Cancer Letters 375 (2016) 31–38

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Articles

eIF4E-phosphorylation-mediated Sox2 upregulation promotes pancreatic tumor cell repopulation after irradiation

Yang Yu [a,1], Ling Tian [b,1], Xiao Feng [a,1], Jin Cheng [a], Yanping Gong [a], Xinjian Liu [c], Zhengxiang Zhang [a], Xuguang Yang [a], Sijia He [a], Chuan-Yuan Li [c,*], Qian Huang [a,**]

[a] The Comprehensive Cancer Center and Shanghai Key Laboratory for Pancreatic Diseases, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 201620, China
[b] Institute of Translational Medicine, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 201620, China
[c] The Department of Dermatology, Duke University Medical Center, Durham, NC 27710, USA

ARTICLE INFO

Article history:
Received 30 December 2015
Received in revised form 23 February 2016
Accepted 24 February 2016

Keywords:
X-irradiation
Repopulation
Sox2
eIF4E
Caspase 3

ABSTRACT

Pancreatic cancer is a devastating disease characterized by treatment resistance and high recurrence rate. Repopulation of surviving tumor cells undergoing radiotherapy is one of the most common reasons for recurrence. Our previous studies have discovered a novel mechanism for repopulation after irradiation that activation of caspase-3 in irradiated tumor cells activates PKCδ/p38 axis to transmit proliferation signals promoting repopulation of surviving tumor cells. Here we found Sox2 expression is up-regulated in irradiated pancreatic cancer cells, which played a major role in tumor cell repopulation after irradiation. Over-expression of Sox2 strongly enhanced the growth-stimulating effect of irradiated dying tumor cells on living tumor cells through a paracrine modality. Furthermore, we identified activated eIF4E, which is phosphorylated by MNK1, as a regulator of Sox2 expression after irradiation, and pharmacologic inhibition of eIF4E with CGP57380 and Ribavirin significantly weakened Sox2-mediated tumor cell repopulation. Finally, we showed the activation of caspase 3/PKCδ/p38/MNK1 signal pathway in irradiated pancreatic tumor cells. Together, we showed a novel pathway regulating Sox2 expression and Sox2 may be a promising target to reduce recurrence due to repopulation of surviving tumor cells after radiotherapy.

Introduction

Pancreatic cancer is one of the most lethal cancers. In spite of decades of effort, the 5-year survival rate remains at only 5% [1]. Due to the lack of effective early diagnostic approaches and typical symptoms or signs, most patients with pancreatic cancer are usually diagnosed at advanced stages. For patients with advanced pancreatic cancer, radiation is an effective therapy [2]. However, even though radiation may kill the great majority of tumor cells, the accelerated repopulation of surviving tumor cells could reestablish the tumor [3]. Tumor repopulation, of which the molecular mechanisms are poorly understood, plays a significant role in tumor recurrence after radiotherapy. Our previous study has demonstrated that caspase 3, which is a master executioner during cell apoptosis, promotes tumor repopulation in breast cancer through activating calcium-independent phospholipase A2 (iPLA2) and increasing subsequent release of prostaglandin E2 (PGE2) from apoptotic cells [4]. Another recent study in our laboratory has manifested that PKCδ in apoptotic pancreatic cancer cells, which can be cleaved and activated by activated caspase 3, stimulates tumor repopulation by activating p38, an important kinase mediating growth factor production [5]. These studies indicate that caspase 3/PKCδ/p38 axis is an initiating signaling pathway for tumor repopulation, but the downstream factors of this pathway is still unclear.

Sox2, containing a distinctive high-mobility-group (HMG) domain which binds DNA in a sequence-specific manner, has been identified as a key transcription factor required for lineage specification, morphogenesis and differentiation in development of mouse embryos [6–8]. Notably, Takahashi et al. discovered that transduction of Sox2 with other "stemness" related transcription factors induces adult fibroblast reprogramming [9]. Recently, accumulating evidence has suggested that dysregulation of Sox2 plays an important role in epithelial cancers. Sox2 regulates cancer stem cell

Abbreviations: Sox2, SRY (sex determining region Y)-box2; eIF4E, eukaryotic initiation factor 4E; 4EBP1, eIF4E binding protein 1; PKC, protein kinase C; MNK, mitogen-activated protein kinases interacting kinases; Oct4, octamer-binding transcription factor 4; CC3, cleaved caspase 3; Fluc, firefly luciferase; IR, ionizing radiation.

* Corresponding author. Tel.: +1 919 613 8754; fax: +1 919 681 0909.
E-mail address: chuan.li@duke.edu (C.-Y. Li).
** Corresponding author. Tel.: +86 21 37798905; fax: +86 21 63240825.
E-mail address: Qhuang@sjtu.edu.cn (Q. Huang).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.canlet.2016.02.052
0304-3835/

(CSCs) function in skin squamous-cell carcinoma by directly promoting transcription of genes associated with stemness, proliferation and survival [10]. In addition, one group reported that Sox 2 upregulation induced by fractionated ionizing radiation is a key marker of radiation-surviving prostate cancer cell lines [11]. However, the specific role of Sox2 in tumor cell repopulation after irradiation is still unclear.

Translational control of malignancy-related mRNAs has been considered as a major part in malignant progression [12,13]. In most cases, translational control is conducted at the initiation step. eIF4E (eukaryotic initiation factor 4E) is thought to be the rate-limiting factor in translation initiation progression. Accumulating evidence suggested that eIF4E plays a significant role in radiotherapy resistance and chemotherapy resistance. For instance, one group reported that both genetic and pharmacologic inhibition of eIF4E enhanced tumor radiosensitivity [14]. Another study showed that MNK2 dependent phosphorylation of eIF4E is responsible for Gemcitabine resistance in pancreatic cancer cells [15]. MNK1 regulates eIF4E activity by phosphorylating Ser209 of eIF4E [16,17]. MNK1 is directly phosphorylated by mitogen-activated protein kinases p38 and ERK [18].

In this study, we showed that upregulation of Sox2 promoted pancreatic tumor cell repopulation in a paracrine modality, and eIF4E phosphorylation induced by caspase 3/PKCδ/p38/MNK1 signaling pathway is required for Sox2 upregulation in irradiated pancreatic tumor cells.

Materials and methods

Cell culture

SW1990 and BxPc-3 cells were purchased from the Chinese Academy of Science Cell Bank (Shanghai, China) and cultured in RPMI 1640 medium (HyClone) containing 10% fetal bovine serum (FBS) (Tianhang Biological Technology, China) at 37 °C under 5% CO_2.

Gene transduction

pLEX lentiviral vector system (Thermo, USA) was used to transduce exogenous genes into SW1990 and BxPc-3 cells. The vectors contains firefly luciferase (Fluc) green fluorescent protein (GFP) fusion gene and sox2 gene were kindly provided by Prof. Chuan-Yuan Li. Live, replication-deficient recombinant lentiviral vectors expressing exogenous genes were packaged in 293T cells following manufacturer's instructions. The stably transduced SW1990 or BxPc-3 cells were obtained by lentivirus infection and puromycin selection at 2 μg/ml.

Colony formation assay

SW1990 and BxPc-3 cells were irradiated with various doses of X-ray, then seeded in 6-well plates in different numbers in triplicates (100, 200, 750, 1250, 5000 and 50,000 SW1990 cells with 0 Gy, 2 Gy, 4 Gy, 6 Gy, 8 Gy and 10 Gy, respectively; 150, 500, 1000, 2000, 10,000 and 15,000 BxPc-3 cells with 0 Gy, 2 Gy, 4 Gy, 6 Gy, 8 Gy and 10 Gy, respectively). 14 days later, colonies were stained with crystal violet and then the number of colonies of 50 or more cells was counted. The surviving fraction was analyzed following published instructions [19].

Irradiation and tumor cell repopulation model in vitro and bioluminescence imaging

Irradiation was performed with X-ray using a linear accelerator (Siemens, Germany) at a dose rate of 3.6 Gy/min. Pancreatic cancer cells (feeder cells) were seeded into two 24-well plates at a density of $0.8–1.2 \times 10^5$/well. After the cells became attached (overnight), one of the 24-well plates was irradiated with 10 Gy of X-ray. Before irradiation, we replaced the 1640 medium containing 10% FBS in the two plates by 1640 containing 2% FBS. After irradiation, the non-irradiated pancreatic cancer cells with firefly luciferase (Fluc) and GFP fusion gene (reporter cells) were seeded into both plates at a density of 1000 cells/well for SW1990 cells and 500 cells/well for BxPc-3. Then, feeder cells and reporter cells co-cultured for 8–12 days. During the co-cultured period, the medium was replaced with fresh 1640 medium containing 2% FBS every two days. For the transwell assay, the cells with firefly luciferase and GFP fusion gene (reporter cells) were seeded into hanging cell culture inserts of 0.4 μm pore size (Millipore, USA).

Bioluminescence imaging machines used in this study were SPECTRAL Ami X (Spectral InstrumentsImaging, USA) and IVIS Lumina Series III (PerkinElmer, USA).

Western blot

Western blot was done as described [5]. The primary antibodies were used: PKCδ (Santa Cruz, USA); c-Myc (Abcam, USA); β-actin, GAPDH, p38, phospho-p38, Akt, phospho-Akt, MNK1, phospho-MNK1, eIF4E, phospho-eIF4E, Sox2, Nanog, Oct4, phosphor-mTOR, phosphor-4EBP1 (Cell Signaling Technology, USA).

Immunofluorescence staining

Immunofluorescence staining was done as described [20]. The cleaved caspase 3 antibody was purchased from Cell Signaling Technology. Immunofluorescence was visualized by confocal microscope (Leica, Germany) located in the Institute of Translational Medicine of Shanghai General Hospital.

Quantitative real-time PCR

Sox2 mRNA expression was quantified using the LightCycler 480 (Roche, USA) with SYBR *Premix Ex Taq II* (Takara, Japan). The following primers were used for the amplification of *sox2* mRNA: 5′- ATGGGTTCGGTGGTCAAGTC-3′ and 5′- GCTCTGG TAGTGCTGGGACA-3′ and *GAPDH* mRNA: 5′-GGAGCGAGATCCCTCCAAAAT-3′ and 5′-GGCTGTTGTCATACTTCTCATGG-3′.

Other drugs used

CGP57380 and Ribavirin were purchased from Selleckchem (TX, USA).

Statistical analysis

Statistical analysis was undertaken using GraphPad Prism 6 (GraphPad Software, USA). Data were presented as mean ± SEM (standard error of the mean). For parameter tests, significance was evaluated with unpaired 2-tailed student's *t* test. For nonparametric tests, significance was analyzed with Mann–Whitney *U* test. The $p < 0.05$ was considered statistically significant.

Results

IR-induced dying pancreatic cancer cells stimulate repopulation

As irradiation-induced cancer cell death has been considered as an initiating factor in driving tumor cell repopulation in our previous studies, we first carried out colony formation assay to determine the lethal dose of X-ray for pancreatic cancer cells. Two pancreatic cancer cell lines, SW1990 and BxPc-3, were irradiated with X-ray at various doses, and subsequently seeded into 6-well plates. The results of colony formation assay showed that the survival fraction of 10 Gy-irradiated SW1990 and BxPc-3 cells were approximately 0.028 ± 0.003% and 0.513 ± 0.03%, respectively (Fig. 1A and B), indicating that 10 Gy irradiation eliminated the majority of these two cell lines. Therefore, we employed 10 Gy irradiation in our following investigations. Next, we confirmed that the bioluminescence signal intensity of the two Fluc-labeled pancreatic cancer cell lines was linearly correlated with cell numbers (Fig. 1C and D, $R^2 = 0.9912$ in Fluc-labeled SW1990 cells and $R^2 = 0.9899$ in Fluc-labeled BxPc-3 cells), so we used the photon values to represent the number of living reporter cells in our tumor repopulation model *in vitro*. To simulate the actual tumor cell repopulation after radiotherapy, whereas a small number of tumor cells survive and repopulate the tumor, we seeded a small number of non-irradiated, Fluc-labeled SW1990 cells or BxPc-3 cells onto a bed of a much larger number of lethally irradiated, unlabeled SW1990 cells or BxPc-3 cells in 24-well plates. Growth of reporter cells was then monitored through bioluminescence imaging. Notably, bioluminescence signals from Fluc-labeled SW1990 cells co-cultured with 10 Gy-irradiated SW1990 cells were approximately 3.8-fold and 3.7-fold higher than co-cultured with non-irradiated SW1990 cells and seeded alone (Fig. 1E). Bioluminescence signals from Fluc-labeled BxPc-3 cells co-cultured with 10 Gy-irradiated BxPc-3 cells were approximately 1.6-fold and 3.0-fold higher than co-cultured with non-irradiated BxPc-3 cells and seeded alone (Fig. 1F). Therefore, these results demonstrated that irradiation-induced dying pancreatic cancer cells greatly stimulate the proliferation of living cells.

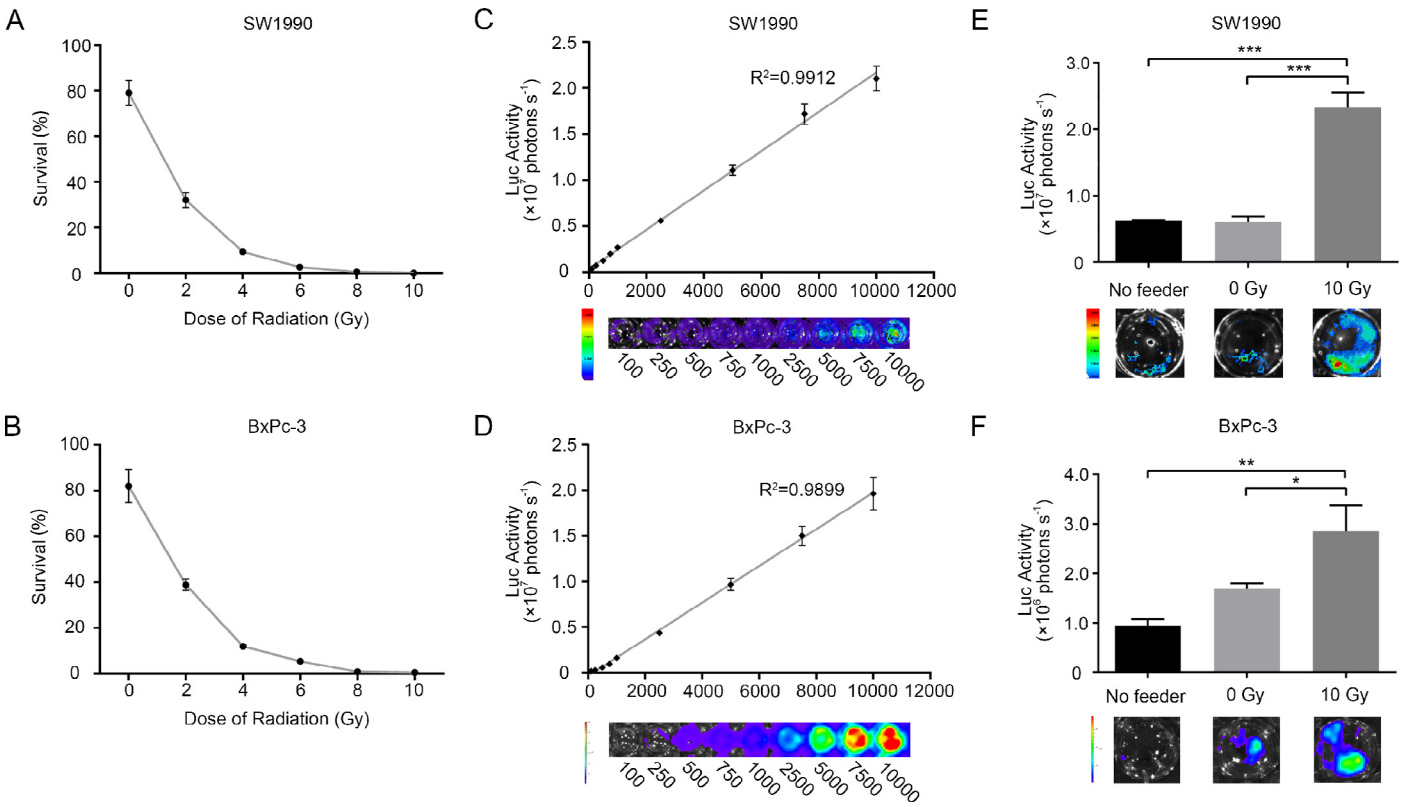

Fig. 1. Irradiated tumor cells enhance the proliferation of living tumor cells. (A, B) Survival fraction of SW1990 and BxPc-3 cells treated with 2 Gy, 4 Gy, 6 Gy, 8 Gy and 10 Gy X-ray irradiation, and then seeded into 6-wells for clone formation assay. Error bars represent the standard error of the mean (SEM). (C, D) Linearity relation between luciferase activity (photons/sec) of Fluc-labeled SW1990 ($R^2 = 0.9912$) or Fluc-labeled BxPc-3 ($R^2 = 0.9899$) and cell number plated. (E) Growth-stimulating effect of irradiated SW1990 cells on SW1990 cells. Upper panel, growth of Fluc-labeled SW1990 cells co-cultured with 10 Gy-irradiated SW1990 cells or non-irradiated SW1990 or cultured alone (No feeder) was tested by bioluminescence imaging. Lower panel, representative bioluminescence images. $^{***}p < 0.001$. (F) Growth-stimulating effect of irradiated BxPc-3 cells on SW1990 cells. Upper panel, growth of Fluc-labeled SW1990 cells co-cultured with 10 Gy-irradiated BxPc-3 cells or non-irradiated BxPc-3 or cultured alone (No feeder) was tested by bioluminescence imaging. Lower panel, representative bioluminescence images. $^{*}p < 0.05$, $^{**}p < 0.01$.

Sox2 mediates pancreatic tumor cell repopulation after IR

As stem cell-like gene signature identified in ionizing radiation-treated cancer cells [20], we wondered whether IR induces the upregulation of some "stemness" related transcription factors in irradiated cancer cells. We carried out western blot analysis to examine expression of Sox2, c-Myc, Oct4 and Nanog. Results manifested that Sox2 expression significantly increased at 24 hours after irradiation in both two pancreatic cancer cell lines (Fig. 2A). However, there was no obvious elevation of Oct4 in these two pancreatic cancer cell lines exposed to irradiation. Although c-Myc increased in both pancreatic cancer cell lines in a short time, it descended to the pre-irradiation level within 48 hours (Fig. 2A). Subsequently, pancreatic cancer cell lines stably overexpressing Sox2 were used in the co-culture model to further identify the role of Sox2 in tumor cell repopulation. Western blot analysis confirmed the overexpression in SW1990 and BxPc-3 cells (Fig. 2B). Bioluminescence imaging showed that these two pancreatic cancer cell lines overexpressing Sox2 receiving 10 Gy irradiation had stronger growth-promoting effect on reporter cells when compared with wild-type pancreatic cancer cells (Fig. 2C). It has been reported that Sox2 could transcriptionally enhance expression of some soluble growth factors to stimulate tumor cell proliferation. In addition, our previous study showed that dying tumor cells during radiotherapy stimulate tumor cell repopulation via a paracrine modality [4]. Thereby, we wondered whether radiation-induced Sox2 elevation mediates growth of living tumor cells through a paracrine modality. Results from transwell assay showed that irradiated SW1990 cells also exhibited potent ability to enhance the growth of reporter cells, when reporter cells were seeded into hanging transwell, and irradiated SW1990 cells overexpressing Sox2 had much stronger proliferation-stimulating effect on reporter cells compared with wild-type SW1990 cells (Fig. 2D). Similar tendency can be seen in BxPc-3 cells (Fig. 2D). Taken together, these data strongly indicated that Sox2 mediates tumor cell repopulation by promoting the secretion of some transmissible pro-proliferation factors from dying tumor cells.

Inhibition of eIF4E represses IR-induced Sox2 upregulation

Having shown that Sox2 mediates pancreatic tumor cell repopulation after IR, we subsequently explored mechanisms underlying IR-induced Sox2 upregulation. Therefore, the phosphorylation status of eIF4E in irradiated pancreatic cancer cells has been monitered with western blot analysis. We observed that phosphorylation of eIF4E gradually increased and peaked at 48 hours in SW1990 and BxPc-3 cells exposed to 10 Gy irradiation (Fig. 3A). To confirm whether the specific small molecular inhibitor of MNK1 CGP57380 could counteract eIF4E phosphorylation in response to irradiation, we detected the phosphorylation status of eIF4E in pancreatic cancer cells 12 hours after 10 Gy irradiation with various doses of CGP57380. Results showed that CGP57380 effectively inhibited radiation-induced eIF4E phosphorylation in a dose-dependent manner (Fig. 3B). Subsequently, we treated pancreatic cancer cells with or without CGP57380 four hours before irradiation, and then cells were harvested at 4 hours, 8 hours, 12 hours and 24 hours after irradiation. Western blot showed that CGP57380 significantly

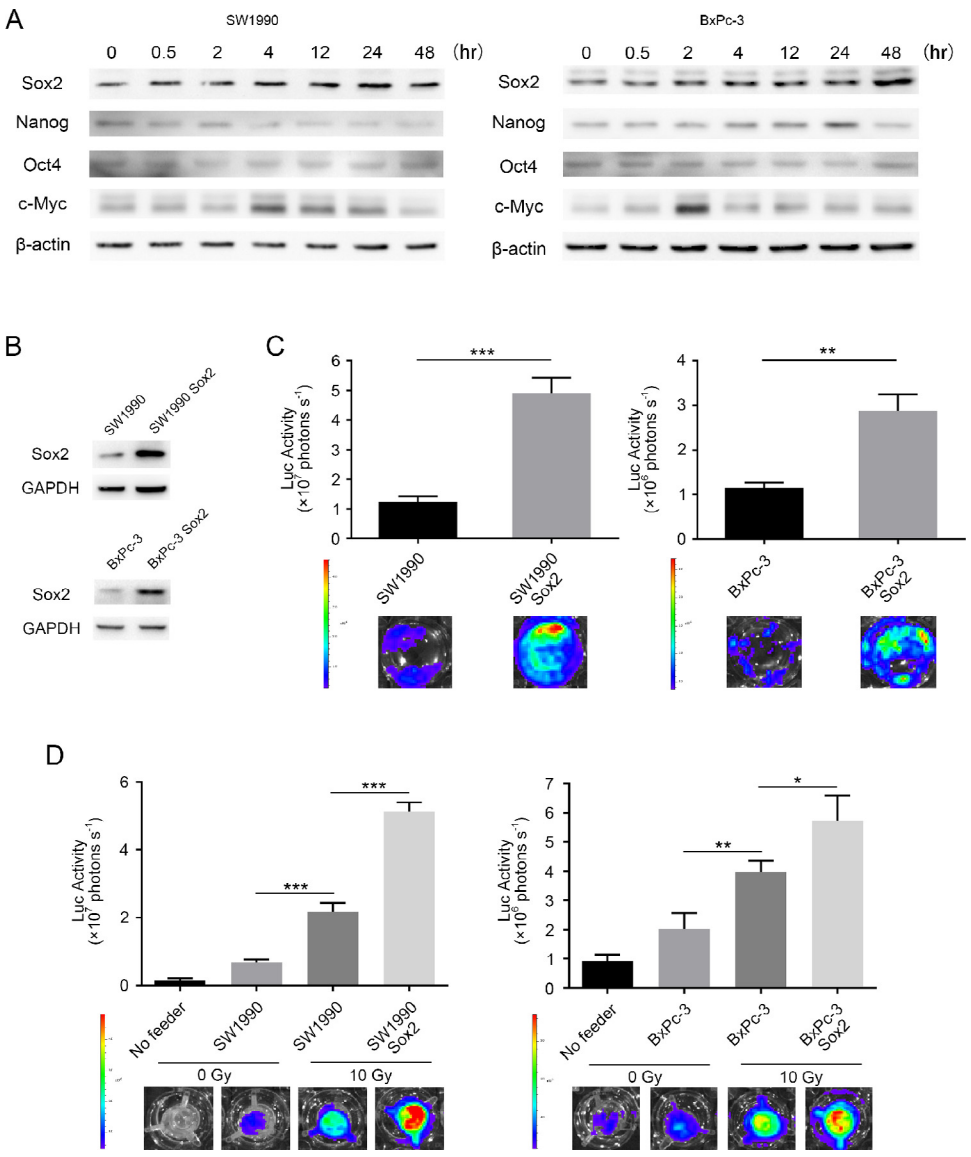

Fig. 2. Sox2 promoting living tumor cell proliferation through a paracrine modality. (A) Expression changes of Sox2, Nanog, Oct4 and c-Myc in 10 Gy-irradiated SW1990 and BxPc-3 cells at indicated time points. (B) Sox2 overexpression was confirmed by western blot analysis. (C) Growth-simulating effect of SW1990 and BxPc-3 cells overexpressing Sox2 was much stronger than that of parental SW1990 and BxPc-3 cells. $^{**}p < 0.01$, $^{***}p < 0.001$. (D) Left panel, Growth-stimulating effect of irradiated SW1990 cells overexpressing Sox2 and parental SW1990 cells on Fluc-labeled SW1990 seeded in hanging cell culture inserts. Right panel, Growth-stimulating effect of BxPc-3 cells overexpressing Sox2 and parental BxPc-3 cells on Fluc-labeled BxPc-3 seeded in hanging cell culture inserts. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

inhibited Sox2 upregulation as well as eIF4E phosphorylation in irradiated tumor cells, indicating that eIF4E phosphorylation regulated Sox2 expression (Fig. 3C). To address whether eIF4E phosphorylation regulates Sox2 expression at translational level rather than at transcriptional level, we tested the mRNA level of Sox2 before and after irradiation. qPCR analysis showed no statistically significant difference between non-irradiated and irradiated group. Meanwile, CGP57380 did not inhibit transcription of *sox2* mRNA (Fig. 3C). The similar tendency can be seen in BxPc-3 cells (Fig. 3D). To further confirm the conclusion, we used Ribavirin, which has been shown to mechanistically inhibit eIF4E activity by competing with the 7-methylguanosine mRNA cap [21], to repeat the above-mentioned experiments. As expected, the results indicated that Ribavirin also inhibit eIF4E induced upregulation of Sox2 expression (Fig. 3E and F). Taken together, these findings demonstrated that eIF4E phosphorylation is required for IR-induced Sox2 upregulation in a translational manner.

Inhibition of eIF4E mitigates pancreatic tumor cell repopulation

Our aforementioned results showed that Sox2 mediates tumor cell repopulation and inhibition of eIF4E phosphorylation significantly reduced IR-induced Sox2 upregulation. We subsequently investigated whether inhibition of eIF4E activity mitigates tumor cell repopulation after irradiation. We used CGP57380, which effectively counteracts eIF4E phosphorylation, in our tumor cell repopulation model. Results showed that CGP57380 strongly suppressed the growth of Fluc-labeled SW1990 cells co-cultured with irradiated SW1990 cells compared to vehicle control (Fig. 4A). Meanwhile, administration of CGP57380 did not interfere with proliferation of Fluc-labeled SW1990 cells (Fig. 4A). The similar tendency can be seen in BxPc-3 cells (Fig. 4B). To further confirm our hypothesis, we also used Ribavirin to repeat the above experiments. As Ribavirin was dissolved in distilled water, we did not set vehicle control group. We observed that the pro-proliferation effect of ir-

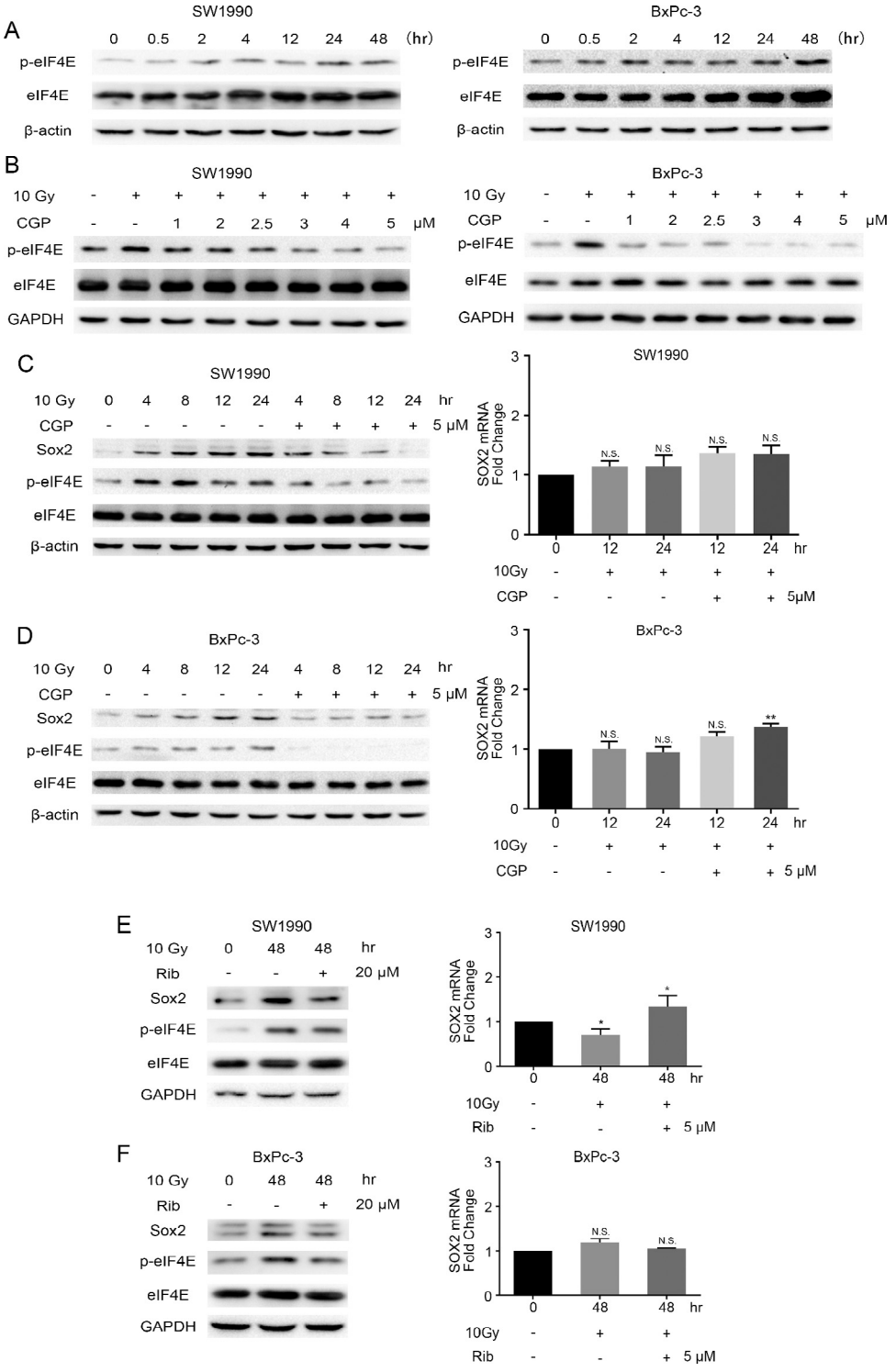

Fig. 3. EIF4E phosphorylation regulates Sox2 expression. (A) Phosphorylation status of eIF4E in 10 Gy-irradiated SW1990 and BxPc-3 cells at different time points. (B) Suppressive effect of different concentration CGP57380 on radiation-induced eIF4E phosphorylation, samples were harvested at 12 hours after irradiation. (C) Left panel, Western blot showed 5 μM CGP57380 effectively inhibits elevated Sox2 expression in irradiated SW1990 cells. Right panel, qPCR analysis showed that there is no significant fluctuation of *sox2* mRNA after irradiation, and CGP57380 did not influence *sox2* mRNA level in irradiated SW1990 cells. N.S. = No Significance. (D) Left panel, Western blot showed 5 μM CGP57380 effectively inhibits elevated Sox2 expression in irradiated BxPc-3 cells. Right panel, qPCR analysis showed that there is no significant fluctuation of *Sox2* mRNA after irradiation. *sox2* mRNA level slightly increased at 24 hours in irradiated BxPc-3 cells treated with CGP57380 compared to irradiated BxPc-3 cells treated without CGP57380, $^{**}p < 0.01$. (E) Left panel, Western blot showed that 20 μM Ribavirin effectively inhibits elevated Sox2 expression in irradiated SW1990 cells. Right panel, qPCR analysis showed that Sox2 mRNA level in irradiated SW1990 cells at 48 hours was lower than that in non-irradiated SW1990 cells, $^{*}p < 0.05$. (F) Left panel, Western blot showed 20 μM Ribavirin inhibits elevated Sox2 expression in irradiated BxPc-3 cells. Right panel, qPCR analysis showed that there is no significant fluctuation of *Sox2* mRNA in irradiated BxPc-3 cells and Ribavirin did not influence *sox2* mRNA level in irradiated BxPc-3 cells.

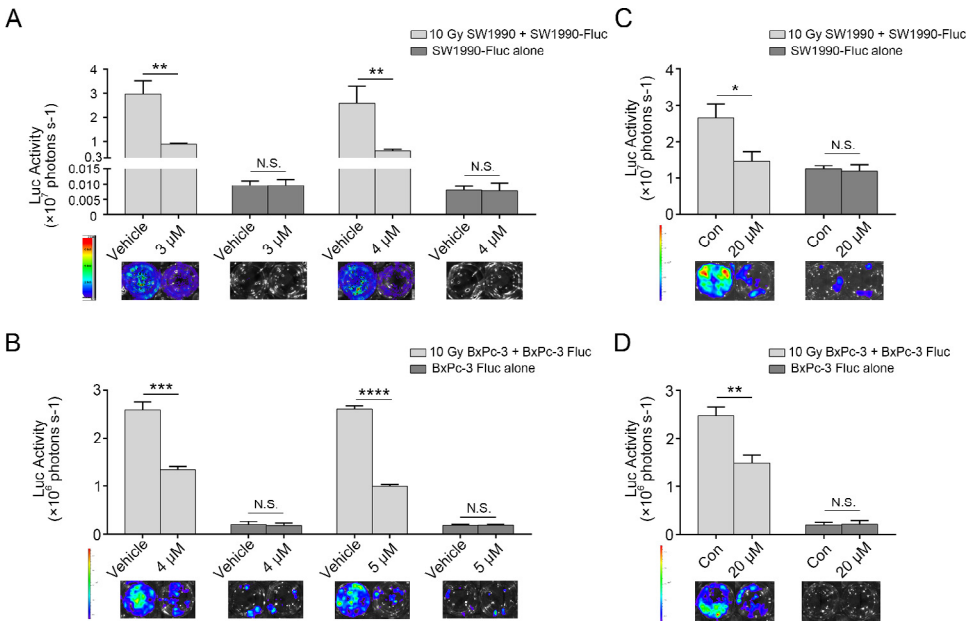

Fig. 4. Suppression of eIF4E activity inhibits proliferation of living tumor cells. (A) CGP57380 significantly inhibits the ability of 10 Gy irradiated SW1990 cells to promote proliferation of Fluc-labeled SW1990 cells. $^{**}p < 0.01$. (B) CGP57380 diminished the ability of 10 Gy irradiated BxPc-3 cells to promote proliferation of Fluc-labeled BxPc-3 cells. $^{***}p < 0.001$, $^{****}p < 0.0001$. (C) Ribavirin extenuated the ability of 10 Gy irradiated SW1990 cells to promote proliferation of Fluc-labeled SW1990 cells and did not interfere with Fluc-labeled SW1990 growth, $^{*}p < 0.05$. (D) Ribavirin diminished the ability of 10 Gy irradiated BxPc-3 cells to promote proliferation of Fluc-labeled BxPc-3 cells and did not interfere with Fluc-labeled BxPc-3 growth, $^{**}p < 0.01$.

radiated SW1990 cells on Fluc-labeled SW1990 cells was weakened by 20 μM Ribavirin (Fig. 4C). Similar experiments were conducted using BxPc-3 cells (Fig. 4D). We used two drugs to inhibit eIF4E activity through different mechanisms, results manifested that inhibition of eIF4E activity weakened Sox2-mediated stimulatory effect of irradiated dying cells on living tumor cells.

Ionizing irradiation induces eIF4E phosphorylation through caspase 3/PKCδ/p38/MNK1 signaling pathway

Having shown that eIF4E phosphorylation regulates Sox2 expression, we subsequently explored which pathway leads to elevated phospho-eIF4E. As it has been demonstrated that activation of p38/MNK1 pathway induces phosphorylation of eIF4E at S209, we tested the phosphorylation status of MNK1 and p38 after irradiation. Western blot showed that p38 and MNK1 were significantly activated at 48 hours in 10 Gy-irradiated SW1990 and BxPc-3 cells (Fig. 5B). Another route mediating eIF4E activity is AKT/mTOR/4EBP1 signaling [22,23]. The current paradigm suggests that mammalian target of rapamycin complex1 (mTORC1) is phosphorylated by AKT and one critical function of mTORC1 activity is to upregulate translational initiation through restricting activity of 4EBP1 (eIF4E-binding protein 1). As mTOR kinase is the functional component of mTORC1, we detected the phosphorylation status of mTOR kinase, as well as AKT and 4EBP1. Although western blot showed phosphorylation of AKT was elevated after irradiation, phosphorylation of mTOR kinase and 4EBP1 did not increase (Fig. S1). In our above-mentioned results CGP57380 attenuated the elevated eIF4E phosphorylation in response to irradiation, we draw a conclusion that p38/MNK1 signaling pathway leads to elevated phosphorylation eIF4E. Recently, our study has shown that caspase 3/PKCδ/p38 signaling pathway induced tumor cell repopulation after irradiation. Our results from immunofluorescence assay and western blot showed that activation of caspase 3 existed in most irradiated pancreatic cancer cells and cleaved PKCδ also significantly increased, indicating that eIF4E is a downstream factor of caspase 3/PKCδ/p38 signaling pathway (Fig. 5A and B).

In summary, based on above results, we would like to propose a novel molecular mechanism in tumor cell repopulation, of which a schematic representation was shown (Fig. 5C).

Discussion

Although radiotherapy effectively induces tumor cells death, radiation-induced dying tumor cells stimulate the repopulation of tumor cells from a small number of surviving cells, leading to failure of treatment or recrudescence. Apoptosis is a major death modality for irradiated tumor cells. Our results showed that activation of caspase 3 occurred in most irradiated tumor cells. Active caspase 3 in radiation-induced apoptotic tumor cells stimulates tumor cell repopulation during radiotherapy through different molecular mechanisms. In breast cancer, activation of caspase 3 induces secretion of PGE_2 from irradiated tumor cells, promoting repopulation of surviving cells [4]. In pancreatic cancer, PKCδ is cleaved by active-caspase 3 after irradiation and then phosphorylates p38, a vital promoter of tumor cell proliferation, promotes tumor cell repopulation [5]. In this study, we found that activation of caspase 3/PKCδ/p38 signaling, an initiating signaling of tumor cell repopulation after irradiation, existed in radiation-treated pancreatic cancer cells. In addition, we found that phosphorylation level of MNK2 increased in irradiated tumor cells. p38 directly phosphorylates MNK1 in tumor cells exposed to extracellular stimuli such as UV-irradiation or IL-1β [18]. Regulating the activity of eIF4E is a key function of MNK1. Our results showed that radiation-induced phosphorylation of eIF4E markedly inhibited by MNK1-specific inhibitor CGP57380 in a dose-dependent manner. In B cells, p38/MNK1/eIF4E translation initiation pathway is required for CpG-induced IL-6 secretion [24]. Here, we observed that pharmacologic inhibition of eIF4E activity attenuates pancreatic tumor cell repopulation after irradiation.

eIF4E is a core component of eIF4E complex (translation initiation complex). In translation initiation progression, eIF4E complex initiates mRNA translation by binding to 7-methylguanosine cap structure of mRNA cap via phosphorylated eIF4E. Growing

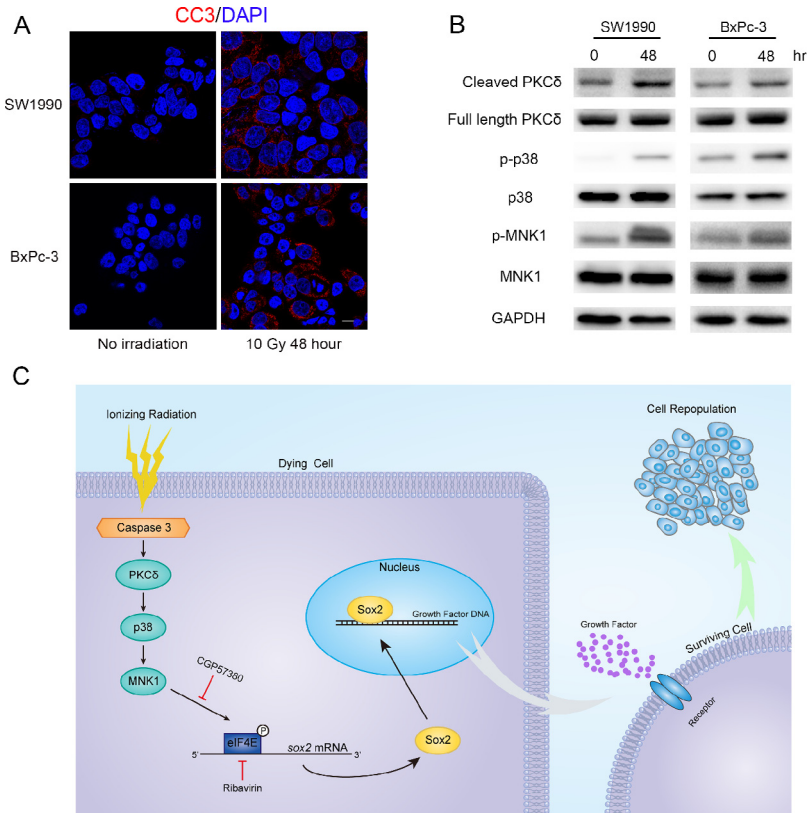

Fig. 5. Activation of caspase 3/PKCδ/p38/MNK1 in irradiated pancreatic tumor cells. (A) Immunofluorescence analysis confirming caspase-3 activation in 10 Gy-irradiated SW1990 and BxPc-3cells. Scale bar: 10 μm. CC3, cleaved caspase 3. (B) Activation of PKCδ, p38 and MNK1 in 10 Gy-irradiated SW1990 and BxPc-3 cells was confirmed by western blot. (C) Schematic overview summarizing caspase 3/PKCδ/p38/MNK1/eIF4E induced Sox2 expression promoting tumor cell repopulation after irradiation through a paracrine modality.

evidence indicates that eIF4E-regulated translational control of specific mRNAs contributes importantly to survival, angiogenesis and malignancy. Translation of VEGF-A, a key factor in tumor vascularization, is under the control of mTORC1-mediated 4EBP1/eIF4E axis [25,26]. In *Eμ-Myc*/+ transgenic mouse, eIF4E promotes the translation of *Prps2* mRNA, which is required for protein and nucleotide biosynthesis, to drive Myc-dependent tumorigenesis [27]. Moreover, c-Myc, which is required for adult fibroblast reprogramming, has been reported to be tightly regulated by eIF4E at translational level [28]. Unexpectedly, we found that Sox2, another transcription factor required for generating iPS cells, is regulated by phosphorylated eIF4E. Quantitative real-time PCR demonstrated that regulation of Sox2 expression occurs in post-transcription level. In addition, pharmacologic inhibition of eIF4E with CGP57380 and Ribavirin both restrains radiation-induced Sox2 upregulation. Repopulation of surviving tumor cells during or after radiotherapy is a main reason for failure of treatment. However, mechanism of repopulation has not been fully understood. Although our previous studies have demonstrated that caspase 3/PKCδ/p38 axis is a novel signaling pathway to initiate repopulation, it is still important to find out downstream factors. The downstream factors could be used as treatment target to restrain repopulation. In this study, we further demonstrated eIF4E and Sox2 are downstream factors of caspase 3/PKCδ/p38 signaling, which deepen the understanding of caspase 3-mediated tumor cell repopulation. Many studies have proved that eIF4E is associated with prognosis of different kinds of cancers such as melanoma and non-small lung cancer [29–31]. Our data showed that pharmacologic inhibition of eIF4E effectively restrains Sox2 upregulation and pancreatic tumor cell repopulation, indicating that eIF4E may be a treatment target to increase radiosensitivity or control recurrence rate of radiotherapy.

Growing evidence suggested that the molecular function of Sox2 in tumorigenesis is involved in pro-proliferation, pro-survival and mediating CSC function. It has been reported that amplification of *sox2* locus and hypomethylation of Sox2 promoter are responsible for Sox2 dysregulation in cancer [32]. Overexpression of Sox2 in cutaneous SCC (squamous cell carcinoma) TIC (tumor initiating cells) is due to reduction in H3K27m3 of Sox2 promoter, which is required for the self-renewing potential of TICs and SCC growth [33]. Unexpectedly, we observed a post-transcriptional regulation modality of Sox2 expression in irradiated tumor cells and the elevated Sox2 in dying tumor cells enhances the proliferation of living tumor cells. Repopulation of living tumor cells after radiotherapy is the main reason for recurrence. Cancer stem cells have been considered as an initial source of repopulation. As Sox2 is a key regulator of cancer stem cells function we wonder whether radiation-induced Sox2 enhances the “stemness” of irradiated tumor cells. It has been reported that a subpopulation of pancreatic cancer cells expressing the cell surface markers CD44 and CD24 have highly tumorigenic potential [34,35]. Our data showed that there was no increase of $CD24^{+}/CD44^{+}$ pancreatic cells after 10 Gy irradiation (Fig. S2). Growing evidence suggested that Sox2 transcriptionally enhances expression of some secretory growth factors to promote tumor cell growth [36]. In addition, our previous studies demonstrated that irradiated tumor cells could secrete some soluble pro-proliferative factors such as PGE_2 and VEGF to enhance repopulation of living tumor cells [4,37]. In order to confirm the paracrine modality in tumor cell repopulation, we separated reporter cells and

feeder cells using transwell chamber. In spite of being separated by transwell, the proliferation-stimulating effect of irradiated tumor cells on reporter cells still exists. What is more notable is that overexpression of Sox2 endows irradiated tumor cells with greater ability to promote living tumor cells growth. Taken together, our data demonstrated that Sox2 elevation in irradiated tumor cells stimulates living tumor cells growth through promoting secretion of soluble pro-proliferative factors.

In conclusion, we have shown that activation of caspase 3/PKCδ/p38/MNK1 signaling induces eIF4E phosphorylation, which mediates upregulation of Sox2 in irradiated tumor cells. Importantly, Sox2 upregulation in irradiated dying tumor cells promotes proliferation of living tumor cells through a paracrine modality, indicating that Sox2 may be a target to reduce tumor cell repopulation after radiotherapy.

Acknowledgments

We feel thankful for the funding of National Natural Science Foundation of China (81120108017, 81572951) (Qian Huang) and (81172030) (Ling Tian) and (81572788) (Xinjian Liu) and grants from National Institutes of Health, USA (ES024015, CA155270) (Chuan-Yuan Li).

Conflict of interest

The authors declare no conflict of interest.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2016.02.052.

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