Identification of C2orf18, termed ANT2BP (ANT2-binding protein), as one of the key molecules involved in pancreatic carcinogenesis

Kotoe Kashiwaya,[1,2] Masayo Hosokawa,[1,2] Hidetoshi Eguchi,[3] Hiroaki Ohigashi,[3] Osamu Ishikawa,[3] Yasuhisa Shinomura,[2] Yusuke Nakamura[1] and Hidewaki Nakagawa[1,4,5]

[1]Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, The University of Tokyo, Tokyo 108-8639; [2]First Department of Internal Medicine, Sapporo Medical University, Sapporo 060-8556; [3]Department of Surgery, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka 537-8511; [4]Laboratory of Biomarker Development, Center for Genomic Medicine, RIKEN, Tokyo 108-8639, Japan

(Received September 10, 2008/Revised November 5, 2008/Accepted November 12, 2008/Online publication December 18, 2008)

Pancreatic ductal adenocarcinoma (PDAC) shows one of the worst mortality rates among the common malignancies, and the great majority of PDAC patients are diagnosed at an advanced stage where no effective therapy is presently available. Hence, identification of novel molecular targets and development of molecular therapy for PDAC are urgently required. Through our genome-wide gene expression profiles of microdissected PDAC cells, we here identified a novel gene *C2orf18* as a molecular target for PDAC treatment. Transcriptional and immunohistochemical analysis validated its overexpression in PDAC cells and limited expression in normal adult organs. Knockdown of *C2orf18* by small-interfering RNA in PDAC cell lines resulted in induction of apoptosis and suppression of cancer cell growth, suggesting its essential role in maintaining viability of PDAC cells. We showed that C2orf18 was localized in the mitochondria and it could interact with adenine nucleotide translocase 2 (ANT2), which is involved in maintenance of the mitochondrial membrane potential and energy homeostasis, and was indicated some roles in apoptosis. These findings implicated that C2orf18, termed ANT2-binding protein (ANT2BP), might serve as a candidate molecular target for pancreatic cancer therapy. (*Cancer Sci* 2009; 100: 457–464)

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer death in the Western world and reveals one of the worst mortality rates among the common malignancies, with a 5-year survival rate of only 5%.[1,2] In the United States, 37 680 new cases of pancreatic cancer are expected to be diagnosed in 2008 and nearly 34 290 patients are expected to die of this disease.[3] Currently, the only curative treatment for PDACs is surgical resection, but the majority of PDAC patients are diagnosed at a very advanced stage, and only 15–20% of patients are candidates for surgical resection at the time of diagnosis. Of those who undergo potentially curative surgery, most patients eventually relapse and die of their disease.[2] Some approaches such as the combination of surgery with chemotherapy, including gemcitabine or 5-FU, with or without radiation, can improve patients' quality of life,[1,2] but those treatments have very limited effects on the long-term survival of PDAC patients due to the extremely aggressive and chemo-resistant nature of this disease. To improve the clinical outcomes of the patients, development of novel molecular therapies for PDACs through identification of molecular targets is now an urgent issue.

We have been performing genome-wide expression profile analysis of various types of cancers including PDAC using cDNA microarrays in combination with microdissection to enrich populations of cancer cells for obtaining the high-quality data.[4] Among dozens of genes being *trans*-activated commonly in PDAC cells, we here focused on one novel gene, *C2orf18*, as an over-expressing one in PDAC cells and demonstrated that the protein coded by this gene could interact with ANT2 (adenine nucleotide translocase 2) in the mitochondria. The mitochondrial adenine nucleotide translocases (ANTs) catalyze ADP/ATP exchange across the mitochondrial inner membrane and play a critical role in energy homeostasis by regulating the ADP/ATP ratio in mitochondrial oxidative phosphorylation.[5,6] ANTs have three isoforms (ANT1, ANT2, and ANT3), and ANT1 and ANT3 export ATP produced by mitochondrial oxidative phosphorylation to the cytosol.[5,7] On the other hand, ANT2 is likely to be involved in the glycolysis-dependent ATP production, allowing the uptake of glycolytic cytoplasmic ATP in exchange with intramitochondrial ADP.[8] This process plays an important role in maintaining mitochondrial membrane potential ($\Delta\psi m$) in glycolysis-dependent cells, such as cancer cells.[8] In fact, expression of ANT2 was shown to be up-regulated in cancer cells and its expression levels seemed to be concordant to the growth rates of cancer cells.[4,9]

In this study, we demonstrated overexpression of C2orf18, termed ANT2BP (ANT2-binding protein), in PDAC cells and its involvement in maintenance of $\Delta\psi m$ as well as apoptosis. Our findings implicated that C2orf18/ANT2BP might be a good molecular target for pancreatic cancer therapy.

Materials and Methods

Cell lines and clinical samples. PDAC cell lines MIA-PaCa2 and Panc-1, and NIH3T3, HEK-293, and COS-7 cells were purchased from ATCC (American Type Culture Collection). PDAC cell lines KLM-1, PK-59, PK-45P, SUIT-2, and PK-1, were provided by the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). All cells were cultured under their respective depositors' recommendations: RPMI-1640 for Panc-1, KLM-1, PK-59, PK-45P, SUIT-2, and PK-1, and Dulbecco's modified Eagle's medium for COS-7, NIH3T3, HEK-293, and MIA-PaCa2. All cell lines were grown in monolayers in appropriate media supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic solution, and maintained at 37°C in air containing 5% CO_2. Frozen and paraffin-embedded PDAC tissues were obtained from surgical specimens that were resected at the Osaka Medical Center for Cancer and Cardiovascular Diseases under the appropriate informed consent,

[5]To whom correspondence should be addressed.
E-mail: hidewaki@ims.u-tokyo.ac.jp

doi: 10.1111/j.1349-7006.2008.01058.x

and this study using these clinical samples was approved by the institutional review board of the Institute of Medical Science, University of Tokyo, and Osaka Medical Center for Cancer and Cardiovascular Diseases.

Semi-quantitative reverse transcription–polymerase chain reaction (RT-PCR). The purification of PDAC cells and normal ductal epithelial cells from frozen PDAC tissues by micodissection has been described previously.[4] RNAs from the purified PDAC cells and normal pancreatic ductal epithelial cells were subjected to two rounds of RNA amplification using T7-based *in vitro* transcription and subsequently reversely transcribed to single-strand cDNA. Total RNAs from human pancreatic cancer cell lines were extracted using Trizol reagent (Invitrogen) according to the manufacturer's recommended procedures. Extracted RNA was treated with DNase I (Nippon Gene, Osaka, Japan) and reversely transcribed to single-stranded cDNAs using oligo (dT) primer with Superscript II reverse transcriptase (Invitrogen). We prepared appropriate dilutions of each single-stranded cDNA for subsequent PCR amplification by monitoring α-tubulin (*TUBA*) as a quantitative control. The primer sequences we used were 5′-AAGGATTATGAGGAGGTTGGTGT-3′ and 5′-CTTGGGTCTGTAACAAAGCATTC-3′ for *TUBA*, and 5′-GGTAGCTCAGTCATAAAACACCG-3′ and 5′-GTCTCT-CCATCATCCTCACTGTC-3′ for *C2orf18* (NM_017877). All reactions involved initial denaturation at 94°C for 2 min followed by 23 cycles (for *TUBA*) or 28 cycles (for *C2orf18*) at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, on a GeneAmp PCR system 9700 (PE Applied Biosystems, Foster, CA, USA).

Northern blot analysis. We extracted total RNAs from 14 pancreatic cancer cell lines using Trizol reagent (Invitrogen) and performed northern blot analysis. After treatment with DNase I (Nippon Gene), mRNA was purified with a mRNA Purification Kit (GE Healthcare, Piscataway, NJ, USA), according to the manufacturer's protocols. One μg of each mRNA from pancreatic cancer cell lines, as well as those isolated from normal human adult heart, lung, liver, kidney, bone marrow, and pancreas, were separated on 1% denaturing agarose gels and transferred onto a nylon membrane. The cancer membrane and Human Multiple Tissue blots (Clontech, Palo Alto, CA, USA) were hybridized for 16 h with ^{32}P-labeled *C2orf18* cDNA, which was labeled using a Mega Label kit (GE Healthcare). A 232-bp PCR product of *C2orf18* cDNA was prepared as a probe using primers 5′-GGTAGCTCAGTCATAAAACACCG-3′ and 5′-GTCTCTCCAT-CATCCTCACTGTC-3′. Prehybridization, hybridization, and washing were performed according to the manufacturer's instructions. The blots were autoradiographed at –80°C for 10 days.

Expression constructs for C2orf18 and ANT2. Full-length cDNA encoding human *C2orf18* (NM_017877) was PCR-amplified by the use of a set of primers: forward primer, 5′-ATTTGAGGAAGATCATGGCCTGGACCAAGTACCA-3′; and reverse primer, 5′-CCGCTCGAGGCTGGCATCATTGATGGGA-3′. Subsequently the cDNA was cloned into the *Not1* and *Xho1* sites of the pCAGGS vector (pCAGGS-C2orf18-HA). Similarly, full-length cDNA fragment encoding human *ANT2* (*SLC25A5*, NM_001152) was PCR-amplified by following primers: forward primer, 5′-ATTCGCGGCCGCTCATGACAGATGCCGCTGTGTC-3′; and reverse primer, 5′-CCGCTCGAGTGTGTACTTCTTGA-TTTCA-3′; and was cloned into the *Not1* and *Xho1* sites of the pCAGGS vector (pCAGGS-ANT2-Flag). DNA sequences of these plasmids were confirmed by DNA sequencing.

Generation of antibodies specific to C2orf18 protein and immunohistochemical staining. The two peptides (CRAAGQSDSSVDP-QQPF and AEESEQERLLGGTRTPINDAS) corresponding to codons 70–86 and codons 351–371, respectively, of the C2orf18 protein (NP_060347) were generated by Sigma-Aldrich Japan (Ishikari, Japan), and their mixture was used for immunization of two rabbits. The sera from the immunized rabbits were purified by means of affinity-columns packed with Affi-Gel 10 activated affinity media (Bio-Rad Laboratories, Hercules, CA, USA) conjugating each of the peptide antigens according to the manufacturer's protocols. Conventional tissue sections from PDACs were obtained from surgical specimens that were resected at the Osaka Medical Center for Cancer and Cardiovascular Diseases under the appropriate informed consent. The sections were deparaffinized and autoclaved at 108°C in citrate buffer pH 6.0 for 15 min. Endogenous peroxidase activity was quenched by incubation in Peroxidase Blocking Reagent (Dako Cytomation, Carpinteria, CA, USA) for 30 min. After incubation in fetal bovine serum for blocking, the sections were incubated with rabbit anti-C2orf18 polyclonal antibody (dilution 1:1500) at room temperature for 1 h. After washing with phosphate-buffered saline (PBS), immunodetection was performed with peroxidase labeled antirabbit immunoglobulin (Envision kit; Dako Cytomation). Finally, the reactants were developed with 3, 3′-diaminobenzidin. Counterstaining was performed using hematoxylin.

Small-interfering RNA (siRNA) specific to *C2orf18*. To down-regulate endogenous *C2orf18* expression in PDAC cells, we used psiU6BX3.0 vector for expression of short hairpin RNA against a target gene as described previously.[10] The U6 promoter was cloned upstream of the gene-specific sequence (19-nt sequence from the target transcript, separated from the reverse complement of the same sequence by a short spacer, TTCAAGAGA), with five thymidines as a termination signal and a neo cassette for selection by Geneticin (Invitrogen). The target sequences for *C2orf18* were 5′-GGAGCACAGCTTCCAGCAT-3′ (#196), 5′-GCACGACAGTCAGCACAAG-3′ (#574), 5′-GTGACTTCCTC-TTTATGGA-3′ (#3254), and 5′-GAAGCAGCACGACTTCTTC-3′ (siEGFP) as a negative control. The human PDAC cell lines, MIA-PaCa2 and Panc-1, were seeded on 10-cm dishes, and transfected with each of #196, #574, #3254, or siEGFP expression vectors using FuGENE6 (Roche, Basel, Switzerland) according to the manufacturer's instructions. Cells were selected by 0.8 mg/mL (for MIA-PaCa2), or 1.0 mg/mL (for Panc-1) of Geneticin (Invitrogen). The cells were harvested at 7 days after the transfection to examine the knockdown effect on *C2orf18* by RT-PCR using the primers described for semiquantitative RT-PCR. After the culture in the appropriate medium containing Geneticin for 9 days, the cells were fixed with 100% methanol and stained with 0.1% of crystal violet-H_2O for colony formation assay. In the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay, cell viability was measured using the Cell-counting kit-8 (DOJINDO, Kumamoto, Japan) at 11 days after transfection. Absorbance was measured at 490 nm, and at 630 nm as reference, with a Microplate Reader 550 (Bio-Rad).

Immunocytochemical analysis. Panc-1 cells were treated with RNA duplex corresponding to #196 or siEGFP described above by using Lipofectamin RNAiMAX (Invitrogen) according to the manufacturer's recommended procedures. Seventy-two hours after the siRNA treatment, 200 nM MitoTracker Red (Invitrogen) was added to the culture medium for 30 min, and the cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS for 1 min at room temperature. Non-specific binding was blocked by treatment with PBS containing 3% bovine serum albumin (BSA) for 30 min at room temperature. The cells were incubated for 60 min at room temperature with rabbit anti-C2orf18 antibody diluted in PBS containing 1% BSA (1:500). After washing with PBS, the cells were stained by fluorescein-isothiocyanate-conjugated secondary antibody for 60 min at room temperature. After washing with PBS, specimen was mounted with VECTASHIELD (Vector Laboratories, Burlingame, CA, USA) containing 4′, 6′-diamidine-2′-phenylindolendihydrochrolide (DAPI) and visualized with Spectral Confocal Scanning Systems (Leica, Bensheim, Germany).

doi: 10.1111/j.1349-7006.2008.01058.x

Cell fractionation and localization of C2orf18 protein. To further investigate the subcellular localization of endogenous C2orf18 in PDAC cells, we first fractionated the cell lysate of Panc-1 cells to separate the cytoplasmic fraction from the nuclear fraction using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL, USA). Then, we homogenized Panc-1 cells in the homogenate buffer (0.25 M sucrose, 10 mM Tris-HCl [pH 7.4], 1 mM EDTA) and ultracentrifuged the cell lysate at 2300 *g* for 10 min at 4°C to remove the nuclei and debris. The supernatant was ultracentrifuged at 17 000 *g* for 20 min at 4°C to collect the fraction of the mitochondria. The supernatant was ultracentrifuged again at 100 000 *g* for 30 min at 4°C to separate the microsome fraction (supernatant) from other cytoplasmic components (pellet). Each of the cell fractions was separated on SDS-PAGE (sodium dodecylsulfate–polyacrylamide gel electrophoresis) and the endogenous C2orf18 was detected by anti-C2orf18 polyclonal antibody as described above, and the mitochondria fraction was detected specifically by antimitofilin antibody.

Immunoprecipitation and mass-spectrometric analysis for C2orf18-interacting proteins. To identify a protein(s) which could interact with C2orf18, we performed immunoprecipitation experiments. The pCAGGS-C2orf18-HA or empty pCAGGS-HA mock were transfected into pancreatic cancer cell line PK-1 using FuGENE6 (Roche). Forty-eight hours after the transfection, the cells were collected and lyzed in lysis buffer (50 mmol/L Tris-HCl [pH 8.0], 0.4% NP-40, 150 mmol/L NaCl, Protease Inhibitor Cocktail Set III [Calbiochem, San Diego, CA, USA]). Total proteins were incubated at 4°C for 15 min with 17.5 μg of rat monoclonal anti-HA antibody (clone3F10; Roche). Immunocomplexes were incubated with 300 μL of protein G Sepharose for 15 min and washed with lysis buffer. Co-precipitated proteins were separated in 12% SDS-PAGE gel and stained with a silver-staining kit. We excised bands that specifically appeared in the precipitates with anti-HA antibody in PK-1 cells transfected with C2orf18-HA, but not in those in the cells transfected with the mock clone, and then digested them in-gel with trypsin and analyzed for peptide-mass fingerprints using an AXIMA-CFRMALDI-TOF mass spectrometer (Shimadzu, Tsukuba, Japan). Peptide masses were searched with 10-p.p.m. mass accuracy, and protein database searches were done using the database-fitting program IntelliMarque (Shimadzu). To confirm the interaction between C2orf18 and ANT2 proteins, C2orf18-HA expression vector and/or ANT2-Flag expression vector were cotransfected into COS-7 cells. The transfected cells were lyzed as described above and immunoprecipitated with rat anti-HA antibody (clone3F10; Roche) or rabbit polyclonal anti-Flag antibody (F-7425; Roche). To examine the interaction of C2orf18-HA and ANT-2-Flag proteins, we analyzed these immune complexes by western blotting with rabbit anti-FLAG or anti-HA antibodies.

Detection of $\Delta\Psi_m$. KLM-1 cells were transfected with siRNA duplex targeting ANT2, 5′-GCAGATCACTGCAGATAA-3′, C2orf18 siRNA duplex 5′-GGAGCACAGCTTCCAGCAT-3′ (#196), or siEGFP duplex 5′-GAAGCAGCACGACTTCTTC-3′ (as a control). Forty-eight hours after the transfection, the cells were collected, and knockdown effect of siRNA duplex targeting C2orf18 was confirmed by western blot analysis with anti-C2orf18 polyclonal antibody as described above. The collected cells were washed twice with cold PBS, then incubated with 10-μM Rhodamine123 (Rh123) (Wako, Osaka, Japan) in PBS for 15 min at 37°C in dark conditions, washed with fluorescence activated cell sorting (FACS) buffer, and resuspended in 0.5 mL of FACS buffer containing 10 μg/mL of propidium iodide (PI). Rh123 is known to accumulate into the mitochondria following the electrochemical gradient. Once the unincorporated Rh123 is removed, the incorporated Rh123 is preferentially retained in the mitochondria in an amount proportional to $\Delta\psi$m.[11] The loss of the mitochondrial integrity of the opening of the permeability transition pore channel results in the leakage of this probe from the mitochondria and the consequent fluorescence decreases.[11,12] Fluorescence intensities from Rh123 (530 nm) and PI (600 nm) were measured by flow cytometry (FACSCalibur; BD Biosciences, Palo Alto, CA, USA).

TdT-mediated dUTP-biotin nick end labeling (TUNEL) *assay.* Panc-1 cells were transfected with siRNA#196 duplex 5′-GGAGCAC-AGCTTCCAGCAT-3′ and siEGFP duplex 5′-GAAGCAGCAC-GACTTCTTC-3′ as a negative control. Seventy-two hours after the transfection, the cells were collected, resuspended with PBS, and fixed by 1% paraformaldehyde in PBS (pH 7.4) for 15 min. After washing by PBS, the cells were permeabilized by precooled ethanol + acetic acid (2:1) for 5 min at –20°C for immunocytochemical analysis, or by precooled 70% ethanol for flow cytometry. To detect apoptosis by TUNEL assay, we used the ApopTag Fluorescein Direct *In situ* Apoptosis Detection Kit (Millipore, Bedford, MA, USA) according to the manufacturer's protocol, and visualized with Spectral Confocal Scanning Systems (Leica), or measured fluorescence by flow cytometry (Cell Laboratory Quanta SC MPL; Beckman Coulter, Fullerton, CA, USA). The permeabilized cells treated with DNase I were prepared as positive controls for TUNEL assay.

Results

Overexpression of C2orf18 in PDAC cells. Among dozens of genes that were identified to be *trans*-activated in PDAC cells through our genome-wide cDNA microarray analysis[4] we focused on one uncharacterized gene, *C2orf18*, for this study. RT-PCR analysis confirmed *C2orf18* overexpression in seven of the nine PDAC cells (Fig. 1a). Northern-blot analysis using a *C2orf18* cDNA fragment as a probe identified an about 4.4-kb transcript to be expressed highly in the prostate and the thyroid, and very faintly in vital organs including the lung, the heart, the liver, and the kidney (Fig. 1b). Its high level of expression was observed in most of the PDAC cell lines we examined (Fig. 1c). To examine its expression at the protein level, we generated a polyclonal antibody specific to C2orf18 protein, and performed western blot analysis that detected endogenous C2orf18 in all of the PDAC cell lines we examined (Fig. 1d). C2orf18 expression in PDAC cell lines was higher than that in non-cancerous cell lines (NIH3T3, HEK-293, and COS7). We also performed immunohistochemical analysis using clinical PDAC tissue sections and found its strong positive staining as small vesicles in the cytoplasm of PDAC cells (Panels C1–3 in Fig. 1e). Although no staining was detected in normal pancreas tissue (N in Fig. 1e), 10 out of 22 PDAC tissues revealed positive staining for C2orf18.

Knockdown of C2orf18 by siRNA attenuated PDAC cell viability. To investigate the biological function of C2orf18 in PDAC cells and its potential as a molecular target for PDAC treatment, we constructed several siRNA-expression vectors specific to *C2orf18* and transfected each of them into a PDAC cell line, MIA-PaCa2 that endogenously expressed high levels of C2orf18. RT-PCR indicated a significant knockdown effect of endogenous *C2orf18* when we transfected #196 and #574 siRNA-expression constructs (Fig. 2a left). Colony-formation assays (Fig. 2b left) and MTT assays (Fig. 2c left) after #196 or #574 transfection revealed a drastic reduction in the number of viable PDAC cells, compared with transfection of #3254 or a negative control siEGFP for which no knockdown effect was observed. When we used another C2orf18-positive PDAC cell line, Panc-1, we observed similar effects (Fig. 2a–c right).

Interaction of C2orf18 with ANT2 in the mitochondria. *In-silico* analysis predicted several *trans*-membrane domains in 371 amino-acid sequences of C2orf18 protein. Immunocytochemical analysis using anti-C2orf18 antibody revealed vesicular patterns of positive signals in the cytoplasm of PDAC cells and we observed the disappearance of these fluorescent signals by

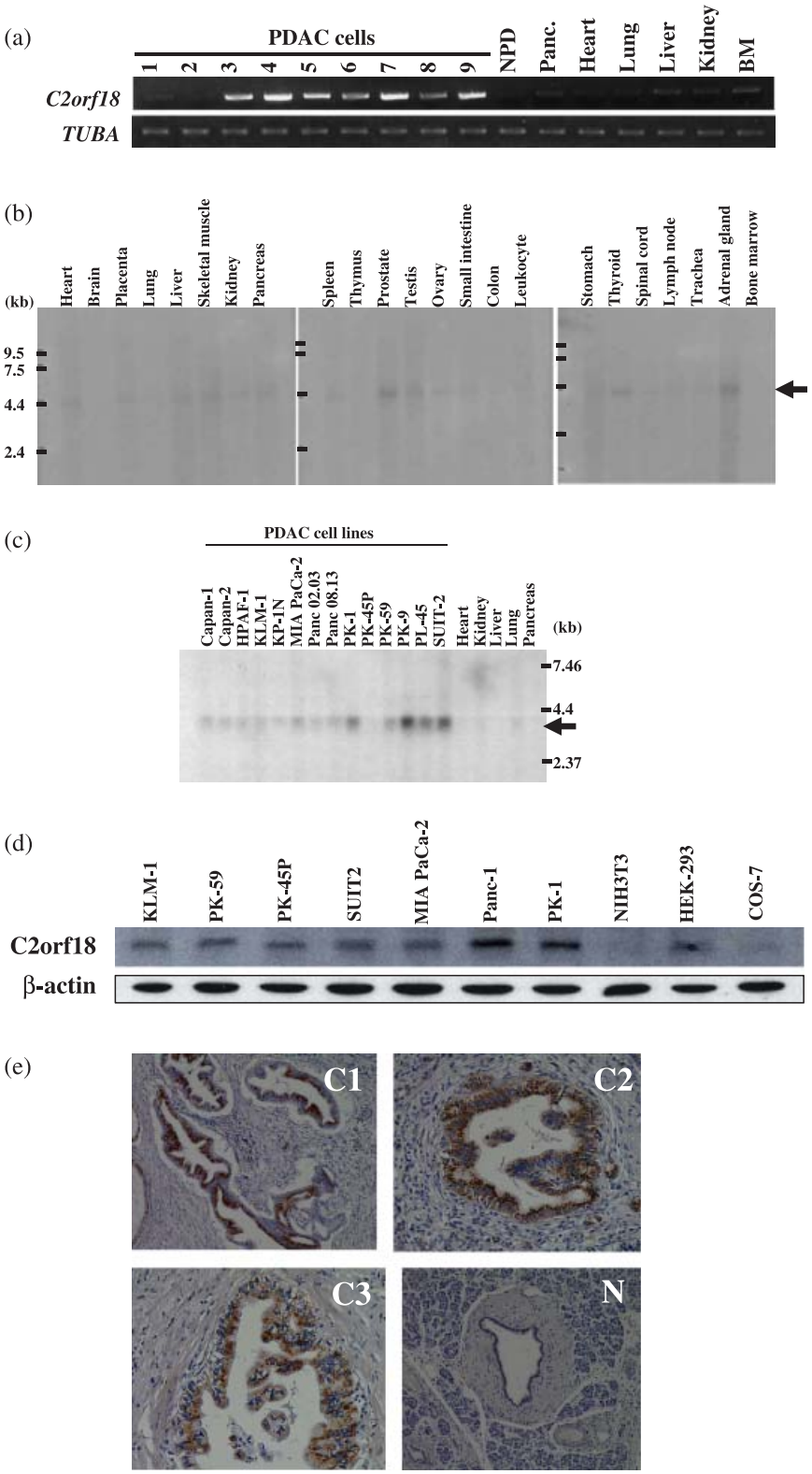

Fig. 1. C2orf18 overexpression in pancreatic ductal adenocarcinoma (PDAC) cells. (a) Rerverse transcription–polymerase chain reaction for RNA expressions of *C2orf18* and α-tubulin (*TUBA*) in the microdissected PDAC cells[1–9] comparing with normal pancreatic ductal epithelial cells (NPD) which were also microdissected and vital adult organs. Panc, normal pancreas; BM, bone marrow. (b, c) Northern blot analysis revealed that *C2orf18* was expressed highly in almost all pancreatic cancer cell lines we examined, and faintly in prostate, thyroid, adrenal gland tissues, but very limited in vital organs. (d) Western blot analysis using anti-C2orf18 antibody we generated detected endogenous C2orf18 in all PDAC cell lines examined. C2orf18 expression in PDAC cell lines was higher than that in non-cancerous cell lines (NIH3T3, HEK-293, and COS7). β-Actin served as the loading control. (e) In immunohistochemical study using anti-C2orf18 antibody, strong positive staining of C2orf18 was observed as a small vesicle pattern in the cytoplasm of PDAC cells (C1 ×200, C2 ×400, and C3 ×400). In normal pancreatic tissue, acinar cells and normal ductal epithelium cells showed no staining (N, ×400). In total, 10 of 22 (45%) PDAC tissues showed positive staining for C2orf18.

knockdown of endogenous C2orf18 using siRNA duplex (Fig. 3a). These signals were partially merged with the signals of MitoTracker, the mitochondria-specific probe (Fig. 3a), indicating its localization in the mitochondria. To define the subcellular localization of C2orf18 protein precisely, we fractionated the lysate of Panc-1 cells into the mitochondria, the endoplasmic reticulum (ER), and other cytoplasmic fraction. As shown in Fig. 3(b), western blot analysis detected C2orf18 protein only in the mitochondrial fraction, as similar to the mitochondria-specific protein, mitofilin. *In-silico* analysis on C2orf18 protein predicted its possible function as a permease related to nucleotide-sugar transporters. To investigate C2orf18 functions in cancer cells, we attempted to identify proteins interacting with C2orf18. We immune-precipitated a protein

doi: 10.1111/j.1349-7006.2008.01058.x

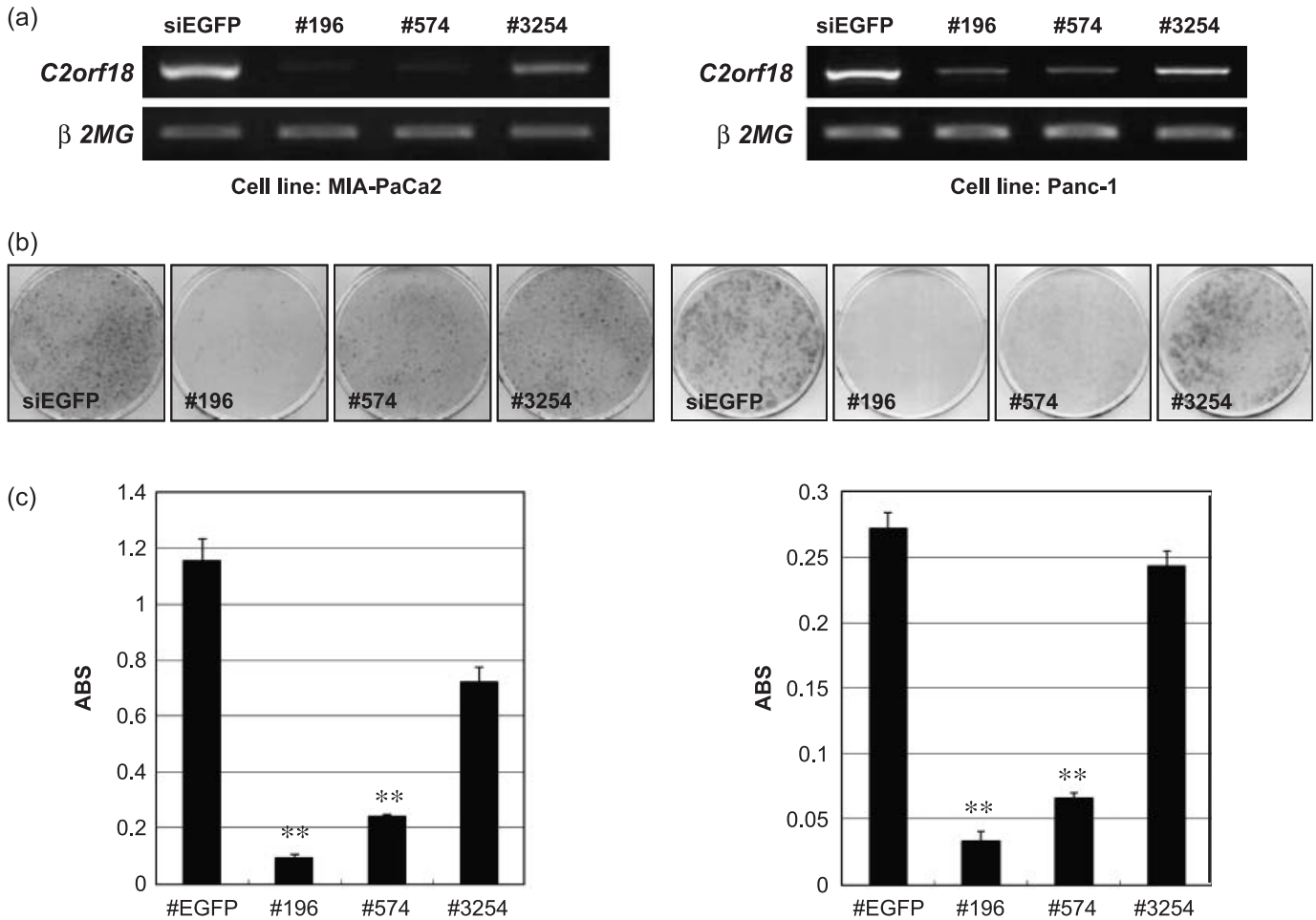

Fig. 2. Effect of C2orf18–small-interfering RNAs (siRNA) on growth of pancreatic ductal adenocarcinoma (PDAC) cells. (a) Knockdown effect of siRNA on *C2orf18* in PDAC cell lines MIA-PaCa2 (left) and Panc-1 (right). Semiquantitative reverse transcription–polymerase chain reaction was performed using cells transfected with each of siRNA-expressing vectors to *C2orf18* (#196, #574, and #3254) as well as the negative control siEGFP, which confirmed the knockdown effect by #196 and #574, but not by siEGFP and #3254. *β2-MG* was used to quantify RNAs. (b) Colony formation assay of MIA-PaCa2 (left) and Panc-1 (right) cells transfected with each of indicated siRNA-expressing vectors to *C2orf18* (#196, #574, and #3254) and the negative control siEGFP. Cells were visualized with 0.1% crystal violet staining after 9-days incubation with Geneticin. (c) MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay of each of MIA-PaCa2 (left) and Panc-1 (right) cells transfected with indicated siRNA-expressing vectors to *C2orf18* (#196, #574, and #3254) and the negative control siEGFP. Each average is plotted with error bars indicating the SD after 11-days incubation with Geneticin. ABS on the y-axis means absorbance at 490 nm, and at 630 nm as reference, measured with a microplate reader. These experiments were carried out in triplicate. ***P*-value of < 0.01 (Students' *t*-test).

complex including C2orf18 from the lysates of cells that overexpressed exogenous C2orf18-HA. We then characterized a protein that was likely to interact with C2orf18 by mass spectrometry and identified ANT2 protein (SLC25A5, NP_00143) to be a candidate interacting with C2orf18 protein. To validate the interaction between C2orf18 and ANT2, we transfected either of the vectors expressing C2orf18-HA, ANT2-Flag, or both vectors together into COS-7 cells, and a protein complex containing C2orf18-HA and/or ANT2-Flag was immunoprecipitated from the cell extracts by anti-HA antibody (Fig. 3c left) or anti-Flag antibody (Fig. 3c right). In Fig. 3c (left), western blotting using anti-Flag antibody indicated that ANT2-Flag was coimmunoprecipitated with C2orf18-HA when the both expression vectors were cotransfected. Furthermore, in Fig. 3c (right), western blotting using anti-HA antibody indicated that C2orf18-HA was coimmunoprecipitated with ANT2-Flag when the both expression vectors were coexpressed. Hence, we termed this uncharacterized protein as ANT2-binding protein (ANT2BP) and suspected that it had possible role as a nucleotide-sugar transporter as well as ANT2.

C2orf18/ANT2BP was involved with $\Delta\Psi_m$ and apoptosis. In cancer cells or cells with mitochondrial defects, the generation of $\Delta\psi m$ is suspected to be dependent mainly on the ATP^{4-}/ADP^{3-} exchange by ANT2.[8,13–15] Hence, ANT2 silencing facilitated apoptosis by modulating $\Delta\psi m$.[16,17] To examine whether ANT2BP could regulate $\Delta\psi m$ and be involved in mitochondrial apoptosis, we knocked down ANT2BP expression in PDAC cells and examined $\Delta\psi m$ and apoptosis by staining with Rh123 and TUNEL assay, respectively. Western blot analysis using anti-C2orf18/ANT2BP antibody confirmed the knockdown effect of C2orf18/ANT2BP siRNA on KLM-1 cells (Fig. 4a). In Fig. 4(b), Rh123 intensity on the x-axis reflects $\Delta\psi m$, and PI permeability on the y-axis reflects the cell membrane destruction in dead cells. The low level of Rh123 intensity and negative-permeability of PI indicate the early apoptotic cells where $\Delta\psi m$ is decreased but apoptosis does not finish completely, while the low level of Rh123 intensity and positive-permeability of PI indicate dead cells.[18] The numbers of the cells showing low $\Delta\psi m$ and negative-permeability of PI were increased when ANTBP2 (25%) or ANT2 (26%) were knocked down, compared with the

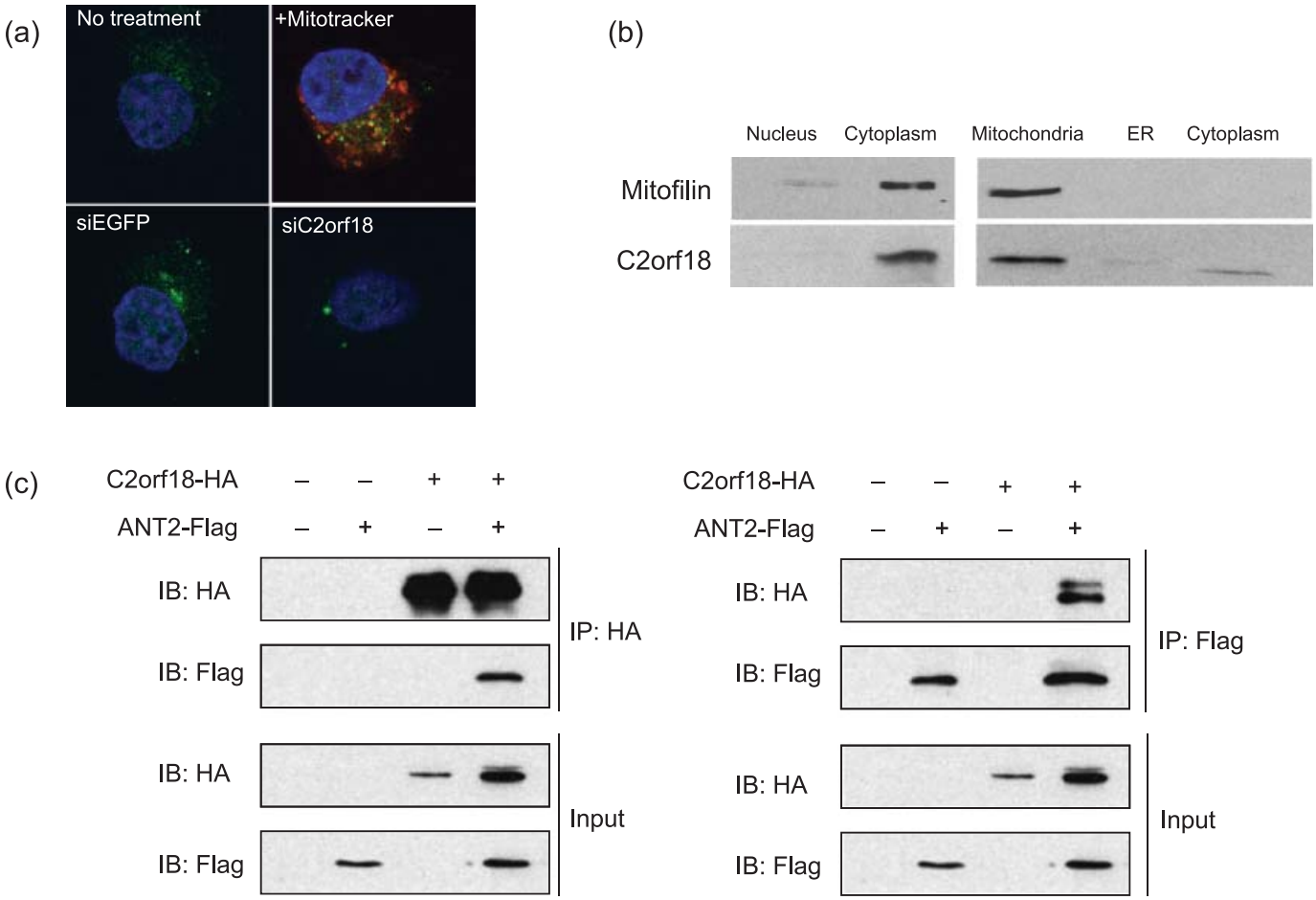

Fig. 3. Interaction of C2orf18 with adenine nucleotide translocase 2 (ANT2) in the mitochondria. (a) Immunocytochemical analysis using anti-C2orf18 antibody detected the positive signals (green) as vesicular patterns in the cytoplasm of pancreatic ductal adenocarcinoma (PDAC) cells (upper left panel). These signals for anti-C2orf18 antibody (green) were partially merged with MitoTracker signals (red: upper right panel). These signals for anti-C2orf18 antibody disappeared when knocking down C2orf18 by siRNA (lower right panel), while they were retained as vesicular patterns in the cytoplasm by treating the control siRNA siEGFP (lower left panel). (b) Western blot analysis using anti-C2orf18 antibody following the cellular fractionation showed that C2orf18 was localized in the cytoplasm and only in the mitochondrial fraction. Anti-mitofilin antibody was used to detect the mitochondrial fraction. ER, endoplasmic reticulum. (c) ANT2 was identified as a candidate of C2orf18-interacting proteins by mass spectrometry following immunoprecipitation. C2orf18-HA expression vector and/or ANT2-Flag expression vector were cotransfected into COS7 cells. Protein complexes containing C2orf18-HA or ANT2-Flag were immunoprecipitated from cell extracts by anti-HA antibody (left panel) or anti-Flag antibody (right panel), respectively. Western blotting using anti-Flag antibody indicated that ANT2-Flag was coimmunoprecipitated with C2orf18-HA when both expression vectors were cotransfected (left panel). Western blot using anti-HA antibody indicated that C2orf18-HA was coimmunoprecipitated with ANT2-Flag when both expression vectors were coexpressed (right panel).

control (siEGFP, 19%). This FACS analysis implicated that ANT2BP knockdown induced reduction of $\Delta\psi m$, as well as ANT2 knockdown. Furthermore, TUNEL staining (Fig. 4c) and FACS analysis (Fig. 4d) showed a significant increase in the number of apoptosis cells when ANT2BP was knocked down. These data indicated ANT2BP could play a critical role in maintaining $\Delta\psi m$ and be involved in the mitochondrial apoptotic pathway, similar to ANT2, probably through its interaction with ANT2.

Discussion

In this study, we found evidence indicating the novel gene *C2orf18/ANT2BP* to be one of key molecules involved in pancreatic carcinogenesis through the genome-wide gene expression profile analysis of PDAC cells. Knockdown of C2orf18/ANT2BP using siRNA in PDAC cell lines resulted in drastic suppression of cancer cell viability and induced apoptosis following the breakdown of $\Delta\psi m$, implicating its essential role in maintaining viability of PDAC cells.

We found some evidence indicating that the interaction of C2orf18 with ANT2, which was shown to have a function to catalyze the exchange of mitochondrial ADP with cytosolic ATP, as one of the components of mitochondrial permeability transition pore complex (PTPC). PTPC plays a key role in the regulation of mitochondrial membrane permeabilization during apoptosis, necrosis, and autophagy, and ANT family members have critical roles in the maintenance of $\Delta\psi m$ as well as ATP exchange or metabolism related with respiration.[6,19] In cancer cells, energy metabolism or ATP production is dependent mainly on glycolysis, which has been recognized as the Warburg effect.[20] Among ANT family members, only ANT2 is likely to be associated with this glycolysis-dependent ATP production and its transport, and was shown to be up-regulated in cancer cells and proliferating cells under hypoxia as well as cells with mitochondrial defects.[8,13–15] Since it interacts with ANT2, C2orf18/ANT2BP is suspected to be involved in glycolysis-dependent ATP production and transport as a member of the ANT2 complex, and to make pancreatic cancer cells resistant to hypoxia or chemotherapy, although further functional analysis

doi: 10.1111/j.1349-7006.2008.01058.x

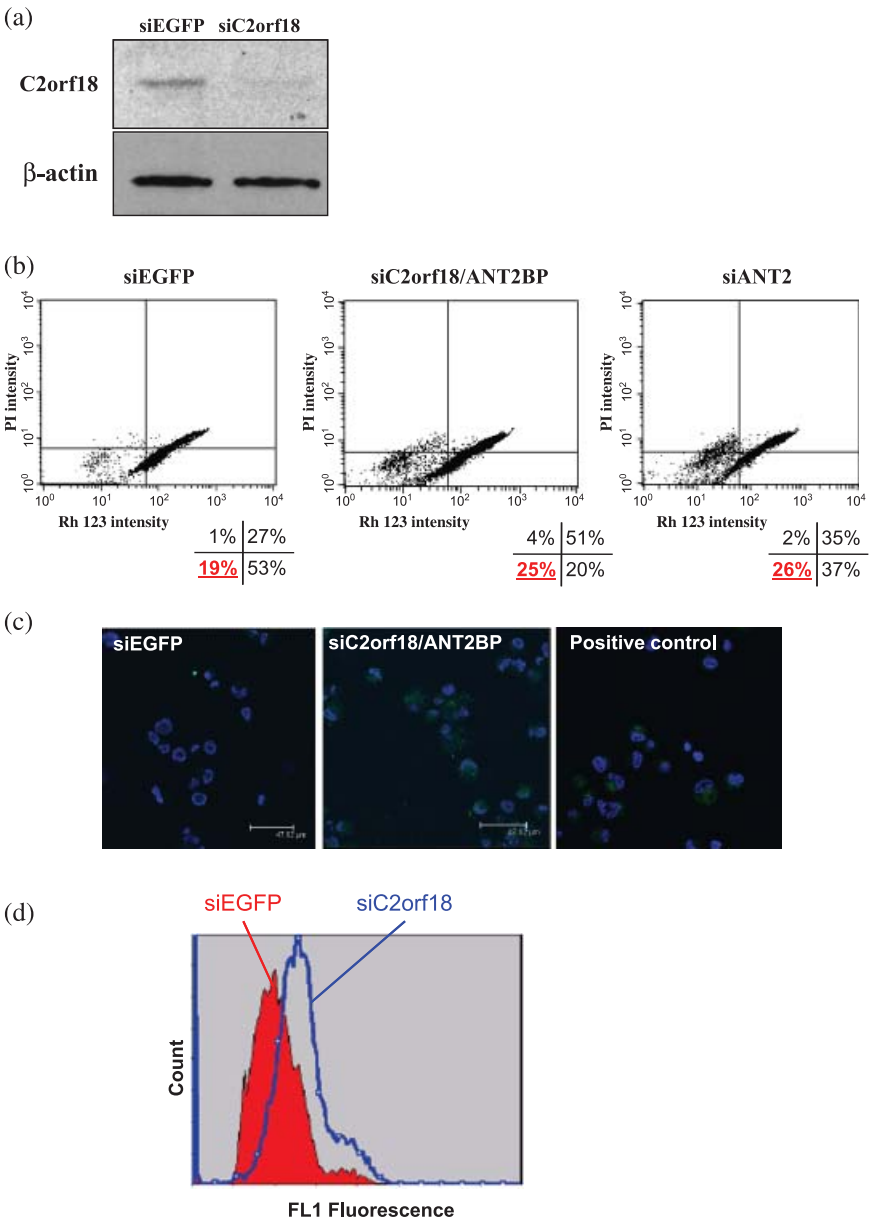

Fig. 4. C2orf18/ANT2BP was involved with mitochondrial membrane potential (Δψm) and apoptosis. (a) Pancreatic ductal adenocarcinoma (PDAC) cells KLM-1 were transfected with adenine nucleotide translocase 2 (ANT2) small-interfering RNA (siRNA), C2orf18/ANT2BP siRNA, or siEGFP (as a control) and collected 48 h after transfection. Western blot analysis using anti-C2orf18/ANT2BP antibody confirmed the knockdown effect of C2orf18/ANT2BP siRNA on KLM-1 cells. (b) The cells were incubated with Rhodamine123 (Rh123) and propidium iodide (PI), and fluorescence was measured by FACS analysis. Rh123 intensity on the x-axis reflects Δψm, and PI permeability on the y-axis reflects the cell membrane destruction in dead cells. The low level of Rh123 intensity and negative-permeability of PI indicate the early apoptotic cells where Δψm is decreased but apoptosis does not finish completely, while the low level of Rh123 intensity and positive-permeability of PI indicate dead cells. The numbers of the cells showing low Δψm and negative-permeability of PI were increased when ANTBP2 (25%) or ANT2 (26%) were knocked down, compared with the control (siEGFP, 19%). (c) TUNEL assay showed that knockdown of C2orf18 in Panc-1 cells increased the number of apoptosis cells (TUNEL-positive cells indicated by green) compared with siEGFP-transfected cells. The permeabilized cells treated with DNase I were prepared as positive controls for TUNEL assay. (d) The number of TUNEL-positive cells was counted by flow cytometry. In the histogram plot, the x-axis reflects the intensity of the green signals of TUNEL-positive cells. The shift to the right of the histogram indicates that knockdown of C2orf18 increased the number of TUNEL-positive cells compared with siEGFP-transfected cells.

of C2orf18/ANT2BP in the mitochondria or energy metabolism of cancer cells is required. Some small compounds are already established to inhibit an ANT transporter as anticancer drugs.[21–23] Considering the possible transporter function of C2orf18/ANT2BP which was predicted by *in-silico* analysis and was supported by our data, further functional analysis could lead to development of an inhibitor to C2orf18/ANT2BP as a novel anticancer drug. In summary, our expressional and functional findings implicated that C2orf18/ANT2BP could be a promising molecular target for PDAC therapy and other cancers.

Acknowledgments

We would like to thank Mr Yasuo Mochizuki and Ms Hiromi Kato for their technical assistance. This work was supported in part by a Research for the Future Program Grant #00L01402 from the Japan Society for the Promotion of Science.

References

1 DiMagno EP, Reber HA, Tempero MA. AGA technical review on the epidemiology, diagnosis, and treatment of pancreatic ductal adenocarcinoma. *Gastroenterology* 1999; **117**: 1464–84.
2 Wray CJ, Ahmad SA, Matthews JB, Lowy AM. Surgery for pancreatic cancer: recent controversies and current practice. *Gastroenterology* 2005; **128**: 1626–41.
3 Jemal A, Siegel R, Ward E *et al*. Cancer statistics, 2008. *CA Cancer J Clin* 2008; **58**: 71–96.
4 Nakamura T, Furukawa Y, Nakagawa H *et al*. Genome-wide cDNA microarray analysis of gene-expression profiles in pancreatic cancers using populations of tumor cells and normal ductal epithelial cells selected for purity by laser microdissection. *Oncogene* 2004; **23**: 2385–400.
5 Stepien G, Torroni A, Chung AB *et al*. Differential expression of adenine nucleotide translocator isoforms in mammalian tissues and during muscle cell differentiation. *J Biol Chem* 1992; **267**: 14592–7.
6 Crompton M. Mitochondrial intermembrane junctional complexes and their role in cell death. *J Physiol* 2000; **529**: 11–21.
7 Zamora M, Granell M, Mampel T, Viñas O. Adenine nucleotide translocase 3 (ANT3) overexpression induces apoptosis in cultured cells. *FEBS Lett* 2004; **563**: 155–60.
8 Chevrollier A, Loiseau D, Chabi B *et al*. ANT2 isoform required for cancer cell glycolysis. *J Bioenerg Biomembr* 2005; **37**: 307–16.

9 Battini R, Ferrari S, Kaczmarek L *et al*. Molecular cloning of a cDNA for a human ADP/ATP carrier which is growth-regulated. *J Bio Chem* 1987; **262**: 4355–9.
10 Hosokawa M, Kashiwaya K, Furihata M *et al*. Over-expression of cysteine proteinase inhibitor cystatin 6 promotes pancreatic cancer growth. *Cancer Sci* 2008; **99**: 1626–32.
11 Darzynkiewicz Z, Staiano-Coico L, Melamed MR. Increased mitochondrial uptake of rhodamine 123 during lymphocyte stimulation. *Proc Natl Acad Sci USA* 1981; **78**: 2383–7.
12 Johnson LV, Walsh ML, Chen LB. Localization of mitochondria in living cells with rhodamine 123. *Proc Natl Acad Sci USA* 1980; **77**: 990–4.
13 Bonod-Bidaud C, Chevrollier A, Bourasseau I *et al*. Induction of ANT2 in liver of patients with mitochondrial DNA depletion. *Mitochondria* 2001; **1**: 217–24.
14 Loiseau D, Chevrollier A, Douay O *et al*. Oxygen consumption and expression of the adenine nucleotide translocator in cells lacking mitochondria. *Exp Cell Res* 2002; **278**: 12–8.
15 Chevrollier A, Loiseau D, Gaurier F *et al*. ANT2 expression under hypoxia conditions produces opposite cell-cycle behavior 143B and HepG2 cancer cells. *Mol Carcinog* 2005; **42**: 1–8.
16 Jang JY, Choi Y, Jeon YK, Kim CW. Suppression of adenine nucleotide translocase-2 by vector-based siRNA in human breast cancer cells induces apoptosis and inhibits tumor growth *in vitro* and *in vivo*. *Breast Cancer Res* 2008; **10**: R11.
17 Le Bras M, Borgne-Sanchez A, Touat Z *et al*. Chemosensitization by knockdown of adenine nucleotide translocase-2. *Cancer Res* 2006; **66**: 9143–52.
18 Shimizu S, Eguchi Y, Kamiike W *et al*. Bcl-2 blocks loss of mitochondrial membrane potential while ICE inhibitors act at a different step during inhibition of death induced by respiratory chain inhibitors. *Oncogene* 1996; **13**: 21–9.
19 Verrier F, Deniaud A, Lebras M *et al*. Dynamic evolution of the adenine nucleotide translocase interactome during chemotherapy-induced apoptosis. *Oncogene* 2004; **23**: 8049–64.
20 Wallace DE. Mitochondria and cancer: Warburg addressed. *Cold Spring Harb Symp Quant Biol* 2005; **70**: 363–74.
21 Galluzzi L, Larochette N, Zamzami N, Kroemer G. Mitochondria as therapeutic targets for cancer chemotherapy. *Oncogene* 2006; **25**: 4812–30.
22 Don AS, Kisker O, Dilda P *et al*. A peptide trivalent arsenical inhibits tumor angiogenesis by perturbing mitochondrial function in angiogenic endothelial cells. *Cancer Cell* 2003; **3**: 497–509.
23 Machida K, Hayashi Y, Osada H. A novel adenine nucleotide translocase inhibitor, MT-21, induces cytochrome c release by a mitochondrial permeability transition-independent mechanism. *J Bio Chem* 2002; **277**: 31243–8.

doi: 10.1111/j.1349-7006.2008.01058.x