Pannexin-1 promotes the invasion of pituitary adenoma by modulating ATP release

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Research

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Abstract

Background

Pannexin-1 (Panx1) channel participates in the development of many tumor subtypes, however, its role of Panx1 in pituitary adenoma (PA) remains largely unknown. The current study was designed to investigate the role of Panx1 in PA, especially in the invasion of PA.

Methods

We examined the expression of Panx1 in 56 surgical PA samples, which were divided into invasive and noninvasive groups. We also established Panx1-overexpressing (Panx1-OE) PA cells using the GH3 and MMQ cell lines in vitro.

Results

We found that the expression of Panx1 in invasive PA was significantly upregulated compared with noninvasive PA and pituitary gland and that Panx1-OE could promote the invasion of these cell lines but had no effect on their proliferation. Further metabolomics experiments confirmed that Panx1-OE could trigger changes in several metabolic pathways of GH3 cells. Among the dysregulated cellular metabolites, we found that adenosine triphosphate (ATP) could be released to the extracellular matrix through the Panx1 channel and thereby enhance the invasion of GH3 cells. Mechanistically, ATP promoted extracellular Ca^{2+} influx and upregulated the expression of secretagogin and MMP-2/9 by activating the P2X7 receptor (P2X7R). We also found that the Panx1-ATP-P2X7R signaling pathway may promote GH3 cell invasion by remodeling the actin cytoskeleton.

Conclusion

Taken together, our findings point to a pivotal role of Panx1 in promoting PA invasion, indicating its use as a potential therapeutic target for invasive PA.

Introduction

Pannexin-1 (Panx1) is a type of membrane channel located in cellular membranes, and has unique conductance properties ranging from non-selective ion permeability to extracellular release of signaling molecules (e.g. ATP, amino acids and glucose) [1–3]. In the central nervous system (CNS), Panx1 is expressed in both neurons and glial cells, and is thought to mediate intercellular connections and interactions. A growing body of research has implicated Panx1-mediated pathological activity in diseases, including epilepsy, stroke, migraine, tumors, and cognitive impairment [4–6].

Interestingly, Panx1 has also been mechanistically implicated in the pathogenesis of different tumor types. For example, Panx1 plays an antitumor role in gliomas of the brain [7], whereas it plays a tumor-promoting role in melanoma of the skin [8]. These effects may result from differences in the specific tumor origin and tumor microenvironment. Panx1 exerts its physiological function mainly through the formation of molecular channels in the cell membrane rather than intercellular gap-connecting channels, which leads to the exchange of metabolites with the extracellular tumor microenvironment [9]. The distribution and expression of Panx1 in the pituitary gland was first reported in 2011, where Panx1 was predominantly expressed in anterior pituitary cells and mediated the release of ATP into the extracellular medium [10]. However, whether Panx1 affects the development of PA remains unknown. In the current study, we focus on the underlying mechanism by which Panx1 affects the progression of PA and propose a new point for targeting Panx1 in PA therapy.

Methods

Patients and tumor samples

A total of 56 surgical tumor samples were obtained from PA patients who underwent transsphenoidal lumpectomy in Department of Neurosurgery, Second Affiliated Hospital of Army Medical University from 2017 to 2019. Their ages ranged from 22 to 76 year, with a mean age of 45.05 year. The tumor volumes were between 0.4 cm × 0.2 cm × 0.3 cm and 5.9 cm × 6.3 cm × 5.5 cm, with a mean volume of 37.9 cm^3. The tumor tissues were taken during surgery and were rapidly frozen in liquid nitrogen and subsequently stored at -80°C. Tumor samples were divided into 3 groups according to postoperative pathology, preoperative hormone levels, and clinical manifestation. Moreover, according to Knosp's classification, the degree of invasion of the 56 specimens was classified into 4 grades: I to IV degree. Clinical data about patient sex, age, and PA subtype are listed in Supplementary file 1: Table S1.

Cell culture and transfection

Rat MMQ cells (ATCC, CRL-10609) and GH3 cells (ATCC, CCL-82.1) were originally purchased from American Type Culture Collection (ATCC), cultured in an atmosphere with 5% CO_2 at 37°C, and maintained in Ham's F-12K medium (Hyclone, sh30526.01) containing 15% horse serum (MRC, Shanghai,China, CCS30035.01), 2.5% fetal bovine serum (BioInd, 04-001-1A) and 1% Penicillin-Streptomycin Solution (Beyotime, Shanghai, China, C0222). To obtain Panx1-OE stable clones, first, GH3 and MMQ cells were transfected with Panx1-OE lentiviral vector and a control vector, respectively. Cells were then cultivated in puromycin (5 μg/mL) for 48 h and the isolated clones were expanded. Subsequently, the transfection efficiency was assessed by RT-qPCR and western blotting (Fig. 1C).

RT-qPCR

The total RNA of PA samples was extracted by using TRIzol Reagent (Takara, Dalian, China, 9180); RNA standard of human pituitary gland was purchased from Takara (Clontech, 636157), the reverse transcription reaction was conducted using 2 μg total RNA with a PrimeScript RT Reagent Kit (TaKaRa,

PR047A). The cDNA samples were used for qPCR with TB Green Premix Ex Taq II (TaKaRa, RR802A) and the CFX96 Real-time System (Bio-Rad Laboratories). The relative expressions were calculated by the $2^{-\triangle\triangle ct}$ method; the primers used in the study are listed in Supplementary file 1: Table S2. Standard curves showed that the amplification efficiency of every gene was between 90% and 110% (Supplementary file 2: Fig S1).

Western blotting

Total protein was extracted using a total protein extraction kit (BestBio, BB3101). 40 μg of protein was subjected and separated by Tris-glycine SDS-PAGE (Boster, Wuhan, China, AR0138) and then transferred onto PDVF membranes (Millipore, ISEQ00010). After blocking in QuickBlock solution, the membranes were incubated overnight at 4°C with the following primary antibodies: Pannexin-1 (1:1000, Abcam, ab233479), MMP-2 (1:1000, ab92536), MMP-9 (1:1000, Abcam, ab76003), and GAPDH (1:5000, Abcam, ab181602). On the second day, after washing three times with TBST, the membranes were deposited in secondary antibodies (1:10000, abcam, ab205718). Eventually, the antigen/antibody complexes were detected by an ECL chemiluminescence kit (Bio-Rad).

Immunohistochemistry (IHC)

PA tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Each section of 6 μm was prepared for subsequent IHC staining, and the anti-Panx1 antibody was used according to manufacturer's instruction. According to the standard protocols, negative controls were prepared by incubation with a specific control mouse IgG, with no incubation with primary antibody. By using a semiquantitative method, the positive cells in every specimen were evaluated within the scope of IR, staining intensity was graded as follows: absent, 0, weak, 1, moderate, 2 and strong, 3, these scores were calculated into an average of selected samples.

CCK-8 assay

Cell proliferation was assessed in Panx1-OE GH3 cells and MMQ cells; cells were seeded in 96-wells plates (1×10^4 cells/well and 5×10^3, respectively), and 10 μL of Cell Counting Kit-8 (CCK-8, Dojindo, CK04) was added into each well at timepoints of 0 h, 24 h, 48 h and 72 h, and left to incubate for 2 h at 37°C. Absorbance was measured at 450 nm according the manufacturer's instruction.

Transwell assay

Approximately 5×10^4 cells were suspended in serum-free medium for 24 h and then seeded into Matrigel (Corning, 356234) precoated transwell chambers (Costar, 3422), with 500 μL of F-12K complete medium added into the lower chamber as a chemoattractant. After incubation for 48 h, cells on the other side of the chamber were stained with crystal violet staining solution (Beyotime, C0121) and at least five random fields were imaged under a microscope at 200 × magnification.

ATP measurement

Panx1-OE GH3 cells and controls were suspended in six-wells plates with F-12K complete medium for 48 h, then incubated for 30 min with probenecid (PBN, MedchemExpress, HY-B0545). Supernatants were collected through centrifugation at a speed of 1500 rpm at 0°C, and a nATP bioluminescence kit was used to assess the ATP concentration according to standard protocol.

Fluo-4AM Assay

The fluorescence indicator Fluo-4AM (Beyotime, Shanghai, China, S1060) was used to measure the change of free intracellular calcium concentration. Cells were seeded into glass-bottom dishes and washed three times with HBSS. 200 μL Fluo-4AM working solution was then added and incubated at 37°C for 30 min, followed by three washes with HBSS. Cells were then incubated for an additional 10 min in HBSS. After treatment with agonists and antagonists, laser confocal microscopy (Leica, SP5, Germany) was used to detect Fluo-4 fluorescence signals, and the averaged Fluo-4 fluorescence signal was acquired by three separate experiments.

Co-immunoprecipitation

anx1-OE GH3 cells and an IP/Co-IP kit (absin, abs955, Shanghai, China) were used to assess the relationship between Panx1 and Arp3. Co-IP was performed according to standard instructions. The total protein was extracted and preabsorbed with 20 μL of protein A/G Sepharose (50%) for 15 min, then IP was performed using 4 μg/mL anti-FLAG at 4°C for 1 h and incubated with 50 μL of A/G Sepharose overnight. After boiling, the entire supernatant was collected for western blotting analysis.

Immunofluorescence analysis

Actin-Tracker Green (Beyotime, Shanghai, China, C1033) was diluted with Immunol Fluorescence Staining Secondary Antibody Dilution Buffer (Beyotime, Shanghai, China, P0108) to 1:100. Approximately 5×10^3 cells were seeded in glass-bottom cell culture dishes (NEST, Wuxi, China, 801001), then fixed with 4% paraformaldehyde for 30 min and incubated in diluted Actin-Tracker Green for 1 h at 25℃ in the dark. After washing with PBS three times, the dish was incubated with Antifade Mounting Medium with DAPI (Beyotime, Shanghai, China, P0131). Actin viability with different drug treatments was determined using fluorescence microscopy.

Metabolomics analysis

Cells were divided into two groups: Panx1-OE GH3 cells (n = 6) and wide GH3 cells (n = 6). Pre-cooled extractant (80% methanol in water) was added, samples were vortexed for 2 min, snap frozen in liquid nitrogen for 5 min, then thawed, centrifuged for 20 min, and the supernatant was transferred into sample bottles for LC-MS/MS analysis. The data acquisition instrument system mainly included ultra performance liquid chromatography (UPLC) and tandem mass spectrometry (MS/MS). Based on the self-built target standard database metware database (MWDB), qualitative analysis was carried out according to the retention time (RT) of the detected substance, the information of the daughter and parent ion pairs, and the secondary spectrum data. Analyst1.6.3 software was then used to process the mass spectrometry data.

Statistical analysis

Data are expressed as mean ± standard error of the means unless otherwise stated. Differences among distributed groups were tested using a 2-tailed unpaired Student's t-test or 1-way ANOVA followed by post hoc Holm-Sidak test when multiple groups were compared. Significance testing was performed using GraphPad Prism 8.2.1 software (GraphPad, United States). Differences were considered significant statistically where $p < 0.05$.

Results

Panx1 expression was upregulated in invasive PA samples

We collected 56 surgical PA samples and divided them into invasive and noninvasive groups according to Knosp classification. The expression of Panx1 in the two groups was detected by RT-qPCR, and the results showed that the expression level of Panx1 mRNA was significantly upregulated in the invasive group when compared with the non-invasive group (Fig. 1A). Similarly, IHC results showed that Panx1 protein mainly expressed in the cytoplasm and membrane of tumor cells, and the invasive tumors presented with high staining intensity (Fig. 1B, left panel). Further statistical results confirmed significantly upregulated expression of Panx1 protein in invasive PA samples (Fig. 1B, right panel), which supported an important role for Panx1 in PA invasion.

Panx1-OE promoted the invasion of PA cell lines

To further investigated the role of Panx1 in the invasive PA, we established a model of Panx1-OE PA cells using GH3 and MMQ cell lines, RT-qPCR and western blotting confirmed stable upregulation of Panx1 mRNA and protein in Panx1-OE cells compared with vector cells (Fig. 1C). Subsequently, CCK-8 assays were performed to investigate whether Panx1 impacts the proliferation of GH3 cells. Probenecid (PBN), a specific and selective Panx1 channel inhibitor [11], was added to the Panx1-OE GH3 cells, MMQ cells and Vector. The results demonstrated that neither Panx1 nor PBN could impact the proliferation of PA cell lines (Fig. 1D). Then, transwell assays were performed to investigate whether Panx1 impact the invasion of GH3 and MMQ cells. We found that compared with Vector, Panx1-OE remarkably promoted invasion of GH3 cells and PBN could reverse the effect (Fig. 1E). Likewise, Panx1-OE also promoted the invasion of MMQ cells (Fig. 1F).

Panx1-OE triggered a series of metabolites changes

It has been well established that Panx1 acts as membrane channel, resulting intracellular metabolite release and extracellular metabolite influx. The up-regulated expression of Panx1 mediated changes of intracellular and extracellular metabolites, which may cause dysregulated cellular biological behaviors [12]. To investigate the mechanism by which Panx1 promoted the invasion of PA cells, we used

metabolomics analysis to assess differences in metabolites between Panx1-OE GH3 cells and wild-type GH3 cells. We found that levels of various metabolites were significantly increased in Panx1-OE GH3 cells, including adenosine, L-lactate, pyruvic acid, dUMP, L-alanine and glyceraldehyde 3-phosphate, while levels of ATP and uracil were decreased (Fig. 2). Based on KEGG pathway analysis, the differentially expressed metabolites were mainly enriched in 12 pathways with a p value<0.05 and gene count ≥ 3, of which the enrichment ratio of four pathways were at least 12, including the Warburg effect, pentose phosphate pathway and gluconeogenesis (Fig. 3A). In addition, according to KEGG pathway analysis, pathways could be divided into 5 groups including organismal systems (e.g. glucagon signaling pathway, insulin secretion and prolactin signaling pathway), metabolism (e.g. metabolic pathway, glycolysis/gluconeogenesis), human diseases (e.g. type II diabetes mellitus, Parkinson's disease), genetic information processing (sulfur relay system) and environment information processing (HIF-1, cAMP, and AMPK signaling pathway and ABC transporters) (Fig. 3B).

Panx1-mediated ATP release promoted GH3 cell invasion by activating P2X7R

It has been reported in many cells, including various tumor cells, that Panx1 could promote cell migration by releasing ATP to the extracellular space [13]. According to the results of our metabolomics analysis, which showed downregulation of intracellular ATP levels, we proposed the hypothesis that Panx1 could promote invasion of GH3 cells by releasing ATP to the extracellular matrix. We measured the extracellular ATP concentration and found that Panx1-OE could significantly increase extracellular ATP concentration; nevertheless, when incubated with PBN, extracellular ATP of Panx1-OE GH3 cells decreased obviously (Fig. 4A). Subsequently, we further investigated whether ATP could influence the invasion of GH3 cells. Compared with Panx1-OE GH3 cells group, when added ATPase (MedchemExpress, HY-19808) into Panx1-OE GH3 cells group, the effect of Panx1-OE on invasion of GH3 cells decreased. Moreover, BzATP trithylammonium salt (BzATP), a kind of ATP analogue, was used to activate P2XR in Vector GH3 cells group and exerted an invasive effect on GH3 cell similar to the Panx1-OE group (Fig. 4B). It has been reported that the role of ATP as an extracellular transmitter of cell surface receptors is mediated by ATP-gated ionotype P2XR to purinergic transmission, which is believed to have a wide range of important functions, including activation of intracellular signaling pathways to trigger a series of physiological and pathological changes [14-16]. We then investigated which type of receptor predominantly affected the invasion of GH3 cells. When treated with PPADS tetrasodium (PPADS, broad-spectrum P2XR antagonist), 5-BDBD (P2X4R specific antagonist) and JNJ-47965567 (JNJ, P2X7R specific antagonist), the number of invasive Panx1-OE GH3 cells was decreased, especially in the JNJ-treated group (Fig. 4C, left panel). This was also confirmed by statistical analysis of cell number (Fig. 4C, right panel), which indicated that P2X7R plays a more important role in the promotion of GH3 cell invasion by Panx1.

It has been reported that Panx1 could directly interact with actin-related protein 3 (Arp3) to impact the actin cytoskeleton and invasion of tumor cells [17-18]. Therefore, we detected the relationship between Panx1 and Arp3, and the Co-IP results showed that Panx1 might not straightly interact with Arp3 in GH3 cells (Fig. 4D). Moreover, to further investigate whether Panx1 could influence the expression of Arp3

protein through ATP-P2X7R pathway, we assessed the level of Arp3 protein after adding PBN, JNJ, and BzATP into GH3 cells respectively. The results illustrated that the protein levels of Arp3 did not change regardless of the antagonists or agonists added to GH3 cells (Fig. 4E).

Activated P2X7R facilitated Ca^{2+} influx and increased MMP-2/9 levels

P2X7R could be activated by ATP and open a pore on the cell membrane for influx of sodium and calcium ions, and efflux of potassium ions, allowing small molecules to pass freely. This could further affect the stability of cell membrane skeleton and fluidity [19]. In our study, we found that antagonists (PBN, JNJ and ATPase) inhibited Ca^{2+} influx into cytoplasm, while BzATP could trigger Ca^{2+} influx (Fig. 5A). It is well known that cytosolic Ca^{2+} is an important secondary messenger and regulates several cell functions via Ca^{2+} binding proteins. We detected two general Ca^{2+} binding proteins, secretagogin and calmodulin kinase II (CaMK II). Western blotting results confirmed that expression of secretagogin protein levels in Panx1-OE GH3 cells obviously increased compared with vector groups. PBN, JNJ, and ATPase could reverse this effect in Panx1-OE GH3 cells. Similarly, the expression of secretagogin was significantly upregulated in the vector GH3 cells after addition of BzATP to the medium (Fig 5B). However, the expression of CaMK II was not affected by the above factors (Fig 5B). We further detected the expression of matrix metalloproteinases (MMPs), which have been confirmed to play an important role in PA invasion [20-21]. As a result, the concentration of MMP-2 and MMP-9 proteins was assessed in GH3 cells when overexpressing Panx1, and when incubated in agonists and antagonists, respectively. The results showed that MMP-2 and MMP-9 increased respond to Panx1-OE and P2X7R activation; in contrast, they decreased when PBN, ATPase or JNJ were added (Fig. 5C). These findings suggest that regulation of Ca^{2+} influx by Panx1 may influence the expression of secretagogin, thereby promoting invasion of GH3 cells.

The Panx1-ATP-P2X7R signaling pathway might promote invasion of GH3 cells by remodeling the actin cytoskeleton

Tumor cell migration is a multi-stage process, in which the actin cytoskeleton participates through actin polymerization or depolymerization, cell adhesion, and myosin contraction. The actin cytoskeleton includes globular actin (G-actin) and actin filaments (F-actin). G-actin is a type of monomeric protein, which requires Ca^{2+} binding and induces the formation of F-actin, further influencing cell migration capacity [22-24]. To investigate whether the Panx1-ATP-P2X7R signaling pathway could modulate the actin cytoskeletal network, immunofluorescence staining was used, and suggested that actin cytoskeleton of Panx1-OE GH3 cells group was significantly more robust and tight, while Vector group cells presented a moderate and loose actin network. When adding PBN, ATPase, and JNJ to block the *Panx1-ATP-P2X7R* signal, the effect of Panx1-OE on the actin cytoskeleton could be reversed, while BzATP functioned similarly to Panx1-OE to make actin cytoskeleton become more robust (Fig. 6A). However, the expression of actin protein levels were not affected by Panx1, PBN, JNJ, and BzATP. (Fig. 6B). Reports have shown that cytochalasin B (cyto B) mainly acts on the cytoskeleton, promotes its depolymerization, destroys its structure, and affects cell proliferation, apoptosis, and movement [25-26].

Subsequently, in order to verify whether the status of the actin cytoskeleton related to GH3 cell invasion, cytoB was used to destroy the actin cytoskeletal structure and invasion of GH3 cells was assessed by transwell assay. The results showed that invasion of Panx1-OE GH3 cells decreased sharply when cultured with cyto B (Fig. 6C), indicating the importance of the actin cytoskeleton in the invasion of PA.

Discussion

Patients with invasive PA present poor clinical outcomes; therefore, it was essential to clarify the mechanisms underlying the invasion process. In our study, we initially found that Panx1 was highly expressed in invasive PA samples, and Panx1-OE promoted invasion of GH3 and MMQ PA cell lines significantly. Further, results of the metabolomics analysis showed that Panx1-OE mediates the dysregulation of various metabolites, altering several cellular metabolic pathways. Among these metabolites, we focused on ATP and found that Panx1-OE promoted ATP release in GH3 cells. ATP then enhanced the invasion of GH3 cells by activating P2X7R, with the underlying mechanism that extracellular Ca^{2+} influx activated the downstream secretagogin and MMP-2/9. Additionally, the Panx1-ATP-P2X7R pathway might promote invasion of GH3 cells by reorganizing the actin cytoskeleton. Above all, our findings suggest that Panx1 may participate in the development of invasive PA.

Over the past decade, many roles of Panx1 in physiology and pathology have emerged, such as immune cell death, cell apoptosis, autophagy, proliferation, invasion, and migration. Previous studies have shown that Panx1-OE is related to the poor prognosis of hepatocellular carcinoma (HCC), providing new clues for effective intervention in HCC metastasis. Panx1-OE promotes the invasion and migration of HCC cells by regulating EMT in vitro and in vivo [27]. Liu et al. found that the increase of Panx1 promoted the migration and invasion of testicular cancer cells, and its effect may be related to the activity of ERK1/2 kinase [28]. Similarly, in our study, we first reported that Panx1 was upregulated in invasive PA samples. In vitro, Panx1-OE promoted invasion of GH3 and MMQ PA cell lines. These results suggest that Panx1-OE plays a vital role in promoting PA invasion.

Panx1 is one of the membrane channels involved in the proliferation and invasion of tumor cells via multiple different signaling pathways. In HCC cells and breast cancer cell lines, Panx1 enhances EMT, contributing to a tumor-promoting effect and poor prognosis [27, 29]. In melanoma cell lines, Panx1 may regulate signals through the Wnt/β-catenin pathway [30–31]. In addition, as a membrane channel, Panx1 plays a key role in ATP and amino acid release, resulting in a change in cell biological behaviors [32]. Through metabolomics analysis, we found that Panx1 might influence the metabolism of GH3 cells by altering the expression of ATP, adenosine, and uracil. Furthermore, KEGG enrichment results showed that Panx1 was related to various pathways in GH3 cells. Subsequently, we demonstrated that Panx1-OE indeed increased extracellular ATP concentration, while extracellular ATP decreased as Panx1 channel was blocked by PBN; at the same time, the invasion of GH3 cells weakened as ATP decreased. Above all, Panx1 might promote invasion of GH3 cells by increasing ATP release.

ATP is an endogenous small molecule that activates downstream signaling pathways via its binding to classical P2XR or P2YR and promotes tumor invasion and metastasis [33–34]. ATP production and purinergic receptor activation have been reported to induce inflammatory cell infiltration and promote the migration of T cells [35–36]. Additionally, P2XR could promote the invasion and migration of Colon Cancer Cells via STAT3 signaling [37]. Mechanically, N-terminal tagging of P2XR affects calcium influx and dye uptake, further triggering downstream reactions [38]. Our results illustrated that P2X7R was activated and Ca^{2+} influx increased upon the increase of ATP release. Secretagogin, a type of protein that could be activated by cytosolic Ca^{2+}, further triggers various biological behaviors including cell migration and invasion. For example, secretagogin-dependent MMP-2 release from neurons regulates neuroblast migration [39]. In addition to enhancing the invasion ability of GH3 cells, we considered there might be some environmental factors that lead to the enhancement of invasion. EMT is already considered to be essential for tumors to acquire aggressive features and be able to metastasize, while matrix metalloproteinases (MMPs) are involved in normal development and pathological processes such as EMT; among these, MMP-2 and MMP-9 are the MMPs most commonly related to EMT [40]. Thus, in our study, we found that activated P2X7R triggered Ca^{2+} influx and further impacted expression of secretagogin and MMP-2/9. Additionally, recent studies have shed light on the control of actin cytoskeleton status by intracellular Ca^{2+} concentration in tumor invasion. Due to the increase of Ca^{2+} and secretagogin concentration, the organization and stability of actin filament structures would be promoted [41]. Studies have shown that hypoxia can induce remodeling of the actin cytoskeleton by regulating CAPZA1 in HCC cells. In addition, the low expression of CAPZA1 drives EMT by regulating actin cytoskeleton remodeling, thereby promoting HCC cell invasion and migration in vitro and in vivo [42]. These studies indicate that remodeling of the actin cytoskeleton is closely related to the migration and invasion of cells. Our results suggest that increased Ca^{2+} is a key determinant of cell invasion via recognition of the actin cytoskeleton.

Conclusions

Herein, we demonstrated, for the first time, that Panx1 expression is upregulated in invasive PA and it plays a key role in promoting the invasion of PA cells. ATP is released from Panx1 in an autocrine or paracrine manner, and it promotes the invasion of PA cells by activating P2X7R and enhancing Ca^{2+} influx to increase expression of secretagogin and MMP2/9 and remodel the actin cytoskeletal, as depicted in Fig. 7. Our study points to a pivotal role of the Panx1 channel in the development of PA invasiveness, indicating that Panx1 could be a potential therapeutic target for invasive PA.

Declarations

Ethics approval and consent to participate

The collection procedure of patient tissue samples in this study was approved by laboratory animal welfare and ethics committee of Xinqiao Hospital (the ethical review number: 2018-049-012). All

procedures performed involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

XT, SL and HY designed the study. XT, XZ, HY, PY, XD, QC and KF contributed to carry out PA tissues and cell experiments. XT, PY, XZ conducted the molecular biology experiment. XT, XZ, HY, XP, ZZ analyzed the data. TS and XZ wrote the manuscript. TS, SL and HY revised the manuscript. All authors read and approved the final manuscript.

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Figures

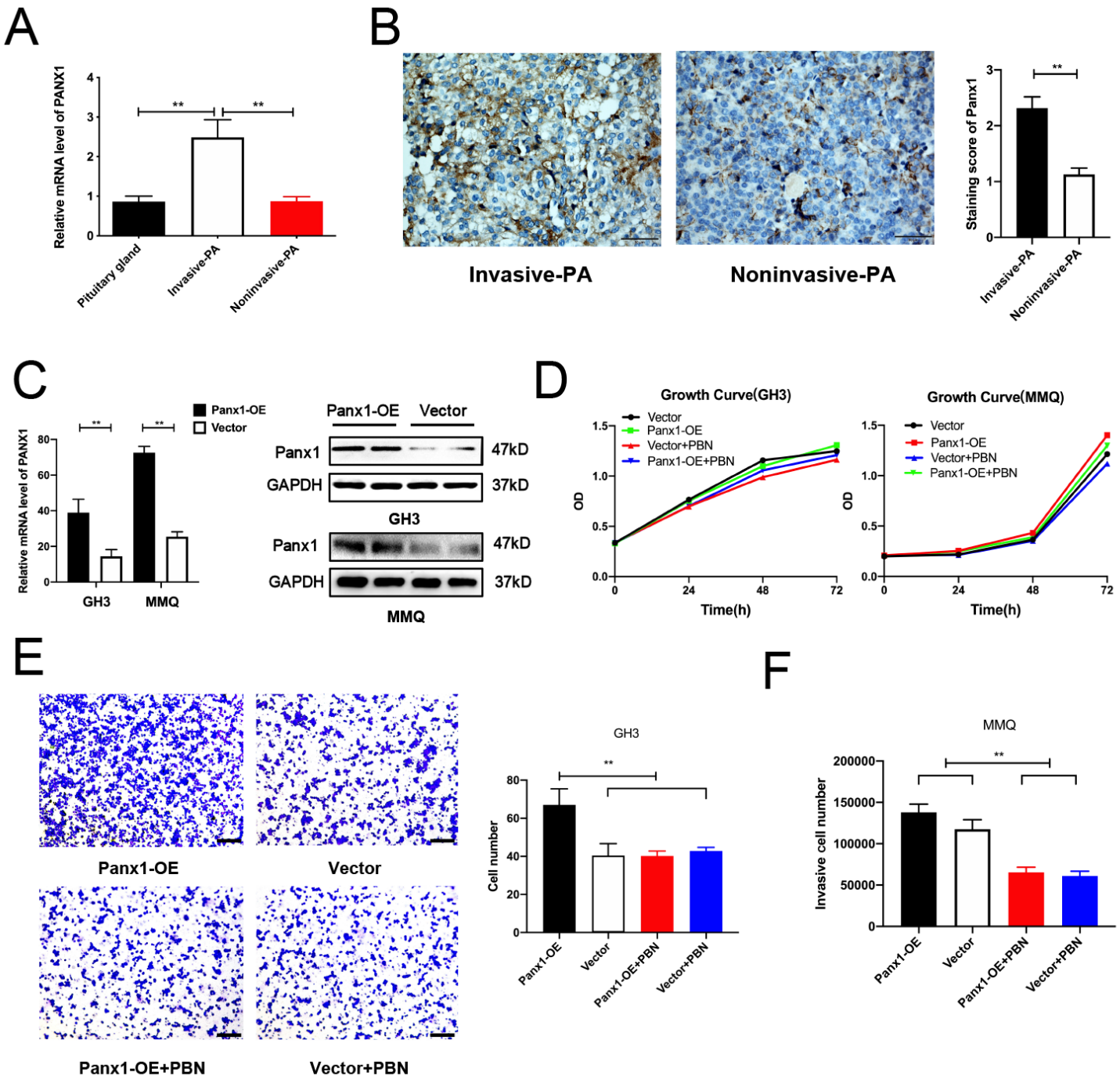


Figure 1

Expression of Panx1 in human PA samples and Panx1-OE promoted the invasion of PA cell lines. A. Expression of Panx1 mRNA in invasive PA human tissues, noninvasive PA human tissue and human pituitary gland RNA standard was evaluated by qRT-PCR. B. Expression of Panx1 protein in noninvasive PA human tissue was assessed by IHC staining (left panel). Statistical analysis of the IHC staining (right panel). Scale bar = 50 μm. C. Panx1 mRNA and protein of GH3 cells Panx1-OE and control (Vector) groups was measured by qRT-PCR and Western blotting (n = 3, ±standard error of the mean (SEM). D. Panx1-OE GH3 and MMQ cells and Vector groups were incubated with 200 μM probenecid (PBN, 200 μM)

for 0, 24, 48, and 72 h, and the proliferation of cells was assessed through a CCK-8 assay at different time points (n = 6, ±SEM). E. Invasion of Panx1-OE GH3 cells and Vector groups treated with PBN (200 μM) was assessed by transwell assay. Scale bar = 100 μm (n = 3, ±SEM). A Kruskal-Wallis test was used to evaluate statistical significance. **P<0.01. F. Invasion of Panx1-OE MMQ cells and Vector groups treated with PBN (200 μM) was assessed by transwell assay. The number of invasive MMQ cells was counted by automatic cell counter. **P<0.01.

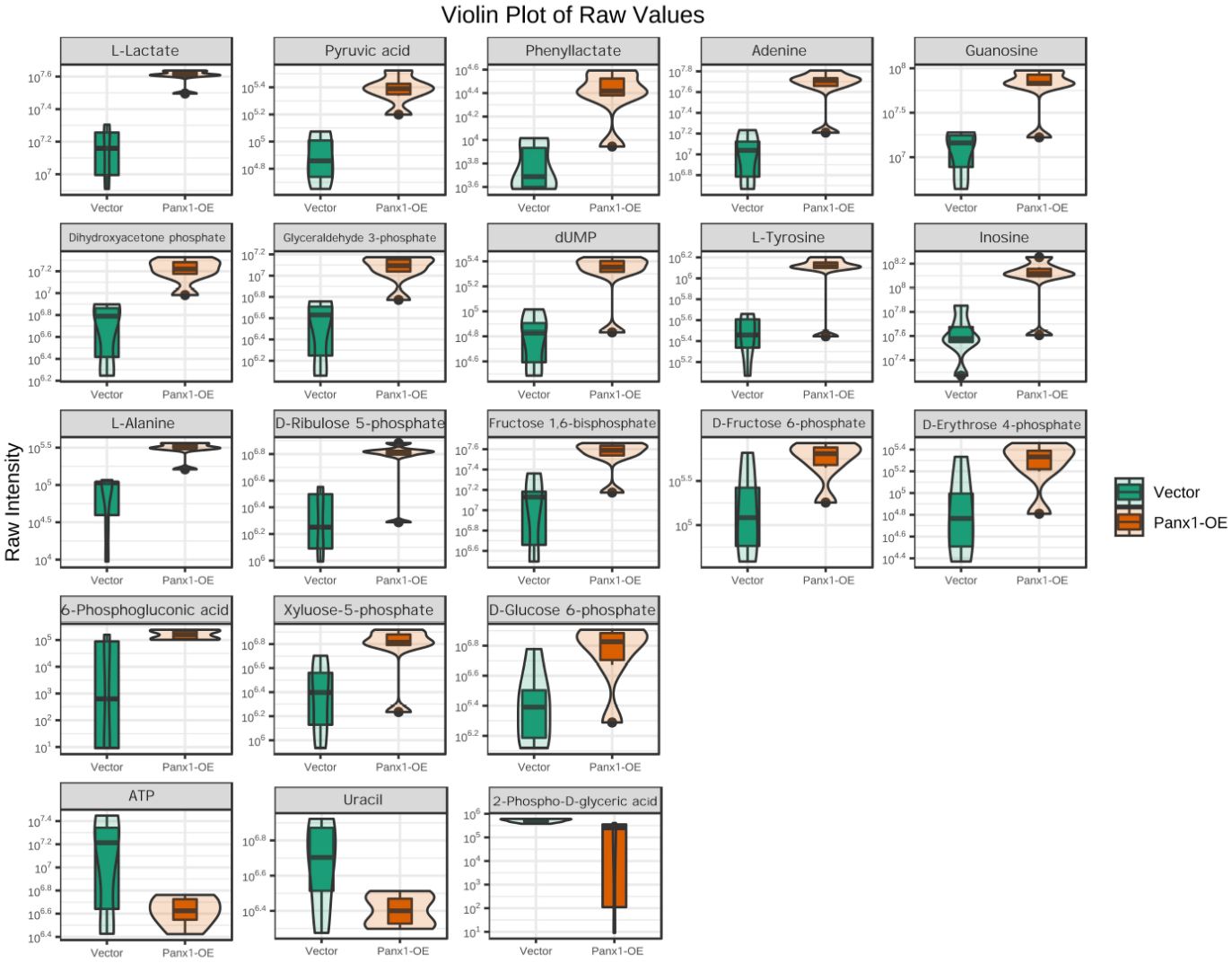
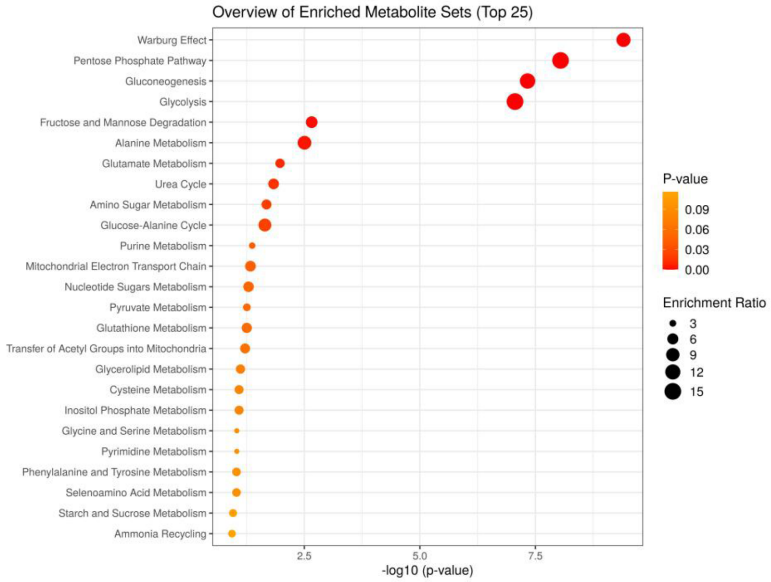


Figure 2

Full violin diagram of different metabolites between Panx1-OE GH3 cells group and Vector group. The box shape represents the interquartile range, the black line extending up and down represents the 95% confidence interval, the horizontal line in the middle is the median, and the outer shape represents the distribution density of the metabolite concentration. According to p value<0.05, the 18 top metabolites were increased in the Panx1-OE GH3 cell group and the top two of all decreased metabolites are shown in Panx1-OE GH3 cells.

A



B

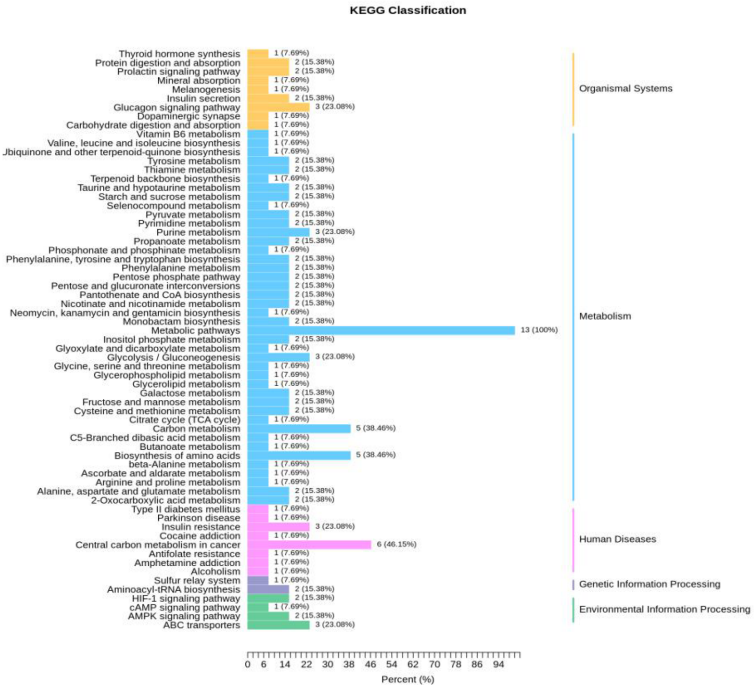


Figure 3

Statistics of KEGG enrichment (Top25) and KEGG classification. A. The size of the point represents the number of enriched differences, the color of the point is p value, the abscissa represents the rich factor of each pathway, and the ordinate is pathway name. B. The ordinate is the name of the KEGG metabolic pathway, and the abscissa is the number and proportion of metabolites in the pathway.

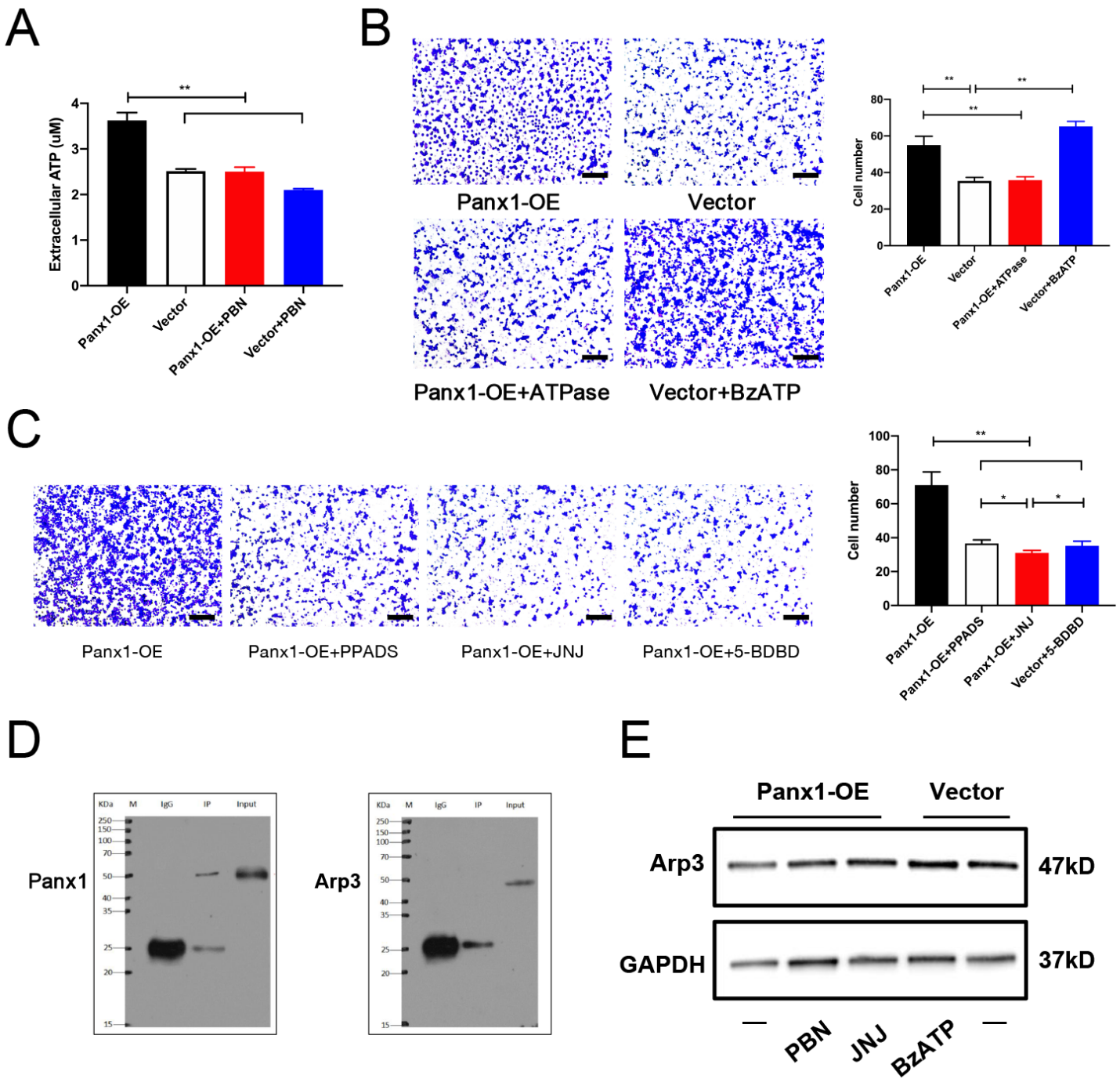


Figure 4

Panx1-mediated ATP release promoted GH3 cell invasion by activating P2X7R. A. Extracellular ATP concentrations in Panx1-OE GH3 cells and Vector groups treated with PBN (200 μM) for 10 min. B. Invasion of Panx1-OE GH3 cells and Vector groups incubated in ATPase (50 μM) and BzATP (50 μM), respectively was assessed by the transwell assay (left panel), scale bar = 100 μm. An unpaired t-test was used to evaluate statistical significance. **P<0.01. C. Invasion of Panx1-OE GH3 cells incubated in P2XR broad-spectrum antagonist PPADS (50 μM), P2X7R specific antagonist JNJ (50 μM) and P2X4R specific antagonist 5-BDBD (50 μM), respectively was assessed by transwell assay (left panel), scale bar = 100

μm (n = 3, ±standard error of the means. A Kruskal-Wallis test was used to evaluate statistical significance. **P<0.01. D. Western blotting of immunoprecipitation for Panx1 from Panx1-OE GH3 cells. Both of expected Panx1 and Arp3 bands were present at 47 kDa singly, and Arp3 was absent at the expected position in IP assay. E. The level of Arp3 protein was assessed by western blotting after adding PBN, JNJ, and BzATP into Panx1-OE GH3 cells and Vector groups, respectively.

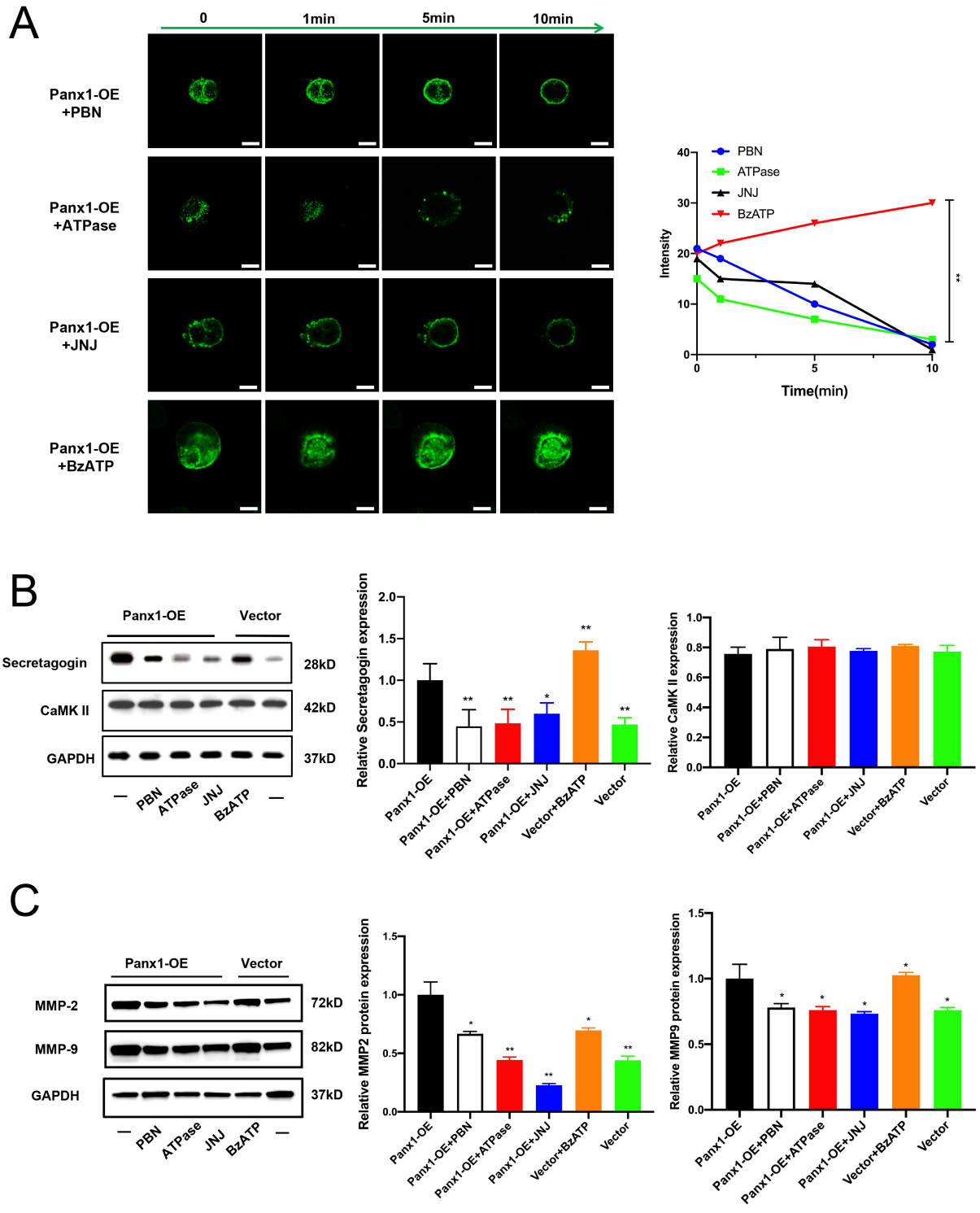


Figure 5

Activated P2X7R facilitated Ca2+ influx and increased MMP-2/9 levels. A. Live cell Ca2+ ion images were captured by confocal microscopy at 0, 1, 5, and 10 min after the addition of PBN, ATPase, JNJ, and BzATP to Panx1-OE GH3 cells, respectively. Scale bar, 400 μm (n = 3, ±standard error of the means. B. Expression of secretagogin and CaMK II was assessed by western blotting after adding PBN, JNJ, and BzATP into Panx1-OE GH3 cells and Vector groups, respectively (left panel), A Kruskal-Wallis test was used to evaluate statistical significance. **P<0.01 (right panel). C. MMP-2 and MMP-9 protein levels of Panx1-OE and Vector cells was evaluated after treatment with PBN, ATPase, JNJ, and BzATP, respectively by western blotting (left panel), A Kruskal-Wallis test was used to evaluate statistical significance.**P<0.01 (right panel).

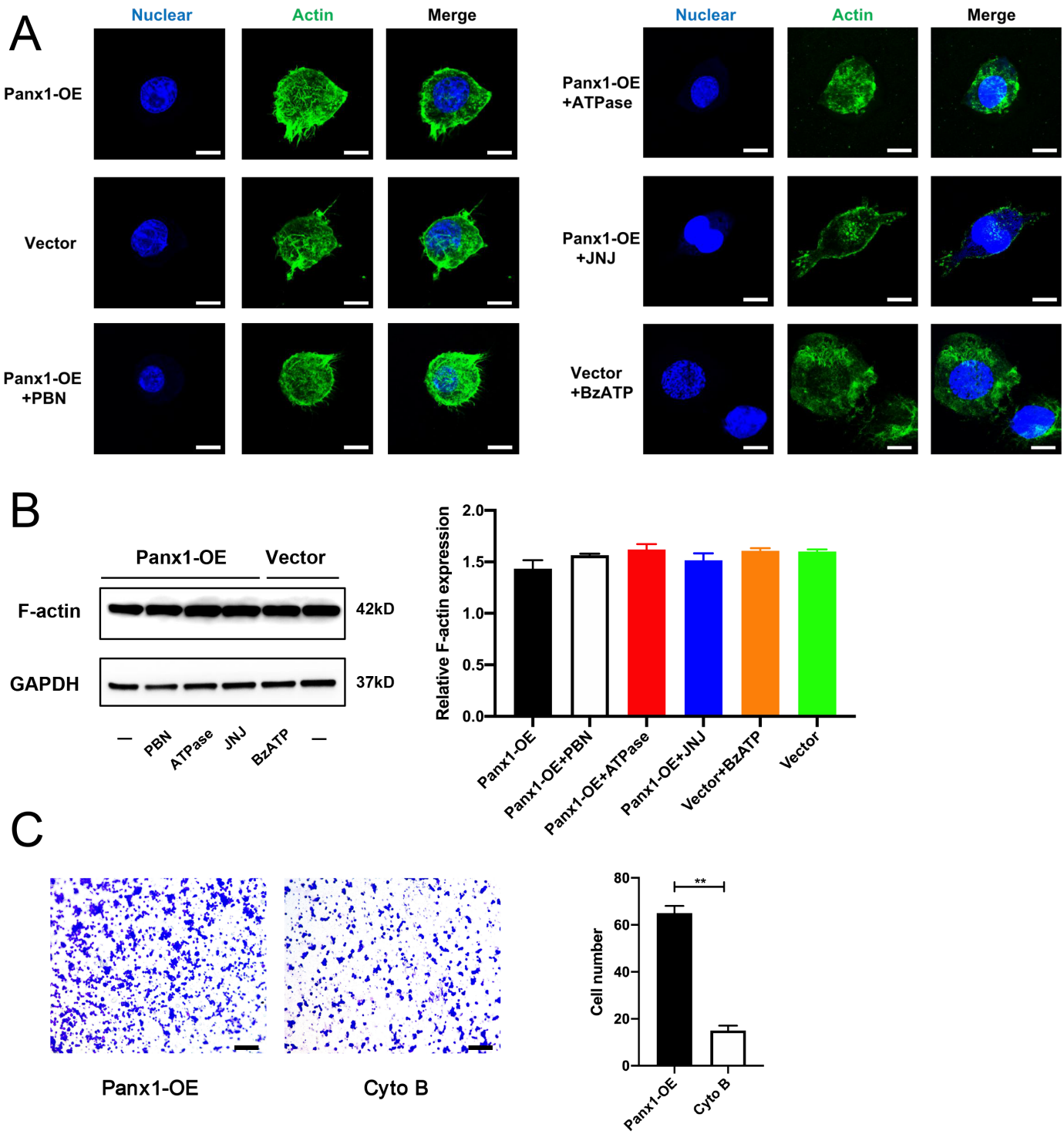


Figure 6

The Panx1-ATP-P2X7R signaling pathway might promote GH3 cell invasion by remodeling the actin cytoskeleton. A. Cell morphology and status were captured by confocal microscopy after addition of PBN, JNJ, and ATPase into Panx1-OE GH3 cells and addition of BzATP into Vector group GH3 cells. Scale bar: 400 μm (n = 3, ±standard error of the mean (SEM). B. The level of F-actin protein was assessed by western blotting after adding PBN, JNJ, and ATPase into Panx1-OE GH3 cells and adding BzATP into

Vector group, respectively (left panel), A Kruskal-Wallis test was used to evaluate statistical significance (right panel). C. The invasion of Panx1-OE GH3 cells was evaluated by transwell assay after incubating in cytochalasin B (50 μM) for 10 minutes. Scale bar, 100 μm (n = 3, ±SEM).

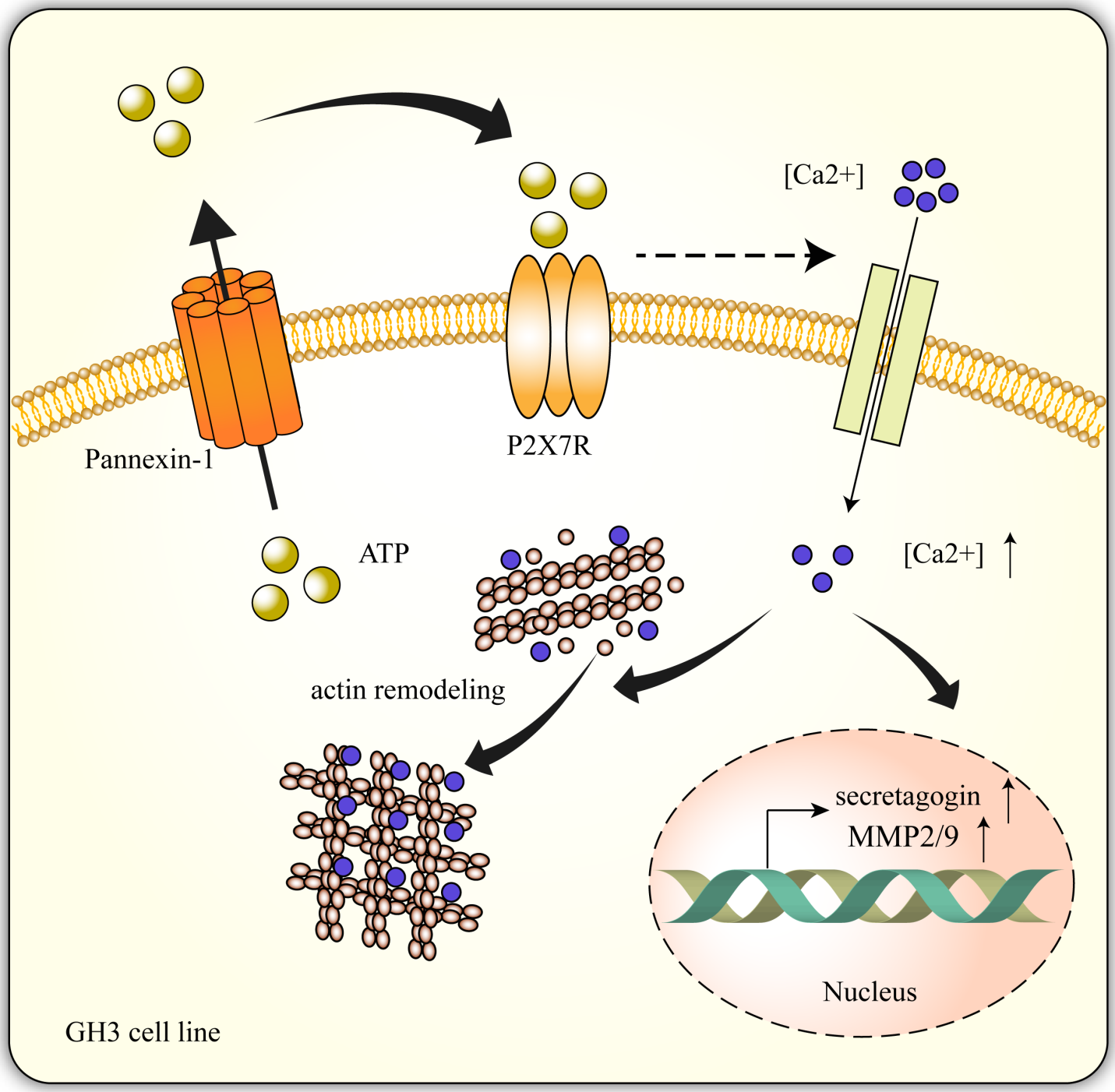


Figure 7

Diagram of the mechanism of Panx1 promoting the invasion of PA cells

Supplementary Files

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