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A disintegrin and metalloproteinase 22 activates integrin β1 through its disintegrin domain to promote the progression of pituitary adenoma

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Abstract

Background. Approximately 35% of pituitary adenoma (PA) display an aggressive profile, resulting in low surgical total resection rates, high recurrence rates, and worse prognosis. However, the molecular mechanism of PA invasion remains poorly understood. Although "a disintegrin and metalloproteinases" (ADAMs) are associated with the progression of many tumors, there are no reports on ADAM22 in PA.

Methods. PA transcriptomics databases and clinical specimens were used to analyze the expression of ADAM22. PA cell lines overexpressing wild-type ADAM22, the point mutation ADAM22, the mutated ADAM22 without disintegrin domain, and knocking down ADAM22 were generated. Cell proliferation/invasion assays, flow cytometry, immunohistochemistry, immunofluorescence, co-immunoprecipitation, mass spectrometry, Reverse transcription-quantitative real-time PCR, phos-tag SDS-PAGE, and Western blot were performed for function and mechanism research. Nude mice xenograft models and rat prolactinoma orthotopic models were used to validate in vitro findings.

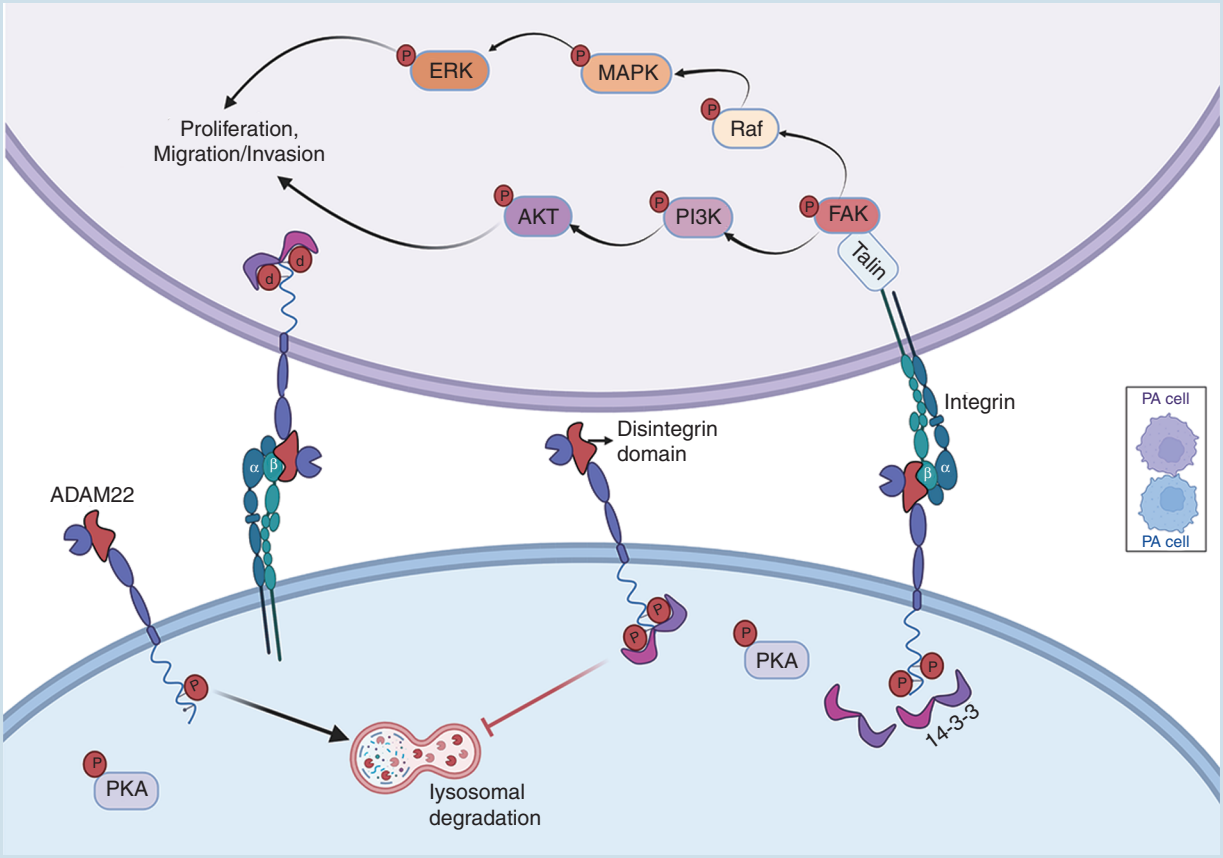
Results. ADAM22 was significantly overexpressed in PA and could promote the proliferation, migration, and invasion of PA cells. ADAM22 interacted with integrin β1 (ITGB1) and activated FAK/PI3K and FAK/ERK signaling pathways through its disintegrin domain to promote PA progression. ADAM22 was phosphorylated by PKA and recruited 14-3-3, thereby delaying its degradation. ITGB1-targeted inhibitor (anti-itgb1) exerted antitumor effects and synergistic effects in combination with somatostatin analogs or dopamine agonists in treating PA.

Conclusions. ADAM22 was upregulated in PA and was able to promote PA proliferation, migration, and invasion by activating ITGB1 signaling. PKA may regulate the degradation of ADAM22 through post-transcriptional modification levels. ITGB1 may be a potential therapeutic target for PA.

Key Points

1. A disintegrin and metalloproteinase (ADAM) 22 was upregulated in pituitary adenoma (PA) and was able to promote PA progression by activating ITGB1 signaling through its disintegrin domain.
2. Phosphorylation of ADAM22 by PKA protects ADAM22 from degradation.
3. ITGB1 may be a potential therapeutic target for PA.

Graphical Abstract



Importance of the Study

A disintegrin and metalloproteinase (ADAM) 22 was highly expressed in pituitary adenoma (PA), but its mechanism in the development of PA remains unknown. This study clarified ADAM22 interacted with ITGB1 and activated FAK/PI3K/AKT and FAK/MAPK/ERK signaling pathways through its disintegrin domain to promote PA progression. ADAM22 was phosphorylated by PKA and recruited 14-3-3, thereby delaying its degradation. ITGB1-targeted inhibitor (anti-itgb1) exerted antitumor effects and synergistic effects in combination with somatostatin analogs or dopamine agonists in treating PA.

Pituitary adenoma (PA) is the second most common intracranial tumor, accounting for 17.4% of all primary central nervous system tumors,[1] of which 35%–60% exhibit invasion into dura, bones, or adjacent structures, including the cavernous or sphenoid sinuses.[2,3] Invasive pituitary adenoma (IPA) usually has a low rate of total surgical resection and exhibits more frequent recurrence, posing a challenge to patient survival and prognosis.[3–5]

Numerous oncogenes (such as PTTG1, EGFR) and tumor suppressor genes (such as MGMT, CDKN1B) have been identified in IPA due to the development of genomic or proteomic technologies.[4,6,7] To date, cumulative studies have established that the development and progression of IPA may be due to dysregulation of cell proliferation and apoptosis, degradation of the extracellular matrix, neovascularization, and epigenetic effects.[4,6,7] However, the specific molecular mechanisms underlying PA invasion remains poorly understood.

A disintegrin and metalloproteinases (ADAMs) are a class of type I transmembrane glycoproteins containing 7 different domains, including the metalloproteinase and disintegrin domain involved in proliferation, invasion, metastasis, and chemotherapeutic drug resistance of a variety of tumors.[8–13] While ADAM22, a specific isoform of ADAMs with inactivated metalloproteinases, has been studied mainly in epilepsy and peripheral nerve diseases,[14–18] and recently there have been several reports demonstrating its high expression in breast cancer and

its promoting effect on proliferation, invasion, metastasis and chemoresistance of breast cancer cells.[19–21] However, the function of ADAM22 in IPA progression remains unknown.

Integrins are a family of heterodimeric (composed of an α-subunit and a β-subunit) transmembrane glycoprotein receptors that are widely involved in various cellular biological processes through their specific bidirectional signal transduction mechanisms (inside-out signaling and outside-in signaling) and are involved in the regulation of proliferation, invasion, migration, and angiogenesis in a variety of tumors.[22–27] Interestingly, ADAMs can also participate in tumor progression by activating the integrin signaling pathway through their disintegrin domain.[24–27] However, whether ADAM22 can affect the progression of PA by its less investigated disintegrin domain remains elusive.

In this study, we clarified the expression level of ADAM22 in PA through publicly available transcriptomic data and clinical specimen analysis, and then validated the biological function of ADAM22 as well as its downstream effect mechanism through integrin signaling and upstream regulation mechanism based on phosphorylation by PKA in vivo and in vitro experiments, and finally explored the efficacy of molecularly targeted drugs targeting integrins in PA, which provided potential therapeutical targets for IPA.

Methods and Materials

Human PA Samples

Fifty PA samples were obtained from patients who underwent surgery at the Department of Neurosurgery, Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology from July 2021 to June 2022 (Supplementary Table 1). All patient-related studies were approved by the ethical committees at Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (TJ-IRB20220325).

Cell Lines and Primary Cell Culture

GH3, AtT20, TtT/GF, 293T, and human PA primary cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. MMQ cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. See Supplementary Methods for details.

Plasmids and Transfection

The overexpression plasmids of wild-type (WT) ADAM22 (WT-ADAM22), the point mutation ADAM22 (S832A-ADAM22; S860A-ADAM22), and the mutated ADAM22 without disintegrin domain (ΔPA-ADAM22) were constructed by Tsingke (Supplementary Table 2). All target sequences were fused with His_6 tags in the 5′ terminals. The ADAM22 knockdown plasmids, ITGB1 knockdown plasmids, and select small interfering RNA (siRNA) against 14-3-3ζ were produced by Tsingke (Supplementary Table 3). See Supplementary Methods for details.

Immunohistochemistry and Immunofluorescence

Immunohistochemistry (IHC) and immunofluorescence (IF) were performed as described previously.[12] See Supplementary Methods for details.

Cell Proliferation Assay, Colony Formation Assay, Wound Healing Assay, and Cell Invasion Assay

Cell proliferation assay, colony formation assay, wound healing assay, and cell invasion assay were performed as described previously.[12,13] See Supplementary Methods for details.

Flow Cytometry

The cell cycle was investigated by using a PI Cell Cycle assay kit (Yeasen) following the manufacturer's protocol. The cell apoptosis was investigated by using an Annexin V-FITC/PI apoptosis assay kit (Yeasen) according to the manufacturer's instructions. See Supplementary Methods for details.

Reverse Transcription-Quantitative Real-Time PCR

Reverse transcription-quantitative real-time PCR was performed as described previously.[12] Primers used in this study are listed in Supplementary Table 5. See Supplementary Methods for details.

Western Blot

Western blot (WB) was performed as described previously.[12] See Supplementary Methods for details and antibodies.

Phos-Tag SDS-PAGE

Phos-tag SDS-PAGE was performed according to the manufacturer's protocols. Briefly, 25 mM Phos-tag acrylamide (Wako) and 50 mM MnCl2 were added into the 6% separating gel when preparing SDS-PAGE. After electrophoresis, the gel was washed with transfer buffer containing 10 mM EDTA for 10 minutes, and proteins were transferred to the PVDF membrane for WB.

Co-Immunoprecipitation (Co-IP)

Cells were lysed in IP assay buffer (Servicebio) containing phosphatase inhibitor and protease inhibitors on ice for 30 min and then centrifuged. The supernatants were immunoprecipitated with antibodies prebound with Protein A/G Magnetic Beads (MCE). The above immune complexes

were separated by SDS-PAGE, followed by WB with indicated antibodies. See Supplementary Methods for details.

Mass Spectrometry

Magnetic beads from Co-IP were digested by trypsin overnight at 37°C. The next day, digested eluate was purified and vacuum dried and stored at –20°C for mass spectrometry (MS) detection. MS data acquisition was carried out on a Q Exactive HF mass spectrometer. See Supplementary Methods for details.

Xenograft Models and Rat Prolactinoma Orthotopic Models In Vivo

All animal studies in this study were approved by Tongji Hospital Committee for the care of animals (process number: TJH-202206015). Subcutaneous xenograft models were performed as described previously.[12]

Intracranial pituitary tumor xenograft models were constructed by injecting PA cells into the frontal lobe base of nude mice with a stereotactic frame.

The rat prolactinoma orthotopic models were induced by 17β-estradiol (MCE), as reported by Cao et.al.[28]

Xenograft and prolactinoma orthotopic models were divided into groups according to the different treatments. The survival status of the tumor-bearing mice/rats was observed daily, and the body weight and the size of the subcutaneous tumors were measured regularly. The tumor volumes were defined as 0.5 x (longest diameter) × (shortest diameter) × (shortest diameter). After the experiment, tumors were harvested and weighed. See Supplementary Methods for details.

Statistical Analyses

If the sequencing data are from different databases, the data were integrated and then normalized using the preprocessCore package. GraphPad Prism 8.0 was used for statistical analysis. Schematic diagrams were created in Biorendor (https://app.biorender.com). An unpaired *t*-test was carried out when comparing the 2 groups. One-way ANOVA was used for multiple comparisons. The data are expressed as the mean ± standard deviation. *P* values less than .05 were considered significant (denoted as $*P < .05$, $**P < 001$, $***P < .001$, $****P < 0001$).

Results

ADAM22 was Highly Expressed in PA Samples and Promoted Proliferation, Migration, and Invasion of PA Cell Lines

Analyzing the expression pattern of the ADAMs family in 2 different publicly available transcriptomics databases (GEO database, Accession number: GSE147786/GSE51618), we identified that ADAM22 was significantly overexpressed in PAs compared to normal pituitary tissues (Figure 1A and Supplementary Figure 1A–B).To investigate the role of ADAM22 in PA, we first performed IHC of ADAM22 in 50 surgical resected PA samples and divided them into different groups according to the proliferation index (Ki-67 <3% or ≥3%; and other cutoffs of Ki-67, Supplementary Figure 1C), Knosp grade (KG, KG<III or ≥III) and tumor size (TS, TS <1cm or ≥1 cm) (Figure 1B). The results showed that tumors with high proliferation index (Ki-67 ≥3%), high (KG ≥III), and promotes or non-microadenoma group (TS ≥1 cm) exhibited higher ADAM22 expression (Figure 1C). These results suggested that ADAM22 might be associated with the progression of pituitary tumors.

To further assess the potential tumor-promoting effect of ADAM22, we next verified the basal expression level of ADAM22 in 4 common PA cell lines (GH3, MMQ, AtT20, and TtT/GF; Supplementary Figure 1D), proving that ADAM22 was highly expressed in all 4 PA cell lines relative to homologous normal pituitary which was consistent with the previous transcriptomic results. ADAM22 of PA cell lines were then knocked down or overexpressed (Supplementary Figure 1E–F). The results of the CCK-8 (Figure 1D and Supplementary Figure 2A), colony formation (Figure 1E and Supplementary Figure 2B, Ki-67 staining (Figure 1F and Supplementary Figure 2C–E), flow cytometry analysis assays (Figure 1G and Supplementary Figure 2F) confirmed that overexpression of ADAM22 significantly promoted cell proliferation, while knockdown of ADAM22 inhibited cell proliferation in PA cells. Moreover, we further performed the wound healing assay (Figure 1H) and transwell invasion assay (Figure 1I) to verify whether ADAM22 could affect PA cell migration and invasion. These results illustrated that ADAM22 knockdown notably attenuated cell migration and invasion. Xenografts in vivo (Figure 1J and Supplementary Figure 2G) and immunofluorescence of Ki-67 and CD31 (a marker of angiogenesis) staining on tumor sections (Supplementary Figure 3A–D) further verified that knockdown of ADAM22 inhibited tumor proliferation and angiogenesis. In summary, ADAM22 is highly expressed in PA and promotes the proliferation and invasion of PA cells.

ADAM22 Increased PA Progression by Interacting With ITGB1 Through Its disintegrin Domain

Next, we are eager to understand the beneath mechanisms of ADAM22-mediated PA growth. Since, PSD95, the major scaffolding protein in the postsynaptic density, and epilepsy-related secreted protein LGI1 are the 2 most common proteins that can bind to ADAM22 and participate in cell signaling transduction in CNS,[14–16,18] so we initially examined the expression levels of these 2 proteins in PAs. Unfortunately, LGI1 and PSD95 were expressed at extremely low levels in PA (Supplementary Figure 4A–C). Therefore, they may not be involved in the tumor-promoting effects of ADAM22 in PAs. Unlike other ADAMs members, such as ADAM8 or ADAM12 as we previously reported,[11–13] the metalloproteinase domain of ADAM22 is inactivated. Therefore, another important structure – the disintegrin domain raised our interest. Indeed, it has been proved that many other members of the ADAMs family (with a similar disintegrin domain to ADAM22) can promote cell proliferation and invasion by binding to integrin proteins and activating integrin-related signaling pathways.[24,26,27] To verify this potential interaction

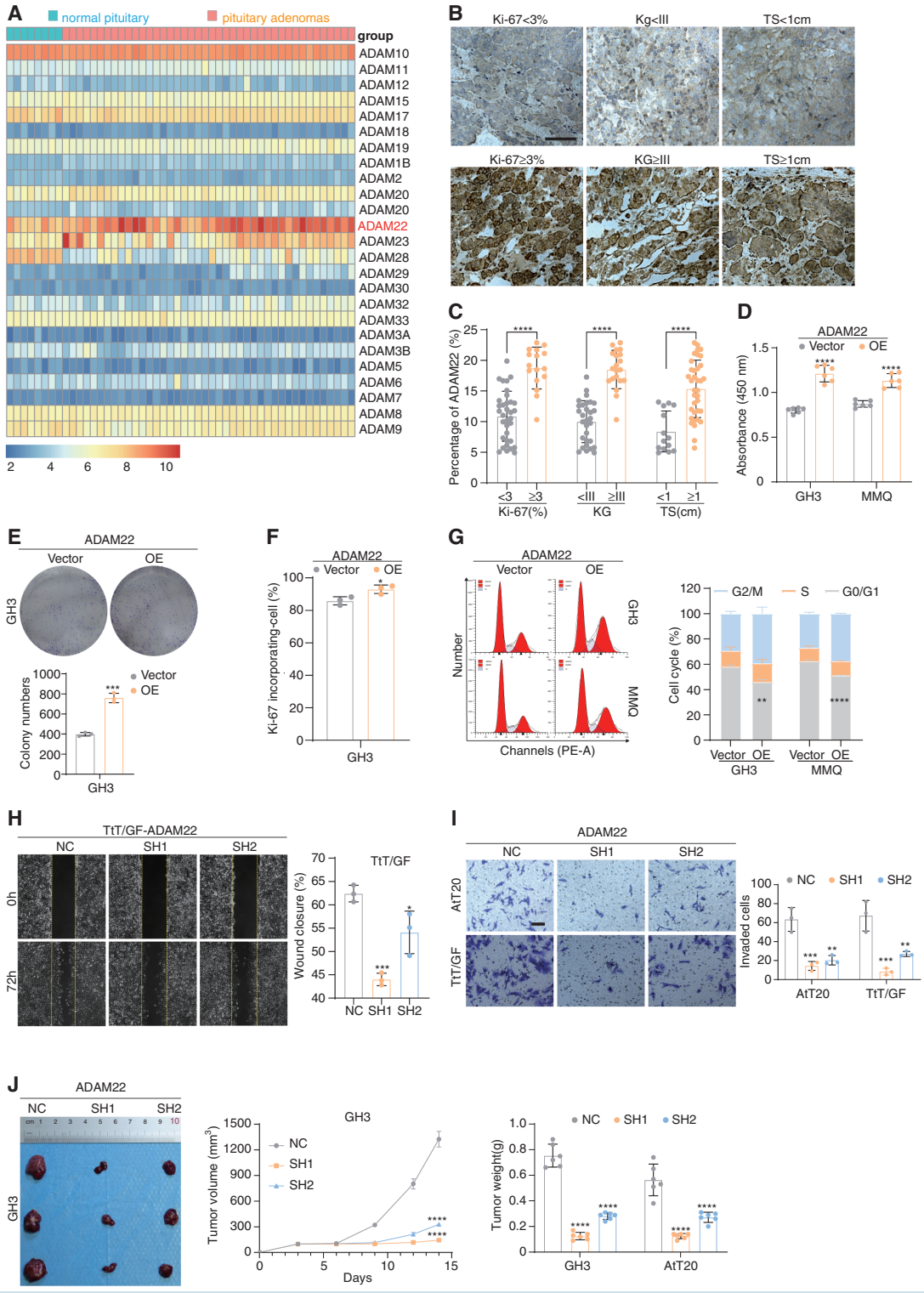


Figure 1. ADAM22 promotes the proliferation, migration, and invasion of pituitary tumor cells. (A) Heatmap of ADAMs RNA sequencing data between normal pituitary and pituitary adenomas (GSE147786). (B–C) Representative IHC staining images of ADAM22 expression in 50 pituitary tumor tissues and its relationship with Ki-67, Knosp grade (KG), and tumor size (TS). Scale bar, 50 μm. (D) CCK-8 cell viability assay of indicated cells with overexpression of ADAM22. (E) Colony formation of GH3 with overexpression of ADAM22. (F) The Ki-67 staining of GH3 with overexpression of ADAM22. (G) Flow cytometry analysis of the cell cycle in indicated cells with overexpression of ADAM22. (H) Wound healing assay of shADAM22 in TtT/GF cells. (I) Transwell invasion assay of shADAM22 in AtT20 and TtT/GF cells. Scale bar, 100 μm. (J) Images of shADAM22 in GH3 subcutaneous xenografts from nude mice (left). Tumor volumes were measured every 3 days (center). The average weight of excised tumors from shADAM22 in GH3 and AtT20 subcutaneous xenografts (right). *$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .001$.

in PA, we performed Co-IP MS in GH3 with ADAM22 as the bait, the results showed that, several integrin family members including Itgav, Itga3, Itga6, Itgb1, and Itgb8 may interact with ADAM22, among which Itgb1 (Fold change >4) was the most likely interactor (Supplementary Figure 5A–B and Supplementary Table 6). Besides, we also analyzed 2 previous PA transcriptomics datasets, and we revealed that the ITBG1 was the most redundant expressed integrins both in PA and the normal pituitary (Supplementary Figure 6A–B), which was consistent with previous reports.[29] Furthermore, we confirmed the expression of ITGB1 in PAs (both cell lines and primary PA from patients) by flow cytometry and immunofluorescence (Supplementary Figure 6C–D), and observed a strong co-localization between ITGB1 and ADAM22 on the cell membrane of PAs (Supplementary Figure 6E). Taken together, we selected ITGB1 as an ADAM22 downstream effector and proceeded with investigating the role of ITGB1 in ADAM22-induced PA progression.

To confirm the hypothesis that ADAM22 may bind ITBG1 through its disintegrin domain and thus exerts its tumor-promoting effect, we first performed IHC for ITGB1 on the 50 previous PA specimens and regrouped them according to the previous criteria (Figure 2A, left). The results showed that ITGB1 was indeed highly expressed in PA and its expression level was positively correlated with Knsop grade, but not with Ki-67 or TS (Figure 2A, right), which was consistent with the analysis of PA transcriptomics datasets (Supplementary Figure 7A). Next, to verify the role of ITGB1 in the tumor-promoting effect of ADAM22, we first constructed cell lines with ITGB1 stably knocking down in ADAM22 overexpressing cell lines (Supplementary Figure 7B–C). In vitro (CCK-8 and transwell; Figure 2B–C and Supplementary Figure 7D) and in vivo assays (Figure 2D–E) showed that the knockdown of ITGB1 in PA cells significantly reversed the ADAM22 overexpression-mediated cell proliferation and invasion. These results suggested that ADAM22 exerts its tumor-promoting effects on PA cells depending on ITGB1.

To further investigate whether ADAM22 directly interacts with ITBG1, we performed Co-IP in GH3 and AtT20 cell lines (Figure 2F). Indeed, the results showed that ADAM22 was able to bind to ITGB1 in PA cells. To further validate whether ADAM22 binds ITGB1 by its disintegrin domain, we constructed stable cell lines overexpressing mutant ADAM22 without disintegrin domain (ΔPA-ADAM22; Figure 2G–H) and performed Co-IP (Figure 2I and Supplementary Figure 7E). Unlike WT ADAM22 (WT-ADAM22) with disintegrin domain, we observed that ΔPA-ADAM22 was unable to bind to ITBG1. In addition, we again performed CCK-8 (Figure 2J), transwell (Figure 2K) as well as in vivo experiments with ΔPA-ADAM22 overexpressed cell lines (Figure 2I and Supplementary Figure 7F–G), and the results showed that overexpression of ΔPA-ADAM22 could not exert pro-proliferative and pro-invasive effects as WT-ADAM22, indicating that ADAM22 binding to ITGB1 relying on its disintegrin domain to promote the proliferation and invasion of PA cells.

ADAM22 Activates FAK/PI3K and FAK/ERK Signaling Pathways by Binding to ITGB1

Integrin signaling is involved in a variety of physiological and pathological processes, including cell survival, proliferation, migration, and invasion.[22,23] Considering that ADAM22 mainly promotes cell proliferation and invasion after binding to ITGB1 in PA, we detected downstream, molecules of the integrin signaling pathway associated with cell proliferation and invasion. WB showed that ADAM22 overexpression led to elevated phosphorylation levels of FAK, PI3K, AKT, B-Raf, and ERK, while conversely, the phosphorylation levels of these key proteins were decreased after ADAM22 knockdown (Figure 3A and Supplementary Figure 8A–E). To further verify that ADAM22 activates ITGB1 signaling, we again performed WB with ΔPA-ADAM22 cells and found that overexpression of ΔPA-ADAM22 failed to activate downstream related proteins as WT-ADAM22 (Figure 3A, and Supplementary Figure 8F–J). Thus, ADAM22 indeed relies on its disintegrin domain to activate integrin-related signaling pathways in PA cells. In addition, we performed CCK-8 (Figure 3B), transwell (Figure 3C), and in vivo xenograft experiments (Figure 3D) including IF of the tumor sections (Figure 3E–F) on ADAM22 overexpressed PA cells treated with an inhibitor of FAK (1,2,4,5-Benzenetetraamine tetrahydrochloride, Y15, HY-12444, MCE). The results showed that FAK inhibition significantly attenuated the proliferation and invasion of PA cells induced by ADAM22 overexpression.

To better elucidate the mechanistic detail of the observed phenotype, we integrated PA RNA-seq cohorts (GSE147786/GSE51618) and performed the in silico correlation analysis. Indeed, ADAM22 is positively correlated with signatures that belong to invasion (Matrix metalloproteinases: MMPs) and proliferation (Cycling-dependent kinases: CDKs, Cycling D: CCNDs) (Supplementary Figure 9A). We have also further determined some of the markers (CCND1 and MMP9) which were all revealed as down effectors of FAK signaling in other types of cancers and displayed as important players participating in tumor invasion or proliferation.[30,31] Indeed, both FAK inhibition and ADAM22 knockdown can suppress their expression while overexpression of ADAM22 induced elevated expression in PA (Supplementary Figure 9B). These results are consistent with our phenotypic experiments.

Taken together, ADAM22 activates ITGB1 and its downstream FAK/PI3K/AKT and FAK/MAPK/ERK signaling pathways to drive PA growth.

Phosphorylation of ADAM22-S832(m)/S860(r) by PKA Protected ADAM22 From Degradation

GSP (stimulatory G-protein alpha subunit, also known as GNAS) mutation-mediated activation of GPCR/PKA or GPCR/PKC signaling was observed in most PA, especially PKA signaling according to others and our previous studies.[32–34] To further clarify whether these reported signals exert a regulative effect on ADAM22 in PA, we first stimulated PA cells with Phorbol 12-myristate 13-acetate (PMA, HY-18739, MCE; an activator of PKC) and forskolin (HY-15371, MCE, an activator of adenylate cyclase) which are classical drugs that promote PA proliferation. Results showed that both PMA and forskolin were able to promote the proliferation and invasion of PA cells (data not shown), which was consistent with previous reports.[34,35] Interestingly, mRNA levels of ADAM22 were not altered after PMA or forskolin stimulation (Supplementary Figure 10A–B), while protein levels of ADAM22 increased

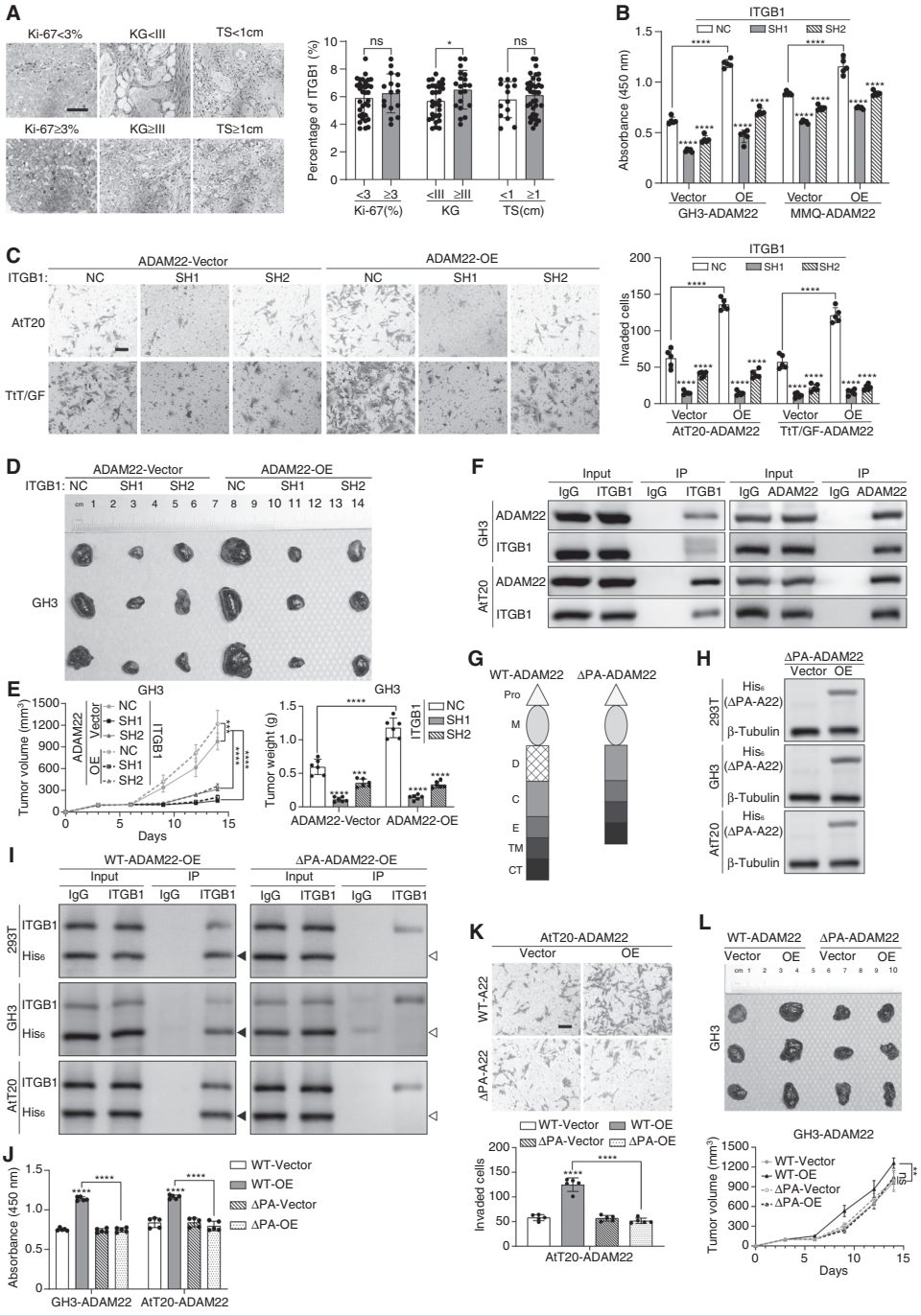


Figure 2. The biological function of ADAM22 depends on the interaction with ITGB1 through its disintegrin domain. (A) IHC staining images of ITGB1 expression in 50 pituitary tumor tissues and its relationship with Ki-67, Knosp grade (KG), and tumor size (TS). Scale bar, 50 μm. (B) CCK-8 cell viability assay of indicated cells with shITGB1 and overexpression of ADAM22. (C) Transwell invasion assay of indicated cells with shITGB1 and overexpression of ADAM22. Scale bar, 100 μm. (D–E) Images of shITGB1 and overexpression of ADAM22 in GH3 subcutaneous xenografts from nude mice (D). Tumor volumes were measured every 3 days (E, left). The average weight of excised tumors (E, right). (F) Co-IP between ADAM22 and ITGB1 in GH3 and AtT20 cells. (G–H) Construct the mutant ADAM22 without the disintegrin domain (ΔPA-A22) in indicated cells. The general structure of wild-type ADAM22 (WT-ADAM22) and mutant ADAM22 without the disintegrin domain (ΔPA-ADAM22). (I: Pro = Prodomain, M = Metalloproteinase domain, D = Disintegrin domain, C = Cysteine-ich domain, E = EGF-like domain, TM = Transmembrane domain, CT = Cytoplasmic domain). (I) Co-IP between His_6-tagged wild-type ADAM22 (WT-ADAM22, closed arrowheads) or mutant ADAM22 without the disintegrin domain (ΔPA-ADAM22, open arrowheads) and ITGB1 in indicated cells (with anti-ITGB1 antibody pulling down). (J) CCK-8 cell viability assay of indicated cells with the overexpression of wild-type ADAM22 (WT) or mutant ADAM22 without the disintegrin domain (ΔPA). (K) Transwell invasion assay of AtT20 cells with the overexpression of wild-type ADAM22 (WT-A22) or mutant ADAM22 without the disintegrin domain (ΔPA-A22). Scale bar, 100 μm. (L) Images of GH3 with the overexpression of wild-type ADAM22 (WT) or mutant ADAM22 without the disintegrin domain (ΔPA) subcutaneous xenografts from nude mice (upper). Tumor volumes were measured every 3 days (lower). ns (nonsignificant), $*P < .05$, $**P < .01$, $***P < .001$, $****P < .001$.

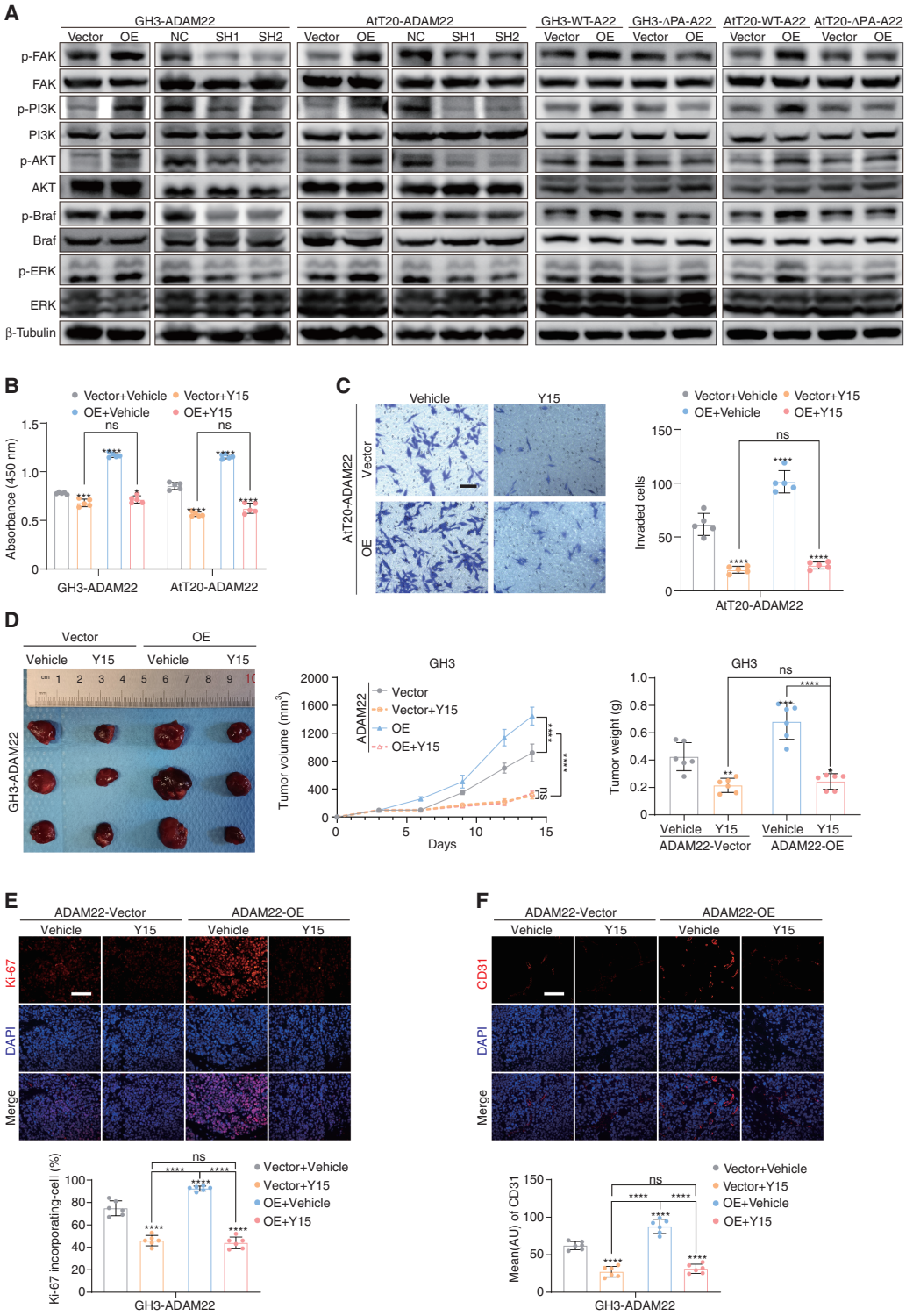


Figure 3. ADAM22 activates downstream FAK/PI3K and FAK/ERK signaling pathways by binding to ITGB1. (A) Western blot analysis of FAK, PI3K, AKT, B-raf, and ERK and their phosphorylation level expression in indicated cells (WT-A22: wild-type ADAM22; ΔPA-A22: mutant ADAM22 without the disintegrin domain). (B) CCK-8 cell viability assay of indicated cells with the overexpression of ADAM22 after Y15 (10 Mm, 24 hours) or vehicle control administration. (C) Transwell invasion assay of AtT20 cells with the overexpression of ADAM22 after Y15 (10 Mm, 24 hours) or vehicle control administration. Scale bar, 100 μm. d Images of GH3 with the overexpression of ADAM22 subcutaneous xenografts from nude mice after Y15 (30 mg/kg/d, intraperitoneal injection, 5 days/week) or vehicle control administration for 2 weeks (left). Tumor volumes were measured every 3 days (center). The average weight of excised tumors (right). (E–F) Immunofluorescence images of sections sliced from indicated subcutaneous xenograft tumors and stained with DAPI (blue), anti-Ki-67 (E, red), or anti-CD31 (F, red) antibodies. Scale bar, 50 μm. ns (nonsignificant), $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .001$.

significantly, especially after forskolin administration for 48 hours (Figure 4A and Supplementary Figure 10C). In contrast, PKA inhibitor (H89, HY-15979, MCE) remarkably attenuated forskolin-mediated upregulation of ADAM22 protein levels (Figure 4B and Supplementary Figure 10D) as well as the pro-proliferative and pro-invasive ability of PA cells (Figure 4C–E).

The above results suggested that the elevation of ADAM22 protein levels promoted by PKA was likely to occur at the post-transcriptional level. Indeed, a previous study illustrated that PKA activation resulted in a decrease in ADAM22 degradation.[15] To examine the above speculation in PA, GH3, and AtT20 were incubated with the translational inhibitor cycloheximide (CHX, HY-12320, and MCE) alone or in combination with forskolin. Results showed that forskolin indeed notably slowed down ADAM22 protein degradation in the presence of CHX in PA cells, compared to those without forskolin, which confirmed our previous speculation (Figure 4F and Supplementary Figure 10E).

Given that PKA is a kinase capable of phosphorylating substrate proteins, we inferred that the specific mechanism by which PKA leads to a delayed degradation of ADAM22 may be associated with the phosphorylation of ADAM22 by PKA. Next, to clarify the phosphorylation sites of ADAM22, we reviewed the comprehensive phosphorylation-site database PhosphoSitePlus (http://www.phosphosite.org), Gene database of NCBI (https://www.ncbi.nlm.nih.gov/gene) and the universal protein knowledge base UniProt (https://www.uniprot.org), and identified the 2 most likely sites for phosphorylation of ADAM22: S832/S855 in mouse (m) or S860/S883 in rat (r), with S832(m)/S860(r) being the most common and predominant phosphorylation residue site. Therefore, we constructed stable point mutant cell lines: S832A(m)/S860A(r)-ADAM22 in 293T, GH3, and AtT20, in which serine 860 was mutated to alanine (S860A) in 293T and rat PA cell line (GH3) and serine 832 was mutated to alanine (S832A) in mouse PA cell line (AtT20) (Figure 4G–H). Phos-tag SDS-PAGE and common WB were performed to detect protein phosphorylation levels and total protein of ADAM22, respectively (Figure 4I). The results showed that (1) in normal cells, the vast majority of ADAM22 was in the phosphorylated form (open arrowheads), (2) both overexpression of ADAM22 and forskolin stimulation led to an increase in the total ADAM22 protein level, (3) both overexpression of WT-ADAM22 and forskolin administration led to an increase in the phosphorylated ADAM22 protein level, and (4) in overexpression of S832A(m)/S860A(r)-ADAM22 cell line, most of the ADAM22 proteins were in the non-phosphorylated state (closed arrowheads). The above results indicated that ADAM22-S832A(m)/S860A(r) impaired intracellular and forskolin-mediated phosphorylation of ADAM22 in PA cells, implying that phosphorylation of ADAM22 by PKA was dependent on the S832(m)/S860(r) of ADAM22.

To further verify our hypothesis, WT-ADAM22 and S832A(m)/S860A(r)-ADAM22 cell lines were incubated with CHX alone or together with forskolin. Results of WB proved that forskolin retarded the degradation of ADAM22, while point mutation of S832(m)/S860(r) significantly attenuated the forskolin-mediated ADAM22 degradation retardation (Figure 4J and Supplementary Figure 11A). CCK-8 (Figure 4K) and transwell (Figure 4L) assays with the S832A(m)/S860A(r)-ADAM22 cell line showed that overexpression of S832A(m)/S860A(r)-ADAM22 did not promote PA cell proliferation and invasion as WT-ADAM22 did.

Based on the above results, we concluded that ADAM22 depends on the phosphorylation of its S832(m)/S860(r) site by PKA, which protected it from degradation and exerts its pro-proliferative and invasive effects on PA cells.

Phosphorylation of ADAM22-S832(m)/S860(r) Protects ADAM22 From Degradation Depending on Binding to 14-3-3

Next, we asked how the degradation of ADAM22 was retarded after phosphorylation. The 14-3-3 proteins are a family of evolutionarily highly conserved phosphoprotein-binding proteins that exist as homodimers or heterodimers and are involved in regulating a wide range of cellular processes by modulating the conformation/stability of target proteins or by facilitating protein-protein interactions as scaffolding proteins.[15,36–38] Although it has been previously reported that phosphorylated ADAM22 can bind to 14-3-3 and that their binding promotes the membrane localization of ADAM22 and inhibits the lysosomal degradation pathway of ADAM22,[15,38] these interactions remain unclear in PA cells. To investigate whether 14-3-3 regulates the degradation of ADAM22 in PA cells, we first queried the Gene database of NCBI (https://www.ncbi.nlm.nih.gov/gene) and the universal protein knowledge base UniProt (https://www.uniprot.org), it was clear that a common motif sequence (-RSXpSXP-) recognizing 14-3-3 does exist in the cytoplasmic domain of ADAM22 (829-RSNpSWQ-834 and 852-RSNpSTE-857 in mice, Figure 5A; 857-RSNpSWQ-862 and 880-RSNpSTE-885 in rat), while the Co-IP MS (Supplementary Figure 5A) and Co-IP also confirmed that 14-3-3 can bind to ADAM22 in PA cells (Figure 5B). In contrast, if Co-IP was performed again with S832A(m)/S860A(r)-ADAM22 cells, either the anti-ADMA22 or the anti-14-3-3 antibody was used to pull down the cell lysates, the S832A(m)/S860A(r)-ADAM22 could no longer be immunoprecipitated with 14-3-3 (Figure 5C–D). The above results indicated that the binding of ADAM22 to 14-3-3 was dependent on its phosphorylation at S832(m)/S860(r) (Figure 5A).

To further verify the regulation of 14-3-3 on the degradation of ADAM22, GH3, and AtT20 were incubated with CHX alone or together with BV02 (HY-101985, MCE; an inhibitor of 14-3-3), and the cell lysates were applied to WB. Results showed that BV02 significantly accelerated the degradation of ADAM22 (Figure 5E and Supplementary Figure 11B). Similarly, interference with the endogenous expression of 14-3-3 could also accelerate the degradation of ADAM22 in PA cells (Figure 5F and Supplementary Figure 11C–D).

To further verify the role of 14-3-3 in the regulation of ADAM22 in PA cells, we performed CCK-8 on 14-3-3ζ silenced GH3/AtT20 (Figure 5G) or cell lines overexpressing ADAM22 incubated with BV02 (Figure 5H), and we found that BV02 not only completely abrogated the proliferative effect mediated by overexpression of ADAM22 but even inhibited cell proliferation, which may be related to the involvement of 14-3-3 in multiple biological functions.[37] The

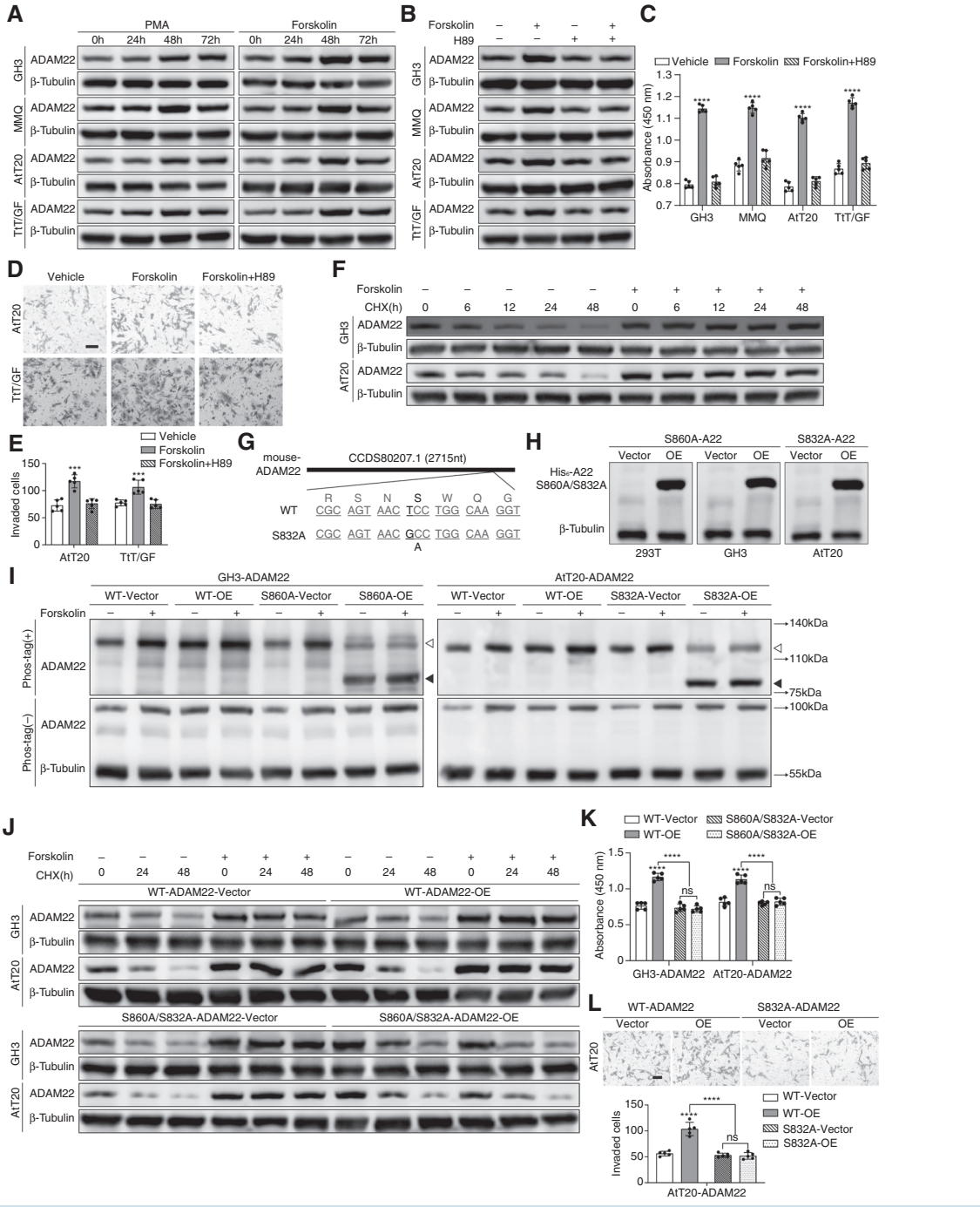


Figure 4. Phosphorylation of ADAM22-S832/ADAM22-S860 by PKA delays ADAM22 degradation. (A) Western Blot (WB) analysis ofADAM22 expression in indicated cells after PMA (150 nM) or forskolin (50 μM) administration for 0 hour, 24 hours, 48 hours, and 72 hours. (B) WB analysis of ADAM22 expression in indicated cells after treatment with either forskolin (50 μM), H89 (20 μM), or forskolin(50 μM) + H89(20 μM) for 48 hours. (C) CCK-8 cell viability assay of indicated cells after treatment with either vehicle control or forskolin (50 μM) or forskolin (50 μM) + H89 (20 μM) for 48 hours. (D–E) Transwell invasion assay of AtT20 and TtT/GF cells after treatment with either vehicle control or forskolin (50 μM) or forskolin (50 μM) + H89 (20 μM) for 48 hours.Scale bar, 100 μm. (F) WB analysis of ADAM22 expression in indicated cells after CHX (20 mg/mL) or CHX (20 mg/ml) + forskolin (50 μM) administration for 0 hour, 6 hours, 12 hours, 24 hours, and 48 hours. (G–H) Construction of point mutant cell lines with a serine point mutation to alanine at position 832 (S832A, mouse)/860 (S860A, rat) of ADAM22 in indicated cells (A22 represents ADAM22). i WB analysis of ADAM22 expression in indicated cells after vehicle control or forskolin (50 μM) administration for 48 hours. Phos-tag SDS-PAGE (upper) displays a quantitative shift of phosphorylated ADAM22 (open arrowheads). ADAM22 in S860A/S832A groups stayed in its original position (closed arrowheads). Normal SDS-PAGE (lower) of ADAM22 shows no migration. (J) WB analysis of ADAM22 expression in indicated cells after CHX (20 mg/mL) or CHX (20 mg/mL) + forskolin (50 μM) administration for 0 hour, 24 hours, and 48 hours. (K) CCK-8 cell viability assay of indicated cells. (L) Transwell invasion assay of AtT20 cells with the overexpression of wild-type (WT) or S832 mutant (S832A) ADAM22. Scale bar, 100 μm. ns (nonsignificant), ***$P < .001$, ****$P < .001$.

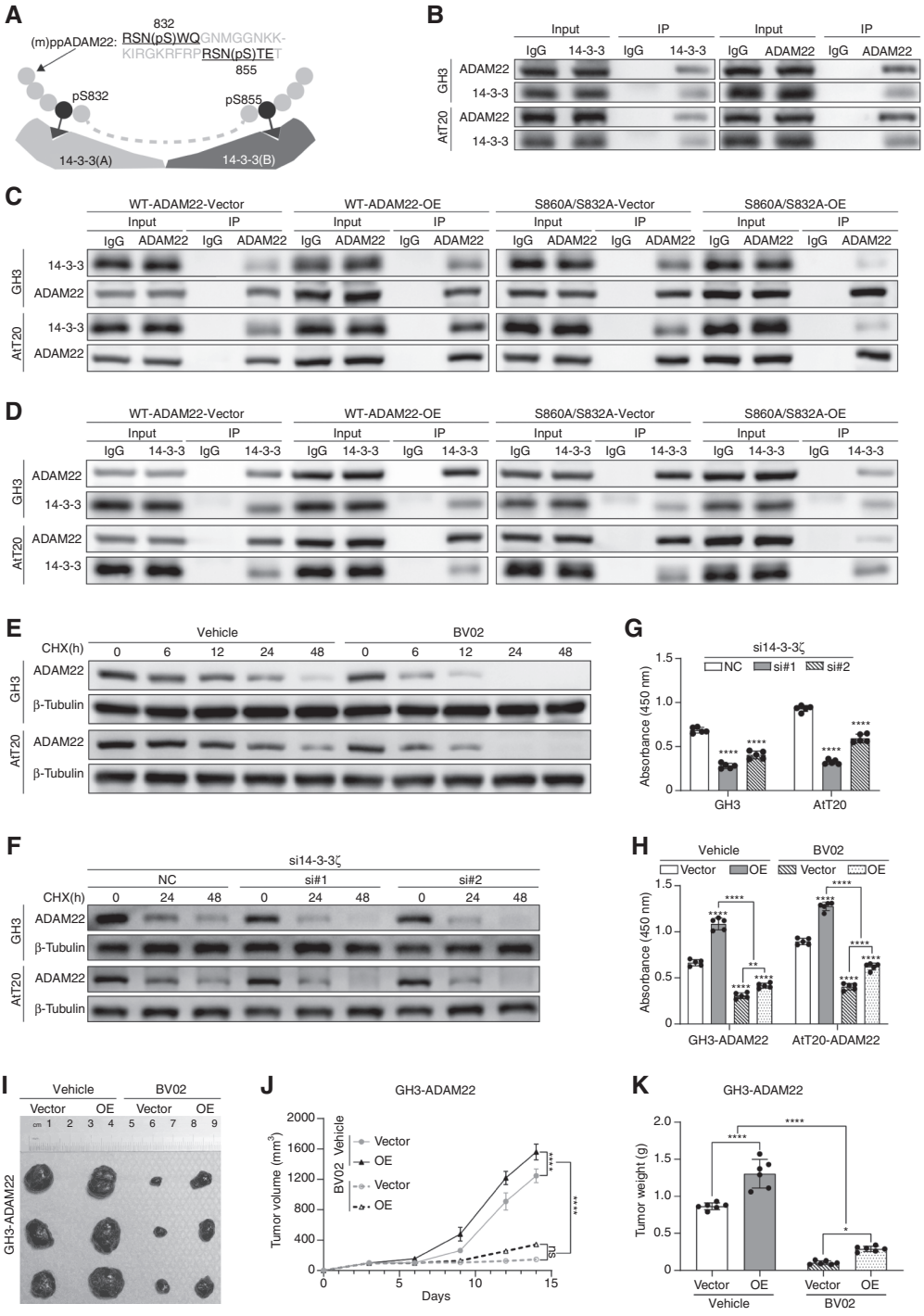


Figure 5. Phosphorylation of ADAM22-S832(m)/S860(r) protects ADAM22 from degradation depending on binding to 14-3-3. (A) Schematic diagram for the interaction of phosphorylated ADAM22 cytoplasmic domain with 14-3-3 in mice. The underlined amino acid sequences represent the common motif sequences that recognize 14-3-3. (B) Co-IP between ADAM22 and 14-3-3 in GH3 and AtT20 cells. (C–D) Co-IP between ADAM22 and 14-3-3 in indicated cells with the overexpression of wild-type ADAM22 or ADAM22 with point mutation (S860A in GH3; S832A in AtT20). (E) Western Blot (WB) analysis of ADAM22 expression in indicated cells after CHX (20 mg/mL) or CHX (20 mg/mL) + BV02 (5 μM) administration for 0 hour, 6 hours, 12 hours, 24 hours, and 48 hours. (F) WB analysis of ADAM22 expression in indicated cells with si14-3-3ζ after CHX (20 mg/mL) administration for 0 hour, 24 hours, and 48 hours. (G) CCK-8 cell viability assay of indicated cells with si14-3-3ζ. (H) CCK-8 cell viability assay of indicated cells with overexpression of ADAM22 after being treated with vehicle control or BV02 (5 μM) for 48 hours. (I–K) Images of GH3 with the overexpression of ADAM22 subcutaneous xenografts from nude mice after vehicle control or BV02 (25 mg/kg/d, intratumoral injection, every 2 days) administration for 2 weeks. (I). Tumor volumes were measured every 3 days (J). The average weight of excised tumors (K). ns (nonsignificant), *$P < .05$, **$P < .01$, ****$P < .001$.

above results were then further verified by xenografts in vivo (Figure 5I–K).

In summary, phosphorylation of ADAM22-S832(m)/S860(r) protects ADAM22 from degradation depending on 14-3-3 binding in PA cells.

Anti-itgb1 Antibody Synergies With SSTA/DAs to Exert Anti-Pituitary Tumor Effect

ITGB1(also known as CD29), is a cell-adhesion molecule implicated in tumor proliferation, invasion, therapeutic resistance, and metastasis.[39–43] Since our above data have shown that ITGB1 is a key molecule in ADAM22 promoting PA progression, and the therapeutic β1 integrin-neutralizing antibody has shown promising results in numerous solid cancers including breast, ovarian, and brain cancers.[42–44] Therefore, we first performed dose-response tests to elucidate the effect of anti-itgb1 on PA cells (Supplementary Figure 12A–B). The results showed that the concentration of 10 μg/ml may be the lowest dose to achieve the maximum inhibitory effect in vitro. In addition, at low concentrations (<10 μg/mL), overexpression or knockdown of ADAM22 could affect the effect of anti-itgb1 on PA cells. However, modification of ADAM22 expression failed to influence the antitumor effect of anti-itgb1 treatment with high concentrations (≥10 μg/mL). (Supplementary Figure 12A–D). Transwell invasion assay further verified the effect of anti-itgb1 on the invasion of PA cells (Supplementary Figure 12C–D), and the results were consistent with its effect on proliferation. Moreover, the subcutaneous xenografts in vivo experiments further validated the above results (Supplementary Figure 13A).

Next, to better clarify the effect of overexpression or knockdown of ADAM22 on the efficacy of anti-itgb1, we constructed nude mice intracranial pituitary tumor xenograft models and rat prolactinoma orthotopic models for in vivo experiments (Supplementary Figure 13B–D). The results were consistent with the above results in vitro experiments and the subcutaneous xenografts *in vivo*. Taken together, these results illustrated that anti-itgb1 treatment suppressed PA growth.

Since anti-itgb1 has antitumor effects in PA, we were interested in comparing the difference in efficacy between anti-itgb1 and the current clinical first-line PA therapeutics (somatostatin analog, SSTA; dopamine agonists, DAs) to provide evidence for its possible future clinical use in PA. First, we compared the effects of octreotide (OCT)/bromocriptine (BRC), anti-itgb1, and the combination of both in the 2 most common representative cell lines of functional PA (GH3 and MMQ) and their primary PA cells, respectively. CCK-8 (Figure 6A–F) and flow cytometry apoptosis assays (Figure 6G–J) showed that anti-itgb1 has an anti-pituitary tumor proliferation effect, and more importantly, synergies with OCT or BRC. We next compared the effects of pasireotide (PAT), cabergoline (CAB), anti-itgb1, and PAT plus anti-itgb1 in another relatively rare ACTH adenoma cell line (AtT20), and CCK-8 (Supplementary Figure 13E), as well as flow cytometry apoptosis assays (Supplementary Figure 13F–G), showed that anti-itgb1 has anti-pituitary tumor proliferation effect and synergies with PAT. The xenografts in vivo experiments also further validated the above results (Figure 6K–L and Supplementary Figure 13H.

Taken together, anti-itgb1 efficiently suppresses and synergies with SSTA/DAs to suppress the growth of PA cells, which might be able to be translated into PA treatment.

Discussion

Although PA is defined as a benign tumor, IPA that possesses a high proliferation index and invades surrounding structures, often making it difficult for total surgical resection, more prone to recurrence, and leading to a poorer patient prognosis,[3–5] which requires extensive investigation illustrating the promoting signals in IPA.

In the present study, we demonstrated that ADAM22 was significantly overexpressed in PA and promoted the proliferation, migration, and invasion of PA cells. Meanwhile, in breast cancer, ADAM22 was also highly expressed and promoted breast cancer cell migration, and dedifferentiation and was associated with endocrine therapy resistance, and metastasis as well as poor prognosis.[19–21] Moreover, it has been proved that ADAM22 is involved in cell adhesion and spreading,[36] which might contribute to migration and invasion. In addition, ADAM22 drives proliferation, migration, and phenotypic transformation in vascular smooth muscle cells.[45] All the above reports support that ADAM22 may have a tumor-promoting effect. However, conversely, decreased level of ADAM22 was observed in glioblastoma (GBM) compared to normal brain tissue,[46] which might be explained by the existence of more pro-tumor mechanisms in GBM (such as overexpression of integrins[47]) and thus progressive silencing during the malignant evolution of gliomas (such as ADAM23, which is highly homologous to ADAM22, was hypermethylated in breast cancer[48]).

Mechanically, in this study, we also clarified that ADAM22 exerts its tumor-promoting effects by binding to integrin receptors through its disintegrin domain. Other ADAMs with the same disintegrin domain as ADAM22 (containing a conserved consensus integrin-binding motif xCD, such as ADAM8, ADAM9, and ADAM17) have also been shown to exert pro-tumorigenic effects by binding integrin molecules, including ITGB1.[24,26,27] Moreover, ADAM23 and ADAM11, which are very homologous to ADAM22, can also bind integrin molecules to promote cell proliferation.[49] However, some groups have also found that ADAM22 or ADAM23 can also negatively regulate the activation of certain integrins through their disintegrin domains.[46,48] This may be explained by their competitive inhibition of certain integrin-activating ligands, and this possibility is supported by the fact that subsequent reactivation of the integrin signaling reverses this inhibitory effect.[46] This means the effect of ADAM22 on cell behavior is dependent on its activation or inhibition of integrin signalings.

Our results showed that ITGB1 is highly expressed in both PA and normal pituitary glands and may be associated with PA invasion. Meanwhile, ITGB1 also exhibits pro-proliferative and pro-invasive effects in a variety of tumors.[39,40] Integrins are involved in promoting cell survival, proliferation, migration, invasion, and angiogenesis by

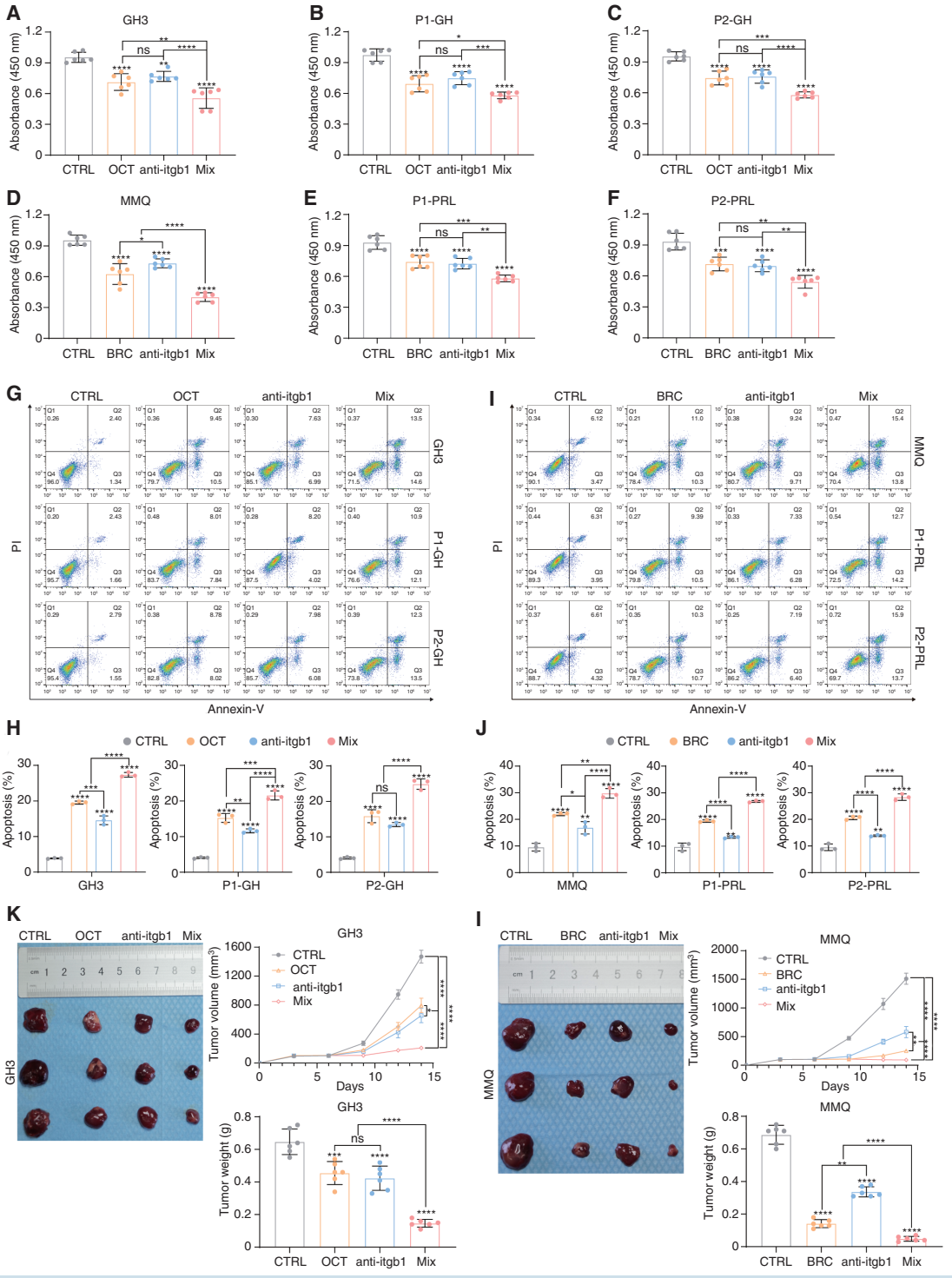


Figure 6. Anti-itgb1 has an anti-pituitary tumor proliferation effect and synergies with SSTA/BRC. a-f CCK-8 cell viability assay of indicated cells after treatment with either vehicle control or OCT (200 nM), PAT (200 nM), anti-itgb1 (10 μg/mL), BRC (10 μM), or Mix (A–C) OCT (200 nM) + anti-itgb1 (10 μg/mL); (D–F) BRC (10 μM) + anti-itgb1 (10 μg/mL) for 24 hours. GH3 cell line (A), GH adenoma primary cells (B–C), MMQ cell line (D), PRL adenoma primary cells (E–F). (G–J) Flow cytometry analysis of indicated cells after treatment with either OCT (200 nM), PAT (200 nM), anti-itgb1 (10 μg/mL), BRC (10 μM), or Mix (G–H) OCT (200 nM) + anti-itgb1 (10μg/mL); (I–J) BRC (10 μM) + anti-itgb1 (10 μg/mL) for 24 hours. GH3 cell line and GH adenoma primary cells (G–H), MMQ cell line and PRL adenoma primary cells (I–J). (K–L) Images of GH3 (L) and MMQ (L) subcutaneous xenografts from nude mice after treatment with either vehicle control or OCT (30 μg/kg/d, intraperitoneal injection), anti-itgb1 (10 mg/kg/d, intraperitoneal injection), BRC (12 mg/kg/d, intraperitoneal injection), or Mix (K) OCT (30 μg/kg/d, intraperitoneal injection) + anti-itgb1(10 mg/kg/d, intraperitoneal injection); (L) BRC (12 mg/kg/d, intraperitoneal injection) + anti-itgb1 (10 mg/kg/d, intraperitoneal injection) for 2 weeks. ns (nonsignificant), *$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .001$.

activating multiple signaling pathways.[22,23,25,26,41,43,47] We found in PA that the pro-tumorigenic effects of ADAM22 were dependent on the interaction with ITGB1, activating mainly 2 signaling pathways, FAK/PI3K and FAK/ERK which was consistent with the binding and activation of the ITGB1 signaling pathway by ADAM8 in pancreatic cancer.[26] Integrins have been considered potential targets for cancer treatment for a long time, and 90 integrin-targeting therapies are currently in clinical trials.[23] Among them, OS2966, a targeted inhibitor of ITGB1, has shown promising results in breast cancer, ovarian cancer, and glioblastoma (GBM).[42–44] In addition, OS2966 holds FDA Orphan Drug Designation for the treatment of GBM and ovarian cancer and is already in Phase I clinical trial for recurrent GBM. In our study, we found that anti-itgb1 is also effective in the treatment of PA, especially the synergistic effect shown in combination with current clinical first-line drugs, which provides a new direction for the future therapeutics of PA.

The translocation or post-translational regulation of ADAMs members has previously been elucidated to have a pivotal role in maintaining ADAM functions.[8–10,15,38] This regulation is closely related to the phosphorylation of the cytoplasmic domain of ADAMs,[8,10] where the serine/threonine phosphorylation sites of the cytoplasmic domain of ADAM22 can be phosphorylated by various kinases, including PKA/PKC.[15,38] We demonstrated that in PA, phosphorylation of the cytoplasmic domain of ADAM22 was mainly dependent on PKA, probably due to the presence of a similar PKA catalytic subunit recognition common sequence (R-R-X-S/T-Φ) at the phosphorylation site of ADAM22,[15] and the fact that sustained activation of adenylate cyclase occurs in many PAs due to GSP mutations.[32] Phosphorylated ADAM22 then recruits 14-3-3 and thus delays its degradation, which is consistent with the results in neurons.[15] Furthermore, since 14-3-3 overrides the endoplasmic reticulum retention signals (di-arginine RXR motifs) and AP2 (the initiator protein of the lysosomal degradation pathway) binding signals (YXXF motifs) of ADAM22,[15,37] it could promote the membrane localization of ADAM22 and protects ADAM22 on the cell membrane from degradation by the lysosomal pathway,[15,38] but the above mechanisms do warrant further exploration.

In summary, our study demonstrates that ADAM22 was upregulated in PA and was able to bind to ITGB1 through its disintegrin domain, thereby activating FAK/PI3K and FAK/ERK signaling pathways to promote PA proliferation, migration, and invasion. Finally, we revealed possible regulatory mechanisms of ADAM22 at the level of post-transcriptional phosphorylation modifications. Our results illustrated the mechanism of aggressive behavior of PA, which might be translated into clinical application.

The present study reveals a key role of the disintegrin domain of ADAM22 in PA progression. However, it remains unclear whether other extracellular domains of ADAM22 (such as EGF-like and cysteine-rich domains) are also involved in its pro-tumorigenic effects in other ways. In PA, although we proposed regulation of ADAM22 from the perspective of protein phosphorylation, the results of ADAM22 transcriptomics were not explained from the level of transcriptional regulation.

Supplementary material

Supplementary material is available online at *Neuro-Oncology* (http://neuro-oncology.oxfordjournals.org/).

Keywords

ADAM22 | integrin β1 | invasion | PKA | pituitary adenoma

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Conflict of interest statement

The authors declare no potential conflicts of interest.

Authorship statement

Conception and design: T.L., Y.H. Conducting experiments: B.X., Z.L. Data analysis and figure preparation: Z.W., Q.W., Z.Z., X.L., S.L., Collection and assembly of data: Y.Q., Y.L., X.L., K.S., H.Z. The overall research supervision: J.W.B., C.N., T.L. Financial support: T.L. Manuscript writing: B.X., Y.H. Final approval of manuscript: All authors.

Data availability

The sequencing data that support the findings of this study are openly available in GEO DataSets (https://www.ncbi.nlm.nih.gov/gds) by pasting the accession number: GSE147786/GSE51618 into the text search box or through the URL: https://www.ncbi.nlm.nih.gov/gds/?term=GSE147786 and https://www.ncbi.nlm.nih.gov/gds/?term=GSE51618.

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