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Long noncoding RNA PANDA promotes esophageal squamous carcinoma cell progress by dissociating from NF-YA but interact with SAFA

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ABSTRACT

Esophageal squamous cell carcinoma (ESCC) is one of the major global health problems, especially in Asia. Long non-coding RNAs (lncRNAs) have been increasingly identified and characterized in almost every aspect of biology, especially in cancer biology. This research desires to explore the regulatory mechanism of lncRNA PANDA (PANDA) on ESCC process. Quantitative real-time PCR (qRT-PCR) was carried out to detect the PANDA expression, which was up-regulated in matched cancerous tissues and adjacent noncancerous tissues from 134 patients and 9 ESCC cell lines. Higher expression of PANDA in ESCC tissues was associated with TNM stage, advanced clinical stage, and shorter overall survival of ESCC patients by MTT, EDU, colony formation assay and flow cytometry in KYSE180 and KYSE450 cells. Exogenous down-regulation of PANDA expression significantly suppressed ESCC cells proliferation and colony formation by arresting G1-S checkpoint transition in vitro, and retarded the development of tumors in vivo. Meanwhile, qRT-PCR and western blot assays showed that depletion of PANDA reduced E2F1, cyclinD1, cyclinD2, cyclinE1 and Bcl-2 expression. RIP showed the interaction between PANDA and NF-YA or SAFA. Our findings suggested that, PANDA drifted away from NF-YA to promote the expression of NF-YA-E2F1 co-regulated proliferation-promoting genes, and to limit the cell apoptosis. In addition, PANDA binds SAFA to switch on the tumor proliferation program through CyclinD1/2-Cyclin E1 and Bcl-2 pathways. PANDA could serve as a potential prognostic biomarker and therapeutic target for ESCC.

1. Introduction

Esophageal cancer (EC) is the eighth most common malignant tumor worldwide and the sixth most common cause of death from cancer [1]. Esophageal cancer, derived from epithelia, consists of two subtypes: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma, which have different etiologic and pathologic characteristics [2]. In Asia, the predominant pathological type is ESCC, which is often diagnosed at the advanced stage, and China is one of the high-risk esophageal cancer areas [3]. To date, the prognosis for ESCC patients is not well improved with a rate of less than 10% 5-year survival [4]. Therefore, it is urgent to understand the detailed interactions and regulatory mechanisms of key pathways involved in the tumor-genesis and progression of ESCC and find molecular markers for early detection and diagnosis.

Recent studies have revealed that epigenetic regulation also participates in cancer development and progression [5]. Long noncoding RNAs (LncRNAs), which occupy the majority of human genome, have been shown to play an important role in the regulation of gene transcription, translation and widespread regulators involved in cell proliferation, migration and apoptosis [6–10]. Mounting evidence indicate that lncRNAs expression is misregulated and contributes to the

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development and progression of multiple cancers. Their dysregulation has been found in various types of carcinomas, including breast cancer, colon cancer, hepatocellular carcinoma and lung cancer. LncRNAs are often up-regulated and down-regulated, and may serve as oncogenes or tumor suppressors in cancers [11–13]. However, the roles of lncRNAs in ESCC are still not well documented and needed to be further explored.

LncRNA PANDA (PANDA), a 5-capped and polyadenylated non-spliced lncRNA, is transcripted from approximately 5 kb upstream of the CDKN1A transcriptional start site. PANDA expression could be induced by DNA damage and limit expression of pro-apoptotic genes by interacting with the transcription factor NF-YA in a p53-dependent manner [14]. Moreover, PANDA deletion could increase human fibroblasts cells sensitivity to doxorubicin and induce cell apoptosis. However, its expression and biological function in ESCC remain poorly understood. In this study, the expression pattern and biological functions of PANDA in ESCC development were explored. The correlations between PANDA and the clinical outcomes of ESCC patients were evaluated, and the effects of PANDA on proliferation and apoptosis of ESCC cells were also investigated both in vitro and in vivo. Moreover, knockdown of PANDA could affect multiple gene expression involved in regulating phase checkpoints of cell cycle, such as E2F1. This study might provide a novel mechanism and potential therapeutic target for ESCC.

2. Materials and methods

2.1. Tissue sample collection and patient data

All samples were collected from informed consent individuals according to protocols approved by the ethics committee of Nanjing Hospital affiliated with Nanjing Medical University. A total of 134 paired samples of ESCC (TNM Stage 0 to IV), including cancer tissues and corresponding adjacent non-cancer tissues were obtained from patients who underwent surgery in our hospital from 2005 to 2012. Only 76 cases of 134 patients had follow-up materials of 5 yrs used for survival analysis for 5 yrs. No patients received radiotherapy, chemotherapy or targeted therapy prior to operation. Tissue samples were immediately preserved in liquid nitrogen after removal from the individual and stored at −80 °C until RNA extraction. All species were histopathologically confirmed as patients with ESCC.

2.2. Cell lines and cell culture procedures

The cell lines of human ESCC including KYSE30, KYSE70, KYSE140, KYSE150, KYSE180, KYSE450, KYSE510 and 9706 were generous gifts by Dr. Zhihua Liu at the State Key Laboratory of Molecular Oncology [57,58], Cancer Institute, Chinese Academy of Medical Sciences (Beijing, China). Eca109 cell line of ESCC and Human Esophageal Epithelial Cells Het1A were obtained from American Type Culture Collection (Manassas, VA, USA). All cells were cultured in RPMI-1640 (GIBCO) medium with 10% fetal bovine serum (GIBCO, USA) supplemented with 100units/ml penicillin and 100 mg/ml streptomycin in a humidified atmosphere containing 5% CO2 at 37 °C.

2.3. RNA extraction and qPCR analyses

Total RNA was extracted from tissue samples or cultured cells with TRIzol reagent (Invitrogen). RNA was reversely transcribed into cDNAs by using a Reverse Transcription Kit of the Prime-ScriptTM one step (Takara, Dalian, China). Quantitative reverse transcriptase polymerase chain reactions (qRT-PCR) were performed using the ABI7500 System (Applied Biosystems, CA, USA) and the SYBR Green PCR Master Mix kit (TaKaRa, Dalian, China) according to the supplied protocol of the manufacturer's instructions. Each experiment was repeated in triplicate at least. The primer sequence was attached in supplement Table 1. Finally, KYSE180 and KYSE450 cells of the highest expression PANDA in 9 ESCC cell lines (KYSE30, KYSE70, KYSE140, KYSE150, KYSE180, KYSE450, KYSE510, 9706 and ECA109 cell lines) were selected for next biology assays.

Table 1
Correlation between PANDA expression and clinicopathological characteristics of esophageal squamous cell carcinoma (ESCC).

Factors	High expression group N (≥Median 1.80 N = 67)	Low expression group N (<Median 1.80 N = 67)	*p*-value
Age group, N			0.119
≦59 yrs	27	36	
59 yrs	40	31	
Gender, N			0.125
Male	44	52	
Female	23	15	
Smoking status, N			0.863
Ever and current	36	31	
Never	35	32	
Alcohol consumption, N			0.729
Ever and current	36	34	
Never	31	33	
Lymph node metastasis, N			0.032[a]
Yes	32	19	
No	35	48	
TNM group stage, N			0.008[a]
0-II	44	58	
III- IV	23	9	

(Two side chi-square test), [a] $p < 0.05$.

2.4. Screening siRNA for lncRNA PANDA

Three pairs of small interference RNAs (siRNA) that targeted PANDA (stealth_75 with the sequence 5′-CAGCUGGCAAUCUACAACC UGUCUU-3′, 5′-AAGACAGGUUGUAGAUUGCCAGCUG-3′, stealth_164 with the sequence 5′-GCUUGUUCCAGAGCCAGGAUGAAUU-3′, 5′-AAUUCAUCCUGGCUCUGGAACAAGC-3′, and stealth_250 with the sequence 5′-GCAUUGAGGAUGACCUUCGGGUUAA-3′, 5′-UUAACCCGA AGGUCAUCCUCAAUGC-3′) were designed and purchased from Life Technologies (Carlsbad, California, USA). Simultaneously, synthetic sequence-scrambled siRNA and 1 × PBS (phosphate buffered solution) were used as negative control (si-NC) and mock transfection, respectively. Human ESCC cells were plated in 6-well plates (4 × 105 cells/well) with antibiotic-free medium till cell density reached 50–60% and then were transiently transfected with either 50 nmol/l siRNAs or si-NC/mock using LipofectamineTM 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocols. After 48 h, the Mock group received no intervention, the control groups were transfected with siRNA-NC, and three experimental groups were transfected with three pairs of designed siRNA-1 (stealth_75), siRNA-2 (stealth_164), and siRNA-3 (stealth_250) respectively. RNA was extracted from KYSE180 and KYSE450 cells transfected. Real-time PCR was used to detect the lncRNA PANDA gene levels, thereby to screen the effective siRNA (siRNA-2 and siRNA-3).

2.5. MTT and EDU assay

KYSE180 and KYSE450 cells that had been transfected with siRNAs (siRNA-2 and siRNA-3) for 48 h were seeded into 96-well plates. Cell density was adjusted to 5 × 103 cells/well for six replicate wells and the final volume was 150 μl/well. MTT (3- (4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) solution (20 μL) was added to the plates 6, 24, 48, 72 and 96 h later. The cells were cultured for 4 h at 37 °C. Then the medium was discarded and 150 μL DMSO was added and oscillated for 15 min. Optical density (OD) was detected at a wavelength of 490 nm using an enzyme-labeled analyzer. Experiments were performed in triplicate.

KYSE180 and KYSE450 cells that had been transfected with siRNA-3 against PANDA for 48 h were seeded into 24-well plates with cover slips in bottom. After 12 h of culture, EdU immunofluorescence staining was performed with an EdU kit (RIBOBIO C10310-3), and cells were grown on cover slips until they reached approximately 50% confluency. Cell proliferation was assessed using EdU immunofluorescence according to the manufacturer's protocols.

2.6. Cell apoptosis analysis and cell cycle analysis

KYSE180 and KYSE450 cells were plated in 6-well plates (4 × 105 cells/well) in antibiotic-free medium and transfected with 50 nmol/l siRNAs (siRNA-2 and siRNA-3) targeting PANDA or a scrambled negative control (si-NC) using Lipofectamine 2000. After 24 h, KYSE180 and KYSE450 cells were collected and washed twice and stained with fluorescein isothio-cyanate (FITC)-Annexin V and propidium iodide (PI, BD Bioscience), using the FITC Annexin V Apoptosis Detection Kit (BD Biosciences) according to the manufacturer's manual. Apoptotic cells were analyzed by flow cytometry (CYTOMICS FC 500, Beckman Coulter, Miami, FL).

KYSE180 and KYSE450 cells were plated in 6-well plates (4 × 105 cells/well) in antibiotic free medium and transfected with 50 nmol/l siRNA (siRNA-2 or siRNA-3) targeting PANDA or si-NC with Lipofectamine 2000 (Invitrogen). After 48 h, cells were harvested and washed three times with ice-cold PBS and fixed with ice–cold 75% ethanol overnight. Then, cell cycle distribution was quantified by flow cytometry (CYTOMICS FC 500, Beckman Coulter, Miami, FL). The percentage of the cells in G0–G1, S, and G2–M phases were counted and compared. All experiments were conducted in triplicate.

2.7. Scratch wound assay

KYSE180 and KYSE450 cells were transfected with either 50 nmol/l siRNA targeting PANDA or si-NC. Wounds were created in adherent cells using a 10 μl pipette tip after transfected 24 h. The cells were then washed three times with PBS to remove floating cells and debris. Culture medium without serum was added, and the cells were incubated under normal conditions. Wound healing was observed after 24 h under light microscopy. Representative scrape lines were photographed using digital microscopy after culture medium was removed. Each experiment was repeated in triplicate.

2.8. Construction of vector and shRNA-PANDA

SiRNA were chemically synthesized (Invitrogen). Synthesized DNA nucleotide fragment encoding short hairpin RNA (shRNA) for knockdown of endogenous PANDA was inserted into pENTRTM/U6 (pENTRTM/U6-shRNA) (Invitrogen, Carlsbad, CA, USA). The KYSE180 and KYSE450 cells were transfected with plasmid pENTRTM/U6 (vector) and pENTRTM/U6-shRNA (shRNA) selected with neomycin (1000 μg/ml) for two weeks using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.9. Plate colony formation assay

KYSE180 and KYSE450 cells were transfected with vector and shRNA for 24 h. Cells were harvested and seeded in 6-well plates (800 cells/well, 3 wells/group). After incubation with 5% CO2 at 37 °C for 14 days, the cells were washed twice with PBS, fixed with methanol and stained with crystal violet. Cells colonies were counted under a microscope and photographed. Plate colony formation efficiency = (number of colonies/ number of cells inoculated) × 100%.

2.10. Xenografts in nude mouse

Ten, four-week-old, male BALB/c nude mice weighing between 18 and 21 g were maintained in SPF animal breeding room of Nanjing Hospital affiliated to Nanjing Medical University. KYSE450 cells were optimized for vivo assays according to the results of colony formation assay and KYSE450 cells transfected with vector or sh-PANDA (shRNA) for 48 h were collected. Each mouse was subcutaneously inoculated with 200 μl of cell suspension containing 4 × 106 cells/ml. Ten mice were divided into two groups and injected respectively with KYSE450 cells transfected with vector or shRNA. The volumes of tumor were examined once a week. Four weeks after injection, mice were sacrificed and examined the growth of subcutaneous tumors.

Tumor volume was determined by the formula: volume = length × width2 × 0.5. Tumor tissues were cut into small pieces about 0.1 × 0.1 cm, then were fixed in 10% buffered formalin and processed for Ki67 protein expression by immunohistochemistry next. Hematoxylin and eosin (H&E) staining was performed.

All of the mouse experimental procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee. The protocol was approved by the Committee on the Ethics of Animal Experiments of Nanjing Hospital affiliated to Nanjing Medical University. All surgery was performed under anaesthesia, and all efforts were made to minimize suffering.

2.11. Immunohistochemistry

Totally 22 of 134 pairs of ESCC tissues and their matched normal tissues were immunostained for E2F1 and SAFA. Ki67 was also immunostained in subcutaneous tumor tissues of nude mice transplant. The signal was amplified and visualized with 3,3′-diaminobenzidine chromogen, followed by counterstaining with hematoxylin. Expression was considered to be positive with more than 50% cancer cells stained. The concentration of anti-E2F1, anti-SAFA, and anti-Ki67 diluted was 1:50 for the assays.

2.12. Western-blot assay

The cell proteins were extracted after KYSE180 and KYSE450 cells were transfected with siRNA-3 targeting PANDA or si-NC for 48 h. KYSE180 and KYSE450 cells were washed with 1 × PBS and lysed for protein extraction by reagent RIPA (Beyotime) supplemented with a protease inhibitor cocktail (Roche, Basel, Switzerland) and PMSF (Roche). The concentration of total protein was quantitated by BCA Protein Assay Kit (Beyotime). Then protein was electrophoresed by 4–12% SDS-PAGE, transferred onto nitrocellulose membranes (Sigma), and incubated with 5% defatted milk including specific primary antibody. Autoradiograms were quantified by densitometry (Quantity One software; Bio-Rad, Hercules, California).

β-actin antibody was used as control. In addition, rabbit anti-Bcl2 (1:1,000) was purchased from Abcam; rabbit anti-caspase9, rabbit anti-caspase3, anti-E2F1 (1:1,000) were from Sigma; rabbit anti-cyclinD1, rabbit anti-cyclinD2, rabbit anti-cyclinE1, and rabbit anti-P53 (1:400) were from Santa Cruz, CA.

2.13. RNA-binding protein immunoprecipitation (RIP) assay

RNA immunoprecipitation was performed using an EZMagna RIP kit (Millipore, Billerica, MA, USA) following the manufacturer's protocol. Briefly, KYSE450 cells at 80–90% confluency were scraped off of the plates and then lysed in complete RIP lysis buffer, after which 100 μl of whole cell extract was incubated with RIP buffer containing magnetic beads conjugated with human anti-SnRNP70, anti-NFYA, anti-EZH2, anti-SUZ12, and anti-SAFA antibody, the negative control was normal rabbit IgG (Millipore). Samples were incubated with Proteinase K with shaking to digest the protein, and then immunoprecipitated RNA was isolated. The RNA concentration was measured using a NanoDrop spectrophotometer (Thermo Scientific), and the RNA quality was assessed using a bioanalyser (Agilent, Santa Clara, CA, USA).

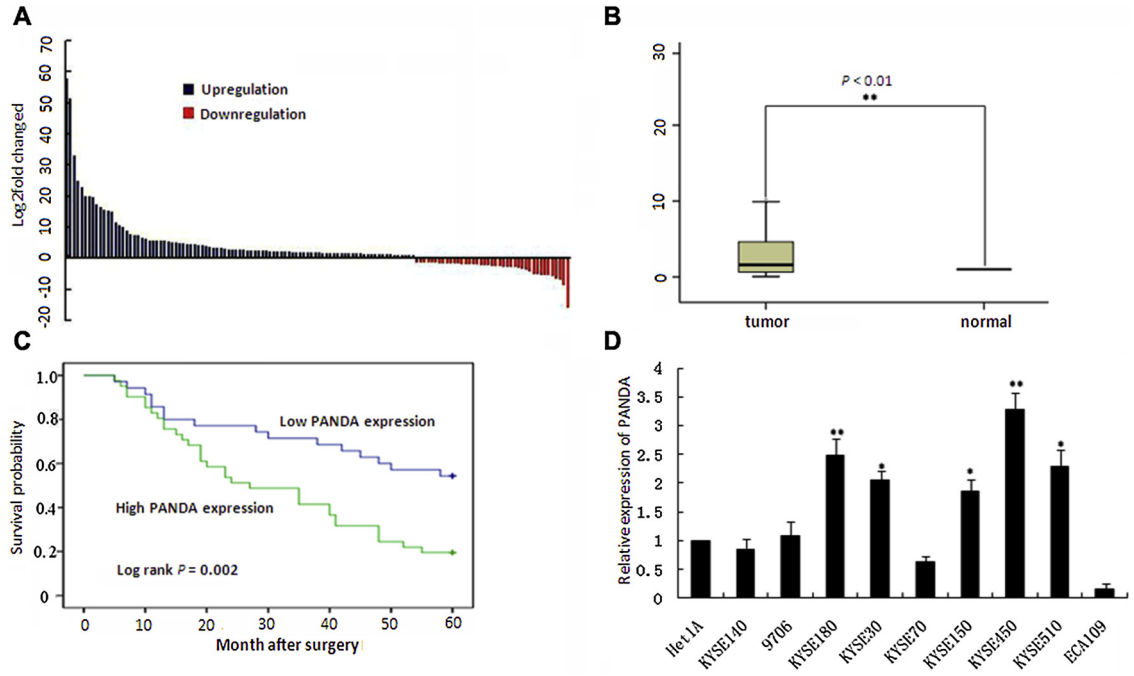

Fig. 1. PANDA expression and its clinical significance in tissues and cell lines of ESCC (A) PANDA expression in matched cancerous tissues and adjacent non-cancerous tissues from 134 ESCC patients were measured by qRT-PCR. Data are presented as fold change in tumor tissues relative to adjacent noncancerous tissues. (B) qRT-PCR was performed to detect PANDA expression in 134 pair of ESCC tissues and adjacent tissues. (C) Kaplane-Meier curves indicate 76 of 134 patients with high levels of PANDA expression (N = 41, ≥ Median 1.80) showed reduced survival times compared with patient with low levels of PANDA expression (N = 35, < Median 1.80) (p = 0.002, log-rank test). (D) The relative expression levels of PANDA were confirmed via qRT-PCR in 9 ESCC cell lines compared to non-tumorigenic esophageal epithelial cell line Het-1A in human. Results are expressed as mean ± SD for three replicate determinations. All data were analyzed using Mann-Whitney U test. *p < 0.05, **p < 0.01.

Table 2
Multivariate Analysis for Overall Survival in clinicopathological factors of ESCC.

Factors	OR (95%CI)	p-value
PANDA expression (low/high)	0.457 (0.230-0.909)	0.026[a]
Lymph node metastasis (yes/no)	0.259 (0.127-0.530)	0.000[a]

Cox proportional hazards regression, [a] $p < 0.05$.
OR, risk ratio; 95%CI, 95% confidence interval.

Furthermore, purified RNA was subjected to qRT-PCR analysis using the respective primers to demonstrate the presence of the binding targets.

2.14. Statistical analysis

All statistical analyses were performed using SPSS version 17.0 software. Data were analyzed using independent two-tailed t-tests in vitro and in vivo. Categorical data were analyzed using the chi-square test and Kruskal-Wallis. P-values less than 0.05 were considered significant.

3. Results

3.1. LncRNA PANDA is up-regulated in of ESCC tissues

To validate levels of PANDA expression, we conducted qRT-PCR analysis to measure the PANDA expression in 134 paired cancerous and adjacent noncancerous tissues of ESCC. PANDA expression was up-regulated in 67.5% ESCC sample (91 of 134 cases, p < 0.05) 91 cancerous tissues and down-regulated in 43 cancerous tissues compared with their paired adjacent noncancerous tissues (Fig. 1A and 1B). All ESCC sample were classified into two groups based on the median value of relative PANDA expression. The high expression group had PANDA expression level ≥ median value (n = 67) and the low expression group had a PANDA expression level < median value (n = 67). Next, we analyzed the relationship between PANDA expression level and clinical pathological information in ESCC patients, and found that up-regulated PANDA expression was closely related to the depth of tumor invasion (p = 0.003), lymph nodes metastasis (p = 0.019) and TNM staging (p = 0.007). However, PANDA expression was not correlated with distant metastasis, tumor cell differentiation and other clinical characteristics, including age, gender, smoking status, and alcohol consumption, etc.

Additionally, we collected information of five years survival from 76 of 134 patients. Kaplan-Meier's survival analysis and log-rank test were conducted to evaluate the association of PANDA expression with prognosis of patients (Table 2). The patients with higher levels of PANDA (n = 41) had poorer survival time than those with lower levels of PANDA (n = 35, p = 0.002, log-rank test, Fig. 1C). By Multivariate cox analysis, PANDA expression and lymph node metastasis were important prognostic factors (p = 0.026, OR 0.457, 95% CI 0.230 to 0.909 and p < 0.01, OR 0.259, 95% CI 0.127-0.530). Taken together, these data suggested that over-expression of PANDA might have important roles in ESCC development and progression.

3.2. PANDA expressed in ESCC cell lines

To investigate the roles of PANDA in ESCC, we performed qRT-PCR to evaluate the level of PANDA in nine ESCC cell lines and one non-malignant esophageal epithelial cell line (Het1A cells). The expression of PANDA in five ESCC cell lines was significantly higher than the levels observed in Het1A cells. The expression of PANDA in KYSE180 and KYSE450 cell lines was found to be the highest and selected for further PANDA cell biological assays (Fig. 1D).

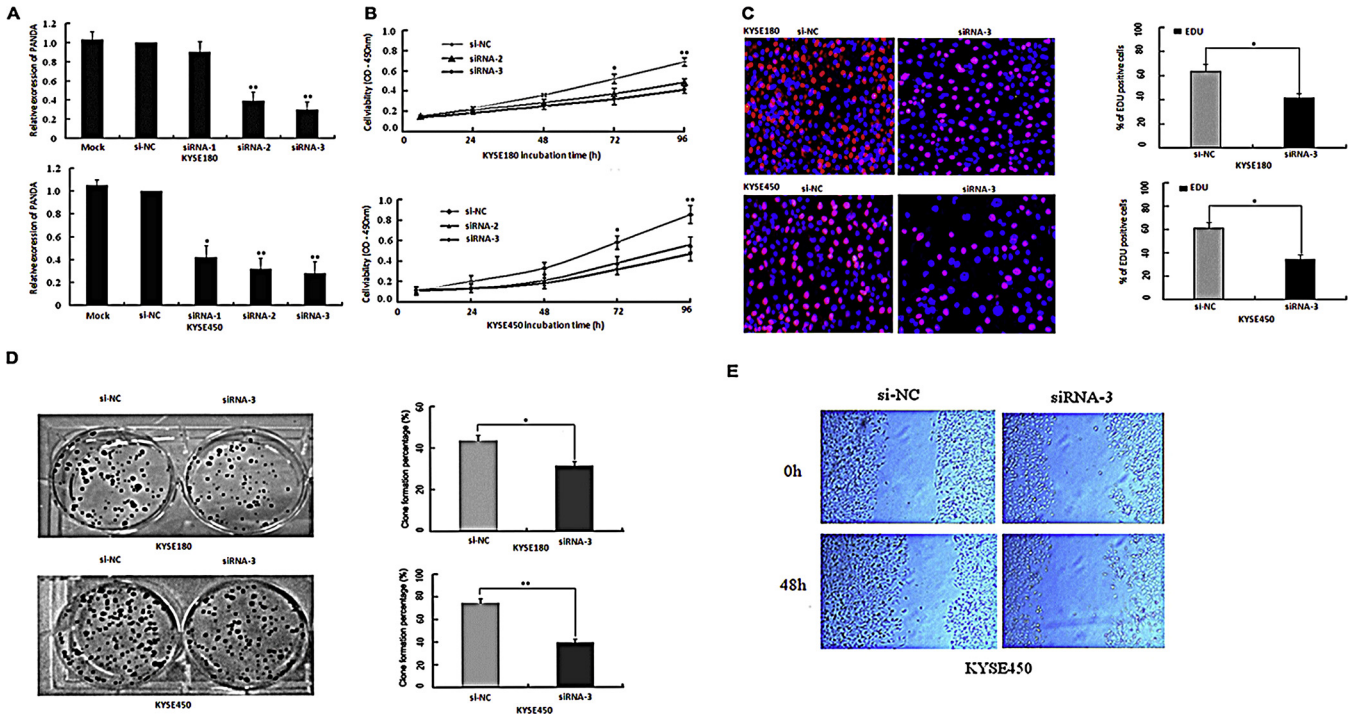

Fig. 2. Knockdown efficiency of PANDA-specific siRNAs in ESCC cell lines and proliferation viability were measured in vitro.
(A) Depletion of PANDA siRNA in KYSE180 and KYSE450 cell lines (**$p < 0.01$). (B) KYSE180 and KYSE450 cells proliferation were detected by MTT assay with siRNA-2 and siRNA-3 of PANDA. (C) KYSE180 and KYSE450 cells proliferation was assessed using EdU immunofluorescence staining with siRNA-3 or si-NC transfection. The graphs on the right show the percentage of EdU-positive nuclei. The data are the mean of EdU-positive nucleus number in three independent experiments. (D) Clone formation assays of KYSE180 and KYSE450 cells after transient transfection with PANDA siRNA-3 or NC siRNA (left panels) and the histogram of the clone formation in each group (right panels). *$p < 0.05$; **$p < 0.01$. (E) Scratch wound assay of KYSE450 cells with PANDA siRNA-3.

3.3. Silence of PANDA promoted cell apoptosis and inhibited cell proliferation in vitro

Malignant proliferation is a well-known and critical cellular event of cancer cells. To investigate the potential function of PANDA in ESCC cells, RNA interference (RNAi) was employed to knockdown endogenous PANDA in KYSE180 and KYSE450 cells. Three different siRNAs were transfected into KYSE180 and KYSE450 cells. The relative expression level of PANDA was measured by qRT-PCR analysis after 24 h. As compared with siRNA-NC, PANDA expression was effectively down-knocked about 60.1% and 69.8% in KYSE180 cells, and about 68.7% and 72.5% in KYSE450 cells transfected with siRNA-2 and siRNA-3 ($p < 0.05$ and $p < 0.01$, Fig. 2A). Thus, siRNA-2 and siRNA-3 were chosen for further cell biological assays.

MTT assay was performed to evaluate the impact of PANDA on cell proliferation in ESCC. Knockdown of PANDA expression inhibited KYSE180 and KYSE450 cell proliferation, and the inhibition rates were 33.25% and 42.38% in KYSE180, and 38.85% and 47.89% at 96 h in KYSE450 transfected with siRNA-2 and siRNA-3 (Fig. 2B). Furthermore, EdU assays were performed in KYSE180 and KYSE450 cells transfected with siRNA-3 or si-NC. The results showed that the number of EDU-positive nuclei was lower in PANDA down-regulated cells than control cells (Fig. 2C).

We also performed colony formation assay, and found that KYSE180 and KYSE450 cells transfected with shRNA-PANDA resulted in decreased cell proliferation compared with cells treated with control. After incubated for 14 days, the colony formation efficiency was 43.75% and 75% in KYSE180 and KYSE450 cells transfected with empty vector, while 22.5% and 31.8% in KYSE180 and KYSE450 cells transfected with shRNA-PANDA ($p < 0.01$, Fig. 2D).

Wound examinations showed that KYSE450 cells transfected with siRNA-3 had not apparently changed scratch wound compared with control cells (Fig. 2E). These findings demonstrated that PANDA might have no effect on the migration of ESCC cell lines

We next evaluated whether PANDA could regulate cell-cycle progression. The results showed that cell population was increased in G1 phase, while reduced in S phase after knockdown of PANDA. The fraction of cells in G1 phase increased from 36.66% to 43.96% and 40.83% to 49.53%, while the fraction of cells in S phase decreased from 40.58% to 33.42% and 40.09% to 32.18% compared with control cells (Fig. 3A and 3B).

We subsequently characterized the role of PANDA in cell apoptosis with flow cytometry assays. Rates of apoptosis in KYSE180 and KYSE450 cells were increased by 6.53%, 8.76% and 7.39%, 8.26% after effective knockdown of PANDA ($p < 0.05$. Fig. 3C and 3D). Thus, PANDA could promote cell proliferation and inhibit cell apoptosis of ESCC.

3.4. PANDA knockdown attenuated xenograft growth and inhibited cell proliferation in vivo

We constructed KYSE450 cells with stable knockdown of PANDA to investigate whether PANDA would affect cell proliferation in vivo according to the results of plate colony formation assay. Xenograft mice models were used to identify the oncogenic role of PANDA in vivo. KYSE450 cells transfected sh-PANDA (shRNA) and vector (control) were subcutaneously injected into Xenograft mice. 24 days after injection, we found that tumor volumes and weights were significantly less in sh-PANDA group than control group (0.052 ± 0.011 g and 0.174 ± 0.045 g, $p < 0.01$, Fig. 4A). As a nuclear antigen associated with cell proliferation-specificity, the Ki-67 can be expressed in proliferating cells in all phases during cell cycle except in phase G0 [15]. Next, collected tumor tissues from sh-PANDA group and control group were analyzed by testing Ki67 expression via immunochemistry (Fig. 4B). The results showed that tissues from mice in sh-PANDA group exhibited lower number of Ki67-positive nuclei compared to controls

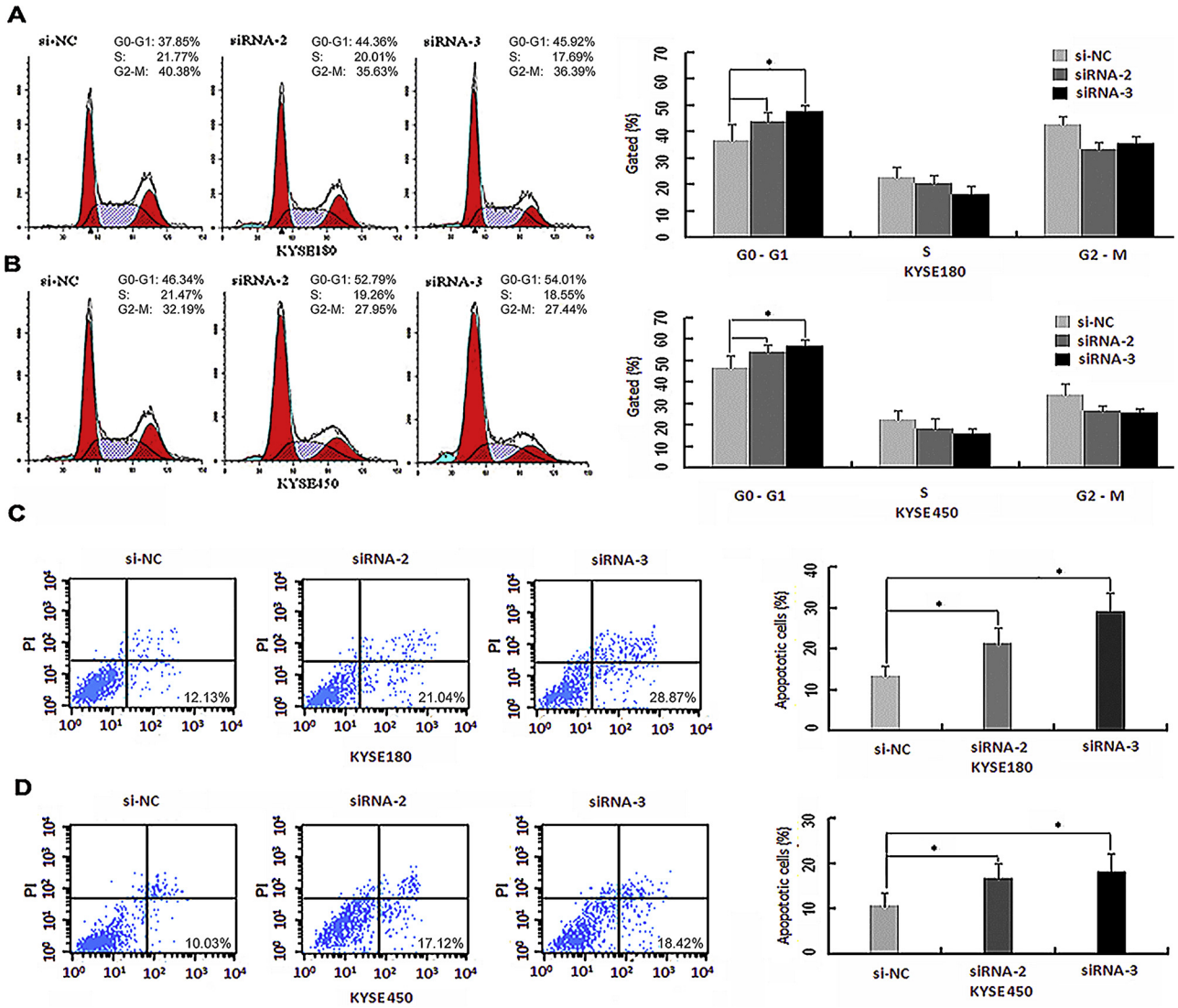

Fig. 3. Inhibition of PANDA promoted apoptosis in esophageal squamous cell carcinoma (ESCC) cells and resulted in cell cycle arrest in G1/S(A–B) KYSE180 (A) and KYSE450 (B) cells transfected with siRNA-2 and siRNA-3 displayed effectively increased response to apoptosis compared with those infected with si-NC (6.53%, 9.26% and 7.39%, 8.26%). (C–D) Knockdown of PANDA resulted in cell cycle arrest in G1/S transition checkpoint. KYSE180 (C) and KYSE450 (D) cells transfected with siRNA-2 and siRNA-3 or si-NC for 48 h. Cell-cycle distribution was measured by flow cytometry. The percentage of cells in G1, S or G2 phases transfected with si-NC was defined as control. Data are represented as mean ± SD from three independent experiments.

and reflected that cell proliferation viability in groups without PANDA decreased [16]. These findings revealed that PANDA might be closely associated with the growth of ESCC cell lines in vivo.

3.5. PANDA knockdown down-regulated cell cycle genes involved in G1-S phase checkpoint

According to the above results, we further investigated whether PANDA knockdown might have an effect on the expression of cell cycle genes. Therefore, we analysis the expression levels of E2F1, cyclinD1, cyclinD2, cyclinE1, IL-12A, PLK1and the anti-apoptosis gene Bcl-2, which involved in regulating G1-S phase checkpoint transition and cell apoptosis in ESCC cells by qRT-PCR [17–19]. The results showed that E2F1, cyclinD1, cyclinD2, cyclinE1 and Bcl-2 expressions were down-regulated significantly, whereas p53, IL-12A and PLK1 had no significant changes in ESCC cells after PANDA knockdown (Fig. 5A). Furthermore, the results of western blot indicated the same change of protein levels that is consistent with change of mRNA expression (Fig. 5B). We also investigated the caspase-9 and caspase-3 protein levels, and the results showed that the expression of cleaved caspase-9 and the ratio of cleaved caspase-3 were increased after PANDA knockdown (Fig. 5C). Altogether, these data suggested that PANDA might promote cell proliferation and suppress cell apoptosis in ESCC by regulating E2F1 and downstream target genes.

3.6. PANDA regulated E2F1 to depress apoptosis in ESCC through dissociating from NF-YA

In order to reveal the clinical relevance of E2F1 in ESCC, we first assessed the expression of E2F1 in ESCC specimens using Immunohistochemistry assay. E2F1 showed higher expression in the cancerous tissues compared with that of the adjacent normal tissues (Fig. 6A). To investigate whether E2F1 expression was driven by PANDA, we detected E2F1 expression in 22 paired primary cancerous and adjacent noncancerous in which PANDA expression had been measured. The results indicated that E2F1 expression was remarkably up-regulated in ESCC tissues compared with adjacent noncancerous (17 of 22 cases, $p < 0.01$) (Fig. 6B). Further analysis revealed that PANDA expression was positively correlated with E2F1 mRNA expression in ESCC ($p = 0.012$, Fig. 6C).

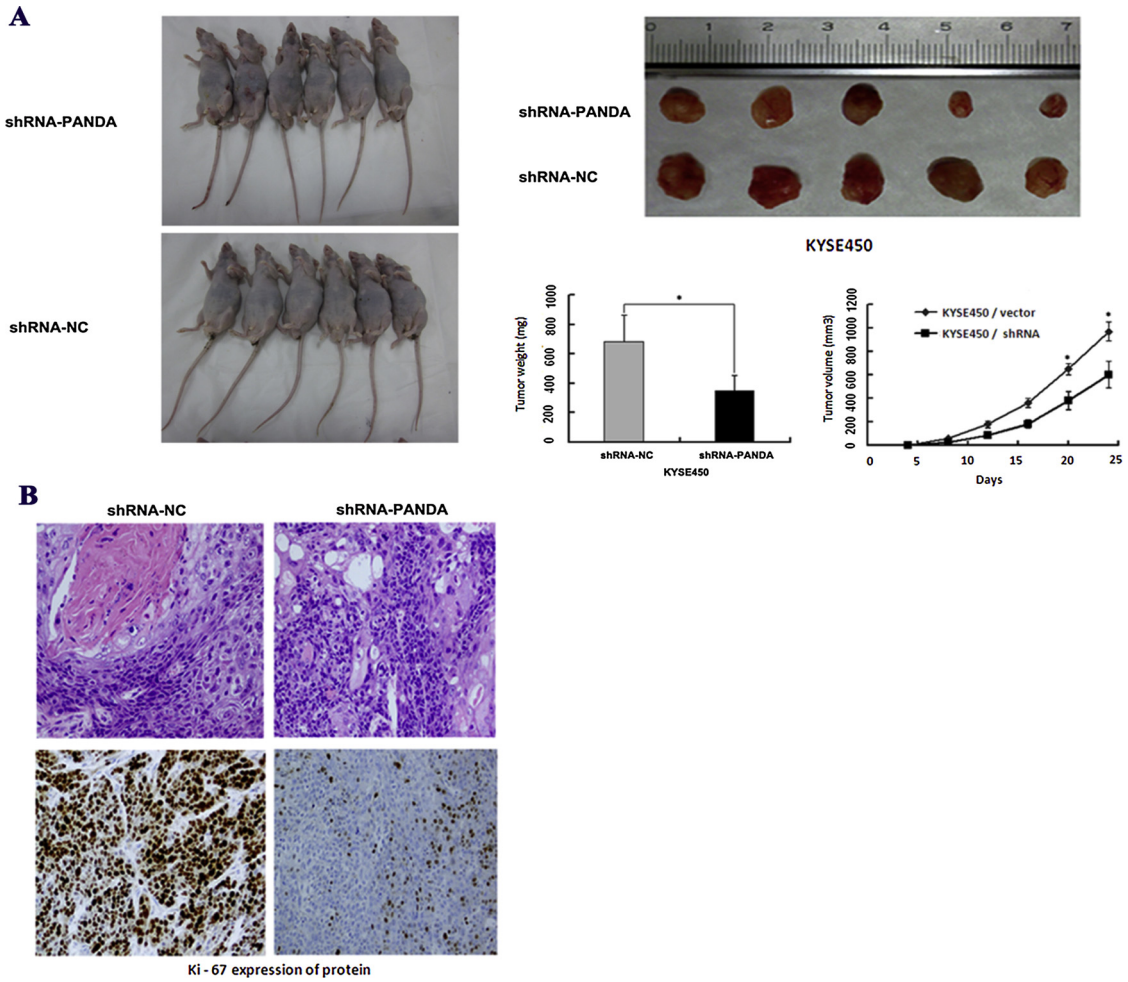

Fig. 4. The effect of PANDA on tumor growth in vivo. (A) shRNA-PANDA or shRNA-NC was transfected into KYSE450 cells, which were injected in the nude mice, respectively. Tumor volumes were calculated after injection every 5 days. Tumor volumes were calculated once a week after injection. Points, mean (n = 5); bars indicate SD. At 24 days, tumor weights of sacrificed nude mice are represented as means of tumor weights ± SD. (B) KYSE450 cell proliferation was analyzed by testing Ki67 expression in shRNA groups and control which coincided with the peak of DNA synthesis in vivo by immunochemistry. The nude mice with PANDA deleted exhibited lower number of Ki67-positive nuclei compared to controls (** $p < 0.01$).

Next, we focused on how PANDA activate E2F1 and regulate downstream genes to promote cell proliferation in ESCC. As we known, DNA damage induced PANDA to interact with NF-YA to promote cell survival by impeding the apoptotic gene expression program [20]. It was interested that the expression of NF-YA had no significant in ESCC (Fig. 6D) and no localization with PANDA by RIP (Fig. 6I). Finally, given the missing of NF-YA in RIP with PANDA, our data point to a potential involvement of PANDA regulating E2F1 by dissociating from NF-YA.

3.7. PANDA interacted with SAFA to promote ESCC cell proliferation

SAFA is a very abundant multimodular nuclear protein that is able to bind DNA and RNA including several classes of noncoding RNA [21–23]. Recently, SAFA was shown to negative regulate PANDA in RAS and RS cells [24]. In ESCC, the expression of SAFA was significant higher in tumor than it in normal tissues (Fig. 6E and F). Same as E2F1, the expression of SAFA was up-regulated in 24 paired primary cancerous and adjacent noncancerous in which PANDA expression had been measured (Fig. 6G and 6 H). RIP was conducted to explore the interaction between PANDA and proteins. PANDA was preferentially enriched in SAFA relative to control immunoglobulin G (IgG) immunoprecipitates (Fig. 6I). This finding suggested that PANDA can SAFA and regulate their expression in ESCC.

To summarize, a PANDA shift model was proposed to describe the PANDA-SAFA-tumor proliferation pathway (Fig. 7). In ESCC cells, PANDA drifted away from NF-YA to promote the expression of NF-YA-E2F1 coregulated proliferation-promoting genes, and limits the cell apoptosis. In addition, PANDA binding SAFA to switch on the tumor proliferation programme though CyclinD1/2-Cyclin E1 and Bcl-2 pathways.

4. Discussion

The human genome contains a large number of lncRNAs that are dynamically expressed in a tissue-, differentiation-, cell type- or developmental stage-specific manner, indicating specific functions of lncRNAs in the development of diseases [25–27]. The cellular functions of most recently discovered lncRNAs then needed to be elucidated. For each individual molecule, it needs to be established whether it executes important functions or just represents “transcriptional noise” or background transcription. In fact, some lncRNAs show clear evolutionary conservation or strict regulation function, implying that they are of functional importance [28–31]. The aberrant expression of lncRNA has been found in a wide variety of tumors [32–36]. Nevertheless, our knowledge of the roles of lncRNAs might play in cancer is still very limited.

A recent study identified 216 putative lncRNAs derived from

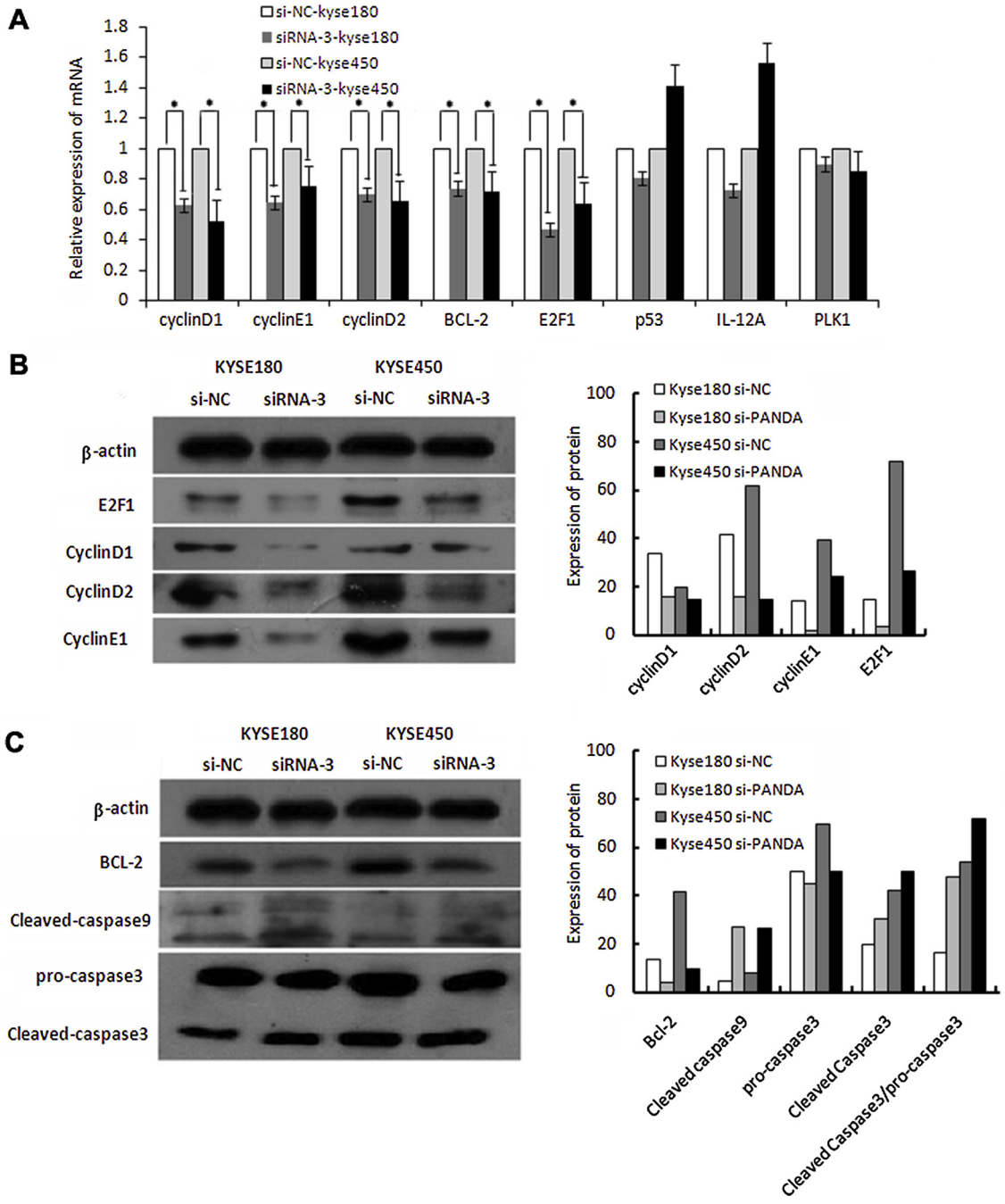

Fig. 5. The effect of PANDA on proliferation and apoptosis related genes(A)The fold changes in mRNA expression levels of P53, E2F1, cyclinD1, cyclinE1,clinD2, Bcl-2, PLK1 in KYSE180 and KYSE450 cell lines after PANDA knockdown by qRT-PCR. β-actin was used as the internal control. * $p < 0.05$. (B–C) The expression levels of cyclinD1, cyclinE1, cyclinD2, E2F1 (B) and BCL2, cleaved-caspase9, pro-caspase3 and cleaved-caspase3 proteins (C) were analyzed by western-blot analysis and β-actin was used as control. The imagery gray values indicated the protein levels (right panels).

promoter regions of cell cycle genes [14]. Many of these transcripts showed periodic expression during the cell cycle and altered expression in human cancers, and their expressions could be regulated by specific oncogenic stimuli or DNA damage. PANDA is one of the lncRNAs that transcript from promoter regions of cell cycle genes, which might be regulated by p53 [37]. However, previous studies have revealed that p53 mutation is the most common genetic alteration in ESCC and p53 function is typically lost early in esophageal squamous cell carcinogenesis [38–40]. P53 mutation accelerated the cell cycle and decreased the expression of p21Waf1/Cip1 [41,42], supporting that PANDA might be divorced from p53-dependent manner regulation in ESCC. Therefore, we speculated that PANDA may be independent of p53 in the regulation of downstream genes to promote cell growth and suppress apoptosis in ESCC progression. In this study, we found that PANDA was up-regulated in ESCC cancerous tissues when compared with corresponding noncancerous tissues. Furthermore, we also showed that the increased expression level of PANDA was correlated with lymph node metastasis and advanced pathologic stage of ESCC patients. In addition, higher PANDA expression was correlated with lower overall survival rates and could be an independent prognostic factor in patients with ESCC. These findings indicated that PANDA might play an important role in the development and progression of ESCC and it could be used to develop as a candidate prognostic biomarker for ESCC.

The balance between cell proliferation and apoptosis plays an

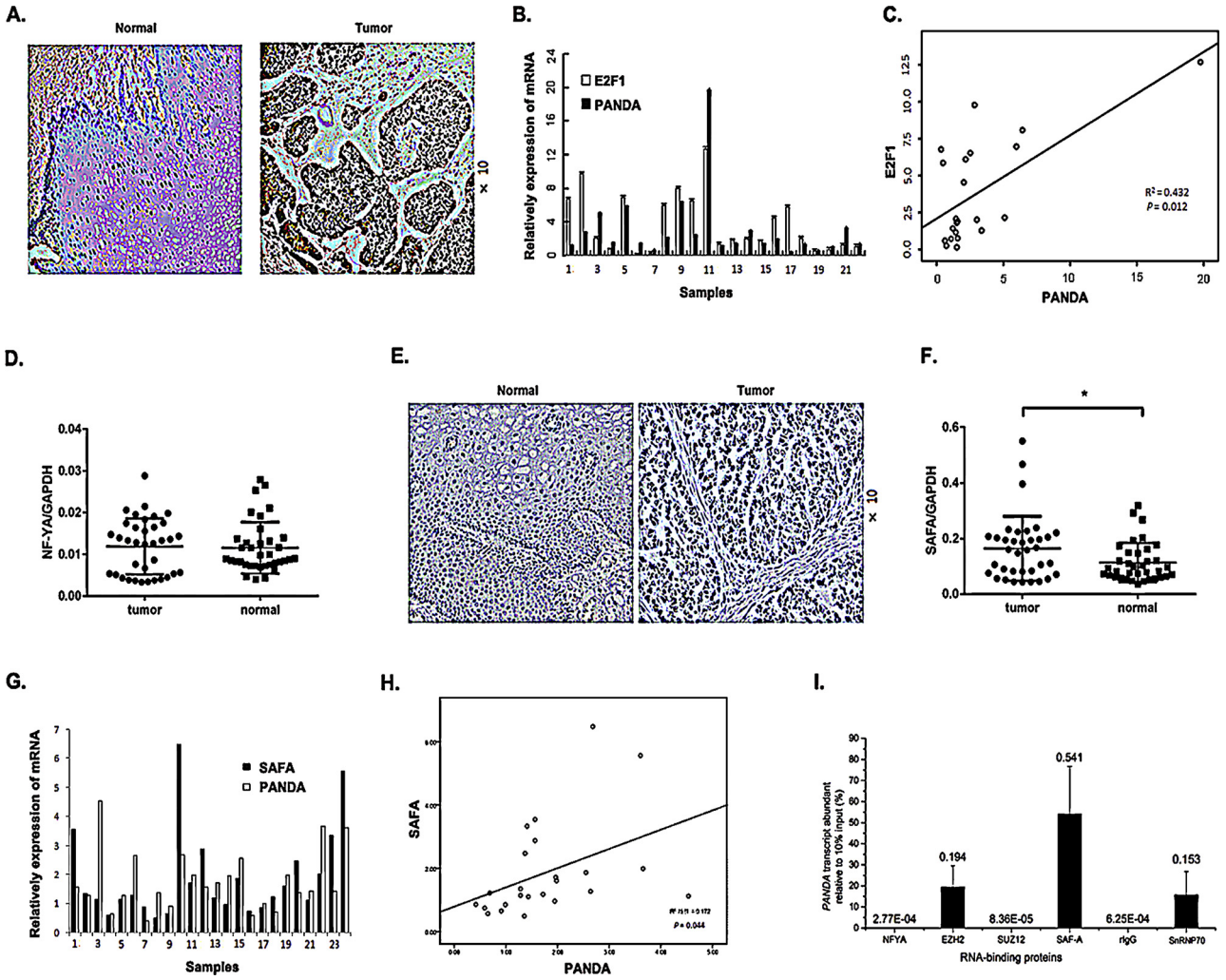

Fig. 6. PANDA regulated E2F1 to depress apoptosis in ESCC through dissociating from NF-YA while interacted with SAFA to promote ESCC cell proliferation (A) Immunohistochemistry assay showed that E2F1 elevated in cancerous esophageal tissues. Representative IHC image is shown. (B) Relative mRNA levels of E2F1 and PANDA in cancerous tissues of ESCC were analyzed by qPCR and normalized to β-actin. (C) The positive correlation between E2F1 and PANDA was examined in 22 cases of ESCC tissue samples ($p = 0.012$). *$p < 0.05$. (D) RT-qPCR was also performed to analyze the expression of NF-YA in 22 matched cancerous tissues and adjacent noncancerous tissues of ESCC and normalized to GAPDH. (E) Immunohistochemistry assay showed that SAFA elevated in cancerous esophageal tissues. Representative IHC image is shown. (F) RT-qPCR was performed to analyze the expression of SAFA in 22 matched cancerous tissues and adjacent noncancerous tissues of ESCC and normalized to GAPDH. *$p < 0.05$. (G) Relative mRNA levels of SAFA and PANDA in cancerous tissues of ESCC were analyzed by qPCR and normalized to β-actin. (H) The positive correlation between SAFA and PANDA was analyzed in 24 cases of ESCC tissue samples. ($p = 0.044$). *$p < 0.05$. (I) The relative RNA level was detected in the substrate of a RIP assay by qRT-PCR. Triplicate experiments were analyzed, and the mean ± SD is shown. P and r values were calculated using Spearman's correlation test.

important role in the control of tissue homeostasis. Increasing evidence had shown that both increased proliferation and decreased apoptosis were pivotal in the formation and progression of cancer [43]. Tumor cells proliferation promotes malignant phenotype and speeds up the tumor invasion and migration in a way. In this study, knockdown of PANDA expression significantly decreased proliferation and promoted apoptosis of ESCC cells by arresting G1-S checkpoint transition. Moreover, knockdown of PANDA could also inhibit tumor growth in vivo. To further assess the potential mechanism involved in PANDA mediated biological function in ESCC cells, we detected genes that contribute to G1-S checkpoint transition and cell apoptosis. Our study showed that knockdown of PANDA reduced E2F1 and Bcl-2 expressions, while increased cleaved Caspase3 and Caspase9 expressions in ESCC cells. We also found that changes of E2F1 downstream genes cyclinE1, cyclinD1 and cyclinD2 were consistent with E2F1. Moreover, a positive correlation between PANDA and E2F1 expression was noted in cancer tissues of ESCC.

E2F factors are potent regulators of cell-cycle checkpoints in mammalian cells, and ectopic expression of individual E2F family member is sufficient to modulate cell proliferation and apoptosis [44]. The transcription factor E2F-1 plays an important role in regulating cell proliferation, and its activity is tightly regulated in a cell-cycle-dependent manner to enable programs of gene expression. This period in the cell cycle, from late G1 to early S phase, defines a window of time during which E2F-1 can activate S-phase genes [45]. Recent studies have shown that E2F-1 expression increases at the transcriptional level in multiple cancers without localized gene amplification. Generally, E2F-1 is accumulated in the late G1 phase of the cell cycle, and rapidly degraded in S/G2 phase. Its expression is closely associated with tumor cell proliferation and might influence clinical outcome mainly via regulating cell cycle progression [46].

Our study demonstrated that aberrant over-expression of PANDA in ESCC tissues and consequent up-regulation of E2F1 could 'drive' tumorigenesis in progression of ESCC by promoting cell proliferation and suppressing cell apoptosis. Thus, we concluded that over-expression of PANDA might contribute to up-regulation of E2F1 which exerts its

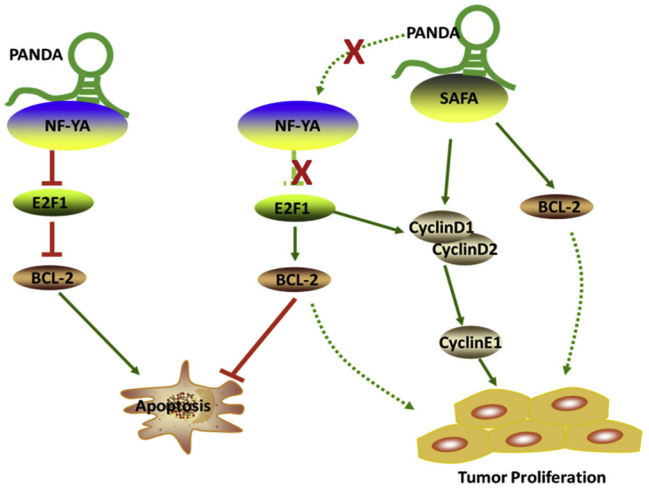

Fig. 7. The mechanism of PANDA in ESCC cells.

oncogene functions. Recent studies have clearly shown that a number of lncRNAs contribute to gene regulation by various mechanisms [10]. (1) lncRNAs 'guide' chromatin-modifying complexes to specific genomic loci and activate and repress specific genes in cis and in trans [47–51]. (2) lncRNAs serve as structural links in ribonucleoprotein complexes (RNPs), and disruption of such lncRNAs may lead to undesired biological consequences [48,52]. (3) LncRNAs regulate distinct transcriptional programs. The ability of an lncRNA to modulate the effects of a transcription factor can lead, in some cases, to significant changes in gene expression and subsequently profound effects on the cells ability to respond to external stimuli [53]. (4) Regulation of microRNAs by lncRNAs. Recently, evidence came to light suggesting that some mammalian lncRNAs may also regulate gene expression post-transcriptionally by binding to miRNAs, and consequently preventing specific miRNAs from binding to their target mRNAs [54].

Another major finding of our study is how PANDA regulates E2F1 in ESCC. As we known, DNA damage triggers p53-dependent G1 arrest, but not apoptosis in human fibroblasts [55,56]. PANDA inhibits the expression of apoptotic genes by sequestering NF-YA from occupying target gene promoters. Furthermore, PANDA promotes cell survival by impeding the apoptotic gene expression program [20]. It was consistent with our study that PANDA regulated E2F1 expression by competitive combination with NF-YA in ESCC cells. Accumulation of DNA damage could result in carcinogenesis. Here, we might assume that DNA damage induced the expression of PANDA in ESCC cells, which leaded cells to proliferation, but not apoptosis. Meanwhile, in proliferating cells PANDA and SAFA interact and recruit PRC2 and BMI1–PRC1 complexes to generate repressive histone marks H3K27me3 and H2AK119Ub1 and dampen transcription of prosenescence PRC target genes [24]. In ESCC, PANDA was up-regulated and interacted with SAFA to promote the tumor proliferation.

In summary, our study showed that PANDA was dramatically up-regulated in ESCC tissues and cell lines. The high expression of PANDA was significantly associated with poor pT stage, pN stage, and patients' survival time. Moreover, down-regulation of PANDA had the effect of suppressing ESCC cell proliferation both in vitro and in vivo. PANDA drifted away from NF-YA to promote the expression of NF-YA-E2F1 coregulated proliferation-promoting genes, and limits the cell apoptosis in ESCC. In addition, PANDA binding SAFA to switch on the tumor proliferation programme though CyclinD1/2-Cyclin E1 and Bcl-2 pathways. Further insights into the functional and clinical implications of PANDA and its target E2F1 might contribute to therapeutic targets for ESCC and further develop as potential prognostic factors. We speculated that PANDA signatures might provide a novel and promising alternative therapeutic approach to future cancer treatment with down-regulation of such oncogenic lncRNAs. Also, our findings indicated that PANDA possessed potential as a cancer biomarker.

5. Conclusions

In this study, we figured out the lncRNA PANDA who could promote the ESCC progression in vitro and in vivo. We reported that PANDA drifted away from NF-YA to promote the expression of NF-YA-E2F1 coregulated proliferation-promoting genes, and to limit the ESCC cell apoptosis. In addition, PANDA binding SAFA to switch on the tumor proliferation programme though CyclinD1/2-Cyclin E1 and Bcl-2 pathways in ESCC.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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Declaration of Competing Interest

The authors declare that they have no competing interests

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.prp.2019.152604.

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