

Development of a *Pde6b* Gene Knockout Rat Model for Studies of Degenerative Retinal Diseases

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PURPOSE. To describe the phenotypes of a newly developed *Pde6b*-deficient rat model of retinal degeneration.

METHODS. *Pde6b* knockout rats were produced by CRISPR-Cpf1 technology. *Pde6b* knockout rats were evaluated for ocular abnormalities by comparison with wild-type eyes. Eyes were imaged using fundus photography and optical coherence tomography (OCT), stained by hematoxylin and eosin (H&E), and examined by TUNEL assay. Finally, eyes were functionally assessed by electroretinograms (ERGs).

RESULTS. *Pde6b* knockout rats exhibited visible photoreceptor degeneration at 3 weeks of postnatal age. The fundus appearance of mutants was notable for pigmentary changes, vascular attenuation with an irregular vascular pattern, and outer retinal thinning, which resembled retinitis pigmentosa (RP) in humans. OCT showed profound retinal thinning in *Pde6b* knockout rats; the outer nuclear layer (ONL) was significantly thinner in *Pde6b* knockout rats, with relative preservation of the inner retina at 3 weeks of postnatal age. H&E staining confirmed extensive degeneration of the ONL, beginning at 3 weeks of postnatal age; no ONL remained in the retina by 16 weeks of postnatal age. Retinal sections of *Pde6b* knockout rats were highly positive for TUNEL, specifically in the ONL. In ERGs, *Pde6b* knockout rats showed no detectable a- or b-waves at 8 weeks of postnatal age.

CONCLUSIONS. The *Pde6b* knockout rat exhibits photoreceptor degeneration. It may provide a better model for experimental therapy for RP because of its slower progression and larger anatomic architecture than the corresponding mouse model. Further studies in this rat model may yield insights into effective therapies for human RP.

Keywords: *Pde6b*, rat model, retinal degeneration, retinitis pigmentosa

Retinitis pigmentosa (RP) is a type of hereditary retinal disease that causes slow, progressive retinal degeneration with night blindness and visual field loss. Worldwide, more than 1 million individuals are affected; the age of onset and rate of retinal degeneration vary among the different forms of RP.¹ Importantly, RP can be inherited in a dominant, recessive, or X-linked fashion and shows considerable locus heterogeneity, with mutations in more than 80 genes known to cause nonsyndromic RP (RetNet: <https://sph.uth.edu/retnet/sum-dis.htm>; in the public domain). The gene encoding the β subunit of rod cGMP-phosphodiesterase type 6 (*Pde6b*) was the first gene identified with a causative mutation for autosomal recessive RP (in 1993); it has been shown to underlie cases of autosomal recessive RP, comprising 1% to 2% of all cases of RP.^{2,3} Because cGMP-PDE is pivotal in photoreceptor conversion of light to neural impulses, mutations in *Pde6b* gene result in permanent opening of cGMP-gated cation channels in the membrane of rod photoreceptors, allowing an excess of extracellular ions to enter the cell; this ultimately leads to cell death by apoptosis.^{4,5}

Mouse strains harboring *Pde6b* mutant alleles (*rd1* [*Pde6b*^{rd1}] and *rd10* [*Pde6b*^{rd10}]) are considered animal models of RP and are the most widely used models of RP.^{6–8}

Although no effective therapy for RP is currently available, gene therapy offers unprecedented opportunities for the development of strategies for RP treatment.^{9–11} The availability of a suitable and easily generated animal model is important for preclinical assessment of potential therapies intended for use in human patients. Although small rodents are easy to manage and genetically manipulate for investigations, surgical treatments can lead to variable intraoperative complications because of the extremely small anatomic architecture. Larger animals have been used in recent years (e.g., rabbits, dogs, and monkeys) because surgical treatments, such as subretinal injection of cells for replacement therapy, can be easily performed.^{12–16} However, these animals are difficult to handle and expensive to breed. Therefore, a rat model with *Pde6b* deficiency may offer a more suitable non-mouse model for experimental therapy for RP.



To provide a novel, non-mouse model for the investigation of experimental therapies related to RP, we developed a stable *Pde6b* knockout rat model by using clustered regulated interspaced short palindromic repeats (CRISPR)-Cpf1 technology, which cause retinal degeneration. In the present study, we describe the phenotypes of this newly developed rat model of retinal degeneration with *Pde6b* deficiency.

METHODS

Generation of *Pde6b* Knockout Rats

All animals were used and cared for in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the Guidelines for Animal Experiments of Asan Medical Center; all protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Asan Institute for Life Sciences (approval reference number, 2017-12-169). All rats were maintained in the specific pathogen-free (SPF) facility of the Laboratory of Animal Research in Asan Medical Center (AMCler).

Rat genomic DNA sequences were analyzed and target sequences were selected by using a Web tool, Benchling (<https://benchling.com/>; in the public domain). CRISPR RNA (crRNA) and *Cpf1* mRNAs were prepared by using oligomers listed in Supplementary Table S1, together with pcDNA3.1-hAsCpf1 (Addgene #69982), as previously described.^{17,18}

For the generation of mutant rats, Sprague-Dawley (SD) female rats (OrientBio, Gyeonggi, Republic of Korea) were used to prepare embryo donors and foster mothers. Briefly, superovulated fertilized eggs were isolated from female SD rats (5 weeks old) treated with 30 IU pregnant mare serum gonadotropin (PMSG; Sigma-Aldrich Corp., St. Louis, MO, USA) and 100 IU human chorionic gonadotropin (hCG; Daesung Microbiological Labs Co., Ltd., Gyeonggi, Republic of Korea) at 50-hour intervals. Subsequently, *Cpf1* mRNA (50 ng/μL) and crRNA (100 ng/μL) were co-microinjected into the cytoplasm of pronuclear stage embryos; surviving embryos were transplanted into the oviducts of pseudo-pregnant surrogate mothers to generate live animals. To avoid possible off-target cleavages, we selected a CRISPR RNA sequence in the rat *Pde6b* gene exhibiting three or more mismatches and at least one mismatch residing in the 5' PAM-proximal region (Supplementary Table S2).

Founder (F₀) rats with targeted mutations were screened by using PCR primers (listed in Supplementary Table S1) as previously described.¹⁹ Mutant alleles were identified by Sanger sequencing of cloned PCR products (generated by using a T-Blunt PCR Cloning Kit [SolGent Co., Ltd., Daejeon, Republic of Korea]). To establish *Pde6b* knockout rat lines, mutant rat founders with desired knockout alleles were crossed with wild-type SD rats, and the F₁ heterozygotes were screened and sequenced (data not shown). After breeding heterozygous knockout rats by crossing with wild-type SD rats, homozygous *Pde6b* knockout rats were generated by crossing male and female heterozygotes. For routine PCR genotyping, a primer pair that produced a short PCR product (173 base pairs [bp] in the wild-type mouse) was used to detect the deletion with frameshift mutation in the knockout allele.

In this study, the retinas of "F2 and F3" generation rats were examined morphologically and functionally for signs of pathological changes.

Fundus Photography

All rats were evaluated by fundus photography at 3 and 8 weeks of postnatal age. Topical anesthesia was achieved using

proparacaine hydroxychloride (Alcaine; Alcon, Fort Worth, TX, USA). The eyes were dilated with 1% tropicamide and 2.5% phenylephrine drops (mydrin-P; Santen, Osaka, Japan) and lubricated with methylcellulose. Fundus photographs were taken with the Micron IV (Phoenix Research Laboratories, Pleasanton, CA, USA), with wavelength range between 450 and 650 nm; the acquired images were stored in Micron IV software (StreamPix; NorPix, Inc., Montreal, QC, Canada). For each period, retinal morphology was compared between knockout rats (two males, two females) and age-matched wild-type rats (one male, one female).

Optical Coherence Tomography (OCT)

Sectional images of the retina were acquired by using image-guided OCT (OCT2; Phoenix Research Laboratories) at 3 and 8 weeks of postnatal age. Six scans, centered on the optic nerve, were obtained. Retinal thickness and morphology were compared between *Pde6b* knockout rats (two males, two females) and age-matched wild-type rats (one male, one female).

Electroretinograms

Animals were dark-adapted overnight and prepared for recording under dim red illumination. General anesthesia was induced by intraperitoneal administration of a 0.6 mL/kg mixture of tiletamine hydrochloride and zolazepam hydrochloride (Zoletil; Virbac, Carros, France) and 0.4 mL/kg xylazine hydrochloride (Rompun; Bayer Korea, Seoul, Korea). Topical anesthesia was achieved by using proparacaine hydroxychloride (Alcaine; Alcon). After anesthesia was complete, the animal was placed in an earthed aluminum recording chamber. The pupils were fully dilated with eye drops containing 1% tropicamide and 2.5% phenylephrine hydrochloride (mydrin-P; Santen). A drop of methylcellulose was placed on the corneal surface to ensure electrical contact and to maintain corneal integrity. During electroretinogram recording, rats were maintained on a heating pad to maintain appropriate body temperature.

Electroretinograms (ERGs) were recorded from the right eye by using the Ganzfeld ERG system (Phoenix Research Laboratories) with a gold-wire loop placed on the right cornea. Reference electrodes were placed in the center of the scalp; ground leads were placed in the skin at the base of the tail. Rod-dominated responses to white flashes of light over a 4.0 to 5.0 log-unit range of intensities were recorded. Cone-dominated responses were obtained with white flashes over a 2.0 log-unit range of intensities at 2.1 Hz on a rod-saturating background (1.46 log cd/m²) after 10 minutes of exposure to the background light to allow for complete light adaptation.

Signals were sampled every 0.5 ms over a response window of 240 ms. For each stimulus condition, responses were computer averaged; up to 10 records were averaged for the weakest signals. Responses were recorded from four *Pde6b* knockout rats (two males, two females) at the ages of 3 and 8 weeks. Comparisons with age-matched wild type were performed on two (one male, one female) rats.

Histopathological Examination

At 1 day, 1 week, and 2, 3, 4, 8, 10, and 16 weeks of postnatal age, eyes were enucleated under general anesthesia, induced by administering a 125 mg/mL mixture of tiletamine hydrochloride and zolazepam hydrochloride (Zoletil; Virbac). After enucleation, animals were killed in a CO₂ chamber. Enucleated eyes were fixed in 4% buffered formaldehyde and embedded in paraffin. Vertical sections of 4-μm thickness were cut, passing

through the optic disc. Specimens were stained with hematoxylin and eosin (H&E) and photographed under a microscope (Olympus CX41; Olympus America, Center Valley, PA, USA) at $\times 20$ and $\times 40$ magnification. Histologic morphology was compared between *Pde6b* knockout rats (two males, two females) and age-matched wild-type rats (one male, one female) at each time point. Additionally, thicknesses of the inner nuclear layer (INL) and outer nuclear layer (ONL) of the superior and inferior retina were measured at 500, 750, and 1000 μm from the optic disc. Statistical analyses were performed to determine differences between the two groups by using the Mann-Whitney *U* test. Data were analyzed by using SPSS version 20.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was defined as *P* value < 0.05 .

TUNEL Assay

Photoreceptor apoptosis was determined by using the terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) assay with a DeadEnd Fluorometric TUNEL System (Promega, Madison, WI, USA). For TUNEL, sections were deparaffinized, rehydrated, treated with Proteinase K, reacted with TdT/nucleotide mix (containing fluorescein-12-dUTP), and counterstained with DAPI-blue. All specimens were examined on a Zeiss LSM 780 confocal microscopy system (Carl Zeiss Meditec AG, Jena, Germany). The TUNEL-positive nuclei within a section of the superior and inferior retina 500 to 750 μm from the optic disc were compared between *Pde6b* knockout rats (two males, two females) and age-matched wild-type rats (one male, one female) at each time point.

Western Blot Analysis

Whole eyes were homogenized in lysis buffer (50 mM Tris, 100 mM NaCl, 5 mM EDTA, 0.1% SDS, 1% Triton X-100, 2.5% glycerol) supplemented with complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA). The samples were cooled for 30 minutes on ice at -4°C and then centrifuged at 13,000g for 15 minutes at 4°C , after which the supernatant was collected. Equal quantities of 35 μg of each sample were resolved by electrophoresis in a running buffer on 10% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel. Samples were electrophoretically transferred to a polyvinylidene fluoride membrane (PVDF) (Bio-Rad Laboratories, Hercules, CA, USA), which was blocked in Tris-buffered saline with Tween 20 (TBST) (10 mM Tris pH 8.0, 150 mM NaCl, 0.2% Tween 20). The PVDF membrane containing the transferred proteins was blocked with 5% lyophilized skim milk in PBS for 1 hour at room temperature. After an overnight incubation with a primary antibody against *Pde6b* (Santa Cruz Biotechnology, Santa Cruz, CA, USA), the membranes were washed with TBST and subsequently incubated with anti-mouse peroxidase-linked secondary antibodies (Amersham Pharmacia, Baie d'Urfé, QC, Canada). Detection of protein signals was performed by using a chemiluminescent reagent (SuperSignal West Dura Extended Duration Substrate; Thermo Fisher Scientific, Fairlawn, NJ, USA), after which membranes were exposed to autoradiograph imaging film (X-OMAT; Eastman Kodak, Rochester, NY, USA). To ensure equal protein loading, the same blot was subsequently incubated with an α -tubulin antibody (Cell Signaling Technology, Danvers, MA, USA).

RESULTS

Generation of *Pde6b* Knockout Rats

The rat model has greater translational relevance than previously established mouse models due to its similarities to

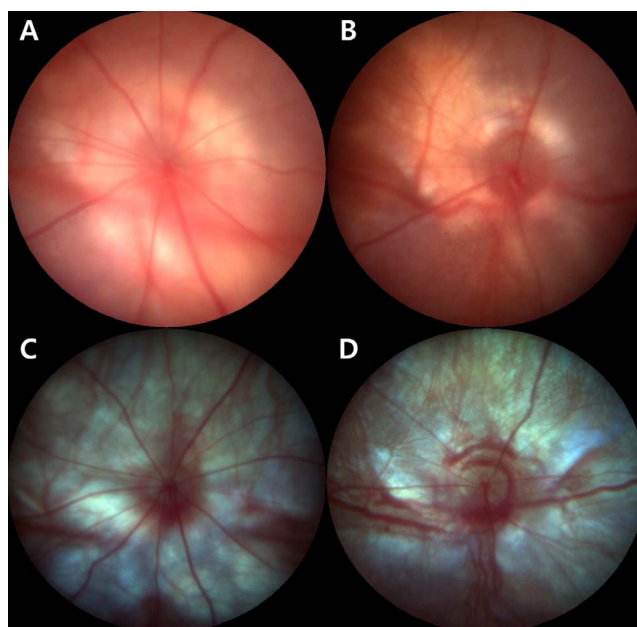


FIGURE 1. Fundus photographs of wild-type rats and *Pde6b* knockout rats at 3 weeks (A, B) and 8 weeks of postnatal age (C, D). The fundus appearance of wild-type rats (A, C) showed a radial pattern of arterioles and venules. Irregular radial patterns of retinal vessels with attenuated retinal arterioles are easily detected in *Pde6b* knockout rats (B, D). In addition, retinal pigmentary changes appear in the fundus at 3 weeks of postnatal age; retinal thinning, resulting in prominent choroidal vessels, is visible at 8 weeks of postnatal age, which was not present in wild-type rats.

humans in many biological aspects.^{20,21} To target the *Pde6b* gene in rats, we adopted the recently developed CRISPR-Cpf1 system, which exhibits better gene-targeting efficiencies and lower off-target activities than CRISPR-Cas9.²² We generated *Pde6b*-mutant founders with a crRNA targeting the coding region of exon 1 (Supplementary Figs. S1A, S1B). After confirming the frameshift mutations of founder rats #26 and #33, we established a *Pde6b*-mutant rat line with an 11-bp deletion (Supplementary Figs. S1C, S1D). Western blot analysis confirmed deficiency of *Pde6b* protein in the homozygous mutant retina, indicating successful generation of *Pde6b*-deficient rats with Cpf1-mediated gene targeting (Supplementary Fig. S2). No phenotypic differences between generations “F2 and F3” were found (data not shown).

Clinical Phenotype

The retinal appearance of the *Pde6b* knockout rats was distinguished from that of wild-type rats by fundoscopy (Fig. 1). The fundus photographs of wild-type rats showed radial patterns of arterioles and venules. *Pde6b* knockout rats showed irregular radial patterns of retinal vessels with attenuated retinal arterioles. In addition, at 8 weeks of postnatal age, *Pde6b* knockout rats showed a thinned retina, resulting in prominent choroidal vessels.

Optical Coherence Tomography

OCT showed profound thinning of the whole retina in *Pde6b* knockout rats, compared with wild-type rats (Fig. 2); retinal thickness gradually decreased in *Pde6b* knockout rats. By 3 weeks of postnatal age, the ONL was significantly thinner in *Pde6b* knockout rats, and the inner retina, including the ganglion cell layer and INL, was similar to that of wild-type

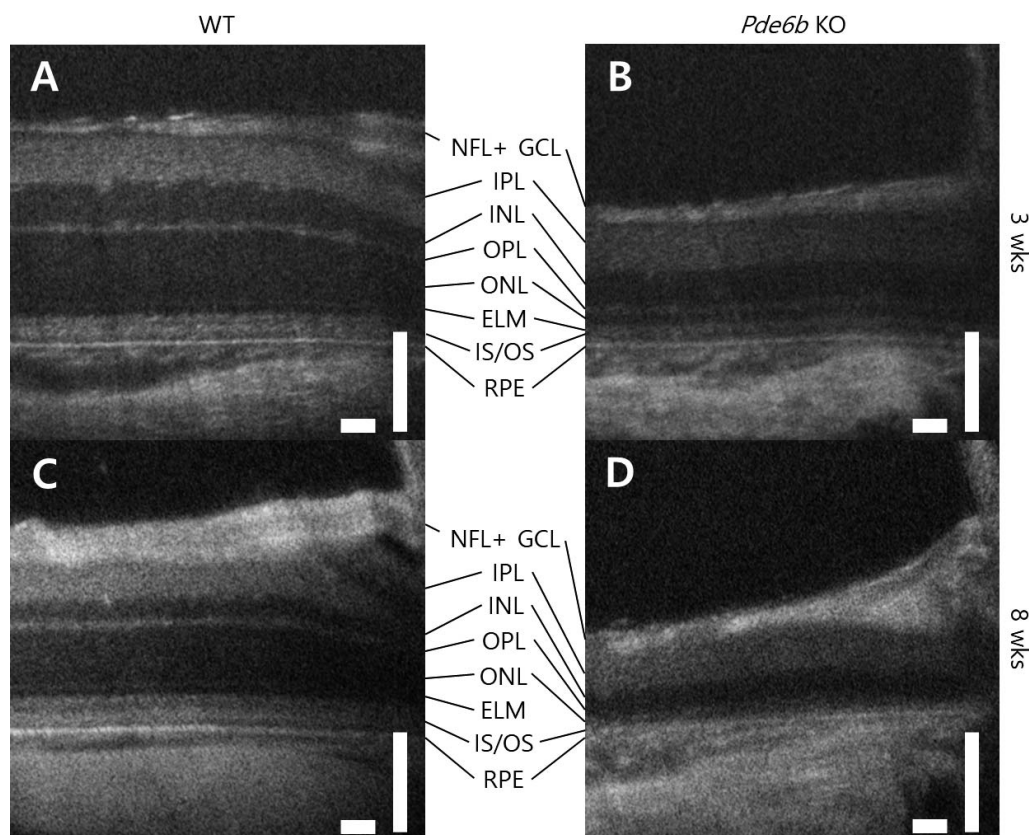


FIGURE 2. Optical coherence tomography of wild-type rats and *Pde6b* knockout rats at 3 weeks (**A, B**) and 8 weeks of postnatal age (**C, D**). OCT showed profound thinning of the whole retina in *Pde6b* knockout rats. At 3 weeks of postnatal age, OCT showed a thinned ONL in *Pde6b* knockout rats, compared to that of wild-type rats. The thickness of the ONL gradually decreased until 8 weeks of postnatal age, with relative preservation of the inner retina. NFL, nerve fiber layer; GCL, ganglion cell layer; IPL, inner plexiform layer; OPL, outer plexiform layer; ELM, external limiting membrane; IS/OS, inner and outer segments; RPE, retinal pigment epithelium. Scale bars: 100 μ m.

controls. OCT showed a marked thinning of the whole retina by 8 weeks of postnatal age, with relative preservation of the inner retina. These results suggested that retinal thinning predominantly occurs in the photoreceptor layer.

Investigation of Retinal Function With ERG

Under dark-adapted and light-adapted conditions, the ERG amplitude was lower in *Pde6b* knockout rats than in wild-type rats at 3 and 8 weeks of postnatal age. Figure 3 shows a series of ERG waveforms recorded in response to increasing flash intensities under dark- and light-adapted conditions. The same flash intensities resulted in much smaller rod responses in *Pde6b* knockout rats at 3 and 8 weeks of postnatal age. The cone response was smaller at all flash intensities at the age of 3 weeks, compared with wild-type responses; there was continued loss of cone function by the age of 8 weeks. There were no recordable a- and b-waves in both dark-adapted and light-adapted conditions at 8 weeks of postnatal age.

These results suggest that *Pde6b* knockout in rats resulted in the functional induction of photoreceptor cell loss.

Histologic Phenotype

Consistent with the OCT findings, histologic analysis confirmed that INL and ONL thicknesses gradually decreased in *Pde6b* knockout rats but did not change in wild-type rats (Fig. 4). Although both layers showed decreased retinal thickness in *Pde6b* knockout rats, the decrease was more severe in the ONL (90–93%) than in the INL (47–53%).

At 1 day of postnatal age, INL was indistinguishable from ONL in both *Pde6b* knockout and wild-type rats (Fig. 5). The retinal lamination of *Pde6b* knockout and wild-type rats developed normally and demonstrated no significant difference among any of the retinal layers until 2 weeks of postnatal age. At 3 weeks of postnatal age, *Pde6b* knockout rats showed only five to six layers of ONL nuclei; progressive thinning of ONL was observed, compared with that of wild-type rats. As a result, ONL at 16 weeks of postnatal age was not evident. From 4 to 10 weeks of postnatal age, some thinning of the INL was observed, which may have been secondary to primary photoreceptor degeneration.

Retinal sections from *Pde6b* knockout rats were TUNEL positive, compared with wild-type rats (Fig. 6). Apoptosis began after 2 weeks of postnatal age, reached a peak at 3 weeks of postnatal age, and then declined. The signal was specific to ONL, with very little or no signal in INL, whereas wild-type rats showed very little TUNEL signal only in INL between 1 and 10 weeks of postnatal age.

DISCUSSION

By using CRISPR-Cpf1 technology, we developed a *Pde6b* knockout rat model that exhibits retinal degeneration. We investigated the ophthalmic appearance, time-dependent changes of retinal morphology, and visual functions by using fundus photography, SD-OCT, ERG, and histologic analyses. A major finding of our study is that rats deficient in *Pde6b* develop retinal degeneration similar to human RP. In *Pde6b*

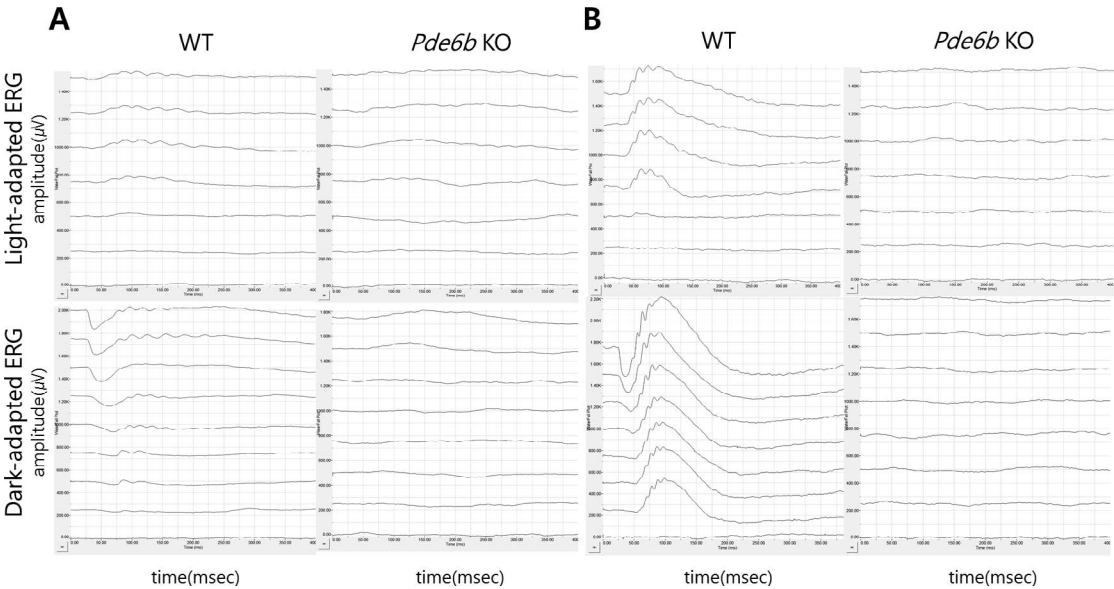


FIGURE 3. Electoretinograms of wild-type rats and *Pde6b* knockout rats at 3 weeks (A) and 8 weeks of postnatal age (B) in light-adapted and dark-adapted conditions. The ERG amplitudes of *Pde6b* knockout rats were relatively preserved at 3 weeks of postnatal age. However, at 8 weeks of postnatal age, *Pde6b* knockout rats showed no recordable a- or b-waves in all conditions.

knockout rats, initial signs of retinal degeneration, which include decreased ONL thickness, were observed at 3 weeks of postnatal age; at 4 weeks of postnatal age, the thickness of ONL was reduced by more than 90%; and at 16 weeks, complete loss of ONL was observed. The stratification and structural organization of the inner retina of *Pde6b* knockout rats were

relatively preserved. At 4 and 10 weeks of postnatal age, however, thinning of the INL (secondary to the photoreceptor degeneration) was observed. Similar morphologic alterations have been found in other mouse models that lack functional cones and/or rods.^{23,24} These findings suggest that the *Pde6b* knockout rat may provide a good model to study the

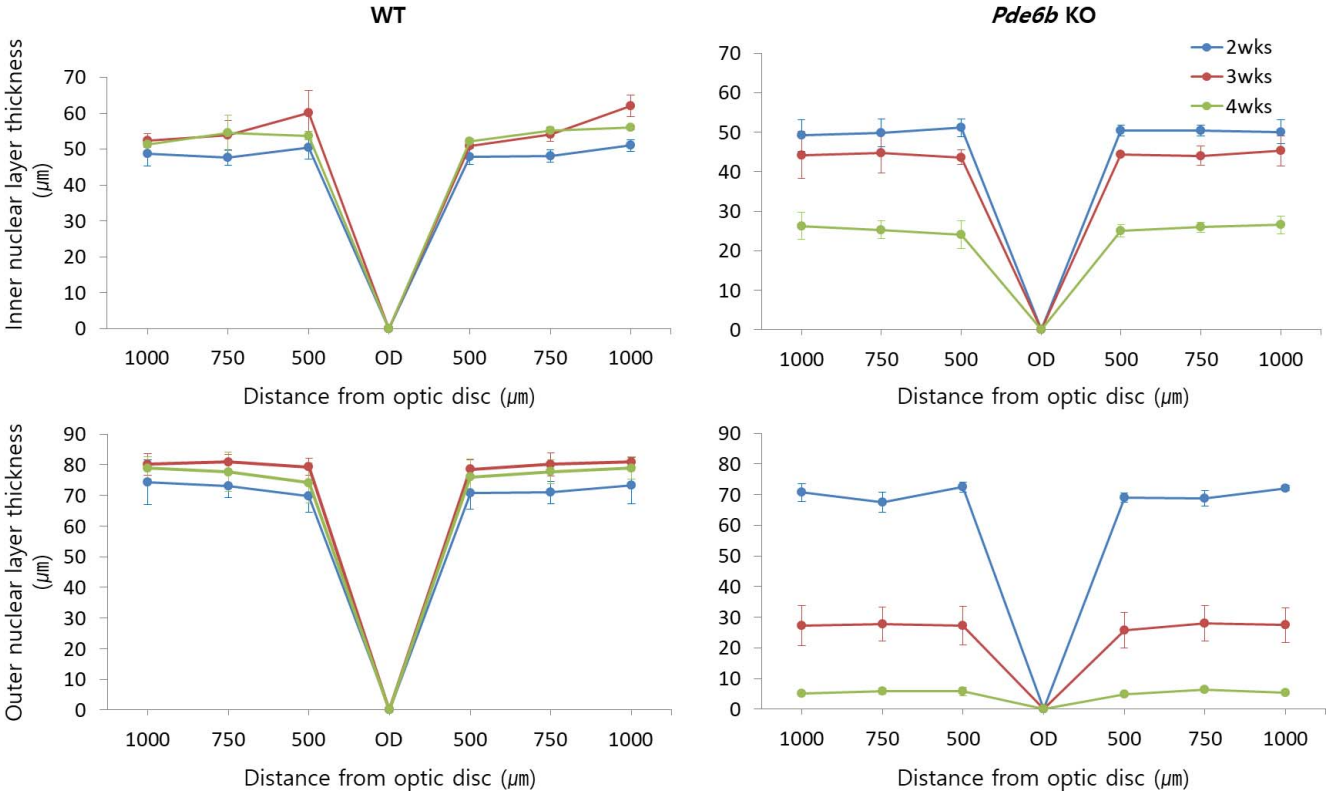


FIGURE 4. The thickness of the INL and ONL of wild-type rats and *Pde6b* knockout rats. The thickness of INL and ONL gradually decreased in *Pde6b* knockout rats but did not change over time in wild-type rats. By 3 weeks of postnatal age, the INL and ONL were significantly thicker in wild-type rats at all measured points, compared with those of *Pde6b* knockout rats ($P < 0.001$ for all). *Statistical analysis with a Mann-Whitney U test.

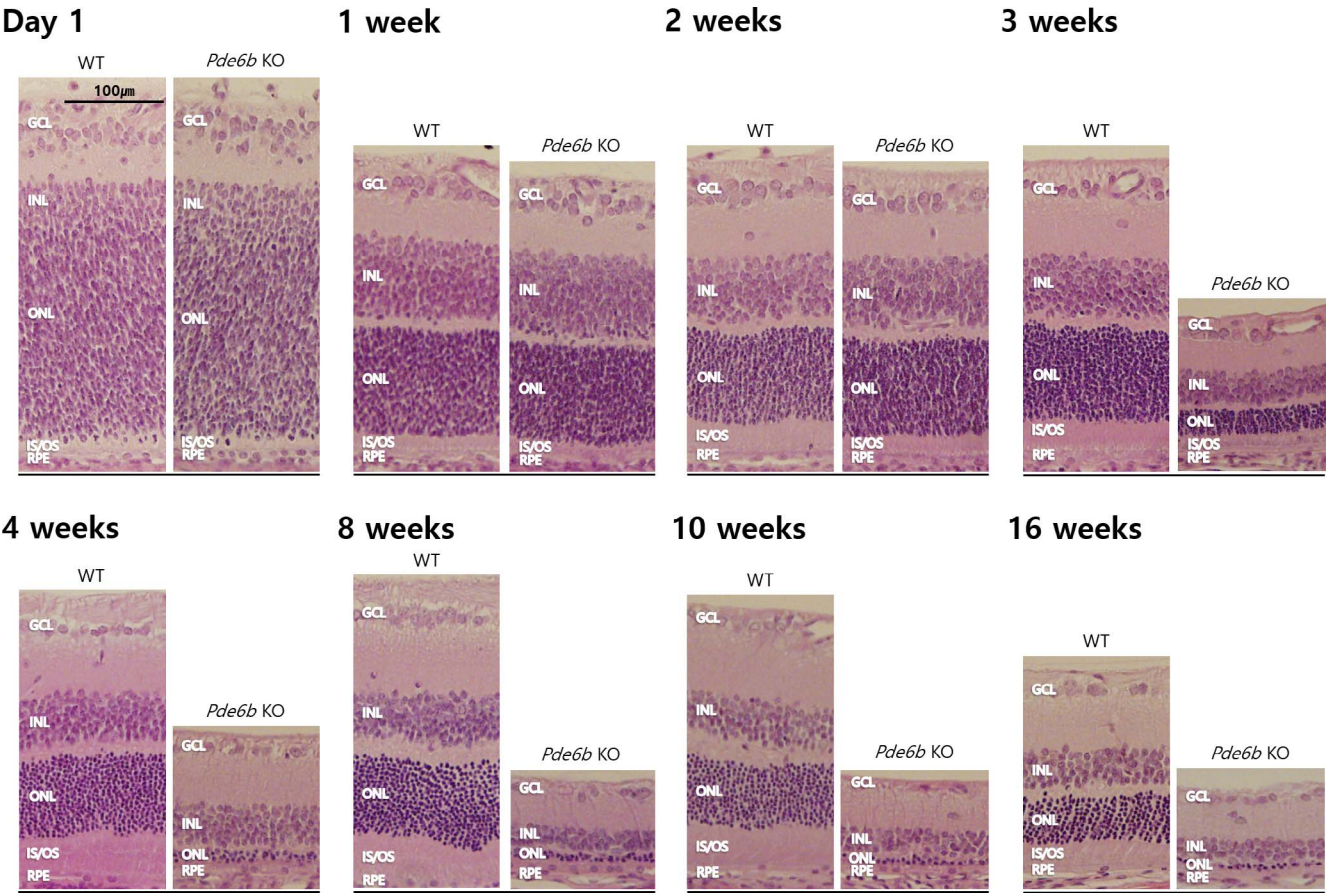


FIGURE 5. Histologic sections of wild-type rats and *Pde6b* knockout rats at each time point (hematoxylin and eosin staining). At 1 day of postnatal age, the lamination of normal retina was not observed in either wild-type rats or *Pde6b* knockout rats. Until 2 weeks of postnatal age, retinal thickness showed a decrease, especially in INL and ONL, which was thought to be normal retinal development. Retinal lamination of any retinal layers was not consistently different between wild-type rats or *Pde6b* knockout rats until 2 weeks of postnatal age. At 3 weeks of postnatal age, compared with wild-type rats, *Pde6b* knockout rats showed only five to six layers of ONL nuclei. Two to three layers at 4 weeks, one to two layers at 8 weeks, and only one layer at 10 weeks of postnatal age were observed. At 16 weeks of postnatal age, the ONL was not evident (zero layers to one layer of ONL nuclei), with relative preservation of the INL in *Pde6b* knockout rats. In wild-type rats, however, the ONL, INL, and retinal lamination were maintained at all experimental time points.

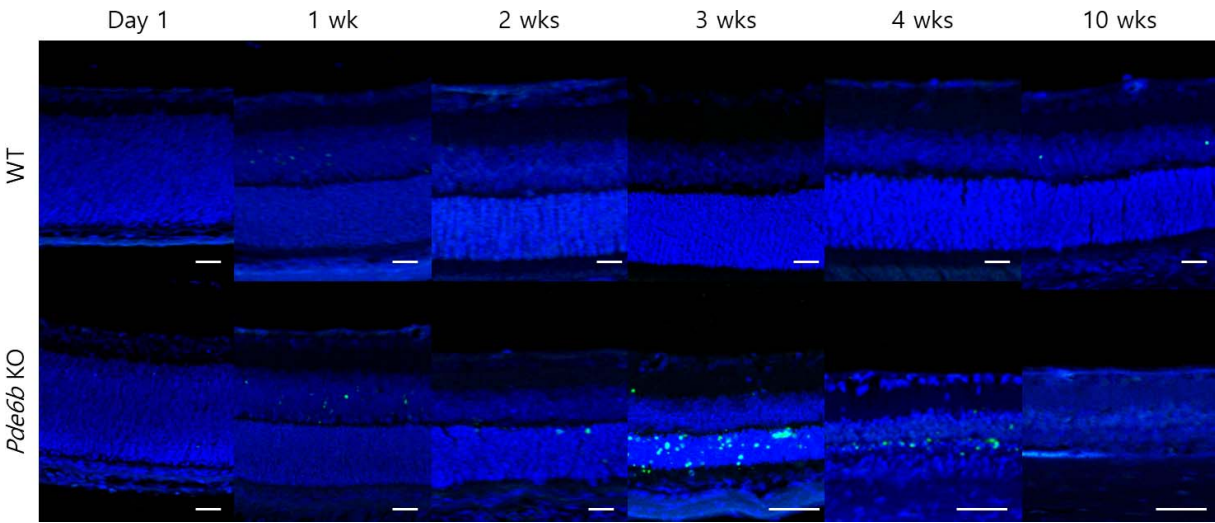


FIGURE 6. TUNEL staining of wild-type rats and *Pde6b* knockout rats at each time point. TUNEL-positive cells fluoresced yellow-green. Wild-type rats showed almost no TUNEL signal in the ONL at all experimental time points, whereas *Pde6b* knockout rats showed abundant TUNEL signals in the ONL and occasionally in the INL. Apoptosis began after 2 weeks of postnatal age, reached a peak at 3 weeks of postnatal age, then declined. Scale bars: 50 μm.

pathogenesis of RP, which is characterized by preferential loss of the photoreceptor layer at an early stage.

Pde6b knockout rats failed to develop normal photoreceptor function, as assessed by ERG, likely due to a lack of cGMP-PDE enzymatic activity that resulted in failure of rod phototransduction. Dark-adapted and light-adapted ERG responses of *Pde6b* knockout rats exhibited reduced a- and b-wave amplitudes at 3 weeks of postnatal age; ERG responses were absent at 8 weeks of postnatal age, consistent with histologic data.

Although the *Pde6b* knockout rat model demonstrated progressive retinal degeneration, our results indicated phenotypic features that are distinguishable from typical mouse models, such as *rd1* and *rd10*. The *rd1* mouse contains a null allele due to a nonsense mutation in exon 7 of the *Pde6b* gene; these mutant mice exhibit abnormal photoreceptor differentiation and subsequent rapid rod degeneration, both of which begin as early as postnatal day 8 and are complete by 4 weeks of postnatal age.^{25,26} The *rd10* mouse displays autosomal recessive hereditary retinal degeneration caused by a missense mutation in exon 13 of the *Pde6b* gene. Rod cells begin degenerating at 16 days of postnatal age; by 60 days (approximately 9 weeks) of postnatal age, no photoreceptors remain.²⁷ Many attempts to treat *rd1* mice with gene replacement therapy have shown limited success.^{28–31} In particular, long-term functional restoration of retinal signaling has not been achieved, perhaps as a result of the rapid nature of degeneration in *rd1* mice. Because the *Pde6b* knockout rat model in this study showed extensive degeneration of ONL beginning at 3 weeks of postnatal age, and no ONL remained in the retina by 16 weeks of postnatal age, this model displays a later onset and slower retinal degeneration process than the *rd1* and *rd10* mice; therefore, the novel rat model provides a longer therapeutic window for intervention. Thus, our results suggest that the *Pde6b* knockout rat model represents a more physiologically relevant animal model to use in the development of treatment strategies for human RP.

To confirm that the *Pde6b* knockout does disrupt *Pde6b* expression in the retina, we performed Western blotting analyses and compared its protein expression with that of wild-type rats; the *Pde6b* knockout rats showed a complete absence of detectable *Pde6b* protein in the retina. In comparison, *rd1* and *rd10* mice produce *Pde6b* protein, despite the presence of mutations in exon of the *Pde6b* gene.³² Based on these results, although the mode of mutation is different, both the *Pde6b*^{rd1} allele and the indel mutation in our rat model are functionally compatible and completely block the expression of *Pde6b* proteins in their retinas. This provides further support for the use of the *Pde6b* knockout rat to study the effects of *Pde6b* expression on retinal degeneration, both after virally mediated gene delivery and after stem cell transplantation.

As gene therapy emerged as treatment for RP, subretinal injections in preclinical animal models became increasingly important. Although there are several studies that optimize the procedures for subretinal delivery in mice, because of the relatively small size of the mouse eye, success of a subretinal injection is not easy to achieve, particularly for less experienced investigators.^{33–36} Extensive subretinal injection-related complications such as accidental perforation of the eyeball, intraocular bleeding, excessive retinal detachment, and unwanted cataract formation may interfere with the therapeutic outcome. In addition, it is difficult to detach a significant portion of the mouse retina after subretinal injection with a minimal injection-related damage.³⁷ Moreover, mice with these complications or with inadequate intraoperative manipulation should be excluded from studies. Therefore, the efficient use of a rat model with a larger anatomic architecture is more desirable, from both animal research ethics and economic

perspectives. Furthermore, compared with mice, a larger volume of injection can be delivered into the subretinal space in the rat; therefore, concentration-dependent treatment effect and toxicity can be evaluated more easily.

Although the advantages of using rodents, including rats and mice, to study ocular diseases are well documented, these exhibit many genetic, reproductive, developmental, morphologic, and anatomic differences, which makes it difficult to perform some experimental procedures. During the course of some experiments with mice in our laboratory, we noted that the use of eye drops containing 1% tropicamide and 2.5% phenylephrine hydrochloride for dilating the pupil of mice developed lens opacification in a number of animals. It has been reported that the use of some mydriatic and general anesthetic drugs may induce reversible cataracts in mice.³⁸ Although there is no clear explanation regarding the mechanism by which this mydriatic agent could induce lens opacification, it is clear that this effect may disturb or fully obstruct performance of necessary studies.

This study has some limitations. Although histologic analysis of retinal tissue has been considered the gold standard for characterizing retinal degeneration, artifacts from histologic processing are unavoidable, including fixation and dehydration, which can alter layer thicknesses and mask features of in vivo degenerative progression. In addition, the area imaged with OCT was quite restricted, such that the degeneration in the retina obtained with OCT did not always correlate with the overall retinal function. Furthermore, a larger sample size and more frequent follow-up intervals may provide more accurate data to analyze functional and anatomic changes.

In conclusion, we developed a stable *Pde6b* knockout rat model and have described the phenotypes of this rat model of retinal degeneration. The *Pde6b* knockout rat shows promise as a larger rodent animal model for the investigation of experimental therapies, and we expect that the *Pde6b* knockout rat model will shed further light on the development of effective therapies for RP.

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