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# Chronic Alcohol Exposure Affects Mammary Stemness and Alters the Mammary and Tumor Transcriptomes of MMTV-Wnt1 Adult Mice

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## ABSTRACT

**Background:** Alcohol consumption is a known risk factor for breast cancer, but the mechanisms that cause this increased risk remain elusive. Mammary epithelial stem-like and progenitor cells exhibit plasticity and are susceptible to malignant transformation that may contribute to tumorigenesis.

**Methods:** Seven-week-old MMTV-Wnt1 female mice were given water ± alcohol, and preneoplastic mammary glands (MGs) were collected after 8 weeks of treatment. Tumors were collected at a diameter of 1.5 cm and analyzed by hematoxylin and eosin. Dissociated cells from tumors and MGs were analyzed by flow cytometry and used for sphere-forming assays. Transcriptomic analysis was performed on RNA isolated from tumor tissue, dissociated MGs, and mammospheres.

**Results:** Alcohol increased the proportion of luminal progenitor cells in preneoplastic MGs while mammosphere forming efficiency (MFE) was decreased. Over 1000 genes were changed by alcohol in the mammospheres, producing an environment of increased keratinization and cell junction organization. Alcohol affected 30 genes in dissociated mammary gland (dMG) cells, including ion channel proteins, transcription factors, immune genes, and ncRNA. *Gabrp*, a marker of triple-negative breast cancer (TNBC), was increased by alcohol in both dMG cells and mammospheres. Alcohol decreased expression of the tumor suppressor *Ptprd* and mitotic count in tumors.

**Conclusions:** Alcohol consumption in adulthood induced a less proliferative phenotype in mammary stem-like/progenitor cells and tumors and caused major changes in the transcriptome of stem-like cells. This may have implications for the types of breast cancers that form in women who drink alcohol.

## 1 | Introduction

Breast cancer is the most common cancer in women worldwide. According to the World Health Organization, approximately 2.3 million women were diagnosed with breast cancer in 2022 (World Health Organization 2024). The American Cancer Society predicts that in 2026, about 321,910 new cases of invasive breast cancer will be diagnosed in women and about 42,140 women will die from the disease (Siegel et al. 2026). Although many known risk factors for breast cancer are nonmodifiable, including sex, age, and family history, some risk factors such as alcohol consumption can be managed through lifestyle choices. According to the U.S. Surgeon General's Advisory on Alcohol and Cancer Risk 2025, alcohol use contributes to approximately 100,000 cancer cases and 20,000 cancer deaths each year. The absolute risk of breast cancer increases from 11.3% in women who consume less than 1 drink per week to 15.3% in those who consume 2 drinks per day. In addition, there is a direct link between alcohol use and increased risk for at least seven different cancers,

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including breast cancer (Murthy 2025). The fact that 48.6% of adult women reported drinking alcohol in the past month (SAMHSA 2023) coupled with the recent report that young women ages 18–25-years of age are now binge drinking more than their male peers (Shuey et al. 2025) emphasizes the critical need to research and understand the link between alcohol use and breast cancer.

The mammary gland is a dynamic organ capable of repeated rounds of proliferation due to the presence of mammary stem cells (MaSCs) (Dontu et al. 2003; Kordon and Smith 1998). These long-lived, multipotent cells have the ability to self-renew and proliferate, making them particularly vulnerable to tumor-promoting insults and/or mutations. Such changes are thought to transform these MaSCs into tumor-initiating cells from which breast cancers may arise (Al-Hajj et al. 2003). In the MMTV-Wnt1 model of basal-like breast cancer (Tsukamoto et al. 1988), tumors arise from an expansion of the stem cell population (Li et al. 2003; Liu et al. 2004). Several studies have used this model to show that alcohol enhances mammary tumorigenesis, metastasis, and the cancer stem cell population (Li et al. 2024; Xu et al. 2022, 2023). The goal of the current study was to further investigate the effect of alcohol on stem-like cells by analyzing the proportion of basal, luminal, and luminal progenitor cells in the preneoplastic mammary glands and tumors of MMTV-Wnt1 adult mice and characterize the transcriptome of the stem-like cells that comprise mammospheres relative to that of the dissociated MG and whole tumor tissue.

## 2 | Materials and Methods

### 2.1 | Experimental Design

All animal protocols were approved by Rutgers University Institutional Animal Care and Use Committees (IACUC 15-023). MMTV-Wnt1 female mice on an FVB/NJ background (FVB.Cg-Tg(Wnt1)1Hev/J) were obtained from an in-house breeding colony for this study (Saboya et al. 2020). Animals were housed in a controlled environment with a 12-h light:dark cycle. Mice were group housed with a maximum of 4 mice per cage and were given ad libitum access to standard mouse chow. At 7 weeks of age, mice were assigned to either the alcohol or control group. Animals were acclimated to the alcohol by providing 5% alcohol with 0.2% (w/v) saccharin (Fisher Scientific, Hampton, NH) in place of water from days 1 to 3 and 10% alcohol with 0.2% (w/v) saccharin from days 4 to 9. Starting on day 10, mice in the alcohol group were given 20% alcohol with 0.2% (w/v) saccharin for the remainder of the study. Control animals were given 0.12% (w/v) saccharin in place of water for the duration of the study. Mice ($n = 25$ for alcohol and $n = 24$ for control) were palpated twice a week to track tumor formation, and tumors were harvested when they reached an average diameter of 1.5 cm. Body weights of all animals were recorded once per week. Fluid intake was monitored for each cage daily. To ensure the recovery of uncontaminated tissue, mice were euthanized by rapid decapitation in accordance with guidelines of the American Veterinary Medical Association. Half of the tumor was fixed in 10% neutral buffered formalin (NBF, Thermo Fisher Scientific, Waltham, MA) and the other half was either flash frozen in liquid nitrogen for subsequent gene expression analysis or partially dissociated for flow cytometry analysis. A subset of mice was sacrificed after 8 weeks on treatment (including 9 days of acclimation, $n = 11$ for alcohol and $n = 10$ for control). Mammary glands were harvested and dissociated as described below.

### 2.2 | Histology

Tissues were fixed, dehydrated, and embedded in paraffin as previously described (Saboya et al. 2020). Tumors were sectioned at 5 μm, placed on Superfrost Plus microscope slides (VWR/Avantor, Allentown, PA), and processed and stained with hematoxylin and eosin (Electron Microscopy Sciences, Hatfield, PA) as previously described (Saboya et al. 2020). Tumor pathology was evaluated by a toxicological pathologist at the Environmental Occupational Health Sciences Institute at Rutgers University (New Brunswick, NJ).

### 2.3 | Tumor and Mammary Gland Dissociation

Tumors were processed as described previously (Saboya et al. 2020) with the following modifications: tumors were dissociated in Dulbecco's Modified Eagle Medium (DMEM)/F12 (GE Life Sciences, Pittsburgh, PA) containing 300 U/mL collagenase, 100 U/mL hyaluronidase (STEMCELL Technologies, Vancouver, BC, Canada) and 50 μg/mL gentamicin (Sigma-Aldrich, St. Louis, MO). After dissociation and red blood cell lysis, tumor organoids were frozen in DMEM/F12 media containing 10% FBS (R&D Systems Inc., Minneapolis, MN) and 10% DMSO (Sigma-Aldrich) for a slow freeze and stored at −80°C until further use. Upon thawing, organoids were resuspended in 10 mL prewarmed DMEM/F12 media and spun at 1200× g for 10 min and further dissociated into a single-cell suspension as previously described (Saboya et al. 2020) with the following modifications: samples were centrifuged for 10 min at 1400 rpm at 4°C and resuspended in prewarmed 5 U/mL dispase plus 1 mg/mL DNase I. Once isolated, tumor epithelial cells were stained for flow cytometry, as well as plated for tumorsphere culture.

At sacrifice, left and right thoracic and inguinal mammary glands were harvested, and lymph nodes were removed. Dissociated mammary gland (dMG) cell isolation followed procedures as previously described (Saboya et al. 2020) with the following modification: mammary glands were dissociated in gentle collagenase (1 mg/mL)/hyaluronidase (100 U/mL) solution (STEMCELL Technologies) in DMEM/F12 containing 50 μg/mL gentamicin. Cells were further dissociated into a single-cell suspension as described previously (Saboya et al. 2020) and above. Freshly isolated cells were immediately stained for flow cytometry analysis, as well as plated for mammosphere culture. The remaining cells were stored at −80°C for RNA isolation.

### 2.4 | Flow Cytometry Analysis

Flow cytometry analysis of tumor and dMG cells was performed as previously described (Saboya et al. 2020). Briefly,

cells were gated for debris (based on forward and side scatter properties), doublets, lineage-positive cells (TER1119, CD45, and CD31), and viability (DAPI). Tumor epithelial cells were analyzed by flow cytometry to analyze the proportion of luminal ($CD24^{+}CD29^{lo}$), basal ($CD24^{+}CD29^{hi}$), and luminal progenitor ($CD24^{+}CD29^{hi}CD61^{+}$) populations. dMG cells were analyzed by flow cytometry to analyze the proportion of luminal ($CD24^{+}CD49f^{lo}$), basal ($CD24^{+}CD49f^{hi}$), and luminal progenitor ($CD24^{+}CD49f^{lo}CD61^{+}$) populations. All antibodies used for flow cytometry were obtained from BD Biosciences (San Jose, CA), except for CD49f and CD61, which were purchased from BioLegend (San Diego, CA).

### 2.5 | Sphere Forming Assays

Mammosphere/tumorsphere forming efficiencies (MFE/TFE) were calculated by culturing dMG and tumor cells as previously described (Saboya et al. 2020) with the following modifications: freshly isolated dMG cells were plated in serum-free DMEM/F12 medium containing B27 supplement (without vitamin A) (Thermo Fisher Scientific), 20 ng/mL human recombinant epidermal growth factor, 20 ng/mL human recombinant basic fibroblast growth factor, 10 μg/mL heparin (STEMCELL Technologies), and 50 μg/mL gentamicin. For all assays, tertiary sphere formation was analyzed.

### 2.6 | RNA Isolation

For RNA isolation from flash frozen tumor tissue, 10 mg tissue was placed in Beadbug tubes (Benchmark Scientific Inc., Sayreville, NJ) with 1 mL Trizol (Thermo Fisher Scientific) and dissociated in a Beadbug Microtube Homogenizer (Benchmark Scientific). Tumor RNA was then isolated using the Macherey-Nagel NucleoSpin RNA kit (Bethlehem, PA). RNA was isolated from dMG cells using the Qiagen RNeasy Micro kit per manufacturer instructions (Germantown, MD). RNA was isolated from mammospheres using the Arcturus PicoPure RNA Isolation Kit (Thermo Fisher Scientific). RNA concentration was measured using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). RNA integrity was assessed using an Agilent 2100 Bioanalyzer with the Agilent RNA 6000 Nano or Pico kit (Agilent Technologies, Santa Clara, CA).

### 2.7 | RNA Sequencing

Standard (tumor tissue and dMG cells) and ultra-low input (mammospheres) RNA sequencing were performed by Azenta Life Sciences (South Plainfield, NJ).

For standard RNA sequencing, total RNA samples were quantified using a Qubit 3.0 Fluorometer (Life Technologies, Carlsbad, CA), and RNA integrity was checked using Agilent TapeStation 4200 (Agilent Technologies). RNA sequencing libraries were prepared using the NEBNext Ultra II RNA Library Prep Kit for Illumina using the manufacturer's instructions (NEB, Ipswich, MA). Briefly, mRNAs were initially enriched with oligo(dT) beads. Enriched mRNAs were fragmented for 15 min at 94°C. First-strand and second-strand cDNA were subsequently synthesized. cDNA fragments were end-repaired and adenylated at 3′ ends, and universal adapters were ligated to cDNA fragments, followed by index addition and library enrichment by PCR with limited cycles.

For ultra-low input RNA sequencing, SMART-Seq v4 Ultra Low Input Kit for Sequencing was used for full-length cDNA synthesis and amplification (Clontech, Mountain View, CA), and Illumina Nextera XT library (Illumina, San Diego, CA) was used for sequencing library preparation. Briefly, cDNA was fragmented, and an adaptor was added using transposase, followed by limited-cycle PCR to enrich and add an index to the cDNA fragments.

The sequencing libraries (both standard and ultra-low input) were validated on the Agilent TapeStation (Agilent Technologies), and quantified by using the Qubit 3.0 Fluorometer (Invitrogen, Carlsbad, CA) as well as by quantitative PCR (KAPA Biosystems, Wilmington, MA). The sequencing libraries were then multiplexed and clustered onto a flow cell on the Illumina NovaSeq instrument according to the manufacturer's instructions. The samples were sequenced using a 2×150bp Paired-End (PE) configuration. Image analysis and base calling were conducted by the NovaSeq Control Software (NCS). Raw sequence data (.bcl files) generated from Illumina NovaSeq were converted into FASTQ files and de-multiplexed using Illumina bcl2fastq 2.20 software. One mismatch was allowed for index sequence identification.

### 2.8 | Bioinformatics Analysis

Standard RNA analysis was performed by Azenta Life Sciences. Briefly, sequences were evaluated for quality using FASTQC, and poor-quality regions and possible adaptor sequences were trimmed from the reads using Trimmomatic v.0.36. Trimmed reads were aligned to the *Mus musculus* GRCm38 reference genome with the STAR aligner v.2.5.2b, generating BAM files. Hit counts were calculated with FeatureCounts from the Subread package v.1.5.2. The gene hit counts table was used for downstream differential expression analysis between groups using DESeq2 (Love et al. 2014). The Wald test was used to generate multiplicity-adjusted *p*-values (*p*adj) and $\log_2$ fold changes ($\log_2$FC); genes with *p*adj $<0.05$ and an absolute $\log_2\text{FC} > 1$ were considered statistically significant differentially expressed genes (DEGs).

### 2.9 | Statistical Analysis

For data other than RNA sequencing, GraphPad Prism version 10.0 (Boston, MA) was used for statistical analyses and $p \leq 0.05$ was considered significant. For analysis of the tumor mitotic count, the Mann–Whitney test was used. Other comparisons between alcohol and control groups were analyzed using an unpaired Student's *t*-test. Two-tailed correlation analysis was performed, assuming a Gaussian distribution. For analysis of the composition of epithelial cell populations, the Holm-Šídák correction for multiple comparisons was used. Significance of the Kaplan–Meier curve was determined using the log-rank Mantel-Cox test.

## 3 | Results

### 3.1 | Effect of Alcohol on Tumor Latency and Phenotype

To determine if alcohol consumption alters the time course of tumor development, animals were palpated twice weekly following the initiation of treatment. Tumors in alcohol and control animals developed with median latencies of 23 and 31 weeks, respectively (Figure 1). While alcohol tended to decrease the latency of tumors that formed prior to 30 weeks of treatment, overall tumor latency was not significantly affected. Both treatment groups presented with tumors classified as adenoma or carcinoma (Figure 2a); however, tumors from alcohol animals had significantly decreased mitotic counts relative to controls (Figure 2b).

### 3.2 | Alcohol Increases Luminal Progenitor Cells and Decreases Mammosphere Forming Efficiency in Hyperplastic Mammary Glands

Since luminal progenitor cells are reported to be the cell of origin for Wnt-1 tumors (Vaillant et al. 2008), dissociated mammary cells isolated from preneoplastic glands after 8 weeks of treatment were analyzed by flow cytometry and plated for mammosphere forming efficiency. Alcohol treatment had no effect on the proportion of luminal or basal cells (Figure 3a) but significantly increased the percentage of luminal progenitor cells (Figure 3b). Alcohol did not affect the luminal, basal, or luminal progenitor cell populations within the tumors (Supplementary Figure 1a,b). Dissociated mammary gland (dMG) cells isolated from alcohol animals had a lower tertiary mammosphere forming efficiency compared to the control animals (Figure 3c). There was no difference in tumorsphere forming efficiency between the two treatment groups (Supplementary Figure 1c).

### 3.3 | Alcohol Affects the Transcriptome of Mammary Gland Cells and Mammospheres

Transcriptomic analysis was performed on control and alcohol dMG cells ($n = 9$ and 10, respectively) and the mammospheres ($n = 3$ alcohol and 3 control) cultured from those cells. Results show that there were 30 significant DEGs ($p$adj $< 0.05$ and absolute $\log_2$ fold change $> 1$) in the dMG cells of animals exposed to alcohol for 8 weeks (Figure 4 and Table 1). Twenty-one of the significant DEGs in the dMG cells were upregulated and 9 were downregulated. Significant DEGs of interest included homeobox transcription factors *Hoxb9* and *Hoxb7*; noncoding RNAs *Dnm3os* and *Snora34*; tumor suppressors *Spink5*, *Slurp1*, and *Synpo2*; *Ccnd1* (cyclin D1); *Muc16* (Ca125); and voltage-gated ion channels *Kcnab1* and *Gabrp*. Ingenuity pathway analysis (IPA; Qiagen) (Kramer et al. 2014) was used to analyze the data set. Since only 30 DEGs were identified, a relaxed threshold of $p$adj $< 0.11$ was applied to uncover potential important links that might guide further research. This provided 457 analysis ready molecules for IPA (Kramer et al. 2014). As shown in Figure 5, *Dnmt1* was the top upstream regulator with 36 out of 440 known target genes in the data set, including *Ptprd*; *Cacna1c*, *Cacnb1*, and *Cacng4* (calcium voltage-gated ion channels); *Ccnd1*, *Hoxb7*, *Hoxb9*, and *Meg3*. The top pathways identified by IPA contained genes that encode calcium and potassium channel proteins, collagen proteins, and solute carrier (SLC) member proteins (Figure 6).

As shown in Figure 7, there were 1031 significant DEGs in the mammospheres cultured from dMG cells of animals exposed to alcohol for 8 weeks (Supplementary Table 1). The top 10 upregulated and top 10 downregulated significant DEGs are listed in Table 2. Seven hundred thirty-five of the significant DEGs in the mammospheres were upregulated and 296 were downregulated by alcohol exposure relative to controls. When IPA was used to analyze the data set ($p$adj $< 0.05$), the top pathways activated by alcohol were keratinization and cell junction organization (Figure 8).

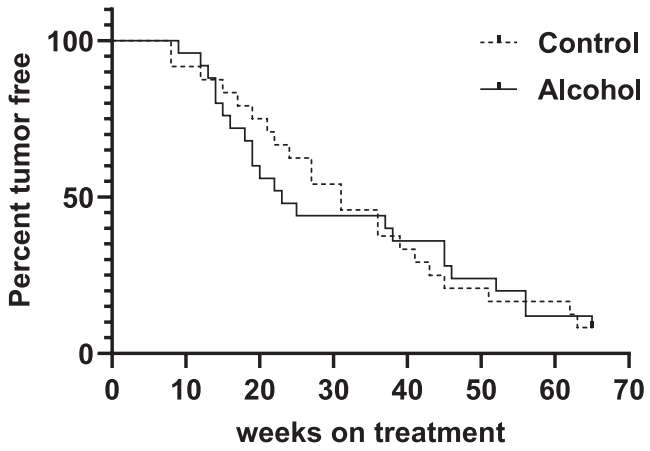


**FIGURE 1** | Effect of alcohol treatment on tumor latency. Mice were palpated twice per week to detect mammary tumor formation. Graph depicts time on treatment (in weeks) when a tumor was first found ($n = 25$ and 24 for alcohol and control groups, respectively).

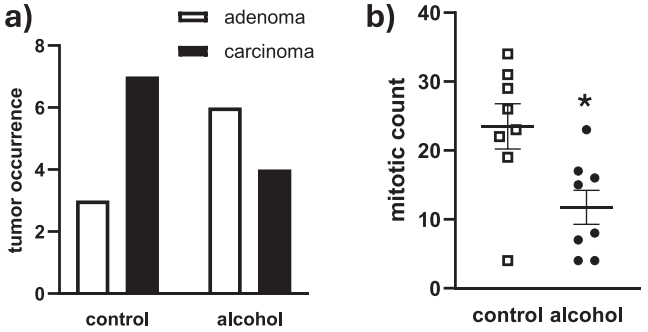


**FIGURE 2** | Effect of alcohol treatment on tumor pathology. Mammary tumors stained with hematoxylin and eosin were analyzed by a pathologist. (a) Tumors were designated by a pathologist to contain either adenomatous glandular elements (adenoma) or foci of unequivocal carcinoma ($n = 10$ for both alcohol and control groups). (b) Mitotic count is the number of cells undergoing mitosis counted per 10 high power fields. ($n = 8$ for both alcohol and control groups). Bars represent mean ± SEM. Mann–Whitney test, * indicates $p < 0.05$.

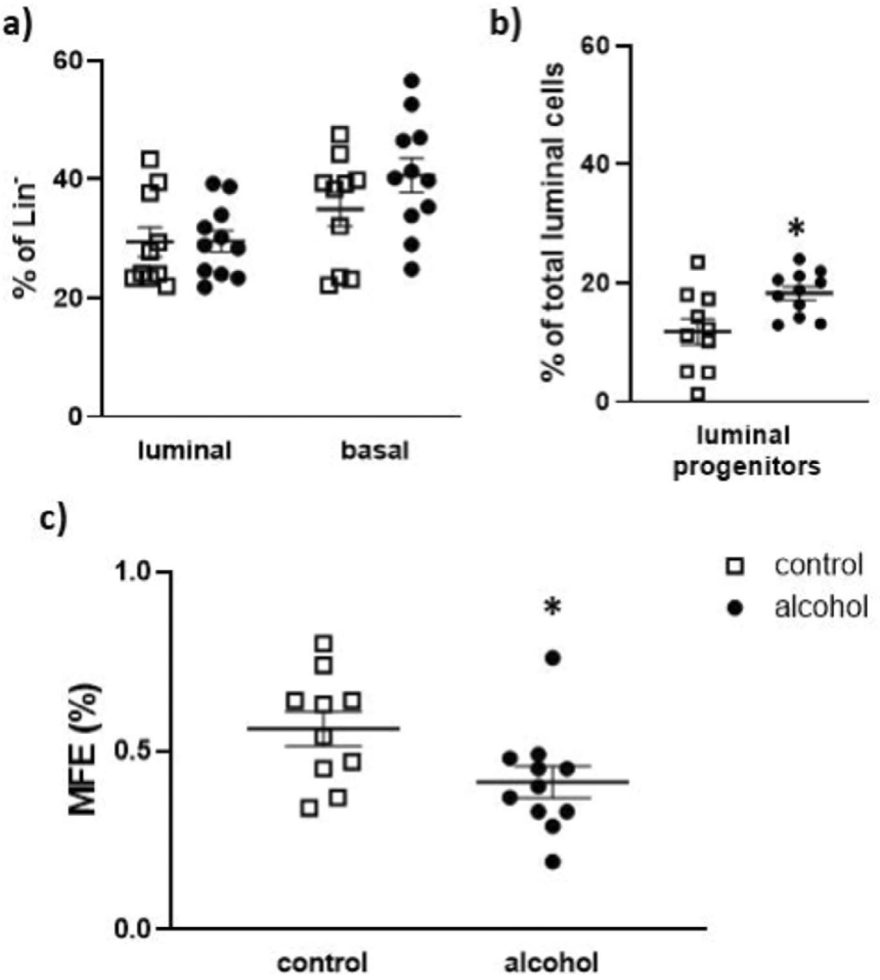


**FIGURE 3** | Composition of the mammary epithelial cell populations and mammosphere forming efficiency (MFE) of epithelial cells isolated from mammary glands (MGs) after 8 weeks of treatment. Mouse mammary epithelial cells were isolated from MGs of alcohol ($n = 11$) and control ($n = 10$) groups. (a) Approximately 500,000 cells were analyzed by flow cytometry. All cells that were lineage negative ($Lin^-$) and stained positive for CD24 were included for analysis. Luminal cells were identified as $Lin^-CD24^+CD49f^{lo}$. Basal cells were identified as $Lin^-CD24^+CD49f^{hi}$. (b) Luminal progenitor (LP) cells were identified as $Lin^-CD24^+CD49f^{lo}$ $CD61^+$. Unpaired Student's *t*-test, Holm-Šídák correction for multiple comparisons, * indicates adj $p < 0.05$. (c) Isolated mammary epithelial cells from alcohol ($n = 11$) and control ($n = 10$) animals were plated for mammosphere culture. MFE was calculated as a percentage of the average number of tertiary spheres per well divided by the number of cells plated. Bars represent mean ± SEM. Unpaired Student's *t*-test, * indicates $p < 0.05$.

### 3.4 | Alcohol Consumption Decreases Expression of Tumor Suppressor Ptprd in MMTV-Wnt1 Tumors

Transcriptomic analysis was performed on a subset of control (12–27 weeks on treatment; $n = 5$) and alcohol (12–20 weeks on treatment; $n = 6$) tumors. Chronic alcohol exposure resulted in one significant DEG, which was the tumor suppressor *Ptprd*, with expression decreased by alcohol ($p$adj = 0.000424; $\log_2$FC = −4.1). When a more relaxed cutoff of $p$adj < 0.11 was used for IPA analysis only one additional DEG was identified (Gm11413; $p$adj = 0.105915; $\log_2$FC = 4.04). Therefore, IPA analysis was not performed for tumors.

## 4 | Discussion

Alcohol is a known carcinogen, and alcohol consumption has been shown to increase the risk of several cancers including breast cancer (Li et al. 2010; Murthy 2025; Williams and Horm 1977). In the current study, chronic alcohol exposure did not significantly affect tumor latency. The window of exposure to alcohol is a critical factor affecting long-term breast cancer risk in women (Donat-Vargas et al. 2021). To this point, in another study using the MMTV-Wnt1 model, exposure to alcohol in adolescence decreased tumor latency, while, like the current study, latency was not affected when the treatment was initiated in adulthood (Xu et al. 2023). Interestingly, the latency curves appeared to be separated between treatment and control groups early in the present study, converging at approximately 31 weeks. This pattern has been reported previously for tumor latency in MMTV-Wnt1 mice where focal adhesion kinase was knocked out (Paul et al. 2020). Confirming whether alcohol exposure during adulthood in mice affects tumor latency will require studies with larger sample sizes.

While tumor characteristics such as grade and receptor status are informative for treatment decisions, transcriptomic molecular signatures are providing tremendous insight into breast

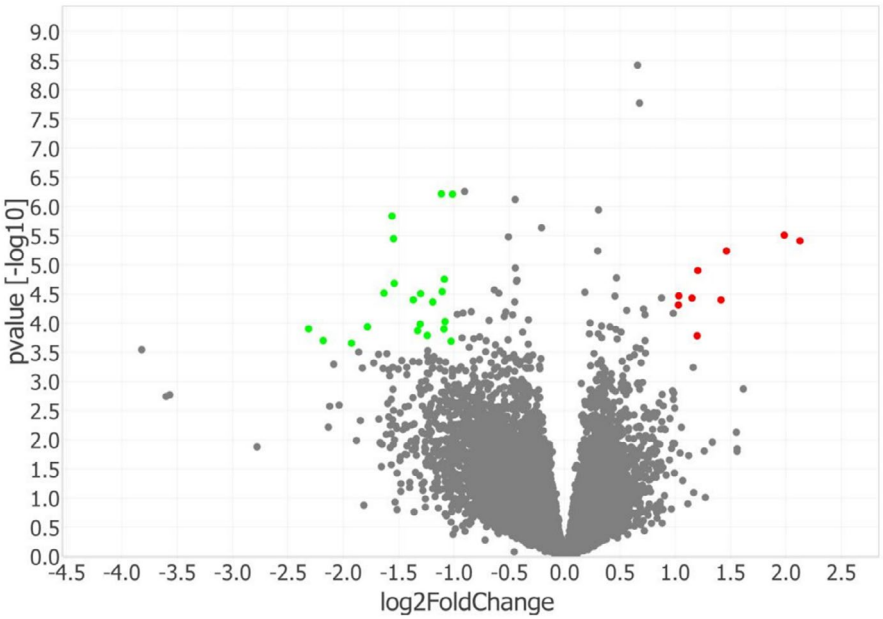


**FIGURE 4** | Volcano plot showing differentially expressed genes in dissociated mammary gland (dMG) cells isolated from alcohol exposed female MMTV-Wnt1 mice. Green dots represent downregulated genes, and red dots represent upregulated genes. Genes with an adjusted $p$-value $< 0.05$ and absolute $\log_2$ fold change $> 1$ were called as significant differentially expressed genes (DEGs).

tumor heterogeneity. In humans, pathway analysis of tumor transcriptomes coupled with reported information on alcohol consumption has added insight into alcohol-related breast tumorigenesis (Wang et al. 2017). Interestingly, the one gene found to be significantly affected ($p$adj = 0.000424) in the current study was the tumor suppressor *Ptprd*, which was decreased by alcohol. It should be noted that the tumor samples analyzed by RNA sequencing correspond to tumors that appeared relatively early on the tumor latency curves, where the alcohol and control curves appear to be separated. *Ptprd* has been shown to be inactivated in many human cancers including breast cancer (Cancer Genome Atlas 2012; Chan et al. 2008; Veeriah et al. 2009; Yu et al. 2017). In the current study, *Ptprd* expression also tended to decrease in alcohol-exposed dMG cells ($p$adj = 0.106; $\log_2$FC = −0.957). The importance of *Ptprd* in human breast cancer is reflected by the fact that even though only a small percentage of tumors analyzed have altered *Ptprd*, a greater percentage of those tumors are classified as basal type tumors compared to the unaltered group (Curtis et al. 2012; Pereira et al. 2016). In addition, a greater percentage of tumors in the *Ptprd* altered group are both ER and PR negative compared to the unaltered group (Curtis et al. 2012; Pereira et al. 2016; Razavi et al. 2018). IPA analysis indicated DNMT1 as the top upstream regulator in alcohol-exposed dMG cells, with *Ptprd* listed as one of 36 downstream targets. The alcohol-induced decrease in *Ptprd* expression could be via an epigenetic mechanism, as inactivation of *Ptprd* has been shown to occur through both mutation and hypermethylation (Chan et al. 2008; Veeriah et al. 2009). The possible epigenetic regulation of *Ptprd* by DNMT1 is supported by the finding that the expressions of *Ptprd* and *DNMT1* are negatively correlated in the tumors analyzed in the METABRIC dataset (Curtis et al. 2012; Pereira et al. 2016). *Ptprd* normally suppresses programmed death-ligand 1 (PD-L1) such that decreased *Ptprd* increases PD-L1 expression (Meng et al. 2020), leading to a better response to immune checkpoint inhibitor (ICI) therapy (Zhang et al. 2022). The PD-1/PD-L1 pathway has been shown to be increased by alcohol exposure in both neuroimmune cells and the mammary tumor microenvironment (Mishra et al. 2020; Xu et al. 2022). A connection between alcohol exposure and decreased *Ptprd* in tumors is potentially valuable information that could be utilized in the clinic when assessing tumors for appropriate treatments.

Tumor transcriptomic data provide a detailed picture of the end result of alcohol exposure. However, we hypothesized that analysis of the expanded and vulnerable stem and progenitor cell population in the MMTV-Wnt1 preneoplastic mammary gland might reveal alcohol-induced changes that preceded tumor formation. In support of this, flow cytometric analysis of dMG cells showed an increase in the luminal progenitor population in the mammary glands of mice exposed to chronic alcohol. Previous results from our laboratory showed a similar increase in the luminal progenitor population in dMG cells from pubertal MMTV-Wnt1 offspring exposed to alcohol in utero (Saboya et al. 2020). However, in the current study, these cells formed mammospheres with a lower efficiency than the control cells. This was unexpected since luminal progenitor cells are presumed to be stem-like in nature and mammosphere-forming efficiency is presumed to be a measure of stemness (Dontu et al. 2003). Interestingly, RNA-seq analysis of the spheres showed increased expression of six type I and 8 type II cytokeratins, including markers of both luminal (*Krt8*, *Krt18*) and basal (*Krt5*, *Krt14* and *Krt17*) cells, illustrating an altered state of the population. Thus, while luminal progenitor cells were increased by alcohol, the treatment induced changes in these cells that decreased their stem-like properties. Garbe et al. found that in the mammary gland, the process of aging also leads to an altered luminal progenitor population with increased basal marker keratin 14 (Garbe et al. 2012). In fact, the effect of alcohol on cellular age is an active field of research (Topiwala et al. 2022; Wang et al. 2023).

**TABLE 1** | Significant DEGs in dMG cells isolated from chronic alcohol-exposed female MMTV-Wnt1 mice.

| Symbol | Entrez gene name | $Log_2$ FC | *p*adj |
|---|---|---|---|
| CD300LG | CD300 molecule like family member g | −2.31 | 0.03 |
| LRAT | Lecithin retinol acyltransferase | −2.18 | 0.04 |
| HOXB9 | Homeobox B9 | −1.93 | 0.04 |
| SPINK5 | Serine peptidase inhibitor Kazal type 5 | −1.78 | 0.03 |
| DNM3OS | DNM3 opposite strand/antisense RNA | −1.63 | 0.02 |
| CNFN | cornifelin | −1.56 | 0 |
| KCNAB1 | Potassium voltage-gated channel subfamily A regulatory beta subunit 1 | −1.55 | 0 |
| HDC | Histidine decarboxylase | −1.54 | 0.01 |
| Krt78 | Keratin 78 | −1.37 | 0.02 |
| HOXB7 | Homeobox B7 | −1.33 | 0.03 |
| KIF1A | Kinesin family member 1A | −1.31 | 0.03 |
| Gm17275 | Predicted gene, 17275 | −1.30 | 0.02 |
| SLURP1 | Secreted LY6/PLAUR domain containing 1 | −1.24 | 0.04 |
| CCM2L | CCM2-like scaffold protein | −1.19 | 0.02 |
| PRR18 | Proline rich 18 | −1.11 | 0 |
| GEM | GTP-binding protein overexpressed in skeletal muscle | −1.11 | 0.02 |
| Snora34 | Small nucleolar RNA, H/ACA box 34 | −1.09 | 0.03 |
| IFITM5 | Interferon-induced transmembrane protein 5 | −1.09 | 0.01 |
| SYNPO2 | Synaptopodin 2 | −1.08 | 0.03 |
| IGSF6 | Immunoglobulin superfamily member 6 | −1.03 | 0.04 |
| Gm44243 | Predicted gene, 44243 | −1.01 | 0 |
| RAB19 | RAB19, member RAS oncogene family | 1.03 | 0.02 |
| CCND1 | cyclin D1 | 1.03 | 0.02 |
| TIGIT | T-cell immunoreceptor with Ig and ITIM domains | 1.15 | 0.02 |
| GABRP | Gamma-aminobutyric acid type A receptor subunit pi | 1.20 | 0.04 |
| LRATD1 | LRAT domain containing 1 | 1.20 | 0.01 |
| Krt4 | Keratin 4 | 1.41 | 0.02 |
| ITPRID1 | ITPR interacting domain containing 1 | 1.46 | 0.01 |
| MUC16 | Mucin 16, cell surface associated | 1.99 | 0 |
| Cd163 | CD163 antigen | 2.13 | 0 |

*Note:* Genes with an adjusted *p*-value $<0.05$ and absolute $\log_2$ fold change $>1$ were called as significant DEGs.
Abbreviations: dMG, dissociated mammary glands; $Log_2FC$, $\log_2$ fold change; *p*adj, adjusted *p*-value.

Interestingly, the 1031 DEGs found in the mammospheres are in stark contrast to the single DEG found in the tumors. However, caution should be taken in directly comparing the two analyses since mammospheres represent a relatively homogeneous clonal population (Dontu et al. 2003) while tumors are a heterogeneous mixture of many types of cells (de Visser and Joyce 2023), which could mask differences. In addition, while the tumors represent a sample size of 9–10 tumors per treatment, technical challenges in obtaining the required quantity and quality of RNA from the tertiary mammospheres restricted the analysis to $n=3$ per group, potentially limiting generalizability to a larger population. With these caveats recognized, chronic alcohol exposure had a marked effect on the transcriptome of the mammospheres overall. *Gabrp*, a chloride ion channel protein upregulated in TNBC (Symmans et al. 2005), was increased in both alcohol-exposed mammospheres (*p*adj = 0.0008; $\log_2FC=5.46$) and dMG cells (*p*adj = 0.037; $\log_2FC=1.198$). IPA analysis showed that keratinization and cell junction organization pathways were both significantly activated in alcohol-exposed spheres, suggesting a less metastatic environment. KLF4 (*p*adj = 7.94e-05;

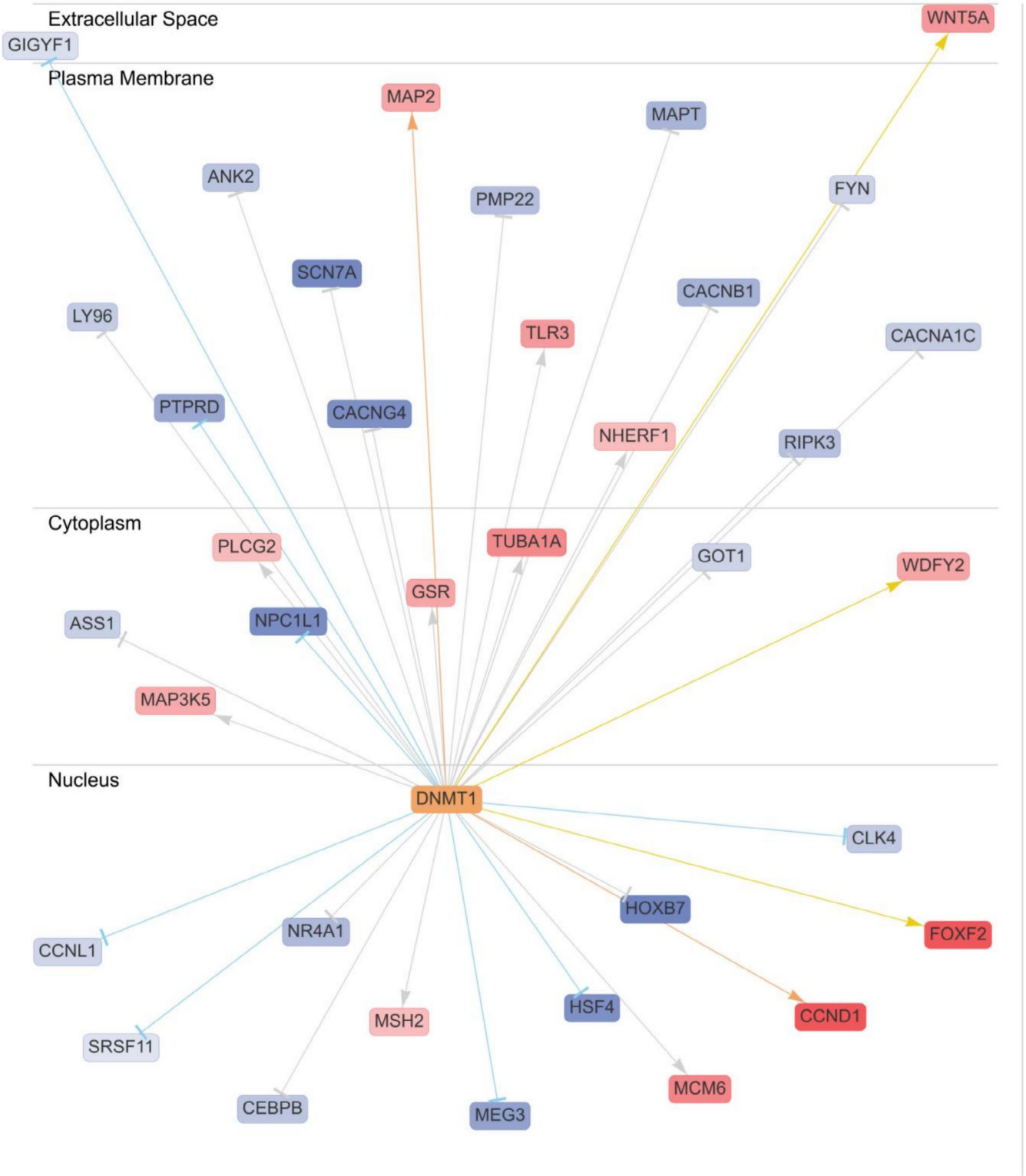


**FIGURE 5** | The effect of chronic alcohol exposure on the transcriptome of MG cells. RNA sequencing was performed on dMG cells and Ingenuity Pathway Analysis (IPA, Qiagen) was used to analyze DEGs (457 analysis ready molecules; *p*adj $<0.11$). DNMT1 was the top predicted upstream regulator (*p*adj $\leq 0.05$; absolute value z-score $>1.5$). Red rectangles indicate upregulated, blue rectangles indicate downregulated, red lines indicate activity consistent with downstream activation, blue lines indicate activity consistent with downstream inhibition, orange lines indicate activity inconsistent with downstream activity, and gray lines indicate neutral interaction. Darker colors indicate a stronger prediction.

$\log_2$FC = 1.3), a zinc-finger containing transcription factor whose expression accompanies growth arrest, has been shown to regulate expression of cytokeratins, promote epithelial cell differentiation, and prevent epithelial-mesenchymal transition (EMT) and metastasis (Chen et al. 2003; Shields et al. 1996; Yori et al. 2011). Gene expression data (cytokeratins, e-cadherin and Elf5 up; Snail down) suggest that EMT is inhibited in the alcohol-exposed spheres. These transcriptomic data taken

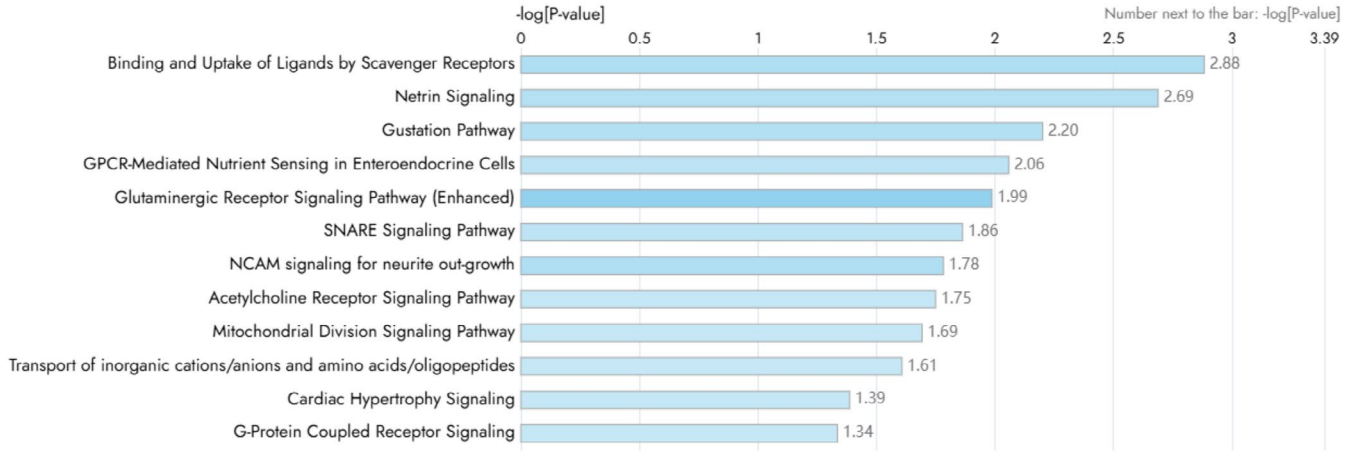


**FIGURE 6** | The effect of chronic alcohol exposure on the transcriptome of MG cells. RNA sequencing was performed on dMG cells and IPA was used to analyze DEGs (457 analysis ready molecules; padj $<0.11$). Top pathways ($p \leq 0.05$; absolute value z-score $>1.5$) were enriched for plasma membrane and extracellular space associated proteins. Blue bars indicate predicted inhibition. Darker blue indicates a stronger prediction.

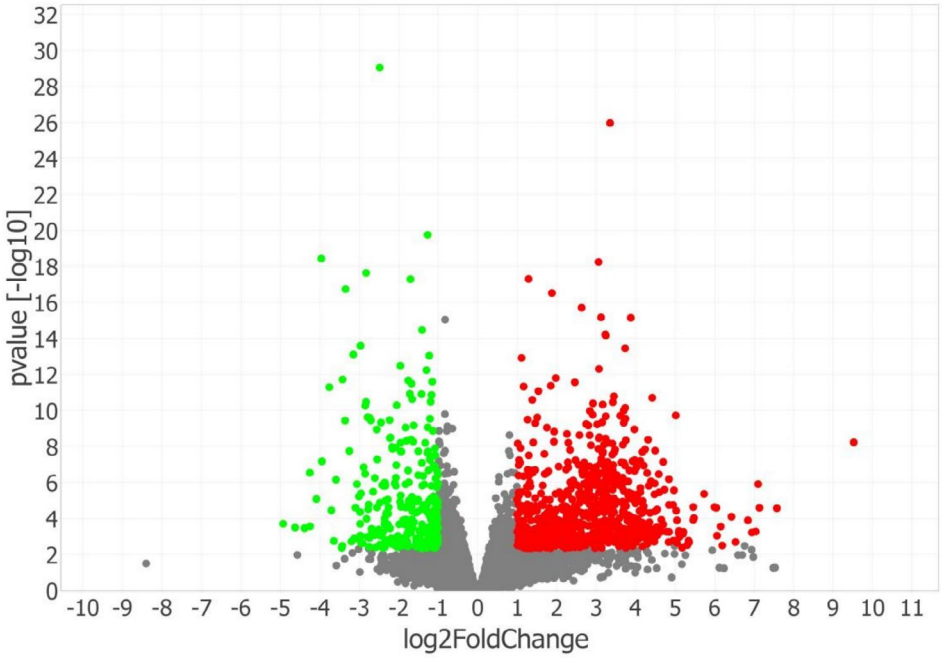


**FIGURE 7** | Volcano plot showing DEGs in mammospheres isolated from alcohol-exposed female MMTV-Wnt1 mouse dMG cells. Green dots represent downregulated genes, and red dots represent upregulated genes. Genes with an adjusted $p$-value $<0.05$ and absolute $\log_2$ fold change $>1$ were called as significant DEGs.

together with the reduced MFE suggest that alcohol promotes a less stem-like and more differentiated environment. Alcohol decreases proliferation in several cell types, including stem cells (Di Rocco et al. 2019), which are thought to comprise mammospheres (Dontu et al. 2003). In addition, in the present study, tumors arising in alcohol-exposed mice appeared less proliferative than the control tumors when analyzed for mitotic counts by a pathologist. These data, which illustrate the anti-proliferative effects of alcohol, support the study which found that consumption of 18% alcohol decreased metastasis to the lungs and distant mammary glands (Vorderstrasse et al. 2012). Alcohol has also been shown to decrease metastasis of melanoma cells (Spitzer et al. 2000). Interestingly, a small percentage of TNBC cases present with low proliferation, defined as a Ki-67 index of $\leq 30\%$ (Srivastava et al. 2022).

Similar to the tumors, the gene expression data from the dMG cells is from a mixed population of cells. In addition to mammary epithelial cells, the samples contain stromal cells including fibroblasts, immune cells and endothelial cells. It is interesting to note that chronic alcohol exposure significantly changed several immune genes in these cells. T-cell immunoreceptor with Ig and ITIM domains (TIGIT) is an immune checkpoint inhibitory receptor expressed on the surface of T cells and NK cells. TIGIT, which has been shown to be upregulated in various cancers (Cari et al. 2018), was upregulated in

**TABLE 2** | Significant DEGs in chronic alcohol-exposed mammospheres.

| Symbol | Entrez gene name | $Log_2$ FoldChange |
|---|---|---|
| Gm3764 | Predicted gene, 3764 | −4.93 |
| DLK1 | Delta-like noncanonical Notch ligand 1 | −4.63 |
| MEPE | Matrix extracellular phosphoglycoprotein | −4.39 |
| COL2A1 | Collagen type II alpha 1 chain | −4.25 |
| CYP1A1 | Cytochrome P450 family 1 subfamily A member 1 | −4.25 |
| SP7 | SP7 transcription factor | −4.09 |
| ATOH8 | Atonal bHLH transcription factor 8 | −3.96 |
| CD300LB | CD300 molecule-like family member b | −3.95 |
| LGI1 | Leucine-rich glioma inactivated 1 | −3.76 |
| ADIPOQ | Adiponectin, C1Q, and collagen domain containing | −3.71 |
| KLK9 | Kallikrein-related peptidase 9 | 6.19 |
| Stfa1 | Stefin A1 | 6.43 |
| ACER1 | Alkaline ceramidase 1 | 6.53 |
| Lipm | Lipase, family member M | 6.84 |
| ATP12A | ATPase H+/K+ transporting non-gastric alpha2 subunit | 6.94 |
| Dsg1c | Desmoglein 1 gamma | 7.04 |
| USP43 | Ubiquitin-specific peptidase 43 | 7.10 |
| ELMOD1 | ELMO domain containing 1 | 7.14 |
| BARX2 | BARX homeobox 2 | 7.58 |
| Gm42047 | Predicted gene, 42,047 | 9.52 |

*Note:* Top 10 significantly upregulated and top 10 significantly downregulated genes in mammospheres isolated from alcohol-exposed female MMTV-Wnt1 mouse dMG cells.

alcohol-exposed dMG cells ($p$adj = 0.02; $\log_2$FC = 1.15). TIGIT is a promising new target for immunotherapy, and anti-TIGIT monoclonal antibodies are currently in clinical trials, both as a monotherapy or in combination with other therapies (Zhang et al. 2024). These data, together with the decrease in *Ptprd* in tumors, which, as noted above, increases PD-L1, points toward a more therapy-sensitive tumor state induced by alcohol exposure. An increase in CD163 ($p$adj = 0; $\log_2$FC = 2.13), which is exclusively expressed on cells of the monocyte lineage, suggests an increase in either macrophage infiltration of the mammary gland or expression of this gene in mammary gland tissue-resident macrophages. Chronic alcohol exposure increases recruitment of monocytes and macrophages to the tumor microenvironment (Molina et al. 2023). Additionally, CD163+ macrophages in the triple-negative breast tumor microenvironment are associated with improved survival (Omilian et al. 2024). Chronic alcohol treatment of rats produces insulin resistance, which is associated with increased macrophage infiltration of adipose tissue (Kang et al. 2007). CD300LG ($p$adj = 0.03; $\log_2$FC = −2.31) is an Ig domain-containing vascular endothelial adhesion molecule implicated in lymphocyte transendothelial migration (TEM) (Jin et al. 2008). IGSF6 ($p$adj = 0.04; $\log_2$FC = −1.03) is a member of the immunoglobulin superfamily of genes whose members have been shown to be biomarkers for breast and colorectal cancer (Li et al. 2017; Rong et al. 2023).

The transcripts of several noncoding RNAs (ncRNAs) were changed in the alcohol-exposed dMG cells. The long ncRNA (lncRNA) DNM3OS (Dynamin 3 Opposite Strand; $p$adj = 0.016; $\log_2$FC = −1.63), which encodes for the microRNA cluster miR-199-214, has been shown to be dysregulated in a variety of cancers by several different mechanisms (Fellah et al. 2023). Specifically, miR-199-214 is downregulated in the triple-negative breast cancer subtype (Cantini et al. 2019). It is important to note that poly-A selection was the RNA selection method used in the current RNA-sequencing project as opposed to rRNA depletion. Therefore, the complete set of alcohol-induced differentially expressed lncRNAs is not reported, since not all lncRNAs are polyadenylated. Small nucleolar RNAs (snoRNAs) are a class of ncRNAs that are involved in RNA modification and ribosome biogenesis and have been shown to be dysregulated in breast cancer (Su et al. 2014). Snora34 ($p$adj = 0.032; $\log_2$FC = −1.09) is downregulated in the aging mouse ovary, where its expression is also affected by diet and toxin treatment (Cuomo et al. 2018). Additional genes that are of interest also changed, as shown in Table 1 but are beyond the scope of this discussion.

Alcohol is physically disruptive to cellular membranes (Freund 1979; Goldstein 1986). Results from the present study show that chronic alcohol exposure also affects the expression of proteins located in the cellular membrane. *Ptprd*, *Gabrp*, and the four immune genes discussed above are all expressed in the membrane on the cell surface. The top 12 affected pathways in the dMG cells were all inhibited by alcohol and were all enriched for plasma membrane proteins (receptors, transporters, ion channels), suggesting an alcohol-induced disruption of signaling and transport across the membrane. Nine of the 12 pathways contained calcium voltage-gated ion channel proteins. Calcium signaling proteins have altered expression in breast cancer (So et al. 2019) and have been shown to be regulated by alcohol (Ho and Lin 2021). Additionally, expression of *Kcnab1*, a voltage-gated potassium channel subunit protein, is significantly decreased in dMG cells with alcohol exposure ($p$adj = 0; $\log_2$FC = −1.55). The pathway involving transport of inorganic cations/anions and amino acids/oligopeptides, which includes several members of the large solute carrier (SLC) family of transporters, was inhibited by alcohol. Expression of the SLC transporter family has been shown to be changed with chronic alcohol exposure (Pan et al. 2011).

In conclusion, chronic alcohol exposure affected preneoplastic mammary glands by increasing the luminal progenitor population and altering the transcriptome. Genes changed included transcription factors, immune genes (including an increased immunotherapy target), and ncRNA. The mammary stem-like/

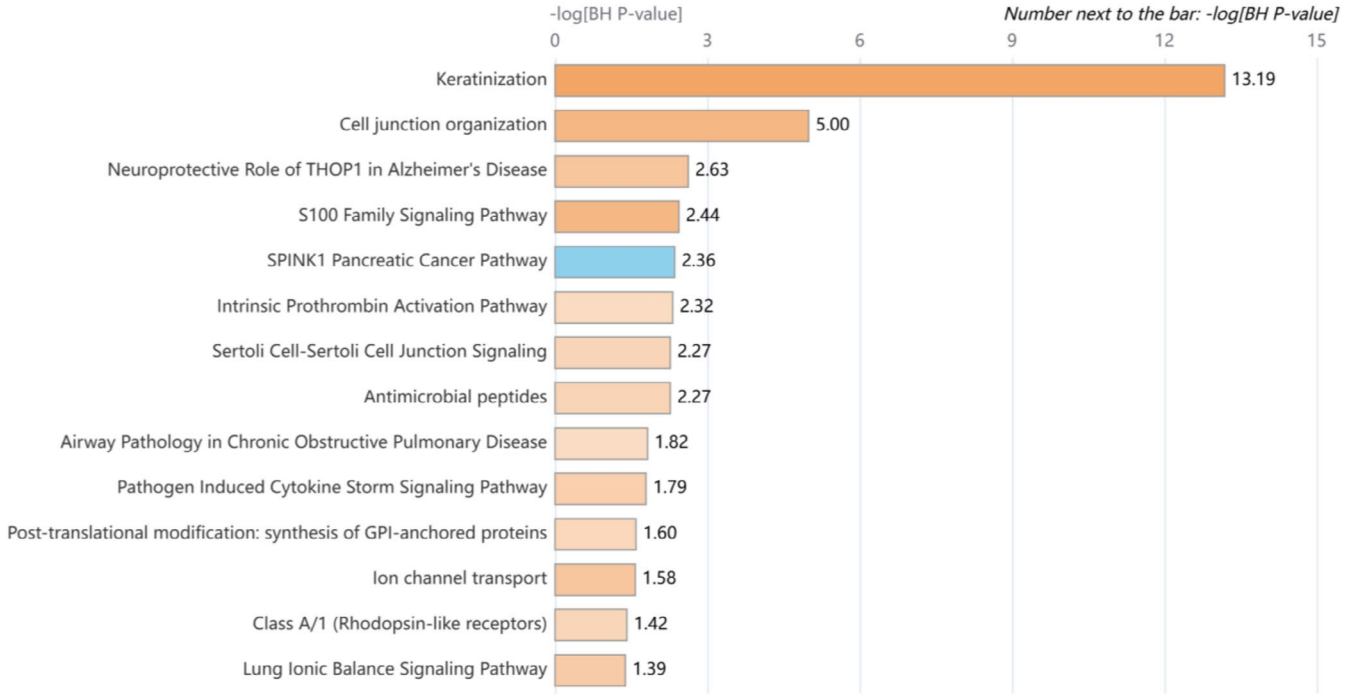


**FIGURE 8** | The effect of direct alcohol exposure on the transcriptome of mammosphere cells isolated from dMG cells. RNA sequencing was performed and IPA was used to analyze DEGs (1025 analysis ready molecules; *p*adj $< 0.05$; absolute $\log_2$ fold change $> 1$). Top pathways (*p*adj $\leq 0.05$; absolute value z-score $> 2$) are shown. Orange bars indicate predicted activation; blue bars indicate predicted inhibition. Darker colors indicate a stronger prediction.

progenitor cells seemed to be the most affected by alcohol, producing fewer mammospheres in culture with a gene expression signature indicative of more differentiation, more cell–cell interactions, less epithelial–mesenchymal transition, and less movement. Collectively, these data suggest that tumors from alcohol-exposed animals may have a less negative prognosis.


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### Conflicts of Interest

The authors declare no conflicts of interest.

### Data Availability Statement

The data that support the findings of this study are openly available in NCBI Gene Expression Omnibus (GEO) at https://www.ncbi.nlm.nih.gov/geo/, reference number GSE316001.

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## Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Composition of tumor epithelial cell populations and tumorsphere-forming efficiency. **Supplementary Table 1:** Significant differentially expressed genes (DEG) in the mammospheres cultured from dMG cells of animals exposed to alcohol for 8 weeks. Genes with an adjusted $p$-value $< 0.05$ and absolute $\log_2$ fold change $> 1$ were called significant DEGs. dMG, dissociated mammary glands; $p_{adj}$, adjusted $p$-value.