Hypoxia-inducible factor-1 drives annexin A2 system-mediated perivascular fibrin clearance in oxygen-induced retinopathy in mice

by

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Short title: Hif-1 Regulation of Annexin A2 in Retinal Angiogenesis
Abstract

Oxygen-induced retinopathy (OIR) is a well-characterized model for retinopathy of prematurity (ROP), a disorder that results from rapid microvascular proliferation following exposure of the retina to high oxygen levels. Here, we report that the proliferative phase of oxygen-induced retinopathy (OIR) requires transcriptional induction of the annexin A2 (A2) gene through the direct action of the hypoxia-inducible factor-1 (HIF-1) complex. We show, additionally, that A2 stabilizes its binding partner, p11, and promotes OIR-related angiogenesis by enabling clearance of perivascular fibrin. Adenoviral-mediated restoration of A2 expression restores neovascularization in the oxygen-primed Anxa2−/− retina, and reinstates plasmin generation and directed migration in cultured Anxa2−/− endothelial cells. Systemic depletion of fibrin repairs the neovascular response to high oxygen treatment in the Anxa2−/− retina, while inhibition of plasminogen activation dampens angiogenesis under the same conditions. These findings reveal that the A2 system enables retinal neoangiogenesis in OIR by enhancing perivascular activation of plasmin and remodeling of fibrin. These data suggest new potential approaches to retinal angiogenic disorders based upon modulation of perivascular fibrinolysis.
Introduction

ROP is the major cause of severe visual impairment in children in the developed world^1^.

A vascular proliferative disorder that affects preterm and low-birth weight infants upon exposure to supraphysiologic oxygen tension, ROP is increasing in incidence with the greater availability of neonatal intensive care, and more frequent survival of very-low-birth-weight infants. In the initial, vaso-obliterative phase of ROP, high oxygen exposure provokes endothelial cell death due to oxidative injury, nitrative stress, and suppression of oxygen-related growth factors^2^.

In the second phase, the resulting retinal ischemia leads to neovascularization characterized by excessive proliferation and vitreal invasion of blood vessels. This pathologic response arises when neurons and supporting astrocytes become severely metabolically deprived due to vaso-obliteration, and produce exaggerated amounts of oxygen-regulated angiogenic factors, such as vascular endothelial cell growth factor (VEGF) and erythropoietin. These agents stimulate the regrowth of abnormal vessels that proliferate toward the vitreous, can form a fibrous scar, and, upon contraction, can apply tractional forces that ultimately may detach the retina from its underlying pigment epithelium. Scarring and retinal detachment can lead to vision loss and blindness. Current preventive measures for ROP include restriction of tissue oxygenation and the use of antioxidants, while standard treatment for established disease requires ablative laser photocoagulation or cryotherapy. Newer therapies with VEGF neutralizing antibodies appear promising, but their effects on retinal ganglion cell integrity, the developing cerebral vasculature, and long term visual acuity and visual fields are unknown^2^.

Annexin A2 is a cell surface phospholipid-binding protein that forms a heterotetrameric complex with its partner S100A10 (p11)^3^-^5^.

The receptor complex binds both plasminogen, the precursor of the major fibrinolytic protease, plasmin, and its activator, tissue plasminogen activator (tPA), thereby accelerating the generation of plasmin^6^-^8^.

Mice deficient in annexin A2 (Anxa2^-/-^), which also lack normal levels of endothelial cell p11^9^, display fibrin accumulation.
within microvessels, and their isolated endothelial cells are unable to support tPA-dependent plasminogen activation in vitro. Although they sustain normal spontaneous angiogenesis in embryonic life, \textit{Anxa2}\textsuperscript{-/-} mice exhibit reduced angiogenesis in growth factor stimulated assays in adulthood. These findings are recapitulated in adult hyperhomocysteinemic mice, in which the A2 protein becomes derivatized upon disulfide linkage with homocysteine, thus preventing tPA binding and plasmin generation.

Here, we examined the A2 system in OIR, a model of pathologic, hypoxia-driven angiogenesis. We found that hypoxia induces the expression of A2 through direct transcriptional induction by hypoxia-inducible factor-1. A2 enabled retinal neovascularization, and the angiogenic block observed in the \textit{Anxa2}\textsuperscript{-/-} retina reflected impairment of cell surface fibrinolysis and reduced cell migration in a fibrin-rich milieu. We demonstrated that depletion of fibrinogen exaggerates, while inhibition of plasminogen activation attenuates, oxygen-induced neoangiogenesis in the retina. Our findings highlight the importance of A2-related fibrinolysis in oxygen-induced neoangiogenesis, and could serve as the basis for targeting the A2 system as a potentially novel therapeutic intervention for ROP and other proliferative retinal vascular disorders.
Materials and Methods

Mice. Anxa2+/− and Anxa2−/− mice were generated on the C57Bl/6 background as previously described 10. These mice were intercrossed for at least 10 generations with wild type 129/SVJ mice to generate both genotypes on the 129/SVJ background. Anxa2+/− and Anxa2−/− mice exhibited no significant differences in body mass in the course of the experiments. All animal procedures were approved by the Institutional Animal Care and Use Committee of Weill Cornell Medical College.

Oxygen-induced retinopathy. OIR was induced as previously described using a BioSpherix ProOX A chamber equipped with ProOx P110 single setpoint oxygen controller, E702 oxygen sensor, and ProCo2 carbon dioxide sensor. P7 mouse pups together with their dam were placed for 5 consecutive days (P7-P12) in a chamber in which the ambient oxygen level was maintained at 75 ± 2%. On P12, they were returned to room air (21% O2). Control pups were maintained in room air throughout the experiment12. Mouse retinas were isolated and assayed by immunoblot, RT-PCR, qRT-PCR, immunostaining, and ELISA on days 2-6 post-hyperoxia (P14-P18).

Retinal whole mounts. Eyes from P17 Anxa2+/− and Anxa2−/− mice subjected to room air (21% O2) or hyperoxia (75% O2) were enucleated, and retinas harvested for analysis by whole mount assay as described 13. Areas of retinal vaso-obliteration and neovascularization from both Anxa2+/− and Anxa2−/− mice were quantified according to Connor et al.14.

Cryosection preparation. Under general anesthesia, P17 mice were perfused intracardially with cold PBS containing 5 U/μl heparin sodium (Sigma Aldrich) at 80 cm H2O pressure (10 min) as described 10. The mouse eyes were collected and fixed in 2% PFA after creating a nick in the anterior cornea. The lenses were removed, and the remaining tissues were cryoprotected in 30% sucrose in PBS (24h), and then transferred to cryosection buffer (30% sucrose: OCT = 1:
2; 4°C) for infiltration. The samples were transferred to foil molds filled with cryosection buffer, oriented with the optical nerve facing up, frozen in 2-methylbutane, chilled in liquid nitrogen, and stored in -80°C prior to sectioning. Cryosections were prepared by cutting the embedded tissue along its vertical axis, and stored at -20°C prior to further analysis.

**Immunohistochemistry.** Cryosections were air dried at 21°C for 30 min and then refixed with ice-cold acetone (10 min). After three washes with PBS, the sections were boiled in antigen retrieval buffer (0.01 M sodium citrate, pH 6.0; 20 min) using Antigen Retriever (PickCell Laboratories), and then processed for staining using monoclonal anti-A2 IgG (Zymed) and the MOM kit (Vector Laboratories) according to the manufacturer’s instructions. For fibrin staining, cryosections were incubated with rabbit IgG directed against fibrinogen (2h, 21°C; Dako), and then incubated with Cy3-conjugated donkey anti-rabbit IgG (30 min, 21°C; Jackson Immunoresearch). Finally, the sections were counterstained with fluorescein-conjugated isolectin B4 and DAPI (Invitrogen). Antibodies for immunohistochemistry are listed in **Supplemental Table 2.**

**Tuft counting.** Retinal cryosections were stained with fluorescein-conjugated isolectin B4 and DAPI as described. Neovascular nuclei within isolectin-positive cells were enumerated under fluorescence microscopy (200x, merge filter; Nikon 80i).

**Cell culture.** Anxa2<sup>+/+</sup> and Anxa2<sup>−/−</sup> mouse cardiac microvascular endothelial cells (CMECs) were isolated and evaluated in migration assays as previously described<sup>10</sup>. Human umbilical vein endothelial cells (HUVECs) were isolated and propagated in M199 Medium (Invitrogen) supplemented with 20% fetal bovine serum (FBS, Invitrogen), 20 μg/ml endothelial cell growth supplement (ECGS, Sigma Aldrich), 100 μg/ml heparin (Sigma Aldrich), 2 mM L-glutamine, 100 U/ml penicillin G, and 0.1 mg/ml streptomycin sulfate at 37°C in a 5% CO<sub>2</sub> atmosphere, as previously described<sup>15</sup>. Hif1a<sup>+/+</sup> and Hif1a<sup>−/−</sup> mouse embryonic fibroblasts<sup>16</sup> (courtesy of Dr.
Gregg Semenza; Johns Hopkins University School of Medicine); HEK 293 and HEK 293AD cells (Stratagene) were cultured in DMEM (Invitrogen) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin G, and 0.1 mg/ml streptomycin sulfate at 37°C in 5% CO₂.

Nuclear and cytoplasmic protein extraction. Nuclear protein was isolated from cultured cells and retinas using an extraction kit (NE-PER Nuclear and Cytoplasmic Extraction Kit, Thermo Scientific) according to the manufacturer’s instructions. Cytoplasmic retinal proteins were extracted as described using 3 repetitions of 6-second sonication pulses (30% maximum output) on ice using a Branson Sonifier (VWR). Protein concentration was estimated using the BCA reagent (Thermo Scientific).

Immunoblotting. Proteins extracted from cultured cells and harvested retinas were resolved on 10% or 15% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad). The blots were probed with mouse IgG directed against A2, human p11, HIF-1α (all from BD Transduction Labs); HIF-1β (Novus); Erk1/2, Tyr 416 phospho-Src, Src (all from Cell Signaling Technology); and fibrinogen (Dako) (Supplemental Table 2). For detection of mouse p11, a peptide representing a portion of the mouse p11 protein (CGDKDHLTKEDLRVLMERE) was conjugated to KLH and used to immunize rabbits (Covance Research Products Inc, Denver, PA); IgG was purified from antisera by protein G affinity. α-actin (Santa Cruz), and pan-cadherin (Cell Signaling Technology) served as cytoplasmic and cell surface protein loading controls, respectively. Fibrin immunoblotting of mouse retinas was carried out as described. Immunoblots were incubated with secondary antibodies, conjugated to horseradish peroxidase, and directed against mouse or rabbit IgG. Immunoreactivity was visualized by chemiluminescence (ECL or ECL plus; GE Healthcare), and quantified by Scion Image.

Quantitative RT-PCR. Total RNA was extracted from cultured cells and retinas using Trizol (Invitrogen) according to manufacturer’s protocol. The concentration and purity of RNA were
estimated by measuring OD260/280. mRNA levels of A2, p11, VE-cadherin 5, and HIF-1α were assessed using a one-step RT-PCR kit (Qiagen) according to the manufacturer’s instructions. GAPDH or β-actin was employed as a loading control. Primers used for RT-PCR are listed in Supplemental Table 1. For quantitative RT-PCR, mRNA was converted to cDNA by reverse transcription (Qiagen). Equal quantities of cDNA were subjected to real-time PCR using the Sybre-green real-time PCR kit (Applied Biosystems). Each sample was run in duplicate. The results were expressed as the mean relative expression compared to control samples. The primers (RealTimePrimers.com) employed are listed in Supplemental Table 1.

Recombinant adenovirus construction and infection. A recombinant adenovirus encoding either A2 or p11 was constructed using the pAdEasy system. Using the following primers: 5'-CTCTCTCGGTACCCTTCAAAATGTCTACTGTCCACG-3' (sense) and 5'-CTACTCTTTCTAGATCAGTCATCCCCACCACACAGGTAC-3' (antisense) for Anxa2 and 5'-CTCTCTCAAAGCTTCTTCAAAATGCCATCCCAAATGG-3' (sense) and 5'-TGTGCTGTCTAGACTATTTCTTCCCCTTCTGCTTC-3' (antisense) for S100a10, A2 or p11 cDNAs were amplified and subcloned into a shuttle vector containing a GFP reporter gene, pAdTrack-CMV. This plasmid was recombined with pAdEasy-1, the replication-deficient adenovirus backbone. The recombinant adenovirus encoding A2 or p11 was allowed to proliferate, and then packaged in HEK 293 cells, and purified via CsCl gradient ultracentrifugation. The shuttle vector (pAdTrack-CMV) and replication-deficient adenovirus backbone (pAdEasy-1) were kindly provided by Dr. Bert Vogelstein (John Hopkins University School of Medicine). Anxa2-/- mouse CMECs were infected with empty, A2-, or p11-encoding recombinant adenoviruses at a concentration of 1000 particles per cell. The efficiency of infection, monitored by fluorescence microscopy, was approximately 60%.
**Cell surface protein biotinylation.** Analyses of cell surface expression of A2 and p11 were conducted as described \(^\text{15}\). Gel loading was assessed by evaluation of immunoreactivity of IgG directed against pan-cadherin (Cell Signaling Technology).

**Immunoprecipitation.** Detection of Tyr\(^{23}\)-phosphorylated A2 was carried out described using antibodies described in Supplemental Table 2 \(^\text{15}\).

**Plasmin generation assay.** tPA-dependent plasmin generation was assayed as previously described \(^\text{18}\).

**Cell migration assay.** Directed migration of HUVECs, and Anxa2\(^{+/+}\) and Anxa2\(^{-/-}\) CMECs through fibrin barriers in response to VEGF-A was quantified as described \(^\text{10}\).

**Enzyme-linked immunosorbent assay (ELISA).** Plasma fibrinogen, as well as VEGF and placental growth factor (PIGF) levels in mouse retinas and other organs, were estimated using ELISA kits (R & D Systems) according to the manufacturer’s instructions.

**mRNA stability assay.** Stability of A2 mRNA was assessed by pre-treating HUVECs with or without actinomycin D (10 \(\mu\)g/ml; 6 h), prior to assaying mRNA and protein by RT-PCR and immunoblot \(^\text{19}\).

**Electrophoretic mobility shift assay (EMSA).** Interaction between HIF-1 protein and a portion of the human A2 promoter containing a putative hypoxia responsive element (Supplemental Figure 3C) was examined by EMSA as described \(^\text{20}\). Probes for the ANXA2 gene consisted of 5’- GAGCTCGACGTGGCACTTAAG -3’ (wild type) and 5’- GAGCTCGAAAAAGCAGCTTAAG -3’ (mutant). Probes for the VEGFA gene were 5’- TGCATACGTGGCCTCCAACAG -3’ (wild type) and 5’- TGCATAAAAGGGCTCCAACAG -3’ (mutant).
Chromatin immunoprecipitation (ChIP). HUVECs were cultured in 150 mm²-dishes and treated with 200 µM CoCl₂ or PBS for 16 h, and then harvested. Interaction between HIF-1α protein and human A2 promoter was tested using the Easy ChIP assay kit (Millipore). Primers for amplification of the human A2 promoter fragment were 5’-CCACTTAATCAAGCCCAGAGT-3’ (sense) and 5’-CGCCACGCGGAGCGGCCCCAG-3’ (antisense).

Promoter reporter assay. A 40-bp A2 promoter probe containing either the wild type (5’-GAGCTCGACGTGGCAGCCTAGCTCTGCAGCATTGTG-3’) or mutant (5’-GAGCTCGAAAAGGCACTTAAGGCTCTGCAGCATTGTG-3’) hypoxia-responsive element was subcloned into the luciferase reporter vector pGL3-luc. The HEK 293 cells, cultured in 48 well dishes, were transfected in triplicate using lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions using a total of 1.5 µg DNA, consisting of different combinations of plasmids. HIF-1α (pcDNA3.1/Hif-1a) and HIF-1β (pBM5/Hif-1b) expression vectors, kindly provided by Dr. Gregg Semenza (Johns Hopkins University School of Medicine) and Dr. Oliver Hankinson (UCLA Medical Center), respectively, induced robust protein expression (Supplemental Figure 4B). A luciferase reporter vector containing the wild type VEGF promoter was kindly provided by Dr. Lee Ellis (MD Anderson Cancer Center). Reporter activity was quantified as luciferase activity (Promega).

Flow cytometry. Surface expression of A2 on HUVEC cells cultured in 21% or 0.5% O₂ for 16h was measured by flow cytometry using either mouse monoclonal IgG1 directed against human/mouse A2 (BD Biosciences), PE-conjugated VE-cadherin 5 (BD Pharmingen), or non-immune monoclonal mouse IgG1 isotype control (R&D Systems) (Supplemental Table 2). Secondary antibodies consisted of Alexa Fluor 647-conjugated goat anti-mouse IgG (Invitrogen/Molecular Probes). Labeled cells were analyzed using a BD LSR II flow cytometer (BD Biosciences). Nonviable cells were excluded from the analysis using 7-AAD (BD Biosciences).
Ancrod and tranexamic acid treatment. For fibrinogen depletion, ancrod (NISBC, UK) was administered from P12 to P16 (2 U/day, SQ), immediately following transfer of the mice from 75% oxygen to room air. To inhibit plasminogen activation, mice were given tranexamic acid (2.5 mg, IP, Q8h; Sigma Aldrich).

Statistical analysis. Statistical significance was assessed by the two-tailed Student’s t-test. P values of ≤ 0.05 were considered statistically significant.
Results

**Annexin A2 promotes oxygen-induced retinal neovascularization.** Using two independent criteria, we quantified retinal neovascularization in P17 *Anxa2<sup>+/−</sup>* and *Anxa2<sup>−/−</sup>* neonatal mice subjected to 75% oxygen for five days (P7-P12) \(^{13,14}\). We found no significant difference in the ratio of the area of vaso-obliteration to total retinal area in *Anxa2<sup>+/−</sup>* (25.6 ± 1.0%) and *Anxa2<sup>−/−</sup>* (27.5 ± 1.1%) mice (P = 0.23). However, we did detect a 2.5-fold reduction in the ratio of retinal neovascularization to total retinal area in *Anxa2<sup>−/−</sup>* mice (4.7 ± 0.4%) as compared to *Anxa2<sup>+/−</sup>* mice (12.1 ± 0.5%) (P = 1.3x10\(^{-7}\)) (Figure 1A-C). In addition, enumeration of neovascular tufts by immunofluorescent isolectin B4 staining of retinal sections (Figure 1D) revealed a greater than 50% reduction in the number of vascular structures extending beyond the internal limiting membrane in *Anxa2<sup>−/−</sup>* (87.1 ± 4.6) versus *Anxa2<sup>+/−</sup>* (194.5 ± 5.2) retinas at P17, five days after release from post-hyperoxia (P = 1.12x10\(^{-10}\)) (Figure 1E). There was no difference in VEGF or placental growth factor (PIGF) induction in *Anxa2<sup>+/−</sup>* versus *Anxa2<sup>−/−</sup>* mice (Supplemental Figure 1A, B), and no difference in neonatal retinal vascularization when *Anxa2<sup>+/−</sup>* and *Anxa2<sup>−/−</sup>* mice were maintained in room air (0-2 tufts per section; Supplemental Figure 2).

Subretinal injection of a replication-deficient A2-encoding adenovirus restored A2 expression in *Anxa2<sup>−/−</sup>* mouse retinas, as demonstrated by RT-PCR and immunoblot of retinal tissue (Figure 2A). At the same time, restoration of A2 expression augmented OIR-related neovascularization (4.7 ± 0.15% vs. 8.6 ± 0.12%; P = 6.4x10\(^{-5}\)) of *Anxa2<sup>−/−</sup>* retinas without changing the degree of vaso-obliteration (19.5 ± 0.64% vs. 19.4 ± 0.79%; P = 0.91) (Figure 2B, C). Viral transduction of A2 also increased by 60% the number of neovascular tuft nuclei in *Anxa2<sup>−/−</sup>* eyes in comparison to contralateral *Anxa2<sup>−/−</sup>* eyes injected with empty virus (88 ± 4.2 vs. 134 ± 3.5; P = 9.0x10\(^{-6}\)) (Figure 2D, E). Together, these data indicate that the complete OIR response depends upon the presence of an intact A2 system.
Hypoxia-inducible factor-1 directly regulates annexin A2 gene expression. We determined whether retinal A2 and p11 expression is regulated by oxygen tension. While p11 mRNA levels remained relatively constant in the retina following high oxygen exposure, A2 mRNA levels increased by 2- to 4-fold at P14-P18 (Figure 3A). Protein levels of both A2 and p11 increased by 2-5-fold at P16-P18 in comparison to the corresponding RA value, as demonstrated by immunoblot analysis of retinal lysates (5.4 ± 1.2-fold at P16 and 2.0 ± 0.2-fold at P18, n=6, for A2; 4.1 ± 0.6-fold at P16 and 2.2 ± 0.3-fold at P18, n=4, for p11, SE; Figure 3B). The increased A2 was further confirmed by immunofluorescence staining of retinal sections (Figure 3C). We showed previously that A2 stabilizes p11 post-translationally in endothelial cells by masking a polyubiquitination site in the C-terminal region of p11, a finding that explains the increase in p11 protein in the absence of any alteration in p11 mRNA.

To ascertain whether A2 synthesis might be governed by the HIF-1 transcription complex, a master regulator of oxygen-sensitive genes, we first confirmed that HIF-1α was stabilized during OIR. Indeed, from P12-P18, HIF-1α mRNA did not change (Supplemental Figure 3A, B), but protein levels increased 2-4-fold in comparison to the corresponding RA value (0.4 ± 0.1-fold at P12; 2.3 ± 0.3-fold at P14; 4.3 ± 0.9-fold at P16; 4.0 ± 0.5-fold at P18; n=3; SE; Figure 3D). In human umbilical vein endothelial cells (HUVECs) treated either with CoCl₂, a heavy metal salt that mimics hypoxia, or with hypoxia itself (0.5% O₂), protein levels of HIF-1α (29.4 ± 1.3-fold for 0.5% O₂ and 90.6 ± 7.0-fold for CoCl₂, n=3), A2 2.4 ± 0.6-fold for 0.5% O₂, n=6 and 2.6 ± 0.5-fold for CoCl₂, n=4), and p11 (2.3 ± 0.4-fold, n=6 for 0.5% O₂ and 2.3 ± 0.3-fold for CoCl₂, n=4) all increased (Figure 3E). CoCl₂ proved to be an even stronger stimulus than hypoxia for stabilization of HIF-1α; this is most likely because CoCl₂ acts by inhibiting the prolyl hydroxylase that allows HIF-1α to be ubiquitinated and destroyed in the proteasome, whereas hypoxia reduces, but does not eliminate oxygen, a key co-substrate for
the hydroxylating enzyme\(^22\). Elevations in mRNA levels for A2, but not HIF-1\(\alpha\) or p11, were observed over the same time frame (Figure 3E).

mRNA stability assays demonstrated that hypoxia-related induction of A2 in HUVECs reflected transcriptional activation. Because A2 was upregulated in \(\text{Hif1a}^{+/+}\) (2.6 ± 0.4-fold for 0.5% O\(_2\) and 2.4 ± 0.3-fold for CoCl\(_2\), n=6, SE), but not \(\text{Hif1a}^{-/-}\) (1.0 ± 0.1-fold for 0.5% O\(_2\) and 0.9 ± 0.1-fold for CoCl\(_2\), n=6, SE), mouse embryonic fibroblasts \(^16\) upon treatment with either CoCl\(_2\) or hypoxia (Figure 3F), we concluded that its induction was HIF-1\(\alpha\)-dependent. Similarly, p11 was upregulated in \(\text{Hif1a}^{+/+}\) MEFs (2.3 ± 0.3-fold for 0.5% O\(_2\) and 2.7 ± 0.5-fold for CoCl\(_2\), n=6, SE), but not in \(\text{Hif1a}^{-/-}\) MEFs (1.0 ± 0.1-fold for 0.5% O\(_2\) and 1.1 ± 0.1-fold for CoCl\(_2\), n=6, SE). Examination of the A2 promoter sequence revealed a nearly complete hypoxia responsive element (Supplemental Figure 3C). In addition, A2 expression was stable in the presence of actinomycin D, suggesting that increased mRNA levels induced by hypoxia reflected an increased rate of transcription. (Supplemental Figure 4A). An electrophoretic mobility shift assay \(^20\) demonstrated that A2 probes containing wild type, but not mutant, hypoxia response elements from the A2 promoter, interacted specifically with nuclear components from HEK 293 cells that over-expressed Hif-1\(\alpha\) and HIF-1\(\beta\) (Figure 3G; Supplemental Figure 4B). A chromatin immunoprecipitation assay \(^16\), furthermore, confirmed direct binding of HIF-1\(\alpha\) to the A2 promoter in HUVECs treated with CoCl\(_2\) (Figure 3H). A similar A2 promoter sequence was activated by the HIF-1 complex in luciferase assays (Figure 3I). Together these experiments revealed that A2 synthesis is regulated by hypoxia at the transcriptional level through the action of HIF-1.

**Enhanced annexin A2 expression during hypoxia promotes plasminogen activation and endothelial cell migration.** We showed previously that cell surface expression of A2, a Src substrate, depends upon both the presence of p11 and phosphorylation of Tyr\(^{23}\) by active pp60Src kinase \(^15\). Here, cell surface biotinylation showed a clear increase in endothelial cell
surface expression of A2, as well as p11, following true hypoxia (Figure 4A). This increase was further quantified by flow cytometry, which revealed an increase of 3- to 4-fold (mean fluorescence intensity 10.4 at 21% O₂ vs 36.1 units in 0.5% O₂; Figure 4B). At the same time, cell surface VE-cadherin 5 remained constant (mean fluorescence intensity 171.4 at 21% O₂ vs 169.2 units in 0.5% O₂), indicating the specificity of the A2 response.

Total cellular levels of both Tyr²³ phosphorylated A2 (phospho-A2) and active Src (Tyr⁴¹⁶ phosphorylated Src) increased along with HIF-1α and p11 (Figure 4C). We noted that hypoxia-induced increases in both tPA-dependent plasmin generation and cell migration through a fibrin barrier were A2-dependent, being observed in Anxa2⁺/⁺, but not Anxa2⁻/⁻, mouse microvascular endothelial cells (Figure 4D, E). Both plasmin generation and cell migration through a fibrin barrier nearly doubled after restoring A2 and p11 expression in Anxa2⁻/⁻ mouse endothelial cells through transduction with an A2-encoding adenovirus (Figure 4F-H). These data indicate that hypoxia enhances both A2 expression and plasminogen activation at the endothelial cell surface. The blunted response of Anxa2⁻/⁻ endothelial cells to hypoxia was not due to impairment of the HIF system, since HIF-1α was stabilized equally in Anxa2⁺/⁺ and Anxa2⁻/⁻ retinal tissue during OIR and in endothelial cells following exposure to hypoxia (Supplemental Figure 5A, B).

Annexin A2 system-mediated proteolysis relieves the fibrin blockade to retinal angiogenesis. When maintained in room air, Anxa2⁻/⁻ retinas exhibited fibrin that was readily detectable immunologically. Anxa2⁺/⁺ retinas, on the other hand, displayed only trace amounts of detectable fibrin (Supplemental Figure 6). During OIR, Anxa2⁻/⁻ retinas exhibited high levels of perivascular fibrin deposition compared with Anxa2⁺/⁺ retinas, in which only small amounts of fibrin were identified (Figure 5A, B). When A2 expression was restored in Anxa2⁻/⁻ upon subretinal injection of an A2-encoding adenovirus, fibrin deposition decreased to the level observed in the Anxa2⁺/⁺ retina (Figure 5C, D).
Furthermore, systemic treatment of mouse pups upon release from hyperoxia with the defibrinating agent, ancrod,\textsuperscript{23} reduced plasma fibrinogen by more than 80% (Figure 6A). This treatment, which eliminated fibrin deposition in both Anxa2\textsuperscript{−/−} and Anxa2\textsuperscript{+/+} retinas (Figure 6B, C and Supplemental Figure 7A-C), more than doubled retinal neovascularization in Anxa2\textsuperscript{−/−}, but not A2\textsuperscript{+/+}, retinas (Figure 6D, E and Supplemental Figure 7D, E). Ancrod also nearly doubled the number of neovascular tuft nuclei in Anxa2\textsuperscript{−/−} (Figure 6F), but not A2\textsuperscript{+/+} (Supplemental Figure 7F), retinas at P17, while the retinal vaso-obliteration phase of OIR remained unchanged.

Conversely, we treated mouse pups, immediately upon their release from hyperoxia, with tranexamic acid (TA), an inhibitor of plasminogen activation\textsuperscript{24}. Under conditions where systemic TA impaired plasma clot lysis (Figure 7A), fibrin deposition increased dramatically in the retina (Figure 7B, C). At the same time, both retinal neo-vascularization and neovascular tuft nuclei decreased by more than half by P17 (Figure 7D-F). Similar reductions in tPA-dependent clot lysis activity, retinal neoangiogenesis, and neovascular nuclei, accompanied by increases in fibrin deposition, were observed in P17, TA-treated Anxa2\textsuperscript{−/−} mice, even though OIR was already depressed (Supplemental Figure 8). Together, these data suggest that fibrin is a potent modulator of oxygen-induced retinal neoangiogenesis.
Discussion

Metazoan cells sense tissue oxygen tension through the action of prolyl-4-hydroxylases, which use oxygen as a substrate to hydroxylate and mark hypoxia-inducible factor-1α (HIF-1α) for proteasomal destruction. Essentially all critical angiogenic factors, including secreted vascular endothelial growth factor, stromal derived growth factor, angiopoietin 2, placental growth factor, platelet-derived growth factor B, stem cell factor, and erythropoietin, as well as many growth factor receptors, signal transduction molecules, and transcription factors, are induced by hypoxia, either directly or indirectly, through HIF-1. The current study places annexin A2 within this group by presenting the first evidence that the annexin A2 gene is transcriptionally regulated in hypoxia through the direct action of HIF-1.

HIF-1 is a ubiquitous, heterodimeric protein consisting of HIF-1α, which is oxygen-regulated, and HIF-1β, which is constitutively expressed. Under normoxic conditions, HIF-1α undergoes hydroxylation at proline 402 and/or 564, which allows it to bind to the von Hippel-Lindau protein and recruit an E3 ubiquitin ligase. Ubiquitination of HIF-1α results in its degradation in the proteasome. Hypoxia inhibits prolyl hydroxylase activity, allowing the intracellular accumulation of HIF-1α, which translocates to the nucleus and dimerizes with HIF-1β. On target genes, the HIF-1α/β complex binds to cis-acting hypoxia response elements (HREs), which contain the consensus sequence 5'-(A/G)CGTG-3'.

The oxygen-induced retinopathy (OIR) model has long been used to study the effects of retinal ischemia on the subsequent angiogenic response. In this respect, it is a robust model of retinopathy of prematurity (ROP), as well as other retinal vascular proliferative disorders, such as diabetic retinopathy. In both OIR and ROP, high-level oxygen causes regression of developing or newly developed blood vessels (vaso-oblitration), resulting in a zone of ischemia within the central retina. Retinal ischemia leads to rapid upregulation of HIF-1α, especially
within the ganglion cell layer, which houses the retina’s vascular plexus\textsuperscript{30, 31}. HIF-1 then drives transcriptional induction of oxygen-regulated genes.

Our data describe a novel pathway for the regulation of A2 within the hypoxic endothelial cell (\textit{Supplemental Figure 9}). We show that, upon stabilization, HIF-1\textsubscript{\alpha}, presumably in complex with HIF-1\textsubscript{\beta}, interacts directly with a putative hypoxia responsive element within the 5’-untranslated region of the A2 gene, leading to activation of the A2 promoter. This interaction resulted in an increase in promoter activity similar to that seen with HIF-\textalpha\ induction of VEGF. Increased transcription of A2 mRNA resulted in higher A2 protein levels and stabilization of p11\textsuperscript{9}. Cell surface A2 and p11 also increased in response to hypoxia-induced phosphorylation of pp60\textsubscript{src} kinase activation\textsuperscript{32} and A2 tyrosine phosphorylation\textsuperscript{15}, and was associated with enhanced plasmin generation and migration through a fibrin barrier. Our previous data have revealed that annexin A2 undergoes tyrosine phosphorylation through the action of pp60\textsubscript{src} kinase, which is activated in response to heat shock, and that this event is required for translocation of A2 to the cell surface\textsuperscript{15}. In the present study, we found that activation of src increases dramatically under hypoxia, and, along with increased total expression of both A2 and p11, likely contributes to the enhanced surface expression of both proteins. Plasmin may activate selected MMPs\textsuperscript{33}, further facilitating the directed migration of endothelial cells and other vascular cells. Plasmin and/or MMPs may also release extracellular matrix-associated angiogenic growth factors that further support neovascularization of the retina\textsuperscript{34}.

The present work is also the first, to our knowledge, to identify fibrin and the vascular fibrinolytic system as a critical regulator of retinal angiogenesis \textit{in vivo}. Fibrin deposition was dramatically increased in the post-hyperoxic \textit{Anxa2\textsuperscript{-/-}}, but not \textit{Anxa2\textsuperscript{+/+}}, retina, and prevention of fibrin deposition restored retinal angiogenesis, while inhibition of fibrinolysis curtailed it. The precise mechanism by which fibrin may block neoangiogenesis is unclear. Individual degradation products of fibrin and fibrinogen have been reported to stimulate such diverse and
even opposing activities as vascular contractility\textsuperscript{35}, leukocyte activation and inhibition\textsuperscript{36}, endothelial cell proliferation and apoptosis\textsuperscript{37, 38}, and smooth muscle cell proliferation\textsuperscript{39}. In addition, fibrin might constitute a physical barrier to the directed migration of plasmin-deficient endothelial cells, or could harbor essential growth factors that are released only upon localized proteolysis by plasmin.

The mouse retina has long served as a model for studying both physiologic and pathologic angiogenesis without the need for surgical manipulation of tissues\textsuperscript{40}. Indeed, the importance of a number of pathways involving VEGF, erythropoietin, insulin-like growth factor, bone marrow progenitor cells, and lipid-derived mediators of angiogenesis have emerged from work based upon the mouse OIR model. While OIR, as a model of human retinal proliferative disease, may be most relevant to retinopathy of prematurity, it also recapitulates several key features of proliferative diabetic retinopathy. These include the development of pathologic neovessels in response to earlier vessel loss\textsuperscript{41}, loss of integrity of the blood-retinal barrier with vascular leak\textsuperscript{42}, and concomitant glial and neuronal damage in association with retinal vascular disease\textsuperscript{43}. Therefore, our findings in the \textit{Anxa2}\textsuperscript{-/-} mouse may have implications that extend beyond retinopathy of prematurity to diabetic retinopathy or possibly age-related macular degeneration.

For high risk ROP, laser ablative therapy and, more recently, anti-VEGF modalities are the mainstays of treatment\textsuperscript{44}. Left untreated, unfavorable outcomes in ROP may be as high as 56\%\textsuperscript{45}. Even with laser therapy, undesirable visual and structural sequelae may be seen in 10-15\% of treated patients\textsuperscript{46}, some of whom progress to retinal detachment\textsuperscript{47}. Although anti-VEGF-based treatments to prevent or reverse ROP are encouraging, further randomized control trials are needed to assess dosage regimens and potential late neurologic and ocular complications\textsuperscript{48}. Clinical trials to evaluate treatment with insulin-like growth factor-1 were
recently initiated, and dietary supplementation of omega-3-polyunsaturated fatty acids may also be tested as a preventative measure in the near future 47.

Our results suggest a potential new therapeutic target for ROP, and possibly other proliferative retinopathies. Although systemic antifibrinolytic therapy with tranexamic acid, aprotinin, or epsilon aminocaproic acid has been used to treat fibrinolysis in association with dental procedures, major surgery, menorrhagia, and traumatic hyphema 24, enthusiasm for its systemic use has been dampened by reports of seizure activity, myocardial infarction, and stroke in adults 49, 50, and aortic thrombosis in a child 51. The stressed premature newborn, moreover, may have exaggerated fibrinolytic activity, with elevated tPA and/or urokinase levels 52, 53, whose activity might be overcome only with large doses of antifibrinolytics, thus increasing the likelihood of untoward effects, such as thrombosis. Therefore, local, intraocular blockade of the annexin A2 system might provide a safer alternative by attenuating cell surface fibrinolysis specifically in the perivascular compartment, and without disrupting systemic hemostatic balance. Indeed, a recent study suggests that chlorotoxin, an agent that interacts with annexin A2, may prevent or reverse ocular neovascularization in several mouse models 54.
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Authorship Contributions and Disclosure of Conflicts of Interests

B.H. designed and conducted the experiments, analyzed the data, and prepared the manuscript; A.D. conducted experiments and analyzed the data; K.H. conducted experiments; K.C. conducted experiments and analyzed the data; G.S., D.A., P.H. and P.B. assisted with the experiments; A.J. provided unpublished promoter sequence data; K.A.H. supervised the study, analyzed the data, and revised the manuscript. All authors claimed no conflict of interest.
References


Figure Legends

Figure 1. The annexin A2 system promotes retinal neo-angiogenesis in OIR. (A) Schematic diagram illustrating areas occupied by total retina (green), regions of vaso-obliteration (yellow) and regions of neovascularization (red) in mice maintained in room air (RA) or treated with oxygen from P7-P12 (OIR). (B) Representative retinal images showing total retinal area, and regions of vaso-obliteration and neovascularization in P17 oxygen-treated Anxa2+/+ and Anxa2-/- mice. Pixel number corresponding to each compartment is indicated below each image. (C) Ratios of vaso-obliterative (VO) to total retinal area (25.6 ± 1.0% vs. 27.5 ± 1.1%) and neovascular (NV) to total retinal area (12.1 ± 0.5% vs. 4.7 ± 0.4%) in oxygen-treated P17 Anxa2+/+ (n = 7) and Anxa2-/- (n = 9) mice (NS: no significant difference; *** p<0.001). (D) Representative retinal cross-sections (40x), with close-up views of peripheral region (200x) of retinas from oxygen-treated P17 Anxa2+/+ and Anxa2-/- mice stained with DAPI (blue) and isolectin B4 (green). Neovascular tufts penetrating the inner limiting membrane (highlighted with a white dashed line) are indicated by arrowheads. E. Enumeration of neovascular nuclei in retinal sections from P17 oxygen-treated Anxa2+/+ vs. Anxa2-/- (194.5 ± 5.2; n = 8 vs. 87.4 ± 4.6; n = 10; ***p<0.001) mice. Scale bars, 200 μm (B, 50x), 500 μm (D, 40x), 100 μm (D, 200x).

Figure 2. Re-expression of annexin A2 restores neo-angiogenesis in OIR. (A) RT-PCR of retinal tissue at P14 following subretinal injection of A2-encoding (A2; OD) or empty (V; OS) adenovirus in Anxa2-/- mice at P12, and immunoblot analysis of identically treated Anxa2-/- mouse retinas two days (P14) after virus injection. The data are representative of three independent experiments. (B) Representative retinal images showing total retinal area, and regions of NV and VO in oxygen-treated P17 Anxa2-/- mice injected with either A2-encoding or empty virus on P12. Pixel number corresponding to each compartment is indicated below each image. (C) Ratios of VO to total retinal area (19.5 ± 0.64% vs.19.4 ± 0.79%) and NV to total
retinal area (4.7 ± 0.15% vs. 8.6 ± 0.12%) in P17 oxygen-treated Anxa2−/− mice injected with either empty or A2-encoding virus (n = 8; NS: no significant difference, *** p< 0.001). (D) Representative retinal cross-sections (40x), with a close-up view of the peripheral region (200x), of retinas from P17 oxygen-treated Anxa2−/− mice injected with empty or A2-encoding virus stained with DAPI (blue) and isolectin B4 (red). Neovascular tufts penetrating the inner limiting membrane (highlighted with a white dashed line) are indicated by arrowheads. (E) Enumeration of neovascular nuclei in retinal sections from P17 oxygen-treated Anxa2−/− mice injected with empty vs. A2-encoding virus (88 ± 4.2 vs. 134 ± 3.5; n = 5; ***p<0.001). S, sclera; V, vitreous body. Scale bars, 200 μm (B, 50x), 500 μm (D, 40x), 100 μm (D, 200x).

Figure 3. Annexin A2 is upregulated during hypoxia through Hif-1α-mediated signaling. (A) RT-PCR determination of the fold change in Anxa2 and S100a10 mRNA levels in P12-P18 retinas from Anxa2+/+ mice following treatment with oxygen. GAPDH mRNA served as a loading control. (B) Representative immunoblot of Anxa2+/+ retinal A2 and p11 at P12, P14, P16, and P18 following treatment with oxygen (O2) or room air (RA). Actin was used as loading control. (C) Representative images of retinal sections from P17 Anxa2+/+ mice either maintained in room air (RA) or treated with oxygen (O2), and stained with anti-A2 (red), isolectin B4 (green), and DAPI (blue). GCL: ganglion cell layer; INL: inner nuclei layer. Scale bars, 75 μm (200x). (D) Immunoblot of HIF-1α in P12, P14, P16, and P18 retinas from Anxa2+/+ mice maintained in RA or treated with oxygen. Actin served as the loading control. (E) Immunoblot and RT-PCR analyses of HIF-1α, A2, and p11 in HUVECs after treatment with either 0.5% O2 or 200 μM CoCl2 for 16h. Actin served as the loading control. (F) Immunoblot and RT-PCR analyses of A2 and p11 in Hif1a+/+ and Hif1a−/− mouse embryonic fibroblasts after treatment with either 0.5% O2 or 200 μM CoCl2 for 16h. Actin served as the loading control. (G) Electrophoretic mobility shift assay showing interaction between the HIF-1 complex and ANXA2 or VEGFA promoter probes.
(C: control, WT: ANXA2 or VEGFA probe containing wild type hypoxia responsive element (HRE), MUT: ANXA2 or VEGFA probe containing mutant HRE, FP: free unbound probe) (H) Chromatin immunoprecipitation assay showing interaction between HIF-1α and the ANXA2 promoter. Preimmune IgG and anti-RNA polymerase II served as negative and positive controls, respectively. (I) Luciferase assay showing activation of ANXA2 and VEGFA promoters by HIF-1 complex in HEK 293 cells (n = 3; *p<0.05, **p<0.01, ***p<0.001). The data are representative of three (A, C, D, G, H and I) and six (B, E, and F) separate experiments.

**Figure 4.** Hypoxia promotes functional endothelial cell surface expression of A2. (A) Immunoblot of cell surface biotinylated A2 and p11 on HUVECs treated with hypoxia (0.5% O2, 16h). Pan-cadherin served as the loading control. (B) Flow cytometric analysis of HUVEC surface A2 and VE-cadherin 5 following normoxia (21% O2, 16h; blue histograms) or hypoxia (0.5% O2, 16h; red histograms). Isotype-matched IgG served as control (shaded histogram). (C) Immunoblot showing total cellular HIF-1α, total A2, Tyr 416 phospho-Src, total Src, p11, and actin following hypoxia (0.5% O2, 16 h). Phospho-A2 was captured by immunoprecipitation, followed by immunoblot. (D) Anxa2+/+ and Anxa2−/− CMEC-associated plasmin generation for cells maintained in 21% vs 0.5% O2 (0.22 ± 0.014 vs. 0.404 ± 0.005 vs. 0.13 ± 0.01 vs. 0.11 ± 0.007; n = 6; **p<0.01, NS: no significant difference). (E) Migration of Anxa2+/+ (8.35 ± 0.074% vs. 18.3 ± 1.36%) and Anxa2−/− (3.9 ± 0.34% vs. 4.4 ± 0.5%) CMECs maintained in 21% or 0.5% O2 (16h, n = 3; **p<0.01). (F) Immunoblot of surface and intracellular A2 and p11 in Anxa2−/− CMECs 48h after infection with either empty or A2-encoding viruses. Pan-cadherin and actin served as surface and intracellular protein loading controls, respectively. (G) Anxa2−/− CMEC-associated plasmin generation following infection with empty (V; 0.195 ± 0.014 RFU/ min²) or A2-encoding virus (A2; 0.312 ± 0.025 RFU/ min²; 48 h, n = 3; *p<0.05, **p<0.01). (H) Migration of Anxa2−/− CMECs infected with either empty (V; 2.6 ± 0.14%) or A2-encoding virus (A2; 5.8±
0.23%; 48h, n = 3; *p<0.05, **p<0.01). The data are representative of three independent experiments (A-H).

Figure 5. The annexin A2 system promotes retinal neoangiogenesis in OIR by enhancing fibrin clearance. (A) Representative cross-sections (40x) of retinas, from P17 oxygen-treated Anxa2+/+ and Anxa2−/− mice, stained with DAPI (blue), isolectin B4 (green), and anti-fibrinogen (red). Close-up views of the peripheral retina are also shown (200x). Fibrin deposition is indicated by arrowheads. (B) Representative immunoblot of fibrin within retinal tissue from P17 oxygen-treated Anxa2+/+ and Anxa2−/− mice. (C) Representative cross-sections (40x) of retinas from P17 oxygen-treated Anxa2−/− mice injected with either empty (V) or A2-encoding (A2) virus, stained with DAPI (blue), isolectin B4 (green) and anti-fibrinogen (red). Close-up views of the peripheral retina are also shown (200x). Fibrin deposition is indicated by arrowheads. (D) Representative immunoblot of fibrin within retinal tissue from P17 oxygen-treated Anxa2−/− mice injected with empty or A2-encoding virus. S, sclera; V, vitreous body. Scale bars, 500 and 100 μm for A and C, 40x and 200x, respectively. The data are representative of five (A and C) and three (B and D) independent experiments.

Figure 6. Fibrinogen depletion enables neovascularization of the Anxa2−/− retina. (A) Enzyme-linked immunosorbent assay of fibrinogen levels in plasma from P17 Anxa2−/− mice treated with (0.19 ± 0.04 mg/ml; n = 7) or without (1.17 ± 0.09 mg/ml; n = 8) ancrod (***p<0.001). (B) Representative cross-sections (40x) of retinas from P17 oxygen-treated Anxa2−/− mice, treated thereafter with or without ancrod, and stained with DAPI (blue), isolectin B4 (green) and anti-fibrinogen (red). Close-up views of the peripheral retina are also shown (200x). S, sclera; V, vitreous body. Scale bars 500 and 100 μm, for 40x and 200x, respectively. (C) Representative immunoblot of fibrin in retinal tissue from P17 oxygen-treated Anxa2−/− mice, treated thereafter
with or without ancrod. (D) Representative images showing total retinal area, and regions of AV and VO from P17 oxygen-treated Anxa2−/− mice, treated thereafter with or without ancrod. Pixel number corresponding to each compartment is indicated below each image. Scale bars, 200 μm (50x). (E) Ratios of areas of VO to total retina (27.2 ± 1.7% vs. 30.1 ± 1.3%) and NV to total retina (4.3 ± 0.09% vs. 9.7 ± 0.2%) in P17 oxygen-treated Anxa2−/− mice, treated thereafter with or without ancrod (n = 5; NS: no significant difference, *** p< 0.001). (F) Enumeration of neovascular nuclei in retinal sections from P17 oxygen-treated Anxa2−/− mice, treated thereafter with (131.8 ± 5.3) or without ancrod (79.4 ± 2.2 nuclei per section; n = 5; ***p<0.001). The data are representative of five (B) or three (C) independent experiments.

Figure 7. Interruption of fibrinolysis blocks retinal neoangiogenesis during OIR. (A) Clot lysis curves representative of at least 3 assays (n = 4 for each) performed using plasma from P17 Anxa2+/+ mice treated with or without tranexamic acid (TA). (B) Representative cross-sections (40x) of retinas from P17 oxygen-treated Anxa2+/+ mice, treated with or without TA, and stained with DAPI (blue), isolectin B4 (green), and anti-fibrinogen (red). Close-up views of the peripheral retina (200x) are also shown. Fibrin deposition is indicated by arrowheads. S, sclera; V, vitreous body. Scale bars 500 and 100 μm for 40x and 200x, respectively. (C) Representative immunoblot of fibrin in retinas from P17 oxygen-treated Anxa2+/+ mice, treated with or without TA. (D) Representative images from P17 oxygen-treated Anxa2+/+ mice, treated with or without TA, showing total retinal area, and regions of VO and NV. Pixel number corresponding to each compartment is indicated below each image. Scale bars, 200 μm (50x). (E) Ratios of VO to total retinal area (19.8 ± 1.3% vs.22.2 ± 3.3%) and NV to total area (11.4 ± 0.9% vs. 4.6 ± 0.3 %) in retinas from P17 oxygen-treated Anxa2+/+ mice, treated with or without TA (n = 4; NS: no significant difference, ***p< 0.001). (F) Enumeration of neovascular nuclei in retinas from P17 oxygen-treated Anxa2+/+ mice, treated with (92 ± 4.0; n = 5) or without TA (207
± 5.9; n = 3; ***p < 0.001). The data are representative of five (B) or three (C) independent experiments.
Figure 1

A

RA

OIR

Total area

Vaso-obliteration

Neovascularization

Anxa2^{+/+}

Anxa2^{−/−}

Pixels

373800

93689

54350

377842

109615

22243

B

Whole retina
Vaso-obliteration
Neovascularization
Merge

C

NS

VNO/whole retina (%)

Anxa2^{+/+}

Anxa2^{−/−}

D

Anxa2^{+/+}

Anxa2^{−/−}

E

Tufts (nucleus per section)

Anxa2^{+/+}

Anxa2^{−/−}
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Hypoxia-inducible factor-1 drives annexin A2 system-mediated perivascular fibrin clearance in oxygen-induced retinopathy in mice

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