Molecular Therapy
Nucleic Acids
Original Article

Novel miR-5088-5p promotes malignancy of breast cancer by inhibiting DBC2

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Breast cancer is the most common female cancer in the world. Despite the active research on metastatic breast cancer, the treatment of breast cancer patients is still difficult because the mechanism is not well known. Therefore, research on new targets and mechanisms for diagnosis and treatment of breast cancer patients is required. On the other hand, microRNA (miRNA) has the advantage of simultaneously regulating the expression of many target genes, so it has been proposed as an effective biomarker for the treatment of various diseases including cancer. This study analyzed the role and mechanism of DBC2 (deleted in breast cancer 2), which is known to inhibit its expression in breast cancer, and proposed microRNA (miR)-5088-5p, which regulates its expression. It was revealed that the biogenesis of miR-5088-5p was upregulated by hypomethylation of its promoter, promoted by Fyn, and was involved in malignancy in breast cancer. With the use of the cellular level, clinical samples, and published data, we verified that the expression patterns of DBC2 and miR-5088-5p were negatively related, suggesting the potential as novel biomarkers for the diagnosis of breast cancer patients.

INTRODUCTION

Breast cancers are the most common female cancer worldwide.[1] Breast cancer is divided into luminal A, luminal B, Her2+, and triple-negative types through gene-expression profiling based on estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2).[2] Metastasis in breast cancer patients is reported to be an important factor affecting the survival rate of breast cancer patients.[1] Despite progressive research, the proportion of metastatic breast cancer (MBC) patients for whom no effective treatment is available remains high.[3] Even with the development of diverse therapeutic regimens, chemo-resistance and disease relapse, which are predominantly attributed to the existence of EMT (epithelial-mesenchymal transition) and cancer stem-like cells (CSCs) in tumor tissues, continue to increase.[4–6] Therefore, biomarkers that facilitate prediction or diagnosis of breast cancer should be developed, and the associated mechanisms to optimize treatment options for patients with MBC should be identified.

DBC2 (deleted in breast cancer 2), also known as RHOBTB2 (Rho-related BTB domain-containing protein 2), is low expressed in 60% of breast cancer patients[7] and is known to function as a tumor suppressor.[8,9] However, the correlation between downregulation of DBC2 and malignant breast cancer mechanisms has not been identified. Therefore, the purpose of this study was to analyze the relationship between the cause of the inhibition of DBC2 expression in breast cancer and the malignancy of breast cancer and to elucidate the possibility that DBC2 can be used as an important biomarker for diagnosis and treatment of malignant breast cancer.

MicroRNAs (miRNAs), as 22−24 nucleotide fragments, affect post-transcriptional activity by degrading or inhibiting mRNA expression through the binding 3′ untranslated region (3′ UTR) of target genes. These molecules play key roles in diverse biological processes, such as cell proliferation, differentiation, and apoptosis. In addition, miRNAs are involved in the production of cancer-related target genes that induced tumor suppression or carcinogenesis. To date, microRNA (miR)-21,[10] miR-155,[11] miR-10b,[12] miR-23b,[13] miR-27b,[13] and miR-9[11] have been identified as carcinogenic and let-7 family,[14] miR-145,[15] miR-200 family,[16] miR-205,[17] and miR-15b[18] as tumor-suppressor miRNAs in breast cancer. Another miRNA, miR-206, downregulated in breast cancer, is reported to suppress ER-α levels in breast and lymph node metastatic breast tumors.[19] miRNAs circulate in the blood that can be easily collected from patients using non-invasive methods and therefore serve as useful biomarkers for diagnosis and treatment,[3,20,21] as well as monitoring surgical responses of patients with cancer.[22]

In this study, we identified miR-5088-5p as a novel oncogenic miRNA that directly regulates expression of DBC2 and is described for a potential signaling factor involved in breast cancer metastasis. In view of our collective results, miR-5088-5p is recommended as a potential biomarker for early diagnosis and treatment of metastatic breast malignancy.

Received 23 September 2020; accepted 5 May 2021;
https://doi.org/10.1016/j.omtn.2021.05.004.

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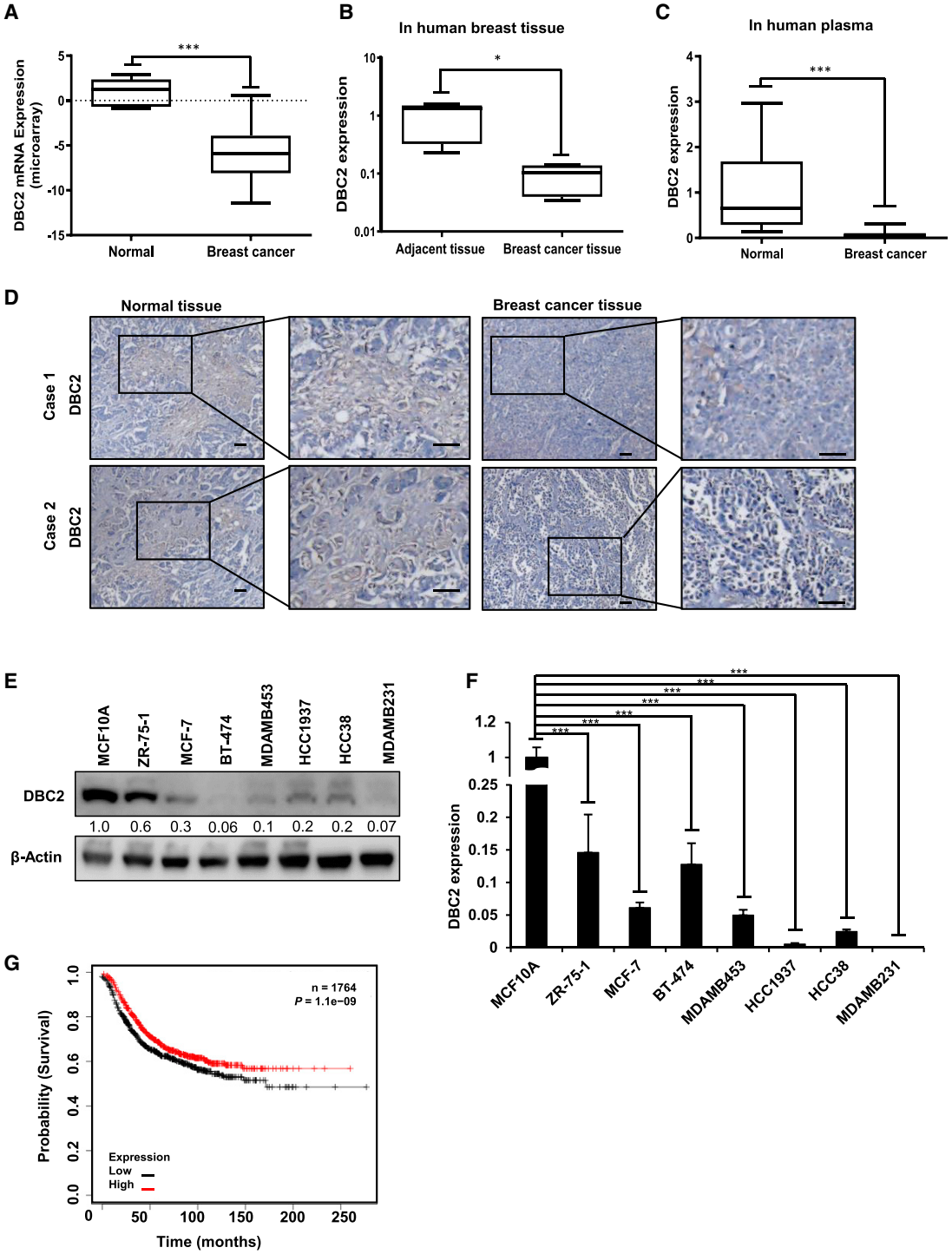

Figure 1. DBC2 expression is downregulated in breast cancer

(A) DBC2 expression was confirmed using public microarray data of breast cancer patients (normal, n = 6; breast cancer patients, n = 53; RePORTER: A_23_P20427; https://www.oncomine.org/resource/login.html). (B) In breast cancer patients, mRNA levels of DBC2 in adjacent and cancer tissues (n = 10, paired) were measured using qRT-PCR. (C) Levels of DBC2 expression in plasma of normal and breast cancer patients were confirmed by qRT-PCR (n = 14, paired). (D) The expression of DBC2 in normal and cancerous tissues of breast cancer patients was performed using IHC (scale bars, 100 μm). (E and F) Expression of DBC2 in various breast cancer cells was confirmed by

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RESULTS

DBC2 is downregulated in breast cancer

DBC2, which has a tumor-suppressor function,[7] is known to be downregulated in breast cancer,[8,9] so we used a public dataset to confirm that the expression of DBC2 was reduced in breast cancer patients rather than normal patients (Figure 1A). Examination of expression patterns of DBC2 (also known as RHOBTB2) in both tumor and normal adjacent tissues of patients with breast cancer revealed lower DBC2 levels in cancerous relative to adjacent tissues (Figure 1B). Histological analysis of used tumors and normal adjacent tissues of breast cancer patients was confirmed by hematoxylin and eosin (H&E) staining (Figure S1). Consistently, DBC2 expression in blood plasma of breast cancer patients was lower than that of normal controls (Figure 1C). The expression of DBC2 in adjacent normal and cancerous tissues in 2 sets of breast cancer patients was confirmed using immunohistochemistry (IHC), and its expression in breast cancer tissues was lower than that in normal tissues (Figure 1D). These results are consistent with previous studies showing that DBC2 expression is suppressed in 60% of breast cancer patients.[7] In addition, the expression of DBC2, according to the type of breast cancer cells, was confirmed. As a result, its expression was significantly lower in various breast cancer cells than in the normal breast cells, MCF10A. In particular, its expression levels were the lowest in HCC1937, HCC38, and MDA-MB-231, which are a triple-negative type of breast cancer cells (Figures 1E and 1F). According to The Cancer Genome Atlas (TCGA) analysis, DBC2 expression was lower in basal type ($Her2^+$) compared to luminal A and luminal B breast cancer subtypes (Figure S2). Kaplan-Meier (KM) curves using a public dataset showed poor prognosis of patients with low expression of DBC2 (Figure 1G) (http://kmplot.com/). The collective data clearly demonstrate downregulation of DBC2 in more aggressive breast cancer cell types.

DBC2 attenuates tumorigenicity in breast cancer

To determine the specific role of DBC2 in breast cancer, tumorigenic phenotypes of two breast cancer cell lines overexpressing DBC2 were examined. Migratory ability (Figure 2A) and invasiveness (Figure 2B) of DBC2-overexpressing MCF-7 and MDA-MB-231 cells was inhibited through suppression of mRNA levels of the invasion-related matrix metalloproteinases (MMP)-2 and MMP-9 (Figure 2C). DBC2 overexpression additionally induced a decrease in sphere formation capacity (Figure 2D), mesenchymal marker proteins (vimentin, Snail, and Twist), stemness-related factors (Oct4 and Sox2) (Figure 2E), and the angiogenesis-related markers (vascular endothelial growth factor [VEGF] and angiopoietin-2 [Ang2]) (Figure 2F). To examine the effects of DBC2 on metastasis *in vivo*, metastatic MDA-MB-231 cells overexpressing the gene were injected into mammary fat pads of nude mice. Notably, mice injected with DBC2-overexpressing cells exhibited significantly decreased lung nodule formation (Figure 2G). Our findings strongly indicate that DBC2 acts as a tumor suppressor and reduces tumorigenicity and metastasis of breast cancer, both *in vitro* and *in vivo*.

DBC2 expression is directly inhibited by miR-5088-5p highly expressed in breast cancer cells

TargetScan and Diana were employed to predict targeting miRNAs, with a view to elucidating the mechanisms underlying low expression of DBC2 in breast cancer (Figure 3A). Among the 16 miRNAs identified, the prediction probability of miR-5088-5p was highest using both tools. Higher expression of miR-5088-5p in breast cancer tissues was clearly detected, compared with adjacent normal tissues (Figure 3B). In addition, the expression of miR-5088-5p was highest in breast cancer patients with metastasis than in normal and breast cancer patients without metastasis (Figure 3C). We found that normal human mammary epithelial cells MCF10A and lumen A (ZR-75-1 and MCF-7), lumen B (BT-474), $Her2^+$ (MDA-MB-453), and triple-negative (HCC1937, HCC38, and MDA-MB-231) types of breast cancer cells were further investigated for miR-5088-5p expression (Figure 3D). Expression of miR-5088-5p was significantly higher in breast cancer cell lines relative to normal MCF10A cells. In addition, miR-5088-5p was more highly expressed in triple-negative-type cells compared with luminal-type cells. Kaplan-Meier curves (http://kmplot.com) showed poorer survival in patients with high expression of miR-5088-5p (Figure 3E). To ascertain whether miR-5088-5p targets DBC2, we examined DBC2 protein and mRNA levels after transfection with a miR-5088-5p mimic. The miR-5088-5p mimic induced a decrease in DBC2 protein (Figure 3F) and mRNA (Figure 3G) levels. Experiments were further performed using reporter constructs containing either wild type (WT) or mutant type (Mut) of DBC2 3′ UTRs predicted to bind miR-5088-5p (Figure 3H). Transduction of miR-5088-5p inhibited the activity by binding to the WT DBC2 3′ UTR but had no change in the Mut (Figure 3I). These results strongly suggest that DBC2 is a direct target of miR-5088-5p in breast cancer cells. It is reported that the mature miRNA binds with Ago2 protein to form RISC (RNA-induced silencing complex) and then binds to the 3′ UTR of the target to downregulate the expression of the target protein.[23] Since miR-5088-5p discovered in this study is a novel miRNA that has not been reported, Ago2 immunoprecipitation (IP) was performed as an additional experiment to verify DBC2 as a target of miR-5088-5p. Because RISC formed by binding of miR-5088-5p and Ago2 bound to target protein DBC2 3′ UTR, DBC2 expression in the miR-5088-5p-overexpression group was higher than that of the control group (Figure 3J) These results reconfirmed that miR-5088-5p directly targets and interacts with DBC2 in MCF-7 cells. Based on the results, we propose that miR-5088-5p is a direct negative regulator of DBC2 in breast cancer cells.

western blotting (E) and qRT-PCR (F) (MCF10A, human mammary epithelial cells; ZR-75-1 and MCF-7, luminal A; BT-474, luminal B; MDA-MB-453, $Her2^+$; HCC1937, HCC38, and MDA-MB-231, triple-negative type). β-actin using a loading control at western blotting. The qRT-PCR results were normalized by U6. (G) Kaplan-Meier analysis (http://kmplot.com; Affymetrix: 1554586) estimating the probability of survival of breast cancer patients based on DBC2 expression status. The data are presented as the mean ± SD. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. Student's t test.

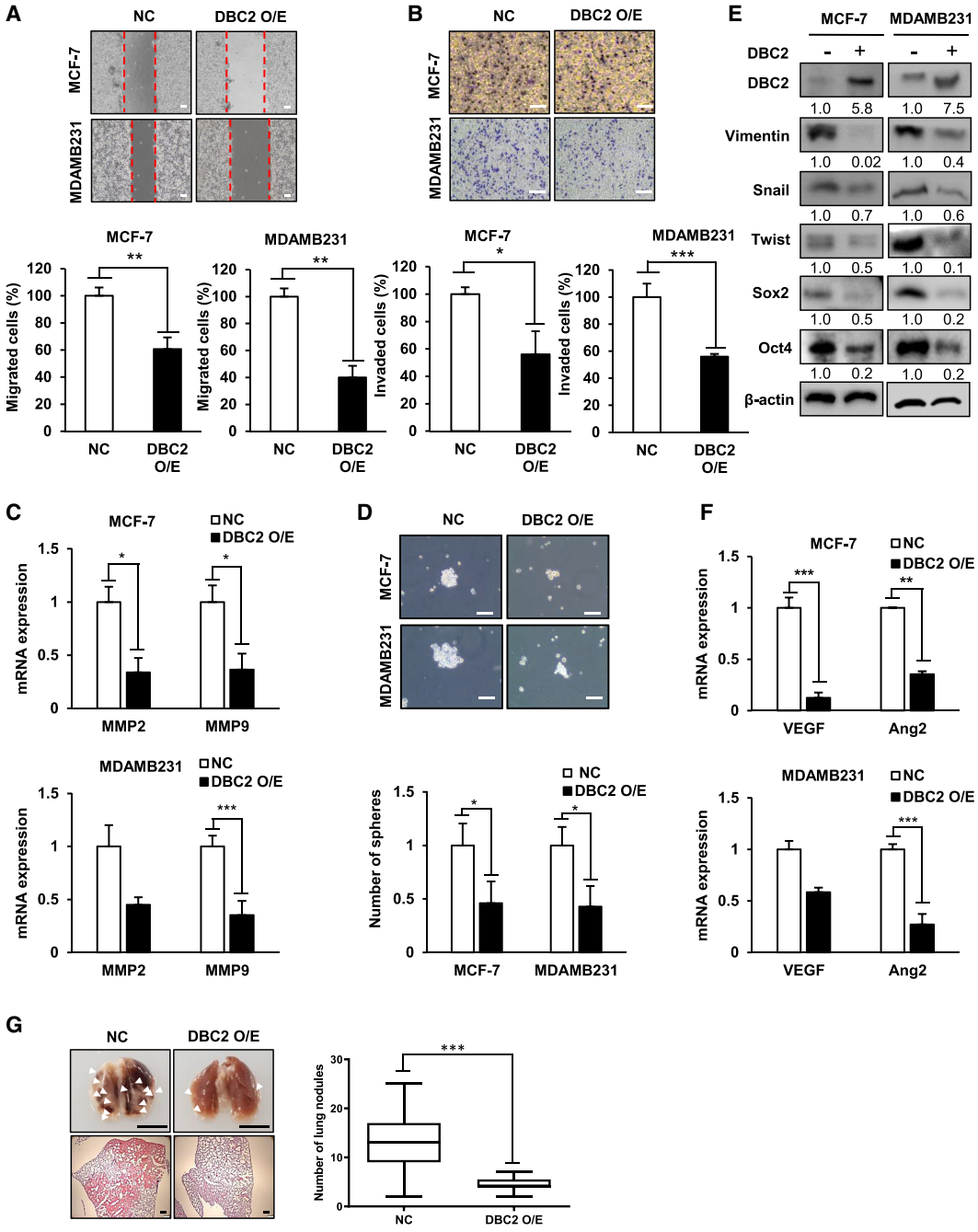

Figure 2. DBC2 decreases tumorigenicity and metastasis in MCF-7 and MDA-MB-231

(A–F) After MCF-7 and MDA-MB-231 cells were transfected with DBC2-overexpressing vector, migratory ability (A) and invasiveness (B) were confirmed by wound-healing assay and Matrigel invasion assay. (C) Invasiveness-related factors, MMP2 and MMP9, were tested by qRT-PCR. (D) Stemness was determined by sphere-formation assay. (E) Mesenchymal trait and stemness markers were confirmed by western blot assay. (F) Angiogenesis-related factors VEGF and Ang2 were confirmed by qRT-PCR. (G) After metastatic MDA-MB-231, cells overexpressed with DBC2 (1×10^6 cells/mouse) were injected into the mammary fat pads of nude mice. After 8 weeks, the metastatic pulmonary tissues were observed (scale bars, 0.5 cm), and hematoxylin and eosin (H&E) images (scale bars, 100 μm) (left panel) were stained. The number of metastatic lung nodules in xenograft mice (right panel) was counted (n = 11, paired). The data are presented as the mean ± SD. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. Student's t test.

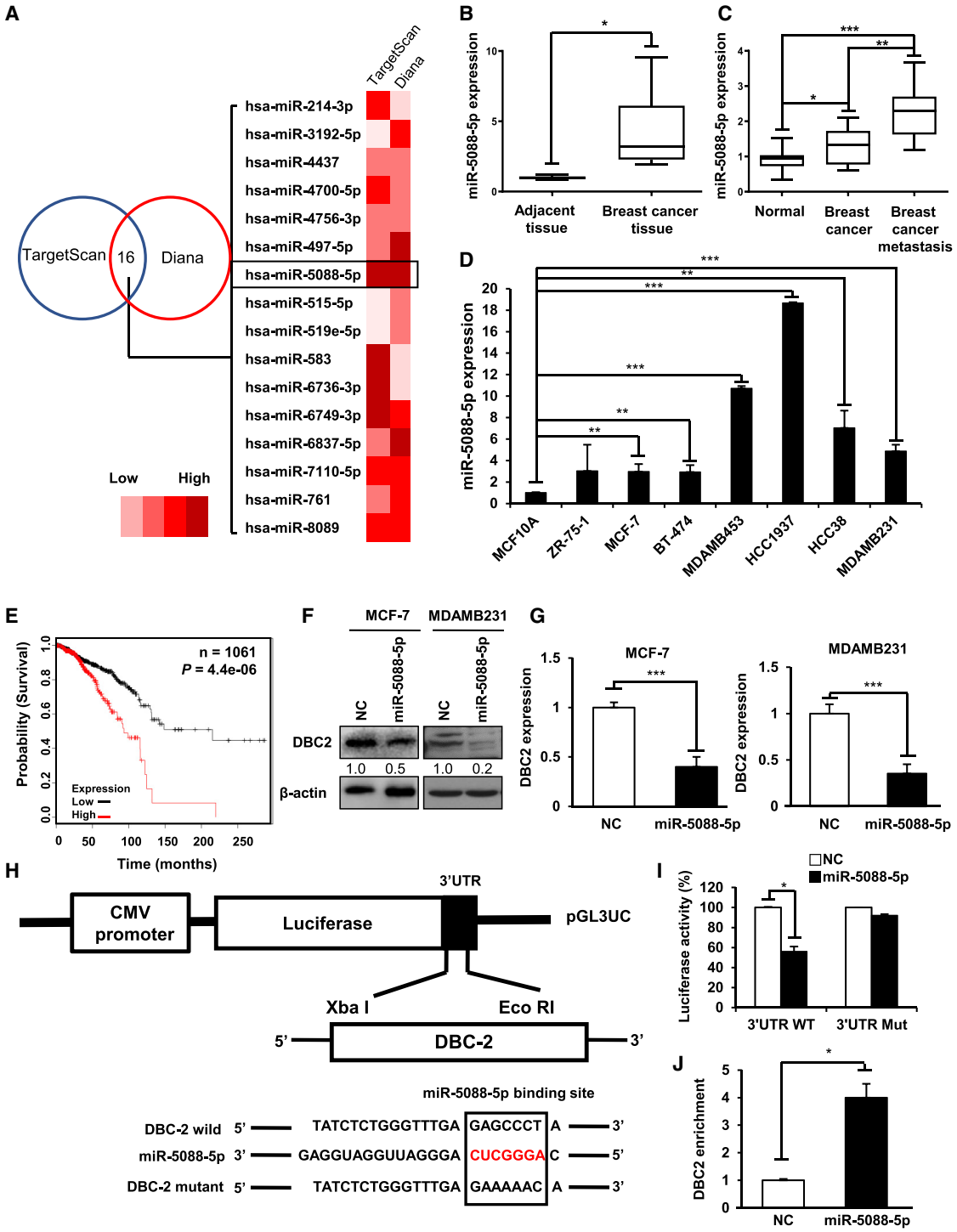

Figure 3. miR-5088-5p directly targets DBC2

(A) To predict miRNAs targeting DBC2, TargetScan (http://www.targetscan.org) and the Diana (http://diana.imis.athena-innovation.gr) were used as prediction tools and expressed as a heatmap. (B) In breast cancer patients and adjacent tissues (n = 6, paired), the level of miR-5088-5p expression was compared by qRT-PCR. (C) The expression of miR-5088-5p was confirmed by qRT-PCR in the plasma of normal, non-metastatic breast cancer patients and metastatic breast cancer patients (normal, n = 14; non-metastatic breast cancer patients, n = 10; metastatic breast cancer patients, n = 11). (D) Expression of miR-5088-5p in MCF10A, human mammary epithelial cells;

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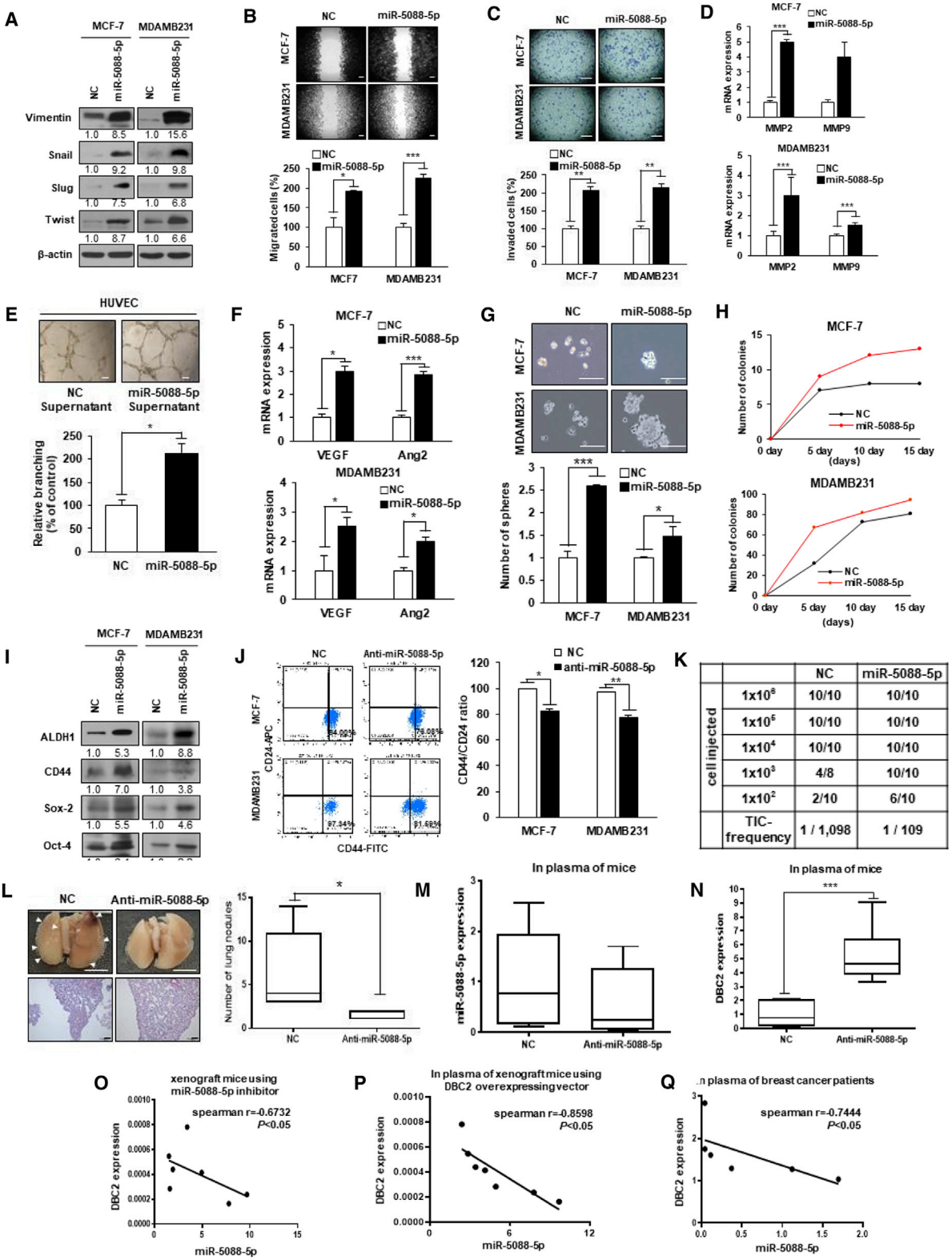
miR-5088-5p promotes tumorigenicity and metastasis in breast cancer *in vitro* and *in vivo*

To determine whether miR-5088-5p contributes to the aggressive properties of breast cancer cells, we evaluated the effects on miR-5088-5p-mediated tumorigenicity, including mesenchymal-related traits, migratory activity, invasiveness, angiogenesis, maintenance of stemness, and metastasis in both MCF-7 and MDA-MB-231 cell lines. Overexpression of miR-5088-5p enhanced the expression of EMT marker proteins, such as vimentin, Snail, Slug, and Twist (Figure 4A). Furthermore, migratory potential (Figure 4B) and invasive properties (Figure 4C) were increased through upregulation of the tumor invasion-associated enzymes MMP-2 and MMP-9 (Figure 4D). The effects of miR-5088-5p on angiogenesis were evaluated via the tube-formation assay with human umbilical vein endothelial cells (HUVECs) widely utilized as a model for the reorganization stage of angiogenesis.[24] Tube-forming capacity was increased in HUVECs treated with the supernatant secreted from MDA-MB-231 cells overexpressing miR-5088-5p compared to negative controls (NCs) (Figure 4E). Expression levels of VEGF and Ang2 were upregulated in miR-5088-5p-overexpressing cells (Figure 4F). Our findings indicate that miR-5088-5p is involved in acquisition of angiogenic properties in human breast cancer cells. To confirm the role of miR-5088-5p on the maintenance of stemness, both cell lines were transfected with miR-5088-5p mimic, followed by sphere-forming and three-dimensional culture assays, expression of CSC markers, analysis of subpopulation ($CD44^+/CD24^-$), and limiting dilution assay being performed. Notably, sphere-formation abilities were increased in miR-5088-5p-overexpressing cells compared to their negative control counterparts (Figure 4G). In anchorage-independent growth, colony-forming ability was enhanced in miR-5088-5p-overexpressing cells (Figure 4H). Moreover, expression levels of CSC markers, such as ALDH1, CD44, Sox2, and Oct4, were increased in miR-5088-5p-overexpressing cells (Figure 4I). The analysis of the $CD44^+/CD24^-$ subpopulation utilizes CD44, which is known as a representative marker of breast CSCs and is confirmed by fluorescence-activated cell sorting (FACS).[25] The $CD44^+/CD24^-$ population of MCF-7 and MDA-MB-231 cells that knocked down miR-5088-5p using the miR-5088-5p inhibitor decreased by 17.5% and 19.6%, respectively, compared to negative control (Figure 4J). Limiting dilution analysis was performed to prove the effect of miR-5088-5p on the ability of tumor formation as tumor-initiating cells. MDA-MB-231 cells overexpressing miR-5088-5p with Matrigel were orthotopically injected into a mammary fat pad of a BALB/c mouse with various cell numbers (1×10^6, 1×10^5, 1×10^4, 1×10^3, and 1×10^2 cells/mouse), and tumor initiation ability was confirmed. As a result, it was confirmed that the tumor-initiation ability of the group overexpressing miR-5088-5p was increased compared to the control group (Figure 4K). In view of these findings, we propose that miR-5088-5p mediates the maintenance of stemness in a stem-like cell population of human breast cancer cells. To determine the effects of miR-5088-5p on metastasis *in vivo*, metastatic MDA-MB-231 cells transfected with miR-5088-5p inhibitor or negative control were implanted orthotopically into nude mice that were sacrificed 55 days after implantation. Histological examination of metastatic pulmonary tissues via H&E staining revealed a lower density in mice injected with miR-5088-5p inhibitor-transfected cells compared to those administered negative control cells (Figure 4L, left). Moreover, the number of metastatic nodules in the lung was significantly decreased in mice administered miR-5088-5p knockdown cells compared with the WT cell-injected group (negative control) (Figure 4L, right). mRNA and miRNA are known as a circulating factor in the plasma,[3,26,27] as well as the cytoplasm, which presents the advantage of being easy to obtain from cancer patients.[28,29] Therefore, it is known that mRNA and miRNA are used as markers to confirm the prognosis of patients.[30] The relationship between DBC2 and miR-5088-5p expression was further verified using plasma from mice and breast cancer patients. Expression of miR-5088-5p was suppressed in plasma of mice treated with the miR-5088-5p inhibitor compared with negative control (Figure 4M). Conversely, DBC2 was upregulated in plasma of mice transfected with the miR-5088-5p inhibitor compared with negative control, as observed using qRT-PCR (Figure 4N). Data from Spearman's correlation analysis showed a negative correlation between DBC2 and miR-5088-5p expression patterns in plasma of xenograft mice injected with cells transfected with the miR-5088-5p inhibitor (Figure 4O) or DBC2-overexpressing vector (Figure 4P), as well as breast cancer patients (Figure 4Q). These findings strongly support the feasibility of clinical application of DBC2 and miR-5088-5p expression analysis in blood of cancer patients. In addition, miR-5088-5p-overexpressing cells displayed increased colony-formation ability (Figure S3A) and proliferation (Figure S3B), as determined from clonogenicity and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, respectively. To establish the effect of miR-5088-5p on drug resistance, tumor cell growth was measured over time after treatment with etoposide on MCF-7 and MDA-MB-231 cells. As a result, two cells overexpressing miR-5088-5p showed resistance to drugs by inhibiting etoposide-induced cell death (Figure S3C). To verify an *in vivo* etoposide-resistance assay, MDA-MB-231 cells overexpressing miR-5088-5p were injected subcutaneously into a nude mice model to create tumors. As a result of treatment with the etoposide, 3 times every 5 days, it was confirmed that tumor weight did not decrease in the miR-5088-5p-overexpressed group compared to the control group. This result confirmed *in vivo* that overexpression of miR-5088-5p increased resistance to etoposide

ZR-75-1 and MCF-7, luminal A; BT-474, luminal B; MDA-MB-453, $Her2^+$; HCC1937, HCC38, and MDA-MB-231, triple-negative type of breast cancer cells) was confirmed by qRT-PCR. The results were normalized by U6. (E) Kaplan-Meier curves estimated the probability of survival of breast cancer patients based on miR-5088-5p expression status. (F and G) After overexpression of miR-5088-5p mimic in MCF-7 and MDA-MB-231 cells, protein (F) and mRNA (G) levels of DBC2 were confirmed by western blot analysis and qRT-PCR, respectively. (H) Structure of reporter constructs containing DBC2 3′ UTR downstream of the luciferase open reading frame (ORF). (I) Luciferase activity was determined in MCF-7 cells transfected with firefly luciferase constructs containing wild-type (WT) or mutant (Mut) putative binding sites of DBC2 3′ UTR, and negative control (NC) or miR-5088-5p is shown. Firefly luciferase activity was normalized to *Renilla* luciferase activity for each sample. (J) To confirm the direct binding of DBC2 and miR-5088-5p, an AGO2 immunoprecipitation assay was conducted in MCF-7.



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(Figure S3D). In an earlier study, Ma et al.[31] reported the involvement of several miRNAs in drug resistance of cancer cells. These results support the hypothesis that miR-5088-5p is linked to treatment resistance and high mortality of patients through promoting cancer cell survival. Our study provides compelling evidence that miR-5088-5p enhances primary breast cancer metastasis to the lung by stimulating expression of the components related to mesenchymal traits, migration, invasiveness, angiogenesis, stemness maintenance, and proliferation and mediates the metastatic capacity of cancer cells *in vivo*.

DBC2 attenuates miR-5088-5p-induced tumorigenicity

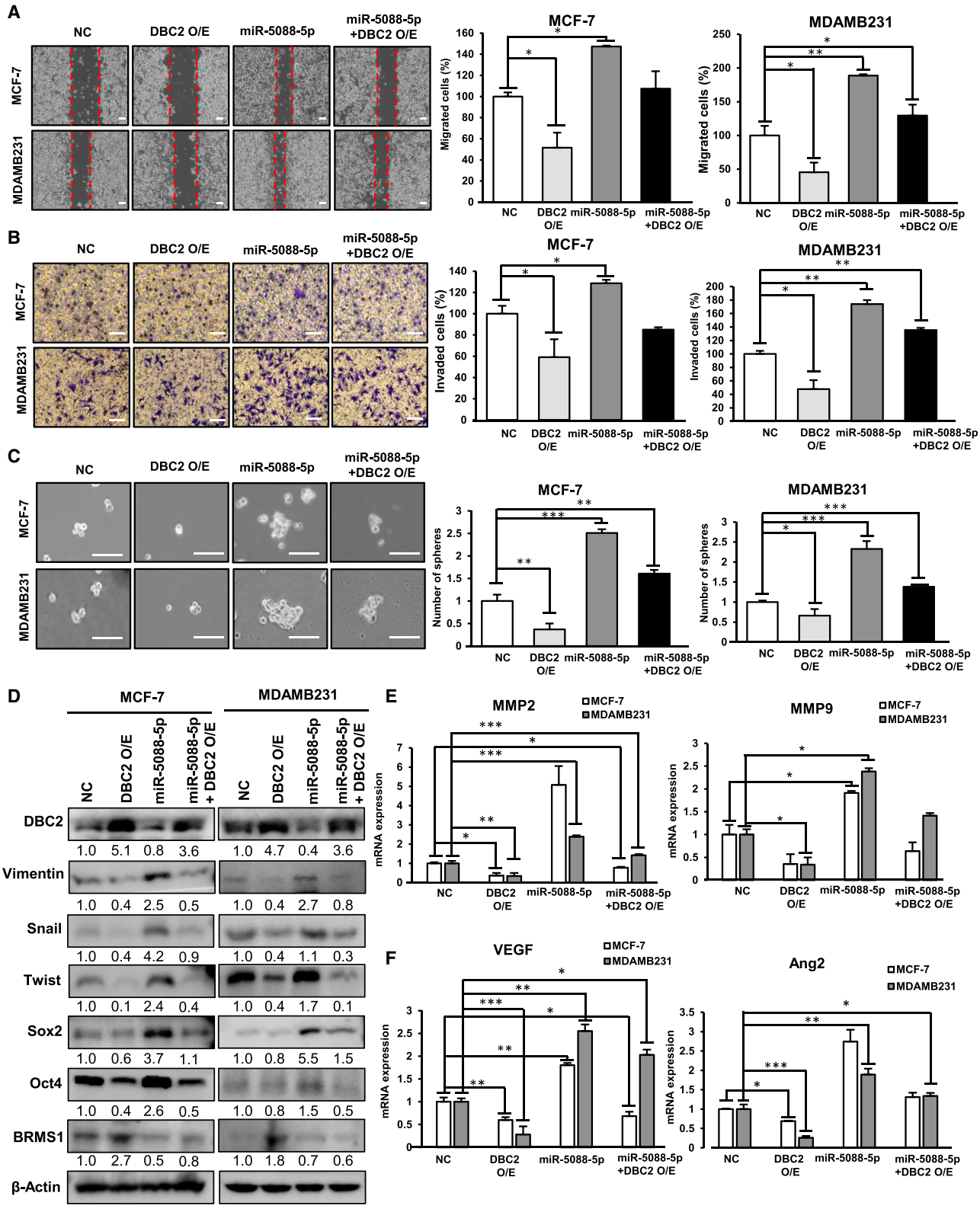
DBC2-overexpression experiments were performed to confirm its relationship with miR-5088-5p in breast cancer. Overexpression of DBC2 inhibited miR-5088-5p-induced cell migration (Figure 5A) and invasion (Figure 5B) through suppressing the expression of MMP-2 and MMP-9 transcripts (Figure 5E); stemness maintenance, including sphere-formation ability (Figure 5C); and CSC markers, EMT-related proteins (Figure 5D), and angiogenesis-related factors (Figure 5F). BRMS1 is known to be involved in the mechanism of suppressing migration and invasion as a downstream signaling factor of DBC2.[32] To elucidate the relationship between miR-5088-5p or DBC2 and BRMS1, the expression of BRMS1 was confirmed after overexpression of miR-5088-5p or DBC2. As a result, it was confirmed that DBC2-induced BRMS1 was inhibited by miR-5088-5p mimic (Figure 5D). Our results suggest that miR-5088-5p directly interacts with and downregulates DBC2 to enhance tumorigenic properties of breast cancer cells, supporting the theory that miR-5088-5p promotes tumor cell aggressiveness through suppressing DBC2 expression.

Fyn promotes biogenesis of miR-5088-5p by inducing its hypomethylation

To identify the factors affecting biogenesis of miR-5088-5p, its expression was examined in MCF-7 and MDA-MA-231 cells under conditions of knockdown of oncogenes (using small interfering [si] RNAs against Lyn, Fyn, and EGFR; LY294002, a phosphatidylinositol 3-kinase [PI3K] inhibitor; SB203580, a p38 inhibitor; and SP600125, a JNK] inhibitor) activated in various cancers. miR-5088-5p expression was dramatically decreased in siFyn-transfected MCF-7 and MDA-MB-231 cells (Figure 6A). Fyn is a Src family tyrosine kinase that participates in numerous biological processes and acts as an oncogene in breast cancer and other tumor types.[33–37] We showed that the expression of Fyn was higher in breast cancer patients than in normal using public microarray data (Figure 6B). In addition, the expression of Fyn was examined in four breast cancer types using an *in silico* TCGA dataset. Our data showed higher expression of Fyn in triple-negative type compared with luminal type in breast cancer patients (Figure 6C). To further examine the potential relationship between biogenesis of miR-5088-5p and Fyn, expression levels of primary, precursor, and mature forms of miR-5088-5p were assessed after knockdown or overexpression of Fyn in MCF-7 and MDA-MB-231 cells. Notably, expression of all three forms of miR-5088-5p was decreased upon suppression of Fyn (Figure 6D) and conversely increased with overexpression of Fyn (Figure 6E). To establish the underlying cause of the increase in miR-5088-5p expression by Fyn, the methylation status of miR-5088-5p CpG islands was determined using methylation-specific PCR (MSP) and quantitative MSP (qMSP). We obtained a schematic map of the miR-5088-5p promoter including the locations of CpG islands (Figure S4A). Knockdown of Fyn increased the methylation of its promoter in MCF-7 and MDA-MB-231 cells (Figure S4B). In contrast, overexpression of Fyn significantly reduced miR-5088-5p promoter methylation (Figure S4C). We investigated whether DNA methyltransferases (DNMTs) are involved in the hypomethylation of the miR-5088-5p promoter by Fyn. It was confirmed that overexpression of Fyn decreased the expression of DNMT1 and DNMT3b (Figure S4D). From these results, it could be suggested that overexpression of Fyn could inhibit the methylation of the miR-5088-5p promoter by reducing the expression of DNMT1 and DNMT3b.

Figure 4. miR-5088-5p enhances tumorigenicity and metastasis *in vitro* and *in vivo*

(A–I) After MCF-7 and MDA-MB-231 cells were transfected with negative control or miR-5088-5p mimic, expressions of EMT-related proteins were detected by western blot analysis (A), migration (B), and invasion assay (C). Expressions of MMP-2 and MMP-9 mRNA were determined using qRT-PCR (D), performed in indicated cells. β-actin and U6 were used as loading control in western blotting and qRT-PCR, respectively. (E) HUVECs were seeded into Matrigel-coated 96-well plates (1×10^4 cells/well) and then incubated with supernatants of MDA-MB-231 cells transfected with miR-5088-5p mimics or negative control for 16 h (scale bars, 100 μm). Averaged numbers of capillary tube branches in eight random fields are counted and shown as a graph. (F) Angiogenesis-related factors, VEGF and Ang2 mRNA, were detected by qRT-PCR analysis. (G) For sphere-formation assay, indicated treated cells were seeded onto 100 mm culture dishes (1×10^5 cells/dish) and cultured for 5∼10 days. These experiments were performed in triplicate, and the mean value was shown (scale bars, 500 μm). (H) To determine anchorage-independent cell growth, indicated cells were seeded on precoated 24-well plates with 60 μL of Matrigel and grown for 15 days in MCF-7 and MDA-MB-231 cells. Colony number was counted every 5 days. These experiments were performed in triplicate. (I) Cancer stem-like cell marker proteins containing ALDH1, CD44, Sox2, and Oct4 were determined in MCF-7 and MDA-MB-231 cells by western blot analysis. β-actin was used as loading control. (J) After the $CD44^+/CD24^-$ analyzed by FACS (left panel), the graph quantified the $CD44^+/CD24^-$ ratio (right panel). (K) For limiting dilution analysis, MDA-MB-231 cells overexpressing miR-5088-5p with Matrigel were orthotopically injected into a mammary fat pad of the BALB/c mouse with various cell numbers (1×10^6, 1×10^5, 1×10^4, 1×10^3, and 1×10^2 cells/mouse). After 4 weeks, tumor formation was confirmed, and mice were sacrificed. Tumor-initiating frequency (TIF) was calculated using ELDA software (http://bioinf.wehi.edu.au/software/elda/). (L) Anti-miR-5088-5p-transfected metastatic MDA-MB-231 cells (1×10^6 cells/mouse) were orthotopically injected into the mammary fat pads of 8-week-old nude mice. After implantation, mice were sacrificed at 8 weeks. Pulmonary metastatic nodules (scale bars, 0.5 cm) are shown as box-and-whisker plots (L, left panel), and pulmonary tissues of mice were stained with H&E (scale bar, 100 μm) (L, right panel) (n = 5, animals for group), respectively. (M and N) Expression of levels of miR-5088-5p (M) and DBC2 (N) in the plasma of mice (negative control and anti-miR-5088-5p, n = 5) was confirmed by qRT-PCR. (O–Q) With the use of Spearman's correlation analysis, the negative correlation between DBC2 and miR-5088-5p was analyzed in plasma of a xenograft mouse model using the miR-5088-5p inhibitor (negative control and anti-miR-5088-5p, n = 7; O) or DBC-overexpressing vector (negative control and DBC overexpression, n = 7; P) and in normal and breast cancer patients' plasma (normal and breast cancer patients, n = 6; Q). The data are presented as the mean ± SD. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Student's t test.



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As confirmed at the cellular level as well as in patient samples, the methylation of the miR-5088-5p promoter was lower in cancer tissues than in adjacent normal tissues in breast cancer patients (Figure S4F).

To validate this finding, cells were treated with 5-Aza-2′-deoxycytidine (5-Aza), an inhibitor of DNA methylation, followed by western blot analysis and qRT-PCR. As a result, the expression of DNMT1 and DNMT3b proteins and mRNA was reduced (Figures S5A and S5B). Two cells were treated with 5-Aza and analyzed by MSP (Figure S5C) and qMSP (Figure S5D). For the accuracy of MSP analysis, double-knockout (DKO) DNMT1$^{-/-}$ DNMT3B$^{-/-}$ in HCT116 cells[38] as unmethylation control, IVD (*in vitro*-methylated DNA) as methylation control, and double-distilled water (ddH_2O) as PCR-negative control were used. As a result, the methylation of the miR-5088-5p promoter was decreased (Figures S5C and S5D), and miR-5088-5p expression was increased (Figure S5E). These results showed that Fyn decreased DNMT, leading to hypomethylation of the miR-5088-5p promoter and eventually increasing the expression of miR-5088-5p. Our findings are consistent with those of a previous report that Fyn-related kinase is associated with methylation at specific CpG islands of the promoter site.[39]

To ascertain the association between signaling mechanisms of miR-5088-5p and Fyn, an miR-5088-5p inhibitor and Fyn-overexpressing vector were used. EMT- and stemness-related markers suppressed by the miR-5088-5p inhibitor were restored upon Fyn overexpression (Figure 6F). As shown in Figure 6G, Fyn suppressed DNA methylation of the miR-5088-5p promoter, ultimately leading to increased expression, which in turn, promoted malignant activity and metastasis through inhibition of DBC2 expression in breast cancer.

DISCUSSION

MBC spreads to other organs of the body, including lung, liver, brain, and bone, through the circulatory and lymphatic systems and increases mortality rates accounting for 90% of deaths in patients with solid tumors.[40] Metastasis to bone is reported in up to 50% breast cancer patients, which is the first site of distal metastasis, with the second and third metastatic sites identified as lung and liver, respectively[1,41].

DBC2 is a tumor-suppressor gene with low expression in breast cancer[42] and a target gene related to tumor development and progression.[43] In the current study, we focused on the role and mechanism of action of DBC2 in breast cancer. DBC2 expression was markedly lower in breast cancer patients than normal controls (Figures 1A and 1B) and in the Her2^{+} type than luminal type of breast cancer (Figure S2). These phenomena are potentially with DBC2-mediated suppression of cell migration, invasion, and cancer stemness (Figure 2). Consistent with our findings, DBC2 gene expression is reported to be significantly reduced in breast and bladder cancer via methylation[43,44] and classified as a tumor suppressor[42,45] that inhibits malignant activities, such as cell migration and invasion.[9,43,46] DBC2 may therefore serve as an effective biomarker for cancer therapy.[42,47]

Recently, dysregulation of circulating miRNAs was shown to contribute to metastatic progression in breast cancer.[3,27] Since miRNAs target multiple genes and circulate in the blood that can be easily collected via non-invasive methods, they present useful sources of biomarkers for diagnosis, treatment,[3,20,21] and monitoring surgical responses of patients with cancer.[22] Here, we identified miR-5088-5p, a miRNA targeting DBC2, as a tumor-suppressor gene (Figures 3A and 3F–3J). Expression of miR-5088-5p was higher in breast cancer than adjacent tissues (Figure 3B). Since circulating miRNAs have the potential to be used as biomarkers for disease diagnosis,[22] the expression of miR-5088-5p discovered in this study was confirmed in the plasma of breast cancer patients. As a result, the expression of miR-5088-5p was higher in breast cancer patients than in normal, and its expression was highest in breast cancer patients with metastasis (Figure 3C). When this was verified in breast cancer cell lines, the expression of miR-5088-5p was increased in various breast cancer cells compared to normal human mammary epithelial cells (Figure 3D). This miRNA is associated with mesenchymal traits, cell migratory ability, invasiveness, angiogenesis, stemness, proliferation, chemoresistance, and metastasis. We observed a clear negative correlation between miR-5088-5p and DBC2. Upregulation of miR-5088-5p and conversely downregulation of DBC2 promoted tumorigenesis, ultimately leading to metastasis (Figure 4; Figure S2). The association between miR-5088-5p and DBC2 was further confirmed using the DBC2-overexpressing vector and miR-5088-5p mimic (Figure 5). Our collective results clearly suggest that miR-5088-5p functions as an onco-miRNA by suppressing DBC2 expression. Consistent with previous findings, oncogenic miRNAs in breast cancer, such as miR-181a,[48] promote tumorigenesis in animal models.

The mechanisms underlying alterations in miRNA expression patterns are regulated by several transcription factors[49] or methylation of gene-regulatory regions.[50] For instance, hypermethylation of miR-132 is associated with poor prognosis of colorectal cancer.[51] The tumor suppressors miR-34b and miR-34c are methylated, especially in chronic lymphocytic leukemia.[52] Methylation of the promoter of miR-210 inhibits its expression and is critical in angiogenesis.[53] In addition, growth factors, including platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β, and brain-derived neurotrophic factor (BDNF), stimulate primary miRNA

Figure 5. Overexpression of DBC2 inhibits miR-5088-5p-induced tumorigenic phenotypes in breast cancer cells

(A–E) After transfection of miR-5088-5p mimic and/or DBC2-overexpressing vector to MCF-7 and MDA-MB-231 cells, the migratory (A), invasive (B), and sphere-formation abilities (C) were detected by wound-healing assay (A; scale bars, 100 μm), Matrigel invasion assay (B; scale bars, 100 μm), and sphere-formation assay (C; scale bars, 100 μm), respectively. (D) Expressions of mesenchymal-related proteins; cancer stem-like, cell-related marker proteins; and BRMS1, known as the factor regulated by DBC2, were confirmed by western blotting. The mRNA levels of invasion-related markers MMP2 and MMP9 (E) and angiogenesis-related markers VEGF and Ang2 (F) were identified by qRT-PCR. The data are presented as the mean ± SD. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. Student's t test.

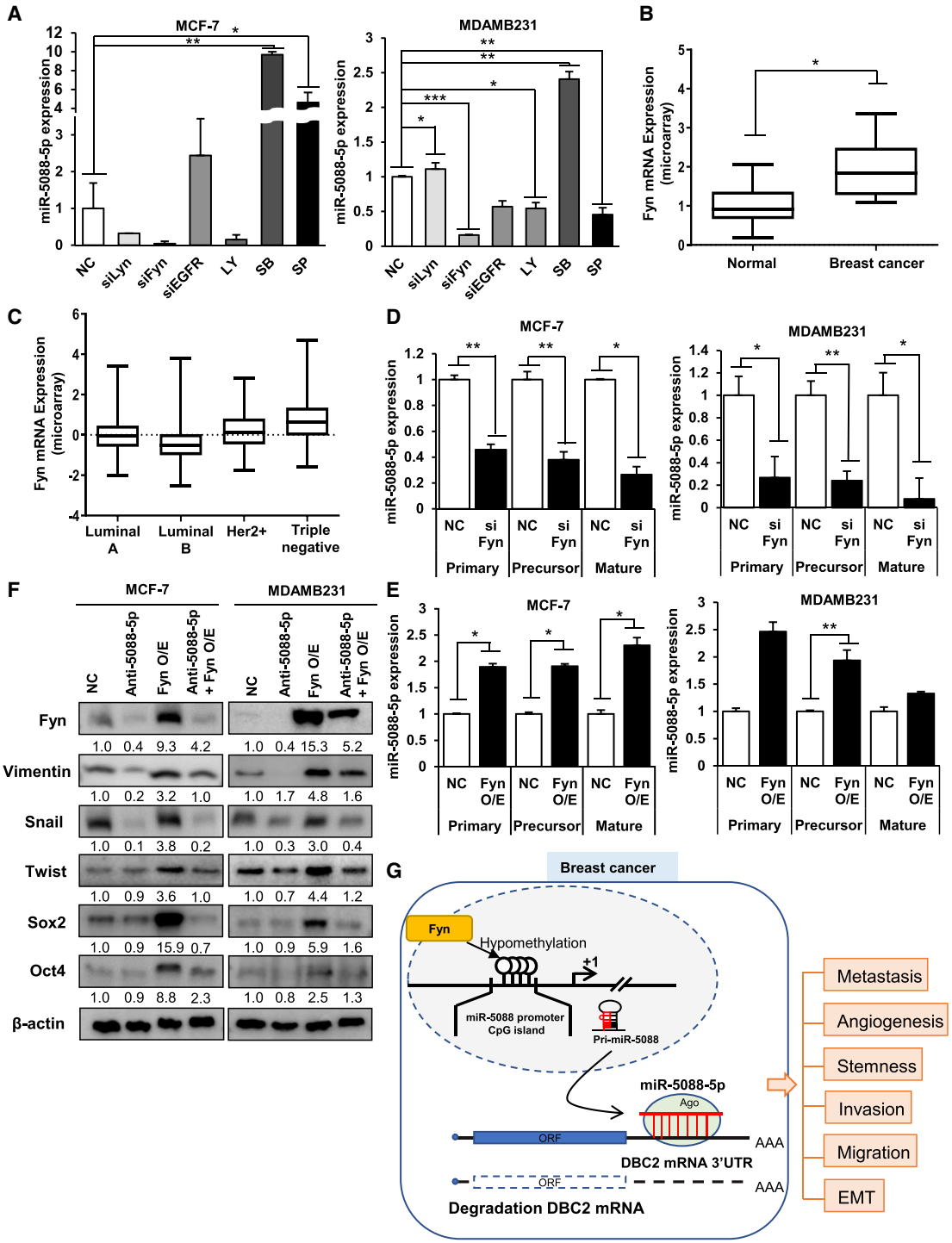

Figure 6. Fyn promotes biogenesis of miR-5088-5p by inhibiting its methylation

(A) Levels of miR-5088-5p expression were measured in MCF-7 and MDA-MB-231 cells, which were transfected with siRNAs against Lyn, Fyn, and EGFR (10 nM) and treated with inhibitors of PI3K (LY294002), p38 (SB203580), and JNK (SP600125) (20 nM) by qRT-PCR. (B) Fyn expression was confirmed in public microarray data of breast cancer patients (normal, n = 14; breast cancer patients, n = 11; RePORTER: Hs.169370.1.A2_3p_a_at; https://www.oncomine.org/resource/login.html). (C) The pattern of

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(pri-miRNA) transcription and contribute to modulation of miRNAs and regulatory gene transcription.[54,55]

Fyn, characterized as a regulator of miR-5088-5p, is highly expressed in the triple-negative type, compared with luminal-type breast cancer patients, as observed using an *in silico* TCGA dataset (Figure 6C). A member of the protein-tyrosine kinase oncogene family, Fyn is involved in several biological processes and acts as an oncogene.[33–37] Our data strongly suggest that Fyn induced the biogenesis of miR-5088-5p by reducing its promoter methylation through inhibiting DNMTs (Figures 6D and 6E; Figures S4 and S5).

Theoretical and experimental studies on miRNA expression mechanisms are essential since these molecules serve as potential targets for diagnosis, prognosis, and treatment of various diseases. In this study, we have identified a novel miRNA that directly targets DBC2 and promotes malignancy in breast cancer, miR-5088-5p, which may be effectively utilized as a diagnostic biomarker for metastatic disease (Figure 6G).

MATERIALS AND METHODS

Cell cultures

The human mammary epithelial cells (MCF10A) were obtained from American Type Culture Collection (Manassas, VA, USA). Breast cancer cell lines (ZR-75-1, MCF-7, BT-474, MDA-MB-453, HCC1937, HCC38, and MDA-MB-231) were obtained from the Korea Cell Line Bank (KCLB). MCF10A cells are cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 media (Thermo Fisher Scientific, Invitrogen, USA) supplemented with 100 ng/mL cholera toxin, 20 ng/mL EGF, 0.01 mg/mL insulin, 500 ng/mL hydrocortisone, and 5% Chelex-treated horse serum. ZR-75-1, BT-474, MDA-MB-453, and MDA-MB-231 cells were maintained in DMEM (Corning, Corning, NY, USA), and MCF-7, HCC1937, and HCC38 cells were maintained in RPMI (Corning, Corning, NY, USA). HUVECs were grown in endothelial cell growth medium MV2 with supplement mix (Promo Cell, Heidelberg, Germany). All medium was supplemented with 10% fetal bovine serum (FBS; Tissue Culture Biologicals, Tulare, CA, USA) and 1% penicillin-streptomycin antibiotics (Corning, Corning, NY, USA). Cells were maintained in a humidified incubator of 5% CO_2 at 37°C.

Antibodies and pharmacological inhibitors

Antibodies against β-catenin, vimentin, Snail, Sox2, Oct4, and DNMT1 were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against β-actin and Fyn were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against Twist was purchased from Abcam (Cambridge, MA, USA). Antibody against DBC2 was purchased from Bioss Antibodies (Woburn, MA, USA). The pharmacological inhibitors were used in this study; inhibitors of PI3K (LY294002) and JNK (SP600125) were purchased from Merck Millipore (Darmstadt, Germany). The inhibitor of p38 (SB203580) was obtained from Enzo Life Sciences (Lausen, Switzerland). The inhibitor of DNMT1 (5-Aza) was obtained from Abcam (Cambridge, MA, USA).

Plasmid DNA, RNA oligoribonucleotides, and transfection

To make DBC2- or Fyn-overexpressing vector, the DBC2 or Fyn gene was inserted to the pCMV-tag2 vector, respectively. Each sequence of primers for plasmid constructs was as follows: DBC2 (forward) 5′-CGCGGATCCATGCAAGCCTGGAGAAAAGGCCC-3′ and (reverse) 5′-GAATTCTCAGACCACAGCCGAGGAGGAAG-3′; Fyn (forward) 5′-CGCGGATCCATGGGCTGTGTGCAATGTAAG-3′ and (reverse) 5′-CGGAATTCTTACAGGTTTTCACCAGGTTGG-3′.

Synthetic miRNA mimics or inhibitors were synthesized by IDT (Integrated DNA Technologies; Coralville, IA, USA) as RNA duplexes designed from the sequences of miR-5088-5p (5′-CAGGGCUCAGGGAUUGGAUGGAGG-3′) using 5′-UUCUUCGAACGUGUCACGUTT-3′ for the negative control. The inhibitor of miR-5088-5p was a 2′-O-methyl-modified oligoribonucleotide single strand with the sequence as 5′-CUCCAUCCAAUCCCUGAGCCCU-3′. All siRNAs (Lyn, Fyn, and EGFR) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). siRNA (20 μM), miRNAs (10 μM), and plasmids were introduced into cells using Lipofectamine 2000 reagent (Thermo Fisher Scientific, Invitrogen, USA) according to the manufacturer's instructions.

Western blotting

Cells were lysed using radioimmunoprecipitation assay (RIPA) buffer with a protease inhibitor cocktail tablet (Roche, Indianapolis, IN, USA). Protein concentration was analyzed using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). Total protein extract was separated by SDS-PAGE (polyacrylamide gel electrophoresis), transferred to the PVDF (polyvinylidene difluoride) membrane (Millipore, Bedford, MA, USA), and blocked in 5% BSA (bovine serum albumin) in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20). The indicated primary and secondary antibodies were reacted as 1:1,000∼10,000 and detected chemiluminescence with an ECL (enhanced chemiluminescence) system (Thermo Scientific, Pierce, USA) by an Amersham Imager 600 system (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA).

RNA extraction and quantitative real-time PCR

Total RNA was isolated from MCF-7 and MDA-MB-231 using TRIzol reagent (Molecular Research Center, Cincinnati, OH, USA) following the manufacturer's introduction. The cDNAs were synthesized using

Fyn expression was confirmed in four types of breast cancer patients using TCGA dataset (luminal A, 619 cases; luminal B, 603 cases; $Her2^+$, 188 cases; triple-negative type, 290 cases). (D and E) Primary, precursor, and mature forms of miR-5088-5p were identified in MCF-7 and MDA-MB-231 after knockdown (D) or overexpression (E) of Fyn using siRNA or overexpressing vector, respectively. (F) After transfection with miR-5088-5p inhibitor and/or Fyn-overexpressing vectors in MCF-7 and MDA-MB-231 cells, the expression of EMT- and stemness-related proteins was measured by western blot analysis. The data are presented as the mean ± SD. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. Student's t test. (G) Scheme of miR-5088-5p-induced tumorigenicity and metastasis by suppressing DBC2 in breast cancer.

the Tetro cDNA Synthesis Kit (Bioline, London, UK), and the miRNAs were synthesized using the Mir-X miRNA First-Stand cDNA Synthesis Kit (Takara, Japan) for mRNA or miRNA detection, respectively. The real-time PCR was performed using the DNA Engine Optocon2 System (Bio-Rad Laboratories, Hercules, CA, USA) following the manufacturer's instructions. The primers of GAPDH and U6 small nuclear RNA (snRNA; Takara, Japan) were used for normalized mRNA and miRNA, respectively. All data were analyzed by the $2^{-\Delta\Delta Cr}$ method. The sequences of primers were as follows: DBC2 (forward) 5′-CAG AGCAGTAGACAGTGACC-3′ and (reverse) 5′-TGTAGTTGGTGC AGATGTGG-3′; MMP-2 (forward) 5′-CATCAAGGGCATTCAGGA GC-3′ and (reverse) 5′-AGAACACAGCCTTCTCCTCC-3′; MMP-9 (forward) 5′-AGGACGACGTGAATGGCATG-3′ and (reverse) 5′-AT CGTCCACCGGACTCAAAG-3′; VEGF (forward) 5′-GACAGACAG ACAGACACCGCC-3′ and (reverse) 5′-GAACAGCCCAGAAGTTG GACG-3′; Ang2 (forward) 5′-GCAAGTGCTGGAGAACATCA-3′ and (reverse) 5′-CACAGCCGTCTGGTTCTGTA-3′; pri-miR-5088-5p (forward) 5′-CCTCTGCATG TTTGCTGCCA-3′ and (reverse) 5′-TGAGGGCCCAGGAAGAAGGGA-3′; precursor-miR-5088-5p (forward) 5′-CAGGGCTCAGGGATTGGATGGAGG-3′ and (reverse) 5′-TGAGGGCCCAGGAAGAAGGGA-3′; mature-miR-5088-5p 5′-C AGGGCTCAGGGATTGGATGGAGG-3′; and GAPDH (forward) 5′-CATCTCTGCCCCCTCTGCTGA-3′ and (reverse) 5′-GGATGAC CTTGCCCACAGCCT-3′.

H&E staining and IHC

Mouse and patient breast cancer tissues were fixed with 4% paraformaldehyde, followed by paraffin fixation and sectioning. Paraffin slides were dissolved, rehydrated, and stained with H&E (Thermo Fisher Scientific, Waltham, MA, USA).

For IHC, slides of mouse and patient breast cancer tissues were fixed with 4% paraformaldehyde for 10 min at room temperature and were blocked with 3% BSA in phosphate-buffered saline (PBS). The samples were incubated with primary antibody DBC2 (Bioss Antibodies, Woburn, MA, USA) overnight, washed with Tris-PBS buffer (TPBS) three times, then washed with TBST and performed with an avidin-biotin complex (ABC) kit and 3,3′-diaminobenzidine (DAB; Vector Labs, Burlingame, CA, USA) according to the manufacturer's protocol, and assessed with a confocal laser-scanning microscope (LSM710; Carl Zeiss, Oberkochen, Germany).

Wound-healing assay

Cells were seeded on 6-well culture plates and scratched with a plastic tip for mimic wound injury. Cells were washed using PBS to remove cellular debris and allowed to migrate for 16−24 h. Then migrating cells were assessed by the closure of the wound area under a light microscope, as described previously.[56]

Transwell invasion assay

Invasion assay was performed using Matrigel (BD Biosciences, San Jose, CA, USA)-coated Transwell chambers (8 μm pore; Corning, Corning, NY, USA). Cells were placed in the upper Transwell chamber, and medium containing 0.1% BSA was added to the lower chamber. The next steps were done according to the manufacturer's instructions. After incubation for 16 h, the invaded cells in the lower Transwell chambers were fixed and stained with Hemacolor solution (Merck, Billerica, MA, USA). The stained cells were counted under a light microscope (AE31 series, Trinocular-inverted microscope; Motic, Hong Kong).

Tube-formation assay

96-well plates coated with Matrigel (BD Biosciences, San Jose, CA, USA) were prepared according to the manufacturer's instructions. Supernatants from MDA-MB-231 transfected with negative control and miR-5088-5p mimetics using G-fectin were harvested 72 h later (Genolution, Seoul, Korea). HUVECs (1×10^4 cells/well) were seeded on a Matrigel-coated 96-well plate with the supernatant obtained from MDA-MB-231. After 16 h, tube formation was observed under a microscope (AE31 series; Motic, Hong Kong).

Sphere-formation assay

Sphere-formation assay was performed as described previously.[56] The indicated cells (1×10^5/dish) were resuspended in DMEM/F12 (Cellgro, Manassas, VA, USA) containing B27 (1:50) (Gibco, USA) and grown for 7−10 days. Spheres were attached by 15% added FBS for 1 day and stained with Coomassie Brilliant Blue R-250 solution (Bio-World, USA). Spheres were counted with a diameter >20 μm under an inverted microscope (AE31 series; Motic, Hong Kong).

Anchorage-independent assay

24-well plate pre-coated with Matrigel (BD Biosciences, San Jose, CA, USA) was prepared according to the manufacturer's instructions. Indicated cells (50−100 cells/dish) suspended in growth medium with Matrigel were seeded on the top of the Matrigel layers. After incubation in 37°C to form colonies, colonies were taken a photo for 15 days.

In vivo metastasis assay

Human breast cancer cells, highly metastatic MDA-MB-231 cells (kindly provided by S. J. Lee, Department of Life Sciences, Hanyang University, Korea) with control vector (pCMV-tag2), DBC2-overexpressing vector, negative control, or anti-miR-5088-5p (1×10^6 cells/mouse) (n = 5) were injected into the mammary fat pad of 6-week female BALB/c nude mice (Orient Bio, Seongnam, Korea). The mice were anesthetized and sacrificed at 5 or 10 weeks, respectively.

GraphPad Prism (version 5.0; GraphPad Software) was used to analyze Spearman correlation between miRNAs and mRNAs of interest, calculated, and graphed after collecting mouse plasma from the heart. After fixation with 4% paraformaldehyde, the number of metastatic pulmonary nodules was counted. The pulmonary tissues were formalin fixed and paraffin embedded for H&E staining (Shandon; Thermo Scientific, Pierce, USA). This experiment was reviewed and approved by the Care and Use Committee (IACUC) of Korea Institute of Radiological & Medical Sciences (KIRAMS).

Limiting dilution assay

MDA-MB-231 cells overexpressing miR-5088-5p with Matrigel were orthotopically injected into mammary fat pads of BALB/c mouse.

After 4 weeks, tumor formation was confirmed, and mice were sacrificed. Tumor-initiating frequency (TIF) was calculated using extreme limiting dilution analysis (ELDA) software (http://bioinf.wehi.edu.au/software/elda/).

Flow cytometry

After knockdown of miR-5088-5p in MCF-7 and MDA-MB-231 cells, they were washed with PBS. Then, the fluorescently attached CD44 and CD24 antibodies were incubated for 30 min in the dark at 4°C. Combinations of fluorochrome-conjugated monoclonal antibodies against human CD44-fluorescein isothiocyanate (FITC) and CD24-allophycocyanin (APC) were obtained from BioLegend (San Diego, CA, USA). After washing 3 times using FACS buffer, the subpopulation of $CD44^+/CD24^-$ was analyzed with a Beckman Coulter Cytoflex S flow cytometer (Beckman Coulter, Brea, CA, USA).

Dual-luciferase reporter assay

MDA-MB-231 cells were seeded in 24-well culture plates and then co-transfected with reporter plasmid (200 ng), pRL-CMV-*Renilla* plasmid (Promega, Madison, WI, USA) (2 ng), and miR-5088-5p using Lipofectamine 2000 (Thermo Fisher Scientific, Invitrogen, USA). Luciferase activity was measured using a dual-luciferase reporter assay system (Promega, Madison, WI, USA) according to the manufacturer's instructions and normalized to *Renilla* luciferase activity. All experiments were performed in triplicate. pGL3UC vector was kindly provided by V. N. Kim (School of Biological Sciences, Seoul National University, Korea).[57] A DNA fragment (WT or Mut) of human DBC2 3′ UTRs containing the putative miR-5088-5p binding site (7 bp) was constructed and cloned into pGL3UC. The nucleotide sequences of primers for the amplification of the WT of DBC2 3′ UTR are 5′-GAGTGCTCGAGCCCTGGCATTTTATCTCTGGGTTTGAGAGCCCTA-3′ (forward) and 5′-CAGCGGAATTCCGAGGCTGGGAGGCTTTCCCTTTGACTCTAGGGCTCTC-3′ (reverse), and the Mut of DBC2 3′ UTR is 5′-GAGTGCTCGAGCCCTGGCATTTTATCTCTGGGTTTGAGAAAAAACA-3′ (forward) and 5′-CAGCGGAATTCCGAGGCTGGGAGGCTTTCCCTTTGACTCTGTTTTTTCC-3′ (reverse), which contained the binding site of miR-5088-5p (5′-CUCGGGA-3′). The Mut vector constituted the directed binding site of miR-5088-5p, "AAAC."

Ago2 IP assay

MDA-MB-231 cells were transfected with negative control or miR-5088-5p mimic. After 48 h, cells were washed by ice-cold PBS for 10 min to washing. Ago2-IP assay was performed using the RiboCluster Profiler/RIP (Ribonucleoprotein IP)-Assay Kit for miRNA (MBL International, Nagoya, Japan). The samples were lysed with lysis buffer containing a protease inhibitor cocktail tablet (Roche, Indianapolis, IN, USA) and centrifugation at 12,000 × *g* at 4°C for 5 min. The precleared lysates and beads were prepared and mixed with anti-human Ago2 (Sigma-Aldrich, St. Louis, MO, USA) and anti-immunoglobulin G (IgG) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as control overnight at 4°C using a rotator. Ago2-IP beads were washed with ice-cold wash buffer 3 times. After RNA extraction by the manufacturer's instructions, a real-time PCR assay was performed using miR-5088-5p primer.

Clinical specimen

Normal and breast cancer patient samples were provided from the Radiation Tissue Resources Bank of Korea Cancer Center Hospital and KIRAMS Radiation Biobank (KRB). All samples used in this experiment have completed the Institutional Review Board (IRB; K-1608-002-048) in KIRAMS. Total RNA isolation and qRT-PCR were performed as indicated protocol.

Clonogenic assay

The indicated cells were seeded on 60 mm dishes as the number of 100, 500, 1,000, and 2,000 cells, respectively. After 2 weeks, colonies were fixed and stained with 0.05% crystal violet. The colonies were counted and graphed.

Proliferation and drug resistance

MDA-MB-231 and MCF-7 cells (5×10^3 cells/well) were seeded on 96-well plates. At the indicated time points, cell proliferation assays were performed using MTT (Sigma-Aldrich, St. Louis, MO, USA). The number of cells in triplicate wells was measured at 450 nm using an automatic plate reader. To describe the drug resistance of miR-5088-5p, the MTT assay was performed after two breast cancer cells transfected with miR-5088-5p were treated with/without 40 μM of etoposide (Sigma-Aldrich, St. Louis, MO, USA).

Statistical analysis

TCGA dataset was used (https://www.cbioportal.org) to analyze DBC2 expression by breast cancer type. Kaplan-Meier analyses for the effect of the expression of DBC2 on survival of breast cancer patients were performed by the open source Kaplan-Meier Plotter (http://kmplot.com).[58] The Spearman correlation was calculated to verify the relationship of DBC2 and miR-5088-5p level using GraphPad Prism (version 5.0; GraphPad Software, San Diego, CA, USA). All data are presented as mean ± SD. Statistical calculations were performed with Student's t test. Differences were considered significant at $p < 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2021.05.004.

ACKNOWLEDGMENTS

Biospecimens and data used in this study were provided by the Korea Institute of Radiological & Medical Sciences (KIRAMS) Radiation Biobank (KRB) and by the Radiation Tissue Resources Bank of Korea Cancer Center Hospital (KRB-2016-I005: TB-2016-07-L/P15,B/P15; TB-2016-07-A-L/P15,B/P15; TB-2016-07-A-L/P15,B/P15-2) in the Republic of Korea. This work was supported by grants of the National Research Foundation of Korea and KIRAMS, funded by Ministry of Science and ICT (MSIT) in the Republic of Korea (number NRF-2021R1A2C2005966 [50698-2021 and 50531-2021]).

AUTHOR CONTRIBUTIONS

I.H.B. supervised the work. H.J.S. and Y.E.C. performed research and analyzed data. I.H.B., H.J.S., and Y.E.C. designed the experiments and drafted the manuscript. I.H.B., H.J.S., Y.E.C., J.Y.C., J.M.Y., E.J.K., M.Y.C., and S.-J.L. designed and performed animal experiments. I.H.B., H.J.S., J.M.Y., and E.J.K. designed and performed promoter methylation experiments. All authors discussed the results and commented on the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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