

MicroRNA-584 and the Protein Phosphatase and Actin Regulator 1 (PHACTR1), a New Signaling Route through Which Transforming Growth Factor- β Mediates the Migration and Actin Dynamics of Breast Cancer Cells^{*[5]}

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Background: TGF- β promotes cell migration in advanced breast cancer.

Results: TGF- β down-regulates miR-584, leading to a PHACTR1 overexpression, and both are involved in cell migration and actin reorganization.

Conclusion: The regulation of miR-584 and regulation of its novel target PHACTR1 are necessary steps for breast cancer cell migration.

Significance: MicroRNAs offer an interesting therapeutic target in the treatment of advanced breast malignancy.

TGF- β plays an important role in breast cancer progression as a prometastatic factor, notably through enhancement of cell migration. It is becoming clear that microRNAs, a new class of small regulatory molecules, also play crucial roles in mediating tumor formation and progression. We found TGF- β to down-regulate the expression of the microRNA miR-584 in breast cancer cells. Furthermore, we identified PHACTR1, an actin-binding protein, to be positively regulated by TGF- β in a miR-584-dependent manner. Moreover, we found TGF- β -mediated down-regulation of miR-584 and increased expression of PHACTR1 to be required for TGF- β -induced cell migration of breast cancer cells. Indeed, both overexpression of miR-584 and knockdown of PHACTR1 resulted in a drastic reorganization of the actin cytoskeleton and reduced TGF- β -induced cell migration. Our data highlight a novel signaling route whereby TGF- β silences the expression of miR-584, resulting in enhanced PHACTR1 expression, and further leading to actin rearrangement and breast cancer cell migration.

Breast cancer remains the most frequent malignancy affecting women and is the second biggest cause of cancer mortality. A major player in the progression of breast cancer is transforming growth factor- β (TGF- β). The TGF- β superfamily of ligands contains over 40 members involved in many critical processes that encompass embryogenesis and adult homeostasis (1). The founding member of the superfamily, TGF- β , and its receptors, are widely expressed in all tissues, and the regula-

tory role played by this growth factor is of central importance to human diseases. Loss of function of TGF- β signaling leads to hyper-proliferative disorders and has been linked to cancer development, inflammatory diseases, and autoimmune diseases, whereas a gain of function has been implicated in immunosuppression and tumor metastasis (2–5). TGF- β displays a dual role in breast cancer carcinogenesis, where it acts against initiation and progression in normal cells and at early stages of cancer, but transforms into a prometastatic factor at later stages of carcinogenesis (2, 6–9). These tumor-promoting effects occur in different types of cancers as TGF- β was shown to promote epithelial-to-mesenchymal transition, migration, invasion, and metastasis in gliomas, colon, pancreatic, and breast cancers (10–16).

Canonical TGF- β signaling is triggered through a complex of two serine/threonine kinase receptors, which in turn activates and phosphorylates the intracellular mediators, Smad2 and Smad3 (17). Phosphorylated Smad2 and Smad3 oligomerize with the co-mediator Smad4 (Co-Smad). The Smad hetero-complex translocates to the nucleus where it binds DNA with low affinity. To achieve high affinity binding, the Smads associate with various DNA binding partners. It is thought that these partner proteins, which act as co-activators or co-repressors, are differentially expressed in different cell types, thus providing a basis for tissue- and cell type-specific functions of TGF- β (17). One such co-factor is the p68 helicase of the microprocessor complex, which participates in the processing of microRNA (miRNA)² maturation. As such, it was determined that TGF- β was involved in the regulation of a specific subset of miRNAs (18, 19).

MicroRNAs are a form of endogenous RNA interference; they are small noncoding RNAs of 18–25 nucleotides, tran-

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[5] This article contains supplemental Fig. 1.

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² The abbreviations used are: miRNA, microRNA; miR, microRNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PHACTR1, protein phosphatase and actin regulator 1; PRRX1, paired-related homeobox 1; DMSO, dimethyl sulfoxide; qRT-PCR, quantitative RT-PCR.

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scribed from the genome, that inhibit protein expression by binding to the 3'-UTR of a specific target mRNA to block its translation (20). Over time, an incredibly vast number of miRNAs have been discovered, shedding light on their important regulatory function in the cell, and particularly in cancer. Indeed, miRNAs are thought to control over one-third of the human genome (21). Mapping of human miRNAs uncovered that their distribution is nonrandom; most are located at fragile sites or sites that are often altered in cancer (22). In fact, many profiling studies have determined an altered miRNA expression pattern in cancer tissues when compared with normal (23–29), and an overall decrease in miRNA expression in malignant tissues (26). Several miRNAs have been identified to play a role in cancer progression. For instance, microRNA-10b (miR-10b) was found to promote tumor metastasis (30), whereas miR-335 was shown to act in an opposite manner and suppress breast cancer metastasis (31).

Considering the role of TGF- β in breast cancer cell migration and the emerging role played by these miRNAs in human cancer, we investigated whether they acted downstream of TGF- β -mediated tumor progression. In this study, we identified miR-584, a potential tumor suppressor (32), as a novel target of TGF- β and found that miR-584 expression was negatively regulated by this growth factor in a number of breast cancer cells. We found the inhibition of miR-584 expression by TGF- β to be required for cell migration as overexpression of ectopic miR-584 could reverse TGF- β -induced cell migration. We further identified the protein phosphatase and actin regulator 1 (PHACTR1) as a downstream target of miR-584 and found that TGF- β -mediated inhibition of miR-584 leads to increased expression of PHACTR1. Furthermore, blocking PHACTR1 expression induced a dramatic reorganization of the actin cytoskeleton and potently antagonized TGF- β promigratory effects in invasive breast cancer cells. Collectively, our results highlight miR-584 and its downstream target, PHACTR1, as a central regulatory axis, which participates in TGF- β -induced cell migration in invasive breast cancer cells.

EXPERIMENTAL PROCEDURES

Reagents—siRNAs for control and Smads was obtained from Ambion, whereas siRNA for PHACTR1 was obtained from Sigma-Aldrich. Mimics and inhibitors for miR-584 were purchased from Shanghai GenePharma Co., Ltd. Rat PHACTR1-overexpressing construct was purchased from Thermo Scientific. RNase-OUT recombinant ribonuclease inhibitor and primers for mRNA qRT-PCR were all purchased from Invitrogen. The pMIR-REPORT reporter construct was from Invitrogen, and oligonucleotides were ordered from Integrated DNA Technologies. Chemical inhibitor SB431542 was obtained from Calbiochem.

Antibodies and Phalloidin—The antibodies used were: F-actin (Invitrogen, Alexa Fluor 488 phalloidin), Vimentin (BD Pharmingen 550513), Rhodamine-conjugated anti-goat antibody (Santa Cruz Biotechnology, sc-2094), Texas Red-conjugated anti-rabbit antibody (Molecular Probes, T2767), β -tubulin (Santa Cruz Biotechnology, sc-53140), PHACTR1 (SIGMA, HPA029756), anti-mouse (Santa Cruz Biotechnology, sc-2005), and anti-rabbit (Santa Cruz Biotechnology, sc-2004).

Cell Culture—HaCaT, HEK293, HuH7, MCF7, MDA-MB-231, and SCP2 were cultured in DMEM (Wisent) with 10% FBS (Gibco), 2 mM solution L-glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin (Thermo Scientific HyClone Laboratories). Serum-starved medium consisted of DMEM and 2 mM L-glutamine.

PC3 were cultured in RPMI (Wisent) with 10% FBS, 2 mM solution L-glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin. Serum-starved medium consisted of RPMI and 2 mM L-glutamine.

SUM149PT and SUM159PT were cultured in Ham's F-12 serum (Wisent) with 5% FBS, 5 μ g/ml insulin, and 1 μ g/ml hydrocortisone. Serum-free medium consisted of Ham's F-12 with 5 μ g/ml insulin and 1 μ g/ml hydrocortisone (Sigma-Aldrich). Cells were cultured at 37 °C with 5% CO₂.

Bioinformatics Search—Potential targets were predicted by performing a search in the following online databases: TargetScan (33); MicroCosm Targets Version 5 (34); miRanda database (35); and PITA catalogs of predicted microRNA targets (Pitap) (36).

Transfections—Cells were cultured to 90% confluence in 6-well plates and transfected with 80 nM siRNA or varying concentrations of mimic and inhibitors. Transfections were carried out using 6 μ l of Lipofectamine 2000 (Invitrogen) in 500 μ l Opti-MEM® (Gibco) with the desired concentration of miRNA or siRNA. Cells were left overnight with transfection reagents in starvation medium, which were removed the next morning. They were then starved overnight and stimulated with the appropriate TGF- β concentration for the indicated period of time.

Reverse Transcription-Polymerase Chain Reaction—Cells were transfected or not with siRNA, mimics, or inhibitors and treated or not with 100 pM TGF- β (PeproTech) for various amounts of time. Total RNA was extracted with TRIzol (Invitrogen). Reverse transcription was carried out using the M-MLV RT (Invitrogen) as per the manufacturer's instructions. Amplification of cDNA product was performed using GAPDH as an internal control with the SsoFast EvaGreen Supermix PCR amplification kit (Bio-Rad Laboratories) and the RotorGene 6000 software from Corbett Life Science.

Primer sequences were as follows: PHACTR1, GATTG-GACCAAGCTCACCA (sense); ACCGTGGGCCTTTGACTGAG (antisense); GAPDH, GCCTCAAGATCATCAGCAATGCCT (sense); TGTGGTCATGAGTCCTTCCACGAT (antisense). Cycling was as follows for mRNA: activation: 30 s, 95 °C; denaturation, 5 s, 95 °C; extension, 20 s, 60 °C.

For miRNAs, reverse transcription and amplification were performed with the miScript PCR system with RNA, U6 small nuclear 2 (RNU6B) as an internal control (Qiagen). Cycling was as follows: activation, 5 min, 95 °C; denaturation, 15 s, 94 °C; annealing, 30 s, 55 °C; extension, 30 s, 70 °C.

Protein Extraction and Western Blot Analysis—Cells were transfected or not with siRNAs, mimics, or inhibitors, and treated or not with 100 pM TGF- β for various amounts of time. Total proteins were extracted with radioimmune precipitation buffer containing 1% Triton (1% Triton x-100, 1% sodium deoxycholate, 150 mM sodium chloride, 50 mM Tris, pH 7.2, 0.1% SDS, 5 mM EDTA, pH 8.0, supplemented with 5 μ g/ml

aprotinin, 1 $\mu\text{g/ml}$ pepstatin, 2 $\mu\text{g/ml}$ leupeptin, 1 mM PMSF). Total cell extracts were separated by SDS-PAGE, semidry transferred to a 0.45- μm nitrocellulose membrane (Bio-Rad Laboratories), and incubated overnight with primary antibodies at 4 °C. Immunoreactivity was revealed by chemiluminescence using an Alpha Innotech FluorChem imaging system (Packard Canberra) with the FluorChem SP software.

Wound-healing Assay—Cells were grown to confluence and transfected with 80 nM siRNA, mimics, or inhibitors and/or the indicated amount of rat PHACTR1-overexpressing construct. Cells were starved overnight. A scratch was performed using a sterile 20–200- μl pipette tip, and pictures were taken at time 0. Cells were then stimulated or not with 200 pM TGF- β , and pictures were taken 24 h after stimulation. Photographs were obtained under bright field light microscopy with a Nikon Eclipse E600 microscope and a RS Photometrics CoolSNAP camera. Images were analyzed using ImageJ.

Wound-healing Assay with SB431542—Cells were grown to confluence and starved overnight. A scratch was performed using a sterile 20–200- μl tip, and cells were pretreated with 10 nM SB431542 in DMSO. Pictures of the wound were taken at time 0 and 24 h after stimulation. Photographs were obtained under bright field microscopy with a Nikon Eclipse E600 microscope and an RS Photometrics CoolSNAP camera. Images were analyzed using ImageJ.

Immunofluorescence—Cells were grown on coverslips, transfected or not with 80 nM siRNA, mimics, or inhibitors, and stimulated or not with 200 pM TGF- β for 24 h. They were then fixed in 3.7% paraformaldehyde (Electron Microscopy Sciences) for 10 min and permeabilized with 0.1% Triton for 3 min. The coverslips were blocked with 1% BSA (Bioshop) for 30 min. Cells were then stained 1 h with primary antibody against vimentin (1/800) followed by 1 h of staining with secondary antibody. They were then incubated in 1/100 Alexa Fluor 488 phalloidin solution (Invitrogen) for 30 min and 1/100 DAPI (Invitrogen) staining for 5 min. Slides were mounted with ProLong Gold mounting reagent and viewed by confocal microscopy with a Zeiss LSM 510 META microscope.

Kinetic Cell Migration Assay—Cells were plated in Essen ImageLock 96-well plates (Essen Bioscience, Ann Arbor, MI) at 50,000 cells/well. The use of ImageLock 96-well plates ensures that images/videos of the wound are automatically taken at the exact same location by the IncuCyteTM software (Essen Bioscience). Cells were then serum-starved for 6 h, and confluent cell layers were scratched using the Essen WoundMaker to generate ~800- μm -width wounds. After wounding, cells were washed two times with PBS and stimulated or not with 5 ng/ml TGF- β . ImageLock 96-well plates were then placed into IncuCyte (Essen Bioscience) and a picture was taken every 3 hours for 24 h. The data were analyzed by wound width automatically measured by the IncuCyte software.

MTT Assay—Following transfection, 5000 cells were plated in 96-well plates in 2% FBS and stimulated with 200 pM TGF- β for 48 h. Cells were then incubated for 2 h and 30 min in 25 μl of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml MTT powder in PBS). MTT solution was removed to incubate the cells in 200 μl of DMSO and 25 μl of Sorensen buffer (0.1 M glycine, 0.1 M sodium chlo-

ride, pH 10.5). Absorbance was read at 570 nm using a BioTek Powerwave XS plate reader and the Gen5 1.10 software.

MicroRNA Target Analysis—The pMIR-REPORT system was used to determine whether PHACTR1 is a target of miR-584. A portion of 70 bp of the PHACTR1 3'-UTR consisting of the putative miR-584 binding site and surrounding nucleotides was synthesized chemically and flanked with overhangs for the HindIII and MluI restriction enzymes. The insert was cloned in the pMIR-REPORT reporter construct and amplified in chemically competent DH5 α . HEK293 cells were transfected with 0.2 μg of the reporter vector or the construct with PHACTR1 3'-UTR insert, with or without 20 nM miR-584, and 0.1 μg of β -galactosidase. Cells were lysed 24 h after transfection, and luciferase activity was measured by incubating cell lysates with luciferin and reading luminescence with an E&G Berthold microplate luminometer L96V. β -Galactosidase activity was measured reading absorbance at 420 nm, using the Powerwave XS plate reader.

Statistical Analysis—Values represent the mean of three or more experiments. Error bars were graphed as the standard error.

RESULTS

miR-584 Is a Novel TGF- β Target—TGF- β is well known to regulate cell migration and invasion of breast cancer cells, thereby promoting tumor progression and metastasis (3, 9, 15, 16, 37). Interestingly, miRNAs have recently been shown to be involved in the process of tumor progression (30, 38, 39). In particular, miR-584 was recently described as a potential tumor suppressor that could decrease cellular invasion in human clear renal cell carcinoma by targeting the Rock-1 pathway (32). To investigate whether miR-584 could act downstream of TGF- β , we first examined whether TGF- β could regulate miR-584 expression using real-time qRT-PCR in a panel of breast cancer cell lines of various phenotypic origins. We have used the luminal cell line MCF7, extracted from the pleural effusion of an invasive ductal carcinoma (41), which is under the cytostatic and apoptotic control of TGF- β (42). We also used a panel of basal breast cancer cell lines, including MDA-MB-231, a highly invasive cell line extracted from the pleural effusion of an adenocarcinoma that relapsed several years after the removal of the primary tumor (41, 43), SCP2 cells that were derived from MDA-MB-231 cells and that express a bone metastatic gene signature, SUM159PT, a highly invasive cell line derived from the anaplastic carcinoma of a primary breast cancer (41), and SUM149PT, a cell line with both basal-like and luminal-like features extracted from the inflammatory ductal carcinoma of a primary breast tumor (41, 44). Interestingly, as shown in Fig. 1, we found miR-584 expression to be inhibited by TGF- β in all tested cell lines but the SUM149PT. This lack of regulation of miR-584 by TGF- β in SUM149PT could be due to the low abundance of the TGF- β type 1 receptor (T β RI) in these cells, as determined by the Gene Expression-Based Outcome for Breast Cancer Online (GOBO) database analysis (supplemental Fig. 1C) (45). The strongest inhibition was observed in the invasive and metastatic cell line SCP2, and this inhibition was as early as 30 min, suggesting a direct regulation of TGF- β over miR-584 (Fig. 1B). Interestingly, we also observed a correlation

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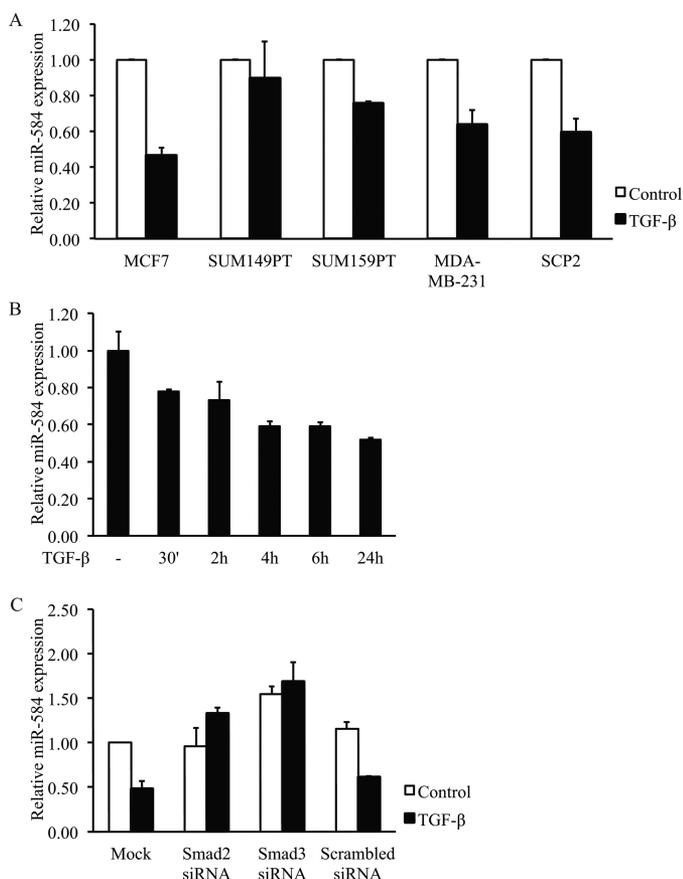


FIGURE 1. miR-584 is a novel TGF- β target. A, several cell lines were stimulated or not with 100 pM TGF- β for 24 h, and total RNA was extracted. TGF- β effect on miR-584 was evaluated by qRT-PCR in breast cancer cells of various origins. B, SCP2 cells were stimulated with 200 pM TGF- β for various periods of time. Total RNA was extracted, and qRT-PCR was performed to evaluate gene expression. C, SCP2 cells were transfected with 80 nM siRNA against Smad2, Smad3, or a scrambled siRNA sequence. Total RNA was extracted, and qRT-PCR was performed to evaluate gene expression.

between the migratory phenotype of the cells and the miR-584 expression levels. Indeed, although miR-584 is highly expressed in normal cells and nonmigratory cancer cells, its expression is diminished in the migratory cell lines, with the lowest expression in the more migratory and invasive cell lines (SCP2 and SUM159PT), suggesting that decreased miR-584 expression is associated with increased cell migration (supplemental Fig. 1, A and B). Together, these results highlight miR-584 as a novel target for TGF- β in breast cancer cells of various phenotypes and suggest that miR-584 may play a role downstream of TGF- β -signal transduction in human breast cancer cells.

TGF- β signals canonically through the Smad effector molecules (9, 46, 47). To determine whether the regulation of miR-584 occurs through the Smad pathway, we have knocked down the receptor Smads with siRNA and measured miR-584 expression by qRT-PCR. As seen in Fig. 1C, the down-regulation of miR-584 is observed in SCP2 with a mock transfection or transfection with a scrambled siRNA sequence; however, knocking down Smad2 or Smad3 abrogated the TGF- β inhibition, indicating that regulation of miR-584 is occurring through the canonical Smad pathway.

miR-584 Antagonizes TGF- β Promigratory Effects in Human Breast Cancer Cells—TGF- β is known to play an important role in breast cancer; in particular, TGF- β was shown to promote cellular migration in human breast cancer cells (9, 48). To next investigate whether TGF- β -mediated down-regulation of miR-584 could regulate breast cancer cell migration, we used miRNA mimics and inhibitors of miR-584. Mimics are synthetic double-stranded modified RNA molecules that imitate the function of endogenous miRNA (49), whereas inhibitors are 2'-O-methyl modified single-stranded molecules that interfere with miRNA function by making them unavailable through irreversible binding (50, 51). As a proof of principle, transient transfection of 80 nM mimic in SCP2 resulted in an overexpression of miR-584 of over 50-fold (Fig. 2A), whereas the transfection of 80 nM inhibitor significantly reduced the expression of endogenous miR-584 (Fig. 2B). These results indicate that the miR-584 mimics and inhibitors are functional as designed and proved valuable tools to analyze miR-584 functions.

Cell migration was measured using the wound-healing assay in SCP2 cells (37, 52). Cells were grown into a monolayer, transfected with the miR-584 mimic or a scrambled mimic as a negative control, and stimulated or not with TGF- β . Cell migration was calculated as the percentage of the surface area that has been covered by the cells after 24 h of stimulation. As shown in Fig. 2C, in the mock and control SCP2 cells transfected with a scrambled mimic, TGF- β induced cell migration. However, overexpression of the miR-584 mimic almost completely blocked the TGF- β effect, indicating that inhibition of miR-584 expression by TGF- β is required for this growth factor to induce cell motility (Fig. 2C). In parallel, to further assess the role of miR-584 in breast cancer cell migration under basal and TGF- β -stimulated conditions, miR-584 expression was blocked using a specific inhibitor. A scrambled inhibitor sequence was used as a negative control. As shown in Fig. 2D, TGF- β induced cell migration in both the mock and the control cells transfected with a scrambled inhibitor, and this effect was strongly potentiated in cells in which miR-584 was blocked with the specific inhibitor. Moreover, blocking expression of miR-584, under basal conditions, also potently enhanced wound closure, similar to what is observed when cells were stimulated by TGF- β (Fig. 2D). To then investigate whether the antimigratory effects of miR-584 downstream of TGF- β are restricted to cells derived from the breast tissue, we examined the microRNA on TGF- β -mediated cell migration in other cell types, including HaCaT, an immortalized human keratinocyte cell line, HuH7, an epithelium-like tumorigenic cell line derived from a liver tumor, and PC3, a cell line derived from bone metastasis of a stage IV prostate cancer patient. Interestingly, as shown in Fig. 2E, our results clearly show that overexpression of the miR-584 mimic but not that of a scrambled mimic completely antagonized the TGF- β effects on cell migration in all cell types tested (skin, liver, and prostate), indicating that the role of miR-584 is not restricted to the breast tissue and that this microRNA plays a pluripotent function downstream of TGF- β in regulating cell migration of multiple human cancer cell types. Together, these results highlight miR-584 as a potent antagonist of TGF- β -induced cell migration in various cancer cell types and indicate

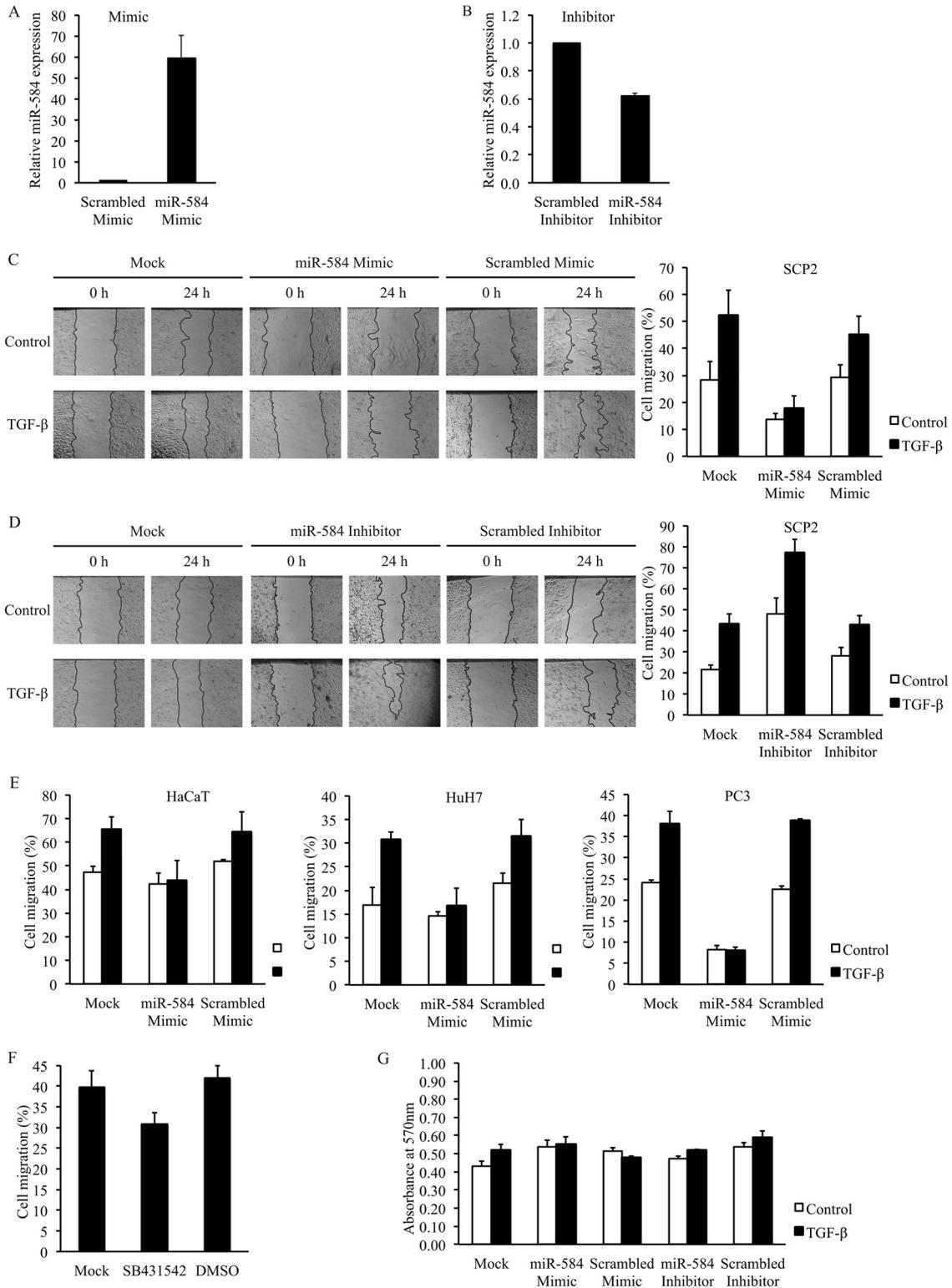


FIGURE 2. miR-584 impairs TGF- β -mediated migration. *A* and *B*, miR-584 was either overexpressed (*A*) or inhibited (*B*) with 80 nM mimic and inhibitor respectively. miR-584 levels were measured by qRT-PCR. *C* and *D*, SCP2 cells were transfected with 80 nM miR-584 mimic (*C*) or inhibitor (*D*) and seeded to confluence as a monolayer. A wound was performed with a tip, and the surface area of the scratch was measured at the time of the scratch and 24 h after stimulation with TGF- β . The surface area covered by migrating cells was graphed. *E*, a wound healing assay was performed as in *C* and *D* in HaCaT, HuH7, and PC3 cells. *F*, SCP2 cells were treated with 10 nM SB431542 and basal cell migration was measured 24 h after stimulation. *G*, cells were transfected with 80 nM mimic or inhibitor for miR-584, as well as mimic and inhibitor negative controls, and an MTT assay was performed 72 h after transfection. Cells were stimulated or not with 100 pM TGF- β for 24 h.

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that down-regulation of miR-584 by TGF- β is a prerequisite step for its cell promigratory effects.

Interestingly, overexpression of miR-584 also led to a decrease in basal cell migration, suggesting that autocrine TGF- β could play a role in regulating this event. To address this, we have performed a migration assay using a specific TGF- β type I kinase inhibitor (SB431542). As shown in Fig. 2F we do observe a decrease in cell migration under basal conditions in cells treated with the inhibitor, suggesting that autocrine TGF- β signaling regulates cell migration under basal conditions. This could explain why basal migration is reduced upon miR-584 overexpression because we found the miRNA to antagonize TGF- β migration. However, this does not exclude the possibility that miR-584 also has a TGF- β -independent effect on basal cell migration.

To verify that the observed effects of the mimics and inhibitors on cell migration were not due to a change in cell proliferation or cell death, we performed a cell viability (MTT) assay (53, 54). As shown in Fig. 2G, altering the levels of miR-584, by means of overexpression and silencing, did not change the proliferative properties or viability of the cells, indicating that the results obtained on the wound-healing assay are due to the antimigratory properties of miR-584 (Fig. 2, C and D).

miR-584 Alters the Actin Cytoskeleton—The changes in the motility properties of the cell require changes in the actin cytoskeleton (55). Hence, to further investigate the role and requirement of miR-584 downstream of TGF- β -mediated cell migration, miR-584 expression was altered using the specific miR-584 mimic and inhibitor, and cells were stimulated or not with TGF- β . Cell morphology was observed using confocal microscopy after actin staining (*green*) with a 63 \times objective. Staining was also carried out for vimentin (*red*), a protein widely used as a marker for migration, as it co-localizes with actin in filopodia (56). DAPI staining was performed for nuclear staining and cell counting purposes (57). For negative controls, cells were mock-transfected or transfected with scrambled mimic and scrambled inhibitor. As shown in Fig. 3A, TGF- β induced an elongated phenotype with a relocalization of vimentin and cortical actin toward the extensions in the mock condition and in cells transfected with the scrambled mimic. These cells are indicated with *yellow arrows*. Quantification of the data, using ImageJ software, indicated that TGF- β stimulation doubled the number of cells with filopodia formation from 18 to 31% (Fig. 3B). In addition, treatment with TGF- β reduced the appearance of stress fibers by about half in control conditions from 24 to 10% (Fig. 3C). The formation of filopodia and the loss of stress fibers are characteristics of motile cells (56, 58), and confirmed the migratory phenotype induced by TGF- β stimulation. Interestingly, overexpression of the miR-584 mimic completely abrogated the TGF- β -induced vimentin relocalization and filopodia formation (Fig. 3, A and B). Moreover, the formation of stress fibers, indicated in Fig. 3A with *white arrows*, were very apparent in the miR-584 mimic-treated cells. Indeed, both the nontreated and the TGF- β -treated conditions display over 40% stress fiber formation upon miR-584 overexpression (Fig. 3C). Hence, increasing the expression of miR-584 with a mimic prevented the cells from acquiring the characteristics of motile cells.

The morphology of the cells was also observed upon blockage of miR-584 expression. In contrast to what we observed with the mimic, blocking the expression of miR-584 with the specific inhibitor strongly induced vimentin relocalization and filopodia formation to a greater extent than what is observed when cells are stimulated with TGF- β (Fig. 4A). In fact, 52% of cells present with filopodia when miR-584 expression is inhibited (Fig. 4B). As for stress fiber formation, the effect of the inhibitor was similar to that of TGF- β , with 11% of cells presenting stress fiber formation (Fig. 4C), demonstrating once again the role of miR-584 in acquisition of features necessary for cell migration. Altogether, these results highlight miR-584 as a potent inhibitor of filopodia formation and an inducer of stress fiber formation in breast cancer cells, downstream of TGF- β .

PHACTR1 Is a Novel TGF- β Target Acting Downstream of miR-584—Having found that TGF- β -mediated down-regulation of miR-584 is required for cell migration and actin remodeling, we next sought to identify the downstream miR-584-regulated genes that could relay these effects. Pairing of a specific miRNA to the 3'-UTR of its target mRNA often leads to cleavage of the message and results in change of expression levels (59, 60). Thus, it is expected that TGF- β -mediated repression of miR-584 will lead to an increased expression of its downstream mRNA targets.

We first performed bioinformatics analyses using various algorithms, namely TargetScan, MicroCosm Targets Version 5, miRanda, and Pitatop (33–36), and identified the top 12 potential predicted targets for miR-584. We investigated whether these candidate genes were regulated by TGF- β , as shown in Fig. 5A. Using real-time quantitative PCR, we found that out of the 12 potential candidates, only two target genes, the paired-related homeobox 1 and the protein phosphatase and actin regulator 1 (*PRRX1* and *PHACTR1*), showed the expected up-regulation in response to TGF- β . To then investigate whether TGF- β -mediated *PHACTR1* and *PRRX1* up-regulation was miR-584-dependent, we examined the TGF- β response on their respective mRNA levels in SCP2 cells transfected or not with the miR-584 mimic. Interestingly, as shown in Fig. 5B, although miR-584 mimic expression did reverse the TGF- β effect on *PHACTR1* mRNA level, it did not significantly affect *PRRX1* levels. Although these results highlight both *PRRX1* and *PHACTR1* as novel TGF- β downstream targets, they also indicate that only *PHACTR1* gene expression is regulated through miR-584.

To further demonstrate the requirement of miR-584 downstream of TGF- β -mediated *PHACTR1* expression, breast cancer cells were transfected with increasing concentrations of either a scrambled mimic or the miR-584 mimic. As shown in Fig. 5C, overexpression of the miR-584 mimic strongly decreased the TGF- β effect on *PHACTR1* mRNA expression, when compared with cells expressing the scrambled mimic. These effects of the miR-584 mimic were further confirmed at the protein level. As shown in Fig. 5D, overexpression of increasing concentrations of the miR-584 mimic almost completely inhibited *PHACTR1* protein levels, as measured by immunoblotting.

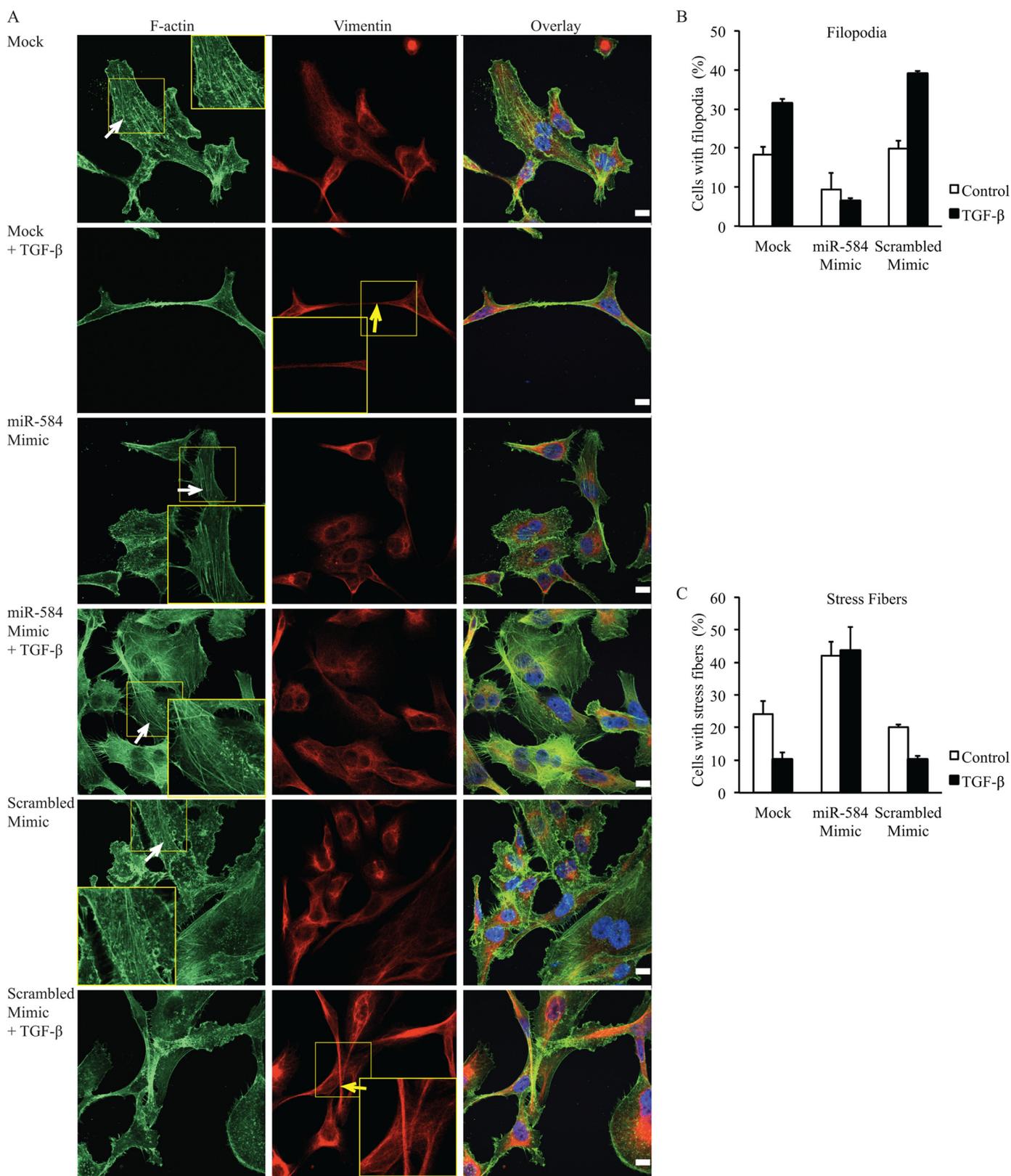


FIGURE 3. miR-584 overexpression impedes TGF- β -mediated filopodia formation and promotes stress fiber formation. *A*, SCP2 cells were transfected with 80 nM miR-584 mimic and were stimulated with TGF- β for 24 h. Cells were fixed and stained with actin (green), vimentin (red), and DNA (blue). *B* and *C*, the percentage of cells with stress fibers (white arrows) and filopodia (yellow arrows) was counted and graphed. Scale bars, 50 μ m.

The previous results demonstrate a regulatory effect of miR-584 over PHACTR1, but do not determine whether cleavage of the PHACTR1 3'-UTR occurs. To this purpose, we have made

the use of the pMIR-REPORT system, where we have inserted the 3'-UTR of PHACTR1 downstream of a luciferase reporter as a regulatory element. Three constructs were synthesized:

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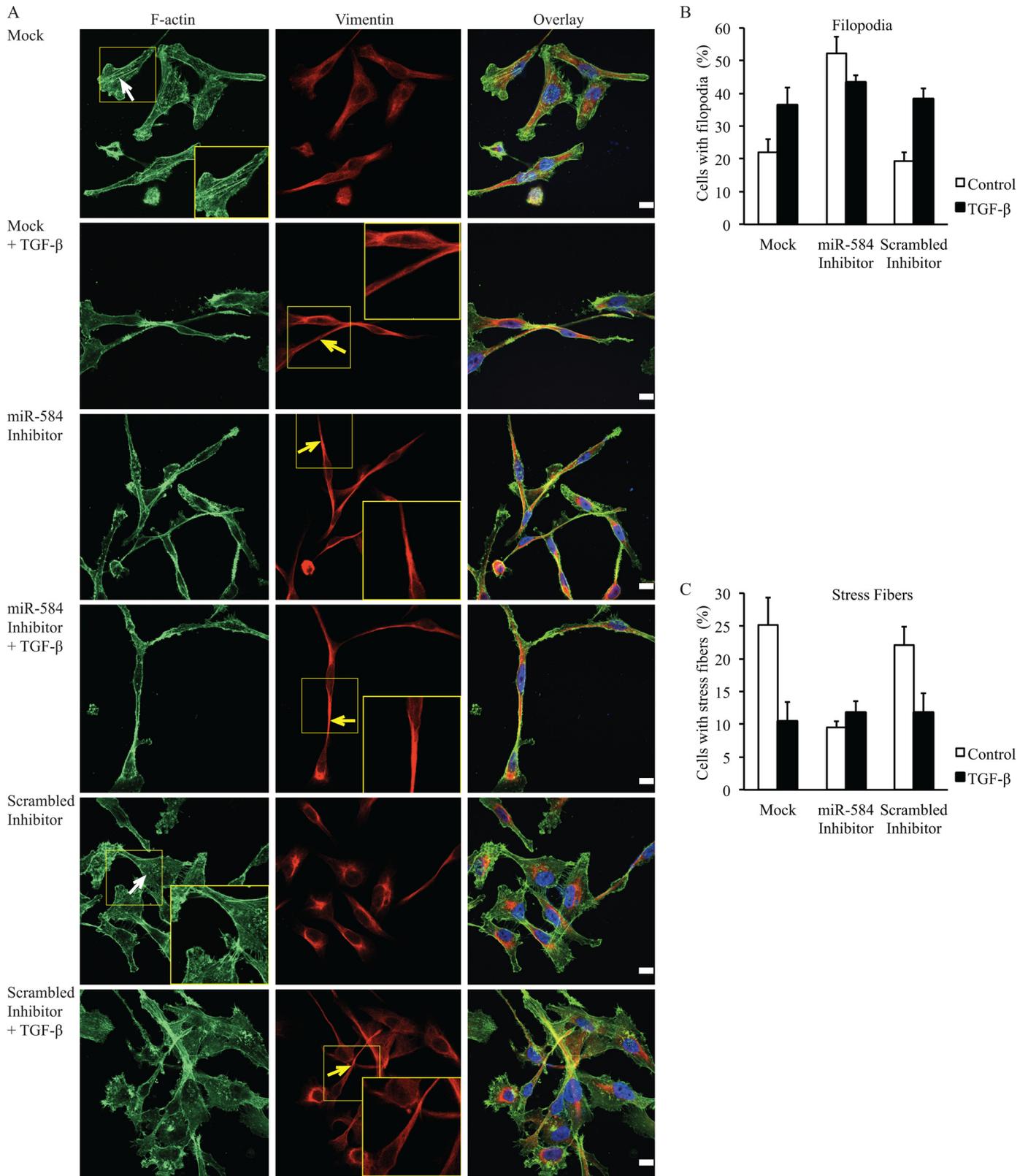


FIGURE 4. miR-584 inhibition promotes TGF- β -mediated filopodia formation and impedes stress fiber formation. A, SCP2 cells were transfected with 80 nm miR-584 inhibitor and were stimulated with TGF- β for 24 h. Cells were fixed and stained with actin (green), vimentin (red), and DNA (blue). B and C, the percentage of cells with stress fibers (white arrows) and filopodia (yellow arrows) was counted and graphed. Scale bars, 50 μ m.

pMIR-REPORT-PHACTR1-WT, with the wild-type putative miR-584 binding site; pMIR-REPORT-PHACTR1-"A", where the two adenosines surrounding the seed sequence have been

changed to cytosines; and pMIR-REPORT-PHACTR1-Mu, where the 8-bp seed sequence for miR-584 has been completely altered (Fig. 5E). These constructs were transfected in HEK293

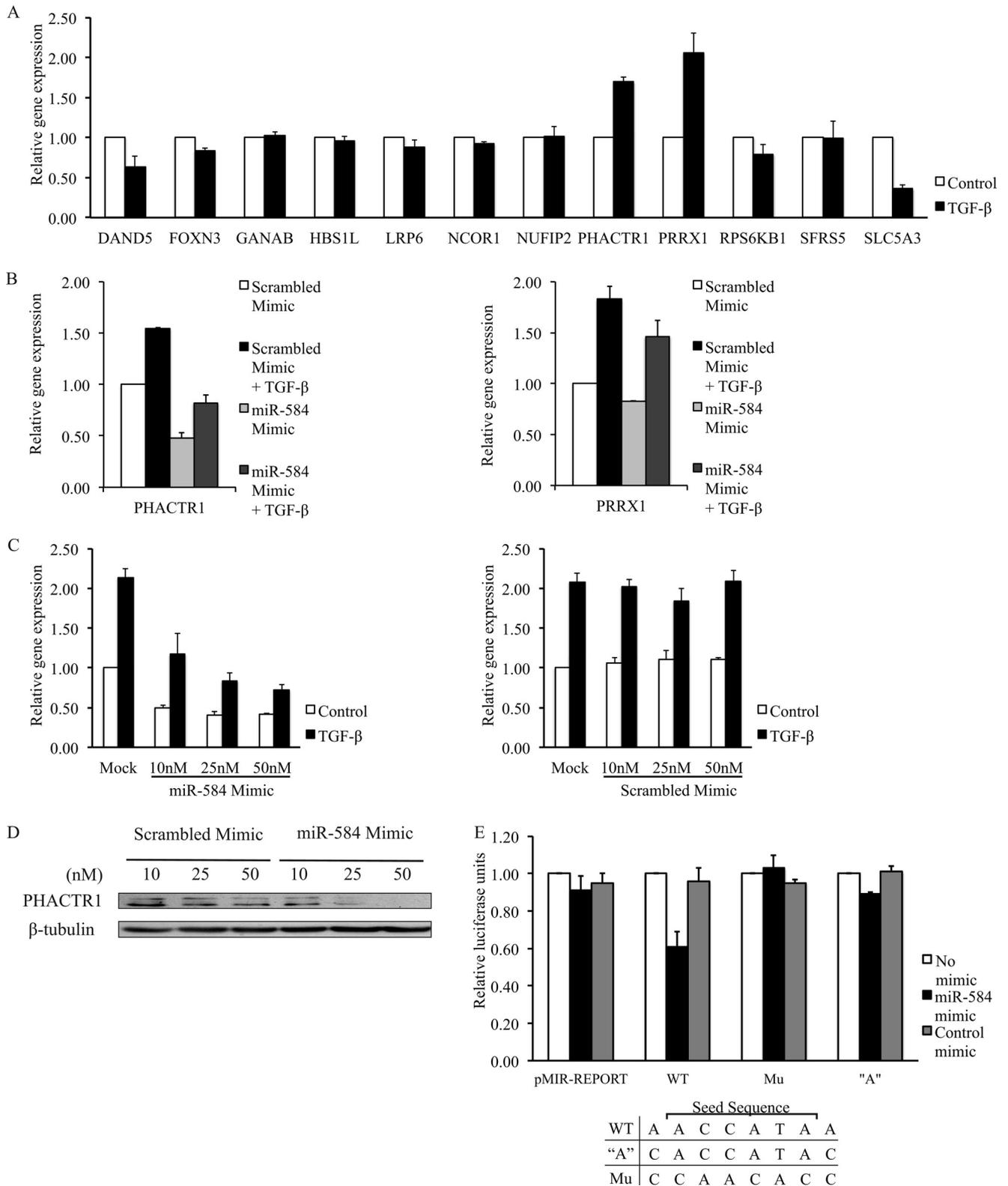


FIGURE 5. **PHACTR1 is regulated by miR-584.** *A*, total RNA was extracted from MDA-MB-231 cells that were stimulated with 100 μ M TGF- β for 24 h. Gene expression of several miR-584 target candidates was measured by qRT-PCR. *B*, SCP2 cells were transfected with 80 nM negative control mimic and miR-584 mimic and stimulated or not with 100 μ M TGF- β for 24 h. Total RNA was extracted, and gene expression of TGF- β -regulated potential miR-584 targets was measured by qRT-PCR. *C*, SCP2 cells were transfected with increasing concentrations of scrambled or miR-584 mimic and stimulated with 100 μ M TGF- β for 24 h. Total RNA was extracted, and PHACTR1 expression was measured by qRT-PCR. *D*, SCP2 cells were transfected with increasing concentrations of scrambled or miR-584 mimic, and total protein was extracted. PHACTR1 expression was measured by Western blot. *E*, HEK293 cells were transfected with pMIR-REPORT luciferase reporter construct containing one of the three synthesized PHACTR1 3'-UTR inserts, with or without mimic, and luciferase assay was performed. *Mu*, pMIR-REPORT-PHACTR1-Mu; "A", pMIR-REPORT-PHACTR1-"A".

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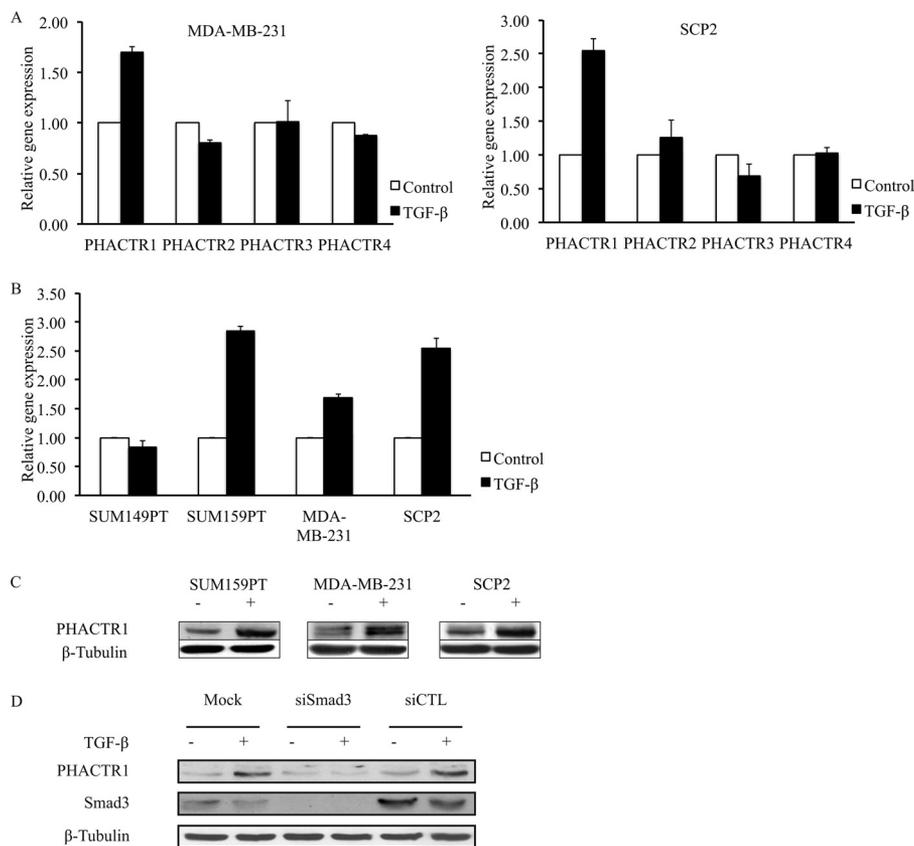


FIGURE 6. PHACTR1 is a novel TGF- β target. *A*, MDA-MB-231 and SCP2 cells were stimulated with 100 pM TGF- β for 24 h, and total RNA was extracted. Quantitative RT-PCR was performed to measure the TGF- β effect on PHACTR family members. *B*, several breast cell lines were stimulated with 100 pM TGF- β for 24 h. Total RNA was extracted, and the TGF- β effect on PHACTR1 was measured by qRT-PCR. *C*, several breast cell lines were stimulated with 100 pM TGF- β for 24 h. Total proteins were extracted, and 50 μ g of protein was loaded for Western blot analysis. *D*, SCP2 cells were transfected with siRNA against Smad3 and stimulated with TGF- β for 24 h.

cells, in the presence or the absence of the miR-584 mimic or a scrambled mimic, as negative control, before being assessed for luciferase activity. As shown in Fig. 5E, overexpression of the miR-584 significantly decreased the luciferase activity of the wild-type reporter construct but did not affect the activity of the mutant construct, in which the microRNA seed sequence was mutated (pMIR-REPORT-PHACTR1-Mu). These data demonstrate that miR-584 directly interacts with PHACTR1 3'-UTR, thereby controlling its gene expression. Furthermore, weakening of the microRNA binding to its sequence, by mutating the two anchoring adenosine residues, which allegedly strengthen the interaction between the miRNA and its target, led to a strong inhibition of the miR-584 mimic effect, when compared with the wild type, further demonstrating the specificity of the binding of miR-584 to the PHACTR1 3'-UTR. Altogether, these results clearly indicate that miR-584 specifically binds to the PHACTR1 3'-UTR sequence to control PHACTR1 gene expression.

PHACTR1 Regulation by TGF- β —PHACTR1 is part of a family of proteins that binds actin, the major component of the cytoskeleton important for motility (61). The biological significance of the interaction between PHACTR1 and actin remains unknown, although it is known that PHACTR3, another family member, prevents polymerization of actin by binding to the latter, increasing cell motility and cell spreading (62). As the four family members show some degree of sequence homology,

the role of the other PHACTR family members downstream of TGF- β signaling could not be excluded. To address this, we examined their response to TGF- β in the breast cancer cell lines MDA-MB-231 and SCP2. Interestingly, TGF- β specifically up-regulates PHACTR1 but not the other family members in both cell lines (Fig. 6A).

As we previously found TGF- β to efficiently down-regulate miR-584 expression in various human breast cell lines of different molecular background (Fig. 1A), we next investigated the effect of TGF- β on PHACTR1 expression in these same cell lines. Interestingly, PHACTR1 mRNA expression was detectable only in invasive triple negative cell lines MDA-MB-231, SCP2, SUM159PT, and SUM149PT (Fig. 6B), but not in MCF7 (data not shown). Furthermore, TGF- β potently induced PHACTR1 expression in all invasive cell lines except SUM149PT, where there was also no regulation of miR-584 (Fig. 1A). These results were then confirmed at the protein level. Indeed, TGF- β -induced protein expression of PHACTR1 is observed in MDA-MB-231, SCP2, and SUM159PT (Fig. 6C), whereas the protein was undetectable in SUM149PT and MCF7 cells (data not shown).

The requirement for Smad proteins in TGF- β -mediated regulation of PHACTR1 was investigated. Smad3 was knocked down with siRNA, and PHACTR1 levels were measured by immunoblot. In Fig. 6D, we see that Smad3 knockdown substantially annihilates the TGF- β -mediated up-regulation of

PHACTR1 when compared with a mock transfection or a transfection with a scrambled siRNA sequence. These results show that PHACTR1 regulation by TGF- β is performed through the canonical Smad pathway.

PHACTR1 Is Involved in TGF- β -mediated Migration—Because PHACTR1 is a downstream target of miR-584 and because down-regulation of miR-584 is required for TGF- β -mediated cell migration, we next investigated whether PHACTR1 expression is required for TGF- β -induced cell migration. For this, expression of PHACTR1 was silenced using an siRNA specific to human PHACTR1 (Fig. 7A). As shown in Fig. 7, C and D, knocking down PHACTR1 expression significantly antagonized the TGF- β promigratory effect in the SCP2 cells but also in HaCaT, HuH7, and PC3 cells. Interestingly, the antagonistic property of the PHACTR1 siRNA on migration was very potent and in fact similar to what we observed when overexpressing the miR-584 mimic (Fig. 2, C and E). To rule out any off-target effects of the PHACTR1 siRNA, a rescue experiment was also performed, where increasing levels of rat PHACTR1 cDNA were co-transfected into cells that had been silenced for endogenous expression of the human PHACTR1, using the siRNA. The proper expression of the rat PHACTR1 cDNA was ensured by Western blot (Fig. 7B). As shown in Fig. 7E, the PHACTR1 inhibitory effect on TGF- β -induced cell migration was progressively restored with expression of increasing concentration of the PHACTR1 cDNA. Again, we verified that the migratory properties of PHACTR1 were not due to an increase in cell proliferation through an MTT assay. In Fig. 7F, we showed that PHACTR1 has no effect on cell proliferation. These results indicate that miR-584 and PHACTR1 are part of the same pathway downstream of TGF- β to regulate cellular migration in various cancer cell types.

PHACTR1 Is Required for TGF- β -regulated Filopodia and Stress Fiber Formation—We then investigated the role and contribution of PHACTR1 in cytoskeletal rearrangement by performing F-actin (green), vimentin (red), and DAPI staining (Fig. 8). In the control siRNA transfection, TGF- β induced the relocalization of vimentin and the formation of an elongated cell shape, and filopodia formation doubled from 19 to 43%, as indicated with yellow arrows. However, in the PHACTR1 siRNA-transfected cells, the influences of TGF- β on cell shape and cytoskeleton rearrangement and on the vimentin relocalization as well as filopodia formation were completely blocked and remained below 20%. These effects are very similar to what was observed on the miR-584 overexpression, where less than 10% of the cells displayed filopodia formation, both with and without TGF- β treatment (Fig. 3B). Additionally, the absence of the actin-binding protein promoted an increase in stress fiber formation, as can be seen in cells indicated with white arrows. Although the control conditions showed a reduction of stress fiber formation upon TGF- β treatment (from 27 to 14% for both the mock and the scrambled siRNA), knockdown of PHACTR1 strikingly induces stress fiber formation for up to 44% of cells (Fig. 8C), a phenotype identical to what was observed in cells overexpressing miR-584 (Fig. 3C). Thus, these results indicate that the TGF- β promigratory effects in breast cancer cells required the presence of the protein phosphatase and actin regulator 1, PHACTR1. Altogether, these results

showed that TGF- β stimulation of invasive breast cancer cells leads to down-regulation of miR-584, further leading to increased expression of the miR-584 target PHACTR1 and increased cell migration.

High PHACTR1 Expression Correlates with Basal-like Breast Cancer Subtype—As PHACTR1 is a specific downstream target of TGF- β in basal-like breast cancer cell lines, we next investigated whether PHACTR1 expression showed any correlation with breast cancer subtypes. We thus examined distribution of PHACTR1 gene expression in tissue samples from subtypes of various breast cancer patients. Using The Cancer Genome Atlas (TCGA) expression profiling database from the National Center for Biotechnology Information (NCBI), we were able to examine PHACTR1 gene expression in 599 tumor samples as determined by an Agilent 244K custom gene expression G4502A_07_3 microarray assay (63, 64).

We first separated these tumors based on the molecular classification performed through a PAM50 test, which is a qRT-PCR assay that uses 50 classifier genes and five control genes to determine the breast cancer subtype of a tumor (65). As shown in Fig. 9, color-coded PHACTR1 gene expression revealed high PHACTR1 expression in basal breast cancer subtype (red bars) when compared with all other types of breast cancer that showed lower expression (green bars). We also separated the tumors based on their receptor status; although there did not seem to be a clear-cut segregation of PHACTR1 distribution between tumors expressing varying levels of HER2 receptor, we clearly observed an over-representation of PHACTR1-expressing tumors within those that are negative for progesterone expression or negative for estrogen expression. The observation that PHACTR1 is mostly expressed in basal tumors strongly suggests that the protein might be playing a role in cancer progression and is consistent with our observations demonstrating a role for PHACTR1 downstream of TGF- β -mediated cell migration. Together, these results suggest that TGF- β -mediated PHACTR1 expression through miR-584 may specifically play a role in the invasive basal-like human breast cancer cells and may contribute to the promigratory function of this growth factor.

DISCUSSION

The implication of TGF- β in the establishment of breast metastases has been well documented (66, 67). Blocking TGF- β expression has been successful in experimental models (15, 16), but because TGF- β is involved in a variety of processes, including negatively regulating cell growth in normal cells, specifically targeting the TGF- β prometastatic signaling arm is critical if it is to be used for therapy. Achieving specificity is possible with the use of miRNAs. The first link between miRNAs and TGF- β was established on the discovery of an interaction between Smad3, a component of the signaling pathway of the cytokine, and the p68 helicase, a protein involved in the biogenesis of the miRNA (19). It was later found that this interaction led to the regulation of a specific subset of miRNAs (18). MicroRNAs were also implicated in TGF- β -mediated multiple processes that eventually lead to metastasis. For example, the miR-200 family and miR-205 were demonstrated to reverse epithelial-to-mesenchymal transition in breast cancer through regulation

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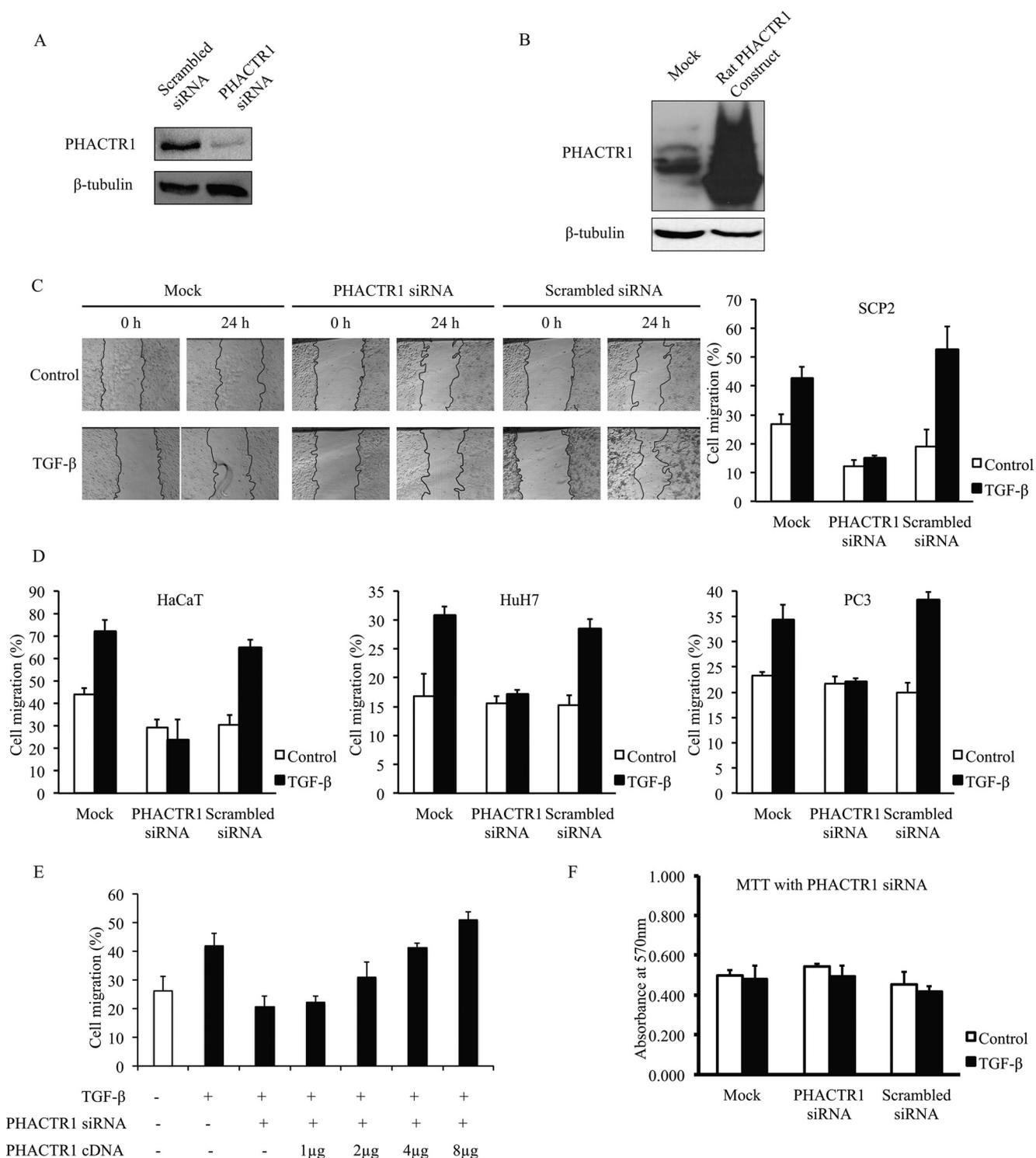


FIGURE 7. PHACTR1 promotes TGF- β migration. *A*, 80 nM PHACTR1 siRNA was transfected in SCP2, and total protein was extracted. Protein expression was evaluated by loading 50 μ g of proteins for Western blot. *B*, SCP2 cells were transfected or not with 8 μ g of rat PHACTR1-overexpressing construct. Total protein was extracted, and 50 μ g was loaded for immunoblot analysis. *C* and *D*, 80 nM PHACTR1 siRNA was transfected in SCP2 (*C*) or HaCaT, HuH7, or PC3 (*D*). Cells were grown into a monolayer, and a scratch was performed. The surface area of the scratch was measured. Cell migration was measured as the surface area covered by migrating cells 24 h after stimulation. *E*, 80 nM PHACTR1 siRNA was transfected along with increasing concentrations of rat PHACTR1-overexpressing construct in SCP2 cells, and a migration assay was performed as described previously. *F*, 80 nM PHACTR1 siRNA was transfected in SCP2, and an MTT assay was performed 72 h after transfection.

of ZEB1/2 (68), whereas the miR-520/373 family was shown to inhibit metastasis through direct inhibition of the TGF- β receptor type II (69). Here, we report a novel signaling pathway downstream of TGF- β , involving the down-regulation of miR-

584 and the subsequent up-regulation of its target PHACTR1, leading to TGF- β -mediated cell migration. Together, these results uncover the importance of microRNAs in development of new strategies for the treatment of cancer.

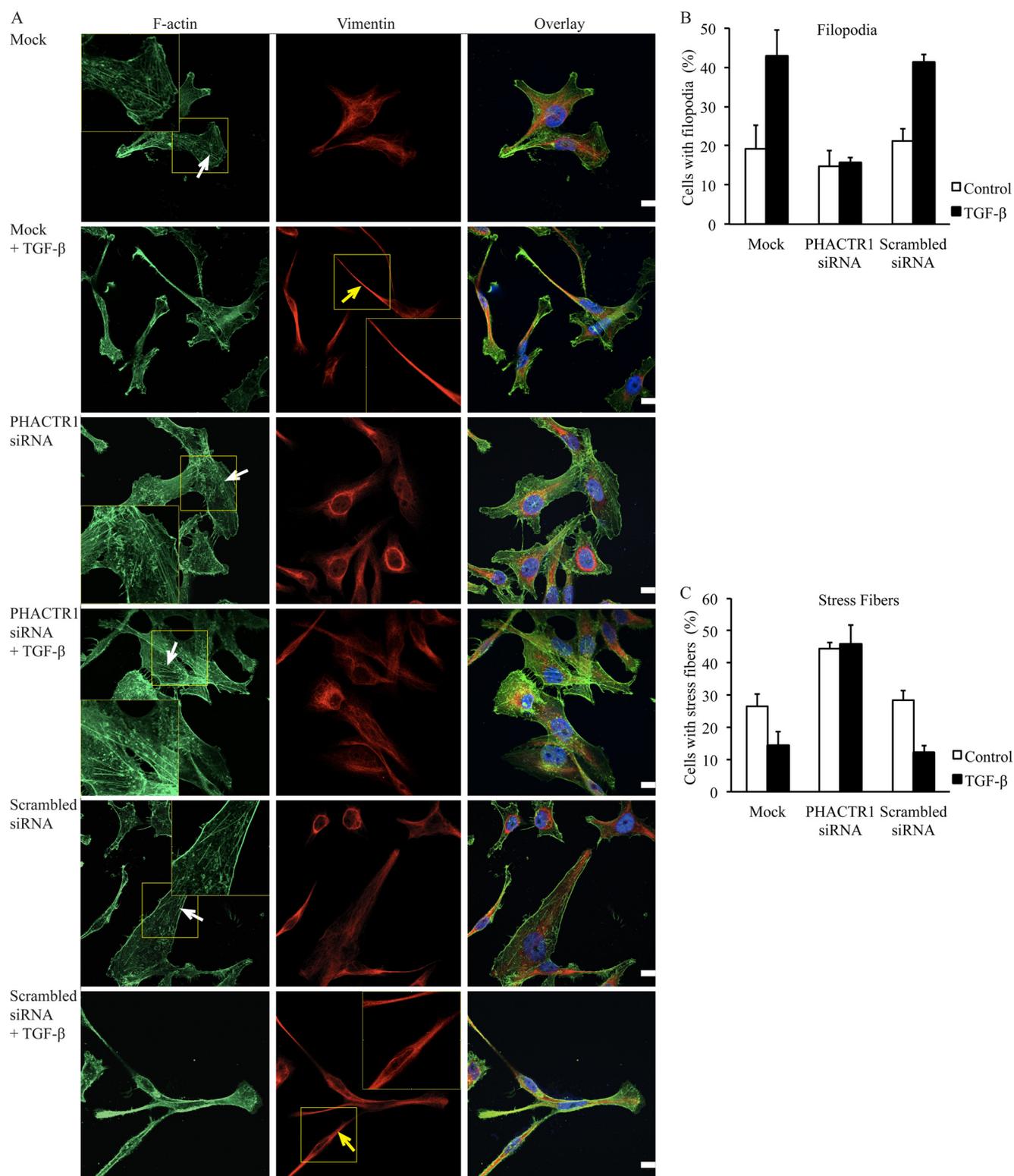


FIGURE 8. **PHACTR1 knockdown impedes TGF- β -mediated filopodia formation and promotes stress fiber formation.** A, 80 nM PHACTR1 siRNA was transfected in SCP2 and cells were stimulated with 200 pM TGF- β for 24 h, fixed, and stained for actin (green), vimentin (red), and DNA (blue) to be visualized by confocal microscopy. B and C, the percentage of cells with filopodia (yellow arrows) or stress fiber (white arrows) formation was counted and graphed. Scale bars, 50 μ m.

Previous studies have highlighted the chromosomal region where miR-584 is located, 5q32, to be deleted in myelodysplastic syndromes that lead to malignant transformation (70, 71). It was proposed that this region contains tumor-suppressive genes involved in actin dynamics (71). Moreover, a recent study

indicated that miR-584 antagonizes the motility of renal carcinoma cells, suggesting that this microRNA may act as a tumor suppressor (32). Our results clearly demonstrate that altering miR-584 levels in human breast cancer cells impacts TGF- β -mediated migration through modulation of actin dynamics

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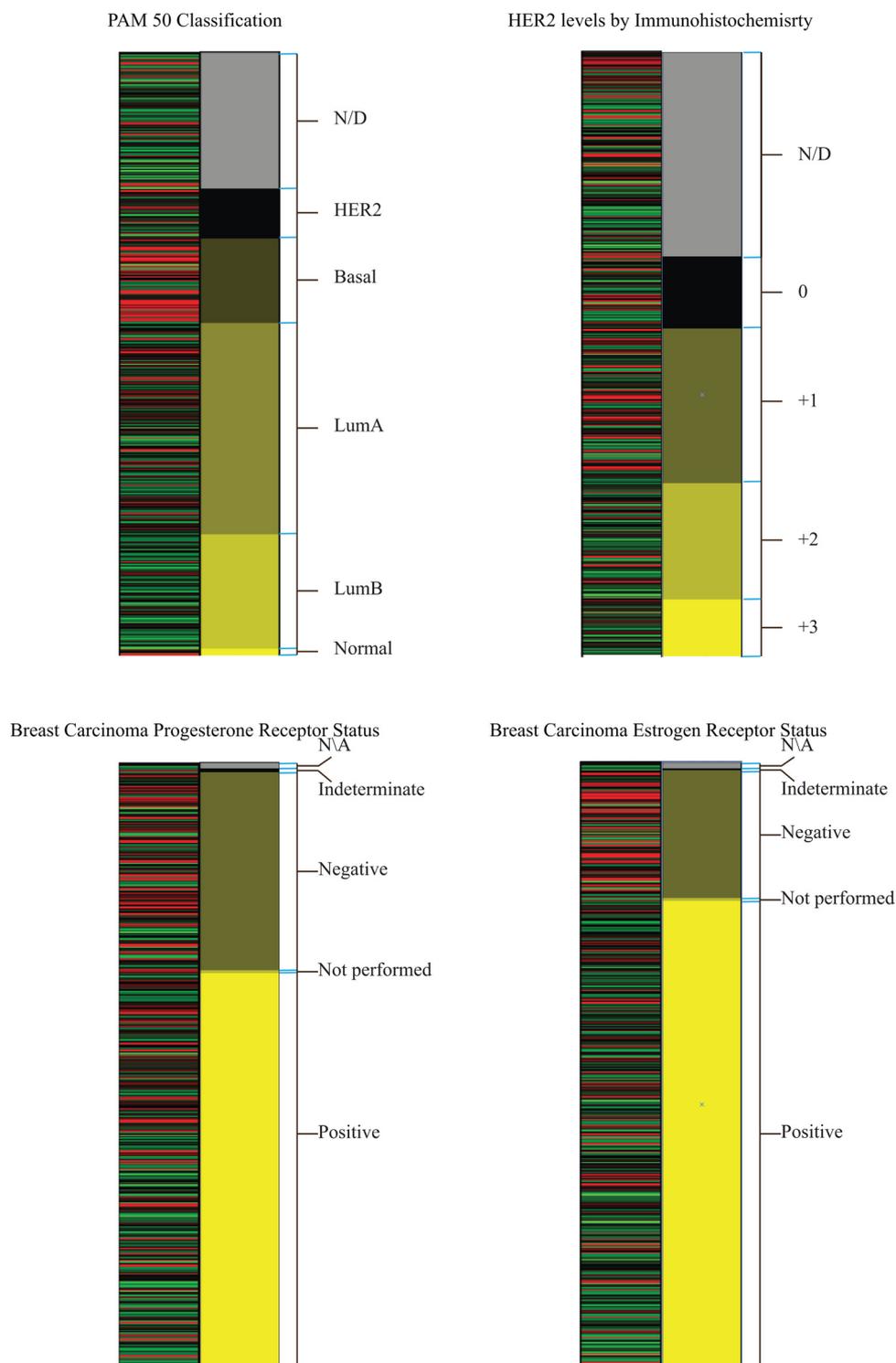


FIGURE 9. Messenger RNA expression of *PHACTR1* was evaluated from a microarray analysis of 599 tumor samples on an Agilent 244K custom gene expression G4502A_07_3 microarray assay available on the University of California Cancer Genomics Browser website. Tumors were separated according to subtype or receptor status. *N/D*, not detected. *N/A*, not applicable.

(Figs. 2–4). We thus propose that down-regulation of miR-584, a putative tumor suppressor, is a prerequisite to TGF- β -mediated migration as it promotes stress fiber turnover and the formation of filopodia that help in cellular motility.

To determine the mechanism of action of miR-584, we have made the use of available online algorithms to establish a list of potential targets and retained those that were common in sev-

eral algorithms. The criteria used for target prediction include seed sequence complementarity and conservation, target site conservation, 3'-pairing, the presence of anchoring adenosines around the seed sequence (21, 33, 72), thermodynamic stability of the miRNA/mRNA duplex, and target site accessibility (34–36, 73–76). This approach allowed us to identify *PHACTR1* as a potential target. The binding site of miR-584 occurs on a con-

served site of PHACTR1 as an 8-mer, within the first quarter of the 3'-UTR, and after the first 15 nucleotides. All these criteria are favorable in binding of the miRNA to its target (21, 72). Moreover, the target site on the 3'-UTR has anchoring adenines at positions 1 and 8, further increasing the possibility of PHACTR1 as being a real target (33). We demonstrated that modulating the miR-584 levels tightly regulated PHACTR1 expression and further confirmed PHACTR1 as a miR-584 direct target by showing the direct binding of miR-584 to the PHACTR1 3'-UTR sequence.

After confirming that PHACTR1 was a target of miR-584, we looked at its regulation by TGF- β in a panel of breast cell lines. At the protein level, it was undetectable in SUM149PT, where miR-584 is not regulated and where 90% of the cells are in fact of luminal classification (Fig. 6) (77). However, we have observed the up-regulation of PHACTR1 by TGF- β in MDA-MB-231, SCP2, and SUM159PT, where miR-584 is down-regulated, suggesting that the function of PHACTR1 downstream of TGF- β is exclusive to invasive basal-like breast cancer cells. This result is in agreement with patient clinical data, where *PHACTR1* was over-represented in tumors of basal classification, as well as estrogen-negative and progesterone-negative tumors (Fig. 9).

PHACTR1 is the founding member of a recently discovered family of proteins with RPEL repeats for actin binding (61). It was previously demonstrated that PHACTR3 interacts directly with actin monomers to prevent their polymerization and that PHACTR3 overexpression induces cell spreading and motility of HeLa cells through reorganization of the cytoskeleton (62). Moreover, introduction of mouse homolog family members in the mouse myoblasts C2C12 modifies the cell shape by adding hair-like cytoplasmic extensions (78). The previous data and the ability to bind actin suggested a possible role in cytoskeletal dynamics; indeed, we found that knocking down PHACTR1 expression in the invasive breast cancer cells impaired the ability of TGF- β to promote migration and completely reshaped the cytoskeleton, similar to what is observed in cells overexpressing the miR-584 mimic.

Both the overexpression of miR-584 and the knockdown of PHACTR1 resulted in the formation of long actin filaments. To explain the role of these stress fibers in cell movement, we need to first understand the molecular mechanisms of cell movement. Motility of a cell requires polarization for the lamellipodium to protrude at the leading edge of the cell, where a focal contact can be established to provide the necessary pulling forces for traction. At the end of the cell, proteolysis occurs to dissociate from the extracellular matrix, and the cell body contracts for retraction of the tail (79). Several lines of evidence had suggested that stress fibers were responsible for this contraction, but other studies have shown that they were more present in nonmotile cells (58). This paradox can be explained by the facts that: (i) there is diversity in the types of stress fibers observed; (ii) depending on the cell type and environment, the involvement of stress fibers in one direction or another will differ; and (iii) contractile forces may be provided by elements other than stress fibers (80). In our study, when overexpressing miR-584 or when knocking down PHACTR1, an important formation of stress fibers was observed, and at the same time, TGF- β migration was impaired. Moreover, TGF- β stimulation

of the cells, associated with increased motility, led to decreased stress fiber formation. Thus, it appears that in our model, the appearance of stress fibers is a characteristic of stationary cells, as evidenced by the data obtained from F-actin and vimentin staining and from the migration assays. Cell migration requires a highly dynamic actin, which can be severed, capped, polymerized, or bundled through the action of actin-binding proteins (81). As mentioned previously, PHACTR1 is known to bind actin monomers (61). It seems that this allows for PHACTR1 to participate in the reorganization of actin during cell movement. We can hypothesize that when we knock it down, stress fibers are able to form, but actin is no longer treadmill. Hence, instead of having cycles of actin polymerization and depolymerization, which are necessary for cell motility, we observe an accumulation of stress fibers and a halt of cell migration. This was confirmed through the observation that abolishing PHACTR1 expression or overexpressing miR-584 resulted in a loss of the formation of filopodia, which are necessary for cell motility, by TGF- β . Moreover, a recent study highlighted a role for PHACTR1 in endothelial cells, where it is required for the control of cycles of actin polymerization and depolymerization (40). Combined with our study, these data may indicate that PHACTR1 plays a central role in regulating cell migration in multiple tissue and cell types, including breast cancer.

With this study, we report, for the first time, the down-regulation of miR-584 by TGF- β in aggressive breast cancer cells, where the cytokine acts as a promigratory factor. From our results, it is clear that the observation of stress fiber formation induced by miR-584 in breast epithelial cancer cells results in a halt of migration. We identified PHACTR1 as a downstream target of miR-584 and determined that it was also required for TGF- β -mediated migration. The discovery of PHACTR1 downstream of miR-584 introduces this poorly characterized protein as a potential major actor in actin dynamics. Further investigation will be necessary to follow the evolution of actin dynamics and associated proteins in response to miR-584 and PHACTR1 downstream of TGF- β signaling.

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MicroRNA-584 and the Protein Phosphatase and Actin Regulator 1 (PHACTR1), a New Signaling Route through Which Transforming Growth Factor- β Mediates the Migration and Actin Dynamics of Breast Cancer Cells

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