Original Article

Trichostatin A Restores Expression of Adherens and Tight Junction Proteins during Transforming Growth Factor β-Mediated Epithelial-to-Mesenchymal Transition

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Abstract

Purpose: Adherens junctions and polarity markers play an important role in maintaining epithelial phenotype but get altered during the epithelial-mesenchymal transition (EMT). Alterations of these markers during EMT of lens epithelial cell (LEC) can lead to vision compromising conditions. The aim of this study was to examine if Trichostatin-A (TSA), a histone deacetylase inhibitor, can prevent EMT by restoring the adherens junction complex in LEC.

Methods: Fetal human lens epithelial cell line (FHL124) was used. Cells were treated with 10 ng/ml TGF-β2 in the presence or absence of TSA. Real time-PCR and western blotting were carried out for *HDAC1*, *HDAC2*, *CDH1* (E-cad), *TJP1 (*ZO-1) and *CTNNB1 (*β-cat). Level of histone acetylation was analyzed by western blotting. Chromatin Immunoprecipitation was carried out to study the level of acetylated histone H4 and HDAC2 at the promoter regions of *CDH1*, *TJP1*, and *CTNNB1*. E-cad, ZO-1, and β-cat were localized using immunofluorescence. Kruskal-Wallis test was used for statistical analysis.

Results: TSA down-regulated HDAC1 and HDAC2 and led to an increase in global acetylation. The mRNA and protein levels of E-cad, ZO-1, and β-cat decreased during EMT but were up-regulated by TSA treatment. TSA also helped in stabilizing these proteins at cell-cell junctions during EMT. TSA decreases association of HDAC2 at the promoter regions of adherens junction genes while increasing histone H4 acetylation status.

Conclusion: TSA increases histone acetylation and restores the adherens junction complex in LECs. TSA helps in preventing EMT and thus shows potential against lens fibrosis and vision compromising conditions.

Keywords: E-cadherin; β-catenin; Epithelial-mesenchymal Transition; Trichostatin-A; ZO-1

J Ophthalmic Vis Res 2018; 13 (3): 274-283

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Received: 27-05-2017 Accepted: 26-12-2017

Access this article online

Quick Response Code:

Website: www.jovr.org

DOI: 10.4103/jovr.jovr_110_17

INTRODUCTION

The adherens junction protein E-cadherin (E-cad) is a transmembrane glycoprotein that plays a crucial role in

How to cite this article: Ganatra DA, Vasavada AR, Vidya NG, Gajjar DU, Rajkumar S. Trichostatin a restores expression of adherens and tight junction proteins during transforming growth factor β-mediated epithelial-to-mesenchymal transition. J Ophthalmic Vis Res 2018;13:274-83.

maintaining the epithelial phenotype.[1] E-cad and the tight junction protein zonula occludens-1 (ZO-1) are involved in the preservation of apical-basal polarity of the epithelia. Beta-catenin (β-cat) also binds to E-cad and helps in cell-cell adhesion.[2] Together, E-cad, ZO-1 and β-cat form the adherens junction complex. The loss of this complex is characteristic of epithelial-to-mesenchymal transition (EMT), a process by which epithelial cells trans-differentiate into motile myofibroblast-like cells.[3] Transforming growth factor beta (TGF-β) is considered to be a key inducer of EMT-related changes in the human lens, and is also associated with complications like subcapsular cataracts and posterior capsular opacification (PCO).[4,5] During EMT, lens epithelial cells (LECs) trans-differentiate into spindle-shaped cells that express a high level of mesenchymal markers and a reduced level of epithelial markers.[4,6-8]

Histone acetylation, an epigenetic modification, is regulated by the opposing effects of enzymes such as histone acetyltransferases (HATs) and histone deacetylases (HDACs).[9,10] Class I and II HDACs have been implicated in TGF-β-mediated EMT in different cell types.[11-14] Trichostatin A (TSA) is a potent and reversible inhibitor of Class I and II HDACs.[15] Several *in vivo* and *in vitro* studies have used TSA as an anti-fibrotic agent to prevent fibrosis of the skin, lung, liver, cornea, and subconjunctiva.[11,16-19] Prevention of EMT by TSA has been confirmed using lens explants and also the LEC cell lines SRA01/04 and HLEB3.[6,7] It has been previously shown that TSA helps in preventing EMT of the LECs by preserving its morphological features, preventing migration, and down-regulating the mesenchymal marker αSMA by modulating the acetylation status of its promoter.[20] However, the effect of TSA on epithelial markers such as E-cad, ZO-1, and β-cat, which are altered during EMT, has not been established. Also, information is lacking on the acetylation status of the promoter regions of these genes during EMT. The aim of the present study was to determine whether TSA helps in preserving adherens junction complexes during TGF-β-induced EMT in LECs.

METHODS

Cell culture

The fetal human lens epithelial cell line (FHL124) was a kind gift from Prof. John Reddan, Oakland University, USA. FHL124 was authenticated using short tandem repeat (STR) profiling[20] and passages 15-18 were used. Cells were cultured in Eagle's minimum essential media (EMEM) (Sigma-Aldrich, St. Louis, USA), supplemented with 10% fetal bovine serum (FBS) (Himedia, Mumbai, India) and 50 μg/ml gentamycin (Himedia, India), and maintained at 35°C in a humidified atmosphere with 5% CO2. On 70% confluence, cultures were serum starved for 24 hours and treated with 10 ng/ml of TGF-β2 (Invitrogen, Carlsbad, CA, USA), or TSA (Sigma-Aldrich), or both for an additional 24 hours. Quantities of TSA were as indicated for specific experiments. After each treatment, the cells were washed with phosphate buffer saline (PBS) and collected for subsequent experiments. Cell morphology was examined under an inverted microscope (Axiovert 200, Carl Zeiss, Gottingen, Germany).

Cell viability assay

The 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used to determine the viability of LECs after TSA treatment.[20] Briefly, 7×10^3 cells per well were added to 96-well plates and incubated for 24 hours. The cells were treated with a wide range of TSA concentrations (0.1-5 μM) for 24 hours. Cells were then incubated with MTT (5 mg/ml in PBS) (Himedia). After four hours, the MTT solution was replaced with 100 μl of dimethyl sulfoxide (DMSO). The absorbance was read at 570 nm and the half-maximal inhibitory concentration (IC_{50}) of TSA was determined.

Real-time polymerase chain reaction

Total RNA was extracted using TRIzol (Invitrogen) and a 4-μg portion was reverse transcribed according to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was carried out to determine expression levels of *CDH1* (E-cad), *TJP1* (ZO-1), *CTNNB1* (β-cat), *HDAC1*, and *HDAC2*, with *ACTB* (β-actin), a housekeeping gene, used for normalization. The list of primers is given in Table 1. The cycle threshold (Ct) value of the target gene was normalized to that of the housekeeping gene and the relative expression was determined.

Western blotting

Western blotting was carried out to measure the protein levels. The detailed protocol has been previously described.[19] Briefly, cells were collected in lysis buffer and equal amounts of protein (20 μg) were resolved using 8-15% SDS-PAGE. Proteins were transferred onto nitrocellulose membranes (Pall Corporation, Port Washington, NY, USA). Membranes were blocked and then incubated overnight at 4°C with a primary antibody against E-cad (BD Transduction Laboratories™, Franklin Lakes, New Jersey, USA), ZO-1 (Zymed Laboratories®, San Francisco, CA, USA), β-cat (BD Transduction Laboratories™), HDAC1 (Abcam, Cambridge, MA, USA), HDAC2 (Abcam), acetylated histone H3 (AcH3) (Millipore, Temecula, CA, USA), AcH4 (Millipore), or β-actin (used as a loading control). A secondary antibody (Abcam) (1:5000 dilution) conjugated with horseradish peroxidase (HRP), was used

Table 1. qRT-PCR primers

Gene Name	Forward primer (5′-3′)	Reverse primer (5′-3′)
ACTB	GTT GTC GAC GAC GAG CG	GCA CAG AGC CTC GCC TT
CDH1	CTT GCG GAA GTC AGT TCA GA	CAG AGC CAA GAG GAG ACC TG
TJP1	CCC CAC TCT GAA AAT GAG GA	GGG AAC AAC ATA CAG TGA CGC
CTNNB1	ATT GTC CAC GCT GGA TTT TC	TCG AGG ACG GTC GGA CT
HDAC1	CAT CTC CTC AGC ATT GGC TT	GAC GGG GAT GTT GGA AAT TA
HDAC2	ATG AGG CTT CAT GGG ATG AC	ATG GCG TAC AGT CAA GGA GG

and blots were developed by chemiluminescence using the ECL western blotting substrate (Pierce Biotechnology, Rockford, IL, USA). Image J software (NIH image) was used to determine band intensity.

Chromatin immunoprecipitation (ChIP) assay

Levels of AcH4 and HDAC2 at the promoter regions of CDH1, TJP1, and CTNNB1 were assessed by ChIP assay. The detailed protocol has been reported previously.[20] Briefly, histones were crosslinked to DNA by incubating cells with 1% formaldehyde for ten min. Cells were then lysed and chromatin was sheared using a Branson SLPe150 sonifier (Branson Ultrasonics, Kowloon, Hong Kong). Input DNA samples were reserved for normalization. Samples from each treated group containing 20 µg of DNA were subjected to immunoprecipitation with antibodies against AcH4 (Millipore), HDAC2 (Millipore), or normal rabbit IgG (Cell Signaling Technology Inc., Danvers, MA, USA). Samples were then incubated with salmon sperm DNA and protein A agarose slurry (Millipore) for three hours at 4°C. Antigen-antibody complexes were pelleted along with the slurry by centrifugation. RIPA buffer [0.1% SDS, 10% sodium deoxycholate, 150 mM sodium chloride, 10 mM sodium phosphate (pH 7.2), 2 mM EDTA, 0.2 mM sodium orthovanadate, 1x protease inhibitor cocktail (Roche Diagnostics GmbH)] and TE buffer [10 mM Tris–HCl (pH 8.0), 1 mM EDTA (pH 8.0)] were consecutively used to wash the pellet and then incubation with elution buffer [1% SDS, 0.1 M sodium bicarbonate, 10 mM DTT] was carried out for one hour at room temperature. After de-crosslinking histone-DNA complexes with 5 M sodium chloride, DNA was deproteinized using the phenol/chloroform/isoamyl alcohol extraction method. qRT-PCR was used to measure levels of AcH4 and HDAC2 at the promoter regions of *CDH1* (forward primer: 5′-TAGAGGGTCACCGCGTCTAT-3′; reverse primer: 5′-TCACAGGTGCTTTGCAGTTC-3′),[21] *TJP1* (forward primer: 5′-CTTGAGGTCTAATGTGGGGTG-3′; reverse primer: 5′-CATGGCTTTCATCTCCGAG-3′),[22] and *CTNNB1* (forward primer: 5′-GCCCCAAAGCGCACTATAATTT-3′; reverse primer: 5′-ACAAATTCTGCACGCGCCAT-3′). The relative DNA level in each immunoprecipitated group was normalized to the input DNA level.

Immunofluorescence

The spatial organization of E-cad was observed by immunofluorescence using goat lens epithelial cells (gLECs). Institutional ethical clearance was obtained and two goat eyes were obtained from the slaughterhouse within thirty minutes of death. Epithelial cells were isolated from the lens capsule as previously described,[23] pooled, and plated in six wells for each treatment group. On harvesting, cells were fixed with 2% paraformaldehyde and permeabilized using 0.25% Triton X-100 (Sigma-Aldrich) and 0.25% saponin (Sigma-Aldrich). A 5% blocking solution was used, then cells were incubated overnight with an antibody against E-cad at 4°C. Blots were incubated with a secondary antibody tagged with Alexa Fluor 488 (Invitrogen) and DAPI (Sigma-Aldrich) for one hour at 37°C. Cells were mounted, proteins were visualized using a fluorescence microscope (Axioskope II, Carl Zeiss, Oberkochen, Germany), and images were captured with a Cohu cool CCD camera (Cohu, San Diego, USA). ZO-1 and β-cat were localized by the same procedure using FHL124 cells.

Statistical analysis

All experiments were repeated at least thrice and the results reported as mean ± standard error. A non-parametric test, the Kruskal-Wallis, was used for statistical analysis and *P* values of less than 0.05 were considered significant. TGF-β, TSA0.5, and TSA1 data (see Results) were compared to controls, and data for TSA0.5+TGF-β and TSA1+TGF-β were compared to TGF-β.

RESULTS

TSA treatment and viability of LECs

The MTT assay showed a dose-dependent decrease in cell viability with TSA addition [Figure 1]. The IC_{50} of TSA was calculated to be 2.5 µM. Thus, concentrations of 0.5 µM and 1 µM were used to avoid compromising cell viability.[20]

Maintenance of the LEC epithelial phenotype

Lens epithelial cells in the control group appeared cuboidal but became elongated and spindle-shaped

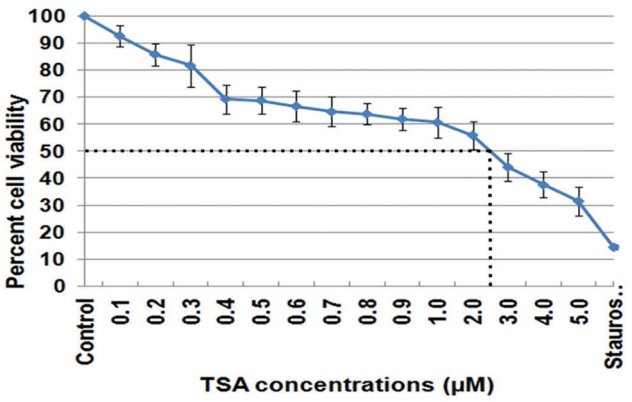

Figure 1. Cell viability assay for FHL124 cells after TSA treatment. 50% viability was observed at around 2.5 μM TSA. Data are presented as mean ± standard error (SE).

on TGF-β2 treatment [Figure 2]. Cells treated with TSA at both 0.5 μM and 1 μM concentrations retained an epithelial morphology similar to that of the control group. During EMT, TSA treatment maintained the cuboidal morphology of LECs and suppressed the effects of TGF-β2. (Credit: Source- Eye (*Lond)* 2015; 29(6)**:** 828-838.)[20]

Acetylated histone levels

In response to TGF-β2, there was a marked increase in mRNA levels for *HDAC1* ($P < 0.05$) and *HDAC2* ($P < 0.01$) [Figure 3]. During EMT, TSA at 0.5 μM down-regulated *HDAC2* mRNA by 39% ($P < 0.05$). TSA at 1 μM down-regulated both *HDAC1* (35%, $P < 0.05$) and *HDAC2* (50%, $P < 0.05$) mRNA. At the protein level, TSA at 0.5 μM and 1 μM abrogated up-regulation of HDACs. The reduction in HDAC levels by TSA also led to a change in histone acetylation status. TSA at 0.5 μM and 1 μM led to a 37% and 63% increase in AcH3, respectively, and a 43% and 64% increase in AcH4, respectively, during EMT [Figure 4].

Epithelial marker levels

Expression of *CDH1, TJP1,* and *CTNNB1* was down-regulated ($P < 0.05$) during EMT [Figure 5]. TGF-β2 reduced expression of *CDH1, TJP1,* and *CTNNB1* by 57.41%, 53.98%, and 33.11%, respectively compared to controls. Although TSA treatment increased levels of *CDH1* and *TJP1* mRNA during EMT, a significant up-regulation was observed only with the higher concentration (1 μM) ($P < 0.05$). TSA treatment did not result in a significant difference in expression of *CTNNB1*. Consistent with the mRNA results, western blots showed a reduction of these proteins upon TGF-β2 treatment ($P < 0.05$). However, co-treatment with TSA significantly elevated the levels of all the three proteins even in the presence of TGF-β2 [Figure 5].

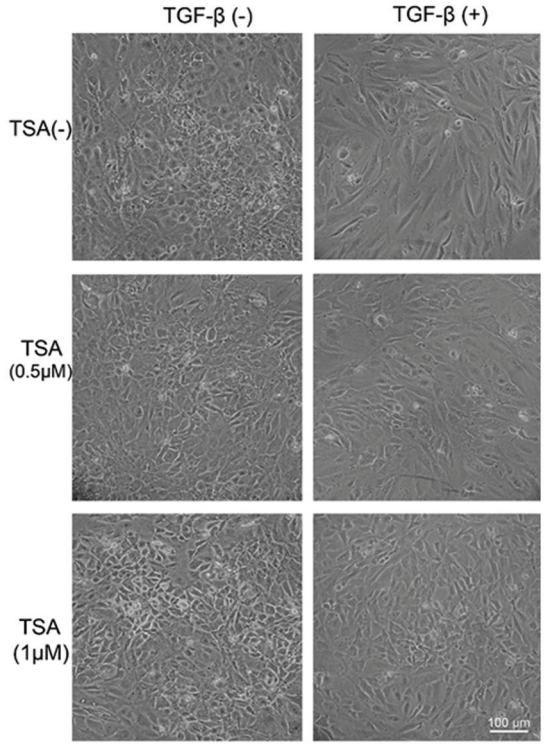

Figure 2. Morphology of FHL124 cells after different treatments. The figure shows phase contrast images of cells treated with 0.5 μM and 1 μM of TSA (labels on left) ±10 ng/ml TGF-β2 (labels above).[20]

To check whether acetylation levels were related to the transcriptional activity of genes, ChIP was carried out. Promoter regions of *CDH1*, *TJP1*, and *CTNNB1* were examined for their involvement with acetylated histone H4 and HDAC2. Treatment of cells with TGF-β2 led to a significant increase ($P < 0.05$) in HDAC2 activity at the promoter regions of all three genes which correlated with a significant decrease ($P < 0.05$) in the level of acetylated histone H4 as compared to controls [Figure 6]. A lower concentration of TSA (0.5 μM) increased acetylation status at *the CDH1* promoter but did not result in a significant change at the promoter region of *TJP1* or *CTNNB1*. However, treatment of cells with the higher concentration of TSA (1 μM) reduced association of HDAC2 at the promoter regions of all three genes and raised levels of AcH4 association [Figure 6].

Epithelial marker localization

In normal epithelial cells, E-cad was distributed at cell membranes, but on induction of EMT, E-cad was lost from cell-cell interfaces and localized in the cytoplasm [Figure 7]. The cells also appeared elongated and had lost cell-cell contacts. On TSA treatment, the cells displayed cuboidal morphology and regained cell-cell contacts. Intense staining of E-cad was observed at cell-cell contacts, and co-treatment with TSA and TGF-β2

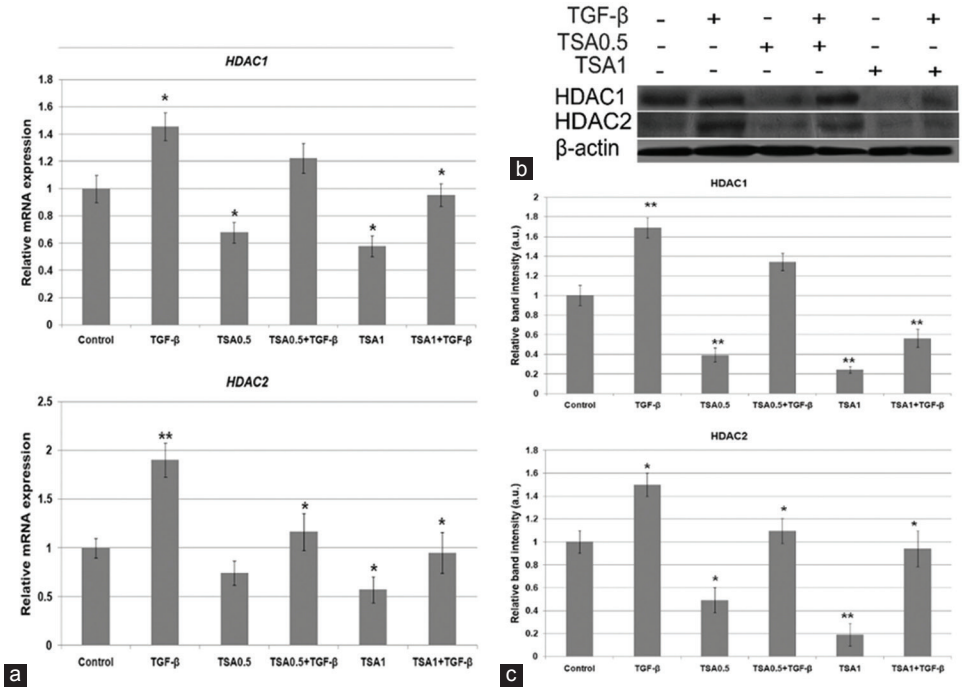

Figure 3. Levels of *HDAC1* and *HDAC2* mRNA and protein for various experimental conditions. (a) Relative expression of *HDAC1* and *HDAC2* mRNA. (b) Western blots of HDAC1, HDAC2, and β-actin (used as a loading control). (c) Graphical representation of relative western blot band intensities (*$P < 0.05$, **$P < 0.01$).

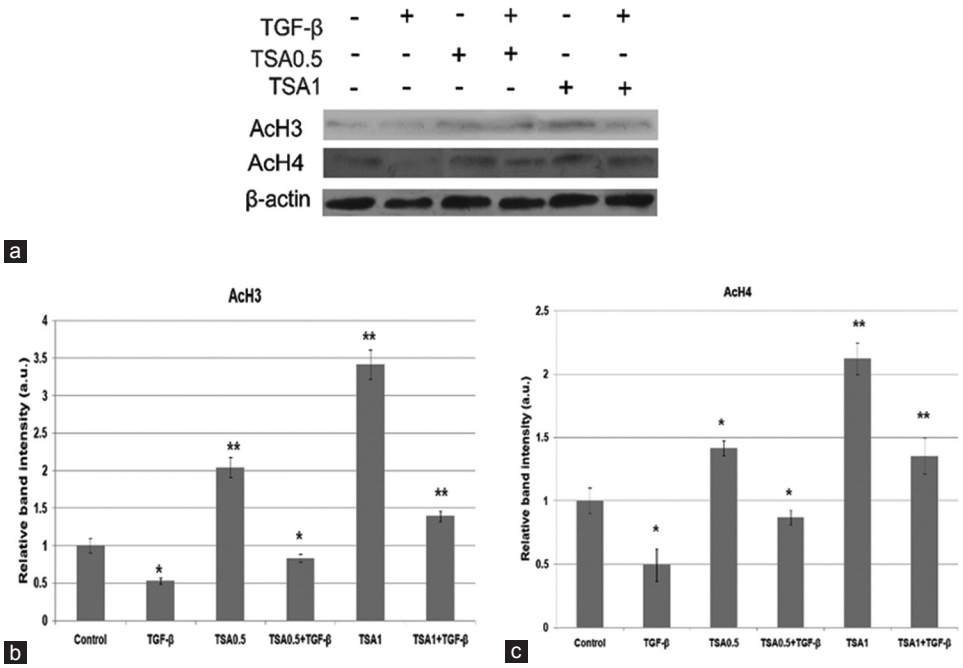

Figure 4. Relative levels of AcH3 and AcH4. (a) Western blots of AcH3, AcH4, and β-actin (used as a loading control). (b) Graphical representation of relative band intensities of AcH3. (c) Graphical representation of relative band intensities of AcH4 (*$P < 0.05$; **$P < 0.01$).

also resulted in confinement of E-cad to the cell membrane. As seen in Figure 8, both ZO-1 and β-cat were primarily present at cell junctions in control cells, but during EMT several cells showed reduced and disrupted staining. TGF-β treatment led to nuclear localization of β-cat and relocation of ZO-1 to the cytoplasm. However, on TSA treatment, these proteins were detected along the cell-cell interface.

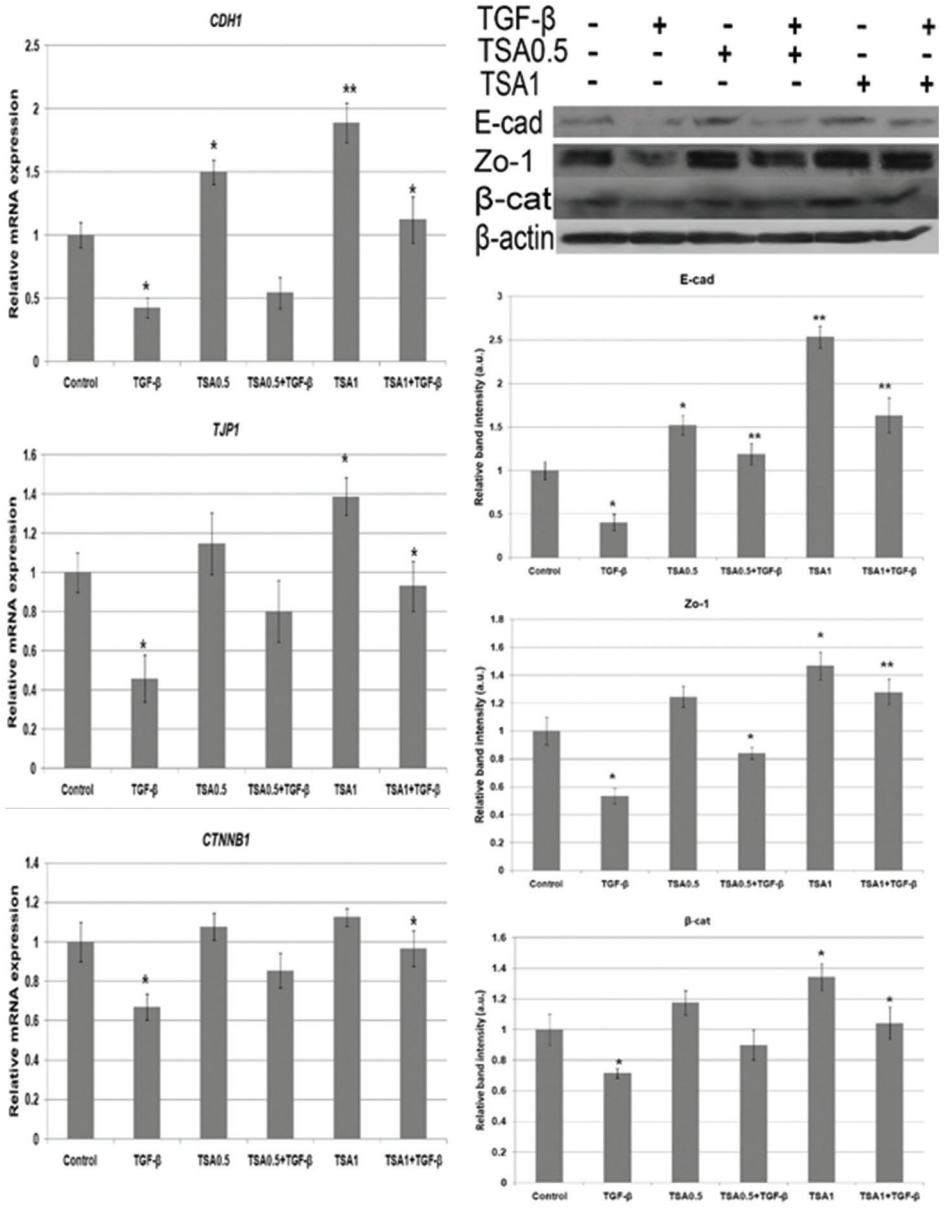

Figure 5. Levels of *CDH1*, *TJP1*, and *CTNNB1* mRNA and protein products (E-cad, ZO-1, and β-cat, respectively) for various experimental conditions. Relative expression of *CDH1*, *TJP1*, and *CTNNB1* mRNA (left column). Western blots of E-cad, ZO-1, β-cat, and the reference protein β-actin, and graphical representations of relative band intensities (right column) (*$P < 0.05$; **$P < 0.01$).

DISCUSSION

Epigenetic changes regulate gene expression through chromatin remodeling. We report here that epigenetic modifications such as histone acetylation help in restoring adherens junction complexes and thus help in EMT prevention. To our knowledge, results presented here are the first to support the proposal that TSA, an HDAC inhibitor, can regulate gene expression for E-cad, ZO-1, and β-cat by modulating histone acetylation status at their promoter regions during TGF-β-mediated EMT in LECs. TSA treatment also stabilized these proteins at cell-cell junctions and maintained the epithelial morphology of LECs. These results thus emphasize the importance of histone acetylation in the regulation of EMT.

Acetylation of lysine present on the amino terminal of the histones is an important epigenetic modification which controls the accessibility of the chromatin and its associated transcription activity. HATs catalyze acetylation of lysine residues at the N-terminal of histones, which destabilizes the histone–DNA bond and makes DNA accessible to RNA polymerase and other transcription factors. HDACs remove acetyl groups from histones and cause compaction of chromatin, leading to reduced transcription. The HDACs are divided into

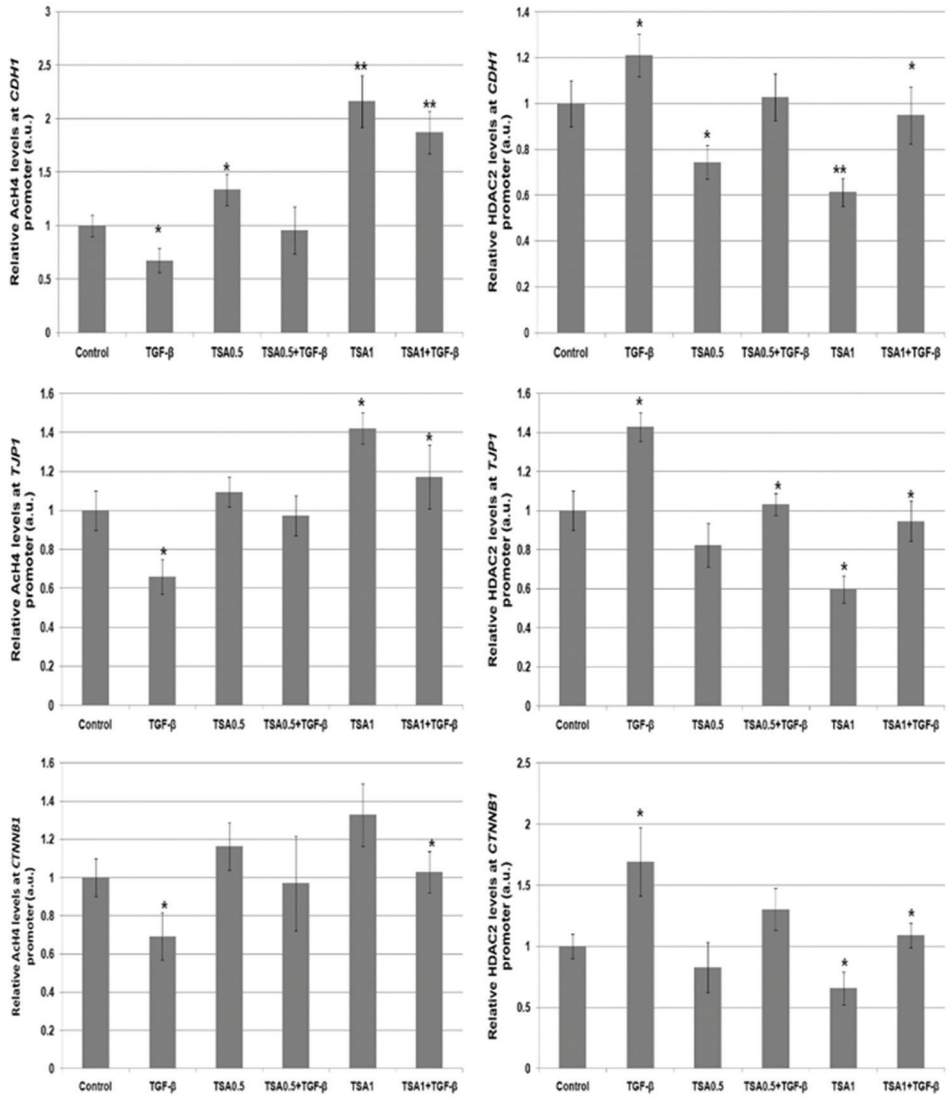

Figure 6. ChIP assay determinations of relative levels of AcH4 and HDAC2 at the promoter regions of *CDH1*, *TJP1*, and *CTNNB1* for various experimental conditions, normalized to input DNA (see Methods) (*$P < 0.05$; **$P < 0.01$).

four groups based on their primary structure. TSA is known to be a competitive inhibitor of HDACs; it aborts the deacetylation reaction by fitting into the enzyme-substrate binding site.[24,25] Currently, there is a lot of interest in modulating gene expression by regulating histone acetylation.

Intercellular junctions, such as tight junctions and adherens junctions play an important role in the regulation of cell function. β-Cat has been shown to bind to E-cad and to play an important role in cell-cell adherens junctions.[2] Tight junction proteins such as ZO-1 contribute to the maintenance of epithelial cell polarization by separating the apical and basolateral domains. Cytoskeletal rearrangement and loss of cell-cell adhesion are considered to be important features of EMT. Remodeling of several junction protein complexes takes place during EMT,[26] followed by disruption of the complexes, leading to loss of epithelial cell polarity and increased migration. It has been shown that treatment with TGF-β disrupts cell-cell junctions and results in EMT[13] in hepatocytes. Previous reports regarding LECs have also found that E-cad is down-regulated during TGF-β-mediated EMT.[6,8,27] According to Choi et al, TGF-β can lead to induction of Slug and suppression of E-cad by altering SP1 DNA binding activity.[8] In the present study, TGF-β also led to disrupted staining of E-cad at cell-cell contacts. E-cad was not detectable by immunostaining in FHL124 cells, so goat LECs were used for observations of E-cad. This characteristic of native LECs has been previously reported.[28,29] FHL124 cells, generated from human capsular epithelial explants, have 99.5% homology with native human LECs.[30]

TGF-β treatment also led to a marked decrease in protein and mRNA levels and altered subcellular

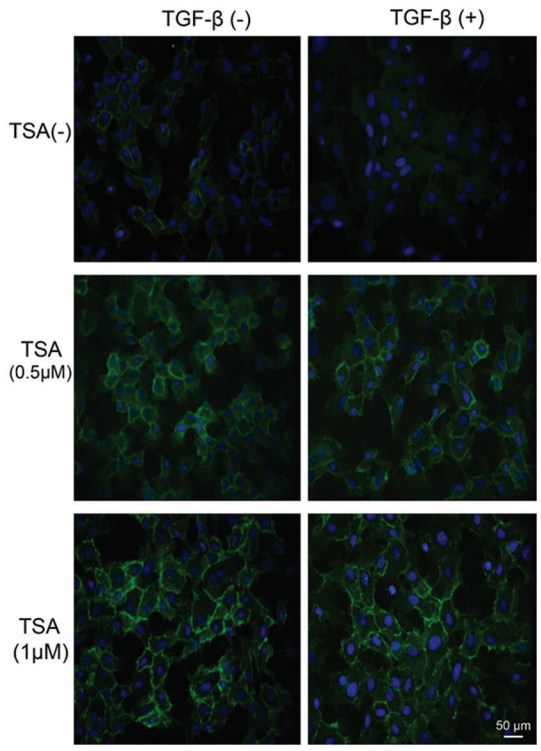

Figure 7. Immunolocalization of E-cad in goat lens epithelial cells. Nuclei are stained blue with DAPI and E-cad is immunostained green. The bar shown is 50 μm.

locations for β-cat and ZO-1. β-cat, which is found at cell junctions in control epithelial cells, showed evidence of nuclear translocation during EMT with decreased and discontinuous staining. Unphosphorylated β-cat is associated with E-cad at cell margins, but during EMT it switches roles from that of an adhesion molecule to that of a signaling molecule, thus activating the Wingless (Wnt) pathway involved in TGF-β-induced EMT.[31] We found that treatment with TSA prevented disruption of junction protein organization and preserved the epithelial phenotype by stabilizing junction proteins the at cell-cell interface. TSA treatment increased the β-cat protein level but not its mRNA level. Levels of signaling proteins, such as β-cat, can be poorly correlated with their corresponding mRNA levels.[32] TSA also increased expression of ZO-1 and stabilized its location at cell-cell interfaces. An increased level of ZO-1 may explain the decreased cell migration reported in an earlier study using FHL124 cells,[33] as it may affect migration by either strengthening the scaffold underlying the plasma membrane or by increasing cell-cell adhesion.

The involvement of HDACs in regulating E-cad expression has been well established in other cell types in which the Snail-HDAC1/HDAC2 transcriptional repressor complex is recruited to the E-cad promoter and leads to E-cad down-regulation.[34,35] Prior to the present study, there were no reports that either HDAC1 or HDAC2 is recruited to the promoter regions of ZO-1 or β-cat. We report that TSA abrogated up-regulation of HDAC1 and HDAC2 during EMT of LECs. Lower levels of HDACs were accompanied by increased levels of AcH3 and AcH4. ChIP assays showed that, during EMT, HDAC2 was associated with promoter regions of genes for E-cad, ZO-1, and β-cat, while associated AcH4 was decreased. TSA inhibited the association of HDAC2 at the promoter region of E-cad and led to increased acetylation of histone H4. Increased acetylation at the E-cad promoter region during TSA treatment helps in the binding of E-cad to β-cat and inhibits its translocation to the nucleus.[36] Also, it was reported that increased histone acetylation at the increased histone acetylation at t total levels of β-cat, but β-cat localization at the cell membrane increased.[37]

TGF-β2, the predominant form of TGF-β in the eye,[38] was used in this study to induce EMT-like changes in the FHL124 cell line. TGF-β can lead to vision-compromising conditions like anterior subcapsular cataract (ASC) and posterior capsular opacification (PCO).[39,40] Cataract is the leading cause of blindness in the world and PCO remains the main postoperative complication that causes visual loss in adults and in pediatric cases.[41-44] TSA is emerging as a strong anti-fibrogenic agent, and *in vivo* and *in vitro* studies have shown its effect on fibrosis of the subconjunctiva[19] and the cornea.[18,45] TSA also prevents myofibroblastic differentiation of LECs, reduces their migration, and abrogates increased αSMA expression, thus suppressing TGF-β-induced EMT.[20] Since lack of intercellular junction complexes is also an important feature of EMT, this study along with others indicates the importance of TSA in inhibiting TGF-β-induced EMT.[6,7,20]

In summary, we present evidence that down-regulation of *CDH1*, *TJP1*, and *CTNNB1* during EMT is HDAC-dependent, and that TSA can restore the adherens junction complexes by increasing histone acetylation at their promoter regions. We conclude that epigenetic modifications play an important role in TGF-β-induced EMT in LECs and that TSA may therefore be helpful in suppressing fibrosis. The limitation of the present study was that it was carried out using only the FHL124 cell line. Further studies using *in vivo* models may provide insight into the selectivity of TSA and ultimately develop it as a promising drug for treatment of lens fibrosis.

Acknowledgement

The authors will like to thank Dr. KaidJohar for his general support.

Financial Support and Sponsorship

This work was funded by the WOS—A scheme of the Department of Science and Technology (SR/WOS-A/LS-272/2010).

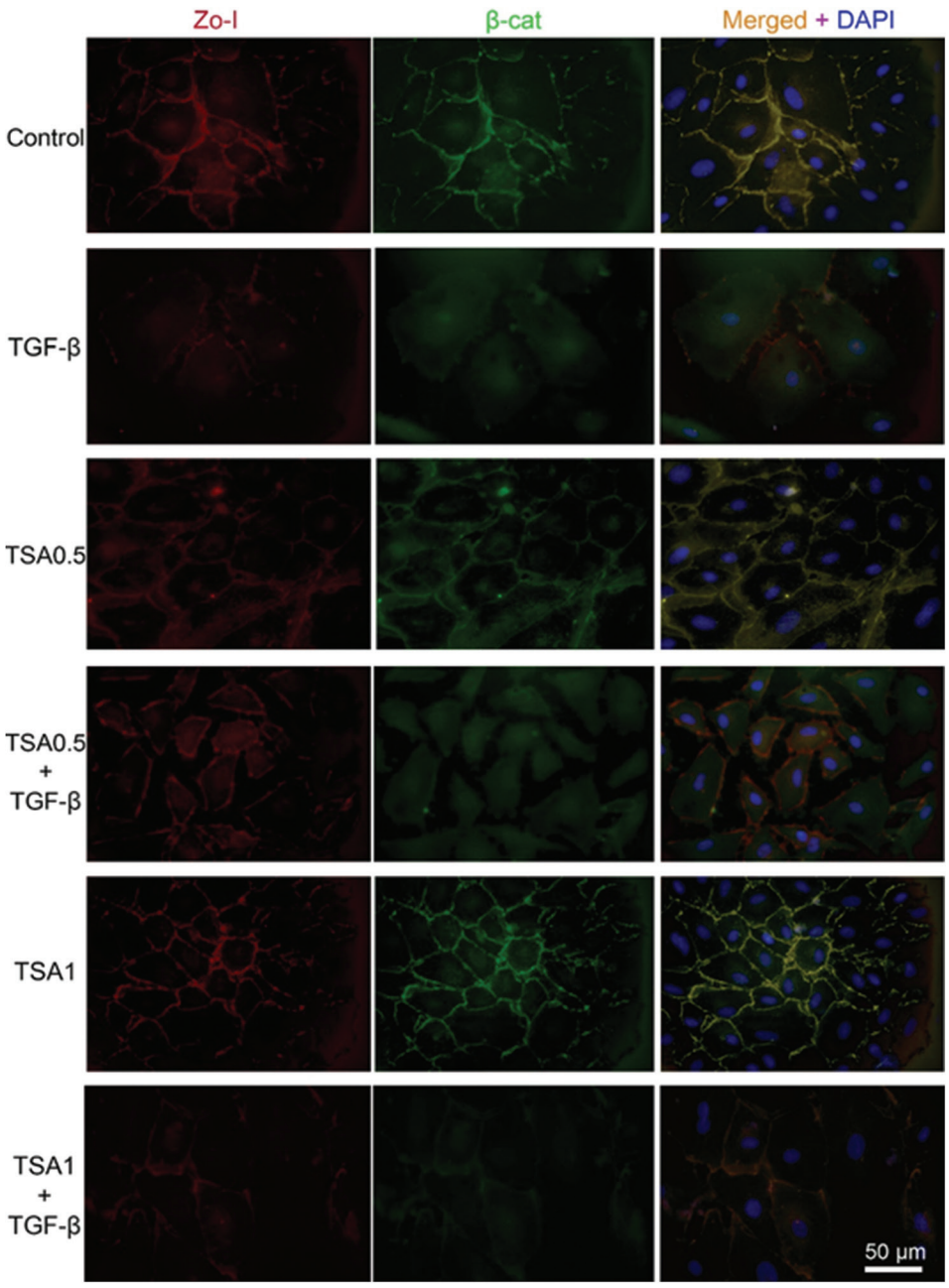

Figure 8. Immunolocalization of ZO-1 and β-cat in FHL124 cells. The polarity marker ZO-1 is immunostained in red, β-cat in green, and nuclei are stained blue with DAPI. The bar shown is 50 µm.

Conflict of Interest

There are no conflicts of interest.

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