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The Effect Of Histone Methyltransferase In Orbital Fibroblast From Graves' Ophthalmopathy

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ABSTRACT

Graves' ophthalmopathy (GO) is an extra-thyroidal complication of Graves' disease (GD). The clinical presentations of GO are upper eyelid retraction, erythema of periorbital tissue, conjunctivitis, proptosis and fibrosis. Some patients have severe symptoms such as optic neuritis leading to sight-threatening symptoms such as exposure keratitis and optic nerve compression or dysthyroid optic neuropathy. Platelet-derived growth factor-BB (PDGF-BB) is an important growth factor in the pathogenesis of GO. As orbital fibroblasts in orbital tissue express platelet-derived growth factor receptors (PDGF-R) on the surface, PDGF-BB stimulates orbital fibroblasts activities such as cytokine and hyaluronan production, proliferation and adipogenesis. Presently, active GO is treated with high-dose systemic glucocorticoids (GCs) as first line treatment which also carries several serious side effects, including hyperglycemia, fulminant hepatitis and a generalized state of immunosuppression. Therefore, novel therapeutic strategies with fewer complications are needed for GO. Epigenetic modifications control gene expression. One of the mechanisms is histone modification at the N-terminal amino acid. Histone methylation is the process of transferring methyl groups to lysine or arginine residues on the histone tail by a group of enzymes called

histone methyltransferases (HMTs). In this study, we investigated the role of histone lysine methyltransferases (KHMTs) and histone arginine methyltransferase (PRMT5) in PDGF-BB-induced orbital fibroblasts activation by using specific inhibitors. Inhibition of KHMT (with DZNeP, BIX01294 or Pinometostat) as well as PRMT5 (with GSK591) inhibited PDGF-BB–induced hyaluronan production by orbital fibroblasts, both in a prophylactic and more treatment related experimental set-up. Inhibition of KHMT with DZNeP also prevented PDGF-BB-induced production of IL-6 and IL-8 by orbital fibroblasts, while BIX01294 DNNeP, pinometostat and GSK591 also inhibited PDGF-BB-induced orbital fibroblasts proliferation, again both in a prophylactic and more treatment related experimental set-up. These data from inhibitors screening suggested that the KHMT enzymes (G9a, EZH2 and DOT1L) and PRMT5 induce orbital fibroblasts proliferation, cytokine and hyaluronan production.

Keywords: Fibrosis, Graves' ophthalmopathy, Histone methylation, Orbital fibroblast

INTRODUCTION

Graves' disease (GD) is an autoimmune disease that is characterized by hyperthyroidism induced by stimulatory autoantibodies directed against thyroid stimulating hormone receptor (TSHR) (De Leo, Lee, & Braverman, 2016). Graves' ophthalmopathy (GO) is an extra-thyroidal complication found in 25-50% of GD patients (Prabhakar, Bahn, & Smith, 2003). Upper eyelid retraction, edema, erythema of periorbital tissue, conjunctivitis, limitation of extraocular movement, proptosis and fibrosis are the symptoms of GO (Prabhakar et al., 2003). Severe symptoms such as corneal ulcers and optic neuritis leading to loss of vision occurs in some patients (R. S. Bahn, 2010).

Orbital fibroblasts are important cells in the pathogenesis of GO where they contribute to the orbital inflammation and tissue expansion (Virakul et al., 2014). Orbital fibroblasts express TSHR at their membrane surface and the expression of TSHR is even higher on orbital fibroblasts from GO patients and consequently these cells become activated by the TSHR stimulatory autoantibodies causing this extra-thyroidal complication (Rebecca S. Bahn, 2015). In GO the orbital fibroblasts produce excessive amounts of hyaluronan and various cytokines, and can differentiate into adipocytes (R. S. Bahn, 2010; Virakul et al., 2014). Platelet-derived growth factor-BB (PDGF-BB) is produced by macrophages, monocytes and mast cells that infiltrated the orbital tissue in GO and represents an important growth factor in GO pathogenesis

(Virakul et al., 2014). Orbital fibroblasts stimulated with PDGF-BB enhance their proliferation, increase the production of hyaluronan and cytokines like IL-6 and IL-8, differentiate into adipocytes and upregulate TSHR expression which makes these cells even more susceptible to activation with TSHR stimulatory autoantibodies (van Steensel, Hooijkaas, et al., 2012; van Steensel, Paridaens, et al., 2012; Virakul et al., 2014).

The common treatments for active GO are high-dose systemic glucocorticoids (GCs) and radiotherapy (Bartalena et al., 2016; Iñigo San Miguel et al., 2018). In sight-threatening GO patients who fail to respond to GCs require decompression surgery to reduce orbital tissue content (Bartalena et al., 2016). The current treatments of GO have many negative effects on the patients (I. San Miguel et al., 2018). For example, patients treated with high-dose systemic GCs for longer than three months developed a long lasting immunosuppressive condition that makes them vulnerable to infections (I. San Miguel et al., 2018). Recently, rituximab, a monoclonal antibody targeting CD20 on B-lymphocyte, was tested for treatment in GO patients; however, rituximab also caused ulcerative colitis and urinary system infections (Şimşek, 2017). In addition, teprotumumab, an anti-IGF-1 receptor, was tested in GO patients; however, 70% of GO patients responded to teprotumumab and patients with diabetes can show hyperglycemia (Smith et al., 2017). So, new therapeutic strategies are in need to effectively treat GO patients.

Epigenetic modification of functionally relevant DNA alterations, including for instance DNA methylation and histone modification, has been proposed to control gene expression in manner independent of the DNA sequence (Wang, Shao, Song, Xu, & Zhang, 2017). The gut microbiome influences epigenetic regulation and may have a role in GD as well as other diseases (Brix, Kyvik, Christensen, & Hegedus, 2001; Czyz, Morahan, Ebers, & Ramagopalan, 2012; Qin & Wade, 2018). Moreover, the gut microbiota was reported to be associated with the development of GO in TSHR-immunized mice (Masetti et al., 2018). Thus, epigenetic regulation may be involved in GD and GO.

Histone modification is the epigenetic mechanism where histone tails modifications occur at the N-terminal amino acids leading to several changes of the chromatin structure and function (Araki & Mimura, 2017). Histone methylation is the process of transferring methyl groups to lysine or arginine residues on the histone tail by enzymes belonging to the family of histone methyltransferases (HMTs) (Araki & Mimura, 2017). Histone lysine methyltransferases (KHMTs) can induce mono-, di- and tri-methylation, while arginine methyltransferases (PRMTs) can induce mono- and di-methylation to

the histone tails (Araki & Mimura, 2017). The number of methyl groups attached to lysine or arginine residues on the histone tail can determine either increased or decreased gene transcription (Araki & Mimura, 2017).

HMT inhibitors are tested for the treatment of several fibrotic diseases. In a pulmonary fibrosis mouse model, DZNep, an inhibitor that targets EZH2 lysine methyltransferase on histone H3 on lysine 27 (H3K27), reduced TGF-β1-induced myofibroblasts differentiation (X. Xiao et al., 2016). Moreover, in a mice peritoneal fibrosis model, BIX01294, an inhibitor that specifically targets the G9a lysine methyltransferase on histone H3 on lysine 9 (H3K9), reduced macrophage infiltration the number of TGF- β1 producing cells and fibroblast numbers, which went along with thinning of the of peritoneal tissue (Maeda et al., 2017). We postulate that histone methyltransferases contribute to GO pathogenesis by modulating orbital fibroblast effector functions and consequently may represent potential novel targets for therapy in GO.

MATERIAL AND METHODS

Reagents

Histone methyltransferases inhibitors were obtained from Selleckchem, US and details are given in table 1. Recombinant human PDGF-BB (Biolegend, San Diego, CA), hyaluronan ELISA (R&D system, Minnesota, US), human IL-6 ELISA max standard kit (Biolegend, San Diego, CA), human IL-8 ELISA max standard kit (Biolegend, San Diego, CA), LDH-Cytotoxicity Colorimetric Assay Kit II (BioVision, Milpitas, CA) and GenElute Universal Total RNA Purification Kit (Sigma-Aldrich, St Louis, MO) and primer-probe combinations (Thermo Fisher Scientific, Vantaa, Finland, see for details table 2)

Isolation of primary Graves' orbital fibroblasts

Orbital fibroblasts were isolated from orbital tissue of GO patients at an inactive stage of disease who underwent orbital decompression surgery at King Chulalongkorn Memorial Hospital (Bangkok, Thailand). Informed consent was obtained in accordance with the principles of the Declaration of Helsinki. Ethical approval was obtained from the local medical ethics committee with the protocol number 401/61. For primary cell isolation, tissue explants were cut and placed in 6-well plates with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% fetal bovine serum (FBS) and antibiotics (Gentamicin). After that, orbital fibroblasts from explants, were

cultured in DMEM with 10% FBS and antibiotics and incubated in humidified 5% CO_2 incubator at 37°C.

Cytotoxicity assay

The cytotoxicity of the histone methyltransferase inhibitors was determined by lactate dehydrogenase (LDH) release in the culture medium. Orbital fibroblasts were treated with different concentrations of histone methyltransferase inhibitor for 24 hours. Then, supernatant was collected and analyzed for LDH (LDH assay, BioVision, Milpitas, CA) according to the manufacturer's protocol. Optical density was measured by microplate reader at 450 nm.

Effects of histone methyltransferase inhibitors on orbital fibroblast proliferation

Orbital fibroblasts were plated at 5.0×10^3 cells/well onto 96-well plates with DMEM containing 1% FBS and antibiotics and incubated in humidified 5% CO_2 incubator at 37°C overnight. After that, orbital fibroblasts were pre-incubated with histone methyltransferase inhibitors (Table 1) for 24 hours and stimulated with 50 ng/ml of PDGF-BB, either with or without the histone methyltransferase inhibitors. To explore the effect the histone methyltransferase inhibitors in an experimental set-up more representative for disease treatment, orbital fibroblasts were stimulated for 24 hours with 50 ng/ml PDGF-BB and histone methyltransferase inhibitor was added at the same time as the PDGF-BB. After 24 hours orbital fibroblast proliferation was determined. Six replicates were performed per condition by colorimetric method based on uptake and subsequent release of methylene blue dye. Briefly, culture medium was removed. Plates were washed with phosphate buffered saline (PBS; 200 µl/well) and fixed with 20% formaldehyde in PBS (50 µl/well) at 4°C. After 48 hours, orbital fibroblasts were stained with 1% methylene blue in 0.01M boric acid (50 µl/well) for 30 minutes. After that orbital fibroblasts were washed five times with 0.1M boric acid pH 8.5 using siphon and the methylene blue dye was eluted by addition of ice-cold 1:1 (v/v) ethanol absolute in 0.1M HCL (100 µl/well). After 5 minutes, the optical density was measured at 650 nm using a microplate reader. %Proliferation was calculated by (Test sample/negative control) *100.

Effects of histone methyltransferase inhibitors on cytokine and hyaluronan production by orbital fibroblasts

Orbital fibroblasts were plated at a density of 1.0×10^5 cells/well in 12-well plates in DMEM/1% FBS and incubated in a humidified 5% CO_2 incubator at 37°C overnight to allow adherence. After that, the orbital fibroblasts were pre-incubated with a histone methyltransferase inhibitor for 24 hours and then stimulated with PDGF-BB (50 ng/ml). In another experimental set-up that may better represent actual disease treatment, the orbital fibroblasts were stimulated with PDGF-BB (50 ng/ml) and treated with histone methyltransferase inhibitor at the same time for 24 hours. Supernatant was then collected hyaluronan, IL-6, and IL-8 levels were measured by ELISA according to the manufacturer's protocol.

Histone methyltransferases mRNA expression in PDGF-BB-stimulated orbital fibroblasts

Orbital fibroblasts were plated at 4.0×10^5 cells/well onto 6-well plates with DMEM/1 %FBS and incubated in humidified 5% CO_2 incubator at 37°C overnight to allow adherence. After that, orbital fibroblasts were stimulated with PDGF-BB (50ng/ml) for 0, 1, 2, 4, 6 and 24 hours. Total RNA were isolated using GenElute Universal Total RNA Purification Kit (Sigma-Aldrich, St. Louis, MO, USA) and converted into cDNA using iScript™ Reverse Transcription Supermix for RT-qPCR according to the manufacturer's protocol (Bio-Rad Laboratories, California, US). *DOT1L*, *G9a*, *EZH1*, *EZH2* and *PRMT5* mRNA expression levels were determined by RQ-PCR and normalized to the control gene ABL (CFX96 Touch™ Real-Time PCR Detection System). Other primer-probe combinations (Thermo Fisher Scientific, Vantaa, Finland) used are shown in Table 2.

Statistical analyses

Data were analyzed by using the paired Student's *t*-test. $P < 0.05$ was considered statistically significant. Data are presented as the mean ± standard error of the mean (SEM).

Table 1. Histone methyltransferase inhibitor library

HMTs	HMT inhibitor	Low concentrations (µM)	High concentration (µM)
Histone lysine methyltransferase			
EZH2	EI1	0.500	3.5
	DZNeP	3	6
EZH1	CPI-360	1.5	3
EZH2, EZH1	CPI-169	5	10
G9a	A-366	0.900	1.8
	BIX 01294	2	5.4
MLL	MI-2	1	4
	MM-102	0.800	1.6
SETD7	PFI-2 HCl	0.004	1
DOT1L	Pinometostat	0.600	4.5
SETD8	UNC0379	7.9	15.8
COMT	Entacapone	10	20
Histone arginine methyltransferase			
PRMT3	SGC707	0.445	3.2
PRMT5	GSK591	0.112	5
PRMT Type 1 [PRMT1,3,4,6 and 8]	MS023	0.238	1.4

Table 2. Other primer-probe combinations

Gene	Forward primer (5' - 3')	Forward primer (5' - 3')	Probe (5' FAM – 3' TAMRA)
ABL	TGGAGATAAC ATCTAAGCAT AACTAAAGGT	GATGTAGTTGC TTGGGACCCA	CCATTTTTGG TTTGGGCTTC ACACCATT
G9a		hs01053846_m1	
DOT1L		hs01579928_m1	
EZH1		hs00157470_m1	
EZH2		hs00544830_m1	
PRMT5		hs01047356_m1	

RESULTS

EZH2, G9a, DOT1L and PRMT5 inhibition reduced PDGF-BB-induced orbital fibroblasts proliferation

To investigate the role of histone methyltransferase (HMT) in PDGF-BB- induced orbital fibroblasts proliferation, specific HMT inhibitors at different concentrations were used. Two groups of HMT inhibitors were tested, histone lysine methyltransferases (KHMTs) inhibitors and histone arginine methyltransferases (PRMTs) inhibitors as shown in Table 1. To obtain the maximum pharmacological effect of the inhibitors, orbital fibroblasts (n=3-9) were pre- treated (mimicking a prophylactic treatment setting) with HMT inhibitors. Inhibition of EZH2, G9a or DOTL1 with the highest concentrations of their inhibitors DZNeP (6 µM), BIX01294 (5.4 µM) and Pinometostat (4.5 µM), respectively, all significantly inhibited PDGF- BB- induced orbital fibroblast proliferation Yet, inhibition of these histone lysine methyltransferases never resulted in complete abrogation of PDGF- BB- induced orbital fibroblast proliferation. None of the tested lysine methyltransferase inhibitors inhibited basal proliferation of the orbital fibroblasts (Figure1). Only inhibition of the histone arginine methyltransferase PRMT5 with the inhibitor GSK591 at the highest concentration tested (5 µM) inhibited PDGF- BB- induced orbital fibroblast proliferation, although this inhibition was not complete (Figure 1B). The HMT inhibitors at the concentration tested were not toxic to orbital fibroblasts as determined by LDH release (data not shown).

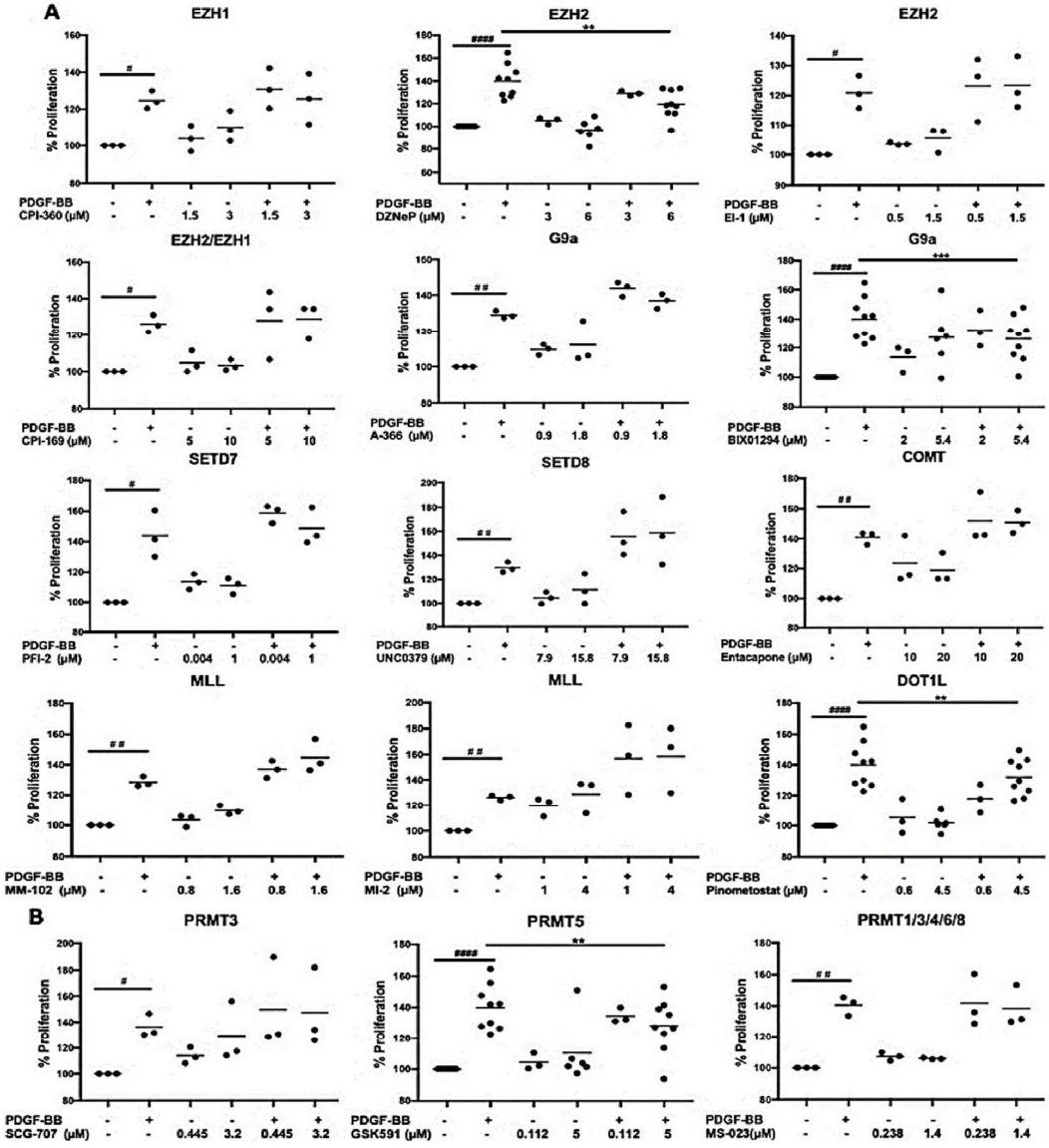
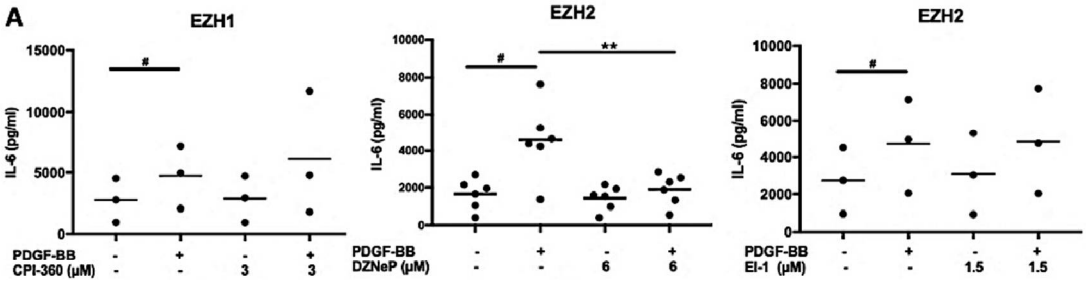


Figure 1. EZH2, G9a, DOT1L and PRMT5 inhibition reduced PDGF-BB-induced orbital fibroblasts proliferation. GO (n=3-9) orbital fibroblasts were pre-incubated with specific histone lysine methyltransferase inhibitors (A) or arginine methyltransferase (B) inhibitors for 24 h, and then stimulated with PDGF-BB (50 ng/ml) in the presence or absence specific HMT inhibitors. After 24 h, proliferation was determined by methylene blue staining. ## and # indicate a p-value of <0.01 and <0.05, respectively, compared to the unstimulated condition, * represents a p-value of <0.05, compared to PDGF-BB stimulation.

EZH2 inhibition inhibits PDGF-BB-induced cytokine production by orbital fibroblasts

As observed previously (Virakul et al., 2014) PDGF-BB induced the production of IL-6 and IL-8 by orbital fibroblasts. Pre-incubation with the histone lysine methyltransferase inhibitors, DZNeP (targeting EZH2), BIX01294 (targeting G9a) and CPI169 (targeting EZH2/EZH1) significantly reduced PDGF-BB-induced IL-6 production by PDGF-BB-induced orbital fibroblasts. Inhibition of EZH2 with DZNeP appeared to abrogate PDGF-BB-induced IL-6 production most efficiently (Figure 2A). Inhibition of the histone arginine methyltransferases PRMT3, PRMT5 and PRMT1/3/4/6/8 with SGC-707, GSK591 or MS023, respectively, did not reduce PDGF-BB-induced IL-6 production (Figure 2B).

Moreover, inhibition of EZH2 with DZNeP significantly reduced PDGF-BB induced IL-8 production (Figure 2C), whereas inhibition of G9a with BIX01294 did not significantly reduce PDGF-BB-IL-8 production (Figure 2C).



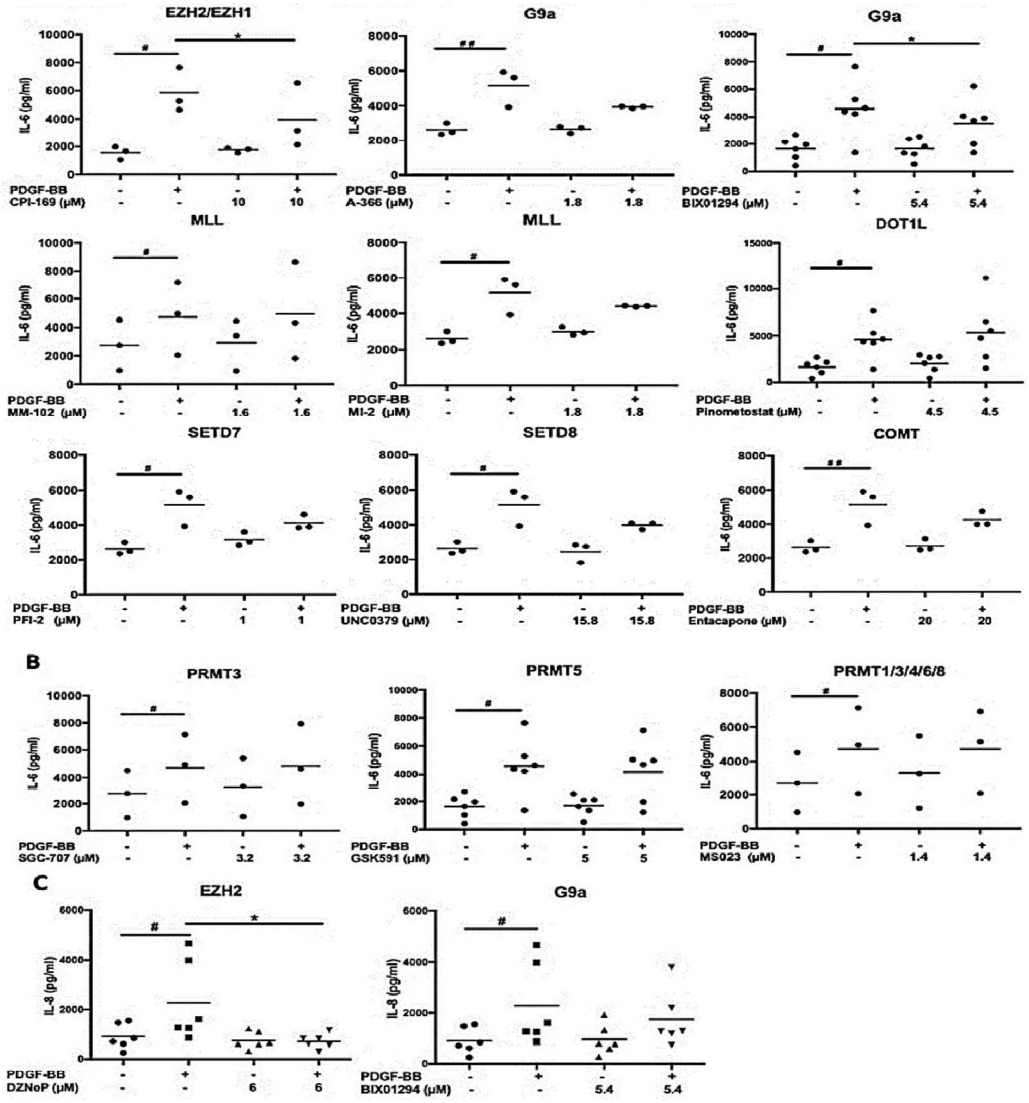


Figure 2. EZH2 inhibition reduced PDGF-BB-induced cytokine production by orbital fibroblasts. GO (n=3-6) orbital fibroblasts were pre-incubated with the highest concentration (as described in figure 1) of specific histone lysine methyltransferase (A, C) or arginine methyltransferase (B) inhibitors for 24 h, and then stimulated with PDGF-BB (50 ng/ml) in the presence or absence specific HMT inhibitors. After 24 h, supernatant was collected, and IL-6 (A, B) and IL-8 (C) levels were measured by ELISA. # indicates a p-value of <0.05, compared to the unstimulated condition, ** and * represent a

p-value of <0.01 and <0.05, respectively, compared to PDGF-BB stimulation.

SETD8, EZH2, DOT1L, G9a, MLL, PRMT5 and PRMT type 1 inhibition reduced PDGF-BB-induced hyaluronan production by orbital fibroblasts

Next, we investigated the contribution of histone lysine methyltransferases and histone arginine methyltransferases in PDGF-BB-induced hyaluronan production by orbital fibroblasts. Pre-incubation with the histone lysine methyltransferase inhibitors UNC0379 (targeting SETD8), CPI169 (targeting EZH2/EZH1), DZNeP (targeting EZH2), A-366 (targeting G9a), BIX01294 (targeting G9a) ML-2 (targeting MLL), Entacapone (targeting COMT), and Pinometostat (targeting DOT1L) significantly reduced PDGF-BB-induced hyaluronan production by orbital fibroblasts (Figure 3A). Inhibition of the histone arginine methyltransferases PRMT5 and PRMT1/3/4/6/8 with GSK591 and MS023, respectively, also significantly reduced PDGF-BB-induced hyaluronan production by orbital fibroblasts (Figure 3B). Yet the inhibition of either histone lysine methyltransferases or histone arginine methyltransferases never reduced PDGF-BB-induced hyaluronan production up to the basal hyaluronan synthesis of the orbital fibroblasts.

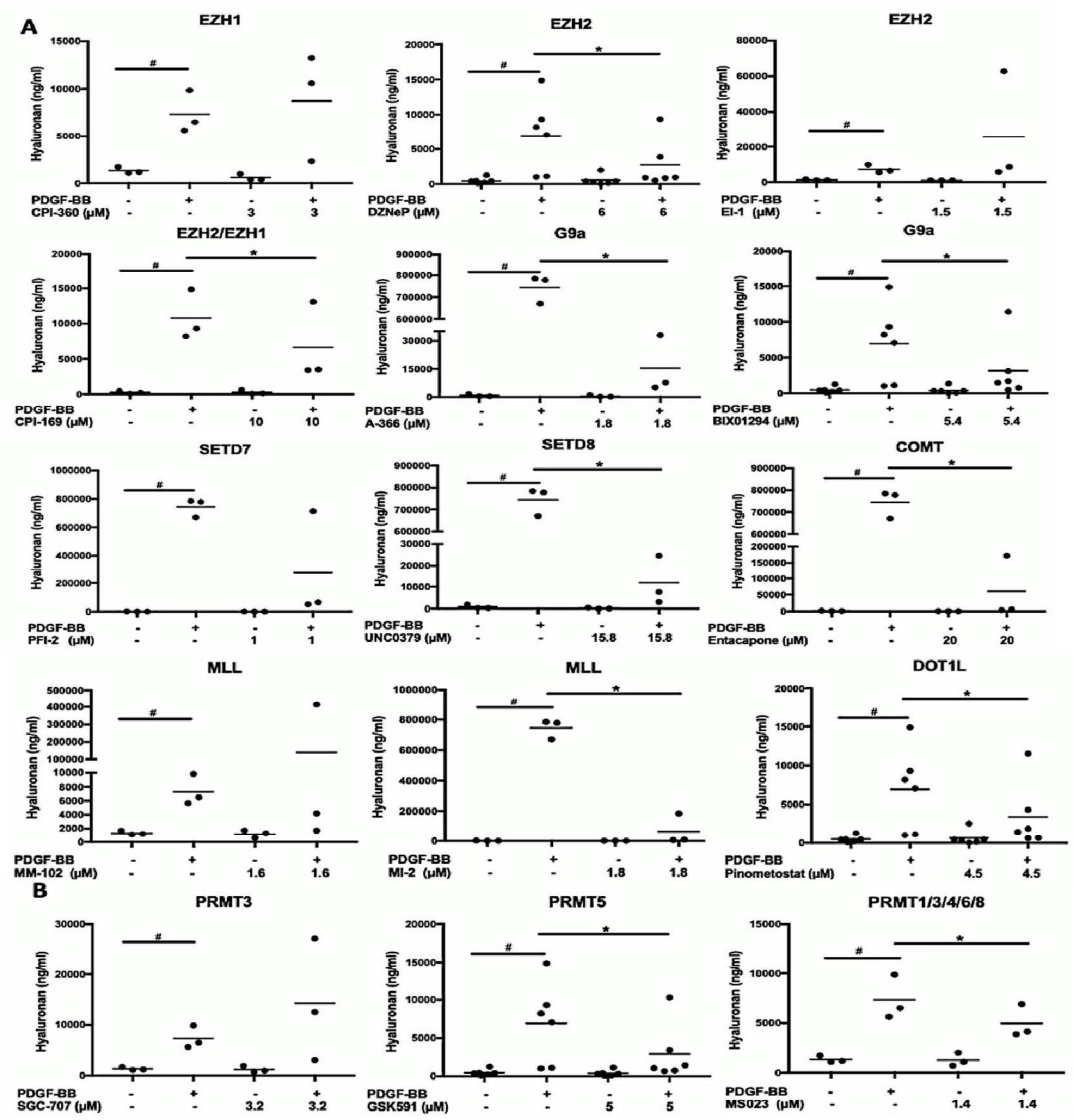
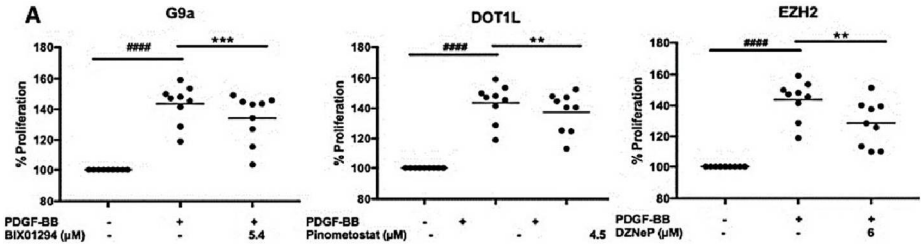


Figure 3. Inhibition of SETD8, EZH2, DOT1L, G9a, MLL, PRMT5 and PRMT type 1 reduces PDGF- BB- induced hyaluronan production by orbital fibroblasts. GO (n=3-6) orbital fibroblasts were pre-incubated with highest concentration of specific histone lysine methyltransferase (A) and arginine methyltransferase (B) inhibitors for 24 h, and then stimulated with PDGF-BB (50 ng/ml) in the presence or absence specific HMT inhibitors. After 24 h, supernatant was collected, and hyaluronan levels were measured by ELISA. # indicates a p- value of <0. 05, compared to the

unstimulated condition, * represents a p-value of <0.05, compared to PDGF-BB stimulation.

DOT1L, EZH2, G9a and PRMT5 inhibitions exhibited the effect in blocking PDGF-BB-induced orbital fibroblast activation

We further examined the effect of Pinometostat (DOT1L inhibitor), DZNeP (EZH2 inhibitor), GSK591 (PRMT5 inhibitor) and BIX01294 (G9a inhibitor) on PDGF-BB-induced orbital fibroblast activation without pre-incubation step, thus being more representative of an actual disease treatment situation. For this only the effects of the highest concentration of histone methyltransferases inhibitors tested so far were explored. PDGF-BB-induced orbital fibroblast proliferation was significantly inhibited by blocking G9a, DOT1L or EZH2 with BIX01294, Pinometostat or DZNeP, respectively. Yet the level of inhibition was never complete (up to the level of basal proliferation) (Figure 4A). In addition inhibition of EZH2 with DZNeP also significantly suppressed PDGF-BB-induced IL-6 (Figure 4B) and IL-8 (Figure 4C) up to basal production levels while inhibition of G9a, DOTL1, or EZH2 with BIX01294, Pinometostat or DZNeP, respectively, also significantly but not fully reduced PDGF-BB-induced hyaluronan production (Figure 4D). Inhibition of the histone arginine methyltransferase PRMT5 with GSK591 significantly reduced PDGF-BB-induced orbital fibroblasts proliferation and hyaluronan production, yet never up to basal levels (Figure 4E).



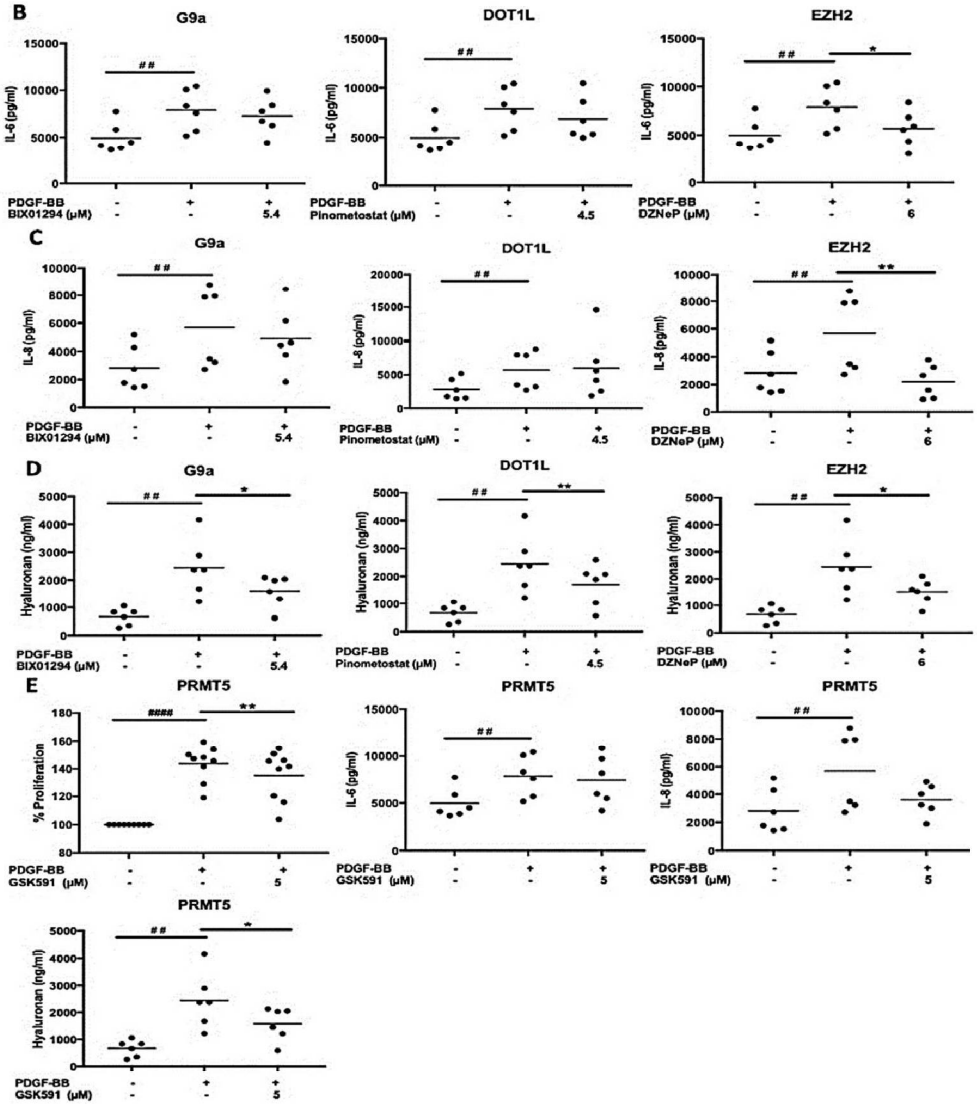


Figure 4. Inhibition of DOT1L, EZH2, G9a and PRMT5 reduce PDGF-BB-induced orbital fibroblast activities. GO (n=6-9) orbital fibroblasts were stimulated with PDGF-BB (50 ng/ml) with simultaneous addition of of specific histone lysine methyltransferase (A-D) or arginine methyltransferase (E) inhibitors for 24 h before evaluating proliferation (A) and the IL-6 production(B), IL-8 production (C) and hyaluronan production (D).. ## indicates a p-value of <0.01, compared

to the unstimulated condition, ** and * represent a p-value of <0.01 and <0.05, respectively, compared to PDGF-BB stimulation.

The effect of PDGF-BB on *DOT1L, EZH2, EZH1, G9a and PRMT5* mRNA expression by orbital fibroblasts

Based on our observations in the HMT inhibition experiments we further explored the effect of PDGF-BB on *HMTs* expression by orbital fibroblasts. Stimulation with PDGF-BB (50 ng/ml) time-dependently and significantly reduced the mRNA expression of the histone lysine methyltransferase *EZH1* (Figure 5A) in orbital fibroblasts. In contrast mRNA expression of the histone lysine methyltransferases *DOT1L*, *G9a* and *EZH2* was in the orbital fibroblasts was significantly and time-dependently enhanced by PDGF-BB. Also, mRNA expression levels of the histone arginine methyltransferase *PRMT5* were significantly enhances in a time dependent manner by PDGF-BB (Figure 5B).

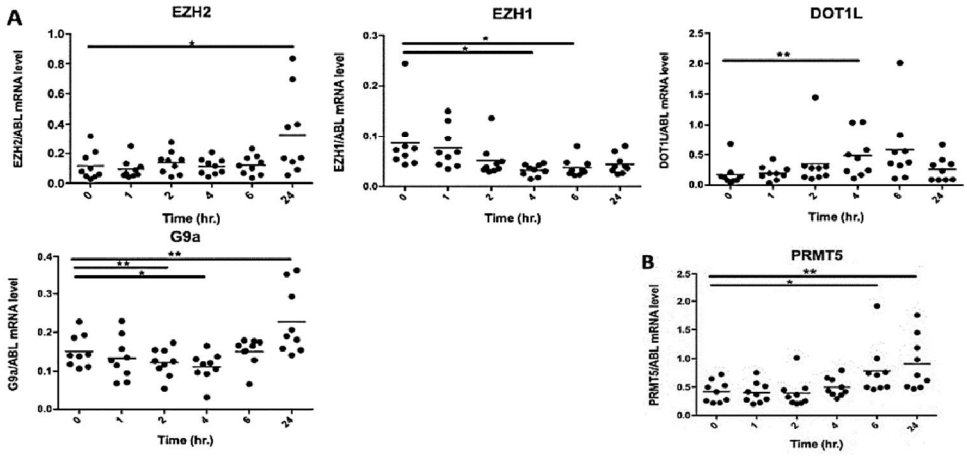


Figure 5. The effect of PDGF-BB on *DOT1L*, *EZH2*, *EZH1*, *G9a* and *PRMT5* mRNA expression by orbital fibroblasts. Orbital fibroblasts from nine GO patients were stimulated with PDGF-BB (50 ng/ml) for 1,2,4,6 or 24 hours. Transcript levels were determined by RT-PCR and normalized to the control gene *ABL*. Each dot represents the orbital fibroblast strain from one individual and horizontal bars represent the mean values. ** and * represent a p-value of <0.01 and <0.05, respectively, compared to PDGF-BB stimulation.

DISCUSSION

PDGF-BB is considered to stimulate several orbital fibroblast effector functions in the pathogenesis of GO. The purpose of this current study was to examine whether specific histone methyltransferases are involved in mediating these activating effects of PDGF-BB. Our results demonstrated that both histone lysine and arginine methyltransferases contribute to PDGF-BB-induced proliferation, IL-6, IL-8 and hyaluronan production by GO orbital fibroblasts.

In line with previous observations we found PDGF-BB to induce proliferation of GO orbital fibroblasts (Virakul et al., 2014). However, in this study we are the first to link this to epigenetic regulation. Our data point out that histone lysine methyltransferases, including EZH2, DOT1L and G9a, are involved in mediating the pro-mitogenic effects of PDGF-BB in orbital fibroblasts from GO patients. Both in our prophylactic and treatment orientated experimental set-ups, we found that G9a inhibition by BIX01294 significantly suppressed orbital fibroblast proliferation. BIX01294 is inhibitor highly specific to G9a and suppresses the methylation of H3K9. Previous study reported that treatment with a G9a inhibitor attenuated renal fibrosis (Irifuku et al., 2016). Also, EZH2 inhibition with the specific inhibitor DZNeP significantly reduced orbital fibroblast proliferation in both experimental set-ups conducted. DZNeP suppresses the methylation of H3K27 and inhibition of EZH2 was previously reported to reduce cell proliferation and renal fibrosis (Shi et al., 2019; Zeybel et al., 2017). Furthermore, Tsou PS., et al. reported that DZNep significantly reduced dermal thickness in scleroderma (Tsou et al., 2019). We also observed that inhibition of DOT1L by Pinometostat significantly reduced orbital fibroblast proliferation. Consistent with our findings, Nassa G., et al. reported that inhibition DOT1L reduced proliferation of breast cancer cells (Nassa et al., 2019). However, cells from different regions display different characteristics. Our data also indicate that histone arginine methyltransferases may be associated with PDGF-BB-induced proliferation in orbital fibroblasts from GO as well. Inhibition of PRMT5 with GSK591 significantly reduced orbital fibroblast proliferation. Previous study reported that PRMT5 inhibition suppressed proliferation of fibroblast-like synoviocytes (FLSs) from rheumatoid arthritis patients (Chen et al., 2017). These finding reported that PRMT5 played roles in progression of several human cancers by promoting cell proliferation (W. Xiao et al., 2019). Although we clearly observed that blocking certain histone lysine methyltransferases or histone arginine methyltransferases reduced PDGF-BB-

induced orbital fibroblast proliferation this inhibition was never complete. Cooperation between the different histone lysine methyltransferases or between histone lysine methyltransferases and histone arginine methyltransferases may very well be involved in establishing the pro-mitogenic effect of PDGF-BB on orbital fibroblast proliferation. Therefore, future studies into this are certainly warranted.

Histone lysine methyltransferase inhibition, including EZH2 also inhibited PDGF-BB-induced IL-6 and IL-8 production by orbital fibroblasts. Previous study showed that inhibition of EZH2 suppressed proinflammatory cytokine levels in renal fibrosis and liver failure (Shi et al., 2019; T. Zhou et al., 2018). Based on these findings, we hypothesize that inhibition of EZH2 prevents phosphorylation of NF-κB signaling and attenuates inflammatory cytokine release (Shi et al., 2019). CPI-169 is a less specific inhibitor that targets both EZH2 and EZH1. CPI-169 significantly reduced IL-6 production in the prophylactic experimental set-up, whereas CPI-169 did not reduce IL-6 production in the treatment setting. In further studies we will confirm the result of CPI-169 by gene depletion of EZH2 and EZH1. Also, G9a inhibition by pre-incubating the orbital fibroblasts with BIX01294 before PDGF-BB stimulation reduced IL-6 production but not IL-8 production. Without pre-incubation BIX01294 also did not reduce PDGF-BB-induced IL-6 production by the orbital fibroblasts. This observation may very well be related to the specificity of BIX01294 as it also non-specifically targets the GLP enzyme. We found no effect of PRMT5 inhibition on PDGF-BB-induced IL-6 and IL-8 production. This is in contrast to findings by Chen D., et al., reported that inhibition of PRMT5 by EPZ015666 or siRNA-mediated knockdown reduced IL-6 and IL-8 production of fibroblast-like synoviocytes in rheumatoid arthritis (Chen et al., 2017). Despite the fact that orbital fibroblasts are well recognized to differ from fibroblasts from other anatomical regions, including embryonic origin, phenotype and behavioral responses (Sacco et al., 2019; Sriram, Bigliardi, & Bigliardi-Qi, 2015). We will further explore the effect of *PRMT5* gene depletion in orbital fibroblast to evaluate its contribution to PDGF-BB-induced cytokine production.

Our study also points at a role for histone lysine methyltransferases including EZH2, G9a, MLL, DOT1L and SETD8 and histone arginine methyltransferases including PRMT5 and PRMT type 1 in PDGF-BB-induced hyaluronan production by orbital fibroblasts. G9a inhibition with BIX01294 significantly suppressed the hyaluronan production. Previous study reported that G9a inhibition attenuated renal fibrosis and peritoneal fibrosis (Irifuku et al., 2016; Maeda et al., 2017). EZH2 inhibition with DZNep significantly

reduced PDGF-BB-induced hyaluronan production by the GO orbital fibroblasts. Consistent with our findings, Tsou PS., et al. reported that DZNep reduced the expression of several well know profibrotic genes and dermal thickness in scleroderma (Tsou et al., 2019). Furthermore, Shi Y., et al. and Zhou X., et al., reported that EZH2 inhibition reduced ECM deposition in renal fibrosis (Shi et al., 2019; X. Zhou et al., 2016). We also found that inhibition of DOT1L by Pinometostat significantly inhibited the production PDGF-BB-induced hyaluronan production by orbital fibroblasts. Inhibition of PRMT5 only suppressed PDGF-BB-induced hyaluronan production by the orbital fibroblasts when they were first pre-incubated with GSK591.

As our inhibition experiments indicated involvement of G9a, DOT1L, EZH2 and PRMT5 in mediating the effects of PDGF-BB on orbital fibroblasts we explored their expression levels. *G9a, DOT1L, EZH2* and *PRMT5* expression significantly increased in orbital fibroblasts upon PDGF-BB stimulation, although at different kinetics, being maximal at 24, 4, 24 and 6 h following stimulation, respectively. On the other hand, *EZH1* mRNA expression significantly decreased in orbital fibroblasts at 4 hours following PDGF-BB stimulation. Although we still need to confirm our observations at the protein level (EZH1, EZH2, DOT1L, G9a and PRMT5). The mRNA data on the histone and arginine methyltransferases are largely in line with the results from the inhibition experiments. Therefore, our data strongly imply that PDGF-BB has to induce expression of certain histone lysine and arginine methyltransferases to establish its activating in effects in orbital fibroblasts form GO patients.

CONCLUSION

Our current data suggest involvement of EZH2, G9a, DOT1L and PRMT5 in regulating PDGF-BB-induced proliferation and hyaluronan production by orbital fibroblasts from GO patients, while EZH2 also controls PDGF-BB-induced IL-6 and IL-8 production by GO orbital fibroblasts. Gene depletion experiments of the specific HMTs are required to further elucidate the contribution and differential involvement of HMTs to the different PDGF-BB-induced effector mechanisms in orbital fibroblast. Increased understanding into these mechanisms may provide rationales to target HMTs in the treatment of GO.

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