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Ethanol differentially regulates Snail family of transcription factors and invasion of premalignant and malignant pancreatic ductal cells

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Abstract

Pancreatic cancer is one of the deadliest of cancers with a dismal 5-year survival rate. Epidemiological studies have identified chronic pancreatitis as a risk factor for pancreatic cancer. Pancreatic cancer cells also demonstrate increased expression of the transcription factor Snail, a key regulator of epithelial-mesenchymal transition. As ethanol is one of the major causes of pancreatitis, we examined the effect of ethanol on Snail family members in immortalized human pancreatic ductal epithelial (HPDE) cells and in pancreatic cancer cells. Ethanol induced Snail mRNA levels 2.5-fold in HPDE cells, with only 1.5-fold mRNA induction of the Snail-related protein Slug. In contrast, ethanol increased Slug mRNA levels 1.5-2-fold in pancreatic cancer cells, with minimal effect on Snail. Because Snail increases invasion of cancer cells, we examined the effect of ethanol on invasion of HPDE and pancreatic cancer cells. Surprisingly, ethanol decreased invasion of HPDE cells, but had no effect on invasion of pancreatic cancer cells. Mechanistically, ethanol increased adhesion of HPDE cells to collagen and increased expression of the collagen binding $\alpha 2$ - and $\beta 1$ -integrins. In contrast, ethanol did not affect collagen adhesion or integrin expression in pancreatic cancer cells. Also in contrast to HPDE cells, ethanol did not attenuate ERK1/2 phosphorylation in pancreatic cancer cells; however, inhibiting ERK1/2 decreased pancreatic cancer cell invasion. Overall, our results identify the differential effects of ethanol on premalignant and malignant pancreatic cells, and demonstrate the pleiotropic effects of ethanol on pancreatic cancer progression.

Keywords

Ethanol; Snail; Slug; ERK1/2; integrins; collagen; adhesion

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related death in the US, with most patients presenting with locally advanced or metastatic disease,

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and a 5-year survival of less than 5% [Hidalgo, 2010; Li et al., 2004]. Epidemiological studies have shown an association between chronic pancreatitis and pancreatic cancer [Ekbom et al., 1994; Lowenfels et al., 1993]. Ethanol abuse is a frequent cause of chronic pancreatitis [Apte et al., 2009a; Apte et al., 2009b; Haber et al., 2001]; however, there are conflicting data for ethanol as a risk factor for pancreatic cancer. In the Netherlands cohort study, Cancer Prevention Study II and the NIH-AARP diet and health study, high ethanol consumption was associated with an increased risk for pancreatic cancer [Gapstur et al., 2011; Heinen et al., 2009; Jiao et al., 2009]; however, a European prospective investigation into cancer and nutrition (EPIC) study and a recent pooled analysis from the pancreatic cancer cohort consortium (PanScan) did not identify any overall association between total ethanol intake and pancreatic cancer [Michaud et al., 2010; Rohrmann et al., 2009].

Ethanol can modulate a number of signaling pathways to regulate cellular behavior [Aroor and Shukla, 2004]. Ethanol can suppress epidermal growth factor (EGF) receptor (EGFR) signaling by inhibiting EGF-induced EGFR autophosphorylation and ERK1/2 activation in fibroblast cells [Ma et al., 2005]. Conversely, ethanol can increase EGFR signaling by promoting EGFR expression in colon cells [Tong et al., 1999], or by transactivating EGFR to enhance ERK1/2 phosphorylation in breast cancer, colon cancer and pancreatic ductal cells [Askari et al., 2006; Forsyth et al., 2010]. The EGFR transactivation induced by ethanol in colon and breast cancer cells was shown to mediate expression of the transcription factor Snail [Forsyth et al., 2010], one of the key regulators of epithelial-mesenchymal transition (EMT) [Thiery et al., 2009]. Significantly, Snail and its related protein Slug play an important role in pancreatic cancer progression by facilitating tumor invasion and metastasis [Horiguchi et al., 2009; Hotz et al., 2007; Shields et al., 2011; Yin et al., 2007].

In this study, we examined the effect of ethanol on Snail levels in immortalized human pancreatic ductal epithelial (HPDE) cells and in pancreatic cancer cells. We show that despite induction of Snail, ethanol decreases invasion of HPDE cells. We also show that ethanol primarily increases Slug levels in pancreatic cancer cells and has no effect on invasion of pancreatic cancer cells. We also demonstrate that ethanol decreases basal ERK1/2 phosphorylation only in HPDE cells and that inhibiting ERK1/2 signaling in pancreatic cancer cells decreases invasion. Overall, our results identify the differential effects of ethanol on premalignant and malignant pancreatic cells, and demonstrate the pleiotropic effects of ethanol on pancreatic cancer progression.

MATERIALS & METHODS

Chemicals/Reagents

General tissue culture materials were obtained from VWR International (West Chester, PA). Molecular grade ethanol was purchased from Sigma-Aldrich. Antibodies against p-ERK1/2 and Snail were purchased from Cell Signaling (Danvers, MA), while α -tubulin antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Taqman gene expression assays for quantitative real time PCR analysis were procured from Applied Biosystems. Broad-spectrum metalloproteinase inhibitor GM6001 was purchased from EMD, the MEK1/2 inhibitor U0126 from Cell Signaling, the EGFR inhibitor AG1478 and the proteasome inhibitor MG132 were from Calbiochem. Lithium chloride (LiCl) was obtained from Sigma. Nucleofector electroporation kit was obtained from Lonza. For adhesion assay, type I collagen-coated plates were purchased from BD Biosciences.

Cell culture

Premalignant HPDE cells (HPV16-immortalized normal pancreatic ductal epithelium) were generously provided by Dr. M. Tsao (Ontario Cancer Institute)[Liu et al., 1998; Ottaviano et al., 2006]. Cells were maintained in 'complete medium', which was composed of keratinocyte serum-free medium (SFM) supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml bovine pituitary extract (supplied with the medium) and 5 ng/ml epidermal growth factor (supplied with the medium). The malignant Panc1 cells were purchased from American Type Culture Collection (Manassas, VA), while the malignant COLO 357-variant FG-Met2 cells were kindly provided by Jonathan C. Jones (Northwestern University) [Baker et al., 1997]. Panc1 and FG-Met2 were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 µg/ml streptomycin[Dangi-Garimella et al., 2011; Shields et al., 2011].

Quantitative Real Time-PCR analysis

Quantitative gene expression analysis was performed for Snail, Slug, E-cadherin, vimentin, fibronectin, α 2-integrin, β 1-integrin and GAPDH as previously published[Ottaviano et al., 2006]. The data were then quantified with the comparative C_T method for relative gene expression [Ottaviano et al., 2006].

Immunoblotting

Immunoblotting was done as previously described [Munshi et al., 2002; Munshi et al., 2004], and detected by enhanced chemiluminescence Western blotting reagents (Pierce Biotechnology).

Invasion assay

Invasive activity was analyzed using Matrigel-coated BD inserts in a Boyden chamber (8 µm pore size) [Joseph et al., 2009; Sun et al., 2008]. Two hundred thousand HPDE cells were added to the upper chamber in 1 ml of keratinocyte-SFM and 1 ml of complete medium was added to the lower well as a chemo-attractant. Similarly, 100,000 Panc1 and FG-Met2 cells were added to the upper chamber in 1 ml of serum-free DMEM and 1 ml of DMEM containing 10% FBS was added to the lower well as a chemo-attractant [Shields et al., 2011]. Cells were left untreated or treated with 1% ethanol and allowed to invade for 24 hours. Non-invasive cells were then removed from the upper chamber and the invasive cells were fixed, stained, photographed and the relative invasion quantified.

Adhesion assay

HPDE, Panc1 or FG-Met2 cells were untreated or treated with ethanol for 24 hours and then 50,000 cells seeded onto tissue culture plates pre-coated with type I collagen. Cells were allowed to adhere at 37 °C for 15 minutes, washed once with PBS, and then fixed and stained [Munshi and Stack, 2002]. Cells were imaged using a Zeiss microscope and photographed using a Nikon camera.

Downregulation of Snail expression

Snail expression was transiently downregulated using a predesigned and validated Silencer siRNA sequences specific for Snail (Ambion siRNA IDs s13186, 50 nmol and s13187, 50 nmol)[Shields et al., 2011]. HPDE cells were transfected with Snail siRNA or control siRNA using the Nucleofector kit R (Amaxa/Lonza). The transfected cells were allowed to recover on plastic for 24 hours, treated with ethanol for an additional 24 hours and changes in gene expression analyzed by real time PCR.

RESULTS

Ethanol induces Snail expression in pancreatic ductal epithelial cells

It was previously shown that the EMT-inducing transcription factor Snail is overexpressed in pancreatic cancer, and overexpression of Snail increases invasion of pancreatic cancer cells [Hotz et al., 2007; Shields et al., 2011; Yin et al., 2007]. Ethanol has been shown to be a major risk factor for chronic pancreatitis [Apte et al., 2009a; Apte et al., 2009b; Haber et al., 2001], and as ethanol increases expression of Snail in breast and colon cancer cells [Forsyth et al., 2010], we assessed the effect of ethanol on the expression of Snail family transcription factors in human pancreatic ductal epithelial (HPDE) cells. HPDE cells were treated with 1% ethanol daily for 3 days and the effect on the mRNA levels of Snail (Snai1) and the Snail-related protein Slug (Snai2) was determined by quantitative real-time PCR. Under basal conditions, HPDE cells express relatively low Snail levels and high Slug levels (Supplemental Fig. 1). Ethanol increased Snail levels by ~2.5-fold (Fig. 1A) and increased Slug levels by ~1.5-fold (Fig. 1B). In addition, we characterized the effect of chronic ethanol treatment on markers of EMT. Ethanol increased fibronectin levels without affecting E-cadherin or vimentin levels in HPDE cells (Fig. 1C). In agreement with the mRNA findings, ethanol did not affect E-cadherin protein levels (Fig. 1C, inset).

We next examined the kinetics of Snail expression following ethanol treatment of HPDE cells. As shown in Fig. 1D, ethanol transiently increased Snail mRNA expression at 2 hours to ~2.0 fold, with a decrease to ~1.5-fold at 24 hours. Additionally, ethanol-induced Snail mRNA expression increased with increasing doses of ethanol treatment (Fig. 1E). We also examined the effect of ethanol on Snail protein at 2 hours. Since Snail has a short half-life as a result of GSK-3 β -dependent and ubiquitin-mediated degradation [Yook et al., 2005; Zhou et al., 2004], HPDE cells were pre-treated with GSK-3 β inhibitor LiCl and proteasome inhibitor MG132 for 30 minutes and then induced with ethanol for 2 hours. In agreement with the mRNA findings, ethanol also increased Snail protein in HPDE cells (Fig. 1F).

Ethanol-induced Snail expression in pancreatic ductal cells is regulated by ERK1/2 signaling

As it was previously shown that ethanol can enhance EGFR-dependent ERK1/2 phosphorylation and that ethanol-dependent EGFR activation can involve metalloproteinases [Forsyth et al., 2010; Ma et al., 2005], and since Snail can be regulated by ERK1/2 [Peinado et al., 2003], we examined the effect of blocking metalloproteinases, EGFR and ERK1/2 on ethanol-induced Snail expression. HPDE cells were serum-starved for 24 hours, pre-treated with the metalloproteinase inhibitor GM6001 (MMPi), the MEK1/2 inhibitor U0126 to block ERK1/2 phosphorylation, or the EGFR inhibitor AG1478 (AG) for 30 minutes and then treated with ethanol for 4 hours. Although these inhibitors did not significantly affect basal Snail levels, ethanol-induced Snail expression was significantly attenuated by U0126 and AG1478 (Fig. 2A).

We also examined the effect of ethanol on ERK1/2 phosphorylation in HPDE cells at 4 hours. In contrast to previous reports [Askari et al., 2006; Forsyth et al., 2010], ethanol did not induce ERK1/2 phosphorylation, but instead decreased basal phosphorylation (Fig. 2B). Although MMPi did not affect basal ERK1/2 phosphorylation, treatment with MMPi further reduced ERK1/2 phosphorylation following ethanol treatment (Fig. 2B). U0126 and AG1478 completely blocked ERK1/2 phosphorylation under basal conditions and in ethanol treated samples (Fig. 2B). These results suggest that even though ethanol decreases basal ERK1/2 phosphorylation, ethanol-induced Snail expression requires certain level of ERK1/2 activity.

We also characterized the effect of MMPi, U0126 and AG1478 on the cellular morphology of ethanol treated HPDE cells at 4 hours. In the absence of ethanol treatment, HPDE cells treated with vehicle control (DMSO) or with MMPi grow as individual cells, while treatment with U0126 and AG1478 causes cellular aggregation (Fig. 2C). Ethanol treatment of HPDE cells for 4 hours also causes cellular aggregation, with the effect more pronounced in cells that were also co-treated with U0126 or AG1478.

Ethanol primarily induces Slug expression in pancreatic cancer cells

We next characterized the effect of ethanol on malignant pancreatic cancer cells. Panc1 cells express high Snail levels while FG-Met2 cells express moderate Slug levels (Supplemental Fig. 1). Panc1 and FG-Met2 cells were treated with 1% ethanol daily for 3 days and the effect on Snail and Slug expression determined. Ethanol had minimal effect on Snail levels in Panc1 (Fig. 3A) and FG-Met2 cells (Fig. 3C); however, ethanol increased Slug levels by ~1.6-fold in Panc1 cells (Fig. 3B) and by ~2-fold in FG-Met2 cells (Fig. 3D). Ethanol did not affect E-cadherin, vimentin or fibronectin expression in FG-Met2 cells (Fig. 3E) or in Panc1 cells (data not shown).

Ethanol *inhibits* invasion of pancreatic ductal epithelial cells, but not that of pancreatic cancer cells

Previously it was shown that Snail family members promoted invasion of pancreatic cancer cells [Horiguchi et al., 2009; Hotz et al., 2007; Yin et al., 2007]. Since ethanol increases Snail and/or Slug in premalignant and malignant pancreatic cells, we examined the effect of ethanol on invasion. HPDE cells were untreated or treated with ethanol for 24 hours, and equal numbers of the cells were then allowed to invade for 16 hours through Matrigel-coated transwell chambers in the presence or absence of ethanol. As shown in Figs. 4A and 4B, HPDE cells invade through Matrigel; however, treatment with ethanol decreases invasion of HPDE cells. We also examined the effect of ethanol on invasion of Panc1 and FG-Met2. As shown in Figs. 4C and 4D, both Panc1 and FG-Met2 cells invade through Matrigel, but ethanol has no effect on the invasive ability of these cells.

Ethanol increases adhesion and collagen-binding integrins in pancreatic ductal cells, but not in pancreatic cancer cells

Since adhesion to the underlying matrix can affect the ability of cells to invade [Lauffenburger and Horwitz, 1996], we examined the effect of ethanol on adhesion of ductal cells to type I collagen. HPDE cells were untreated or treated with ethanol for 24 hours, trypsinized and plated onto collagen-coated tissue culture plates for 15 minutes for the cells to adhere, and were then washed with PBS to remove non-adherent cells. As shown in Fig. 5A, HPDE cells treated with ethanol demonstrate increased adhesion to collagen. We also examined the effect of ethanol on the ability of FG-Met2 and Panc1 cells to adhere to collagen. In contrast to the effect of ethanol on premalignant cells, ethanol did not affect adhesion of FG-Met2 (Fig. 5B) or Panc1 cells (data not shown).

We next evaluated the effect of ethanol on the expression of α 2- and β 1-integrins, which mediate binding to collagen [Kadler et al., 2007; Leitingner and Hohenester, 2007]. HPDE and FG-Met2 cells were treated with ethanol for 24 hours and changes in integrin expression were determined by real time PCR. As shown in Fig. 5C, ethanol increased α 2-integrin expression by ~2-fold and β 1-integrin by ~1.5-fold in HPDE cells. In contrast, ethanol did not affect the levels of these integrins in FG-Met2 (Fig. 5D) or in Panc1 cells (data not shown).

Since Snail can regulate integrin expression [Haraguchi et al., 2008], we next examined whether ethanol-induced α 2-integrin expression was being regulated by Snail. HPDE cells

were transfected with control siRNA (CtrlSi) or Snail-specific siRNA (SnSi), allowed to recover and then treated with ethanol for 24 hours. Although the Snail siRNA successfully down-regulated Snail expression, it did not affect basal or ethanol-induced α 2-integrin expression (Fig. 5E).

Inhibiting ERK1/2 signaling in pancreatic cancer cells decreases pancreatic cancer cell invasion

Since ethanol decreases ERK1/2 phosphorylation in HPDE cells (Fig. 2B), we examined the effect of ethanol on ERK1/2 phosphorylation in Panc1 cells. Panc1 cells were pre-treated with the MMP inhibitor GM6001, the MEK1/2 inhibitor U0126 or the EGFR inhibitor AG1478 for 30 minutes and then treated with ethanol for 4 hours. In contrast to the effect of ethanol on HPDE cells (Fig. 2B), ethanol did not affect ERK1/2 phosphorylation in Panc1 cells co-treated with DMSO or the MMPi (Fig. 6A). Treatment with U0126 abrogated phospho-ERK1/2 levels in both the untreated and the ethanol treated samples; however, treatment with AG only partially attenuated phospho-ERK1/2 levels in Panc1 cells (Fig. 6A).

We also examined the effect of inhibiting ERK1/2 phosphorylation on Panc1 invasion. As shown previously (Fig. 4), treatment with ethanol did not affect invasion of Panc1 cells (Figs. 6B and 6C). In contrast, both U0126 and AG1478 decreased Panc1 invasion in both the untreated and the ethanol treated cells (Figs. 6B and 6C). These results demonstrate that ERK1/2 signaling regulates invasion and suggests that the effect of ethanol on HPDE invasion may be due to attenuation of ERK1/2 signaling in HPDE cells.

DISCUSSION

The Snail family of transcription factors, which are key regulators of EMT [Thiery et al., 2009], promotes tumor progression in a variety of cancers. Although the contribution of Slug to pancreatic cancer progression is not well studied, Snail enhances pancreatic cancer invasion and promotes metastasis in xenograft mouse models [Nishioka et al., 2010; Shields et al., 2011; Yin et al., 2007]. Clinically, Snail and Slug expression are increased in human pancreatic tumors relative to normal tissue [Hotz et al., 2007]. In this report, we show ethanol increases Snail and Slug expression in HPDE cells, and that the effect of ethanol on Snail was more pronounced compared to the effect on Slug. There was no effect on E-cadherin following ethanol treatment, suggesting that either sustained and/or higher expression of Snail may be necessary for E-cadherin repression [Zhou et al., 2004]. Previously we had published that Snail and Slug induction also varied following treatment with TGF- β 1 [Joseph et al., 2009; Sun et al., 2008]. In contrast to the premalignant cells, ethanol increased expression of Slug to a larger extent than Snail in both Panc1 and FG-Met2 cells. Since different signaling pathways can regulate expression of Snail and Slug [Joseph et al., 2009; Sun et al., 2008], it is possible that the differences in signaling between premalignant and cancer cells may account for the differential induction of these transcription factors following ethanol treatment.

We show that ethanol-induced Snail expression was abrogated following treatment with EGFR and MEK1/2 inhibitors. This is consistent with a recent report demonstrating that ethanol-induced Snail expression in colon and breast cancer cells was also blocked by treatment with an EGFR inhibitor [Forsyth et al., 2010]. Although ethanol was shown to increase ERK1/2 phosphorylation in colon and breast cancer cells [Forsyth et al., 2010], we have found that ethanol in fact suppresses basal phospho-ERK1/2 levels in HPDE cells. Previously, it was shown that ethanol attenuates ERK1/2 signaling in vascular smooth muscle cells, in hepatocytes and in neurons [Chen et al., 1998; Hendrickson et al., 1998; VanDemark et al., 2009]. Since ethanol did not affect phospho-ERK1/2 levels in Panc1

cells, this suggests that modulation of ERK1/2 signaling by ethanol may depend on whether the cells are normal, premalignant or malignant [Aroor and Shukla, 2004].

Despite the increased Snail levels, ethanol blocked invasion of HPDE cells. This may possibly be due to an ethanol-mediated increase in the adhesion of HPDE cells to collagen. In contrast, ethanol did not affect invasion of pancreatic cancer cells and also did not affect adhesion to collagen. Expression of $\alpha 2$ - and $\beta 1$ -integrins, which promotes binding to collagen [Kadler et al., 2007; Leitinger and Hohenester, 2007], was increased in HPDE cells but not in cancer cells following ethanol treatment. Previously, it was shown that chronic treatment of hepatocytes with ethanol also increased $\beta 1$ -integrin levels [Schaffert et al., 2001]. Although Snail in HPDE cells did not mediate ethanol-induced $\alpha 2$ -integrin expression, it was previously shown that expression of Snail in MDCK and A431 cells altered adhesion by modulating integrin expression [Haraguchi et al., 2008]. Snail increased expression of $\alpha 5$ -integrin, while repressing $\alpha 3$ -, $\alpha 6$ - and $\beta 4$ -integrin expression, resulting in decreased attachment to basement membrane proteins and increased binding to fibronectin [Haraguchi et al., 2008]. Slug has also been shown modulate integrin expression. Slug represses expression of $\alpha 3$ -, $\beta 1$ - and $\beta 4$ -expression in keratinocytes to decrease attachment to laminin-5 matrix [Turner et al., 2006].

It is also possible that ethanol inhibition of HPDE cells is due to repression of ERK1/2 signaling in HPDE cells. ERK1/2 signaling has been clearly demonstrated to regulate invasion. Blocking ERK1/2 signaling using U0126 was shown to inhibit invasion of melanoma, gall bladder and breast cancer cells [Chen et al., 2009; Ge et al., 2002; Horiuchi et al., 2004]. Although ethanol did not affect invasion or phospho-ERK1/2 levels in pancreatic cancer cells, inhibiting ERK1/2 phosphorylation with U0126 or the EGFR inhibitor AG1478 decreased invasion of Panc1 cells. This is in agreement with our recent paper demonstrating that ERK1/2 signaling is important for invasion of pancreatic cancer cells [Shields et al., 2011].

Interestingly, even though ethanol has been shown in a carcinogen-induced model of pancreatic cancer to enhance the development of pancreatic ductal adenocarcinomas [Wendt et al., 2007], it is still debated whether ethanol increases the risk of pancreatic cancer in humans [Gapstur et al., 2011; Michaud et al., 2010]. Since we demonstrate the differential effect of ethanol on premalignant and malignant pancreatic cells, it is possible that the varying effects of ethanol may negate the tumor promotion and inhibition effects of ethanol.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Ethanol induces Snail expression in pancreatic ductal epithelial cells

A-B. Human pancreatic ductal epithelial (HPDE) cells were treated with 1% ethanol daily for 3 days. The mRNA isolated from the cells was analyzed for relative expression of Snail, Slug and GAPDH (endogenous control) by qRT-PCR, quantified and normalized to an untreated sample arbitrarily set at 1. *C.* HPDE cells were treated with 1% ethanol daily for 3 days. The mRNA isolated was analyzed for expression of E-cadherin, vimentin, fibronectin and GAPDH by qRT-PCR and normalized to an untreated sample arbitrarily set at 1. *, $p < 0.05$. The protein lysates were immunoblotted for E-cadherin and tubulin (inset). *D.* HPDE cells were treated with 1% ethanol for the times indicated and the mRNA isolated was analyzed for Snail and GAPDH by qRT-PCR. *E.* HPDE cells were treated with increasing doses of ethanol for 4 hours and the mRNA isolated was analyzed for Snail and GAPDH by qRT-PCR. The results are an average of at least 3 independent experiments with error bars indicating SEM. *F.* HPDE cells were pretreated with the GSK-3 β inhibitor LiCl (40 mM) and proteasome inhibitor MG132 (5 μ M) for 30 min and then induced with ethanol for 2 hours. The protein lysates were immunoblotted for Snail and tubulin. NS, non-specific band. The results are representative of 3 independent experiments.

Figure 2. Ethanol-induced Snail expression in pancreatic ductal cells is regulated by ERK1/2 signaling

HPDE cells were pretreated with vehicle control (DMSO), MMP inhibitor (MMPi, GM6001, 10 μ M), MEK 1/2 inhibitor (U0126, 10 μ M) or EGFR inhibitor (AG1498, AG, 10 μ M) for 30 minutes before being induced with 1.0% ethanol for 4 hours. **A.** The mRNA isolated was analyzed for expression of Snail and GAPDH by qRT-PCR and normalized to the vehicle control (DMSO) sample arbitrarily set at 1. **B.** The protein lysates were immunoblotted for p-ERK1/2 and tubulin (loading control). **C.** Morphology of cells was examined by phase contrast microscopy, and pictures taken with Nikon camera. The results are an average of at least 3 independent experiments.

Figure 3. Ethanol primarily induces Slug expression in pancreatic cancer cells

A, B. The malignant Panc1 cells were treated with 1% ethanol daily for 3 days. The mRNA isolated from the cells was analyzed for relative expression of Snail, Slug and GAPDH (endogenous control) by qRT-PCR, quantified and normalized to an untreated sample arbitrarily set at 1. *C, D.* The effect of 1% ethanol on Snail and Slug mRNA was also determined in the malignant FG-Met2 cells. *E.* FG-Met2 cells were treated with 1% ethanol daily for 3 days. The mRNA isolated was analyzed for expression of E-cadherin, vimentin, fibronectin and GAPDH by qRT-PCR and normalized to an untreated sample arbitrarily set at 1. *, $p < 0.05$. The results are an average of at least 3 independent experiments with error bars indicating SEM.

Figure 4. Ethanol inhibits invasion of HPDE cells, but not that of pancreatic cancer cells
A, B. Equal numbers of HPDE cells were plated onto Matrigel-coated transwell chambers, left untreated or treated with 1% ethanol for 24 hours and allowed to invade. Non-invading cells were removed from the upper chamber and filters were fixed and stained. Invading cells were photographed (*A*) and counted (*B*). *C, D.* Similarly, the relative invasion of Panc1 and FG-Met2 cells through Matrigel-coated transwell chambers was also determined, with the invading cells photographed (*C*) and counted (*D*). *, $p < 0.05$. The results are an average of at least 3 independent experiments with error bars indicating SEM.

Figure 5. Ethanol increases adhesion and collagen-binding integrins in pancreatic ductal cells, but not in pancreatic cancer cells

A, B. HPDE and FG-Met2 cells were left untreated or treated with 1% ethanol for 24 hours, and then equal numbers of the cells were seeded onto BD Biocoat tissue culture plates coated with thin layer collagen. Cells were allowed to adhere at 37°C for 15 minutes, washed and then stained. Adherent cells were photographed (*top*) and counted (*bottom*). *C, D.* HPDE and FG-Met2 cells were treated with 1% ethanol for 24 hours and the mRNA analyzed for expression of $\alpha 2$ -integrin, $\beta 1$ -integrin and GAPDH expression by qRT-PCR. *, $p < 0.05$. *E.* HPDE cells were transfected with control siRNA (CtrlSi) or Snail-specific siRNA (SnSi), allowed to recover overnight and then treated with ethanol for 24 hours. The cells were analyzed for Snail, $\alpha 2$ -integrin, and GAPDH expression by qRT-PCR. *, $p < 0.05$. The results are an average of at least 3 independent experiments with error bars indicating SEM.

Figure 6. Inhibiting ERK1/2 signaling in pancreatic cancer cells decreases pancreatic cancer cell invasion

A. Panc1 cells were pretreated with vehicle control (DMSO), MMP inhibitor (MMPi, GM6001, 10 μ M), MEK 1/2 inhibitor (U0126, 10 μ M) or EGFR inhibitor (AG1498, AG, 10 μ M) for 30 minutes before being treated with 1% ethanol for 4 hours. The protein lysates were immunoblotted for phospho-ERK1/2 and tubulin (loading control). B, C. Equal numbers of Panc1 cells were plated onto Matrigel-coated transwell chambers, left untreated or treated with 1% ethanol and allowed to invade in the presence of DMSO, U0126 or AG for 24 hours. Non-invading cells were removed from the upper chamber and filters were

fixed and stained. Invading cells were photographed (*B*) and counted (*C*).*, $p < 0.05$. The results are an average of at least 3 independent experiments with error bars indicating SEM.