

Association of histone acetylation at the *ACTA2* promoter region with epithelial mesenchymal transition of lens epithelial cells

DA Ganatra, S Rajkumar, AR Patel, DU Gajjar, K Johar, AI Arora, FB Kayastha and AR Vasavada

Abstract

Purpose Epithelial mesenchymal transition (EMT) plays a central role in the development of fibrotic complications of the lens. The current study is designed to check whether EMT of lens epithelial cells (LECs) is regulated by epigenetic modifications and to evaluate the effect of Trichostatin-A (TSA) on the transforming growth factor- β (TGF- β)-induced EMT.

Methods Fetal human LECs (FHL124) were treated with TGF- β 2 in the presence or absence of TSA. Levels of mRNA, protein, as well as localization of α -smooth muscle actin (α SMA) were studied along with migration of LECs. Acetylation of histone H4 was analyzed and chromatin immunoprecipitation (ChIP) was carried out to study the level of acetylated histone H4 at the promoter of α SMA gene (*ACTA2*). Student's *t*-test was used for statistical analysis.

Results TGF- β 2 treatment resulted in myofibroblast-like changes and increased migratory capacity of FHL124. Protein and mRNA expression of α SMA increased, and immunofluorescence revealed presence of extensive stress fibers. TSA treatment preserved epithelial morphology, retarded cell migration, and abrogated an increase in α SMA levels. TSA led to the accumulation of acetylated histone H4 that was reduced on TGF- β 2 treatment. However, increased level of histone H4 acetylation was found at the *ACTA2* promoter region during TGF- β treatment.

Conclusions The increased level of α SMA, a hallmark of EMT in LECs, is associated with increased level of histone H4 acetylation at its promoter region, and TSA helps in

suppressing EMT by epigenetically reducing this level. TSA thus shows promising potential in management of fibrotic conditions of the lens.

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Introduction

Epithelial mesenchymal transition (EMT) is a fundamental process that is critical during embryogenesis. However, it is also involved in fibrotic conditions of the kidney, liver, lung, and several ocular diseases. EMT leads to conversion of epithelial cells into spindle-shaped myofibroblast-like cells through downregulation of epithelial markers and upregulation of mesenchymal markers like α -smooth muscle actin (α SMA).¹ Transforming growth factor- β (TGF- β) can mediate these EMT-related changes and it is considered a key inducer of EMT in human lenses involved in the development of subcapsular cataracts and posterior capsular opacifications (PCO).^{2–4} Cataracts are currently the leading cause of blindness worldwide and PCO is the most common postoperative complication of cataract surgery that causes visual loss in adults and pediatric cases.^{5–8} Therefore, there is a definite need for better preventive measures for PCO.

Epigenetic modifications can lead to chromatin remodeling, affect gene expression, and have been shown to regulate EMT in different cell types.^{9–12} Histone (de) acetylation is one such epigenetic mechanism that controls transcription. Opposing effects of histone acetyltransferases (HATs) and their counterpart histone deacetylases (HDACs) mediate histone acetylation. Histones are compactly arranged around the DNA and acetylation by HAT

Cell and Molecular Biology,
Iladevi Cataract and IOL
Research Centre,
Ahmedabad, India

Correspondence:
AR Vasavada, Iladevi
Cataract and IOL Research
Centre, Gurukul road,
Memnagar, Ahmedabad,
Gujarat 380052, India
Tel: +91 79 27492303;
Fax: +91 79 27411200.
E-mail: icirc@
abhayvasavada.com

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dissociates the histone–DNA complex, thereby opening it up to transcription factors.¹³ On the other hand, HDACs remove the acetyl group from histones, condensing the chromatin and reducing gene expression. The role of HDACs in mediating myofibroblast differentiation driven by TGF- β has been previously documented using different cell types.^{10–12,14}

Several HDAC inhibitors (HDACis) have been developed that block the catalytic reaction of HDAC, leading to hyperacetylation of histones. Trichostatin-A (TSA), an HDACi, is a fermentation product derived from *streptomyces* species and is a potent and specific inhibitor of HDACs.¹⁵ In recent years, HDACis like TSA have received special attention as potential antifibrogenic agents for different diseases such as nasal polyposis, idiopathic pulmonary fibrosis, and chronic liver diseases.^{10,11,16} Several *in vivo* and *in vitro* studies on abrogation of TGF- β -mediated fibrosis of ocular surface tissues like cornea^{17,18} and subconjunctiva¹⁹ have also used TSA. Therefore, TSA shows promising potential as an antifibrotic agent and there is need for better understanding of its mode of action. In a recent study, TSA was found to suppress proliferation and EMT of lens epithelial cells (LECs) using SRA01/04 and HLEB3 lines.²⁰ As transdifferentiated LECs show increased expression of α SMA, a marker for subcapsular cataracts and PCO,^{3,4,21,22} the current study was designed to study the effect of histone acetylation by TSA on α SMA and the changes that take place at its promoter region during TGF- β -induced EMT of LECs.

Materials and methods

LEC culture and treatments

The fetal human LEC line (FHL124) (a kind gift from Professor John Reddan, Oakland University, Rochester, MI, USA) was cultured using Eagle's minimum essential media (EMEM) (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Himedia, Mumbai, India) and 50 μ g/ml gentamycin at 35 °C in a humidified atmosphere with 5% CO₂. Passages 15–18 were used for the study. FHL124 was authenticated by short tandem repeat (STR) profiling using AmpFISTR identifier PCR amplification kit (LifeTechnologies, Carlsbad, CA, USA). STR analysis was performed over 16 marker panel for human identification. Peaks were obtained for the autosomal markers as well as two peaks for X and Y (Supplementary Figure 1), confirming that the cell line is human and of male origin.

At 70% confluence, the cells were serum starved for 24 h. This was followed by 24 h of treatment. EMT was induced by exposing the culture to 10 ng/ml of TGF- β 2 (Invitrogen, Carlsbad, CA, USA). TSA (Sigma-Aldrich)

was given either alone or along with TGF- β 2 treatment. Cell morphology after each treatment was examined using an inverted microscope (Axiovert 200, Carl Zeiss, Göttingen, Germany) and photographed. After treatment, the cells were washed with phosphate buffer saline (PBS) and collected for subsequent experiments.

Cell viability assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was carried out to study the effect of TSA on the viability of LECs. In this method, MTT (Himedia), a tetrazolium compound, is converted by the active mitochondrial dehydrogenases present in viable cells, into purple formazan crystals that can be measured by spectrophotometry. One hundred microliter media containing 7×10^3 cells were added in each well of a 96-well plate and incubated for 24 h. The cells were then treated with either a vehicle or a wide range of TSA concentrations (0.1–5 μ M) for an additional 24 h. Thereafter, 20 μ l of MTT (5 mg/ml in PBS) was added to each well and incubated for 4 h. Medium containing MTT was removed from the wells and 100 μ l of dimethyl sulfoxide (DMSO) was added. The plates were agitated for 10 min and absorbance was read at 570 nm. The half-maximal inhibitory concentration (IC₅₀) of TSA was determined.

Cell migration assay

A confluent monolayer of LECs was serum starved for 24 h and cell migration was studied by wound healing assay. The center of each well was scratched using a 200 μ l sterile tip. Thereafter, the wells were rinsed with PBS to remove suspended cells and treated with TGF- β 2 and/or TSA for 24 h. The wound was photographed under an inverted microscope before and after the treatment and cell migration was analyzed. In all, 5 microscopic fields were analyzed and a quantitative assessment of the migration rate was done by counting the number of cells that had migrated onto the wound after 24 h.

Immunofluorescent staining

Proteins of interest were localized as previously described.²³ Briefly, fixed cells were permeabilized using 0.25% Triton-X 100 (Sigma-Aldrich) and 0.25% saponin (Sigma-Aldrich). Nonspecific sites were blocked using 5% goat serum in PBS. The cells were incubated with 1 : 200 dilution of a monoclonal antibody against α SMA (Sigma-Aldrich) or polyclonal anti-acetyl histone H4 antibody (Millipore, Temecula, CA, USA) at 4 °C overnight. After washing the cells with PBST (PBS + Tween-20), they were probed with a corresponding secondary antibody tagged with either Alexa Fluor 488 or

Alexa Fluor 546 (Invitrogen) for 1 h at 37 °C. DAPI (Sigma-Aldrich) was used to counterstain the nuclei. A fluorescence microscope (Axioskope II, Carl Zeiss, Oberkochen, Germany) was used to visualize the proteins and the images were captured using a Cohu cool CCD camera (Cohu, San Diego, CA, USA).

Real-time reverse transcription-PCR (RT-PCR)

Total RNA was extracted from LECs using TRIzol reagent (Invitrogen). Then, 4 µg of total RNA was reverse transcribed using 250 ng oligo (dT) (Merck, Genei, Bangalore, India), 250 ng random hexamer (Merck), 10 mM dNTPs (Merck), 4 µl 5× reaction buffer (Merck), 20 U Recombinant RNasin ribonuclease inhibitor, and 20 U of reverse transcriptase (Merck) in a 20 µl reaction according to the manufacturer's instructions. Quantitative real-time PCR (qPCR) was carried out with 20 µl of the reaction mixture (100 ng of cDNA, 2× SYBR green (Roche Diagnostics GmbH, Mannheim, Germany), 2.5 µM of forward and reverse primers) using LightCycler480 (Roche Diagnostics GmbH) at 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and at 60 °C for 60 s. Quantitative PCR was carried out for *ACTA2* (α SMA) (forward primer: 5'-CCAGAGCCATTGTCACACAC-3', reverse primer: 5'-CAGCCAAGCACTGTCAGG-3') with *ACTB* (β -actin) (forward primer: 5'-GTTGTGCGACGACGAGCG-3', reverse primer: 5'-GCACAGAGCCTCGCCTT-3') used as a housekeeping gene. The relative expression of the gene was calculated by normalizing the cycle threshold (Ct) values of the target gene with that of the housekeeping gene.

Western blot analysis

Western blot analysis was carried out to measure protein levels of α SMA. The cells were collected in cell lysis buffer (50 mM Tris (pH 7.4), 150 mM sodium chloride, 1% Triton-X 100, 0.1% SDS, 0.5% sodium deoxycholate, protease and phosphatase inhibitor cocktail (Roche Diagnostics GmbH)). The final protein concentration was quantified using a BCA assay kit (Pierce Biotechnology, Rockford, IL, USA). An equal amount of protein (20 µg) was resolved using 12% SDS-PAGE. The proteins were then transferred onto a nitrocellulose membrane (Pall Corporation, Port Washington, NY, USA). The membranes were blocked with 5% skimmed milk in TBST (10 mM Tris-HCl (pH 7.6), 150 mM sodium chloride, 0.05% Tween-20) for 1 h followed by overnight incubation in primary antibody against α SMA (Sigma-Aldrich) (1 : 2000 dilution) at 4 °C. After washing with TBST, the membranes were probed with a horseradish peroxidase (HRP)-conjugated secondary antibody (Abcam, Cambridge, MA, USA) (1 : 2500 dilutions). Blots were

developed by chemiluminescence using ECL western blotting substrate (Pierce Biotechnology). In order to use the same blot for other proteins, it was stripped using a mild stripping buffer (0.2 M glycine, 0.1% SDS, 1% Tween 20, pH 2.2). After washing with PBS and TBST, the blot was blocked and reprobated with primary antibody against β -actin and the above procedure was repeated. Band intensity was determined using Image J software (NIH image, Bethesda, MD, USA).

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was carried out in accordance with an earlier reported method²⁴ to check the level of acetylated histone H4 (AcH4) at the *ACTA2* promoter region. Briefly, treated cells as well as controls were incubated with 1% formaldehyde for 10 min in order to crosslink histones to the DNA. The cells were lysed and chromatin was sheared between 400 and 600 bp using Branson SLPe150 (Branson Ultrasonics, Kowloon, Hong Kong). Then, 20 µg of DNA was used for immunoprecipitation with an antibody against acetyl histone H4 (Millipore) and normal rabbit IgG (Cell Signaling Technology Inc., Danvers, MA, USA) at 4 °C overnight. After incubation with Salmon sperm DNA and protein A agarose slurry (Millipore) for 3 h at 4 °C, the antibody and histone complex along with the slurry was pelleted. The pellet was washed with 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, 1× protease inhibitor cocktail) and TE buffer and then incubated with elution buffer (1% SDS, 0.1 M sodium bicarbonate, and 10 mM DTT) for 1 h at room temperature. The DNA-histone complex was then decrosslinked by adding 5 M sodium chloride and incubated at 65 °C overnight. Simultaneously, 1% inputs of all the samples were decrosslinked. DNA was deproteinized using the phenol/chloroform/isoamylalcohol extraction method. qPCR was carried out for the *ACTA2* promoter region (forward primer 5'-ATTCCTATTTCCACTCAC-3' and reverse primer 5'-ACTTGCTTCCCAAACA-3'). The relative DNA levels in each immunoprecipitated group were normalized to the input DNA level.

Statistical analysis

Each treatment set was repeated thrice. Results were reported as mean ± SE. Student's *t*-test was used for statistical analysis and *P*-values of <0.05 were considered significant.

Results

Cell viability of LECs after TSA treatment

A wide range of TSA concentrations (0.1–5 μM) were used to study its cytotoxicity on FHL124 cells by MTT assay. The proportion of viable cells was calculated as percentage of control absorbance. TSA led to a dose-dependent decrease in cell viability and IC_{50} for TSA was calculated to be 2.5 μM .

TSA inhibits TGF- β 2-induced morphological changes in LECs

Cell morphology was analyzed under an inverted microscope (Figure 1). Cultured LECs without treatment look cuboidal, but when exposed to TGF- β 2, the cells become elongated and spindle shaped. TSA treatment alone showed cells with morphology similar to that of control group. On cotreatment, TSA suppressed the morphological changes induced by TGF- β 2 to a great extent and maintained them in the epithelial phenotype. F-actin localization was carried out (see Supplementary Figure 2) and cell density was calculated after chromatin staining with DAPI (see Supplementary Figure 3).

TSA prevents migration of LECs during wound healing

Representative images of wound healing, 24 h after the scratch, are shown in Figure 2a. FHL124 cells possess migratory capacity in serum-free conditions but show delayed wound healing as compared with the TGF- β 2-treated group in which the cells rapidly migrated and covered the wound area after 24 h (Figure 2b). Conversely, treatment with TSA in the presence of TGF- β 2 succeeded in significantly restricting the migration of LECs into the wound as compared with the TGF- β 2-treated group. Cell migration was inhibited up to 48%, resulting in reduced wound healing.

TSA inhibits TGF- β 2-induced upregulation of αSMA

Immunofluorescence staining (Figure 3) revealed αSMA present as fibrous projections in the TGF- β 2-treated group with enhanced staining intensity as compared with the control. TSA-treated groups showed almost control-like arrangement with weakened staining and complete reduction in stress fibers. Cotreated groups had a heterogeneous population of cells that showed both fiber-like and diffused arrangement of the protein.

αSMA was quantified at the mRNA and protein levels during EMT and after TSA treatment. TGF- β 2 upregulated αSMA mRNA by fourfold as compared with the control group. This increase was abrogated by treatment of TSA. During EMT, TSA at 0.5 and 1 μM

concentrations was able to downregulate the increased gene expression ($P < 0.05$ and $P < 0.01$, respectively; Figure 4a). Western blotting showed similar results with TGF- β 2 leading to increased protein levels and TSA suppressing TGF- β 2-induced increase of αSMA (Figure 4b and c). Cotreatment with 0.5 and 1 μM TSA decreased protein concentration by 50% and 60%, respectively, as compared with the TGF- β 2 group.

TSA increases the level of histone H4 acetylation

As TSA is known to inhibit HDACs and increase acetylation status, we analyzed histone H4 acetylation by means of immunofluorescence staining. TSA-treated samples showed hyperacetylation of histone H4 with both the concentrations as compared with the control group (Figure 5a). However, the intensity of the nuclear protein was considerably reduced on TGF- β 2 treatment alone. In the cotreated groups, TSA led to accumulation of acetylated histone H4 in most of the cells.

Level of histone H4 acetylation increases during EMT at the αSMA promoter region

In order to check whether the level of acetylation is related with the transcriptional activity of αSMA , ChIP was carried out. We examined the promoter region 500 bp upstream of *ACTA2* and its involvement with acetylated histone H4. Ct values were not obtained for IgG precipitated samples. As compared with the controls, the level of acetylation at the promoter region increased significantly ($P < 0.01$) during EMT (Figure 5b). Cotreatment of TSA during EMT suppressed the involvement of histone H4 acetylation with the *ACTA2* promoter region ($P < 0.01$).

Discussion

Of the three TGF- β isoforms in mammals, TGF- β 2 is the predominant form in the eye.²⁵ In the present study, TGF- β 2 was used to induce EMT-like changes in the human LEC line (FHL124). FHL124 exhibits 99.5% homology with native human LECs and expresses LEC-specific marker FoxE3 as well as phenotypic markers α -A-crystallin and pax6.²⁶ As epigenetic mechanisms have been shown to regulate EMT,^{9,11,20} we checked the effect of histone acetylation by TSA on FHL124 during EMT. IC_{50} of TSA was determined and lower concentrations of 0.5 and 1 μM were used for further experiments without compromising cell survival.

αSMA is known to increase during EMT,^{2-4,11,18} and we found similar results after TGF- β 2 treatment. The αSMA stress fibers observed in our study during TGF- β 2

treatment are also present in spindle-shaped cells of donor lenses with anterior subcapsular fibrosis.²¹ TSA cotreatment suppressed morphological changes induced by TGF- β 2. It was also effective in abrogating the increased level of α SMA during EMT and disturbed its stress fiber arrangement. Our data are consistent with previous studies where TSA caused a significant decrease in the expression of other profibrotic

genes,^{11,17-20} thereby suggesting the antifibrogenic properties of TSA.

Migration of residual LECs along with TGF- β 2-induced EMT is a wound healing response triggered by cataract surgery and plays a crucial role in the development of PCO. Hence, we examined the migratory capacity of cells during EMT and after TSA treatment by wound assay. α SMA expression correlates with matrix contraction

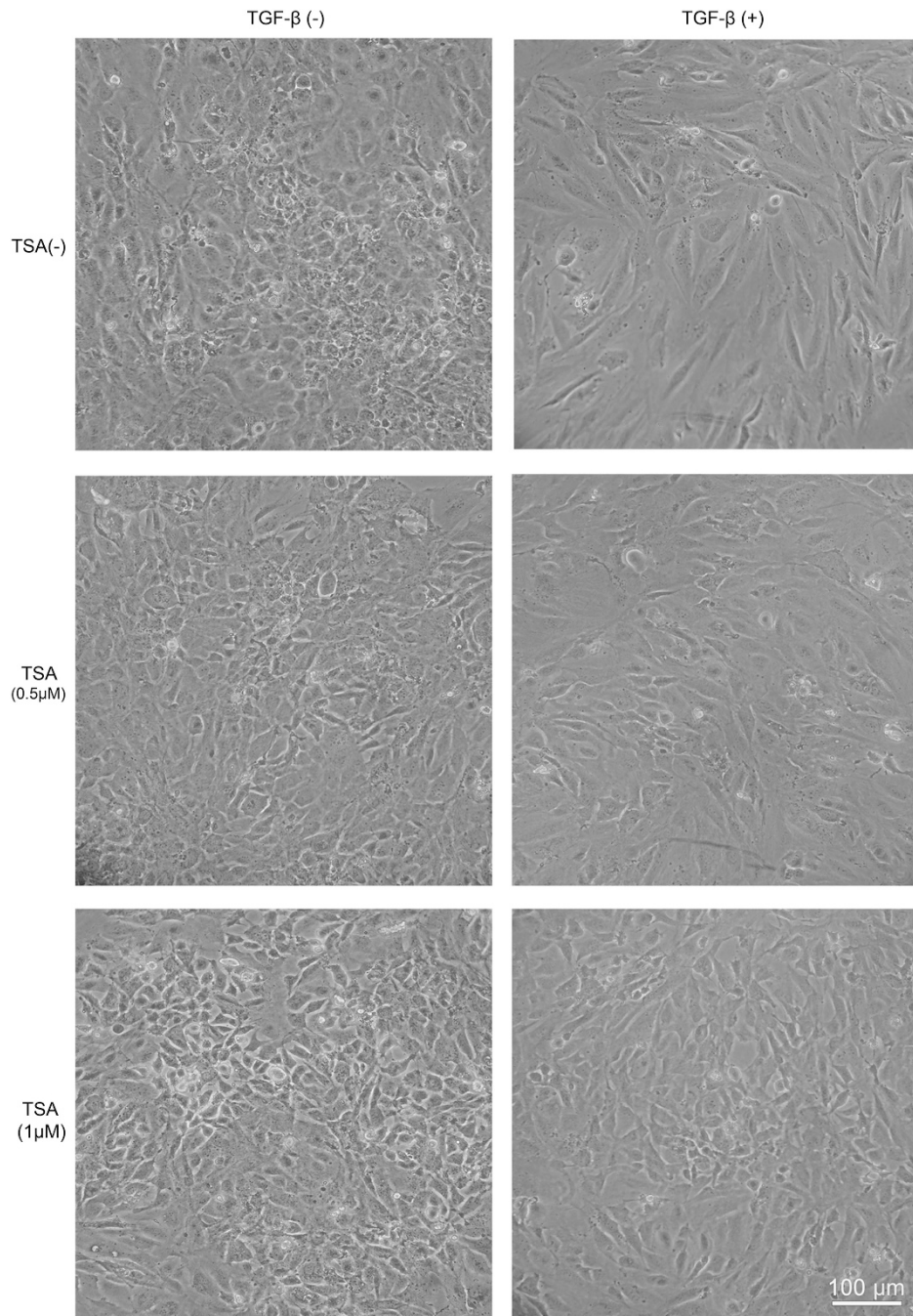


Figure 1 TSA inhibits TGF- β 2-induced morphological changes in FHL124 cells. TSA was used at two concentrations (0.5 and 1 μ M), given either alone or alongside 10 ng/ml TGF- β 2.

necessary for wound healing,^{27,28} and we found increased migration of lens cells during EMT coinciding with the higher expression of α SMA and prevalence of increased

stress fiber arrangement. Myofibroblasts are central to wound healing and α SMA expressed by these myofibroblasts can increase cell adhesion necessary for

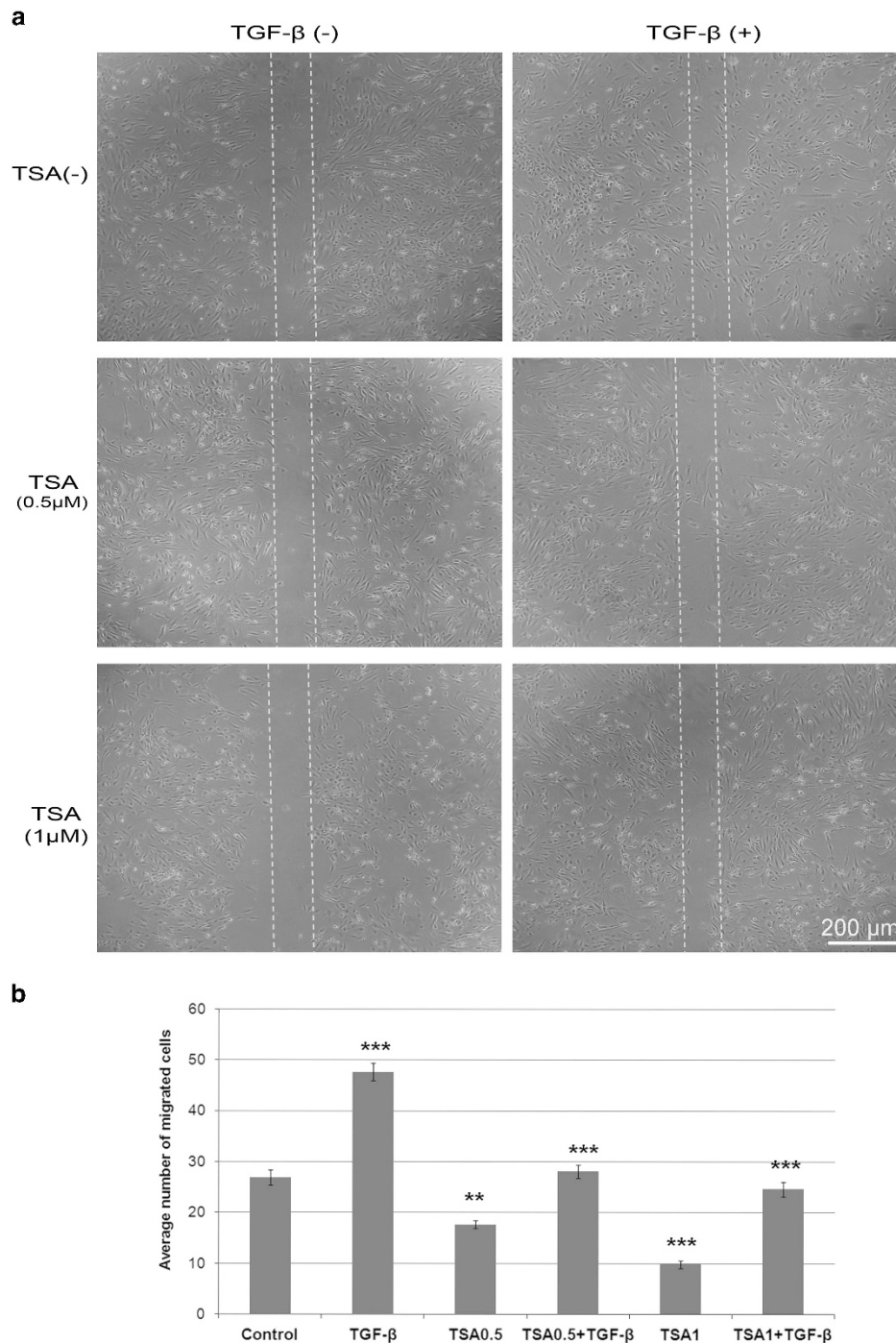


Figure 2 TSA blocks migration of wounded FHL124 cells. Confluent cells on the coverslip were scratched with a 200 μ l tip and treated with TGF- β 2 (10 ng/ml) and/or TSA. (a) The migration of cells into the wound was examined under an inverted microscope. The broken white line in the image shows the wounded edge. (b) The bar graph represents the average number of cells that have migrated into the wound area for each treatment group. TGF- β 2 treatment led to increased migration (*** P < 0.001). TSA at 0.5 and 1 μ M concentrations significantly inhibited migration (** P < 0.01 and *** P < 0.001, respectively) as compared with the control. On cotreatment with TGF- β 2, TSA significantly blocked migration of LECs (*** P < 0.001) as compared with TGF- β 2 treatment alone and kept the wound area wide.

cell spreading and migration of cells, thus playing an important role in the wound healing response.^{29–31} According to Zhou *et al*,¹⁷ reduced expression of α SMA

and disturbed stress fiber arrangement is responsible for reduced migration of cells. In our study, during wound healing, TSA treatment prevented migration of cells and this

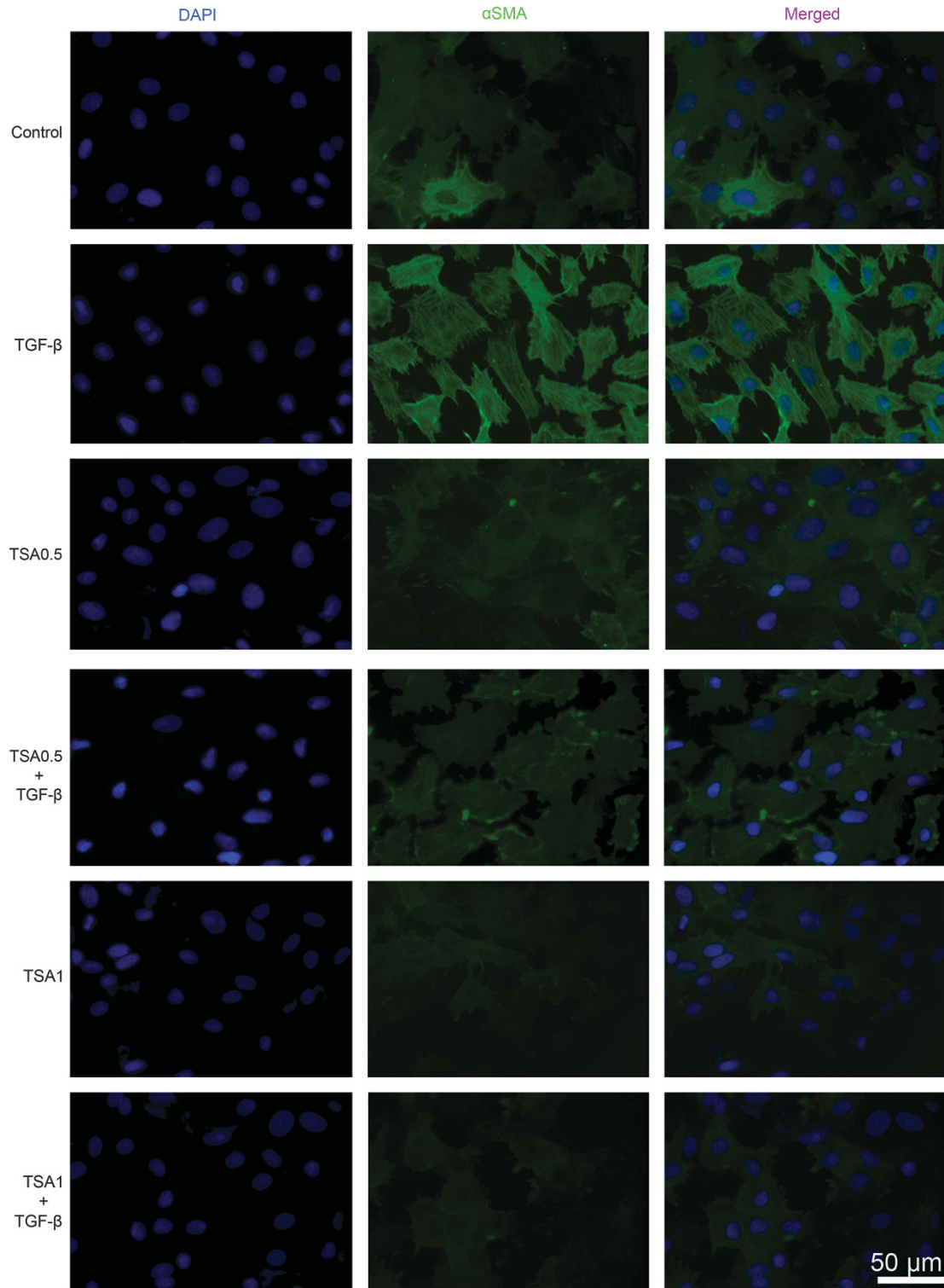


Figure 3 TSA disturbs localization of α SMA during EMT. Lane 1 shows the nuclei stained with DAPI (blue) in all the groups, lane 2 shows localization of α SMA (green), and lane 3 shows merged images of DAPI and α SMA. Bar = 50 μ m.

could be because of the reduction in expression levels of α SMA and disruption of stress fibers on TSA treatment. This indicates the importance of TSA as an antimigratory agent.

Chen *et al*²⁰ reported increased histone H4 acetylation with TSA in LECs but it was specific for Lys8. In our study, we looked at global acetylation of histone H4 and found that during EMT this level reduced significantly whereas TSA treatment led to hyperacetylation. We then checked the level of global histone H4 acetylation at the *ACTA2* promoter region that showed an upregulated transcript level during EMT. For this, a region 500 bp upstream of the gene was analyzed by qPCR after ChIP assay. We found that although the level of global H4 acetylation decreased during EMT, this level was significantly higher at the *ACTA2* promoter region. This opened chromatin explains the increased expression of α SMA on TGF- β 2 treatment. TSA, on the other hand, led to decreased levels of H4 acetylation at the promoter region and this probably caused TSA to significantly inhibit TGF- β 2-induced α SMA expression at the transcript level. Similar results were reported by Cho *et al*¹¹ using

nasal polyp-derived fibroblasts where TSA significantly reduced the opening of the α SMA promoter. HDACi prevents removal of acetyl groups from histones, leading to increased acetylation that is associated with transcriptionally active chromatin.¹³ We found that histone deacetylase inhibition by TSA led to hyperacetylation of H4 and this increase was accompanied by inhibition of α SMA synthesis. However, the question arises as to how TSA strongly suppresses expression of myofibroblast marker α SMA in spite of increasing histone acetylation. For this, several possible mechanisms have been suggested. TSA might interfere with the Smad pathway that is considered to be primarily involved in TGF- β signal transduction from the receptor to the nucleus and responsible for inducing EMT.^{11,20} TSA can also lead to up-regulation of genes that can interfere with the signaling pathway leading to fibrosis like the inhibitory Smad, Smad7, and co-repressor of Smad, TGIF.^{9,19} Alternatively, TSA might abrogate EMT by downregulating other pathways like Notch, MAPK, and PI3K pathways.^{12,20}

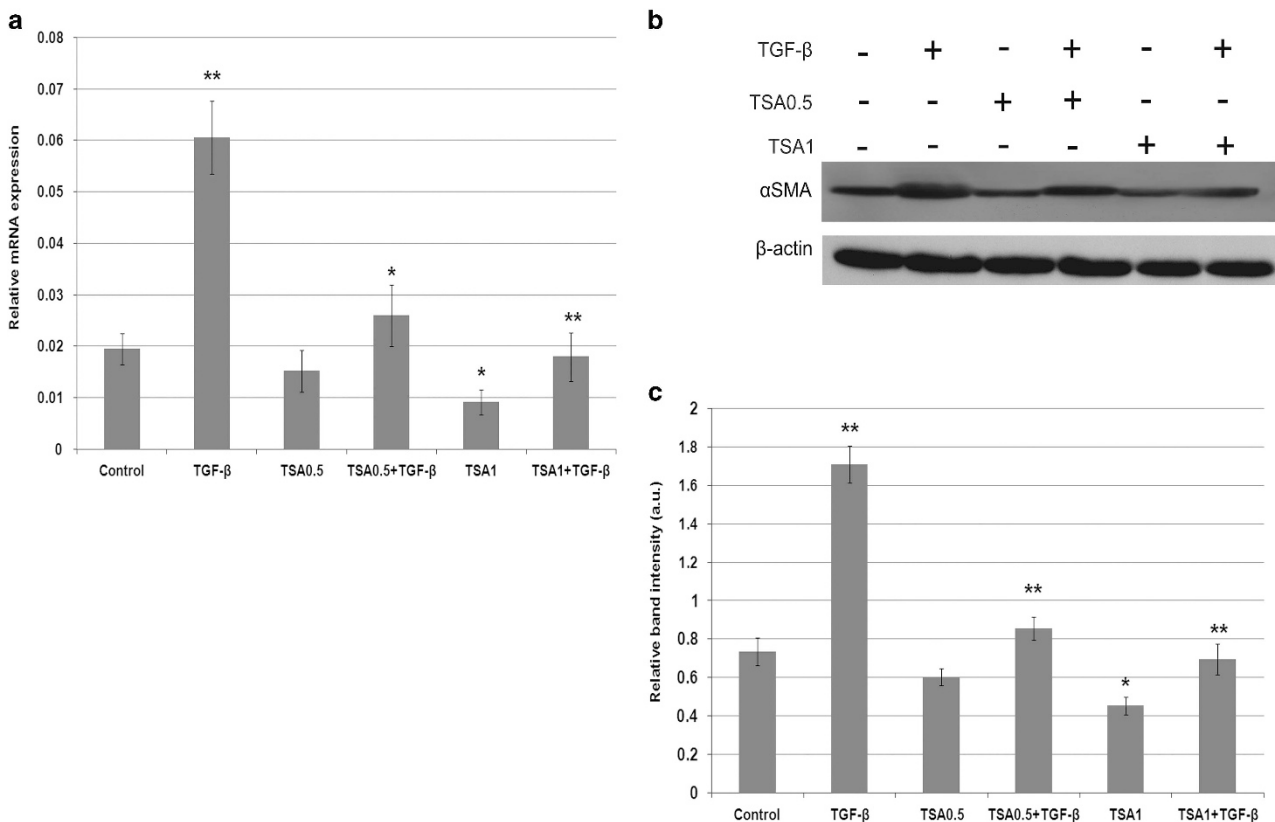


Figure 4 TSA abrogates the level of α SMA during EMT in FHL124 cells. (a) Gene expression of α SMA was quantified by real-time PCR after each treatment. TGF- β 2, TSA0.5, and TSA1 are compared with control and cotreated groups are compared with TGF- β 2-treated group. * $P < 0.05$ and ** $P < 0.01$. (b) Whole-cell extracts were prepared and western blot analysis of α SMA was carried out. β -Actin served as the loading control. (c) A graphical representation of relative α SMA protein levels (a.u. = arbitrary units). TGF- β 2 increased α SMA protein levels (** $P < 0.01$) as compared with the control. TSA at 1 μ M decreased the α SMA protein levels versus the control (* $P < 0.05$). Cotreatment of TSA with TGF- β 2 significantly reduced levels of α SMA by (** $P < 0.01$) compared with TGF- β 2.

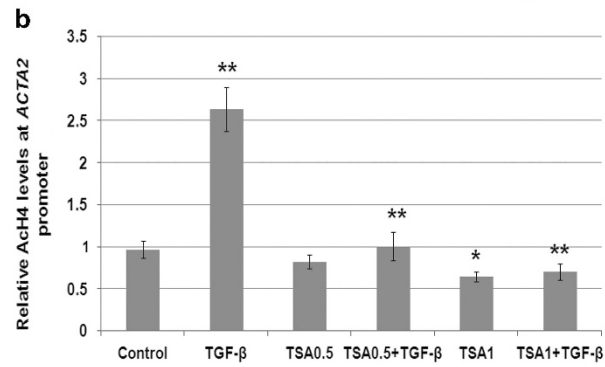
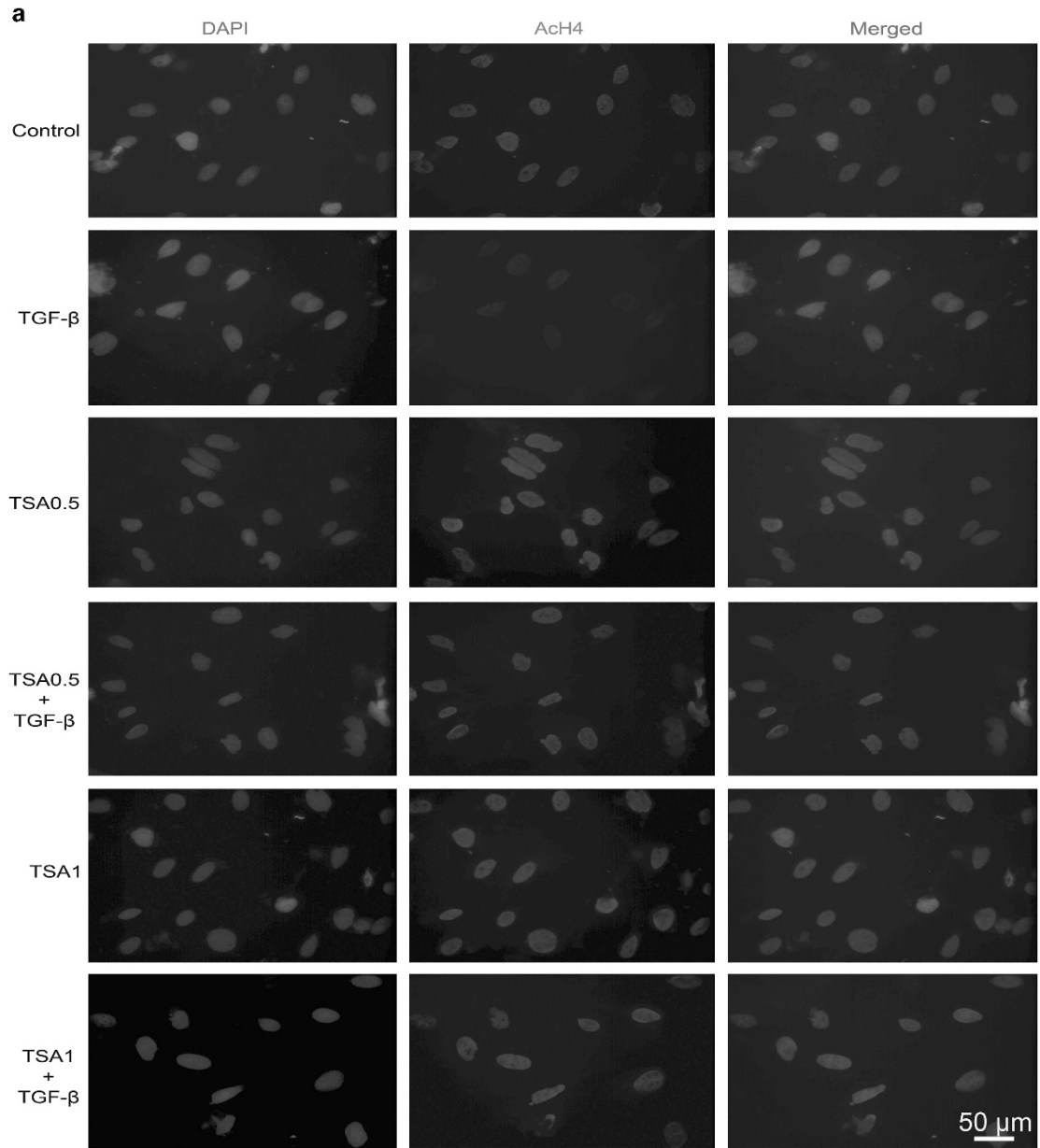


Figure 5 Status of histone H4 acetylation (AcH4) in FHL124 cells. (a) Representative fluorescent images are shown where lane 1 shows the nuclei stained with DAPI (blue) in all the groups, lane 2 shows localization of AcH4 (red), and lane 3 shows merged images of DAPI and AcH4. Bar = 50 μm . (b) Level of AcH4, 500 bp upstream of the *ACTA2* promoter region, was analyzed using ChIP and qPCR. The figure shows relative levels of AcH4 normalized with 1% input in different experimental groups. TGF- β 2 treatment led to significant upregulation of the AcH4 level at α SMA promoter (** $P < 0.01$) as compared with the control. TSA at 1 μM , but not 0.5 μM , significantly reduced acetylation status versus the control group (* $P < 0.05$). Cotreatment of FHL124 with TSA and TGF- β 2 inhibited increase in acetylation levels at the α SMA promoter (** $P < 0.01$) as compared with treatment with only TGF- β 2. A full color version of this figure is available at the *Eye* journal online.

TSA is emerging as a strong therapeutic candidate for many inflammatory diseases as well as cancer.³² Our results clearly suggest that epigenetic modifications are involved during myofibroblastic differentiation of LECs and TSA suppresses the main features of EMT induced by TGF- β , in terms of preserving the morphological features, reducing migratory capacity of cells, and inhibiting increased α SMA expression. We can conclude that the increased gene expression of α SMA during EMT is associated with the increased acetylation status at its promoter region and TSA epigenetically reduces this level and suppresses myofibroblastic differentiation of LECs. Our study along with other *in vivo* and *in vitro* studies thus show the promising potential of TSA in management of vision compromising fibrosis of cornea,^{17,18} subconjunctiva,¹⁹ and lens conditions like subcapsular cataracts and PCO. However, the precise mechanism by which TSA exerts these antifibrotic effects needs to be further elucidated.

Summary

What was known before

- Levels of α SMA increases during EMT.
- TSA is a known inhibitor of histone deacetylases.

What this study adds

- TSA reduces α SMA levels and helps in suppressing EMT of LECs.
- Acetylation of histone H4 is increased at the α SMA promoter region during EMT.

Conflict of interest

The authors declare no conflict of interest.

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