Ethanol Modulates the Growth of Human Breast Cancer Cells *In Vitro*

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The role of ethanol or its metabolites on breast neoplasm has not been characterized. We hypothesized that ethanol may alter the growth rate of human breast tumor epithelial cells by modulating putative growth-promoting signaling pathways such as p44/42 mitogen-activated protein kinases (MAPKs). The MCF-7 cell line, considered a suitable model, was used in these studies to investigate the effects of ethanol on [^{3}H]thymidine incorporation, cell number, and p44/42 MAPK activities in the presence or absence of a MAPK or extracellular signal-regulated kinase ERK-1, and (MEK1) inhibitor (PD098059). Treatment of MCF-7 cells with a physiologically relevant concentration of ethanol (0.3% or 65 m*M*) increased p44/42 activities by an average of 400% ($P < 0.02$), and subsequent cell growth by 200% ($P < 0.05$) in a MEK1 inhibitor (PD098059)-sensitive fashion, thus suggesting that the Ras/MEK/MAPK signaling pathways are crucial for ethanol-induced MCF-7 cell growth.

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Key words: human breast cancer; MAP-kinases; ethanol

Evidence suggests that moderate consumption of ethanol may protect against cardiovascular diseases (1). Chronic abuse is, however, associated with deleterious health problems such as increased incidence of heart diseases (2), hepatic injuries that subsequently lead to liver diseases (3–6), and breast cancer (7, 8). Several mechanisms have been postulated to mediate ethanol signaling. For example, Hankinson and colleagues (7) showed a positive correlation between alcohol consumption and blood estrogen levels in menopausal women. The increase in blood estrogen level accompanied by increase in estrogen receptors (9) may contribute to the risk of breast cancer development (10). Other mechanisms of ethanol action include impaired immune systems due alterations in the levels of cytokines (11–13) and aberrant expression of carcinogen-metabolizing enzymes such as cytochrome P450s in the liver (14, 15). Cell proliferation plays an important role in the maintenance of normal and healthy breast tissues, hence unregulated breast epithelial cell growth is a characteristic feature reported in breast cancer patients (16). Consequently, there is an increasing quest to identify novel regulators of human breast epithelial cell growth. The role of ethanol on human breast epithelial cell proliferation is poorly understood. Because previous studies have shown that activation of the mitogen-activated protein kinase (MAPK) signaling pathway is required for the growth of MCF-7 cells (17–20), we hypothesized that ethanol may modulate MCF-7 cell growth through the MAPK pathway. We chose this cell line, a suitable model for the study of breast cancer (21), to study the effects of ethanol on cell growth and MAPK signaling pathway. In the present study, we demonstrate that physiologically relevant concentrations of ethanol stimulated the growth of quiescent MCF-7 cells in a MEK-1-dependent fashion, thus suggesting that the MAPK signaling pathway is crucial for ethanol to elicit its mitogenic effect(s).

Materials and Methods

MCF-7 human breast cancer cell line was a generous gift from Dr. Adrian Senderowicz (National Institute of Dental and Craniofacial Research/National Institutes of Health). Fetal bovine serum (FBS), RPMI 1640 medium, and phosphate-buffered saline (PBS) were purchased from Gibco BRL (Grand Island, NY). BCA protein assay kits were obtained from Pierce (Rockford, IL). PD 098059 was obtained from Calbiochem (La Jolla, CA). [^{3}H]thymidine (1 mCi/ml) was purchased from ICN Pharmaceutical (Irving, CA) p44/42 MAP kinase assay kits were purchased from Cell Signaling Technology (Beverly, MA). All other chemicals were obtained from Sigma Chemical (St. Louis, MO).

Cell Culture. Human breast tumor cells (MCF-7) were propagated in RPMI 1640 medium containing 10% FBS and 1% pen/strep/fungisome mixture and were grown in a humidified incubator under an atmosphere of 95% air

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and 5% CO_2 at 37°C to subconfluency. Fresh medium was supplied every 48 hr. For cell count experiments, cells were plated at 3×10^5 cells in a 100-mm tissue culture plate, and cells grow to 60%–65% confluent in 5 days. For the [^{3}H]thymidine incorporation experiments, cells were seeded at a density of 4×10^4 cells in a 35-mm tissue culture plate.

Cell Count. Subconfluent cells were serum-starved for 24 hr before treatment with different concentrations of ethanol (0.1%–10%) with the appropriate controls. Twenty-four hours following ethanol treatment, triplicate 100-mm wells/treatment were randomly selected for cell number determination. The medium was aspirated from cell monolayers and washed with PBS, pH 7.4, for easier de-attachment of cells from the substratum of the culture. The resulting cell monolayers were treated with 1 ml of trypsin/100-mm well and were incubated briefly at 37°C. Cells were viewed microscopically to ensure a complete de-attachment of cells, then they were resuspended in DMEM and counted with a hemocytometer.

[^{3}H]Thymidine Incorporation Studies. Cell proliferation was measured by [^{3}H]thymidine incorporation studies as we have previously described (22–24). For the [^{3}H]thymidine incorporation studies, subconfluent cells were serum-starved overnight, and then treated with different concentrations (0.1%–10%) of ethanol with or without an inhibitor of MEK-1, PD098059. Positive control cultures received 10% FBS, whereas negative controls received serum-free medium alone. Cells were incubated for 18 hr before 1 μCi/ml [^{3}H]thymidine/ml was added to each 35-mm diameter dish for an additional 4- to 6-hr period. All incubations were terminated by aspirating the culture medium and doing sequential washes (three times) with cold PBS, followed by the addition of 2 ml/35-mm dish of ice-cold 10% TCA for 20 min at 4°C. After washing the cells three times with ice-cold water, they were solubilized with 1 ml of 0.5 *M* NaOH/35-mm dish at 37°C for 30 min. Upon solubilization, 0.5-ml/well aliquot samples were removed and transferred to scintillation vials, 5 ml of scintillation cocktail was added to each vial, and radioactivity was quantified by liquid scintillation counter

MAPK Assay. Cells at approximately 80% confluence were serum-starved overnight and were stimulated with different concentrations of ethanol (0.1%–10%) for the dose-response experiments, or 0.3%, 3%, and 10% ethanol for 5-, 10-, 20-, and 40-min time-course experiments. After incubation, the culture medium was aspirated, and cells were washed with cold PBS and lysed in a buffer containing 20 m*M* Tris HCl (pH 7.5), 150 m*M* NaCl, 1 m*M* EDTA, 1 m*M* EGTA, 1 m*M* β-glycerophosphate, 1% Triton X-100, 2.5 m*M* $MgCl_2$, 1 m*M* dithiothreitol (DTT), 1 m*M* sodium vanadate, 1 m*M* phenylmethylsulfonyl fluoride, 2.5 m*M* sodium pyrophosphate, and 10 μg/ml leupeptin. Cells were scraped, transferred to Eppendorf tubes, and centrifuged. The clarified supernatants were recovered and immunoprecipitated with immobilized phospho-p44/42 kinase (Thr202/Tyr204) monoclonal antibody with gentle rocking overnight at 4°C. Pellets were recovered and washed twice with lysis buffer and again twice with kinase buffer containing 25 m*M* Tris (pH 7.5), 5 m*M* β-glycerophosphate, 2 m*M* DTT, 0.1 m*M* sodium vanadate, and 10 m*M* $MgCl_2$. The suspended pellets, in 50 μl of kinase buffer supplemented with 200 μ*M* ATP and 2 μl of ELK-1 fusion protein (substrate for MAPK), were incubated for 30 min at 37°C. The reactions were terminated by the addition of 15 μl of 5× Laemmli buffer before samples were boiled and electrophoresed in 12% polyacrylamide gel electrophoresis. The resulting gels were transferred onto a nitrocellulose membrane in buffer containing 25 m*M* Tris base, 0.2 *M* glycine, and 25% methanol (pH 8.5) at 70 mA overnight.

Western Immunoblotting. After transfer, the membrane was washed with Tris-buffered saline (TBS) for 5 min at room temperature, followed by incubation in blocking buffer for 2 hr at room temperature. The membrane was then incubated with primary antibody (ELK-1 at 1:1000 dilution) in antibody dilution buffer containing TBS, 0.1% Tween-20, and 5% bovine serum albumin (BSA) with gentle agitation overnight at 4°C. The membrane was washed three times for 5 min each with TBS-Tween (TBST), followed by incubation with a secondary antibody conjugated to horseradish peroxidase at 1:2000 dilution in blocking buffer containing TBS, 0.1% Tween, and 5% (w/v) non-fat dry milk with gentle agitation for 1 hr at room temperature. Finally, the membrane was washed with TBST three times each for 5 min at room temperature.

Detection of Phospho-ELK-1 (Serine 383). Per the manufacture's instructions, the membrane was incubated with 10 ml of LumiGLO (purchased from Cell Signaling Technology, Inc., Beverly, MA) (chemiluminescent reagent) with gentle agitation for 1 min at room temperature. The membrane was drained of excess developing solution, wrapped in plastic wrap, and exposed to X-OMAT AR film (Eastman-Kodak, Rochester, NY). Phosphorylated ELK-1 fusion protein was visualized by autoradiography and was quantitated by densitometry.

Statistical Analysis. Results are expressed as the mean ± SD of values obtained in triplicate from at least three different experiments. Differences between groups were compared by Student's *t* test; *P* values less than 0.05 were considered significant. When more than two means were compared, significance was determined by one-way analysis of variance (ANOVA) followed by multiple comparisons using the Student-Neuman-Keul's test.

Results

Ethanol Stimulation of [^{3}H]Thymidine Incorporation in Human Breast Tumor Cells (MCF-7). Exposure of MCF-7 cells to 65 m*M* (0.3% ethanol) increased incorporation of [^{3}H]thymidine into MCF-7 cells by approximately 2-fold over control (Fig. 1). Similar ethanol effective doses have been previously reported to enhance DNA synthesis (25). In contrast to the growth stimulatory

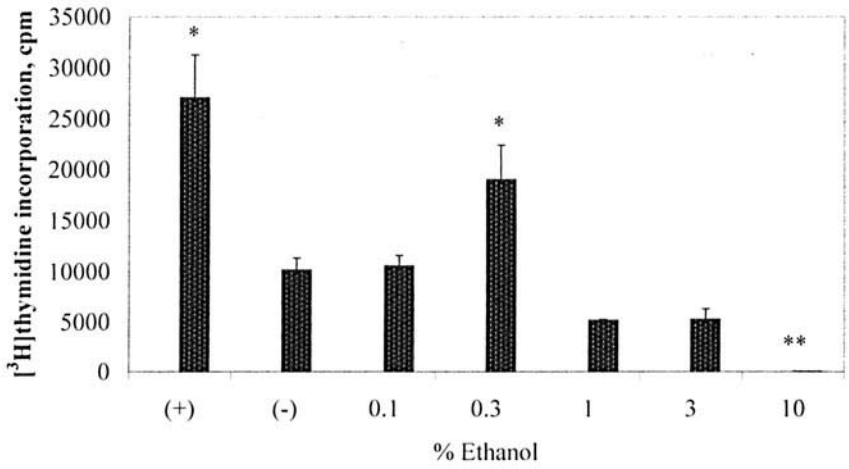

Figure 1. Ethanol-induced [^{3}H]thymidine incorporation in MCF-7 cells. Subconfluent cells were serum-starved overnight and then treated with various concentrations of ethanol (0.1%–10%) or serum (+) and serum (–) for 18 hr. After incubation, cells were pulsed with 1 μCi/ml [^{3}H]thymidine for an additional 4–6 hr as described in "Materials and Methods." The results represent the mean ± SD of three independent experiments. *P < 0.05; **P < 0.01.

effect of 0.3% ethanol, both 3% and 10% ethanol significantly inhibited cell growth.

Mitosis. The mitogenic effects of 0.3% ethanol were further confirmed by cell counts using a hemocytometer (Fig. 2). These findings are consistent with those reported by Przylipiak *et al.* (26) who observed that the addition of ethanol (0.001%–10%) to MCF-7 cell cultures stimulated cell growth. More recently, Singletary and colleagues (9) reported that 10–100 mM, which is approximately (0.05%–0.5%) ethanol, significantly increased MCF-7 cell growth. On one hand, the present results corroborate these previous findings of the stimulatory effect of ethanol (9, 26), on the other hand, they extend previous findings and provide mechanisms to explain ethanol mode of action on MCF-7 cells. In contrast to previous findings (26), we did not observe detectable increases in the growth of cells treated with concentrations of ethanol less or higher than 0.3%. Our finding that ethanol concentrations greater than 0.3% inhibited MCF-7 cell growth has been seen previously in other cell types. In regenerating hepatocytes, 100 mM ethanol inhibited cell growth (6). Because exposure of MCF-7 cells to ethanol increased cell proliferation, we examined whether ethanol-induced MCF-7 cell growth was MAPK dependent.

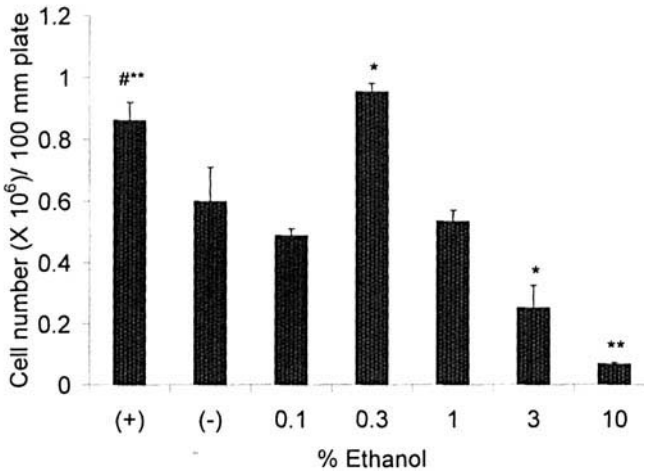

Figure 2. Ethanol-induced MCF-7 cell proliferation. Subconfluent cells were serum-starved overnight and then treated with various concentrations of ethanol (0.1%–10%) or serum (+) and no serum (–) for 24 hr. Medium was aspirated from cells, washed with PBS, pH 7.4, and then trypsinzed. Aliquot samples were removed from triplicate dishes, and cell numbers were determined using a hemocytometer. The results represent mean of three independent experiments. *P < 0.05; **P < 0.01; # indicates serum-stimulated cell growth (8.2 × 10^6).

Effect of Ethanol on MAPK Activity. Exposure of cells to all concentrations of ethanol used (0.1%–10%) markedly stimulated MAPK activity compared with untreated cells (Fig. 3). Similar MAPK stimulatory or potentiation by ethanol in other cell types has been previously reported (6, 27, 28). Treatment of cells with 25 μM concentration of 25 μM PD098059 significantly inhibited ethanol-induced MAPK activation (Fig. 4). Because ethanol-induced MAPK activation was PD098059 sensitive, we reasoned that such inhibition in MAPK activity should result in decreased MCF-7 cell [^{3}H]thymidine incorporation. Treatment of cells with 25 μM PD098059 significantly impeded cell growth (Fig. 5). Because ethanol increased MAPK activity at concentrations up to 10%, but only 0.3% ethanol showed an increase in cell proliferation, it is possible that MAPK is required, but is not sufficient, to regulate MCF-7 cell growth. We then asked: Why are MAPK activities induced by ethanol concentrations greater than 0.3% not mitogenic? To answer this question, we conducted time-dependent experiments using a mitogenic concentration (0.3% ethanol) and non-mitogenic concentrations (3% and 10% ethanol).

Time-Dependent Stimulation of MAPK by Ethanol. Results from the time-dependent studies suggest that like FBS (positive control), 0.3% ethanol stimulated MAPK activity maximally at 5 min and then declined sharply at 20 min (Fig. 6). In contrast, 3% ethanol treatment also stimulated MAPK activity at 5 min, but persisted for 20 min and declined at 40 min. Although 10% ethanol treatment resulted in sustained MAPK activation, MCF-7 cell growth was markedly inhibited at that concentration of ethanol. Others have observed that prolonged activation of the p44/42 MAPK pathway is associated with decreased cell growth

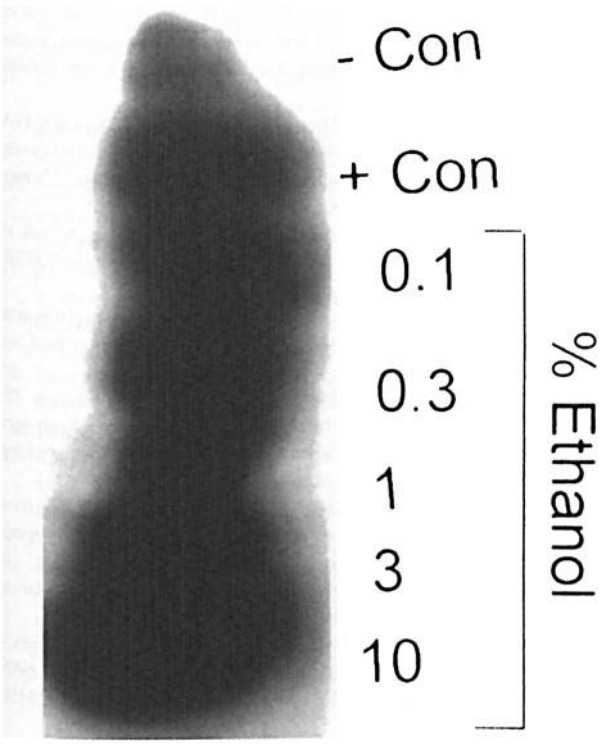

Figure 3. Ethanol-induced MAPK activity. MCF-7 cells propagated to subconfluency were serum-starved overnight and treated with various concentrations of ethanol (0.1%–10%) or serum (+) and were incubated at 37°C for 10 min. After incubation, MAPK activity was determined in an immobilized dual phospho-MAPK monoclonal antibody immunoprecipitate using ELK-1 fusion protein as substrate. The result shown here is representative of at least three independent experiments.

(29–32). The observed inhibition may be due to increased expression of cyclin-dependent kinase (CDK) inhibitor, p21WAF1/Cip-1, and decreased expression and activities of cyclin-dependent kinases, CDK2 and CDK4 (29–31).

Discussion

In these studies, we sought to determine the action of ethanol on human breast tumor cell growth and the MAPK signaling pathway. We report here that physiologically relevant concentrations of ethanol stimulated MCF-7 cell growth in a MAPK-dependent fashion. To the best of our knowledge, this is the first report to show ethanol stimulatory effects of on MAPK activity and subsequent MCF-7 cell proliferation. These findings are consistent with previous epidemiological studies that demonstrate a correlation between ethanol consumption and risks of developing breast cancer (33, 34). Modulating the proliferative activity of breast cells may represent one mechanism by which ethanol contributes to breast cancer development. It is also possible that ethanol may also contribute to the formation of new capillaries, known as angiogenesis (27). In addition, other investigators have previously reported that ethanol synergizes with other growth factors to promote cell proliferation (35, 36). Consistent with previous reports (17–20), we have also shown that activation of MAPK is required, but not

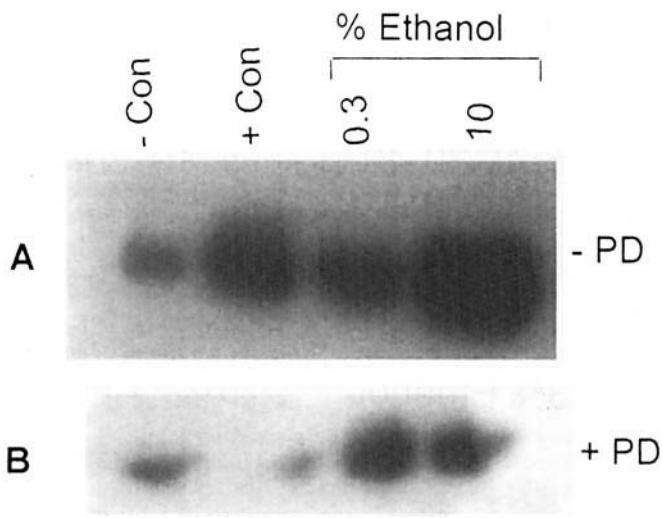

Figure 4. (A and B) Effect of a MEK-1 inhibitor (PD098059) on ethanol-induced MAPK activity in MCF-7 cells. MCF-7 cells propagated to subconfluency were serum-starved overnight and treated with either 0.3% or 10% ethanol in the presence or absence of 25 μM PD098059. Ten percent FBS served as positive control, and untreated cells served as negative control. Cells were incubated at 37°C for 10 min. After incubation, MAPK activity was determined in an immobilized dual phospho-MAPK monoclonal antibody immunoprecipitate using ELK-1 fusion protein as substrate. The result shown here is representative of at least three independent experiments.

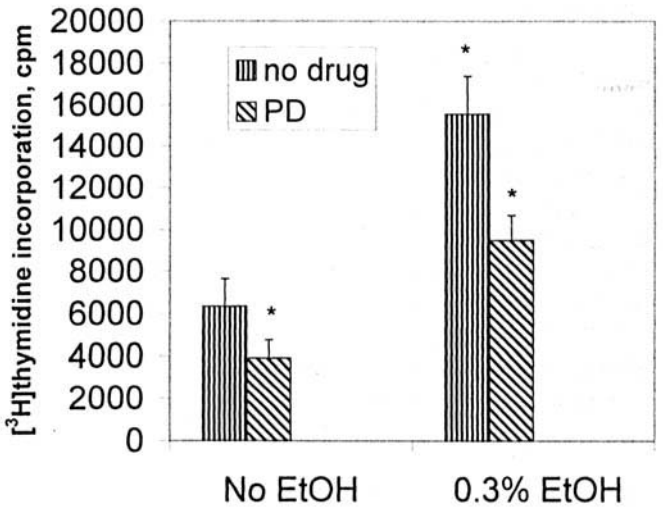

Figure 5. Reversal of ethanol-induced MCF-7 cell proliferation by MEK-1 inhibition. Subconfluent cells were serum-starved overnight, followed by incubation in the presence or absence of ethanol (0.3%) for 18 hr. After incubation, cells were pulsed with 1 μCi/ml [^{3}H]thymidine for an additional 4–6 hr before medium was aspirated from cell monolayers. Following washes of monolayers with PBS, pH 7.4, treatment with 10% TCA, and solubilization with 0.5 M NaCl, aliquot samples were removed and counted with a scintillation counter. The results represent the mean ± SD of three independent experiments. *$P < 0.05$.

sufficient, for MCF-7 cell proliferation. Although a wide range of ethanol concentrations (0.1%–10%) stimulated MAPK activity, only a 0.3% dose was mitogenic. To understand why ethanol concentrations greater than 0.3% stimulated MAPK activity and yet were non-mitogenic, we conducted time-dependent studies to examine the patterns of activation of these doses of ethanol. Results revealed that

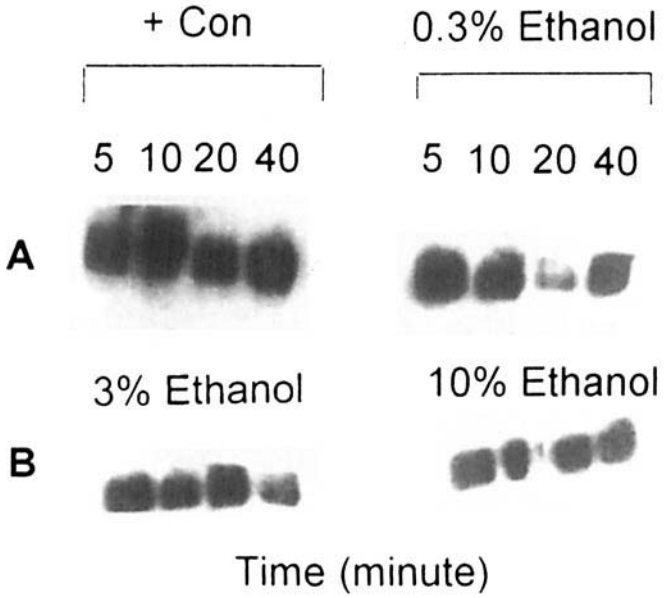

Figure 6. Time- and dose-dependent ethanol-induced MAPK activity in MCF-7 cells. MCF-7 cells propagated to subconfluency were serum-starved overnight and treated with either 0.3% or 10% ethanol. Ten percent FBS served as positive control, and untreated cells served as negative control. Cells were incubated at 37°C for the time period indicated. After incubation, MAPK activity was determined in an immobilized dual phospho-MAPK monoclonal antibody immunoprecipitate using ELK-1 fusion protein as substrate. The result shown here is representative of at least three independent experiments.

like FBS, 0.3% ethanol stimulated MAPK in acute/phasic fashion. In other cell systems, activation of MAPK may either stimulate or inhibit cell proliferation depending on whether the stimulation of MAPK was acute or long lasting (31). In those studies, the authors showed that inhibition of cell proliferation resulted from reduced cdk2 and cdk4 activities (31). It is possible that ethanol at high concentrations exerts a similar regulatory effect on cdk2 and cdk4 activities in MCF-7 cells. Experiments are currently underway in our laboratory to elucidate the mechanism by which high concentrations of ethanol inhibit MC7-7 cell growth.

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