

PDCD5 regulates cell proliferation, cell cycle progression and apoptosis

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Abstract. Programmed cell death (PDCD)5 is cloned from human leukemia cell line TF-1. PDCD5 is one of the members of the programmed cell death protein family that is frequently involved in tumor growth and apoptosis. To investigate the molecular and cellular functions of PDCD5, the present study established a PDCD5 stably overexpressing A431 cell line and examined the role of PDCD5 in cell proliferation, cell cycle progression and apoptosis. The data demonstrated that overexpression of PDCD5 significantly inhibited cell proliferation, induced cell cycle arrest at G2/M phase and apoptosis in A431 cells. The expression profiles of certain key regulators of these cellular events were further investigated, including P53, B cell lymphoma (BCL)-2, BCL-2 associated X protein (BAX) and caspase (CASP)3. The data demonstrated that at the transcript and protein levels, P53, BAX and CASP3 were all upregulated in the PDCD5 stably overexpressing A431 cells whereas BCL-2 was downregulated, indicating that PDCD5 acts as an important upstream regulator of P53, BCL-2, BAX and CASP3. The data suggest that PDCD5 regulates cell proliferation, cell cycle progression and apoptosis in A431 cells. PDCD5 may be a novel tumor suppressor gene, and may be potentially used for cancer treatment in the future.

Introduction

Cell proliferation and apoptosis are key cellular events in the development of organisms (1). A number of biological processes, such as tissue development and homeostasis, require a balance between cell proliferation and apoptosis, dysregulation of which would result in different types of human diseases (1). In eukaryotes, the cell cycle consists of four stages: G1/G0, S, G2 and M. Each stage needs to be monitored by specific checkpoint to ensure that the genetic information of the cell

is faithfully transmitted to the next generation. The G2/M checkpoint (DNA damage checkpoint) is an important cell cycle checkpoint to prevent the cell from entering the mitosis with DNA damage (2). In other words, cells will be arrested at G2/M phase when genomic DNA is damaged and needs to be repaired. All somatic cells proliferate via a mitotic process determined by successful progression of the cell cycle (3).

Programmed cell death 5 (PDCD5) is a one of the members of programmed cell death protein family. The gene *PDCD5*, alternatively named *TFAR19*, was originally cloned from human leukemia cell line TF-1 (4). This gene is localized on chromosome 19q12-q1311 and spans around 6 kb of genomic DNA that contains 5 introns and 6 exons. The open reading frame of *PDCD5* encodes a 125-aa protein that is highly conserved ranging from yeast to human (4). *PDCD5* is ubiquitously expressed in different tissues and involved in the regulation of apoptosis in different cell types (4-8). The apoptotic potential of PDCD5 may be partially resulted from its phosphorylation at serine 118 by CK2, which is required for the nuclear translocation of PDCD5 in response to genotoxic stress (9,10). Recently, it was shown that PDCD5 is also an important regulator of the non-apoptotic programmed cell death (PCD), designated 'paraptosis' (11). More recently, it was reported that PDCD5 also regulates autophagy to protect against cardiac remodeling (12). Dysregulation of *PDCD5* has been found to be involved in different type of tumors (13-22). The antitumor activity of PDCD5 has been also proposed (23-29) and low expression level of PDCD5 has been suggested to be a prognostic indicator for cancers (30). PDCD5 was also indicated to have the therapeutic potential in the treatment of rheumatoid arthritis and other autoimmune diseases because of its inflammatory effects (31,32). Knockout of *PDCD5* can also protect the brain from ischemic injury by inhibiting the PDCD5-VHL pathway (33).

PDCD5 is downregulated in the lung adenocarcinoma patients compared to the healthy controls, which indicates PDCD5 is a tumor suppressor gene associated with lung cancer (34). Single nucleotide polymorphism in the *PDCD5* gene locus was also found to be associated with non-small cell lung cancers (35). Recently, a few important interacting partners of PDCD5 have been discovered, including Tip60, CK2, CTT, p53, tumor suppressor protein pVHL and YY1-associated factor 2 (YAF-2) (9,36-41). In the genotoxic conditions, PDCD5 selectively mediates HDAC3 dissociation

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from p53, and induces HDAC3 degradation through the ubiquitin-dependent proteasomal pathway, which subsequently activates p53 as a result in response to the stress (42,43). The promoter activity of *PDCD5* is activated by the transcription factor NF- κ B p65 (44) and the protein stability of *PDCD5* are positively regulated by YAF2 and OTUD5 (41,45), and negatively regulated by DNAJB1 (46).

In the present study, we investigate the roles of *PDCD5* in cell proliferation, cell cycle progression and apoptosis by using a *PDCD5* stably overexpressing A431 cell line. We further examine whether these changes of cellular processes caused by overexpression of *PDCD5* are related to the P53 signaling pathway.

Materials and methods

Reagents and cell line. DMEM [10% fetal bovine serum (FBS), 2 mM glutamine, 1% penicillin/streptomycin]. The A431 cells were cultured at 37°C incubator supplemented with 5% CO₂. dNTP (10 mM) and One Step SYBR® PrimeScript™ RT-PCR kit were purchased from Takara Bio (Dalian, China); Primers were synthesized by GeneCreate Biological Engineering Co., Ltd. (Wuhan, China); TRIzol was purchased from Invitrogen (Carlsbad, CA, USA); MTT was purchased from Sigma (St. Louis, MO, USA; cat. no. m5655); FBS was purchased from Gibco; PI and Annexin V-FITC were purchased from Beyotime. Antibodies were purchased from Cusabio. The *PDCD5* overexpressing A431 cell line was established by GeneCreate Biological Engineering Co., Ltd. (Wuhan, China). The cell line stably transfected empty vector was used as a control.

MTT assay. Cells splitted into each well of 96-well plate with the cell density ~1000-10000 cells/well. 180 μ l of diluted cells was added into each well. 5 different time points including 12, 24, 48, 72 and 96 h were set-up and each time point has 5 replicates for *PDCD5* overexpressing and control cells. The cells were cultured in the 37°C incubator supplemented with 5% CO₂. 20 μ l of MTT (5 mg/ml, 0.5% MTT) was added to each well and the cells were cultured for additional 4 h. The culture media were carefully removed and 100 μ l DMSO was added into each well. The plate was gently shaken on the shaker at low speed for 10 min to dissolve the crystal completely. The absorbance of each well was measured at OD490 nm by using the ELISA reader.

Flow cytometry. The cells were synchronized with serum withdrawal (media without serum) for 24 h and then replenished with 10% FBS containing DMEM media for additional 48 h. The cells were then trypsinized and transferred to the collection tube. Cells were pelleted by centrifuge at 1000 rpm for 5 min. The supernatant was removed and cells were resuspended with ice-cold PBS and then transferred into 1.5 ml tube, and repelleted by centrifugation. The cells were then fixed in 1 ml of 70% cold ethanol for >2 h. The cells were pelleted again by centrifugation at 1000 rpm for 5 min. The supernatant was carefully aspirated and the cells were resuspended with 1 ml ice-cold PBS. The cells were then repelleted and the supernatant was carefully removed. 0.5 ml PI solution (PI 5 mg, RNase 2 mg, 1.0% Triton X-100 0.25 ml,

saline 65 ml, sodium citrate 100 mg, ddH₂O was added to bring total volume to 100 ml and the pH value was adjusted to 7.2-7.6; Stored at 4°C in brown bottle and keep away from light) was added into the cells and incubated at 37°C for 30 min without light exposure. The cells can be stored at 4°C or kept on ice. Flow experiments should proceed within 24 h after PI staining. Flow cytometer (Beckman Moflo XDP) was set at 488 nm (excitation wavelength) to detect red fluorescence and light scattering. DNA content and light scattering analyses were performed by using Modfit software.

Real-time quantitative PCR (qPCR). Total RNA was extracted by using TRIzol method (Invitrogen) according to the instructions of the manual. 1 ml TRIzol was added to the cell pellet (containing ~1x10⁷ cells), mixed well and incubated at RT for 5 min. 0.2 ml chloroform was then added, vortexed for 15 sec and incubated for 3 min. The lysates were centrifuged at 12,000 rpm, 4°C for 10 min. The supernatant was removed and mixed well with 0.5 ml cold isopropanol, and kept on ice for 20~30 min. The mixture was then centrifuged at 12,000 rpm, 4°C for 10 min to pellet the RNA. The supernatant was removed and the pellet was washed with 1 ml 75% ethanol. The RNA/ethanol mixture was centrifuged again at 7,500 g for 5 min, and the supernatant was discarded. The RNA was air-dried and dissolved in ddH₂O. The qPCR reaction was as follows: RNA (template): 2 μ l; SYBR® PrimeScript Master Mix (2x), 12.5 μ l; forward primer (20 μ M): 0.5 μ l; reverse primer (20 μ M): 0.5 μ l; ddH₂O: 11.5 μ l; total volume, 25 μ l. GAPDH or β -actin was used as an internal control. Primer sequences are presented below in Table I. qRT-PCR program was as follows: 45°C, 15 min; 95°C, 5 min; 95°C, 20 sec; 60°C, 20 sec; 72°C, 30 sec; 40 cycles. The data were analyzed by using 2^{- $\Delta\Delta C_q$} method.

Western blot analysis. Total protein was extracted from about 1x10⁷ A431 cells. The lysate was equally mixed with 2X loading buffer and boiled at 100°C for 5 min, and then subjected to 15% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE). Proteins were then electrotransferred onto a PVDF (Bio-Rad) membrane, which was then blocked with 5% non-fat milk in PBST (1xPBS with 0.05% Tween-20) at RT for 1 h. The membrane was then incubated with each individual primary antibody, including *PDCD5*, P53, BAX, BCL-2, CASP3 and GAPDH, at 37°C for 1 h. GAPDH was used as a loading control. The membrane was then washed with PBST for 5 min/3 times and proceeded to incubate with secondary antibody (goat anti-mouse or goat anti-rabbit) at 37°C for 1 h. After washing with PBST for 5 min/3 times, membrane-bound antibodies were detected with ECL (enhanced chemiluminescence) kit.

Results

Establishment of *PDCD5* stably overexpressing A431 cell line. A431 is a cell line derived from human epidermoid carcinoma and has been widely used in the studies on cell cycle progression and tumor-related signaling pathways. Therefore, we introduced this cell line to our studies. The cells were transfected with *PDCD5* construct and its empty vector, respectively. To confirm whether the stable cell line was successfully

Table I. Primers used for quantitative RT-PCR.

Gene	Primer	Sequence	T _m (°C)
<i>PDCD5</i>	Forward	5'-ACAGATGGCAAGATATGGACA-3'	60
	Reverse	5'-TCCTAGACTTGTTCGGTTAAG-3'	
<i>P53</i>	Forward	5'-CAGCCAAGTCTGTGACTTGCA-3'	60
	Reverse	5'-GTGTGGAATCAACCCACAGCT-3'	
<i>BAX</i>	Forward	5'-CCCTTTTGCTTCAGGGTTTCATCCA-3'	60
	Reverse	5'-CTTGAGACACTCGCTCAGCTTCTTG-3'	
<i>BCL-2</i>	Forward	5'-CTGCACCTGACGCCCTTCACC-3'	60
	Reverse	5'-CACATGACCCACCGAACTCAAAGA-3'	
<i>CASP3</i>	Forward	5'-CATGGAAGCGAATCAATGGACT-3'	60
	Reverse	5'-CTGTACCAGACCGAGATGTCA-3'	

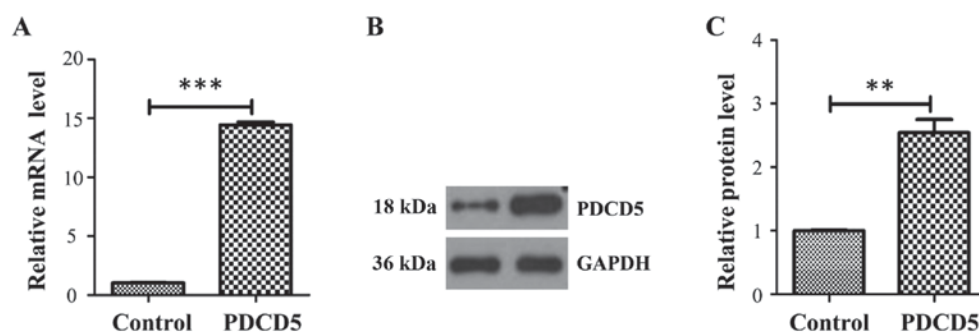


Figure 1. Verification of PDCD5 overexpression at the transcript and protein levels in the A431 overexpressing cells. (A) The transcript levels in the PDCD5 overexpressing A431 cells and its empty vector-transfected cells were analyzed by qRT-PCR. (B) The expression levels in the PDCD5 overexpressing A431 cells and its empty vector-transfected cells were detected by western blot. The protein level of PDCD5 is strikingly increased in the PDCD5 overexpressing A431 cells compared with the empty vector control. GAPDH was used as a loading control. (C) Quantification for the western blot data in (B) from three independent experiments. ** $P < 0.01$ and *** $P < 0.001$. Control, empty vector-transfected A431 cells; PDCD5, PDCD5 overexpressing A431 cells.

established, we performed quantitative RT-PCR and western blot. At the transcript level, PDCD5 was increased ~15-fold in the PDCD5 stably overexpressing A431 cells compared with the empty vector control (Fig. 1A). At the protein level, PDCD5 was also strikingly increased in the PDCD5 overexpressing cells compared with the control (Fig. 1B and C).

Overexpression of PDCD5 inhibits A431 cell proliferation. To examine whether overexpression of PDCD5 affects cell proliferation, MTT assay was performed for the PDCD5 overexpressing cells and control cells at different time points, including 12, 24, 48, 72 and 96 h. The growth curves for these two cell lines were generated according to the OD values at these time points. The data indicated that at the time points of 72 and 96 h, cell proliferation in the PDCD5 stably overexpressing cells was significantly slower than that in the empty vector control cells (Fig. 2).

Overexpression of PDCD5 induces cell cycle arrest at G2/M phase in A431 cells. To further understand the inhibitory effect of PDCD5 on cell proliferation, we performed flow cytometry to investigate the distribution of specific phases of cell cycle in the PDCD5 stably overexpressing A431 cells and its empty vector control. The results showed that PDCD5 overexpressing cells were strikingly arrested at the G2/M phase of the cell cycle, compared with the control cells (Fig. 3A and B).

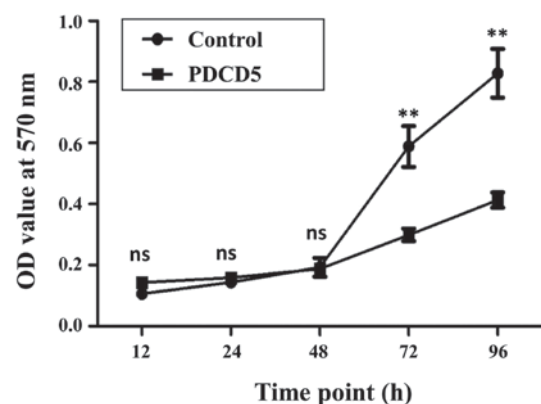


Figure 2. MTT assay was performed to monitor cell proliferation in PDCD5 overexpressing A431 cells and its control cells. The cells were splitted at the similar density for the PDCD5 overexpressing cells and the control cells. Cell growth was monitored at multiple time points, including 12, 24, 48, 72 and 96 h. From 72 h, the cell viability was significantly lower in the PDCD5 overexpressing cells compared with the control cells. ** $P < 0.01$. Control, empty vector-transfected A431 cells; PDCD5, PDCD5 overexpressing A431 cells.

Overexpression of PDCD5 induces apoptosis in A431 cells. Cell cycle arrest at G2/M is generally resulted from DNA damage. To investigate whether the PDCD5 overexpressing cells with DNA damage underwent apoptosis, we performed flow cytometric analysis as well. The data indicated that

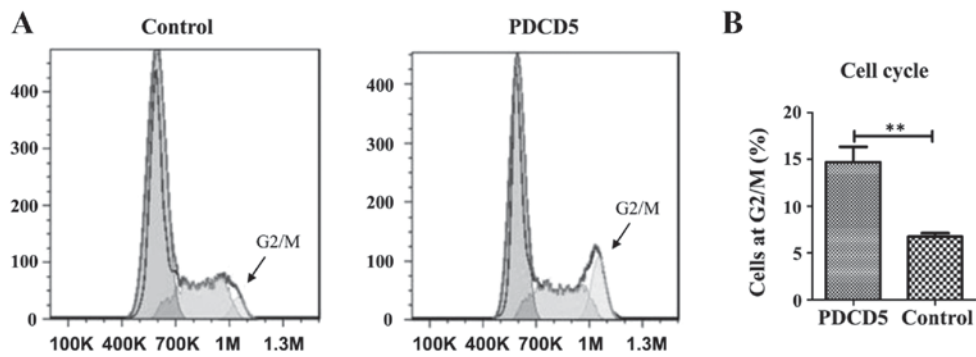


Figure 3. Overexpression of PDCD5 induces G2/M cell cycle arrest in A431 cells. After propidium iodide staining, flow cytometry was performed to determine the distribution of cell cycle phases, including G0/G1, S and G2/M, in the PDCD5 overexpressing cells and its control cells. (A) Cell cycle was strikingly arrested at the G2M phase in the PDCD5 overexpressing cells. Representative images from three independent experiments are shown. (B) Quantification of G2/M arrest in the PDCD5 overexpressing A431 cells and its control cells from three independent experiments. **P<0.01. Control, empty vector-transfected A431 cells; PDCD5, PDCD5 overexpressing A431 cells.

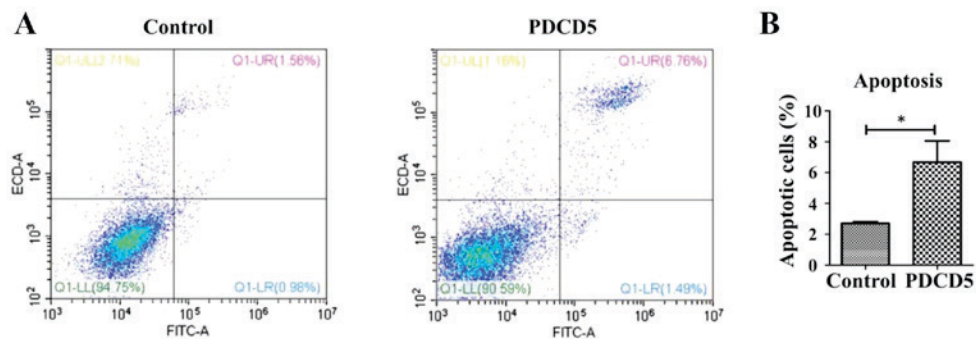


Figure 4. Overexpression of PDCD5 induces apoptosis in A431 cells. Flow cytometry was performed to determine apoptosis in the PDCD5 overexpressing cells and its control cells. (A) More apoptotic cells were clearly observed in the PDCD5 overexpressing cells compared with the control cells. Representative images from three independent experiments are shown. (B) Quantification for the percentage of apoptosis in the PDCD5 overexpressing A431 cells and its control cells from three independent experiments. *P<0.05. Control, empty vector-transfected A431 cells; PDCD5, PDCD5 overexpressing A431 cells.

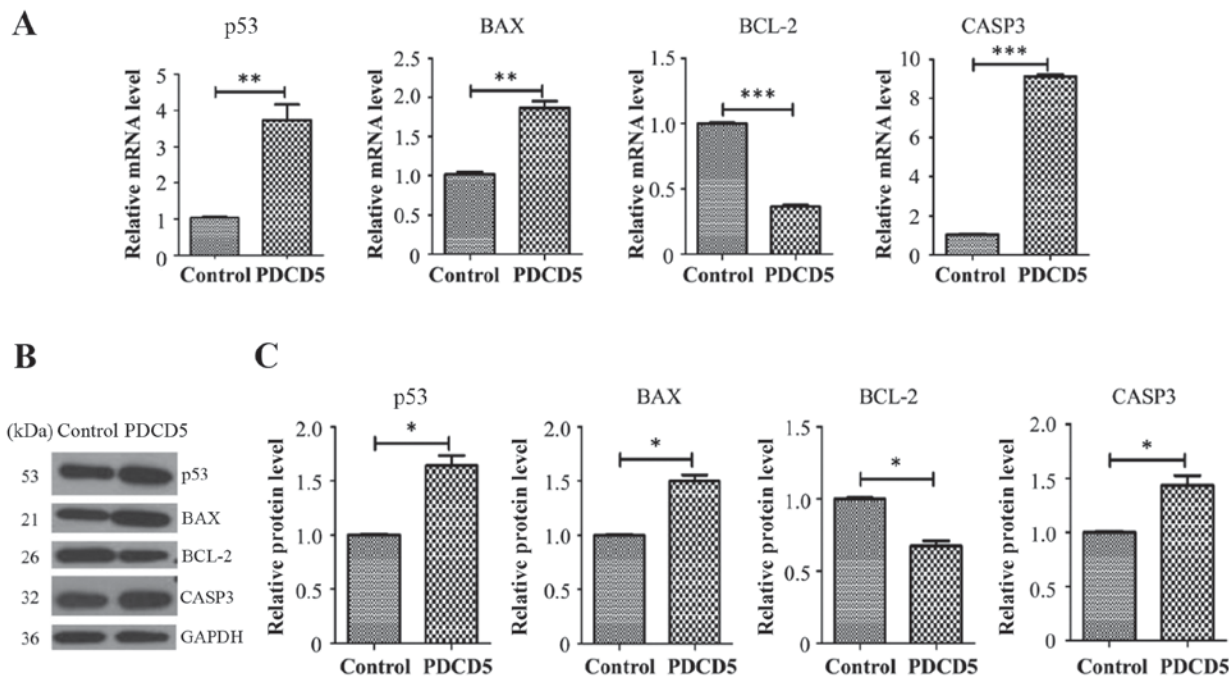


Figure 5. P53, BAX and CASP3 are upregulated while BCL-2 is downregulated in the PDCD5 overexpressing A431 cells. (A) The transcript levels of P53, BAX, BCL-2 and CASP3 in the PDCD5 overexpressing A431 cells and its control cells were analyzed by real-time RT-PCR. (B) The expression levels of P53, BAX, BCL-2 and CASP3 in the PDCD5 overexpressing A431 cells and its control cells were examined by western blot. GAPDH was used as a loading control. (C) Quantification for western blot data in (B) from there independent experiments. *P<0.05, **P<0.01 and ***P<0.001. Control, empty vector-transfected A431 cells; PDCD5, PDCD5 overexpressing A431 cells.

overexpression of PDCD5 strikingly induces apoptosis, compared with the control cells (Fig. 4A and B).

Overexpression of PDCD5 leads to dysregulation of P53, BAX, BCL-2 and CAPS3 in A431 cells. To further understand the molecular underpinnings that might be relevant to decreased proliferation, increased apoptosis and G2/M arrest in the PDCD5 overexpressing A431 cells, we examined the transcript and protein levels of tumor suppressor P53 and key molecules of apoptosis including BCL-2, BAX and CAPS3. Real-time RT-PCR results showed that *P53*, *BAX* and *CAPS3* were all upregulated in the PDCD5 stably overexpressing A431 cells while *BCL-2* is downregulated, compared with the empty vector control cells (Fig. 5A). Western blot by using specific antibodies against these proteins was then performed to further confirm RT-PCR results (Fig. 5B and C). The data showed that the pattern of protein dysregulations was basically consistent with that observed in the qRT-PCR data.

Discussion

Programmed cell death (PCD) is the death of a cell mediated by a series of intracellular programs. There are three forms of PCD: Apoptosis, autophagy and programmed necrosis (47). It is well known that apoptosis is an orchestrated cellular process that can occur in physiological and pathological conditions (48). Cell proliferation is a cellular event that causes an increase of cell number. In human cancers, cell proliferation is out of control and apoptosis is suppressed (49). Cell proliferation is decreased when cell cycle arrest occurs. In the condition of DNA damage, cell cycle arrest will be initiated as an attempt to repair the damage, however, if the damage is too extensive to be repaired, the cell will undergo cell death in a way of apoptosis (50).

According to the NCBI database, there are currently 12 members in total in the PDCD protein family, namely PDCD1~PDCD12. Among them, PDCD8 and PDCD9 are officially known as AIFM1 and MRPS30, respectively. PDCD1, often known as PD-1, is the member that has been most extensively studied and shown to negatively regulate T cell responses, in collaboration with its two ligands, PD-L1 and PD-L2 (51-53). In addition to PDCD5, other programmed cell death proteins are also known to play important roles in apoptosis and/or cell cycle progression (54-57), and are also dysregulated in many types of human cancers (13,16,58-63). Opposite to what we observed for PDCD5, depletion of PDCD2 in human acute leukemia cells impairs their proliferation, induces cell cycle arrest and p53 activation while overexpression of PDCD2 facilitates cell growth in cancers (55,64). However, in gastric cancer cells, expression of PDCD2 seems to induce cell cycle arrest and apoptosis, which are also found to be p53-dependent (54). This suggests the connection between PDCD2 and cell cycle arrest might be tissue and cancer type-dependent.

In the present study, we used A431 cells as a cell model to investigate the role of PDCD5 in cell proliferation, cell cycle progression and apoptosis. As a human model epidermoid carcinoma cell line, A431 has been widely used in studies on the cell cycle and tumor related cell signaling pathways because epidermal growth factor receptor (EGFR) is known to

be strikingly upregulated in these cells (65-68). In this study, we found that in the A431 cell, overexpression of PDCD5 inhibits cell proliferation, induces cell cycle arrest at G2/M phase and apoptosis. We next attempted to examine the molecular underpinnings of such dysregulations in these cellular events described above. Some key molecules involved in cell proliferation, cell cycle progression and apoptosis, including *P53*, *BAX*, *BCL-2* and *CASP-3*, were found to be dysregulated when PDCD5 was stably overexpressed in A431 cells.

P53 mutation has been found in over 50% of all human cancers (69). Loss of *p53* was recently found to induce cell proliferation via Ras-independent activation of the Raf/Mek/Erk signaling pathway (70). In line with these findings, overexpression of *p53* is known to inhibit cell proliferation (71,72). *p53*-dependent G1 and G2/M arrests of the cell cycle are important components of the cellular response to a variety of stresses, including DNA damage (73). In the PDCD5 overexpressing A431 cells, we observed upregulation of *P53*, reduced cell proliferation and G2/M arrest, which is consistent with previous reports. *p53* activates Bax to mediate mitochondrial membrane permeabilization and apoptosis (74), and inhibits Bcl-2 in some conditions such as apoptotic response to DNA damage (75). Activation of *p53* signaling pathway, including upregulation of *p53*, Bax, caspase-3 and downregulation of Bcl-2, was accompanied with G2/M cell cycle arrest in different cell types when treated with different drugs (76-78). These lines of evidences are in line with our observations in the PDCD5 stably overexpressing A431 cells.

It is noted that all our data in this study were generated by using a PDCD5 stably overexpressing cell model. In our future study, we might need to establish a PDCD5 knockdown cell model and knockout animal model to further confirm the roles of PDCD5 in cell proliferation, cell cycle progression and apoptosis. Moreover, the detailed molecular mechanism underlying the regulation of cell proliferation, cell cycle progression and apoptosis by PDCD5 also needs to be further investigated.

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