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Ethanol exposure induces the cancer-associated fibroblast phenotype and lethal tumor metabolism

Implications for breast cancer prevention

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Little is known about how alcohol consumption promotes the onset of human breast cancer(s). One hypothesis is that ethanol induces metabolic changes in the tumor microenvironment, which then enhances epithelial tumor growth. To experimentally test this hypothesis, we used a co-culture system consisting of human breast cancer cells (MCF7) and hTERT-immortalized fibroblasts. Here, we show that ethanol treatment (100 mM) promotes ROS production and oxidative stress in cancer-associated fibroblasts, which is sufficient to induce myofibroblastic differentiation. Oxidative stress in stromal fibroblasts also results in the onset of autophagy/mitophagy, driving the induction of ketone body production in the tumor microenvironment. Interestingly, ethanol has just the opposite effect in epithelial cancer cells, where it confers autophagy resistance, elevates mitochondrial biogenesis and induces key enzymes associated with ketone re-utilization (ACAT1/OXCT1). During co-culture, ethanol treatment also converts MCF7 cells from an ER(+) to an ER(-) status, which is thought to be associated with "stemness," more aggressive behavior and a worse prognosis. Thus, ethanol treatment induces ketone production in cancer-associated fibroblasts and ketone re-utilization in epithelial cancer cells, fueling tumor cell growth via oxidative mitochondrial metabolism (OXPHOS). This "two-compartment" metabolic model is consistent with previous historical observations that ethanol is first converted to acetaldehyde (which induces oxidative stress) and then ultimately to acetyl-CoA (a high-energy mitochondrial fuel), or can be used to synthesize ketone bodies. As such, our results provide a novel mechanism by which alcohol consumption could metabolically convert "low-risk" breast cancer patients to "high-risk" status, explaining tumor recurrence or disease progression. Hence, our findings have clear implications for both breast cancer prevention and therapy. Remarkably, our results also show that antioxidants [such as N-acetyl cysteine (NAC)] can effectively reverse or prevent ethanol-induced oxidative stress in cancer-associated fibroblasts, suggesting a novel strategy for cancer prevention. We also show that caveolin-1 and MCT4 protein expression can be effectively used as new biomarkers to monitor oxidative stress induced by ethanol.

Introduction

It is now well-recognized that alcohol consumption is a significant risk factor for the development of a number of human cancers, and especially breast cancer.[1,2] Over the years, numerous epidemiological studies have shown that increased alcohol consumption and binge-drinking are dose-dependently associated with the development of human breast cancers.[3-9] These findings have also been recapitulated using mouse animal models, where ethanol-fed mice showed increased rates of breast cancer tumor growth.[10] Importantly, alcohol consumption also significantly promotes breast cancer tumor recurrence[11] and, hence, disease progression.

However, the exact mechanism(s) by which ethanol functionally promotes the onset and progression of breast cancer remains largely unknown. Metabolically, ethanol is first converted to acetaldehyde, which is a toxic intermediate.[12,13]

Then, acetaldehyde is further converted to acetic acid (acetate), which is transformed into acetyl-CoA, a mitochondrial fuel (www.en.wikipedia.org/wiki/Acetaldehyde). Acetyl-CoA can then enter the TCA/citric acid cycle, in cells with functional mitochondria (www.en.wikipedia.org/wiki/

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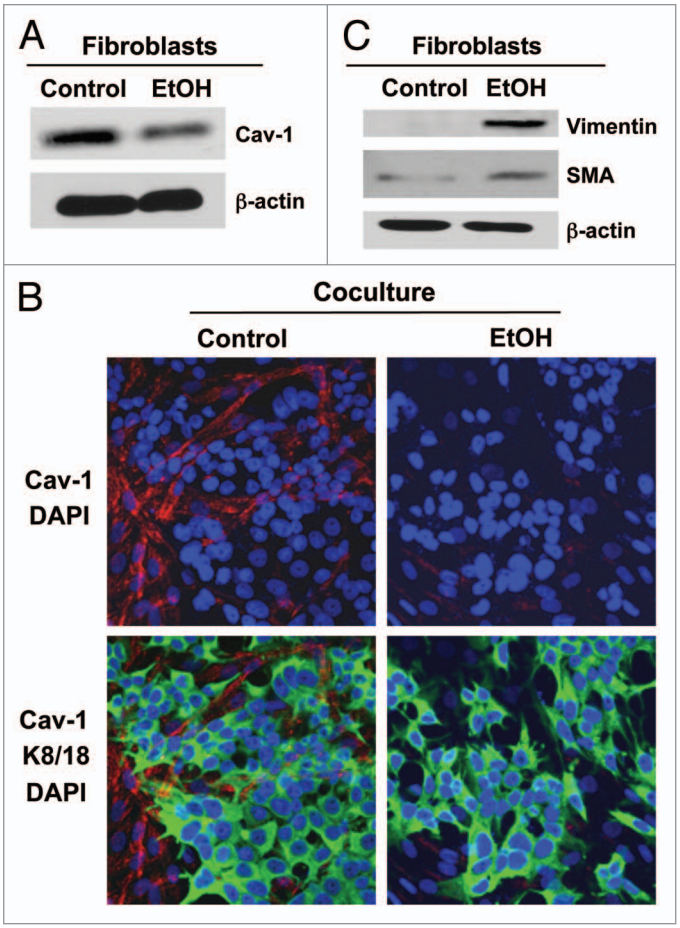

Figure 1. Ethanol induces Cav-1 downregulation and myofibroblastic differentiation in the stromal fibroblast compartment. (**A**) Fibroblasts were cultured for 2 d. To study the effect of ethanol (EtOH), cells were cultured in the presence or in the absence of 100 mM EtOH for additional 72 h. Western blot analysis was performed using antibody directed against Cav-1. Note that EtOH exposure induces a downregulation of Cav-1 in fibroblasts. β-actin was used as an equal loading control. (**B**) Immunofluorescence. MCF7 cells were co-cultured with fibroblasts for 2 d and treated with 100 mM EtOH for additional 72 h. Then, cells were fixed and immunostained with antibody probes against Cav-1 (red, upper panels) and keratin-8/18 (K8/18, green, lower panels). Nuclei were counterstained with DAPI (blue). K8/18 staining is specific for MCF7 cells and Cav-1 is specific for fibroblasts, since MCF7 cells lack Cav-1 expression. Note that Cav-1 expression is downregulated in the fibroblast compartment after ethanol treatment, relative to untreated cells. Original magnification, 40x. (**C**) Fibroblasts were allowed to growth for 2 d, and then were cultured in the presence or in the absence of 100 mM EtOH for 72 h. Western blot analysis was performed using antibodies against vimentin and SMA. β-actin was used as equal loading control. Note that ethanol-treated fibroblasts display an upregulation of the myofibroblast markers SMA and vimentin.

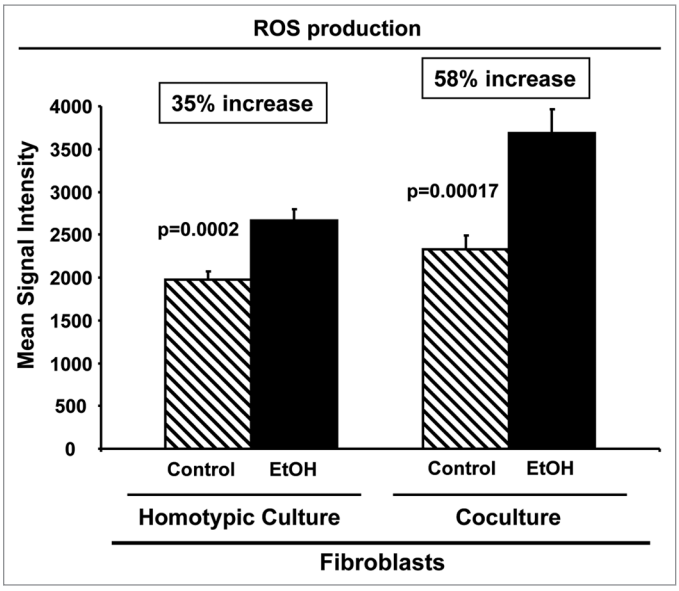

Figure 2. Ethanol treatment drives oxidative stress in fibroblasts. Fibroblasts were plated in homotypic culture or in co-culture with RFP(+)-MCF7 cells. After 2 d, cells were treated with 100 mM EtOH for 72 h. Untreated cells served as controls. ROS production was determined by flow cytometry using CellROX Deep Red Reagent probe. Fibroblasts were identified as the RFP(-)-cell population. Note that in fibroblasts cultured alone, EtOH treatment increases ROS production by 35%, as compared with control cells. When fibroblasts were co-cultured with cancer cells, the increase in ROS production is even higher, rising up to 58%. p values are as indicated.

Ethanol_metabolism). Or, in cells with mitochondrial dysfunction, acetyl-CoA is converted to ketone bodies (acetoacetate and β-hydroxy-butyrate).

The tumor-promoting effects of ethanol have been largely attributed to the production of acetaldehyde, which is an unstable molecule that generates reactive oxygen species (ROS) and can directly interact with DNA, driving the onset of DNA-damage and the DNA-damage/repair response.[12,13] In this regard, acetaldehyde has also been classified as a bona fide chemical mutagen or carcinogen.

Despite these findings, little is known about the interaction(s) of ethanol with the tumor microenvironment. There should be a significant etiological connection, as alcohol consumption also predisposes to the development of fibrotic liver disease and clearly enhances the process of fibrosis. And, fibrosis is a significant risk factor in the pathogenesis of human breast cancers. Importantly, fibrosis is associated with the generation of tissue-associated myofibroblasts in the liver, which are thought to be highly related to cancer-associated fibroblasts, and represent a fibrotic component of human breast cancers.[14-20]

Here, we show that ethanol treatment induces the generation of ROS species in human fibroblasts, which has certain metabolic consequences that are known to drive tumor growth and metastasis. This resulting oxidative stress promotes autophagy, mitochondrial dysfunction and ketone production in cancer-associated fibroblasts. Conversely, ethanol has just the opposite effects on epithelial cancer cells, where it functions as a mitochondrial fuel via ketone re-utilization and promotes mitochondrial biogenesis. Thus, ethanol serves as "fertilizer" for the tumor microenvironment, where it helps drive the onset of "two-compartment tumor metabolism" and stromal-epithelial metabolic coupling.[21,22]

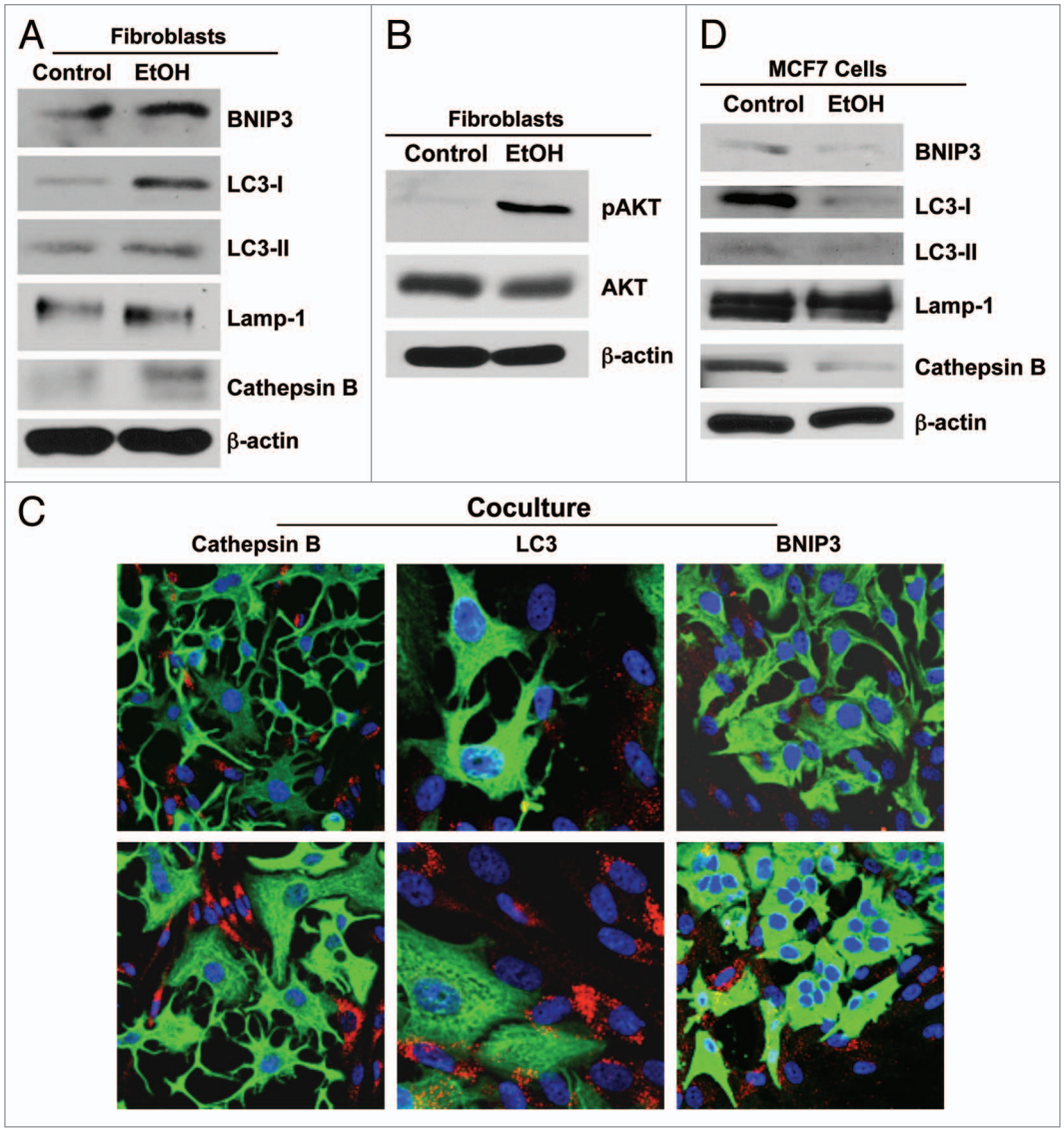

Figure 3. Ethanol induces autophagy in fibroblasts but promotes autophagy resistance in MCF7 cancer cells. (**A and B**) Fibroblasts were cultured in the presence or in the absence of 100 mM EtOH for 72 h. (**A**) Western blot analysis using antibodies against a panel of autophagy markers. β-actin was used as equal loading control. Note that EtOH treatment increases the expression of several key autophagy markers, namely BNIP3, LC3, Cathepsin B and, to a lesser extent, Lamp-1. LC3 activation is also increased, as detected by cleavage (LC3-II). (**B**) Western blot analysis using antibodies against phospho-AKT. The membranes were then reprobed with total AKT antibodies. Note that ethanol treatment promotes activation of the AKT pathway. This reaction is probably an anti-apoptotic response to protect the fibroblasts against autophagy-induced cell death. (**C**) Immunofluorescence. MCF7 cell-fibroblast co-cultures were maintained in the presence or absence of 100 mM EtOH for 72 h. Cells were fixed and immunostained with antibody probes directed against K8/18 (green, to identify the MCF7 cell population), and Cathepsin B, LC3 or BNIP3 (all red, to detect autophagy). Nuclei were counterstained with DAPI (blue). To detect LC3 prior to fixation, cells were incubated with HBSS in the presence of 25 μM chloroquine for 6 h. The upper panels show the control, untreated conditions. The bottom panels show the ethanol-treated samples. Note that autophagy markers (Cathepsin B, LC3 and BNIP3) were all selectively upregulated in the fibroblast compartment in response to ethanol treatment. Original magnification, 40x, except for LC3, 63x. (**D**) MCF7 cells were cultured in the presence or in the absence of 100 mM EtOH for 72 h. Western blot analysis was performed using antibodies against a panel of autophagy markers. β-actin was used as equal loading control. Note that EtOH treatment decreases the expression of several key autophagy markers, namely BNIP3, LC3-I (precursor form), LC3-II (active cleaved form), Cathepsin B and, to a lesser extent, Lamp-1, suggesting that ethanol promotes autophagy resistance in MCF7 cancer cells.

Results

Ethanol induces the downregulation of Cav-1, promotes the myofibroblast phenotype and drives oxidative stress in the fibroblast compartment. To study the effect(s) of ethanol on stromal cell behavior, fibroblasts were treated with ethanol for 72 h and analyzed by immunoblotting with antibodies directed against Cav-1. **Figure 1A** shows that ethanol exposure induces the downregulation of Cav-1 in fibroblasts. To confirm these results in a more physiological cell model that includes cancer cells and their tumor microenvironment, co-cultures of MCF7 cells and fibroblasts were treated with ethanol and immunostained with

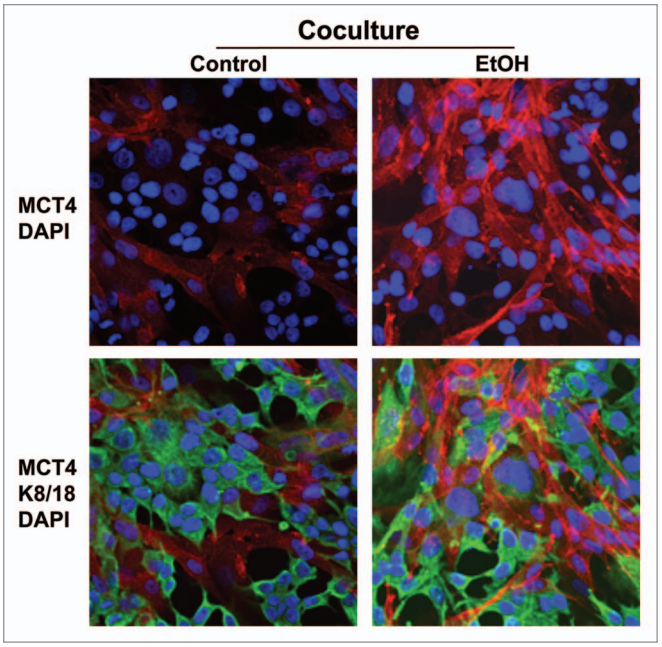

Figure 4. Ethanol increases the expression of MCT4 in cancer-associated fibroblasts. MCF7 cells were plated in co-culture with fibroblasts, and cells were treated with 100 mM EtOH for 72 h. Cells were fixed and immunostained with antibodies against MCT4 (red, upper panels) and K8/18 (green, lower panels). Nuclei were counterstained with DAPI (blue). Note that treatment with EtOH greatly increases the expression of MCT4 in cancer-associated fibroblasts. Original magnification, 40x.

antibodies directed against Cav-1 and keratin-8/18 (specific for MCF7-epithelial cells). **Figure 1B** shows that Cav-1 expression is significantly downregulated in the fibroblast compartment after treatment with ethanol.

It is well-known that a loss of Cav-1 is sufficient to promote a fibroblast-to-myofibroblast conversion.[23,24] Thus, we next evaluated if ethanol exposure induces myofibroblast differentiation by immunoblot analysis, with antibodies directed against the myofibroblast markers vimentin and SMA. **Figure 1C** shows that fibroblasts treated with ethanol display the upregulation of SMA and vimentin, relative to untreated cells.

As a loss of Cav-1 in fibroblasts is a marker of oxidative stress, we next asked if ethanol exposure induces a rise in ROS levels in fibroblasts in homotypic culture or in co-culture with MCF7 cells. **Figure 2** shows that exposure to ethanol promotes ROS generation in fibroblasts cultured alone and, even more prominently, in fibroblasts co-cultured with cancer cells, as compared with untreated control cells.

Ethanol induces autophagy in fibroblasts, but promotes autophagy resistance in MCF7 cancer cells. A rise in ROS levels is a well-established trigger for the initiation of autophagy. Thus, we next asked if ethanol exposure promotes an autophagic program, using immunoblot analysis with antibodies directed against a panel of autophagy markers. **Figure 3A** shows that ethanol treatment increases the levels of several key autophagy markers, namely BNIP3, LC3 (including the active cleaved form LC3-II), Cathepsin B and, to a lesser extent, Lamp-1. We then assessed if increased autophagy leads to a compensatory activation of the

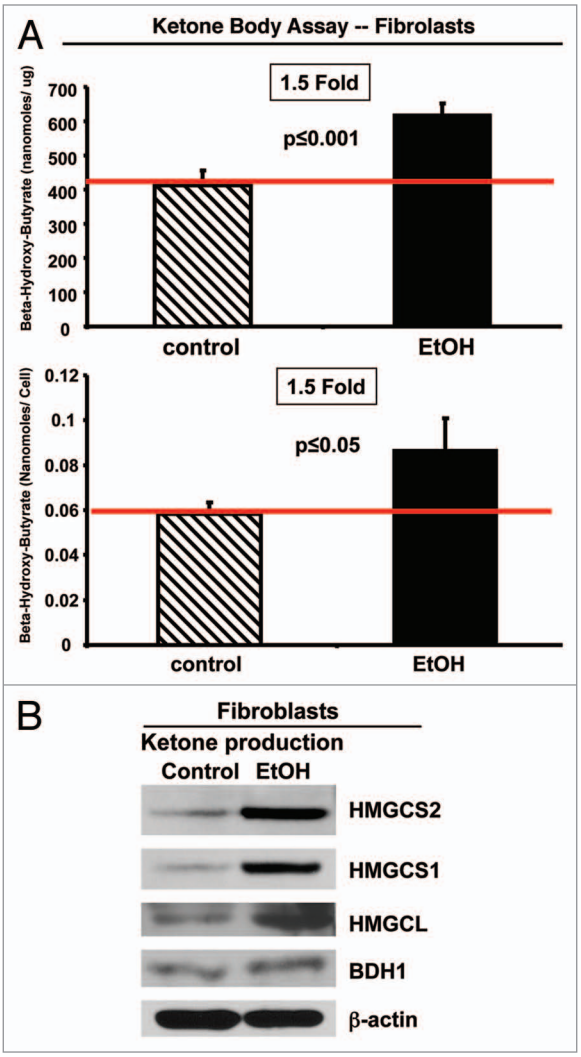

Figure 5. Ethanol promotes ketone body generation in fibroblasts. (**A**) β-hydroxybutyrate (β-OH-butyrate) concentration was measured in the cell culture media derived from fibroblasts cultured in the presence or absence of 100 mM EtOH for 72 h. Values were normalized either for protein concentration (left) or cell number (right). Note that EtOH treated-fibroblasts display a 1.5-fold increase in β-OH-butyrate accumulation, relative to untreated fibroblasts. (**B**) Western blot analysis. Control and EtOH-treated fibroblasts were analyzed by immunoblotting with a panel of antibodies against key enzymes of ketone body production. β-actin was used as equal loading control. Note that treatment with EtOH induces the expression of the enzymes involved in ketone production (HMGCS1, HMGCS2 HMGCL and BDH1). These data indicate that ethanol increases ketone body generation in fibroblasts.

AKT pathway, by immunoblot analysis using phospho-specific AKT antibodies. **Figure 3B** shows that ethanol treatment promotes the activation of the AKT pathway, likely as an anti-apoptotic response, to protect fibroblasts against autophagy-induced cell death. To independently assess if ethanol promotes autophagy, we next evaluated autophagic markers by immunofluorescence of ethanol-treated co-cultures of MCF7 cells and fibroblasts. **Figure 3C** shows that autophagy markers (Cathepsin B, LC3 and BNIP3) are all selectively upregulated in the fibroblast compartment, in response to ethanol treatment, relative to untreated control cells.

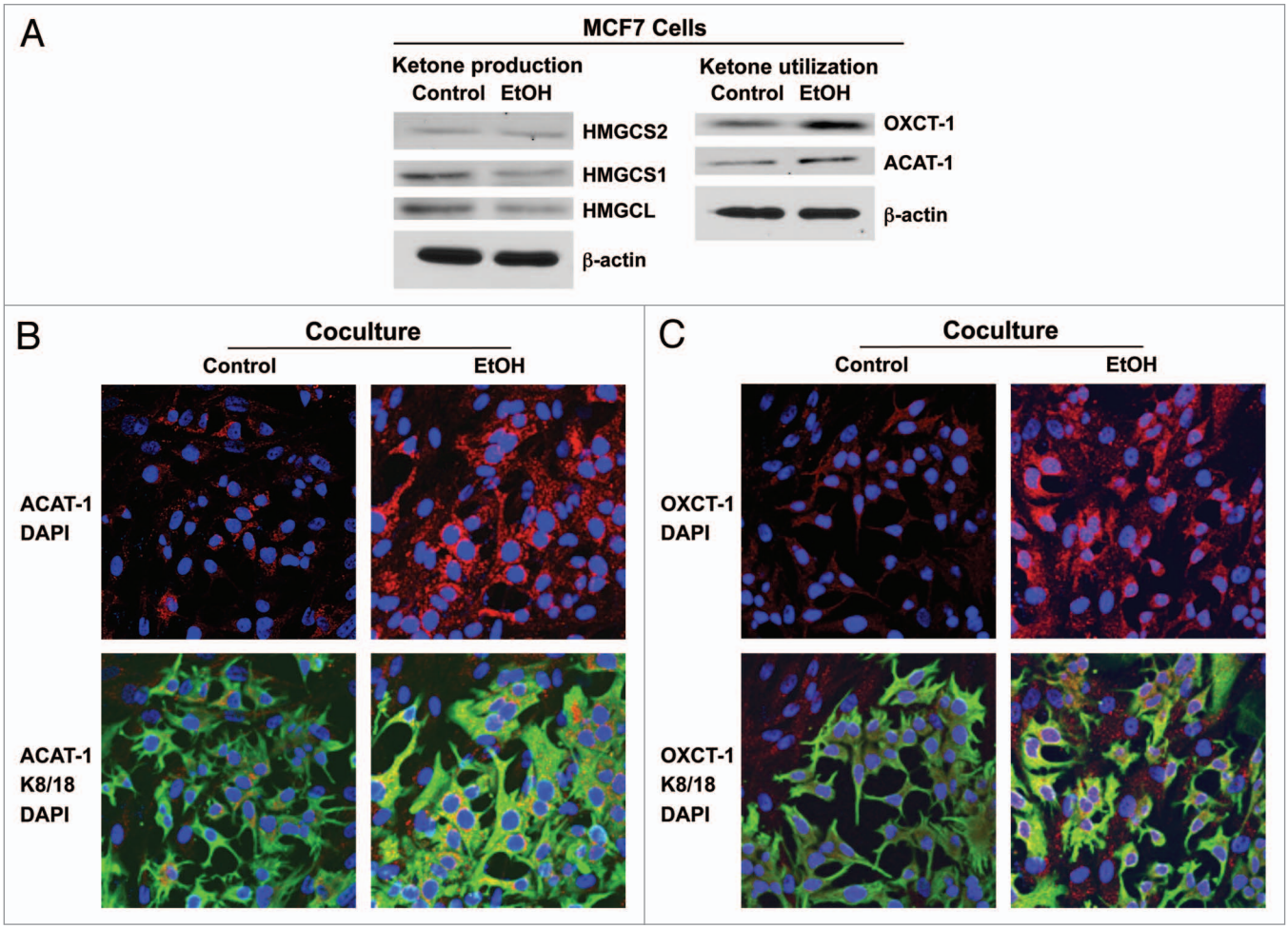

Figure 6. Ethanol promotes ketone body utilization in MCF7 cells. (**A**) Western blot analysis. Control and EtOH-treated MCF7 cells were analyzed by immunoblotting with a panel of antibodies directed against key enzymes of ketone body metabolism (production and utilization). β-actin was used as an equal loading control. Note that treatment with EtOH induces the expression of key enzymes involved in ketone utilization (ACAT-1 and OXCT1). The expression of key enzymes involved in ketone body production (HMGCS1, HMGCS2 and HMGCL) is unchanged or slightly decreased. (**B and C**) Immunofluorescence. Fibroblast-MCF7 cell co-cultures were treated with 100 mM EtOH for 72 h. Cells were then fixed and immunostained with antibodies against K8/18 (green) and ACAT-1 [red, (**B**) or OXCT-1 red, (**C**)]. Nuclei were counterstained with DAPI (blue). The upper panels show ACAT-1 or OXCT-1 staining (red). The bottom panels show also the K8/18 staining (green) to identify the MCF7 cell population. Note that ethanol increases the expression of enzymes for ketone bodies utilization [ACAT-1, (**B**); and OXCT-1, (**C**)] in cancer cells in co-culture with fibroblasts. Original magnification, 40x. These data indicate that ethanol increases ketone body utilization in MCF7 cancer cells.

We have previously shown that the induction of autophagy is cell type- and cell compartment-specific.[25] Thus, we explored the possibility that ethanol exposure protects MCF7 cells against autophagy. **Figure 3D** shows that ethanol treatment decreases the expression of several key autophagy markers, namely BNIP3, LC3, Cathepsin B and, to a lesser extent, Lamp-1, suggesting that ethanol promotes autophagy resistance in epithelial cancer cells.

Ethanol promotes MCT4 expression and ketone body generation in cancer-associated fibroblasts. Elevated ROS levels induce the expression of MCT4.[26] MCT4 is a HIF1 target and a marker of oxidative stress. MCT4 is also the transporter that mediates the efflux of monocarboxylates (L-lactate and ketone bodies) out of cells. As ethanol induces ROS production, we next asked if ethanol exposure drives the expression of MCT4 in fibroblasts. To this end, MCF7-fibroblast co-cultures were treated with ethanol and immunostained with antibodies directed against MCT4. **Figure 4** shows that treatment with ethanol greatly increases the expression of MCT4 in cancer-associated fibroblasts.

As ethanol increases the stromal MCT4 expression, we next asked if ethanol exposure also promotes the generation of the metabolites whose efflux is mediated by MCT4, namely ketone bodies. To this end, the concentration of β-OH-butyrate was measured in the cell culture media derived from fibroblasts cultured in the presence or absence of ethanol. **Figure 5A** shows that ethanol-treated fibroblasts display a 1.5-fold increase in β-OH-butyrate accumulation relative to untreated fibroblasts. Consistent with these findings, immunoblot analysis demonstrates that key enzymes involved in ketone body production (HMGCS1, HMGCS2, HMGCL and BDH1) are clearly upregulated in ethanol-treated fibroblasts, relative to untreated cells (**Fig. 5B**).

Ethanol promotes ketone body re-utilization in epithelial cancer cells. These data indicate that ethanol increases ketone

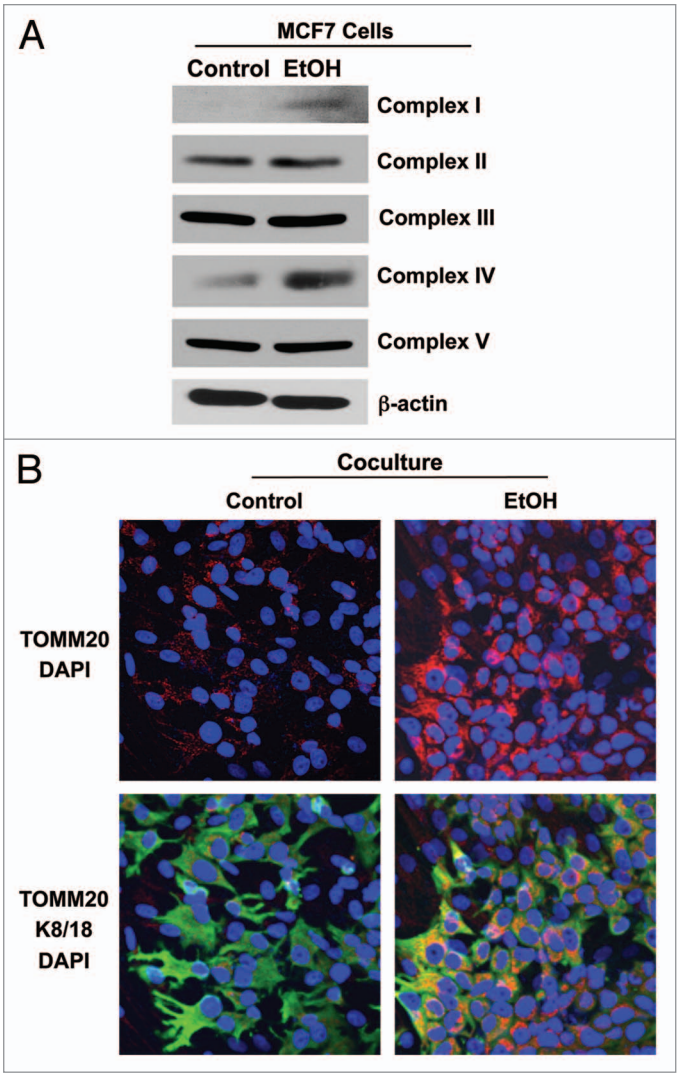

Figure 7. Ethanol increases the mitochondrial activity in MCF7 cancer cells. (**A**) MCF7 cells were cultured in the presence or in the absence of 100 mM EtOH for 72 h. Western blot analysis was performed using antibodies against subunits of the mitochondrial OXPHOS respiratory chain (complexes I–V). β-actin is shown as an equal loading control. Note that ethanol significantly increases the expression of OXPHOS complex I (20 kDa subunit) and IV (COX-II) in MCF7 cancer cells. (**B**) Immunocytochemistry. MCF7 cell-fibroblast co-cultures were treated with 100 mM EtOH for 72 h. Cells were fixed and immunostained with antibodies against the mitochondrial membrane marker TOMM20 and K8/18. Nuclei were counterstained with DAPI (blue). The upper panels show the mitochondrial staining (red). The bottom panels show also the K8/18 staining (green), to identify the MCF7 cell population. Note that ethanol increases the mitochondrial mass specifically in MCF7 cells, as compared with untreated cells. However, ethanol does not promote mitochondrial biogenesis in fibroblasts. Original magnification, 40x.

body generation and the expression of the ketone body transporter MCT4, in cancer-associated fibroblasts. Thus, in the presence of ethanol in the tumor microenvironment, fibroblast-derived ketones may serve as energy-rich substrates for breast cancer cells. To test this hypothesis, we first asked if ethanol promotes ketone body utilization in breast cancer cells. We analyzed the expression levels of key enzymes of ketone body metabolism (production and utilization) in control- and ethanol treated-MCF7 cells by immunoblotting. **Figure 6A** shows that treatment with ethanol induces the expression of key enzymes involved in ketone utilization (ACAT-1 and OXCT-1). However, the expression of enzymes involved in ketone body production (HMGCS1, HMGCS2 and HMGCL) is unchanged or slightly decreased.

We next tested the idea that ethanol accentuates fibroblast-epithelial cell coupling and facilitates the ability of cancer cells to utilize fibroblast-derived ketone bodies. Control and ethanol-treated co-cultures of fibroblasts and MCF7 cells were immunostained with antibodies directed against enzymes for ketone body utilization (ACAT-1 and OXCT-1). **Figure 6B and C** show that ethanol greatly increases the expression of ACAT-1 and OXCT-1 in cancer cells during co-culture with fibroblasts. These data indicate that ethanol increases the utilization of fibroblast-derived ketone bodies by MCF7 cancer cells.

Ethanol increases the mitochondrial activity of MCF7 cancer cells. As ketone bodies are high-energy mitochondrial fuels, we next asked if ethanol exposure might increase the mitochondrial activity of epithelial cancer cells. To this end, control and ethanol-treated MCF7 cells were analyzed by immunoblotting with antibodies directed against subunits of the mitochondrial OXPHOS respiratory chain (complexes I-V). **Figure 7A** shows that ethanol significantly increases the expression of OXPHOS complex I (20 kDa subunit) and IV (COX-II), in MCF7 cancer cells. To independently validate these results, control and ethanol-treated co-cultures of fibroblasts and MCF7 cells were immunostained with antibodies directed against the mitochondrial membrane marker TOMM20. **Figure 7B** shows that ethanol amplifies mitochondrial mass specifically in MCF7 cells, as compared with untreated cells. However, ethanol does not promote mitochondrial biogenesis in adjacent fibroblasts.

Ethanol increases the amount of ATP-filled vesicles in MCF7 cancer cells. ATP accumulation in the tumor microenvironment is associated with a variety of processes, including increased cell migration, proliferation and the immune response. As ethanol increases the mitochondrial activity in epithelial cancer cells, we next asked if ethanol exposure could also augment the release of ATP-enriched vesicles, delivering ATP to the extracellular matrix. To this end, the relative amount of ATP-rich vesicles was measured in MCF7 cells alone and in co-culture, after ethanol treatment. **Figure 8** shows that ethanol treatment promotes a 45% increase in ATP-enriched vesicles, but only when cancer cells are co-cultured with fibroblasts. No differences were noted in MCF7 cells cultured alone.

Ethanol suppresses the expression of ERα in MCF7 cancer cells. Our results indicate that ethanol treatment promotes the aggressive behavior of estrogen receptor (ERα)-positive MCF7 breast cancer cells. A loss of ERα is associated with increased aggressiveness in breast cancer. Thus, we next asked if ethanol may suppress ERα expression. Control and ethanol-treated co-cultures of MCF7 cells and fibroblasts were immunostained with antibodies directed against ERα. **Figure 9** shows that ethanol suppresses the expression of ERα specifically in MCF7 cells, as compared with untreated cells.

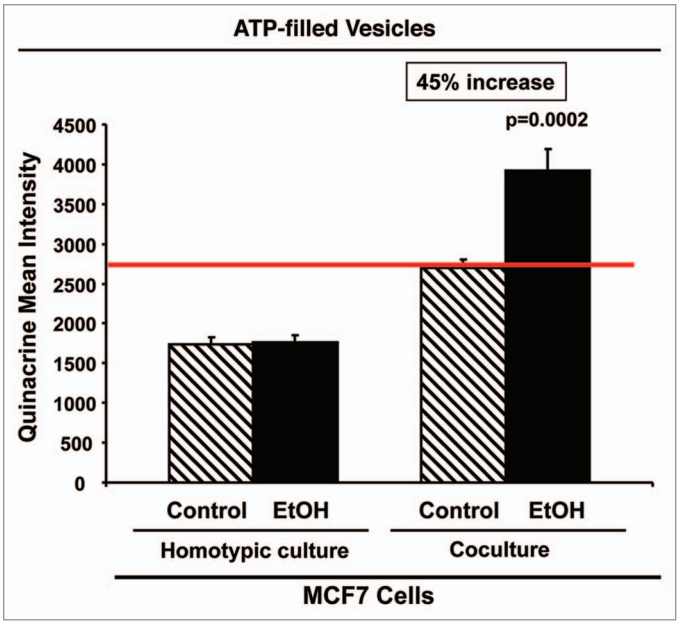

Figure 8. Ethanol increases the amounts of ATP-rich vesicles in MCF7 cancer cells. RFP(+)-MCF7 cells were plated in homotypic culture or in co-culture with fibroblasts. Cells were treated with 100 mM EtOH for 72 h. To detect ATP-rich vesicles, cells were incubated with 30 μM quinacrine in phenol-red-free DMEM for 1 h at 37°C. The green fluorescence of ATP-filled vesicles was recorded by flow cytometry. Note that ethanol treatment promotes a 45% increase in ATP-enriched vesicles when cancer cells are co-cultured with fibroblasts, without affecting quinacrine uptake of MCF7 cells cultured alone. p values are as indicated.

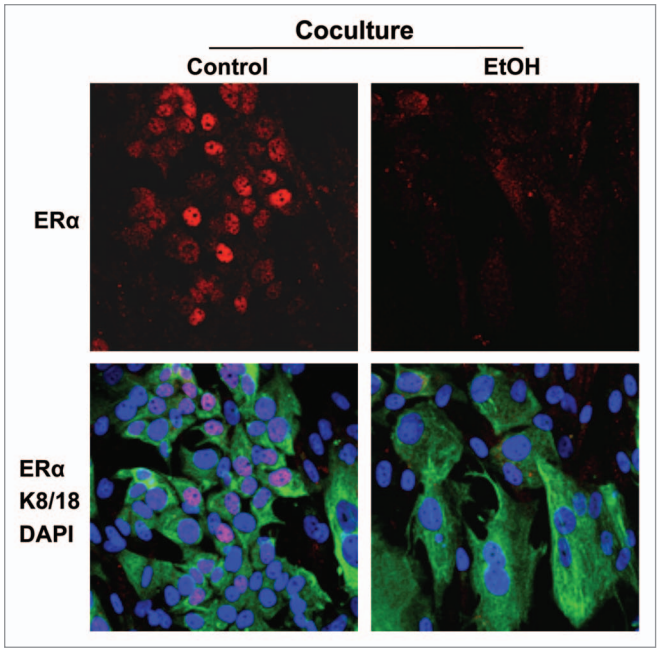

Figure 9. Ethanol suppresses the expression of ERα in MCF7 cancer cells. MCF7 cell-fibroblast co-cultures were treated with 100 mM EtOH for 72 h. Cells were fixed and immunostained with antibody probes directed against ERα (red) and K8/18 (green). Nuclei were counterstained with DAPI (blue). The upper panels show ERα staining only (red). The bottom panels show also the K8/18 staining (green) to identify the MCF7 cell population. Note that ethanol suppresses the expression of ERα specifically in MCF7 cells, as compared with untreated cells. Original magnification, 40x.

Ethanol does not promote apoptotic cell death. To rule out the possibility that ethanol induces apoptosis, MCF7 cells in homotypic culture or in co-culture with fibroblasts were treated with ethanol, and apoptosis was detected with annexin V-APC and PI staining by flow cytometry. As we have previously shown, **Figure 10A** demonstrates that MCF7 cells are protected against cell death when co-cultured with fibroblasts. However, ethanol does not significantly promote apoptosis in MCF7 cells (**Fig. 10A**) or in fibroblasts (**Fig. 10B**).

Is ethanol-induced ROS production in fibroblasts strictly dependent on ethanol metabolism? To determine whether ethanol-induced ROS production could be attributed to ethanol metabolism, we treated MCF7-fibroblast co-cultures with pyrazole, an inhibitor of alcohol dehydrogenase (ADH) and microsomal CYP2E1, and amino-triazole (ATZ), which inhibits catalase. These experiments were performed both at 100 mM and 20 mM EtOH. In all these situations, neither of these inhibitors prevented ROS production, indicating that it may not be strictly dependent on ethanol metabolism (data not shown). In this context, increased ROS in organs that do not metabolize significant amounts of ethanol have been shown to demonstrate augmented oxidative stress. Whether physical effects of EtOH on biological membranes,[27] non-oxidative metabolites of ethanol[28] or other factors play a role, will require further study.

Antioxidant treatment prevents ethanol-induced oxidative stress, autophagy and glycolysis in cancer-associated fibroblasts. Here, we show that loss of stromal Cav-1 and upregulation of MCT4 are biomarkers for the ethanol-mediated induction of autophagy and glycolysis in cancer-associated fibroblasts (**Figs. 1A and B and 4**). To determine if these microenvironmental effects are strictly dependent on enthanol-induced ROS production and the resulting oxidative stress, we next treated MCF7-fibroblasts co-cultures with a powerful antioxidant, namely N-acetyl cysteine (NAC). **Figure 11** directly shows that NAC treatment prevents or reverses the ethanol-induced loss of Cav-1 in cancer-associated fibroblasts. Similarly, NAC treatment also blocks the induction of MCT4 in the fibroblast compartment (**Fig. 12**). Thus, ethanol induced oxidative stress, autophagy and glycolysis can be effectively prevented by using simple antioxidant therapies.

Discussion

Ethanol, ketones and two-compartment tumor metabolism. Here, we investigated the mechanism(s) underlying the tumor-promoting activity of alcohol consumption. Numerous epidemiological studies have all shown that ethanol consumption and binge-drinking are significantly associated with an increased risk of developing breast cancer in a dose-dependent fashion (see Introduction). However, it remains unknown how ethanol participates in tumor initiation and cancer progression. One hypothesis is that ethanol somehow affects the metabolic behavior of stromal cells and/or the tumor microenvironment. In support of

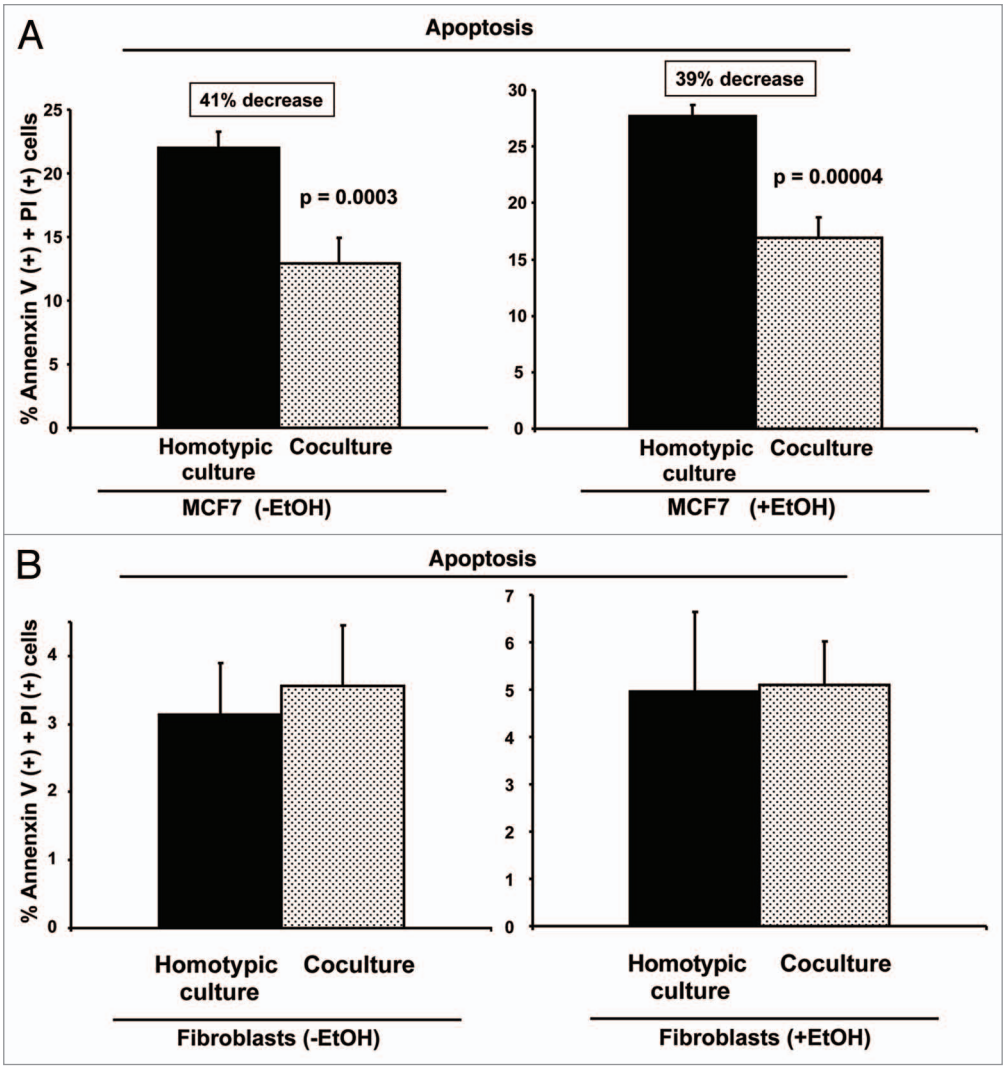

Figure 10. Ethanol does not promote apoptotic cell death. RFP(+)-MCF7 cells were plated in homotypic culture or in co-culture with fibroblasts. Cells were treated with 100 mM EtOH for 72 h. Apoptosis was detected with annexin V-APC and PI by flow cytometry. Note that MCF7 cells are protected against cell death when co-cultured with fibroblasts (**A**). However, ethanol does not significantly promote apoptosis in MCF7 cells (**A**) or in fibroblasts (**B**).

this notion, alcohol consumption promotes the onset of fibrotic liver disease and, thus, fibrosis.

To address this issue experimentally, we studied the effects of ethanol in a co-culture setting to better mimic the effects of the tumor microenvironment. More specifically, ER(+) human breast cancer cells (MCF7) were co-cultured with hTERT-immortalized fibroblasts, and the effects of ethanol on both cell types were simultaneously assessed. Interestingly, our results indicate that ethanol treatment is sufficient to induce oxidative stress in cancer-associated fibroblasts, resulting in ROS production and the onset of autophagy and mitophagy as well as myofibroblastic differentiation (**Fig. 13**).

A loss of mitochondrial function is normally associated with ketone production. As predicted, ethanol induced the expression of several ketogenic enzymes in fibroblasts (HMGCS1/2/HMGCL/BDH1) and also resulted in elevated ketone production. These observations are consistent with the idea that ethanol is converted to ketones (acetoacetate/β-hydroxy-butyrate) in fibroblasts, as it is normally detoxified to acetyl-CoA (ethanol → acetaldehype → acetate → acetyl-CoA → ketone bodies).

However, ethanol had just the opposite effect in the epithelial cancer cell compartment. In MCF7 cells, ethanol conferred autophagy resistance and increased mitochondrial biogenesis, as seen using TOMM20, which is marker of mitochondrial mass. In addition, ethanol increased the expression of enzymes associated with ketone re-utilization (ACAT1/OXCT1; ketone bodies → acetyl-CoA → mitochondrial TCA cycle/OXPHOS). Thus, ethanol has opposing compartment-specific effects, depending on the cell type, facilitating a form of metabolic-symbiosis or communication between epithelial cancer cells and their surrounding stromal microenvironment.

Remarkably, establishment of ethanol-enhanced "two-compartment tumor metabolism" also resulted in conversion of MCF7 cells from ER(+) to ER(-) status. This observation may

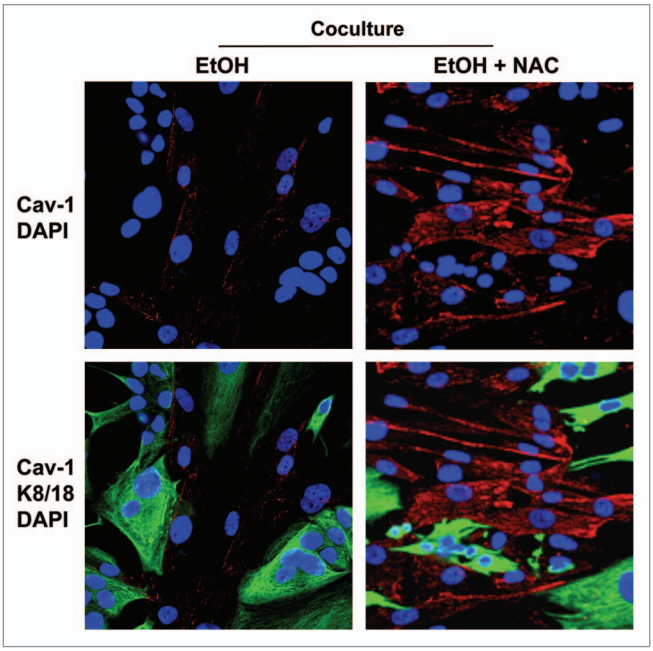

Figure 11. Antioxidant treatment prevents the ethanol-induced loss of Cav-1 in cancer-associated fibroblasts. MCF7 cells were co-cultured with fibroblasts for 2 d and treated with 100 mM EtOH for an additional 72 h. Then, 10 mM NAC or vehicle was added for 24 h. The cells were fixed and immunostained with antibody probes against Cav-1 (red, upper panels) and keratin-8/18 (K8/18, green, lower panels). Nuclei were counterstained with DAPI (blue). K8/18 staining is specific for MCF7 cells and Cav-1 is specific for fibroblasts, since MCF7 cells lack Cav-1 expression. Note that treatment with the antioxidant NAC prevents the downregulation of Cav-1 in the fibroblast compartment, as compared with co-cultured fibroblasts in the absence of NAC. Original magnification, 40x.

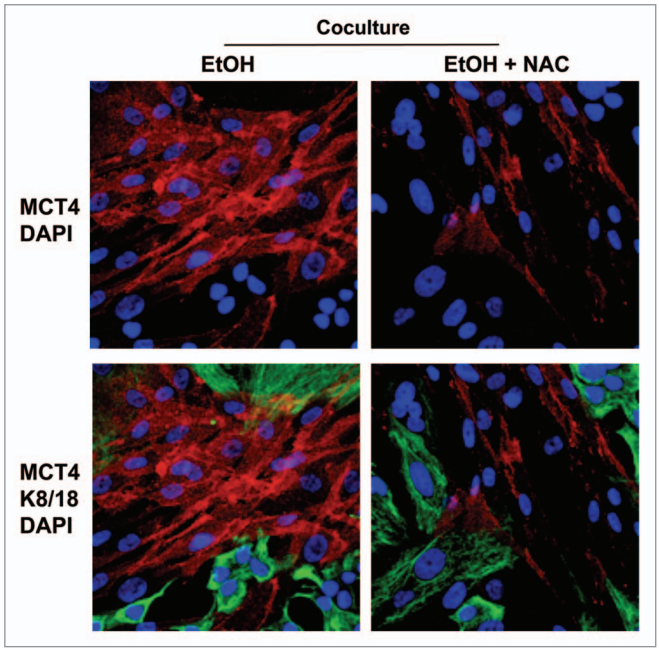

Figure 12. Antioxidant treatment prevents the ethanol-induced upregulation of MCT4 in cancer-associated fibroblasts. MCF7 cells were plated in co-culture with fibroblasts, and cells were treated with 100 mM EtOH for 72 h. Then, 10 mM NAC or vehicle was added for 24 h. Cells were fixed and immunostained with antibodies against MCT4 (red, upper panels) and K8/18 (green, lower panels). Nuclei were counterstained with DAPI (blue). Note that treatment with the antioxidant NAC prevents the upregulation of MCT4 in fibroblasts compartment, as compared with co-cultured fibroblasts in the absence of NAC. Original magnification, 40x.

have clinical implications for understanding how ethanol affects breast cancer progression in ER(+) breast cancer patients, and how it may affect the response to anti-estrogen therapies, such as tamoxifen.

We also observed that ethanol induced a loss of Cav-1 and an elevation of MCT4 in cancer-associated fibroblasts. Loss of Cav-1 is a marker for the onset of stromal autophagy (Cav-1 is destroyed by autophagy-induced lysosomal degradation), while increased MCT4 is a marker of oxidative stress and glycolysis in the tumor microenvironment.[24,26] This has important clinical implications, as we and others have previously shown that a loss of stromal Cav-1 in human breast patients is a strong predictor of early tumor recurrence, metastasis, tamoxifen resistance and overall poor clinical outcome.[29-39] Similarly, elevated MCT4 in cancer-associated fibroblasts is predictive of a poor clinical outcome in breast cancer patients, and strictly correlates with a loss of Cav-1.[40] Thus, our current results with co-culture directly mirror what occurs in a specific subset of high-risk human breast cancers in patients in vivo. Perhaps, more importantly, our current results imply that alcohol consumption in women with breast cancer can affect their tumor's biomarker status, shifting it from Cav-1(+)/MCT4(-)/ER(+) to Cav-1(-)/MCT4(+)/ER(-), which would be strongly predicted to negatively affect clinical outcome. Thus, presumably alcohol consumption could metabolically convert "low-risk" breast cancer patients to "high-risk" status, suggesting a role for ethanol in tumor promotion (**Fig. 14**).

We have recently shown that five genes involved in either stromal ketone production (HMGCS2) or ketone-body re-utilization (ACAT1/2 and OXCT1/2) all function as bona fide metabolic oncogenes in vivo, using a xenograft tumor cell implantation model.[41,42] In addition, expression of ACAT1/2 in epithelial cancer cells effectively promotes metastasis.[42] Similarly, ketogenic fibroblasts promote metastasis, in a paracrine fashion.[43] Finally, BRCA1-deficient fibroblasts are autophagic and produce ketone bodies, which then promote tumor growth via ketone-mediated energy transfer.[44] Thus, alcohol consumption induces that same metabolic phenotype, which is related to the onset of hereditary ovarian and breast cancers, by promoting oxidative mitochondrial metabolism (OXPHOS) in epithelial cancer cells.[45,46]

Ketone body re-utilization has also been shown to confer tamoxifen resistance upon MCF7 cells, consistent with our current findings that ethanol can suppress ER-α expression in MCF7 cells during co-culture with fibroblasts.[47] And, loss of stromal Cav-1 is a predictor of treatment failure in tamoxifen-treated ER(+) breast cancer patients.[34] Consistent with these clinical findings, mammary fat pads derived from Cav-1-deficient mice show a 4-fold increase in ketone body production,[48] and they promote tumor growth up to 5-fold, relative to wild-type

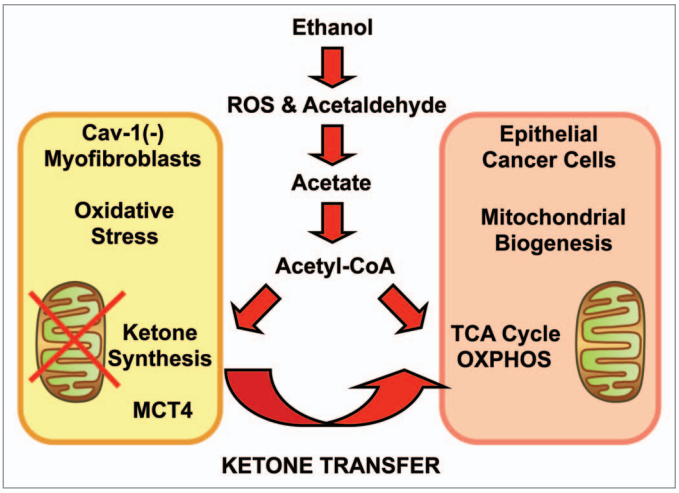

Figure 13. Alcohol consumption and two-compartment tumor metabolism. Ethanol is normally detoxified to acetyl-CoA, which functions as a mitochondrial fuel. However, the first metabolic intermediate is acetaldehyde, which is known to be a chemical carcinogen that results in DNA damage and increased ROS production. In fibroblasts, this initiates the onset of oxidative stress, driving a loss of Cav-1 (via autophagy) and promoting the myofibroblast phenotype [SMA(+)]. Ultimately, this leads to mitophagy and mitochondrial dysfunction in fibroblasts. In cells with dysfunctional mitochondria (fibroblasts), acetyl-CoA is converted to ketone bodies, which are then exported via MCT4 (monocarboxylate transporter 4). In contrast, in cells with functional mitochondrial (epithelial cancer cells), acetyl-CoA is burned in the TCA cycle via OXPHOS. Thus, fibroblasts convert ethanol to mitochondrial fuel (ethanol → acetyl-CoA → ketone bodies), which is exported to "feed" adjacent cancer cells. Then, cancer cells upregulate the necessary enzymes (ACAT/OXCT) to convert ketone bodies back into acetyl-CoA, fueling oxidative mitochondrial metabolism.

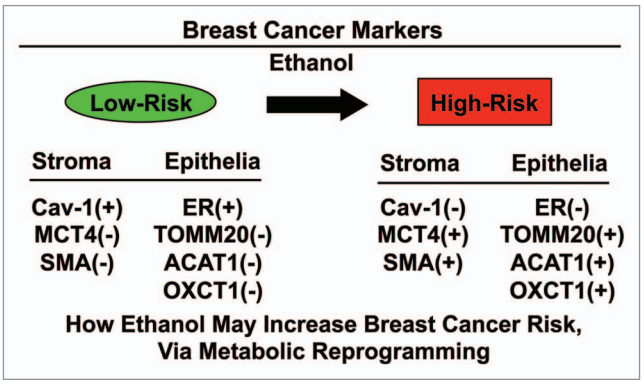

Figure 14. Ethanol may increase breast cancer risk via metabolic reprogramming. In summary, we show that ethanol exposure dramatically affects the expression status of both stromal and epithelial biomarkers functionally associated with poor clinical outcome. For example, ethanol induces myofibroblastic differentiation [SMA(+)], autophagy [Cav-1(-)], as well as oxidative stress and mitochondrial dysfunction [MCT4(+)] in the tumor microenvironment, which fuels metabolic-coupling. Conversely, ethanol drives a shift toward ER(-) status, as well as mitochondrial biogenesis [TOMM20(+)] and ketone body re-utilization [ACAT1(+)/OXCT1(+)] as a mitochondrial energy source. Thus, ethanol "fertilizes" the microenvironment, leading to "two-compartment tumor metabolism" and field cancerization.

mice.[49] Similarly, I.P. injection of ketone bodies in tumor-bearing xenografted mice, increases tumor growth up to 2.5-fold, independently of tumor angiogenesis.[50] Thus, ethanol and ketone bodies both function as onco-metabolites.

Ethanol, ketones, stemness and "neuron-glia" metabolic coupling. Just as stromal fibroblasts (glycolytic) and epithelial cancer cells (oxidative) show two-compartment metabolic-coupling, similar "metabolic symbiosis" occurs in the brain. This is known as "neuron-glia metabolic coupling."[51] In this model, astrocytes are glycolytic and neurons are oxidative.[51] Remarkably, ethanol exposure increases astrocytic differentiation and increases the stemness of the neuronal compartment,[52,53] akin to what we have observed here. Thus, cancer-associated myofibroblasts and astrocytes may have unexpected similarities, and both cell types have been reported to be GFAP(+). Similarly, we have previously shown that treatment of human breast cancer cells with ketone bodies increases their "stemness," as observed by genome-wide transcriptional profiling.[54] In addition, Cav-1-deficient mice show the same phenotype, with increased ketone body production systemically[48] and increased numbers of stem cells in the skin, gut, mammary gland and brain.[55-60]

In the brain, astrocytes produce mitochondrial fuels (L-lactate, ketone bodies and glutamine), which are then consumed by oxidative mitochondrial metabolism in neurons.[61] In addition, neurons are privileged cells that specifically express ACAT1/2 and OXCT1/2 isoforms, that allows them to convert ketone bodies to acetyl-CoA.

Here, we show that ethanol induces ketone body production in fibroblasts and drives ACAT1 and OXCT1 expression in human breast cancer cells (for ketone re-utilization), so cancer cells can use the same metabolic principles as neurons, to fuel their mitochondria.[62]

Materials and Methods

Materials. Antibodies and their source were as follows: Cav-1 (sc-894, Santa Cruz); ACAT-1 (HPA004428, Sigma-Aldrich); OXCT-1 (sc-133988, Santa Cruz); HMGCS1 (sc-33829, Santa Cruz); HMGCS2 (sc-33828, Santa Cruz); HMGCL (WH0003155M1, Sigma-Aldrich); BDH-1 (Sigma-Aldrich, SAB1400024); vimentin (550513, BD Pharmigen); α-SMA (sc-53142, Santa Cruz); BNIP3 (ab10433, Abcam); Cathepsin B (sc-13985, Santa Cruz); LC3B (ab48394, Abcam); Lamp-1 (611043, BD Transduction Laboratories); MCT4 (sc-50329, Santa Cruz); AKT (2967, Cell Signaling); pAKT (9271, Cell Signaling); Mitoprofile total OXPHOS cocktail (MS601, MitoSciences); ERα (sc-7207, Santa Cruz); β-actin (A5441, Sigma-Aldrich).

Cell culture. Human skin fibroblasts immortalized with human telomerase reverse transcriptase (hTERT-BJ1 cells) were purchased originally from Clontech, Inc. The breast cancer cell line, MCF7, was from ATCC. All cells were maintained in DMEM, with 10% fetal bovine serum (FBS) and penicillin 100 units/mL-streptomycin 100 μg/mL (Pen-Strep, Invitrogen). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Fibroblasts and MCF7 cells were co-plated

or plated as homotypic cell cultures in 12-well plates in 1 ml of complete media. The total number of cells per well in co-culture was 1×10^5 cells, with a 5:1 fibroblast-to-epithelial cell ratio. In co-culture experiments, fibroblasts were plated first and MCF7 cells were plated within 2 h of fibroblast plating. As controls, homotypic cultures of fibroblasts and MCF7 cells were plated in parallel, using the same number of a given cell population as the corresponding co-cultures. The day after plating, the media was changed to DMEM with 10% NuSerum (a low protein alternative to FBS; BD Biosciences) and Pen-Strep. Cells were maintained in this media for up to 5 d, until further analysis. For flow cytometry, GFP (+) fibroblasts or RFP (+) MCF7 cells were used to distinguish fibroblasts and MCF7.

Ethanol treatment. To study the effect of EtOH, homotypic cultures and fibroblast-MCF7 co-cultures were treated with 100 mM EtOH for 72 h in 10% NuSerum. Tissue culture plates were placed in chambers saturated with EtOH to mitigate EtOH evaporation from the culture media. The dose of EtOH was selected based on concentrations used previously by others in cell culture.[52,53,63]

Immunoblot analysis. Cells were harvested in lysis buffer (10 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100 and 60 mM n-octyl-glucoside), containing protease (Roche Applied Science) and phosphatase inhibitors (Sigma). After rotation at 4°C for 40 min, cell lysates were centrifuged at 10,000 × g for 15 min at 4°C to remove insoluble debris. Protein concentrations were measured using the BCA reagent (Pierce). Cell lysates were then separated by SDS-PAGE (12–15% acrylamide) and transferred to nitrocellulose membranes. After blocking for 1 h in TBST (10 mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween 20) supplemented with 5% nonfat dry milk, membranes were incubated with primary antibodies diluted in TBST with 1% bovine serum albumin (BSA). Horseradish peroxidase-conjugated secondary antibodies [anti-mouse, 1:6,000 dilution (Pierce) or anti-rabbit, 1:5,000 dilution (BD PharMingen)] were used to visualize bound primary antibodies with enhanced chemiluminescence (ECL) substrate (Pierce).

Immunofluorescence. Cells were rinsed with PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS/CM) and fixed with 2% paraformaldehyde in PBS/CM for 30 min. After fixation, cells were washed three times with PBS/CM and permeabilized with IF buffer (PBS/CM, 0.1% Triton-X100, 0.2% BSA) for 10 min. Then, cells were quenched with 50 mM NH_4Cl in PBS/CM for 10 min, rinsed and incubated with primary antibodies for 1 h at room temperature. Cells were washed with IF buffer and incubated with fluorescent secondary antibodies (Molecular Probes) for 30 min. After washing, nuclei were counterstained with DAPI, and samples were mounted using a Prolong Gold antifade reagent (P36934, Invitrogen).

Confocal microscopy. Images were collected with a Zeiss LSM510 meta confocal system equipped with oil immersion lens (40X). Images of fluorophore-labeled cells were acquired using a 405-nm Diode excitation laser with a band pass filter of 420–480-nm, a 488-nm Argon excitation laser with a band pass filter of 505–550-nm and a 543-nm HeNe excitation laser with a 561–604-nm filter.

ROS assay. Cells were washed and incubated with 5 μM CellROX Deep Red Reagent (C10422, Invitrogen) for 30 min at 37°C. Then, cells were washed with PBS, trypsinized and resuspended in PBS. The signal was analyzed by FACS using the BD LSRII (BD Bioscience). The results were analyzed using FlowJo software (Tree star Inc.).

β-hydroxybutyrate assay. Fibroblasts were plated at a density of 8.3×10^4 in 12-well plates in complete media. The next day, media was changed to phenol-red-free DMEM containing 2% FBS and cells were treated with 100 mM EtOH. After 72 h, the cell culture media was collected. β-OH-butyrate concentration was determined using the β-hydroxybutyrate Assay Kit (K632–100, BioVision), and was normalized either for protein concentrations or cell numbers.

Quinacrine uptake. The assay is based on the selective binding of the fluorescent dye quinacrine (Quinacrine mustard dihydrochloride, Q2876–5MG, Sigma) to ATP-filled vesicles. When excited, quinacrine emits green fluorescence that can be measured by flow cytometry. To this end, cells were washed with PBS and incubated with 30 μM quinacrine in phenol-red-free DMEM for 1 h at 37°C. After three washes with PBS, cells were trypsinized, harvested and resuspended in PBS. RFP(+) MCF7 cells were used in co-culture experiments to identify MCF7 cells. The resulting signal was quantified by FACS using the BD LSRII (BD Bioscience). The results were analyzed using FlowJo software (Tree star Inc.).

Apoptosis detection. Cell death was quantified by flow cytometry using propidium iodide (PI) and annexin-V-APC, as previously described with minor modifications.[64] Briefly, GFP(+) fibroblasts were plated with MCF7 cells in 12-well plates. After ethanol treatment, cells were collected by centrifugation and resuspended in 500 μL of PBS. Then, the annexin-V-APC conjugate (4 μL) and PI (1 μL) was added and incubated in the dark at room temperature for 5 min. Cells were then analyzed by flow cytometry using a GFP signal detector to detect fibroblasts, a PE Texas Red signal detector for PI staining and an APC signal detector to detect annexin-V binding. GFP(-) cells were the MCF7 cells. Data were analyzed using FlowJo software (Tree star Inc.).

Statistical analysis. Data were analyzed with the Student's t-test. p values lower than 0.05 were considered statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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