

# Quantification and Localization of the IGF/Insulin System Expression in Retinal Blood Vessels and Neurons during Oxygen-Induced Retinopathy in Mice

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**PURPOSE.** Retinopathy is a result of pathologic angiogenesis influenced by insulinlike growth factor (IGF)-1. The authors examined the local expression of the IGF/insulin family.

**METHODS.** In retinas with and without oxygen-induced retinopathy, the authors assessed with real-time RT-PCR mRNA expression of the IGF-1 receptor (IGF-1R), insulin receptor (IR), IGF-1, IGF-2, insulin (Ins2), and IGF-binding protein 1 (IGFBP1) to IGFBP6 in total retina from postnatal day (P) 7 to P33 to examine changes over time with the induction of retinopathy and at P17 on laser-captured retinal components to quantitatively localize mRNA expression in the ganglion cell layer, the outer nuclear layer, the inner nuclear layer, normal blood vessels, and neovascular tufts.

**RESULTS.** IGF-1R and IR are expressed predominantly in photoreceptors and in vessels, with scant expression in the rest of the neural retina. IGF-1R expression is more than 100-fold greater than IR. The major local growth factor (expressed in photoreceptors and in blood vessels) is IGF-2 (approximately 1000-fold greater than IGF-1). IGF-1 (approximately 600 copies/10<sup>6</sup> cyclophilin) is expressed throughout the retina. IGFBP2, IGFBP4, and IGFBP5 expression is unchanged with increasing retinal development and with the induction of retinopathy. In contrast, IGFBP3 expression increased more than 5-fold with hypoxia, found in neovascular tufts.

**CONCLUSIONS.** IGF-1R, IR, and the ligand IGF-2 are expressed almost exclusively in photoreceptors and blood vessels. IGFBP3 and IGFBP5 expression increases in neovascular tufts compared with normal vessels. IGF-1 is expressed throughout the retina at much lower levels. These results suggest cross-talk between vessels and photoreceptors in the development of

retinopathy and retinal vasculature. (*Invest Ophthalmol Vis Sci.* 2009;50:1831-1837) DOI:10.1167/iovs.08-2903

Many ocular diseases, including diabetic retinopathy and retinopathy of prematurity, are a result of pathologic angiogenesis. At present, we do not understand the contribution of local versus systemic regulation of normal and pathologic vessel growth. Insulinlike growth factor (IGF)-1 is an important growth factor involved in retinal angiogenesis.<sup>1,2</sup> Low systemic levels of IGF-1 correlate with the degree of retinopathy of prematurity in the clinical setting,<sup>3,4</sup> and IGF-1 is implicated in diabetes.<sup>5-7</sup> Insulin receptors and IGF-1 receptors in retinal vascular endothelial cells have been implicated in pathologic angiogenesis.<sup>8</sup> Although mRNAs for IGF-1, IGF-1 receptor (IGF-1R), and IGF-binding protein 2 (IGFBP2) through IGFBP6 have been described in specific histologic layers of the normal rat retina, choroid, ciliary body, and cornea,<sup>9</sup> there has been to date no comprehensive survey of the relative local expression (and, by implication, contribution) of IGF-1 and the IGF/insulin family, in retinal blood vessels and neurons during the induction of retinopathy. This is important because neural retinal signaling through the insulin receptor and the IGF receptor might influence retinal vascular growth. There is also evidence that IGF-2 is expressed in hemangiomas, proliferative vascular tumors of infancy. The role of IGF-2 in other neovessels has not been established. We sought to find the localization and quantification of insulin, IGF-1R, and the ligands IGF-1, IGF-2, and their binding proteins in the retina to better understand the role of the IGF family in retinopathy.

Diabetes, which is characterized by abnormal insulin levels and function, leads to retinal microvascular disease and diabetic retinopathy. Insulin signaling may play a role in this process, and mice lacking either insulin receptor (*IR*) or *IGF-1* receptor (*IGF-1R*) in endothelial cells undergo normal vascular development but are protected from hypoxia-induced retinal neovascularization.<sup>8</sup> Total loss of *IGF-1R* in the neural retina and in blood vessels, however, does suppress normal retinal vascular development,<sup>4,10</sup> suggesting that *IGF-1R* in the neural retina influences vascular development. However, localization and quantification of *IGF-1R* and *IR*<sup>11</sup> in the neural retina have not been completely established.<sup>12</sup>

IGF-1 and IGF-2 are potent mitogens that stimulate proliferation but not differentiation in vivo.<sup>13</sup> Genetic evidence suggests that in vivo, IGF-1 signals only through the tyrosine kinase IGF-1R, whereas IGF-2 signals through both IGF-1R and IR.<sup>13</sup> In contrast, the IGF-2 receptor (IGF-2R) does not signal but acts as an IGF-2 sink.<sup>14</sup> The IGFs also have high affinity for a family of six structurally related IGFBPs, IGFBP1 to IGFBP6, which regulate the bioavailability of the IGFs in the circulation. The IGFBPs also modulate the activity of IGFs at the cellular level, either inhibiting or enhancing IGF action.<sup>15</sup> There are also independent actions of the IGFBPs. In vivo, with decreasing IGFBP3, there is a dose-dependent increase in oxygen-induced retinal vessel loss and vascular regrowth after vascular destruc-

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TABLE 1. Sequences of Mouse Primer Sets for Quantitative Real-Time PCR

	Forward Primer (5'-3')	Reverse Primer (5'-3')
<i>Igfbp1</i>	GAC CTC AAG AAA TGG AAG GA	GAT GTC TCA CAC TGT TTG CT
<i>Igfbp2</i>	GCC CGC TGG AAC ATC TCT ACT	TCC GTT CAG AGA CAT CTT GCA
<i>Igfbp3</i>	CCA GGA AAC ATC AGT GAG TCC	GGA TGG AAC TTG GAA TCG GTC A
<i>Igfbp4</i>	TTC ATC ATC CGG ATT GCA AAC	ACC CCT GTC TTC CGA TCC A
<i>Igfbp5</i>	GAT GAG ACA GGA ATC CGA ACA AG	AAT CCT TTG CGG TCA CAG TTG
<i>Igfbp6</i>	AGG AGA GCA AAC CCC AAG GA	TGA ACA GGA TTG GGC CGT ATA
<i>Igfbp7</i>	AAG AGG CGG AAG GGT AAA GC	TGG GGT AGG TGA TGC CGT T
<i>Igf1</i>	TCA TGT CGT CTT CAC ACC TCT TCT	CCA CAC ACG AAC TGA AGA GCA T
<i>Igf2</i>	ACA ACT TCG ATT TGA ACC ACA TTC	GAG AGC TCA AAC CAT GCA AAC T
<i>Igf1 receptor</i>	GTG GGG GCT CGT GTT TCT C	GAT CAC CGT GCA GTT TTC CA
<i>Igf2 receptor</i>	GGG AAG CTG TTG ACT CCA AAA	GCA GCC CAT AGT GGT GTT GAA
<i>Insulin receptor</i>	ATG GGC TTC GGG AGA GGA T	GGA TGT CCA TAC CAG GGC AC

tion, resulting in less retinal neovascularization.<sup>16</sup> IGFBP-7 constitutes a low-affinity member of the IGFBP superfamily and primarily functions as a modulator of cell growth in an IGF-independent manner.<sup>17</sup>

It is not possible to accurately assess local protein levels within retinal neuronal layers and vessels because immunohistochemistry is not quantitative and laser-capture microdissection of retinal layers does not provide sufficient quantities of protein for quantitative analysis. In addition, other methods of protein analysis are problematic because many of the proteins are soluble and diffusible and because the levels in serum are up to 20 to 50 times greater than in tissue,<sup>18</sup> making accurate assessment of tissue contribution difficult. We have quantitatively evaluated mRNA expression of the IGF-1 family with real-time RT-PCR on laser-captured retinal components. With the time course of local production of *IGF-1R*, *IR*, *IGF-1*, *IGF-2*, *Ins2*, and *IGFBP* mRNA and their quantified localization in the retina, we can begin to address the question of the contributions and functions of the IGF system during retinopathy.

## MATERIALS AND METHODS

### Animals

These studies adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by Children's Hospital Animal Care and Use Committee. C57BL/6N (Taconic Farms, Germantown, NY) mice were used for the study.

### Model of Ischemia-Induced Proliferative Retinopathy

Retinopathy was induced in pups (with nursing mothers) by exposure to 75% ± 2% O<sub>2</sub> from postnatal day (P) 7 to P12, followed by exposure to room air until kill.<sup>19</sup> Age-matched control C57BL/6 mice were housed in room air continuously.

### Isolation of Retinal RNA and cDNA Preparation

At selected time points (P8, P10, P12, P15, P17, P33), animals were killed by lethal doses of tribromoethanol (Avertin; Sigma, St. Louis, MO), and retinas were dissected immediately, flash frozen, and stored at -80°C. RNA was extracted (RNeasy Mini RNA extraction kit; Qiagen, Chatsworth, CA) in accordance with the manufacturer's instructions incorporating a mortar and pestle followed by Qiagen column (QIAshredder) for homogenization. At each time point, total mRNA from 12 pooled retinas (to reduce biological variability) from 12 mice with ischemia-induced proliferative retinopathy or normal age-matched control mice was analyzed. The purified RNA was suspended in diethyl pyrocarbonate (DEPC)-treated H<sub>2</sub>O. To generate cDNA, RNA was first treated with DNase I (Ambion, Austin, TX) to remove contaminating genomic DNA. The DNase-treated RNA (100 ng) was then converted to cDNA using murine leukemia virus reverse transcriptase

(Gibco BRL Life Technologies, Bethesda, MD). cDNA samples were portioned into aliquots and stored at -20°C.

### Laser-Capture Microdissection

For laser microdissection, eyes with oxygen-induced retinopathy (OIR) or control eyes at P17 were enucleated and embedded in OCT compound, cryosectioned at 10 μm, mounted on RNase-free polyethylene naphthalate (PEN) foil glass slides (11505189; Leica Microsystems, Deerfield, IL), and immediately stored at -80°C. Frozen sections were immersed in 50% ethanol, 75% ethanol, and DEPC H<sub>2</sub>O for 15 seconds each, followed by 30 seconds of staining solution (Arcturus HistoGene; Molecular Devices, Eugene, OR) and a rinse in DEPC H<sub>2</sub>O. After they were air dried for 10 minutes, the sections were laser microdissected for vessels and retinal neuron layers (LMD 6000; Leica Microsystems). Each population was estimated to be greater than 95% homogenous as determined by microscopic visualization of the captured cells. Material was collected directly into lysis buffer (RNeasy Micro kit; Qiagen). RNA was subsequently isolated according to the manufacturer's protocol, treated with DNase, and converted to cDNA.

### Real-Time Quantitative RT-PCR

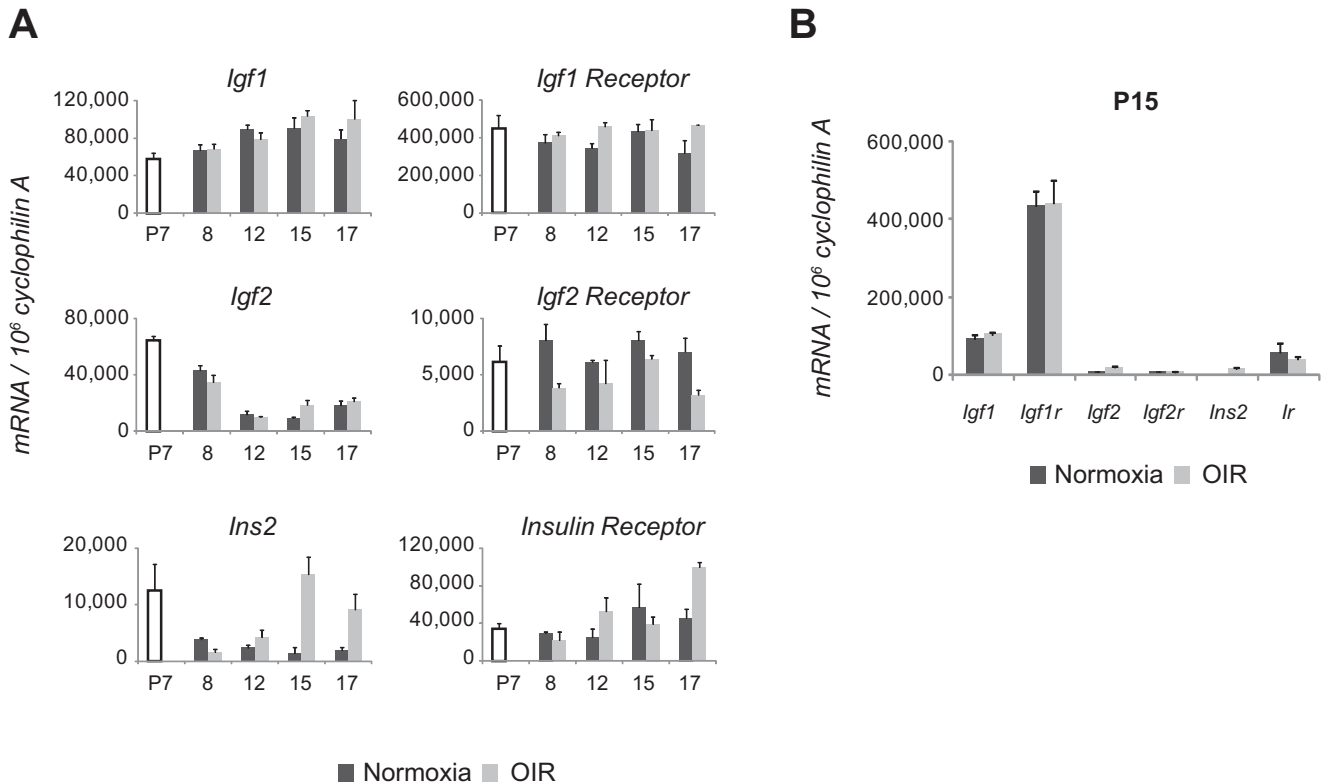
Real-time quantitative RT-PCR primer sets targeting murine *IGF-1*, *IGF-2*, *IGF-1R*, *IGF-2R*, *IR*, and *IGFBP1* to *IGFBP7* were designed using the primer bank verified database<sup>20</sup>; sequences (5' to 3') are listed in Table 1. Primers for murine *Ins2* were difficult to design, and a verified set was purchased from Qiagen (Quantitect Assays). Reactions including master mix (SYBR Green I; Qiagen) and 1 ng cDNA template were run in triplicate on a PCR system (7300 Real-Time; Applied Biosystems, Foster City, CA), and each target gene was run concurrently with *cyclophilin A*, a constitutively expressed control gene for normalization. After amplification, first-derivative melting curve analysis was used to confirm the specificity of the target product. Copy numbers for each target gene were normalized to 1 million copies of *cyclophilin A*. By comparison to a known dilution of *cyclophilin A* copies, the data approximate absolute copy numbers. However, some data have been scaled to match parallel experiments based on empirically determined ratios.

For analysis of biological variability, pooling RNA from 12 samples was a more pragmatic approach than processing individual retinas. From our experience, genes that are moderately expressed (>100 copies/10<sup>6</sup> cyclophilin) vary less than 2-fold between individual retinas within an experimental group. Generally, expression levels below 100 copies/1 million *cyclophilin A* and fold changes of less than 2 cannot be reliably interpreted, as reflected in the Discussion.

## RESULTS

### Temporal Expression in Total Retina of *IGF-1*, *IGF-2*, *Ins2*, *IGF-1R*, *IR*, *IGF-2r* mRNA during Oxygen-Induced Retinopathy

We examined regulation of the IGF-I/insulin system in whole retina of total mRNA from 12 pooled retinas at each time point



**FIGURE 1.** Retinal mRNA expression profiles of *IGF1*, *IGF2*, *Ins2*, and receptors in OIR compared with normoxic controls. (A) Mouse litters were exposed to 75% O<sub>2</sub> from P7 to P12, and retinas from selected times were isolated and used for quantitative real-time PCR analysis. Target genes were normalized to 10<sup>6</sup> copies of *cyclophilin A*. P7 samples (*white bars*) were collected immediately before OIR and are common to both treatments. (B) Data from P15 replotted for comparison. Error bars represent  $\pm$ SD.

between P7 and P33 during the induction of OIR and in untreated age-matched controls. Retinas became hypoxic after return to room air at P12 following hyperoxia-induced vessel loss from P7 to P12.

In total retina, *IGF-1*, *IGF-1R*, and *IGF-2R* mRNA expression was essentially unchanged with the development of retinopathy and unchanged with retinal development from P7 to P17. Retinal *Ins2* mRNA increased more than 5-fold at P15 and P17 with the induction of retinopathy (Fig. 1A). *IGF-2* mRNA decreased with development between P7 and P17, and local *IR* mRNA production increased modestly (approximately 2-fold) at the peak of neovascularization at P17. However the IGF-1/IGF-1R system predominated. The relative ratio of *IR/IGF-1R* mRNA production in total retina was approximately 1:10, as was the ratio of local *Ins2/IGF-1* mRNA production. In total retina, *IGF-2* was not a dominant as *IGF-1*. The relative amount of *IGF-2R* was negligible (Fig. 1B).

#### Temporal Expression in Total Retina of *IGFBP1* to *IGFBP7* during Oxygen-Induced Retinopathy

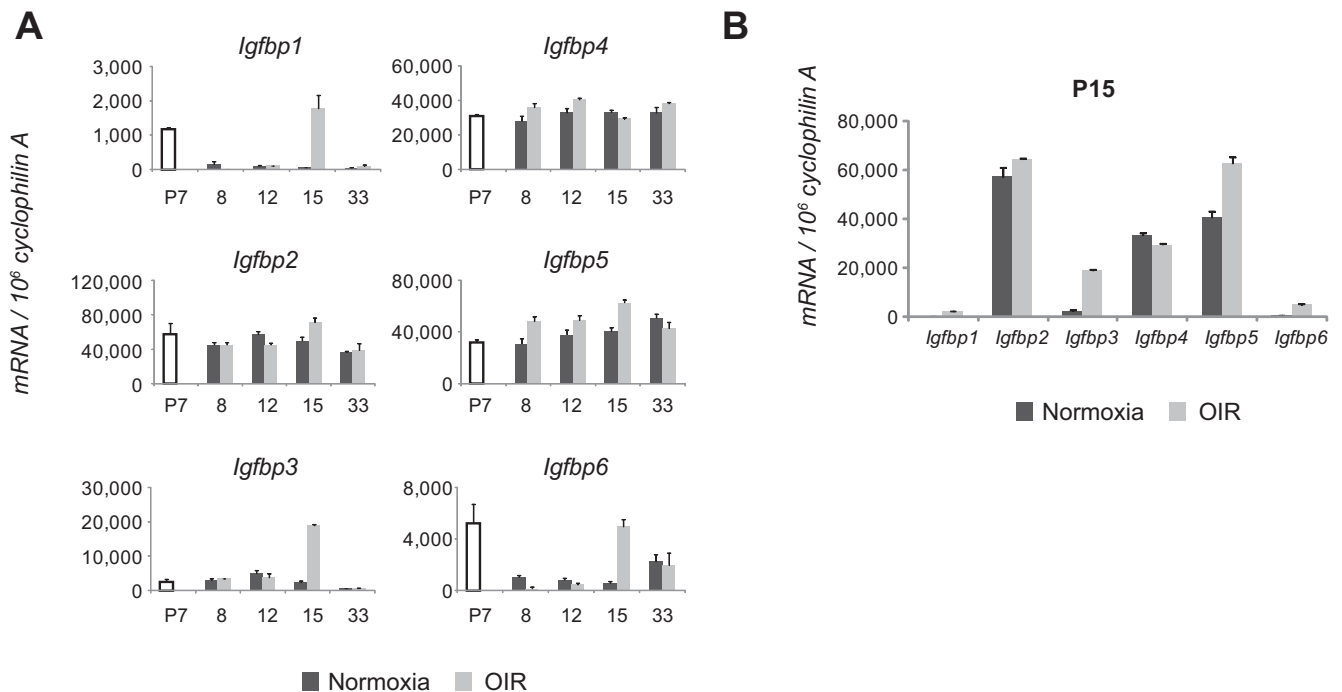
*IGFBP2*, *IGFB4*, and *IGFBP5* mRNA expression was essentially unchanged with increasing retinal development between P7 and P33, and was there no change of *IGFBP2*, *IGFB4*, and *IGFBP5* expression with the induction of retinopathy. In contrast *IGFB1*, *IGFBP3*, and *IGFBP6* all had greater than 5-fold increases in mRNA at P15 with the induction of retinopathy that then decreased to baseline at P33 (adult; Fig. 2A). However, when relative mRNA production was examined at P15, *IGFBP1* and *IGFBP6* were expressed at very low levels (even after a large induction with retinopathy) compared with the essentially unchanged *IGFBP2*, *IGFB4*, and *IGFBP5* mRNA expression. In contrast, *IGFBP3* mRNA levels were low during

development but were upregulated with the onset of hypoxia to a level within 3-fold that of *IGFBP2*, *IGFB4*, and *IGFBP5* (Fig. 2B).

#### Localization and Abundance of the IGF and Insulin Systems in Retinal Cross-Section with OIR and Normal Retina at P17

To localize and quantify mRNA in retina, we laser captured with microdissection retinal vessels and neovascular vessels extending into the vitreous (tufts) and then captured the ganglion cell layer (GCL), inner nuclear layer (INL), and outer nuclear layer (ONL) or photoreceptors in normal retinopathy and OIR at P17. RNA was then quantified to measure absolute abundance in copy numbers per 10 million cyclophilin mRNA copies (Fig. 3).

*IGF-1R* and *IR* are expressed predominantly in the photoreceptors in the neural retina, to a lesser extent in normal vessels, and to an even more reduced level in neovascular tufts, with little expression in other parts of the retina (Fig. 4A). However, *IGF-1R* predominates. The relative abundance of *IGF-1R* to *IR* in photoreceptors and vessels was approximately 100:1 (Fig. 4B). In contrast, *IGF-1* was expressed in all neural retinal layers and in vessels and tufts (with slightly lower expression in the ONL (photoreceptors; Fig. 4A). *IGF-2* mRNA was expressed at a much higher level (up to a 1000-fold difference) than *IGF-1* in photoreceptors and in blood vessels. *IGF-2* mRNA expression was increased more than 6-fold in neovascular tufts compared with normal vessels. The relative abundance of *IGF-1/Ins2* mRNA in photoreceptors in normal retina was approximately 1:10. However, with retinopathy at P17, there was approximately a 5-fold decrease in photorecep-



**FIGURE 2.** Retinal mRNA expression profiles of *IGFBP1* to *IGFBP6* in OIR compared with normoxic controls. (A) Mouse litters were exposed to 75% O<sub>2</sub> from P7 to P12, and retinas from selected times were isolated and used for quantitative real-time PCR analysis. Target genes were normalized to 10<sup>6</sup> copies of *cyclophilin A*. P7 samples (white bars) were collected immediately before OIR and are common to both treatments. (B) Data from P15 replotted for comparison. Error bars represent  $\pm$ SD.

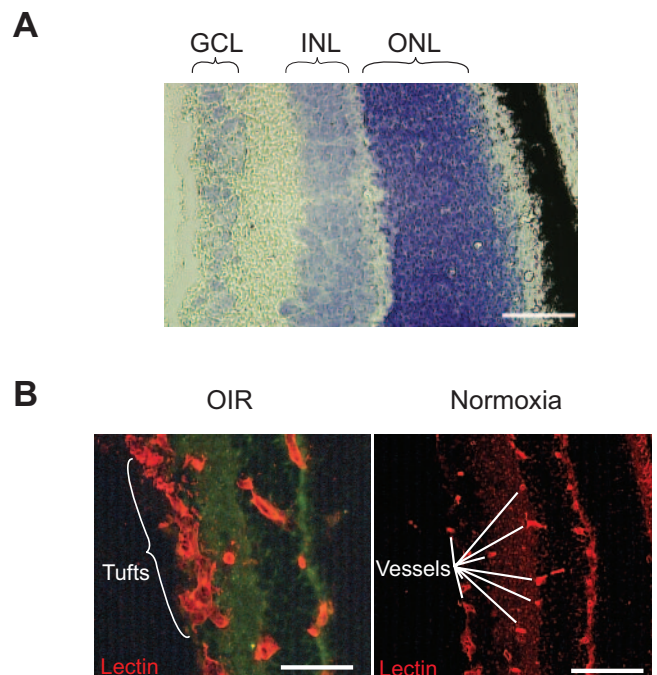
tor insulin production such that the *IGF-1/Ins2* ratio was closer to 1. Nevertheless, the most prominent components of the *IGF-1/Ins2* system were *IGF-2* and *IGF-1R* in photoreceptors and vessels.

The localization of the IGFBPs shows large increases in neovascular tufts in *IGFBP3*, *IGFBP5*, and *IGFBP7* mRNA compared with normal vessels (Fig. 5). The GCL predominantly expresses *IGFBP2*, *IGFBP3*, and *IGFBP5*, as do the ONL and INL (Fig. 5).

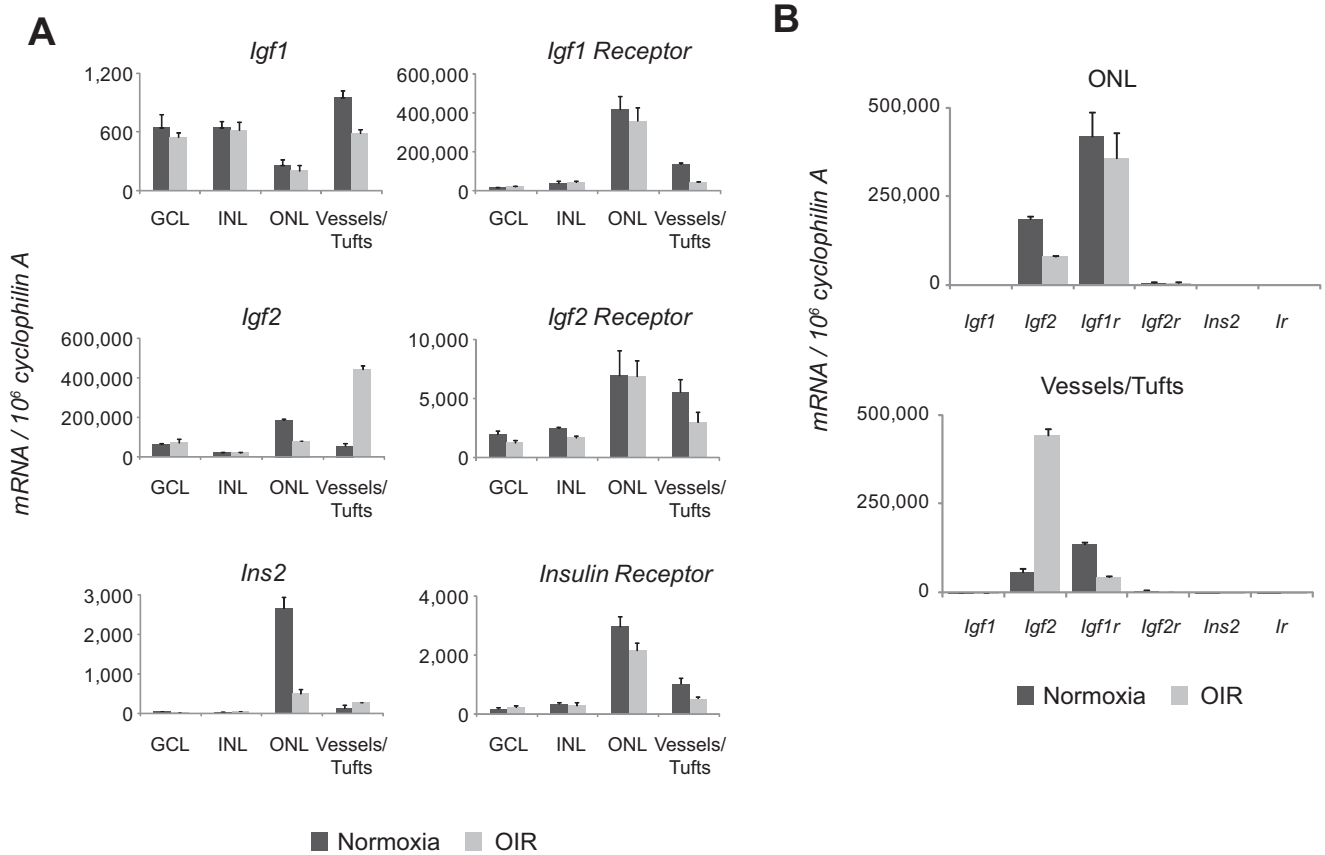
## DISCUSSION

Knowing the retinal distribution and relative expression levels of IGF family components (*IGF-1R* and *Insr*, *IGF-1*, *IGF-2*, *Ins2* and *IGFBP1* to *IGFBP7*) in vessels and specific neuronal layers (the equivalent of quantitative *in situ* hybridization) as well as changes in mRNA expression in total retina during retinopathy may enhance our understanding of the local IGF system control. Using the technique of quantitative RT-PCR on laser-capture microdissected retinal elements and vessels (normal and neovascular vessels), we found that in the retina the major local IGF system receptor expressed is *IGF-1R* rather than *IR*. Surprisingly, the localization of *IGF-1R* (and *IR*) showed that these receptors are expressed predominantly in photoreceptors and more modestly in vessels (*IGF-1R* at approximately 100-fold greater level than *IR*) with little expression in the rest of the neural retina.

Interestingly, this suggests that *IGF-1R* in photoreceptors is required for retinal vascular development because of decreased normal retinal vascular development in the *IGF-1R* null mouse, with its lack of *IGF-1R* in endothelial cells and in photoreceptors, but normal vascular development in a mouse lacking *IGF-1R* in vascular endothelial cells alone.<sup>8</sup> Vascular loss occurs when photoreceptors degenerate, and the IGF system in photoreceptors is likely to be one of the growth factor systems required for photoreceptor stability<sup>21,22</sup> and for



**FIGURE 3.** Representative micrographs of P17 mouse retinal sections for laser-capture microdissection. (A) Ethanol-fixed sections stained with hematoxylin and eosin for capture of three neural layers (GCL, INL, ONL). (B) To capture endothelium, sections were stained with isolectin B<sub>4</sub> (red). Physiologic vessels were isolated from normoxic samples, and angiomatous tufts in the superficial layer protruding into the vitreous were selectively captured from samples exposed to OIR. Scale bar, 50  $\mu$ m.



**FIGURE 4.** Intraretinal mRNA expression profiles of *IGF1*, *IGF2*, *Ins2*, and receptors in OIR compared with normoxic controls. (A) Mouse litters were exposed to 75% O<sub>2</sub> from P7 to P12. At P17, eyes were collected and cryosectioned, and laser-capture microdissection was used to enrich for GCL, INL, ONL, and normoxic blood vessels and angiomatous tufts (Vessels/Tufts). RNA was isolated from samples and used for quantitative real-time PCR analysis. Target genes were normalized to 10<sup>6</sup> copies of *cyclophilin A*. (B) Data replotted for comparison of the ONL and Vessels/Tufts. Error bars represent +SD.

normal vascular development.<sup>8</sup> Less is known about *IR* in retinal neurons and vasculature, though *IR* is also important in retinal neovascularization<sup>8</sup> despite the fact that the expression level is low compared with *IGF-1R*. Insulin receptor signaling provides a trophic signal for transformed retinal neurons in culture,<sup>23</sup> but the role of *IR* in retinal neuronal activity in vivo is less understood. In *Drosophila*, *IR* guides retinal photoreceptor axons from the retina to the brain during development,<sup>24</sup> and *IR* influences the size and number of photoreceptors.<sup>25</sup> The lack of *IR* activation leads to neurodegeneration in brain/neuron-specific *IR* knockout mice.<sup>26</sup>

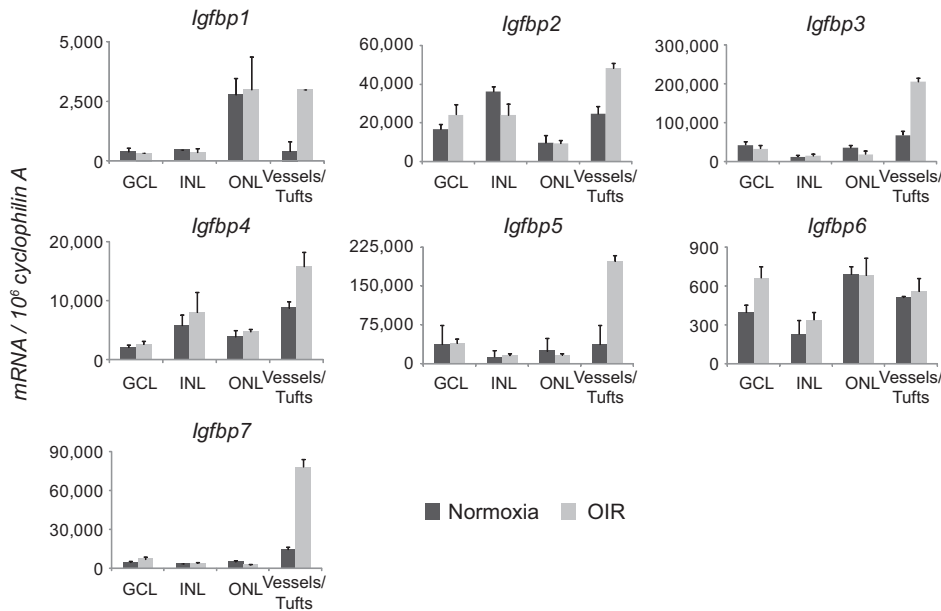
We found that the major local retinal IGF-related growth factor expressed in photoreceptors and in blood vessels is *IGF-2* rather than *IGF-1* (or *Ins2*).<sup>27</sup> Local production of *IGF-2* mRNA at these sites is 100- to 1000-fold greater than *IGF-1* or *Ins2*. *IGF-2* signals through *IGF-1R* and *IR*, suggesting that *IGF-2* may be important for photoreceptor cell survival and for vessel growth. Importantly, *IGF-2* is increased 8-fold in neovascular tufts compared with normal vessels. *IGF-2* is also highly expressed in a wide variety of neoplasms and hemangiomas,<sup>28</sup> vascular tumors of infancy. Thus, *IGF-2* expression may be characteristic of neovessels. We found a very low level of expression of the *IGF-2* scavenging receptor, *IGF-2R*, in photoreceptors and vessels. *IGF-2R* binds *IGF-2* on the cell surface and transports it to the lysosome for degradation, acting as a sink because it does not transduce any signal.<sup>14</sup>

We found that the major IGF-binding proteins expressed in the total retina are *IGFBP2*, *IGFBP3*, *IGFBP4*, and *IGFBP5*. *IGFBP2*, *IGFBP4*, and *IGFBP5* are expressed at constant levels

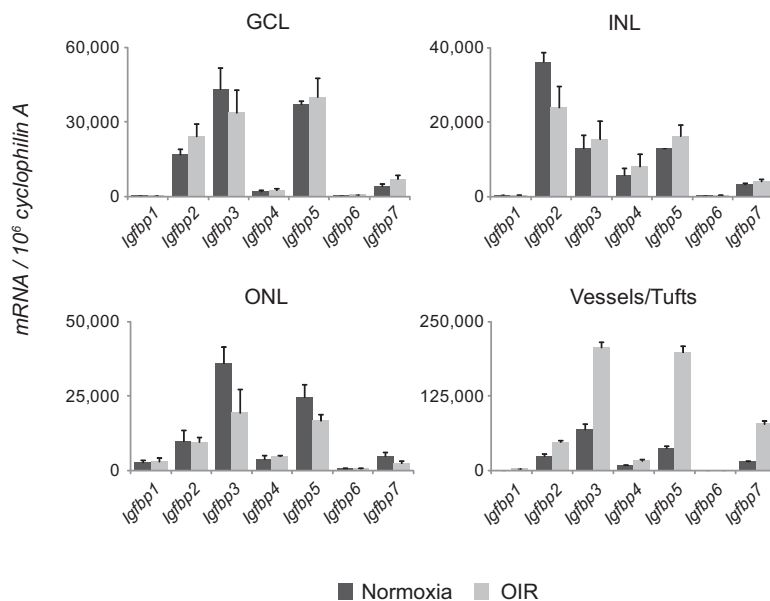
with development and with oxygen-induced retinopathy. *IGFBP3* is strongly induced by hypoxia and rises to levels comparable to those of *IGFBP2*, *IGFBP4*, and *IGFBP5*. Laser-capture localization shows that neovessels compared with normal vessels strongly express *IGFBP3* and *IGFBP5*, with increases also in *IGFBP2* and *IGFBP-7* (though to much lower levels) over normal vessels. Ischemia is not a stimulus for the local production of *IGF-1*, *IGF-2*, *IGFBP2*, *IGFBP4*, or *IGFBP5* but is a strong stimulus of *IGFBP3* in the eye. Although expressed at much lower levels, mRNA of *IR*, *Ins2*, *IGFBP1*, and *IGFBP6* also appears to be upregulated with hypoxia.

In cell culture, *IGFBP5* controls cell survival, differentiation, and apoptosis independently of *IGF-1* or *IGF-2*.<sup>29</sup> The expression pattern of *IGFBP5* in retina in vivo points to a significant role during retinal development.<sup>30</sup> In primary porcine retinal Müller glial cells cultured in vitro, *IGFBP5* increases photoreceptor survival.<sup>31</sup> In cell culture, *IGFBP2* and *IGFBP4* are generally inhibitory, whereas *IGFBP5* potentiates *IGF-1*.<sup>32</sup> *IGFBP3*, depending on culture conditions, can either inhibit or potentiate the actions of *IGF-1*. In serum, *IGFBP3* is by far the most abundant binding protein, with concentrations in the range of 100 nM, compared with the 2 to 15 nM concentrations of other binding proteins.<sup>33</sup> Thus, serum and local *IGFBP3* production appear to play a role in the *IGFBP3* effects on retinopathy.<sup>16</sup> *IGFBP3* has actions independent of *IGF-1*.<sup>34-37</sup> In vivo, *IGFBP3*, independent of *IGF-1*, helps to prevent oxygen-induced vessel loss and to promote vascular regrowth after vascular destruction in vivo, resulting in less retinal neovascularization.<sup>16</sup>

## A



## B



**FIGURE 5.** Intraretinal mRNA expression profiles of *IGF1* to *IGF7* in OIR compared with normoxic controls. (A) Mouse litters were exposed to 75% O<sub>2</sub> from P7 to P12. At P17, eyes were collected and cryosectioned, and laser-capture microdissection and laser-capture microdissection was used to enrich for GCL, INL, and ONL and for normoxic blood vessels and angiomatous tufts (Vessels/Tufts). RNA was isolated from samples and used for quantitative real-time PCR analysis. Target genes were normalized to 10<sup>6</sup> copies of *cyclophilin A*. (B) Data replotted for comparison of binding proteins within each retinal layer. Error bars represent ±SD.

A number of studies have examined the serum contribution of the IGF system by comparing levels in vitreous from patients with proliferative retinopathy to vitreous levels obtained from patients with nonproliferative eye disease. IGF-1, IGF-2, IGFBP2, and IGFBP3 were elevated in vitreous 1.5- to 13-fold with proliferative disease. These changes may partially be explained by leakage of serum into the vitreous because IGFs and IGFBPs are 20- to 50-fold higher in serum than in vitreous but may also reflect local production given that vitreous protein content was 1.5- to 5-fold elevated in ischemia patients compared with control subjects.<sup>18,38,39</sup> Our results suggest that local IGF-2, IGFBP2, IGFBP3, and IGFBP5 may contribute to vitreous levels because these are produced by blood vessels close to the vitreous and are increased with vasoproliferation.

Measurement of protein levels should enable further elucidation of mechanisms. Interpretation of mRNA results is limited by the purity of laser-captured cells, and mRNA levels may not reflect protein levels. Even low expression levels of some IGF components may have profound effects, and results must be interpreted in that light. However, this is the first study that examines the IGF system in retinopathy, and it improves our understanding of local IGF control with hypoxia and the development of retinopathy. We have shown the importance of local *IGF-2* production in blood vessels. We have also shown that *IGF-1R* is much more abundant than *IR* and is found almost exclusively in photoreceptors and blood vessels. This system may be part of the cross-talk between vessels and neurons during retinopathy. These studies will help form the basis of future studies of the IGF system in retinopathy.

## References

- Wilkinson-Berka JL, Wraight C, Werther G. The role of growth hormone, insulin-like growth factor and somatostatin in diabetic retinopathy. *Curr Med Chem*. 2006;13:3307-3317.
- Bergman PB, Moravski CJ, Edmondson SR, et al. Expression of the IGF system in normal and diabetic transgenic (mRen-2)27 rat eye. *Invest Ophthalmol Vis Sci*. 2005;46:2708-2715.
- Hellstrom A, Engstrom E, Hard AL, et al. Postnatal serum insulin-like growth factor I deficiency is associated with retinopathy of prematurity and other complications of premature birth. *Pediatrics*. 2003;112:1016-1020.
- Hellstrom A, Perruzzi C, Ju M, et al. Low IGF-I suppresses VEGF-survival signaling in retinal endothelial cells: direct correlation with clinical retinopathy of prematurity. *Proc Natl Acad Sci U S A*. 2001;98:5804-5808.
- Poulaki V, Joussen AM, Mitsiades N, Mitsiades CS, Iliaki EF, Adamis AP. Insulin-like growth factor-I plays a pathogenetic role in diabetic retinopathy. *Am J Pathol*. 2004;165:457-469.
- Ruberte J, Ayuso E, Navarro M, et al. Increased ocular levels of IGF-I in transgenic mice lead to diabetes-like eye disease. *J Clin Invest*. 2004;113:1149-1157.
- Chantelau E, Kimmeler R, Meyer-Schwickerath R. Insulin, insulin analogues and diabetic retinopathy. *Arch Physiol Biochem*. 2008;114:54-62.
- Kondo T, Vicent D, Suzuma K, et al. Knockout of insulin and IGF-I receptors on vascular endothelial cells protects against retinal neovascularization. *J Clin Invest*. 2003;111:1835-1842.
- Burren CP, Berka JL, Edmondson SR, Werther GA, Batch JA. Localization of mRNAs for insulin-like growth factor-I (IGF-I), IGF-I receptor, and IGF binding proteins in rat eye. *Invest Ophthalmol Vis Sci*. 1996;37:1459-1468.
- Hellstrom A, Carlsson B, Niklasson A, et al. IGF-I is critical for normal vascularization of the human retina. *J Clin Endocrinol Metab*. 2002;87:3413-3416.
- Hitchcock PF, Otterson DC, Cirenza PF. Expression of the insulin receptor in the retina of the goldfish. *Invest Ophthalmol Vis Sci*. 2001;42:2125-2129.
- Kuang H, Zou W, Liu D, et al. The potential role of IGF-I receptor mRNA in rats with diabetic retinopathy. *Chin Med J (Engl)*. 2003;116:478-480.
- Nakae J, Kido Y, Accili D. Distinct and overlapping functions of insulin and IGF-I receptors. *Endocr Rev*. 2001;22:818-835.
- Gary-Bobo M, Nirde P, Jeanjean A, Morere A, Garcia M. Mannose 6-phosphate receptor targeting and its applications in human diseases. *Curr Med Chem*. 2007;14:2945-2953.
- Murphy IJ. Insulin-like growth factor-binding proteins: functional diversity or redundancy? *J Mol Endocrinol*. 1998;21:97-107.
- Lofqvist C, Chen J, Connor KM, et al. IGFBP3 suppresses retinopathy through suppression of oxygen-induced vessel loss and promotion of vascular regrowth. *Proc Natl Acad Sci U S A*. 2007;104:10589-10594.
- Rosenfeld RG, Hwa V, Wilson E, Plymate SR, Oh Y. The insulin-like growth factor-binding protein superfamily. *Growth Horm IGF Res*. 2000;10(suppl A):S16-S17.
- Pfeiffer A, Spranger J, Meyer-Schwickerath R, Schatz H. Growth factor alterations in advanced diabetic retinopathy: a possible role of blood retina barrier breakdown. *Diabetes*. 1997;46:S26-S30.
- Smith LE, Wesolowski E, McLellan A, et al. Oxygen-induced retinopathy in the mouse. *Invest Ophthalmol Vis Sci*. 1994;35:101-111.
- Wang X, Seed B. A PCR primer bank for quantitative gene expression analysis. *Nucleic Acids Res*. 2003;31:e154.
- Zygar CA, Colbert S, Yang D, Fernald RD. IGF-I produced by cone photoreceptors regulates rod progenitor proliferation in the teleost retina. *Brain Res Dev Brain Res*. 2005;154:91-100.
- Rother KI, Accili D. Role of insulin receptors and IGF receptors in growth and development. *Pediatr Nephrol*. 2000;14:558-561.
- Barber AJ, Nakamura M, Wolpert EB, et al. Insulin rescues retinal neurons from apoptosis by a phosphatidylinositol 3-kinase/Akt-mediated mechanism that reduces the activation of caspase-3. *J Biol Chem*. 2001;276:32814-32821.
- Song J, Wu L, Chen Z, Kohanski RA, Pick L. Axons guided by insulin receptor in *Drosophila* visual system. *Science*. 2003;300:502-505.
- Brogio W, Stocker H, Ikeya T, Rintelen F, Fernandez R, Hafen E. An evolutionarily conserved function of the *Drosophila* insulin receptor and insulin-like peptides in growth control. *Curr Biol*. 2001;11:213-221.
- Schubert M, Gautam D, Surjo D, et al. Role for neuronal insulin resistance in neurodegenerative diseases. *Proc Natl Acad Sci U S A*. 2004;101:3100-3105.
- Louvi A, Accili D, Efstratiadis A. Growth-promoting interaction of IGF-II with the insulin receptor during mouse embryonic development. *Dev Biol*. 1997;189:33-48.
- Ritter MR, Dorrell MI, Edmonds J, Friedlander SF, Friedlander M. Insulin-like growth factor 2 and potential regulators of hemangioma growth and involution identified by large-scale expression analysis. *Proc Natl Acad Sci U S A*. 2002;99:7455-7460.
- Beattie J, Allan GJ, Lochrie JD, Flint DJ. Insulin-like growth factor-binding protein-5 (IGFBP5): a critical member of the IGF axis. *Biochem J*. 2006;395:1-19.
- de la Rosa EJ, Bondy CA, Hernandez-Sanchez C, et al. Insulin and insulin-like growth factor system components gene expression in the chicken retina from early neurogenesis until late development and their effect on neuroepithelial cells. *Eur J Neurosci*. 1994;6:1801-1810.
- Hauck SM, Gloeckner CJ, Harley ME, et al. Identification of paracrine neuroprotective candidate proteins by a functional assay-driven proteomics approach. *Mol Cell Proteomics*. 2008;7:1349-1361.
- Clemmons DR, Jones JJ, Busby WH, Wright G. Role of insulin-like growth factor binding proteins in modifying IGF actions. *Ann N Y Acad Sci*. 1993;692:10-21.
- Mohan S, Baylink DJ. Insulin-like growth factor system components and the coupling of bone formation to resorption. *Horm Res*. 1996;45(suppl 1):59-62.
- D'Ercole AJ, Ye P, O'Kusky JR. Mutant mouse models of insulin-like growth factor actions in the central nervous system. *Neuropeptides*. 2002;36:209-220.
- Mohan S, Baylink DJ. IGF-binding proteins are multifunctional and act via IGF-dependent and -independent mechanisms. *J Endocrinol*. 2002;175:19-31.
- Franklin SL, Ferry RJ Jr, Cohen P. Rapid insulin-like growth factor (IGF)-independent effects of IGF binding protein-3 on endothelial cell survival. *J Clin Endocrinol Metab*. 2003;88:900-907.
- Firth SM, Baxter RC. Cellular actions of the insulin-like growth factor binding proteins. *Endocr Rev*. 2002;23:824-854.
- Spranger J, Buhnen J, Jansen V, et al. Systemic levels contribute significantly to increased intraocular IGF-I, IGF-II and IGF-BP3 [correction of IFG-BP3] in proliferative diabetic retinopathy. *Hormone Metab Res*. 2000;32:196-200.
- Meyer-Schwickerath R, Pfeiffer A, Blum WF, et al. Vitreous levels of the insulin-like growth factors I and II, and the insulin-like growth factor binding proteins 2 and 3, increase in neovascular eye disease: studies in nondiabetic and diabetic subjects. *J Clin Invest*. 1993;92:2620-2625.