

# The Mouse Retina as an Angiogenesis Model

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The mouse retina has been used extensively over the past decades to study both physiologic and pathologic angiogenesis. Over time, various mouse retina models have evolved into well-characterized and robust tools for *in vivo* angiogenesis research. This article is a review of the angiogenic development of the mouse retina and a discussion of some of the most widely used vascular disease models. From the multitude of studies performed in the mouse retina, a selection of representative works is discussed in more detail regarding their role in advancing the understanding of both the ocular and general mechanisms of angiogenesis. (*Invest Ophthalmol Vis Sci*. 2010;51:2813–2826) DOI:10.1167/iovs.10-5176

In 2006, intraocular anti-VEGF therapy was ranked among the top 10 science breakthroughs of the year,<sup>1</sup> reflecting the increased attention retinal angiogenesis has received in recent years. Epidemiologic studies provide evidence that this attention is well deserved: Proliferative diabetic retinopathy (PDR) accounts for the highest incidence of acquired blindness in the working-age population<sup>2,3</sup>; choroidal (subretinal) neovascularization in age-related macular degeneration (AMD) represents the leading cause of blindness in people over the age of 65.<sup>4,5</sup>

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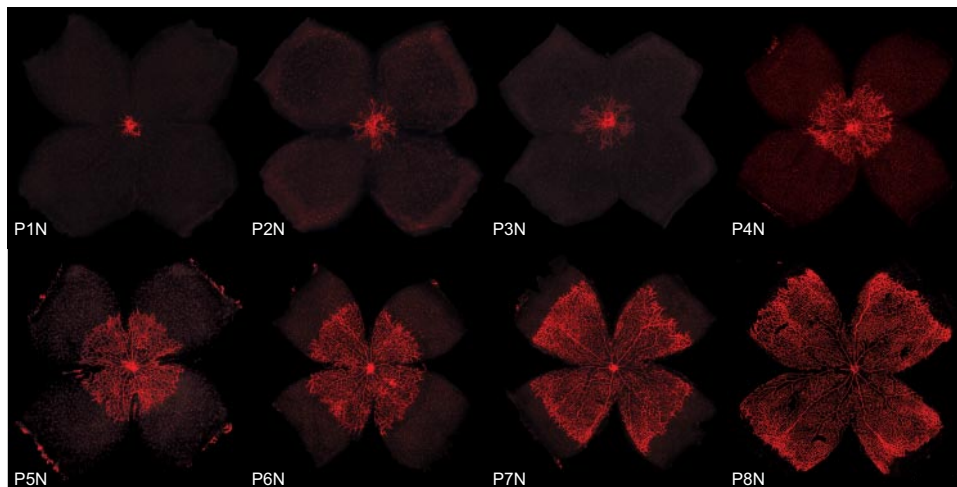
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and retinopathy of prematurity (ROP) is a major cause of acquired blindness in children.<sup>6</sup> In addition, there are many other ocular diseases that have a vascular component, such as Norrie disease (ND), familial exudative vitreoretinopathy (FEVR), Coats' disease, sickle cell retinopathy, retinitis pigmentosa, and many others. Various animal models have been developed in the effort to understand the underlying mechanisms of these sight-threatening conditions. Among the numerous animal species used in these models, the mouse offers a unique combination of affordable cost, well-established platforms for creating systemic or conditional transgenic strains, and access to a wide availability of recombinant proteins and antibodies. In this article, we review mouse models of retinal angiogenesis and provide brief discussions of some of the most relevant lessons learned from these models.

## DEVELOPMENTAL RETINAL ANGIOGENESIS IN HUMANS

Any animal model that is intended to advance the understanding of a human disease state must be interpreted with regard to the clinical picture. In humans, the retina begins to vascularize *in utero* at approximately 16 weeks of gestational age. During the early stages, vascular supply to the eye is partially provided by the hyaloid vasculature originating from the optic nerve and traversing the primitive vitreous toward the anterior segment.<sup>7</sup> To attain a transparent visual axis, the hyaloid vasculature must regress when ocular development proceeds.<sup>8</sup> Hyaloid regression is usually completed before 34 weeks of gestation in humans. By this time, development of the intraretinal vasculature is already well advanced. The nasal retina becomes fully vascularized at approximately 36 weeks of gestational age, and the temporal vessels reach the ora serrata at approximately week 40.<sup>7</sup> A human term infant is thus born with fully developed retinal vessels and with regressed hyaloid vasculature. Several developmental disorders, however, can interfere with normal growth or maturation of the retinal vasculature. One example is Norrie's disease, an inherited eye disease that is associated with persistent hyaloid vessels, incomplete development of the retinal vasculature, and pathologic retinal neovascular proliferation, often resulting in blindness from birth.<sup>9</sup>

Besides developmental disorders, premature birth is the most common condition leading to altered retinal vascular development. Human preterm infants with ROP show delayed maturation of the retinal vasculature that can be associated with pathologic extraretinal vessel formation. In recent years, there has been an increase in the prevalence of ROP, probably as a result of improved survival among the most premature infants.<sup>10</sup> This alarming increase in ROP represents a challenge for clinicians who care for these infants and a challenge for researchers to further intensify their efforts to understand the mechanisms behind ROP. Clinically, ROP is characterized by



**FIGURE 1.** Development of the superficial vascular plexus in C57Bl/6 mouse retinas. Retinal whole mounts from postnatal day (P)1 to P8 were stained for endothelial cells with isolectin B<sub>4</sub>-Alexa 594 (red). N (normoxia) signifies normal development, as opposed to the hyperoxia time course shown in Figures 4 and 5. At P1N, the mouse retina is almost completely devoid of blood vessels. The superficial vascular plexus can be seen originating from the optic nerve head. During the first week of postnatal development, the superficial plexus extends radially from the optic nerve head into the surrounding tissue, reaching the retinal periphery at ~P8N.

two stages: an early phase of vascular injury with obliteration of immature vessels and a second phase of vascular repair. In most premature infants, the retina successfully vascularizes during this second phase of repair. However, in some (usually the most immature ones with the least vascularization of the retina at birth), there is pathologic NV formation with angiogenic sprouting of abnormal vessels from the retina into the vitreous. This NV sprouting leads to complications such as plasma leakage, bleeding, and, in some cases, tractional retinal detachment and blindness.<sup>11–14</sup> The severity of pathologic NV formation is dictated to a large extent by the amount of avascular retina present in an individual infant.<sup>15</sup> Consequently, in infants with only limited nonvascularized retina, the sequence of events from initial vascular injury to the deleterious consequences of NV formation does not develop to its full extent<sup>16</sup> and the pathologic NVs either never fully form or regress over time.<sup>17</sup>

Even if pathologic neovascularization develops, the initially damaged intraretinal vasculature often resumes physiologic growth during the second phase of ROP, and vascularization of the avascular areas with functional vessels may ensue.<sup>18</sup> Ultimately, the final functional outcome of ROP in eyes is largely dependent on which of the two processes, pathologic NV formation or physiologic vascularization, prevails. However, it is important to note that even in cases of mild ROP, in which no or only minimal overt NV formation occurs, physiologic vascularization of the retina is significantly delayed compared with that in term-born infants. Although retinal vascularization is complete by 40 weeks' gestational age in term infants, complete retinal vascularization in infants with ROP can be delayed until 48 to 52 weeks of gestational age.<sup>7</sup> In this context, the same growth factors deemed responsible for pathologic NV formation are likely to be crucial for physiologic vascularization of the retina, albeit with different timing and at different levels.<sup>19–24</sup>

## DEVELOPMENTAL RETINAL ANGIOGENESIS IN MICE

In contrast to humans, mouse (and other rodent) pups have an immature retinal vasculature and persistent hyaloid vessels at birth.<sup>25</sup> The highly organized pruning of hyaloid vessels in the postnatal mouse eye has been a very useful tool in the study of mechanisms of physiologic vessel regression. In 2005, seminal work from the Lang laboratory<sup>26</sup> identified WNT7b as a short-range paracrine signal for vessel regression using the mouse hyaloid system, and important work from Liu and Nathans<sup>27</sup> identified an essential role for the putative WNT-receptor frizzled 5 in hyaloid regression. Of interest, two human conditions

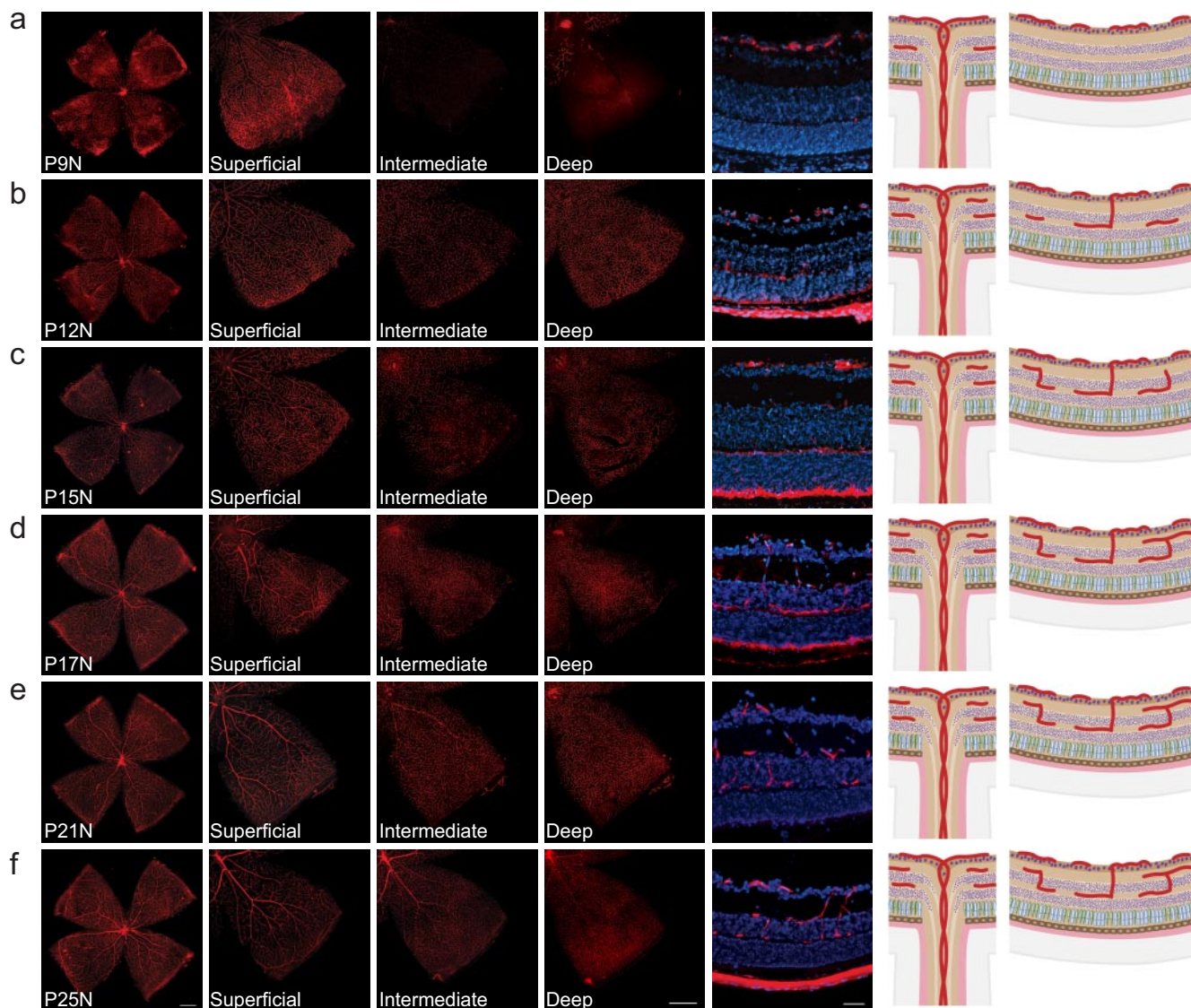
with disturbed retinal development, FEVR and ND, have both been linked to defects in the Wnt signaling cascade.<sup>9,28–34</sup>

Parallel to hyaloid regression, development of the intraretinal vasculature in mice occurs postnatally in a tightly regulated temporal and spatial pattern that can readily be observed and manipulated experimentally.<sup>35</sup> Most of the data obtained to date were collected from different wild-type strains, among which C57Bl/6 is the most widely used. It is important to note that the time course of normal vascular development in wild-type mice varies considerably among the strains. Normal retinal vascular development in BALB/cByJ mice, for example, is delayed by a few days compared with that in C57Bl/6 mice.<sup>36</sup> The time course illustrated in Figures 1 and 2 therefore applies only to retinal vascular development in C57Bl/6 mice: In this strain, the superficial vascular plexus forms during the first week after birth by radial outgrowth of vessels from the optic nerve into the periphery, reaching the retinal edges at approximately P8 (Fig. 1). From P7 onward the superficial capillaries start sprouting vertically to form first the deep and then the intermediate vascular plexus (Fig. 2 and Movie S1, <http://www.iovs.org/cgi/content/full/51/6/2813/DC1>). The deep plexus, located in the outer plexiform layer, forms rapidly and reaches the retinal periphery at approximately P12 (Fig. 2b), followed by the intermediate plexus in the inner plexiform layer between P12 and P15 (Figs. 2b, 2c). By the end of the third postnatal week, all three vascular layers are fully mature with multiple interconnecting vessels between layers (Figs. 2e, 2f).

## THE MOUSE RETINA AS A MODEL FOR PHYSIOLOGIC ANGIOGENESIS

The cellular and molecular mechanisms underlying the developmental time course just described have been studied extensively.<sup>37–41</sup> One of the major advantages of studying postnatal retinal development in mice lies in the ease of accessibility of the developing vasculature for imaging and intervention without the difficulties associated with investigating embryonic development.<sup>36,37,42,43</sup> In pioneering work from the Keshet laboratory,<sup>22</sup> for example, the developing mouse retina was used to meticulously analyze VEGF expression during developmental angiogenesis. The Carmeliet and D'Amore groups<sup>44</sup> further investigated the specific roles of different VEGF isoforms during retinal vascular development and Gerhardt et al.<sup>45</sup> investigated how endothelial cells respond to VEGF gradients by tip cell formation and guided migration in the developing mouse retina. More recently, the important role of the notch signaling pathway in vascular development has been extensively studied in the mouse retina model.<sup>46–48</sup>





**FIGURE 2.** Development of the deep and intermediate vascular plexus in C57Bl/6 mouse retinas. Retinal whole mounts and cross sections from postnatal day (P)9 to P25 were stained for endothelial cells with isolectin B<sub>4</sub>-Alexa 594 (red) and for cell nuclei with DAPI (blue). N (normoxia) signifies normal development, as opposed to the hyperoxia time course shown in Figures 4 and 5. (a) At P9N, the superficial plexus has fully extended to the peripheral retina. The deep vascular plexus begins forming centrally from vertical vessels diving down from the superficial plexus. The intermediate vascular plexus has not begun to form yet. On cross section, the three neuronal layers of the retina and the superficial vascular plexus can be identified. Representative drawings on the right illustrate both superficial and deep vascular plexus in the immediate vicinity of the optic nerve, whereas in the periphery the superficial plexus remains the only vascular network. (b) At P12N, the intermediate vascular plexus becomes visible on retinal whole mounts. The deep vascular plexus is nearly fully developed. On cross section, vertical sprouting of vessels toward the intermediate plexus can be observed. Representative drawings illustrate the beginning of a three-layered vascular plexus around the optic nerve and superficial and deep vascular plexus with interconnecting vessels in the periphery. (c) At P15N, the intermediate vascular plexus continues to develop throughout the retina as illustrated both on retinal whole mounts and cross sections. Representative drawings illustrate complete formation of superficial and deep plexus with continued development of the intermediate vascular plexus in the periphery. (d) At P17N, the superficial, intermediate, and deep plexus can be seen both centrally and in the peripheral retina. Cross sections and representative drawings illustrate the three-layered vascular system in all parts of the retina along with multiple interconnecting vessels. (e, f) Between P21N and P25N, further maturation of especially the intermediate plexus can be observed. Cross sections and representative drawings illustrate the mature retinal vasculature. Note that isolectin B<sub>4</sub> binds to choroidal vessels as well as nonspecifically to RPE and the scleral wall in some of the cross sections (a-f).

As postnatal vascular development in the retina proceeds in a very tightly regulated and organized pattern, it also allows for reliable detection of any developmental abnormality, both in transgenic animals and pharmacologic studies.<sup>41</sup> A very useful tool in this respect is an analysis of global gene expression during normal retinal development published by the Friedlander group.<sup>49</sup> The same group has also used the developing mouse retina to investigate the role of

bone-marrow-derived stem cells in angiogenic development, which has significantly advanced our understanding of how bone-marrow-derived cells contribute to retinal vessel formation and how endothelial progenitor cells (EPCs) can attenuate vessel regression in the rd/rd mouse model of retinal degeneration.<sup>50-52</sup>

As organized vascularization is essential not only for physiologic development of the retina but also for the normal de-

velopment of most other tissues, many observations made in the developing mouse retina also apply to developmental angiogenesis in other organs. In this respect, the eye has been used by many researchers to decipher the general angiogenic mechanisms related to developmental angiogenesis or tumor vascularization.<sup>53,54</sup>

## ANIMAL MODELS OF ROP

Besides modeling normal development, the mouse retina can be used as a tool to investigate conditions in which normal vascular development becomes unbalanced, leading to sight-threatening diseases, as occurs in human ROP. Successful modeling of human ROP in animals necessitates replication of most of the hallmarks of the human disease. The animal model should accurately reproduce the two phases of ROP—initial vaso-obliteration (VO), and subsequent neovascularization (NV)—along with some of the more frequent complications like vascular leakage. An adequate model system should furthermore display revascularization of the avascular area with functional intraretinal vasculature and regression of pathologic neovascularization during the later disease stages. Several animal models have been developed that fulfill these criteria. All these models use exposure of neonatal animals to high oxygen tension to induce loss of immature retinal vasculature.<sup>55</sup> Because of the causal role of oxygen exposure and considering that the animals used in these models were not born prematurely, oxygen-induced retinopathy (OIR) is a more accurate term than ROP for describing these models. However, because term neonates in these species are born with an immature retinal vasculature, they replicate the cardinal aspects of human ROP and the two characteristic phases of ROP: vaso-obliteration followed by neovascularization. These two phases have been reported in feline,<sup>56–58</sup> canine,<sup>59</sup> rat,<sup>60</sup> and mouse models of OIR.<sup>61</sup> The mouse model of OIR will be described in more detail, along with a review of some of its most relevant contributions to *in vivo* angiogenesis research.

## THE MOUSE MODEL OF OIR

One of the most widely used animal models of ROP is the mouse model of OIR,<sup>61</sup> with more than 15,000 publications since it was first published in 1994 (Google Scholar search on keywords “oxygen induced retinopathy mice,” June 15, 2009). Using the mouse as a model animal enables access to a wide range of genetically manipulated animals, recombinant proteins, and antibodies. In addition, animal costs and procedure times in the mouse model compare favorably to those of other animal species, and the mouse retina very reliably develops pathologic vaso-obliteration and neovascularization. However, certain caveats should be kept in mind when using the OIR mouse model: Different severities of OIR have been found to develop in different wild-type strains.<sup>62,63</sup> Similarly, OIR severity can vary within one wild-type strain because of vendor-related substrain differences. This variability has also been observed in the rat OIR model.<sup>64</sup> From our experience with the OIR mouse model, C57Bl/6 wild-type mice from Jackson Laboratories (Bar Harbor, ME) display slightly higher retinal NV than do their C57Bl/6 counterparts from Taconic Farms (Germantown, NY). The reason for these differences is most likely the many generations of inbreeding at an individual vendor's facility during which spontaneous mutations can occur that persist in the colony undetected and translate into measurable phenotypic differences in the OIR model. On the basis of this possibility, it can be speculated that even animals from the same vendor may vary, depending, for example, on whether

the animals were bought from the same vendor's Asian, European, or American breeding facility. The differences in wild-type strains, however, can easily be controlled by using the same strain and vendor source for all animals throughout an entire experimental protocol in both treatment and control groups. For studies involving transgenic mice, however, it becomes crucial to obtain the correct wild-type control animal, based on the genetic background of each transgenic strain investigated.

Another variable that should be carefully controlled in the OIR mouse model is postnatal weight gain during the OIR period. Clinical observations from ROP infants have identified postnatal weight gain as a strong predictor of the severity of ROP.<sup>65–71</sup> The same appears to be true in the OIR mouse model.<sup>72</sup> Therefore, weights should be measured both at P7 and P17, and all experimental groups in the OIR mouse model should be adjusted for equal body weights. Ideally, weight recordings should be reported in publications. In addition, to control for other confounding variables, sufficient animal numbers per experimental group are essential. To account for possible litter-specific differences, at least 10 eyes from three different litters should be collected per experimental group (based on power analysis with  $\alpha = 0.05$ ,  $\beta = 0.2$ , treatment effect = 25%, and SD = 23%).

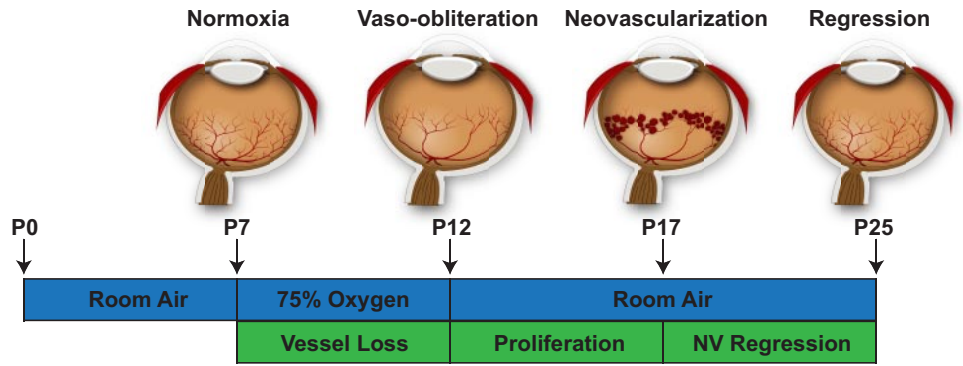
## Using the OIR Mouse Model

When these variables are controlled, the OIR mouse model<sup>61</sup> produces robust and reliable data in a straightforward and time-efficient experimental setting. It also reproduces the defining disease characteristics observed during the development of human ROP. An extensive step-by-step protocol for the OIR mouse model has recently been published.<sup>73</sup> In brief, neonatal mice are exposed to 75% oxygen from postnatal day (P)7 until P12 and returned to room air (21% oxygen) from P12 to P17 (Fig. 3). During the first phase of hyperoxic exposure (P7–P12), retinal vessels constrict to regulate retinal  $Po_2$  levels<sup>74</sup> and immature capillaries in the central retina regress leading to a central zone of vaso-obliteration (Figs. 4a–c). As hyperoxia exposure overlaps with the phase of physiologic vertical sprouting of vessels from the superficial layer into the deep and intermediate plexus, a marked delay in the formation of deeper retinal vasculature can also be observed. With regard to the central VO area, it is important to note that the oxygen-induced vaso-obliteration is rapid, with peak VO being reached 48 hours after onset of oxygen exposure.<sup>75</sup> Although still exposed to oxygen, revascularization of the VO zone begins from P9 to P12,<sup>75,76</sup> likely reflecting increasing oxygen demands of the developing retina.<sup>77</sup> On return to room air at P12, the VO area becomes hypoxic<sup>78</sup> and significant upregulation of Hif-1 $\alpha$ -dependent, proangiogenic pathways ensues.<sup>20,77,79</sup> Of note, the avascular retina in the VO zone of OIR mice differs from the avascular retina during normal development in regard to oxygen tension: whereas the VO zone stains positively for conjugated pimonidazole (Hypoxyprobe; Chemicon, Temecula, CA), a marker for tissue oxygen tensions of less than 10 mm Hg, the peripheral avascular retina during normal development remains unstained.<sup>78,80</sup> These findings indicate a balance between oxygen supply and demand in the avascular areas of the developing peripheral retina, as opposed to an oxygen undersupply in the VO zone of the OIR model.

The hypoxia-triggered angiogenic growth factors in the second phase of OIR are not only essential for revascularization of the VO zone but also induce formation of pathologic NV (Figs. 5a–c). These pathologic neovessels leave the



**FIGURE 3.** The mouse model of OIR. Neonatal mice and their nursing mother are kept at room air from birth through postnatal day (P)7. From P7 to P12, the mice are exposed to 75% oxygen, which induces loss of immature retinal vessels and slows development of the normal retinal vasculature, leading to a central zone of vaso-obliteration (VO). After returning mice to room air at P12, the central avascular retina becomes hypoxic, triggering both normal vessel regrowth and a pathologic formation of extraretinal neovascularization (NV). Maximum severity of NV is reached at P17. Shortly thereafter, NV starts to regress and by P25 almost no VO or NV remains visible. This figure was first published in Connor KM, Krah NM, Dennison RJ, et al. Quantification of oxygen-induced retinopathy in the mouse: a model of vessel loss, vessel regrowth and pathologic angiogenesis. *Nat Protoc.* 2009;4:1565-1573.

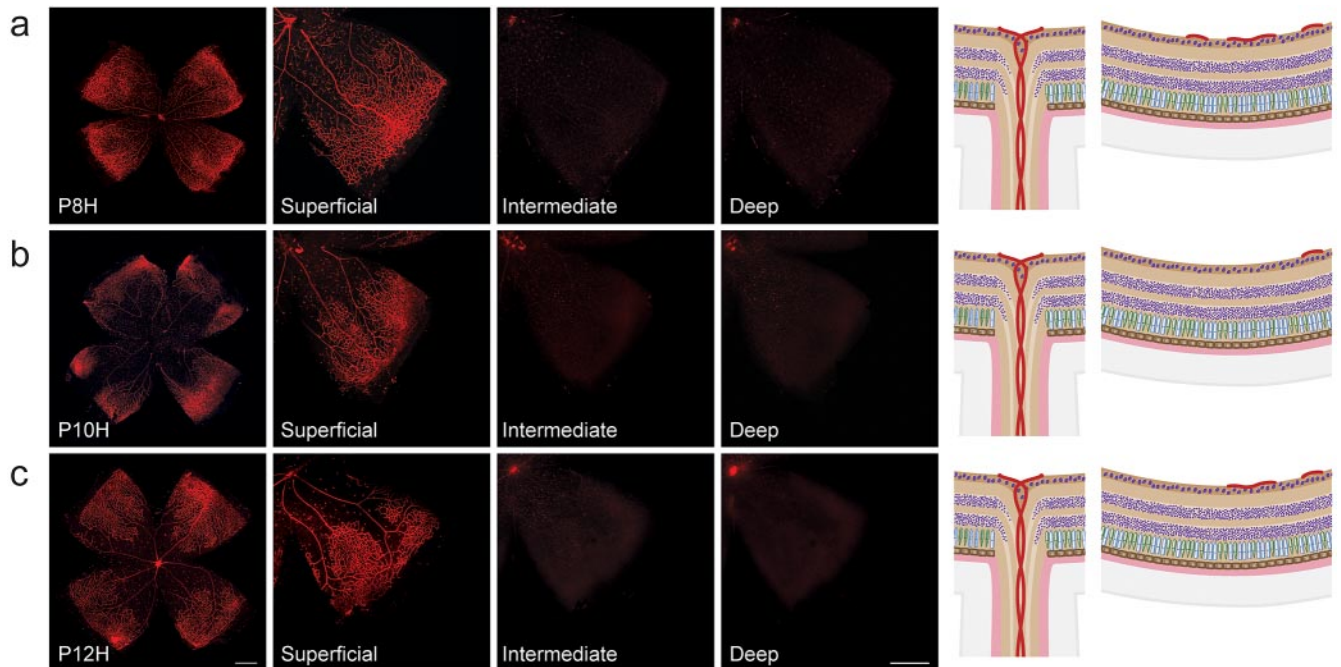


highly organized, layered structure of the retina and grow into the normally avascular vitreous cavity to form unorganized, small-caliber vessels, termed preretinal tufts, that resemble the pathologic neovascularizations seen in human ROP or PDR.<sup>61</sup> Whereas neovascular tufts form in the superficial layer, the intermediate and deep layers also show considerable vascular abnormalities compared with normoxic age-matched control retinas (see Fig. 2 for comparison). In the OIR mouse model, the maximum severity of the proliferative phase is reached at P17, marked by the greatest extent of pathologic NV and associated plasma leakage from the preretinal neovessels. From P17 onward, neovessels start to regress, until at P25, almost no VO or NV remains visible<sup>81</sup> (Figs. 5d, 5e). The tight interdependence of VO and NV, with the former dictating the time course and severity

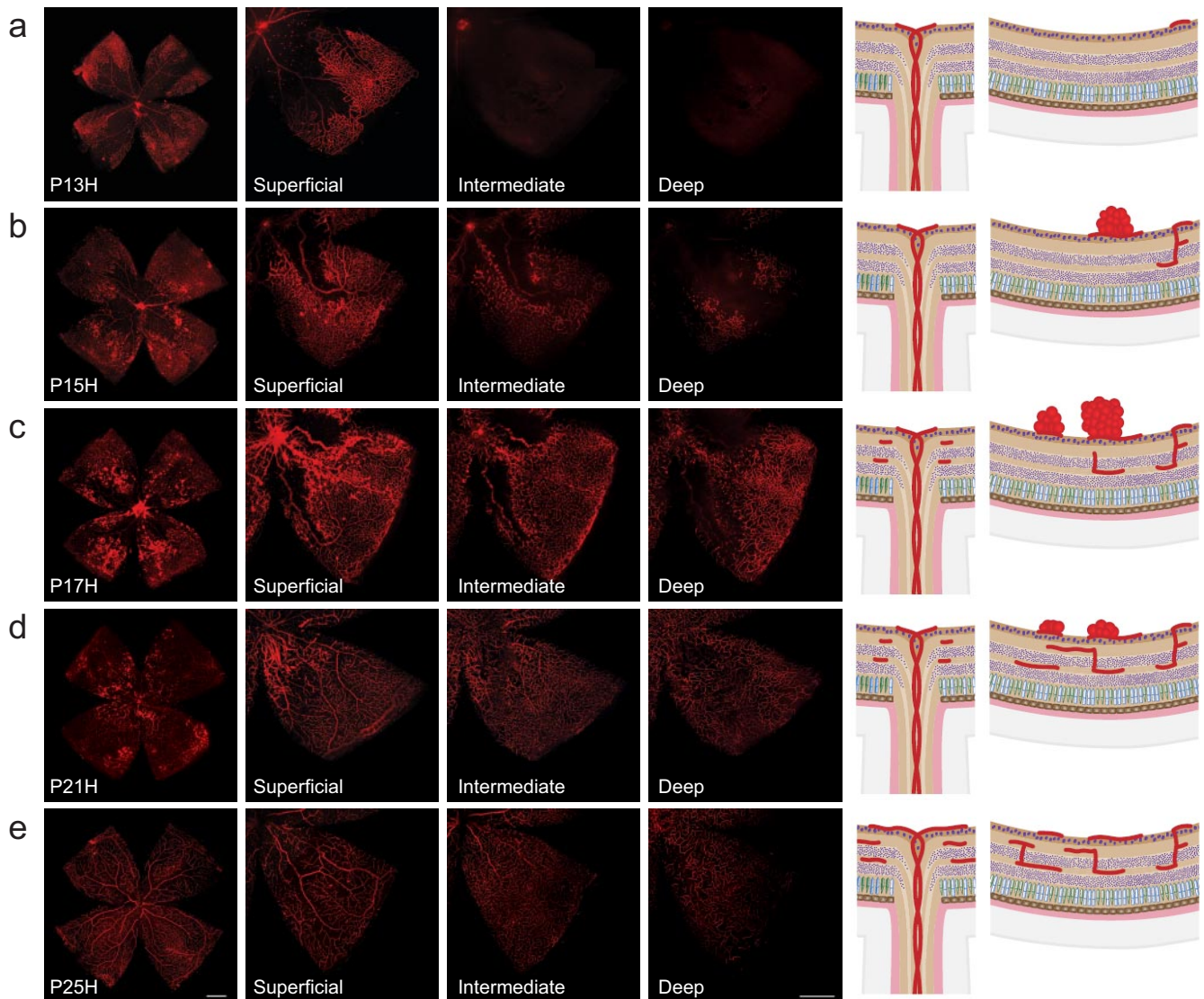
of the latter to a considerable degree, emphasizes the importance of publishing data on both VO and NV, with the OIR mouse model.

**Quantifying VO and NV in the OIR Mouse Model**

The two main features to quantify in the OIR mouse model are VO and subsequent NV. VO develops during the first, hyperoxic, phase of the model, and NV formation follows from P14 onward. In studies designed to investigate vaso-protective strategies, the extent of VO can be measured at P12 (or even as early as P8), to determine the amount of vasculature lost during hyperoxia. For most other studies, VO is measured together with neovascularization at P17, when the severity of the proliferative change is at its maximum.<sup>61</sup> Regrowth



**FIGURE 4.** Vessel loss during hyperoxia in the OIR model. Retinal whole mounts from postnatal day (P)8 to P12 were stained for endothelial cells with isolectin B<sub>4</sub>-Alexa 594 (red). H for hyperoxia is used to signify the OIR time course as opposed to the normal development shown in Figures 1 and 2. (a) At P8H, only 24 hours after onset of hyperoxia, a central zone of vaso-obliteration (VO) develops in the superficial vascular plexus. As hyperoxia coincides with formation of the intermediate and deep vascular plexus, development of these layers is significantly impeded. *Right:* representative drawings of the central VO zone showing preserved superficial vessels in the periphery and the absence of deep and intermediate vessel formation. (b) At P10H, the central VO zone has increased in size, extending farther into the peripheral retina. Formation of both deep and intermediate plexus is suppressed. (c) At P12H, the central VO zone has slightly decreased in size, probably due to revascularization in response to increased oxygen demand of the developing retina. The deep and intermediate plexus, however, are still absent.



**FIGURE 5.** Neovascularization during relative hypoxia in the OIR model. Retinal whole mounts from postnatal day (P)13 to P25 were stained for endothelial cells with isolectin B<sub>4</sub>-Alexa 594 (red). H for hyperoxia is used to signify OIR time course as opposed to the normal development shown in Figures 1 and 2. (a) At P13H, 24 hours after moving mice back to room air, the superficial vascular plexus shows a central area of vaso-obliteration (VO) without morphologic signs of neovascularization (NV). Intermediate and deep vascular plexus are absent. (b) At P15H, the VO zone has decreased in size through revascularization by normal vessels. At the same time, NV formation in the superficial vascular plexus starts to emerge between the VO zone and the peripheral vascularized retina. Vertical vessels diving down to form the deep and intermediate plexus can be observed in the peripheral retina where the superficial plexus was preserved during hyperoxia. Both this growth pattern and the time course of deeper plexus formation differ significantly from normal development. Representative drawings on the right illustrate vessel regrowth and extraretinal NV formation in the superficial plexus along with development of the deep and intermediate plexus. (c) At P17H, revascularization of the VO zone has progressed farther and pathologic NV formation is at its maximum. The intermediate and deep vascular plexus have continued to form, covering now the same area as the superficial plexus. The remaining central VO zone is devoid of all three vascular layers. (d) At P21H, revascularization of the VO area has progressed farther in all layers and NV has begun to regress. (e) At P25H, the VO area is fully revascularized in all layers and NV has completely resolved.

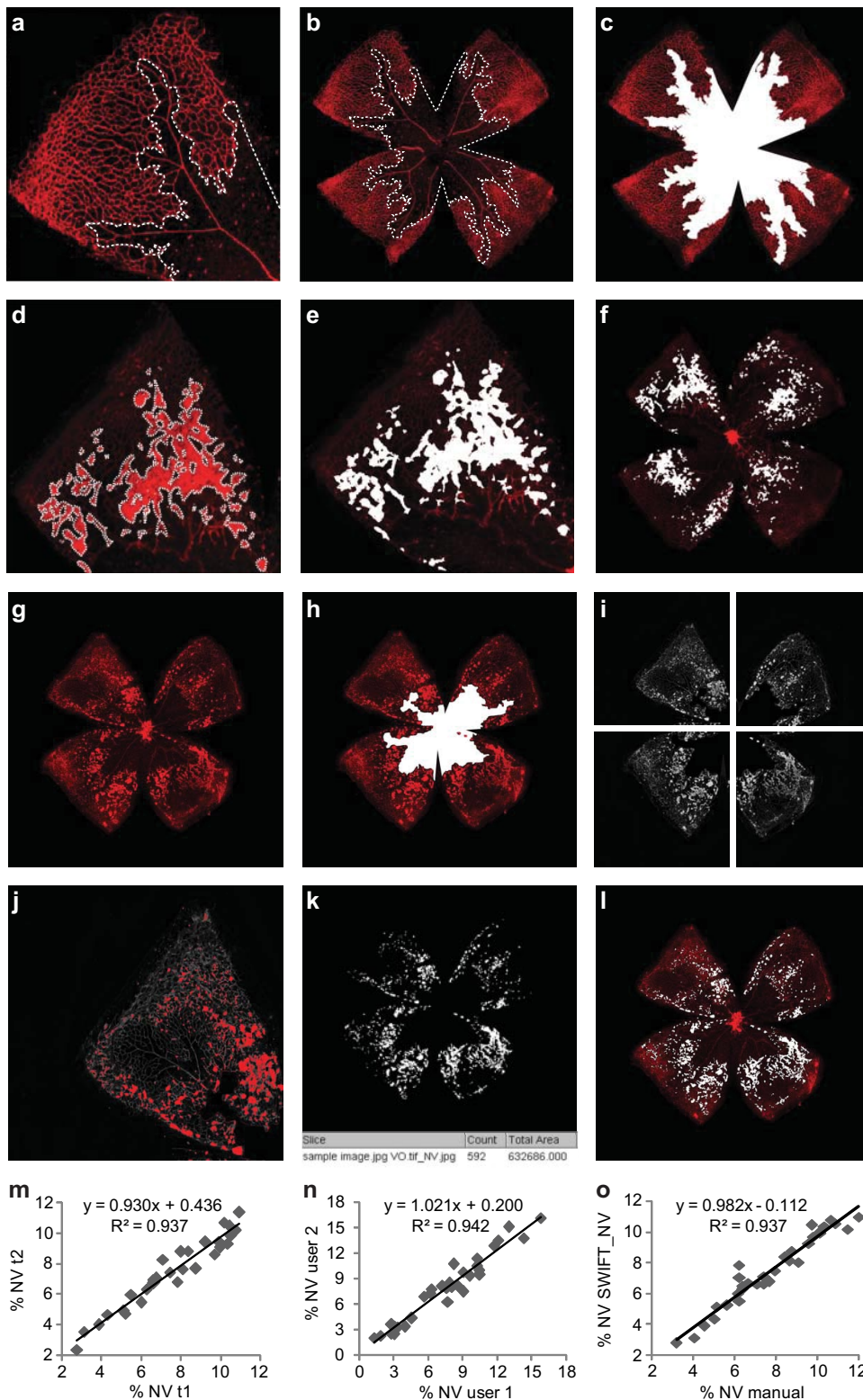
of normal vasculature occurs together with NV formation between P12 and P17. Two groups with identical VO areas at P12 may therefore have different VO areas at P17 if they differ in their capability to repair physiologic vasculature. In studies of vascular regrowth, the extent of VO can therefore be compared between P12 and P17. VO is most frequently measured by outlining the vaso-obliterated area with image-processing software (e.g., Photoshop; Adobe Systems, San Jose, CA) in relation to total retinal area (%VO; Figs. 6a-c).<sup>73</sup>

Similar to VO, quantification of NV is performed on retinal whole mounts, usually at the peak of proliferative retinopathy at P17. It is important to note that NV formation

and regression are rapid processes in the OIR mouse model: At P14, almost no NV is visible, peak NV is observed only 3 days later, and by P25 NV has completely regressed (Smith laboratory, unpublished observations, 2009). For NV quantification, various approaches have been developed over time. In the earliest OIR studies, retinal cross sections were used to count preretinal nuclei of extraretinal neovessels.<sup>20,61,79,82</sup> Subsequent studies moved to using retinal whole mounts to score retinal NV in a grading system.<sup>83,84</sup> In 2006, Banin et al.<sup>85</sup> introduced a quantification method, using image-processing software (e.g., Photoshop; Adobe) to manually outline neovascular tufts on retinal whole



**FIGURE 6.** Quantification of vasoobliteration (VO) and neovascularization (NV) in the OIR mouse model. Retinal whole-mounts from postnatal day (P)17 were stained for endothelial cells with isolectin B<sub>4</sub>-Alexa 594 (red), with VO and NV outlined in white. (a–c) For measurement of VO, the central avascular area was outlined with image-processing software (Photoshop; Adobe Systems, San Jose, CA) in relation to total retinal area (%VO). (d–f) For manual quantification of neovascularization, each individual neovascularization tuft and cluster was outlined using image-processing software (Photoshop; Adobe Systems) and the total neovascular area is expressed in relation to total retinal area (%NV). (g, h) For computer-aided NV quantification, both the original image and the VO image were imported into NIH's free-access ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>). (i) The macro set SWIFT\_NV divides the VO image into four quadrants, isolates the red color channel, and subtracts background fluorescence. (j) SWIFT\_NV then allows the user to set a fluorescence threshold for each quadrant, marking NV tufts but not normal vessels. (k, l) Using the user-defined thresholds, SWIFT\_NV quantifies all NV pixels from all four quadrants, reports the result as neovascular total area and creates an overlay of NV and original image. (m–o) Results obtained with the SWIFT\_NV macros show reliable intra- and interindividual reproducibility and very good correlations to the established hand measurement protocols. Parts (a) through (f) of this figure were first published in Connor KM, Krah NM, Dennison RJ, et al. Quantification of oxygen-induced retinopathy in the mouse: a model of vessel loss, vessel regrowth and pathologic angiogenesis. *Nat Protoc.* 2009;4:1565-1573; parts (g) through (o) were reproduced with permission from Stahl A, Connor KM, Sapieha P, et al. Computer-aided quantification of retinal neovascularization. *Angiogenesis.* 2009;12:297-301. © Springer.



mounts (Figs. 6d–f). Recently, a computer-aided technique has been published that allows semiautomated quantification of retinal NV (Figs. 6g–l).<sup>86</sup> This approach greatly accelerates NV quantification along with reliable interindividual reproducibility and very good correlations to the established hand measurement protocols (Figs. 6m–o and Movie S2, <http://www.iovs.org/cgi/content/full/51/6/2813/DC1>).

### THE OIR MOUSE MODEL IN ANGIOGENESIS RESEARCH

Over the past 15 years, the OIR model<sup>61</sup> has been used to investigate many relevant pathways involved in pathologic retinal angiogenesis: Several different groups have for example used the OIR mouse model to investigate the effects of

reactive oxygen species like NO,<sup>87–90</sup> leukocytes, and their inflammatory mediators like tumor necrosis factor (TNF)- $\alpha$ <sup>91–94</sup> or angiogenic modulators like angiopoietin 2 (Ang-2).<sup>95–98</sup> Of the large number of important pathways investigated, some work will be discussed in more detail in the following sections.

### Vascular Endothelial Growth Factor

During the first phase of OIR (hyperoxic phase from P7 to P12), retinal VEGF expression is significantly suppressed, contributing to vessel loss.<sup>19,21,99</sup> During the second, hypoxic phase of the OIR model, however, VEGF becomes upregulated mostly in glial and Müller cells of the inner retina<sup>77</sup> and strongly contributes to pathologic NV formation.<sup>19,20,100,101</sup> Early studies using the OIR model showed the beneficial effects of anti-VEGF treatment on hypoxia-driven retinal neovascularization.<sup>82,102–104</sup> In addition, differential roles for the two main VEGF receptors, VEGFR1 and -2 were identified during vasoobliteration in the OIR model.<sup>105,106</sup> Together, these studies have significantly advanced our understanding of VEGF in pathologic neovascularization and have helped to lay the foundations for today's clinical application of anti-VEGF therapies in patients.

### Erythropoietin

Another potent angiogenic factor expressed in the retina is erythropoietin (Epo).<sup>107</sup> Epo, like VEGF, is induced via Hif-1 $\alpha$  in a hypoxia-dependent fashion<sup>108</sup> and has been found to have neuroprotective properties in the rat model of retinal ischemia and reperfusion injury.<sup>109</sup> In the OIR mouse model, Epo shows expression patterns similar to those of VEGF, with initial downregulation during the hyperoxic phase and subsequent upregulation in relative hypoxia.<sup>110</sup> Exogenous Epo administration during the early phase of vessel loss was found to reduce the area of VO and subsequent neovascularization. However, when administered during the later stages of retinal hypoxia, Epo conversely appears to contribute to pathologic neovascularization.<sup>110</sup> This biphasic role with protective Epo effects during VO and proangiogenic effects during neovascularization was further confirmed in a subsequent study showing that siRNA-mediated knockdown of ocular Epo during the neovascular phase attenuates NV formation.<sup>78</sup> The relevance of this work for clinical applications is illustrated by the fact that in patients with proliferative retinopathy and retinal vein occlusion vitreal levels of Epo are significantly elevated.<sup>107,111,112</sup>

### Insulin-like Growth Factor and Insulin-like Growth Factor–Binding Protein

While VEGF is rightfully considered a master switch for angiogenesis,<sup>113</sup> both the major proliferative retinopathies—ROP and PDR—have underlying pathomechanisms that are regulated by extensive metabolic pathways, both locally in the retina and systemically. It is therefore essential to investigate the underlying mechanisms of ROP and PDR that function upstream of VEGF expression. One of the mediators that has been found to act upstream of retinal VEGF is IGF-1. In ROP, IGF-1 appears to act as a permissive factor for retinal neovascularization, with inhibition of IGF-1 during the neovascular phase reducing hypoxia-induced retinal NV despite high levels of intraocular VEGF.<sup>79</sup> Similarly, vessel-specific knockout of either insulin or IGF-1 receptors was found to protect from retinal neovascularization in the OIR mouse model.<sup>114</sup> However, the complete role of IGF-1 in ROP appears to be significantly more complex: During the early phases of VO, low levels of IGF-1 seem to be associated with poor retinal vascularization, precipitating increased hypoxia. Since premature infants have very low levels of serum IGF-1 and the level is

inversely correlated with the severity of ROP,<sup>67,115,116</sup> the goal during neonatal care must be early *normalization* of IGF-1 levels, matching as closely as possible those levels found during normal development in utero. Normalization may also be critical during proliferative disease, as abnormally high IGF-1 levels during the proliferative stages can have equally detrimental effects.<sup>67,115–117</sup> One important regulator of IGF-1 is the main IGF-binding protein, IGFBP-3. In the OIR mouse model, systemic IGFBP-3 administration was successfully used to reduce retinopathy through suppression of oxygen-induced vessel loss and promotion of vascular regrowth.<sup>118</sup> IGFBP-3 was also found to be significantly increased in neovascular tufts of the OIR retina, suggesting not only systemic but also possibly local regulatory functions of IGFBP-3 in the retina.<sup>119</sup> In addition, recent work from the Grant laboratory<sup>120</sup> found that IGFBP-3 prevents cell death of endothelial cells in the retina during OIR. Combined, these observations have helped lay the foundation for clinical trials intended to correct IGF-1 deficiency in premature infants by using equimolar combinations of IGF-1 and IGFBP-3.<sup>121</sup>

### Bone-Marrow–Derived Progenitor Cells

Similar to the regulatory roles of IGF-1/IGFBP-3, the search for modifiable pathomechanisms of proliferative retinopathies upstream of VEGF have led to research into the role of bone-marrow–derived progenitor cells during OIR. One of the homing factors for bone-marrow–derived endothelial progenitor cells (EPCs) to the retina is IGFBP-3.<sup>122</sup> Once recruited to the retina, bone-marrow–derived cells can facilitate normalization of retinal vasculature after OIR and reduce the hypoxic VO area and subsequently attenuate pathologic NV.<sup>63</sup> However, in a mouse model of retinal vein occlusion and ocular VEGF overexpression, progenitor cells were found to play a role both in physiologic revascularization as well as in pathologic neovascularization.<sup>123</sup> The possible therapeutic implications of targeting bone-marrow–derived progenitor cells for retinal vascular repair have been reviewed in more detail by Friedlander et al.<sup>124</sup>

### Lipid-Derived Mediators of Angiogenesis

The  $\omega$ -3 polyunsaturated fatty acids ( $\omega$ -3 PUFAs) are essential fatty acids and key structural components of retinal cell membranes that are most abundant in the rod and cone outer segments. Besides their crucial role in photoreceptor function,  $\omega$ -3 PUFAs have been found to bind to nuclear receptors involved in transcriptional regulation of pro- and anti-inflammatory mediators.<sup>125</sup> With regard to ROP,  $\omega$ -3 PUFAs are essential fatty acids that are abundant during intrauterine development, but are deficient after premature birth.<sup>126</sup> Increasing  $\omega$ -3 PUFAs in the OIR mouse model either by dietary supplementation from birth or by using transgenic *fat-1* mice that are able to convert  $\omega$ -6 into  $\omega$ -3 PUFAs resulted in a 50% reduction of pathologic NV through improved regrowth of physiologic vasculature and increases in resolvins and neuroprotectins.<sup>127</sup> Cyclooxygenase (Cox)-2, one of the key metabolizing enzymes for  $\omega$ -3 and  $\omega$ -6 PUFAs, has been independently identified as a strong modulator of proliferative retinopathy in the OIR retina<sup>128</sup>; Serhan et al.<sup>129</sup> showed that Cox-2 modulation via aspirin can generate anti-inflammatory lipid mediators of the resolvins class. If clinical trials that are currently in the planning phase, find that increasing  $\omega$ -3 PUFA levels in preterm infant nutritional intake is effective at reducing the severity of ROP, this highly cost-effective intervention could benefit millions of prematurely born infants.

### Neurovascular Cross-talk

Clinicians have observed that PDR regresses after the onset of photoreceptor loss in diabetic patients with retinitis pigmen-



tosa.<sup>130</sup> Similarly, both experimental diabetic retinopathy and hypoxia-induced neovascularization are reduced in mouse models of outer retinal degeneration.<sup>131,132</sup> These findings may be explained by the fact that retinal photoreceptors are among the most highly metabolically active cells in the body, consuming significant amounts of oxygen delivered to the retina.<sup>75</sup> When photoreceptors are lost, more oxygen is available to the remaining retina that subsequently reduces its expression of hypoxia-mediated proangiogenic mediators.<sup>133–137</sup> This interdependence of vascular and neurosensory retina is further illustrated by studies showing that rod photoreceptor function can predict vascular anomalies<sup>138</sup> and vice versa, that retinal vascularization can determine neuronal and glial changes as evidenced, for example, by specific upregulation of GFAP in Müller cells of the vaso-obiterated zone.<sup>139</sup>

Similar to photoreceptors, the role of glial cells of the inner retina in maintaining or directing retinal vascularization has increasingly gained interest,<sup>140–144</sup> and it has been suggested that protection of inner retinal glial cells has beneficial effects on the recovery of physiologic vasculature after VO.<sup>76</sup> In a recent publication, Dorrell et al.<sup>145</sup> found that maintaining retinal astrocytes normalizes physiological revascularization and reduces secondary NV in the OIR mouse model. Similarly, the specific importance of retinal ganglion cells (RGCs) for both physiologic and pathologic retinal angiogenesis has been elucidated by the work of Sapieha et al.<sup>146</sup> Retinas of transgenic mice engineered to specifically lack RGCs were found to be completely devoid of a retinal vascular plexus. More important, RGCs were found to express the GPR91 receptor for the Krebs cycle intermediate succinate, which accumulates in the hypoxic retina. Knockdown of GPR91 on RGCs substantially diminished pathologic NV formation, suggesting a regulatory role for RGCs during the proliferative phase of the OIR model.<sup>146</sup>

## OTHER MODELS OF PATHOLOGIC ANGIOGENESIS IN THE MOUSE RETINA

Besides the OIR mouse model, many other models replicate various aspects of pathologic angiogenesis in the mouse retina. Some of the most relevant of these models will be discussed in the following sections.

### Proliferative Diabetic Retinopathy

To date, there is no perfect animal model of diabetic retinopathy (DR).<sup>36</sup> Models using streptozotocin-induced diabetes is used or genetic models resembling type II diabetes replicate aspects of the early stages of DR—namely, leukocyte adherence, capillary dropout, and pericyte loss—but do not proceed to the proliferative stages of DR.<sup>147–150</sup> Many insightful reviews cover these models of non-PDR.<sup>151–154</sup> However, as these models lack the proliferative stage seen in diabetic patients, results from other animal models of proliferative retinopathies have frequently been extrapolated to human PDR.<sup>36</sup> One of the most frequently used models of proliferative retinopathies is the OIR mouse model. Although OIR mice do not exhibit the metabolic changes associated with DR, they share key features of human PDR. Most important, both in the OIR model and in human PDR, pathologic neovascularization develops as a consequence of earlier vessel loss.<sup>155</sup> Similarly, breakdown of the blood-retinal barrier and plasma leakage can be observed in both the OIR model and human DR.<sup>156,157</sup> Finally, both the OIR models and PDR show glial and neuronal damage associated with the vascular disease.<sup>145,158,159</sup> The feasibility of this approach of using nondiabetic surrogate models for PDR has been repeatedly confirmed by clinical studies that have shown

many of the same mechanisms to be involved in PDR that had earlier been identified in the OIR mouse model.<sup>160–162</sup>

### Wet AMD

The mouse model of laser-induced choroidal neovascularization has been extensively used to study the pathogenesis and possible treatment of subretinal neovascularization commonly associated with the wet form of AMD.<sup>36</sup> Different from all other model systems described in this article, the laser-CNV model induces subretinal (choroidal) neovascularization rather than neovessels originating from the intraretinal vasculature. Although laser-induced CNV has been performed in various animal species,<sup>163–165</sup> it is now most widely used in the mouse, and important insights into both the pathogenesis of choroidal neovascularization and the general angiogenic mechanisms have been gained from the mouse model of laser-induced CNV.<sup>166,167</sup> Work from both the Cousins and Grant groups,<sup>168–171</sup> for example, have illustrated the role of bone-marrow-derived progenitor cells during choroidal neovascularization and a recent study from the D'Amato group<sup>172</sup> identified genetic loci that control the size of laser-induced CNV. Similarly, the Ambati laboratory has used the laser CNV mouse model to investigate the role of macrophages and their inflammatory mediators in CNV formation.<sup>173–175</sup> The same group also identified the chemokine CCR3 as an important contributor to laser-induced CNV formation.<sup>176</sup> Of interest, CCR3 was found to be specifically expressed in choroidal neovascular endothelial cells from human patients with wet AMD, and blocking CCR3 in the mouse model has been found to be more effective at reducing CNV than is inhibition of VEGF.<sup>176</sup>

In addition to the laser-induced model of CNV formation, several genetic models have been described that replicate aspects of AMD. The Ambati group reported that mice deficient in the macrophage chemokine CCL2 or its receptor CCR2 develops cardinal features of AMD.<sup>177</sup> This was the first mouse model of spontaneous AMD and promoted the concept that inflammation is critical in AMD pathogenesis. This work also spurred later reports of AMD-like disease in mice deficient in related macrophage chemoattractants.<sup>178,179</sup> Work from the Chan laboratory found that  $\omega$ -3 fatty acids can slow and even reverse the progression of AMD-like neovascular lesions in these models,<sup>180</sup> thus confirming earlier results obtained in epidemiologic AMD studies<sup>181</sup> and in the OIR mouse model.<sup>127</sup>

### Retinal Vascular Occlusion and Ischemia-Reperfusion Injury

Retinal vascular occlusion in the mouse retina can be achieved by laser photocoagulation of retinal vessels. The resulting reduction in retinal blood flow leads to retinal ischemia, vascular leakage and retinal neovascularization resembling the clinical picture of patients with retinal vein occlusions.<sup>123</sup> Schepke et al.,<sup>182</sup> for example, have used this model to investigate the effect of a novel VEGFR2/Src kinase inhibitor on retinal vascular permeability. Another model for impeded retinal circulation uses instillation of sterile saline into the anterior chamber to increase the intraocular pressure until retinal circulation ceases.<sup>183</sup> After normalization of intraocular pressure, reperfusion of the retina ensues, and postischemic injury/repair mechanisms can be investigated. The Kern laboratory,<sup>184</sup> for example, has used this model to show that death of retinal ganglion cells (RGCs) can precede vascular degeneration. Similarly, the Grant laboratory<sup>185</sup> has shown that ischemia-reperfusion injury can be repaired by healthy, but not diabetic endothelial progenitor cells (EPCs).

### Transgenic Mice Overexpressing VEGF or IGF-1

Elevated levels of VEGF have been reported both for ROP and PDR.<sup>18,186</sup> The Campochiaro<sup>187–189</sup> group developed a model

with transgenic VEGF overexpression in the retina under the photoreceptor-specific rhodopsin promoter. With this driver, the neovascular changes mainly localize to intra- and subretinal areas of neovascularization. A mouse model with targeted very high overexpression of retinal IGF-1 resulting in very high VEGF expression has also been developed.<sup>190</sup> This model has been found to reproduce some of the vascular changes observed in diabetic retinopathy—namely, loss of pericytes, thickening of basement membranes and microvascular abnormalities. These models overexpressing VEGF or IGF-1 can be useful to study the effects of genetic manipulation or antiangiogenic treatment in a controllable and defined in vivo system.

## CONCLUSION

The mouse retina represents a well-characterized, readily accessible tissue for angiogenesis research. It allows the detailed investigation of both developmental and pathologic vessel growth in vivo. Because of the postnatal development of the retinal vasculature, both the formation and regression of blood vessels can be studied in a tightly controlled setting. The importance of various in vivo models using the postnatal mouse retina is emphasized by the large number of studies employing the mouse retina and their significant contribution to our understanding of the angiogenic pathomechanisms underlying some of the most sight-threatening human diseases.

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