Regulation of Lysyl Oxidase-like 1 (LOXL1) and Elastin-Related Genes by Pathogenic Factors Associated with Pseudoexfoliation Syndrome

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PURPOSE. Pseudoexfoliation (PEX) syndrome/glaucoma is a complex, late-onset disorder of the elastic fiber system. Strong genetic risk is conferred by the lysyl oxidase-like 1 (LOXL1) gene, but additional comodulating factors are necessary for the manifestation of the disease. The aim of this study was to analyze the effect of various PEX-associated pathogenic factors on the genotype-correlated expression of LOXL1 and elastin-related genes.

METHODS. Cultured human Tenon’s capsule fibroblasts with high- and low-risk LOXL1 haplotypes were exposed to transforming growth factor (TGF)-β1, interleukin (IL)-6, homocysteine, oxidative stress, hypoxia, or ultraviolet (UV) radiation. Changes in the expression of LOXL1 and elastic constituents of PEX material and TGF-β1 were assessed by quantitative real-time PCR, Western blotting, immunohistochemistry, and electron microscopy.

RESULTS. Treatment of fibroblasts with TGF-β1, oxidative stress, UV light, and hypoxia induced a significant increase in expression levels of LOXL1 and elastic proteins, whereas the effect of IL-6 was limited to induction of elastic constituents. Immunohistochemistry and electron microscopy confirmed an upregulation of LOXL1 and elastic fiber proteins and their assembly into extracellular microfibrillar networks with focal aggregation of microfibrils into PEX-like fibrils on stimulation with TGF-β1 and oxidative stress. Basal and stimulated expression of LOXL1 mRNA and protein was slightly decreased in cells carrying the high-risk compared with the low-risk haplotype of LOXL1, but the differences between groups were statistically not significant.

CONCLUSIONS. The findings support the notion that both genetic and nongenetic fibrogenic factors, particularly TGF-β1 and oxidative stress, may cooperate in the stable accumulation of PEX fibrils.1 It accounts for a majority of glaucoma cases in some countries and for approximately 25% of open-angle glaucoma cases worldwide.2 Progressive obstruction of the aqueous humor outflow pathways by abnormal PEX material deposits is considered the primary cause of chronic pressure elevation and development of open-angle glaucoma in eyes with PEX syndrome.3 Moreover, PEX deposits are found in a multitude of intra- and extraocular tissues, including conjunctiva, skin, and connective tissue compartments of visceral organs.4 As shown by biochemical and immunohistochemical studies, PEX fibrils predominantly contain elastic fiber components, such as elastin, fibrillin-1, latent transforming growth factor binding proteins (LTBP-1/2), and fibulins (fibulin-2/4), as well as lysyl oxidase-like 1 (LOXL1).5–7 A key cross-linking matrix enzyme normally required for elastic fiber formation and stabilization.8 However, the exact composition of the abnormal extracellular material as well as the mechanisms responsible for its excessive production and accumulation still remain elusive.

PEX syndrome is generally considered as a complex, multifactorial, late-onset disease involving a combination of genetic and nongenetic factors in its etiopathogenesis.9 Genetic studies in multiple geographical populations have provided conclusive evidence that single nucleotide polymorphisms (SNPs) in exon 1 of the LOXL1 gene represent the principal genetic risk factor for both PEX syndrome and PEX glaucoma.10,11 A common haplotype (G-G) composed of the two coding SNPs rs1048861 (R141L) and rs3825942 (G153D) appears to be the strongest associated risk factor in Caucasian populations with PEX.12 but also occurs in approximately 50% of the normal population. Due to the high prevalence of the PEX-associated LOXL1 variants in the general population, it has been suggested that, in addition to the LOXL1 risk alleles, other genetic variants and/or environmental factors are necessary for manifestation of the PEX phenotype. Additional genetic factors with smaller effect sizes appear to include SNPs in the genes for clusterin (CLU) and contactin-associated protein-2 (CNTNAP2), which may act as modifying genes in certain populations.13–15 Moreover, several stress conditions associated with PEX, including oxidative stress,16 hypoxia,17 ultraviolet (UV) radiation,18 as well as elevated levels of profibrotic growth factors and cytokines such as transforming growth factor-β1 (TGF-β1)19 and interleukin-6 (IL-6)20 may potentially act as comodulating external factors. Finally, elevated homocysteine levels in plasma and aqueous humor of patients with PEX may further contribute to PEX pathophysiology.21

LOXL1 is a key enzyme involved in elastic fiber synthesis and homeostasis by catalyzing the covalent cross-linking of tropoelastin monomers into elastin polymers.22 Recently, we provided evidence that LOXL1 and elastic fiber components are transiently upregulated in ocular tissues during the early stages of the fibrotic PEX process, suggesting their participation in the formation and aggregation of abnormal PEX fibrils.3 This observation is in agreement with published studies dem-
onstrating that LOXL1, in conjunction with its putative extra-
cellular substrates, becomes transiently upregulated and acti-
ated at early stages of fibrogenesis (e.g., in liver fibrosis). Pro-
fiobrotic stimuli, such as TGF-β1 or oxidative stress, have been
demonstrated to regulate the expression of LOXL1 and extracel-
lar matrix molecules in various experimental settings and may therefore be considered candidate comodulating factors in
PEx pathophysiology. Consistently, it may be hypothesized that
the abnormal matrix process characteristic of PEX syn-
drome can be activated by certain fibrogenic stimuli and, in
the background of the high-risk LOXL1 haplotype, participate in
the formation and accumulation of PEX aggregates within intra-
and extraocular tissues.

The purpose of the present study, therefore, was to analyze
the effect of a number of PEX-associated pathogenic factors
and stress conditions, including TGF-β1, IL-6, homocysteine,
oxidative stress, hypoxia, and UV radiation, on the expression
of LOXL1 and elastic fiber proteins by human Tenon’s capsule
fibroblasts obtained from patients with different LOXL1 haplo-
types in vitro. The potential influence on expression of TGF-
β1, a key mediator in PEX fibrogenesis, was analyzed in
addition. Here we show that most pathogenic factors, partic-
ularly TGF-β1, oxidative stress, and UV light, have the ability
to upregulate the expression of LOXL1 and major elastic fiber
proteins present in PEX deposits, on both the mRNA and the
protein level, in a dose-dependent manner. These factors were
also found to stimulate the assembly of an elastic microfibrillar
network and the formation of microfibrillar aggregates resem-
bles PEX fibrils in the extracellular space. Although significant
differences between LOXL1 haplotype groups could not be
established, the findings support the notion that both genetic
and nongenetic fibrogenic factors may cooperate in the stable
accumulation of PEX aggregates.

Materials and Methods

Cell Culture

Tenon’s capsule biopsies from five patients with cataract and PEX
syndrome (18.2 ± 6.4 years old; 2 females, 3 males) and three
patients with cataract without PEX syndrome (38.0 ± 3.9 years old;
1 female, 2 males) were obtained during cataract surgery. Informed
counsel to tissue donation was obtained from the patients and the
protocol of the study was approved by the local Ethics Committee
and adhered to the tenets of the Declaration of Helsinki for exper-
iments involving human tissues and samples. For establishing ex-
plant cultures, the biopsies were dissected, placed in 50-mL tissue-
culture flasks in Dulbecco’s modified Eagle’s medium (DMEM/Ham’s
F12; Invitrogen, Darmstadt, Germany) containing 15% (vol/vol) fetal
calf serum and antibiotic-antimycotic solution (Invitrogen), main-
tained at 37°C in a humidified 95% air/5% CO2 atmosphere, and cell
layers were passaged at confluence. For all experiments Tenon’s
capsule fibroblasts were used at passage three to four. The LOXL1
haplotype, formed by the two nonsynonymous coding SNPs
rs1048661 and rs3825942 in exon 1 of the
LOXL1 gene, was determined through direct sequencing of cDNA from cultured cells as previously described.

To study the regulation of expression of LOXL1 and elastic fiber
components, cells were grown to subconfluence (90%), kept in serum-
free medium for 24 hours, and then exposed to either TGF-β1 (1–10
ng/mL; R&D Systems, Wiesbaden, Germany), IL-6 (5–50 ng/mL; Pep-
rotech, Hamburg, Germany), homocysteine (5–500 μM; Sigma-Aldrich,
Munich, Germany), or oxidative stress (50–500 μM hydrogen peroxide
[H2O2]; Sigma) for 48 hours under serum-free conditions. Exposure to
hypoxia (2% oxygen) for 6, 12, 24, or 48 hours was carried out as previously described. Exposure to UV radiation (5–40 mJ/cm2) was
also studied on once in PBS with a previously described UV-ABL source (wavelength: 300–400 nm), followed by replacement of PBS with

Regulation of LOXL1 and Elastin-Related Genes

Cell viability was assessed using a fluorescent kit (Live/Dead Viability/Cytotoxicity kit; Molecular Probes, Eugene, OR) and a plate
reader (Fluoroscan Ascent 2.4; Thermo Scientific, Bonn, Germany) as previously described. Total RNA was extracted from cultured cells using a commercial kit
(RNeasy kit; Qiagen, Hilden, Germany), including an on-column DNase digestion step. First-strand cDNA synthesis from 1 μg of total RNA and quantitative real-time PCR were performed using a thermal cycler and software (MyQ; BioRad, Munich, Germany) as previously described. Total cellular protein (10 μg) and concentrated cell culture supernatant (20 μL), respec-
tively, were separated by SDS–polyacrylamide gel electrophoresis un-
der reducing conditions and transferred onto nitrocellulose mem-
branes (Hybond ECL; GE Healthcare, Munich, Germany) with a semi-dry blotting unit (BioRad). Membranes were blocked with 5% low-fat milk powder (Sigma-Aldrich) for 1 hour and incubated over-
night at 4°C with rabbit polyclonal antibody against LOXL1 (kindly
provided by Ion Hornstra, St. Louis, MO), diluted 1:500 (Superblock;
Thermo Scientific). Equal loading was verified with mouse anti-human β-actin antibody (clone AC-15; Sigma-Aldrich), 1:5000 (in Superblock). In negative control experiments, the primary antibody was replaced by PBS. Immunodetection was performed with a horseradish peroxidase-
conjugated secondary antibody, diluted 1:10000 (Superblock, Super Signal West Pico ECL kit; Thermo Scientific), and a chemiluminescence
detection system (ChemilSmart 5000; Vilber Lourmat, Eberhardzell,
Germany) according to the manufacturer’s instructions.

Immunohistochemistry

Immunofluorescence labeling was performed on cultured fibroblasts in
two-well chamber slides (0.5 × 105 cells/well) grown to subconflu-
ence (90%), kept in serum-free medium for 24 hours, and then exposed
to TGF-β1 (10 ng/mL) or oxidative stress (100 μM H2O2) for 72 hours as described earlier. Cells were fixed in 70% methanol for 10 minutes at
4°C, blocked with a 1:10 dilution of normal goat serum (Dako,
Glostrup, Denmark) for 30 minutes, and incubated with antibodies
against LOXL1 (kindly provided by Ian Hornstra, St. Louis, MO), fibril-
lin-1 (kindly provided by Dieter Reinhardt, Montreal, Canada), elastin
(PR533, Elastin Products Company), fibrillin-2 and fibrillin-4 (kindly
provided by Takako Sasaki, Erlangen, Germany), LTBP-1 (BD Biosci-
ences), and LTBP-2 (Takako Sasaki) for 2 hours at room temperature. Antibody binding was detected by secondary antibodies (Alexa 488–
conjugated; Molecular Probes) and nuclear counterstaining was per-
formed with propidium iodide or 4′,6-diamidino-2-phenindole (DAPI; Sigma-Aldrich). In negative control experiments, the primary antibody was replaced by PBS or equimolar concentrations of an irrelevant primary antibody.

**Transmission Electron Microscopy of Three-Dimensional Cultures**

To study extracellular matrix production and assembly, fibroblasts were suspended in basement membrane matrix (Matrigel; BD Biosciences, San Jose, CA), which was prepared according to the manufacturer’s protocol, at a final concentration of 500,000 cells/mL. After solidification, the gels were overlayed with medium containing 10 ng/mL TGF-β1 or 100 µM H2O2 for 72 hours. Parallel cultures maintained under serum-free conditions without exposure to pathogenic stimuli served as controls. After incubation the gels were fixed in 2.5% buffered glutaraldehyde, postfixed in 2% buffered osmium tetroxide, dehydrated in a graded ethanol series, and embedded in epoxy resin according to standard protocols. Ultrathin sections were examined with a transmission electron microscope (LEO 906E; Carl Zeiss NTS, Oberkochen, Germany).

**Statistics**

Data are presented as mean ± SD and were analyzed using analytical statistical software (SPSS, Inc., Chicago, IL). Statistical evaluation of differences between groups was performed using the Student’s t-test, and a P-value of P < 0.05 was considered statistically significant.

**RESULTS**

**Effect of Fibrogenic Stimuli on mRNA Expression of LOXL1 and Elastic Proteins**

To investigate the effect of PEX-associated fibrogenic stimuli on the expression of LOXL1 and elastic constituents of PEX material, cultures of human Tenon’s capsule fibroblasts obtained from PEX patients (n = 4) were treated with either TGF-β1 (1–10 ng/mL), IL-6 (5–50 ng/mL), or homocysteine (5–500 µM), or exposed to oxidative stress (50–500 µM H2O2), hypoxia (2% oxygen), or UV radiation (5–40 mJ/cm2) up to 48 hours. To exclude potential genotype-related expression differences of LOXL1,5,10 all cell cultures used in these experiments carried the high-risk haplotype (G-G) in the homozygous state. To further exclude cytotoxic effects of stress conditions, cell viability assays were performed in parallel after stimulation with homocysteine, H2O2, hypoxia, and UV radiation. Live/dead assays revealed ratios of living versus dead cells that were not significantly different from untreated control cells within the applied time periods and intensities of stress (data not shown).

Quantitative real-time PCR demonstrated a time- and dose-dependent upregulation of most gene products in response to the various external factors applied. TGF-β1 consistently caused a significant upregulation of all gene products analyzed (Fig. 1). Peak stimulatory effects were reached with 5 ng/mL for LOXL1 (4.3-fold; P < 0.005), LTBP-1 (3.5-fold; P < 0.0005), and TGF-β1; with 10 ng/mL for elastin (3.4-fold; P < 0.0005), and with 10 ng/mL for elastin (3.4-fold; P < 0.0005), fibulin-1 (3.9-fold; P < 0.0005), LTBP-1/2 (6.7-fold; P < 0.0005), and fibulin-2/4 (up to 2.9-fold; P < 0.0005).

IL-6 induced an overall weaker and more selective stimulation of the genes of interest (Fig. 2), with maximum levels of

**FIGURE 1.** Effect of TGF-β1 on the expression of LOXL1, elastic microfibrillar components, and TGF-β1 in cultured human Tenon’s capsule fibroblasts as determined by quantitative real-time PCR. The expression levels were normalized against GAPDH and the results are expressed as molecules of interest per molecules GAPDH, together with the relative fold change in treated cells compared with untreated control cells (expression levels of fibulin-1: × 105; LOXL1, elastin, LTBP-1: × 104; fibulin-2, TGF-β1: × 105). The values represent mean values ± SD of four separate experiments; *P < 0.005, **P < 0.0005.
Homocysteine had a comparatively weak effect on the expression of the selected gene products (Fig. 3). At the highest concentration of 500 μM, only fibrillin-1 (2.1-fold; \( P < 0.0005 \)) and TGF-β1 (2.2-fold; \( P < 0.0005 \)) were significantly upregulated. A tendency to increased expression of LOXL1, fibrillin-1, and LTBP-2 (1.5 to 1.7-fold) by 500 μM homocysteine, however, did not reach statistical significance.

Oxidative stress caused a consistent upregulation of all genes of interest (Fig. 4). Peak stimulatory effects were reached at 100 and 250 μM of \( \text{H}_2\text{O}_2 \) for LOXL1 (3.5-fold; \( P < 0.0005 \)), fibrillin-1 (3.1-fold; \( P < 0.0005 \)), LTBP-1/2 (up to 6.9-fold; \( P < 0.0005 \)), fibulin-2/4 (up to 5.2-fold; \( P < 0.0005 \)), and TGF-β1 (2.6-fold; \( P < 0.0005 \)), followed by a decline in expression at 500 μM of \( \text{H}_2\text{O}_2 \). Only elastin (2.9-fold; \( P < 0.0005 \)) reached maximum expression levels at 500 μM of \( \text{H}_2\text{O}_2 \).

Similarly, exposure to UV radiation resulted in a consistent upregulation of all genes of interest (Fig. 5). Peak stimulatory effects were reached at intensities of 20 and 40 mJ/cm² for LOXL1 (3.8-fold; \( P < 0.0005 \)), elastin (2.4-fold; \( P < 0.0005 \)), fibrillin-1 (2.5-fold; \( P < 0.0005 \)), LTBP-1/2 (up to 2.7-fold; \( P < 0.0005 \)), fibulin-2/4 (up to 2.7-fold; \( P < 0.0005 \)), and TGF-β1 (2.2-fold; \( P < 0.0005 \)).

Under hypoxic conditions (Fig. 6), a significant upregulation of LOXL1 (3.4-fold; \( P < 0.0005 \)) mRNA expression was observed.
detected as early as 12 hours after exposure to 2% of oxygen. Maximum levels of upregulation were reached after 24 hours (4.9-fold; \( P < 0.005 \)). The induction of elastin expression reached significance after 24 hours (2.2-fold; \( P < 0.005 \)). In contrast, elastic microfibrillar components including fibrillin-1, LTBP-1, and fibrulins were unaffected by reduced oxygen partial pressure.

Comparative analysis revealed that expression of LOXL1 was predominantly stimulated by TGF-\( \beta \)-1, hypoxia, oxidative stress, and UV light, whereas expressions of fibrillin-1, LTBP-1, and fibrulin-2/4 could be mainly localized to prominent microfibrillar networks in the extracellular space (Figs. 7B, 7C). Extracellular elastin matrix assembly was not observed within the short culture period. Specific immunostaining was abolished when an irrelevant primary antibody or PBS was used instead of the primary antibodies (not shown).

Ultrastructural investigation of fibroblast-containing matrices (\( n = 3 \)), simulating a natural three-dimensional (3D) environment, disclosed individual fibroblasts in association with small bundles of microfibrils, approximately 8 to 10 nm in diameter, within electron-lucent pericellular spaces (Fig. 8A). Stimulation with TGF-\( \beta \)-1 (10 ng/mL) or \( \mathrm{H}_2\mathrm{O}_2 \) (100 \( \mu \mathrm{M} \)) for 72 hours appeared to increase production of microfibrils, which were occasionally observed to aggregate into thicker fibrils, approximately 35 to 45 nm in diameter, resembling mature PEX fibrils in vivo (Figs. 8B, 8C).

**Influence of LOXL1 Haplotypes on the Stimulatory Effect of Fibrogenic Factors**

To investigate the combined effects of fibrogenic stimuli and genetic background, cultured Tenon’s capsule fibroblasts carrying the high-risk LOXL1 haplotype G-G in the homozygous state, derived from PEX patients (\( n = 3 \)), were compared with fibroblasts carrying a low-risk LOXL1 haplotype T-G, obtained from patients without PEX syndrome (\( n = 3 \)). Comparative analysis showed that the basal mRNA expression levels of LOXL1 displayed a tendency toward reduced expression (approximately 20%) in the presence of the high-risk haplotype G-G compared with the T-G haplotype (Fig. 9). On stimulation with TGF-\( \beta \)-1 (10 ng/mL) or oxidative stress (100 \( \mu \mathrm{M} \) \( \mathrm{H}_2\mathrm{O}_2 \)) for 48 hours, LOXL1 expression levels showed a similar decline in G-G cultures compared with T-G cultures, but these differences between the haplotype groups were statistically not significant. In addition, no significant differences were obtained for expression levels of elastin, fibrillin-1, LTBP-1/2, and fibrulin-2/4 in both groups (data not shown).

**FIGURE 7.** Immunofluorescence labeling of LOXL1, fibrillin-1, LTBP-2, and fibrulin-2 in cultured human Tenon’s capsule fibroblasts without stimulation (A) and in response to 5 ng/mL TGF-\( \beta \)-1 (B) or 250 \( \mu \mathrm{M} \) \( \mathrm{H}_2\mathrm{O}_2 \) (C). Cell nuclei were stained with propidium iodide (red fluorescence) or DAPI (blue fluorescence) (magnification bars: 100 \( \mu \mathrm{m} \)).
To assess potential differences in LOXL1 protein synthesis and secretion, Western blot analyses were performed on lysates and supernatants of cells carrying the LOXL1 G-G or T-G haplotype without and with stimulation by TGF-β1 (10 ng/mL). LOXL1 was detected in cell lysates and media with an apparent molecular mass of approximately 62 kDa, probably representing the proenzyme, and with an additional band in culture media at 53 kDa, probably representing the active enzyme (Fig. 10). Whereas LOXL1 was barely detectable in unstimulated cells and media, stimulation with TGF-β1 resulted in a marked upregulation of LOXL1 in both cell lysates and supernatants. Similar to mRNA expression levels, expression levels of LOXL1 protein appeared slightly reduced in cells carrying the G-G haplotype compared with the T-G haplotype on stimulation with TGF-β1, but levels of secreted LOXL1 revealed no obvious differences between the groups.

**DISCUSSION**

PEX syndrome is one of the most common causes of glaucoma worldwide. This disorder has been characterized as a generalized fibrotic matrix process, a stress-induced elastosis, asso-

![Figure 8](image-url)

**FIGURE 8.** Transmission electron micrograph showing human Tenon’s capsule fibroblasts within matrigels without stimulation (A) and after exposure to 5 ng/mL TGF-β1 (B, C). The cells are associated with small bundles of microfibrils (asterisks), which focally aggregate into thicker fibrils (arrows) on stimulation with TGF-β1 (Fb, fibroblast; Ma, Matrigel; magnification bars: 0.5 μm).

![Figure 9](image-url)

**FIGURE 9.** Influence of the LOXL1 haplotype on mRNA expression of LOXL1 in cultured human Tenon’s capsule fibroblasts carrying the high-risk haplotype G-G or a low-risk haplotype T-G with or without 10 ng/mL TGF-β1 and oxidative stress (250 μM H2O2), respectively, as determined by quantitative real-time PCR. The expression levels were normalized against GAPDH. The values represent mean values ± SD of three separate experiments; *P < 0.005 compared with unstimulated cells.

![Figure 10](image-url)

**FIGURE 10.** Western Blot analysis of LOXL1 protein in cell lysates and cell culture supernatants from Tenon’s capsule fibroblasts carrying the high-risk haplotype G-G or a low-risk haplotype T-G with or without stimulation by 10 ng/mL TGF-β1. Equal loading of samples was verified by immunodetection of β-actin.

As previously shown, the contribution of multiple genetic factors and environmental conditions has been suggested. To date, circumstantial evidence exists for the contribution of several genes with relatively small effect sizes, such as *CLU* (clusterin), *CNTNAP2* (contactin-associated protein-like 2), and *APOE* (apolipoprotein E), in some study populations indicating a modifying rather than a direct genetic effect. In contrast, LOXL1 has been identified as a major contributor and principal genetic risk factor for PEX throughout all geographical populations examined. Although the PEX-associated LOXL1 missense variants showed a different allele frequency within different geographical populations, a high-risk haplotype (G-G) formed by the two coding SNPs rs1048661 (R141L) and rs3825942 (G153D) appeared to be the strongest associated risk factor for PEX in Caucasian and European populations, whereas the T-G and G-A haplotypes were associated with lower risks. However, approximately 50% of the normal population was also found to carry the high-risk haplotype, indicating that, in addition to LOXL1 risk alleles, other PEX-specific genetic variants or environmental factors may contribute to the risk of developing the PEX phenotype. Recently, novel polymorphisms in the promoter region of LOXL1 have been identified to be associated with PEX syndrome/glaucoma in a U.S. Caucasian population and were suggested to influence LOXL1 gene expression by causing a reduction in LOXL1 protein expression and activity.

In fact, dysregulated expression of LOXL1 has been previously shown to be substantially involved in PEX pathophysiology. The available data indicate that LOXL1 is transiently upregulated in anterior segment tissues at early stages of PEX fibrogenesis, together with elastic fiber constituents, to participate in the formation of the aberrant fibrillar aggregates. Thus, LOXL1 and elastic fiber components, such as elastin, fibrillin-1, LTBP-1/2, and fibulin-2/4, were found to be prominent components of fibrillar PEX aggregates in the anterior segment. In the posterior segment, lamina cribrosa tissue of PEX eyes revealed a site-specific downregulation of LOXL1 and elastic fiber constituents, which was associated with a pronounced elastosis of the laminar beams and which has been suggested to represent a major susceptibility factor for a PEX-associated risk of glaucoma development and progression.
These differential expression patterns indicate that other PEX-associated factors may modulate local LOXL1 expression levels in addition to genetic predisposition.

In view of these considerations, it is reasonable to assume that the combined effect of LOXL1 genotype and external factors or stress conditions with fibrogenic potential, which are known to be present in the anterior segment of PEX eyes, might influence the manifestation of the disease (i.e., the accumulation of abnormal fibrillar aggregates). Candidate factors, which might stimulate the synthesis of abnormal PEX fibrils, include profibrotic growth factors (TGF-β1),19 cytokines (IL-6),20 and amino acids (homocysteine),21 as well as various stress conditions such as oxidative stress,16 UV radiation,18 and hypoxia.17 In response to these profibrotic triggering factors, LOXL1 may become upregulated in PEX tissues together with elastic matrix components serving as putative substrates for an abnormal cross-linking action of the enzyme. It is likely that through such protein–protease interactions the effects of the PEX-associated LOXL1 variants become more significant.20

Using human Tenon’s capsule fibroblasts in both 2D- and 3D-cell culture models, we first analyzed the capacity of PEX-associated pathogenic factors to regulate the expression of LOXL1 and major integral components of PEX material, TGF-β1, oxidative stress, hypoxia, and UV light were found to induce a significant increase in expression levels of LOXL1 and elastic proteins in a time- and dose-dependent manner compared with untreated controls, whereas the effect of IL-6 was limited to induction of elastic constituents. TGF-β1 and oxidative stress proved to be the most potent factors to stimulate the coordinated expression of LOXL1, fibrillin-1, the main component of PEX fibrils, and TGF-β1, a key cytokine in the fibrotic PEX process. In contrast, hardly any effect on expression levels could be seen for homocysteine. Immunohistchemistry confirmed an upregulation of LOXL1 and elastic fiber proteins and their assembly into extracellular microfibrillar networks on stimulation with TGF-β1 and oxidative stress after 3 days in culture. Moreover, focal aggregation of microfibrils into thicker fibrils resembling PEX fibrils could be observed in 3D-culture systems by electron microscopy.

These findings are consistent with previous studies demonstrating a broad responsiveness of extracellular matrix genes to a wide array of profibrotic stimuli including TGF-β1, oxidative stress, UV light, hypoxia, IL-6, and homocysteine in various cell types in vitro.20,31-35 Lysyl oxidase family members have also been shown to be upregulated by a variety of external factors including TGF-β1,36-38 oxidative stress,39 hypoxia,40 and mechanical stress.41 Homocysteine was found to suppress LOX expression and activity in vascular endothelial cells32 and inhibited LOX activity in the vitreous body.41 Thus, upregulation of lysyl oxidases was shown to underlie the onset and progression of various fibrotic disorders, such as liver fibrosis,23 whereas reduced expression of these cross-linking enzymes has been associated with the pathophysiology of diseases affecting connective tissues (e.g., cardiovascular diseases).44 In contrast, IL-6 did not significantly regulate LOX expression by gingival fibroblasts,45 which is in agreement with the present findings on LOXL1.

To further investigate the combined effect of external factors and LOXL1 genotype on expression levels of LOXL1 and elastic proteins, cell cultures carrying the high-risk haplotype G-G in the homozygous state were compared with cell cultures carrying a low-risk LOXL1 haplotype T-G. Although basal and stimulated expression levels of LOXL1 displayed a tendency toward reduced expression in cells with the G-G haplotype compared with the T-G haplotype, the differences were statistically not significant, which may be due to the small sample size analyzed (n = 3) or to in vitro conditions only imperfectly mimicking the in vivo situation.

In conclusion, the present findings support the notion that both genetic and nongenetic fibrogenic factors, particularly TGF-β1 and oxidative stress, may cooperate in the induction of PEX fiber formation and cross-linking and the stable accumulation of PEX aggregates within anterior segment tissues. The 2D- and 3D-cell cultures established may also serve as model systems to further analyze the molecular pathophysiology of PEX syndrome and to develop new strategies for an antibifibrotic therapy of this potentially blinding disease.

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