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GABA(A) Receptor Pi (GABRP) Stimulates Basal-like Breast Cancer Cell Migration through Activation of Extracellular-regulated Kinase 1/2 (ERK1/2)*

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Background: *GABRP* correlates with the basal-like breast cancer (BLBC)/triple negative subtype, but its function in this disease is poorly understood.
Results: Silencing GABRP in BLBC cells decreases migration, BLBC-associated cytokeratins and ERK1/2 activation.
Conclusion: A GABRP-ERK1/2-cytokeratin axis maintains BLBC migration.
Significance: GABRP is a component of a cell-surface receptor, thus, targeting this signaling axis may have therapeutic potential in BLBC.

Breast cancer is a heterogeneous disease comprised of distinct subtypes predictive of patient outcome. Tumors of the basal-like subtype have a poor prognosis due to inherent aggressiveness and the lack of targeted therapeutics. Basal-like tumors typically lack estrogen receptor-α, progesterone receptor and HER2/ERBB2, or in other words they are triple negative (TN). Continued evaluation of basal-like breast cancer (BLBC) biology is essential to identify novel therapeutic targets. Expression of the pi subunit of the GABA(A) receptor (*GABRP*) is associated with the BLBC/TN subtype, and herein, we reveal its expression also correlates with metastases to the brain and poorer patient outcome. *GABRP* expression in breast cancer cell lines also demonstrates a significant correlation with the basal-like subtype suggesting that GABRP functions in the initiation and/or progression of basal-like tumors. To address this postulate, we stably silenced *GABRP* in two BLBC cell lines, HCC1187 and HCC70 cells. Decreased *GABRP* reduces *in vitro* tumorigenic potential and migration concurrent with alterations in the cytoskeleton, specifically diminished cellular protrusions and expression of the BLBC-associated cytokeratins, *KRT5*, *KRT6B*, *KRT14*, and *KRT17*. Silencing *GABRP* also decreases phosphorylation of extracellular regulated kinase 1/2 (ERK1/2) in both cell lines and selective inhibition of ERK1/2 similarly decreases the basal-like cytokeratins as well as migration. Combined, these data reveal a GABRP-ERK1/2-cytokeratin axis that maintains the migratory phenotype of basal-like breast cancer. GABRP is a component of a cell surface receptor, thus, these findings suggest that targeting this new signaling axis may have therapeutic potential in BLBC.

Breast cancer is a heterogeneous disease with at least five distinct molecular subtypes, including luminal-A, luminal-B, HER2/ERBB2, basal-like, and normal-like (1, 2). Most striking is the correlation between disease subtype and patient outcome: patients with luminal disease have the most favorable outcome, while basal-like disease is associated with poor outcome (2). Differences in patient prognosis are partly due to intrinsic aggressiveness of distinct subtypes as well as the relative availability of effective targeted therapies. Basal-like breast cancers (BLBCs)[5] tend to be estrogen receptor-α (ERα), progesterone receptor (PR), and HER2/ERBB2 negative (*i.e.* triple negative), and therefore lack the therapeutic molecular targets used in treating women with the other disease subtypes (3). Moreover, women with BLBC have a higher propensity of developing visceral metastases to the lung, liver, and brain (3–6). The absence of established targets in BLBC underscores the need to identify pathways that drive this disease to facilitate the development of BLBC-specific pharmacologic strategies.

The pi subunit of the GABA(A) receptor (*GABRP*) correlates with the BLBC subtype (7) and is up-regulated in the normal luminal progenitor population in the breast, the predicted cell-of-origin of BLBCs (8). Although its functional role in the breast is unknown, the *GABRP* subunit has been suggested to induce the growth of pancreatic cancer cell lines through calcium mobilization and ERK1/2 signaling (9, 10). GABA(A) receptors are heteropentameric ligand-gated chloride channels whose traditional role is to mediate synaptic inhibition in the central nervous system. There are multiple GABA(A) receptor subunits (α, β, γ, δ, ϵ, θ, and π), with α, β, and γ all having multiple isoforms (α1–6, β1–3, γ1–3). At least two α and two β subunits comprise most functional receptors. Interestingly, unlike other GABA(A) subunits, *GABRP* is not abundant in the brain, but is

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[5] The abbreviations used are: BLBC, basal-like breast cancer; ERBB2, erythroblastic leukemia viral oncogene homolog 2; ERα, estrogen receptor-α; ERK1/2, extracellular-regulated kinase 1/2; GABA, γ-aminobutyric acid; GABRP, GABA(A) receptor pi; GAD, glutamic acid decarboxylase; HER2, human epidermal growth factor receptor; MMP, matrix metalloprotease; PR, progesterone receptor; qRT-PCR, quantitative real-time polymerase chain reaction; TN, triple negative.

detectable in multiple non-neuronal normal human tissues, including the uterus and mammary gland (11, 12). Its proposed uterine roles are inhibition of contractility and regulation of endometrial receptivity (11, 13, 14).

While GABRP function in either the normal or cancerous breast has not been defined, the GABA(A) receptor ligand, γ-aminobutyric acid (GABA), and glutamine acid decarboxylase (GAD), the enzyme that catalyzes the synthesis of GABA, are detected in the normal mammary gland (15). Most importantly, breast cancer patient brain metastases exhibit a GABAergic phenotype including the up-regulation of GABA(A) receptors, GABA transporters and GAD expression (16). A putative role for GABA(A) in breast cancer is further supported by the observation that propofol, a multifunctional drug with agonist activity for the GABA(A) receptor, induces actin reorganization and migration of breast cancer cells through collagen matrices (17, 18). GABA treatment correspondingly increases cell number, matrix metalloprotease (MMP) expression and *in vitro* invasiveness in prostate cancer cells (19, 20). These results have been corroborated in patient samples where the primary tumor expression of GABA and GAD are positively correlated with high MMP expression and lymph node metastases in prostate cancer (19). Comparable *in vitro* results have also been observed in renal cell carcinoma and hepatocellular carcinoma cell lines (21, 22). In contrast, GABA agonists inhibit migration and invasion of colon cancer cells (23, 24). While contradictory, these findings suggest a critical role for GABAergic signaling in cancer, and enforce the need for continued evaluation of this pathway in distinct cancer types.

Given the significant and subtype-specific elevation of *GABRP* in the BLBC subtype (7), we hypothesized that GABRP is functionally relevant in this disease. Utilizing publicly available gene expression data, we confirmed the correlation of *GABRP* with the BLBC subtype. Our studies also reveal, for the first time, that *GABRP* is associated with metastases to the brain and poorer patient survival. We further describe a requirement for GABRP in BLBC secondary tumorsphere formation and cell migration, both functional readouts of tumor aggressiveness. Silencing of GABRP expression in BLBC cell lines concomitantly diminishes basal-like cytokeratin expression and ERK1/2 activity, suggesting GABRP may mediate a pro-migratory cytoskeletal structure through the ERK1/2 signaling pathway. These findings illustrate a previously unrecognized role for GABRP in promoting BLBC aggressiveness, and support further analysis of GABRP as a putative therapeutic target in this disease.

EXPERIMENTAL PROCEDURES

Statistical Methods—Significance was determined by Student's *t* test assuming a two-tailed distribution and equal variance among sample populations.

Gene Expression Microarray Analyses—Twenty-six cDNA microarray datasets of human breast tumors were retrieved from Oncomine (oncomine.org) for *in silico* analyses. *GABRP* expression was compared between tumors defined as BLBC *versus* all other molecular subtypes in the Curtis (25) and Esserman (26) datasets. For determining if *GABRP* in the primary tumor correlates with metastases to the brain, we evaluated the Bos dataset (27) for *GABRP* primary tumor expression (mean ± 95% confidence) in patients with metastases that went to the brain (includes brain only, brain plus bone, brain plus lung, as well as brain plus lung and bone) *versus* those that went to other sites (lung only, bone only, as well as lung plus bone). For GABRP-related survival analyses, patients in the Curtis (25) and Esserman (26) datasets were separated into high (upper quartile) and low (remaining) *GABRP* expressing groups and overall survival evaluated over a 5 year period. Kaplan-Meier survival curves were generated and statistical significance determined using log-rank. For co-expression analysis, only breast cancer datasets that utilized Affymetrix HG-U133A or U133 Plus 2.0 arrays were included to eliminate platform-related variability. This list was then further refined to exclude sets where the samples were stroma or non-invasive breast cancer. These criteria provided 24 datasets for co-expression analysis (Table 1). Each dataset was evaluated for genes that positively correlate with *GABRP* using a Pearson Correlation Coefficient of ≥0.5. Pearson correlation coefficients between *GABRP* and *KRT5*, *KRT6B*, *KRT14*, and *KRT17* were calculated and used to determine covariance of these factors in each data set. The overall significance of covariance between *GABRP* and basal keratin was determined by Fisher's combined probability test with values ≥0.05 considered significant.

Cell Culture and Lentiviral Transduction—HCC1187 and HCC70 cells were obtained from ATCC and cultured in RPMI media (Invitrogen) supplemented with 10% FBS and 1% penicillin-streptamycin (Invitrogen). MISSION shRNA (Sigma) were used to stably silence GABRP. shRNAs targeting either control (shLuciferase; SHC007) or *GABRP* (shGABRP#2 = TRCN0000063094; shGABRP#4 = TRCN0000063096; shGABRP#5 = TRCN0000063097) were engineered within the pLKO.1-puro lentiviral vector. Lentivirus was generated by the Protein Expression Facility at the Wistar Institute, and used to transduce cells at a multiplicity of infection of one in the presence of 8 μg/ml polybrene. After 48–72 h, cells were selected for approximately one month with 1 μg/ml puromycin to generate stable knockdown populations.

Tumorsphere Assay—Primary and secondary tumorsphere formation was performed similar to previous studies (28). Briefly, 10,000 viable cells, as determined by trypan blue exclusion, were seeded per well into ultra low attachment flat bottom 6 well plates (Costar). Cells were seeded in Biowhittaker Inc. Mammary Epithelial Basal Medium (Fisher Scientific) supplemented with 1xB-27 (Invitrogen) and 10 ng/ml epidermal growth factor (Sigma). After 2 weeks, the primary tumorspheres were collected by centrifugation (800rpm), trypsinized with 0.05% trypsin/EDTA, and strained (40 μm filter) to generate a single cell suspension. 2,500 viable cells were then reseeded per well in ultra low attachment 6 well plates to generate secondary tumorspheres. At least five unique fields (×4 magnification) were quantified per experimental condition.

Migration—Migration assays were performed as described (29). Briefly, 50,000 viable cells, as determined by trypan blue exclusion, were seeded into the top of uncoated 24-well transwell inserts (Corning) in either serum-free RPMI media

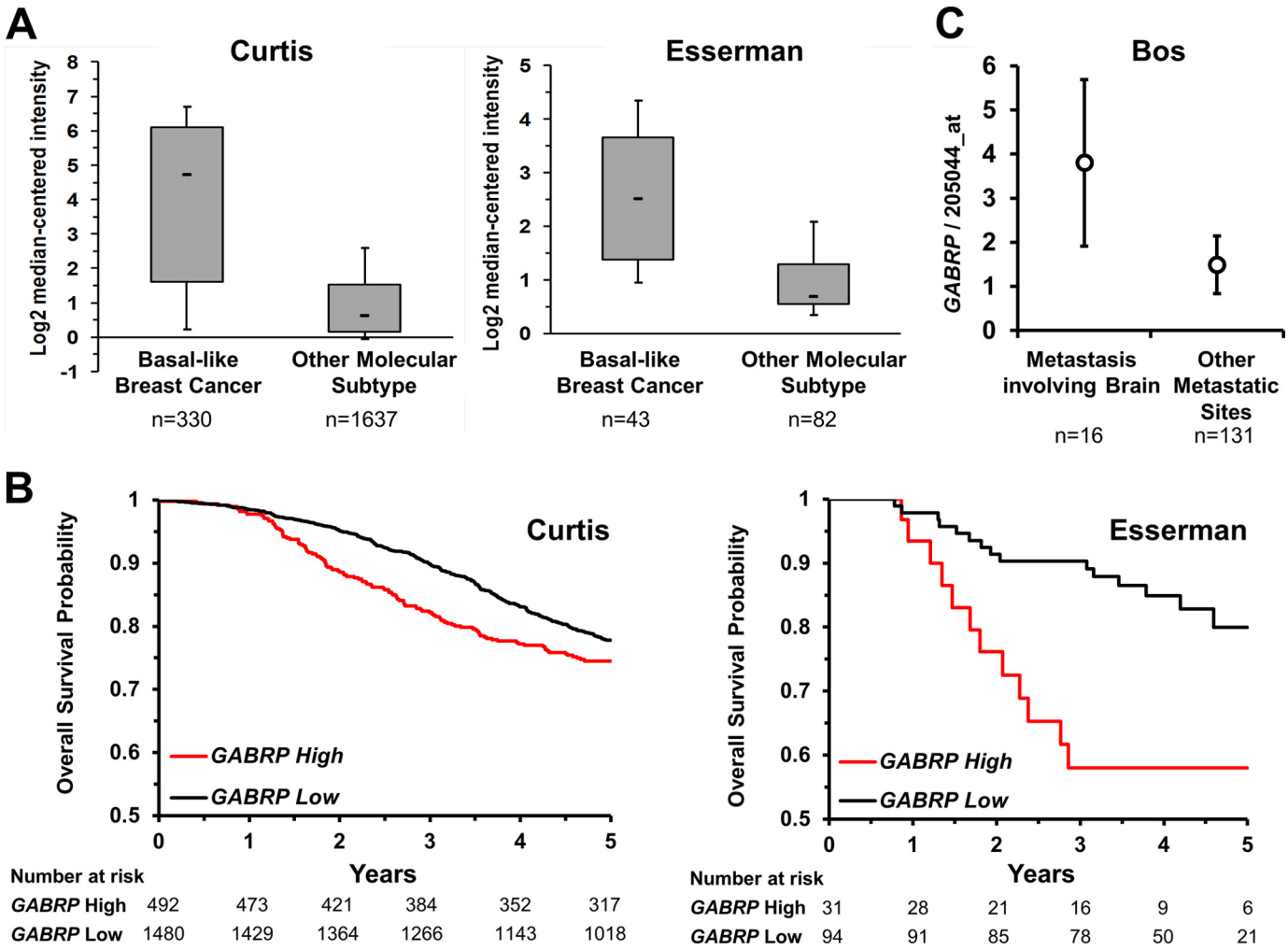

FIGURE 1. **High *GABRP* correlates with the BLBC molecular subtype and shorter overall breast cancer patient survival.** *A–C*, breast tumor expression of *GABRP* was obtained from the Curtis, Esserman, and Bos cDNA microarray datasets via Oncomine. *A*, high *GABRP* correlates with the basal-like breast cancer subtype (Curtis, BLBC $n = 330$, Other $n = 1637$, $p = 1 \times 10^{-20}$; Esserman, BLBC $n = 43$, Other $n = 82$, $p = 8.6 \times 10^{-13}$). *B*, patients were separated into high (upper quartile) and low (remaining) *GABRP* expressing groups. Kaplan-Meier survival analyses indicates patients whose tumors have high *GABRP* expression have a significantly worse prognosis *versus* patients whose tumors have low levels of *GABRP* (Curtis, *GABRP* high $n = 492$, low $n = 1480$, $p = 0.044$; Esserman, *GABRP* high $n = 31$, low $n = 94$, $p = 0.002$). *C*, primary tumors with metastases involving lesions in the brain have higher *GABRP* than those that metastasized to other metastatic sites (involving brain, $n = 16$; other, $n = 131$; $p = 0.02$).

(HCC70) or RPMI media containing 1% FBS (HCC1187). Cells migrated toward complete media with 10% FBS for ~24 h. For experiments including treatment with PD98059 (Tocris, #1213), HCC1187 cells were pre-treated for 48 h with 30 μM PD98059, trypsinized, and 50,000 viable cells seeded in RPMI media containing 1% FBS and 30 μM PD98059. Cells migrated toward complete media as above. Migrated cells were fixed and stained using the DiffQuik staining procedure. Three technical replicates were performed per experiment, where five unique fields (×10 (HCC70) and ×4 (HCC1187) magnification) were quantified per replicate.

Immunofluorescence—Cells were seeded onto sterile glass coverslips, fixed with 4% paraformaldehyde and permeabilized with acetone. Nonspecific binding was blocked with 1% BSA/1× PBS/0.1% azide. F-actin was visualized using Texas Red-X Phalloidin (Molecular Probes) and nuclei counter-stained with Vectashield mounting media with DAPI (Vector Labs). Quantification of cell protrusions was performed using the count tool in Adobe Photoshop. A protrusion was counted if it was greater than 30 pixels (or ~14 μm) in length. At least five unique fields (20× magnification) were quantified per experimental condition.

Quantitative Real-time PCR—RNA processing, DNase I treatment and reverse transcription was performed as described (29). Applied Biosystems TaqMan Gene Expression Assays included: *GAPDH*, Hs99999905_m1; *GABRP*, Hs00959454_m1; *KRT5*, Hs00361185_m1; *KRT6B*, Hs00745492_s1; *KRT14*, Hs00559328_m1; *KRT17*, Hs00356958_m1; *KRT19*, Hs00761767_s1.

Immunoblots—Western analyses were performed as described (29) using primary antibodies diluted in 5%-milk-1× TBST (ERK1/2-Thr202/Tyr204, Cell Signaling 9101; total ERK1/2, Cell Signaling 4695). Prior to probing for total ERK1/2, blots were stripped at 55 °C for 30 min in a buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 100 mM β-mercaptoethanol. After washing, blots were reblocked with 5% milk. Quantitation of protein levels was performed using Image J (30).

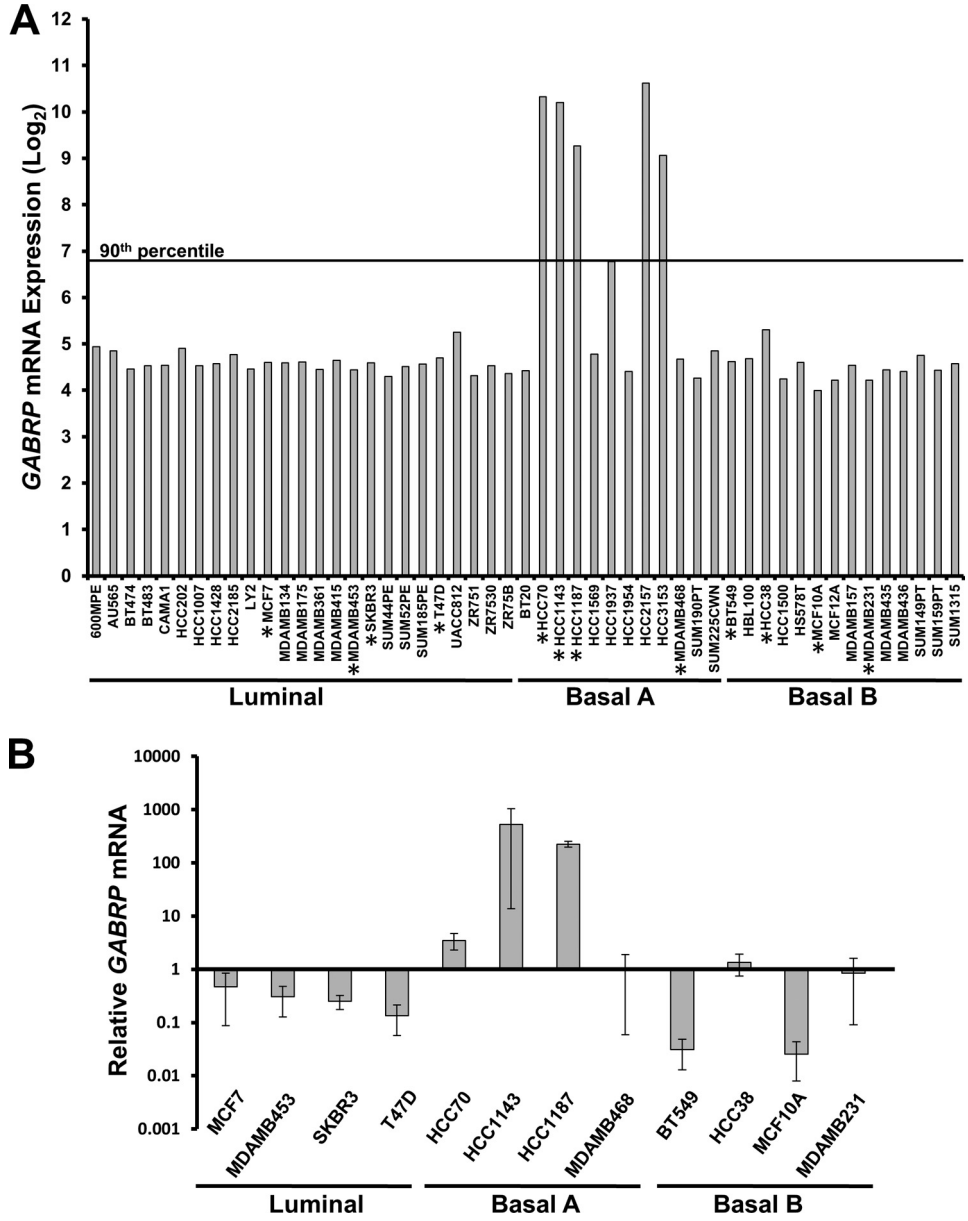

FIGURE 2. **GABRP expression is only observed in breast cancer cell lines characterized as basal A.** *A*, publicly available cDNA microarray expression data from Neve and colleagues (31) were evaluated for *GABRP*. Expression levels are $\log_2$-scale. *B*, *GABRP* was confirmed by qRT-PCR in a representative panel of cell lines (marked by *asterisks* in *A*). Bars represent the mean of three experiments ± S.D. relative to *GAPDH*.

RESULTS

GABRP Correlates with the BLBC Subtype and Decreased Patient Survival—GABRP has been reported to correlate with the BLBC subtype, but the analysis was limited to a single population (7). To confirm these findings in additional independent patient cohorts, we utilized publicly available cDNA microarray expression data. In two distinct datasets, *GABRP* expression is significantly higher in tumors defined as basal-like *versus* the other molecular subtypes (Fig. 1*A*). More importantly, we found that *GABRP* correlates with reduced overall patient survival in these patient cohorts (Fig. 1*B*). Since the poorer outcome observed in BLBC patients is the result of visceral metastases (lung, liver, and brain) (3–6), we reasoned that the presence of *GABRP* in the primary tumor may facilitate breast cancer cell homing to sites with high GABA expression, *i.e.* the brain. To test this, we evaluated whether primary tumors that metastasized to sites including the brain had higher *GABRP* than those metastasizing to other sites (lung and bone). Interestingly, *GABRP* is up-regulated in primary tumors that resulted in brain metastases (Fig. 1*C*). Combined, these data indicate that *GABRP* is correlated with BLBC patient outcome including the site of metastatic spread.

To begin to gain a better understanding of the functional role of GABRP, we evaluated *GABRP* in a publicly available gene expression dataset encompassing a large cohort of breast cancer cell lines (31). Unlike tumors, the molecular subtype of breast cancer cell lines is designated as luminal, basal A and basal B (31, 32). Basal A lines are representative of the human BLBC phenotype, while basal B lines are more closely related to the claudin-low subtype (31, 32). Of the 12 cell lines designated

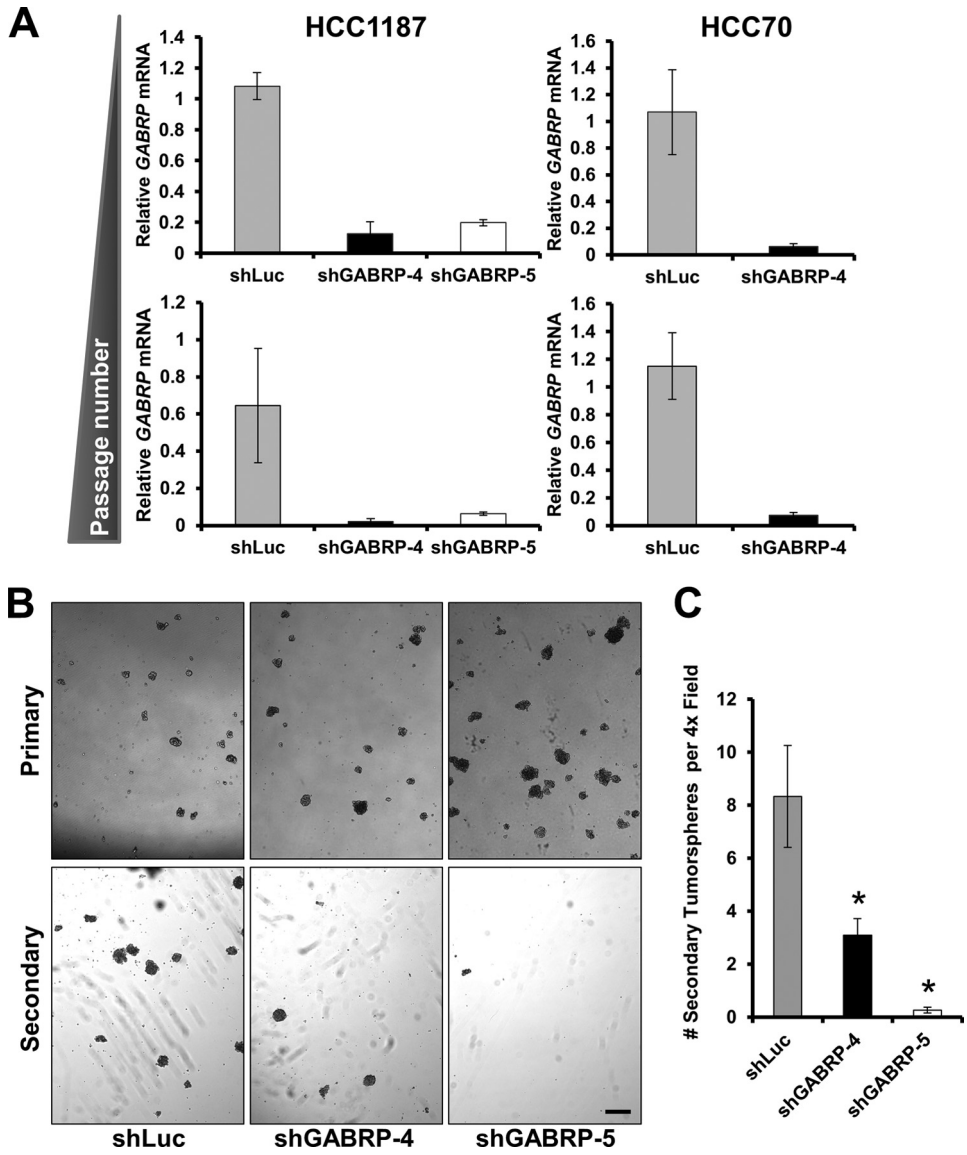

FIGURE 3. **Stable silencing of GABRP decreases basal A breast cancer cell secondary tumorsphere formation.** *A*, HCC1187 and HCC70 cells were transduced with lentiviral shRNA expression plasmids targeting either luciferase (shLuc) or *GABRP* (shGABRP-4 and -5). Cells were selected with puromycin for at least 3 weeks and stable populations generated. Silencing of *GABRP* mRNA was evaluated by qRT-PCR at early and later passages. Bars represent the range of three technical replicates ± S.D. relative to *GAPDH*. *B*, HCC1187 cells were cultured under non-adherent conditions to generate primary (*top*) and secondary (*bottom*) tumorspheres (Scale bar, 300 μm). *C*, quantification of the number of secondary tumorspheres. Bars represent the mean ± S.D. of a representative experiment repeated three times (*, $p < 0.05$).

as basal A, five (HCC70, HCC1143, HCC1187, HCC2157, and HCC3153) have *GABRP* expression in the 90th percentile (Fig. 2*A*). We confirmed these findings by quantitative real time PCR (qRT-PCR) of a representative panel of the subtypes. Similar to the microarray data, *GABRP* mRNA expression is elevated in the basal A cell lines HCC70, HCC1143, and HCC1187 cells (Fig. 2*B*). These findings affirm that *GABRP* expression is primarily restricted to basal breast cancers.

GABRP Is Necessary for BLBC Cell Line Tumorigenic Potential and Migration—To determine if GABRP plays a role in the aggressiveness of BLBCs, we stably silenced GABRP in representative BLBC cell lines, HCC1187 and HCC70 cells. Antibodies that are specific for GABRP immunodetection are not available, thus *GABRP* mRNA silencing was confirmed directly after antibiotic selection and after several passages (Fig. 3*A*). As a measure of tumorigenic potential, we assessed both primary and secondary tumorsphere formation in the HCC1187 cells. Silencing *GABRP* with either of two independent shRNAs (shGABRP-4 or shGABRP-5) resulted in significantly reduced secondary tumorsphere formation in HCC1187 cells relative to controls (shLuc) (Fig. 3, *B* and *C*). As a second readout of BLBC aggressiveness, we also evaluated whether GABRP knockdown altered migration toward serum. HCC1187 cells with silenced GABRP exhibited significantly decreased migration (Fig. 4, *A* and *C*). Similar findings were observed in a second BLBC cell line, HCC70, in which *GABRP* was stably silenced with shGABRP-4 (Fig. 4, *B* and *D*). Combined, these results indicate that GABRP expression is necessary to maintain BLBC tumorigenic potential and cellular motility. Furthermore, the GABRP knockdown cells have significantly fewer cellular protrusions

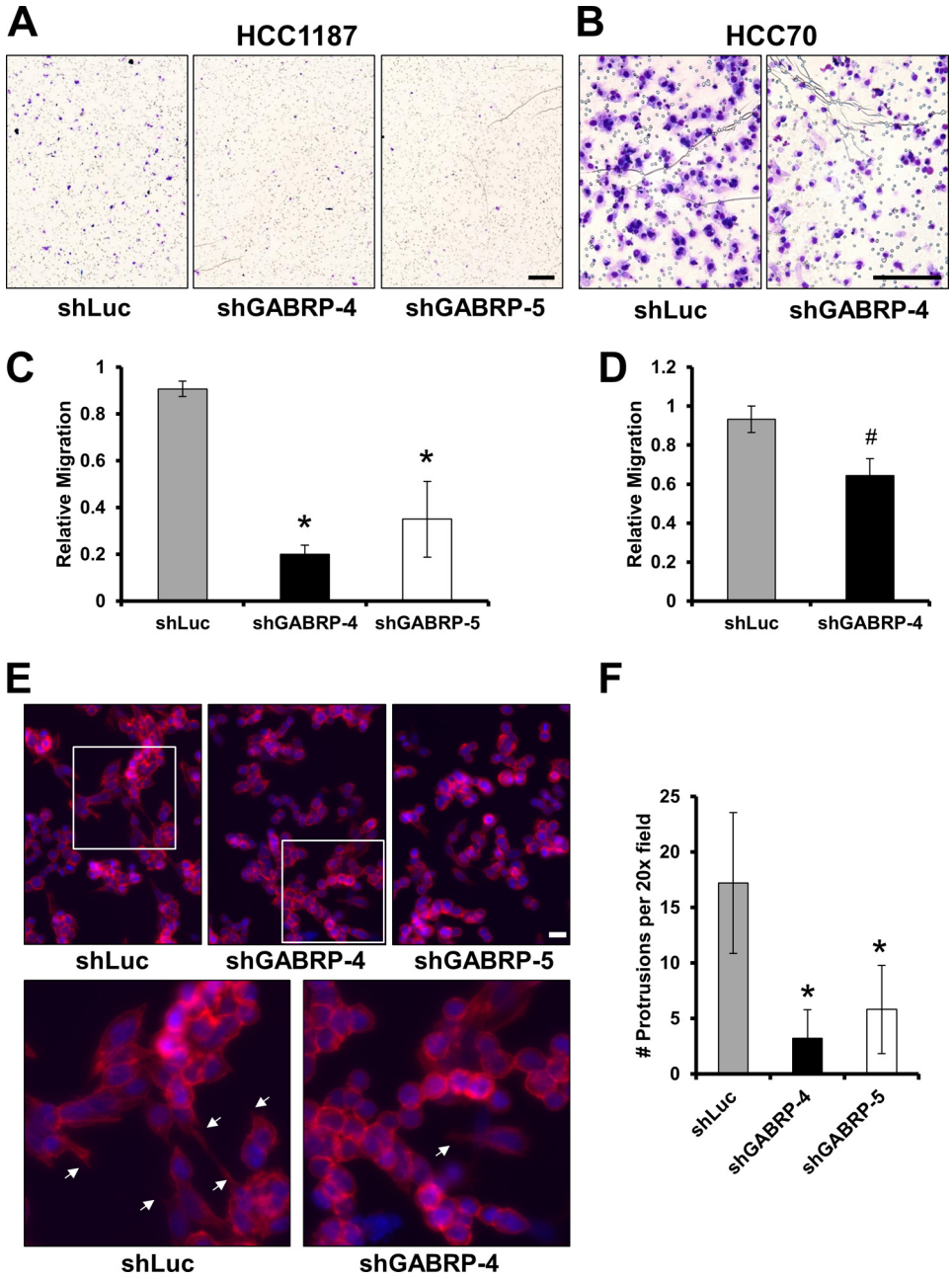

FIGURE 4. **Stable silencing of GABRP decreases basal A breast cancer cellular protusions and migration.** HCC1187 (*A*, *C*) and HCC70 (*B*, *D*) cells were plated in modified Boyden chambers to analyze migration over 24 h. Cells were plated in RPMI supplemented with 1% FBS (HCC1187) or under serum-free conditions (HCC70). RPMI with 10% FBS was used as a chemoattractant. Representative images of migrated cells (*A–B*) and quantification (*C–D*) are shown, where *bars* represent the mean of at least three experiments ± S.E. (*, $p < 0.05$; #, $p = 0.058$; Scale bars, 200 μm).*E*, HCC1187 cells were labeled with Texas-red-conjugated phalloidin (*red*) to visualize F-actin and counterstained with DAPI (*blue*). Magnified images for shLuc and shGABRP-4 exhibit cellular protrusions (*arrows*) (Scale bar, 40 μm). *F*, quantification of the number of cell protrusions represented in *E*. *Bars* represent the mean ± S.D. of representative staining repeated twice (*, $p < 0.05$).

when compared with control cells (Fig. 4, *E* and *F*) indicating a less motile morphology that corroborates the decrease in migration.

GABRP Maintains Basal-like Cytokeratin Expression—To begin to delineate the mechanism underlying GABRP-mediated BLBC migration, we evaluated 24 publicly available human breast cancer datasets (Table 1) to identify genes that positively correlate with *GABRP* and are established regulators of cellular migration. Within these datasets, 14 genes are significantly co-expressed with *GABRP* (Pearson Correlation Coefficient >0.5; frequency >0.42) (Table 2). Interestingly, four of these genes encode cytokeratins (*KRT5*, *KRT6B*, *KRT14*, and *KRT17*). The Pearson correlation coefficients between *GABRP* and *KRT5*, *KRT6B*, *KRT14*, and *KRT17* for each of the 24 datasets are shown in Fig. 5*A*. As intermediate filaments, the cytokeratins are critical components of the cytoskeleton and cancer cell motility (33, 34). Moreover, *KRT5*, *KRT6B*, *KRT14*, and *KRT17* are all well-described BLBC markers (reviewed in Ref. 35). Having identified that reduced *GABRP* expression decreases BLBC migration, we then tested whether stable *GABRP* knockdown

TABLE 1
Human breast cancer gene expression datasets used for *GABRP* co-expression analysis

Study	Affymetrix Platform[a]	Sample *n*	Refs.
Bittner	U133 Plus 2.0	336	Not published
Bos	U133 Plus 2.0	204	*Nature* 459: 1005–1009 (2009)
Chin	U133A	118	*Cancer Cell* 10(6):529–541 (2006)
Desmedt	U133A	198	*Clin. Cancer Res.* 13(11):3207–3214 (2007)
Farmer	U133A	49	*Oncogene* 24(29):4660–4671 (2005)
Ginestier	U133 Plus 2.0	55	*Clin. Cancer Res.* 12(15):4533–4544 (2006)
Hatzis	U133A	508	*JAMA* 305(18):1873–1881 (2011)
Hoeflich	U133 Plus 2.0	30	*Clin. Cancer Res.* 15(14):4649–4664 (2009)
Ivshina	U133 Plus 2.0	289	*Cancer Res.* 66(21):10292–10301 (2006)
Kao	U133 Plus 2.0	327	*BMC Cancer* 11:143 (2011)
Korde	U133 Plus 2.0	61	*Breast Cancer Res. Treat.* 119:685–699 (2010)
Loi	U133 Plus 2.0	87	*J. Clin. Onco.* 25(1):1239–1246 (2007)
Loi_3	U133 Plus 2.0	77	*BMC Genomics* 9:239 (2008)
Lu	U133 Plus 2.0	129	*Breast Cancer Res. Treat.* 108(2):191–201 (2008)
Miller_2	U133A	116	*Pharmacogenetics and Genomics* 17(10):813–826 (2007)
Minn_2	U133A	121	*Nature* 436(7050):518–524 (2005)
Miyake	U133 Plus 2.0	115	*Cancer Science* 103(5):913–920 (2012)
Pawitan	U133A and U133B	159	*Breast Cancer Res* 7:R953-R964 (2005)
Richardson 2	U133 Plus 2.0	47	*Cancer Cell* 9(2):121–132 (2006)
Schmidt	U133A	200	*Cancer Res.* 68(13):5405–5413 (2008)
Symmans	U133A	195	*J. Clin. Onco* 28(27):4111–4119 (2010)
Symmans_2	U133A	103	*J. Clin. Onco* 28(27):4111–4119 (2010)
Tabchy	U133A	178	*Clin. Cancer Res* 16(21):5351–5361 (2010)
Wang	U133A	286	*Lancet* 365(9460):671–679 (2005)

[a] The same reporter ID for *GABRP* (205044_at) was used for each study.

TABLE 2
Genes co-expressed with *GABRP* in twenty-four cDNA microarray datasets of human breast tumors

Freq[a]	Gene Symbol			
1	***GABRP***			
0.79	*MIA*	*SOX10*		
0.75	*SFRP1*			
0.63	*SYNM*			
0.58	*ROPN1*			
0.54	*DSC3*	*ELF5*		
0.5	***KRT6B***	*TRIM29*		
0.46	*CRYAB*			
0.42	***KRT14***	***KRT17***	***KRT5***	*MMP7*
0.38	*BBOX1*	*FOXC1*	*SERPINB5*	*SLC6A14*
0.33	*SOSTDC1*	*TRIM2*		
0.29	*ACTG2*	*GPM6B*	*PROM1*	*PTX3*
0.25	*BCL11A*	*EN1*	*MFGE8*	*TTYH1*

[a] Frequency is the number of datasets in which the gene is significantly co-expressed with GABRP divided by the total number of datasets analyzed ($n = 24$). The cutoff for significant correlation was set at a Pearson Correlation Coefficient of ≥0.5.

alters cytokeratin expression in HCC1187 and HCC70 cells. Reducing *GABRP* significantly decreases *KRT5*, *KRT6B*, *KRT14*, and *KRT17* in HCC1187 cells (Fig. 5*B*) and *KRT6B*, *KRT14*, and *KRT17* in HCC70 cells (Fig. 5*C*). Although we and others have observed subtype plasticity in breast cancer cell lines following alterations in subtype-specifying genes (29, 36), the decrease in the basal-like cytokeratins with *GABRP* knockdown was not accompanied with an up-regulation of *KRT19*, a luminal-specific cytokeratin (Fig. 5, *B* and *C*). Hence *GABRP* silencing does not appear to induce a molecular switch to a more differentiated state. These data do reveal that the correlation between *GABRP* and *KRT5*, *KRT6B*, *KRT14*, and *KRT* 17 is due to the ability of GABRP to induce their expression. Furthermore, these studies reveal a novel function for GABRP in maintaining the BLBC phenotype by regulating intermediate filament expression and the resulting migratory phenotype.

A GABRP-ERK1/2-Cytokeratin Axis Is Pro-migratory in BLBC—GABA(A) receptor signaling leads to chloride influx (hypopolarization) and subsequent calcium mobilization from intracellular stores (hyperpolarization) in immature neurons (37). Treating breast cancer cell lines with GABA(A) agonists induces migration (17, 18) and although these studies did not evaluate specifically whether altered ionic flux was responsible for changes in cell migration, calcium signaling alone induces breast cancer cell line motility (38). To test whether the GABRP-mediated BLBC migration described herein is due to changes in intracellular calcium signaling, we treated parental HCC1187 cells with 100 μM GABA and evaluated changes in intracellular calcium levels by FURA2. GABA treatment did not induce calcium mobilization (data not shown). These data suggest that intracellular calcium in HCC1187 cells is not responsive to GABA, and thus, is unlikely to underlie the effects of GABRP silencing.

GABA(A) agonists can also induce ERK1/2 phosphorylation in cancer cell lines (10, 21). Like calcium signaling, ERK1/2 signaling is pro-migratory (33, 39, 40), and importantly, regulates cytokeratin expression in non-breast cancer cell lines (34, 41). These findings, in combination with the observation that BLBC cells are sensitive to ERK1/2 inhibition (42, 43), led us to predict that in BLBC, GABRP may maintain basal-like cytokeratin expression through the ERK1/2 pathway. Indeed, stable silencing of *GABRP* significantly reduces basal ERK1/2 phosphorylation in both HCC1187 and HCC70 cells (Fig. 6). To directly test whether ERK1/2 inhibition could replicate the same effect as GABRP silencing on basal-like cytokeratin expression in basal breast cancer cells, HCC1187 cells were treated with a selective MEK inhibitor (PD98059). PD98059 significantly decreased *KRT5*, *KRT6B*, and *KRT14* expression (Fig. 7, *A* and *B*) suggesting that GABRP may maintain the basal-like cytokeratin profile in BLBC cells through ERK1/2 signaling. We further found that PD98059 inhibits migration of these cells, further mimicking the effects of *GABRP* knockdown (Fig. 7*C*). Notably, the decrease in migration does not appear to be due to a loss in cell viability following MEK inhibition as equal numbers of viable cells were plated during the migration assays. Moreover, decreased migration is not secondary to treatment-

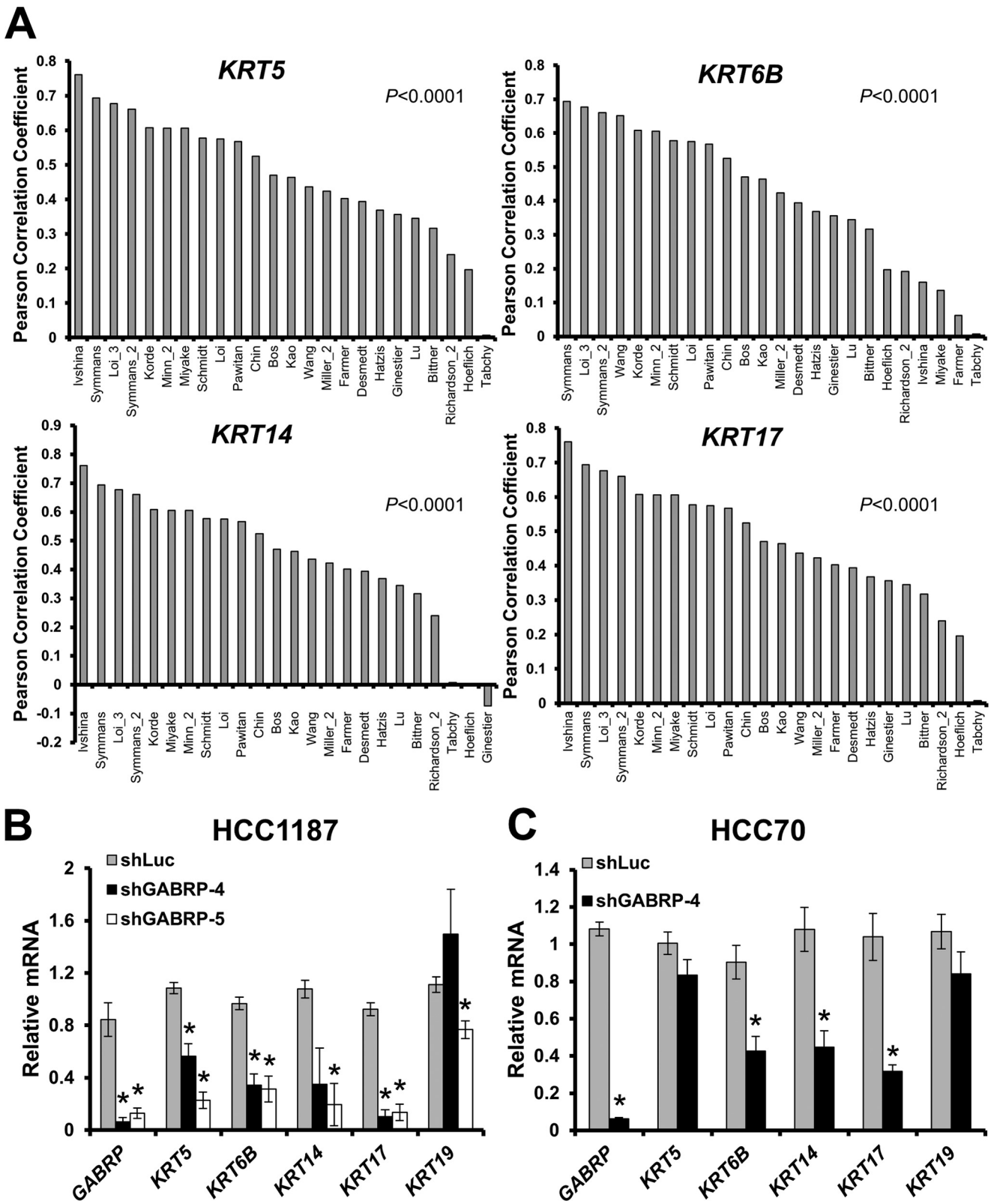

FIGURE 5. **Stable silencing of GABRP diminishes cytokeratin expression in basal A breast cancer cells.** *A*, bar graphs representing the Pearson correlation coefficients between *GABRP* with *KRT5*, *KRT6B*, *KRT14*, and *KRT17* in 24 independent breast cancer patient gene expression datasets. The overall *p* value for each cytokeratin is $p < 0.0001$ by Fisher's combined probability test. *B*, HCC1187 and *C*, HCC70 cells with and without *GABRP* knockdown were evaluated for changes in cytokeratin mRNA expression by qRT-PCR. Bars represent the mean of three experiments ± S.E. relative to *GAPDH* (*, $p < 0.02$ *versus* shLuc).

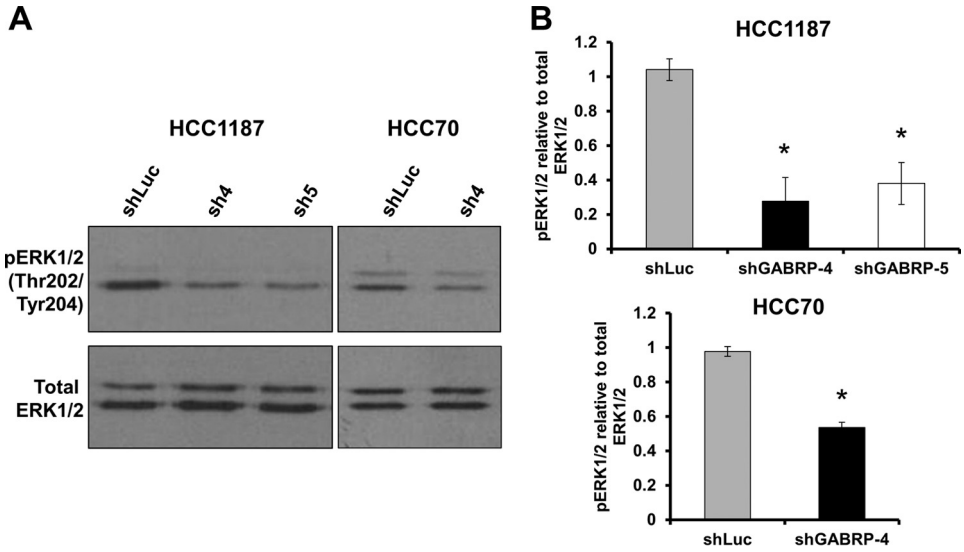

FIGURE 6. **Stable silencing of GABRP reduces ERK1/2 activation.** *A*, representative western analyses of phosphorylated (active) and total ERK1/2 expression in HCC1187 and HCC70 cells with and without GABRP expression. *B*, quantification of phosphorylated ERK1/2 relative to total ERK1/2 expression shown in *A*. *Bars* represent the mean of three experiments ± S.E. (*, $p < 0.01$).

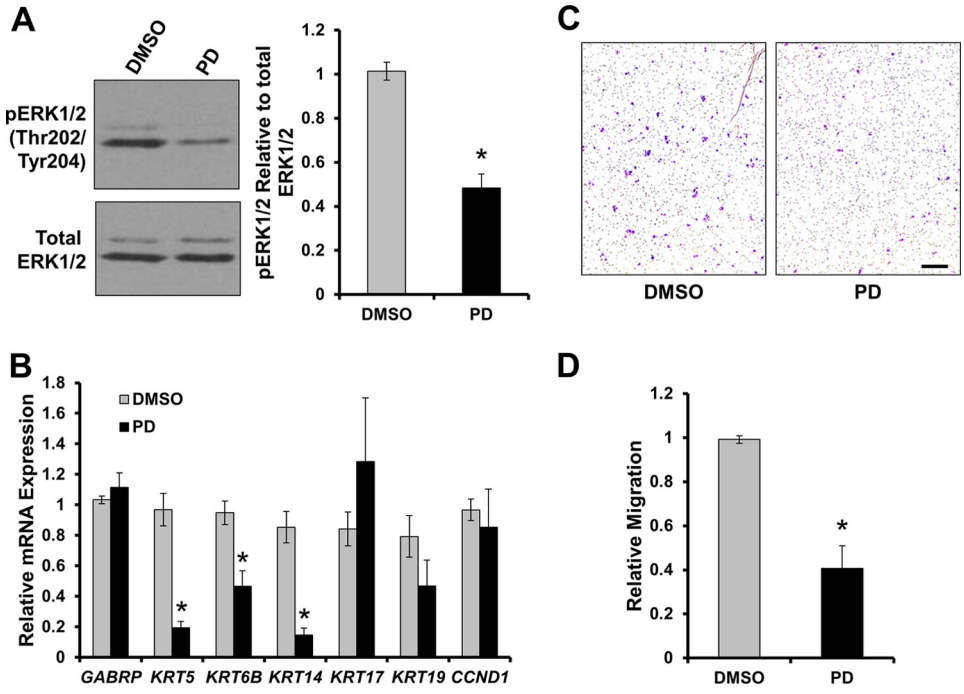

FIGURE 7. **Inhibition of ERK1/2 diminishes basal cytokeratin expression and migration in HCC1187 cells.** *A*, representative western analyses (*left*) and quantification (*right*) of phosphorylated ERK1/2 relative to total ERK1/2 expression confirming the efficacy of the PD98059 compound. *Bars* represent the mean of three experiments ± S.E. (*, $p < 0.001$). *B* and *C*, cells treated with PD98059 for 48 h were analyzed for mRNA expression by qRT-PCR (*bars* represent the mean of three experiments ± S.E. relative to *GAPDH* (*, $p < 0.05$ *versus* shLuc)) (*B*) and cell migration over 24 h (*C*). *D*, quantification of the migration shown in *C* (*bars* represent the mean of at least three experiments ± S.E. *, $p < 0.01$; *scale bar*, 200 μm).

induced growth arrest, as evidenced by the maintenance of cyclin D1 expression (*CCND1*) in the presence of PD98059 (Fig. 7*B*). Combined, these data reveal that GABRP acts through ERK1/2 to regulate basal-like cytokeratin expression and enhanced BLBC cellular motility.

DISCUSSION

GABRP expression was initially reported to correlate with the BLBC subtype nearly a decade ago (7); however its functional role in this disease has not been investigated. The results reported herein reveal that *GABRP* expression not only correlates with this breast cancer subtype, but it is also highly correlated with metastatic dissemination to the brain and poorer patient outcome. These results led us to delineate the pro-tumorigenic function for GABRP using cell lines representative of the BLBC molecular subtype. Through lentiviral knockdown, we generated stable populations of two BLBC cell lines with greatly reduced levels of *GABRP*. Use of these lines revealed that GABRP is required for maintaining basal-like cytokeratin expression, ERK1/2 phosphorylation and the pro-migratory

phenotype of BLBC cells. Small molecule inactivation of ERK1/2 recapitulated this phenotype suggesting GABRP mediates basal-like intermediate filament expression and cellular motility through ERK1/2 signaling.

Our observation that GABRP functions in BLBC migration is supported by previous reports indicating GABA(A) receptor signaling is pro-migratory in cancer cell lines (17–19). Interestingly, the studies evaluating propofol-induced migration in breast cancer used the MDA-MB-468 cell line. Although these cells are characterized as basal A (31, 32), they have relatively low *GABRP* mRNA compared with other basal A lines (Fig. 2). While it is possible that this cell line expresses GABRP protein in the absence of high levels of mRNA, it is more likely that propofol induces migration of these cells through GABA(A) receptors lacking the pi subunit. Alternatively, propofol has been reported to inhibit sodium channels as well as impact endocannabinoid metabolism, and these pathways may also contribute to its effects on MDA-MB-468 cells (44, 45). Our studies reveal that HCC1187 cells do not mobilize calcium in response to GABA stimulation suggesting that GABRP may act in a ligand-independent manner, or that these cells intrinsically produce high quantities of GABA. While we cannot exclude the possibility that the GABRP-containing GABA(A) receptors in the HCC1187 and HCC70 cells would induce calcium flux in response to propofol or allopregnanolone, another GABA(A) receptor agonist, our findings reveal a previously unrecognized requirement for the pi subunit of the GABA(A) receptor in the absence of exogenous ligand for maintaining the migratory phenotype of BLBC. There is substantial evidence supporting aberrant GABAergic signaling in carcinogenesis (reviewed in Ref. 46), thus, future studies should delineate how differential GABA(A) isoform expression correlates with ligand dependence and resulting tumorigenicity. These studies will be critical to explain why GABA is pro-migratory in certain cancer cells (17, 18), but may be anti-migratory in others (23).

The decreased migration observed in GABRP-silenced BLBC cells is concomitant with diminished ERK1/2 activity and basal-like cytokeratin expression. Moreover, *KRT5*, *KRT6B*, *KRT14*, and *KRT17* positively correlate with *GABRP* in 24 patient datasets, as well as in a previously described correlation analysis of a single patient cohort (7). The cytokeratins, along with vimentin, comprise the intermediate filament protein family. The cytoskeleton is comprised of a combination of intermediate filaments, actin microfilaments and microtubules and alterations in any of these elements can affect cellular motility (47, 48). Specifically, cytokeratins have been implicated in the migratory potential of multiple cancer types, including breast (33, 34, 49). However, to our knowledge, GABAergic signaling has not previously been shown to regulate intermediate filament expression. In contrast, ERK1/2 is an established regulator of both cellular motility (33, 39, 40) and cytokeratin expression (34, 41). GABA induces ERK1/2 activation in pancreatic (10) and renal cancers (21), hence we postulated that GABRP stimulates BLBC motility through ERK1/2 regulation of basal-like cytokeratin expression. Indeed, treatment with an ERK1/2 inhibitor recapitulated the decrease in migration and cytokeratin expression observed with diminished GABRP. While beyond the scope of the current study, identification of the signaling intermediates between GABRP and ERK1/2 activation will be necessary to fully characterize this novel signaling pathway. It will also be important to determine if the changes in cytokeratin expression are due to phosphorylation by ERK1/2, which has been shown for other intermediate filaments (50). Regardless, our findings reveal a functional GABRP-ERK1/2-cytokeratin axis in BLBC, uncovering the basis for the simultaneous correlation of GABRP, ERK1/2 activity and basal-like cytokeratins with this breast cancer subtype.

BLBCs are intrinsically aggressive and women with BLBC have an increased propensity to develop visceral metastases including lesions in the brain (3–6). Following Paget's seed/soil hypothesis, our studies suggest that GABRP expression in BLBC may facilitate disseminated cells preferential homing to the central nervous system where GABA is most concentrated. Taken together with the correlation of *GABRP* with the BLBC subtype, metastatic spread to the brain, overall patient survival, and the migratory ability of BLBC cells described herein, GABRP may be a viable therapeutic target for treating BLBC. Supporting this possibility, *GABRP* is consistently detected in circulating breast tumor cells (51–54) and in lymph nodes isolated from breast cancer patients (55). Targeting GABRP should be achievable because it is a cell surface receptor subunit. Furthermore, systemic GABRP inhibition is likely to display minimal neurotoxicity due to its decreased abundance in neuronal tissues relative to other organs. That said, a GABRP-specific inhibitor will require negligible cross reactivity with the other GABA(A) receptor isoforms to minimize off-target side effects. Testing the full impact of GABRP's significance in metastatic progression awaits the development of BLBC-specific *in vitro* model systems, which will provide the crucial next step in evaluating the function and clinical utility of GABRP targeting in BLBC.

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