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Research Paper

Alcohol Attenuated the Epithelial Phenotype in Liver HepG2 Cells Co-Cultured with K562 Cells

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ABSTRACT

Background: Excessive drinking can cause liver damage and even liver fibrosis and cirrhosis. This study aimed to investigate the influence of alcohol and its related mechanisms on the transformation of growth factor-β1 (TGF-β1) and the loss of epithelial phenotype in hepatocytes.

Methods: In the present study, HepG2 cells (2×10^5 cells/ml) were co-cultured with K562 cells (2×10^5 cells/ml) and treated with 50 mM, 100 mM, and 200 mM alcohol for 24 hours. Cell morphology, as well as the expression of endothelial markers E-cadherin, Desmoplakin, CK18, and TGF-β1, were detected.

Results: The study showed that alcohol induced the loss of epithelial phenotype, considerably decreasing the mRNA levels of E-cadherin, Desmoplakin, and CK18, as well as E-cadherin protein expression. In contrast, alcohol increased the mRNA levels and concentrations of TGF-β1 in the cultured media of co-cultured HepG2 cells in a concentration-dependent manner. These results demonstrated that alcohol attenuated the epithelial phenotype in HepG2 cells co-cultured with K562 cells; meanwhile, K562 cells played an essential role in enhancing the expression of TGF-β1 in HepG2 cells.

Conclusion: Our research has established an in vitro model of alcohol-induced epithelial phenotype loss in hepatocytes and partly explained the mechanisms of alcohol-induced liver fibrosis.

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Introduction

Excessive alcohol consumption can cause a wide range of liver diseases, ranging from simple steatosis to severe liver injury [1]. Liver fibrosis gradually alters the liver's physiological structure and hemodynamics, eventually progressing to liver cancer or liver failure [2]. Previous studies have indicated that resident epithelial cells in the liver may undergo a process known as epithelial-to-mesenchymal transition (EMT) in response to injury [3]. In adults, liver EMT has been linked to chronic inflammation, cancer, primary biliary fibrosis, and nonalcoholic fatty liver disease in patients and rodents [4].

During EMT, mature epithelial cells lose their typical epithelial characteristics, including the expression of E-cadherin, cytokeratin-18 (CK18), and desmoplakin, as well as the expression of tight junction proteins. They detach from the epithelial layer, gain increased motility, and acquire a myofibroblastic phenotype [3, 5–7]. E-cadherin played a key role in forming stable physical connections and maintaining cell polarity. Abnormal structural or reduced expression of E-cadherin can trigger the occurrence of epithelial phenotype loss [8–11]. Desmoplakin, encoded by the DSP gene, is a cell adhesion molecule and a member of the cadherin family. CK18, the primary substrate of caspase, constitutes the hepatic cytoskeleton and is associated with the apoptosis of liver cells and various liver diseases [12].

Human chronic myeloid leukemia K562 cells are malignant tumor cells derived from the hematopoietic system with multi-directional differentiation potential and precursor cells of granulocytic, mononuclear, and erythroid lines, which have been widely used in studies of natural killer cells and mononuclear macrophages [13, 14]. Additionally, K562 cells can secrete various inflammatory cytokines, including TGF-β1, IL-1, IL-6, and IL-8 [14]. TGF-β1 is not only a key profibrotic cytokine but also the most potent growth factor promoting EMT and EndMT [15–17]. Notably, TGF-β signaling pathways are upregulated in alcoholic liver disease models [18, 19]. Moreover, alcohol has been shown to induce epithelial phenotype loss in breast cancer cells and stimulate mesenchymal marker expression in breast, colon cancer cells, and normal intestinal epithelial cells [20, 21].

Human hepatocellular carcinoma HepG2 cells, derived from the liver tissue of a 15-year-old Caucasian male with well-differentiated hepatocellular carcinoma, are extensively used to study drug metabolism, cytotoxicity, genotoxicity, and hepatotoxicity in vitro. In this study, an in vitro model of alcohol-induced epithelial phenotype loss in hepatocytes was established by co-culturing HepG2 cells with K562 cells. This model aims to elucidate the molecular mechanisms underlying alcohol-induced epithelial phenotype loss and provide evidence for the role of cytokines in alcohol-induced liver fibrosis.

Materials and Methods

Cell culture

K562 cells and HepG2 cells were maintained in DMEM culture medium (GIBCO, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone), 100 U/mL penicillin (Sigma-Aldrich), and 100 µg/mL streptomycin (Sigma-Aldrich) in a humidified 5% CO_2 incubator at 37°C. Exponentially growing cells at passage 4–8 after recovery were collected and re-suspended in fresh culture medium.

Cell morphology analysis

To measure the effects of alcohol on cell morphology, HepG2 cells were seeded and cultured for 4 hours. The cells were co-cultured with K562 cells and treated with 50–200 mM ethanol (ETOH) for 24 h. Then, the spindle-like cells were photographed using a phase contrast microscope (ELWDN.A.0.30) and counted using a haemocytometer.

Cell viability analysis

Cell viability was detected using trypan blue dye exclusion. HepG2 cells were exposed to various concentrations of alcohol (0 mM–1000 mM) for 24 h. Later, the cells were collected and stained with trypan blue (Sigma-Aldrich). The unstained living cells were counted under a light microscope with a hemocytometer.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

RT-PCR was conducted as described previously [17]. The PCR primer sequences are listed in Table 1.

Immunofluorescence

Immunofluorescence of E-cadherin was conducted as described previously [17].

Quantification of TGF-β1 by ELISA

Quantification of TGF-β1 was conducted by ELISA as described previously [17].

Table 1. Sequences of primers used in reverse transcriptase PCR.

Gene	Sequence of primers (5'-3')	Size (bp)
TGF-β1	Forward primer: CTAATGGTGGAAACCCACAACG Reverse primer: TATCGCCAGGAATTGTTGCTG	209
E-cadherin	Forward primer: CCAAAAAGTGATTGCAGGGT Reverse primer: GGGGGTTAAGTTGAGGGGTA	170
Desmoplakin	Forward primer: GTGCCCAATAGAACTACTGC Reverse primer: GGCTTACCCAGCCCTGCTGAA	179
CK18	Forward primer: AGGAGTATGAGGCCCTGCTGAA Reverse primer: TTGCATGGAGTTGCTGCTGTC	128
β-actin	Forward primer: TGGACTTCGAGCAAGAGATGG Reverse primer: ATCTCTTCTGCATCCTGTCG	289

Statistical analyses

Experimental data were expressed as the mean±standard deviation (SD) and statistically compared using the Student's t-test. $P < 0.05$ was considered statistically significant.

Results

The cytotoxicities of alcohol to HepG2 cells and K562 cells

Our previous study showed that treating HepG2 cells with >200 mM alcohol for 24 h induced a key morphological change: from anti-aliasing cell clusters to elongated spindle shapes (a hallmark of epithelial phenotype loss). In contrast, K562 cells showed no such typical change (Figure 1A). When HepG2 and K562 cells were treated with 0–1000 mM alcohol for 24 h, respectively, viable cell numbers decreased and mortality increased in a concentration-dependent manner (Figure 1B, C). At 200 mM alcohol, HepG2 and K562 cell mortality were <10% and <5% respectively, supporting the use of low alcohol concentrations (50–200 mM) in subsequent experiments.

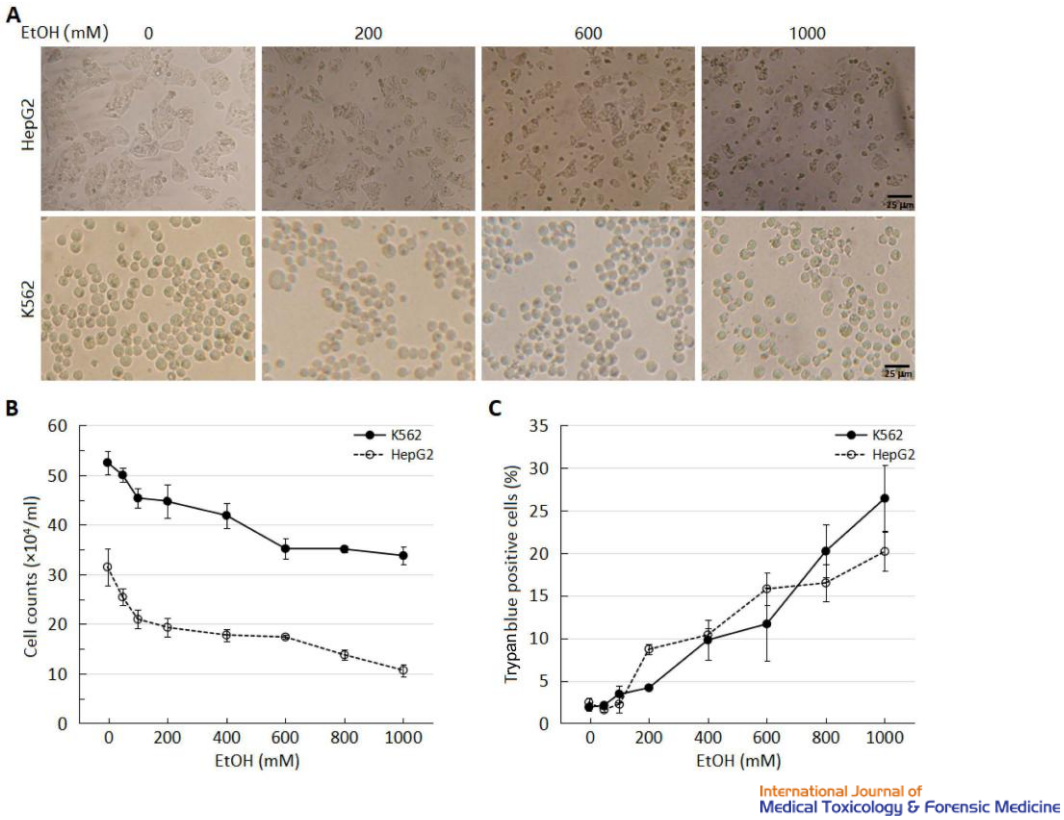

Figure 1. The proliferation and cytotoxicity in alcohol-induced HepG2 cells and K562 cells. (A) Morphological changes of HepG2 cells and K562 cells. (B) The number of viable cells. (C) The number of dead cells. EtOH: alcohol.

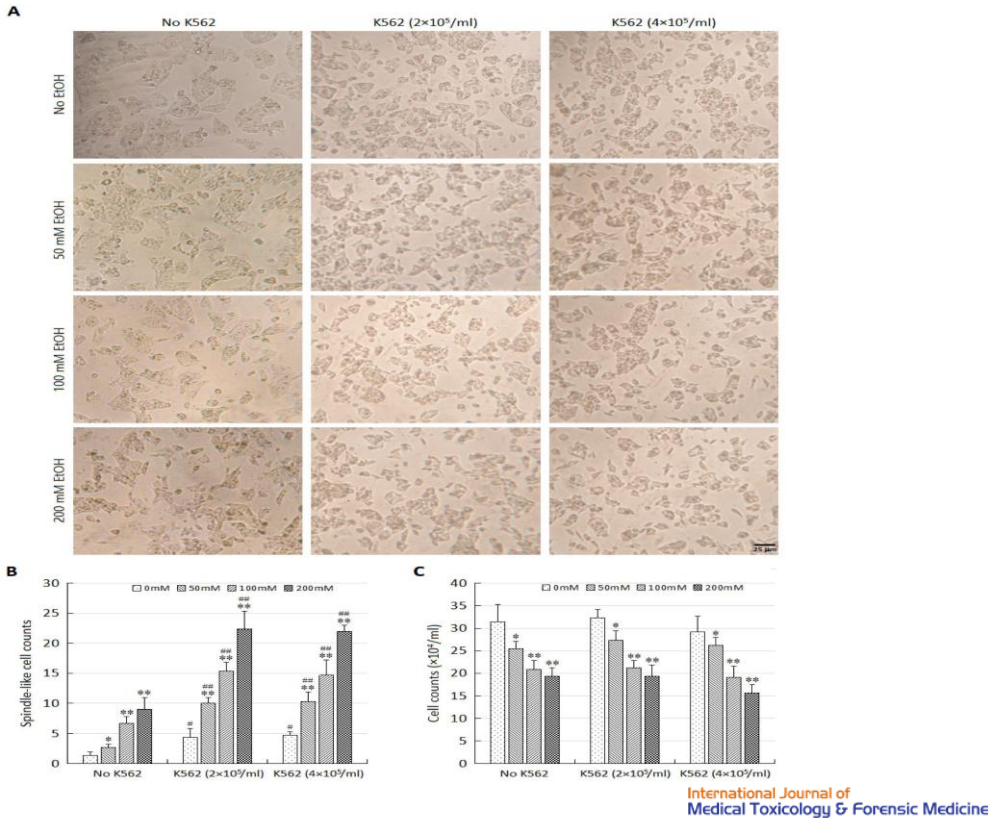

Figure 2. Morphological changes and the percentages of spindle-like cells in alcohol-induced HepG2 cells. (A) Morphological changes of HepG2 cells. (C) The number of viable HepG2 cells. EtOH: alcohol. No K562: single HepG2 cells; K562 (2×10^5 cells/ml): HepG2 cells co-cultured with K562 cells (2×10^5 cells/ml); K562 (4×10^5 cells/ml): HepG2 cells co-cultured with K562 cells (4×10^5 cells/ml). *P< 0.05 and **P< 0.01 (vs corresponding "0 μM" EtOH control group). #P<0.05 and ##P<0.01 (vs corresponding No K562 group.

The effect of co-culture with K562 cells on alcohol-induced HepG2 cells

After the alcohol-induced HepG2 cells were co-cultured with 2×10^5 cells/ml and 4×10^5 cells/ml K562 cells for 24 h, the cell morphology is shown in Figure 2A. Alcohol could considerably increase the number of spindle-like HepG2 cells in a concentration-dependent manner, whereas co-culture with K562 cells enhanced the number of spindle-like HepG2 cells. However, no significant differences were detected between the two K562 cell co-culture groups, which supports further investigation using only the lower density (Figure 2B). Consistently, alcohol could considerably inhibit the proliferation of HepG2 cells in a concentration-dependent manner, whereas co-culture with K562 cells had no crucial change in the proliferation of HepG2 cells (Figure 2C).

Alcohol attenuated the epithelial phenotype in HepG2 cells co-cultured with K562 cells.

When HepG2 cells were co-cultured with 2×10^5 cells/ml K562 cells, 50–200 mM alcohol significantly decreased the mRNA levels of E-cadherin, Desmoplakin, and CK18 in a concentration-dependent manner, and 200 mM alcohol also significantly decreased E-cadherin protein expression. (Figure 3A–E). These findings indicated that alcohol leads to loss of epithelial phenotype in HepG2 cells co-cultured with K562 cells.

The role of TGF-β1 in alcohol-induced HepG2 cells co-cultured with K562 cells.

When K562 cells or HepG2 cells were co-cultured with 2×10^5 Cells/ml, K562 cells were treated with 50–200 mM alcohol. The mRNA levels of TGF-β1 and the TGF-β1 concentrations in the cultured media both increased considerably in a concentration-dependent manner (Figure 4A–E). These indicated that co-culture with K562 cells activated the secretion and mRNA expression of TGF-β1 in HepG2 cells, which was enhanced by alcohol treatment.

Discussion

A summary of the signaling mechanisms underlying alcohol-induced epithelial phenotype loss is outlined in Figure 5. The study found that exposure to 0–200 mM alcohol for 24 h attenuated epithelial phenotype in HepG2 cells co-cultured with K562 cells. There is no contradiction between the typical changes

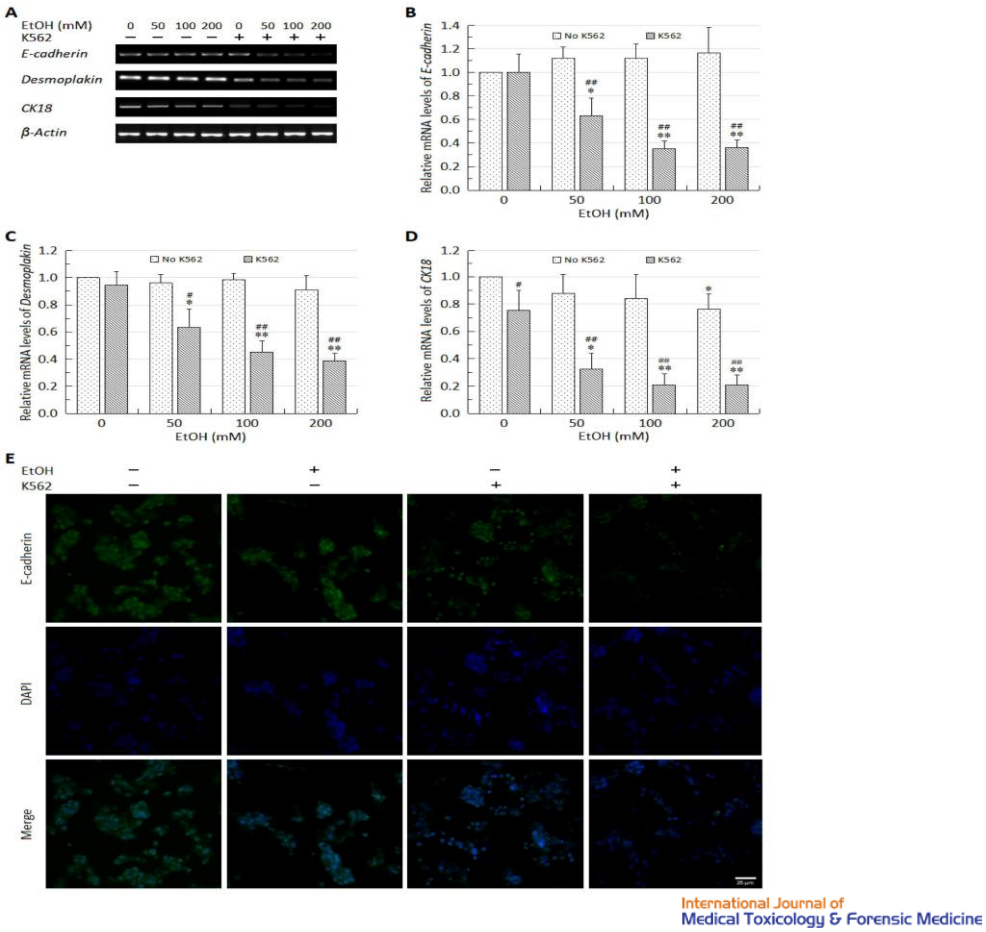

Figure 3. The expression of epithelial markers in alcohol-induced HepG2 cells. (A–D) The mRNA levels of *E-cadherin*, *Desmoplakin* and *CK18*. (E) The protein expression of E-cadherin. EtOH: alcohol. No K562: single HepG2 cells; K562: HepG2 cells co-cultured with K562 cells (2×10^5 cells/ml). *P< 0.05 and **P< 0.01 (vs corresponding "0 μM" EtOH control group). #P<0.05 and ##P<0.01 (vs corresponding No K562 group).

in cell morphology and the epithelial markers when HepG2 cells are treated with 50–200 mM alcohol for 24 h. The alcohol-treated HepG2 cells are probably in the intermediate stage of EMT [5,22]. Many pedigree tracking studies on the mechanisms of renal and lung fibrosis have confirmed a similar process [23].

In our study, co-culture with K562 cells activated the expression of TGF-β1 in HepG2 cells. Alcohol may lead to the loss of epithelial phenotype in HepG2 cells by enhancing the expression of TGF-β when co-cultured with K562 cells. K562 cells could secrete various inflammatory cytokines, including TGF-β, IL-1, IL-6, and IL-8 [14]. TGF-β1 secreted by K562 cells stimulated the related signaling pathways, such as the Smad pathway and the Wnt pathway in HepG2 cells [24-26]. And then activated the autocrine pathway of TGF-β1, which promotes the EMT process, leading to the loss of epithelial phenotype in HepG2 cells [29].

How to rule out the effects of other cytokines on HepG2 cells or whether the alcohol-induced loss of epithelial phenotype could be reversed by removing K562 cells or neutralizing TGF-β1 remains to be further studied. The mechanisms of long-term exposure to low concentrations of alcohol on the epithelial phenotype of HepG2 cells and the role of inflammatory cells require further study. Further assessment of suitable animal models or other tools would be a significant advantage for the therapeutic potential.

Conclusion

This study indicates that exposure to 0–200 mM alcohol for 24 hours attenuated the epithelial phenotype in HepG2 cells co-cultured with K562 cells, where K562 cells enhanced the expression of TGF-β in HepG2 cells, playing important roles. Our research has established an in vitro model of alcohol-induced epithelial phenotype loss in hepatocytes, using HepG2

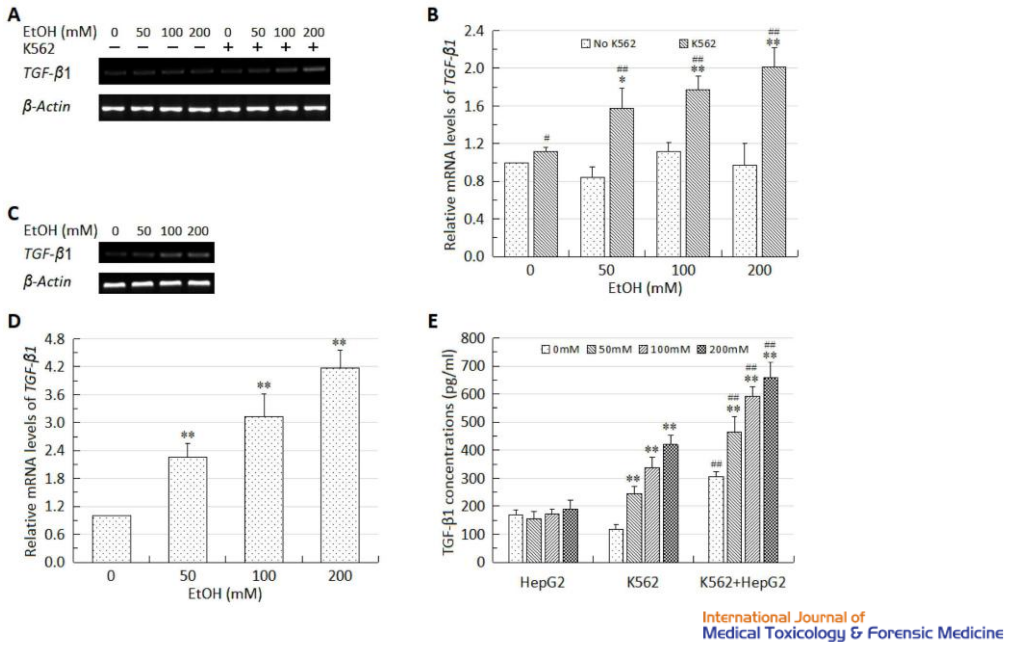

Figure 4. The expression of TGF-β1 in alcohol-induced HepG2 cells and K562 cells. (A–B) The mRNA levels of TGF-β1 in HepG2 cells. (C-D) The mRNA levels of TGF-β1 in K562 cells. (E) The concentrations of TGF-β1 in the culture medium. EtOH: alcohol. K562+HepG2: HepG2 cells co-cultured with K562 cells. *P< 0.05 and **P< 0.01 (vs corresponding "0 μM" EtOH control group). #P<0.05 and ##P<0.01 (vs corresponding No K562 group in B; vs corresponding K562 group in E).

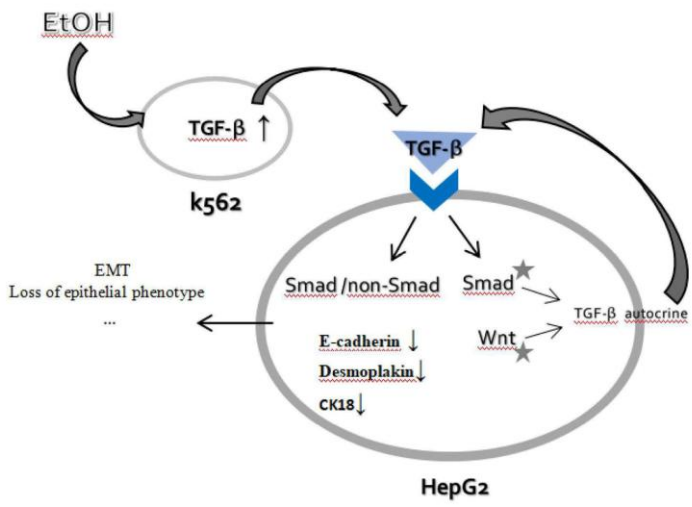

Figure 5. Mechanisms of alcohol attenuated the epithelial phenotype in HepG2 cells co-cultured with K562 cells.

cells co-cultured with K562 cells, which allows for the examination of the mechanism of cytokines in alcohol-induced epithelial phenotype loss. This molecular mechanism may enable us to understand alcohol addiction-induced liver fibrosis better, but more studies are required to make it a prime candidate for future therapeutic manipulation.

Conflicts of Interest

The authors declare that there is no conflict of interest to indicate.

Ethical Statement

This is an in vitro study. No animals or human subjects were used.

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