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Alteration of BRCA1 expression affects alcohol-induced transcription of RNA Pol III-dependent genes

Qian Zhong[1,2], **Ganggang Shi**[3], **Yanmei Zhang**[3], **Lei Lu**[1], **Daniel Levy**[1], and **Shuping Zhong**[1,3,*]

[1]Department of Biochemistry and Molecular Biology, Keck School of Medicine, University of Southern California, Los Angeles, CA, USA

[2]State Key Laboratory of Oncology in South China, Sun Yat-sen University Cancer Center, China

[3]Shantou University Medical College, Shantou, Guangdong, China

Abstract

Emerging evidence has indicated that alcohol consumption is an established risk factor for breast cancer. Deregulation of RNA polymerase III (Pol III) transcription enhances cellular Pol III gene production, leading to an increase in translational capacity to promote cell transformation and tumor formation. We have reported that alcohol intake increases Pol III gene transcription to promote cell transformation and tumor formation *in vitro* and *in vivo*. Studies revealed that tumor suppressors, pRb, p53, PTEN and Maf1 repress the transcription of Pol III genes. BRCA1 is a tumor suppressor and its mutation is tightly related to breast cancer development. However, it is not clear whether BRCA1 expression affects alcohol-induced transcription of Pol III genes. At the present studies, we report that restoring BRCA1 in HCC 1937 cells, which is a BRCA1 deficient cell line, represses Pol III gene transcription. Expressing mutant or truncated BRCA1 in these cells does not affect the ability of repression on Pol III genes. Our analysis has demonstrated that alcohol induces Pol III gene transcription. More importantly, overexpression of BRCA1 in estrogen receptor positive (ER+) breast cancer cells (MCF-7) decreases the induction of $tRNA^{Leu}$ and 5S rRNA genes by alcohol, whereas reduction of BRCA1 by its siRNA slightly increases the transcription of the class of genes. This suggests that BRCA1 is associated with alcohol-induced deregulation of Pol III genes. These studies for the first time demonstrate the role of BRCA1 in induction of Pol III genes by alcohol and uncover a novel mechanism of alcohol-associated breast cancer.

Keywords

BRCA1; Alcohol; Pol III genes; Breast cancer

*Correspondence: Dr. S. Zhong, szhong@usc.edu, Phone: (323) 442-1141, FAX: (323) 442-1224.

Conflict of interest

The authors declare no conflict of interest.

1. Introduction

Studies have indicated that RNA polymerase III-dependent (Pol III) transcriptional products are elevated in both transformed and tumor cells suggesting that they play a crucial role in tumorigenesis (White, 2001; Winter *et al*, 2007). Consistent with this idea, enhanced Pol III transcription is required for oncogenic transformation (Zhang *et al*, 2013; Johnson *et al*. 2008, Zhong *et al*, 2013). RNA Pol III transcribes a variety of untranslated RNAs, including tRNAs, 5S rRNAs, 7SL RNA, 7SK RNA and U6 RNA (Ullu *et al*. 1984; Dieci *et al*. 2007; Raha *et al*. 2010), while tRNA and 5S rRNA control the translational and growth capacity of cells (White, 2001; Goodfellow *et al*. 2006). Oncogenic proteins, such as Ras, c-Jun, and c-Myc, stimulate RNA Pol III gene transcription (Zhong *et al*, 2004; Johnson *et al*. 2008); whereas tumor suppressors, such as pRb, p53, PTEN and Maf1 repress transcription of this class of genes (White, 2004; Johnson *et al*. 2008; Woiwode *et al*. 2008). The ability of these oncogenic proteins and tumor suppressor to alter Pol III transcription results from their capacity to regulate the TFIIIB complex. The TFIIIB complex consists of TATA box-binding protein (TBP) and its associated factors, Brf1 and Bdp1. TFIIIB, together with TFIIIC and RNA Pol III, are required to transcribe tRNA genes, whereas TFIIIB, together with TFIIIA, TFIIIC and RNA Pol III, are required to transcribe 5S rRNA genes. Our studies have demonstrated that alcohol intake causes changes in Pol III gene transcription to promote cell transformation and tumor formation (Zhong *et al*, 2011; Zhang *et al*, 2013)

Alcohol consumption is most consistently associated with breast cancer risk (Hamajima *et al*. 2002; MacMehon, 2006; Petri, *et al*. 2004; Singletary *et al*. 2001). This association involves the estrogen receptor (ER), which is over-expressed in approximately 80% of breast cancer cases (Deandrea *et al*. 2008; Suzuki *et al*. 2008). Alcohol is known to promote mammary tumorigenesis (Singletary *et al.* 1995, 1991; Watabiki *et al*. 2000). Cancer cells have a consistent cytological feature of nucleolar hypertrophy, rRNAs are synthesized by RNA polymerase (Pol) I and III within this nucleolar compartment. Pathologists have been using enlarged nucleoli 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 genes, because the size of the nucleolus reflects the levels of rRNA synthesis (White, 2004). Although alcohol-associated breast cancer is widely studied, the molecular mechanism remains to be addressed. To explore the role of alcohol in Pol III gene transcription, we treated normal and breast cancer cell lines with ethanol. Our results indicate that ethanol-induced tRNA and 5S rRNA transcription in breast cell lines is correlated with ER expression. These studies demonstrated that ERα may mediate the regulation of ethanol-induced Pol III gene transcription and that alcohol induces deregulation of Pol III gene transcription via ERα.

BRCA1 (breast cancer susceptibility gene 1) is a human tumor suppressor (Jump *et al*, 1998 and 2004), called by the synonym breast cancer type 1 susceptibility protein. BRCA1 is normally expressed in the cells of breast and other tissues, where it repairs damaged DNA (Check W, 2006). If BRCA1 itself is mutated, damaged DNA is not repaired properly, and this increases the risk for breast cancer (Friedenson B, 2007). Certain variations of the *BRCA1* gene lead to an increased risk for breast cancer. Women with an abnormal *BRCA1*

gene have up to an 80% risk of developing breast cancer (Jump *et al*, 2012; Kuznetsov *et al*, 2008).

Accumulation of Pol III gene transcripts around the nucleolus is especially evident in transformed cells (Wang *et al*. 2003). Consistent with the idea that a high translational capacity is necessary for rapid growth and proliferation of tumor cells, Pol III gene transcripts have been found to be increased in ovarian tumors (Winter *et al*, 2007). Furthermore, expression of the Pol III gene, BC200, was elevated in breast squamous cell carcinoma tissues (Chen *et al*. 1997). Our recent studies using both cell culture models and animal models have revealed that alcohol increases transcription of Pol III genes (Zhong *et al*, 2011, Zhang *et al*, 2013). This induction in mice fed with ethanol is associated with tumor development (Zhong *et al.* 2011). This implies that alcohol-induced deregulation of Pol III genes may play a critical role in tumor development. To explore the role of BRCA1 in Pol III gene transcription, we restored BRCA1 expression construct in BRCA1 deficient cell line, HCC1937, to determine Pol III gene activity. The results indicate that BRCA1 expression in HCC1937 cells represses Pol III gene transcription. Further analysis reveals that ethanol treatment of MCF-7 cells, an ER+ (estrogen receptor positive) breast cancer cell line, increases Pol III gene transcription. Overexpression of BRCA1 reduces ethanol-induced $tRNA^{Leu}$ and 5S rRNA transcription in the cells. Repealing function of BRCA1 by mutation or truncating enhances Pol III gene transcription. These studies demonstrate, for the first time, that BRCA1 mediates alcohol-induced deregulation of Pol III genes. These novel findings provide a potential approach of treatment for alcohol-associated breast cancer patients.

2. Materials and methods

2.1. Reagents and antibodies

HCC 1937 and MCF-7 cell lines were from ATCC. Cell culture medium DMEM/F12, Lipofectamine 2000, TRizol reagent and OPTI-MEM were from Invitrogen. Antibodies against BRCA1 and β-actin were obtained from Santa Cruz Biotech. The sequences of primers of Pol III genes were described previously (Zhong *et al*, 2011). BRCA1 siRNAs were from Dharmacon Inc. The kit of RNase protection assay was from Ambion, Inc. The reagent of real time PCR was from Bio-Rad Biotech.

2.2. RNase protection assay

HCC 1937 cells were transfected with the pArg-maxi expression plasmid, a Pol III gene, for 48 h (Zhong *et al*, 2004). RNase protection assays (RPAs) were carried out as described in the protocol of Ambion, Inc. Briefly, HCC 1937 cells were harvested after transfection 48 h. The cell pellets were resuspended with 1 ml of TRIzol reagent (Invitrogen) and incubated at room temperature for 10 min. Chloroform was added (200 μl), and the mixture was incubated at room temperature for 3 min. Suspensions were centrifuged at 14,000 rpm for 20 min at 4°C.

The supernatant was mixed with an equal volume of isopropanol, incubated on dry ice for 15 min, and centrifuged at 14,000 rpm for 20 min at 4°C. The pellet was rinsed with 70% ethanol and resuspended in 20 μl of water. For the preparation of probe, the pArg-maxi gene

plasmid was digested with XbaI, and labeled RNA probe was synthesized using the Maxi-script T7 kit (Ambion) with [^{32}P] CTP. RNA (1 μg) was hybridized with the labeled RNA probe and incubated at 42°C overnight. The samples were digested by RNase A/T_1 at 37°C for 30 min and precipitated. The samples were incubated on dry ice for 15 min and centrifuged at 14,000 rpm for another 15 min. The pellets were resuspended in gel loading buffer (Ambion) and separated on 8% acrylamide-8 M urea denaturing gels. The bands were visualized by autoradiography, and the RNA products were quantified by a phosphorimager.

2.3 RNA isolation and RT-qPCR

MCF-7 cells were cultured in 10% FBS/DMEM-F12 medium and transfected with BRCA1 siRNAs or mismatch (mm) RNA for 48 h. The cells were starved in FBS-free medium for 3–4 h and then treated with 25 mM ethanol for 1 h. The total RNA was isolated from the cells using single step extraction method TRIzol reagent (Invitrogen). The RNA samples were quantified and reverse-transcribed in a 20 μl 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 as described before (Zhong *et al*, 2011) and PCR reagent kits (Bio-Rad Biotech) in the ABI prism 7700 Sequence Detection System. 5S rRNA and precursor of $tRNA^{Leu}$ transcripts were measured by RT-qPCT as described previously (Zhong *et al*. 2011).

2.4. SDS-PAGE and immunoblot analysis

MCF-7 cells were transfected with BRCA1 expression construct or siRNAs for 48 h. The cells were incubated with 25 mM ethanol for 1 h as described above. Cells were collected with lysis buffer and sonicated. The suspensions were centrifuged to save the supernatants. Protein concentrations were determined by the Bradford method. Lysates (50 μg 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. Membranes were probed with either antibodies against BRCA1 or β-actin. Bound primary antibody was visualized using horseradish peroxidase-conjugated secondary antibody (Vector Laboratories) and enhancing chemiluminscence reagents (Santa Cruz Biotech).

3. Results

3.1. BRCA1 expression represses Pol III gene transcription in HCC 1937 cells

Studies have demonstrated that oncoproteins, c-Jun, c-Fos and Myc, stimulate Pol III gene transcription. In contrast, tumor suppressors, pRb, p53, PTEN and Maf1, repress the activity of the class of genes. BRCA1 is a tumor suppressor and its mutation is tightly related to breast cancer. However, it remains to be established whether BRCA1 modulates Pol III gene transcription. To investigate the role of BRCA1 in Pol III gene transcription, we transfected HCC 1937 cells with a wild type (wt) BRCA1 expression construct plus pArg Maxi gene plasmid for 48 h to perform RPAs. The results indicate that restoring BRCA1 in HCC 1937 cells decreases pArg Maxi gene transcription, compared to the cells transfected with control vector construct (Fig. 1). It suggests that BRCA1 may modulate Pol III gene transcription. Next, we further estimated the role of BRCA1 in Pol III genes. HCC 1937 cells were

transfected with the construct of dominant negative mutant (mt) BRCA1. The result indicates that the transcription levels of pArg Maxi gene is not reduced by expressing DNM BRCA1, compared to expressing wt BRCA1 (Fig. 2). Further analysis reveals that expressing truncated BRCA1 could not repress pArg Maxi gene (Fig. 3). This shows that BRCA1 by mutation or truncated destroys its ability to modulate Pol III gene transcription.

3.2. Alteration of BRCA1 affects alcohol-induced Pol III gene transcription

Alcohol consumption is associated with development of human breast cancer (Wang *et al*, 2012; Wong *et al*, 2012). Recent, we have demonstrated that ethanol treatment increases Pol III gene transcription and cell transformation in breast cells (Zhang *et al*, 2013). Therefore, we further investigate whether BRCA1 expression affects the induction of Pol III genes by alcohol. Our recent studies have demonstrated that ethanol dramatically enhances transcription of pre-tRNA^{leu} and 5S rRNA in MCF-7 cells (Zhang *et al*, 2013). To determine changes in alcohol-induced transcription of Pol III genes by BRCA1, MCF-7 cells were transfected with wt-BRCA1 expression construct for 48 h and treated with 25 mM ethanol. The results reveal that cellular levels of BRCA1 protein is increased in the cells transfected with its expression construct, compared to the cells with vector alone (Fig. 4A). Overexpression of BRCA1 decreases the induction of pre-tRNA^{leu} and 5S rRNA by ethanol (Fig. 4B and 4C). This implies that BRCA1 mediates alcohol-induced Pol III gene transcription, whereas ethanol treatment does not obviously change cellular level of BRCA1 (Fig. 5A, Lane 1 and Lane 3). To confirm the observation of repressing the induction of Pol III genes by BRCA1, we further determine whether reduction of BRCA1 by its siRNA is able to affect alcohol-induced Pol III gene transcription. The result shows that BRCA1 siRNA efficiently decreases the cellular level of its protein and mRNA (Fig. 5A and 5B). Next, we estimated the changes of Pol III genes by BRCA1 siRNA. The results indicate that reduction of BRCA1 by the siRNA slightly increases the induction of pre-tRNA^{leu} and 5S rRNA by ethanol in MCF-7 cells, compared to mm (mismatch) RNA (Fig. 5C and 5D). These results of MCF-7 cells are consistent with ones in HCC 1937 cells. This shows that BRCA1 negatively modulates Pol III gene transcription and the overexpression of BRCA1 decreases the induction of Pol III genes by alcohol.

Discussion

BRCA1 status is tightly associated with breast cancer. In this study, we used both RPA and RT-qPCR approaches to determine whether alteration of BRCA1 expression affects Pol III gene transcription. These results indicate that restoring BRCA1 expression represses pArg Maxi gene transcription in HCC 1937 cells. Alcohol dramatically increases transcription of pre-tRNA^{leu} and 5S rRNA in MCF-7 cells. Overexpressing BRCA1 markedly inhibits the induction of Pol III genes by alcohol. In contrast, reduction of BRCA1 expression by its siRNA slightly enhances the induction of Pol III genes. These studies for the first time demonstrate that BRCA1 modulates Pol III gene transcription induced by alcohol. The alteration of cellular level of BRCA1 affects alcohol-induced deregulation of Pol III genes in breast cancer cells.

Breast cancer is the most common malignant disease in females. Individuals harboring germline mutations in the breast cancer susceptibility gene *BRCA1* carry an 80% lifetime risk of developing breast cancer (Ford, *et al*. 1998). The protein product of the *BRCA1* gene has many important cellular functions including DNA repair, cell cycle regulation and transcriptional regulation. Accordingly, deficiency in BRCA1 leads to accelerated proliferation, aberrant mitosis, increased chromosome instability and tumorigenesis (Deng CX., 2006; Mullan *et al*, 2006). *BRCA1* transcription is regulated by diverse environmental stimuli including genotoxic agents, hypoxia and mitogenic hormone stimulation. The best-characterized stimulant of *BRCA1* expression is estrogen, which induces the highest elevations in *BRCA1* mRNA levels that routinely peaks just before the onset of DNA synthesis (Spillman *et al*, 1996; Marks *et al*, 1997). Studies from our lab and others have demonstrated that tumor suppressors repress Pol III gene transcription (White, 2004; Johnson *et al*. 2008; Woiwode *et al*. 2008) and deregulation of Pol III gene tightly links to cell transformation and tumor formation (Zhang *et al*, 2011, 2013; Zhong *et al*, 2011, 2013; Johnson *et al* 2008). Although a study showed that BRCA1 decreased Pol III gene transcription in HCC1937 cells (Veras *et al*, 2009), the role of BRCA1 in alcohol-induced deregulation of Pol III genes remains to be addressed. Here, this present study indicates that BRCA1 negatively mediates Pol III gene transcription and decreases the induction of the genes by alcohol. The mutational and truncated BRCA1 lost its function on Pol III genes.

Epidemiological studies have indicated alcohol consumption has consistently been associated with an increased risk for breast cancer in women (Petri *et al*. 2004; Singletary *et al*. 2001). Studies by Wang *et al* have demonstrated that alcohol increased MCP-1 and CRR2 expression, which promoted mammary tumor growth in alcohol-fed mice (Wang *et al*. 2012). Alcohol intake was associated with ER+ (estrogen receptor positive) breast cancer cases more than to ER− cases (Deandrea *et al*. 2008; Suzuki *et al*. 2008; Dumitrescu *et al.* 2005). A study indicated that alcohol increased ERα expression to promote breast tumor formation in mice (Wong *et al* 2012). However, the exact mechanism, by which alcohol promotes development of ER+ breast cancer, is still unknown. A previous study demonstrated that alcohol down-regulated the expression of BRCA1, a potent inhibitor of ERα, thereby contributing to breast cancer (Dumitrescu *et al*. 2005). Alcohol consumption was also shown to increase the transcriptional activity of ERα (Fan *et al*. 2000, Zhang *et al*, 2013). Recently, we reported that alcohol increases Pol III gene transcription in both normal and cancer breast cell lines. The induction in ER+ breast cancer cell lines (MCF-7 and T47D) is significantly higher (5~6 fold) than in ER− normal breast cell lines (MCF-10A, MCF-10F and MCF-12A) and ER− breast cancer cells (MAD-MB231 and SKRB-3). Ethanol increases ERα expression, resulting in an increase in products of Pol III genes (Zhang *et al*, 2013). These results support the idea that alcohol increases ERα expression to elevate Pol III gene transcription to bring about greater phenotypic changes. Given that alcohol down-regulates BRCA1 expression (Dumitrescu *et al*. 2005), this implies that BRCA1 may mediate alcohol-induced deregulation of Pol III genes. Therefore, it is critically important to determine the role of BRCA1 in alcohol-induced Pol III gene transcription. In the present studies, our studies indicate that overexpression of BRCA1 reduces the induction of Pol III genes by alcohol. Inhibiting BRCA1 function by mutation, truncation or its siRNA destroys the repression of BRCA1 on Pol III genes. Next, we will

further investigate whether BRCA1 affects TFIIIB function or it interacts with subunits of TFIIIB complex to modulate Pol III gene transcription.

In summary, the present study provides evidence that BRCA1 represses Pol III gene transcription. Overexpression of BRCA1 decreases the induction of Pol III genes by alcohol. It is the first report that BRCA1 modulates RNA Pol III-dependent transcription induced by alcohol. The novel findings indicate that BRCA1 may play critical role in alcohol-associated breast cancer.

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Abbreviation

Pol III genes	RNA polymerase III-dependent genes
BRCA1	breast cancer 1, early onset
RPA	RNase protection assay
Mm	mismatch

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Research highlights

1. Overexpression of BRCA1 represses Pol III gene transcription;
2. Mutant or truncated BRCA1 lost the function of modulation on Pol III genes;
3. Alcohol increases Pol III gene transcription;
4. BRCA1 represses the induction of Pol III genes elevated by alcohol

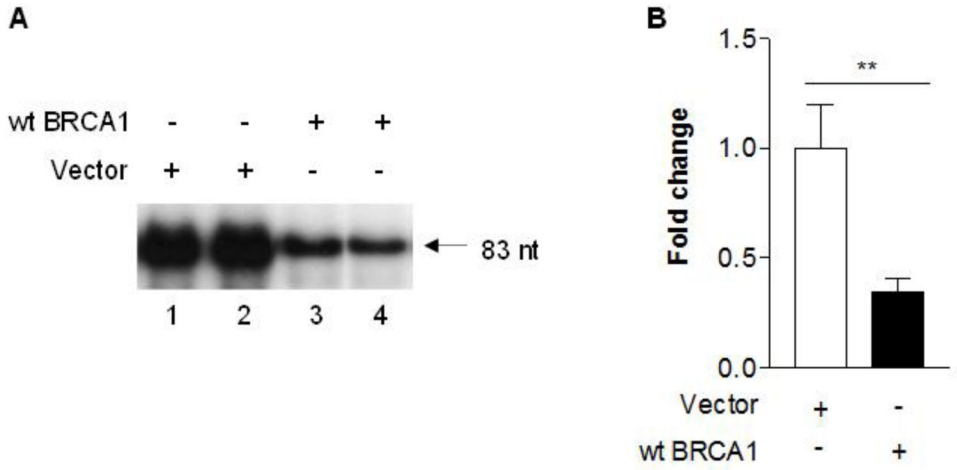

Fig. 1. RNA Pol III transcription is modulated by BRCA1

HCC 1937 cells, a BRCA1 deficient line, were transfected with vector (lane 1–2) or wild type (wt) (lane 3–4) BRCA1 expression construct plus a tRNA gene reporter plasmid, pArg Maxi gene for 48 h. RNAs ware isolated from the cells. RNase protection assay was carried out to determine the transcription levels of pArg Maxi gene (83nt). An example of autoradiogram is shown in (**A**). Quantification of 3 independent experiments of transfection with the constructs is showed (**B**). **: $p<0.01$.

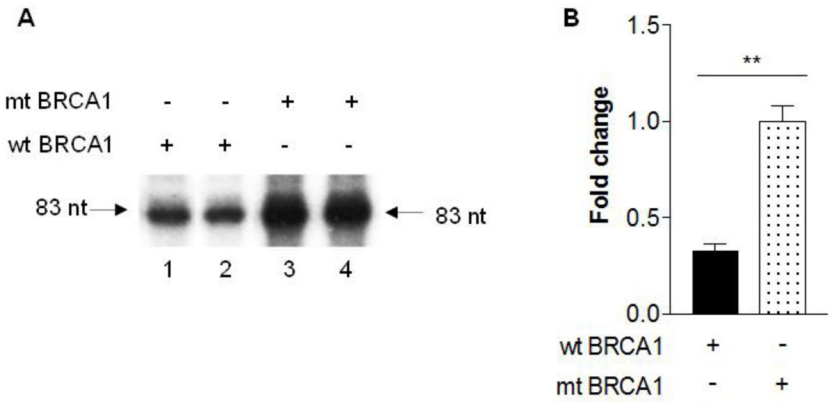

Fig. 2. Expression of a mutational BRCA1 does not affect the Pol III gene

HCC 1937 cells were transfected with wt BRCA1 (lane 1–2) or a dominant negative mutant (mt) BRCA1 (lane 3–4) expression construct plus pArg Maxi gene as described in Fig. 1. RNase protection assay was carried out to determine the transcription levels of pArg Maxi gene (83nt). An example of autoradiogram is shown in (**A**). Quantification of 3 independent experiments of transfection with the constructs is showed (**B**). **: $p<0.01$.

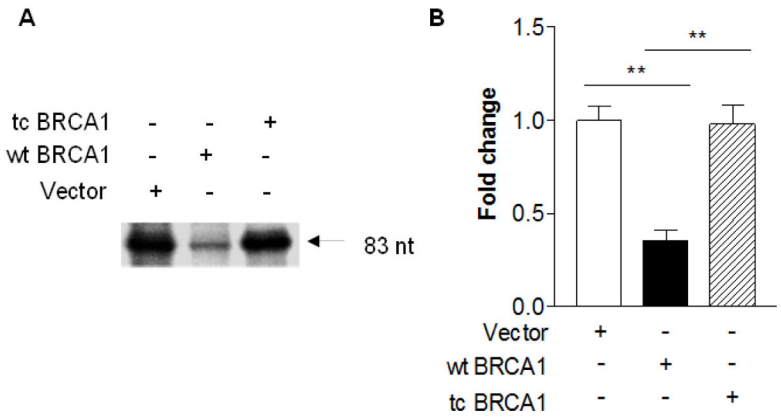

Fig. 3. Expression of a truncated BRCA1 does not repress the activity of Pol III genes

HCC 1937 cells were transfected with wt BRCA1 or a truncated BRCA1 expression construct plus pArg Maxi gene as described in Fig. 1. RNase protection assay was carried out to determine the transcription levels of pArg Maxi gene (83nt). An example of autoradiogram is shown in (**A**). Quantification of 3 independent experiments of transfection with the constructs is showed (**B**). Truncated BRCA1 lost its function repressing Pol III gene transcription. **: $p<0.01$.

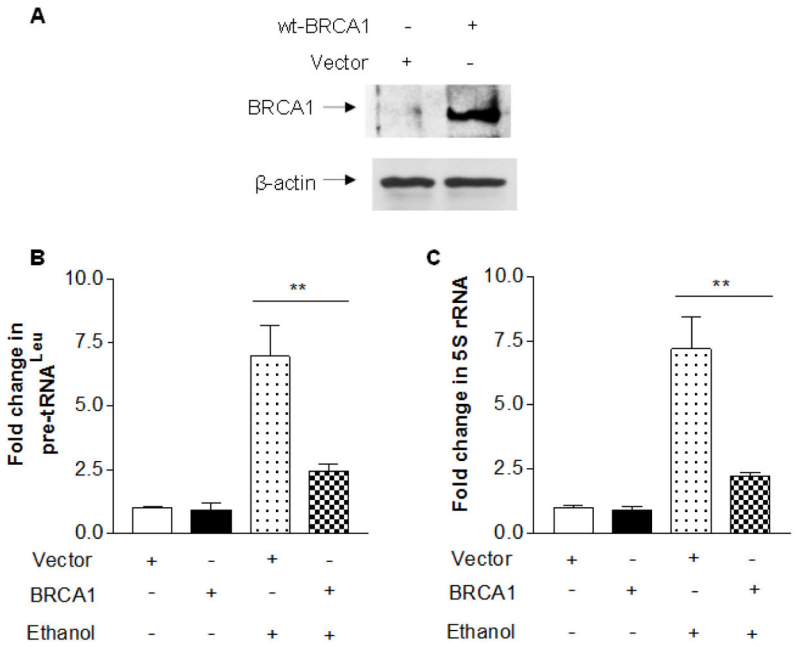

Fig. 4. Overexpression of BRCA1 represses the induction of Pol III genes by alcohol

MCF-7 cells were transfected with vector or wt BRCA1 expression construct as described in Fig. 1. The resultant cell lysates and total RNA from these cells was extracted. Immunoblot analysis was carried out to determine protein level of BRCA1 (**A**). RT-qPCR was performed to measure the levels of pre-tRNA^{Leu} (**B**), 5S rRNA (**C**) transcription. The fold change was calculated by normalizing to the amount of GAPDH. The bars represent Mean ± SE of at least three independent determinations of transfection with the constructs. The results indicate that overexpression of BRCA1 decreases the induction of Pol III gene transcription. **: $p<0.01$.

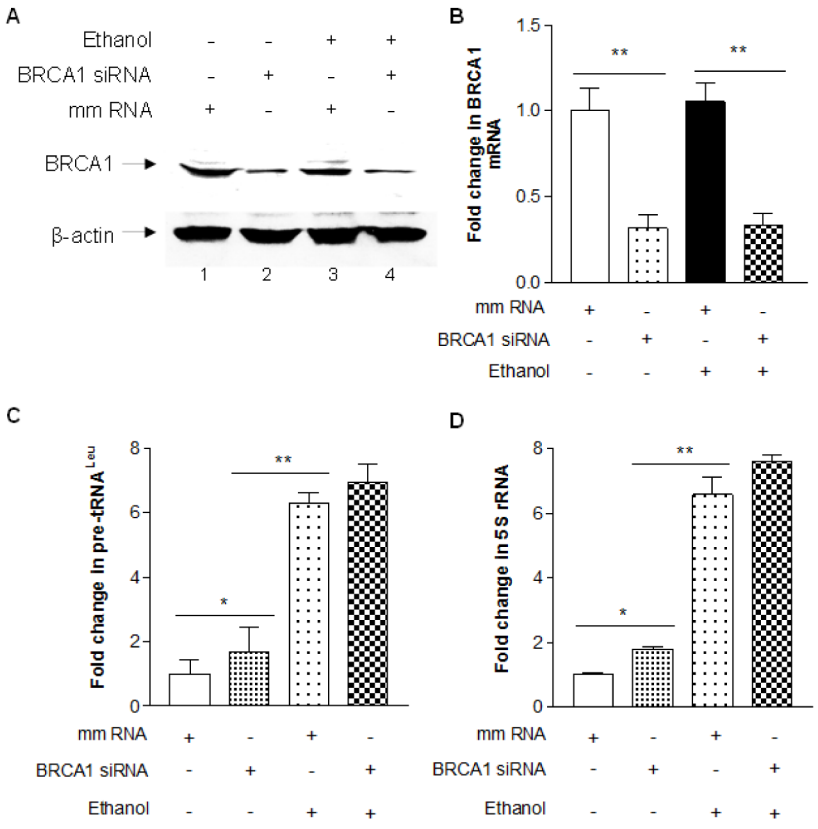

Fig. 5. Reduction of BRCA1 increases Pol III gene transcription

MCF-7 cells were transfected with mismatch RNA (mm RNA, as a control) or BRCA1 siRNA for 48 h. The resultant cell lysates and total RNAs from these cells was extracted. Immunoblot analysis was carried out to determine protein level of BRCA1 (**A**). RT-qPCR was performed to measure the levels of BRCA1 mRNA (**B**), pre-tRNA^{Leu} (**C**), 5S rRNA (**D**) transcription. The fold change was calculated by normalizing to the amount of GAPDH. The bars represent Mean ± SE of at least three independent determinations of transfection with mm RNA or BRCA1 siRNA. The results indicate that repression of BRCA1 by its siRNA increases the induction of Pol III gene transcription, compared to mm RNA. **: $p<0.01$.