γ-Aminobutyric Acid (GABA) Stimulates Pancreatic Cancer Growth through Overexpressing $GABA_A$ Receptor π Subunit

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Abstract

γ-Aminobutyric acid (GABA) functions primarily as an inhibitory neurotransmitter in the mature central nervous system, and GABA/GABA receptors are also present in nonneural tissues, including cancer, but their precise function in nonneuronal or cancerous cells has thus far been poorly defined. Through the genome-wide cDNA microarray analysis of pancreatic ductal adenocarcinoma (PDAC) cells as well as subsequent reverse transcription-PCR and Northern blot analyses, we identified the overexpression of GABA receptor π subunit (GABRP) in PDAC cells. We also found the expression of this peripheral type $GABA_A$ receptor subunit in few adult human organs. Knockdown of endogenous *GABRP* expression in PDAC cells by small interfering RNA attenuated PDAC cell growth, suggesting its essential role in PDAC cell viability. Notably, the addition of GABA into the cell culture medium promoted the proliferation of GABRP-expressing PDAC cells, but not GABRP-negative cells, and $GABA_A$ receptor antagonists inhibited this growth-promoting effect by GABA. The HEK293 cells constitutively expressing exogenous GABRP revealed the growth-promoting effect of GABA treatment. Furthermore, GABA treatment in GABRP-positive cells increased intracellular Ca^{2+} levels and activated the mitogen-activated protein kinase/extracellular signal–regulated kinase (MAPK/Erk) cascade. Clinical PDAC tissues contained a higher level of GABA than normal pancreas tissues due to the up-regulation of glutamate decarboxylase 1 expression, suggesting their autocrine/paracrine growth-promoting effect in PDACs. These findings imply that GABA and GABRP could play important roles in PDAC development and progression, and that this pathway can be a promising molecular target for the development of new therapeutic strategies for PDAC. [Cancer Res 2007;67(20):9704–12]

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer death in the western world and reveals the worst mortality among common malignancies, with a 5-year survival rate of only 5% (1, 2). In 2007, it is estimated that 37,170 new cases of pancreatic cancer will be diagnosed and a roughly equal number of deaths will be attributed to pancreatic cancer in the United States (3). The majority of patients with PDAC are diagnosed at an advanced stage, for which no effective therapy is available at present. Although surgical resection offers only a little possibility for cure, 80% to 90% of patients with PDAC who undergo curative surgery die from their disseminated or metastatic diseases (1, 2). Recent advances in surgery and chemotherapy including 5-fluorouracil or gemcitabine, with or without radiation, could improve the patients' quality of life (1, 2), but those treatments have a very limited effect on the long-term survival of patients with PDAC due to its extremely aggressive and chemoresistant nature. Hence, the management of most patients is focused on palliative measures (1).

To overcome this dismal situation, the development of novel molecular therapies against good molecular targets is an urgent issue. Toward this direction, we previously generated detailed expression profiles of PDAC cells using our genome-wide cDNA microarrays consisting of ~30,000 genes, in combination with laser microbeam microdissection to enrich populations of cancer cells as much as possible (4). Among genes that overexpressed in PDAC cells, here, we focused on one peripheral type γ-aminobutyric acid (GABA) receptor subunit, the π subunit (GABRP), as a novel molecular target for this disease. The $GABA_A$ receptor is a multisubunit chloride channel that mediates the fastest inhibitory synaptic transmission in the mature central nervous system (CNS). It consists mainly of α, β, and γ units; six α subunits, three β subunits, and three γ subunits have thus far been reported. Atypical GABRP can assemble with these known $GABA_A$ receptor subunits and the presence of this subunit may alter the sensitivity of $GABA_A$ receptors to GABA or modulator agents (5). Although GABA primarily functions as an inhibitory neurotransmitter in the mature CNS, it can also act as a trophic factor during CNS development to modulate the proliferation, migration, and differentiation of neuronal cells (6–8). GABA and $GABA_A$ receptors are also present and function in peripheral tissues other than the CNS, but their precise function in nonneuronal cells, including cancerous cells, is poorly defined at present.

In this study, we report GABRP overexpression in PDAC cells and show that GABA and $GABA_A$ receptors associated with GABRP are involved in promoting cancer cell growth through an increase of intracellular Ca^{2+} level and activation of the mitogen-activated protein kinase (MAPK) / extracellular signal–regulated kinase (Erk) cascade, implicating that the GABA pathway could be a promising molecular target for the development of a novel treatment for PDAC.

Materials and Methods

Cell lines. PDAC cell lines KLM-1, SUIT-2, KP-1N, PK-1, PK-45P, and PK-59 were provided from Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). MIAPaCa-2 and Panc-1 were purchased from the American Type Culture Collection. Flp-In-293 cells were purchased

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from Invitrogen. The cell lines KLM-1, SUIT-2, PK-1, PK-45P, PK-59, and Panc-1 were grown in RPMI 1640 (Sigma-Aldrich), whereas MIAPaCa-2 and Flp-In-293 cells were grown in DMEM (Sigma-Aldrich), all with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic solution (Sigma-Aldrich). Cells were maintained at 37°C in an atmosphere of humidified air with 5% CO_2.

Semiquantitative RT-PCR. Purification of PDAC cells and normal pancreatic ductal epithelial cells from frozen PDAC tissues was described previously (4). RNA from the purified PDAC cells and normal pancreatic ductal epithelial cells were subjected to two rounds of RNA amplification using T7-based *in vitro* transcription (Epicentre Technologies). Total RNA from human PDAC cell lines were extracted using Trizol reagent (Invitrogen) according to the manufacturer's recommendations. Extracted RNA were treated with DNase I (Roche Diagnostic) and reverse-transcribed to single-stranded cDNA using oligo(dT) primer with Superscript II reverse transcriptase (Invitrogen). We prepared appropriate dilutions of each single-stranded cDNA for subsequent PCR amplification by monitoring tubulin α (*TUBA*) as a quantitative control. The sets of primer sequences were 5′-AAGGATTATGAGGAGGTTGGTGT-3′ and 5′-CTTGGGTCTGTAACAAAGCATTC-3′ for *TUBA*, 5′-CTCTCCAAATCCAGCCAGAG-3′ and 5′-ATGATTGGCTCATACAACCACA-3′ for *GABRP*, 5′-TGCATTTGTGAGCCAAAGAG-3′ and 5′-CCTTAGGTTTCAGCTAAGCGAG-3′ for glutamate decarboxylase 1 (*GAD1*), 5′-ATGGACAAAGAAGGCACAGG-3′ and 5′-GTTGGGGGAATGTTGATGTC-3′ for *GAD2*. All reactions involved initial denaturation at 94°C for 2 min followed by 23 cycles (for *TUBA*), 28 cycles (for *GABRP*), or 35 cycles (for *GAD1* and *GAD2*) at 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min on a GeneAmp PCR system 9700 (PE Applied Biosystems).

Northern blotting analysis. One microgram of poly(A)+ RNAs from seven PDAC cell lines (KLM-1, PK-59, PK-45P, MIAPaCa-2, Panc-1, PK-1, and SUIT-2) and six adult normal tissues (heart, lung, liver, kidney, brain, and pancreas; from BD Bioscience) were blotted onto the membrane. This Northern blot membrane and human MTN blot membrane (Multiple Tissue Northern blot; BD Bioscience) were hybridized for 16 h with ^{32}P-labeled GABRP probe, which was labeled using Mega Label kit (GE Healthcare). Probe cDNA of *GABRP* was prepared as a 958-bp PCR product by using primers 5′-AAGGACTCTGAGGCTTTATTCCC-3′ and 5′-ATGATTGGCTCATACAACCACA-3′. Prehybridization, hybridization, and washing were done according to the instructions of the manufacturer. The blots were autoradiographed at −80°C for 10 days.

Small interfering RNA–expressing vectors specific to *GABRP* and *GAD1*. To knock down endogenous *GABRP* or *GAD1* expression in PDAC cells, we used psiU6BX3.0 vector for the expression of short hairpin RNA against a target gene as described previously (9). The U6 promoter was cloned upstream of the gene-specific sequence (19-nt sequence from the target transcript, separated from the reverse complement of the same sequence by a short spacer, TTCAAGAGA), with five thymidines as a termination signal and a neo cassette for selection by Geneticin (Sigma-Aldrich). The target sequences for *GABRP* were 5′-ACCAGCGACAAGTTCAAGT-3′ (si-pi1), 5′-GATGGGCAGGATTGTTGAT-3′ (si-pi2), 5′-AGGAAGTAGAAGAAGTCAG-3′ (si-pi3), and 5′-GAAGCAGCACGACTTCTTC-3′ (si-EGFP) as a negative control. The target sequences for *GAD1* were 5′-CCTTTGGTTGCATGTCGA-3′ (si-G1), 5′-GTTCTGGCTGATGTGGAAA-3′ (si-G2), 5′-GGGGACAAGGCCAACTTCT-3′ (si-G3), and 5′-GAAGCAGCACGACTTCTTC-3′ (si-EGFP) as a negative control. Human PDAC cell lines, PK-45P and KLM-1, were plated onto glass coverslips within 10 cm dishes, and transfected with these small interfering RNA (siRNA) expression vectors using FuGENE6 (Roche) according to the instructions of the manufacturer, followed by 500 μg/mL of Geneticin selection. The cells from 10 cm dishes were harvested 7 days later to analyze the knockdown effect on *GBARP* or *GAD1* by RT-PCR using the above primers. After culturing in appropriate medium containing Geneticin for 2 weeks, the cells were fixed with 100% methanol, stained with 0.1% of crystal violet/water for colony formation assay. In 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, cell viability was measured using Cell counting kit-8 (DOJINDO) at 6 days after the transfection. Absorbance was measured at 490 nm, and at 630 nm as reference, with a Microplate Reader 550 (Bio-Rad).

GABA and GABA receptor agonist/antagonists treatment and PDAC cell proliferation. GABRP-positive cell lines, KLM-1 and PK-45P, and GABRP-negative cell lines, PK-59 and KP-1N, were incubated with GABA (Sigma-Aldrich) or GABA receptor agonist Muscimol (Sigma-Aldrich) at serial concentration (0, 1, 10, 100 μmol/L) in appropriate medium supplemented with 1% FBS for 6 days. To inhibit the GABA-mediated pathway, cells were incubated with 250 μmol/L of $GABA_A$ receptor antagonist bicuculline methiodide (BMI; Sigma-Aldrich) or 1 mmol/L of $GABA_B$ receptor antagonist CGP-35348 (Sigma-Aldrich). After 6 days of exposure to either of these drugs, cell viability was measured by MTT assay as described above.

Establishment of GABRP-HA/HEK293 cells and growth assay. Full-length *GABRP* cDNA (accession no. NM_014211) was amplified by using the primer pair with restriction enzyme sites; 5′-CGGGATCCATGAACTACAGCCTCCACTTG-3′ and 5′-CCGCTCGAGTCAAGCGTAGTCTGGGACGTCGTATGGGTAAAAATACATGTAGTATGCCCA-3′, which contained *Bam*HI and *Xho*I restriction sites indicated by the first and second underlines, respectively. The product was inserted into the *Bam*HI and *Xho*I sites of pcDNA 5/FRT (Invitrogen) to express a HA-tagged GABRP protein. Then the pOG44 plasmid and the pcDNA5/FRT-GABRP vector or mock vector was cotransfected into the Flp-In-293 cells. Cells were selected with appropriate medium containing 0.2 mg/mL of hygromycin B (Invitrogen) for 2 weeks, and Western blot analysis using the membrane fraction of the selected clones confirmed GABRP-HA protein expression. The membrane fractions were isolated by differential centrifugation with modifications to a procedure described by Chen et al. (10). Briefly, the cells were suspended in homogenization buffer [0.25 mol/L sucrose, 10 mmol/L Tris-HCl (pH 7.4), 1 mmol/L EDTA, and 0.1% protease inhibitor cocktail III (Calbiochem)] and disrupted using a Microson ultrasonic cell disruptor. Cell homogenates were centrifuged at 600 × *g* for 10 min at 4°C, the supernatant was then centrifuged at 10,000 × *g* for 4°C, the resulting supernatant was then centrifuged at 60,000 × *g* for 30 min at 4°C in Beckman TLA 100.2 rotor, and pellets were suspended in lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 1 mmol/L EDTA, 20% glycerol, and protease inhibitor cocktail III]. The protein content of each fraction was determined by Bio-Rad Protein Assay (Bio-Rad) with bovine serum albumin as a standard. Fifty micrograms of each membrane fraction was resolved on a 10% polyacrylamide gel and transferred electrophoretically to a polyvinylidene difluoride membrane (GE Healthcare). After blocking with 3% nonfat dry milk in TBS-T, the membrane was incubated with anti-HA high-affinity antibody (3F10, Roche) for 1 h at room temperature and anti-rat IgG-HRP antibodies (Santa Cruz Biotechnologies) for 1 h at room temperature. After washing with TBS-T, the reactants were developed using the ECL Plus Western Blotting Detection System (GE Healthcare). The loading quantity of the membrane fraction was evaluated by anti–E-cadherin antibody (BD Biosciences). Proliferation of HEK293 cells that stably expressed GABRP (GABRP-HA/HEK293) or those transfected with empty pcDNA 5/FRT (Mock/HEK293) were examined by MTT assay. GABRP-HA/HEK293 or Mock/HEK293 cells (4,000 cells/well) were seeded on a 24-well plate, and 48 h after seeding, medium was changed to DMEM with 2% FBS in the presence or absence of 100 μmol/L of GABA. The MTT assay was done every 24 h for 5 days, using the Cell counting kit-8 described above. Cell proliferation activity was also evaluated by (BrdU) incorporation assay. Cells were seeded onto a 96-well plate (3,000 cells/well). After incubation for 48 h, medium was changed to 2% FBS medium with GABA (0, 1, 10, 100 μmol/L) in the presence or absence 100 μmol/L of BMI, and cells were cultured for 48 h. BrdU incorporation was measured using cell proliferation ELISA, version 2 (GE Healthcare) according to the manufacturer's instructions.

Intracellular calcium detection. GABRP-HA/HEK293 and Mock/HEK293 cells were incubated in 5 μmol/L of Fura-2 (Molecular Probes) dissolved in Krebs buffer (125 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $MgSO_4$, 1 mmol/L KH_2PO_4, 6 mmol/L glucose, 25 mmol/L HEPES, 2 mmol/L $CaCl_2$; pH 7.4) for 45 min at 37°C. Then the cells were washed and harvested with Krebs buffer. A CAF-110 Intracellular Ion Analyzer (Jasco Corp.) was used to measure the Fura-2 fluorescence emission (11). Changes in $[Ca^{2+}]i$ in KLM-1 cells were measured by the Fura-2 method as

described above with minor modifications. The culture medium of cells grown on 35 mm of poly-L-lysine–coated glass-bottomed dish (MATSUNAMI) was replaced with HBSS (Life Technologies) with 20 mmol/L of HEPES. The cells were loaded with Fura-2 by incubation with 5 μmol/L of Fura-2 at 37°C for 45 min with or without 100 μmol/L of picrotoxin (Sigma-Aldrich) or 10 μmol/L of nifedipine (Sigma-Aldrich), and pulsed with 100 μmol/L of GABA. Measurements were carried out at room temperature using an inverted fluorescence microscope (Ix70, Olympus) and bandpass filters of 340 and 380 nm wavelengths. Image data were analyzed using a Ca^{2+} analyzing system (Aquacosmos/Ratio, Hamamatsu Photonics).

MAPK/Erk cascade evaluation. To assess the activity of the MAPK/Erk cascade, KLM-1 cells were seeded onto six-well microtiter plates (2.5×10^5 cells/well). After 24 h of preincubation, the medium was replaced with serum-free medium. On the next day, the cells were maintained in medium containing 100 μmol/L of GABA with or without 100 μmol/L of picrotoxin or 10 μmol/L of nifedipine for 6 h, then washed with ice-cold PBS, and harvested in a lysis buffer containing 500 mmol/L of Tris-HCl (pH 7.4), 150 mmol/L of NaCl, 0.25% deoxycholic acid, 1% NP40, 1 mmol/L of EDTA, 1 mmol/L of NaF, 1 mmol/L of $NaVO_4$, and 1 mmol/L of phenylmethylsulfonyl fluoride. Samples were centrifuged and the pellets were discarded. After 10% SDS-PAGE, the proteins were subjected to Western blot analysis using phosho-Erk1/2 (Thr^{203}/Tyr^{204})–specific antibody (Cell Signaling Technology). The amount of each sample was normalized by total Erk1/2 protein level by using Erk1/2 antibody (Cell Signaling Technology).

GABA content in PDAC tissues and normal pancreas. Fresh human PDAC tissues and normal pancreas tissues were obtained from surgical specimens that were resected in Osaka Medical Center for Cancer and Cardiovascular Diseases under the appropriate informed consent. The frozen tissue samples were disrupted using a mortar and dissolved with cold methanol. Suspended samples were homogenized by sonication and centrifuged at 15,000 rpm for 15 min at 4°C. Then the supernatants were subjected to measurement of GABA contents. GABA measurement was carried out by a high-performance liquid chromatography method with fluorimetric detection using *O*-phthalaldehyde (12). Pellets were dried up using centrifugal concentrator and its dry weight was measured.

Results

Overexpression of *GABRP* in PDAC cells. Among dozens of transactivated genes that were screened by our genome-wide cDNA microarray analysis of PDAC cells (4), we focused on *GABRP* for this study. *GABRP* overexpression was confirmed by RT-PCR in five of the nine microdissected PDAC cell populations (Fig. 1*A*). Northern blot analysis using a *GABRP* cDNA fragment as the probe identified an ~4.0 kb transcript specifically in the trachea, prostate, and stomach but no expression was observed in any other organs including lung, heart, liver, kidney, and brain (Fig. 1*B*). We also examined *GABRP* expression in several PDCA cell lines and found its expression evidently in KLM-1, PK-45P, and PK-1 cells but not in other PDAC cell lines (Fig. 1*C*).

Effect of *GABRP*-siRNA on the growth of PDAC cells. To investigate the biological significance of GABRP overexpression in PDAC cells, we constructed three siRNA expression vectors (si-pi1, si-pi2, and si-pi3) specific to *GABRP* transcripts and transfected them into KLM-1 or PK-45P cells that endogenously expressed high levels of *GABRP* as shown in Fig. 1*C*. A knockdown effect was observed by RT-PCR when we transfected si-pi2 or si-pi3, but not si-pi1 or a negative control si-EGFP (Fig. 2*A, left*). Colony formation and MTT assays (Fig. 2*B* and *C, left*) using KLM-1 revealed a drastic reduction in the number of cells transfected with si-pi2 and si-pi3, compared with si-pi1 and si-EGFP for which no knockdown effect was observed. Similar results were obtained when we transfected these siRNA expression vectors into PK-45P cells (Fig. 2*A, B,* and *C, right*).

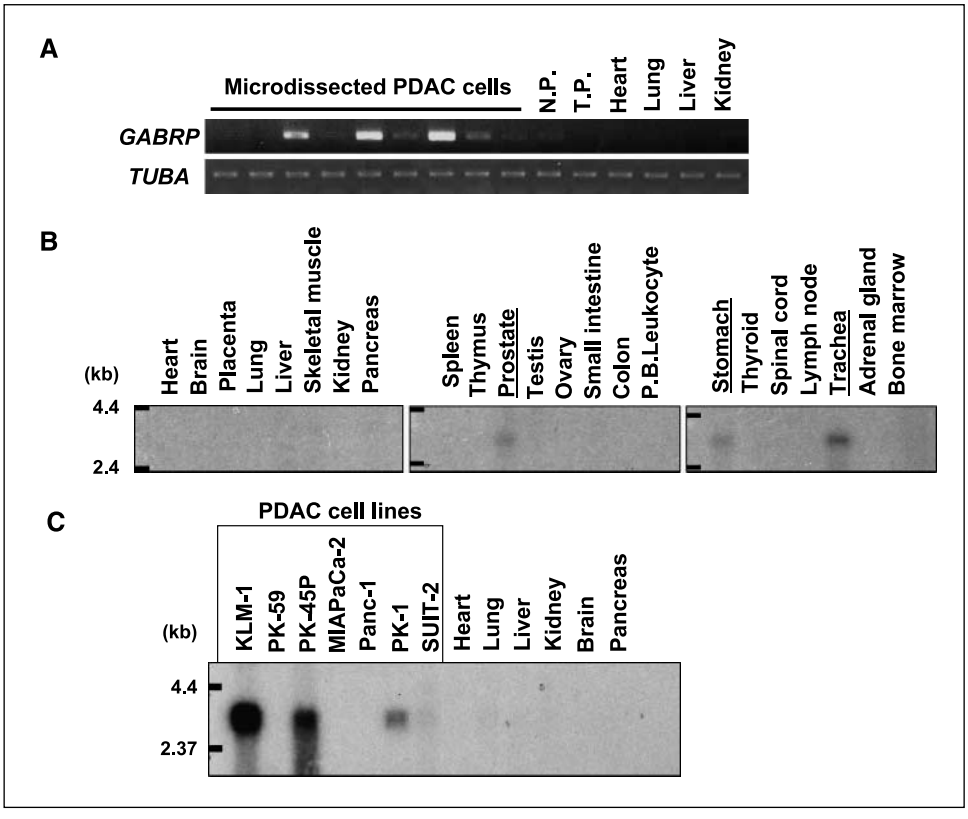

Figure 1. *GABRP* overexpression in PDAC cells. *A,* semiquantitative RT-PCR validated that *GABRP* expression was overexpressed in the microdissected PDAC cells (*lanes 1–9*), compared with normal pancreatic ductal cells which were also microdissected (*N.P.*), whole normal pancreatic tissue (*T.P.*), and vital organs (*heart, lung, liver,* and *kidney*). Expression of *TUBA* served as the quantitative control. *B,* MTN blot analysis for *GABRP* expression showed a faint band at the trachea, prostate, and stomach among the adult human organs. *C,* Northern blot analysis for *GABRP* expression showed that several PDAC cell lines (KLM-1, PK-45P, and PK-1) strongly expressed *GABRP*, whereas other normal adult organs did not express *GABRP*.

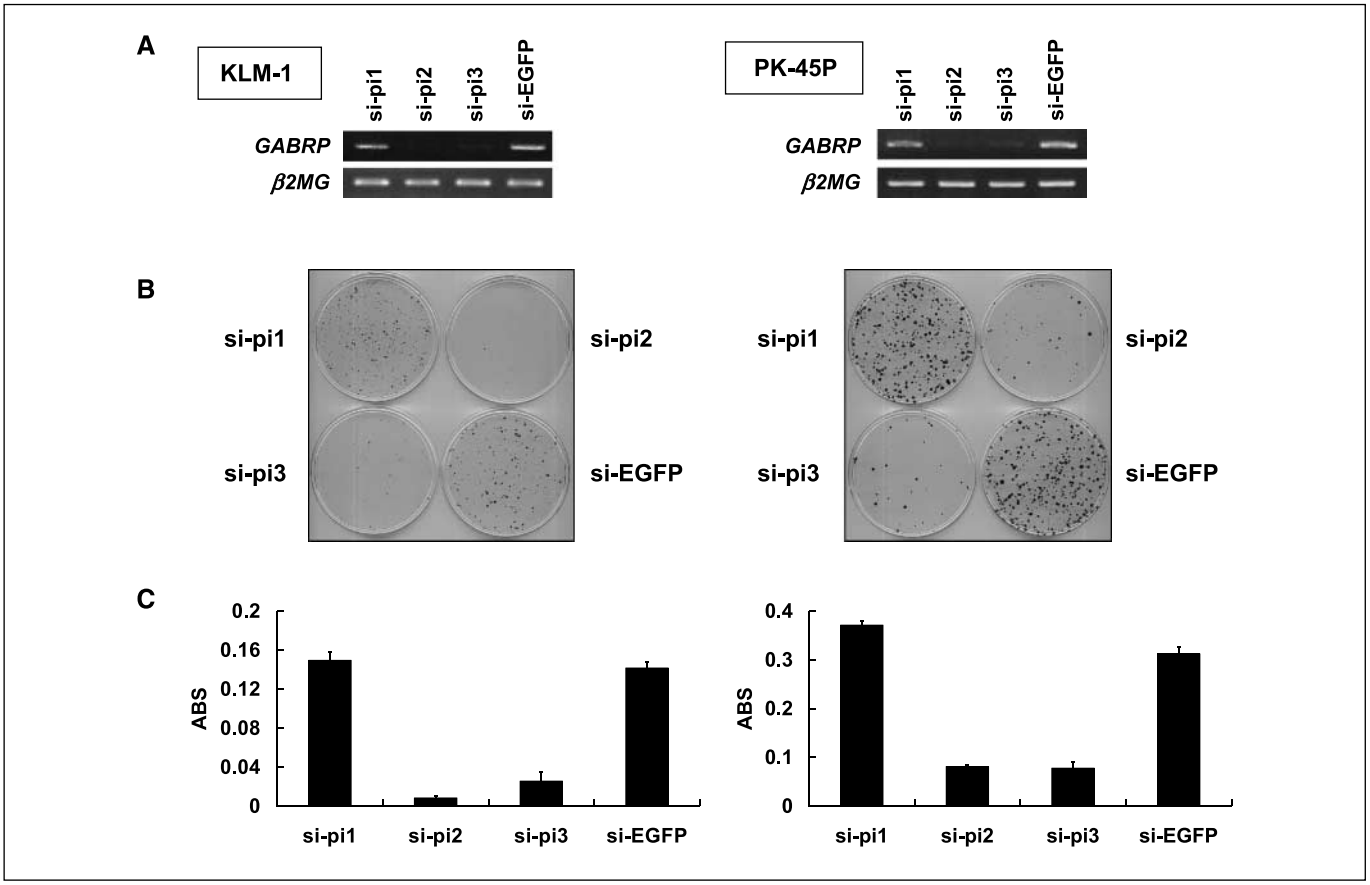

Figure 2. Effect of *GABRP*-siRNA on the growth of PDAC cells. *A,* RT-PCR confirmed the knockdown effect on *GABRP* expression by si-pi2 and si-pi3, but not by si-pi1 and a negative control si-EGFP in KLM-1 (*left*) and PK-45P (*right*) cells. *β2MG* was used to quantify RNAs. *B,* colony formation assay of KLM-1 (*left*) and PK-45P (*right*) cells transfected with each of the indicated siRNA-expressing vectors to *GABRP* (*si-pi1, si-pi2,* and *si-pi3*) and a negative control vector (*si-EGFP*). Cells were visualized with 0.1% crystal violet staining after 2 wk of incubation with Geneticin. *C,* MTT assay of each of KLM-1 (*left*) and PK-45P (*right*) transfected with the indicated siRNA-expressing vectors to *GABRP* (*si-pi1, si-pi2,* and *si-pi3*) and a negative control vector (*si-EGFP*). Each average is plotted with error bars indicating SD after 6 d incubation with Geneticin. *Y*-axis, absorbance (*ABS*) at 490 nm, and at 630 nm as reference, measured with a microplate reader. These experiments were carried out in triplicate. Transfected with si-pi2 and si-pi3 in KLM-1 cells (*left*) and PK-45P cells (*right*) resulted in a drastic reduction in the number of viable cells, compared with si-pi1 and si-EGFP for which no knockdown effect was observed ($P < 0.01$, Student's *t* test).

GABA stimulated PDAC cell proliferation through $GABA_A$ receptor. To examine the function of GABRP as a GABA receptor and the effect of GABA on the growth of *GABRP*-expressing PDAC cells, we treated *GABRP*-positive or -negative PDAC cells with GABA at several concentrations. As shown in Fig. 3*A*, the addition of GABA in the culture media enhanced the proliferation of GABRP-positive KLM-1 cells in a dose-dependent manner, although it did not enhance the proliferation of GABRP-negative PK-59 cells (Fig. 3*B*). Treatment with the GABA agonist Mucimol also promoted the proliferation of KLM-1 in a dose-dependent manner, but such an effect was not observed in GABRP-negative PK-59 cells (data not shown). Similar results were obtained when we used other GABRP-positive PK-45P cells and GABRP-negative KP-1N cells (data not shown).

Then, we treated GABRP-positive KLM-1 cells with GABA receptor antagonists in the presence or absence of GABA, and examined the growth-promoting effect of GABA. $GABA_A$ receptor antagonist BMI hammered the growth-promoting effect by GABA, but $GABA_B$ receptor antagonist CGP-35348 did not affect the growth-promoting effect by GABA (Fig. 3*C*). On the other hand, GABRP-negative PK-59 cells did not respond to $GABA_A$ or $GABA_B$ receptor antagonists (Fig. 3*D*). These findings indicated that GABA stimulated PDAC cell growth through $GABA_A$ receptors with which GABRP is likely to be incorporated.

Acquirement of GABA-dependent cell growth of HEK293 cells by introduction of exogenous GABRP. $GABA_A$ receptor in the mature CNS is a heteropentamer consisting mainly of α, β, and γ units. However, because we observed the expression of peripheral type GABRP subunit alone in PDAC cells, the mechanism of how the $GABA_A$ receptor is formed in its incorporation with GABRP in PDAC cells is completely unknown. To investigate whether only GABRP overexpression could contribute to the growth-promoting effect by GABA, we generated the HEK293-derivative clones that constitutively expressed exogenous GABRP (GABRP-HA/HEK293, C1, and C3). In the initial screening, we found that, in most of the transfected cells, the majority of exogenous GABRP was localized in the cytoplasm and only a small proportion of the protein was located in the plasma membrane. Hence, we selected the clones that constitutively expressed exogenous GABRP-HA in the plasma membrane at a relatively high level, which was examined by Western blot analysis using the plasma membrane fractions (Fig. 4*A*), and also by immunocytochemical analysis (data not shown). As shown in Fig. 4*B*, the growth rates of C1 and C3 were similar to those of the control mock-transfected clones

(Mock/HEK293, M1, and M2) in the absence of GABA. However, the treatment with GABA clearly enhanced the proliferation of C1 and C3 clones, whereas it did not enhance the growth of M1 and M2 clones. This growth-promoting effect by GABA was observed in a dose-dependent manner (Fig. 4*C*), and this growth-promoting effect was inhibited by $GABA_A$ receptor antagonist BMI (Fig. 4*D*). Taken together, our findings implicated the possibility that overexpression of GABRP alone could form $GABA_A$ receptor and contribute to the GABA-dependent growth promotion in PDAC cells.

GABA changed intracellular Ca^{2+} in *GABRP*-expressing cells. Although GABA hyperpolarizes the membrane of mature neurons, it depolarizes the membrane in immature neurons and glial tumor cells. It could also influence the proliferation or differentiation of these cells through membrane depolarization (8, 13). The GABA receptor is an ionotropic receptor permeable Cl^- ion and GABA stimulation is able to hyperpolarize or depolarize the cell, depending on the intracellular Cl^- concentration. The depolarizing action by GABA activates voltage Ca^{2+} channels, leading to an elevation of $[Ca^{2+}]i$ (6). Therefore, we characterized the intracellular Ca^{2+} response triggered by GABA by using a fluorescent Ca^{2+} indicator. In Ca^{2+} mobilization experiments, GABA treatment clearly increased $[Ca^{2+}]i$ in GABRP-HA/HEK293 cells at a concentration of 100 μmol/L (Fig. 5*A*). In contrast, GABA did not increase $[Ca^{2+}]i$ in Mock/HEK293 cells (Fig. 5*A*). These findings indicated that GABRP could form the GABA-responsive channel which could induce $[Ca^{2+}]i$. Then, we investigated Ca^{2+} mobilization in GABRP-positive PDAC cell line KLM-1 by treatment with GABA, and picrotoxin (*PTX*), or nifedipine (*NIF*). GABA treatment significantly increased $[Ca^{2+}]i$ in KLM-1 cells (Fig. 5*B, top*) as well as in GABRP-HA/HEK293 cells. This increase of $[Ca^{2+}]i$ was completely blocked by $GABA_A$ chloride channel blocker picrotoxin treatment (Fig. 5*B, middle*). This $[Ca^{2+}]i$ change triggered by GABA was also blocked by nifedipine, a voltage-gated calcium channel of the L-subtype ($VGCC_L$) blocker (Fig. 5*C, bottom*), clearly indicating that GABA increased $[Ca^{2+}]i$ in KLM-1 through the activation of $GABA_A$ receptor, in which GABRP played a key role.

GABA activated MAPK/Erk cascade through the intracellular Ca^{2+}. One of the consequences of $[Ca^{2+}]i$ involved in cell proliferation is considered to be the activation of the MAPK/Erk cascade (13). Hence, we examined the activity of the MAPK/Erk cascade in KLM-1 cells in the presence of GABA, and picrotoxin (*PTX*) or nifedipine (*NIF*). As shown in Fig. 5*C*, GABA treatment induced the phosphorylation of Erk1/2 in PDAC cells, and $GABA_A$ receptor chloride channel blocker picrotoxin inhibited this GABA-induced phosphorylation of Erk1/2. Similarly, Ca^{2+} channel blocker nifedipine also blocked GABA-induced phosphorylation of Erk1/2.

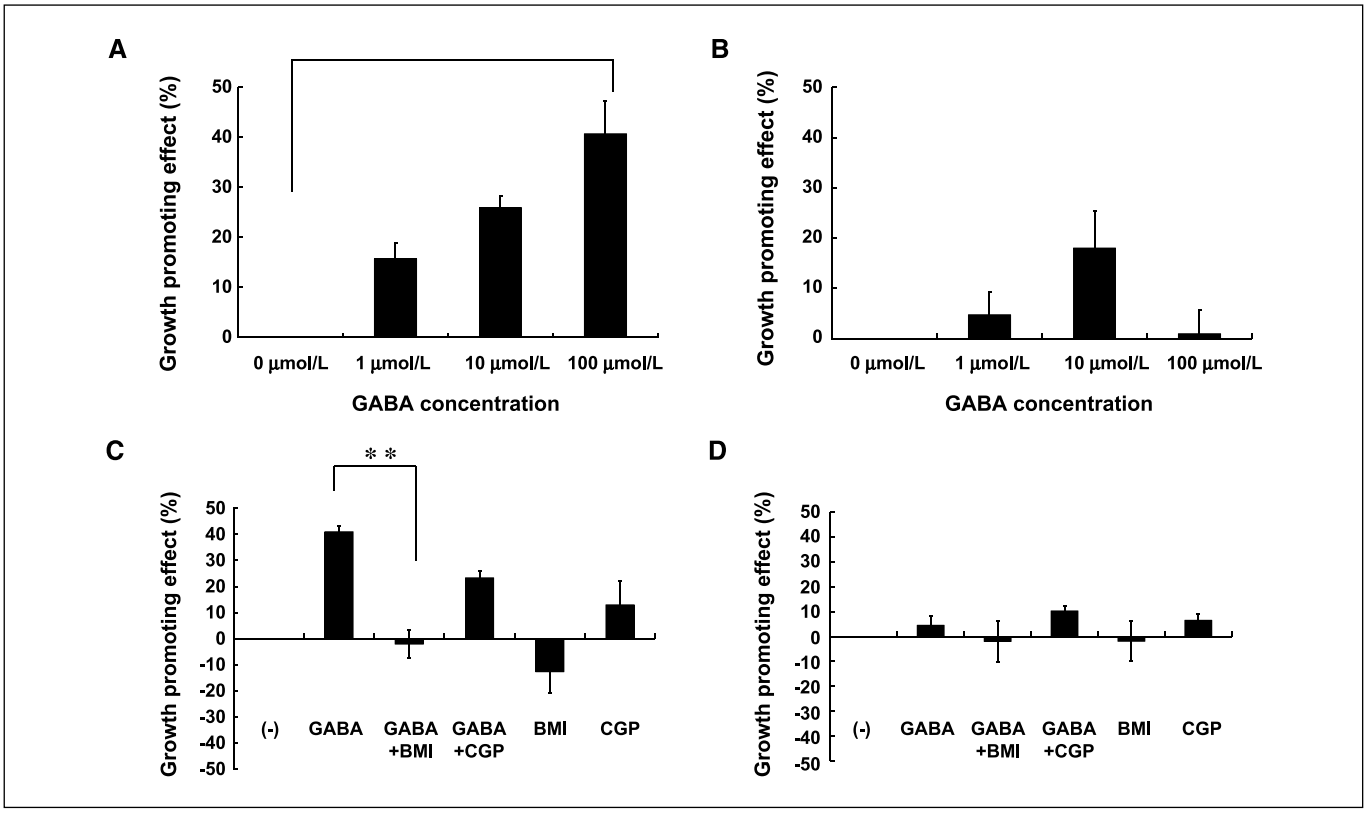

Figure 3. GABA treatment stimulated GABRP-positive PDAC cell proliferation. *A,* GABRP-positive KLM-1 cells were incubated with GABA at serial concentrations (0, 1, 10, 100 μmol/L) for 6 d, and the growth-promoting effect of each GABA concentration was shown (*Y*-axis). GABA treatment promoted the growth of GABRP-positive cells dose-dependently (*, $P < 0.01$, Student's *t* test). *B,* GABRP-negative PK-59 cells were incubated with GABA at serial concentrations (0, 1, 10, 100 μmol/L) for 6 d, and the growth-promoting effect of each GABA concentration was shown (*Y*-axis). GABA treatment did not significantly promote the growth of GABRP-negative cells. *C,* GABRP-positive KLM-1 cells were incubated with $GABA_A$ receptor antagonist BMI at 250 μmol/L or $GABA_B$ receptor antagonist CGP-35348 (*CGP*) at 1 mmol/L, in the presence or absence of 100 μmol/L of GABA, and cell viability was measured by MTT assay after 6 d of exposure. $GABA_A$ receptor antagonist BMI, but not $GABA_B$ receptor antagonist CGP-35348, hammered the growth-promoting effect by GABA (**, $P < 0.01$, Student's *t* test). *D,* GABRP-negative PK-59 cells were incubated with $GABA_A$ receptor antagonist BMI at 250 μmol/L or $GABA_B$ receptor antagonist CGP-35348 (*CGP*) at 1 mmol/L, in the presence or absence of 100 μmol/L of GABA, and cell viability was measured by MTT assay after 6 d of exposure. GABRP-negative PK-59 cells did not respond to $GABA_A$ or $GABA_B$ receptor antagonists.

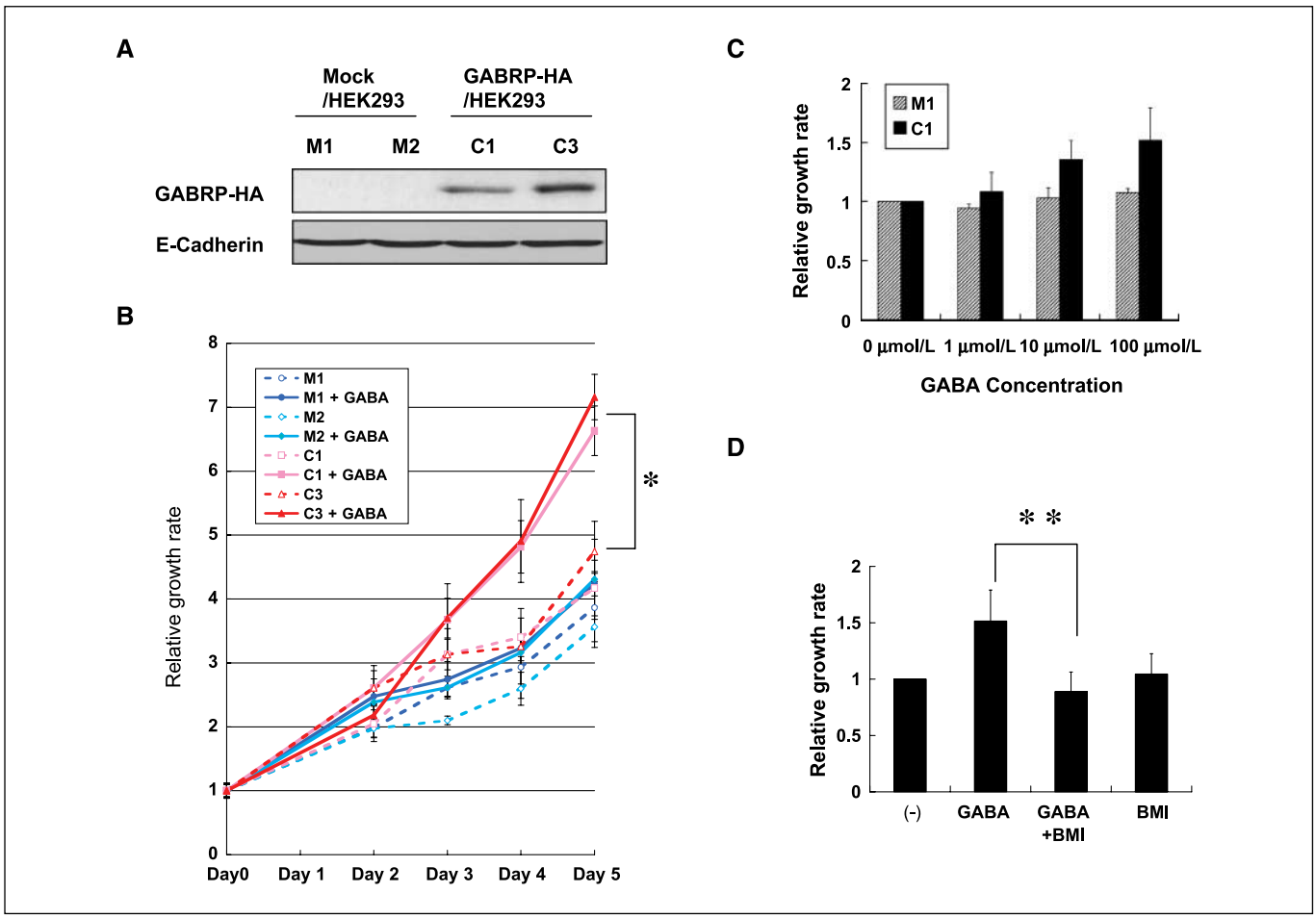

Figure 4. Growth-promoting effect of GABA on GABRP-HA/HEK293. *A,* Western blot analysis with anti–HA-tag antibody validated the exogenous HA-tagged GABRP expression at the plasma membrane fraction of C1 and C3 clones of GABRP-HA/HECK293. M1 and M2 were the clones transfected with the mock vector (*Mock/HECK293*). E-Cadherin served as a loading control of the plasma membrane fraction. *B,* GABRP-HA/HEK293 cells (*C1* and *C3*) and Mock/HEK293 cells (*M1* and *M2*) were incubated with or without 100 μmol/L of GABA, supplied with 3% FBS. *X*-axis, days after GABA treatment; *Y*-axis, relative growth rate, which was calculated in absorbance of the diameter by comparison with the absorbance value of day 0 as a control. *Points,* averages of experiments done in triplicate; *bars,* SD. The growth rates of C1 and C3 were similar to those of the controls M1 and M2 in the absence of GABA. GABA treatment stimulated the proliferation of C1 and C3 clones (*, $P < 0.01$, Student's *t* test), whereas GABA did not stimulate the growth of M1 and M2 clones. *C,* BrdU incorporation assay showed GABA treatment (1, 10, 100 μmol/L) stimulated the proliferation of GABRP-HA/HEK293 cells (*C1, closed columns*) dose-dependently, but not Mock/HEK293 cells (*M1, open columns*). *Y*-axis, relative growth rate. *D,* growth-promoting effect by 100 μmol/L of GABA on GABRP-HA/HEK293 was hammered by BMI (250 μmol/L) treatment (**, $P < 0.01$, Student's *t* test). *Y*-axis, relative growth rate.

Figure 5*D* showed the relative ratio of the phosphorylated Erk1/2 with the total Erk1/2, which were quantified by the densitometric analyses of Fig. 5*C*. These results supported the hypothesis that GABA stimulation activated the MAPK/Erk cascade through $GABA_A$ receptor activation and Ca^{2+} influx in PDAC cells.

GABA ligand content in PDAC tissues and GAD1 expression in PDAC cells. We showed that $GABA_A$ receptor involved with GABRP functioned in a growth-promoting pathway in PDAC cells. To further validate the significance of the GABA/GABA receptor pathway in PDAC, we measured GABA ligand content in 15 human PDAC tissues and 12 normal pancreatic tissues by using a high-performance liquid chromatography method with fluorimetric detection using *O*-phthalaldehyde. As a result, the mean value of GABA ligand in normal pancreatic tissues was 277.3 nmol/g of dry tissue, and none of the 12 normal pancreas tissues contained 500 nmol/g of dry tissue (Fig. 6*A*). In contrast, PDAC tissues had significantly higher content of GABA ligand (554.6 nmol/g of dry tissue, $P < 0.05$) in their mean values, and five of them contained >500 nmol/g of dry tissue of GABA, indicating that PDAC tissues were abundant in GABA ligand. GABA ligand is produced mainly by GAD1 (or GAD67) or GAD2 (or GAD65) enzymes in the CNS (14, 15), or in the islet cells in the pancreas. Our microarray analysis on PDAC cells suggested high transcriptional levels of GAD1 in PDAC cells, and we validated the expression of *GAD1* and *GAD2* by RT-PCR using the microdissected PDAC cells. As shown in Fig. 6*B, GAD1* expression was significantly up-regulated in PDAC cells, compared with that of normal pancreatic duct cells (*N.P.*) or total normal pancreas (*T.P.*). These findings implicated that PDAC cells could produce GABA ligand by themselves and GABA/GABA receptors could stimulate PDAC cell growth in an autocrine/paracrine manner.

Knocking down *GAD1*-suppressed PDAC cell viability. To investigate whether GABA production by GAD1 could stimulate PDAC cell growth in an autocrine/paracrine manner, we constructed siRNA-expressing vectors specific to *GAD1* and transfected them into GABRP-positive and *GAD1*-positive PDAC cell KLM-1. A knockdown effect was observed by RT-PCR when we transfected si-G4 and si-G3, but not si-G1 and the negative control si-EGFP

(Fig. 6*C*). Colony-formation assays (Fig. 6*D*) and MTT assays (Fig. 6*E*) revealed a drastic reduction in the number of cells transfected with si-G2 and si-G3, compared with si-G1 and a negative control si-EGFP for which no knockdown effect was observed. Similar effects were obtained with the PK-45P cell line (data not shown).

Discussions

In this study, we validated the overexpression of GABRP in nearly half of the PDACs and found that GABRP was moderately expressed in few normal organs, implicating that GABRP is a good molecular target for the development of novel PDCA therapies with a minimal risk of side effects, with regards to its expression pattern. Functional analysis using siRNA as well as exogenous introduction of GABRP strongly supported its involvement in the development and progression of PDAC. Primary, GABA and GABA receptors function as an inhibitory neurotransmitter in the mature CNS, but their precise functions in nonneuronal cells or tumor cells are unknown. Azuma et al. reported that the GABA and $GABA_B$ receptor pathway could involve prostate cancer metastasis or invasion through the regulation of metalloproteinase production (16). On the other hand, another report showed that GABA could inhibit colon cancer migration associated with the norepinephrine-induced pathway (17). Thus, it is controversial whether GABA-associated pathways could act positively or negatively in the regulation of cancer cell behavior. However, our findings in this study could clearly indicate evidence supporting the theory that GABA and $GABA_A$ receptor with GABRP promoted PDAC cell proliferation. Although GABA usually induces hyperpolarization in adult neurons, GABA has been shown to exert depolarizing responses in the immature CNS structures and CNS tumors (6, 18). In particular, GABA increased the proliferation of immature cerebellar granule cells through the activation of $GABA_A$ receptors and voltage-dependent calcium channels (8). Several pieces of evidence support the trophic action of GABA during CNS development, and the purported mediator of these trophic effects is a depolarizing response triggered by GABA, which elicits a calcium influx in immature CNS cells (19). In our study on PDAC cells, GABA-inducing Ca^{2+} influx was observed in GABRP-positive cells, but not in GABRP-negative cells. This GABA-inducing Ca^{2+}

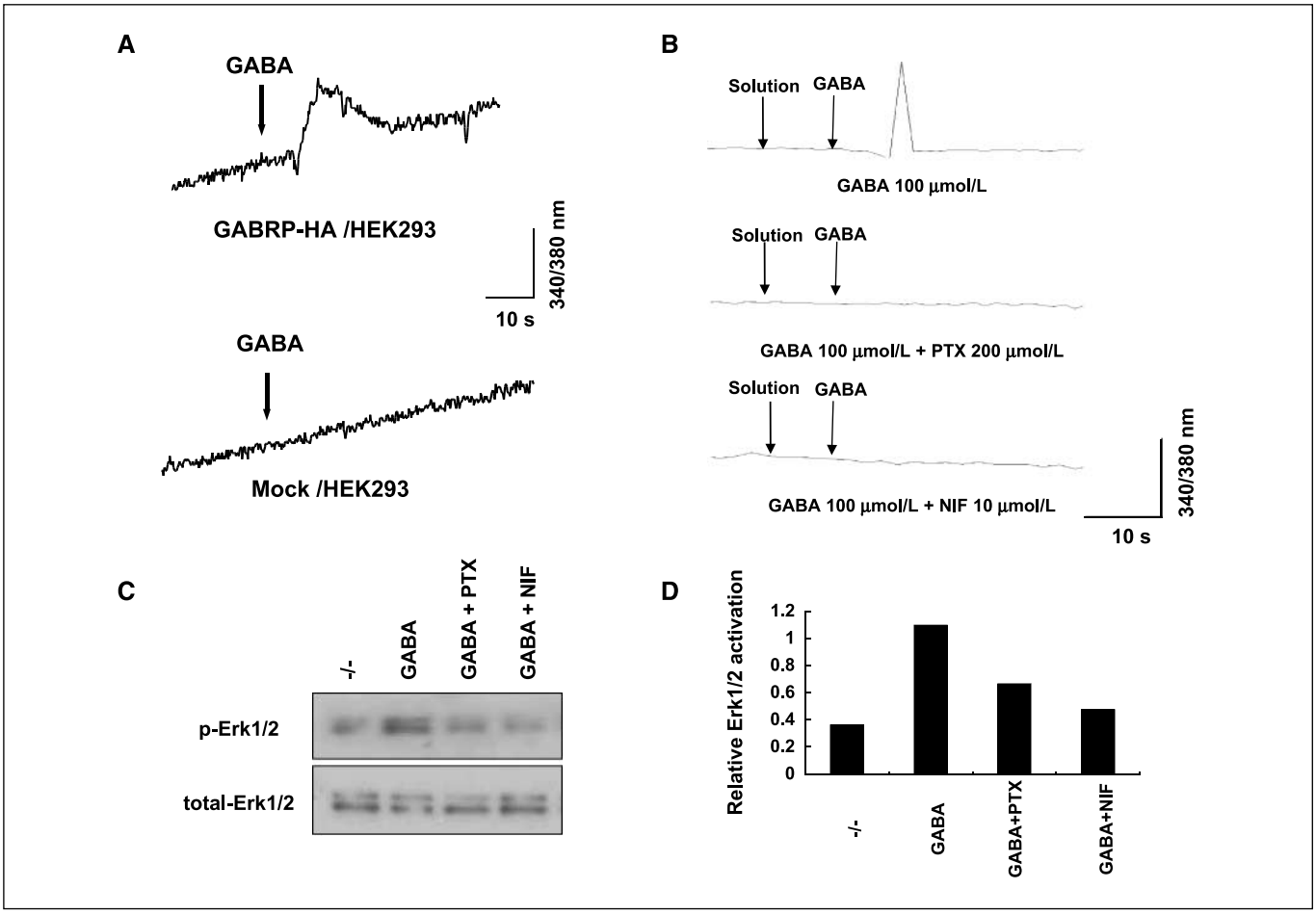

Figure 5. GABA induced Ca^{2+} influx and activated MAPK/Erk cascade. *A,* GABA treatment induced calcium mobilization in GABRP-HA/HEK293, but not in Mock/HEK293. *B,* calcium mobilization in GABRP-positive KLM-1 cells was induced by 100 μmol/L of GABA, but not with solution only. In the presence of 200 μmol/L of picrotoxin (*PTX*, GABA Cl^- channel blocker) or 10 μmol/L of nifedipine (*NIF*, Ca^{2+} channel blocker), GABA treatment did not induce calcium mobilization in KLM-1 cells. *C,* GABA treatment increased the phosphorylation level of Erk1/2 in KLM-1 cells, compared with nontreatment (−/−). In the presence of 200 μmol/L of picrotoxin (*PTX*) or 10 μmol/L of nifedipine (*NIF*), GABA treatment did not increase the phosphorylation level of Erk1/2 in KLM-1 cells. Erk1/2 was detected by Western blot analysis using the antibody specific to phosphorylated Erk1/2, and the antibody to Erk1/2 to evaluate the total level of Erk1/2 proteins. *D,* the relative ratios of phosphorylated Erk1/2 with the total levels of Erk1/2, which were quantified by the densitometric analysis of (*C*).

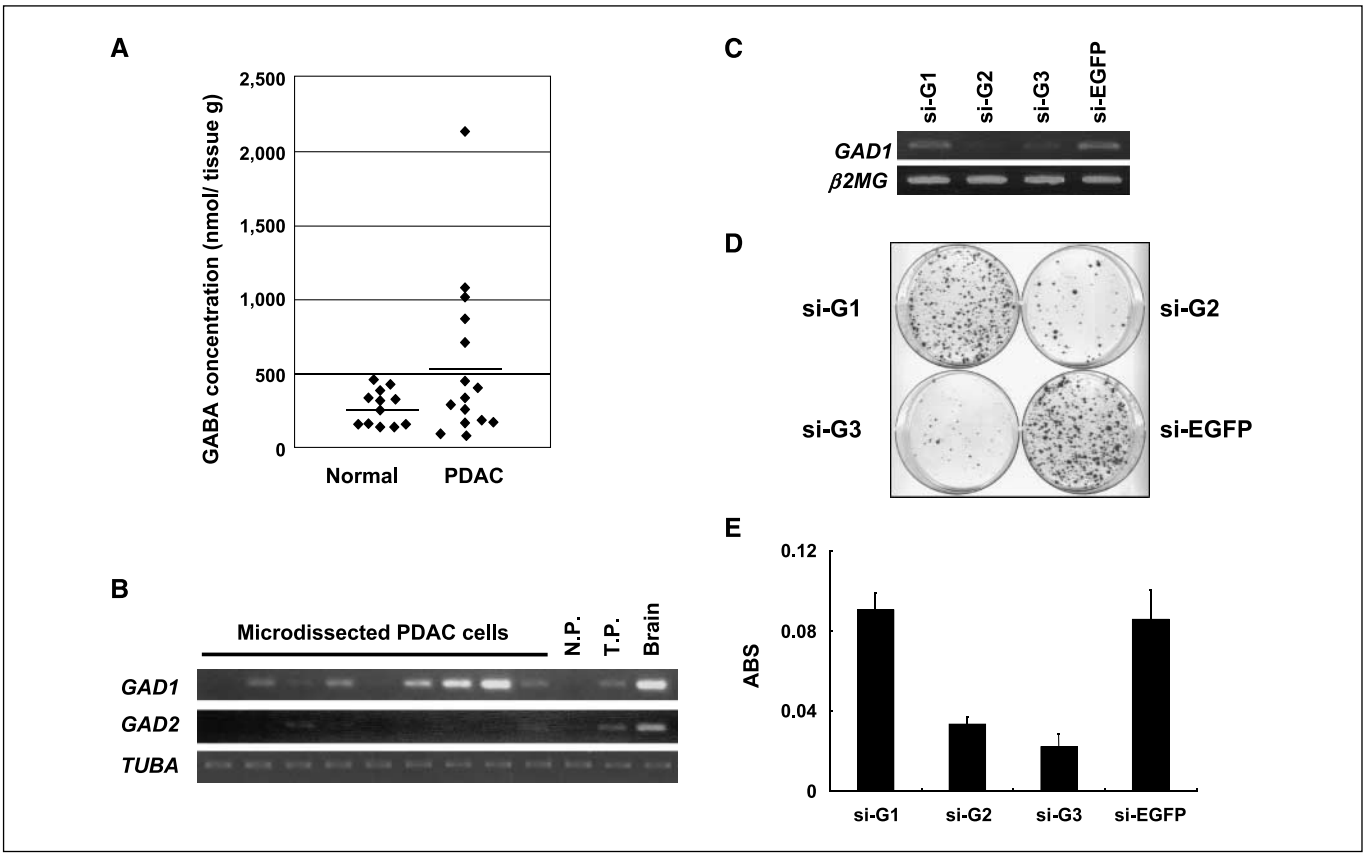

Figure 6. GABA ligand content and GAD expression in PDACs and effect of *GABRP*-siRNAs on the growth of PDAC cells. *A,* GABA ligand content in the normal pancreas tissues (n = 12) and PDAC tissues (n = 15). Normalized by dry weight of each sample. Means of GABA ligand content in PDAC tissues (554.6 nmol/g of dry tissue) were significantly higher than those in normal pancreas (277.3 nmol/g of dry tissue, Student's t test; $P < 0.05$). *B,* RT-PCR analysis of GABA-producing enzymes *GAD1* and *GAD2. GAD1* expression, but not *GAD2* expression, was up-regulated in PDAC cells compared with normal pancreatic ductal cells (*N.P.*). Expression of *TUBA* served as the quantitative control. *C,* semiquantitative RT-PCR confirmed the knockdown effect on *GAD1* expression by si-G2 and si-G3, but not by si-G1 and a negative control si-EGFP. *β2MG* was used to quantify RNAs. *D,* colony formation assay of KLM-1 cells transfected with each of indicated siRNA-expressing vectors to *GAD1* (*si-G1, si-G2,* and *si-G3*) and a negative control vector (*si-EGFP*). Cells were visualized with 0.1% crystal violet staining after 2 wk of incubation with Geneticin. *E,* MTT assay of KLM-1 cells transfected with indicated siRNA-expressing vectors to *GAD1* (*si-G1, si-G2 and si-G3*) and a negative control vector (*si-EGFP*). *Columns,* averages plotted after 2 wk of incubation with Geneticin; *bars,* SD. *Y*-axis, absorbance (*ABS*) at 490 nm, and at 630 nm as reference, measured with a microplate reader. These experiments were carried out in triplicate.

influx in PDAC cells was hammered by a $GABA_A$ receptor chloride channel inhibitor (picrotoxin) as well as a Ca^{2+} blocker (nifedipine). The significant activation of the MAPK/Erk cascade was also observed after GABA treatment on PDAC cells, and this activation by GABA was inhibited by the Ca^{2+} blocker nifedipine, as well as by the $GABA_A$ receptor chloride channel inhibitor picrotoxin. Therefore, it is evident that GABA can induce Ca^{2+} influx through GABRP-associated $GABA_A$ receptor and $VGCC_L$, and subsequently activate MAPK/Erk cascade in PDAC cells, resulting in the growth promotion of PDAC cells. Increases of the intracellular Ca^{2+} activate various signaling pathways which are essential for cell growth and survival. It was reported that Ca^{2+} influx could activate the MAPK/Erk cascade through calmodulin in neural cells and promote neural growth and synaptic plasticity (19, 20), and Ca^{2+}/calmodulin may be critical for the link between Ca^{2+} influx and the activation of the MAPK/Erk cascade in PDAC cells as well. Further studies are required to clarify this link between Ca^{2+} influx and the activation of the MAPK/Erk cascade or other growth/survival signaling pathways in PDAC cells.

$GABA_A$ receptor in the CNS forms a heteropentamer consisting mainly of $\alpha\beta\gamma$ subunits, and a few reports indicated that the GABRP subunit could assemble with these known $GABA_A$ receptor subunits, and the incorporation of GABRP into $GABA_A$ receptor altered its affinity to GABA or modulatory agents (5). In PDAC cells, however, the expressions of other main $GABA_A$ receptor subunits such as α, β, and γ subunits were very limited in our RT-PCR analysis (data not shown), and it is largely unknown how GABRP could form the $GABA_A$ receptor in PDAC cells. In this study, we established the HEK293 clones in which GABRP alone was exogenously overexpressed and found that these clones showed the intracellular Ca^{2+} change and the growth-promoting effect in response to GABA treatment, including the possibility of the homopentamer formation of GABRP as well as GABA rho subunit (21), although the possibility that other endogenous $GABA_A$ receptor subunits in HEK293 cells could form a functional heteropentamer together with overexpressing GABRP was not excluded.

We also found an abundance of GABA ligands in clinical PDAC tissues. Elevated expression of GABA-producing enzymes, GADs, was indicated in certain types of human tumors such as colon cancer, gastric cancer, and breast cancer (22–24). GAD was reported to be expressed in pancreas tissues, mainly in islet cells (25), but our study showed its up-regulation in PDAC cells. Interestingly, knocking down of GAD1 expression in PDAC cells resulted in the suppression of PDAC cell growth, similar to the

knocking down of GABRP. Therefore, it should be suggested that GABA/GABRP could function in an autocrine/paracrine manner in PDAC cells and promote cell growth.

Blocking of GABRP or GABA function on PDAC cell by small molecules or antibody can provide a promising new approach to molecular therapy for deleterious PDACs. However, the presently available $GABA_A$ inhibitors such as bicuculline and picrotoxin can affect $GABA_A$ receptor in the CNS inhibitory neurons and induce severe convulsions *in vivo* (26). Hence, to avoid the risk of severe adverse reactions, it should be a key issue to develop antagonistic drugs that are very specific to the receptor, in which GABRP is the major component, and/or ones that have no ability to penetrate the blood-brain barrier.

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