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# PAF-Mediated MAPK Signaling Hyperactivation *via LAMTOR3* Induces Pancreatic Tumorigenesis

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## SUMMARY

Deregulation of mitogen-activated protein kinase (MAPK) signaling leads to development of pancreatic cancer. Although *Ras* mutation-driven pancreatic tumorigenesis is well understood, the underlying mechanism of Ras-independent MAPK hyperactivation remains elusive. Here, we identified a distinct function of PAF (PCNA-associated factor) in modulating MAPK signaling. PAF is overexpressed in pancreatic cancer, and required for pancreatic cancer cell proliferation. In mouse models, PAF expression induced pancreatic intraepithelial neoplasia with expression of pancreatic cancer stem cell markers. PAF-induced ductal epithelial cell hyperproliferation was accompanied by extracellular signal-regulated kinase (ERK) phosphorylation, independent of *Ras* or *Raf* mutations. Intriguingly, PAF transcriptionally activated the expression of late endosomal/lysosomal adaptor, MAPK and mTOR activator 3 (*LAMTOR3*) that hyperphosphorylates MEK and ERK, which was necessary for pancreatic cancer cell proliferation. Our results reveal an unsuspected mechanism of mitogenic signaling activation *via LAMTOR3*, and suggest that PAF-induced MAPK hyperactivation contributes to pancreatic tumorigenesis.

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**AUTHOR CONTRIBUTIONS**

Conceived and designed the experiments: J.I.P.; performed the experiments: S.J., S.H.L., H.C.K., A.M.S., C.N., H.J., and J.I.P.; analyzed the data: S.H.L., H.C.K., C.N., H.W. and J.I.P.; contributed materials: H.Y. and R.A.D.; wrote the paper: J.I.P.

**Keywords**

PAF; KIAA0101; pancreatic cancer; LAMTOR3; MAPK

## INTRODUCTION

Pancreatic cancer is the fourth-leading cause of cancer death in the United States, with a five-year survival rate of less than 6% (Siegel et al., 2012). Pancreatic cancer is characterized by highly aggressive potential and the absence of a distinct biomarker, which leads to poor early diagnosis (Iovanna et al., 2012). Thus, understanding the molecular mechanism of pancreatic tumorigenesis is imperative to develop efficient treatment, prevention, and early diagnosis of pancreatic cancer.

Pancreatic tumorigenesis is driven by genetic and epigenetic deregulation of oncogenes, tumor suppressor genes, and developmental signaling pathways (Abraham et al., 2002). Among them, *K-Ras* oncogenic mutations occur in 90% of pancreatic cancers (Bos, 1989; Thomas et al., 2007). It was also shown that the *K-Ras* genetic mutation is required not only for the initiation but also for the maintenance of pancreatic cancer (Collins et al., 2012; Ying et al., 2012). These evidences highlight the crucial role of K-Ras-mediated signaling in pancreatic cancer (Bardeesy and DePinho, 2002). K-Ras transduces mitogen-activated protein kinase (MAPK) signaling, which controls cell proliferation, differentiation, and apoptosis (Malumbres and Barbacid, 2003). However, mutation in the *K-Ras* gene constitutively hyperactivates the downstream signaling, including extracellular signal-regulated kinase (ERK), phosphoinositide 3-kinase (PI3K), and the Ral guanine nucleotide exchange factor (Rajalingam et al., 2007; Schubbert et al., 2007; Sweet et al., 1984), which subsequently leads to cell transformation and tumorigenesis (Campbell et al., 2007; Rajalingam et al., 2007; Schubbert et al., 2007; Sweet et al., 1984). Despite the pivotal roles of K-Ras-mediated MAPK signaling in pancreatic tumorigenesis, cancer therapies targeted directly against Ras have not been successful (Surade and Blundell, 2012), which has led to seek alternative strategies such as inhibiting the downstream molecules of Ras or using synthetic lethal interactions (Chan and Giaccia, 2011). Thus, it is important to understand the full spectrum of regulatory mechanisms of Ras/MAPK signaling in pancreatic cancer.

In association with proliferating cell nuclear antigen (PCNA), PAF (PCNA-associated factor, *KIAA0101/NS5ATP9/OEACT-1*) plays roles in translesion DNA synthesis (TLS) during error-prone DNA repair and homologous recombination (Emanuele et al., 2011; Povlsen et al., 2012). Here, we found that PAF overexpression was associated with MAPK signaling activation and pancreatic cancer cell proliferation. Our mouse models demonstrated that PAF ectopic expression induces pancreatic neoplasia. Interestingly, PAF hyperactivates MAPK signaling *via* transactivation of *LAMTOR3* (*MAP2KIP1/MAPBP/MAPKSP1/MP1*), a scaffolding protein that facilitates interaction between MEK and ERK, and hyperphosphorylates MEK and ERK (Schaeffer et al., 1998). Our results reveal the novel mechanism of MAPK signaling activation *via* PAF-mediated LAMTOR3 transactivation in pancreatic cancer.

# RESULTS

## Mitogenic role of PAF in pancreatic cancer cells

To identify genes playing pivotal roles in pancreatic tumorigenesis, we analyzed multiple datasets of human pancreatic cancer using Oncomine database (www.oncomine.org). Among several genes highly overexpressed in pancreatic cancer, we focused on the *PAF* gene, based on high expression of *PAF* in pancreatic cancer cells (Fig. S1) (Emanuele et al., 2011; Logsdon et al., 2003). Consistent with the previous studies (Emanuele et al., 2011), we observed that PAF is significantly overexpressed in human pancreatic adenocarcinoma but not expressed in normal pancreas including ductal epithelial, acinar, and islet cells (data not shown), which led us to hypothesize that PAF expression is associated with pancreatic tumorigenesis. First, we asked whether PAF expression contributes to proliferation of pancreatic cancer cells. Consistent with *in silico* analysis, Panc-1 cells expressed a high level of PAF protein, which prompted us to perform PAF loss-of-function analysis in Panc-1 cells. To deplete the endogenous PAF protein, we used lentiviruses encoding short hairpin RNA (shRNA) against green fluorescent protein (GFP) (shGFP) (control) or PAF (shPAF) (Fig. 1A) and examined the effects of PAF knockdown on Panc-1 cell proliferation. Intriguingly, shRNA-mediated PAF knockdown inhibited proliferation of Panc-1 cells (Figs. 1B and 1C). Also, we observed that PAF knockdown increased the proportion of cells in the G1 phase of the cell cycle (Fig. 1D). Additionally, ectopic expression of non-targetable wild-type PAF (ntPAF) reverted the shPAF-induced cell growth inhibition (Fig. 1E, lane 5), which confirms the specific effect of shPAF on *PAF* transcripts.

PAF was initially identified as a PCNA interacting protein (Yu et al., 2001). Thus, we tested whether PAF-PCNA association is dispensable for PAF-mediated pancreatic cancer cell proliferation, using a PAF mutant harboring mutations in the PIP motif (mutPIP-PAF; I65A:F68A:F69S). Consistent with ntPAF, mutPIP-PAF also rescued shPAF-induced cell growth inhibition (Figs. 1E and 1F), indicating that PAF-PCNA interaction is dispensable for PAF-mediated pancreatic cancer cell proliferation. These results suggest that PAF expression is required for pancreatic cancer cell proliferation, independent of PCNA interaction.

## Pancreatic intraepithelial neoplasia by PAF

Given (1) overexpression of PAF in pancreatic cancer cell and (2) the mitogenic role of PAF in pancreatic cancer cells, we hypothesized that PAF conditional expression induces pancreatic tumorigenesis. To address this, we assessed *in vivo* effects of PAF overexpression on pancreatic cell proliferation using genetically engineered mouse models. In order to mimic the overexpression of PAF in pancreatic cancer, we utilized doxycycline (doxy)-inducible *PAF* (*iPAF*) transgenic mice (Jung et al., 2013a). Then, we bred an *iPAF* with a *Rosa26-rtTA* strain. Upon doxy treatment, reverse tetracycline transactivator (*rtTA*) expressed from the *Rosa26* promoter becomes active and binds to TetO, which then transcriptionally induces the expression of *PAF* (Fig. 2A). After Doxy administration (8 mos), we examined the pancreatic tissues of control (*iPAF* + doxy) and experimental (*iPAF:Rosa26-rtTA* + doxy) group of mice (Fig. 2B). Intriguingly, PAF-induced mice exhibited pancreatic intraepithelial neoplasias (PanINs), with characteristic features of

columnar cell morphology, aberrant foci, papillary infoldings, and chronic pancreatitis, whereas the pancreas of control mice displayed normal cuboidal ductal epithelium (Figs. 2C and 2D). Given that PAF is required for pancreatic cancer cell proliferation *in vitro* (Fig. 1), we examined whether PAF-induced PanINs is due to hyperproliferation of pancreatic ductal epithelial cells. Indeed, PAF-induced mice exhibited mitogenic activation of pancreatic ductal epithelial cells, as manifested by Ki67, a cell mitotic marker, positive cells, whereas control mice did not show any Ki67 positive cells (Figs. 2E and 2F). Moreover, PAF-induced pancreatic lesion exhibited marked expression of several pancreatic tumor markers including Alcian blue, Mucin1 (MUC1), cytokeratin 19 (CK19), and Cyclooxygenase-2 (COX2) (Figs. 2G–2J), as shown in *Pdx1-Cre:K-RasLSL*$^{G12D}$ pancreatic cancer mouse model (Fig. 2K). These results suggest that conditional expression of PAF is sufficient to initiate PanINs, the precursor lesion of human pancreatic cancer.

## Pancreatic cancer stem cell marker expression by PAF

A growing body of evidence suggests that cancer stem cells (CSCs), the origin of cancer cells, are mainly responsible for tumor heterogeneity, metastasis, recurrence, and therapy resistance (Magee et al., 2012). Given that PAF is specifically expressed in pancreatic cancer cells (Fig. S1), and its ectopic expression is sufficient to develop pancreatic neoplasia (Fig. 2), we asked whether PAF expression is also associated with positive regulation of pancreatic CSCs. We analyzed the expression of pancreatic CSC markers including CD24, C44, CD133, and CXC chemokine receptor 4 (CXCR4) (Hermann et al., 2007; Li et al., 2007), in the setting of PAF ectopic expression or depletion. First, we assessed the expression of *CXCR4* and *CD24* in Panc-1 cells (shGFP and shPAF), using quantitative reverse transcriptase PCR (qRT-PCR). Interestingly, depletion of endogenous PAF downregulated the expression of *CXCR4* and *CD24* (Fig. 3A). Additionally, the activity of aldehyde dehydrogenase (ALDH), another pancreatic CSC marker (Rasheed et al., 2010), was significantly inhibited by PAF knockdown in Panc-1-shPAF cells (Fig. 3B). Next, we further examined the effects of PAF ectopic expression on pancreatic CSC marker expression in our *iPAF* mouse model. Consistent with *in vitro* results using Panc-1 cells, pancreatic CSC markers (CD133, CD44, and CD24) were induced in neoplastic lesions of PAF-induced mice, compared with normal pancreatic ducts of control mice (Figs. 3C and 3D). These results imply that PAF induction may also contribute to the development or maintenance of pancreatic CSCs during pancreatic tumorigenesis.

## PAF induces MAPK hyperactivation *via LAMTOR3* transactivation

Having observed the mitogenic role of PAF in pancreatic cancer cells, we then sought to determine the underlying mechanism of PAF-induced pancreatic neoplasia. Most pancreatic cancer cells exhibit hyperactivation of the Ras/MAPK signaling pathway (Bardeesy and DePinho, 2002). Moreover, *K-Ras* oncogenic mutation is sufficient to initiate pancreatic cancer in genetically engineered mouse models (Aguirre et al., 2003; Hingorani et al., 2003), which led us to hypothesize that PAF positively modulates Ras/MAPK signaling activity. To test this, we examined whether PAF ectopic expression affects the phosphorylation status of ERK1/2, MAPK signaling components. Intriguingly, immunostaining for phosphorylated ERK1/2 showed that PAF expression induced phosphorylation of ERK1/2 in neoplastic ductal epithelial cells, while the control mice displayed no phosphorylation of ERK1/2 in

ductal epithelial cells (Fig. 4A). Next, we tested whether PAF expression *per se* is sufficient to activate ERKs in pancreatic cancer cells. Due to *K-Ras* mutation (G12D) in Panc-1 cells, we utilized BxPC-3 pancreatic cancer cells that harbor the wild-type *K-Ras* gene. Indeed, PAF ectopic expression hyperphosphorylated ERK and MEK in BxPC-3 cells (Fig. 4B). Additionally, to complement gain-of-function approach, we tested whether PAF knockdown downregulates ERK1/2 phosphorylation in Panc-1 cells exhibiting constitutive activation of MAPK by *K-Ras* mutation. Interestingly, PAF knockdown (shPAF) significantly suppressed phosphorylation of ERK1/2 in Panc-1 cells (Fig. 4C). However, Raf pull-down assays showed that PAF ectopic expression did not affect Ras GTPase activity in BxPC-3 cells (Fig. 4D), suggesting that PAF acts at the downstream of Ras to activate MAPK signaling. Due to the canonical role of PAF-PCNA complex in facilitating TLS (Emanuele et al., 2011; Povlsen et al., 2012), it is plausible that PAF ectopic expression might compromise the DNA repair pathway and cause genetic mutations in Ras/MAPK signaling components. Thus, we performed sequencing analysis of *Ras* and *Raf* genes in PAF-induced neoplastic lesions of pancreas. However, we found no mutations in *Ras* (*K*-, *H*-, and *N*-) and *Raf* genes (Fig. S2), indicating that PAF-mediated TLS is not involved in PAF-induced pancreatic neoplasia. This is also consistent with our results showing the PCNA-independent mitogenic function of PAF in pancreatic cancer cells (Figs. 1E and F). These *in vitro* and *in vivo* results suggest that PAF activates MAPK signaling at the downstream of Ras.

In our recent study, we observed that PAF occupies the proximal promoter and transactivates β-catenin target genes (Jung et al., 2013a). Hence, we examined whether PAF hyperactivates MAPK signaling by transcriptional regulation of MAPK signaling components. To test this, we performed gene expression analysis of Ras/MAPK signaling components and regulators including LAMTOR3, kinase suppressor of Ras (KSR), protein kinase A (PKA), and dual specificity phosphatase 1/6 (DUSP1/6). Intriguingly, PAF-depleted Panc-1 cells (shPAF) exhibited the specific downregulation of *LAMTOR3* transcription (Figs. 4E and 4F). Moreover, immunostaining for LAMTOR3 showed that LAMTOR3 protein was significantly upregulated in ductal epithelial cells of PAF-induced neoplastic pancreatic lesions (Fig. 4G). It has been shown that, as a scaffold protein, LAMTOR3 facilitates MEK-ERK interaction and hyperphosphorylates MEK and ERK *via* complex formation with p14 and MEK1 (Schaeffer et al., 1998; Wunderlich et al., 2001). Thus, our data suggest that PAF-induced ERK activation might be mediated by LAMTOR3. Next, we asked how PAF upregulates *LAMTOR3* gene expression. Based on the recruitment of PAF to promoters of Wnt targets including *c-Myc* (Jung et al., 2013a), we tested whether *c-Myc* or Wnt signaling activation mediates PAF-induced LAMTOR3 upregulation. We found that c-Myc ectopic expression or Wnt signaling activation did not transactivate *LAMTOR3* (Fig. S3), suggesting that both Wnt signaling and c-Myc are not involved in PAF-mediated *LAMTOR3* transactivation. Next, we tested whether LAMTOR3 is upregulated in human pancreatic cancer. Immunostaining of tissue microarray showed that pancreatic adenocarcinoma cells exhibited marked upregulation of LAMTOR3 in the perinucleus and cytosol (15 out of 20 pancreatic adenocarcinoma samples) (Fig. 4H), consistent with *in silico* analysis of *LAMTOR3* expression of cDNA microarray datasets (Iacobuzio-Donahue et al., 2003) (Fig. 4I). We also observed that PAF and LAMTOR3 were co-expressed in PAF-expressing cells of PAF-induced pancreatic lesion (Fig. 4J) and human

pancreatic cancer cells (Fig. 4K). Next, we asked whether PAF-induced pancreatic cell hyperproliferation is due to PAF-mediated transactivation of *LAMTOR3*. Indeed, LAMTOR3 expression rescued PAF depletion-induced growth arrest of Panc-1 shPAF cells (Fig. 4L). Moreover, shPAF-induced hypophosphorylation of ERK1/2 was also reverted by LAMTOR3 expression (Fig. 4M). Also, using constitutively active ERK mutant (ERK2-MEK1-LA) (Robinson et al., 1998), we tested whether ERK activation restores PAF depletion-induced growth inhibition of Panc-1 cells. Given that ERK2-MEK1-LA mutant is a fusion protein of MEK1 and ERK2 (Robinson et al., 1998), ERK2-MEK1-LA mutant does not need a scaffolding protein such as LAMTOR3 for signal transduction. Indeed, ERK2-MEK1-LA mutant rescued PAF depletion-induced cell growth arrest in Panc-1 cells (Figs. 4N and 4O). Additionally, we observed that LAMTOR3-depleted Panc-1 cells displayed the decreased cell proliferation (Fig. 4P). These results strongly suggest that PAF activates MAPK signaling *via LAMTOR3* transactivation, which may contribute to pancreatic cancer cell proliferation (Fig. 4Q).

## DISCUSSION

The prevailing view of pancreatic cancer models is that the constitutive activation of K-Ras/MAPK signaling leads to hyperproliferation and transformation of pancreatic ductal epithelial cells (Aguirre et al., 2003; Hingorani et al., 2003). Nonetheless, the etiology of pancreatic cancer not carrying genetic mutations in *K-Ras* or *Raf* has remained elusive. In our pancreatic cancer model, PAF induces hyperproliferation of pancreatic ductal epithelial cells, independently of *Ras* or *Raf* oncogenic mutations. Ras-independent MAPK activation is due to the PAF-induced transactivation of *LAMTOR3*, a scaffolding protein for ERK and MEK. Our results reveal the novel mechanism of Ras-independent MAPK activation in pancreatic cancer.

In the setting of PAF conditional expression, we observed the overall induction of pancreatic CSC markers (Fig. 3) in the pancreatic ductal epithelial cells. However, we detected Ki67 positive cells only in a small portion of neoplastic cells. This discrepancy might be explained by the differential effects of PAF on cell proliferation and cell fate property. For example, in intestine, Ki67 only marks transit-amplifying cells located at the crypt-villi boundary, whereas CD44, a β-catenin target gene, is expressed in most intestinal epithelial cells of the crypt base (Wielenga et al., 1999). Alternatively, different expression pattern between pancreatic CSC markers and the Ki67 might be due to additional intrinsic or extrinsic factors that are only required for PAF-induced cell proliferation. Although we here focused on the role of PAF in regulating cell proliferation, PAF-induced pancreatic CSC marker expression implies that PAF may play an additional role in modulating epithelial cell plasticity beyond its mitogenic role, which should be addressed in future studies.

We observed that PAF induces *LAMTOR3* transcriptional activation. Although we recently found that PAF-β-catenin-EZH2 transcriptional complex hyperactivates Wnt target genes in colon cancer (Jung et al., 2013a), in pancreatic cancer cells, Wnt signaling did not transactivate *LAMTOR3* (Fig. S3), showing the different function of PAF in regulating MAPK signaling between pancreatic and colorectal cancer. Thus, it is necessary to understand the detailed molecular mechanism of PAF-induced *LAMTOR3* transactivation.

In addition to its function in activating MEK-ERK (Schaeffer et al., 1998), *LAMTOR3* was rediscovered as a regulator of mammalian target of rapamycin (mTOR) signaling (Sancak et al., 2010). mTOR signaling regulates cell growth by upstream stimuli, including growth factors, oxygen levels, intracellular energy levels, and amino acids (Zoncu et al., 2011). As a component of the trimeric protein complex (LAMTOR3, ROBLD3, and C11orf59), LAMTOR3 recruits mTORC1 to Rheb, a Ras-related GTP-binding protein, and activates mTORC1 (Sancak et al., 2010). Thus, it is likely that both MAPK and mTOR signaling might be activated by PAF-induced *LAMTOR3*. We observed that PAF ectopic expression indeed hyperphosphorylated p70 S6 kinase, a downstream effector of mTOR, in BxPC-3 cells (data not shown). Thus, it will be interesting to study the convergent roles of PAF in regulating both MAPK and mTOR signaling pathway. Additionally, it is necessary to determine whether genetic ablation of PAF suppresses pancreatic tumorigenesis using *PAF* conditional knockout mice, which will further validate our gain-of-function mouse model results. Taken together, our study reveal an unsuspected function of PAF in activating the MAPK signaling pathway, and propose that PAF overexpression contributes to pancreatic tumorigenesis *via LAMTOR3* transactivation.

## EXPERIMENTAL PROCEDURES

### Mouse models

*iPAF* (Jung et al., 2013a):*Rosa26-rtTA* (Jackson Laboratory) strain was administered with doxy (2 mg/ml in 5% sucrose drinking water). All mice were maintained under institutional guidelines and Association for Assessment and Accreditation of Laboratory Animal Care International standards.

### Mammalian cell culture

Panc-1 and BxPC-3 cells were maintained with Dulbecco's modified eagle medium containing 10% fetal bovine serum. Lentiviruses encoding shRNAs for PAF and LAMTOR3 (Sigma-Aldrich) were stably transduced into target cells using puromycin selection (2 mg/ml). shRNAs and mammalian expression plasmids were transfected using polyethyleneimine reagent, as previously performed (Jung et al., 2013b).

### Constructs

Wild-type, nt-, and mutPIP-PAF constructs were generated via polymerase chain reaction (PCR) from complementary DNA, as previously performed (Jung et al., 2013a). ERK2-MEK1-LA construct was kindly provided by Melanie Cobb (University of Texas Southwestern).

### Gene expression analysis

Gene expression analysis was performed, as previously performed (Park et al., 2009).

### Immunoblotting

Immunoblotting was performed as previously described (Jung et al., 2013b).

### Immunohistochemistry

Mouse pancreatic tissues were collected and fixed with 10% formalin and processed for paraffin embedding. The sectioned samples were immunostained according to the standard protocols. Pancreatic cancer tissue microarray slides were purchased from Biomax (PA242a). Immunostained samples were analyzed using Observer.Z1m microscope (Zeiss) and Axiovision software (Zeiss).

### Alcian blue staining

Deparaffinized slides were stained in 1% (w/v) Alcian blue 8GX dissolved in 3% acetic acid for 30 min and then washed with distilled water.

### ALDH activity quantification

The Aldefluor assay was carried out following the manufacturer's guidelines (StemCell Technologies).

### Ras activity assay

Ras activity was measured using a Ras Activation Assay Kit (Millipore).

### Statistical analysis

For comparison of two samples, we employed Student's *t*-test. The calculation was from at least three biological replicates. A $P$ value $< 0.05$ was considered significant. Error bars indicate standard deviation.

Full Experimental Procedures are available in Supplemental Information.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## HIGHLIGHTS

- PAF overexpressed in pancreatic cancer is required for pancreatic cancer cell proliferation
- PAF ectopic expression develops pancreatic ductal neoplasia in mouse models
- PAF hyperactivates MAPK signaling *in vitro* and *in vivo*
- PAF-induced MAPK activation is mediated by *LAMTOR3* transactivation

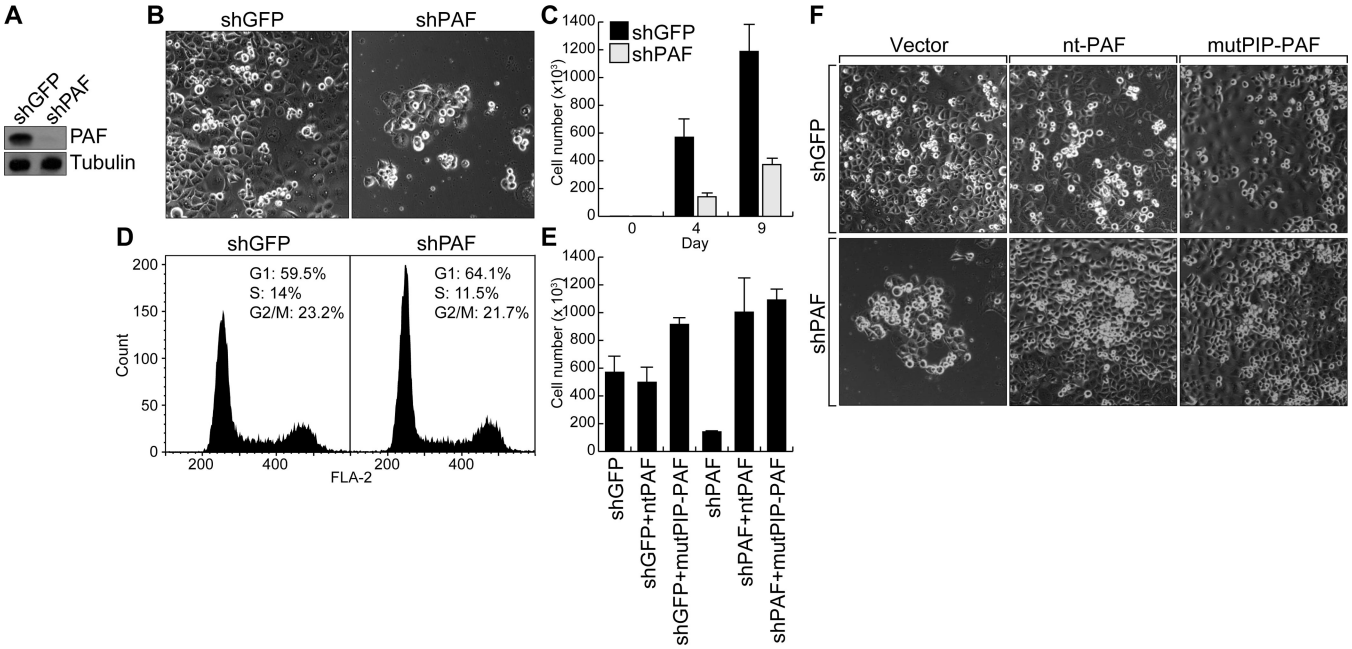


**Fig. 1. Mitogenic role of PAF in pancreatic cancer cells**

(A) Depletion of endogenous PAF in Panc-1 cells.

Immunoblot of Panc-1 stably expressing shGFP or shPAF.

(B and C) Growth inhibition of Panc-1 cells by PAF depletion.

Panc-1 (shGFP or shPAF) cells were plated and analyzed for phase contrast imaging (B) and cell proliferation by cell counting (C) (N = 3).

(D) G1 cell cycle arrest by PAF depletion.

Cell cycle analysis of Panc-1 using flow cytometry. The representative was shown (N = 3).

(E and F) PCNA-independent mitogenic role of PAF.

Panc-1 (shGFP or shPAF) were stably transfected with mutPIP-PAF or nt-PAF. Then, the same number of each group of cells were plated and counted after 4 days. Cell counting (E) (N = 3); phase-contrast images (F).

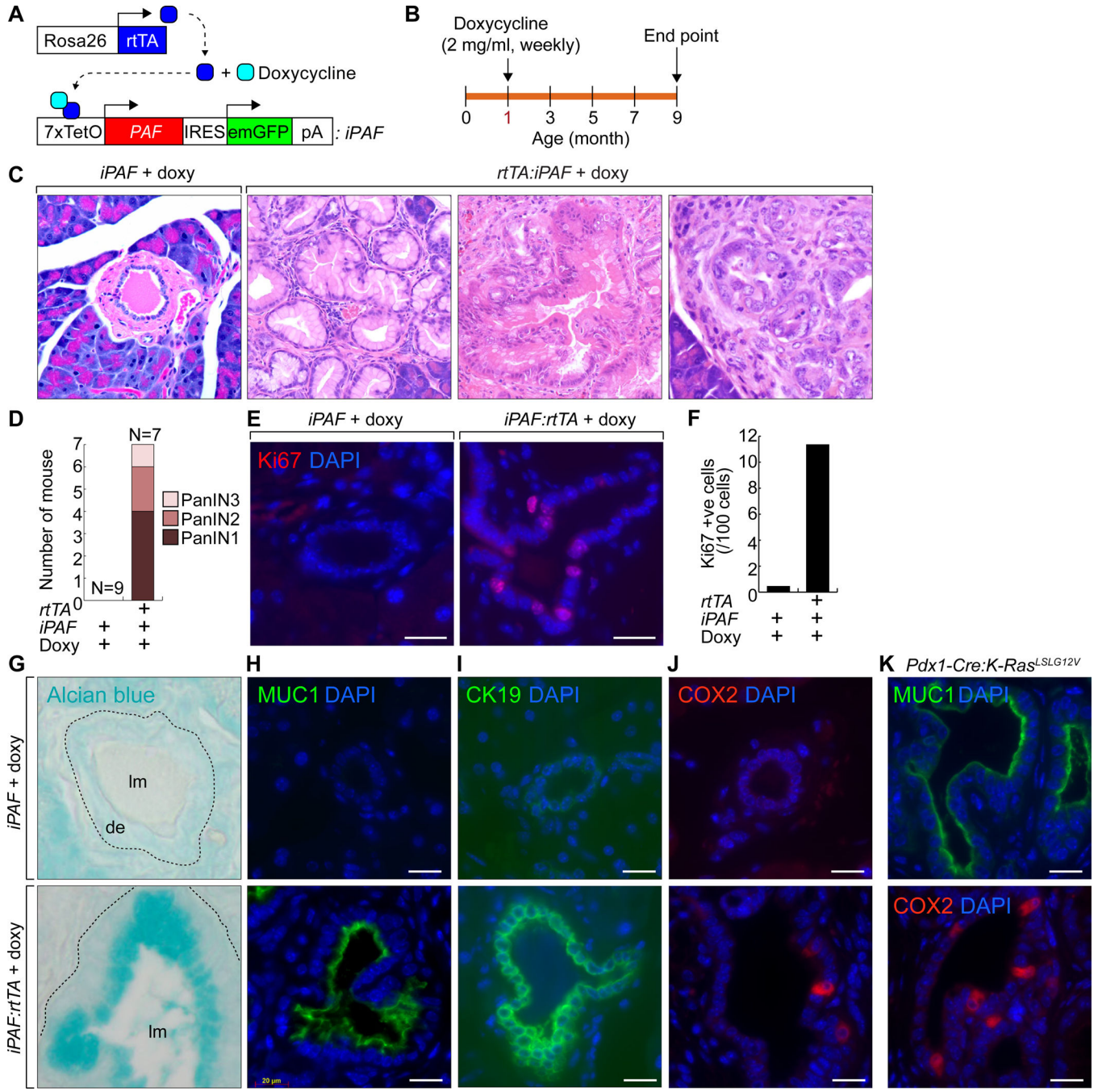


**Fig. 2. PAF expression induces pancreatic intraepithelial neoplasia**

(A) PAF-inducible mouse model.

(B) PAF induction strategy.

(C and D) PanINs by PAF ectopic expression.

Hematoxylin and eosin staining (PanIN1-3)(C); quantitative analysis of PanINs (D).

(E and F) Hyperproliferation of pancreatic ductal epithelial cells by PAF.

Ki67 immunostaining (E) and quantification of Ki67 positive cells (F). Doxy: 2 mos.

(G–J) Tumorigenic marker expression by PAF.

Alcian blue (G) (doxy: 2 mos.) and MUC1 (H); CK19 (I); COX2 (J) (Doxy: 8 mos.) (K) MUC1 and CK19 expression in PanINs of *Pdx1-Cre:K-Ras*$^{LSLG12D}$ mouse. Scale bar = 20 μm.

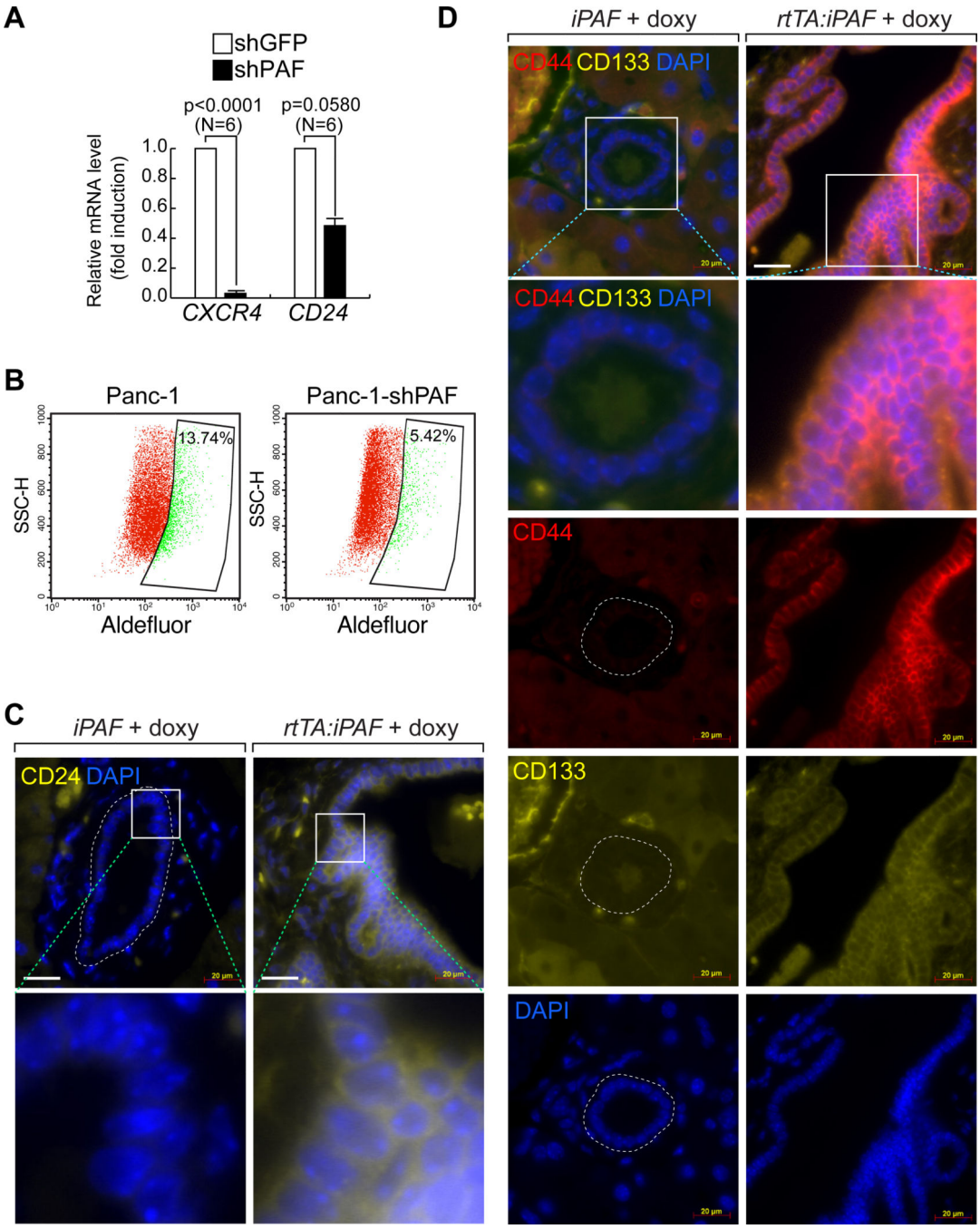


**Fig. 3. Pancreatic cancer stem cell marker expression by PAF**

(A) Downregulation of *CXCR4* and *CD24* by PAF knockdown.

qRT-PCR of Panc-1-shGFP and –shPAF.

(B) Downregulation of ALDH activity by PAF depletion.

ALDH activity analysis of Panc-1 shGFP and –shPAF cells. The representative was shown (N = 2).

(C and D) Expression of CD44, CD133, and CD24 by PAF.

Pancreatic tissues of control (*iPAF*) and PAF-induced mice (*Rosa26-rtTA:iPAF*; doxy, 4 mos) were immunostained for CD24 (C), CD44, and CD133 (D).
Scale bar = 20 μm.

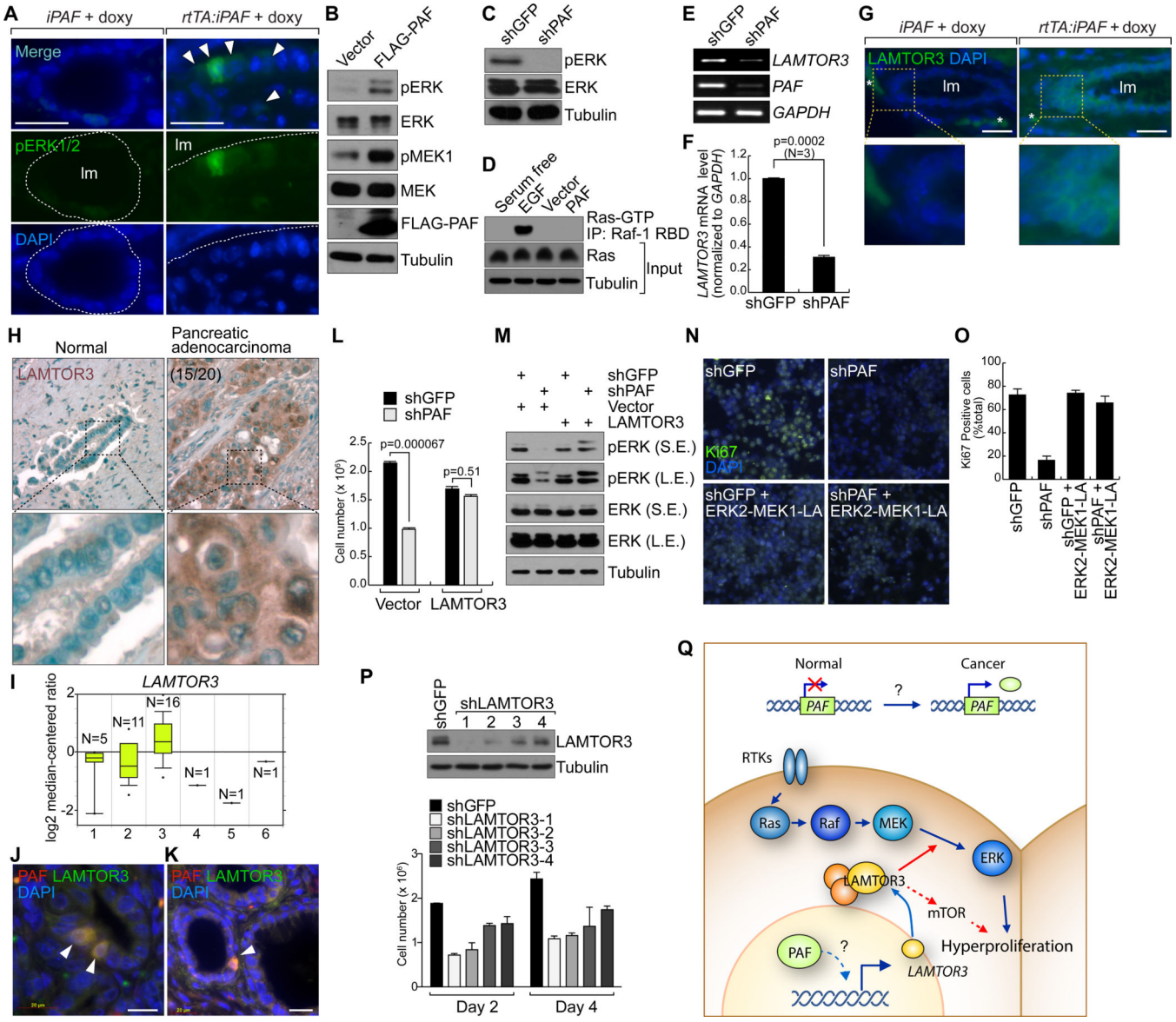


**Fig. 4. PAF-induced MAPK signaling activation *via LAMTOR3* transactivation**

(A) *In vivo* ERK phosphorylation by PAF.

Immunostaining of pancreatic tissues from control and PAF-induced mice (doxy for 2 mos). Arrowheads: pERK-positive pancreatic ductal epithelial cells.

(B) Hyperphosphorylation of ERK by PAF.

Immunoblot analysis of BxPC-3 cells transfected with empty vector or FLAG-PAF expression plasmids.

(C) Dephosphorylation of ERK by PAF knockdown.

Immunoblot of Panc-1-shGFP and –shPAF cells.

(D) No Ras activation by PAF.

BxPC-3 cells were transiently transfected for PAF expression and analyzed for Ras activity. Serum-free and EGF treatment conditions served as negative and positive controls, respectively.

(E and F) Downregulation of *LAMTOR3* by PAF knockdown.
Panc-1 cells (shGFP and shPAF) were analyzed using semiquantitative reverse transcription PCR (E) and qRT-PCR (F).
(G) LAMTOR3 upregulation by PAF *in vivo*.
Pancreatic tissues of control and PAF-induced mice (doxy for 2 mos) were immunostained for LAMTOR3. All images were taken with the same exposure time for quantification. Of note, artifacts (asterisks) by tissue autofluorescence were equally observed in both control and experimental samples.
(H) LAMTOR3 expression in pancreatic cancer.
Pancreatic cancer tissue microarray was immunostained. 3,3'-Diaminobenzidine (DAB) (brown): LAMTOR3; Hematoxylin (blue): nuclear counterstaining.
(I) *In silico* analysis of *LAMTOR3* expression in pancreatic cancer.
Oncomine analysis of *LAMTOR3* expression in pancreatic cancer. NCBI Gene Expression Omnibus (GEO) accession number: GSE3654. 1: normal pancreas; 2: pancreatic adenocarcinoma; 3: pancreatic ductal adenocarcinoma; 4: pancreatic endocrine carcinoma; 5: pancreatic intraductal papillary mucinous carcinoma; 6: pancreatic osteoblast-like giant cell carcinoma.
(J and K) Co-expression of PAF and LAMTOR3 in pancreatic cancer.
Co-immunostaining of PAF-induced lesions (J) (PAF-induced mice; doxy for 2 mos; N = 3) and human pancreatic cancer tissue microarray (K) (15 out 20 pancreatic adenocarcinoma samples showed co-expression of PAF and LAMTOR3). The representative images were shown.
(L) LAMTOR3 rescues PAF depletion-induced pancreatic cancer cell growth inhibition.
Panc-1 cells (shGFP or shPAF) were transduced with retroviruses (LAMTOR3 or empty [control]) were plated ($8\times10^5$ cells) and cultured (4 days) for cell counting (N = 3).
(M) LAMTOR3 expression restores shPAF-induced ERK dephosphorylation.
Panc-1 cells (shGFP or shPAF) were stably transduced with retrovirus encoding *LAMTOR3*. Immunoblotting.
(N and O) ERK2-MEK1-LA rescues PAF depletion-induced growth inhibition.
Panc-1 cells (shGFP or shPAF) were transfected with ERK2-MEK1-LA.
Immunofluorescent staining (N); Ki67 positive cell quantification (O) (N = 2).
(P) LAMTOR3 depletion inhibits Panc-1 cell proliferation.
Panc-1 cells were transduced with lentiviruses encoding shGFP or shLAMTOR3 (total four different shRNAs [1–4] targeting *LAMTOR3*). LAMTOR3 depletion was validated by immunoblot (upper panel). Then, each group of cells was plated ($8\times10^5$ cells) for cell proliferation analysis by cell counting (N = 3).
(Q) Illustration of PAF-induced MAPK signaling activation.
In normal pancreas, PAF is not expressed. However, during pancreatic tumorigenesis, PAF upregulated by unknown factors transactivates *LAMTOR3*, which facilitates MEK-ERK assembly and phosphorylates MEK-ERK. Subsequently, hyperactivation of ERK signaling induces pancreatic ductal epithelial cell hyperproliferation, which may contribute to pancreatic cancer development. PAF-induced *LAMTOR3* might also activate mTOR signaling.
Scale bar = 20 μm.