Mechanistic Contribution of Ubiquitous 15-Lipoxygenase-1 Expression Loss in Cancer Cells to Terminal Cell Differentiation Evasion

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Abstract

Loss of terminal cell differentiation promotes tumorigenesis. 15-Lipoxygenase-1 (15-LOX-1) contributes to terminal cell differentiation in normal cells. The mechanistic significance of 15-LOX-1 expression loss in human cancers to terminal cell differentiation suppression is unknown. In a screen of 128 cancer cell lines representing more than 20 types of human cancer, we found that 15-LOX-1 mRNA expression levels were markedly lower than levels in terminally differentiated cells. Relative expression levels of 15-LOX-1 (relative to the level in terminally differentiated primary normal human-derived bronchial epithelial cells) were lower in 79% of the screened cancer cell lines than relative expression levels of p16 (INK4A), which promotes terminal cell differentiation and is considered one of the most commonly lost tumor suppressor genes in cancer cells. 15-LOX-1 was expressed during terminal differentiation in three-dimensional air-liquid interface cultures, and 15-LOX-1 expression and terminal differentiation occurred in immortalized non-transformed bronchial epithelial but not in lung cancer cell lines. 15-LOX-1 expression levels were lower in human tumors than in paired normal lung epithelia. Short hairpin RNA-mediated downregulation of 15-LOX-1 in Caco-2 cells blocked enterocyte-like differentiation, disrupted tight junction formation, and blocked E-cadherin and ZO-1 localization to the cell wall membrane. 15-LOX-1 episomal expression in Caco-2 and HT-29 colon cancer cells induced differentiation. Our findings indicate that 15-LOX-1 downregulation in cancer cells is an important mechanism for terminal cell differentiation dysregulation and support the potential therapeutic utility of 15-LOX-1 reexpression to inhibit tumorigenesis. Cancer Prev Res; 4(12): 1961–72. ©2011 AACR.

Introduction

Loss of terminal cell differentiation promotes tumorigenesis (1–3); tumors exhibit aberrant cell differentiation, especially suppression of terminal cell differentiation (4, 5). Tumor suppressor genes, such as p16 (INK4A; p16), promote terminal cell differentiation (6, 7). Efforts are being made to develop anticancer therapeutic interventions to restore terminal differentiation in cancer cells (5, 8–10).

15-Lipoxygenase-1 (15-LOX-1) contributes to terminal cell differentiation in normal cells via promoting maturational degradation of organelles (11, 12). 15-LOX-1 expression is downregulated in various major human cancers including colon cancer (13–15), esophageal cancer (16), breast cancer (17), and pancreatic cancer (18). Sodium butyrate-induced terminal differentiation in Caco-2 colon cancer cells is associated with 15-LOX-1 reexpression (19, 20), and 15-LOX-1 genetic episomal expression in Caco-2 cells induces apoptosis (20). Nevertheless, the mechanistic contribution of 15-LOX-1 to terminal cell differentiation dysregulation in tumorigenesis and the mechanism by which 15-LOX-1 downregulation contributes to terminal cell differentiation suppression in cancer cells are unknown. We examined these important questions using the following 3 approaches. First, we compared 15-LOX-1 expression in 128 randomly collected cancer cell lines from more than 20 different types of human cancer grown in monolayer (immersion) cultures with 15-LOX-1 expression in 3 different types of differentiated cells: primary normal human-derived bronchial epithelial (NHBE) cells and normal human-derived epidermal keratinocytes (NHEK) grown in

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a terminally differentiated state in 3-dimensional organotypic air–liquid interface cultures and Caco-2 cells terminally differentiated with sodium butyrate treatment. Second, we examined the ability of immortalized nontransformed human bronchial epithelial cells (HBECl) and lung cancer cells to undergo terminal cell differentiation and to express 15-LOX-1 in air–liquid interface cultures. Third, we studied the effects of 15-LOX-1 downregulation, induced using a stable transfection short hairpin RNA (shRNA) knockdown approach, on the induction of spontaneous (without sodium butyrate treatment) terminal differentiation in Caco-2 cells in protracted cell culture.

Materials and Methods

Materials
We obtained rabbit polyclonal antiserum to recombinant human 15-LOX-1 as described previously (21). The human colon cancer cell lines were obtained from American Type Culture Collection (ATCC) and donating academic laboratories. 13-S-HODE enzyme immunoassay kits were purchased from Assay Designs Inc. Other reagents, molecular-grade solvents, and chemicals were obtained from commercial manufacturers or as specified.

We obtained the large collection of human cancer cell lines tested for 15-LOX-1 expression from either ATCC or various academic laboratories at the University of Texas MD Anderson Cancer Center. Authentication of cells lines was conducted on a sample of the large number of the cell lines that were surveyed. The sample of authenticated cancer cell lines included: HT-29, Caco-2, HCT-116, LoVo, and DLD-1. The authenticity of these cell lines was validated by short tandem repeats (STR) DNA fingerprinting using the AmpF/STR Identifier Kit according to manufacturer instructions (Applied Biosystems; catalog no. 4322288). The STR profiles were compared with known ATCC fingerprints (http://www.atcc.org/), with the Cell Line Integrated Molecular Authentication database (CLIMA) version 0.1.200808 (http://bioinformatics.tigem.it/clima/; ref. 22), and with the MD Anderson fingerprint database. The STR profiles matched known DNA fingerprints or were unique. In addition, LNCaP and SW480 cell lines were obtained directly from ATCC and passed for less than 1 month in our laboratory before testing.

Cell cultures
We obtained from MatTek Corporation primary NHBE cells (originally provided by Clonetics) grown in an in vitro system to form a pseudostratified, highly differentiated mucociliary epithelium that closely resembles the epithelial tissue of the respiratory tract (differentiated NHBE cells; Supplementary Fig. S1A) and NHBE cells grown in an undifferentiated state in submerged (immersion) cultures (undifferentiated NHBE cells; Supplementary Fig. S1B; refs. 23, 24). The differentiated NHBE cells were cultured on 0.8-cm Millipore Millicell CM cell culture inserts (Millipore) at the air–liquid interface at 37°C in 5% CO₂ according to the manufacturer’s specifications. The culture medium was composed of serum-free Dulbecco’s Modified Eagle’s Medium with proprietary growth factors and hormones (24). The undifferentiated NHBE cells were cultured in submerged fashion without a cell culture insert or addition of proprietary hormones or growth factors to the medium. We also purchased from MatTek Corporation NHEKs cultured in 3-dimensional orientation to maintain air–liquid interface and to form a multilayered, highly differentiated structure that highly resembles human epithelium (differentiated NHEKs; Supplementary Fig. S1C; ref. 25).

For the screen of the 128 cancer cell lines (Supplementary Tables S1–S3), cells were cultured in RPMI medium with 10% FBS, except for Caco-2 cells, which were cultured in Eagle’s Minimal Essential Medium supplemented with 15% FBS. Cells were grown as attached monolayers and incubated in a humidified atmosphere with 5% CO₂ at 37°C. 15-LOX-1 expression was induced in Caco-2 cells by sodium butyrate for 48 hours, similar to what was described previously (Supplementary Fig. S1D and S1E; ref. 19).

Four nontransformed HBEC cell lines (HBEC3KT, HBEC6KT, HBEC12KT, and HBEC24KT) immortalized by hTERT and Cdk4 transfection without a viral oncoprotein (26) and H460 and A549 human non–small-cell lung cancer cells were grown up to 28 days in air–liquid interface cultures with air–liquid interface to induce terminal differentiation and then harvested and fixed for histology studies as described previously (27, 28).

Acquisition of clinical samples
We obtained surgically resected paired specimens of normal and non–small-cell lung cancer tissues from patients at The University of Texas MD Anderson Cancer Center through the Institution’s Tissue Procurement and Banking Facility. The Institutional Review Board Of The University of Texas MD Anderson Cancer Center approved this study. For each patient, samples were procured from both the tumor area and the normal-appearing mucosa. Tissue blocks were kept frozen at −70°C until processed.

15-LOX-1 immunohistochemical staining
For staining of samples from organotypic cultures, paraffin-embedded tissue blocks were cut into 5-mm thick sections and deparaffinized in xylene. Antigen retrieval was conducted using sodium citrate, pH = 6.0, at subboiling temperature for 20 minutes, and the slides were allowed to cool before incubation with the primary antibody. Sections were stained for 15-LOX-1 as described previously (20). For staining of paired normal and lung cancer tissue samples, frozen sections were stained in a manner similar to that described previously (13). Two experienced pathologists (M.G. Raso and I.I. Wistuba) quantified 15-LOX-1 expression in clinical lung samples in a manner similar to that described previously (29). Briefly, the cytoplasmic expression of 15-LOX-1 was quantified by using a scoring system in which scores were the product of multiplying staining intensity scores (0, 1+, 2+, and 3+) by percentage of stained epithelial cells (0%–100%).
Quantitative real-time reverse transcription PCR
Total RNA was extracted from cells using TRI reagent (Molecular Research Center Inc.). The integrity of the total RNA was verified using the RNA 6000 Nano LabChip Kit and an Agilent 2100 Bioanalyzer (Agilent Technologies). Five hundred nanograms of RNA from each sample was then reverse transcribed in a 20-µl reaction using an iScript cDNA synthesis kit (BioRad Laboratories). Quantitative real-time reverse transcription PCR and measurement of relative RNA expression levels were carried out as described previously (20) using a comparative Ct method. Primers and probes for human p16 (assay ID: 00233655_m1), 15-LOX-1 (HS00609608_m1), and HPRT1 (4326321E; internal control for cell line expression studies) were purchased from Applied Biosystems.

Western blot analysis of proteins
Cell lysates were subjected to SDS-PAGE, transferred, probed with antibody solutions (e.g., human 15-LOX-1), and analyzed by the enhanced chemiluminescence method as described previously (20). Preparation of cytoskeleton-associated E-cadherin (insoluble) and total fractions (equal amounts of soluble and insoluble fraction) was conducted in a manner similar to that described previously (30). We quantified the amount of blotted E-cadherin using an IRDye 680–conjugated goat anti-mouse IgG secondary antibody (LI-COR Biosciences). Fluorescence was measured using an Odyssey fluorescent imager (LI-COR Biosciences), band intensities were quantified using Odyssey software, and images were converted to gray scale.

Measurement of 13-HODE
13-HODE, the primary product of 15-LOX-1, was measured by enzyme immunoassay or by high-performance liquid chromatography (Agilent 1200 series) and tandem quadrupole mass spectrometry (Agilent) using methods similar to that described previously (20).

Immunofluorescence and confocal microscopy analysis
Cells grown on glass coverslips for the specified time periods were fixed with 4% formaldehyde at 4°C for 15 minutes, rinsed in PBS, and then blocked with 5% milk for 1 hour at room temperature. Overnight incubations were conducted with primary antibodies diluted in PBS solution containing 0.1% bovine serum albumin and 0.3% Triton X-100. Monoclonal mouse anti-human E-cadherin antibody (Invitrogen) dilution was 1:100, and polyclonal rabbit antihuman ZO-1 antibody (Invitrogen) dilution was 1:100. The secondary antibodies used were Alexa Fluor 488–conjugated donkey anti-mouse IgG (Molecular Probes) for E-cadherin staining. Nuclei were stained with DAPI (DAKO). Images were captured by an Olympus IX-81 DSU confocal microscope using a 60× water immersion objective and 3I’s SlideBook software (Intelligent Imaging Innovations, Inc.). SlideBook software was used to conduct analysis including the creation of projection images and the addition of scale bars. Exposure times and dynamic range values were consistent for all images to allow for comparison.

Stable 15-LOX-1 shRNA transfection
PRS 15-LOX-1 shRNA vectors (OriGene) containing 29-bp shRNA forward and reverse complementary sequences that are connected by a 7-bp loop and driven by the human U6 promoter were designed to target human 15-LOX-1. The vectors were named for the various 15-LOX-1 shRNA constructs (e.g., 15-LOX-1-shRNA-83). We selected the pRS-15-LOX-1-shRNA-83 vector (OriGene) containing 29-bp shRNA forward sequence of 15-LOX-1: AGGCCTCTCTC-CAGATGTCCATCACTTGG for the stable transfection experiments on the basis of preliminary studies showing efficient 15-LOX-1 gene downregulation. The pRS-15-LOX-1-shRNA-83 vector and a nonspecific shRNA vector (control) were transfected into Caco-2 cells using FuGene 6 (Roche). Clones with stable transfection were selected by using puromycin (Invitrogen) and then isolated and expanded.

We also tested the effects of 15-LOX-1 downregulation on colon epithelial cell differentiation and tight junction formation using 2 Caco-2 stable clones transfected with a different 15-LOX-1 shRNA construct, which were generated as described previously (31). Clone 19 maintained effective 15-LOX-1 knockdown when treated with sodium butyrate and was named 15-LOX-1 KD (+), whereas clone 33 had ineffective 15-LOX-1 knockdown when treated with sodium butyrate and was named 15-LOX-1 KD (−) as shown in Fig. 5A.

Colon cancer cell transfection with 15-LOX-1 adenoviral and plasmid vectors
HT-29 colon cancer cells were cultured and transfected with modified 5/3 adenoviral vectors that expressed either 15-LOX-1 (Ad-15-LOX-1) or luciferase (Ad-luciferase) at 500, 1,000, and 2,000 viral particles per cell as described previously (32). Caco-2 colon cancer cells were cultured and transfected with pAdenoVator-CMV5-GFP plasmid vectors carrying either 15-LOX-1 cDNA or green fluorescent protein (GFP) alone (control vector), similar to that described previously (21).

Electron microscopy
Samples were fixed with a solution containing 3% glutaraldehyde plus 2% paraformaldehyde in 0.1 mol/L cacodylate buffer, pH = 7.3, for 1 hour. After fixation, the samples were washed and treated with 0.1% Millipore-filtered cacodylate-buffered tannic acid, postfixed with 1% buffered osmium tetroxide for 30 minutes, and stained en bloc with 1% Millipore-filtered uranyl acetate. The samples were dehydrated in increasing concentrations of ethanol and was named 15-LOX-1 KD (−) as shown in Fig. 5A.
EM stainer, and examined in a JEM 1010 transmission electron microscope (JEOL, Inc.) at an accelerating voltage of 80 kV. Digital images were obtained using an AMT imaging system (Advanced Microscopy Techniques Corp.).

Macromolecule permeability assay
Wild-type Caco-2, 15-LOX-1 KD (+), and 15-LOX-1 KD (−) cells were grown on polycarbonate permeable inserts (0.4-µm pores, 24-mm diameter; Costar), and permeability was assessed 14 days after confluence. Inserts were washed with Hank’s balanced salt solution (HBSS). At time 0, 1.5 mL of HBSS was added to the lower wells, and 0.5 mL of HBSS containing 3.5 µmol/L fluorescein isothiocyanate (FITC)-labeled 3-kDa dextran (NANCOS) was added to the inserts. At 10, 20, 30, and 40 minutes after addition of FITC-labeled dextran, 100 µL samples were taken from the lower wells. Sample volumes were replaced with equal volumes of HBSS. The fluorescence intensity of each sample was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a FLUOstar Omega microplate reader (BMG Labtech).

Alkaline phosphatase assay
Caco-2 cells were cultured, harvested, lysed, and assayed for alkaline phosphatase activity using a StemTAG alkaline phosphatase activity assay kit (Cell Biolabs, Inc.) according to the manufacturer’s directions. Enzymatic activity was calculated and expressed as diethanolamine (DEA) units (enzymatic activity that hydrolyzes 1 µmol/L of p-nitrophenyl phosphate per minute at pH of 9.8 and 37°C in DEA buffer).

Statistical analyses
We used the Student t test for 2-group comparisons. For analyses involving single factors and more than 2 groups, we conducted a one-way ANOVA. If the overall ANOVA test result was significant, we conducted pairwise comparisons, adjusting for multiplicities using the Bonferroni correction. We analyzed data involving the simultaneous consideration of 2 factors using 2-way ANOVA. Relative expression levels of 15-LOX-1 and p16 in cancer cell lines had a nonnormal distribution; therefore, means and 95% confidence intervals (CI) were calculated using log-transformed data. Relative expression levels of 15-LOX-1 and p16 in cancer cell lines and clinical samples were compared using the non-parametric sign test.

Results
15-LOX-1 expression during NHBE cell differentiation
Primary NHBE cells grown in air–liquid interface culture to produce a terminally differentiated growth pattern of human bronchial epithelium with pseudostratified mucociliary structure (Supplementary Fig. S1A) had much higher 15-LOX-1 mRNA expression (Fig. 1A) and protein expression (Fig. 1B) than NHBE cells grown in undifferentiated immersion cultures (Supplementary Fig. S1B). Terminal differentiation of NHBE cells was

Figure 1. 15-LOX-1 and differentiation of primary NHBE cells. Primary NHBE cells were grown for 3 weeks in an undifferentiated state in immersion cultures or in air–liquid interface cultures to induce terminal differentiation into bronchial-epithelial-like structures. Cells were then harvested and processed for quantitative real-time reverse transcription PCR (A, differentiated vs. undifferentiated NHBE cells, P = 0.0005), Western blotting (B), and 13-HODE levels by liquid chromatography tandem mass spectroscopy (C, differentiated vs. undifferentiated NHBE cells, P = 0.0002). 15-LOX-1−positive control (HCT-116 colon cancer cells transfected with 15-LOX-1 expression vector).
associated with increased expression of 13-HODE: levels were 0.974 ± 0.156 ng/mg protein (mean ± SD) in undifferentiated cells versus 4.79 ± 0.46 ng/mg protein (P = 0.0002) in differentiated cells (Fig. 1C).

**15-LOX-1 and p16 expression in cancer cell lines**

We next compared 15-LOX-1 expression levels between 128 randomly collected cancer cell lines and terminally differentiated cells.

In all 128 screened cancer cell lines, 15-LOX-1 mRNA expression level was markedly lower than the level in the terminally differentiated NHBE cells (Fig. 2A and Supplementary Table S1; mean relative expression level: 0.000027, 95% CI, 0.000017–0.000041). In approximately 90% of the screened cancer cell lines, the mRNA expression level of p16, one of the most commonly lost tumor suppressors in human cancers (33–35), was lower than the level in the terminally differentiated NHBE cells (Fig. 2B and Supplementary Table S1; mean relative expression level: 0.01, 95% CI, 0.00428–0.0234). Relative mRNA expression levels of 15-LOX-1 were lower than relative mRNA expression levels of p16 in 79% of tested cancer cell lines (P < 0.0001).

Similarly, 121 (95%) of the 128 cancer cell lines had 15-LOX-1 expression levels lower than those of differentiated NHEK (mean relative expression level: 0.0049, 95% CI, 0.0026–0.009), and 71 (56%) of the 128 cancer cell lines had p16 levels lower than those of NHEK (Supplementary Fig. S2A and S2B and Table S2; mean relative expression level: 0.0411, 95% CI, 0.016–0.1055). Relative mRNA expression levels of 15-LOX-1 were lower than relative mRNA expression levels of p16 in 70% of tested cancer cell lines (P < 0.0001).

In addition, 96% of the 128 screened cancer cell lines had 15-LOX-1 expression levels lower than the level in Caco-2 colon cancer cells terminally differentiated by sodium butyrate treatment (Fig. 2C and Supplementary Table S3; mean relative expression level: 0.0035, 95% CI, 0.0022–0.0055).

![Figure 2. 15-LOX-1 and p16 mRNA and protein expression levels in cancer cell lines.](image-url)

A and B, a total of 128 cancer cell lines (Supplementary Table S1) were cultured and processed for 15-LOX-1 (A) and p16 (B) mRNA by quantitative real-time reverse transcription PCR. Dots in the dot plots are means of triplicate measurements from each cell line. The relative expression levels were calculated relative to expression of the calibrator sample (differentiated NHBE cells). Solid lines represent the median value for each group. C, 15-LOX-1 relative expression levels in cancer cell lines compared with the level in Caco-2 cells terminally differentiated by sodium butyrate treatment. 15-LOX-1 mRNA measurements are as in (A) but with terminally differentiated Caco-2 cells as the calibrator sample. Dots in the dot plots are means of triplicate measurements from each cell line. The solid line represents the median value for the relative expression levels. D, 15-LOX-1 protein expression in cancer cell lines. Cell lines—including cell lines with 15-LOX-1 mRNA expression levels nearly equal to or greater than the level in differentiated Caco-2 cells or NHEK—were cultured, processed for Western blotting, and probed with 15-LOX-1 antibody. Three repeated experiments yielded similar results.
Of the cancer cell lines that had 15-LOX-1 mRNA expression levels equal to or greater than the level in differentiated Caco-2 cells or NHEK (LNCaP, 14B3, 183A, MCF-7, MDAMB-453, SK-BR-3, and MKN28), all had nondetectable protein expression, except for 1483 cells, which had a faint band (Fig. 2D). In 1483 cells, 13-S-hydroxyoctadecadienoic acid (13-S-HODE) levels by enzyme immunoassays were lower than those in differentiated Caco-2 cells (mean ± SD: 1.57 ± 0.09 ng/μg protein vs. 2.59 ± 0.13 ng/μg protein; P < 0.0001).

We further investigated the nature of this faint band in 1483 cells by measuring the expression in these cells of 12-LOX and 15-LOX-2, enzymes that have significant homology with 15-LOX-1 (36). The 1483 cells had higher 12-LOX and 15-LOX-2 levels than 183A and MKN28 cells (Supplementary Fig. S2C and S2D), 2 of the other cell lines that had 15-LOX-1 mRNA expression levels equal to or higher than the level in differentiated Caco-2 cells (Supplementary Fig. S2E).

**15-LOX-1 expression in three-dimensional air–liquid interface cultures of normal and cancerous cell lines and patients with non–small-cell lung cancer**

Immortalized HBEC cells grown in organotypic 3-dimensional air–liquid interface culture conditions expressed 15-LOX-1 and showed features of terminal bronchoepithelial differentiation, whereas H460 and A549 human non–small-cell lung cancer cells grown in the same 3-dimensional culture conditions failed to express 15-LOX-1 or undergo epithelial differentiation (Fig. 3A and Supplementary Fig. S3). 15-LOX-1 expression scores were significantly lower in non–small-cell lung cancer tissue than in paired normal tracheobronchial epithelium in 16 (80%) of the 20 examined cases (Fig. 3B; P = 0.02).

**15-LOX-1 downregulation blocks terminal differentiation**

At day 14 after confluence, Caco-2 cells transfected with nontargeted shRNA (control shRNA) had significant 15-LOX-1 mRNA and protein expression (Fig. 4A and B). Stable 15-LOX-1 shRNA transfection into Caco-2 cells markedly reduced 15-LOX-1 mRNA and protein expression (Fig. 4A and B).

Caco-2 cells transfected with control shRNA exhibited ultrastructural differentiation features of well-organized intestinal microvilli on the apical side of cells (brush borders) and tight junctions between adjacent cells (Fig. 4C) on electron microscopy examination at day 14 after confluence. In contrast, 15-LOX-1 downregulation by 15-LOX-1 shRNA suppressed the formation of these differentiation features including tight junctions (Fig. 4C).

Sodium butyrate treatment was unable to induce terminal differentiation as indicated by alkaline phosphatase activity levels in Caco-2 cells that were stably transfected with a different shRNA and that had effective 15-LOX-1 knockdown during treatment with sodium butyrate [15-LOX-1 KD (+) cells; Fig. 5A and B]. These cells also failed to express 15-LOX-1 mRNA or protein during spontaneous differentiation induced with cell growth for 14 days after confluence (Fig. 5C and Supplementary Fig. S5C). 15-LOX-1 reexpression in 15-LOX-1 KD (+) cells via a plasmid vector (Supplementary Fig. S6A and S6B) significantly increased alkaline phosphatase activity level (Supplementary Fig. S6C). The mean ± SD level for cells transfected with 15-LOX-1 plasmid vector was 6.85 ± 0.32 DEA unit/g protein, which was significantly higher (P < 0.0001) than that for cells transfected with the control GFP plasmid vector and treated with (4.52 ± 0.13) or without (3.95 ± 0.15) sodium butyrate (Supplementary Fig. S6C).

Transfection of HT-29 colon cancer cells with 15-LOX-1 adenoviral vector (Ad-15-LOX-1) induced 15-LOX-1 mRNA and protein expression in a concentration-dependent manner, whereas transfection of cells with the Ad-luciferase control vector failed to induce 15-LOX-1 expression (Supplementary Fig. S7A and S7B). Alkaline phosphatase activity, a well-established marker of HT-29 cell differentiation (37), increased in Ad-15-LOX-1-transfected, but not Ad-luciferase–transfected, cells in a concentration-dependent manner (Supplementary Fig. S7C).

**15-LOX-1 downregulation suppresses tight junction formation and ZO-1 and E-cadherin localization to the cell wall membrane**

15-LOX-1 downregulation by 15-LOX-1 shRNA in 15-LOX-1 KD (+) Caco-2 clone increased relative FITC-labeled dextran permeability by more than 3-fold at 20 (P = 0.007), 30 (P = 0.0003), and 40 minutes (P = 0.0002) compared with the values in the control 15-LOX-1 KD (−) clone (Fig. 5D), which indicated that 15-LOX-1 downregulation disrupted the formation of tight junctions in 15-LOX-1 KD (+) cells.

We investigated the potential mechanism by which 15-LOX-1 downregulation suppressed formation of tight junctions by examining the impact of 15-LOX-1 downregulation on expression of E-cadherin and ZO-1, which are important proteins for tight junction formation. At 14 days of cell culture after confluence, control shRNA–transfected Caco-2 cells showed the typical honeycomb immunofluorescence staining of cell–cell interaction sites for both E-cadherin and ZO-1 that is observed with terminal differentiation (Fig. 4D and E). In contrast, 15-LOX-1 shRNA–transfected Caco-2 cells showed features of disorganized intercellular borders with redistribution of E-cadherin and ZO-1 from the cell wall membrane into the cytoplasm (Fig. 4D and E). We also found that in control 15-LOX-1 KD (−) Caco-2 cells, terminal differentiation upregulated the total expression of E-cadherin and cytoskeleton-associated (insoluble) E-cadherin fraction involved in intercellular junction formation; this effect started 3 to 7 days after confluence and continued as terminal differentiation progressed (Fig. 5E). In contrast, in 15-LOX-1 KD (+) Caco-2 cells, there was no significant increase in cytoskeleton-associated E-cadherin levels during the same postconfluence cell culture period (Fig. 5E).
Discussion

We have found that ubiquitous downregulation of 15-LOX-1 expression in cancer cells plays a mechanistically important role in their loss of terminal cell differentiation, especially by dysregulating key events, such as tight junction formation, via the disruption of E-cadherin and ZO-1 functions.

15-LOX-1 expression was ubiquitously downregulated in cancer cells. 15-LOX-1 mRNA expression levels were markedly diminished in 128 randomly collected cancer cell lines (mean expression level relative to expression level in differentiated NHBE cells: <1 in 10,000). In contrast, we observed that terminally differentiated cells (e.g., NHBE and NHEK cells) expressed 15-LOX-1, in agreement with prior studies showing that 15-LOX-1 expression is inducible in normal cells with terminal cell differentiation (11, 12). The markedly low 15-LOX-1 relative expression levels in cancer cell lines were most likely not due to a high 15-LOX-1 expression level unique to one model of normal epithelial cells (i.e., differentiated NHBE cells) given our finding that 95% of the screened cell lines also had 15-LOX-1 mRNA expression levels below the mean.

Figure 3. 15-LOX-1 expression in normal and cancerous lung cells. A, nontumorigenic immortalized normal HBECs (HBEC3KT, HBEC6KT, HBEC12KT, and HBEC24KT) and H460 lung cancer cells were grown in air-liquid interface cultures that were paraffin embedded, sectioned, and examined with hematoxylin and eosin (H&E) staining and 15-LOX-1 immunohistochemical (IHC) staining. B, paired normal and nonsmall-cell lung cancer tissues were immunohistochemically stained for expression of 15-LOX-1. Values are the intensity scores for 15-LOX-1 immunohistochemical cytoplasmic staining (IHC intensity scores) in each individual case as listed in the table. NA, tissue samples were not available.
levels below than that in differentiated normal NHEK (mean relative expression level: <0.005). Even when the screened cancer cell lines were compared with a terminally differentiated colon epithelial cancer cell line (differentiated Caco-2 cells), 96% of the screened cancer cell lines had 15-LOX-1 mRNA levels lower than that in differentiated Caco-2 cells (mean relative expression level: <0.01). Furthermore, in the cancer cell lines that had 15-LOX-1 mRNA levels equal to or higher than the level in differentiated NHEK or Caco-2 cells, 15-LOX-1 protein expression was
undetectable except for a faint band in 1483 cells. Two different lines of evidence indicate that this faint band in 1483 cells is unlikely to be related to 15-LOX-1 expression. First, the enzymatic activity of 15-LOX-1, measured by its primary product (13-S-HODE), was significantly lower in 1483 cells than in differentiated Caco-2 cells. Second, 1483 cells expressed higher levels of 12-S-LOX and 15-LOX-2 than Caco-2 cells. Both 12-S-LOX and 15-LOX-2 have significant protein sequence homology with 15-LOX-1 (36), and their high expression levels in 1483 cells are likely to have caused cross-reaction with the 15-LOX-1 polyclonal antibody.

These new findings show for the first time that 15-LOX-1 expression is universally suppressed in cancer cells. Although prior reports have shown loss of 15-LOX-1 expression in colorectal, pancreatic, and gastric cancer, and lymphoma cell lines (38, 39), our study is, to our knowledge, the first to show ubiquitous loss of 15-LOX-1 expression in a very large collection of cancer cell lines (128 cell lines from more than 20 types of human cancer, including all of the more common types—lung, breast, colorectal, prostate, esophageal, gastric, pancreas, liver, head and neck, cervical, ovarian, and endometrial). Furthermore, our current study adds to the list of several major human cancer clinical...
studies showing 15-LOX-1 downregulation in tumors [e.g., colon (refs. 13–15), esophageal (ref. 16), breast (ref. 17), and pancreatic cancers (ref. 18)] by showing the loss of 15-LOX-1 in human lung cancer. The frequency of loss of expression of 15-LOX-1 in cancer cells exceeded the frequency of loss of expression of p16, a tumor suppressor gene that promotes terminal cell differentiation (6, 7) and is considered one of the most frequently downregulated genes in human cancers (33–35). The ubiquitous loss of 15-LOX-1 expression in human cancers in our study supports its role as a tumor suppressor gene.

In the current study, terminal differentiation evasion by cancer cells was associated with 15-LOX-1 expression downregulation not only in monolayer immersion cultures but also in 3-dimensional organotypic air–liquid interface cultures. In air–liquid interface cultures, nonviral immortalized HBEC cells, which exhibit a nontransformed phenotype and retain the ability to undergo terminal differentiation to produce normal airway epithelial maturation in organotypic air–liquid interface cultures (40), expressed 15-LOX-1 when undergoing terminal differentiation to airway epithelium-like structures, whereas lung cancer cells grown under the same conditions failed to show features of terminal differentiation or to express 15-LOX-1. The clinical relevance of these findings was confirmed by our demonstration of 15-LOX-1 downregulation in human non–small-cell lung cancers. Interestingly, expression of 15-LOX-2, a related but distinct 15-LOX isoenzyme, is also reduced in human non–small-cell lung cancers, especially in poorly differentiated cases (41). During the review of the manuscript, another independent group reported that levels of 15-LOX-1 mRNA expression and its primary product (13-S-HODE) are suppressed in human non–small-cell lung cancer, in agreement with our currently reported findings (42). Taken together, our findings from air–liquid interface cultures and examination of human tissue samples indicate that loss of 15-LOX-1 expression is unlikely to be related to an artificially generated in vitro phenomenon and support the link between 15-LOX-1 expression loss in cancer cells and their ability to escape terminal differentiation.

Our current study showed for the first time that specific 15-LOX-1 downregulation was sufficient to disrupt terminal cell differentiation. Prior studies have reported on the association between sodium butyrate–induced terminal differentiation and 15-LOX-1 expression (19, 20), but whether 15-LOX-1 downregulation is sufficient to disrupt terminal differentiation was unknown prior to the current study. In our current study, we used specific 15-LOX-1 downregulation via shRNA to examine the specific role of 15-LOX-1 in terminal cell differentiation. In addition, instead of using sodium butyrate treatment, which can induce the expression of a large number of genes independently of terminal cell differentiation, we used a well-established model in which terminal differentiation is induced spontaneously by protracted cell culture (43) to specifically examine 15-LOX-1 mechanistic role in terminal differentiation. Protracted culture of Caco-2 cells induced typical cell differentiation features, including formation of microvilli and tight junctions, in agreement with what was previously reported (43). The ability of 15-LOX-1 downregulation to disrupt tight junction formation in colon cancer cells was further confirmed by quantitative macromolecule permeability assay. Furthermore, our findings show for the first time that through this spontaneous terminal differentiation of Caco-2 cells, 15-LOX-1 expression was induced independent of drug treatment, and suppression of 15-LOX-1 expression via shRNA was sufficient to block terminal cell differentiation. Thus, our findings show that 15-LOX-1 expression is necessary for terminal differentiation and tight junction formation. Furthermore, we have confirmed that these results are not due to shRNA off-target effects by using a second 15-LOX-1 shRNA construct with a different sequence and by comparing 2 stably transfected clones of this second 15-LOX-1 shRNA construct, one clone with and one clone without successful 15-LOX-1 knockdown. In addition, we used a complementary approach via 15-LOX-1 expression in cancer cells through either plasmid- or adenoviral vector-induced cell differentiation. Therefore, findings from experimental models of gain and loss of 15-LOX-1 function indicate that 15-LOX-1 loss of expression in cancer cells mechanistically contributes to their ability to escape cell differentiation.

The findings from our current study also indicate that 15-LOX-1 expression is mechanistically important for E-cadherin and ZO-1 localization, which are critical for tight junction formation. Formation of tight junctions is an important step in the terminal differentiation of epithelial cells, especially intestinal epithelial cells, that allows them to maintain their polarity and barrier function (44). E-cadherin and ZO-1 are critical proteins for the formation of the large protein complexes of tight junctions (44). Disruption of tight junction formation and E-cadherin function is thought to propel various tumorigenic mechanisms (45), especially epithelial-mesenchymal transition (46–48). Because we observed loss of tight junctions with 15-LOX-1 downregulation in Caco-2 cells, we examined the effects of specific 15-LOX-1 downregulation by shRNA on E-cadherin and ZO-1. 15-LOX-1 downregulation blocked the upregulation of E-cadherin and its cytoskeleton association during terminal differentiation. In addition, we found by immunofluorescence studies in this experimental model that 15-LOX-1 downregulation blocked E-cadherin and ZO-1 recruitment into the cell–cell contact areas, where they play their critical role in tight junction formation, and redistributed these proteins into the cytoplasm. These results show for the first time how the loss of 15-LOX-1 in cancer cells leads to the disruption of E-cadherin function in epithelial cells, a potential mechanism to promote tumorigenesis.

Our current results establish the significance of the mechanistic role of 15-LOX-1 in terminal cell differentiation and establish a mechanistic link between the pervasive loss of
15-LOX-1 expression in cancer cells and their ability to evade terminal cell differentiation. Given the role of termina- l differentiation loss as an important mechanism early in tumorigenesis, we believe that the new findings reported here are highly relevant to cancer prevention research because they show how 15-LOX-1 expression loss serves as a mechanism for terminal differentiation loss in cancer cells and thereby allows important early tumorigenic changes, such as loss of tight cell junction formation to promote epithelial-mesenchymal transition. The ubiquity of 15- LOX-1 expression loss in the very large number of cancer cell lines screened in our study, taken together with the ability of 15-LOX-1 expression loss to dysregulate terminal differentiation, supports the need for molecularly targeted interventions to reexpress 15-LOX-1 in cancer cells and thus inhibit tumorigenesis via reestablishment of terminal cell differentiation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


Cancer Prevention Research

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