Wnt5a catalyzes phosphorylation of ß-catenin. Phosphorylation of ß-catenin is a key step in the regulation of its intracellular levels and its transcriptional activity (28). To detect phosphorylated ß-catenin, Western blotting was carried out using an anti-ß-catenin phospho-sepcific antibody (Thr41, Ser37, and Ser33) (Fig. 4). The strongest immunoreaction for phosphotyrosine occurred in extracts from cells treated with Wnt5a. In contrast, no immunoreactivity for phosphotyrosine occurred in the control groups.

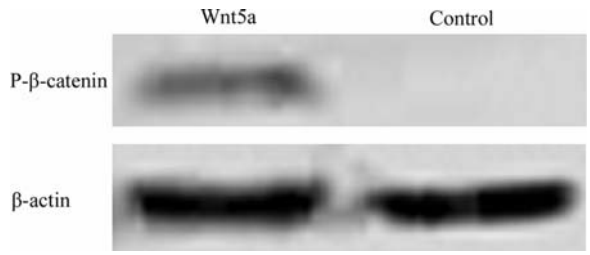


Figure 4. Tyrosine phosphorylation of ß-catenin was detected in K562 cells. Phosphotyrosine was detected in K562 cells treated with Wnt5a. Cell lysates were analyzed using the Thr41, Ser37, Ser33 phospho-specific antibody to confirm expression. ß-actin was used as a loading control.

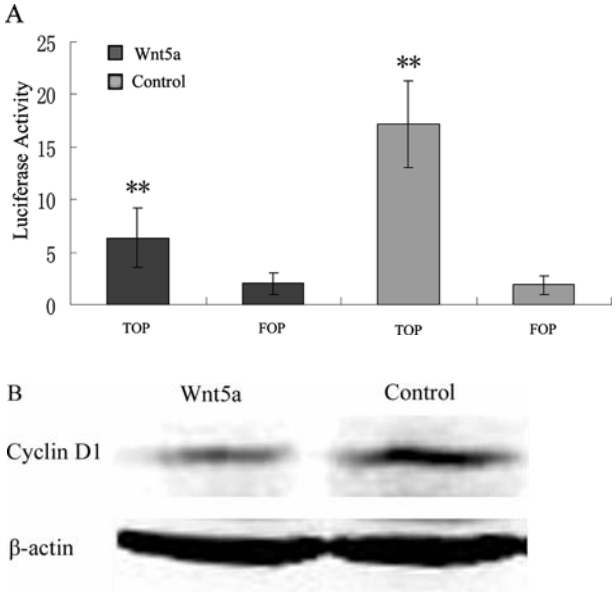


Figure 5. Evaluation of ß-catenin/TCF-dependent transcriptional activity and cyclin D1 expression. (A) TOPflash reporter assay demonstrated significant down-regulation of ß-catenin transcriptional activity in Wnt5a-treated groups. Luciferase activity in FOPflash remained unaffected, confirming the absence of nonspecific activation of the reporter system (**P<0.01, compared with the control). (B) Western blotting demonstrates that Wnt5a suppresses cyclin D1 expression. Lysates were probed with anti-cyclin D1 and anti-ß-actin antibodies.

Wnt5a inhibits ß-catenin transcriptional activity and down-regulates the downstream target gene cyclin D1 in K562 cells. The TOPflash reporter assay is a direct and reliable measure of ß-catenin/TCF-dependent transcriptional activity. TOPflash activity was significantly down-regulated in Wnt5a-treated groups compared with control groups; there was no effect on FOPflash activity (Fig. 5A). These results indicate a reciprocal relationship between the transcriptional activity of ß-catenin and Wnt5a protein levels. This study demonstrates that ß-catenin transcriptional activity was negatively regulated by Wnt5a via decreasing the localization of ß-catenin in the nucleus.

Cyclin D1 is a downstream target gene of the canonical Wnt pathway and critical for the development of a variety of human tumors. Western blotting demonstrated a weak expression of cyclin D1 in Wnt5a-treated groups compared with control groups (Fig. 5B). These results could be due to a decrease in the transcriptional activity of ß-catenin, resulting in the down-regulation of the expression of the target gene, cyclin D1.

Discussion

Our previous studies have reported that Wnt5a was not expressed or down-regulated in myeloid leukemia. However, it was strongly expressed in cases that were in complete remission, which were associated with Wnt5a promoter methylation. In addition, we have demonstrated that exogenous Wnt5a inhibits the proliferation of K562 cells and induces differentiation (13,14). Some reports have tested this hypothesis in human peripheral blood B and myeloid cells, demonstrating that Wnt5a expression was absent or greatly down-regulated in the majority of acute leukemia patients (9,13). Wnt5a methylation is common and tumor-specific in lymphomas (29). Combining our previous studies, we suggest that Wnt5a is a tumor suppressor in leukemia and that the down-regulated expression of Wnt5a correlates with leukemogenesis. However, the mechanism of the Wnt5a-mediated tumorigenesis is unclear. In this study, we aimed to elucidate the role of the tumor suppressor Wnt5a in the K562 cell line.

Wnt5a expression is variably regulated in a variety of human primary tumors. There are conflicting data concerning the role of Wnt5a in the noncanonical Wnt signaling. Wnt5a noncanonical signaling is dependent on a multitude of variables including the availability of various types of cell surface receptors. Furthermore, Wnt5a has been reported to inhibit ß-catenin/Wnt canonical signaling activity (6,30,31). Many studies concerning signal transduction and membrane trafficking have suggested that the sorting of signaling molecules and their receptors to different membrane-bound compartments has a critical function in regulating signaling. Therefore, noncanonical Wnts are antagonistic or synergistic to the canonical Wnt signaling pathway, depending on their receptor context (6).

Ror2 specifically interacts with the noncanonical Wnt ligand. Depending on the cellular context, Ror2 proteins can activate or repress transcription of Wnt target genes and can modulate Wnt signaling. In addition, Wnt5a regulates distinct pathways through receptor internalization-dependent and -independent mechanisms. Some reports suggest that Ror2 synergizes with Wnt5a and mediates inhibition of canonical Wnt signaling (6,32). Current data from our laboratory indicate that Ror2 and Wnt5a co-localize in the observed nuclear invagination area foci and that expression of Wnt5a resulted in increased Ror2 expression levels. Ror2 has a role in the Wnt5a-mediated noncanonical Wnt pathway and is internalized upon ligand binding. A conclusive experiment to determine that Wnt5a actually binds to the Ror2 receptor in K562 cells is to perform co-immunoprecipitation studies. These results suggest the Wnt5a signaling via the Ror2 recipient in K562 cells. Ror2 could compete with other co-activators of the canonical Wnt pathway and it is involved in leukemogenesis.

In the canonical Wnt signaling, a Wnt ligand binds to a Frizzled receptor and to the LRP5/6 co-receptor. This interaction results in stabilization of cytoplasmic ß-catenin,

enabling it to translocate to the nucleus and function as a transcriptional co-activator with TCF. Nuclear localization of ß-catenin is indispensable for canonical Wnt signaling (33,34). Moreover, the extracellular CRD of Ror is similar to the Wnt binding domain found in Frizzled receptors (35). Our results show that Wnt5a reduced binding to Frizzled4 and LRP5 receptors in Wnt5a-treated K562 cells. We thus think, that Ror2 controls competitive binding of Frizzled4 and LRP5 to Wnt5a in K562 cells. Ror2 can bind to Wnt5a and inhibit its interaction with the Frizzled4 and LRP5 receptors.

Abnormal Wnt signaling is a molecular mechanism of tumorigenesis. ß-catenin plays a role in the signal transduction of the Wnt canonical pathway and regulates the expression of downstream genes in agreement with its oncogenic properties (36). We have observed that Wnt3a activates this pathway, resulting in the accumulation of ß-catenin in the cytoplasm, and localization of the protein in the nucleus. In addition, ß-catenin levels in the whole cell remained unchanged in the presence or absence of Wnt5a; however, nuclear ß-catenin levels were significantly lower in Wnt5a-treated cells than in controls. Therefore, ß-catenin protein was stabilized and translocated to the nucleus due to Wnt3a signal activation but Wnt5a had opposite effects in K562 cells. Active Wnt5a can tyrosine-phosphorylate ß-catenin. The expression of the target gene cyclin D1 and the results of the TOPflash reporter assay indicated that Wnt5a results in a decrease in ß-catenin transcriptional activity. These results are in agreement with observations that Wnt5a reduced the localization of ß-catenin in the nucleus due to its effect on ß-catenin phosphorylation, but not via the direct down-regulation of ß-catenin. We conclude that the Wnt5a/Ror2 signaling pathway inhibits the Wnt/ß-catenin signaling cascade in K562 cells.

In view of the above findings, we suggest that Wnt5a and its receptor Ror2 act synergistically to increase autocrine signaling in K562 cells. We hypothesized that Wnt5a plays a role in the activation of Ror2-mediated noncanonical Wnt signaling to inhibit the canonical pathway. We examined the effect of Wnt5a on the expression of the canonical Wnt pathway downstream target genes, and we demonstrated that Wnt5a inhibited the ß-catenin/TCF-dependent transcriptional activity in K562 cell lines. The present data, together with previous reports, indicate that Wnt5a can function as a tumor suppressor in K562 myeloid leukemia cells and that this tumor-suppressive activity is mediated by the Wnt5a/Ror2 noncanonical signaling inhibition of the canonical Wnt signaling pathway.

Acknowledgments

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