VASP is upregulated by WDR5-MYC nexus and promotes cell migration in breast cancer

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Abstract

Backguroud: Breast cancer is one of the most threatening diseases for women, whose metastasis and recurrence are important causes of death in breast cancer patients. Vasodilator-stimulated phosphoprotein (VASP) is a cytoskeletal regulatory protein that promotes invasion and metastasis of tumor cells by regulating cell migration. Bioinformatics data indicated that H3K4me3, WDR5 and MYC co-enriched in the VASP promoter region.

Aims: The purpose of this study is to demonstrate the regulatory function and mechanism of WDR5-MYC nexus complex on VASP in breast cancer.

Method: In this present study, the expression of VASP in breast cancer and adjacent normal tissues was detected by RT-qPCR. The enrichment of H3K4me3, WDR5 and MYC on the VASP promoter was analyzed by ChIPseeker R package and verified by ChIP-PCR. The interaction of H3K4me3, WDR5 and MYC in breast cancer cells was detected by immunoprecipitation and immunofluorescence. Transcriptional activation function of MYC on VASP was detected by site-directed mutagenesis and dual fluorescence reporter system. The regulatory effect of WDR5 on breast cancer cell migration was tested by wund healing and transwell.

Result: VASP is up-regulated in breast cancer tissues as compared with adjacent normal tissues. There is interaction between H3K4me3, WDR5 and MYC and co-enrichment on the VASP promoter. MYC can activate VASP transcription by binding to the VASP promoter-842 binding site. WDR5 and MYC can enhance the migration ability of breast cancer cells by up-regulating VASP

Conclusion: Our results suggest that WDR5-MYC nexus can activate the transcription of VASP by binding to the VASP promoter region, and promote the migration of breast cancer by up-regulating the expression level of VASP.

Background:

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females worldwide. According to the latest cancer statistics in 2018, the number of breast cancer cases ranked first in all female cancer cases, accounting for about 30% of them. And the number of breast cancer deaths accounted for 14% of total female cancer deaths, only lower than lung cancer deaths. Distal metastasis and recurrence are the leading causes of death in breast cancer patients. At present, the 5 years survival rate of patients with distant metastasis of breast cancer was only 27%(Siegel, Miller, & Jemal, 2018). Therefore, it is of great significance to clarify the mechanism of breast cancer metastasis and to develop the corresponding targeted therapeutic drugs for the clinical treatment of breast cancer. However, up to now, the mechanism of metastasis and recurrence of breast cancer has not reached a comprehensive understanding.

Researches have shown that the metastasis and recurrence of tumors are closely related to the cell migration ability. The migration process of tumor cells is a dynamic process that the filamentous pseudopods and flaky pseudopods continue to form and extend. The most critical molecular of this dynamic processes is the constant aggregation and assembly of actin to form F-actin, and the process was regulated by the cytoskeletal regulatory pathway(Khan & Steeg, 2018). Among the many proteins in the cytoskeletal regulatory pathway, Vasodilator stimulated phosphoprotein (VASP) is a protein that binds to actin and promotes its assembly into F-actin(Barzik, McClain, Gupton, & Gertler, 2014). VASP has been found to be overexpressed and promoted cell migration in a variety of tumors, including gastric cancer(J. Wang et al., 2012) and colorectal cancer (Tu et al., 2015). In addition, Research has shown that VASP is overexpression and associated with the tumor metastasis, recurrence and poor prognosis in breast cancer(Padilla-Rodriguez et al., 2018). Therefore, VASP might be involved in the migration of tumor cells and played an important role in the metastasis and recurrence of breast cancer. However, there is still a lack of comprehensive understanding of the regulatory mechanisms of VASP in breast cancer.

MYC is a classic oncogene that has been found to be abnormally expressed in a variety of malignant tumors including gastric cancer, colon cancer and malignant glioma. It can play a role in promoting tumorigenesis, cell proliferation, differentiation, apoptosis and angiogenesis(Chen & Hu, 2016; Stine, Walton, Altman, Hsieh, & Dang, 2015). In addition, studies have shown that MYC was also overexpressed in breast cancer and was associated with the development of breast cancer(E. Wang et al., 2018). In this study, we found that MYC could regulate the transcription of VASP and promoted the migration of breast cancer cells as a transcription factor.

Previous studies have shown that MYC prefers to activate the cancer-promoting pathway. For example, it can upregulate the expression of cyclins to promote cell proliferation (Blevrakis et al., 2018), but at the same time can inhibit bcl-2 expression and inhibit apoptosis (Song et al., 2016). It show a selective enrichment for the target genes which have active chromatin context, such as the histone modifications H3K4me3, H3K79me, H3ac and H2A.Z enrichment regions(Fernandez et al., 2003; Guccione et al., 2006; Stine et al., 2015)。It is also known that activation of oncogenes in tumors depends on the interaction of co-activators such as WDR5 and BPTF(Richart et al., 2016; Thomas, Foshage, Weissmiller, & Tansey, 2015). However, there is no relevant studies have described the relationship between MYC and histone modifications and coactivators in gene expression regulation. Through studies of protein interactions, we found that WDR5 can not only binds to the MbIIIb domain of MYC(Thomas, Wang, et al., 2015) but also recognizes H3K4me3 via the WD40 repeat domain (Trievel & Shilatifard, 2009). In this study, we also found that H3K4me3, WDR5 and MYC are co-enriched in the VASP promoter region, and the could interact with each other. These results suggest that MYC may regulate VASP expression and promote breast cancer cell migration by interacting with WDR5 and H3K4me3, however, the mechanism remains to be unclear.

Therefore, this study focused on the regulation of VASP in breast cancer cell migration. And we found that MYC could act as a transcription factor to regulate the expression of VASP. Meanwhiles, this regulation was related to the level of H3K4me3 modification in the VASP promoter region, and this

process might be mediated by the H3K4me3 recognizer, WDR5. Subsequently, we demonstrated the co-enrichment of the VASP promoter regions H3K4me3, WDR5 and MYC as well as their interactions. We further found that they could act as a complex to promote the cell migration of VASP-mediated in breast cancer. In conclusion, the success of this research might provide a theoretical basis for the diagnosis of breast cancer and the development of targeted drugs.

2. Materials And Methods:

2.1 Human breast cancer sample and bioinformatics analysis

20 pairs of breast cancer tissues and adjacent normal tissues were collected from Affiliated Zhongnan Hospital of Wuhan University and diagnosed by the Department of Pathology. All patients were informed and agreed. Our research was supported by the Ethics Board of School of Basic Medical Sciences, Wuhan University and was based on all relevant principles of the Declaration of Helsinki.

To analyze WDR5, MYC and VASP mRNA expression level in breast cancer. The mRNA and clinical data were download from The Cancer Genome Atlas (TCGA).

To analyze the correlation between WDR5, MYC, H3K4me3 expression levels and breast cancer cell migration ability, the ChIP-seq data of WDR5, MYC, and H3K4me3 in breast cancer cells were downloaded from the GEO databases (GEO dataset number is GSE60897, GSE70098, GSE75169) (Messier et al., 2016; Sun et al., 2015; Thomas, Wang, et al., 2015). The ChIP-seq data of the WDR5, MYC, and H3K4me3 in reported breast cancer were analyzed to visualize Genomic Annotation and distribution of transcription factor-binding loci relative to Transcription Start Site (TSS) using the Chipseeker R package, and the genes enriched by WDR5, MYC and H3K4me3 were intersected and the signal pathway analysis was performed on the co-enriched genes. The co-enrichment sites of WDR5, MYC, and H3K4me3 on the gene of VASP were analyzed and determined by Integrative Genomics Viewer (IGV).

2.2. Plasmids, siRNA, and antibody

To overexpress a protein in cells, the coding sequence of WDR5 (NM_017588.2) or MYC (NM_002467.5) was cloned into the pCMV5 vector (Clontech Laboratories Inc.) and pFLAG-CMV (Clontech Laboratories Inc.). In order to label proteins in cells, the coding sequences of WDR5 or MYC were cloned into pEGFP-C1 (Clontech Laboratories Inc.) vector and pDsRed2-C1(Clontech Laboratories Inc.), respectively. For the luciferase assay, different lengths of VASP transcription start sites upstream sequences were cloned into pGL3-Basic vector (Clontech Laboratories Inc.), the mutation vector was constructed based on pGL3-VASP995, the sequence -852 GGC**CA**G**GTGG**C -842 was muted into -852 GGC**CG**G**CATG**C -842.

WDR5 and MYC shRNA interference sequence (WDR5 target sequence: 5’-CCAACCTTATTGTCTCAGGAT-3’; MYC target sequence: 5’-CCTGAGACAGATCAGCAACAA-3’) was constructed into pLKO.1 vector (Clontech Laboratories Inc.). VASP was interfered by three pairs of siRNAs (siRNA#1, sense: 5’-AAGGAAAGCCACGCAAGUUTT -3’, antisense: 5’- AACUUGCGUGGCUUCCUUTT -3’; siRNA#2, sense: 5’-

GGAGCCAAACUCAGGAAAGTT -3', antisense: 5'- CUUCCUG AGUUGGCUCCTT-3; siRNA#3, sense: 5'- GCCUCUACUUGACUUGGAATT -3', antisense: 5'- UCCAAGUCAAGUAG AGGCTT-3').

The following primary antibodies were used for Western blotting：H3K4me3 (Proteintech, USA), WDR5 (Proteintech, USA), MYC (Proteintech, USA), H3 (Proteintech, USA), GAPDH (Proteintech, USA); and following ChIP grade antibodies were used for chromatin immunoprecipitation (ChIP) and co-immunoprecipitation (Co-IP)：H3K4me3 (Abclonal, USA), WDR5 (Abclonal, USA), MYC (Abclonal, USA), H3 (Abclonal, USA) and rabbit IgG(Abclonal, USA).

2.3 Cell culture

Human normal mammary epithelial cells (MCF-10A) and the breast cancer cell (MCF-7, MDA-MD-231) were used in this study. The cell lines were purchased from the China Centerfor Type Culture Collection (CCTCC, Chinese Academy of Sciences, Shanghai, China). Their culture conditions are not exactly same. MCF-10A used a special medium (DMEM/F12 medium), 5% horse serum, 20 ng/ml EGF, 100 ng/ml cholera, 0.01 mg/ml insulin, and 500 ng/ml cortisolwere extraly added. MCF-7 and MDA-MB-231 were cultured in DMEM medium, and this cell culture medium was included 10% fetal bovine serum (FBS)and 1% penicillin and streptomycin. The cells were cultured in a incubator with 5% CO2, 37 °C (Esco, Singapore).

2.4 Reverse transcription and quantitative polymerase chain reaction (RT-qPCR)

The mRNA expression level of gene was examined by qPCR (SYBR Green Supermix, Bio-rad, China) normalized to expression of GAPDH. First, Total RNAwas extracted from cells using Trizol reagent (Applied Biosystem Inc., USA according to the manufacturer's protocol. The cDNA was obtained by RevertAid^{TM} First Strand cDNA Synthesis Kit (Fermentas：Canada). Then the expression level of target genes was analyzed by cDNA. Mixed 2×SYBR Green PCR Master Mix 5 μl, forward and reverse primers 2 μl (MYC Forward: GGCTCCTGGCAAAAGGTCA, Reverse: CTGCGTAGTTGTGCTGATGT; WDR5 Forward: AATTCAGCCCGAATGGAGAGT, Reverse: AGGCTACATCGGATATTCCCAG; VASP Forward: CTGGGAGAAGAACAGCACAACC, Reverse: AGGTCCGAGTAATCACTGGAGC; GAPDH Forward: CCCAGCCATCAGTTATTCAG, Reverse: GAGTTGGCACCGTTACAGTG), appropriate amount of cDNA, and ddH_2O to 10μl volume. qPCR conditions consisted of the following: 95˚C for 3 min for denaturation; 95˚C for 20 sec for annealing; and 72˚C for 5 min for extension, for 40 cycles. The relative expression of each gene was calcuated by using the $2^{-\Delta\Delta Ct}$ method. Each experiment was repeated three times.

2.5 Western blot

Cells were collected, and the protein was extracted with RIPA lysate. Then theprotein was denatured by boiling at 99 °C for 5 min, and quantified with BCA Protein Assay Kit (Beyotime Biotechnology Co： Jiangsu：China). After SDS-PAGE electrophoresis and transmembrane, the proteins were binded to PVDF membrane, then blocked the nonspecific sites with 5% Skimmed milk powder. Meanwhile, a primary antibody (Including MYC dilution of 1:1000, WDR5 dilution of 1:1000, VASP dilution of 1:1000, GAPDH

dilution of 1:500) should be prepared, and then incubating the PVDF membrane overnight at 4 °C. The membrane was incubated with the corresponding secondary antibody horseradish peroxidase (dilution of 1:1000) for 1 h at room temperature, and finally detected by ECL reagents (Tanon, Shanghai, China). The optical density of bands was measured by a computer-assisted imaging analysis system (Tanon, Shanghai, China) and the relative protein expression levels were normalized to GAPDH.

2.6 Co-Immunoprecipitation

Co-immunoprecipitation (Co-IP) is one of the most widely used methods to identify novel proteins that associate with a protein of interest or to determine complex formation between known proteins. In this experiment, the interactions between the proteins H3K4me3, MYC and WDR5 were identified. Cells were lysed with IP lysis buffer. After centrifugation, 2% of the supernatant was taken as Input. The rest of the supernatant was used as test groups, then H3K4me3, MYC or WDR5 specific antibodies were added to capture the target protein, and IgG was used as a negative control group. The antibody-bound protein and proteins that bind to the protein of interest were precipitated using immunomagnetic beads. Proteins that were not bound to the protein of interest were removed by a series of washes. The resulting immunocomplexes were analyzed by western blot.

2.7 Immunofluorescence

The immunofluorescence is used to detect the distribution of protein MYC and WDR5 in the cell to detect whether there is a co-location between the two proteins. After the cells in the logarithmic growth period are inoculated on the circular slide, the cells are co-transfected with pEGFP-WDR5 and pDsRed-MYC. Then the protein labeled fluorescent (WDR5 with green fluorescence, MYC with red fluorescence) was observed under fluorescence microscope (Olympus, Janpan) after 48h.

2.8 Wound healing assay

Wound healing assay can be used to detect the ability of cell migrate. In this experiment, It was used to detect changes in the migration ability of breast cancer cell lines after cells were treated. Cells (2×10^5 cells/mL,) were cultured in a 6-well plate, meanwhile a set of negative controls was set up. When the cells reached 80% to 90% confluence, cells were scratched. And the cells were continued to culture for 0, 24, and 48 h in the serum-free medium. Then cells were fixed and photographed under a microscope (Olympus, Japan), the ability of the cells to migrate was determined by calculated the number of cells that migrated to the scratched area.

2.9 Transwell assay

Some 24-well plates and a polyvinyl-pyrrolidone-free polycarbonate filter (8 μm pore size) were needed in the experiment. when the transwell assay was used to detect the ability cell invasion, the 8 micronpore was coated with Matrigel Matrix (BD, USA). Cells at a density of 1×10^5 in 100 μL serum-free medium, and the lower chamber contained 700 μL of culture medium with 20% FBS, a set of negative controls also was set up. After cells were cultured for 20 h, cells were fixed, stained, and counted the cell number in the surface of the lower chamber under light microscope (Olympus, Japan).

2.10 Chromatin immunoprecipitation (ChIP)

The cells were treated with a final concentration of 1% formaldehyde for DNA and protein cross-linking. The lysed nuclear extract was fragmented by microsphere enzyme and ultrasound. 2% volume of lysate was used as inputs, and the remaining lysate was co-immunoprecipitated with proteinG beads and corresponding antibody overnight to enrich DNA protein cross-linking complex. The DNA fragment was purified after washing and elution. Two enrichment sites on VASP promoter region were detected by PCR. The primers were designed as follows: VASP (-54~-194) Forward: 5'-CCCGGCATTCGCCCG-3', Reverse: 5'-ATTGGGGAGAGGAGCGA

GAT-3'; VASP (-754~-1102) Forward: 5'-CCAAAATTCCACTCCCCGCA-3', Reverse: 5'-CCAAAATTCCACTCCCCGCA-3'.

2.11 Luciferase assay

Based on the predicted binding site of MYC and the promoter of VASP using JASPAR, we constructed wild-type and mutant plasmids carrying different lengths of the VASP promoter including pGL3-VASP362、pGL3-VASP995、pGL3-VASP1527、pGL3-VASP748 and pGL3-VASP842mut. pGL3-Basic (Promega, Madison, WI, USA) acts as the blank control. HEK293T was cultured in 24-well plates and transfected with the mixture containing 400 ng of pGL3-VASP/pGL3-Basic, 400 ng of pEGFP-MYC/pEGFP-C1 and 10 ng of pRL-TK using Lipofectammine 2000 (Invitrogen). Cells were harvested 48 hours after transfection. The activity of firefly luciferase and Renilla luciferase were detected by the Dual Luciferase Reporter Assay System (Promega, Wisconsin, USA). The value of firefly luciferase was standardized with the corresponding Renilla luciferase activity.

2.12 Statistical analysis

Statistical analysis was performed using software SPSS. Repeat 3 times for each set of experiments. The data was expressed as the mean ± standard deviation (SD). Differences between groups were analyzed using Student's t-test. *P<0.05* was considered statistically significant.

3. Results:

3.1 VASP is upregulated in breast

To explore the differential expression of VASP in different subtypes of breast cancer tissues, we analyzed the mRNA expression levels of VASP in breast cancer tissues from data downloaded from the TCGA database. The results showed that VASP was highly expressed in breast cancer tissues compared with normal breast tissue, especially highly expressed in breast metastasis tumors and HER2-enriched and balsal-like breast cancer tissues (Fig. 1A and 1B). Further, we analyzed the correlation between VASP expression and clinicopathological features in breast cancer patients. The results indicate that breast cancer patients with high mRNA levels of VASP are more likely to develop lymph node metastasis and distant metastasis (Table 1). Subsequently, the results of the survival analysis showed that patients with high VASP mRNA expression had a lower overall survival ratio (Fig. 1C). To verify the above results, we analyzed VASP mRNA levels in 20 pairs of breast cancer tissues and adjacent normal tissues via RT-qPCR. VASP mRNA levels were significantly increased in 18 pairs of breast cancer tissues compared to adjacent tissues (Fig. 1D). In addition, western-blot results showed that the mRNA and protein levels of VASP in breast cancer cells MCF-7 and MDA-MB-231 were significantly higher than those in normal breast cells MCF-10A (Fig. 1E). The above experimental results indicate that VASP plays a role in proto-oncogenes in breast cancer.

Table 1
VASP mRNA expression and clinicopathologic characteristics of breast cancer.

Various		Number	VASP		P value
			Low experssion	High experssion	
Age					
	< 60	655	305	300	0.230
	≥ 60	562	304	258	
ER status					
	Negtive	143	62	81	0.066
	Postive	322	171	151	
Pathologic M					
	M0	1020	565	455	< 0.001
	M1/M2	196	43	153	
Pathologic N					
	N0/N1	620	349	271	0.131
	N2/N3	598	260	338	
Pathologic T					
	T1	311	167	144	0.612
	T2/T3/T4	907	474	433	
Distant metastasis					
	NO	342	172	170	0.829
	YES	17	7	10	
P values were based on χ^2-test, P < .05 was considered statistically significant.					

3.2 MYC is a transcription factor of VASP that promotes VASP expression and cell migration

Through the JASPAR database, the promoter of VASP was found to have a binding site to MYC from −842 bp to -852 bp from the transcription start site (Fig. 2A). Therefore, we hypothesize that MYC up-regulates the expression level of VASP by promoting its transcription which promotes the migration of breast cancer cells.

To explore the effect of MYC on the invasive ability of breast cancer cells, according to the expression level of MYC in different breast cancer cells,We overexpress MYC in MCF-7 cells and knock down MYC in MDA-MB-231 cells (Fig. 2B, 2C). Further, we examined changes in VASP protein levels after overexpression and knockdown of MYC. The results of western blot revealed that the mRNA and protein level of VASP was upregulated after overexpression of MYC (Fig. 2D), while the protein level of VASP decreased after knocking down MYC (Fig. 2E).

Subsequently, we used the wound-healing assay and transwell assay to detect the effect of overexpressing MYC and knocking down MYC on the invasion ability of breast cancer cells. The results showed that the migration of MCF-7 cells was enhanced after overexpression of MYC, and the invasion ability of MDA-MB-231 was inhibited after knocking down MYC (Fig. 2F, 2G).

To detect the effect of exogenous MYC on transcriptional activity of VASP promoter, a series of truncated VASP promoter luciferase reporter vectors were constructed (Fig. 2F). The results of Luciferase assay revealed that MYC can significantly activate transcription of the luciferase reporter gene when the promoter length is greater than or equal to 995 bp (Fig. 2H). Subsequently, to verify that MYC binds to the promoter of VASP via the − 852~-842 site, we constructed the pGL3-VASP995 wild-type and pGL3-VASP-842 mutant luciferase reporter plasmids. Comparing with the wild type pGL3-VASP995, the results of luciferase assay indicated that the mutation of the binding site causes the pGL3-VASP-842 to lose its ability to promote transcription of the VASP promoter (Fig. 2I).

3.3 H3K4me3, WDR5 and MYC may regulate cytoskeletal pathway in genome-wide range, and co-enrich on the VASP promoter.

To analyze the correlation between WDR5, MYC, H3K4me3 and breast cancer cell migration ability, the ChIP-seq data of WDR5, MYC, and H3K4me3 in breast cancer cells were downloaded from the GEO databases (GEO number are GSE60897, GSE70098, GSE75169). The ChIP-seq data of the WDR5, MYC, and H3K4me3 in reported breast cancer were analyzed to visualize Genomic Annotation and distribution of transcription factor-binding loci relative to Transcription Start Site (TSS) using the ChIPseeker R package(Yu, Wang, & He, 2015), and the genes enriched by WDR5, MYC and H3K4me3 were intersected and the signal pathway analysis was performed on the co-enriched genes. The co-enrichment sites of WDR5, MYC, and H3K4me3 on the gene of VASP were analyzed and determined by Integrative Genomics Viewer (IGV).

To explore the association between WDR5, MYC, H3K4me3 and breast cancer cell migration, we firstly used the ChIPseeker R package to analyze ChIP-seq data of WDR5, MYC, and H3K4me3 from breast cancer cells. Visual genomic annotation and binding site analysis demonstrated that WDR5, MYC and H3K4me3 were mainly distributed in the genome at the Promoter position and within 2 kb from the Transcription Start Site (TSS), indicating that they may be involved in the transcriptional regulation of the gene (Fig. 3A, 3B). The genes enriched by WDR5, MYC and H3K4me3 were intersected and the signal pathways of the co-enriched genes were analyzed. It was found that the three genes were co-enriched in 10013 genes and may be involved in the regulation of cytoskeletal pathway (Fig. 3C, 3D). Then, IGV was

used to analyze and determine the co-enrichment sites of WDR5, MYC and H3K4me3 in the VASP promoter region. All of the three have significantly higher peaks in the VASP promoter region (-54~-194 and − 754~-1102 range from VASP TSS), indicating that H3K4me3, WDR5 and MYC, have colocalization on the VASP promoter (Fig. 3E). The binding sites were verified by ChIP-PCR (Fig. 3F) and the interactions between H3K4me3, WDR5 and MYC were verified by Co-IP and immunofluorescence (Fig. 3G, 3H).

3.4 H3K4me3, WDR5 and MYC are associated with breast cancer metastasis

Previous studies have shown that VASP is overexpressed in breast cancer and can participate in the cell invasion and migration by regulating cell matrix rearrangement. And based on our findings, there were enrichment of H3K4me3, WDR5 and MYC in the VASP promoter region, it was crucial to further explore the expression of H3K4me3, WDR5 and MYC in breast cancer and research on whether they regulate the expression of VASP. First, we analyzed the expression of MYC and WDR5 in breast cancer by TCGA database. The results (Fig. 4A) indicated that the mRNA of MYC and WDR5 were overexpressed in breast cancer tissues, especially in metastatic breast cancer tissues. At the same time, we also analyzed the relationships between MYC, WDR5 expression levels and clinicopathology by multivariate analysis. As shown in tables (Table 2, Table 3), there was a statistically significant correlations among high levels of MYC and WDR5 expression were found with tumor size ($P = 0.002$), distal metastasis ($P = 0.001$) and depth of invasion ($P = 0.038$). Survival analysis (Fig. 4B) revealed that breast cancer patients with high MYC and WDR5 expression exhibited poorer overall survival than those with low MYC and WDR5 expression. Next, we analyzed the enrichment of H3K4me3 on the VASP promoter in breast normal epithelial cells MCF-10A and breast cancer cells MCF-7 and MDA-MB-231 by RNA-sEq. The results (Fig. 4C) indicated that the enrichment level of H3K4me3 in the VASP promoter region of MDA-MB-231 and MCF-7 was significantly higher than that of MCF-10A. qPCR and Western blot were used to analysis WDR5, MYC and VASP expression levels in MCF-10A, MCF-7 and MDA-MB-231, The results (Fig. 4D) indicated that the mRNA and protein of WDR5, MYC and VASP were highly expressed in MCF-7 and MDA-MB-231. In conclusion, the above results showed that H3K4me3, WDR5 and MYC were overexpressed in breast cancer, and the expression level was positively correlated with the expression level of VASP. And they were all enriched in the promoter region of VASP, and might participate in the regulation of breast cancer development by regulating the expression of VASP.

Table 2
WDR5 mRNA expression and clinicopathologic characteristics of breast cancer.

Various		Number	WDR5		P value
			Low experssion	High experssion	
Age					
	< 60	654	316	338	0.195
	≥ 60	561	292	269	
ER status					
	Negtive	143	73	70	
	Postive	322	160	162	0.865
Pathologic M					
	M0	1021	531	490	0.002
	M1	195	77	118	
Pathologic N					
	N0/N1	979	491	488	0.828
	N2/N3	237	117	120	
Pathologic T					
	T1	310	164	146	0.236
	T2/T3/T4	906	444	462	
Distant metastasis					
	NO	442	275	167	0.080
	YES	17	7	10	

P values were based on χ^2-test, P < .05 was considered statistically significant.

Table 3
MYC mRNA expression and clinicopathologic characteristics of breast cancer.

Various		Number	MYC		P value
			Low experssion	High experssion	
Age					
	<60	654	276	378	0.001
	≥60	561	289	272	
ER status					
	Negtive	143	62	81	0.066
	Postive	322	171	151	
Pathologic M					
	M0	1020	566	454	< 0.001
	M1	196	42	154	
Pathologic N					
	N0/N1	979	444	535	0.132
	N2/N3	237	121	116	
Pathologic T					
	T1	277	155	112	< 0.001
	T2/T3/T4	907	410	497	
Distant metastasis					
	NO	251	126	115	0.013
	YES	7	3	14	

P values were based on χ^2-test, $P < 0.05$ was considered statistically significant.

3.5 H3K4me3 and WDR5 regulate VASP expression levels and migration of breast cancer cells

By analyzing the KEEG database, we found that H3K4 trimethylation, WDR5 and MYC may be involved in the regulation of cytoskeletal regulatory pathways. Therefore, MDA-MB-231 cells were treated with different concentrations of H3K4 trimethylation modifying enzyme inhibitor MM102, western blot was used to detect the effect of different H3K4me3 modification levels on VASP protein expression levels. The results showed that with the increase of MM102 concentration, the level of H3K4me3 modification

decreased, and the expression level of VASP was significantly inhibited (Fig. 5A). Furthermore, and the effect of MM102 on the migration ability of triple negative breast cancer MDA-MB-231 cells was detected by wound healing assay. Compared with the control group, MDA-MB-231 cells treated with 100 μM MM102 for 24 h showed a significant decrease in cell migration ability (Fig. 5F).

We examined changes in VASP expression levels and cell migration capacity after overexpression or knockdown of WDR5 in breast cancer cells. The results indicate that WDR5 can significantly up-regulate the expression level of VASP and promote the migration of breast cancer. To explore the effect of WDR5 on the invasive ability of breast cancer cells, we overexpressed WDR5 in MCF-7 cells and knocked down WDR5 in MDA-MB-231 cells according to the expression level of WDR5 in different breast cancer cells. Subsequently, western blot and RT-qPCR were used to detect the overexpression and knockdown efficiency of WDR5 in breast cancer cells (Fig. 5B, 5C). We used the wound healing and transwell assay to examine the effect of WDR5 on cell invasion. The results showed that the invasive ability of MCF-7 cells was enhanced after overexpression of WDR5 (Fig. 5G), while the invasion ability of MDA-MB-231 was inhibited after knockdown of WDR5 (Fig. 5H). Further, we examined the effect of overexpression and knockdown of WDR5 on VASP expression levels. Western blot and Q-PCR results showed that the mRNA and protein expression levels of vasp increased in MCF-7 cells after overexpression of WDR5, while mRNA and protein expression levels of vasp after MB-MDA-231 cells knocked down WDR5(Fig. 5D, 5E).

3.6 H3K4me3, WDR5 and MYC jointly regulate VASP-mediated breast cancer cell migration

In previous study, we found that the interaction between H3K4me3, WDR5 and MYC and co-enrichment on the VASP promoter, on the other hand, proved that they can promote VASP mRNA and protein expression levels in breast cancer cells. And enhance the migration ability of breast cancer. So, what we want to make clear is whether their regulation of VASP depends on the interaction of the three. First, in order to clarify the key role of H3K4me3 in the regulation of VASP expression by WDR5 and MYC, we treated the breast cancer cell MCF-7 with H3K4me3 methylase inhibitor MM102 to inhibit H3K4me3 modification level, and overexpress WDR5 or MYC, respectively. Western-blot was used to detect the protein expression level of VASP. The results showed that the level of H3K4me3 modification of cells treated with MM102 was decreased, and the up-regulation of VASP expression level caused by overexpression of MYC or WDR5 was reversed (Fig. 6A). To demonstrate whether WDR5 and MYC are independent of each other in the regulation of VASP expression, we have established a shRNA-stable cell line that interferes with WDR5 or MYC, overexpressing MYC or interfering with MYC based on interference with WDR5 in breast cancer cell MCF-7 respectively. On the basis of overexpression of WDR5, western-blot detected the expression level of VASP protein. The results showed that the interference of WDR5 expression can reverse the up-regulation of VASP expression caused by MYC overexpression (Fig. 6B). Down-regulation of MYC expression can reverse the up-regulation of VASP expression caused by WDR5 overexpression (Fig. 6C). These results indicate that H3K4me3, WDR5 and MYC are interdependent in the regulation of VASP expression. To further illustrate the key role of VASP in H3K4me3, WDR5 and MYC in promoting breast cancer cell migration, we designed and synthesized VASP siRNA to interfere with the expression level of VASP in breast cancer cell MCF-7, and overexpress WDR5 or MYC, respectively.

Scratch and Transwel detect cell migration ability. Scratch and Transwel results indicate that interference with VASP expression can partially reverse the effect of WDR5 or MYC overexpression on breast cancer cell migration (Fig. 6D). This result indicates that VASP acts as a downstream target gene and mediates the regulation of WDR5 and MYC on breast cancer cell migration.

4. Discussion:

Breast cancer is one of the most threatening diseases for women, while recurrence and metastasis is the leading cause of death in breast cancer patients. In many breast cancer metastasis-related pathways, the cytoskeletal regulatory pathway plays an important role in the migration of tumor cells by regulating the formation of microfilaments and microtubules, participating in cytoskeletal rearrangement, and regulating the formation of pseudopods. Vasodilating stimulating phosphoprotein (VASP), a member of the cytoskeletal regulatory pathway, promotes the migration of cells by promoting action assembly to form F-actin(Krause, Dent, Bear, Loureiro, & Gertler, 2003; Kwiatkowski, Gertler, & Loureiro, 2003). VASP plays an important role in various tumors such as breast cancer (Padilla-Rodriguez et al., 2018), gastric cancer (J. Wang et al., 2012), and colorectal cancer (Tu et al., 2015). The specific mechanism of VASP promoting migration hasn't been proved. In this study, we focused on one of the pathways that regulate VASP expression and cell migration in breast cancer. In order to clarify the role of VASP in the invasion and metastasis of breast cancer, we analyzed the RNA-seq data and corresponding clinical data of the published breast cancer samples downloaded from the TCGA database. We founded that comparing with normal breast tissues VASP is highly expressed in breast cancer tissues and metastatic tissues. Subsequently, we analyzed mRNA expression levels of VASP in different molecular subtypes of breast cancer. The results indicated that VASP is highly expressed in HER2-enriched and Basal-like breast cancer, while these two subtypes are considered to be highly invasive. Subsequently, we analyzed the effect of VASP expression level on the clinicopathological features and prognosis of breast cancer patients. The results showed that patients with high expression of VASP not only had a worse prognosis, but also were more prone to lymph node metastasis of breast cancer. Furthermore, we examined the expression level of VASP in breast cancer tissues and corresponding adjacent tissues. The results showed that the expression level of VASP in breast cancer tissues was higher than that in adjacent tissues. Subsequently, we examined the expression levels of VASP in breast cancer cells and normal breast cells, respectively, and the results showed that the expression level of VASP in breast cancer cells was higher than that in normal breast cells. Compared with MCF-7 cells, MDA-MB-231 cells with high expression of VASP have stronger invasive ability. Although we have initially explored the mechanism of VASP promoting breast cancer migration in previous studies (Hu, Zou, Zhan, & Cao, 2008; J. Wang et al., 2012), the existing research still lacks a comprehensive understanding of the regulation of VASP expression regulation in breast cancer.

Early studies of VASP have revealed the mechanism by which VASP promotes cell migration by regulating the assembly of Actin, so most of the subsequent studies are more concerned with the regulation of VASP. In these studies, VASP has been shown to be regulated by various transcription factors such as HIF1-α, ERα, and CREB in various tumors(Kan et al., 2008; J. Wang et al., 2012; Yoneda et

al., 2017). In the present study, we predicted a binding site for the transcription factor MYC at the promoter of VASP. As a classical proto-oncogene, MYC has been well known for its role in promoting tumorigenesis. However, whether MYC can be used as a transcription factor of VASP to regulate tumor invasion and metastasis has not been reported. In order to clarify this issue, we carried out a series of studies around MYC's regulation of VASP. By over-expressing or knocking down MYC in breast cancer cells, we founded that MYC not only promotes the expression levels of VASP but also promotes the migration of breast cancer cells. Through luciferase reporter assay, we demonstrated that MYC acts as a transcription factor to regulate VASP transcription and identifed the binding site of MYC and VASP promoters.

With the development of epigenetics research, more and more studies have revealed that the transcription of a transcription factor-regulated gene depends not only on the presence or absence of its binding site on the promoter of the gene, but also on the openness of the chromosomal structure to the transcription factor at the position of the gene including loose or dense chromosomes, active histone modifications, etc. MYC is also selective for the activation of gene transcription as a cancer-promoting factor. This selectivity for cancer-promoting target genes depends not only on the genomically-existing binding sequences, but also on the chromosomal-active histone modifications at the gene locus. For example, MYC can promote the translation of cyclin D2 by acetylation of histones by recruiting TRRAP (Bouchard et al., 2001), while MYC can recruit CBP to regulate the transcriptional translation of downstream genes by histone acetylation (Vervoorts et al., 2003). In addition, MYC stimulates its activity by recruiting P-TEFb, phosphorylating the C-terminal domain (CTD) of serine 2 of RNA polymerase II (POL II) (Ullius et al., 2014). H3K4me3 is a currently recognized active histone modification, and abnormal H3K4me3 can promote the invasion and metastasis of tumor by causing epigenetic changes and regulating the expression of oncogenes (Low, Mizoguchi, & Mizoguchi, 2013). Studies have shown that H3K4me3 is significantly associated with the prognosis of colorectal cancer and prostate cancer (Ellinger et al., 2010; Liu, Li, Li, Liu, & Cui, 2018). MYC is ecotropic for H3K4me3 modified regions within the genome. However, there is no research showing that MYC has direct interaction with H3K4me3 modification. Therefore, the functions and related mechanisms of MYC and H3K4me3 need to be further studied. In previous studies, we founded that H3K4me3 exerts its function as an active histone modification depending on a series of proteins that recognize it. These proteins contain specific domains that specifically recognize H3K4me3 modifications such as PHD finger, WD40 domain, etc(Klein & Gay, 2015; Meloche et al., 2015; Patel & Wang, 2013). WDR5 is a protein containing WD40 domain, and its ability to specifically bind to H3K4me3 has been revealed by many studies. What is more interesting is that WDR5 can bind a variety of proteins in its spatial structure. It can bind to H3K4me3 and can also bind to various transcription factors including MYC. However, no relevant studies have clearly indicated that it can act as an "adapter" for H3K4me3 and MYC to regulate gene transcription and play a role in tumorigenesis. Therefore, in the follow-up study, we focused on the regulation of VASP expression by H3K4me3, WDR5 and MYC, and explored their role in the invasion and metastasis of breast cancer.

The transcriptional regulation of genes by proteins or transcription factors depends on their interaction with the promoter of the gene. To illustrate the gene regulation of H3K4me3, WDR5 and MYC in breast

cancer, we analyzed ChIP-seq data for H3K4me3, WDR5 and MYC in breast cancer cell lines. We found that H3K4me3, WDR5 and MYC is mainly enriched in the promoter region of 10013 genes within 2kbp of the gene transcription initiation site. These genes are mainly involved in the regulation of MAPK, ErbB, mTOR and other signaling pathways including VASP involved cytoskeletal regulatory pathway related to tumorigenesis and development. This result suggests that H3K4me3, WDR5 and MYC may be involved in the regulation of some key cancer-promoting pathways in breast cancer. Further visualizing the genetic enrichment levels of H3K4me3, WDR5 and MYC, we found that H3K4me3, WDR5 and MYC have co-enrichment sites on the VASP promoter. We then verified this prediction by ChIP-PCR. Our results reveal that H3K4me3, WDR5 and MYC are close to each other in spatial position, and these three factors may play a role in regulating VASP transcription through interaction. To confirm this conjecture, we detected their interaction by co-immunoprecipitation. The results showed that there was indeed an interaction between H3K4me3, WDR5 and MYC. However, the mechanisms by which these three factors regulate VASP in breast cancer cells remain to be studied.

Through previous studies, we have learned that H3K4me3 is one of the markers of genomic transcriptional activity, and its cancer-promoting effect has been studied in many cancers. For example, high expression of H3K4me3 in early colon cancer has been shown to be associated with tumor recurrence and poor prognosis in patients (Benard et al., 2014). Upregulation of H3K4me3 mediated by low expression of ARID5B in prostate cancer promotes expression of androgen receptor leading to cell proliferation (Yamakawa, Waer, & Itakura, 2018). These studies suggest that H3K4me3 may play a role in the development of tumorigenesis by regulating the gene transcription. In breast cancer, H3K4me3 is highly expressed in HER2-enriched and Basal-like subtypes of breast cancer which have a higher invasive ability than the other breast cancer subtypes. and H3K4me3 is also associated with lymph node metastasis and poor prognosis in breast cancer patients(Elsheikh et al., 2009). Therefore, H3K4me3 is highly likely to be involved in the transfection of breast cancer. In this study we analyzed the modification level of H3K4me3 in VASP promoter region using normal mammary epithelial cell MCF-10A, low invasive Luminal breast cancer cell MCF-7, and highly invasive Basal-like breast cancer cell MDA-MB-231. The results showed that the level of modification of H3K4me3 in the VASP promoter region was also higher in cells with stronger invasive ability. Therefore, it is also meaningful to further explore the mechanism by which H3K4me3 regulates VASP expression. WDR5 is reported as an adapter for H3K4me3 and its role in tumors has also been extensively studied. At present, most studies proved that WDR5 acts as a core component of histone methylase to promote H3K4me3 modification. For example, in colon cancer and acute myeloid leukemia, high expression of WDR5 directly regulates the modification level of H3K4me3 and enhances the survival ability of tumor cells in chemoradiotherapy (Neilsen et al., 2018; Zhang et al., 2018). However, the regulatory mechanism does not explain how H3K4me3 selectively opens oncogenes and plays a role in promoting cancer.

Some studies about WDR5 shows that WDR5 plays a role in promoting cancer and is closely related to MYC. Since the MbIIIb domain of MYC can bind to WDR5, WDR5 and myc can act as a co-activator to promote cancer in a variety of tumors(Thomas, Wang, et al., 2015). Co-activation of WDR5 and MYC in pancreatic ductal adenocarcinoma allows cells to cross cell replication checkpoints and promotes cell

proliferation. In neuroblastoma, the interaction between WDR5 and N-myc inhibits cell apoptosis by up-regulating MDM2 expression. Early formation of neuroblastoma. In breast cancer, WDR5 has been reported to promote breast cancer proliferation through CBX8 interaction activation of the Notch pathway(Neilsen et al., 2018). WDR5 expression levels are associated with poor prognosis in breast cancer patients(Dai et al., 2015). However, there are no relevant reports indicating the relationship between WDR5 and MYC interaction and the development of breast cancer(Thomas, Foshage, et al., 2015). In this study, we analyzed the relationship between WDR5, MYC and breast cancer, and analyzed the mRNA expression data and clinical data of WDR5 and MYC from TCGA database. The results showed that the expression of WDR5 and MYC in breast cancer tissues was significantly higher than that in adjacent tissues. And the expression of WDR5 and MYC is associated with lymph node metastasis and poor prognosis in breast cancer patients. The mRNA and protein levels of WDR5 and MYC in breast cancer cells MCF-7 and MDA-MB-231 were also significantly higher than those of normal breast epithelial cells MCF-10A. These results suggest that WDR5 and MYC are likely to be involved in the development of breast cancer.

To further clarify that H3K4me3, WDR5 and MYC together enhance the migration of breast cancer cells via promoting the expression of VASP, we inhibited intracellular H3K4me3 levels via H3K4me3 modified enzyme inhibitors MM102. After treated with MM102 or negative control, WDR5 were overexpressed or knocked down in breast cancer cells. The results suggest that the expression level of VASP and the invasion ability of breast cancer cells can be positively regulated by H3K4me3 and WDR5. Our previous studies have confirmed that H3K4me3, WDR5 and MYC are co-enriched and co-localized on the VASP promoter, so we suspect that the three factors are involved in the regulation of VASP transcription. After treated with MM102, we found that overexpression of WDR5 or MYC did not upregulate the expression level of VASP in breast cancer cells. Overexpression of MYC did not upregulate the expression level of VASP after knockdown of WDR5 in breast cancer cells. Overexpression of WDR5 did not affect the expression level of VASP after knockdown of MYC in breast cancer cells.

The above results indicate that H3K4me3, WDR5 and MYC are interdependent in the regulation of VASP. To further elucidate whether the regulation of breast cancer migration by H3K4me3, WDR5 and MYC is mediated by VASP, we used siRNA to knock down VASP. The results suggested that overexpression of WDR5 or MYC didn’t promote the invation of breast cancer cell after knocking down VASP. Therefore, WDR5 and MYC promote the invasion of breast cancer cells via regulating VASP. Taken together, this study demonstrates that H3K4me3, WDR5, and MYC have co-enrichment and interactions in the VASP promoter region in breast cancer cells. The complex formed by H3K4me3, WDR5 and MYC regulates VASP transcription and participates in VASP-mediated invasion in breast cancer cells. Therefore, inhibitors that specifically inhibit the interaction between MYC and WDR5 might specifically inhibit cancer-promoting pathways including VASP-mediated cell migration pathway. This study provides a theoretical basis for the development of such targeted anticancer drugs.

Declarations

Ethics approval and consent to participate

The experimental protocol was established, according to the ethical guidelines of the Helsinki Declaration and was approved by the Human Ethics Committee of Zhongnan Hospital of Wuhan University. Written informed consent was obtained from individual or guardian participants.

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Author contribution statement

Xiaolong Xu, Xiaoning Yuan and Lei Wei designed and performed the study; Yang Gao, Weinan Yin and Jing Guo collected and analyzed the clinical data; Xiaolong Xu, Jiali Ni, Jingwei Zhang and Feng Li analyzed the results and wrote the manuscript. All authors read and approved the final manuscript.

Data availability statement

Publicly available datasets were analyzed in this study. Source data of this study were derived from the public repositories, as indicated in the section of “Materials and Methods” of the manuscript. And all data that support the findings of this study are available from the corresponding author upon reasonable request.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

Not applicable.

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41. Legends.

Figures

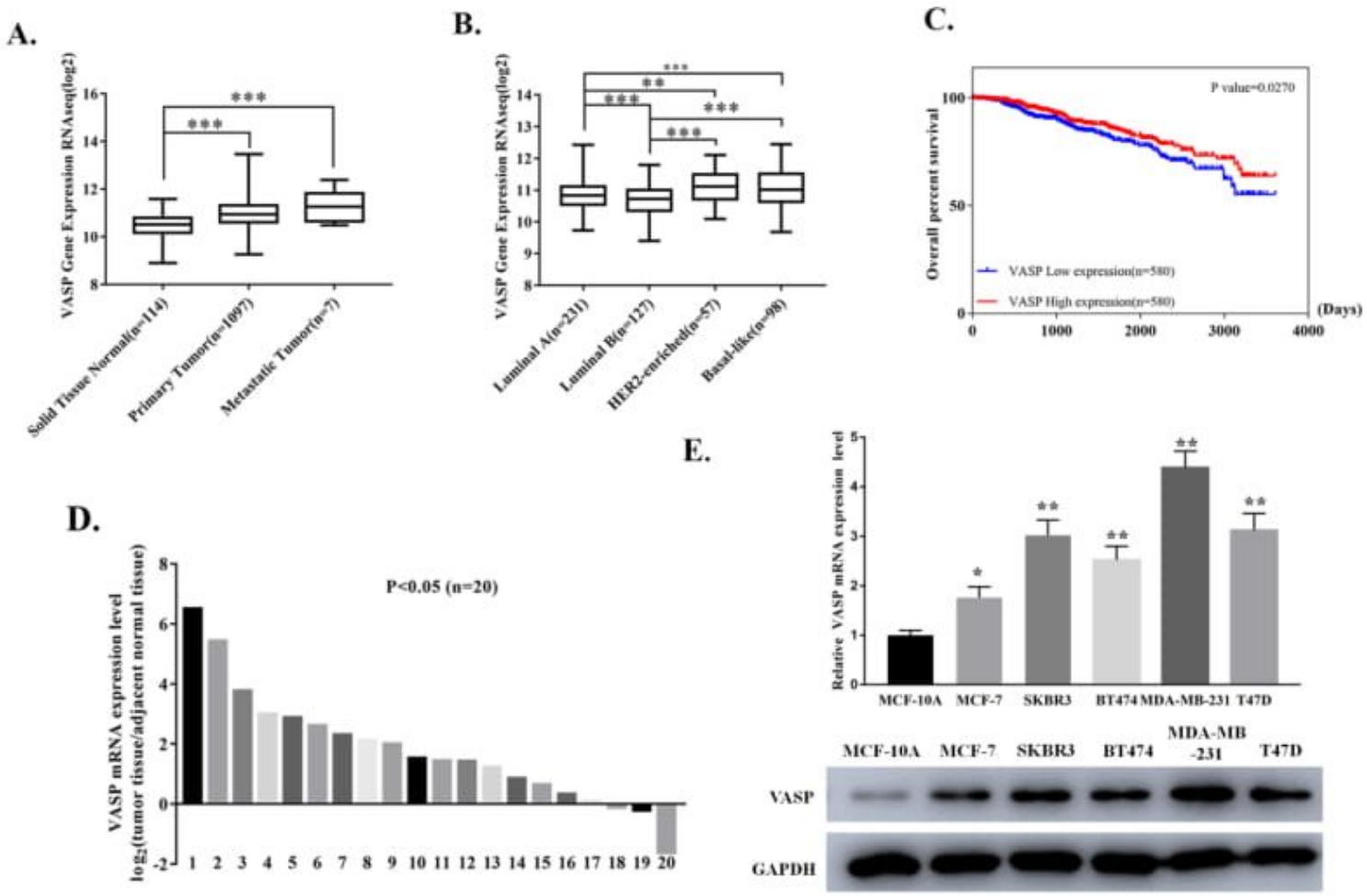

Figure 1

VASP is upregulated in human breast cancer tissues and cells. A VASP mRNA expression level in different sample types of breast cancer tisssues the data was extractsed from TCGA-BRCA dataset, mRNA expression level was normalized by log2(counts+1). B, VASP mRNA expression level in different molecular subtypes of breast cancer tissues, the data was extractsed from TCGA-BRCA dataset, mRNA expression level was normalized by log2(counts+1). C, Overall survival probability of breast cancer patient with VASP mRNA in high or low expression level. The data was extractsed from TCGA-BRCA dataset, patients were grouped by median mRNA expression level of VASP. D, VASP mRNA expression level in 20 pairs of breast tumor and adjacent normal tissues. The VASP expression level was measued by qPCR and normalized by log2(fold change of VASP mRNA level in tumor relative to adjacent normal tissues). E, VASP mRNA and protein expression level in three breast cell lines. MCF-10A is breast normal epithelial cell line, MCF-7 is luminal subtype breast cancer cell line, MDA-MB-231 is basal-like subtype breast cancer cell line. *P <.05, **P <.01, ***P <.001.

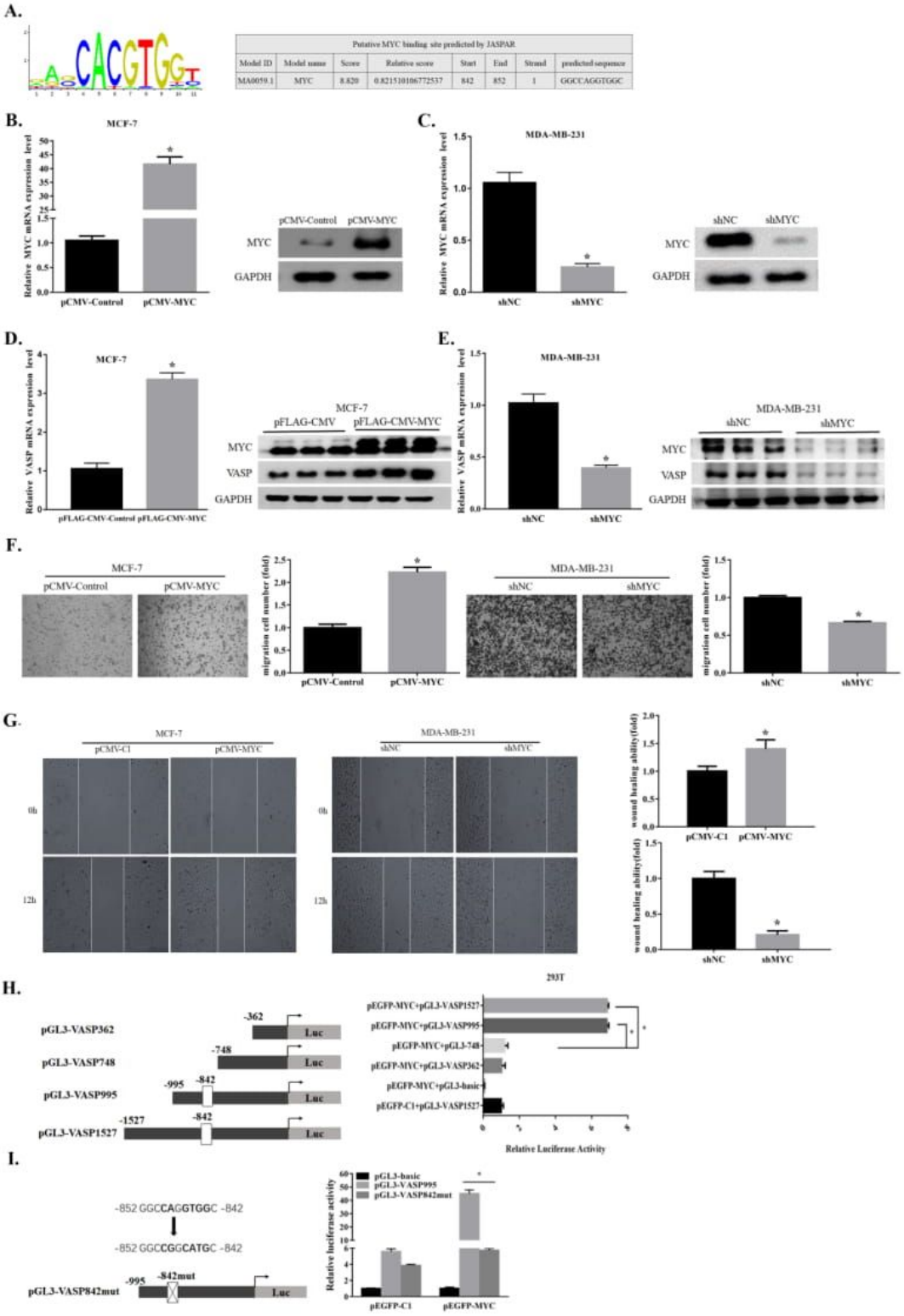

Figure 2

MYC is a transcription factor of VASP that promotes VASP expression and cell migration. A, The promoter of VASP was found to have a binding site to MYC from -842bp to -852 bp from the transcription start site through the JASPAR database. B, The pCMV-MYC overexpression vector or the pCMV-control vector were transfected into MCF-7 cells, RT-qPCR and western blot were performed to detect the expression of MYC after transfection. C, shMYC (for Knocking down MYC) was transfected into MDA-MB-231 cells with shNC

plasmids as control. RT-qPCR and western blot were performed to detect the expression of MYC after transfection. D, The results of RT-qPCR and western blot revealed that overexpression of MYC in MCF-7 cells significantly up-regulated VASP mRNA and protein levels. E, Knockdown of MYC in MDA-MB-231 cells significantly inhibited VASP mRNA and protein expression. F, Results of transwell assay suggested that MYC knockdown enhances the migration ability of MDA-MB-231 cells, while MYC overexpression suppress the migration of MCF-7 cells. G, Results of wound healing assay suggested that MYC knockdown enhances the migration ability of MDA-MB-231 cells, while MYC overexpression suppress the migration of MCF-7 cells. H, A series of truncated VASP promoter luciferase reporter vectors were constructed. MYC overexpression vector and reporter vectors were co-transfected into 293T cells, luciferase assay detected the transcriptional activity. The results of Luciferase assay revealed that MYC can significantly activate transcription of the luciferase reporter gene when the promoter length is greater than or equal to 995 bp. I, The results of luciferase assay indicated that MYC reduced transcriptional regulation of the VASP promoter after mutating the -842bp~-852 bp site. *P <.05, **P <.01, ***P <.001.

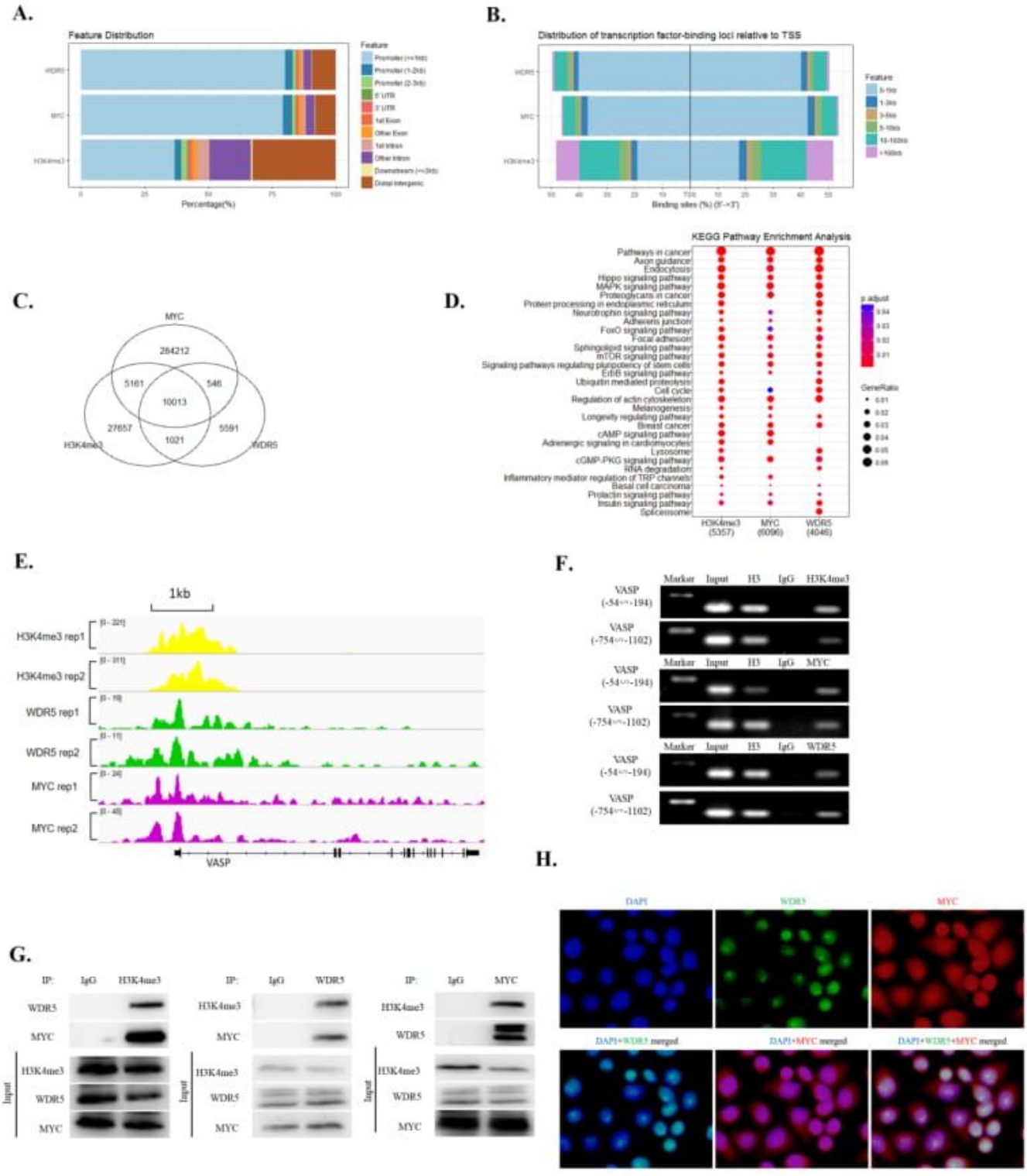

Figure 3

WDR5, MYC, H3K4me3 regulate cytoskeletal pathway in breast cancer. A, Visualized genome annotation of WDR5, MYC, H3K4me3. Visual genomic annotation of WDR5, MYC, and H3K4me3 was performed using the Chipseeker R package, indicating that the three genes were mainly distributed in the promoter position in the genome. B, The positional distribution of the WDR5, MYC, and H3K4me3 transcription factor-binding sites from the Transcription Start Site (TSS). The binding site analysis of WDR5, MYC and

H3K4ME3 by Chipseeker R package showed that the they were mainly distributed in the genome within distance of 2kb from TSS, indicating that they may be involved in the transcriptional regulation of genes. C, H3K4me3, WDR5 and MYC enriched genes were found to be intersections, and it was found that they were enriched in 10013 genes in common. D, Enrichment of WDR5, MYC, H3K4me3 related KEGG pathways. For the KEGG signal pathway analysis, the left side is the enriched signal pathways, and the right side shows the enrichment degrees of WDR5, MYC, H3K4me3 in each signal pathway. The degree of enrichment is positively related to the diameter of the solid point. The results demonstrated that WDR5, MYC and H3K4me3 were enriched in the regulatory of actin cytoskeleton pathway. E, Enrichment of H3K4me3, WDR5, MYC in the VASP promoter region. At the bottom of the figure is the VASP full-gene reference sequence pattern, with the promoter region of VASP on the left side, and the peaks in the upper genes which are significantly higher than other regions are possible binding sites, indicating the three protein have co-enrichment on the VASP promoter. The co-enrichment site was on -54~-194 and -754~-1102 upstream of the VASP transcription start site which were analyzed by Integrative Genomics Viewer (IGV). F, H3K4me3, WDR5 and MYC co-enrichment site on VASP promoter was proved by ChIP-PCR, rabbit IgG as negtive control. G, H3K4me3, WDR5 and MYC could interact with each other which were proved by Co-Immunoprecipitation (Co-IP), rabbit IgG as negtive control. H, WDR5 and MYC colocalized in MCF-7 breast cancer cell. WDR5 was overexpressed with GFP tag (green), and MYC was overexpressed with DsRed tag (red).

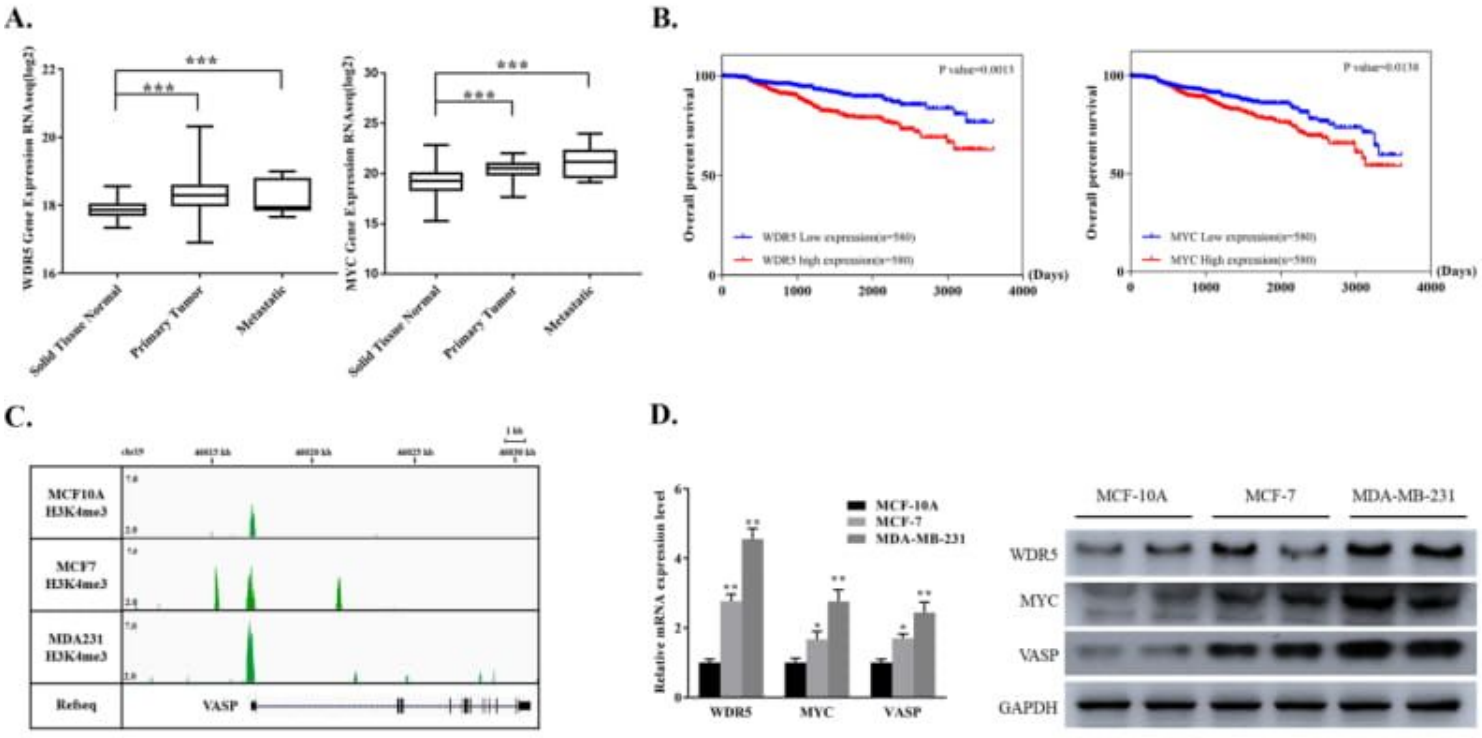

Figure 4

WDR5, MYC are upregulated in breast cancer. A, WDR5 and MYC mRNA expression level in different sample types of breast cancer tisssues the data was extractsed from TCGA-BRCA dataset, mRNA expression level was normalized by log2(counts+1). B, Overall survival probability of breast cancer

patient with WDR5 or MYC mRNA in high or low expression level. The data was extractsed from TCGA-BRCA dataset, patients were grouped by median mRNA expression level of VASP. C, ChIP-seq visualized analysis of the enrichment H3K4me3 on the VASP promoter in breast normal epithelial cells MCF-10A and breast cancer cells MCF-7 and MDA-MB-231. The data was extracted from GSE69377, GSE71003 and GSE60897 dataset in GEO and analyzed by MACS2 and Integrative Genomics Viewer. D, qPCR and western-blot analysis of WDR5, MYC and VASP mRNA and protein levels in MCF-10A, MCF-7 and MDA-MB-231. *P <.05, **P <.01, ***P <.001.

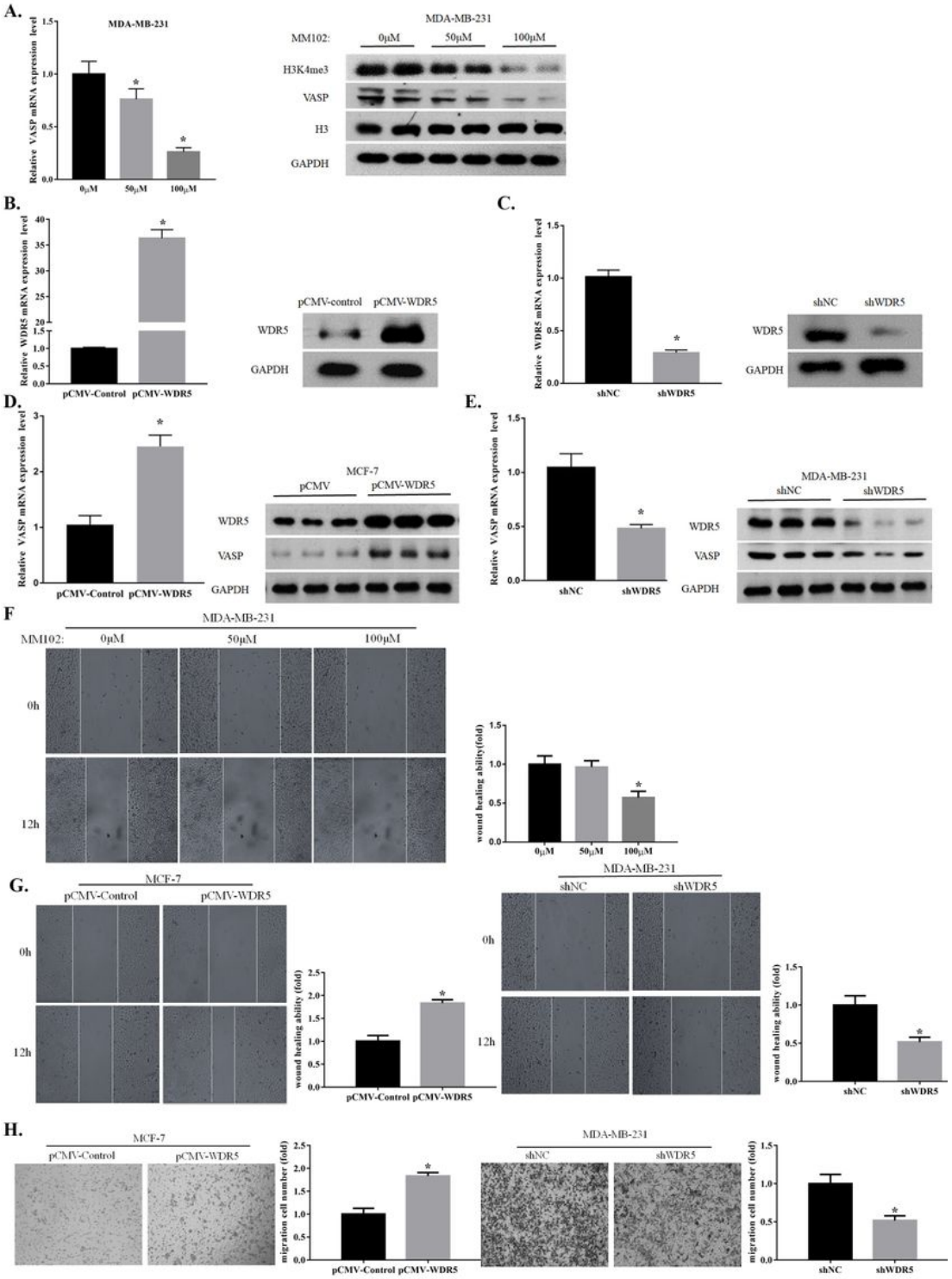

Figure 5

H3K4me3, WDR5 up-regulate the expression of VASP and promote the migration of breast cancer cells. A, H3K4me3 can regulate mRNA and protein expression level of VASP. H3K4me3 is down-regulated by H3K4 histone methylase specific inhibitor MM102 in concentration of 50μM and 100μM. B, The pCMV-WDR5 overexpression vector or the pCMV-control vector were transfected into MCF-7 cells, RT-qPCR and western blot were performed to detect the expression of WDR5 after transfection. C, shWDR5 (for Knocking down WDR5) was transfected into MDA-MB-231 cells with shNC plasmids as control. RT-qPCR and western blot were performed to detect the expression of WDR5 after transfection. D, The results of RT-qPCR and western blot revealed that overexpression of WDR5 in MCF-7 cells significantly up-regulated VASP mRNA and protein levels. E, Knockdown of MYC in MDA-MB-231 cells significantly inhibited VASP mRNA and protein expression. F, Cell migration was down regulated when H3K4me3 level was inhibited by MM102. Cell migration ability was detected by wound healing assay. G, Results of transwell assay suggested that WDR5 knockdown enhances the migration ability of MDA-MB-231 cells, while WDR5 overexpression suppress the migration of MCF-7 cells. H, Results of wound healing assay suggested that WDR5 knockdown enhances the migration ability of MDA-MB-231 cells, while WDR5 overexpression suppress the migration of MCF-7 cells. *P <.05, **P <.01, ***P <.001.

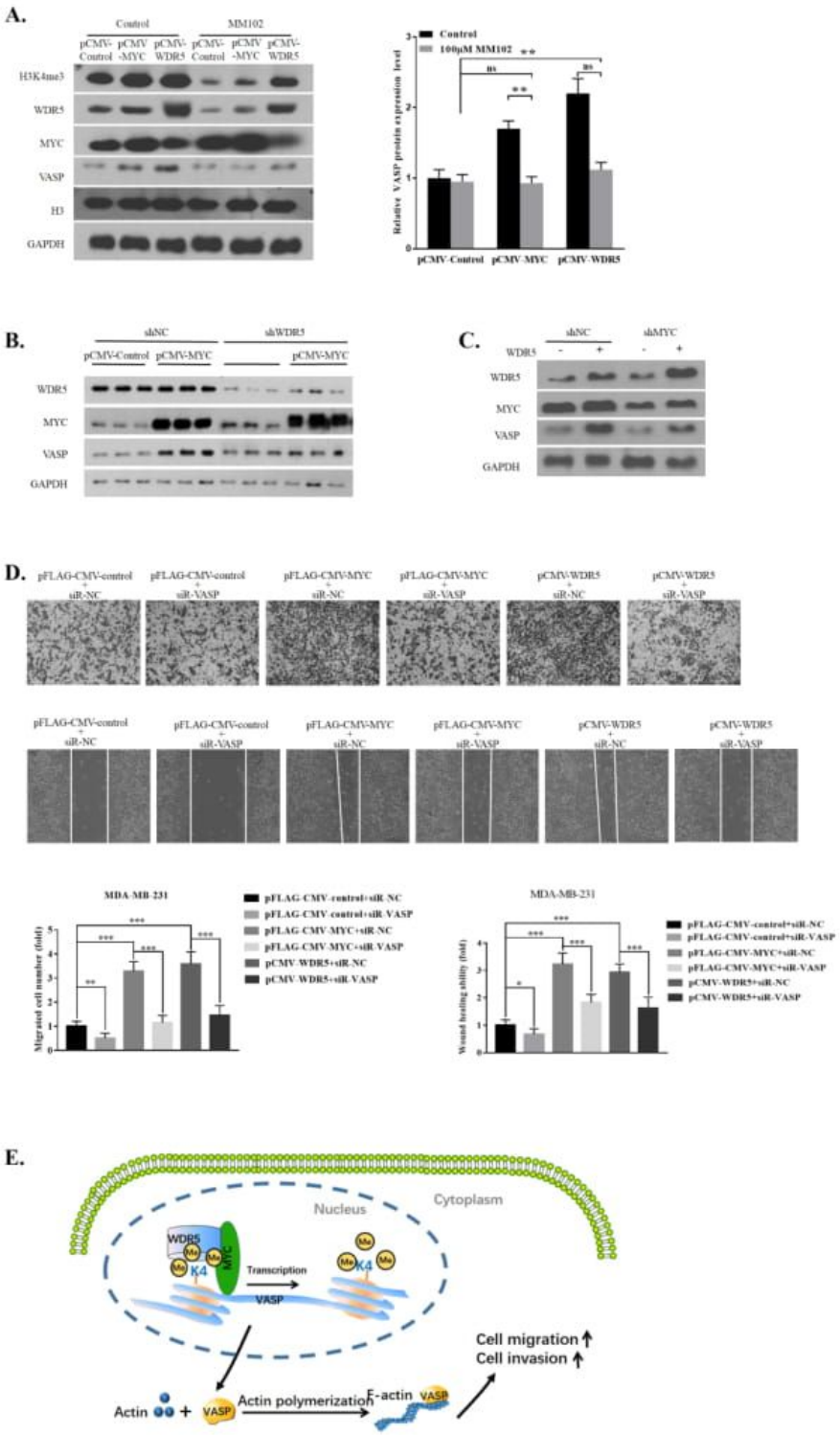

Figure 6

H3K4me3, WDR5 and VASP work together regulating VASP-mediated breast cancer cell migration. A, Inhibition of H3K4me3 expression can reverse the effect of WDR5 or MYC up-regulating VASP expression. B, Inhibition of WDR5 expression can reverse the effect of MYC up-regulating VASP expression. C, Inhibition of MYC expression can reverse the effect of WDR5 up-regulating VASP expression. D, Inhibition

of VASP expression can reverse the effect of WDR5 and MYC up-regulating cell migration ability which was detected by wound healing assay and transwell. *P <.05, **P <.01, ***P <.001.