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Ethanol Inhibits B16-BL6 Melanoma Metastasis and Cell Phenotypes Associated with Metastasis

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Abstract. *Background: Every year, approximately 68,000 new cases of malignant melanoma are diagnosed in the US. Ethanol consumption inhibits metastasis of melanoma in mice, but the mechanism is not well understood. Materials and Methods: C57BL/6J ob/+ mice, given either water or 20% ethanol, were injected intravenously with B16-BL6 melanoma cells to determine pulmonary metastasis. The effects of ethanol on cell phenotypes and markers of the epithelial-to-mesenchymal transition were determined in cell culture. Results: In mice, ethanol consumption inhibited experimental pulmonary metastasis. This inhibition was associated with decreased body weight, and levels of systemic leptin, and insulin. In cell culture, ethanol inhibited B16-BL6 cell motility, invasion, and anchorage-independent growth. Additionally, ethanol reduced Snai1 expression and increased E-cadherin expression. Lastly, ethanol increased the expression of Kiss1 metastasis-suppressor and the metastasis suppressor Nm23/nucleoside diphosphate kinase. Conclusion: In both animal and in cell culture conditions, ethanol inhibited the metastatic ability of B16-BL6 melanoma cells.*

Malignant melanoma is a type of cancer that arises from melanocytes that have acquired the ability to metastasize and colonize secondary organs such as the lungs, liver, and brain (1). In the US, approximately 1 million cases of skin cancer are reported annually (2). According to the American Cancer Society, melanoma comprises about 4% of all skin cancer cases diagnosed every year; however, malignant melanoma accounts for 75% of deaths associated with skin cancer (2). Therefore, there is a need to understand the mechanism by which melanomas metastasize so that better preventative and treatment strategies can be established.

According to a survey by the National Institute of Alcohol Abuse and Alcoholism, about 60% of US adults consider themselves chronic consumers of alcohol, consuming on average more than three drinks a week (3). Alcohol consumption is a risk factor for many types of cancer, including breast and colorectal cancer (4). Epidemiological data exists for both positive association and lack of correlation between alcohol consumption and the risk of developing melanoma (5, 6). Data regarding the effects of alcohol on metastasis of melanoma is difficult to collect; this may be because tumors are treated or removed immediately after detection. This may explain why there is currently no epidemiological data available for the association of alcohol consumption and metastasis of melanoma.

Animal models have been used to determine the effect of alcohol on metastasis of melanoma. Tan *et al*. showed that consumption of 1% alcohol increased angiogenesis and promoted growth of B16-F10 tumors in mice; however, these investigators did not determine the effects of alcohol on metastasis (7). On the other hand, studies by Meadows *et al*. have consistently showed that consumption of 20% ethanol inhibits the ability of B16-BL6 melanomas to metastasize (8, 9). The inhibition of metastasis of melanoma by 20% ethanol is not due to toxic effects of ethanol on the mice or due to a negative effect of ethanol on the body weight of the animals (8). Consistent with Meadows *et al*., in our previous studies we have shown that 20% ethanol does not affect the body weight of the mice (10). Furthermore, our group and Meadows *et al*. have shown that consumption of 20% ethanol by the mice leads to blood alcohol levels of approximately 40-60 mg/dl, which are levels found in individuals who consumed 3-5 alcoholic beverages (11-13).

Even though animal studies show that ethanol inhibits the ability of melanoma to metastasize, the mechanism by which ethanol mediates this effect is not known. There is evidence suggesting that the phenotype of the cancer cells influences their ability to metastasize (14). Specifically, cancer cells that transition from an epithelial to a mesenchymal phenotype have a

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higher metastatic ability than those with an epithelial phenotype (15). The transition from an epithelial to a mesenchymal phenotype is known as the epithelial-to-mesenchymal transition or EMT (16). The mesenchymal phenotype is associated with high expression levels of Snai1, a zinc-finger transcription factor that regulates metastasis by down-regulating the expression of genes such as E-cadherin (17). E-Cadherin is a cell membrane protein that keeps cancer cells attached to one another; however, as cancer cells gain the ability to metastasize, E-cadherin expression is significantly reduced, which allows the cancer cells to disseminate to form distant metastases (18). Among the many effects of alcohol, modulation of the activity of various transcription factors has been shown (19, 20). Therefore, it is feasible that ethanol may affect the expression of various genes, including those associated with EMT, such as the transcription factor Snai1, to contribute to the observed inhibition of metastasis of melanoma in mice. The effect of ethanol on Snai1 expression in melanoma cells is currently not known.

Alternatively, ethanol may also inhibit metastasis of melanoma by affecting other genes that regulate the ability of cancer cells to metastasize. These metastasis-suppressor genes encode proteins that have the ability to slow down or prevent the invasiveness of cancer cells, thus inhibiting the establishment of metastases (21). Metastasis suppressor genes include *Kiss1 metastasis-suppressor* and *Nm23/nucleoside diphosphate kinase*; both *Kiss1* and *Nm23* have been shown to inhibit the ability of melanoma cells to metastasize (22, 23). Furthermore, experimental evidence shows that as melanoma cells transition from an epithelial to a mesenchymal phenotype, there is significant reduction in the expression of both *Kiss1* and *Nm23* (24). Thus, ethanol may consequently affect the expression of these metastasis-suppressors by inhibiting the process of EMT.

In the present study, we determined the effect of 20% ethanol on the metastatic ability of B16-BL6 melanoma cells in mice; additionally, in cell culture conditions we determined the effect of ethanol on cell phenotypes and on the expression of metastasis suppressor genes and genes associated with EMT.

Materials and Methods

Cancer cells and cell culture reagents. B16-BL6 melanoma cells (kindly provided by Dr. Isiah J. Fidler, University of Texas at MD Anderson, Houston, TX, USA) were cultured in Dulbecco's modified minimum essential medium (DMEM; Invitrogen, Carlsbad, CA, USA), pH 7.4, containing 10% heat-inactivated fetal bovine serum (FBS; Invitrogen) and 1% antibiotic-antimycotic solution (CellGro, Manassas, VA, USA). The cells were grown at 37˚C in a humidified atmosphere of 5% CO_2. For cell culture studies, B16-BL6 cells were exposed to 0.1%, 0.2% or 0.5% v/v ethanol (Sigma Aldrich, St. Louis, MO, USA) as indicated.

Animal studies. All animal procedures and methods employed in our studies were approved by the Animal Care and Use Committee at the University of Texas at Austin. Pathogen free male ob/+ heterozygous C57BL/6J mice were purchased from The Jackson Laboratory (JAX, Bar Harbor, MN, USA) at 6-8 weeks old and housed according to NIH guidelines (25). They were singly housed and acclimated for a week before being randomized into either water or ethanol-drinking groups (n=12). Ethanol-consuming mice were acclimated to 20% v/v ethanol as follows: they received 5% v/v ethanol in the water for the first 2 weeks, 10% v/v ethanol for the next 2 weeks, 15% v/v ethanol for next 2 weeks, and 20% v/v ethanol for the rest of the study. Previously, we showed that mice consuming 20% ethanol in their drinking water had an average blood alcohol level of ~40-50 mg/dl, which are the physiological blood alcohol levels found in people who regularly consume alcohol (11). Additionally, these alcohol levels are lower than 80 mg/dl, which is considered the level for an individual to be legally intoxicated in most states in the US (26). Both water- and ethanol- consuming mice were fed a low-fat (5% fat) chow diet (Research Diets, New Brunswick, NJ, USA). We measured body weight, food, and liquid consumption on a weekly basis. After 10 weeks on either water or 20% v/v ethanol, about 150 μl of blood was collected from each mouse *via* retro-orbital bleeding to determine blood alcohol levels.

B16-BL6 pulmonary metastasis. After mice had consumed water or 20% ethanol for over 10 weeks, they were anesthetized using isoflurane, then injected *via* the retro-orbital vein with 100ul DMEM containing 1×10^5 B16-BL6 melanoma cells; mice continued to consume water or 20% ethanol throughout the study. After cancer cell injection, mice were euthanized at 10, 16, 19, or 21 days to determine pulmonary metastases (n=3). For this purpose, the lungs were inflated and fixed in 10% formalin for 24 h, at which point they were stored in 70% ethanol. Pulmonary metastases for each mouse were determined by counting the total number of metastatic foci in each lung.

Cytokine analysis and blood chemistry. Serum collected from each mouse was used to determine the effect of ethanol on systemic levels of leptin, insulin, interleukin 6 (IL-6), tumor necrosis factor-alpha (TNF-α), resistin, tissue plasminogen activator inhibitor-1 (tPAI-1), and monocyte chemotactic protein-1 (MCP-1). For this purpose, Multiplex MAP Mouse Serum Adipokine Panel (Millipore, Billerica, MA, USA) was used. Blood alcohol levels were measured using a NAD-ADH kit (Sigma-Aldrich) (n=12).

Invasion assay. We used a Boyden chamber assay to determine the effect of ethanol on the ability of B16-BL6 melanoma cells to invade (27). Briefly, the top chamber membrane was coated with a 1:10 dilution of BD Matrigel™ (BD Biosciences, Franklin Lakes, NJ, USA), then 5×10^4 B16-BL6 melanoma cells were placed in the top chamber in serum-free DMEM with 0.1% bovine serum albumin (BSA) containing either no ethanol (0%), 0.1% ethanol, 0.2% ethanol, or 0.5% ethanol. The lower chamber was filled with DMEM containing 5% FBS and the dose of ethanol. Cells were allowed to migrate from the top towards the bottom of each chamber. After 24 h, cells that remained on the top were removed using a Q-tip. Cells that had invaded to the bottom were fixed and stained using Diff-Quick Stain Set (Siemens, Malvern, PA, USA). Stained cells were visualized and quantified by microscopy. To determine the average number of cells that migrated for each well, the number of cells in 3 random fields in each well were counted at ×200. Each treatment had 6 wells per experiment, with each

experiment being carried out three times (n=3). The above experiment was repeated using 0.5% ethanol and 5% mouse serum as the chemoattractant instead of 5% FBS.

Methylthiazol tetrazolium (MTT) proliferation assay. The MTT assay was used to determine the effect of ethanol on B16-BL6 cell viability and growth. Cells were plated in a 96-well plate and allowed to attach overnight. Cells were subsequently FBS-starved overnight before being treated with either control DMEM or 0.5% ethanol for 24 h. Cell viability and proliferation were measured according to the manufacturer's instructions (no. 30-1010K; ATCC, Chicago, IL, USA). Briefly, 10 μl of MTT reagent was added to the wells containing the cells given the different treatments. The plate was subsequently incubated for 4 h until purple precipitates were observed. Then 100 μl of Detergent Reagent was added to each well. Next, the plate was left at room temperature in the dark for 2 h and finally, the absorbance at 570 nm was measured for each well. Each experiment was repeated three times with each group having 4 wells per experiment (n=3).

Apoptosis assay. The apoptosis assay was used to determine if ethanol affected B16-BL6 cell death. B16-BL6 cells were grown to 70% confluency in 6 cm^3 plates. Cells were rinsed with Phosphate buffered saline (PBS) and FBS-starved overnight. The cells were then treated with control DMEM or 0.5% v/v ethanol in DMEM for 24 h. Afterwards, cells were rinsed with PBS, trypsinized, and centrifuged at 10,000 rpm for 5 min at 4˚C. The supernatant was decanted and the cell pellet was subsequently vortexed thoroughly to make a single-cell suspension. The percentage of apoptotic cells was determined by an assay-kit according to the manufacturer's instructions (no. 10010-02; Southern Biotech, Birmingham, AL, USA). Briefly, 1×10^7 cells were resuspended in 1 ml 1× binding buffer, then 100 μl of this cell suspension was incubated with 10 μl of Annexin V-FITC for 15 min on ice. Next, 380 μl of 1× binding buffer was added to the suspension and lastly, 10 μl of propidium iodide (PI) was added and the percentage of apoptotic cells determined using flow cytometry. Appropriate negative controls (unstained cells) and single stained controls (Annexin V-FITC or PI alone) were used to determine the pro-apoptotic quadrants. Unstained cells in the lower left quadrant are the population of viable cells. Cells in the lower right quadrant that stained for Annexin V are in the early stages of apoptosis while the cells in the upper right quadrant that stained for Annexin V and PI are in the late stages of cell death. Cells in the upper left quadrant that stained positive for PI but negative for Annexin V consist of necrotic or dead cells. For our study, apoptotic cells were defined as the sum of the percentage of Annexin V-positive cells in both the lower and upper right quadrants. Three separate experiments were performed (n=3).

Wounding assay. The wounding assay was used to determine the effect of ethanol on the ability of B16-BL6 melanoma cells to migrate. In the wounding assay, cells were plated to 100% confluency and a scratch was drawn in the middle of the plate. The decrease in gap distance was measured over time and quantified; the larger the gap distance that remains, the less the cells have migrated. Briefly, B16-BL6 cells were grown on a 24-well plate until they were confluent. Then the cells were grown in FBS-free DMEM overnight. The next day, wells were washed twice with PBS and a scratch was drawn on each well using a p200 pipette tip. After two more washes with PBS to remove cell debris, the following cell culture media were added: DMEM supplemented with 1% FBS containing either no ethanol or 0.5% v/v ethanol. Each well was photographed at the time of treatment (0h) and after 9 h of incubation at ×40. The difference in gap distance was measured to quantify cell motility. Each experiment was repeated three times with each group having 6 wells per experiment (n=3).

Soft-agar colony formation assay. Soft-agar colony formation assay was used to determine the effect of ethanol on the ability of B16-BL6 melanoma to grow in an anchorage-independent manner (28). Cancer cells with a higher ability to grow in an anchorage-independent manner have a higher propensity to metastasize (29). Briefly, a 24-well plate was plated with 500 μl of DMEM containing 20% FBS and 1% agar. The bottom agar was allowed to solidify at room temperature, and the plate was stored at 4˚C for less than one week before use.

During this time, B16-BL6 cells were grown to 70% confluency, washed with PBS twice, FBS-starved for 24 h, and then treated with or without 0.5% ethanol in DMEM supplemented with 1% FBS for 24 h. Subsequently, the cells were harvested using trypsin and counted. Meanwhile, the plate that was previously coated with 1% agar was allowed to warm to room temperature in an incubator. Then 1,000 B16-BL6 cells in a total volume of 500 μl of DMEM containing 10% FBS and 0.5% agar solution, with or without 0.5% v/v ethanol, were added to each well. Once the agar solidified, 200 μl of fresh DMEM containing 10% FBS, with or without 0.5% v/v ethanol, was added to the respective wells. The cell culture media (with or without ethanol) was replaced every day for one week. At the end of the week, three random fields from each well were photographed at ×40 and the number of visible colonies were counted. The experiment was carried out in triplicates: 3 wells for control and 3 wells for the 0.5% v/v ethanol treatment. The experiment was repeated three times (n=3).

RT^2 Profiler™ EMT PCR array. To determine the ability of ethanol to affect the epithelial or mesenchymal cell phenotype, we measured the expression of genes associated with EMT. The mouse EMT RT^2 Profiler PCR Array (no. PAMM-090; SA Biosciences, Frederick, MD, USA) measures the expression of 84 key genes that are increased or decreased during EMT. Details of each gene measured by our array can be found on the SA Bioscience website http://www.sabiosciences.com/rt_pcr_product/HTML/PAMM-090A.html. To obtain the RNA for our array, B16-BL6 cells were FBS-starved overnight and treated with DMEM or DMEM containing 0.5% v/v ethanol for 24 h. Cells were subsequently harvested using trypsin and pelleted by spinning for 5 min at 1,000 rpm at 4˚C. After removing the supernatant, cell pellets were stored at –80˚C before being transported to MD Anderson Science Park Molecular Biology Facility Core for mRNA extraction, cDNA synthesis, and RT^2 Profiler EMT PCR array analysis.

Briefly, cells were lysed in Qiagen's RLT lysis buffer and total RNA was extracted using the RNeasy Kit with optional DNaseI treatment (Qiagen, Valencia, CA, USA). An Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) was used to confirm RNA integrity. One microgram of total RNA was used as a template for cDNA synthesis using High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). qRT-PCR was performed using ABI 7900HT Fast Real Time PCR System (Applied Biosystems) to analyze 84 genes related to EMT. Subsequent data analysis was performed using the Sequence

Detection System software from ABI, version 2.2.2. Endogenous control products provided in the array such as β-glucuronidase (*Gusb*), hypoxanthine phosphoribosyltransferase 1 (*Hprt1*), heat shock protein 90 kDa alpha (cytosolic), class B member 1 (*Hsp90ab1*), glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*), and β-actin (*Actb*) were averaged and used to calculate experimental Ct (cycle threshold). The ΔΔCt method was used to determine the amount of gene up- or down-regulation relative to genes expressed by non-ethanol treated B16-BL6 cell-derived RNA (1-fold). Four independent samples of no ethanol treatment (control) and ethanol treatment were compared for analysis (n=4).

Quantitative real-time PCR (qRT-PCR). To validate the array results for *Snai1* and its target E-cadherin, qRT-PCR was performed. Total RNA was collected from cells that were serum starved overnight and then treated with control media or with 0.5% ethanol for 24 h. RNA was extracted using an RNeasy Mini Kit according to the manufacturer's instructions (Qiagen). Using 1 μg of RNA for each sample, reverse transcription was performed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). We also measured the expression of genes known to regulate *Snai1*, such as *Il6* and *Nfkb*. Primers for *18S, Snai1, Il6, Nfkb, E-cadherin, Kiss1* and *Nm23* (isoforms *Nm23-m1* and *Nm23-m2*) were purchased from Integrated DNA Technologies (IDT, Coralville, IA, USA) and were as follows: 18S forward, F: GCATGGCCGTTCTTAGTTGGTGGA, backward, B: TCTCGGGTGGCTGAACGCCA; *Il6* F: GCTGGTG ACAACCACGG CCT, B: AGCCTCCGACTTGTGAAGTGGT; *Nfkb* F: AGATC TTCTTGCTGTGCGACAA, B: GTGCCTCC CAGCCTGGT; *Snai1* F: CACCTCCAGACCCACTCAGAT, B: CCTGAGTGGGGTGGGAGCT TCC; E-cadherin F: TTGAGGAGT TGAATGCTGAC, B: AGCTC GAACTTTCCAAGCAG; *Kiss1* F: GCAAGCCTGGGT CTGCAGGG, B: CGACTGCGGGAGGCA CACAG; *Nm23-m1* F: AGGACC AGTGGTTGCTATGG, B: CGCACAGCTCTTGTACTCCA; *Nm23-m2* F: GGCCTCTG AAGAACACCTGA, B: GATGGTGCCTGGTTT TGAAT.

We used primers for the p65 subunit of Nfkb (RelA) because other studies have shown that it is up-regulated in many metastatic melanomas compared to normal melanocytes (30).

Quantitative RT-PCR was performed with a SYBR GreenER qPCR kit (Invitrogen) in a Mastercycler® ep Realplex Real-time PCR thermocycler (Eppendorf North America, Hauppauge, NY, USA). The relative expression levels of target genes were normalized to that of the housekeeping *18S* rRNA. Amplification specificity was confirmed by melting curve analysis. Each gene was measured in quadruplicate and the average ΔCt was taken from the three wells before fold change was calculated using the ΔΔCt method. Analysis of at least three independent sets of samples w performed for each gene (n=3).

Immunofluorescence microscopy. To determine protein localization of Snai1 and E-cadherin, immunofluorescence microscopy was used. Wax pencils were used to mark a closed circle on microscope coverslips. B16-BL6 cells were plated into the circles and allowed to attach for 24 h before being FBS-starved overnight. They were subsequently treated with control DMEM or DMEM with 0.5% ethanol for 24 h. Cells were washed twice with PBS and fixed in 4% formalin/PBS for 10 min, then cells were subsequently washed twice with PBS before being stored in PBS at 4˚C overnight. The next day, cells were permeabilized with 0.1% Triton-X100/PBS and neutralized with 100 μM glycine/PBS, before being treated with antibodies against Snai1 or E-cadherin (Cell Signaling, Danvars, MA, USA and Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA, respectively) and appropriate fluorescently-conjugated secondary antibodies (Cell Signaling, and Abcam, Cambridge, UK, respectively) as recommended by the manufacturer. After three washes with 0.2% Tween20 in PBS, cells were counterstained with two drops of 4',6-diamidino-2-phenylindole (DAPI)/antifade (Millipore) according to the manufacturer's instructions for detection of cellular nuclei. After 15 min incubation, coverslips were placed onto microscope slides and sealed with nail polish, and images were taken with a Zeiss Axiovert 200 M fluorescent microscope at UT Austin's ICMB Core Facility. Images of control and experimental cells were acquired under identical exposure conditions for comparative analysis (n=3).

Statistical analysis. All experiments were analyzed for significance using the independent Student's *t*-test in SPSS (PAWS version 18; International Business Machines (IBM) Corporation, Armonk, NY, USA). *P*-values ≤0.05 were considered significant, and all data is represented as the mean±SEM.

Results

Ethanol consumption inhibited pulmonary metastasis. To determine the effect of ethanol on the metastatic ability of melanoma cells, C57BL/6J ob/+ mice consuming either water or 20% ethanol in the drinking water were injected *via* the retro-orbital vein with 1×10^5 B16-BL6 melanoma cells. We measured pulmonary metastasis at 10, 16, 19, and 21 days after tumor cell injection. Results showed that there was a trend for a smaller number of total metastatic foci at days 16 and 19 post tumor injection, which reached statistical significance at day 21 $p=0.01$ (Figure 1A and 1C). Ethanol-consuming mice were also less prone to developing lung metastases since all water-consuming mice had visible metastases by day 16; however, some of the ethanol-consuming mice did not develop metastases until day 21 (Figure 1B).

Effects of ethanol on body weight and serum cytokine levels. Ethanol-consuming mice had a lower body weight than water-consuming mice (Figure 2A). The lower body weight in ethanol-consuming mice was accompanied by lower circulating insulin and leptin levels (Figure 2B and 2C). Results also showed that mice consuming 20% ethanol on average had 9 mg/dl blood alcohol compared to mice consuming water which had 0 mg/dl blood alcohol levels ($p<0.05$). We also measured systemic levels of MCP-1, IL-6, TNF-α, tPAI1, and resistin; however, none of these factors were affected by ethanol consumption ($p>0.05$).

Effects of ethanol on migration, invasion, and anchorage-independent growth of B16-BL6 melanoma cells. To determine if acute ethanol exposure affected the tumorigenicity of melanoma cells independently of its effect on body weight, we determined the direct effect of ethanol on B16-BL6 melanoma

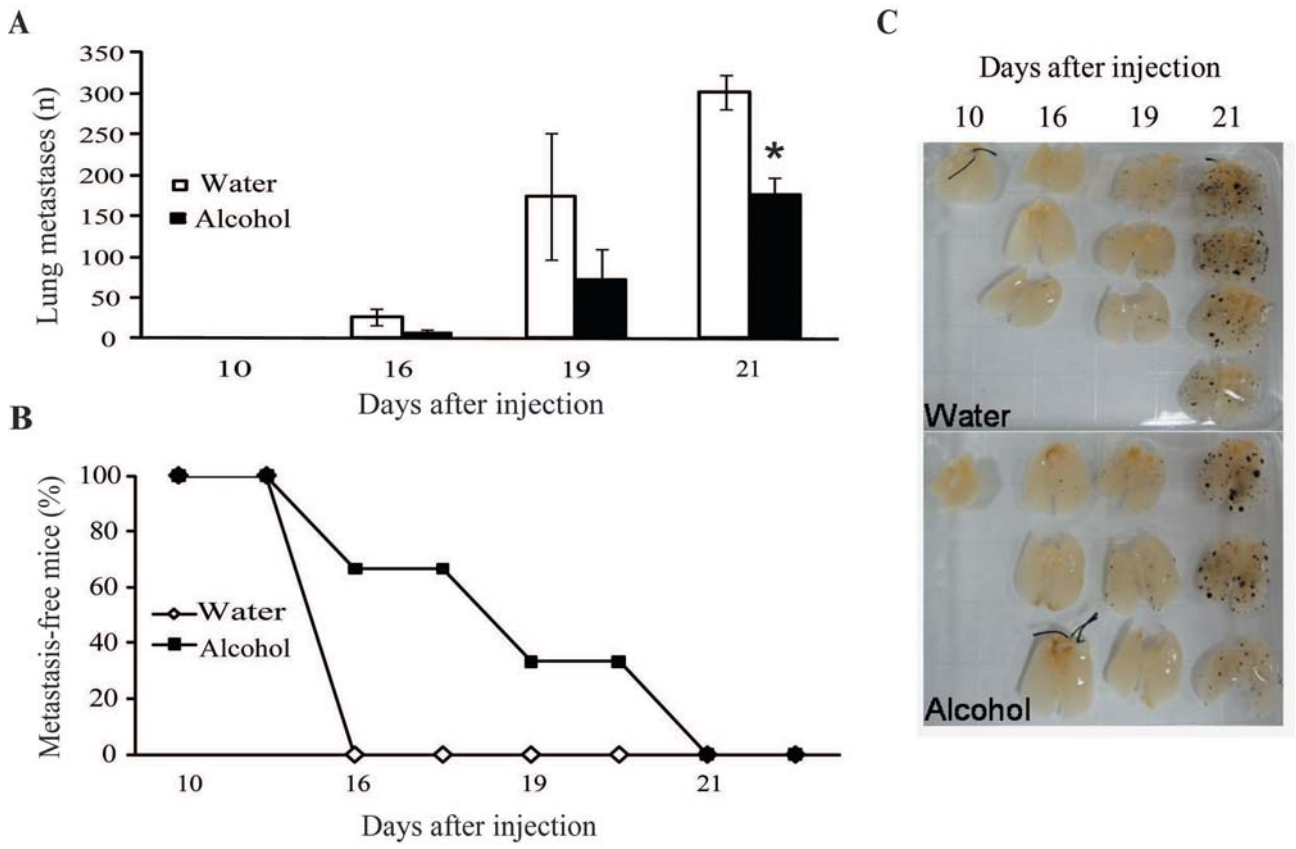

Figure 1. *Ethanol consumption and metastasis. Mice were administered 20% v/v ethanol in their water for at least 10 weeks before they were injected with B16-BL6 melanoma cells. A: Effects of ethanol on number of pulmonary metastases. B: Effects of ethanol on incidence of pulmonary metastasis. C: Visual depiction of lung metastases quantified in A and B. Values are given as means±SEM (n=3; *p<0.05).*

cells in cell culture conditions. Cells were exposed to 0.1%, 0.2%, and 0.5% ethanol for 24 h to determine B16-BL6 cell invasive ability. Results showed that all three doses of ethanol inhibited B16-BL6 cell invasion; 0.5% ethanol treatment inhibited cell invasion the most (Figure 3A). To determine if 0.5% ethanol inhibited the invasive ability of B16-BL6 cells by reducing cell proliferation or by increasing cell death, we determined both proliferation and apoptosis in the cells. We showed that 0.5% ethanol did not significantly affect cell proliferation after 24 h (Figure 3B). Furthermore, 0.5% ethanol did not affect the number of apoptotic cells after 24 h (Figure 3C). We also determined that 0.5% ethanol did not affect the number of necrotic cells after 24h (control: 0.43%±0.26 *vs*. ethanol: 0.30±0.15, $p>0.05$). Thus, we showed that 0.5% ethanol is not toxic to B16-BL6 cells. Others have used similar ethanol levels in cell culture experiments ranging from 0.5% to 1%, and showed that these ethanol levels are not toxic (31, 32). For the rest of our cell culture condition experiments, we used 0.5% ethanol.

To further characterize if ethanol affected the tumorigenicity of B16-BL6 cells, we determined the ability of 0.5% ethanol to affect cell migration, invasion and the ability of cells to grow in an anchorage-independent manner. Results show that ethanol exposure inhibited the ability of melanoma cells to migrate (Figure 4A) and to invade (Figures 4B). Because ethanol may also reduce the ability of melanoma cells to establish metastases by inhibiting their ability to grow at secondary sites, we determined the effects of ethanol on the ability of melanoma cells to grow in an anchorage-independent manner by using the soft-agar colonization assay. Results showed that ethanol inhibited the ability of B16-BL6 melanoma cells to establish colonies in soft agar (Figure 4C). Thus, it is possible that acute ethanol treatment reduces the metastatic ability of melanomas by inhibiting their ability to migrate and invade, as well as their ability to attach and propagate once they have reached distant secondary sites. These results suggest that ethanol may also affect metastasis of melanoma through other means besides its effect on body weight and circulating leptin and insulin levels.

Ethanol affected the expression of Snai1 and E-cadherin in B16-BL6 cells. To better understand the mechanism by which

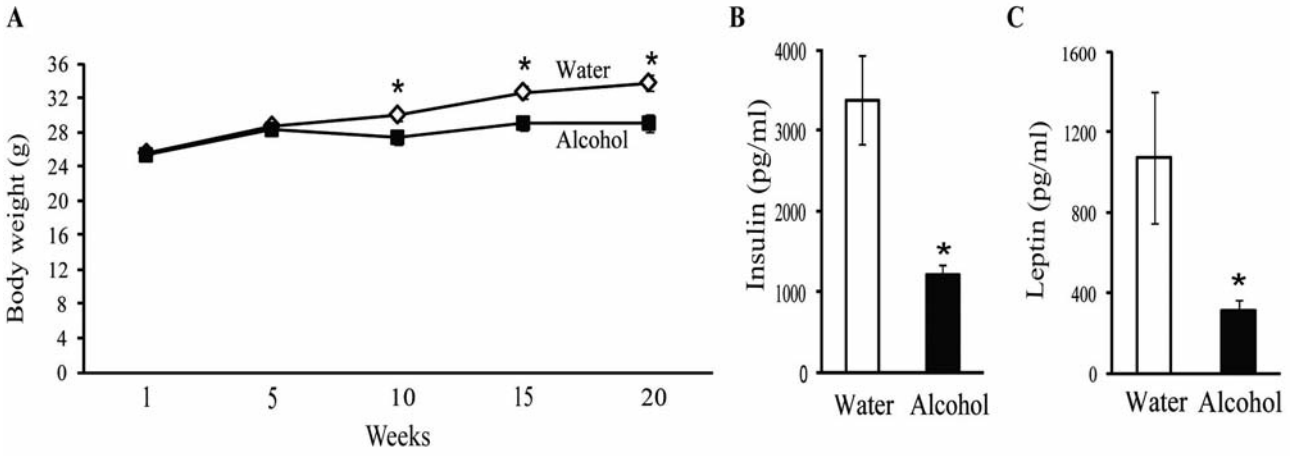

Figure 2. *Effects of ethanol consumption on body weight, insulin, and leptin levels. Mice were administered 20% v/v ethanol in their water for at least 10 weeks. A: Body weight. Systemic levels of insulin (B) and leptin (C). Values are given as means±SEM (n=12; *p<0.05).*

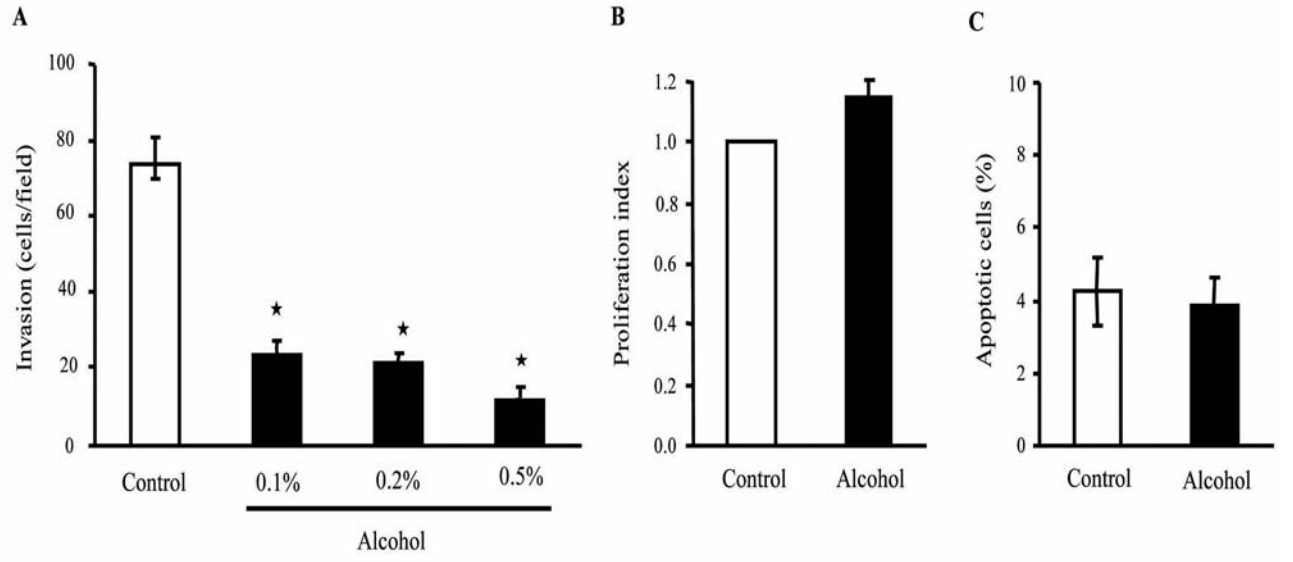

Figure 3. *Effects of ethanol on B16-BL6 cell invasion, cell proliferation and apoptosis. In the invasion assay, cells were treated with no ethanol, 0.1%, 0.2%, or 0.5% ethanol for 24 h and the number of cells that invaded in response to 5% FBS as chemoattractant was quantified. Cell proliferation was determined by the MTT assay, and cell apoptosis was determined by Annexin V staining using flow cytometry. Effects of ethanol on B16-BL6 cell invasion (A), proliferation (B), and apoptosis (C). The data is representative of at least three separate experiments. Values are given as means±SEM (n=3; *p<0.05).*

ethanol affects the tumorigenicity of melanoma, we measured the expression of genes associated with EMT and also known to modulate cell motility, invasion, and anchorage-independent growth (33). Many genes associated with the mesenchymal phenotype, including integrins, matrix metalloproteinases (MMPs), vimentin, and transcription factors, were reduced by ethanol exposure, although no change was significant (data not shown, $p>0.05$). The genes that were down-regulated by ethanol included *Snai1*, *Integrin, alpha V* (Itgav), and *Mmp9*. One reason for the lack of significance from the array results may be due to the fact that the sample size for each group was only four. Thus, in order to validate genes of interest on the array and to determine if the effects of ethanol were significant, we analyzed the expression of *Snai1* and its target E-cadherin by qRT-PCR. Results showed that *Snai1* expression was significantly reduced by ethanol (Figure 5A). Others have suggested that Snai1 increases the aggressiveness of cancer cells by down-regulating the expression of E-cadherin (34). Because Snai1 is known to repress E-cadherin expression and we showed that expression of Snai1 is decreased by ethanol,

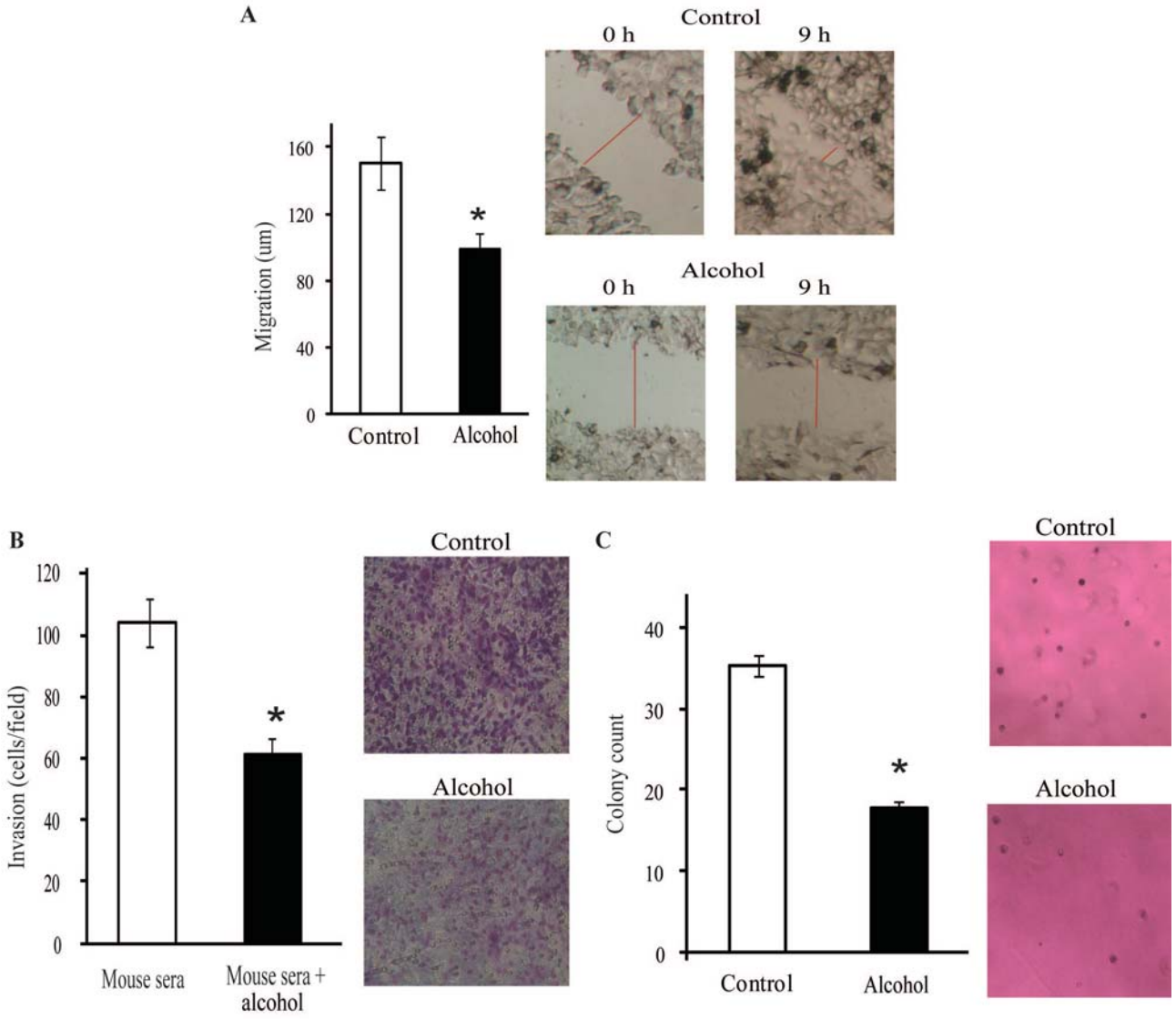

Figure 4. *Effects of ethanol on B16-BL6 cell migration, invasion, and anchorage-independent growth. In each assay, we treated B16-BL6 cells with 0.5% ethanol. In the migration assay, we determined the decrease in gap distance that occurred after 9 h as a measure of cell migration. In the invasion assay, 5% mouse serum was used as the chemoattractant instead of 5% FBS. Anchorage-independent growth was assessed by the soft-agar assay. Effects of ethanol on B16-BL6 cell migration (A), invasion (B), and anchorage-independent growth (C). The data are representative of at least three separate experiments. Values are given as means±SEM (n=3; *p<0.05).*

we assessed the effect of ethanol on the levels of E-cadherin. Indeed, results showed that E-cadherin expression was increased by ethanol exposure (Figure 5A). Interestingly, we observed that *E-cadherin* mRNA expression was increased as early as 12 h (with a 1.6-fold increase, $p<0.05$) and persisted for 36 h (with a 4.1-fold increase, $p<0.05$) compared to control. Furthermore, protein levels of Snai1 and E-cadherin were determined by immunofluorescense microscopy. We showed that protein levels of Snai1 in the nucleus were reduced by ethanol treatment, while E-cadherin expression at the cell membrane was increased by ethanol (Figure 5B).

Next, to determine how ethanol reduces *Snai1* expression, we measured the expression of *Nfkb*, which has been shown to increase the expression of Snai1 in some cancer cells (35). For this purpose, we determined the mRNA expression level of the p65 subunit of *Nfkb* (RelA). Furthermore, because Il6 has been shown to up-regulate Nfkb expression in epithelial cells, we also measured *Il6* mRNA levels in our B16-BL6 cells (36). Our results showed that the mRNA expression of *Nfkb* and *Il6* were reduced by ethanol exposure (Figure 5A). Thus, ethanol may reduce the expression of *Snai1* by decreasing both *Nfkb* and *Il6*.

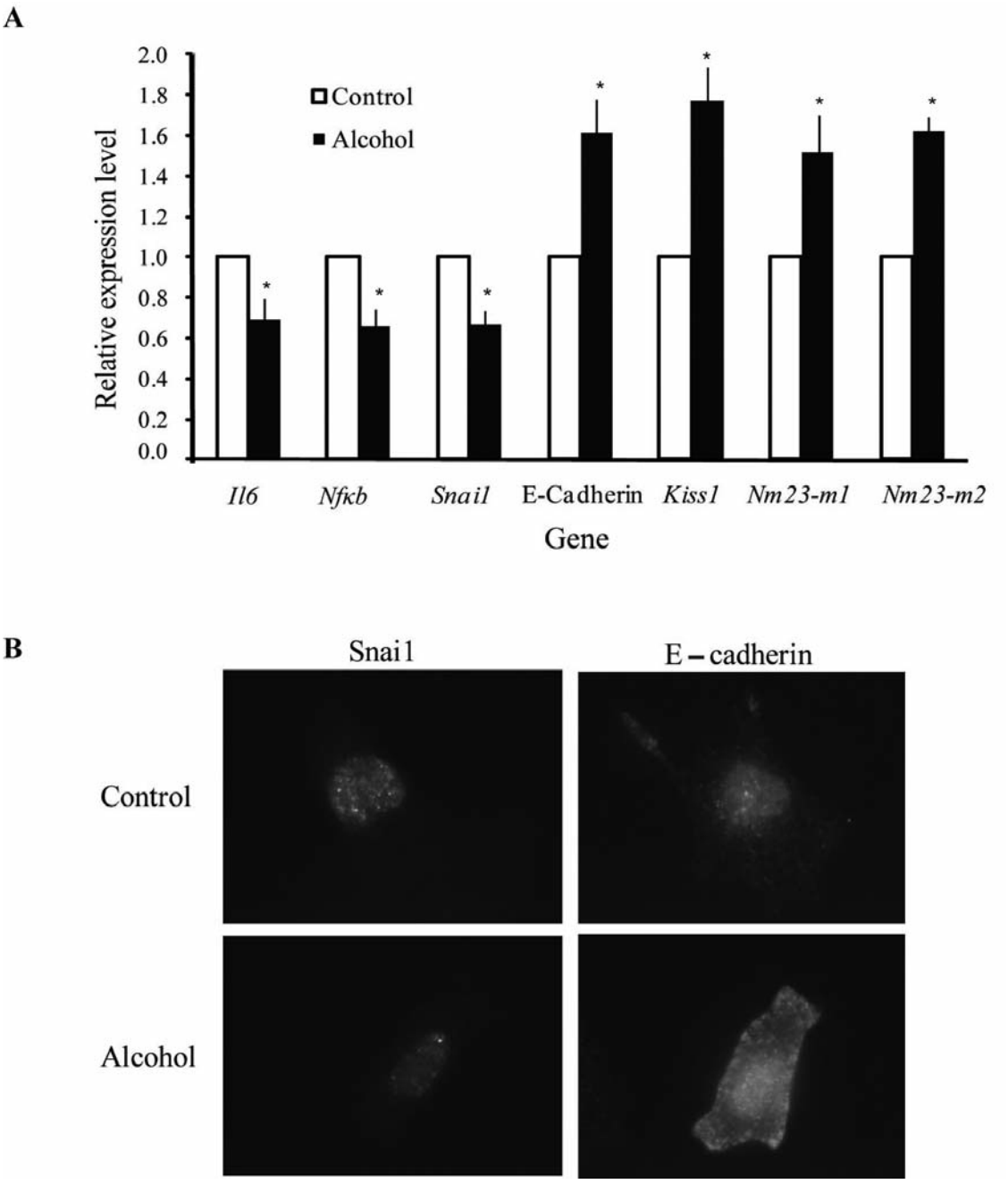

Figure 5. *Effects of ethanol on gene expression. B16-BL6 cells were treated with 0.5% ethanol for 24 h before being subjected to qRT-PCR or immunofluorescence microscopy. A: Effects of ethanol on Il6, Nfkb, Snai1, E-cadherin, Kiss1, Nm23-m1, and Nm23-m2 mRNA levels. B: Effects of ethanol on Snai1 and E-cadherin protein levels as measured by immunofluorescence. The data are representative of at least three separate experiments. Values are given as means±SEM (n=3; *p<0.05).*

Effects of ethanol on the metastasis suppressor genes Kiss1 and Nm23. Alternatively, it is feasible that ethanol affects the expression of *Snai1* and *E-cadherin*, while simultaneously increasing the expression of genes known to inhibit metastasis. Metastasis suppressor genes encode proteins that have the ability to slow down or prevent cancer metastasis (21). *Kiss1* and *Nm23* are metastasis suppressor genes known to inhibit metastasis of melanoma (37, 38). Furthermore, it has been suggested that the mesenchymal phenotype is associated with a down-regulation of *Kiss1* and *Nm23* (24). Our results showed that expression of mRNA for both *Kiss1* and *Nm23* was increased by ethanol in B16-BL6 melanoma (Figure 5A).

Discussion

Consistent with previous reports, we showed that consumption of 20% ethanol inhibited metastasis of melanoma in mice (8). This inhibition was associated with a significant lower body weight in ethanol-consuming mice. Ethanol-consuming mice also had lower systemic insulin and leptin levels. Consistent with our findings, other groups have reported that chronic ethanol users have lower levels of insulin and leptin (39, 40). A lower body weight has been associated with the inhibition of the progression of many other types of cancer, and obesity has been shown to promote metastasis of melanoma (41, 42). Thus, it is conceivable that ethanol inhibited metastasis of melanoma in our study by promoting a lower body weight. However, Meadows *et al*. showed that the inhibition of metastasis by ethanol is not associated with a lower body weight in 20% ethanol-consuming C57BL/6J mice (8). The discrepancy between our results and those of Meadows *et al*. may be due to the fact that the mice in our study were C57BL/6J ob/+ mice and those in the study of Meadows *et al*. were C57BL/6J +/+ mice: the former have only one functional copy of leptin compared to the latter, which have two copies of leptin. We show that ethanol reduced circulating levels of insulin and leptin in our mice. Other studies have shown, however, that ethanol consumption can increase levels of leptin in C57BL/6J mice (43). This discrepancy could be due to the difference in leptin copy number. In our mouse model, however, ethanol may inhibit the availability of leptin to melanoma cells; leptin is a risk factor for melanoma (44). Furthermore, other studies have shown that leptin promotes invasiveness of kidney and colonic epithelial cells (45), and we hypothesize that it may have a similar effect on melanomas. However, in a leptin-deficient ob/ob mouse model, metastasis of melanoma is still greatly increased (42); thus, there may be more important factors besides leptin that are responsible for increased metastasis.

In regards to insulin, other groups have shown that melanoma tumor-bearing mice that consumed ethanol did indeed have lower levels of insulin than their water-drinking counterparts (43). Other studies show that melanoma cells are insensitive to the mitogenic effects of insulin (46); however, still others show that insulin may also be a pro-metastatic factor because overexpression of the insulin receptor substrate-1 (IRS-1) leads to increased mammary tumorigenesis and metastasis (47). Similarly, insulin-like growth factor (IGF-1) can stimulate melanoma cell invasion (48), and there is much crosstalk between the insulin and IGF-1 pathways (49). Thus, it currently remains to be determined if weight loss and alterations in these factors, due to ethanol consumption, may be a contributing factor in the effect of ethanol on metastasis of melanoma.

To determine if the effect of ethanol on the tumorigenicity of B16-BL6 melanoma cells can be observed in the absence of the systemic effects of ethanol on body weight and circulating factors discussed previously, we determined if acute ethanol exposure affected the ability of melanoma cells to migrate, invade, and grow in an anchorage-independent manner under cell culture conditions (50). Our results showed that ethanol inhibited the ability of melanomas to migrate, invade and grow in an anchorage-independent manner without affecting cell proliferation or cell death.

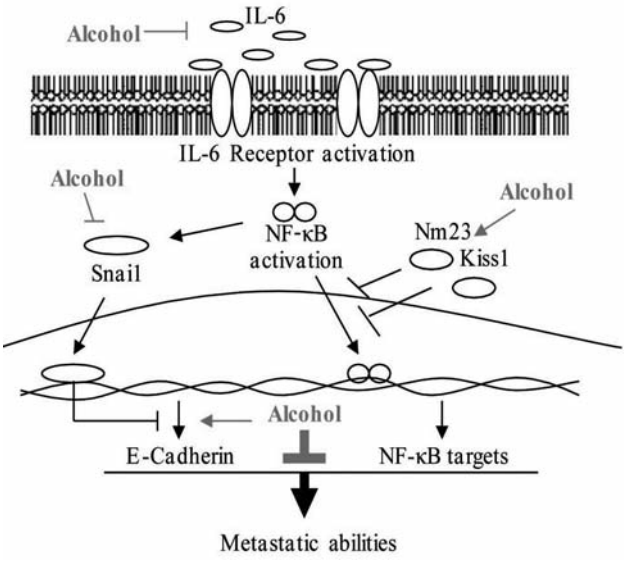

Figure 6. *Hypothesized mechanisms by which ethanol inhibits B16-BL6 melanoma metastasis. Ethanol reduces available IL-6, which lowers IL-6 receptor activation in melanoma cells. This may lead to lower NF-kB activation and subsequent reduced stabilization of pro-metastatic Snai1. Snai1 repression of E-cadherin is alleviated, allowing more cell-to-cell contact and thus inhibiting invasion. Ethanol may also simultaneously increase the expression of metastasis suppressors Nm23 and Kiss1, which may repress NFkB activation of Itgav and matrix metalloproteinases. However, further studies are required to confirm this hypothesis. ⊣ inhibition, → promotion.*

We proceeded to determine if ethanol affects the expression of genes associated with EMT and those also known to modulate the aggressiveness of the cancer cells (*e.g*. invasion) (33). EMT plays an important role in the metastatic ability of cancer cells (51). Melanomas with an epithelial phenotype are less aggressive than those with a mesenchymal phenotype (52). Genetic markers for the epithelial phenotype are high expression of genes such as *E-cadherin* and various cytokeratins (53). Cancer cells that transition to the mesenchymal phenotype usually lose the expression of these genes and acquire the expression of genes that makes them more metastatic (54). Genes linked to the mesenchymal phenotype are high expression of *Snai1, Itgav,* and *Mmp9*, which have been shown to increase the metastatic ability of melanoma (52, 55). We show that these genes were down-regulated by ethanol in the array. Of interest, the transcription factor Snai1 has been shown to

increase the metastatic ability of melanomas; Snai1 increases the metastatic ability of cancer cells by reducing the expression of E-cadherin (34). Studies by others show that overexpression of Snai1 in cancer cells promotes the transition from an epithelial to a mesenchymal phenotype; moreover, overexpression of Snai1 increases the migration and invasive ability of cancer cells (34). E-Cadherin plays an important role in cell-to-cell adhesion; thus, loss of E-cadherin decreases the adhesion of cancer cells to each other, which increases their aggressiveness and ability to metastasize (56). We showed that ethanol reduced the expression of *Snai1* in B16-BL6 melanoma cells and, in contrast, increased the expression of *E-cadherin* in B16-BL6 melanoma cells. It is not clear how ethanol modulates the expression of these genes, however, others have shown that ethanol can increase cell membrane fluidity and that ethanol itself can modulate the activity of various transcription factors (19, 20). Currently, the effect of alcohol on *Snai1* expression in melanoma cells is not known. Thus, it is feasible that ethanol alters the tumorigenicity of melanoma *via* transcription factors, such as Snai1. On the other hand, ethanol may reduce the expression of *Snai1* in an indirect manner, by reducing the expression of factors known to increase its expression. Both Nfkb and Il6 have been shown to increase the expression of *Snai1*, and our results showed that both are significantly reduced by ethanol exposure in B16-BL6 melanoma cells (57, 58). IL-6 may be an important factor for melanoma cells as others show some human melanoma cell lines endogenously produce detectable levels of IL-6 in an autocrine fashion (59). Thus, it is possible that ethanol inhibits the metastatic ability of melanomas by reducing the levels of IL-6, Nfkb, and ultimately Snai1.

Alternatively, ethanol may affect EMT and consequently increase the expression of genes known to inhibit metastasis. Genes that have been identified to inhibit or slow down metastasis are known as metastasis suppressor genes (60). We measured the expression levels of *Kiss1* and *Nm23*, both of which have been shown to inhibit the metastasis of melanoma and whose expression has been shown to be reduced during EMT (24, 37-38). Kiss1 may inhibit the invasive ability of melanomas by reducing the expression of MMPs, such as Mmp9 (61). MMPs are critical for the degradation of extracellular matrix proteins which facilitate metastasis (62). Kiss1 may reduce Mmp9 expression by preventing the binding of Nfkb to the *Mmp9* promoter (61). Others have shown that overexpression of Nm23 inhibits tumor cell motility, invasion, and anchorage-independent growth in some cancer cells (63, 64). Nm23 may inhibit metastasis by reducing the expression of genes that aid in the establishment of tumors at secondary tissue, such as the *Itgav* (65). Itgav is an integrin protein that helps melanomas attach to endothelium cells in the blood vessels, which is an essential step in the metastatic cascade. Nm23 may block the expression of Itgav by inhibiting the binding of Nfkb to the *Itgav* promoter (66). Consistent with the inhibition of the metastatic ability of melanomas by ethanol, both *Kiss1* and *Nm23* expressions were increased by ethanol exposure.

Figure 6 depicts our proposed mechanism by which ethanol exposure may inhibit the metastatic ability of melanomas. First, we propose that ethanol reduces IL-6 expression, which leads to down-regulation of Nfkb. This effect of ethanol may lead to a lower expression of Snai1 and subsequent increase in E-cadherin. Furthermore, we showed that the expression of both metastasis suppressor genes *Kiss1* and *Nm23* are increased by ethanol in B16-BL6 melanoma cells, which may reduce the expression of factors that promote metastasis, such as *Itgav* and MMPs (61, 65).

Conclusion

In summary, we propose possible means by which ethanol may inhibit B16-BL6 metastasis of melanoma in the present study. Ethanol consumption may reduce pulmonary metastasis of melanoma by reducing body weight and the availability of circulating leptin and insulin in the serum of these mice. In cell culture, ethanol exposure also reduced the metastatic cell phenotypes of migration, invasion, and anchorage-independent growth. Among the many effects of ethanol, it may reduce the metastatic ability of melanoma cells by affecting the expression of EMT-associated genes such as *Snai1*. Furthermore, we showed that ethanol exposure may be used as a tool in the identification of proteins involved in metastatic processes of melanomas such as migration and anchorage-independent growth, as it correctly identified and strengthened the importance of known genes, such as *Snai1*, and the metastasis suppressors in our study.

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