Clinical Cancer Research

Cancer-associated Fibroblast–promoted LncRNA *DNM3OS* Confers Radioresistance by Regulating DNA Damage Response in Esophageal Squamous Cell Carcinoma

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Abstract

Purpose: Our study aimed to investigate whether CAF (cancer-associated fibroblasts) were involved in long noncoding RNAs (lncRNA)-regulated radioresponse in esophageal squamous cell carcinoma (ESCC).

Experimental Design: By use of lncRNAs PCR array, 38 lncRNAs were screened in esophageal cancer cells and in normal esophageal epithelial cells Het-1A. LncRNA DNM3OS was detected in tumor tissues of patients with ESCC and in matched normal esophageal epithelial tissues by qRT-PCR analysis and *in situ* hybridization assay. The association of DNM3OS and tumor radioresistance was investigated *in vitro* and *in vivo*. The influences of DNM3OS on DNA damage response (DDR) was investigated by Western blotting, immunofluorescence imaging, and comet assay. The mechanisms by which CAFs promoted DNM3OS expression was investigated by kinase inhibitors' screening, luciferase assay, and chromatin immunoprecipitation.

Results: Among the 38 lncRNAs tested, DNM3OS was found to have a much higher expression level in esophageal cancer cells than in Het-1A. In tumor tissues of 16 patients with ESCC, the expression level of DNM3OS showed an average increase of 6.3429-fold compared with that in matched normal tissues. DNM3OS conferred significant radioresistance *in vitro* and *in vivo* by regulating DDR. CAFs promoted the expression of DNM3OS with a 39.2554-fold and 38.3163-fold increase in KYSE-30 and KYSE-140, respectively. CAFs promoted the expression of DNM3OS in a PDGFβ/PDGFRβ/FOXO1 signaling pathway–dependent manner. FOXO1, a transcription factor downstream of PDGFβ/PDGFRβ signaling pathway, initiated the transcription of DNM3OS by binding to DNM3OS promoter.

Conclusions: Our study highlighted CAF-promoted DNM3OS as an attractive target to reverse tumor radioresistance in ESCC.

Introduction

The 5-year survival rate of patients with esophageal squamous cell carcinoma (ESCC) treated with radiotherapy, one major curative treatment choice for patients with ESCC, is less than 20% due to radioresistance (1). Accumulating evidences have suggested that tumor microenvironment, especially its major component cancer-associated fibroblasts (CAF), were closely associated with tumor initiation and progression (2, 3). The complicated interaction between tumor cells and their host microenvironment has important influences on their biological behavior. Tumor cells promote the growth and survival of stromal fibroblasts; in parallel, the microenvironment potentiates the activation of signaling pathways that are involved in proliferation, invasion, and survival of tumor cells (4–6). Our previous study revealed that the cross-talk of esophageal cancer cells and CAFs induced TGFβ1 expression in an autocrine/paracrine signaling loop, which resulted in the development of chemoresistance (7). Furthermore, we found CAF-secreted CXCL1 conferred ESCC-significant radioresistance *in vitro* and *in vivo* by regulating DNA damage response (DDR) in a reactive oxygen species–dependent manner (8).

Long noncoding RNAs (lncRNA) are a group of transcripts comprising more than 200 nucleotides and lacking protein-coding potential (9). LncRNAs exerted their functions through chromatin modification, transcriptional regulation, and post-transcriptional regulation. Aberrant expressions of lncRNAs were associated with human diseases such as cancer (10). LncRNAs were involved in many cellular biological processes including cell-cycle progression, apoptosis, and pluripotency of stem cells. Recent studies showed that lncRNAs were involved in DDR by sensing DNA damage, transducing damage signals, repairing damaged DNA, activating cell-cycle checkpoints, and inducing apoptosis (11). Our study aimed to investigate whether lncRNAs were involved in DNA damage repair following irradiation in ESCC and whether CAFs regulated the expressions of those functional lncRNAs in esophageal cancer cells. By screening lncRNAs PCR array, we found lncRNA DNM3OS

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Translational Relevance

Radiotherapy is one of major curative treatment modalities for patients with esophageal squamous cell carcinoma (ESCC). However, the 5-year survival rate of patients with ESCC treated with radiotherapy is less than 20% due to radioresistance. By screening long noncoding RNAs (lncRNA) PCR array, our study identified that lncRNA DNM3OS had a much higher expression level in esophageal cancer cells than in normal esophageal epithelial cells Het-1A. DNM3OS was also highly expressed in tumor tissues of ESCC patients than in matched normal esophageal epithelial tissues. CAFs, as one major component of tumor microenvironment, promoted the expression of DNM3OS in esophageal cancer cells in a PDGFβ/PDGFRβ/FOXO1 signaling pathway–dependent manner. CAF-promoted DNM3OS conferred significant radioresistance *in vitro* and *in vivo* by regulating DNA damage response. These findings highlighted CAF-promoted DNM3OS as an attractive therapeutic target to reverse tumor radioresistance in ESCC and might provide novel insights into CAF-induced tumor radioresistance.

conferred significant radioresistance by regulating DDR in ESCC. More importantly, we found CAFs significantly promoted the expression of DNM3OS in esophageal cancer cells by activating the PDGFβ/PDGFβR/FOXO1 signaling pathway.

Materials and Methods

Isolation and culture of CAFs

To isolate stromal CAFs, tumor tissues were obtained with the permission of patients with ESCC who had not been treated with any preoperative chemoradiotherapy before esophagectomy. The use of tumor tissues was in accordance with the guidelines of the Committees for Ethical Review of Research at Hangzhou Cancer Hospital (Hangzhou, China). The isolation of CAFs in ESCC was accomplished according to the method described in our previous study (8). CAFs were cultured in RPMI1640 medium supplemented with 10% FBS.

Preparation of the conditioned medium

CAFs were seeded into 75-cm^2 culture flask. Forty-eight hours later, when the cells were grown at approximately 80% confluence, the culture medium was collected and centrifuged at 3,000 rpm at 4°C for 30 minutes. The supernatant was collected as conditioned medium and kept at −80°C until use. The conditioned medium from CAFs was defined as CAFs medium. Normal medium was fresh RPMI1640 medium with 10% FBS.

Culture of ESCC cells

The human ESCC cells KYSE-30, KYSE-140, KYSE-150, KYSE-180, and KYSE-450, as well as normal esophageal epithelial cells Het-1A were kindly provided by Dr. Xiaonan Sun of Zhejiang University Medical College, Zhejiang University (Hangzhou, China) in 2015. The radioresistant esophageal cancer cells KYSE-150R had been established from the parental KYSE-150 cells by multiple fractionated irradiations (12). Cells were cultured in RPMI1640 medium (Gibco, Life Technologies Inc.) supplemented with 10% FBS (Gibco, Life Technologies Inc.) at 37°C in 5% CO_2/95% air. All of the cells were authenticated and routinely tested for *Mycoplasma* contamination with the MycoAlert Mycoplasma Detection Kit (Lonza). The use of cells was no longer than 2 months after thawing early-passage cells.

Animals and tumor tissues of patients with ESCC

Six-week-old female BALB/c nude mice were purchased from Vital River and maintained under standard conditions in Experimental Animal Center in Zhejiang Chinese Medicine University (Zhejiang, China). All of animal protocols in our study were in accordance with the institutional animal welfare guidelines of Zhejiang Chinese Medicine University (Zhejiang, China). Tumor tissues of patients with ESCC used in our study were collected with the informed consent of each patient. All of human studies in our study were in accordance with Declaration of Helsinki and the guidelines of the Committee for Ethical Review of Research at Hangzhou Cancer Hospital (Hangzhou, China).

In vivo tumor experiments

To develop xenograft tumors in mice, *in vitro* growing KYSE-30 cells and KYSE-150R cells transfected with lentivirus vectors containing DNM3OS siRNA-1 or scrambled siRNA were harvested by treatment with trypsin-EDTA, washed with ice-cold PBS twice, and implanted into the right flanks of female BALB/c nude mice (1.0×10^5 cells in 100 μL PBS). When xenograft tumors had reached a mean diameter of approximately 0.5 cm, the mice were randomly assigned into different groups (7 mice in each group). Tumors were treated with fractionated irradiation at a total dose of 12 Gy, once every 2 days with 2 Gy per fraction for 12 days (experimental group). The tumors without exposure to irradiation were chosen as a control. Each animal was earmarked and followed individually throughout the experiments. Tumor volume (mm^3) was calculated using the following formula: $V\,(mm^3) = A\,(mm) \times B\,(mm)^2/2$, where A and B were the longest and widest diameter of tumor, respectively, and measured every 2 days with a caliper. The day when fractionated irradiation was delivered for the first time was defined as day 1 and the treatment ended on day 12. When the experiment ended, all of the mice were sacrificed according to the institutional guidelines and the tumors were resected and weighted. The tumor inhibition rates (IR) were calculated using the following equation: IR = 100% × (mean tumor weight of control group − mean tumor weight of experimental group)/mean tumor weight of control group.

Statistical analysis

All of the experiments in our study were independently performed in triplicate and the data were presented as mean ± SD. Statistical analyses of 138 patients were performed with χ^2 goodness-of-fit test and the others with Student t test unless otherwise specified using SPSS software 16.0. Differences were considered statistically significant at a level of $P < 0.05$.

Results

LncRNA DNM3OS was highly expressed in esophageal cancer cells by screening lncRNAs PCR array

Because lncRNAs were involved in tumor initiation and progression, our study investigated which lncRNAs had higher expression levels in esophageal cancer cells than in normal esophageal epithelial cells Het-1A. By screening lncRNA PCR array, we detected 38 lncRNAs expressions in three esophageal cancer cells

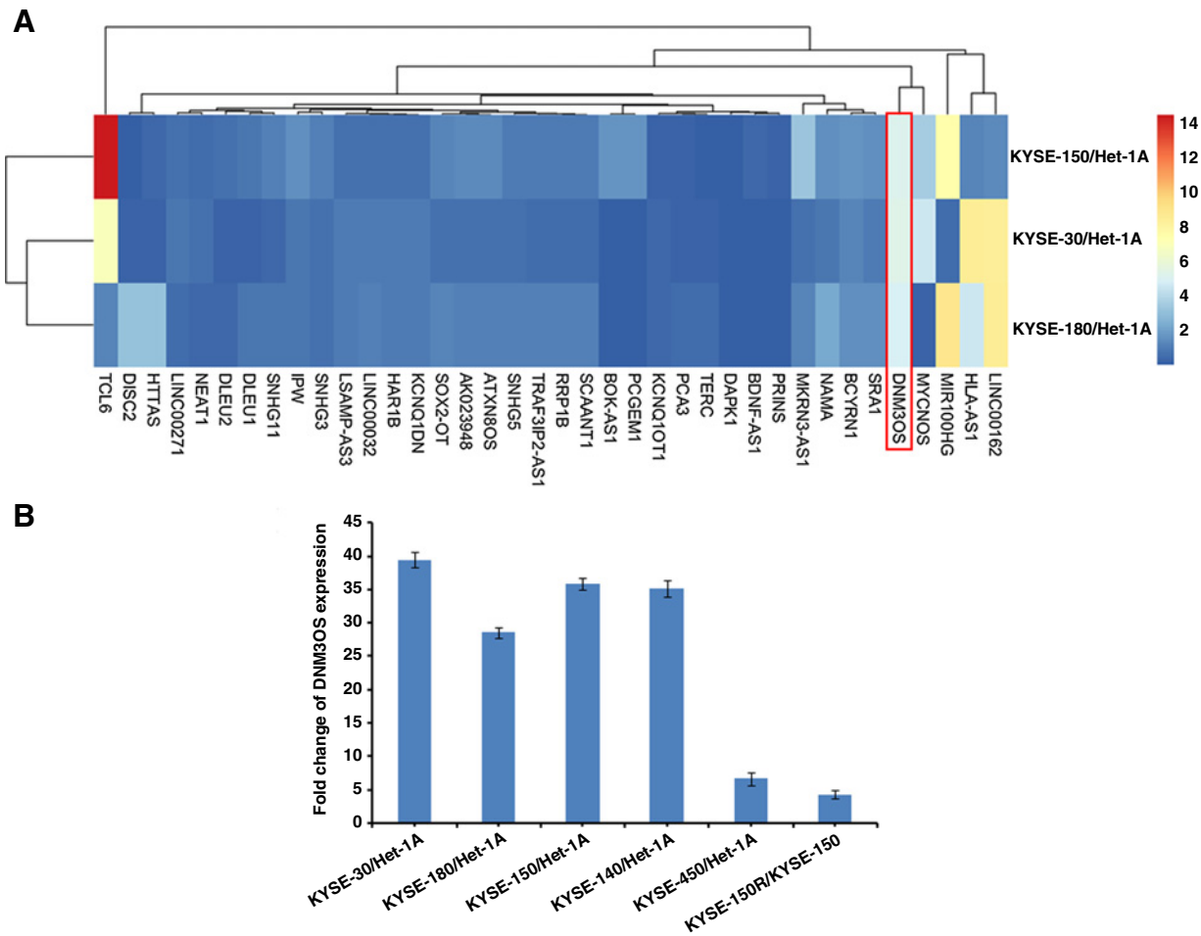

Figure 1.

The screening of lncRNAs PCR array revealed DNM3OS was highly expressed in esophageal cancer cells. **A,** The expressions of 38 lncRNAs in esophageal cancer cells including KYSE-150, KYSE-30, and KYSE-180 compared with that in normal esophageal epithelial cells Het-1A by qRT-PCR analysis, and the heatmap of these lncRNAs expressions was generated with the $\log_2$-transformed data. Blue (red) coloring indicates expressions levels of the genes ($\log_2$ transformed). **B,** The fold change of DNM3OS expression in esophageal cancer cells including KYSE-30, KYSE-180, KYSE-150, KYSE-140, and KYSE-450 compared with that in normal esophageal epithelial cells Het-1A, and in radioresistant KYSE-150R cells compared with that in parental KYSE-150 cells by qRT-PCR analysis.

including KYSE-30, KYSE-150, and KYSE-180, which were often adopted in the literature (13–15), and in normal esophageal epithelial cells Het-1A. As shown in Fig. 1A, among the lncRNAs tested, we found lncRNA DNM3OS had a much higher expression level in esophageal cancer cells than in normal esophageal epithelial cells Het-1A. In KYSE-30 cells, the expression level of DNM3OS showed a 39.4553-fold increase compared with that in Het-1A (Fig. 1B). In KYSE-180 cells, the expression level of DNM3OS showed a 28.5365-fold increase compared with that in Het-1A (Fig. 1B). In KYSE-150 cells, DNM3OS exhibited a 35.8426-fold increase compared with that in Het-1A (Fig. 1B). Furthermore, DNM3OS was also highly expressed in other esophageal cancer cells compared with that in Het-1A (Fig. 1B). Because DNM3OS was highly expressed in esophageal cancer cells, we then investigated its pathologic roles. We compared the expression of DNM3OS in parental KYSE-150 cells and radioresistant KYSE-150R cells, which had been established by multiple fractionated irradiations and used in our previous study (12). The result showed that the expression level of DNM3OS exhibited a 4.3169-fold increase in radioresistant KYSE-150R cells compared with that in parental KYSE-150 cells (Fig. 1B), suggesting that DNM3OS was possibly associated with tumor radioresistance.

LncRNA DNM3OS was highly expressed in tumor tissues of patients with ESCC and associated with tumor stage

To further define the association of DNM3OS and esophageal cancer, we examined the expression of DNM3OS in tumor tissues and matched normal esophageal epithelial tissues of 16 patients with primary ESCC who had not been treated with any chemoradiotherapy before surgery. As shown in Fig. 2A, by use of *in situ* hybridization assay, we found DNM3OS had a much higher expression level in tumor tissues of patients with ESCC than in matched normal esophageal epithelial tissues. By qRT-PCR analysis, DNM3OS was found to be expressed with an average 6.3429-fold increase in tumor tissues compared with that in matched normal esophageal epithelial tissues (Fig. 2B). We further

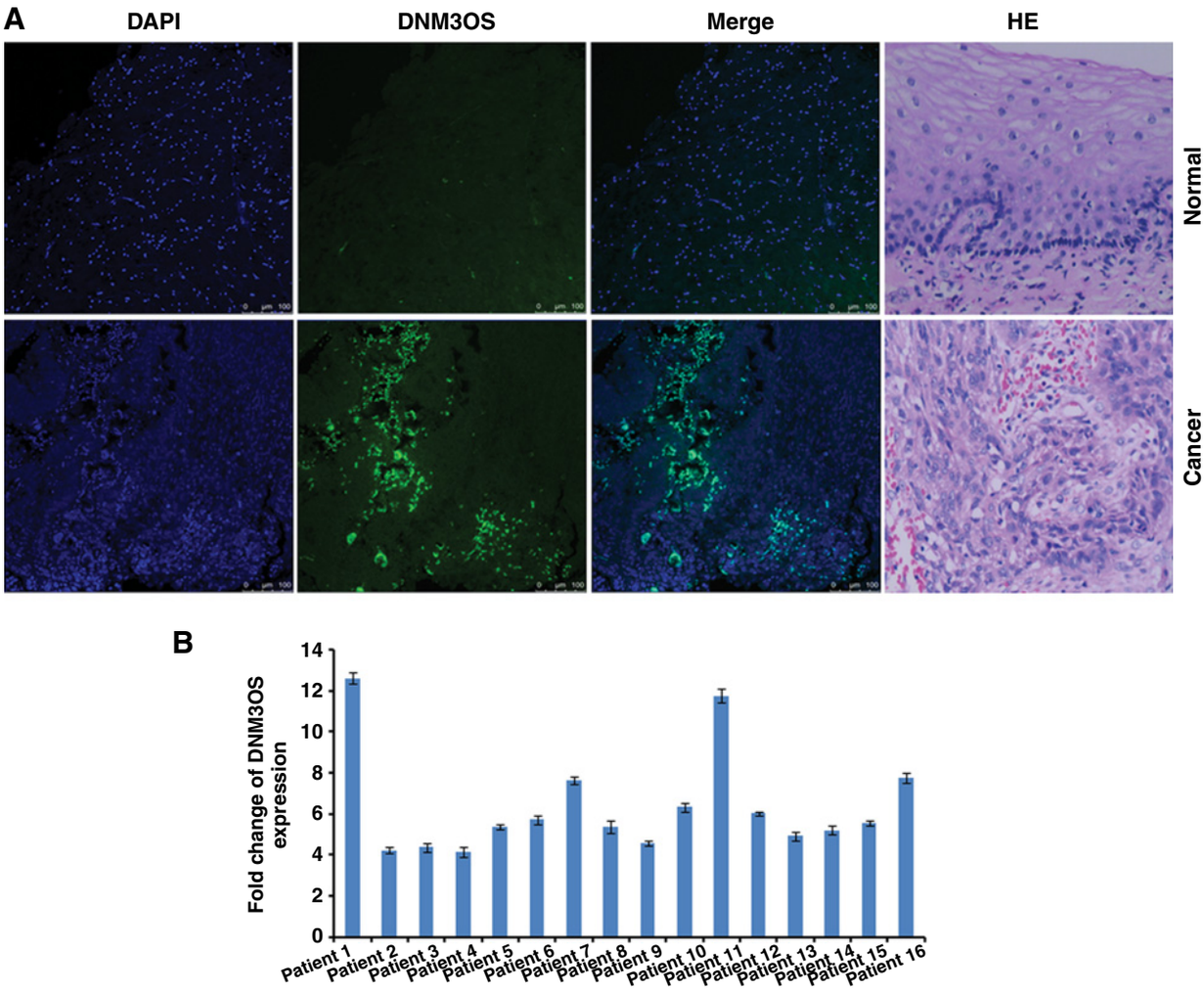

Figure 2.

DNM3OS was highly expressed in tumor tissues of patients with ESCC. **A,** One representative result of DNM3OS expression in tumor tissues of patients with ESCC compared with that in matched normal esophageal epithelial tissues by *in situ* hybridization assay. **B,** The fold change of DNM3OS expression in tumor tissues of 16 patients with ESCC compared with that in matched normal esophageal epithelial tissues by qRT-PCR analysis.

investigated the clinical significance of DNM3OS in patients with ESCC. By use of *in situ* hybridization assay, we detected the expression of DNM3OS in tumor tissues of 138 patients with ESCC with the patients' information shown in Supplementary Table S1. The results showed that the expression level of DNM3OS in tumor tissues was significantly associated with tumor stage ($P = 0.018$), suggesting that DNM3OS was closely associated with tumor progression.

LncRNA DNM3OS conferred significant radioresistance *in vitro* and *in vivo*

By clonogenic survival assay, we investigated the effect of DNM3OS on the radiosensitivity of esophageal cancer cells. When DNM3OS was stably downregulated by transfection with lentivirus vectors containing DNM3OS siRNA-1, the radiosensitivity of KYSE-30 cells and KYSE-140 cells was significantly increased (Fig. 3A and B; Supplementary Fig. S1). The dose enhancement ratios (DER_{10}) defined as the ratio of doses required to achieve 10% surviving fraction for indicated cells were determined. The DER_{10} for KYSE-30 cells without or with siRNA-1 transfection was 3.4492 (Fig. 3A). The DER_{10} for KYSE-140 cells without or with siRNA-1 transfection was 2.589 (Fig. 3B). Because DNM3OS was involved in the radioresponse of KYSE-30 cells and KYSE-140 cells, we then investigated whether irradiation affected the expression of DNM3OS in these cells. As shown in Supplementary Fig. S2, 24 hours after exposure to irradiation, the expression level of DNM3OS showed an increase of 1.0396-fold and 3.7442-fold in KYSE-30 cells and in KYSE-140 cells, respectively, suggesting that irradiation had differential influences on DNM3OS expression in cancer cells, and irradiation-induced high DNM3OS expression may also be involved in the radioresponse of KYSE-140 cells. In addition to KYSE-30 cells and KYSE-140 cells, we also investigated the radiosensitivity of KYSE-150 cells, KYSE-150R cells, KYSE-450 cells, and Het-1A cells in which the expression levels of DNM3OS

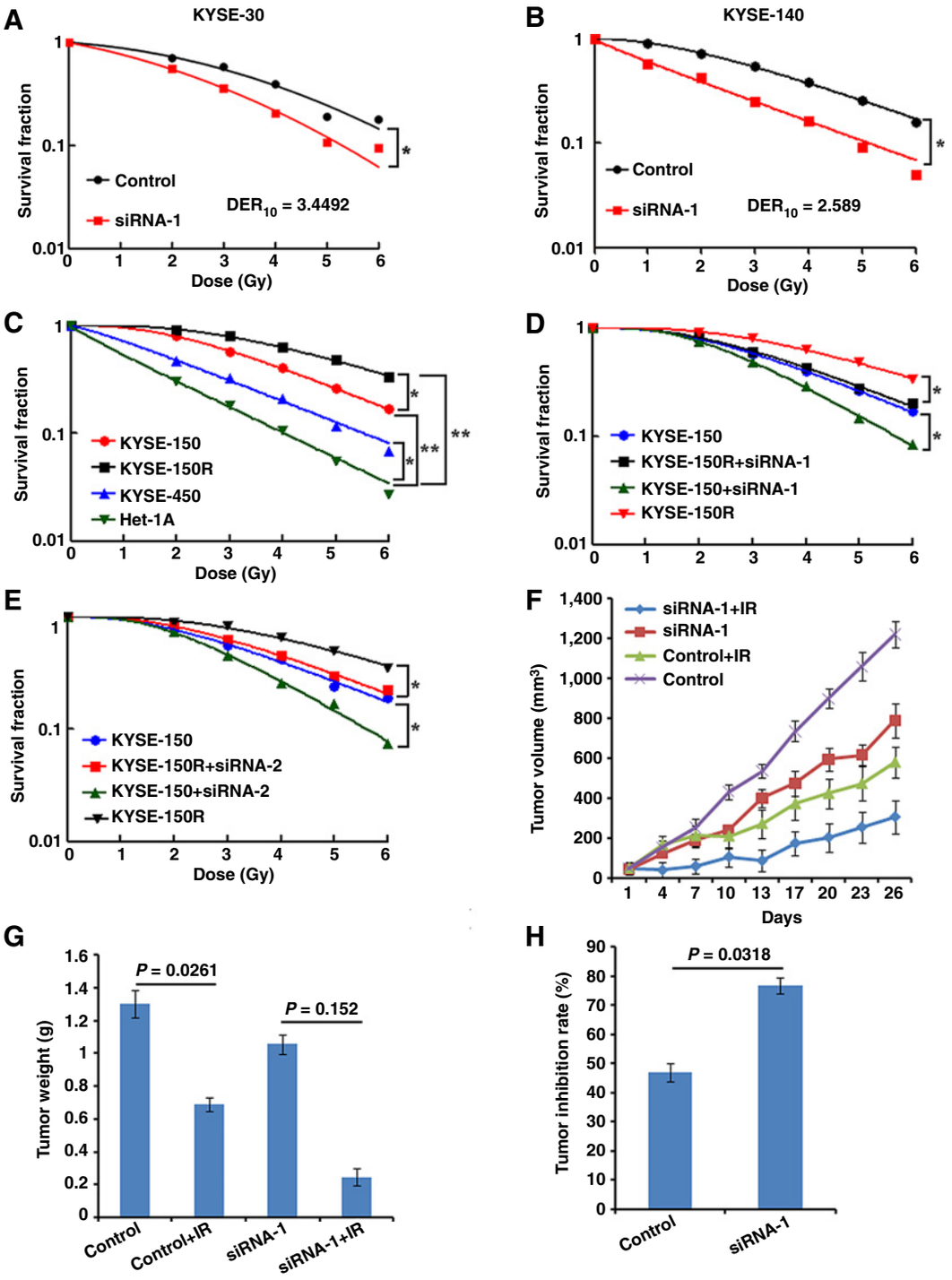

Figure 3.

DNM3OS conferred significant radioresistance *in vitro* and *in vivo*. Radiation survival curves are shown for KYSE-30 cells (**A**) and KYSE-140 cells (**B**) which were transfected with lentivirus vectors containing DNM3OS siRNA-1 or scrambled siRNA (Control). *, $P < 0.05$, using ANOVA. The DER_{10} was defined as the ratio of doses required to achieve 10% surviving fraction for cells without or with siRNA transfection. **C,** Radiation survival curves are shown for KYSE-150 cells, KYSE-150R cells, KYSE-450 cells, and Het-1A cells. *, $P < 0.05$, using ANOVA. **D** and **E,** Radiation survival curves are shown for KYSE-150 cells and KYSE-150R cells with or without siRNA transfection. *, $P < 0.05$, using ANOVA. **F,** The growth curve of xenograft tumors implanted with KYSE-30 cells which were transfected with lentivirus vectors containing DNM3OS siRNA-1 or scrambled siRNA (Control). The day when fractionated irradiation (FIR) was delivered for the first time was defined as day 1 and the treatment ended on day 12. **G,** The weight of xenograft tumors in **F** at the end of experiment. **H,** The tumor inhibition rate of fractionated irradiation in xenograft tumors transfected with DNM3OS siRNA-1 or scrambled siRNA (Control).

were significantly different. As shown in Fig. 3C, the expression levels of DNM3OS were negatively correlated with the radiosensitivity. KYSE-150R cells with the highest expression level of DNM3OS showed the most radioresistance, whereas Het-1A cells with the lowest expression level of DNM3OS were the most radiosensitive. The DER_{10} was 3.1742, 19.9795, 6.2943, and 3.3456 for KYSE-150R versus KYSE-150, KYSE-150R versus Het-1A, KYSE-150 versus Het-1A, and KYSE-450 versus Het-1A, respectively. When lentivirus vectors containing DNM3OS siRNA-1 or siRNA-2 were transfected, the radiosensitivity of KYSE-150 cells and KYSE-150R cells was both significantly increased (Fig. 3D and E; Supplementary Fig. S1). The DER_{10} for KYSE-150R cells without or with DNM3OS siRNA-1/siRNA-2 transfection was 2.6399 and 2.6165, respectively. The DER_{10} for KYSE-150 cells without or with DNM3OS siRNA-1/siRNA-2 transfection was 4.1698 and 5.0445, respectively. These results suggested that DNM3OS conferred esophageal cancer cells significant radioresistance *in vitro*.

Our study further investigated the effect of DNM3OS on the radiosensitivity *in vivo* by the establishment of xenograft tumor models in BALB/c nude mice. As shown in Fig. 3F, when lentivirus vectors containing DNM3OS siRNA-1 were transfected, KYSE-30 xenograft tumors grew slower than control group after fractionated irradiation (Fig. 3F). At the end of experiment, the tumors were weighted (Fig. 3G). The tumor IR of fractionated irradiation in siRNA-transfected group was 76.5900%, which was significantly higher than 47.0330% in control group (Fig. 3H). We also investigated the radiosensitivity of KYSE-150R xenograft tumors in which DNM3OS was stably downregulated by transfection with DNM3OS siRNA. As shown in Supplementary Fig. S3A, when lentivirus vectors containing DNM3OS siRNA-1 were transfected, KYSE-150R xenograft tumors grew slower than control group after fractionated irradiation. At the end of experiment, the tumors were weighted (Supplementary Fig. S3B) and the tumor IR was calculated. As shown in Supplementary Fig. S3C, the tumor IR of fractionated irradiation in siRNA-transfected group was significantly higher than control group. These results suggested that DNM3OS conferred esophageal cancer cells significant radioresistance *in vivo*.

LncRNA DNM3OS regulated cellular DDR in esophageal cancer cells

Because DDR directly regulates cell fate in response to death stimuli, our study investigated whether DDR was involved in DNM3OS-regulated radioresponse. DNA double-strand breaks (DSB) are the major form of cellular damage induced by irradiation. Herein, we detected the expression of γH2AX, a marker of DSBs following irradiation in KYSE-30 cells and in KYSE-140 cells, which were transfected with DNM3OS siRNA or scrambled siRNA (negative) before the exposure to irradiation. As shown in Supplementary Figs. S4 and S5, when DNM3OS siRNA-1 was transfected, KYSE-30 cells and KYSE-140 cells showed increased γH2AX expression following irradiation, suggesting that the inhibition of DNM3OS expression enhanced irradiation-induced DNA damage. As shown in Fig. 4A and B and Supplementary Fig. S6, when DNM3OS siRNA-1 was transfected, KYSE-30, KYSE-140, and KYSE-150R exhibited increased expressions of DNA damage markers γH2AX and cleaved PARP, but decreased expressions of DNA repair proteins including p-ATM, Rad50, p-Chk2, Ku80, MRE11, NBS1, DNA-PKcs, and P53 following irradiation. These results suggested that the inhibition of DNM3OS expression enhanced irradiation-induced DNA damage, but attenuated DNA repair response. By the comet assay, we also confirmed DNM3OS regulated DDR in KYSE-30 cells and KYSE-140 cells. As shown in Fig. 4C and D, when DNM3OS siRNA-1 was transfected, the DNA repair ability of KYSE-30 cells and KYSE-140 cells was significantly inhibited following irradiation. These results suggested that DNM3OS regulated DDR in esophageal cancer cells.

CAFs significantly increased the expression of lncRNA DNM3OS in a PDGFβ/PDGFβR/FOXO1 signaling pathway–dependent manner in esophageal cancer cells

CAFs, as one major component of tumor microenvironment, were involved in tumor radioresponse as evidenced by our previous study (8). In this study, we investigated whether CAFs regulated the expression of DNM3OS in esophageal cancer cells. When KYSE-30 cells and KYSE-140 cells were cultured in the conditioned medium (CM) of CAFs for 48 hours, the expression of DNM3OS was significantly upregulated, with a 39.2554-fold and 38.3163-fold increase, respectively, compared with that cultured in normal medium (Fig. 5A). We then investigated the effect of CAFs-induced high DNM3OS expression on the radiosensitivity of KYSE-30 cells and KYSE-140 cells. As shown in Fig. 6, after the treatment with CAF CM for 48 hours, KYSE-30 cells and KYSE-140 cells displayed significant radioresistance compared with that cultured in normal medium. The DER_{10} was 3.3015 for KYSE-30 cells cultured in CAF medium versus cultured in normal medium. The DER_{10} was 1.8368 for KYSE-140 cells cultured in CAF medium versus cultured in normal medium. In contrast, when KYSE-30 cells and KYSE-140 cells were transfected with DNM3OS siRNA before the treatment with CAF CM, their increase of radioresistance was partially reversed. The DER_{10} was 2.6687 and 3.2615 for KYSE-30 cells without or with DNM3OS siRNA-1/siRNA-2 transfection before the treatment with CAF CM, respectively. The DER_{10} was 1.7127 and 1.7268 for KYSE-140 cells without or with DNM3OS siRNA-1/siRNA-2 transfection before the treatment with CAF CM, respectively. These results suggested that CAFs conferred esophageal cancer cells significant radioresistance by promoting DNM3OS expression.

We then explored the molecular mechanisms by which CAFs promoted DNM3OS expression in esophageal cancer cells. As shown in Fig. 5B and C and Supplementary Table S2, by screening 14 kinds of small-molecule kinase inhibitors that target multiple critical kinases, we found crenolanib, an inhibitor of PDGFRα/β signaling, had potent ability of inhibiting DNM3OS expression. When KYSE-30 cells and KYSE-140 cells were cultured in CAFs CM, which contained crenolanib, the expression of DNM3OS was decreased to 42.19% and 37.15%, respectively, compared with cells cultured in CAF CM without crenolanib (Fig. 5B and C). We then examined the expressions of PDGFRα, PDGFRβ, PDGFα, and PDGFβ in KYSE-30 cells and in KYSE-140 cells, which were cultured in CAFs CM for 48 hours. The results showed that the expressions of PDGFRβ and PDGFβ were highly upregulated, with a 6.5812-fold and 8.1062-fold increase, respectively, in KYSE-30 cells, which were cultured in CAF CM for 48 hours (Fig. 5D). Similarly, PDGFRβ and PDGFβ were expressed with a 5.1694-fold and 6.4225-fold increase, respectively, in KYSE-140 cells, which were cultured in CAF CM for 48 hours (Fig. 5E).

To further confirm whether the PDGFβ/PDGFRβ signaling pathway was associated with CAF-induced DNM3OS upregulation, we detected the expression of DNM3OS in KYSE-30 cells and KYSE-140 cells, which were transfected with PDGFRβ or PDGFβ

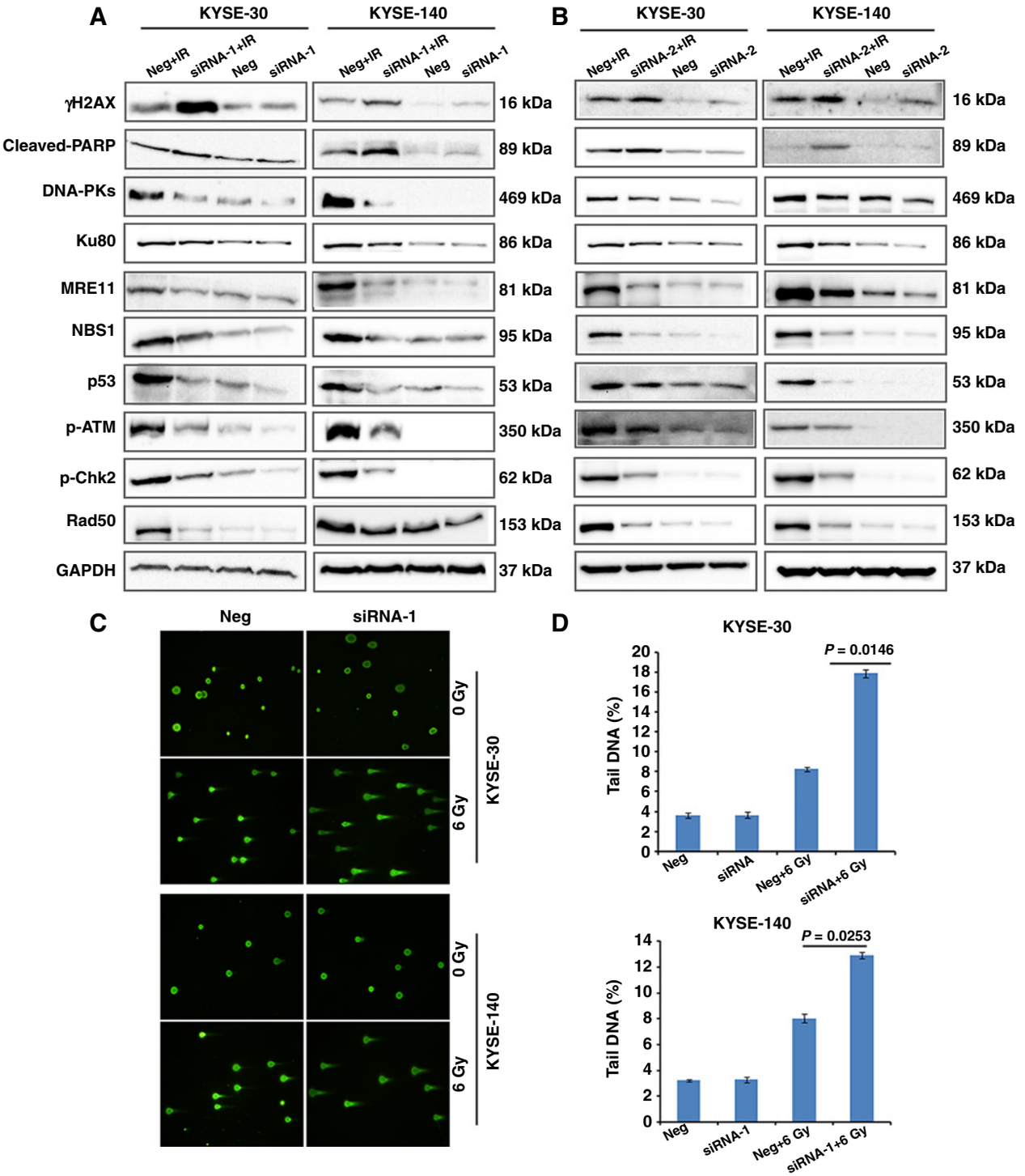

Figure 4.

DNM3OS regulated cellular DDR in esophageal cancer cells. **A** and **B,** The expressions of DDR proteins including γH2AX, cleaved-PARP, DNA-PKcs, Ku80, MRE11, NBS1, p53, p-ATM, p-Chk2, and Rad50 24 hours following 6 Gy of irradiation in KYSE-30 cells and in KYSE-140 cells, which were transfected with DNM3OS siRNA-1/siRNA-2 or scrambled siRNA (negative, Neg) by Western blotting analysis. GAPDH was used as a loading control. **C,** Irradiation-induced DNA damage in KYSE-30 cells and in KYSE-140 cells which were transfected with DNM3OS siRNA-1 or scrambled siRNA (negative, Neg) by the comet assay. **D,** Quantitative analysis of irradiation-induced DNA damage in **C** by the tail moment in the comet assay.

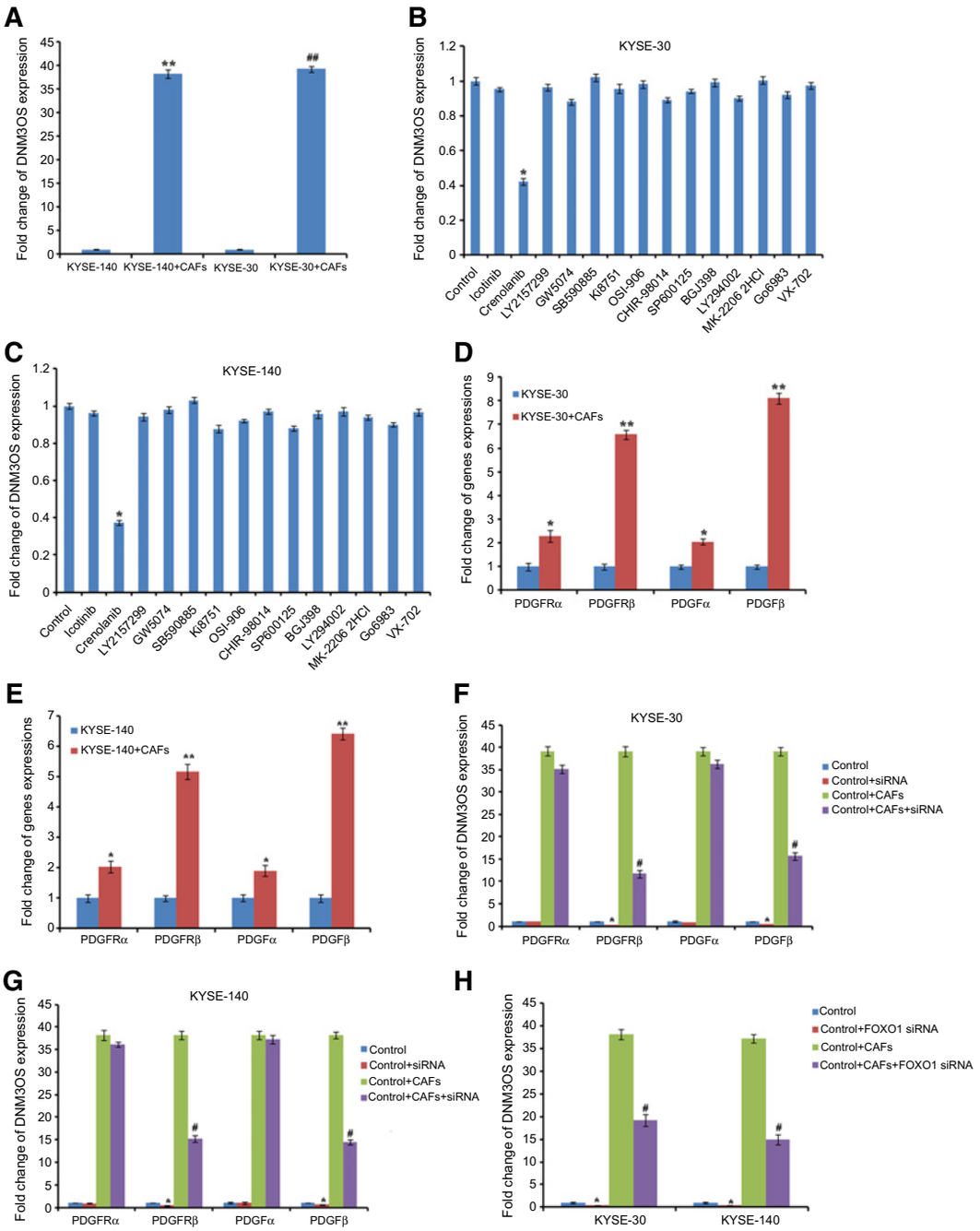

Figure 5.

CAFs promoted the expression of DNM3OS in esophageal cancer cells in a PDGFβ/PDGFRβ/FOXO1 signaling pathway–dependent manner. **A,** qRT-PCR analysis of DNM3OS expression in KYSE-140 cells and in KYSE-30 cells that were cultured in normal medium or CAFs medium for 48 hours. **, $P < 0.01$; ##, $P < 0.01$, compared with KYSE-140 cells and KYSE-30 cells that were cultured in normal medium, respectively. **B** and **C,** qRT-PCR analysis of DNM3OS expression in KYSE-30 cells and KYSE-140 cells that were cultured in medium with or without kinase inhibitors. The cells without kinase inhibitor treatment were used as a control. *, $P < 0.05$, compared with control group. **D** and **E,** qRT-PCR analysis of the expressions of PDGFRα, PDGFRβ, PDGFα, and PDGFβ in KYSE-30 cells and KYSE-140 cells which were cultured in normal medium or CAFs medium for 48 hours. *, $P < 0.05$; **, $P < 0.01$, compared with KYSE-30 cells or KYSE-140 cells that were cultured in normal medium for 48 hours. **F** and **G,** qRT-PCR analysis of DNM3OS expression in KYSE-30 cells and KYSE-140 cells that were transfected with indicated siRNA before the treatment with CAFs medium for 48 hours. The cells that were transfected with scrambled siRNA were used as a control. *, $P < 0.05$; #, $P < 0.05$, compared with control group and control group cultured in CAFs medium, respectively. **H,** qRT-PCR analysis of DNM3OS expression in KYSE-30 cells and KYSE-140 cells that were transfected with FOXO1 siRNA before the treatment with normal medium or CAFs medium for 48 hours. The cells which were transfected with scrambled siRNA were used as a control. *, $P < 0.05$; #, $P < 0.05$, compared with control group and control group cultured in CAFs medium, respectively.

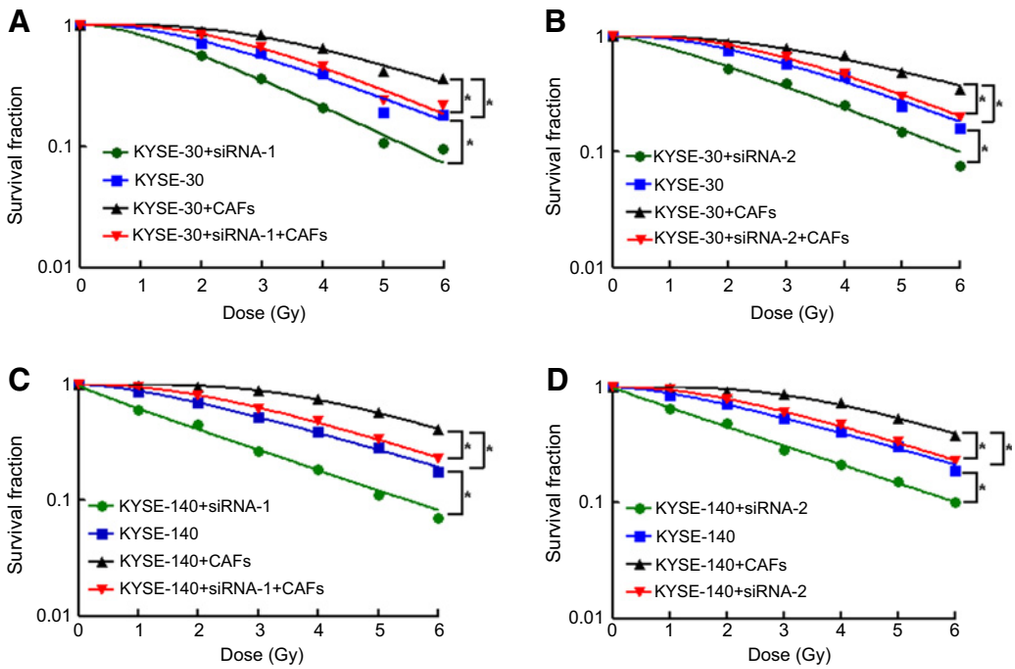

Figure 6.

CAF-induced DNM3OS upregulation significantly increased the radioresistance of esophageal cancer cells. Radiation survival curves are shown for KYSE-30 cells (**A** and **B**) and KYSE-140 cells (**C** and **D**) that were transfected with lentivirus vectors containing DNM3OS siRNA or scrambled siRNA (Control) before the treatment with normal medium or CAFs medium for 48 hours. *, $P < 0.05$, using ANOVA.

siRNA before the treatment with CAF CM. As shown in Fig. 5F and Supplementary Fig. S7, when KYSE-30 cells were transfected with PDGFRβ siRNA before the treatment with CAF CM, the increase of DNM3OS expression was decreased from 39.106-fold to 11.732-fold compared with cells transfected with scramble siRNA. When KYSE-30 cells were transfected with PDGFβ siRNA before the treatment with CAF CM, the increase of DNM3OS expression was decreased from 39.106-fold to 15.642-fold compared with cells transfected with scramble siRNA (Fig. 5F; Supplementary Fig. S7). When KYSE-140 cells were transfected with PDGFRβ siRNA before the treatment with CAF CM, the increase of DNM3OS expression was decreased from 38.204-fold to 15.282-fold compared with cells transfected with scramble siRNA (Fig. 5G; Supplementary Fig. S7). When KYSE-140 cells were transfected with PDGFβ siRNA before the treatment with CAF CM, the increase of DNM3OS expression was decreased from 38.204-fold to 14.518-fold compared with cells transfected with scramble siRNA (Fig. 5G; Supplementary Fig. S7). However, transfection with siRNA against PDGFRα or PDGFα had no significant reversal effects on CAF-induced DNM3OS upregulation in KYSE-30 cells and in KYSE-140 cells (Fig. 5F and G; Supplementary Fig. S7).

There were several transcription factors downstream of the PDGFβ/PDGFRβ signaling pathway (16). By use of promoter prediction tool eGPMiner, FOXO1, a transcription factor downstream of the PDGFβ/PDGFRβ signaling pathway, was predicted to have high affinity with two regions in the DNM3OS promoter. Our previous study had confirmed the expression of FOXO1 was obviously increased at protein level in esophageal cancer cells, which were cultured in CAF CM (7). Herein, we investigated whether FOXO1 was involved in CAF-induced DNM3OS upregulation. When KYSE-30 cells were transfected with FOXO1 siRNA before the treatment with CAF CM, the increase of DNM3OS expression was decreased from 38.162-fold to 19.261-fold compared with cells transfected with scrambled siRNA (Fig. 5H). When KYSE-140 cells were transfected with FOXO1 siRNA before the treatment with CAF CM, the increase of DNM3OS expression was decreased from 37.251-fold to 14.902-fold compared with cells transfected with scrambled siRNA (Fig. 5H). These results suggested that FOXO1 was involved in CAF-induced DNM3OS upregulation. By chromatin immunoprecipitation (ChIP) assay, we analyzed whether FOXO1 regulated the transcription of DNM3OS by binding to its promoter region. As shown in Fig. 7A and B, FOXO1 exhibited strong ability to bind to two regions in the DNM3OS promoter in KYSE-30 cells and in KYSE-140 cells. We further investigated whether FOXO1 initiated the transcription of DNM3OS when binding to the promoter regions of DNM3OS using luciferase reporter gene assay. As shown in Fig. 7C, when FOXO1 cDNA was transfected, the transcription activity of DNM3OS promoter in HEK293 cells was obviously increased, suggesting FOXO1 could initiate the transcription of DNM3OS upon binding to its promoter regions. Together, these results suggested that CAFs promoted DNM3OS expression in a PDGFβ/ PDGFRβ/FOXO1 signaling pathway–dependent manner in esophageal cancer cells.

Discussion

During the past few years, one of the most important findings of the genomics era of biology is that less than 2% of the human genome is coding sequences, whereas more than 90% of the

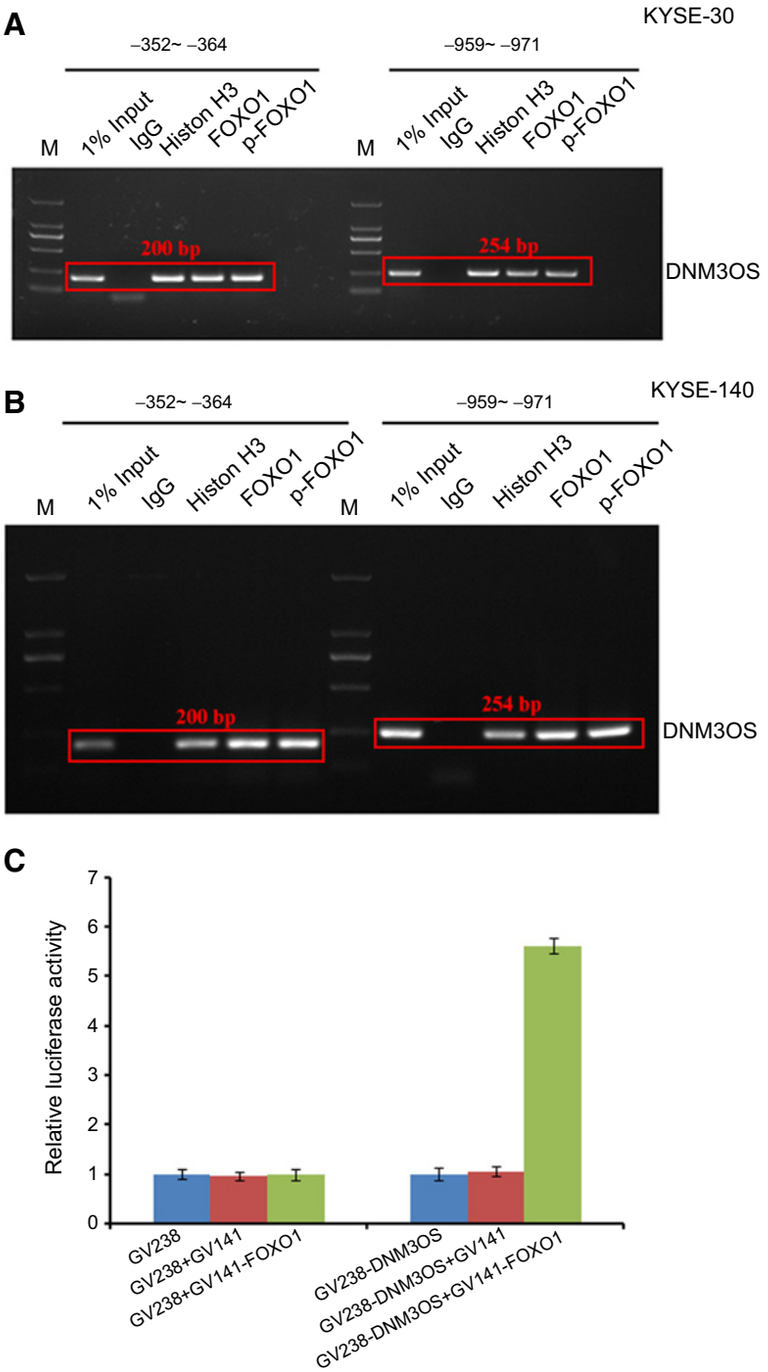

Figure 7.

FOXO1 regulated the expression of DNM3OS by binding to the promoter as a transcription factor. **A** and **B,** The binding activity of FOXO1 and p-FOXO1 to two regions (−352 ∼ −364; −951 ∼ −971) in the DNM3OS promoter was assessed by ChIP assay. The antibodies against IgG and Histone H3 were used as a negative and positive control, respectively. **C,** Relative luciferase activity in HEK293 cells transfected with plasmids of DNM3OS promoter (GV238-DNM3OS), empty GV238 vector, GV141-FOXO1, or empty GV141 vector, alone or in their combinations.

genome is transcribed into nonprotein-coding RNA (17). This suggests those nonprotein-coding RNAs such as lncRNAs, and miRNAs exist mainly as regulatory RNAs in human. LncRNAs are endogenous RNAs with more than 200 nucleotides in length. These RNAs control many cellular functions by interaction with chromatin-modifying complex, regulation of transcriptional enhancers, and posttranscriptional regulation (18). LncRNAs were found to be involved in DDR by regulating the expressions of DDR relevant genes, and the inhibition of lncRNAs expressions improved the radiosensitivity of some cancers (19–22). Our study investigated whether lncRNAs were involved in the radioresponse in esophageal cancer cells for the first time. By PCR screening of 38 cancer-related lncRNAs, we found DNM3OS had a much higher expression level in esophageal cancer cells and tumor tissues of patients with ESCC than in normal esophageal epithelial cells Het-1A and matched normal esophageal epithelial tissues, respectively. Furthermore, we showed that DNM3OS had a much higher expression level in radioresistant KYSE-150R cells than in parental

KYSE-150 cells, suggesting that DNM3OS may be involved in tumor radioresponse.

Loss-of-function studies showed that the downregulation of DNM3OS expression significantly enhanced the radiosensitivity of esophageal cancer cells both *in vitro* and *in vivo*. DNA DSBs are the major form of irradiation-induced DNA damage (23). Following irradiation, γH2AX, a marker of DNA DSBs, is highly expressed and initiates the activation of DDR in cancer cells. γH2AX interacts with the MRE11-RAD50–NBS1 (MRN) complex, leading to the activation of ataxia telangiectasia–mutated (ATM) kinase and/or ataxia telangiectasia and Rad3-related (ATR) kinase. ATM and ATR kinases phosphorylate their downstream target genes such as *BRCA1*, *p53*, *Chk1*, and *Chk2* to induce cell-cycle arrest and/or cell apoptosis (24). Our study revealed that the downregulation of DNM3OS expression significantly increased irradiation-induced DNA damage, but inhibited DNA repair response by decreasing the expressions of DNA repair proteins. Therefore, we proposed that DNM3OS conferred ESCC radioresistance possibly through regulating DDR.

The members of tumor microenvironment, such as CAFs, transfer energy and biomass to support tumor cells growth and survival in response to death stimulus (3). In parallel, tumor cells facilitate the transformation of normal fibroblasts into CAFs, which in turn enhance the malignant ability of tumor cells (4, 25). Our previous studies revealed that CAFs were involved in tumor chemoradioresponse by secretion of chemokines and cytokines (7, 8). We found that the cross-talk between CAFs and tumor cells caused the expressions of CXCL1 and TGFβ1 in an autocrine/paracrine signaling loop. These findings suggest that the crosstalk between CAFs and tumor cells exerted significant influences on the expression patterns of genes. Previous studies focused on the pathologic roles of lncRNAs that were expressed in tumor cells or CAFs. However, whether CAFs regulated the expressions of functional lncRNAs in tumor cells has never been studied. Our study revealed that CAFs upregulated the expression of lncRNA DNM3OS in KYSE-30 cells and KYSE-140 cells with an increase of 39.2554-fold and 38.3163-fold, respectively, suggesting that CAFs were the major reason for the high expression of DNM3OS in esophageal cancer cells.

By screening 14 types of kinase inhibitors that target many critical kinases, we found crenolanib, an inhibitor of PDGFRα/β signaling pathway, significantly attenuated CAF-induced DNM3OS upregulation in esophageal cancer cells. We further explored the direct evidence that the PDGFRα/β signaling pathway was involved in CAF-induced DNM3OS upregulation. Upon inhibition of PDGFβ or PDGFRβ expression in tumor cells by transfection with specific siRNAs, CAF-induced DNM3OS upregulation was significantly attenuated, suggesting that the PDGFβ/PDGFRβ pathway played a direct role in CAF-induced DNM3OS upregulation. FOXO1, a transcription factor downstream of PDGFβ/PDGFRβ signaling pathway, activated the transcription of DNM3OS by binding to the promoter regions of DNM3OS. These results suggested that CAFs promoted the expression of DNM3OS in esophageal cancer cells in a PDGFβ/ PDGFRβ/ FOXO1 signaling pathway–dependent manner. Taken together, our study highlighted CAF-promoted DNM3OS as an attractive therapeutic target to reverse radioresistance in ESCC and these findings might provide novel insights into CAF-induced tumor radioresistance.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: S. Wu, H. Zhang
Development of methodology: Y. Hua, M. Shi, X. Zheng, R. Zhou
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Zhang, Z. Jiang, J. Yue
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Hua, M. Shi, R. Zhou, L. Yang
Writing, review, and/or revision of the manuscript: S. Wu, H. Zhang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Shi, R. Zhou

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References

1. Minsky BD, Pajak TF, Ginsberg RJ, Pisansky TM, Martenson J, Komaki R, et al. INT 0123 (Radiation Therapy Oncology Group 94-05) phase III trial of combined-modality therapy for esophageal cancer: high-dose versus standard-dose radiation therapy. J Clin Oncol 2002;20:1167–74.
2. Ostman A, Augsten M. Cancer-associated fibroblasts and tumor growth - bystanders turning into key players. Curr Opin Genet Dev 2009;19:67–73.
3. Martinez-Outschoorn UE, Lisanti MP, Sotgia F. Catabolic cancer-associated fibroblasts transfer energy and biomass to anabolic cancer cells, fueling tumor growth. Semin Cancer Biol 2014;25:47–60.
4. Yang Z, Yang X, Xu S, Jin P, Li X, Wei X, et al. Reprogramming of stromal fibroblasts by SNAI2 contributes to tumor desmoplasia and ovarian cancer progression. Mol Cancer 2017;16:163.
5. Glentis A, Oertle P, Mariani P, Chikina A, El Marjou F, Attieh Y, et al. Cancer-associated fibroblasts induce metalloprotease-independent cancer cell invasion of the basement membrane. Nat Commun 2017;8:924.
6. Sun Y, Campisi J, Higano C, Beer TM, Porter P, Coleman I, et al. Treatment-induced damage to the tumor microenvironment promotes prostate cancer therapy resistance through WNT16B. Nat Med 2012;18:1359–68.
7. Zhang HF, Xie CH, Yue J, Jiang ZZ, Zhou RJ, Xie RF, et al. Cancer-associated fibroblasts mediated chemoresistance by a FOXO1/TGF1 signaling loop in esophageal squamous cell carcinoma. Mol Carcinog 2017;56:1150–63.
8. Zhang HF, Yue J, Jiang ZZ, Zhou RJ, Xie RF, Xu YP, et al. CAF-secreted CXCL1 conferred radioresistance by regulating DNA damage response in a ROS-dependent manner in esophageal squamous cell carcinoma. Cell Death Dis 2017;8:e2790.

9. Marchese FP, Raimondi I, Huarte M. The multidimensional mechanisms of long noncoding RNA function. Genome Biol 2017;18:206.
10. Hosono Y, Niknafs YS, Prensner JR, Iyer MK, Dhanasekaran SM, Mehra R, et al. Oncogenic role of THOR, a conserved cancer/testis long non-coding RNA. Cell 2017;171:1559–72.
11. Shen L, Wang Q, Liu R, Chen Z, Zhang X, Zhou P, et al. LncRNA lnc-RI regulates homologous recombination repair of DNA double-strand breaks by stabilizing RAD51 mRNA as a competitive endogenous RNA. Nucleic Acids Res 2018;46:717–29.
12. Jing Z, Gong L, Xie CY, Zhang L, Su HF, Deng X, et al. Reverse resistance to radiation in KYSE-150R esophageal carcinoma cell after epidermal growth factor receptor signal pathway inhibition by cetuximab. Radiother Oncol 2009;93:468–73.
13. Sutter AP, Hopfner M, Huether A, Maaser K, Scherubl H. Targeting the epidermal growth factor receptor by erlotinib (Tarceva) for the treatment of esophageal cancer. Int J Cancer 2006;118:1814–22.
14. Al-Taie OH, Graf T, Illert B, Katzenberger T, Mork H, Kraus MR, et al. Differential effects of PPARgamma activation by the oral antidiabetic agent pioglitazone in Barrett's carcinoma *in vitro* and *in vivo*. J Gastroenterol 2009;44:919–29.
15. Yang C, Zheng S, Liu T, Liu Q, Dai F, Zhou J, et al. Down-regulated miR-26a promotes proliferation, migration, and invasion via negative regulation of MTDH in esophageal squamous cell carcinoma. FASEB J 2017;31:2114–22.
16. Farooqi AA, Siddik ZH. Platelet-derived growth factor (PDGF) signalling in cancer: rapidly emerging signalling landscape. Cell Biochem Funct 2015; 33:257–65.
17. Tehrani SS, Karimian A, Parsian H, Majidinia M, Yousefi B. Multiple functions of long non-coding RNAs in oxidative stress, DNA damage response and cancer progression. J Cell Biochem 2018;119:223–36.
18. Schmitt AM, Chang HY. Long noncoding RNAs in cancer pathways. Cancer Cell 2016;29:452–63.
19. Wang XY, Li M, Wang ZQ, Han SC, Tang XH, Ge YX, et al. Silencing of long noncoding RNA MALAT1 by miR-101 and miR-217 inhibits proliferation, migration, and invasion of esophageal squamous cell carcinoma cells. J Biol Chem 2015;290:3925–35.
20. Shen YM, Liu YY, Sun T, Yang W. LincRNA-p21 knockdown enhances radiosensitivity of hypoxic tumor cells by reducing autophagy through HIF-1/Akt/mTOR/P70S6K pathway. Exp Cell Res 2017;358:188–98.
21. Jiao Y, Liu C, Cui FM, Xu JY, Tong J, Qi XF, et al. Long intergenic non-coding RNA induced by X-ray irradiation regulates DNA damage response signaling in the human bronchial epithelial BEAS-2B cell line. Oncol Lett 2015;9:169–76.
22. Lai Y, Chen Y, Lin YH, Ye L. Down-regulation of LncRNA CCAT1 enhances radiosensitivity via regulating miR-148b in breast cancer. Cell Biol Int 2018;42:227–36.
23. Srivastava M, Raghavan SC. DNA double-strand break repair inhibitors as cancer therapeutics. Chem Biol 2015;22:17–29.
24. Hosoya N, Miyagawa K. Targeting DNA damage response in cancer therapy. Cancer Sci 2014;105:370–88.
25. Fang T, Lv HW, Lv GS, Li T, Wang CZ, Han Q, et al. Tumor-derived exosomal miR-1247-3p induces cancer-associated fibroblast activation to foster lung metastasis of liver cancer. Nat Commun 2018;9:191.