ETS Proto-oncogene 1 Transcriptionally Up-regulates the Cholangiocyte Senescence-associated Protein Cyclin-dependent Kinase Inhibitor 2A

Received for publication, January 18, 2017, and in revised form, February 6, 2017. Published, JBC Papers in Press, February 8, 2017, DOI 10.1074/jbc.M117.777409

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Primary sclerosing cholangitis (PSC) is a chronic, fibroinflammatory disease of bile ducts of unknown pathogenesis. We reported that cholangiocyte senescence features prominently in PSC and that neuroblastoma RAS viral oncogene homolog (NRAS) is activated in PSC cholangiocytes. Additionally, Persistent microbial insult (e.g. LPSs) induces cyclin-dependent kinase inhibitor 2A (CDKN2A/p16INK4a) expression and senescence in cultured cholangiocytes in an NRAS-dependent manner. However, the molecular mechanisms involved in LPS-induced cholangiocyte senescence and NRAS-dependent regulation of CDKN2A remain unclear. Using our in vitro senescence model, we found that LPS-induced CDKN2A expression coincided with a 4.5-fold increase in ETS1 (ETS proto-oncogene 1) mRNA, suggesting that ETS1 is involved in regulating CDKN2A. This idea was confirmed by RNAi-mediated suppression or genetic deletion of ETS1, which blocked CDKN2A expression and reduced cholangiocyte senescence. Furthermore, site-directed mutagenesis of a predicted ETS-binding site within the CDKN2A promoter abolished luciferase reporter activity. Pharmacological inhibition of RAS/MAPK reduced ETS1 and CDKN2A protein expression and CDKN2A promoter-driven luciferase activity by ~50%. In contrast, constitutively active NRAS expression induced ETS1 and CDKN2A protein expression, whereas ETS1 RNAi blocked this increase. Chromatin immunoprecipitation-PCR detected increased ETS1 and histone 3 lysine 4 trimethylation (H3K4Me3) at the CDKN2A promoter following LPS-induced senescence. Additionally, phospho-ETS1 expression was increased in cholangiocytes of human PSC livers and in the Abcb4−/−Mdr2−/− mouse model of PSC. These data pinpoint ETS1 and H3K4Me3 as key transcriptional regulators in NRAS-induced expression of CDKN2A, and this regulatory axis may therefore represent a potential therapeutic target for PSC treatment.

Primary sclerosing cholangitis (PSC) is an idiopathic, progressive cholangiopathy characterized by peribiliary fibroinflammatory destruction of bile ducts and a predisposition to cholangiocarcinoma (CCA) (1–4). Currently, no effective pharmacotherapy exists to slow progression of the disease, and the median liver transplant-free survival remains at ~12 years (5). Although liver transplant can be an effective therapy for this disease, PSC and/or CCA can occur post-transplant. Given the morbidity and mortality, progressive nature, and lack of effective pharmacotherapy for PSC, a better understanding of the pathogenesis and identification of novel therapeutic targets is needed.

Senescence, an irreversible cell cycle arrest in G1 or G2 phase of the cell cycle (6, 7), is an important pathologic cellular phenotype in a number of conditions of diverse etiologies, including atherosclerosis, osteoarthritis, and chronic obstructive pulmonary disease (8, 9). Senescence can be triggered by a number of stimuli, including strong mitogenic or oncogenic signals, telomere shortening, and/or non-telomeric DNA damage (9, 10). In recent work, we demonstrated that senescent cholangiocytes are abundant in PSC livers, and these senescent cells exhibit characteristics of the senescence-associated secretory phenotype (SASP) (11), a potentially pathologic state of proinflammatory cytokine, chemokine, and growth factor hypersecretion (12). We also previously demonstrated that LPS promotes the cholangiocyte proinflammatory phenotype through TLR4-dependent NRAS activation (13), likely through the transactivation of the epidermal growth factor receptor (14). Given the observation that enterically derived LPS is excreted in bile in a bioactive form (15, 16) and accumulates in cholangiocytes in PSC liver tissue (15), we further assessed whether LPS could induce cholangiocyte senescence in vitro. We dem-

1 This work was supported by National Institutes of Health Grants AI089713 (to S. P. O.), DK057993 (to N. F. L.), the Mayo Foundation, Clinical and Optical Microscopy Cores of the Mayo Clinic Center for Cell Signaling in Gastroenterology Grant P30DK084567, and the Chris M. Carlos and Catharine Nicole Jockisch Carlos Endowment Fund in Primary Sclerosing Cholangitis.

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* The abbreviations used are: PSC, primary sclerosing cholangitis; CCA, cholangiocarcinoma; SASP, senescence-associated secretory phenotype; SA-β-gal, senescence-associated β-galactosidase; qPCR, quantitative PCR; SASP, senescence-associated secretory phenotype; DDR, DNA damage response; NHC, normal human cholangiocyte; SDM, site-directed mutagenesis.
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shown that prolonged LPS treatment (10 days) of cultured cholangiocytes induced expression of the tumor suppressor, CDKN2A, and an associated senescence cellular phenotype (11). Using this novel culture model, we found that insulin-induced cholangiocyte senescence required the small GTPase, NRAS. Finally, we showed that NRAS activation and CDKN2A mRNA is increased in cholangiocytes of PSC livers, supporting a role for the RAS/MAPK signaling axis and CDKN2A expression in cholangiocyte senescence in this disease. However, the precise molecular mechanisms regulating insulin-induced cholangiocyte senescence remain ambiguous. It is known, however, that the ETS1/2 transcription factors are effectors of the RAS/MAPK signaling pathway (17–19), and both ETS1 and ETS2 have been implicated in CDKN2A expression and the induction of senescence (20). Whether either or both of these transcription factors mediate CDKN2A expression in patients with PSC or an experimental model system of insulin-induced cholangiocyte senescence is not known and is a focus of this manuscript.

To address this knowledge gap in PSC and improve our general understanding of insulin-induced cellular senescence, we tested the hypothesis that NRAS/MAPK promotes ETS1/2–dependent transcription of CDKN2A and insulin-induced senescence. Furthermore, we examined epigenetic (i.e. histone methylation) modifications at the CDKN2A locus associated with cellular senescence induction. Finally, we addressed the expression of phospho-ETS1 in cholangiocytes of human PSC livers. Our collective data suggest that during the process of cholangiocyte senescence, the RAS/MAPK pathway induces expression and phosphorylation of ETS1, but not ETS2, promotes CDKN2A transcription via loss of repressive chromatin marks (i.e. histone 3 lysine 27 trimethylation (H3K27me3)) and the induction of permissive chromatin remodelling (H3K4me3). These novel results provide mechanistic insight into insulin-induced cholangiocyte senescence, a cellular state with a potential pathophysiological role in the development and progression of PSC and other diseases. Moreover, a greater understanding of these pathways may provide new therapeutic strategies for PSC and perhaps other conditions where senescent cells likely contribute to disease progression.

Results

Persistent LPS Treatment of Cholangiocytes Increases ETS1 and CDKN2A Expression—The ETS1/2 transcription factors are effectors of the RAS/MAPK signaling pathway (17–19), and both ETS1 and ETS2 have been implicated in CDKN2A expression and the induction of senescence (20). Hence, we assessed ETS expression in our culture model of induced cholangiocyte senescence. We first performed qRT-PCR on cells treated with LPS for 1, 2, 6, or 10 days compared with control (no LPS) cells. CDKN2A mRNA expression was increased (~9-fold, p < 0.05), as was ETS1 (~5-fold, p < 0.05) compared with control cells after 10 days of LPS treatment (Fig. 1A). In contrast, ETS2 expression was not altered in the presence of LPS treatment at any time point. We next determined whether protein expression for ETS1 or ETS2 was altered in the presence of LPS. Following 10-day LPS treatment, ETS1 protein increased compared with control cells (>2-fold), whereas ETS2 protein expression remained unchanged (Fig. 1, B and C). We further show that ETS1 is activated (phosphorylated at threonine 38) following 10-day LPS treatment, whereas ETS2 shows no change in expression or phosphorylation status (Fig. 1, B and C). Given that ETS1, and not ETS2, is altered in our culture model, we stably transfected our cholangiocytes with ETS1-shRNA or an empty vector control. After 10-day LPS treat-
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Depletion of Cholangiocyte ETS1 Prevents LPS-induced CDKN2A Expression and Senescence—To further demonstrate the functional role of ETS1 during LPS-induced cholangiocyte senescence, ETS1-deficient cultured cholangiocytes were generated using Crispr Cas9 technology. ETS1-deficient cholangiocytes were resistant to LPS-induced CDKN2A expression and senescence, whereas forced expression of functional ETS1 (ETS1-GFP) in these cells promoted both increased CDKN2A and senescence (Fig. 2, A and B). Moreover, reconstitution of ETS1-deficient cells with constructs encoding either ETS1 lacking the transactivation domain (ETS-ΔTrans) or a non-phosphorylatable mutant (ETS1-T38A) were resistant to LPS-induced CDKN2A expression and senescence (Fig. 2, A and B). In contrast, forced expression of CDKN2A in ETS1-depleted cells promoted senescence in cells cultured in the presence or absence of LPS (Fig. 2, C and D). Together, these results demonstrate that functional ETS1 is necessary for LPS-induced cholangiocyte senescence, and CDKN2A overexpression is sufficient for cholangiocyte senescence.

To address whether resistance to LPS-induced senescence (i.e. ETS1-deficient cells) alters the secretion of proinflammatory mediators associated with the SASP, we assessed the expression of two known SASP components, IL6 and IL8. NHCs treated with LPS for 10 days exhibited an increase in IL6 and IL8 expression compared with control NHCs treated with LPS as determined by qPCR (Fig. 2E). Secreted IL6 and IL8, as measured by ELISA of cell culture supernatant, also increased in LPS-treated NHCs compared with control NHCs not treated with LPS (~4- and ~2.5-fold, respectively; Fig. 2F). ETS1-depleted cells, cultured in the absence of LPS exhibited an increase in both IL6 (2-fold) and IL8 (3-fold) mRNA compared with NHCs cultured in the absence of LPS. ETS1-depleted cells cultured in the presence of LPS also exhibited an increase, the control cells showed increased ETS1 and CDKN2A protein compared with no LPS treatment control, whereas ETS1-shRNA transfected cells exhibited reduced CDKN2A protein expression in both the LPS-treated and untreated cells (Fig. 1D). We next assessed whether ETS1-shRNA prevented LPS-induced cholangiocyte senescence. In agreement with our previously published data (11), empty vector transfected control cells treated with LPS exhibited a 9-fold increase (p < 0.05) in senescence compared with untreated cells as assessed by senescence-associated β-galactosidase (SA-β-gal) activity (Fig. 1E). In contrast, RNAi-mediated suppression of ETS1 blocked LPS-induced senescence (6-fold decrease, p < 0.05 compared with empty vector transfected LPS-treated cells).

FIGURE 2. Genetic deletion (CRISPR-Cas9 double nickase) of ETS1 suppresses LPS-induced CDKN2A expression and cholangiocyte senescence. A and B, ETS1 expression was prevented in NHC cells by cotransfection with the CRISPR-Cas9 double nickase system plasmids, and the cells were maintained in culture in the presence or absence of LPS for 10 days. Phospho-ETS1, ETS1, and CDKN2A expression was not detected in these cells in the presence or absence of LPS. Accordingly, no increase in cholangiocyte senescence was detected by SA-β-gal assay. Reconstitution of ETS1 expression via transfection with an ETS1-GFP expression construct promoted phospho-ETS1 detection, promoted CDKN2A expression, and increased SA-β-gal detection in both the presence and absence of LPS. Reconstitution of ETS1-deficient cells with forced expression of a mutant ETS1 lacking the transactivation domain (ETS1-Trans) resulted in increased phospho-ETS1 detection in the presence and absence of LPS yet prevented CDKN2A expression and associated cholangiocyte senescence (SA-β-gal detection). Additionally, reconstitution of ETS1-deficient cells with forced expression of a non-phosphorylatable mutant ETS1 (T38A) prevented phospho-ETS1 detection, CDKN2A expression, and cholangiocyte senescence. C and D, forced overexpression of CDKN2A in ETS1-deficient NHCs promotes CDKN2A detection in the presence and absence of LPS and increases cholangiocyte senescence (SA-β-gal detection). The Western blots shown are representative from three separate experiments. SA-β-gal data are expressed as the percentages of SA-β-gal-positive cells per total cell counts (n = 4). E, the expression of IL6 and IL8 was assessed by qPCR in NHCs and NHCs depleted of ETS1 cultured in the presence or absence of LPS. NHCs cultured in the presence of LPS exhibited ~2-fold increase in IL6 and IL8 mRNA expression compared with NHCs not treated with LPS (Ctrl). ETS1-deficient cells cultured in the absence of LPS exhibited an increase in both IL6 (2-fold) and IL8 (3-fold) mRNA compared with Ctrl NHCs. ETS1-deficient cells cultured in the presence of LPS also exhibited an increase in both IL6 (3-fold) and IL8 (3-fold) mRNA compared with Ctrl NHCs. The data represent fold change ± standard deviation compared with NHCs cultured in the absence of LPS (n = 3). F, the expression of IL6 and IL8 was assessed by ELISA in NHCs and NHCs depleted of ETS1 cultured in the presence or absence of LPS. NHCs cultured in the presence of LPS exhibited an increase in IL6 and IL8 expression (4- and 2.5-fold, respectively) compared with control NHCs. ETS1-deficient cells cultured in the absence of LPS exhibited an increase in both IL6 (6-fold) and IL8 (3-fold) compared with Ctrl NHCs. ETS1-depleted cells cultured in the presence of LPS also exhibited an increase in IL6 (~15-fold) and IL8 (~6-fold) compared with control NHCs. The data are presented as mean pmol/ml ± standard deviation (n = 3). Ctrl, control.
increase in IL6 (>3-fold) and IL8 (>3-fold) mRNA compared with NHCs cultured in the absence of LPS (Fig. 2E). ETS1-deficient cells cultured in the absence of LPS exhibited an increase in both IL6 (~6-fold) and IL8 (~3-fold) secreted protein compared with NHCs cultured in the absence of LPS (Fig. 2F). Moreover, ETS1-depleted cells cultured in the presence of LPS exhibited an increase in IL6 (~15-fold) and IL8 (~6-fold) compared with NHCs cultured in the absence of LPS (Fig. 2F). These results suggest that although ETS1 promotes LPS-induced senescence, this transcription factor may function to negatively regulate proinflammatory chemokine/cytokine production.

Expression of Constitutively Active NRAS Promotes ETS1 Expression and Senescence—To assess the effects of NRAS activation on ERK signaling, ETS1 and CDKN2A expression, and senescence, cultured cholangiocytes were stably transfected with a cumate-inducible constitutively active NRAS construct (SparQ NRAS 12D). Constitutive NRAS expression was induced for 0, 4, or 8 days. Expression of activated NRAS was confirmed by precipitating activated RAS protein with RAF beads, followed by an NRAS immunoblot (Fig. 3A). Western blotting demonstrates that in the absence of the ETS1 shRNA, constitutive active NRAS promotes the up-regulation of CDKN2A compared with control NHCs (Ctrl) with or without the ETS1 shRNA and pCGN empty vector transfected cells (EV Ctrl) without the ETS1 shRNA. The activated NRAS-dependent up-regulation of CDKN2A is blocked in the presence of the ETS1 shRNA. F, Western blotting quantitation (densitometry) of the CDKN2A to β-actin (ACTB) ratio demonstrates that RNAi depletion of ETS1 (shRNA) prevents NRAS 12D-dependent up-regulation of CDKN2A (n = 3).
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A. LPS

p-ERK1/2

Total ERK1/2

B. Ratio of p-ERK1/2 to Total ERK1/2

C. ETS1

CDKN2A

ACTB

D. Total ETS1 protein expression (normalized to ACTB)

E. Total CDKN2A protein expression (normalized to ACTB)

FIGURE 4. Pharmacologic inhibitors of Ras (manumycin A)/MAPK (UO126 or PD98059) block LPS-induced ERK phosphorylation and Ets1 and CDKN2A expression. A. phospho-ERK1/2 is increased in persistent LPS-treated cells; this increase is blocked in the presence of both Ras (manumycin A) and MAPK inhibitors (UO126 and PD98059). B. densitometry of the phospho-ERK1/2 and total ERK 1/2 blots demonstrate a significant increase of phospho-ERK1/2 following persistent LPS treatment compared with control cells (no LPS) and a suppression of this increase in the presence of the Ras and MEK inhibitors (n = 3). C, total ETS1 and CDKN2A protein expression is increased in persistent LPS-treated cells; this increase is reduced when the cells are treated with manumycin A, UO126, or PD98059 (−50% decrease, p < 0.05) (Fig. 4, C and E). Collectively, these data suggest that Ras/ERK signaling is required for LPS-induced cholangiocyte ETS1 and CDKN2A expression.

ERK1/2 protein remains unchanged (Fig. 3, B and C). Additionally, ETS1 protein is increased, indicating that NRAS activation is sufficient for the induction of ETS1 expression (Fig. 3, B and C). We further demonstrate that overexpression of activated NRAS promotes increased CDKN2A protein expression and cholangiocyte senescence (Fig. 3, B–D). To confirm a functional role for ETS1 in NRAS-induced cholangiocyte senescence, cholangiocytes were cotransfected with a constitutively active NRAS construct (pCGN NRAS 12D) and an ETS1 shRNA. The ETS1 shRNA suppressed NRAS-induced CDKN2A expression (Fig. 3, E and F). These data support that NRAS activation promotes CDKN2A expression and cholangiocyte senescence via ETS1.

Pharmacologic Inhibitors of RAS/MAPK Block LPS-induced CDKN2A Expression—To better understand the connection between the RAS/MAPK and LPS induction of CDKN2A, we performed immunoblots on cells in the presence or absence of manumycin A (RAS inhibitor), UO126 (MEK1/2 inhibitor), or PD98059 (MEK inhibitor) with or without LPS for 10 days. LPS-treated cells exhibited an approximate 3-fold increase in pERK1/2 compared with untreated cells (Fig. 4A). This increase in pERK1/2 was diminished in the presence of the Ras and MEK inhibitors (~50% decrease, p < 0.05; Fig. 4, A and B). Having demonstrated that the RAS and MEK inhibitors suppress ERK1/2 phosphorylation, we next assessed ETS1 and CDKN2A expression in the presence or absence of these inhibitors. Total ETS1 was increased ~4-fold in the presence of persistent LPS (Fig. 4, C and D), whereas the presence of the inhibitors diminished ETS1 expression by ~50% (p < 0.05; Fig. 4, C and D). LPS-treated cells also demonstrated a 2.5-fold (p < 0.05) increase in CDKN2A compared with untreated cells (Fig. 4, C and E). This LPS-induced CDKN2A protein increase was diminished when the cells were treated with manumycin A, UO126, or PD98059 (~50% decrease, p < 0.05) (Fig. 4, C and E). Collectively, these data suggest that RAS/ERK signaling is required for LPS-induced cholangiocyte ETS1 and CDKN2A expression.

NRAS and ETS1 Drive CDKN2A Promoter-induced Luciferase Expression—To further assess the functional role of RAS in CDKN2A transcription in our LPS-induced senescence model, we generated a CDKN2A promoter-driven luciferase construct. The DNA elements upstream of the CDKN2A transcriptional start site were analyzed for putative transcription factor binding sites using the website-based search engine TFSearch. Two ETS1 binding sites were identified in the CDKN2A promoter (−518 and −279 bp). Approximately 780 bp (the full length) of the CDKN2A promoter were cloned into the luciferase reporter plasmid, pGL4.22 (CDKN2A-pGL4.22). Truncation constructs were also generated deleting the distal (truncation 1) or both (truncation 2) ETS1 sites (Fig. 5A). Cholangiocytes were transfected with each of these constructs. In the absence of LPS, none of these constructs promoted increased luciferase detection. In contrast, in the presence of LPS (10-day culture), the full-length construct resulted in increased luciferase detection ~3.5-fold, whereas no increase in luciferase was detected with either truncation 1 or 2 construct (Fig. 5A), supporting that the full-length construct, including the distal ETS1 site of CDKN2A promoter, is required for LPS-induced expression of the lucif-
We next asked whether RAS or MEK inhibitors suppressed full-length CDKN2A promoter-driven luciferase expression in the presence of LPS. Transfected cholangiocytes, cultured in the absence of LPS and in the presence or absence of the inhibitors, exhibited no increase in reporter luciferase detection compared with empty vector (EV) control (Fig. 5B), further supporting a mechanistic role of RAS/MAPK in CDKN2A transcription. We next assessed whether overexpression of ETS1 is sufficient to drive CDKN2A transcription. We again utilized the full-length CDKN2A promoter luciferase construct, CDKN2A-pGL4.22. Transfection of this construct into control NHC or cotransfection of this reporter construct with the ETS1-shRNA and subsequent dual luciferase reporter assay resulted in no change in luciferase detection compared with the empty pGL4.22 vector (Fig. 5C). Luciferase activity was not increased above control EV for any of the constructs in the absence of LPS. Persistent LPS treatment induced luciferase activity in cells transfected with both the full-length (FL) and mutated proximal predicted ETS1 sites (Δ2). Luciferase detection was diminished in persistent LPS-treated cells transfected with either the Δ1 or Δ1,2 constructs, suggesting that the distal ETS1 site is required for LPS-induced CDKN2A expression (Fig. 5C).
of the CDKN2A promoter to ETS1 and activated NRAS. Additionally, this increase in luciferase detection was suppressed in the presence of the ETS1-shRNA. Site-directed mutagenesis was performed to eliminate the distal (Δ1), proximal (Δ2), or both (Δ1,2) predicted ETS1 binding sites, and dual luciferase assays were again performed (Fig. 5D). Cholangiocytes were transfected with each of these constructs. As with previous assays, in the absence of LPS, none of the constructs exhibited increased luciferase detection. In the presence of LPS (10-day culture), the full length and Δ2 resulted in increased luciferase detection (~3-fold increase), whereas no increase in luciferase detection was observed in the cells transfected with either the Δ1 or Δ1,2 constructs (Fig. 5D). These results further support that the distal ETS1 site is required for LPS-induced expression of the luciferase reporter. Together, these results suggest that either NRAS or ETS1 activation is sufficient to induce transcription from the CDKN2A promoter, and ETS1 is involved in NRAS-induced CDKN2A expression via the distal (~518 bp) ETS1 binding site within the CDKN2A promoter.

**Prolonged LPS Treatment Induces ETS1 Binding and Chromatin Remodeling within the CDKN2A Promoter**—The DNA elements upstream of the CDKN2A transcriptional start site were analyzed for potential epigenetic regulation (i.e. histone methylation marks). To perform these analyses, we used the Encyclopedia of DNA Elements (ENCODE) annotation data (21) through the UCSC Genome Browser (22) and the Blast-like alignment tool (BLAT) (23). This search tool identified a number of putative H3K27me3 (repressive chromatin) and H3K4me3 (permissive chromatin) marks throughout the CDKN2A promoter region. Next, we performed ChIP-PCR on control (no LPS) and LPS-treated cells from our induced senescence model (Fig. 6, A–C). We demonstrated that phospho-ETS1 does not bind to the CDKN2A locus in control conditions (i.e. no LPS). However, after 10-day LPS treatment, the CDKN2A promoter can be PCR-amplified following ETS1 immunoprecipitation, suggesting that this transcription factor occupies this site at the CDKN2A promoter following prolonged LPS treatment. We also performed ChIP-PCR for activated RNA polymerase 2 (POLR2), using an antibody specific for phosphorylated serine 2 of the POLR2 carboxyl-terminal domain (Ser(P)-2 POLR2). Although we were unable to amplify the CDKN2A locus following Ser(P)-2 POLR2 immunoprecipitation in control conditions, CDKN2A DNA elements were successfully amplified following 10-day LPS treatment, supporting active transcription from this locus. Given that our *in silico* analysis also identified potential epigenetic regulation at the CDKN2A promoter region, we performed ChIP PCR analysis with H3K27me3 and H3K4me3 antibodies (Fig. 6, B and C). Our data demonstrate that H3K27me3 is present at the CDKN2A locus during control conditions (i.e. no LPS) but is absent following 10-day LPS treatment, supporting the loss of this repressive chromatin mark at the CDKN2A locus. We next investigated the H3K4me3 chromatin mark at this locus. We demonstrate that H3K4me3 chromatin marks are increased at the CDKN2A locus following 10 days of LPS treatment (Fig. 6, B and C). Moreover, two inhibitors (WDR50103 and OICR9429) of the H3K4 methyltransferase, lysine methyltransferase 2A (KMT2A/MLL), prevented persistent LPS-induced CDKN2A expression.
RNA and protein expression (Fig. 6, D and E), suggesting a crucial role for KMT2A during LPS-induced H3K4 methylation and CDKN2A expression. Taken together, these data indicate that, following 10-day treatment with LPS, ETS1 interacts with genetic elements and promotes transcription of CDKN2A. Moreover, these data suggest that persistent LPS treatment promotes chromatin remodeling in which the repressive chromatin mark, H3K27me3, is removed and is replaced with the permissive H3K4me3 mark at the CDKN2A promoter.

Phosphorylated ETS1 Protein Expression Is Increased in Cholangiocytes of Human PSC Liver Tissue and in a Murine Model of PSC—We recently demonstrated that cholangiocyte senescence is increased in livers of patients with PSC. In these cholangiocytes, CDKN2A is increased as observed by in situ hybridization (11). Given that our in vitro data support a functional role of phospho-ETS1 in cholangiocyte senescence and CDKN2A expression, we performed immunofluorescence for phospho-ETS1 and confirmed CDKN2A expression by fluorescent in situ hybridization. Very little phospho-ETS1 or CDKN2A mRNA was observed in control human liver sections (Fig. 7A). In contrast, cholangiocytes from PSC liver sections exhibited nuclear staining and increased total expression of phospho-ETS1 (~3.5-fold increase, p < 0.01; Fig. 7, A and B) in CDKN2A positive cholangiocytes. We next asked whether cholangiocyte phospho-Ets1 and Cdkn2a were up-regulated in a murine model of PSC (ATP binding cassette subfamily B member 4 knock-out, Abcb4 (Mdr2)−/− mice), a model that exhibits increased cholangiocyte senescence (11). As with human PSC liver tissue, we observed cholangiocyte nuclear localization and increased expression of phospho-Ets1 (~10-fold increase; Fig. 7, C and D) in Cdkn2a positive cholangiocytes. Similar to our in vitro findings, these in vivo data support a functional role for ETS1 in cholangiocyte senescence, a potential pathophysiological cellular state associated with initiation or progression of PSC.

Discussion

Using an in vitro model of stress-induced cholangiocyte senescence, we demonstrated that: (i) prolonged LPS treatment of cholangiocytes increased ETS1, but not ETS2, expression and phosphorylation; (ii) overexpression of constitutively active NRAS promoted Cdkn2a expression and cholangiocyte senescence in an ETS1-dependent manner; (iii) in prolonged LPS-treated cells, pharmacologic inhibition of RAS/
MAPK reduced ETS1 and CDKN2A protein expression and CDKN2A promoter-driven luciferase detection; (iv) suppression of ETS1 (shRNA or genetic deletion) blocked persistent LPS-induced CDKN2A protein expression, diminished cholangiocyte senescence, and suppressed ETS1 overexpression and NRAS-induced CDKN2A promoter-driven luciferase expression; (v) site-directed mutagenesis or deletion of the predicted distal ETS binding site within the CDKN2A promoter prevented LPS-induced luciferase reporter detection; and (vi) LPS treatment-induced chromatin modification including H3K4me3, ETS1, and active POLR2 (Ser(P)-2) occupation of the CDKN2A locus. Additionally, inhibitors of the histone methyltransferase, KMT2A, suppressed CDKN2A expression. Moreover, we demonstrated the relevance of our in vitro data by demonstrating in vivo that phospho-ETS1 protein expression was increased in cholangiocytes of both human PSC liver samples and in a murine model of PSC. These data show for the first time that cholangiocyte ETS1, but not ETS2, promotes CDKN2A expression and senescence in vitro, and this pathway is likely operative in cholangiocytes in PSC liver. Hence, we provide mechanistic insight into stress-induced (i.e., prolonged LPS treatment) cholangiocyte senescence, a cellular process that may contribute to the pathogenesis of PSC. Moreover, these novel data suggest that ETS1 interaction with the predicted ETS binding site in the CDKN2A promoter is the principle activator of CDKN2A expression in insult-induced (e.g., LPS) or oncogene-induced (i.e., NRAS) senescence in general. Finally, cells lacking ETS1 express increased IL6 and IL8 both in the presence and absence of LPS suggesting that although ETS1 promotes cholangiocyte CDKN2A expression and senescence, this transcription factor may function as a negative regulator of proinflammatory cytokine production.

Cellular senescence was described over 50 years ago when it was demonstrated that normal human cells had a limited ability to proliferate in culture (24). Until recently, the physiological relevance of this process has been unclear. It is now increasingly recognized that senescence prevents continued proliferation of aged (i.e., shortened telomeres) or damaged (i.e., DNA double-stranded breaks) cells, supporting the notion that senescence is a barrier to proliferation of precancerous or potentially neoplastic cells. A common mechanism for senescence induction involves the initiation of the DNA damage response (DDR). Although exceptions to DDR-induced senescence exist (25–27), both telomere shortening and RAS-induced senescence occur as a result of detection of genomic damage and the induction of the DDR (28–30). Indeed, it has been shown that aberrant expression of oncogenic RAS promotes cultured cell senescence through DNA damage induced during the initial period of hyperproliferation and the induction of the DDR (30). In previous work, we demonstrated that LPS-induced senescence in cultured cholangiocytes in a RAS-dependent manner. We further demonstrated that activated NRAS increased CDKN2A, maintenance of telomere length, and the accumulation of γH2A.X positive foci, which accumulate at DNA double-stranded breaks, in cholangiocytes from PSC livers. These data are consistent with oncogene-induced (i.e., RAS) senescence in PSC. These observations, coupled with the known role of ETS transcription factors in the expression of CDKN2A (20), was the underlying rationale for our hypothesis that the RAS/MAPK pathway induction of ETS was operative in our cell culture model and in human and animal models of PSC.

Prolonged withdrawal from the cell cycle promotes the transcriptional up-regulation of CDKN2A (31). CDKN2A blocks the activation of cyclin-dependent kinases CDK4 and CDK6, preventing the phosphorylation of pRB and promoting permanent cell cycle arrest. In this study, we show that an exogenous insult (i.e., LPS treatment) in the presence of RAS or ERK1/2 inhibitors prevented CDKN2A expression confirming the importance of RAS/ERK in CDKN2A expression (32, 33). We further demonstrate that treatment with LPS in the presence of an ETS1–shRNA strongly abrogated LPS-induced senescence through transcriptional regulation of CDKN2A. Together, these data suggest that activated RAS may not only induce DNA damage but is required for the induction of ETS1-dependent CDKN2A expression and, hence, the induction of senescence.

Moreover, deletion of the predicted distal ETS binding domain in the CDKN2A promoter luciferase construct prevented LPS-induced luciferase detection. Thus, our data support the novel observation that insult-induced RAS activation promotes cholangiocyte senescence via phospho-ETS1-dependent CDKN2A expression. Moreover, we show for the first time that the RAS–ETS1 pathway is likely operative in a progressive, premalignant human disease, PSC. Why senescent cholangiocytes accumulate in PSC livers remains unanswered yet may involve the balance between DDR-dependent apoptosis and senescence, as well as immunity-mediated removal of senescent cells.

Although we demonstrated the transition from repressive to permissive chromatin at the CDKN2A promoter over time in our culture model of senescence, the mechanisms driving chromatin modification and the engagement of the senescence pathway (i.e., CDKN2A expression) remain obscure. Enhancer of Zeste 2 (EZH2), a core member of the polycomb repressive 2 complex, maintains CDKN2A repression (H3K27Me3) under normal physiologic conditions (34). We demonstrated the loss of this chromatin mark and the gain of the permissive H3K4me3 mark following long term LPS treatment. A likely contributor to the transition to permissive chromatin is the H3K4me3 methyltransferase, KMT2A (35). We demonstrated that both expression of a constitutive active NRAS mutant and prolonged activation of NRAS via persistent LPS treatment promote CDKN2A expression, whereas inhibitors of KMT2A suppressed persistent LPS-induced CDKN2A expression. Whether and how the RAS/MAPK pathway influences chromatin modifier function and the transition from repressive to permissive chromatin at this locus is not addressed in this work but merits further investigation.

Many of the hallmarks of senescence, including the signaling pathways involved, have been defined in vitro, yet the question remains as to how these in vitro observations translate to animal models of disease and in human pathologic states. In previous work, we demonstrated that NRAS is activated and CDKN2A is overexpressed in cholangiocytes in both PSC and an animal model of PSC. Here we demonstrate that ETS1 is not only active in stress-induced cholangiocyte senescence in culture, but phospho-ETS1 is highly expressed in cholangiocytes...
in human PSC and in a mouse model of PSC. These observations suggest functionality of this tumor-suppressive pathway in a premalignant state, i.e. PSC, and provide pathophysiological insight into the transition of PSC to CCA. Indeed, close to 50% of all human cancers show some level of CDKN2A inactivation (36–38). In these tumors, several mechanisms of CDKN2A inactivation have been described including mutation, deletion, and promoter methylation. Moreover, genetic analysis has demonstrated that allelic loss of the CDKN2A locus (9p21) and CDKN2A epigenetic silencing through DNA methylation are common in PSC-associated CCA (39). Together, our data support a model in which cholangiocyte senescence, via stress-induced mechanisms involving RAS and ETS1-dependent expression of CDKN2A, is increased in PSC livers, and accumulation of these senescent cells and transition to the senescence-associated secretory phenotype contribute to the fibroinflammatory processes of PSC. We further propose that escape from this senescent state through inactivation of CDKN2A is likely to contribute to PSC-associated CCA.

Senescent cells frequently transition to the SASP, and it is proposed that the SASP contributes to pathogenesis in certain disease states (12). Given this link and the proposed link between SASP and PSC, we assessed the SASP-associated molecules IL6 and IL8 in cells that are resistant to LPS-induced senescence (i.e. ETS1-deficient cells). Our finding that cells lacking ETS1 showed increased IL6 and IL8 mRNA and protein expression irrespective of the presence of LPS suggests that although ETS1 promotes cholangiocyte CDKN2A expression and senescence, this transcription factor may function as a negative regulator of proinflammatory cytokine production. Indeed, in studies using ETS1-deficient mice, numerous cytokines (including IL6) are up-regulated (40, 41). Hence, cholangiocyte senescence and SASP are likely activated by distinct, yet interdependent pathways. In this case, activation of the protective function of cholangiocyte senescence via ETS1 (i.e. tumor suppression) may also limit the hypersecretion of proinflammatory cytokine/chemokines associated with the cholangiocyte SASP. This interesting observation merits further consideration in additional model systems.

In summary, we have defined a molecular pathway regulating LPS-induced cholangiocyte senescence in vitro and provide compelling evidence for the function of this pathway in human and animal models of PSC (Fig. 8). These insights provide a better mechanistic understanding of cholangiocyte senescence in the cholangiopathies, particularly PSC. Moreover, these observations may be generalizable to other progressive, fibroinflammatory diseases that predispose to malignancy, especially those that implicate cellular senescence in pathogenesis, for example idiopathic pulmonary fibrosis (42, 43). Indeed, the functional importance of CDKN2A in cancer and the frequent genetic and epigenetic inactivation of this locus in CCA suggest that senescence is a functional tumor suppressive mechanism in PSC. Furthermore, our results provide direction for targeted interventions that may include selective elimination of senescent cells (i.e. “senolytics”) as a therapeutic approach for PSC-associated fibrosis and inflammation, as well as a potential preventative therapy for the progression to CCA.

Experimental Procedures

This study was approved by the Mayo Clinic Institutional Review Board and Institutional Animal Care and Use Committee.

Cell Culture and In Vitro Model of Senescence—The well characterized normal human cholangiocyte (NHC) cell line was provided to us by Dr. Medina (University of Navarra, Pamplona, Spain) (44). All experiments were carried out with mycoplasma-free (PCR-tested) low passage (less than passage 15) plasma-free, (PCR-tested) low passage (less than passage 15) NHCs. For our in vitro model of senescence, the NHC cells were treated with LPS (200 ng/ml) (Invivogen). Medium containing vehicle or LPS was replaced every 48 h for 10 days (11).

Total RNA Extraction and Quantitative RT-PCR—Following total RNA extraction from NHCs with TRIzol (Invitrogen), 2.0 μg of RNA were reverse-transcribed using the SuperScript III first strand synthesis system (Invitrogen) according to the manufacturer’s protocol. After synthesis of the cDNA, quantitative RT-PCR was completed with the Light Cycler Fast Start DNA
**PCR primers were designed using the NEBaseChanger (New England BioLabs). Briefly, CDKN2A primers and spark plasmid (Sino Biologic, Inc.), which contains the ETS1 gene, were used in this study.**

**Cloning of the ETS1 Mutant Constructs—**To construct the SparQ NRAS 12D cumate switch vector, a series of subclonings were performed. First, the pCGN NRAS 12D plasmid (Addgene) was digested with the BamHI restriction enzyme (New England BioLabs) to obtain the NRAS 12D insert. After gel purification, the NRAS 12D insert was ligated into the corresponding BamHI site in the pCDNA 3.1 (+) vector (Invitrogen). The resulting pCDNA 3.1 (+) NRAS 12D plasmid was propagated in 10-bla.b competent cells (New England BioLabs), and the plasmid was purified using the Quantum Prep kit (Bio-Rad) and digested with the NheI and NotI restriction enzymes (New England Biolabs) per the manufacturer’s instructions. The NRAS 12D insert was gel-purified and ligated into the corresponding NheI and NotI sites in the SparQ All-in-One Cumate Switch inducible vector (System Biosciences, Inc.). The resultant SparQ NRAS 12D Cumate Switch Vector was propagated, purified, and sequenced (Mayo Clinic Sequencing Core, Rochester, MN) to confirm proper insertion of the NRAS 12D coding sequence into the SparQ vector.

**protein Isolation, Western Blotting, and ELISA—**Protein was extracted from NH cells using Mammalian Protein Extraction Reagent (M-PER) (Roche) containing protease and phosphatase inhibitors. Total cell lysate was subjected to electrophoresis on SDS-PAGE gels and transferred to nitrocellulose membranes. After blocking, the membranes were incubated overnight at 4 °C with the following primary antibodies (1:500): ETS1 (sc-350), ETS2 (sc-255), IL6 (sc-377), and IL8 (sc-104). Over 10 days in culture, the medium was removed, and IL6 and IL8 ELISAs were performed according to the manufacturer’s instructions (Qiagen).

**Transfection and Generation of Stable Cell Lines—**NH cells were transfected with the short hairpin RNA ETS1 (ETS1-shRNA, TRC 1.5; Sigma-Aldrich) or pCGN (NRAS 12D; Addgene) using FuGENE HD (Promega). On the day of transfections, the NH cells were 60–70% confluent. Each of the two plasmids contain a D10Amutated Cas9 (Chapel Hill, NC) and the plasmid was purified using the Quantum Prep kit (Bio-Rad) and digested with the NheI and NotI restriction enzymes (New England Biolabs) per the manufacturer’s instructions. The NRAS 12D insert was gel-purified and ligated into the corresponding NheI and NotI sites in the SparQ All-in-One Cumate Switch inducible vector (System Biosciences, Inc.). After gel purification, the NRAS 12D insert was ligated into the corresponding BamHI site in the pCDNA 3.1 (+) vector (Invitrogen). The resulting pCDNA 3.1 (+) NRAS 12D plasmid was propagated in 10-bla.b competent cells (New England BioLabs), and the plasmid was purified using the Quantum Prep kit (Bio-Rad) and digested with the NheI and NotI restriction enzymes (New England Biolabs) per the manufacturer’s instructions. The NRAS 12D insert was gel-purified and ligated into the corresponding NheI and NotI sites in the SparQ All-in-One Cumate Switch inducible vector (System Biosciences, Inc.). The resultant SparQ NRAS 12D Cumate Switch Vector was propagated, purified, and sequenced (Mayo Clinic Sequencing Core, Rochester, MN) to confirm proper insertion of the NRAS 12D coding sequence into the SparQ vector.
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CymR repressors, thus allowing for regulated promoter-induced transcription of the NRAS 12D open reading frame.

SA-β-Galactosidase Staining—Cells grown in 6-well plates were washed, fixed, and stained using the SA-β-galactosidase staining kit (Cell Biolabs) according to the manufacturer’s directions. The percentage of senescent cells was determined for each condition using a 20× objective and bright field illumination of no fewer than five randomly selected areas (11).

NRAS Activation—NHCs were grown in 6-well plates in the presence of pCGN (NRAS 12D), the empty vector control, or WT (untransfected cells). The cells were lysed, and immunoprecipitations were performed using RAF1-RBD beads (Cyto-skeleton), which binds GTP-bound RAS protein. The precipitate was separated by SDS-PAGE followed by Ponceau Red total protein staining, and an NRAS immunoblot was performed.

**RAS and MAP Kinase Inhibitor Assays—Manumycin (5 μM), UO126 (5 μM), PD980159 (10 μM), or DMSO as vehicle was added to the culture medium in the presence or absence of LPS.**

To achieve stable transfections for constitutively activated NRAS, pCGN (N-RAS 12D) (Addgene) and MISSION TRC2-pLKO.puro (ETS1-shRNA) (Sigma-Aldrich) were transfected into NHCs cells using FuGENE HD (Promega) and subsequently selected with hygromycin (200 ng/ml) for the NRAS 12D plasmid and puromycin (1.5 μg/ml) for the ETS1 shRNA plasmid.

**Cloning of the CDKN2A Promoter Luciferase Constructs—**Genomic DNA was extracted from cultured NHC using the DNA Blood & Cell Culture DNA Midi Kit (Qiagen) according to the manufacturer’s protocol. PCR was performed using the primer pairs found in Table 1 that contain a XhoI restriction enzyme site in the forward primer (underlined) and a HindIII site in the reverse primer (bold type). The reverse primer was used in the generation of all the constructs. Following PCR amplification (95 °C for 10 min; 30 cycles of 95 °C for 30 s and 55 °C for 60 s; and 72 °C for 30 s) the PCR amplicons were digested with XhoI and HindIII, electrophoresed, and gel-puriﬁed by QIAquick gel extraction kit (Qiagen). These digested inserts were then ligated into the XhoI and HindIII linearized pGL4.22 luciferase plasmid (Promega). To obtain the ETS1 site-directed mutagenesis clones in the CDKN2A promoter, we used a Q5 SDM kit (New England BioLabs). Briefly, PCR primers were designed using the NEBaseChanger (New England BioLabs) algorithm. The Δ1 SDM construct deleted 3 bp within the −518 Ets1 site. Primers used for SDM are found in Table 1. PCR for the site-directed mutants was carried out using the following parameters: 98 °C for 10 s, 25 cycles of 98 °C for 10 s, 65 °C (Δ1) or 72 °C (Δ2) for 60 s, 72 °C for 3 min, and 72 °C for 30 s. The ΔΣ SDM construct was then used as template for Δ1/2 SDM by targeting the −518 ETS1 site (Δ1 SDM) as above. DNA sequencing was performed on all plasmid constructs at the Mayo Clinic DNA Sequencing Core Facility.

**Promoter Luciferase Assay—CDKN2A promoter luciferase constructs and TK-Renilla (Promega), an internal control, were cotransfected into NHC cells using FuGENE HD (Promega) when the cultured cells were 20% confluent. As a baseline control, the pGL4.22 empty vector was cotransfected with TK-Renilla. After 24 h, complete medium or medium containing LPS and/or inhibitors was placed on the cells and incubated for 10 days changing the respective media every 48 h. At the end of incubation, the dual-luciferase reporter assay system (Promega) was used to measure CDKN2A promoter-driven firefly luciferase and Renilla luciferase. The values are expressed as fold change between the relative CDKN2A promoter-driven firefly luciferase to control Renilla luciferase compared with empty pGL4.22 empty vector firefly luciferase to Renilla luciferase.

**Chromatin Immunoprecipitation and PCR—**CHIP was performed according to the Abcam cross-linking ChIP protocol. Briefly, NHCs were treated with or without LPS for 10 days to induce senescence. The cells were incubated at room temperature for 10 min with formaldehyde, and glycine was added at a final concentration of 125 mM to terminate the cross-linking reaction. The cells were then scraped, lysed, and subsequently sheared to generate genomic DNA fragments. Immunoprecipitations were performed with the following antibodies: anti-ETS1 (Thr(P)-38, ab59179; Abcam), anti-RNA POL2 (Ser(P)-2, MABE953; Millipore), H3K27me3 (61017; Active Motif), and H3K4me3 (39159; Active Motif).

**Liver Tissues—**Upon Institutional Animal Care and Use Committee approval, FVB/N background Abcb4−/− mice were obtained as a gift from Dr. Oude Elferink (Tytgat Institute, Amsterdam, The Netherlands). FVB/N background (wild type) mice were obtained from the Jackson Laboratory (FVB/NJ). The mice were housed at the Mayo Clinic animal care facility with a standard 12:12 light/dark cycle and ad libitum access to water and standard rodent diet. The mice were sacrificed under general anesthesia at 60 days of age.

Following institutional review board approval, three human PSC patient samples, which fulfilled clinical, serological, histological, and/or cholangiographic criteria for stage IV PSC, were obtained at the time of transplant. Three normal liver samples from surgical resection or explant were also utilized. Liver specimens were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned (4 μm) for dual in situ hybridization and immunofluorescence.

**Dual in Situ Hybridization and Immunofluorescence—**Dual in situ hybridization and immunofluorescence was performed as previously published (45). Briefly, tissue sections were deparaffinized in xylene and rehydrated through a series of increasing ethanol dilutions. An antigen retrieval step was performed by immersing slides in boiling sodium citrate buffer (pH 6.0) for 10 min. The slides were then incubated at 55 °C in a prehybridization solution for 20 min followed by incubation with the CDKN2A FITC-conjugated in situ LNA probe (Exiqon) in hybridization solution for 1 h. A series of wash steps in SSC buffer were then performed with a final wash in PBS. Next, slides were subjected to a signal amplification step using a tyramide signal amplification Kit (PerkinElmer Life Sciences). Slides were then washed in buffer containing Tris base, NaCl, and Triton X-100; blocked in 4% BSA solution; and incubated with primary antibodies to pETS1 (Abcam) and CK19 (Santa Cruz Biotechnology) overnight at 4 °C. After primary incubation, the slides were washed in PBS and incubated with Alexa Fluor secondary antibodies (Life Technologies) for 30 min at room temperature. The slides were washed again in PBS and mounted using Prolong-Gold Antifade with DAPI (Life Tech-
nologies). The slides were analyzed using a Zeiss 780 laser scanning confocal microscope.

Statistical Analysis—All data are reported as the means (or fold change in means) ± standard deviation from a minimum of three independent experiments. Statistical analyses were performed with Student’s t test or analysis of variance when appropriate. p < 0.05 was considered statistically significant.

Author Contributions—S. P. O. conceived the idea for the project, analyzed the results, and wrote most of the manuscript. P. L. S. conceived the idea for the project with S. P. O., conducted most of the experiments, analyzed results, and wrote portions of the paper. N. S. S. and B. F. S. generated several constructs used throughout the manuscript. M. J. P. L. generated the ETS1-deficient cell line and performed the ELISAs. L. L. and C. E. T conducted experiments on the expression of CDKN2A and Ets1 in mouse and human tissues. N. F. L. analyzed results, oversaw project development, and wrote the manuscript with S. P. O.

Acknowledgments—We thank Drs. M. Fernandez-Zapico, J. Kirkland, and G. Gores for encouragement and critical reading of the manuscript. We also acknowledge D. Hintz for assistance in preparation of the manuscript.

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