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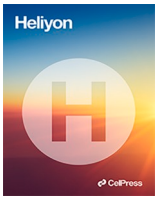

Research article

Pancreatic cancer cells hijack tumor suppressive microRNA-26a to promote radioresistance and potentiate tumor repopulation

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ABSTRACT

Pancreatic cancer is one of the most lethal cancers with significant radioresistance and tumor repopulation after radiotherapy. As a type of short non-coding RNA that regulate various biological and pathological processes, miRNAs might play vital role in radioresistance. We found by miRNA sequencing that microRNA-26a (miR-26a) was upregulated in pancreatic cancer cells after radiation, and returned to normal state after a certain time. miR-26a was defined as a tumor suppressive miRNA by conventional tumor biology experiments. However, transient upregulation of miR-26a after radiation significantly promoted radioresistance, while stable overexpression inhibited radioresistance, highlighting the importance of molecular dynamic changes after treatment. Mechanically, transient upregulation of miR-26a promoted cell cycle arrest and DNA damage repair to promote radioresistance. Further experiments confirmed HMGA2 as the direct functional target, which is an oncogene but enhances radiosensitivity. Moreover, PTGS2 was also the target of miR-26a, which might potentiate tumor repopulation via delaying the synthesis of PGE2. Overall, this study revealed that transient upregulation of miR-26a after radiation promoted radioresistance and potentiated tumor repopulation, highlighting the importance of dynamic changes of molecules upon radiotherapy.

Radiotherapy is one of the main modalities for cancer treatment. More than half of the cancer patients will receive radiotherapy at different stage of their disease for various purposes, from disease cure to palliative care [1]. In certain cancers such as early stage of non-small cell lung cancer, the effect of radiotherapy is almost as powerful as surgical removal of cancers [2]. However, several obstacles remain, such as radioresistance of cancer cells, which is a particularly important cause for radiotherapy failure [3]. Resistance to radiotherapy in gastrointestinal cancer occurs in 70–96 % of patients [4]. Resistance to radiation therapy has been found to be associated with changes in DNA repair, cellular energetics, signaling pathways, *etc.* [4]. Nevertheless, mechanisms of radioresistance are far from clear. Tumor repopulation after radiotherapy is considered to be the important cause of treatment failure [5,6]. We have

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previously revealed that irradiated dying tumor cells released PGE2 after a latent time, which was necessary for the survival of damaged tumor repopulating cells [7]. However, the reason why the upregulation of COX-2, the key enzyme controlling PGE2 synthesis, was delayed after radiation remained to be revealed.

Pancreatic cancer is one of the most malignant cancers [8]. Despite decades of efforts, the treatment of pancreatic cancer has not achieved much progress, with the five-year survival rate of just reached 12 % [9]. Surgery is currently the only cure strategy for pancreatic cancer, but the majority of patients lost the opportunity to receive surgery due to locally unresectable or metastasis when diagnosed. Moreover, radiotherapy, which should be the standard-of-care for localized disease, showed little effect in pancreatic cancer due to the significantly radioresistance [10]. Therefore, to investigate radioresistance and tumor repopulation of pancreatic cancer is of great significance.

microRNAs (miRNAs), a kind of small non-coding RNAs that may simultaneously regulate multiple targets, have been found to modulate a variety of biological and pathological processes [11]. It has been reported that miRNAs promote or inhibit radioresistance by repressing the translation of targeted mRNAs, regulating DNA damage response [12], cell cycle [13], cell stemness [14], *etc*. Tumor repopulation of pancreatic cancer was also regulated by miRNAs [7,15]. Generally, the biological effects of altered miRNAs are mostly studied by lentivirus-mediated stable overexpression/inhibition of miRNAs. However, changes of miRNAs and signaling upon radiation are rapid and dynamic, and stable overexpression/inhibition of miRNAs might leave out or misinterpret some important information of the changed molecules.

In this study, we aimed to investigate the effect and underlying mechanisms of miRNA on radioresistance of pancreatic cancer. We would first find the target miRNA and then investigate its dynamic changes, function and mechanisms. Surprisingly, we revealed that miR-26a showed dynamic changes upon radiation in pancreatic cancer cells. Further mechanistic investigations revealed that pancreatic cancer cells could hijack tumor suppressive microRNA-26a to promote radioresistance and potentiate tumor repopulation. These results uncovered the "double-edged sword" role of miR-26a on pancreatic cancer and might innovate more meaningful research.

1. Materials and methods

1.1. Cancer cell lines and cell culture

Human pancreatic cancer cell line PANC-1 (ATCC® CRL-1469), MiaPaCa-2(ATCC® CRL-1420), BxPC3(ATCC® CRL-1687) and SW1990 (ATCC® CRL-2172) was purchased from Cell Bank of Chinese Academy of Sciences. Cells were cultured in DMEM (PANC-1 and MiaPaCa-2) or RPMI 1640 (SW1990 and BxPC3) with 10 % FBS (Gibco) and 100 U/ml Penicillin and 100 μg/ml Streptomycin, and incubated in a humidified incubator containing 5 % CO_2 at 37 °C. All the cell lines were identified by STR test and routinely tested for mycoplasma-free.

1.2. Plasmid construction

The methods used for plasmid constructed were as previous reported [7,16]. In brief, gene of MIR26A-1 was amplified from genomic DNA of 293T cells using the primer F: 5′-GACGGATCCTGTACCACGTGACTGTAAGC-3′, and R: 5′-CTGC TAGCCACAAGAC TCCTCGTTGCCA-3′ to construct pLex-MIR26A; The primer F: 5′-CTGGCTAGCTCCTTTGTACCACGTGACTGT-3′, and R: 5′-ACG-GATCCCAC AAGACTCCTCGTTGCCA-3′ to construct pCW-MIR26A. The plasmids used for dual luciferase reporter assay was constructed as previously described [7], and the sequence used was showed in Supplementary Fig. S1. The miR-26a inhibitor sequence was 5′-GAGTCGACGGCGCTAGGATCATCAACTTCAAGTAATCATCT CAGGATAGGCTCAAGTATTCTGGTCACAGAATACAACTTCAAGTAAT CATCTCAGGATAGGCTCAAGATGATCCTAGCGCCACCTTTTTTGCTAGCTAG-3′ and cloned into pLV-U6 plasmid using *SalI*/*Nhe*I double enzyme digestion as previously described [15,17]. Lentivirus production and infection was also performed as described [7].

1.3. RNA extraction and quantification

Total RNAs were extracted using RNAiso Plus (Takara, 9108Q). Reverse transcription of RNAs was performed with PrimeScript™ RT reagent Kit (Takara, RR037A). Random primers and oligo dTs were used for reverse transcribing the mRNA; Specific RT primers were used for miR-26a (5′-GTCGTATCCAGTGCAGG GTCCGAGGTATTCGCACTGGATACGACAGCCTA-3′) and internal control U6 (5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAAAAATAT-3′). Quantitative PCR was performed with TB Green™ Premix Ex Taq™ (Takara, RR820Q) in QuantStudio 6 Flex (Applied Biosystems) follow the manufacturer's instructions. The sequence of qPCR primers was as follows, miR-26a forward primer: 5′-CGGCAGGTTCAAGTAATCCAGGA-3′, miR-26a reverse primer: 5′-ATCCAG TGCAGGGTCCGAGG-3′, U6 forward primer: 5′-CAAGGATGACACGCAAA-3′, U6 reverse primer: 5′-TCAACTGGTGTCGTGG-3'. The primers were synthesized by Sangon Biotech (Shanghai, China). The relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ algorithm.

1.4. RNA sequencing

Total RNA used for sequencing was extracted by TRI Reagent® (Sigma, T9424). RNA-Seq was performed by Sangon Biotech (Shanghai, China). A NanoPhotometer® spectrophotometer (IMPLEN) and an Agilent 2100 Bioanalyzer (Agilent Technologies) were used to assess the quality and quantity of cellular RNAs. Total RNAs were used to prepare the sequencing libraries, and ~150 bp PCR amplicons (corresponding to ~22 nt miRNAs) were selected. The libraries were then applied for RNA sequencing with Illumina HiSeq sequencer (Illumina). All the original sequencing data were submitted to SRA database (Accession No PRJNA748461).

1.5. Western blot

Proteins were collected by RIPA lysis buffer (plus the complete protease inhibitor cocktail). The protein concentration was determined using BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai). Proteins were then separated by regular SDS-PAGE electrophoresis and transferred to nitrocellulose membrane. 5 % milk in TBS was used to block the membrane and then the primary antibodies were used, including anti-GAPDH (CST, 2118), anti-Phospho-Histone H2AX (Ser 139) (CST, 2577), anti-HMGA2 (CST, 8179), anti-COX-2 (CST, 12282), anti-PCNA (CST, 13110), anti-phospho-Histone H3 (CST, 53348). All antibodies were used as recommended by the manufacturer. After washing three times with TBST, the secondary antibodies, IRDye® 800CW Goat anti-Rabbit IgG and IRDye® 680RD Goat anti-Mouse IgG (LI-CDR), were incubated. The membranes were then imaged by Odyssey® Imaging Systems (LI-CDR).

1.6. Dual luciferase reporter assay

To verify the target site of miR-26a, the Dual-Luciferase® Reporter (DLR™) Assay System (Promega) was performed [18]. PANC-1 cells were co-transfected with pmirGLO plasmids containing miR-26a target sites or 3′UTR of HMGA2 or PTGS2, and miR-26A mimics or mimic NC. Cells were collected 48 h later and subjected to analyze the dual luciferase activities according to the manufacturer's protocol, and measured by Varioskan Flash (Thermo Scientific).

1.7. Cell cycle analysis

Routine flow cytometric analysis was performed to detect cell cycle. Briefly, cells were collected by trypsinization and fixed by cold 75 % ethanol overnight, then subjected to cell staining with BD Pharmingen™ PI/RNase Staining Buffer (BD Bioscience) [19] following the manufacturer's protocol. The stained cells were further filtered with 70 μm nylon cell strainer (Falcon) and subjected to examine cell cycle by using BD LSRFortessa™ Cell Analyzer. The ModFit LT 5.0 software was used to analyze the distribution of cell cycles.

1.8. Real-time cell analysis assay (RTCA)

RTCA was performed by using xCELLigence RTCA DPlus (ACEA Bioscience) according to the manufacturer's instructions [20]. Briefly, cells were plated into E-Plate 16 (ACEA Bioscience) at 1500 cells/well, and kept at room temperature for 30 min for cell seeding. Cell proliferation was detected immediately after the plates were put into the machine. Data were collected every 15 min.

1.9. Apoptosis assay

Cell apoptosis was detected by the PE Annexin V Apoptosis Detection Kit I (BD Bioscience) according to the manufacturer's instructions. In brief, cells were collected by trypsinization and washed twice with PBS, then suspended in the 1 × binding buffer at the concentration of 1×10^7/ml. 100 μl suspended cells was stained with 5 μl PE-Annexin V and/or 7-AAD for 15 min at room temperature. 300 μl binding buffer was then added, and the sample was analyzed using BD LSRFortessa™ Cell Analyzer. The data was analyzed with FlowJo V10.

1.10. Colony formation assay

Cells were seeded into 6-well plate at a density of 1×10^5 cells/well or 12-well plate at 4×10^4 in triplicate. After 6 h incubation, cells were treated with Doxycycline (1 μg/ml, MedChemExpress), or transfected with miR-26a inhibitor (100 nM, RiboBio) or corresponding controls, and further exposed to 10Gy irradiation. Cell culture media were changed once every 3–4 days. After 12–14 days, the colonies were fixed using paraformaldehyde and stained by crystal violet. Colonies were scanned and counted by using Image J software.

1.11. Transwell assay

20,000 cells were suspended in 200 μl fresh media without FBS, and then were added to the hanging cell culture inserts (PIEP12R48, Millipore), and 900 μl fresh media containing 10 % FBS were added to the lower chamber. After 24 h culture, cells in the hanging cells were fixed with 4 % paraformaldehyde, and stained with crystal violet. Cells in the inner side of the inserts were removed with cotton swabs. Images were taken by routine microscopy (Leica).

1.12. Survival analysis

We obtained 172 tumor samples of TCGA Pancreatic Cancer (PAAD) along with corresponding clinical data from the UCSC Xena database (https://xenabrowser.net/datapages/) [21]. Subsequently, we categorized the patients into high and low MIR26A expression groups based on the cutoff value of MIR26A expression. We then examined the survival outcomes of these two groups by generating Kaplan-Meier (KM) curves using the "survival" package in R version 4.2.1 software.

2. Statistics

All data were analyzed with software GraphPad Prism 7. Normally distributed data were presented as mean with SD. Differences between means were assessed using unpaired student's *t*-test. $p < 0.05$ was considered statistically significant.

3. Results

1 miR-26a was transiently upregulated in irradiated pancreatic cancer cells

To investigate the changed miRNAs in pancreatic cancer cells upon radiation, we performed miRNA sequencing on irradiated or unirradiated pancreatic cancer cells. The results showed that 21 miRNAs were significantly upregulated, while 12 were downregulated (Fig. 1A). We noticed that both miR-26a-5p and miR-26a-1-3p were upregulated after radiation, indicating that miR-26a was specifically transcriptionally upregulated. Previous massively parallel sequencing also identified that miR-26a exhibited induction peaks at 8 h and 24 h post-irradiation [22]. Moreover, earlier meta-analysis revealed that miR-26a was one of the most frequently dysregulated miRNAs in response to hypoxic stress in eukaryotic cells [23].

We further performed qPCR assay to figure out the changes of miR-26a in several pancreatic cancer cells. The results revealed that miR-26a was upregulated in pancreatic cancer cells at 2–4 days post-irradiation, but soon returned to almost pristine levels after 8 days of irradiation (Fig. 1B). Similar dynamic changes were also examined in irradiated PDX tissues that we previously established [7]. It was found that miR-26a was upregulated in PDX tissues after 4 days of irradiation, then began to fall back, and returned to the original level at 28 days after irradiation (Fig. 1C). Overall, these results indicated that miR-26a was transiently upregulated after radiation in pancreatic cancer.

2 miR-26a suppressed the malignant behavior of pancreatic cancer cells

We further investigated the biological role of miR-26a on pancreatic cancer. Pancreatic cancer cells with doxycycline-induced miR-26a expression were constructed (Supplementary Fig. S1A). After doxycycline treatment, the expression of miR-26a in pancreatic cells was significantly upregulated (Supplementary Fig. S1B). MiR-26a upregulation significantly induced G1/S cell cycle arrest (Fig. 2A). And RTCA (Real-time cell analysis) analysis revealed that miR-26a upregulation significantly inhibited pancreatic cancer cell proliferation (Fig. 2B). Then pancreatic cancer cells with miR-26a stable overexpression were also established (Supplementary Fig. S2C).

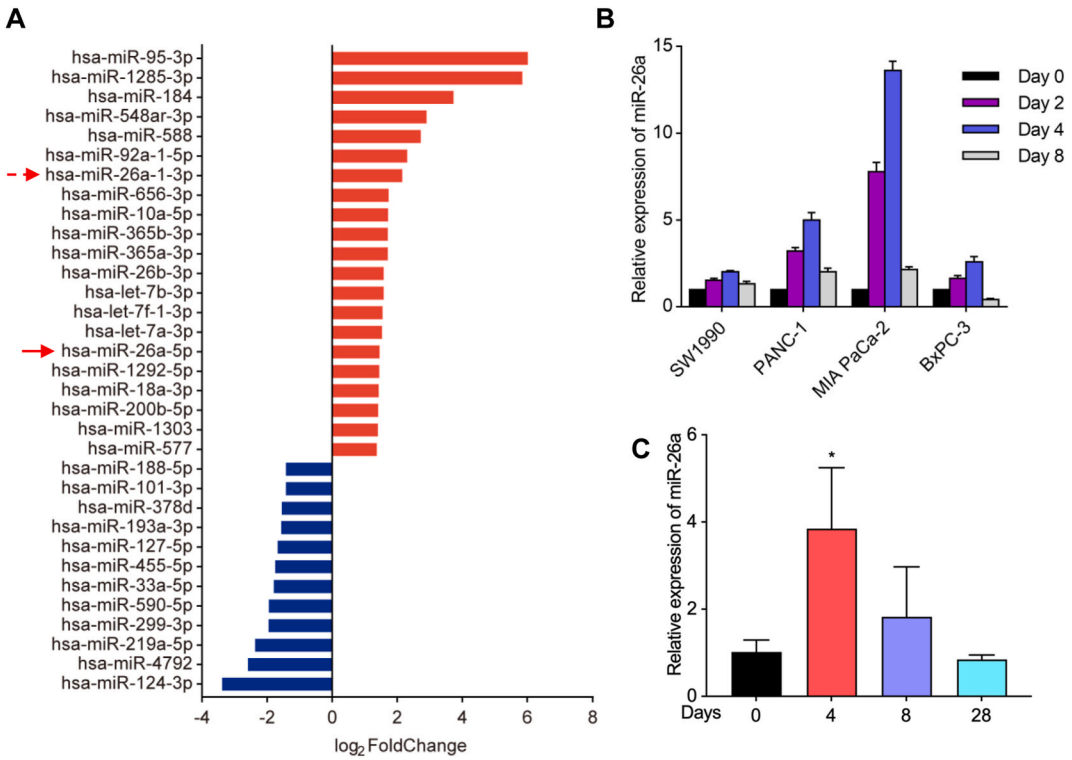

Fig. 1. miR-26a was transiently upregulated in irradiated cancer cells. (A) Statistically significantly changed miRNAs after radiation in pancreatic cancer cells. (B) qPCR detection of miR-26a in pancreatic cancer cells at indicated time points after radiation. (C) qPCR detection of miR-26a in pancreatic cancer PDX tissues at indicated time points after radiation. *p < 0.05 from unpaired Student's *t*-test.

Remarkably, the colony formation ability of pancreatic cancer cells was inhibited by miR-26a overexpression (Fig. 2C). Transwell assay revealed the migration ability of pancreatic cancer cell was reduced after miR-26a overexpression (Fig. 2D).

We further analyzed TCGA database to figure out the relationship between miR-26a expression and pancreatic cancer survival. It was found that higher miR-26a expression was associated with better prognosis in pancreatic cancer patients (Fig. 2E). These data indicated that miR-26a was a tumor suppressive miRNA that inhibited the malignant behavior of pancreatic cancer cells.

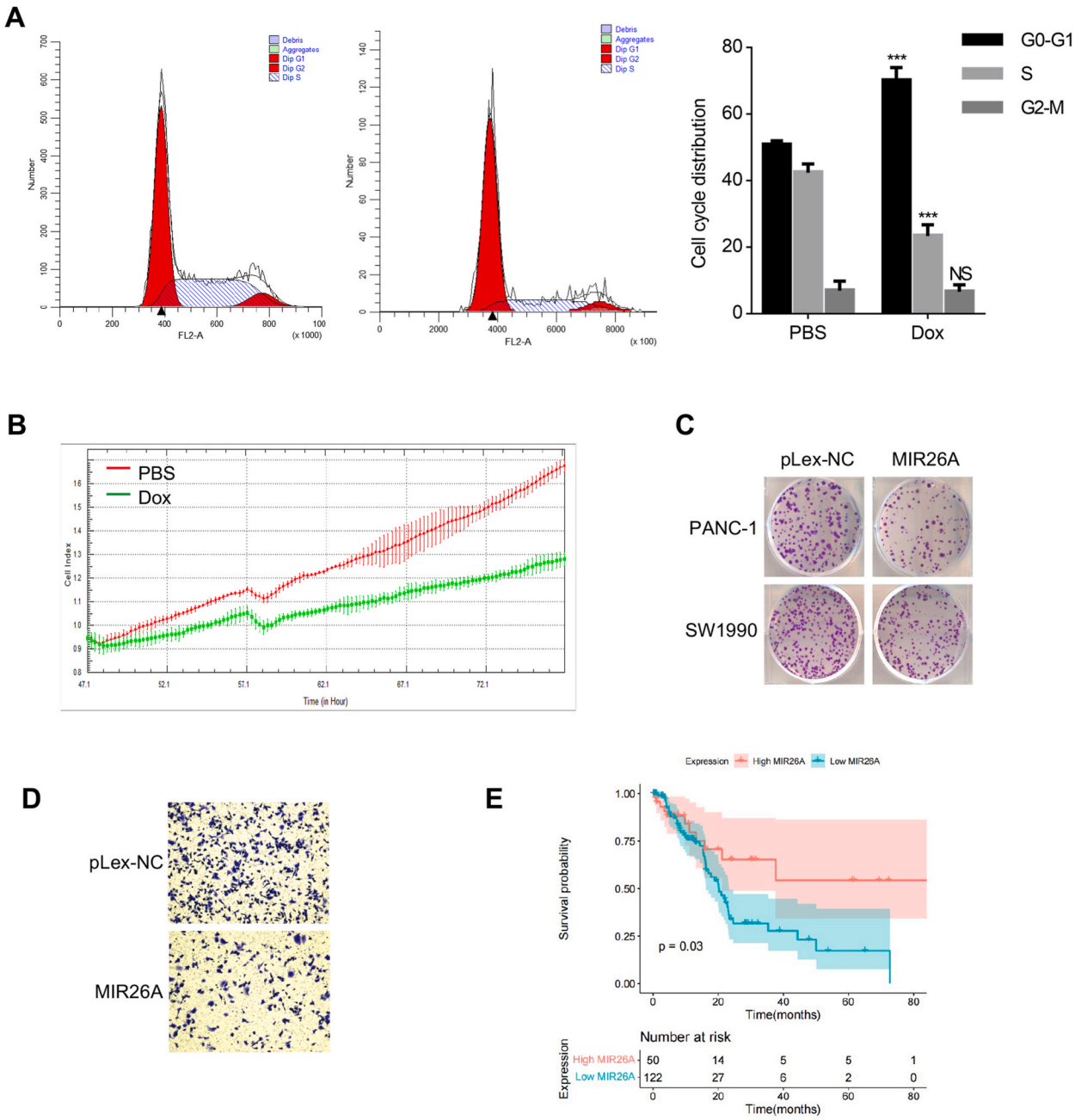

Fig. 2. miR-26a suppressed the malignant behavior of pancreatic cancer cells. (A) Cell cycle distribution of SW1990 tet-on cells that were treated with PBS or Doxycycline (Dox) for 48 h. (B) RTCA analysis of SW1990 tet-on cells that were treated with PBS or Doxycycline. (C) Colonies of SW1990 and PANC-1 cells that were stably overexpression of miR-26a or control sequence. (D) Transwell assay of PANC-1 cells that were stably overexpression of miR-26a or control sequence. (E) Survival analysis of TCGA pancreatic cancer patients with differed miR-26a expression level.

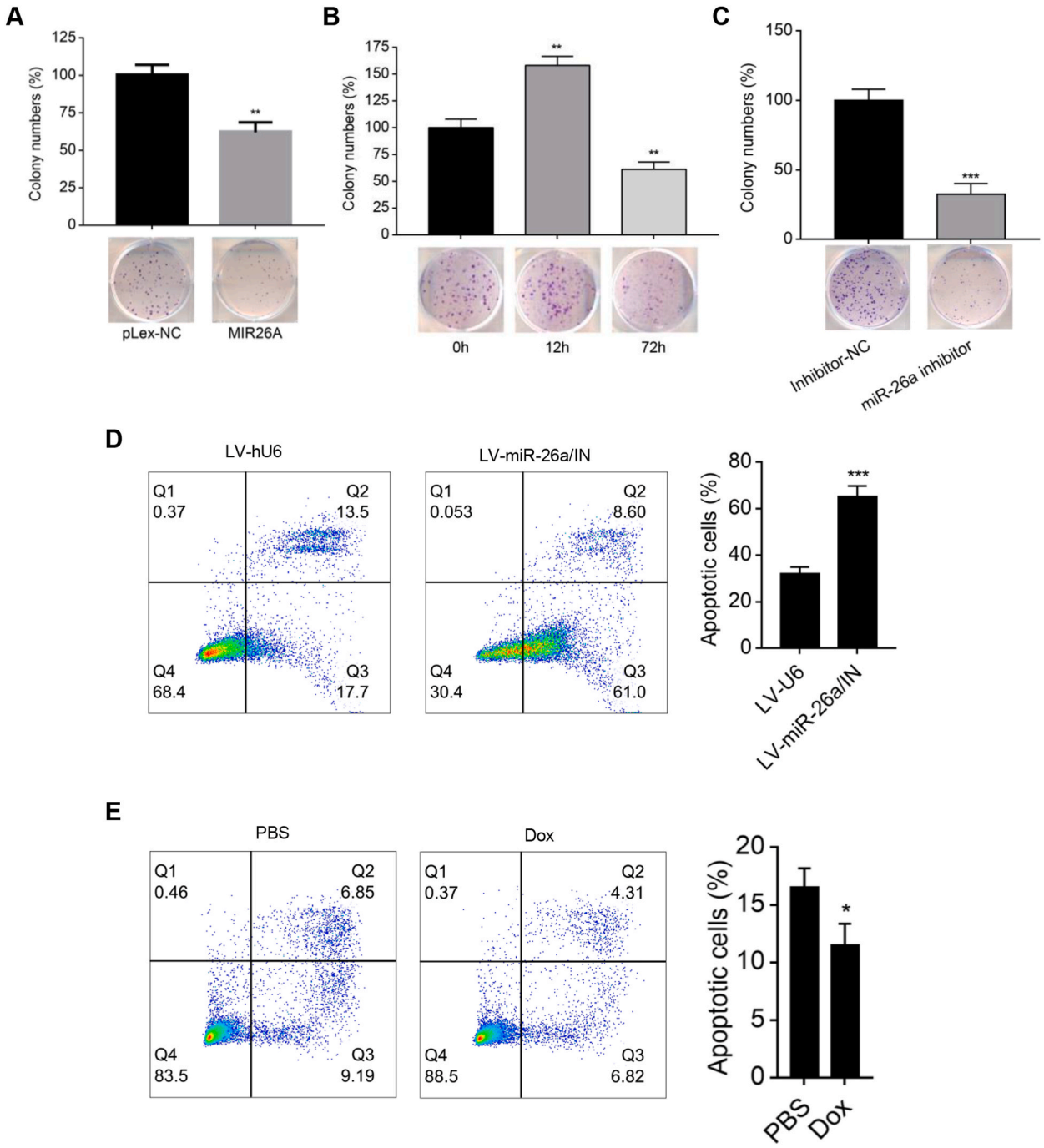

Fig. 3. Stably miR-26a overexpression inhibits while transiently overexpression promotes radioresistance of pancreatic cancer cells. (A) Colony formation assay of SW1990 cells that were stably overexpression of miR-26a or control sequence. (B) Colony formation of SW1990 tet-on cells after radiation. Cells were treated with doxycycline for indicated times. (C) Colony formation of miR-26a inhibitor or NC transfected SW1990 cells after radiation. (D) Cell apoptosis analysis of SW1990 cells that were stably overexpression of miR-26a inhibitor sequence or NC and subjected to radiation. (E) Cell apoptosis analysis of and SW1990 tet-on cells after radiation. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, determined using unpaired student's *t*-test.

3 Stable overexpression of miR-26a inhibited, while transient overexpression promoted, radioresistance of pancreatic cancer cells

To further illustrate the role of miR-26a upregulation after radiation, we performed plate colony formation assay of irradiated pancreatic cancer cells. Stable overexpression of miR-26a significantly inhibited colony formation after radiation (Fig. 3A). Surprisingly, however, short-term overexpression of miR-26a after 12 h of doxycycline treatment, which would induce miR-26a overexpression for less than 72 h (Supplementary Fig. S2A), instead promoted colony formation after radiation (Fig. 3B). When doxycycline treatment was prolonged to 72 h, the ability of colony formation of irradiated pancreatic cancer cells was significantly reduced (Fig. 3B). Meanwhile, treatment of doxycycline in SW1990 cells without tet-on inducible expression system did not influence their ability of colony formation (Supplementary Fig. S2B). These data indicated that transiently miR-26a upregulation upon radiation might promote radioresistance in pancreatic cancer cells.

Then we treated pancreatic cancer cells with miR-26a inhibitor, and found that miR-26a inhibitor dramatically promoted radiosensitivity of pancreatic cancer cells (Fig. 3C), indicating that miR-26a upregulation upon radiation was necessary for radioresistance.

We further performed apoptosis assay to evaluate the effect of miR-26a on irradiated pancreatic cancer cells. MiR-26a inhibition significantly promoted cell death after radiation (Fig. 3D), while short-term miR-26a overexpression inhibited apoptosis after radiation (Fig. 3E, Supplementary Fig. S3). These data validated that miR-26a transient upregulation upon radiation promoted pancreatic cancer cell survival.

4 miR-26a promoted DNA damage response

To further figure out the mechanisms underlying the biological function of miR-26a, we performed GSEA analysis from two datasets from GEO database [24,25]. It was revealed that miR-26a overexpression in intestinal epithelial cells (Fig. 4A) or breast cancer cells (Supplementary Fig. S4) significantly enriched signaling related to cell cycle and DNA repair, indicating that miR-26a might involve in DNA damage response, which is characterized as cell cycle arrest and DNA damage repair.

We further carried out Western blot analysis to detect DNA damage response, and found that doxycycline treatment in tet-on inducible pancreatic cancer cells downregulated phosphor-Histone H3, PCNA and γ-H2AX (Fig. 4B), demonstrating that miR-26a inhibited cell proliferation and DNA damage.

We then transfected pancreatic cancer cells with miR-26a mimics, and found that the DNA repair process was significantly promoted as evidenced by faster downregulation of γ-H2AX (Fig. 4C). These data indicated that miR-26a enhanced DNA damage response, including inducing cell cycle arrest and promoting DNA damage repair.

5 HMGA2 was the direct target of miR-26a and mediated the biological effect of miR-26a

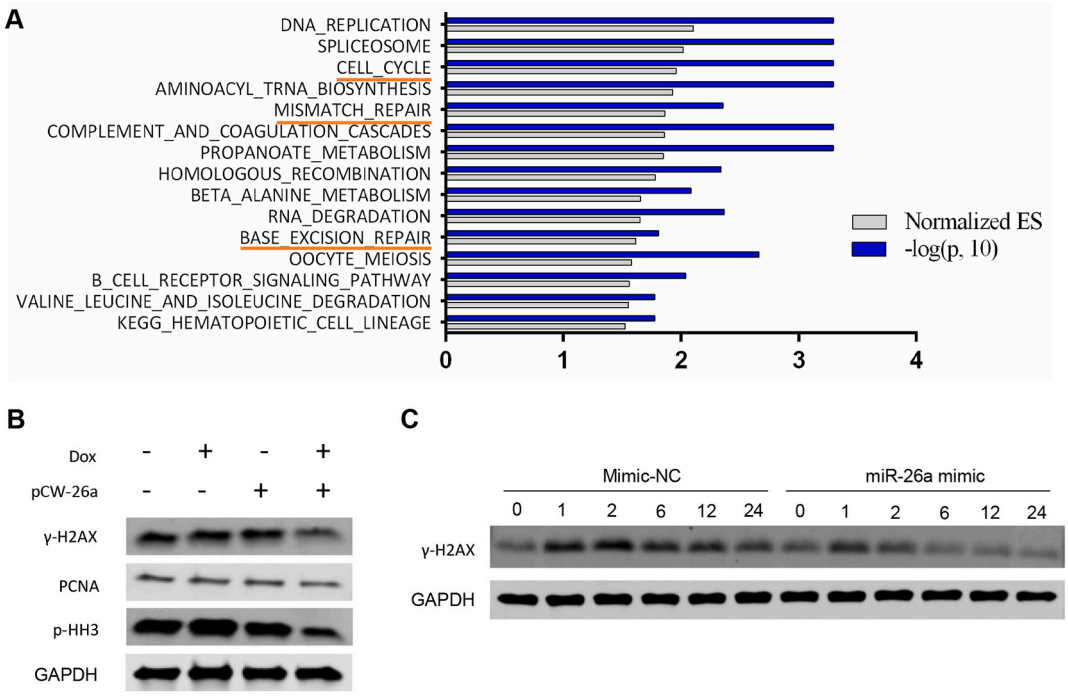

Fig. 4. miR-26a promoted DNA damage response. (A–B) GSEA analysis reveals enriched signaling in intestinal epithelial cells after miR-26a overexpression. (B) Expression of indicated markers with/without miR-26a conditional overexpression (Original gel image; Fig. 4B). (C) Relative expression of γH2AX in PANC-1 cells with transient miR-26a overexpression (Original gel image; Fig. 4C).

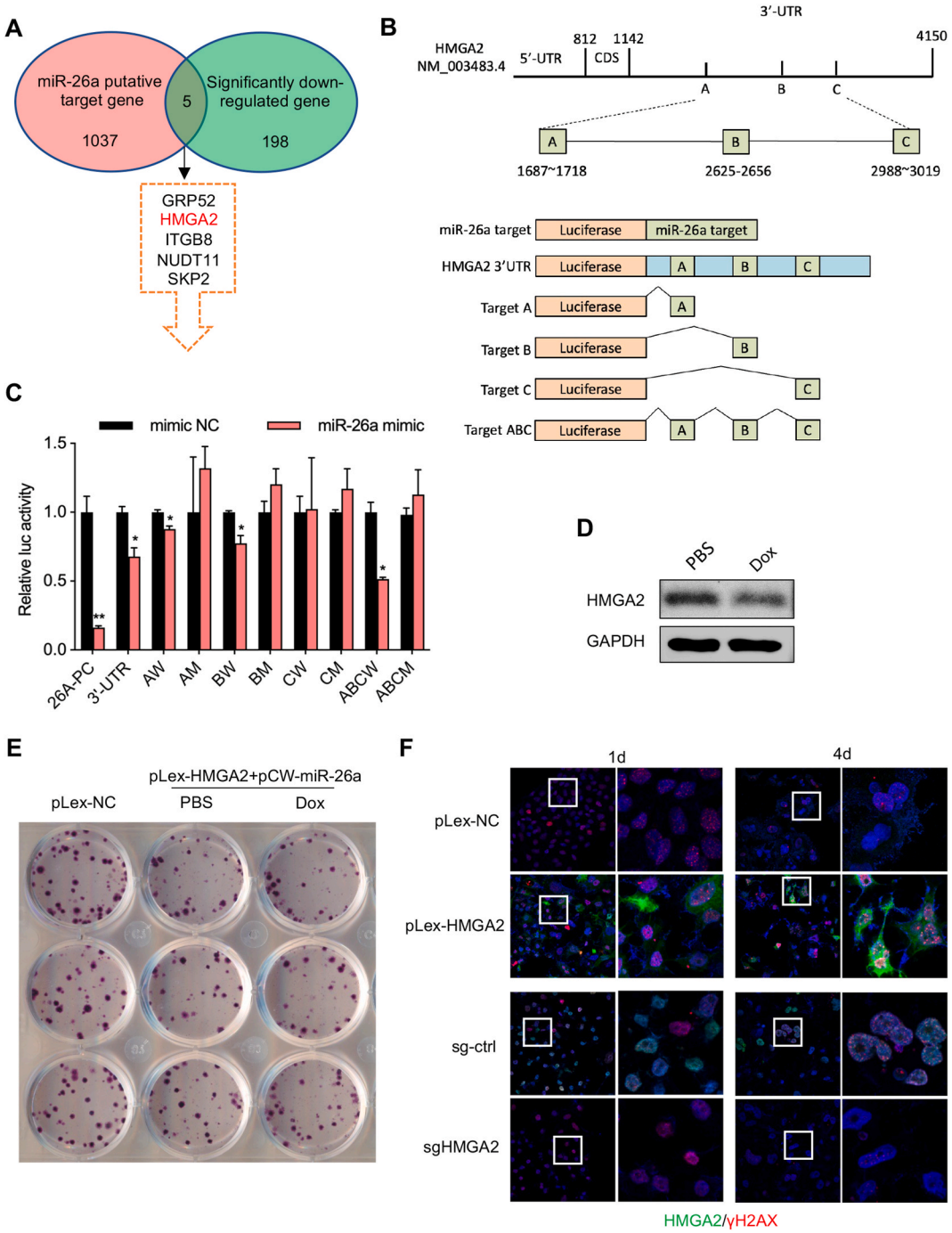

Fig. 5. HMGA2 was the direct target of miR-26a and mediate the effect of miR-26a. (A) Venn diagram shows the overlap of miR-26a predicted targets and down-regulated mRNAs after radiation. (B) Schematic diagram of the construct to detect the functional binding site of miR-26a in HMGA2. (C) Relative luciferase activity of the dual-luciferase reporter assay. 26A-PC: positive control which contain the complementary sequence of miR-26a; 3′-UTR: 3′ untranslated region of HMGA2; AW: target A wild type sequence; AM: target A mutant sequence; BW: target B wild type sequence; BM: target B mutant sequence; CW: target C wild type sequence; CM: target C mutant sequence; ABCW: tandem wild type sequence of target A, B and C. ABCM: tandem mutant sequence of target A, B and C. (D) Expression of HMGA2 in PANC-1 cells with/without miR-26a over-expression by doxycycline induction in tet-on cells (Original gel image; Fig. 5D). (E) Colony formation of SW1990 cells with/without HMGA2 knock-out and miR-26a overexpression. (F) Fluorescent image showing HMGA2 and γH2AX in SW1990 cells with HMGA2 overexpression or PANC-1 cells with HMGA2 knock-out. *p < 0.05, **p < 0.01, determined using unpaired student's *t*-test.

We further explored the direct target of miR-26a. 1042 miR-26a putative target genes were predicted with the online tool TargetScan, while our previous results showed that 203 mRNAs were statistically significantly downregulated in pancreatic cancer cells after radiation [15], which jointly predicted 5 downregulated putative targets of miR-26a (Fig. 5A). Coincidentally, HMGA2, which we and others have previously revealed to promote cell proliferation but inhibit DNA damage repair [7,26,27], happened to be the predicted target of miR-26a. Hence, based on dual-luciferase reporter assay, we proved that HMGA2 was the direct target of miR-26a, with site A and site B as the direct binding sites (Fig. 5B & C, Supplementary Fig. S5A).

Overexpressing miR-26a by doxycycline in tet-on pancreatic cancer cells or transfecting miR-26a mimics into pancreatic cancer cells suppressed HMGA2 expression (Fig. 5D). Moreover, consistent with our previous results [7], HMGA2 overexpression inhibited colony formation of irradiated pancreatic cancer cells (Fig. 5E). More importantly, HMGA2 overexpression inhibited DNA damage repair, while knock-out of HMGA2 enhanced it (Fig. 5F), which was consistent with the role of miR-26a. In addition, as the overexpressed HMGA2 by lentivirus did not contain its wild-type 3′-UTR and thus miR-26a could not inhibit its expression, miR-26a overexpression under the context of HMGA2 overexpression did not enhance radioresistance (Fig. 5E).

6 miR-26a inhibited PTGS2 and might contribute to potentiating tumor repopulation

We have previously demonstrated that the delay of PGE2 secretion was vital for the survival and repopulation of tumor repopulating cells, and that the key enzyme cyclooxygenase 2 (COX-2, encoded by prostaglandin-endoperoxide synthase 2 (PTGS2) gene) was also upregulated after a certain delay [7]. The consistency of expression between COX-2 and miR-26a made us wonder whether there existed some relationships or not. Thereupon, we applied TargetScan algorithm and found that miR-26a was the strong candidate to suppress PTGS2 (Fig. 6A). The dual-luciferase reporter assay also revealed that miR-26a directly inhibited the expression of PTGS2 via binding to the 3' UTR of PTGS2 mRNA at the predicted binding site (Fig. 6B, Supplementary Fig. S5B). Moreover, overexpression of miR-26a was found to suppress the expression of COX-2, while inhibition of miR-26a enhanced the expression of COX-2 (Fig. 6C & D). These data indicated that the upregulation of miR-26a after radiation could suppress the expression of COX-2, and may thus potentiate the survival and repopulation of pancreatic cancer cells.

4. Discussion

Pancreatic cancer is one of the most lethal cancers with significant therapy resistance, especially radiotherapy [10]. We found that pancreatic cancer cells upon radiotherapy transiently upregulated tumor suppressive miR-26a to promote their radioresistance partly via targeting HMGA2, and the upregulated of miR-26a might further potentiate tumor repopulation via suppressing PTGS2 (Fig. 7).

As a type of short non-coding RNA with ~22 nt, miRNA mainly exert its biological function through binding to the 3′-UTR of the targeted mRNAs and thus inhibiting their translation or inducing their degradation [28]. A miRNA might exert its function by simultaneously regulating several targets [29], or several miRNAs could synergistically inhibiting one target to generate enhanced bioeffects [30]. In this study, we demonstrated that miR-26a exerted its function partly by suppressing HMGA2 via binding to several binding sites of the 3′UTR, and might promote tumor repopulating through suppressing PTGS2. Nevertheless, miR-26a might further regulate other targets and influence other biological processes, which may need further investigations.

Many studies revealed that miR-26a enhanced therapy response. In breast cancer, miR-26a promoted the sensitivity of cancer cells to cisplatin chemotherapy by targeting FEN1 [31]. miR-26a was also found to promote chemosensitivity of hepatocellular carcinoma

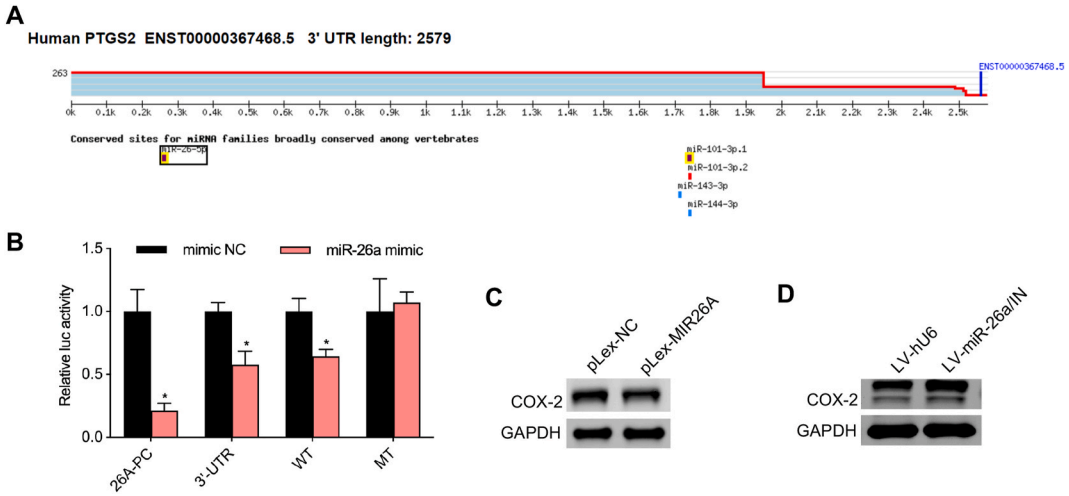

Fig. 6. PTGS2 was the direct target of miR-26a. (A) predicted target miRNAs of the 3′UTR of PTGS2. (B) Relative luciferase activity of the dual-luciferase reporter assay. (C–D) Expression of PTGS2 after miR-26a overexpression (C) or inhibition (D) (Original gel image; Fig. 6C and D). *$p < 0.05$ from unpaired Student's *t*-test.

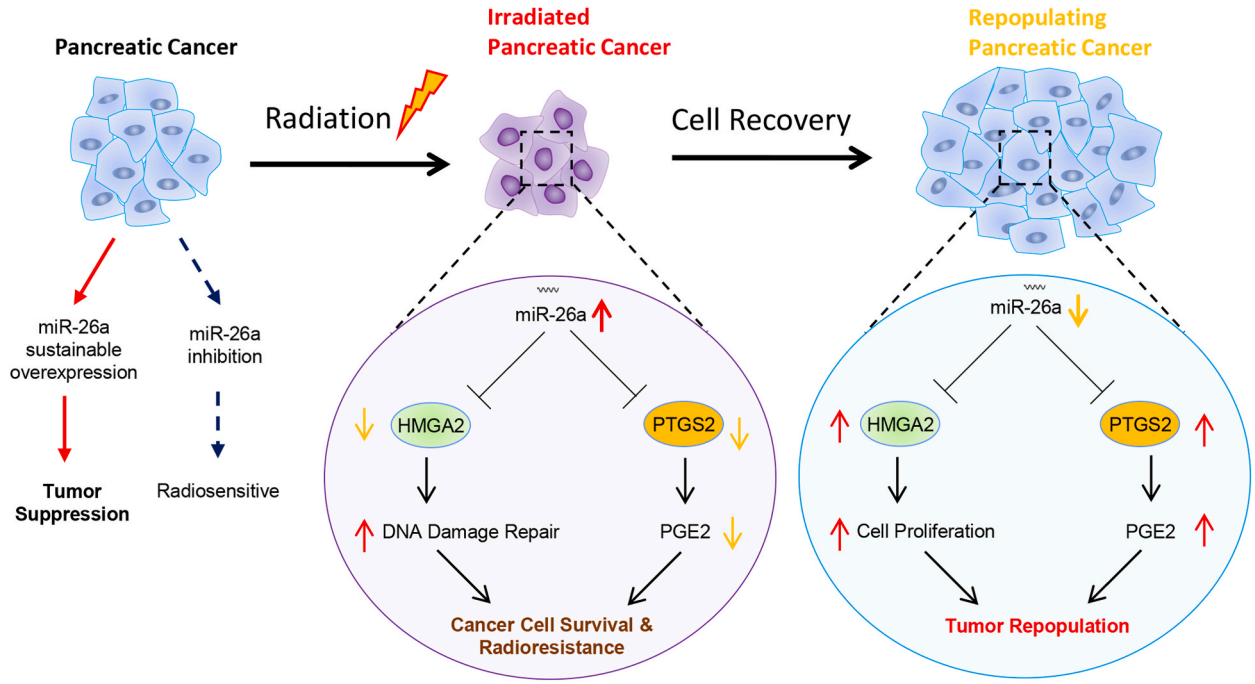

Fig. 7. Schematic diagram shows that pancreatic cancer cells hijack miR-26a to promote radioresistance and tumor repopulation. Irradiated pancreatic cancer cells transiently upregulate the expression of miR-26a, which inhibited the expression of HMGA2 and PTGS2 to promote cell DNA damage response and cell survival and radioresistance. After cell recovery, miR-26a was back to low-expression level, the expression of HMGA2 and PTGS2 fast recovered, which promote cell proliferation and production of PGE2, eventually lead to fast tumor repopulation. Sustained overexpression or inhibition of miR-26a both promoted radiosensitivity via different mechanisms.

cells via inhibiting autophagy by targeting ULK1 [32]. Stable overexpression of miR-26a enhanced radiosensitivity of glioblastoma multiforme cells via targeting ATM [33]. Herein, we also found that stable overexpression of miR-26a significantly promoted radiosensitivity in pancreatic cancer. However, we were surprised to note that miR-26a transient overexpression upon irradiation in pancreatic cancer promoted radioresistance. These results remind us that the

miR-26a has been regarded as a tumor suppressive miRNA. miR-26a expression was associated with liver cancer survival and therapy response to interferon alfa [34]. Therapeutic miR-26a was found to suppress liver cancer progression without toxicity [35]. Overexpression of miR-26a was also revealed to suppress tumorigenesis in Apc^{min} transgenic mice [24]. Besides directly exerts the function in cancer cells, miR-26a was further revealed to suppress the intestinal inflammatory response and attenuated colitis-associated cancer [36]. Moreover, the oncogenic function of many long non-coding RNA or circular RNA are mediated by sponging miR-26a [37–39], highlight the tumor suppressive role of miR-26a. We herein also revealed that miR-26a inhibited pancreatic cancer progression. There are also some investigations identified miR-26a as an oncogenic miRNA [40,41], indicating the context-dependent function of miRNA.

Meanwhile, miR-26a was also found to be upregulated upon therapy and be related with DNA damage response. p53-dependent augmentation of miR-26a expression levels mediated cancer cell cycle arrest and increased apoptosis by targeting critical checkpoint kinases, Chk1 and Wee1 [42]. miR26a-knockout HeLa cells showed increased cell growth and altered proliferation, and SWATH-MS (sequential window acquisition of all theoretical mass spectra) proteomics technology identified miR-26a-induced proteins were mainly involved in stress response, proliferation, localization establishment, and repopulation *etc* [43]. In pancreatic cancer, we also revealed that miR-26a promoted DNA damage response. Of note, miR-26a was used as the therapeutic agents to promote bone regeneration [44], highlighting the role of miR-26a in promoting cell recovering.

HMGA2 was identified as one of the eight key regulator hubs of pancreatic cancer [45]. In the long time, HMGA2 was widely revealed to be an oncogene [46]. We have previously revealed that HMGA2 was an oncogene but inhibited DNA damage response in pancreatic cancer [7], as previously indicated by others [26,27]. miR-26a transient upregulation upon radiotherapy resulted in HMGA2 transient downregulation, which is vital for the survival of irradiated cancer cells. miR-26a was also found to inhibit hepatocellular carcinoma and gallbladder cancer progression via suppressing HMGA2 [47,48]. Moreover, in contrast to the conventional tumor-promoting function of PGE2 [49,50], immediate PGE2 upregulated upon radiation was found to suppress cancer cell fitness [7]. The biological effects of miR-26a were also partly mediated by the suppression of PTGS2 expression, which delay the upregulation of COX2 and the PGE2, thus leaving time for the recovering of damaged cells.

However, needless to say, there are some limitations of the study. Firstly, although we tested the expression level of miR-26a after radiation in PDX models, we were unable to validate it in the clinical samples. Generally, pancreatic cancer is radioresistant and radiotherapy is not a standard therapeutic option. Additionally, due to the function of miR-26a is time-dependent, when repeating the

experiments, the time of treatment should be stringently followed, otherwise the results might get weird. Besides, the mechanism underlying the transient upregulation of miR-26a after radiation remains unclear and further exploration is needed. Moreover, we harnessed the capabilities of TransmiR v2.0 [51] to pinpoint seven pivotal transcription factors that may regulate the expression of miR-26a: CEBPA, E2F3, E2F7, MYC, SMAD3, TAL1, and TP53 (Supplementary Fig. S6). Nevertheless, the details are far from clear. In a word, our results just suggested that transient upregulation of miR-26a might contribute to radioresistance of pancreatic cancer, and further efforts are essential.

5. Conclusion

In conclusion, we found that tumor suppressive miR-26a showed dynamic changes upon radiation in pancreatic cancer cells. miR-26a, on the one hand, was hijacked to promote cell cycle arrest and DNA damage repair via suppressing HMGA2 and ulteriorly promoted radioresistance. On the other hand, miR-26a suppressed PTGS2 to delay the production of PGE2 and thus promote cancer cell survival and contributed to potentiating tumor repopulation.

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Declaration of ethics

This study doesn't use data from clinical patients and doesn't perform animal experiment.

Consent for publication

Written informed consent for publication was obtained from all participants.

Data availability statement

All the original sequencing data were submitted to SRA database (Accession No PRJNA748461).

CRediT authorship contribution statement

Ming-jie Jiang: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Chen-jing Lin:** Visualization, Validation, Software, Project administration, Investigation, Formal analysis, Data curation. **Fu-rao Liu:** Validation, Software, Investigation. **Zhu Mei:** Validation, Software, Investigation. **Dian-na Gu:** Writing – review & editing, Validation, Investigation, Conceptualization. **Ling Tian:** Writing – review & editing, Writing – original draft, Validation, Resources, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e31346.

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