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Cigarette Smoke Induces Stem Cell Features of Pancreatic Cancer Cells via PAF1

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Abstract

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Conflicts of interest

The authors disclose no conflicts

Data Resources

RNA sequencing data: GSE101726 (<https://www.ncbi.nlm.nih.gov/geo/>); Flow cytometry data flow repository IDs: FR-FCM-ZYJ3, FR-FCM-ZYJ4, FR-FCM-ZYJ5, FR-FCM-ZYJ6, FR-FCM-ZYJ7, FR-FCM-ZYJ8 and FR-FCM-ZYJ9. (<http://flowrepository.org/>).

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Background & Aims—Cigarette smoking is a major risk factor for pancreatic cancer.

Aggressive pancreatic tumors contain cancer cells with stem cell features. We investigated whether cigarette smoke induces stem cell features in pancreatic cancer cells.

Methods—Kras^{G12D}; Pdx1-Cre (KC) mice were exposed to cigarette smoke or clean air (controls) for up to 20 weeks; pancreata were collected and analyzed by histology, quantitative reverse transcription PCR, and confocal immunofluorescence microscopy. HPNE and Capan1 cells were exposed to cigarette smoke extract (CSE), nicotine and nicotine-derived carcinogens (NNN or NNK), or clean air (controls) for 80 days and evaluated for stem cell markers and features using flow cytometry-based autofluorescence, sphere formation, and immunoblot assays. Proteins were knocked down in cells with small interfering RNAs. We performed RNA sequencing analyses of CSE-exposed cells. We used chromatin immunoprecipitation assays to confirm the binding of FOS like 1, AP-1 transcription factor subunit (FOSL1) to RNA polymerase II-associated factor (*PAF1*) promoter. We obtained pancreatic ductal adenocarcinoma (PDAC) and matched non-tumor tissues (n=15) and performed immunohistochemical analyses.

Results—Chronic exposure of HPNE and Capan1 cells to CSE caused them to increase markers of stem cells, including autofluorescence and sphere formation, compared to control cells. These cells increased expression of ABCG2, SOX9 and PAF1, via cholinergic receptor nicotinic alpha 7 subunit (CHRNA7) signaling to mitogen-activated protein kinase 1 and FOSL1. Pancreatic cell lines with knockdown of PAF1 did not develop features of stem cells upon exposure to CSE. Exposure of cells to NNN and NNK led to increased expression of CHRNA7, FOSL1, and PAF1 along with stem cell features. Pancreata from KC mice exposed to cigarette smoke had increased levels of PAF1 mRNA and protein, compared with control mice, as well as increased expression of SOX9. Levels of PAF1 and FOSL1 were increased in PDAC tissues, especially those from smokers, compared with non-tumor pancreatic tissue. CSE exposure increased expression of PHD finger protein 5A, a pluripotent transcription factor and its interaction with PAF1.

Conclusions—Exposure to cigarette smoke activates stem cell features of pancreatic cells, via CHRNA7 signaling and FOSL1 activation of PAF1 expression. Levels of PAF1 are increased in pancreatic tumors of humans and mice with chronic cigarette smoke exposure.

Graphical Abstract

Keywords

PHF5A; ERK; pancreatic carcinogenesis; nicotine receptor signaling

Introduction

Pancreatic cancer (PC) is recognized as one of the deadliest diseases¹. Of various established risk factors, cigarette smoking causes 30% of all cases of PC¹, and therefore there is an

urgent need to develop novel therapeutic strategies to specifically treat PC patients who have a history of smoking. Currently, efforts for the development of such strategies are limited because there is no mechanistic understanding of how cigarette smoking is involved in PC initiation and progression.

A previous study from our lab showed that cigarette smoke and its major addictive component, nicotine induces progression and metastasis of PC through cholinergic receptor nicotinic alpha 7 subunit (CHRNA7)-mediated MUC4 upregulation². Cigarette smoke contains over 4,000 chemical components, and among them, nicotine and nicotine-derived carcinogens, 4-(methyltyramine)-1-(3-pyridyl)-1-butanone (NNK), N-Nitrosornicotine (NNN) are associated with carcinogenesis³⁻⁵. Recent studies provide evidence for the association of cigarette smoke and its ingredients with the enrichment of cancer stemness population in various cancers^{6, 7}. In mice, nicotine promotes initiation and progression of Kras-induced PC via Gata6-dependent de-differentiation of acinar cells⁸. In our editorial commentary, we have proposed a role for nicotine in pancreatic stemness induction and acinar cell de-differentiation⁹. These studies suggest that cigarette smoke and its particular ingredients induce cancer stemness in various cancers. However, whether and how cigarette smoking induces pancreatic stemness or cancer stemness remains unexplored.

Recent studies have shown that FOS like 1, AP-1 transcription factor subunit (FOSL1) is significantly overexpressed in PC¹⁰. Loss of FOSL1 reduces stemness properties in hepatocellular carcinoma¹¹. FOSL1 belongs to the Fos gene family, which consists of 4 members: FOS, FOSB, FOSL1 (or FRA1) and FOSL2. FOS family members dimerize with JUN family proteins such as c-Jun, JunB, and JunD forming a dimeric transcription factor called Activator Protein-1 (AP-1) transcription factor. FOSL1 transcription factor levels are increased in response to a variety of stimuli including smoking¹². However, to our knowledge, the role of FOSL1 in smoking-induced activation of pancreatic stemness has not been investigated before.

PAF1, Human RNA polymerase II-associated factor also known as pancreatic differentiation protein 2 (PD2) is a subunit of the PAF1 complex (PAF1C), which is composed of PAF1, Ctr9, Cdc73, Rtf1, Ski8 and Leo1, and regulates multiple processes, including transcription initiation and elongation¹³⁻¹⁶. We and others have demonstrated that PAF1 plays an important role in the maintenance of embryonic stem cell (ESC) signature in a complex independent manner. The PAF1 maintains pluripotency and self-renewal of mouse embryonic stem cells (ESCs)^{17, 18}. PHD-finger protein 5A (PHF5A) has recently been shown to regulate the self-renewal of ESCs. The endogenous PAF1, by interacting with PHF5A activates over 600 pluripotent genes in ESCs by regulating RNA polymerase II elongation in pluripotent loci¹⁹. We also showed that PAF1 maintains cancer stemness population and induces tumorigenesis and metastasis in PC^{20, 21}. Overall, these studies suggest that PAF1 is a major ESC maintenance factor, and its levels are increased in PC. To our knowledge, the role of PAF1 in cigarette smoke induced PC is unknown.

In the present study, we sought to determine whether chronic exposure to cigarette smoke and its ingredients, Nicotine, NNN, and NNK could enrich pancreatic stemness. We investigated the mechanism involved in the smoking-induced promotion of pancreatic

stemness and cancer stemness population, using *in vitro* and *in vivo* models. We observed that chronic exposure to cigarette smoke increases PAF1, a major ESC signature protein through CHRNA7/ERK/FOSL1/cJun (AP1) signaling pathway. We concluded that chronic cigarette smoke exposure promotes pancreatic stemness and that NNN and NNK are the major contributing factors for the smoking-induced stemness induction.

Materials and Methods

Cell lines and cell culture

An immortalized human pancreatic nestin-positive epithelial (hTERT-HPNE) cell line was obtained as a generous gift from Dr. Ouellette at the University of Nebraska Medical Center (UNMC). It was cultured in DMEM (low glucose) with 25% M3 Base Media (Incell), supplemented with 5% (v/v) fetal bovine serum (FBS), 10ng/ml EGF, and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin). The Capan1 cell line was cultured in DMEM (4.5 mg/ml glucose), supplemented with 10% FBS and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin). Cells were incubated in a humidified incubator at 37°C supplied with 5% CO₂.

Animal Studies

Animal experiments were carried out in compliance with the regulations of University of Nebraska Medical Center Institutional Animal Care and Use Committee (IACUC). Ten-week old LSL-Kras^{G12D}(Control) and Kras^{G12D};Pdx-1Cre animals were used for cigarette (University of Kentucky Reference Cigarette, 3R4F) smoke exposure and the process of smoke exposure was performed for 20 weeks, 3 h twice a day, using Teague TE-10C system (Davis, CA, USA) by delivering smoke at a rate of 150 mg total suspended particles/m³,²² (Figure 3A). CS exposure was started when Kras^{G12D}; Pdx-1Cre animals begin to form low-grade PanIN lesions. Sham animals, exposed to filtered air for the same number of hours and days were used as controls. Additional methods can be found in the Supplementary Materials.

Results

Chronic exposure to cigarette smoking increases stemness in normal pancreatic duct cells and PC cells

To study the effect of cigarette smoking on pancreatic stemness, we prepared CSE using a standard protocol in our laboratory^{2, 23}. Since Nicotine is a major cancer-causing agent in cigarette smoke, we first sought to determine the levels of Nicotine in CSE. Using LC MS/MS analysis, we determined the concentration of nicotine in CSE to be 33.073 ± 0.698µg/mL (Supplementary Figure 1A, 1B and 1C).

To determine whether cigarette smoke induces stemness signature in pancreatic duct epithelial cells and PC cells, we exposed HPNE and Capan1 cells to CSE (1%) for up to 80 days (Figure 1A). Lorenzo et al. recently developed a novel autofluorescence based technique to identify cancer stemness population²⁴. The source of AF is riboflavin-loaded intracellular vesicles: the riboflavin enters vesicles through ABCG2 transporters using an

ATP-dependent process. Since the technique is mainly based on riboflavin, we sought to investigate whether the addition of riboflavin increases AF. Flow cytometry analysis of AF showed that the addition of riboflavin (30 μ M) increased the AF⁺ population to 0.4 % and 1% in HPNE and Capan1 cells, respectively (Supplementary Figure 2A). We were also interested to observe, using confocal microscopy, that riboflavin loaded intracellular vesicles with membranous ABCG2 expression (Supplementary Figure 2B). Confocal microscopy also revealed that CSE significantly increases AF-positive (AF⁺) content in HPNE and Capan1 cells (Figure 1B). AF analysis using flow cytometry on days 20, 40, and 80 of CSE treatment showed a time-dependent increase in the percentage of AF⁺ cells as compared to respective time point controls (Figure 1C).

To confirm whether AF⁺ cells are indeed a stemness population, we sorted AF⁺ and non-autofluorescent (AF⁻) cells using FACS. As expected, AF⁺ cells show elevated expression of major stemness signature genes, such as Sox2, PAF1, Oct3/4 and ABCG2 (Figure 1D). Further, flow cytometry analysis showed more ABCG2⁺ and CD44^{High} population in AF⁺ cells as compared to AF⁻ cells (Figure 1E).

Previous studies have shown that ability to form spheres *in vitro* is one of the standard ways to assess the self-renewal potential of normal and cancer cells^{25, 26}. To determine the self-renewal property of AF⁺ and AF⁻ populations, we performed the sphere assay, and observed that AF⁺ cells showed increased number of spheres as compared to AF⁻ cells. In addition, AF⁺ cells showed the capacity to form secondary and tertiary spheres (Supplementary Figure 3A). Next, to determine *in vivo* tumorigenicity of AF⁺ and AF⁻ cells, 4000 cells were injected subcutaneously into the left and right flanks of athymic nude mice. AF⁺ cells sorted from CSE exposed Capan1 cells started forming tumor nodules two weeks after implantation. After 27 days, mice were euthanized and tumors were excised. Compared with AF⁻ cells, AF⁺ cells sorted from CSE-induced Capan1 cells showed increased tumor weight (Supplementary Figure 3B). However, the AF⁺ population sorted from CSE-induced HPNE cells could not form tumors even after 4 months (Supplementary Figure 3C).

Smoking induces Stemness Signature in Pancreatic Ductal Epithelial Cells and Cancer Cells

To determine the ability of sphere formation by the CSE-induced whole cell population, we performed sphere assay on CSE-exposed HPNE and Capan1 cells on day 20, 40, and 80 of CSE exposure. Compared with respective time point controls, CSE-exposed cells showed an increase in the number and size of spheres (Figure 2A). In agreement with this observation, Liu and colleagues reported that chronic treatment with CSE increases the number of spheres in normal human bronchial epithelial cells²³. To determine whether cells within the spheres show AF and stemness marker expression, we performed immunofluorescence assay for Oct3/4 and AF. Cells within spheres formed by CSE-induced HPNE and Capan1 cells showed co-localization of AF and Oct3/4 expression (Figure 2B).

To further investigate the impact of CSE on pancreatic stemness, we analyzed protein expression levels of major stemness signature proteins, SOX2, Oct3/4, KLF4, BMI1, Nanog and PAF1, the pancreatic progenitor cell marker, SOX9, and the cancer stemness markers, ALDH1 and CD44. In HPNE and Capan1 cells, CSE markedly induced the expression of

stemness signature and cancer stemness markers (Figure 2C). However, BMI1 did not show any variation (Supplementary Figure 4A). Of interest, CSE induced an over 3-fold increase in the protein expression levels of PAF1, a master regulator of stemness¹⁹, suggesting its importance in smoking-mediated stemness enrichment (Figure 2C). To investigate whether commercial CSE (C-CSE) also shows a similar effect on stemness marker expressions, HPNE and Capan1 cells were exposed to 20µg/mL C-CSE for 80 days. Increased expression of PAF1 and SOX9 was observed in C-CSE-exposed cells as compared to vehicle- (DMSO) treated control cells (Supplementary Figure 4B).

Next, we also observed increased expression of ABCG2, SOX9, PAF1, β -catenin, CD44, and CD24 in CSE-exposed cells, as revealed by immunofluorescence staining (Figure 2D and Supplementary Figure 5A). CSE also significantly upregulated PAF1 and SOX9 mRNA levels (Figure 2E). Aldefluor assay²⁷ showed that CSE induces ALDH activity up to 2% in HPNE cells, but up to 50% in Capan1 cells (Supplementary Figure 5B). These data suggest that cigarette smoke induces a stemness signature, specifically PAF1, in human pancreatic ductal epithelial cells and in PC cells.

Cigarette Smoking Induces PAF1 in Kras^{G12D}; Pdx-1 Cre Mouse

The findings described above suggest that cigarette smoke significantly upregulates PAF1 *in vitro*. Previously, we and others showed the importance of PAF1 in the maintenance of stemness in ESCs and PC stemness populations^{17–19}, and this triggered us to further explore its role in cigarette smoke-enriched stemness *in vivo*. We observed significantly elevated expression of PAF1 in the pancreas of 20 weeks cigarette smoke-exposed control and Kras^{G12D}; Pdx-1Cre mice²² (Figure 3B and 3C). Of note, PAF1 mRNA levels are also significantly higher in cigarette smoke -exposed mouse groups (Figure 3D). We also observed an increased expression of SOX9 (Supplementary Figure 6A) and an elevated co-expression of PAF1 with CD44 (Figure 3E) and SOX9 (Supplementary Figure 6B) in cigarette smoke -exposed mice. In addition, cigarette smoke-exposed pancreas tissues showed an increased AF content as compared to controls (Figure 3F). In addition to riboflavin, another source of AF is lipid content, or lipofuscin²⁸. Therefore, we analyzed co-localization of AF and Nile red (lipid content marker) staining in the pancreas of cigarette smoke-exposed mice. Results indicated that AF does not emanate from lipid content or lipofuscin, and this provides evidence for the purity of riboflavin-derived AF. Furthermore, AF is co-localized with PAF1 and ABCG2 (Figure 3G). Co-localization of PAF1 expression with AF+ in CSE-exposed HPNE cells was also shown in Supplementary Figure 6B. These *in vivo* data clearly correlated with *in vitro* findings, suggesting that smoking induces PAF1 and stemness markers *in vivo*.

Cigarette Smoke Induces the Enrichment of Stemness through PAF1

Next, we investigated whether up-regulation of the stemness signature in response to cigarette smoke exposure is regulated through PAF1. To elucidate this hypothesis, we performed CRISPR/Cas9-based KO and siRNA-based KD of PAF1 in Capan1 and HPNE cells (Figure 4A). We were interested to find that loss of PAF1 reduced protein expression of SOX9 and β -catenin (Figure 4A). Further, silencing of PAF1 reduced the percentage of cigarette smoke -induced AF cells (Figure 4B and C) and the ABCG2+ population (Figure

4D–F) as compared to scramble control. These results suggest that smoking induces pancreatic stemness through PAF1.

Smoking-Induced PAF1 is Regulated through FOSL1 Transcription Factor

To unveil the mechanism involved in the smoking-mediated induction of pancreatic stemness, whole transcriptome analysis was performed using RNA sequencing (GEO database accession number: GSE101726) in CSE-treated HPNE and Capan1 cells (Supplementary Figure 7A and B). We found that four stemness-associated genes including LIF (maintains stemness in ESCs), ALDH1A3 (cellular detoxifying enzyme and cancer stemness marker), ABCC4, ABCG2 (associated with drug resistance), and FOSL1 are commonly overexpressed in CSE-exposed HPNE and Capan1 cells. We further validated the precision of our RNA sequencing data by performing qRT-PCR on these stemness genes (Supplementary Figure 7C).

We focused on FOSL1 given that it showed upregulation in both HPNE and Capan1 cells, due to its involvement in a variety of cellular events mediated by smoking (Supplementary Figure 4A; Supplementary Table 6 and 7) and to its association with stemness¹¹. We thus performed PAF1 gene promoter analysis for potential transcription factor binding sites of FOSL1, using TRANSFAC software, and observed 10 FOSL1 binding sites (Supplementary Figure 8). Further, we silenced FOSL1 and observed downregulation of cigarette smoking-induced PAF1 in both HPNE and Capan1 cells (Figure 5A). However, FOSL1 knockdown did not show any effect on untreated HPNE and Capan1 cells, suggesting that FOSL1-mediated PAF1 upregulation is specific to cigarette smoking (Figure 5A). To determine whether cigarette smoke-induced FOSL1 binds to PAF1 promoter, we performed chromatin immunoprecipitation (ChIP) assay. ChIP results indicated that FOSL1 specifically binds to three binding sites (BS9: -2162TGACTGACTGAC-2150; BS1: -67TGAGCAT-61; BS3: -836TCACTCAGT-828) among 10 predicted binding sites (Figure 5B and Supplementary Figure 8). This observation was further confirmed by sequencing of ChIP PCR products for these specific sites (Supplementary Figure 9A and B). These results clearly indicate that cigarette smoke-induced FOSL1 is involved in the upregulation of PAF1.

We also observed the overexpression of FOSL1 (Figure 5C) and its co-localization with PAF1 (Figure 5C) and CD44 (Supplementary Figure 10) in the pancreas of smoke-exposed control and Kras^{G12D}; Pdx Cre mice. Taken together, accumulating data suggest that smoking-induced FOSL1 regulates the PAF1 gene.

PAF1 and FOSL1 co-overexpressed in Human PDAC

To study the relevance of PAF1 and FOSL1 to the human pancreatic ductal adenocarcinoma (PDAC), we analyzed the expression of FOSL1 and PAF1 in PDAC tissues with smoking history. PAF1 and FOSL1 showed significantly increased expression in PDAC tissues as compared to normal pancreas (Figure 5D). Of interest, increased expression of PAF1 and FOSL1 was observed in smoker PDAC tissues as compared to non-smoker PDAC (Figure 5D). FOSL1 and PAF1 also showed increased co-localization in PDAC tissues of smokers compared to non-smokers (Figure 5D).

Smoking Induces PAF1 through nACHR α 7/ERK/FOSL1-cJUN (AP1) Signaling Pathway

The observations presented above indicate that FOSL1 regulates PAF1 under smoking treatment. We next investigated the mechanism involved in this process. The up-regulation of phospho-FOSL1 was confirmed in HPNE and Capan1 cells following chronic exposure to CSE and C-CSE (Figure 5E and Supplementary Figure 4B). We further examined whether CSE would also activate FOSL1 interacting partner (phospho c-Jun, AP-1 family member). We found that the expression of phospho c-Jun (Ser 73) is significantly elevated in response to CSE treatment, demonstrating that chronic exposure to cigarette smoke induces upregulation of the AP1 (FOSL1-c-Jun) transcription factor (Figure 5E). Next, we aimed to study the upstream regulators that mediate cigarette smoke-induced AP1 activation by examining Erk1/2, known upstream regulators of AP1^{29,30}. The inhibition of pErk1/2 abolished cigarette smoke-induced phospho-FOSL1, phospho-cJUN, and PAF1 in CSE-exposed cells (Figure 5F). This data suggested that cigarette smoke induces Erk1/2 phosphorylation and that the subsequent phosphorylation of FOSL1 may lead to direct transcriptional activation of PAF1.

Our next goal was to find the upstream receptor that activates the ERK1/2-FOSL1 signaling pathway. We analyzed the activation of smoking-associated receptors, including CHRNA1, CHRNA7, and TLR4 and observed an increased expression only in CHRNA7 in CSE-exposed cell line models (Figure 5E and Supplementary Figure 11A). Furthermore, inhibition and knockdown of CHRNA7 resulted in decreased expression of phospho-ERK, phospho-FOSL1 and PAF1 in CSE-exposed cell line models (Figure 5G and Supplementary Figure 11B). These results clearly indicate that smoking induces PAF1 through the CHRNA7/ERK/FOSL1-cJUN (AP1) signaling pathway.

Specific Cigarette Smoke Components, Nicotine, NNK, and NNN Induce Pancreatic Stemness and PAF1

To determine the major cigarette smoke components responsible for pancreatic stemness induction, we treated HPNE and Capan1 cells with cigarette smoke components, Nicotine, NNK, and NNN for 80 days and analyzed for stemness enrichment. Specifically, NNN and NNK treatment significantly enriched the AF+ population (Figure 6A) and significantly increased sphere formation (Figure 6B) in HPNE and Capan1 cells. Further, treatment with NNN and NNK increased the protein expression levels of CHRNA7, p-FOSL1, FOSL1 and PAF1, and this effect is similar to that observed with CSE treatment (Figure 6C and 6D). These data suggest that NNK and NNN specifically contribute to cigarette smoke-induced pancreatic stemness through the PAF1 mechanism.

Smoking Induces Interaction between PAF1 and PHF5A, an Event Required for Stemness Enrichment

Recent evidence shows that PAF1 interacts with PHF5A, and that this complex regulates over 600 pluripotent genes in ESCs¹⁹. PHF5A is a PHD finger protein required for PAF1 complex recruitment, complex stabilization, release of RNA polymerase II proximal promoter pause, serine 2 phosphorylation of RNA Polymerase II (Ser-2-P-Pol II) and elongation of pluripotency gene transcription in ESCs^{19,31-33}. The PAF1-PHF5A complex-mediated activation of pluripotent genes was well studied in ESCs; however, its role in

smoking induced enrichment of stemness in PC has not been investigated. Based on this study, we developed a hypothesis that PAF1 interacts with PHF5A and regulate the smoking mediated stemness enrichment. Our results showed an increased expression of PHF5A in CSE- and C-CSE-treated cells (Figure 7A and Supplementary Figure 4B) as well as in the pancreas of control and Kras^{G12D}; Pdx-Cre mice (Figure 7B and C). A clear co-localization of PAF1 and PHF5A was also observed by immunofluorescence staining in the pancreas tissues of cigarette smoke-exposed mice and in CSE-exposed cells (Figure 7B, D and Supplementary Figure 12). In addition, cigarette smoke increased the interaction between PAF1 and PHF5A compared to untreated controls as evidenced by immunoprecipitation assay (Figure 7E). We also observed increased protein expression of Ser-2-P-Pol II and Leo1, a PAF1 complex molecule; however, other PAF1 complex subunits, including CTR9, CDC73, and SKI8, did not show any variation (Figure 7A). Taken together, our data suggest that smoking induces PHF5A and increases the interaction between PAF1 and PHF5A, an event required for the activation of stemness genes.

Discussion

Cigarette smoking is a well-established risk factor for PC; however, a mechanistic understanding of PC development in response to cigarette smoking is limited. Accumulating evidence indicates that the stemness signature is induced in various cancers that initiate and aggravate tumors^{34, 35}. However, whether and how cigarette smoking induces pancreatic stemness is unknown. Here, we establish for the first time a novel PAF1-mediated mechanism of cigarette smoke-induced pancreatic stemness.

Despite several studies showing an association between cigarette smoking and stemness induction in various cancers^{36, 37}, it remains unclear whether exposure to cigarette smoke induces stemness in PC. To explore this critical gap in understanding, we performed chronic exposure to cigarette smoke in pancreatic normal and cancer cells and found that cigarette smoking increases pancreatic stemness. Cigarette smoke exposure increased the pancreas-specific multipotent stemness factor, SOX9, and the oncogenic stemness factors, ALDH1 and CD44 in pancreatic normal and cancer cells, suggesting that exposure to cigarette smoke induces multipotent stemness fraction and cancer stemness in the pancreas.

Cigarette smoke-exposed pancreatic normal and cancer cells also showed SOX2, Oct3/4, Nanog and KLF4 expression along with significant overexpression of PAF1, an ESC signature maintenance factor, suggesting that cigarette smoking augments an ESC-like stemness program. We further found that cigarette smoke-induced stemness signatures in normal cells are almost similar to that of PC cells. However, the cigarette smoke-induced stemness population of normal pancreatic duct cells did not show any oncogenic potential. Cigarette smoke-induced stemness population isolated from normal duct cells failed to form subcutaneous tumors, while the stemness population of cigarette smoke-induced cancer cells did produce tumors. These data suggest that the observed stemness induction under cigarette smoke stress in normal duct cells is an early step towards cancerous reprogramming. Consistent with this observation, a recent report indicated that airway basal cells of healthy smokers acquire an ESC-like phenotype or signature, itself an early step toward malignant transformation³⁸.

Our data suggest the possibility that cigarette smoking expands stemness population in normal tissues, and that this mechanism may be responsible for PC initiation. There are two possibilities for the expansion of stemness population in response to cigarette smoke exposure. First, a cigarette smoke-induced increase in stemness may be due to promotion of an existing stemness population within normal pancreatic tissues. Second, a benign and differentiated cell may also take on the stemness phenotype upon chronic exposure to cigarette smoke. In the first possibility, long-term, continuous, and rapid cell divisions in the stemness population in response to extracellular stress may eventually induce mutations leading to complete malignant transformation³⁹. Investigation of this possibility by increasing the length and/or amount of cigarette smoke exposure would provide clues to PC initiation caused by cigarette smoke. However, we conclude that cigarette smoking does induces stemness signatures in normal and PC cells.

Previous studies suggested PAF1 as a major stemness signature maintenance factor in embryonic stem cells (ESCs) and cancers cells^{18–20, 40}. Observations presented above also show that PAF1 is one of the significantly overexpressed stemness factors under the oncogenic stress of cigarette smoke. Based on these, we sought to further explore its mechanistic role in cigarette smoke-induced stemness. As an important piece of evidence that PAF1 maintains stemness, PAF1 loss showed reduced expression levels of stemness markers, SOX9 and β -catenin in normal pancreatic cells and PC cells. Further, we also identified overexpression of PAF1 in 20 weeks cigarette smoke-exposed normal and Kras^{G12D}; Pdx-Cre mice. Additional evidence that PAF1 is associated with stemness is found in the fact that cigarette smoke-induced PAF1 expression correlates with the expression of other stemness markers, SOX9 and CD44, in cigarette smoke-exposed normal and Kras^{G12D}; Pdx-Cre mice. Of note, the loss of PAF1 decreased cigarette smoke-induced pancreatic stemness, suggesting that PAF1 is a major regulator of cigarette smoke-induced pancreatic stemness.

Furthermore, elevation of FOSL1, a critical transcription factor, was observed in cigarette smoke-exposed cells along with other stemness genes, including LIF, ALDH1, and ABCG2. Consistent with this finding, cigarette smoke was found to activate the AP1(FOSL1-cJun) signaling pathway to induce EMT in bladder cancer³⁰. The loss of FOSL1 also downregulated stemness marker expressions and inhibited sphere formation in hepatocellular carcinoma cells, suggesting a role for FOSL1 in the maintenance of stemness¹¹. We also demonstrated that cigarette smoke increases the expression of the FOSL1 gene and that it activates the AP1 transcription factor. Given the association between the FOSL1 transcription factor and stemness, we investigated whether the cigarette smoke-induced PAF1 gene is regulated by the FOSL1 transcription factor. We observed ten FOSL1 binding sites near or on the PAF1 gene promoter, suggesting the potential of FOSL1 (AP1) binding on the PAF1 promoter and FOSL1-mediated regulation of the PAF1 gene. Our data suggest that FOSL1 specifically binds to 3 out of 10 predicted binding sites: two are at the distal (-²¹⁶¹TGACTGACTGAC-²¹⁵⁰ and -⁸³⁶TCACTCAGT-⁸²⁸) and the other at the proximal promoter region (-⁶⁷TGAGCAT-⁶¹) of the PAF1 gene. Of interest, cigarette smoke-exposed cells with FOSL1 depletion also displayed reduced expression of PAF1, indicating that cigarette smoke-activated FOSL1 regulates PAF1.

Our data also demonstrated that the pancreas of PDAC patients shows increased PAF1 and FOSL1 expression as compared to normal healthy persons. In addition, PAF1 and FOSL1 expressions are higher in the pancreas of PDAC patients who have a smoking history, suggesting a strong co-relation of PAF1 and FOSL1 expressions with smoking history in PDAC patients.

We also proved that cigarette smoke induces PAF1 through an CHRNA7-ERK1/2-FOSL1 signaling pathway. The FOSL1 is a known downstream effector of the ERK pathway²⁹ and a previous study from our lab showed that cigarette smoke and its component, nicotine, induces PC by activating CHRNA7-ERK1/2-MUC4 pathway². Consistent with these findings, CHRNA7 and its downstream effector ERK show upregulation in response to cigarette smoke treatment. Inhibition of CHRNA7 and ERK reduces expression levels of the downstream signaling molecules, phospho-FOSL1 and PAF1. Thus, our data suggest that one mechanism by which cigarette smoke promotes pancreatic stemness is through the CHRNA7-ERK1/2-FOSL1-PAF1 signaling pathway.

We next sought to investigate the cigarette smoke ingredients most responsible for activation of the CHRNA7-ERK1/2-FOSL1-PAF1 signaling pathway. We demonstrated that cigarette smoke components, specifically NNN and NNK, induce pancreatic stemness, as evidenced by the increased percentage of AF and up-regulation of stemness markers, SOX9 and KLF4. Moreover, these cigarette smoke components induced signaling molecules of CHRNA7-ERK1/2-FOSL1-PAF1 signaling pathway. Overall, NNK and NNN are the major cigarette smoke components that are responsible for cigarette smoke-mediated stemness enrichment.

We further found that cigarette smoke induces PHF5A, a PAF1 complex recruiting and stabilizing protein, and increases the interaction between PHF5A and PAF1. A recent study showed that PAF1 interacts with PHF5A, and that the PAF1-PHF5A complex regulates 686 stemness genes in ESCs¹⁹. Another study demonstrated that pluripotent genes in differentiated somatic cells are in an inactive state due to the RNA polymerase II proximal promoter pause³¹. Loss of PHF5A in ESCs increased RNA polymerase pausing and reduced Ser-2-P-Pol II levels in the promoter regions of PAF1 target or pluripotent genes, thereby reducing the expression of these genes¹⁹. These studies indicate that PHF5A and PAF1 are required for the pause release and for maximum levels of Ser-2-P-Pol II at the promoter regions of pluripotent genes. In agreement with this, cigarette smoke increased the levels of Ser-2-P-Pol II in pancreatic normal and cancer cells. Overall, our observations suggest that cigarette smoking increases the PAF1-PHF5A interaction that is required for the activation of stemness genes.

Altogether, our results show that cigarette smoke induces the PAF1 gene through the CHRNA7-ERK-FOSL1 signaling pathway, resulting in the induction of a stemness signature (Figure 7F). Upon induction of PAF1 in response to cigarette smoke, PAF1 interacts with PHF5A and may reprogram a differentiated cell or cancer cell population into stemness or a cancer stemness population by upregulating stemness genes; however, this suggestion requires further research. However, our study shows for the first time a novel mechanism of cigarette smoke-induced expansion of the pancreatic stemness population. In future, our

findings may contribute to the development of targeted therapy for PC patients who have a smoking history.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
ANTIBODIES (see Supplementary Table 2 and 3)	This paper	N/A
Experimental Models: Cell Lines		
hTERT-HPNE cells	Dr. Ouellette (UNMC)	
Capan1	ATCC	ATCC (HTB-79)
Experimental Models: Animals		
Mouse: LSL-Kras ^{G12D} and Kras ^{G12D} ; Pdx-1cre	In house developed	N/A
Critical commercial assays		
ALDEFLUOR assay kit	Stem Cell Technologies	Cat #01700
Bio-Rad DC Protein Assay kit	Bio-Rad	Cat # 500-0114
ECL chemiluminescence kit	Thermo Scientific	Cat#32209
Illumina TruSeq RNA Sample Preparation Kit	Illumina	CAT# RS-122-2001
Double stain IHC kit: M&R on human tissue	Abcam	AB210061
Bloxall blocking solution	Vector laboratories, Inc.	SP-6000
Chip DNA Clean & Concentrator kit	The Epigenetics Company	CAT# D5205
Software		
TRANSFAC	gene X plain	www.genexplain.com
Signalplot	Systat Software, Inc.	www.sigmaplot.co.uk
ImageJ	NIH	https://fiji.sc
BD FACS Diva	BD Biosciences	N/A
Zen Imaging	Zeiss	https://www.zeiss.com/microscopy/us/products/microscope-software/zen-lite.html

REAGENT or RESOURCE	SOURCE	IDENTIFIER
PRISM GRAPHPAD	Graphpad	https://www.graphpad.com/scientific-software/prism/
Multiquant	AB Sciex	https://sciex.com/products/software/multiquant-software
Finch TV	Geospiza, Inc.	https://digitalworldbiology.com/FinchTV
Chemicals & Reagents		
3R4F research cigarettes	University of Kentucky	https://ctrp.uky.edu/
PD98059	Sigma	Cat# P215
Macamylamine	Sigma	Cat# M9020
Hoechst 33342	Ana Spec	Cat#AS-83218
ChromPure Rabbit IgG, whole molecule	Jackson Immunoresearch Laboratories, Inc.	Cat# 011-000-003
Optima™ LC/MS water	Fisher Chemical	W6212
Methanol, Optima™ LC/MS Grade	Fisher Chemical	A456-1
Acetonitrile, Optima™ LC/MS Grade	Fisher Chemical	A955-1
Ammonium bi-carbonate	Sigma	09830
Lipofectamine 2000	Invitrogen	Cat# 11668027
Nile Red	ThermoFisher Scientific	Cat# N1142
DAPI, FluoroPure™ grade	ThermoFisher Scientific	Cat# D21490
N'-Nitrosonornicotine (NNN) solution	Sigma	Cat# N-075
4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) solution	Sigma	Cat#N-076
NICOTINE	Sigma	Cat#N-3876
Commercial Cigarette smoke extract (C-CSE)	Murthy pharmaceuticals,	N/A
Riboflavin	Sigma	R9504

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Matrigel	Fisher Scientific (Corning)	08-774-552
Nicotine D4 solution	Sigma	N048
Plasmids/siRNAs		
PAF1 (ID 54623) Trilencer-27 Human siRNA	Origene	SR310177
FOSL1 (ID 8061) Trilencer-27 Human siRNA	Origene	SR322327
CHRNA7 Trilencer-27 Human siRNA	Origene	SR300818

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AF	autofluorescence
ALDH	aldehyde dehydrogenase
CS	cigarette smoke
CSE	cigarette smoke extract
C-CSE	commercial CSE
EMT	epithelial-mesenchymal transition
ERK	extracellular signal-regulated kinase
ESC	embryonic stem cell
FACS	fluorescence activated cell sorter
hPAF1	human RNA polymerase II-associated factor
KC	KRas ^{G12D} ; Pdx-1Cre
LPS	lipopolysaccharide
CHRNA7	cholinergic receptor nicotinic alpha 7 subunit
NNN	N-Nitrosornicotine
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
PanIN	pancreatic intraepithelial neoplasia
PC	pancreatic cancer
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
PD2	pancreatic differentiation protein 2
SC	stem cell
LC MS	Liquid chromatography Mass spectrometry
PDAC	Pancreatic ductal adenocarcinoma

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Figure 1. Exposure to cigarette smoke increases pancreatic stemness

(A) HPNE and Capan1 cells were exposed to CSE (1%), commercial CSE (C-CSE) (20µg/ml) or to cigarette smoke components, Nicotine (2µM), NNK (2µM) and NNN (2µM) for 80 days (two days exposure per passage; thus, up to 40 passages). (B) Confocal imaging for AF in CSE-treated (CSE-T) cells compared to untreated controls. AF (Green) and nuclei were stained using DAPI (Blue) (n=4). Scale bars: 50µm. (C) Left: Flow cytometry analysis of AF content in CSE exposed cells at different treatment time points. AF cells were excited using a 488-nm blue laser and filters, 530/30 and 585/42 1 550LP Blue Det A-A. Right: Percentage of AF cells at different time points of CSE exposure as compared to respective time point controls. (n=3). (D) Quantitative reverse transcription polymerase chain reaction assays for stemness genes in AF- and AF+ population sorted from CSE exposed cells. Data shown are normalized with β -actin expression. (n=3). (E) Left: Flow cytometry analysis for ABCG2⁺ and CD44^{High} population in AF- and AF+ cells. CSE exposed cells were subjected to immunophenotyping using anti-ABCG2-APC or anti-CD44-APC antibody. AF + and AF- population were selected and analyzed for ABCG2 or CD44 positivity using flow cytometer. Right: Percentage of ABCG2⁺ and CD44^{High} population in selected AF- and AF + population of CSE exposed cells. (n=3). (B-E) Data represent mean $\pm$ SD (p values were

calculated by Student's t test). * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$. DAPI, 4',6-diamidino-2-phenylindole.

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Figure 2. Cigarette smoke exposure augments pancreatic stemness *in vitro*

(A) Sphere formation assays performed on CSE exposed and unexposed HPNE and Capan1 cells. 2000 cells/well were seeded in 96 well ultra-low attachment plates in stem cell medium. Left: Morphology of 10-day old spheres. Scale bar: 200 μm. Right: Number of spheres per 2000 cells at different time points of CSE exposure as compared to respective time point controls. Data represent mean $\pm$ SD (n=6). (p values were calculated by Student's t test). * p < 0.05, *** p < 0.001. (B) Confocal imaging. Spheres were immunostained for Oct3/4. Confocal images were captured for Oct3/4 (red staining), AF (green staining) and DAPI (blue staining for nuclei). Scale bar: 100 μm. (C) Immunoblotting assays for stemness or cancer stemness markers in untreated and CSE treated HPNE and Capan1 cells. β -actin was used as a loading control. (D) Immunofluorescence staining on chronically CSE exposed and unexposed cells for stemness markers, ABCG2, SOX9 and PAF1. Nuclei were stained in blue (DAPI) and red staining indicates ABCG2, SOX9 and PAF1 expression. Scale bar: 100 μm. (E) Quantitative reverse transcription polymerase chain reaction assay on CSE exposed and unexposed HPNE and Capan1 cells for PAF1 and SOX9 genes. Data shown are normalized for β -actin expression. Data represent mean $\pm$ SD (n=3). (p values

were calculated by Student's t test). * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$. DAPI, 4',6-diamidino-2-phenylindole.

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Figure 3. Cigarette smoke exposure induces PAF1 *in vivo*

(A) Ten-week-old control and Kras^{G12D} Pdx-Cre mice were exposed to cigarette smoke for 20 weeks (3hrs; twice a day) using Teague TE-10C system. Filtered air was used to expose control animals. (B) Immunohistochemistry staining of PAF1 protein in pancreatic tissues obtained from cigarette smoke-exposed (CS-T) control and Kras^{G12D} Pdx-Cre mice. Scale bars, 100µm. (C) Histo score (H-Score) of PAF1 protein expression. Data represent mean ± SD (n=6). (p values were calculated by Student's t test). **p < 0.01. (D) Quantitative reverse transcription polymerase chain reaction assay on pancreatic tissues obtained from cigarette smoke exposed control and Kras^{G12D} Pdx-Cre mice for PAF1. Data shown are normalized for β-actin expression. Data represent mean ± SD (n=3). (p values were calculated by Student's t test). *p < 0.05, **p < 0.01. (E) Immunofluorescence staining for PAF1 (Green) and CD44 in cigarette smoke-exposed pancreatic tissues. Scale bar: 50µm. (F) Confocal images of pancreatic tissues obtained from cigarette smoke exposed mice showing the AF (Green) and localization of Nile red (lipid droplet staining). Scale bar: 50µm. Enlarged boxed image shows the absence of co-localization of AF with Nile red (Lipofuscin) (G) Confocal images of pancreatic tissue collected from cigarette smoke exposed floxed mice showing the co-localization of PAF1 with ABCG2 and AF (Green). Arrowheads point to co-

localization of PAF1, ABCG2 and AF. Enlarged boxed image shows the co-localization. Scale bar: 50µm. (E–G) Nuclei were stained with DAPI, 4',6-diamidino-2-phenylindole.

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Figure 4. Smoking-mediated induction of pancreatic stemness is regulated by PAF1

(A) Immunoblotting assay for PAF1, SOX9 and β -catenin in PAF1 KD HPNE and PAF1 CRISPR/Cas9 KO Capan1 cells. (B) Flow cytometry analysis of AF cells in CSE exposed HPNE and Capan1 cells transfected with PAF1 siRNA and control siRNA. (C) Percentage of AF cells in scramble control and PAF1 KD CSE exposed cells. Data represent mean $\pm$ SD (n=3). (p values were calculated by Student's t test). * $p < 0.05$, ** $p < 0.01$. (D and E) Flow cytometry analysis for ABCG2+ population in scramble control and PAF1 KD CSE exposed HPNE and Capan1 cells. (F) Percentage of ABCG2+ population in scramble control and PAF1 KD CSE exposed cells. (n=3). Data represent mean $\pm$ SD (n=3). (p values were calculated by Student's t test). ** $p < 0.01$.

Figure 5. Smoking induces PAF1 through the nACHR α 7-ERK1/2-FOSL1/cJun (AP1) signaling pathway

(A) Small interfering RNA (siRNA) knockdown of FOSL1. Western blot analysis showing FOSL1 knockdown and its effect on PAF1 in CSE-treated and untreated HPNE and Capan1 cells. GAPDH was used as loading control. (B) Chromatin immunoprecipitation (ChIP) assays were performed using chromatin from CSE exposed cells and the control IgG or phospho-FOSL1 antibodies. Phospho-FOSL1 enriched DNA was used in PCR assay using primers specific to FOSL1 or AP1 binding sites (see Supplementary Figure 8) on the promoter region of PAF1 gene. Chip DNA PCR product was resolved on 2% agarose gel, and the DNA bands for BS1-9 were shown. (C) Left: Representative images of immunohistochemistry for FOSL1 in pancreatic tissues obtained from cigarette smoke-exposed control and *Kras*^{G12D} Pdx-Cre mice. Scale bar, 100 μ m. Middle: Immunofluorescence staining for PAF1 (stained in red) and p-FOSL1 (stained in green) in cigarette smoke exposed control and *Kras*^{G12D} Pdx-Cre tissues (Nuclei were stained with DAPI). Scale bar, 50 μ m. Right: Bar chart represents the H score of FOSL1 staining. Data represent mean $\pm$ SD (n=6). (p values were calculated by Student's t test). *p < 0.05, **p < 0.01. (D) Left: Immunohistochemical staining for FOSL1 and PAF1 in human PDAC tissues (with and without smoking history) and in normal pancreas. Scale bar, 100 μ m. Right:

Confocal images showing the co-expression of FOSL1 (stained in red) and PAF1 (stained in green) in these tissues. Scale bar, 100 μ m. Nuclei were stained in blue using DAPI. Bar charts below show quantification of FOSL1 and PAF1 staining in normal pancreas (n=15), PDAC without (n=15) and with (n=15) smoking history. Data represent mean $\pm$ SD. (p values were calculated by Student's t test). ***p < 0.001. (E) Immunoblotting assays for CHRNA7, p-ERK1/2, ERK1/2, p-FOSL1, FOSL1, p-cJun and cJun signaling molecules in CSE-treated cells as compared to untreated controls. (F) Immunoblotting assays for p-ERK1/2, ERK1/2, p-FOSL1, FOSL1, PAF1 in CSE exposed HPNE and Capan1 cells with or without ERK1/2 inhibition using PD98059. (G) Small interfering RNA (siRNA) knock down of nACHR α 7 in CSE treated cells. Western blot analysis showing the effect of nACHR α 7 knockdown on p-FOSL1 and PAF1. (E–G) β -actin was used as loading control. DAPI, 4',6-diamidino-2-phenylindole; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Figure 6. Cigarette smoke components, Nicotine, NNN, and NNK are highly responsible for augmentation of pancreatic stemness

HPNE and Capan1 cells were left untreated or treated with Nicotine (2μM), NNK (2μM) and NNN (2μM) for 80 days. (A) Left: Flow cytometry analysis of AF content. Right: Percentage of AF+ population in HPNE and Capan1 cells exposed to cigarette smoke components as compared to respective controls. Data represent mean ± SD (n=3) (p values were calculated by Student's t test). * p < 0.05, ** p < 0.01. (B) Sphere formation assay was performed on HPNE and Capan1 cells exposed to cigarette smoke components. 2000 cells/well were seeded in 96 well ultra-low attachment plates in stem cell medium. Left: Morphology of 10-day old spheres. Scale bar: 200μm. Right: Number of spheres per 2000 cells in HPNE and Capan1 cells exposed to cigarette smoke components as compared to untreated controls. Data represent mean ± SD (n=6). (p values were calculated by Student's t test). *** p < 0.001. (C–D) Immunoblotting assay for CHRNA7, p-FOSL1, PAF1, KLF4 and SOX9. β-actin was used as loading control.

Figure 7. Cigarette smoke exposure increases PHF5A and augments interaction between PAF1 and PHF5A

(A) HPNE and Capan1 cells were left untreated or treated with CSE (1%) for 80 days. Immunoblot analysis showing the protein expression levels of PAF1 complex (PAF1C) molecules along with serine 2 phosphorylated RNA polymerase II (S2-Phos RNA Pol II) and PHF5A. β-actin was used as loading control. (B) Left: Representative images of IHC for PHF5A in pancreatic tissues obtained from 20 weeks cigarette smoke-exposed control and Kras^{G12D} Pdx-Cre mice. Scale bar, 100 μm. Right: Immunofluorescence staining for PAF1 (stained in red) and PHF5A (stained in green) in pancreas of cigarette smoke-exposed control and Kras^{G12D} Pdx-Cre mice models (Nuclei were stained with DAPI). Scale bar, 50 μm. Bar chart below (C) represents the H score of PHF5A staining in the pancreas of cigarette smoke exposed mice. Data represent mean ± SD (n=6). (p values were calculated by Student's t test). *p < 0.05, **p < 0.01. (D) Immunofluorescence staining for PAF1 and PHF5A on HPNE and Capan1 cells exposed to CSE. Co-expression of PAF1 with PHF5A was shown. Nuclei were stained with DAPI. (E) Immunoblots showing that PAF1 interacts with PHF5A in untreated and CSE treated HPNE and Capan1 cells. Pull down was performed using PAF1 antibody, and immunoprecipitates were probed with PHF5A antibody. IgG control and input, 10% of total lysate, were used as negative and positive

controls, respectively. (F) Schematic showing overall mechanism involved in the cigarette smoke mediated induction of pancreatic stemness. Exposure of human pancreatic ductal cells and cancer cells to cigarette smoke and its components increases induce stemness by increasing PAF1 through CHRNA7-ERK1/2-AP1 (FOSL1-cJUN) signaling pathway. Cigarette smoke induced PAF1 and PHF5A interacts and form PAF1-PHF5A complex, required for the activation of stemness or cancer stemness genes.

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