Received: 22 February 2020 | Revised: 11 June 2020 | Accepted: 13 July 2020

DOI: 10.1111/cas.14589

ORIGINAL ARTICLE

Cancer Science WILEY

Hypoxia-inhibited miR-338-3p suppresses breast cancer progression by directly targeting ZEB2

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Abstract

Hypoxia plays an essential role in the development of various cancers. The biological function and underlying mechanism of microRNA-338-3p (miR-338-3p) under hypoxia remain unclarified in breast cancer (BC). Herein, we performed bioinformatics, gain and loss of function of miR-338-3p, a luciferase reporter assay, and chromatin immunoprecipitation (ChIP) in vitro and in a tumor xenograft model. We also explored the potential signaling pathways of miR-338-3p in BC. We detected the expression levels and prognostic significance of miR-338-3p in BC by qRT-PCR and in situ hybridization. MiR-338-3p was lowly expressed in BC tissues and cell lines, and BC patients with underexpression of miR-338-3p tend to have a dismal overall survival. Functional experiments showed that miR-338-3p overexpression inhibited BC cell proliferation, invasion, migration, and epithelial-mesenchymal transition (EMT) process, whereas miR-338-3p silencing abolished these biological behaviors. Zinc finger E-box-binding homeobox 2 (ZEB2) was validated as a direct target of miR-338-3p. ZEB2 overexpression promoted while ZEB2 knockdown abolished the promoted effects of miR-338-3p knockdown on cell biological behaviors through the NF-κB and PI3K/Akt signal pathways. HIF1A can transcriptionally downregulate miR-338-3p under hypoxia. In total, miR-338-3p counteracts hypoxia-induced BC cells growth, migration, invasion, and EMT via the ZEB2 and NF-κB/PI3K signal pathways, implicating miR-338-3p may be a promising target to treat patients with BC.

KEYWORDS

breast cancer, epithelial-mesenchymal transition, HIF1A, miR-338-3p, ZEB2

Abbreviations: ANT, adjacent noncancerous tissue; BC, breast cancer; ChIP, chromatin immunoprecipitation; DFS, disease-free survival; E-cad, E-cadherin; EMT, epithelial-mesenchymal transition; EV, empty vector; HIF1A, Hypoxia-inducible factor 1A; IHC, immunohistochemistry; ISH, in situ hybridization; MiR-338-3p, microRNA-338-3p; NC, negative control; N-cad, N-cadherin; TMA, tissue microarray; TT, tumor tissue; UTRs, untranslated regions; Vim, vimentin; ZEB2, Zinc finger E-box-binding homeobox 2.

1 | BACKGROUND

Breast cancer (BC) is the most common cancer and the second cause of cancer deaths in women after lung cancer.[1] MicroRNAs (miRNAs), a group of noncoding RNA molecules (about 22 nucleotides in length), form a ribonucleoprotein complex acting as an inhibitor of target gene expression via binding to their 3′-untranslated regions (UTRs).[2] Recent studies have confirmed that miRNAs participate in the development of various types of human malignancies.[3-5] In human cells, miR-338-3p (previously named miR-338) is located on chromosome 17q25, which commonly acts as a tumor suppressor in some aggressive types of cancers.[6-10] For example, some authors have described that the knockdown of miR-338-3p promotes BC cell growth, migration, and metastasis[8] and reverses the inhibitory effect of baicalin, a Chinese herbal medicine, on BC cell biological functions.[11] However, the expression levels and clinical significance of miR-338-3p in human BC tissues, especially from a large cohort, and the potential mechanisms in BC aggression have yet to be clarified.

Recently, many studies have observed that epithelial-to-mesenchymal transition (EMT) plays a critical role in a variety of malignant traits in cancer cells such as motility, invasion, metastasis, and chemoresistance.[12,13] During the EMT process, epithelial cells are transformed into an invasive mesenchymal cell phenotype,[14] and the expression of the epithelial marker, E-cadherin (E-cad), is downregulated. In contrast, the expressions of mesenchymal markers, including vimentin (Vim) and N-cadherin (N-cad) as well as EMT-related transcription factors, such as Twist, the Slug/Snail family, and ZEB1/ZEB2, are upregulated.[13] In cancers, EMT-related miRNAs have been recently reported,[15] such as miR-9, miR-21, miR-200 family, and so on. However, the relationship between miR-338-3p and EMT in BC is poorly determined.

Hypoxia, an important feature of solid tumors, can induce tumor cells to acquire more aggressive phenotypes, such as proliferation, invasion, metastasis, and resistance to therapy.[16] Hypoxia-inducible factors (HIFs), *HIF1A* and *HIF2A*, which mediate the cellular response to hypoxia,[17] can facilitate coordinated regulation of multiple genes involved in tumor EMT, angiogenesis, dissemination, and shifting cancer cells towards a metastatic phenotype.[18,19] Accumulating data have suggested that miRNA expression patterns participate in the molecular mechanisms triggered by hypoxia.[20,21] In particular, various hypoxia-related miRNAs, which are termed as hypoxamiRs, are highly or lowly expressed in human malignancies, implicating that these molecules are also involved in malignancy development and aggressiveness.[22] Thus, further researches should be focused on the functions and potential mechanisms of hypoxamiRs on BC progression.

In this study, we examined miR-338-3p expression in human BC tissues and identified the consistent underexpression of miR-338-3p in BC tissues. Furthermore, miR-338-3p knockdown enhanced the cell growth and migration as well as the invasion and EMT process in cell culture and mouse BC xenograft models, while overexpression of miR-338-3p led to opposite effects. miR-338-3p inhibits ZEB2 expression by binding to the 3′-UTR of ZEB2. Overexpression of ZEB2 activated the NF-κB and PI3K/Akt pathways, while the inhibition of NF-κB or PI3K abolished the effects of ZEB2 overexpression on cellular functions. MiR-338-3p was transcriptionally suppressed by HIF1A and counteracted tumor progression induced by hypoxia.

2 | MATERIALS AND METHODS

2.1 | Sample collection and follow-up

A total of 148 women with histologically confirmed BC were recruited from the Department of Breast Surgery, First Affiliated Hospital, Zhengzhou University (Henan, China). Written informed consent for the use of human samples was obtained from all patients before surgery. The research protocols were also approved by the ethics committee of the First Affiliated Hospital, Zhengzhou University. Each cancer specimen contained at least 80% tumor cells, as confirmed by microscopic examination. The demographic and clinical characteristics of patients are summarized in Table S1. Follow-up for all patients was performed every 3 months for the first year and then every 6 months. Overall survival (OS) was determined as the interval between the date of surgery and the time of the last follow-up or death due to any cause. Disease-free survival (DFS) was defined as the interval between the date of surgery and the date of having any of the following events: invasive recurrence in regional or distant sites and/or any second diagnosis of malignancy.

2.2 | In situ hybridization (ISH), immunohistochemistry (IHC), and scoring

A commercial tissue microarray (TMA) with 116 pairs of breast samples was available (Zuocheng Ltd.). Briefly, after the sections had been blocked in 3% hydrogen peroxide, they were ready for ISH and IHC. ISH was conducted on these using miRCURY DIG-labelled miRNA detection probe specific for miR-338-3p according to the manufacturer's protocol (Exiqon). For IHC staining, the slides were incubated with polyclonal antibodies to E-cad and Vim using the DAKO Envision system (DAKO) as described previously.[23]

The staining results were scored by multiplying the staining intensity (none, weak, intermediate, and robust staining represented 0, 1, 2, and 3 points, respectively) by the percentage of positive staining cells (0, 1, 2, and 3 represented no, <10%, 10% to 50%, and >50% positive tumor cells, respectively). Two pathologists blinded to the clinicopathological data for the samples independently assessed the score. Other detailed methods are shown in Appendix S1.

3 | RESULTS

3.1 | miR-338-3p inhibits BC cells proliferation, migration, invasion, and EMT in vitro

We first conducted qRT-PCR to detect the expression levels of miR-338-3p in five BC cell lines and a breast epithelial cell line

(MCF10A; Figure 1A). Compared with MCF10A, the expression of miR-338-3p in the five BC cell lines was significantly downregulated. MCF7 had the lowest levels, and hepatocellular carcinoma (HCC)1937 cells had the highest levels of miR-338-3p; we therefore transfected miRNA mimics in MCF7 and inhibitors in HCC1937 cells, respectively, and then verified the efficiency of transfection by qRT-PCR. As shown in Figure S1A,B, miR-338-3p expression levels were significantly changed after transfecting corresponding vectors in the two BC cells. We then investigated the biological roles of miR-338-3p in BC cells in vitro. We showed that miR-338-3p overexpression significantly inhibited MCF7 cell proliferation (Figure 1B), migration (Figure 1D), and invasion (Figure 1F), while silencing miR-338-3p significantly increased these biological behaviors in HCC1937 cells, as determined by MTT, wound healing, and Transwell assays (Figure 1C,E,G). We further evaluated whether miR-338-3p modulated BC progression via regulating the EMT process and found that the IHC score of E-cad expression in BC tissues with highly expressing miR-338-3p was notably higher than that with lowly expressing miR-338-3p (Figure 1H). Conversely, the IHC score of Vim in BC tissues with highly expressing miR-338-3p was markedly lower than that with lowly expressing miR-338-3p (Figure 1I). Moreover, qRT-PCR and Western blot results showed that upregulation of miR-338-3p increased the expression of epithelial marker (E-cad) and decreased the levels of mesenchymal marker (N-cad, Vim, and Twist1) in MCF7 (Figures 1J and S2A), whereas silencing miR-338-3p led to the contrary changes in HCC1937 cells (Figures 1K and S2B). Taken together, these results suggest that miR-338-3p suppressed BC cell proliferation, migration, invasion, and EMT process in vitro.

3.2 | miR-338-3p inhibits BC tumor growth and EMT in vivo

To evaluate the effects of miR-338-3p on in vivo tumor growth, MCF7 and HCC1937 cells were implanted subcutaneously into the mammary fat pads of mice, and the tumor was treated with agomir NC, agomir miR-338-3p (10 nmol), antagomir NC, and antagomir miR-338-3p (10 nmol), respectively (Figure 2A). We showed that antagomir miR-338-3p treatment significantly enhanced while agomir miR-338-3p treatment suppressed both tumor volume (Figure 2B,C) and weight (Figure 2D,E). Further, qRT-PCR assay verified decreased or increased levels of miR-338-3p expression in tumors treated with miR-338-3p antagomir or agomir-treated BC cells, respectively, compared with control tumors (Figure 2F,G). The expression levels of Ki-67 staining by IHC were increased or decreased in tumors from miR-338-3p-antagomir– or -agomir–treated mice compared with those in tumors from control mice (Figure 2H,I). Western blot was also used to evaluate the effects of miR-338-3p on the EMT process of BC in vivo. As shown in Figure 2J, the EMT process was increased or decreased by antagomir- or agomir-miR-338-3p–treated xenografts, compared with control tumors, respectively. Collectively, these findings indicate that miR-338-3p inhibits tumor growth and EMT in vivo.

3.3 | miR-338-3p could directly target ZEB2 in BC cells

We then performed an analysis of three independent algorithmic programs to define putative targets, and 16 common predicted targets were identified (Figure 3A). Among these, ZEB2 was selected because it was previously known as a transcriptional factor that could regulate tumor EMT process and progression and had been determined as one of the target genes of miR-338-3p in gastric cancer.[10] Hence, our subsequent analyses were focused on ZEB2 in BCs. We showed that the upregulation of miR-338-3p dramatically decreased while silencing miR-338-3p enhanced the expression levels of ZEB2 mRNA and protein in MCF7 (Figure 3B,C) and HCC1937 (Figure 3D,E) cells. Moreover, we found that the expression of ZEB2 mRNA had a significant inverse relationship with miR-338-3p expression by Spearman's correlation analysis in human BC tissues from the Zhengzhou cohort (Figure 3F) and the cancer genome atlas (TCGA) data set. (Figure 3G). Additionally, higher protein levels of ZEB2 were shown in human BC tissues (n = 6) with low expression of miR-338-3p than in those with high expression of miR-338-3p (Figure 3H). To determine whether ZEB2 is an authentic target of miR-338-3p, we generated luciferase reporter gene constructs wherein the 3′-UTR sequences of ZEB2 were fused with the Renilla luciferase coding sequence (Figure 3I). We demonstrated that the upregulation of miR-338-3p significantly decreased while miR-338-3p silencing increased the luciferase activity of ZEB2 wt 3′-UTR (Figure 3J,K). Nevertheless, changing the miR-338-3p expression showed no significant effects on mut construct (Figure 3J,K). Totally, our data suggested that ZEB2 could be a direct target of miR-338-3p in BC cells.

3.4 | Modulation of ZEB2 partially rescues miR-338-3p-mediated cellular processes in BC cell lines

Next, rescue experiments were performed to determine whether miR-338-3p executed its functions by suppressing its target gene ZEB2. On successful knockdown (Figure S3A) or upregulation (Figure S3B), we found that ZEB2 overexpression promoted while the knockdown of ZEB2 inhibited BC cell proliferation (Figure S3C,D) and migration (Figure S3E,F). Rescue experiments showed that upregulation of ZEB2 restored the miR-338-3p mimics-inhibited proliferation (Figure S4A), migration (Figure 4A), invasion (Figure 4C), and EMT process (Figure 4E) in MCF7 cells, while the knockdown of ZEB2 blocked the anti-miR-338-3p-enhanced biological effects on HCC1937 cells (Figures 4B,D,F and S4B). These data demonstrated that ZEB2 is not only a downstream target but also a functional mediator of miR-338-3p-induced functions in BC cells.

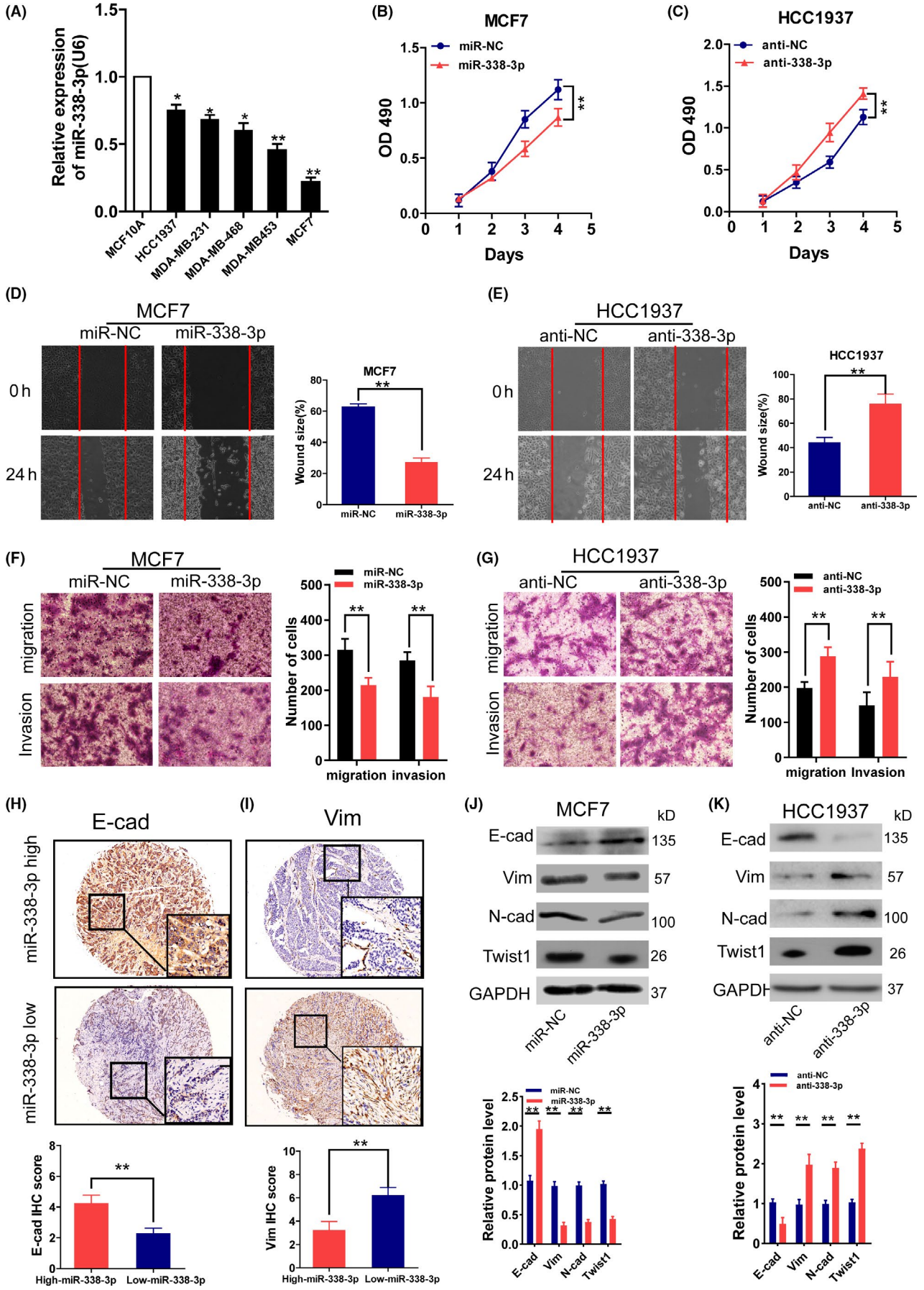
(A)
Relative expression of miR-338-3p(U6)
MCF10A
HCC1937
MDA-MB-231
MDA-MB-468
MDA-MB453
MCF7
(B)
MCF7
miR-NC
miR-338-3p
OD 490
Days
(C)
HCC1937
anti-NC
anti-338-3p
OD 490
Days
(D)
MCF7
miR-NC
miR-338-3p
0 h
24 h
Wound size(%)
(E)
HCC1937
anti-NC
anti-338-3p
0 h
24 h
Wound size(%)
(F)
MCF7
miR-NC
miR-338-3p
migration
Invasion
Number of cells
migration invasion
(G)
HCC1937
anti-NC
anti-338-3p
migration
Invasion
Number of cells
migration Invasion
(H)
E-cad
miR-338-3p high
miR-338-3p low
E-cad IHC score
High-miR-338-3p Low-miR-338-3p
(I)
Vim
Vim IHC score
High-miR-338-3p Low-miR-338-3p
(J)
MCF7
kD
E-cad
Vim
N-cad
Twist1
GAPDH
135
57
100
26
37
miR-NC
miR-338-3p
Relative protein level
E-cad
Vim
N-cad
Twist1
(K)
HCC1937
kD
E-cad
Vim
N-cad
Twist1
GAPDH
135
57
100
26
37
anti-NC
anti-338-3p
Relative protein level
E-cad
Vim
N-cad
Twist1

FIGURE 1 MiR-338-3p inhibits proliferation, migration, invasion, and EMT process of BC cells in vitro. A, qRT-PCR assay analyses of miR-338-3p expression in five human BC cell lines and normal breast cell line MCF10A. B-C, MTT assay analyses of (B) MCF7 and (C) HCC1937 cells after transfection with the indicated miRNA vectors. D-E, Wound healing assay analyses of migrative potential in (D) MCF7 and (E) HCC1937 cells after transfection with the indicated miRNA vectors. F-G, Transwell assay analyses of migrative and invasive potential in (D) MCF7 and (E) HCC1937 cells after transfection with the indicated miRNA vectors. H-I, Representative images and quantitative results of immunohistochemistry assay of E-cad (H) and Vim (I) expression in human BC tissues (n = 116). J-K, Western blotting assay analyses of expression levels of EMT markers (E-cad, Vim, Twist1, and N-cad) in MCF7 (J) and HCC1937 (K) cells after transfection with the indicated miRNA vectors. Data are presented as means ± SD from triplicate experiments. BC, breast cancer; E-cad, E-cadherin; EMT, epithelial-mesenchymal transition; MiR-338-3p, microRNA-338-3p; NC, negative control; N-cad N-cadherin; Vim, Vimentin. $^{*}P < .05$; $^{**}P < .01$

3.5 | ZEB2 regulates BC progression through the NF-κB and PI3K/Akt signal pathways

Previous studies have shown that ZEB2 overexpression enhanced tumor progression through the activation of the NF-κB and PI3K/Akt signal pathways in several types of human cancer, such as gastric cancer and BC.[10,24] We first examined whether ZEB2 expression had a significant association with both pathways according to the TCGA dataset and found that ZEB2 expression was positively associated with the majority constituents of the NF-κB and PI3K/Akt pathways (Figure S5A-F). Then, Western blotting assays showed that ZEB2 silencing significantly decreased while ZEB2 overexpression increased the protein levels of phosphorylated IκBα, P65, P85, and Akt in MCF7 cells. Modulating ZEB2 expression did not change the protein levels of total IκBα, P65, P85, and Akt (Figure 5A-D). These data indicated that ZEB2 could enhance the activation of the NF-κB and PI3K/Akt pathways in BC cells. Next, by treatment with Bay117082 and LY294002, we showed that ZEB2 overexpression significantly enhanced cancer cell proliferation (Figure 5E,F), migration (Figure 5G), and EMT process (Figure 5H,I), but Bay117082 and LY294002 partially abrogated the effects of ZEB2 overexpression on BC cells. Thus, our findings demonstrated that NF-κB signaling was involved in the miR-338-3p/ZEB2-axis–regulated BC cell mobility and EMT process.

3.6 | Hypoxia regulates the miR-338-3p/ZEB2 axis at the transcriptional level in BC

To explore miR-338-3p expression under hypoxia, MCF7 and HCC1937 cells were incubated in hypoxic (1% and 5% O_2) or normoxic (20% O_2) conditions for 12 hours, or in hypoxia (1% O_2) for 0, 12, and 24 hours. We found that the miR-338-3p level was decreased in a dose- and time-dependent manner (Figure 6A,B). We also determined whether the downregulation of miR-338-3p was related to HIF1A expression. After HIF1A was successfully knocked down or overexpressed (Figure S6A,B), we found that overexpression of HIF1A decreased while the knockdown of HIF1A enhanced miR-338-3p expression in both BC cells (Figure 6C,D). We then investigated ZEB2 expression in response to hypoxia and found that expression of ZEB2 mRNA (Figure S7A,B) and protein in BC cells (Figure 6E-H) was also increased in a time- and dose-dependent manner. Results from TCGA also validated a significant association between HIF1A and the miR-338-3p/ZEB2 axis in BC (Figure S7C,D). Moreover, overexpression of HIF1A enhanced while HIF1A knockdown decreased ZEB2 expression in BC cells (Figure 6I,J). Collectively, our findings implicate that the regulation of the miR-338-3p/ZEB2 axis by hypoxia is HIF1A-dependent.

We then identified whether HIF1A regulated miR-338-3p expression at the transcriptional level. We searched the potential hypoxia response element (HRE) in a 2-kb region upstream of the MIR-338 sequence using the JASPER bioinformatics software program. Two predicted HIF1A binding motifs at ~514 to ~523 and ~1643 to ~1652, named P1 and P2, were identified inside the putative MIR-338 promoter region (Figure 6K), and ChIP assays confirmed that HIF1A protein was indeed recruited to these two binding sites in both cells (Figure 6L,M). Furthermore, reduced luciferase activity in the wt miR-338 promoter was observed after overexpression of HIF1A in MCF7 (Figure 6N) and HCC1937 cells (Figure 6O). These effects were not observed when the P1 and/or P2 sites were mutated. These data demonstrated that HIF1A directly suppressed miR-338-3p at transcript levels via directly binding to the miR-338 promoter region.

3.7 | MiR-338-3p/ZEB2 mediates hypoxia-induced cell growth, migration, and EMT in BC

We then explored whether hypoxia-inhibited miR-338-3p played a vital role in the above phenotypes. We first determined the effects of miR-338-3p on HIF1A expression under hypoxic conditions (1% O_2). Western blotting assay showed that hypoxia significantly increased HIF1A expression, while overexpression of miR-338-3p markedly reversed these effects in BC cells (Figure S8). Also, hypoxia significantly increased MCF7 and HCC1937 cell growth (Figure 7A,B), migration (Figure 7C,D), and EMT process (Figure 7E,F), while miR-338-3p overexpression partially reversed these effects. Taken together, our findings indicate that miR-338-3p overexpression counteracts hypoxia-induced BC growth and EMT process via targeting ZEB2.

3.8 | Association of miR-338-3p/ZEB2 expression with clinical features and prognosis in patients with BC

We then examined the expression levels of miR-338-3p in a commercial TMA consisting of 116 human BC specimens by using ISH and found that the expression levels of miR-338-3p according to

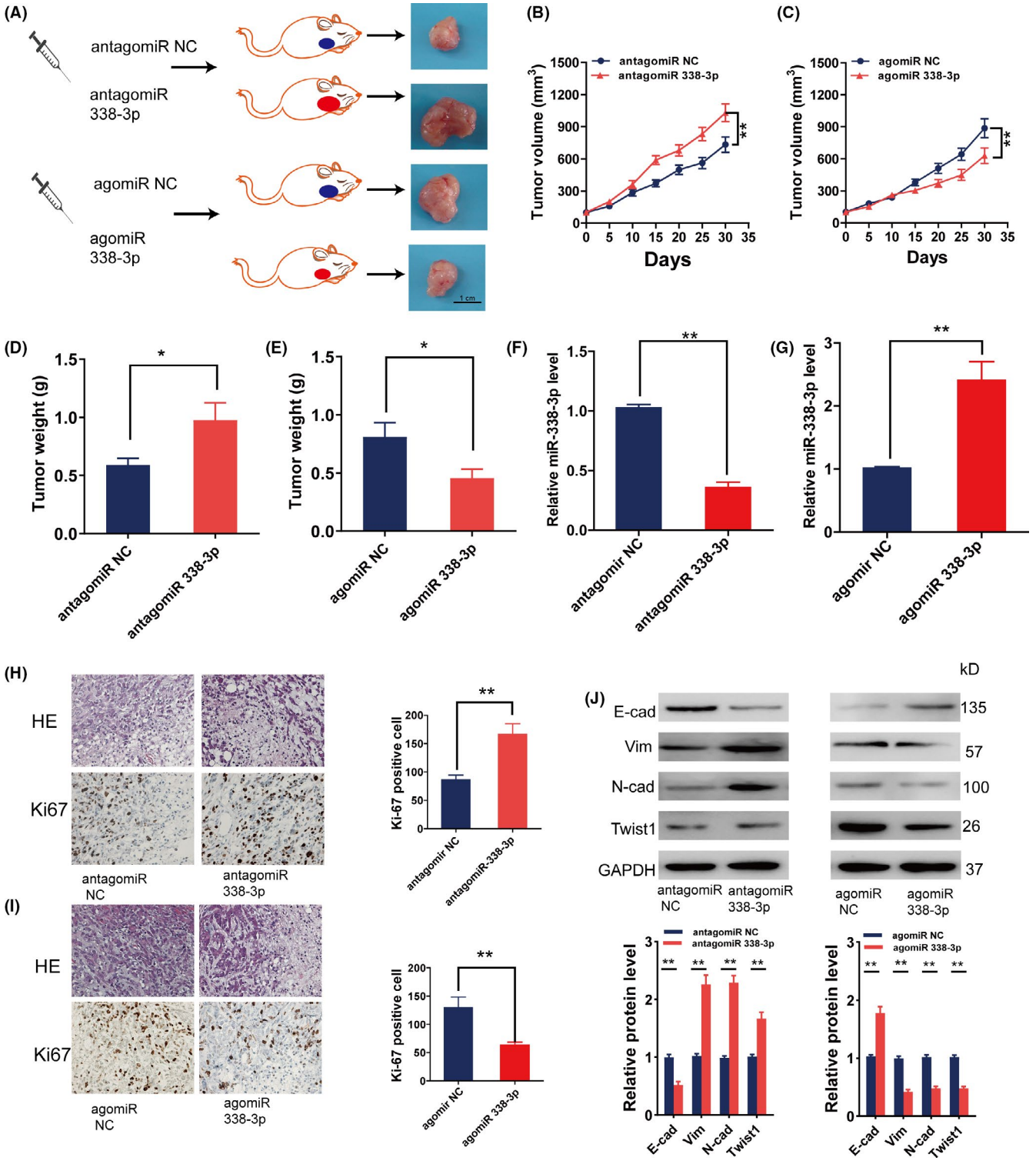

FIGURE 2 MiR-338-3p inhibits tumor growth and EMT of BC in vivo. A, Diagram depicting the overall xenograft study. B-E, The volumes (B-C) and weights (D-E) of xenograft tumors from mice in the four experimental groups. F-G, qRT-PCR analyses of miR-338-3p expression levels in xenograft tumors from mice in the four experimental groups. H-I, HE and Ki67 staining in xenograft tumor in the four experimental groups (magnification ×100). J, Western blotting analyses of E-cad, Vim, and N-cad expression in xenograft tumor samples in the four experimental groups. Data are shown as the mean ± SD of three replicates. BC, breast cancer; E-cad, E-cadherin; EMT, epithelial-mesenchymal transition; MiR-338-3p, microRNA-338-3p; NC, negative control; N-cad, N-cadherin; Vim, vimentin. $^{*}P < .05$, $^{**}P < .01$

the ISH score were significantly lower in the tumor tissues (TTs) than those in the adjacent non-TTs (ANTs, Figure 8A,B). To explore the effects of the miR-338-3p/ZEB2 axis on BC aggression, we used qRT-PCR assay to determine the expression levels of the miR-338-3p/ZEB2 axis in the TT and matched ANT samples from our cohort containing 148 BC patients. We showed that the

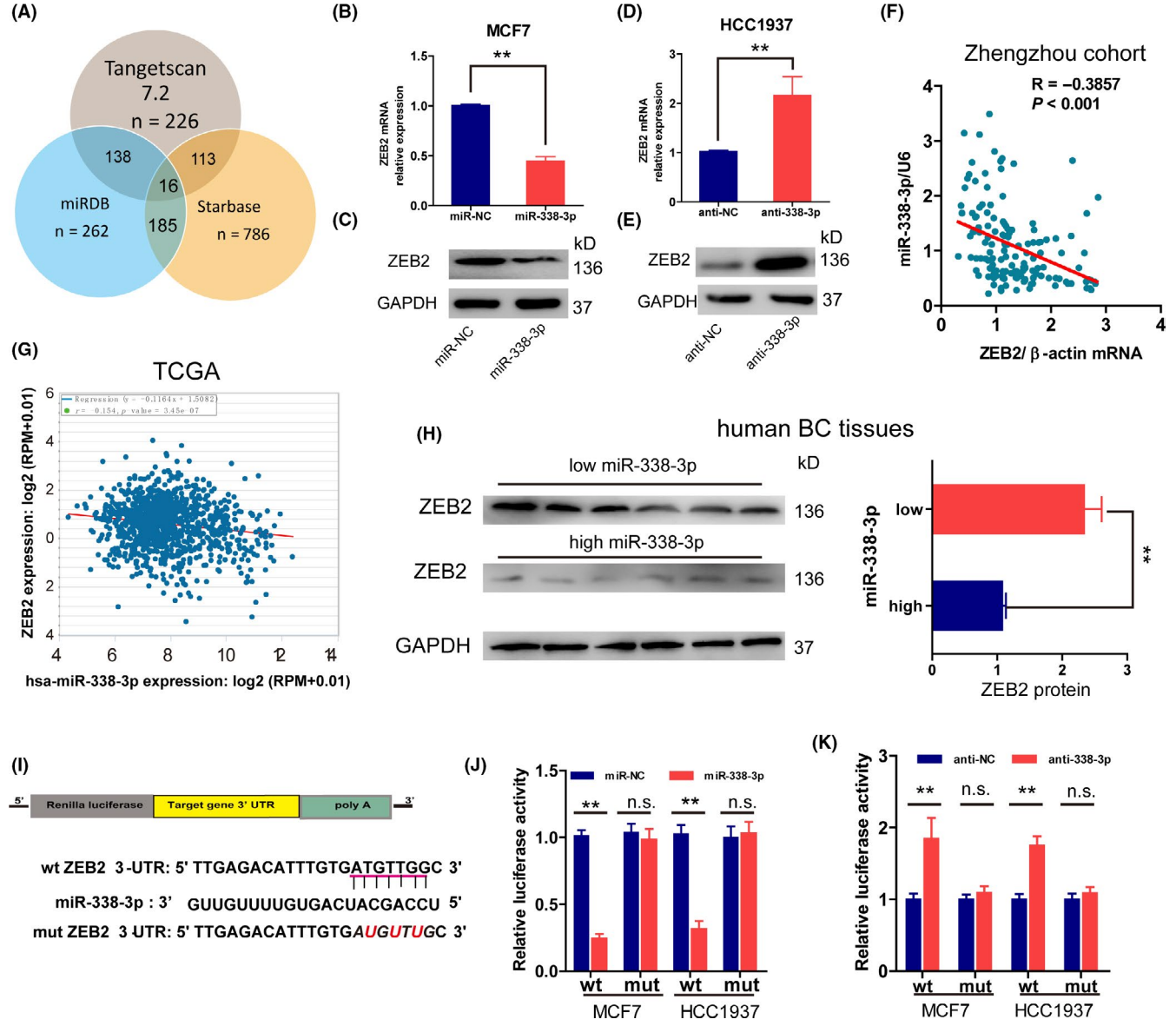

FIGURE 3 ZEB2 is a direct target gene of miR-338-3p. A, A Venn diagram showed the number of genes identified as potential targets of miR-338-3p. B-E, The expression levels of ZEB2 mRNA and protein were measured by qRT-PCR and western blot analyses in MCF7 (B-C) and HCC1937 (D-E) cells after transfection with the indicated vectors. F-G, Significantly inverse correlations between expression levels of miR-338-3p and ZEB2 mRNA in human BC tissues from the Zhengzhou cohort (F) and TCGA data set (G). H, Western blot analyses of ZEB2 protein expression in human BC tissues with high- and low-miR-338-3p expression. I, Diagrams showed the miR-338-3p putative binding sequences and corresponding mutant sites of ZEB2. (J-K) Dual-luciferase reporter assays in MCF7 (J) and HCC1937 (K) cells. The relative luciferase activity was normalized to the Renilla luciferase activity. Data are presented as means ± SD from triplicate experiments. wt, wild-type; mut, mutant-type; n.s., not significant. $^{**}P < .01$

expression of miR-338-3p in TT was significantly lower than that in ANT (Figure 8C), while the expression of ZEB2 in TT was significantly higher than that in ANT (Figure 8D). Furthermore, Table S2 shows that low expression of miR-338-3p was significantly associated with aggressive T stage, node invasion, and lymphovascular invasion, while high expression of ZEB2 was statistically associated with node invasion and poor tumor grade. Kaplan-Meier survival curves showed that low miR-338-3p expression was related to dismal DFS (Figure 8E) and OS (Figure 8F) in patients with BC from the Zhengzhou cohort, and also high ZEB2 expression was related to dismal DFS (Figure 8G) and OS (Figure 8H). These results were also verified according to metaBric data set (Figure 8I). Further multivariate Cox survival analyses also showed that low miR-338-3p or high ZEB2 expression was an independent predictor of dismal DFS (miR-338-3p: hazard ratio [HR] = 2.07, 95% confidence interval [CI]: 1.21-3.54; ZEB2: HR = 2.03, 95% CI: 1.04-3.97) and OS (miR-338-3p: HR = 2.48, 95% CI: 1.05-5.85; ZEB2: HR = 1.61, 95% CI: 1.04-2.49; Table 1). These results unambiguously demonstrated that miR-338-3p and ZEB2 might act as prognostic tumor markers in BC patients.

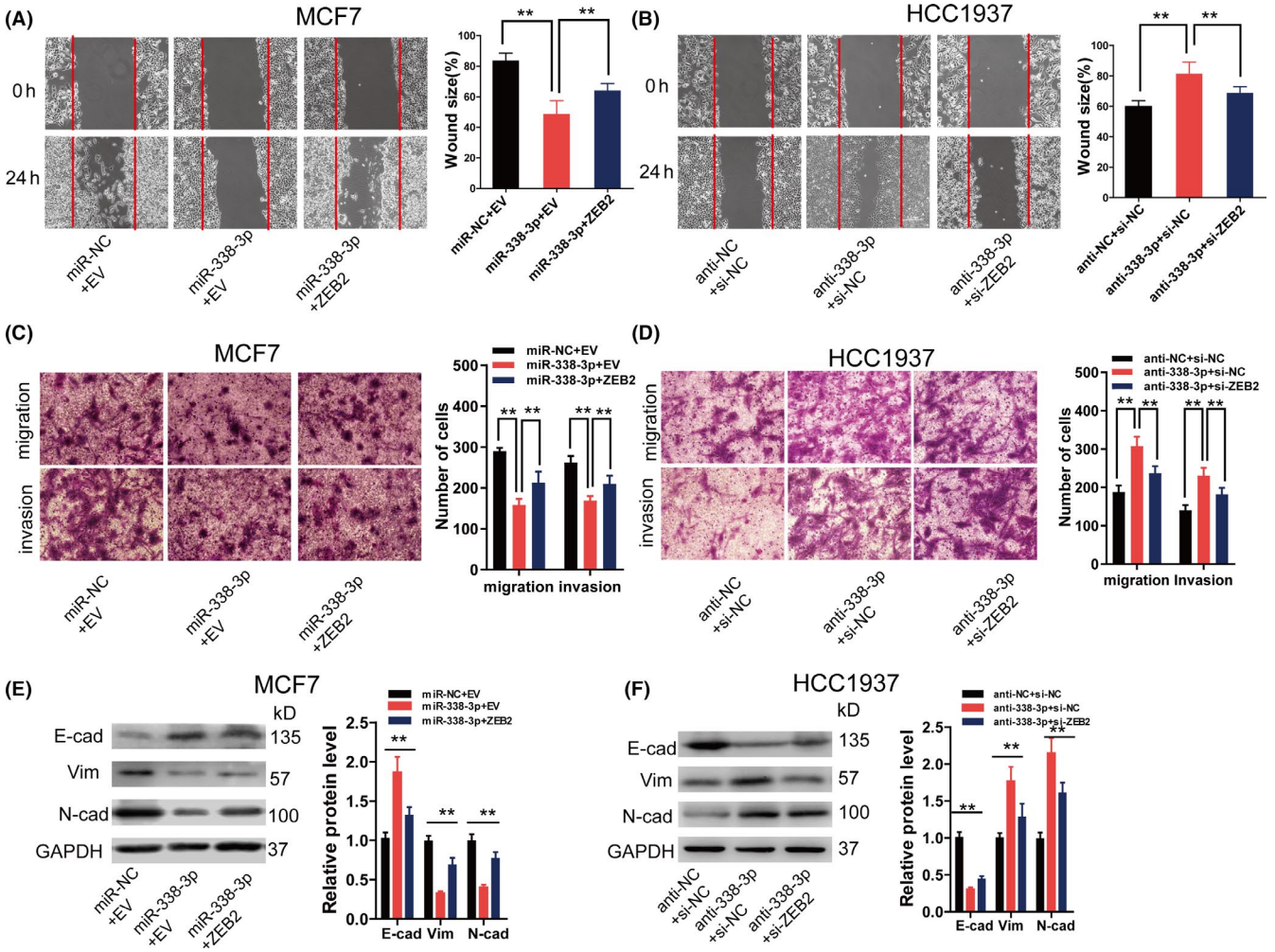

FIGURE 4 Modulation of ZEB2 partially abolishes miR-338-3p-mediated cellular processes in BC cell lines. A-B, Wound healing assay analyses of cell migration in MCF7 (A) and HCC1937 (B) cells after transfection with the indicated vectors. C-D, Transwell assay (with or without matrigel) analyses of cell migrative or invasive potential in MCF7 (C) and HCC1937 (D) cells after transfection with the indicated vectors. E-F, Western blot assay analyses of expression of EMT markers (E-cad, Vim, and N-cad) in MCF7 (E) and HCC1937 (F) cells after transfection with the indicated vectors. E-cad, E-cadherin; N-cad N-cadherin; Vim, Vimentin. Data are presented as means ± SD from triplicate experiments. $^{**}P < .01$

4 | DISCUSSION

In the current research, we verified that miR-338-3p was significantly downregulated in BC tissues and cell lines and was negatively related to tumor aggression and survival of human BC. Functional experiments have shown that miR-338-3p inhibited the proliferation, migration, invasion, and EMT process via targeting ZEB2. Moreover, the activation of the NF-κB and PI3K/Akt pathways was mediated by the miR-338-3p/ZEB2 axis, and the inactivation of the NF-κB and PI3K/Akt pathways ameliorated the effects of ZEB2 overexpression on BC cellular behaviors. Specifically, miR-338-3p expression was inhibited under hypoxia and by HIF1A at the transcriptional level and was involved in hypoxia-induced cell biological functions.

Accumulating data demonstrates that miR-338-3p plays important roles in many types of human malignancy. For example, overexpression of miR-338-3p led to the attenuated cell proliferation, migration, and invasion of ovarian cancer cells.[6] In gastric cancer and esophageal squamous cell carcinoma, miR-338-3p was downregulated compared to non-TTs,[25,26] and associated with poor OS of these cancer patients. Regarding BC, Liang et al described that overexpression of miR-338-3p inhibited cell growth, migration, and invasion in BC cell lines and contributes to lung metastasis in vivo.[8] Results from Duan et al showed that miR-338-3p knockdown reversed the effects of baicalin on BC cell viability, apoptosis, and invasion.[11] Hence, qRT-PCR and ISH analyses were performed to determine the expression level of miR-338-3p in human BC samples as well as in a panel of BC cell lines and normal breast cells. Our data revealed that miR-338-3p expression was downregulated in BC tumor tissue samples and cell lines. Our in vitro and in vivo findings implicated that miR-338-3p overexpression suppressed the proliferation, migration, invasion, and EMT process of MCF7 and HCC1937 cells, while the knockdown of miR-338-3p had the opposite effects. We further evaluated the prognostic roles of miR-338-3p and found that higher miR-338-3p

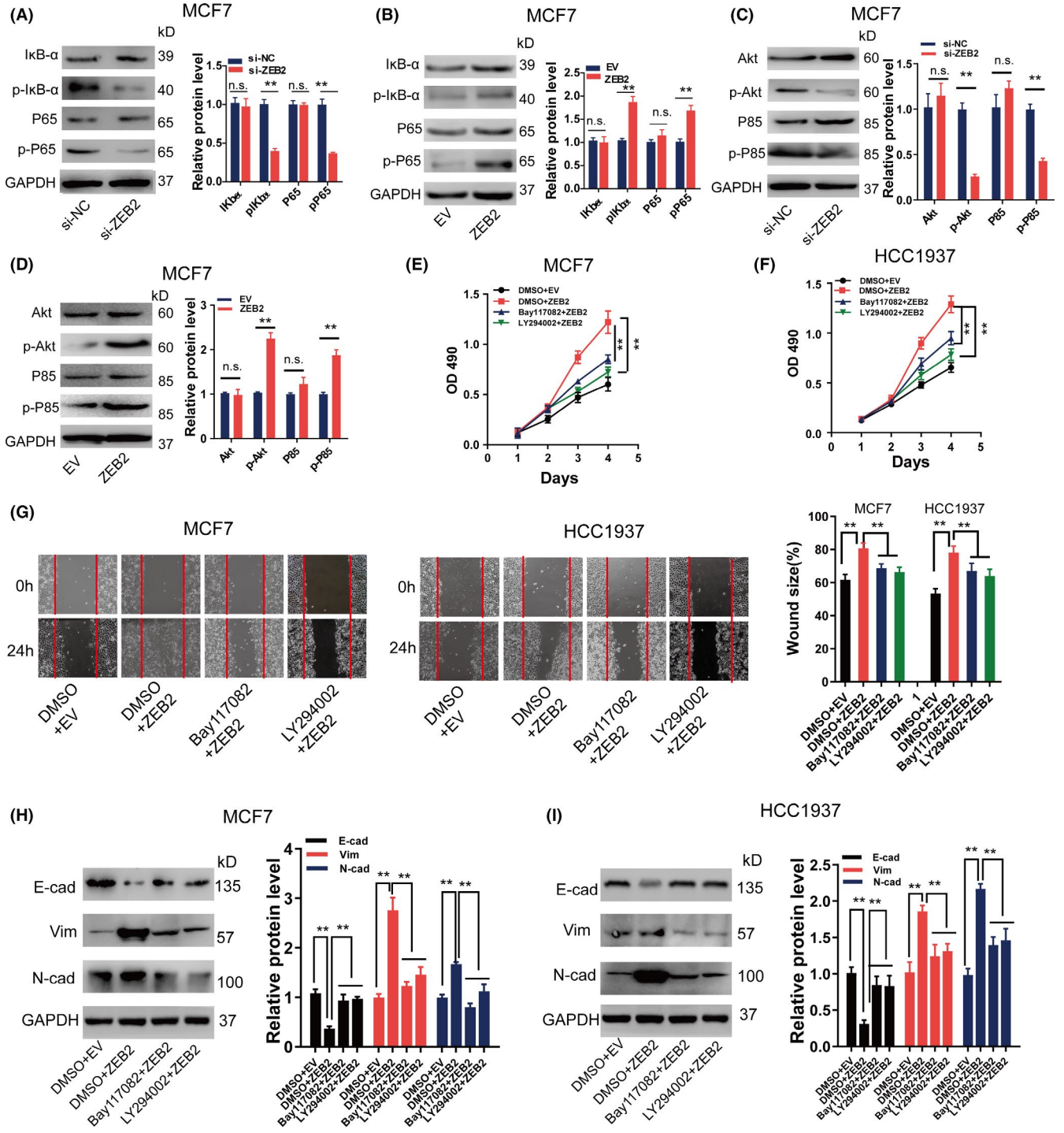

FIGURE 5 ZEB2 overexpression regulates BC progression through the NF-κB and PI3K/Akt signal pathways. A-D, Western blot assay analyses of expression levels of NF-κB (total and phosphorylated IκBα and P65) and PI3K/Akt (total and phosphorylated Akt and P85) signal pathway protein in MCF7 cells after transfection with the indicated vectors. E-F, MTT assay analyses of BC cell proliferation after treatment of ZEB2 overexpressing MCF7 (E) and HCC1937 (F) cells with the inhibitor of the NF-κB (Bay117082) or PI3K (LY294002) pathway. G, Wound healing assay analyses of BC cell migration after treatment of ZEB2 overexpressing MCF7 and HCC1937 cells with Bay117082 or LY294002. H-I, Western blot assay analyses of expression of EMT markers (E-cad, Vim, and N-cad) of BC cells after treatment of ZEB2 overexpressing MCF7 (H) and HCC1937 (I) cells with Bay117082 or LY294002. Data are presented as means ± SD from triplicate experiments. BC, breast cancer; E-cad, E-cadherin; N-cad N-cadherin; n.s., not significant; Vim, Vimentin; ZEB2, Zinc finger E-box-binding homeobox 2. $^{**}P < .01$

expression is an independent prognostic factor for better OS, suggesting that miR-338-3p might act as a tumor suppressor in BC progression.

Physiologically, ZEB2, a zinc finger homeodomain, functions as a molecular master switch during the EMT process that occurs in tumor aggression, including BC.[27-29] Recently, ZEB2 has gained attention for

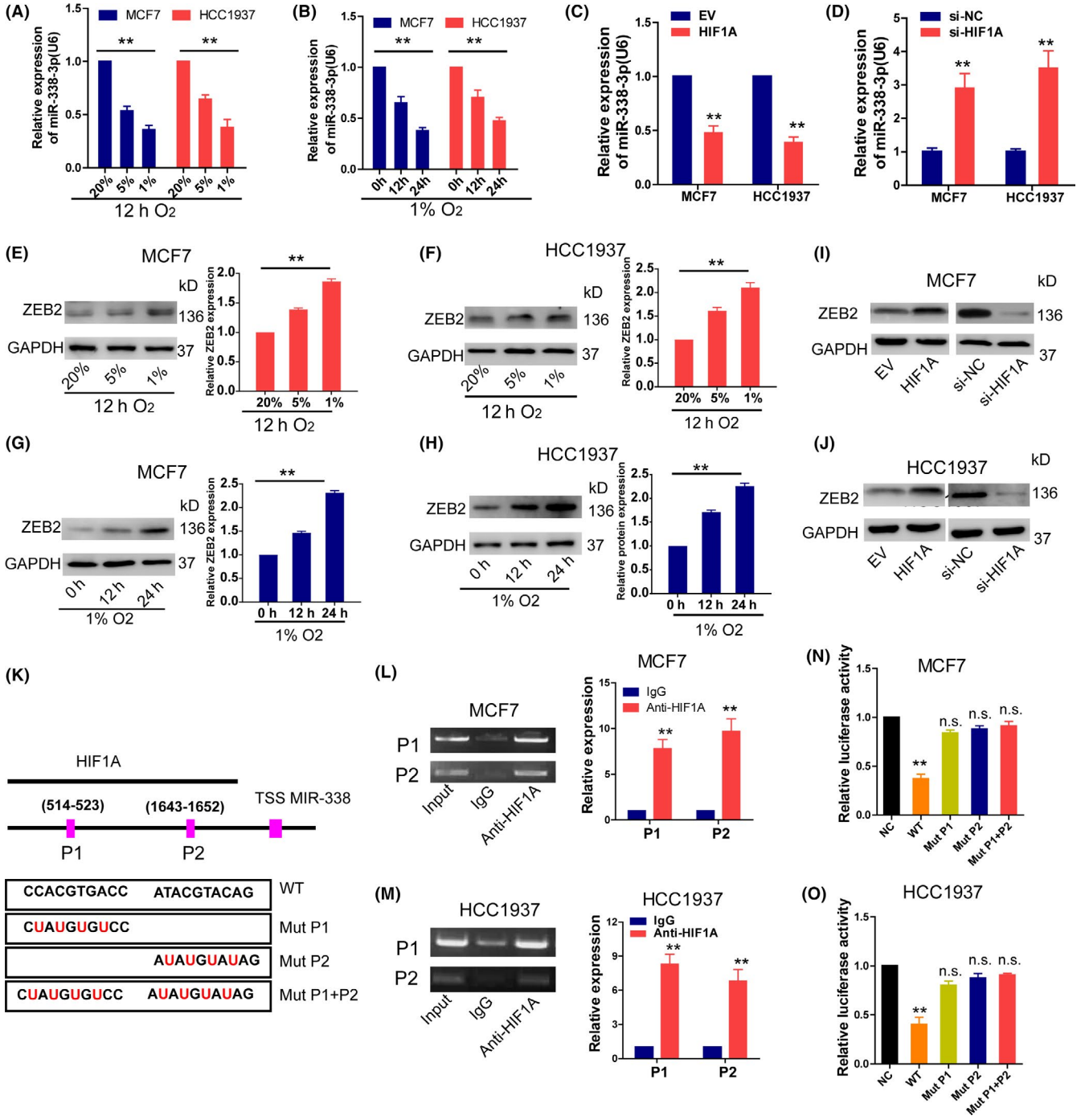

FIGURE 6 Hypoxia regulates the miR-338-3p/ZEB2 axis at the transcriptional level in BC. A, B, qRT-PCR assay analyses of miR-338-3p expression in BC cells exposed to normoxia (20%) and hypoxia at different concentrations (1%, 5%, A) and time points (0, 12, 24 h; B). C-D, qRT-PCR assay analyses of miR-338-3p expression in BC cells after transfection with either HIF1A overexpressing plasmid (C) or HIF1A siRNA (D) or controls. E-H, Western blot assay analyses of ZEB2 expression in BC cells exposed to normoxic and hypoxic conditions in a different concentration (1%, 5%, E-F) and time points (0, 12, 24 h; G-H). I-J, Western blot assay analyses of ZEB2 expression in BC cells after transfection with either HIF1A overexpressing plasmid (I) or HIF1A siRNA (J) or controls. K, Schematic of the promoter regions of miR-338-3p with potential HIF1A binding sites (P1 and P2) and the structure of the wild-type (WT) and mutant binding sites (Mut P1, Mut P2, and Mut P1 + P2). L-M, ChiP assay analyses of chromatin-bound DNA with the antibody against HIF1A in MCF7 (L) and HCC1937 (M) cells. Anti-IgG antibody was used as a negative control. N-O, Dual-luciferase reporter assay in MCF7 (N) and HCC1937 (O) cells. The relative luciferase activity was normalized to the Renilla luciferase activity. Data are presented as means ± SD from triplicate experiments. BC, breast cancer; MiR-338-3p, microRNA-338-3p; n.s., not significant, WT, wild type; ZEB2, Zinc finger E-box-binding homeobox 2. $^{**}P < .01$

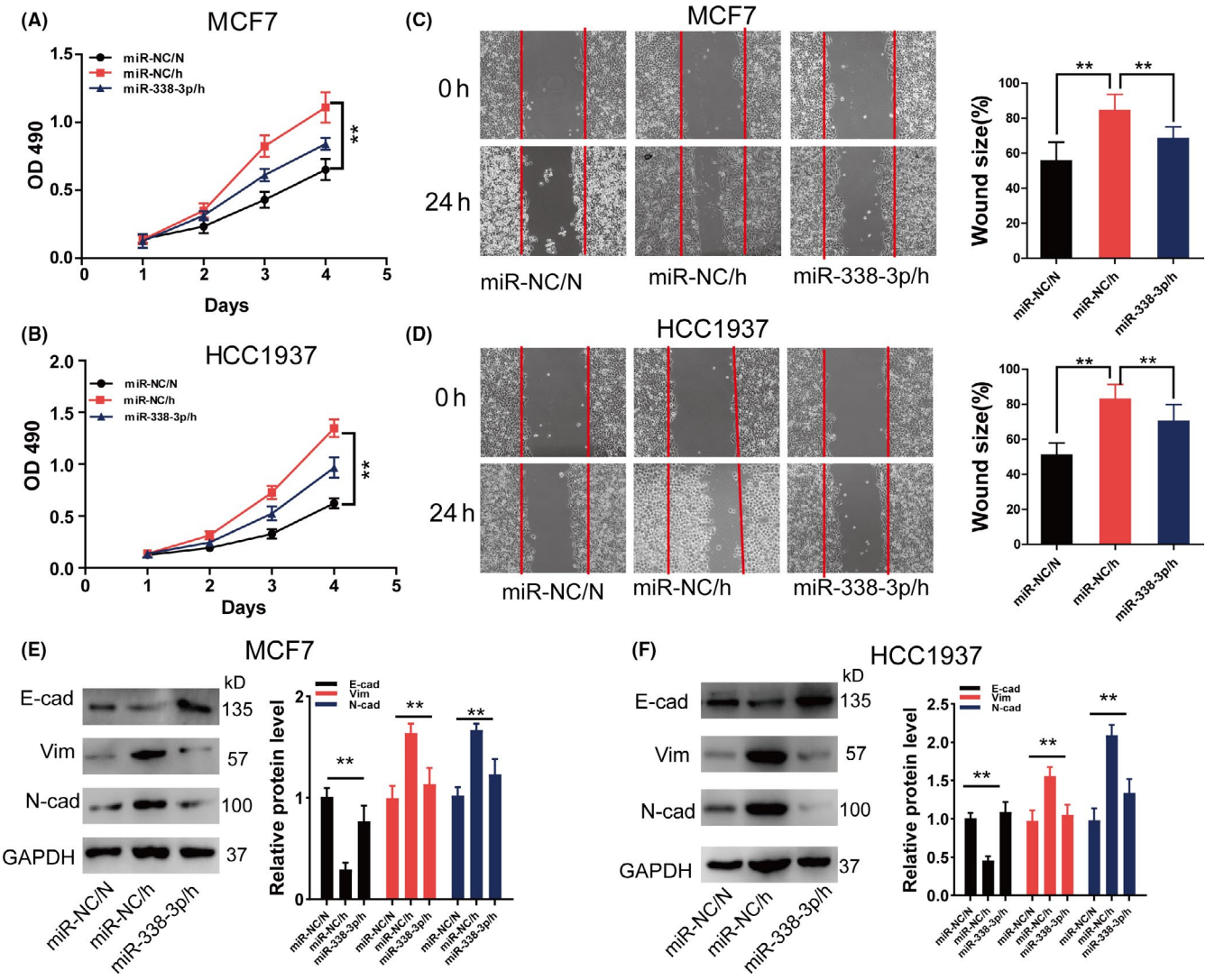

FIGURE 7 MiR-338-3p overexpression reverses hypoxia-induced cell growth, migration, and EMT process. A-B, MTT assay analyses of cell proliferation in miR-338-3p overexpressing MCF7 (A) and HCC1937 (B) cells exposed to normoxia or hypoxia (1% O_2). C-D, Wound healing assay analyses of cell migration in miR-338-3p overexpressing MCF7 (C) and HCC1937 (D) cells exposed to normoxia and hypoxia (1% O_2). E-F, Western blot assay analyses of expression of EMT markers (E-cad, Vim, and N-cad) in miR-338-3p overexpressing MCF7 (E) and HCC1937 (F) cells exposed to normoxia and hypoxia (1% O_2). Data are presented as means ± SD from triplicate experiments. E-cad, E-cadherin; EMT, epithelial-mesenchymal transition; h, hypoxia; MiR-338-3p, microRNA-338-3p; N-cad N-cadherin; N, normoxia; Vim, Vimentin. $^{**}P < .01$

its pro-oncogenic functions: overexpression of ZEB2 contributes to the degree of malignancy, rapid cell proliferation, and poor patient survival in different tumors, and knockdown of ZEB2 expression inhibits cell proliferation, migration, and invasion.[28,30,31] Based on biomedical databases query and previous reports,[10,32] we confirmed that miR-338-3p could directly bind to ZEB2 3'UTR, as evidenced by dual-luciferase reporter assay and Western blot assay. Moreover, ZEB2 expression was upregulated in human BC tissues and inversely related to miR-338-3p expression. Silencing of ZEB2 expression reproduced the miR-338-3p-induced phenotype in BC cells, while restored ZEB2 expression abolished the tumor-suppressive roles of miR-338-3p in BC cells. Collectively, ZEB2 was a direct target gene of miR-338-3p and mediated the biological effects of miR-338-3p on BC cells.

To detect the underlying mechanism of miR-338-3p-regulated EMT and the invasion of BC cells, we focused on ZEB2 and its downstream signals. The constitutive activation of NF-κB and PI3K/Akt plays a pivotal role in tumorigenesis.[33,34] In the present study, ZEB2 overexpression significantly increased while ZEB2 silencing significantly decreased the tyrosine kinase phosphorylation of NF-κB (IκBα and P65) and PI3K/Akt (P85 and Akt) in BC cells. Moreover, ZEB2 overexpression enhanced cancer cell proliferation, migration, and EMT process, but Bay117082 and LY294002 abrogated the effects of ZEB2 overexpression on BC cells, indicating that the NF-κB and PI3K/Akt signaling pathways were involved in miR-338-3p/ZEB2-axis–regulated biological behaviors in BC cells.

One of the predominant mechanisms causing miRNA loss in BC cell lines is the altered tumor microenviroment.[35] HIF-1A is known to regulate several hypoxamiRs.[36] For instance, Ying et al have found that through

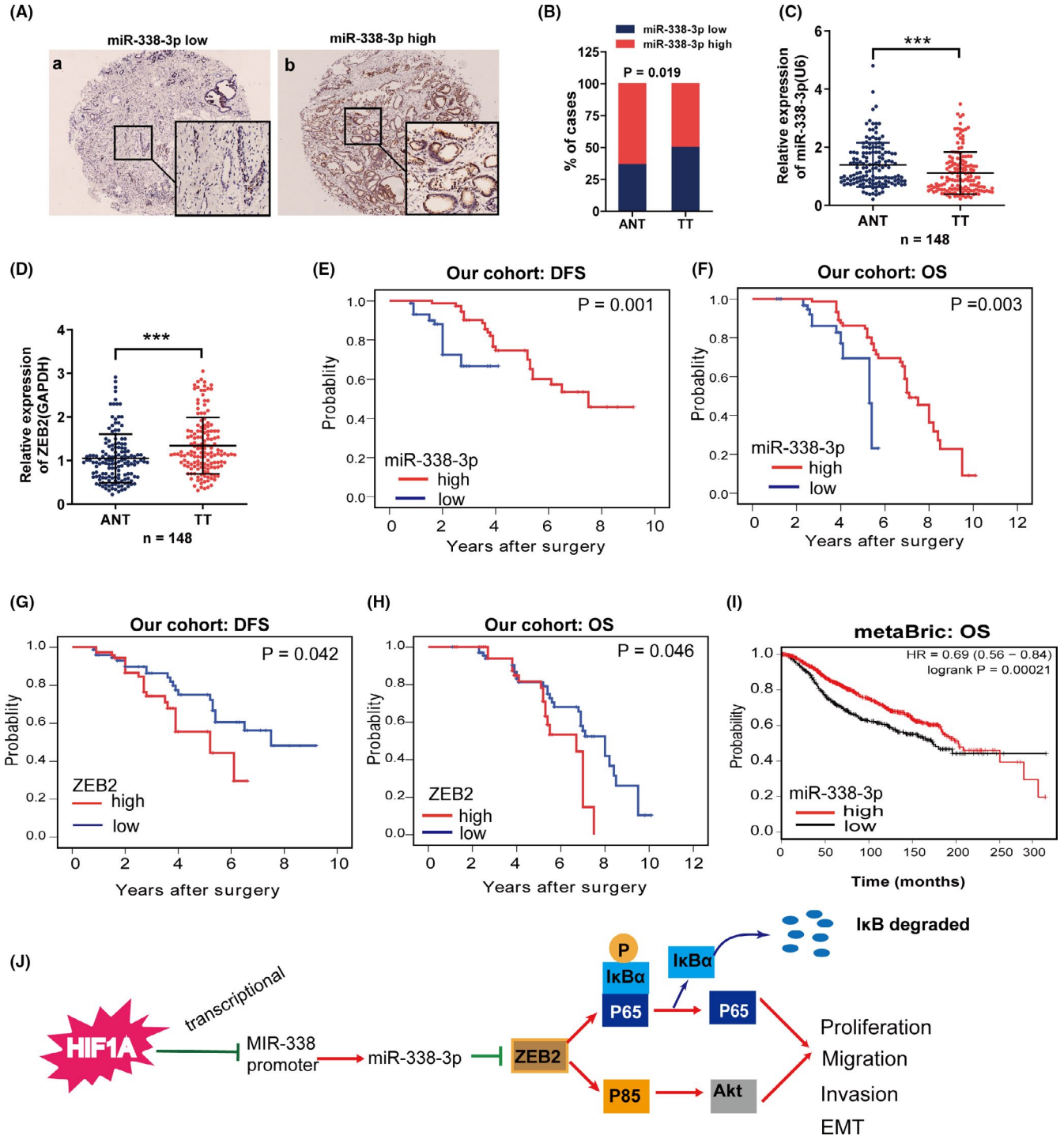

FIGURE 8 MiR-338-3p and ZEB2 expression in human BC tissue samples, and association with prognosis of BC patients. A-B, In situ hybridization assay (A) and semi-quantitative results (B) of miR-338-3p expression in matched human BC tumor samples. a, low expression; b, strong expression. C-D, qRT-PCR assay analyses of miR-338-3p (C) and ZEB2 (D) expression in TT and ANT human breast tissues (n = 148). Data were obtained using the $2^{-\Delta\Delta C_t}$ method. E-H, Kaplan-Meier DFS and OS curves according to high and low miR-338-3p and ZEB2 expression in patients with BC from our cohort. Log-rank test was used. I, Kaplan-Meier OS curves according to high and low miR-338-3p expression in patients with BC from metaBric data set. J, A working model depicting the mechanism of transcriptional regulation of HIF1A on miR-338-3p targeting ZEB2 and the subsequent activation of the AKT/NF-κB signal pathways contribute to BC progression. ANT, adjacent non-cancerous tissues; BC, breast cancer; DFS, disease-free survival; MiR-338-3p, microRNA-338-3p; OS, overall survival; TT, tumor tissues; Zinc finger E-box-binding homeobox 2. ***$P < .001$

TABLE 1 Clinicopathologic characteristics for overall survival from univariate and multivariate Cox regression analyses

Clinical and pathologic indexes	Univariate analysis	*P*	Multivariate analysis	*P*
OS				
T stage, T3 + T4 vs T1 + T2	1.72 (1.04-2.86)	.036	1.24 (1.01-1.52)	.040
Node involvement, yes vs no	1.91 (1.10-3.31)	.021	1.18 (1.00-1.39)	.048
Grade: G3 vs G1 + G2	1.89 (0.97-3.69)	.061	1.19 (0.33-4.29)	.796
Lymphovascular invasion, yes vs no	1.39 (0.66-2.92)	.387		
Adjuvant chemotherapy, yes vs no	0.50 (0.26-0.97)	.041	0.55 (0.30-1.00)	.049
Adjuvant radiotherapy, yes vs no	0.89 (0.46-1.76)	.735		
MiR-338-3p, high vs low	2.50 (1.30-4.81)	.006	2.48 (1.05-5.85)	.038
ZEB2, high vs low	1.91 (1.03-3.55)	.041	1.61 (1.04-2.49)	.033
DFS				
T stage, T3 + T4 vs T1 + T2	2.37 (1.53-3.66)	<.001	1.62 (1.13-2.31)	.008
Node involvement, yes vs no	1.78 (1.22-2.60)	.004	1.70 (1.11-2.61)	.015
Grade: G3 vs G1 + G2	2.12 (1.18-3.81)	.012	1.39 (0.94-2.05)	.095
Lymphovascular invasion, yes vs no	1.54 (0.75-3.16)	.238		
Adjuvant chemotherapy, yes vs no	0.43 (0.20-0.93)	.031	0.058 (0.34-0.98)	.041
Adjuvant radiotherapy, yes vs no	0.99 (0.53-1.88)	.986		
MiR-338-3p, low vs high	2.36 (1.37-4.07)	.002	2.07 (1.21-3.54)	.008
ZEB2, high vs low	1.94 (1.01-3.75)	.048	2.03 (1.04-3.97)	.038

binding to the HRE in the miR-210 promoter, HIF1A increased the expression of miR-210, resulting in HCC tumor aggressiveness.[37] Likewise, miR-155 has been observed to inhibit the expression of Von Hippel-Lindau, a tumor suppressor involved in the cellular response to hypoxia, lead to increased angiogenesis, and facilitate cancer cell survival.[38] In this study, we have validated that HIF1A overexpression or hypoxic conditions led to a decrease, while HIF1A silencing led to an increase in expression of miR-338-3p. Furthermore, our study showed that HIF1A overexpression increased while the silencing of HIF1A inactivated the miR-338 promoter, as evidenced by luciferase assay. When mutations were produced within the HRE, the effects of hypoxia or HIF1A on miR-338 promoter activation disappeared. Moreover, the ChIP assay also validated a specific PCR product flanking the HRE of miR-338. Additionally, a previous study[9] from Xu et al showed that HIF1A might act as a direct targeting gene of miR-338-3p, mediating the inhibitory effects on HCC biological behaviors. Given that our data also showed overexpression of miR-338-3p markedly reversed hypoxia-induced HIF1A expression in BC cells, we suppose that HIF1A not only inhibits miR-338-3p at the transcriptional levels but also acts as a direct targeting gene of miR-338-3p, thus promoting ZEB2 expression and the subsequent BC progression.

To conclude, miR-338-3p is underexpressed in BC tissue and cells, and its expression may help predict the malignancy of BC. Functionally, miR-338-3p inhibits the proliferation, invasion, and EMT process of BC cells, probably by directly targeting ZEB2 and the subsequent NF-κB and PI3K/Akt signaling pathways. Our study also validates that HIF1A inhibits miR-338-3p at the transcription level (Figure 8J). Thus, our findings suggest that miR-338-3p could play essential roles in hypoxia-regulated cancer progression, and may act as a therapeutic target for hypoxic BC.

ACKNOWLEDGMENTS

Not applicable.

DISCLOSURE

The authors disclose no conflict of interest.

ETHICAL CONSIDERATIONS

The present study was approved by the Ethics Committee of the First Affiliated Hospital, Zhengzhou University. The research has been carried out in accordance with the World Medical Association Declaration of Helsinki. All patients and healthy volunteers provided written informed consent prior to their inclusion within the study. All animal procedures were performed following approval from the Animal Care and Use Committee of the First Affiliated Hospital, Zhengzhou University.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: He J, Wang J, Li S, Li T, Chen K, Zhang S. Hypoxia-inhibited miR-338-3p suppresses breast cancer progression by directly targeting ZEB2. *Cancer Sci*. 2020;111:3550–3563. https://doi.org/10.1111/cas.14589