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Analysis of transcription profile to reveal altered signaling pathways following the overexpression of human desumoylating isopeptidase 2 in pancreatic cancer cells

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Abstract. Human desumoylating isopeptidase 2 (DESI-2) is a member of the DESI family and contains a conserved PPPDE1 domain. Previous studies have demonstrated that DESI-2 overexpression may induce cell apoptosis. In the present study, differentially expressed genes were analyzed using a transcription microarray in DESI-2 overexpressing PANC-1 pancreatic cancer cells. A total of 45,033 genes were examined by microarray, which identified 1,766 upregulated and 1,643 downregulated genes. A series of altered signaling pathways were analyzed, in which certain essential signaling factors, including retinoid X receptor (RXR), BH3 interacting-domain death agonist, Ras homolog gene family member A (RhoA) and Rho-associated protein kinase, were further investigated at the protein level. The release of cytochrome *c* and the activation of caspase-3 were also detected by western blot analysis. Immunohistochemistry further revealed the expression features of RXR and RhoA in pancreatic ductal adenocarcinoma tissues with various DESI-2 expression levels. The results serve as a valuable reference for the further elucidation of the functions of DESI-2 in pancreatic cancer.

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Introduction

Human desumoylating isopeptidase 2 (DESI-2), also known as PPPDE peptidase domain-containing protein 1 (PPPDE-1) was initially identified through large-scale genome sequencing (1-3). DESI-2 is ubiquitously distributed in the majority of organs and tissues, and its altered expression has been detected in certain cancer types (4). Bioinformatics have indicated that DESI-2 contains a PPPDE domain and belongs to the DESI protein family (5). As another member of the DESI family, DESI-1 has been reported to be a deSUMOylase that reverses protein SUMOylation (6). Therefore, DESI-2 is expected to possess deSUMOylase activity to regulate protein SUMOylation, which may serve important roles in diverse cellular processes (7,8).

Although previous studies have determined certain functions of DESI-2 (9-11), its precise underlying molecular mechanisms have yet to be elucidated. A previous study demonstrated that the overexpression and depletion of DESI-2 in embryos was able to induce developmental defects in *Xenopus* and zebrafish animal models, indicating that DESI-2 is crucial for the maintenance of basic cellular activities (9,10). In our previous study, it was demonstrated that DESI-2 is located at the Golgi apparatus in the cytoplasm and that its expression levels are decreased in pancreatic cancer tissues (4). It has been identified that DESI-2 overexpression may induce apoptosis in A549 human lung adenocarcinoma cells, HeLa cervical cancer cells and PANC-1 pancreatic cancer cells (11). In the current study, the alterations in the cell transcriptional profile following the overexpression of DESI-2 were evaluated using an mRNA microarray; the key signaling pathways in pancreatic cancer tissues were also examined. The aim of the present study was to elucidate the biological functions of DESI-2 in cancer development and progression.

Materials and methods

Materials. The anti-DESI-2 primary antibody (#20517-1-AP) was purchased from Proteintech Group, Inc. (Rosemont, IL,

USA). Primary antibodies targeting BH3 interacting-domain death agonist (BID; #sc-373939), retinoid X receptor (RXR; #sc-553), cytochrome *c* (#sc-13156), caspase-3 (#sc-271028), Rho-associated protein kinase (ROCK; #sc-17794) and β-actin (#sc-81178) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The anti-Ras homolog gene family member A (RhoA) primary antibody (#ab54835) was purchased from Abcam (Cambridge, MA, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies, including goat anti-rabbit immunoglobulin (Ig)G (#ZB-2301) and goat anti-mouse IgG (#ZB-2305), were purchased from ZSGB-Bio (Beijing, China). The radioimmunoprecipitation assay (RIPA) lysis buffer was from the Beyotime Institute of Biotechnology (Haimen, China). A Histostain-Plus kit and an Immobilon Western Chemiluminescent HRP Substrate were purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and EMD Millipore (Billerica, MA, USA), respectively.

Cell culture and transfection. PANC-1 human epithelial pancreatic cancer cells (ATCC, Manassas, VA, USA) were cultured in Gibco Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (both from Thermo Fisher Scientific, Inc.) at 37˚C in a humidified incubator with an atmosphere containing 5% CO_2. The open reading frames of DESI-2 (GenBank accession: NM_016076) were subcloned into a plasmid pcDNA3.1(+) vector (Invitrogen; Thermo Fisher Scientific, Inc.) between the *Bam*HI and *Xho*I sites according to the manufacturer's instructions, in order to obtain the recombinant plasmid vector pcDNA3.1-DESI-2. The pcDNA3.1-DESI-2 and empty control vector were transfected into PANC-1 cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Following a 24-h transfection, the cells were collected and prepared for subsequent experiments.

Microarray. The Human 12x135K Gene Expression Array was manufactured by Roche NimbleGen, Inc. (Madison, WI, USA). Briefly, total RNA was isolated from PANC-1 cells using Trizol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.). The integrity of the RNA was assessed by electrophoresis on a 1% denaturing agarose gel. Double-stranded cDNA (ds-cDNA) was then synthesized from 1-μg RNA samples using a SuperScript ds-cDNA Synthesis kit (Invitrogen; Thermo Fisher Scientific, Inc.) and labeled with Cy3 fluorescence (Roche NimbleGen, Inc.). The microarrays were hybridized with Cy3-labeled ds-cDNA (Hybridization System; Roche NimbleGen, Inc.) and imaged using an Axon GenePix 4000B microarray scanner (Molecular Devices LLC, Sunnyvale, CA, USA). The images were imported into NimbleScan software version 2.5 (Roche NimbleGen, Inc.) for grid alignment and expression data analysis. Expression data were normalized through quantile normalization and the Robust Multichip Average algorithm included in the NimbleScan software. The differentially expressed genes (fold change ≥2.00) were identified through fold change filtering by Agilent GeneSpring GX software version 11.5.1 (Agilent Technologies, Inc., Santa Clara, CA, USA). Hierarchical clustering analysis was also performed using the Agilent GeneSpring GX software version 11.5.1. Gene Ontology (GO; www.geneontology.org) and Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/) analyses were performed to determine the respective roles of the differentially expressed genes.

Western blot analysis. To extract the proteins, PANC-1 cells were collected and lysed using RIPA lysis buffer on ice prior to being centrifuged at 12,000 x *g* for 30 min at 4˚C. Following the determination of protein concentration using a Pierce bicinchoninic acid assay kit (Thermo Fisher Scientific, Inc.), the protein samples (50 μg/lane, reduced with 50 mM dithiothreitol) were separated by 12% SDS-PAGE under denaturing conditions. Subsequently, the separated proteins were transferred onto polyvinylidene fluoride membranes and blocked in phosphate-buffered saline with 0.05% Tween® 20 (PBST) containing 5% skimmed milk for 2 h. The membranes were washed three times with PBST and incubated with the primary antibodies against DESI-2, RhoA, ROCK, RXR, BID, cytochrome *c*, caspase-3 and β-actin (dilution, 1:800) for 2 h at room temperature. The membranes were then washed three times with PBST and probed with the HRP-conjugated secondary antibodies (dilution, 1:10,000) for 1 h at room temperature. Following three washes with PBST, the target blots were visualized using Immobilon Western Chemiluminescent HRP Substrate. In order to detect cytochrome *c* in the cytoplasm, a Mitochondria Isolation kit (Beyotime Institute of Biotechnology) was used to remove the mitochondria, according to the manufacturer's instructions, and these were subsequently subjected to western blotting. The western blot experiments were each performed in triplicate to assess the relative protein abundance.

Immunohistochemistry. Pancreatic ductal adenocarcinoma tissue specimens were obtained from 96 patients with pancreatic cancer (age range, 31-74 years; 54 males and 42 females) who had undergone surgery at the West China Hospital of Sichuan University (Chengdu, China). The institutional ethics committee of Sichuan University approved the present study and informed consent was obtained from all patients enrolled in the study. The formalin-fixed and paraffin-embedded tissue samples were cut into 5-μm-thick sections using a microtome. The sections were dewaxed in xylene and rehydrated in graded alcohol. Following heat-induced epitope retrieval, the sections were probed for DESI-2, RXR and RhoA with the corresponding primary antibodies (dilution, 1:200) at 4˚C overnight. After washing for 3x5 min in PBS, the sections were stained using an avidin-biotin-peroxidase complex method and visualized with diaminobenzidine (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. The sections were imaged with a Leica DM2500 microscope and analyzed using Leica Application Suite software version 3.8 (Leica Microsystems, Inc., Buffalo Grove, IL, USA). DESI-2 expression level was scored as a staining intensity score (0, negative; 1, mild; 2, moderate; and 3, strong) multiplied by a score representing the percentage of positively staining cells (0, none; 1, 1-25%; 2, 26-50%; 3, 51-75%; and 4, 76-100%), resulting in an overall scoring scale of 0-12 (12). According to the median expression level of DESI-2, the cancer samples were divided into DESI-2-higher and DESI-2-lower tissue samples.

Statistical analysis. GO analysis was used to analyze differentially expressed genes with respect to GO categories. The P-value denotes the significance of the GO term enrichment in the differentially expressed gene list (threshold for significance, $P<0.05$). Biological pathways that were significantly enriched among the differentially expressed genes were determined by pathway analysis based on the KEGG database ($P<0.05$).

Results

Quality control and analysis of mRNA microarray. The recombinant vector pc-DNA3.1(+) containing DESI-2 was transiently overexpressed in PANC-1 cells, with an empty vector as the control. The total RNA was isolated from the cells and subjected to quality control prior to mRNA microarray. Agarose gel electrophoresis demonstrated that the isolated RNA samples were able to be separated into three distinct bands of 28s RNA, 18s RNA and 5s RNA (Fig. 1A). The gene expression variations were assessed and visualized using the scatter-plot method to identify differential genes (Fig. 1B). Hierarchical cluster analysis was performed based on their expression levels and presented in the dendrogram. The results revealed distinguishable gene expression profiles between DESI-2 overexpressing cells and the control cells (Fig. 1C).

Alterations in cell signaling pathways following the overexpression of DESI-2. A total of 45,033 genes were examined by NimbleGen Human Gene Expression Microarrays. The genes were considered to be differentially expressed when the mRNA abundance exceeded the fold change threshold (fold change ≥2.0). The altered expression included a total of 1,766 upregulated and 1,643 downregulated genes. These differential genes were subsequently subjected to pathway analysis using the KEGG database. The upregulated genes were primarily distributed in protein degradation, cell death and differentiation (Fig. 2A; only the top 10 altered pathways are presented). It is noteworthy that a number of genes associated with apoptosis signaling, including Fas, caspase-10, BID and TRADD, exhibited increased expression levels (Fig. 2B). The downregulated genes were principally distributed in metabolism, cell movement and adhesion (Fig. 3A; only the top 10 altered pathways are presented). The critical genes Rho-guanine nucleotide exchange factor (GEF), RhoA and ROCK in the Rho signaling pathway exhibited decreased expression levels (Fig. 3B).

Western blotting to determine the differential expression of key genes. As changes to the mRNA must be evaluated at the protein level, the key signaling pathways that were altered in DESI-2 overexpressing cells were further examined. Western blotting demonstrated that RXR and BID are upregulated in PANC-1 cells that overexpress DESI-2 (Fig. 4). BID has an essential role in the apoptosis pathway and, therefore, its downstream signaling pathways, including the release of cytochrome *c* and the activation of caspase-3, were further investigated. As hypothesized, cleaved caspase-3 and the emergence of cytochrome *c* in cytoplasm were detected in the cells overexpressing DESI-2, but not in the control PANC-1 cells (Fig. 4). These results suggest that endogenous apoptosis may be induced by the overexpression of DESI-2 in PANC-1 cells.

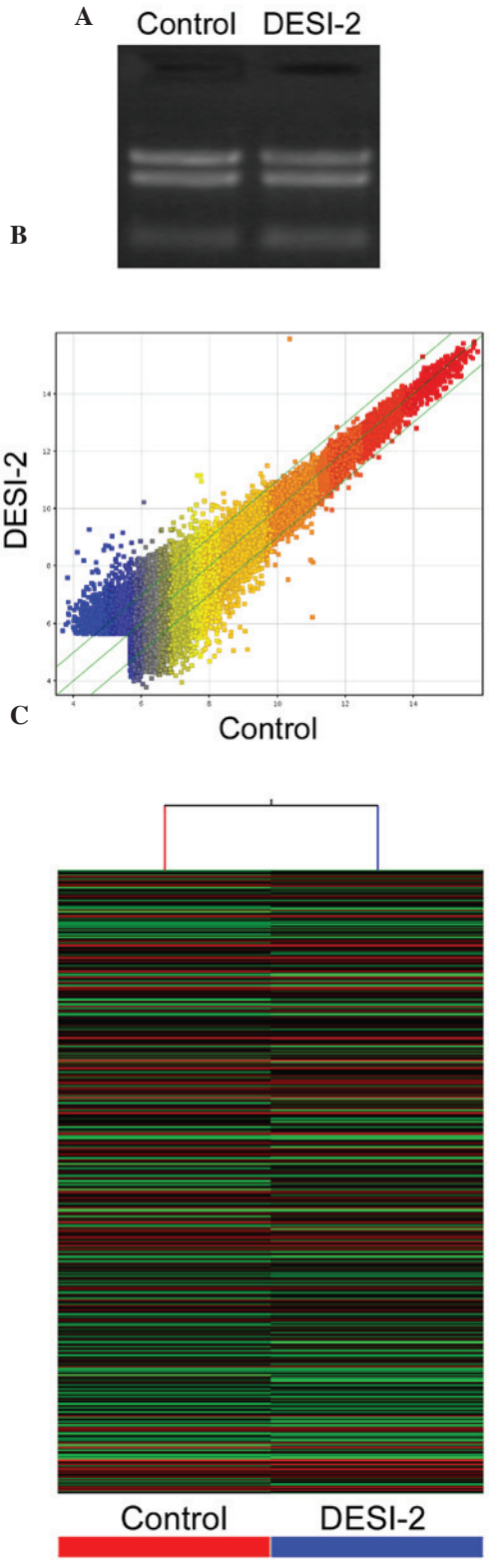

Figure 1. Quality control and analysis of the mRNA microarray. (A) Agarose gel electrophoresis of the isolated RNA samples. Following a 24 h transient transfection with a DESI-2-expressing plasmid vector into PANC-1 cells, the total RNA was isolated for analysis by agarose gel electrophoresis. The RNA samples were separated into 28s RNA, 18s RNA and 5s RNA bands. (B) A scatter-plot was used to assess the variations in gene expression between the two microarrays of DESI-2-overexpressing PANC-1 cells and control PANC-1 cells. The green lines represent the fold change analysis. The genes above the top green line and below the bottom green line indicate a ≥2.0 fold change between the two compared microarrays. (C) Hierarchical clustering was performed based on **all target values. The results demonstrated dis**tinguishable gene expression profiles between the cells that overexpressed DESI-2 and the control cells. DESI-2, desumoylating isopeptidase 2.

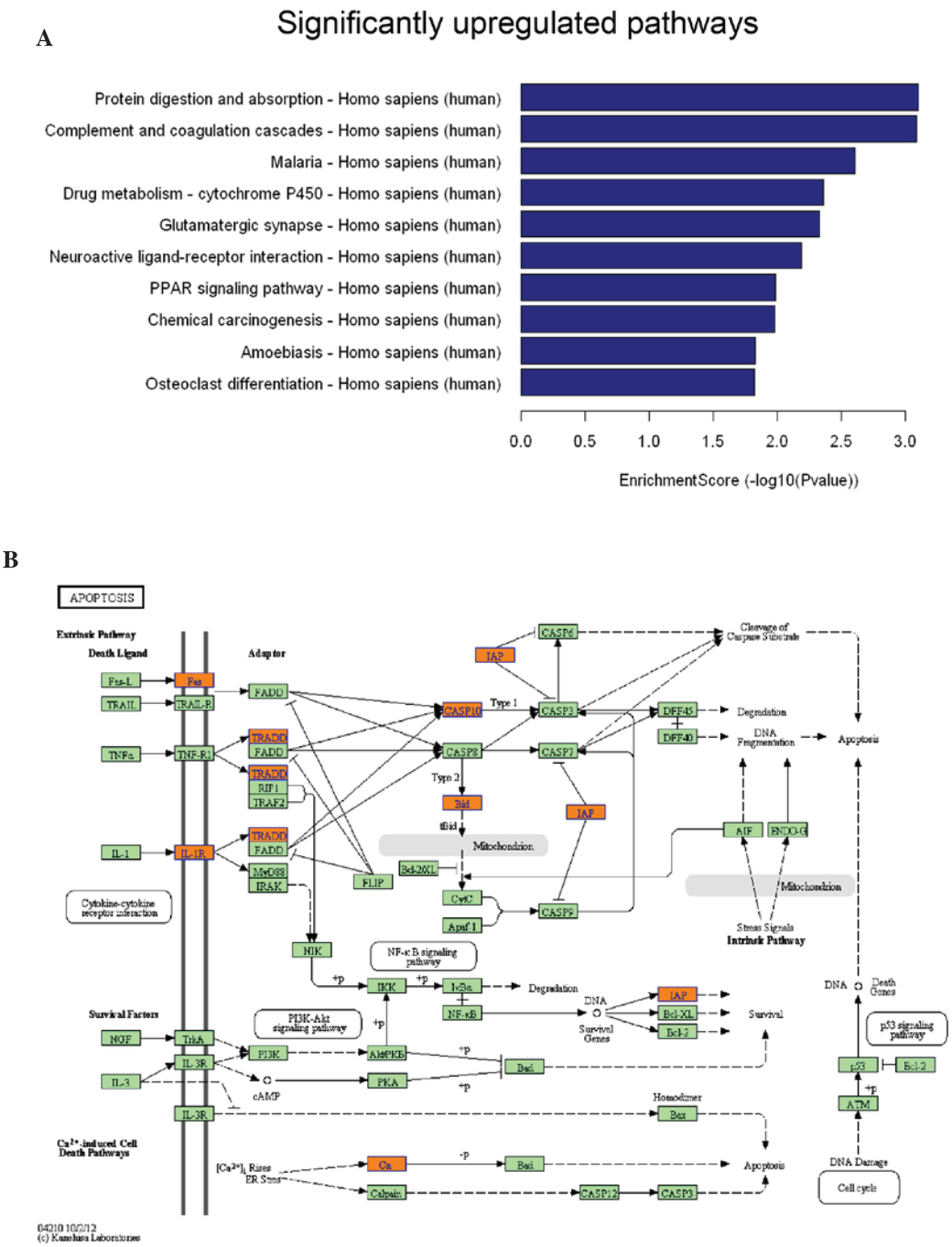

Figure 2. Upregulated signaling pathways following the overexpression of DESI-2 in PANC-1 cells. (A) The top 10 upregulated signaling pathways. (B) KEGG analysis demonstrated certain factors associated with the apoptosis signaling pathways were upregulated (orange boxes). DESI-2, desumoylating isopeptidase 2; KEGG, Kyoto Encyclopedia of Genes and Genomes.

With regard to the downregulated mRNA, western blotting demonstrated that RhoA and ROCK exhibited decreased expression levels in DESI-2 overexpressing PANC-1 cells, corresponding with the changes in their mRNA (Fig. 4). These results indicated that the RhoA signaling pathway was downregulated following the overexpression of DESI-2 in PANC-1 cells.

Expression features of RhoA and RXR in pancreatic cancer tissues with various DESI-2 levels. RhoA and RXR exhibit marked changes in their expression profiles in DESI-2 overexpressing cells; thus, the expression features of RhoA and RXR in pancreatic cancer tissues were further investigated. A total of 96 pancreatic ductal adenocarcinoma tissue specimens were collected and subjected to immunohistochemistry to analyze the expression levels of DESI-2, RhoA and RXR. The tissue samples were divided into two groups according to the expression levels of DESI-2, which included DESI-2 abundant and DESI-2 scarce pancreatic tumor tissues (Fig. 5). Immunohistochemistry demonstrated that RXR exhibited similar changes in expression levels to DESI-2, with higher expression levels in DESI-2 abundant pancreatic tumor tissues (Fig. 5). However, RhoA expression exhibited lower expression levels in DESI-2-abundant pancreatic tumor tissues, contrary to the expression profile of DESI-2 (Fig. 5).

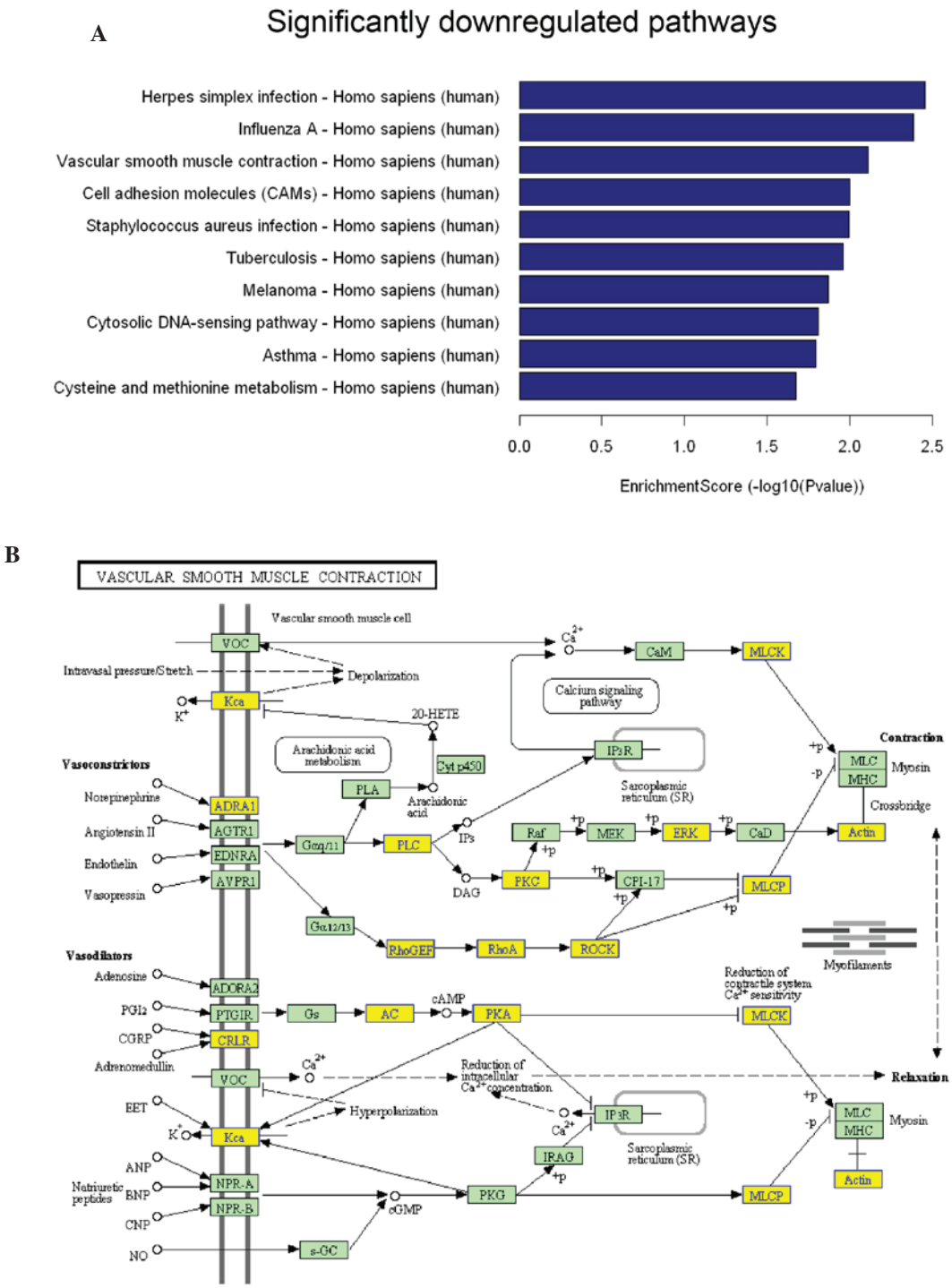

Figure 3. Downregulated signaling pathways following the overexpression of DESI-2 in PANC-1 cells. (A) The top 10 downregulated signaling pathways. (B) KEGG analysis revealed that certain cytoskeleton-associated kinases were downregulated (yellow box). DESI-2, desumoylating isopeptidase 2; KEGG, Kyoto Encyclopedia of Genes and Genomes.

These results were established by further analysis. The 96 tissue samples were divided into the DESI-2-higher (52 tissue samples) and DESI-2-lower (44 tissue samples) cancer groups, according to the median expression level of DESI-2 present. The tissue samples with RXR or RhoA expression levels higher or lower than the median expression level are presented in Fig. 6. The proportion of tissues with RXR expression levels exceeding the median is markedly increased in DESI-2-higher cancer (31/52), compared with DESI-2-lower cancer (11/44). However, the proportion of tissues with RhoA expression levels that exceed the median is markedly decreased in DESI-2-higher cancer (15/52), compared with DESI-2-lower cancer (29/44).

Discussion

In the present study, the transcription profiles of a set of specific genes in DESI-2-overexpressing PANC-1 cells were

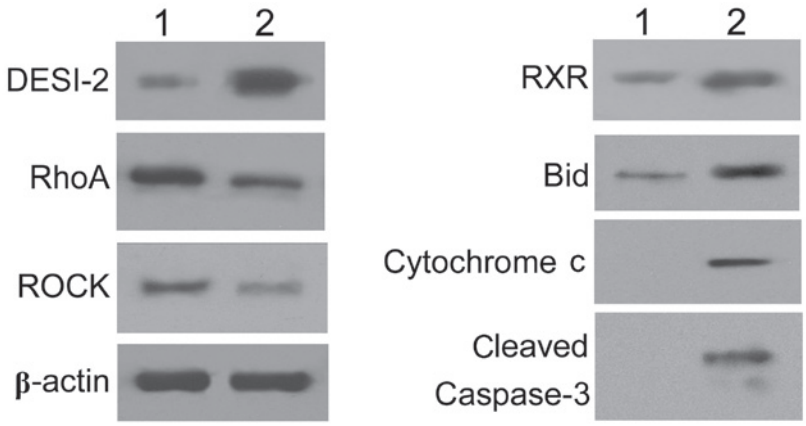

Figure 4. The essential factors involved in cell movement and apoptosis were detected by western blotting (1, untreated cells; 2, DESI-2-overexpressing cells). Following the overexpression of DESI-2 in PANC-1 cells, the key factors identified as significantly altered in the mRNA microarray were selected for examination at the protein level. Western blot analysis revealed that RhoA and ROCK were downregulated, whereas DESI-2, RXR and BID were upregulated in DESI-2-overexpressing cells. Additionally, cytoplasmic cytochrome *c* and cleaved caspase-3 were detected. β-actin was used as the internal control. DESI-2, desumoylating isopeptidase 2; RhoA, Ras homolog gene family member A; ROCK, Rho-associated protein kinase; RXR, retinoid X receptor; BID, BH3 interacting-domain death agonist.

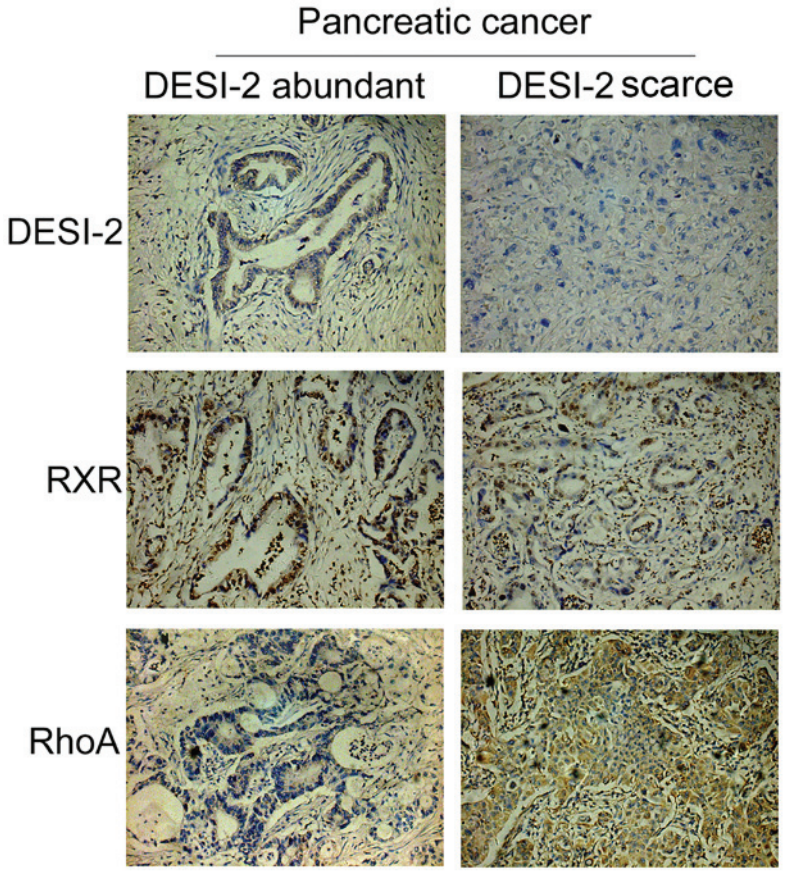

Figure 5. Immunohistochemistry revealed the expression features of RXR and RhoA in pancreatic cancer tissues samples with various DESI-2 expression levels. In DESI-2-scarce pancreatic cancer tissue, RXR expression was decreased, compared with DESI-2-abundant pancreatic cancer tissues. However, RhoA exhibited increased expression levels in DESI-2-scarce pancreatic cancer tissues. DESI-2, desumoylating isopeptidase 2; RhoA, Ras homolog gene family member A; RXR, retinoid X receptor.

investigated using analysis of mRNA microarrays. To the best of our knowledge, this is the first study to comprehensively analyze the associated signaling pathways following the induced overexpression of DESI-2 in pancreatic cancer. Our previous study (12) investigated the altered protein expression profiles in A549 human lung adenocarcinoma cells with DESI-2 overexpression, using a proteomic strategy consisting of two-dimensional electrophoresis (2-DE) coupled with mass spectrometry (13). Although several proteins were identified with the aforementioned proteomic strategy, it was challenging

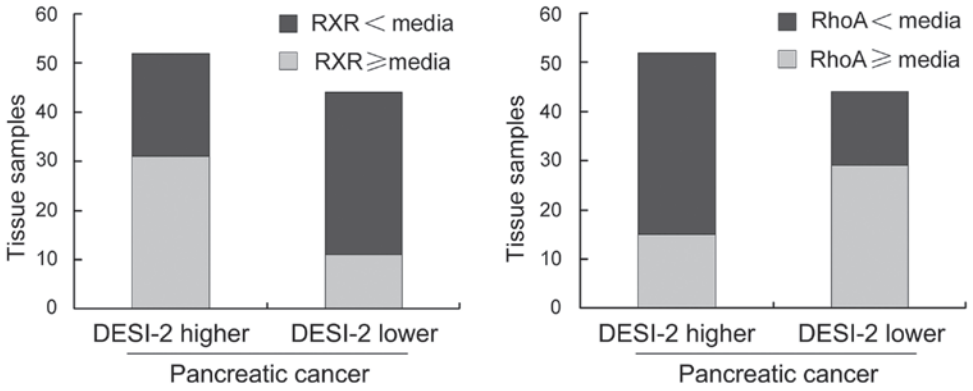

Figure 6. Assessment of RXR and RhoA expression profiles in pancreatic ductal adenocarcinoma tissues with various DESI-2 expression levels. The tissue samples were divided into DESI-2-higher and DESI-2-lower cancer groups according to the calculated median expression level of DESI-2. The number and scale of the samples with RXR or RhoA expression levels higher or-lower than the respective median levels are presented. DESI-2, desumoylating isopeptidase 2; RhoA, Ras homolog gene family member A; RXR, retinoid X receptor.

to elucidate the comprehensive pathways due to the limitations of 2-DE, including protein solubility, throughput and abundance. Therefore, mRNA microarray data may facilitate further investigations into the functions of DESI-2 from alternative perspectives.

As mRNA microarrays reflect the transcription profile of the genome, the protein expression levels must be further examined to determine the actual changes in the expression levels of specific genes. In our previous study, it was identified that the overexpression of DESI-2 was able to induce cell apoptosis through a mitochondrial apoptosis pathway; decreased B-cell lymphoma-2 (Bcl-2) expression levels, increased of Bcl-2-associated x-protein (Bax) expression levels, the release of cytochrome *c* and the activation of caspase-3 were observed (11). In the current study, microarray analysis identified that upstream BID expression levels were increased, which was corroborated at the protein level. As an essential apoptosis-associated protein, BID may be cleaved into tBID, which then activates Bax and Bcl-2 antagonist/killer, triggering the mitochondrial apoptosis pathway (14-16). The results suggest a mechanism that may underlie the DESI-2-induced apoptosis observed in our previous study (11).

RXR was another identified upregulated gene in DESI-2 overexpressing PANC-1 cells. The changes in RXR expression are important due to its essential role in the peroxisome proliferator-activated receptor (PPAR) signaling pathway. As a transcription factor, RXR binds to PPAR-α, -β and -γ to form RXR-PPAR complexes, which may subsequently induce tumor protein p53 transcription and nitric oxide production to further activate the mitochondrial apoptosis pathway (17-19). The results in this study suggest that a significant decrease in RXR expression levels in DESI-2-lower pancreatic cancer tissues indicates a potential association between RXR and DESI-2 expression levels in the development of pancreatic cancer.

With regard to the downregulated signaling pathways revealed by microarray analysis, it was identified that RhoGEF, RhoA and ROCK all exhibited decreased protein expression in DESI-2-overexpressing PANC-1 cells. RhoA has an important role in cell adhesion and proliferation (20-22). Numerous previous studies have reported associations between RhoA expression and development of pancreatic cancer (23,24). The relatively low RhoA expression levels in DESI-2-higher pancreatic cancer tissues, which were identified in this study, suggested that high DESI-2 expression levels may be a marker for a lower degree of malignancy in pancreatic cancer.

In conclusion, the results of the present study revealed a comprehensive gene expression and transcription profile in DESI-2-overexpressing cells, and also evaluated the essential signaling factors at the protein level. The altered signaling pathways may be considered as potential molecular mechanisms underlying the functions of DESI-2 in the regulation of certain biological processes. Additionally, the gene expression analysis of pancreatic cancer tissues demonstrated that DESI-2 may be an indicator of malignancy in pancreatic cancer.

Acknowledgements

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