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Roles of Histone Deacetylases in Orbital Fibroblasts from Graves' Ophthalmopathy Patients

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ABSTRACT

Graves' ophthalmopathy (GO) is an autoimmune thyroid eye disease which mainly causes eyelid retraction and proptosis. Orbital fibroblasts play an important role in GO pathogenesis by increasing cells proliferation, cytokines and hyaluronan production and adipogenesis. Nevertheless, current GO treatments remain limited and novel treatments are needed. Histone deacetylases (HDACs) are enzymes that remove acetyl group from histone tails which plays a role in the regulation of gene expression. HDACs are involved in the pathogenesis of various diseases, including cancer and autoimmune diseases. Therefore, treatment with HDAC inhibitors (HDACis) was conducted. Currently, the roles of HDACs in orbital fibroblasts from GO remain unidentified. Hence, the aim of this study was to investigate the roles of HDACs in PDGF-BB-induced orbital fibroblast activation using isoform-selective HDACis. The results showed that prophylactic effect of HDAC3i (RGFP966), HDAC4i (Tasquinimod) and HDAC4/5i (LMK235) significantly decreased PDGF-BB-induced cell proliferation. In addition, HDAC3i, HDAC4i and HDAC4/5i significantly reduced PDGF-BB-induced hyaluronan production. Unexpectedly, prophylactic effect of HDAC1i (Quisinostat), HDAC3i and HDAC4i significantly enhanced PDGF-BB-induced IL-6

production, while IL-8 production was not affected by HDACis. The level of *HDAC3* and *HDAC4* expressions increased in PDGF-BB-stimulated orbital fibroblasts. Thus, the data from this study suggested that aberrant HDAC4 levels are involved in PDGF-BB-induced orbital fibroblasts activation.

Keywords: Graves' ophthalmopathy, orbital fibroblasts, platelet-derived growth factor-BB, histone deacetylases

INTRODUCTION

Graves' ophthalmopathy (GO) is an autoimmune eye disease which develops in ~25-50% of Graves' disease patients (Bahn, 2010). GO patients have symptoms including upper eyelid retraction (91%), proptosis (62%), dysfunction of extraocular muscles (42%), conjunctiva edema (34%) and diplopia (31%) (Sahli & Gunduz, 2017). Orbital fibroblasts play a critical role in GO pathogenesis. Orbital fibroblasts, which mainly influence the orbital sites, express thyroid-stimulating hormone receptor (TSHR) on their surface leading to TSHR autoantibody stimulation and resulting in orbital tissue expansion and remodeling (van Steensel & Dik, 2010). Moreover, orbital fibroblasts can be activated by platelet-derived growth factor-BB (PDGF-BB) leading to proliferation, adipogenesis, increased TSHR expression, cytokine and hyaluronan production (Virakul, van Steensel, et al., 2014). In general, treatments of GO consist of steroids, surgery or orbital radiation (Sahli & Gunduz, 2017). Not only that recent treatments have long-term side effects, including hepatotoxicity and diabetes mellitus, but they also non-specifically target the activated immune component in this disease (Salvi, 2014; Salvi & Campi, 2015). Hence, GO therapy needs improvement in both the specificity and efficiency.

Histone deacetylases (HDACs) are enzymes that play role in the alteration of gene expression by removing the acetyl group from lysine residues on histone tails (Xu et al., 2007). There are 11 classical HDACs, which depend on the zinc molecule as co-factor. These HDACs can be divided on the basis of sequence homology into different classes, class I HDACs (HDAC1-3, 8), class IIa HDACs (HDAC4-5, 7, 9), class IIb HDACs (HDAC6, 10) and class IV HDACs (HDAC11) (Tang et al., 2013). Previous studies found that up-regulation of HDACs expression is associated with the development of various diseases, including fibrotic disorders, cancer and autoimmune diseases (Pang & Zhuang, 2010; Yoon & Eom, 2016). For example, HDAC3 is associated with the expression of inflammatory genes in fibroblast-like synoviocytes

obtained from rheumatoid arthritis patients (Angiolilli et al., 2017). Previous study also demonstrates that upregulation of *HDAC6* correlates with increasing neuroblastoma cell migration (Zhang et al., 2014). Thus, the discoveries of HDACs function in disease potentially leads to the development of HDACs inhibition as treatment.

HDAC inhibitors (HDACis) consist of pan-, class-selective and isoform-selective HDACis. Pan- and class-selective HDACis, such as Panobinostat and Vorinostat, have been used as anticancer drugs with US FDA approval (Eckschlager et al., 2017). In addition, isoform-selective HDACis are novel inhibitors that target a specific HDAC enzyme with less side effects and were studied in several clinical trials including prostate cancer, metastatic gastric carcinoma and lymphoma (M Reilly, 2014; Wang et al., 2018). For instance, Quisinostat (HDAC1i) and Tasquinimod (HDAC4i) were studied in patients with relapsed multiple myeloma and prostate cancer, respectively (Gong et al., 2018; Moreau et al., 2016). Therefore, the findings on specific HDAC involvement on the pathogenesis of the diseases may be helpful for improving effective treatment of diseases with long-term safety by the inhibition with isoform-selective HDACis.

In this study, the roles of each HDAC in PDGF-BB-activated orbital fibroblasts from GO patients were investigated by using various isoform-selective HDACis (Quisinostat, CAY10683, RGFP966, PCI-34051, Tasquinimod, LMK-235 and Tubastatin A).

MATERIAL AND METHODS

Reagents

Isoform-selective HDACis including Quisinostat (HDAC1i), CAY10683 (HDAC2i), RGFP966 (HDAC3i), Tasquinimod (HDAC4i), LMK-235 (HDAC4/5i), Tubastatin A (HDAC6i) and PCI-34051 (HDAC8i) were purchased from Selleck Chemicals Inc., TX, USA. Recombinant Human PDGF-BB was obtained from BioLegend Inc., CA, USA.

Isolation of orbital fibroblasts from orbital tissues

Orbital tissues were obtained from GO patients after decompression surgery at King Chulalongkorn Memorial Hospital (Bangkok, Thailand) with the approval by the Institutional Review Board of the Faculty of Medicine (Protocol number 401/61), Chulalongkorn University, (Bangkok, Thailand). The tissues were cultured for isolation of orbital fibroblasts in medium containing Dulbecco's modified Eagle's medium (DMEM) supplemented with

20% fetal bovine serum (FBS) and 50 µg/ml gentamicin. After orbital fibroblasts isolation, 10% FBS, DMEM and 50 µg/ml gentamicin was used for further culture at 37 °C and 5% CO_2. Orbital fibroblasts were used for experiments between the 4th and 12th passages.

Lactate dehydrogenase (LDH) cytotoxicity assay

Orbital fibroblasts were seeded at 6 x 10^3 cells/well into 96-cell plate with 1% FBS DMEM for 24 hours to allow the cells to attach overnight. After that, cells were pretreated with HDACis for 24 hours. The cell supernatants were collected to determine cell toxicity by LDH assay according to the manufacturer's protocol (BioVision Inc., CA, USA). Briefly, 10 µl of cell culture supernatant was mixed with 100 µl of LDH reaction mix for 30 minutes. Then 10 µl of stop solution was added and the absorbance was measured with microplate reader at 450 nm. Then % cytotoxicity was calculated as follows:

$$\%\ Cytotoxicity = \frac{(\text{Test Sample} - Negative\ Control)}{(Positive\ Control - Negative\ Control)}\ x\ 100$$

Orbital fibroblasts proliferation assay

Orbital fibroblasts were seeded at 6 x 10^3 cells/well into 96-cell plate with DMEM containing 1% FBS and antibiotics for 24 hours to allow the cells to attach overnight. For prophylactic effect, cells were pretreated with HDACis at concentrations that were not toxic to the cells for 24 hours and followed by further stimulation with PDGF-BB (50 ng/ml) for 24 hours. Another experiment on the treatment effect, cells were treated with HDACis, simultaneously with 50 ng/ml PDGF-BB stimulation for 24 hours. Cell numbers were detected by colorimetric assay using methylene blue dye as described previously study (Virakul, Dalm, et al., 2014). Briefly, cell culture supernatant was removed, followed by cell fixation with 50 µl of 20% formaldehyde for 48 hours. After removing of the 20% formaldehyde, cells were stained with 50 µl of 1% methylene blue for 30 minutes. Cell bound dye was eluted with ice-cold 1:1 of HCI: absolute ethanol solution and the absorbance was measured with microplate reader at 620 nm. % Proliferation was calculated as follows:

$$\%\ Proliferation = \frac{\text{Test Sample}}{Negative\ Control}\ x\ 100$$

Hyaluronan, IL-6 and IL-8 production by orbital fibroblasts

Orbital fibroblasts were seeded at 1 x 10^5 cells/well into 12-cell plate with DMEM containing 1% FBS and antibiotics for 24 hours. Subsequently, HDACis were added into the cultures for another 24 hours. Cells were further stimulated with PDGF-BB (50 ng/ml) for 24 hours. Another experiment on the treatment effect, cells were treated simultaneously with HDACis and PDGF-BB (50 ng/ml) for 24 hours. The cell culture supernatants were collected for hyaluronan, IL-6 and IL-8 measurement. Hyaluronan level was measured by ELISA kit according to the manufacturer's protocol (R&D system Inc., MN, USA). IL-6 and IL-8 concentrations were determined by ELISA according to the manufacturer's protocol (BioLegend Inc., CA, USA).

***HDACs* mRNA expression in orbital fibroblasts**

Orbital fibroblasts were seeded at 4 x 10^5 cells/well into 6-well plates in DMEM containing 1% FBS and antibiotics overnight. The cultures were stimulated with PDGF-BB (50 ng/ml) for 1, 2, 4, 6 and 24 hours. Total RNA was extracted from the cells using GenElute™ Total RNA Purification Kit according to the manufacturer's protocol (Sigma-Aldrich Inc., MO, USA) and converted to cDNA using iScript™ cDNA Synthesis Kit according to the manufacturer's protocol (Bio-Rad Inc., CA, USA). Expression levels of *HDAC1, 2, 3, 4, 5, 6* and *8* were determined with TaqMan® Gene Expression Assays (Thermo Fisher Scientific Inc., NY, USA; Hs00606262_g1, Hs00231032_m1, Hs00187320_m1, Hs01041648_m1, Hs00608351_m1, Hs00997427_m1 and Hs00218503_m1, respectively) with SsoAdvanced™ Universal Probes Supermix (Bio-Rad Inc., CA, USA) by real-time PCR (CFX96 Touch™ Real-Time PCR Detection System, Bio-Rad Inc., CA, USA). *HDACs* expression level was normalized with *ABL*.

Statistical analysis

Data was presented as mean ± the standard error of the mean (SEM) and analyzed by paired t-test. A *P*-value <0.05 was considered to indicate a statistically significant difference.

RESULTS

Prophylactically applied HDAC3i, 4i and 4/5i decreased PDGF-BB-induced orbital fibroblasts proliferation

Orbital fibroblasts were pre-treated with different concentrations of HDACis from other previous studies and their effect on cell toxicity was

measured by LDH assay. The results indicated that the different concentrations of Quisinostat (0.02, 0.04, 0.08 μM), CAY10683 (0.1, 1, 10 μM), RGFP966 (10, 15, 20 μM), Tasquinimod (1, 10, 20 μM), LMK-235 (0.25, 0.5, 1 μM), Tubastatin A (1, 2, 3 μM) and PCI-34051 (0.02, 0.1, 1 μM), tested did not induce cytotoxicity (Figure 1).

Pre-treatment of the orbital fibroblasts with different concentrations of class I HDACis; HDAC1i (Quisinostat), HDAC2i (CAY10683), HDAC8i (PCI-34051) and class IIb HDACis; HDAC6i (Tubastatin A) did not affect PDGF-BB-induced orbital fibroblast proliferation (Figure 2A and C). In contrast, pre-treatment with the maximum concentration of the class I HDACi; HDAC3i (RGFP966) significantly reduced PDGF-BB-induced orbital fibroblast proliferation ($P < 0.01$; Figure 2A). Moreover, the maximum concentration of class IIa HDACis; HDAC4i (Tasquinimod) and HDAC4/5i (LMK-235) also significantly decreased PDGF-BB-induced orbital fibroblasts proliferation ($P < 0.05$; Figure 2B).

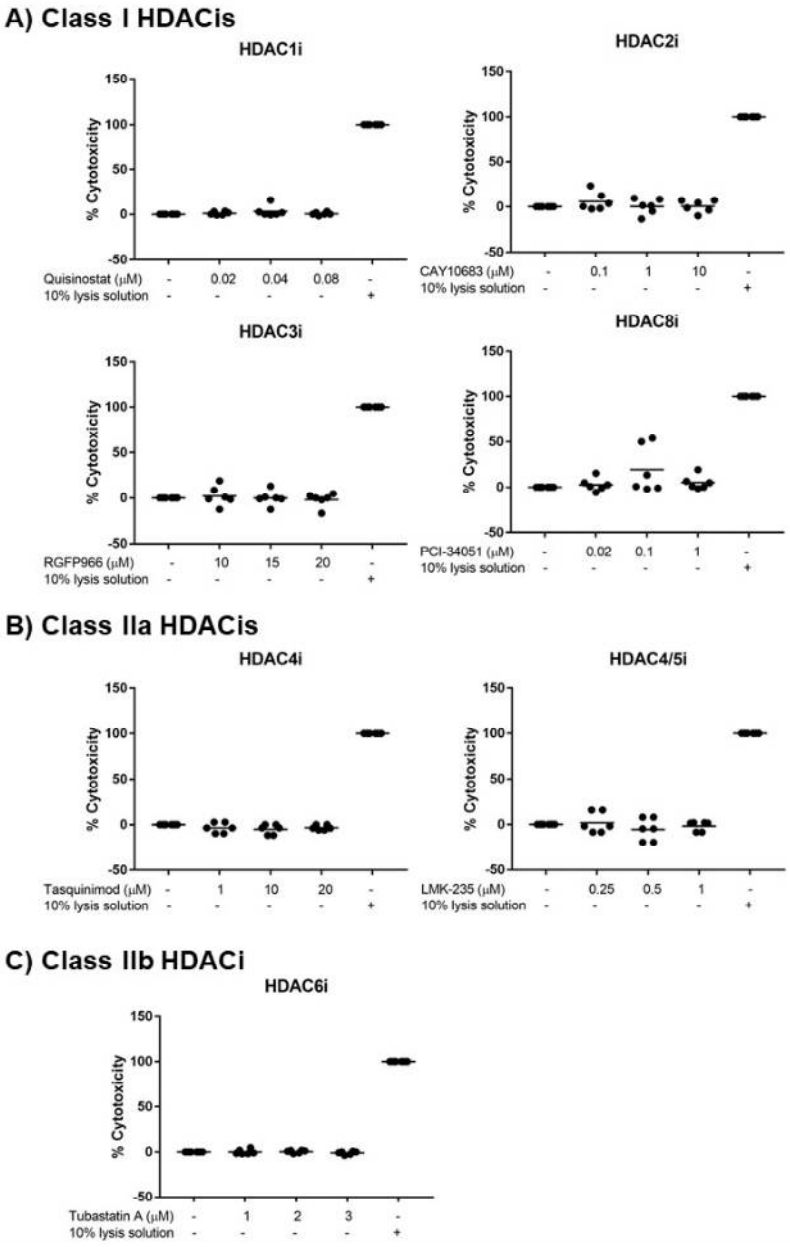

Figure 1. Cytotoxicity of isoform-selective HDACis on orbital fibroblasts. Orbital fibroblasts (n=6) were pre-treated with (A) class I HDACis; Quisinostat, CAY10683, RGFP966, PCI-34051 (B) class IIa HDACis; Tasquinimod, LMK-235 and (C) class IIb HDACi; Tubastatin A for 24 hours and then LDH cytotoxicity assay was measured. 10% lysis solution was used for positive control of the test.

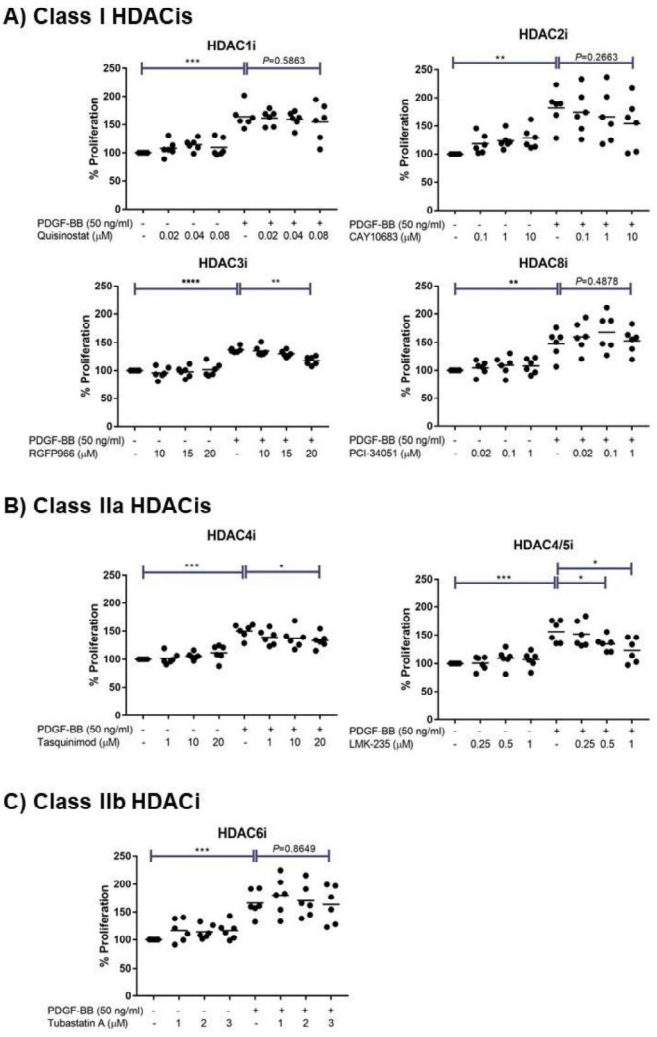

Figure 2. Isoform-selective HDACis affected on PDGF-BB-induced orbital fibroblasts proliferation. Orbital fibroblasts (n=6) were pre-treated for 24 hours with different HDACi concentrations; (A) class I HDACis; Quisinostat, CAY10683, RGFP966, PCI-34051 (B) class IIa HDACis; Tasquinimod, LMK-235 and (C) class IIb HDACi; Tubastatin A, followed by 50 ng/ml PDGF-BB stimulation. Orbital fibroblasts proliferation was determined by proliferation assay. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 and *****P* < 0.0001 compared with unstimulated orbital fibroblasts or orbital fibroblasts stimulated by PDGF-BB alone.

Prophylactic effect of HDAC1i, 3i and 4i on PDGF-BB-induced IL-6 by orbital fibroblasts stimulation

To investigate the effect of specific HDACi on pro-inflammatory cytokines production, the highest non-toxic concentration of each HDACi was used. Pre-treatment of the orbital fibroblasts with class I HDACis; HDAC1i (Quisinostat), HDAC3i (RGFP966) and class IIa HDACi; HDAC4i (Tasquinimod) significantly enhanced the PDGF-BB-induced IL-6 production by the orbital fibroblasts ($P < 0.05$; Figure 3A and B). On the other hand, class I HDACis; HDAC2i (CAY10683), HDAC8i (PCI-34081) and class IIb HDACi; HDAC6i (Tubastatin A) did not affect PDGF-BB-induced IL-6 production (Figure 3A and C). None of the HDACis tested significantly affected the PDGF-BB-induced IL-8 production by orbital fibroblasts (Figure 4).

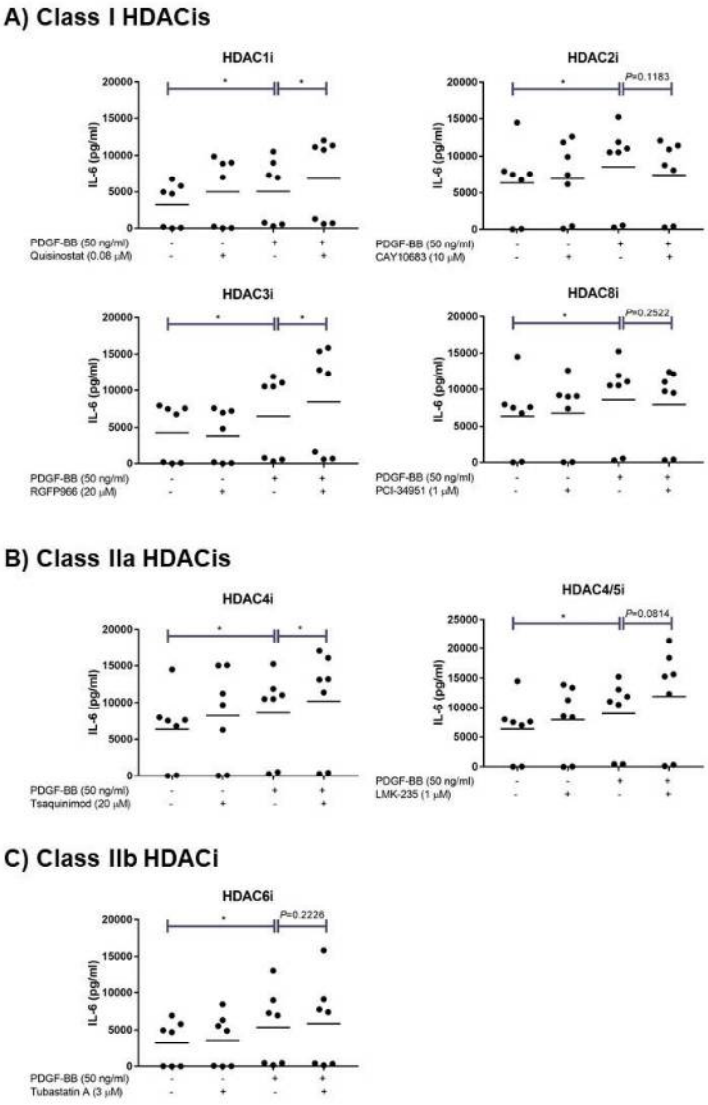

Figure 3. Isoform-selective HDACis influenced IL-6 production from orbital fibroblasts after PDGF-BB stimulation. Orbital fibroblasts (n=7) were pre-treated with non-toxic and maximum HDACis concentration including (A) class I HDACis; Quisinostat, CAY10683, RGFP966, PCI-34051 (B) class IIa HDACis; Tasquinimod, LMK-235 and (C) class IIb HDACi; Tubastatin A for 24 hours, followed by 50 ng/ml PDGF-BB stimulation. IL-6 production was determined by ELISA. * $P < 0.05$, compared with unstimulated orbital fibroblasts or orbital fibroblasts stimulated by PDGF-BB alone.

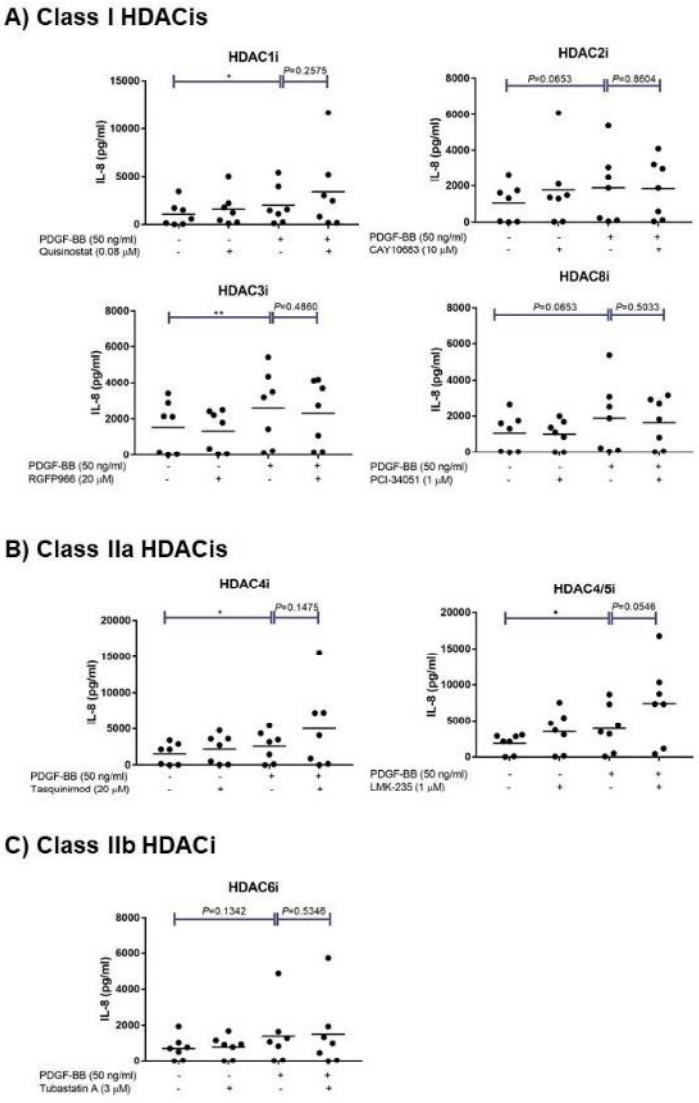

Figure 4. Isoform-selective HDACis affected on IL-8 produced by PDGF-BB-induced orbital fibroblasts activation. Orbital fibroblasts (n=7) were pre-treated with non-toxic and maximum concentration of HDACi; (A) class I HDACis; Quisinostat, CAY10683, RGFP966, PCI-34051 (B) class IIa HDACis; Tasquinimod, LMK-235 and (C) class IIb HDACi; Tubastatin A for 24 hours, then 50 ng/ml PDGF-BB was stimulated for 24 hours. IL-8 production was determined by ELISA. * $P < 0.05$, ** $P < 0.01$, compared with unstimulated orbital fibroblasts or orbital fibroblasts stimulated by PDGF-BB alone.

Prophylactic effect of HDAC2i, 4i and 4/5i on PDGF-BB-induced hyaluronan production by orbital fibroblasts

The class I HDACi directed against HDAC2 (CAY10683) significantly reduced PDGF-BB-induced hyaluronan production by orbital fibroblasts ($P < 0.05$; Figure 5A). In addition, the class IIa HDACis HDAC4i (Tasquinimod) and HDAC4/5i (LMK-235) significantly decreased PDGF-BB-induced hyaluronan production by orbital fibroblasts ($P < 0.05$; Figure 5B). The class I HDACis directed against HDAC1, 3 and 8 (Quisinostat, RGFP966 and PCI-34051, respectively) and the class IIb HDACi against HDAC6 (Tubastatin A) did not affect PDGF-BB-induced hyaluronan production (Figure 5A and C).

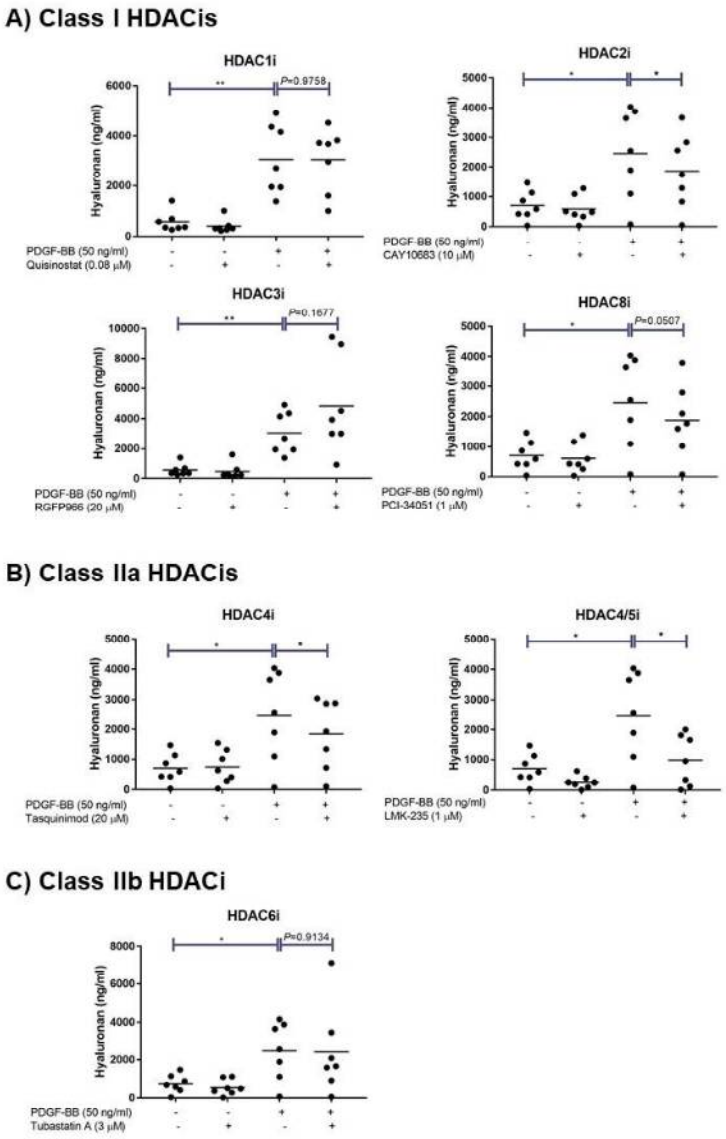

Figure 5. Isoform- selective HDACis regulated PDGF- BB- mediated hyaluronan in orbital fibroblasts. Orbital fibroblasts (n=7) were pre- treated with HDACis including (A) class I HDACis; Quisinostat, CAY10683, RGFP966, PCI-34051 (B) class IIa HDACis; Tasquinimod, LMK-235 and (C) class IIb HDACi; Tubastatin A for 24 hours, followed by stimulated with 50 ng/ml PDGF-BB. Hyaluronan production was analyzed by ELISA. *$P <$ 0.05, **$P < 0.01$ compared with unstimulated orbital fibroblasts or orbital fibroblasts stimulated by PDGF-BB alone.

Treatment effect of HDAC4/5i on PDGF-BB-induced orbital fibroblast activation

HDACis found effective in the prophylactic experimental set-up were subsequently tested in a set of experiments more representative for treatment. Hereto, orbital fibroblasts were stimulated with PDGF-BB (50 ng/ml) and simultaneously the HDACi of interest was added to the culture. In this setting only the class IIb HDACi HDAC4/5i (LMK-235) significantly decreased PDGF-BB-induced orbital fibroblasts proliferation ($P < 0.05$; Figure 6A).

Moreover, class I HDAC1i (Quisinostat), and the class IIb HDACis HDAC4i (Tasquinimod) and HDAC4/5i (LMK-235), but not HDAC3i (RGFP966), significantly enhanced the PDGF-BB-induced IL-6 production by the orbital fibroblasts (HDAC1i; $P < 0.01$, HDAC4i and 4/5i; $P < 0.05$; Figure 6B), while IL-8 production was significant elevated by class I HDACi; HDAC1 (Quisinostat) and class IIa HDACi; HDAC4 (Tasquinimod) (HDAC1i; $P <$ 0.05, HDAC4i; $P < 0.01$; Figure 6C), but not HDAC3i (RGFP966) and HDAC4/5i (LMK-235).

Furthermore, class IIa HDACi HDAC4/5 (LMK-235) significantly reduced PDGF-BB-induced hyaluronan production by the orbital fibroblasts ($P < 0.01$; Figure 6D).

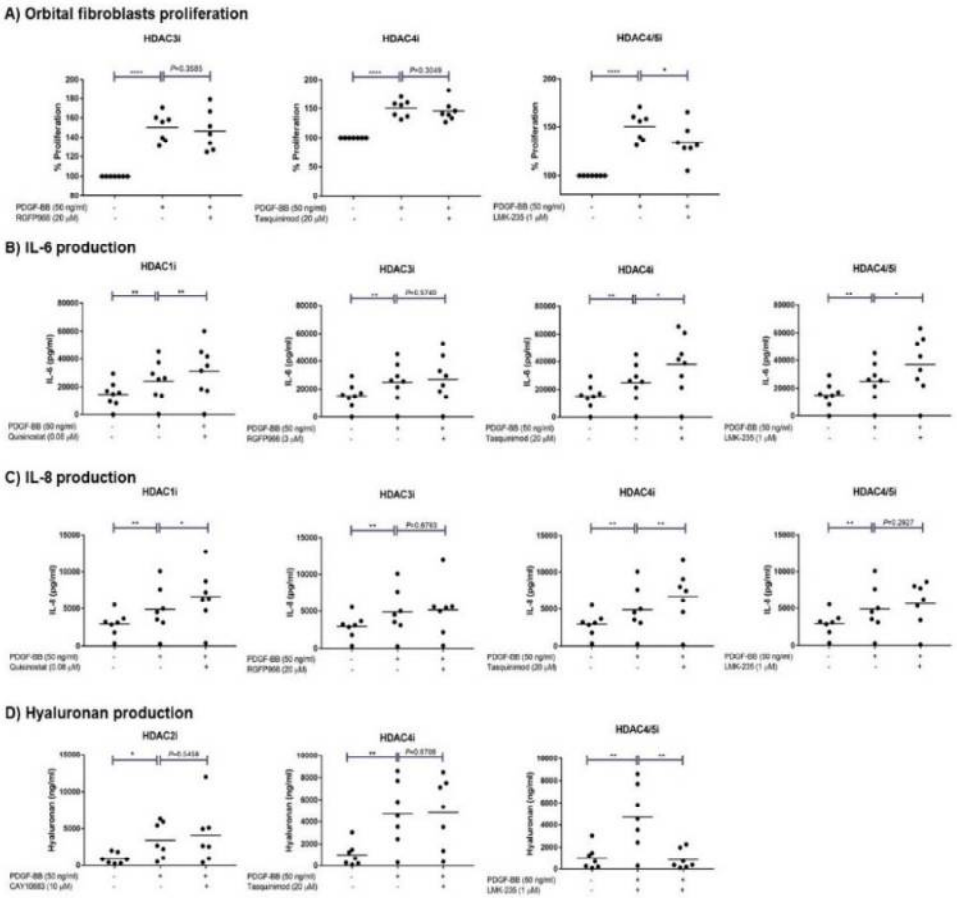

Figure 6. Treatment effect of selected HDACis in PDGF-BB-induced orbital fibroblasts stimulation. (A) Orbital fibroblasts (n=7) were treated with maximum concentration of RGFP966, Tasquinimod or LMK-235, simultaneously with 50 ng/ml PDGF-BB stimulation for 24 hours, then orbital fibroblasts proliferation was analyzed. (B) IL-6 production from orbital fibroblasts (n=8) was determined by ELISA after co-stimulation for 24 hours with 50 ng/ml PDGF-BB and Quisinostat, RGFP966, Tasquinimod or LMK-235. (C) Quisinostat, RGFP966, Tasquinimod or LMK-235 was treated in PDGF-BB-induced orbital fibroblasts activation (n=7) for 24 hours, finally IL-8 ELISA was performed. (D) PDGF-BB-induced orbital fibroblasts activation (n=7) was treated with CAY10683, Tasquinimod or LMK-235 for 24 hours and hyaluronan ELISA was performed. *$P <$ 0.05, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$ compared with unstimulated orbital fibroblasts or orbital fibroblasts stimulated by PDGF-BB alone.

The effect of PDGF-BB on *HDAC* expression by orbital fibroblasts

All tested HDACs were expressed by the orbital fibroblasts under basal culture conditions, with HDAC2 being expressed highest and HDAC4 being expressed at the lowest level. Stimulation of orbital fibroblasts with PDGF-BB resulted in slightly yet significantly higher expression levels of *HDAC3* and *4* mRNA at 24 hours after stimulation ($P < 0.05$; Figure 7A and B), whereas *HDAC5* expression was significantly decreased after 24 hours of PDGF-BB stimulation ($P < 0.05$; Figure 7B). Moreover, *HDAC6* expression showed significantly lower expression after 2 hours of PDGF-BB stimulation ($P < 0.05$; Figure 7C).

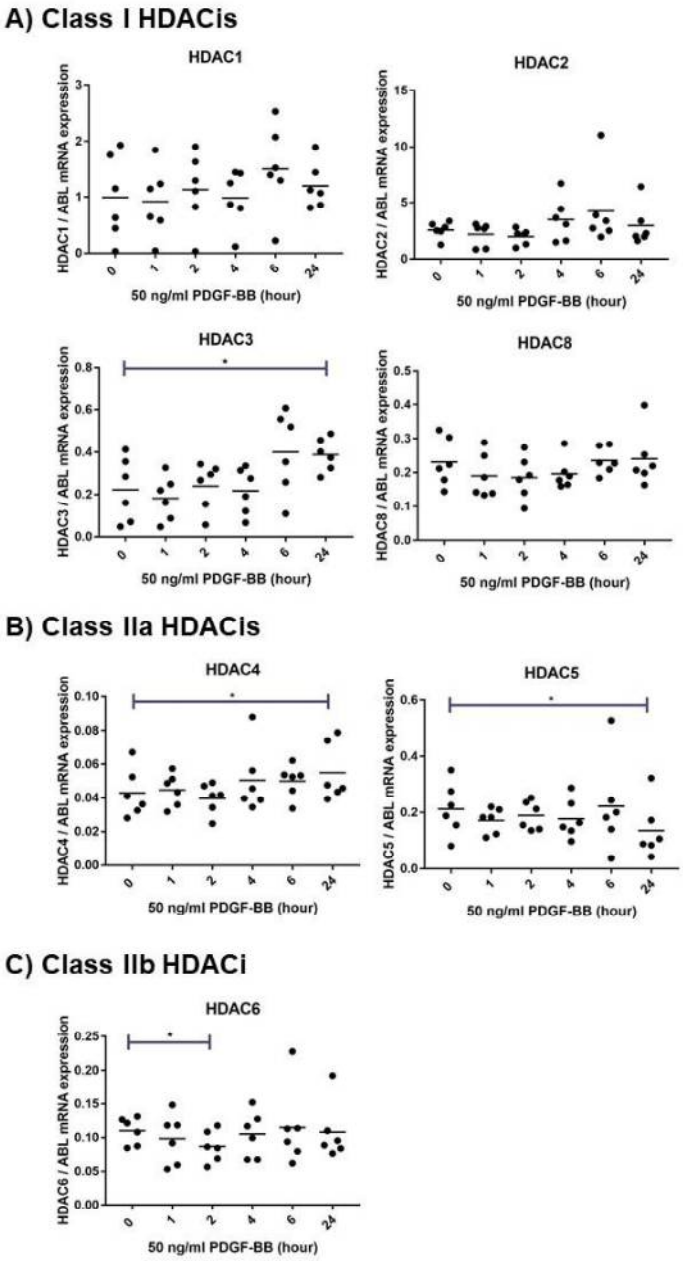

Figure 7. *HDACs* mRNA expression upon PGDF-BB stimulation. Orbital fibroblasts (n=6) were induced by PDGF-BB for 0, 1, 2, 4, 6 and 24 hours and then (A) class I *HDACs*; *HDAC1, 2, 3, 8* (B) class IIa *HDACs*; *HDAC4, 5* and (C) class IIb *HDAC*; *HDAC6* expressions were determined in relation to *ABL* by RT-qPCR. $*P < 0.05$, compared with untreated orbital fibroblasts.

DISCUSSION

This study used isoform-selective HDACis to find an association between HDACs and orbital fibroblasts activation. The results indicated that specific HDAC isoforms are involved in the stimulatory effect of PDGF-BB on proliferation and hyaluronan production by orbital fibroblast. In contrast, they seem to inhibit PDGF-BB-induced IL-6 and IL-8 production by the orbital fibroblasts.

HDAC3i (RGFP966), HDAC4i (Tasquinimod) and HDAC4/5i (LMK-235) significantly decreased PDGF-BB-induced orbital fibroblasts proliferation in a prophylactic setting ($P < 0.05$; Figure 2A and B). Of these, only HDAC4/5i (LMK-235) also significantly reduced PDGF-BB-induced orbital fibroblasts proliferation ($P < 0.05$; Figure 6A) when added simultaneously with PDGF-BB to the orbital fibroblast cultures. Previous studies found that HDAC3 inhibition decreased multiple myeloma proliferation (Ho et al., 2019). Additionally, inhibition of class IIa HDACs (HDAC4, 5, 7 and 9) significantly decreased proliferation of pulmonary artery endothelial cells and fibroblasts from idiopathic pulmonary fibrosis (Kim et al., 2015; Korfei et al., 2015). Although down-regulation of HDAC1 was found to suppress proliferation by fibroblast-like synoviocytes and cardiac fibroblasts (Hawtree et al., 2015; Lin et al., 2019), the present study found no inhibitory effect of HDAC1i (Quisinostat) on PDGF-BB-induced orbital fibroblasts proliferation. Our results suggest involvement of especially the class IIa HDAC4 and/or 5 in PDGF-BB-induced GO orbital fibroblasts proliferation.

Unexpectedly, the experiments in the prophylactic set-up revealed that HDAC1i (Quisinostat), HDAC3i (RGFP966) and HDAC4i (Tasquinimod) significantly elevated PDGF-BB-induced IL-6 production ($P < 0.05$; Figure 3A and B), while in the treatment orientated experiments, HDAC1i (Quisinostat) and HDAC4i (Tasquinimod) were found to increase both PDGF-BB-induced IL-6 and IL-8 production ($P < 0.05$; Figure 6B and C) and HDAC4/5i (LMK-235) significantly enhanced the IL-6 production ($P < 0.05$; Figure 6B). This data indicates that HDAC3, 4 and 5 somehow prevent PDGF-BB-induced IL-6 and IL-8 production. A major transcription factor which controls the expression of pro-inflammatory cytokines is NF-*k*B (Liu et al., 2017). Previous studies reported involvement of HDAC1, 3 and 4 in NF-*k*B deacetylation of the NF-*k*B Rel domain of p65, they promoted p65 to interact with the inhibitory protein I*k*Bα, and consequently inhibited NF-*k*B translocation into the nucleus (Ashburner et al., 2001; Pham et al., 2016). These processes cause repression of NF-*k*B-dependent gene expression in TNF-

activated HeLa cells as well as LPS-activated macrophages (Ashburner et al., 2001; Pham et al., 2016). Moreover, in line with our findings pan-HDACi was found to increase NF-kB-regulated IL-8 gene expression in TNF-activated HeLa cells (Ashburner et al., 2001). Our current data corroborate the previous notion that activation of the NF-kB signaling pathway in orbital fibroblasts is involved in shaping the orbital inflammatory milieu in GO (van Steensel et al., 2010). The observation that HDAC1, 3, 4 and/or 5 inhibit inflammatory responses in orbital fibroblasts from GO patients were not found in previous studies and overall, these data imply that suppression of these HDACs might aggravate orbital inflammation in those GO patients that have excess PDGF-BB present in their orbital tissue, yet this requires further study.

The studies on hyaluronan production showed that pre-treatment with HDAC2i (CAY10683), HDAC4i (Tasquinimod) and HDAC4/5i (LMK-235) significantly reduced PDGF-BB-induced hyaluronan production by orbital fibroblasts ($P < 0.05$; Figure 5A and B). Moreover, in the experimental set-up more representative for actual disease treatment HDAC4/5i (LMK-235) was found to significantly decrease PDGF-BB-induced hyaluronan production ($P < 0.01$; Figure 6D). Previous studies found that HDAC2 knockdown inhibited TGF-β1-induced production of PAI-1, fibronectin, collagen I, and collagen IV in Peyronie's plaque fibroblasts (Ryu et al., 2013). HDAC4 and 5 were found to contribute to the accumulation of extracellular matrix proteins (including collagen type 1 and fibronectin) in human renal epithelial cells (Choi et al., 2016). Although our current study did not find an inhibitory effect of HDAC1i (Quisinostat) on PDGF-BB-induced hyaluronan production by orbital fibroblasts activation, HDAC1 knockdown has previously been shown to result in reduced expression of hyaluronate synthase 2 (HAS2) in human rheumatoid arthritis fibroblasts (Hawtree et al., 2015). Collectively the data here presented suggest involvement of class IIa HDACs (HDAC4 and 5) in regulating PDGF-BB-induced hyaluronan production by orbital fibroblasts in GO.

Differential expression of *HDACs* might participate in regulating PDGF-BB-induced orbital fibroblasts activation. Therefore, this study also examined m RNA expression levels of *HDAC1, 2, 3, 4, 5, 6* and *8* mRNA. We showed that PDGF-BB enhanced the expression levels of *HDAC3* and *4* in orbital fibroblasts ($P < 0.05$; Figure 7A and B), while it decreased the expression levels of *HDAC5* and *6* ($P < 0.05$; Figure 7B and C). Previous studies described higher expression of *HDAC3* and *4* mRNA in primary lung fibroblasts from idiopathic pulmonary fibrosis patients compared to non-diseased control (Korfei et al., 2015). Down-regulation of *HDAC5* has been reported in type I interferon activated rheumatoid arthritis fibroblast-like

synoviocytes (Angiolilli et al., 2016), while a down-regulated *HDAC6* in fibroblasts is to our knowledge never reported before. We propose that aberrant *HDAC3*, *4* and *5* expression can be associated with GO pathogenesis, yet this requires further study.

This study found that HDAC4 strongly regulated PDGF-BB-induced orbital fibroblasts activation. It is possible that class IIa HDACs rather than class I and IIb HDACs might involve in the pathogenesis of GO in PDGF-BB-induced orbital fibroblasts activation. Furthermore, HDAC4 found to predominantly regulate the disease which is confirmed by the results of Tasquinimod, LMK-235 and *HDAC4* mRNA expression. However, HDACis might have non-target activity and showed different results between prophylactic and treatment effects. Further experiments require the validation in more orbital fibroblast samples. In addition, increasing HDACis concentration and specific HDAC knockdown experiments might subsequently be investigated in orbital fibroblasts in response to PGDF-BB stimulation. Moreover, our study illustrates that the pathogenic contribution of HDACs might be disease specific.

CONCLUSION

To our knowledge, this is the first study to show the important role of HDAC4 in PDGF-BB-induced orbital fibroblasts activation. These findings might lead to understanding of HDACs activity in GO and may be helpful for the development of novel treatment for GO patients.

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