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MicroRNA-182 Promotes Cell Growth, Invasion, and Chemoresistance by Targeting Programmed Cell Death 4 (PDCD4) in Human Ovarian Carcinomas

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ABSTRACT

As an important tumor suppressor, programmed cell death 4 (PDCD4) influences transcription and translation of multiple genes, and modulates different signal transduction pathways. However, the upstream regulation of this gene is largely unknown. In this study, we found that microRNA-182 (miRNA-182, miR-182) was upregulated, whereas PDCD4 was downregulated in ovarian cancer tissues and cell lines. Blocking or increase of miR-182 in ovarian cancer cell lines led to an opposite alteration of endogenous PDCD4 protein level. Using fluorescent reporter assay, we confirmed the direct and negative regulation of PDCD4 by miR-182, which was dependent on the predicted miR-182 binding site within PDCD4 3′ untranslated region (3′ UTR). MTT and colony formation assays suggested that miR-182 blockage suppressed, whereas miR-182 mimics enhanced viability and colony formation of ovarian cancer cells. These effects may partly be attributed to the cell cycle promotion activity of miR-182. miR-182 also contributed to migration and invasion activities of ovarian cancer cells. Furthermore, miR-182 reduced the chemosensitivity of ovarian cancer cells to CDDP and Taxol, possibly by its anti-apoptosis activity. Importantly, all the alterations of the above cellular phenotypes by blocking or enhancing of miR-182 could be alleviated by subsequent suppression or ectopic expression of its target PDCD4, respectively. We conclude that in ovarian cancer cells, miR-182 acts as an oncogenic miRNA by directly and negatively regulating PDCD4. J. Cell. Biochem. 114: 1464–1473, 2013.

KEY WORDS: OVARIAN CARCINOMA; microRNA; miR-182; PDCD4; CELL GROWTH; CHEMORESISTANCE

Ovarian cancer is the sixth most lethal gynecologic malignancy, which has a highly aggressive natural history and causes estimated 125,000 deaths all over the world every year. Despite advances in detection and chemotherapies, only 30% of patients with advanced-stage ovarian cancer survive 5 years after initial diagnosis [Greenlee et al., 2001]. The high mortality of this disease is due to late-stage diagnosis for more than 70% of cases, and is also due to the common resistance to the current chemotherapeutic regimens [Fung-Kee-Fung et al., 2007].

In the recent years, many studies focused on the molecular mechanism of ovarian cancer initiation and development. For example, as in many other types of malignancies, *TP53* mutations are common in ovarian cancer [Ahmed et al., 2010; Kuhn et al., 2011]. *Breast cancer 1* and *2* (*BRCA1/2*) mutations are also observed in ovarian cancer, as either germline mutations [Zhang et al., 2011] or somatic mutations [Moskwa et al., 2011]. Importantly, the tumor suppressor *programmed cell death 4* (*PDCD4*) has been found implicated in the initiation and progression of ovarian cancer [Wei et al., 2009ab, 2012]. PDCD4 was originally identified as the neoplasmic transformation inhibitor in the JB6 mouse epidermal cell line [Cmarik et al., 1999]. As an inhibitor of activator protein 1 (AP-1), PDCD4 inhibits AP-1-dependent transcriptional activity [Yang et al., 2001]. Then, PDCD4 was found to directly interact with and inhibit the helicase activity of the eukaryotic translation initiation factor 4A (eIF4A) by competing its binding to the scaffold protein eIF4G, thus subsequently inhibiting translation [Yang et al., 2003]. PDCD4 has intimate connection with malignancies, because down-regulation of PDCD4 was discovered in a variety of tumors

Abbreviations: 3′ UTR 3′, untranslated region; ASO, antisense oligonucleotide; CDDP, *cis*-diaminodichloroplatinum (II), cisplatin; miR-182, microRNA-182; miRNA, microRNA; PDCD4, programmed cell death 4; shR/shRNA, small hairpin RNA.

We declare that we have no conflict of interest.

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[Lankat-Buttgereit and Goke, 2009]. In ovarian cancer, PDCD4 plays a significant tumor suppressor role. It is suggested that PDCD4 inhibits proliferation and cell cycle progression, and induces apoptosis in ovarian cancer cells [Wei et al., 2009b]. Thus, it is reasonable that loss of PDCD4 is a common abnormality at molecular level in ovarian cancer [Wei et al., 2009a]. Furthermore, the tumor suppressive function of PDCD4 is linked to the phosphoinositide 3-kinase-protein kinase B (PI3K-Akt) pathway [Wei et al., 2012].

Despite increasing evidences to suggest the functions of PDCD4 in ovarian cancer, the upstream regulation of this interesting gene is still largely unknown. MicroRNAs (miRNAs) are a recently discovered class of noncoding small regulatory RNAs that interfere with the translation of coding messenger RNAs (mRNAs) in a sequence-specific manner [Bartel, 2004]. MiRNAs are frequently deregulated in cancer and have been suggested to have important roles in cancer initiation and development [Farazi et al., 2011; Lujambio and Lowe, 2012]. Until now, the mainly identified miRNA that directly targets PDCD4 is microRNA-21 (miR-21). MiR-21 has been found to negatively regulate PDCD4 expression in breast cancer [Frankel et al., 2008; Zhu et al., 2008], colorectal cancer [Asangani et al., 2008], and malignant peripheral nerve sheath tumor [Itani et al., 2012]. However, other potential PDCD4-targeting miRNAs remains to be defined. The identification of more miRNAs that directly and functionally adjust PDCD4 represents a hurdle in our understanding of the regulation of this gene. This prompted us to identify and functionally validate additional PDCD4-associated miRNAs.

In this study, we suggest that the microRNA miR-182 plays oncogenic role by directly targeting and negatively regulating PDCD4 in ovarian cancer. We first found an overexpression of miR-182 and a downregulation of PDCD4 in ovarian tissues and cell lines. PDCD4 was confirmed to be a direct target gene of miR-182. Subsequently, we detected that miR-182 enhanced various malignant phenotypes of ovarian cancer cells, and these effects were PDCD4-dependent. We demonstrated here a new and functional upstream regulator of PDCD4 in ovarian cancer cells.

MATERIALS AND METHODS

TISSUE SAMPLES, CELL LINES, AND TRANSFECTION

Fifteen ovarian tissue samples, including two normal ovarian tissues, five ovarian serous cystadenocarcinomas, four ovarian endometrioid carcinomas, and four ovarian clear cell adenocarcinomas were obtained from patients in the Gynecology Department of The Second Hospital of Tianjin Medical University with the patients' informed consent. The class of all the clinical samples was confirmed by pathological analysis. This study was approved by the Ethics Committee of Tianjin Medical University.

Two immortalized normal OSE (ovarian surface epithelial) cell lines T29 and T80 were maintained in special medium as previously described [Liu et al., 2004]. Eight ovarian carcinoma cell lines, OVCAR3, SKOV3, OV2008, HEY, 3AO, A2780, HO8910, and C13, were used in this study. The HEY cells were maintained in Dulbecco's modified eagle's medium (DMEM) supplemented with 2 mmol/L of L-gluatamine, and the other cell lines were maintained in RPMI-1640 medium. All the cell culture media were supplemented with 10% fetal bovine serum (FBS), 100 IU/ml of penicillin and 100 mg/ml of streptomycin. Cells were cultured at 37°C in a humidified chamber supplemented with 5% CO_2. Transfection of plasmids and oligonucleotides was performed with Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

EXTRACTIONS OF RNA AND PROTEIN

The large and small RNA fractions of the tissue samples or cell lines were isolated using the *mir*Vana™ miRNA Isolation Kit (Ambion, Austin, TX) according to the manufacturer's instructions. Large RNA (larger than 200 nt) and small RNA (smaller than 200 nt) were separated and purified. The integrity of the large RNA was confirmed by resolution on a 1% agarose gel by gel electrophoresis.

The residual organic phase in RNA extraction of tissue samples was subsequently used to extract the tissue protein following the protein isolation procedure of the TRIzol Reagent. To extract the cellular protein, the cells were lysed with RIPA lysis buffer (150 mM NaCl, 50 mM Tris–HCl pH 7.2, 1% Triton X-100 and 0.1% SDS) supplemented with the Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland). After centrifuging, the undissolved cell components were removed and the cellular proteins were obtained.

NORTHERN BLOT ASSAY

Forty micrograms of small RNAs were separated in 8 mol/L urea denaturing 18% polyacrylamide gel, electrophoretically transferred to a BrightStar-Plus+ Nylon Membrane (Ambion), 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC)-cross-linked, prehybridized, and then hybridized to the labeled probes. A fragment generated with probes against the mature miRNA was γ-^{32}P-labeled using T4 polynucleotide kinase (Fermentas, Shenzhen, China). The membrane was then washed and exposured to the X-ray film (Fujifilm, Tokyo, Japan). U6 snRNA was simultaneously measured to serve as the loading control.

WESTERN BLOT ASSAY

The proteins were resolved on an SDS denaturing polyacrylamide gel and then transferred onto a nitrocellulose membrane. Antibody to PDCD4 or GAPDH was incubated with the membranes overnight at 4°C. The membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. Protein expression was assessed by enhanced chemiluminescence and exposure to chemiluminescent film. LabWorks™ Image Acquisition and Analysis Software (UVP, Upland, CA) were used to quantify the band intensities. All the antibodies were purchased from Abcam (Cambridge, MA).

QUANTITATIVE RT-PCR FOR miRNAs

Quantitation of miRNAs was performed using stem-loop RT-PCR [Chen et al., 2005]. Briefly, 2 μg of small RNA was reverse transcribed into cDNA using M-MLV reverse transcriptase (Promega, Madison, WI) with the following RT primers: miR-182-RT: 5′-GTCGTATCCAGTGCAGGGTCCGAGGTGCACTGGATACGACAGTGT GA-3′; and U6-RT: 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTC GCACTGGATACGACAAAATATGGAAC-3′, which can fold into a stem-loop structure. The cDNA was used for the amplification of

mature miR-182 and the endogenous control, U6 snRNA, by PCR. The corresponding PCR primers were as follows: gene-specific forward primers miR-182-Fwd: 5′-TGCGGTTTGGCAATGGTAGAAC-3′, U6-Fwd: 5′-TGCGGGTGCTCGCTTCGGCAGC-3′, and a universal downstream primer Reverse: 5′-CCAGTGCAGGGTCCGAGGT-3′. The PCR conditions were: initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 30 s. Quantitative PCR was performed using the SYBR Premix Ex Taq (TaKaRa, Otsu, Shiga, Japan) on an iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA). All of the primers were purchased from AuGCT Inc. (Beijing, China).

PREDICTION OF miRNAs THAT TARGET A PROTEIN-CODING GENE

Bioinformatics method was used to predict the miRNAs that directly target PDCD4. The databases used were TargetScanHuman (Release 6.2, http://www.targetscan.org) and miRanda (Release August 2010, http://www.microrna.org/microrna/getGeneForm.do).

VECTOR CONSTRUCTION

To construct the fluorescent reporter vectors, two about 30-bp double-stranded fragments were obtained via annealing reactions. The oligonucleotides are: wt-mRNA-Top: 5′-CTCTGTGCTAATTTAAACTGCCAAA-3′, wt-mRNA-Bottom: 5′-AGCTTTTGGCAGTTTAAATTAGCACAGAGAGCT-3′, mut-mRNA-Top: 5′-CTCTGTGCTAATTTAAACAGGCTAA-3′, and mut-mRNA-Bottom: 5′-AGCTTTAGCCTGTTAAATTAGCACAGAGAGCT-3′. The two fragments were then cloned into pMIR-REPORT vector (Ambion) at the SacI and Hind III sites, respectively. The constructed vectors were named pMIR/PDCD4-wt-mRNA and pMIR/PDCD4-mut-mRNA.

To construct the PDCD4-siRNA expression vector, a 64-bp double-stranded fragment was obtained via an annealing reaction using the following two single-strands: PDCD4-shR-Top: 5′-GATCCGTGAAAAGAGGTTATGAGAGTTCAAGAGACTCTCATAACCTCTTTTCATTTTTTGGAAA-3′, and PDCD4-shR-Bottom: 5′-AGCTTTTCCAAAAAATGAAAAGAGGTTATGAGAGTCTCTTGAACTCTCATAACCTCTTTTCACG-3′. The fragment was then cloned into a pSilencer 2.1 U6 neo vector (Ambion) at the *Bam*HI and *Hin*dIII sites. The constructed vector was named pSilencer/shR-PDCD4.

To construct the PDCD4 ectopic expression vector, the complete coding sequence of PDCD4 was amplified by PCR using a cDNA library from T29 cells as the template. The PCR primers were as follows: PDCD4-sense: 5′-GTCGACGCGTATGGATGTAGAAAATGAGCAG-3′, and PDCD4-antisense: 5′-GTCTGCTCGAGGTAGCTCTCTGGTTTAAGAC-3′. The amplified fragment was cloned into the pCMV6-Entry vector (AMS Biotechnology, Abingdon, UK) at the *Mlu*I and *Xho*I sites. The constructed vector was named pCMV6/PDCD4.

FLUORESCENT REPORTER ASSAY

The cells were transfected with one of the fluorescent reporter vector (pMIR/PDCD4-wt-mRNA or -mut-mRNA) along with miR-182 ASO (2′-*O*-methyl modified, 5′-agugugaguucuaccauugccaaa-3′, purchased from GenePharma, Shanghai, China, for SKOV3) or miR-182 mimics (double strand RNA, sense: 5′-uuuggcaaugguagaacucacacu-3′, antisense: 5′- agugugaguucuaccauugccaaa-3′, purchased from GenePharma, for OV2008). Identical amount of Renilla expression vector pRL-TK (Promega) was co-transfected into every group to serve as the normalization. At 48 h after transfection, cells were harvested and luciferase activity was measured using the Dual-Glo Luciferase Assay System (Promega).

MTT AND CYTOTOXICITY (IC50) ASSAYS

In MTT assay, cells were seeded and transfected in 96-well plate. At 24, 36, 48, and 72 h after transfection, the MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added into the cells and the absorbance at 490 nm was measured using a μQuant Universal Microplate Spectrophotometer (BioTek, Winooski, VT).

The viability of the transfected SKOV3 and OV2008 cells treated with *cis*-diaminodichloroplatinum (II) (CDDP, also known as cisplatin) and Taxol (both were purchased from Sigma–Aldrich, Shanghai, China) was measured using cytotoxicity (IC50) assay. Briefly, cells were transfected and plated into 96-well plate. For each drug, five or four concentrations were used on the next day, covering a 5- or 4-log concentration range that was chosen to span the 50% inhibitory concentration determined by preliminary assays. The cellular viability was detected using MTT assay 48 h later, and a standard curve between cellular viability and drug concentration was drawn. The IC50 of each drug was calculated according to the standard curve.

COLONY FORMATION ASSAY

To measure the colony formation activity of the ovarian cells, transfected SKOV3 and OV2008 cells were counted and seeded in 12-well plates at 100 cells per well. Culture medium was replaced every 3 days. At the 12th day after seeding, colonies were counted only if they contained more than 50 cells. Finally, the cells were stained using crystal violet and images were taken.

FLOW CYTOMETRY ANALYSES FOR CELL CYCLE AND APOPTOSIS

For detection of cell cycle, transfected cells were detached from the plates by trypsin incubation, rinsed with PBS and fixed in 70% ethanol. Before detection, the cells were rehydrated in PBS and incubated with RNase (100 μg/ml) and propidium iodide (PI, 60 μg/ml, Sigma–Aldrich, MO). Cells in different phages were analyzed using the Beckman coulter flow cytometer.

For detection of apoptosis, cells were stained with Annexin V and 7-AAD using ApoScreen Annexin V Apoptosis Kit (Southern Biotech, Birmingham, AL) as instructed by the manufacturer. The stained cells were analyzed using a Beckman coulter flow cytometer.

WOUND HEALING ASSAY

Cells were transfected and cultured to confluence or near (>90%) confluence in 24-well dishes. A sterile 200 μl pipette tip was used to scratch a straight wounds through the cells. Then the medium was removed and replaced with fresh medium. Images were taken at 0, 24, 36, 48, and 72 h after scratching, respectively. Two parallel lines were drawn at the border of the wound, and the distance between the lines were measured.

TRANSWELL INVASION ASSAY

Cell migration activity was detected using Transwell assay. Briefly, 40 μl Matrigel (BD, Franklin Lakes, NJ) at a concentration of 1 mg/ml

was added into the upper chamber. The lower chamber of the Transwell (Corning, NY) was filled with 600 μl of medium with 10% FBS. The transfected cells were then trypsinized, washed once using serum-free medium and resuspended. An amount of 1×10^5 cells within 100 μl suspensions was added to the upper chamber of the well, and the cells were allowed to migrate at 37°C, 5% CO_2 for 24 h. Then, the upper surface of the membrane was wiped with a cotton tip to mechanically remove non-invasion cells, and the invasive cells attached to the lower surface were stained with crystal violet for 20 min. The membranes were carved and embedded under cover slip with cells on the top side. Cells in 5 random fields of view at 100× magnification were counted and expressed as the average number of cells/field of view.

STATISTICAL ANALYSIS

All the experiments were carried out in triplicate. The quantitative values were expressed as mean ± standard deviation (SD), and the hypothesis test for significance between two groups utilized the Student's *t*-test. Statistical significance was set as $P \leq 0.05$.

RESULTS

PDCD4 IS DOWN-REGULATED, WHEREAS miR-182 IS UP-REGULATED IN OVARIAN CANCER

To test whether the tumor suppressor PDCD4 is aberrantly expressed in ovarian cancer, we first detected PDCD4 protein level in 15 tissue samples, including 2 normal ovarian tissues, 5 ovarian serous cystadenocarcinomas, 4 ovarian endometrioid carcinomas, and 4 ovarian clear cell adenocarcinomas. According to the Western blot analysis, PDCD4 showed high level in the two normal ovarian tissues. However, PDCD4 level in ovarian cancer tissues was significantly lower, especially in the Number 6, 9, 11, and 15 tissues, where PDCD4 was extremely low or undetectable (Fig. 1A). These data indicated that PDCD4 was down-regulated in ovarian cancer.

In our previous study, using miRNA microarray analyses, we found that miR-182 was one of the elevated miRNAs in ovarian cancer tissues compared to the adjacent normal ovarian tissues (unpublished data), which is in accordance with previous findings [Liu et al., 2012]. To further confirm it, we used Northern blot assay to evaluate miR-182 level in these 15 tissues. As a result, miR-182 was almost undetectable in the two normal ovarian tissues, whereas its level in ovarian cancer tissues was higher, although a relative low level was obtained in the Number 4, 13, and 15 tissues (Fig. 1A). These results provide evidence that PDCD4 showed contrary expression pattern to miR-182.

Consequently, we similarly detected miR-182 and PDCD4 levels in the two OSE cell lines and eight ovarian cancer cell lines. Consistent with above data, PDCD4 showed lower level, whereas miR-182 exhibited higher level expression in ovarian cancer cells (Fig. 1B). Among these data, we noticed that in the ovarian cancer cell lines, miR-182 was extremely low in SKOV3 cells and relatively

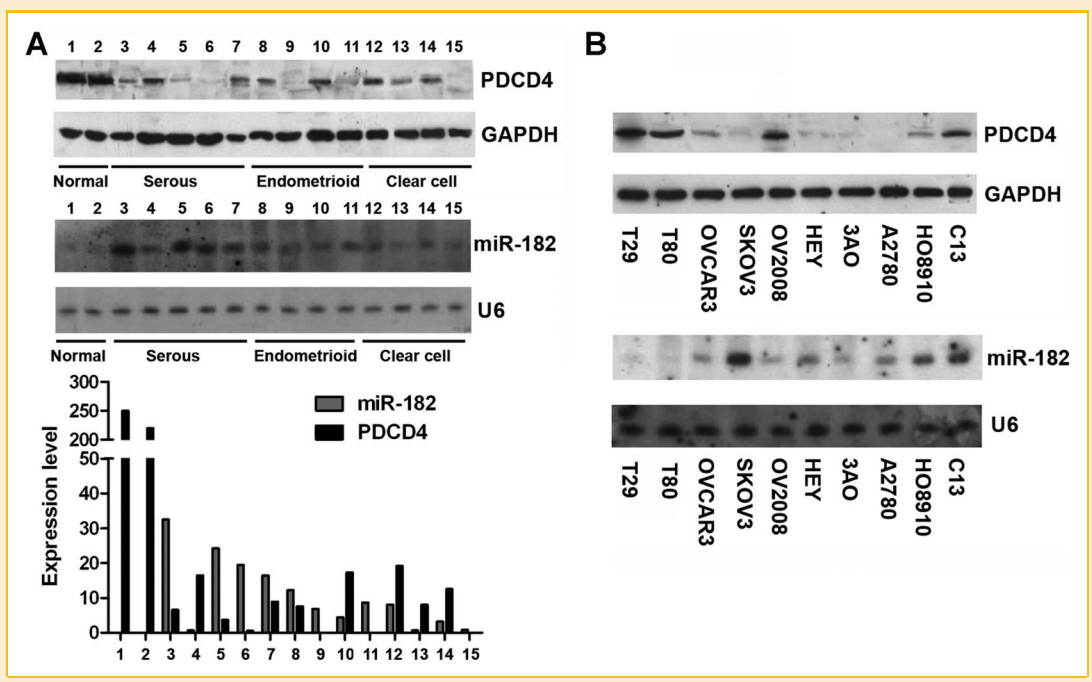

Fig. 1. miR-182 was up-regulated, while PDCD4 is down-regulated in ovarian cancer cell. A: The PDCD4 protein levels in two normal ovarian tissues (normal, 1, 2), five ovarian serous cystadenocarcinomas (serous, 3–7), four ovarian endometrioid carcinomas (endometrioid, 8–11) and four ovarian clear cell adenocarcinomas (clear cell, 12–15) were measured using Western blot assay. Also, the miR-182 level in these 15 samples was measured using Northern blot assay. GAPDH and U6 snRNA were served as the endogenous normalizer, respectively. The normalized PDCD4 and miR-182 levels are shown in the histogram. B: The PDCD4 and miR-182 levels in two normal ovarian epithelial derived cell lines (T29 and T80), and eight ovarian carcinoma cell lines (OVCAR3, SKOV3, OV2008, HEY, 3AO, A2780, HO8910 and C13) were detected by Western or Northern blot assays, respectively.

higher in OV2008 cells (Fig. 1B). Thus, we chose these two cell lines in the following functional experiments.

miR-182 DIRECTLY AND NEGATIVELY REGULATES PDCD4 EXPRESSION IN OVARIAN CANCER CELLS

To further validate the negative correlation between miR-182 and PDCD4, the ASO of miR-182 was introduced to inhibit the endogenous miR-182 level in ovarian cancer cells (Fig. 2A), which resulted in an elevation of PDCD4 level in these cells (Fig. 2B). In contrast, transfection of miR-182 mimics into the two OSE cell lines as well as two ovarian cancer cells (Fig. 2C) led to a decline of PDCD4 level (Fig. 2D). These data provide further evidence that PDCD4 was negatively correlated with miR-182.

Next, we utilized bioinformatics databases to confirm that PDCD4 mRNA 3′ untranslated region (3′UTR) bears a potential binding site of miR-182 (Fig. 2E), which provided a valuable clue to elucidate the mechanism of negative correlation between miR-182 and PDCD4. To achieve experimental evidences to support the assumption that miR-182 directly regulates PDCD4, we created two luciferase reporter vectors with one bearing a wild type and the other bearing a mutated sequence of the predicted miR-182 binding site (Fig. 2E) downstream the PDCD4 coding region. In SKOV3 cells, suppression of miR-182 resulted in an elevation of luciferase intensity, whereas introduction of exogenous miR-182 caused depression of luciferase expression in OV2008 cells (Fig. 2F). These effects depend on the presence of miR-182 binding site within the PDCD4 3′ UTR, because when the reporter vectors bearing mutated binding site was used, alteration of miR-182 aborted to influence the luciferase expression (Fig. 2F). These data highlight the direct regulation of PDCD4 by miR-182.

miR-182 PROMOTES OVARIAN CANCER CELL GROWTH THROUGH NEGATIVELY REGULATING PDCD4

To further evaluate the effect of miR-182 on malignant phenotypes in ovarian cancer cells, we constructed a small hairpin RNA (shRNA) expression vector (shR-PDCD4) to specially suppress endogenous PDCD4 expression, and a pCMV6-Entry based PDCD4 ectopic expression vector (pCMV6/PDCD4) to overexpress this gene. In SKOV3 cells, inhibition of miR-182 resulted in an elevation of PDCD4 level, which could be alleviated by following transfection with PDCD4 shRNA. In contrast, miR-182 mimics induced PDCD4 low level could also be restored by ectopic expression of PDCD4 (Fig. 3A). In addition, the shRNA or expression vectors had no effects on miR-182 level (Fig. 3B). From the MTT and colony formation assays, we observed that miR-182 ASO suppressed SKOV3 cell viability and colony formation activity, whereas miR-182 mimics

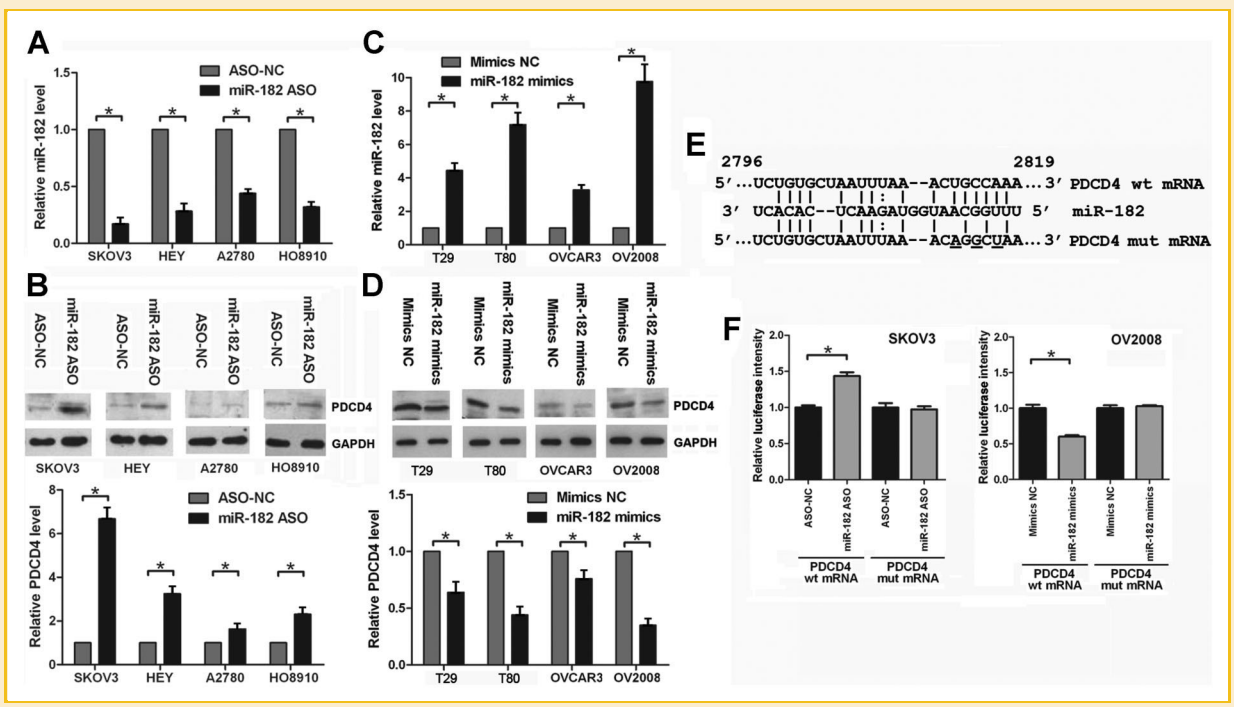

Fig. 2. miR-182 directly and negatively regulates PDCD4 expression. A: Four ovarian carcinoma cell lines, SKOV3, HEY, A2780, and HO8910, were transfected with miR-182 ASO, and miR-182 level was measured using quantitative RT-PCR assay. U6 snRNA was regarded as the endogenous normalizer. B: The PDCD4 protein level in the four cell lines transfected with miR-182 ASO was detected using Western blot assay. C,D: The miR-182 (C) and PDCD4 protein (D) levels in T29, T80, OVCAR3 and OV2008 cells treated with miR-182 mimics were detected by quantitative RT-PCR or Western blot assays, respectively. E: As is predicted by miRanda database, the 3′ UTR of PDCD4 wild type mRNA (PDCD4 wt mRNA) bears a potential miR-182 binding site. A mutated PDCD4 mRNA (PDCD4 mut mRNA), which has three mutated nucleotides within the predicted binding site, is also shown. F: The PDCD4 wt and mut mRNA fragments were synthesized and cloned into the pMIR-REPORT vector, respectively. The constructed reporter vectors were co-transfected into SKOV3 or OV2008 cells with miR-182 ASO or mimics, respectively. Then the luciferase intensity was measured. The histograms show the normalized luciferase intensity (luciferase/Renilla). $^{*}P < 0.05$.

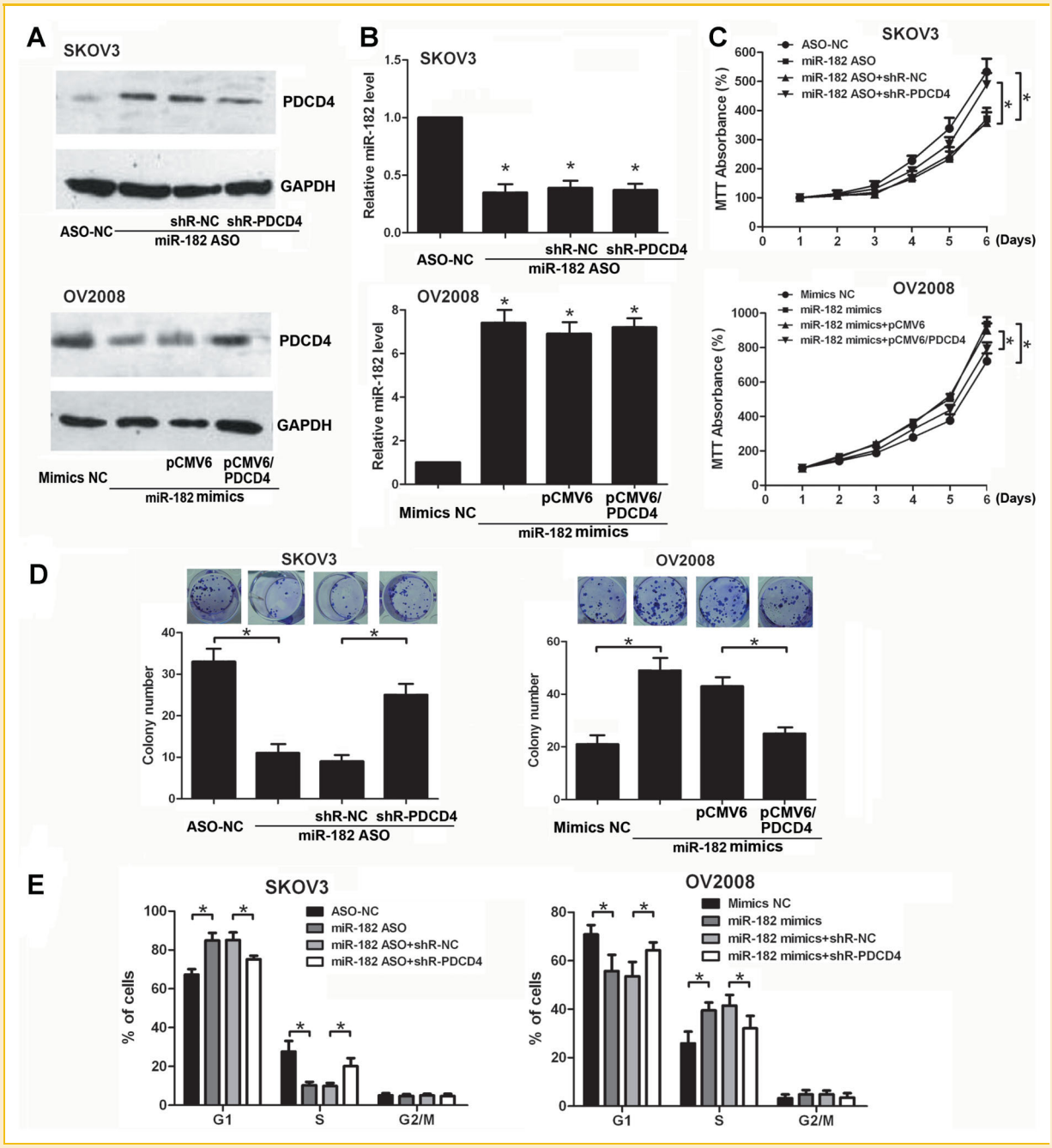

Fig. 3. miR-182 promotes ovarian cancer cell growth via negatively regulating PDCD4. A: SKOV3 cells were transfected with miR-182 ASO and subsequently with PDCD4 siRNA expression vector pSilencer/shR-PDCD4. Conversely, OV2008 cells were sequentially transfected with miR-182 mimics and pCMV6/PDCD4. PDCD4 protein level was measured using Western blot assay in these cells. B: miR-182 level in the SKOV3 and OV2008 cells treated as indicated in (A) was detected by quantitative RT-PCR. C: SKOV3 and OV2008 cells were transfected as indicated in (A), and MTT assay was performed to measure the cellular viability. The line charts show the relative MTT absorbance, which indicates the cellular viability, at 1–6 days after transfection. D: SKOV3 and OV2008 cells were transfected as indicated in (A), and colony formation assay was used to detect the colony formation activity. E: SKOV3 and OV2008 cells were transfected as indicated in (A), and cell cycle progression was measured using flow cytometry method. $^{*}P < 0.05$.

promoted these phenotypes of OV2008 cells. Importantly, alterations of these cellular phenotypes could be reverted by sequential transfection of PDCD4 shRNA or expression vectors, respectively (Fig. 3C,D). Furthermore, cell cycle process is an underlying mechanism for the effects of miR-182 on cell growth. Our data also demonstrated that inhibition of miR-182 delayed, while miR-182 mimics accelerated cell cycle progression mainly by interfering G1/S phases. These effects could also be eliminated by reduction or supplement of PDCD4 (Fig. 3E). We suggest here a PDCD4-dependent positive role of miR-182 on ovarian cancer cell growth.

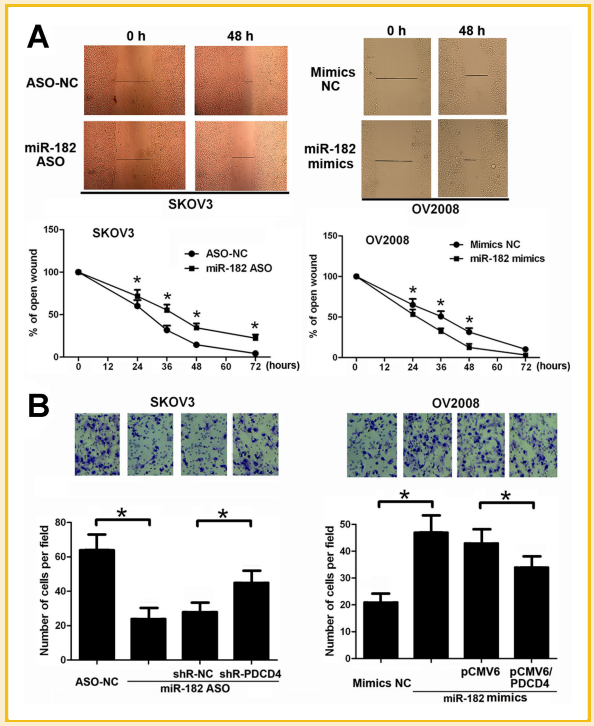

Fig. 4. miR-182 promotes migration and invasion activities of ovarian cancer cells via negatively regulating PDCD4. A: SKOV3 and OV2008 cells were transfected with miR-182 ASO or mimics, respectively. Then, the cell migration activity was detected using wound healing assay. The images show the wound at 0 and 48 h after scratching, and the line charts show the relative open wound at 0, 24, 36, 48, and 72 h after scratching. B: SKOV3 and OV2008 cells were treated as in Figure 3A, and cell invasion activity was measured using Transwell assay. The histograms show the average migrated cells per field of view. $^{*}P < 0.05$.

miR-182 PROMOTES MIGRATION AND INVASION ACTIVITIES OF OVARIAN CANCER CELLS THROUGH NEGATIVELY REGULATING PDCD4

Metastasis is a major obstacle in ovarian cancer therapy [Balbi et al., 2009]. To evaluate the role of miR-182 in regulating migration activity of ovarian cancer, we performed the wound healing assay in cultured cells, where miR-182 promoted the migration activity of ovarian cancer cells (Fig. 4A). Importantly, in Transwell invasion assay, miR-182 also enhanced invasion activity of ovarian cancer cells in vitro by using Matrigel as the simulant of extracellular matrix. Suppression or overexpression of PDCD4 could also alleviate the miR-182 ASO or mimics induced alteration of invasion activity, respectively (Fig. 4B). These data indicate that miR-182 exhibited a PDCD4-dependent stimulated activity in regulating migration and invasion of ovarian cancer cells.

miR-182 ENHANCES CHEMORESISTANCE OF OVARIAN CANCER CELLS TO CDDP AND TAXOL THROUGH NEGATIVELY REGULATING PDCD4

Chemotherapy for ovarian cancer is often ineffective due to common resistance to the current chemotherapeutic regimens [Fung-Kee-Fung et al., 2007]. This prompted us to investigate whether miR-182 was involved in chemoresistance of ovarian cancer. We found that miR-182 ASO treated SKOV3 cells were more sensitive to the cytotoxicity of CDDP, and subsequent suppression of PDCD4 could reverse this effect. This was repeatable when CDDP was replaced by Taxol, another regular chemotherapeutic agent in ovarian cancer therapy. Correspondingly, enhancing of miR-182 results in a low sensitivity of OV2008 cells to CDDP and Taxol, which is also PDCD4-dependent (Fig. 5A). Next, we used Annexin V- and 7-AAD-based flow cytometry analyses to evaluate the effect of miR-182 on apoptosis of ovarian cancer cells. It was indicated that suppression of miR-182 enhances, while increase of miR-182 reduces apoptosis in CDDP or Taxol treated ovarian cancer cells. Also, inhibition or overexpression of PDCD4 could reverse the miR-182-associated effects, respectively (Fig. 5B). Therefore, we suggested that miR-182 reduces sensitivity of ovarian cancer cells to CDDP and Taxol via suppression PDCD4 expression.

DISCUSSION

Despite great efforts in clinical and basic research, the survival rate of patients with ovarian cancer has remained the same in the past several decades [Kurman and Shin Ie, 2010]. Revealing the molecular changes is responsible for overcoming this deadly disease. The key role of the important tumor suppressor PDCD4 in initiation and progression of ovarian cancer has recently been elucidated [Wei et al., 2009ab, 2012]. However, there is a need for elucidating the upstream regulation of PDCD4 expression in ovarian cancer. In this study, we linked PDCD4 to the miRNA miR-182, and demonstrated their roles in regulating malignant phenotypes of ovarian cancer cells. We confirmed here that miR-182 directly and negatively regulates PDCD4 in ovarian cancer. The evidence for this comes from the following sources. First, the endogenous miR-182 level was higher, whereas PDCD4 level was lower in both the ovarian-derived cell lines and tissue samples, indicating the inverse correlation of them. Second, alteration of miR-182 in ovarian cancer cells led to opposite change of PDCD4, highlighting their negatively regulation. Third, PDCD4 mRNA 3′ UTR bears a binding site of miR-182. This 6-mer binding sequence was efficient because the expression of the reporter gene followed by this site could be suppressed when miR-182 binds to this regulating element. Here, we should notice that despite significant overexpression, miR-182 level was unequal in the ovarian cancer tissues used in this study, and we did not observe consistent difference of miR-182 expression level between the three pathologic types. Similarly, miR-182 level also varied in the eight ovarian cancer cell lines. A higher level of miR-182 was detected in SKOV3, HO8910 and C13 cells, and in the other cells, its level was relatively low. Interestingly, although in some of the cases, higher miR-182 level was associated with lower PDCD4 level, and vice versa, it was not strict in the other samples. We presumed that a given protein-coding gene, that is, PDCD4, may be precisely regulated by several miRNAs and other factors, and the mainly functional miRNAs that target a gene are usually cell type dependent [Lujambio and Lowe, 2012]. The underlying mechanism of this phenomenon is worthwhile to be elucidated in further studies.

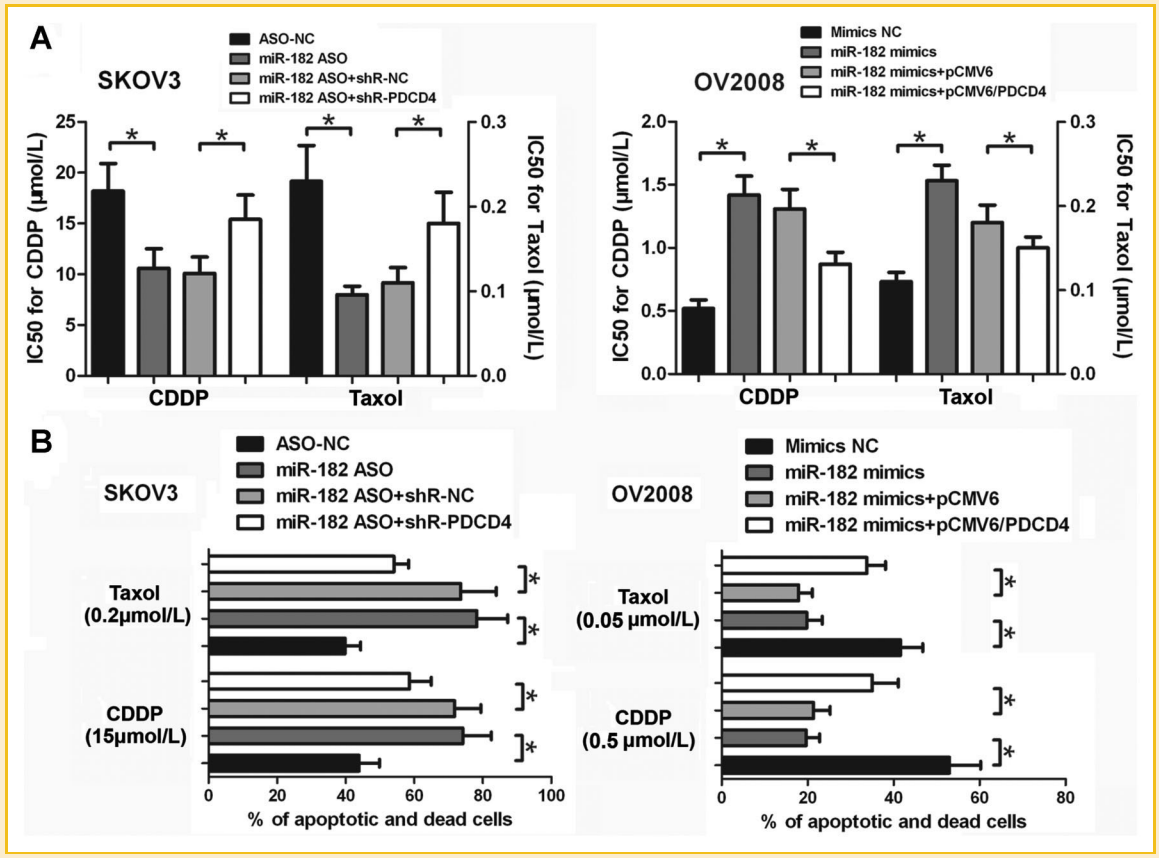

Fig. 5. miR-182 enhances chemoresistance of ovarian cancer cells to CDDP and Taxol via negatively regulating PDCD4. A: SKOV3 and OV2008 cells were treated as in Figure 3A, and then seeded into 96-well plates with different concentration of CDDP or Taxol. The cell viability was detected 48 h later, and IC50 of each drug was calculated and shown in the histograms. B: SKOV3 and OV2008 cells were transfected as in Figure 3A, and then treated with CDDP (15 μmol/L for SKOV3 or 0.5 μmol/L for OV2008) or Taxol (0.2 μmol/L for SKOV3 or 0.05 μmol/L for OV2008). The apoptosis of these cells was detected 48 h later using flow cytometry analyses. The ratio of apoptotic and dead cells in every group was shown. $^{*}P < 0.05$.

The human miR-182 is located in the 7q32 region and is transcribed from the cluster of the miR-183 family. There is emerging knowledge about the role of miR-182 in cancers. On the one hand, miR-182 acts as an oncogenic miRNA, which is frequently overexpressed in many solitary tumors, including ovarian cancer [Segura et al., 2009; Liu et al., 2012]. The oncogenic properties of miR-182 are exhibited by negatively regulating many tumor suppressor genes. In melanoma, miR-182 promotes cancer cell metastasis by repressing FOXO3 and some associated transcription factors [Segura et al., 2009]. miR-182 was overexpressed in high-grade serous ovarian carcinoma and confers powerful oncogenic potential by suppressing breast cancer 1 (BRCA1) and metastasis suppressor 1 (MTSS1). Furthermore, in breast cancer cells, miR-182-mediated downregulation of BRCA1 impedes repair of DNA double-strand breaks [Moskwa et al., 2011]. Similar phenomenon was also observed in HG-SOC [Liu et al., 2012]. The overexpression of miR-182 was reported to be associated with genomic amplification and to be regulated by the IL2/STAT5 pathway [Stittrich et al., 2010]. TP53 may regulate miR-182 expression [Suzuki et al., 2009], although some other studies suggest overexpression of miR-182 to be TP53-independent [Chang et al., 2007]. On the other hand, however, miR-182 shows tumor-suppressive activity in human gastric adenocarcinoma by negatively regulating cAMP-responsive element-binding protein 1 (CREB1) [Kong et al., 2012]. The functions and experimental targets of miR-182 varied depending on the cellular types used in the experiment, suggesting a significant influence of the molecular background on miRNA target selection. In this study, we observed that miR-182 contributed to multiple malignant phenotypes in ovarian cancer cells. First, miR-182 enhances viability and colony formation activity of ovarian cancer cells. These are possibly due to the cell cycle promotion and apoptosis-inhibition effects of miR-182. Second, miR-182 enhances migration and invasion activities of ovarian cancer cells, which was verified by the evidence that miR-182 accelerates wound healing of cultured cells and promotes invasion of cells to pass through the simulated extracellular matrix. Third, miR-182 reduces the sensitivity of ovarian cancer cells to the cytotoxicity of CDDP and Taxol. The anti-apoptosis effect induced by miR-182 may, at least in part, contribute to its chemoresistance function. Importantly, all the alterations of cellular phenotypes caused by miR-182 blockage could be restored by suppression of PDCD4, and in contrast, those caused by miR-182 mimics were able to restored by

ectopic expression of PDCD4 (lack of 3′ UTR to avoid being regulated by any miRNA). These results give strong evidence that miR-182 affects cellular phenotypes through negatively regulating PDCD4. Interestingly, we were led to ask whether the parental SKOV3 cells exhibited more obvious malignant phenotypes than OV2008 cells due to its higher miR-182 and lower PDCD4 expression levels. We found that although SKOV3 cells showed more active invasion activity than OV2008 cells, no consistent differentiation of the other malignant phenotypes was detected between these two cell lines (data not shown). We presumed that the cellular phenotypes were not only affected through miR-182-PDCD4 axis. However, complicated regulatory mechanisms exist in the regulation of ovarian cancer development.

It is reasonable that miR-182 influences intensive phenotypes of ovarian cancer cells. The potential mechanism is that its functional target gene PDCD4 acts as a translation inhibitor, thereby widely influencing the protein pattern in the cells. In cancer cells, PDCD4 regulates multiple proteins which are involved in tumor progression, cell cycle and differentiation [Lankat-Buttgereit and Goke, 2009]. Interestingly, the action of PDCD4 may be cell-type specific, and no correlation was discovered between PDCD4 levels and expression of different proteins involved in cell cycle and apoptosis in various cancer cell lines [Lankat-Buttgereit et al., 2008]. In colon cancer cells, PDCD4 inhibits the expression of a kinase upstream of JNK, known as MAPK kinase (MAPKKK), and suppresses cell invasion [Yang et al., 2006]. Another PDCD4-regulated protein is uPAR (urokinase receptor), which modifies cell invasion and metastasis [Leupold et al., 2007]. In breast cancer, regulation of uPAR by PDCD4 was not detected. However, PDCD4 increases tissue inhibitor of metalloproteinase 2 (TIMP2) and thereby inhibits cell invasion [Nieves-Alicea et al., 2009]. Another example is that reduced dUTPase level associated with high PDCD4 amounts may contribute to the tumor suppressor function of PDCD4 in pancreatic neuroendocrine tumor cell line Bon-1, but could not be observed in colon cancer cell line HCT116 [Lankat-Buttgereit et al., 2008]. In ovarian cancer cells, it is worthwhile to discover and confirm the main target regulated by PDCD4 in further studies, thus to elucidate the mechanism of tumor suppression role of PDCD4, because our findings have suggested that miR-182 widely influences the malignant phenotypes of ovarian cancer cells via suppressing this tumor suppressor.

Considering the complexity of the regulation network between miRNA and protein coding genes, we also expect further studies to discover additional miRNAs targeting PDCD4, and more functional target genes of miR-182 in ovarian cancer. According to the previous studies, other miRNAs such as miR-21 may also target and regulate PDCD4 expression. We observed that the binding site of miR-21 on PDCD4 3′ UTR is different from miR-182. Thus, it is possible that these two miRNAs cooperatively regulate PDCD4, which needs to be elucidated in the future.

Collectively, we found that miR-182 directly and negatively regulates tumor suppressor PDCD4 and enhances multiple malignant phenotypes in ovarian cancer cells. This knowledge may shed light on the mechanism of miRNA in regulating cancer cells, and may also aid the development of new therapeutic strategies to target ovarian cancer.

REFERENCES

Ahmed AA, Etemadmoghadam D, Temple J, Lynch AG, Riad M, Sharma R, Stewart C, Fereday S, Caldas C, Defazio A, Bowtell D, Brenton JD. 2010. Driver mutations in TP53 are ubiquitous in high grade serous carcinoma of the ovary. J Pathol 221:49–56.

Asangani IA, Rasheed SA, Nikolova DA, Leupold JH, Colburn NH, Post S, Allgayer H. 2008. MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. Oncogene 27:2128–2136.

Balbi G, Manganaro MA, Monteverde A, Landino I, Franzese C, Gioia F. 2009. Ovarian cancer: lymph node metastases. Eur J Gynaecol Oncol 30:289–291.

Bartel DP. 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116:281–297.

Chang TC, Wentzel EA, Kent OA, Ramachandran K, Mullendore M, Lee KH, Feldmann G, Yamakuchi M, Ferlito M, Lowenstein CJ, Arking DE, Beer MA, Maitra A, Mendell JT. 2007. Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. Mol Cell 26:745–752.

Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, Barbisin M, Xu NL, Mahuvakar VR, Andersen MR, Lao KQ, Livak KJ, Guegler KJ. 2005. Real-time quantification of microRNAs by stem-loo p RT-PCR. Nucleic Acids Res 33:e179.

Cmarik JL, Min H, Hegamyer G, Zhan S, Kulesz-Martin M, Yoshinaga H, Matsuhashi S, Colburn NH. 1999. Differentially expressed protein Pdcd4 inhibits tumor promoter-induced neoplastic transformation. Proc Natl Acad Sci USA 96:14037–14042.

Farazi TA, Spitzer JI, Morozov P, Tuschl T. 2011. miRNAs in human cancer. J Pathol 223:102–115.

Frankel LB, Christoffersen NR, Jacobsen A, Lindow M, Krogh A, Lund AH. 2008. Programmed cell death 4 (PDCD4) is an important functional target of the microRNA miR-21 in breast cancer cells. J Biol Chem 283:1026–1033.

Fung-Kee-Fung M, Oliver T, Elit L, Oza A, Hirte HW, Bryson P. 2007. Optimal chemotherapy treatment for women with recurrent ovarian cancer. Curr Oncol 14:195–208.

Greenlee RT, Hill-Harmon MB, Murray T, Thun M. 2001. Cancer statistics, 2001. CA Cancer J Clin 51:15–36.

Itani S, Kunisada T, Morimoto Y, Yoshida A, Sasaki T, Ito S, Ouchida M, Sugihara S, Shimizu K, Ozaki T. 2012. MicroRNA-21 correlates with tumorigenesis in malignant peripheral nerve sheath tumor (MPNST) via programmed cell death protein 4 (PDCD4). J Cancer Res Clin Oncol 138:1501–1509.

Kong WQ, Bai R, Liu T, Cai CL, Liu M, Li X, Tang H. 2012. MicroRNA-182 targets cAMP-responsive element-binding protein 1 and suppresses cell growth in human gastric adenocarcinoma. FEBS J 279:1252–1260.

Kuhn E, Kurman RJ, Vang R, Sehdev AS, Han G, Soslow R, Wang TL, Shih Ie M. 2011. TP53 mutations in serous tubal intraepithelial carcinoma and concurrent pelvic high-grade serous carcinoma–evidence supporting the clonal relationship of the two lesions. J Pathol 226:421–426.

Kurman RJ, Shih Ie M. 2010. The origin and pathogenesis of epithelial ovarian cancer: a proposed unifying theory. Am J Surg Pathol 34:433–443.

Lankat-Buttgereit B, Goke R. 2009. The tumour suppressor Pdcd4: recent advances in the elucidation of function and regulation. Biol Cell 101: 309–317.

Lankat-Buttgereit B, Lenschen B, Schmidt H, Goke R. 2008. The action of Pdcd4 may be cell type specific: evidence that reduction of dUTPase levels might contribute to its tumor suppressor activity in Bon-1 cells. Apoptosis 13:157–164.

Leupold JH, Yang HS, Colburn NH, Asangani I, Post S, Allgayer H. 2007. Tumor suppressor Pdcd4 inhibits invasion/intravasation and regulates urokinase receptor (u-PAR) gene expression via Sp-transcription factors. Oncogene 26:4550–4562.

Liu J, Yang G, Thompson-Lanza JA, Glassman A, Hayes K, Patterson A, Marquez RT, Auersperg N, Yu Y, Hahn WC, Mills GB, Bast RC, Jr. 2004. A genetically defined model for human ovarian cancer. Cancer Res 64:1655–1663.

Liu Z, Liu J, Segura MF, Shao C, Lee P, Gong Y, Hernando E, Wei JJ. 2012. MiR-182 overexpression in tumourigenesis of high-grade serous ovarian carcinoma. J Pathol 228:204–215.

Lujambio A, Lowe SW. 2012. The microcosmos of cancer. Nature 482: 347–355.

Moskwa P, Buffa FM, Pan Y, Panchakshari R, Gottipati P, Muschel RJ, Beech J, Kulshrestha R, Abdelmohsen K, Weinstock DM, Gorospe M, Harris AL, Helleday T, Chowdhury D. 2011. miR-182-mediated downregulation of BRCA1 impacts DNA repair and sensitivity to PARP inhibitors. Mol Cell 41:210–220.

Nieves-Alicea R, Colburn NH, Simeone AM, Tari AM. 2009. Programmed cell death 4 inhibits breast cancer cell invasion by increasing tissue inhibitor of metalloproteinases-2 expression. Breast Cancer Res Treat 114:203–209.

Segura MF, Hanniford D, Menendez S, Reavie L, Zou X, Alvarez-Diaz S, Zakrzewski J, Blochin E, Rose A, Bogunovic D, Polsky D, Wei J, Lee P, Belitskaya-Levy I, Bhardwaj N, Osman I, Hernando E. 2009. Aberrant miR-182 expression promotes melanoma metastasis by repressing FOXO3 and microphthalmia-associated transcription factor. Proc Natl Acad Sci USA 106:1814–1819.

Stittrich AB, Haftmann C, Sgouroudis E, Kuhl AA, Hegazy AN, Panse I, Riedel R, Flossdorf M, Dong J, Fuhrmann F, Heinz GA, Fang Z, Li N, Bissels U, Hatam F, Jahn A, Hammoud B, Matz M, Schulze FM, Baumgrass R, Bosio A, Mollenkopf HJ, Grun J, Thiel A, Chen W, Hofer T, Loddenkemper C, Lohning M, Chang HD, Rajewsky N, Radbruch A, Mashreghi MF. 2010. The microRNA miR-182 is induced by IL-2 and promotes clonal expansion of activated helper T lymphocytes. Nat Immunol 11:1057–1062.

Suzuki HI, Yamagata K, Sugimoto K, Iwamoto T, Kato S, Miyazono K. 2009. Modulation of microRNA processing by p53. Nature 460:529–533.

Wei NA, Liu SS, Leung TH, Tam KF, Liao XY, Cheung AN, Chan KK, Ngan HY. 2009a. Loss of Programmed cell death 4 (Pdcd4) associates with the progression of ovarian cancer. Mol Cancer 8:70.

Wei ZT, Zhang X, Wang XY, Gao F, Zhou CJ, Zhu FL, Wang Q, Gao Q, Ma CH, Sun WS, Fu QZ, Chen YH, Zhang LN. 2009b. PDCD4 inhibits the malignant phenotype of ovarian cancer cells. Cancer Sci 100:1408–1413.

Wei N, Liu SS, Chan KK, Ngan HY. 2012. Tumour suppressive function and modulation of programmed cell death 4 (PDCD4) in ovarian cancer. PLoS ONE 7:e30311.

Yang HS, Jansen AP, Nair R, Shibahara K, Verma AK, Cmarik JL, Colburn NH. 2001. A novel transformation suppressor, Pdcd4, inhibits AP-1 transactivation but not NF-kappaB or ODC transactivation. Oncogene 20:669–676.

Yang HS, Jansen AP, Komar AA, Zheng X, Merrick WC, Costes S, Lockett SJ, Sonenberg N, Colburn NH. 2003. The transformation suppressor Pdcd4 is a novel eukaryotic translation initiation factor 4A binding protein that inhibits translation. Mol Cell Biol 23:26–37.

Yang HS, Matthews CP, Clair T, Wang Q, Baker AR, Li CC, Tan TH, Colburn NH. 2006. Tumorigenesis suppressor Pdcd4 down-regulates mitogen-activated protein kinase kinase kinase kinase 1 expression to suppress colon carcinoma cell invasion. Mol Cell Biol 26:1297–1306.

Zhang S, Royer R, Li S, McLaughlin JR, Rosen B, Risch HA, Fan I, Bradley L, Shaw PA, Narod SA. 2011. Frequencies of BRCA1 and BRCA2 mutations among 1,342 unselected patients with invasive ovarian cancer. Gynecol Oncol 121:353–357.

Zhu S, Wu H, Wu F, Nie D, Sheng S, Mo YY. 2008. MicroRNA-21 targets tumor suppressor genes in invasion and metastasis. Cell Res 18:350–359.