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Differential Lysyl oxidase like 1 expression in pseudoexfoliation glaucoma is orchestrated via DNA methylation

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ABSTRACT

Pseudoexfoliation syndrome (PXF) is the most common cause of secondary open angle glaucoma worldwide. Single nucleotide polymorphisms (SNPs) in the gene Lysyl oxidase like 1 (*LOXL1*) are strongly associated with the development of pseudoexfoliation glaucoma (PXFG). However, these SNPs are also present in 50–80% of the general population, suggestive of other factors being involved in the pathogenesis of PXFG. In this study, we aimed to investigate the influence of epigenetic regulation, specifically DNA methylation, on *LOXL1* expression in PXFG using human tenons fibroblasts (HTFs), aqueous humour and serum samples from donors with and without PXFG. *LOXL1* expression in HTFs was measured by qPCR and Western Blotting and *LOXL1* concentration in aqueous humour was determined by ELISA. Global DNA methylation levels were quantified using an ELISA for 5-methylcytosine. MeDIP assays assessed the methylation status of the *LOXL1* promoter region. Expression of methylation-associated enzymes (*DNMT1*, *DNMT3a* and *MeCP2*) were determined by qPCR and inhibited by 0.3 μM 5-azacytidine (5-aza). Results showed that *LOXL1* expression was significantly decreased in PXFG HTFs compared with Control HTFs at gene (Fold change 0.37 ± 0.05 , $P < 0.01$) level and showed a decrease, when measured at the protein level (Fold change 0.65 ± 0.42 , $P = 0.22$), however this was not found to be significant. *LOXL1* concentration was increased in the aqueous of PXFG patients compared with Controls (2.76 ± 0.78 vs. 1.79 ± 0.33 ng/ml, $P < 0.01$). Increased global methylation ($56.07\% \pm 4.87\%$ vs. $32.39\% \pm 4.29\%$, $P < 0.01$) was observed in PXFG HTFs compared with Control HTFs, as was expression of methylation-associated enzymes (*DNMT1* 1.58 ± 0.30 , $P < 0.05$, *DNMT3a* 1.89 ± 0.24 , $P < 0.05$, *MeCP2* 1.63 ± 0.30 , $P < 0.01$). Methylation-associated enzymes were also increased when measured at protein level (*DNMT1* 5.70 ± 2.64 , $P = 0.04$, *DNMT3a* 1.79 ± 1.55 , $P = 0.42$, *MeCP2* 1.64 ± 1.33 , $P = 0.45$). *LOXL1* promoter methylation was increased in patients with PXFG compared to Control patients in both blood (3.98 ± 2.24 , 2.10 ± 1.29 , $P < 0.05$) and HTF cells (37.31 ± 22.0 , 8.66 ± 10.40 , $P < 0.01$). Treatment of PXFG HTFs with 5-azacytidine increased *LOXL1* expression when compared with untreated PXFG HTFs (Fold change 2.26 ± 0.67 , $P < 0.05$). These data demonstrate that *LOXL1* expression is altered in PXFG via DNA methylation and that reversal of these epigenetic changes may represent future potential therapeutic targets in the management of PXFG.

1. Introduction

Pseudoexfoliation (PXF) syndrome is systemic disorder of the extracellular matrix, characterised by the production and accumulation of fibrillar material in the anterior segment of the eye, skin and visceral organs (Schlötzer-Schrehardt and Naumann, 2006). When these extra-cellular matrix (ECM) materials obstruct aqueous outflow this

leads to an elevation of intraocular pressure (IOP) and subsequent glaucomatous optic nerve damage. Pseudoexfoliation glaucoma (PXFG) is an aggressive subtype of glaucoma and is the most common cause of secondary open angle glaucoma worldwide (Heijl et al., 2009; Ritch, 1994).

PXF and PXFG have a strong hereditary component and variants in the gene Lysyl oxidase-like 1 (*LOXL1*) have been associated with PXF/PXFG risk (Thorleifsson et al., 2007). *LOXL1* is required for elastin

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Abbreviations

DMEM	Dulbecco's modified Eagle's medium	MeDIP	Methylated DNA immunoprecipitation
DNMT	DNA Methyltransferase	NAD	No abnormality detected
DNMTi	DNA Methyltransferase inhibitors	ONH	Optic Nerve Head
ECM	Extracellular Matrix	POAG	Primary Open Angle Glaucoma
GWAS	Genome Wide Association Study	PXF	Pseudoexfoliation Syndrome
HIF1 α	Hypoxia Inducible Factor 1 alpha	PXFG	Pseudoexfoliation Glaucoma
HTF	Human Tenons Fibroblast	RASAL1	Ras GTPase-Activating-Like Protein
IOP	Intraocular Pressure	RGC	Retinal Ganglion Cell
LC	Lamina Cribrosa	SNP	Single Nucleotide Polymorphism
LOXL1	Lysyl oxidase like 1	Sp1	Transcription factor specific protein 1
MECP2	Methyl CpG Binding Protein 2	TGF β 1	Transforming Growth Factor Beta 1
		TM	Trabecular Meshwork
		5-aza	5-azacytidine

maturation (Greene et al., 2020; Mäki, 2009) and *LOXL1* knockdown mice develop multiple abnormalities of elastic tissue including increased skin laxity, abdominal aortic aneurysms, intestinal diverticula, emphysema and pelvic and rectal prolapse (Alsofi et al., 2016; Gauthier and Liu, 2017; Lee et al., 2008; Liu et al., 2004, 2007; Wiggs et al., 2009). In 2007, a genome-wide association study performed in a Scandinavian population identified three single nucleotide polymorphisms (SNPs) in the gene Lysyl oxidase-like 1 (*LOXL1*) which strongly associated with developing PXF/PXFG (Thorleifsson et al., 2007). Two of the SNPs were identified in the coding regions of *LOXL1* in exon 1 (rs1048661 and rs3825942) while the third SNP is located nearby in the non-coding region in intron 1 (rs2165241). These coding SNPs may be carried together and individuals with the high-risk (G-G) haplotype were found to have an estimated 700-fold increased risk for PXFG compared to those with the low risk haplotype (Thorleifsson et al., 2007). The association of these *LOXL1* coding variants with PXFG risk has been replicated in populations around the world, including Australian Caucasian (Hewitt et al., 2007), US Caucasian (Fan et al., 2008; Fingert et al., 2007), Chinese (Chen et al., 2010), German (Pasutto et al., 2017), Italian (Pasutto et al., 2017), Japanese (Pasutto et al., 2017), Latin American (Tang et al., 2014) and many other ethnic groups. Our group has recently shown similar results in the Irish population (Eivers et al., 2020). However, these risk variants were also reversed in certain populations, meaning the same variant may be either protective or a risk variant depending on ethnicity. (Fuse et al., 2008; Hayashi et al., 2008; Mabuchi et al., 2008; Mori et al., 2008; Ozaki et al., 2008; Park et al., 2013; Rautenbach et al., 2011; Sagong et al., 2011; Tanito et al., 2008; Williams et al., 2010).

Currently, the mechanism whereby these *LOXL1* variants contribute to PXFG pathogenesis is poorly understood. These coding variants were present in up to 98% of PXF cases but were also found in up to 85% of unaffected individuals (Thorleifsson et al., 2007), indicating that these variants alone may not be solely responsible for PXF development.

Several studies have focused on the *LOXL1* promoter region, indicating the contribution of both alternatively spliced *LOXL1* and long non-coding RNA to *LOXL1* expression. *LOXL1* levels were decreased due to reduced binding of the transcription factor RXR α (retinoid X receptor alpha). RXR α is known to be a repressor of transcription in the absence of a ligand (Szanto et al., 2004). Downregulation of *LOXL1* was also demonstrated via alternative splicing of *LOXL1* pre-mRNA (Pasutto et al., 2017) The alternatively spliced transcript *LOXL1*- α has a premature stop codon in exon 2 resulting in the formation of an inactive isoform. Increased formation of *LOXL1*- α has the secondary effect of reducing formation wild type *LOXL1*. At the post-transcriptional level, the alternatively spliced *LOXL1* transcript is associated with nonsense-mediated decay (NMD), a degradation pathway that removes spliced unproductive mRNA to regulate gene expression. The combination of reduced RXR α binding, increased splicing and higher levels of NMD resulted in an up to 50% reduction in levels of wild-type *LOXL1*

mRNA in the cells carrying the risk allele. It was later found that this “alternative splicing coupled to NMD” can be modulated by PXF associated stressors like oxidative stress, caffeine and retinoic acid (Bernier et al., 2017). Post-transcriptional *LOXL1* gene regulation may also occur via long non-coding RNAs (lncRNA). *LOXL1*-AS1 is a lncRNA which is encoded on a DNA strand opposite *LOXL1* (Hauser et al., 2015; Wiggs et al., 2012). A haplotype containing 3 SNPs from intron 1 reduced *LOXL1*-AS1 promoter activity by 43% in lens epithelial cells. *LOXL1*-AS1 was also reduced in response to cellular stressors, oxidative stress and cyclical mechanical stress in Schlemm's canal endothelial cells in PXF. This study also identified *LOXL1*-AS1 in multiple ocular and systemic tissues associated with PXF (Hauser et al., 2015). Although *LOXL1*-AS1 is not directly involved in the regulation of *LOXL1*, it may regulate distant genes involved in PXF pathogenesis. *LOXL1*-AS1 expression has also been found to be upregulated in many cancers stimulating cell proliferation, and invasion via the NF-kB and P13k/Akt pathways (Chen et al., 2019; Gao et al., 2018; Long et al., 2018; Wang et al., 2018) with *LOXL1*-AS1 knockdown inhibiting tumorigenesis (Gao et al., 2018; Long et al., 2018). The role of this lncRNA in PXF pathophysiology needs to be further delineated.

For the purpose of this investigation, we aimed to examine the influence of epigenetic factors, specifically DNA methylation on *LOXL1* expression in PXFG.

Epigenetics is the study of changes in gene function that do not involve changes in the DNA sequence itself (Wu and Morris, 2001) with an example being DNA methylation, or the addition of a methyl group to a cytosine base (Jin et al., 2011). When this occurs at a gene promoter, it may lead to transcriptional repression. The transfer of these methyl groups is orchestrated by DNA methyltransferases that create (DNMT3A, DNMT3B) or maintain (DNMT1) patterns of methylation.

DNA methylation has been implicated in the regulation of fibrotic genes in glaucoma, and our group has previously demonstrated increased global DNA methylation and decreased *TGF β 1* promoter methylation in POAG Lamina Cribrosa cells (F. S. McDonnell et al., 2016). *LOXL1* has been previously decreased via a number of methylation related mechanisms. It has been shown to be silenced due to hypermethylation of CpG islands in the *LOXL1* promoter (Debret et al., 2010; Moulin et al., 2017; Wu et al., 2007; Ye et al., 2015). In the Uighur population, hypermethylation of CpG islands in the *LOXL1* promoter region has been observed in the anterior lens capsules of PXF patients compared to controls. *LOXL1* mRNA levels were reduced in the PXF lens capsules, suggesting that hypermethylation downregulates gene expression (Ye et al., 2015).

In systemic disease, *LOXL1* was silenced via DNA hypermethylation in a case of autosomal recessive Cutis Laxa. This *LOXL1* methylation was reversed by inhibition using the DNA methyltransferase inhibitor 5-azacytidine (Debret et al., 2010). 5-azacytidine is currently licenced for the treatment of Myelodysplastic syndromes (Roboz et al., 2016; Tran et al., 2011) and has also been shown to ameliorate fibrosis in cardiac and

renal disease (Bechtel et al., 2010; Kim et al., 2014). *LOXL1* promoter transcriptional activity has been increased using 5-azacytidine in skin fibroblasts (Moulin et al., 2017) and human bladder cancer cell lines (Wu et al., 2007). This suggests that targeting *LOXL1* methylation may provide a therapeutic target in the management of PXFG.

In this study, we aimed to investigate the role of epigenetics, specifically methylation, in regulating *LOXL1* expression in PXFG patients using human tenons fibroblasts (HTFs), aqueous humour and blood samples from PXFG and Control (cataract) donors. *LOXL1* expression was found to be decreased in HTFs from PXFG patients compared to Control patients, while *LOXL1* concentration was increased in aqueous humour samples from PXFG patients. Global methylation and methylation-associated enzymes were upregulated in HTFs from PXFG patients while *LOXL1* promoter methylation was increased in both HTFs and blood samples from PXFG donors. Furthermore, treatment of HTFs from PXFG patients with an inhibitor of DNA methylation (5-azacytidine) increased *LOXL1* expression. These data support the hypothesis that differential *LOXL1* expression in PXFG is orchestrated via DNA methylation.

2. Material and methods

2.1. Donor recruitment

Donors were recruited from the Mater Misericordiae University Hospital, Dublin and the Mater Private Hospital, Dublin following ethical approval from the Hospital Institutional Review Board in accordance with the Declaration of Helsinki (Study approval number: 1/378/1956). Written and informed consent was obtained from all subjects prior to sampling and all patients received a patient information leaflet. The patient classification was in accordance with the guidelines of the European Glaucoma Society ([European Glaucoma Society Terminology and Guidelines for Glaucoma, 4th Edition, 2017](#)). All patients were ≥ 60 years of age. The diagnosis of pseudoexfoliation glaucoma (PXFG) was given based on a full anterior segment and fundoscopic exam on slit lamp biomicroscopy, along with Goldmann applanation tonometry (GAT) and previous Humphrey Visual Field 24-2 tests and optical coherence tomography of the optic disc (if available) (Table 1). Patients with a previous traumatic eye injury, previous history of intraocular surgery, retinal venous occlusion, age-related macular degeneration, uveitis and other ocular inflammatory disease, malignancy and a history of diabetes mellitus were excluded. Patient

Table 1
Clinical patient classification into Control, PXF and PXFG cohorts.

	Control	PXF	PXFG
IOP	<21 mmHg	>/<21 mmHg	>21 mmHg
Visual Field Loss (HVF-24-2)	Absent	Absent	Present
Exfoliation material present (post-dilation)	Absent	Yes (pupillary border, anterior lens capsule)	Yes (pupillary border, anterior lens capsule)
Gonioscopy	NAD	Trabecular pigmentation + exfoliation material + Sampaolesi's line	Trabecular pigmentation + exfoliation material + Sampaolesi's line
Iris	NAD	± Central/mid-iris pigmentary loss/iris transillumination	± Central/mid-iris pigmentary loss/iris transillumination
Optic disc (OCT disc + posterior segment exam)	No optic neuropathy	No optic neuropathy	Neuronal Rim loss/Retinal Ganglion cell layer loss/disc haemorrhages/peripapillary atrophy/increased cup disc ratio.

Demographics of the total cohort recruited (Table 2) and the patient sample size used in each experimental technique are included (Table 3).

2.2. Cell culture

An approximately 2 mm Tenon's sample was obtained by blunt dissection in theatre, placed on a spear and transported to the lab in 10 mls of Dulbecco's modified Eagle's medium (DMEM)/F12 carrier media (D8437, Sigma, Ireland) supplemented with 1% Penicillin/Streptomycin (P4333, Sigma, Ireland). 1 ml of digestion media containing DMEM/F12 with 20% FBS (F9665, Sigma, Ireland), 0.25% Liberase TL (05401020001, Roche), 0.05% DNase1 (DN25, Sigma, Ireland) and 1% Penicillin/Streptomycin, was added. The sample was then placed in tissue culture for 24 h maintained at 37 °C in 5% CO₂. Following this the sample was sheared by vortexing and transported to a flask containing DMEM/F12 supplemented with 10–20% (v/v) fetal calf serum (F9665, Sigma, Ireland), 200 mM L-glutamine (G7513, Sigma, Ireland) and 1–2% Penicillin/Streptomycin (P4333, Sigma, Ireland). Confluent HTF cells were used in experimental procedures between passages 3 and 6, and 3 control and 3 PXFG donors were used for each experiment.

2.3. 5- Azacytidine treatment

To establish the treatment effect of 5-azacytidine, cells were cultured to confluence and serum starved for 24 h prior to treatment. 5-azacytidine (A2385, Sigma, Ireland) was reconstituted in serum free media. Cells were treated with 5-azacytidine at 0.3 μ M for 24 h. This concentration was optimised for HTFs based on previous research from our lab group in trabecular meshwork cells (F. McDonnell et al., 2016) and following a cell viability assay.

2.4. Quantitative real-time PCR (qPCR)

Total RNA was extracted using TRIzol Reagent Solution (15596026, Life Technologies, Ireland) extraction, chloroform phase separation and isopropanol precipitation. The RNA was reverse transcribed to complementary DNA (cDNA) using AMV reverse transcriptase (A4464, Sigma, Ireland), oligo dT (04387, Sigma, Ireland) and deoxyribonucleotide triphosphates (dNTPs) (D7295, Sigma, Ireland).

Gene expression was analysed using primers designed on BLAST and manufactured by Sigma (Table 4). A LightCycler® 480 Instrument II (Roche) and LightCycler® 480 SYBR Green I Master (04707516001, Roche) was used. Standard qPCR cycling conditions were used for *18S*, *LOXL1*, *MeCP2*, *DNMT1* and *DNMT3a*.

Fold change in gene expression was assessed using the $2^{\Delta\Delta Ct}$ equation in which the value of the normal donors is set to an arbitrary value of 1. All qPCR results are given as the mean fold change in gene expression of the experimental compared to control \pm standard deviation (SD) (n = 3). *18S* was used as a reference gene for normalization. A minimum of three PXFG and control donors was used for each experiment with technical triplicates for each donor.

Table 2
Patient Demographics of total cohort.

	Control (n = 26)	PXFG (n = 27)	P-value ^a
Gender (Male/Female)	8/18	16/11	<0.01
Age (mean, range)	77.48 [66.5–90.1]	75.56 [63.9–86.7]	0.24
Ethnicity (Caucasian/Other)	25/1	26/1	0.98
Family History of Glaucoma (Y/N)	2/24	5/22	0.03

^a Calculated using Pearson's chi squared test.

Table 3
Patient sample size used in each experimental technique.

	Control (M: F, age [mean ± SD])	PXFG (M: F, age [mean ± SD])
Clinical database	26 (2.25:1 [77.5 ± 6.3])	27 (1.45:1 [75.6 ± 6.5])
Aqueous ELISA	6 (1:1 [80.0 ± 2.6])	6 (1.5:1 [80.0 ± 2.0])
Molecular techniques (ELISA, qPCR, Western Blot)	3 (1:1.5 [80.5 ± 2.2])	3 (1.5:1 [72.3 ± 8.6])

2.5. Western Blotting

Cells were treated as indicated and scraped into ice-cold PBS followed by centrifugation to pellet the cells. The cells were re-suspended in RIPA buffer (R0278, Sigma, Ireland) containing 1% protease inhibitor cocktail (P8340, Sigma, Ireland), and then incubated on ice for 5 min and clarified by centrifugation as per the manufacturers' instructions. The total protein concentration of the cell lysates was determined using a Bradford assay (B6916, Sigma, Ireland) using bovine serum albumin as the standard. Protein samples were added to sample buffer, boiled at 95 °C for 5 min, and stored at -80 °C prior to electrophoresis. SDS-PAGE was then used for 90 min to separate the proteins, and they were transferred to nitrocellulose membranes (N8267, Sigma, Ireland) for 20 min using the Thermo Scientific™ Pierce™ G2 Fast Blotter. The membranes were incubated overnight in primary antibodies at 4 °C as listed in Table 5.

Membranes were then washed in TBST and incubated with antibody dilutions of HRP-linked secondary antibody (anti-rabbit IgG- 7074S/ anti-mouse IgG A1014) and developed using enhanced chemiluminescence (ECL) reagents (10005943, Fisher Scientific, Ireland) according to standard protocols. β -actin was used as a loading control.

2.6. LOXL1 ELISA

LOXL1 concentration was measured using the Human Lysyl oxidase like 1 ELISA kit (MBS931742) as per manufacturers' instructions. Aqueous samples were diluted with 1X PBS to bring the sample to a volume sufficient for the ELISA kit. 100 μ l of the samples and standards were added to each well in triplicate and incubated. The samples and standard were removed and 100 μ l of 1x Biotin antibody was added and incubated. Following this, 100 μ l of HRP-avidin was added to each well and incubated. This was then removed and 90 μ l of 3,3',5,5'-tetramethylbenzidine (TMB) substrate was added to each well and colour change to blue was observed in all samples. Stop solution was added and samples were read at 450 nm absorbance.

Concentration in ng/ml was calculated by comparing the samples with the standard. Each experiment was performed in triplicate. Results are presented as the mean value of optical density at absorbance 450 nm \pm standard deviation.

2.7. Global methylation DNA quantification

Genomic gDNA was isolated from confluent cells using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma G1N70) according to manufacturers' instructions. Briefly, the cells were lysed in a chaotropic

Table 4
Primer Sequences for quantitative real-time PCR.

Primer	Sequence	Primer	Sequence	Amplicon size
LOXL1 Forward	5' - GCACCTCTCATAACCCAGGGC-3'	LOXL1 Reverse	5'-TGGCAGTCGATGTCCGCAT-3'	68bp
MeCP2 Forward	5'-GATCAATCCCCAGGAAAAGC-3'	MeCP2 Reverse	5-CCTCTCCCAGTTACCGTGAAG-3'	118bp
DNMT1 Forward	5'-AGCCCGTAGAGTGGGAATGGCA-3'	DNMT1 Reverse	5-ACGCTTAGCCTCTCCATCGGACT-3'	101bp
DNMT3a Forward	5'- GTAACCCGTTGAACCCCAT-3'	DNMT3a Reverse	5'- CCATCCAATCGGTAGTAGC-3'	151bp

salt-containing solution to ensure denaturation, and ethanol was used to precipitate DNA when the lysate was spun through a silica membrane. The DNA was then eluted in a Tris-EDTA solution (10 mM Tris-HCl, 0.5 mM EDTA, pH 9.0), and DNA concentration was determined by spectrometry (260 nm). The equation $(OD_{260} \times 100(\text{dilution factor}) \times 50 \mu\text{g/ml})$ was used to determine the DNA concentration. Gel electrophoresis was conducted on a 1% agarose gel to confirm the integrity of the eluted DNA.

The Imprint Methylated DNA Quantification Kit (Sigma MDQ1) was used to detect global methylation levels using the protocol recommended by the manufacturer. 200 ng of gDNA was bound to the wells and then an antibody for 5-methylcytosine was used to bind the methylated DNA. A secondary detection antibody was used to create the colorimetric change that quantifies the level of methylated DNA and measured at 450 nm.

Each experiment was performed in triplicate. Results are presented as the mean \pm SD value percentage of global methylation relative to the methylated control DNA. The Control Methylated DNA contains 50ng/ μ l-methylated DNA.

2.8. Methylated DNA immunoprecipitation (MeDIP) assay

The methylated DNA immunoprecipitation (MeDIP) assay was carried out using the MeDIP assay kit (Active Motif 55009) as per the kit's standard protocol. Briefly, total gDNA was extracted as outlined above using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma G1N70) and as per the standard protocol for either whole blood samples or HTF cells. For sonication, 2 μ g of total gDNA was used and mechanically fragmented for 35 cycles (20s on, 20s off). Fragmented DNA was resolved on an agarose gel to confirm fragments were between 100 and 600 base pairs. 1 μ g of fragmented DNA was used for immunoprecipitation with a 5-methyl cytosine antibody as per the MeDIP kit protocol. An input sample consisting of 0.1 μ g of fragmented DNA was kept back prior to immunoprecipitation. As a negative control, 1 μ g of fragmented gDNA was incubated with mouse IgG and was carried out alongside the test immunoprecipitations. Elutes were subjected to quantitative PCR analysis using the LightCycler® 480 SYBR Green I Master system (Roche Diagnostics 04707516001). Primers used for MeDIP analysis are as follows, LOXL1 forward, 5'-CTCAGAGGCAGGTCTGTGGCTT-3', reverse, 5'-GCGCTTCCCCTCTGAATG-3'. Relative methylation (% Input) was calculated using the formula: $2^{-\Delta Ct} \times 100$. The MeDIP assay was carried out on at least 3 individual patients (blood n = 4, HTF cells n = 3). Internal triplicates were performed for each MeDIP elute when carrying out PCR analysis.

Table 5
Antibodies for western blotting.

Target Protein	Host Species	Product Code
LOXL1	Rabbit	ab81488 (Abcam, UK)
B-Actin	Mouse	A5441 (Sigma, Ireland).
DNMT1	Rabbit	ab19905 (Abcam, UK)
DNMT3a	Rabbit	ab2850 (Abcam, UK)
MeCP2	Rabbit	ab2828 (Abcam, UK)

2.9. Statistical analysis

Data was analysed using IBM SPSS statistics Version 24 and are presented as mean \pm standard deviation (SD). Laboratory data was analysed using unpaired 2-tailed *t*-test for analysis between two groups. Linear correlation was assessed using Pearson correlation coefficient. Statistical significance was set at (* $P < 0.05$ and ** $P < 0.01$) of differences between the mean values.

3. Results

3.1. *LOXL1* expression is decreased in pseudoexfoliation glaucoma (PXFG) human tenons fibroblast (HTFs) compared to control HTFs

As *LOXL1* expression has been found to be a primary susceptibility factor in the development of PXFG (Schlötzer-Schrehardt et al., 2012), the expression of this gene was examined in HTF cells cultured from Control and PXFG donors. Quantitative real time PCR results showed that levels of *LOXL1* expression were significantly reduced in PXFG HTFs compared with levels observed in Control cells (Fig. 1A) with a fold change in gene expression of 0.36 ± 0.05 ($P < 0.01$). Immunoblot analysis of *LOXL1* protein expression confirmed a decrease in PXFG compared with controls (Fig. 1B and C). The fold change in protein expression between PXFG and controls was 0.65 ± 0.42 ($P = 0.22$). PXFG HTFs showed wide variability in expression levels, so the overall decrease in protein expression did not reach statistical significance. However, a downward trend in *LOXL1* protein expression was observed in the PXFG group.

3.2. *LOXL1* concentration is elevated in the aqueous humour of patients with pseudoexfoliation glaucoma (PXFG) compared with controls

Next, we aimed to investigate *LOXL1* concentration in the aqueous humour of patients with PXFG compared with Controls using an ELISA specific for *LOXL1* (Fig. 2A). Our results show *LOXL1* concentration was increased in PXFG compared to levels detected in Controls (Fig. 2A). The mean concentration of *LOXL1* in Control aqueous humour was 1.79 ± 0.33 ng/ml compared with 2.76 ± 0.78 ng/ml in PXFG patients ($P < 0.01$). *LOXL1* concentration in the aqueous humour from PXFG patients showed far greater variability compared to Control. This was found to correlate with disease severity. PXFG patients with a higher mean deviation (MD) and Pattern Standard Deviation (PSD) were found to have higher levels of *LOXL1* in their aqueous humour (Fig. 2B, $n = 5$, $P < 0.05$, Fig. 2D, $n = 5$, $P < 0.01$). Patients with a higher Visual Field Index (VFI) were found to have lower levels of *LOXL1* (Fig. 2C, $n = 5$, $P < 0.01$).

3.3. Global DNA methylation and expression of methylation-associated enzymes are increased in PXFG HTFs compared to control HTFs

To investigate whether DNA methylation levels could underlie the decreased expression of *LOXL1* observed in PXFG HTFs, we examined the global methylation profile of HTFs cultured from PXFG and Control donors using an ELISA assay for 5-Methylcytosine (5-MeC), an indicator of total cellular DNA methylation status (Fig. 3A). Total cellular DNA methylation was examined in Control ($n = 3$), and PXFG ($n = 3$) patients by ELISA (absorbance at 450 nm). Results showed a significant increase ($P < 0.01$) in global methylation in HTFs from PXFG patients ($56.07\% \pm 4.87\%$) compared to Controls ($32.39\% \pm 4.29\%$). Following this, we aimed to investigate the expression of genes responsible for DNA methylation, including the DNA methyltransferases (DNMTs) and methyl CpG binding proteins. Quantitative real time PCR analysis (Fig. 3B) showed that there was a significant increase in expression of *DNMT1* (fold change 1.58 ± 0.30 ; $P < 0.05$), *DNMT3A* (fold change 1.89 ± 0.24 ; $P < 0.05$) and *MeCP2* (1.63 ± 0.30 ; $P < 0.01$) in HTFs isolated from PXFG donors compared to levels in Control HTFs. Western blotting

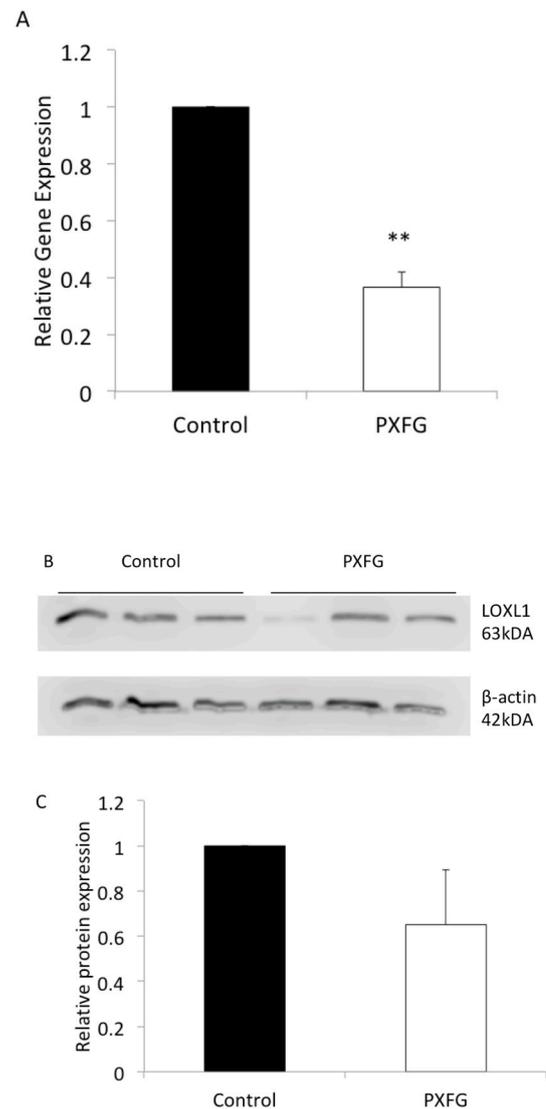


Fig. 1. Expression of *LOXL1* is decreased in Human Tenon's Fibroblasts (HTFs) cultured from PXFG patients compared with Control.

A: Quantitative real time PCR analysis of *LOXL1* mRNA expression in HTFs isolated from either Control or PXFG patients. Data is presented as mean *LOXL1* mRNA expression (\pm SD, $n = 3$) relative to levels in Control cells, set to a value of 1. The asterisks indicate that *LOXL1* mRNA expression was significantly decreased in PXFG (fold change 0.36 ± 0.05 , ** $P < 0.01$). +Patient groups had no statistically significant differences in age (Control = 80.5 ± 2.2 , PXFG = 72.3 ± 8.6 , $P = 0.35$) or gender ($P = 0.22$). **B:** Immunoblot analysis of *LOXL1* expression in HTFs isolated from either Control or PXFG patients. To confirm uniform protein loading, immunoblots were screened with an anti- β -Actin antibody. Data presented is representative of 3 independent patient samples experiments. The relative molecular size of the proteins (kDa) is indicated to the right of the panels. **C:** Densitometric analysis of immunoblots. Data is presented as relative *LOXL1* protein expression, relative to levels in Controls, set to a value of 1. *LOXL1* protein expression was decreased in PXFG HTFs compared with Control HTFs (fold change 0.65 ± 0.24 , $P = 0.22$).

(Fig. 3C) and densitometry (Fig. 3D) showed that there was an increase in protein expression of *DNMT1* (fold change 5.70 ± 2.64 , $P = 0.04$), *DNMT3A* (fold change 1.79 ± 1.55 , $P = 0.42$) and *MeCP2* (1.64 ± 1.33 , $P = 0.45$) in HTFs isolated from PXFG donors compared to levels in Control HTFs.

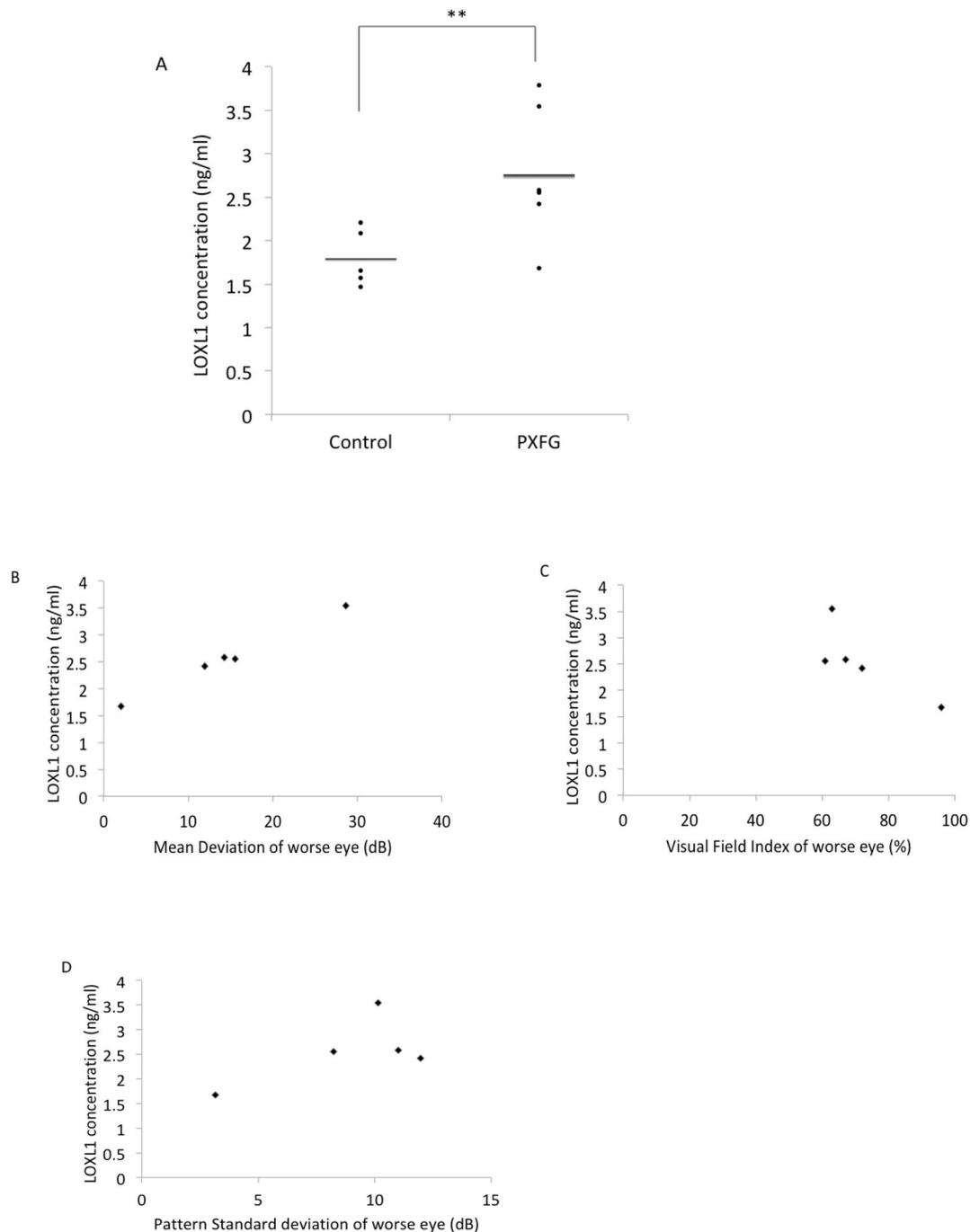


Fig. 2. Concentration of LOXL1 in the aqueous humour is elevated in PXFG patients and correlates with disease severity.

A: Enzyme-linked immunosorbent assay (ELISA) of LOXL1 concentration was carried out in aqueous humour from Control and PXFG patients. Graph shows concentration of LOXL1 detected compared to known standards. Each dot represents one individual patient (Control $n = 5$, PXFG $n = 6$)[†]. The solid line depicts the mean concentration, where mean LOXL1 concentration in Control aqueous was 1.79 ± 0.32 ng/ml compared with 2.76 ± 0.78 ng/ml in PXFG patients. Data shows mean LOXL1 concentration was increased in PXFG (** $P < 0.01$). [†] $n = 1$ Control eliminated following Tukey's test + Patient groups had no statistically significant differences in age (Control 80 ± 2.6 , PXFG 80 ± 2.0 , $P = 0.97$) or gender ($p = 0.42$) **B:** The relationship between LOXL1 concentration (ng/ml) in the aqueous humour and the Mean Deviation (MD) of the Patient's worse eye on Humphrey visual field analysis. There was a linear relationship between increasing LOXL1 concentration and higher mean deviation. This was statistically significant ($P = 0.02$). **C:** The relationship between LOXL1 concentration (ng/ml) in the aqueous humour and the Visual Field Index (VFI) of the Patient's worse eye on Humphrey visual field analysis. There was a linear relationship between increasing LOXL1 concentration and decreasing Visual Field Index. This was statistically significant ($P < 0.01$). **D:** The relationship between LOXL1 concentration (ng/ml) in the aqueous humour and the Pattern Standard Deviation (PSD) of the Patient's worse eye on Humphrey visual field analysis. There was a linear relationship between increasing LOXL1 concentration and higher pattern standard deviation. This was statistically significant ($P < 0.01$).

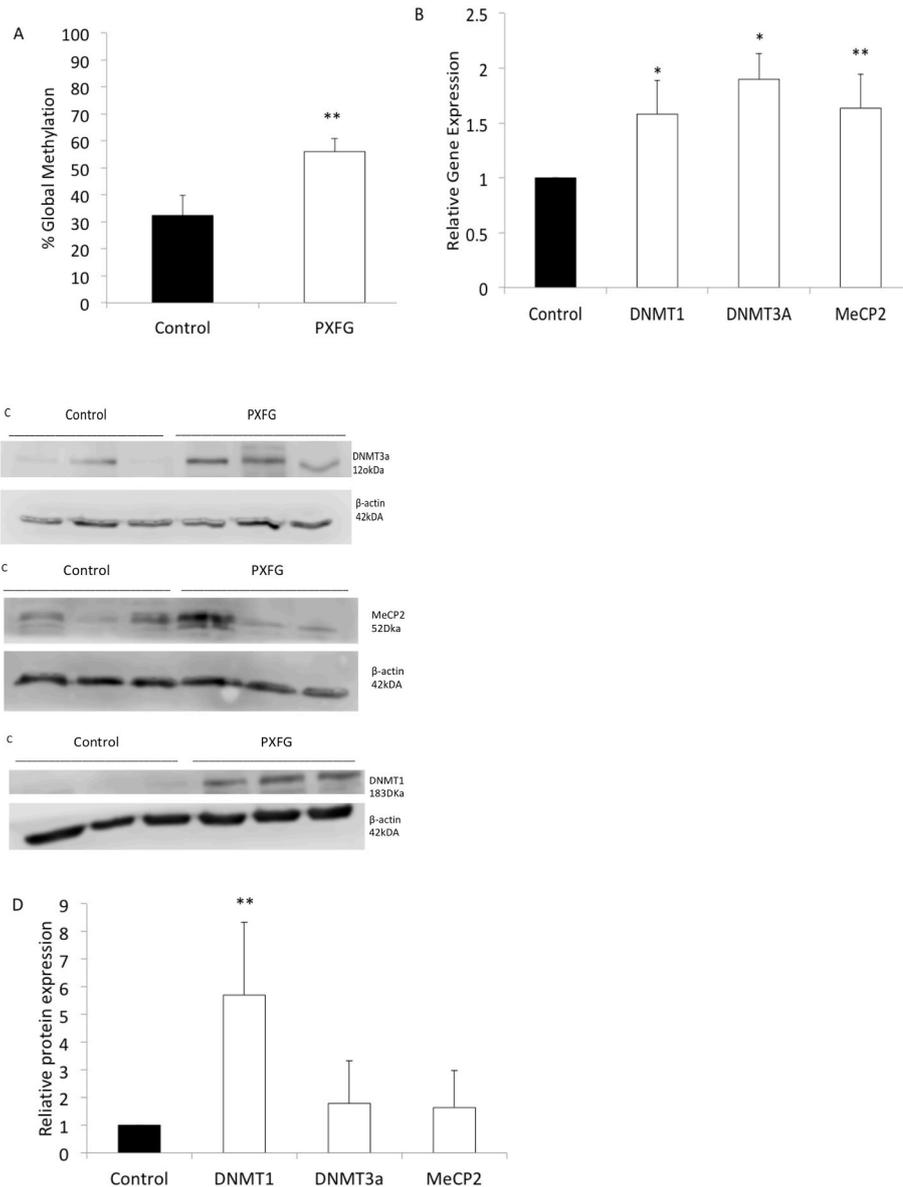


Fig. 3. Global methylation levels and expression of methylation associated enzymes are increased in HTF cells isolated from PXFG patients compared with Control.

A: Global methylation levels were measured in Control and PXFG HTF cells using an ELISA assay. Data is presented as the mean percentage (%) global methylation ($n = 3; \pm SD$) in Control and PXFG samples as compared to a methylated DNA standard, set to 100%. Bar chart shows that % global methylation was increased in PXFG compared with Controls (56.07 ± 4.87 vs. 32.39 ± 7.45 , $P < 0.01$). +No statistically significant difference in age (Control = 81 ± 2.4 , PXFG: 78 ± 2.51 , $p = 0.65$) or gender ($p = 0.52$). B: Quantitative RT-PCR analysis of DNA methyl transferase (*DNMT1*), *DNMT3a* and Methyl CpG binding protein (*MECP2*) mRNA expression. Data is presented as mean change in gene mRNA expression ($\pm SD$, $n = 3$) relative to levels in Control cells, set to a value of 1. There was increased mRNA expression of *DNMT1* (fold change 1.58 ± 0.30 , $P < 0.05$), *DNMT3a* (fold change 1.89 ± 0.23 , $P < 0.05$) and *MeCP2* (fold change 1.63 ± 0.31 , $P < 0.01$) in PXFG HTFs compared to Control HTFs. The asterisks indicate that *DNMT1*, *DNMT3a* and *MECP2* mRNA expression was significantly increased in PXFG (* $P < 0.05$, ** $P < 0.01$). +No statistically significant difference in age (Control = 81.7 ± 2.75 , PXFG: 72.27 ± 8.61 , $p = 0.50$) or gender ($p = 0.12$). C: Immunoblot analysis of *DNMT1*, *DNMT3a* and *MeCP2* expression in HTFs isolated from either Control or PXFG patients ($n = 3$ for both). To assess protein loading, immunoblots were probed with an anti- β -Actin antibody. The relative molecular size of the proteins (kDa) is indicated to the right of the panels. D: Densitometric analysis of immunoblots. Data is presented as relative *DNMT1*, *DNMT3a* and *MeCP2* protein expression, relative to levels in Controls, set to a value of 1. *DNMT1* protein expression was increased in PXFG HTFs compared with Control HTFs (fold change 5.70 ± 2.64 , $P = 0.04$). *DNMT3a* protein expression was increased in PXFG HTFs compared with Control HTFs (fold change 1.79 ± 1.55 , $P = 0.42$). *MeCP2* protein expression was increased in PXFG HTFs compared with Control HTFs (fold change 1.64 ± 1.33 , $P = 0.45$). (* $P < 0.05$, ** $P < 0.01$).

3.4. *LOXL1* promoter is hypermethylated in PXFG

LOXL1 was previously reported to be hypermethylated in Cutis Laxa (Debret et al., 2010). Using the Meth Primer 2.0 software we identified a CpG island within the *LOXL1* promoter region spanning the region -419 to -248 . The numbering system relates to the genomic positioning relative to the translational initiation codon (ATG) of the *LOXL1* translated region is at $+1$. Using a methylated DNA immunoprecipitation (MeDIP) assay we found significantly increased DNA methylation of the *LOXL1* promoter region in patients with PXFG compared to Control patients in both blood (Fig. 4A) (3.98 ± 2.24 , 2.10 ± 1.29) ($P < 0.05$) and HTF cells (Fig. 4B) (37.31 ± 22.0 , 8.66 ± 10.40) ($P < 0.01$) when calculated relative to the input. Levels of *LOXL1* promoter methylation varied between patients, but overall mean levels of methylation were increased in PXFG compared to Controls.

3.5. The DNA methylation inhibitor 5-azacytidine increases expression of *LOXL1* in PXFG HTFs

PXFG HTFs were treated with 5-azacytidine to investigate if inhibition of DNA methylation could restore *LOXL1* expression in PXFG HTFs.

LOXL1 mRNA expression was significantly increased ($P < 0.05$) in PXFG HTFs treated with 5-azacytidine compared with untreated PXFG HTFs (fold change 2.27 ± 0.68) while methylation associated enzymes were decreased (*DNMT1* 0.63 ± 0.10 , ** $P < 0.01$, *DNMT3a* 0.60 ± 0.41 , *MeCP2* 0.95 ± 0.13) (Fig. 5A). Immunoblot analysis (Fig. 5B and C) demonstrated that *LOXL1* protein levels were increased in 5-aza treated PXFG HTF cells compared with untreated (fold change 1.95 ± 1.79), however, upon analysis by densitometry statistical significance was not reached ($P = 0.62$).

4. Discussion

Here we have demonstrated that decreased *LOXL1* expression in PXFG is orchestrated via DNA methylation of the *LOXL1* promoter.

Firstly, we aimed to establish if *LOXL1* expression was altered in PXFG. *LOXL1* was decreased in HTFs of patients with PXFG at both the RNA and protein level (Fig. 1). Albeit, the PXFG HTFs showed wide patient variability in protein expression so the decrease did not reach statistical significance. *LOXL1* was reported to be decreased in the LC (Schlötzer-Schrehardt et al., 2012), lens capsules (Khan et al., 2010) and aqueous humour (Gayathri et al., 2016) of PXFG patients. It is known

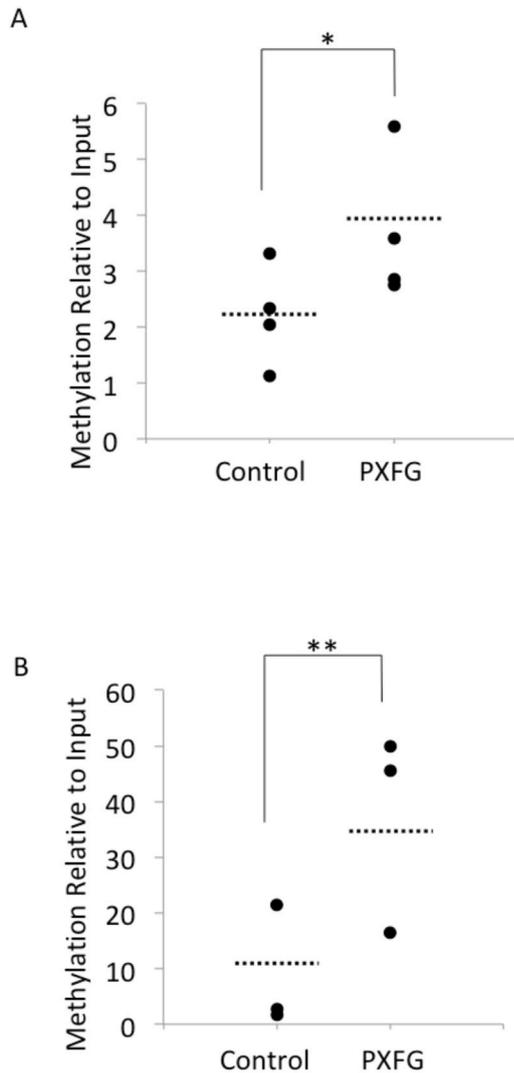


Fig. 4. Altered methylation status of CpG island identified within the *LOXL1* promoter between PXFG and Control. Quantitative real time PCR analysis of MeDIP assay elutes. Graphs show levels of amplification of methylated DNA in Control and PXFG elutes following MeDIP assay, relative to that and as a percentage of their respective inputs, where gDNA was extracted from either blood (**A**; $n = 4$) or HTFs (**B**; $n = 3$). DNA methylation of the *LOXL1* promoter region was significantly increased in patients with PXFG compared to Control patients in both blood (3.98 ± 2.24 , 2.10 ± 1.29) ($P < 0.05$) and HTF cells (76.13 ± 5.89 , 74.21 ± 7.08 , $P = 0.69$, Gender $P = 0.54$). The tenons had a significant difference in age profile but no statistically significant difference in gender (Age; Control = 83.75 ± 3.04 , PXFG = 75.41 ± 1.57 , $P = 0.01$, Gender $P = 1.00$). Dashed line represents the mean amplification of all patients. Each point represents one patient assayed. Asterisk signifies that the *LOXL1* promoter was significantly methylated in PXFG patients compared to Controls (* $P < 0.05$, ** $P < 0.01$).

that *LOXL1* deficiency results in elastinopathy. *LOXL1* is downregulated in the PXF LC region, along with the main elastic fibre components of PXF material (elastin, fibrillin-1, fibulin-4) (Schlötzer-Schrehardt et al., 2012). This global reduction in elastic fibre components compromises the LC and ONH structure and may explain why IOP independent optic neuropathy tends to be more severe in PXFG than in other forms of glaucoma (Grødem et al., 2005) and how the altered biomechanics in the elastic tissue of the ONH can accelerate this damage. This *LOXL1* related elastinopathy also provides an explanation for some of the surgical complications commonly seen in PXF such as lens subluxation and

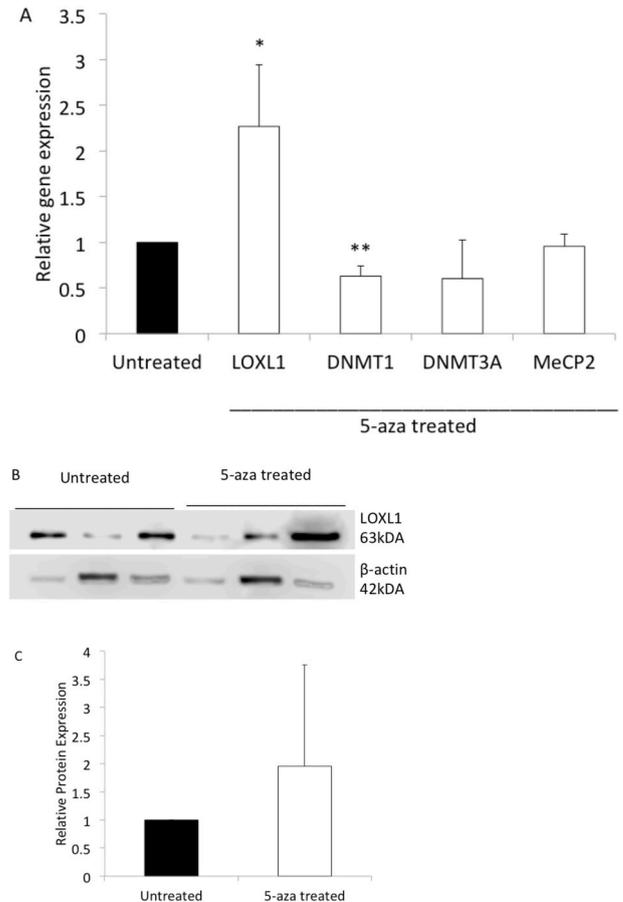


Fig. 5. The methylation inhibitor 5-azacytidine increases expression of *LOXL1* in HTF from PXFG patients

A: Quantitative RT-PCR analysis of *LOXL1*, *DNMT1*, *DNMT3a* and *MeCP2* mRNA expression in HTFs isolated from PXFG patients. Cells were treated with 5-azacytidine (5-aza; $0.3 \mu\text{M}$) for 24 h. Data is presented as mean *LOXL1* mRNA expression (\pm SD, $n = 3$) relative to levels in untreated cells, set to a value of 1. *LOXL1* mRNA expression was increased in 5-aza treated cells compared with untreated cells (2.27 ± 0.68 , * $P < 0.05$) while methylation associated enzymes were decreased (DNMT1 0.63 ± 0.10 , ** $P < 0.01$, DNMT3a 0.60 ± 0.41 , MeCP2 0.95 ± 0.13).

B: Immunoblot analysis of *LOXL1* expression in PXFG HTF cells treated with 5-aza ($0.3 \mu\text{M}$; 24 h). To confirm uniform protein loading, immunoblots were probed with an *anti*- β -Actin antibody. Each lane represents an independent patient sample. The relative molecular size of the proteins (kDa) is indicated to the right of the panels. **C:** Densitometric analysis of immunoblots. Data is presented as *LOXL1* protein expression, relative to expression levels in untreated samples, set to a value of 1. *LOXL1* protein expression was increased in 5-aza treated PXFG HTFs compared with untreated PXFG HTFs, however it was not significant (1.95 ± 1.79 , $P = 0.62$).

zonule dehiscence. Systemic manifestations of elastinopathy such as pelvic organ prolapse (Drewes et al., 2007; Wirostko et al., 2016), inguinal hernias (Besch et al., 2018) and abdominal aortic aneurysms (Djordjevic-Jocic et al., 2012; Gonen et al., 2013; Schumacher et al., 2001) have all been associated with PXF and similar findings have been described in *LOXL1* knockout mice (Alsofi et al., 2016; Gauthier and Liu, 2017; Lee et al., 2008; Liu et al., 2004, 2007; Wiggs et al., 2009).

While *LOXL1* concentration in the aqueous was previously reported to be decreased in PXFG (Gayathri et al., 2016), we found it to be increased in our patient cohort (Fig. 2A). This may be due to the fact that *LOXL1* expression has been found to vary by disease state, with expression decreased in late disease compared with early disease in tissue from donor eyes (Schlötzer-Schrehardt et al., 2008). However, in this case disease severity was based on the amount of macroscopically

visible PXF material seen rather than a clinical parameter. We compared the concentration of LOXL1 in the aqueous with our patients Mean Deviation (MD), Pattern Standard Deviation (PSD) and Visual Field Index (VFI) on visual field testing. The MD gives an overall value of the patients total visual field loss, with higher values representing a poorer visual field defect. The PSD shows more focal and early loss while correcting for global changes in sensitivity, which occur in patients with Cataract. The Visual Field Index (VFI) is a staging index representing the total amount of visual loss. We found that patients with more severe visual field loss had a higher concentration of LOXL1 in the aqueous (Fig. 2B–D). It is also important to note that the source of LOXL1 in the aqueous is the ciliary epithelium while the source of LOXL1 in our primary cells was fibroblasts. LOXL1 levels may be inherently different in cells of epithelial origin compared with those of mesenchymal origin. The main areas of ECM dysregulation in PXF and glaucoma are the trabecular meshwork (Sethi et al., 2011a) and LC (Schlötzer-Schrehardt et al., 2012) which are both of mesenchymal origin and show decreased LOXL1 expression similar to what was observed in our HTFs. This increased LOXL1 aqueous expression could also be due to our geographical location and colder Irish climate. Cold weather has also been found to lead to increased aggregation of PXFG material in the aqueous (Lee, 2008; Pasquale et al., 2014) and LOXL1 has been identified by proteomic analysis as a component of PXF material (Ronci et al., 2013; Sharma et al., 2009).

These data indicate that LOXL1 exists in a dysregulated state in PXFG; however, it does not explain the cause of this gene dysregulation. LOXL1 has been reported as being decreased in Cutis Laxa (Debret et al., 2010), aged human skin fibroblasts (Moulin et al., 2017) and bladder cancer (Wu et al., 2007) in association with increased promoter methylation (Debret et al., 2010; Wu et al., 2007, 2015) and increased binding of methylation associated enzymes (Moulin et al., 2017). While LOXL1 has not been reported to be decreased secondary to methylation in PXFG, It has been shown to be silenced due to hypermethylation of CpG islands in the LOXL1 promoter in anterior lens capsules in PXF (Ye et al., 2015). With this in mind our research focus was determining whether the LOXL1 decrease, seen in our PXFG HTFs was due to epigenetic silencing by DNA methylation.

We found that there was increased global DNA methylation in PXFG compared with Control cells (Fig. 3). Increased DNA methylation has previously been demonstrated in POAG. Our group reported increased global DNA methylation and decreased *TGFβ1* promoter methylation in POAG LC cells (F. S. McDonnell et al., 2016). These data suggested to us that DNA methylation with reduced gene expression could also provide an explanation as to why expression of LOXL1 is reduced in PXFG HTFs.

Gene expression of methylation-associated genes was increased in PXFG HTFs compared to control HTF cells (Fig. 3). Increased DNMT1 has been shown to alter the methylation status of genes associated with fibrosis (Bechtel et al., 2010) and is increased in POAG LC cells (F. S. McDonnell et al., 2016). The increased expression of DNMT1 in PXFG may indicate a similar mechanism. DNMT3a was also found to be increased in PXFG HTFs compared with Control HTFs. DNMT3a expression and global methylation were upregulated in response to damaged collagen in smooth muscle cells (Jiang et al., 2013). This is of particular interest as the collagen matrix is altered and damaged in glaucoma (Lütjen-Drecoll, 2005; Schlötzer-Schrehardt et al., 2012) and these matrix changes may be a cause for the increased expression of DNMT3a in PXFG. DNMT3a has been previously found to be upregulated in PXF but not PXFG lens capsules indicating that increased de novo methylation may occur at this disease stage (Hayat et al., 2020).

MeCP2 levels were also examined. MeCP2 is a protein that binds to methylated DNA and can either activate or repress gene expression. MeCP2 is thought to recognise methylated CpG motifs and counteract binding of the transcription factor Sp-1 by DNA accessibility competition leading to reductions in LOXL1 expression (Debret et al., 2010; Kudo, 1998). Sp-1 is the main inducer of LOXL1 promoter activation in healthy fibroblasts (Debret et al., 2010). These Sp-1 binding motifs are

located in a GC rich area of LOXL1 prone to methylation and methylated DNA has been shown to counteract Sp-1 binding preventing transcription (Clark et al., 1997). MeCP2 expression was increased in PXFG HTFs compared with control HTFs (Fig. 3) indicating that this may be the mechanism of LOXL1 repression in PXFG.

Finally, we found there was increased LOXL1 promoter methylation in PXFG compared with Controls (Fig. 4). Hypermethylation of CpG islands in the LOXL1 promoter region has been seen in the anterior lens capsules of PXF patients compared to controls (Ye et al., 2015). More importantly, these methylation patterns seemed to influence LOXL1 expression with LOXL1 mRNA levels reduced in PXF lens capsules compared with controls. This paper highlights that similar patterns of methylation may also occur in PXFG. It also highlights that different ocular tissues have similar patterns of methylation in disease, with methylation seen in both the anterior lens capsule and HTFs. In systemic disease higher binding of DNMT3a to the LOXL1 promoter has also been previously observed in aged skin fibroblasts which reduced LOXL1 transcription (Moulin et al., 2017). Overall, these alterations in methylation profile and the associated enzymes observed indicate that the LOXL1 decrease observed in PXFG HTFs was possibly a result of epigenetic silencing by DNA methylation and LOXL1 promoter hypermethylation.

The impact of LOXL1 promoter hypermethylation to associated transcription factors and proteins remains largely unknown. A deletion of the LOXL1 SNP rs7173049 was shown to enhance transcription of STRA6 and ISLR2 (Berner et al., 2019). STRA6 is a retinol transporter and is important in the regulation of vitamin A and its derivatives, including retinoic acid. While this receptor is important in maintaining photoreceptor homeostasis and retinal thickness, its overexpression has been shown to lead to insulin resistance and SNPs in this gene are associated with type 2 Diabetes. ISLR2 is required for axon extension and navigation during neural development, and more specifically in the guidance of RGC axons at the optic chiasm (Panza et al., 2015). Abnormalities in this protein may have the potential to exacerbate the optic neuropathy previously observed in glaucoma. Other transcription factors such as Sp-1 and RXRα are negatively impacted by reduced LOXL1 expression and this may lead to more generalised repression of transcription pathways in PXFG.

The current treatment options for PXF and PXFG are limited to lowering the IOP either medically or surgically. However, PXFG presents with more severe IOP independent optic neuropathy and patients often have a poorer medication response compared with POAG. If the LOXL1 reduction observed in PXFG HTFs is due to methylation, there is the potential that methylation inhibition could represent an additional therapeutic target in the management of PXFG. The DNA methyltransferase inhibitor 5-azacytidine, in particular, could potentially be used to counter the hypermethylation observed in PXFG. 5-azacytidine has been shown to increase LOXL1 expression in aged skin fibroblasts (Moulin et al., 2017) and in Cutis Laxa (Debret et al., 2010) and has also been shown to decrease fibrosis in kidney fibroblasts and renal fibrosis (Bechtel et al., 2010) and in a mouse model of myocardial infarction (Kim et al., 2014). Furthermore, our lab has previously demonstrated that this inhibitor has the potential to reduce the fibrosis seen in glaucoma (F. McDonnell et al., 2016).

LOXL1 expression was increased in 5-azacytidine treated PXFG HTFs compared with untreated PXFG HTFs (Fig. 5), reinforcing our hypothesis that LOXL1 expression is regulated by methylation in PXFG. It also indicates that 5-azacytidine may reverse the changes in LOXL1 expression seen in PXFG and highlights the therapeutic potential of this approach. However, there are several limitations associated with this concept such as the high levels of associated nephrotoxicity. Other alternative inhibitors of LOXL1 and LOXL1 stimulatory pathways could also be investigated as possible future treatment options. There is increasing evidence that Smad inhibitors could be effective in reducing fibrosis in PXFG by attenuating TGFβ signaling pathways (Sethi et al., 2011a, 2011b). Based on these data and the above literature LOXL1

downregulation may be occurring due to DNA methylation and LOXL1 promoter methylation. Inhibitors of DNA methylation, *LOXL1* and *LOXL1* stimulatory pathways may represent potential future treatment options in the management of PXFG.

5. Conclusion

The pathogenesis of PXFG is likely due to a combination of genetic and epigenetic factors. LOXL1 expression is downregulated in PXFG due to DNA methylation and LOXL1 promoter methylation. The DNA methyltransferase inhibitor 5-azacytidine restores LOXL1 expression in PXFG. These data have added to our understanding of how LOXL1 expression is altered in PXFG and has provided evidence to explore future ways of exploiting chromatin-modifying interventions for therapeutic benefits.

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Declaration of competing interest

None.

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