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Original Article

Role of TGF-β1/SMADs signalling pathway in resveratrol-induced reduction of extracellular matrix deposition by dexamethasone-treated human trabecular meshwork cells

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ABSTRACT Deposition of extracellular matrix (ECM) in the trabecular meshwork (TM) increases aqueous humour outflow resistance leading to elevation of intraocular pressure (IOP) in primary open-angle glaucoma, which remains the only modifiable risk factor. Resveratrol has been shown to counteract the steroid-induced increase in IOP and increase the TM expression of ECM proteolytic enzymes; however, its effects on the deposition of ECM components by TM and its associated pathways, such as TGF-β-SMAD signalling remain uncertain. This study, therefore, explored the effects of *trans*-resveratrol on the expression of ECM components, SMAD signalling molecules, plasminogen activator inhibitor-1 and tissue plasminogen activator in dexamethasone-treated human TM cells (HTMCs). We also studied the nature of molecular interaction of *trans*-resveratrol with SMAD4 domains using ensemble docking. Treatment of HTMCs with 12.5 μM *trans*-resveratrol downregulated the dexamethasone-induced increase in collagen, fibronectin and α-smooth muscle actin at gene and protein levels through downregulation of TGF-β1, SMAD4, and upregulation of SMAD7. Downregulation of TGF-β1 signalling by *trans*-resveratrol could be attributed to its effect on the transcriptional activity due to high affinity for the MH2 domain of SMAD4. These effects may contribute to resveratrol's IOP-lowering properties by reducing ECM deposition and enhancing aqueous humour outflow in the TM.

INTRODUCTION

Glaucoma is a neurodegenerative illness involving the optic nerve and is currently the leading cause of irreversible visual loss, worldwide. The most prevalent type, primary open-angle glaucoma (POAG) is clinically and pathologically akin to steroid-induced glaucoma, except for the history of steroid use. Elevated intraocular pressure (IOP) in both these types of glaucomas is associated with enhanced resistance to the aqueous humour (AH) drainage at the trabecular meshwork (TM) [1]. Considering these

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similarities, both glaucomas are treated in similar ways [2]. These similarities have also led to the utilisation of steroid-induced *in vitro* and *in vivo* models for glaucoma-related research including drug development, drug delivery and mechanisms of action [3,4].

Under physiological conditions, changes in the IOP are sensed as signals by the TM tissue, and TM cells respond to these changes by modulating resistance across the aqueous outflow facility by modifying the turnover of extracellular matrix (ECM) in TM. ECM is a highly dynamic structure comprised of non-cellular elements such as proteoglycans, collagens (COL) and fibronectin (FN) [5] and its accumulation in TM is linked to IOP elevation in glaucoma [6,7]. Maintaining ECM homeostasis largely refers to striking a balance between its synthesis and degradation.

Since elevated IOP is the most important and the only adjustable risk factor for glaucoma development and progression, its reduction continues to be the only therapeutic target for antiglaucoma agents [8]. Currently available antiglaucoma medications primarily reduce aqueous production or augment its minor outflow pathway, the uveoscleral route. Since a larger fraction of the AH exits the body *via* the TM routes, drugs that can lessen ECM deposition are presently the main focus of research.

TGF-β regulates many cellular processes including ECM remodelling and is known to be involved in the pathogenesis of POAG [9-11]. TGF-β1 level is elevated in the AH of patients with POAG [12] and has recently been observed to correlate positively with the IOP [13]. TGF-β also influences the level of plasminogen activator inhibitor (PAI), a specific inhibitor of the active form of tissue plasminogen activator (tPA). Increased PAI level in TM [14] and retinal glial cells [15] inhibits the activity of enzymes responsible for ECM degradation. TGF-β ligands activate this downstream signalling *via* SMAD molecules. This SMAD-dependent signalling is associated with ECM deposition and the pathogenesis of glaucoma [16]. Therefore, this pathway has attracted attention as a potential target for new antiglaucoma drug development.

Resveratrol is a dietary polyphenol which can be obtained from grapes, pines, and berries. Resveratrol has been shown to attenuate ECM deposition, including COL, FN and α-smooth muscle actin (α-SMA) in various tissues [17-19]. It has also been found to lower IOP in rats with normal IOP and steroid-induced ocular hypertensive rats by increasing the matrix metalloproteinases (MMP) level and reducing the TM thickness [20-22]. Additionally, treatment of primary human trabecular meshwork cells (HTMCs) with *trans*-resveratrol after exposure to dexamethasone has been demonstrated to significantly increase the expression of MMPs, enzymes responsible for ECM degradation [23]. However, whether treatment with resveratrol also reduces the expression of ECM proteins at gene and protein levels by TM cells remains unclear. Hence, we investigated if *trans*-resveratrol affects the ECM protein expression by steroid-treated primary HTMCs and possible involvement of TGF-β-SMAD signalling pathway. Since SMAD4, the co-SMAD, that has homology with receptor-regulated SMADs (R-SMADs) for amino and carboxyl terminals, is the most important regulator of the TGF-β signalling [24], we also performed ensemble docking experiments to understand the nature of molecular interaction of *trans*-resveratrol with SMAD4 domains.

METHODS

Cell culture

Primary cells of HTMCs, removed from the juxtacanalicular and corneoscleral regions of human eye were obtained from the ScienCell Research Laboratories. They were cultured in low glucose Dulbecco's modified Eagle medium (DMEM) (Gibco, Life Technologies). The media was also complemented with 10% fetal bovine serum (Gibco, Life Technologies) together with 1% of penicillin/streptomycin (Gibco, Life Technologies). Dexamethasone was obtained from Enzo Life Science , dimethyl sulfoxide (DMSO) and *trans*-resveratrol was procured from Sigma Aldrich. The authenticity of cells has previously been confirmed by studying the expression of myocilin in response to dexamethasone treatment [23].

Study design

HTMCs in the 5th passage were chosen for the experiment. Before treatment, the HTMCs were incubated in 2% DMEM overnight and maintained in a CO_2 incubator at 37°C. For the treatment, the media was substituted with fresh media comprising of 12.5 μM *trans*-resveratrol in 0.1% DMSO with or without 100 nM dexamethasone [25]. The vehicle-treated group consisted of DMEM with 0.1% DMSO. The cells were seeded and divided into five groups:

- Group 1: HTMC in 2% DMEM (untreated)
- Group 2: HTMC in 2% DMEM + 0.1% DMSO (vehicle control)
- Group 3: HTMC in 2% DMEM + 0.1% DMSO + 100 nM dexamethasone
- Group 4: HTMC in 2% DMEM + 0.1% DMSO + 12.5 μM *trans*-resveratrol
- Group 5: HTMC in 2% DMEM + 0.1% DMSO + 100 nM dexamethasone + 12.5 μM *trans*-resveratrol

MTS assay was performed to determine the cell viability after 3 and 7 days of incubation. Further experiments were divided into two studies. Study one explored the impact of *trans*-resveratrol treatment on the expression of ECM proteins by HTMCs, with and without dexamethasone. Cells were collected after treatment for 3 days to determine the expressions of *COLIA1*, *COLIA2*, *COLIIIA1*, *COLIVA1*, *COLIVA2*, *FN1* and *ACTA2* genes using qPCR. The media and cells were also collected for estimation of COLI, COLIII, COLIV, FN and α-SMA proteins after 7 days of incubation using ELISA.

Study two explored the effect of *trans*-resveratrol on TGF-β1-SMAD signalling in HTMCs. The expressions of TGF-β1, SMAD4, SMAD7, PAI-1 and tPA genes and proteins were determined using the same duration and method as Study one. All experiments were performed with three technical replicates from three biological samples for each experiment.

MTS assay

CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) (Promega) was utilised to ascertain the cell viability of HTMCs. In brief, a 20 µl of CellTiter MTS mixture was pipetted into each well and incubated at 37°C for 1 h while being covered with aluminium foil to avoid light exposure. The absorbance was then quantified using a plate reader (Perkin Elmer) at 490 nm. The assay was performed after 3 and 7 days of incubation.

Quantitative real-time (qPCR)

The qPCR was done following the MIQE guidelines [26]. Extraction of total RNA from cultured cells was performed using an RNA Purification Kit (MACHEREY-NAGEL), which enabled consecutive elution of DNA with low salt buffer and RNA NucleoSpin Column with water. Reverse transcription was performed to generate the cDNA template utilising OneScript Hot cDNA Synthesis Kit (Applied Biological Materials). Subsequently, qPCR was performed using 4X CAPITAL qPCR Green Master Mix (BiotechRabbit). Briefly, the qPCR reactions were programmed on BioRad iCycler PCR machine (Bio-Rad Laboratories) consisting of 3 steps, including 45 cycles at 95°C for 2 min (initial activation), 95°C for 5 sec (denaturation) and 60°C for 30 sec (annealing). All samples were analyzed in triplicate. For each gene, standard curve was plotted to ascertain the PCR efficiencies. Relative fold expressions of the genes of interest were calculated using the ΔΔCT method with the data normalized to both reference genes, β-actin (ACTB) and GAPDH. Primers were all sourced by Sigma Life Science and Integrated DNA Technologies (Table 1).

ELISA

The target protein concentrations were measured using commercially available ELISA kits. These proteins included COLI, total and active TGF-β1, SMAD4, SMAD7, PAI-1 and tPA (Finetest), COLIII, COLIV, FN (Elabscience Biotechnology Co.) and α-SMA (BioAssay Technology Laboratory). For COLI, COLIII, COLIV and FN measurement, media from HTMC culture were gathered at the end of 7 days incubation phase and transferred into a 15 ml

Table 1. qPCR primers

Target genes	Primer sequences (5′→3′)	GenBank accession
ACTA2 F	AGA TCA AGA TCA TTG CCC C	NM_001613
ACTA2 R	TTC ATC GTA TTC CTG TTT GC	
COLIA1 F	GCT ATG ATG AGA AAT CAA CCG	NM_000088
COLIA1 R	TCA TCT CCA TTC TTT CCA GG	
COLIA2 F	GTG GTT ACT ACT GGA TTG AC	NM_000089
COLIA2 R	CTG CCA GCA TTG ATA GTT TC	
COLIIIA1 F	ATT CAC CTA CAC AGT TCT GG	NM_000090
COLIIIA1 R	TGC GTG TTC GAT ATT CAA AG	
COLIVA1 F	AAA GGG AGA TCA AGG GAT AG	NM_001845
COLIVA1 R	TCA CCT TTT TCT CCA GGT AG	
COLIVA2 F	AAA AGG AGA TAG AGG CTC AC	NM_001846
COLIVA2 R	GTA TTC CGA AAA ATC CAG CC	
FN1 F	CCA TCG CAA ACC GCT GCC AT	NM_002026
FN1 R	AAC ACT TCT CAG CTA TGG GCT T	
GAPDH F	GTC TCC TCT GAC TTC AAC AGC G	NM_002046
GAPDH R	ACC ACC CTG TTG CTG TAG CCA A	
ACTB F	TGG CAC CCA GCA CAA TGA A	NM_001101
ACTB R	CTA AGT CAT AGT CCG CCT AGA AGC A	
TGF-β1 F	GCC CTG GAC ACC AAC TAT TGC T	NM_000660
TGF-β1 R	AGG CTC CAA ATG TAG GGG CAG G	
SMAD 4 F	ACT GCA GAG TAA TGC TCC ATC AAG T	NM_001407041
SMAD 4 R	GGA TGG TTT GAA TTG AAT GTC CTT	
SMAD 7 F	TAG CCG ACT CTG CGA ACT AGA GT	NM_001190821
SMAD R	GGA CAG TCT GCA GTT GGT TTG A	
PAI-1 F	AGG ACC GCA ACG TGG TTT TCT C	NM_001018067
PAI-1 R	AGT GCT GCC GTC TGA TTT GTG	
tPA F	CAG GAA ATC CAT GCC CGA TTC	NM_000930
tPA R	TTC TTC AGC ACG TGG CAC CA	

F, forward sequence; R, reverse sequence.

centrifuge tube. Cell debris was then taken out by centrifuging the samples at 3,500 rpm for 20 min at 4°C. Supernatants were then collected and aliquoted into 1.5 ml microcentrifuge tubes and were kept until further use at –80°C. The cell lysate was used to determine total and active TGF-β1, SMAD4, SMAD7, PAI-1, tPA and α-SMA concentration. Following the media removal, the cells were washed and trypsinized with trypsin-EDTA solution (Gibco, Life Technologies). The pellet formed after centrifugation was gently washed using phosphate buffered saline (Sigma Aldrich) to eliminate the residual trypsin solution. One ml of 1X RIPA buffer (Elabscience Biotechnology Co.) was added to the cell pellet. The cell lysate was sonicated for one minute and then incubated on ice for 15 min. They were subsequently centrifuged at 3,500 rpm for 20 min at 4°C, and the supernatant was garnered and stored at –80°C until further analyses. ELISA for all proteins was performed as per the manufacturer's protocol. For TGF-β1 analysis, the supernatant was diluted first with the reference standard and sample diluent. Then, the sample was activated using the activator reagent 1 (1 M HCL) and was neutralized by adding the activator reagent 2 (1.2 M NaOH/ 0.5 M HEPES) to activate the latent TGF-β1.

Molecular mechanism of interaction between *trans*-resveratrol and SMAD4 domains

Hypothesis: SMAD4 plays a crucial role as the central protein in the canonical TGF-β signalling pathway orchestrating signal transduction through a positive feedback mechanism [27]. Expanding on this pivotal function, we explored the molecular interaction between *trans*-resveratrol and the primary domains of SMAD4 using multiple docking techniques [28]. The SMAD4 protein spans a total of 552 amino acids (UniProt, 2023). Amino acids 18-142 constitute the MH1 domain, responsible for DNA binding. Meanwhile, the MH2 domain, formed by amino acids 323-552, engages in diverse interactions with other proteins and contributes to the regulation of transcription.

Data preparation: Two experimental X-ray heteromeric models were sourced from the UniProt (UniProt, 2023) and PDBe (PDBe, 2023) databases: 5MEY, encompassing the MH1 domain, and 1U7F encompassing the MH2 domain. For subsequent use in docking, isolated subunits of the MH1 and MH2 domains were extracted from these models. The optimized 3D model of *trans*-resveratrol was constructed in a sequential process, first utilizing molecular mechanics with the MarvinSketch 17.1.23 program (MarvinSketch, 2018) followed by the PM7 semi-empirical quantum-chemical method using the MOPAC2012 program (MOPAC, 2018), as per the methodology described by Vassiliev *et al*. [29]. To identify the most significant binding regions, 27 docking spaces were established on the models of the MH1 and MH2 domains using the MSite v21.04.22 program [28].

Docking: Ensemble docking was conducted using the AutoDock Vina 1.1.1 program [30]. Each compound was docked in 10 conformers, repeated 5 times within each docking space, with the calculation of the minimum binding energies ΔE from 50 obtained values, following the procedure outlined in [29]. The docking process was executed independently in each of the 27 spaces created for multiple docking.

Statistical analysis

The statistical comparisons among groups for protein and gene expressions were performed using one-way analysis of variance (ANOVA) with Tukey *post-hoc* analysis. All data were reported as mean ± standard deviation and $p < 0.05$ was considered significant.

RESULTS

Effect of *trans*-resveratrol on HTMCs viability following treatment for 3 and 7 days

The cell viability was determined after treatment for 3 and 7 days with vehicle, dexamethasone alone, resveratrol alone or a combination of dexamethasone with *trans*-resveratrol. No significant differences in the HTMCs viability were observed among all groups ($p > 0.05$) at the end of both incubation periods (Fig. 1). The cells treated with 100 nM dexamethasone alone exhibited the highest cell viability at both 3- and 7-day incubation periods, with percentage viability of 114% and 113%, respectively. However, these differences, were not statistically significant.

Study 1

Effects of *trans*-resveratrol on the gene and protein expression for ECM components in dexamethasone-treated HTMCs: The gene expressions for collagen type I α1 chain (*COLIA1*) and collagen type I α2 chain (*COLIA2*) were significantly upregulated in the dexamethasone-only group compared to the control and vehicle group ($p < 0.05$) (Fig. 2A, B). Moreover, the protein expression of collagen type I was significantly higher in the dexamethasone group than in other groups ($p < 0.05$). Cells co-treated with *trans*-resveratrol and dexamethasone exhibited a significant upregulation of the *COLIA1* gene compared to the control group ($p < 0.05$), however, the gene expression was significantly lower compared to the dexamethasone-only group ($p < 0.05$) (Fig. 2A). The *COLIA2* gene expression in cells co-treated with *trans*-resveratrol and dexamethasone was lower than the dexamethasone-only group, although not significantly different ($p > 0.05$) (Fig. 2B). The collagen type I in cells co-treated with *trans*-resveratrol and dexamethasone showed a significant decrease compared to the control and vehicle group ($p < 0.05$). The expression was also significantly lower compared to the dexamethasone-only group ($p < 0.05$) (Fig. 2C).

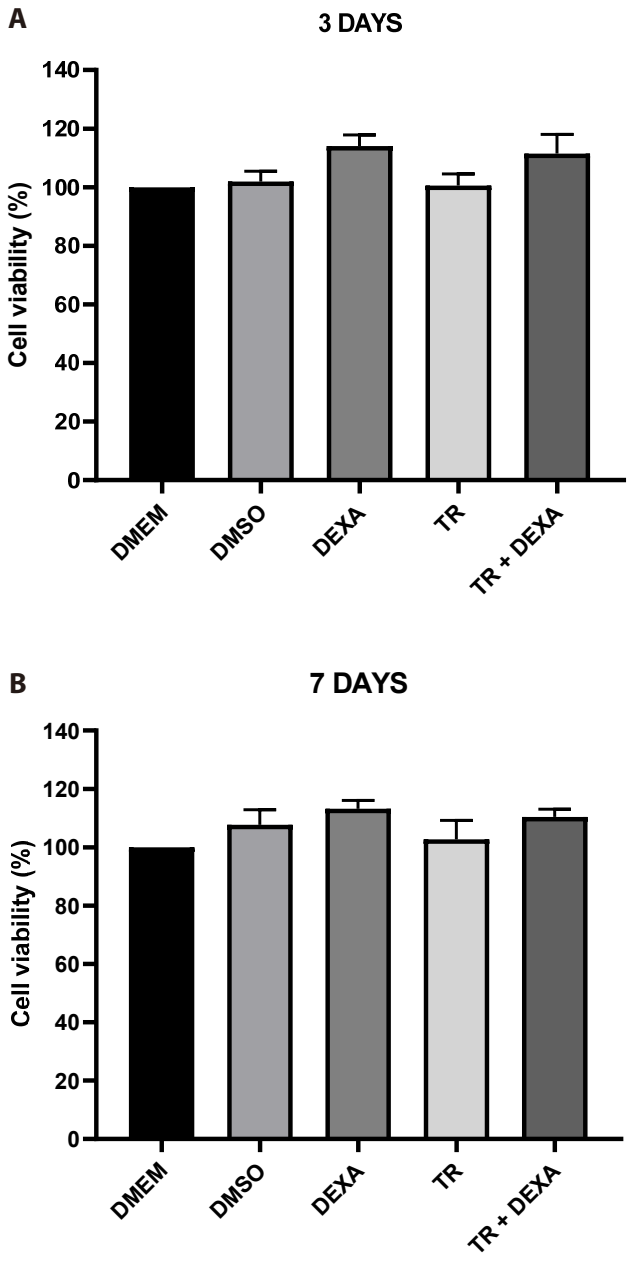

Fig. 1. TR treatment with or without DEXA were non-toxic to HTMC viability. Percentage viability of HTMC following treatment for (A) three days and (B) seven days. Bars represent mean ± SD (n = 3). $p > 0.05$, ANOVA with Tukey's *post-hoc*. TR, resveratrol; DEXA, dexamethasone; HTMC, human trabecular meshwork cell; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulfoxide.

The gene expression of collagen type III α1 chain (*COLIIIA1*) in the dexamethasone-only group was significantly upregulated compared to all groups ($p < 0.05$). The protein expression of collagen type III was also higher in the dexamethasone-only group compared to the control and vehicle group ($p < 0.05$). HTMCs co-treated together with *trans*-resveratrol and dexamethasone showed higher *COLIIIA1* gene expression when compared to the control and vehicle groups ($p < 0.05$) but significantly lower compared to the dexamethasone-only group ($p < 0.05$) (Fig. 2D). The collagen type III protein expression in HTMCs co-treated with *trans*-resveratrol was significantly reduced compared to the dexamethasone-only group ($p < 0.05$) (Fig. 2E).

The gene expression of collagen type IV α1 chain (*COLIVA1*) in the dexamethasone-only group was significantly upregulated compared to the control group ($p < 0.05$). Treatment of HTMCs with *trans*-resveratrol and dexamethasone downregulated the *COLIVA1* level, but the difference from the dexamethasone-only group was not significant ($p > 0.05$) (Fig. 2F). The gene expression for collagen type IV α2 chain (*COLIVA2*) and protein expression for collagen type IV was significantly upregulated in the dexamethasone-only group compared to all groups ($p < 0.05$). *COLIVA2* gene expression significantly decreased in cells co-treated with *trans*-resveratrol and dexamethasone compared to the dexamethasone-only group ($p < 0.05$) (Fig. 2G). Additionally, collagen type IV protein expression in HTMCs co-treated with *trans*-resveratrol and dexamethasone was significantly reduced compared to dexamethasone-only and the control group ($p < 0.05$) (Fig. 2H).

In the group treated with dexamethasone-only, the gene expression of fibronectin 1 (*FN1*) and the protein expression of FN was significantly upregulated compared to all groups ($p < 0.05$). Cells co-treated with *trans*-resveratrol and dexamethasone had significant upregulation of *FN1* gene expression compared to the control and vehicle group ($p < 0.05$), however, the level was lower compared to dexamethasone-only group ($p < 0.05$) (Fig. 2I). The FN protein level in cells co-treated with *trans*-resveratrol and dexamethasone was also significantly reduced compared dexamethasone-only group ($p < 0.05$) (Fig. 2J).

HTMCs showed significantly greater expression of the *ACTA2* gene and α-SMA protein in the dexamethasone-only group in comparison to all other groups ($p < 0.05$). Cells treated with *trans*-resveratrol and dexamethasone significantly showed upregulation of *ACTA2* gene expression compared to the control and vehicle group ($p < 0.05$) but reduced significantly in comparison with dexamethasone only group ($p < 0.05$) (Fig. 2K). Cells incubated with *trans*-resveratrol together with dexamethasone also showed significantly lower α-SMA protein expression compared to dexamethasone only and the control group ($p < 0.05$) (Fig. 2L and Table 2).

Study 2

Effects of *trans*-resveratrol on the TGF-β1 and SMADs gene and protein expressions in HTMCs: To further explain the mechanisms behind the results of the study 1, the effect of *trans*-resveratrol on the gene and protein expression of TGF-β1 and SMADs was carried out using the same study design. The gene and protein expression of TGF-β1 in the dexamethasone-only group were significantly greater than all other groups ($p < 0.05$). Treatment with *trans*-resveratrol and dexamethasone significant-

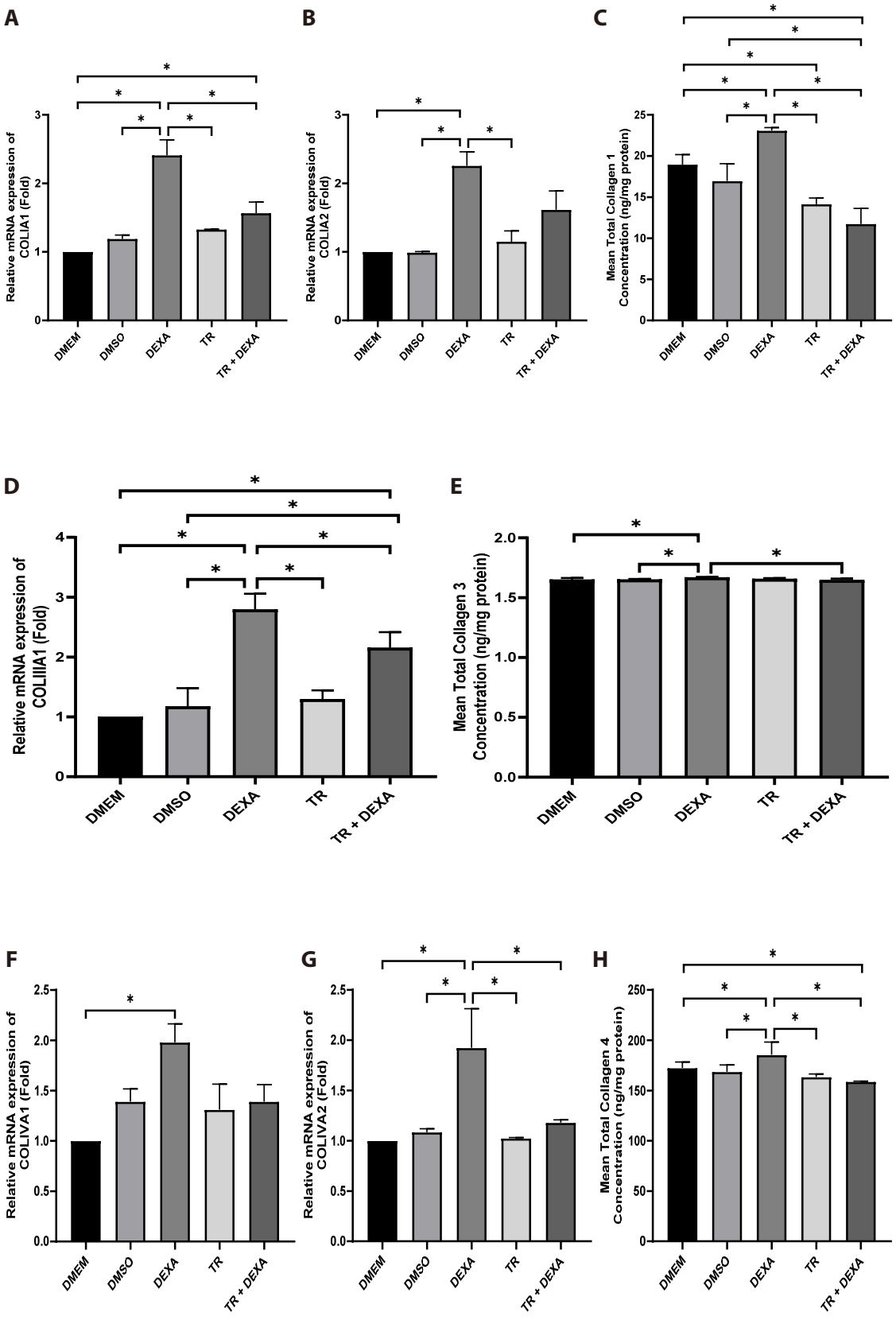

Fig. 2. TR reduces ECM expressions induced by DEXA in HTMCs. Effect of TR on the (A) *COLIA1* gene (B) *COLIA2* gene (C) COLI protein (D) *COLIIIA1* gene (E) COLIII protein (F) *COLIVA1* gene (G) *COLIVA2* gene (H) COLIV protein (I) *FN1* gene (J) Fibronectin protein (K) *ACTA2* gene and (L) Alpha smooth muscle actin protein expressions by the DEXA-treated HTMCs after treatment for three and seven days. Bars represent mean ± SD (n = 3). *p < 0.05, ANOVA with Tukey's *post-hoc*. TR, resveratrol; ECM, extracellular matrix; DEXA, dexamethasone; HTMC, human trabecular meshwork cell; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulfoxide.

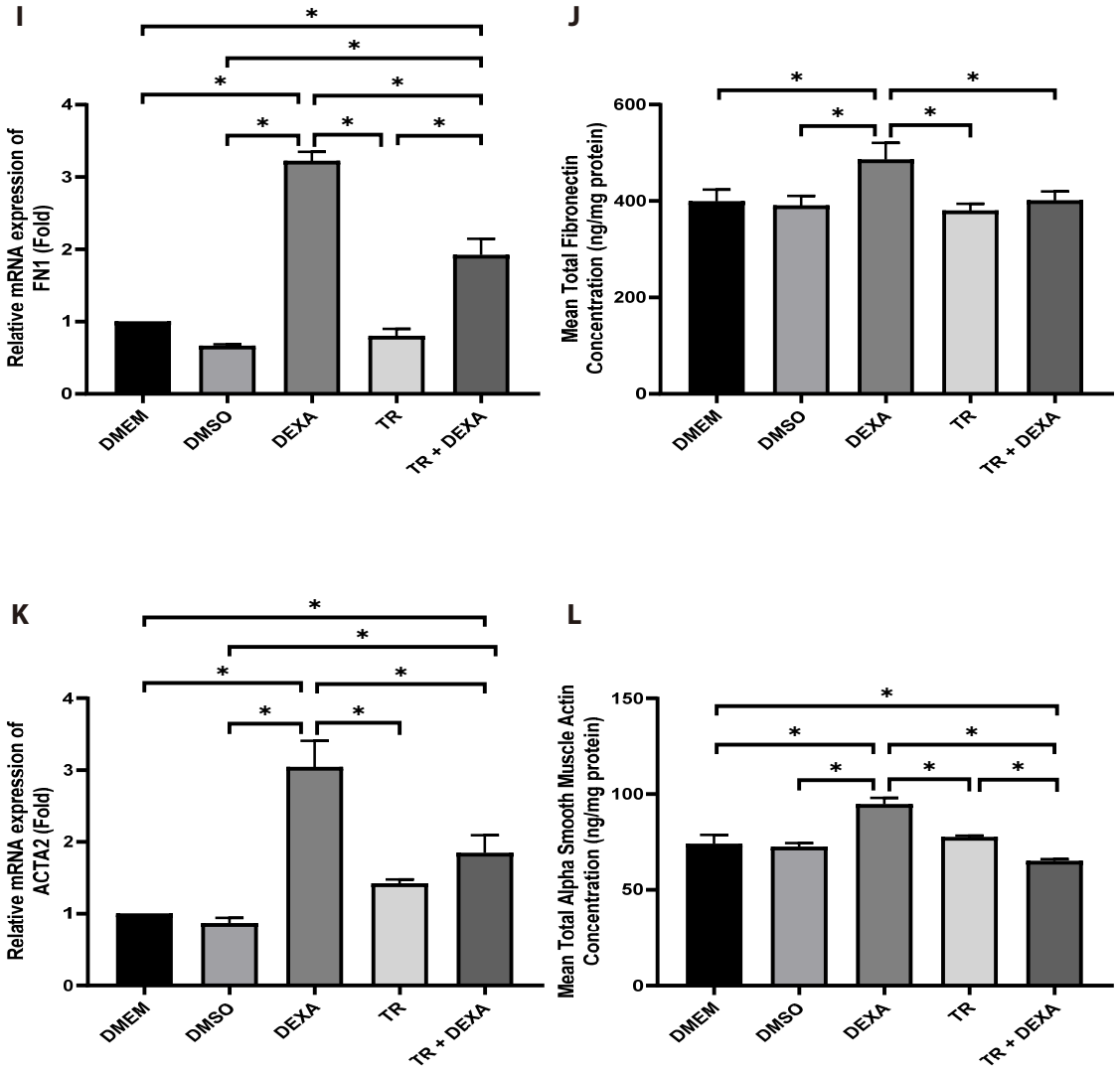

Fig. 2. Continued.

Table 2. ECM gene expressions in HTMC after three days of incubation with resveratrol in the presence and absence of dexamethasone

Gene	*COLIA1*	*COLIA2*	*COLIIIA1*	*COL4A1*	*COL4A2*	*FN1*	*ACTA2*
Group 1	1.0*	1.0*	1.0*	1.0*	1.0*	1.0*	1.0*
Group 2	1.19*	0.99*	1.18*	1.39	1.09*	0.67*	0.87*
Group 3	2.41	2.23	2.80	1.98	1.93	3.22	3.04
Group 4	1.30*	1.15*	1.30*	1.31	1.03*	0.80*	1.42*
Group 5	1.57*	1.61	2.16*	1.39	1.18*	1.92*	1.85*

Data presented as fold change relative to the dexamethasone-only group (N = 3; ANOVA; *p < 0.05). Group 1, control; Group 2, vehicle; Group 3, dexamethasone only; Group 4, resveratrol only; Group 5, resveratrol and dexamethasone; ECM, extracellular matrix; HTMC, human trabecular meshwork cell.

ly reduced the *TGF-β1* gene expression in HTMCs compared to the dexamethasone-only group ($p < 0.05$) (Fig. 3A). Comparable effects were also seen for both levels of total and active TGF-β1 (Fig. 3B, C) ($p < 0.05$).

The gene and protein expression of SMAD4 were also significantly upregulated in the dexamethasone-only group compared to all other groups ($p < 0.05$). Cells co-treated with *trans*-resveratrol and dexamethasone showed significant downregulation of the SMAD4 gene (Fig. 3D) and reduction of its protein (Fig. 3E) compared to the dexamethasone-only group ($p < 0.05$).

In the dexamethasone-only group, the the expression of the SMAD7 gene was significantly downregulated compared to both the control and *trans*-resveratrol-only group ($p < 0.05$). Interestingly, the SMAD7 protein expression in the dexamethasone-only

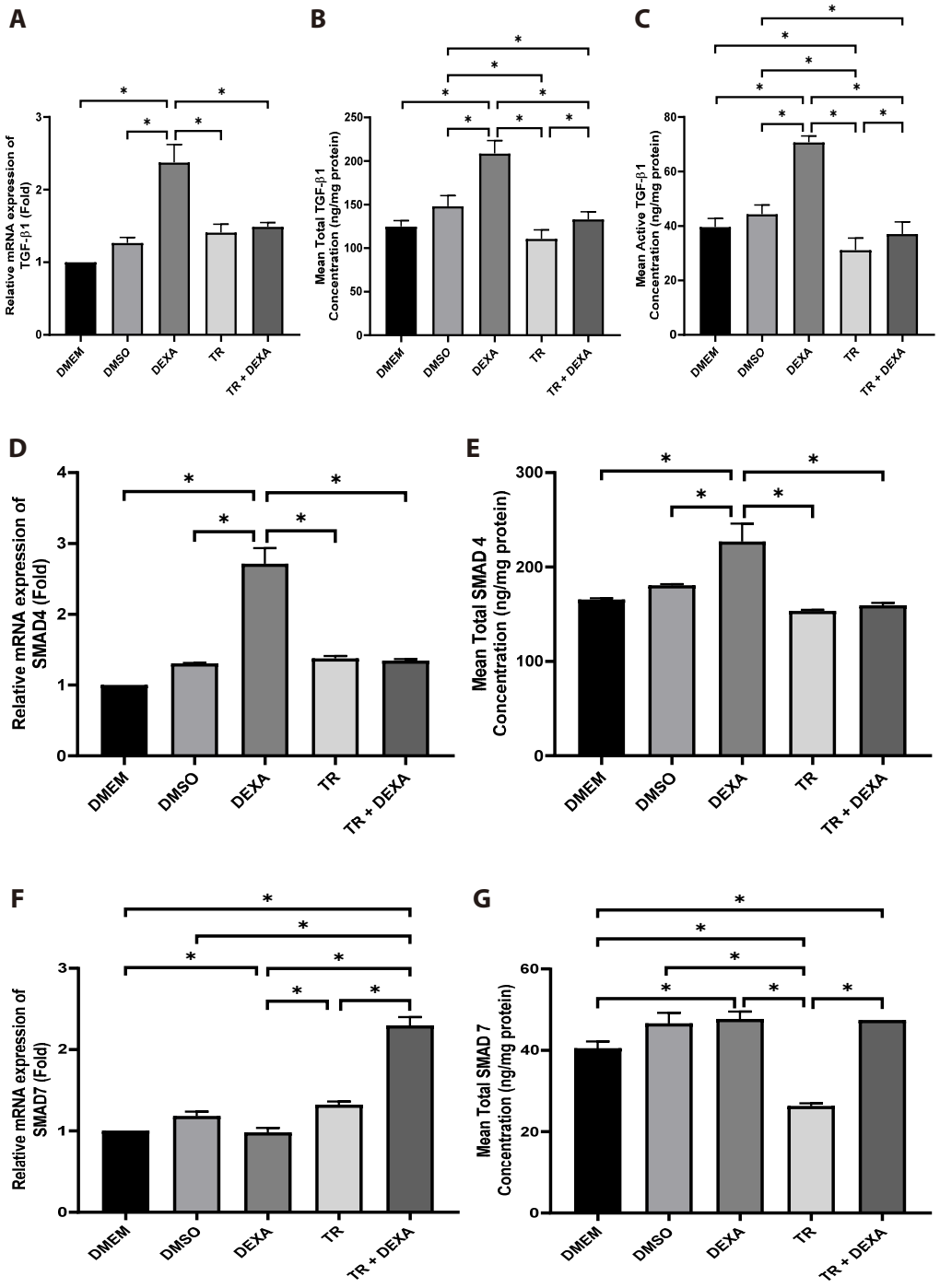

Fig. 3. TR alleviates TGF-β1 and SMAD expressions induced by DEXA but increases inhibitory SMAD 7. Effect of TR on the (A) *TGF-β1* gene (B) Total TGF-β1 protein (C) Active TGF-β1 protein (D) *SMAD4* gene (E) SMAD4 protein (F) *SMAD7* gene (G) SMAD7 protein by the DEXA-treated HTMCs after treatment for three and seven days. Bars represent mean ± SD (n = 3). *p < 0.05, ANOVA with Tukey's *post-hoc*. TR, resveratrol; DEXA, dexamethasone; HTMC, human trabecular meshwork cell; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulfoxide.

group showed a significant upregulation compared to the same groups ($p < 0.05$). Co-treatment with *trans*-resveratrol and dexamethasone resulted in a significant upregulation of the *SMAD7* gene expression in HTMCs compared to dexamethasone-only and other groups ($p < 0.05$) (Fig. 3F). Additionally, the SMAD7 protein level was also significantly increased in cells incubated with *trans*-resveratrol and dexamethasone compared to the control group ($p < 0.05$). However, the mean concentration was not significantly different when compared to the dexamethasone-only group ($p > 0.05$) (Fig. 3G and Table 3).

Effects of resveratrol on the PAI-1 and tPA secretion in HTMCs: The effect of *trans*-resveratrol on the gene and protein ex-

pressions of PAI-1 and tPA are shown in Fig. 4. The gene expression and protein level of PAI-1 in the dexamethasone-only group were significantly elevated than all other groups ($p < 0.05$). Co-treatment with *trans*-resveratrol and dexamethasone significantly downregulated the PAI-1 gene and protein expression in HTMCs compared to the dexamethasone-only (Fig. 4A) as well as other groups (Fig. 4B) ($p < 0.05$).

There was no significant difference in the *tPA* gene expression in the dexamethasone-only group compared to the control and vehicle groups ($p > 0.05$). The tPA protein, however, was significantly downregulated in the dexamethasone-only group in comparison to the control and vehicle group ($p < 0.05$). Cells incubated with *trans*-resveratrol and dexamethasone showed significant upregulation of the tPA gene (Fig. 4C) and protein (Fig. 4D) expressions compared to dexamethasone-only and *trans*-resveratrol only group ($p < 0.05$) (Table 3).

Table 3. The expression of *TGFβ1, SMAD 4, SMAD 7, PAI-1* and *tPA* genes in HTMC after three days of incubation with resveratrol in the presence and absence of dexamethasone

Gene	*TGFβ1*	*SMAD 4*	*SMAD 7*	*PAI-1*	*tPA*
Group 1	1.0*	1.0*	1.0*	1.0*	1.0
Group 2	1.27*	1.31*	1.18	0.93*	1.04
Group 3	2.38	2.71	0.98	3.26	1.20
Group 4	1.41*	1.38*	1.32*	1.47*	1.13
Group 5	1.49*	1.35*	2.30*	1.05*	3.47*

Data presented as fold change relative to the dexamethasone-only group (N = 3; ANOVA; *$p < 0.05$). Group 1, control; Group 2, vehicle; Group 3, dexamethasone only; Group 4, resveratrol only; Group 5, resveratrol and dexamethasone; HTMC, human trabecular meshwork cell.

Multiple docking results

***Affinity for MH1*:** Based on the simulation results, it appears that *trans*-resveratrol does not exhibit significant affinity for the MH1 domain of SMAD4. Among the 27 indicators, the minimum docking energy value of docking energy for *trans*-resveratrol was $\Delta E_5 = -6.1$ kcal/mol. Considering that the MH1 domain is primarily associated with DNA binding [31], it is presumed that *trans*-resveratrol does not affect the binding of SMAD4 to DNA.

***Affinity for MH2*:** In accordance with the simulation results, *trans*-resveratrol demonstrated a high affinity for the MH2 domain of SMAD4. Multiple docking analyses revealed two distinct binding sites for *trans*-resveratrol, characterized by minimum docking energies of $\Delta E_4 = -7.2$ kcal/mol and $\Delta E_7 = -7.2$ kcal/mol. Given that the, MH2 domain plays a role in regulating transcriptional activity [31], the potent inhibition of the MH2 domain by *trans*-resveratrol suggests a potential hindrance to the interaction between SMAD4 and proteins in the TGF-β signalling pathway

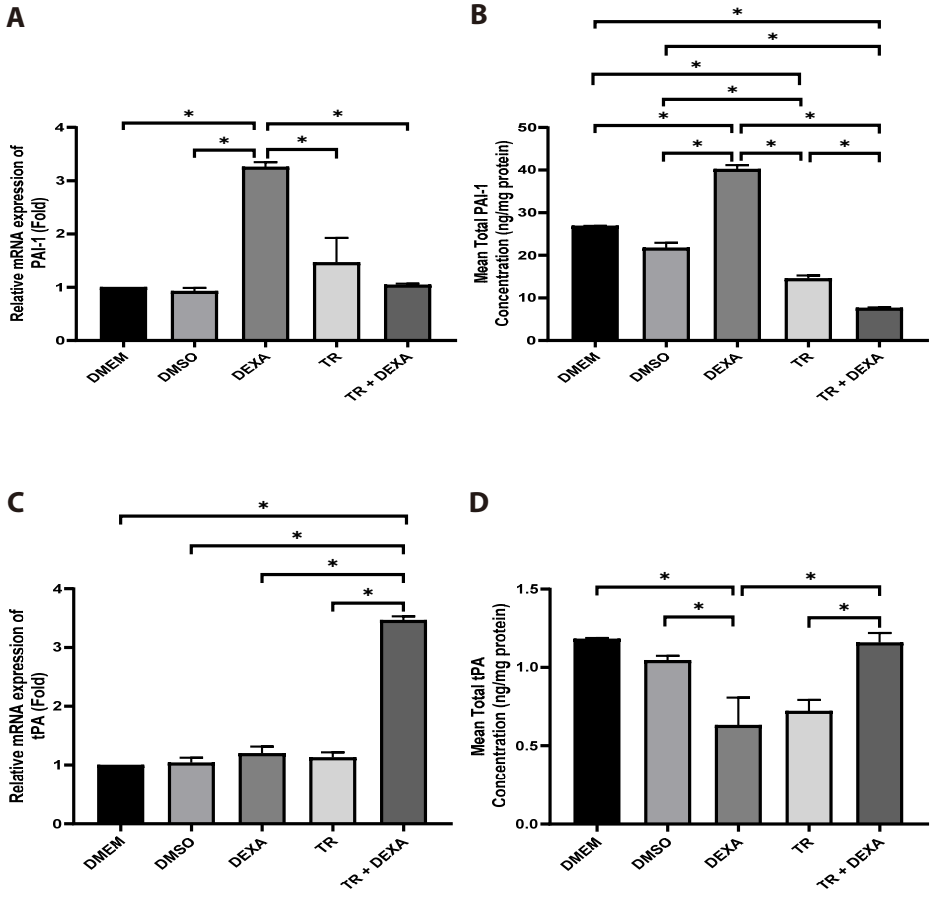

Fig. 4. TR reduces PAI-1 expression induced by DEXA and increases tPA expression. Effect of TR on the (A) *PAI-1* gene (B) PAI-1 protein (C) *tPA* gene and (D) tPA protein expressions by the DEXA-treated HTMCs after treatment for three and seven days. Bars represent mean ± SD (n = 3). *$p < 0.05$, ANOVA with Tukey's *post-hoc*. TR, resveratrol; PAI-1, plasminogen activator inhibitor-1; DEXA, dexamethasone; tPA, tissue plasminogen activator; HTMC, human trabecular meshwork cell; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulfoxide.

consequently impeding transcriptional activation.

Identification of key binding amino slots

***Determination of all possible binding amino acids*:** The LigPlot+ 2.2.5 program [32], was used to generate lists of all potential amino acids that contribute to the affinity of resveratrol for two crucial MH2 binding sites within the SMAD4 domain. The corresponding results are presented in Table 4.

***Key binding amino acids*:** The intersection of two lists of binding amino acids, acquired for two important binding regions, is also presented in Table 4. Fig. 5 illustrates a graphical representation of the overlay of these two binding regions for resveratrol.

DISCUSSION

The treatment of primary HTMCs using 12.5 μM *trans*-resveratrol for 3 and 7 days in the presence and absence of dexamethasone caused no detrimental effect on TM cell viability. The dose of *trans*-resveratrol used in this study was established in an earlier study by Mohd Nasir *et al*. [25], which showed that the effect of *trans*-resveratrol on the viability of HTMCs was dose-dependent but not time-dependent. The use of *trans*-resveratrol up to 25 μM for 3, 5 and 7 days had no significant effect on the TM cell's viability, however, with higher doses of 50 μM and above, *trans*-resveratrol significantly reduced HTMC viability with or without dexamethasone. Moreover, a shorter 24-h treatment with *trans*-resveratrol for up to 100 μM has been shown not to affect the survival of glaucomatous HTMCs [33].

Table 4. Amino acids that ensure the binding of resveratrol to the MH2 domain of SMAD 4

Area4	Area7	General
TYR412	TYR412	TYR412
TYR413	TYR413	TYR413
ARG416	ARG416	ARG416
PHE438	PHE438	PHE438
GLN442	GLN442	GLN442
GLN446	GLN446	GLN446
LEU464	LEU464	LEU464

The numbering of amino acids has been reduced to the standard one, taking into account the shift in the numbers of amino acids in the 1U7F 3D model.

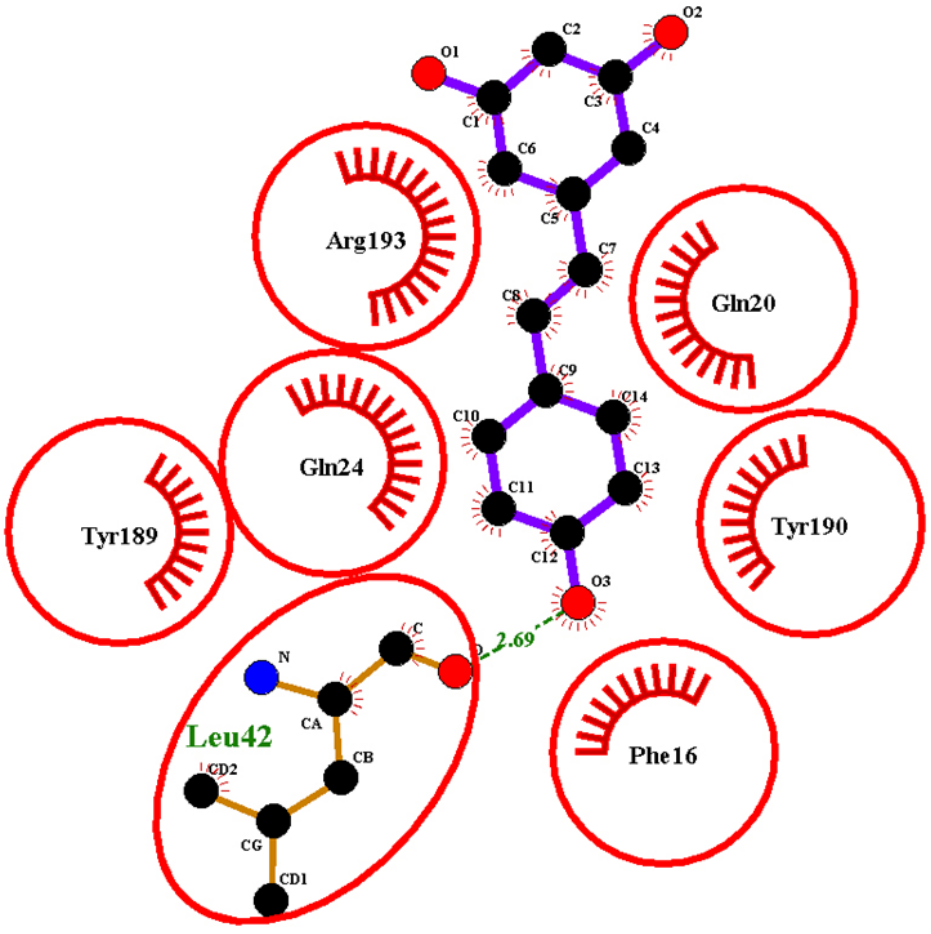

Fig. 5. Key binding amino acids that ensure the affinity of resveratrol to the MH2 domain of SMAD4 (amino acid numbers correspond to the numbering in the 1U7F 3D model).

In study one, the effect of *trans*-resveratrol on the expression of ECM components, including COL, FN and α-SMA, by steroid-treated HTMCs was determined. Dexamethasone affects ECM homeostasis by interrupting the equilibrium between its synthesis and degradation, which is evident in glaucomatous eyes [34]. Previous studies have observed that HTMCs and bovine TM cells treated with dexamethasone showed an increased accumulation of ECM constituents such as collagen type I, III, IV, FN and α-SMA in a time-dependent manner [35-37]. Apart from HTMCs, exposure to dexamethasone has also been demonstrated to intensify the expression of ECM constituents in other cell types, such as human ovarian cancer cell line [38] and primary human glioblastoma cells [39]. In addition, dexamethasone exerts a variety of effects on TM cells, such as increased cell stiffness [37], cytoskeletal reorganization [40], suppression of phagocytic activity [41] and abnormal ECM accumulation [34]. Following these observations, the current study also showed that treatment with dexamethasone causes HTMCs to upregulate gene and protein expression of ECM components compared to other groups. Honjo *et al*. [42] in 2018 also showed that dexamethasone-treated HTMCs have significantly upregulated mRNA expression of *COLIA1*, *COLIVA1* and *FN1* and protein expression of α-SMA, FN and COL.

Notably, we observed that HTMCs incubated with *trans*-resveratrol in the presence of dexamethasone affects the gene and protein expressions of collagen type I, III, IV, FN and α-SMA. Previously, treatment of HTMCs with 12.5 μM *trans*-resveratrol in the presence of dexamethasone for 5 days was shown to cause a significant elevation in the level of MMP-2 and -9 through NFkB activation [23] indicating that *trans*-resveratrol enhances degradation of ECM proteins. However, it remained unclear if *trans*-resveratrol also affects the synthesis of ECM proteins by downregulating the protein and gene expressions of the COL, FN and α-SMA. For the first time, the present research demonstrates that *trans*-resveratrol also reduces the dexamethasone-induced increase in the expression of ECM components by HTMCs. In accordance with our findings, *trans*-resveratrol was also shown to downregulate the expression of type I and III procollagen mRNA by human hypertrophic scar fibroblasts after a 24-h treatment [43]. *Trans*-resveratrol has been shown to reduce the expression of various ECM components in other cell types such as human primary lung and prostate fibroblasts [44], the uterine smooth muscle cell line and leiomyoma cell line [45]. The treatment with *trans*-resveratrol also lowered the level of hydroxyproline, an amino acid traditionally used to quantify collagen levels [46]. The effects of *trans*-resveratrol on ECM components have also been widely studied using rodent *in vivo* models. Administration of *trans*-resveratrol downregulated the expression of various ECM components in the kidneys of rats with tubulointerstitial fibrosis [47], and in the rat peritoneum causing reduced intra-abdominal adhesion formation and fibrin accretion [48]. Similar anti-fibrotic effects of *trans*-resveratrol have also been observed in the liver [49], gastrointestinal tract [50,51], urinary tract [52], lungs [53], pancreatic stellate cells [54] and skin [43]. Hence, as observed in the current study, by reducing the expression of ECM components by HTMCs along with previous observations of its ability to enhance ECM degradation [23], *trans*-resveratrol seems to restore dexamethasone-induced dysregulation of ECM homeostasis.

To clarify the mechanisms underpinning these findings in more detail, study two was conducted to assess the effects of *trans*-resveratrol on the TGF-β1 SMAD signalling pathway and secretion of PAI-1 and tPA. In this study, dexamethasone-treated HTMCs showed significantly increased TGF-β1, SMAD4 and PAI-1 genes and proteins expression compared to other groups. Dexamethasone has been shown to significantly increase the expressions of the TGF-β1 gene and protein, such as in human T cells [55] and rat kidneys [56]. Exposure to dexamethasone after 48 h also led to a notable increase in the expression of TGF-β1 protein in A549 cells, epithelial cells of lung carcinoma [57]. Furthermore, in accordance with our findings, dexamethasone, has previously been shown to cause significantly increased expression of SMAD4, a TGF-β signalling molecule in HTMCs [58]. In contrast to the findings of this study, we observed a significant increase in the expression of SMAD7 in response to treatment with *trans*-resveratrol, which was in line with preceding work [59]. This suggests that the TGF-β1/SMADs pathway might play a role in the fibrotic effect induced by dexamethasone in HTMCs. In the SMAD-dependent pathway of TGF-β, type I receptors stimulate SMAD2 and SMAD3 (R-SMADs) *via* phosphorylation at carboxyl termini, creating a complex with SMAD4. This complex translocates into the nucleus to positively affect the profibrotic gene expression. SMAD7, on the other hand inhibits profibrotic signaling of TGF-β by inhibiting phosphorylation of R-SMADs. Elevated levels of TGF-β1 lead to in the activation of SMAD signalling. Within this pathway, TGF-β1 plays a role either directly or indirectly, in regulating the pro-fibrotic overexpression of critical genes associated with the ECM such as COL, fibrillin, FN and thrombospondin [60]. Recent investigations into miRNAs have revealed that the fibrotic process involving TGF-β occurs by regulating the expression of multiple miRNAs including miR-192 [61,62]. The TGF-β mediated alteration of gene expression is known to involve miRNA. In fact, TGF-β modulates miRNA expression by affecting transcriptional activity of Smad complexes or by affecting posttranscriptional activity of R-Smads [63]. Notably, the present study shows that the incubation of HTMCs with *trans*-resveratrol in the presence of dexamethasone causes significantly reduced expression of TGF-β1 and SMAD4 and a significant upregulation of SMAD7 expression. The upregulation of the cellular SMAD7 gene was also observed in the HTMCs after 48-h of treatment with *trans*-resveratrol, though in the absence of dexamethasone [20]. In a study by Zhai *et al*. [64], *trans*-resveratrol treatment negatively affected the protein and mRNA expression of TGF-β and SMAD 2,3,4 and positively affected the SMAD7 expression. *Trans*-resveratrol was also shown to down-

regulate TGF-β1 and SMAD signaling in the lung [65], heart [46] and kidney [47].

To further understand the nature of interaction of *trans*-resveratrol with TGF-β1 signalling we conducted docking studies to analyse the affinity of *trans*-resveratrol for SMAD4 domains. The choice of SMAD4 for these studies was based on the observation that SMAD4 is the major regulator of the binding and transcriptional activity in TGF-β1 signalling. It is homologues with R-SMADs in its amino and carboxyl terminals, called the Mad homology domain (MH) 1 and 2, respectively. Moreover, SMAD7, the inhibitory SMAD, largely acts as R-SMAD decoy by inhibiting their phosphorylation and activation and does not directly impact the transcriptional activity of TGF-β1 signalling [31]. It was interesting to note that *trans*-resveratrol has strong affinity for MH2 domain of SMAD4. Hence, *trans*-resveratrol seems to affect transcriptional activity of TGF-β1 signalling and hence modulating expression of various signalling molecules and ECM proteins.

The raised level of TGF-β is linked with enhanced expression of PAI-1, an established downstream target of TGF-β. A study by Kimura *et al*. [66] found that the expression of PAI-1 mRNA and protein is increased by glucocorticoid in human proximal tubular epithelial cells. This overexpression induces ECM deposition by averting the generation of plasmin and MMP. tPA and urokinase plasminogen activator (uPA) convert plasminogen into plasmin, leading to activation of MMPs that degrade ECM proteins. As a serine protease, tPA is inhibited primarily by its serine protease inhibitor superfamily, PAI-1, at the level of plasminogen activation. The activation of PAI-1 supressed uPA and tPA, increasing the ECM deposition and lowering the AH outflow resistance. tPA was shown to form complexes with PAI more readily than uPA [67]. Our result revealed that the tPA secretion in the dexamethasone group was significantly downregulated compared to other groups indicating a dysregulation of ECM homeostasis in favour of its greater deposition. Notably, in the current study, *trans*-resveratrol in the presence of dexamethasone significantly reduced the secretion of PAI-1 and increased the expression of tPA genes and proteins. *Trans*-resveratrol has also been shown to reduce the PAI-1 mRNA and protein expression in human adipose tissue *in vitro* [68,69]. Additionally, tPA and uPA expression were found to be elevated in the AH of steroid-induced ocular hypertensive rats and in HTMCs cultured without dexamethasone in response to *trans*-resveratrol treatment. Hence, it is likely that a reduced level of PAI-1 and raised level of tPA by *trans*-resveratrol facilitate

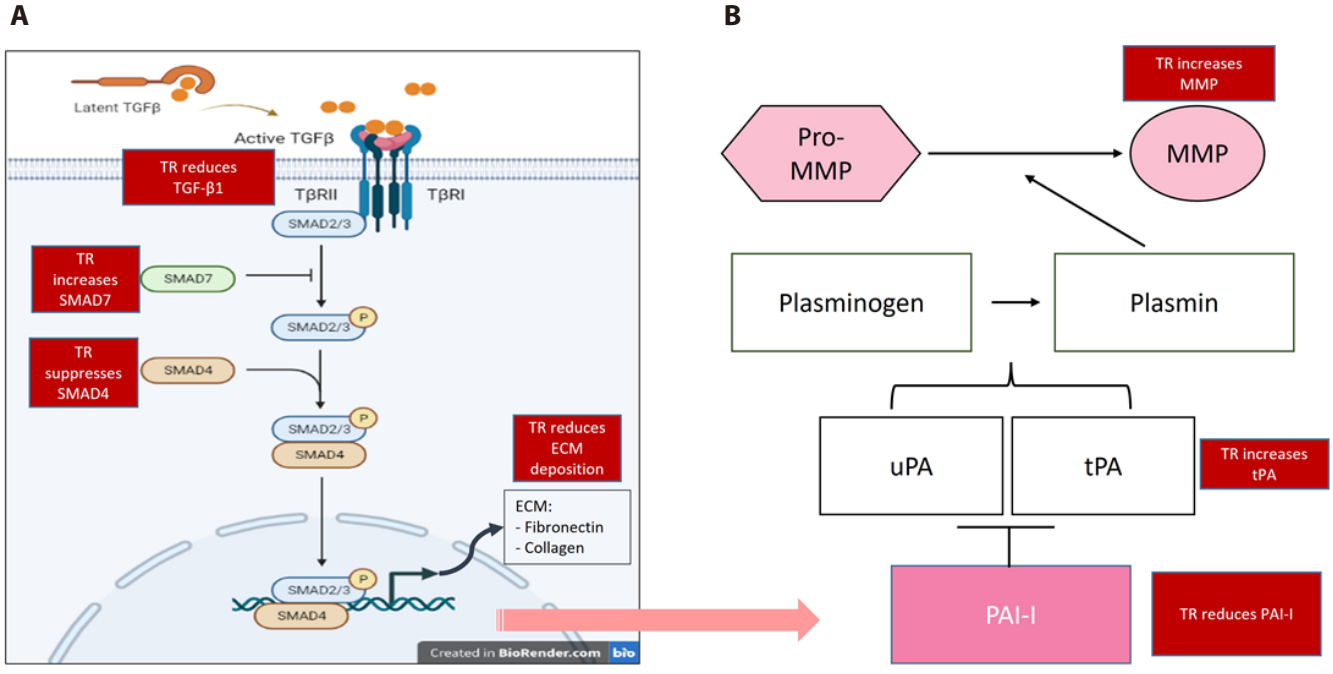

Fig. 6. Schematic representation of the possible mechanisms of action of TR for altering ECM turnover. (A) After latent TGF-β being activated, it binds to the TGF-BRII and phosphorylates TGF-BRI, forming a tetrameric complex. Activation of TGF-BRI leads to the downstream signalling of TGF-β involving SMAD proteins called TGF-β-SMAD dependent signalling pathway or also known as canonical signalling pathway. The phosphorylated receptors cause SMAD2 and SMAD3 to phosphorylate and subsequently attach to SMAD4, which amplifies signalling. The heteromeric SMAD complex accumulates in the nucleus and serves as a transcription factor to activate the transcription of fibrotic genes such as collagen, fibronectin, and α-SMA. SMAD7 is a negative regulator of SMAD 2/3 and inhibits fibrosis. (B) PAI-1 is a member of the serine protease inhibitor superfamily that binds to and inhibits the activity of uPA and tPA. tPA and uPA convert plasminogen into plasmin, which then activates the pro or latent MMPs into active form of MMPs that degrade ECM proteins. TR, resveratrol; ECM, extracellular matrix; TGF-β, transforming growth factor β; TGF-BRI, trans-membrane receptor type I; TGF-BRII, trans-membrane receptor type II; SMAD, suppressor of mothers against decapentaplegic; α-SMA, α-smooth muscle actin; PAI, plasminogen activator inhibitor; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator; MMP, matrix metalloproteinase.

the degrading action of MMP, tipping the balance in favour of increased deposition of ECM which translates into reduced aqueous outflow *via* the TM pathway. Further studies using siRNA or techniques for overexpression of target proteins may be beneficial in confirming the current finding and yielding new information. Since the current study for the first time explored the potential molecular targets of resveratrol, the experimental approach has given useful information.

In conclusion, *trans*-resveratrol reduces dexamethasone-induced increase in the expression of ECM components by HTMCs both at the protein and gene levels indicating reduced ECM synthesis. It is likely that this effect of *trans*-resveratrol involves the repression of TGF-β1 and SMAD4 and the enhancement of SMAD7 signalling. This effect of *trans*-resveratrol on transcriptional activity of TGF-β1 signalling may be attributed to its high affinity for MH2 domain of SMAD4. *Trans*-resveratrol also reduced PAI-1 and increased tPA levels, which could lead to its ability to increase the MMP secretion, and hence enhanced ECM degradation. Overall, the effects of *trans*-resveratrol both on the synthesis and degradation of ECM are likely to restore dexamethasone-induced dysregulation of ECM homeostasis. These findings serve as a foundation for additional research aimed at developing *trans*-resveratrol as a therapeutic approach in glaucoma management. A schematic diagram representing the summary of possible mechanisms of action of resveratrol for altering ECM turnover is shown in the Fig. 6.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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