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Article

miR-221/222 activate the Wnt/β-catenin signaling to promote triple-negative breast cancer

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Triple-negative breast cancer (TNBC), characterized by the lack of expression of the estrogen receptor, the progesterone receptor, and the human epidermal growth factor receptor 2, is an aggressive form of cancer that conveys unpredictable and poor prognosis due to limited treatment options and lack of effective targeted therapies. Wnt/β-catenin signaling is hyperactivated in TNBC, which promotes the progression of TNBC. However, the molecular mechanism of Wnt/β-catenin activation in TNBC remains unknown. Here, we report the drastic overexpression of miR-221/222 in all of four TNBC cell lines and TNBC primary tumor samples from patients. Furthermore, we demonstrate by both *ex vivo* and xenograft experiments that inhibiting miR-221/222 expression in a TNBC cell line (MDA-MB-231) suppresses its proliferation, viability, epithelial-to-mesenchymal transition, and migration; whereas expressing miR-221/222 in a non-TNBC line (MCF7) promotes all of the above cancer properties. miR-221/222 achieve so by directly repressing multiple negative regulators of the Wnt/β-catenin signaling pathway, including WIF1, SFRP2, DKK2, and AXIN2, to activate the pathway. Notably, the level of miR-221/222 expression is inversely correlated whereas that of WIF1, DKK2, SFRP2, and AXIN2 expression is positively correlated with the patient survival. Last, we show that anti-miR-221/222 significantly increases apoptotic cells with tamoxifen/Wnt3a treatment but not with cyclophosphamide/Wnt3a treatment. These results demonstrate that miR-221/222 activate the Wnt/β-catenin signaling to promote the aggressiveness and TNBC properties of breast cancers, and thus reveal a new prospect for TNBC treatment.

Keywords: miR-221/222, Wnt/β-catenin, triple-negative breast cancer

Introduction

Breast cancer is the most invasive cancer in women and is the second leading cause of death in women (King et al., 2012). Breast cancer subtypes based on clinical or molecular characteristics are typically referred to as hormone-receptor positive (HR^+, a.k.a. luminal), human epidermal growth factor receptor 2 (HER2)-amplified, and triple-negative breast cancer (TNBC, a.k.a. basal-like). TNBC is characterized by a lack of immunohistochemical expression of estrogen receptors (ER), progesterone receptors (PR), and HER2. It accounts for approximately 15%–20% of breast cancer patients. TNBC does not respond well to standard hormone therapy and is associated with a higher grade, undifferentiated metaplastic histology, stem cell-like characteristics, invasiveness, higher metastatic potential, and inconsistency in effectiveness of therapies. Although considerable progress has been made in breast cancer research, the mortality rate of TNBC has remained unchanged in the last decade primarily due to the lack of specific target (O'Toole et al., 2013).

The Wnt/β-catenin signaling pathway plays a major role in embryonic growth and can lead to tumor formation when aberrantly activated (Polakis, 2012). Under normal circumstances, the free cytosolic pool of β-catenin is rapidly degraded by the action of a multiprotein complex that includes APC, Axin, and the GSK3β kinase. This complex phosphorylates β-catenin on key N-terminal residues and thereby targeting the protein for ubiquitination and proteolysis (Howe and Brown, 2004). Mutations in β-catenin or other Wnt pathway components that result in β-catenin in accumulation are found in a wide range of human cancers. The prime example thus far is in colorectal cancer, in which most of the cases display loss-of function

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mutations in the tumor-suppressor gene APC—an essential component of the APC/Axin/GSK3β complex that degrades β-catenin. Thus APC is a negative regulator of intracellular Wnt signaling (Polakis, 2000; Giles et al., 2003). However, APC mutations have been rarely found in breast cancer (Howe and Brown, 2004; Geyer et al., 2011), which suggests that Wnt/β-catenin activation in breast cancer is caused by other factors. More recently, several studies demonstrated that Wnt/β-catenin signaling is particularly activated in TNBC or in a subgroup of invasive breast cancers of TNBC. It is associated with poor prognosis (Khramtsov et al., 2010; Geyer et al., 2011; King et al., 2012; Dey et al., 2013), such that the Wnt receptor frizzled-7 (FZD7) and the Wnt co-receptor LRP6 were found to be upregulated in TNBC (King et al., 2012).

MicroRNAs (miRNAs) are a class of short single-stranded noncoding RNAs (typically 21 nt) involved in modulating many biological processes by imperfect base-pairing with the 3′ untranslated region (3′UTR) of target mRNAs. miRNA binding mediates mRNA cleavage and/or translational repression (Lewis et al., 2003; Djuranovic et al., 2011), and is known to regulate a variety of genes involved in development, proliferation, and apoptosis (Fabian et al., 2010). Accumulating evidence has demonstrated the function of miRNAs in both physiological and pathological conditions, including cancer (Esquela-Kerscher and Slack, 2006; Liu et al., 2014; Sun et al., 2016; Xue et al., 2016). Bioinformatic data indicate that each miRNA can control hundreds of mRNAs, underscoring the potential influence of miRNAs on almost every genetic pathway. Therefore, to investigate the function and action mechanism of the Wnt/β-catenin pathway in breast cancer, it is important to identify miRNAs that might regulate Wnt/β-catenin signaling to promote cancer progression, especially miRNAs that target multiple regulators of the pathway.

Among many miRNAs identified to regulate neoplastic transformation, invasion and metastasis, miR-221 and miR-222 are two highly homologous miRNAs among the best-studied miRNAs (Volinia et al., 2006; Lupini et al., 2013). miR-221/222 are overexpressed in the majority of epithelial tumors in humans but they have also been reported to suppress erythroleukemia by inhibiting erythropoiesis through the downregulation of c-Kit receptor. Stinson et al. (2011) reported that miR-221 and miR-222 are specifically expressed in basal-like but not luminal breast cancer cell lines. miR-221 and miR-222 decrease the expression of epithelial-specific genes and increase the expression of mesenchymal-specific genes. Despite intensive studies on the oncogenic properties of miR-221/222 (Di Leva et al., 2010; Stinson et al., 2011; Gan et al., 2014; Li et al., 2017), it still remains unknown how they regulate their target signaling pathways to achieve their function. This understanding is crucial to unravel oncogenic processes and to develop novel therapeutic molecules. Here, we report the potent oncogenic effect of miR-221/222 in breast cancer. Furthermore, we demonstrate that miR-221/222 achieve such effect by suppressing the expression of multiple negative regulators of the Wnt/β-catenin pathway, which leads to the activation of the pathway and consequently the promotion of breast cancer progression.

Results

miR-221/222 are markedly upregulated in human TNBC cells and negatively correlated with TNBC patient survival

To assess the role of miR-221/222 in breast cancers, we examined the expression level of miR-221 and miR-222 in four TNBC cell lines (MDA-MB-468, Hs 578T, HCC1937, and MDA-MB-231), two ER-positive lines (MCF7, T47D), one HER2 positive line (SKBR3), and one PR and HER2 positive line (BT-474). In addition, we also examined its expression in a mouse cell line, 4T1, which is derived from spontaneous mammary tumor in a BALB/cfC3H mouse. Both miR-221 and miR-222 were significantly upregulated in all the TNBC lines but downregulated or unchanged in the non-TNBC lines (Figure 1A and B), indicating that the overexpression of miR-221/222 might be specifically correlated to TNBCs. To further test this hypothesis, we examined the expression of miR-221 and miR-222 in 20 pairs of normal and breast cancer samples from 20 patients by quantitative RT-PCR. miR-221 and miR-222 were overexpressed in 9 out of 20 breast cancer samples, including all six TNBC samples. These results support that the miR-221/222 overexpression is specifically correlated with TNBC (Supplementary Figure S1).

To further assess the role of miR-221/222 in breast cancer, we conducted a Kaplan–Meier survival analysis to examine the correlation between miR-221 and miR-222 levels and prognosis of breast cancer patients, based on clinical data from bioinformatics websites (www.kmplot.com) (Gyorffy et al., 2010). When patient samples were stratified by a median cutoff of miR-221 or miR-222 expression (Figure 1C and D), patients with higher (i.e. >median) miR-222 expression were found to have shorter overall survival (OS) than those with lower (≤median) levels of miR-222 (Figure 1D). However, the expression level of miR-221 was not associated with overall survival (Figure 1C). When analyzed with TNBC samples, it was found that both miR-221 and miR-222 were negatively correlated with survival rate (Figure 1C and D). Therefore, miR-221/222 might play an important role in maintaining the characteristics of TNBCs.

miR-221 and miR-222 promote the proliferation, viability, epithelial-to-mesenchymal transition, and migration of breast cancer cells

To investigate the function of miR-221/222 in breast cancer cells, we overexpressed miR-221/222 by transfecting mature miR-221 and/or miR-222 sequences (hereafter called miR-221/222 mimics) to ER-positive MCF7 cells to examine whether this will transform MCF7 into a more aggressive TNBC-like cell line. In addition, we inhibited the expression miR-221/222 in the triple-negative MDA-MB-231 cells by transfecting antisense RNA against miR-221/222 (hereafter called miR-221/222 inhibitors) to examine whether this will cause MDA-MB-231 cells to lose aggressive TNBC characteristics. We first examined the cell proliferation and viability of the treated cells by growth curve assay (Figure 2A), colony formation assay (Figure 2B), and flow cytometry analysis using Annexin V and 7AAD as early and late apoptotic markers, respectively (Figure 2C). Overexpression of miR-221/222 significantly promoted the proliferation and viability of MCF7

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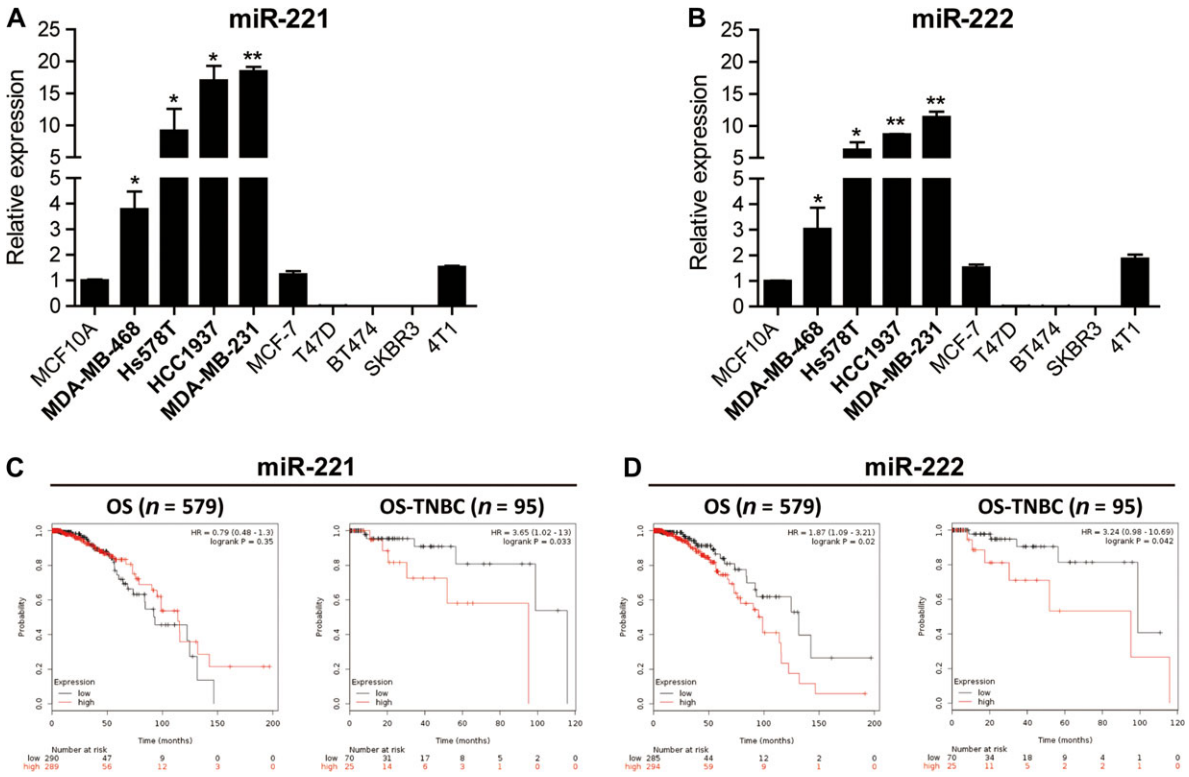

Figure 1 miR-221 and miR-222 are markedly overexpressed in TNBCs. (**A** and **B**) The expression of miR-221 (**A**) and miR-222 (**B**) in nine breast cancer cell lines and a noncancerous breast cell line MCF-10A. The bold font represents TNBC cell lines. Error bars in **A** and **B** represent SEM. *$P < 0.05$, **$P < 0.01$. (**C** and **D**) Clinical OS and OS of TNBC samples of breast cancer patients with different levels of miR-221 (**C**) and miR-222 (**D**). OS, overall survival; OS-TNBC, overall survival of TNBC.

cells; whereas miR-221/222 inhibitor treatment significantly inhibited the proliferation and viability of MDA-MB-231 cells (Figure 2A–C). These results indicate that miR-221 and miR-222 are potent promoters of proliferation and survival of breast cancer cells.

We then examined whether miR-221/222 also have a role in the migration of cancer cells by conducting transwell migration and wound healing assays on both MCF7 and MDA-MB-231 cells. Overexpression of miR-221/222 significantly promoted the migration ability of MCF7; whereas miR-221/222 inhibitors compromised the migratory ability of MDA-MB-231 cells (Figure 2D and E). These observations indicate that miR-221/222 also promote the migratory ability of the cancer cells.

The divergent function of miR-221/222 on proliferation, viability, and migratory ability of cancer cells points to the possibility that these miRNAs might have a general role in promoting the fundamental properties of breast cancer, such as epithelial-to-mesenchymal transition (EMT). To investigate this possibility, we examined the levels of E-cadherin, a key epithelial marker, and N-cadherin, a key mesenchymal marker, in both MCF7 and MDA-MB-231 cells when miR-221/222 expression was altered. Western blotting analysis revealed that overexpression of miR-221/222 in MCF7 cells significantly reduced E-cadherin expression but increased N-cadherin expression (Figure 2F). In contrast, inhibition of miR-221/222 in MDA-MB-231 cells increased E-cadherin expression but inhibited N-cadherin expression (Figure 2F). These data indicate that miR-221/222 have a general impact in promoting the mesenchymal fate of breast cancer cells, which is a key feature of TNBC.

To further establish the *in vivo* relevance of the above findings, we transplanted miR-221/222 antagomirs-transfected MDA-MB-231 cells into BALB/c nude mice, using MDA-MB-231 cells transfected with antagomirs against randomized sequences as a negative control. The tumor size was measured twice every week. After 25 days, the mice were euthanized and the tumors were dissected. In mice injected with the antagomir-transfected MDA-MB-231 cells, tumor growth was significantly suppressed throughout the 25 days of observation, with the difference in tumor size between the experimental and the control mice consistently increasing (Figure 2G–I). These data confirm that miR-221/222 promote proliferation, viability, and migration of TNBC cells.

miR-221 and miR-222 activate Wnt/β-catenin expression in breast cancer cells

To further investigate the molecular mechanism of miR-221/222 regulation in breast cancer, we conducted a pathway enrichment analysis of the 3091 targets of miR-221/222 predicted by the TargetScan program (Lewis et al., 2003). Multiple signaling pathways, including the Wnt pathway, are enriched among the miR-221/222 targets (Figure 3A). These results were confirmed by

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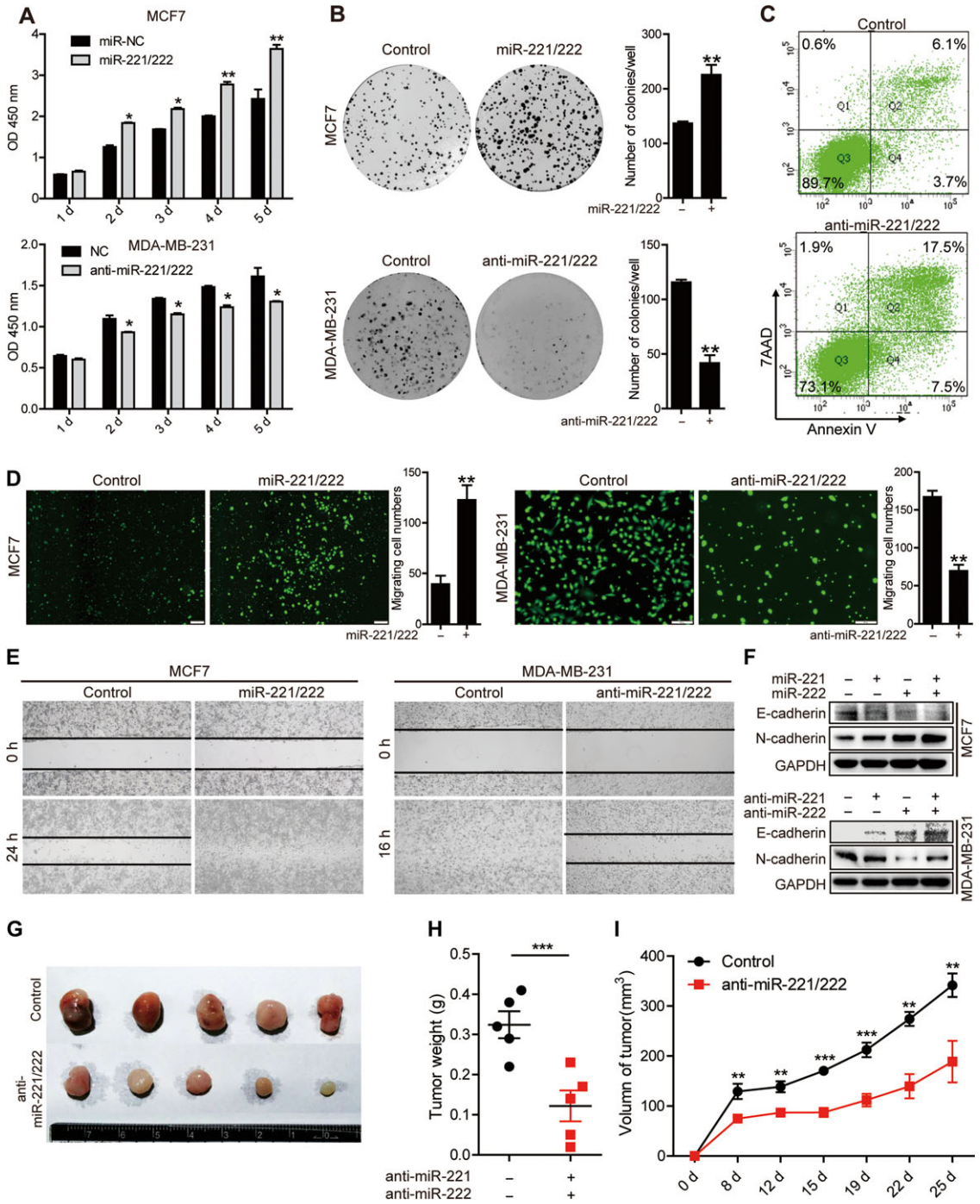

Figure 2 miR-221 and miR-222 promote proliferation and EMT of breast cancer cell lines. (**A**) Growth curve of MCF7 (upper) and MDA-MB-231 (lower) cells transfected with miR-221/222 mimics (100 nM) and inhibitors (200 nM), respectively. (**B**) Colony formation assay of MCF7 (upper) and MDA-MB-231 (lower) cells transfected with miR-221/222 mimics (100 nM) and inhibitors (200 nM), respectively. Three independent biological replicates were conducted. (**C**) Flow cytometry analysis of cell apoptosis in MDA-MB-231 cells using Annexin V and 7AAD. (**D**) Transwell assay of MCF7 (left) and MDA-MB-231 (right) cells transfected with miR-221/222 mimics (100 nM) and inhibitors (200 nM), respectively. Scale bar, 1 mm for MCF7 and 100 μm for MDA-MB-231. (**E**) The wound healing results of MCF7 (left) and MDA-MB-231 (right) cells transfected with miR-221/222 mimics (100 nM) and inhibitors (200 nM), respectively. Original magnification, 100×. (**F**) Expression of epithelial cell marker (E-cadherin) and mesenchymal cell marker (N-cadherin) of MCF7 (upper) and MDA-MB-231 (lower) cells as examined by western blot analysis. GAPDH was used as a loading control. (**G**) Tumor growth in mice injected with MDA-MB-231 cells transfected with miR-NC or miR-221/222 antagomirs. (**H** and **I**) Tumor weight (**H**) and volume (**I**) in mice injected with MDA-MB-231 cells that were transfected with miR-NC or miR-221/222 antagomirs. Error bars in **A**, **B**, **D**, **H**, and **I** represent SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

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another bioinformatics tool, miRanda (Enright et al., 2003). Particularly, miR-221 and miR-222 are the only miRNAs that target multiple suppressors of the Wnt/β-catenin pathway (Figure 3B). These results indicated that the Wnt/β-catenin pathway might be regulated by miR-221/222. Considering the critical role of Wnt/β-catenin pathway in tumorigenesis and metastasis, we selected Wnt/β-catenin pathway for the further investigation.

Subcellular fractionation assay showed that the overexpression of miR-221 or/and miR-222 in MCF7 cells resulted in substantial nuclear accumulation of β-catenin in these cells, indicating that miR-221/222 might contribute to the activation of Wnt/β-catenin signaling (Figure 3C and E). Furthermore, miR-221 or/and miR-222 overexpression in MCF7 cells also markedly increased the transactivating activity of β-catenin, as determined by β-catenin reporter assay (Figure 3F). Conversely, transfection of miR-221 or/and miR-222 inhibitors to MDA-MB-231 and HCC-1937 cells reduced the nuclear translocation of β-catenin (Figure 3D and E) and transactivating activity of β-catenin with or without Wnt3a stimulation in these two types of cells (Figure 3G and Supplementary Figure S2). Accordingly, decreased level of β-catenin was detected in xenograft tumors from cells treated with miR-221/222 antagomirs (Supplementary Figure S3). Consistently, miR-221/222 mimics and inhibitors influenced the expression of Wnt/β-catenin target genes in MCF7 and MDA-MB-231 cells (Figure 3H–I). Notably, treatment with miR-222 or miR-221/222 mimics or inhibitor was more effective than miR-221 in the regulation of Wnt/β-catenin signaling in both MCF7 and MDA-MB-231 cells (Figure 3F and G), indicating that miR-222 may play a more important role than miR-221 in TNBC.

In addition, we examined whether the expression of miR-221 and miR-222 is regulated by Wnt/β-catenin signaling. First, Wnt3a stimulation upregulated miR-221/222 expression in MDA-MB-231 cells (Supplementary Figure S4A). Second, Wnt/β-catenin signaling inhibitor XAV-939 or depletion of β-catenin using short hairpin RNA decreased miR-221/222 expression (Supplementary Figure S4B–D). These results suggesting that there may be a positive feedback loop between Wnt/β-catenin signaling and miR-221/222, which exacerbates tumorigenesis.

miR-221/222 repress multiple suppressors of β-catenin signaling

Considering that the predicted miR-221/222 target mRNAs regulate the Wnt/β-catenin signaling pathway at different levels, several representative target mRNAs, WIF1, DKK2, SFRP2, AXIN2, BTRC, NLK, and CHD8, were selected for validation (Figure 4A). Western blotting analysis revealed that the treatment of miR-221/222 mimics or inhibitors did not change the levels of BTRC, NLK, and CHD8 proteins in MCF7 or MDA-MB-231 cells (data not shown), but increased that of WIF1, DKK2, SFRP2, and AXIN2 in MDA-MB-231 cells (Figure 4B). In addition, overexpression of miR-221 and/or miR-222 reduced the levels of WIF1, DKK2, SFRP2, and AXIN2 mRNAs as well as proteins in MCF7 cells (Figure 4B and Supplementary Figure S5; the mRNA levels in MDA-MB-231 cells were too low to be detected). Furthermore, reporter assays demonstrated that the activity of luciferase with the 3′UTR of WIF1, DKK2, SFRP2, or AXIN2 was repressed in MCF7 cells transfected with miR-221 and/or miR-222 mimics (Figure 4D); whereas mutations in the seeds sequence of miR-221 or miR-222 (Figure 4C) abolished the suppressive effects (Figure 4D). Conversely, inhibition of miR-221 and/or miR-222 significantly increased luciferase reporter activities of WIF1, DKK2, SFRP2, or AXIN2 in MDA-MB-231 cells (Figure 4E). Finally, treatment with miR-222 mimics or inhibitors more strongly affected target gene expression than miR-221 mimics or inhibitors, indicating that miR-222 might have a stronger role in regulating the Wnt/β-catenin pathway (Figure 4B, D, and E).

To assess the clinical relevance of the above regulations, we used a public database (Simonyan and Mazumder, 2014) to compare the expression of WIF1, DKK2, SFRP2, and AXIN2 in 18 different kinds of human cancers. WIF1 and AXIN2 are downregulated in more than 90% of breast cancer patients, DKK2 in 50% of patients, and SFRP2 in only 27% of the patients (Supplementary Figure S6). Based on mRNA levels of a public database (Anaya, 2016), there is also a weak negative correlation between miR-221/222 expression and that of WIF1, DKK2, SFRP2, or AXIN2, but not CTNNB1 (Supplementary Figure S7). Taken together, these data indicate that miR-221/222 promote oncogenes in breast cancer cell lines by partially directly suppressing WIF1, DKK2, SFRP2, and AXIN2 expression.

miR-221/222 repression of multiple suppressors of β-catenin signaling promotes oncogenesis

To test the hypothesis that miR-221/222 directly suppress WIF1, DKK2, SFRP2, and AXIN2 expression in breast cancer cell lines to promote oncogenesis, we expressed the WIF1, DKK2, SFRP2, or AXIN2 mRNAs lacking 3′UTR in miR-221/222 transduced MCF7 cells (Figure 5A–C). As expected, the 3′UTR-truncated mRNAs no longer subject to miR-221/222 suppression and the resulting MCF7 cells loss the miR-221/222-induced robust ability in colony formation and cell migration (Figure 5A–C). In addition, depletion of WIF1, DKK2, SFRP2, or AXIN2 dramatically restored the cell colony formation and migration ability in miR-221/222-inhibited MDA-MB-231 cells (Figure 5A, B, and D). These results confirm that miR-221/222 regulate the expression WIF1, DKK2, SFRP2, and AXIN2 to promote the proliferation and migration of the breast cancer cell lines.

WIF1, DKK2, SFRP2, and AXIN2 expression is negatively correlated with patient survival in breast cancer

In order to analyze the association of WIF1, DKK2, SFRP2, and AXIN2 with breast cancer progression, we conducted a Kaplan–Meier survival analysis to examine the correlation between WIF1, DKK2, SFRP2, and AXIN2 levels and prognosis of breast cancer patients, based on clinical data from bioinformatics websites (www.kmplot.com) (Gyorffy et al., 2010), using the parameters of overall survival (OS) and relapse-free survival (RFS). This analysis included 153–3554 samples for each of WIF1, DKK2, SFRP2, and AXIN2 analyses. The results showed that patients with lower WIF1, DKK2, SFRP2, and AXIN2

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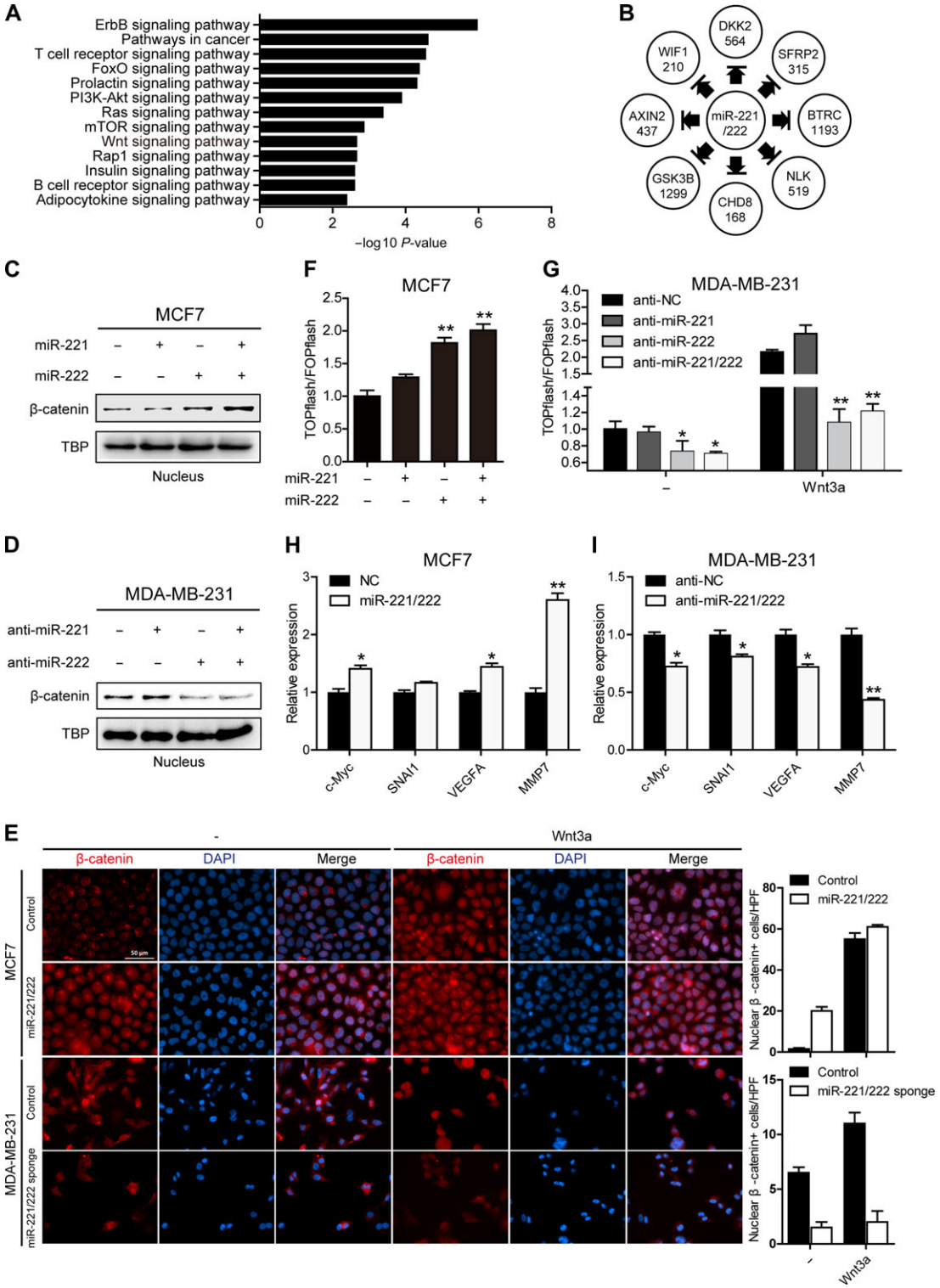

Figure 3 miR-221 and miR-222 activate Wnt/β-catenin signaling. (**A**) Significantly enriched pathways ($P < 0.01$) among the 3091 predicted targets of miR-221/222. (**B**) Predicted targets of miR-221/222 are negative regulators of Wnt signaling. The number under each gene name represents the number of predicted miRNAs that regulate its expression (www.targetscan.org; www.microrna.org). (**C**) The expression of β-catenin in the nucleus of MCF7 cells transfected with 100 nM miR-NC, miR-221, miR-222, or miR-221/222 mimics, as indicated by subcellular fractionation and western blotting analysis, in which TBP was nuclear marker. (**D**) The expression of β-catenin in the nucleus of MDA-MB-231 cells transfected with 200 nM miR-NC, miR-221, miR-222, or miR-221/222 inhibitors. (**E**) Subcellular β-catenin localization in MCF7 and MDA-MB-231 cells as assessed by immunofluorescence staining. Scale bar, 50 μm. (**F**) The effect of increasing miR-221/222

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expression had shorter RFS and overall survival than those expressing higher levels of WIF1, DKK2, SFRP2, and AXIN2, especially for the TNBC patients except for WIF1 (Figure 6A–D). These analyses further validate the importance of the miR-221/222 regulation of Wnt/β-catenin pathway in promoting breast cancer.

miR-221/222 knockdown abolishes the antagonistic effect of Wnt3a towards tamoxifen treatment of breast cancer cells

Given the lack of a suitable treatment option for patients with TNBC, there is an urgent need to identify new therapeutic targets to improve the outlook for these patients. The previous study has shown that miR-221/222 negatively regulates estrogen receptor α (ERα) expression and is associated with tamoxifen (TAM) resistance in breast cancer (Zhao et al., 2008). And TNBC also lacks the expression of ERα. We speculate that if the expression of ER is restored, TAM will be effective for TNBC treatment. Our FACS analysis revealed that anti-miR-221/222 or TAM treatment slightly promoted apoptosis in MDA-MB-231 cells with Wnt3a (Figure 7A and B). Remarkably, when miR-221/222 inhibitors were added to TAM/Wnt3a-treated cells, the number of apoptotic cells was significantly increased (Figure 7A and B). Cyclophosphamide (CTX) is a commonly used drug in breast cancer treatment (von Minckwitz, 2007; Fischer et al., 2015). Recently, it was reported that the expression of β-catenin is significantly suppressed by the CTX-helvolic acid combination treatment in murine sarcoma S180 tumor-bearing mice (Xiao et al., 2017). However, CTX can only play an anti-tumor function in the body, and it does not work in the cells *in vitro*. Therefore, we selected CTX as a negative control for MDA-MB-231 cell treatment, we used the same strategy for CTX treatment in MDA-MB-231 cells. The results showed that CTX had no effect on MDA-MB-231 cells (Figure 7C and D). These results indicate that miR-221/222 inhibitors alone, especially combined with TAM might be effective agents for the treatment of TNBC.

Discussion

Recent studies indicate that β-catenin accumulation is more frequently observed in TNBC than other breast cancer subtypes, suggesting that activation of the Wnt/β-catenin pathway might be an early event in TNBC development (Khramtsov et al., 2010) and might provide as novel therapeutic targets for the treatment of TNBC. Unlike in other cancer types, the mutations in Wnt/β-catenin signaling are rare in breast cancer (Howe and Brown, 2004). Therefore, the molecular mechanism of Wnt/β-catenin signaling activation in TNBC remains elusive. In this study, we report that miR-221 and miR-222 are preferentially overexpressed in both TNBC cell lines and clinical samples to activate Wnt/β-catenin signaling pathway by inhibiting WIF1, DKK2, SFRP2, and AXIN2, thereby promoting the development of TNBC. Thus, our current study provides new insights into this area of research by identifying miR-221/222 as a clinically relevant promoter of TNBC.

Our data show that downregulation of WIF1, DKK2, SFRP2, and AXIN2 mediates the effects of miR-221/222 on β-catenin activation. All of these four molecules have been shown to play important roles in the modulation of β-catenin signaling in cancers. WIF1 has been shown to be downregulated in various human cancers, including breast cancer, and has been regarded as a tumor suppressor (Wissmann et al., 2003). Furthermore, Trifa et al. (2013) have reported that the loss of WIF1 expression is related to aggressiveness of sporadic breast cancer. Rubin et al. (2010) demonstrated that loss of WIF1 could trigger Wnt/β-catenin signaling and thereby contributes to tumor invasion and metastasis. More recently, two studies have identified DKK2 as a target gene for miR-221/222 in glioma and esophageal cancers (Li et al., 2013; Wang et al., 2016a). Hassan et al. (2012) showed that miR-218 enhances Wnt activity in metastatic breast cancer cells by downregulating DKK2 and SFRP2, which contribute to homing and growth of cells metastatic to bone. AXIN2, an important regulator in Wnt/β-catenin pathway, which was involved in cell proliferation, migration, apoptosis, and other important functions in many kinds of human cancers (Li et al., 2015). AXIN2 and SOX7 might also play important roles as co-regulators through the Wnt/β-catenin pathway in the breast tissue to affect the carcinogenesis process (Liu et al., 2016). A study by Yook et al. (2006) has elegantly demonstrated that Wnt/β-catenin signaling engages tumor cell dedifferentiation and tissue-invasive activity through an AXIN2-dependent manner in human breast cancer. Bao et al. (2012) showed that the small molecule tankyrase inhibitor, XAV939, increases AXIN1 and AXIN2 protein levels and attenuates Wnt-induced cell migration and growth in MDA-MB-231 cells. In addition, it has been reported that AXIN2 is a target gene of Wnt/β-catenin signaling, and form a negative feedback loop with Wnt in colorectal cancer and liver cancer (Lustig et al., 2002). Our study suggests that this negative feedback loop may be disrupted in TNBC due to the high expression of miR-221/222. A recent study also demonstrated that miR-221 promotes pulmonary artery smooth muscle cells proliferation by targeting AXIN2 (Nie et al., 2017), which is consistent with our results. The expression of WIF1, DKK2, SFRP2, and AXIN2, in addition to miR-221/222 regulation, is subjected to epigenetic regulation (Suzuki et al., 2008; Veeck et al., 2008; Luo et al., 2016). This indicates that there are different

expression on Wnt signaling in MCF7 cells. MCF7 cells transfected with TOPflash or FOPflash and Renilla pRL-TK plasmids were subjected to dual-luciferase assays 48 h after transfection. Reporter activity was normalized to the Renilla luciferase activity. (**G**) The effect of reducing miR-221/222 expression on Wnt signaling in MDA-MB-231 cells. The cells were transfected with TOPflash or FOPflash and Renilla pRL-TK plasmids. Wnt3a (100 ng/ml) was added 24 h later, and cells were subjected to dual-luciferase assays 48 h after transfection. (**H** and **I**) The mRNA levels of target genes of the Wnt/β-catenin signaling in MCF7 cells treated with 100 nM miR-221/222 mimics for 48 h (**H**) or MDA-MB-231 cells treated with 200 nM miR-221/222 inhibitors for 48 h (**I**). Error bars in **F**–**I** represent SEM. $*P < 0.05$, $**P < 0.01$.

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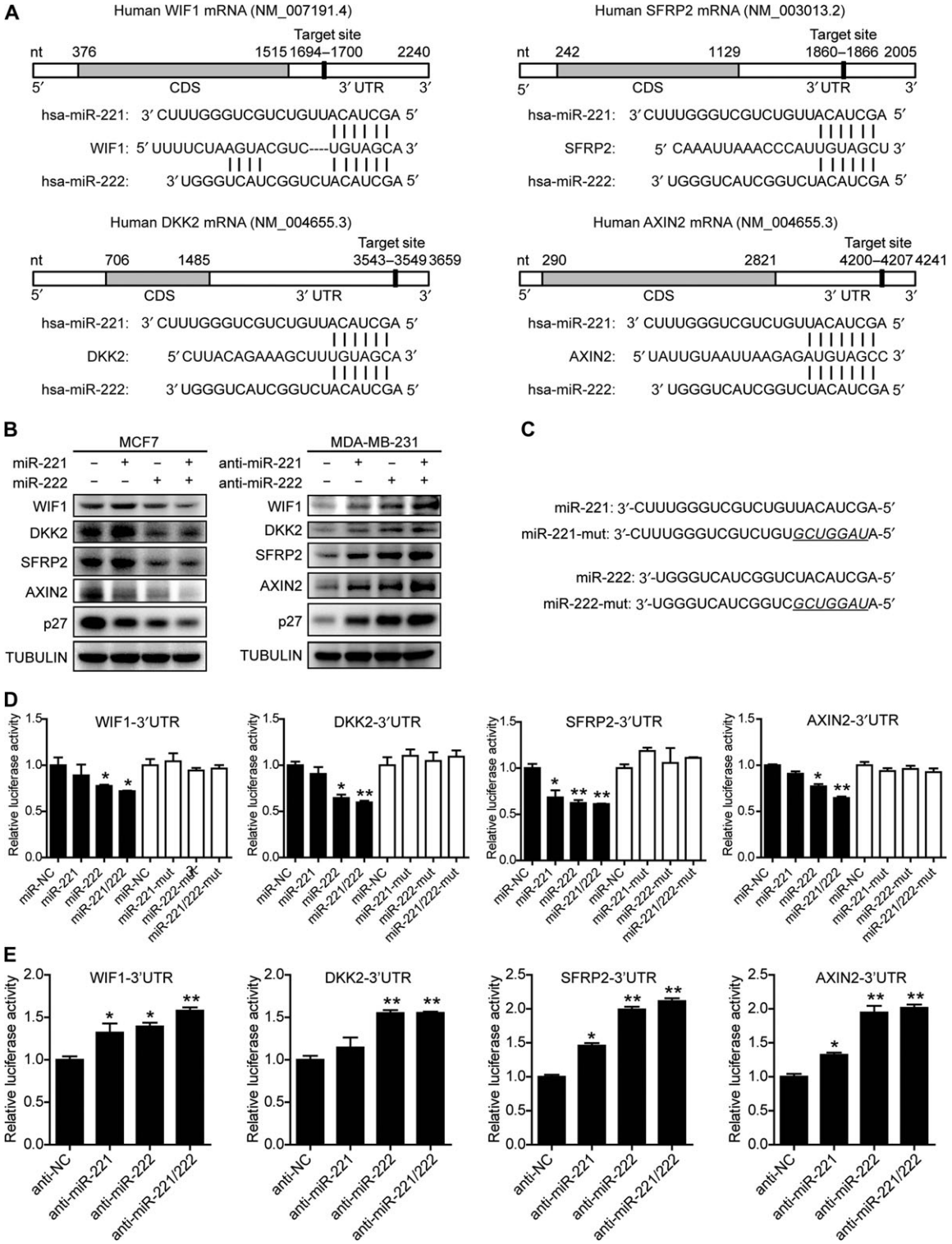

Figure 4 WIF1, DKK2, SFRP2, and AXIN2 are direct targets of miR-221/222. (**A**) Schematic putative miR-221/222 target sites in 3′UTRs of WIF1, DKK2, SFRP2, and AXIN2. (**B**) Western blotting analysis of the protein levels of WIF1, DKK2, SFRP2, and AXIN2 in response to deregulated miR-221/222 expression of the indicated cells. (**C**) Sequence of miR-221-mut and miR-222-mut. The underlined italics indicate the seed sequence mutation. (**D** and **E**) Luciferase assay of 100 ng psi-CHECK2-WIF1-3′UTR, psi-CHECK2-AXIN2-3′UTR, psi-CHECK2-DKK2-3′UTR, and psi-CHECK2-SFRP2-3′UTR reporters in MCF7 cotransfected with 100 nM miR-221, miR-222, miR-221/222 mimics, or mutant oligonucleotides (**D**), or in MDA-MB-231 cotransfected with 200 nM miR-221, miR-222, or miR-221/222 inhibitors (**E**). Error bars in **D** and **E** represent SEM. $*P < 0.05$, $**P < 0.01$.

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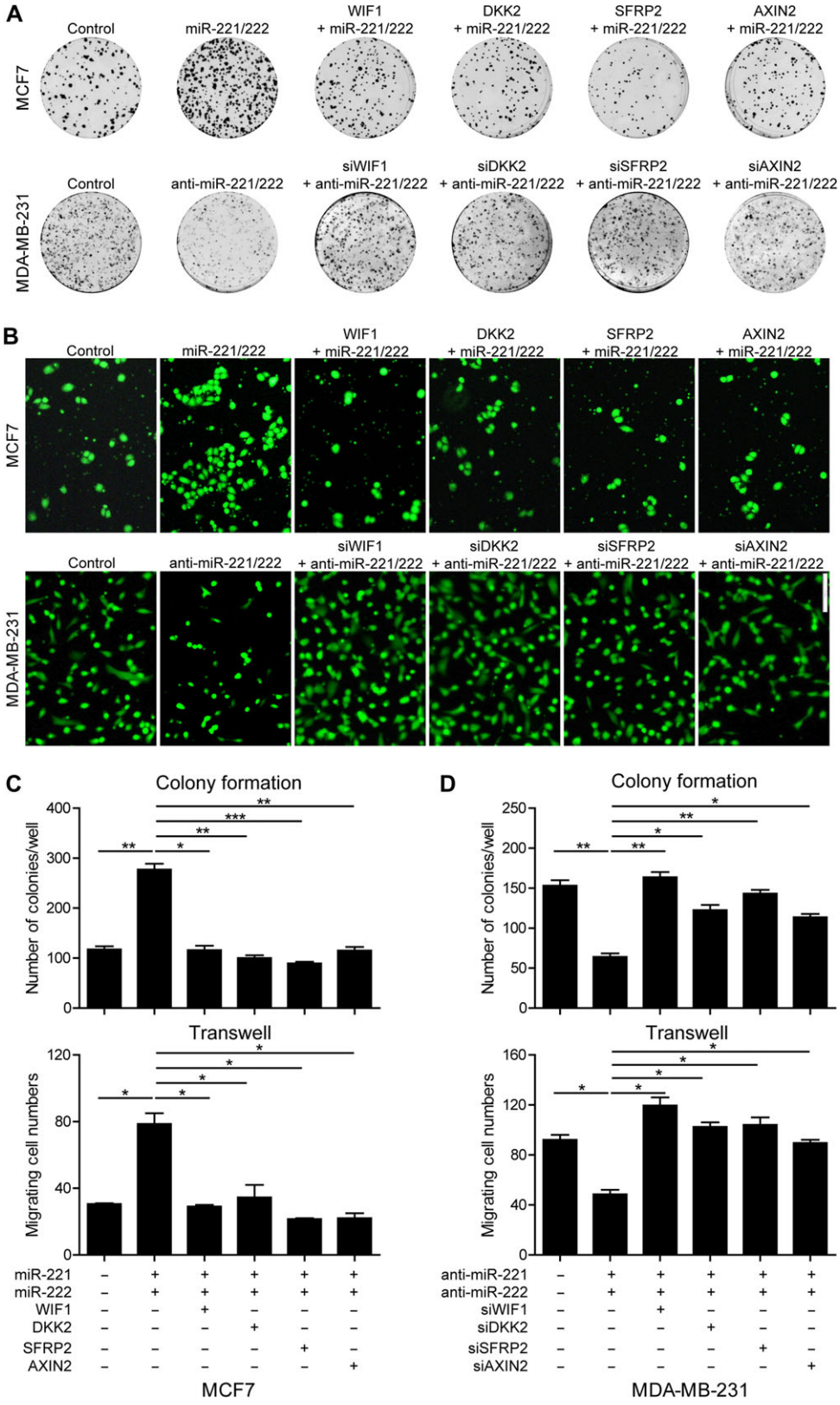

Figure 5 WIF1, DKK2, SFRP2, and AXIN2 mediate miR-221/222-induced migration of breast cancer cells. (**A**) Colony formation assay of MCF7 or MDA-MB-231 cells transfected with the indicated mRNAs with plasmids carrying the indicated genes lacking the 3′UTR-encoding sequences. (**B**) Transwell assay of MCF7 or MDA-MB-231 cells with the same transfection as in **A**. The transfected cells were divided into 24-well plates after 24 h of transfection. Transwell assay was conducted after 20 h for MCF-7 and after 14 h for MDA-MB-231, respectively. (**C** and **D**) Quantification of colonies (upper) and migrating cells (lower) of MCF7 (**C**) and MDA-MB-231 (**D**). Error bars in **C** and **D** represent SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Scale bar, 100 μm.

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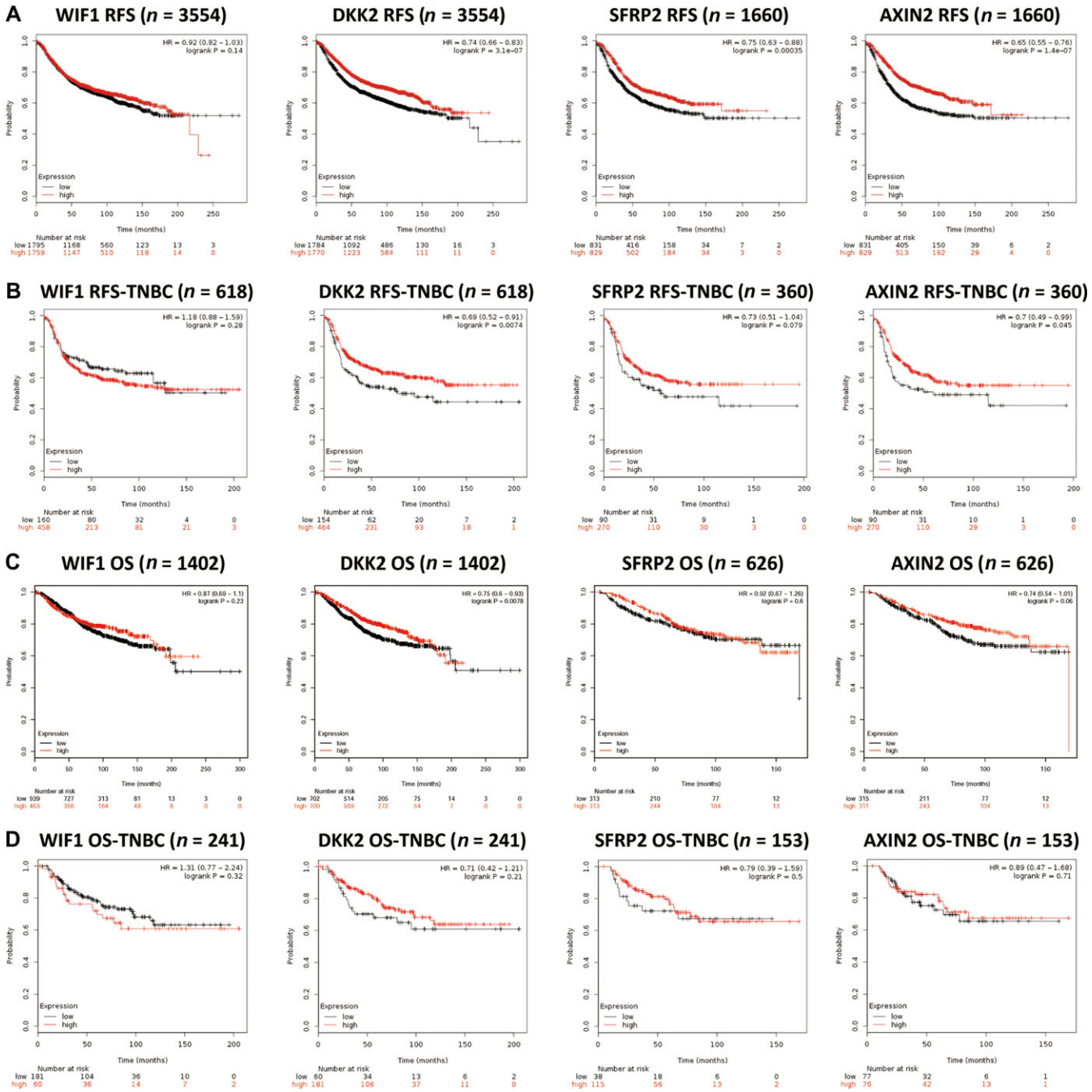

Figure 6 Correlation analysis of patient overall survival vs. expression of WIF1, AXIN2, DKK2, and SFRP2 in breast cancer. Clinical RFS (**A**), RFS of TNBC samples (**B**), OS (**C**), and OS of TNBC samples (**D**) of breast cancer patients with different levels of WIF1, DKK2, SFRP2, and AXIN2. RFS, relapse-free survival; RFS-TNBC, relapse-free survival of TNBC; OS, overall survival; OS-TNBC, overall survival of TNBC.

ways to silence tumor-suppressor genes in tumor cells. Our finding demonstrated that upregulated miR-221/222 in TNBC may confer simultaneous suppression of multiple inhibitors of Wnt/β-catenin signaling, supporting the notion that miRNA represents a potent activator of the pathway. It remains to be clarified whether miR-221/222 also regulate other signaling pathways to promote oncogenesis.

When TNBC occurs, there are a few treatment options in this setting. Considering that ER is a target gene of miR-221/222 (Yoshimoto et al., 2011), we speculate whether restoration of ER expression by miR-221/222 inhibitors will increase the efficacy of tamoxifen treatment in TNBC. Consistent with several studies showing that miR-221/222 confers tamoxifen or fulvestrant resistance by targeting p27Kip1, TIMP3, or activating

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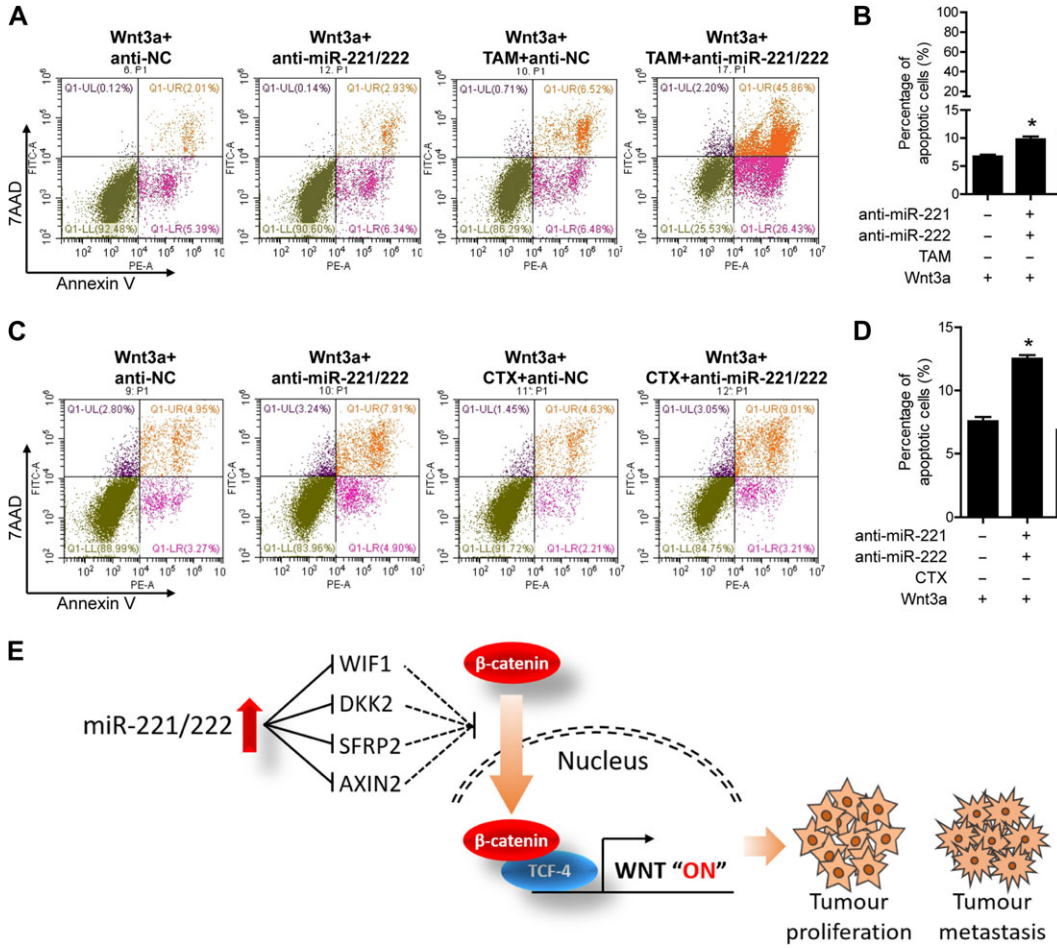

Figure 7 miR-221/222 inhibitors abolish the Wnt3a-induced resistance to apoptosis due to tamoxifen treatment. (**A**) Flow cytometry analysis of cell apoptosis using Annexin V and 7AAD with the indicated treatment. NC or miR-221/222 inhibitors (200 nM) were transfected into MDA-MB-231 cells. After 24 h, these cells were treated with Wnt3a (100 ng/ml) with or without tamoxifen (100 μM) for 24 h, and then analyzed for apoptosis by flow cytometry. (**B**) Quantitative results of **A**. Error bars represent SEM. *$P < 0.05$, **$P < 0.01$. (**C**) Flow cytometry analysis of cell apoptosis using Annexin V and 7AAD with the indicated treatment. NC or miR-221/222 inhibitors (200 nM) were transfected into MDA-MB-231 cells. After 24 h, these cells were treated with Wnt3a (100 ng/ml) with or without CTX (200 μg/ml) for 24 h, and then analyzed for apoptosis by flow cytometry. (**D**) Quantitative results of **C**. Error bars represent SEM. *$P < 0.05$. (**E**) Working model of miR-221/222 regulation of Wnt/β-catenin signaling in promoting breast cancer.

Wnt/β-catenin signaling in ER-positive breast cancer cells (Miller et al., 2008; Rao et al., 2011; Gan et al., 2014), our study demonstrates that Wnt3a counteracts by tamoxifen-induced apoptosis but adding miR-221/222 inhibitors can abolish the Wnt3a effect. These results suggest that targeting either miR-221/222 or Wnt/β-catenin signaling is crucial in overcoming the resistance of TNBC to tamoxifen treatment.

Materials and methods

Cell culture and clinical samples

MDA-MB-231 and MCF7 cells were cultured according to a previously described standard method (Wang et al., 2016b). MCF10A, MDA-MB-468, Hs578T, HCC1937, T47D, BT474, SKBR3, 4T1 cells were cultured according to the culture methods of ATCC. Twenty pairs of clinical samples were purchased from the tissue bank of the Institute of Health Sciences, Chinese Academy of Sciences. The study was approved by the University Research Ethics Committee.

miR-221/222 knockdown and overexpression

A hundred nanomolar miR-221 (5′-AGCUACAUUGUCUGCUGGGUUUC-3′, Ribobio) and/or miR-222 mimics (5′-AGCUACAUCUGGCUACUGGGU-3′, Ribobio) was transfected to MCF7 cells for miR-221 and/or miR-222 overexpression. Two hundred nanomolar miR-221 (5′-GAAACCCAGCAGACAAUGUAGCU-3′, Ribobio) and/or miR-222 inhibitor (5′-ACCCAGUAGCCAGAUGUAGCU-3′, Ribobio) or antagomir (Ribobio) (for xenograft) was transfected to MDA-MB-231 cells for miR-221 and/or miR-222 knockdown. In general, the follow-up analyses were performed after 48 h transfection.

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Water-soluble tetrazolium-1 (WST-1) assay and colony formation

For WST-1 assay, MCF7 or MDA-MB-231 cells were seeded at a density of 1×10^3 per well in 96-well plates, triplicate wells were seeded. A450 was measured at 1, 2, 3, 4, and 5 days after seeding using a WST-1 Cell Proliferation and Cytotoxicity Assay Kit (C0036, Beyotime) according to the manufacturer's protocol. For colony formation assay, a total of 1000 MCF7 or MDA-MB-231 cells per well were seeded in 6-well plate, triplicate wells were seeded. After 10 days, cells were stained with Crystal Violet Staining Solution (C0121, Beyotime).

Apoptosis assay

MDA-MB-231 cells were stained with the FITC Annexin V apoptosis detection kit (556547, BD Biosciences) or PE Annexin V apoptosis detection Kit (559763, BD Biosciences) according to the manufacturer protocol and analyzed early- and late-stage apoptosis by FACS (FACS AriaTM IIII, BD Biosciences).

Wound healing and transwell migration assays

Indicated cells were plated to confluence in 6-well plates. Streaks were created in the monolayer with a pipette tip. Progression of migration was observed and photographed at 24 h (MCF7) or 16 h (MDA-MB-231) after wounding. The transwell assay was done using Corning FluoroBlokTM cell culture inserts (351152, Falcon) according to the protocol of the manufacturer.

Animal experiments

Five-week-old BALB/c2 nude mice (female) were purchased from Shanghai Model Organisms Center. All experiments were performed under the guidelines for the care and use of laboratory animals. BALB/c nude mice were randomly divided into two groups (n = 5 per group). Negative control miRNA (miR-NC) or miR-221/222 antagomirs (all at 200 nM concentration) were firstly transfected into MDA-MB-231 cells for 24 h. Then, 5×10^5 transfected cells in 0.1 ml of medium mixture (medium: matrigel = 1 : 1) were subcutaneously injected into the nude mice. The tumor size was measured at time intervals as indicated in Figure 2I. After 25 days, the mice were sacrificed and the tumors were dissected. The tumor weight was measured, and the tumor volume was calculated using the following formula: $D \times d^2/2$, with 'D' representing the longest diameter and 'd' representing the shortest diameter.

RNA extraction and real-time PCR

Total RNA including miRNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. To obtain cDNA, reverse transcription was performed using ABI high-capacity kit for mRNA (4368814, Life Technologies) and 1 μg RNA as template. For miRNA, 1 μg total RNA from each sample was reverse transcribed into complementary DNA by a specific miRNA stem loop primer. Real-time PCR reactions were performed according to the protocol of the Bio-Rad real-time PCR system (iQTM SYBR Green Supermix and CFX96^{TM} real-time system). The relative quantification (RQ) was derived from the difference in cycle threshold (Ct) between the target gene and internal control (GAPDH or miR-U6) compared to control using the formula $RQ = 2^{-\Delta\Delta Ct}$. The mRNA and miRNA levels were assessed by SYBR Green-based quantitative real-time PCR with gene-specific primers (Supplementary Table S1).

Western blot analysis

Western blot analysis was performed according to our previous method (Wang et al., 2016b). The following primary antibodies were used: anti-AXIN2 (5863S, 1:500), anti-p27 Kip1 (3686S, 1:500) anti-E-cadherin (3195S, 1:500), anti-GAPDH (2118S, 1:1000), anti-TUBULIN (2125S, 1:1000) and anti-TBP (8515S, 1:1000) were all purchased from Cell Signaling Technology; anti-WIF1 (sc-25520, 1:500) was purchased from Santa Cruz Biotechnology; anti-DKK2 (3802A-100, 1:500) was purchased from Biovision; anti-SFRP2 (ab137560, 1:500) and anti-N-cadherin (ab18203, 1:500) were purchased from Abcam; anti-β-catenin (610153, 1:500) was purchased from BD Transduction Laboratories.

Immunofluorescence microscopy

Immunofluorescence microscopy assay was performed according to a previously described standard method (Wang et al., 2016b).

Colony formation assay on the effect of miR-221/222 on WIF1, DKK2, SFRP2, and AXIN2

The ORFs of WIF1, DKK2, SFRP2, and AXIN2 in pReceiver-M13 vector were purchased from GeneCopoeia Inc. For Colony formation assay of MCF7 or MDA-MB-231 cells, 100 nM miR-221/222 mimics with or without 2.5 μg pReceiver-M13-WIF1, pReceiver-M13-DKK2, pReceiver-M13-SFRP2, or pReceiver-M13-AXIN2 were transfected into MCF7, 200 nM miR-221/222 inhibitors with or without 100 nM si-WIF1, si-DKK2, si-SFRP2, or si-AXIN2 were transfected into MDA-MB-231. A total of 1000 cells were used for colony formation assay after 24 h of transfection.

Luciferase reporter assay

The reporter plasmids containing wild-type (CCTTTGATC; TOPflash) or mutated (CCTTTGGCC; FOPflash) TCF/LEF DNA binding sites were purchased from Upstate Biotechnology. In TOP/FOP reporter assay, MCF7 or MDA-MB-231 cells were grown to 70%–80% density in 24-well plates and then transfected with 100 ng TOP reporter, 100 ng FOP reporter, 20 ng of the transfection control Renilla vector (pRL-TK, Promega), and 100 nM miRNA mimics or 200 nM miRNA inhibitor (RiboBio) along with 1 μl Lipofectamine 2000 (Invitrogen).

The 3′UTRs of WIF1, DKK2, SFRP2, and AXIN2 were amplified and cloned into psiCHECK2 vector (C8021, Promega) using primers specified in Supplementary Table S1. In the 3′UTR reporter assay, MCF7 or MDA-MB-231 cells in 24-well plates were transfected with 100 ng of the psiCHECK2-WIF1-3′UTR or psiCHECK2-DKK2-3′UTR or psiCHECK2-SFRP2-3′UTR or psiCHECK2-AXIN2-3′UTR and 100 nM mimics or 200 nM antisense (RiboBio) along with 2 μl Lipofectamine 2000 (Invitrogen). Lysates were harvested after 48 h of transfection. The reporter activity was measured with the Dual Luciferase Assay (E1910, Promega).

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Supplementary material

Supplementary material is available at *Journal of Molecular Cell Biology* online.

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Conflict of interest: none declared.

Author contributions: H.L. conceived the project. H.L., S.L., Z.W., Z.L., and S.S. designed the experiments. S.L., Z.W., and Z.L. conducted most experiments. S.S., Z.Z., and J.Z. conducted some of the pilot experiments. H.L. and S.L. supervised the experiments. S.L., Z.W., Z.L., and H.L. analyzed the data. S.L. and H.L. wrote the manuscript.

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