Rhodopsin Transgenic Pigs as a Model for Human Retinitis Pigmentosa

Zong-Yi Li, Fulton Wong, Jean H. Chang, Daniel E. Possin, Ying Hao, Robert M. Petters, and Ann H. Milam

PURPOSE. To further characterize the retinas of Pro347Leu rhodopsin transgenic pigs, a model for human retinitis pigmentosa.

METHODS. Retinas from normal and transgenic pigs, newborn to 20 months old, were processed for light and electron microscopic immunocytochemical examination.

RESULTS. At birth, rod numbers were normal in the transgenic retinas, but their outer segments were short and disorganized and their inner segments contained stacks of rhodopsin-positive membranes. The newborn rod synapses lacked synaptic vesicles and ribbons and had numerous rhodopsin-positive, filopodia-like processes that extended past the cone synapses into the outer plexiform layer. Rod cell death was apparent by 2 weeks and was pronounced in the mid periphery and central regions by 6 weeks. Far peripheral rods were initially better preserved, but by 9 months virtually all rods had degenerated. Cones degenerated more slowly than rods, but by 4 weeks the cone synapses were shrunken and some mid peripheral cones had lost their immunoreactivity for phosphodiesterase-β, arrestin, and recoverin. From 9 months to 20 months, the cone outer segments shortened progressively, and more cones lost immunoreactivity for these proteins.

CONCLUSIONS. The rhodopsin transgenic pig retina shares many cytologic features with human retinas with retinitis pigmentosa and provides an opportunity to examine the earliest stages in photoreceptor degeneration, about which little is known in humans. The finding of abnormal rhodopsin localization in newborn rods is consistent with misrouting of mutant rhodopsin as an early process leading to rod cell death. Novel changes in the photoreceptor synapses may correlate with early electrophysiological abnormalities in these retinas. (Invest Ophthalmol Vis Sci. 1998;39:808-819)

The inherited retinal dystrophy, retinitis pigmentosa, causes primary degeneration of rod photoreceptors, which is followed by slowly progressive degeneration of the cones. The cones are essential for daytime vision, and, in some cases, death of these critical cells leads to total blindness. In recent years, many studies have added information on the genetics of retinitis pigmentosa, with identification of causative mutations in a number of photoreceptor-specific genes, such as rhodopsin and peripherin-RDS.1 However, many gaps remain in our understanding of how these mutations lead to dysfunction and, ultimately, degeneration of the rods and then the cones. This information is essential for understanding the pathophysiology of retinitis pigmentosa and for establishing a basis from which therapies for this disease can be developed.

Much of our understanding of inherited retinal degeneration has come from the study of animal models for retinitis pigmentosa. These include naturally occurring inherited retinal degenerations in the dog,2-3 cat,4 chicken,5 rat, and mouse.6 In addition, genetically engineered animal models have been produced, including transgenic mice7-11 and rats.12 To meet the need for a retinitis pigmentosa model with a high percentage of cones and a human-sized eye, transgenic pigs were produced that carry a porcine Pro347Leu rhodopsin transgene.13 Similar to retinitis pigmentosa in patients with the same mutation,14-16 these pigs have early, severe rod degeneration that is nearly complete by 8 postnatal weeks.13 Some cones also degenerate in this early period, but most undergo slowly progressive death, with preservation of a monolayer of cones with tiny outer segments at 87 weeks of life. The article by Petters et al.13 emphasized that additional studies are needed to discern how the mutant rhodopsin gene causes early rod cell death. More specifically, it raised the question of the possible role of abnormal rhodopsin accumulation in the cells. Finally, further work is required to elucidate the secondary process of cone cell degeneration.

To gain additional insight into the process of rod and cone photoreceptor degeneration, we have evaluated the retinas of the rhodopsin transgenic pigs at various ages by light and electron microscopic immunocytochemical examination. Detailed immunocytochemical observations on the rods and cones at various ages indicated that the rhodopsin transgenic pigs have early, severe rod degeneration that is nearly complete by 8 postnatal weeks.13 Some cones also degenerate in this early period, but most undergo slowly progressive death, with preservation of a monolayer of cones with tiny outer segments at 87 weeks of life. The article by Petters et al.13 emphasized that additional studies are needed to discern how the mutant rhodopsin gene causes early rod cell death. More specifically, it raised the question of the possible role of abnormal rhodopsin accumulation in the cells. Finally, further work is required to elucidate the secondary process of cone cell degeneration.

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Tissue samples from the central, superior mid and far periphery of the retinas were treated with 1% sodium borohydride, cryosectioned at 12 μm, and processed for indirect immunofluorescence analysis using published methods. Control sections were treated in the same way but with the omission of the primary antibody. The sections were examined with a microscope (Nikon, Tokyo, Japan) equipped for epifluorescence or with a laser scanning confocal microscope (MRC-600; Bio-Rad, Richmond, CA). Scanned images and confocal files were imported into a graphics program (Photoshop 4.0; Adobe, San Jose, CA), and dye sublimation prints were generated.

Antibodies

Cell-specific markers were used to analyze the various retinal cell types. Rods were identified with anti-rhodopsin: monoclonal antibody (mAb) 1D4 (1:40) from R. Molday (University of British Columbia, Vancouver, Canada); and a polyclonal antibody (pAb) (1:10,000) from E. Kean (Case Western Reserve University, Cleveland, OH). Photoreceptor outer segments and cytoplasm were labeled with pAb anti-phosphodiesterase γ (PDE-γ) (1:2,000 to 1:10,000; B. K. Fung, University of California at Los Angeles), pAb anti-recoverin (1:100; A. Dizhoor, University of Washington, Seattle), and pAb anti-arrestin (1:500; H. Shichi, Wayne State University, Detroit, MI). Cone outer segments were identified with pAb anti-red/green or anti-blue cone opsin (1:5,000; J. Nathans, Johns Hopkins University, Baltimore MD), pAb anti-red/green cone opsin (1:200; J. Saari, University of Washington, Seattle), or biotinylated peanut agglutinin lectin (PNA) (1:200 to 1:400; Vector Laboratories, Burlingame, CA). Rod and cone outer segments were labeled with mAb 3B6 against rd6-peripherin (undiluted; R. Molday). Photoreceptor synapses were labeled with mAb anti-synaptophysin (1:200; Sigma Chemical, St. Louis, MO) and mAb anti-SV2 protein (1:400; K. Buckley, Harvard University, Boston, MA). The retinal pigment epithelium (RPE) and Müller cells were labeled with mAb anti-cellular retinaldehyde binding protein (CRALBP) (1:200; J. Saari). Intermediate filaments in astrocytes and Müller cells were identified with anti-gial fibrillary acidic protein (GFAP) (pAb: 1:400; Dako, Carpinteria, CA; mAb: 1:10; Boehringer Mannheim Biochemical Products, Indianapolis, IN). The interphotoreceptor matrix and cone matrix sheaths were labeled with biotinylated PNA, and the interphotoreceptor matrix was also labeled with pAb anti-interphotoreceptor retinoid binding protein (IRBP) (1:500; J. Saari). Basic fibroblast growth factor (bFGF) was detected with pAb anti-bFGF (1:773 directed against amino acids 1-24 of human bFGF; 1:500; A. Baird, Prizm Pharmaceuticals, San Diego, CA, and A. Hanneken, Scripps Research Institute, La Jolla, CA). Fibrous actin in the external limiting membrane was labeled using fluorescein isothiocyanate-phallolidin (10-20 mg/ml, Sigma), and the transgenic rod synapses were evaluated using anti-GAP-43, a marker for growth cones (1:1000; Boehringer Mannheim Biochemical Products).

Cell-specific primary antibodies were localized using secondary antibodies labeled with fluorescein isothiocyanate (green), Cy-2 (green), or Cy-3 (red) (Jackson ImmunoResearch Laboratories, West Grove, PA). The biotinylated-PNA was localized with fluorescein isothiocyanate-avidin (Jackson ImmunoResearch Laboratories). In some sections, nuclei were counterstained (blue) with 4',6'-diamidino-2-phenylindole (1 mg/ml; Molecular Probes, Eugene, OR) added to the mixture of secondary antibodies.

**TABLE 1. Features of Pig Retinas Used in Study**

<table>
<thead>
<tr>
<th>Age</th>
<th>Normal No. of Retinas</th>
<th>TG No. of Retinas</th>
<th>TG Genotype</th>
<th>Fixative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Newborn</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>A</td>
</tr>
<tr>
<td>2 weeks</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>A</td>
</tr>
<tr>
<td>3 weeks</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>A</td>
</tr>
<tr>
<td>4 weeks</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>A</td>
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<td>5 weeks</td>
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<td>6 weeks</td>
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<td>1</td>
<td>1</td>
<td>A</td>
</tr>
<tr>
<td>8 weeks</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>B</td>
</tr>
<tr>
<td>4 months</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>B</td>
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<tr>
<td>6 months</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>B</td>
</tr>
<tr>
<td>9 months</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>C</td>
</tr>
<tr>
<td>20 months</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>B</td>
</tr>
<tr>
<td>Total</td>
<td>21</td>
<td>26</td>
<td></td>
<td></td>
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</tbody>
</table>

*Fixatives used: A, 4% paraformaldehyde (P) + 0.5% glutaraldehyde (G); B, 1% F + 1.25% G; C, 4% P.

TG, transgenic.
Electron Microscopy
Retinal samples from newborn, 2-, 4-, and 6-week-old pigs were postfixed in 1% OsO₄ in phosphate buffer and embedded in Medcast (Ted Pella, Redding, CA). Ultrathin sections were stained with uranyl acetate and lead citrate. Samples were also treated with sodium borohydride,²¹ embedded in LR-White (Ted Pella), and processed for immunocytochemical examination using the secondary antibody labeled with 5-nm gold particles and silver enhancement.¹⁸ Control sections were processed in the same way but with the omission of the primary antibody.

Morphometric Analysis
Color prints were prepared of sections labeled with anti-rhodopsin and counterstained with 4',6'-diamidino-2-phenylindole. For pigs 2, 4, and 6 weeks old, three each of normal and transgenic retinas were analyzed morphometrically. For newborn pigs, one normal and three transgenic retinas were analyzed. All rhodopsin-positive outer nuclear layer (ONL) cells were counted within 0.35-mm segments of retina in the superrior quadrant at intervals of 2, 4, 6, 8, 10, and 12 mm from the ora serrata.

RESULTS
Normal Pig Retinas
Rod Photoreceptors. The ONL of the newborn pig contained the same number of rod nuclei as the ONL of the fully developed pig (4 months)—four to five layers in the far periphery and four to six layers in the mid periphery and central retina. However, the rod outer segments in newborn retinas were shorter (approximately 12 μm) than the rod outer segments at 4 months (approximately 22 μm). At all ages, the rod outer segments were strongly labeled with anti-rhodopsin, and the rod inner segments and somata were weakly labeled (Figs. 1A, 1E). Rhodopsin was also present in elongated Golgi complexes in the rod inner segments (Fig. 1D). These observations are similar to rhodopsin localization in normal human rod photoreceptors. However, in normal pigs, the somata and bulb-shaped synapses (spherules) of a few rods and displaced rods in the inner nuclear layer were immunoreactive for rhodopsin (Fig. 1A). Displaced rods, although infrequent, were more numerous in younger (newborn to 6-week-old) animals and in peripheral than in central retina.

Starting from birth, the rod spherules closely resembled those of human rods and formed a row just scleral to the cone synapses in the outer plexiform layer (OPL) (Fig. 2A). The rod spherules were immunoreactive for the synaptic vesicle proteins, synaptophysin, and SV-2. From birth to 4 weeks, the OPL showed a punctate pattern of PNA labeling.

Specific labeling of the rods by several other photoreceptor-specific antibodies were observed: outer segments (anti-red/green and rod outer segments, somata and spherules (anti-PDE-γ, anti-arrestin, and anti-recoverin). Unlike most normal human peripheral rods,²³ porcine rods were bFGF negative (Fig. 3A).

Cone Photoreceptors. The normal porcine retina contains a large number of cones,²³ the nuclei of which form a monolayer most scleral in the ONL. Two populations of cones were identified on the basis of outer segments labeled with anti-red/green (Fig. 3E) and anti-blue cone opsin. The outer segments of the red/green cones were longer (approximately 13 μm) than those of the blue cones (approximately 10 μm).

At all ages studied, the cone cell compartments (outer segments, inner segments, somata, and synaptic pedicles) were immunoreactive throughout for PDE-γ (Fig. 3C), arrestin, and recoverin, and the cone pedicles were immunoreactive for synaptophysin and SV-2. The porcine cones, like the rods, were bFGF negative (Fig. 3A).

Interphotoreceptor Matrix. The interphotoreceptor matrix surrounding the rod and cone outer segments in the subretinal matrix was IRBP immunoreactive and PNA positive, and the cone matrix sheaths were PNA positive (Fig. 3C).

Glial Cells. The astrocytes and Müller cells showed a filamentous pattern of labeling with anti-GFAP, and their nuclei were strongly bFGF immunoreactive (Fig. 3A). All regions of the Müller cells were CRALBP immunoreactive, and fibrous
2. Rod and cone synapses in porcine retinas. (A) Rod spherules (arrows) and a cone pedicle (C) in a 6-week normal pig retina. The cone pedicles lie vitreal to the rod spherules. R, rod nucleus. Original magnification, ×8820. (B) Six-week transgenic retina of type 1 genotype. The rod synapses, which contain few vesicles and no synaptic ribbons, are enlarged with numerous surface protrusions (arrows) corresponding to the filopodia-like structures observed by immunofluorescence. No normal rod spherules are present in the outer plexiform layer in this or any other transgenic retina. Original magnification, ×17,500. (C) Immunogold labeling of rhodopsin in 6-week-old transgenic retina, type 1 genotype. Rhodopsin is delocalized to the surface membranes (small arrows) of the rod somata and filopodia-like structures (arrows) that extend vitreal to the cone pedicles. Some rod processes contact the cone synapses (arrowheads). M, Müller cell cytoplasm. Original magnification, ×9525. (D) Cone pedicles in a 6-week transgenic retina, type 1 genotype. The cone pedicles (arrows) are narrower than normal and some have increased electron density. The spaces between the shrunken cone pedicles are occupied by electron-lucent Müller cell processes (M). Original magnification, ×6858.
actin in the external limiting membrane was phalloidin positive.

Retinal Pigment Epithelium. The cytoplasm of the RPE cells was CRALBP immunoreactive, and their nuclei were bFGF positive (Fig. 3A). The choriocapillaris was intact throughout the eye.

Rhodopsin Transgenic Pig Retinas

Rod Photoreceptors. Regional differences in rod cell degeneration were examined. The number of rods in the central, mid and far peripheral regions of the transgenic retinas was near normal at birth, but their outer segments were disorganized and shorter than normal (transgenic, approximately 7 μm; normal, 12 μm) (Fig. 4A). By 2 weeks, rod cell loss was evident in all retinal regions, and the remaining rod outer segments were shorter than normal (transgenic, approximately 7 μm; normal, approximately 14 μm). Regional differences in rod cell degeneration were apparent at 4 weeks, and the mid peripheral and central rods were degenerate by 6 weeks, with relative sparing of the rods in the far periphery (within 4 mm of the ora serrata). Figure 5 summarizes the regional differences in rod degeneration from birth to 6 weeks of age. At 4 months of age (Fig. 3F) only one to two layers of rods remained in the ONL throughout the retina, and at 9 months (Fig. 3G) only scattered rods were retained. No rods survived at 20 months (Fig. 3H).

We examined the abnormalities in rhodopsin distribution by electron microscopy and found that the newborn transgenic rod outer segments were disorganized (Fig. 4A), and scattered degenerating rods had dense cytoplasm. In addition to the abnormal outer segments, rhodopsin immunoreactivity was present in the inner segments and somata of most transgen-
genomic rods at birth (Fig. 1F) and in all rods thereafter. The transgenic rods (Fig. 1F) lacked the prominent rhodopsin-positive Golgi complexes found in the inner segments of normal rods (Fig. 1E). Instead, rhodopsin-immunoreactive tubulovesicular structures, which resembled endoplasmic reticulum, were present in the cytoplasm of the transgenic rods (Fig. 1F).

Rhodopsin immunoreactivity was also found in abnormal inclusions in the inner segments of scattered rods from birth to 6 weeks (Figs. 1F, 4B, 6A, 6B). These inclusions were most prominent in the newborn transgenic rods and consisted of rhodopsin-immunoreactive membrane stacks that resembled those in the rod outer segments (Fig. 4B), except these stacks were rdS-peripherin negative. Similar inclusions were found in nearby Müller cells (Fig. 4C). In older animals, the rod inner segment inclusions were membrane bounded and had dense, heterogeneous contents (Fig. 6A) that were rhodopsin positive by immunogold labeling (Fig. 6B). These rhodopsin-immunoreactive inclusions usually occurred singly and were also found in the processes of nearby Müller cells. On the basis of their fine structure, these electron-dense inclusions in the rod inner segments can be identified as autophagosomes,24 whereas those in Müller cells can be interpreted as phagocytosed rhodopsin-immunoreactive debris from nearby degenerating rod cells.

We observed that rhodopsin immunoreactivity was also prominent in the transgenic rod synapses, which differ from the normal bulb-shaped rod spherules because of their delicate surface protrusions resembling growth cone filopodia that extend vitreal to the cone synapses in the OPL (Figs. 1B, 1C, 1D, 1F, 2B, 2C). These rhodopsin-immunoreactive protrusions were prominent from birth onward but, unlike filopodia on axonal growth cones, were negative for fibrin actin. The transgenic rod synapses were also negative with the growth cone marker, anti-GAP-43.

Electron microscopic examination revealed that the rod spherules from birth onward were replaced by larger membranous structures with numerous rhodopsin-positive processes, corresponding to the filopodia identified by immunofluorescence (Figs. 2B, 2C). Some of these rhodopsin-positive processes extended past the cone synapses in the OPL (Fig. 2C). These abnormal rod synapse structures lacked synaptic vesicles and ribbons and did not make recognizable contact with the postsynaptic processes. Few normal rod spherules were found among these abnormal synapse structures from birth onward.

Peripheral rods in human retinas with retinitis pigmentosa undergo robust sprouting of rhodopsin-positive, axon-like neurites.18 We searched for comparable rod neurites in the transgenic retinas but identified only a few in newborn to 4-week-old animals. These rod neurites were beaded and branched and extend through the inner nuclear layer into the inner plexiform layer (Fig. 1B).

The transgenic rod outer segments and cytoplasm at birth were positive for PDE-7, recoverin, and arrestin. However, by 2 weeks, some rods in the mid periphery had lost immunoreactivity for these proteins. By 9 months, the few remaining rods lacked outer segments and were reduced to rhodopsin-positive somata (Fig. 3C), which lacked immunoreactivity for PDE-7, recoverin, and arrestin. As in normal pig retinas, the OPL showed a punctate pattern of PNA labeling from birth to 4 weeks; however, unlike normal retinas, this punctate pattern persisted in the transgenic retinas until 20 months (Fig. 3D). Most rods in the periphery of human retinas with retinitis pigmentosa are bFGF positive,25 but the transgenic rods were consistently bFGF negative (Fig. 3B).

Cone Photoreceptors. In the period of active rod cell death, from 2 to 4 weeks, near normal numbers of red/green
FIGURE 6. Electron microscopic examination of rod inner segment inclusions. C, cone inner segments. (A) Rod inner segment inclusions (arrows) in a 6-week transgenic retina (type 1 genotype) are electron dense and membrane bounded. (*) External limiting membrane. Original magnification, ×9800. (B) Immunogold labeling in a 6-week transgenic retina (type 1 genotype). The rod inner segment inclusions are rhodopsin immunoreactive (arrow). The rod inner segment (R) is identified by the rhodopsin immunoreactivity of the surface membrane (arrowheads). M, mitochondria in cone inner segment. (*) External limiting membrane. Original magnification, ×9800.

and blue cones were retained. At 4 months, the cones had outer segments of normal length, as visualized by immunolabeling (Figs. 3E, 3F). By 9 months, the cone outer segments were shorter (Fig. 3G) (transgenic red/green cone outer segments: approximately 6 μm [normal, approximately 13 μm]; transgenic blue cone outer segments: approximately 5 μm [normal, approximately 10 μm]). At 20 months, the cone outer segments were tiny, and most were immunoreactive for red/green cone opsin (Fig. 3H); only a few were immunoreactive for blue cone opsin.

After 6 weeks, most cones in the central and far peripheral retina remained immunoreactive for PDE-γ, arrestin, and recoverin, but scattered cones in the mid periphery (4 to 8 mm from the ora serrata) had lost their immunoreactivity for these proteins (Fig. 3D). At 9 and 20 months, more cones in the mid periphery, but not elsewhere, lacked immunoreactivity for PDE-γ, arrestin, and recoverin. The cone pedicles remained synaptophysin and SV-2 positive at all times, and the cones were consistently bFGF negative (Fig. 3B), as in human retinas with retinitis pigmentosa.

Electron microscopic examination of the 4-week-old transgenic retinas revealed that most cones have cytoplasm of normal density, but the cytoplasm of occasional mid peripheral cones was electron dense. The cone synapses were shrunken by 6 weeks, and some had increased electron density (Fig. 2D). Müller cell processes expanded to occupy the spaces left after rod cell degeneration and cone synapse shrinkage (Fig. 2D).

Interphotoreceptor Matrix. The subretinal space shrinks as the rod and cone outer segments shorten. At 9 months, when virtually all rods had been lost, the interphotoreceptor matrix remained positive for PNA and IRBP, and the matrix sheaths of the short cone outer segments were PNA positive.

Glial Cells. The astrocytic and Müller glial cells in the transgenic retinas were not markedly different from those in normal porcine retinas. At 2 to 4 weeks, Müller cell labeling intensity with anti-GFAP in the transgenic retinas was slightly increased, but labeling with anti-CRALBP was the same at all ages. Unlike human retinas with retinitis pigmentosa,17,19 the older transgenic retinas (6 weeks to 20 months) did not show an increase in GFAP labeling in the Müller glia (Fig. 3B). As in normal porcine retinas, normal human retinas, and human retinas with retinitis pigmentosa,17,19 the nuclei of the astrocytes and Müller cells were bFGF positive (Fig. 3B). The external limiting membrane in the transgenic retinas remained intact and phalloidin positive at all ages examined.

Retinal Pigment Epithelium. In human retinitis pigmentosa,19 retinal regions in which all photoreceptors have degenerated usually show RPE cell migration into the inner retina. In even the oldest transgenic pigs, no RPE cell migration into the retina was noted, which correlates with the retention of a monolayer of cone somata. The cytoplasm of the RPE cells remained CRALBP immunoreactive, and their nuclei were bFGF positive (Fig. 3B). No alterations were found in the choriocapillaris.

DISCUSSION

As in human patients with retinitis pigmentosa caused by the Pro347Leu rhodopsin mutation,14,15 the transgenic pigs...
showed early loss of their rods and slowly progressive degeneration of their cones. Because the normal pig eye is similar to that of the human in size and percentage of cones, it was suggested in the initial report on the rhodopsin transgenic pigs\textsuperscript{15} that these animals constitute a useful animal model for retinitis pigmentosa in humans. Because cellular changes in retinas of patient donors with retinitis pigmentosa have been well documented by light and electron microscopic immunocytochemistry, \textsuperscript{17}~\textsuperscript{21}~\textsuperscript{26} we used these techniques to analyze the cellular alterations in the transgenic pig retinas and to compare them with human retinitis pigmentosa.

In the pigs, the loss of rods was evident by 2 weeks and severe in the central and mid peripheral regions, by 6 weeks, with relative sparing of the far periphery at this time (Fig. 5). Rod cell death was progressive. By 9 months, only scattered rods remained throughout the retina and by 20 months, all had died. The underlying cause of the early geographic pattern of rod (and cone) degeneration is unknown, but it resembles closely that found in many patients with retinitis pigmentosa.\textsuperscript{20}

The transgenic pig and human retinitis pigmentosa rods, along with those of transgenic mice with Pro347His\textsuperscript{9} and Gln344Arg\textsuperscript{10} rhodopsin mutations, all have short outer segments and delocalization of rhodopsin immunoreactivity to the surface membranes of their inner segments, somata, and synapses. As in human retinitis pigmentosa rods,\textsuperscript{27} other early abnormalities in the transgenic pig rods are lack of rhodopsin-immunoreactive Golgi complexes and presence throughout the cytoplasm of numerous rhodopsin-immunoreactive tubulovesicular structures interpreted as profiles of endoplasmic reticulum. The absence of rhodopsin-immunoreactive Golgi organelles in the transgenic rods suggests that protein trafficking is defective in these cells, presumably a result of the presence of mutant rhodopsin. This interpretation is supported by the finding of inner segment inclusions resembling stacks of outer segment membranes in newborn transgenic rods, which have disorganized outer segments. Similar membrane inclusions were found in\textit{Drosophila} photoreceptors expressing mutant rhodopsin,\textsuperscript{28} and hypothesized to represent mutant and wild type rhodopsin blocked from transport out of the endoplasmic reticulum. Verification of this hypothesis for the transgenic pig rods awaits development of an antibody specific for Pro347Leu porcine rhodopsin.

The large, electron-dense, rhodopsin-immunoreactive inclusions in the inner segments of some transgenic rods from birth to 6 weeks, like those in human retinas with retinitis pigmentosa,\textsuperscript{29} may represent autophagosomes for degradation of rhodopsin not recognized for transport to the outer segments. Extracellular rhodopsin-immunoreactive vesicles, as noted in transgenic mice with the Pro347Ser mutation,\textsuperscript{14} have not been found in the transgenic pig rods at any stage of degeneration.

In addition to outer segment and organelle abnormalities, the transgenic pig rods show a remarkable alteration in their synapses from birth onward. Although early accumulation of rhodopsin in synapses has been noted in rhodopsin transgenic mice,\textsuperscript{3} we are unaware of synapse abnormalities comparable to those in the transgenic pig rods. Although normal porcine rod spherules, like those of humans, have a regular bulb shape and form a row just scleral to the cone pedicles in the OPL, the transgenic pig rod synapses are enlarged and their surface membranes are greatly expanded by the presence of rhodopsin-positive protrusions resembling filopodia.

Filopodia are surface protrusions on growth cones, the motile tips of elongating axons and neurites, and are thought to function for signal detection, guidance, and movement.\textsuperscript{30} In many neuron types, the growth cone filopodia are phalloidin positive, indicating that they contain fibrous actin,\textsuperscript{31} and the elongating axon tips are positive for GAP-43, a protein associated with growth cones.\textsuperscript{32} However, the transgenic rod synapses with filopodia-like protrusions are negative for fibrous actin and GAP-43, suggesting that they are not true growth cones. What is the significance of these novel structures? At present we can only speculate. Because rhodopsin delocalization is an early change in the transgenic rods, accumulation of excess rhodopsin in the synapse membranes may lead to filopodia formation by an unknown mechanism. The filopodia represent an increase in synapse surface membrane area so may also form as a default pathway for excess surface membrane generated when mutant rhodopsin cannot be inserted into the outer segment. Whatever their cause, it seems likely that these synaptic abnormalities, including lack of synaptic vesicles, ribbons, and membrane specializations, account for the early absence of recordable rod ERG b-waves in the transgenic pigs.\textsuperscript{13}

After initial rod cell loss in \textit{rdx} mice, the remaining rod synapses enlarge and accumulate synaptic ribbons.\textsuperscript{33} However, to our knowledge, filopodia-like protrusions on rod synapses have not been noted previously in a dystrophic human or animal retina. Filopodia form in other types of neurons in response to electrical stimulation\textsuperscript{34} and increases in intracellular calcium,\textsuperscript{35} but it is unknown how such changes relate to the abnormalities in the transgenic pig rod synapses.

Rods in the periphery of human retinas with retinitis pigmentosa sprout prominent neurites, rhodopsin-positive processes that project through the inner retinal layers to the inner limiting membrane.\textsuperscript{18} Filopodia are thought to represent the first step in neurite formation by mature amphibian rods in culture,\textsuperscript{36} so the filopodia-like structures on the rod synapses may be related to the neurite sprouting noted in the transgenic pig retinas. However, the abnormal synapses persist as long as the rods survive, and few rod neurites are found in these retinas. The relative paucity of rod neurites in the transgenic pig retinas may be a result of the lack of a robust gliotic response by the Müller cells\textsuperscript{16} or of the absence of bFGF in the porcine rods, a factor present in rods in human retinitis pigmentosa retinas\textsuperscript{25} and associated with neurite sprouting in other neuron types. The paucity of neurites may also reflect the rapid rate of rod cell death in the transgenic pig retinas, whereas rods in the periphery of human retinas with retinitis pigmentosa typically survive for many decades.

Transgenic cones also show a regional pattern of degeneration, with cytoplasmic densification observed in some mid peripheral cones by 4 weeks. Cone cell death is slowly progressive thereafter, but a monolayer of cone somata persists throughout the retina at 20 months of age. In addition to the shortening of outer segments, as occurs in cones of patients with retinitis pigmentosa caused by rhodopsin mutations,\textsuperscript{17}~\textsuperscript{19}~\textsuperscript{20} some mid-peripheral transgenic cones have lost immunoreactivity for the cytoplasmic proteins, PDE-γ, arrestin, and recoverin by 6 weeks. This abnormality in the cones, like that in rods in their early stages of degeneration, becomes more widespread at 9 months and 20 months. The loss of immunoreactivity for these phototransduction proteins suggests that protein synthesis is decreased in the cones, and this
correlates well with the observed outer segment shortening. Immunoreactivity for PDE-γ is also decreased in photoreceptors in experimental retinal detachment,\textsuperscript{37} for PDE-γ and arrestin in prcd retinas,\textsuperscript{5} and for α-transducin after damage from light.\textsuperscript{38} However, a similar loss of immunoreactivity has not been noted in degenerate rods or cones in human retinas with retinitis pigmentosa, even though these cells also have shortened outer segments. Further information is needed on photoreceptor gene expression and its control to understand the degenerative changes that precede the final death of these critical cells.

It was suggested that rods secrete a factor essential for cone cell survival,\textsuperscript{39} and, based on the known degeneration of cones that occurs in vitamin A deficiency, it was hypothesized\textsuperscript{19} that IRBP, a major component of the interphotoreceptor matrix secreted mainly by the rods,\textsuperscript{40,41} may represent such a survival factor. Support for this idea came from the observation that some human retinas with retinitis pigmentosa lack IRBP immunoreactivity in regions in which rod cells are missing and the surviving cones have shortened outer segments.\textsuperscript{19} However, areas of rod cell loss in the transgenic pig retinas show normal IRBP immunoreactivity in the interphotoreceptor matrix around surviving cones. This suggests that IRBP loss does not account for cone outer segment shrinkage and cell death, although it is unknown whether the IRBP in the degenerate transgenic retinas carries normal levels of vitamin A. It is anticipated that an increased understanding of the cause and mechanism of cone degeneration in the transgenic pig retinas will be useful for developing therapy for cones in human retinas with retinitis pigmentosa that undergo a similar pattern of degeneration.\textsuperscript{20}

Information is unavailable on the morphology of mutant rods at birth in retinas of patients with retinitis pigmentosa. The observation that the newborn transgenic pig rods have disorganized outer segments, rhodopsin delocalization, abnormal rhodopsin-positive inner segment inclusions, and synapse filopodia indicates that prenatal expression of mutant rhodopsin has a significant effect on these cells. In addition to altering their function, the early changes in the transgenic rod synapses may inhibit synapse formation with second-order neurons. It remains to be determined whether failure to make appropriate synaptic connections also affects the viability of the mutant rods, as is known to occur in other neuron types.\textsuperscript{42-45}

In summary, the many similarities between the retinas of transgenic pigs and those of patients with retinitis pigmentosa validate the use of this animal model for studying mechanisms of rod and cone cell death and testing new therapies. This model should also be valuable for correlating functional changes in degenerating retinas with the histopathologic changes found in the involved cells, offering new insight into the basis of visual function abnormalities in patients with retinitis pigmentosa.

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References


