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ORIGINAL ARTICLE

MiR-429 suppresses proliferation and invasion of breast cancer via inhibiting the Wnt/β-catenin signaling pathway

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Breast cancer; epithelial-mesenchymal transition; invasion and metastasis; MiR-429.

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Abstract

Background: microRNAs (miRNAs) have been verified as molecular targets for regulating tumor proliferation, invasion, and metastasis in tumor progression. However, the relationship between miRNAs and cellular energy metabolism in breast cancer still needs to be clarified. This study aimed to investigate the role of miR-429 in breast cancer progression.

Methods: Bioinformatic analyses were employed to detect the relationship between miR-429 and cancer-related signaling pathways. We used a Kaplan-Meier curve to analyze survival rate in patients with high or low expression of miR-429. We used real-time quantitative PCR (RT-qPCR) to detect the expression of miR-429 in different cell lines. Sh-con, over-miR-429, miR-429 inhibitor, and sh-inhibitor control were transfected. Colony formation and EDU assay were used to detect the proliferation of transfected cells. Wound healing and transwell assays were performed to detect the mobility and invasion ability of transfected cells. Western blot assay was used to detect relative protein expression in transfected cells and different tissues. Bioinformatic analyses were conducted to detect the target proteins expression in different breast cancer databases. Dual luciferase reporter assay was used to confirm the binding site between miR-429 and fibronectin 1 (FN1).

Results: The results of our study indicate that MiR-429 and its target genes are associated with cancer-related signaling pathways and that higher miR-429 expression corresponds with a better prognosis. When miR-429 was overexpressed, the proliferation, invasion of MDA-MB-231 were inhibited. MiR-429 was able to suppress the Wnt/β-catenin signaling pathway, and FN1 overexpression could rescue the influence of over-miR-429.

Conclusions: The results of our study suggest that miR-429 suppresses the proliferation and invasion of breast cancer via inhibiting the Wnt/β-catenin signaling pathway.

Introduction

Breast cancer is recognized as one of the most diagnosed cancers and the leading cause of cancer deaths in females.[1] The carcinogenesis in breast tissue involves the accumulation of germline and somatic abnormalities.[2] Early diagnosis and preventive interventions could improve the survival rate in patients with breast cancer.[3] Therefore, numerous studies have focused on the molecular biological mechanisms underlying the tumorigenesis and progression of breast cancer.

MicroRNAs (miRNAs) play a part in various cellular and physiological processes such as cell proliferation and differentiation,[4–6] and their aberrant expression has been reported to be associated with progression and metastasis in various human tumors.[7–9] In breast cancer, several miRNAs have been reported to exert their effects on cell proliferation, invasion, migration, and apoptosis, etc.[9–11] MiR-429, a member of the miR-200 family, has been shown to be a tumor suppressor in many carcinomas including ovarian, non-small cell lung, and gastric cancers.[12–15] However, the molecular mechanisms by which miR-429 plays a part in breast cancer still needs to be explored. There is increasing evidence which indicates that aberrant activation of the Wnt/β-catenin signaling plays an important role in epithelial-to-mesenchymal transition (EMT) activation and CSC reinitiation, leading to tumor growth and metastasis.[16] Hence, inhibition of Wnt signaling through genetically modifying Wnt-related genes has been used as a novel therapeutic approach for cancer treatment. In this study, bioinformatic analyses was first performed in order to predict the possible function of miR-429 in breast cancer and revealed that higher expression of miR-429 corresponded to better prognosis in patients. Bioinformatic prediction also showed that it could bind to FN1. Therefore, we focused on miR-429 and FN1. Next, we found that miR-429 was downregulated in breast cancer cells. However, overexpression of miR-429 inhibited the proliferation, migration, and invasion abilities of breast cancer cells.

Based on the findings in our study, we propose a hypothesis that miR-429 inhibits the invasion and metastasis of breast cancer by targeting FN1. To further explore the significance of miR-429 and FN1 in breast cancer, we performed experiments to investigate how miR-429 regulates the invasion and metastasis of breast cancer. Our results propose a mechanism by which miR-429 mediates cell proliferation in breast cancer, and our research may offer a new target for anticancer therapies of breast cancer.

Methods

Enrichment analysis and prediction of target genes

Kyoto encyclopedia of genes and genomes (KEGG) analyses were utilized to explore the functional annotation of potential target genes through DIANA tools. Gene ontology (GO) analyses were carried out through the DAVID website. The GO analyses consist of biological processes (BPs), cellular components (CCs), and molecular functions (MFs). The bioinformatic websites of TargetScan, miRDB and miRWalk were employed for predicting the potential target genes of miR-429. Consensus results derived from the three tools were selected for subsequent analyses.

Cell culture and reagents

Cells (MCF-10A, MCF-7, MDA-MB-231, and 293T) were purchased from the American Type Culture Collection (ATCC, Manassas, Virginia, USA). MCF-10A cells were cultured in mammary epithelial growth medium (MEGM) (Catalog No. CC-3150). MCF-7 were cultured in Eagle's Minimum Essential Medium (EMEM) (ATCC, 30-2003), with MDA-MB-231 in Roswell Park Memorial Institute-1640 solution (RPMI) (Solarbio, Catalog No. 3180) and 293T in Dulbecco's modified Eagle's medium (DMEM) (Solarbio, Catalog No.11995).

All four kinds of cells were cultured at 37°C with 5% CO_2, and their media was supplemented with 10% fetal bovine serum (FBS).

Plasmid construction and cell transfection

MiR-429 mimics, miR-429 negative control, mimic hsa-miR-429, inhibitor (inhi)-miR-429, inhibitor (inhi)-FN1, and inhibitor(inhi)LRP6, were purchased from Sigma-Aldrich Shanghai GenePharma Co., Ltd. (Shanghai, China). Transfection was performed with Lipofectamine 2000 reagent (Thermo Fisher Scientific) following the manufacturer's instructions.

Western blot and immunofluorescence

Total proteins were extracted from breast cancer cell lines as previously stated.[17] The following antibodies were used in this study: β-actin (1:5000), vimentin (1:500), E-cadherin (1:500), fibronectin 1 (FN1) (1:500), β-catenin (1:500), c-Myc (1:500), c-Jun (1:500), LEF1 (1:500), MMP2 (1:500). β-actin was used for normalization. The antibodies were purchased from Abcam (www.abcam.cn). For immunofluorescence, 1×10^4 cells were seeded in 24-well plates and grown in regular medium after 12 hours. The cells were washed with PBS, and then treated in succession with 4% paraformaldehyde for fixation, 0.1% Triton X-100 for permeabilization, and serum working fluid for blocking nonspecific bindings. Cells were then incubated with fluorescence secondary antibodies and counterstained with Hoechst the next day. Representative photos were collected and analyzed using a confocal laser scanning microscope (TCS SP8, Leica, Germany). All the experiments were repeated independently three times. For all human experiments, the operators were blinded to the group allocation. All human experiments were approved by the Ethics Committee of Weifang Medical University.

Cell proliferation and wound healing assays

After being digested with 0.25% trypsinase supplemented with 0.02% EDTA for three minutes, 500 cells were seeded into six-well plates and cultured for seven days without disturbing the formation of colonies. Colonies were fixed with formaldehyde and stained with Giemsa. Colonies carrying over 50 cells were counted. Cell proliferation was assayed by an EDU cell proliferation detection kit after culture for 12 hours. Cells were labeled with 250 μL EDU at 50 μM for two hours, fixed with 250 μL paraformaldehyde at 4% for 30 minutes at room temperature, and washed successively with 250 μL PBS for five minutes. Cells were then stained with 250 μL 1 × Apollo for 30 minutes. After three rinses in 250 μL PBS (0.5% Triton X-100) for 10 minutes, they were treated with 100 μL 1× Hoechst 33342 staining solution for 30 minutes, followed by microscopic observation under a confocal laser scanning microscope (TCS SP8, Leica, Germany).

The MDA-MB-231 cells were seeded in six-well plates. When the cells reached a confluency of 30%, a pipette tip of 10 μL was used to generate a straight wound. The cells were then cultured in serum-free medium at 37°C with 5% CO_2 in a humidified incubator. At 0, 24, 48, and 72 hours, an inverted microscope was utilized for microscopic observation, respectively. All experiments were repeated independently three times.

Transwell assay

Cell suspensions for each group were added to a matrigel-covered compartment, with FBS added to the upper and lower compartments. After being cultured for 24 hours, cells were fixed with 4% paraformaldehyde and then stained with Giemsa. Five representative fields were randomly selected for microscopic observation and the experiment was repeated three times independently to calculate the means.

Table 1 Sequence of primers used for RT-qPCR

Name	Sequence (5′-3′)
miR-429 (RT primer)	GTCGTATCCAGTGCAGGGTCC GAGGTATTCGCACTGGATACGACACGGTT
miR-429 (reverse)	AGTGCAGGGTCCGAGGTATT
miR-429 (forward)	CGCGCGTAATACTGTCTGGTAA
U6 (reverse)	AACGCTTCACGAATTTGCGT
U6 (forward)	CTCGCTTCGGCAGCACA

Immunohistochemistry analysis

Based on the high specificity of an antibody binding to an antigen, immunohistochemistry revealed the relative distribution and abundance of proteins. The Human Protein Atlas (HPA, https://www.proteinatlas.org) is the world's largest and most comprehensive human tissue cell protein spatial distribution database, possessing great features and potential application prospects. We used HPA to observe differences of FN1 expression in normal and breast cancer tissues.

RNA extraction and real-time quantitative PCR (RT-qPCR)

TRIzol reagent (Invitrogen, CA, USA) was used for extracting total RNA from breast cancer cells, followed by determining the RNA purity and concentration. Stem ring primers of miR-429 were used for reverse transcription. U6 was used as an internal reference for RT-qPCR. The cDNA of miRNA was synthesized employing a one-step PrimerScript miRNA cDNA Synthesis Kit (Takara Bio, Inc., Shiga, Japan). RT-qPCR was performed on an ABI 7500Fast RT-qPCR system (Applied Biosystems, Foster City, CA, USA) using a kit with SYBR Green and low Rox (BioEasy Master Mix, Hangzhou Bioer Technology Co.). Gene expression levels were normalized to the housekeeping gene β-actin as the control and calculated as 2 – [(Target gene Ct) – (β-actin Ct)], where Ct represents the threshold cycle for each transcript. The relative expression levels were calculated as 2 – [(miR-429 Ct) – (U6 Ct)] following normalization with reference to the expression of small nuclear RNA U6. The primer sequences used in this study are presented in Table 1.

Luciferase reporter assay

We constructed a luciferase reporter gene containing wild-type (WT) or mutant (MU) 3′UTR of FN1 with a mutant miR-429 binding site. Luciferase activity was tested using a dual-luciferase reporter assay system (Promega, Madison, WI, USA). Activities were normalized to that of Renilla luciferase. All experiments were performed independently three times.

Bioinformatic analyses

PPI network was built on STRING (https://string-db.org/), and Cytoscape was used for seeking the potential target gene of miR-429, namely the hub gene. The hub gene of miR-429 was identified by gene expression profile interaction acquisition analysis (GEPIA). Meta-analysis of global gene expression data from the Oncomine database

(Compendia Bioscience, Ann Arbor, USA) was performed using primary filters of "breast cancer" and "cancer versus normal analysis", sample filter of "clinical specimens", and data type filter using "mRNA" data sets. Patients of all ages, gender, disease stages and treatments were included. Data were acquired in an unbiased manner by compiling all the Oncomine studies with significantly altered FN1 expression at the threshold settings (P-value = 0.05, fold-change = 1.5, and gene rank = all). Significant studies with three or less patients in at least one analyzed group were excluded. All data were reported as log 2 median-centered intensity in the Oncomine database. The datasets were exported from Oncomine and analyzed using GraphPad Prism V8 software. The relationship between hsa-miR-429 and FN1 was constructed using starBase V2.0 (http://starbase.sysu.edu.cn/). The prognostic value of candidate genes were analyzed using the Kaplan-Meier Plotter (KM-plot) (http://kmplot.com/analysis/), an online database that assesses the effects of 54 675 genes on survival involving 5143 breast cancer samples.[18] The correlation between mRNA expression of candidate genes and relapse-free survival (RFS) in patients with breast cancer was assessed.

Statistical analysis

A survival curve was generated by Kaplan-Meier survival plot and analyzed with a log-rank test. The differences between two groups were analyzed by paired or unpaired Student's t-test. $P < 0.05$ was considered statistically significant and all statistical analyses were conducted using IBM SPSS software 19.0.

Results

MiR-429 and its target genes associated with cancer-related signaling pathways and higher miR-429 expression corresponds with better prognosis

We predicted the potential biological function of miR-429 through KEGG analysis. The results indicated that KEGG pathways enriched by miR-429 targeted protein was mainly related to cancer-specific pathways, including Wnt, TGF-beta, and RAS, etc (Fig **1**a). To explore the molecular mechanism underlying the oncogenic activity of miR-429 in breast cancer, miRDB, Targetscan, and miRwalk were employed to predict the interaction probabilities between miR-429 and targeted genes. A total of 148 common targeted genes were predicted in this way (Fig **1**b). We then conducted KEGG and GO analyses on the 148 target genes of miR-429, and the results showed that the target protein of miR-429 was related to various cancer-specific pathways (Fig **1**c, d). The prediction results of target genes were consistent with the prediction results of miR-429, suggesting that miR-429 is closely related to the occurrence and development of cancer. Kaplan-Meier plots showed that higher expression of miR-429 was correlated with a better overall survival probability for patients with breast cancer (Fig **1**e). These findings suggest that miR-429 negatively regulates the targeted genes in breast cancer.

MiR-429 inhibits proliferation, migration, and invasion of breast cancer cells

Next, we further investigated the correlation between miR-429 and breast cancer. First, the results of RT-qPCR showed that the expression of miR-429 in breast cancer cells decreased significantly, compared with that in MCF-10A cells (Fig 2a). In addition, miR-429 expression in MCF-7 cells with low invasion was significantly higher than in MDA-MB-231 cells with high invasion. MiR-429 expression in MDA-MB-231/miR-429 cells was also increased, confirming successful transfection (Fig 2b). Colony formation and EDU assays were used to detect the proliferation capacity of breast cancer cells. The colony formation assay showed that overexpressed miR-429 resulted in fewer formed colonies in MDA-MB-231 cells than those in control (Fig 2c). EDU assay showed that overexpression of miR-429 inhibited cell proliferation in MDA-MB-231 cells (Fig 2d). These results suggested that miR-429 inhibited the proliferation ability of breast cancer cell lines. Wound healing assay showed suppressed mobility ratio of breast cancer cells by overexpression of miR-429 (Fig 2e,f). Invasive and migratory abilities were further evaluated via transwell assays, demonstrating the significantly suppressed invasion and migration abilities of breast cancer cells due to upregulated miR-429 (Fig 2g). These results suggest that miR-429 may play an antioncogenic role and regulate the capabilities of proliferation, migration, and invasion of breast cancer cells.

MiR-429 inhibits EMT and cytoskeleton rearrangement in breast cancer cells

Since EMT is widely regarded as a crucial process in tumor invasion and metastasis,[19–21] we hypothesized that miR-429 functions as an antioncogene through regulating the EMT progression. Western blot, RT-qPCR, and immunofluorescence analyses were performed to detect the protein levels of EMT-related markers. Western blot data showed that miR-429 inhibited the expression of vimentin, but upregulated E-cadherin (Fig 3a). RT-qPCR analysis showed that overexpressed miR-429 reduced the mesenchymal marker of vimentin in MDA-MB-231 cells and immunofluorescence analyses showed that overexpressed miR-429 elevated the expression of the epithelial marker of E-

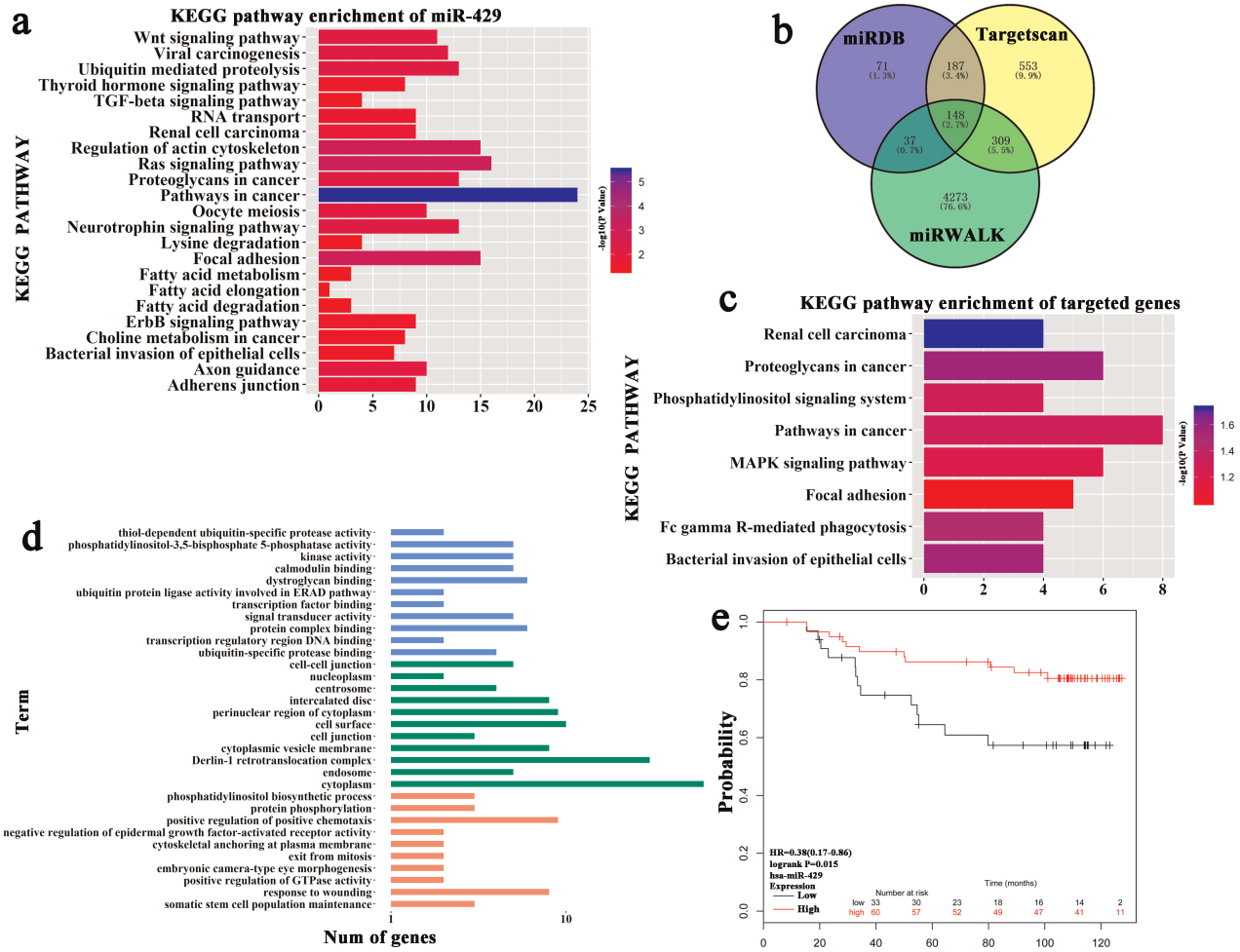

Figure 1 miR-429 and its target genes are associated with cancer-related signaling pathways and higher miR-429 expression corresponds with a better prognosis. (**a**) KEGG analyses of miR-429. (**b**) 148 common target genes predicted by miRDB, TargetScan, and miRwalk. (**c**) KEGG analyses of miR-429 targeted genes. (**d**) Results of GO analyses: biological process (red), cellular component (green), and molecular function (blue) (■) GOTERM_BP-DIRECT, (■) GOTERM_CC-DIRECT, (■) GOTERM_MF-DIRECT. (**e**) Kaplan-Meier plotter (KM-plot) showed that overall survival corresponded with miR-429 expression level (——) Low, (——) High. Dataset: GSE19783, Log-rank *P*-values were calculated in KM-plot database.

cadherin while it reduced that of the mesenchymal marker of vimentin in MDA-MB-231 cells (Fig 3b,c), which confirmed that miR-429 inhibited EMT in breast cancer cells. MDA-MB-231/con cells also exhibited higher levels of F-actin compared with MDA-MB-231/miR-429 cells (Fig 3d), suggesting that miR-429 inhibited the characteristics of tumor cells via reducing F-actin accumulation in cytoplasm. Taken together, our data suggest a potential link between miR-429 and the EMT status as well as F-actin response in tumor cells. Wnt signaling is an important regulatory pathway involved in different biological processes such as embryonic development, cell differentiation, cell proliferation, and tissue maintenance in adults.[22] KEGG pathway enrichment of miR-429 (Fig **1**a) showed that the Wnt signaling pathway demonstrated a higher correlation with miR-429. To identify the molecular pathways mediated by miR-429, we tested the important cancer signaling pathways with luciferase reporter assays, including Wnt/β-catenin, NF-κB, MAPK, TGF-β, JNK, and PI3K/AKT. Among these pathways, Wnt/β-catenin signaling pathway was the most significantly suppressed pathway upon ectopic expression of miR-429 (Fig S1a). MiR-429 suppressed the Wnt signaling TOPflash reporter activity down to 45% but not the FOPflash reporter in 293T cells (Fig 3e). We also found that miR-429 could alter the nuclear accumulation of β-catenin and the activation of Wnt/β-catenin signaling (Fig 3f). In addition, miR-429 overexpression inhibited Wnt signaling activation (Fig 3g).

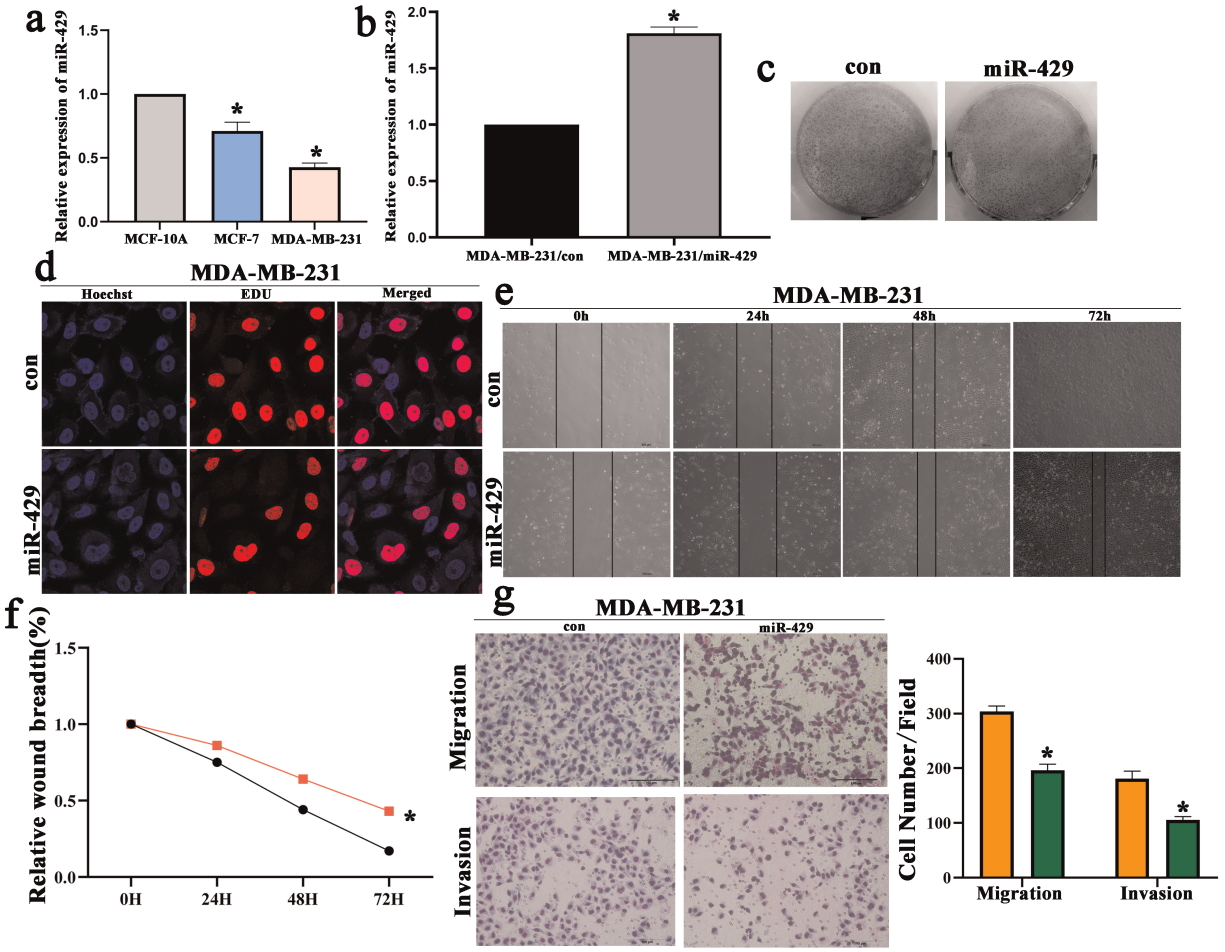

Figure 2 MiR-429 inhibits the proliferation, migration, and invasion of breast cancer cells. (**a**) RT-qPCR analysis of miR-429 in MCF-10A and human breast cancer cells. (**b**) RT-qPCR analysis of miR-429 in transfected cells. (**c**) Colony formation of MDA-MB-231 cells after transfection with con and miR-429. (**d**) EDU assay of MDA-MB-231 cells after transfection with con and miR-429. (**e**) and (**f**) Wound healing assay of MDA-MB-231 cells after transfection with con and miR-429 (—●—) MDA-MB-231/con, (—■—) MDA-MB-231/miR-429. (**g**) Migration and invasion assays of MDA-MB-231 cells after transfection with con and miR-429 (■) MDA-MB-231/con, (■) MDA-MB-231/miR-429.

MiR-429 suppresses Wnt signaling pathway by targeting FN1

We then used PPI analysis and the cytoHubba algorithm of Cytoscape to detect the potential targets for miR-429. The results revealed 10 genes: SOX2, CBL, FN1, KDR, KLF4, NR3C1, QK1, RBFOX3, SYNJ1, and VEGFA as the hub genes of miR-429 (Fig **4**a). GEPIA analysis of FN1 within the central region of PPI network, indicated that FN1 was significantly upregulated in the tumor group when compared with the control, while the remaining nine genes exhibited lower expression in breast cancer tissues (Fig **4**b). Therefore, we mainly focused on FN1 among the targets for miR-429. We then used the GEO and TCGA database to detect the expression of FN1 in tumor tissues. The data collected by GSE9014 showed FN1 had a higher mRNA level in tumor tissues compared with normal tissues (Fig **4**c). The protein expression level was then detected by immunohistochemical analysis (Fig **4**d), and we found that FN1 also presented higher protein expression in breast cancer tissues. The results of western blot also showed higher expression of FN1 in MDA-MB-231 cells (Fig **4**e, f). We then examined whether miR-429-mediated Wnt/β-catenin signaling activation in breast cancer cells was clinically relevant. As shown in Fig **4**g, miR-429 expression in six freshly collected breast cancer samples was inversely correlated with the mRNA levels of Wnt/β-catenin downstream targets, including cyclin D1, MYC, as well as LRP6. Collectively, our results demonstrated that miR-429 downregulation activates Wnt/β-catenin signaling, resulting in breast cancer tumorigenicity and poorer clinical outcomes. LRP6 is a transmembrane Wnt coreceptor essential for Wnt/β-catenin signaling.[23]

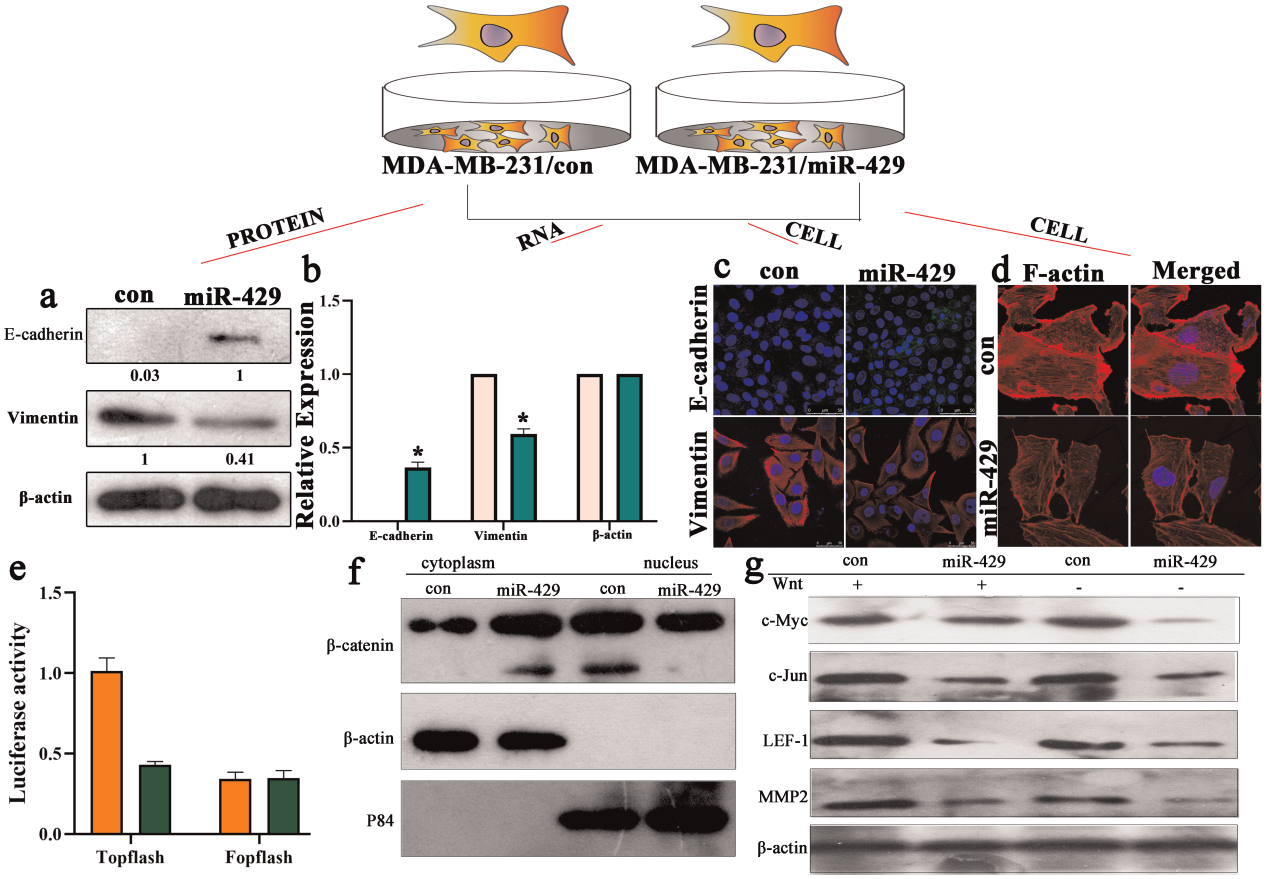

Figure 3 MiR-429 inhibits EMT, cytoskeleton rearrangement and suppresses Wnt/β-catenin signaling pathway in breast cancer cells. (**a**) Expression of EMT markers tested by western blot, with β-actin as internal reference. (**b**) Expression of EMT markers evaluated by RT-qPCR analysis, with β-actin as internal reference (□) MDA-MB-231/con, (■) MDA-MB-231/miR-429. (**c**) Expression of EMT markers tested by immunofluorescence. (**d**) Representative images of F-actin (red) and nucleus (blue) staining in MDA-MB-231/con and MDA-MB-231/miR-429 cells; scale bars = 50 μm. (**e**) MiR-429 suppressed the Wnt signaling TOPflash reporter activity but not the FOPflash reporter. 293T cells were cotransfected with either con plasmid or miR-429 plasmid, in combination with each pathway luciferase reporter and pRL-CMV control reporter vectors for 24 hours. A dual-luciferase assay was performed and results are expressed as fold change (□) con, (■) miR-429. (**f**) The protein levels in the cytoplasm and nucleus of transfected cells. (**g**) The protein levels of Wnt/β-catenin signaling target genes in MDA-MB-231 cells transfected with con or over-miR-429.

Moreover, FN1 protein was positively correlated with LRP6 in breast cancer tissues. MiR-429 expression was negatively correlated with Wnt/β-catenin genes (Fig 4g). We further investigated whether miR-429 could inhibit the Wnt signaling pathway by downregulating FN1. As shown in Fig 4h and Fig S1b,c, overexpression of FN1 activated Wnt signaling, which could, however, be suppressed by overexpression of miR-429.

FN1 upregulated in human breast cancer and elevated expression corresponds to worsen overall survival probability

To investigate the expression of FN1 in human breast cancer, a meta-analysis was performed based on data from the Oncomine database. We compared the FN1 expression in 597 breast cancer samples and 409 adjacent normal ones derived from 12 datasets. It was upregulated in breast cancer samples (gene median rank: 60, $P = 1.41e^{-4}$) in all included datasets (Fig 5a). Further analyses showed higher mRNA levels of FN1 in breast cancer samples including ductal breast, invasive lobular breast (Fig 5b), invasive ductal (Fig 5c), ductal breast in situ (Fig 5d), invasive breast (Fig 5e), and ductal breast carcinomas (Fig 5f), compared with their respective control ($P < 0.05$). Kaplan-Meier plots suggested that elevated FN1 corresponded to lower overall survival probability for patients with breast cancer (Fig 5g). Collectively, these data suggest that FN1 plays a role as an oncology gene and its mRNA level is negatively associated with the prognosis for patients with breast cancer.

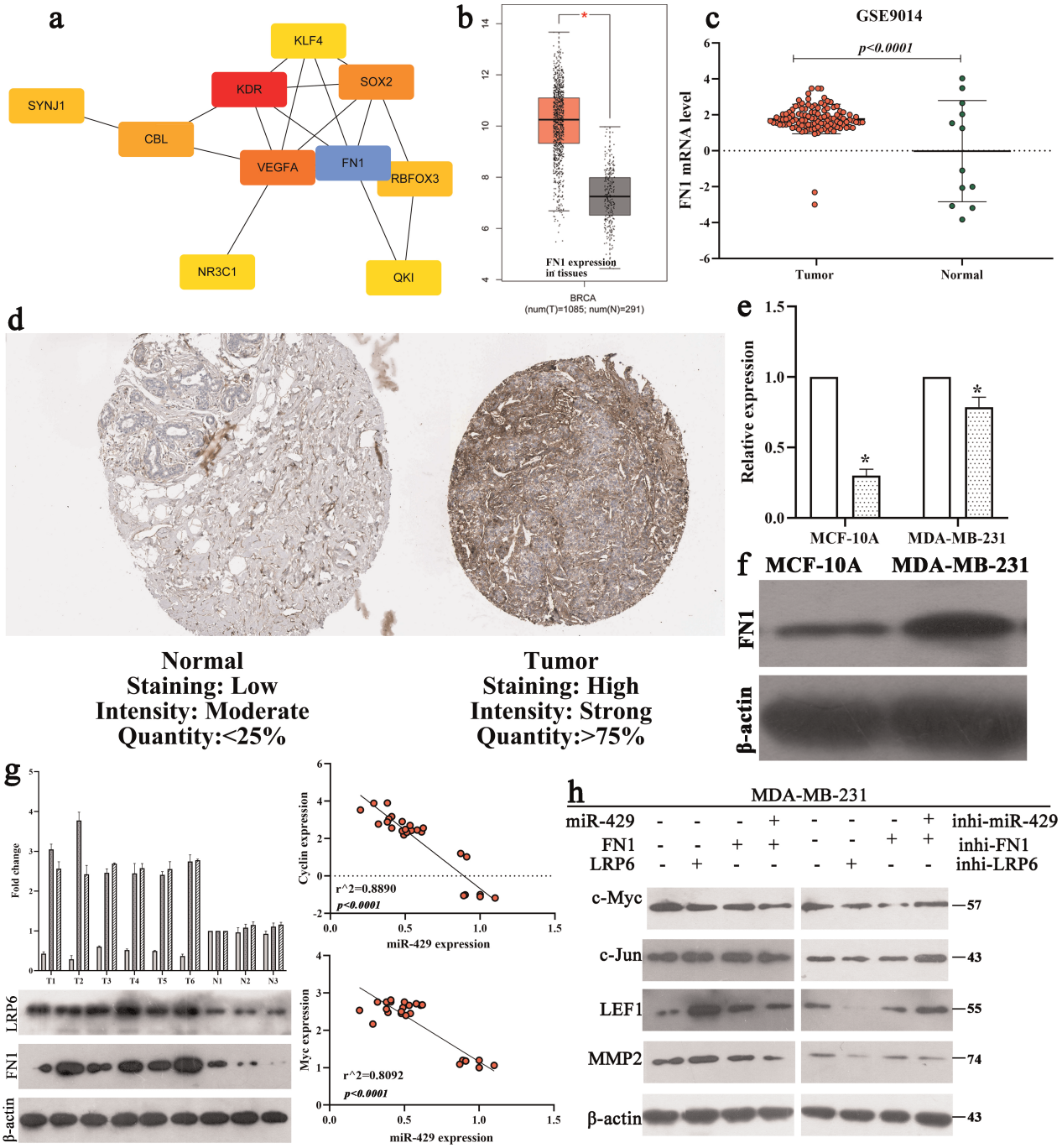

Figure 4 miR-429 target FN1 to suppress the Wnt signaling pathway. (**a**) Hub genes of miR-429 obtained by cytoHubba algorithm of cytoscape. (**b**) GEPIA analysis of FN1. (**c**) mRNA level of FN1in GSE9014. (**d**) protein level of FN1 in tumor tissues. (**e**) mRNA level of FN1 in breast cancer cells (▭) β-actin, (▭) FN1. (**f**) protein level of FN1 in breast cancer cells. (**g**) Real-time PCR analysis of miR-429, cyclin D1, MYC, and western blot analysis of LRP6 and FN1 expression in breast cancer tissues (▭) miR-429, (▭) cyclin D1, (▨) MYC. (**h**) The protein levels of Wnt/β-catenin signaling target genes in MDA-MB-231 cells transfected with over-miR-429, over-LRP6, over-FN1, si-miR-429, si-LRP6, and si-FN1.

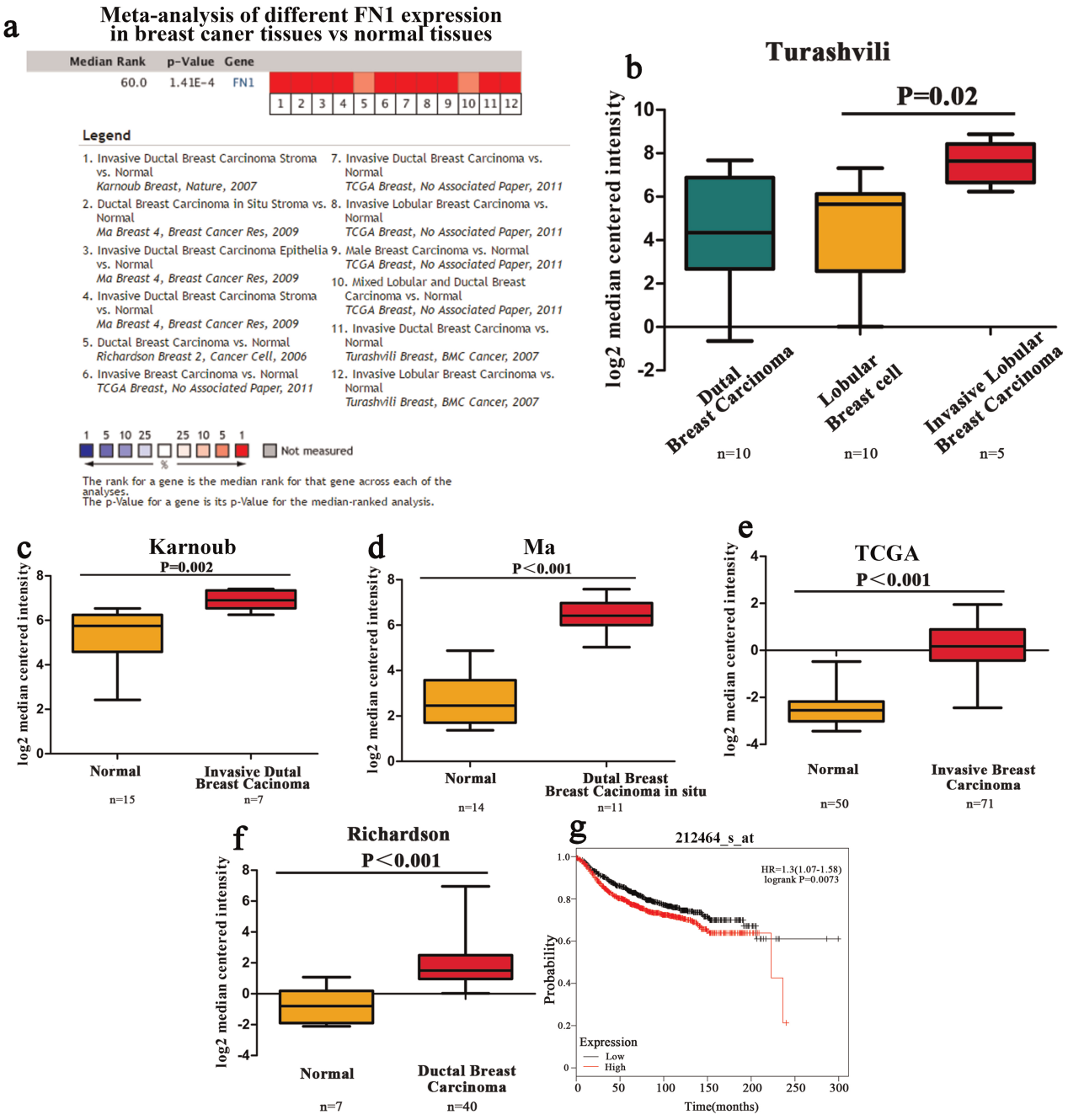

Figure 5 FN1 is upregulated in human breast cancers and its elevated expression corresponds to worsen overall survival probability. (**a**) mRNA level of FN1 analyzed by Oncomine microarray database and meta-analysis from five microarray datasets. Data is shown as median rank of FN1 expression through each dataset analysis. The *P*-value for FN1 was determined by the median ranked analysis of breast cancer and normal tissues. (**b**) mRNA levels of FN1 in the Turashvili database. (**c**) mRNA levels of FN1 in the Karnoub database. (**d**) mRNA levels of FN1 in Ma database. (**e**) mRNA levels of FN1 in TCGA database. (**f**) mRNA levels of FN1 in the Richardson database. Differential expression of FN1 mRNA in the five datasets included in the meta-analysis (Normal: normal adjacent breast tissue). Median and interquartile range (10th and 90th percentiles). Two-sided *t*-test was used for two class differential expression analyses and Pearson's correlation for multiclass analyses. FDR-corrected *P*-values. (**g**) Kaplan-Meier plots showing the overall survival corresponding to FN1 expression (probe: 212464_s_at) (——) Low, (——) High. Log-rank *P*-values were calculated in the KM-plot database.

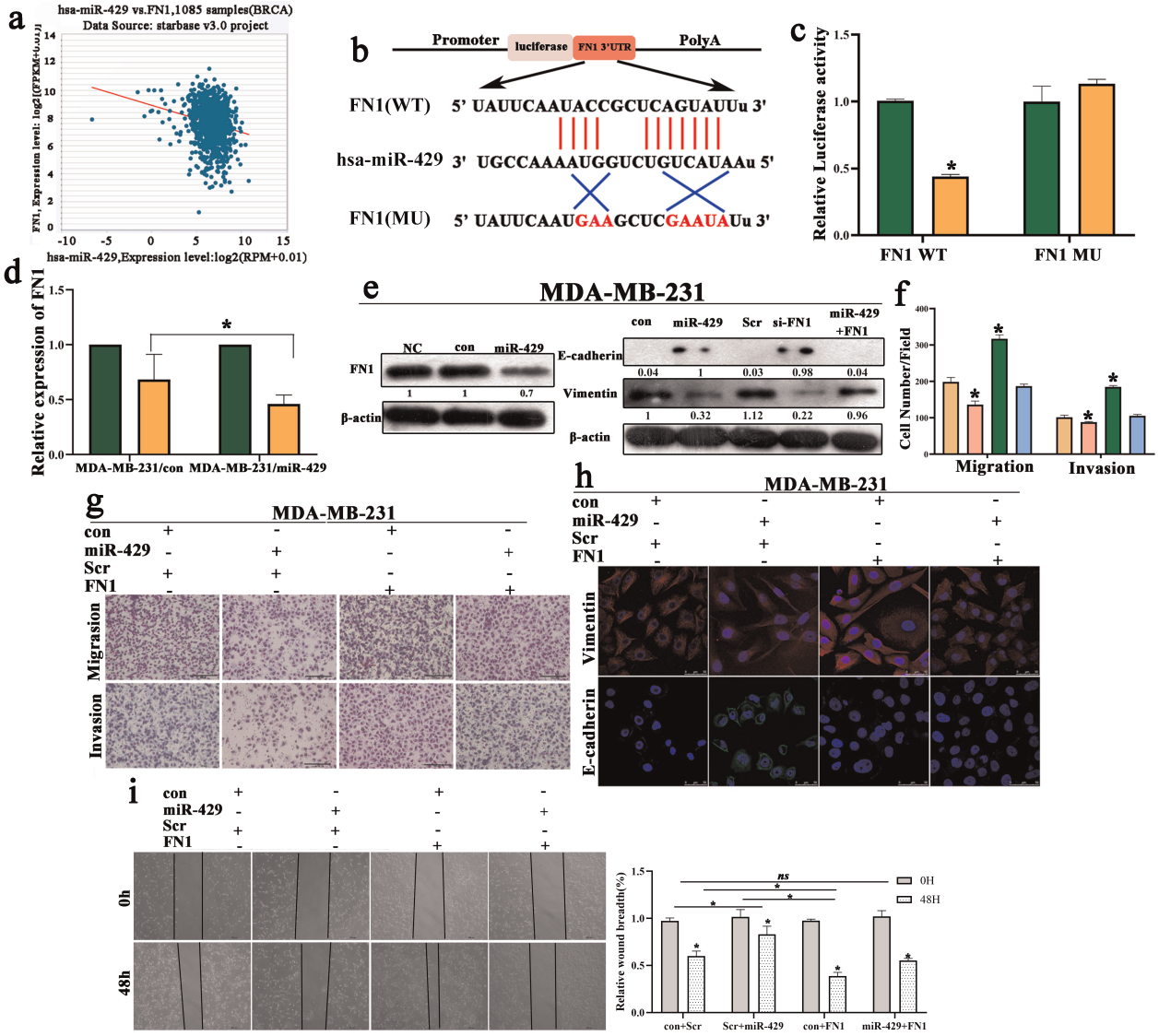

Figure 6 MiR-429 was able to directly bind to FN1 whose overexpression could restore the effects of miR-429 on the invasion, migration, EMT and mobility of breast cancer cells in vivo. (**a**) Correlation between miR-429 and FN1 in breast cancer tissues analyzed by starBase v2.0 (——) Regression (y = −0.1938x + 8.9021), (●) r = −0.193, *p*-value = $1.36e^{-10}$. (**b**) Predicted target sites of miR-429 to 3′-UTRs of FN1, with the corresponding sequence in the mutated (MU) version. (**c**) The luciferase activity in transfected cells (■) 293T/con, (■) 293T/miR-429. (**d**) RT-qPCR analysis of FN1 in transfected cells (■) β-actin, (■) FN1. (**e**) Expression of FN1 and EMT markers determined by western blot, with β-actin as internal reference. (**f** and **g**) Migration and invasion assays for transfected cells (■) con + Scr, (■) miR-429 + Scr, (■) con + FN1, (■) miR-429 = FN1. (**h**) Immunofluorescence assays for transfected cells. (**i**) The mobility ability of transfected cells (▭) 0H, (▭) 48H.

MiR-429 was able to directly bind to FN1 whose overexpression could restore the effects of miR-429 on invasion, migration, EMT and mobility of breast cancer cells in vivo

Although the interaction between miR-429 and FN1 has been predicted by computational predictive algorithms, it is unknown whether miR-429 can effectively regulate FN1 in breast cancer cells. The interactions between FN1 and miR-429 were predicted using starBase v2.0 database, with the corresponding sequence in the mutated (MU) version (Fig 6a,b) and confirmed by dual-luciferase reporter assay (Fig 6c). The luciferase activity in 293T/miR-429 cell line was lower than that in 293T/con. Subsequent RT-qPCR analysis presented the suppression of FN1 by miR-429 in breast cancer cells (Fig 6d). Compared with MDA-MB-231/con, the miR-429 mimics significantly downregulated

FN1 and overexpressed FN1 could restore the effects of miR-429 on the EMT of breast cancer cells (Fig 6e). The MDA-MB-231/miR-429 cells were found to possess a lower ability of migration and invasion compared with MDA-MB-231/con cells, and overexpressed FN1 could restore the effects of miR-429 on the invasion and migration ability of MDA-MB-231 ones (Fig 6f,g). As shown in Fig 6h, miR-429 could decrease the expression of vimentin but increase that of E-cadherin, and overexpressed FN1 could restore the effects of it. Taken together, these data suggested that miR-429 could directly bind to FN1 and subsequently inhibit the invasion and metastasis of human breast cancer. Wound healing assay also showed suppressed mobility ratio of breast cancer cells by overexpression of miR-429, which could also be restored by FN1 (Fig 6i). To conclude, miR-429 was able to directly bind to FN1 whose overexpression could restore the effects of miR-429 on the invasion, migration, EMT and mobility of breast cancer cells in vivo.

Discussion

Breast cancer is a clinically challenging disease involving complicated genomic alterations.[24] To date, changes in miRNAs of cancer-related genes are being given more and more attention. The role of miRNAs in breast cancer has been widely reported in several studies over recent years.[17, 25, 26] In most cases, miRNAs are regarded as negative regulators of gene expression.[27-29] FN1 has previously been well documented to be overexpressed in tumor tissues[30, 31] and its downregulation has been reported to contribute to its antitumor activity in breast cancer[32] however, there have been fewer studies focusing on its regulation by miRNAs. Consistent with the findings in these studies, the present study also demonstrated significantly downregulated miR-429, but upregulated FN1 in breast cancer.

This purpose of this study was to investigate the role of miR-429 in breast cancer by bioinformatic assays. KEGG and GO analyses showed that miR-429 and its target genes were associated with some cancer-related pathways, and elevated miR-429 was predicted to correspond with a better prognosis based on the analyses of miRNA expression and clinical data from GSE19783. Stationary and adherent cells can acquire the ability to migrate through EMT which belongs to a developmental process, and tumor cells can increase their aggressiveness through reactivating EMT.[33] Therefore, we studied the expression of EMT-related biomarkers and the Wnt/β-catenin signaling pathway. The results showed that overexpressed miR-429 in MDA-MB-231 cells upregulated E-cadherin but downregulated vimentin, and suppressed activity of the β-catenin signaling pathway. Thus, understanding the role of miR-429 in EMT of breast cancer may be an effective therapeutic strategy. Our results were consistent with those from bioinformatic assays. To further explore the participation of miR-429 in breast cancer, bioinformatic analyses were performed to predict its hub genes. A total of 10 hub genes were found by Cytoscape, and only FN1 among them exhibited a higher expression in various breast cancer tissues. We then identified miR-429 as a regulator suppressing FN1 expression by directly targeting its 3′UTR. An inverse correlation between levels of FN1 and miR-429 was reached in breast cancer cells in vivo. Therefore, miR-429 may be involved in the proliferation, invasion, and migration by regulating FN1 expression.

In summary, our work suggests that miR-429 inhibits the proliferation, invasion, and migration by regulating FN1 expression in breast cancer cells. MiR-429 serves as an important molecular marker with the potential to be a novel promising candidate for the prognosis and therapy of breast cancer. It should be noted that the functional research conducted was based on exogenous overexpression studies, while the exogenous and endogenous levels of edited miRNA might differ significantly. Therefore, the effects of miR-429 in multiple mouse models and its collaborative application with other anticancer therapies remain to be evaluated.

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Disclosure

No authors report any conflict of interest.

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Supporting Information

Additional Supporting Informationmay be found in the online version of this article at the publisher's website:

Figure S1 (**a**) Effects of miR-429 on six cancer related pathways were determined by luciferase reporter assay. (**b** & **c**) A histogram of the gray value of Fig 4h (the protein levels of Wnt/β-catenin signaling target genes in transfected cells).

