Girdin regulates both migration and angiogenesis in pancreatic cancer cell lines

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Abstract. Girdin, an actin-binding protein, is reportedly involved in the invasion and angiogenesis of various cancers. It has been suggested that the flavonoid Scutellarin (SCU) inhibits Girdin signaling. In the present study, the function and therapeutic applications of Girdin in pancreatic cancer (PaCa) were investigated. Immunohistochemical staining of Girdin in resected PaCa specimens from the Department of Gastroenterological Surgery, Nagoya City University Graduate School of Medical Science showed that high Girdin expression was associated with poor overall survival and relapse-free survival, as well as with T factor, indicating invasion into the surrounding tissues. On the other hand, Girdin was highly expressed in almost all PaCa cell lines, and the migration ability of Girdin-knockdown cell lines was decreased even under epidermal growth factor (EGF) stimulation. In addition, SCU suppressed PaCa cell migration by inhibiting the phosphorylation of Girdin. The expression and production of vascular endothelial growth factor A (VEGF-A) was significantly decreased in Girdin-knockdown cell lines. Furthermore, in Matrigel tube formation assays performed using culture supernatant, the lumen-forming ability of vascular endothelial cells was also decreased in Girdin-knockdown cell lines. However, SCU treatment did not significantly alter the expression or production of VEGF-A. These results suggested that Girdin is involved in EGF signaling-mediated migration of PaCa cells, that SCU inhibits PaCa invasion by suppressing Girdin activity, and that Girdin is also involved in angiogenesis via an activation pathway different from the action site of SCU. Girdin may be a prognostic biomarker, and the development of a novel molecular-targeted drugs for Girdin may improve the prognosis of PaCa in the future.

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Abbreviations: APE, Akt phosphorylation enhancer; CM, conditioned medium; EGF, epidermal growth factor; GIV, guanine nucleotide-binding protein α subunit (Gα)-interacting vesicle-associated protein; HUVEC, human umbilical vein endothelial cell; OS, overall survival; RT-qPCR, reverse transcription-quantitative PCR; RFS, relapse-free survival; STAT3, signal transducer and activator of transcription 3; VEGF-A, vascular endothelial growth factor A

Key words: girdin, scutellarin, pancreatic cancer, cancer migration, cancer angiogenesis

Introduction

Pancreatic cancer (PaCa) is one of the most intractable malignancies in the digestive system. The 5-year overall survival (OS) rate for PaCa is ~11%, one of the lowest among the various carcinomas (1). One likely reason is the high local invasive and metastatic potential in PaCa. PaCa can easily invade adjacent tissues (e.g., major blood vessels and nerves), and it is not rare for PaCa to be unresectable or to have metastasized at the time of diagnosis. Therefore, suppressing the migratory ability of cancer cells (which is an important process in tissue invasion and metastasis), is important in the treatment of PaCa. In addition, in a previous study, it was reported that there is a correlation between the metastatic potential and angiogenic potential of PaCa (2). However, the molecular biological mechanisms involved in PaCa invasion and angiogenesis have not yet been fully elucidated.

In the present study, Girdin was the protagonist, which was identified in 2005, as a novel substrate of Akt, serine/threonine kinase, a product of the proto-oncogene AKT1. Girdin is activated by Akt and is involved in the formation of lamellipodia, which are important for cell motility, through the reorganization of actin fibers that form the cytoskeleton (3-5). Girdin also has a wide range of other functions, including cell proliferation and microangiogenesis (6-8). Girdin is the product of the *CCDC88A* gene (Coiled-Coil Domain Containing 88A). It is also called Akt phosphorylation enhancer (APE) (9-11) or guanine nucleotide-binding protein α subunit (Gα)-interacting vesicle-associated protein (GIV) and is involved in a wide

variety of functions. Previously, Girdin has been reported to be involved in migration invasion and angiogenesis in some carcinomas (12,13). It has been previously reported that the expression of Girdin in esophageal carcinoma correlates with the depth and prognosis of the tumor (14). In the present study, tumor migration and angiogenesis in PaCa was the primary objective, which is more aggressive than esophageal cancer.

To the best of our knowledge, there are only a few studies on substances that inhibit Girdin. Scutellarin (SCU; PubChem ID: 185617) is a flavonoid that inhibits signal transducer and activator of transcription 3 (STAT3)/Girdin/Akt signaling in hepatocellular carcinoma, and it suppresses tumor invasion and metastasis (15). SCU is a natural compound known for its antitumor, hepatoprotective and neuroprotective effects, and has long been used in China for the treatment of cerebrovascular diseases (16). There have been studies reporting its tumor suppressive effects in other carcinomas (17-19). However, there are no studies focusing on the antitumor effects of SCU in PaCa.

In the present study, the function of Girdin in PaCa was analyzed and the role of Girdin in tumor progression was elucidated. The inhibitory effect of SCU on Girdin in PaCa and its tumor suppressive effect was also investigated.

Materials and methods

Antibodies and chemicals. The primary antibodies polyclonal rabbit anti-Girdin (cat. no. ab113890), for immunohistochemical (IHC) staining, and anti-GIV [EPR18433] (cat. no. ab179481), for western blotting, were purchased from Abcam. In addition, the polyclonal rabbit anti-phosphate-Girdin (Tyr-1746) antibody (cat. no. GP5801) was purchased from ECM Biosciences, monoclonal mouse anti-β-Actin (8H10D10) antibody (cat. no. 3700) from Cell Signaling Technology, Inc., and monoclonal mouse anti-GAPDH (0411) antibody (sc-47724) from Santa Cruz Biotechnology, Inc. The secondary antibodies used in the present study included polyclonal goat anti-rabbit immunoglobulins/HRP (cat. no. P0448), polyclonal rabbit anti-mouse immunoglobulins/HRP (P0161) (Dako A/S; Agilent Technologies, Inc.) goat anti-rabbit IgG H&L (Cy3®) (cat. no. ab6939) and goat anti-mouse IgG H&L (Alexa Fluor® 488; cat. no. ab150113; Abcam). SCU analytical standard (cat. no. 73577) was purchased from Sigma-Aldrich and recombinant human epidermal growth factor (EGF) (236-EG) was purchased from R&D Systems, Inc.

Patients and tumor samples. The patients included in the present study underwent surgery due to a diagnosis of PaCa at Nagoya City University Hospital from June 2006 to August 2016. All patients were monitored for at least 5 years after surgery, except for death and data censoring. In addition, in the study of clinicopathological characteristics, cases with no pathological findings described were excluded. The present retrospective study was approved (approval no. 60-18-0025) by the Institutional Review Board (Ethical Review Committee for Medical Research, Nagoya City University Hospital, Nagoya, Japan). Written informed consent was obtained from all patients. Clinicopathological characteristics of patients are listed in Table I.

IHC staining. IHC staining using an anti-Girdin antibody was performed in PaCa tissues obtained from surgically resected specimens at Nagoya City University Graduate School of Medical Sciences. The IHC staining procedure was performed as previously described (20).

Survival Kaplan-Meier analysis. Kaplan-Meier plots were generated based on the results of immunohistochemistry at our own institution and validated for OS and RFS with logrank test. In addition, prognostic predictions based on mRNA were also generated using the Kaplan-Meier plotter online tool (https://kmplot.com/analysis/, accessed September 8, 2020).

Cell culture. Human PaCa cell lines [MIA PaCa-2 (cat. no. CRL-1420™), SW 1990 (cat. no. CRL-2172™), AsPC-1 (cat. no. CRL-1682™), BxPC-3 (cat. no. CRL-1687™), PANC-1 (cat. no. CRL-1469™), Capan-2 (cat. no. HTB-80™) (21)] and the human pancreatic duct epithelial cell line H6c7 (cat. no. PCS-600-010) were obtained from the American Type Culture Collection (ATCC). The EA.hy926 cell line (cat. no. CRL-2922™), used as immortalized human umbilical vein endothelial cell (HUVECs) in the present study, was also purchased from ATCC. The AsPC-1 and BxPC-3 cell lines were cultured in RPMI-1640 medium, whereas the other PaCa cell lines and the EA.hy926 cell line were cultured in DMEM (both media from Sigma-Aldrich; Merck KGaA). Each medium was supplemented with 10% FBS, 10 mg/ml streptomycin, 10,000 U/ml penicillin and 25 μg/ml amphotericin B (all from Gibco; Thermo Fisher Scientific, Inc.). The H6c7 cell line was cultured in keratinocyte SFM basal medium with 2.5 μg/ml EGF and 25 mg/ml bovine pituitary extract (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 1X antibiotic-antimycotic (Gibco; Thermo Fisher Scientific, Inc.). All the cells were incubated at 37˚C in a humidified incubator containing 5% CO_2.

Immunofluorescence staining. MIA PaCa-2, AsPC-1 and PANC-1 cell lines were seeded on glass slides at $2x10^5$ cells per slide and allowed to adhere in optimal medium for 24 h. The cells were then incubated for 2 h at 37˚C in medium with or without reagents. Cells were fixed and permeabilized by incubation with cold methanol on ice for 20 min and blocked with 3% BSA (Wako Chemicals USA, Inc.) for 1 h at room temperature. Immunofluorescence staining was performed using anti-β-actin mouse antibody and anti-Girdin (phospho Y1764) rabbit antibody as primary antibodies for 1 h at room temperature. Anti-mouse goat IgG (Alexa Fluor® 488; green) and anti-rabbit goat IgG (Cy3; red) were used as secondary antibodies for 1 h at room temperature under shading, and DAPI for nuclear staining (blue) [ProLong® Gold Antifade Reagent with DAPI (cat. no. 8961; Cell Signaling Technology, Inc.) was applied a few drops per slide]. Fluorescence intensities were observed using a fluorescence microscope BZ-X710 at x100 field of view (Keyence Co., Ltd.).

Small interfering RNA (siRNA)-mediated knockdown of Girdin. Girdin was knocked down by RNA interference. Thermo Fisher's Silencer® Select pre-Designed and Validated siRNA was used. Cells were transfected with Girdin siRNA (assay ID: s31296, cat. no. 4392420) or negative control

Table I. Association of Girdin expression with clinicopathological characteristics. The clinicopathological characteristics of the two patient groups whose tumors stained strongly and weakly in immunohistochemistry for Girdin. Pathological findings were not available in two cases, and a total of 88 cases were analyzed (Mean age: Mann-Whitney U test, Others: Fisher's exact test).

	Expression level of Girdin		
Clinicopathological characteristics	Weakly stained (n=28)	Strongly stained (n=60)	P-value
Sex			
Male	21	40	
Female	7	20	0.47
Mean age, years	69	67	0.48
IQR, years	63-74	60-75	
TNM factors			
T1/2/3/4	4/3/17/4	4/3/29/24	
T1-3	24	36	
T4	4	24	0.02
N0/1/2/3	14/11/2/1	22/25/10/3	
N-	14	22	
N+	14	38	
M0/1	28/0	56/4	0.3
TNM stage			
1/2/3/4	4/2/15/7	4/2/21/33	
1-3	21	27	
4	7	33	0.01
Venous invasion			
v0/1/2/3	6/13/7/2	12/22/15/11	
v-	6	12	
v+	22	48	1
Lymphatic invasion			
ly0/1/2/3	3/19/3/3	6/33/10/11	
ly-	3	6	
ly+	25	54	1
Neural invasion			
ne0/1/2/3	3/13/9/3	15/16/11/18	
ne-	3	15	
ne+	25	45	0.16

siRNA (Select Negative Control No.1, cat. no. 4390844) using Lipofectamine® RNAiMAX (Both reagents were purchased from Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The sequences of siRNAs were not available from the supplier due to confidentiality issues. Concretely, the transfection of siRNA into cells was performed as follows: $1x10^6$ cells were seeded per well using Falcon six-well Clear Flat Bottom TC-treated Multiwell Cell Culture Plates. A total of 10^6 cells were seeded and left overnight to settle, then 25 mol siRNA and 7.5 μl Lipofectamine RNAiMAX per well were mixed at room temperature (Opti-MEM medium was used as solvent). After 5 min, the cells were added to each well and incubated for 48 h before being used as a knockdown cell line.

Reverse transcription-quantitative PCR (RT-PCR) analysis. The total RNA extraction from cells, purification of cDNA obtained by reverse transcription, and subsequent real-time PCR procedures were performed as previously described (22). The primer probes used were from TaqMan Gene Expression Assays and were purchased from Thermo Fisher Scientific, Inc. (*CCDC88A*: H01554974_m1, *GAPDH*: Hs9999999905_m1, *VEGF-A*: Hs00900055_m1).

Western blotting. Proteins were extracted from cultured cells (H6c7, MIA PaCa-2, SW 1990, AsPC-1, BxPC-3, PANC-1 and Capan-2) using RIPA buffer containing Protease Inhibitor Single Use Cocktail and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The protein concentrations were measured using the Pierce BCA protein assay kit (Thermo Fisher Scientific, Inc.). A total of 20 μg of each protein extract was denatured at 90˚C for 5 min and separated on 10% Mini-PROTEAN TGX Precast gels (Bio-Rad Laboratories, Inc.). The protein bands were transferred to nitrocellulose membranes and blocked in iBind Flex Solution (iBind Flex Buffer, iBind Flex Additive and distilled water; Thermo Fisher Scientific, Inc.) for 15 min at room temperature. The primary and secondary antibody reactions were performed using the iBind Flex Western System (Thermo Fisher Scientific, Inc.) for 3 h at room temperature according to the manufacturer's instructions. The membranes were incubated with anti-GIV (EPR18433; (1:1,000), polyclonal rabbit anti-phosphate-Girdin (Tyr-1746; 1:500), or anti-GAPDH (0411) (1:2,000) primary antibody, followed by polyclonal HRP-conjugated goat anti-rabbit or rabbit anti-mouse secondary antibody (1:2,000). The protein-antibody complexes were visualized using SuperSignal West Pico Chemiluminescent Substrate, SuperSignal West Femto Chemiluminescent Substrate, or Pierce ECL Western Blotting Substrate (all from Thermo Fisher Scientific, Inc.). The immunoreactive protein bands were detected using the Amersham Imager 600 (Cytiva), and the densities of the detected bands were calculated using ImageJ software 1.52v (National Institutes of Health).

In vitro cell migration assays (Boyden chamber assays) and wound healing assays. Transwell assays were performed by the Boyden double chamber method using Falcon 24-well Clear Flat Bottom TC-treated Multiwell Cell Culture Plates and Falcon Cell Culture Inserts (pore size, 8.0-μm diameter). After knockdown of Girdin in MIA PaCa-2, AsPC-1 and PANC-1, the cells were seeded into the upper chamber at 50,000 cells/insert. The bottom chamber was loaded with medium containing 10% FBS. In the EGF-stimulated group, 0.1 ng/ml EGF was added. After 24 h of incubation at 37˚C, the cells on the upper surface of the insert filter were removed, and the cells migrating to the bottom side of the insert were fixed and stained using the Diff-Quik staining kit (Dade Behring; Siemens Healthineers) (~3 min total, at room temperature).

The migration cells were quantified by counting in nine randomly selected high-power (x200) fields of view using a compound light microscope.

Wound healing assays were performed as follows: The treated cells were seeded in Falcon 24-well plates at 100,000 cells/well so that the cells were relatively dense, and after 24 h, the monolayer was scratched using a pipette tip. Following 48 h of incubation at 37˚C with medium containing 2% FBS and reagents, the scratched gaps were measured in low-power (magnification, x40) fields of view with a phase contrast microscope (BZ-X710, Keyence Co., Ltd.). For quantification, the company's optional Measurement Module (BZ-H3M) was used.

Enzyme-linked immunosorbent assay (ELISA). The following procedure was used to prepare the treated cell supernatant. Each PaCa cell line was seeded at 50,000 cells/well in six-well plates and incubated overnight at 37˚C. Then, the knockdown of Girdin was performed or SCU was administered, and the cell supernatant was collected after 72 h of incubation at 37˚C. The supernatant was centrifuged to remove particulate matter. ELISA was performed using the VEGF-A ELISA kit (cat. no. DVE00; R&D Systems, Inc.) according to the manufacturer's protocol.

Matrigel tube formation assay. To assess the angiogenic activity of vascular endothelial cells *in vitro*, a tube formation assay using Matrigel (Corning, Inc.) with reduced growth factors was used as previously described (23-25). The EA.hy926 cell line (immortalized vascular endothelial cells) was established by fusion of primary human umbilical vein cells with a thioguanine-resistant clone of A549 using polyethylene glycol. It has been screened for factor VIII-related antigens and has been used to evaluate angiogenic function *in vitro* (26). The conditioned medium (CM) was prepared from the culture supernatants of Girdin-knockdown PaCa cell lines (MIA PaCa-2, AsPC-1 and PANC-1), and DMEM containing 2% FBS Matrigel was applied to 96-well plates (50 μl/well). EA.hy926 cell lines were incubated in this CM. The number of endotubes was then measured by phase contrast microscopy. The assay was repeated three times for evaluation.

Cytotoxicity assay. The cytotoxicity of SCU against PaCa cell lines was evaluated using the Premix WST-1 Cell Proliferation Assay System (Takara Bio, Inc.) according to the manufacturer's protocol. MIA PaCa-2 and PANC-1 cell lines were seeded in 96-well plates at $2x10^3$ cells/100 μl/well and cultured for 1 day. Various concentrations of SCU (1-200 μM) were then added to the cells; after 72 h of incubation at 37˚C, absorbance was measured at 450 nm using a spectraMax 340 spectrophotometer (Molecular Devices, LLC).

Statistical analysis. All experimental data are shown as the mean ± SEM. Comparisons between two groups were assessed using unpaired t-tests. One-way ANOVA was used to compare multiple groups, followed by Dunnett's or Bonferroni's multiple comparison test for individual comparisons. $P<0.01$ was considered to indicate a statistically significant difference. Regarding clinical data, the age was expressed as median and quartiles, and comparisons between the groups were made using the Mann-Whitney U test. Other clinical data were analyzed using Fisher's test. All statistical analyses were performed with EZR (27), which is based on R software (https://www.r-project.org/). More precisely, it is a modified version of R commander designed to add statistical functions frequently used in biostatistics. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of Girdin in human PaCa tissues. IHC was used to evaluate the expression of Girdin in surgical specimens from 90 PaCa patients in Nagoya City University Hospital between 2006 to 2016. In all PaCa tissues, Girdin staining consistent with the cytoplasm of cancer cells was observed. The degree of staining was graded as follows: No staining was designated as '-'; staining weaker than that in isles of Langerhans was designated as '+'; staining stronger than that in islets of Langerhans was designated as '+++'; and those other than those were designated '++'. Staining intensity was determined by three independent observers blinded to the disease stage and patient outcome. The concordance rate was >90%. Differences in opinion were resolved by consensus with a fourth evaluator (Fig. 1A).

Association of Girdin expression with OS and relapse-free survival (RFS). The associations between the intensity of Girdin expression and postoperative survival of PaCa patients was investigated and the OS and RFS of patients in the weakly stained (- and +) and the strongly stained (++ and +++) groups were compared using the log-rank test. Both OS and RFS suggested a significantly poorer prognosis in the group that stained strongly for Girdin (both P=0.02) (Fig. 1B). An online database (https://kmplot.com/analysis/) was utilized to confirm the clinical significance of Girdin expression in PaCa. The gene expression data and OS and RFS sources of the Kaplan-Meier plotter website are based on NCBI Gene Expression Omnibus, The European Genome-phenome Archive and The Cancer Genome Atlas (28). The gene expression of Girdin was evaluated at the level of mRNA. As found in the present study, the online databases also showed that strong expression of Girdin in PaCa is a poor prognostic factor for both OS and RFS (OS and RFS were examined in 177 and 69 patients, respectively) (Fig. 1C).

In PaCa, high Girdin expression is associated with the invasion of cancer cells into the surrounding tissues, including major blood vessels. Clinicopathological characteristics were analyzed according to the intensity of Girdin staining in 88 PaCa patients (two patients lacking histopathological findings were excluded). At the time of surgery, there were no differences in sex or age ratios between the two groups. Evaluation was performed based on the T4 classification, which indicates the invasion into major organs adjacent to the pancreas (General Rules for the Study of PaCa in Japan). Accordingly, the progression of the cancer from Stage 4 or less than Stage 4 was also evaluated. As a result, the T factor was significantly greater in the strongly stained group than in the weakly stained group (T1-3 vs. T4, P=0.02). The TNM stage was also significantly higher in the strongly stained group (Stages 1-3 vs. Stage 4, P=0.01). However, there was no significant difference in venous invasion, lymphatic invasion, or neural invasion (Table I).

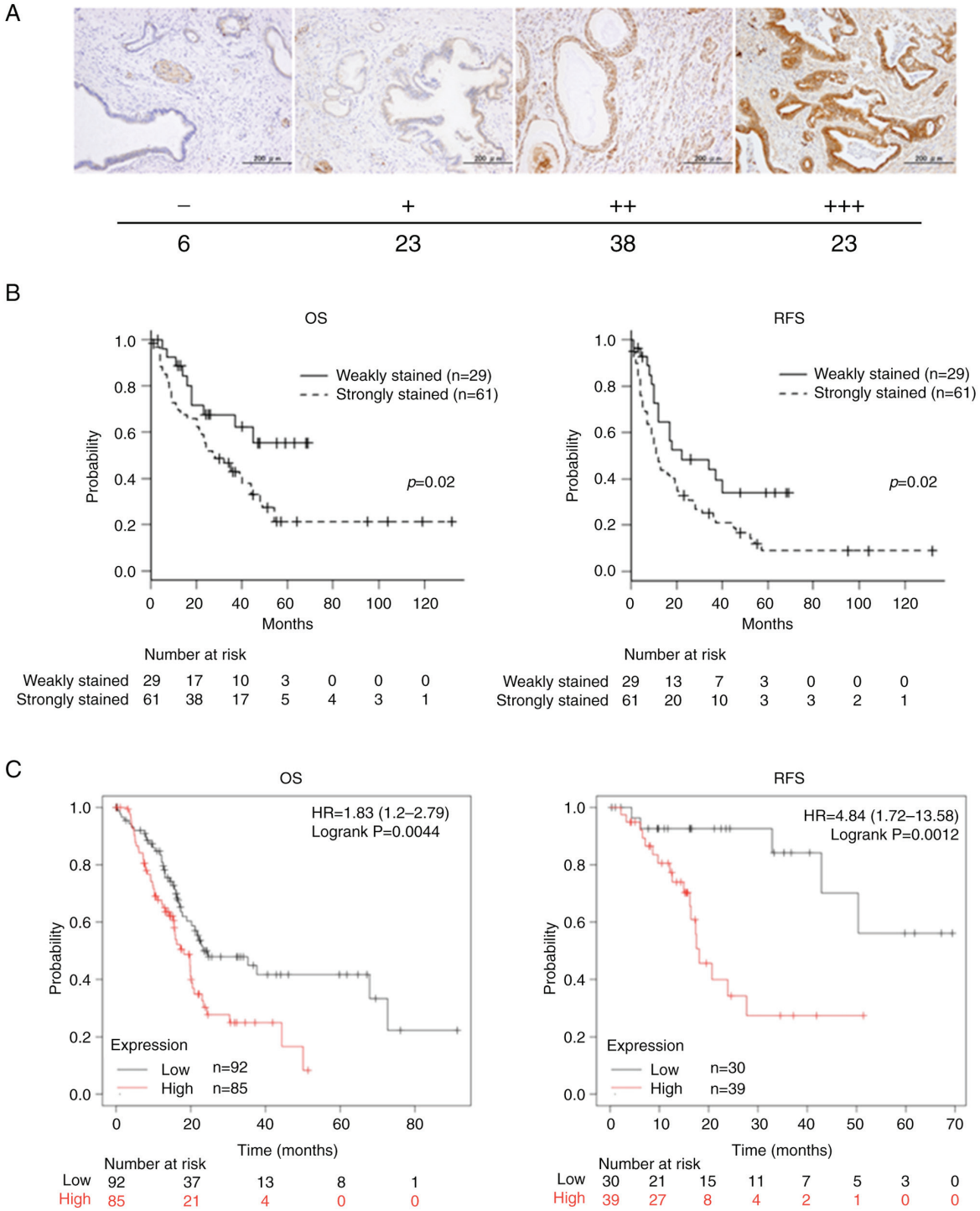

Figure 1. Relationship between Girdin expression in PaCa specimens and its prognosis. (A) IHC of tissues resected from patients with PaCa at Nagoya City University Graduate School of Medical Sciences. Paraffin-embedded PaCa tissues were analyzed for Girdin expression by IHC using an anti-Girdin antibody. In the PaCa tissues, there were differences in the staining intensity for Girdin, and they were classified into four types according to the intensity (-, +, ++ and +++). The number of cases in each of the four staining intensity categories was follows: -, 6 cases; +, 23 cases; ++, 38 cases; and +++, 23 cases. (B) Kaplan-Meier curves showing OS RFS in 90 PaCa patients at Nagoya City University Graduate School of Medical Sciences. The tissues in both survival groups were classified into the weakly stained (n=29) and the strongly stained (n=61) according to the staining intensity. (i.e. '-' and '+' represent weak staining, '++' and '+++' represent strong staining). (C) Kaplan-Meier curves showing OS and RFS of 177 and 69 PaCa patients, respectively, from the database in the Kaplan-Meier plotter website (https://kmplot.com/analysis/). PaCa, pancreatic cancer; IHC, Immunohistochemistry; OS, overall survival; RFS, relapse-free survival.

Girdin expression and the knockdown of Girdin in PaCa cells. RT-qPCR and western blotting detected Girdin expression in all cell lines, upregulation of Girdin was observed in several PaCa cell lines, particularly in MIA PaCa-2, AsPC-1 and PANC-1 cells, compared with the human pancreatic ductal epithelial cell line H6c7 (Fig. 2A and B). Although the Capan-2 cell line also had high Girdin expression, it was not used in this experiment because its proliferation cycle is longer

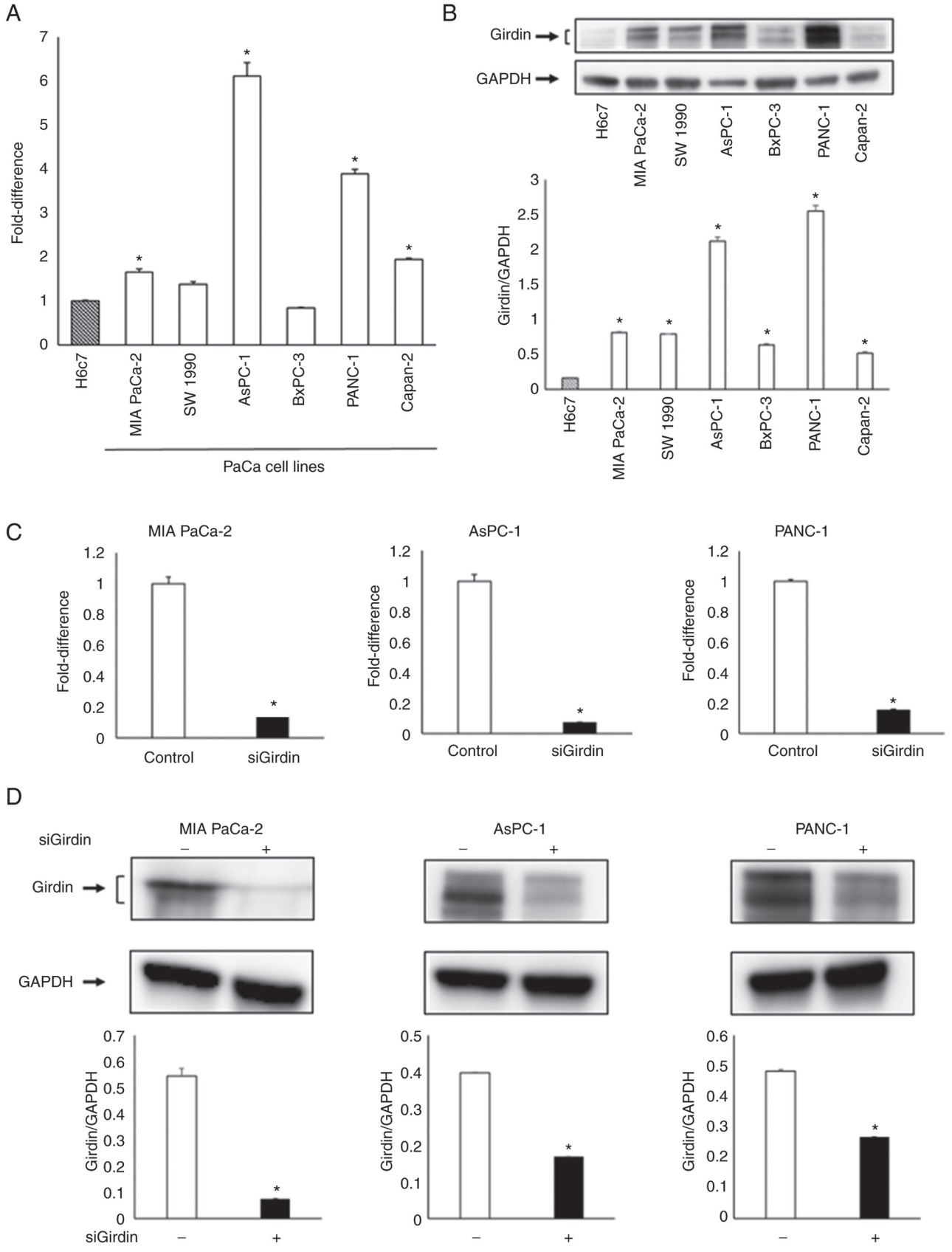

Figure 2. Expression and knockdown of Girdin in PaCa cell line. (A) Expression of Girdin mRNA in normal pancreatic and PaCa cell lines. The reverse transcription-quantitative PCR experiments were calculated using a comparative quantitative method with calibration curves. The vertical axis represents the ratio of Girdin expression to that in the human pancreatic ductal epithelial cell lines. (B) Western blotting was performed using Girdin and GAPDH antibodies. The density of bands detected was measured, and the ratio of Girdin to GAPDH was plotted. (C) Transfection of PaCa cell lines with negative control siRNA or Girdin siRNA. Girdin was knocked down by transfection of Girdin siRNA for MIA PaCa-2, AsPC-1 and PANC-1. (D) The knockdown of Girdin in these cell lines was confirmed by western blotting. The data are presented as the mean ± SEM. All experiments were performed in triplicate and repeated three times. *P<0.01 [A: One-way ANOVA with Dunnett's multiple comparison test. B: Student's t-test (unpaired), two-tailed]. PaCa, pancreatic cancer; siRNA, small interfering RNA.

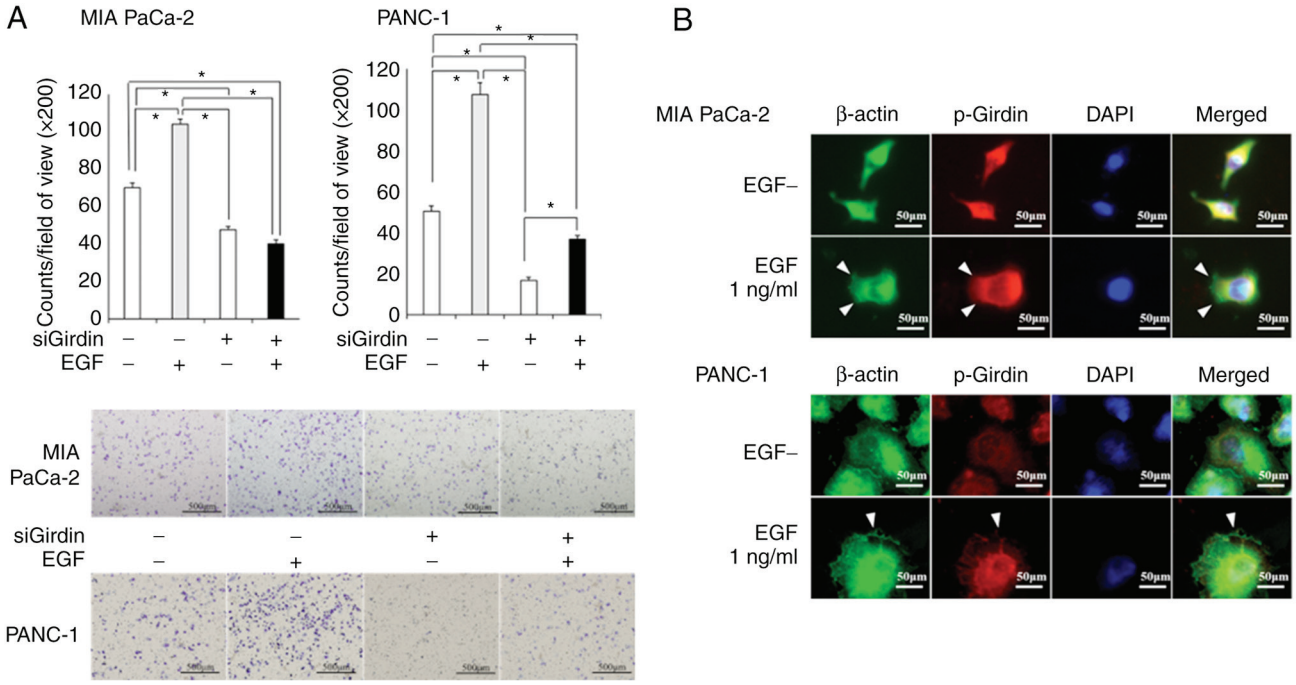

Figure 3. PaCa cells migration is enhanced by EGF stimulation and decreased by suppression of Girdin activation. (A) *In vitro* cell migration assays in Boyden double chambers. The Transwell assays were performed as described in Materials and Methods. The data are presented as the mean ± SEM. All of the experiments were performed in triplicate and repeated three times. (B) Immunocytochemistry and IF with anti-phosphate-specific Girdin antibody. MIA PaCa-2 cells were cultured for 2 h in the presence or absence of EGF, followed by fixation and IF staining. Anti-β-actin mouse and anti-Girdin (phospho Y1764) rabbit antibodies were used as primary antibodies. Anti-mouse goat IgG (Alexa Fluor® 488; green) and anti-rabbit goat IgG (Cy3; red) were used as secondary antibodies, and DAPI was used for nuclear staining (blue). *P<0.01 (one-way ANOVA with Bonferroni's multiple comparison test, two-tailed). IF, immunofluorescence; si-, small interfering.

than that of other cell lines, and this line was therefore considered unsuitable for this experiment [(Capan-2, 96 h; MIA PaCa-2, 26 h; AsPC-1, 38-40 h; PANC-1, 25.83 h) based on Cellosaurus (https://www.cellosaurus.org/) data]. RT-qPCR and western blotting confirmed that Girdin expression was suppressed after Girdin knockdown in the aforementioned cell lines (Fig. 2C and D).

PaCa cell migration is enhanced by EGF stimulation and decreased by suppression of Girdin activation. The Transwell assay revealed that EGF stimulation increased the migratory potential of cells. Moreover, the knockdown of Girdin inhibited the migration that was induced by EGF stimulation in PaCa cells (Fig. 3A). PaCa cells form lamellipodia by restructuring actin fibers that form the cytoskeleton upon EGF stimulation (white arrowheads). It is suggested that the phosphorylation of Girdin, which binds to actin fibers, is promoted by the EGF signaling system (Fig. 3B).

SCU, a flavonoid, suppresses the migration ability of PaCa cells by inhibiting the phosphorylation of Girdin. SCU is a chemical compound found in *Scutellaria barbata* and *Scutellaria lateriflora*, which are medicinal herbs, and it is a flavonoid glycoside with glucuronic acid attached to the flavone skeleton (Fig. 4A). SCU did not inhibit the proliferation of MIA PaCa-2 or PANC-1 cells at any concentration (Fig. 4B). SCU at 100 μM was used for further experiments. Immunocytochemistry (ICC)/Immunofluorescence (IF) showed that stimulation of PaCa cell lines with 1 ng/ml EGF resulted in the formation of lamellipodia and the expression of phosphorylated Girdin in the same regions; however, 100 μM SCU inhibited the formation of lamellipodia and the phosphorylation of Girdin even under EGF stimulation (Fig. 4C). Western blotting also revealed that EGF stimulation increased, whereas SCU treatment decreased, the level of phosphorylated Girdin (Fig. 4D). The number of cells that formed lamellipodia was evaluated using ICC/IF. The proportion of cells that formed lamellipodia was significantly increased after EGF stimulation, while it was suppressed after SCU treatment (Fig. S1). In addition, the wound healing assay suggested that migration ability was significantly decreased in the 100 μM SCU group after EGF stimulation. However, SCU alone did not significantly affect the migration ability of PaCa cells (Fig. 4E).

Girdin regulates angiogenic activity by upregulating the gene expression of VEGF-A, an angiogenic factor. Both gene expression and secretion of the angiogenic factor VEGF-A were suppressed in three PaCa cell lines (MIA PaCa-2, AsPC-1 and PANC-1) with knockdown of Girdin (Fig. 5A and B). In the Matrigel tube formation assay using EA.hy926 cells, tube formation was enhanced in the CM with the culture supernatant of those PaCa cell lines, whereas tube formation was significantly suppressed in the PaCa cell line with knockdown of Girdin (Fig. 5C). As demonstrated in Fig. 4C, SCU inhibited the phosphorylation of Girdin (tyrosine 1764). When the effect of SCU treatment on the expression of VEGF-A was evaluated, no difference was observed in the expression and secretion of VEGF-A treated with SCU with or without EGF (Fig. 5D). These results suggested that Girdin regulates the

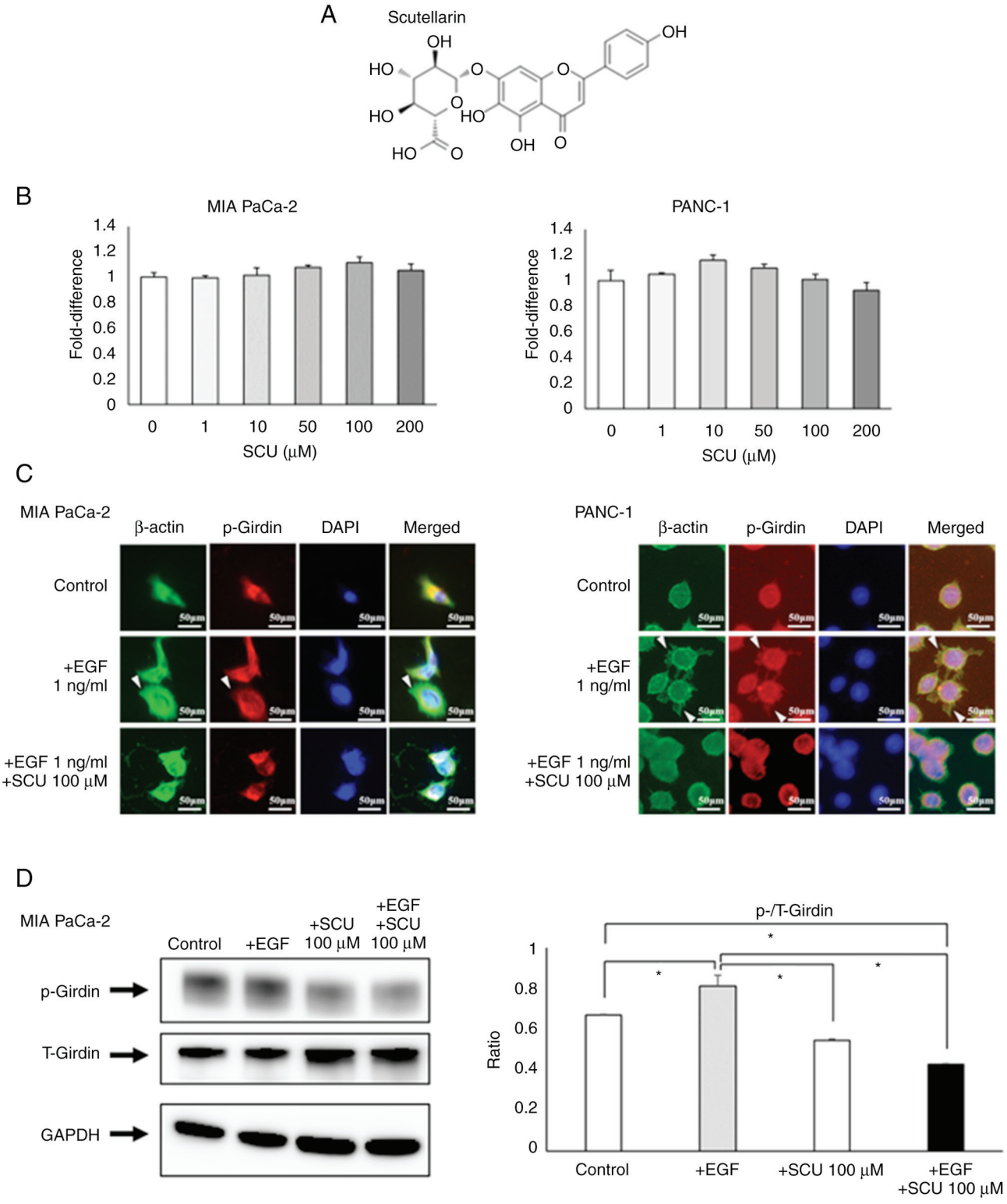

Figure 4. Continued.

angiogenic factor VEGF-A through a pathway different from that of tyrosine 1764 phosphorylation, which is considered to be at least related to migration ability.

Discussion

The malignant potential of cancer cells is likely to be defined by their migratory, invasive and micro-angiogenic potentials. Various intracellular and extracellular signaling networks in the cancer microenvironment are involved. One of the key processes in cancer cell invasion and metastasis is cell motility. Three steps are important for cells to crawl between tissues: Protrusion, adhesion, and traction (29). In protrusion, different cell types generate various protruding structures such as filopodia, lamellipodia and pseudopodia (30). Epithelial cells, certain fibroblasts, and some

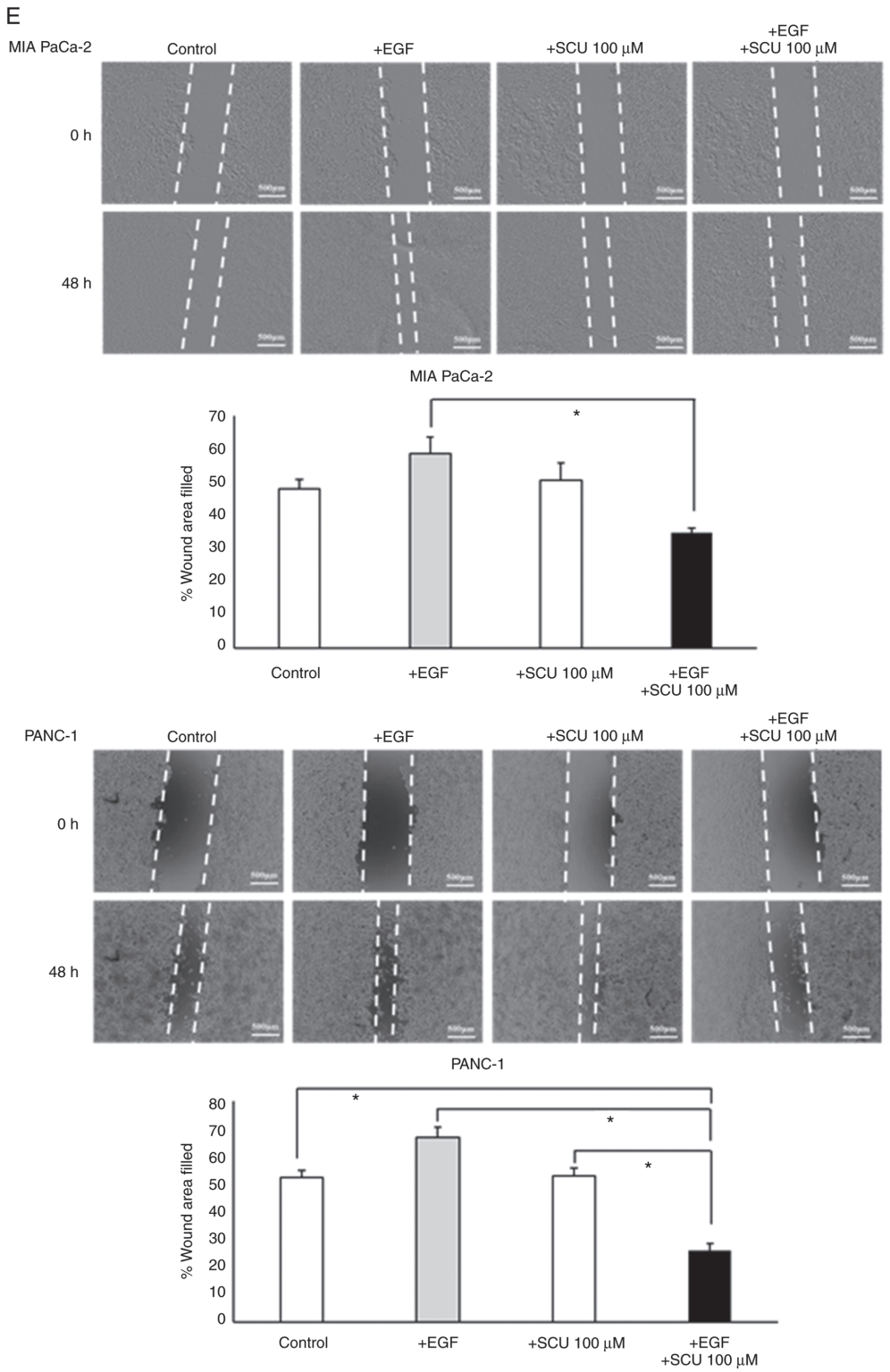

Figure 4. SCU suppresses PaCa cell migration by inhibiting Girdin phosphorylation. (A) The chemical structure of SCU. SCU is a flavonoid with a molecular weight of 462.36 g/mol. (B) The toxicological effects of SCU on PaCa cells were evaluated using the WST-1 assay. SCU was administered to MIA PaCa-2 and PANC-1 at various concentrations and incubated for 72 h, followed by addition of WST-1 reagent and measurement of absorbance. The values are expressed relatively to the control. (C) Immunocytochemistry/Immunofluorescence was used to evaluate the level of phosphorylated Girdin in MIA PaCa-2 and PANC-1 cells with or without EGF and 100 μM SCU. The cells were incubated with each reagent for 30 min and then stained. White arrows indicate the location of the lamellipodia enhanced by EGF stimulation. In addition, western blotting was used to quantify phosphorylated Girdin (Y1764). (D) The changes in Girdin and p-Girdin expression with SCU treatment were evaluated by western blotting. The ratio of p-Girdin to total Girdin (T-Girdin) is shown in the graph. The expression of p-Girdin was enhanced by EGF (1 ng/ml) stimulation, whereas p-Girdin expression was significantly suppressed under SCU treatment, even under EGF stimulation. (E) The effects of SCU on the migration ability of PaCa cells was evaluated by wound healing assay. MIA PaCa-2 and PANC-1 cells were seeded in 24-well plates at 100,000 cells per well, respectively. On the following day, after cell settlement, scratches were made in a monolayer using a pipette tip. Each reagent was added to medium containing 2% FBS, and the cells were incubated for 48 h and then observed under a phase contrast microscope. The length of the gaps at three random locations were measured and averaged. The data are presented as the mean ± SEM. All experiments were performed in triplicate and repeated three times. *P<0.01 (B: One-way ANOVA with Dunnett's multiple comparison test. D and E: One-way ANOVA with Bonferroni's multiple comparison test, two-tailed). SCU, scutellarin; PaCa, pancreatic cancer; p-, phosphorylated.

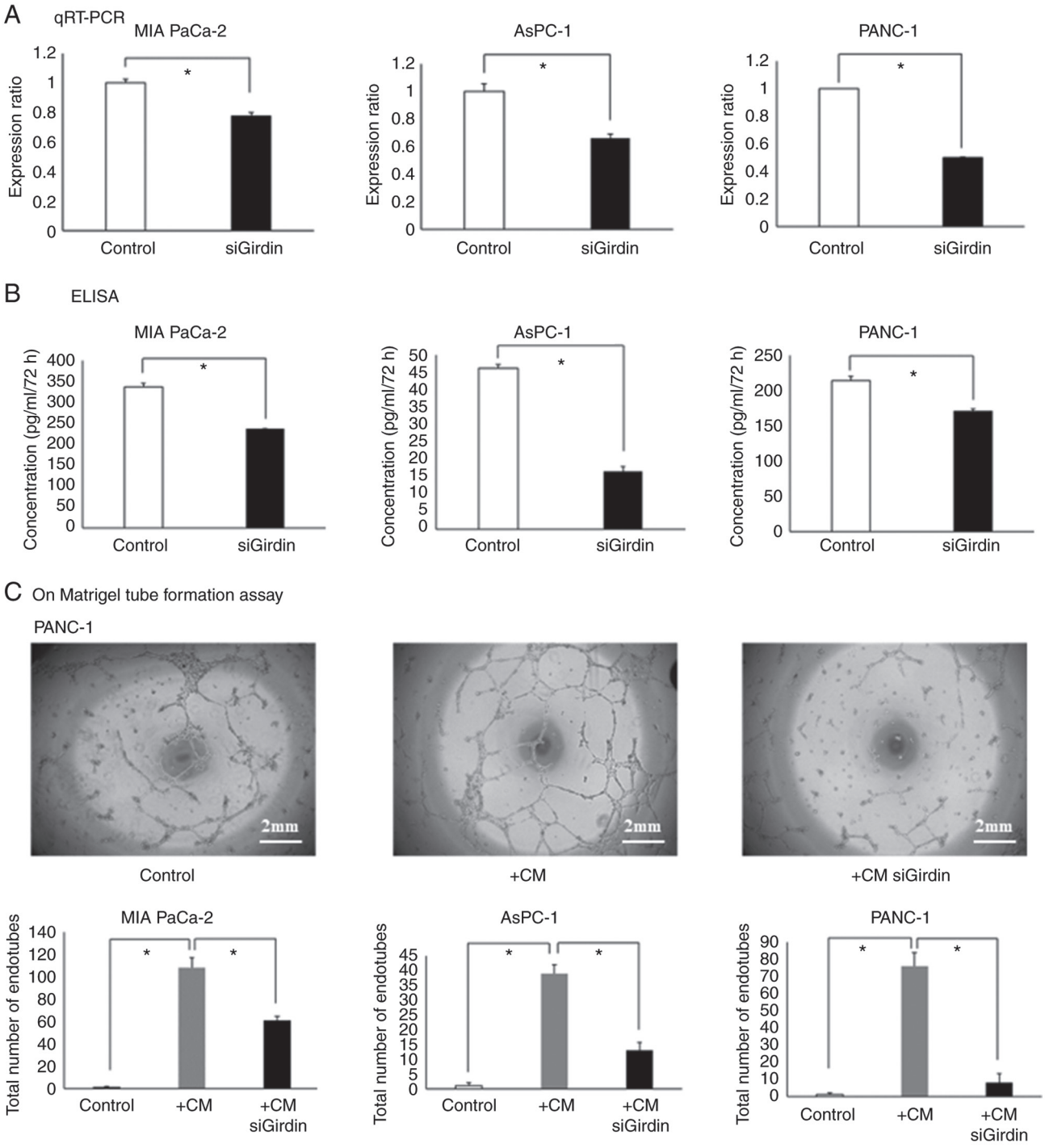

Figure 5. Continued.

neurons are known to form lamellipodia, two-dimensional sheet-like structures containing a cross-linking network of actin filaments (31).

Girdin, a novel substrate of Akt identified by Enomoto *et al* (3), is a protein bound to actin filaments that form the cytoskeleton. It is activated by Akt, a serine/threonine kinase, and is involved in the formation of lamellipodia, which are important for cytoskeletal remodeling and cell migration. Girdin is also an APE (9-11), G-alpha-interacting vesicle-associated protein (GIV) (32-34) and Hook-related protein 1 (HkRP1) (35). For example, GIV is transcriptionally induced by STAT3 and functions as a non-receptor guanine nucleotide exchange factor for the Gai protein, promoting tumor invasion and metastasis via STAT3 activation (32,36). Thus, Girdin likely plays multiple physiological roles in cells.

Girdin is highly expressed in various human malignancies, including breast, colon, lung, thyroid and cervical cancer (37-40). The Department of Gastroenterological Surgery, Nagoya City University Graduate School of Medical Sciences also reported that high Girdin expression was present in esophageal cancer and it is correlated with invasive potential and prognosis (14).

The purpose of the present study was to clarify the role of Girdin in the migration and progression of PaCa. In the present

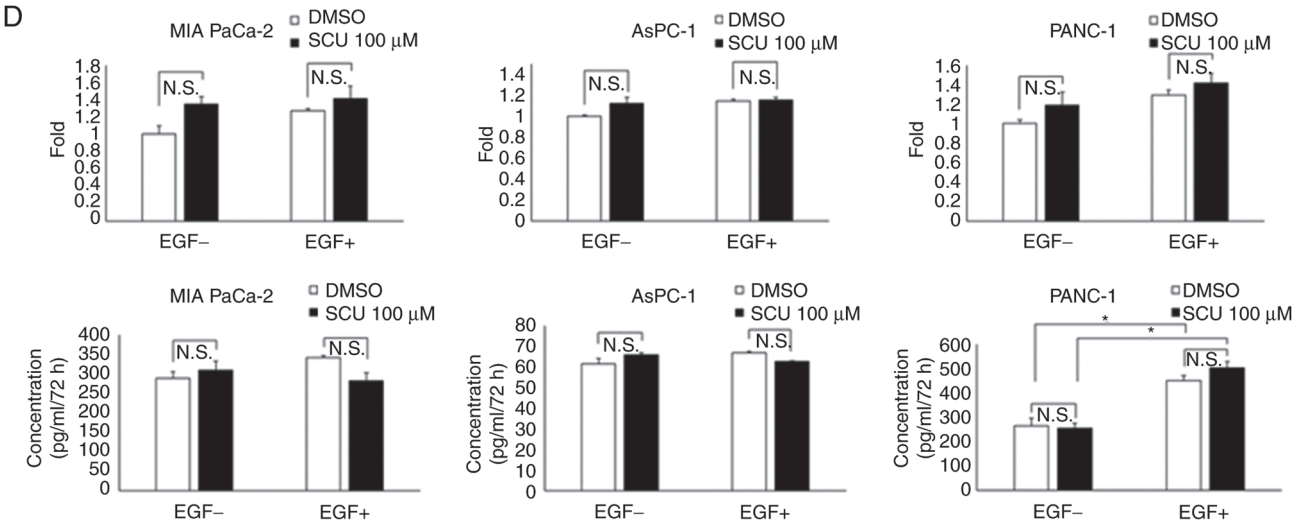

Figure 5. Girdin regulates angiogenic activity by upregulating VEGF-A gene expression. (A and B) Evaluation of VEGF-A (A) expression and (B) secretion in PaCa cell lines (MIA PaCa-2, AsPC-1 and PANC-1) with knockdown of Girdin. (A) RT-qPCR experiments were calculated using a comparative quantitative method with calibration curves. The vertical axis of the figure shows the ratio of VEGF-A expression to that of the respective control group. (B) After knockdown of Girdin in each cell line, the cells were cultured in medium containing 2% FBS for 72 h, and the supernatant was collected to measure VEGF-A by ELISA. The vertical axis of the figure shows the absolute concentration of VEGF-A. (C) The HUVEC tube formation assay was used to evaluate the angiogenic ability *in vitro*. The PaCa cell lines after knockdown of Girdin were cultured in 2% FBS-containing medium for 72 h, and then the cell supernatant was mixed with the same volume of 2% FBS-containing medium to make conditioned medium (CM). In the present study, the EA.hy926 cell line was used as immortalized HUVECs. EA.hy926 cells were cultured in the prepared conditioned medium for 16 h on Matrigel, and the number of endotubes was counted by phase-contrast microscopy. (D) Effect of SCU on the angiogenic ability of PaCa cell lines. MIA PaCa-2, AsPC-1 and PANC-1 cells were treated with 1 ng/ml EGF and 100 μM SCU in 2% FBS-containing medium to verify the alteration of VEGF-A expression and secretion. For RT-qPCR, cDNA prepared 2 h after the addition of reagents was used; for ELISA, the cell supernatant collected 72 h after the addition of reagents was used. The data are presented as the mean ± SEM. All experiments were performed in triplicate and repeated three times. *P<0.01 (A and B: Student's t-test. C and D: One-way ANOVA with Bonferroni's multiple comparison test, two-tailed). PaCa, pancreatic cancer; SCU, scutellarin; RT-qPCR, reverse transcription-quantitative PCR; HUVEC, human umbilical vein endothelial cell.

study of resected PaCa specimens, the expression of Girdin varied in intensity. The results were divided into strong and weak Girdin expression groups and it was found that the strong Girdin expression group had a significantly poorer prognosis in OS and RFS than the weak Girdin expression group, similar to previous studies on other carcinomas. For confirmation of the data that were provided, a web database (Kaplan Meier plotter) was addressed for mRNA levels, and the results were similar. Evidently, expression of Girdin in cancer cells is associated with greater invasiveness of organs. On the other hand, there was no significant difference in lymph node metastasis or distant metastasis, and the effect of the T factor significantly correlated with the intensity of Girdin expression at stage 4 and lower levels. However, no significant difference was obtained for venous or lymphatic invasion. Neural invasion was not significantly different but tended to be slightly more common in the Girdin-expressing group. It was hypothesized that this is because Girdin is involved in not only migration, but also angiogenesis, as suggested in the present study, as well as in proliferation and inhibition of apoptosis (10). Thus, Girdin expression could be a biomarker for PaCa prognosis.

In vitro, Girdin was more highly expressed in human pancreatic ductal cells than in numerous other PaCa cell lines. MIA PaCa-2, AsPC-1 and PANC-1 also expressed Girdin at high levels. It was found that the migration ability of these PaCa cell lines was enhanced by EGF stimulation. By contrast, migration was inhibited by EGF stimulation when Girdin was suppressed. In addition, it was confirmed that EGF stimulation enhanced the phosphorylation of Girdin, resulting in cell morphological changes and the formation of lamellipodia. These results indicated that Girdin is involved in cell migration via EGF signaling in PaCa. On the other hand, the present ICC/IF results revealed that phosphorylated Girdin was also expressed in the absence of EGF. Enomoto *et al* (3) reported that phosphorylated Girdin is also expressed in the absence of EGF. This may indicate that phosphorylated Girdin is necessary but not sufficient for lamellipodia formation. It was hypothesized that phosphorylated Girdin has other functions besides lamellipodia formation. Unfortunately, one of the limitations of the present study was that it was not possible to search for phosphorylated Girdin in all cell lines due to time constraints and the only results that were obtained were from the MIA PaCa-2 cell line. It is aspired to obtain similar results by conducting experiments on other cell lines in the future.

There are a few substances that inhibit Girdin signaling. In the present study the main focus was SCU, a type of flavonoid, which it has long been used in Chinese medicine as a treatment for cerebrovascular diseases, and is known for its antitumor, hepatoprotective and neuroprotective effects. Natural compounds have been used in China for the treatment of cerebrovascular and other diseases (16). SCU was reported to inhibit cancer invasion and metastasis by suppressing STAT3/Girdin/Akt signaling in hepatocellular carcinoma (15). SCU was selected for evaluation of its effects on Girdin suppression in PaCa cells. As in the case of Girdin knockdown, the PaCa migration enhanced by EGF was significantly reduced by SCU. ICC/IF demonstrated that EGF-induced

increase in lamellipodia formation was suppressed by SCU treatment, and western blotting indicated that this effect was mediated by phosphorylation of Tyr-1764 in Girdin. On the other hand, knockdown of Girdin also reduced cell migration ability. It was hypothesized that SCU competitively interacts with the phosphorylation pathway of Girdin.

Since Girdin also increases the activity of Akt and STAT3, as aforementioned, the effect of Girdin was examined on angiogenesis during tumor progression. In non-small cell lung cancer, the production of VEGF was increased by IL-17, and it was suppressed by knockdown of Girdin (41). It was also reported to be involved in the level of mRNA expression for the angiogenic factor VEGF in breast cancer (42). PaCa is commonly referred to as an ischemic tumor based on microscopic-level imaging findings, but there are numerous studies showing an association between microvascular density and the prognosis of PaCa (43). In fact, certain studies have demonstrated the efficacy of anti-angiogenic therapy for PaCa (44,45). Furthermore, with regard to novel therapies targeting angiogenesis, it has been previously demonstrated by the authors that inhibition of PaCa proliferation at the *in vivo* level suppressed gene expression and the production/secretion of the angiogenic factor VEGF-A. It was also demonstrated that inhibition of Girdin expression suppressed the angiogenic potential of VEGF-A *in vitro*. In the Matrigel tube formation assay performed to evaluate angiogenic potential *in vitro*, a significant decrease in tube formation was found in the cell supernatants from Girdin-knockdown cell lines. No obvious involvement of EGF stimulation in the expression and production of these angiogenic factors and in angiogenesis could be noted. On the other hand, the addition of SCU, which inhibits Girdin phosphorylation, did not significantly alter the expression, production or secretion of angiogenic factors. This suggests that the regulatory mechanism of angiogenic potential of Girdin uses a different pathway than the control of migratory activity without EGF signaling. Further detailed investigation of these mechanisms is warranted.

In the present study, experiments were performed to suppress Girdin in order to analyze the function of Girdin. On the other hand, it is necessary to examine the effects of strong Girdin expression in order to further support the present findings, as it was not possible to perform Girdin knock-in experiments due to limited time, technology and facilities in the authors' laboratory. In addition, a limitation to the study on phosphorylated Girdin is that it was conducted only in MIA PaCa-2 cells and that the multiple phosphorylation sites were not verified sufficiently. These research limitations will be further developed and studied in the future.

In conclusion, the results of the present study suggested that Girdin is involved in EGF signal-mediated migration in PaCa and that SCU may suppress cancer invasion by inhibiting it (Fig. 6). Moreover, Girdin levels associated with PaCa angiogenesis by regulating VEGF-A production from PaCa. As the expression of Girdin is associated with PaCa prognosis, Girdin may be a prognostic biomarker. SCU, a novel targeting Girdin, may improve the prognosis of PaCa.

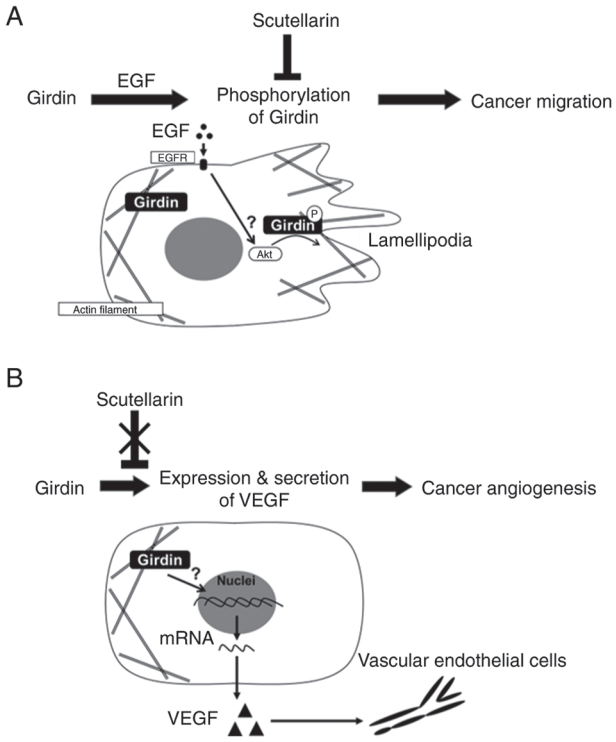

Figure 6. Working model of Girdin for migration and angiogenesis in PaCa cells. (A) A working model of the involvement of Girdin in the migratory ability of PaCa and the inhibitory effect of SCU. Girdin, which binds to actin fibers, is likely activated through phosphorylation via the EGF signaling pathway and involved in the migration ability of PaCa. SCU suppressed the migration ability of PaCa by inhibiting the activation of Girdin through EGF signaling. (B) A working model for the involvement of Girdin in the angiogenic ability of PaCa. Girdin appears to enhance angiogenesis of human umbilical vein endothelial cells by enhancing transcription and secretion of VEGF-A. SCU, which inhibits the phosphorylation of Girdin, had no effects on the expression of VEGF-A. Therefore, Girdin was suggested to be involved in the expression of VEGF-A through a different pathway. PaCa, pancreatic cancer; SCU, scutellarin.

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Availability of data and materials

All data generated or analyzed during this study are included in the published article.

Authors' contributions

YH and YM contributed to the conception and design of the study, analyzed and interpreted the data, and wrote and reviewed the manuscript. YH, YM, GU, YA, TK, KO, HI, KS, MM, HT and ST designed the study. YH, YD, KN, HM, GU, YA and TK performed the experiments and acquired the data. YM, HI, KS, MM and HT provided technical support in performing the experiments. YH, YM, AM and MK confirm the authenticity of all the raw data. YM, RO, HT and ST supervised the study. YH and YM planned all experiments, analyzed and interpreted the data, and drafted the manuscript. All authors read and approved the final version of the manuscript and are equally responsible for all aspects of the study, ensuring that the integrity or accuracy of all part of the study.

Ethics approval and consent to participate

Ethics approval (approval no. 60-18-0025) for the use of human tissue was granted by the Institutional Review Board (Ethical Review Committee for Medical Research, Nagoya City University Hospital, Nagoya, Japan). Written informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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