Human Gene Transfer Protocol #0410-677:

Phase I Trial of Ocular Subretinal Injection of a Recombinant Adeno-Associated Virus (rAAV-RPE65) Gene Vector in Patients with Retinal Disease Due to RPE65 Mutations

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Phase I Trial of Ocular Subretinal Injection of a Recominant Adeno-Associated Virus (rAAV-RPE65) Gene Vector in Patients with Retinal Disease Due to RPE65 Mutations

→ Background – human retinal disease, RPE65

→ Pre-clinical proof-of-concept studies

→ Models versus human patients

→ Pre-clinical safety studies

Phase I Trial of Ocular Subretinal Injection of a
Recominant Adeno-Associated Virus (rAAV-RPE65) Gene Vector
in Patients with Retinal Disease Due to RPE65 Mutations
→ Background – **human retinal disease**, RPE65

Q. What is this “Retinal Disease”?

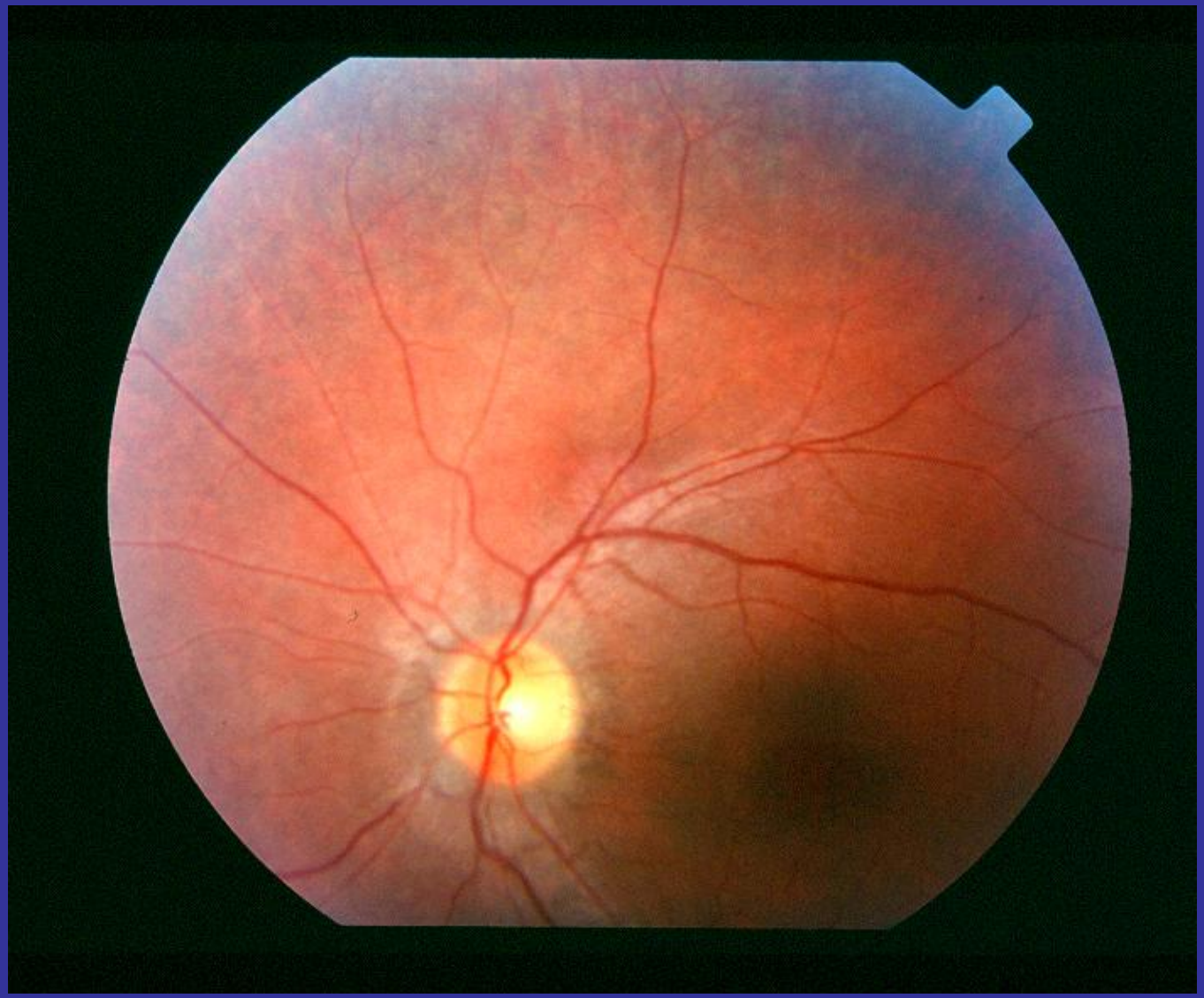
A. Leber congenital amaurosis (LCA) is an untreatable and incurable group of early-onset molecularly heterogeneous retinal degenerations that cause severe visual loss.

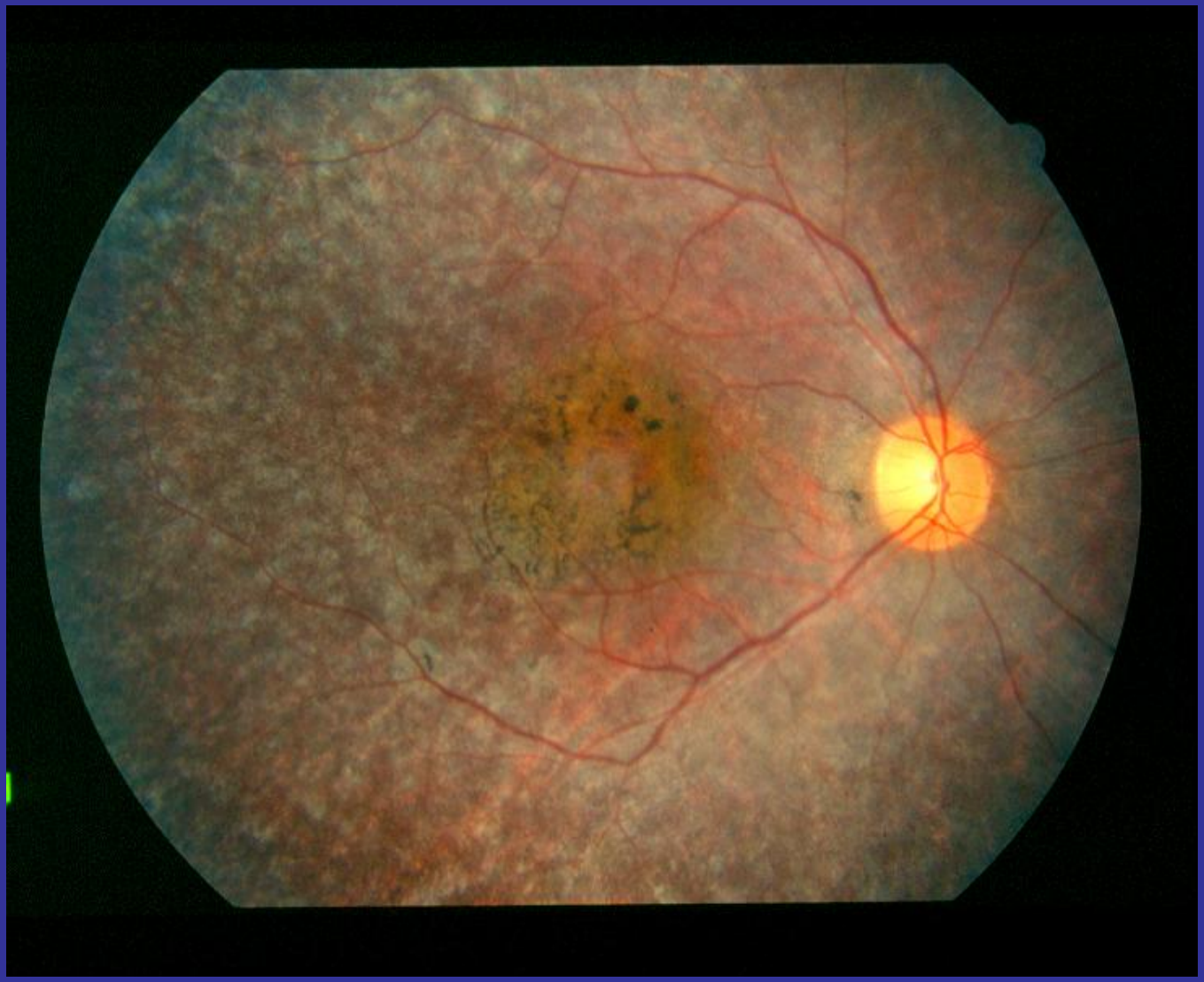
CASE

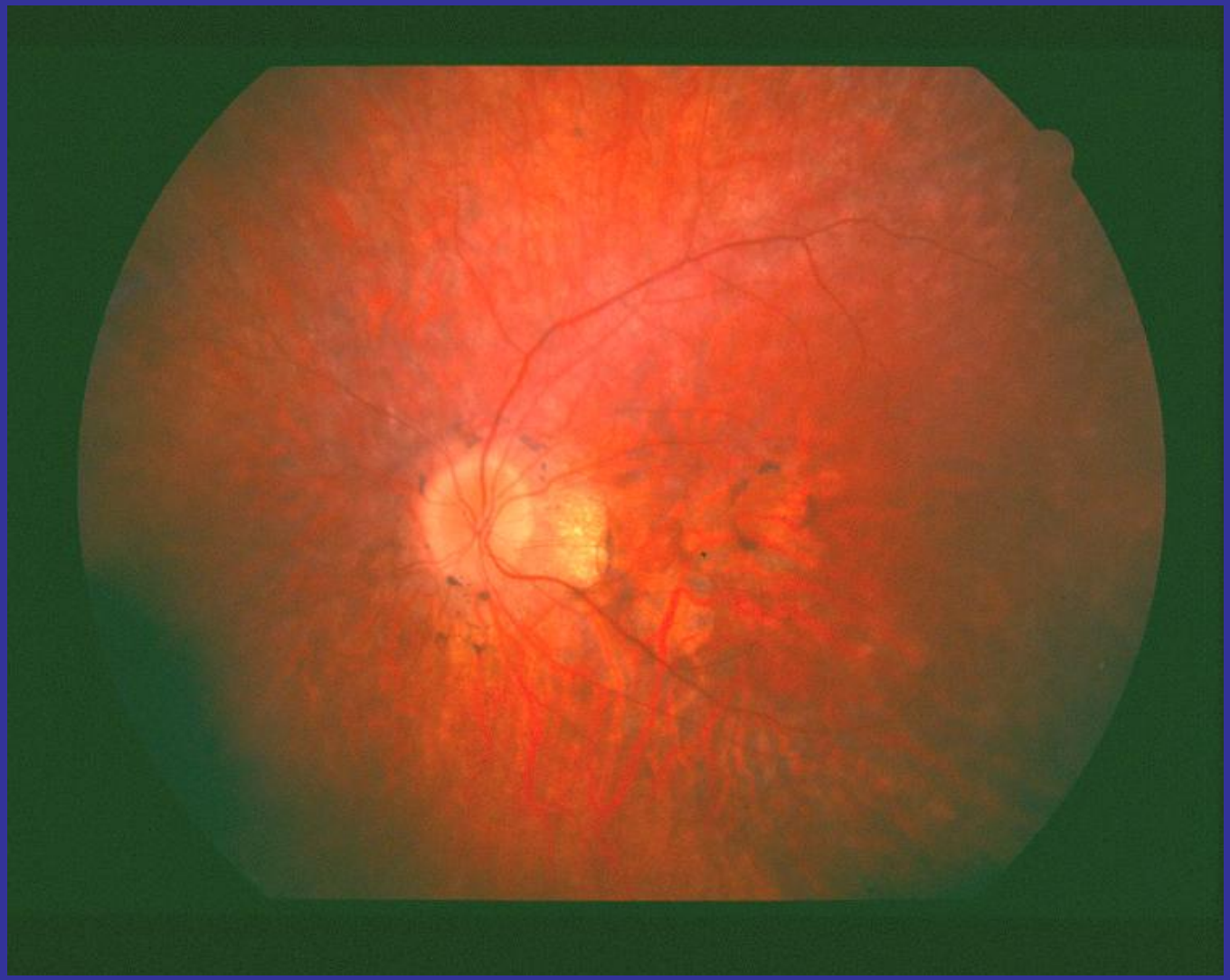
1991 – 3-year-old female patient with nystagmus and reduced vision from infancy
Pertinent history – parental consanguinity; healthy child
Vision – Fix and followed bright penlight with each eye
Cycloplegic retinoscopy - +2.50 OU
Fundus – attenuated retinal vessels
ERG – standard stimuli elicited no detectable responses
Diagnosis: Leber congenital amaurosis (LCA), autosomal recessive (AR)

1995 – Follow-up visit at age 7 years
Complaint – declining night vision
Visual acuities – 20/200
Fundus – attenuated retinal vessels and central and peripheral pigmentary change
Diagnosis: LCA, AR

2000 – Follow-up visit at age 12 years
Complaint – further reading difficulties
Visual acuities – 20/200
Visual field – kinetic field reduced in extent
ERG – submicrovolt flicker protocol shows detectable signal
Diagnosis: LCA, homozygous 20-bp deletion in *RPE65* gene







Leber Congenital Amaurosis

Molecular causes:

GUCY2D (RETGC1)
CRX
RPE65
AIPL1
TULP1
CRB1
RPGRIP1
LRAT
and more.

Human Molecular Genetics, 2002, Vol. 11, No. 10 1169–1176

Molecular genetics of Leber congenital amaurosis

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Received March 1, 2002; Revised and Accepted March 4, 2002

Leber congenital amaurosis (LCA) is the most common inherited cause of blindness in childhood and is characterised by a severe retinal dystrophy before the age of one year. Six genes have been identified that together account for approximately half of all LCA patients. These genes are expressed preferentially in the retina or the retinal pigment epithelium. Their putative functions are quite diverse and include retinal embryonic development (CRX), photoreceptor cell structure (CRB1), phototransduction (GUCY2D), protein trafficking (AIPL1, RPGRIP1), and vitamin A metabolism (RPE65). The molecular data for *CRB1* and *RPE65* support previous hypotheses that LCA can represent the severe end of a spectrum of retinal dystrophies. Given the diverse mechanisms underlying the disease, future therapies of LCA may need to be tailored to certain genetically defined subgroups. Based on experimental evidence in mice and dogs, patients with disturbed retinal metabolism of vitamin A through a mutation in the *RPE65* gene will likely be the first candidates for future therapeutic trials.

Table 1. Chromosomal location, size, and mutation frequency of LCA genes

Gene	Chromosomal location	Coding region (aa)	Exons	Amplicons for SSCA	Mutation frequency (%)
AIPL1	17p13.1	384	6	6	5.8
CRB1	1q31.3	1406	12	26	9.0; 13.5
CRX	19q13.3	299	3	4–7	2.0; 2.8
GUCY2D	17p13.1	1051	20	20	6.0; 6.3; 20.3
RPE65	1p31	533	14	10–15	3.0; 6.8; 8.2; 11.4; 15.6
RPGRIP1	14q11	1259	25	24	5.3; 5.6

Human Molecular Genetics, 2002, Vol. 11, No. 10

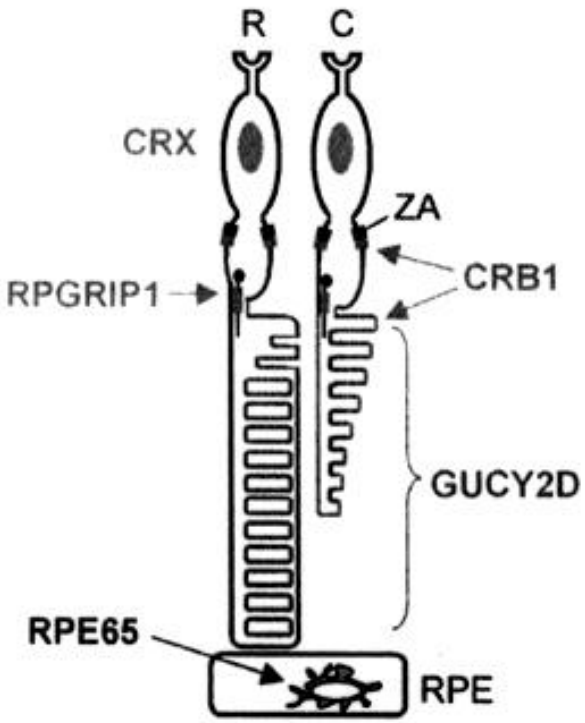

Figure 1. Subcellular localisation of proteins encoded by LCA disease genes in the photoreceptor cells and RPE. The CRX transcription factor has a nuclear localisation (52). CRB1 has been localised adjacent to the zonula adherens (ZA) of rod (R) and cone (C) inner segments and in the cone outer segment plasma membrane (35). GUCY2D was localised in different retinal cell types, but most abundantly in the marginal (membrane-rich) region of cone outer segments and to a lesser degree in rod outer segments (39). RPGRIP1 was localised in rod outer segments (25) and at the ciliary axoneme at the junction between inner and outer segments (26). RPE65 has been associated with microsomal membranes, which are presumably derived from the endoplasmic reticulum of the RPE (90,91).

Phase I Trial of Ocular Subretinal Injection of a
Recominant Adeno-Associated Virus (rAAV-RPE65) Gene Vector
in Patients with Retinal Disease Due to RPE65 Mutations
→ Background – human retinal disease, **RPE65**

Q. What is the role in vision of RPE65 (retinal pigment epithelium-specific protein 65-kDa)?

A. The *RPE65* gene encodes a 533 amino acid protein in RPE cells critical to the retinoid (visual) cycle: specifically, RPE65 is necessary for 11-cis retinal to be synthesized and thus available to form the light-absorbing visual pigment in photoreceptors.

nature genetics volume 20 december 1998

Rpe65 is necessary for production of 11-*cis*-vitamin A in the retinal visual cycle

T. Michael Redmond[1], Shirley Yu[1], Eric Lee[2], Dean Bok[3], Duco Hamasaki[4], Ning Chen[5], Patrice Goletz[5], Jian-Xing Ma[5], Rosalie K. Crouch[5] & Karl Pfeifer[2]

Mutation of *RPE65* can cause severe blindness from birth or early childhood, and RPE65 protein is associated with retinal pigment epithelium (RPE) vitamin A metabolism. Here, we show that *Rpe65*-deficient mice exhibit changes in retinal physiology and biochemistry. Outer segment discs of rod photoreceptors in $Rpe65^{-/-}$ mice are disorganized compared with those of $Rpe65^{+/+}$ and $Rpe65^{+/-}$ mice. Rod function, as measured by electroretinography, is abolished in $Rpe65^{-/-}$ mice, although cone function remains. $Rpe65^{-/-}$ mice lack rhodopsin, but not opsin apoprotein. Furthermore, all-*trans*-retinyl esters over-accumulate in the RPE of $Rpe65^{-/-}$ mice, whereas 11-*cis*-retinyl esters are absent. Disruption of the RPE-based metabolism of all-*trans*-retinyl esters to 11-*cis*-retinal thus appears to underlie the $Rpe65^{-/-}$ phenotype, although cone pigment regeneration may be dependent on a separate pathway.

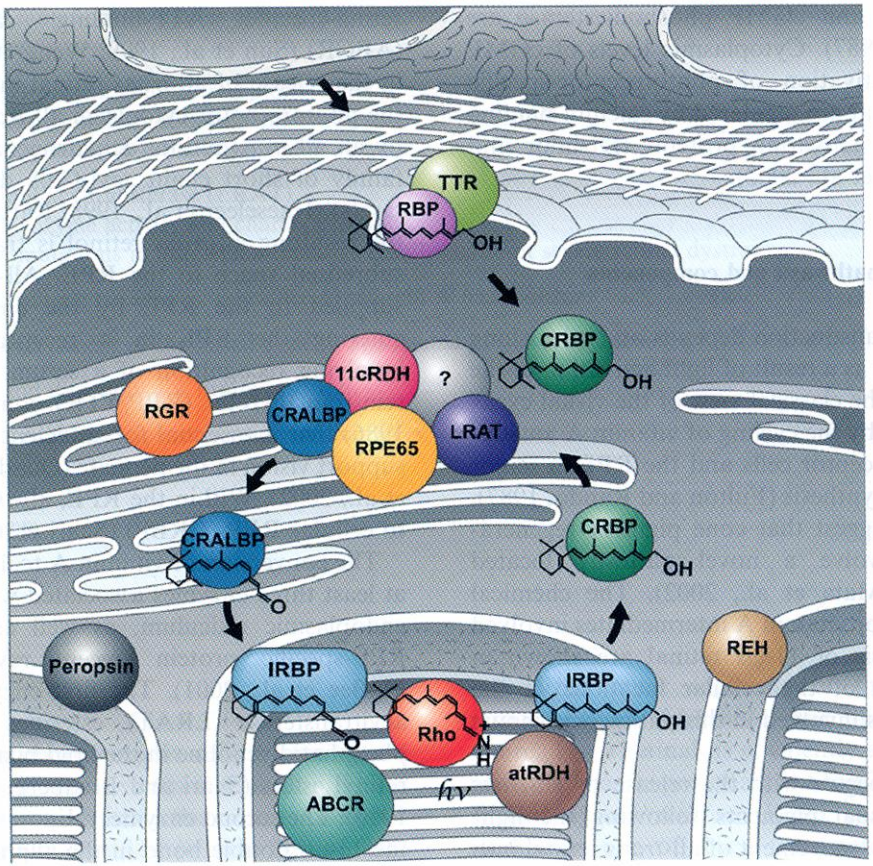

Fig. 2. Depiction of the visual cycle proteins present in rod photoreceptors and RPE. 11cRDH, 11-*cis* retinol dehydrogenase; atRDH, all-*trans* retinal dehydrogenase; Rho, rhodopsin; all other abbreviations as in the text.

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in Patients with Retinal Disease Due to RPE65 Mutations
→ Pre-clinical proof-of-concept studies

Q. What proof-of-concept studies have been performed to warrant consideration of this proposal to perform a Phase I gene vector trial?

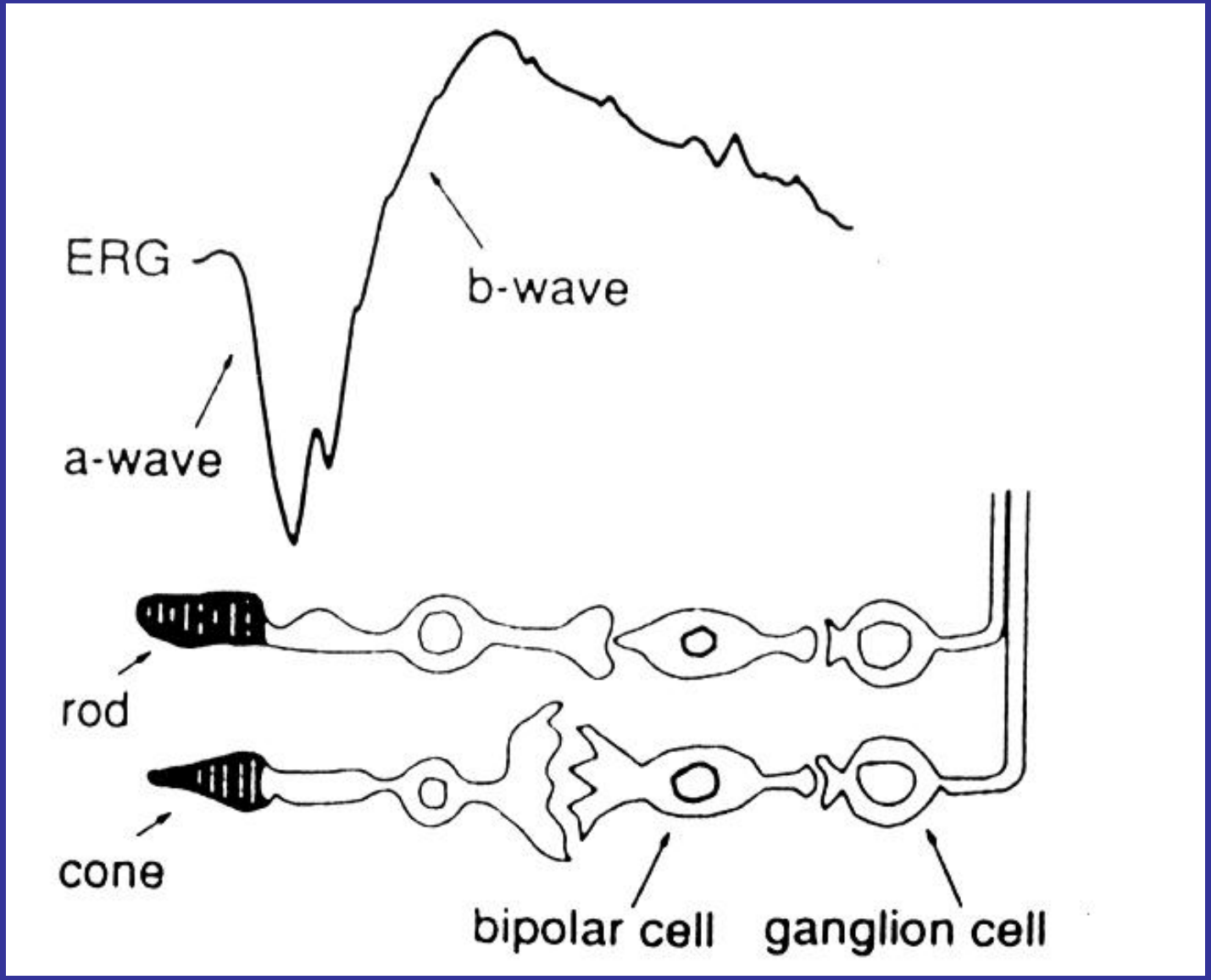
A. Ocular subretinal injections of rAAV-RPE65 in a naturally-occurring dog *RPE65* mutation and in *Rpe65* knockout or mutant mice restored visual function to these blind animals.

nature genetics • volume 28 • may 2001

Gene therapy restores vision in a canine model of childhood blindness

Gregory M. Acland[1], Gustavo D. Aguirre[1], Jharna Ray[1], Qi Zhang[1], Tomas S. Aleman[2], Artur V. Cideciyan[2], Susan E. Pearce-Kelling[1], Vibha Anand[2], Yong Zeng[2], Albert M. Maguire[2], Samuel G. Jacobson[2], William W. Hauswirth[3] & Jean Bennett[2]

The relationship between the neurosensory photoreceptors and the adjacent retinal pigment epithelium (RPE) controls not only normal retinal function, but also the pathogenesis of hereditary retinal degenerations. The molecular bases for both primary photoreceptor[1] and RPE diseases[2–4] that cause blindness have been identified. Gene therapy has been used successfully to slow degeneration in rodent models of primary photoreceptor diseases[5,6], but efficacy of gene therapy directed at photoreceptors and RPE in a large-animal model of human disease has not been reported. Here we study one of the most clinically severe retinal degenerations, Leber congenital amaurosis (LCA). LCA causes near total blindness in infancy and can result from mutations in *RPE65* (LCA, type II; MIM 180069 and 204100). A naturally occurring animal model, the *RPE65*$^{-/-}$ dog, suffers from early and severe visual impairment similar to that seen in human LCA. We used a recombinant adeno-associated virus (AAV) carrying wild-type *RPE65* (AAV-*RPE65*) to test the efficacy of gene therapy in this model. Our results indicate that visual function was restored in this large animal model of childhood blindness.


ERG
b-wave
a-wave
rod
cone
bipolar cell
ganglion cell

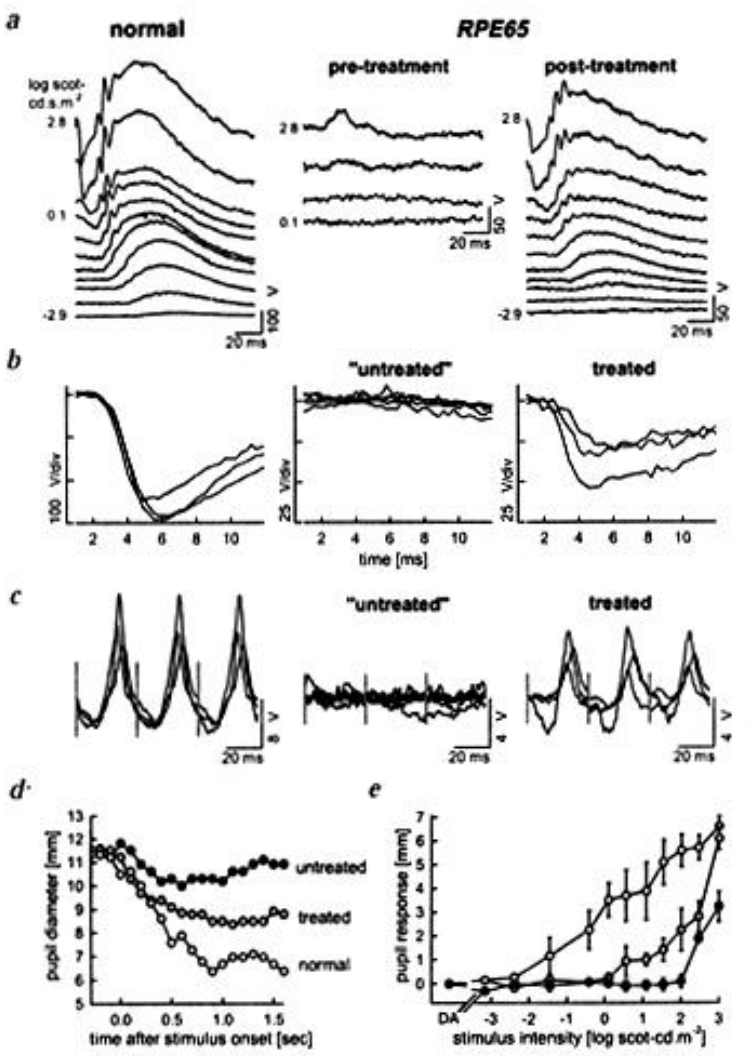
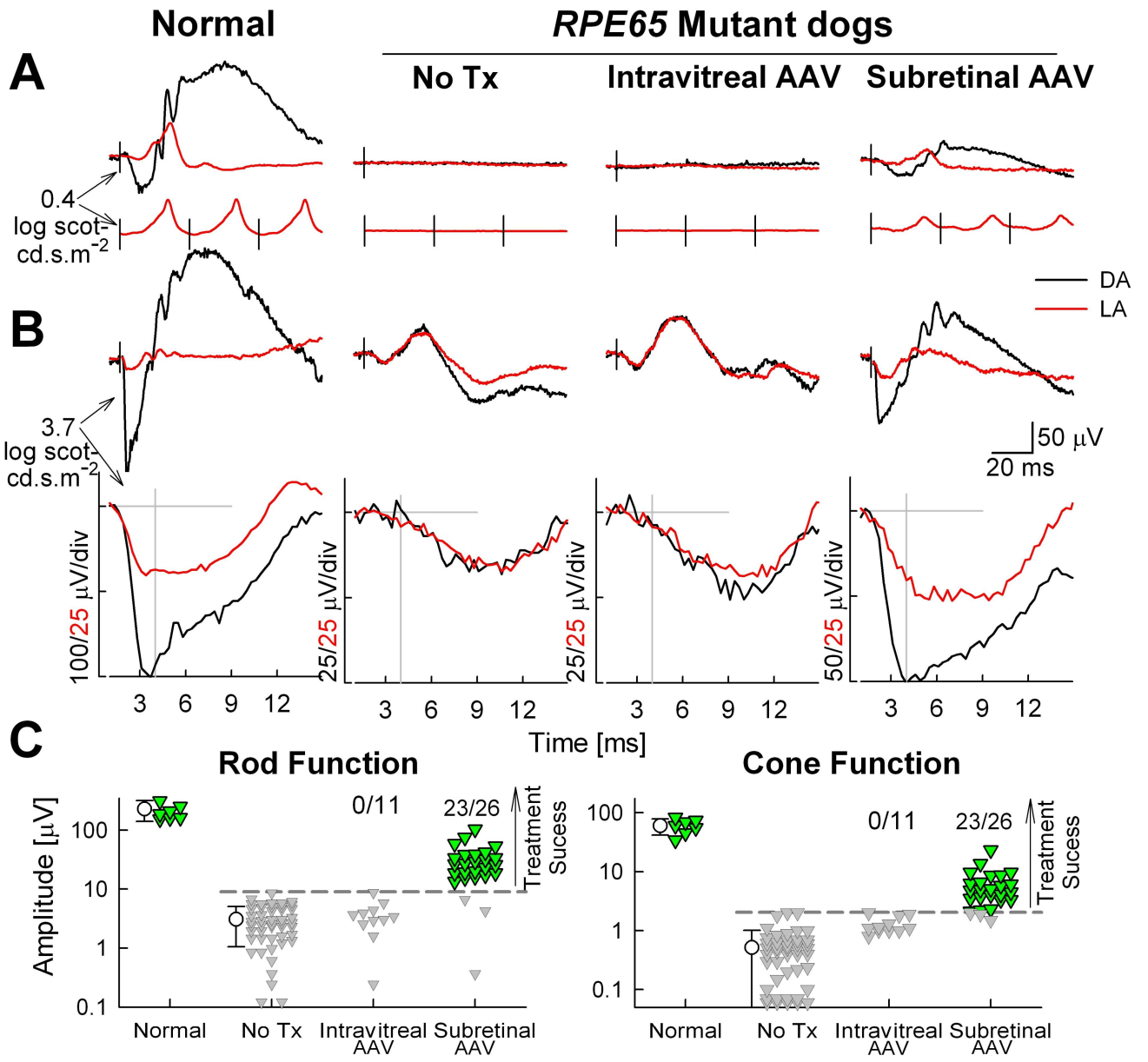
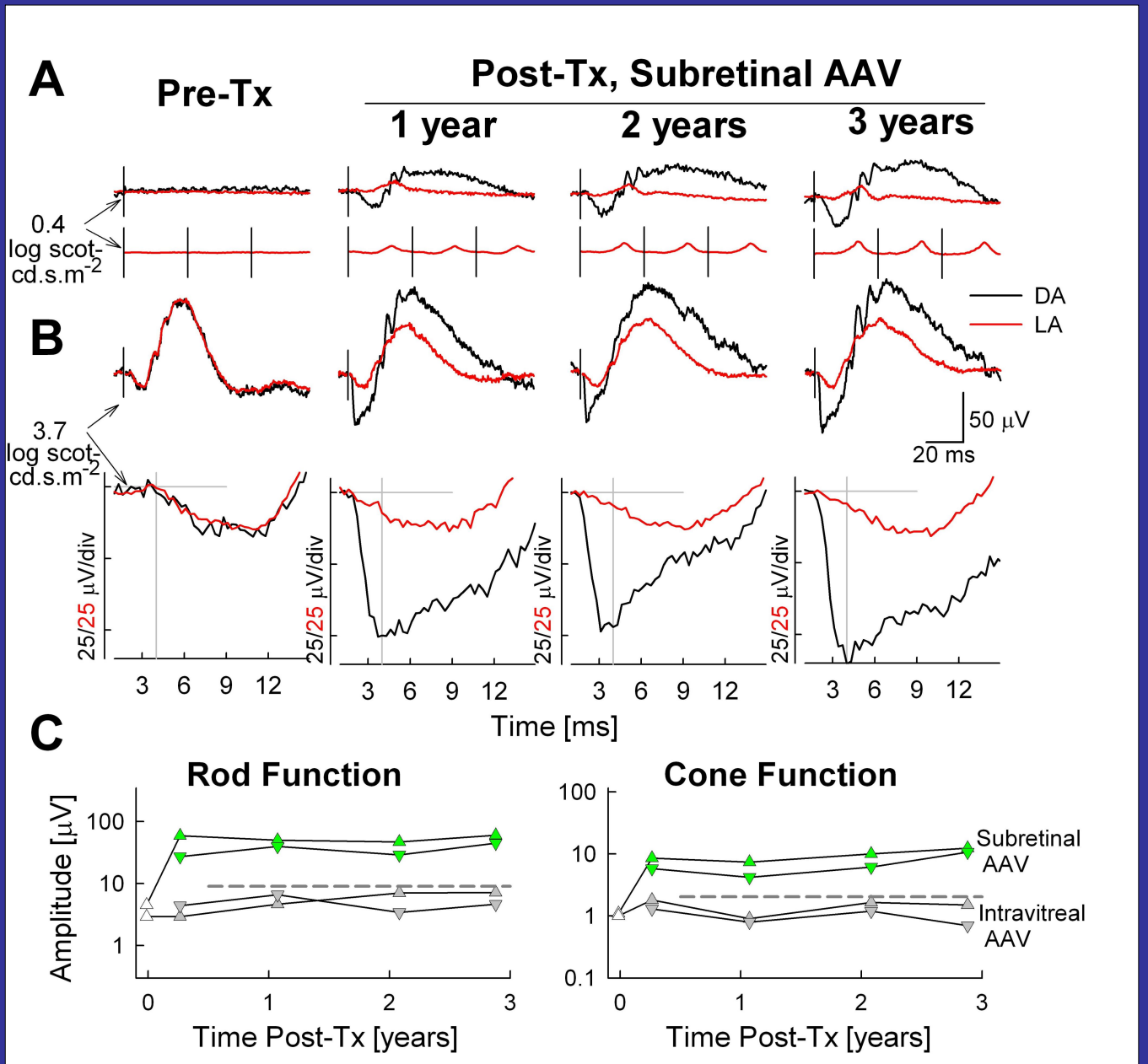


Fig. 3 Restoration of retinal and visual function in *RPE65* mutant dogs by subretinal AAV-*RPE65*. **a**, Comparison of dark-adapted ERGs evoked by increasing intensities of blue light stimuli in a control dog (left) with ERGs with the same stimuli in *RPE65* mutant dog BR33 (middle). The affected animal has elevated thresholds, reduced amplitudes and waveform shape changes (b-waves but no detectable a-waves). After subretinal AAV-*RPE65* therapy (right), the mutant dog shows an improved b-wave threshold, a large increase of a- and b-wave amplitudes (although not to normal levels), and an ERG waveform shape that is now similar to that of controls. Traces start at stimulus onset, stimulus luminance is to the left of key traces. **b**, Details of photoreceptor function were analyzed by the amplitude and timing of the ERG photoresponses evoked by 2.8 log scot-cd s m^{-2} flashes; recordings from 3 control dogs (left) show ~250 μV saturated amplitudes peaking between 4.5 and 6 ms. Photoreceptor function was near noise level in three untreated eyes of *RPE65* mutant dogs and two eyes treated with intravitreal AAV-*RPE65* (middle). Photoresponses (of reduced amplitude but normal timing) were present in all three eyes that received subretinal AAV-*RPE65* (right). **c**, Flicker ERGs in the same eyes as in (*b*) demonstrate a lack of detectable cone-mediated responses from *RPE65* mutant dogs with untreated or intravitreally treated eyes (middle). All eyes with subretinal AAV-*RPE65* treatment recovered cone flicker responses (right). Vertical gray bars denote stimulus onset. **d**, Change in pupil diameter in response to 2.5 log cd.m^{-2} green stimulus in one eye of three representative dogs: untreated (BR46), subretinal AAV treated (BR33) and a normal control. **e**, Pupil response as a function of stimulus intensity showed 3.8 log unit elevation of threshold (1 mm response criterion) in untreated eyes (black symbols, n=3; two eyes of BR46 and one eye of BR29) compared with normal eyes (white symbols, n=3). Eyes treated with subretinal AAV (gray symbols; n=2; BR33 and BR47) had 0.8 log unit lower thresholds compared with untreated eyes. Error bars denote s.e.m.

But did it work in more than these 3 well-publicized dogs?


Normal
RPE65 Mutant dogs
No Tx
Intravitreal AAV
Subretinal AAV
A
0.4 log scot-cd.s.m-2
B
3.7 log scot-cd.s.m-2
DA
LA
50 µV
20 ms
100/25 µV/div
25/25 µV/div
25/25 µV/div
50/25 µV/div
3 6 9 12
Time [ms]
C
Rod Function
Cone Function
Amplitude [µV]
100
10
1
0.1
0/11
23/26
Treatment Sucess
Normal
No Tx
Intravitreal AAV
Subretinal AAV

But does the restored vision from gene transfer last?


A
Pre-Tx
Post-Tx, Subretinal AAV
1 year
2 years
3 years
0.4 log scot-cd.s.m-2
B
3.7 log scot-cd.s.m-2
DA
LA
50 µV
20 ms
25/25 µV/div
3 6 9 12
Time [ms]
C
Rod Function
Cone Function
Amplitude [µV]
100
10
1
0.1
0 1 2 3
Time Post-Tx [years]
Subretinal AAV
Intravitreal AAV

MOLECULAR THERAPY Vol. 9, No. 2, February 2004

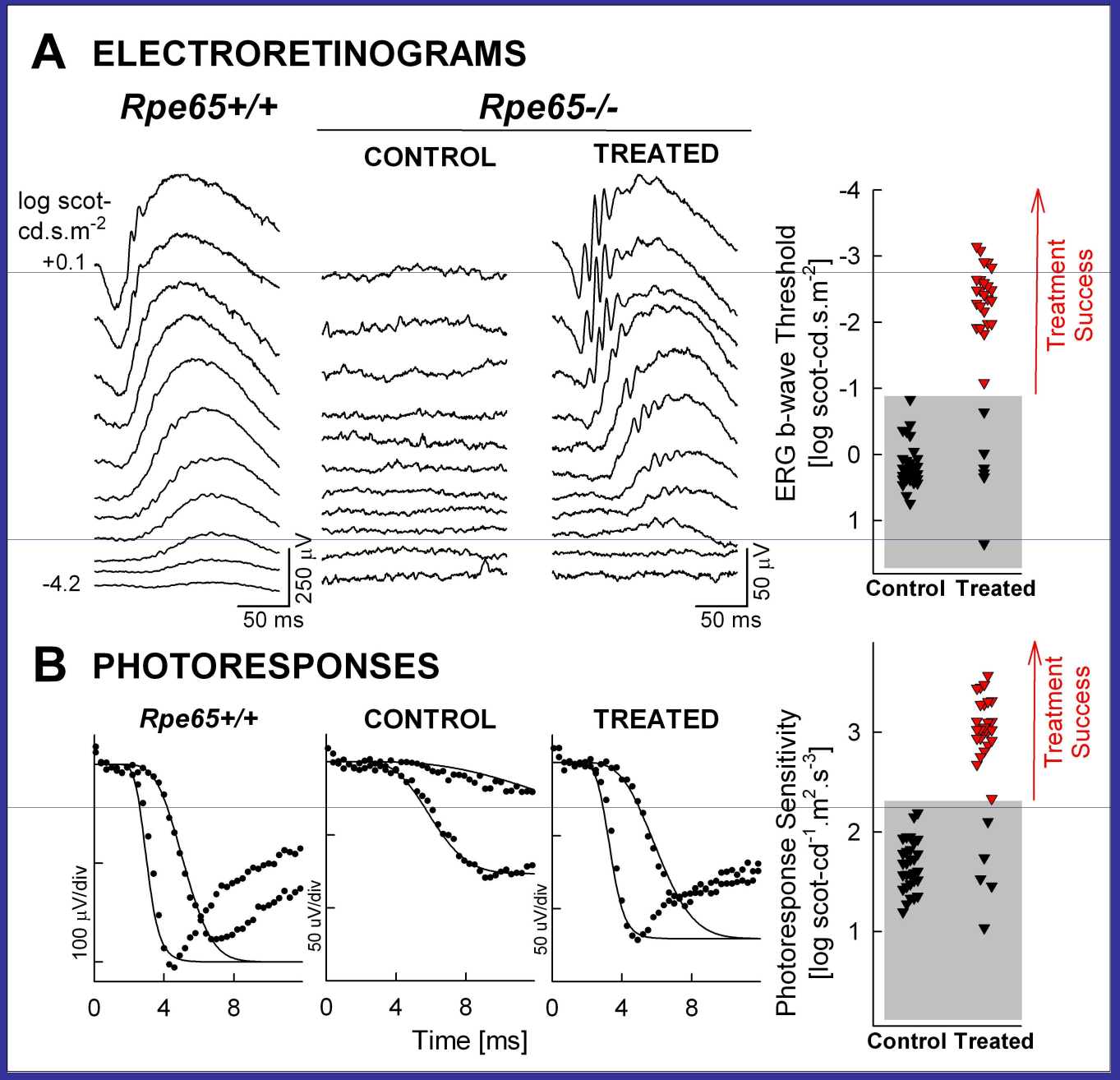
In Utero Gene Therapy Rescues Vision in a Murine Model of Congenital Blindness

Nadine S. Dejneka,[1,*] Enrico M. Surace,[1,*] Tomas S. Aleman,[1] Artur V. Cideciyan,[1] Arkady Lyubarsky,[1] Andrey Savchenko,[1] T. Michael Redmond,[2] Waixing Tang,[1] Zhangyong Wei,[1] Tonia S. Rex,[1] Ernest Glover,[1] Albert M. Maguire,[1] Edward N. Pugh Jr.,[1] Samuel G. Jacobson,[1] and Jean Bennett[1,†]

[1] *F.M. Kirby Center and Scheie Eye Institute, Department of Ophthalmology, University of Pennsylvania, 51 N. 39th Street, Philadelphia, PA 19104-2689, USA*

[2] *Laboratory of Retinal Cell and Molecular Biology, National Eye Institute, National Institutes of Health, 6 Center Drive, MSC 2740, Bethesda, MD 20892-2740, USA*

The congenital retinal blindness known as Leber congenital amaurosis (LCA) can be caused by mutations in the *RPE65* gene. RPE65 plays a critical role in the visual cycle that produces the photosensitive pigment rhodopsin. Recent evidence from human studies of LCA indicates that earlier rather than later intervention may be more likely to restore vision. We determined the impact of *in utero* delivery of the human *RPE65* cDNA to retinal pigment epithelium cells in a murine model of LCA, the *Rpe65*$^{-/-}$ mouse, using a serotype 2 adeno-associated virus packaged within an AAV1 capsid (AAV2/1). Delivery of AAV2/1-CMV-*hRPE65* to fetuses (embryonic day 14) resulted in efficient transduction of retinal pigment epithelium, restoration of visual function, and measurable rhodopsin. The results demonstrate AAV-mediated correction of the deficit and suggest that *in utero* retinal gene delivery may be a useful approach for treating a variety of blinding congenital retinal diseases.


A ELECTRORETINOGRAMS
Rpe65+/+
Rpe65-/-
CONTROL
TREATED
log scot-cd.s.m-2
+0.1
-4.2
250 µV
50 ms
50 µV
50 ms
ERG b-wave Threshold [log scot-cd.s.m-2]
-4
-3
-2
-1
0
1
Control Treated
Treatment Success
B PHOTORESPONSES
Rpe65+/+
CONTROL
TREATED
100 µV/div
50 uV/div
50 uV/div
0
4
8
Time [ms]
Photoresponse Sensitivity [log scot-cd-1.m2.s-3]
3
2
1
Control Treated
Treatment Success

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in Patients with Retinal Disease Due to RPE65 Mutations
→ Models versus human patients

Q. Is there any evidence that human LCA from *RPE65* mutations show any similarities to the animal models treated successfully with ocular subretinal rAAV-RPE65?

A. Now there is. We used high resolution retinal imaging and co-localized measures of vision to determine the relationship of structure to function in patients.

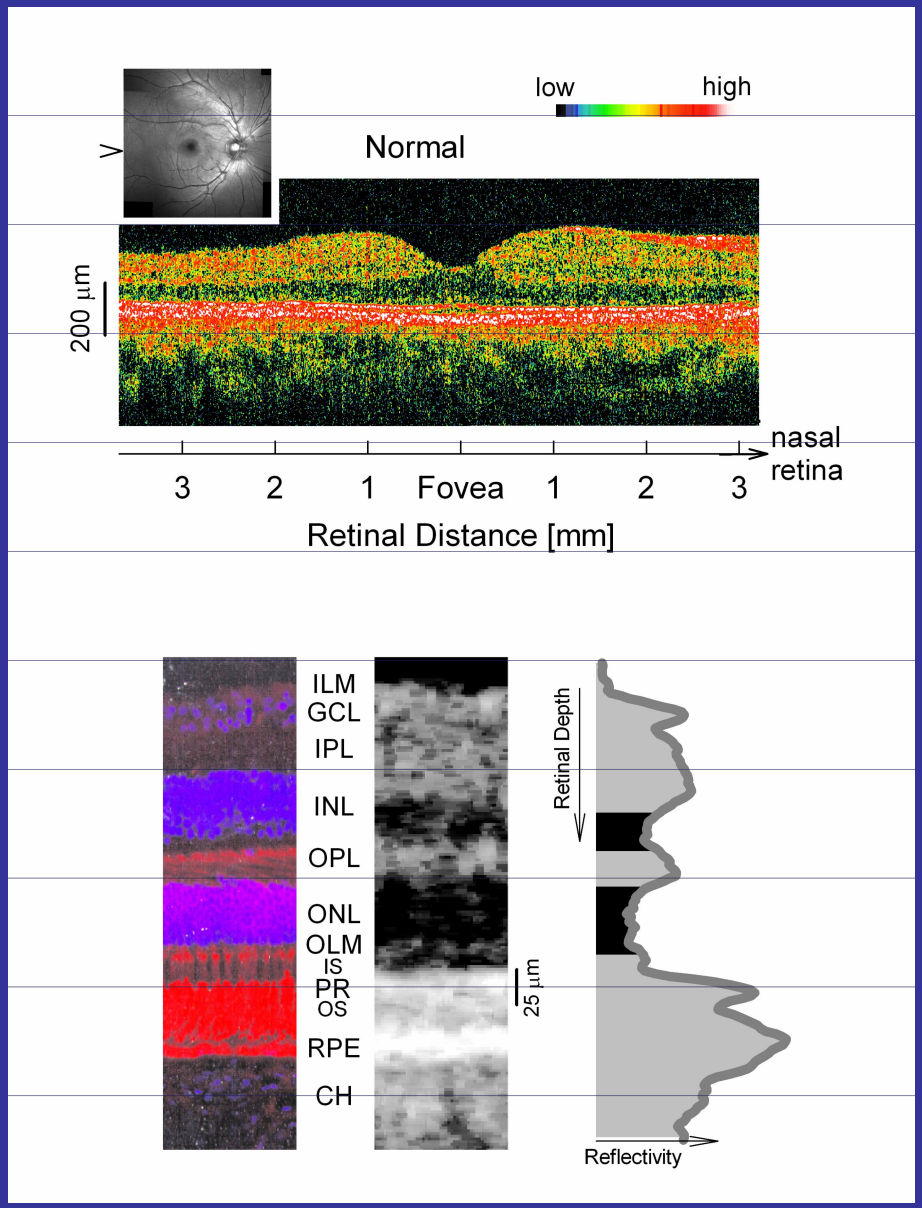
PNAS | April 26, 2005 | vol. 102 | no. 17 | 6177–6182

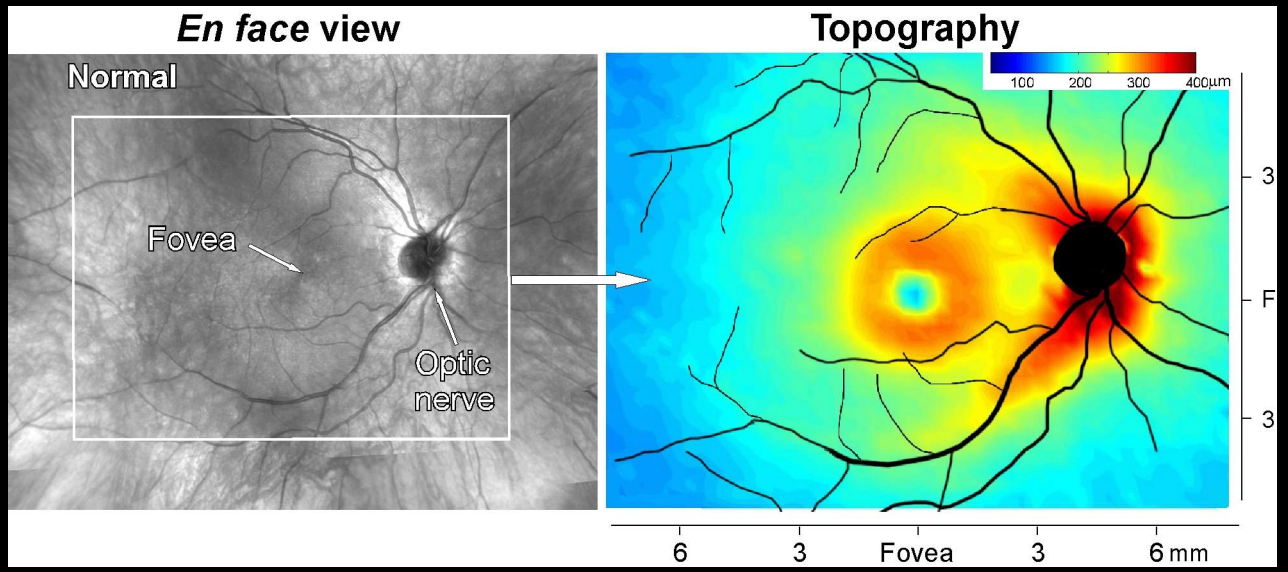
Identifying photoreceptors in blind eyes caused by *RPE65* mutations: Prerequisite for human gene therapy success

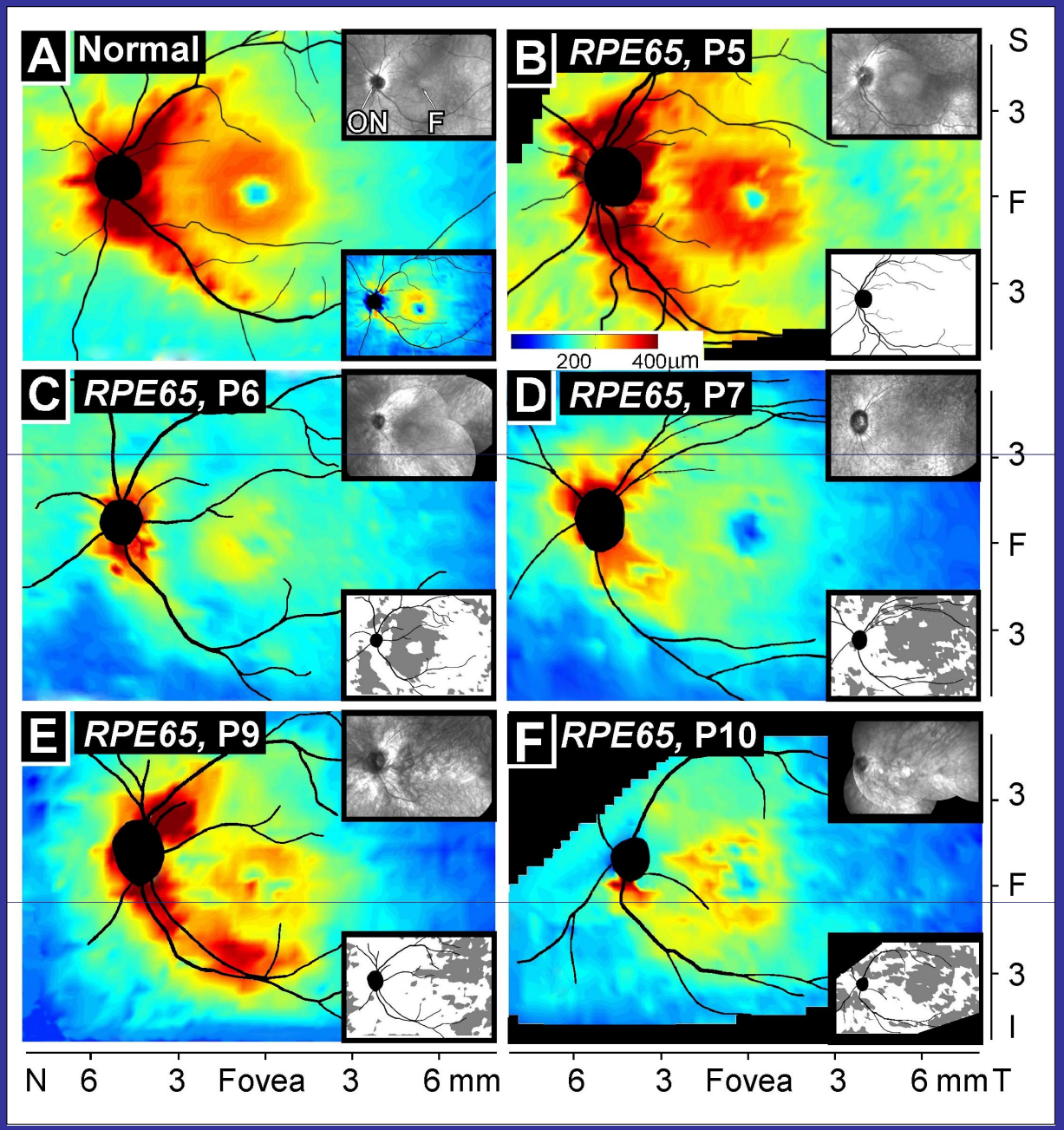
Samuel G. Jacobson*†, Tomas S. Aleman*, Artur V. Cideciyan*, Alexander Sumaroka*, Sharon B. Schwartz*, Elizabeth A. M. Windsor*, Elias I. Traboulsi‡, Elise Heon§, Steven J. Pittler¶, Ann H. Milam*, Albert M. Maguire*, Krzysztof Palczewski‖, Edwin M. Stone**, and Jean Bennett*

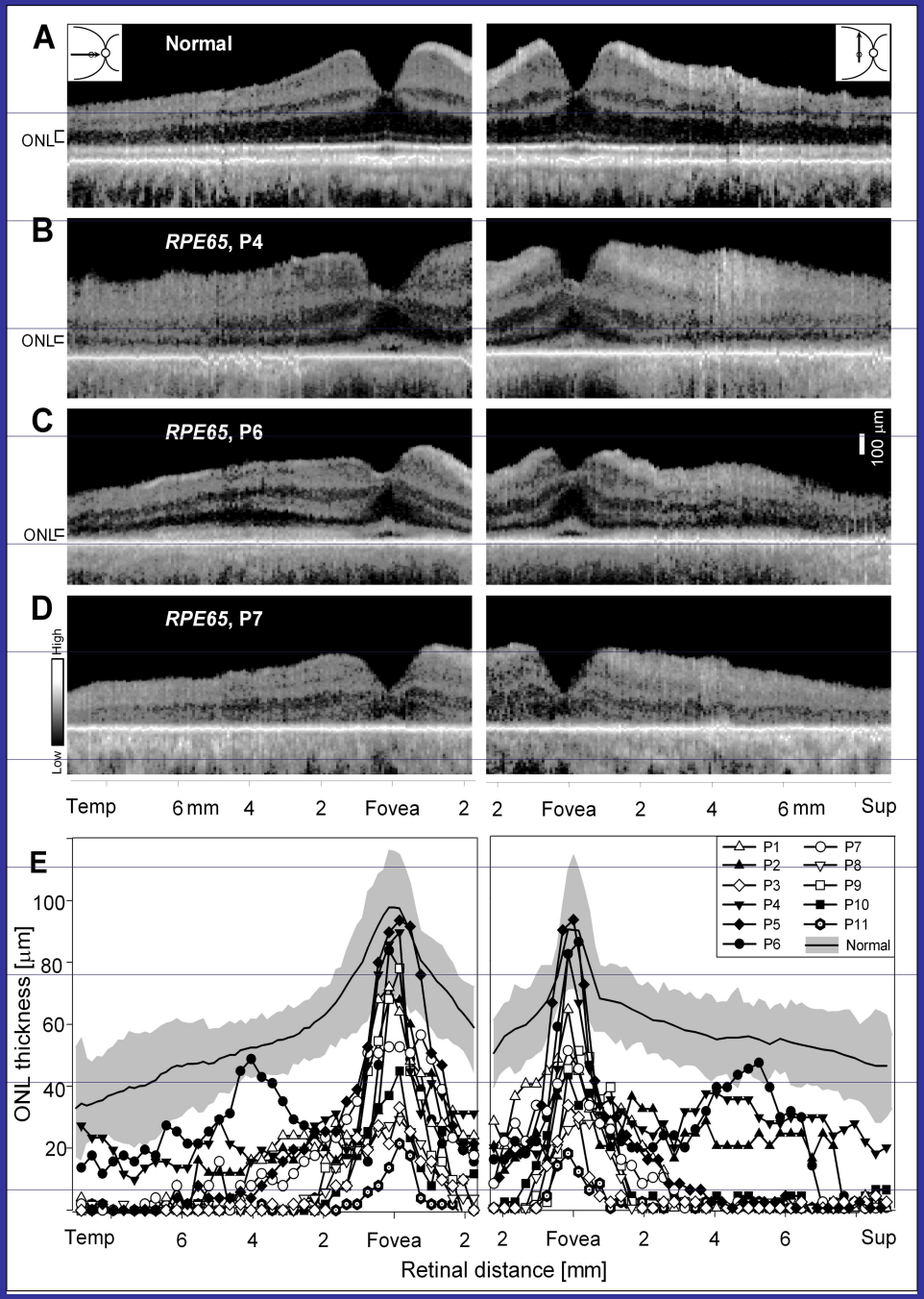
*Department of Ophthalmology, University of Pennsylvania, Philadelphia, PA 19104; ‡Cole Eye Institute, Cleveland Clinic Foundation, Cleveland, OH 44195; §Department of Ophthalmology and Vision Sciences, Hospital for Sick Children, Toronto, ON, Canada M5G 1X8; ¶Department of Physiological Optics, University of Alabama at Birmingham, Birmingham, AL 35294; ‖Department of Ophthalmology, University of Washington School of Medicine, Seattle, WA 98195; and **Howard Hughes Medical Institute and Department of Ophthalmology, University of Iowa Carver College of Medicine, Iowa City, IA 52242

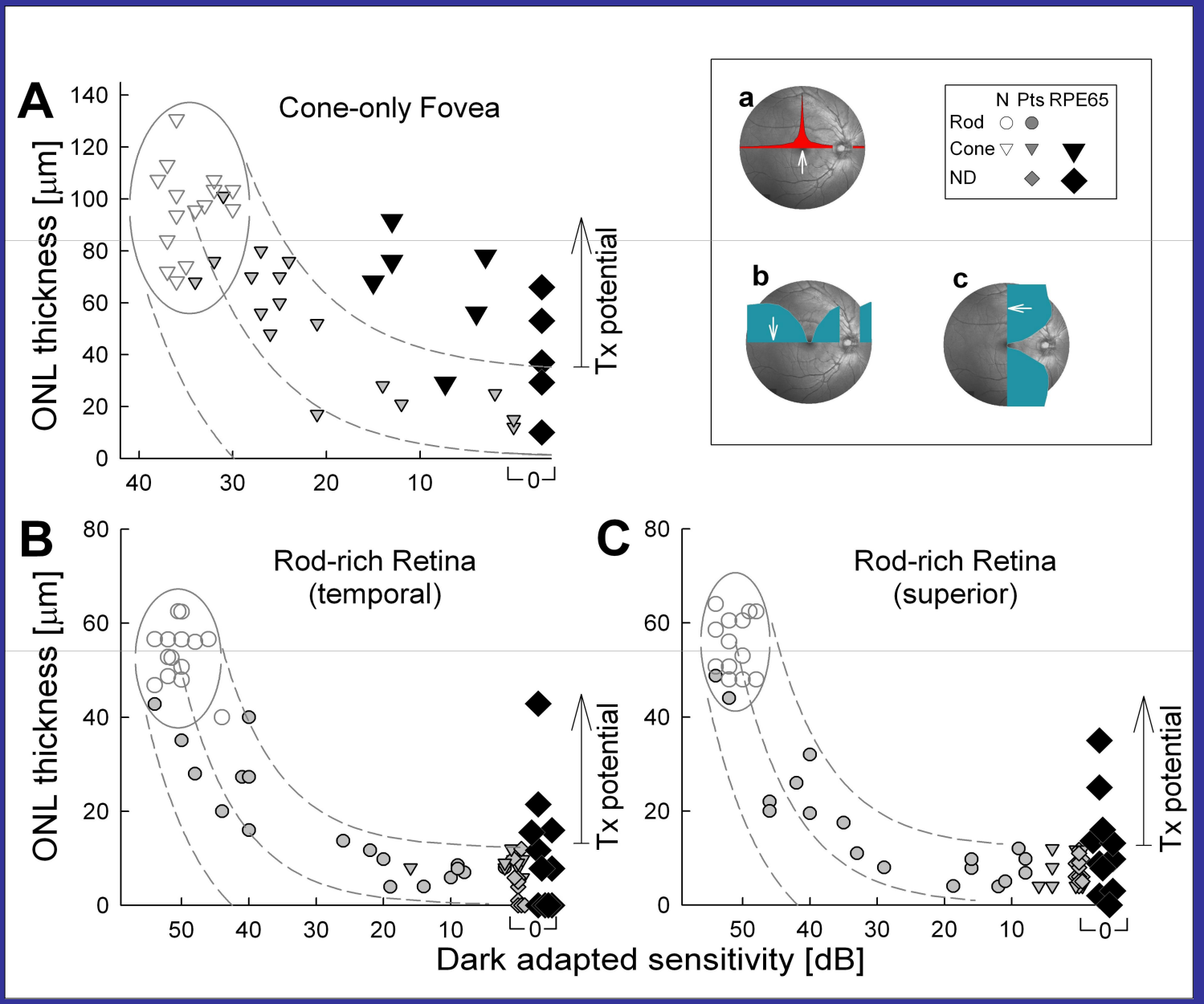
Mutations in *RPE65*, a gene essential to normal operation of the visual (retinoid) cycle, cause the childhood blindness known as Leber congenital amaurosis (LCA). Retinal gene therapy restores vision to blind canine and murine models of LCA. Gene therapy in blind humans with LCA from *RPE65* mutations may also have potential for success but only if the retinal photoreceptor layer is intact, as in the early-disease stage-treated animals. Here, we use high-resolution *in vivo* microscopy to quantify photoreceptor layer thickness in the human disease to define the relationship of retinal structure to vision and determine the potential for gene therapy success. The normally cone photoreceptor-rich central retina and rod-rich regions were studied. Despite severely reduced cone vision, many *RPE65*-mutant retinas had near-normal central microstructure. Absent rod vision was associated with a detectable but thinned photoreceptor layer. We asked whether abnormally thinned *RPE65*-mutant retina with photoreceptor loss would respond to treatment. Gene therapy in *Rpe65*$^{-/-}$ mice at advanced-disease stages, a more faithful mimic of the humans we studied, showed success but only in animals with better-preserved photoreceptor structure. The results indicate that identifying and then targeting retinal locations with retained photoreceptors will be a prerequisite for successful gene therapy in humans with *RPE65* mutations and in other retinal degenerative disorders now moving from proof-of-concept studies toward clinical trials.

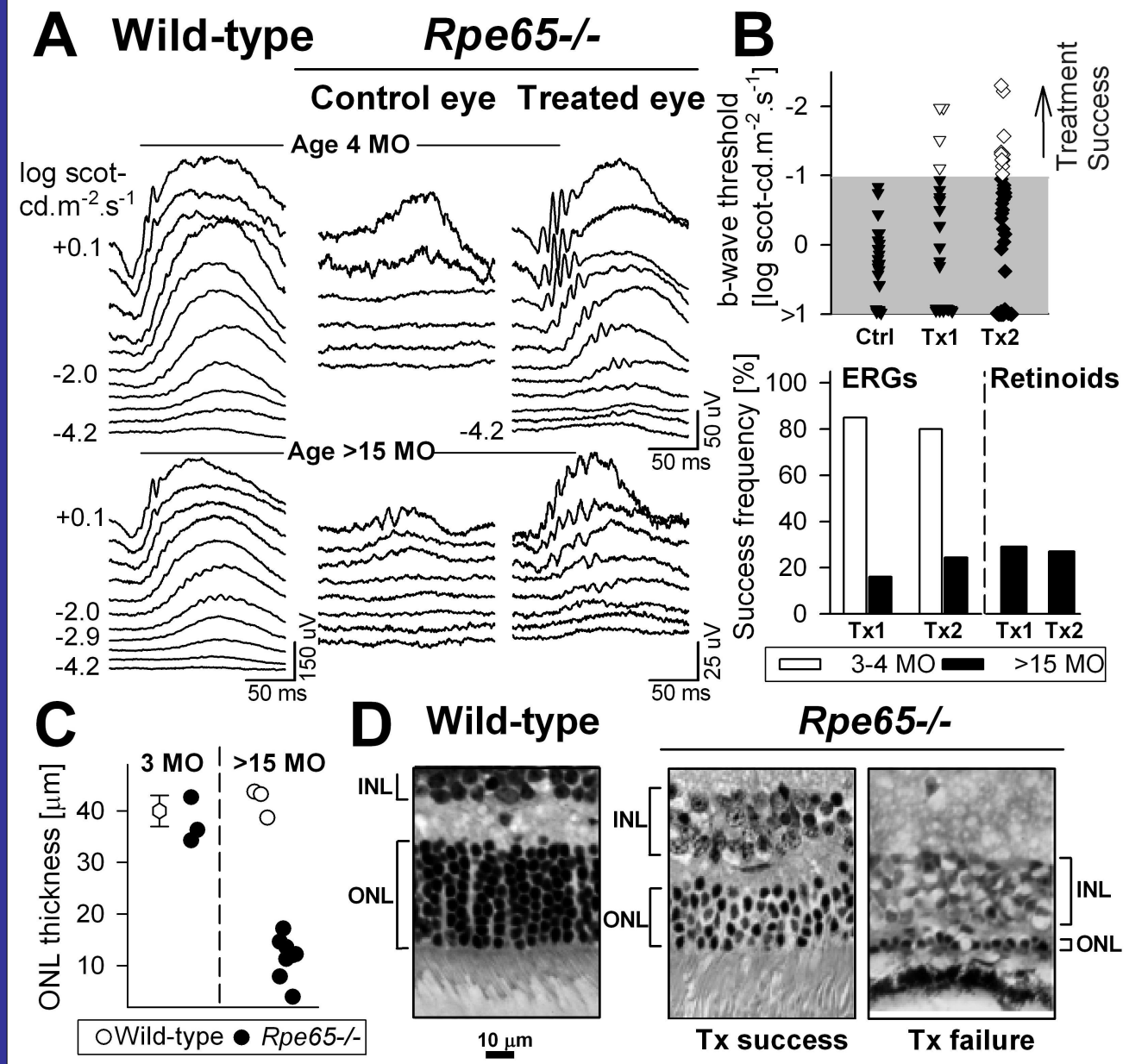

low
high
V
Normal
200 µm
nasal
retina
3
2
1
Fovea
1
2
3
Retinal Distance [mm]
ILM
GCL
IPL
INL
OPL
ONL
OLM
IS
PR
OS
RPE
CH
25 µm
Retinal Depth
Reflectivity


En face view
Topography
Normal
Fovea
Optic nerve
100
200
300
400µm
3
F
3
6
3
Fovea
3
6 mm


A Normal
ON
F
B RPE65, P5
200
400µm
C RPE65, P6
D RPE65, P7
E RPE65, P9
F RPE65, P10
S
3
F
3
3
F
3
3
F
3
I
N 6 3 Fovea 3 6 mm
6 3 Fovea 3 6 mm T


A
Normal
ONL
B
RPE65, P4
ONL
C
RPE65, P6
ONL
100 µm
D
RPE65, P7
High
Low
Temp
6 mm
4
2
Fovea
2
2
Fovea
2
4
6 mm
Sup
E
ONL thickness [µm]
100
80
60
40
20
P1
P2
P3
P4
P5
P6
P7
P8
P9
P10
P11
Normal
Temp
6
4
2
Fovea
2
2
Fovea
2
4
6
Sup
Retinal distance [mm]


A
Cone-only Fovea
ONL thickness [μm]
140
120
100
80
60
40
20
0
40
30
20
10
0
Tx potential
a
b
c
N Pts RPE65
Rod
Cone
ND
B
Rod-rich Retina
(temporal)
ONL thickness [μm]
80
60
40
20
0
50
40
30
20
10
0
Tx potential
C
Rod-rich Retina
(superior)
80
60
40
20
0
50
40
30
20
10
0
Tx potential
Dark adapted sensitivity [dB]


A
Wild-type
Rpe65-/-
Control eye
Treated eye
Age 4 MO
log scot-cd.m-2.s-1
+0.1
-2.0
-4.2
50 uV
50 ms
Age >15 MO
+0.1
-2.0
-2.9
-4.2
150 uV
25 uV
50 ms
B
b-wave threshold [log scot-cd.m-2.s-1]
-2
-1
0
>1
Ctrl
Tx1
Tx2
Treatment Success
Success frequency [%]
100
80
60
40
20
0
ERGs
Retinoids
Tx1
Tx2
Tx1
Tx2
3-4 MO
>15 MO
C
ONL thickness [µm]
40
30
20
10
3 MO
>15 MO
Wild-type
Rpe65-/-
D
Wild-type
Rpe65-/-
INL
ONL
10 µm
Tx success
Tx failure

Phase I Trial of Ocular Subretinal Injection of a
Recominant Adeno-Associated Virus (rAAV-RPE65) Gene Vector
in Patients with Retinal Disease Due to RPE65 Mutations
→ Pre-clinical Safety Studies

Q. What pre-clinical safety studies have been performed for ocular subretinal injections of rAAV-RPE65?

A. To date, there have been a 3-month non-GLP study in RPE65-mutant dogs and two non-human primate GLP studies, a 1-week study and an ongoing 3-month study.

rAAV2-Cβ-h*RPE65*

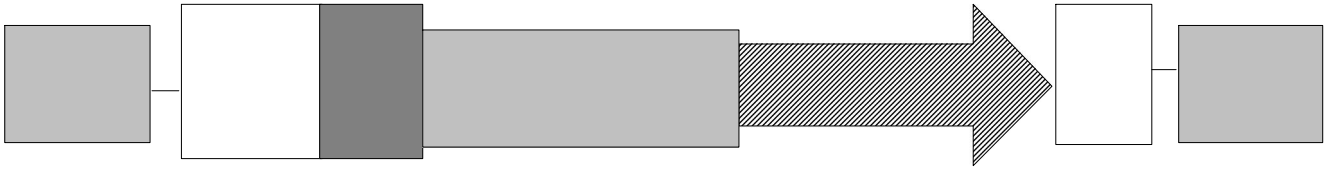
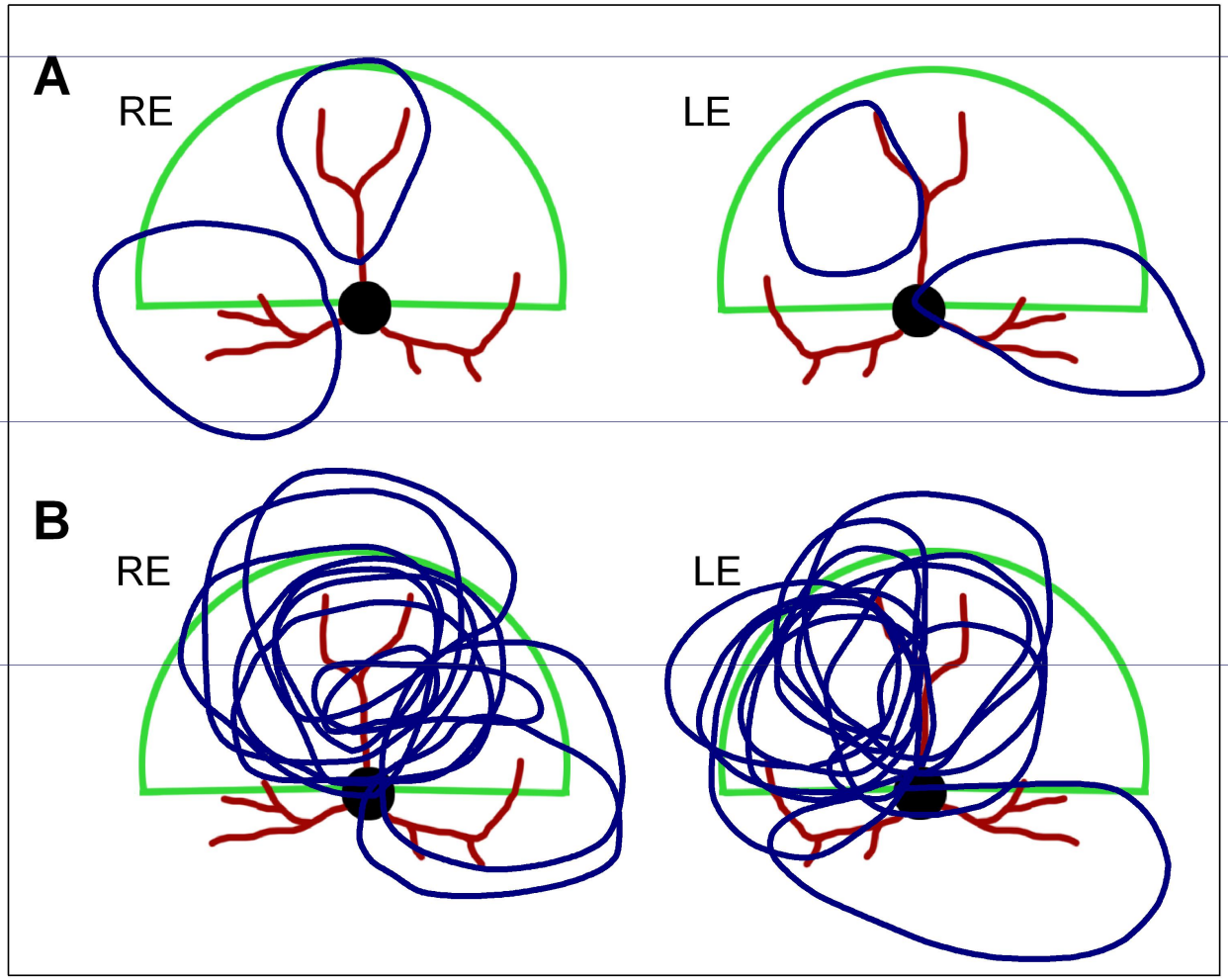
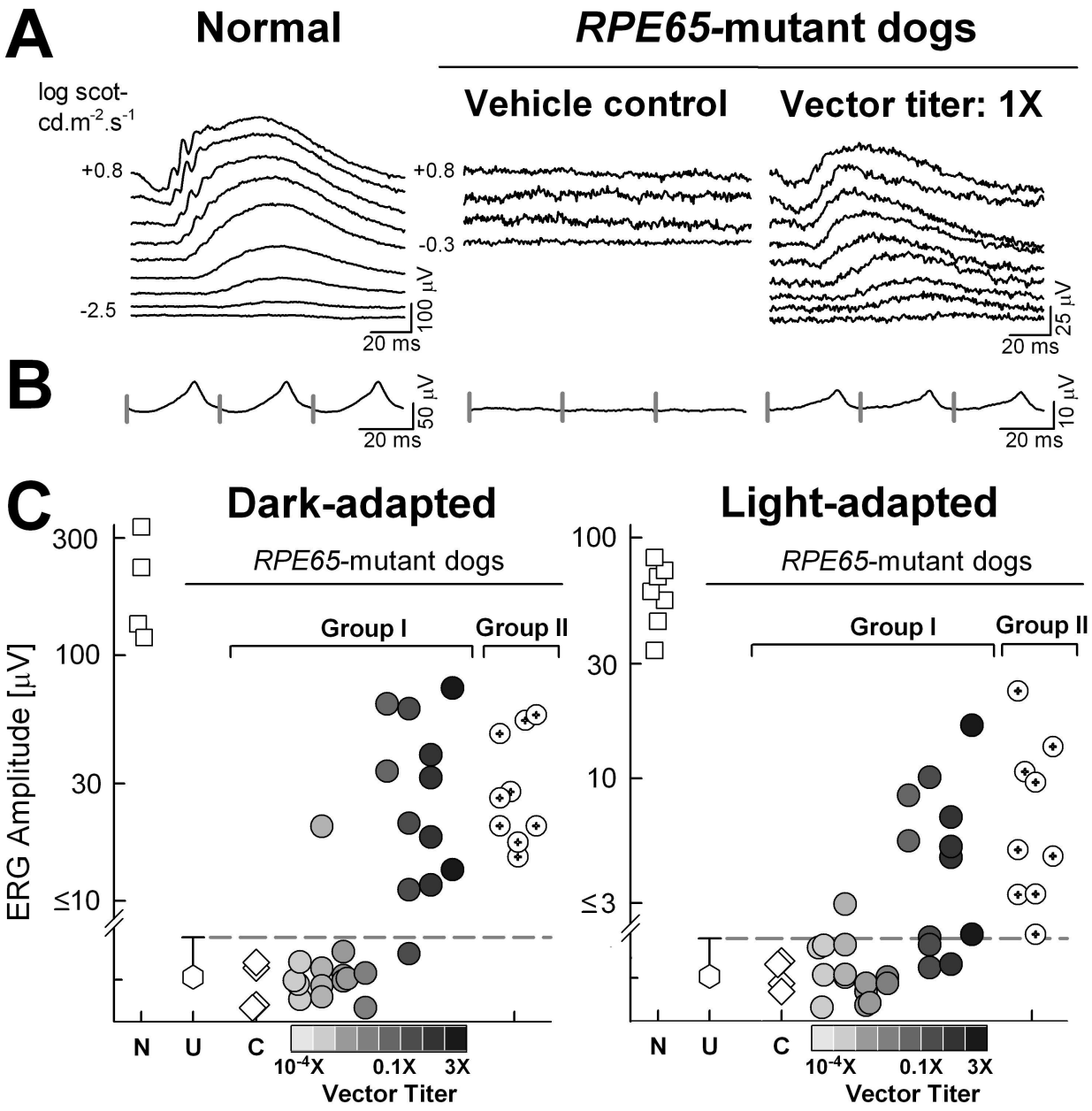


TABLE: Characteristics of *RPE65-/-* dogs and ocular rAAV doses injected

Animal	Gender	Age at injection (months)	Eye	Vector	Titer[a]	Volume (µl)	Age at necropsy (months)
BR246	M	2.7	R	Vehicle	0	150	6.2
			L	Vehicle	0	150	
EMB31	F	7.4	R	Vehicle	0	150	11.1
			L	Vehicle	0	150	
BR244	M	2.7	R	AAV2-CB-h*RPE65*	0.0001X	150[b]	6.2
			L	AAV2-CB-h*RPE65*	0.0001X	150	
BR239	F	7.0	R	AAV2-CB-h*RPE65*	0.0001X	150	10.5
			L	AAV2-CB-h*RPE65*	0.0001X	150	
BR256	F	5.0	R	AAV2-CB-h*RPE65*	0.001X	150[b]	7.9
			L	AAV2-CB-h*RPE65*	0.001X	100	
EMB33	F	7.4	R	AAV2-CB-h*RPE65*	0.001X	150	11.1
			L	AAV2-CB-h*RPE65*	0.001X	150	
BR251	F	2.7	R	AAV2-CB-h*RPE65*	0.01X	150	6.2
			L	AAV2-CB-h*RPE65*	0.01X	150	
EMB28	M	7.4	R	AAV2-CB-h*RPE65*	0.01X	150[c]	10.9
			L	AAV2-CB-h*RPE65*	0.01X	150	
BR257	F	5.0	R	AAV2-CB-h*RPE65*	0.03X	150	7.9
			L	AAV2-CB-h*RPE65*	0.03X	150[d]	
BR263	F	4.1	R	AAV2-CB-h*RPE65*	0.1X	150	7.0
			L	AAV2-CB-h*RPE65*	0.1X	150	
BR266	F	3.8	R	AAV2-CB-h*RPE65*	0.3X	150	6.7
			L	AAV2-CB-h*RPE65*	0.3X	150	
BR264	F	4.1	R	AAV2-CB-h*RPE65*	0.3X	150[e]	7.0
			L	AAV2-CB-h*RPE65*	0.3X	100	
BR248	M	2.7	R	AAV2-CB-h*RPE65*	1X	150	6.2
			L	AAV2-CB-h*RPE65*	1X	150[b]	
BR235	M	7.0	R	AAV2-CB-h*RPE65*	1X	150	10.5
			L	AAV2-CB-h*RPE65*	1X	150	
BR265	F	4.1	R	AAV2-CB-h*RPE65*	3X	150	7.0
			L	AAV2-CB-h*RPE65*	3X	150	

R, right; L, left; Vehicle, balanced salt solution; [a]1X=1.0 x 10^{10} vg/µl; [b]subretinal/intravitreal; [c]intravitreal; [d]intratapetal; [e]sub-RPE.


A
RE
LE
B
RE
LE

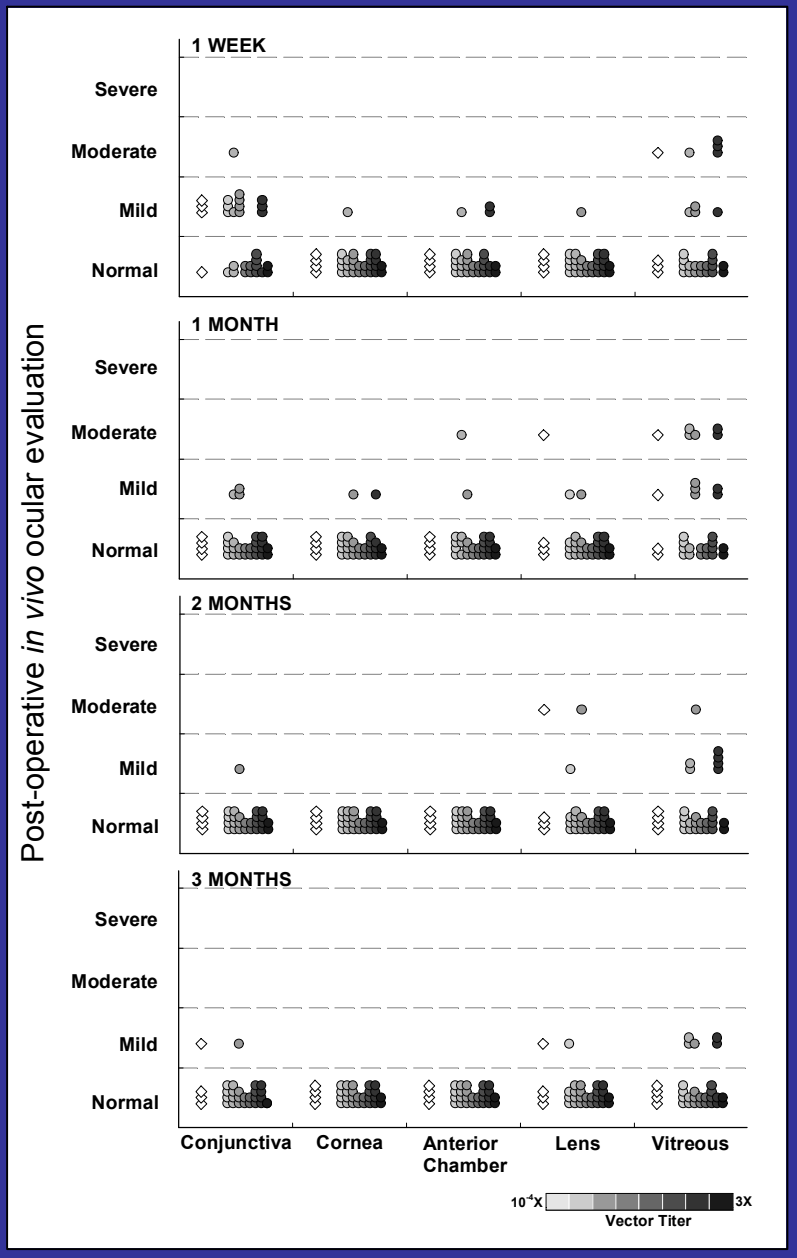

A
Normal
RPE65-mutant dogs
log scot-cd.m-2.s-1
+0.8
-2.5
100 µV
20 ms
Vehicle control
Vector titer: 1X
+0.8
-0.3
25 µV
20 ms
B
50 µV
20 ms
10 µV
20 ms
C
Dark-adapted
Light-adapted
RPE65-mutant dogs
Group I
Group II
ERG Amplitude [µV]
300
100
30
≤10
100
30
10
≤3
N
U
C
10-4X
0.1X
3X
Vector Titer

Phase I Trial of Ocular Subretinal Injection of a
Recominant Adeno-Associated Virus (rAAV-RPE65) Gene Vector
in Patients with Retinal Disease Due to RPE65 Mutations
→ Pre-clinical Safety Studies: non-GLP RPE65-mutant dogs

In-Life Observations: No early deaths; no clinical signs of toxicity.
Clinical Pathology: Details given in 5/10/05 letter, but there were no changes in hematology or clinical chemistry parameters that were clearly test article related.

Clinical Ocular Examinations:
[Performed by Drs. Acland, Aguirre and Komaromy at RDS Facility of UPenn/Cornell]
Graded as 3 levels of severity for conjunctiva, cornea, anterior chamber, lens and vitreous.

Findings:
1 week: Mild to moderate inflammatory changes were noted in both vehicle-injected and vector-injected eyes, especially in conjunctiva and vitreous.
1 month: Conjunctiva clearing but persistent mild-moderate vitreous cellularity;
2-3 months: Mild vitreous cellularity persisted in 5 eyes (1 had vitreous hemorrhage at surgery) and mild posterior subcapsular cataract.


1 WEEK
1 MONTH
2 MONTHS
3 MONTHS
Severe
Moderate
Mild
Normal
Post-operative *in vivo* ocular evaluation
Conjunctiva
Cornea
Anterior Chamber
Lens
Vitreous
10^{-4}X
3X
Vector Titer

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→ Pre-clinical Safety Studies: non-GLP RPE65-mutant dogs

Clinical Funduscopic Examinations:
[Performed by Drs. Acland, Aguirre and Komaromy]

Findings:
At 3 months: Flat retinas with linear or curvilinear pigmented scars in the tapetal retina at/near retinotomy sites (25 eyes). One dog with an inferior retinotomy showed depigmentation in the region of the injection. Three eyes had areas within or near the retinotomy site that suggested retinal thinning, but the contralateral eyes did not show this. There was an intratapetal injection in one eye and this was associated with marked pigmentary change throughout the area of injection. No vector-dose related complications were evident.

Phase I Trial of Ocular Subretinal Injection of a
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→ Pre-clinical Safety Studies: non-GLP RPE65-mutant dogs

Postmortem Observations:

No gross necropsy findings at termination of study.

Histological findings were common findings in canine toxicology studies.

No vector dose-related increases in incidence or severity of any histological finding.

Optic nerves and chiasm were normal.

Ocular histopathology – main findings (to date, by Dr. Caroline Zeiss):

- Traumatic retinal lesions attributable to the surgical intervention (e.g. focal retinal perforation; segmental retinal disruption and fragmentation; segmental retinal atrophy).
- Inflammatory lesions noted in two dogs (EMB31, vehicle control; EMB33, vector dose 0.001X), consisting of fibrin accumulation in vitreous with small numbers of macrophages and lymphocytes.
- Lesions attributable to the RPE65-associated retinal disease (RPE inclusions; modest outer nuclear layer thinning particularly in the inferior retina.

Animal	Sex	Eye	Vector	Titer[a]	Positive biodistribution results by tissue[f] (copy number)[g]
BR246	M	R	Vehicle	0	---
		L	Vehicle	0	
EMB31	F	R	Vehicle	0	---
		L	Vehicle	0	
BR244	M	R	AAV2-CB-h*RPE65*	0.0001X[b]	---
		L	AAV2-CB-h*RPE65*	0.0001X	
BR239	F	R	AAV2-CB-h*RPE65*	0.0001X	Mandibular node (100)[h]
		L	AAV2-CB-h*RPE65*	0.0001X	
BR256	F	R	AAV2-CB-h*RPE65*	0.001X[b]	---
		L	AAV2-CB-h*RPE65*	0.001X	
EMB33	F	R	AAV2-CB-h*RPE65*	0.001X	Diaphragm (1441)[i]
		L	AAV2-CB-h*RPE65*	0.001X	L heart (565)[i]
BR251	F	R	AAV2-CB-h*RPE65*	0.01X	---
		L	AAV2-CB-h*RPE65*	0.01X	
EMB28	M	R	AAV2-CB-h*RPE65*	0.01X[c]	---
		L	AAV2-CB-h*RPE65*	0.01X	
BR257	F	R	AAV2-CB-h*RPE65*	0.03X	---
		L	AAV2-CB-h*RPE65*	0.03X[d]	
BR263	F	R	AAV2-CB-h*RPE65*	0.1X	L optic nerve (220)[h]
		L	AAV2-CB-h*RPE65*	0.1X	
BR266	F	R	AAV2-CB-h*RPE65*	0.3X	---
		L	AAV2-CB-h*RPE65*	0.3X	
BR264	F	R	AAV2-CB-h*RPE65*	0.3X[e]	---
		L	AAV2-CB-h*RPE65*	0.3X	
BR248	M	R	AAV2-CB-h*RPE65*	1X	Diaphragm (196)[h]
		L	AAV2-CB-h*RPE65*	1X[b]	R mandibular node (580)[h]
					Optic chiasm (105)[h]
BR235	M	R	AAV2-CB-h*RPE65*	1X	---
		L	AAV2-CB-h*RPE65*	1X	
BR265	F	R	AAV2-CB-h*RPE65*	3X	---
		L	AAV2-CB-h*RPE65*	3X	

R, right; L, left; Vehicle, balanced salt solution; [a]1X=1.0 x 10^{10} vg/µl; [b]subretinal/intravitreal; [c]intravitreal; [d]intratapetal; [e]sub-RPE; [f] Positive biodistribution result defined as >40 copies/1.0µg genomic DNA; [g]At termination organs sampled: L&R optic nerve, L&R periocular muscle, spleen, pancreas, L&R kidney, L&R liver, diaphragm, L&R heart, lung, skeletal muscle, L&R mandibular node, L parotid node, L brain, optic chiasm, jejunum, and L&R gonad; [h]Single replicate value only, negative second replicate value; [i]Average of both replicate values

Phase I Trial of Ocular Subretinal Injection of a
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in Patients with Retinal Disease Due to RPE65 Mutations
→ Pre-clinical Safety Studies

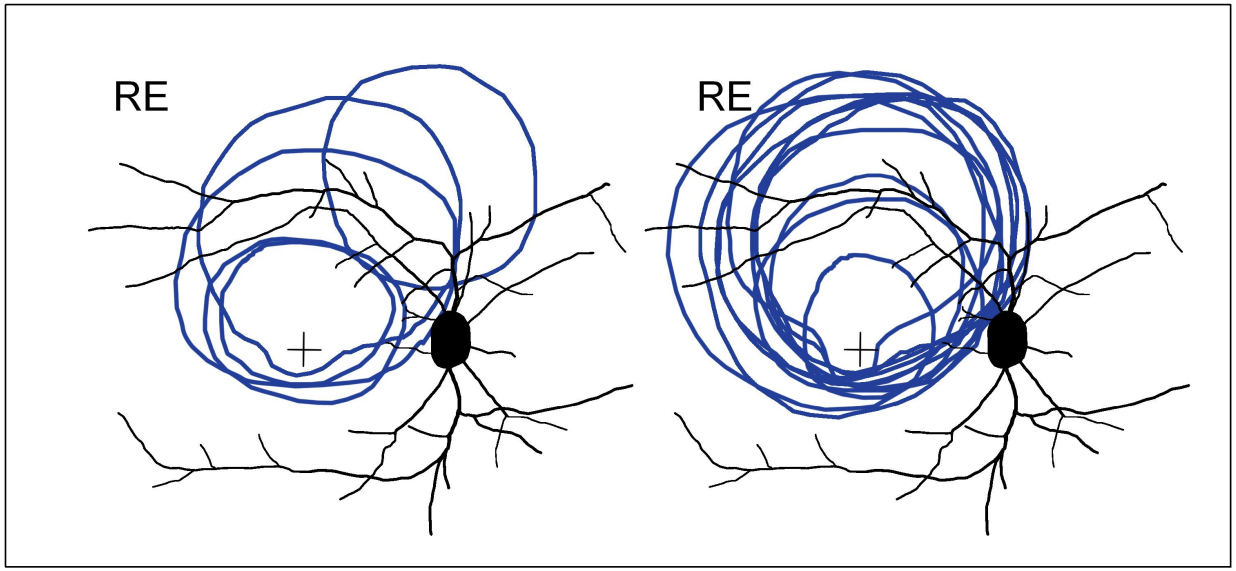
Q. What pre-clinical safety studies have been performed for ocular subretinal injections of rAAV-RPE65?

A. To date, there have been a 3-month non-GLP study in RPE65-mutant dogs and two non-human primate GLP studies, a 1-week study and an ongoing 3-month study.

TABLE: Characteristics of non-human primates and ocular* rAAV doses injected

Study	Animal	Gender	Vector	Titer[a]	Volume (µl)/ Injection route	Current Status
I	20515	F	Vehicle	0	150/SR	Necropsy (1wpi)
	20564	M	Vehicle	0	150/SR	Necropsy (1wpi)
	20507	F	AAV2-CB-h*RPE65*	1X	150/SR	Necropsy (1wpi)
	20566	M	AAV2-CB-h*RPE65*	1X	150/SR	Necropsy (1wpi)
	20480	F	AAV2-CB-h*RPE65*	3X	100/SR	Necropsy (1wpi)
	20552	M	AAV2-CB-h*RPE65*	3X	110/SR	Necropsy (1wpi)
II	20877	M	Vehicle	0	150/SR	Alive
	20547	M	Vehicle	0	150/SR	Alive
	C06987	F	Vehicle	0	150/SR	Alive
	20730	M	AAV2-CB-h*RPE65*	1X	150/SR	Alive
	C06992	F	AAV2-CB-h*RPE65*	1X	150/SR	Alive
	C08913	F	AAV2-CB-h*RPE65*	1X	150/SR	Alive
	20725	M	AAV2-CB-h*RPE65*	1X	150/IV	Alive
	20777	M	AAV2-CB-h*RPE65*	3X	150/SR	Alive
	20732	M	AAV2-CB-h*RPE65*	3X	150/SR	Alive
	C09033	F	AAV2-CB-h*RPE65*	3X	150/SR	Alive
	20747	M	AAV2-CB-h*RPE65*	3X	150/IV	Alive

*Only right eyes were injected in all animals; Vehicle, 0.65PBS (Phosphate Buffered Saline) + 85mM NaCl. [a]1X, 1.0×10^{10} vg/µl; wpi, week post injection; SR, subretinal; IV, intravitreal; all monkeys were ~2 years of age at time of ocular surgery.


RE
RE

Phase I Trial of Ocular Subretinal Injection of a
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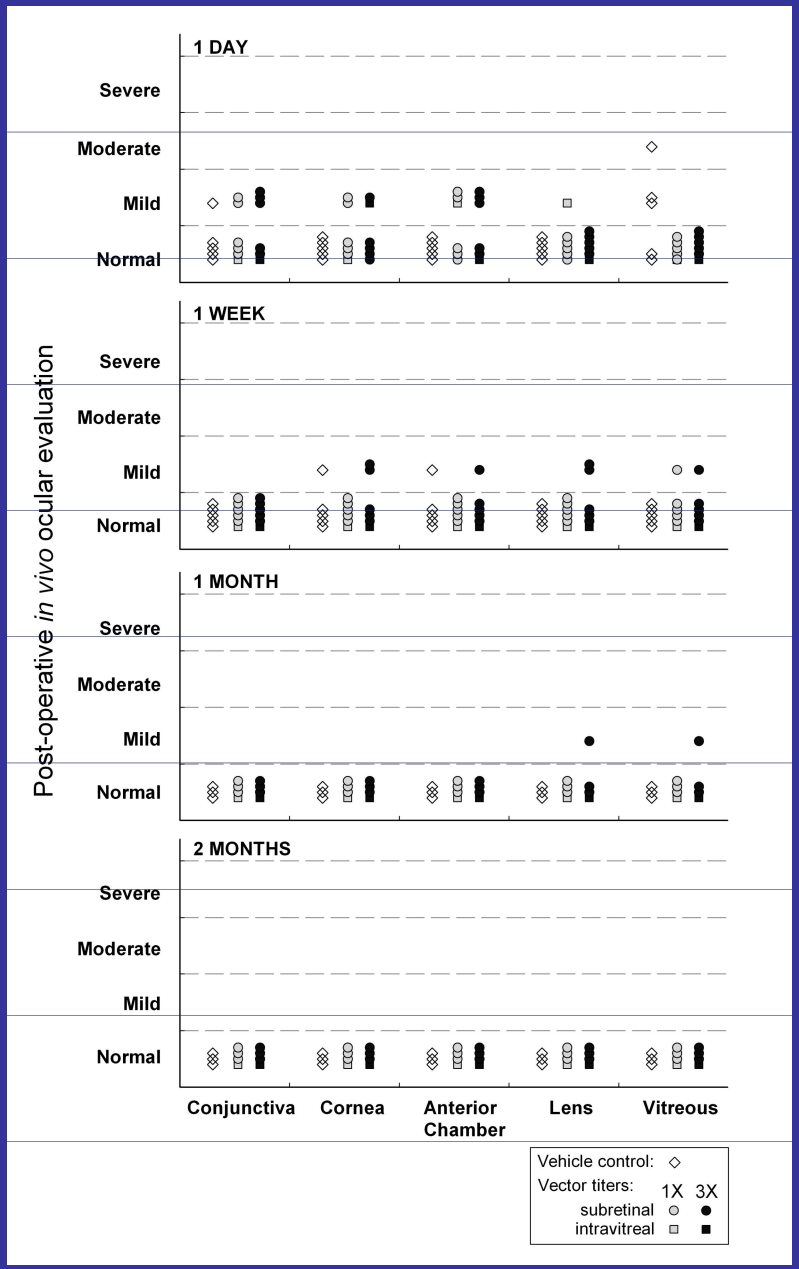
in Patients with Retinal Disease Due to RPE65 Mutations
→ Pre-clinical Safety Studies: GLP non-human primates

In-Life Observations (Study I): No early deaths; no clinical signs of toxicity;
no effects on food consumption or body weight changes
Clinical Pathology (Study I): No test article related changes in hematology or
clinical chemistry parameters.

Clinical Ocular Examinations (Study I; Study II until 2 months post-injection):
[Performed by Drs. Jacobson and Kaushal; independent veterinary opinion to follow.]
Graded as 3 levels of severity for conjunctiva, cornea, anterior chamber, lens and vitreous.

Preliminary Findings to date:
1 day and 1 week: Mild inflammatory changes were noted in both vehicle-injected
and vector-injected eyes (less than half of the animals).
1 month: one 3X vector-injected animal (eye) showed anterior surface lens changes
(presumed to be residual from paracentesis) and this cleared by 2 months.
Another 3X vector-injected animal had mild vitreous cellularity,
but this was no longer detected at 2 months.
2 months: No abnormalities in conjunctiva, cornea, anterior chamber, lens and vitreous.

Note: There is a further clinical examination planned for early July and necropsy will occur on the following day.


1 DAY
1 WEEK
1 MONTH
2 MONTHS
Severe
Moderate
Mild
Normal
Post-operative *in vivo* ocular evaluation
Conjunctiva
Cornea
Anterior Chamber
Lens
Vitreous
Vehicle control:
Vector titers:
1X
3X
subretinal
intravitreal

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Clinical Funduscopic Examinations (Study I; Study II until 2 months post-injection):
[Performed by Drs. Jacobson and Kaushal; independent veterinary opinion to follow.]

Preliminary Findings to date (subretinal-injected eyes):
1 day: Elevated retina and small intraretinal hemorrhages with RPE changes at/near the retinotomy site. Findings confined to site of subretinal injection (based on drawings at surgery).
1 week: Flattening of the retina or less prominent elevations. Demarcations between previously-elevated retina and surrounding uninjected retina.
1 month and 2 months: Flat retinas with RPE changes (likely scarring) mainly at/near retinotomy sites. Demarcation lines persist between previously-elevated retina and surrounding uninjected retina.

Note: A further clinical examination is planned for early July and necropsy will occur the next day.

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→ Pre-clinical Safety Studies: GLP non-human primates

Postmortem Observations (Study I):

No gross necropsy findings at termination of study.

No test article related changes in organ weights.

No microscopic lesions (non-ocular tissues) considered related to the test article.

Ocular histopathology (Study I) – main retinal findings (by Dr. Caroline Zeiss):

- Distinct pathology in all right eyes vs. post-mortem mild changes in left eyes;
- Retinal detachment near injection site as well as some distance away;
- Foci of pycnotic photoreceptor nuclei, probably resulting from retinal detachment;
- No necrosis, inflammation, proliferation or extensive hemorrhage.

Table:Biodistribution AAV2-CB-h*RPE65* study in non-human primates (Study I)						
Animal ID, gender	20515, F	20564, M	20507, F	20566, M	20480, F	20552, M
Vector Titer	Vehicle	Vehicle	1X	1X	3X	3X
Volume (ul)/injection route	150/SR	150/SR	150/SR	150/SR	100/SR	110/SR
Kidney	-	na	na	-	na	-
Spleen	na	na	-	-	-	-
Lung	na	na	na	na	na	-
Liver	-	-	-	na	-	-
Pancreas	-	-	-	-	-	-
Jejunum	-	-	na	-	-	-
Heart	-	-	na	-	-	-
Muscle	-	-	+(152)[a]	+(3781)[a]	-	-
Tracheobronch lymph node	-	-	-	-	-	-
Mesenteric lymph node	na	na	na	-	-	-
Preauricular lymph node	-	na	-	+(180)[b]	-	na
Mandibular lymph node	-	-	na	-	-	-
Cerebellum	-	-	na	-	na	na
ACF (L, at necropsy)	na	na	no sample[c]	no sample[c]	no sample[c]	-
Vitreous (L)	no sample[c]	-	na	-	no sample[c]	no sample[c]
Retina (L)	-	-	-	-	-	-
Optic nerve (L)	-	na	na	-	-	na
Orbital un-injected	na	-	na	-	na	na
ACF (R, pre-op)	na	na	na	na	na	na
ACF (R, at necropsy)	na	-	no sample[c]	no sample[c]	no sample[c]	+(608)[a]
Vitreous (R)	-	na	+(6545)[a]	+(25565)[a]	+(920700)[a]	no sample[c]
Retina (R)	-	-	+(2930674)[a]	+(436307)[a]	+(470375)[a]	+(2459501)[a]
Optic nerve (R)	na	-	+(601)[a]	+(127)[a]	+(1360)[a]	+(665)[a]
Orbital injected	-	-	na	-	-	na
Optic chiasma	-	-	-	-	-	-
Optic tract (L)	-	+(124)[a]	-	+(439)[a]	+(106)[b]	-
Lateral geniculate nucleus (L)	-	-	-	-	-	-
Thalamus (L)	-	-	-	-	-	-
Visual cortex (L)	-	-	-	-	+(200)[a]	-
Superior colliculus (L)	-	-	-	-	-	+(184)[a]
Optic tract (R)	-	na	-	+(155)[b]	+(114)[a]	+(264)[a]
Lateral geniculate nucleus (R)	-	-	-	+(144)[b]	+(123)[b]	-
Thalamus (R)	-	-	na	-	-	-
Visual cortex (R)	-	na	-	-	-	-
Superior colliculus (R)	na	na	-	-	-	-

L, left; R, right; -, no PCR amplification; + (copy number per 1.0 µg of genomic DNA), PCR amplification of vector sequences; [a]Average of 2 replicates; [b]Replicate PCR negative; [c] No DNA in extraction as determined by UV spectrophotometery; na, not available (not determinable because of an unacceptable spike-in).

		NHP Gonad Biodistribution (Study I) Copy #		
	ug of DNA loaded	Replicate # 1	Replicate # 2	Spike in control
Female 20515 (Vehicle)	0.5	-	-	90
Male 20564 (Vehicle)	0.5	-	-	48
Female 20507 (1X)	0.5	-	-	56
Male 20566 (1X)	0.5	-	-	40
Female 20480 (3X)	0.5	-	-	70
Male 20552 (3X)	0.5	-	-	90

"-", below detection level of 100 copies per µg genomic DNA

We are grateful to:

our patients

[for their trust and tolerance,
as we grapple with all this
and try to get it right]