

# EphA4 receptor, overexpressed in pancreatic ductal adenocarcinoma, promotes cancer cell growth

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To isolate novel diagnostic markers and drug targets for pancreatic ductal adenocarcinoma (PDAC), we previously performed expression profile analysis of PDAC cells using a genome-wide cDNA microarray combined with laser microdissection. Among dozens of up-regulated genes identified in PDAC cells, we herein focused on one tyrosine kinase receptor, Eph receptor A4 (EphA4), as a molecular target for PDAC therapy. Immunohistochemical analysis validated EphA4 overexpression in approximately half of the PDAC tissues. To investigate its biological function in PDAC cells, we knocked down EphA4 expression by siRNA, which drastically attenuated PDAC cell viability. In concordance with the siRNA experiment, PDAC-derivative cells that were designed to constitutively express exogenous EphA4 showed a more rapid growth rate than cells transfected with mock vector, suggesting a growth-promoting effect of EphA4 on PDAC cells. Furthermore, the expression analysis for ephrin ligand family members indicated the coexistence of ephrinA3 ligand in PDAC cells with EphA4 receptor, and knockdown of ephrinA3 by siRNA also attenuated PDAC cell viability. These results suggest that the EphA4–ephrinA3 pathway is likely to be a promising molecular target for pancreatic cancer therapy. (*Cancer Sci* 2006; 97: 1211–1216)

**P**ancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer death in the developed world and has one of the worst mortality rates among the common malignancies, with a 5-year survival rate of only 4%.<sup>(1,2)</sup> Approximately 33 730 new patients are expected to be diagnosed with pancreatic cancer in 2006 in the USA, and nearly 32 300 to die of the disease;<sup>(3)</sup> in Japan nearly 18 000 PDAC patients are expected to die each year. The great majority of PDAC patients are diagnosed at an advanced stage where no effective therapy is available. Surgical resection offers the only possibility for cure at present, but 80–90% of patients who undergo curative surgery suffer from relapse and die due to the metastatic or disseminated disease.<sup>(1,2)</sup> Some approaches that combine surgery and chemotherapy based on gemcitabine or 5-fluorouracil, with or without radiation therapy, can improve the quality of life of patients.<sup>(1,2)</sup> However, such treatments have a very limited effect on long-term survival because PDAC is biologically extremely aggressive, and usually chemo- and radiation-resistant. Hence, the management of most PDAC patients is now focused on palliative measures.

To overcome this dismal situation, development of novel molecular-targeted therapies for PDAC through identification of cancer-specific drug-amenable molecules is urgently desired. We previously performed detailed and accurate expression profile analysis of PDAC using a genome-wide cDNA microarray consisting of approximately 27 000 genes, in combination with laser microdissection to purify the cancer cell population.<sup>(4)</sup> Among the genes we identified as being trans-activated in PDAC cells, we herein investigated one of the Eph receptors, EphA4, as a possible molecular target for this disease.

The Eph receptor family constitutes one of the largest groups of transmembrane receptor tyrosine kinases.<sup>(5)</sup> They are activated by a second family of cell surface-anchored ligands, the ephrins, that are attached to the plasma membrane via either a glycosylphosphatidylinositol (GPI) linkage (type A) or a transmembrane sequence (type B). The Eph receptors are also divided into type A or type B according to their ligand-binding specificities. In general, type A receptors bind type A ephrin ligands and type B ephrin ligands stimulate type B receptors. One molecule that shows an exception to this rule is EphA4, which can bind and respond to type B as well as type A ephrin ligands.<sup>(6)</sup> These Eph receptors and their ligands have been implicated in playing important roles in a variety of biological activities including axon guidance and migration of neural crest cells in the nervous system, establishment of segmental boundaries, and formation of angiogenic capillary plexi.<sup>(7–11)</sup> Among the Eph receptor family members, EphA2 and EphB2 were shown to be frequently overexpressed or functionally altered in many types of cancers, and to be involved in tumor progression or angiogenesis.<sup>(12–18)</sup>

The aim of this study was to determine the role of EphA4 in pancreatic carcinogenesis. We identified a possible candidate for a ligand to the EphA4 receptor, ephrinA3, which coexisted in PDAC cells with EphA4, and our second aim was to determine the effects of EphA4 and ephrinA3 on the viability of PDAC cells.

## Materials and Methods

**Cell lines.** Pancreatic ductal adenocarcinoma cell lines MIA-PaCa2 and Panc-1 were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA), and were grown in Delbecco's modified Eagle's medium or RPMI1640 (Sigma-Aldrich, St Louis, MO, USA). PK-59, KLM-1, PK-45P, and PK-1 were provided by the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan) and maintained in RPMI1640; both media were supplemented with 10% fetal bovine serum (Cansera, Ontario, Canada) and 1% antibiotic/antimycotic solution (Sigma-Aldrich). Cells were maintained at 37°C in an atmosphere of humidified air with 5% CO<sub>2</sub>.

**Semi-quantitative RT-PCR.** Microdissection of PDAC cells and normal pancreatic ductal cells were as described previously.<sup>(4)</sup> RNAs from the PDAC cells and normal pancreatic ductal cells were subjected to two rounds of T7-based RNA amplification (Epicentre Technologies, Madison, WI, USA) and subsequent synthesis of single-strand cDNA. For reverse transcription-polymerase chain reaction (RT-PCR), total RNA from human

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PDAC cell lines was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Extracted RNA was treated with DNase I (Roche, Mannheim, Germany) and reverse-transcribed to single-stranded cDNA using oligo (dT) primer with Superscript II reverse transcriptase (Invitrogen). The primer sequences used were 5'-CATCCACGAACTACTC-TTCAACT-3' and 5'-TCTCCTTAGAGAGAAGTGGGGTG-3' for *β-actin* (*ACTB*), 5'-GAAGGCGTGGTCACTAAATGTAA-3' and 5'-TTTAATTTTCAGAGGGCGAAGAC-3' for *EphA4*, 5'-GAGT-CCCTTCCCTCTTTAACC-3' and 5'-TATGAAAGTCACAGCCA-AAGC-3' for *ephrinA3*. The primer sequences for other ephrin ligands are available on request.

The RT-PCR exponential phase was determined to allow semiquantitative comparisons among cDNAs developed from identical reactions. Each PCR regime involved a 95°C, 5-min initial denaturation step followed by 23 cycles (for *ACTB*), 28 cycles (for *EphA4*), or 30 cycles (for *ephrinA3*) at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, on a Gene Amp PCR system 9600 (PE Applied Biosystems, Foster, CA, USA).

**Northern blot analysis.** We extracted total RNA from several PDAC cell lines using Trizol reagent and performed Northern blot analysis. After treatment with DNase I (Nippon Gene, Osaka, Japan), mRNA was purified with Micro-FastTrack (Invitrogen), according to the manufacturer's protocols. A 1-μg aliquot of each mRNA from PDAC cell lines, as well as those isolated from normal human heart, lung, liver, kidney, brain, and pancreas (BD Biosciences, Palo Alto, CA, USA), were separated on 1% denaturing agarose gels and transferred onto nylon membranes. The 1014-bp probe specific to *EphA4* was prepared by PCR using the following primer set: forward 5'-GAAGGCGTGGTCACTAAA-TGTAA-3' and reverse 5'-CTTTAATTTTCAGAGGGCGAAGAC-3'.

Hybridization with a random-primed,  $\alpha^{32}\text{P}$ -dCTP-labeled probe was carried out according to the instructions for Megaprime DNA labeling system (Amersham Biosciences, Buckinghamshire, UK). Prehybridization, hybridization and washing were performed according to the supplier's recommendations. The blots were auto-radiographed with intensifying screens at -80°C for 10 days.

**Immunohistochemical staining.** Conventional sections from PDAC tissues were obtained from surgical specimens that were resected in Osaka Medical Center for Cancer and Cardiovascular Diseases under the appropriate informed consent. Sections from normal pancreas were purchased from Biochain (Hayward, CA, USA). Tissue microarray sections of pancreatic carcinoma (AccuMax Array) were purchased from ISU ABXIS (Seoul, Korea), where 31 PDAC tissues, two endocrine tumor tissues, and two normal pancreas tissues were spotted duplicated.

The sections were deparaffinized and autoclaved at 108°C in Dako Cytomation Target Retrieval Solution High pH (Dako, Carpinteria, CA, USA) for 15 min. After blocking of endogenous peroxidase and proteins, the sections were incubated with anti-EphA4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA, diluted by 1:200) or anti-ephrinA3 antibody (Santa Cruz Biotechnology, diluted by 1:150) at room temperature for 30 min. After washing with PBS, immunodetection was performed with peroxidase labeled anti-rabbit immunoglobulin (Envision kit, Dako). Finally, the reactants were developed with 3,3'-diaminobenzidine (Dako). Counterstaining was performed using hematoxylin.

**siRNA-expressing constructs and transfection.** To knock down endogenous *EphA4* or *ephrinA3* expression in PDAC cells, we used psiU6BX3.0 vector for expression of short hairpin RNA against a target gene as described previously.<sup>(19)</sup> The target sequences of the synthetic oligonucleotides for small interfering RNA (siRNA) for *EphA4* were as follows: EphA4-198si, 5'-TCCGAACCTACCAAGTGTG-3'; EphA4-486si, 5'-TCATGAAGCTGAACACCGA-3'; EphA4-1313si, 5'-GCAGCACCATCATCCATTG-3'; and EGFPsi, 5'-GAAGCAGCAGACTTCTTC-3' (as a negative control). The target sequences for siRNA for *ephrinA3* were: ephrinA3-539si,

5'-GTGTCTGAGGATGAAGGTG-3'; ephrinA3-689si, 5'-GCTTG-AGAAGAGCATCAGC-3'; and ephrinA3-1584si, 5'-CGCACAG-ACACTTTTGGAG-3'.

The PDAC cell lines MIA-PaCa2 and PK-59, which expressed both *EphA4* and *ephrinA3*, were plated onto six-well plates, and transfected with plasmid designed to express siRNA to either *EphA4* or *ephrinA3* (10 μg or 20 μg) using FuGENE6 (Roche) or Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions. Cells were selected by 0.15 mg/mL (for PK-59) or 0.8 mg/mL (for MIA-PaCa2) of Geneticin (Sigma-Aldrich) for 5 days, and then harvested to analyze the knockdown effect on *EphA4* or *ephrinA3* expression.

RT-PCR for *EphA4* or *ephrinA3* knockdown was performed using the primers described above, and Western blot analysis for *EphA4* or *ephrinA3* knockdown was performed by using the antibodies to *EphA4* or *ephrinA3* described above and anti- $\beta$ -actin (*ACTB*) antibody (Sigma-Aldrich) as a loading control.

For colony formation assay, transfectants expressing siRNA were grown for 14 days in media containing Geneticin. After fixation with 4% paraformaldehyde, transfected cells were stained with Giemsa solution to assess colony formation. Cell viability was quantified using Cell counting kit-8 (Dojindo, Kumamoto, Japan). After 14 days of culture in the Geneticin-containing medium, the solution was added at a final concentration of 10%. Following incubation at 37°C for 3 h, absorbance at 450 nm was measured with a Microplate Reader 550 (Bio-Rad, Hercules, CA, USA).

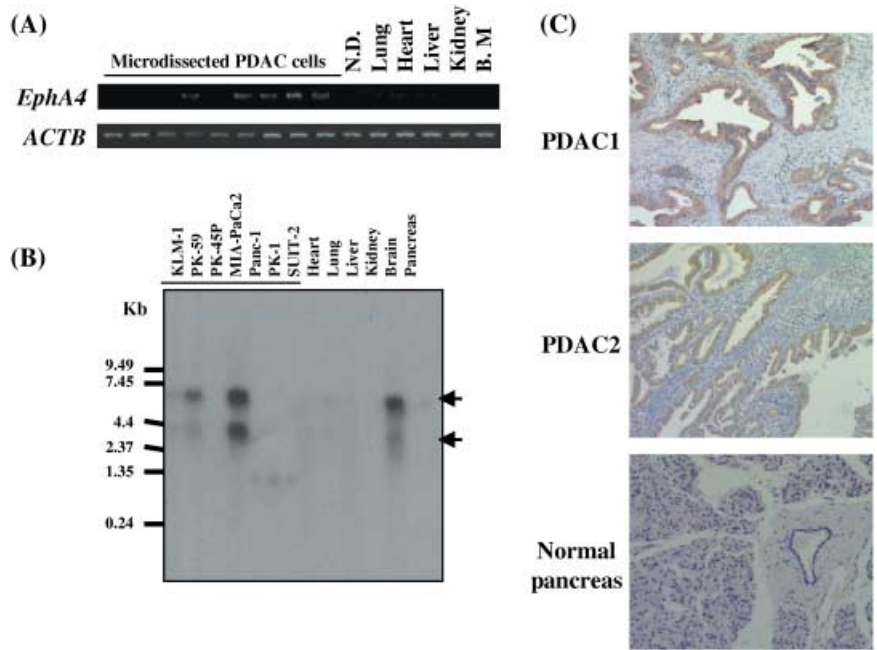
**Establishment of EphA4-expressing cells and their growth.** *EphA4* cDNA was prepared by PCR amplification using the forward primer that included the Kozak sequence and *EcoRI* linker, and the reverse primer including an *XhoI* linker. The PCR product was inserted into the *EcoRI* and *XhoI* sites of the mammalian expression vector, pCAGGS/FLAG for expressing a FLAG-tagged protein.<sup>(20)</sup> The pCAGGS-EphA4-FLAG or empty pCAGGS/FLAG mock vector was transfected into Panc-1 cell line, which exhibited barely detectable expression of *EphA4* among pancreatic cancer cell lines we examined, by FuGENE6 (Roche) according to the manufacturer's protocol. Then, the Geneticin-resistant clones were selected in the culture medium containing 1.0 mg/mL of Geneticin.

The exogenous *EphA4* expression in each clone was confirmed by Western blot analysis using anti-FLAG tag and anti- $\beta$ -actin antibodies (Sigma-Aldrich). For growth assay, 50 000 cells of each of *EphA4* expressing clone (Panc1-EphA4) or control clone (Panc1-Mock) was seeded into each well of a six-well culture dish and incubated in the medium containing 10% fetal bovine serum. Cell viability was quantified with MTT assay every day. The experiment was repeated at least three times.

## Results

**EphA4 overexpressed in PDAC cells.** Among the dozens of up-regulated genes in PDAC identified previously through detailed expression profile analysis,<sup>(4)</sup> we here focused on further expressional and functional analysis of *EphA4*. The semiquantitative RT-PCR analysis using RNA from microdissected PDAC cells and normal pancreatic ductal cells demonstrated that expression of *EphA4* was significantly up-regulated in PDAC cells, compared with that of normal pancreatic ductal cells that were believed to be the origins of PDAC cells (Fig. 1A). Further comparisons of *EphA4* expression patterns in PDAC cells and normal tissues by Northern blot analysis revealed apparently strong and specific expression of *EphA4* in PDAC cells although its expression was observed in normal brain (Fig. 1B). Two bands (about 6.5 kb and 3 kb) were observed, which are differential splicing patterns of 3' UTR of human *EphA4*.

To confirm overexpression of *EphA4* protein in PDAC, we performed immunohistochemical staining using anti-EphA4 antibody and validated overexpression of *EphA4* protein in PDAC



**Fig. 1.** Overexpression of EphA4 in pancreatic ductal adenocarcinoma (PDAC) cells. (A) Semi-quantitative RT-PCR validated that *EphA4* expression was up-regulated in the microdissected PDAC cells compared with normal pancreatic duct cell (ND) that were also microdissected and several vital organs (lung, heart, liver, kidney and bone marrow). Expression of *ACTB* served as the quantitative control. (B) Northern blot analysis showed the strong expression of *EphA4* in PDAC cell lines, MIA-PaCa2 and PK-59, and the normal brain, while no expression was observed in vital organs including heart, lung, liver, and kidney. (C) Immunohistochemical study using anti-EphA4 antibody. Intense staining was observed in PDAC cells in two representative specimens (PDAC1 and PDAC2), while acinar cells and normal ductal epithelium in normal pancreatic tissue showed no staining (original magnification  $\times 200$ ).

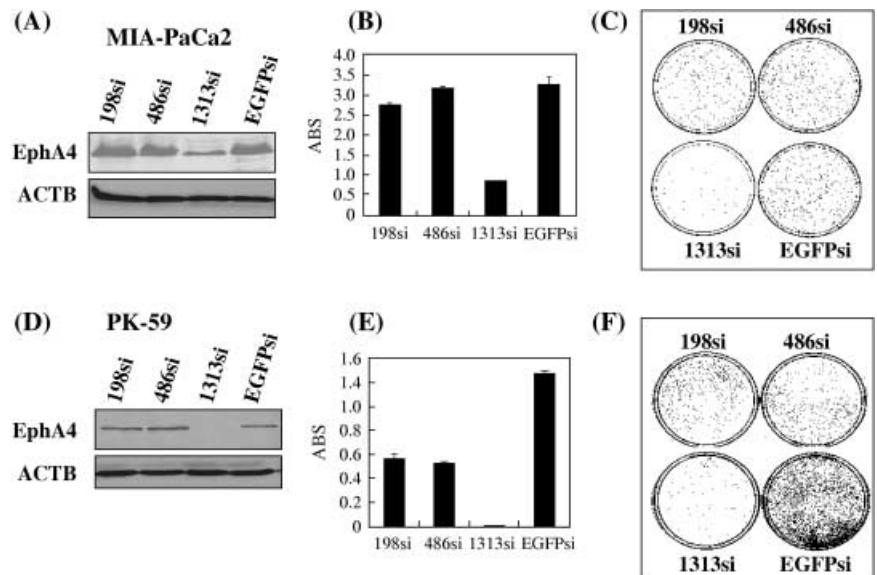
cells, but no staining in normal pancreas (Fig. 1C). The immunohistochemical analysis on the tissue microarray combined with conventional tissue sections showed that EphA4 protein was overexpressed in 48% (22/46) of PDAC specimens we examined. There was no significant correlation between EphA4 expression and differentiation of PDAC in our analysis.

**Knockdown of EphA4 by siRNA on PDAC cells.** To investigate a potential growth-promoting role of EphA4 aberrant expression, we constructed several siRNA-expression vectors to examine their knockdown effects on two *EphA4*-overexpressing PDAC cell lines, MIA-PaCa2 and PK-59. After transfection of each of these siRNA-expressing constructs into them, semiquantitative RT-PCR (data not shown) and Western blot revealed that 1313si construct, but no other constructs, significantly knocked down EphA4 expression (Figs 2 A,D). After 14-day selection in culture medium containing Geneticin, MTT assay (Figs 2B,E) and colony formation assay (Figs 2C,F) demonstrated that introduction of 1313si in MIA-PaCa2 and PK-59 cells drastically attenuated

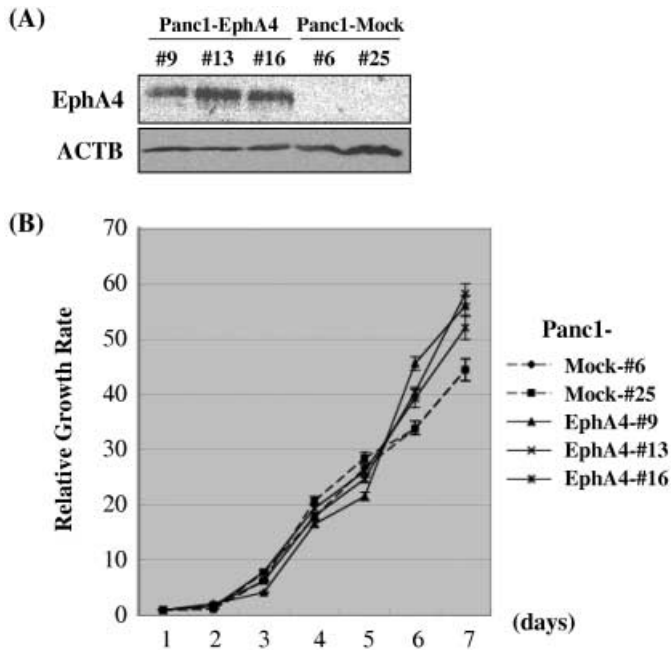
their cell growth or viability; however, other siRNAs that could not affect *EphA4* expression did not affect cell growth.

**Growth promoting effect of EphA4 expression on PDAC cells.** To further explore the potential oncogenic property of *EphA4*, we established the Panc1-derivative cell line Panc1-EphA4, in which exogenous EphA4 expressed constitutively. We also prepared control Panc-1 cells transfected with the mock vector (Panc1-Mock) and compared their growth rates. Western blot analysis (Fig. 3A) validated exogenous EphA4 expression in three Panc1-derivate clones. The growth curve measured by MTT assay demonstrated that the three Panc1-EphA4 clones (#9, #13 and #16) grew significantly more rapidly than the two Panc1-mock clones (#6, and #25;  $P = 0.0358$ ) (Fig. 3B), indicating the EphA4 expression enhanced proliferation of PDAC cells.

**Expression pattern of ephrin ligands in PDAC cells.** Type A Eph receptors usually bind to type A ligands that are known to be GPI-anchored to the plasma membrane, and type B Eph receptors bind to type B ligands that have a transmembrane domain.



**Fig. 2.** Knockdown effect on EphA4 by siRNA attenuated pancreatic ductal adenocarcinoma (PDAC) cell viability. Three *EphA4* siRNA expression vectors (EphA4-198si, -486si, and -1313si) and an EGFP siRNA expression vector (EGFPsi) as a negative control were transfected into (A, B, C) MIA-PaCa2 and (D, E, F) PK-59 cells. (A and D) Knockdown effect on EphA4 was validated by Western blot analysis, with ACTB level as a quantitative control. EphA4-1313si revealed a strong knockdown effect, while EphA4-198si, EphA4-486si and EGFPsi did not show any effect on the level of EphA4. Transfection with EphA4-1313si vector resulted in drastic reduction of the numbers of viable cells measured by (B and E) MTT assay and (C and F) the number of colonies formed, compared with the cells transfected with other siRNA expression vectors that did not showed their knockdown effect on *EphA4*. ABS, absorbance at 490 nm (630 nm reference), measured with a microplate reader.



**Fig. 3.** Expression of exogenous EphA4 promoted pancreatic ductal adenocarcinoma (PDAC) cell growth. (A) Western blot analysis of three Panc-1 derivative cells (Panc1-EphA4 #9, #13, and #16) expressing exogenous EphA4 constitutively and those transfected with mock vector (Panc1-Mock #6 and #25). Exogenous introduction of EphA4 expression was validated with anti-FLAG tag antibody. ACTB served as a loading control. (B) The growth measurement by MTT assay demonstrates that the three Panc1-EphA4 clones (#9, #13 and #16, solid lines) grew significantly more rapidly than the two Panc1-mock clones (#6, and #25, dashed lines) ( $P = 0.0358$ ). X-axis represents day point after seeding and Y-axis represents relative growth rate that was calculated in absorbance of the diameter by comparison with the absorbance value of day 1 as a control. Each average is plotted with bars representing standard error. These experiments were performed in triplicate.

However, there is one exception to this rule; the EphA4 receptor has been shown to bind to both type A and type B ephrin ligands.<sup>(6)</sup> The ligand-receptor combinations for the Eph family are considered to occur in a cancer-type or tissue-type specific manner.<sup>(16,18)</sup>

To search for potential candidate ligands for the EphA4 receptor in PDAC cells, we examined the expression patterns of members in the ephrin A (A1–5) and B (B1–3) ligand families by semiquantitative RT-PCR using RNA from microdissected PDAC cells, and found that the expression patterns of *ephrinA1* and *ephrinA3* were concordant with that of *EphA4* (Fig. 4A). However, the expression of *ephrinA1* was not concordant with that of *EphA4* in PDAC cell lines. As better concordance between *EphA4* and *ephrinA3* expression was observed in PDAC cell lines than between *EphA4* and *ephrinA1* (Fig. 4B), we considered that *ephrinA3* might be a better candidate as a ligand for EphA4. Immunohistochemical staining in 31 PDAC samples spotted on tissue microarray and five conventional sections of PDAC also showed concordant expression of ephrinA3 and EphA4 (Fig. 4C) ( $P > 0.001$  by  $\chi^2$  test).

#### Knockdown of ephrinA3 by siRNA attenuated PDAC cell growth.

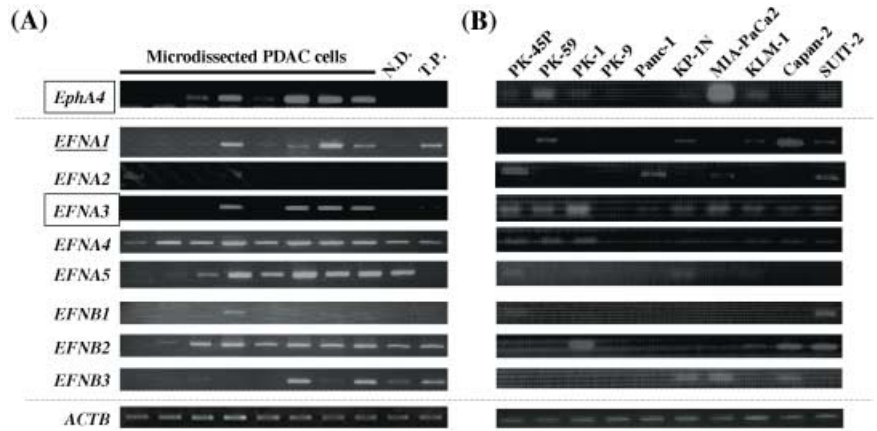
To investigate possible roles of ephrinA3 in PDAC, we constructed several expression vectors that were designed to express siRNA to *ephrinA3* and examined knockdown effects in MIA-PaCa2 cells, in which both EphA4 and ephrinA3 ligand were expressed at a high level. Semi-quantitative RT-PCR (data not shown) and Western blot analysis revealed that 539si and 689si, but no other

siRNAs, clearly knocked down ephrinA3 expression (Fig. 5A). After 14-day culture in the medium containing Geneticin, we observed that introduction of 539si or 689si into MIA-PaCa2 cells drastically attenuated cell viability measured by colony formation assay (Fig. 5B) and by MTT assay (Fig. 5C), while no growth-suppressive effect was observed by either 1584si or EGFPsi, which showed no knockdown effect on *ephrinA3* expression. Similar results were also obtained when we used PK-59 cells, in which *EphA4* and *ephrinA3* were co-expressed (data not shown). These findings indicate that, in addition to the EphA4 receptor, the ephrinA3 ligand could also play an important role in cell viability of PDAC cells.

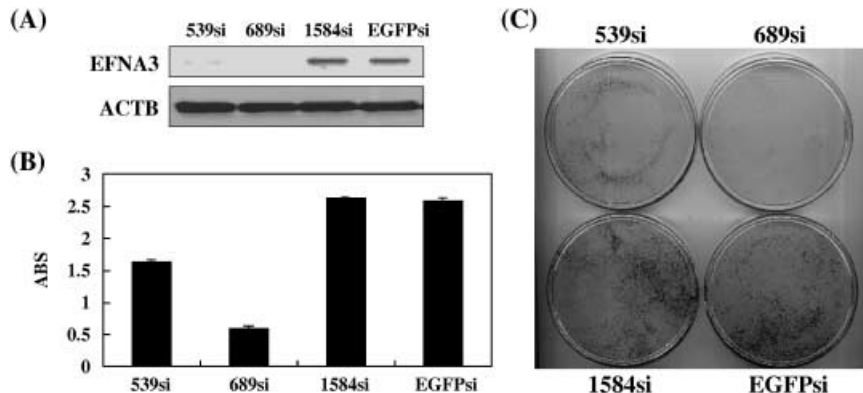
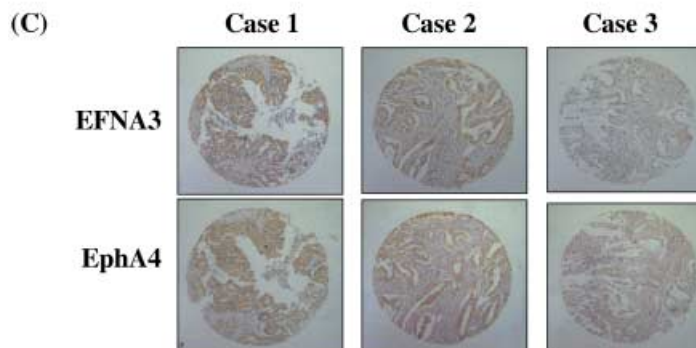
#### Discussion

In this study, we found overexpression of EphA4, a member of the Eph receptor kinase family, in PDAC cells, which indicates that molecules involved in the EphA4-signaling pathway might be very promising molecular targets for the development of anticancer drugs. In general, receptor tyrosine kinases and their ligands play critical roles in the regulation of a variety of cell activities including cellular survival, proliferation, differentiation, and tissue organization.<sup>(21)</sup> Eph receptors and their ligands, ephrins, are indeed involved in several cell processes during embryonic development, including pattern formation, cell aggregation and migration, segmentation, neural development, angiogenesis, and vascular hierarchical remodeling.<sup>(7–11)</sup> The overexpression of some members of the Eph receptor family in various cancers was found to play an important role in the development and progression of cancer cells. In particular, EphA2 and EphB2 overexpression was found frequently in human invasive cancers including PDAC<sup>(12–18)</sup> and they are considered to be good targets for antibody treatment.<sup>(22,23)</sup> In addition to these two members, we here demonstrated a critical role of one member of the Eph receptor family, EphA4, in pancreatic tumorigenesis. We also identified ephrinA3 as a candidate ligand for EphA4 in PDAC cells. In our gene-expression profile studies for other types of cancer, we found that EphA4 was also overexpressed in a subset of prostate cancer and soft tissue sarcomas.<sup>(24,25)</sup> Hence, we suspect it has a carcinogenic role in a wide range of malignancies like other Eph family members.

A unique feature of the signaling of the Eph receptors and ephrin ligands is its bi-directionality.<sup>(26,27)</sup> In contrast to other receptor tyrosine kinases, Eph receptors are activated by interaction with membrane-attached ephrin ligands; interaction between cells expressing Eph receptors and those expressing ephrin ligand is required for activation of the Eph receptor signaling pathway. In addition, ephrin ligands on plasma membrane can also be activated and lead to separate signaling into ephrin-expressing cells. In this case, the ephrin signaling in ephrin-expressing cells is activated by the phosphorylation of tyrosine residing in the cytoplasmic tail of ephrins B or by ephrin A interaction with integrins or other membrane proteins.<sup>(27,28)</sup> Our expression data by RT-PCR and immunohistochemical analysis indicated that ephrinA3 ligand co-expressed with EphA4 in PDAC cells, and that ephrinA3 is likely to be a possible candidate for the ligand of EphA4 receptor in PDAC cells. Furthermore, experiments using siRNA to *EphA4* and *ephrinA3* have indicated that both EphA4 and ephrinA3 could play essential roles in PDAC cell viability, and EphA4 introduction into PDAC cells lacking in EphA4 expression resulted in growth promotion. These findings suggest that interactions between the EphA4 receptor and ephrinA3 in PDAC cells function as growth-promoting factors for PDAC cells in an autocrine/paracrine manner and probably through the EphA4–ephrinA3 bi-directional signaling in PDAC cells. Indeed EphA4 has been studied for its association in axon guidance or repulsion and regeneration in the central nervous system,<sup>(27,29)</sup> and the interaction



**Fig. 4.** Expression pattern of ephrin ligands in pancreatic ductal adenocarcinoma (PDAC) cells. Semi-quantitative RT-PCR in (A) clinical PDAC cells and (B) PDAC cell lines demonstrated that, among ephrin ligands A1-5 and B1-3, *ephrinA3* (*EFNA3*) expression pattern was relatively correlated with that of *EphA4*. *ACTB* served as a quantitative control. ND, normal pancreatic ductal cells; TP, total pancreas. (C) Immunohistochemical staining in PDAC tissue microarray demonstrated that ephrinA3 (*EFNA3*) expression (upper panel) was correlated with *EphA4* expression (lower panel). Three representative specimens with both *EFNA3* and *EphA4* positive (Cases 1 and 2) and with both negative (Case 3) are shown (original magnification  $\times 200$ ).



**Fig. 5.** Knockdown effect on ephrinA3 (*EFNA3*) by siRNA attenuated pancreatic ductal adenocarcinoma (PDAC) cell viability. Three *EFNA3* siRNA expression vectors (*EFNA3*-539si, -689si, and -1584si) and an EGFP siRNA expression vector (EGFPsi) as a negative control were transfected into MIA-PaCa2 cells. (A) Knockdown effect on *EFNA3* was validated by Western blot analysis with *ACTB* level as a quantitative control. *EFNA3*-689si and *EFNA3*-539si revealed a strong knockdown effect on *EFNA3* expression, while *EFNA3*-1584si and EGFPsi did not show any effect on *EFNA3* expression level. Transfection with *EFNA3*-689si and *EFNA3*-539si resulted in a drastic reduction of (B) the numbers of viable cells measured by MTT assay and (C) the number of colonies formed, compared with the cells transfected with siRNA expression vectors that did not show a knockdown effect on *EFNA3*. ABS, absorbance at 490 nm (630 nm reference), measured with a microplate reader.

between *EphA4* and ephrinA3 was implicated to be essential in spinal morphology and synaptic connections at neural development.<sup>(30)</sup> Our results are the first evidence indicating that this specific interaction between *EphA4* and ephrinA3 might play a critical role in promoting cancer cell proliferation in PDAC carcinogenesis or development.

We demonstrated that overexpression of ephrinA3 was involved in PDAC cell viability as well as *EphA4*. However, other Eph receptors and ephrin ligands have been implicated in altering cellular environment; in particular, neovascularization in fetal tissues and pathogenic angiogenesis in tumor are well known.<sup>(31,32)</sup> Our results do not exclude the possibility that other ephrin

ligands expressed in vascular cells or mesenchymal cells as well as cancer cells in pancreatic cancer tissues could promote tumor growth by cross-communication of these cells, and further *in vitro* and *in vivo* investigations are required to make clear the interaction between overexpressing *EphA4* and ephrin ligands in PDAC.

Our previous microarray data obtained by gene expression profile analysis of 29 normal human tissues<sup>(33)</sup> as well as our Northern blot analysis in Fig. 1B clearly indicate that *EphA4* expression in normal adult tissues is very restricted. *EphA4* was found to be expressed in the adult brain, but it has been suggested that therapeutic antibody targeting membrane molecules could

not pass through the blood–brain barrier and were unlikely to affect the central nervous system.<sup>(34)</sup> Considering the expression pattern of EphA4 together with its oncogenic function, EphA4 receptor could be an attractive and promising target for drug design by targeting its kinase property and also by an antibody-based strategy.

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