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Long-Term Ethanol Exposure-Induced Hepatocellular Carcinoma Cell Migration and Invasion through Lysyl Oxidase Activation Are Attenuated by Combined Treatment with Pterostilbene and Curcumin Analogues

Ching-Shui Huang,[†,§] Chi-Tang Ho,[○] Shih-Hsin Tu,[†,§] Min-Hsiung Pan,[△] Chien-Hui Chuang,[‡] Hui-Wen Chang,[#] Chien-Hsi Chang,[#] Chih-Hsiung Wu,*[,⊥,∥,¶] and Yuan-Soon Ho*[,‡,#,∥]

[†]Graduate Institute of Medical Sciences, Taipei Medical University, Taipei, Taiwan

[§]Department of Surgery, Cathay General Hospital, Taipei, Taiwan

[○]Department of Food Science, Rutgers University, New Brunswick, New Jersey, United States

[△]Department of SeaFood Science, National Kaohsiung Marine University, Kaohsiung, Taiwan

[‡]School of Medical Laboratory Science and Biotechnology, College of Medical Science and Technology, Taipei Medical University, Taipei, Taiwan

[#]Department of Laboratory Medicine, Taipei Medical University Hospital, Taipei, Taiwan

[⊥]Department of Surgery, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan

[∥]Center of Excellence for Cancer Research, Taipei Medical University, Taipei, Taiwan

[¶]Department of Surgery, Taipei Medical University-Shuang Ho Hospital, Taipei, Taiwan

ABSTRACT: Ethanol consumption induces hepatocellular carcinoma (HCC) cell metastasis by changing the extracellular matrix (ECM). Lysyl oxidase (LOX) catalyzes the cross-linkage of collagen or elastin in the ECM. LOX protein and mRNA overexpression (>21-fold compared with controls, $n = 6$) was detected in cirrhotic HCC patients with a history of alcoholism. LOX protein expression was induced in HCC cells after long-term treatment with ethanol (10 mM) for 20−40 passages (denoted E20−E40 cells). Pterostilbene (PSB, 1 μM) displayed significant potency to reduce LOX-mediated activity in E40 cells when combined with curcumin and its analogues. The ability of E40 cells to form colonies in soft agar was reduced by both genetic depletion of LOX and by chemical inhibitors of LOX expression. This study suggests that targeting LOX expression with food components such as PSB and curcumin may be a novel strategy to overcome ethanol-induced HCC cell metastasis in liver cancer patients.

KEYWORDS: *lysyl oxidase, hepatocellular carcinoma, metastasis, pterostilbene, alcoholism*

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant primary liver tumors in the world and accounts for more than 500,000 deaths each year.[1] More than 80% of HCC patients have primary liver tumors arising from the transformation of hepatic parenchymal cells (hepatocytes). In Asian and African countries, the incidence of HCC is as high as 20−150 cases per 100,000 people, with underlying cirrhosis in 50−60% of HCC cases.[2] In Taiwan, HCC is primarily associated with risk factors such as aflatoxin B1 exposure, hepatitis B virus (HBV) infection, and alcoholism.[3] However, epidemiological studies have demonstrated that ethanol-dependent patients with viral hepatitis have a poorer prognosis than patients who abstain from ethanol.[4] Consequently, we postulated that combined viral infection and risk factors (such as ethanol consumption) contribute to the development of cirrhosis.

HCC is a highly metastatic cancer that accounts for approximately 83% of all liver cancer cases and is the third leading cause of cancer-related death worldwide.[5] HCC frequently displays early invasion into blood vessels as well as intrahepatic metastasis and, later, extrahepatic metastasis, which is indicated by the spread of cancer cells from the primary neoplasm to distant sites and subsequent growth at those sites. Cancer metastasis is the major cause of poor clinical outcomes, including death, in liver cancer patients.[6] Recurrence and metastasis are the main causes of treatment failure and the high fatality rate of HCC.[7,8] Cancer metastasis is a multistep process that is responsible for most cancer-related deaths and can be influenced by both the immediate microenvironment (cell−cell or cell−matrix interactions) and the extended tumor microenvironment (such as vascularization). Metastasis is also involved in various cytophysiological changes such as the overexpression of matrix metalloproteinases (MMPs) in the extracellular matrix (ECM). Hypoxia (low oxygen) is clinically associated with metastasis and poor patient outcomes, although the underlying processes remain unclear. Microarray studies

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have shown that lysyl oxidase (LOX) expression is elevated in hypoxic human liver tumor cells.[9] LOX is an ECM enzyme that catalyzes the cross-linkage of collagen or elastin in the extracellular compartment, thereby regulating the tensile strength of tissues.[10,11]

Five LOX family genes have been identified thus far in mammalian genomes that encode the prototypical LOX and four different LOX-like proteins (LOXL1, LOXL2, LOXL3, and LOXL4).[12] The gene encoding the LOX isoenzyme has been localized to human chromosome 5q23.3-q31.2.[13,14] The enzyme is synthesized as a 48 kDa precursor that is N-glycosylated, resulting in an apparent molecular mass of 50 kDa. Pro-lysyl oxidase is then processed extracellularly to a 32 kDa form by proteolysis of a Gly−Asp bond. This reaction, which leads to activation of the proenzyme, is catalyzed by a metalloproteinase identified as PCP/BMP-1. LOX and LOX-like proteins are copper-containing enzymes that catalyze the oxidative deamination of the ε-amino group on certain peptidyl lysine residues promoting covalent cross-linking of proteins.[12] All members of the LOX family have a highly conserved C-terminal region that contains the catalytic domain. The N-termini of the LOX isoforms are less conserved among the different members and are thought to determine the individual role and tissue distribution of each isoenzyme.[12] A previous study demonstrated that forkhead box M1b transcription factor-induced LOX protein secretion by metastatic liver tumor cells enhances the invasion and recruitment of metastasizing tumor cells.[15] Recent studies have demonstrated novel roles for LOX, including the regulation of gene transcription,[16] cell motility and migration, and cell adhesion.[17] Biopsies of human tumors and fibrotic liver tissues have revealed an increase in LOXL2 expression in disease-associated stroma and limited expression in healthy tissues.[18] Targeting LOXL2 with an inhibitory monoclonal antibody (AB0023) is efficacious in both primary and metastatic xenograft models of cancer as well as in liver fibrosis models.[18]

Serum LOX activity has been shown to be a more sensitive indicator of liver fibrosis than serum prolyl hydroxylase and laminin P1.[19] However, the unavailability of sufficient samples has hindered the investigation of the mechanisms underlying LOX-induced liver fibrosis. Epidemiological studies have shown that the consumption of alcoholic beverages is associated with an increased risk of fibrotic HCC.[20,21] Although ethanol itself is not genotoxic, chronic ethanol consumption increases the risk of hepatocarcinogenesis.[22] However, the mechanism by which ethanol exerts a carcinogenic effect on hepatic cells is not well established. Our previous studies focused on the ability of natural polyphenolic compounds such as pinocembrin[23] and phloretin to inhibit tumor formation and ethanol-induced liver cirrhosis.[24,25] The aim of this study was to test the hypothesis that long-term ethanol consumption plays an important role in HCC metastasis and the associated up-regulation of ECM genes (such as LOX expression) involved in human liver cancer metastasis. To test this hypothesis, we established human hepatocellular carcinoma cell lines (Hep 3B) containing integrated HBV sequences by consecutively culturing these cells for 20−40 passages in the presence or absence of 10 mM ethanol (designated E20−E40 and C20−C40, respectively).[26] We then tested the effects of natural compounds, including dibenzoylmethane (DBM), a phytochemical that is a minor constituent of the root extract of licorice (*Glycyrrhiza glabra* of the family Leguminosae), and pterostilbene (PSB; see chemical structure in Figure 1), a stilbenoid chemically related to

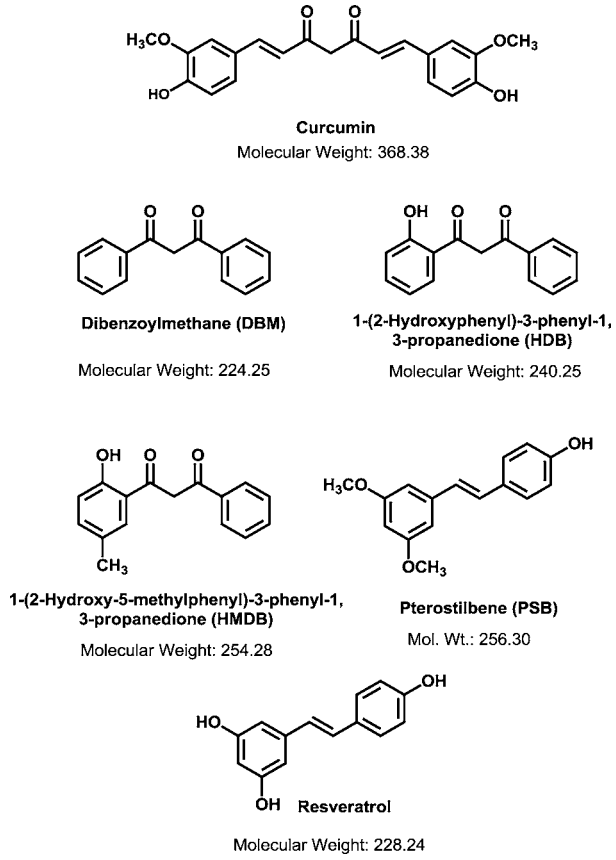

Figure 1. Chemical structures of PSB, curcumin, and their analogues.

resveratrol (RES) found in blueberries and grapes, on the metastatic ability of E40 cells. The results showed that diverse functions of LOX have led researchers to hypothesize that this enzyme may represent a potential target for HCC therapy.[9,15] There are various natural DBM analogues in plants. For example, curcumin (Figure 1) is isolated from both *Belamcanda chinensis* and turmeric.[27,28] The antitumor effects of RES and its methoxy derivative on human cancer cell lines have been evaluated,[29,30] revealing that the difference in the bioactivity of the methoxy derivative is due to the presence and position of the methoxy groups on the basic curcumin chemical structure. 1-(2-Hydroxyphenyl)-3-phenyl-1,3-propanedione (HDB) and 1-(2-hydroxy-5-methylphenyl)-3-phenyl-1,3-propanedione (HMDB) have a basic skeleton identical to that of DBM and are similar to curcumin in that they possess a β-diketone (1,3-diketone), but HDB and HMDB possess a hydroxyl group and a methyl group on the aromatic ring, respectively.

MATERIALS AND METHODS

Establishment of Hep 3B Cell Lines Chronically Treated with Ethanol. The Hep 3B cell line was derived from a human HCC cell line (ATCC HB-8064) that secretes major plasma proteins and the hepatitis B surface antigen.[31] This cell line, which contains integrated HBV sequences, is a good model to study whether long-term exposure to ethanol can promote tumor formation in virally infected HCC cells. To demonstrate the suitability of this system as a model for exploring the mechanisms of ethanol-promoted HCC development, we derived chronically ethanol-treated cells from Hep 3B cells by continuously culturing the cells in medium containing 10 mM ethanol (Sigma-Aldrich, Dorset, UK).[26] The ethanol-treated cells were harvested at 10, 20, 30, and 40 passages (designated E10, E20, E30, and E40, respectively). Cells that were not treated with ethanol were harvested

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as controls (designated C10, C20, C30, and C40). Ethanol evaporation from the culture medium may complicate the interpretation of the results of long-term cell exposure to ethanol. To avoid evaporation, the culture dishes were placed in a closed system that permitted CO_2 and O_2 exchange in the incubator with an ethanol-saturated atmosphere over the culture medium.[32] Cell proliferation and viability were determined using a hemocytometer and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay.

Chemicals and Reagents. Curcumin was purchased from E. Merck Co. (Darmstadt, Germany). RES, DBM, HDB, and HMDB were purchased from Sigma-Aldrich Chemical Co. (Milwaukee, WI, USA). The chemicals used in this study were dissolved in dimethyl sulfoxide (DMSO) as described previously.[33,34] Pterostilbene (PSB, 96% purity) was a gift from Sabinsa Corp. (East Windsor, NJ, USA).[35]

Patient Samples. Human HCC samples (n = 14) were obtained as anonymous specimens from Taipei Medical University Hospital through a protocol approved by the Institutional Review Board (P950023) and were embedded in paraffin for immunohistochemistry. The samples were obtained from 10 male patients with a mean age of 58.4 years (range, 39−77 years) and 4 female patients with a mean age of 56.2 years (range, 53−62 years). Among the 14 patients, 9 were hepatitis B carriers, 2 were hepatitis C carriers, and 1 had simultaneous hepatitis B and C infections. Two patients were free of hepatitis B and C infection. Six patients also presented with a history of heavy alcohol drinking; among these patients, one was a hepatitis B carrier, and another was a hepatitis C carrier.

Tissue Microarray (TMA). A TMA containing human tissues was purchased from Biomax Co. (Rockville, MD, USA). In accordance with the manufacturer's information, all tissue samples were fixed in formalin, processed in a tissue processor (no more than 24 h of fixation), and embedded in paraffin. The TMA #IC03001 contained 80 spots (1.5 mm diameter) of the following three tissue types: adjacent normal (from A1 to C10), viral hepatitis inflammation (from D1 to D10), and active hepatic cirrhosis (from E1 to H10).

Immunohistochemistry. The localization of LOX protein in TMA and HCC tumor tissues was determined by immunohistochemistry. HCC tumor tissues excised from patients were embedded in paraffin and cut into 8 μm sections. After deparaffinization, the tissue sections were preincubated in 3% H_2O_2 and 0.3% Triton X-100, followed by microwaving in Tris buffer (pH 6) for 10 min to retrieve the antigen. The sections were then blocked in 5% horse serum (Chemicon, Temecula, CA, USA) for 30 min and incubated with LOX antibody (diluted 1:400) for 2 h at room temperature. Following incubation with secondary antibodies, the bound antibodies were visualized using the streptavidin−biotin−peroxidase method with an LSAB 2 Kit from DAKO (Carpinteria, CA, USA) as previously described.[36] The fibrotic hepatic tissues were stained with hematoxylin and eosin (H&E) and confirmed using Masson's stain methods.

Laser Capture Microdissection (LCM).[25] The sections stained with H&E were subjected to LCM using a PixCell IIe system (Arcturus Engineering, Mountain View, CA, USA). Areas of interest were selected under microscopic guidance and covered with ethylene vinyl thermoplastic film mounted on an optically transparent cap. The infrared laser was activated, and the laser melted the film directly above the target cells. Laser-induced melting causes the cells to bind and transfer to the film because this interaction is stronger than the association between the cells and the slide. The parameters used for LCM included a laser diameter of 7.5 μm and a laser power of 48−65 mW. Per specimen, 15000 laser pulse discharges were used to capture ∼10000 morphologically normal epithelial cells or malignant carcinoma cells for each case. Each population was visualized under a microscope to ensure that the captured cells were homogeneous. The caps with the captured cells were then fitted onto 0.5 mL Eppendorf tubes containing 42 μL of DNA lysis buffer.

RNA Isolation and Real-Time RT-PCR. Total RNA was isolated from human cell lines, and LCM-dissected liver tumor tissue samples were acquired directly from patients using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.[36] The primers used were as follows: sense (LOX), TATACATAAGGCAGCCGTGAA; antisense (LOX), GAGACAGTTGTGGTTTGGG; sense (GUS), AAACAGCCCGTTTACTTGAG; and antisense (GUS), AGTGTTCCCTGCTAGAATAGATG. Real-time PCR reactions were performed in a LightCycler thermocycler (Roche Molecular Biochemicals, Mannheim, Germany). LOX mRNA fluorescence intensity was measured and normalized to β-glucuronidase (GUS) expression using the built-in Roche LightCycler Software, version 4. LOX mRNA levels in 14 patient samples were classified according to their history with (group 1, n = 6) or without (group 2, n = 8) alcoholism. The copy numbers (1×10^5 per μg of mRNA) were calculated from the mean real-time RT-PCR data; the error bars indicate 95% confidence intervals. The fold ratios of LOX mRNA expression detected in tumor versus normal samples with different clinical staging criteria were compared using the Scheffe test; all P values are two-sided.

Protein Extraction, Western Blot Analysis, and Antibodies. To examine protein expression, human HCC tumor cells were thawed in lysis buffer containing protease inhibitors, and protein (50 μg) from each sample was separated by 12% SDS−polyacrylamide gel electrophoresis (SDS-PAGE), transferred, and analyzed by Western blot analysis. Primary antibodies (anti-LOX, anti-p-FAK576, anti-FAK, antipaxillin, anti-p-paxillin Tyr-118, and anti-GAPDH) and secondary antibodies (alkaline phosphatase-coupled antimouse and anti-rabbit IgG) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). GAPDH expression was used as a loading control.

RNA Interference and the Generation of Stable LOX-Knockdown Cell Lines. LOX expression was depleted in HCC cancer cells with at least three independent small interfering RNAs (siRNAs).[36] After a BLAST analysis was performed to verify that the LOX sequence shared no significant sequence homologies with other human genes, the selected sequences were inserted into *Bgl*II/*Hin*dIII-cut pSUPER vectors to generate the pSUPER-Si (Si-LOX) and pSUPER-scramble (Sc-LOX) vectors. Three different antisense siRNAs targeted against different parts of the *LOX* sequence are as follows: Si-1, 5′-GATCCCCCACCCAGAGGAGAGTGGCTTTCAAGAGAAGCCACTCTCCTCTGGGTGTTTTTA-3′; Si-2, 5′-GATCCCCCACCCAGAGGAGAGTGGCTTTCAAGAGAAGCCACTCTCCTCTGGGTGTTTTTA-3′; Si-3, 5′-GATCCCCCACCCAGAGGAGAGTGGCTTTCAAGAGAAGCCACTCTCCTCTGGGTGTTTTTA3′; and Sc, 5′-GATCCCCAGATAGAGACGCGCGCTCGTTCAAGAGACGAGCGCGCGTCTCTATCTTTTTTA-3′. The pSUPER-Si-LOX and pSUPER-Sc-LOX constructs were confirmed by DNA sequencing. The transfection protocol has been described previously.[37,38]

At least three clones of the Hep 3B cell line that stably expressed siRNA were generated. The pSUPER-Si-LOX and pSUPER-Sc-LOX vectors were transfected into cells, and stable clones were selected using G418 (Geneticin; 4 mg/mL) starting at 72 h. After 30 days of growth in selective medium, G418-resistant clones (referred to as Si-LOX) were isolated; these clones demonstrated >80% reduction in LOX mRNA and protein levels compared with the control clones (referred to as Sc-LOX).

Wound-Healing Cell Migration Assay. The Hep 3B (p40-C and p40-E) and Si-LOX cells were seeded in 6-well plates and allowed to grow to 70% confluence in complete medium. The cell monolayers were wounded with a plastic pipet tip (1 mm in diameter). The wounded monolayers were washed several times with PBS to remove cell debris, and the cells were incubated in medium in the absence or presence of HMDB (1 μM) or PSB (1 μM) for 24 h. The average number of cells migrating into the wound area was determined by examination by inverted microscopy at various time points.

Confocal Microscopy. To investigate whether LOX protein was overexpressed in migratory human HCC cancer cell lines, a wound-healing migration assay was performed, and images were subsequently captured via confocal microscopy. The cell monolayers were wounded with a plastic tip (1 mm), as described above. After washing with PBS, the monolayers were fixed for 15 min with 4% formaldehyde in PBS and incubated in blocking solution (PBS containing 15% fetal bovine serum, 2% bovine serum albumin, and 0.1% saponin) for 45 min at room temperature. This procedure was followed by incubation with

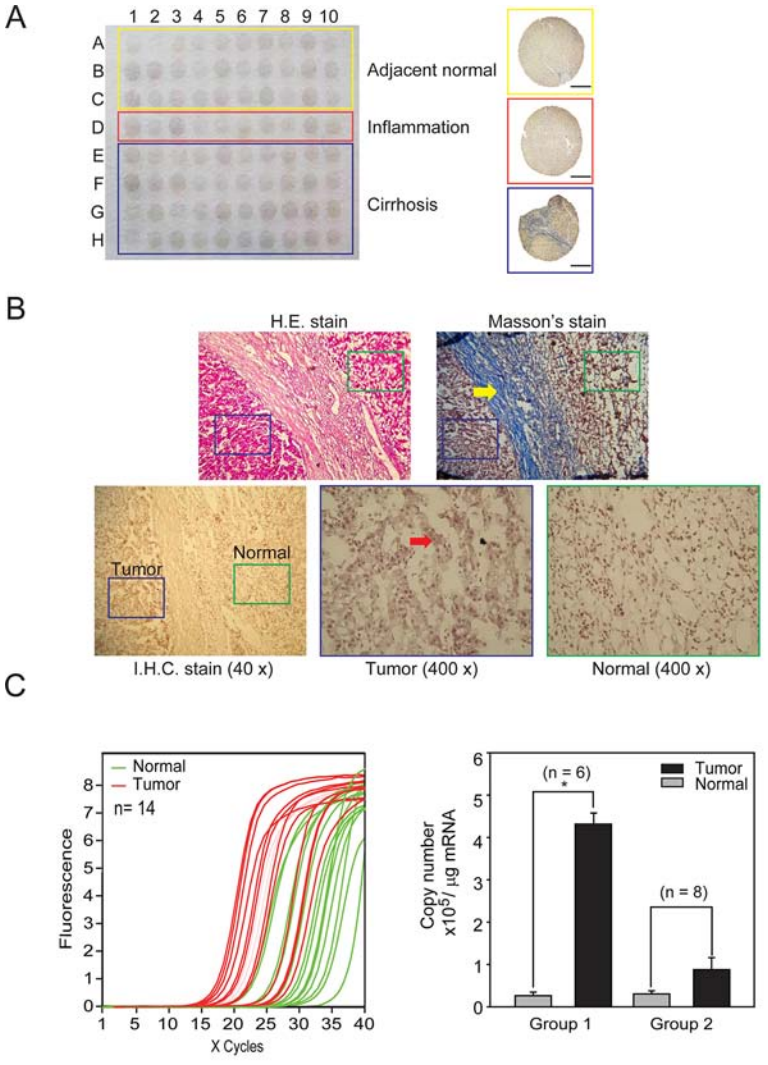

Figure 2. Immunohistochemical detection of the LOX protein in liver tissues. (A) IHC staining of a TMA containing human liver tissues was performed with a LOX antibody,[36] and the results illustrate the LOX protein expression levels in the adjacent normal (A1−C10, yellow box), inflammatory virus-infected (D1−D10, red box), and cirrhotic tissues (E1−H10, blue box). To the right are representative immunolabeled data. (B) LOX protein expression in HCC liver tissues with cirrhotic changes (upper right picture, indicated with a yellow arrow). Serial sections were obtained from the same tissue. The tissue sections were separately stained with H&E and Masson's and LOX-specific IHC stains. (C) LOX mRNA expression profiles in LCM-dissected paired human HCC tumor (red lines) and normal (green lines) tissues (n = 14) were investigated using real-time RT-PCR.

primary monoclonal (anti-LOX conjugated with FITC) antibody (Santa Cruz) at a 1:100 dilution in blocking solution for 1 h at room temperature. The samples were washed three times with PBS and mounted with Gel Mount (Sigma), and images were acquired with a Leica TCS SP5 Confocal Spectral Microscope Imaging System (Leica Microsystems, Wetzlar, Germany) as described previously.[36]

Immunoprecipitation Assay. A possible association between phosphorylated paxillin (pTyr-118) and phosphorylated FAK (pTyr-576) was investigated using immunoprecipitation assays. Briefly, using an anti-*p*-paxillin antibody (2 μg) and protein A−agarose beads (20 μL), the *p*-paxillin-associated proteins were precipitated from 200 μg of protein lysate from each sample. The immunoprecipitates were washed five times with extraction buffer and once with PBS. The pellets were resuspended in sample buffer (50 mM Tris, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol) and incubated for 10 min at 90 °C to release the proteins from the beads. The *p*-paxillin-immunoprecipitated p-FAK protein was then electrophoresed on 12% SDS-PAGE gels and evaluated by Western blot analysis. The gel was dried and subjected to autoradiography.

In Vitro Invasion Assays. In vitro invasion assays were performed using Transwell chambers 10 mm in diameter containing an 8 μm pore polycarbonate membrane (Corning Costar, Cambridge, MA, USA) coated with Matrigel as described previously.[36] The cells were trypsinized and suspended at a final concentration of 5×10^5 cells/mL in serum-free L15 medium. The HCC cells (p40-C and p40-E) were incubated in medium in the absence or presence of HMDB (1 μM) or PSB (1 μM) for 24 h or LOX-directed siRNA for 24 h before seeding. The cell suspension was added into each upper Transwell chamber. The bottom chamber contained medium with 5% FBS, which served as a chemoattractant. After a 24 h incubation at 37 °C in a 5% CO_2 and 95% air atmosphere, all of the noninvaded cells were removed from the upper face of the Transwell membrane with a cotton swab. The invaded cells were fixed with 100% methanol, stained with H&E (Nanjing Sunshine Biotechnology Ltd., Nanjing, China), and subsequently counted under a microscope. Ten fields were counted for each assay.

Soft Agar Cloning Assay. The base layer consisted of 0.9% low-gelling-point SeaPlaque agarose (Sigma, St. Louis, MO, USA) in culture medium.[36] Soft agar (0.4% SeaPlaque agarose in culture

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medium) was mixed with 1×10^4 HCC (p40-E), Si-LOX, or Sc-LOX cells and plated on top of the base layer in 60 mm culture dishes. The soft agar cultures were maintained at 37 °C, and colonies that appeared were counted using a Leica DMI 4000B Microscope Imaging System (Leica Microsystems).

Statistical Methods. All data are expressed as the mean ± standard deviation (SD); the error bars in the figures represent the SD from at least three determinations unless stated otherwise. The fold ratios of LOX mRNA expression in tumor versus normal samples at various clinical stages were compared using the Scheffe test. The numbers of paired samples at each stage were analyzed with an overall nonparametric test (Kruskal–Wallis test). Multiple comparisons were assessed using the Mann–Whitney test. All P values given are two-sided. Significant differences in the tumor cell migration and invasion assays were analyzed using the Kruskal–Wallis (nonparametric) test, and each pairwise comparison was performed with the Mann–Whitney test. The fold ratios of LOX mRNA expression detected in cells treated with different regimens were compared to the untreated E40 cells (bar 2) using the paired t test. All statistical comparisons were performed with SigmaPlot graphing software (San Jose, CA, USA) and SPSS version 11.0.0 (SPSS, Chicago, IL, USA). A P value of <0.05 was considered to be significant, and all statistical tests used were two-sided.

RESULTS AND DISCUSSION

Long-term ethanol consumption confers therapeutic resistance in human liver cancer patients with HBV infection.[26] Within the liver, hepatocytes play the most salient role in ethanol metabolism. Hepatocytes regulate ethanol metabolism through three main pathways: ethanol dehydrogenase, catalase, and the microsomal ethanol-oxidizing system, which predominantly comprises inducible cytochrome P450 2E1.[39] The metabolism of ethanol by these systems results in an increased generation of free radicals and cellular damage through peroxidation. In addition, ethanol consumption generates highly reactive compounds that increase DNA oxidation and inversely inhibit the DNA damage repair system.[40] Although ethanol itself is not genotoxic, chronic ethanol consumption increases the risk of hepatocarcinogenesis.[22] However, the mechanism by which ethanol exerts a hepatocellular cocarcinogenic effect is not well established. In this study, we examined whether the effects of long-term ethanol exposure had mechanisms different from those of chemical-induced effects (such as free radical generation). We first tested whether LOX activation could be detected in cirrhotic liver tissues. The expression of activated LOX was detected by IHC staining of a commercially available tissue microarray (TMA) obtained from Biomax Co. (Rockville, MD, USA). The TMA contained spots of adjacent normal, viral-induced inflammation, and cirrhotic tissues, as listed under Materials and Methods (Figure 2A). We observed that LOX was preferentially overexpressed in cirrhotic liver tissues (Figure 2A, spots E1–H10). To confirm this observation, IHC staining of the tumor tissue of an HCC patient with a history of long-term alcoholism was performed to determine LOX protein expression (Figure 2B). As shown in Figure 2B, the patient exhibited severe changes in the liver associated with cirrhosis (indicated by the yellow arrow). The results indicated that LOX was overexpressed in HCC cells (red arrow) compared with adjacent normal cells.

The aim of this study was to determine whether LOX plays a role in the effects of long-term ethanol consumption, which induces human HCC cell proliferation and metastasis. To ascertain whether overexpression of the LOX gene in HCC tumor tissues of Asian patients was associated with a history of alcoholism, LOX mRNA levels were examined by real-time RT-PCR of LCM-dissected tumor/normal paired tissue samples (Figure 2C, n = 14). The cases were classified into two groups according to their alcohol consumption habits (Figure 2C). Within group 1 (with alcoholism history, n = 6), a higher level (>21-fold) of LOX mRNA expression was detected in the HCC tumors compared with adjacent normal tissues (Figure 2C, bar 1 vs bar 2). However, in group 2 (without alcoholism history, n = 8), the LOX mRNA expression levels in tumor tissues were <3-fold greater than those in normal tissues (Figure 2C, bar 3 vs bar 4).

The HCC metastatic process is thought to involve multiple steps, including epithelial–mesenchymal transition,[41] the loss of cell–cell adhesion,[42] and increased cell motility. These steps all involve dynamic interactions among migratory tumor cells, stromal cells, and the ECM. LOX is an ECM enzyme that catalyzes the cross-linkage of collagen or elastin in the extracellular compartment, thereby regulating the tensile strength of tissues.[10,11] The identification of functional proteins (such as LOX) that are induced by long-term ethanol exposure in HCC cells with migratory ability may be important for clinical applications. To establish a cell research model, we previously described the creation of a human HCC cell line (Hep 3B) containing integrated HBV sequences by consecutively culturing these cells for 20–40 passages with or without 10 mM ethanol (designated E20–E40 and C20–C40, respectively). Interestingly, profound LOX mRNA overexpression was detected in the long-term ethanol-treated Hep 3B cells (E30 and E40; Figure 3A, lanes 4 and 6).

We previously studied the inhibitory effects of natural polyphenolic compounds such as pinocembrin[23] and phloretin[24,25] on ethanol-induced liver cirrhosis. We then tested therapeutic strategies aimed at reducing LOX production or activity, such as genetic or chemical inhibitors (Figure 3B,C), in E40 cells. As shown in Figure 3B, overexpression of LOX mRNA was detected in E40 cells compared with C40 cells (lane 1 vs lane 2). The E40 cells were treated with PSB, curcumin, and its analogues (1 μM each). With the exception of DBM, most of the compounds had significant effects individually on the inhibition of LOX mRNA expression in E40 cells (Figure 3B, lane 2 vs lanes 3–7). Interestingly, we found that PSB (1 μM) had a significant potency to synergistically reduce LOX mRNA expression levels when combined with these compounds (Figure 3B, lane 3 vs lanes 8–11). We identified PSB plus HMDB as the most effective regimen (Figure 3B, lane 2 vs lane 11). On the basis of this observation, we tested whether the LOX protein levels in the E40 cells were down-regulated by PSB plus HMDB (Figure 3C). LOX is synthesized and secreted as a 50 kDa inactive proenzyme (pro-LOX), which is processed by proteolytic cleavage to a functional 32 kDa enzyme (LOX) and an 18 kDa propeptide (LOX-PP).[43] We previously demonstrated that LOX mRNA is up-regulated in invasive breast cancer cells and that catalytically active LOX (32 kDa) facilitates cell invasion in vitro.[36] In this study, we observed that the LOX proteins (pro-LOX and LOX) were significantly increased in E40 cells (Figure 3C, lane 1 vs lane 2). E40 cells treated with a combination of PSB plus HMDB showed significantly reduced pro-LOX protein expression levels (Figure 3C, lane 2 vs lane 3). We further demonstrated that the proteolytic cleavage of pro-LOX to a functional LOX enzyme (32 kDa) in the combination-treated E40 cells was also inhibited (Figure 3C, lane 2 vs lane 3).

We next explored the mechanisms by which chemical inhibitors or genetic knockdown (siRNA; this cell line is

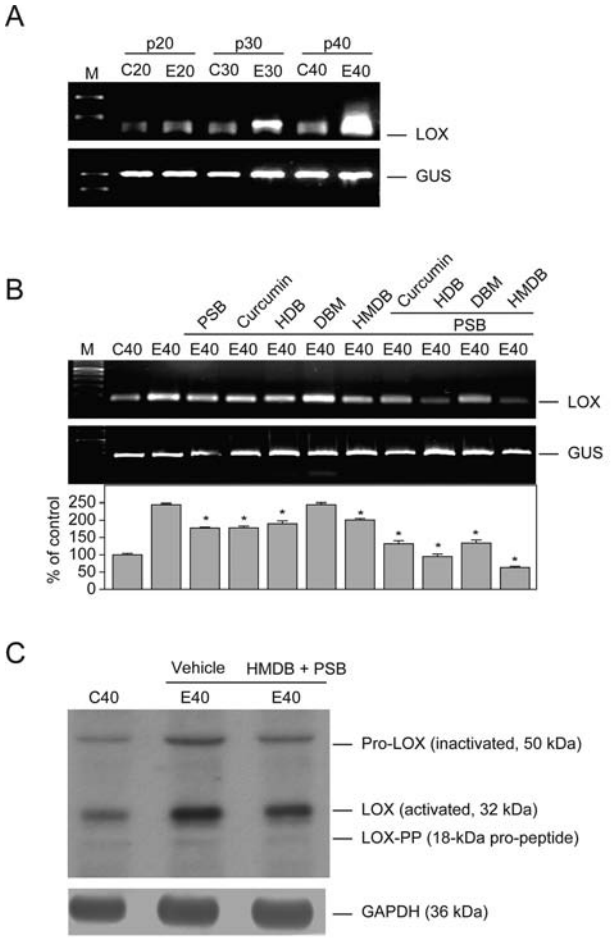

Figure 3. Long-term ethanol exposure-induced LOX protein and mRNA expression were attenuated by the combined treatment of PSB plus HMDB in E40 cells. (A) LOX mRNA expression was detected by RT-PCR in the long-term ethanol-induced HCC (E20−E40) and solvent control-treated (C20−C40) cells. (B) Combined treatment with PSB and HMDB attenuated LOX mRNA expression in E40 cells. E40 cells were treated with curcumin, HDB, DBM, or HMDB alone or in combination with PSB (1 μM each) for 24 h. LOX mRNA expression was detected by RT-PCR analysis. (C) Expression of LOX protein was attenuated by PSB plus HMDB treatment (1 μM each for 24 h) in long-term ethanol-treated E40 HCC cells. The cells treated with solvent only (C40) were used as a control to compare the expression levels of pro-LOX, activated LOX, and LOX-PP in E40 cells. Immunoblotting was performed, and the results shown are representative of three independent experiments.

hereinafter referred to as Si-LOX; Figure 4A, lanes 3 and 4) inhibited LOX expression in E40 HCC cells, and cellular migration and invasion levels were evaluated. Cell migration and invasion were increased in E40 cells compared with control (C40) cells (Figure 4B, both panels; bar 11 vs bar 12; $*$, $P < 0.01$). The combination of HMDB (1 μM) and PSB (1 μM) significantly inhibited the migration and invasion of E40 but not C40 cells compared with untreated cells (Figure 4B, both panels; bar 8 vs bar 12; $*$, $P < 0.01$). However, such effects were not observed with the combination treatment of both HMDB (1 μM) and RES (1 μM) in E40 cells (Figure 4B, both panels; bar 10 vs bar 12). The chemical inhibition of LOX protein expression inhibited cellular migration and invasion in both E40 and C40 Si-LOX cells (Figure 4B, both panels; bar 17 vs bar 19 and bar 18 vs bar 20; $*$, $P < 0.01$).

To confirm the association between LOX protein levels and tumor cell migration, wound-healing migration assays were performed, and LOX protein expression in the migratory cells was detected by IF staining (Figure 4C). High migratory activity was observed in E40 cells at 24 h compared with C40 cells (Figure 4C, left panel, middle pictures). However, combined treatment of HMDB plus PSB (1 μM each) significantly inhibited cell migration in E40 but not C40 cells (Figure 4C, left panel, right pictures; Figure 4B). We then detected Lox expression levels in migratory E40 cells with or without HMDB plus PSB treatment (Figure 4C, right panel, indicated as yellow and red boxes, respectively). The results demonstrated that LOX protein expression was inhibited in the HMDB plus PSB-treated E40 cells compared with the DMSO-treated control (Figure 4C, right panel, red arrow). These results suggest that LOX may represent a potential target molecule for the prevention of long-term ethanol-induced HCC cell metastasis.

Several recent studies have demonstrated that HCC cell migration is influenced by the tumor microenvironment, which includes stromal cells and the ECM.[44,45] Targeting LOXL2 results in a marked reduction in activated fibroblasts, desmoplasia, and endothelial cells.[18] Recent studies have demonstrated novel roles for LOX, including the ability to regulate gene transcription,[16] cell motility and migration, and cell adhesion.[17] However, investigating the mechanisms underlying LOX-induced HCC cancer and metastasis has been challenging because a sufficient number of samples are not readily available.[9] The demonstration of diverse functions of LOX has led researchers to hypothesize that this enzyme may represent a potential target for cancer therapy.[17,18,46]

The adherence of tumor cells to the ECM is mediated by integrin binding; this binding triggers the phosphorylation of two major integrin-binding proteins, FAK and Src, which are both present at focal adhesions (FA), leading to FA disassembly and cell migration.[47] Our recent study indicated that LOX facilitates migration via the FAK/Src signaling pathway in invasive breast cancer cells.[36] To determine whether the activation of the FAK/Src signaling pathway is involved in long-term ethanol-induced HCC metastasis, E40 and C40 cells were treated with HMDB or PSB alone or in combination (1 μM each). As shown in Figure 5A, levels of phosphorylated FAK (p-FAK; Tyr-576) and phosphorylated Src (p-Src; Tyr-418) were increased in E40 cells compared with control (C40) cells (Figure 5A, lane 1 vs lane 4). These results suggest that LOX facilitated migration and the formation of cell−matrix adhesions in long-term ethanol-treated HCC (E40) cells as a result of FAK/Src signaling pathway activation. Combination treatment with HMDB and PSB attenuated the ethanol-induced effects (Figure 5A, lane 4 vs lane 6).

Previous studies have demonstrated that the FAK/Src complex-mediated phosphorylation of Tyr-31 and Tyr-118 of paxillin, an adaptor protein that is a component of the FA complex,[48] is enhanced by hydrogen peroxide.[49] Mechanistic investigations also revealed that LOX promotes invasion by increasing the generation of hydrogen peroxide and the interaction between FAK and paxillin, two proteins that are key for the regulation of dynamic cell migratory behavior.[36,50] We and others have demonstrated that hydrogen peroxide is a byproduct of the activated (32 kDa) form of LOX,[17,36,51] and excess intracellular hydrogen peroxide can facilitate the

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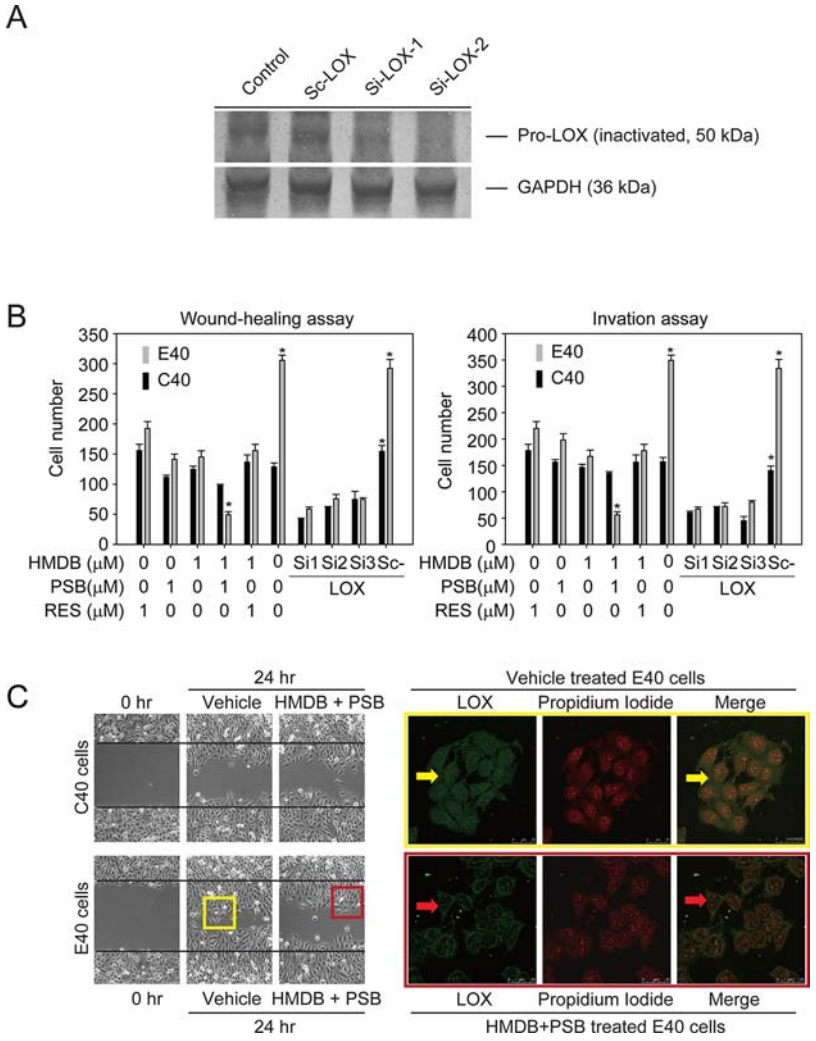

Figure 4. Down-regulation of LOX protein expression suppresses the migration and invasion of long-term ethanol-treated HCC cells. (A) Expression of the pro-LOX (50 kDa) protein in E40 cells was inhibited by siRNA knockdown. (B) E40 and C40 cells were treated with HMDB, PSB, or RES (1 μM) alone or in combination for 24 h (bars 1−12). LOX protein levels were reduced in the Si-LOX cells compared with the Sc-LOX control (bars 13−20). The migration and invasion of the cells under the various treatment regimens were determined using a wound-healing assay and Matrigel invasion assay, respectively. (∗) A P value of <0.05 was considered to be significant, and all statistical tests were two-sided. (C) C50 and E50 cells were used in wound-healing migration assays, and expression levels of the LOX protein in the migratory cells were determined by confocal microscopy. Migrating E40 cells treated with either solvent alone (indicated with a yellow box) or HMDB and PSB (indicated with a red box) were stained with a LOX antibody. Confocal microscopy was performed as described under Materials and Methods.

phosphorylation and activation of Src.[52] In addition to modification of the local ECM via increased collagen cross-linking, increases in active LOX species may contribute to the hydrogen peroxide-mediated activation of the FAK/paxillin complex and thereby promote breast cancer cell migration and invasion. To examine whether the decrease in FAK phosphorylation (Figure 5A) induced by LOX inhibition affects the formation of the FAK/paxillin complex, the interaction between FAK and paxillin was examined (Figure 5B). Immunoprecipitations (IPs) were performed with an antibody specific for paxillin phosphorylated at Tyr-118. The formation of complexes containing paxillin phosphorylated at Tyr-118 and FAK phosphorylated at Tyr-576 was detected by immunoblotting (IB). As shown in Figure 5B, the formation of the *p*-paxillin/p-FAK complex was reduced by HMDB plus PSB combination treatment in E40 cells (Figure 5B, lane 4). Moreover, combination treatment with both agents almost completely inhibited the phosphorylation of paxillin at Tyr-118 in E40 cells (Figure 5B, lane 4).

Enhanced anchorage-independent proliferation has been suggested to be a hallmark of tumor cell metastasis.[53] In this study, we found that long-term ethanol treatment (Figure 6) increased the expression of the LOX protein in E40 cells. To determine whether LOX protein expression is involved in anchorage-independent proliferation of HCC cells, in vitro soft-agar experiments were performed. As shown in Figure 5C, the colonies formed by the HCC (E40) and Sc-LOX (E40) cells were 2−3 times larger, on average, than those of the Si-LOX (E40) group. In addition, it is unclear whether these effects of the LOX protein on HCC (E40) cells could be attenuated. The colonies formed by Si-LOX cells exhibited subtle morphological differences, including slight disaggregation, compared with colonies formed by Sc-LOX-treated and untreated E40 cells (data not shown). The colony-forming ability of the Si-LOX and the HMDB plus PSB-treated E40 cells was profoundly reduced compared with untreated E40 cells (Figure 5C, lane 1 vs lane 4 and lane 5 vs lanes 6−8; ∗, P = 0.01).

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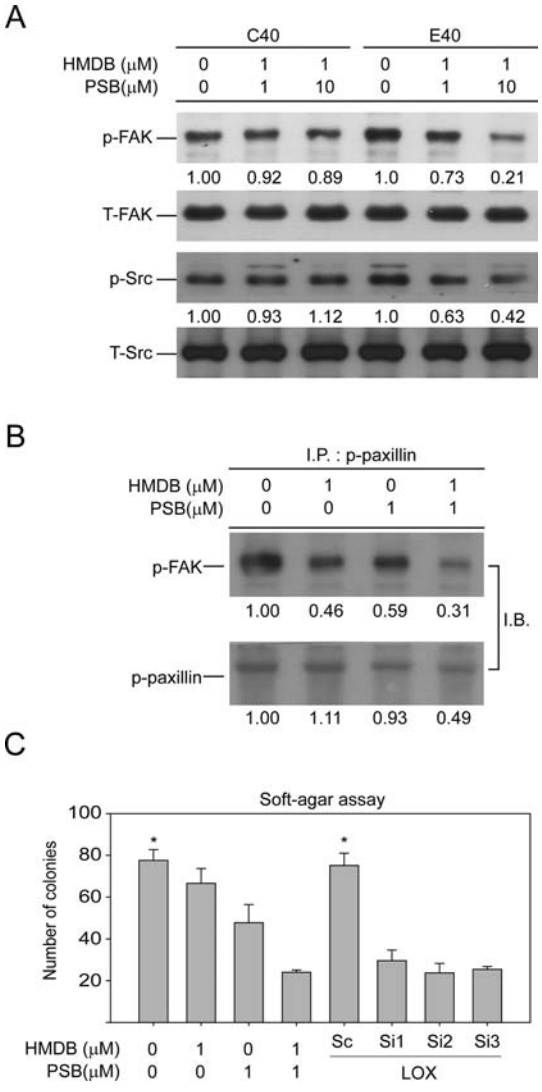

Figure 5. LOX-mediated effects on cellular migration are mediated through the p-FAK/*p*-paxillin-signaling pathway. (A) E40 and C40 cells were treated with HMDB (1−10 μM) or PSB (1 μM) alone or in combination for 24 h. The levels of FAK, p-FAK (Tyr-576), Src, and p-Src (Tyr-418) were detected by immunoblotting. (B) E40 cells were treated with HMDB (1 μM) or PSB (1 μM) alone or in combination for 24 h. After the various treatment regimens, the cells were assessed for the presence of interactions between p-FAK and *p*-paxillin by immunoprecipitation. (C) Anchorage-independent growth of E40 cells treated with HMDB, PSB, both agents, or Si-LOX was evaluated in soft agar. The data shown represent the mean ± SD of three different experiments. Significance was set at $P < 0.05$. An asterisk indicates that the Sc-LOX and solvent-treated cells were significantly different from the LOX inhibition groups.

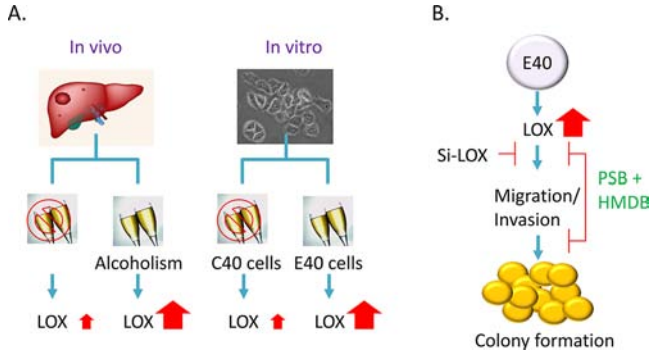

Figure 6. Schematic diagram of the role of long-term ethanol-induced LOX expression in HCC cell migration and invasion. (A) In this study, we detected LOX overexpression in vivo in human liver cancer tissues from individuals with a history of alcoholism as well as in vitro in long-term ethanol-treated human HCC (E40) cells. (B) The results demonstrated that LOX plays a positive role in HCC metastasis by E40 cells. Such effects were significantly attenuated by combined treatment with PSB and HMDB or by reducing LOX activity via genetic knockdown (siRNA). This study suggests that targeting LOX expression with food components may be a novel strategy to overcome ethanol-induced HCC cell metastasis in liver cancer patients with long-term alcoholism.

In this study, we determined that PSB synergistically inhibits LOX mRNA and protein expression mediated by natural compounds with similar chemical structures (DBM, HMDB, HDB, RES, curcumin). These compounds have been demonstrated to be antitumor agents through various mechanisms. For example, previous papers have shown that DBM and HMDB induce growth inhibition and apoptosis in A431, A549, and CH27 cells through the modulation of mitochondrial functions regulated by reactive oxygen species (ROS)[54] and DNA-damaging effects.[30] Another study showed that HMDB induced apoptosis of many types of cancer cells through the activation of the tumor suppressor gene CEBPD (CCAAT/enhancer-binding protein delta).[55] However, PSB has been shown to protect vascular endothelial cells against oxidized low-density lipoprotein-induced apoptosis in vitro and in vivo.[56] It has been proposed that PSB attenuates 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-stimulated metastasis in HCC cells through the inhibition of nuclear factor kappa B (NF-κB)- and activator protein-1 (AP-1)-dependent transcriptional activity.[57] NF-κB and AP-1 are transcription factors that activate the LOX gene promoter; LOX then confers abnormal collagen and elastin cross-linking and plays an essential role in coordinating the control of the tumor ECM.[58] Such results support the idea that PSB and structural analogues such as curcumin, DBM, HMDB, and RES may have clinical relevance for HCC cell metastasis induced by long-term ethanol exposure by blocking ROS generation and abnormal ECM formation through the inhibition of LOX activity. Our findings highlight the importance of LOX activity in ethanol-induced HCC cancer progression and support the idea that the development of anti-LOX therapeutics may lead to an effective treatment for metastatic HCC cancer.

AUTHOR INFORMATION

Corresponding Author

*(Y.-S.H.) Mailing address: School of Medical Laboratory Science and Biotechnology, College of Medical Science and Technology, Taipei Medical University, No. 250 Wu-Hsing Street, Taipei 110, Taiwan. Phone: +886-2-27361661, ext. 3327. Fax: +886-2-2739-3422. E-mail: hoyuansn@tmu.edu.tw. (C.-H.W.) Mailing address: Department of Surgery, School of Medicine, Taipei Medical University-Shuang Ho Hospital, No. 291 Jhongjheng Road, Jhonghe City, Taipei County 23561, Taiwan. Phone: +886-2-2249-0088, ext. 8810. Fax: +886-2-2248-0900. E-mail: chwu@tmu.edu.tw.

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Notes

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■ ABBREVIATIONS USED

AP-1, activator protein-1; DBM, dibenzoylmethane; DMSO, dimethyl sulfoxide; ECM, extracellular matrix; FA, focal adhesions; GUS, glucuronidase; HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HDB, 1-(2-hydroxyphenyl)-3-phenyl-1,3-propanedione; H&E, hematoxylin and eosin; HMDB, 1-(2-hydroxy-5-methylphenyl)-3-phenyl-1,3-propanedione; IB, immunoblotting; IP, immunoprecipitation; LOX, lysyl oxidase; MMPs, matrix metalloproteinases; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; NF-κB, nuclear factor kappa B; PSB, pterostilbene; TMA, tissue microarray; LCM, laser capture microdissection

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