SWATH-MS Proteomic Analysis of Oxygen-Induced Retinopathy Reveals Novel Potential Therapeutic Targets

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PURPOSE. Oxygen-induced retinopathy (OIR) is the most widely used model for ischemic retinopathies such as retinopathy of prematurity (ROP), proliferative diabetic retinopathy (PDR), and retinal vein occlusion (RVO). The purpose of this study was to perform the most comprehensive characterization of OIR by a recently developed technique, sequential window acquisition of all theoretical mass spectra (SWATH-MS) proteomics.

METHODS. Control and OIR retina samples collected from various time points were subjected to SWATH-MS and detailed data analysis. Immunohistochemistry from mouse retinas as well as neovascular membranes from human PDR and RVO patients were used for the detection of the localization of the proteins showing altered expression in the retina and to address their relevance to human ischemic retinopathies.

RESULTS. We report the most extensive proteomic profiling of OIR to date by quantifying almost 3000 unique proteins and their expression differences between control and OIR retinas. Crystallins were the most prominent proteins induced by hypoxia in the retina, while angiogenesis related proteins such as Filamin A and nonmuscle myosin II A stand out at the angiogenesis related proteins level.

CONCLUSIONS. The results reveal new potential therapeutic targets to address hypoxia-induced pathological angiogenesis taking place in number of retinal diseases. The extensive proteomic profiling combined with pathway analysis also identifies novel molecular networks that could contribute to the pathogenesis of retinal diseases.

Keywords: proteomics, mass spectrometry, oxygen induced retinopathy, angiogenesis, hypoxia.
position (DDA), which iTRAQ and targeted data analysis uses, extending the throughput of proteins and data completeness. Here we report the most extensive proteomic profiling of OIR to date by relatively quantifying almost 3000 unique proteins. The novel methodology used in the present study also allowed the temporal profiling of OIR proteome for the first time, while in the previous studies, it has been possible to compare the protein levels in a single time point. Immunohistochemistry was used to study the localization of differently expressed proteins in control and OIR retinas, as well as in neovascular membranes obtained from PDR and RVO patients to address the relevance of these findings to human ischemic retinopathies.

MATERIALS AND METHODS

Mouse OIR Model

WT C57BL/6 mice were used for the study. Mice were housed under standard conditions with 12-hour dark/12-hour light cycle and fed with standard laboratory pellets and water ad libitum. The OIR model was generated as described in detail previously. Briefly, to induce OIR, the pups at postnatal day 7 (P7) and their nursing mothers were exposed to 75% oxygen (ProOx P110 oxygen controller; Biospherix Ltd., Parish, NY, USA) for 5 days until P12 when they were returned to normal room air. Mice were sacrificed and retinas collected at P13 (early hypoxic phase), at P17 (late hypoxic phase and the peak of neovascularization), and at P42 (after vascular recovery) to assess the effect of OIR on retinal proteome. Control animals were housed under normal room air conditions and retinas were harvested at corresponding days. The study design is illustrated in Figure 1. As postnatal weight gain has been shown to affect outcome in the OIR model, only the pups weighing between 6.3 and 7.5 g at P17 were included in the study. All animal experiments were conducted under ARVO Statement for the Use of Animals in Ophthalmic and Vision Research guidelines in accordance with protocols approved by the National Animal Ethics Committee of Finland.

For the proteomic analysis, eye balls were harvested into cold PBS and retinas were dissected immediately under the dissection microscope, and retinas were snap frozen with liquid nitrogen. Samples were stored in 

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FIGURE 1. Outline of the study. OIR was generated by exposing the mice pups for 75% oxygen for 5 days from P7 to P12. Control and OIR retinas were collected to SWATH-MS analysis, while some retinas from each litter were used for histological examination to confirm the changes in the vasculature in OIR. Retinal vasculature was stained with Isolectin GS-IB4. OIR samples were collected during hypoxia at P13 when central retina is avascular, at P17 when retina is partially revascularized and the neovascularization (preretinal tufts) peaks, and at P42 when retina is completely revascularized and neovascularization is regressed. Age-matching control samples were collected from mice housed in normal room air: P13 n = 5; P17 n = 9; P42 n = 6; P13 OIR n = 5; P17 OIR n = 6; P42 OIR n = 7.

Proteomics

Chemicals and Materials. Acetonitrile (ACN), formic acid (FA), water (UHPLC-MS grade), triethylammonium bicarbonate buffer 1M (TEAB), sodium dodecyl sulfate (SDS), iodoacetamide (IAA), trifluoroacetic acid (TFA), ammonium bicarbonate (ABC), and urea were all purchased from Sigma Aldrich Corp. (St. Louis, MO, USA). Radioimmunoprecipitation assay (RIPA) cell lysis buffer, a cocktail (Halt Protease Inhibitor Cocktail), and sample clean up tips (C18) were from Thermo Fisher Scientific (San Jose, CA, USA). A kit (Bio-Rad DC) and bovine serum albumin standard were purchased from Bio-Rad (Hercules, CA, USA), and 30 kDa molecular weight cut off (MWCO) centrifugal devices were from PALL (Port Washington, NY, USA).
Sample Preparation and Analysis. Proteins were extracted by submerging tissues to 300 μL RIPA cell lysis buffer in ice and homogenized using plastic pestles and centrifuged (2,400g/min, 5 minutes). After centrifugation, tissue samples were set in cold ultrasonic bath for 5 minutes and incubated on ice for 25 minutes. After incubation, samples were centrifuged at 21,000g for 20 minutes and the supernatant containing the proteins were transferred to new tube. Protein concentration was measured using Bio-Rad DC protein quantification kit. Average amount of protein recovered per sample was 253.8 ± 32.9 μg (± SD). Fifty micrograms of total protein was taken from each sample to trypptic digestion.

Samples were then subjected to reduction, alkylation, and trypptic digestion. These steps were performed as described in detail in the Supplementary Methods. For MS analysis, the samples were eluted to the same concentration and 4 μg sample was injected into NanoLC-TripleTOF (Sciex 5600+). Two replicate MS analyses were produced from each sample. Analysis of the samples was done by NanoLC-TripleTOF MS using SWATH acquisition as described in the Supplementary Methods.

Protein Identification and Quantification. As part of the SWATH analysis method, a relative protein quantification library, consisting of >3500 retinal proteins, was created using retina samples from this study. Overall library consisted of 32 different samples and 45 data-dependent analysis (DDA) runs with same LC gradient and instrument settings that were used for SWATH analyses. Library was created using Protein Pilot 4.7 (Sciex, Redwood City, CA, USA) and all DDA runs spectra were identified against Mouse UniprotKB/SwissProt protein library. Quantification was done by Peak Viewer and Marker Viewer (Sciex). False discovery rate (FDR) 1% was used in the library creation and only distinctive peptides were used in the quantification. Retention time calibration was done for all samples using 15 representative peptides from two different proteins, α-enolase (ENO1) and albumin (ALB). Five transitions per peptide and 1 to 15 peptides were used for protein quantification calculations. All statistically significant (adjusted P < 0.05) and other interesting proteins discussed further were subjected to manual inspection of peptides. This consisted of checking correct peak selection in the chromatogram (FDR 1%, 99% peptide confidence level), sufficient signal to noise ratio inspection (>7), and chromatogram inspection in relation to library chromatogram. Also, variation of replicate MS analysis results was calculated as means to all samples/protein. All peptides were eliminated from results processing if manual inspection requirements were not fulfilled. Proteins with missing values were excluded from consideration. Checked proteins whose quantification was based on only one specific peptide are marked with †-symbol to the graphs and tables. Most of these proteins were small proteins, meaning a small likelihood of having more than one specific peptide identified for quantification.

Western Blotting. Retinal protein lysates were run into SDS PAGE gels, transferred to polyvinylidene difluoride (PVDF) membranes, and immunoblotted with anti-Flna, anti-Myh9, and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies, as described in the Supplementary Methods.

Immunohistochemistry. For the retinal flat mount analysis, the eye balls were harvested at P13, P17, and P42. Eye balls were fixed with 4% paraformaldehyde (PFA) for 1 hour where after retinas were dissected, stained with isolecitin (Isolectin GS-IB4; Invitrogen, Carlsbad, CA, USA), flat mounted, and imaged via confocal microscope (LSM 700; Carl Zeiss, Oberkochen, Germany).

For immunohistochemistry, the eye balls were harvested, fixed with 4% PFA for 4 hours, and processed for paraffin embedding. Five-micrometer thick sections were subjected to antigen retrieval (Tris-EDTA, pH9), blocked and incubated either with anti-Filamin-A antibody (Cat#ab76289; Abcam, Cambridge, UK) or anti-Myh9 antibody (Cat#11128-1-AP; Proteintech, Rosemont, IL, USA) followed by horseradish peroxidase (HRP) conjugated secondary antibodies. For immunofluorescence (IF) double-staining, sections were pretreated with trypsin and incubated with anti-Myh9 antibody (Proteintech) and anti-CD31 (Cat#550274; BD Biosciences, San Jose, CA, USA) or anti-Myh9 and anti-NG2 (provided by W. Stallcup, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA, USA), followed by appropriate Alexa Fluor dye conjugated secondary antibodies (Invitrogen). Samples were imaged via confocal microscope (LSM700; Carl Zeiss).

Neovascular membranes were obtained from nine PDR and one RVO patients, who were undergoing pars plana vitrectomy. At the time of surgery, patients’ mean age was 37 years (range, 27–56 years) and mean duration of diabetes was 26 years (range, 21–32 years). The protocol for collecting human tissue samples was approved by the institutional review boards of the Pirkkamaa Hospital District, and the study was conducted in accordance with the Declaration of Helsinki. All patients gave written informed consent. The fibrovascular membranes were isolated, grasped with vitreous forceps, and pulled out through a sclerotomy. Samples were immediately fixed with 10% formalin for 3 hours, transferred to 70% ethanol, and processed for paraffin embedding and immunohistochemistry. Samples were stained with anti-Myh9 antibody (Proteintech) and anti-HSA (cat #LS-B6178; LSBio, Seattle, WA, USA) followed by HRP conjugated secondary antibodies.

Statistical Analysis. Proteins were quantified and log2-transformation was applied to the data, and in addition, geometric means of replicate MS analyses were taken. No further normalization was deemed necessary. The quality of the replicate MS analyses was analyzed by analyzing the intraclass correlation (ICC), and Spearman’s rank correlation was used to generate P values in permutation tests (n = 1000 permutations / replicate MS analyses).

Principal component analysis (PCA) was used to cluster the samples based on full proteomic profiles. Two-sample t-test was used to analyze differences between the relative protein expression levels of control and OIR retinas. Levene’s test was performed for the statistically significant proteins (after P value adjustment), and two proteins had a P value < 0.05. For these proteins, the statistical significance was checked with Wilcoxon rank sum test.

Benjamini-Hochberg adjustment was used to account for multiple testing, and the significance threshold, α, was set at 0.05. All statistical analyses for the proteomics data were performed using R software version 3.2.3 (R Core Team, Foundation for Statistical Computing, Vienna, Austria) and IPA software (IPA; QIAGEN, Redwood City, CA, USA).

RESULTS

Protein Profiles Are Associated With the Developmental Stage of the Retina

To study the protein profiles of control and OIR retinas, we first confirmed the development of OIR by staining the vasculature from retinal flat mounts at different time points (Fig. 1). With SWATH-MS quantification, we then used a library of 121,145 peptides from 32 samples, corresponding to 1,576,233 identified spectra in an assembly of 3516 protein groups using FDR of 1.0%. From this library, 2944 proteins had distinct peptides sequences with matching spectra to SWATH-MS analysis, and these proteins were quantified in all samples. Out of 2944 relatively quantified proteins, the quantification
was based on more than one peptide in 2177 proteins. Proteins quantified using a single peptide are marked in the figure legends. The replicate MS analysis quality checks showed that the ICC coefficient was 0.99 between the replicates. Permutation tests using Spearman’s correlation showed that 94.4% of the replicate MS analyses had a $P<0.05$, which suggests that they were of excellent quality.

For the proteomic data, we first wanted to evaluate the underlying patterns and differences between time points. PCA was performed for the whole data, and the plotting results based on the first two components suggested that there was a clear division between different time points of both control and OIR retina samples (Fig. 2). In addition, some separation was seen between control and OIR retinas at P13 and P17. At P42, the two groups were overlapping and there was no longer detectable division between control and OIR protein profiles. Taken together, the strongest cause for the differences in protein expression levels based on the first component appears to be the developmental stage of mouse retina.

**Differential Expression Analysis of OIR and Control Retinas: The Induction of Crystallins by Hypoxia at P13 and Angiogenesis-Related Proteins at P17**

Next, we performed differential expression analysis in order to identify proteins, which differed between control and OIR samples in specific time points. Comparison of control and OIR samples resulted in 364 differentially expressed proteins at P13, 387 at P17, and 104 at P42 ($P<0.05$) prior to further $P$ value adjustments. These proteins are listed in Supplementary Table S1 for P13, Supplementary Table S2 for P17, and Supplementary Table S3 for P42.

$P$ value adjustments to account for the multiple hypotheses testing reduced the number of statistically significant results and led to 17 differentially expressed proteins at P13, 22 at P17, and none at P42. To visualize the differential expression in response to hypoxia at P13, P17, and P42, volcano plots are shown (Figs. 3A–C). In addition to visualizing the statistically significant (adjusted $P<0.05$) proteins and their associated up- or downregulation, the plots revealed a highly upregulated group of proteins in response to hypoxia at P13 OIR (Fig. 3A). These proteins are crystallins, including members of $\alpha$-, $\beta$-, and $\gamma$-crystallins. The expression levels of individual crystallins did not reach statistical significance after $P$ value adjustment, but they are the most upregulated proteins in the whole data according to fold change (FC) ($\log_2$ FC $2.67$–$4.32$) (Supplementary Fig. S1 and Supplementary Table S1). It is also noteworthy that based on FC alone, the most upregulated ($\log_2$ FC $>2$) proteins at the peak of the
angiogenesis (at P17), were vitamin D-binding protein (Gc), α-2HS-glycoprotein/fetuin-A (Ahsg), α-lactalbumin 1-4 (SerpinA1d), and carboxylesterase 1C (Ces1c) (nonadjusted \( P < 0.05 \)) (Supplementary Table S2). Of the aforementioned proteins, one (Ahsg) was also statistically significant (Fig. 3B). At P17, cocaine- and amphetamine-regulated transcript protein (Carrpt) was initially found to be statistically significant after the \( P \) value adjustment (Fig. 3C). However, this change was deemed unreliable after manual peak checking.

The heat maps of the statistically significant results at P13 and P17 show the relative expression of the proteins and the differences between control and OIR samples (Figs. 4A, 4B). Four proteins had statistically significant difference at P13 (absolute \( \log_2 \text{FC} > 0.585 \); i.e., \( \text{FC} > 1.5 \); abs(\( \log_2(1.5) \))) glutamine synthetase (Glu), guanylyl cyclase GC-E (Gucy2c), cGMP-gated cation channel α1 (Cnga1) and rod outer segment membrane protein 1 (Rom1) (Fig. 4). Substantially more proteins showed similar differences (\( \log_2 \text{FC} > \text{abs}(\log_2(1.5)) \)) in the expression levels between OIR and control at P17: Ahsg, annexin A2 (Anxa2), transgelin-2 (Tagln2), cell surface glycoprotein MUC18 (Mcam), tropomyosin α-4 chain (Tpm4), filamin-B (Flnb), protein SON (Son), Gpx1, vinculin (Vcl), myosin-9 (Myh9), serpin H1 (Serpinh1), filamin-A (Flna), moesin (Msn), and annexin A6 (Anxa6) (Figs. 4B, 5). Similarly, downregulated proteins at P17 OIR were Glul and retindexe-binding protein-1 (Rlp1). The mean expression values are illustrated in Figure 5 and in Supplementary Figure S2 for all of the statistical significant proteins (adjusted \( P \) value \(< 0.05 \)). There were no proteins with altered expression level at P42 OIR after \( P \) value adjustment.

**Hyoxia Induces Filamin-A Cleavage at P17 OIR**

In order to validate the results from proteomic analysis of OIR, Flna and Myh9 were selected for immunoblotting. Immunoblotting with C-terminal anti-Flna antibody confirmed increased Flna expression at P17 OIR (4.6-fold compared to P17 control). In hypoxia, Flna is cleaved by calpain-proteases to generate 90 kDa C-terminal fragment that interacts with hypoxia-inducible factor-1α (HIF-1α) and promotes angiogenesis. The expression of C-terminal fragment of Flna (Flna(CT)) generated in the presence of hypoxia was increased 14-fold in OIR at P17 OIR (Figs. 5C, 5D). Next, the expression of Myh9 was studied in OIR by immunoblotting. A 9.6-fold increase in the expression of the Myh9 was seen at P17 OIR when compared to the expression at P17 controls (Figs. 5E, 5F).

**Filamin-A and Myh9 Expression Is Increased in the Angiogenic Blood Vessels in OIR Retinas**

While proteomic analysis and immunoblotting revealed an increased expression of Flna and Myh9 at P17 OIR, immuno-histochemical stainings confirmed substantially stronger expression of these proteins from P17 OIR retinas than from control retinas (Fig. 5G). We observed marked increase in Flna expression in preretinal tufts and blood vessels of inner retina in the OIR retinas in comparison to controls. Also, a faint staining is seen in others cell types, presumably retinal neurons, but the expression level in neurons looks identical in control and OIR retinas. Thus, the induction of Flna expression seen in OIR at P17 is clearly from the angiogenic blood vessels.

Myh9 expression in OIR retinas was studied in detail by performing double-immunofluorescent stainings for Myh9 and endothelial cell (CD31), and pericyte (NG2) markers. Strong Myh9 expression was localized to endothelial cells and pericytes in OIR at the peak of the angiogenesis at P17. All endothelial cells expressed Myh9, while there were some pericytes negative for Myh9 at P17 OIR (Fig. 6).

**Abundant Expression of Myh9 on Neovascular Membranes From Human PDR Patients**

Generally, the expression levels of Myh9 reflect the overall stiffness of the tissue.\(^{19–21}\) The increased matrix stiffness destabilizing endothelial cell–cell junctions impairs the endothelial barrier function and leads to enhanced endothelial permeability.\(^ {22}\) We next studied Myh9 and human serum albumin (HSA; i.e., marker of vascular leakage) expression on human fibrovascular membranes (using adjacent tissue sections) from vitrectomized patients with PDR and RVO. Strong fibrosis formation leads to retinal traction and ultimately to retinal detachment in RVO. The represent the end stage of these diseases, where substantial amount of fibrosis has been formed, but the samples still contain also regions with active pathological angiogenesis. When only a faint expression of Myh9 is seen outside of the blood vessels, the intensity of Myh9 staining in blood vessels is also faint one (Fig. 7). Strong Myh9 expression was seen both in the blood vessels and in the matrix in some samples and then HSA to accumulate in the tissue. Particularly strong Myh9 expression was seen all over the fibrotic membrane in the RVO sample that contains blood vessels and massive scarring (Fig. 7). Comparison of Myh9 expression to HSA expression showed a strong positive correlation between the two; more Myh9 expressing cells (increased matrix stiffness) outside the blood vessels, more Myh9 expression (contractility and destabilization of cell–cell junctions) in the blood vessels and the more abundant vascular leakage outside the blood vessels (Fig. 7).

**Ingenuity Pathway Analysis (IPA) Identifies Angiogenesis and Transforming Growth Factor-β1 (TGF-β1) Pathway As the Most Prominent Features of OIR at P17.**

In order to connect the differentially expressed proteins to specific pathways and biological functions, pathway analysis was performed using IPA on the proteins showing statistically significant differences in their expression between control and OIR retinas (adjusted \( P < 0.05 \)) in each time point. The top canonical pathways connected to the 17 proteins at P13 (Fig. 4A) were phototransduction pathway (\( P = 4.95e-10 \)) (Supplementary Fig. S3), protein kinase A signaling (\( P = 2.29e-04 \)), and glutamine biosynthesis I (\( P = 8.05e-04 \)). There were no unbiased activation scores \(< -2 \) or \( > 2 \) for disease and biological functions or for upstream regulators.

For the 22 proteins identified in differential expression analysis at P17 (Fig. 4B), the top canonical pathways were actin cytoskeleton signaling (activation score = 2.1, \( P = 3.18e-06 \)), ILK signaling (activation score = −1, \( P = 4.61e-05 \)), and glutamine biosynthesis I (\( P = 1.04e-03 \)). We then performed the disease and biological function enrichments analyses, which showed both necrosis and cell death reduced (\( P = 7.25e-04 \) and \( P = 6.94e-05 \), both with activation score \(< -2 \)), while angiogenesis was increased (\( P = 2.53e-04 \), activation score \(\geq 2 \)). According to IPA, the increase in angiogenesis was due to the upregulation of Anxa2, Flna, Flnb, Gpx1, Mcam, Myh9, and Serpinh1. For further classification, upregulated proteins Tagln2, Vcl, Mcam, Flnb, ATP-dependent 6-phosphofructokinase (Pikp), Myh9, and Flna are proteins involved in cell–cell adhesion (Amigo2). In addition, the upstream regulator analysis identified TGF-β1 (Tgfb1), MAP kinase-interactive serine/threonine-protein kinase 1 (Mnk1), and...
MKL/myocardin-like protein 2 (Mkl2) as potential upregulated enhancers of angiogenesis (for all $P < 0.001$ and activation score $>2$), while brain-derived neurotrophic factor (Bdnf), Krueppel-like factor 3 (Klf3), and Myc proto-oncogene protein (Myc) were inhibited (for all $P < 0.001$ and activation score $< -2$) (Supplementary Table S4).

Marginal Long-Term Changes in the Protein Expression Persist in OIR Retina

There were no proteins with statistically significant difference in the expression levels between OIR and control retina after $P$ value adjustment at P42. We then performed the IPA analysis.
Figure 5. Differentially expressed proteins in OIR during hypoxia and angiogenesis. Mice pups were exposed to hyperoxia-induced OIR, and retinas were harvested at P13, P17, and P42. All retina samples were analyzed by MS-SWATH and the data underwent statistical analysis to compare control and OIR samples in each time point. The means and standard error bars are shown for control (red) and OIR (blue) samples for chosen proteins during hypoxia at P13 (A) and at the peak of neovascularization at P17 (B). The asterisks identify statistically significant differences between the two groups before P value adjustment (*), and after P value (**) adjustment. Note that Glul (A) is different both at P13 and at P17. (C) Western blot membrane immunoblotted with anti-Flna antibody shows increased Flna expression at P17 OIR and hypoxia-induced cleavage of C-
terminal fragment of Flna (FlnaCT). (D) Densitometric quantification revealed 4.6-fold induction in total Flna protein levels, while FlnaCT levels were increased 14-fold at P17 OIR compared to P17 control samples. (E, F) Western blotting against Myh9 and densitometric quantification showed 9.6-fold induction at P17 OIR compared to P17 control samples. (*P < 0.05, **P < 0.01, ***P < 0.001) (G) Control and OIR retinas collected at P17 were processed for immunohistochemical analysis and stained for Flna and Myh9 as described in the Materials and Methods section. Both Flna and Myh9 expression is increased in OIR retinas compared to controls. Strong expression of Flna and Myh9 can be seen from preretinal tufts (arrows) and from the blood vessels in inner retina in OIR. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer, OPL, outer plexiform layer; ONL, outer nuclear layer; IS/OS, photoreceptor inner/outer segments; RPE, retinal pigment epithelium. Scale bars represent 50 μm in panel A and 20 μm in panels B and C.

**DISCUSSION**

We have carried out the most comprehensive proteomics analysis of the commonly used experimental retinal angiogenesis model, OIR. The results reveal that in addition to the changes detected on proteins responding to hypoxia and those regulating angiogenesis, some changes in protein expression take place in the neuronal tissue of the retina. This is in accordance with the fact that human retinal vascular diseases are associated with neuropathy and gliopathy. Furthermore, the dysregulation of the cross talk between vasculature and retinal neuroglia and neuronal cells has been shown to contribute to the pathogenesis of DR.23–25 During the hypoxic phase, the most upregulated proteins were crystallins, which are small heat shock proteins that act as molecular chaperones by binding misfolded proteins to prevent their denaturation and aggregation.26,27 They protect cells from hypoxia and maintain mitochondrial integrity.26,28 In addition to their neuroprotective role, crystallins also have other roles in vascular biology.29,30 αB-crystallin functions as a chaperone for VEGFA and is crucial for its proper secretion, which in turn, is crucial for endothelial cell survival in hypoxia.28,31,32 Thus, αB-crystallin knockout mice had less VEGFA and neovascularization than WT in OIR.31 In the eye, crystallins were originally characterized as abundant lens proteins, but they are also expressed in developing and mature retina.28,32–34 Their expression is dramatically upregulated in numerous retinal diseases, such as mechanical injury, ischemic insults, age-related macular degeneration (AMD), uveoretinitis, and DR.28 Concerning the potential therapeutic value of crystallins in retinal diseases, their exact functions are emerging.27,35 Namely, the recently discovered roles for αB-crystallin in mediating TGF-β induced epithelial-mesenchymal transformation (EMT) and subretinal fibrosis in AMD,37 while αA-crystallin providing neuroprotection for retina in DR,35 point out the opposite therapeutic values for these crystallin-family members.7,36

A large number of proteins was also downregulated in response to hypoxia in retina. Among these proteins were Cnga1, Gucy2e, Rom1, and Grk1, proteins associated with phototransduction. Since rod photoreceptors are the most oxygen-dependent cells in the retina,37 the downregulation of proteins associated with rod function is a plausible outcome in hypoxia.
The most abundant changes in the retinal protein expression in OIR were seen at the peak of neovascularization at P17. Among the most upregulated proteins were plasma proteins Ahsg, Gc, Apoa1, Alb, and Tf (Supplementary Table S2). This is most probably due to increased permeability of the angiogenic blood vessels, which leads to the accumulation of plasma proteins in retinal tissue by leakage. However, some of these proteins could also have relevant biological functions, not just be bystanders by leakage, in OIR. Fetuin-A (Ahsg) as well as its cellular receptors, Anxa2 and -6, were highly upregulated in OIR. Fetuin-A is an adhesive glycoprotein that binds to Anxa2 and -6 and induces cell proliferation in its target cells.\(^{38}\) Anxa2, in turn, drives angiogenesis in OIR.\(^{38}\) Gc is a multifunctional glycoprotein that acts as a carrier protein for vitamin D but can also modulate certain immune and inflammatory responses.\(^{39}\) Vitamin D, in turn, is known to inhibit retinal neovascularization in OIR,\(^{40}\) and vitamin D receptor agonists inhibit developmental retinal angiogenesis.\(^{41}\) Thus, the accumulation of Gc by the leakage from the angiogenic blood vessels could be a natural, endogenous signal to suppress angiogenesis at the time angiogenesis reaches its peak in OIR.

In line with the extensive angiogenesis taking place in retina at P17, IPA analysis revealed proteins “related to angiogenesis” being the most significantly upregulated proteins. Among these proteins were Filamins, Flna and Flnb. In hypoxia, oxygen-sensing prolyl hydroxylase domain protein 2 (PHD2) inactivation rapidly upregulates Flna expression.\(^{42}\) Flna, in turn, interacts physically with HIF-1\(\alpha\) and promotes angiogenesis.\(^{43}\) Hypoxia induces calpain-dependent cleavage of Flna, and its C-terminal fragment (Flna\(_{CT}\)) accumulates in the nucleus and facilitates the nuclear localization of HIF-1\(\alpha\).\(^{44}\) Our study shows that hypoxia induces Fina cleavage, that is, Fina\(_{CT}\)
generation in OIR. Very recently it was shown that blocking the calpain-dependent cleavage of Flna impairs tumor cell proliferation and migration. Moreover, it is worth noting that Flna also interacts with small GTPase, R-Ras, and the gene needed for proper endothelial lumenogenesis. The interaction between Flna and R-Ras is crucial for controlling vascular permeability in angiogenesis. R-Ras, in turn, regulates vascular permeability in OIR and the reduced expression of R-Ras is associated with vascular leakage in PDR. The interaction between R-Ras and Flna takes place with the N-terminal part of Flna. Thus, selective blocking of the C-terminal cleavage of Flna (Flna CT) could potentially be therapeutic in ischemic retinal diseases with pathological angiogenesis.

Another angiogenesis-related protein that stands out is Myh9. Myh9 gene encodes for Myosin 9, a heavy chain of Myosin IIA, a cytoskeletal contractile protein. In general, the expression levels of Myh9 reflect the stiffness of the tissue; cells in stiff tissues express plenty of Myh9, while the cells in nonstiff tissues express low levels of Myh9. In addition to the stiffness of the tissue, cell migration induces high mechanical strains on the cells as their ‘‘pierce’’ through extracellular matrix. Thus, the migrating cells upregulate the expression of Myh9 to make themselves more rigid to withstand the mechanical forces placed on them during the migratory process, but their enhanced contractility destabilizes endothelial cell–cell junctions and impairs endothelial barrier function, resulting in increased vascular permeability.22,53–55 Angiogenesis is essentially a cell migratory process and as shown in this study, the upregulation of the Myh9 takes place in the angiogenic endothelial cells in the retina. Thus, the induction of Myh9 during the peak of angiogenesis in OIR is plausible phenomenon, but could also be highly relevant as the increased vascular leakage is a hallmark in OIR. We also studied samples obtained from human PDR and RVO patients and showed that the vascular leakage in these diseases correlates with the amount of Myh9 expression in this area. Endothelial cell contractility (i.e., enhanced Myh9 expression) increases not only with cell migration, but also with the increased matrix stiffness resulting in increased endothelial permeability due to impaired endothelial barrier function.22,53–55 Thus, the changes in matrix stiffness and the resultant endothelial cell contractility could be highly relevant for large number of neovascular retinal diseases associated with pathological leakage from the blood vessels.

Myh9 also regulates angiogenesis via controlling the production of VEGFA in ischemia-driven arteriogenesis as well as controlling nucleolin translocalization. Nucleolin is primarily a nuclear protein that translocates to cell surface in angiogenesis. The different antagonists of nucleolin, among them Myh9 antibodies, have been shown to inhibit tumor angiogenesis by stabilizing the pathological vasculature, which enhances tissue oxygenation. The modest increase (log2 FC 0.23) in nucleolin expression seen by MS in OIR at P17 suggests that the effects of nucleolin in angiogenesis is merely related to its translocation from nucleus to cell surface than increase in its expression level.

The present proteomics approach quantifies complete expression pattern of almost 3000 proteins and thus provides strong value to identify pathways involved in OIR. Using the upstream regulator analysis with IPA, the most potential and activated upstream regulator driving angiogenesis was TGF-β1. This is in accordance with previous studies showing TGF-β1 upregulation in OIR. Interestingly, conditional ocular deletions of TGF-β signaling results in pronounced structural changes of retinal capillaries and a phenotype similar to human DR. The upstream regulator analysis identified Mkl2 and...
Mknk1 as enhancers of angiogenesis in OIR. Mkl2, a transcriptional coactivator, regulates conserved TGF-β signaling pathway.65 Mice deficient for Mkl2 die at the early embryonic state, and Mkl2-/- ECs have defects in cytoskeletal organization and cell adhesion.65 Mkl2/TGF-β pathway is required for the maturation and stabilization of embryonic vasculature,65,66 but also for the myofibroblast transformation and EMF induction.66,67 The enhanced TGF-β signaling taking place in vasculature leads to the endothelial-mesenchymal transition (EndMT), a process that has many similarities with EMT.68,69 The transformed endothelial cells have high vascular permeability, further driving inflammation and by thus perpetuating the incomplete repair state.69 This inflammation–TGF-β circuit in vasculature also promotes fibrosis in the surrounding tissue, and it is largely irreversible.68,69 The recently identified association between TGF-β signaling and β-crystallin in the EMT and subretinal scarring,27 the massive induction of crystallins by hypoxia and the subsequent activation of TGF-β/Mkl2-signaling pathways in OIR provide clues about the potential interplay that leads to retinal fibrosis in the human neovascular diseases of retina.

Another upstream regulator potentially enhancing angiogenesis in OIR is Mknk1/MNK1. Mknk1 is a kinase that exclusively phosphorylates a cap-binding subunit of the elf4F translation initiation complex, elf4E, and selectively facilitating translation of proliferation, migration, and survival promoting mRNAs, among them VEGFA.70 Inhibition of elf4F phosphorylation, in turn, suppressed angiogenesis.71 In retina, elf4E interacts with 4E-bp1, which expression is enhanced in retina by diabetes-induced hyperglycemia and necessary for VEGFA expression.72,73 Our results suggest that blocking MNK-elf4E pathway could be a potential target for blocking pathological angiogenesis in retinal diseases.

We included the late OIR-time point, P42, in our study to assess whether hypoxic exposure and subsequent neovascularization cause permanent changes in the protein expression in the retina. Very little is known about the long-term effects of OIR on retina, although the information could be useful to understand the prognosis ofROP. The disruption of the retinal morphology as well as decrease in neuronal function have been reported after OIR.74 Our iPA analysis revealed decreased neurotransmission at P42 OIR retinas. Further examination revealed changes in the synaptic vesicle cycle pathway (KEGG) (Supplementary Fig. S4).

The present proteomics analysis revealed novel pathways that might contribute to the development of pathological angiogenesis in OIR. Furthermore, we were able to identify molecular interplay between the proteins induced by hypoxia and then by subsequent angiogenesis in OIR. They could be potential druggable targets of retinal diseases inflicted with hypoxia and neovascularization.

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