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3 The influence of subretinal injection pressure on the microstructure of the monkey retina

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5 Short title: The influence of subretinal injection pressure on the monkey retina

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25

26 **Abstract**

27 **PURPOSE:** To investigate the influence of subretinal injection pressure on the
28 microstructure of the retina in a monkey model.

29 **METHODS:** After vitrectomy, balanced salt solution was injected subretinally into one
30 eye each of four cynomolgus monkeys while controlling the injection pressure. Initially,
31 a pressure of 2 psi was used, and this was gradually increased to determine the
32 minimum required pressure. Subsequent injections were performed at two pressures:
33 minimum (n=13) and high (n=6). To compare the influence of these injection pressures
34 on retinal structure, optical coherence tomography (OCT) was performed before
35 surgery and every week afterwards. The monkeys were euthanized and their eyes were
36 enucleated at 1 or 6 weeks after the injections. The eyes were processed for light
37 microscopy and transmission electron microscopy (TEM) as well as for TdT-mediated
38 dUTP nick end labeling.

39 **RESULTS:** The minimum pressure required to perform subretinal injection was 6 psi.
40 After injection at this pressure, both OCT and microscopy showed that the retinal
41 structure was well-preserved throughout the experimental period at all injection sites.
42 Conversely, after injection at high pressure (20 psi) OCT images at all injection sites
43 showed disruption of the ellipsoid zone (EZ) after 1 week. Microscopy indicated damage
44 to the photoreceptor outer segment (OS) and stratification of the retinal pigment
45 epithelium (RPE). After 6 weeks, OCT demonstrated that the EZ had become
46 continuous and TEM confirmed that the OS and RPE had recovered. Photoreceptor
47 apoptosis was absent after subretinal injection at both pressures.

48 **CONCLUSIONS:** The retinal damage caused by subretinal injection increases
49 depending on pressure, indicating that clinicians should perform subretinal injection at
50 pressures as low as possible to ensure safety.

51

52 **Introduction**

53 Surgical subretinal injections are used to displace subretinal hemorrhages [1-4],
54 deliver gene therapy for retinal degeneration [5-8], perform macular translocation in
55 patients with age-related macular degeneration [9,10], remove hard foveal exudates
56 [11], and resolve diffuse diabetic macular edema during planned foveal detachment
57 procedures [12,13]. To ensure that visual function is preserved after such procedures,
58 clinicians must not damage the macula, and a safe and reliable technique for subretinal
59 injection must therefore be developed.

60 To deliver a subretinal injection to a patient with an attached retina, the
61 injection pressure applied must exceed the adhesion force between the retina and
62 retinal pigment epithelium (RPE) [14-16]. However, when a liquid is injected into the
63 subretinal space, the stream may physically damage the structure of the outer retina
64 and the RPE [17]. According to Poiseuille's law, when a liquid of consistent viscosity is
65 injected through a cannula with a consistent diameter, the flow rate is determined by the
66 injection pressure [14]. However, the relationship between injection pressure and
67 consequent retinal damage has not been sufficiently elucidated.

68 On the basis of Laplace's law, patients with an attached retina require the
69 highest injection pressure to initiate retinal detachment, and this required pressure
70 decreases as the area of retinal detachment expands [18]. Therefore, in the present
71 study, we investigated the effect of injection pressure, which is required to initiate retinal
72 detachment, on the microstructure of the monkey retina using *in vivo* optical coherence
73 tomography (OCT), light and electron microscopy, and TdT-dUTP terminal nick-end
74 labeling (TUNEL).

75

76 **Methods**

77 **Ethics statement**

78 All animal experiments were reviewed and approved by the Institutional Animal
79 Care and Use committee of Santen Pharmaceutical Co., Ltd. (approval no.
80 DR-2016-0157). Male cynomolgus monkeys (*Macaca fascicularis*) aged 3–4 years and
81 weighing 3.0–5.0 kg were purchased from Eve Bioscience Ltd. (Wakayama, Japan).
82 The animal welfare and steps taken to ameliorate suffering were in accordance with
83 Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan) and
84 the recommendations of the Weatherall report on the use of non-human primates in
85 research. The animals were housed individually in stainless steel cages (width: 47 cm,
86 depth: 89 cm, height: 76 cm) at the animal facility of Santen Pharmaceutical Co., Ltd.,
87 where the environmental conditions were as follows: room temperature, 24°C; relative
88 humidity, 60%; illumination, 12-hour lighting (7 a.m. to 7 p.m.) at 300 lux. They were fed
89 100 g/animal/day of pellet food for monkeys (Monkey Bit; Nosan Corporation,
90 Yokohama, Japan). Tap water from a feed-water nozzle was supplied ad libitum. During
91 subretinal injection and OCT examination, monkeys were anesthetized by intramuscular
92 injection of ketamine hydrochloride (10 mg/kg). Respiratory rate was monitored
93 frequently and used to maintain adequate anesthesia using ketamine. Topical drops of
94 oxybuprocaine were used for analgesia. Topical drops of 0.5% phenylephrine
95 hydrochloride and 0.5% tropicamide were used for mydriasis. The monkeys were
96 sedated and humanely euthanized by intravenous pentobarbital by a licensed
97 veterinarian, either 1 week or 6 weeks after subretinal injections. Confirmation of death
98 was determined by monitoring for absence of pulse, respiration, and neural reflexes.
99 The eyes were enucleated immediately after euthanasia, either 1 week or 6 weeks after
100 subretinal injections.

101

102 **Subretinal injection with local removal of the internal**
103 **limiting membrane**

104 In total, four monkeys were subjected to surgical subretinal injections in one
105 eye. Specifically, transconjunctival, 25-gauge, 3-port, pars plana vitrectomies were
106 performed using a commercially available vitrectomy machine (Accurus; Alcon
107 Laboratories Inc., Fort Worth, TX, USA). Each injection site was at a mid-peripheral
108 location within the eye. The internal limiting membrane (ILM) was then removed locally
109 over approximately 1/4 of the disc area at each injection site, and balanced salt solution
110 (BSS) was injected using a 38-gauge cannula (MedOne, Sarasota, FL, USA) until the
111 area of retinal detachment had expanded to approximately one disc area (Fig 1), as
112 described in our previous report [19]. By using this method, the BSS could be injected
113 subretinally by placing the cannula tip in contact with the retinal nerve fiber layer
114 exposed by ILM removal; thus, penetration of the retina with the cannula was
115 unnecessary.

116

117 **Fig 1. Local removal of internal limiting membrane and subretinal injection of**
118 **balanced salt solution.** (A) Schematic drawing of local internal limiting membrane
119 (ILM) removal showing the removed ILM (arrow), retina (asterisk), and intact ILM
120 (arrowhead). (B) Schematic drawing of the subretinal injection procedure. Balanced salt
121 solution (BSS) was injected with a 38-gauge cannula by placing the cannula tip in
122 contact with the retinal nerve fiber layer. The arrow indicates the flow of the injected
123 BSS; the asterisk and arrowhead indicate the retina and ILM, respectively. (C) Surgical
124 photograph after local ILM removal. The arrow indicates the 38-gauge cannula; the
125 arrowhead indicates the area of peeled ILM. (D) Surgical photograph during subretinal

126 injection. The arrow indicates the 38-gauge cannula; arrowheads indicate the area of
127 retinal detachment due to subretinal injection of BSS. (E) Optical coherence
128 tomography 30 minutes after subretinal injection. The asterisk indicates retinal
129 detachment caused by the procedure. Scale bar = 200 μm .

130

131 **Investigation of minimum required injection pressure** 132 **and establishment of experimental groups**

133 To ensure a constant pressure, subretinal injection was performed using the
134 Viscous Fluid Control System (VFC; Alcon Laboratories Inc.), which allows the operator
135 to raise the injection pressure from 2 psi to 80 psi in increments of 2 psi. Therefore, to
136 identify the minimum required injection pressure, we began at 2 psi and increased the
137 pressure until subretinal injection became possible. After the minimum required
138 injection pressure was established (6 psi), we performed subretinal injection at this
139 pressure at three or four sites in each eye (minimum-pressure group), for a total of 13
140 sites (S1–S4 Figs). To compare the influence of the procedure on the retina at different
141 pressures, we also performed high-pressure subretinal injection (20 psi) at one or two
142 points in each eye (high-pressure group), for a total of six sites (S1–S4 Figs). We also
143 performed local removal of the ILM, but not subretinal injection, at a total of three sites
144 in two eyes (control group). We identified all injection sites after the surgery by using a
145 recorded surgical video and conducted subsequent investigations.

146

147 **Optical coherence tomography**

148 OCT (Spectralis; Heidelberg Engineering GmbH, Heidelberg, Germany) was
149 performed before surgery and every week after surgery until the 5th week. OCT images
150 were first obtained 1 week after injection because this was the time point when

151 postoperative inflammation was reduced and clear OCT images were stably obtained in
152 all cases. To improve reproducibility, the exact injection site and area of retinal
153 detachment were identified based on the surgical video (Figure 1D) and infrared fundus
154 images (Fig 2 and S1–S4 Figs).

155

156 **Fig 2. Optical coherence tomography images of monkey retina after subretinal**
157 **injection of balanced salt solution.** Optical coherence tomography (OCT) images of
158 both control (no injection of balanced salt solution; BSS) and minimum-pressure (BSS
159 injection at 6 psi) groups show a well-preserved retinal structure throughout the
160 experimental period, including continuity of the ellipsoid zone (EZ) (A to F). OCT image
161 from the high-pressure group (BSS injection at 20 psi) show EZ disruption (asterisk in
162 G) at 1 week after the injection (G). At 3 weeks after the injection (H), OCT image from
163 the high-pressure group shows partial recovery of the EZ (asterisk). The EZ finally
164 became continuous (asterisk in I) at 5 weeks after the injection. Arrows in A, D, and G
165 indicate the EZ. Scale bars = 200 μm .

166

167 **Light and transmission electron microscopy**

168 Two eyes, which were enucleated at either 1 week (S3 Fig) or 6 weeks (S1 Fig)
169 after injections, were investigated with light and transmission electron microscopy
170 (TEM). The eyes were fixed in a solution of 1% glutaraldehyde and 1%
171 paraformaldehyde in phosphate-buffered saline. The specimens were post-fixed in
172 veronal acetate buffer containing osmium tetroxide (1%), dehydrated in ethanol and
173 water, and then embedded in Epon resin (Epon 812 Resin; TAAB Laboratories,
174 Aldermaston, UK). The eyes were cut into 1- μm -thick sections, stained with toluidine
175 blue, and observed by light microscopy. Tissue sections were prepared based on a
176 surgical video and retinal blood vessels of enucleated eyeballs. For TEM, ultrathin

177 sections were cut from the Epon resin blocks and mounted on copper grids. The
178 specimens were observed by using an H-7770 transmission electron microscope
179 (Hitachi, Tokyo, Japan).

180

181 **TdT-dUTP terminal nick-end labeling**

182 Apoptosis of photoreceptor cells was investigated using TUNEL staining. For
183 this analysis, two eyes were enucleated at 1 week (S4 Fig) and 6 weeks (S2 Fig) after
184 injections and were fixed by using a mixture of methanol and formalin (Superfix; Kurabo,
185 Osaka, Japan) at room temperature for the first 2.5 hours and at 4°C for the next 3 days.
186 The eyes were then embedded in paraffin and cut into 3- μ m sections. Tissue sections
187 were prepared based on a surgical video and retinal blood vessels of enucleated
188 eyeballs. TUNEL staining was performed by using an *in situ* apoptosis detection kit
189 (Takara Bio Inc., Shiga, Japan), in accordance with the manufacturer's protocol.

190

191 **Results**

192 **Minimum pressure required for subretinal injection with** 193 **local removal of ILM**

194 The minimum pressure required for subretinal injection was 6 psi, and the
195 procedure was thus performed at this pressure in the minimum-pressure group. In the
196 high-pressure group, subretinal injection was performed at 20 psi, based on the upper
197 limit of the range of clinically-used injection pressures for gene therapy [15].

198

199 **Influence of minimum and high-pressure injection on** 200 **the structure of the retina**

201 *In vivo* OCT examination revealed that there were no defects in the ellipsoid
202 zone (EZ) at any of the 13 injection sites in the minimum-pressure group. Additionally,
203 the retinal structure was identical between the minimum-pressure group and the control
204 group, indicating that subretinal injection at 6 psi did not damage the retina (Fig 2A–2F
205 and S1–S4 Figs). Conversely, in the high-pressure group, all six injection sites showed
206 defects in the EZ at 1 week after injection (Fig 2G and S1–S4 Figs). These defects had
207 begun to resolve at 3 weeks after injection (Fig 2H, S1 and S2 Figs) and had almost
208 completely recovered at 5 weeks after injection (Fig 2I, S1 and S2 Figs).

209 To further investigate our OCT findings that damaged EZ was observed 1 week
210 after injection and had almost completely recovered by 5 weeks after injections (Fig 2G,
211 Fig 2I, S1 and S2 Figs), eyeballs were enucleated at 1 or 6 weeks after surgery. We
212 then examined the influence of subretinal injection on retinal structure by using light
213 microscopy and TEM. In the minimum-pressure group, light microscopy showed normal
214 retinal structure at both 1 and 6 weeks after injections (Fig 3C and 3D); this result was
215 similar to that in the control group (Fig 3A and 3B). However, TEM analysis showed that
216 the photoreceptor outer segment (OS) lengths were slightly shorter in the
217 minimum-pressure group than in the control group at 1 week after injections (Fig 4A and
218 4C). At 6 weeks after the injections, the OS lengths had recovered and were identical to
219 that of the control group (Fig 4B and 4D). No RPE damage was observed by either light
220 microscopy or TEM. In the high-pressure group, disappearance of the OS and
221 stratification of the RPE were observed at 1 week after injections by both light
222 microscopy and TEM (Fig 3E and 4E). These defects had partially resolved at 6 weeks

223 after injections, when reconstruction of the OS and flattening of the RPE were observed
224 (Fig 3F and 4F).

225

226 **Fig 3. Light microscopy images of monkey retina after subretinal injection of**
227 **balanced salt solution.** The retinal structures of both the control (no injection of
228 balanced salt solution; BSS) and the minimum-pressure (BSS injection at 6 psi) groups
229 are well-preserved at 1 week (A and C) and 6 weeks (B and D) after injection. The high
230 injection pressure group (BSS injection at 20 psi) shows thinning of the photoreceptor
231 outer segment layer (OS, arrows in E) and thickening of the retinal pigment epithelium
232 (RPE) layer (asterisk in E) at 1 week after injection (E), while the photoreceptor cells are
233 well-preserved. At 6 weeks after injection, the high-pressure group shows restoration of
234 the OS (arrows in F) and flattening of the RPE (asterisk in F). Scale bars = 100 μm .

235

236 **Fig 4. Transmission electron microscopy images of monkey retina after**
237 **subretinal injection of balanced salt solution.** Retinal structures of the control group
238 (no injection of balanced salt solution; BSS) are well-preserved at 1 week (A) and 6
239 weeks (B) after injection. The minimum-pressure group (BSS injection at 6 psi) shows a
240 shorter photoreceptor outer segment (OS) than the control group at 1 week after
241 injection (C, bidirectional arrow). The OS is restored at 6 weeks after injection (D,
242 bidirectional arrow). The retinal pigment epithelium (RPE) is well-preserved throughout
243 the experimental period (C and D). Conversely, the high-pressure group (BSS injection
244 at 20 psi) shows degeneration of the OS (bidirectional arrow in E) and migration of RPE
245 cells (arrows in E), leading to multiple RPE layers at 1 week after injection.
246 Regeneration of the OS (bidirectional arrow in F) and flattening of the RPE (arrows in F)
247 were observed at 6 weeks after the injection. Scale bars = 10 μm .

248

249 **Photoreceptor cell death after subretinal injection at 6** 250 **psi and 20 psi**

251 Light microscopy images with toluidine blue staining showed normal
252 morphology of photoreceptor cells at 1 and 6 weeks after injections in both groups (Fig
253 5A, 5D, 5G, and 5J). TUNEL staining showed no positive cells at either 1 or 6 weeks
254 after injections in either group (Fig 5B, 5E, 5H, and 5K). Consistent with this result, TEM
255 images showed that no chromatin condensation or nuclear fragmentation, which are
256 characteristic features of apoptotic cell death, had occurred in the photoreceptor cells of
257 either group at either time point (Fig 5C, 5F, 5I, and 5L).

258

259 **Fig 5. The effect of subretinal injection on photoreceptor cells.** In the
260 minimum-pressure (injection at 6 psi with balanced salt solution; BSS) and
261 high-pressure (BSS injection at 20 psi) groups, both light microscopy images with
262 toluidine blue staining and transmission electron microscopy (TEM) images show
263 normal morphology of photoreceptor cells at 1 week (A, C, G and I) and 6 weeks (D, F,
264 J, and L) after injection. TdT-dUTP terminal nick-end labeling (TUNEL) showed no
265 positive photoreceptor cells in either the minimum-pressure or high-pressure groups
266 throughout the experimental period (B, E, H, and K). Black and white scale bars = 20
267 μm . Positive control of TUNEL staining is shown in S5 Fig.

268

269 **Discussion**

270 In the present study, we showed that the photoreceptor cells and the RPE of
271 the monkey retina can be damaged by subretinal injection. To our knowledge, this is the
272 first report to reveal that higher injection pressure causes greater retinal damage. Our

273 OCT (Fig 2D) and TEM (Fig 4C) results show that the retinal structure was relatively
274 well-preserved when subretinal injection was applied at 6 psi. Conversely, severe EZ
275 disruption was observed by OCT following subretinal injection at 20 psi (Fig 2G).
276 However, the EZ had become continuous at 6 weeks after 20 psi subretinal injection
277 (Fig 2I). Reflecting the OCT results, TEM images showed severe OS damage (Fig 4E)
278 after 20 psi subretinal injection. However, as shown in Fig 5, the photoreceptor cells
279 were intact; thus, the OS had recovered (Fig 4D and 4F) at 6 weeks after subretinal
280 injection. These results are in agreement with the OS recovery observed after
281 experimental short-term retinal detachment in monkey eye [20,21] and are similar to the
282 OS recovery observed after retinal reattachment in patients with retinal detachment
283 [22-24]. Considering that the severity of retinal damage was dependent on the injection
284 pressure, subretinal injection should be performed at pressures as low as possible to
285 ensure safety.

286 The resistance associated with subretinal injection arises from the stiffness of
287 the neural retina tissue and the adhesion force between the retina and the RPE; thus,
288 the injection pressure must exceed this resistance [14,15]. Alternatively, the resistance
289 caused by tissue stiffness can be eliminated by puncturing the neural retina with the
290 injection cannula. However, this can damage the retina, RPE, and choroidal vessels,
291 thereby causing serious complications, such as subretinal bleeding and choroidal
292 neovascularization. Most tissue resistance within the neural retina originates from the
293 ILM [25]. Therefore, to resolve the problems of subretinal injection, we recently
294 attempted local removal of the ILM at the subretinal injection site [19]. This method
295 enabled us to perform subretinal injection at a much lower pressure (6 psi) without
296 puncturing the retina. Based on this finding, the present study investigated subretinal
297 injection at the site of local ILM removal, revealing that subretinal injection could be
298 performed at a pressure of 6 psi without puncturing the retina. These results are

299 consistent with our recent findings in the human eye [19] indicating that local removal of
300 the ILM eliminates the resistance caused by the tissue stiffness of the neural retina,
301 thus removing the need for retinal puncture.

302 Although we focused on injection pressure in the present study, several other
303 factors should be considered with respect to the retinal damage caused by subretinal
304 injection. The first is the volume of the liquid to be injected under the retina, which differs
305 depending on the aim and content of the subretinal injection (tPA [2,4,26], adenovirus
306 vector [5,6,8], cell suspension [27-29], or BSS [9-13]). It has been reported that
307 excessive stretching of the retina by the injected liquid can cause photoreceptor cell
308 death by extension stress [15]. Secondly, the duration of retinal detachment should be
309 considered. Several studies have reported that, in cases of long-term retinal
310 detachment, apoptotic cell death of photoreceptor cells occurs due to a deficiency of
311 nutrients from the choroid [20,30-32]. Thirdly, if the subretinal injection location includes
312 the fovea, a macular hole may occur due to the injection pressure [6,7,33]. Finally, the
313 presence or absence of adhesion between the retina and the RPE can influence the risk
314 of retinal damage after subretinal injection. In cases of gene therapy or cell
315 transplantation therapy to treat retinal degenerative diseases, subretinal injection must
316 sometimes be performed at a site of retinal–RPE adhesion [5-8,15,34]. In such cases,
317 the possibility of damaging the retina and RPE increases because higher injection
318 pressures must be applied to exceed the adhesive force.

319 This study has several important limitations. Firstly, it is unclear whether our
320 results from monkey eyes can be generalized to humans. Secondly, although we
321 revealed the effects of subretinal injection outside the macula, the effects of subfoveal
322 injection on the fovea remain unknown. In the present study, we performed subretinal
323 injections at several mid-peripheral locations at different injection pressures in the same
324 eye because we wished to examine the influence of injection pressure on the retina by

325 establishing identical injection conditions. However, both the presence or absence of
326 the foveal depression and the proportion of cone and rod photoreceptor cells differ
327 between the fovea and midperiphery. Thirdly, although the approximate injection
328 volumes were determined using the area of retinal detachment, the injected volumes
329 were not necessarily the same at each injection site. In future studies, it will be
330 necessary to adjust the injection volumes of all retinal detachments, which could be
331 made possible by monitoring the injection volume with intraoperative OCT. Fourthly, the
332 time point to evaluate photoreceptor cell death by TUNEL was limited to 1 week after
333 injections due to the number of available monkeys. Considering the peak of TUNEL
334 staining in previous reports on retinal detachment in animal models [35,36], evaluation
335 at an earlier time point, such as 1–3 days after injection, will be necessary in future
336 studies. Finally, the present study examined the influence of injection pressure on retina
337 histology, but the influence of injection pressure on retinal function remains unknown.
338 Future studies are needed to investigate functional changes in the retina after damage
339 to the retinal outer layer and RPE as well as after their recovery.

340 In summary, our results show that the photoreceptor layer and RPE can be
341 damaged by subretinal injection and that the degree of damage depends on the
342 injection pressure. To perform subretinal injection safely, clinicians must inject at the
343 lowest possible pressure.

344

345

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References

- 350
- 351 1. Hauptert CL, McCuen BW, Jaffe GJ, Steuer ER, Cox TA, Toth CA, et al. Pars
352 plana vitrectomy, subretinal injection of tissue plasminogen activator, and
353 fluid-gas exchange for displacement of thick submacular hemorrhage in
354 age-related macular degeneration. *Am J Ophthalmol.* 2001;131: 208–215.
- 355 2. Kimura S, Morizane Y, Hosokawa M, Shiode Y, Kawata T, Doi S, et al.
356 Submacular hemorrhage in polypoidal choroidal vasculopathy treated by
357 vitrectomy and subretinal tissue plasminogen activator. *Am J Ophthalmol.*
358 2015;159: 683–689. doi: 10.1016/j.ajo.2014.12.020
- 359 3. Inoue M, Shiraga F, Shirakata Y, Morizane Y, Kimura S, Hirakata A. Subretinal
360 injection of recombinant tissue plasminogen activator for submacular
361 hemorrhage associated with ruptured retinal arterial macroaneurysm. *Graefes*
362 *Arch Clin Exp Ophthalmol.* 2015;253: 1663–1669. doi:
363 10.1007/s00417-014-2861-6
- 364 4. Kimura S, Morizane Y, Matoba R, Hosokawa M, Shiode Y, Hirano M, et al.
365 Retinal sensitivity after displacement of submacular hemorrhage due to
366 polypoidal choroidal vasculopathy: effectiveness and safety of subretinal tissue
367 plasminogen activator. *Jpn J Ophthalmol.* 2017;61: 472–478. doi:
368 10.1007/s10384-017-0530-0
- 369 5. Bainbridge JWB, Smith AJ, Barker SS, Robbie S, Henderson R, Balaggan K, et
370 al. Effect of Gene Therapy on Visual Function in Leber's Congenital Amaurosis.
371 *N Engl J Med.* 2008;358: 2231–2239. doi: 10.1056/nejmoa0802268
- 372 6. Maguire AM, Simonelli F, Pierce EA, Pugh EN, Mingozzi F, Bennicelli J, et al.
373 Safety and efficacy of gene transfer for Leber's congenital amaurosis. *N Engl J*
374 *Med.* 2008;358: 2240–2248. doi: 10.1056/NEJMoa0802315

- 375 7. Jacobson SG, Cideciyan AV, Ratnakaram R, Heon E, Schwartz SB, Roman AJ,
376 et al. Gene therapy for leber congenital amaurosis caused by RPE65 mutations:
377 safety and efficacy in 15 children and adults followed up to 3 years. *Arch*
378 *Ophthalmol.* 2012;130: 9–24. doi: 10.1001/archophthalmol.2011.298
- 379 8. MacLaren RE, Groppe M, Barnard AR, Cottrill CL, Tolmachova T, Seymour L, et
380 al. Retinal gene therapy in patients with choroideremia: initial findings from a
381 phase 1/2 clinical trial. *Lancet.* 2014;383: 1129–1137. doi:
382 10.1016/S0140-6736(13)62117-0
- 383 9. Machemer R. Macular translocation. *Am J Ophthalmol.* 1998;125: 698–700. doi:
384 10.1371/journal.pone.0177241
- 385 10. Morizane Y, Shiraga F, Takasu I, Yumiyama S, Okanouchi T, Ohtsuki H.
386 Selection for inferior limited macular translocation on the basis of distance from
387 the fovea to the inferior edge of the subfoveal choroidal neovascularization. *Am J*
388 *Ophthalmol.* 2002;133: 848–850. doi: 10.1016/S0002-9394(02)01413-7
- 389 11. Takagi H, Otani A, Kiryu J, Ogura Y. New surgical approach for removing
390 massive foveal hard exudates in diabetic macular edema. *Ophthalmology.*
391 1999;106: 249–257. doi: 10.1016/S0161-6420(99)90054-4
- 392 12. Morizane Y, Kimura S, Hosokawa M, Shiode Y, Hirano M, Doi S, et al. Planned
393 foveal detachment technique for the resolution of diffuse diabetic macular edema.
394 *Jpn J Ophthalmol.* 2015;59: 279–287. doi: 10.1007/s10384-015-0390-4
- 395 13. Toshima S, Morizane Y, Kimura S, Shiraga F. Planned Foveal Detachment
396 Technique for the Resolution of Diabetic Macular Edema Resistant to
397 Anti-Vascular Endothelial Growth Factor Therapy. *Retina.* 2017. doi:
398 10.1097/IAE.0000000000001771

- 399 14. Fischer MD, Hickey DG, Singh MS, MacLaren RE. Evaluation of an Optimized
400 Injection System for Retinal Gene Therapy in Human Patients. *Hum Gene Ther*
401 *Methods*. 2016;27: 150–158. doi: 10.1089/hgtb.2016.086
- 402 15. Xue K, Groppe M, Salvetti AP, MacLaren RE. Technique of retinal gene therapy:
403 delivery of viral vector into the subretinal space. *Eye*. 2017;31: 1308–1316. doi:
404 10.1038/eye.2017.158
- 405 16. Ochakovski GA, Peters T, Michalakakis S, Wilhelm B, Wissinger B, Biel M, et al.
406 Subretinal Injection for Gene Therapy Does Not Cause Clinically Significant
407 Outer Nuclear Layer Thinning in Normal Primate Foveae. *Invest Ophthalmol Vis*
408 *Sci*. 2017;58: 4155–4160. doi: 10.1167/iovs.17-22402
- 409 17. Nork TM, Murphy CJ, Kim CBY, Ver Hoeve JN, Rasmussen CA, Miller PE, et al.
410 Functional and anatomic consequences of subretinal dosing in the cynomolgus
411 macaque. *Arch Ophthalmol*. 2012;130: 65–75. doi:
412 10.1001/archophthalmol.2011.295
- 413 18. Kita M, Negi A, Kawano S, Honda Y, Maegawa S. Measurement of retinal
414 adhesive force in the in vivo rabbit eye. *Invest Ophthalmol Vis Sci*. 1990;31:
415 624–628.
- 416 19. Okanouchi T, Toshima S, Kimura S, Morizane Y, Shiraga F. Novel Technique for
417 Subretinal Injection Using Local Removal of the Internal Limiting Membrane.
418 *Retina*. 2016;36: 1035–1038. doi: 10.1097/IAE.0000000000001029
- 419 20. Guerin CJ, Anderson DH, Fariss RN, Fisher SK. Retinal reattachment of the
420 primate macula. Photoreceptor recovery after short-term detachment. *Invest*
421 *Ophthalmol Vis Sci*. 1989;30: 1708–1725.
- 422 21. Guerin CJ, Lewis GP, Fisher SK, Anderson DH. Recovery of photoreceptor outer
423 segment length and analysis of membrane assembly rates in regenerating

- 424 primate photoreceptor outer segments. *Invest Ophthalmol Vis Sci.* 1993;34:
425 175–183.
- 426 22. Menke MN, Kowal JH, Dufour P, Wolf-Schnurrbusch UE, Ceklic L, Framme C, et
427 al. Retinal layer measurements after successful macula-off retinal detachment
428 repair using optical coherence tomography. *Invest Ophthalmol Vis Sci.* 2014;55:
429 6575–6579. doi: 10.1167/iovs.14-14412
- 430 23. Dell'omo R, Viggiano D, Giorgio D, Filippelli M, Di Iorio R, Calo R, et al.
431 Restoration of foveal thickness and architecture after macula-off retinal
432 detachment repair. *Invest Ophthalmol Vis Sci.* 2015;56: 1040–1050. doi:
433 10.1167/iovs.14-15633
- 434 24. Kobayashi M, Iwase T, Yamamoto K, Ra E, Murotani K, Matsui S, et al.
435 Association Between Photoreceptor Regeneration and Visual Acuity Following
436 Surgery for Rhegmatogenous Retinal Detachment. *Invest Ophthalmol Vis Sci.*
437 2016;57: 889–898. doi: 10.1167/iovs.15-18403
- 438 25. Candiello J, Cole GJ, Halfter W. Age-dependent changes in the structure,
439 composition and biophysical properties of a human basement membrane. *Matrix*
440 *Biol.* 2010;29: 402–410. doi: 10.1016/j.matbio.2010.03.004
- 441 26. Doi S, Kimura S, Morizane Y, Shiode Y, Hosokawa M, Hirano M, et al. Successful
442 displacement of a traumatic submacular hemorrhage in a 13-year-old boy treated
443 by vitrectomy, subretinal injection of tissue plasminogen activator and intravitreal
444 air tamponade: a case report. *BMC Ophthalmol.* 2015;15: 94. doi:
445 10.1186/s12886-015-0090-3
- 446 27. Schwartz SD, Hubschman J-P, Heilwell G, Franco-Cardenas V, Pan CK, Ostrick
447 RM, et al. Embryonic stem cell trials for macular degeneration: a preliminary
448 report. *Lancet.* 2012;379: 713–720. doi: 10.1016/S0140-6736(12)60028-2

- 449 28. Schwartz SD, Regillo CD, Lam BL, Elliott D, Rosenfeld PJ, Gregori NZ, et al.
450 Human embryonic stem cell-derived retinal pigment epithelium in patients with
451 age-related macular degeneration and Stargardt's macular dystrophy: follow-up
452 of two open-label phase 1/2 studies. *Lancet*. 2015;385: 509–516. doi:
453 10.1016/S0140-6736(14)61376-3
- 454 29. Song WK, Park KM, Kim HJ, Lee JH, Choi J, Chong SY, et al. Treatment of
455 macular degeneration using embryonic stem cell-derived retinal pigment
456 epithelium: preliminary results in Asian patients. *Stem Cell Reports*. 2015;4:
457 860–872. doi: 10.1016/j.stemcr.2015.04.005
- 458 30. Lewis GP, Charteris DG, Sethi CS, Leitner WP, Linberg KA, Fisher SK. The
459 ability of rapid retinal reattachment to stop or reverse the cellular and molecular
460 events initiated by detachment. *Invest Ophthalmol Vis Sci*. 2002;43: 2412–2420.
- 461 31. Arroyo JG, Yang L, Bula D, Chen DF. Photoreceptor apoptosis in human retinal
462 detachment. *Am J Ophthalmol*. 2005;139: 605–610. doi:
463 10.1016/j.ajo.2004.11.046
- 464 32. Kogachi K, Wolfe JD, Kashani AH. Surgically Induced Focal Retinal Detachment
465 Does Not Cause Detectable SD-OCT Retinal Changes in Normal Human Retina.
466 *Invest Ophthalmol Vis Sci*. 2017;58: 5270–5279. doi: 10.1167/iovs.17-22737
- 467 33. Campochiaro PA, Lauer AK, Sohn EH, Mir TA, Naylor S, Anderton MC, et al.
468 Lentiviral Vector Gene Transfer of Endostatin/Angiostatin for Macular
469 Degeneration (GEM) Study. *Hum Gene Ther*. 2017;28: 99–111. doi:
470 10.1089/hum.2016.117
- 471 34. Ochakovski GA, Bartz-Schmidt KU, Fischer MD. Retinal Gene Therapy: Surgical
472 Vector Delivery in the Translation to Clinical Trials. *Front Neurosci*. 2017;11: 174.
473 doi: 10.3389/fnins.2017.00174

- 474 35. Hisatomi T, Sakamoto T, Murata T, Yamanaka I, Oshima Y, Hata Y, et al.
475 Relocalization of apoptosis-inducing factor in photoreceptor apoptosis induced by
476 retinal detachment in vivo. *Am J Pathol.* 2001;158: 1271–1278. doi:
477 10.1016/S0002-9440(10)64078-3
- 478 36. Besirli CG, Chinskey ND, Zheng Q-D, Zacks DN. Inhibition of retinal
479 detachment-induced apoptosis in photoreceptors by a small peptide inhibitor of
480 the fas receptor. *Invest Ophthalmol Vis Sci.* 2010;51: 2177–2184. doi:
481 10.1167/iovs.09-4439

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484 **Supporting information**

485 **S1 Fig. The sites of subretinal injections and the areas of retinal detachment in**
486 **the monkey eye-1.** A and B: The same fundus picture taken 3 weeks after subretinal
487 injection. In B, the area of internal limiting membrane removal (a) and areas of retinal
488 detachment due to subretinal injection (b–e) are illustrated as circles. Cross marks
489 indicate the sites of subretinal injection at b–e. C–E: B-scan optical coherence
490 tomography (OCT) images captured at “a”. F–H: B-scan OCT images captured at “b”.
491 I–K: B-scan OCT images captured at “c”. L–N: B-scan OCT images captured at “d”.
492 O–Q: B-scan OCT images captured at “e”. OCT images of both the control (removal of
493 the ILM without subretinal injection of balanced salt solution; BSS) and
494 minimum-pressure groups (BSS injection at 6 psi) show a well-preserved retinal
495 structure, including continuity of the ellipsoid zone (EZ) throughout the experimental
496 period (C–N). OCT images of the high-pressure group (BSS injection at 20 psi) show
497 EZ disruption at 1 week after injection (asterisk in O). At 3 weeks after injection, OCT

498 images show partial recovery of the EZ (asterisk in P). The EZ finally became
499 continuous at 5 weeks after injection (asterisk in Q). The eye was enucleated 6 weeks
500 after subretinal injections and used for light and transmission electron microscopy.
501 Scale bars = 200 μm .

502 **S2 Fig. The sites of subretinal injections and the areas of retinal detachment in**
503 **the monkey eye-2.** A and B: The same fundus picture taken 4 weeks after subretinal
504 injection. In B, the areas of internal limiting membrane removal (a and b) and the areas
505 of retinal detachment due to subretinal injection (c–f) are illustrated as circles. Cross
506 marks indicate the sites of subretinal injection at c–f. C–E: B-scan optical coherence
507 tomography (OCT) images captured at “a”. F–H: B-scan OCT images captured at “b”.
508 I–K: B-scan OCT images captured at “c”. L–N: B-scan OCT images captured at “d”.
509 O–Q: B-scan OCT images captured at “e”. R–T: B-scan OCT images captured at “f”.
510 OCT images of both control (no injection of balanced salt solution; BSS) and
511 minimum-pressure (BSS injection at 6 psi) groups show a well-preserved retinal
512 structure throughout the experimental period, including continuity of the ellipsoid zone
513 (EZ) (C to Q). OCT images of the high-pressure group (BSS injection at 20 psi) show EZ
514 disruption at 1 week after injection (asterisk in R). The EZ became continuous at 3 and
515 5 weeks after injection (asterisks in S and T). The eye was enucleated 6 weeks after
516 subretinal injections and used for TdT-dUTP terminal nick-end labeling. Scale bars =
517 200 μm .

518 **S3 Fig. The sites of subretinal injections and the areas of retinal detachment in**
519 **the monkey eye-3.** A and B: The same fundus picture taken 1 week after subretinal
520 injection. In B, the areas of retinal detachment due to subretinal injection (a–e) are
521 illustrated as circles. Cross marks indicate the sites of subretinal injection at a–e. C:
522 B-scan optical coherence tomography (OCT) images captured at “a”. D: B-scan OCT

523 images captured at “b”. E: B-scan OCT images captured at “c”. F: B-scan OCT images
524 captured at “d”. G: B-scan OCT images captured at “e”. OCT images of
525 minimum-pressure (BSS injection at 6 psi) group show a well-preserved retinal structure
526 at 1 week after injection, including continuity of the ellipsoid zone (EZ) (C–E). OCT
527 images of the high-pressure group (BSS injection at 20 psi) show EZ disruption at 1
528 week after injection (asterisks in F and G). The eye was enucleated 1 week after
529 subretinal injections and used for light and transmission electron microscopy. Scale
530 bars = 200 μm .

531 **S4 Fig. The sites of subretinal injections and the areas of retinal detachment in**
532 **the monkey eye-4.** A and B: The same fundus picture taken 1 week after subretinal
533 injection. In B, the areas of retinal detachment due to subretinal injections (a–f) are
534 illustrated as circles. Cross marks indicate the sites of subretinal injection at a–f. C:
535 B-scan optical coherence tomography (OCT) images captured at “a”. D: B-scan OCT
536 images captured at “b”. E: B-scan OCT images captured at “c”. F: B-scan OCT images
537 captured at “d”. G: B-scan OCT images captured at “e”. H: B-scan OCT images
538 captured at “f”. OCT images of minimum-pressure (BSS injection at 6 psi) group show a
539 well-preserved retinal structure at 1 week after injection, including continuity of the
540 ellipsoid zone (EZ) (C to F). OCT images of the high-pressure group (BSS injection at
541 20 psi) show EZ disruption at 1 week after injection (asterisk in G and H). The eye was
542 enucleated 1 week after subretinal injections and used for TdT-dUTP terminal nick-end
543 labeling. Scale bars = 200 μm .

544 **S5 Fig. Positive control of TdT-dUTP terminal nick-end labeling.** Lymph nodes
545 taken simultaneously at the time of enucleation of monkey eyes were used as the
546 positive control for TdT-dUTP terminal nick-end labeling (TUNEL). Arrows show
547 TUNEL-positive cells. Scale bar = 20 μm .