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LETTER TO THE EDITOR

SMAD4 depletion enhances NHEJ by regulating BRCA1 expression through ARIH1 in pancreatic cancer



KEY WORDS

SMAD4;
BRCA1;
Pancreatic cancer;
Non-homologous end joining;
Homologous recombination

To the editor:

Pancreatic ductal adenocarcinoma (PDAC) represents the most prevalent form of malignancy affecting the pancreas and is associated with a very grim prognosis¹. PDAC is often diagnosed at advanced stages due to the lack of early symptoms and biomarkers¹. Understanding the molecular mechanisms underlying PDAC initiation and development is vital for improving early diagnosis and treating for the patients². Whole-genome sequencing revealed that SMAD4 is one of the most frequently mutated genes implicated in PDAC progression³. SMAD4 mutations occur in approximately 30%–50% of PDAC tissues and are associated with tumor metastasis, radiotherapy resistance, and poor prognosis^{4,5}.

In this study, we reveal a novel role of SMAD4 in DNA damage repair through regulating BRCA1 expression. SMAD4 directly interacts with BRCA1 within its degron region located in the N-terminus, thereby inhibiting ARIH1-mediated ubiquitination and subsequent degradation of BRCA1. As a result, depletion of SMAD4 inhibited homologous recombination (HR), while promoting non-homologous end joining (NHEJ) in PDAC cells.

To investigate the role of SMAD4 in PDAC, we first analyzed SMAD4 expression in a TCGA PDAC cohort. Intriguingly, we found that patients with low SMAD4 expression levels exhibited significantly higher HR deficiency (HRD) scores, suggesting that SMAD4

might exert a potential function in HR repair (Fig. 1A). Next, we interfered SMAD4 expression in Panc-1 PDAC cells and examined the expressions of HR repair markers. We observed that the expression of BRCA1 was significantly reduced in SMAD4-depleted cells (Fig. 1B). However, the expression levels of other HR repair-related proteins, MRE11 and RAD50, remained unchanged in SMAD4-depleted PDAC cells (Fig. 1B). Moreover, the expression levels of SMAD4 were positively correlated with those of BRCA1 in PDAC cell lines (Fig. 1C). Similar results were observed in PDAC tissues (Supporting Information Fig. S1A and S1B). These data suggest that SMAD4 regulates BRCA1 expression in PDAC.

SMAD4 is a transcriptional factor component of TGF- β signaling pathway. To investigate the potential mechanisms by which SMAD4 regulates BRCA1 expression, we first examined the mRNA levels of BRCA1 in SMAD4-depleted Panc-1 cells. The result of real-time qPCR revealed that *BRCA1* mRNA levels remained unchanged in SMAD4-depleted Panc-1 cells (Fig. S1C), suggesting that SMAD4 may regulate BRCA1 at the post-transcriptional level rather than at the transcriptional level. Next, we treated SMAD4-knockdown Panc-1 cells with MG132 to inhibit ubiquitin and proteasome-related protein degradation. We observed a significant increase in BRCA1 expression levels after MG132 treatment (Fig. 1D). Moreover, the result of CHX chase assay showed that SMAD4 knockdown impaired BRCA1 stability in PDAC cells (Fig. 1E). These data indicate that SMAD4 may regulate BRCA1 ubiquitination and degradation.

We next performed co-immunoprecipitation assay and found that SMAD4 interacted with BRCA1 at both exogenous and endogenous levels (Fig. 1F and G). The results of the immunofluorescence assay demonstrated that SMAD4 colocalizes with BRCA1 in PDAC cells, and their interaction is significantly enhanced following DNA damage induction (Fig. S1D). Pull-down assay also revealed a direct interaction between SMAD4 and BRCA1 (Fig. 1H). To determine the precise interaction

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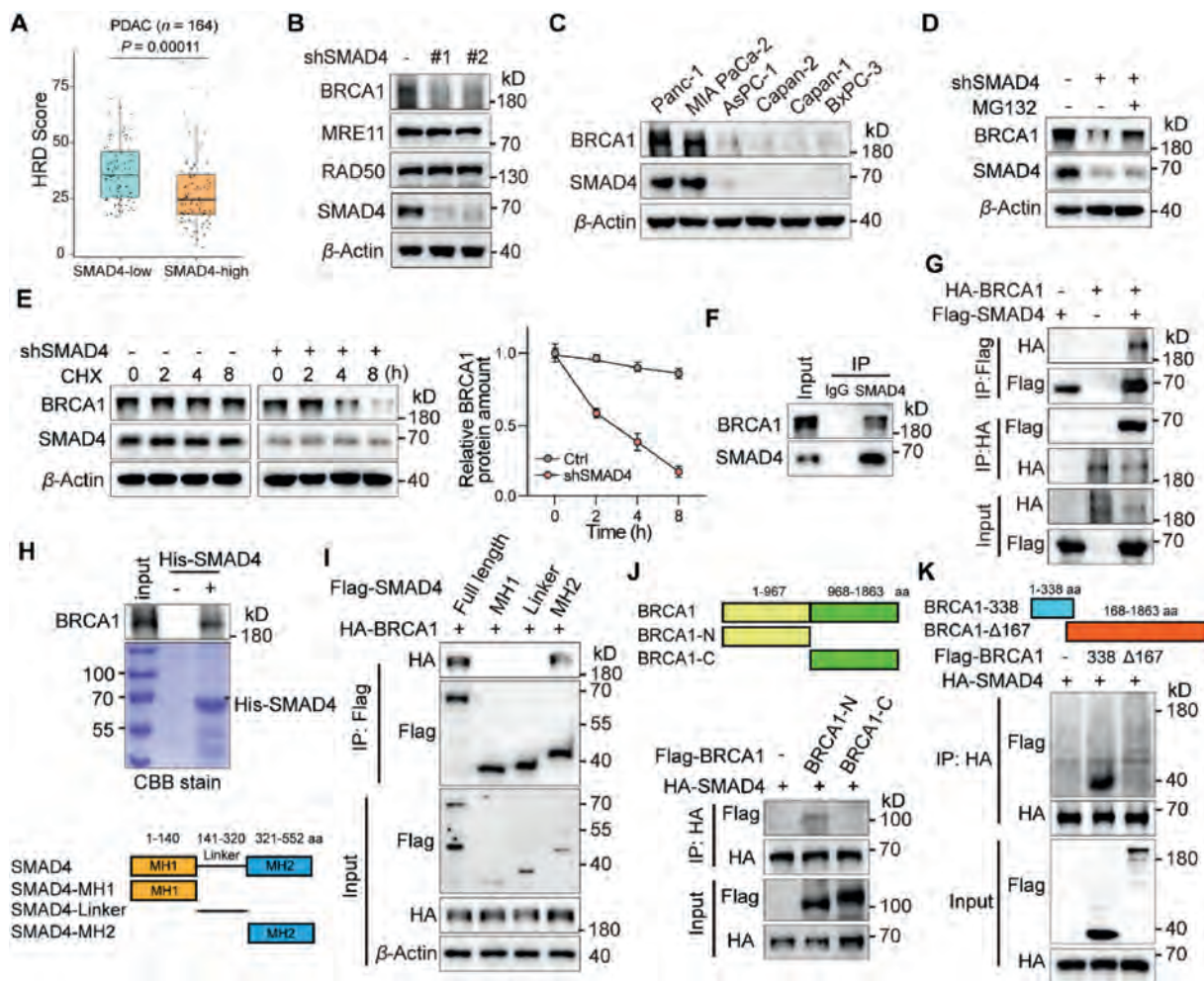


Figure 1 SMAD4 binds to BRCA1 and enhances its stability. (A) Box plot of HRD score of TCGA pancreatic cancer cohort ($n = 164$). Box outlines the 25th and 75th percentiles. (B) Western blot showing the expression of HR markers in SMAD4-depleted pancreatic cancer cells. (C) Western blot analysis of BRCA1 expression in different pancreatic cancer cell lines. (D) Western blot analysis of BRCA1 expression in SMAD4 knockdown Panc-1 cells treated with MG132. (E) Western blot analysis of BRCA1 expression over time in SMAD4 knockdown Panc-1 cells treated with CHX. Data are shown as mean $\pm$ SD ($n = 3$). (F, G) Co-immunoprecipitation assay showing the endogenous (F) and exogenous (G) interaction of SMAD4 and BRCA1. (H) Pull-down assay indicating the direct interaction of SMAD4 and BRCA1. (I) Co-immunoprecipitation assay detecting the interaction between SMAD4 truncations and BRCA1 (right). Illustration of SMAD4 truncations (left). (J) Co-immunoprecipitation assay detecting the interaction between SMAD4 and BRCA1 truncations (bottom). Illustration of BRCA1 truncations (top). (K) Co-immunoprecipitation analysis of the interaction between SMAD4 and BRCA1 fragments (bottom). Illustration of BRCA1 truncations (top).

domains in each protein, we constructed a series of truncations of SMAD4 and BRCA1 (Fig. 1I and J). The results of co-immunoprecipitation assays showed that MH2 domain of SMAD4 and N-terminal half of BRCA1 were essential for their interaction (Fig. 1I and J). To further explore the mechanism that SMAD4 enhances BRCA1 stability, we generated truncations that contains the degron (aa 1–338) region of BRCA1. Co-immunoprecipitation assay showed that SMAD4 bound to the degron of BRCA1 (Fig. 1K). Collectively, our results indicate that SMAD4 binds to BRCA1 and enhances its stability in PDAC cells.

To investigate the effect of SMAD4 on BRCA1 ubiquitination, we transfected HEK293FT cells with HA-BRCA1 and His-ubiquitin expression vectors. Our results demonstrated that overexpression of SMAD4 inhibited the ubiquitination of BRCA1 (Fig. 2A). Thus, we hypothesized that the binding of SMAD4 to BRCA1 may competitively inhibits the interaction between BRCA1 and E3 ubiquitin

ligases. To test this hypothesis, we co-transfected HEK293FT cells with plasmids expressing BRCA1 and SMAD4 or empty control vector. Subsequently, we conducted mass spectrometry analysis to identify the proteins interacting with BRCA1 in each experimental group. We characterized the protein interaction profile of BRCA1 and identified several BRCA1-interacting E3 ligases (Fig. S1E). The result of mass spectrometry analysis showed that the interactions of ARIH1 and SMURF1 with BRCA1 were inhibited upon SMAD4 overexpression (Fig. S1E). Next, we performed co-immunoprecipitation assays to examine the result of mass spectrometry. We found that SMAD4 overexpression specifically inhibited the interaction between ARIH1 and BRCA1, but not that between SMURF1 and BRCA1 (Fig. 2B and Fig. S1F). Subsequent ubiquitination experiments demonstrated that ARIH1 significantly promoted the ubiquitination of BRCA1 (Fig. 2C). Moreover, we found that knockdown of ARIH1 can restore BRCA1 expression in SMAD4-

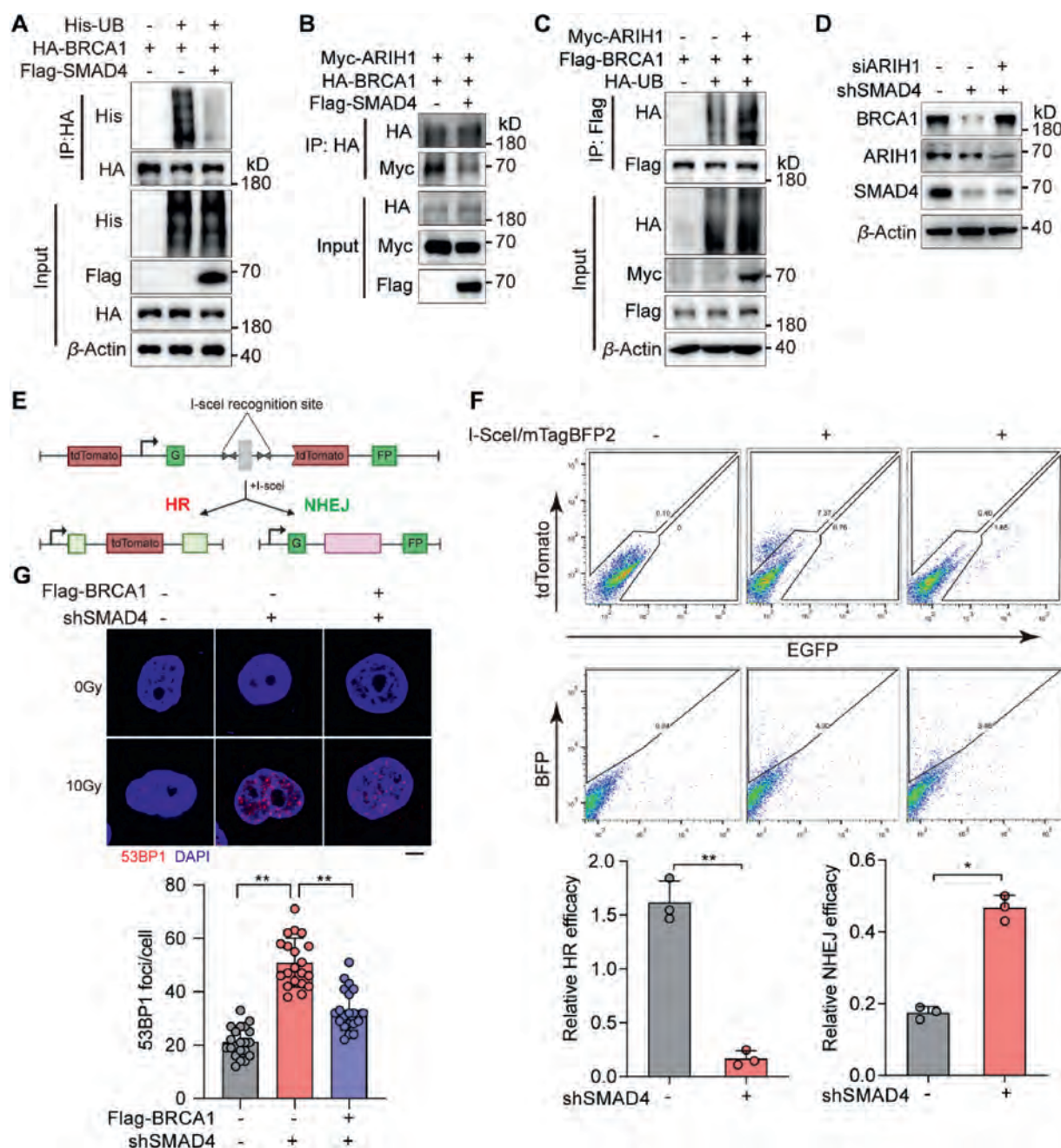


Figure 2 SMAD4 depletion enhances NHEJ by inhibits the interaction of BRCA1 and E3 ligase ARIH1. (A) Co-immunoprecipitation assay to detect BRCA1 ubiquitination in the presence of SMAD4. (B) Co-immunoprecipitation assay showing the interaction between BRCA1 and ARIH1. (C) Co-immunoprecipitation assay to detect BRCA1 ubiquitination in the presence of ARIH1. (D) Western blot of BRCA1 expression in SMAD4 knockdown Panc-1 cells transfected with ARIH1 siRNA. (E) Diagram of the dual fluorescent reporter system for analysis of HR and NHEJ efficiency. (F) FACS analysis of HR and NHEJ efficiency in Panc-1 cell line transfected with the dual fluorescent reporter plasmids. Data are shown as mean $\pm$ SD ($n = 20$). (G) Immunofluorescence assay indicating the 53BP1 foci in Panc-1 cells of indicated groups. Scale bar = 5 μ m. Data are shown as mean $\pm$ SD ($n = 3$). * $P < 0.05$, ** $P < 0.01$.

depleted Panc-1 PDAC cells (Fig. 2D). The above results reveal that SMAD4 inhibits the interaction of BRCA1 and E3 ligase ARIH1 rather than SMURF1.

Given the critical role of BRCA1 in repairing DNA double-strand breaks, we subsequently assessed the levels of HR and NHEJ using a DSB reporter cassette specifically designed to quantify HR and NHEJ events (Fig. 2E). The result of flow cytometry showed that the depletion of SMAD4 enhanced NHEJ while concurrently inhibiting

HR in Panc-1 PDAC cells (Fig. 2F). Next, we investigated the expression of 53BP1, a marker gene for NHEJ, using an immunofluorescence assay. Our results demonstrated that SMAD4 knockdown led to a significant increase in 53BP1 foci formation, which was abrogated by the reintroduction of BRCA1 (Fig. 2G). These data suggest that SMAD4-depletion enhanced NHEJ in PDAC cells. Moreover, knockdown of SMAD4 in Panc-1 cells markedly increased the colony formation efficiency (Fig. S1G) and EdU⁺ cell populations

(Fig. S1H). However, restoration of BRCA1 inhibited the growth rate of SMAD4 knockdown Panc-1 cells (Fig. S1F and S1H).

Next, we established an orthotopic PDAC mouse model using Panc-1 cells to evaluate the effects of ionizing radiation and PARP inhibitor (PARPi) on PDAC. Then the tumor-bearing nude mice underwent oral olaparib and local radiation treatment (Fig. S1I). The results of bioluminescence signals showed that radiation treatment alone or combined with olaparib significantly inhibited the growth of PDAC cells (Fig. S1J and S1K). However, radiation alone had no significant effect on growth of SMAD4-kd pancreatic cells (Fig. S1J and S1K). Interestingly, in the SMAD4-kd group, combined treatment of radiation and olaparib suppressed the growth of the tumor significantly (Fig. S1J and S1K). The tumor weight exhibited consistent outcomes in accordance with the bioluminescence results (Fig. S1L and S1M). These results collectively demonstrate that SMAD4 depletion enhances resistance to radiation while simultaneously increasing sensitivity to the combined treatment of PARPi and radiation in PDAC *in vivo*, corroborating our recent findings⁶.

The two primary pathways responsible for repairing double-strand breaks (DSBs), homologous recombination (HR) and non-homologous end joining (NHEJ), compete with each other in the repair process. However, the equilibrium between these pathways varies significantly among various cell types within the same species and across various cell cycles within a specific cellular type. Typically, NHEJ remains active throughout the entire cell cycle, whereas HR is nearly absent in G1, exhibits limited activity in G2/M, and reaches its highest activity during the S phase. Consequently, NHEJ predominates as the primary pathway for repairing DSBs, while HR primarily addresses DNA breaks that occur during replication. Moreover, NHEJ defects are associated with the sensitivity of tumor cells to radiotherapy. Here, we report a novel regulatory mechanism wherein SMAD4 competes with the BRCA1-associated E3 ligase ARIH1 to reduce BRCA1 ubiquitination and degradation, thereby enhancing BRCA1 expression in PDAC. Consequently, depletion of SMAD4 results in decreased HR and increased NHEJ in PDAC cells, thus promoting ionizing radiation resistance in PDAC cells. Recently, our group reported that SMAD4 interacts with PARP1 to impair its recruitment to DNA damage sites, thus enhancing radiotherapy efficacy⁶. In conclusion, our series of studies comprehensively elucidated the intricate molecular mechanisms underlying SMAD4 deficiency-induced radiotherapy resistance in pancreatic cancer.

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Author contributions

Feng Wang conceived this project. Junyi Ju designed the experimental contents and plans. Yiran Song and Yazhi He performed the experiments and wrote the manuscript. Tianyu Yu, and Yang Wang analyzed the data. Liwei An, Yingqun Zhou, and Yang Shi

revised the original manuscript. All authors commented on this manuscript.

Conflicts of interest

The authors declare no conflict of interest.

Appendix A. Supporting information

Supporting information to this article can be found online at <https://doi.org/10.1016/j.apsb.2025.03.035>.

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