



HHS Public Access

Author manuscript

Gene. Author manuscript; available in PMC 2018 August 30.

Published in final edited form as:

Gene. 2017 August 30; 626: 309–318. doi:10.1016/j.gene.2017.05.048.

Exploring a common mechanism of alcohol-induced deregulation of RNA Pol III genes in liver and breast cells

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Abstract

Alcohol intake is associated with numbers of different human cancers, such as hepatocellular carcinoma (HCC) and breast cancer. However, the molecular mechanism remains to be elucidated. RNA polymerase III-dependent genes (Pol III genes) deregulation elevates cellular production of tRNAs and 5S rRNA, resulting in an increase in translational capacity, which promote cell transformation and tumor formation. To explore a common mechanism of alcohol-associated human cancers, we have comparably analyzed that alcohol causes deregulation of Pol III genes in liver and breast cells. Our results reveal that alcohol enhances RNA Pol III gene transcription in both liver and breast cells. The induction of Pol III genes caused by alcohol in ER+ breast cancer lines or liver tumor lines are significantly higher than in their non-tumor cell lines. Alcohol increases cellular levels of Brf1 mRNA and protein, which is key transcription factor and specifically regulate Pol III gene activity. Alcohol activates JNK1 to upregulate transcription of

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#These authors have contributed equally at the present study.

Declaration of interest

The authors declare that they have no conflict of interest.

Author Contributions

GS, ZW, NX, and SZ involved in conception and design and in analysis and interpretation of data. GS, SZ and NX involved in data acquisition and in writing and reviewing of the manuscript. YY, CH, YZ and JL involved in development of methodology. SC, ST, YZ, YY provided administrative, technical, or material support. SZ and GS supervised the study. All authors read and approved the final manuscript.

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Brf1 and Pol III genes, whereas inhibition of JNK1 by SP600125 or its siRNA significantly decreases the induction of these genes. Furthermore, alcohol increases the rates of transformation of liver and breast cells, repressed JNK1 and Brf1 expression decrease transcription of Pol III genes and reduce the rates of colony formation of AML-12 and MCF-10 cells. Together, these studies support the idea that alcohol induces deregulation of Brf1 and RNA Pol III genes in liver and breast cells, which share a common signaling pathway to promote cell transformation. Through the common mechanism, alcohol-induced deregulation of RNA Pol III genes brings about greater phenotypic changes.

Keywords

Alcohol; Brf1; Pol III genes; HCC; breast cancer; transformation

1. Introduction

Alcohol has been classified as carcinogen to humans (IARC, 2011; Coglianani *et al.*, 2011; Shi *et al.*, 2015). Targeted organs of alcohol-associated cancers include the breast, liver, stomach, pancreas, oral cavity, pharynx, esophagus, larynx, colon and ovary (Shi *et al.*, 2015). Why alcohol is able to promoted cancer development in the different organs? Is there a common mechanism, which mediates this process? The questions remain to be studied. Cancer cells have a consistent cytological feature, nucleolar hypertrophy, where RNA polymerase III-dependent genes (Pol III genes) are transcribed. This feature provides possibility to explore the mechanism of alcohol-associated human cancers by determining the effect of alcohol on Brf1 (TFIIB-related factor 1) and Pol III genes (Shi *et al.*, 2015). To investigate alcohol-induced deregulation of Brf1 and Pol III genes, we focus the studies on alcohol-associated hepatocellular carcinoma (HCC) and breast cancer in present study. Uncovering the underlying molecular mechanisms could lead to improving therapeutic approaches and preventative.

RNA Pol III transcribes a variety of untranslated RNAs, including tRNAs, 5S rRNAs, 7SL RNA, 7SK RNA and U6 RNA (Dieci *et al.*, 2007; Ulu *et al.*, 1984; Zhang *et al.*, 2013), while tRNAs and 5S rRNAs control the translational and growth capacity of cells (Raha *et al.*, 2010; White, 2001; Zhang *et al.*, 2013). Oncogenic proteins (c-Myc, c-Jun, c-Fos) stimulate RNA Pol III gene transcription (Goodfellow *et al.*, 2006; Johnson *et al.*, 2008; Zhong *et al.*, 2004, 2007); whereas tumor suppressors (BRCA1, PTEN, pRb, p53) inhibits transcription of this class of genes (Johnson *et al.*, 2008A; White, 2001; Woiwode *et al.*, 2008; Zhong *et al.*, 2015). Studies have revealed that RNA Pol III transcription products are increased in both transformed and tumor cells, suggesting that they play an important role in tumorigenesis (Johnson *et al.*, 2008; Winter *et al.*, 2000; Zhong *et al.*, 2015). Consistent with this idea, elevated Pol III transcription is required for cell transformation and tumor development (Johnson *et al.*, 2008A; Zhang *et al.*, 2013). The capacity of these oncogenes and tumor suppressor genes to alter Pol III transcription results from their ability to regulate the TFIIB complex, which is composed of Brf1, Bdp1 and TBP (TATA box-binding protein). Brf1 and Bdp1 specifically regulate the transcription of Pol III genes (Zhang *et al.*, 2013; Zhong *et al.*, 2013A).

Alcohol is the dietary factor which is most consistently associated with risk of cancers (Hamajima *et al.*, 2002; Macmahom B, 2006; Petri *et al.*, 2004; Singlwtary KW and Gaspstur S., 2001). Alcohol induces liver injury, including liver steatosis and fibrosis, promotes liver cirrhosis, thereby increasing the risk of development of HCC (Lieber, 2000; Zhong *et al.*, 2011). Alcohol in combination with viruses (hepatitis C or B), carcinogens (aflatoxin), obesity, or diabetes mellitus, promotes liver cancer development (Bagnardi *et al.*, 2001; Seitz *et al.*, 1998; Zhong *et al.*, 2013A; Yuan *et al.*, 2004). Emerging evidence indicates that alcohol intake is associated with breast cancer (Zhang *et al.*, 2013). This association involves the estrogen receptor (ER), which is over-expressed (ER+) in approximately 80% of breast cancer cases (Deandrea *et al.*, 2008). Alcohol is known to promote mammary tumorigenesis (Singletary *et al.*, 1991, 1995; Watabiki *et al.*, 2000). A cytological feature, nucleolar hypertrophy, has been used as a strong diagnostic indicator of cell transformation and neoplasia. This indicates that transformation *in situ* is tightly linked to the deregulation of RNA Pol I and III gene transcription, because the size of the nucleolus reflects the levels of rRNA synthesis (White R, 2001; Zhang *et al.*, 2013). Although alcohol-associated human cancers were widely studied, the molecular mechanism remains to be elucidated (Zhang *et al.*, 2013).

Our recent studies using both cell culture model and animal models have revealed that alcohol induces transcription of tRNA^{Leu} and 5S rRNA (Zhang *et al.*, 2013; Zhong *et al.*, 2011). This induction in mice fed with ethanol is associated with liver tumor development (Machida *et al.*, 2009; Zhong *et al.*, 2011). This implies that alcohol-induced deregulation of Pol III genes may play critical role in tumor development (Zhong *et al.*, 2011). However, very little is known about the comparison study of alcohol-induced deregulation of Pol III genes and transformation in different human cancer cells. To comparably analyze the role of alcohol in this phenomenon, we treated liver and breast cells with ethanol. Our results indicate that ethanol increases tRNA and 5S rRNA transcription in both liver and breast cancer cells. The ER+ breast cancer cells is more sensitive to ethanol than liver cancer cells. Ethanol induces activation of JNK1, which mediates Pol III gene transcription. Further analysis reveals that inhibition of JNK1 by a chemical inhibitor (SP 600125) or JNK1 siRNA reduces Brf1 expression and Pol III gene transcription. Repressing Brf1 decreases alcohol-enhanced Pol III gene transcription, resulting in repression of alcohol-induced transformation of liver and breast cells. These studies support this idea that alcohol activates JNK1 to upregulate Brf1 expression and Pol III gene transcription, leading to transformation of liver and breast cells, which is a common mechanism of alcohol-promoted transformation of different cells. These novel findings will be of great interest both to the basic and clinical research communities and provide a potential approach of treatment for alcohol-associated cancer patients (Zhang *et al.*, 2013).

2. Materials and methods

2.1. Cell lines, reagents and antibodies

Mouse hepatocyte line, AML-12 cell, which is a line with transgenic for human TGF- α ; ER- human breast non-tumorigenic epithelial cell line (MCF-10A), ER+ human breast cancer cell lines (MCF-7 and T47D) were from ATCC. HepG2-ADH (alcohol

dehydrogenase) was provided by Dr. DL Clemens (Nebraska University). Cell culture medium DMEM, DMEM/F12, Lipofectin reagent, Lipofectamine 2000, TRIzol reagent and OPTI-MEM were from Life Technologies. Antibodies against c-Jun, JNK1 (46kD) and β -actin were obtained from Santa Cruz Biotech. Brf1 (90kD) antibody was from Bethyl laboratories Inc. JNK and phosphor-JNK antibodies were from Cell Signaling. JNK inhibitor, SP600125 was from A.G. Scientific, Inc. The sequences of siRNAs of Brf1 and primers of Brf1, tRNA^{Leu} and 5S rRNA were listed in supplements. EGF and E2 (17 β -estradiol) was from Sigma-Aldrich.

2.2. SDS-PAGE and immunoblot analysis

Human liver or breast cells were incubated with 50mM or 25mM ethanol for 60 min after starvation 3h. Cells were collected with lysis buffer and sonicated. The suspensions were centrifuged to save the supernatants. Protein concentrations were determined by the Bradford method using Fluostar Omega spectrometer (Cell Biology Core Laboratory of University of Southern California Research Center for Liver Diseases, P30DK DK048522). Lysates (50 μ g of protein) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred from the SDS-PAGE gel to Hybond-P membrane and immunoblot analysis were performed with specific antibodies (Sun *et al.*, 2016A; Song and Yin, 2016). Membranes were probed with these antibodies against Brf1, JNKs, phosphor-JNK and β -actin as described (Zhong *et al.*, 2004; Zhong *et al.*, 2013A). Bound primary antibody was visualized using horseradish peroxidase-conjugated secondary antibody (Vector Laboratories) and enhancing chemiluminescence reagents (Santa Cruz Biotech).

2.3. RNA isolation and RT-qPCR

Total RNA was isolated from liver and breast cells treated with ethanol using single step extraction method TRIzol reagent (Life Technologies). Total RNA samples were quantified and reverse-transcribed in a 20 μ l reaction containing 1 x RT (reverse transcription) buffer. After first-strand cDNA synthesis, the cDNAs were diluted in DNase-free water and real time qPCR (RT-qPCR) were performed with specific primers (Table S2) and PCR reagent kits (Bio-Rad Biotech) in the ABI prism 7700 Sequence Detection System (Zhou *et al.*, 2016). Precursor of tRNA^{Leu} and 5S rRNA transcripts and mRNAs of Brf1 were measured by RT-qPCR as described previously (Zhong *et al.*, 2013B, 2016).

2.4. Transfection with siRNA and expression construct

For transient transfection assays, cells were transfected with plasmids of JNK1 expression or siRNAs of JNK1 and Brf1 as described previously (Fang *et al.*, 2016; Sun *et al.*, 2016B–C). Serum-free medium was added to each dish with Lipofectin-DNA or Lipofectamine 2000-siRNA complexes, and cells were further incubated for 4h. The medium was changed with 10% FBS DMEM or DMEM/F12 (phenol red-free) (Zhong *et al.*, 2013A) and cells were incubated for 48h before harvesting. Cells were starved in DMEM for 3 h after rising twice with 1X PBS and treated with 50mM or 25mM ethanol for 60min to collect cell lysates or total RNA. Protein concentrations of the resultant lysates were measured by the Bradford method.

2.5. Cell Anchorage-independent growth

AML-12 cells and MCF-10A cells were transfected with mismatch RNA (siMM), JNK1 or Brf1 siRNAs (Table S1) as described (Zhang *et al.*, 2011; Zhong *et al.*, 2016). The transfected AML-12 cells or MCF-10A cells (1×10^4 cells/well in 6well plate) were suspended in 0.35% (w/v) agar in 10% FBS/DMEM/F12. AML-12 cells were treated with 20ng EGF with or without 50mM ethanol or both EGF and ethanol. MCF-10A cells were treated with or without 5nM E2, 25mM ethanol or both E2 and ethanol over a bottom layer of media with 0.5% (w/v) agar. Cells were fed fresh complete media with EGF or/and ethanol, or E2 or/and ethanol twice weekly. Colonies were counted 3 weeks or longer after plating as previously described (Zhang *et al.*, 2011; Zhong *et al.*, 2014, 2016).

3. Results

3.1. Alcohol induces expression of Brf1 and Pol III genes of liver and breast cells

To determine the effect of alcohol on transcription of Pol III genes, non-tumor liver cell line AML-12 and breast cell line MCF-10A were exposed to ethanol. The cellular levels of precursor tRNA^{Leu} and 5S rRNA were tested by RT-qPCR. We performed the time curve and dose-dependent curve of ethanol-induced transcription of Pol III genes. We found that ethanol-caused Pol III gene transcription can reach high levels after 60min ethanol treatment (**data not shown**). Ethanol treatment of both AML12 and MCF-10A cells creates a dose-dependent increase in transcription of pre-tRNA^{Leu} and 5S rRNA genes (Zhang *et al.*, 2013), where ethanol-caused maximum induction of Pol III genes was observed at 50mM ethanol for 60min of liver cells (Fig. 1A), whereas high peak of this induction in breast cells is 25mM (Fig. 1B). Therefore, we have used the conditions for the whole study. This results display that ethanol-caused induction of Pol III genes in breast cells is higher than in liver cells (Fig. 1). We further determined difference in cancer cell lines and non-tumor cells. Alcohol enhances transcription of pre-tRNA^{Leu} and 5S rRNA of liver cancer cells of HepG2-ADH and TSCML (tumor stem cells of mouse liver) (Fig. 2C,D) markedly higher than both non-tumor cells, AML-12 and primary mouse hepatocytes (PMH) (Fig 2A,B). Whereas ethanol-increased transcription of Pol III genes in ER+ breast cancer cell lines, MCF-7 and T47D (Fig 2G,H) is dramatically more than in ER- non-tumor breast cells, MCF-10A and MCF-12A (Fig. 2E,F). More interestingly, the inductions of pre-tRNA^{Leu} and 5S rRNA by ethanol in ER- breast cancer cells are similar to MCF-10A and MCF-12A (data not shown). These results indicate that ethanol-induced transcription of Pol III genes in cancer cells, such as liver HepG2-ADH and TSCML tumor cells, and ER+ breast MCF-7 and T47D cancer cells, is more than non-tumor liver and breast cells. This suggests that alcohol-caused transcription of Pol III genes is associated with oncogenic status (Zhong *et al.*, 2015).

Brf1 is a subunit of TFIIIB, which specifically regulates tRNA and 5S rRNA transcription (Zhang *et al.*, 2013). Our recent studies reveal that Brf1 is overexpression in human biopsies of HCC patients, and the patients of HCC with high expression of Brf1 show shorter period of overall survival (Zhong *et al.*, 2016). Further studies indicate that Brf1 expression is also increases in human tumor foci of breast cancer (data not shown). Above studies show that alcohol is able to enhance Pol III gene transcription. Therefore, we further investigate

whether alcohol affects Brf1 expression in liver and breast cell. The results indicate that ethanol treatments of either HepG2-ADH (Fig. 3A) or MCF-7 cells (Fig. 3B) increase the Brf1 protein and mRNA levels.

3.2. Signal events of alcohol-induced cellular response which mediates Pol III gene transcription

Since ethanol has been shown to induce JNK activation (Luedemann *et al.*, 2005; Zhang *et al.*, 2013) and the JNKs modulates Pol III gene transcription, we have determined whether JNKs mediates ethanol-induced Pol III gene transcription (Zhong *et al.*, 2007; 2009). Ethanol strongly activates JNK1, but a weaker JNK2, in the HepG2-ADH cells (Fig. 4A). Therefore, we further assess whether ethanol induces activation of JNKs in breast cells. The results indicate that ethanol dramatically induced activation of JNK1 in MCF-7 cells (Fig. 4B). Our previous studies have identified that JNK1 and JNK2 have different function in Pol III gene transcription (Zhong *et al.*, 2007; 2009). JNK1 positively, but JNK2 negatively, modulates transcription of Pol III genes (Zhong *et al.*, 2007). This implies that alcohol-activated JNK1 may play a critical role in Pol III gene activity and cell transformation. Next, we investigate whether JNK1 affects Pol III gene transcription induced by alcohol. HepG2-ADH cells and MCF-7 cells were treated with 5 μ M SP600125, a JNK chemical inhibitor for one hour (Zhang *et al.*, 2013). Hereafter, the cells were exposed to ethanol for another one hour. Fig. 5A–D (**left panel**) indicate that this JNK inhibitor significantly decreases transcription of pre-tRNA^{Leu} and 5S rRNA induced by alcohol. Then, we transfected HepG2-ADH and MCF-7 cells with JNK1 siRNA to determine alteration of transcription of Pol III genes. The results reveal that JNK1 siRNA specifically represses JNK1 expression (Fig. 4C–D), whereas JNK1 siRNA dramatically reduces alcohol-induced transcription of Pol III genes (Fig. 5A–D, **Middle panel**). In contrast, we found that increasing JNK1 expression by JNK1 expressing construct elevates alcohol-induced Pol III gene transcription in both HepG2-ADH cells and MCF-7 cells (Fig. 5A–D, **Right panel**). These studies demonstrate that alcohol-caused JNK1 activation indeed mediates transcription of Pol III genes.

3.3. Reduction of Brf1 expression represses cell transformation

As mentioned above that Brf1 overexpression was in human HCC cases (Zhong *et al.*, 2016), we determine whether alcohol-induced activation of JNK1 mediates Brf1 expression. HepG2-ADH cells were transfected with JNK1 and Brf1 siRNA, respectively and Brf1 alteration was determined. The results indicate that repressing JNK1 or Brf1 expression by their siRNA results in reduction of the cellular levels of Brf1 protein (Fig 6A). Then, we transfected HepG2-ADH and MCF-7 cells with Brf1 siRNA to further determine the alteration of Pol III genes. Fig. 6B–C show that repression of Brf1 by its siRNA decrease pre-tRNA^{Leu} (**Left panel**) 5S rRNA transcription (**Right panel**). Previous studies demonstrated that repression of Brf1 was sufficient for inhibition of cell transformation (Johnson S and Johnson D, 2008). Induction of Brf1 expression allowed anchorage-independent colonies to form and promoted tumor formation (Zhang *et al.*, 2011, 2013; Zhong *et al.*, 2013B, 2016). To further assess potential alterations of the rate of cell transformation by alcohol-increased Brf1 expression, we carried out soft agar assay to determine cell anchorage-independent growth. We seeded AML-12 cells in soft agar and

treated the cells with EGF or EGF plus ethanol, or seeded MCF-10A cells in soft agar and treated the cells with E2 or E2 plus ethanol. The results indicate that ethanol at the concentrations alone hardly induce transformation of AML-12 cells (Fig. 7A) and MCF-10A cells (Fig. 7B), whereas EGF with ethanol or E2 with ethanol significantly enhance the rates of transformation of AML-12 cell of MCF-10A cells (Fig. 7). Given that Brf1 siRNA or JNK1 siRNA decreased cellular levels of Brf1 mRNA (Fig. 3 and 6) (Zhang *et al.*, 2013), as well as the levels of Pol III genes (Fig. 5 and 6), repression of Brf1 and JNK1 by their siRNA markedly decreased ethanol-induced colony formation of these cells, compared to control RNA (Fig. 7A–B). These results demonstrate that alcohol activates JNK1 to increase cellular level of Brf1 and enhance transcription of Pre-tRNA^{Leu} and 5S rRNA, thereby promoting alcohol-induced transformation of liver and breast cells.

4. Discussion

Our studies have shown a comparable analysis, which characterizes how alcohol mediates the transcription of endogenous Pol III genes in both liver and breast cells. The ER⁺ breast cells is more sensitive to alcohol than liver cells. The results have indicated that ethanol induces activation of JNK1 of HepG2 and MCF-7 cells. Inhibiting JNK1 by an inhibitor, SP600125 or its siRNA decreases the expression of Brf1 and Pol III genes caused by ethanol, whereas enhancement of JNK1 by its expressing construct augments alcohol-induced transcription of Pol III genes of both liver and breast cells. These studies demonstrate that ethanol induces expression of Brf1 and RNA Pol III genes through the JNK1 pathway. Repression of JNK1 or Brf1 is sufficient to inhibit ethanol-induced anchorage-independent growth of AML-12 cells and MCF-10A cells. Therefore, the comparable analysis reveals a common mechanism, by which ethanol activates JNK1 to increase transcription of Brf1 and Pol III genes and to promote cell transformation, resulting in alteration of cellular phenotype (Fig. 8).

Alcohol-caused liver injury, including liver steatosis and fibrosis, promotes liver cirrhosis, thereby increasing the risk of hepatocellular carcinoma (HCC) development (Hamajima *et al.*, 2002; Shi *et al.*, 2015; Zhong *et al.*, 2013A). Alcohol combining with viruses (hepatitis C or B), carcinogens (aflatoxin), obesity, or diabetes mellitus, promotes liver cancer development (Suzuki *et al.*, 2008; Zhong *et al.*, 2013A). Alcohol intake has consistently been associated with an enhanced risk of breast cancer in both premenopausal and postmenopausal woman (Macmahon B, 2006; Petri *et al.*, 2004; Zhang *et al.*, 2013). Studies have indicated that alcohol enhanced MCP-1 and CRR2 expression, which promoted mammary tumor growth in alcohol-fed mice (Wang *et al.*, 2011). Epidemiologic studies showed that alcohol intake was associated with ER⁺ breast cancer cases more than to ER⁻ cases (Dumitrescu R & Shoelds P, 2005; Wang *et al.*, 2011; Zhang *et al.*, 2013). Ethanol increases ER α expression to promote breast tumor formation in mice (Wong *et al.*, 2012). Literatures reveal that alcohol intake is associated with human cancers in more than ten organs (Bagnardi *et al.*, 2001; Pudohit *et al.*, 2005; Zhang *et al.*, 2013). Why alcohol intake is related to different human cancers (Zhang *et al.*, 2013), this question remains to be elucidated. Nucleolar hypertrophy is a consistent cytological feature of cancer cells, where products of Pol III genes are synthesized (Shi *et al.*, 2015). The nucleolus size reflects the transcription level of Pol III genes. Thus, this cytological feature provides a possibility to

identify a common mechanism of alcohol-associated cancers. Our recent studies have demonstrated that alcohol-feeding mice enhances Pol III gene transcription to promote liver tumor formation (Zhong *et al.*, 2011). In present studies, these studies support the idea that alcohol indeed causes deregulation of Pol III genes to promote transformation of liver and breast cells (Zhong *et al.*, 2016; Zhang *et al.*, 2013).

Oncogenic proteins, c-Myc, cJun, c-Fos and Ras, stimulate Pol III gene transcription (Goodfellow *et al.*, 2006; Johnson D & Johnson S, 2008; White R, 2001; Woiwode *et al.*, 2008; Zhang *et al.*, 2013, Zhong *et al.*, 2004, 2007, 2015), whereas tumor suppressors, BRCA1, PTEN, p53 and pRb repress transcription of Pol III genes (Goodfellow *et al.*, 2006; Johnson D & Johnson S, 2008; White R, 2001; Woiwode *et al.*, 2008; Zhang *et al.*, 2013, Zhong *et al.*, 2004, 2007, 2015). The capacity of these oncogenes and tumor suppressor genes to regulate Pol III gene transcription result from their ability to modulate the TFIIB complex (Zhong *et al.*, 2013A, 2013B). The TFIIB complex includes TATA box-binding protein (TBP) and its associated factors, Brf1 and Bdp1 (Zhang *et al.*, 2013; Zhong *et al.*, 2013B). In the present studies, the results indicate that ethanol increases transcription of Brf1 and Pol III genes (Fig. 2,3). We have demonstrated that the cases of human HCC with alcohol consumption reveal higher expression of Brf1 and Pol III genes (Zhang *et al.*, 2013; Zhong *et al.*, 2011; 2016). The animal studies further support that alcohol feeding mice promotes liver tumor formation (Machida *et al.*, 2009; Zhong *et al.*, 2011). Our recent results indicate that overexpression of Brf1 was in human cases of ER+ breast cancer (data not shown). This shows that alcohol-increased Brf1 expression is critically important during tumor development. Furthermore, these studies reveal that ethanol enhances Brf1 expression and Pol III gene transcription in breast and liver cell lines and the induction of these genes in cancer cell lines are higher than in non-tumor cell lines (Fig 2). Whereas, reduction of Brf1 expression inhibits Pol III gene transcription (Fig. 6) and the rates of transformation (Fig. 7). As Brf1 expression is elevated in human HCC cases (Zhong *et al.*, 2016), high levels of Brf1 expression of HCC patients reveals shorter survival times (Zhong *et al.*, 2016). This suggests that the high level of Brf1 reflects the oncogenesis status of cells. Brf1 may be as a novel biomarker of cancers. Whereas alcohol-caused deregulation of Brf1 and Pol III genes are a common mechanism of alcohol-associated cancers.

Our studies have shown that JNK1 and JNK2 have different roles in Pol III gene transcription (Zhong *et al.*, 2007, 2009). JNK1 positively mediated transcription of these genes (Zhong *et al.*, 2007). In the present studies, the results show that alcohol induces JNK1 activation in both breast and liver cells (Fig. 4). Inhibiting JNK1 reduces the induction of Brf1 and Pol III genes caused by ethanol (Fig. 5). In contrast, enhancement of JNK1 expression increases alcohol-caused Brf1 and Pol III gene induction. Alcohol may go through other MAP kinase subfamily member, such as p38 to promote breast cancer progress (Sakurai *et al.*, 2006; Xu *et al.*, 2016). However, JNK1 knock out mice is able to repress liver tumor development (Kalra *et al.*, 2008; Naugker *et al.*, 2007). Therefore, alcohol induces JNK1 activation to promote cell transformation, which may be a key step of alcohol-associated tumor development (Fig. 8) (Shi and Zhong, 2016).

In summary, the studies provide evidences that ethanol induces JNK1 activation to enhance Brf1 expression and Pol III gene transcription to promote AML-12 and MCF-10A cell

transformation (Zhong *et al.*, 2013B, 2016). The novel findings suggest a common mechanism, which mediates alcohol consumption-associated human cancers. It implies that the possibility that repression of Brf1 may be as a potential approach to inhibit alcohol-induced cell transformation and tumor development (Zhang *et al.*, 2013; Shi & Zhong, 2016).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We want to thank Drs. M. R. Stallcup, Danial Levy and Neil Kaplowitz (University of Southern California) for scientific discussions. This work was supported by NIAAA/NIH grants AA017288, AA021114 and AA02324 to S.Z. and CNJ14C007 in China to Y.Y.

Abbreviation

HCC	Hepatocellular carcinoma
Pol III genes	RNA polymerase III-dependent genes
Brf1	TFIIB-related factor 1
ER	estrogen receptor

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Highlights

1. Alcohol induces deregulation of RNA Pol III genes in liver and breast cells;
2. The induction of Pol III genes by alcohol in breast cancer cells is more sensitive than liver cancer cells;
3. Alcohol-increased Pol III gene transcription is through MAP kinase, JNK1 pathway;
4. Alcohol upregulates Brf1 expression and Pol III gene transcription to promote cell transformation.

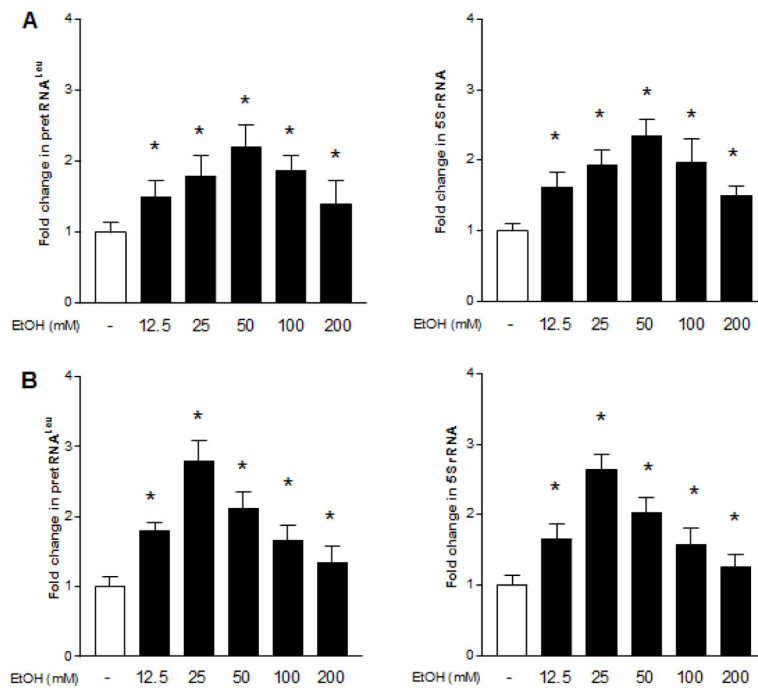


Fig. 1. Alcohol induces RNA Pol III-dependent transcription

AML-12 cells and MCF-10A cells were starved in FBS/DMEM-F12 for 3h. Cells were treated with or without different amounts of ethanol as indicated. RNAs were isolated from these cells and RT-qPCR was performed to measure the amounts of pre-tRNA^{Leu} (**A and B, left panel**), 5S rRNA (**A and B, right panel**). The fold change was calculated by normalizing to the amount of GAPDH mRNA. High peak of the induction of Pol III genes are in 50mM ethanol of Liver cells and at 25mM ethanol of breast cells. *: $P > 0.05$. The columns represent Mean \pm SE of at least three independent determinations.

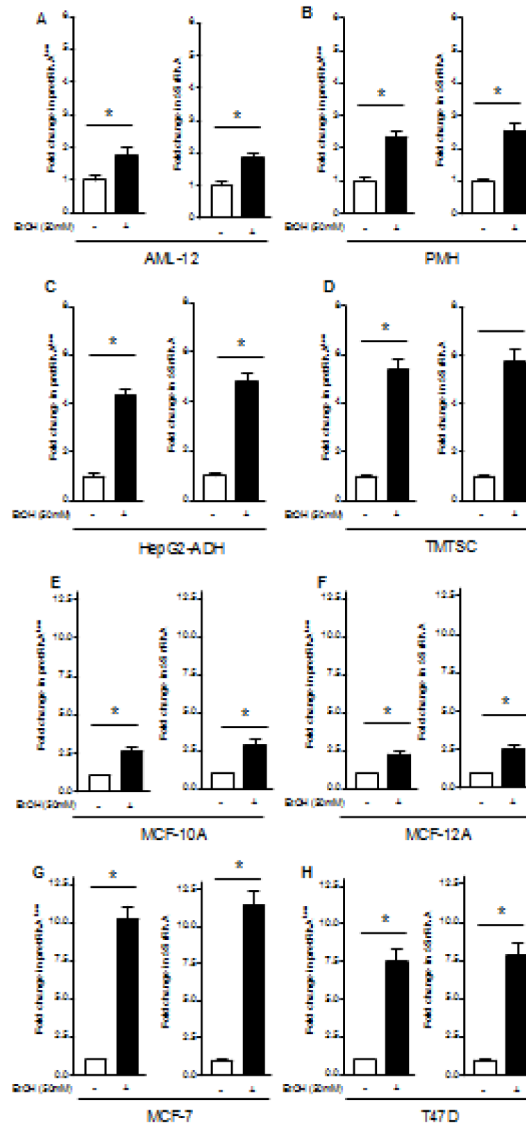


Fig. 2. Pol III gene transcription is increased by alcohol

(A–D): *Ethanol increases Pol III gene transcription in liver cells.* Non-tumor mouse liver line, AML-12 cells and PMH (primary mouse hepatocytes) (A–B), and liver tumor cells (C–D), HepG2 and TSCML (tumor stem cells of mouse liver) were grown to 85% confluency and starved in DMEM-F12 for 3 h and treated with 50mM ethanol for another hour. (E–H): *Ethanol induces Pol III gene transcription in breast cells.* Non-tumor breast cell lines (MCF-10A and MCF-12A) (E–F) and ER+ breast cancer cell lines (MCF-7 and T47D) (G–H) were treated with 25mM ethanol as described above. The total RNAs from these cells were extracted to measure pre-tRNA^{Leu}, 5S rRNA and GAPDH transcripts. The fold change was calculated by normalizing to the amount of GAPDH. *: $P > 0.05$. The columns represent Mean \pm SE of at least three independent determinations.

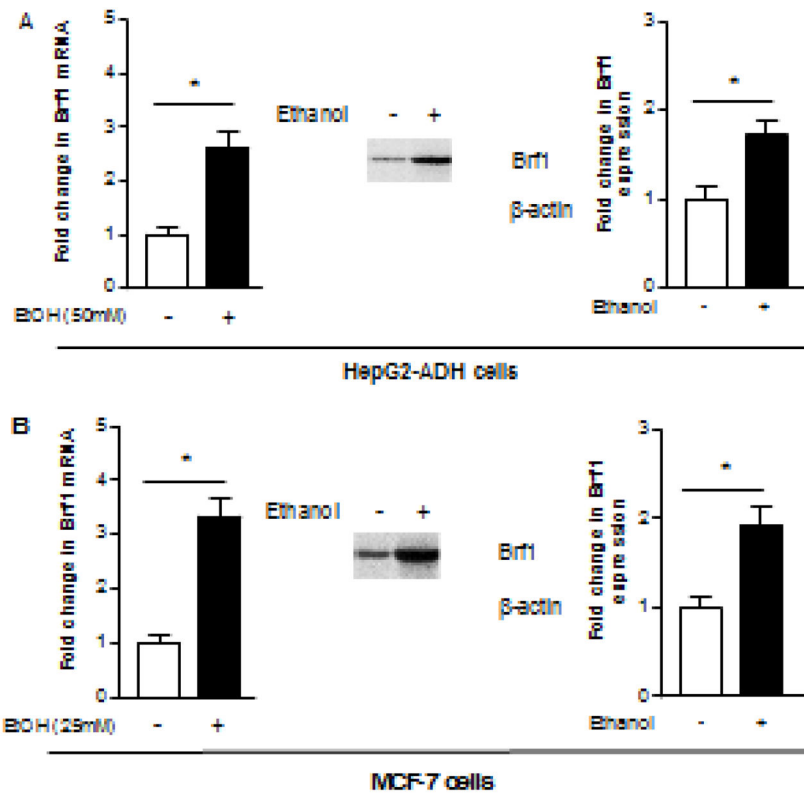


Fig. 3. Alcohol-mediated induction of Brf1 expression in liver and breast cells

HepG2-ADH cells (**A**) and MCF-7 cells (**B**) were treated with ethanol respectively as described above. Resultant cell lysates and total RNAs were isolated from these cells. Immunoblot analysis was performed with a specific antibody against Brf1 (90kD) to determine changes in cellular levels of Brf1 (**A and B, middle and right panel**). A representative blot from three independent determinations and quantitative analysis are shown (**middle and right**) The total RNAs from these cells were extracted to measure mRNA of Brf1 by RT-PCR (**A and B, left panel**). The fold change was calculated by normalizing to the amount of GAPDH. *: $P > 0.05$. The values represent mean \pm SE from three independent experiments.

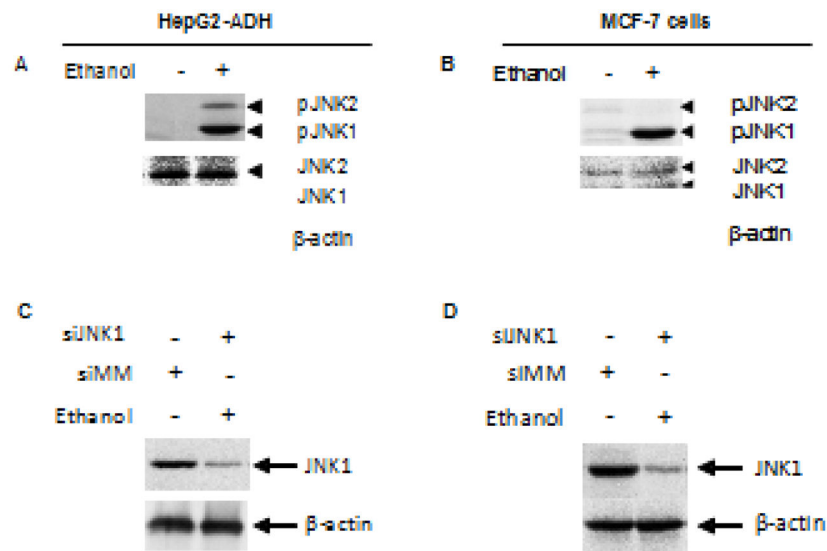


Fig. 4. Alcohol induces activation of JNK1

(A and B): Ethanol induces JNK1 activation. HepG2-ADH cells and MCF-7 cells were treated with or without ethanol as described above. Immunoblot analysis was performed using protein lysates derived from these cells and antibodies against phosphorylated JNK1/2 (46kD/54kD), JNK1/2 and β -actin as designated. **(C and D): JNK1 siRNA decreases cellular levels of JNK1.** HepG2-ADH cells and MCF-7 cells were transfected with mismatch RNA (siMM) and JNK1 siRNA (siJNK1) for 48 hours. The cell lysates were extracted from these cells to determine cellular levels of JNK1 and β actin (**up panel**) and quantitation analysis (**bottom panel**) as indicated. A representative blot from three independent determinations is shown.

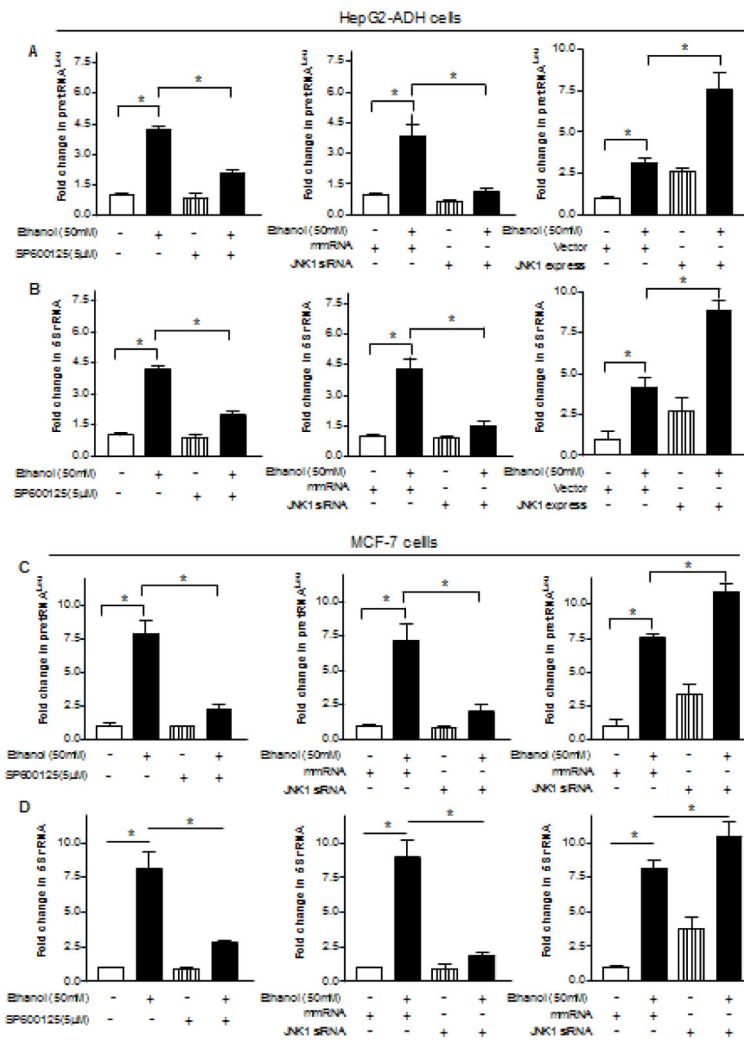


Fig. 5. Alcohol-activated JNK1 mediates transcription of Pol III genes
(A–D, left panel) *JNK inhibitor SP600125 inhibits alcohol-induced Pol III gene transcription.* HepG2-ADH cells and MCF-7 cells were pretreated with 5 μ M SP600125 and then treated with or without ethanol. **(A–D, middle panel):** *Repression of JNK1 decreases transcription.* HepG2-ADH cells and MCF-7 cells were transfected with either mismatch RNA (siMM) or JNK1-specific siRNA (siJNK1) for 48 hours and then treated with ethanol; **(A–D, right panel):** *Overexpression of JNK1 enhances transcription of Pol III genes.* HepG2-ADH cells and MCF-7 cells were transfected with either JNK1 expression construct or vector for 48 hours and treated with ethanol. RNAs were derived from these cells and RT-qPCR was performed to measure the amounts of pre-tRNA^{Leu}, **(A and C)**, 5S rRNA **(B and D)**, and GAPDH transcripts. The fold change was calculated by normalizing to the amount of GAPDH. *: $P < 0.05$. The values represent mean \pm SE from three independent experiments.

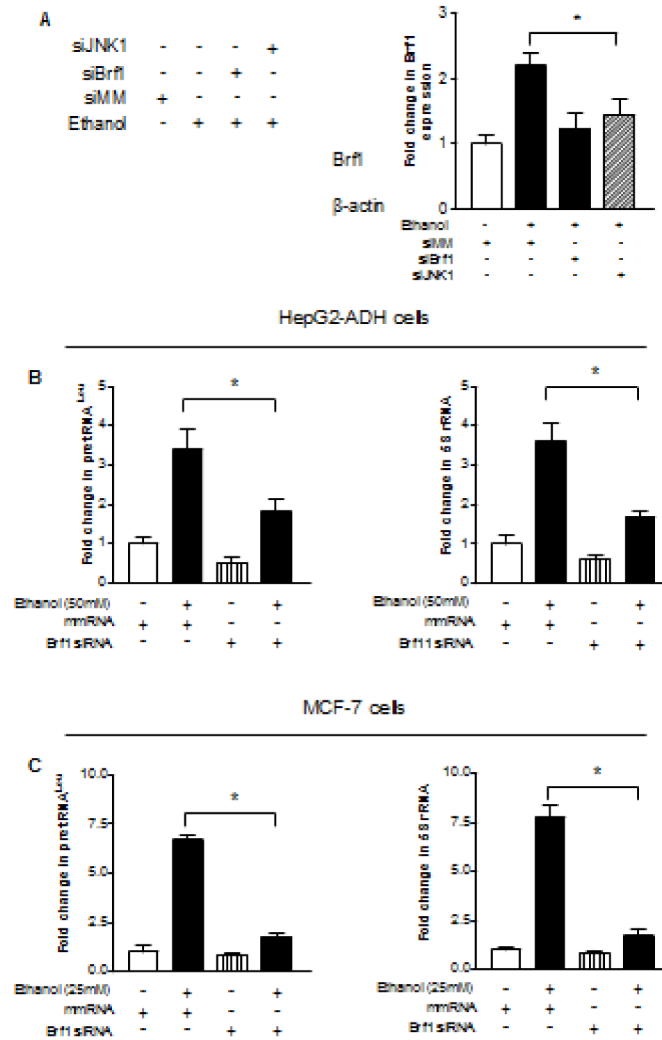


Fig. 6. Repression of Brf1 decreases the induction of Pol III genes

(A) *JNK1* and *Brf1* siRNA reduce the levels of *Brf1* in breast cells. MCF-7 cells were transfected with mismatch RNA (siMM), JNK1 siRNA (siJNK1) or Brf1 siRNA (siBrf1) 48 hours and then treated with ethanol for another 1 hour. The cell lysates were extracted from these cells to determine. Immuno-blot was performed for these sample to determine the cellular levels of Brf1. A representative blot from three independent determinations is shown (left panel) and quantitative analysis (right panel). (B–C) *Pre-tRNA^{Leu}* and *5S rRNA*. HepG2-ADH cells and MCF-7 cells were transfected with siMM or siBrf1 for 48 hours and then treated with ethanol as described above. RNAs was derived from these cells and RT-qPCR was performed to measure the amounts of pre-tRNA^{Leu} (B and C, left panel), 5S rRNA (B–C, right panel), and GAPDH transcripts. The fold change was calculated by normalizing to the amount of GAPDH. *: $P > 0.05$. The values represent mean \pm SE from three independent experiments.

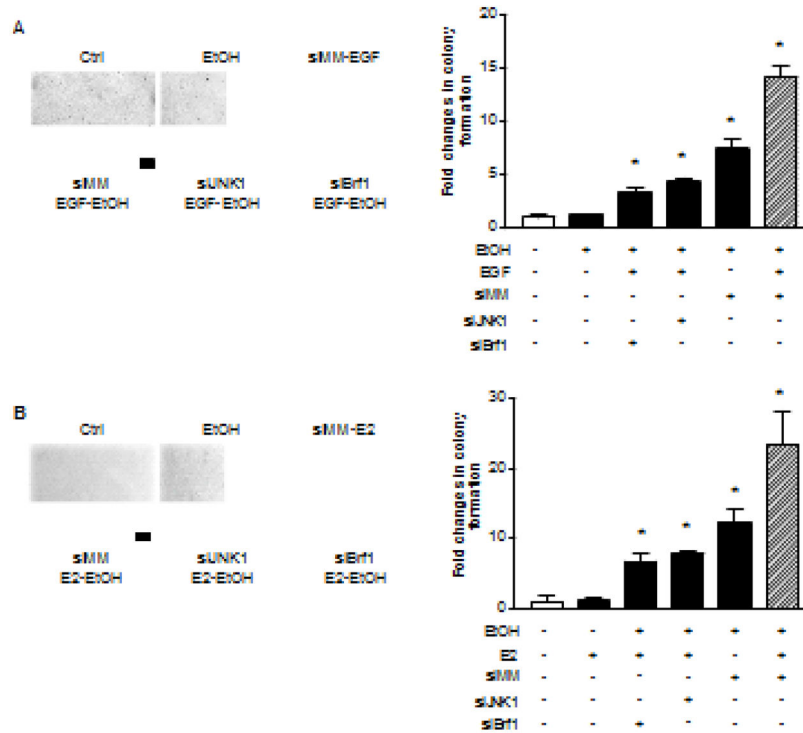


Fig. 7. Down-regulating JNK1 and Brf1 expression decreases ethanol-induced anchorage-independent growth

(A) Alcohol increases the rate of liver cell transformation. Parent AML-12 cells and AML-12 cells expressing JNK1 siRNA or Brf1 siRNAs were poured in triplicate into 6-well plate with 0.35% agar containing 50mM ethanol, 20ng EGF or ethanol plus EGF; (B) Alcohol promotes transformation of breast cells. Parent MCF-10A cells and MCF-10A cells expressing JNK1 siRNA or Brf1 siRNAs were poured in triplicate into 6-well plate with 0.35% agar containing 25mM ethanol, 5nM E2 or ethanol plus E2. AML-12 cells or MCF-10A cells were incubated at 37°C in 5% CO₂ for 3 weeks or longer and were fed with fresh complete media with or without ethanol, ethanol + EGF or ethanol + E2 twice weekly. Colonies were counted at 3 weeks after plating. The fold changes were calculated by normalizing to the colonies amounts control. Bar = 200 μM. *: $P > 0.05$. Values are the means ± SE (n = 3).

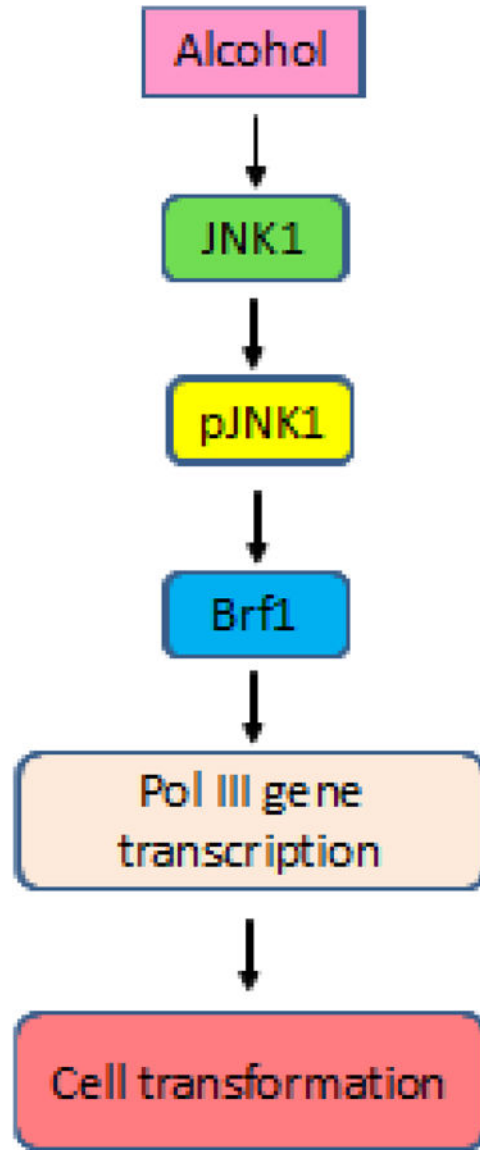


Fig. 8. Schematic illustration of a proposed mechanism of alcohol-induced deregulation of Pol III genes

Ethanol induces activation of JNK1 to upregulate Brf1 expression, which in turn enhances Pol III gene transcription to promote alcohol-induced cell transformation.