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ORIGINAL ARTICLE

Role of STAT3 Phosphorylation in Ethanol-Mediated Proliferation of Breast Cancer Cells

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Purpose: In this study, we investigated the molecular mechanism involved in ethanol (EtOH)-mediated proliferation of breast cancer cells. **Methods:** EtOH concentration was optimized by studying its effect on cell proliferation in MCF-7 and MDA MB-231 cells. We used flow cytometry and immunoblot analysis to evaluate the increased proliferation caused by the optimized concentrations of EtOH. The mechanism of EtOH-mediated proliferation was determined using reactive oxygen species (ROS) release assay, reverse transcription polymerase chain reaction, and immunoblot studies. Gene silencing followed by quantitative real-time polymerase chain reaction studies and inhibitor studies indicated the involvement of signal transducer and activator of transcription 3 (STAT3) in EtOH-mediated breast cancer proliferation. **Results:** Exposure to EtOH caused an increase in cell proliferation and an accumulation of cells in S-phase in MCF-7 (347 μM EtOH) and MDA MB-231 (173 μM EtOH) cells. Additionally, increased release of ROS and the expression of pro-inflammatory cytokines, such as interleukin 6 and tumor necrosis factor α, confirmed that the proliferation was induced by the ROS-linked inflammatory response in breast cancer. The proinflammatory response was followed by phosphorylation of STAT3. The importance of STAT3 activation in EtOH-mediated proliferation was confirmed through the silencing of STAT3, followed by an investigation on the expression of cyclins and matrix metalloproteinases. Finally, studies using specific inhibitors indicated that the EtOH-mediated effect on STAT3 activation could be regulated by phosphoinositide-3-kinase and Janus kinase 2. **Conclusion:** The study demonstrates the involvement of STAT3 signaling in EtOH-mediated breast cancer proliferation.

Key Words: *Breast neoplasms, Cell proliferation, Ethanol, Inflammation*

INTRODUCTION

Inflammation is an important component for the development of all types of cancer. Breast cancers have been reported to be caused due to inflammation triggered by various factors including dietary changes, intake of alcohol, and tobacco. Ethanol (EtOH) has been widely reported to act as a tumor initiator as well as a tumor promoter. Experimental and epidemiological studies have shown that a moderate intake of alcohol had a causal relationship with tumor promotion, which may be associated with activation of the inflammatory response [1]. An increased risk of breast cancer due to alcohol consumption in pre- and postmenopausal women has been hypothesized to be due to a deregulation in the hormonal response of estrogen and progesterone [2]. In addition to activation of the estrogen receptor (ER) signaling pathway, intake of EtOH has been reported to decrease the expression of *BRCA1* [3], facilitate angiogenesis through hypoxia-inducible factor 1 pathway [4], and enhance metastasis by disrupting the vascular endothelial barrier [5] leading to an accumulation of reactive oxygen species (ROS) in breast cancer cells [6].

ROS is a major inflammatory and tumor promoting factor involved in the activation of cytokine and growth factor signaling. Goldberg and Schwertfeger [7] suggested that ROS-linked activation of interleukin 6/Janus kinase 2/signal transducer and activator of transcription 3 (IL-6/JAK2/STAT3), tumor necrosis factor α (TNF-α), and phosphoinositide-3-kinase (PI3K) is the inflammatory pathway responsible for promoting tumor development. Similarly, Carballo et al. [8] suggested that STAT3 is associated with inflammation-induced tumor progression and metastasis in colorectal, hepatocellular, and breast carcinomas. Constitutive activation of STAT3 is in-

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volved in tumor initiation, progression, and maintenance, marking it as a potential target for cancer therapy [9]. Various reports strongly suggest a causal relationship between inflammation and alcohol-mediated breast cancer. However, the exact molecular mechanisms underlying tumor promotion in breast cancer are not clearly understood. The present study investigates EtOH-mediated breast cancer cell proliferation and the role of STAT3 in this process.

METHODS

Reagents and antibodies

All chemicals were obtained from Sigma-Aldrich (St. Louis, USA). All cell culture solutions and supplements were purchased from Life Technologies Inc. (Gaithersburg, USA). Dulbecco's modified Eagle medium (DMEM) was obtained from Gibco, BRL (Carlsbad, USA). All other high performance liquid chromatography and analytical grade solvents were obtained from SRL (Mumbai, India). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent; reverse transcription polymerase chain reaction (RT-PCR) and quantitative real-time polymerase chain reaction (qRT-PCR) reagents; and Lipofectamine 2000 were obtained from Invitrogen (Waltham, USA). Antibodies were purchased from BD biosciences (Gurgaon, India) and Cell Signaling Technologies (Danvers, USA). AG490 and wortmannin were purchased from Merck chemicals (Goa, India). siRNA was purchased from Sigma Aldrich.

Cell culture

MCF-7 and MDA MB-231 cells were maintained in DMEM supplemented with 2 mM glutamine, antibiotics (gentamycin 160 μg/mL and amphotericin B 3 μg/mL), and 10% heat-inactivated fetal bovine serum. All the cell cultures were maintained at 37°C in a humidified incubator with 5% CO_2.

Optimization of EtOH concentration using MTT assay

To determine the effect of EtOH on cancer cells and to optimize the concentration for maximum enhancement of cell proliferation, dose-dependent and time-dependent assessment on cell viability was performed as described by Posa et al. [10]. Cells were seeded onto 96-well plates. After treatment with incremental concentrations of EtOH (400, 800, 1,200, 1,600, and 2,000 μg/dL) for 24, 48, 72, and 96 hours, the cells were washed twice with phosphate-buffered saline (PBS) and incubated with 5 mg/mL MTT reagent in DMEM for 4 hours at 37°C. Next, the medium was removed and the crystals were solubilized using dimethyl sulfoxide (DMSO), and absorbance was measured at a wavelength of 570 nm.

Assessment of cell proliferation using radiolabelled thymidine [^{3}H] incorporation assay

Cells were seeded in a 96-well plate and treated with the previously optimized concentration of EtOH. [^{3}H]-thymidine (0.5 μCi/well) was added to the wells 24 hours prior to harvesting the cells. After incubation, the supernatant was removed and cells were lysed with 10% sodium dodecyl sulphate (SDS)-0.025% sodium hydroxide (NaOH) solution. Radioactivity was then measured in a liquid scintillation counter (MicroBeta^2 LumiJET; Perkin Elmer, Waltham, USA).

Fluorescence-activated cell sorting analysis

Cells were seeded in a 6-well plate. After treatment with EtOH, cell suspensions containing 1 to 5 million cells were washed in ice cold PBS. Cells were dispersed again in 200 μL PBS using a vortex, and 1 mL of ice cold 70% EtOH was added to the cells. Then, the cells were centrifuged and resuspended in 1 mL of 0.01% triton-x in PBS solution, and 1 μL of propidium iodide solution was added. After 1-hour incubation at 37°C, the cells were analyzed using a flow cytometer.

Immunoblotting

Cells were treated with optimized concentrations of EtOH and total cell lysates were prepared using lysis buffer. Immunoblotting was performed as described by Sangeetha et al. [11].

Nitro blue tetrazolium reduction assay for ROS release

Cells were seeded in a 96-well plate and treated with EtOH. After 48 hours, the supernatant was removed and 0.01% nitro blue tetrazolium (NBT) (150 μL) was added to each well. The plate was incubated at 37°C for 1 hour, after which the supernatant was removed and treated with 50 μL of 30% potassium hydroxide for 5 to 10 minutes, followed by addition of 100 μL DMSO. The absorbance was measured at 630 nm.

Reverse transcription polymerase chain reaction

RT-PCR was carried out as described previously [12]. After incubation, cells were lysed using trizol and total RNA was extracted with chloroform followed by precipitation with isopropyl alcohol. The RNA pellet was washed with 70% EtOH and resuspended in 9 μL of diethylpyrocarbonate treated water. Reverse transcription was carried out using 200 units of avian reverse transcriptase enzyme and 200 ng/mL oligo dT. The following primers were used: IL-6 Forward, 5′-ATGACTTCCAAGCTGGCCGTGGCT-3′, Reverse, 5′-TCTCAGCCCTCTTCAAAAACTTCT-3′; TNF-α Forward, 5′-CGGGACGTGGCCGAGGAG-3′; Reverse, 5′-CACCAGCTGGTTATCTCACAGCTC-3′; and glyceraldehyde

3-phosphate dehydrogenase (GAPDH) Forward, 5′-CCACCCATGGCAAATTCCATGGCA-3′, Reverse, 5′-TCTAGACGGCAGGTCAGGTCCACC-3′. For the PCR reaction, we followed the manufacturer's protocol for Taq polymerase (Invitrogen) PCR products were resolved using 1.5% agarose gel and stained with ethidium bromide. The band intensity was measured using Image J.

Gene silencing and quantitative real-time PCR

Gene silencing of STAT3 followed by real time PCR was performed following a previously described method with some modifications [13]. MCF-7 and MDA MB-231 cells were seeded in a 6-well plate and 50 nM siRNA was transfected into the cells using the Lipofectamine (Invitrogen, Waltham, USA) reagent. After a 48-hour incubation in which the maximum transfection efficiency was reached, MCF-7 and MDA MB-231 cells were treated with EtOH (347 μM and 173 μM, respectively). The total RNA was extracted using the trizol method described above. cDNA was prepared using reverse transcription. Amplification was performed using StepOne Real-Time PCR (Applied Biosystems, Waltham, USA). All of the targeted gene cycle threshold values were normalized to that of GAPDH expression in the same sample. Results were expressed as fold change in gene expression.

Assessment of effect of inhibitors on EtOH-mediated proliferation using MTT assay

To determine the effect of inhibitors such as wortmannin

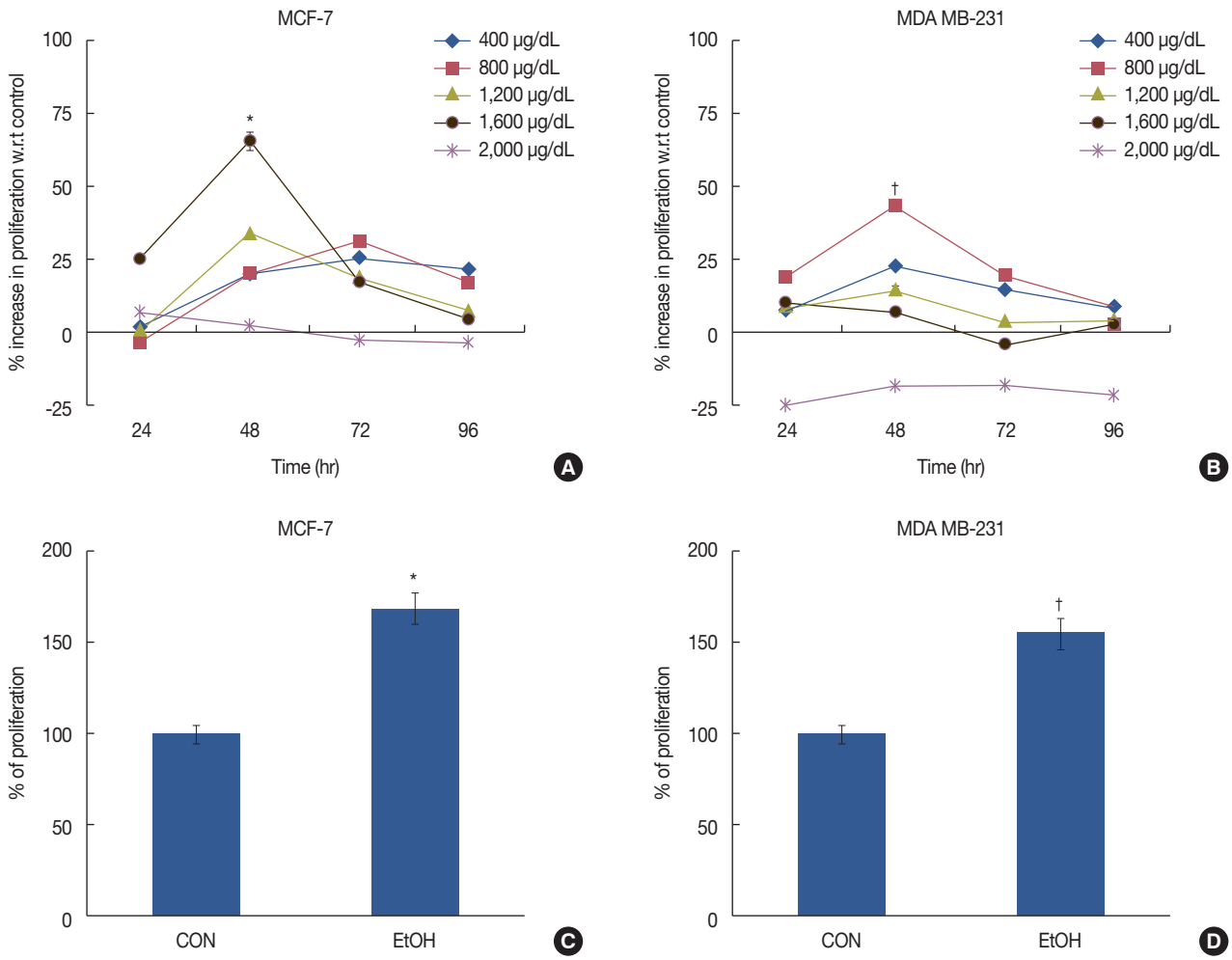

Figure 1. Dose and time course effect of EtOH on proliferation in MCF-7 and MDA MB-231 cells. (A) MTT assay in MCF-7 cells. (B) MTT assay in MDA MB-231 cells. Cells were treated with 400–1,600 μg/dL of EtOH for 24, 48, 72, and 96 hours. (C) Thymidine incorporation assay in MCF-7 cells. (D) Thymidine incorporation assay in MDA MB-231 cells. Cells were treated with the optimized concentration of EtOH 1,600 and 800 μg/dL for 48 hours. Results are expressed as percentage of proliferation. Data expressed as mean ± SD from triplicates of three independent experiments.
CON = control; EtOH = ethanol.
*$p \leq 0.005$; †$p \leq 0.05$.

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(100 nM) and tyrphostin (AG490) (100 μM) in the presence/absence of EtOH, an MTT assay was performed after 48 hours of treatment as described above.

Immunoblotting after treatment with inhibitors

To understand the effect of wortmannin and tyrphostin inhibitors on STAT3 phosphorylation and expression of cyclin D1, cells were incubated with the inhibitors in the presence and absence of EtOH for 48 hours. The protein lysate preparation and protein expression levels were analyzed by immunoblotting analysis.

Statistical analysis

All data are expressed as mean ± standard error. Differences among treatment groups were analyzed by one way ANOVA using GraphPad prism software. Differences in which $p \leq 0.05$ (denoted as†) were considered to be statistically significant, and, differences in which $p \leq 0.005$ (denoted as*) were considered to be highly significant. Each experiment was performed in triplicate.

RESULTS

Effect of EtOH on proliferation of breast cancer cells

Treating breast cancer cells with 400, 800, 1,200, 1,600, and 2,000 μg/dL EtOH for 24, 48, 72, and 96 hours showed a maximum increase in cell proliferation of 65% ± 0.03% in MCF-7 cells at 1,600 μg/dL, (347 μM) (Figure 1A) and a maximum increase in cell proliferation of 45% ± 0.17% in MDA MB-231

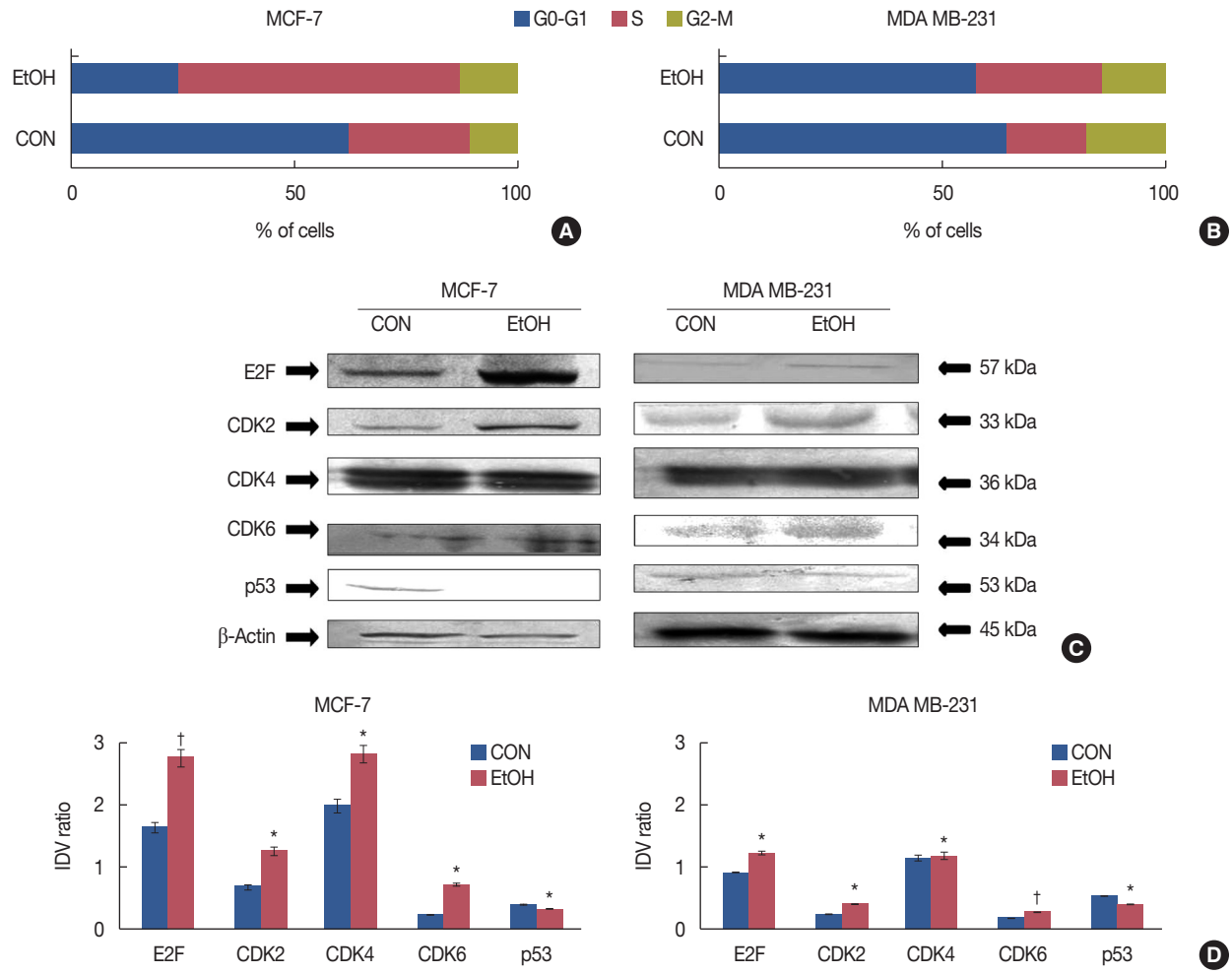

Figure 2. EtOH induced G1-S phase transition at 48 hours. Results of flow cytometry analysis (A) MCF-7. (B) MDA MB-231. (C) Immunoblot analysis of cell cycle proteins. (D) Expression of cell cycle proteins Integrated density value (IDV) in MCF-7 and MDA MB-231 cells. Target protein expressions were measured as IDVs using Image J and was normalized with β-actin expression. Data expressed as mean ± SD from triplicates of three independent experiments.
CON = control; EtOH = ethanol.
*$p \leq 0.005$; †$p \leq 0.05$.

cells at 800 μg/dL, (173 μM) for 48 hours (Figure 1B). No significant increase in proliferation was observed after 48 hours. The increase in proliferation was further confirmed using a radiolabelled thymidine incorporation assay, where a 68% ± 0.17% increase in proliferation of MCF-7 cells (Figure 1C) and 55% ± 0.03% increase in MDA MB-231 cells (Figure 1D) was observed at 48 hours.

Effect of EtOH on cell cycle

Towards understanding the effect of EtOH on breast cancer cell proliferation, we treated MCF-7 (Figure 2A) and MDA MB-231 (Figure 2B) cells with optimized doses of EtOH (1,600 μg/dL and 800 μg/dL, respectively) and analyzed the cells after 48 hours using flow cytometry. The results clearly indicate a significant increase in the population of cells at S-phase. The accumulation of cells in S-phase was further confirmed by measuring the expression of cell cycle proteins. An increase in the expression of eukaryotic transcription factor 2 (E2F), cyclin-dependent kinase 2 (CDK2), cyclin-dependent kinase 4 (CDK4), and cyclin-dependent kinase 6 (CDK6) was

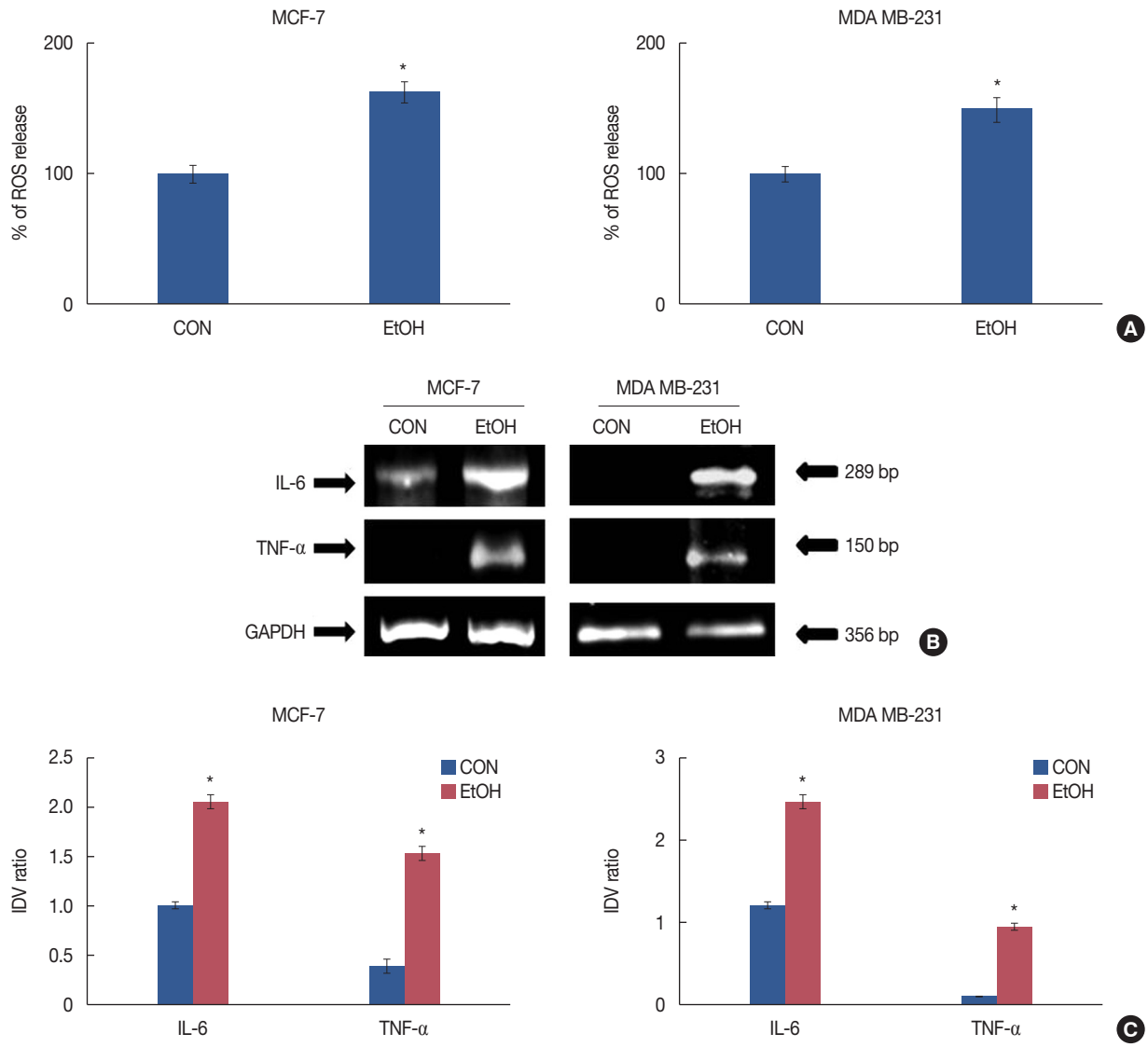

Figure 3. (A) EtOH induced reactive oxygen species (ROS) accumulation at 48 hours in MCF-7 and MDA MB-231 cells. Results of nitro blue tetrazolium assay. (B) Effect of EtOH on the expression of interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α) in MCF-7 and MDA MB-231 cells at 48 hours using reverse transcription polymerase chain reaction. (C) Expression of target genes normalized with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression in MCF-7 and MDA MB-231 cells. Data were observed from three independent experiments performed in triplicates.
CON = control; EtOH = ethanol; IDV = Integrated density value.
*$p \leq 0.005$.

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observed at 48 hours in MCF-7 (Figure 2C, D) and MDA MB-231 cells (Figure 2C, D), whereas a decrease in expression of the tumor suppressor protein p53 was observed, thereby validating the increase in cell proliferation after treatment with EtOH.

EtOH promotes the ROS-mediated inflammatory response

To determine the effect of EtOH on ROS accumulation, NBT reduction assay was performed. The cells were induced with optimized concentrations of EtOH, and we measured the release of ROS after 48 hours. The results showed an increase of ROS accumulation by 65% for MCF-7 and 51% for MDA MB-231 cells (Figure 3A) in comparison to untreated cells. Also, we found an increase in the gene expression of pro-inflammatory cytokines IL-6 and TNF-α in MCF-7 and MDA MB-231cells (Figure 3B, C) at 48 hours following EtOH treatment, suggesting a role for inflammatory cytokines in EtOH-mediated breast cancer cell proliferation.

Role of transcription factor STAT3 in EtOH-mediated breast cancer progression

To determine the downstream effector of the IL-6-mediated pathway, the phosphorylation status of the transcription factor STAT3 was assessed. We found that in both MCF-7 and MDA MB-231 cells, EtOH treatment enhanced the phosphorylation of STAT3 (Figure 4A, B), highlighting the important role played by STAT3 in EtOH-mediated proliferation of breast cancer cells. To further understand the role of STAT3 in this process, specific gene silencing by RNA was performed. Knockdown of the STAT3 gene was performed using siRNA. The transfection efficiency was achieved at 48 hours (Figure 4C, D).

We analyzed the expression of genes regulated by STAT3 using quantitative real-time PCR. The results indicated an in-

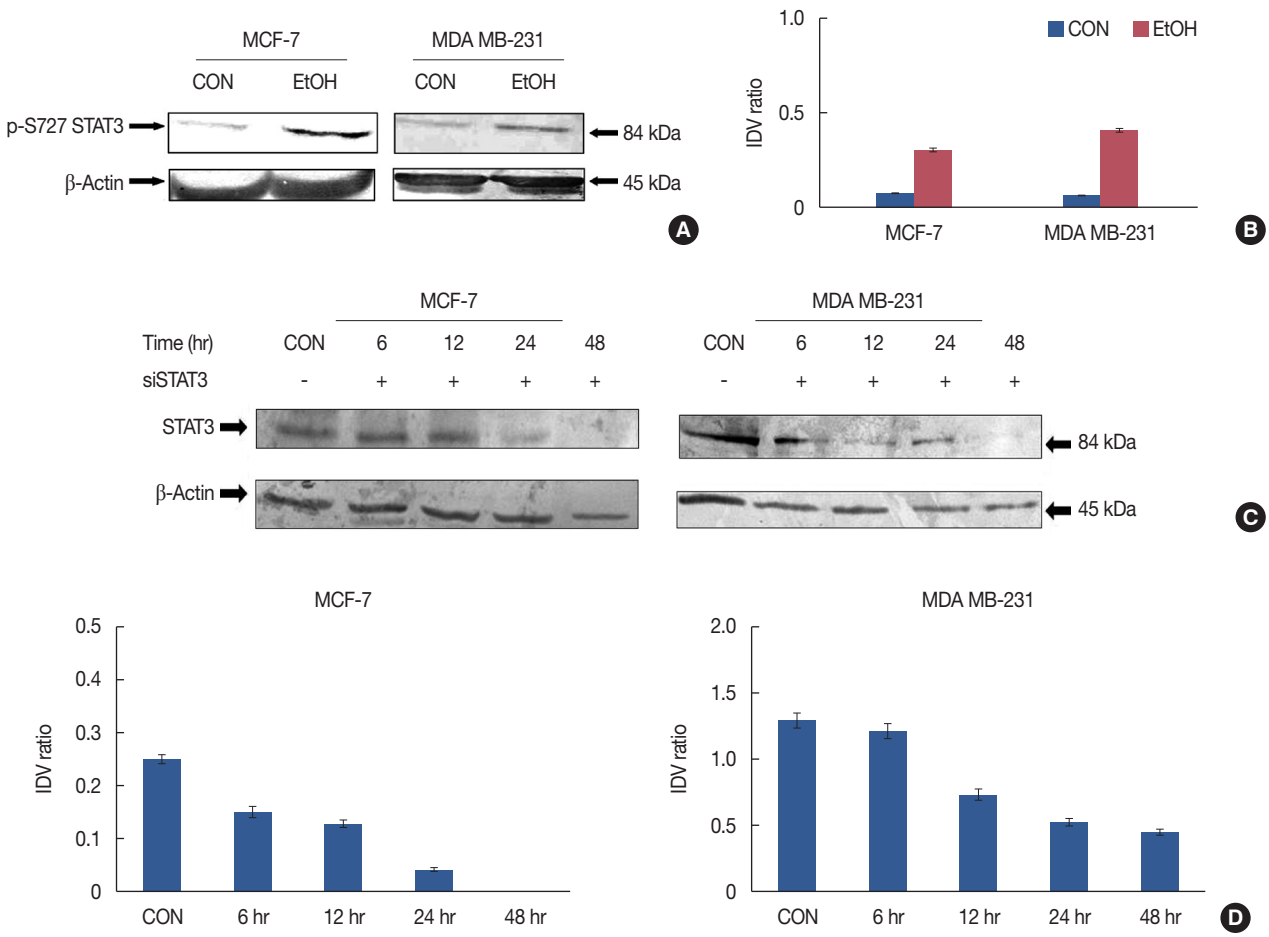

Figure 4. (A) EtOH induced phosphorylation of STAT3 in MCF-7 and MDA MB-231 cells at 48 hours. Results of immunoblot analysis. (B) Expression of target protein normalized with β-actin expression. (C) Gene silencing studies for optimising the maximum transfection efficiency in MCF-7 and MDA MB-231 cells using immunoblot analysis. (D) Expression of target protein normalized with β-actin expression in MCF-7 and MDA MB-231 cells. CON = control; EtOH = ethanol; siSTAT3 = signal transducer and activator of transcription 3 silenced cells; IDV = Integrated density value.

crease in the expression of cyclin D1, D2, and D3; matrix metalloproteinase (MMP)-2; and MMP-9 to about 1.8, 2.1, 1.8, 1.35, and 1.6 fold, respectively, in MCF-7 cells (Figure 5A) and 2.5, 1.79, 2.25, 2.56, and 2.43 fold, respectively, in MDA MB-231 cells (Figure 5B) after incubation with EtOH in comparison to untreated cells. The expression of these genes was significantly reduced with the silencing of STAT3.

In order to determine the effect of EtOH on STAT3, the total protein expression level of STAT3 was assessed using immunoblot analysis in MCF-7 and MDA MB-231 cells. No significant change was observed after treatment with EtOH (Figure 6). These results suggest that EtOH enhances the phosphorylation of STAT3 (posttranslation) without any change in the expression of total STAT3 (translation).

These results confirm that the EtOH-mediated progression of breast cancer cells requires activation of STAT3, upregulation of the transcription of cyclins, and the expression of matrix metalloproteinases.

Effect of inhibitors on STAT3 phosphorylation in EtOH-mediated breast cancer

The transactivation of STAT3 is regulated through both PI3K/Akt and IL-6/JAK2 pathways [14]. Upon treatment with wortmannin, a significant reduction in EtOH-mediated proliferation and STAT3 phosphorylation was observed in MDA MB-231 cells, indicating the involvement of PI3K on STAT3 activation during EtOH-induced proliferation in MDA MB-231 cells. In contrast, no change was observed in MCF-7 cells, suggesting that these cells rely on PI3K-independent activation of STAT3 (Figure 7).

Treatment with the JAK2 inhibitor AG490 resulted in a decrease in EtOH-mediated proliferation in both MCF-7 and MDA MB-231cells (Figure 7A). The antagonistic effect of AG490 was confirmed by evaluating the expression of p-STAT3, and, its association with proliferation was probed by studying the expression of cyclin D1 by immunoblotting (Figure 7).

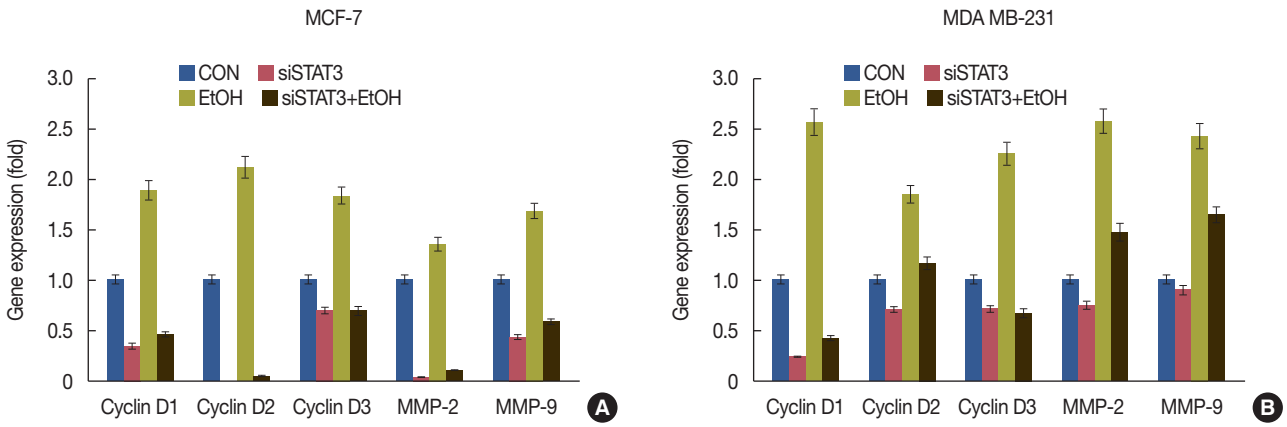

Figure 5. Quantitative real-time polymerase chain reaction analysis for STAT3 targeted proteins (cyclins and MMPs) at 48 hours. (A) MCF-7. (B) MDA MB-231 cells. Results are expressed as mean of gene expression values ± SD from duplicates of two independent experiments.
MMPs = matrix metalloproteinases; CON = control; siSTAT3 = signal transducer and activator of transcription 3 silenced cells; EtOH = ethanol; siSTAT3+EtOH = STAT3 silenced cells in presence of ethanol.

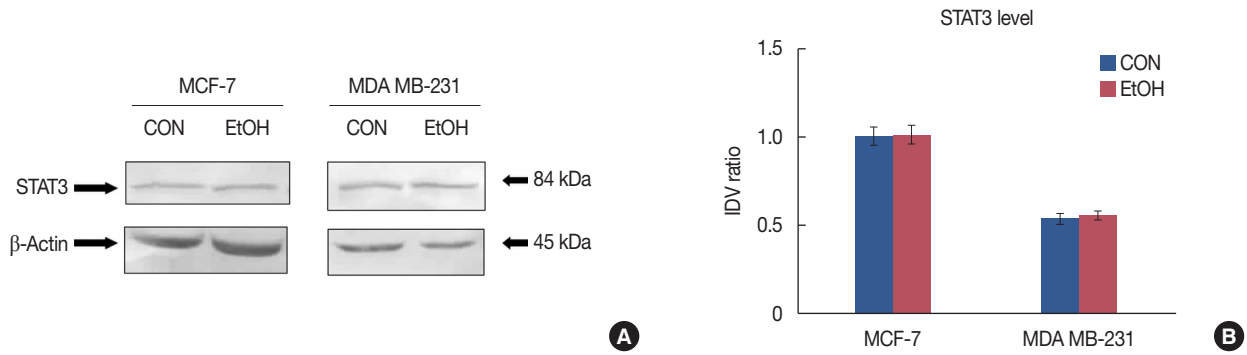

Figure 6. Effect of EtOH on the expression of total signal transducer and activator of transcription 3 (STAT3) in MCF-7 and MDA MB-231 cells at 48 hours. (A) Results of immunoblot analysis. (B) Expression of target protein normalized with β-actin expression in MCF-7 and MDA MB-231 cells.
CON = control; EtOH = ethanol; IDV = Integrated density value.

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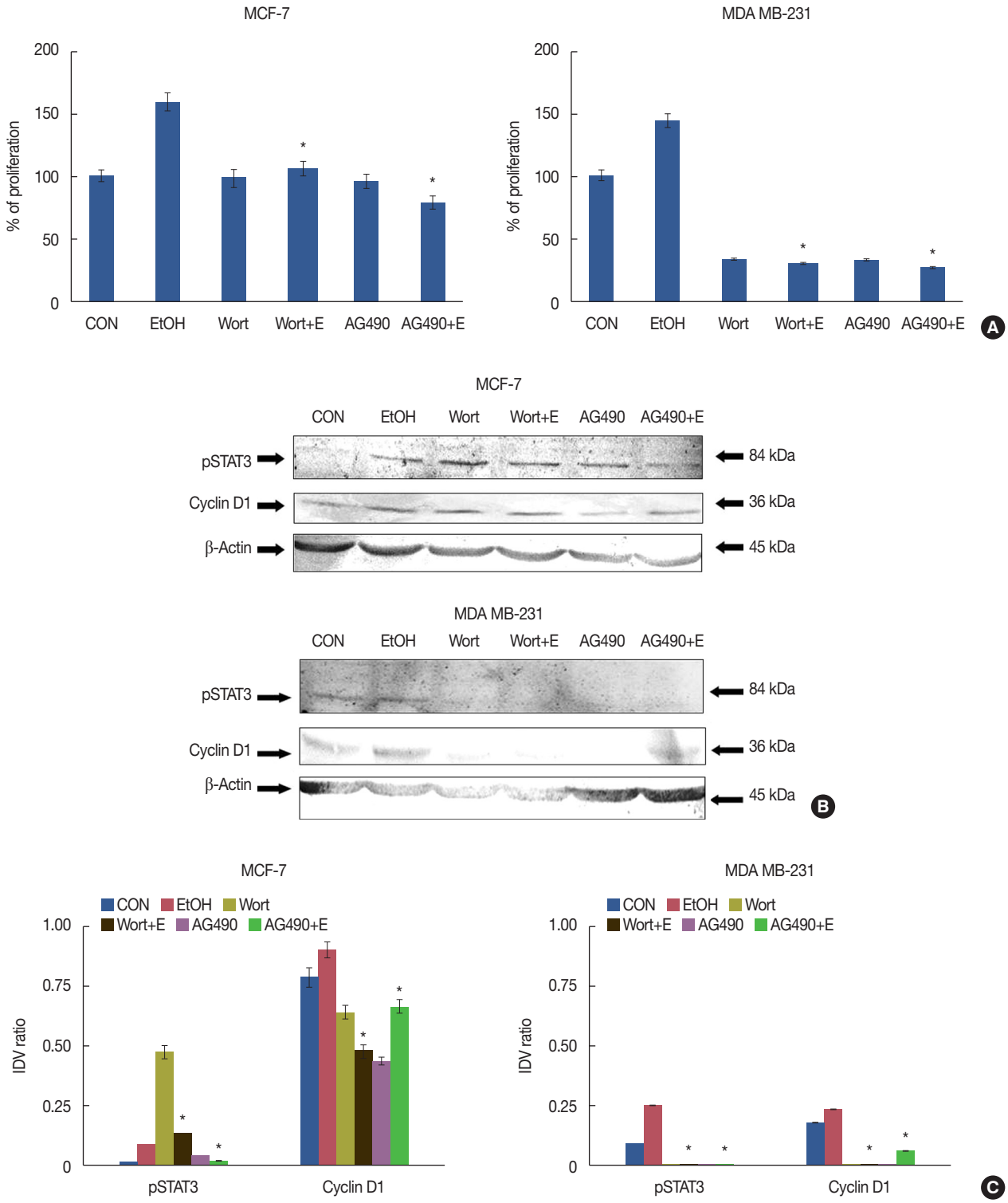

Figure 7. Effect of PI3K and JAK2 inhibitors on EtOH-mediated proliferation at 48 hours. (A) MTT assay in MCF-7 and MDA MB-231 cells. (B) Effect of PI3K and JAK2 inhibitors on STAT3 and cyclin D1 expression using immunoblot analysis. Results were based on three independent experiments performed in triplicates. (C) Expression of target protein normalized with β-actin expression in MCF-7 and MDA MB-231 cells.
CON = control; EtOH = ethanol; Wort = wortmannin; Wort+E = wortmannin in presence of ethanol; IDV = Integrated density value.
*$p \leq 0.005$.

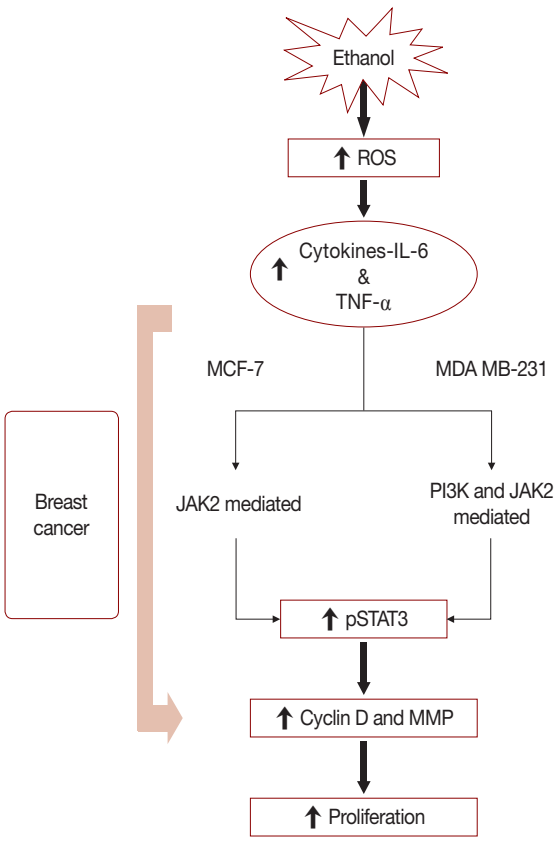

Figure 8. Schematic representation of the proposed mechanism of ethanol-mediated proliferation in MCF-7 (ER+) and MDA MB-231 (ER-) cells.
ER = estrogen receptor; ROS = reactive oxygen species; IL-6 = interleukin 6; TNF-α = tumor necrosis factor α; PI3K = phosphatidylinositol-3-kinases; JAK2 = Janus kinase 2; STAT3 = signal transducer and activator of transcription 3; MMP = matrix metalloproteinase.

Taken together, the results clearly indicate that the EtOH-induced proliferation in both MCF-7 and MDA MB-231 cells are mediated by IL-6/JAK2/STAT3 signaling (Figure 8).

DISCUSSION

Alcohol consumption has long been associated with breast cancer risk through an increased rate of tumorigenic cell proliferation. Hong et al. [15] showed that EtOH does not promote proliferation in peripheral blood mononuclear cells at lower concentrations; however, Seitz [16] reported that acetaldehyde, a metabolite of alcohol, exerts a carcinogenic effect even at low concentrations (approximately 60 μM). In the present study, a dose- and time-dependent analysis of EtOH on breast cancer cells revealed an increase in proliferation at the concentrations of 1,600 μg/dL (347 μM) and 800 μg/dL (173 μM) of EtOH in MCF-7 and MDA MB-231 cells, respectively. Cell death observed at higher concentrations of EtOH could be due to elevated ROS generation by EtOH; although ROS acts as a signal transducer at lower concentrations, it induces cell death at higher concentrations.

The G1/S-phase transition is a crucial checkpoint point in cell division and is often dysregulated in cancer cells. Previous studies have shown that CDKs play an essential role in the G1/S-phase transition by coupling the transition with mitosis and retinoblastoma protein/E2F signaling [17,18]. Mitogenic factors induce the expression of cyclin D, thereby activating CDK4 and CDK6. In tumor cells, the increased expression of CDKs leads to the G1/S-phase transition [19]. In addition, the loss of p53 in cancer cells leads to an uncontrolled cell proliferation and increased translation of proteins [20].

Our results show that EtOH causes an accumulation of cells in S-phase, altered expression of cell cycle proteins CDKs (CDK2, 4, and 6) and E2F, and reduced expression of p53 in breast cancer cells. These results strongly suggest that EtOH promotes proliferation through G1/S-phase transition in both breast cancer cell lines.

Previous clinical studies have shown that increased proliferation of breast cancer cells could also be caused by the secretion of pro-inflammatory cytokines, such as IL-6 and TNF-α [21,22]. In the present study, we found that EtOH causes an accumulation of ROS, as well as the expression of pro-inflammatory cytokines (IL-6 and TNF-α) in breast cancer cells.

Elevated levels of phosphorylated STAT3, a downstream target of IL-6, has been related to different phases of cancer progression, including invasion, proliferation, and metastasis. We found that EtOH caused a significant increase in STAT3 phosphorylation. In breast cancer, hyperactivation of STAT3 is linked to the expression of cell cycle proteins such as cyclin D1, cyclin D2, cyclin D3 [23], MMP-2 [24,25], and MMP-9 [26]. The observed elevation in expression of cyclins and MMPs in both breast cancer cell lines validates the hyperactivation of STAT3 by EtOH. STAT3 knockdown inhibited the expression of cyclin D1, cyclin D2, cyclin D3, MMP-2, and MMP-9 in both cell lines, confirming that EtOH increases the proliferation in breast cancer cells through STAT3 activation with an increased expression of cyclins and MMPs.

Upon treatment with wortmannin, a significant reduction in EtOH mediated proliferation was observed in both cells. However, wortmannin did not affect the phosphorylation of STAT3 in MCF-7 cells and loss of cell viability in MDA-MB-231 cells may rule out the direct involvement of PI3K pathway in the action of STAT3 by EtOH. Treatment with the JAK2 inhibitor (AG490) showed a decrease in the EtOH mediated proliferation in both MCF-7 and MDA MB-231cells. This antagonistic effect of AG490 was confirmed by evaluat-

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ing the expression of p-STAT3, and, its association with proliferation was probed by studying the expression of cyclin D1 by immunoblotting. Taken together, the present study concluded that MCF-7 cells rely on a PI3K-independent and JAK2-dependent way for the activation of STAT3, whereas STAT3 was activated in a PI3K-dependent manner in MDA MB-231 cells.

In summary, we showed that low concentrations of EtOH were able to increase proliferation in breast cancer cells. Exposure to EtOH effectively modulated cell cycle progression, especially the G1/S-phase transition. Our results show that EtOH increases the accumulation of ROS in cells and the expression of pro-inflammatory cytokines. Therefore, the present study suggests that EtOH induces breast cancer cell proliferation through a ROS-mediated inflammatory response. EtOH caused hyperactivation of STAT3, which was responsible for the increased expression of cyclins and MMPs observed with EtOH exposure. Furthermore, PI3K and JAK2 proteins regulate the EtOH-mediated phosphorylation of STAT3 activation, leading to enhanced proliferation. In conclusion, EtOH-mediated breast cancer requires STAT3 activation, and STAT3 could serve as a therapeutic target to treat EtOH-mediated ER-positive and ER-negative breast cancer.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

ACKNOWLEDGMENTS

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