A Pro23His Mutation Alters Prenatal Rod Photoreceptor Morphology in a Transgenic Swine Model of Retinitis Pigmentosa

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PURPOSE. Functional studies have detected deficits in retinal signaling in asymptomatic children from families with inherited autosomal dominant retinitis pigmentosa (RP). Whether retinal abnormalities are present earlier during gestation or shortly after birth in a subset of children with autosomal dominant RP is unknown and no appropriate animal RP model possessing visual function at birth has been available to examine this possibility. In a recently developed transgenic P23H (TgP23H) rhodopsin swine model of RP, we tracked changes in pre- and early postnatal retinal morphology, as well as early postnatal retinal function.

METHODS. Domestic swine inseminated with semen from a TgP23H miniswine founder produced TgP23H hybrid and wild type (Wt) littermates. Outer retinal morphology was assessed at light and electron microscopic levels between embryonic (E) and postnatal (P) day E85 to P3. Retinal function was evaluated using the full field electroretinogram at P3.

RESULTS. Embryonic TgP23H rod photoreceptors are malformed and their rhodopsin expression pattern is abnormal. Consistent with morphological abnormalities, rod-driven function is absent at P3. In contrast, TgP23H and Wt cone photoreceptor morphology (E85–P3) and cone-driven retinal function (P3) are similar.

CONCLUSIONS. Prenatal expression of mutant rhodopsin alters the normal morphological and functional development of rod photoreceptors in TgP23H swine embryos. Despite this significant change, cone photoreceptors are unaffected. Human infants with similarly aggressive RP might never have rod vision, although cone vision would be unaffected. Such aggressive forms of RP in preverbal children would require early intervention to delay or prevent functional blindness.

Keywords: swine, retinitis pigmentosa, photoreceptor morphology, electron microscopy, electroretinography

Retinitis Pigmentosa (RP) is a group of inherited retinal disorders caused by mutations in genes (available in the public domain at www.sph.uth.tmc.edu/RetNet) most frequently expressed in rod photoreceptors. In humans, RP causes a primary degeneration of rod photoreceptors, whereas cone photoreceptor degeneration is protracted.¹ As a consequence, the most frequent first reported symptom of RP is impaired night vision. Most patients with RP report symptoms between adolescence and late middle age.^{2,3} Whether retinal abnormalities are present during gestation and/or shortly after birth in a subset of subjects with RP is unknown, although ERGs have detected visual deficits in asymptomatic children from families with both autosomal dominant (aged 6–8 years)^{4,5} and sex-linked RP (aged 5–13 years).⁶ It may be that many children with RP experience degraded vision at or shortly after birth, but deficits cannot be articulated until they are cognizant that night blindness and/or constriction of their peripheral visual fields are abnormal.

What we know about potential early postnatal changes in RP is limited by a lack of postnatal testing and appropriate animal models that have visual function at birth, similar to man (e.g.,

the rodent retina is immature at birth and reaches maturation over the first postnatal month⁷). We show here that a recently developed transgenic P23H (TgP23H) rhodopsin swine model of RP⁸ is an appropriate model. We show that embryonic TgP23H rod photoreceptors are malformed, their rhodopsin expression pattern is abnormal, and rod-driven function is absent at P3. In contrast, cone photoreceptor morphology (E85–P3) and cone-driven retinal function (P3) are similar between TgP23H and Wt retinæ.

MATERIALS AND METHODS

Swine

All experimental protocols were approved by the University of Louisville Institutional Animal Care and Use Committee and adhere to the ARVO Statement for Use of Animals in Ophthalmic and Vision Research. Methods used for genotyping the offspring have been described previously.⁸ To generate large numbers of progeny, we artificially inseminated multiple Wt domestic sows with semen from TgP23H miniswine

founder 53-1.⁸ Shortly after birth, a blood sample was taken from each piglet and DNA was isolated using a phenol-extraction method.⁹ The DNA was used in PCR with primers specific to the human rhodopsin transgene and under reaction conditions that have been described previously.⁸ Retinal function of most piglets was evaluated with the experimenters masked to the piglet's genotype.

Retinal ERG

Methods to anesthetize and prepare P3 piglets for recording the full-field electroretinogram (ffERG) have been described in our companion study.¹⁰ Briefly, prior to ffERG recordings, anesthesia with isoflurane was induced in piglets by placing a mask over the snout and mouth. An IV catheter was placed in the ear vein for delivery of intravenous fluids and to maintain normal glycemic levels (60–140 mg/dL; with lactated Ringer's solution with 5% dextrose). Anesthesia was maintained with isoflurane (1%–3%)¹¹ by continuous delivery through the mask. Vital signs (SpO₂, CO₂, respiratory rate, heart rate, and blood pressure) and body temperature were monitored and maintained within the normal range throughout the experiment. Topical applications of 2.5% phenylephrine hydrochloride and 1% tropicamide drops were administered to induce mydriasis of the pupils and to inhibit accommodation. The cornea was kept moist with 2.5% hypromellose solution. Size-appropriate, adjustable lid specula held the eyelids open.

The details of ffERG recording have been published previously.^{8,12} Stimuli (flashes of various intensities) were produced and responses recorded using an ERG system (UTAS with a BigShot Ganzfeld stimulator; LKC Technologies, Inc., Gaithersburg, MD, USA). Testing conditions and response analyses have been described previously.¹² Piglets were dark-adapted for 20 minutes and the scotopic ERG recorded first, using a strobe flash intensity of 0.01 cd/m². An averaged response was based on 15 presentations with a 2-second interstimulus interval. To measure cone-driven responses, the retinas were adapted and the stimulus (either a 3 cd/m² flash or 30-Hz flicker) was presented 30 times on a 20 cd/m² adapting background. Measures of a- and b-waves were obtained from averaged responses to a single flash intensity. The a-wave is defined as baseline to trough and the b-wave from a-wave trough to peak.

Retinal Morphology

Embryonic tissue (E85–E105) was retrieved via cesarean section of pregnant sows after induction of anesthesia and euthanasia. The gestation period of the pig is 114 to 116 days.¹³ Embryos also received an injection of euthanasia solution (Beuthanasia-D1 mL/5 kg; Merck Animal Health, Summit, NJ, USA). For postnatal tissue, piglets were euthanized (1 mL/5 kg IV; Merck Animal Health) at the end of the ERG evaluations. From all, eyes were enucleated and prepared for morphological analysis by immediate immersion in fixative (4% paraformaldehyde for immunohistochemistry or 2% paraformaldehyde/2% glutaraldehyde for ultrastructural analyses both in phosphate (PO₄) buffer (0.1 M, pH 7.4) for 24 hours at 4°C.

The results are from a total of 31 eyes at two embryonic ages and at two postnatal ages (See Table). The embryonic ages chosen reflect preliminary studies of photoreceptor development (i.e., E85: the earliest detection of photoreceptor inner segments; E105: the earliest detection of rod and cone outer segments and synaptic terminals in the outer plexiform layer; Scott, unpublished observations, [2013]).

TABLE. Number of Swine Eyes Examined at Each Age

Genotype/Age	E85	E105	P0	P3
Wt	4	3	4	4
Tg	4	5	4	3

Morphometric Analysis of the Outer Retina

Plastic sections were prepared as previously described.¹⁴ Briefly, vertical and horizontal bands of fixed retinal tissue extending from the margin of the optic disc to the ora serrata were dissected, dehydrated in ascending ethanol concentrations, infiltrated, and embedded in resin (JB-4 Plus; Ted Pella, Redding, CA, USA). Sections 4-μm thick were cut on a ultramicrotome (Leica EMUC6 Ultramicrotome; Leica Microsystems, Buffalo Grove, IL, USA), mounted on slides, dried, and stained with 1% cresyl violet (Sigma-Aldrich, St. Louis, MO, USA). Sections were examined at ×40 or ×100 using a commercial microscope (NIKON EFD-3 Episcopic-Fluorescence; Nikon, Inc., Melville, NY, USA). Photomicrographs were taken on a high-resolution camera (Moticam 2500; Motic, British Columbia, Canada) and digitally processed using a graphics editing program (Adobe Photoshop; Adobe Systems, Inc., San Jose, CA, USA) to adjust brightness and contrast.

Thickness of the outer nuclear layer (ONL; E85–P3) was measured at 2-mm increments along the vertical and horizontal meridian, extending from the margin of the optic disc to the ora serrata. To measure the thickness of the ONL a vertical line was drawn through 10 adjacent vertical columns of photoreceptor nuclei using microscope software (Moticam Image Plus 2.0; Motic China Group Co., Ltd., Xiamen, China) in five sections per location/eye and the mean calculated for each location. Overall thickness of ONL was calculated by averaging the mean thickness across all locations in each eye and for each age. ONL thickness was measured without knowledge of the genotype.

Transmission Electron Microscopy

Sections for electron microscopy were prepared as previously described.^{10,14} A vertical strip of 2% paraformaldehyde/2% glutaraldehyde fixed retinal tissue approximately 2-mm wide was removed dorsal to the optic nerve and a 2 × 2-mm piece of tissue was harvested approximately 5 mm above the superior margin of the optic disc. Retinal tissue was rinsed in buffer and then postfixed in 2% osmium tetroxide and 1.5% potassium ferrocyanide in dH₂O for 2 hours. The tissue was dehydrated in a graded series of ethanol and embedded in resin (Epon-Araldite; Electron Microscopy Sciences, Hatfield, PA, USA). Semi-thin sections (4 μm) were cut and stained with 1% cresyl violet. Ultra-thin sections (90 nm) were cut on an ultramicrotome (Ultracut E 701704; Reichert-Jung, Inc., Buffalo, NY, USA) using a diamond knife (Micro Star Technologies, Inc., Huntsville, TX, USA); collected on copper grids; counterstained with 4% methanolic uranyl acetate (Electron Microscopy Sciences); and photoreceptor morphology examined with a transmission electron microscope (TEM, Model 300; Philips, Eindhoven, The Netherlands). Photomicrographs were captured with a digital camera (15-megapixel; Scientific Instruments and Applications, Inc., Duluth, GA, USA) and imaging software (Maxim DL Version 5; Diffraction Limited, Ottawa, Canada).

Immunohistochemistry

Sections from paraformaldehyde fixed retinas were cut (20 μm) on a cryostat and stored at –80°C until further processing. Monoclonal anti-Rho 1D4 (Cat. # MABN5356,

FIGURE 1. Light microscopy and morphometric analysis of Wt and TgP23H swine outer retina (E85-P3). **(A)** Retinal micrographs taken 5 mm above optic disc in Wt versus TgP23H swine. Wt sections (*left column*) show the normal laminar arrangement of the retina during histogenesis. Retinae from TgP23H swine at E85 (*right column*) exhibit no change compared with Wt. TgP23H swine at E105 through P3 lack rod outer segments (ROS); CISs appear enlarged and their outer segments (*black arrows*) abut the RPE, and the ONL appears thinner at P0 and P3. Scale bar: 20 μm and applies to all panels. **(B)** Mean thickness (averaged across all locations and all eyes) of the ONL (E85-P3) in Wt versus TgP23H swine. P0 and P3 exhibit overall significant reduction in ONL thickness. **(C)** Mean thickness (averaged at each tested location) of the ONL along the vertical and horizontal meridians in Wt versus TgP23H. P0 and P3 TgP23H retinae show a central-to-peripheral pattern of thinning of the ONL. * $P \leq 0.05$.

1:500; Millipore Corp., Chicago, IL, USA) antibody was used to label rhodopsin in rod photoreceptors.¹⁵ Retinal sections were then rinsed in PBS buffer and incubated with fluorophore-labeled secondary antibody AlexaFluor 647 goat anti-mouse IgG (H+L, Cat. # A21235, 1:100; Invitrogen, Carlsbad, CA, USA). After incubation in fluorophore-labeled secondary antibody, sections were rinsed in buffer and mounted in either antifade reagent (ProLong Gold; Cat. # P36930; Invitrogen) and coverslipped. Sections were cured in the dark for 48 hours at 30°C before examination with a confocal microscope (Olympus FV1000; Olympus Corp., Tokyo, Japan) using a $\times 40$ objective. Control sections were not exposed to primary antibodies, but were processed simultaneously through all other labeling steps. These no-primary control sections were included in all labeling studies.

Statistics

All statistics related to retinal morphology were calculated and analyzed using scientific statistics software (InStat 3 for Macintosh; GraphPad Software, Inc., La Jolla, CA, USA). Unpaired *t*-tests were used to compare measurements of mean thickness of the ONL (E85–P3) in TgP23H versus Wt, with a *P* value ≤ 0.05 taken as indicating a significant difference from age-matched Wt. One-way ANOVA and post hoc *t*-tests were also used to compare mean ONL thickness across all groups. Statistics for fERG measurements were calculated and analyzed using scientific 2D graphing and statistics software (Prism 5; GraphPad Software, Inc.). Unpaired *t*-tests were used to compare amplitudes of the waveforms and a *P* value ≤ 0.05 was interpreted as being statistically significant.

RESULTS

A Central to Peripheral Gradient of Photoreceptor Degeneration Is Present at Birth in TgP23H Pig Retina

At the light microscopic level, the retinal morphology of the Wt hybrid at P0 (Fig. 1A) is similar to the domestic swine published previously.¹⁶ At E105, Wt photoreceptor nuclei and outer segments are easily identified in both Wt and TgP23H littermates, and the chromatin pattern of all photoreceptor nuclei in their ONL appeared similar.

To quantify the progression of photoreceptor degeneration over time and across the retina, we measured the overall thickness of the ONL (Fig. 1B) by averaging the measured ONL thickness at 2-mm increments along the vertical and horizontal meridian of the retina in each eye and at each age. At E85 and at E105, ONL thickness of Wt and TgP23H piglets were similar. We observed the first significant reduction in mean ONL thickness between P0 Wt and TgP23H retina, where 35% (8/23) of retinal locations in TgP23H retina were reduced relative to Wt. By P3, 74% (17/23) of Tg retinal locations were significantly reduced. The reduction in ONL thickness followed a central-to-peripheral progression pattern, which became more pronounced at P3 (Fig. 1C). When all locations were averaged, the ONL thickness of TgP23H retina was significantly reduced compared with Wt at both P0 and P3 (Fig. 1B; *P* = 0.0018; <0.0001 , respectively).

Rod Photoreceptor Morphological Abnormalities Arise Prior to Birth

At the ultrastructural level at E85, the morphology of Wt and TgP23H photoreceptors were similar. Although outer segments had not developed, their photoreceptors had inner segments

FIGURE 2. Ultrastructure of photoreceptor layer in Wt versus TgP23H swine. (A) TEM image of photoreceptor layer in Wt versus TgP23H E85. Photoreceptor IS and connecting-cilium (black arrowhead) appeared similar, and outer segments have not yet formed in Wt and Tg. Scale bar: 2 μm. (B) TEM image of photoreceptor layer in Wt versus TgP23H swine E105 through P3. E105 Wt, ROS/RIS, and COS/CIS exhibit normal morphology. E105 through P3 TgP23H, truncated ROS (black arrows) extend from RIS, COS appear normal or are lacking, and CIS appear enlarged. IS, inner segment; RIS, rod inner segment. Scale bar: 2 μm.

with connecting cilia (Fig. 2A). Photoreceptor synaptic terminals also had not developed in the outer plexiform layer (Figs. 4A, 4B). E105, Wt and TgP23H rod photoreceptors differed in several ways. Wild-type rod photoreceptors had both inner and outer segments (Fig. 2B), whereas TgP23H rods either lacked outer segments or those present appeared truncated with no evidence of ordered stacked discs. At E105, Wt rod photoreceptor nuclei (RN) had axons and spherules that contained a few synaptic ribbons (Fig. 4B, black arrowheads), although clear triadic profiles were absent. Rod photoreceptors in TgP23H littermates at that age lacked axons and spherules. From P0 onward, Wt rod spherules (Figs. 4C–E, white arrows) contained ribbons, as well as synaptic and triadic profiles. By P0 some TgP23H rod photoreceptor nuclei appeared pyknotic due to densification of chromatin (Fig. 3, black arrowhead). At P0, TgP23H rods failed to develop triadic profiles and their synaptic terminals could not easily be identified (Figs. 4C–E, black arrows). At P3, ribbons were occasionally found in rod photoreceptor terminals; however, no triads were found (Fig. 4D, black arrows).

FIGURE 3. TEM image of outer retina TgP23H swine (P0). Cone outer segments (*black arrows*) abut the RPE and many CIS lack cone outer segments. External limiting membrane is intact (*white arrows*). Outer nuclear layer shows cone nuclei in the outermost row and stacks of rod nuclei (RN1–RN4), with degenerating rod nuclei (*black arrowhead*). Only cone pedicles (CP) can be seen in the outer plexiform layer. Scale bar: 10 μ m.

Cone Photoreceptor Develop Normally Despite Rod Degeneration

At E105, when rod photoreceptor outer segments are absent or grossly abnormal, TgP23H cone photoreceptors with inner and outer segments (CIS and COS, respectively) exhibit COS that appear somewhat enlarged compared with Wt (Fig. 1A) and they abut the RPE (Figs. 1A, 3). At P0 and P3, some cone photoreceptors without outer segments were evident (Fig. 3), but the majority of cone photoreceptor axons and cone pedicles (CPs) exhibited normal morphology (Figs. 3, 4C, 4D), which included ribbons and triad profiles.

Rhodopsin Expression Is Mislocalized in TgP23H Rod Photoreceptors Although Postsynaptic Markers Are Normal

At E85 rhodopsin expression can be detected in both Wt and TgP23H swine rod photoreceptors. At this and all other ages, expression in Wt rod photoreceptors is restricted to the outermost portion of the developing photoreceptor layer (PRL; Fig. 5, left column). In contrast, rhodopsin expression is mislocalized in TgP23H rod photoreceptors from its first detection at E85 and is spread throughout the ONL (Fig. 5, right column).

FIGURE 4. TEM image of outer plexiform layer Wt versus TgP23H swine (E85–P3). (A) E85, no identifiable photoreceptor axons or synaptic terminals in the OPL. (B) E105 Wt, axons extending from RN into OPL, as well as a few ribbons (black arrowheads), while TgP23H show no axons or ribbons. (C) P0 and (D) P3 Wt retinae show rod spherules (white arrows) and CP with synapses and triads in the OPL. E105 through P3 TgP23H retinae show axonal retraction, no spherules or triads, and few ribbon synapses (black arrows), but cone photoreceptor axons and pedicles appear normal. (E) Magnification of boxed region in P0 showing Wt showing normal triadic profiles (white arrows) in Wt and their absence in TgP23H (black arrow). HZCN, horizontal cell nucleus. Scale bar: 2 μm and applies to all panels.

TgP23H Rod-Driven Function Is Absent While Cone-Driven Function Is Normal at P3

To characterize retinal function, we used a standard International Society for Clinical Electrophysiology of Vision (ISCEV) protocol in Wt and TgP23H littermates.^{8,11,17} Figure 6 illustrates representative ERG responses from a P3 Wt and its TgP23H littermate to different flash intensities. In Wt piglets, there are clear b-wave responses at all stimulus intensities. In

FIGURE 5. Immunolabeling with anti-Rho 1D4 (rhodopsin) antibody Wt versus TgP23H (E85–P3). (A–C) Intense staining with anti-Rho 1D4 is restricted to the PRL in Wt retinae (left column). Anti-Rho 1D4 is delocalized to the ONL in TgP23H (right column). Scale bar: 20 μm and applies to all panels. Figures from P0 are not shown but are the same as P3.

contrast, TgP23H littermates lack an ERG response to the rod-isolating stimulus (0.01 cd/m^2), while maintaining responses similar to the Wt in both the cone full field flash stimulus (3.0 cd/m^2) and the 30-Hz flicker stimulus ($P = 0.4555$; $P = 0.2723$, respectively). These results are consistent with the morphological characteristics of these retinae. They suggest that rod photoreceptors are abnormal and rod-driven retinal function, absent at birth, may never develop. In contrast, even in the presence of rod degeneration, cones, and cone-driven function are unaffected.

FIGURE 6. Representative fERG recordings for rod, cone, and 30-Hz flicker in Wt and TgP23H Swine (P3). (A) The rod waveform and histogram show a striking difference, with a nearly extinguished response in the TgP23H, while the Wt has a clearly discernible response. In contrast, the representative cone traces and histograms show how the morphology of the components of the cone and cone flicker waveforms at P3 ([B, C], respectively) are very similar between the TgP23H and Wt.

DISCUSSION

In the present study, we show that in this TgP23H swine RP model, rod photoreceptor morphology is abnormal before birth as is rod-driven function in the perinatal retina. Despite this, cones and cone-driven function develop normally. This analysis is possible because of the physical separation of rods and cones across the swine retina. Consistent with previous studies,^{18,19} our results suggest that abnormal localization of rhodopsin is correlated with the robust loss of rod photoreceptors in swine embryos at birth. The severity of this phenotype may have several causes and we describe the two that we think are most likely. First, the insertion site of the transgene within the genome is known to result in phenotypic variation in transgenic rodents, where numerous lines usually are created from a single construct. In fact, we observed phenotypic variation in our original description of the multiple TgP23H swine lines,⁸ and showed that across lines, the transgene was located on different chromosomes. Equally plausible are modifier effects, which also are known to be related to the severity or penetrance of a phenotype within the human population. This also could occur since these Tg pigs are the filial 1 progeny of a cross of an inbred miniswine with a domestic swine.

The TgP23H swine rod photoreceptors show abnormal localization of rhodopsin, as well as a central-to-peripheral spatial pattern of rod photoreceptor degeneration. In contrast, the spatial pattern of rod photoreceptor degeneration in human RP patients begins in the mid-peripheral retina and then spreads to the peripheral and central retina.¹ The newborn Pro347Leu rhodopsin Tg swine also show rhodopsin mislocalization and abnormal rod spherule morphology at age 4 weeks.²⁴ In that study, rod photoreceptor counts in the superior retinal quadrant did not show a spatiotemporal change, although it is possible that other retinal quadrants might show differences similar to those in the TgP23H swine. The cause of these differences, across species and within species across transgenes/insertion sites remains unclear.

Mislocalization of rhodopsin is commonly reported across hereditary retinal degenerations,^{20–24} as well as in animal models that express a mutant rhodopsin protein.^{21,22,24,25} As a consequence, mislocalization is hypothesized to be an early step in the cell death cascade within rod photoreceptors.^{18,26} In domestic swine embryos, rhodopsin expression can be detected at E85, proceeds in a central-to-peripheral fashion²⁷ and is confined to the developing rod photoreceptor outer segments.²⁷ In TgP23H swine embryos, rhodopsin expression also is detected at E85 and even this early is mislocalized. The P23H point mutation prohibits the differentiation between native and mutant rhodopsin expression. The exact mechanism whereby mislocalization of P23H mutant rhodopsin affects rod outer segments and spherule formation is not fully understood. Numerous studies describe the accumulation and trafficking of P23H rhodopsin and its intracellular fate in other models.^{21,22,28–41} In particular, TgP23H frogs and mice²⁸ show subcellular microstructures (mutant rhodopsin aggregates) that accumulate and destabilize rod outer segments. These studies also suggest misfolded protein accumulates in the endoplasmic reticulum leading to stress that contributes to rod photoreceptor cell death.^{28–30}

In the few studies that examined retinal function among children in families with inherited RP, deficits were found using the ERG, although the children did not report symptoms.^{4–6} It is possible that since they have no experience with normal scotopic vision, that they are not able to articulate visual deficits. Along these same lines, children with undiagnosed color vision deficiency are unaware that their photopic vision is abnormal.⁴² If retinal abnormalities are present during

gestation and/or shortly after birth in a subset of these RP patients, our model will be very beneficial in developing therapeutic intervention strategies targeted at delaying or preventing rod photoreceptor degeneration.

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