

ORIGINAL ARTICLE

FZD7 has a critical role in cell proliferation in triple negative breast cancerL Yang¹, X Wu², Y Wang³, K Zhang³, J Wu⁴, Y-C Yuan², X Deng², L Chen³, CCH Kim¹, S Lau⁵, G Somlo⁶ and Y Yen¹¹Department of Molecular Pharmacology, Beckman Research Institute, City of Hope National Medical Center, Duarte, CA, USA;²Department of Molecular Medicine, Beckman Research Institute, City of Hope National Medical Center, Duarte, CA, USA;³Translational Research Laboratory Core, Beckman Research Institute, City of Hope National Medical Center, Duarte, CA, USA;⁴Division Comparative Medicine, Beckman Research Institute, City of Hope National Medical Center, Duarte, CA, USA;⁵Department of Pathology, Beckman Research Institute, City of Hope National Medical Center, Duarte, CA, USA and⁶Department of Medical Oncology, Beckman Research Institute, City of Hope National Medical Center, Duarte, CA, USA

Breast cancer is genetically and clinically heterogeneous. Triple negative breast cancer (TNBC) is a subtype of breast cancer that is usually associated with poor outcome and lack of benefit from targeted therapy. We used microarray analysis to perform a pathway analysis of TNBC compared with non-triple negative breast cancer (non-TNBC). Overexpression of several Wnt pathway genes, such as frizzled homolog 7 (FZD7), low density lipoprotein receptor-related protein 6 and transcription factor 7 (TCF7) was observed in TNBC, and we directed our focus to the Wnt pathway receptor, FZD7. To validate the function of FZD7, FZD7shRNA was used to knock down FZD7 expression. Notably, reduced cell proliferation and suppressed invasiveness and colony formation were observed in TNBC MDA-MB-231 and BT-20 cells. Study of the possible mechanism indicated that these effects occurred through silencing of the canonical Wnt signaling pathway, as evidenced by loss of nuclear accumulation of β -catenin and decreased transcriptional activity of TCF7. *In vivo* studies revealed that FZD7shRNA significantly suppressed tumor formation, through reduced cell proliferation, in mice bearing xenografts without FZD7 expression. Our findings suggest that FZD7-involved canonical Wnt signaling pathway is essential for tumorigenesis of TNBC, and thus, FZD7 shows promise as a biomarker and a potential therapeutic target for TNBC.

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Introduction

With improvements in the systematic treatment of breast cancer, the survival rate of these patients has improved steadily over the past two decades. However, the heterogeneity of breast cancer makes the treatment of many subtypes, including triple negative breast cancer (TNBC) difficult (Gonzalez-Angulo *et al.*, 2007; Perez *et al.*, 2010). TNBC is characterized by lack of both estrogen and progesterone hormone receptors, and lack of overexpression of human epidermal growth factor receptor 2 (HER2). Although TNBC constitutes ~15% of all breast cancers, its death rate is disproportionately higher than any other subtype of breast cancer, especially among young African-American, Asian and Hispanic patients (Anders and Carey, 2009; Kassam *et al.*, 2009; Rahman *et al.*, 2009). Although, anti-HER2 antibodies, Herceptin, estrogen receptor antagonists and aromatase inhibitors have improved treatment of many breast cancer subtypes, the treatment of TNBC remains a great challenge (Gluz *et al.*, 2009; Amir *et al.*, 2010; Silver *et al.*, 2010). Therefore, it is imperative to identify new potential therapeutic targets for TNBC.

Canonical Wnt signaling regulates cell fate decisions throughout embryonic development and is involved in human cancer (Reya and Clevers, 2005; Clevers, 2006; MacDonald *et al.*, 2009). Activation of the canonical Wnt pathway is transduced through Frizzled (FZD) family receptors and the LRP5/lipoprotein receptor-related protein 6 (LRP6) co-receptor to initiate the β -catenin signaling cascade (Bhanot *et al.*, 1996; Pinson *et al.*, 2000). In the absence of Wnt signal, β -catenin binds to the Axin complex and is constantly degraded. Sequential phosphorylation by casein kinase (CK1) and glycogen synthase kinase 3 (GSK3) results in β -catenin ubiquitination and proteasome-mediated degradation (He *et al.*, 2004; Price, 2006). In the presence of canonical Wnt signaling, FZD binds to Dishevelled (DVL) and LRP5/6-binds to AXIN-FRAT to form a complex. β -catenin is prevented from phosphorylation (Tolwinski *et al.*, 2003) and the stabilized β -catenin translocates from the cytoplasm to the nucleus to activate transcription of Wnt-responsive genes by binding to T-cell factor/lymphoid enhancer factor

Correspondence: Dr Y Yen, Department of Molecular Pharmacology, Beckman Research Institute, City of Hope National Medical Center, Beckman Building, Room 4117, 1500 East Duarte Road, Duarte, CA 91010, USA.

E-mail: yyen@coh.org

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(TCF/LEF) family transcription factors. Activation of tissue-specific Wnt target genes is involved in the development of human tumors, including those of the breast, colon and other cancers (Lee *et al.*, 1995; Jones and Kemp, 2008; Ojalvo *et al.*, 2010).

The goal of this study was to identify the cell proliferation regulator for TNBC tumor cells. Microarray analysis was used to screen differentially expressed genes in TNBC, and pathway analysis was performed to identify the pathway involved in TNBC tumorigenesis. We found that canonical Wnt signaling is more active in TNBC than non-TNBC via microarray screening. Therefore, we investigated the role of Wnt signaling in TNBC.

Results

Wnt pathway genes are overexpressed in TNBC

Microarray analysis using the Human Gene Array ST-1.0 (Affymetrix, Santa Clara, CA, USA) was performed in 19 breast tumor samples (fives TNBC and 14 non-TNBC). Identification of differentially expressed genes was first carried out under the criteria of 1.5-fold upregulation in TNBC with a P -value < 0.01 . Two hundred and six genes, including 169 annotated genes (Supplementary Figure 1) were identified as being differentially expressed and were further subjected to gene ontology analysis (Biological Process, National Institute of Allergy and Infectious Disease (NIAID), NIH, USA) using the DAVID analysis online tool (<http://david.abcc.ncifcrf.gov/>). Sixty-three genes from the most significant cluster ($P < 0.01$), were identified (Figure 1a) and subsequent pathway analysis was performed on these genes. The Wnt signaling pathway was identified as a pathway that was significantly overexpressed in TNBC ($P < 0.05$) (Figure 1b). In addition, FZD7, LRP6 and TCF7 were all upregulated along the Wnt signaling pathway (Figure 1c, Supplementary Figure 2), whereas FZD7 showed the greatest difference in expression as compared with other genes. Notably, FZD7 is the only member of the Frizzled family that significantly overexpressed in TNBC (Figure 1d). The expression levels of Axin, GSK3 and DVL were not significantly different in the microarray study. Reverse transcriptase (RT-PCR) analysis confirmed the lack of differential expression of Axin, GSK3 and DVL between TNBC and non-TNBC (Supplementary Figure 3). We speculate that these proteins might function either through phosphorylation-induced conformational change or mutations.

FZD7 is upregulated in TNBC and TNBC-derived cell lines

To examine the expression levels of FZD7 in TNBC in comparison to non-TNBC tumor, RT-PCR was used. Overexpression of FZD7 was observed in all TNBC samples, whereas four non-TNBC tissues with equal differentiation/stage minimally expressed FZD7 (Figure 2a). To validate the FZD7 overexpression in TNBC, immunohistochemical staining of 20 formalin-fixed paraffin-embedded breast tumor samples (three TNBC and 17 non-TNBC) was performed. We found

that 67% of TNBC samples expressed FZD7; whereas only 5% of non-TNBC samples expressed FZD7 (Figure 2b). We then studied FZD7 expression in a larger human breast tumor cohort reported by Finak *et al.* We found that FZD7 mRNA expression was significantly higher in the TNBC samples ($n = 14$) as compared with non-TNBC samples ($n = 109$; $P = 0.0017$, Wilcoxon test), consistent with the immunohistochemistry results (Supplementary Figure 4) (Finak *et al.*, 2008). RT-PCR was then carried out to detect FZD7 expression in various breast cancer cell lines (Figure 2c). Among the seven cell lines investigated, the MDA-MB-231 and BT-20 cell lines expressed high levels of FZD7, whereas the other five lines either had no or limited FZD7 expression. Although the MDA-MB-231 and BT-20 cell lines are known to be TNBC-derived cell lines, all other cell lines were either derived from normal breast tissue (MCF 10A) or non-TNBC tissues. Notably, FZD7 is the most abundant FZD in TNBC cell lines, MDA-MB-231 and BT-20 (Figure 2d).

FZD7 shRNA suppressed tumor transformation in the TNBC cell lines MDA-MB-231 and BT-20

To evaluate the function of FZD7 in TNBC cells, FZD7shRNA- or control GFPshRNA-expressing constructs were lentivirally transduced into MDA-MB-231 and BT-20 cells. Effective knockdown of FZD7 in MDA-MB-231 and BT-20 cells was confirmed in FZD7shRNA lentivirus-transduced cells. FZD7 expression was reduced by 95–97.5% at the mRNA level in these cells (Figure 3a, left panel), which nearly completely inhibited FZD7 protein expression (Figure 3a, right panel). Specific inhibition of FZD7 expression without affecting the other members of the FZD family was confirmed by RT-PCR of FZD7shRNA-transduced MDA-MB-231 (Figure 3b) and BT-20 cells (Figure 3c).

We also compared the rate of cell growth of FZD7shRNA and GFPshRNA lentivirus-infected MDA-MB-231 cells. Cell growth significantly slowed in FZD7shRNA-transduced cells as compared with GFPshRNA-transduced cells (Figure 4a, left panel). When both groups of transduced cells were treated with the FZD7 ligand Wnt3a, significant acceleration of cell growth was observed in GFPshRNA-transduced MDA-MB-231 cells, however, there no growth advantage was seen in FZD7shRNA-transduced MDA-MB-231. To determine if the FZD7shRNA alone was sufficient to inhibit growth, MDA-MB-231 cells transduced with GFPshRNA or FZD7shRNA were treated with the LRP6 inhibitor DKK1 to block the LRP6 signal. No significant change in the suppression of cell growth by treatment with both FZD7shRNA and DKK1 as compared with FZD7shRNA treatment alone was seen in MDA-MB-231 cells (Figure 4a, right panel).

The effect of FZD7 on the cell invasiveness was examined using an invasion assay. MDA-MB-231 and BT-20 cells that expressed GFPshRNA actively migrated onto the bottom layer of membranes in insert chambers during overnight culture. MDA-MB-231 and BT20 cells with GFPshRNA virus showed aggressive migration from the matrigel to the membrane

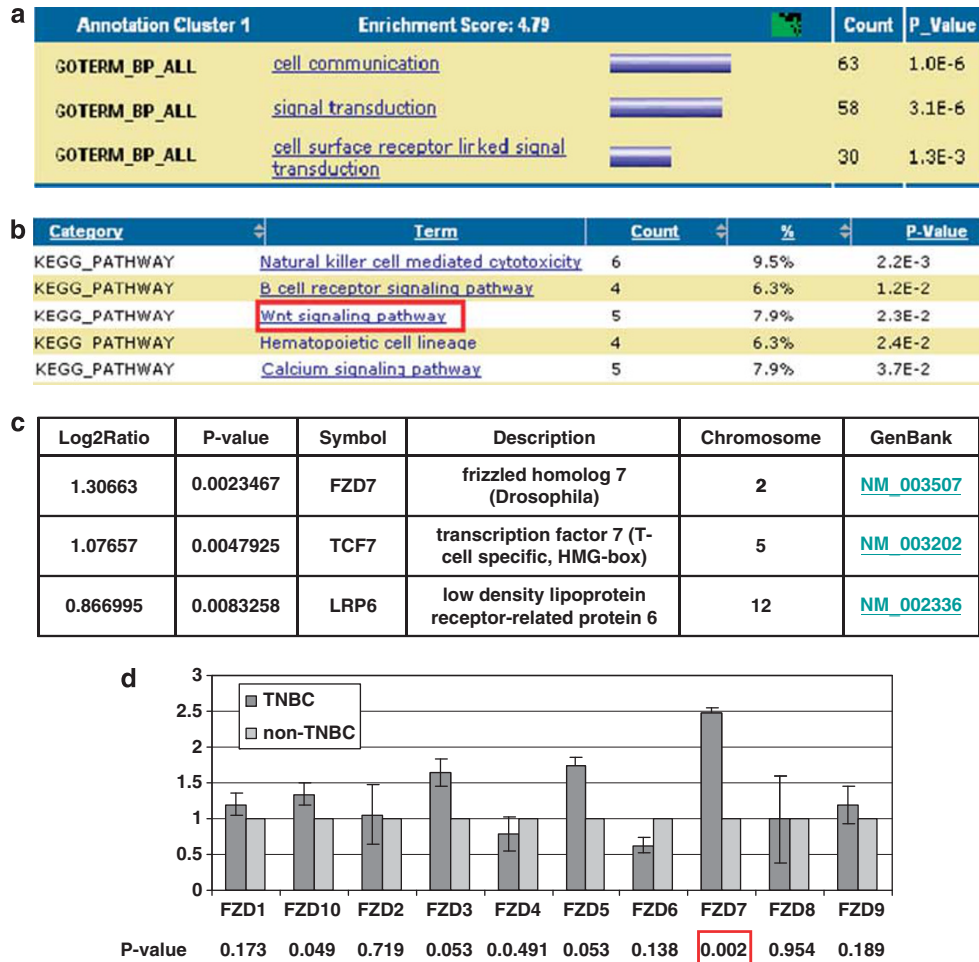


Figure 1 Microarray-based detection of overexpressed Wnt pathway genes in TNBC. (a) Gene ontology analysis (GOTERM_BP_ALL) was performed using the DAVID online tool, Functional Annotation Clustering identified a significantly upregulated cluster of genes in TNBC ($P < 0.01$). (b) Pathway analysis (KEGG_PATHWAY) using the DAVID online tool identified the Wnt pathway as being significantly active in TNBC ($P < 0.05$). (c) Summary of microarray results indicating that LRP6, FZD7 and TCF7 were significantly upregulated in TNBC (> 1.5 fold, $P < 0.01$). (d) Normalized intensity of each FZD for each tumor sample, as obtained from microarray. The average intensity of each FZD in TNBC and non-TNBC is shown. Ratio of TNBC vs non-TNBC was plotted.

(Figure 4b). The numbers of MDA-MB-231 and BT-20 GFPshRNA-transduced cells on the membrane were 2.6 and 1.9 times higher than those of MDA-MB-231 and BT-20 FZD7shRNA-transduced cells, respectively. We also assessed cell motility by a wound-healing assay. MDA-MB-231 cells transduced with GFPshRNA moved to the wound area within 16 h and completely closed the wound within 24 h; whereas with FZD7-inhibited cells, the wound remained open, even after 24 h (Figure 4c). In colony formation assays, more than twice the number of colonies were found in GFPshRNA-expressing cells as were found in MDA-MB-231 FZD7shRNA-transduced cells (Figure 4d). These results indicate that FZD7 is a key factor involved in cell transformation, especially in cell invasion, motility and clonogenicity.

FZD7 regulated cell tumorigenesis through the canonical Wnt signaling pathway

FZD7 is known as the receptor for the Wnt signaling pathway. To investigate if FZD7 affects TNBC tumor

cell biology through this pathway, β -catenin immunofluorescence and DAPI staining was performed to determine the localization of β -catenin and thereby confirm activation of the Wnt/ β -catenin pathway. β -catenin accumulated in the nuclei of MDA-MB-231 cells transduced with GFPshRNA cells (Figure 5a); however, in cells in which FZD7 expression was suppressed by FZD7shRNA, β -catenin staining was attenuated in the nuclei and remained in the cytoplasm. Furthermore, assessment of TCF7 promoter activity by dual luciferase assay revealed that the promoter activity of TCF7 declined by $\sim 50\%$ in FZD7-inhibited MDA-MB-231 and BT-20 cells (Figure 5b).

We then assessed whether expression of target genes of the Wnt pathway (for example, Cyclin D1 and C-myc) changed when FZD7 was inhibited. Western blot analysis revealed decreased expression of Cyclin D1 and C-myc, downstream components of the Wnt pathway, in MDA-MB-231 and BT-20 cells in which FZD7 was knocked down as compared with GFPshRNA-expressing control cells (Figure 5c). To evaluate whether

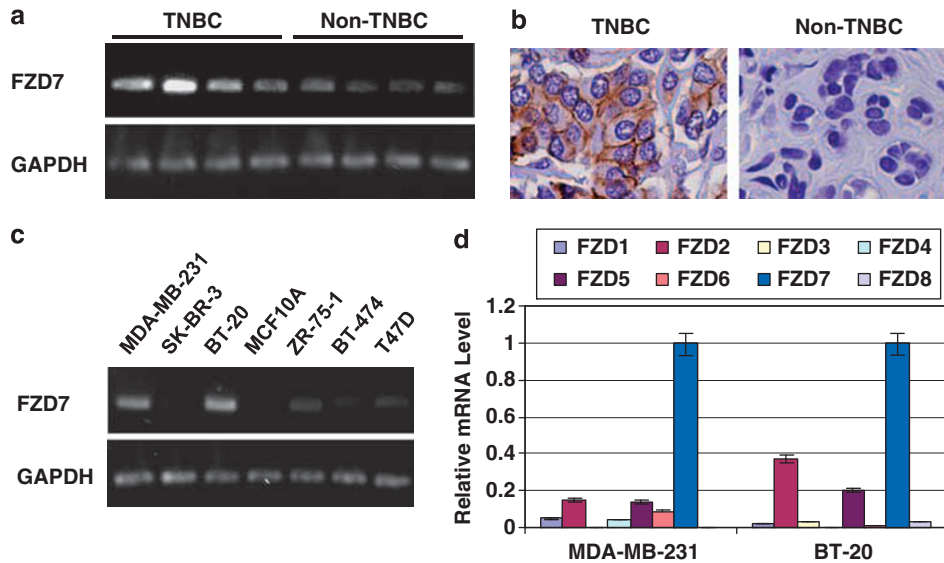


Figure 2 FZD7 is overexpressed in TNBC. (a) RT-PCR of FZD7 in TNBC and other breast tumors. (b) Immunohistochemical staining of FZD7 in TNBC and other breast tumors. (c) RT-PCR analysis of FZD7 expression in breast cancer cell lines. MDA-MB-231 and BT20 are TNBC-derived cell lines. (d) Real-time RT-PCR analysis of each Frizzled in MDA-MB-231 and BT-20 cells.

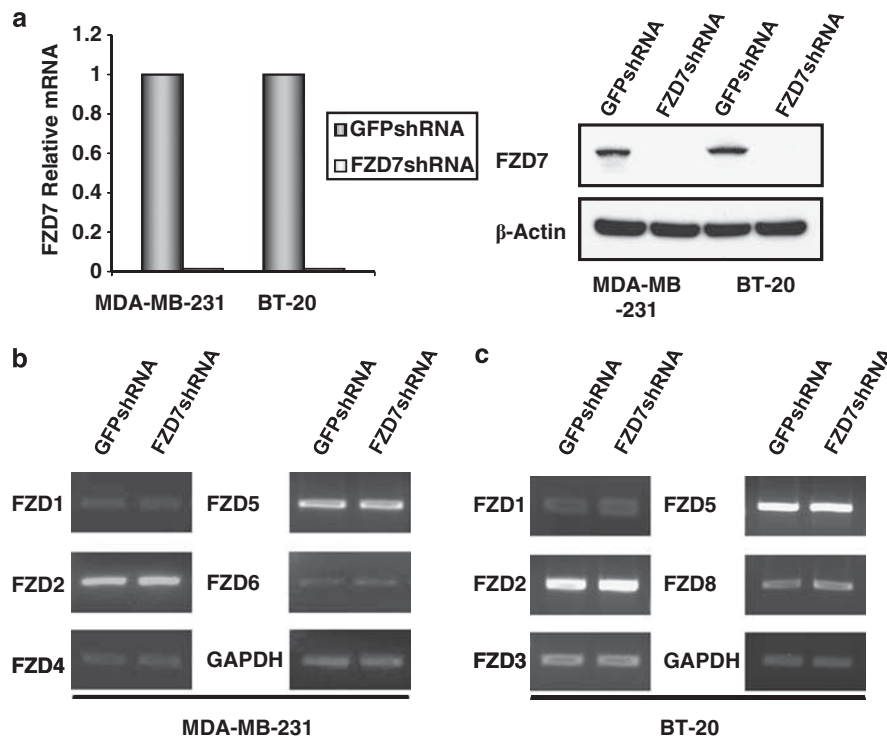


Figure 3 FZD7 knockdown in MDA-MB-231 and BT-20 cells. (a) Real-time RT-PCR (left panel) and western Blot (right panel) of FZD7 in GFPshRNA- and FZD7shRNA-expressing cells. (b) RT-PCR of FZD family members showed that FZD7shRNA was able to specifically knockdown FZD7 but not other FZDs in MDA-MB-231 cells. (c) RT-PCR of FZD family members indicated that FZD7shRNA was able to specifically knockdown FZD7 but not other FZDs in BT-20 cells.

overexpression of FZD7 was sufficient to trigger Wnt/ β -catenin signaling, FZD7 tagged with GFP was transiently expressed in MCF7 cells, which expresses low levels of FZD7. Approximately 70–80% of transfected cells were GFP-positive, indicating they should overexpress FZD7. However, overexpression of FZD7 did

not activate the Wnt canonical pathway (Figure 5d) and did not appear to generate a significant cell proliferation benefit (Figure 5e).

In our microarray data, we noticed that two genes, *FZD* and *PKCB1*, along the non-canonical Wnt/ Ca^{2+} pathway were upregulated in TNBC. In the Wnt/ Ca^{2+}

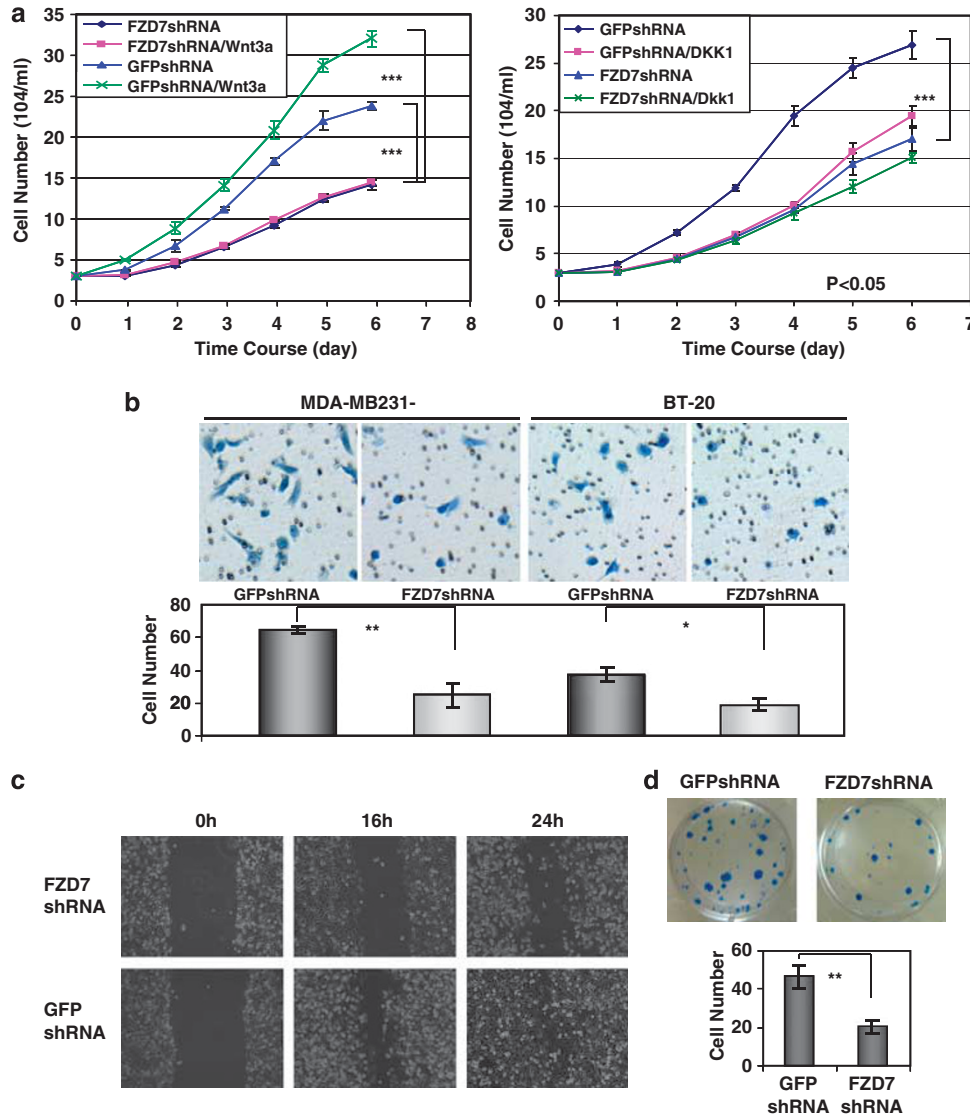


Figure 4 FZD7 knockdown affects the tumorigenesis of MDA-MB-231 and BT-20 cells. (a) Growth curve of MDA-MB-231 cells with FZD7 knockdown and/or Wnt3a treatment (left panel). Growth curve of MDA-MB-231 cells with FZD7 knockdown and/or treated with DKK1 (right panel). Each point represents cell number as mean \pm s.d. ($n=3$), multiple comparison test with Tukey adjustment was used to compare the difference, *** $P<0.001$. (b) FZD7shRNA significantly decreased the invasive ability of MDA-MB-231 and BT-20 cells. Number of cells that migrated into the membrane calculated as mean \pm s.d. ($n=3$ independent experiments). ** $P<0.01$ and * $P<0.05$. (c) FZD7shRNA inhibited wound healing in MDA-MB-231 cells. Wound healing status at 0, 16 and 24h after wound was made in MDA-MB-231 cells transduced with FZD7shRNA or GFPshRNA. (d) FZD7shRNA impaired cell colony formation in MDA-MB-231 cells. Colony number was counted and the graph represents an average of three independent experiments. ** $P<0.01$.

pathway, Wnt binds to FZD to initiate signaling that results in stimulation of protein kinase C and release of intracellular Ca^{2+} . Increased concentrations of Ca^{2+} induce dephosphorylation of NFAT (nuclear factor of activated T cells), which promotes its translocation to the nucleus where it can activate the transcription of target genes of the Wnt/ Ca^{2+} pathway. However, when FZD7 was inhibited in MDA-MB-231 and BT-20 cells, expression of PKCB1 and the phosphorylation status of NFAT did not change (Supplementary Figure 5), suggesting that FZD7 does not have a role in Wnt/ Ca^{2+} signaling in TNBC. Taken together, these data provide strong evidence that Wnt/ β -catenin, but not

Wnt/ Ca^{2+} signaling is active in TNBC, and that blocking the Wnt canonical pathway leads to tumor cells losing their tumorigenicity.

FZD7shRNA suppresses tumor growth in vivo

To explore whether the Wnt pathway contributes to the tumorigenesis of TNBC *in vivo*, MDA-MB-231 cells transduced with FZD7shRNA or GFPshRNA were inoculated into NOD-SCID IL2rg female null mice. FZD7shRNA blocked the Wnt signaling in MDA-MB-231 cells and induced significant ($P<0.001$) suppression of tumor growth *in vivo*. Moreover, growth arrest was observed in MDA-MB-231/FZD7shRNA cell-derived

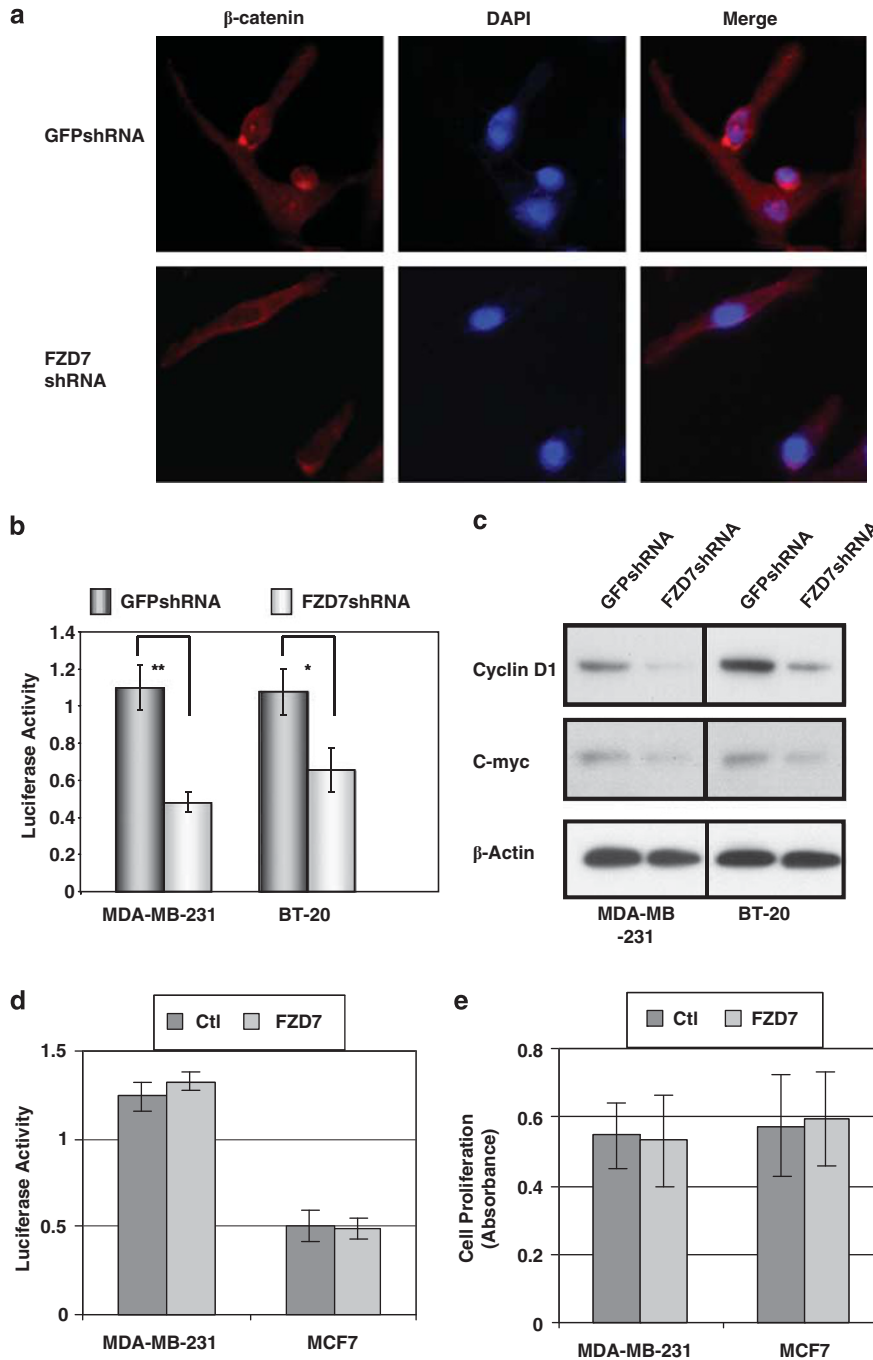


Figure 5 FZD7 regulates TNBC cell tumorigenesis through the Wnt signaling pathway. (a) Immunofluorescence staining of β -catenin (red). β -catenin was detected in the nucleus in GFPshRNA-transduced MDA-MB-231 cells and accumulated in the cytoplasm in FZD7shRNA-expressing cells. Nuclei are counterstained with DAPI (blue). (b) FZD7shRNA decreases TCF transcription activity. Dual luciferase assay of TCF promoter activity 48 h post co-transfection of Renilla luciferase and the reporter plasmid Topflash in MDA-MB-231 and BT-20 cells transduced with FZD7shRNA or GFPshRNA lentivirus. Bars represent the average luciferase activities normalized to internal control ($n=3$). $**P<0.01$ and $*P<0.05$. (c) Western blot of the Wnt pathway responsive genes *C-myc* and *Cyclin D1*. Diminished expression of *C-myc* and *Cyclin D1* in FZD7shRNA-transduced MDA-MB-231 and BT-20 cells. (d) MDA-MB-231 and MCF7 cells were seeded into 48-well plates the day before transfection. The plasmid pCMV6-AC-GFP-FZD7 (FZD7) or pCMV-AC-GFP (Ctl) was co-transfected with Topflash and Renilla luciferase. Cells were collected and dual luciferase assay performed to determine the transcription activity of TCF. (e) MDA-MB-231 and MCF7 cells were spread into 96-well plates. The plasmid pCMV6-AC-GFP-FZD7 (FZD7) or pCMV-AC-GFP (Ctl) was transfected by lipofectamine 24 h after plating the cells. MTS assay was conducted to evaluate cell proliferation 36 h post-transfection.

tumors 1 week after inoculation (Figure 6a). Tumor tissues were harvested and stained with antibodies against the FZD7, β -catenin and Cyclin D1

(Figure 6b). In control tumors, which had normal FZD7 expression, β -catenin localized to the nucleus and Cyclin D1 expression were detected (Figure 5b, left panel).

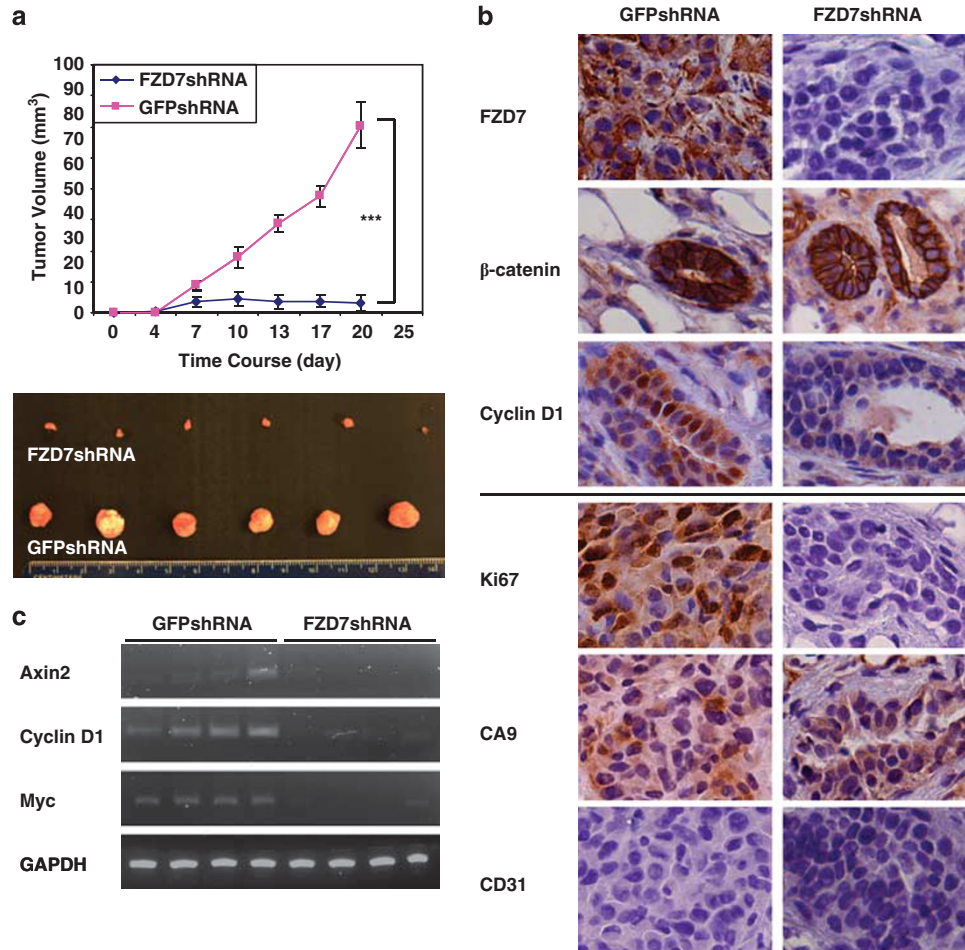


Figure 6 FZD7 knockdown suppressed tumor growth *in vivo*. (a) Tumor size was measured twice per week for 3 weeks. Graph shows mean tumor volume of eight mice; bar: s.d. (upper panel). Multiple comparison test with Tukey adjustment was used to compare the difference, *** $P < 0.001$. All tumors were harvested and photographed (lower panel); (b) immunohistochemical staining, with antibodies against the indicated proteins of MDA-MD-231 tumors with or without FZD7 knockdown. (c) RT-PCR analysis to detect expression of the canonical Wnt pathway target genes *Axin2*, *Cyclin D1* and *Myc* in FZD7+ (GFPshRNA) and FZD7-/low (FZD7shRNA) tumors.

However, in tumors with FZD7 knockdown, most cells expressed β -catenin in the cytoplasm and did not express Cyclin D1 (Figure 5b, right panel). Further examination of the Wnt/ β -catenin target genes in these tumors revealed that *Axin2*, *Cyclin D1* and *Myc* were over-expressed in FZD7-expressing tumors, but not in FZD7 knockdown tumors (Figure 6c). These results are consistent with the *in vitro* findings and confirmed that FZD7shRNA induced growth retardation via canonical Wnt signaling.

We also characterized the FZD7-expressing and FZD7 knockdown tumors for expression of hypoxia cell marker CA9, angiogenesis marker CD31 and cell proliferation marker Ki67 (Figure 6b). Ki67 expression was significantly increased in FZD7-expressing tumors, but not in those in which FZD7 expression was knocked down. CA9 was weakly expressed in both FZD7-expressing tumor and FZD7 knock-down tumors, whereas CD31 was not detected in either. These findings indicate that activation of Wnt signaling in TNBC

induced cell proliferation, but may not directly involve cell hypoxia and angiogenesis.

Discussion

TNBC has been a particular focus of attention because it has no confirmed molecular target for development of targeted therapeutics and a poor prognosis. Basal-like breast cancer is categorized as TNBC because almost all basal-like breast cancers lack hormone receptor expression and HER2 overexpression (Perou *et al.*, 2000; Sotiriou *et al.*, 2003). Although aberrant activation of Wnt signaling has been observed in breast cancer (Brown, 2001; Turashvili *et al.*, 2006), the correlation of Wnt signaling with TNBC has rarely been investigated. However, canonical Wnt pathway activation in basal-like tumors has been observed (DiMeo *et al.*, 2009; Lindvall *et al.*, 2009). More recently, LRP6 over-expression was found in a subtype of ER-negative and

HER2-negative breast tumor (Liu *et al.*, 2010), which is consistent with our microarray results.

In our study, downregulation of FZD7 to inactive Wnt signaling in the TNBC cell lines MDA-MB-231 and BT-20 resulted in impaired cell growth and tumor transformation. Thus, the essential role of canonical Wnt signaling in TNBC makes it an attractive therapeutic target for TNBC. Targeting Wnt pathway genes for development of novel pharmacological agents for other neoplasms has been investigated and great effects have been observed (Yang *et al.*, 2008). Our data, together with other recent findings, suggest that inhibition of either FZD7 or LRP6 will block Wnt signaling and suppress Wnt signal-mediated cell proliferation (Bafico *et al.*, 2001). Because FZD7 and LRP6 are cell surface antigens, siRNA or antibodies against these mRNAs or proteins, respectively, should strongly block canonical Wnt signaling in TNBC cells. Small molecules that inhibit the biological function of these two genes may also act as powerful drugs for TNBC treatment. Notably, any agents that increase the phosphorylation activity of CK1 and GSK3 or block β -catenin nuclear accumulation also offer possible therapeutic approaches for TNBC.

Constitutive expression of Wnt signaling enhances the self-renewal of mammary progenitor cells and continuous stimulation of this pathway leads to formation of breast tumors (Lindvall *et al.*, 2007; Jones and Kemp, 2008). Study of a mouse model of breast cancer indicated that breast cancers that arise from stem-progenitor cells undergo transformation through deregulation of Wnt signaling, whereas epithelial cell derived breast tumors are triggered by oncogenic activation of HER2 (Li and Rosen, 2005). We also noticed that breast cell lines that express high levels of HER2 usually express low levels of FZD7 (Supplementary Figure 6). This association of Wnt signaling and HER2 in breast tumor development requires further exploration.

The Wnt signaling pathway is a highly conserved pathway, and three signaling branches have been identified: canonical Wnt (Wnt/ β -catenin pathway), non-canonical (including planar cell polarity pathway) and Wnt/ Ca^{2+} (Komiya and Habas, 2008). Although upregulation of the Wnt/ Ca^{2+} pathway genes *FZD7* and *PRKCB1* raises the possibility that the non-canonical Wnt pathway may be involved in tumor formation in TNBC, our findings argue that FZD7 may only initiate Wnt/ β -catenin signaling, whereas *PRKCB1* upregulation in TNBC might be induced via other signaling pathways. Therefore, we speculate that the Wnt canonical pathway may provide the unique signaling from Wnt that contributes to tumorigenesis in TNBC.

Materials and methods

Patients and tissues

All tumor tissues were collected under an IRB-approved protocol (IRB 06206) at City of Hope (COH) National Medical Center with assistance from the COH Translational Research Laboratory Core. The tissues were obtained from biopsies and saved

in 1 ml RNAlater solution. The tumor tissues were pathologically diagnosed as ductal or lobular breast cancer. Immunohistochemical staining and FISH confirmed that ER/PR/HER2 were negatively expressed, as assessed by pathologists in the Department of Pathology of COH.

Microarray analysis

The Affymetrix GeneChip Human Gene 1.0-ST array (Affymetrix) was used to define gene expression profiles from the samples. Synthesis and labeling of complementary DNA targets, and hybridization and scanning of GeneChips were carried out by the Microarray Core at COH according to the manufacturer's protocol. Raw intensity measurements of all probe sets were background corrected, normalized and converted into expression measurements using Affymetrix's Expression Console v1.1.1. Statistical analysis was performed using the Bioconductor package 'ArrayTools' (Affymetrix, Santa Clara, CA, USA, <http://bioconductor.org/packages/release/bioc/html/ArrayTools.html>). Differentially expressed genes were identified. Gene ontology analysis was first performed using DAVID Bioinformatics Resource 6.7 online software (NIAID, NIH) and pathway analysis was subsequently performed using the same software.

RNA isolation

An $\sim 0.3 \times 0.5 \text{ cm}^2$ piece of tissue stored in RNAlater was put into RNA lysis buffer (1 ml) and homogenized using polytron (Brinkmann Instrument, Rexdale, ONT, Canada). RNA was isolated according to the manufacturer's protocol (Qiagen, Germantown, MD, USA), during which DNase treatment was carried out. The quality of total RNA isolated from human tissue was analyzed by Agilent Bioanalyzer. Total RNA extraction from MDA-MB-231 was carried out using the RNeasy Mini Kit (Qiagen) and on-column DNase treatment was performed.

RT-PCR and real-time RT-PCR

For complementary DNA synthesis, total RNA (1 μg) was transcribed using random hexamers (Invitrogen, Carlsbad, CA, USA) and SuperScript III reverse transcriptase (Invitrogen) following the manufacturer's protocol. For DNA amplification, complementary DNA was denatured at 94 °C for 30 s, primer was annealed at 55–62 °C for 30 s, and DNA extended at 72 °C for 30 s for 24–30 cycles. RT-PCR for FZD7 was performed using an iQ5 iCycler (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The amplification conditions consisted of an initial incubation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s and 59 °C for 30 s. Data were analyzed using Bio-Rad iQ5 Optical System Software v2.0. Primers for PCR and RT-PCR and their sequences are listed in Supplementary Figure 7 (Sonderegger *et al.*, 2007). All products yielded a single band with the predicted size.

Western blot analysis

Cell protein was extracted from cells using RIPA buffer (Cell Signaling, Danvers, MA, USA) with phosphatase inhibitor (Roche, Indianapolis, IN, USA). Equal amount of protein was loaded and separated by sodium dodecyl sulfate PAGE. After the protein was transferred onto a membrane, the blot was blocked with 5% non-fat milk in Tris-buffered saline and probed overnight at 4 °C using the following antibodies: FZD7 (Sigma-Aldrich, St Louis, MO, USA), Cyclin D1, C-myc (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and β -actin (Bio-Rad). Appropriate antibodies were used for secondary antibody reaction. Signal was detected by the

ECL Plus Western Blot Detecting System (GE Healthcare, Piscataway, NJ, USA).

Cell culture and cell growth assays

The triple negative cell lines MDA-MB-231 and BT-20 were purchased from ATCC and cultured in the recommended media. Specific lentivirus shRNA (Sigma-Aldrich) was used to disrupt the expression of FZD7 while shRNA-targeting GFP (Sigma-Aldrich) served as control. Transduced cells were selected with puromycin (0.8 µg/ml, MDA-MB-231; 0.5 µg/ml, BT-20) 2 days post-viral transduction. FZD7 expression in stable cells was determined by RT-PCR and immunoblot analysis. MDA-MB-231 cells that expressed FZD7 or GFP shRNA (FZD7shRNA or GFPshRNA cells) were cultured in growth medium to observe cell growth. Cells (3×10^4 /ml) were seeded into 24-well plates, and cell number was counted every day for 6 days using a Cellometer Auto T4 (Nexcelom Bioscience, Lawrence, MA, USA). Independent experiments were performed in triplicate. To overexpress FZD7, the plasmid pCMV6-AC-GFP-FZD7 (OriGene Technologies, Inc. Rockville, MD, USA) was transfected into cells by Lipofectamine 2000 (Invitrogen).

Cell invasion and colony formation assay

Cell mobility was determined by a wound closure assay. Cells were placed onto six-well plates at 80% confluence and cultured in serum-depleted media for 24 h. A wound was made by scraping the monolayer cells with a plastic pipette tip and fresh serum-free medium was replenished. Images of wound closure were photographed at 0, 16 and 24 h post-scraping.

A BioCoat Matrigel Invasion Chamber (Becton Dickinson Labware, Bedford, MA, USA) was used to perform the invasion assay according to the manufacturer's protocol. Cells (2.5×10^5) in 2 ml serum-free media were distributed into the inserts of six-well plates. Equal amounts of growth media were placed into the wells. After overnight culture, the cells that remained in the matrigel were removed and the chamber membrane was stained with 50% methanol blue/ethanol overnight. Cells that invaded into the chamber membrane were photographed and quantified using Image-Pro Plus 7.0 software (Media Cybernetics, Inc., Bethesda, MD, USA).

For the colony formation assay, 500 cells were transferred into 12-well plates and fresh growth media were changed every 3–4 days. After 2 weeks of culture, cells were fixed with methanol at 4 °C for 10 min, stained with methylene blue and colonies were photographed.

Immunofluorescence and immunohistochemistry staining

Cells were seeded on cover slips in six-well plates and allowed to adhere to the glass overnight to reach 50% confluence. Cells on the cover slips were fixed with methanol and incubated first with Image-iT FX Signal Enhancer (Invitrogen) for 30 min. After a wash with phosphate-buffered saline, mouse anti-human β-catenin antibody (3.5 µg/ml) (BD Bioscience, San Jose, CA, USA) was added and incubated for 1.5 h. Alex Fluor 568 (Invitrogen) was added as the secondary antibody, and the cover slips incubated in the dark for 1 h. The cover slip was sealed with Prolong Gold Antifade (Invitrogen), which contains

DAPI. Images were taken 5 min after the cover slips were sealed. For immunohistochemical staining, all slides were prepared and stained by the Pathology Core Facility at COH using a standard protocol. Antibodies used in this study were as follows: rabbit polyclonal antibodies FZD7 (1:75 dilution, Sigma-Aldrich) and CA9 (1:50 dilution, R&D System Inc., Minneapolis, MN, USA), mouse monoclonal antibodies β-catenin (1:50 dilution, BD Bioscience), Cyclin D1 (1:100 dilution, Santa Cruz Biotechnology), Ki67 (1:25 dilution, BD Bioscience) and CD31 (1:75 dilution, Cell Marque, Rocklin, CA, USA). All antibodies were titrated with negative and positive controls to obtain optimal staining.

Luciferase assay

ShFZD7- or shGFP-virus-transduced MDA-MB-231 cells were distributed into 12-well plates the day before transfection. Cells at 80% confluence were co-transfected with TCF7-driven Topflash (Millipore, Billerica, MA, USA) reporter (1 µg) and Renilla luciferase (20 ng) using 2.5 µl of Lipofectamine 2000 (Invitrogen). Cells were lysed in 1 × passive lysis buffer for 48 h and the supernatant was collected for Dual-luciferase activity measurement (Promega, Madison, WI, USA). For each sample, firefly luciferase activity was normalized with an internal control, Renilla luciferase activity.

Tumor inoculation

MDA-MB-231 cells with FZD7shRNA or GFPshRNA were trypsinized and cell numbers were determined using a Cellometer AutoT4. The cells were pelleted with a centrifuge and resuspended in a final concentration of 5×10^7 /ml. Tumors were initiated in immunodeficient NOD-SCID IL2rg female null mice (8-week-old) by subcutaneous injection of 5×10^6 cells into the right ventral flank. These humanized mice enable various human cells and tissues to be engrafted and retain the same functions as in humans. The mice were housed in the Animal Resource Center of COH in accordance with the guidelines of City of Hope/Beckman Research Institute Animal Care and Use Committee and the work was done with an IACUC-approved protocol. Tumor size was measured twice a week for about 3 weeks. The mice were then sacrificed and the tumors collected.

Conflict of interest

The authors declare no conflict of interest.

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