Identification of Genes and Pathways Involved in Retinal Neovascularization by Microarray Analysis of Two Animal Models of Retinal Angiogenesis

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Purpose. Comparative retinal gene expression analysis in two rodent models of oxygen-induced retinopathy (OIR) was performed to identify the genes and pathways involved in retinal neovascularization.

Methods. Three independent experimental runs were conducted for each species, according to standard protocols for induction of OIR. Total retinal RNA was isolated at two time points, corresponding to the early response to relative hypoxia (P13 in mouse, P15 in rat) and to the later phase of maximum retinal neovascularization (P18 in mouse, P20 in rat) and was used to prepare labeled probes for hybridization. Gene expression was compared between normal and experimental conditions for each species at each time point. Probesets with a false-discovery rate of ≤0.05 were considered significantly different and were classified as cellular functions or biological pathways. Changes in expression of selected genes were confirmed by quantitative rtPCR.

Results. At the early time point, there were changes in 43 genes in each species, with two in common. Increased expression of members of the VEGF and ephrin receptor signaling pathways were identified in both models. At the later time point, there were changes in 26 genes in the rat and in 1622 in the mouse, with 13 in common. Four pathways were identified in both models.

Conclusions. Genes and pathways known to be involved in angiogenesis, as well as other biologically plausible genes and pathways, were identified. This work serves as a comprehensive resource for the study of retinal neovascularization and identification of potential rational targets for antiangiogenic therapy. (*Invest Ophthalmol Vis Sci.* 2010;51:1098–1105) DOI:10.1167/iovs.09-4006

Retinal neovascularization is the final common pathway of numerous blinding disorders and comprises a complex cascade of molecular and cellular processes. It is hoped that elucidation of the molecular mechanisms underlying pathologic neovascularization will ultimately allow the identification of targets for pharmacologic therapy. To date, vascular endothelial growth factor (VEGF) has attracted the most attention, and its biological role has been validated by clinical response after pharmacologic inhibition of its activity. However, laboratory and clinical observations support the involvement of factors other than VEGF in both normal retinal vasculogenesis and retinal neovascularization.[1]

Several robust animal models have been validated for the in vivo study of retinal neovascularization and for testing of antiangiogenic therapies. Most commonly used among these are two rodent models (rat and mouse) of oxygen-induced retinopathy (OIR). In both models, exposure of the developing retina to fluctuations in oxygen results in a predictable course of retinal avascularity immediately after removal to room air, followed several days later by preretinal neovascularization.[2,3]

Over the past decade, the use of cDNA microarrays has facilitated the identification of individual genes and pathways involved in myriad biological processes.[4] The advantage of such high-throughput analysis is the opportunity to compare gene expression between different cells, tissues, or physiological conditions. Disadvantages include the challenges of interpretation given the voluminous data and the potential for a high incidence of irrelevant expression changes (background noise).

In an effort to identify additional factors involved in retinal neovascularization, we used microarrays to perform comparative gene expression analysis of whole retinal RNA from both rats and mice with oxygen-induced retinopathy (OIR). For each model, gene expression was compared between normal and experimental conditions at each of two time points, corresponding to the early angiogenic response to relative hypoxia and to the later phase of maximum retinal neovascularization. It was hoped that this approach would yield more credible and biologically relevant data by identifying the commonalities from independent models with a similar phenotype.

Materials and Methods

Animals

Experiments involving animals were approved by the Vanderbilt University Institutional Animal Care and Use Committee and were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animals were maintained in fluorescent lighting of 80 lux on a 12-hour light/dark cycle. Litters of Sprague-Dawley rat pups and nursing dams were maintained in environments of either variable oxygen (OIR rats) or room air (control rats). The OIR rats were placed with mothers in infant incubators within 4 hours after birth and exposed to alternating 24-hour periods of 50% oxygen and 10% oxygen for 14 days, whereupon they were removed to room air. Age-matched control animals were reared simultaneously in room air from birth. Seven days after birth (postnatal day [P]7), litters of C57BJ/6L wild-type mice were exposed to 75% oxygen for 5 days until P12, whereupon they were transferred to room air (OIR mice). Age-matched control animals were reared simultaneously in room air. Three independent experimental runs were conducted for each species.

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TABLE 1. Genes with Consistent Change in Expression (≥1.7-fold) at the Early Timepoint of Two Rodent Models of Experimental Retinal Neovascularization

		Change (*I*-Fold)	Step-up *P*-value
Rat genes (P15), of a total of 43			
EGL nine homolog 3 (*C. elegans*)	*Egln3*	2.5	0.008
Chemokine (C-X-C motif) receptor 4	*Cxcr4*	2.4	0.049
Glypican 2 (cerebroglycan)	*Gpc2*	1.9	0.036
Pro-melanin-concentrating hormone	*Pmch*	1.8	0.036
Claudin 5	*Cldn5*	−3.2	0.008
Proteoglycan peptide core protein	*Pgsg*	−2.1	0.016
EGF, latrophilin and seven transmembrane domain containing 1	*Elid1*	−2.1	0.008
Endothelial-specific receptor tyrosine kinase	*Tek*	−2	0.047
von Willebrand factor	*Vwf*	−1.9	0.008
Vitronectin	*Vln*	−1.9	0.010
Tetraspanin 8	*Tspan8*	−1.9	0.049
G protein-coupled receptor 116	*Gpr116*	−1.9	0.033
MAM domain containing 2	*Mamdc2*	−1.8	0.021
SRY-box containing gene 18	*Sox18*	−1.8	0.049
cAMP responsive element binding protein-like 2	*Crebl2*	−1.7	0.049
ATP-binding cassette, sub-family C (CFTR/MRP), member 9	*Abcc9*	−1.7	0.008
Solute carrier family 39 (iron-regulated transporter), member 1	*Slc40a1*	−1.7	0.048
Angiotensin receptor-like I	*Agtrl1*	−1.7	0.049
Mouse genes (PI3), of a total of 43			
Transglutaminase 2, C polypeptide	*Tgm2*	5.4	0.035
NADH dehydrogenase (ubiquinone) I alpha subcomplex, 4-like 2	*Ndufa412*	5.3	0.005
Metallothionein 2	*Mt2*	3.7	0.038
Adrenomedullin	*Adm*	3.5	0.035
EGL nine homolog 3 (*C. elegans*)	*Egln3*	3.5	0.036
Tubulin, beta 6	*Tubb6*	2.8	0.041
Selenium binding protein 1	*Setenbpl*	2.7	0.038
Centrosomal protein 55	*Cep55*	2.5	0.038
Cell division cycle 2 homolog A (*S. pombe*)	*Cdc2a*	2.4	0.044
BCL2/adenovirus EIB interacting protein 1, NIP3	*Bnip3*	2.3	0.003
Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1-like	*Mthfdll*	2.3	0.038
Histocompatibility 2, K1, K region	*H2-K1*	2.2	0.047
Adenylate kinase 3 alpha-like I	*Ak311*	2.2	0.003
Phosphalidylinositol-specific phospholipase C, X domain containing 1	*Plcxdl*	2.1	0.049
Galactokinase 1	*Galk1*	1.9	0.046
Phosphofructokinase, platelet	*Pfkp*	1.9	0.039
Harvey rat sarcoma oncogene, subgroup R	*Rras*	1.8	0.049
EROI-like (*S. cerevisiae*)	*Eroll*	1.8	0.038
Nucleolar and spindle associated protein 1	*Nusap1*	1.8	0.034
EGL nine homolog 1 (*C. elegans*)	*Egln1*	1.8	0.035
Solute carrier family 14 (urea transporter), member 1	*Slcl4a1*	1.7	0.036
Procollagen, type II, alpha 1	*Col2a1*	1.7	0.021
HIG1 domain family, member 1A	*Higdla*	1.7	0.013
Pyruvate dehydrogenase kinase, isoenzyme 1	*Pdkl*	1.7	0.021
Vascular endothelial growth factor A	*Vegfa*	1.7	0.038
ATP-binding cassette, sub-family C (CFTR/MRP), member 9	*Abcc9*	−3.3	0.042
Potassium inwardly-rectifying channel, subfamily J, member 8	*Kenj8*	−3.2	0.034
Solute carrier organic anion transporter family, member Ic1	*Stcolcl*	−2.3	0.044
Aquaporin 4	*Aqp4*	−1.90948	0.038
Ecotropic viral integration site I	*Evil*	−1.70382	0.038

Isolation of Retinal RNA and Assessment of Quality

OIR and control rats were killed at P15 or P20, and OIR and control mice were killed at P13 or P18. Retinas from two eyes of each group were removed, pooled, and immediately frozen in liquid nitrogen. Total RNA was purified (1 mL Trizol reagent; Invitrogen, Carlsbad, CA, and RNeasy Mini kit; Qiagen, Valencia, CA) according to the manufacturers' recommended protocols. All RNA was kept at −80°C until used for microarray hybridization or real-time (rt)PCR. RNA concentration was assessed with a spectrophotometer (NanoDrop 1000; Thermo Scientific, Waltham, MA). All RNA samples were assessed for integrity with a bioanalyzer (Agilent Technologies, Palo Alto CA).

Microarray Hybridization

For quality control, total RNA (1.5 μg, at a concentration of >0.7 μg/μL) was used to prepare labeled probes for microarray analysis with a one-cycle protocol (Affymetrix, Inc., Santa Clara, CA) and hybridized to one of two gene chips (Rat Genome 230 2.0 or Mouse Genome 430 2.0; Affymetrix, Inc.).

Statistical Analysis

After acquisition, each species group was analyzed separately (Genomics Suite ver. 6.4; Partek Inc., St. Louis, MO). All data were RMA (robust multiarray) normalized.[5] Analysis of variance (ANOVA) was performed between each group of interest, accounting for any batch effects caused by different preparation dates. Probesets with a Benjamini-Hochberg step-up false-discovery rate of ≤0.05 were considered significantly different.[6]

Comparisons of gene expression between the following four sets of conditions were loaded into analysis software (Pathway Analysis [IPA]; Ingenuity Systems, Redwood, CA) to classify results

TABLE 2. Genes with Consistent Change in Expression (≥1.7-fold) at the Later Time Point of Two Rodent Models of Experimental Retinal Neovascularization

		Change (*x*-Fold)	Step-up *P*-value
Rat genes (P20), of a total of 26			
Apelin, AGTRLI ligand	*Apln*	3.9	0.016
EGL nine homolog 3 (C. elegans)	*Egln3*	3.0	0.009
Endothelial cell-specific molecule I	*Esm1*	2.8	0.036
Complement component 1, q subcomponent, receptor 1	*Clqrl*	2.8	0.036
Chemokine (C-X-C motif) receptor 4	*Cxcr4*	2.5	0.049
Ceruloplasmin	*Cp*	2.1	0.039
Melanoma cell adhesion molecule	*Mcam*	1.9	0.029
Angiopoietin 2	*Angpt2*	1.8	0.036
Platelet-derived growth factor receptor, alpha polypeptide	*Pdgfra*	1.8	0.049
Interleukin 2 receptor, gamma (severe combined immunodeficiency)	*Il2rg*	1.7	0.023
Potassium voltage-gated channel, Isk-related subfamily, member 3	*Kcne3*	1.7	0.042
Fc receptor, IgE, high affinity I, alpha polypeptide	*Fcerla*	−2.5	0.032
Osteomodulin	*Omd*	−1.8	0.049
Mouse genes (PI8), of a total of 1622			
Endothelin 2	*Edn2*	34.1	0.003
Procollagen C-endopeptidase enhancer protein	*Pcolce*	13.7	0.003
Alpha-2-macroglobulin	*A2m*	10.8	0.002
Lysozyme	*Lyz*	10.7	0.002
NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4-like 2	*Ndufa412*	10.0	0.001
CCAAT/enhancer binding protein (C/EBP), delta	*Cebpd*	9.9	0.003
Adrenomedullin	*Adm*	9.0	0.002
Suppressor of cytokine signaling 3	*Socs3*	8.8	0.003
Metallothionein 2	*Mt2*	6.2	0.003
Insulin-like growth factor binding protein 3	*Igfbp3*	5.8	0.003
EGL nine homolog 3 (*C. elegans*)	*Egln3*	5.3	0.003
Nudix (nucleoside diphosphate linked moiety X)-type motif 6	*Nudt6*	5.1	0.003
H19 fetal liver mRNA	*H19*	4.5	0.002
Tubulin, beta 6	*Tubb6*	4.3	0.003
Oncostatin M receptor	*Osmr*	4.1	0.002
Complement component 1, q subcomponent, alpha polypeptide	*Clqa*	4.1	0.002
Growth arrest and DNA-damage-inducible 45 beta	*Gadd45b*	3.6	0.002
Interferon induced transmembrane protein 3	*Ifitm3*	3.6	0.003
Fibronectin 1	*Fn1*	3.6	0.003
Insulin-like growth factor binding protein 7	*Igfbp7*	3.1	0.003
TYRO protein tyrosine kinase binding protein	*Tyrobp*	3.1	0.002
Peptidylprolyl isomerase C	*Ppic*	3.1	0.003
Jun-B oncogene	*Junb*	3.0	0.003
Beta-2 microglobulin	*B2m*	2.9	0.003
Biglycan	*Bgn*	2.7	0.002
Crystallin, mu	*Crym*	2.7	0.003
Cathepsin C	*Clsc*	2.6	0.002
Signal transducer and activator of transcription 3	*Stat3*	2.6	0.002
Plasminogen activator, tissue	*Plat*	2.5	0.002
Tropomyosin 4	*Tpm4*	2.5	0.003
Lymphocyte antigen 86	*Ly86*	2.4	0.002
Guanine nucleotide binding protein (G protein), gamma 11	*Gngl1*	2.4	0.003
Procollagen, type II, alpha 1	*Col2a1*	2.2	0.002
Procollagen, type V, alpha 3	*Col5a3*	2.1	0.003
Solute carrier family 14 (urea transporter), member 1	*Slcl4a1*	2.0	0.003
Nucleolar and spindle associated protein 1	*Nusapl*	1.9	0.003
Vascular endothelial growth factor A	*Vegfa*	1.9	0.003
SEC22 vesicle trafficking protein-like C (*S. cerevisiae*)	*Sec22c*	1.8	0.002
Transformation related protein 53 inducible protein 11	*Trp53ill*	1.7	0.002
Colony stimulating factor I receptor	*Csflr*	1.7	0.003
Kv channel interacting protein 4	*Kcnip4*	−1.9	0.004
Solute carrier organic anion transporter family, member 1c1	*Slcolc1*	−2.6	0.004
Tachykinin receptor 3	*Tacr3*	−1.7	0.004
Fatty acid binding protein 3, muscle and heart	*Fabp3*	−1.7	0.004
Chemokine (C-X-C motif) ligand 12	*Cxcl12*	−2.1	0.005
Phosphatase and actin regulator 3	*Phactr3*	−1.7	0.005

In the interest of saving space, only the 10% of mouse genes with the lowest step-up *P*-values are included.

as functions or pathways: (1) rat early phase (P15 OIR versus P15 control); (2) rat late phase (P20 OIR versus P20 control); (3) mouse early phase (P13 OIR versus P13 control); and (4) mouse late phase (P18 OIR vs. P18 control). Results from each phase were compared between species, to identify common genes and pathways. In a separate analysis, gene expression at the early and late time points of each OIR model was compared: (1) rat P15 OIR versus P20 OIR; (2) mouse P13 OIR versus mouse P18 OIR.

Confirmation of Changes in Gene Expression by rtPCR

Changes in expression of selected genes (*apln*, *esm1*, and *egln3*) were confirmed by quantitative rtPCR. These three genes code for the proteins apelin, endothelial-specific molecule-1 (endocan), and EGL-nine homolog-3, respectively. These three were chosen because they are common to both species and are thought to have a role in angiogenesis. Gene-specific primers (*Taq*Man; Applied Biosystems, Inc. [ABI], Foster City, CA) were used, and *b-actin* was used a reference gene. Gene expression was measured in OIR and control rat retinas at P15 and P20 and in OIR and control mouse retinas at P13 and P18. The source of the RNA was pooled retinas from the eyes of three animals that had been part of previous experimental runs. The RNA used for rtPCR was different from that used for the microarray analysis. Each quantitation was performed in duplicate from two different pooled samples. Reverse transcription of RNA to complementary (c)DNA was performed (High Capacity cDNA Reverse Transcription Kit; ABI). PCR results were validated by rtPCR (*Taq*Man analysis, Prism 7300 Sequence Detector System; ABI) and gene-specific primers, to produce amplicons of 70 to 100 bp according to the manufacturer's instructions. The relative gene expression levels were calculated by using the comparative Ct (ΔΔCt) method where the relative expression is calculated as $2^{-\Delta\Delta Ct}$ and Ct represents the threshold cycle.[7]

Results

Changes in Gene Expression

For each species, gene expression in OIR and control animals was compared with each other at an early time point and compared with each other at a late time point. This design yielded four comparisons: (1) rat early phase (P15 OIR vs. P15 control); (2) rat late phase (P20 OIR vs. P20 control); (3) mouse early phase (P13 OIR vs. P13 control); and (4) mouse late phase (P18 OIR vs. P18 control). Genes for which *any change in expression* (as little as 1.1-fold up or down) was consistently seen among the multiple experimental runs (corrected $P < 0.05$) were deemed significant. In the interest of space, only genes showing a change of 1.7-fold or more are included in Tables 1 and 2. This arbitrary cutoff of expression change was decided on for the following reasons: Previous microarray studies of retinal gene expression have used cutoffs ranging from 1.5- to 2.0-fold[8–10]; and preliminary review of the rat and mouse data suggested that the expression changes of known mediators of angiogenesis (e.g., vascular endothelial growth factor, angiopoietin, platelet-derived growth factor) were ~1.7-fold.

In P15 OIR rats (Table 1), a significant change in expression was seen in 43 known genes, 21 predicted genes, and 23 ESTs (expressed sequence tags), when compared with that in P15 control rats. Among the known genes, expression increased in 9 (change ranging from +1.1- to +2.5-fold) and decreased in 34 (change ranging from −1.1- to −3.2-fold).

In P13 OIR mice (Table 1), a significant change in expression was seen in 43 known genes and 10 ESTs, when compared with that in the P13 control mice. Among the known genes, expression was increased in 34 (+1.1- to +5.4-fold) and decreased in 9 (−1.1- to −3.3-fold).

In P20 OIR rats (Table 2), significant change in expression was seen in 26 known genes, 4 predicted genes, and 7 ESTs, when compared with P20 control rats. Among the known genes, expression was increased in 20 (+1.3- to +3.9-fold) and decreased in 6 (−1.2- to -2.5-fold).

In P18 mice (Table 2), a significant change in expression was seen in 1622 known genes and 322 transcribed loci or ESTs, when compared with that in P18 control mice. Among the known genes, expression was increased in 933 (+1.1- to 34.1-fold) and decreased in 689 (−1.1- to −2.6-fold). In the interest of space, only the 10% of mouse genes with the lowest step-up *P*-values (≤ 0.003 for upregulated genes and ≤ 0.005 for downregulated genes) are included in Table 2.

All genes showing any statistically significant change in expression (1.1-fold or more) were compared between the two species, to identify common changes. At the early time point (P15 in rat and P13 in mouse), expression of one gene, EGL nine homolog 3 (*egln3*), was increased in both species. Expression of one gene, ATP-binding cassette, subfamily C, member 9 (*abcc9*), was decreased in both species (Table 3).

At the later time point (P20 in rat and P18 in mouse), expression of 13 known genes (*angpt2*, *apln*, *casp1*, *cp*, *cxcr4*, *egln3*, *esm1*, *il2rg*, *mcam*, *pfkp*, *pdgfra*, *col2a1*, and *unc5b*) was significantly increased in both species. No gene showed consistently decreased expression in both species.

When gene expression was compared within each OIR model, 163 genes showed some magnitude of significant change in the rat and the mouse (98 were increased at the later time point and 65 were decreased). Twenty-nine genes showed a change of at least 1.7-fold in both models, with 24 increasing and 5 decreasing (Table 4).

Pathway Analysis

Genes showing significant changes in expression of any magnitude at each of the four conditions were further analyzed (Pathway Analysis software; Ingenuity). From the early time points, 12 distinct pathways were identified in the rat and 15 distinct pathways the mouse (Table 5). Two pathways were common to both species: ephrin receptor signaling and VEGF signaling. From the late time points, 5 distinct pathways were identified in the rat and 48 in the mouse. Four pathways were common to both species: amyotrophic lateral sclerosis (ALS) signaling, axonal guidance signaling, ephrin receptor signaling, and hepatic fibrosis/hepatic stellate cell activation.

Pathway analysis of all genes showing significant change during the course of OIR identified 27 common pathways with a $P < 0.05$: 14-3-3 signaling, androgen signaling, axonal guidance signaling, CCR3 signaling in eosinophils, chemokine signaling, CREB signaling in neurons, CXCR4 signaling, ephrin receptor signaling, ERK/MAPK signaling, fMLP signaling in neutrophils, germ cell-Sertoli cell junction signaling, glioma signaling, glutamate receptor signaling, GM-CSF signaling, GNRH signaling, adrenergic signaling, IL-15 signaling, IL-8 signaling, leukocyte extravasation signaling, melatonin signaling, molecular mechanisms of cancer, nicotinate and nicotinamide me-

Table 3. Genes Showing Consistent Change in Expression of Any Magnitude in Both Rodent Models of Retinal Neovascularization

Increased Expression	Decreased Expression
Early	
EGL nine homolog 3 (*C. elegans*)	ATP-binding cassette, subfamily C, member 9
Late	
Angiopoietin 2	None in common
Apelin	
Caspase 1	
Ceruloplasmin	
Chemokine (C-X-C motif) ligand 4	
EGL nine homolog 3 (*C. elegans*)	
Endothelial cell-specific molecule 1	
Interleukin 2 receptor, gamma chain	
Melanoma cell adhesion molecule	
Phosphofructokinase, platelet	
Platelet-derived growth factor receptor, alpha polypeptide	
Procollagen, type II, alpha 1	
Unc-5 homolog B (*C. elegans*)	

TABLE 4. Genes Showing a Consistent Change in Expression (≥1.7-fold) between Early and Late Phases of OIR in Both Rodent Models

Common Genes (Late OIR vs. Early OIR)	Symbol	Change (Mouse *x*-Fold)	Change (Rat *x*-Fold)
Interferon induced transmembrane protein 3	*Ifitm3*	5.5	3.0
Potassium inwardly-rectifying channel, subfamily J, mem 8	*Kcnj8*	4.9	1.7
von Willebrand factor	*Vwf*	4.8	2.0
Growth arrest and DNA-damage-inducible 45 beta	*Gadd45b*	3.2	2.0
Coagulation factor 5 (mapped)	*F5*	3.1	2.4
Regulator of G-protein signaling 5	*Rgs5*	3.0	3.3
Tachykinin 2	*Tac2*	2.9	2.2
Platelet derived growth factor receptor, beta polypeptide	*Pdgfrb*	2.6	1.8
G protein-coupled receptor 116	*Gprl16*	2.5	2.2
Bone morphogenetic protein 2	*Bmp2*	2.4	1.7
Interferon-induced protein 44	*Ifi44*	2.4	1.8
Intercellular adhesion molecule I	*Icam1*	2.2	1.8
Insulin responsive sequence DNA binding protein-1	*Sned1*	2.1	1.8
Tyrosine kinase with immunoglobulin-like and EGF-like domains 1	*Tie1*	2.1	2.3
Claudin 5	*Cldn5*	2.0	3.3
Fibrillin 1	*Fbn1*	2.0	2.0
Clusterin	*Clu*	1.9	2.0
Hexokinase 2	*Hk2*	1.9	2.2
Selenoprotein P, plasma, 1	*Sepp1*	1.9	2.2
Serum/glucocorticoid regulated kinase	*Sgk*	1.9	2.2
Aquaporin 4	*Aqp4*	1.9	3.8
ATPase, Na+/K+ transporting, alpha 2 polypeptide	*Alpla2*	1.8	2.2
ADP-ribosyltransferase 3	*Art3*	1.7	1.8
Guanine nucleotide binding protein (G protein), gamma 11	*Gngl1*	1.7	1.9
Fatty acid binding protein 7, brain	*Fabp7*	−3.7	−4.3
Tubulin, beta 2b	*Tubb2b*	−3	−2
Doublecortin	*Dcx*	−2.5	−1.8
Achaete-scute complex homolog-like 1 (*Drosophila*)	*Ascl1*	−1.9	−2.1
Dihydropyrimidinase-like 3	*Dpysl3*	−1.8	−1.9

tabolism, reelin signaling in neurons, renin-angiotensin signaling, semaphoring signaling in neurons, synaptic long-term potentiation, and tight junction signaling. Of these, 11 pathways were significant to a level of $P < 0.01$ (Table 6).

Confirmation of Changes in Gene Expression by rtPCR

Quantitative rtPCR was performed for three selected genes with expression that was significantly increased at the later time point in both species: *apln*, *esm1*, and *egln3*. For each gene, the increased expression initially suggested by microarray analysis was confirmed by rtPCR (Table 7).

DISCUSSION

The purpose of this in vivo study was to identify specific genes and pathways involved in retinal angiogenesis. We thought that the most rigorous approach to this end would be to use two different animal models with a similar phenotype, to identify common pathogenic mechanisms. Both the rat and mouse

TABLE 5. Biological Pathways Identified in Both Rodent Models of Retinal Neovascularization after Pathway Analysis of Genes with Significant Changes in Expression

Name of Pathway	Genes in Pathway Identified in Microarrays
Early	
Ephrin receptor signaling	*VEGFA,RRAS,CDC2, MAPK1, CXCR4*
Vascular endothelial growth factor (VEGF) Signaling	*VEGFA,RRAS, MAPK1*
Late	
Amyotrophic lateral sclerosis signaling	*NEFL, VEGFC, CYCS, VEGFA, NEFM, NEFH, TP53, HECW1, GPX1, GRIA3, GRIK2, IGFI, GLUL, BIRC4, BIRC3, GRIA4, CASPI, PGF*
Axonal guidance signaling	*CXCR4, VEGFC, NTNGLPXN, ROBO3, PRKARIB, EFNALFZDI, UNC5D, PFN2, SDCBP, PLXNB2, GNAL, RTN4, GNG2, GNAIL, EPHBI, GNAI3, PRKCBI, VEGFA, SEMA6D, ROCK2, PLCBI, CXCL12, SEMA3A, PRKAR2B, PDGFD, RAPIB, EPHA4, ITGBI, BMP4, ADAM12.BMP1, GNB3, ARHGEF7.GNB2, GNG12, ARPC2, GNG13, WIPFI, IGFI, SEMA3B, MICALI, PDGFB, ARPC5, BMP2, PLXNCI, GNGII, NRPI, GNG4, RRAS.DOCKLACTR3, PLXNDI, SEMA3C, RRAS2, GNG5, UNC5B, NTRK2, CDC42.RHA, DPYSL5, FZD2.FZD3, FZD6, GNG3, MYL9, ARP PGF*
Hepatic fibrosis/hepatic stellate cell activation	*TGFB2, VEGFC, PDGFRB, FLTI, COLIAI, ACTA2, COL3AI, FGF2, PDGFRA, FGFI, TGFBI, MYL9, COLIA2, TGFBR2, IGFI, FGFR2, PDGFB, EDN1, VEGFA, TNFRSFIA, A2M, ICAMI, CTGF, FGFRI, IGFBP5, CCL2, EDN RA, FNI, STATI, MYH9, TIMP2, IGFBP3, PGF*
Ephrin receptor signaling	*CXCR4, VEGFC, CDK6, GNB3, PXN, STAT3, GNB2, CDC2, GNGI2, EFNAI, GNG3, FGFI, DAPKI, ARPC2, GNGI3, WIPF1, CDC42, SDCBP, GNAL, MAP2K3, PDGFB, ARPC5, GNG2, GNAII, EPHBI, GNA13, RHOA, VEGFA, ROCK2, GNG11, MAK, GNG4,CXCL12, RRAS, PDGFD, ACTR3, RAPIB, RRAS2, GNG5, EPHA4, ITGBI, MAP4K4, PCTK3, ARPCIB, PGF*

TABLE 6. Biological Pathways Identified from Comparative Analysis of Gene Expression between Early and Late Time Points of OIR in Both Rodent Models ($P < 0.01$)

Name of Pathway	Representative Genes in Pathway Identified in Microarrays
14-3-3-mediated signaling	*MAPKI, PIK3RI, MAPT, HRAS, KRAS, MAP3K, PLCH2, PLCDLPLCD3, JUN, TUBA8, PLCBI, GFAP, GSK3B, YWHAQ, PIK3R2, PRKCA, TUBB3, YWHAG, YWHAE, TNFRSFIA, YWHAB, RRAS, GRB2, TUBB2A, YWHAZ, MAPK8, MAPK6, MAPK9, BAX, PLCI.L2, PIK 3R3, FOS, PLCB4, TUBAIA, FOXOI, PRKCD, PLCG2, YAPLPRKCH, PIK3CD, MAPK7, SNCA, PRKCB*
Axonal guidance signaling	*DPYSL2, RAC2, BMP4, PIK3RI, HRAS, KRAS, NCKI, ADAM8, NCK2, GNBI, EPHBI, PAKI, BAIAP2, ABLIM3, FIGF, PLCBI, GNAI3, WNTSB, GNG12, EFNA2, CFLI, KALRN, RRAS, SEMA5A, LICAM. MYL9, DOCKI, SRGAP3, ARHGEF6, KLK2, GNB2, RTN4, PAK7, PIK3CD, MAPK7, GNG2.WNT1, GNAL, NRPI, FYN, LRRC4C, RGS3, ARPCIB, BDNF, ARHGEF7, PLXNA2, GNAI4, FZDI, ABLIMI, SEMA6C,*
CCR3 signaling in eosinophils	*MAPKI, PIK3RI, GNB5, HRAS, KRAS, LIMK2, ROCK2, MYLK, GNBI, PAKI, GNB3, PLCBI, PPPICA, GNG12, CALMI, CFLLITPR2, RRAS, GNG3, ITPRI, PIK3R3, PRKCD, CFL2, JMID7-PLA2G4B, GNB2, PIK3CD, PAK7, PRKCH, MAPK7, GNG2, OPNISW, CAMK4, MAPK11, GNG11, PIK3R2, GNG4, PRKCA, PAK2, PAK6, GNAI1, MAPK6, CCL11, GNG5, GNA12, PLA2G4A, GNAS, PLCB4, MAPKI4, PAK3, PRKCB*
CREB signaling in neurons	*POLR2F, POLR2D, MAPKI, GRM3, ADCY4, PIK3R1, GNB5, HRAS, KRAS, PLCH2, GRIA4, GNBI, PLCD3, GNB3, GNATI, ADCY5, PLCBI, GNA13, GNGI2, GRIK1, CALM1, ITPR2, RRAS, CREB3, CREBBP, GRIA2, GNG3, ITPRI, PLCL2, PIK3R3, GRM7, POL CG2, PRKCD, GNAOI, GNB2, PIK3CD, PRKCH, GNG2, MAPK7, POLR2I, GNAL, OPNISW, CAMK2G, CAMK4, POLR2J, GNAI4, PLCDI, SHCI, GNGI1.*
CXCR4 Signaling	*MAPK1, ADCY4, PIK3RI, GNB5, HRAS, KRAS, GNBI, ROCK2, PAKI, MLCI, GNB3, GNATI, ADCYS, PLCBI, MYL4, GNA13, GNGI2, RND2, ITPR2, RRAS, RHOJ, ITPRI, GNG3, MYL9, PIK3R3, DOCKI, PRKCD, GNAOI, GNB2, PIK3CD, PRKCH, PAK7, GNG2, MAPK7, G NAL, FNBPI, OPNISW, GNAI4, GNGII, JUN, RHOTI, GNAT2, PIK3R2, GNG4, PRKCA, PXN, PAK2, PAK6, RHOC, EGRI, GNAII, MAPK8. MAPK6, GNAQMAPK9, GNG5, GNAI2MYL9, GNAS, FOS, RHOV, PLCB4, PAK3, CXCL12, LYN,*
Ephrin receptor signaling	*RAC2, MAPKI, EPHB2, GNB5, HRAS, LIMK2, KRAS, NCKI, GNBI, ROCK2, NCK2, PAKI, EPHBI, GNB3, GNATI, FIGF, GNAI3, EFNB3, GNG12, EPHA7, EFNA2, KALRN, CFLI, RRAS, CREB3, GNG3, STAT3, RAC3, PDGFB, SDCBP, CFL2, GNAOI, GNB2, P AK7, GNG2, MAPK7, GNAL, FYN, RGS3, ARPCIB, GNAI4, JAK2, PGF, EFNB2, SHCLGNG11, ACTR3, WASL, SORBSI, GNAT2.GNG4, ITGBI, GRIN2B, PXN, PAK2, PAK6, GRB2, GNA11, MAPK6.*
Adrenergic signaling	*CAMK4, MAPK1, ADCY4, GNB5, HRAS, KRAS, GNBI, GYSI, GNB3, GNG11, ADCY5, HLA-B, GNG4, GNG12, PRKCA, CALMI, PRAS, ITPR2, GNAQ, MAPK6, SLC8A3, GNA11, GNG3, ITPRI, GNG5, GNAI2, ADRA2A, GNAS, PYGM, PRKAR2B, PRKCD, PLCG2, GNB2, PRKCH, MAPK7, GNG2, ADCY7, OPNISW, PRKCB*
Melatonin signaling	*CAMK4, MAPK1, PLCH2, PLCDI, PLCD3, RORA, PLCBI, CALM1, CAMK2B, PRKCA, GNA11, MAPK6, GNAQ. RORC, PLCL2, GNA12, PLCB4, PRKAR2B, PLCG2, PRKCD, GNAOI, PRKCH, MAP2K3, MAPK7, RORB, OPNISW, MAP2K5, PRKCB, CAMK2G*
Reelin signaling in neurons	*PAFAHIB2, FYN, APOE, PIK3RI, MAPT, DABI, APP, CDK5RI, YESI, CDK5, HCK, ARHGEF2, GSK3B, ARHGEF3, PIK3R2, ITGBI, ARHGEFI2, CNRI, MAPK8IP2, MAPK8, ITGA6, MAPK9, MAPK8IPI, ITGB3, PIK3R3, APBBI, ITGB2, ARHGEF5, ARHGEF10 , ARHGEF6, LYN, ITGAI, LRP8, PIK3CD, PAFAHIBI, DCX, PAFAHIB3*
Synaptic long-tern depression	*MAPKI, PPP2CA, GRM3, ADCY4, HRAS, KRAS, PRKG2, PLA2G2C, GNATI, LCAT, ADCY5, PPMIJ, RYR3, PLCBI, GNA13, GUCYIA3, ITPR2, RRAS, GRIA2, YWHAZ, C7ORF16, ITPRI, GRM7, PPP2R3A, PRKCD, GNAOI, PRKCH, MAPK7, GNAL, PPP2R2A GNA14, PRDX6, GRIDI, IGFI, GNAT2, IGFIR, PPP2R2C, PRKCA, PPP2R5C, PPP2R5D, GNAQ, GNAII, MAPK6, PPP2R5A, LAMB2, GNAI2, GRN, PLA2G4A, GNAS, PLCB4, LYN, PPP2RSE.*
Tight junction signaling	*TGFBRI, PPP2CA, JAM2, MLLT4, CLDN7, PARD6A, OCLN, TGFBR2, MYLK, MPDZ, TGFBI, PPMLI, CGN, MYL4, ACTAI, CSTFLT1, TIAMI, TJP2, MYL9, CLDN23, PPP2R3A, CDK4, PRKCH, CPSF2, RELA, PPP2R2A, PVRL3, CTNNA1, NFKBI, JUN, CEBPA, TGF B2, PPP2R2C, ARHGEF2, STX4, VCL, TNFRSFIB, ACTCI, PPP2R5C, TJPI, TNFRSFIA, PPP2R5D, CASK, ACTGI, PPP2R5A, FIIR, EPB41, FOS, PRKAR2B, CLDN5, CLDNI NUDT21.*

models of retinal neovascularization (OIR) have been validated as experimental systems for studies of pathophysiology as well as preclinical drug development. In both models, fluctuations in oxygen lead to a phenotypically similar, reproducible progression from retinal avascularity to preretinal neovascularization. This well-defined progression represents the presumed early angiogenic response to ischemia and later vasoproliferative phases of angiogenesis, seen clinically in disorders such as retinopathy of prematurity and diabetic retinopathy. In our experimental design, therefore, we also chose two distinct time points, corresponding to these two possibly distinct processes.

Data sets from both models were subjected to identical statistical analysis and compared, to identify common genes and pathways (Tables 1–6). At the early time point, two pathways (ephrin receptor signaling and VEGF signaling) were identified in common. At the later time point, four pathways (ALS signaling, axonal guidance signaling, hepatic fibrosis/stel-

TABLE 7. Changes in Expression of Selected Genes by rtPCR

	OIR vs. RA	
Gene Tested	**Early**	**Late**
esm1		
Mouse	10.96 ± 2.21*	34.04 ± 12.8*
Rat	1.22 ± 0.02	6.67 ± 0.80*
apln		
Mouse	2.41 ± 0.43*	10.32 ± 2.51*
Rat	1.71 ± 0.22	3.57 ± 0.26*
egln3		
Mouse	3.16 ± 0.30*	4.95 ± 0.69*
Rat	2.92 ± 0.42*	3.43 ± 0.35*

Data are expressed as *x*-fold change ± SD.
* $P < 0.01$, by Mann-Whitney U test.

late cell activation, and ephrin receptor signaling) were identified in common. The identification of the VEGF signaling pathway, probably the most studied of all angiogenic pathways, serves as an internal control and lends support to the validity of our approach. Analysis of gene changes during the course of OIR (Tables 4, 6) provide insight into an angiogenic switch and may offer targets of early intervention to prevent neovascularization. Just as careful investigation and pharmacologic targeting of the VEGF pathway have provided novel treatments, it is conceivable that further investigation of other pathways identified herein may yield new therapeutic options.

Of interest, few common genes and pathways were identified from the earlier time point (corresponding to the early angiogenic response to ischemia). This discrepancy is not surprising, given the physiologic differences at this early time point between the two models. These differences include, but are not limited to, differences in oxygen tension (Pao_2 of ~500 mm Hg in the mouse versus ~200 mm Hg in the rat), differences in the primary vascular net (virtually complete in the mouse versus incipient in the rat), differences in oxygen delivery (sustained hyperoxia in the mouse, compared with regular cycling in the rat), as well as possible species-specific reactive mechanisms. The later neovascular response, by contrast, appears to invoke more common pathways.

We identified genes and pathways already targeted by biologics in clinical trials, genes and pathways known to be involved in retinal angiogenesis, and genes with biological plausibility. For example, monoclonal antibodies and aptamers directed against various aspects of VEGF signaling are commonly used in clinical practice or in various phases of clinical trials for the treatment of proliferative retinopathies and neovascular age-related macular degeneration. Inhibition of platelet-derived growth factor signaling has been shown to reduce pathologic retinal neovascularization[11] and is currently under clinical study for the treatment of neovascular age-related macular degeneration (clinicaltrials.gov number NCT00569140). Ephrin signaling and angiopoietin 2 have been implicated in both normal retinal vasculogenesis[12,13] and pathologic neovascularization.[14,15] Genes with biological plausibility include (1) *egln3*, which codes for an intracellular prolyl hydroxylase involved in the cellular response to hypoxia by regulation of the transcription factor HIF-α[16]; (2) *apln*, which codes for apelin, a cytokine known to be necessary for cardiovascular development and mitogenic for retinal endothelial cells[17,18]; and (3) *esm1*, which codes for endocan, a proteoglycan associated with vascular endothelial growth and tumorigenesis.[19,20]

A potential limitation of this study, as with any microarray analysis, is the choice of approach for data interpretation and statistical analysis. Approaches differ by methods of data normalization, thresholds for significance of change in gene expression, or level of statistical significance. A single approach is unlikely to be optimal for all experimental systems. In this study, we sought to emphasize consistency of change (across three independent replicates), by testing all probesets with ANOVA, rather than simply selecting for a high degree of change in expression. Even with this approach, it is possible that we excluded genes (such as transcription factors) that may exert profound downstream effects with little change in expression or genes with a rapid turnover that precludes adequate detection.

It is conceivable that many of the identified genes, especially those identified at the early time point, are not specific to angiogenesis, but represent instead a nonspecific stress response. In addition, since both models rely on extreme manipulations of oxygen for their phenotype, there may be a bias toward oxygen-sensitive genes or oxygen-related mechanisms of neovascularization. To determine whether there is such a bias, a similar experimental paradigm could be applied with other models of neovascularization,[21,22] to delineate oxygen-specific mechanisms and concerted angiogenic pathways.

In summary, we present detailed data designed to serve as a resource for the further study of normal retinal development, angiogenesis, and therapeutics. Comparative gene analyses using two distinct animal models have identified plausible angiogenic pathways, as well as novel aspects of known angiogenic pathways. It is hoped that this work will help guide future investigations into basic mechanisms of retinal angiogenesis, as well as identification of rational therapeutic targets.

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