Published in final edited form as:

Doc Ophthalmol. 2013 August ; 127(1): 13–31. doi:10.1007/s10633-013-9396-8.

Next Generation Sequencing Analysis of Gene Regulation in the Rat Model of Retinopathy of Prematurity

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Abstract

Purpose—To identify the genes, biochemical signaling pathways and biological themes involved in the pathogenesis of retinopathy of prematurity (ROP).

Methods—Next-generation sequencing (NGS) was performed on the RNA transcriptome of rats with the Penn *et al.* (1994) oxygen-induced retinopathy (OIR) model of ROP at the height of vascular abnormality, postnatal day (P) 19, and normalized to age-matched, room-air-reared littermate controls. Eight custom developed pathways with potential relevance to known ROP sequelae were evaluated for significant regulation in ROP: The three major Wnt signaling pathways, canonical, planar cell polarity (PCP), and Wnt/Ca²⁺, two signaling pathways mediated by the Rho GTPases RhoA and Cdc42, which are respectively thought to intersect with canonical and noncanonical Wnt signaling, nitric oxide signaling pathways mediated by two nitric oxide synthase (NOS) enzymes, neuronal (nNOS) and endothelial (eNOS), and the retinoic acid (RA) signaling pathway. Regulation of other biological pathways and themes were detected by gene ontology using the Kyoto Encyclopedia of Genes and Genomes (KEGG) and the NIH's Database for Annotation, Visualization and Integrated Discovery (DAVID)'s GO terms databases.

Results—Canonical Wnt signaling was found to be regulated, but the non-canonical PCP and Wnt/Ca²⁺ pathways were not. Nitric oxide (NO) signaling, as measured by the activation of nNOS eNOS, was also regulated, as was RA signaling. Biological themes related to protein translation (ribosomes), neural signaling, inflammation and immunity, cell cycle and cell death, were (among others) highly regulated in ROP rats.

Conclusions—These several genes and pathways identified by NGS might provide novel targets for intervention in ROP.

Introduction

Complex interactions occur between the neural retina and its vascular supply in normal retinal development and in retinal disease [1]. Retinopathy of prematurity (ROP) occurs when the prematurely born infant is exposed to supplemental oxygen [2-7] during ages at which the retina [8-16] and its vascular supply [17] are both immature and the eye is growing rapidly [18-21]. Although a critical role for the neural retina in the pathogenesis of

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ROP has become increasingly recognized [22, 23], the clinical hallmark of active ROP continues to be abnormal retinal vasculature: tortuous arterioles, dilated venules, and vitreoretinal neovascularization (NV) [24]. Nevertheless, lifelong retinal dysfunction is found even if the ROP was so mild that the vascular disease resolved spontaneously [25-29]. Furthermore, the most common clinical sequela of ROP is altered growth of the eye and its refractive components [18-21, 30-36]. The mechanisms underpinning the altered eye growth in ROP remain poorly understood.

It follows that, to date, most of the ROP research, in patients and in animal models, has been focused on the vascular abnormalities. The critical pro-angiogenesis messenger vascular endothelial growth factor (VEGF) has been targeted in severe, active ROP, with promising therapeutic success [37]. Recently, alternative pathways that impact both vascular and neural tissues rather directly, such as the Wnt signaling pathway, have also been implicated in ROP [38]. The continued discovery of new pathways important to ROP pathology suggests that there are still many more. Because it is so complex, a modern approach is needed to consider the biological factors that lead to the distinct pathology of the ROP eye. Next-generation sequencing (NGS) assesses the relative expression of every gene in an organism's genome and can, therefore, identify a large number of potential targets for intervention. NGS is an hypothesis generator.

We used NGS to evaluate the transcriptome (mRNA) of the Penn *et al.* "50/10" oxygen-induced retinopathy (OIR) rat model of ROP [39] at the height of vasculopathy (postnatal day 19). Thus, we determined all genes that were regulated in the eye afflicted by OIR at this cross-section in time. Our data supplement those already obtained by microarray [40]. In addition to ascertaining individually regulated genes, we conducted gene analysis on pathways annotated by the Kyoto Encyclopedia of Genes and Genomes (KEGG) and the NIH's Database for Annotation, Visualization and Integrated Discovery (DAVID)'s GO terms. We also tested eight systems for which published data and our own preliminary data suggest might be intimately involved in the ROP disease process for likely involvement in ROP pathogenesis (Table 1).

First, we looked at canonical Wnt signaling. This pathway is, by definition, activated when a Wnt ligand binds to a frizzled (Frz) receptor on a cell membrane [41]. Disruption in the canonical Wnt pathway results in reduced neovascularization (NV) in the Smith *et al.* [42] mouse model of ROP [43]. In patients with familial exudative vitreo-retinopathy (FEVR), particular genetic mutations lead to failure of activation of the Frz receptor. Infants with severe FEVR (and Norrie Disease, ND) have tortuous, dilated retinal vessels, an avascular peripheral retina, subretinal exudation, and extraretinal NV with consequent vitreoretinal traction, features common in ROP. Indeed, recent data suggest that as many as 10% of severe ROP cases are complicated by Wnt signaling disorders [44, 45]. Interestingly, as in mild ROP, in mild FEVR/ND, there may be significant retinal and visual dysfunction [46].

Wnt signaling coopts the ubiquitous, versatile, and complex effectors of biochemical activity, the Rho family of GTPases [47]. The Rho GTPases belong to the 'rat sarcoma' (Ras) superfamily. They act as molecular switches. The function of Rho GTPases depends upon their various downstream effectors [48]. There is accumulating evidence that misregulated Rho GTPase signaling is involved in wide variety of vascular and neural pathological processes (*e.g.*, cerebral and coronary spasm [49], hypertension [50], arteriosclerosis [51], nerve injury/regeneration [52-54], neuronal degeneration [55], and a whole host of ocular diseases (*e.g.*, retinal gliosis [56], retinal ischemia and reperfusion injury [57], glaucoma [58-62], diabetic retinopathy[63]), including ROP [64]. RhoA's primary downstream effector is Rho-kinase (ROCK) [48, 65, 66]. The lysophosphatidic acid receptor LPA₁ plays a critical role in oxygen-induced retinal ganglion cell degeneration that is likely

mediated by ROCK [67], and the ROCK inhibitor Y-27632 reduces the severity of vascular abnormality in OIR [64]. Cell division cycle 42 (Cdc42) Rho GTPase isoforms interact with other downstream effectors such as p21-activated kinase (PAK). Collectively, Rho GTPase signaling is essential for fundamental cellular functions including cell cycle progression, gene expression, cytoskeleton dynamics regulation, cell adhesion/migration, cell proliferation and (of particular importance to ROP) angiogenesis *and* neurogenesis [68-77]. Wnt signaling primarily interacts with Rho GTPase signaling *via* two alternative pathways activated upon Frz activation: the non-canonical planar cell polarity (PCP) pathway and the Wnt/Ca²⁺ pathway. In the PCP pathway, Dsh complexes with Dsh-associated activator of morphogenesis (Daam) which activates Rho. Additionally, Dsh also recruits the Rho GTPase Rac. Furthermore, Cdc42 is recruited by the other non-canonical pathway, Wnt/Ca²⁺ [78]. Thus, we also looked at the two major non-canonical Wnt pathways (PCP, Wnt/Ca²⁺), and the downstream RhoA and Cdc42 GTPase signaling pathways.

Next, we considered the nitric oxide (NO) signaling pathway, a signaling system in neurovascular tissues including the retina [79-81]. Regulation of NO was recently shown to be effective at preventing neuropathology in the early stages of a mouse model of diabetic retinopathy [82]. The generation of NO is controlled by a family of enzymes, called NO synthase (NOS), which catalyze its production from L-arginine: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). nNOS is the isoform most involved in regulation of neural function, while eNOS is associated with vascular changes, and iNOS is inducible only in pathological conditions [83]. All classes of retinal neurons are likely affected by NO, principally *via* its activation of soluble guanylyl cyclase (sGC) [84]. NO increases the voltage range of exocytosis in photoreceptors [85, 86], reduces light responses in horizontal cells [87, 88], increases the circulating current of bipolar cells [89, 90], uncouples gap junctions between AII amacrine cells and cone bipolars [91], and more. NO also impacts the retinal vasculature. For instance, NO is involved in helping the choroid respond to changes in oxygen tension [92]. Changes in NOS activity are also associated with profound changes to blood vessels in retinal diseases such as diabetic retinopathy and glaucoma [93].

Finally, mindful of the fact that the most common clinical sequela of ROP is refractive error, we also looked at retinoic acid (RA) signaling, a candidate pathway for mediation of myopia; notably, though, expression of the gene encoding the retinoic acid receptor (RARA) is not likely altered in myopia [94], RA levels are higher in the choroid than any other tissue in the body. Increased levels of RA in the choroid are associated with decreased eye growth while, paradoxically, increased RA in the neural retina is associated with increased eye growth [95]. Vitamin A supplementation at preterm ages is beneficial to retinal function in ROP [96]. It is, therefore, plausible that changes in RA production (and possibly its receptors) are important controllers of eye growth and refractive error in ROP. Furthermore, nNOS is associated with rapid swelling of the choroid and changes in the sclera that alter the size and shape of the ametropic chick eye [97-99]. NOS is strongly up-regulated in mammalian form-deprivation myopia, too, although such dramatic changes in the choroid and sclera are not found [100]. NO nevertheless remains another compelling candidate for controlling eye growth and refractive development [95].

In addition to testing these specific hypothesis, we make the gene expression database available (see online supporting materials) for other labs to inspect and test their own hypothesis against.

Methods

Preparation of Genetic Material

The retinae of ten, 19 day old Sprague-Dawley rats were studied. Half had retinopathy induced following the Penn *et al.* “50/10” paradigm [101], as previously described [102-105]. The other half were room-air-reared (RAR) littermates. Briefly, newborn ROP rat pups were exposed to alternating 24 h periods of 50% and 10% oxygen from the day of birth until P14, when they were returned to room air (~21% oxygen). On P19, when neovascularization and arteriolar tortuosity are most severe, the ROP rats and RAR controls were sacrificed with an overdose of sodium pentobarbital and their retinae quickly dissected from the eye. These experiments complied with the ARVO Statement for the Use of Animals in Research.

Total RNA was obtained using an RNA Mini Prep Kit (Qiagen, Valencia CA). The cells of each retina were disrupted and homogenized by pulling samples, immersed in -mercaptoethanol and Buffer RLT, through sequentially higher-gauged needles. The lysate was centrifuged, the supernatant removed and transferred to a clean container, and 70% ethanol added to precipitate the RNA. The sample was then centrifuged on a column to trap the RNA, rinsed with Buffer RW1 and RPE, and then the RNA was eluted using RNase-free water. RNA purity and concentration were determined using a spectrophotometer (NanoDrop 8000, Thermo Scientific, Wilmington, DE). The contribution of each retina to the final analysis was normalized within group (ROP, RAR); volume was scaled by the inverse of concentration. Thus, each rat contributed the same amount of RNA to the respective group analysis.

Next-Generation Sequencing and Analysis

Whole-genome RNA next-generation sequencing (RNA-seq) was performed at the Harvard Medical School Biopolymers Core Facility (Boston, MA) using a HiSeq2000 Sequencer (Illumina, San Diego CA). Prior to sequencing, 4 µg of RNA was isolated from each sample (ROP, RAR) using a Dynabeads mRNA Purification Kit (Life Technologies, Carlsbad CA) and processed using the NEBNext mRNA Sample Prep Reagent Set 1 (New England Biolabs, Ipswich, MA). The samples were next run on a Bioanalyzer 2100 High Sensitivity DNA chip (Agilent, Santa Clara, CA) and then in a SYBR-based qPCR assay to verify the presence of adapters. RNA library samples of ROP and RAR were sequenced with 100 base single-end reads. Reads were mapped to the *rattus norvegicus* reference genome (Baylor 3.4/rn4) using Tophat 2.0.1 software (<http://tophat.cbcb.umd.edu/>). Quantification for gene expression was performed as reads per kilobase of exon per million mapped reads (RPKM) [106]. A summary of the mapped reads is in Supplementary Table 1.

Derivation of Pathways

To evaluate the eight custom pathways (Table 1), diagrams were produced (Figs. 1 to 8). Qiagen pathway maps were used as an initial template for all pathways except the relatively simple RA synthesis pathway, which is outlined by Cvekl and Wang [107]. Additionally, the PCP pathway map was expanded using information compiled by Katoh [108]. The NCBI gene database was used to confirm the existence and name of each gene in the rat. Protein interactions in all pathways were confirmed to be accurate to current understanding using recent literature [109-143].

Isoforms were removed if they were not present in the retina or if their retinal expression was known to be very low. The up- or down-regulation of each gene in each pathway was culled from the NGS database.

Although several papers have employed gene chips to analyze expression in murine OIR models of ROP [144-147], only one (to our knowledge), Recchia *et al.* [40], did so in the 50/10 ROP rat. Thus, in our study, genes were deemed 'regulated' if the fold change (FC) was greater than 1.7, up ($+0.77 \log_2$) or down ($-0.77 \log_2$), for easy comparison to Recchia *et al.*'s gene chip results wherein this was the cutoff used for significance. The proportion of regulated genes in the ROP genome, G_{reg} , was calculated. To determine whether the activation of each pathway (Table 1) was significantly altered, the probability of finding the observed number (or more) of regulated genes was calculated using the inverse cumulative binomial probability distribution. We assumed that G_{reg} represented the expected proportion of regulated genes in the pathway. Since eight pathways were tested, marginal significance was defined as $P < 0.05$, but full significance only if $P < 0.00625$.

After testing our hypotheses about the eight custom pathways, gene ontology analysis for 'significant genes' ($\pm 0.77 \log_2$) was performed using KEGG and NIH DAVID to identify additional regulated biological themes.

Results

Many individual genes were significantly regulated, up and down, in ROP retinæ. Table 2 shows those protein-coding genes that were regulated more than $\pm 6.64 \log_2$ (100-fold) in ROP. The complete results are given in Supplementary Table 2. In supplementary figure 1, histograms of the pre-alignment quality of all bases from all reads in the ROP and RAR samples (*top*) and the qualities of all the reads in ROP and RAR (*bottom*) are shown; quality of a read is defined as the average of qualities of the bases comprising the read.

Literature-Based Pathway Analyses (Table 1)

Canonical Wnt signaling (fig.1) was significantly altered in ROP. Six isoforms of Wnt were significantly up-regulated (Wnt2b, Wnt3, Wnt4, Wnt6, Wnt10a, Wnt10b) and four down-regulated (Wnt7a, Wnt7b, Wnt11, Wnt16). However, the lipid-binding Wnt-inhibitory factor, Wif1, was up-regulated, and may have counteracted some of the increased Wnt activity. In addition, three dickkopf-related protein encoding genes (Dkk1, Dkk2, Dkk4) and associated kremen gene (Kremen1), which together inhibit low-density lipoprotein receptors (LRPs), were also up-regulated. Indeed, Lrp5, which is a coreceptor for Fzd binding of Wnt, was down-regulated. Consistent with overall down-regulation of the Wnt pathway, two isoforms of Fzd (Fzd2, Fzd4) were under-expressed; none were up-regulated. The downstream cytoplasmic phosphoprotein encoding dishevelled-like gene (Dvl3) was subsequently down-regulated.

The non-canonical PCP Wnt signaling pathway (fig. 2) was not significantly regulated, although two of the ligands most notably involved in the activation of this pathway (Wnt4, Wnt5b) were markedly up-regulated. This is one pathway by which Wnt and Rho GTPase activity interact (*via* Daam) [47], and although Daam2 was markedly down-regulated, there was no significant change in Rho expression.

Despite the down-regulation of Fzd2, the frizzled isoform most important in the activation of the Wnt/ Ca^{2+} pathway, and despite significant changes in the expression of several G-proteins, both up and down, there was no significant change in the overall expression of the non-canonical Wnt/ Ca^{2+} pathway (fig. 3).

The Rho GTPase pathways initiated by Rho and Cdc42 are respectively shown in figure 4 and figure 5. As mentioned, Rho was not regulated in ROP. Despite marked regulation in several genes in these pathways (as indicated), neither Rho nor Cdc42 signaling were regulated.

NO activity, indicated by the combined expression of the nNOS (fig. 6) and eNOS (fig. 7) signaling pathways, was marginally significantly regulated ($P=0.029$). Individually, regulation in the nNOS pathway was marginally significant but in the eNOS pathway not quite so (Table 1). Members of the glutamate (NMDA) receptor superfamily (Grin), which complex to form ligand-gated calcium channels that are instrumental to the activation of nNOS (Nos1), were down-regulated in ROP (Grin2a, Grin2b, Grin2c, Grin2d; fig. 6). Nevertheless, despite the putative decrease in Ca^{2+} flux in ROP retinal neurons, Nos1 was not significantly regulated. Of note, the downstream Rho GTPase, Rasd1, was significantly down-regulated. In contrast, despite the overall pathway not being significantly regulated, eNOS (Nos3) itself was significantly over-expressed in ROP. Therefore, the total amount of NO catabolized from L-arginine by NOS was likely higher in ROP than RAR retinæ.

Lastly, RA synthesis (fig. 8) was marginally significantly regulated in ROP. Multiple enzymes in the retinoid cycle were significantly regulated, some down (Adh4, Rdh5, Aldh1a3, Aldh8a1) and one up (Adh1). The gene that encodes the RA receptor (Rara) was not, itself, regulated.

Digital Gene Analyses

The mRNA expression profile was tested against databases of biological themes, including KEGG and DAVID's GO terms. The significance of each theme was calculated following the Benjamini correction for false discovery. Details of the significantly enriched biological themes are given in Tables 3 (KEGG) and 4 (DAVID).

Discussion

This data set, obtained at the height of retinal vascular abnormalities (*i.e.*, tortuosity, neovascularization), should provide insights into those genes and pathways activated in the pathogenesis of ROP. It may complement an earlier data set obtained in the 50/10 ROP rat by gene chip [40]. Interestingly, while there is substantial overlap between that study and this NGS study, there is also substantial divergence. In addition to the digital gene expression analysis against standardized databases, we investigated genes in specific pathways of interest to our and other groups.

For instance, the Wnt signaling pathway has been recently implicated in ROP and we found it to be regulated in our sample. However, the KEGG pathway database does not differentiate between the canonical Wnt pathway and the alternative PCP and Wnt/ Ca^{2+} pathways. Thus, we studied these pathways individually, and found that while, unsurprisingly, a significant proportion of the genes in the canonical pathway were regulated, a less marked proportion of the PCP and Wnt/ Ca^{2+} pathways were activated (Table 1, figs. 1-3). Indeed, while the PCP pathway showed some evidence of regulation ($=0.056$), the Wnt/ Ca^{2+} pathway showed almost no regulation beyond that which would be expected by chance ($P=0.41$).

It is increasingly clear that Wnt signaling coops Rho GTPase signaling. Therefore, we looked at Rho GTPase activity in the Rho pathway, which has several upstream elements that are regulated by Wnt signaling, and in the Cdc42 pathway, which has equivocal association with Wnt signaling. Neither were regulated.

We have found that, early in the pathogenesis of diabetic retinopathy (DR), NO signaling seems to become activated and be associated with retinal dysfunction [82]. Since both DR and ROP are neovascular diseases, we examined whether genes involved in the activation of NOS, the enzyme that generates NO, were regulated. They were (Table 1): probably those in neurons ($P=0.034$), and possibly those in blood vessels ($P=0.073$). Since the retinal blood

vessels and neurons are in close physical proximity and mature together, NO may help synchronize the neurovascular congruence in the retina [148]. While the evidence suggested that the nNOS pathway was down-regulated, nNOS itself (Nos1) was not regulated. Conversely, the eNOS pathway was not regulated; however, eNOS itself (Nos3) was up-regulated. Since NO freely diffuses through most cells, neurons may have received increased NO from blood vessels.

In addition, we recently completed a study of the refractive state of the ROP rat's eye [149]. Relative to the control rat, the ROP rat is a myope. Important in the regulation of refractive development is RA, and therefore we looked at RA signaling. We found that it was significantly regulated in ROP (Table 1). Furthermore, we note that the SIX family of sine oculis homeodomain-containing DNA-binding proteins, in particular Six3 and Six6, are critical mediators of vertebrate eye growth and development [150] and that one of the most down-regulated genes in the ROP rat retina is Six6os1 (Table 2). This 'opposite strand' natural antisense transcript (NAT) to Six6 is an important mediator of Six6's activity. Six6 and Six6os are expressed almost uniquely in the neural retina. The degree of sequence similarity between murine and human of Six6os is quite high [151]. Also, keratocan (Kera), a gene known for its involvement in corneal development [152], was highly overexpressed in ROP retinæ (Table 2). This might contribute to the increased corneal curvature frequently noted in ROP myopia [153].

In our analysis, we defined "marginally significant regulation" of a pathway as having a number of regulated constituent genes that was less than five percent likely to have occurred by chance given the total number of genes that were regulated in ROP. However, we note that statistically significant regulation of a pathway does not imply that the aggregate activity of the pathway is up or down (or even changed at all). For example, the canonical Wnt signaling pathway, the most regulated of our eight literature-based (*i.e.*, hypothesis-driven) pathways, had eleven up-regulated genes and eight down-regulated genes. Further complicating matters, some of the genes that were up-regulated inhibit the activation of other genes in the pathway. Thus, we are tempted to claim that the net effect on Wnt pathway activation is 'down', but really, the result is equivocal. Focusing on the ligands, in comparison to the mouse model of ROP in which Wnt3a, 7a, and 10a are all down-regulated [43], in our rat data, expression of 3a was not altered (although expression of 3 was up-regulated), 7a was significantly down-regulated, and 10a was significantly up-regulated.

The most significantly regulated biological theme in the KEGG digital gene expression analysis was 'Ribosome' (Table 3). Ribosomes are involved in translation of mRNA into proteins. Ribosomes are involved in so many cellular processes that it is difficult to conclude what their role in ROP might be from this data point alone. Rather, these data suggest that a proteomic approach is likely to yield much new and valuable information.

The KEGG 'Neuroactive ligand receptor interaction' theme (Table 3), which contains many G-protein coupled receptors (GPCRs), was also significantly regulated. GPCRs were excluded from our custom pathway analyses because they are so numerous. If these had been included in the custom analyses, the results for the Rho, Cdc42, and eNOS pathways would have been altered. In any event, it is clear that neurotransmitter activity is importantly altered in the ROP rat retina. Certainly, the ERG results in both animal models and patients with ROP indicated that neural retinal dysfunction is a feature of ROP that persists for life.

Cytokines, proteins that act as intercellular messengers, are involved in varied cellular functions including immune processes like inflammation, repair, and maintenance of homeostasis. They are also involved cell growth, differentiation, and death, including in endothelial cells. Several groups of cytokines, distinguished by their structure, exist.

'Cytokine-cytokine receptor interaction' was regulated in the KEGG database as shown in Table 3.

Seventy GO terms were regulated in the NIH's DAVID database, following Benjamini correction for multiple tests (Table 4). These terms encompass a variety of biological systems, but two classes seem particularly pronounced due to their repeated appearance: immunity and cell-cycle. There is a small but increasing body of evidence that suggests inflammatory and immune system pathways are involved in neovascular retinal diseases. In particular, a role for the complement system has been recently reviewed [154]. Thus, immune system activity may represent an emerging target for ROP therapy. With respect to the cell-cycle, there is little literature to suggest significant changes therein are a feature of ROP. However, changes in mitosis events in endothelial cells, regulated by VEGF and perhaps downstream NO signaling, are associated with the increased arteriolar tortuosity in ROP [155].

There are a few limitations of this kind of study. For instance, while it is fortunate that the output of the HiSeq 2000 sequencing system enables rapid profiling and deep investigation of the transcriptome, we did not perform sequencing on technical replicates. Thus, the degree of certainty in the activation values is somewhat low. Furthermore, while we did have biological replicates in the form of pooled samples, they were sequenced ensemble, which is a less powerful (but much less costly) approach than sequencing individuals' RNA. The declining costs of NGS will make such replication more feasible in needed future studies of this variety. And an additional, final note: The regulation of any given biological theme found to be significant herein may indeed be important to the pathogenesis of ROP, but it may also be *compensatory* to the pathology. Thus, while it is tempting to imagine interventions that would normalize one or more of the several regulated biological themes discovered herein, caution should be applied since the eye may *already* be compensating for some other, deleterious biochemical response. In this case, normalizing activation of the signaling pathway could potentially worsen outcomes. Since we assayed only one time point, there is no evidence in our current dataset to distinguish between pathological and compensatory activation of genes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH EY020308 (JDA) and the Massachusetts Lions Eye Research Fund (RMH).

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Figure 1.

Major elements of the canonical Wnt signaling pathway. Genes that encode membrane proteins are in squares, ligands in hexagons, and scaffolding proteins in rhomboids. All other genes are represented by circles. Lines ending in flat heads represent inhibition. Lines ending in arrowheads represent excitation. Arrowheads alone represent proteins that act together, such as coenzymes. Large boxes surround genes that act at the same step in the pathway. The expression of each gene in the ROP rats' retinae, relative to the RAR rats' retinae, is color-coded to the bar in the lower left. Bold borders around a gene indicate that it was significantly regulated ($\pm 0.77 \log_2$).

Figure 2.
Major elements of the noncanonical PCP Wnt signaling pathway. Symbology as in figure 1.

Figure 3. Major elements of the noncanonical Wnt/Ca²⁺ signaling pathway. Symbology as in figure 1.

Figure 4. Major elements of the Rho signaling pathway. Symbology as in figure 1.

Figure 5.
Major elements of the Cdc42 signaling pathway, Symbology as in figure 1.

Figure 6. Major elements of nitric oxide signaling in neurons (nNOS). Symboly as in figure 1; additionally, genes that encode binding proteins are in diamonds.

Figure 7. Major elements of nitric oxide signaling in blood vessels (eNOS). Symbology as in figures 1 and 6.

Figure 8.
Major elements of retinoic acid signaling. Symbology as in figures 1 and 6.

Table 1
Literature-based biochemical signaling pathways

Name	<i>P</i>
Canonical Wnt	2.55E-04
Noncanonical PCP Wnt	0.0563
Noncanonical Wnt/Ca2+	0.412
Rhoa	0.179
Cdc42	0.315
nNOS	0.0337
eNOS	0.0733
Retinoic Acid	0.0368

Table 2
Protein-expressing ROP genes with >100 fold change (FC) relative to RAR

Downregulated Gene	Entrez ID	FC (Log ₂)	Upregulated Gene	Entrez ID	FC (Log ₂)
'Similar to ferritin light chain'	499244	-9.28	Mobp	25037	8.89
RGD1310371	308794	-8.64	Serpib5	116589	8.74
LOC681301	681301	-7.53	RGD1560723	500448	8.62
Six6os1	500673	-7.48	Tpte	364629	8.37
'Similar to ribosomal protein S15a'	691065	-7.24	Tnni2	29389	8.22
'Rho GTPase activating protein 6'	100363276	-7.12	Tnni3	24838	8.17
RGD1560207	501568	-6.83	Mb	59108	7.95
			Myh4	360543	7.85
			Pfkfb1	24638	7.63
			Myh3	24583	7.55
			Krt14	287701	7.28
			Ehfr	295965	7.27
			Olr1619	295843	7.26
			Pkp3	293619	7.25
			Kera	314771	7.22
			Opalin	361757	7.16
			Olig1	60394	7.07
			Iqub	296936	6.89
			'Similar to golgi matrix protein GMI130'	690485	6.88
			Aspa	79251	6.85
			Plscr2	315883	6.82
			Stc2	63878	6.78

Table 3
Significantly Regulated KEGG Terms

Term	rno	P	Benjamini
Ribosome	03010	1.84E-11	3.51E-09
Neuroactive ligand-receptor interaction	04080	2.73E-05	0.00173
Cytokine-cytokine receptor interaction	04060	2.33E-05	0.00222

Table 4
A. Significantly Regulated DAVID Terms 1-35 (of 70)

Term	GO	P	Benjamini
Immune response	0006955	1.08E-09	5.93E-06
M phase	0000279	2.33E-08	4.26E-05
Translational elongation	0006414	1.93E-08	5.28E-05
Response to wounding	0009611	7.80E-07	0.00107
Response to organic substance	0010033	2.41E-06	0.00264
M phase of mitotic cell cycle	0000087	3.39E-06	0.00265
Positive regulation of immune response	0050778	3.91E-06	0.00268
Chemical homeostasis	0048878	3.15E-06	0.00287
Mitosis	0007067	5.47E-06	0.00299
Nuclear division	0000280	5.47E-06	0.00299
Regulation of transcription, DNA-dependent	0006355	5.26E-06	0.00320
Regulation of RNA metabolic process	0051252	6.84E-06	0.00341
Citamine metabolic process	0006766	1.37E-05	0.00534
Ion homeostasis	0050801	1.48E-05	0.00539
Organelle fission	0048285	1.64E-05	0.00561
Cell cycle phase	0022403	1.37E-05	0.00575
Positive regulation of immune system process	0002684	2.04E-05	0.00588
Regulation of immune effector process	0002697	1.30E-05	0.00591
Hemopoietic or lymphoid organ development	0048534	2.03E-05	0.00618
Regulation of lymphocyte mediated immunity	0002706	2.00E-05	0.00644
Positive regulation of adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains	0002824	3.68E-05	0.00957
Positive regulation of adaptive immune response	0002821	3.68E-05	0.00957
Regulation of cell proliferation	0042127	3.57E-05	0.00974
Cell cycle process	0022402	4.38E-05	0.0109
Immune system development	0002520	6.04E-05	0.0143
Regulation of adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains	0002822	7.40E-05	0.0149
Regulation of adaptive immune response	0002819	7.40E-05	0.0149
Regulation of cAMP biosynthetic process	0030817	7.25E-05	0.0152
Inflammatory response	0006954	6.70E-05	0.0152
Leukocyte differentiation	0002521	8.73E-05	0.0153
Homeostatic process	0042592	7.09E-05	0.0154
Regulation of programmed cell death	0043067	8.33E-05	0.0156
Regulation of cell death	0010941	8.05E-05	0.0156
Regulation of leukocyte mediated immunity	0002703	8.66E-05	0.0157
Regulation of cAMP metabolic process	0030814	1.00E-04	0.0170

B. Significantly Regulated DAVID Terms 36-70 (of 70)

Term	GO	P	Benjamini
Positive regulation of lymphocyte mediated immunity	0002708	1.08E-04	0.0173
Positive regulation of leukocyte mediated immunity	0002705	1.08E-04	0.0173
Positive regulation of immune effector process	0002699	1.08E-04	0.0177
Regulation of transcription from RNA polymerase II promoter	0006357	1.16E-04	0.0180
Regulation of cyclic nucleotide biosynthetic process	0030802	1.23E-04	0.0186
Regulation of nucleotide biosynthetic process	0030808	1.23E-04	0.0186
Regulation of apoptosis	0042981	1.27E-04	0.0187
Ion transport	0006811	1.39E-04	0.0188
Cell activation	0001775	1.33E-04	0.0189
Regulation of nucleotide metabolic process	0006140	1.38E-04	0.0192
Cyclic-nucleotide-mediated signaling	0019935	1.56E-04	0.0206
Myeloid cell differentiation	0030099	1.96E-04	0.0242
Multicellular organism reproduction	0032504	1.91E-04	0.0247
Reproductive process in a multicellular organism	0048609	1.91E-04	0.0247
Positive regulation of response to stimulus	0048584	1.96E-04	0.0247
Cellular ion homeostasis	0006873	2.06E-04	0.0248
Regulation of cyclic nucleotide metabolic process	0030799	2.19E-04	0.0257
Positive regulation of developmental process	0051094	2.40E-04	0.0276
Response to drug	0042493	2.48E-04	0.0280
Cellular chemical homeostasis	0055082	2.62E-04	0.0283
Regulation of transcription	0045449	2.57E-04	0.0284
Cation homeostasis	0055080	2.82E-04	0.0287
Monovalent inorganic cation transport	0015672	2.77E-04	0.0288
Regulation of lyase activity	0051339	2.77E-04	0.0293
Regulation of adenylate cyclase activity	0045761	3.26E-04	0.0326
Hemopoiesis	0030097	3.40E-04	0.0333
Regulation of cyclase activity	0031279	3.50E-04	0.0337
Positive regulation of macromolecule metabolic process	0010604	3.77E-04	0.0356
Gamete generation	0007276	3.95E-04	0.0366
Sexual reproduction	0019953	4.59E-04	0.0418
Elevation of cytosolic calcium ion concentration	0007204	4.86E-04	0.0435
Regulation of immunoglobulin mediated immune response	0002889	5.32E-04	0.0467
Regulation of T cell mediated immunity	0002709	5.32E-04	0.0467
Regulation of B cell mediated immunity	0002712	5.32E-04	0.0467
Epidermal cell differentiation	0009913	5.79E-04	0.0499