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Alcohol Activates the Hedgehog Pathway and Induces Related Pro-carcinogenic Processes in the Alcohol-Preferring Rat Model of Hepatocarcinogenesis

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Abstract

Background—Alcohol consumption promotes hepatocellular carcinoma (HCC). The responsible mechanisms are not well understood. Hepatocarcinogenesis increases with age and is enhanced by factors that impose a demand for liver regeneration. Because alcohol is hepatotoxic, habitual alcohol ingestion evokes a recurrent demand for hepatic regeneration. The alcohol-preferring (P) rat model mimics the level of alcohol consumption by humans who habitually abuse alcohol. Previously, we showed that habitual heavy alcohol ingestion amplified age-related hepatocarcinogenesis in P-rats, with over 80% of alcohol-consuming P rats developing HCCs after 18 months of alcohol exposure, compared to only 5% of water-drinking controls.

Methods—Herein, we used quantitative real time PCR and quantitative immunocytochemistry to compare liver tissues from alcohol-consuming P rats and water-fed P rat controls after 6, 12, or 18 months of drinking. We aimed to identify potential mechanisms that might underlie the differences in liver cancer formation, and hypothesized that chronic alcohol ingestion would activate Hedgehog (HH), a regenerative signaling pathway that is over-activated in HCC.

Results—Chronic alcohol ingestion amplified age-related degenerative changes in hepatocytes, but did not cause appreciable liver inflammation or fibrosis even after 18 months of heavy drinking. HH signaling was also enhanced by alcohol exposure, as evidenced by increased levels of mRNAs encoding HH ligands, HH-regulated transcription factors, and HH-target genes. Immunocytochemistry confirmed increased alcohol-related accumulation of HH ligand-producing cells and HH-responsive target cells. HH-related regenerative responses were also induced in alcohol-exposed rats. Three of these processes (i.e., deregulated progenitor expansion, the reverse-

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Warburg effect, and epithelial-to-mesenchymal transitions) are known to promote cancer growth in other tissues.

Conclusions—Alcohol-related changes in Hedgehog signaling and resultant deregulation of liver cell replacement might promote hepatocarcinogenesis.

Keywords

Liver cancer; Hedgehog; liver progenitors; reverse-Warburg effect; epithelial-to-mesenchymal transitions

Introduction

Alcohol abuse is a highly prevalent condition. In 2002, the lifetime prevalence of alcohol abuse among adults in the United States was 17.8 percent (Hasin et al. 2007). Although average alcohol consumption among Americans has decreased over the past 50 years, the incidence of alcohol related pathologies has not changed (Zhang et al. 2008). Alcohol is a contributing risk factor to a variety of medical conditions including cancers of the mouth, esophagus, pharynx, larynx, and liver (Thun et al. 1997). Indeed, alcohol and cancer development are closely related: Alcohol consumption has been implicated in 3.6% of cancer cases, and 3.5% of cancer deaths, worldwide (Boffetta et al. 2006). While many epidemiological studies demonstrate that alcohol consumption increases the risks for cancer, the mechanism by which alcohol elevates cancer risk remains unknown.

In western countries, habitual alcohol consumption is also the most frequent cause of liver disease (Bruha et al. 2012). For example, over half the cases of advanced liver disease in the US are due to alcohol abuse (Thun et al. 1997). The largest risk factor for developing alcohol-induced liver disease (ALD) is cumulative lifetime alcohol consumption, and relative risk increases in a dose-dependent manner (Bellentani and Tiribelli 2001). Alcohol metabolism in the liver is the principal cause of liver damage, with alcohol metabolic by-products causing a complex disease that can progress from alcoholic steatosis to cirrhosis and hepatocellular carcinoma (HCC). Habitual alcohol consumption also exacerbates liver fibrosis, and increases the risk of developing HCC, in liver diseases that are not caused by alcohol (Rehm et al. 2010). Although over 20% of HCC worldwide can be attributed to alcohol and ALD (Forner et al. 2012), the molecular mechanisms by which alcohol potentiates the evolution of HCC in chronically damaged livers remain unclear.

Dysfunctional liver regeneration is at the root of all chronic liver disease. A key signaling pathway involved in liver regeneration is the Hedgehog signaling pathway. Hedgehog (HH) signaling is essential during development (Omenetti et al. 2011), becomes reactivated during organ injury, and is dysregulated in many types of cancer (Arwert et al. 2012). Certain conditions in adulthood stimulate HH-ligand producing cells to generate and secrete HH ligands into the environment. These ligands bind to the receptor Patched (PTC) on HH-responsive target cells. This relieves PTC of its normal function as an inhibitor of Smoothened (SMO). Once activated, SMO localizes to the primary cilia and initiates an intracellular chain-of-events leading to the activation of Gli-family transcription factors (Teglund and Toftgard 2010). While the HH pathway is not active in healthy adult livers, it

becomes reactivated in response to a variety of liver insults, including chronic alcohol ingestion (Jung et al. 2008), and regulates liver repair (Choi et al. 2009). HH ligands, released from liver epithelia, activate HH signaling in liver epithelial progenitors, a process which promotes their viability and proliferation, thereby enhancing liver regeneration (Sicklick et al. 2006, Fleig et al. 2007). The expansion of these progenitor compartments, however, has also been associated with the progression and maintenance of hepatocellular carcinomas (Roskams et al. 2003, Pereira et al. 2010). HH signaling has been found to be elevated in human HCC tissue, and several human liver cancer cell lines require HH pathway activity for proliferation, viability, and chemoresistance (Sicklick et al. 2006). Furthermore, an HH signaling inhibitor, GDC-0449, significantly reduced both fibrosis and tumor volume in aged mice with advanced liver fibrosis and HCC (Philips et al. 2011). Recently, we demonstrated that malignant hepatocytes release HH ligands that incite neighboring cells in the stromal compartments to secrete viability factors that promote tumor maintenance (Chan et al. 2012). Together these studies suggest that HH signaling contributes to both the pre-neoplastic and neoplastic environment following chronic liver injury in humans and mice.

Despite the strong association of alcohol consumption with HCC development in humans, animal models to study this process are limited. Although alcohol ingestion has been shown to enhance hepatitis C virus (HCV)-related tumorigenesis in mouse models (Szabo et al. 2010), HCCs do not typically develop in wild type mice or rats that consume alcohol in the standard Lieber-DeCarli diet- or that are given alcohol-enriched diets via an intragastric feeding approach (Tsukamoto-French). The reasons for this are not understood, but it has been suggested that insufficient alcohol exposure might have a role because neither alcohol delivery model completely recapitulates the level and duration of alcohol consumption in human alcohol abusers.

In our current study, therefore, we interrogated liver tissues from an alcohol-preferring (P) rat line, selectively bred for high alcohol preference. P rats voluntarily drink 6g alcohol per kg body weight per day, the equivalent of 1.5g alcohol per kg body weight per day in humans (e.g., roughly comparable to the alcohol intake of a 80 kg man who drinks two 6 packs of beer daily). Recently, we reported that P rats develop spontaneous hepatic tumors after 18 months of alcohol consumption. These tumors were further characterized to have increased mitogen-activated protein kinase 1/2 phosphorylation, consistent with findings in clinical samples of human HCC tissue (Yip-Schneider et al. 2011). Given that P rats represent a clinically relevant model for studying the development of HCC in the context of alcohol consumption, our aims were to determine whether HH signaling is elevated in this animal model of alcohol-induced HCC and identify potential mechanisms by which HH pathway activation might contribute to hepatocarcinogenesis.

Materials and Methods

Animals

We studied livers from rats that had been selectively bred for high alcohol preference (P rats). In an earlier study, we randomized 6 month old P rats into one of two treatment groups: Group 1 was allowed free access to water, Group 2 was given free access to water

and alcohol admixed with water (final concentration 10% alcohol by volume) for up to 18 months (the drinking period). Body weights and blood alcohol concentrations were carefully monitored during the drinking period and results demonstrated comparable weight gains in both groups and blood alcohol levels that hovered around 5 mmol/L in alcohol-exposed rats. Rats were sacrificed after 6 months (n=6-7/group), 12 months (n=12/group), or 18 months (n=20/group) of water/alcohol exposure to harvest the liver tissues that we analyzed in the present study. Gross autopsy and liver ultrasonography at the time of tissue harvest demonstrated emergence of HCCs after 12 months of alcohol exposure (prevalence 8.3%), with a dramatic increase in HCC incidence between 12-18 months, such that scattered, small tumors (approximately three 3-4 mm tumors/mouse) were demonstrated in 15 of 18 (83.3%) of alcohol-exposed rats after 18 months of drinking. In contrast, HCCs occurred in 1 of 20 age-matched P rats that drank only water during the drinking period (Yip-Schneider et al. 2011).

Quantitative Immunohistochemistry

Liver specimens fixed in formalin and embedded in paraffin were cut into 4μm sections, dewaxed, hydrated, and subsequently incubated in 3% hydrogen peroxide/methanol for 15 minutes to block endogenous peroxidase. To evaluate tissue architecture, slides were stained with hematoxylin and eosin (H&E) per standard protocol. Antigen retrieval was performed by heating in 10mM sodium citrate buffer or 0.25% pepsin (K19; Invitrogen, Carlsbad, CA) for 10 minutes. Sections were blocked (Dako Envision, Carpinteria, CA) and incubated with primary antibodies overnight at 4°C: Pyruvate kinase M2 (1:1000; Cell Signaling, Danver, MA), Glioblastoma-2 (18-732-292462; 1:2000; GenWay Biotech, Inc., San Diego, CA); Indian Hedgehog (1:750; Abcam, Cambridge, MA); cytokeratin 19 (Troma-III, 1:750; Hybridoma Bank, Iowa City, IA); α-fetoprotein (AFP) (1:1000; Dako); and SOX9 (AB5535, 1:1000; Millipore, Billerica, MA). Polymer-HRP anti-rabbit (K4003; Dako) or anti-mouse (K40011; Dako) were used as secondary antibodies. 3,3'-Diaminobenzidine (DAB) Substrate Chromogen System (K3466; Dako) and/or Vino Green (Biocare Medical, Concord CA) was employed in the detection procedure. Omitting primary antibodies from the reactions eliminated staining which demonstrated staining specificity. Images were acquired on an Olympus IX71 (Tokyo, Japan) inverted microscope using the DP2-BSW (Olympus) image acquisition software system. Numbers of immunoreactive cells were evaluated by examination of coded sections. A minimum of 10 randomly selected 200× field were evaluated for each rat. To quantify degenerative changes in both water and alcohol-fed rats, imaging samples from each slide were taken at 20×, 40×, 100×, 200× and 400×. The number of tumors (if present) were counted and measured. Next, we estimated the percent surface area (non-tumor and tumor counted separately) involved by each of the changes described (macrofat, degeneration). The presence or absence of microfat and megamitochondria in the tumors was also noted.

Quantitative Real-Time Reverse Transcription-PCR

RNA was extracted from snap frozen, whole liver using TriZol (Life Technologies, Grand Island, NY) as previously described (Xie et al. 2013). Fifteen nanograms of cDNAs was used to perform quantitative PCR assay in duplicate with SYBR Green Supermix. Primer sequences listed in Supplemental Table 1. Expression of the examined genes was

normalized against internal control gene (rodent β-actin or human β-actin) based on the threshold cycle (C*t*) and relative fold change calculated by the $2^{-\Delta\Delta C}{}_t$ method.

Statistical Analysis

Results are expressed as mean ± standard error mean (SEM) unless otherwise specified. Mean data were compared using the Student's t-test. Differences were considered significant when $p<0.05$.

Results

Alcohol Consumption Causes Progressive Liver Damage in P Rats

We characterized the histopathological changes that occurred in livers of alcohol- and water-exposed P rats over the 18-month drinking period. Results in alcohol-consuming rats were compared to age- and gender matched control P rats that drank water without added alcohol. Compared to controls (**Fig. 1A**), livers of P rats that drank alcohol demonstrated subtle degenerative changes **(Table 1, Fig.s 1B,D)**. Mild liver damage was observed at the first time point examined (6 months after beginning alcohol-consumption) and progressed with the duration of alcohol ingestion. Degenerative changes include macrofat **(Fig. 1B),** megamitochondria **(Fig. 1C),** and accumulation of cytoplasmic degenerative bodies **(Fig. 1D).** Despite having the afore-mentioned features of mild, chronic liver injury for more than half of their adult lives, however, alcohol drinking P rats did not develop overt steatohepatitis or cirrhosis, Some degenerative changes also emerged with age in water-fed P rats, but alcohol-consuming P rats exhibited greater liver injury at every time point than water-fed controls **(Fig. 1A).**

Hedgehog Signaling is Activated in Aged, Ethanol-Fed Rats

In humans, chronic consumption of alcohol can result in alcoholic hepatitis and liver cancer. In animal models of alcoholic hepatitis, and humans with either alcoholic hepatitis (Jung et al. 2008) or liver cancer (Chan et al. 2012), HH signaling is activated. In our P rat model of alcohol-induced HCC we observed only subtle hepatic degenerative changes indicative of relatively mild liver injury. Because it was unclear that this level of liver injury would be sufficient to provoke HH pathway activation, we compared HH signaling in control- and alcohol-consuming- P rats. We observed that hepatic expression of HH ligands and HH-target genes remained stable or declined with age in water-fed P rats. In contrast, liver levels of HH mRNAs and various HH target genes increased over time in P rats that consumed alcohol. As was noted in mice with genetic liver injury and enhanced susceptibility to aging-related HCC, (Philips et al. 2011), in alcohol-consuming P rats, the increase in HH signaling was modest at younger ages (e.g., during the initial 12 months of alcohol exposure) but became much more pronounced at older ages (during the final 6 months of alcohol drinking) **(Fig. 2).** Increased protein expression of HH ligands and HH-target genes was also demonstrated in alcohol consuming P rats relative to water-fed controls using quantitative immunohistochemistry **(Fig. 3).** For example, the numbers of cells expressing Indian Hedgehog (IHH) ligand were significantly greater in in rats that had been exposed to alcohol for 12 or 18 months than in age-matched controls that drank only water **(Fig. 3A).** Moreover, increased HH ligand expression in the alcohol-consuming group was paralleled

by accumulation of HH-responsive cells (marked by nuclear expression of the HH-regulated transcription factor, GLI2) **(Fig. 3B).** As observed in other types of chronic liver injury (Guy et al. 2012), expression of HH ligand predominated in epithelial-appearing cells (in this case, ductular cells) **(Fig. 3A, Fig. S1A),** while both ductular cells and stromal cells expressed GLI2 **(Fig. 3B, Fig. S1B).** These findings support the concept that chronic alcohol consumption activated autocrine/paracrine signaling via the HH pathway.

Liver Progenitor Populations Expand During Alcohol-Related Hepatocarcinogenesls

HH pathway activation is accompanied by dramatic expansion of progenitor populations in humans and rodents with alcoholic hepatitis (Jung et al. 2008). The HH pathway is over-activated in alcohol-consuming P rats, although these rats do not develop overt histological features of alcoholic hepatitis. Thus, the alcohol-consuming P rat model provides an opportunity to examine the relationship between alcohol exposure, HH pathway activation, and progenitor accumulation, absent confounding influences of alcoholic hepatitis. Quantitative PCR analysis of whole liver RNA revealed age-related changes in hepatic expression of mRNAs encoding different liver progenitor markers in both control- and alcohol-consuming P rats. Specifically, marker expression tended to increase as the rats aged between 6 and 12 months of water exposure in the control group; thereafter, expression of AFP, CK19, and SOX9declined back to comparable levels that were observed at the earliest time point (6 months water-fed control rats) (**Fig. 4A-C**). Alcohol consumption generally enhanced age-related induction of the progenitor markers SOX9 and CD44, potentiating marker gene expression after 18 months of alcohol exposure and blunting the age-related decline in CK19 and AFP expression that occurred between 12 and 18 months of treatment in the water-fed control group **(Fig. 4).** Of note, alcohol appeared to exert differential effects on expression of markers for ductular progenitors (i.e., CK19) and hepatocytic progenitors (i.e., AFP), potentiating induction of CK19 while attenuating induction of AFP between 6 and 12 months of treatment (Fig. 4A, B). This finding is intriguing because the fate of bipotent liver epithelial progenitors is generally modulated to match the relative demand for replacement of hepatocytes and ductular cells (Jung et al. 2010). Alcohol-consuming P rats demonstrated evidence of hepatocyte injury **(Fig. 1),** and this would be predicted to mobilize bipotent progenitors to differentiate along the hepatocytic (as opposed to the biliary) lineage. However, we observed relatively inhibited induction of AFP mRNA and coincident relative hyper-induction of CK19 mRNA in the alcohol-consuming rats **(Fig. 4A,B),** suggesting that alcohol exposure disrupts normal mechanisms that modulate cell fate decisions in bipotent liver epithelial progenitors.

To clarify the effects of alcohol on progenitor cell fate, markers of bipotent progenitors (SOX9), hepatocytic progenitors (AFP), and ductular progenitors (CK19) were examined using quantitative immunocytochemistry **(Fig. 5).** Aging was accompanied by slight expansion of SOX9(+) progenitor populations in periportal areas in water fed rats. At each age, this process was significantly enhanced by alcohol exposure **(Fig. 5A),** confirming other assays which indicated that alcohol exposure enhanced expansion of bipotent progenitor populations **(Fig. 4)** and supporting earlier evidence linking HH pathway activation to outgrowth of such cells during alcoholic hepatitis (Jung et al. 2008). Differential effects of alcohol on hepatocytic and ductular progenitors were observed again

as well. The livers of control rats had more AFP(+) progenitors **(Fig. 5B),** and fewer CK19(+) progenitors **(Fig. 5C),** than age-matched rats that had been exposed to alcohol for 6 months of treatment. Numbers of CK19(+) progenitors remained relatively constant in the alcohol consuming group, and were consistently greater in those rats than in respective age-matched water-fed controls at each time point examined **(Fig. 5C)**. In contrast, aging-related accumulation of AFP-expressing progenitors was delayed in alcohol-consuming rats, exceeding levels observed in water-fed controls only after 12 and 18 months of alcohol exposure **(Fig. 5B),** again suggesting that alcohol exposure might interfere with normal hepatocytic differentiation of bipotent progenitors. This possibility is further supported by demonstration of CK19 (which typically marks ductular cells) in hepatocytic-appearing cells in the parenchyma following alcohol exposure. This is apparent even at the earliest time point, but becomes more pronounced after both 12 and 18 months of treatment **Fig. 5D).** The aggregate data, therefore, demonstrate that HH pathway activation is accompanied by accumulation of liver progenitors in alcohol-consuming P rats, and suggest that progenitor differentiation may also be deregulated in this model.

Alcohol Consumption Stimulates Stromal Metabolic Processes that Enhance Tumorigenesis

Growth of progenitor cells in regenerating livers and in cancers is facilitated by stromal cells. Previously, we demonstrated that HH pathway activation induces glycolytic activity in HH-responsive stromal cells, including liver myofibroblasts (Chen et al. 2012). We also showed that metabolic by-products released by glycolytic stromal cells enhanced the growth of neighboring malignant liver epithelial cells, and suggested that such changes in the hepatic microenvironment contribute to carcinogenesis (Chan et al. 2012). To determine if a similar process might be occurring in P rats during alcohol-related carcinogenesis, we compared expression of mRNAs encoding the muscle-related isoenzyme of pyruvate kinase (Pkm2), an acknowledged marker of glycolytic cells (Christofk et al. 2008), in water-fed and alcohol-consuming P rats **(Fig. 6).** Expression of PKM2 mRNAs remained relatively consistent from 6-18 months of treatment in water-fed rats, but increased from 6-12 months in alcohol-consuming rats and remained significantly higher than in age-matched water-fed controls at 18 months of treatment **(Fig. 6A).** Quantitative immunohistochemistry revealed greater hepatic content of Pkm2-expressing cells in alcohol-fed rats relative to water-fed controls at each time point examined during the 18 month drinking period **(Fig. 6B).** The aggregate data, therefore, suggest a previously-unsuspected mechanism by which alcohol exposure might promote carcinogenesis, namely by activating HH-mediated induction of stromal cell glycolytic activity (i.e., the so-called “reverse Warburg” effect; (Pavlides et al. 2009).

Alcohol Consumption Promotes EMT Responses that Have Been Linked to Regeneration of Cancer Stem/Progenitor Cells

Emerging evidence indicates the epithelial-to-mesenchymal transitions (EMT) play an important role in replenishing cancer stem/progenitor cell populations in certain non-liver cancers (Polyak and Weinberg 2009). Our results demonstrate relative outgrowth of liver progenitor cells in P rats, which develop HCCs related to chronic alcohol consumption **(Fig. 5A-D).** Therefore, we compared expression of various EMT markers in age-matched water-

fed and alcohol-fed P rats to determine if alcohol exposure might influence this process. We found that alcohol exposure increased mRNA expression of Snail and Zeb-1**(Fig. 7A, B),** two transcription factors that have been identified as master regulators of the EMT process (Mani et al. 2008). It also tended to increase mRNA levels of Transforming Growth Factor (TGF)-beta (another key EMT-inducing factor) (Heldin et al. 2012), but that effect did not achieve statistical significance **(Fig. 7C).** As expected (Scheel and Weinberg 2012), induction of EMT regulators was accompanied by increased expression of mesenchymal makers, such as vimentin and alpha smooth muscle actin (αSMA), in the alcohol-consuming group **(Fig. 7D,E).** Thus, our results show that HH pathway activation and EMT accompany repopulation of progenitor populations during alcohol-related hepatocarcinogenesis, as has been documented to occur during oncogene-driven carcinogenesis in the breast and prostate (Karhadkar et al. 2004, O'Toole et al. 2011). HH signaling promotes EMT (Choi et al. 2009, Syn et al. 2009), and over-activation of the HH pathway occurs in HCCs (Sicklick et al. 2006, Pereira et al. 2010, Philips et al. 2011, Chan et al. 2012, Che et al. 2012). The present findings in the P rat model, therefore, suggest another novel, HH-sensitive mechanism that might contribute to alcohol-related carcinogenesis, namely, induction of EMT in liver cell sub-populations. Interestingly, although HH pathway activation and EMT have been linked to fibrosis in many tissues (Cigna et al. 2012, Ding et al. 2012, Horn et al. 2012), including liver (Guy et al. 2012, Machado and Cortez-Pinto 2011), P rats did not develop progressive liver fibrosis despite drinking alcohol-containing water for a year and a half **(Fig. 1, Table 1).** Further research is needed to clarify why alcohol-consuming P rats escaped cirrhosis despite activating EMT and accumulating large numbers of myofibroblastic, αSMA(+) cells. Preliminary evidence for increased expression of matrix metalloproteinases (e.g., MMP9) in alcohol-consuming P rats relative to age-matched water-fed controls **(Fig. 7F)**, suggest that enhanced matrix turnover might help to limit scar accumulation in the P rat model.

Discussion

Alcohol is considered to be a co-carcinogen by the World Health Organization, based on well-documented associations between habitual alcohol consumption and increased odds for cancer in many tissues, including liver (Thun et al. 1997). In this study, we used the P rat model that mimics the level of alcohol consumption by humans who habitually abuse alcohol to determine if (and how) chronic alcohol exposure impacts hepatocarcinogenesis. Hepatocarcinogenesis generally increases with age in rodents (Philips et al. 2011), and the process is accelerated by factors that impose a demand for liver regeneration (Omenetti et al. 2011). Because alcohol is hepatotoxic and also inhibits replication of surviving hepatocytes (Koteish et al. 2002), habitual alcohol ingestion evokes a recurrent demand for hepatic regenerative activity that must be met by mobilizing liver progenitors (Roskams et al. 2003). Progenitor cells are believed to play key roles in the growth of many cancers, including HCC (Wu et al. 2012). However, the mechanisms regulating their growth and differentiation during hepatocarcinogenesis in general, or alcohol-related liver cancers in particular, are not well understood. This issue has been difficult to study in people because HCC typically develop in livers that are already cirrhotic and cirrhosis itself increases progenitor accumulation (Schuppan and Afdhal 2008). Also, although alcohol consumption has been shown to potentiate virus-associated hepatocarcinogenesis in HCV-transgenic mice

(Machida et al. 2009), HCCs do not typically develop in wild type rodents treated with either of the two most commonly used models for ALD (Lieber DeCarli and Tsukamoto-French). Our prior work suggests that P-rats provide an opportunity to investigate how alcohol might modulate carcinogenesis independently of exogenous stressors, such as chronic viral infection.

In P-rats habitual heavy alcohol ingestion amplifies age-related hepatocarcinogenesis without triggering antecedent steatohepatitis, progressive liver fibrosis, or cirrhosis (Yip-Schneider et al. 2011). Never-the-less, more careful inspection of P rat livers in the present study demonstrated that habitual heavy alcohol consumption definitely injures their hepatocytes. Given that P-rats do not develop progressive steatohepatitis or cirrhosis despite ongoing hepatocyte injury and very long follow-up, these rats are capable of compensating for chronic injury to mature liver epithelial cells. Our results suggest that the mechanism likely involves efficient mobilization of hepatic progenitor populations to replace dying liver cells and identifies several mechanisms that are predicted to modulate that process. These findings are relevant to alcohol-related liver cancers in humans because only 10-35% of alcoholics exhibit changes on liver biopsy consistent with steatohepatitis, and the documented prevalence of cirrhosis after 20 years of heavy alcohol consumption is only 5-15% (Scaglioni et al. 2011). However, alcoholism remains one of the largest risk factors for the development of HCC in developed nations and many HCCs occur in alcoholic patients without evidence of cirrhosis (Morgan et al. 2004).

Our results show that the hepatic microenvironment in P rats changes in response to habitual alcohol exposure , and identify several alcohol-induced alterations that are relevant to hepatocarcinogenesis in humans, including reactivation of developmental morphogens that regulate stem/progenitor cell fate (such as Hedgehog) (Pereira et al. 2010, Chan et al. 2012, Che et al. 2012), and induction of several HH-regulated wound healing responses that occur during adult liver injury (i.e., outgrowth of progenitor cells [Zulehner et al. 2010], induction of EMT [van Zijl et al. 2009], and accumulation of glycolytic stromal cells).

We focused our attention on the Hedgehog pathway and putative pro-carcinogenic mechanisms that it is known to regulate because Hedgehog signaling is over-activated in liver cancer (Sicklick et al. 2006) and our earlier work demonstrated striking activation of Hedgehog signaling in the livers of alcoholic patients with cirrhosis and superimposed severe acute alcoholic hepatitis, as well as typical rodent models of progressive ALD (Jung et al. 2008). Here we show that although alcohol-consuming P rats do not develop progressive liver injury or fibrosis, they also activate the Hedgehog pathway. We found that hepatic production of HH ligands and accumulation of HH-responsive cells were significantly increased by chronic alcohol consumption in P rats.

Given that hepatic Hh signaling is increased in rodent models that develop alcohol-related HCC (i.e., P rats) and rodent models that do not develop alcohol-related HCC (i.e., rodents given alcohol via Lieber DeCarli diets or the Tsukamoto-French intragastric infusion approach), it might be argued that Hedgehog is irrelevant for alcohol-related hepatocarcinogenesis.

However, we believe that an alternative interpretation merits consideration, namely that Hedgehog pathway activation is necessary, but not sufficient, for development of liver cancer. This concept is supported by evidence for deregulated Hedgehog signaling in many human liver cancers (Sicklick et al. 2006a,b) and earlier work which proved that treatment with a highly-specific Hedgehog signaling antagonist was sufficient to provoke regression of established liver cancers in another mouse model of HCC (Philips et al. 2011). Our results in the P rat model are also informative about factors that are not obligatory for alcohol-related hepatocarcinogenesis, namely overt hepatic inflammation or progressive liver fibrosis. Like many human alcoholics, alcohol-consuming P rats develop HCCs in the absence of overt steatohepatitis, progressive liver fibrosis, or cirrhosis. Conversely, increased HCCs have not been reported in well-established rodent models of progressive alcohol-related steatohepatitis and fibrosis. On the other hand, our findings raise the possibility that deregulated differentiation of Hedgehog-responsive progenitors might be important for the pathogenesis of alcohol-related liver cancer. We identified subtle qualitative differences in the progenitor populations of alcohol-fed rats, suggesting that habitual ingestion of alcohol might differentially modulate the relative activities of signaling pathways (e.g., Notch and SOX9) that interact with Hedgehog to regulate progenitor differentiation (Xie et al. 2013). Additional research, however, is needed to clarify the latter issue, and to evaluate its implications.

More work is also required to determine how the newly- demonstrated, alcohol-related effects on HH-sensitive tumor promoting mechanisms interface with established inflammatory signaling mechanisms that control hepatocarcinogenesis in alcohol-fed HCV transgenic mice (Machida et al. 2009). Both processes are likely to be important in humans because alcohol is known to promote liver cancer in patients with and without underlying virally-mediated liver disease. In addition, these mechanisms may interact to potentiate growth of virus-associated liver cancers given two recent reports which reveal previously unsuspected interactions between HCV and Hedgehog. An initial study showed that infecting cultured hepatoma cells with HCV activated HH signaling, and that treating such cells with HH signaling antagonists inhibited viral replication (Choi et al. 2011). A second independent study that used an unbiased approach to identify signaling pathways that were upregulated by HCV infection also demonstrated HCV-related induction of Hedgehog (Papic et al. 2012). It is conceivable that alcohol-related activation of the HH pathway might fuel HCV-initiated mechanisms for liver cancer formation by helping to perpetuate HCV infection.

Finally, evidence that HH signaling is involved in alcohol-related hepatocarcinogenesis has potential therapeutic implications because chemical inhibitors of the HH pathway are approved for use in humans. Treatment with such agents dramatically improved the outcomes of patients with advanced basal cell carcinomas, an acknowledged HH-responsive tumor type (Von Hoff et al. 2009), and we've reported that this agent promoted HCC regression in mice (Philips et al. 2011). Other workers have shown that HH inhibition also inhibits the growth of explanted cholangiocarcinomas, another primary liver cancer that derives from bipotent liver epithelial progenitors (Fingas et al. 2011). Whether or not HH inhibitors would be beneficial as a treatment for alcohol-related HCC in humans remains to

be determined, but the results in P rats justify further evaluation of this approach in pre-clinical models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ALD	alcoholic liver disease
HCC	Hepatocellular carcinoma
HH	Hedgehog
Ptc	Patched
IHH	Indian Hedgehog ligand
AFP	alpha fetoprotein
CK19	cytokeratin 19
PKM2	pyruvate kinase M2
OPN	osteopontin
Sfrp-1	soluble frizzled related peptide-1
SOX9	SRY (sex determining region Y)-box 9

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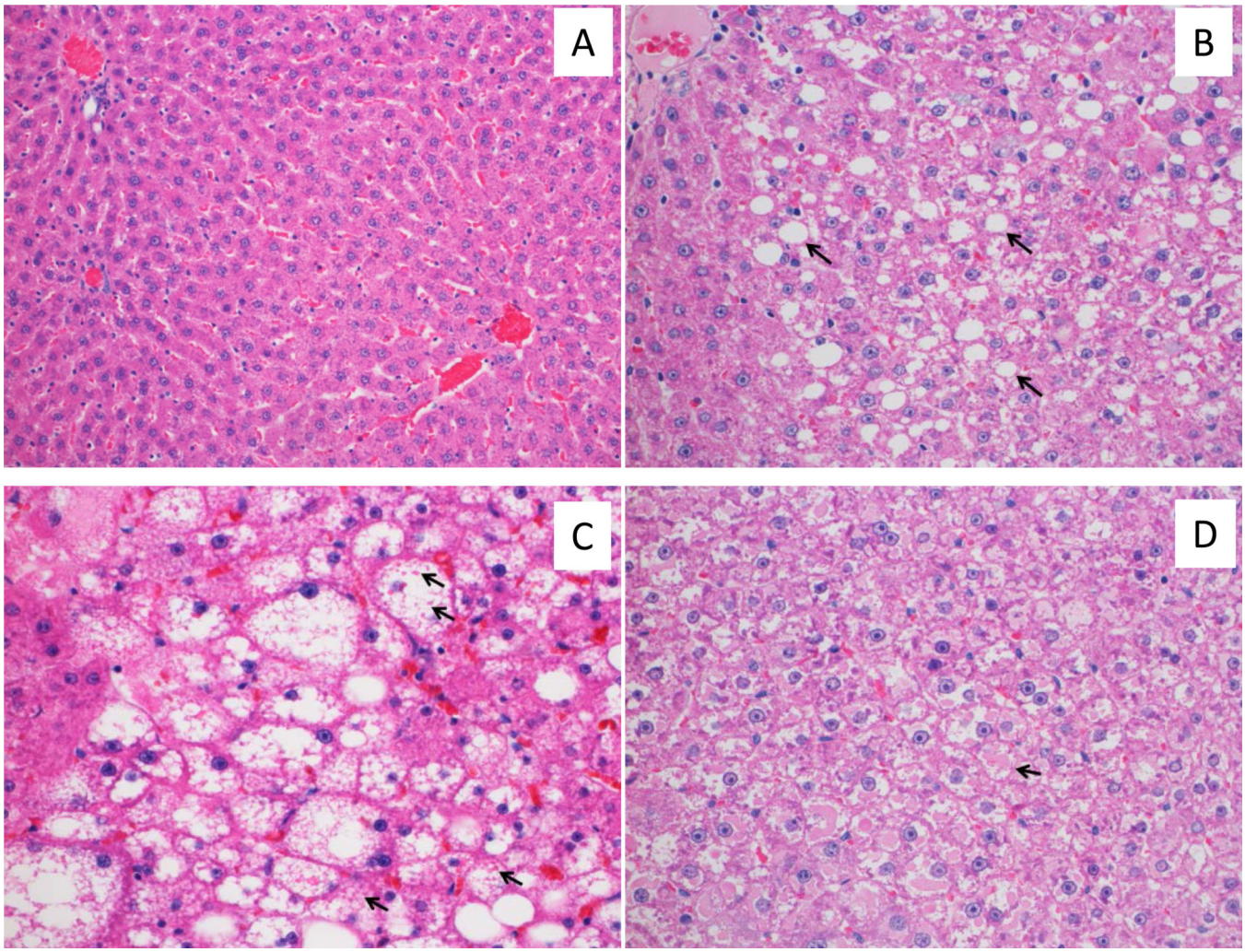


Fig. 1. Alcohol drinking exacerbates age-related hepatocyte degeneration

Livers were harvested from rats that had been drinking either water (control) or alcohol for 18 months. Results of representative H&E-stained sections are shown. **(A)** Control rat (200×); **(B-D)** Age-matched alcohol-drinking rats. Alcohol-drinking rats demonstrated several degenerative changes including **(B)** macrofat (arrows, 400×), **(C)** megamitochondria (arrows, 400×), and **(D)** cytoplasmic degenerative bodies (arrow, 400x).

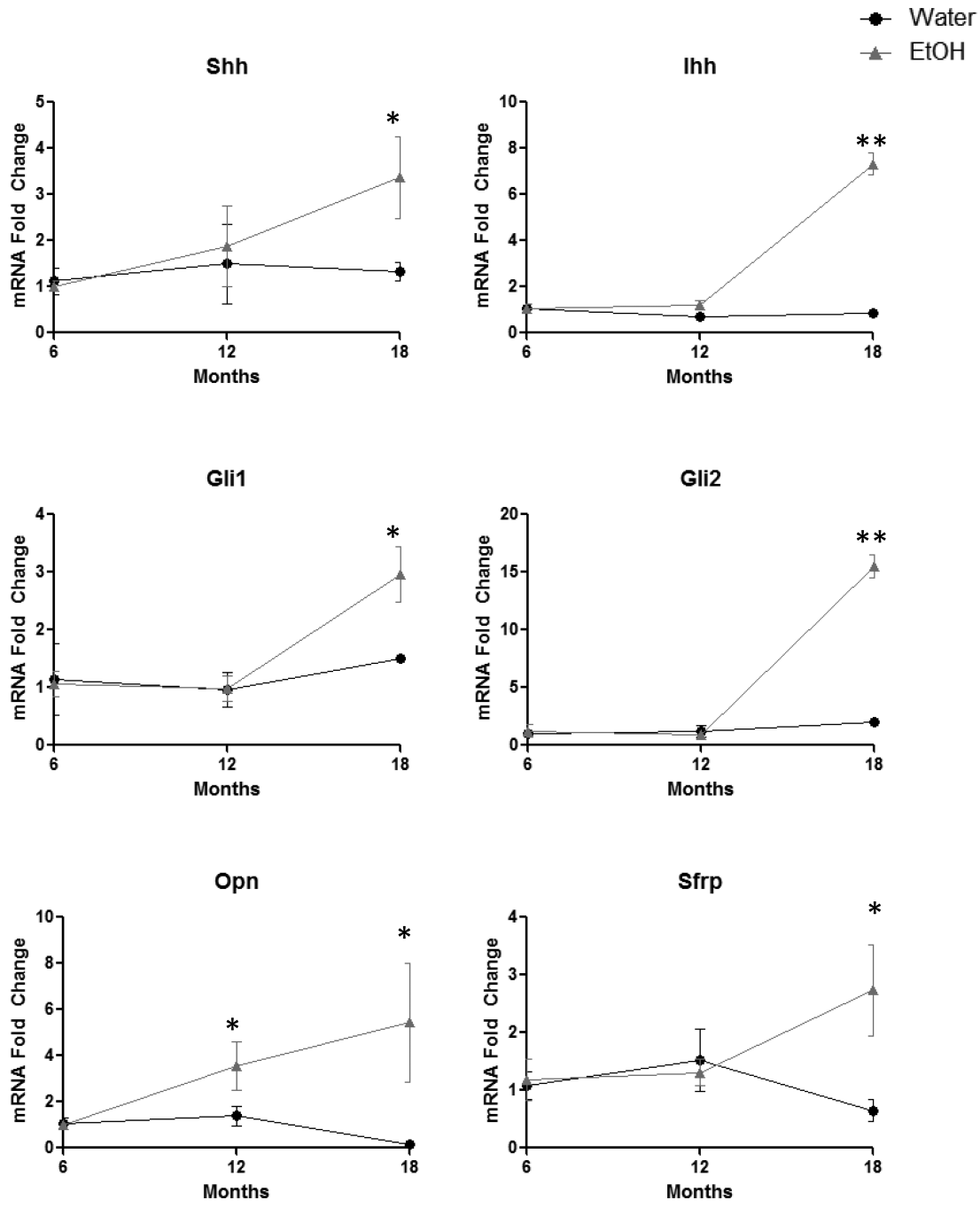


Fig. 2. Alcohol drinking increases hepatic Hedgehog signaling

Rats were randomized to drink either water (control, black) or alcohol (gray) at 6 months of age. Livers were harvested after 6, 12, or 18 months of alcohol and water exposure and evaluated by quantitative real-time PCR for the following Hedgehog-related genes: Sonic Hedgehog (SHH) and Indian Hedgehog (IHH) ligands, Hedgehog-regulated transcription factors, Glioma (GLI1 and GLI2, and Hedgehog-regulated target genes, osteopontin (OPN) and soluble frizzled related peptide-1 (SFRP). Results at 12 and 18 months were normalized

to the respective 6 months values in each group. Mean ± SEM at each time point is graphed for each group. *p<0.05, **p< 0.01 versus gene expression at 6 months.

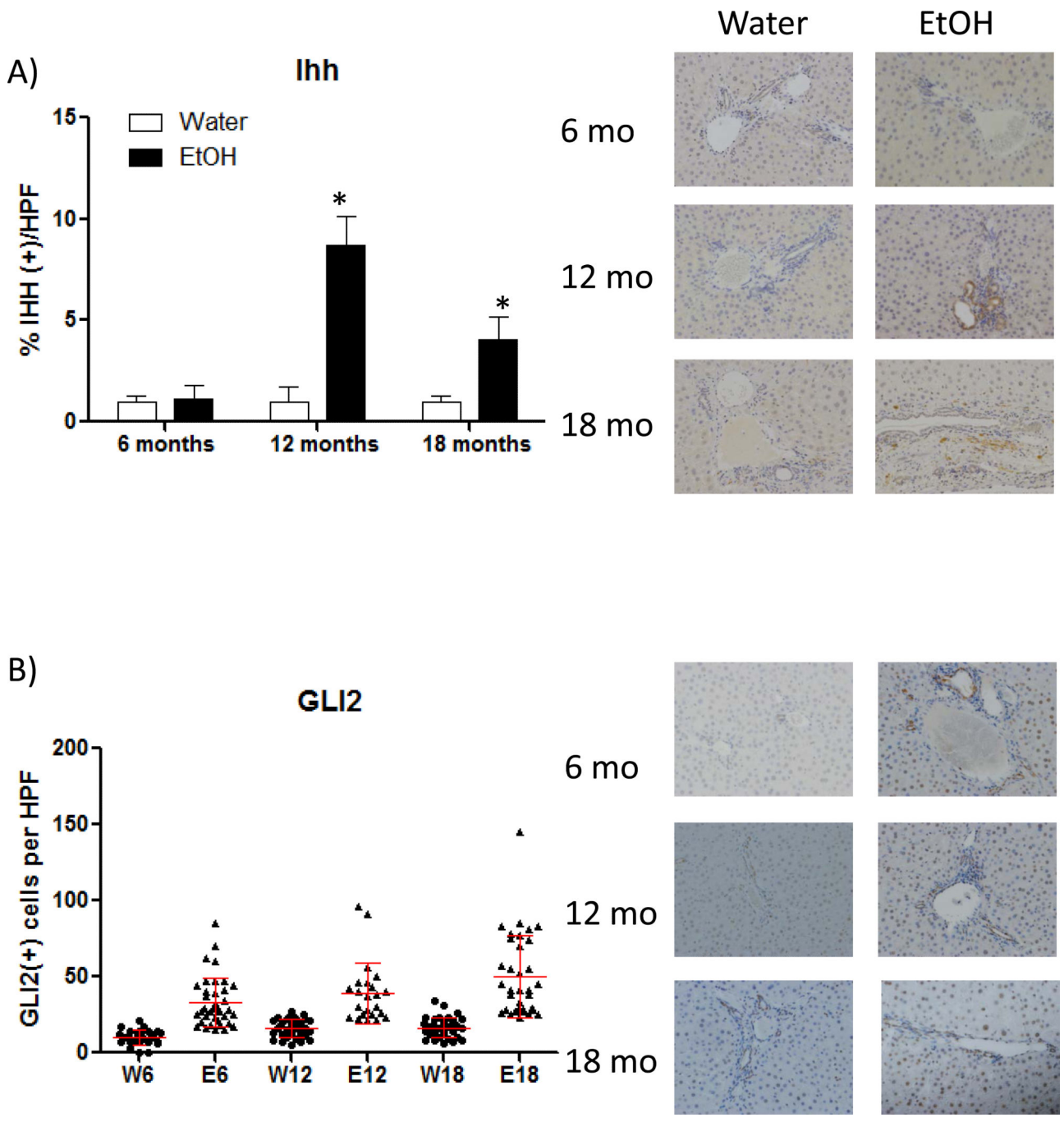


Fig. 3. Alcohol drinking causes hepatic accumulation of Hedgehog ligand-producing cells and Hedgehog-responsive cells

Livers were harvested from rats after 6, 12, or 18 months of drinking either water (control) or alcohol. Sections were immunostained for Indian Hedgehog ligand (IHH) and the Hedgehog-regulated transcription factor, GLI2. Representative images are shown and quantitative data are graphed as mean ± SEM for IHH **(A)** and mean ± SD for GLI2 **(B).** *p<0.05 versus the respective age-matched, water-drinking controls. W, water-fed rats, alcohol-fed rats; HPF, high power field.

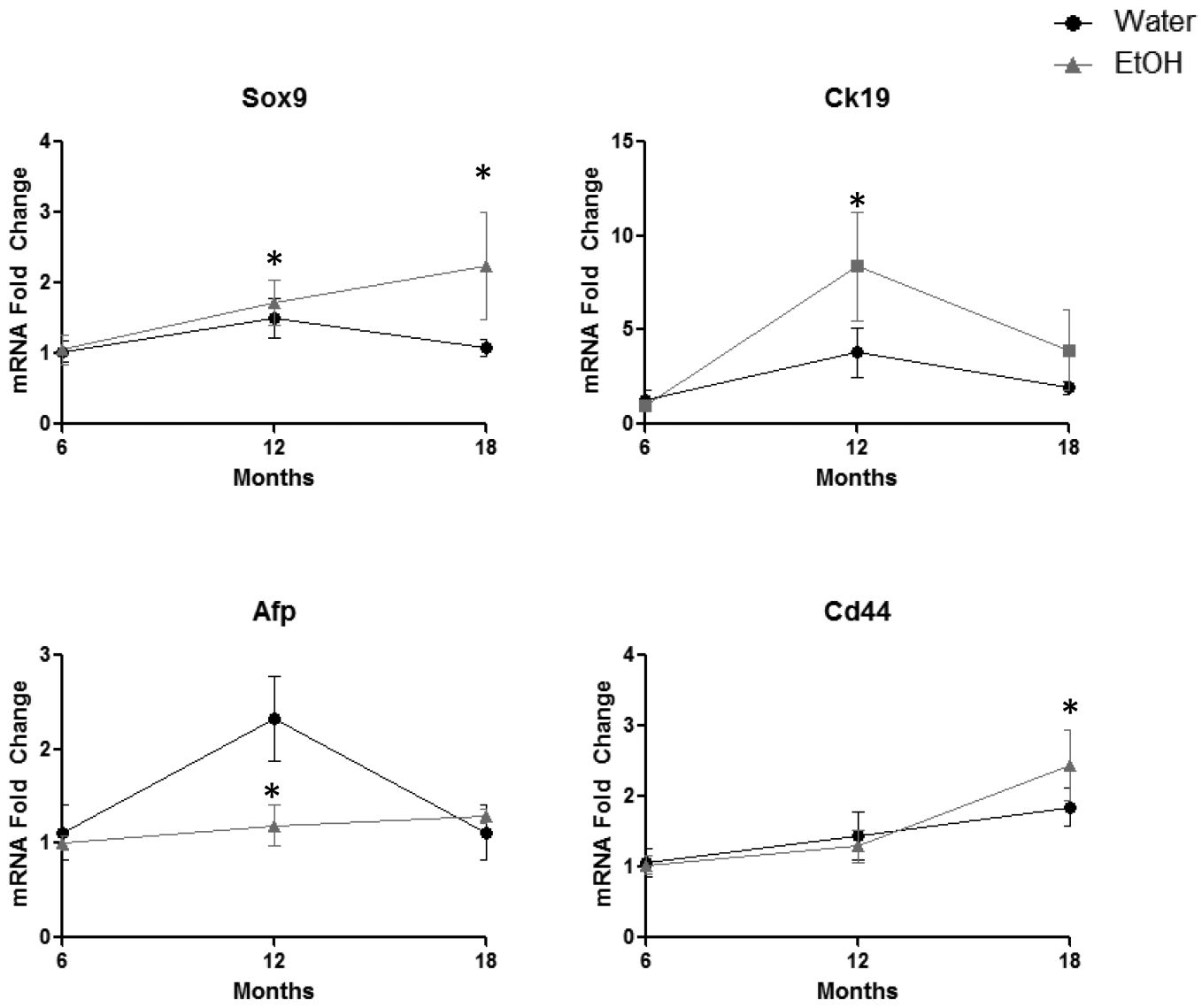
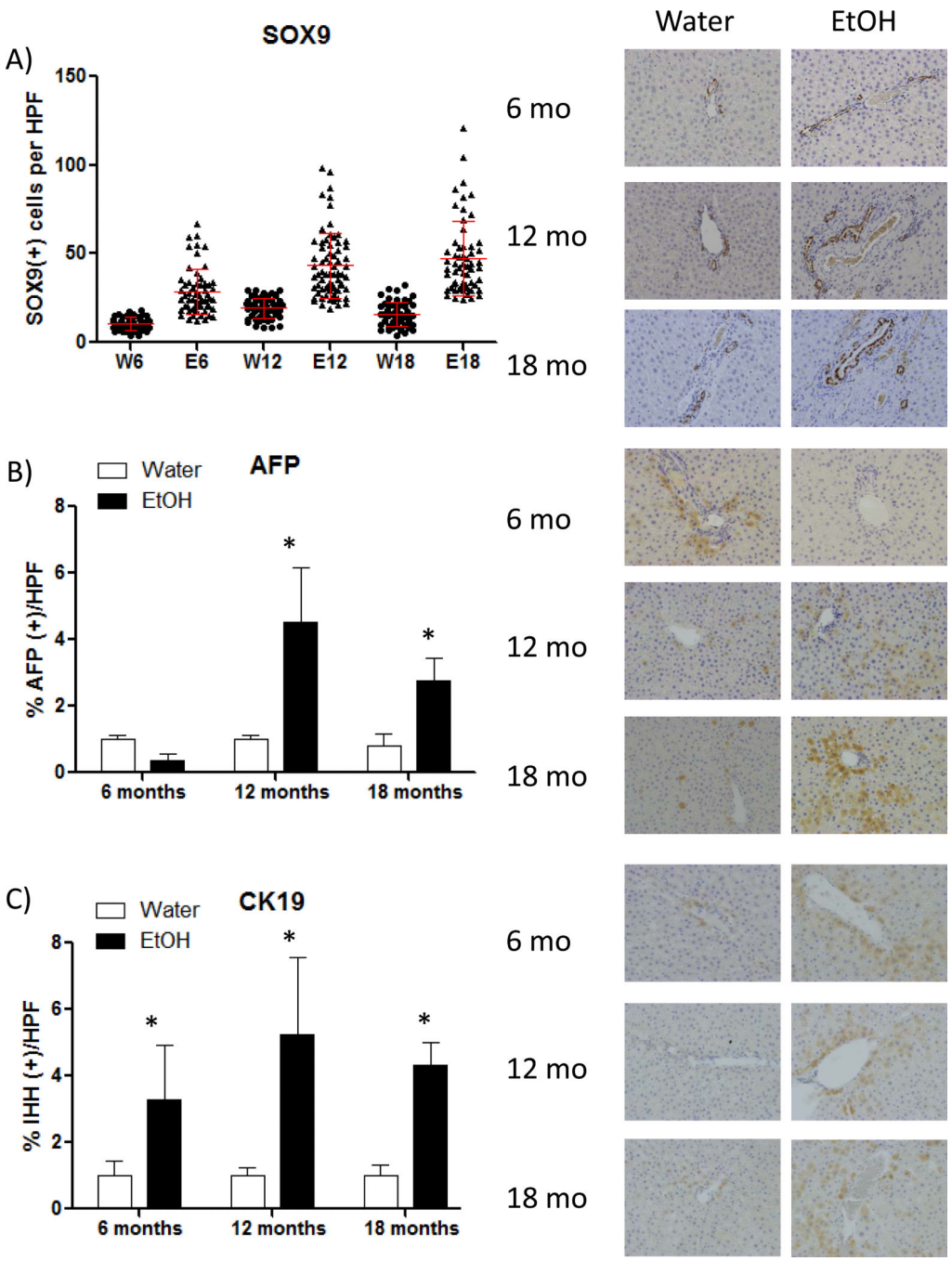


Fig. 4. Alcohol drinking induces hepatic expression of progenitor-associated genes

Rats were randomized to drink either water (control, black) or alcohol (gray) at 6 months of age. Livers were harvested at 6, 12, or 18 months later and evaluated by quantitative real-time PCR for mRNA expression of the following liver progenitor markers: A)SOX9, B) cytokeratin 19 (CK19), C) alpha fetoprotein (AFP), and D) CD44. Results at 12 and 18 months were normalized to the respective 6 months values within each group. Mean ± SEM at each time point is graphed for each group. *$p<0.05$ versus gene expression at 6 months.



D)

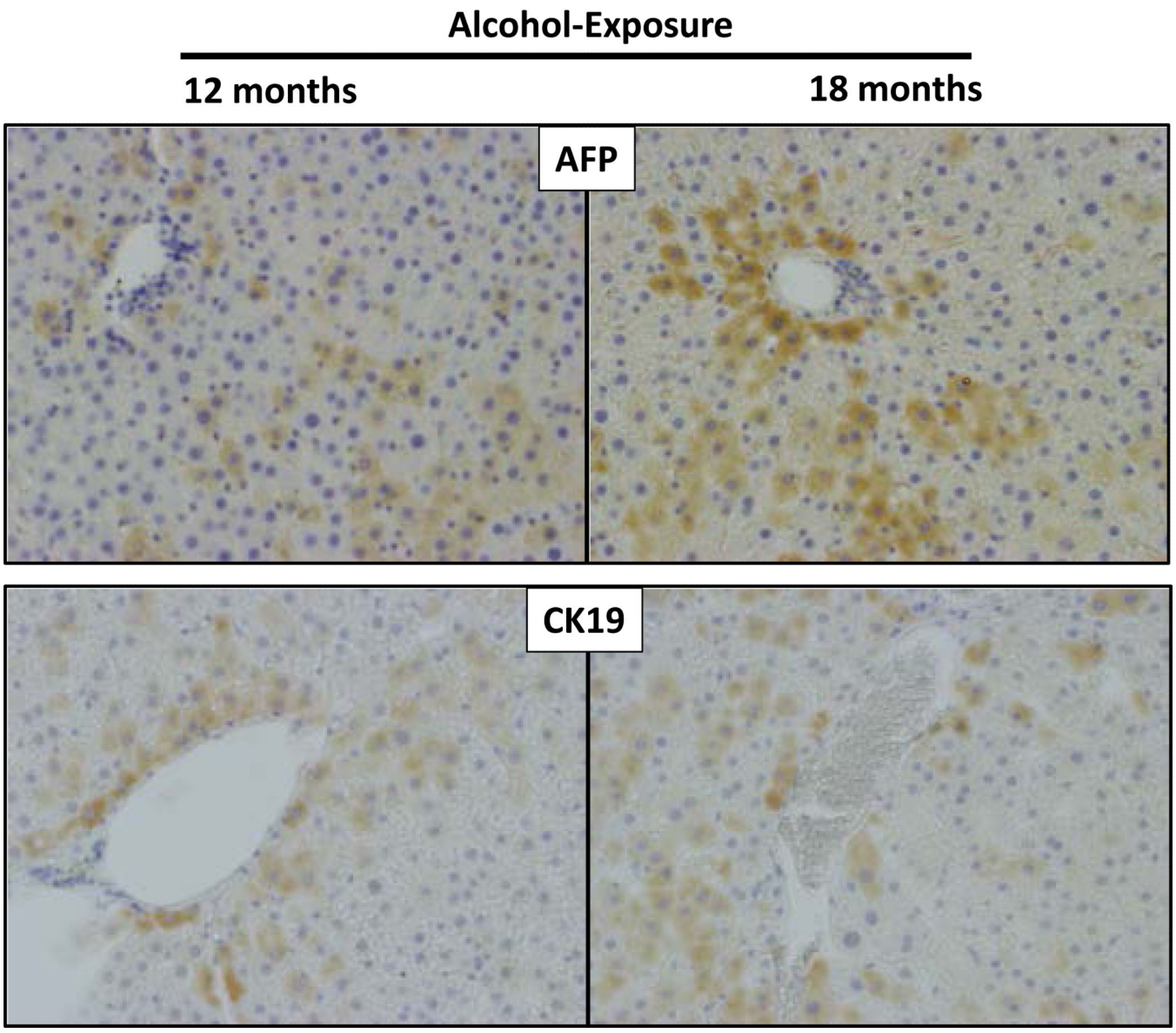


Fig. 5. Drinking alcohol influences hepatic progenitor populations

Livers were harvested from rats after 6, 12, or 18 months of drinking either water (control) or alcohol. Sections were immunostained for SOX9, AFP, and CK19 and representative images are shown. Alcohol drinking increased expression of **A)** SOX9, **B)** AFP, and **C)** CK19. Quantitative data are graphed as A) mean ± SD, B) mean ± SEM, *p<0.05 and C) mean ± SEM, *p<0.05. **D**) Both AFP and CK19 appear in hepatocytic parenchymal cells following both 12 and 18 months of alcohol exposure. HPF, high power field.

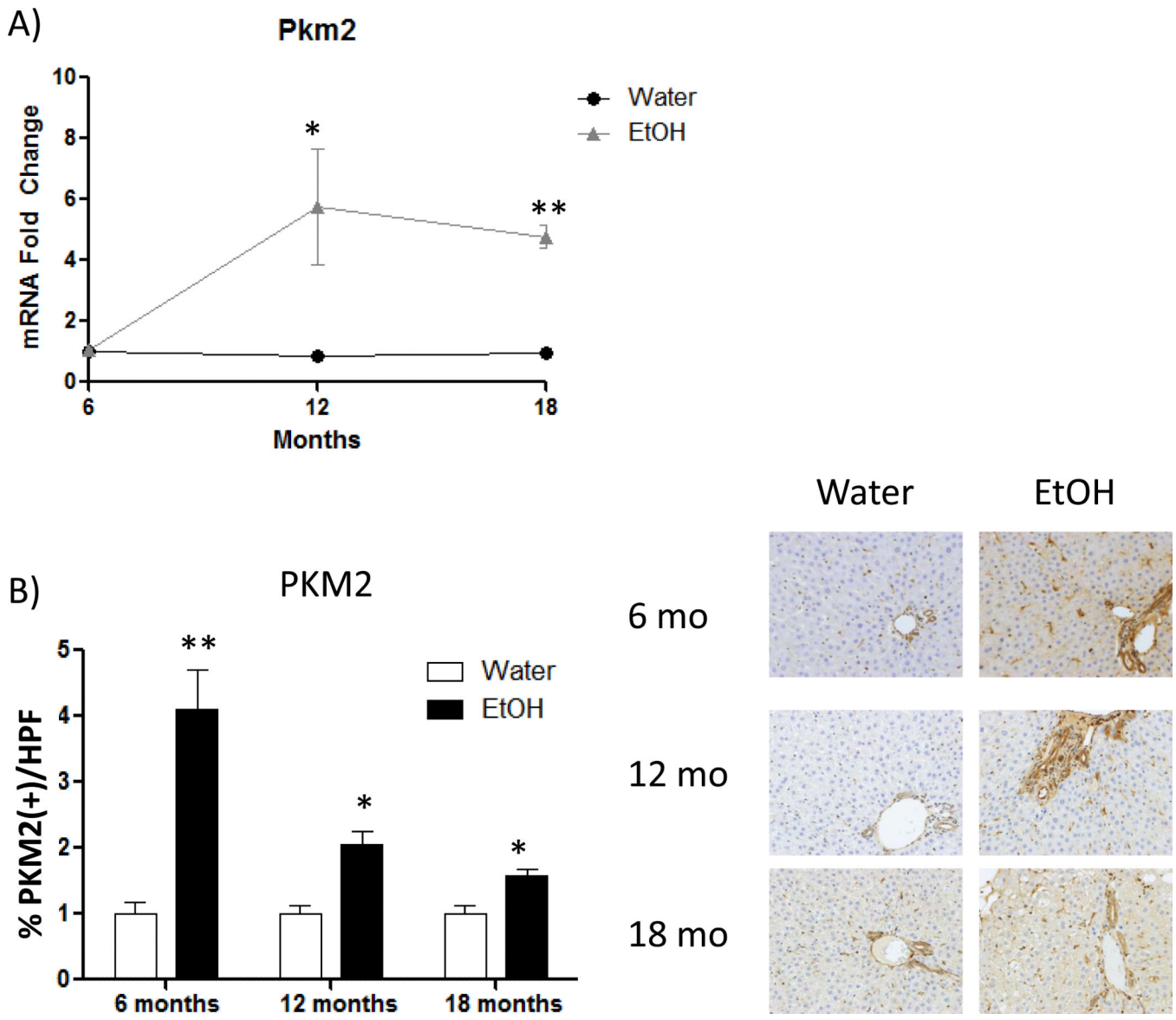


Fig. 6. Alcohol drinking promotes accumulation of glycolytic liver stromal-ductular cells

Rats were randomized to drink either water (black) or alcohol (gray). Livers were harvested 6, 12, or 18 months later and evaluated by quantitative real-time PCR for mRNA expression of Pkm2, a marker of glycolytic activity **(A).** Results at 12 and 18 months were normalized to the respective 6-month values in each group. Mean ± SEM at each time point is graphed for each group, *p<0.05 versus gene expression at 6 months. **B)** Liver sections were also immunostained for PKM2. Representative images are shown and mean ± SEM data are graphed. *p<0.05, **p<0.01 versus respective water-drinking control group. HPF, high power field.

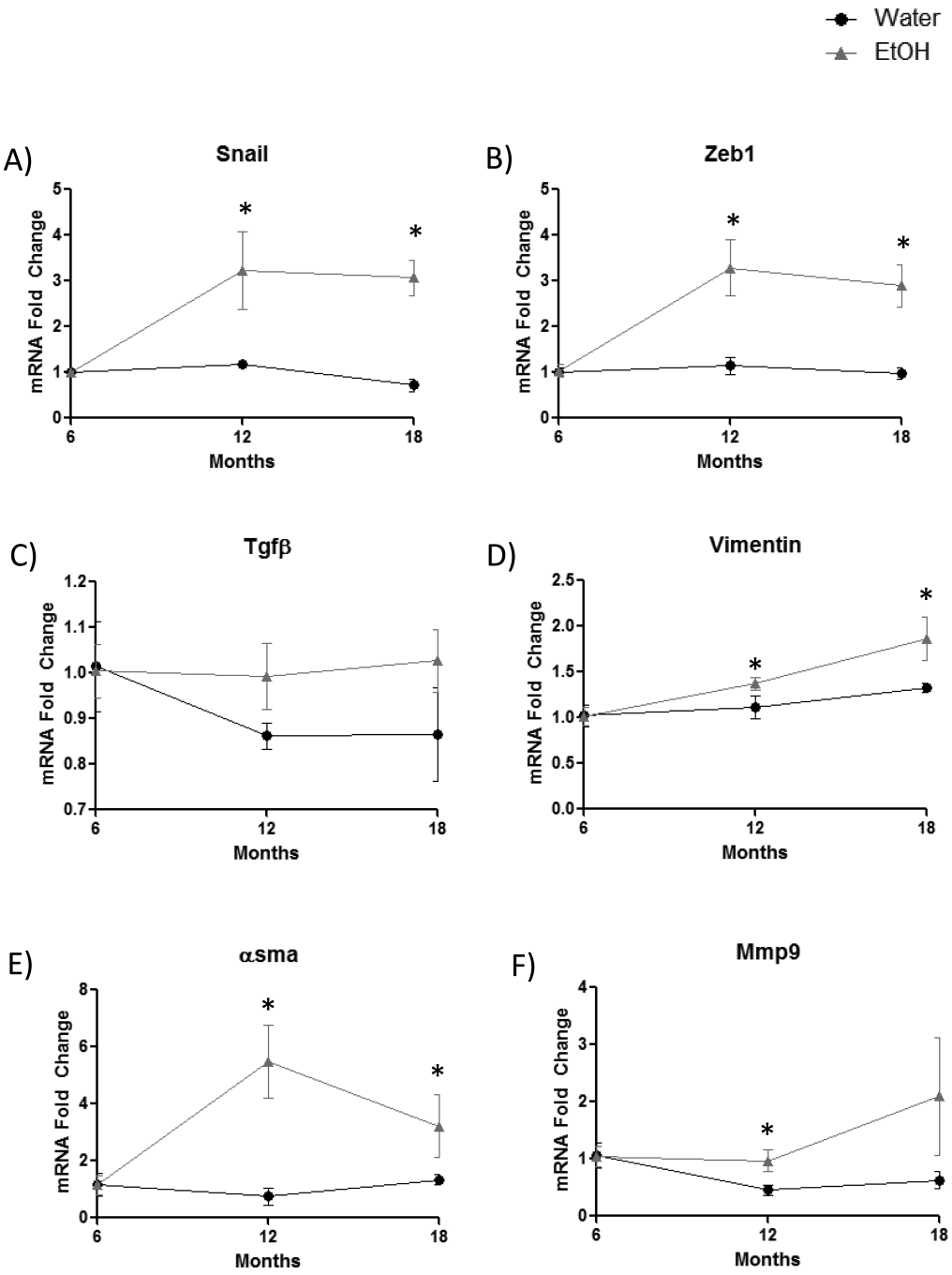


Fig. 7. Drinking alcohol promotes hepatic epithelial-to-mesenchymal transitions (EMT)

Rats were randomized to drink either water (black) or alcohol (gray). Livers were harvested 6, 12, or 18 months later and evaluated by quantitative real-time PCR for mRNA expression of genes related to EMT: **A)** Snail, **B)** Zeb-1, **C)** TGFβ, **D)** Vimentin, **E)** alpha smooth muscle actin (αSMA) and F) matrix metalloproteinase-9 (MMP9). Results at 12 and 18 months were normalized to the respective 6-month values in each group. Mean ± SEM at each time point is graphed for each group. *$p<0.05$ versus gene expression at 6 months.

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Table 1

Histological Evaluation of Alcohol-Fed Rats Versus Water-Fed Rats

Rat ages and feeding group listed. A=alcohol, W=water. N=4/age/group

Feeding group	number of tumors	size of tumors (mm)	% macro fat non-tumor	microfat present non-tumor	megamitochondria present non-tumor	% degen non-tumor	% tumor with fat	% tumor with degen
6W1	0	na	0	No	no	0	na	na
6W2	0	na	0	No	no	0	na	na
6W3	0	na	0	No	no	0	na	na
6W4	0	na	0	No	no	0	na	na
6A1	0	na	<5	No	no	0	na	na
6A2	0	na	5	No	no	10	na	na
6A3	0	na	5	No	no	15	na	na
6A4	0	na	5	No	no	15	na	a
12W1	0	na	0	No	no	0	na	na
12W2	0	na	0	No	no	0	na	na
12W3	0	na	5	No	no	0	na	na
12W4	0	na	5	No	no	5	na	na
12A1	0	na	<5	No	no	60	na	na
12A2	1	4	<5	No	no	60	<5	90
12A3	0	na	<5	No	no	60	na	na
12A4	0	na	<5	No	no	70	na	na
18W1	0	na	5	No	no	10	na	na
18W2	0	na	0	No	no	5	na	na
18W3	0	na	0	No	no	5	na	na
18W4	0	na	0	No	no	5	na	na
18A1	1	1	5	No	no	15	0	100
18A2	7	1, 1, 2, 2, 2, 4, 7	5	Yes	yes	40	40	60
18A3	2	3, 5	<5	No	no	60	5	60
18A4	5	1, 2, 3, 3, 8	<5	No	No	5	50	50