

Wnt Signaling Inhibits the Growth of Primary Cilia and Activates CyclinD1, Snail and VEGFA Expression in Breast Cancer Cell Line MDA-MB-231

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Abstract: Although Wnt/ β -catenin signaling has been shown to be essential in the process of cancer formation, it is unclear how Wnt3a signaling pathway regulates abnormal proliferation and differentiation of breast cancer cells. Here, we found overexpression of Wnt3a stimulated the expression of the Wnt signaling's downstream genes such as LRP6, Naked, Axin1, DVL-2, β -catenin, and TCF-1 in breast cancer cell line MDA-MB-231. Primary cilia is deemed as sensory cell antennae that coordinates a large number of cellular signaling pathways, sometimes coupling cell division and differentiation. Primary cilia were found on the surface of this cell line. Overexpression of Wnt3a decreased the formation of primary cilia. Inhibition of Wnt3a with Calphostin C facilitated growth of primary cilia. Wnt3a activated cell proliferation gene of CyclinD1. In contrary, Calphostin C decreased the promotional effect on proliferation of MDA-MB-231. The Snail family of transcription factor has previously been implicated in the differentiation of epithelial cells into mesenchymal cells (epithelial-mesenchymal transitions) during embryonic development. Wnt3a promoted MDA-MB-231 induced expression of Snail, whose effect was inhibited by Calphostin-C. VEGFA activity was originally referred to as vascular permeability factor and had been shown to stimulate endothelial cell mitogenesis and cell migration and increased microvascular permeability. Wnt3a facilitated MDA-MB-231 induced expression of VEGFA, as well as Calphostin-C suppressed its effect. Taken together, these results suggested that Wnt3a signaling played an important role in regulating the formation of breast cancer.

Keywords: Wnt3a, Wnt Signaling, Breast Cancer, Calphostin C, Primary Cilia

1. Introduction

Wnt family, deems as secreted glycolipoproteins, plays an essential role in cell proliferation, polarity and fate determination during embryonic development and tissue homeostasis(1). Mutations in the Wnt pathways are often linked to human birth defects, cancers and other diseases(2). Among at least three different Wnt pathways, the canonical pathway is considered to have significance in controlling developmental gene expression programs. Studies have shown

that glioblastoma cells over-express Wnt ligands and receptors, such as Wnt3, Wnt6, and Fz9 and Wnt3a (3). Wnt3a has been identified as a critical molecule in Wnt pathway. Some studies indicate that Wnt3a promotes nuclear translocation of FoxM1, and forms a complex with β -catenin/TCF on promoters of Wnt target genes and thereby enhances its transcriptional activity (4,5). In the cancer field, some found that Wnt3a is high expression in colorectal cancer and associated with MMP-9

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expression(6), the Focal adhesion kinase(FAK) protein regulates Wnt3a gene expression to control anterior-posterior cell fate in the developing neural plate and the connecting of the FAK- and Wnt-signaling pathways act to promote cancer when aberrantly activate in mammalian cells(7). Other studies show deregulation of the Wnt signaling pathway is associated with the development and progression of breast cancer. Similar to the observations in *Xenopus* embryos, human Wnt3a and Wnt3 mRNA levels were also strongly reduced in the Focal adhesion kinase depleted MCF7 human breast cancer cells, yet expression of other Wnt ligands was not significantly lowered(8). Wnt3a could stimulate the downregulation of β -catenin acetylation and promote the proliferation of MCF7 cells (9).

Primary cilia is a small microtubulebased organelle projecting from the plasma membrane of cells, sensing mechanical and chemical cues which provide in the cellular environment. Some studies suggest that primary cilia repress cellular Wnt responsiveness(10). Primary cilia restrains the canonical Wnt pathway at least partially downstream of β -catenin cytosolic stabilization and represses nuclear accumulation of β -catenin(11). Inversin, a ciliary protein, has significance in part through transcriptional regulation of genes involved in Wnt signaling and pathways that control cytoskeletal organization and ion transport(12). There is a growing body of evidence to support the notion that ciliary proteins are required not only for regulation of Wnt signaling, but also as downstream effectors of Wnt signaling(13). Reduced primary cilia expression has been observed in human cancers, such as pancreatic cancer, renal cell carcinoma, breast cancer, and melanoma. Studies suggest that primary cilia is dysfunctional in human prostate cancer, and increases Wnt signaling occurred in a subset of cancers(14).

CyclinD1 is a critical gene in cell proliferation process. Notch1 signaling activates CyclinD1 expression and improves cell proliferation in colon cancer(15). Transcription factor snail is essential for individual development, organ fibrosis and tumor occurrence. In tumor occurrence, snail is the important induce-factor of the phenomenon epithelia to mesenchymal transition, and plays an important role in generating and maintaining stem cell properties, cell survival and apoptosis, immunoregulation(16,17,18). A growing body of research suggests that angiogenesis is a prerequisite for tumor growth and metastasis(19). Recently, more than twenty angiogenic factors, such as vascular endothelial growth factor A(VEGFA), transform growth factor- β (TGF- β), fibroblast growth factor(FGF) were found(20,21). Cell proliferation and angiogenesis are the essential characteristic of cancer.

Here, we demonstrated Wnt3a activated the downstream genes of Wnt/ β -catenin signaling pathway in breast cancer cell line MDA-MB-231. Wnt3a decreased the formation of primary cilia, enhanced tumor proliferation gene of CyclinD1, promoted epithelial-mesenchymal transition gene expression of Snail, and facilitated the vascular permeability factor VEGFA. The Antagonist CalphostinC, suppressed these Wnt3a's effects. Thus Wnt3a signaling plays a possibly important role in the pathogenetic mechanism of breast cancer.

2. Materials and Methods

2.1. Cell Culture and Reagents

The MDA-MB-231 cell line was obtained from the American Type Culture Collection (ATCC). Cells were seeded at a density of 1×10^4 cells/cm² and maintained in DMEM growth media supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin and 100 mg/ml streptomycin, in a humidified atmosphere of 5% CO₂ at 37°C. The reagents of Calphostin C (sc-3545) were purchased from Santa Cruz Biotechnology, Inc. As reported, the final concentration of Calphostin C was 10 μ M(22). Adenoviruses of GFP, Wnt3a and β -catenin were sent friendly by the Molecular Oncology Laboratory of the University of Chicago Medical Center.

2.2. RNA Isolation and Quantitative Real Time RT-PCR Analyses

MDA-MB-231 cells were infected with Ad-GFP or Ad-Wnt3a or pretreated with Calphostin C (10 μ M) for 4 days as described above. Then the infected cells were induced with the DMEM medium for 4 days. Total RNA was isolated using TRIZOL Reagents (Invitrogen) and used to generate cDNA templates by RT reaction with hexamer and M-MuLV Reverse Transcriptase (New England Biolabs, Ipswich, MA). The first strand cDNA products were further diluted 5- to 10-fold and used as PCR templates. cDNA were synthesized from 1 μ g total RNA using the Super- Script III reverse transcriptase kit (Invitrogen) in a final volume of 20 μ l. Primers were designed with the Primer 5.0 software and constructed by Beijing Dingguochangsheng Biotechnology COLTD. Quantitative Real-time PCR experiments were performed with a Bio-Rad MJ mini option real-time PCR system and software of with Bio-Rad CFX manager Bio-Rad CFX manager V1.5.534.0511 in triplicate. Sequence and product lengths for each primer pair were followed in Table 1. Samples were analyzed in triplicate and the raw data consisted of PCR cycle numbers required to reach a fluorescence threshold level. The relative expression of target genes were normalized with genorm software (Primer Design Ltd) using GAPDH as reference to determine the normalization factor. The data were expressed as the mean \pm SE.

Table 1. Oligonucleotides used for qRT-PCR.

Gene name	Primer
β -Catenin	Forward primer 5' ATGCCAACGGCACACTC 3'
	Reverse primer 5' CTGGCTGTACTGTCCGTGTT3'
Snail	Forward primer 5' GAGGACAGTGGGAAAGGCTC3'
	Reverse primer 5' TGGCTTCGGATGTGCATCTT3'
VEGFA	Forward primer 5' AGGCCAGCACATAGGAGAGA3'
	Reverse primer 5' ACGCGAGTCTGTGTTTTTGC3'
Cyclin D1	Forward primer 5' GGCCATGCTGAAGCGGAGG3'
	Reverse primer 5' GGCCGTCAGGGGGATGGTCT3'
TCF-1	Forward primer 5' ACCTGGTCACCCAGCAGCCA3'
	Reverse primer 5' TCTCCTTGCGGGCCAGCTCA3'
GAPDH	Forward primer 5' GCATGGCCTTCGTGTCCCC3'
	Reverse primer 5' GAGGGCAATGCCAGCCCCAG3'

2.3. Immunofluorescence

To visualize the primary cilia, MDA-MB-231 cells were infected with Ad-GFP or Ad-Wnt3a or pretreated with Calphostin C (10 μ M) for 4 days and then MDA-MB-231 cells were fixed with 100% ice-cold methanol for 10 min and washed 3 times with PBS. Fixed cells were blocked with 5% bovine serum albumin (BSA) in PBS for 60 min. The cells were then incubated with primary antibody diluted in PBS containing 1% BSA for 1 h. The primary antibodies used were as follows: anti- γ -tubulin (Sigma, T3320, 1:1000) and mouse monoclonal anti-acetylated tubulin antibody (Sigma, 6-11B-1, 1:1000) and anti-snail antibody (proteintech, 13099-1-AP, 1:200), and anti- β -catenin antibody (proteintech, 51067-2-AP, 1:200), anti-VEGFA antibody (proteintech, 19003-1-AP, 1:200). After washing 3 times in PBS, AlexaFluor488, AlexaFluor555 (Invitrogen, Grand Island, NY) conjugated anti-rabbit or anti-mouse IgG was added in PBS with 1% BSA for 1 h. After the final wash, 6-diamidino-2-phenylindole (DAPI) (Sigma) was added and used as a counterstain for nuclei. Fluorescence images were acquired by using an Olympus microscope with DP manager software.

2.4. Western Blotting Analysis

The infected cells were induced with DMEM medium for 4 days and then lysed with NP40 buffer (1% NP-40, 0.15 M NaCl, 50 mM Tris, pH 8.0) containing protease inhibitor cocktail (Sigma, St. Louis, MO.). The cell lysates were centrifuged at 12,000 g for 10 min at 4°C, and the supernatants were stored at -80°C. Protein quantitation was performed with BCA protein assay reagent (Pierce, Rockford, IL, USA). Equal amounts of protein from the different groups were denatured in sodium dodecyl sulfate (SDS) sample buffer and separated on 8–10% polyacrylamide-SDS gel based on the protein molecular weight after electrophoretic separation, proteins were transferred to an Immobilon-P membrane. Membrane was blocked with SuperBlock Blocking Buffer, and the antibodies to cyclinD1 (proteintech, 60186-1-Ig), GAPDH (proteintech,

HRP-60004), Wnt3a (Cell signaling technology, #2915), Naked1 (Cell signaling technology, #2915), Dvl2 (Cell signaling technology, #2915), LRP6 (Cell signaling technology, #2915), Axin1 (Cell signaling technology, #2915), Snail (proteintech, 13099-1-AP), VEGFA (proteintech, 19003-1-AP) were used to detect the target proteins, followed by incubation with a secondary antibody conjugated with horseradish peroxidase. The proteins of interest were detected by using SuperSignal West Pico Chemiluminescent Substrate kit.

2.5. Statistical Analyses

Statistical analysis was performed using SPSS-17.0 software. Data were analyzed using one-way analysis of variance, and Tukey's HSD test was applied as a post hoc test if statistical significance was determined. Statistical significance for the two groups was assessed using Student's *t*-test. The probability level at which differences were considered significant was $P < 0.05$.

3. Result

3.1. Wnt3a Promotes the Wnt/ β -Catenin Signaling Activity in the Breast Cancer Cell Line MDA-MB-231

We investigated the functional significance of Wnt3a in activating Wnt/ β -catenin signaling pathways in breast cancer cell line MDA-MB-231. As the immunofluorescence study, compared with GFP, Wnt3a activated the protein expression of β -catenin in MDA-MB-231. Calphostin C inhibited the translation of key effector β -catenin, then blocked Wnt3a signaling pathway (Fig1A). In addition, Wnt3a activated the mRNA transcription of β -catenin and TCF-1; otherwise Calphostin C inhibited their expression (Fig1B). Wnt3a promoted the protein translation of downstream genes such as DVL2, Axin1, Naked and LRP6. Calphostin C prevented the translation of Wnt3a effectors in MDA-MB-231 (Fig 1 C and D). Similarity, overexpression β -catenin also got the same results as overexpression Wnt3a in the MDA-MB-231 cell line (Fig 1 C and D).

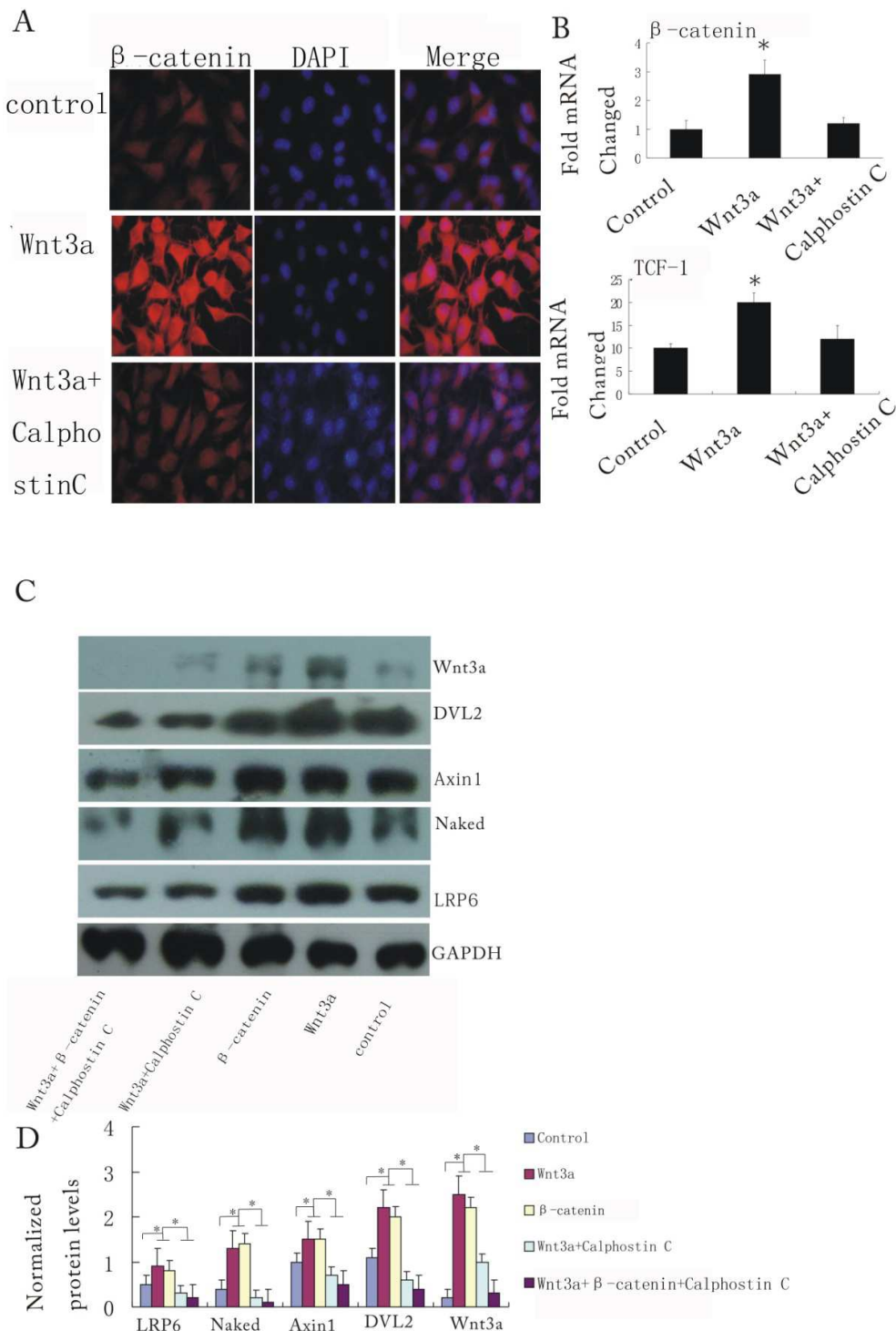


Fig. 1. Wnt3a activated the Wnt/ β -catenin signaling in the breast cancer cell line MDA-MB-231.

(A) Expression of β -catenin upon Wnt3a stimulation using immunofluorescence. Subconfluent MDA-MB-231 cells were infected with Ad-GFP or Ad-Wnt3a, with or without Calphostin C pretreatment. The immunofluorescence staining was manipulated through using anti- β -catenin (β -catenin, red). Thenuclei were labeled with DAPI(DAPI, blue). The nuclei were labeled with DAPI (blue). The cells were imaged on a Zeiss Axiomager microscope (100 \times). (B) Expression of β -catenin and TCF-1 upon Wnt3a stimulation using qRT-PCR analysis. Subconfluent MDA-MB-231 cells were cultured in 0.5% FBS DMEM and infected with Ad-GFP or Ad-Wnt3a, with or without Calphostin C pretreatment. Total RNA was collected and subjected to qRT-PCR analysis. All samples were normalized for GAPDH expression. (C) Western blotting analysis of Wnt3a-induced expression of DVL2, Axin1, Naked and LRP6. Subconfluent MDA-MB-231 cells were infected with Ad-GFP or Ad-Wnt3a or/and Ad- β -catenin, with or without Calphostin C pretreatment. Cells were lysed and subjected to western blotting using anti-Wnt3a, DVL2, Axin1, Naked and LRP6 antibody. The expression level of GAPDH was used as a loading control. (D) Quantification of the protein levels of the immunoblots shown in (C). Each band was quantitated by densitometry using ImageJ software. The bars represent the means \pm SEM. N=3, *P<0.05.

3.2. Wnt3a Blocks the Growth of Primary Cilia of Breast Cancer Cell Line MDA-MB-231

Primary cilia is found to be required to the cell proliferation. Cells lacking cilia have increased Wnt signaling and improved cell proliferation(23,24). To observe expression of primary cilia on the surface of MDA-MB-231, the cells were pretreated with or without Calphostin C for 2h, and then infected with Ad-Wnt3a. The immunofluorescence study was used with the specific antibody of cilia such as γ tubulin and acetylated tubulin, acetylated tubulin antibody stained the

ciliary flagella and γ tubulin antibody stained the ciliary basal body. As shown in Fig 2A, Wnt3a inhibited the expression of γ tubulin and acetylated tubulin. On the contrary, Calphostin C inhibited signaling of Wnt3a and promoted the expression of γ tubulin and acetylated tubulin. Although the length of primary cilia has no apparently change, the number of primary cilia decreased significantly by Wnt3a. Calphostin C rescued this effect of Wnt3a (Fig 2B). It was suggested that Wnt3a blocks the formation of primary cilia on the breast cancer cell line of MDA-MB-231.

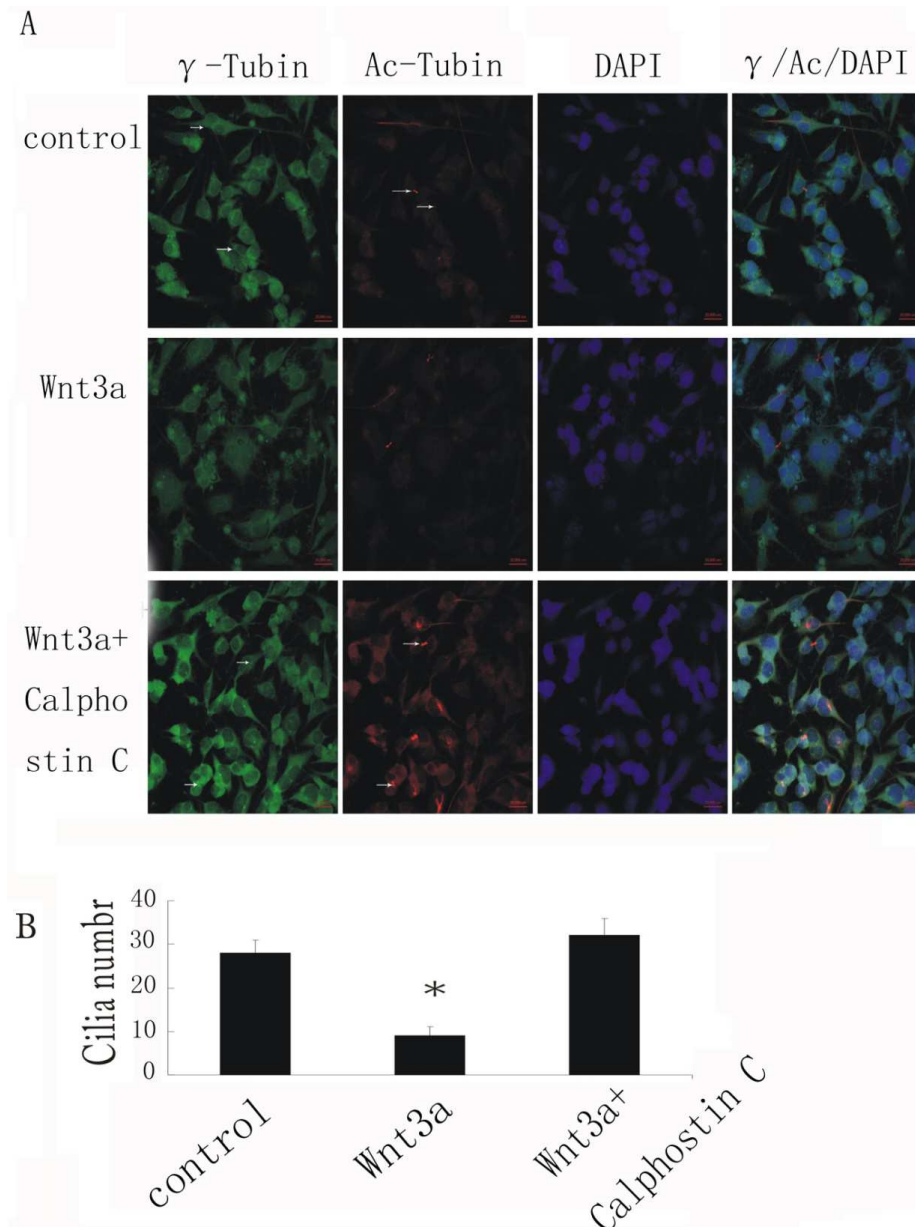


Fig. 2. Wnt3a inhibited primary cilia formation in breast cancer cell line MDA-MB-231.

(A) Expression of γ tubulin and acetylated tubulin upon Wnt3a inhibition using immunofluorescence. MDA-MB-231 cells were infected with GFP or Wnt3a, with or without Calphostin C. The immunofluorescence staining was manipulated through using anti- γ tubulin (γ tubulin, green) and anti-acetylated tubulin (Ac-tub, red). The nuclei were labeled with DAPI (DAPI, blue). The cells were imaged on a Zeiss Axioimager microscope (100 \times). (B) Quantitative analysis of the number of cilia shown in (A). The bars represented the means \pm SEM, * $P < 0.05$.

3.3. Wnt3a Improves CyclinD1 Expression and Promotes the Proliferation of Breast Cancer Cell Line MDA-MB-231

To observe whether Wnt3a promotes the growth of MDA-MB-231, the cell line was infected with Ad-GFP, Ad-Wnt3a, Ad- β -catenin, or pretreated with Calphostin C after 48 hours. MTT assay also displayed that Wnt3a or β -catenin improved the proliferation of the breast cancer cell line from the 48 hours to 144 hours. Furthermore, MTT assay confirmed that Calphostin C inhibited the improved proliferation effect of Wnt3a or β -catenin on MDA-MB-231

cell line (Fig 3A). CyclinD1 is a marker gene in cell proliferation. Over expression CyclinD1 in stem cells shortened G1 and cell cycle (25). Real time RT-PCR showed that Wnt3a or β -catenin facilitated the mRNA and protein expression of CyclinD1 in MDA-MB-231 (Figure 3B), Moreover, Calphostin C inhibited the Wnt/ β -catenin signaling pathway, and significantly down-regulated CyclinD1 expression at protein level (Figure 3C,D). Taken together, the data showed Wnt/ β -catenin signaling played a critical and prerequisite role in promoting the proliferation of MDA-MB-231.

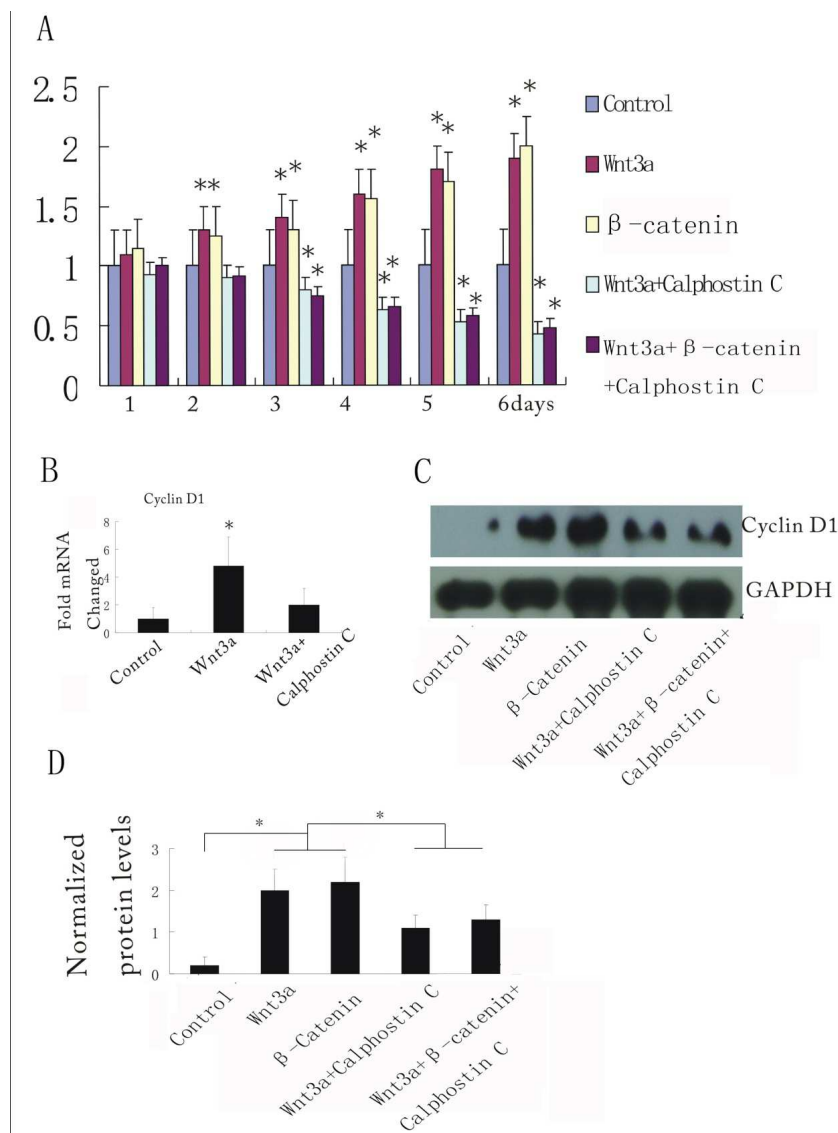


Fig. 3. Wnt3a improved cell proliferation through enhancing CyclinD1 expression in breast cancer cell line MDA-MB-231.

(A) Cell proliferation detected using MTT assay from the 48 to 144 hours. Subconfluent MDA-MB-231 cells were seeded in 96-well plates and infected with Ad-GFP or Ad-Wnt3a or/and Ad- β -catenin, with or without Calphostin C pretreatment. Each assay was done in triplicate and carried out in at least three independent experiment. Representative results are shown, $p < 0.05$. (B) Expression of CyclinD1 upon Wnt3a stimulation using qRT-PCR analysis. Subconfluent MDA-MB-231 cells were cultured in 0.5% FBS DMEM and infected with Ad-GFP or Ad-Wnt3a, with or without Calphostin C pretreatment. Total RNA was collected and subjected to qRT-PCR analysis. All samples were normalized for GAPDH expression. (C) Western blotting analysis of Wnt3a-induced expression of CyclinD1. Subconfluent MDA-MB-231 cells were infected with Ad-GFP or Ad-Wnt3a or/and Ad- β -catenin, with or without Calphostin C pretreatment. Cells were lysed and subjected to western blotting using anti-CyclinD1 antibody. The expression level of GAPDH was used as a loading control. (D) Quantification of the protein levels of the immunoblots shown in (C). Each band was quantitated by densitometry using ImageJ software. The bars represented the means \pm SEM. $N = 3$, $*P < 0.05$.

3.4. Wnt3a Activates Epithelial-Mesenchymal Transition that is associated with High Expression of Snail Gene

To investigate whether Wnt signaling effects epithelial-mesenchymal transition, we tested the key gene of Snail. This gene represents the event of epithelial-mesenchymal transition during embryonic development and cancer process. As shown in Fig 4A, Wnt3a reinforced the much higher fluorescence intensity of Snail expression in MDA-MB-231 cell, compared with the control group. Furthermore, once Wnt signaling was inhibited with the antagonist of Calphostin C, the stimulatory effect on Snail

decreased obviously. We also detected mRNA transcription level of Snail gene by qRT-PCR. Wnt3a improved Snail gene transcription level by 2.5 folds, compared with the control. The inhibitor Calphostin C suppressed transcription level of Snail in MDA-MB-231 (Fig 4B). Similarly, β -catenin also enhanced the translation of Snail gene in MDA-MB-231. Calphostin C blocked Wnt3a or β -catenin induced signaling pathway, finally blocked Snail gene translation (Fig 4 C and D). These results suggested that Wnt/ β -catenin signaling takes the certain role to be effective in inducing epithelial-mesenchymal transition of cancer progress.

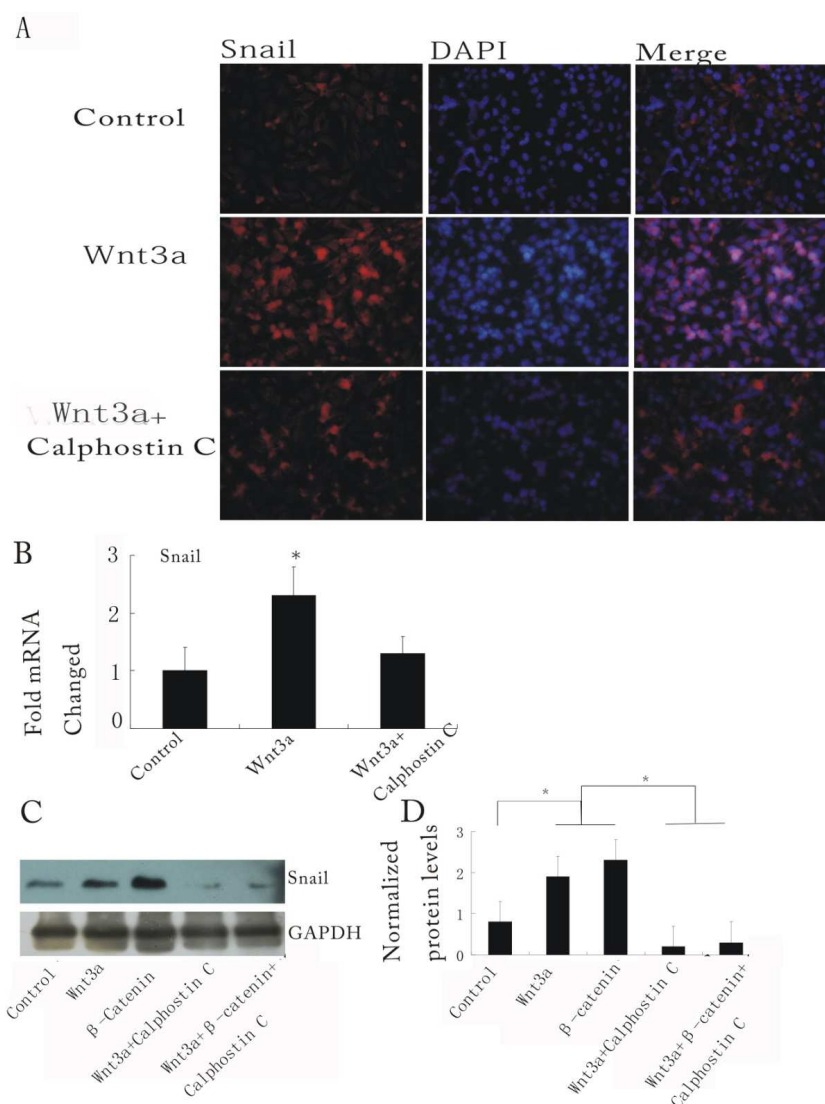


Fig. 4. Wnt3a improved epithelial to mesenchymal transition which was blocked by Wnt signaling inhibitor of Calphostin C.

(A) Expression of Snail upon Wnt3a stimulation using immunofluorescence. MDA-MB-231 cells were infected with GFP or Wnt3a, with or without Calphostin C. The immunofluorescence staining was manipulated through using anti-Snail (snail, red). The nuclei were labeled with DAPI (DAPI, blue). The cells were imaged on a Zeiss Axioimager microscope (100 \times). (B) Expression of Snail upon Wnt3a stimulation using qRT-PCR analysis. Subconfluent MDA-MB-231 cells were cultured in 0.5% FBS DMEM and infected with Ad-GFP or Ad-Wnt3a, with or without Calphostin C pretreatment. Total RNA was collected and subjected to qRT-PCR analysis. All samples were normalized for GAPDH expression. * $p < 0.05$. (C) Western blotting analysis of Wnt3a-induced expression of Snail. Subconfluent MDA-MB-231 cells were infected with Ad-GFP or Ad-Wnt3a or/and Ad- β -catenin, with or without Calphostin C pretreatment. Cells were lysed and subjected to western blotting using anti-Snail antibody. The expression level of GAPDH was used as a loading control. (D) Quantification of the protein levels from the immunoblots shown in (C). Each band was quantitated by densitometry using ImageJ software. The bars represented the means \pm SEM. N=3, * $P < 0.05$.

3.5. Wnt3a Activities Angiogenesis by Improving VEGFA Gene

The formation of cancer is associated with cell proliferation, epithelial-mesenchymal transition and angiogenesis. VEGFA is one of the critical genes in the event of angiogenesis. In the immunofluorescence experiment, we analyzed the effect of Wnt/ β -catenin on the crucially important angiogenic marker VEGFA. Wnt3a was found to enhance the higher fluorescence expression of VEGFA, compared with the control group. The inhibitory effect of Calphostin C on VEGFA activity of

MDA-MB-231 was also confirmed by the immunofluorescence (Fig 5A). Quantitatively, Wnt3a-mediated positive effect on VEGFA transcription was increased by 3.3 folds, compared with the control (Fig 5B). Similarly, β -catenin also enhanced the translation of VEGFA in MDA-MB-231. Furthermore, Calphostin C blocked Wnt3a or β -catenin induced signaling pathway, finally blocked VEGFA translation (Fig 5 C and D). Taking together, Wnt/ β -catenin signaling is shown to potentiate the angiogenic differentiation of breast cancer cells.

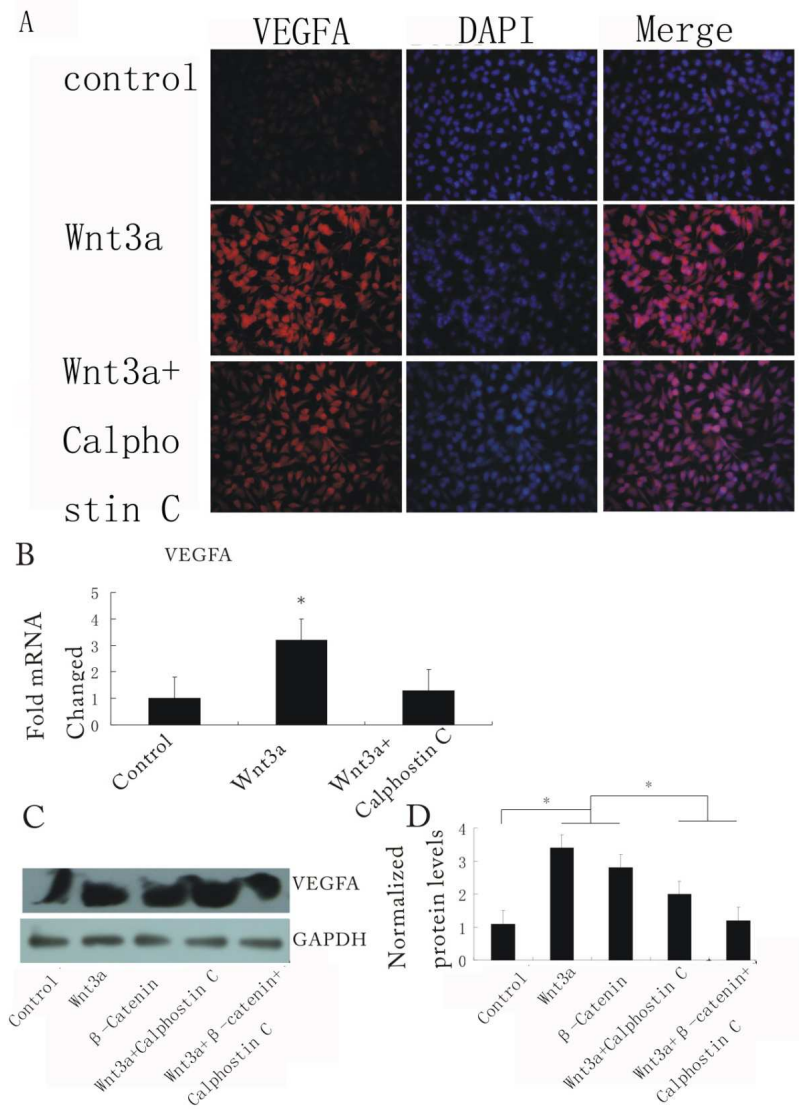


Fig. 5. Wnt3a activated angiogenesis by improving VEGFA expression, whereas Calphostin C blocked its effect.

(A) Expression of Snail upon VEGFA stimulation using immunofluorescence. MDA-MB-231 cells were infected with GFP or Wnt3a, with or without Calphostin C. The immunofluorescence staining was manipulated through using anti-VEGFA (VEGFA, red). The nuclei were labeled with DAPI(DAPI, blue). The cells were imaged on a Zeiss Axioimager microscope (100 \times). (B) Expression of VEGFA upon Wnt3a stimulation using qRT-PCR analysis. Subconfluent MDA-MB-231 cells were cultured in 0.5% FBS DMEM and infected with Ad-GFP or Ad-Wnt3a, with or without Calphostin C pretreatment. Total RNA was collected and subjected to qRT-PCR analysis. All samples were normalized for GAPDH expression.* $p < 0.05$. (C) Western blotting analysis of Wnt3a-induced expression of VEGFA. Subconfluent MDA-MB-231 cells were infected with Ad-GFP or Ad-Wnt3a or/and Ad- β -catenin, with or without Calphostin C pretreatment. Cells were lysed and subjected to western blotting using anti-Snail antibody. The expression level of GAPDH was used as a loading control. (D) Quantification of the protein levels from the immunoblots shown in (C). Each band was quantitated by densitometry using ImageJ software. The bars represented the means \pm SEM. N=3,* $P < 0.05$.

4. Discussion

It is reported that Wnt signaling involves cancer progression apparently(26). Some studies have been shown that Wnt/ β -catenin signaling pathway plays a critical role in malignant transformation and progression of breast epithelial cells(27). Wnt signaling regulates Cystathionine- γ -lyase gene expression and accumulates β -catenin in colon cancer(28). Wnt3a has been identified as a critical molecule in Wnt signaling pathways(29). Recently, RB Clarke reported high expression of Wnt3a in patient-derived metastatic breast cancer stem-like cells. However the mechanism of Wnt3a in breast cancer is not still uncertain(30).

Primary cilia has been known to regulate many of the processes, including cell microenvironmental interaction, Hedgehog and PDGF signaling and cell polarity. Primary cilia regulates cellular homeostasis through Wnt signaling(31). Moreover, the primary cilium integrates Hedgehog and Wnt signaling between dental epithelia and mesenchyme during the proper tooth development(32). In addition, primary cilia may participate in the development and progression of cancer, such as breast cancer(33). At the molecular level, the function of cilia in ciliogenesis is regulated by phosphorylation which in turn depends on Frizzled-2-mediated noncanonical Wnt signaling(34). Here our study suggested Wnt3a inhibited the formation of cilia in breast cancer cells. When Wnt signaling was improved, the length and number of primary cilia decreased.

CyclinD1 is well recognized that it is a critical target of proliferative signals in G1 phase cell cycle progression(35). Wnt3a up-regulated expression of CyclinD1 significantly in the progression of chronic myeloid leukemia(36).

Epithelial-mesenchymal transitions are determinants of the progression of carcinomas, and the Snail family of transcription factors has previously been implicated in the differentiation of epithelial cells into mesenchymal cells during embryonic development. Recently, Snail was reported to trigger epithelial-mesenchymal transition in tumor progression(37). SFRP4, another specifically Wnt antagonist, by inducing apoptosis, decreasing epithelial to mesenchymal transition in head and neck cancer(38).

Wnts are multifunctional factors that act through the frizzled receptors to regulate proliferation, apoptosis, branching morphogenesis, inductive processes, and cell polarity. But all of these processes must occur as developing vascular structures are formed and maintained. The roles of growth factors vascular endothelial growth factor (VEGFA) in angiogenesis have been known for some time, the development and maintenance of vascular structures are dependent on VEGFA. Vascular development and the process of angiogenesis depend on evaluating the function of novel endothelial regulatory pathways such as Wnt/ β -catenin signaling(39). In clinical specimens of colorectal cancer, Forkhead box Q1 (FOXQ1) is overexpression in mRNA level. Overexpression of FOXQ1 upregulates the genes of VEGFA,

Wnt3a, which has positive roles for tumor growth(40).

In summary, this study showed that Wnt3a stimulated downstream genes of Wnt/ β -catenin signaling pathway in breast cancer cell line MDA-MB-231. Overexpression of Wnt3a decreased the formation of primary cilia. Wnt3a activated tumor proliferation gene of CyclinD1, then improved the cancer cells proliferation. Wnt3a promoted MDA-MB-231-induced expression of Snail and took part in differentiation of epithelial cells into mesenchymal cells. Wnt3a facilitated MDA-MB-231-induced expression of VEGFA, and stimulated cell migration, as well as increased microvascular permeability. Inhibition of Wnt/ β -catenin signaling pathway inactivated these effects in this tumor cell line. Taken together, our findings strongly suggest Wnt3a may play an important role in pathogenetic mechanism of breast cancer. Thus Wnt3a inhibitors Calphostin C may be used as novel therapeutics for breast cancer.

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