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FZD7 in Triple Negative Breast Cancer Cells

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1. Introduction

Breast cancer is the most prevalent cancer in women. Although the survival rate of breast cancer has improved steadily in the past two decades, over 40,000 women die from breast cancer related complications each year. The heterogeneity of breast cancer makes the treatment of different subtypes difficult (Gonzalez-Angulo *et al.*, 2007; Perez *et al.*, 2010). Triple negative breast cancer (TNBC) is one of the subtypes. It is negative for both estrogen and progesterone hormone receptors and lack the overexpression of human epidermal growth factor receptor 2 (HER2). TNBC constitutes approximately 15% of all breast cancers. However, its death rate is disproportionately higher than any other subtype of breast cancer, especially among young Black, Asian and Hispanic patients (Anders and Carey, 2009; Kassam *et al.*, 2009; Rahman *et al.*, 2009). Although, HER2 antibody, Herceptin, estrogen receptor antagonist, and aromatase inhibitor have brought hope to breast cancer patients, the treatment of TNBC remains to be a great challenge (Amir *et al.*, 2010; Gluz *et al.*, 2009; Silver *et al.*, 2010). Thus it is imperative to investigate effective therapeutic targets for TNBC patients.

FZD7, a Wnt pathway receptor, is one of the most abundant Frizzled family proteins expressed in TNBC and its cell lines. Wnt canonical signaling regulates cell fate decision throughout embryonic development and is related to human disease (Clevers, 2006; MacDonald *et al.*, 2009; Reya and Clevers, 2005). Activation of Wnt canonical pathway is transduced through Frizzled (FZD) family receptors and LRP5/LRP6 coreceptor to the β-catenin signaling cascade (Bhanot *et al.*, 1996; Pinson *et al.*, 2000). In the presence of canonical Wnt signal, FZD binds to Dishevelled (DVL) and LRP5/6 to AXIN-FRAT to form a complex. β-catenin is protected from phosphorylation (Tolwinski *et al.*, 2003) and the stabilized β-catenin translocates from the cytoplasm to the nucleus to activate the transcription of Wnt responsive genes by binding with T-cell factor/lymphoid enhancer factor (TCF/LEF) family transcription factors. Activation of these tissue-specific Wnt target genes is involved in the development of human tumors, including breast, colon, and other cancers (Jones and Kemp, 2008; Lee *et al.*, 1995; Ojalvo *et al.*, 2010).

To investigate the therapeutic targets for TNBC, microarray has been performed to identify the genes that may be involved in the tumorigenesis of TNBC. FZD7 is differentially expressed in TNBC which raises the possibility that aberrant Wnt signaling might be critical for TNBC development. In this chapter we will address the role that FZD7 plays in cell proliferation of TNBC and its mechanism.

2. FZD7 plays a critical role in cell proliferation of TNBC

Basal-like breast cancer is categorized as TNBC because almost all basal-like breast cancers do not have hormone receptor expression and HER2 overexpression (Perou *et al.*, 2000; Sotiriou *et al.*, 2003). Although aberrant Wnt signaling activation 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 tumor has been observed (DiMeo *et al.*, 2009; Lindvall *et al.*, 2009). Recently, we performed Human Gene Array ST-1.0 (Affymetrix) in 19 breast tumor samples of five triple negative and 14 non-triple negative breast cancers (non-TNBC). Identification of the differentially expressed genes was carried out under the criteria of 1.5 fold up-regulated in TNBC with p-value less than 0.01. FZD7 as well as other two Wnt pathway genes were found to be overexpressed in TNBC tissues. Among these genes, FZD7 showed the greatest change as compared to other genes (Table 1). Although other Frizzled, such as FZD3 and FZD5 showed high level expression in TNBC tissues, it was not significantly higher ($p>0.05$). This result suggested that overexpression of FZD3 and FZD5 in the TNBC samples used in our microarray study may have been due to the variation of individual samples, but not from overall upregulation of FZD3 and FZD5 in TNBC.

Log2Ratio	P-value	Symbol	Description	Chromosome	GenBank
1.30663	0.0023467	FZD7	frizzled homolog 7 (Drosophila)	2	NM_003507
1.07657	0.0047925	TCF7	transcription factor 7 (T-cell specific, HMG-box)	5	NM_003202
0.866995	0.0083258	LRP6	low density lipoprotein receptor-related protein 6	12	NM_002336

Table 1. FZD7 and other Wnt pathway genes were up-regulated in TNBC.

2.1 FZD7 is up-regulated in TNBC and its derived cell lines

Aberrant Wnt signaling in TNBC has been noted by a few research groups. Dr. Bu and his team reported that LRP6 overexpression is found in a subtype of breast tumor that is ER-negative and Her2-negative (Liu *et al.*, 2010). This is consistent with our microarray result. Benefiting from free access to the public database, we analyzed FZD7 expression in a larger human breast tumor cohort study reported by Finak *et al* (20). We found that FZD7 mRNA expression was significantly higher in the TNBC samples (n=14) as compared to non-TNBC samples (n=109; P=0.0017, Wilcoxon Test) (Figure 1A). We further evaluated the FZD7 expression using breast tumor tissues. In Fig 1B, the overexpression of FZD7 was observed in all TNBC samples, whereas the other four non-TNBC tissues with equal differentiation/stage minimally expressed FZD7. To validate the FZD7 overexpression in TNBC, immunohistochemistry staining was performed in 20 formalin-fixed paraffin-embedded breast tumor slides. It was found that 67% of TNBC expressed FZD7; while only 5% non-TNBC weakly expressed FZD7 (Fig. 1C). FZD7 expression in various breast cancer cell lines was also assessed (Fig. 1D). Among the seven cell lines investigated, MDA-MB-231 and BT-20 cell lines expressed high levels of FZD7, while the other five cell lines either had no or limited FZD7 expression. While the MDA-MB-231 and BT-20 cell lines were known to be TNBC-derived, 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 (Fig. 1E).

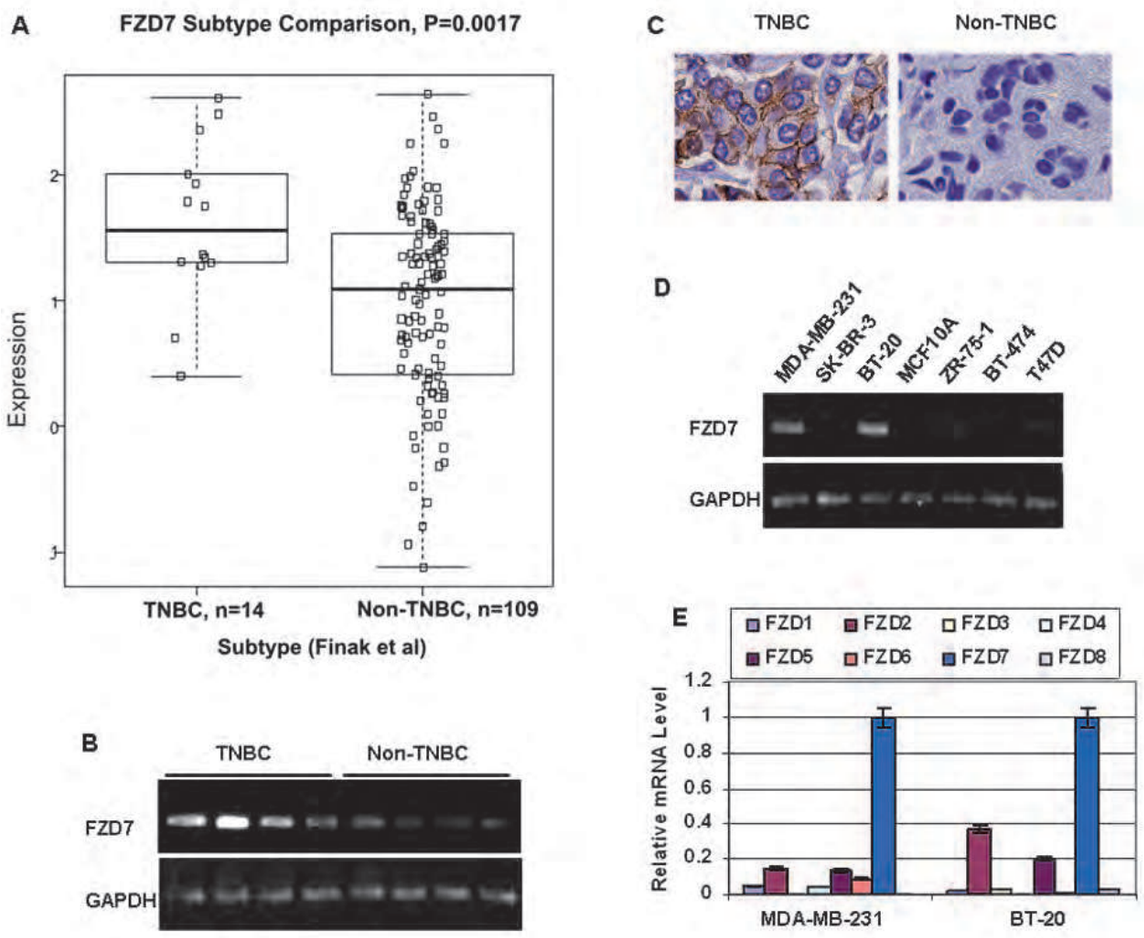

Fig. 1. FZD7 expression in TNBC tissues and TNBC cell lines.

2.2 FZD7shRNA suppressed tumor transformation in TNBC cell lines MDA-MB-231 and BT-20

To evaluate the function of FZD7 in TNBC cells, FZD7shRNA or its control GFPshRNA lentivirus were transduced into TNBC-derived cell lines MDA-MB-231 and BT-20, and were selected with puromycin. Effective knockdown of FZD7 in MDA-MB-231 and BT-20 cells was found in FZD7shRNA lentivirus transduced cells. It was observed that FZD7 expression was reduced by 95% to 97.5% at the mRNA level (Fig.23A, left panel) and almost completely inhibited the protein expression (Fig. 2A, right panel). Specific inhibition of FZD7 expression without affecting the other members of the FZD family was confirmed by RT-PCR in MDA-MB-231(Fig. 2B) and in BT-20 cells (Fig. 2C).

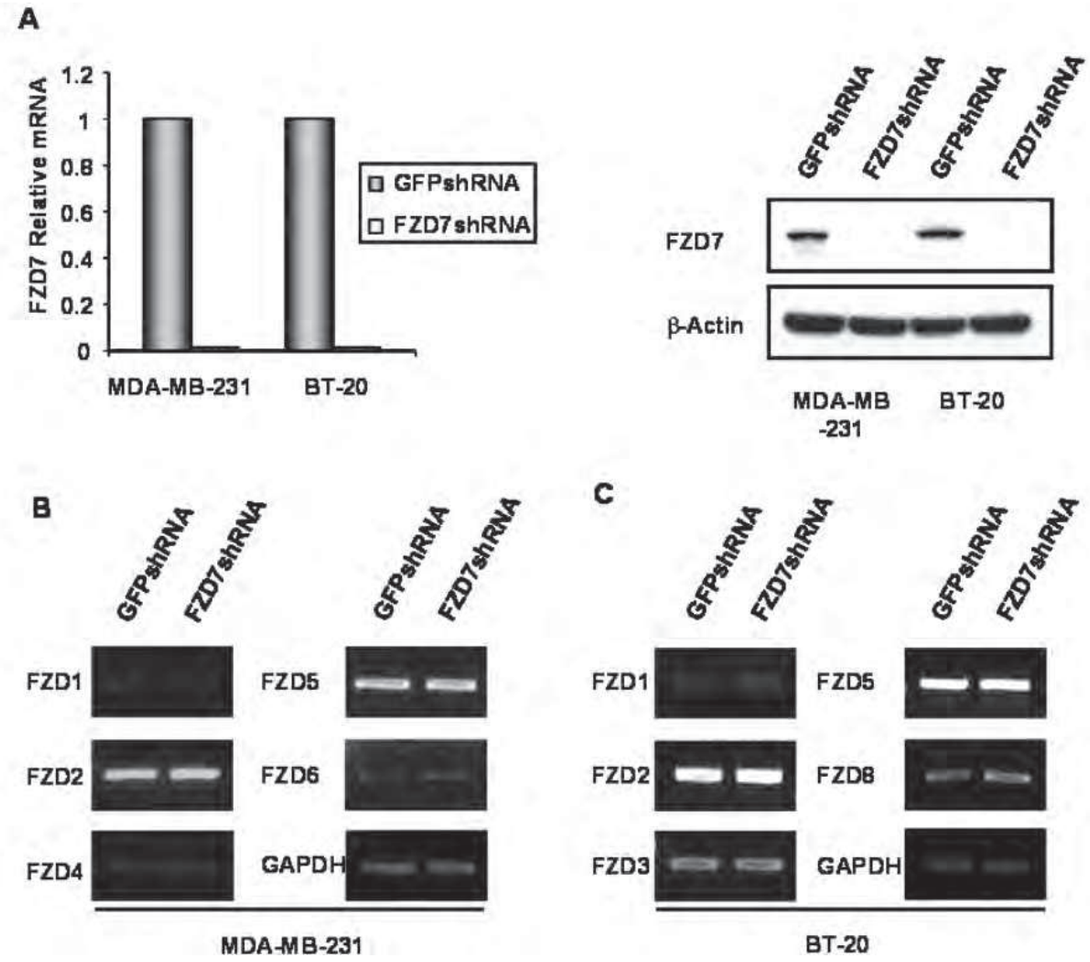

Fig. 2. FZD7 knockdown in MDA-MB-231 and BT-20 cells.

2.2.1 FZD7 is required for cell growth in TNBC cells

Frizzled family gene is commonly up-regulated in various tumors from different organs. Wnt signaling initiated from these genes induce acceleration of cell growth in these tumor cells. Comparisons were made with regards to the rate of cell growth of FZD7shRNA and GFPshRNA lentivirus infected MDA-MB-231 cells. As shown in Fig. 3 left panel, cell growth significantly slowed in FZD7shRNA transduced MDA-MB-231 cells as compared to that of GFPshRNA infected MDA-MB-231 cells. When both cell lines were treated with FZD7 ligand Wnt3a, significant cell growth acceleration was observed in GFPshRNA transduced MDA-MB-231 cells. However, there was no growth advantage seen in FZD7shRNA transduced MDA-MB-231 cells. To determine if the FZD7shRNA alone is sufficient for the growth inhibition, MDA-MB-231/GFPshRNA cells and MDA-MB-231/FZD7shRNA cells were treated with LRP6 inhibitor DKK1 to block the LRP6 signal. As indicated in Fig 3 right panel, there is no significant change in the suppression of cell growth by double treatment with FZD7shRNA and DKK1 as compared with FZD7shRNA treatment alone in MDA-MB-231 cells. In each panel of Fig 3, FZD7 suppressed cells with FZD7shRNA transduction showed significant growth retardation ($P<0.05$). The data indicates that FZD7 plays a critical role in cell growth of TNBC.

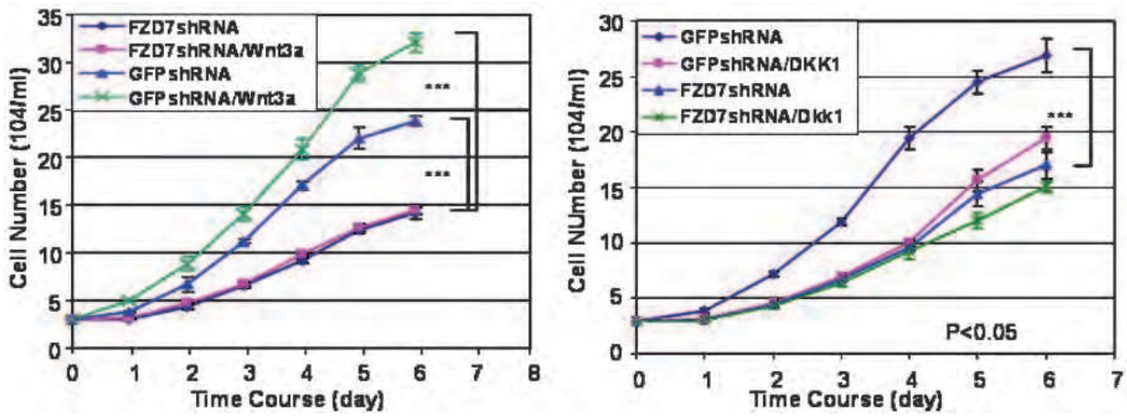

Fig. 3. FZD7 regulated cell growth in MDA-MB-231 cells.

2.2.2 FZD7 plays an important role in cell motility and invasion

We have noted that FZD7 enhances cell growth in TNBC. We then asked whether FZD7 is involved in cell motility and invasion of TNBC. We evaluated the invasive ability of the cells with or without FZD7 by using an invasion assay. MDA-MB-231 and BT-20 cells that express GFPshRNA actively migrated into the bottom layer of the membrane of the insert chamber after overnight culture. A representative area from each well for each cell line cultured was analyzed. The number of invasive cells in the membrane was quantified using the Image-Pro 6.3 software. As shown in Fig. 4A, MDA-MB-231 and BT20 cells treated with

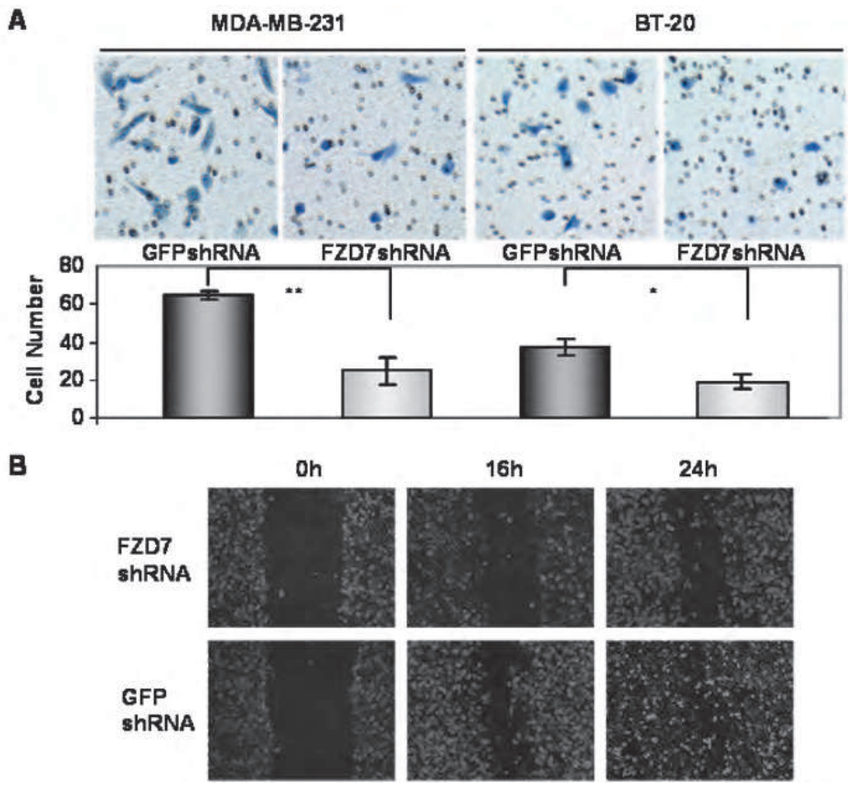

Fig. 4. FZD7 increased cell migration in TNBC.

GFPshRNA virus showed aggressive migration from the metrigel to the membrane. The numbers of cells in the membrane of MDA-MB-231/GFPshRNA and BT-20/GFPshRNA cells were 2.6 and 1.9 times higher than those of MDA-MB-231/FZD7shRNA and BT-20/FZD7shRNA cells respectively. To address the effect of FZD7 in cell motility, we performed a wound healing assay. MDA-MB-231/GFPshRNA cells migrated to the wound area within 16 hours and completely closed the wound within 24 hours; whereas with FZD7 inhibited cells, the wound remained open even after 24 hours of observation (Fig. 4B). These results suggested that FZD7 exert positive effect on cell migration in TNBC.

2.2.3 FZD7 modulated cell tumorigenicity in TNBC

It has been shown that FZD7 is involved in not only enhanced cell growth, but also enhanced cell migration, raising the possibility that FZD7 might be a regulator for cell tumorigenicity in TNBC. To verify this hypothesis, colony formation assay was performed on both FZD7 expressing and FZD7 suppressed MDA-MB-231 cells. It was found that more than twice the number of colonies was observed in GPFshRNA expressing cells as compared to MDA-MB-231/FZD7shRNA cells (Fig. 5). These results indicated that FZD7 is a key factor involved cell colonigenicity in TNBC. Increased colonigenicity in TNBC cells expressing FZD7 lead to accelerated tumor growth in TNBC.

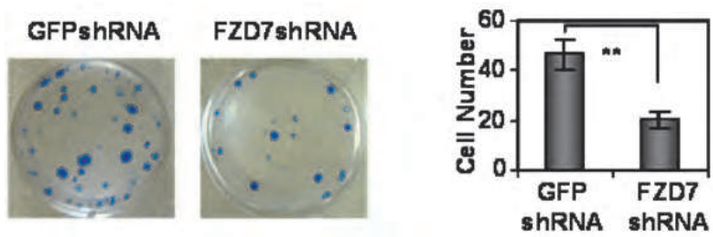

Fig. 5. FZD7 increased cell tumorigenicity in TNBC.

2.3 FZD7 regulated cell transformation through cannonical Wnt signaling pathway

FZD7 is known as the receptor for the Wnt signaling pathway. To investigate if FZD7 affects TNBC tumor cell biology through the canonical Wnt pathway, β-catenin immunofluorescence and DAPI staining was performed to determine the localization of β-catenin thereby confirming the involvement of the Wnt/β-catenin pathway. β-catenin accumulated in the nuclei of MDA-MB-231 cells transduced with GFPshRNA (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 approximately 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 (e.g., 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 to GFPshRNA-expressing control cells (Figure5C). To evaluate whether 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. MDA-MB-231 cells served as

control. Approximately 70% to 80% of transfected cells were GFP-positive, indicating that FZD7 should have been overexpressed. 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).

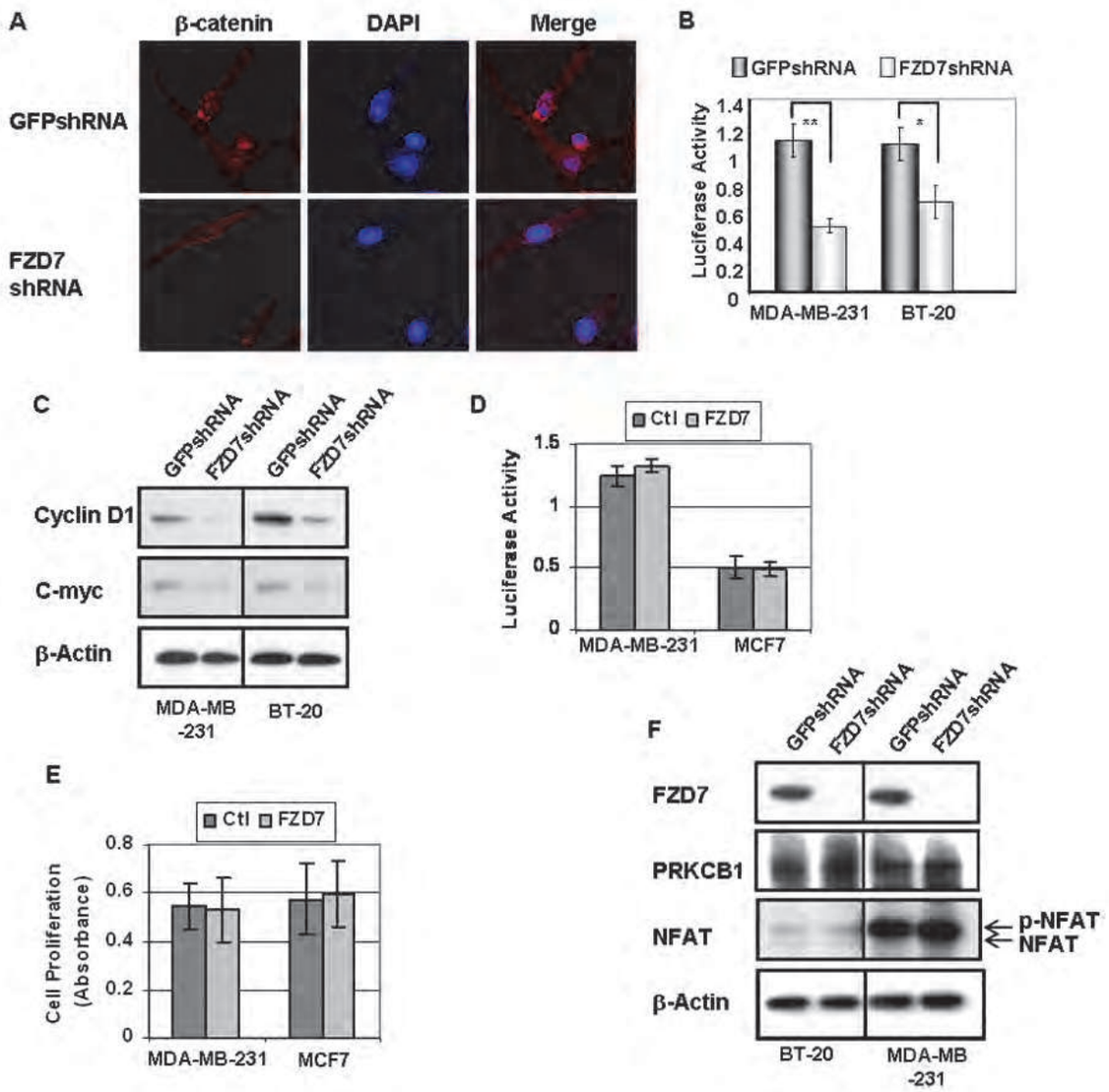

Fig. 6. FZD7 regulated cell transformation through Wnt canonical pathway in TNBC.

In our microarray data, we noticed that two genes, FZD and PKCB1, along the noncanonical Wnt/Ca^{2+} pathway were upregulated in TNBC. In the Wnt/Ca^{2+} pathway, Wnt binds to FZD to initiate signaling that results in the stimulation of protein kinase C (PKC) and the 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 (Figure 7), 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.

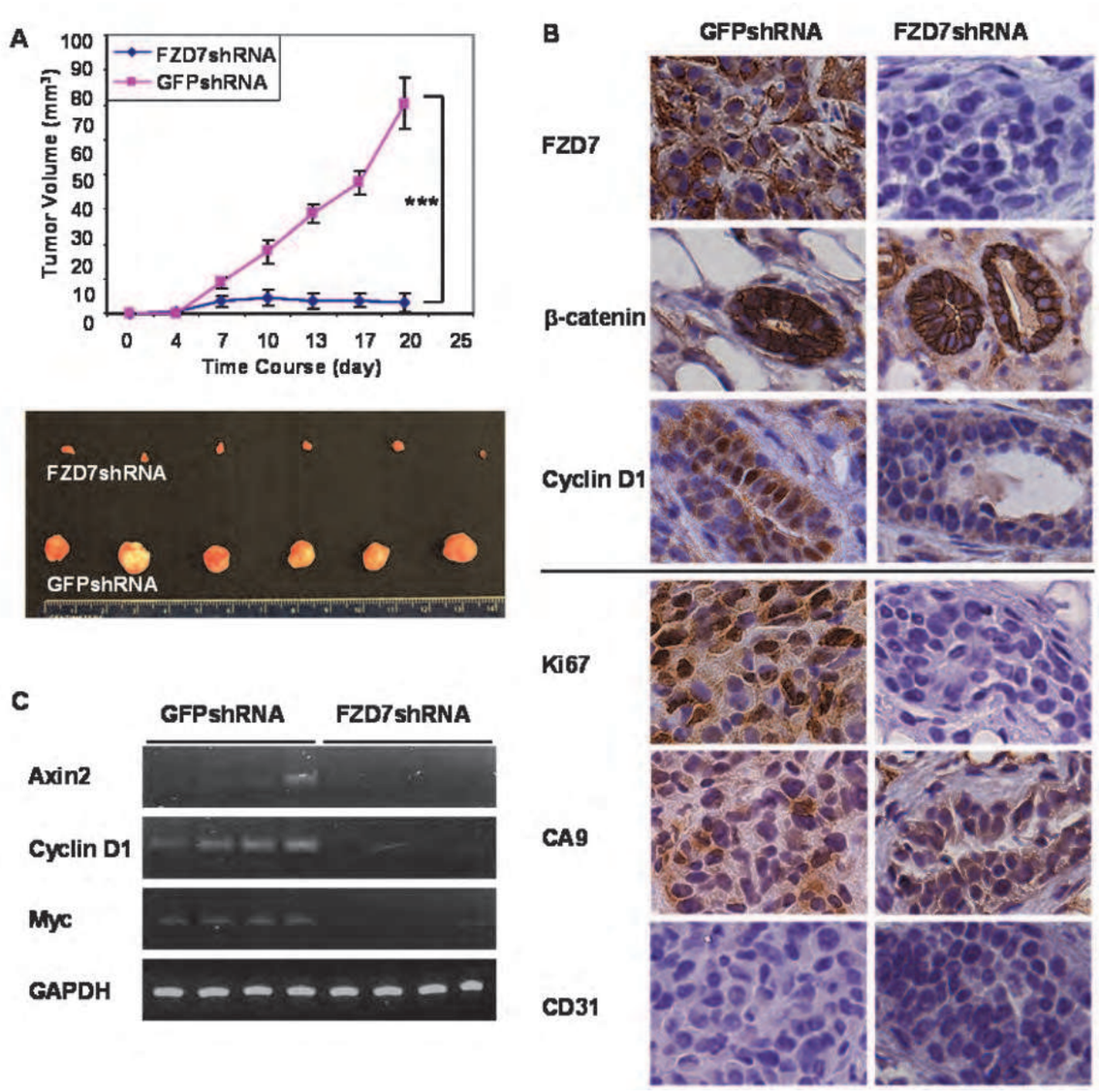

Fig. 7. FZD7 knockdown suppressed tumor growth *in vivo*.

2.4 FZD7shRNA suppresses tumor growth in vivo

To explore whether the Wnt pathway contributes to the tumorigenesis of TNBC in vivo, MDA-MB-231 cells with FZD7shRNA or GFPshRNA were inoculated into NOD-SCID IL2rg female null mice. FZD7shRNA blocked the Wnt signal in MDA-MB-231 cells and induced significant ($p<0.001$) suppression of tumor growth in vivo. Moreover, growth arrest has been observed in MDA-MB-231/FZD7shRNA cell-derived tumors after 1 week of inoculation (Fig. 6A). Gross tissue was harvested and processed with immunohistochemistry (IHC)

staining of Wnt pathway gene FZD7, β-catenin, and Wnt pathway target gene Cyclin D1 (Fig. 6B). In Fig. 5B left panel, the GFPshRNA control group tumor showed normal FZD7 expression, β-catenin locatization in the nucleus, and Cyclin D1 expression. In tumors with FZD7shRNA shown in the right panel, most cells expressed β-catenin in the cytoplasm and did not express Cyclin D1. This result is consistent with the in vitro findings and confirms that FZD7shRNA induces growth retardation via the Wnt canonical signal. Furthermore, the tumors with FZD7 or FZD7 suppression were characterized. Hypoxia cell marker CA9, angiogenesis marker CD31 and cell proliferation marker Ki67 in the cells were stained (Fig. 6B). The data revealed that Ki67 expression was significantly increased in FZD7 expressed tumor but not in FZD7 inhibited tumor. CA9 was weakly expressed in both FZD7 expressed tumor and FZD7 suppressed tumor. CD31 was not detected in either tumor. These findings indicate that the activation of Wnt signal in TNBC induces cell proliferation, but may not directly involve cell hypoxia and angiogenesis.

3. Conclusion

TNBC has been a particular focus of attention because it has no confirmed therapeutic molecular target and poor prognosis. Basal-like breast cancer is categorized as TNBC because almost all basal-like breast cancers have no hormone receptor expression and HER2 overexpression (Perou *et al.*, 2000; Sotiriou *et al.*, 2003). Although aberrant Wnt signaling activation 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 tumor has been observed (DiMeo *et al.*, 2009; Lindvall *et al.*, 2009). More recently, LRP6 overexpression was found in a subtype of breast tumor that is ER-negative and Her2-negative (Liu *et al.*, 2010). This is consistent with our microarray results. In our study, downregulation of FZD7 to inactivate the Wnt signaling in triple negative breast cell line, MDA-MB-231 and BT-20 resulted in impaired cell growth and tumor transformation. The essential role that canonical Wnt signaling plays in TNBC makes it the most attractive therapeutic stratagem in TNBC (Yang *et al.*, 2011). Targeting Wnt pathway genes as novel pharmacological agent for other neoplasms has been investigated and great effect has been observed (Yang *et al.*, 2008). Wnt pathway receptor FZD7 and LRP6 are cell surface antigens. Our data together with other recent findings suggest that with the inhibition of either of these two genes, Wnt signaling pathway will be blocked and Wnt signal-mediated cell proliferation will be suppressed (Bafico *et al.*, 2001). SiRNA or antibodies against these mRNAs or proteins will provide strong blocking of canonical Wnt signaling in TNBC cells. Small molecules that inhibit the biological functions of these two genes may also be powerful drugs for treatment of TNBC. Notably, any agents that increase the phosphorylation activity of CK1 and GSK3 or block β-catenin nuclear accumulation will be 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 the formation of breast tumor (Jones and Kemp, 2008; Lindvall *et al.*, 2007). Mouse model study indicated that breast cancers that arise from stem-progenitor cells undergo transformation through deregulation of the Wnt signal, while epithelial cell derived breast tumors are triggered by oncogenic activation of HER2 (Li and Rosen, 2005). We also noticed that breast cell lines expressing high levels of HER2 usually express low levels of FZD7 (data not shown). The association of Wnt signaling and HER2 in breast tumor development will be further explored.

Wnt signaling pathway is a highly conserved pathway. Three signaling branches have been identified: canonical Wnt pathway (Wnt/β-catenin pathway), non-canonical pathway (including planar cell polarity pathway), and Wnt/Ca^{2+} pathway (Komiya and Habas, 2008). We have observed that two genes, FDZ and PKC, along the Wnt/Ca^{2+} pathway were upregulated in TNBC. Addressing the significance of this branch in TNBC in the future remains important.

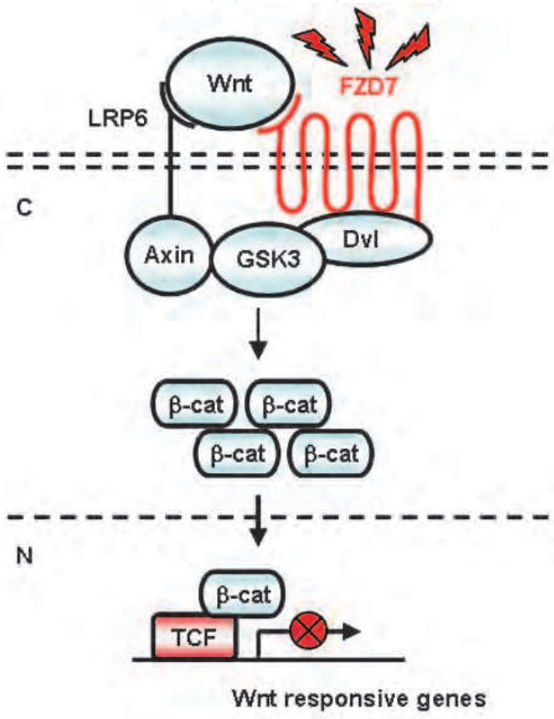

Fig. 8. FZD7 as a target for TNBC.

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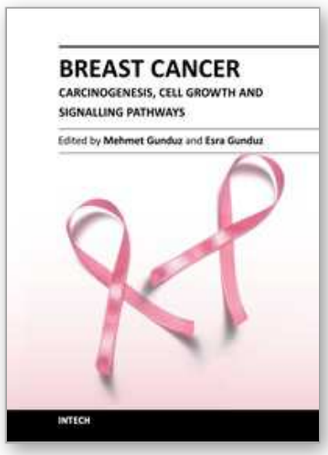

Breast Cancer - Carcinogenesis, Cell Growth and Signalling Pathways

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Cancer is the leading cause of death in most countries and its consequences result in huge economic, social and psychological burden. Breast cancer is the most frequently diagnosed cancer type and the leading cause of cancer death among females. In this book, we discussed various aspects of breast cancer carcinogenesis from clinics to its hormone-based as well as genetic-based etiologies for this deadly cancer. We hope that this book will contribute to the development of novel diagnostic as well as therapeutic approaches.

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