Immunoglobulin superfamily 9 (IGSF9) is trans-activated by p53, inhibits breast cancer metastasis via FAK

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INTRODUCTION

Breast cancer is the most common malignant cancers with high incidence and mortality in women [1], with ~2.3 million new cases and 685,000 deaths in 2020 [2]. Vast majority of breast cancer-related deaths is due to metastasis to distant organs, such as lungs [3–5]. The metastatic breast cancer (mBC) patients are mostly from recurrence after primary treatment of earlier-stage breast cancer, and partially from de novo metastasis [6]. mBC is largely an incurable disease with a 5-year survival rate around 23.8–30% [7]. Thus, understanding the underlying mechanisms of breast cancer metastasis is of paramount importance for developing effective treatment.

Tumor suppressor p53, a sequence-specific DNA binding transcription factor, regulates the expression of genes involved in a variety of cellular functions, including cell-cycle arrest, DNA repair, and apoptosis [8]. In breast cancer, p53 suppressed metastasis by upregulating an early onset breast cancer-associated gene GAS7 [9], p53 negatively regulated the expression of a potential targeted radioiodine therapy candidate, human sodium iodide symporter [10]. TP53 (p53) is most frequently mutated in invasive breast cancer. Mutations in TP53 present in ~25–30% of all breast cancer cases [11], and in 80% of patients with triple-negative breast cancer (TNBC) [12]. The TNBC with mutant p53 usually have poor prognosis [13]. Wei et al. described a global map of p53 transcription-factor binding sites in the human genome [14]. Further dissect and confirm potential p53 targets will be beneficial for mBC diagnosis and treatment.

Cell adhesion molecules (CAMs) are membrane glycoprotein receptors that mediate cell–cell/matrix interaction and transduce intracellular signals for cell adhesion, migration, invasion and organ-specific metastasis [15]. CAMs include cadherins, selectins, integrins, the immunoglobulin superfamily (IgSF), and etc [15, 16]. Immunoglobulin superfamily 9 (IGSF9), first cloned in 2000 [17], is a member of the IgSF. IGSF9 contains extracellular N terminus with five Ig domains followed by two fibronectin type III domains, a signal transmembrane sequence, and a large C terminal cytoplasmic tail [18]. Previous studies reported that IGSF9 was aberrantly expressed in different cancer types, such as down-regulation in melanoma, colorectal familial adenomatous polyposis [19, 20], and upregulation in gallbladder cancer, ovarian cancer, and endometrial cancer [21–23]. Elevated IGSF9 was associated with the poor prognosis of nasopharyngeal carcinoma in Huang et al.’ study [24]. However, the molecular mechanisms underlying IGSF9 expression and cancers remain elusive, especially in breast cancer.

Focal adhesion kinase (FAK), a non-receptor tyrosine kinase, consists of N-terminal 4.1, ezrin, radixin, moesin homology domain FERM, a central catalytic tyrosine kinase domain, and a C-terminal region containing a focal-adhesion targeting domain and a proline-rich region [25, 26]. FAK acts as both a signaling kinase and cell adhesion-associated scaffold to coordinate the positional recruitment and phosphorylation of different cytoskeletal-associated proteins paxillin and p130Cas [27]. FAK could bind with integrins or growth factor receptors to control cell motility, invasion, and survival [28]. Previous studies indicated that FAK was...

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involved in cancer development and progression [29, 30]. FAK is over-expressed and activated in invasion or metastatic breast cancer, and is associated with breast cancer progression and poor prognosis [31–33]. Wu et al.'s study showed that deletion of FAK in mammary epithelial cells suppressed tumor formation and metastasis [34].

In this study, we discovered that wild-type p53 trans-activates IGSF9 transcriptional activity by binding to its promoter. Loss of
IGSF9 correlates with breast cancer metastasis and poor prognosis. IGSF9 inhibits breast cancer metastasis through FAK signaling. IGSF9/FAK axis might serve as a potential target for breast cancer treatment.

RESULTS
p53 trans-activates IGSF9 in breast cancer
p53 as a transcriptional factor regulates significant cellular activities, such as cell cycle, senescence, and apoptosis. We predicted the characteristic of potential transcription binding sites using JASPAR database (Fig. 1A). Then Chip Atlas, HTTarget, GTRD, and Harmonizome were used for potential target genes of p53. Venn diagram analysis revealed 1795 target genes (Fig. 1B). GO and KEGG pathway enrichment analysis indicated these potential genes mainly involved focal adhesion pathway, adhesion junction, and so on (Fig. S1A, B). Metascape was also used for GO function and KEGG pathway enrichment analysis to explore the main functions and pathways of target genes (Fig. S1C). These data suggested that p53 regulated cell adhesion-related genes (CAMs). Given that IGSF9 is a member of CAMs and potential target of p53 (data from GTRD database), we further used GeneMANIA to construct the network of TP53 and IGSF9 to explore their interaction from different aspects (Fig. 1C).

To explore the relationship between p53 and IGSF9, we searched TCGA datasets. The positive correlation between the mRNA levels of TP53 and IGSF9 was observed in breast cancer patients (Pearson $r = 0.17$, $P < 0.001$) (Fig. 1D). Compared with normal mammary cell MCF-10A, lower level of p53 protein was observed, which is consistent with decreased IGSF9 level in breast cancer cell MCF-7 (Fig. 1E), suggesting the potential role of p53 in regulating IGSF9 expression of breast cancer. The chromatin immunoprecipitation (ChIP) assay was then performed with PCR primers flanking the region of the binding site (~3000~500 bp). The results indicated that p53 bound and enriched the promoter of IGSF9 (Fig. 1F, G).

To further confirm the regulating role of p53 in IGSF9 gene expression, luciferase report assay was employed to detect transcription activity of a serial of IGSF9-luc reporter genes ranging from ~3000 to +50 bp of putative promoter. The sequences of ~183~/~116 nt have the highest promoter activity, and this region included p53 binding site (~137~/~131 nt) (Fig. 1H). Deletion and mutation of putative p53 binding site significantly diminished IGSF9 promoter activity (Fig. 1I, J). p53 protein significantly increased IGSF9 transcriptional activity, which was markedly reduced by TP53 knockdown (Fig. 1K). Consistently, TP53 knockdown significantly decreased endogenous and exogenous IGSF9 expression in MCF-7 cells (Fig. 1I). IGSF9 and p53 protein was determined by western blot (Fig. 1O). IGSF9 was over-expressed and TP53 was knocked down in MCF7 cells as indicated. IGSF9 and p53 protein was determined by western blot. Relative intensity of IGSF9 and p53 was quantified by ImageJ and normalized to GAPDH. The Student’s $t$ test was used; ***$P < 0.001$. p53 binds to the promoter of IGSF9. ChIP assay was applied to determine the binding of IGSF9 promoter to p53. p53 antibody enriched the DNA sequence of IGSF9 promoter. PCR (F) and qRT-PCR quantification (G) of the immunoprecipitated DNA were measured. Rabbit IgG was used as a negative control and RNA polymerase II (RNAP II) as a positive control. Values represented enrichment relative to input DNA. Data were represented as the mean ± SD. The Student’s $t$ test was used; ***$P < 0.001$.

Analysis of IGSF9 expression in breast cancer
gRNAi was used to downregulate IGSF9 expression in MCF-7 cells (Fig. 1L). These results indicated that p53 trans-activated IGSF9 by binding to its promoter.
with those of high IGSF9 expression (Fig. 2H, I). Taken together, these data indicated that decreased IGSF9 expression correlated with poor prognosis and metastasis and could be a valuable indicator for poor prognosis in breast cancer patients.

**IGSF9 copy number and mutations do not alter IGSF9 mRNA levels**

Genetic alteration and epigenetic inactivation are two major causes for the loss of suppressor tumor genes. To explore genomic alterations and epigenetic inactivation of IGSF9, we analyzed copy number alterations and mutations of IGSF9 in breast cancer patients. We found that IGSF9 copy number and mutations did not alter IGSF9 mRNA levels in breast cancer patients.
alteration of IGSF9 in breast cancer, we analyzed the sequences in 9555 breast cancer cases from cBioportal database (2012 to 2021). The frequency of IGSF9 copy number alteration was 0–21.26%, which were all amplification (Fig. S3A). Besides, only 5/16 studies from cBioportal database indicated IGSF9 mutation may occur, and the frequency of IGSF9 mutation was <3% in these datasets (Fig. S3B). No significant decrease of IGSF9 mRNA with the shallow deletion of IGSF9 was found (Fig. S3C). These data indicate that gene copy number is not a major factor for reduced IGSF9 expression level in breast cancer. The location of the different mutations within IGSF9 was shown in Fig. S3D, four mutations of which were predicated to be deleterious with the polymorphism phenotyping tool (Table 2). The analysis of COSMIC showed that no mutation at IGSF9 promoter region containing the p53 binding site (Fig. S3E), mRNA expression of IGSF9 mutations was uniformly distributed in three indicated datasets, indicating that IGSF9 mutations did not affect its mRNA level in breast cancer (Fig. S3F).

Proteasome-mediated degradation might contribute to decreased IGSF9 expression in breast cancer cells

To investigate the role of miRNAs in the regulation of IGSF9 expression at a post-transcriptional stage, two potential miRNAs targeting the 3′-UTR of IGSF9 mRNA with high probability, miR-2355-5p and miR-8485, were identified from Target scan, miRDB and miRTarBase (Fig. S4A–C). However, neither hsa-miR-8485 nor hsa-miR-2355-5p inhibited luciferase activity of IGSF9 3′-UTR, suggesting that these two miRNAs were not involved in IGSF9 regulation in breast cancer.

We next tested the stability of IGSF9 protein in breast cancer. CHX chase experiments showed that IGSF9 protein became less stable in the breast cancer cell than that in normal mammary cell MCF-10A (Fig. S4D). MG132 (a proteasome inhibitor), but not NH4Cl (a lysosome inhibitor) substantially increased IGSF9 protein levels in MCF-10A and MCF-7 cells (Fig. S4E, F). These data suggested that proteasome-mediated degradation might contribute to decreased IGSF9 protein level in breast cancer cells.

Loss of IGSF9 promotes breast cancer metastasis

To investigate the role of IGSF9 in proliferation and metastasis of breast cancer cells, we stably over-expressed IGSF9 in MCF-7 and T47D cell lines, and knocked down IGSF9 in MDA-MB-231 and MDA-MB-468 cell lines. IGSF9 protein levels were verified using western blot (Fig. S5A-B and Fig. 3A, B). Both CCK-8 proliferation and colony formation assays indicated that proliferation ability was not altered in IGSF9 over-expression/knockdown cells (Fig. S5C, D and Fig. 3C, D, G). However, over-expression of IGSF9 inhibited the migration of MCF-7 and T47D cells, while IGSF9 knockdown promoted the migration of MDA-MB-231 and MDA-MB-468 cells, as measured by wound-healing and transwell migration assays (Fig. S5E, F and Fig. 3E, F, H, I). Consistently, transwell invasion assays indicated that over-expression of IGSF9 led to a significant decrease of cell invasion ability, while IGSF9 knockdown resulted in an increase of cell invasion in breast cancer cells (Figs. S5G and 3F, J). Together, these data indicated that loss of IGSF9 promoted breast cancer metastasis in vitro. Furthermore, over-expression of IGSF9 dampened TP53-knockdown induced MCF-7 cell migration and invasion (Fig. 3K–N), suggesting that IGSF9 could rescue breast cancer metastasis induced by TP53-knockdown.

IGSF9 inhibits epithelial–mesenchymal transition (EMT) process in breast cancer cells

EMT plays critical and intricate roles in promoting tumor invasion and metastasis in epithelium-derived carcinomas, such as breast cancer, colorectal cancer. The typical EMT phenotypes, down-regulation of E-cadherin and ZO-1, and up-regulation of N-cadherin and MMP2, were found as well in IGSF9-knockdown breast cancer cells with western blot analysis (Fig. 4A, B) and immunofluorescence assay (Fig. 4C–E). In contrary, EMT phenotypes were inhibited in IGSF9-overexpressed breast cancer cells (Fig. 4A, B, F–H). We next generated lung metastasis model via tail vein injection of breast cancer stable cells suspensions. MDA-MB-231 cells with IGSF9-knockdown led to an increase in the lung metastasis burden evidenced by both Micro-CT and hematoxylin-eosin (HE) staining for lung metastases nodes (Fig. 4I–K).

IGSF9 inhibits FAK/AKT signaling via its interaction with FERM domain of FAK

To explore the molecular mechanisms underlying the tumor suppressive effect of IGSF9, we analyzed the differential gene expression between IGSF9 high and low expression samples in GSE27830. KEGG enrichment pathway analysis was conducted using DEGs. Enriched pathways mainly included focal adhesion,
PI3K-AKT signaling, ECM-receptor interaction, and cell adhesion molecules (Fig. 5A, B). FAK acts as both signaling kinase and cell adhesion-associated scaffold protein involved in breast cancer metastasis [27, 36]. The structure of IGSF9 renders it capable of serving as a potential cell-surface receptor and transducing outside signal into intracellular pathway. Immunofluorescence exhibited co-localization of IGSF9 and FAK (Fig. 5C). Moreover, co-immunoprecipitation assay showed the endogenous and exogenous interaction between IGSF9 and FAK in MCF-7 cells and HEK-293T cells, respectively (Fig. 5D). To identify the regions in FAK that interact with IGSF9, we expressed various HA-tagged FAK fragments together with IGSF9 (Fig. 5G), and performed co-immunoprecipitation experiments. As shown in Fig. 5F, FAK (1-355 aa) containing FERM domain can bind IGSF9 (1-493 aa) containing FAK (1-355 aa) fusion protein (Fig. 5I), indicating a direct interaction between IGSF9 (1-493 aa) and FAK (1-355 aa). We predicted IGSF9–FAK complex structural model based on the X-ray crystal structure from Protein Data Bank. Docking modeling data suggested that amino acids R6, L63, L64, D80, E101, and R109 of IGSF9, as well as amino acids S269, H292, L293, and E325 of FAK, are critical for the interaction (Fig. 5I). The activities of key molecules in FAK/AKT pathway were thus investigated in cells with IGSF9 over-expression or knockdown. We found that p-FAK (Y397), p-AKT (S473), and p-AKT (T308) were significantly decreased, indicating an inhibition of FAK/AKT signaling in IGSF9 over-expression MCF7 and T47D cells (Fig. 5K-L). Conversely, FAK/AKT signaling was activated in IGSF9-knockdown MDA-MB-231 and MDA-MB-468 cells (Fig. 5K-L). These results suggested that IGSF9 interacted with FAK and antagonized FAK/AKT signaling.

**PND1186, FAK inhibitor, inhibits breast cancer metastasis induced by IGSF9 knockdown in vitro and in vivo**

PND1186, a specific FAK inhibitor, was used to treat MDA-MB-231 and MDA-MB-468 cells. Western blot analysis indicated that IGSF9 loss-mediated FAK/AKT signaling activation, was remarkably blocked in the presence of PND1186 (Fig. 6A). Transwell migration and invasion assays also demonstrated that administration of PND1186 dramatically reversed IGSF9 knockdown-induced tumor migration and invasion of MDA-MB-231 and MDA-MB-468 cells (Fig. 6B, C). Moreover, we developed a lung metastasis model by injecting MDA-MB-231 cells into the tail vein in 4-6 weeks female NOD-SCID mice (Fig. 6D). After 6 weeks, the mice were sacrificed and lung tissue sections were stained with HE (Fig. 6E). The numbers of metastatic lesions in lungs showed that IGSF9 knockdown accelerated tumor metastasis, which could be reversed by PND1186 treatment (Fig. 6F). Together, these data indicate that IGSF9 loss induces metastasis of breast cancer via activating FAK/AKT signaling. PND1186 might apply for breast cancer treatment in the future.

**DISCUSSION**

In this study, we identified a novel p53 target gene IGSF9, which is frequently lost in breast cancer patients and is associated with poor prognosis. Loss of IGSF9 promotes breast cancer metastasis in vitro and in vivo. In breast cancer cells, reduced wild-type
p53 or mutated p53 (R175H) down-regulated IGSF9 protein expression. A decrease of IGSF9 releases FAK, activates FAK/AKT signaling and promotes EMT process, resulting in cancer metastasis (Fig. 6G). FAK inhibitor PND1186 could inhibit IGSF9 knockdown-induced breast cancer metastasis in vitro and in vivo.

IGSF9 family proteins show limited expression in normal tissues. According to PaxDb, mammalian members of the IGSF9 family belong to the bottom 5% of expression level [37]. The role of IGSF9 in cancer appears to be tissue context-specific. IGSF9 shows low expression in liver hepatocellular carcinoma, skin cutaneous...
IGSF9 shows high expression in bladder urothelial carcinoma, which was reported in the present study. On the other hand, melanoma, testicular germ cell tumors, as well as in breast cancer loss of IGSF9 promotes breast cancer metastasis, but not proliferation. A Fig. 3 and regulates IGSF9 expression in breast cancer.

Alterations of its target gene 

and regulates IGSF9 expression in breast cancer. As a transcription factor, p53 regulates tumor-related genes expression, such as CDKN1A, a cyclin-dependent kinase inhibitor [39], and GAS7, a breast cancer metastasis suppression gene [9]. Alterations of TP53 are the most common genetic changes found in breast cancer and play a central role in cancer process [40]. The vast majority of p53 mutant proteins comes from missense mutations mostly within the DNA-binding domain of TP53 [41]. Here we found that wild-type p53, but not R175H mutation within the DNA binding domain of p53, can bind to IGSF9 promoter, activating the transcription of IGSF9. Compared with wild-type p53, mutant p53 has a prolonged half-life, whereas cannot recognize wild-type p53 DNA-binding sites [42, 43]. Models for mutant p53 regulated transcriptional activity include: i) mutant p53 binds the regulatory regions of its target genes through a specific and yet unknown consensus sequence for DNA binding; ii) mutant p53 interacts with a specific transcription factor that drives its target gene [41]. Chang et al. reported that wild-type p53 trans-regulated GAS7 gene expression; while mutated p53 significantly reduced GAS7 expression [9]. Mutant p53 might sequester and inactivate proteins involving in genomic instability and the resistance to anticancer treatments, leading to increased metastasis.

FAK is a cytoplasmic protein tyrosine kinase. Upon stimulation by integrins and a broad range of growth factors and chemokines, canonical FAK signaling is activated to facilitate the formation and turnover of focal adhesions. FAK is autophosphorylated at Y397 to create a binding site for some SH2-containing molecules, including Src, PI3K, Grb7, and PLCγ [44]. When FAK is associated with Src upon Y397 autophosphorylation, it can (i) lead to Src conformational change and activation, which subsequently phosphorylates other sites of FAK, including Y407, Y576, and Y577, to maximize FAK kinase activity [45]; (ii) provide binding sites for other signaling molecules. The interaction between the FERM and kinase domain can maintain FAK autoinhibition state. IGSF9 was shown to mediate dendrites outgrowth and spine formation by interaction with MRCKβ [46]. Here, we discovered the direct interaction between Ig domain of IGSF9 and FERM domain of FAK. Loss of IGSF9 enhances FAK kinase activity, activates AKT, leading to breast cancer metastasis. It seems that binding IGSF9 to FAK enhances the autoinhibition of FAK kinase activity. Increased FAK expression and activity are associated with breast cancer progression and poor prognosis. Interestingly, nuclear FAK recruits p53, increasing p53 polyubiquitilation (Ub) and degradation [47], thereby preventing p53 transcription activity. This event would further decrease IGSF9 expression and facilitate cancer metastasis.

Small molecule FAK inhibitors are emerging as promising chemotherapeutics. FAK inhibition in mouse models prevents tumor growth, metastasis, vascular permeability and angiogenesis [48]. We found that PND1186, a FAK inhibitor, could inhibit breast cancer metastasis induced by IGSF9 knockdown in vitro and in vivo. In line with our work, Walsh et al. reported that PND1186 blocked FAK Tyr-397 phosphorylation in vivo and exhibited antitumor efficacy in orthotropic breast carcinoma mouse tumor models [36]. However, the scaffolding functions of FAK are not blocked by FAK inhibition, even possibly enhanced by it [47], thereby leading to unpredictable therapy outcomes. The kinase-independent functions of FAK must be taken into consideration when designing or testing approaches to inhibit FAK kinase activity for therapeutic applications.

Collectively, we identified a new p53 target, IGSF9, in promoting breast cancer metastasis through EMT process mediated via FAK/AKT signal. This study discovered a feasible prognostic biomarker IGSF9, and at the same time sheds light on the potential new strategies for developing treatment for breast cancer patients.

MATERIALS AND METHODS

Patient and specimens
Randomly chosen 28 pairs of freshly collected tumor tissues and adjacent normal tissues coming from breast cancer patients who underwent surgical resection and pathologically diagnosed with breast cancer in Zhongshan Hospital of Fudan University (Shanghai, China) were used for quantitative reverse transcription polymerase chain reaction (qRTPCR) and western blot analysis. A total of 160 breast cancer patients tissue microarray and patients’ clinicopathological characteristics: age, tumor size, TNM staging et were obtained from Shanghai superbiotech technology company. RFS was defined as the time interval from surgery to recurrence, and overall survival (OS) as time interval from surgery to death. The study was approved by the Institutional Ethics Committee of Fudan University, and written informed consent was obtained from each patient before clinical data analysis.

Plasmids and transfection
The IGSF9 plasmids have been previously described [46]. FAK sequence were cloned into GV492 vector, and mutant sequences were cloned into pcDNA3.1 for overexpression. pGL3 vector for wild-type and mutant IGSF9 3'-UTR, and pGEX-KG for GST pull-down assays. Deletion and site-directed mutations were incorporated into plasmid DNA using Q5 Site-Directed Mutagenesis kit (New England Biolabs, Massachusetts, USA). Primers used in this study are listed in Table S1.

HEK-293T, human breast cancer cell lines MCF-7, T47-D, MDA-MB-231, MDA-MB-468, MDA-MB-436, BT549, and mammary epithelial cell MCF-10A were all purchased from Chinese Academy of Sciences Cell Bank. MCF-7 and HEK-293T were maintained in Dulbecco’s Modified Eagle Medium (Invitrogen Corporation, CA, USA) supplemented with 10% of Fetal Bovine Serum (F85; Invitrogen Corporation, CA, UA) and with 1% penicillin/ streptomycin (P/S; Invitrogen Corporation, CA, USA) at 37 °C in a 5% CO2.
Fig. 4  IGSF9 inhibits epithelial-mesenchymal transition (EMT) process in breast cancer. IGSF9 knockdown promoted EMT in breast cancer cells. EMT markers in MDA-MB-231 and MDA-MB-468 stable cells were determined by western blot (A) and relative protein levels were normalized to GAPDH (B). Error bars denoted mean ± SD. The Student’s t test was used. *P < 0.05, **P < 0.01, ***P < 0.001. IGSF9 manipulation changes the protein expression of E-cadherin and N-cadherin in breast cancer cells. The mentioned stable cells were subjected to immunofluorescence (C, F). Red: E-cadherin, Green: N-cadherin, Blue: DAPI. Scale bars, 20 μm. Quantification analysis of E-cadherin and N-cadherin foci were performed by Image J (D, E, G, H). Error bars denote mean ± SD. The Student’s t test was used. *P < 0.05, **P < 0.01. I MDA-MB-231 cells were injected into tail vein of NOD-SCID mice to develop the lung metastasis model. At 4 weeks after injection, metastasis tumors were visualized by Micro-CT. J IGSF9 reduction promoted lung metastasis of breast cancer cell in vivo. Hematoxylin-eosin (HE) staining images of lung metastatic nodules from each group were showed. Upper scale bar, 200 μm; lower scale bar, 50 μm. K Relative quantification of lung metastasis nodules from shCtrl and shIGSF9 group. Values were mean ± SD. The Student’s t test was used; n = 5, *P < 0.05, **P < 0.01.
atmosphere. MDA-MB-231, MDA-MB-468, MDA-MB-436 were all maintained in L-15 (Invitrogen Corporation, CA, USA) supplemented with 10% of FBS and with 1% P/S at 37 °C without CO2. MCF-7A were maintained in Ham’s F12 (Sigma-Aldrich, St. Louis. MO) supplemented with 5% of Horse serum, 1% P/S, 10 μg/mL insulin, 0.5 μg/mL dexamethasone, 100 ng/mL cholera toxin (CT), 20 ng/mL Epidermal Growth Factor at 37 °C in a 5% CO2 atmosphere. The MDA-MB-231, MDA-MB-468, MCF-7, and T47D were infected by the shRNA expressing lentivirus for IGSF9 and the IGSF9 overexpression lentivirus respectively, and stable clones were selected by puromycin (Sigma Aldrich, St. Louis. MO). Breast cancer cells and HEK-293T cells were transfected with the over-expression plasmids and siRNA using Lipofectamine 3000 (Invitrogen Corporation, CA, USA).
RT-qPCR
Total RNA from cells and breast tissues was extracted with TRIzol® (Thermo Fisher Scientific). cDNA was retro-transcribed using PrimeScript™ RT Master Mix (Takara, Japan). Quantitative PCR (qPCR) reactions for IGSF9 and GAPDH were performed with an ABI Prism 7500 instrument (Applied Biosystem, USA), and amplified in SYBR Green Mix (Takara, Japan) with the corresponding primers. The primers were listed in Table S1. The relative gene expressions were determined by 2^(-ΔΔCt) method [49]. Experiments were performed in triplicate, and results were presented as mean value ± SD.

Luciferase reporter assays
The promoter sequence of IGSF9 gene (from −3000 to +50 bp) was obtained from the http://genome.ucsc.edu. The sequences of the promoter were amplified by PCR, and the fragments were cloned into the pGL3-basic vector. Cells were co-transfected with pGL3 or pGL3 IGSF9-Luc together with pRL-TK plasmid. Cell samples were harvested at 48 h post-transfection, lysed with lysis buffer and measured luciferase activity using the luciferase detection kits (Promega, USA) according to the manufacturer’s instructions. Relative luciferase activity was detected using a Lumat LB 9507 luminometer (Berthold Technologies, Germany). All experiments were performed in three independent replicates.

Chromatin Immunoprecipitation (ChIP) assays
ChIP experiments were performed using the ChIP assay kit (17-295, Merck Millipore, Darmstadt, Germany) according to the manufacturer’s instructions. Breast cancer cells were seeded at a density of 1 x 10^5 cells in 100 mm cell culture dish, and grown overnight to 95% confluence. Cells treated for 10 min at room temperature were crosslinked by adding formaldehyde into cell culture medium to a final concentration of 1%, and washed with cold 1 x PBS buffer containing protease inhibitors. Then the cells were sonicated to shear DNA for 30 min on ice, and immunoprecipitated with anti-p53 antibody and control IgG overnight at 4 °C. DNA fragments were extracted and purified from the immunoprecipitates by the instructions and analyzed by RT-PCR using the primers designed to recognize and amplify the IGSF9 promoter. See Table S2 for primer information.

Western blotting
Total protein was extracted from cells or tissues were lysed in RIPA buffer (Beyotime Biotechnology, Shanghai, China) with 1 x protease inhibitor cocktail (Roche), incubated for 30 min on ice, and centrifuged 10 min at 12,000 rpm at 4 °C. Protein concentrations in supernatants were determined using a BCA Protein Assay Kit (Vazyme Biotech, Nanjing, China). 10 μg of protein lysates were run on 10–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to 0.45 μm PVDF membranes (Millipore, Darmstadt, Germany). The PVDF membranes were incubated in 5% (w/v) non-fat dry milk resuspended in 0.1% TBST for 1 h at room temperature, and the membranes were incubated with primary antibodies overnight at 4 °C. After washing primary antibodies with TBST, PVDF membranes were incubated in horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. The primary and secondary antibodies, and their dilutions were listed in Table S2. After washing the membranes four times with TBST, bands were visualized using ECL Western Blotting Detection Reagents (Tanon, Shanghai, China).

Immunohistochemical (IHC) staining and scoring
The paraffin-embedded specimens were sliced into 4 μm thick, and mounted on poly-L-lysine-coated glass slides. Then sections were deparaffinized, rehydrated with xylene and alcohols. Antigen retrieval was performed by placing sections in EDTA solution (pH = 9.0) and heating at 100 °C for 20 min, followed by cooling at room temperature for 30 min. Placing the sections in 0.03% H2O2 at 37 °C for 15 min was used to block endogenous peroxidase activity. After blocking in TBS with 5% bovine serum albumin (BSA) at room temperature for 30 min, the sections were incubated with the anti-IGSF9 antibody overnight at 4 °C, and then incubated HRP-conjugated secondary antibodies. The antibodies and dilutions used were listed in Table S2. Immunoreactivity was quantified using the staining intensity (0, negative; 1, weak; 2, moderate; 3, strong) and the percentage of positive stained tumor cells (0, <5%; 1, 6–25%; 2, 26–50%; 3, 50–75%; 4, 76–100%). The scores were obtained by multiplying the staining intensity and the percentage of positive stained cells [50].

Immunofluorescence
The breast cancer cells were seeded on coverslips. After 24 h, cells were fixed with 4% paraformaldehyde at 4 °C for 30 min, and permeabilized/monoclonal antibody and normal IgG followed by adsorption to protein blocking 10 min. After washing in TBS with 5% BSA for 1 h at room temperature, cells were incubated with primary antibodies overnight at 4 °C, then incubated with secondary antibodies at room temperature for 2 h. DAPI was used to dye cell nuclei. The antibodies and dilutions used were listed in Table S2. Images were taken with Confocal microscope.

Co-immunoprecipitation and GST pull-down assay
Cells were cultured in 100 mm dishes, harvested at 90% confluence. After washing three times with cold PBS, the cells were lysed in IP lysis buffer with 1 x protease inhibitor cocktail, and incubated for 90 min on ice, then centrifuged 10 min at 12,000 rpm at 4 °C. For co-immunoprecipitation assays, cells lysates were immunoprecipitated with the anti-myc mouse monoclonal antibody and normal IgG followed by adsorption to protein A/G beads (Santa Cruz, Texas, USA), incubating overnight at 4 °C on a rotating rocker. After washing three times with cold PBS, the samples were analyzed by western blotting and 5% of input samples were used as a positive control. GST pull down assay was performed as described previously [46]. Protein pull down of GST and GST-FAK (1–355) fusion protein were expressed in bacterial BL21 cells. Cells were lysed, and GST fusion proteins were captured by Glutathione-Sepharose 4B beads at 4 °C for 1 h. To investigate the interaction between IGSF9 and FAK (1–355), equal amounts of GST or GST-FAK (1–355) fusion protein beads were incubated with in vitro translated IGSF9. After washing with ice-cold wash buffer, the proteins were eluted from the beads and detected by western blot.

Cell proliferation and colony formation assay
Cells (1 x 10^4) were seeded into 96-well plate. CCK-8 solution was added in each well and incubated for 1 h at 37 °C. The absorbance at 450 nm was measured using a fully automated microplate reader (ELx800TM, BIO-TEK Instruments, Minneapolis, MN, USA). Cells (1 x 10^4) were seeded into six-well plate, and were grown 2 weeks for detecting colony formation ability. The cells were fixed by 4% paraformaldehyde and stained with crystal violet (Beyotime Biotechnology, Shanghai, China).

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Cells (1 x 10^4) were seeded into 96-well plate. CCK-8 solution was added in each well and incubated for 1 h at 37 °C. The absorbance at 450 nm was measured using a fully automated microplate reader (ELx800TM, BIO-TEK Instruments, Minneapolis, MN, USA). Cells (1 x 10^4) were seeded into six-well plate, and were grown 2 weeks for detecting colony formation ability. The cells were fixed by 4% paraformaldehyde and stained with crystal violet (Beyotime Biotechnology, Shanghai, China).
Wound-healing, transwell migrations, and invasion assays

For wound-healing assay, breast cancer cells were seeded in a 6-well plate and cultured to reach 85% confluence. Then cells were scraped by a sterile tip of a 10 μl pipette to generate an artificial wound. Photographed and measured the wound at 0 h, 24 h, 48 h, 72 h, and 96 h, respectively. The cell migration rates were obtained by calculating the wounds width changing.

For the transwell migration assay, 2 × 10^4 cells were resuspended in 200 μl serum-free medium and added to the upper compartments of transwell chambers (8 μm pore filter, Corning, NY, USA). Putting the transwell chamber in 24-well plate, and the lower compartments were filled with 20% serum medium (600 μl/well). After incubation at 37 °C for 12 h or 24 h, the migrated cells had passed through the pore to the lower membrane.
and the transwell chamber lower membranes were fixed and stained with 0.1% crystal violet, photographed using an IX71 inverted microscope (Olympus Corp, Tokyo, Japan). For the transwell invasion assay, the upper compartments of the transwell chamber were coated by 30 μg matrigel (BD Biosciences, Franklin Lakes, NJ), the subsequent procedures were performed as the transwell migration assay. All experiments above were performed in three independent triplicates.

**Animals and lung metastatic tumor model**

The pre-specified minimum sample size was calculated according to the approval of Shanghai Medical Experimental Animal Care Commission. Animals were excluded from the analysis if they died from other reasons than tumor metastasis (for example, sickness). The possibility of these events was considered over the sample size estimation. BALB/c athymic nude mice (female, 6–8 weeks old and specific pathogen free) were purchased from Shanghai SLAC laboratory Animal Co., Ltd (Shanghai, China), and were maintained in a pathogen-free animal facility under standard conditions. All animal procedures were performed according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985). Studies were approved by the Shanghai Medical Experimental Animal Care Commission.

To establish experimental lung metastasis model in BALB/c athymic nude mice, breast cancer cells (1 × 10^6) in 150 μL PBS) from different treated groups were injected through the tail vein. The mice were randomly allocated to four groups according to the breast cancer cells received: MDA-MB-231- shCtrl, MDA-MB-231-shIGSF9, MDA-MB-231-shCtrl + PND1186, MDA-MB-231-shIGSF9 + PND1186. For each group, six mice were recruited. For PND1186 group, 50 mg/kg PND1186 was administered by oral gavage two times a day for 4 weeks. PND1186 abolished the effect of IGSF9 knockdown promoted lung metastasis of breast cancers. Representative HE staining images of lung tissues from each group were shown (E). Upper scale bar, 200 μm; lower scale bar, 50 μm. Quantification of metastatic nodules (F). Error bars denote as mean ± SD. The Student’s t test was used. **P < 0.01, ***P < 0.001. A schematic diagram illustrated the role of IGSF9 in EMT process and metastasis of breast cancer. In the normal tissue, wild-type p53 acting as a transcriptional factor bound to the promoter region of IGSF9. Consequently, EMT and metastasis were halted. In breast cancer, p53 with R175H mutation was decreased. Transcription of IGSF9 was promoted its transcription. Increased IGSF9 interacted with FAK to block its phosphorylation, leading to decreased downstream protein AKT activity in breast cancer (middle panel). Conversely, EMT and metastasis were facilitated. In breast cancer patients with low IGSF9 expression were treated with PND1186, a specific FAK inhibitor, the reduced FAK activity then led to attenuated EMT and metastasis (right panel).

**KEGG pathway analysis for DEGs**

Differentially expressed genes (DEGs) were detected between IGSF9 high and low expression breast cancer group from GSE27830 dataset using package DESeq2. The adjusted P value < 0.05 and |log2FC| > 0.5 as the threshold. Genes positively related with IGSF9 were used to analyze the functional enrichment of IGSF9-interacted genes. Kyoto Encyclopedia of Genes and genomes (KEGG) analysis was conducted with R package “clusterProfiler”.

**The structural analysis of IGSF9/FAK complex**

The structural models of IGSF9 (1–493 aa; Template ID: Q50851) and FAK (1–355 aa; Template ID: Q50397) were modeled with I-TASSER, FR-T5-M, and FALCON software. The IGSF9-FAK docking candidate interfaces were performed using ISPRED4. According to the candidate interfaces, IGSF9-FAK complex were docked with ZDOCK v3.02f. We selected highest-confidence complex model from top 10 candidate models using ZDOCK and SPR. All structure models were exhibited by PyMol Software (www.pymol.org).

**Statistical analysis**

All statistical analyses were carried out using SPSS version 22.0. Pearson’s Chi-square test was performed to analyze the correlations between IGSF9 expression and clinicopathological characteristics. OS and TTR curves were depicted using Kaplan-Meier analyses (log-rank test). Differences between groups were compared by using a two-tailed Student’s t test. All experiments were performed in triplicate, and the data were presented as mean value ± SD. A value of P < 0.05 was considered statistically significant.

**REFERENCES**

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. CA Cancer J Clin. 2019;69:7–34.
2. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2021;71:209–49.
3. Mehlen P, Puisieux A. Metastasis: a question of life or death. Nat Rev Cancer. 2006;6:649–58.
4. Schneider C, Fehr MK, Steiner RA, Hagen D, Haller U, Fink D. Frequency and distribution pattern of distant metastases in breast cancer patients at the time of primary presentation. Arch Gynecol Obstet. 2003;269:9–12.
5. Siegel RL, Miller KD, Fuchs HE, Jemal A. Cancer statistics, 2021. CA Cancer J Clin. 2021;71:7–33.
6. Press DJ, Miller ME, Liederbach E, Yao K, Hsu D. De novo metastasis in breast cancer: occurrence and overall survival stratified by molecular subtype. Clin Exp Metastasis. 2017;34:457–65.
7. Santis C, Siegel R, Bandi P, Jemal A. Breast cancer statistics, 2011. CA Cancer J Clin. 2011;61:409–18.
28. Mitra SK, Hanson DA, Schlaepfer DD. Focal adhesion kinase: in command and control of cell motility. Nat Rev Mol Cell Biol. 2005;6:56–68.

29. Goloubkova VM, Cance WG. Focal adhesion kinase and p53 signaling in cancer cells. Int Rev Cytol. 2007;263:103–53.

30. McLean GW, Carragher NO, Avizienyte E, Evans J, Brunton VG, Frame MC. The role of focal-adhesion kinase in cancer - a new therapeutic opportunity. Nat Rev Cancer. 2005;5:505–15.

31. Cance WG, Harris JE, Iacocca MV, Roche E, Yang X, Chang J, et al. Immunohistochemical analyses of focal adhesion kinase expression in benign and malignant human breast and colon tissues: correlation with preinvasive and invasive phenotypes. Clin Cancer Res. 2000;6:2417–23.

32. Pylayeva Y, Gillen KM, Gerald W, Beggs HE, Reichardt LF, Giancotti FG. Ras- and PI3K-dependent breast tumorigenesis in mice and humans require focal adhesion kinase signaling. J Clin Investig. 2009;119:252–66.

33. Watermann DO, Gabriel B, Jager M, Orlowska-Volk M, Hasenburg A, zur Hausen A, et al. Specific induction of pp125 focal adhesion kinase in human breast cancer. Br J Cancer. 2005;93:694–8.
