



(51) International Patent Classification:

A61K 48/00 (2006.01) C12N 15/63 (2006.01)

A61P 27/02 (2006.01) C12N 15/86 (2006.01)

(21) International Application Number:

PCT/US2017/017469

(22) International Filing Date:

10 February 2017 (10.02.2017)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/294,362 12 February 2016 (12.02.2016) US

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2206 (US).(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY,
BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM,
DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,
HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN,
KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA,
MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG,
NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS,
RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY,
TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN,
ZA, ZM, ZW.(84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,
GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ,
TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU,
TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE,
DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU,

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(54) Title: ANTI-ANGIOGENIC MIRNA THERAPEUTICS FOR INHIBITING CORNEAL NEOVASCULARIZATION

(57) Abstract: The disclosure relates, in some aspects, to compositions and methods for treating corneal disease (e.g., corneal neovascularization). In some embodiments, the disclosure relates to rAAV-mediated delivery of a cornea-associated transgene to a subject. In some embodiments, the rAAV transduces the corneal tissue of a subject.

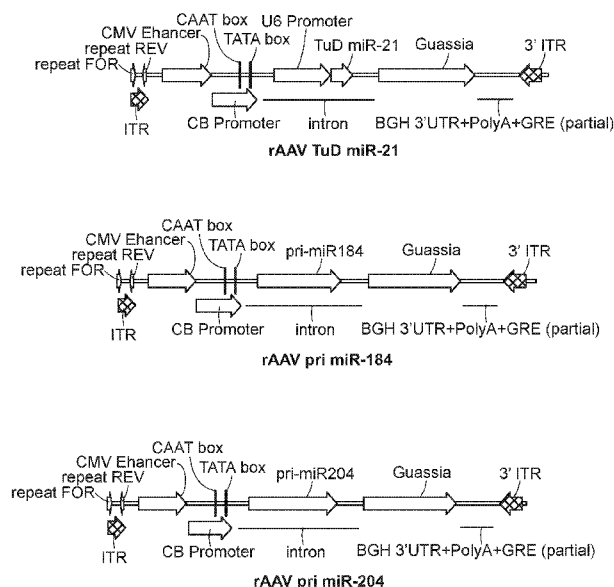


FIG. 1C



LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK,
SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ,
GW, KM, ML, MR, NE, SN, TD, TG).

— *before the expiration of the time limit for amending the
claims and to be republished in the event of receipt of
amendments (Rule 48.2(h))*

Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

ANTI-ANGIOGENIC MIRNA THERAPEUTICS FOR INHIBITING CORNEAL NEOVASCULARIZATION

RELATED APPLICATIONS

5 This Application claims the benefit under 35 U.S.C. 119(e) of U.S. provisional application serial number U.S.S.N. 62/294,362, filed on February 12, 2016, entitled “ANTI-ANGIOGENIC MIRNA THERAPEUTICS FOR INHIBITING CORNEAL NEOVASCULARIZATION”, the entire contents of which are incorporated by reference herein.

GOVERNMENT SUPPORT

10 This invention was made with government support under grant numbers R01NS07699-01 and 1P01AI100263-01 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF INVENTION

15 Cornea, the transparent and avascular tissue of the anterior ocular segment, is the major refractive surface of the eye, as well as a protective barrier to physical and pathogenic injury. Corneal opacities due to disease, infection or injury, is one of the leading causes of
20 blindness worldwide (5.1%). Corneal neovascularization (NV), one of the most common pathological processes in corneal diseases, is a significant and underestimated cause of unilateral blindness, leading to between 1.5 and 2 million new cases each year. Although a variety of treatments are available in clinic, including steroid hormone drugs, non-steroidal anti-inflammatory drugs (NSAIDs), cyclosporine, peroxisome proliferator activated receptor
25 (PPARy) agonists, and anti-VEGF therapies, a safe and effective therapy for corneal visual impairment remains to be an unmet medical challenge, especially the most severe cases, for which corneal transplantation is required. However, even in developed countries, access to this surgery is very difficult for lack of donors.

SUMMARY OF INVENTION

30 Adeno-associated virus (AAV) is a single-stranded DNA virus, and recombinant AAV (rAAV) vectors possess many advantages in gene therapy applications, including low

immunogenicity and genotoxicity, broad tissue tropism and high transduction efficiency *in vivo*, and long-term transgene expression. Aspects of the invention are related to the discovery that rAAV vectors comprising capsid proteins having a certain serotype, including, but not limited to, AAV5, AAV6, AAV6.2, AAV7, AAV8, AAV9, AAVrh.8, AAVrh.10, AAVrh.39, and AAVrh.43, mediate delivery of transgenes to ocular tissue (*e.g.*, corneal tissue) more efficiently than other vectors (*e.g.*, rAAV vectors comprising other capsid protein serotypes).

Accordingly in some aspects, the disclosure provides a method for delivering a transgene to ocular tissue (*e.g.*, corneal tissue), *e.g.*, for treating or preventing eye diseases, such as corneal neovascularization. In some embodiments, methods provided herein comprise administering to ocular (*e.g.*, corneal) tissue of a subject an effective amount of rAAV, wherein the rAAV comprises (i) a capsid protein having a selected serotype (*e.g.*, selected from the group consisting of AAV5, AAV6, AAV6.2, AAV7, AAV8, AAV9, AAVrh.8, AAVrh.10, AAVrh.39, and AAVrh.43), and (ii) a nucleic acid comprising a promoter operably linked to a transgene.

In some aspects, the disclosure provides a method of treating an ocular (*e.g.*, corneal) disease. In some embodiments, the methods comprise: administering to a subject having or suspected of having an ocular (*e.g.*, corneal) disease an effective amount of rAAV, wherein the rAAV comprises (i) a capsid protein having a selected serotype (*e.g.*, selected from the group consisting of AAV5, AAV6, AAV6.2, AAV7, AAV8, AAV9, AAVrh.8, AAVrh.10, AAVrh.39, and AAVrh.43), and (ii) a nucleic acid comprising a promoter operably linked to a transgene.

Aspects of the invention relate, in part, to the discovery that certain genes are highly up-regulated or highly down-regulated (*e.g.*, SEQ ID NOs: 1-3) in a subject having an ocular disease (*e.g.*, a subject having a corneal disease) and modulation of such genes confers a therapeutic benefit to the subject. Accordingly, in some aspects the disclosure provides a method of treating an ocular disease (*e.g.*, a corneal disease) comprising administering to ocular (*e.g.*, corneal) tissue of a subject an effective amount of rAAV, wherein the rAAV comprises (i) a capsid protein, and (ii) a nucleic acid comprising a promoter operably linked to a transgene, wherein the transgene encodes a gene associated with an ocular (*e.g.*, corneal) disease.

In some aspects, the disclosure provides compositions for use in the methods described herein. In some aspects, the disclosure provides a recombinant adeno-associated virus comprising: (i) a capsid protein having a serotype selected from the group consisting of AAV5, AAV6, AAV6.2, AAV7, AAV8, AAV9, AAVrh.8, AAVrh.10, AAVrh.39, and AAVrh.43; and, (ii) a nucleic acid comprising a promoter operably linked to a transgene.

In some aspects, the disclosure provides a recombinant adeno-associated virus (rAAV) comprising: (i) a capsid protein; and, (ii) a nucleic acid comprising a promoter operably linked to a transgene, wherein the transgene encodes a gene associated with an ocular (*e.g.*, corneal) disease.

In some embodiments, the capsid protein of an rAAV described by the disclosure comprises an amino acid sequence that is at least 70%, at least 80%, at least 90%, at least 95%, or at least 99% identical to any one of SEQ ID NO: 7-16. In some embodiments, the capsid protein comprises an amino acid sequence as set forth in SEQ ID NO: 7-16. In some embodiments, the capsid protein is AAVrh.10 capsid protein (SEQ ID NO: 14), or AAVrh.39 capsid protein (SEQ ID NO: 15).

In some embodiments, the transgene encodes a gene associated with an ocular (*e.g.*, corneal) disease. In some embodiments, the ocular (*e.g.*, corneal) disease is selected from corneal neovascularization (NV), corneal dystrophy, corneal inflammation, and corneal fibrosis. In some embodiments, the gene encodes a miRNA, an antagomir, or a miRNA mimic. In some embodiments, the gene encodes a miRNA, optionally a TuD miRNA or a pri miRNA. In some embodiments, the transgene comprises a region of complementarity to a sequence selected from the group consisting of SEQ ID NO: 1, 2, 3, 27, 28, and 29. In some embodiments, the transgene comprises SEQ ID NO: 4, SEQ ID NO: 5, or SEQ ID NO: 6.

In some embodiments, the administration occurs by injection. In some embodiments, the injection is intrastromal injection (intrastromal injection into ocular tissue). In some embodiments, the administration is topical administration (*e.g.*, topical administration to an eye).

In some embodiments, the administration results in transduction of an ocular (*e.g.*, corneal) cell type selected from the group consisting of keratocytes, corneal endothelial cells, corneal basal cells, corneal wing cells, and corneal squamous cells. In some embodiments, the administration results in transduction of keratocytes.

In some embodiments, the rAAV further comprises two AAV inverted terminal repeats (ITRs), wherein the ITRs flank the transgene. In some embodiments, the AAV ITRs are ITRs of one or more serotypes selected from: AAV2, AAV3, AAV4, AAV5, and AAV6.

In some embodiments of methods described herein, the subject is a mammal,
5 optionally a human.

In some aspects, the disclosure provides a composition comprising a recombinant adeno-associated virus as described herein.

Each of the limitations of the disclosure can encompass various embodiments of the disclosure. It is, therefore, anticipated that each of the limitations of the disclosure involving
10 any one element or combinations of elements can be included in each aspect of the disclosure. This disclosure is not limited in its application to the details of construction and the arrangement of components set forth in the following description or illustrated in the drawings. The disclosure is capable of other embodiments and of being practiced or of being carried out in various ways.

15 BRIEF DESCRIPTION OF DRAWINGS

FIGs. 1A-1C show the miR profile of corneal neovascularization in an alkali burn induced mouse model. FIG. 1A shows corneal neovascularization (NV) was observed for 15 days after alkali burn, and the corneas of four time points (before and days 5, 10 and 15 after
20 alkali burn) were harvested for RNA extraction. Nanostring technology was used to detect 618 miRNAs, and the analysis of the results is showed as the heatmap. The color represents the expression fold of miRNA in neovascularized corneas compared to normal corneas. FIG. 1B shows miRNA expression results using nanostring technology is further verified by qRT-PCR. Sample results are showed. (n = 8/group). FIG. 1C shows the top 3 miRNAs (miR-21,
25 miR-184 and miR-204) with over 10-fold expression change were selected as gene therapy candidates. The pri/TuD miRNA constructs, which overexpress or inhibit the target miRNA expression, were cloned and verified. The rAAV genome of the three constructs are showed.

FIGs. 2A-2D show rAAV serotype screening for gene transfer to mouse cornea. FIG. 2A shows the gene transfer efficiency of 14 rAAV serotypes (AAV1, AAV2, AAV3b,
30 AAV4, AAV5, AAV6, AAV6.2, AAV7, AAV8, AAV9, AAVrh.8, AAVrh.10, AAVrh.39, and AAVrh.43) with EGFP were assessed in mouse corneas delivered by intrastromal

injection. rAAVrh.8, rh.10, rh.39 and rh.43 showed stronger EGFP signals in whole corneal flat-mounts. 25×, scale bar = 250 μm. Left lower: high magnification images of central cornea, 100×. FIG. 2B shows gene transfer efficiency of rAAVrh.8, rh.10, rh.39 and rh.43 were further evaluated by topical administration with or without corneal epithelium removed. rAAVrh.10 and rh.39 showed stronger EGFP signals in whole corneal flat-mounts when delivered by topical administration in the condition that the corneal epithelium was removed. 25×, scale bar = 250 μm. Left lower: high magnification images of central cornea, 100×. FIG. 2C shows *in vivo* observation of EGFP expression mediated by rAAVrh.10 and rh.39. EGFP signals could be detected from 1 week till 4 weeks after intrastromal injection or topical administration. FIG. 2D shows confocal images of mouse corneas transduced by rAAVrh.10 and rh.39 EGFP vectors. Corneal stromal cells (keratocytes) were stained as red (keratocan positive). 630×, scale bar = 50 μm.

FIGs. 3A-3D show rAAV mediated miRNA therapeutics inhibit corneal NV by intrastromal injection. FIG. 3A shows an overview of the experimental schedule. FIG. 3B shows CD31-stained whole corneal flat-mounts harvested at day 15 post alkali burn. Blood vessels in corneal stroma were stained as red. No blood vessels were detected in corneas without alkali burn (group of normal). TuD miR-21, pri miR-184 and pri miR-204 inhibited corneal NV when compared to control groups of PBS, Gluc and TuD scramble. Yellow dash circle: avascular areas (area without blood vessels). Gluc: backbone plasmid of all the three miRNA constructs, TuD scramble: same backbone as other miRNA constructs with scramble TuD sequence. 25×, scale bar = 1 mm. FIG. 3C shows corneal NV was observed and measured *in vivo* at days 3, 5, 7, 10 and 14 after alkali burn. The *in vivo* results showed that the inhibitory effects of TuD miR-21, pri miR-184 and pri miR-204 started from day 5 till day 14 after alkali burn. FIG. 3D shows quantification analysis of NV area percentage among CD31-stained whole corneal flat-mounts indicated that all the three constructs effectively inhibited corneal NV. NV: neovascularization. ** $p < 0.01$, *** $p < 0.001$ compared to PBS group.

FIGs. 4A-4D show rAAV mediated miRNA therapeutics inhibit corneal NV by topical administration at days 7 and 10 after alkali burn. FIG. 4A shows an overview of the experimental schedule. FIG. 4B shows CD31-stained whole corneal flat-mounts harvested at day 15 post alkali burn. Blood vessels in corneal stroma were stained as red. No blood

vessels were detected in corneas without alkali burn (group of normal). TuD miR-21, pri miR-184 and pri miR-204 could not inhibit corneal NV at day 15 when compared to control groups of PBS, Gluc and TuD scramble (whole corneas were covered by blood vessels). Yellow dash circle: avascular areas (area without blood vessels). 25×, scale bar = 1 mm.

FIG. 4C shows corneal NV was observed and measured *in vivo* at days 3, 5, 7, 10 and 14 after alkali burn. The *in vivo* results showed that TuD miR-21, pri miR-184 and pri miR-204 could partially suppress the corneal NV at days 7 and 10 after alkali burn. However, the inhibitory effects disappeared till the day 14 post alkali burn. FIG. 4D shows quantification analysis of NV area percentage among CD31-stained whole corneal flat-mounts indicated that no inhibitory effect was found among any of the three miRNA therapeutics. NV: neovascularization. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to PBS group.

FIGs. 5A-5D show rAAV mediated miRNA therapeutics inhibit corneal NV by subconjunctival injection. FIG. 5A shows an overview of the experimental schedule. FIG. 5B shows CD31-stained whole corneal flat-mounts harvested at day 15 post alkali burn.

Blood vessels in corneal stroma were stained as red. No blood vessels were detected in corneas without alkali burn (group of normal). TuD miR-21, pri miR-184 and pri miR-204 inhibited corneal NV when compared to control groups of PBS, Gluc and TuD scramble. Whole cornea of Gluc group was covered by blood vessels. Yellow dash circle: avascular areas (area without blood vessels). 25×, scale bar = 1 mm. FIG. 5C shows corneal NV was observed and measured *in vivo* at days 3, 5, 7, 10 and 14 after alkali burn. The *in vivo* results showed that the inhibitory effects of pri miR-184 and pri miR-204 started from day 5, while TuD miR-21 from day 10, and continued till day 14 after alkali burn. FIG. 5D shows quantification analysis of NV area percentage among CD31-stained whole corneal flat-mounts indicated that all the three constructs effectively inhibited corneal NV. NV: neovascularization. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to PBS group.

FIGs. 6A-6B show the overview of intra-stromal injection and topical administration of rAAV vectors to cornea. FIG. 6A shows an intra-stromal injection. A small incision in the corneal epithelium was first created, and a 33-gauge needle attached to a 5 μ L Hamilton microliter syringe was then introduced through the incision into the corneal stroma and 2.4×10¹⁰ genomic copies (GC) of rAAV vectors in 4 μ L PBS were injected. *In vivo* imaging was conducted at weeks 1, 2, 3 and 4 after injection. Corneas were harvested at 4 weeks post-

injection. FIG. 6B shows topical administration (eye drop). rAAV transduction through topical administration was conducted with or without corneal epithelium removed. For epithelium removal, alcohol soaked filter membrane was applied on each cornea for 20 seconds and epithelium was removed by gentle scraping. One drop of rAAV vectors
 5 (2.4×10¹⁰ GC) in 4 µL PBS was directly applied to the intact cornea or the corneal stroma after epithelium removed.

FIGs. 7A-7C show intra-stromal injection of rAAVrh.8, rAAVrh.10, rAAVrh.39 and rAAVrh.43 efficiently transduce mouse cornea. FIG. 7A shows the gene transfer efficiency of fourteen rAAV serotypes with EGFP was assessed in mouse corneas delivered by intra-
 10 stromal injection. rAAVrh.8, rh.10, rh.39 and rh.43 showed stronger EGFP signals in the immunofluorescence images of whole corneal flat-mounts at the fourth week after injection. Magnification: 25×, scale bar = 250 µm. Lower left: images of central cornea under high magnification, 100×. FIG. 7B shows the percentage of EGFP positive area from whole corneal flat-mount area. Nearly 80% of the whole cornea areas were efficiently transduced by
 15 rAAVrh.8, rh.10 and rh.39 vectors. FIG. 7C shows quantification of EGFP fluorescence intensity of whole corneal flat-mounts presented in arbitrary unit (a.u.). The EGFP intensity in the four rhesus serotype groups of rAAVrh.8, rh.10, rh.39 and rh.43 were almost four folds over other rAAV serotypes tested. **: $p < 0.01$, ***: $p < 0.001$ compared to PBS group (n=3/group).

FIGs. 8A-8C show topical administration of rAAVrh.10 and rAAVrh.39 transduced mouse cornea when corneal epithelium was removed. FIG. 8A shows the gene transfer efficiency of rAAVrh.8, rh.10, rh.39 and rh.43 were further evaluated by topical eye-drop administration without (left column) or with (middle column) corneal epithelium removed. Groups of rAAVrh.10 and rh.39 showed stronger EGFP signals in immunofluorescence
 25 images of whole corneal flat-mounts when delivered by topical administration in the condition that the corneal epithelium was removed. Intra-stromal injection (right column) was set as a positive control. Magnification: 25×, scale bar = 250 µm. Lower left: images of central cornea under high magnification, 100×. FIG. 8B shows percentage of EGFP positive area from whole corneal flat-mount area. FIG. 8C shows quantification of EGFP fluorescence
 30 intensity of whole corneal flat-mounts presented in arbitrary unit (a.u.) (n=3/group).

FIGs. 9A-9C show rAAVrh.10 and rAAVrh.39 transduction in mouse cornea continued at least for 4 weeks *in vivo*. FIG. 9A shows *in vivo* observation of EGFP expression delivered by rAAVrh.10 and rh.39. EGFP signals were detected from 1 to 4-weeks after intra-stromal injection or topical administration (with corneal epithelium removed). FIG. 9B shows quantification of rAAV genome copies in mouse corneas harvested 4-weeks after topical administration or intra-stromal injection. For both serotypes, the intra-stromal injected groups showed higher genome copies than the topical administration groups. Liver DNA from mice that received an intravenous injection of rAAV9 EGFP vectors (1×10^{12} genomic copies) was used as positive control. FIG. 9C shows quantification of EGFP mRNA expression in mouse corneas harvested 4-weeks after topical administration or intra-stromal injection. Higher EGFP expression was detected with intra-stromal injection than topical administration in both serotypes. **: $p < 0.01$, ***: $p < 0.001$. (n=5/group).

FIGs. 10A-10B show rAAVrh.10 and rAAVrh.39 could transduce mouse keratocytes in corneal stroma. FIG. 10A shows representative immunofluorescence images of mouse corneas transduced by rAAVrh.10 and rh.39 EGFP vectors. Corneal stromal cells (keratocytes) were stained as red (keratocan positive). Magnification: 630 $\times$, scale bar = 50 μ m. Squared regions indicate the locations of high magnification images shown on the right. FIG. 10B shows quantification of the percentage of EGFP positive cells among keratocytes. Higher percentage of EGFP positive keratocytes was found in intra-stromal injection groups than topical administration groups of both serotypes. **: $p < 0.01$, ***: $p < 0.001$ (n=3/group).

FIG. 11 shows transduction of rAAVrh.10 and rAAVrh.39 vectors had no adverse effect on cornea histology. Paraffin-embedded sections of the corneas stained with Haematoxylin and Eosin (H&E) displayed normal structures: all layers of the cornea were clear without obvious morphological changes compared to the control group or any other signs of inflammatory and immune reactions. Magnification: 200 $\times$, scale bar = 50 μ m.

FIGs. 12A-12B show candidate miRNA selection. FIG. 12A shows miRNA profiling of alkali-burn induced mouse corneal NV. FIG. 12B shows signaling systems.

FIG. 13 shows candidate miRNA selection and qRT-PCR confirmation of miR-184 and miR-204. The arrow is the time point of alkali burn, *****: $p < 0.0001$.

FIG. 14 shows rAAV serotype screening in mouse cornea of rAAV1, rAAV2, rAAV3b, rAAV4, rAAV5, rAAV6, rAAV6.2, rAAV7, rAAV8, rAAV9, rAAVrh.8, rAAVrh.10, rAAVrh.39, rAAVrh.43, and PBS.

FIG. 15 shows rAAV.rh10 delivered EGFP expression after alkali burn and intrastromal injection (IS). Control and alkali-burned representative immunofluorescence images are shown after 1 and 2 weeks.

FIG. 16 graphically depicts rAAV.rh10 delivered EGFP expression after alkali burn and intrastromal injection (ddPCR). The left panel shows data regarding genomic copies and the right panel shows data regarding mRNA expression.

FIG. 17 shows rAAV.rh10 delivered EGFP expression after alkali burn and subconjunctival injection (SC). Control and alkali-burned representative immunofluorescence images are shown after 1 and 2 weeks.

FIG. 18 graphically depicts rAAV.rh10 delivered EGFP expression after alkali burn and subconjunctival injection (ddPCR). The left panel shows data regarding genomic copies and the right panel shows data regarding mRNA expression.

FIGs. 19A-19D show rAAV.rh10 delivered pri miR-184 and pri miR-204 could inhibit corneal NV as prevention through intrastromal injection (IS). The dashed circle represents avascular area, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$.

FIGs. 20A-20D show rAAV.rh10 delivered pri miR-184 and pri miR-204 could inhibit corneal NV as treatment through subconjunctival injection (SC). The dashed circle represents avascular area, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$.

FIG. 21 shows overexpression of miR-184 inhibit Fzd4 expression (Wnt signaling).

FIG. 22 shows overexpression of miR-184 inhibit Fzd4 expression (Wnt signaling).

FIG. 23 shows overexpression of miR-204 inhibit Angpt-1 expression (Tie2-P13K-Akt pathway).

FIG. 24 shows overexpression of miR-204 inhibit Angpt-1 expression (Tie2-P13K-Akt pathway).

FIGs. 25A-25B show rAAVrh.10 delivered pri miR-184 and pri mir-204 did not induce obvious abnormality in normal mouse eyes.

FIGs. 26A-26C show RNA-seq analysis reveals differential expression of miR target genes. FIG. 26A shows a dendrogram of gene expression profiles between untreated corneas

(day 0), corneas from 5 days post-treatment with alkali burn (day 5), and corneas from 15 days post-treatment (day 15). FIG. 26B shows volcano plots comparing fold-change ($\log_2$ of fold-change) vs. significance values ($-\log_{10}$ of p-value) between day 0 and day 5 (left plot), day 0 and day 15 (middle plot), and day 5 and day 15 (right plot). Lighter shaded data points denote genes that show significant fold-change between compared conditions. FIG. 26C shows a heat map display of the fold-change in alkali-burn treated corneas. The shading scale is displayed to the right. Fold-change is shown as $\log_2$ difference over day-0 values ($\log_2(\text{FPKM}/\text{day } 0)$).

FIGs. 27A-27C show up-regulated miR-204-predicted targets are associated with multiple biological processes and pathways. FIG. 27A shows a heat map of fold-change in expression of miR-204 predicted genes in corneas 5 days and 15 days post-alkali-burn treatment. The shading scale is displayed to the right. Fold-change is shown as $\log_2$ difference over day 0 values ($\log_2(\text{FPKM}/\text{day } 0)$). FIG. 27B shows K-means clustering of miR-204 predicted target gene expression profiles in alkali-burn treated corneas. Three distinct groups were defined: genes with little or no change (left plot, group 1); down-regulated genes (center plot, group 2); and up-regulated genes (right plot, group 3). FIG. 27C shows a gene ontology (GO) network map for the group 3 genes. Genes that enrich for selected terms are displayed as small nodes that connect to the larger GO-term nodes. The relative sizes of the GO-term nodes also reflect their significance levels.

FIG. 28 shows expression of predicted miR-204 target genes that are related to vasculogenesis. Relative expression (FPKM) of predicted miR-204 target genes that are up-regulated after alkali-burn treatment of corneas. The six genes displayed, including their known isoforms, demonstrate an increase in mRNA expression as assessed by RNA-seq analysis. Data for untreated corneas (day 0), corneas 5 days post-treatment (day 5), and 15 days post-treatment (day 15) are shown.

DETAILED DESCRIPTION OF INVENTION

The disclosure relates in some aspects to compositions and methods for tissue-specific delivery of a transgene by a recombinant adeno-associated virus (rAAV). The invention relates, in part, to the discovery that rAAV vectors comprising a capsid protein(s) having a certain serotype (*e.g.*, AAV5, AAV6, AAV6.2, AAV7, AAV8, AAV9, AAVrh.8, AAVrh.10,

AAVrh.39, and AAVrh.43) mediate delivery of transgenes to ocular tissue (*e.g.*, corneal tissue) more efficiently than other vectors (*e.g.*, rAAV vectors comprising other capsid protein serotypes). In some embodiments, the disclosure relates to the discovery of 35 miRNA-encoding genes that are expressionally up-regulated, and 3 miRNA-encoding genes that are expressionally down-regulated, in response to ocular injury (*e.g.*, corneal trauma).

Methods and Compositions for AAV-mediated Delivery of a Transgene to Ocular Tissue

Methods for delivering a transgene to ocular (*e.g.*, corneal) tissue in a subject are provided herein. The methods typically involve administering to a subject an effective amount of a rAAV comprising a nucleic acid for expressing a transgene in the subject. An “effective amount” of a rAAV is an amount sufficient to infect a sufficient number of cells of a target tissue in a subject. In some embodiments, a target tissue is ocular (*e.g.*, corneal) tissue. An effective amount of a rAAV may be an amount sufficient to have a therapeutic benefit in a subject, *e.g.*, to extend the lifespan of a subject, to improve in the subject one or more symptoms of disease, *e.g.*, a symptom of ocular disease (*e.g.*, corneal neovascularization (NV)). In some cases, an effective amount of a rAAV may be an amount sufficient to produce a stable somatic transgenic animal model. The effective amount will depend on a variety of factors such as, for example, the species, age, weight, health of the subject, and the ocular tissue to be targeted, and may thus vary among subject and tissue.

An effective amount may also depend on the rAAV used. The invention is based, in part on the recognition that rAAV comprising capsid proteins having a particular serotype (*e.g.*, AAV5, AAV6, AAV6.2, AAV7, AAV8, AAV9, AAVrh.8, AAVrh.10, AAVrh.39, and AAVrh.43) mediate more efficient transduction of ocular (*e.g.*, corneal) tissue than rAAV comprising capsid proteins having a different serotype. Thus in some embodiments, the rAAV comprises a capsid protein of an AAV serotype selected from the group consisting of: AAV5, AAV6, AAV6.2, AAV7, AAV8, AAV9, AAVrh.8, AAVrh.10, AAVrh.39, and AAVrh.43 (SEQ ID NO: 7-16). In some embodiments, the rAAV comprises a capsid protein of AAVrh.10 serotype (SEQ ID NO: 14) or AAVrh.39 serotype (SEQ ID NO: 15). In some embodiments, the capsid protein comprises an amino acid sequence that is at least 70%, at least 80%, at least 90%, at least 95% , or at least 99% identical to any one of SEQ ID NO: 7-

16. In some embodiments, the capsid protein is AAVrh.10 capsid protein (SEQ ID NO: 14) or AAVrh.39 capsid protein (SEQ ID NO: 15).

In certain embodiments, the effective amount of rAAV is 10^{10} , 10^{11} , 10^{12} , 10^{13} , or 10^{14} genome copies per kg. In certain embodiments, the effective amount of rAAV is 10^{10} , 10^{11} , 10^{12} , 10^{13} , 10^{14} , or 10^{15} genome copies per subject.

An effective amount may also depend on the mode of administration. For example, targeting an ocular (*e.g.*, corneal) tissue by intrastromal administration or subcutaneous injection may require different (*e.g.*, higher or lower) doses, in some cases, than targeting an ocular (*e.g.*, corneal) tissue by another method (*e.g.*, systemic administration, topical administration). The invention is based, in part, on the recognition that intrastromal injection (IS) of rAAV having certain serotypes (*e.g.*, AAV5, AAV6, AAV6.2, AAV7, AAV8, AAV9, AAVrh.8, AAVrh.10, AAVrh.39, and AAVrh.43) mediates efficient transduction of ocular (*e.g.*, corneal) cells. Thus, in some embodiments, the injection is intrastromal injection (IS). In some embodiments, the injection is topical administration (*e.g.*, topical administration to an eye). In some cases, multiple doses of a rAAV are administered.

Generally, the anatomy of an eye (*e.g.*, a mammalian eye) comprises a sclera, choroid, retina, vitreous body, macula, fovea, optic disc, lens, pupil, iris, aqueous fluid, cornea, conjunctiva ciliary body, and optic nerve. The cornea is a transparent, multilayered (*e.g.*, comprising four, five, or six layers) tissue that covers the iris, pupil, and anterior chamber of the eye. Layers of the cornea include, but are not limited to, corneal epithelium, Bowman's layer (*e.g.*, anterior limiting membrane), corneal stroma (*e.g.*, substantia propria), Descemet's membrane (*e.g.*, posterior limiting membrane), and corneal endothelium. Administration of compositions described by the disclosure may result in transduction of one of the foregoing corneal layers, or more than one corneal layer (*e.g.*, 2, 3, 4, 5, or 6 corneal layers). Corneal layers can comprise a single cell type, or multiple cell types. In some embodiments, administration of an rAAV as described herein results in transduction of an ocular (*e.g.*, corneal) cell type selected from the group consisting of keratocytes, corneal endothelial cells, corneal basal cells, corneal wing cells, and corneal squamous cells. In some embodiments, the administration results in transduction of keratocytes.

Ocular (*e.g.*, corneal) tissue can be healthy ocular (*e.g.*, corneal) tissue (*e.g.*, ocular tissue not having a disease, or at risk of developing an ocular disease, such as a corneal

disease) or diseased ocular tissue (*e.g.*, ocular tissue having corneal neovascularization (NV), corneal dystrophy, corneal inflammation, and corneal fibrosis). As used herein, “at risk of developing an ocular disease” refers to a subject having an increased probability of developing an ocular disease (*e.g.*, corneal disease) than the general population due to the presence of a risk factor. Examples categories of risk factors for developing ocular disease include, but are not limited to: exposure to certain microbial pathogens (*e.g.*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*), contact lens wear, ocular trauma, prior ocular surgery, age, race, and family history (*e.g.*, positive family history of ocular disease, high cholesterol, high blood pressure, or diabetes).

Without wishing to be bound by any particular theory, efficient transduction of ocular (*e.g.*, corneal) cells by rAAV described herein may be useful for the treatment of a subject having an ocular disease (*e.g.*, corneal disease). Accordingly, methods and compositions for treating ocular disease are also provided herein. In some aspects, the disclosure provides a method for treating an ocular disease (*e.g.*, corneal disease), the method comprising:

administering to a subject having or suspected of having an ocular disease (*e.g.*, corneal disease) an effective amount of rAAV, wherein the rAAV comprises (i) a capsid protein having a serotype selected from the group consisting of AAV5, AAV6, AAV6.2, AAV7, AAV8, AAV9, AAVrh.8, AAVrh.10, AAVrh.39, and AAVrh.43, and (ii) a nucleic acid comprising a promoter operably linked to a transgene.

As used herein, an “ocular disease” is a disease or condition of the eye. In some embodiments, an ocular disease is a corneal disease (*e.g.*, a disease affecting the cornea or corneal cells). Non-limiting examples of ocular diseases include, but are not limited to, amblyopia, astigmatism, blepharitis, cataract, chalazion, conjunctivitis, diabetic retinopathy, dry eye, glaucoma, keratitis, keratoconus, macular degeneration, ocular hypertension, pinquecula, pterygium, retinitis pigmentosa, and ocular cancer (*e.g.*, retinoblastoma, melanoma of the eye, lymphoma of the eye, medulloepithelioma, squamous cell cancer of the conjunctiva). Examples of corneal diseases include, but are not limited to, corneal neovascularization (NV), corneal dystrophy, corneal inflammation, corneal abrasion, and corneal fibrosis.

Ocular Disease-associated Transgenes

In some aspects, an rAAV described by the disclosure comprises a nucleic acid encoding a transgene (*e.g.*, miR-21, pri miR-184, pri miR-204) associated with ocular disease (*e.g.*, corneal disease, such as corneal neovascularization). Without wishing to be bound by any particular theory, rAAV-based delivery of a transgene encoding a gene associated with a
5 ocular disease is useful for treatment of subjects having an ocular disease (*e.g.*, corneal disease). As used herein, “gene associated with an ocular disease” refers to any gene, wherein expression of that gene that provides a therapeutic benefit in a subject, *e.g.*, to improve in the subject one or more symptoms of disease, *e.g.*, a symptom of ocular disease (*e.g.*, corneal neovascularization (NV), corneal dystrophy, corneal inflammation, corneal
10 abrasion, and corneal fibrosis, etc.).

A gene associated with ocular disease can be a protein, polypeptide, antibody or fragment thereof (*e.g.*, ScFv), toxin, or interfering RNA (*e.g.*, siRNA, dsRNA, miRNA, artificial miRNA (ami-RNA), antagomir). Examples of genes associated with ocular disease include, but are not limited to Frizzled 4 (*Fzd4*; SEQ ID NO: 27), angiopoietin-1 (*Angpt1*,
15 isoform 1 and/or isoform 2; SEQ ID NOs: 28-29, respectively), associated with corneal trauma; transforming growth factor β (TGF- β), Smad and mitogen-activated protein kinases (*e.g.*, MAPK), associated with fibrotic disorders of the eye; IL-1 α , IL-1 β , IL-6, TNF α , interferon γ , transforming growth factor β 1, and CD4, associated with traumatic corneal injury (*e.g.*, alkali burn), protein p27, Cytokeratin 13, interleukin-like growth factor 2 (ILGF-
20 2), junB, Metallothionein hMT-Ie, keratin 6 (*e.g.*, KRT6), and beta 2-microglobulin, associated with corneal disease; and, connective tissue growth factor (CTGF) and vascular endothelial growth factor (VEGF).

In some aspects, the disclosure relates to the discovery that 3 genes encoding miRNA are significantly down-regulated (*e.g.*, miR-184, miR-203, and miR-204) in response to
25 ocular injury (*e.g.*, corneal trauma). Without wishing to be bound by any particular theory, increasing the expression of down-regulated miRNA may provide a useful therapeutic benefit in a subject. Thus, in some embodiments, a rAAV described by the disclosure comprises a nucleic acid that encodes a micro-RNA (miRNA) that is down-regulated in response to ocular injury (*e.g.*, corneal injury) or ocular disease, such as miR-184, miR-203, or miR-204.
30 MicroRNAs are transcribed by RNA polymerase II as large RNA precursors called pri-miRNAs. In some embodiments, a rAAV described by the disclosure comprises a transgene

encoding a pri-miRNA. In some embodiments, the pri-miRNA is pri-miRNA-184 or pri-miRNA-204.

In some aspects, the disclosure relates to the discovery that 35 genes encoding miRNA are significantly up-regulated (*e.g.*, miR-21) in response to ocular injury (*e.g.*, corneal trauma). Without wishing to be bound by any particular theory, decreasing the expression of up-regulated miRNA may provide a useful therapeutic benefit in a subject. Overexpression of miRNA can be reduced by using a “sponge design”, for example a Tough Decoy (TuD) scaffold, as disclosed by Haraguchi *et al.*, Nucleic Acids Res, 37: e43 (2009). A TuD is an ~60-bp long hairpin-shaped RNA with an internal loop exposing two miRNA binding sites. In some embodiments, a rAAV described by the disclosure comprises a transgene that encodes a TuD miRNA (*e.g.*, miR-21 TuD miRNA).

In some embodiments, the gene associated with ocular disease (*e.g.*, corneal disease) is selected from the group consisting of miR-106b, miR-1955, let-7i, miR-126-3p, miR-152, miR-24, miR99b, miR223, miR126-5p, miR146a, miR-150, miR191, miR-140, miR-221, miR301a, miR-484, miR-327, miR-2132, miR-28, miR-27b, miR-423-5p, miR-132, miR-19a, miR-1-3, miR-1-6, miR-17, miR-19b, miR-214, miR-21, miR-350, miR-425, miR-335-5p, miR-382, miR-2146, miR-804, miR-378, miR-184, miR-203, and miR-204.

In some embodiments the molecule that modulates miRNA activity (*e.g.*, antagomir) modulates the activity of a miRNA selected from the group consisting of miR-106b, miR-1955, let-7i, miR-126-3p, miR-152, miR-24, miR99b, miR223, miR126-5p, miR146a, miR-150, miR191, miR-140, miR-221, miR301a, miR-484, miR-327, miR-2132, miR-28, miR-27b, miR-423-5p, miR-132, miR-19a, miR-1-3, miR-1-6, miR-17, miR-19b, miR-214, miR-21, miR-350, miR-425, miR-335-5p, miR-382, miR-2146, miR-804, miR-378, miR-184, miR-203, and miR-204.

In some aspects, the disclosure relates to the discovery that AAV-mediated delivery of molecules (*e.g.*, miR-184, miR-204) that target certain ocular-disease associated genes (*e.g.*, *Fzd4*, *Angpt1*) are useful for treatment of corneal disease, such as corneal neovascularization (NV). Thus, in some embodiments, a rAAV described by the disclosure comprises a nucleic acid encoding a transgene that has a region of complementarity to *Fzd4* or *Angpt1*. A “region of complementarity” refers to a region on a nucleic acid antisense strand (*e.g.*, miRNA) that is substantially complementary (*e.g.*, 60%, 70%, 80%, 90%, 95%,

99%, or 100% complementary) to a sequence, for example a target sequence (*e.g.*, *Fzd4*, *Angpt1*). A region of complementarity can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 40, or 50 nucleotides in length. In some embodiments, a region of complementarity is greater than 50 nucleotides in length. In some
5 embodiments, a rAAV described by the disclosure comprises transgene, wherein the transgene comprises a region of complementarity to a sequence selected from the group consisting of SEQ ID NO: 1, 2, 3, 27, 28, and 29.

Recombinant Adeno-associated Viruses (rAAVs)

In some aspects, the disclosure provides isolated AAVs. As used herein with respect
10 to AAVs, the term “isolated” refers to an AAV that has been artificially produced or obtained. Isolated AAVs may be produced using recombinant methods. Such AAVs are referred to herein as “recombinant AAVs”. Recombinant AAVs (rAAVs) preferably have tissue-specific targeting capabilities, such that a nuclease and/or transgene of the rAAV will be delivered specifically to one or more predetermined tissue(s). The AAV capsid is an
15 important element in determining these tissue-specific targeting capabilities. Thus, an rAAV having a capsid appropriate for the tissue being targeted can be selected.

In some aspects, the disclosure provides an rAAV having a capsid appropriate for targeting ocular tissue (*e.g.*, corneal tissue). In some embodiments, the capsid has a serotype selected from the group consisting of AAV5, AAV6, AAV6.2, AAV7, AAV8, AAV9,
20 AAVrh.8, AAVrh.10, AAVrh.39, and AAVrh.43. In some embodiments, the capsid has an AAVrh.10 serotype (*e.g.*, SEQ ID NO: 14) or an AAVrh.39 serotype (*e.g.*, SEQ ID NO: 15). The skilled artisan also recognizes that rAAV described herein may comprise variants of AAV5, AAV6, AAV6.2, AAV7, AAV8, AAV9, AAVrh.8, AAVrh.10, AAVrh.39, and AAVrh.43 serotype capsid proteins. In some embodiments, the capsid protein comprises an
25 amino acid sequence that is at least 70%, at least 80%, at least 90%, at least 95% , or at least 99% identical to any one of SEQ ID NO: 7-16.

Methods for obtaining recombinant AAVs having a desired capsid protein are well known in the art. (See, for example, US 2003/0138772), the contents of which are incorporated herein by reference in their entirety). Typically the methods involve culturing a
30 host cell which contains a nucleic acid sequence encoding an AAV capsid protein; a functional *rep* gene; a recombinant AAV vector composed of, AAV inverted terminal repeats

(ITRs) and a transgene; and sufficient helper functions to permit packaging of the recombinant AAV vector into the AAV capsid proteins. In some embodiments, capsid proteins are structural proteins encoded by the cap gene of an AAV. AAVs comprise three capsid proteins, virion proteins 1 to 3 (named VP1, VP2 and VP3), all of which are transcribed from a single cap gene via alternative splicing. In some embodiments, the molecular weights of VP1, VP2 and VP3 are respectively about 87 kDa, about 72 kDa and about 62 kDa. In some embodiments, upon translation, capsid proteins form a spherical 60-mer protein shell around the viral genome. In some embodiments, the functions of the capsid proteins are to protect the viral genome, deliver the genome and interact with the host. In some aspects, capsid proteins deliver the viral genome to a host in a tissue specific manner.

The components to be cultured in the host cell to package a rAAV vector in an AAV capsid may be provided to the host cell in *trans*. Alternatively, any one or more of the required components (*e.g.*, recombinant AAV vector, rep sequences, cap sequences, and/or helper functions) may be provided by a stable host cell which has been engineered to contain one or more of the required components using methods known to those of skill in the art. Most suitably, such a stable host cell will contain the required component(s) under the control of an inducible promoter. However, the required component(s) may be under the control of a constitutive promoter. Examples of suitable inducible and constitutive promoters are provided herein, in the discussion of regulatory elements suitable for use with the transgene. In still another alternative, a selected stable host cell may contain selected component(s) under the control of a constitutive promoter and other selected component(s) under the control of one or more inducible promoters. For example, a stable host cell may be generated which is derived from 293 cells (which contain E1 helper functions under the control of a constitutive promoter), but which contain the rep and/or cap proteins under the control of inducible promoters. Still other stable host cells may be generated by one of skill in the art.

In some embodiments, the instant disclosure relates to a host cell containing a nucleic acid that comprises a coding sequence encoding a gene associated with an ocular disease (*e.g.*, corneal disease). In some embodiments, the instant disclosure relates to a composition comprising the host cell described above. In some embodiments, the composition comprising the host cell above further comprises a cryopreservative.

The recombinant AAV vector, rep sequences, cap sequences, and helper functions required for producing the rAAV of the disclosure may be delivered to the packaging host cell using any appropriate genetic element (vector). The selected genetic element may be delivered by any suitable method, including those described herein. The methods used to construct any embodiment of this disclosure are known to those with skill in nucleic acid manipulation and include genetic engineering, recombinant engineering, and synthetic techniques. See, *e.g.*, Sambrook et al, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. Similarly, methods of generating rAAV virions are well known and the selection of a suitable method is not a limitation on the present disclosure. See, *e.g.*, K. Fisher et al, *J. Virol.*, 70:520-532 (1993) and U.S. Pat. No. 5,478,745.

In some embodiments, recombinant AAVs may be produced using the triple transfection method (described in detail in U.S. Pat. No. 6,001,650). Typically, the recombinant AAVs are produced by transfecting a host cell with an recombinant AAV vector (comprising a transgene) to be packaged into AAV particles, an AAV helper function vector, and an accessory function vector. An AAV helper function vector encodes the "AAV helper function" sequences (*i.e.*, rep and cap), which function in *trans* for productive AAV replication and encapsidation. Preferably, the AAV helper function vector supports efficient AAV vector production without generating any detectable wild-type AAV virions (*i.e.*, AAV virions containing functional rep and cap genes). Non-limiting examples of vectors suitable for use with the present disclosure include pHLP19, described in U.S. Pat. No. 6,001,650 and pRep6cap6 vector, described in U.S. Pat. No. 6,156,303, the entirety of both incorporated by reference herein. The accessory function vector encodes nucleotide sequences for non-AAV derived viral and/or cellular functions upon which AAV is dependent for replication (*i.e.*, "accessory functions"). The accessory functions include those functions required for AAV replication, including, without limitation, those moieties involved in activation of AAV gene transcription, stage specific AAV mRNA splicing, AAV DNA replication, synthesis of cap expression products, and AAV capsid assembly. Viral-based accessory functions can be derived from any of the known helper viruses such as adenovirus, herpesvirus (other than herpes simplex virus type-1), and vaccinia virus.

In some aspects, the disclosure provides transfected host cells. The term "transfection" is used to refer to the uptake of foreign DNA by a cell, and a cell has been

"transfected" when exogenous DNA has been introduced inside the cell membrane. A number of transfection techniques are generally known in the art. See, *e.g.*, Graham *et al.* (1973) Virology, 52:456, Sambrook *et al.* (1989) Molecular Cloning, a laboratory manual, Cold Spring Harbor Laboratories, New York, Davis *et al.* (1986) Basic Methods in Molecular Biology, Elsevier, and Chu *et al.* (1981) Gene 13:197. Such techniques can be used to introduce one or more exogenous nucleic acids, such as a nucleotide integration vector and other nucleic acid molecules, into suitable host cells.

A "host cell" refers to any cell that harbors, or is capable of harboring, a substance of interest. Often a host cell is a mammalian cell. A host cell may be used as a recipient of an AAV helper construct, an AAV minigene plasmid, an accessory function vector, or other transfer DNA associated with the production of recombinant AAVs. The term includes the progeny of the original cell which has been transfected. Thus, a "host cell" as used herein may refer to a cell which has been transfected with an exogenous DNA sequence. It is understood that the progeny of a single parental cell may not necessarily be completely identical in morphology or in genomic or total DNA complement as the original parent, due to natural, accidental, or deliberate mutation.

As used herein, the term "cell line" refers to a population of cells capable of continuous or prolonged growth and division *in vitro*. Often, cell lines are clonal populations derived from a single progenitor cell. It is further known in the art that spontaneous or induced changes can occur in karyotype during storage or transfer of such clonal populations. Therefore, cells derived from the cell line referred to may not be precisely identical to the ancestral cells or cultures, and the cell line referred to includes such variants.

As used herein, the terms "recombinant cell" refers to a cell into which an exogenous DNA segment, such as DNA segment that leads to the transcription of a biologically-active polypeptide or production of a biologically active nucleic acid such as an RNA, has been introduced.

As used herein, the term "vector" includes any genetic element, such as a plasmid, phage, transposon, cosmid, chromosome, artificial chromosome, virus, virion, *etc.*, which is capable of replication when associated with the proper control elements and which can transfer gene sequences between cells. Thus, the term includes cloning and expression vehicles, as well as viral vectors. In some embodiments, useful vectors are contemplated to

be those vectors in which the nucleic acid segment to be transcribed is positioned under the transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrases "operatively positioned," "under control" or
5 "under transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the gene. The term "expression vector or construct" means any type of genetic construct containing a nucleic acid in which part or all of the nucleic acid encoding sequence is capable of being transcribed. In some embodiments, expression includes transcription of
10 the nucleic acid, for example, to generate a biologically-active polypeptide product or functional RNA (*e.g.*, guide RNA, miRNA) from a transcribed gene.

The foregoing methods for packaging recombinant vectors in desired AAV capsids to produce the rAAVs of the disclosure are not meant to be limiting and other suitable methods will be apparent to the skilled artisan.

15 *Isolated Nucleic Acids*

A "nucleic acid" sequence refers to a DNA or RNA sequence. In some embodiments, proteins and nucleic acids of the disclosure are isolated. As used herein, the term "isolated" means artificially produced. As used herein with respect to nucleic acids, the term "isolated" means: (i) amplified *in vitro* by, for example, polymerase chain reaction (PCR); (ii)
20 recombinantly produced by cloning; (iii) purified, as by cleavage and gel separation; or (iv) synthesized by, for example, chemical synthesis. An isolated nucleic acid is one which is readily manipulable by recombinant DNA techniques well known in the art. Thus, a nucleotide sequence contained in a vector in which 5' and 3' restriction sites are known or for which polymerase chain reaction (PCR) primer sequences have been disclosed is considered
25 isolated but a nucleic acid sequence existing in its native state in its natural host is not. An isolated nucleic acid may be substantially purified, but need not be. For example, a nucleic acid that is isolated within a cloning or expression vector is not pure in that it may comprise only a tiny percentage of the material in the cell in which it resides. Such a nucleic acid is isolated, however, as the term is used herein because it is readily manipulable by standard
30 techniques known to those of ordinary skill in the art. As used herein with respect to proteins or peptides, the term "isolated" refers to a protein or peptide that has been isolated from its

natural environment or artificially produced (*e.g.*, by chemical synthesis, by recombinant DNA technology, *etc.*).

The skilled artisan will also realize that conservative amino acid substitutions may be made to provide functionally equivalent variants, or homologs of the capsid proteins. In some aspects the disclosure embraces sequence alterations that result in conservative amino acid substitutions. As used herein, a conservative amino acid substitution refers to an amino acid substitution that does not alter the relative charge or size characteristics of the protein in which the amino acid substitution is made. Variants can be prepared according to methods for altering polypeptide sequence known to one of ordinary skill in the art such as are found in references that compile such methods, *e.g.*, Molecular Cloning: A Laboratory Manual, J. Sambrook, *et al.*, eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, or Current Protocols in Molecular Biology, F.M. Ausubel, *et al.*, eds., John Wiley & Sons, Inc., New York. Conservative substitutions of amino acids include substitutions made among amino acids within the following groups: (a) M, I, L, V; (b) F, Y, W; (c) K, R, H; (d) A, G; (e) S, T; (f) Q, N; and (g) E, D. Therefore, one can make conservative amino acid substitutions to the amino acid sequence of the proteins and polypeptides disclosed herein.

Recombinant AAV Vectors (rAAV Vectors)

“Recombinant AAV (rAAV) vectors” of the disclosure are typically composed of, at a minimum, a transgene and its regulatory sequences, and 5' and 3' AAV inverted terminal repeats (ITRs). It is this recombinant AAV vector which is packaged into a capsid protein and delivered to a selected target cell. In some embodiments, the transgene is a nucleic acid sequence, heterologous to the vector sequences, which encodes a polypeptide, protein, functional RNA molecule (*e.g.*, gRNA) or other gene product, of interest. The nucleic acid coding sequence is operatively linked to regulatory components in a manner which permits transgene transcription, translation, and/or expression in a cell of a target tissue.

In some embodiments, the instant disclosure relates to a recombinant AAV (rAAV) vector comprising a nucleic acid sequence including a promoter operably linked to a transgene, wherein the transgene is a gene associated with an ocular disease (*e.g.*, corneal disease). In some embodiments, a rAAV vector further comprises nucleic acid sequences encoding one or more AAV inverted terminal repeat sequences (ITRs), for example AAV2

ITRs. In some embodiments, a rAAV vector further comprises nucleic acid sequences encoding one or more AAV ITRs selected from the group consisting of AAV3, AAV4, AAV5, and AAV6.

The AAV sequences of the vector typically comprise the cis-acting 5' and 3' inverted terminal repeat sequences (See, *e.g.*, B. J. Carter, in "Handbook of Parvoviruses", ed., P. Tijsser, CRC Press, pp. 155 168 (1990)). The ITR sequences are about 145 bp in length. Preferably, substantially the entire sequences encoding the ITRs are used in the molecule, although some degree of minor modification of these sequences is permissible. The ability to modify these ITR sequences is within the skill of the art. (See, *e.g.*, texts such as Sambrook et al, "Molecular Cloning. A Laboratory Manual", 2d ed., Cold Spring Harbor Laboratory, New York (1989); and K. Fisher *et al.*, J Virol., 70:520 532 (1996)). An example of such a molecule employed in the present disclosure is a "cis-acting" plasmid containing the transgene, in which the selected transgene sequence and associated regulatory elements are flanked by the 5' and 3' AAV ITR sequences. The AAV ITR sequences may be obtained from any known AAV, including presently identified mammalian AAV types (*e.g.*, AAV2, AAV3, AAV4, AAV5, or AAV6 ITR sequences).

In addition to the major elements identified above for the recombinant AAV vector, the vector also includes control elements necessary which are operably linked to the transgene in a manner which permits its transcription, translation and/or expression in a cell transfected with the plasmid vector or infected with the virus produced by the disclosure. As used herein, "operably linked" sequences include both expression control sequences that are contiguous with the gene of interest and expression control sequences that act in trans or at a distance to control the gene of interest.

Expression control sequences include appropriate transcription initiation, termination, promoter and enhancer sequences; efficient RNA processing signals such as splicing and polyadenylation (polyA) signals; sequences that stabilize cytoplasmic mRNA; sequences that enhance translation efficiency (*i.e.*, Kozak consensus sequence); sequences that enhance protein stability; and when desired, sequences that enhance secretion of the encoded product. A great number of expression control sequences, including promoters which are native, constitutive, inducible and/or tissue-specific, are known in the art and may be utilized.

As used herein, a nucleic acid sequence (*e.g.*, coding sequence) and regulatory sequences are said to be “operably” linked when they are covalently linked in such a way as to place the expression or transcription of the nucleic acid sequence under the influence or control of the regulatory sequences. If it is desired that the nucleic acid sequences be translated into a functional protein, two DNA sequences are said to be operably linked if induction of a promoter in the 5’ regulatory sequences results in the transcription of the coding sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region to direct the transcription of the coding sequences, or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. Thus, a promoter region would be operably linked to a nucleic acid sequence if the promoter region were capable of effecting transcription of that DNA sequence such that the resulting transcript might be translated into the desired protein or polypeptide. Similarly two or more coding regions are operably linked when they are linked in such a way that their transcription from a common promoter results in the expression of two or more proteins having been translated in frame. In some embodiments, operably linked coding sequences yield a fusion protein. In some embodiments, operably linked coding sequences yield a functional RNA (*e.g.*, gRNA, miRNA).

For nucleic acids encoding proteins, a polyadenylation sequence generally is inserted following the transgene sequences and before the 3’ AAV ITR sequence. A rAAV construct useful in the present disclosure may also contain an intron, desirably located between the promoter/enhancer sequence and the transgene. One possible intron sequence is derived from SV-40, and is referred to as the SV-40 T intron sequence. Another vector element that may be used is an internal ribosome entry site (IRES). An IRES sequence is used to produce more than one polypeptide from a single gene transcript. An IRES sequence would be used to produce a protein that contain more than one polypeptide chains. Selection of these and/or other vector elements may be performed, as appropriate, and many such sequences are available [see, *e.g.*, Sambrook et al, and references cited therein at, for example, pages 3.18 3.26 and 16.17 16.27 and Ausubel *et al.*, Current Protocols in Molecular Biology, John Wiley & Sons, New York, 1989]. In some embodiments, a Foot and Mouth Disease Virus 2A sequence is included in polyprotein; this is a small peptide (approximately 18 amino acids in

length) that has been shown to mediate the cleavage of polyproteins (Ryan, M D *et al.*, EMBO, 1994; 4: 928-933; Mattion, N M *et al.*, J Virology, November 1996; p. 8124-8127; Furler, S *et al.*, Gene Therapy, 2001; 8: 864-873; and Halpin, C *et al.*, The Plant Journal, 1999; 4: 453-459). The cleavage activity of the 2A sequence has previously been

5 demonstrated in artificial systems including plasmids and gene therapy vectors (AAV and retroviruses) (Ryan, M D *et al.*, EMBO, 1994; 4: 928-933; Mattion, N M *et al.*, J Virology, November 1996; p. 8124-8127; Furler, S *et al.*, Gene Therapy, 2001; 8: 864-873; and Halpin, C *et al.*, The Plant Journal, 1999; 4: 453-459; de Felipe, P *et al.*, Gene Therapy, 1999; 6: 198-208; de Felipe, P *et al.*, Human Gene Therapy, 2000; 11: 1921-1931.; and Klump, H *et al.*,
10 Gene Therapy, 2001; 8: 811-817).

The precise nature of the regulatory sequences needed for gene expression in host cells may vary between species, tissues or cell types, but shall in general include, as necessary, 5' non-transcribed and 5' non-translated sequences involved with the initiation of transcription and translation respectively, such as a TATA box, capping sequence, CAAT
15 sequence, enhancer elements, and the like. Especially, such 5' non-transcribed regulatory sequences will include a promoter region that includes a promoter sequence for transcriptional control of the operably joined gene. Regulatory sequences may also include enhancer sequences or upstream activator sequences as desired. The vectors of the disclosure may optionally include 5' leader or signal sequences. The choice and design of an
20 appropriate vector is within the ability and discretion of one of ordinary skill in the art.

Examples of constitutive promoters include, without limitation, the retroviral Rous sarcoma virus (RSV) LTR promoter (optionally with the RSV enhancer), the cytomegalovirus (CMV) promoter (optionally with the CMV enhancer) [see, *e.g.*, Boshart et al, Cell, 41:521-530 (1985)], the SV40 promoter, the dihydrofolate reductase promoter, the β -
25 actin promoter, the phosphoglycerol kinase (PGK) promoter, and the EF1 α promoter [Invitrogen]. In some embodiments, a promoter is an enhanced chicken β -actin promoter.

Inducible promoters allow regulation of gene expression and can be regulated by exogenously supplied compounds, environmental factors such as temperature, or the presence of a specific physiological state, *e.g.*, acute phase, a particular differentiation state of the cell,
30 or in replicating cells only. Inducible promoters and inducible systems are available from a variety of commercial sources, including, without limitation, Invitrogen, Clontech and Ariad.

Many other systems have been described and can be readily selected by one of skill in the art. Examples of inducible promoters regulated by exogenously supplied promoters include the zinc-inducible sheep metallothionein (MT) promoter, the dexamethasone (Dex)-inducible mouse mammary tumor virus (MMTV) promoter, the T7 polymerase promoter system (WO 98/10088); the ecdysone insect promoter (No et al, Proc. Natl. Acad. Sci. USA, 93:3346-3351 (1996)), the tetracycline-repressible system (Gossen et al, Proc. Natl. Acad. Sci. USA, 89:5547-5551 (1992)), the tetracycline-inducible system (Gossen et al, Science, 268:1766-1769 (1995), see also Harvey et al, Curr. Opin. Chem. Biol., 2:512-518 (1998)), the RU486-inducible system (Wang et al, Nat. Biotech., 15:239-243 (1997) and Wang et al, Gene Ther., 4:432-441 (1997)) and the rapamycin-inducible system (Magari et al, J. Clin. Invest., 100:2865-2872 (1997)). Still other types of inducible promoters which may be useful in this context are those which are regulated by a specific physiological state, *e.g.*, temperature, acute phase, a particular differentiation state of the cell, or in replicating cells only.

In another embodiment, the native promoter for the transgene will be used. The native promoter may be preferred when it is desired that expression of the transgene should mimic the native expression. The native promoter may be used when expression of the transgene must be regulated temporally or developmentally, or in a tissue-specific manner, or in response to specific transcriptional stimuli. In a further embodiment, other native expression control elements, such as enhancer elements, polyadenylation sites or Kozak consensus sequences may also be used to mimic the native expression.

In some embodiments, the regulatory sequences impart tissue-specific gene expression capabilities. In some cases, the tissue-specific regulatory sequences bind tissue-specific transcription factors that induce transcription in a tissue specific manner. Such tissue-specific regulatory sequences (*e.g.*, promoters, enhancers, *etc.*) are well known in the art. Exemplary tissue-specific regulatory sequences include, but are not limited to the following tissue specific promoters: an eye-specific retinoschisin promoter or K12 promoter, a liver-specific thyroxin binding globulin (TBG) promoter, an insulin promoter, a glucagon promoter, a somatostatin promoter, a pancreatic polypeptide (PPY) promoter, a synapsin-1 (Syn) promoter, a creatine kinase (MCK) promoter, a mammalian desmin (DES) promoter, a α -myosin heavy chain (α -MHC) promoter, or a cardiac Troponin T (cTnT) promoter. Other exemplary promoters include Beta-actin promoter, hepatitis B virus core promoter, Sandig *et*

al., Gene Ther., 3:1002-9 (1996); alpha-fetoprotein (AFP) promoter, Arbuthnot *et al.*, Hum. Gene Ther., 7:1503-14 (1996)), bone osteocalcin promoter (Stein *et al.*, Mol. Biol. Rep., 24:185-96 (1997)); bone sialoprotein promoter (Chen *et al.*, J. Bone Miner. Res., 11:654-64 (1996)), CD2 promoter (Hansal *et al.*, J. Immunol., 161:1063-8 (1998); immunoglobulin heavy chain promoter; T cell receptor α -chain promoter, neuronal such as neuron-specific enolase (NSE) promoter (Andersen *et al.*, Cell. Mol. Neurobiol., 13:503-15 (1993)), neurofilament light-chain gene promoter (Piccioli *et al.*, Proc. Natl. Acad. Sci. USA, 88:5611-5 (1991)), and the neuron-specific vgf gene promoter (Piccioli *et al.*, Neuron, 15:373-84 (1995)), among others which will be apparent to the skilled artisan.

In some embodiments, one or more binding sites for one or more of miRNAs are incorporated in a transgene of a rAAV vector, to inhibit the expression of the transgene in one or more tissues of an subject harboring the transgene. The skilled artisan will appreciate that binding sites may be selected to control the expression of a transgene in a tissue specific manner. For example, binding sites for the liver-specific miR-122 may be incorporated into a transgene to inhibit expression of that transgene in the liver. The target sites in the mRNA may be in the 5' UTR, the 3' UTR or in the coding region. Typically, the target site is in the 3' UTR of the mRNA. Furthermore, the transgene may be designed such that multiple miRNAs regulate the mRNA by recognizing the same or multiple sites. The presence of multiple miRNA binding sites may result in the cooperative action of multiple RISCs and provide highly efficient inhibition of expression. The target site sequence may comprise a total of 5-100, 10-60, or more nucleotides. The target site sequence may comprise at least 5 nucleotides of the sequence of a target gene binding site.

Recombinant AAV Administration Methods

The rAAVs may be delivered to a subject in compositions according to any appropriate methods known in the art. The rAAV, preferably suspended in a physiologically compatible carrier (*i.e.*, in a composition), may be administered to a subject, *i.e.* host animal, such as a human, mouse, rat, cat, dog, sheep, rabbit, horse, cow, goat, pig, guinea pig, hamster, chicken, turkey, or a non-human primate (*e.g.*, Macaque). In some embodiments, a host animal does not include a human.

Delivery of the rAAVs to a mammalian subject may be by, for example, intraocular injection or topical administration (*e.g.*, eye drops). In some embodiments, the intraocular injection is intrastromal injection, subconjunctival injection, or intravitreal injection. In some embodiments, the injection is not topical injection. Combinations of administration methods (*e.g.*, topical administration and intrastromal injection) can also be used.

The compositions of the disclosure may comprise an rAAV alone, or in combination with one or more other viruses (*e.g.*, a second rAAV encoding having one or more different transgenes). In some embodiments, a composition comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more different rAAVs each having one or more different transgenes.

In some embodiments, a composition further comprises a pharmaceutically acceptable carrier. Suitable carriers may be readily selected by one of skill in the art in view of the indication for which the rAAV is directed. For example, one suitable carrier includes saline, which may be formulated with a variety of buffering solutions (*e.g.*, phosphate buffered saline). Other exemplary carriers include sterile saline, lactose, sucrose, calcium phosphate, gelatin, dextran, agar, pectin, peanut oil, sesame oil, and water. The selection of the carrier is not a limitation of the present disclosure.

Optionally, the compositions of the disclosure may contain, in addition to the rAAV and carrier(s), other pharmaceutical ingredients, such as preservatives, or chemical stabilizers. Suitable exemplary preservatives include chlorobutanol, potassium sorbate, sorbic acid, sulfur dioxide, propyl gallate, the parabens, ethyl vanillin, glycerin, phenol, and parachlorophenol. Suitable chemical stabilizers include gelatin and albumin.

The rAAVs are administered in sufficient amounts to transfect the cells of a desired tissue (*e.g.*, ocular tissue, such as corneal tissue) and to provide sufficient levels of gene transfer and expression without undue adverse effects. Examples of pharmaceutically acceptable routes of administration include, but are not limited to, direct delivery to the selected organ (*e.g.*, intrastromal delivery to the eye), oral, inhalation (including intranasal and intratracheal delivery), intraocular, intravenous, intramuscular, subcutaneous, intradermal, intratumoral, and other parental routes of administration. Routes of administration may be combined, if desired.

The dose of rAAV virions required to achieve a particular "therapeutic effect," *e.g.*, the units of dose in genome copies/per kilogram of body weight (GC/kg), will vary based on

several factors including, but not limited to: the route of rAAV virion administration, the level of gene or RNA expression required to achieve a therapeutic effect, the specific disease or disorder being treated, and the stability of the gene or RNA product. One of skill in the art can readily determine a rAAV virion dose range to treat a patient having a particular disease or disorder based on the aforementioned factors, as well as other factors.

An effective amount of an rAAV is an amount sufficient to target infect an animal, target a desired tissue. In some embodiments, an effective amount of an rAAV is an amount sufficient to produce a stable somatic transgenic animal model. The effective amount will depend primarily on factors such as the species, age, weight, health of the subject, and the tissue to be targeted, and may thus vary among animal and tissue. For example, an effective amount of the rAAV is generally in the range of from about 1 ml to about 100 ml of solution containing from about 10^9 to 10^{16} genome copies. In some cases, a dosage between about 10^{11} to 10^{13} rAAV genome copies is appropriate. In certain embodiments, 10^{10} or 10^{11} rAAV genome copies is effective to target ocular tissue (*e.g.*, corneal tissue). In some cases, stable transgenic animals are produced by multiple doses of an rAAV.

In some embodiments, a dose of rAAV is administered to a subject no more than once per calendar day (*e.g.*, a 24-hour period). In some embodiments, a dose of rAAV is administered to a subject no more than once per 2, 3, 4, 5, 6, or 7 calendar days. In some embodiments, a dose of rAAV is administered to a subject no more than once per calendar week (*e.g.*, 7 calendar days). In some embodiments, a dose of rAAV is administered to a subject no more than bi-weekly (*e.g.*, once in a two calendar week period). In some embodiments, a dose of rAAV is administered to a subject no more than once per calendar month (*e.g.*, once in 30 calendar days). In some embodiments, a dose of rAAV is administered to a subject no more than once per six calendar months. In some embodiments, a dose of rAAV is administered to a subject no more than once per calendar year (*e.g.*, 365 days or 366 days in a leap year).

In some embodiments, rAAV compositions are formulated to reduce aggregation of AAV particles in the composition, particularly where high rAAV concentrations are present (*e.g.*, $\sim 10^{13}$ GC/ml or more). Appropriate methods for reducing aggregation of may be used, including, for example, addition of surfactants, pH adjustment, salt concentration adjustment,

etc. (See, *e.g.*, Wright FR, *et al.*, Molecular Therapy (2005) 12, 171–178, the contents of which are incorporated herein by reference.)

Formulation of pharmaceutically-acceptable excipients and carrier solutions is well-known to those of skill in the art, as is the development of suitable dosing and treatment regimens for using the particular compositions described herein in a variety of treatment regimens. Typically, these formulations may contain at least about 0.1% of the active compound or more, although the percentage of the active ingredient(s) may, of course, be varied and may conveniently be between about 1 or 2% and about 70% or 80% or more of the weight or volume of the total formulation. Naturally, the amount of active compound in each therapeutically-useful composition may be prepared in such a way that a suitable dosage will be obtained in any given unit dose of the compound. Factors such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological considerations will be contemplated by one skilled in the art of preparing such pharmaceutical formulations, and as such, a variety of dosages and treatment regimens may be desirable.

In some embodiments, rAAVs in suitably formulated pharmaceutical compositions disclosed herein are delivered directly to target tissue, *e.g.*, direct to ocular tissue (*e.g.*, corneal tissue). However, in certain circumstances it may be desirable to separately or in addition deliver the rAAV-based therapeutic constructs via another route, *e.g.*, subcutaneously, intraopaneatically, intranasally, parenterally, intravenously, intramuscularly, intrathecally, or orally, intraperitoneally, or by inhalation. In some embodiments, the administration modalities as described in U.S. Pat. Nos. 5,543,158; 5,641,515 and 5,399,363 (each specifically incorporated herein by reference in its entirety) may be used to deliver rAAVs. In some embodiments, a preferred mode of administration is by intrastromal injection.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. Dispersions may also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms. In many cases the form is sterile and fluid to the extent that easy syringability exists. It must be stable

under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (*e.g.*, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and/or vegetable oils. Proper fluidity may be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

For administration of an injectable aqueous solution, for example, the solution may be suitably buffered, if necessary, and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, a suitable sterile aqueous medium may be employed. For example, one dosage may be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the host. The person responsible for administration will, in any event, determine the appropriate dose for the individual host.

Sterile injectable solutions are prepared by incorporating the active rAAV in the required amount in the appropriate solvent with various of the other ingredients enumerated herein, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The rAAV compositions disclosed herein may also be formulated in a neutral or salt form. Pharmaceutically-acceptable salts, include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug-release capsules, and the like.

As used herein, "carrier" includes any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Supplementary active ingredients can also be incorporated into the compositions. The phrase "pharmaceutically-acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a host.

Delivery vehicles such as liposomes, nanocapsules, microparticles, microspheres, lipid particles, vesicles, and the like, may be used for the introduction of the compositions of the present disclosure into suitable host cells. In particular, the rAAV vector delivered transgenes may be formulated for delivery either encapsulated in a lipid particle, a liposome, a vesicle, a nanosphere, or a nanoparticle or the like.

Such formulations may be preferred for the introduction of pharmaceutically acceptable formulations of the nucleic acids or the rAAV constructs disclosed herein. The formation and use of liposomes is generally known to those of skill in the art. Recently, liposomes were developed with improved serum stability and circulation half-times (U.S. Pat. No. 5,741,516). Further, various methods of liposome and liposome like preparations as potential drug carriers have been described (U.S. Pat. Nos. 5,567,434; 5,552,157; 5,565,213; 5,738,868 and 5,795,587).

Liposomes have been used successfully with a number of cell types that are normally resistant to transfection by other procedures. In addition, liposomes are free of the DNA length constraints that are typical of viral-based delivery systems. Liposomes have been used effectively to introduce genes, drugs, radiotherapeutic agents, viruses, transcription factors and allosteric effectors into a variety of cultured cell lines and animals. In addition, several successful clinical trials examining the effectiveness of liposome-mediated drug delivery have been completed.

Liposomes are formed from phospholipids that are dispersed in an aqueous medium and spontaneously form multilamellar concentric bilayer vesicles (also termed multilamellar vesicles (MLVs)). MLVs generally have diameters of from 25 nm to 4 μ m. Sonication of MLVs results in the formation of small unilamellar vesicles (SUVs) with diameters in the range of 200 to 500 $\text{\AA}$, containing an aqueous solution in the core.

Alternatively, nanocapsule formulations of the rAAV may be used. Nanocapsules can generally entrap substances in a stable and reproducible way. To avoid side effects due to intracellular polymeric overloading, such ultrafine particles (sized around 0.1 μ m) should be designed using polymers able to be degraded *in vivo*. Biodegradable polyalkyl-cyanoacrylate nanoparticles that meet these requirements are contemplated for use.

Kits and Related Compositions

The agents described herein may, in some embodiments, be assembled into pharmaceutical or diagnostic or research kits to facilitate their use in therapeutic, diagnostic or research applications. A kit may include one or more containers housing the components of the disclosure and instructions for use. Specifically, such kits may include one or more agents described herein, along with instructions describing the intended application and the proper use of these agents. In certain embodiments agents in a kit may be in a pharmaceutical formulation and dosage suitable for a particular application and for a method of administration of the agents. Kits for research purposes may contain the components in appropriate concentrations or quantities for running various experiments.

In some embodiments, the instant disclosure relates to a kit for producing a rAAV, the kit comprising a container housing an isolated nucleic acid encoding an AAV capsid protein selected from any one of SEQ ID NO: 7-16. In some embodiments, the kit further comprises

instructions for producing the rAAV. In some embodiments, the kit further comprises at least one container housing a recombinant AAV vector, wherein the recombinant AAV vector comprises a transgene (*e.g.*, a gene associated with ocular disease, such as corneal disease).

In some embodiments, the instant disclosure relates to a kit comprising a container
5 housing a recombinant AAV having an isolated AAV capsid protein having an amino acid sequence as set forth in SEQ ID NO: 14 or SEQ ID NO: 15.

The kit may be designed to facilitate use of the methods described herein by researchers and can take many forms. Each of the compositions of the kit, where applicable, may be provided in liquid form (*e.g.*, in solution), or in solid form, (*e.g.*, a dry powder). In
10 certain cases, some of the compositions may be constitutable or otherwise processable (*e.g.*, to an active form), for example, by the addition of a suitable solvent or other species (for example, water or a cell culture medium), which may or may not be provided with the kit. As used herein, "instructions" can define a component of instruction and/or promotion, and typically involve written instructions on or associated with packaging of the disclosure.

15 Instructions also can include any oral or electronic instructions provided in any manner such that a user will clearly recognize that the instructions are to be associated with the kit, for example, audiovisual (*e.g.*, videotape, DVD, *etc.*), Internet, and/or web-based communications, *etc.* The written instructions may be in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological
20 products, which instructions can also reflect approval by the agency of manufacture, use or sale for animal administration.

The kit may contain any one or more of the components described herein in one or more containers. As an example, in one embodiment, the kit may include instructions for mixing one or more components of the kit and/or isolating and mixing a sample and applying
25 to a subject. The kit may include a container housing agents described herein. The agents may be in the form of a liquid, gel or solid (powder). The agents may be prepared sterilely, packaged in syringe and shipped refrigerated. Alternatively it may be housed in a vial or other container for storage. A second container may have other agents prepared sterilely. Alternatively the kit may include the active agents premixed and shipped in a syringe, vial,
30 tube, or other container. The kit may have one or more or all of the components required to administer the agents to an animal, such as a syringe, topical application devices, or iv needle

tubing and bag, particularly in the case of the kits for producing specific somatic animal models.

The kit may have a variety of forms, such as a blister pouch, a shrink wrapped pouch, a vacuum sealable pouch, a sealable thermoformed tray, or a similar pouch or tray form, with the accessories loosely packed within the pouch, one or more tubes, containers, a box or a bag. The kit may be sterilized after the accessories are added, thereby allowing the individual accessories in the container to be otherwise unwrapped. The kits can be sterilized using any appropriate sterilization techniques, such as radiation sterilization, heat sterilization, or other sterilization methods known in the art. The kit may also include other components, depending on the specific application, for example, containers, cell media, salts, buffers, reagents, syringes, needles, a fabric, such as gauze, for applying or removing a disinfecting agent, disposable gloves, a support for the agents prior to administration *etc.*

The instructions included within the kit may involve methods for detecting a latent AAV in a cell. In addition, kits of the disclosure may include, instructions, a negative and/or positive control, containers, diluents and buffers for the sample, sample preparation tubes and a printed or electronic table of reference AAV sequence for sequence comparisons.

EXAMPLES

Example 1. Anti-angiogenic miRNA therapeutics for the prevention and/or treatment of corneal NV

In this example, anti-angiogenic miRNA therapeutics for the prevention and/or treatment of corneal NV are described. First, target miRNAs that play roles in corneal NV were identified. To date, there has been no report on miRNA expression profile of corneal NV. Using Nanostring technologies and the classic alkali-burn induced corneal NV mouse model, small RNAs prepared from corneal tissues harvested from of study mice were profiled for expression levels of 618 mouse miRNAs before and days 5, 10 and 15 after alkali injury (corneal NV in mouse model begins to regress naturally after 2 weeks post alkali burn). 35 up-regulated and 3 down-regulated miRNAs were identified in the mouse neovascularized corneas (FIG. 1A). The expression profiles of 19 miRNAs were further verified by qRT-PCR; examples of data generated are shown in FIG. 1B. Among them, the top 3 miRNAs (miR-21, miR-184 and miR-204) with over 10-fold expression change were selected as

therapeutic candidates. Then, pri/TuD miRNA constructs, which overexpress or inhibit the target miRNA expression, were cloned and verified (FIG. 1C).

rAAV vectors were tested for efficient delivery of candidate miRNA pri-miRNAs or the corresponding TuD RNAs to the corneas of alkali-burn induced corneal NV mice. The

5 potency of the pri-miRNA and TuD RNAs in preventing or treating corneal NV was evaluated. To this end, 14 serotypes of rAAV EGFP (AAV1, AAV2, AAV3b, AAV4, AAV5, AAV6, AAV6.2, AAV7, AAV8, AAV9, AAVrh.8, AAVrh.10, AAVrh.39, AAVrh.43) were assessed for gene transfer efficiency in mouse corneas after delivery by intrastromal injection in order to identify the most efficient AAV serotypes for miRNA
10 therapeutics. Among them, for the first time, rAAVrh.8, rh.10, rh.39 and rh.43 were found to be highly efficient in transgene delivery (FIG. 2A). Transduction efficiency of these four rAAV serotypes (*e.g.*, rh.8, rh.10, rh.39, and rh.43) was tested by topical administration with/without corneal epithelium removed, to further explore the feasibility of using rAAV to deliver transgenes into cornea by eye dropping. Data indicated that rAAVrh.10 and rh.39
15 showed better gene transfer efficiency through topical administration in the condition that the corneal epithelium was removed (FIG. 2B). The *in vivo* observation indicated that both rAAVrh.10 and rh.39 EGFP expression started from as early as 1 week after intrastromal injection or topical administration, reached the peak at around the 2nd week, and continued at least for 2 more weeks (FIG. 2C). Moreover, it was observed that rAAVrh.10 and rh.39
20 efficiently transduced keratocytes, which are the major cells in corneal stroma (FIG. 2D).

Based on these findings, rAAVrh.10 was selected to deliver candidate pri/TuD miRNAs into corneal stroma by intrastromal injection and topical administration to estimate their effects on corneal NV. Considering the time course of transgene expression delivered by rAAV vector and the feasibility of clinical application, intrastromal injection was used as
25 a prevention strategy, delivering the rAAV vectors into mouse corneal stroma 2 weeks before alkali burn (FIG. 3A). Meanwhile, conducting topical administration right after alkali burn was tested as a treatment for injury induced corneal NV (FIG. 4A). In both experiments, the corneal NV was observed and measured *in vivo* at days 3, 5, 7, 10 and 14 after alkali burn (FIG. 3C and FIG. 4C), and then the mice were sacrificed on day 15 post alkali burn with the
30 corneas enucleated for whole flat-mount immunofluorescence staining. The cell marker of vascular endothelial cells, CD31 was used to visualize the new blood vessels in corneas (FIG.

3B and FIG. 4B) and the percentage of neovascularized area was quantified using Imaris 8 software (FIG. 3D and FIG. 4D). Data suggested that all three constructs including TuD miR-21, pri miR-184 and pri miR-204, effectively inhibited corneal NV through intrastromal injection; thus serving as a prevention method (FIG. 3). However, none of them showed sustained inhibitory effect (*e.g.*, post day 10) on corneal NV when delivered by topical administration (FIG. 4).

In sum, this example demonstrates that miRNA-targeted therapeutics can be delivered as either rAAV or synthetic nucleic acid drugs (*e.g.*, miRNA mimics and antagomir) to offer an additional clinical option for preventing and treating corneal NV.

Example 2. Efficient Transduction of Corneal Stroma by Adeno-Associated Virus Serotype Vectors for Implications in Gene Therapy of Corneal Diseases

Materials and Methods

Primary antibody of rabbit anti-mouse keratocan was obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Primary antibody of rabbit anti-mouse GFP, and goat anti rabbit IgG (H+L) secondary antibody Alexa Fluor 488 conjugate, as well as Alexa Fluor 568 conjugate, were purchased from Life Technologies (Grand Island, NY, USA). VECTASHIELD® anti-fade mounting medium with 4', 6-diamidino-2-phenylindole (DAPI) was obtained from Vector Laboratories (Burlingame, CA, USA).

Six-to-eight-week old female C57BL/6J mice (Charles River Laboratories) were maintained and used according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the University of Massachusetts Medical School. Prior to experimental operation, all animals were anesthetized by an intraperitoneal injection of a ketamine-xylazine (100 mg/kg and 10 mg/kg, respectively) mixture. The right eyes of mice were treated as experimental eyes.

rAAV vector production

rAAV vectors were generated by triple plasmid transfection of HEK293 cells. The self-complimentary (sc) pAAV-CB-PI-EGFP plasmid was used for packaging with capsids from 14 different serotypes to produce rAAV1, rAAV2, rAAV3b, rAAV4, rAAV5, rAAV6, rAAV6.2, rAAV7, rAAV8, rAAV9, rAAVrh.8, rAAVrh.10, rAAVrh.39 and rAAVrh.43.

Viruses were purified with CsCl gradient ultracentrifugation and titered by both quantitative polymerase chain reaction (qPCR) and silver staining of SDS-Page.

rAAV transduction of mouse cornea by intra-stromal injection

5 Intra-stromal injection was performed according to the previously published procedure (FIG. 6A). Briefly, a 1.0 mm long incision was first created through the corneal epithelium using the tip of a 26-gauge needle, which was performed equidistant between the cornea-scleral junction and the corneal center. The tip of a 33-gauge needle attached to a 5 μ L Hamilton microliter syringe (Hamilton, Reno, NV, USA) was then introduced through the
10 incision into the corneal stroma and 2.4×10^{10} genomic copies (GC) of vector in 4 μ L of PBS were injected. The antibiotic ointment was applied after injection.

rAAV transduction of mouse cornea by topical administration

 rAAV transduction through topical administration was conducted with or without
15 corneal epithelium removed (FIG. 6B). The corneal epithelium scraping was performed accordingly. Alcohol soaked filter membrane was applied on each cornea for 20 seconds and the whole layer of epithelium covering about 80% corneal area was removed by gentle scraping with a #64 Beaver blade (Beaver Visitec, Waltham, MA, USA) under an operating microscope. 2.4×10^{10} GC of virus vectors in 4 μ L of PBS were directly applied to the intact
20 cornea or the corneal stroma after epithelium removal and allowed to sit for 2 minutes. After drying the cornea with antiseptic cotton swab, an antibiotic ointment was applied afterwards.

In vivo microscopy studies

 Animals of each group were observed *in vivo* at the 1st, 2nd, 3rd and 4th week after
25 rAAV administration. The image of EGFP expression in the mouse eye was captured utilizing the Micron III camera (Phoenix Research Labs, Pleasanton, CA, USA).

Histological and immunofluorescence-histochemical analysis

 Following sacrifice, mouse eyes were enucleated. Eight eyes from each group were
30 fixed in 4% paraformaldehyde. Among them, four corneas with limbus were harvested for corneal flat-mounts, which were blocked in 5% goat serum in PBS and stained with 1:1000

primary antibody of rabbit anti-mouse GFP, followed by 1:1500 secondary antibody of goat anti-rabbit IgG-Alexa Fluor 488. The corneal whole mounts were then mounted for observation and imaging analysis.

The remaining four eyeballs harvested from each group were embedded in O.C.T compound (Fisher Scientific, Pittsburgh, PA, USA) for cryosectioning at a thickness of 10 μm , then blocked in 5% goat serum and stained with 1:50 primary antibody of rabbit anti-mouse keratocan and 1:1500 secondary antibody of goat anti-rabbit IgG-Alexa Fluor 568. All immunofluorescence stained sections were mounted with VECTASHIELD® medium containing DAPI and fluorescence detection of native EGFP expression and stained keratocan in eyeball samples was generated using the Leica DM5500 microscope. The embedded samples were stored at -80°C .

Two eyes from each group were fixed in 10% formalin and embedded in paraffin to be sectioned later at 4- μm thickness and stained with Haematoxylin and Eosin (H&E) for histological analysis. Images were obtained by the Leica DMC2900 microscope.

Quantification analysis of EGFP expression in corneal whole mounts

Digital images of the corneal whole mounts were taken by Leica DM5500. EGFP positive area and fluorescence signal intensity was measured on these flat-mounts using ImageJ software. The total corneal area was outlined using the innermost vessels of the limbal arcade as the border. Total area containing EGFP expression was then normalized to the total corneal area, and the percentage of EGFP positive area was calculated. Mice treated with PBS were used as the negative control.

Quantification analysis of EGFP expression in cryosections

Four eyeballs from each group were fixed and cryosectioned for keratocan immunostaining. For each sample, images of five corneal slides were captured. The EGFP⁺ cells and keratocan⁺ cells on each image were counted separately, and the number of co-localized cells was then obtained using Imaris 8 software (Bitplane, Concord, MA, USA) to determine percentages of EGFP⁺keratocan⁺ cells among keratocan⁺ cells.

Quantification analysis of rAAV genome copy number and RNA expression

Corneas of three eyes in each group were harvested. Genomic DNA was isolated using QIAamp DNA kit (Qiagen, Hilden, Germany) following the manufacturer's instructions, and then digested with Mol Neurobiol SalI (NEB) at >10 U/ μ g of DNA under 37 °C for 1 hour. There are two SalI sites in the rAAV genome, and SalI digestion ensures single copies of EGFP transgene for droplet digital PCR (ddPCR) quantification. Multiplexed ddPCR was performed on a QX200 ddPCR system (Bio-Rad Laboratories, Hercules, CA, USA) using Taqman reagents targeting EGFP (Catalog # 4400293, Life Technologies, Carlsbad, CA, USA) and the reference gene transferrin receptor (Tfrc) (Catalog # 4458367, Invitrogen, Waltham, MA, USA). rAAV genome copy numbers per diploid genome were calculated as EGFP transgene copy numbers per two Tfrc gene copies. Liver DNA from mice with intravenous injection of rAAV9 EGFP vectors (1×10^{12} genomic copies) was used as a reference for a high vector genome copy number per cell.

Total RNA was extracted using the RNeasy 96 QIAcube HT kit with on-column DNase I digestion (Qiagen, Valencia, CA, USA), and then reverse transcribed into cDNA and subjected to multiplexed ddPCR using Taqman reagents targeting EGFP and Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) (Catalog # 4352339E, Life Technologies, Carlsbad, CA, USA). The quantity of EGFP mRNA was normalized to GAPDH mRNA and expressed as EGFP mRNA copy numbers per GAPDH mRNA copy.

Statistical analysis

Results were expressed as mean $\pm$ SD. Analysis was performed with one-way analysis of variance (ANOVA) for multiple variables and Bonferroni's post-hoc multiple-comparison test was used for between-group differences using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA), p values < 0.05 were considered significant in all cases.

Intra-stromal injections of rAAVrh.8, rAAVrh.10, rAAVrh.39 and rAAVrh.43 transduce the cornea efficiently

The transduction efficiency of fourteen different rAAV serotypes was investigated. Mice were injected intra-stromally with rAAV1, rAAV2, rAAV3b, rAAV4, rAAV5, rAAV6, rAAV6.2, rAAV7, rAAV8, rAAV9, rAAVrh.8, rAAVrh.10, rAAVrh.39, or rAAVrh.43 expressing EGFP at a dose of 2.4×10^{10} GCs per eye; PBS was injected as a

negative control into normal mouse corneas (FIG. 6A). Compared to the PBS control, robust EGFP expression was observed in corneas at the fourth week after injection with rAAVrh.8, rAAVrh.10, rAAVrh.39 and rAAVrh.43 (FIG. 7A); the percentage of EGFP positive area and EGFP intensity in the corneal whole mounts of those four groups are presented in FIGs. 7B and 7C. Nearly 80% area of the whole corneas were efficiently transduced by rAAVrh.8, rh.10 and rh.39 vectors (FIG. 7B), while the EGFP intensity in the four rhesus serotype groups of rAAVrh.8, rh.10, rh.39 and rh.43 were approximately 4-fold stronger over those of other rAAV serotypes tested (FIG. 7C). These results demonstrated that rAAVrh.8, rAAVrh.10, rAAVrh.39 and rAAVrh.43 could transduce mouse cornea in a highly efficient manner.

Topical administrations of rAAVrh.10 and rAAVrh.39 transduce the cornea with corneal epithelium removed

To explore the feasibility of delivering certain rAAV serotypes to mouse cornea by an easy and noninvasive method, EGFP transduction of rAAVrh.8, rAAVrh.10, rAAVrh.39 and rAAVrh.43 was evaluated after topical administration to the corneas (FIG. 6B). Since the corneal epithelium is known to be a natural barrier for topical therapeutics to the corneal stroma, topical application of the four leading rAAV vectors were performed either with, or without, corneal epithelium scraping in comparison with intra-stromal injections of the same vectors at the same dose (2.4×10^{10} GCs per eye). Corneal whole mount imaging showed that all four serotypes were unable to transduce mouse cornea when the epithelium was present; however, rAAVrh.10 and rAAVrh.39 efficiently transduced corneal stroma when the epithelium was removed (FIG. 8A). In comparison to intra-stromal injections, topical administration of these two serotypes after epithelium removal produced similar percentages of EGFP positive area (FIG. 8B), but exhibited significantly lower EGFP intensity ($p < 0.001$) (FIG. 8C).

rAAVrh.10 and rAAVrh.39 achieve sustained corneal transduction

Following topical administration (eye drop with corneal epithelium removed) or intra-stromal injection, EGFP expression from rAAVrh.10 and rAAVrh.39 was apparent at 1-week post-treatment, reached peak expression around 2-weeks, and remained detectable at the 4-

week study endpoint (FIG. 9A) when mouse corneas were harvested for ddPCR quantification of vector genome copies and EGFP mRNA levels. Data suggested that on average, more than 5,000 vector genomes per cell persisted at 4-weeks post-treatment for intra-stromally injected rAAVrh.10 and rh.39 serotypes and approximately 200 vector genome copies per cell detected for the topically administered two serotype vectors (FIG. 9B). The vector genome abundance in corneas that received rAAVs intra-stromally is 20- to 30-folds higher than that in mouse liver treated with an intravenous injection of rAAV9.EGFP at approximately 40-folds higher the dosage of rAAVrh.10 and rh.39 (FIG. 9B). This suggests that, at per genome basis, intra-stromal delivery of rAAVrh.10 and rAAVrh.39 to the cornea is much more efficient (800-to 1200-folds) than systemic delivery of a more concentrated highly liver tropic rAAV9 vector. In addition, EGFP mRNA expression levels in the rAAV treated corneas were well correlated with the abundance of vector genomes (FIG. 9C).

rAAVrh.10 and rAAVrh.39 primarily target keratocytes in corneal stroma

As keratocytes with characteristic interconnecting dendritic processes comprise 96% of the cornea in mice and humans, keratocytic tropism of rAAVrh.10 and rAAVrh.39 was characterized by using keratocan as a cell marker for keratocytes (FIG. 10A). Quantitative analysis of EGFP⁺/keratocan⁺ cells in the corneal stroma revealed that rAAVrh.10 and rAAVrh.39 transduced 51.1±3.0% and 55.97±3.5% of keratocytes respectively by intra-stromal injections, and 35.9±8.1% and 33.64±7.7% of keratocytes respectively by eye-drop applications to the epithelium-removed corneas (FIG. 10B). In other words, intra-stromal injection of rAAVrh.10 and rh.39 transduced 1.5- to 2-folds more keratocytes when compared to topical administration (FIG. 10B), which was aligned with results from the ddPCR quantification of EGFP mRNAs (FIG. 9C).

Corneal transduction by rAAVrh.10 and rAAVrh.39 vectors causes no histopathology

To evaluate possible vector-related toxicity caused by rAAV transduction in the corneal stroma, the histopathology of cornea tissues treated with rAAVrh.10 and rAAVrh.39 vectors at 4-weeks post-treatment was analyzed. Histological images of Haematoxylin and Eosin (H&E) stained tissue sections of corneas treated with rAAVrh.10 and rAAVrh.39 via

either topical eye-drops or intra-stromal injections presented structures and morphologies similar to those that received PBS or no treatment; all corneal stromas remained organized into interweaving collagen lamellae with an even distribution of keratocytes (FIG. 11). These findings suggest that rAAV transduction of the corneas induced no adverse effects on corneal stromal morphology throughout the course of 4-weeks, implicating that rAAV did not deter overall health of the cornea tissue.

Example 3. rAAV delivered microRNA therapeutics towards efficacious treatment of corneal neovascularization

Candidate miRNA selection

FIG. 12 and Table 1 below show candidate miRNA selection and miRNA profiling of alkali-burn induced mouse corneal NV. Small RNAs prepared from corneal tissues harvested from study mice were profiled for expression levels of 618 mouse miRNAs before and days 5, 10 and 15 after alkali injury.

FIG. 13 shows candidate miRNA selection and qRT-PCR confirmation. miR-184 and miR-204 are significantly down-regulated post-alkali burn.

Table 1.

GO Term	Nr. Genes	% Associated Genes	Term Pvalue Corrected with Bonferroni step down
MAPK cascade	449	63.328632	4.07E-10
stress-activated MAPK cascade	154	70.31963	1.58E-06
regulation of MAPK cascade	408	62.76923	3.20E-08
positive regulation of MAPK cascade	280	62.92135	4.03E-05
JNK cascade	126	71.18644	1.61E-05
activation of MAPK activity	77	75.4902	2.35E-04
regulation of MAP kinase activity	166	66.666664	1.59E-04
positive regulation of MAP kinase activity	123	70.68965	4.74E-05
regulation of JNK cascade	112	70.440254	2.66E-04
regulation of epithelial cell proliferation	196	63.843647	0.001112793

regulation of cell growth	238	66.111115	6.55E-07
regulation of cell differentiation	1005	62.61682	2.09E-23
cell development	1332	63.33809	5.20E-36
cell morphogenesis	830	68.31276	1.04E-37
eye development	249	64.010284	2.60E-05
eye morphogenesis	111	66.467064	0.025034129
Wnt signaling pathway	257	67.81003	1.99E-09
regulation of Wnt signaling pathway	173	70.04049	2.43E-07
positive regulation of Wnt signaling pathway	73	70.19231	0.044343167
negative regulation of Wnt signaling pathway	99	71.73913	3.33E-04
canonical Wnt signaling pathway	165	69.32773	2.25E-06
negative regulation of canonical Wnt signaling pathway	79	75.2381	2.08E-04
regulation of canonical Wnt signaling pathway	130	72.6257	1.11E-06
vasculature development	421	65.37267	1.40E-12
blood vessel development	403	65	2.25E-11
blood vessel morphogenesis	337	64.80769	7.68E-09
angiogenesis	279	64.73318	6.42E-07
blood circulation	257	61.778847	0.001406428
heart development	370	64.45993	1.58E-09
cardiovascular system development	645	63.988094	4.14E-17
response to transforming growth factor beta	132	65.67164	0.010692643

rAAV serotype screening in mouse cornea

FIG. 14 shows rAAV serotype screening in mouse cornea. Intra-stromal injection of
5 rAAVrh.8, rAAVrh.10, rAAVrg.39 and rAAVrh.43 results in efficient transduction and
EGFP expression.

rAAV.rh10 delivered EGFP expression after alkali burn

FIG. 15 shows rAAV.rh10 delivered EGFP expression after alkali burn; rAAVrh.10
10 was delivered via intrastromal injection. FIG. 16 graphically depicts rAAV.rh10 delivered
EGFP expression administered via intrastromal injection after alkali burn, as measured by
ddPCR. FIG. 17 shows rAAV.rh10 delivered EGFP expression after alkali burn and

subconjunctival injection. FIG. 18 graphically depicts rAAV.rh10 delivered EGFP expression after alkali burn and subconjunctival injection (ddPCR).

rAAV.rh10 delivered pri miR-184 & pri miR-204 inhibit corneal neovascularization (NV)

FIGs. 19A-19D show rAAV.rh10 delivered pri miR-184 and pri miR-204 inhibit corneal neovascularization (NV) as prevention through intrastromal injection (IS). FIGs. 20A-20D show rAAV.rh10 delivered pri miR-184 & pri miR-204 inhibit corneal NV as treatment through subconjunctival injection (SC).

Overexpression of miR-184 inhibit Fzd4 expression

FIGs. 21-22 show overexpression of miR-184 inhibit Fzd4 expression (Wnt signaling). The diagram of the Wnt signaling pathway depicted on the right side of FIG. 21 is adapted from Shen *et al.*, Mol Ther. 2008.

Overexpression of miR-204 inhibit Angpt-1 expression

FIGs. 23-24 show overexpression of miR-204 inhibit Angpt-1 expression (*e.g.*, Tie2-P13K-Akt pathway). The diagram of the miR-204 signaling pathway on the right side of FIG. 23 is adapted from Kather *et al.*, Invest Ophthalmol Vis Sci; 2004.

rAAVrh.10 delivered pri miR-184 & pri mir-204 did not induce obvious abnormality in normal mouse eyes

FIGs. 25A-25B show rAAVrh.10 delivered pri miR-184 & pri mir-204 do not induce obvious abnormality in normal mouse eyes.

Example 4. Ocular delivery of miR-204 by rAAV

Materials and Methods

Mouse corneal neovascularization induced by alkali burn

Alkali-burn treatments were conducted following previously published methods. Only the right eyes of mice were treated. Filter-paper discs (3-mm diameter) were pre-soaked in 1 M NaOH for 15 seconds, and applied to eyes in experimental groups for 20 seconds. The ocular surface was then washed with 15 mL of normal saline for 1 minute.

Mouse corneas of anesthetized animals were imaged and acquired with a Micron III camera

(PhoenixResearch Labs, Pleasanton, CA, USA). The area of corneal NV was calculated by using the following formula: $\text{Area (mm}^2\text{)} = \text{CN}/12 \times 3.1416 \times [\text{R}^2 - (\text{R} - \text{VL})^2]$, where CN is the clock hours of NV (1 clock hour equals 30 degrees of arc); R is the radius of the cornea; and VL is the maximal vessel length, extending from the limbal vasculature. Measurements of
5 corneas in live animals were performed five times each under a Micron III microscope and the area of corneal NV was calculated accordingly.

Nanostring nCounter miRNA assay for miRNA profiling

A total of 100 ng of RNA was extracted from whole mouse corneas. Four corneas
10 were pooled into one sample and profiled for miRNA expression using the nCounter miRNA Expression Assay Kit (NanoString Technologies, Inc., Seattle, WA, USA). The assay was performed according to the manufacturer's instructions, querying 578 mouse miRNA targets, 33 mouse-associated viral miRNA targets, and 6 negative control targets. The mean
expression values of each miRNA were calculated by normalizing across our cohort to filter
15 for expressed miRNAs. The 6 internal negative control probes served as the background threshold cutoff-point (set to 1.0).

RNA-seq

Mouse corneas representing three treatment groups: non-treated (day 0), post-
20 operative(day 5), and regression post-operative (day 15) were treated as above. Total RNA was extracted from tissues and processed for RNA-seq library preparation and high-throughput sequencing on a HiSeq2500 platform. Four corneas were pooled to represent a single sample library, and two libraries represent each treatment condition. Each biological condition therefore reflects eight individual mouse corneas. This strategy was employed to
25 compensate for the low abundance of total RNA in an individual mouse cornea, and to limit the number of animals used for each condition. Primary bioinformatics analysis (Tophat/Cufflinks workflows, differential expression, and ontology enrichment analysis) was performed. Predicted miRNA target genes were selected from differentially expressed genes and analyzed with the CummeRbund (v2.12.1) software package.

Hierarchical Cluster Analysis

Hierarchical clustering was performed with average linkage using Cluster 3.0 (Eisen Lab, University of California at Berkeley, CA, USA). The clustered heat map was visualized using the interactive graphical software, TreeView. A limma algorithm was applied to filter the differentially expressed miRNAs from different experimental groups. After performing
 5 significance ($P < 0.05$) and false discovery rate analysis ($FDR < 0.05$), differentially expressed miRNAs with a ± 2 -fold change cut-off were selected. Selected miRNAs were ranked by fold-change.

miRNA target gene prediction and gene ontology/pathway analysis

10 Differentially expressed miRNAs identified by nCounter Analysis were subjected to target gene prediction analysis using TargetScan and miRTarbase definitions. Gene ontology network maps and term enrichment analyses were performed using Cytoscape_v3.3.0 plug-in tools and ClueGO v2.2.332 with terms defined by GO_BiologicalProcess-GOA_07.12.2015 and KEGG pathways. Significance was defined by a Kappa score threshold of 0.4, with p-
 15 value cutoffs of 0.05 for pathway reporting. Genes and miRNAs enriching for terms related to vasculogenesis, JAK/STAT signaling, Ephrin signaling, eye development, epithelial cell homeostasis, BMP signaling, wound healing, and cell growth were reported.

rAAV vector production

20 pri-miR-184 and pri-miR-204 were amplified using the PrimeSTAR Max DNA Polymerase kit (Takara, Japan) with the following primers:

pri-miR-184: sense 5'-CCGGAATTCTGTGCAGAAACATAAGTGACTCTCCAGGTG-3' (SEQ ID NO: 30)

25 antisense 5'-ATCGGCGGCCGCGCAGAGAGCACATTTTGAATAAGCAAAGTG-3' (SEQ ID NO: 31)

pri-miR-204: sense 5'-CCGGAATTCTTTACCCACAGGACAGGGTGATGGAGAGGA-3' (SEQ ID NO: 32)

30 antisense 5'-ATCGGCGGCCGCGTCACATGGTTTGGACCCAGAACTATTAGT-3' (SEQ ID NO: 33)

PCR products were sub-cloned into the self-complementary (sc) pAAV-CB-PI-GaussiaLuc plasmid by conventional means using NotI and EcoRI restriction sites. The sc-pAAV-CB-PIEGFP plasmid and sc-pAAV-CB-PI-pri-miR184/204-GaussiaLuc plasmids were packaged with rAAVrh.10 capsids by triple plasmid transfection of HEK293 cells. Viruses were purified with CsCl gradient ultracentrifugation and titered by both quantitative polymerase chain reaction (qPCR) and silver staining of SDS-PAGE gels.

rAAV transduction by intrastromal or subconjunctival injection

Intrastromal injections were performed. Briefly, a 1.0 mm long incision using the tip of a 26-gauge needle was first created through the corneal epithelium, equidistant between the cornea-scleral junction and the corneal center. Then, 3.6×10^{10} genomic copies (GC) of vector in 4 μ L of PBS were injected through the incision into the corneal stroma using a 33-gauge needle and a 5 μ L Hamilton syringe (Hamilton, Reno, NV, USA). Subconjunctival injections were performed using a 5 μ L Hamilton syringe to deliver 3.6×10^{10} GC of vector in 4 μ L of PBS. Antibiotic ointment was applied after injections.

Quantitative real-time PCR (qRT-PCR) for microRNA and mRNA expression analyses

RNA extraction and qRT-PCR for miRNA (TaqMan miRNA assay, Life Technologies, Carlsbad, CA, USA: miR-184, miR-204) and mRNAs were performed. Primer sequences for *fzd4*, *vegfa*, and *angpt1* are reported in Table 2. *U6* and β -actin were used as normalization transcripts for miRNAs and mRNAs, respectively.

Table 2

Primer Name	Primer Sequence	SEQ ID NO:
Fzd4 Fwd	TGCCAGAACCTCGGCTACA	34
Fzd4 Rev	ATGAGCGGCGTGAAAGTTGT	35
Vegf-a Fwd	GCCAGCACATAGAGAGAATGAGC	36
Vegf-a Rev	CAAGGCTCACAGTGATTTTCTGG	37
Angpt1 Fwd	CACATAGGGTGCAGCAACCA	38
Angpt1 Rev	CGTCGTGTTCTGGAAGAATGA	39

Droplet digital PCR for rAAV genome copy number and RNA expression analyses

Mouse cornea genomic DNA was isolated using the QIAamp DNA kit (Qiagen, Hilden, Germany), and then digested with >10 U/μg SalI (New England Biolabs, Ipswich, MA, USA) at 37°C for 1 hour. There are two SalI sites in the rAAV genome, and SalI digestion ensures single copy emulsion for droplet digital PCR (ddPCR) quantification.

5 Multiplexed ddPCR was performed on a QX200 ddPCR system (Bio-Rad Laboratories, Hercules, CA, USA) using Taqman reagents targeting EGFP (Catalog # 4400293, Life Technologies) and the reference gene, transferrin receptor (Tfrc) (Catalog # 4458367, Invitrogen, Waltham, MA, USA). rAAV genome copy numbers per diploid genome were calculated as *EGFP* transgene copy numbers per two *Tfrc* gene copies.

10 Total RNA was extracted using the RNeasy 96 QIAcube HT kit with on-column DNase I digestion (Qiagen, Valencia, CA, USA), reverse-transcribed into cDNA, and subjected to multiplexed ddPCR using TaqMan reagents targeting *EGFP* and Glyceraldehyde-3-Phosphate Dehydrogenase (*gapdh*) (Catalog # 4352339E, Life Technologies). The quantity of *EGFP* was normalized to *gapdh* levels.

15 Western blot

Total protein from corneas was extracted on ice with RIPA lysis buffer in the presence of freshly added protease and phosphatase inhibitors (Thermo Fisher Scientific, Waltham, MA, USA). A total of 10 μg / lane protein extract was loaded onto a 4-
20 20% gradient SDS-polyacrylamide gel and transferred to nitrocellulose membranes (Bio-Rad Laboratories). Nonspecific binding was blocked with 5% nonfat milk or 5% BSA in TBST as recommended for each antibody. The membrane was incubated with rabbit anti-
VEGF(ab46154, Abcam, Cambridge, MA, USA), anti-Angpt1 (ab95230, Abcam), anti-Tie2 (Cat.7403, Cell Signaling, Danvers, MA, USA), anti-phospho-Tie2 (AF2720-SP, R&D
25 Systems, Minneapolis, MN, USA), anti-PI3K (p85) (Cat. 4292, Cell Signaling), anti-phospho-PI3K(p85) (Cat. 4228, Cell Signaling), anti-Akt (ab8805, Abcam), anti-phospho-Akt1 (ab81283, Abcam), anti-Fzd4 (ab83042, Abcam), anti-LRP6 (Cat. 3395S, Cell Signaling), antiphospho-LRP6 (Cat. 2568S, Cell Signaling), anti-N-p-β-catenin (Cat. 4270, Cell Signaling), or anti-β-catenin (Cat. 8480S, Cell Signaling) antibodies overnight at 4°C.
30 IRDye 800CW goat anti-rabbit IgG (Cat. 926-32211, LI-COR, Lincoln, NE, USA) was used

as the secondary antibody, and mouse anti-GAPDH antibody (ab8245, Abcam) was used as an internal standard.

Histological and immunofluorescence-histochemical analyses

5 For rAAV transduction efficiency analysis, mouse eyes were enucleated and fixed in 4% paraformaldehyde. Corneas with limbi were then harvested for corneal flat-mounts, and blocked in 5% goat serum in PBS. For detecting EGFP expression in normal mouse corneas, flat-mounts were stained with rabbit anti-mouse GFP primary antibody (1:1000; Life Technologies), followed by goat anti-rabbit IgG-Alexa Fluor 488 secondary antibody
10 (1:1500; Life Technologies). For corneas treated by alkali burn, flat-mounts were stained with rat anti-mouse CD31 (1:500; Abcam) and rabbit anti-mouse keratocan (1:50; SantaCruz Biotechnology, Dallas, TX, USA) primary antibodies, followed by goat anti-rat IgG-Alexa Fluor 568 and goat anti-rabbit IgG-Alexa Fluor 694 secondary antibodies (1:1500; Life Technologies). Corneal whole mounts were set with VECTASHIELD anti-fade
15 mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA) for observation and imaging analysis. For corneal NV detection after alkali-burn treatment, flat-mounts were stained with the rat anti-mouse CD31 primary antibody (1:500; Abcam), followed by goat anti-rat IgG-Alexa Fluor 568 secondary antibody (1:1500).

To evaluate the safety of pri-miRNA vectors, mouse eyes from each group were fixed
20 in 10% formalin, embedded in paraffin, sectioned at a thickness of 4 μ m, and stained with Haematoxylin and Eosin (H&E) for histological analysis. Images were obtained using a Leica DMC2900 microscope (Leica Microsystems, Buffalo Grove, IL, USA).

miR-204 is significantly down-regulated in neovascularized mouse corneas

25 The expression of miRNAs in alkali-burn induced neovascularized mouse corneas was profiled by nCounter analysis. It was observed that among differentially expressed miRNAs, miR-204 is reduced more than 10-fold in response to alkali-burn injury. Whole-transcriptome analyses by RNA-seq and miRNA target prediction identified more than 200 corneal genes that are up-regulated in response to alkali-burn treatment and are predicted to
30 be regulated by miR-204. Data indicate that overexpression of pri-miR-204 in injured corneas inhibited vascularization into the cornea.

Candidate therapeutic miRNAs that may function to inhibitor reverse corneal neovascularization (NV) when overexpressed were identified. First, neovascularized mouse corneas induced by alkali-burn treatment were characterized. Vascularization into the cornea was observed for 15 days following injury (Figure 1A). Notably, corneal NV was observed to originate in the limbus by day 5, and fully expanded into the cornea by days 10 and 15. Untreated corneas and corneas following 5-, 10-, and 15-days after alkali-burn treatments were subjected to miRNA profiling using Nanostring nCounter analysis. 36 highly up-regulated and 3 strongly down-regulated miRNAs were observed in alkali-burn treated corneas (corneal NV miRNAs) compared to non-treated controls (Figure 1A).

The range of angiogenesis-related genes that might be directly regulated by corneal NV miRNAs were investigated. Unbiased miRNA target prediction analysis was performed to identify genes with high likelihoods of being targeted by our panel of corneal NV miRNAs. TargetScan and miRTarbase target prediction yielded a list of 5,520 target genes. In this example, miR-204 was selected as the candidate therapeutic miRNA based on the observation that miR-204 exhibits >10-fold expression reduction in neovascularized corneas. Additionally, miR204 is conserved across several vertebrate species, making it an ideal candidate for translation into humans. The effect of miR-184 mimics on corneal NV was also observed. Differential miR-184 and miR-204 expression was verified by qRT-PCR (Figure 13), confirming nCounter analysis results.

Differential expression of miR-204 target genes in alkali-burn treated corneas indicates that multiple pathways promote corneal angiogenesis.

The extent that miR-204 may impact corneal angiogenesis was investigated. Specifically, whether miR-204 displays characteristics of a potent therapeutic miRNA for corneal NV by targeting multiple genes involved in angiogenesis, wound healing, and related signaling pathways was assessed. Targetscan and miRTarbase analysis indicate that miR-204 is targets 1,729 genes. Whole-transcriptome analysis of alkali-burn treated corneas by RNA-seq analysis was performed. Untreated corneas (day 0), and corneas 5-days and 15-days post-treatment (day 5 and day 15, respectively) were analyzed. Dendrograms reflecting the expression profile relationships across sample libraries indicate that day 5 and day 15 libraries share a higher degree of similarity than the day 0 corneas (FIG. 26A). The fold-

change differences of the predicted target genes of all 39 corneal NV miRs were calculated. Volcano plots of differential miR target gene expression between day 0 and day 5 corneas, and day 0 and day 15 corneas (FIG. 26B) show an abundance of differentially expressed miR target genes, while comparison between day 5 and day 15 exhibit fewer differentially expressed miR target genes. This observation indicates that the majority of gene expression changes due to injury occur within the first five days of treatment. Heatmap analysis (FIG. 26C) further illustrates the range of miR target genes that are differentially expressed as a result of alkali-burn treatment.

Importantly, it was observed that among the 1,729 miR-204 gene targets, 1,232 are expressed in the cornea (FIG. 27A-27B). A set of genes that are up-regulated upon alkali-burn treatment was investigated. To this end, k-means clustering was performed to identify 208 genes that are exclusively up-regulated in corneas after 5 and 15 days post-alkali-burn treatment (FIG. 27B). These 208 miR-204 target genes were subjected to gene ontology (GO)-term enrichment analysis. By selecting on KEGG pathway and ontological terms closely related to angiogenesis and wound healing, several up-regulated miR-204 predicted targets were identified that demonstrate miR-204 as a potent anti-angiogenic effector (FIG. 27C). Specifically, the vasculogenesis-related genes: Hey2, Gjc1, Angpt1, Has2, and Amot were identified (FIG. 28). It was observed that Angpt1, Has2, and Hey2 also enrich for epithelial cell- and keratinocyte-related ontology terms, indicating that miR-204 directly regulates key genes with diverse roles in angiogenesis and cell proliferation in the cornea.

Both intrastromal and subconjunctival delivery of rAAVrh.10 efficiently transduces normal and alkali-burn treated corneas

Delivery of therapeutic miRs into corneal tissues was investigated. It was observed that rAAVrh.10 exhibits the highest transduction efficiency in the corneal stroma by intrastromal injection. Two different routes of administration, intrastromal and subconjunctival, were investigated. Data for rAAVrh.10 transduction efficiency in normal mouse corneas by either intrastromal or subconjunctival injections indicate that both injection methods to deliver rAAVrh.10 EGFP vectors efficiently transduce the entire cornea (FIG.

14).

The effects of alkali-burn on the efficacy of corneal transduction by rAAVrh.10 vectors (schematized in FIG. 15 and FIG. 17) were investigated. Intrastromal injections were performed two weeks prior to alkali-burn induction. Subconjunctival injections were performed directly following alkali-burn. Whole flat-mount immunofluorescence analyses of eyes harvested one or two weeks post-alkali-burn show that EGFP transgene expression in non-alkali-burn treated corneas (control group) is strongly detected at weeks 1 and 2 with little change in expression for both intrastromal and subconjunctival vector injections (FIG. 15 to FIG. 18). Alkali-burn treated corneas showed robust EGFP expression at week 1 following alkali-burn treatment, but exhibited an extreme loss of EGFP expression at week 2 (FIG. 15 and FIG. 17). EGFP was mainly expressed in keratocytes and not in vascular endothelial cells.

The abundance of transduced rAAV genomes and EGFP mRNA expression following alkali-burn treatment was investigated. Quantitative analysis of vector genomes delivered by either intrastromal or subconjunctival injection indicated that rAAV genome copies in alkali-burn treated corneas were significantly lower than control corneas (FIG. 16 and FIG. 18). It was also observed that intrastromal delivery was more potent for transgene expression than subconjunctival delivery. In some embodiments, the relative loss between weeks 1 and 2 was ~50% in both delivery routes. Quantification of EGFP mRNA expression showed that there was no significant difference between alkali-burn and control groups at post-alkali-burn week 1; however, EGFP expression in alkali-burn treated corneas after 2 weeks were significantly lower than control corneas (FIG. 16). Data indicate that, in some embodiments, alkali-burn severely compromises the expression of rAAVrh.10 delivered transgenes following a two-week time course. In some embodiments, differences in transgene expression between normal corneas and treated corneas are negligible for at least one week after alkali burn following only a single treatment.

rAAVrh.10-mediated miR-204 and miR-184 overexpression by subconjunctival injection inhibits corneal NV

A rAAVrh.10 vector that drives the expression of pri-miR-204 was produced. The efficacy of pri-miR-184 when delivered by rAAVrh.10 was also investigated.

To evaluate the anti-angiogenic effects of pri-miR-204 or pri-miR-184 on corneal NV, miRNA vectors were delivered through subconjunctival injection immediately following alkali burn (FIG. 20A). *In vivo* tracking of corneal NV progression indicated an inhibition of NV areas after 7 days of mock (vector backbone), pri-miR-204, and pri-miR-184 treatments (FIG. 20C). The NV area in the mock treatment group recovered to those of PBS treatments by day 10, while pri-miR-204 and pri-miR-184 treatments resulted in significantly less NV. Differences in corneal NV after 15 days of treatment with pri-miR-204 or pri-miR-184 vectors as assessed by immunofluorescence analyses in flat-mounts were more definitive (FIG. 20B and FIG. 20D). Corneas immune-stained with anti-CD31 demonstrated that new blood vessels grew robustly in PBS and mock control groups, while corneas treated with pri-miR-204 and pri-miR-184 vectors effectively inhibited corneal NV.

rAAVrh.10-mediated miR-204 and miR-184 overexpression by intrastromal injection can inhibit corneal NV

Mice were injected with pri-miR-204 vectors intrastromally, two weeks before alkali-burn treatment. Corneal NV was again observed for two weeks following treatment (FIG. 19A). Similar to subconjunctival injections that immediately followed alkali-burn treatment, a lag in NV in mock and pri-miR injections was observed. By day 10, the mock controls and PBS controls exhibited similar degrees of NV areas (FIG. 19C). Strikingly, NV area did not significantly increase between days 10 and 14 indicating that neovascularization can be halted by the exogenous expression of miR-204. Similar efficacies were also observed with pri-miR-184 treatments (FIG. 19C). Immunofluorescence and quantitative analysis of NV areas indicated that both miRNAs could effectively inhibit corneal NV, leading to a 20% reduction of NV area as compared to PBS and mock groups (FIG. 19B and FIG. 19D).

Delivery of the anti-angiogenic miR-204 transgene targets the Angpt1/Tie2/PI3K/Akt pathway

Whether blockage of corneal NV by rAAV delivery of pri-miR-204 directly impinges on neovascularization in the cornea was investigated. Angpt1 was selected as a marker for vasculogenesis, since it was identified as a predicted target for miR-204 and is significantly up-regulated upon alkali-burn treatment (FIG. 27C and FIG. 28). In some embodiments,

Angpt1 has play a role in angiogenesis and wound healing in the cornea via activation of the PI3K/AKT signaling pathway. Alkali-burn treated mice were injected with rAAVrh.10 pri-miR-204 vectors either intrastromally or subconjunctivally.

Analysis of miR-204 expression in NV corneas indicated that miR-204 expression was significantly up-regulated in the pri-miR-204 treatment group compared to the PBS treatment group in both intrastromal and subconjunctival delivery routes (FIG. 23). The levels of exogenous miR-204 after vector injection and alkali-burn were still below those of normal control levels. Nevertheless, data indicate that *angpt1* and *vegf* (a downstream target gene of PI3K/Akt pathway) messages were significantly down-regulated with rAAVrh.10-pri-miR-204 treatments compared to the PBS group (FIG. 24). Western blot analysis confirms that ANGPT-1 and VEGF are significantly reduced by exogenous expression of miR-204, while TIE2 (receptor for ANGPT1), PI3K, and AKT demonstrated a loss of phosphorylation without significant reduction in protein expression (FIG. 24). It was observed that delivery of the pri-miR-184 transgene via rAAV is able to perturb Fzd4/Wnt/ β -catenin signaling in mouse corneas, and in turn, blocks corneal NV (FIG. 22). This finding is significant, as it provides *in vivo* support for miR-184's ability to down-regulate fzd-4, β -catenin, and vegf expression in the cornea, whereas previous evidence by others were resolved in human umbilical vein endothelial cells (HUVECs) and in transformed human corneal epithelial cells (HCEs).

Intrastromal or subconjunctival injection of rAAVrh.10 pri-miR-204 and pri-miR-184 are safe for ocular tissues

The safety profile for the delivery of pri-miR-204 and pri-miR-184 vectors was investigated. Normal mouse eyes were treated with pri-miRNA vectors by intrastromal or subconjunctival injection, and the ocular surface and fundus were observed after two weeks. Gross *in vivo* observation showed no obvious abnormality in either pri-miRNA injected groups when compared to control groups (FIG. 25A). Eyes were then harvested for histopathologic analysis of corneas and retinas by H&E staining (FIG. 25B). No clear pathological outcomes were observed for the exogenous expression of pri-miRNA-184 and pri-miRNA-204 by rAAVrh.10 delivery. Together these results indicate that rAAV delivery

and the exogenous expression of miRNAs do not elicit an inflammatory response and do not on their own drive tissue damage in the eye.

5 Sequences

>mmu-miR-21a (SEQ ID NO: 1)

UGUACCACCUUGUCGGAUAGCUUAUCAGACUGAUGUUGACUGUUGAAUCUCA
UGGCAACAGCAGUCGAUGGGCUGUCUGACAUUUUGGUAUC

10 >mmu-miR-184 (SEQ ID NO: 2)

CCUUUCCUUAUCACUUUUCAGCCAGCUUUGUGACUCUAAGUGUUGGACGGAG
AACUGAUAAGGGUAGG

>mmu-miR-204 (SEQ ID NO: 3)

15 UGGACUUCCCUUUGUCAUCCUAUGCCUGAGAAUAUAUGAAGGAGGCUGGGAA
GGCAAAGGGACGUUCA

>TuD miR-21 (SEQ ID NO: 4)

20 GACGGCGCTAGGATCATCAACTCAACATCAGTCATCTTGATAAGCTACAAGTATT
CTGGTCACAGAATACAACATCAGTCATCTTGATAAGCTACAAGATGATCC
TAGCGCCGTCTTTT

>pri miR-184 (SEQ ID NO: 5)

25 TGTGCAGAAACATAAGTGACTCTCCAGGTGTCAGAGGGAGAGACTGGGGCGAGA
GGCCAGAGCAAAGTAGAAGGGCACAGAGGGGCTTTGAATTTGAGGCAGAGGAG
GAACTGCAGAGAGGGGGCGGGGAGGGCTCGCCGGGAAATCAAACGTCCATTTAC
ATCTTGTCCTGCAAAGCTTCATCAAACTTCTTTGCCGGCCAGTCACGTCCCCTTA
TCACTTTTCCAGCCCAGCTTTGTGACTGTAAGTGTTGGACGGAGAAGTATAAGG
GTAGGTGATTGACACTCACAGCCTCCGGAACCCCGCGCCGCCTGCACTTGCGTG
30 ATGGGGAAAACCTGGCGTTCCCGCTCTGGGTGCCCCGAGGACAGCAGGGGATTCC
AGGAGGAGACCTTGGGCATAGGGGGGCCAGGTATGCGCCCCCTGCCTGAGGATG
CTGGGGTAGCCTTTGTGTTTTGTCAGTGAGATCTCCACTTTGCTTATTCAAATGT
GCTCTCTGC

35 pri miR-204 (SEQ ID NO: 6)

TTTACCCACAGGACAGGGTGATGGAGAGGAGGGTGAGGGTGAGGCAAGCAGA
GGACCTCCTGATCATGTACCCATAGGACAGGGTGATGGAGAGGAGGGTGAGGGT
GGAGGCAAGCAGAGGACCTCCTGATCATGTACCCATAGGACAGGGTGATGGAAA
GGAGGGTGGGGGTGGAGGCAAGCAGAGGACTTCCTGATCGCGTACCCATGGCTA
40 CAGTCTTTCTTCATGTGACTCGTGGACTTCCCTTTGTCATCCTATGCCTGAGAATA

TATGAAGGAGGCTGGGAAGGCAAAGGGACGTTCAATTGTCATCACTGGCATCTT
 TTTTGATCATTGCACCATCATCAAATGCATTGGGATAACCATGACATGAAATTTT
 CCATCATTGGGCCCATAACTGTCCCATAAGAGAGATGAAAAACACTGTATGTTAA
 AGGTCATAGTAGAACTTCATCCAAGCAGCTCTGGAATTAGGAAGGAGTGAAATA
 5 TACTCTCAAAGACTAATAGTTCTGGGTCCAAACCATGTGAC

> AAV5 capsid protein amino acid sequence (SEQ ID NO: 7)

MSFVDHPPDWLEEVGEGREFLGLEAGPPKPKPNQQHQDQARGLVLPGYNYLGPNG
 GLDRGEPVNRADDEVAREHDISYNEQLEAGDNPYLKYNHADADEFQEKLADDTSFSGN
 10 LGKAVFQAKKRVLEPFGLVEEGAKTAPTGKRIDDHFPKRKKARTEEDSKPSTSSDAE
 AGPSGSQQLQIPAPASSLGADTMSAGGGGPLGDNNQGADGVGNASGDWHCDSTW
 MGDRVVTKSTRTWLPSYNNHQYREIKSGSVDGSNANAYFGYSTPWGYFDFNRFH
 SHWSPRDWQRLINNYWGFRPRSLRVKIFNIQVKEVTVQDSTTTIANLSTVQVFTD
 DDYQLPYVVGNTEGCLPAFPQVFTLPQYGYATLNRDNTENPTERSSFFCLEYFPS
 15 KMLRTGNNFEFTYNFEEVPPHSSFAPSQNLFKLANPLVDQYLRFVSTNNTGGVQFN
 KNLAGRYANTYKNWFPGPMGRTQGWNLGSVNRASVSFAFATTNRMELEGASYQV
 PPQPNGMTNNLQGSNTYALENTMIFNSQPANPGTTATYLEGNMLITSESETQPVNRV
 AYNVGGQMATNNQSSTTAPATGTYNLQEIVPGSVWMERDVYLQGPWAKIPETGAH
 FHPSPAMGGFGLKHPPPMMLIKNTPVPGNITSFSDVPVSSFITQYSTGQVTVEMEWEL
 20 KKENSKRWNPEIQYTNNYNDPQFVDFAPDSTGEYRTRPIGTRYLTRPL

>AAV6 capsid protein amino acid sequence (SEQ ID NO: 8)

MAADGYLPDWLEDNLSEGIREWWDLKPGAPKPKANQQKQDDGRGLVLPGYKYL
 PFNGLDKGEPVNAADAAALEHDKAYDQQLKAGDNPYLRYNHADADEFQERLQEDTS
 25 FGGNLGRAVFQAKKRVLEPFGLVEEGAKTAPGKKRPVEQSPQEPDSSSGIGKTGQQP
 AKKRLNFGQTGDSESVDPQPLGEPPATPAAVGPTTMASSGGAPMADNNEGADGV
 GNASGNWHCDSTWLGDREVTTSTRTWALPTYNNHLYKQISSASTGASNDNHYFGYS
 TPWGYFDFNRFHCHFSRPDWQRLINNNWGFRPKRLNFKLFNIQVKEVTTNDGVT
 NNLTSTVQVFSDSEYQLPYVLGSAHQGCLPPFPADVFMIPQYGYLTLNNGSQAVGRS
 30 SFYCLEYFPSQMLRTGNNFTFSYTFEDVPFHSSYAHSQSLDRLMNPLIDQYLYLNRT
 QNQSGSAQNKDLLFSRGSPAGMSVQPKNWLPGPCYRQQRVSKTKTDNNNSNFTWT
 GASKYNLNGRESIINPGTAMASHKDDKDKFFPMMSGVMIFGKESAGASNTALDNVMI
 TDEEEIKATNPVATERFGTVAVNLQSSSTDPATGDVHVMGALPGMVWQDRDVYLQ
 GPIWAKIPHDTGHHFSPMLMGGFGLKHPPPQILIKNTPVPANPPAEFSATKFASFITQYS
 35 TGQVSVEIEWELQKENS KRWNPEVQYTSNYAKSANVDFTVDNNGLYTEPRPIGTRY
 LTRPL

>AAV6.2 capsid protein amino acid sequence (SEQ ID NO: 9)

MAADGYLPDWLEDNLSEGIREWWDLKPGAPKPKANQQKQDDGRGLVLPGYKYL
 40 PFNGLDKGEPVNAADAAALEHDKAYDQQLKAGDNPYLRYNHADADEFQERLQEDTS
 FGGNLGRAVFQAKKRVLEPLGLVEEGAKTAPGKKRPVEQSPQEPDSSSGIGKTGQQP
 AKKRLNFGQTGDSESVDPQPLGEPPATPAAVGPTTMASSGGAPMADNNEGADGV
 GNASGNWHCDSTWLGDREVTTSTRTWALPTYNNHLYKQISSASTGASNDNHYFGYS
 TPWGYFDFNRFHCHFSRPDWQRLINNNWGFRPKRLNFKLFNIQVKEVTTNDGVT
 45 NNLTSTVQVFSDSEYQLPYVLGSAHQGCLPPFPADVFMIPQYGYLTLNNGSQAVGRS
 SFYCLEYFPSQMLRTGNNFTFSYTFEDVPFHSSYAHSQSLDRLMNPLIDQYLYLNRT

QNGSGSAQNKDLLFSRGSPAGMSVQPKNWLPGPCYRQQRVSKTKTDNNNSNFTWT
 GASKYNLNGRESIINPGTAMASHKDDKDKFFPMSGVMIFGKESAGASNTALDNVMI
 TDEEEIKATNPVATERFGTVAVNLQSSSTDPATGDVHVMGALPGMVWQDRDVYLQ
 GPIWAKIPHTDGHFHPSPLMGGFGLKHPPPQILIKNTPVPANPPAEFSATKFASFITQYS
 5 TGQVSVEIEWELQKENS KRWNPEVQYTSNYAKSANVDFTVDNNGLYTEPRPIGTRY
 LTRPL

>AAV7 capsid protein amino acid sequence (SEQ ID NO: 10)

MAADGYLPDWLEDNLSEGIREWWDLKPGAPKPKANQQKQDNGRGLVLPGYKYLG
 10 PFNGLDKGEPVNAADAAALEHDKAYDQQLKAGDNPYLRYNHADADEFQERLQEDTS
 FGGNLGRAVFQAKKRVLEPLGLVEEGA KTAPAKKRPVEPSPQRSPDSSTGIGKKGQQ
 PARKRLNFGQTGDSESVDPQPLGEPPAAPSSVGS GTVAAGGGAPMADNNEGADGV
 GNASGNWHCDSTWLGD RVITTSTRTWALPTYNNHLYKQISSETAGSTNDNTYFGYS
 TPWGYFDFNRFHCHFS PRDWQRLINNNWGFRPKKLRFKLFNIQVKEVTTNDGVTIA
 15 NNLSTIQVFSDSEYQLPYVLGSAHQGCLPPFPADVFMIPQYGYLTLNNGSQSVGRSS
 FYCLEYFPSQMLRTGN NFESYSFEDVPFHSSY AHSQSLDRLMNPLIDQYL YLART
 QSNPGGTAGNRELQFYQGGPSTMAEQAKNWLPGPCFRQQRVSKTLDQNNNSNFAW
 TGATKYHLNGRNSLVNPGVAMATHKDDEDRFFPSSGVLIFGKTGATNKTTLE NVLM
 TNEEEIRPTNPVATEEY GIVSSNLQAANTAAQTQVVNNQGALPGMVWQNRDVYLQ
 20 GPIWAKIPHTDGNFHPSPLMGGFGLKHPPPQILIKNTPVPANPPEVFTPAKFASFITQYS
 TGQVSVEIEWELQKENS KRWNPEIQYTSNFEKQTGVDFAVDSQGVYSEPRPIGTRYL
 TRNL

>AAV8 capsid protein amino acid sequence (SEQ ID NO: 11)

MAADGYLPDWLEDNLSEGIREWWALKPGAPKPKANQQKQDDGRGLVLPGYKYLG
 25 PFNGLDKGEPVNAADAAALEHDKAYDQQLQAGDNPYLRYNHADADEFQERLQEDTS
 FGGNLGRAVFQAKKRVLEPLGLVEEGA KTAPGKKRPVEPSPQRSPDSSTGIGKKGQQ
 PARKRLNFGQTGDSESVDPQPLGEPPAAPSGVGPNTMAAGGGAPMADNNEGADG
 VGSSSGNWHCDSTWLGD RVITTSTRTWALPTYNNHLYKQISNGTSGGATNDNTYFG
 30 YSTPWGYFDFNRFHCHFS PRDWQRLINNNWGFRPKRLSFKLFNIQVKEVTQNEGTKT
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 TQTTGGTANTQTLGFSQGGPNTMANQAKNWLPGPCYRQQRVSTTTGQNNNSNFAW
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 35 LTSEEEIKTTNPVATEEY GIVADNLQQQNTAPQIGTVNSQGALPGMVWQNRDVYLQ
 GPIWAKIPHTDGNFHPSPLMGGFGLKHPPPQILIKNTPVPADPPTTFNQSKLNSFITQY
 STGQVSVEIEWELQKENS KRWNPEIQYTSNYYKSTSVDFAVNTEGVYSEPRPIGTRYL
 TRNL

>AAV9 capsid protein amino acid sequence (SEQ ID NO: 12)

MAADGYLPDWLEDNLSEGIREWWALKPGAPQPKANQQHQDNARGLVLPGYKYLG
 40 PGNGLDKGEPVNAADAAALEHDKAYDQQLKAGDNPYLKYNHADADEFQERLKEDTS
 FGGNLGRAVFQAKKRLLLEPLGLVEEAAKTAPGKKRPVEQSPQEPDSSAGIGKSGAQP
 AKKRLNFGQTGDTE SVDPQPIGEPPAAPSGVGS LTMASGGGAPVADNNEGADGVG
 45 SSSGNWHCDSQWLGD RVITTSTRTWALPTYNNHLYKQISNSTSGGSSNDNAYFGYS
 TPWGYFDFNRFHCHFS PRDWQRLINNNWGFRPKRLNFKLFNIQVKEVTDNNGVKTI
 ANNLTSTVQVFTDSDYQLPYVLGSAHEGCLPPFPADVFMIPQYGYLTLNDGSQAVG

RSSFYCLEYFPSQMLRTGNNFQFSYEFENVPFHSSY AHSQSLDRLMNPLIDQYLYYLS
 KTINGSGQNQQTTLKFSVAGPSNMAVQGRNYIPGPSYRQQRVSTTVTQNNNSEFAWP
 GASSWALNGRNSLMNPGPAMASHKEGEDRFFPLSGSLIFGKQGTGRDNVDADKVM
 TNEEEIKTTNPVATESYGQVATNHQSAQAQAQTGWVQNQGILPGMVWQDRDVYLQ
 5 GPIWAKIPHTDGNFHPSPLMGGFGLKHPPPQILIKNTPVPADPPTAFNKDKLNSFITQ
 YSTGQVSVEIEWELQKENS KRWNPEIQYTSNYYKSNVFEFAVNTEGVYSEPRPIGTR
 YLTRNL

>AAV rh.8 capsid protein amino acid sequence (SEQ ID NO: 13)

10 MAADGYLPDWLEDNLSEGIREWWDLKPGAPKPKANQQKQDDGRGLVLPGYKYLG
 PFNGLDKGEPVNAADAAALEHDKAYDQQLKAGDNPYLRYNHADADEFQERLQEDTS
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 15 STPWGYFDENRFHCHFSRWDQRLINNNWGFRPKRLNFKLFNIQVKEVTNEGTKTI
 ANNLTSTVQVFTDSEYQLPYVLGSAHQGCLPPFPADVFMVPQYGYLTLNNGSQALG
 RSSFYCLEYFPSQMLRTGNNFQFSYTFEDVPFHSSY AHSQSLDRLMNPLIDQYLYYLV
 RTQTTGTGGTQTLAFSQAGPSSMANQARNWVPGPCYRQQRVSTTTNQNNSNFAW
 TGAAKFKLNGRDSL MNPGVAMASHKDDDDRFFPSSGVLIFGKQAGNDGVDYSQV
 20 LITDEEEIKATNPVATEEYGA VAINNQAANTQAQTGLVHNQGVIPGMVWQNRDVYL
 QGPIWAKIPHTDGNFHPSPLMGGFGLKHPPPQILIKNTPVPADPPLTFNQA KLNSFITQ
 YSTGQVSVEIEWELQKENS KRWNPEIQYTSNYYKSTNVDFAVNTEGVYSEPRPIGTR
 YLTRNL

25 >AAVrh.10 capsid protein amino acid sequence (SEQ ID NO: 14)

MAADGYLPDWLEDNLSEGIREWWDLKPGAPKPKANQQKQDDGRGLVLPGYKYLG
 PFNGLDKGEPVNAADAAALEHDKAYDQQLKAGDNPYLRYNHADADEFQERLQEDTS
 FGGNLGRAVFQAKKRVLEPLGLVEEGA KTAPGKKRPVEPSPQSPDSSTGIGKKGQQ
 PAKKRLNFGQTGDSESVDPDQPIGEPPAGPSGLSGTMAAGGGAPMADNNEGADGV
 30 GSSSGNWHCDSTWLGD RVITTSTRTWALPTYNNHLYKQISNGTSGGSTNDNTYFGY
 STPWGYFDENRFHCHFSRWDQRLINNNWGFRPKRLNFKLFNIQVKEVTQNEGTKTI
 ANNLTSTIQVFTDSEYQLPYVLGSAHQGCLPPFPADVFMIPQYGYLTLNNGSQAVGR
 SSFYCLEYFPSQMLRTGNNFEFSYQFEDVPFHSSY AHSQSLDRLMNPLIDQYLYYLSR
 TQSTGGTAGTQQLFSQAGPNNMSAQAKNWLPGPCYRQQRVSTTLSQNNNSNFAW
 35 TGATKYHLNGRDSL VNPGVAMATHKDDEERFFPSSGVL MFGKQAGKDNVDYSSV
 MLTSEEEIKTTNPVATEQYGVVADNLQQQNAAPIVGAVNSQGALPGMVWQNRDVY
 LQGPIWAKIPHTDGNFHPSPLMGGFGLKHPPPQILIKNTPVPADPPTTFSQAKLASFIT
 QYSTGQVSVEIEWELQKENS KRWNPEIQYTSNYYKSTNVDFAVNTDGTYSEPRPIGT
 RYLTRNL

40 >AAVrh.39 capsid protein amino acid sequence (SEQ ID NO: 15)

MAADGYLPDWLEDNLSEGIREWWALKPGAPKPKANQQKQDDGRGLVLPGYKYLG
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 45 PAKKRLNFGQTGDSESVDPDQPIGEPPAGPSGLSGTMAAGGGAPMADNNEGADGV
 GSSSGNWHCDSTWLGD RVITTSTRTWALPTYNNHLYKQISNGTSGGSTNDNTYFGY
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ANNLTSTIQVFTDSEYQLPYVLGSAHQGCLPPFPADVFMIPQYGYLTLNNGSQAVGR
 SSFYCLEYFPSQMLRTGNNFEEFSYTFEDVPFHSSYAHQSQSLDRLMNPLIDQYLYYLSR
 TQSTGGTQGTQQLLFSQAGPANMSAQAKNWLPGPCYRQQRVSTTLSQNNNSNFAW
 TGATKYHLNGRDSLVPNGVAMATHKDDEERFFPSSGVLMFGKQGAGRDNVDYSSV
 5 MLTSEEEIKTTNPVATEQYGVVADNLQQTNTGPIVGNVNSQGALPGMVWQNRDVY
 LQGPIWAKIPHTDGNFHPSPMLMGGFGLKHPPPQILIKNTPVPADPPTTFSQAKLASFIT
 QYSTGQVSVEIEWELQKENS KRWNPEIQYTSNYYKSTNVDFAVNTEGTYSEPRPIGT
 RYLTRNL

>AAVrh.43 capsid protein amino acid sequence (SEQ ID NO: 16)

MAADGYLPDWLEDNLSEGIREWDLKPGAPKPKANQQKQDDGRGLVLPGYKYL
 PFNGLDKGEPVNAADAAALEHDKAYDQQLLEAGDNPYLRYNHADADEFQERLQEDTS
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 ARKRLNFGQTGDSESVDPDQPLGEPPAAPSGVGPNTMAAGGGAPMADNNEGADGV
 15 GSSSGNWHCDSTWLGD RVITTSTRTWALPTYNNHLYKQISNGTSGGATNDNTYFGY
 STPWGYFDENRFHCHFSRDPWQRLINNNWGFPRKRLSFKLFNIQVKEVTQNEGTKTI
 ANNLTSTIQVFTDSEYQLPYVLGSAHQGCLPPFPADVFMIPQYGYLTLNNGSQAVGR
 SSFYCLEYFPSQMLRTGNNFQFTYTFEDVPFHSSYAHQSQSLDRLMNPLIDQYLYYLSR
 TQTTGGTANTQTLGFSQGGPNTMANQAKNWLPGPCYRQQRVSTTTGQNNNSNFAW
 20 TAGTKYHLNGRNSLANPGIAMATHKDDEERFFPVTGSCFWQQNAARDNADYSDVM
 LTSEEEIKTTNPVATEEYGIVADNLQQQNTAPQIGTVNSQGALPGMVWQNRDVYLQ
 GPIWAKIPHTDGNFHPSPMLMGGFGLKHPPPQILIKNTPVPADPPTTFNQSKLNSFITQY
 STGQVSVEIEWELQKENS KRWNPEIQYTSNYYKSTSVDFAVNTEGVYSEPRPIGTRYL
 TRNL

>AAV5 capsid protein nucleic acid sequence (SEQ ID NO: 17)

ATGTCTTTTGTGATCACCTCCAGATTGGTTGGAAGAAGTTGGTGAAGGTCTTC
 GCGAGTTTTTGGGCCTTGAAGCGGGCCACCGAAACCAAAACCCAATCAGCAGC
 30 ATCAAGATCAAGCCCGTGGTCTTGTGCTGCCTGGTTATAACTATCTCGGACCCGG
 AAACGGTCTCGATCGAGGAGAGCCTGTCAACAGGGCAGACGAGGTCGCGCGAGA
 GCACGACATCTCGTACAACGAGCAGCTTGAGGCGGGAGACAACCCCTACCTCAA
 GTACAACCACGCGGACGCCGAGTTTCAGGAGAAGCTCGCCGACGACACATCCTT
 CGGGGGAAACCTCGGAAAGGCAGTCTTTCAGGCCAAGAAAAGGGTTCTCGAACC
 35 TTTTGGCCTGGTTGAAGAGGGTGCTAAGACGGCCCCCTACCGGAAAGCGGATAGA
 CGACCACTTTCCAAAAAGAAAGAAGGCCCGGACCGAAGAGGACTCCAAGCCTTC
 CACCTCGTCAGACGCCGAAGCTGGACCCAGCGGATCCCAGCAGCTGCAAATCCC
 AGCCCAACCAGCCTCAAGTTTGGGAGCTGATACAATGTCTGCGGGAGGTGGCGG
 CCCATTGGGCGACAATAACCAAGGTGCCGATGGAGTGGGCAATGCCTCGGGAGA
 40 TTGGCATTGCGATTCCACGTGGATGGGGGACAGAGTCGTCACCAAGTCCACCCG
 AACCTGGGTGCTGCCCAGCTACAACAACCACAGTACCGAGAGATCAAAAGCGG
 CTCCGTCGACGGAAGCAACGCCAACGCCTACTTTGGATACAGCACCCCTGGGG
 GTACTTTGACTTTAACCGCTTCCACAGCCACTGGAGCCCCCGAGACTGGCAAAGA
 CTCATCAACAATACTGGGGCTTCAGACCCCGGTCCCTCAGAGTCAAAATCTTCA
 45 ACATTCAAGTCAAAGAGGTCACGGTGCAGGACTCCACCACCACCATCGCCAACA
 ACCTCACCTCCACCGTCCAAGTGTTCACGGACGACGACTACCAGCTGCCCTACGT
 CGTCGGCAACGGGACCGAGGGATGCCTGCCGGCCTTCCTCCGCAGGTCTTTACG

CTGCCGCAGTACGGTTACGCGACGCTGAACCGCGACAACACAGAAAATCCCACC
 GAGAGGAGCAGCTTCTTCTGCCTAGAGTACTTTCCCAGCAAGATGCTGAGAACG
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 5 GTACCGCTTCGTGAGCACAAATAAACACTGGCGGAGTCCAGTTCAACAAGAACCT
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 CGCCACGACCAATAGGATGGAGCTCGAGGGCGCGAGTTACCAGGTGCCCCCGCA
 GCCGAACGGCATGACCAACAACCTCCAGGGCAGCAACACCTATGCCCTGGAGAA
 10 CACTATGATCTTCAACAGCCAGCCGGCGAACCCGGGCACCACCGCCACGTACCT
 CGAGGGCAACATGCTCATCACCAGCGAGAGCGAGACGCAGCCGGTGAACCGCGT
 GCGGTACAACGTCGGCGGGCAGATGGCCACCAACAACCAGAGCTCCACCACTGC
 CCCCCGCGACCGGCACGTACAACCTCCAGGAAATCGTGCCCCGGCAGCGTGTGGAT
 GGAGAGGGACGTGTACCTCCAAGGACCCATCTGGGCCAAGATCCCAGAGACGGG
 15 GGCGCACTTTCACCCCTCTCCGGCCATGGGCGGATTCGGACTCAAACACCCACCG
 CCCATGATGCTCATCAAGAACACGCCTGTGCCCGGAAATATCACCAGCTTCTCGG
 ACGTGCCCGTCAGCAGCTTCATCACCAGTACAGCACCGGGCAGGTCACCGTGG
 AGATGGAGTGGGAGCTCAAGAAGGAAAACCTCCAAGAGGTGGAACCCAGAGATC
 CAGTACACAAACAACCTACAACGACCCCCAGTTTGTGGACTTTGCCCCGGACAGC
 20 ACCGGGGAATACAGAACCACCAGACCTATCGGAACCCGATACCTTACCCGACCC
 CTT

> AAV6 capsid nucleic acid sequence (SEQ ID NO: 18)

ATGGCTGCCGATGGTTATCTTCCAGATTGGCTCGAGGACAACCTCTCTGAGGGCA
 25 TTCGCGAGTGGTGGGACTTGAAACCTGGAGCCCCGAAACCCAAAGCCAACCAGC
 AAAAGCAGGACGACGGCCGGGTCTGGTGTCTTCTGGCTACAAGTACCTCGGAC
 CCTTCAACGGA CTGACAAGGGGGGAGCCCGTCAACGCGGCGGATGCAGCGGCCC
 TCGAGCACGACAAGGCCTACGACCAGCAGCTCAAAGCGGGTGACAATCCGTACC
 TGCGGTATAACCACGCCGACGCCGAGTTTCAGGAGCGTCTGCAAGAAGATACGT
 30 CTTTTGGGGGCAACCTCGGGCGAGCAGTCTTCCAGGCCAAGAAGAGGGTTCTCG
 AACCTTTTGGTCTGGTTGAGGAAGGTGCTAAGACGGCTCCTGGAAAGAAACGTC
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>AAV6.2 capsid protein nucleic acid sequence (SEQ ID NO: 19)

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15 >AAV7 capsid protein nucleic acid sequence (SEQ ID NO: 20)

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10 >AAV8 capsid protein nucleic acid sequence (SEQ ID NO: 21)

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 5 CCCGCCCCATTGGCACCCGTTACCTCACCCGTAATCTG

>AAV9 capsid protein nucleic acid sequence (SEQ ID NO: 22)

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>AAVrh.8 capsid protein nucleic acid sequence (SEQ ID NO: 23)

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45 CCCATTGGCACCCGTTACCTCACCCGCAACCTGTAA

>AAVrh.10 capsid protein nucleic acid sequence (SEQ ID NO: 24)

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 40 ACACAGATGGCACTTATTCTGAGCCTCGCCCCATCGGCACCCGTTACCTCACCCG
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>AAVrh.39 capsid protein nucleic acid sequence (SEQ ID NO: 25)

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 35 CTTTATTACGCAGTACAGCACCGGACAGGTACGCGTGGAATCGAGTGGGAGCT
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40 > AAVrh.43 capsid protein nucleic acid sequence (SEQ ID NO: 26)

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> Frizzled 4 (*Fzd4*) Nucleic Acid Sequence (SEQ ID NO: 27)

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5 TCCCGCCCCAGAACGACCACAACCACATGTGCATGGAAGGACCAGGTGATGAAG
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 10 AAACATTTTCATGGATTCCAAATCCCAAATAGGCAAATAGGTGTTCAAATTCTGGA
 AAT

> Angiopoietin-1 Nucleic Acid Sequence (SEQ ID NOs: 28 and 29)

15 Isoform 1 (SEQ ID NO: 28)

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Isoform 2 (SEQ ID NO: 29):

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35

CLAIMS

What is claimed is:

1. A method for delivering a transgene to ocular tissue, the method comprising:
5 administering to ocular tissue of a subject an effective amount of rAAV, wherein the rAAV comprises (i) a capsid protein having a serotype selected from the group consisting of AAV5, AAV6, AAV6.2, AAV7, AAV8, AAV9, AAVrh.10, AAVrh.39, and AAVrh.43, and (ii) a nucleic acid comprising a promoter operably linked to a transgene.
- 10 2. The method of claim 1, wherein the capsid protein comprises an amino acid sequence that is at least 70%, at least 80%, at least 90%, at least 95% , or at least 99% identical to any one of SEQ ID NO: 7-16.
3. The method of claim 1 or 2, wherein the capsid protein comprises an amino acid sequence
15 as set forth in SEQ ID NO: 14 or SEQ ID NO: 15.
4. The method of any one of claims 1 to 3, wherein the capsid protein is AAVrh.10 capsid protein (SEQ ID NO: 14) or AAVrh.39 capsid protein (SEQ ID NO: 15).
- 20 5. The method of any one of claims 1 to 4, wherein the transgene encodes a gene associated with an ocular disease.
6. The method of claim 5, wherein the ocular disease is selected from corneal neovascularization, corneal dystrophy, corneal inflammation, and corneal fibrosis.
25
7. The method of claim 5 or 6, wherein the gene encodes a miRNA, an antagomir, or a miRNA mimic.
8. The method of claim 7, wherein the gene encodes a miRNA, optionally a TuD miRNA or a
30 pri miRNA.

9. The method of claim 7 or claim 8, wherein the transgene comprises a region of complementarity to a sequence selected from the group consisting of SEQ ID NO: 1, 2, 3, 27, 28, and 29.

10. The method of any one of claims 7 to 9, wherein the transgene comprises SEQ ID NO: 4; SEQ ID NO: 5, or SEQ ID NO: 6.

11. The method of any one of claims 1 to 10, wherein the administration occurs by injection.

12. The method of claim 11, wherein the injection is intrastromal injection.

13. The method of any one of claims 1 to 10, wherein the administration is topical administration to an eye.

14. The method of any one of claims 1 to 13, wherein the administration results in transduction of an ocular cell type selected from the group consisting of keratocytes, corneal endothelial cells, corneal basal cells, corneal wing cells, and corneal squamous cells.

15. The method of claim 14, wherein the administration results in transduction of keratocytes.

16. The method of any one of claims 1 to 15, wherein the rAAV further comprises two AAV inverted terminal repeats (ITRs), wherein the ITRs flank the transgene.

17. The method of claim 16, wherein the AAV ITRs are ITRs of one or more serotypes selected from: AAV2, AAV3, AAV4, AAV5, and AAV6.

18. The method of any one of claims 1 to 17, wherein the subject is a mammal, optionally a human.

19. A method for treating an ocular disease, the method comprising: administering to a subject having or suspected of having an ocular disease an effective amount of rAAV, wherein the rAAV comprises (i) a capsid protein having a serotype selected from the group consisting of AAV5, AAV6, AAV6.2, AAV7, AAV8, AAV9, AAVrh.10, AAVrh.39, and AAVrh.43, and (ii) a nucleic acid comprising a promoter operably linked to a transgene.

20. The method of claim 19, wherein the capsid protein comprises an amino acid sequence that is at least 70%, at least 80%, at least 90%, at least 95% , or at least 99% identical to any one of SEQ ID NO: 7-16.

21. The method of claim 19 or 20, wherein the capsid protein comprises an amino acid sequence as set forth in SEQ ID NO: 14 or SEQ ID NO: 15.

22. The method of any one of claims 19 to 21, wherein the capsid protein is AAVrh.10 capsid protein (SEQ ID NO: 14) or AAVrh.39 capsid protein (SEQ ID NO: 15).

23. The method of any one of claims 19 to 22, wherein the transgene encodes a gene associated with an ocular disease.

24. The method of claim 23, wherein the ocular disease is selected from corneal neovascularization, corneal dystrophy, corneal inflammation, and corneal fibrosis.

25. The method of claim 23 or 24 wherein the gene encodes a miRNA, an antagomir, or a miRNA mimic.

26. The method of claim 25, wherein the gene encodes a miRNA, optionally a TuD miRNA or a pri miRNA.

27. The method of claim 23 or claim 24, wherein the transgene comprises a region of complementarity to a sequence selected from the group consisting of SEQ ID NO: 1, 2, 3, 27, 28, and 29.

5 28. The method of claim 25 or 26, wherein the transgene comprises SEQ ID NO: 4, SEQ ID NO: 5 or SEQ ID NO: 6.

29. The method of any one of claims 19 to 28, wherein the administration occurs by injection.

10 30. The method of claim 29, wherein the injection is intrastromal injection.

31. The method of any one of claims 19 to 30, wherein the administration is topical administration to the eye.

15 32. The method of any one of claims 19 to 31, wherein the administration results in transduction of an ocular cell type selected from the group consisting of keratocytes, corneal endothelial cells, corneal basal cells, corneal wing cells, and corneal squamous cells.

20 33. The method of claim 32, wherein the administration results in transduction of keratocytes.

34. The method of any one of claims 19 to 33, wherein the rAAV further comprises two AAV inverted terminal repeats (ITRs), wherein the ITRs flank the transgene.

25 35. The method of claim 34, wherein the AAV ITRs are ITRs of one or more serotypes selected from: AAV2, AAV3, AAV4, AAV5, and AAV6.

30 36. The method of any one of claims 19 to 35, wherein the subject is a mammal, optionally a human.

37. A method for delivering a transgene to ocular tissue, the method comprising:
administering to ocular tissue of a subject an effective amount of rAAV, wherein the rAAV
comprises (i) a capsid protein, and (ii) a nucleic acid comprising a promoter operably linked
to a transgene, wherein the transgene encodes a gene associated with an ocular disease.

5

38. The method of claim 37, wherein the ocular disease is selected from corneal
neovascularization, corneal dystrophy, corneal inflammation, and corneal fibrosis.

39. The method of claim 37 or 38, wherein the transgene encodes a miRNA, an
10 antagomir, or a miRNA mimic.

40. The method of any one of claims 37 to 39, wherein the transgene comprises a region
of complementarity to a sequence selected from the group consisting of SEQ ID NO: 1, 2, 3,
27, 28, and 29.

15

41. The method of any one of claims 37 to 40, wherein the transgene comprises SEQ ID
NO: 4, SEQ ID NO: 5 or SEQ ID NO: 6.

42. The method of any one of claims 37 to 41, wherein the gene encodes a miRNA,
20 optionally a TuD miRNA or a pri miRNA.

43. The method of any one of claims 37 to 42, wherein the capsid protein has a serotype
selected from the group consisting of AAV5, AAV6, AAV6.2, AAV7, AAV8, AAV9,
AAVrh.10, AAVrh.39, and AAVrh.43.

25

44. The method of any one of claims 37 to 43, wherein the capsid protein comprises an
amino acid sequence that is at least 70%, at least 80%, at least 90%, at least 95% , or at least
99% identical to any one of SEQ ID NO: 7-16.

30 45. The method of any one of claims 37 to 44, wherein the capsid protein comprises an
amino acid sequence as set forth in SEQ ID NO: 14 or SEQ ID NO: 15.

46. The method of any one of claims 37 to 45, wherein the capsid protein is AAVrh.10 capsid protein (SEQ ID NO: 14) or AAVrh.39 capsid protein (SEQ ID NO: 15).

5 47. The method of any one of claims 37 to 46, wherein the administration occurs by injection.

48. The method of claim 47, wherein the injection is intrastromal injection.

10 49. The method of any one of claims 37 to 46, wherein the administration is topical administration to an eye.

50. The method of any one of claims 37 to 49, wherein the administration results in transduction of an ocular cell type selected from the group consisting of keratocytes, corneal
15 endothelial cells, corneal basal cells, corneal wing cells, and corneal squamous cells.

51. The method of claim 50, wherein the administration results in transduction of keratocytes.

20 52. The method of any one of claims 37 to 51, wherein the rAAV further comprises two AAV inverted terminal repeats (ITRs), wherein the ITRs flank the transgene.

53. The method of claim 52, wherein the AAV ITRs are ITRs of one or more serotypes selected from: AAV2, AAV3, AAV4, AAV5, and AAV6.

25

54. The method of any one of claims 37 to 53, wherein the subject is a mammal, optionally a human.

55. A method for treating a disease, the method comprising:

administering to ocular tissue of a subject an effective amount of rAAV, wherein the rAAV comprises (i) a capsid protein, and (ii) a nucleic acid comprising a promoter operably linked to a transgene, wherein the transgene encodes a gene associated with an ocular disease.

5 56. The method of claim 55, wherein the ocular disease is selected from corneal neovascularization, corneal dystrophy, corneal inflammation, and corneal fibrosis.

57. The method of claim 55 or 56, wherein the transgene encodes a miRNA, an antagomir, or a miRNA mimic.

10

58. The method of any one of claims 55 to 57, wherein the transgene comprises a region of complementarity to a sequence selected from the group consisting of SEQ ID NO: 1, 2, 3, 27, 28, and 29.

15 59. The method of any one of claims 55 to 58, wherein the transgene comprises SEQ ID NO: 4, SEQ ID NO: 5 or SEQ ID NO: 6.

60. The method of any one of claims 55 to 59, wherein the gene encodes a miRNA, optionally a TuD miRNA or a pri miRNA.

20

61. The method of any one of claims 55 to 60, wherein the capsid protein has a serotype selected from the group consisting of AAV5, AAV6, AAV6.2, AAV7, AAV8, AAV9, AAVrh.10, AAVrh.39, and AAVrh.43.

25 62. The method of any one of claims 55 to 61, wherein the capsid protein comprises an amino acid sequence that is at least 70%, at least 80%, at least 90%, at least 95% , or at least 99% identical to any one of SEQ ID NO: 7-16.

30 63. The method of any one of claims 55 to 62, wherein the capsid protein comprises an amino acid sequence as set forth in SEQ ID NO: 14 or SEQ ID NO: 15.

64. The method of any one of claims 55 to 63, wherein the capsid protein is AAVrh.10 capsid protein (SEQ ID NO: 14) or AAVrh.39 capsid protein (SEQ ID NO: 15).

65. The method of any one of claims 55 to 64, wherein the administration occurs by injection.

66. The method of claim 65, wherein the injection is intrastromal injection.

67. The method of any one of claims 55 to 64, wherein the administration is topical administration to an eye.

68. The method of any one of claims 55 to 67, wherein the administration results in transduction of an ocular cell type selected from the group consisting of keratocytes, corneal endothelial cells, corneal basal cells, corneal wing cells, and corneal squamous cells.

69. The method of claim 68, wherein the administration results in transduction of keratocytes.

70. The method of any one of claims 55 to 69, wherein the rAAV further comprises two AAV inverted terminal repeats (ITRs), wherein the ITRs flank the transgene.

71. The method of claim 70, wherein the AAV ITRs are ITRs of one or more serotypes selected from: AAV2, AAV3, AAV4, AAV5, and AAV6.

72. The method of any one of claims 55 to 71, wherein the subject is a mammal, optionally a human.

73. A recombinant adeno-associated virus comprising:

- (i) a capsid protein having a serotype selected from the group consisting of AAV5, AAV6, AAV6.2, AAV7, AAV8, AAV9, AAVrh.10, AAVrh.39, and AAVrh.43; and,

- (ii) a nucleic acid comprising a promoter operably linked to a transgene.

74. The method of claim 74, wherein the capsid protein comprises an amino acid sequence that is at least 70%, at least 80%, at least 90%, at least 95% , or at least 99% identical to any one of SEQ ID NO: 7-16.

75. The method of claim 73 or 74, wherein the capsid protein comprises an amino acid sequence as set forth in SEQ ID NO: 14 or SEQ ID NO: 15.

76. The method of any one of claims 73 to 75, wherein the capsid protein is AAVrh.10 capsid protein (SEQ ID NO: 14) or AAVrh.39 capsid protein (SEQ ID NO: 15).

77. The method of any one of claims 73 to 76, wherein the transgene encodes a gene associated with an ocular disease.

78. The method of claim 77, wherein the ocular disease is selected from corneal neovascularization, corneal dystrophy, corneal inflammation, and corneal fibrosis.

79. The method of claim 77 or 78, wherein the gene encodes a miRNA, an antagomir, or a miRNA mimic.

80. The method of claim 79, wherein the gene encodes a miRNA, optionally a TuD miRNA or a pri miRNA.

81. The method of claim 79 or claim 80, wherein the transgene comprises a region of complementarity to a sequence selected from the group consisting of SEQ ID NO: 1, 2, 3, 27, 28, and 29.

82. The method of claim 79 or 80, wherein the transgene comprises SEQ ID NO: 4, SEQ ID NO: 5 or SEQ ID NO: 6.

83. The method of any one of claims 73 to 82, wherein the rAAV further comprises two AAV inverted terminal repeats (ITRs), wherein the ITRs flank the transgene.

84. The method of claim 83, wherein the AAV ITRs are ITRs of one or more serotypes selected from: AAV2, AAV3, AAV4, AAV5, and AAV6.

85. A pharmaceutical composition comprising the rAAV of any one of claims 73 to 84 and a pharmaceutically acceptable excipient.

86. A recombinant adeno-associated virus (rAAV) comprising:
(i) a capsid protein; and,
(ii) a nucleic acid comprising a promoter operably linked to a transgene, wherein the transgene encodes a gene associated with an ocular disease.

87. The method of claim 86, wherein the ocular disease is selected from corneal neovascularization, corneal dystrophy, corneal inflammation, and corneal fibrosis.

88. The method of claim 86 or 87, wherein the transgene encodes a miRNA, an antagomir, or a miRNA mimic.

89. The method of any one of claims 86 to 88, wherein the transgene comprises a region of complementarity to a sequence selected from the group consisting of SEQ ID NO: 1, 2, 3, 27, 28, and 29.

90. The method of any one of claims 86 to 89, wherein the transgene comprises SEQ ID NO: 4, SEQ ID NO: 5 or SEQ ID NO: 6.

91. The method of any one of claims 86 to 90, wherein the gene encodes a miRNA, optionally a TuD miRNA or a pri miRNA.

92. The method of any one of claims 86 to 91, wherein the capsid protein has a serotype selected from the group consisting of AAV5, AAV6, AAV6.2, AAV7, AAV8, AAV9, AAVrh.10, AAVrh.39, and AAVrh.43.

5 93. The method of any one of claims 86 to 92, wherein the capsid protein comprises an amino acid sequence that is at least 70%, at least 80%, at least 90%, at least 95% , or at least 99% identical to any one of SEQ ID NO: 7-16.

94. The method of any one of claims 86 to 93, wherein the capsid protein comprises an
10 amino acid sequence as set forth in SEQ ID NO: 14 or SEQ ID NO: 15.

95. The method of any one of claims 86 to 94, wherein the capsid protein is AAVrh.10 capsid protein (SEQ ID NO: 14) or AAVrh.39 capsid protein (SEQ ID NO: 15).

15 96. The method of any one of claims 86 to 95, wherein the rAAV further comprises two AAV inverted terminal repeats (ITRs), wherein the ITRs flank the transgene.

97. The method of claim 96, wherein the AAV ITRs are ITRs of one or more serotypes selected from: AAV2, AAV3, AAV4, AAV5, and AAV6.

20

98. A pharmaceutical composition comprising the rAAV of any one of claims 86 to 97 and a pharmaceutically acceptable excipient.

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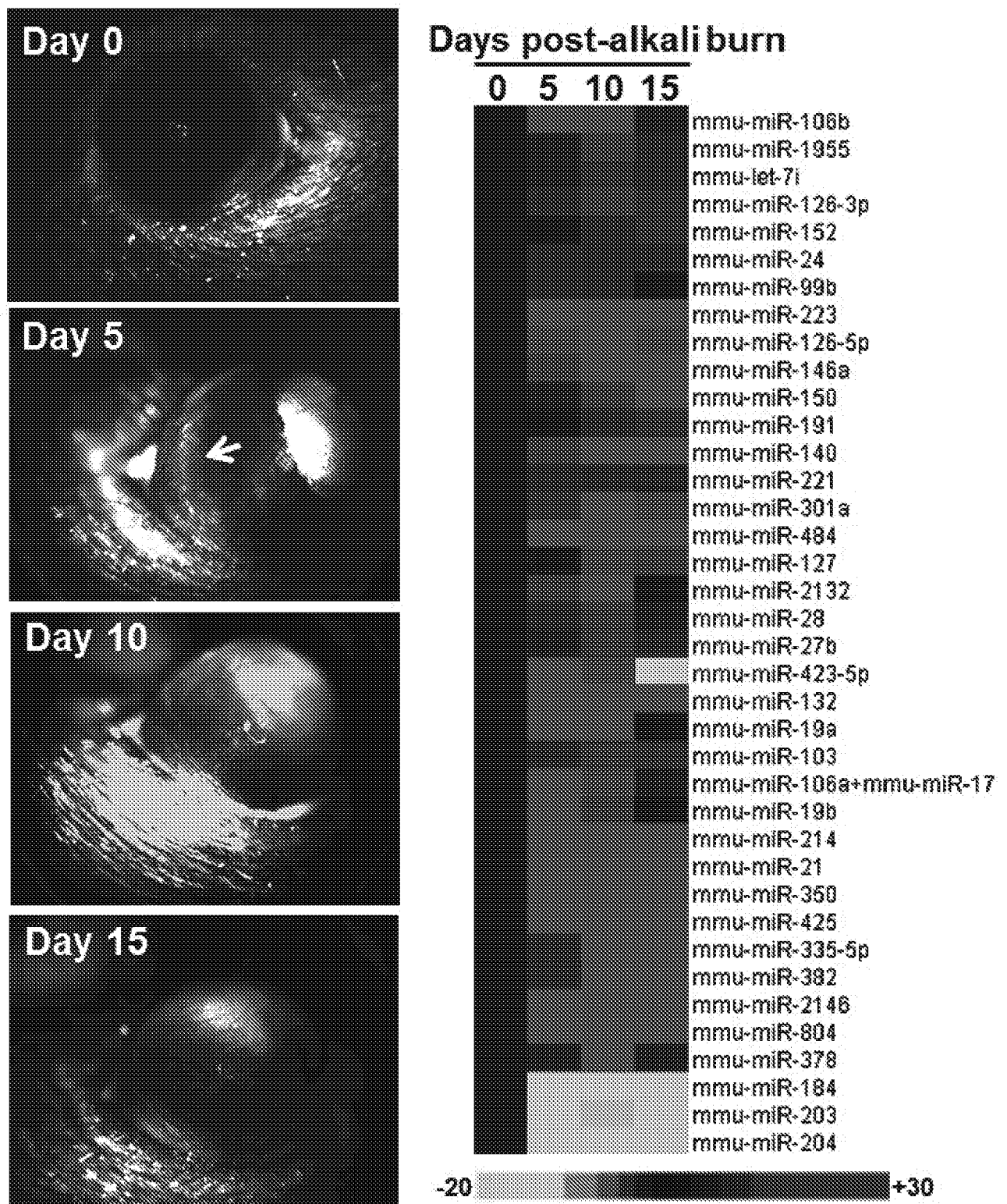


FIG. 1A

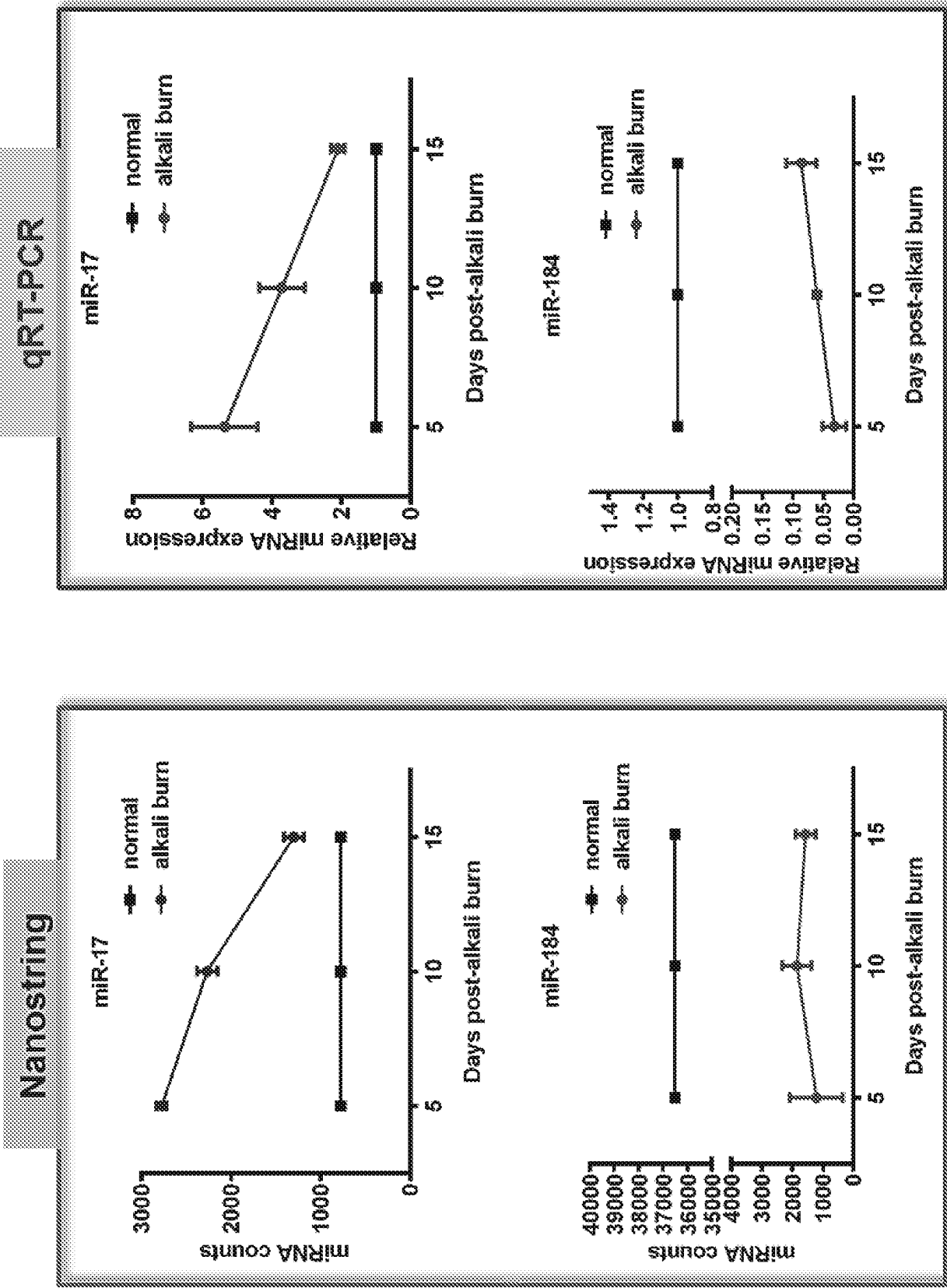


FIG. 1B

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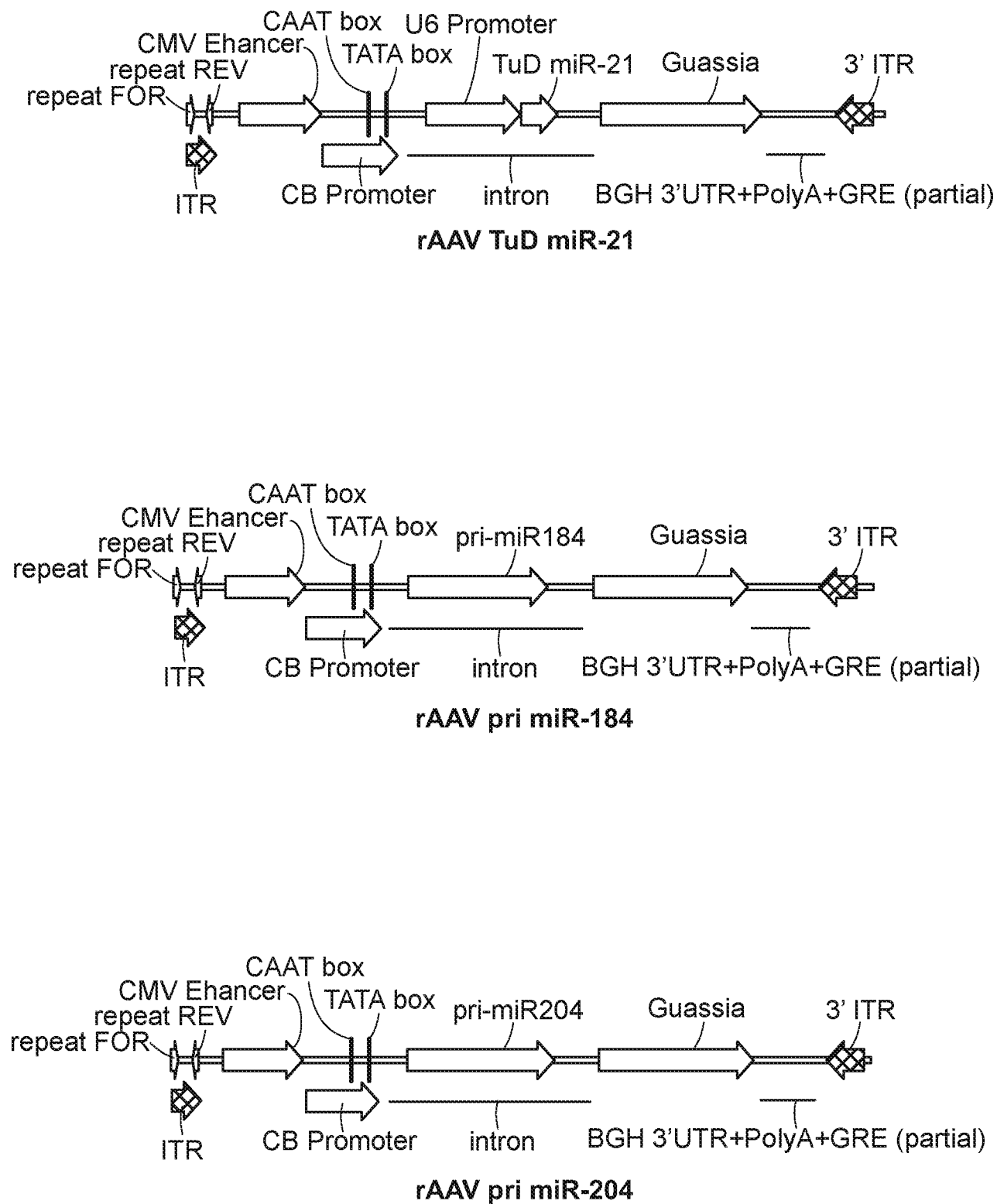


FIG. 1C

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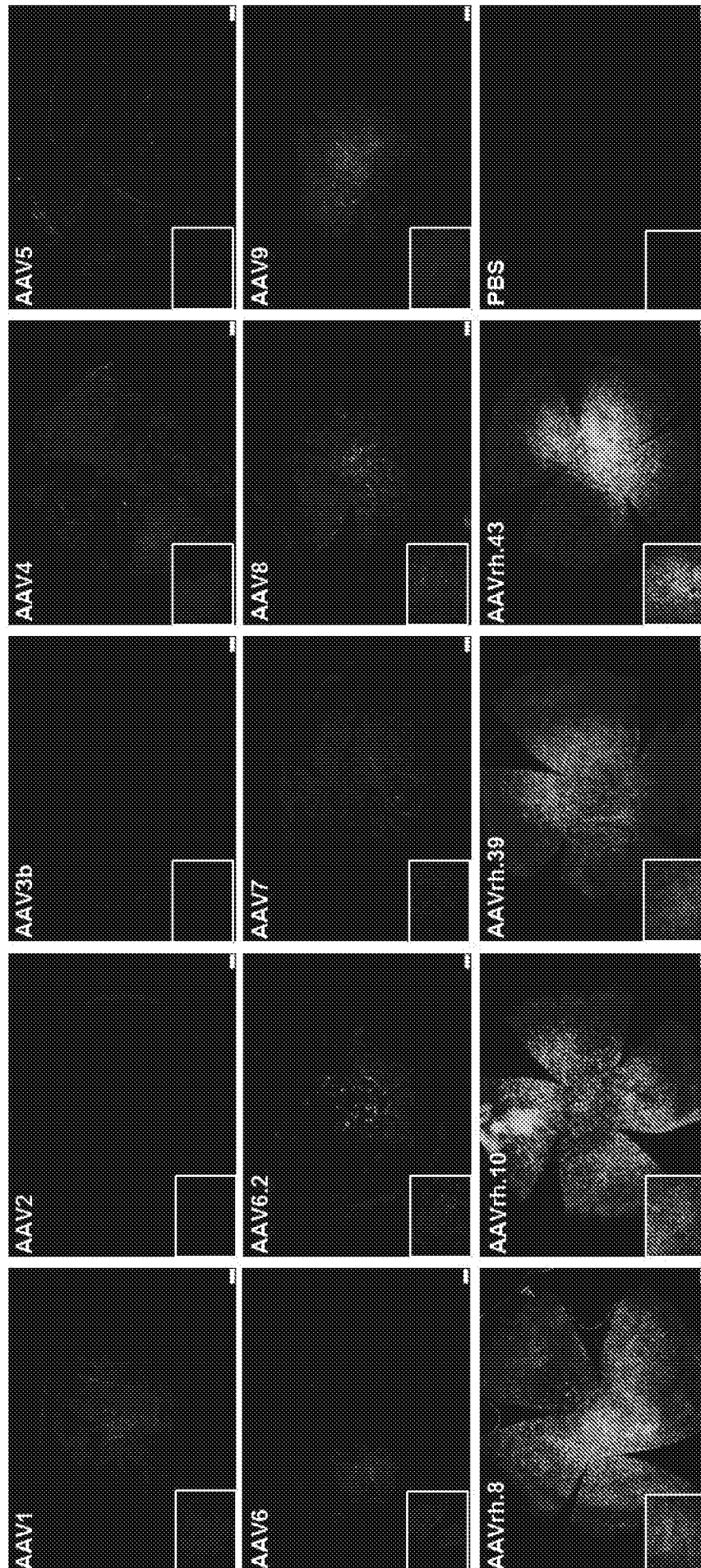


FIG. 2A

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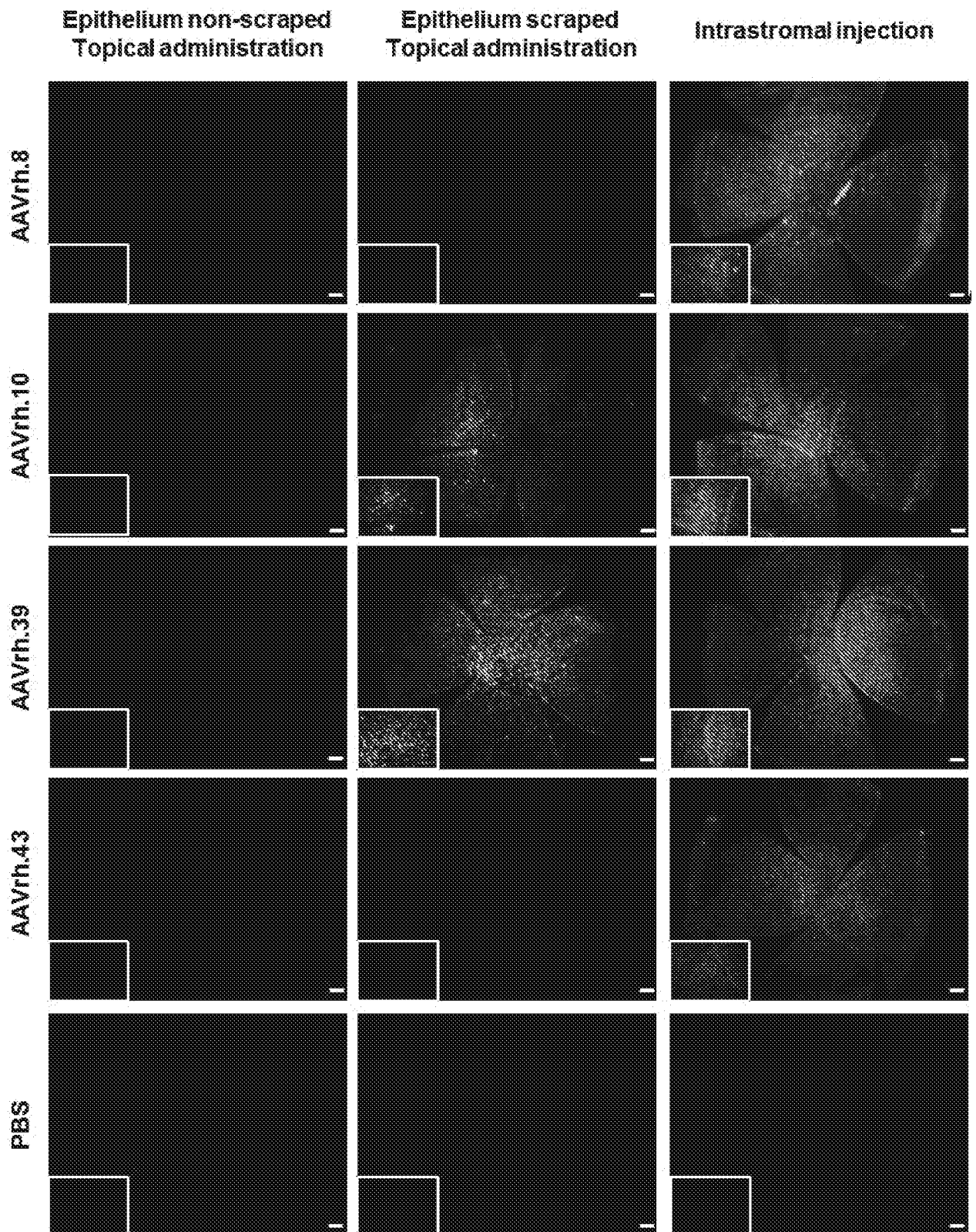


FIG. 2B

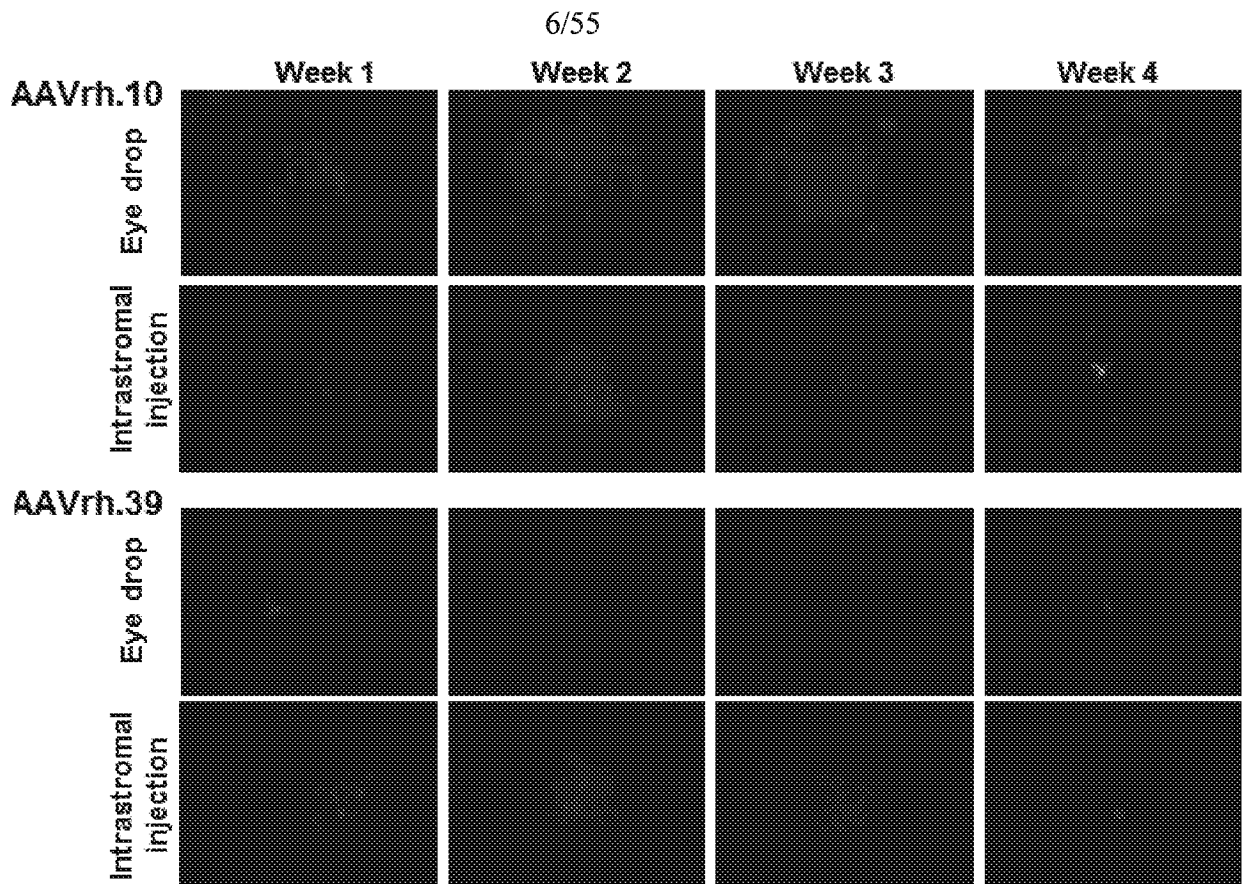


FIG. 2C

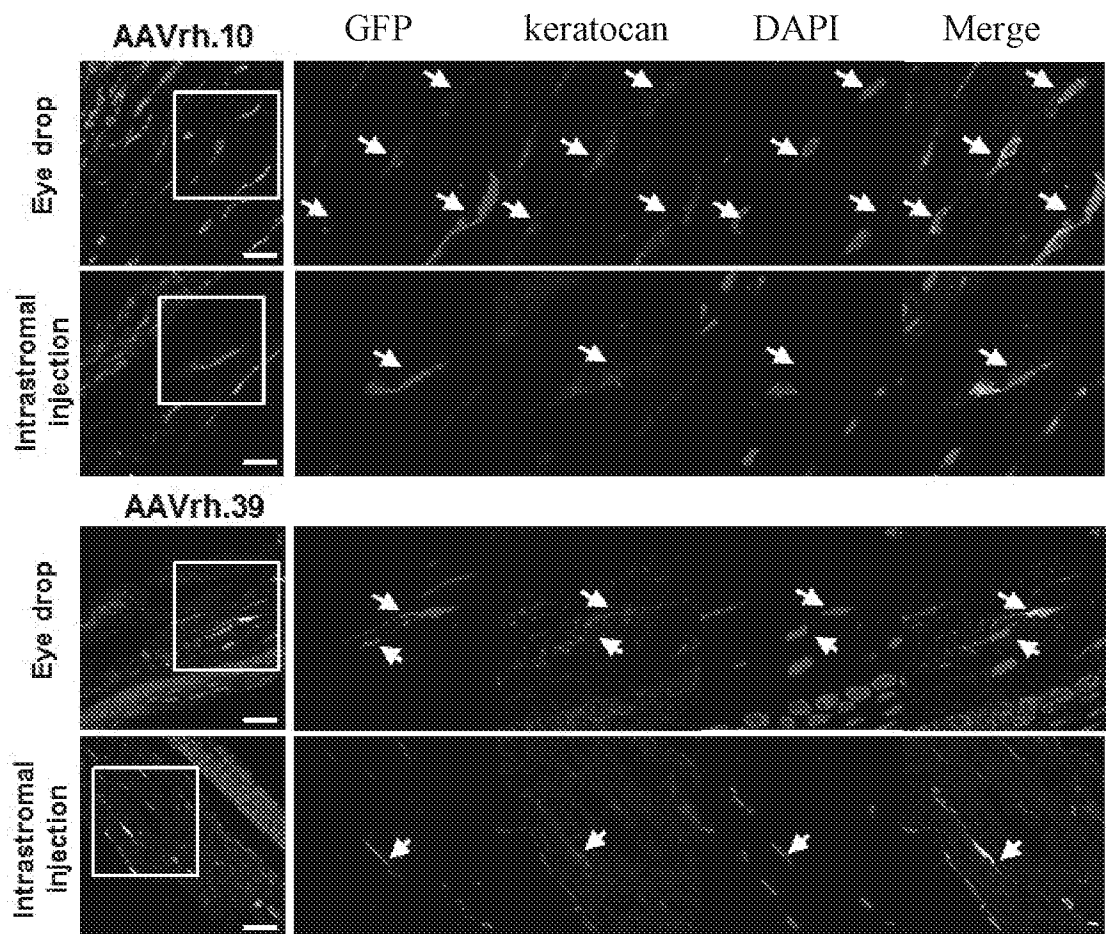
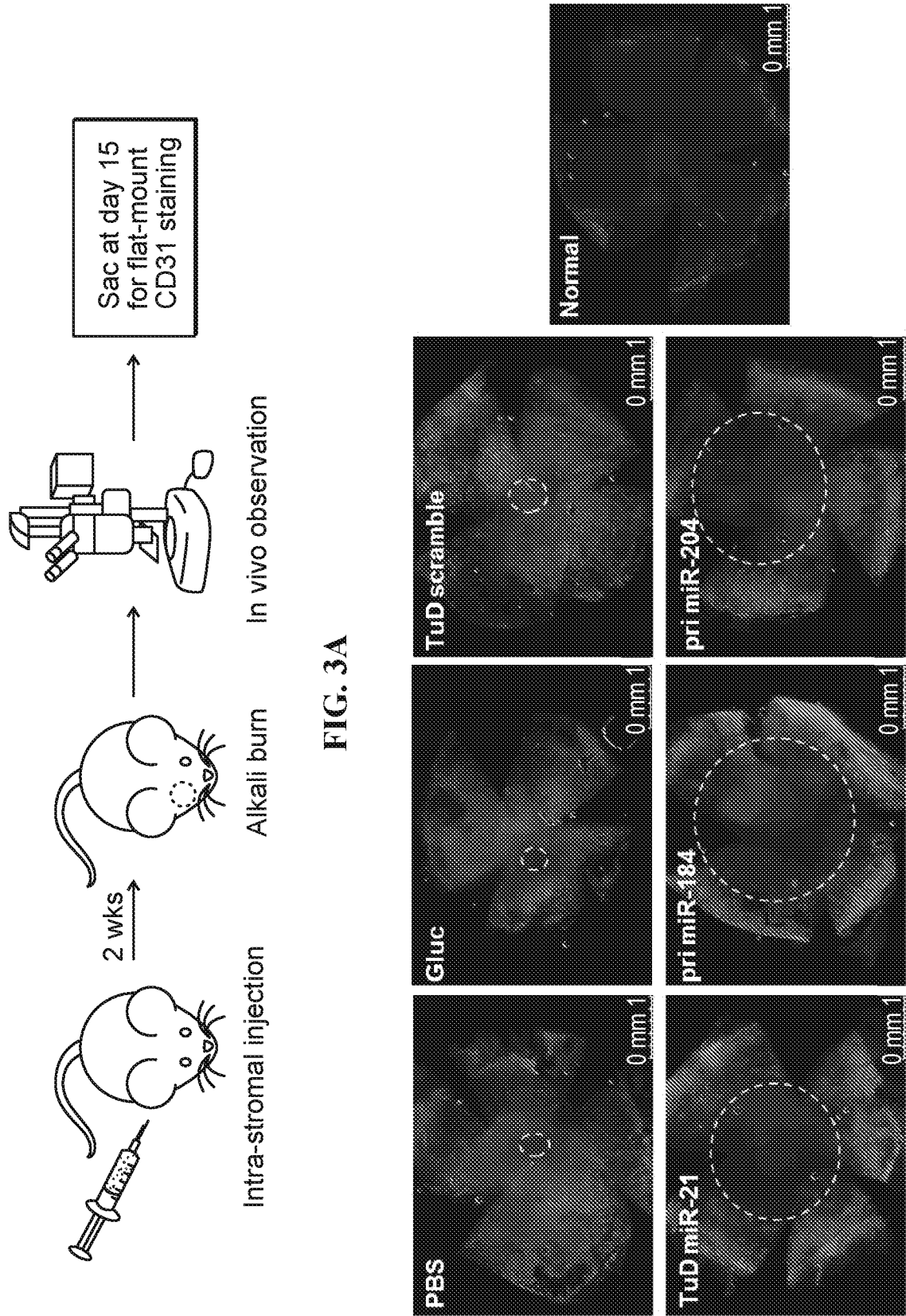


FIG. 2D



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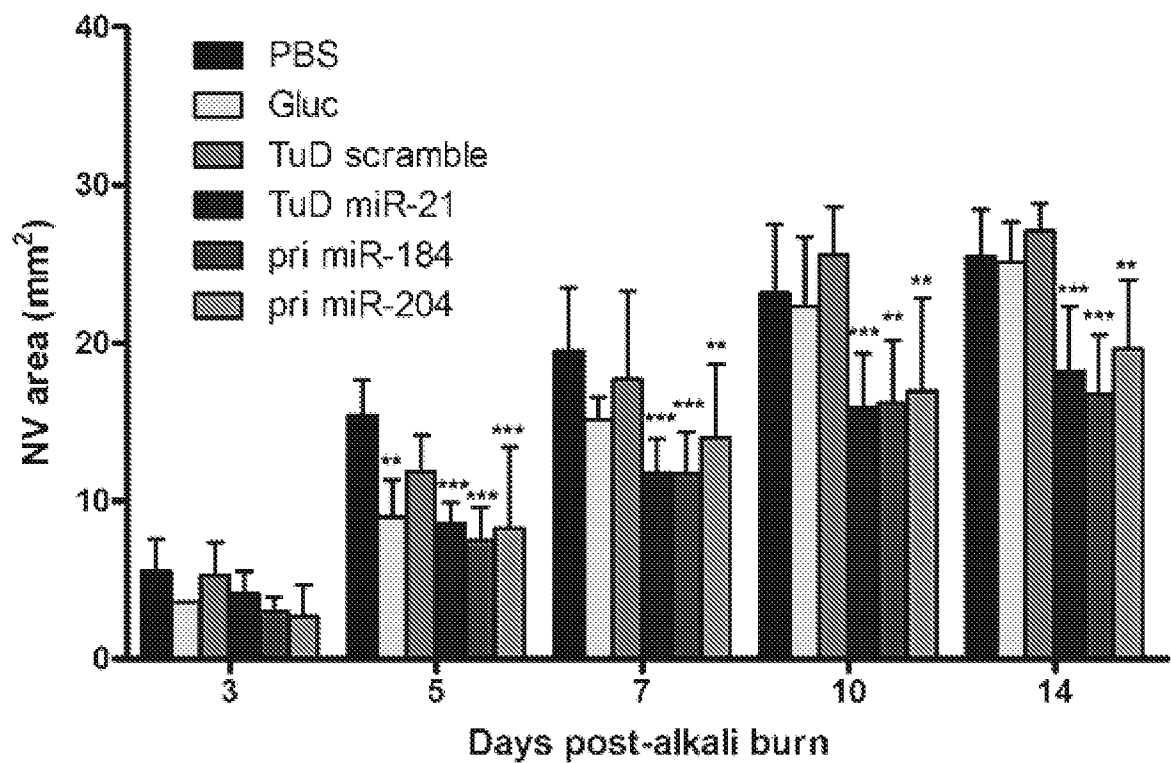


FIG. 3C

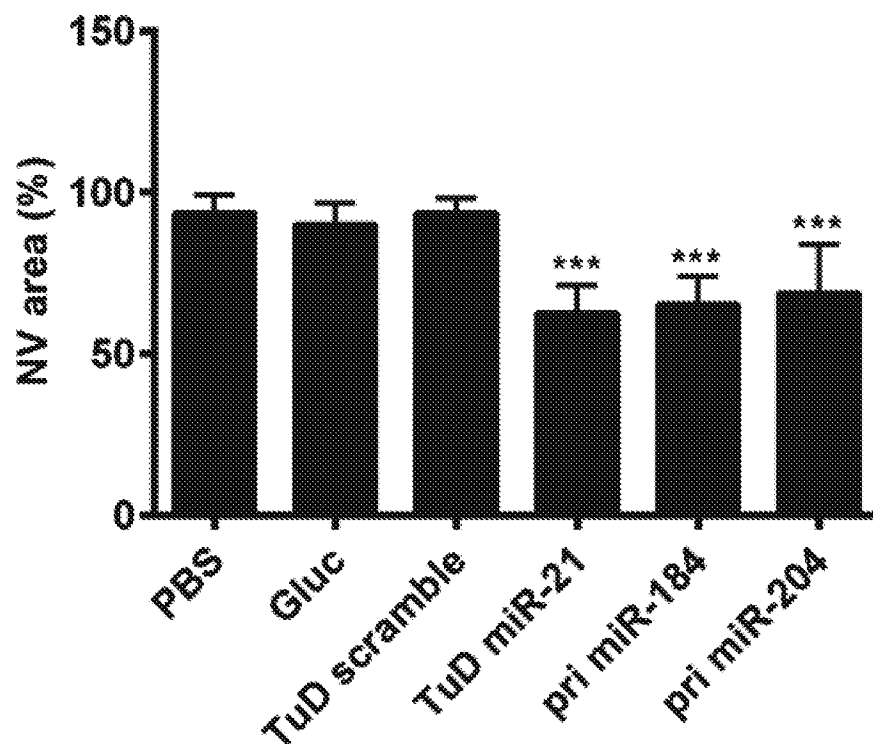


FIG. 3D

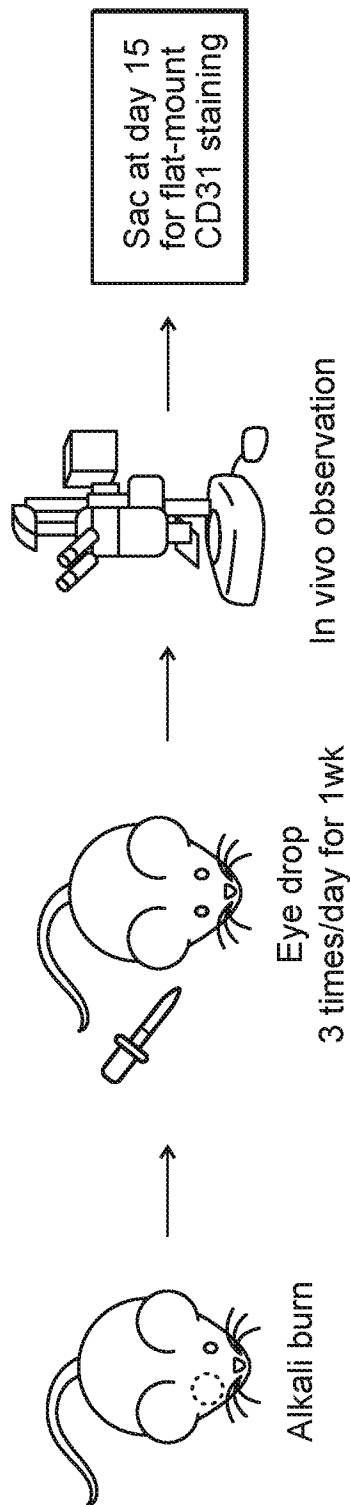


FIG. 4A

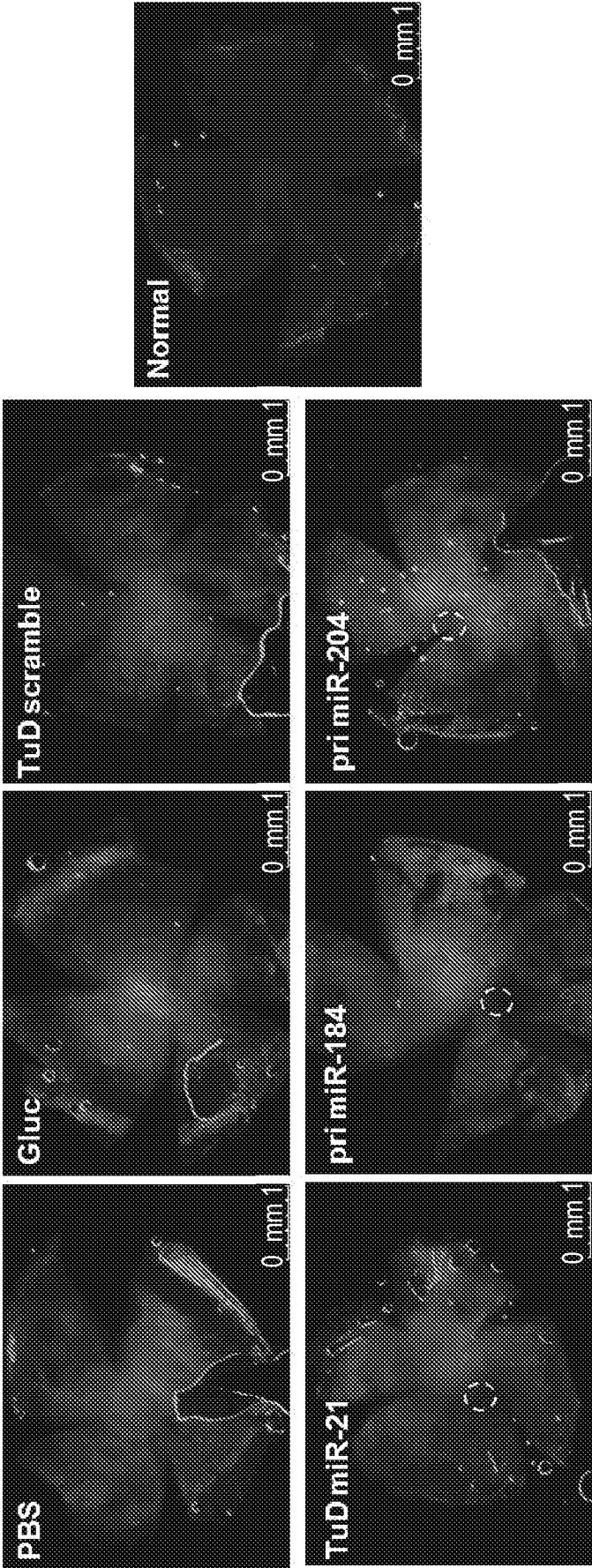


FIG. 4B

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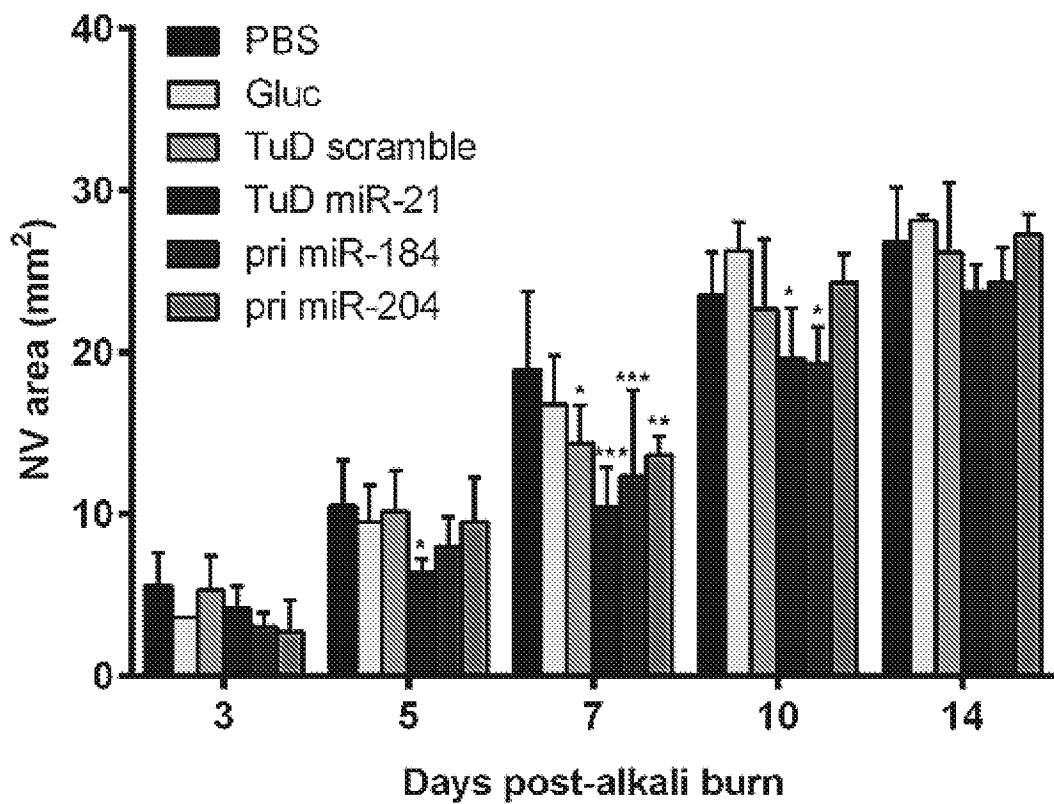


FIG. 4C

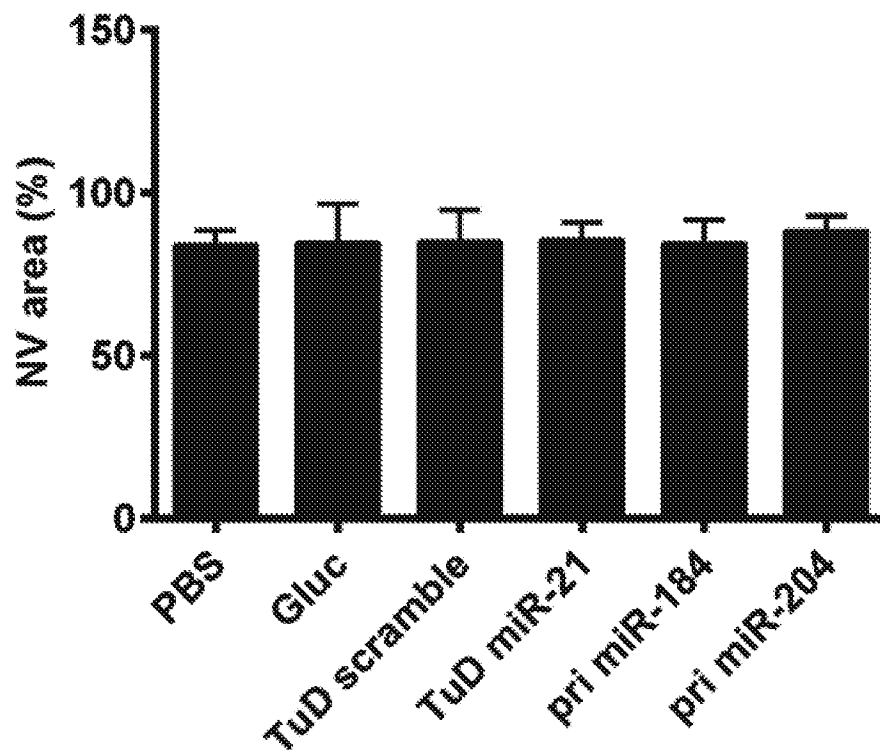


FIG. 4D

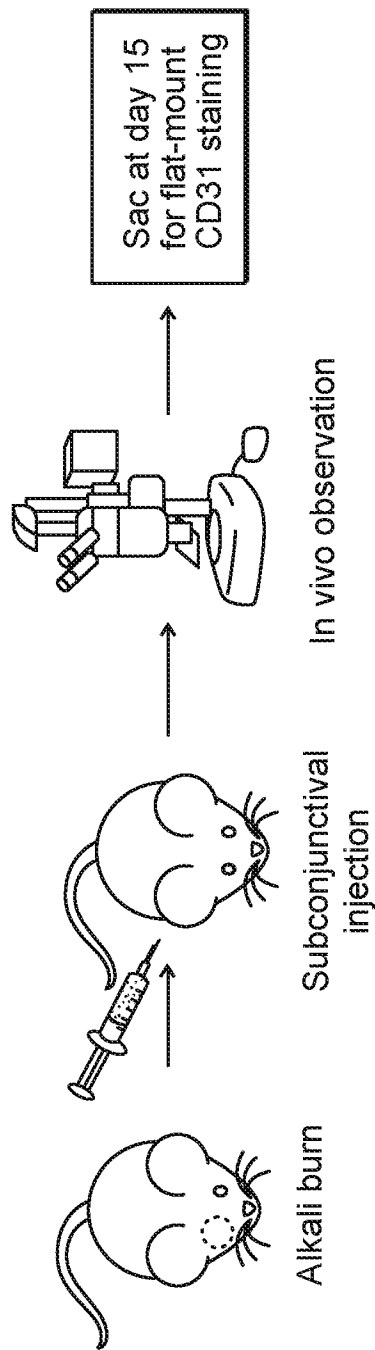


FIG. 5A

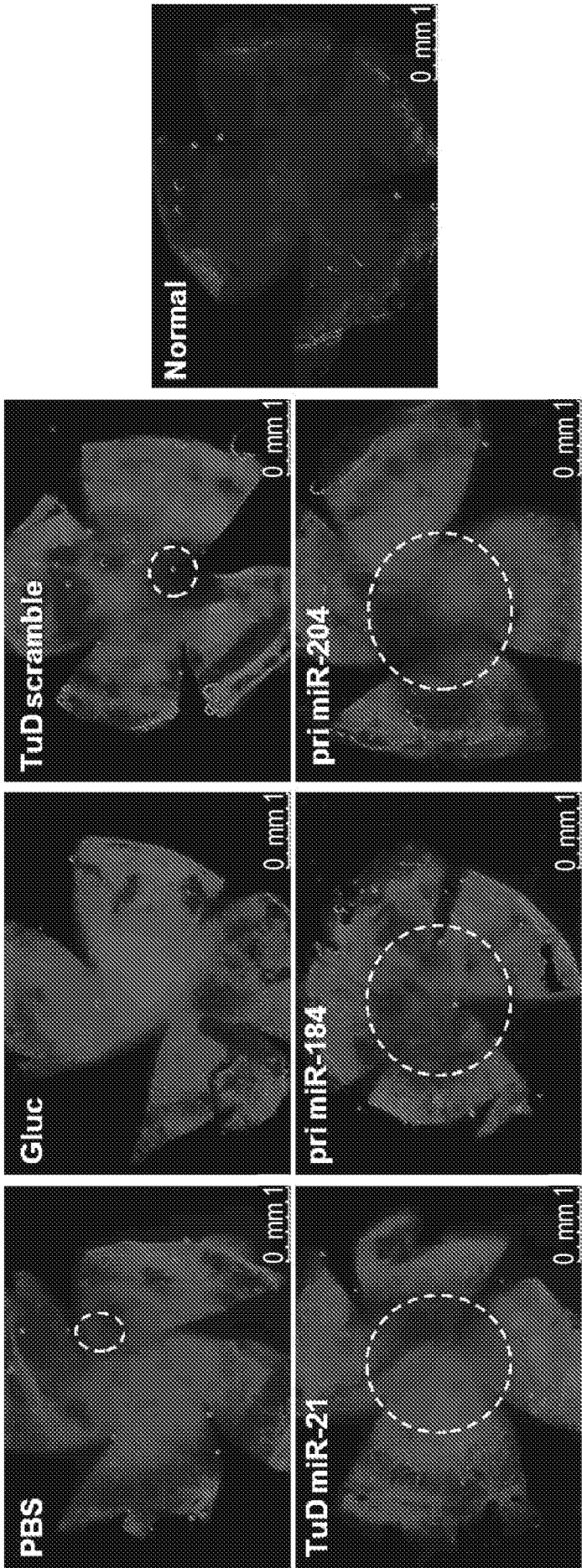


FIG. 5B

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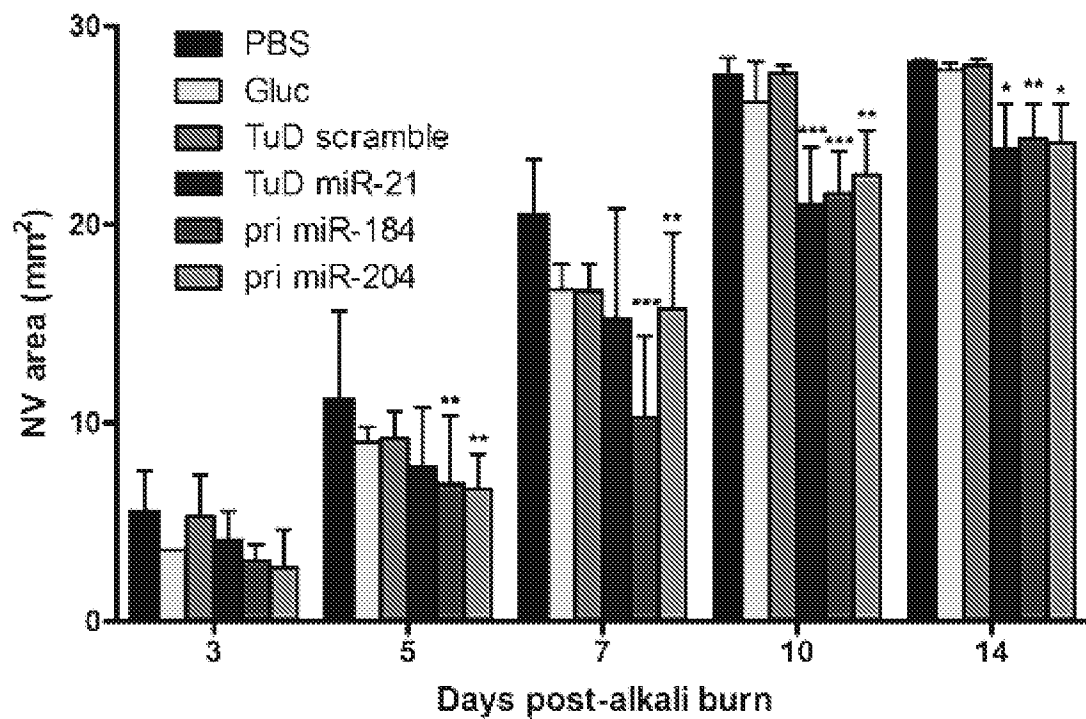


FIG. 5C

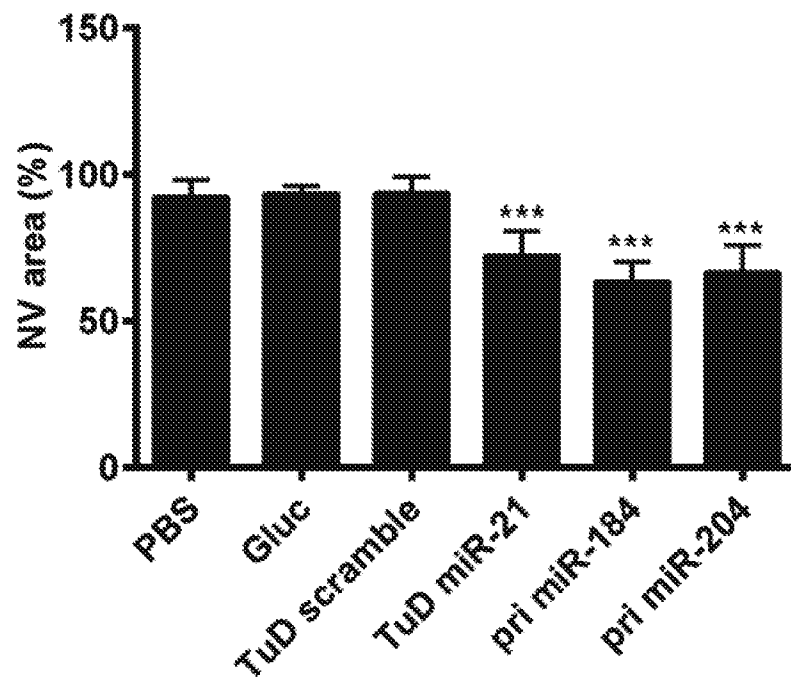


FIG. 5D

FIG. 6A

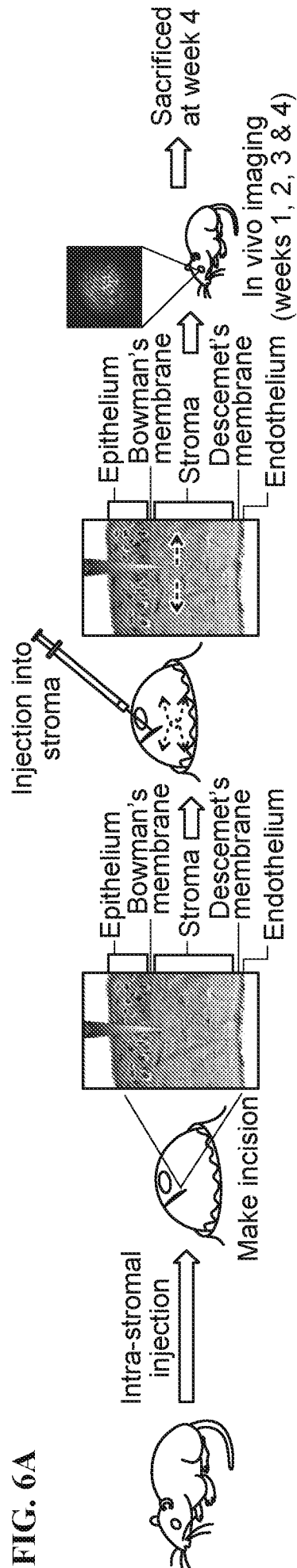
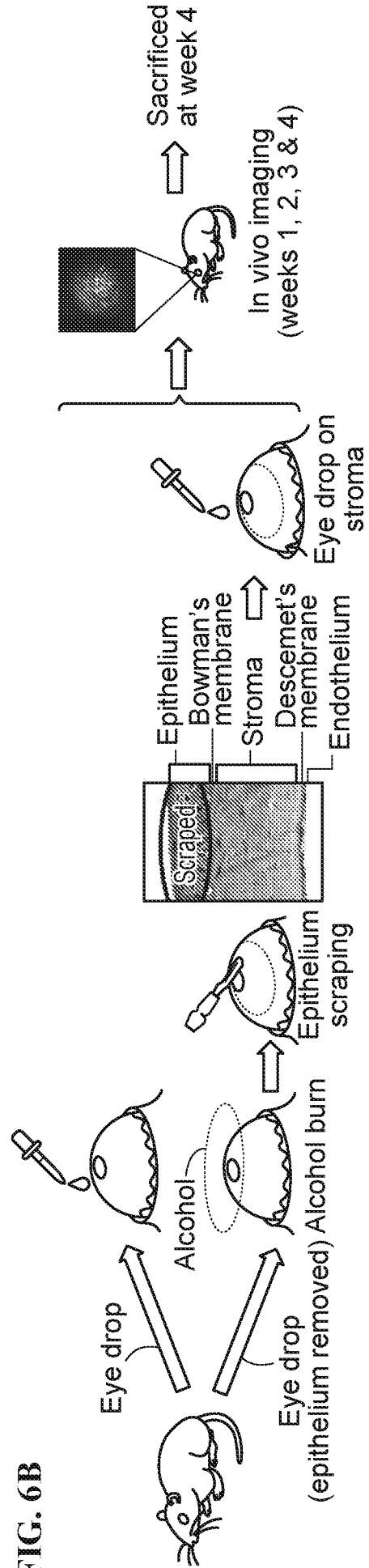


FIG. 6B



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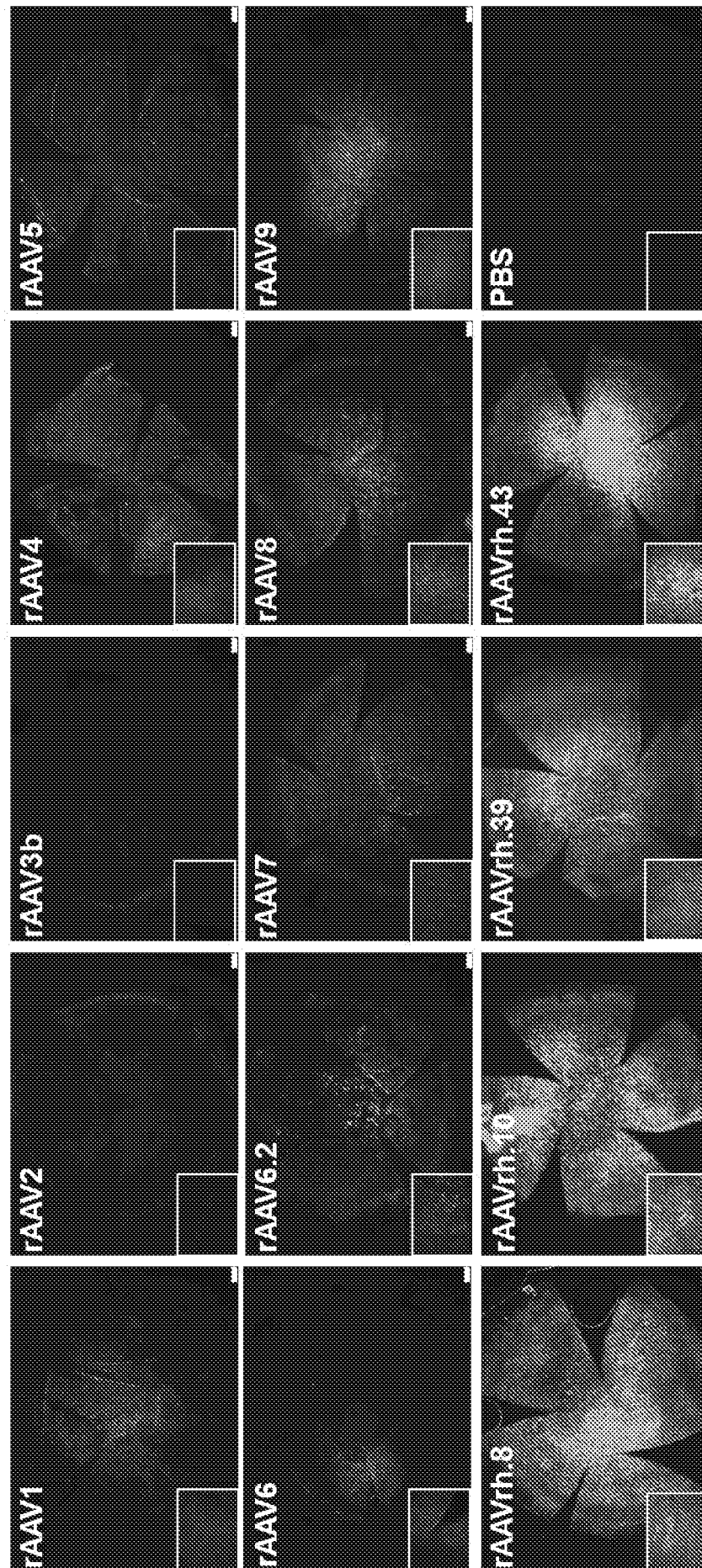


FIG. 7A

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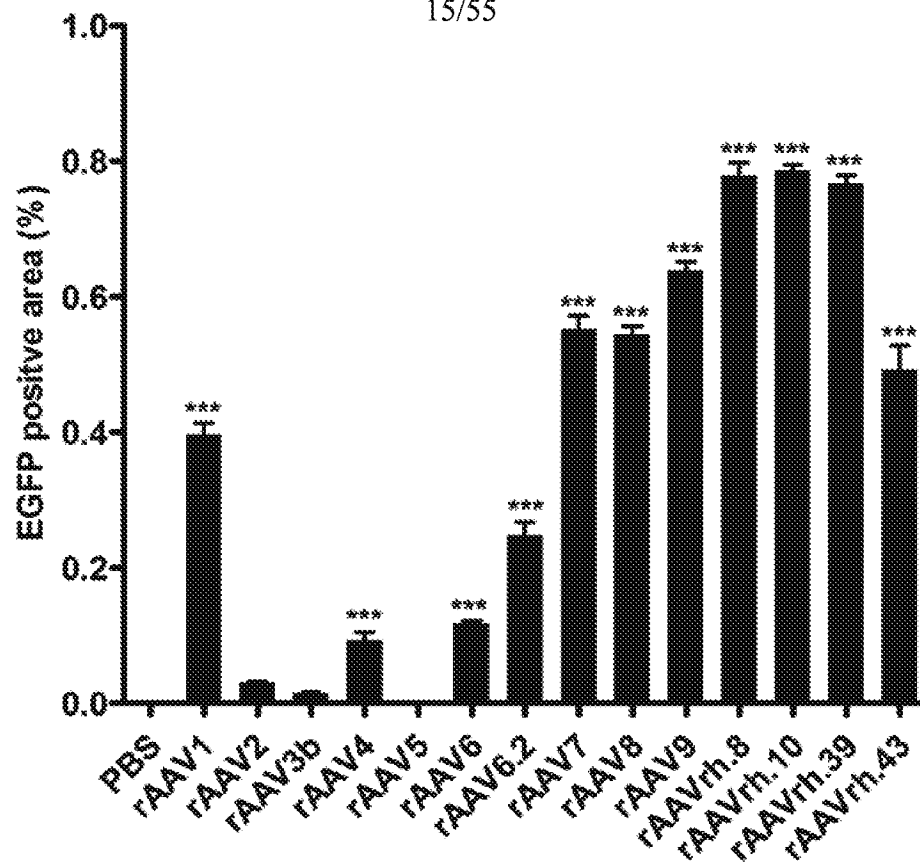


FIG. 7B

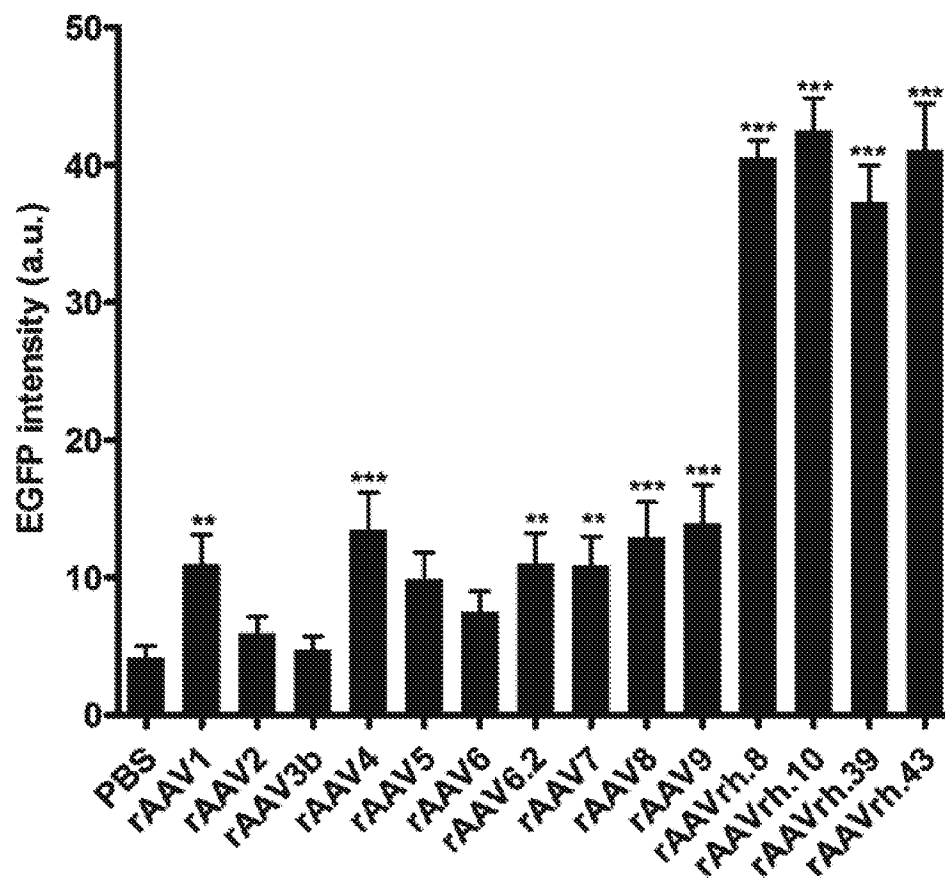


FIG. 7C

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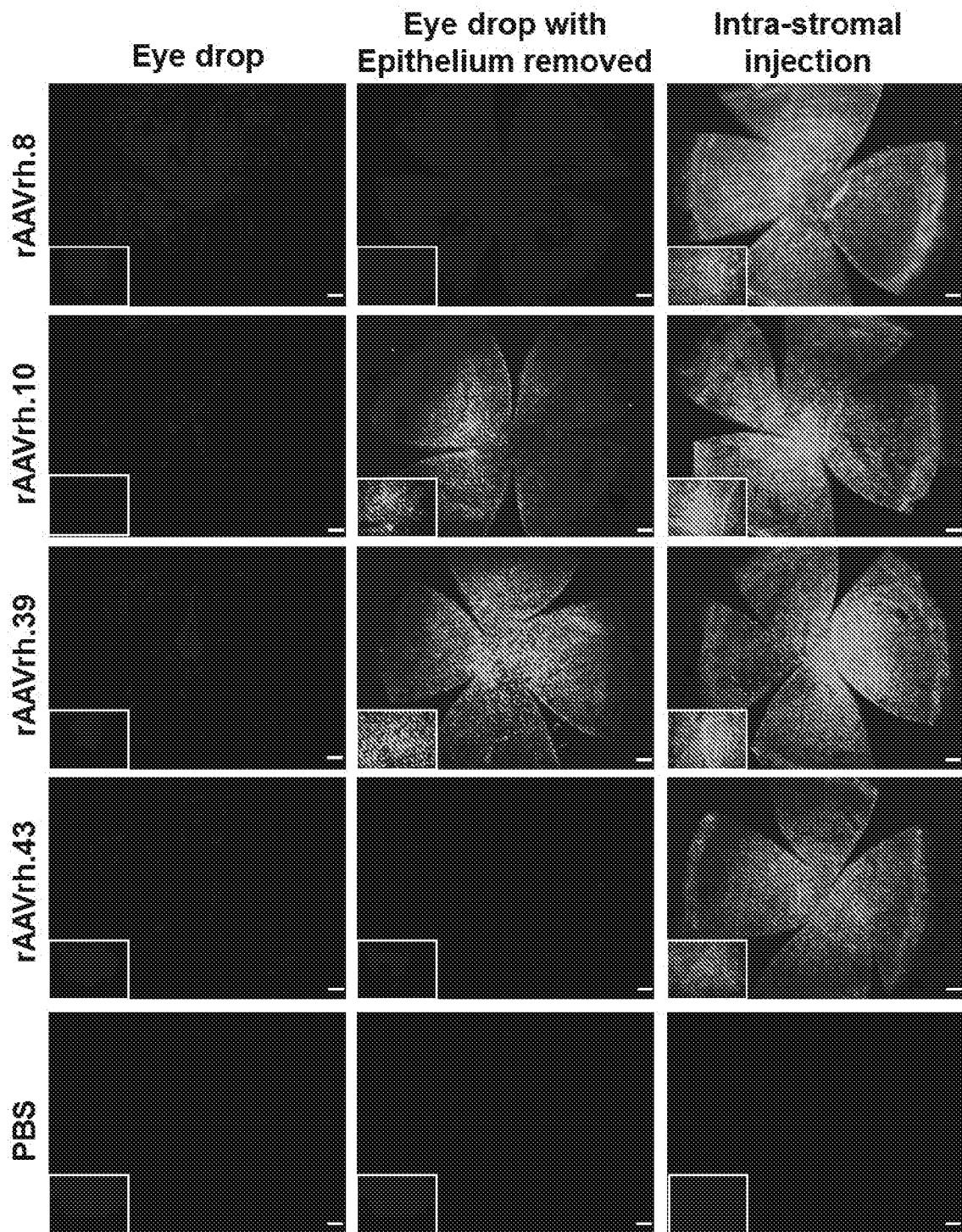


FIG. 8A

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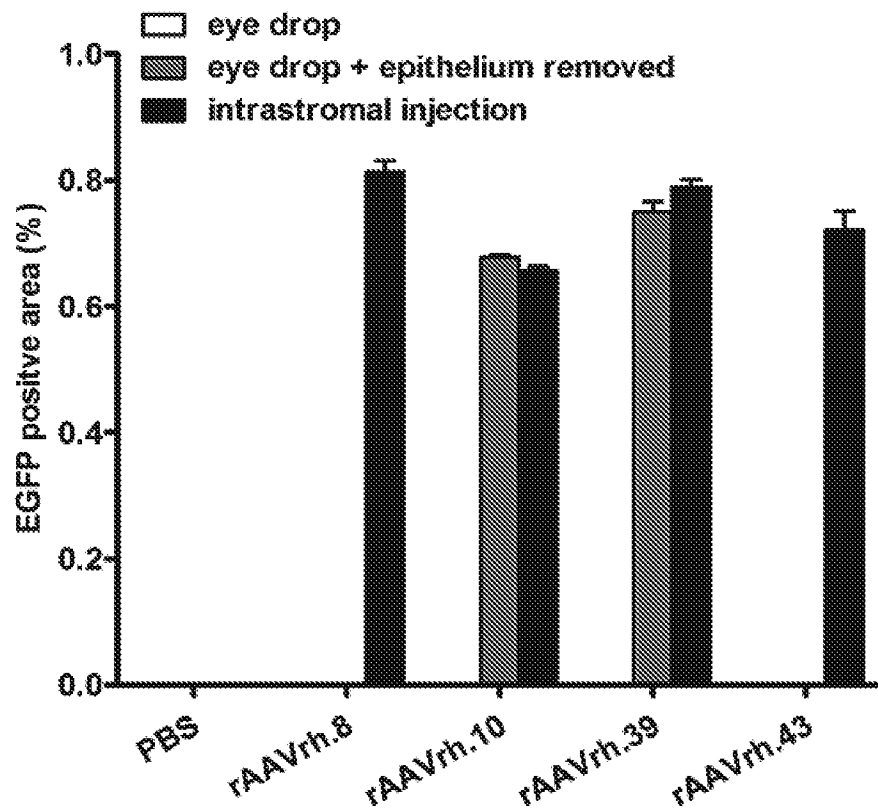


FIG. 8B

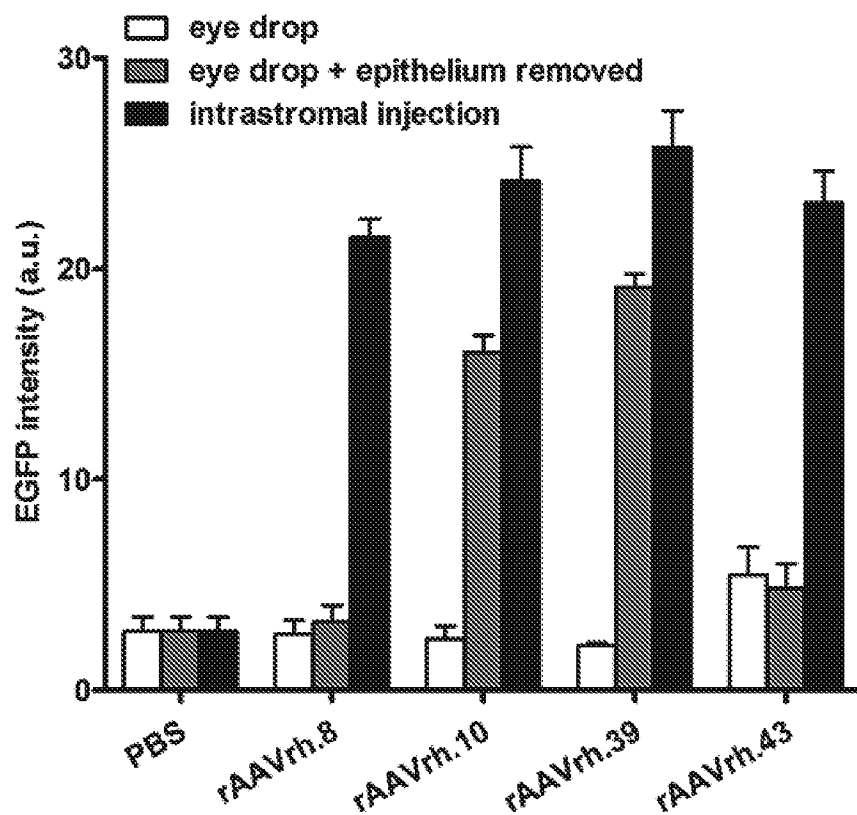


FIG. 8C

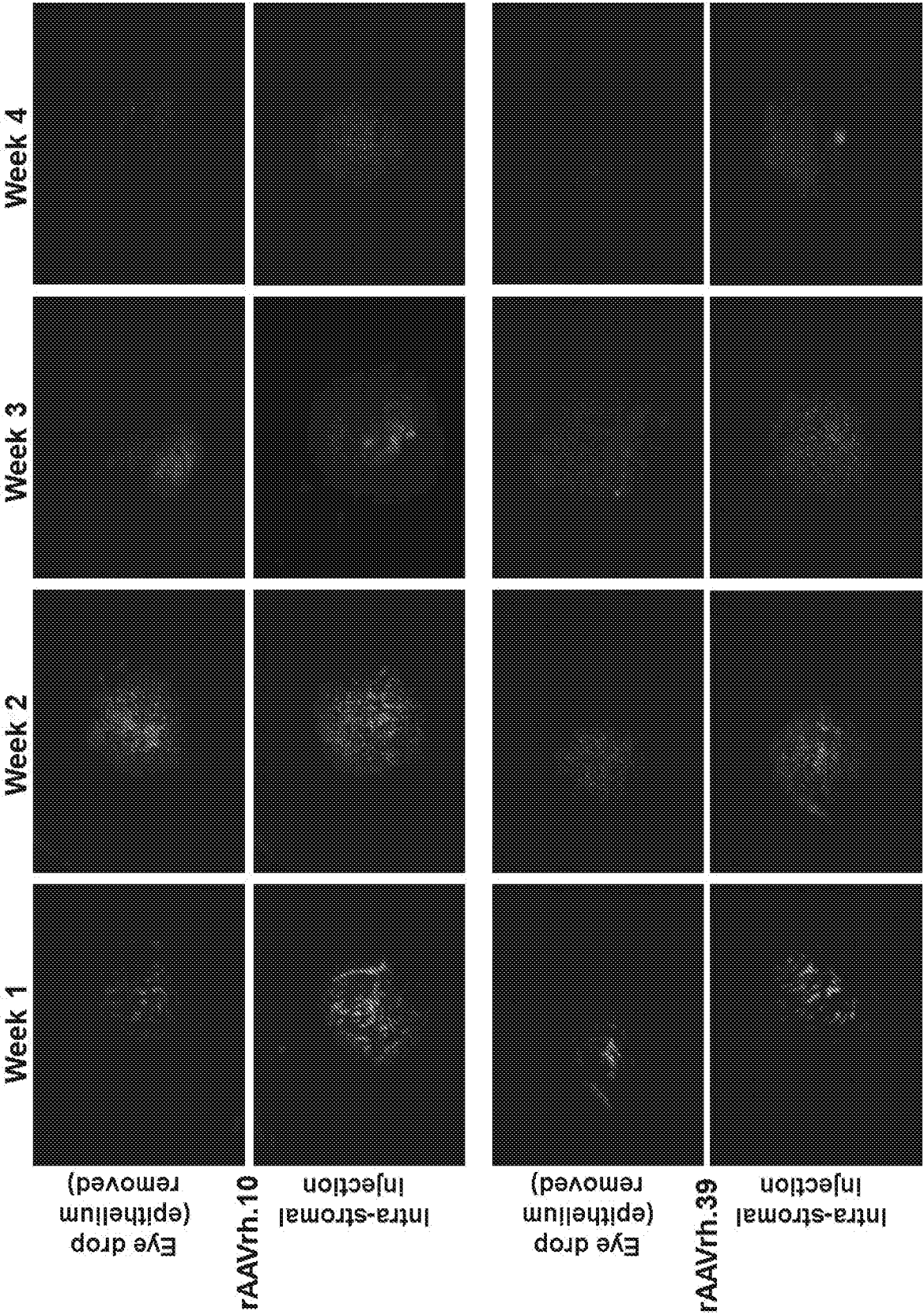


FIG. 9A

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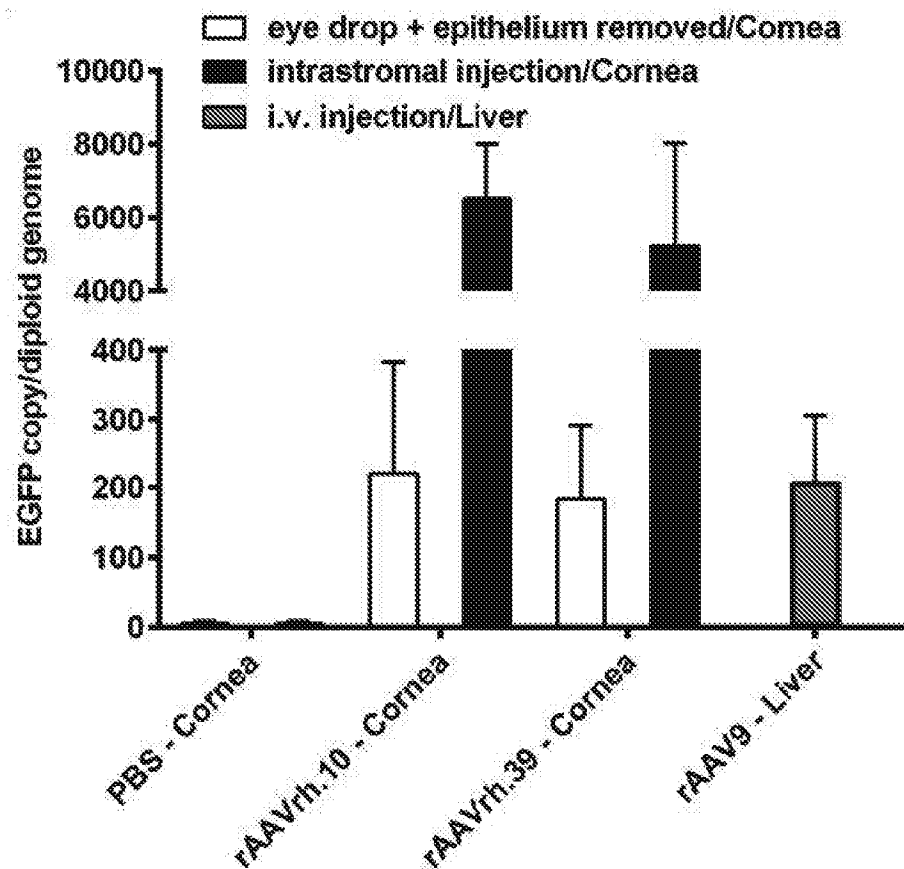


FIG. 9B

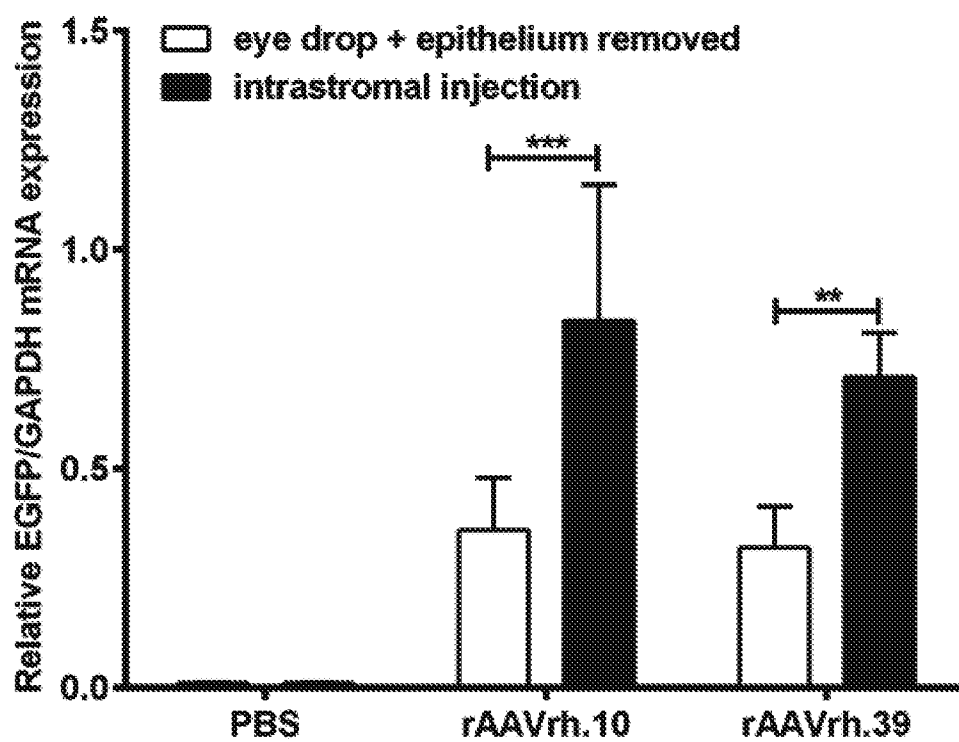


FIG. 9C

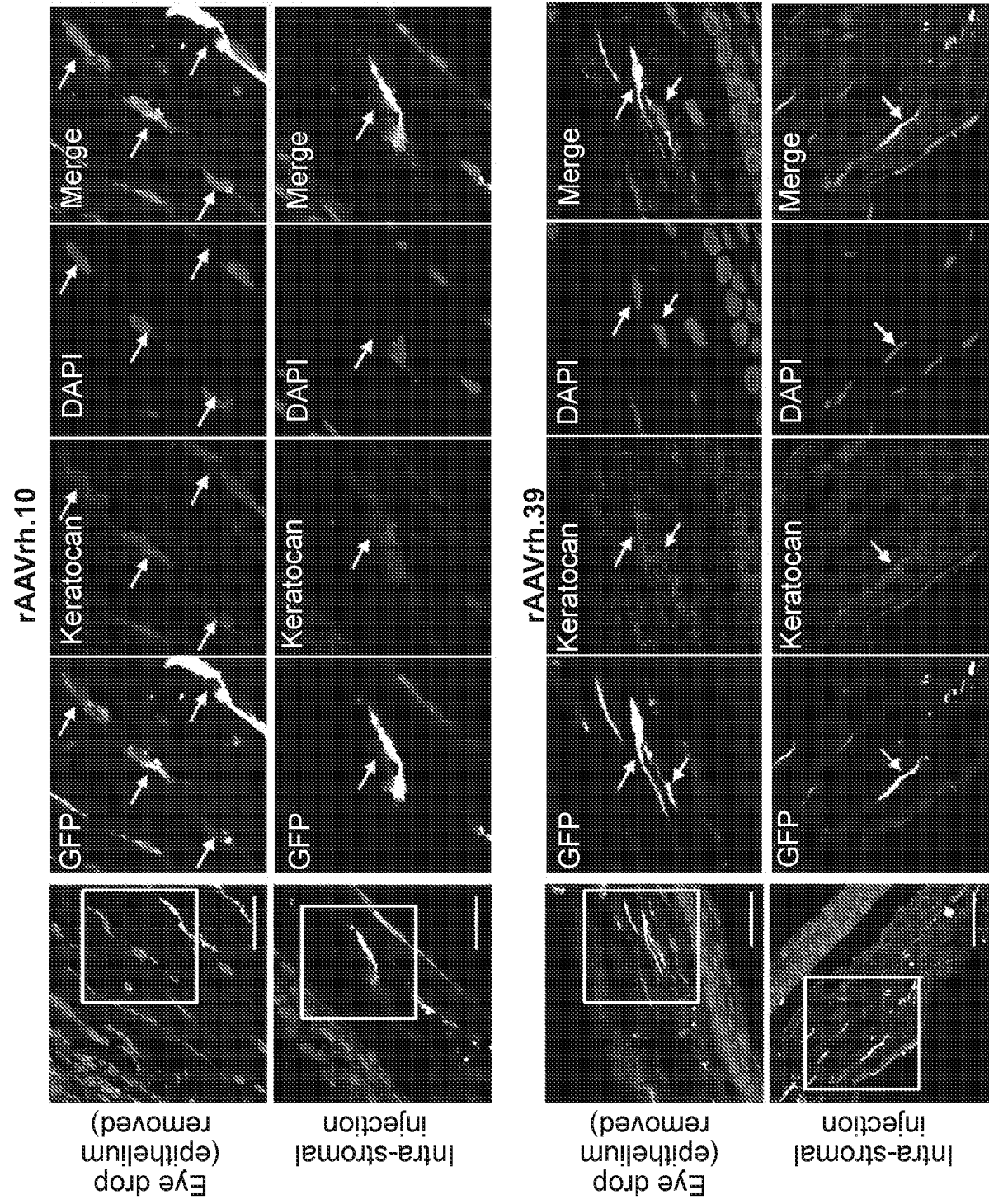


FIG. 10A

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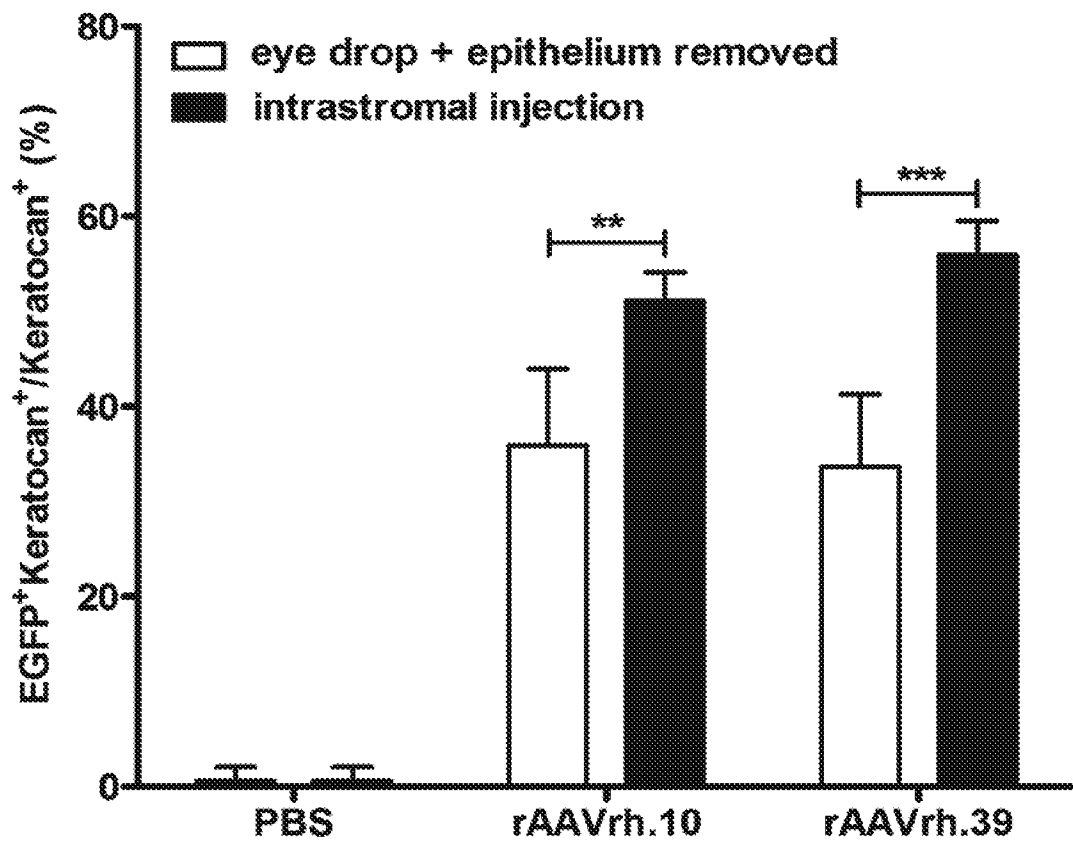


FIG. 10B

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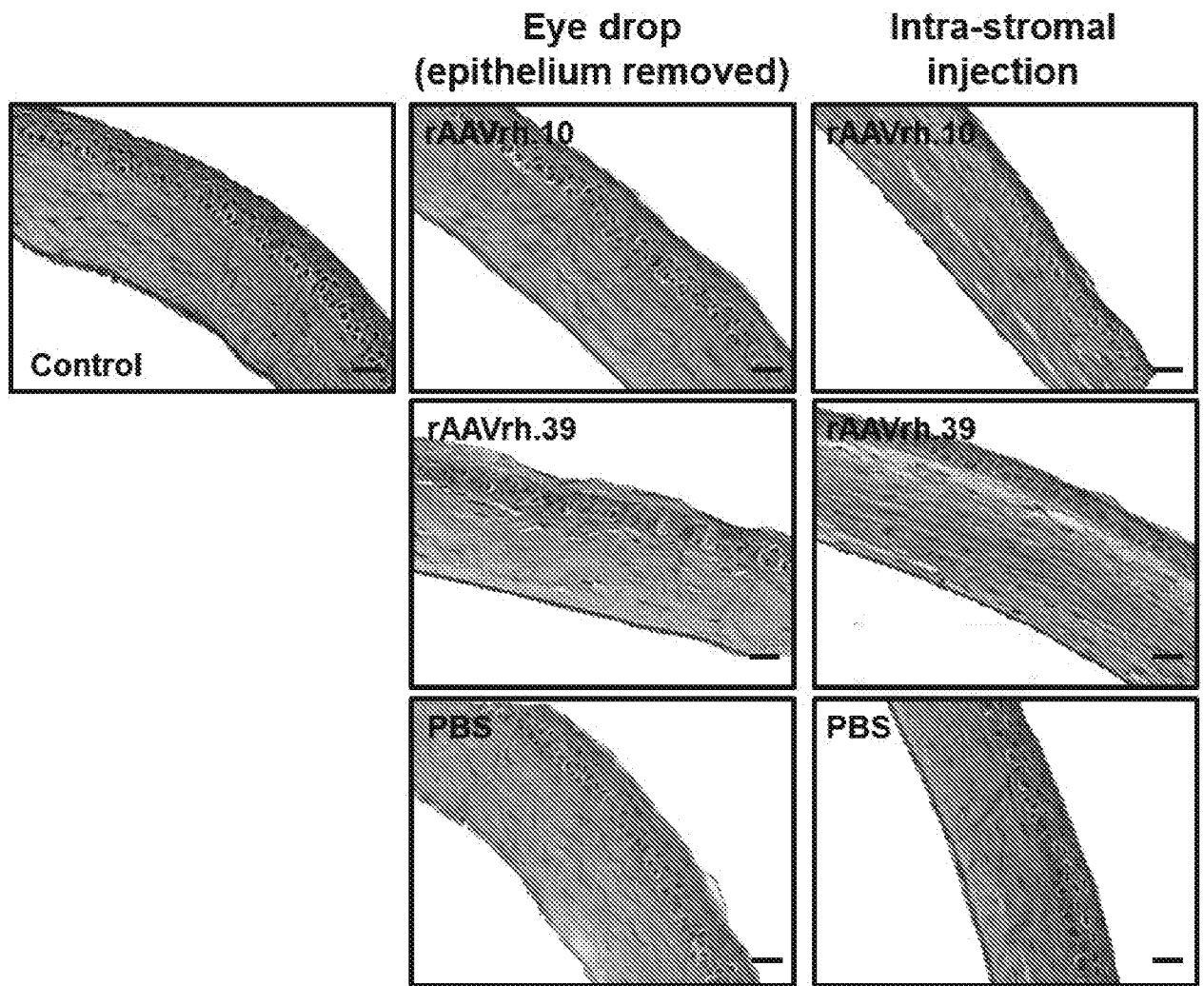


FIG. 11

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Days after alkali burn

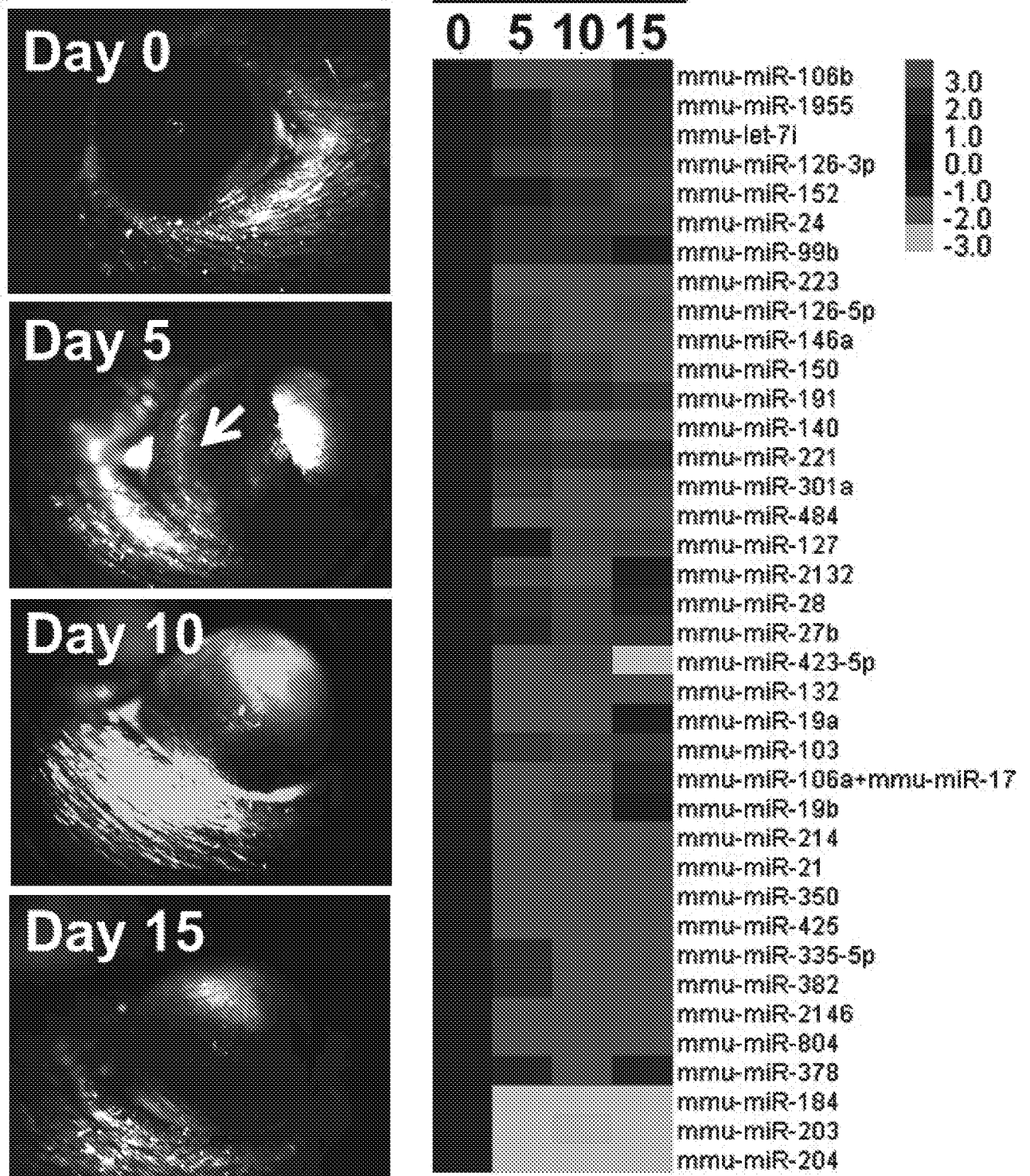
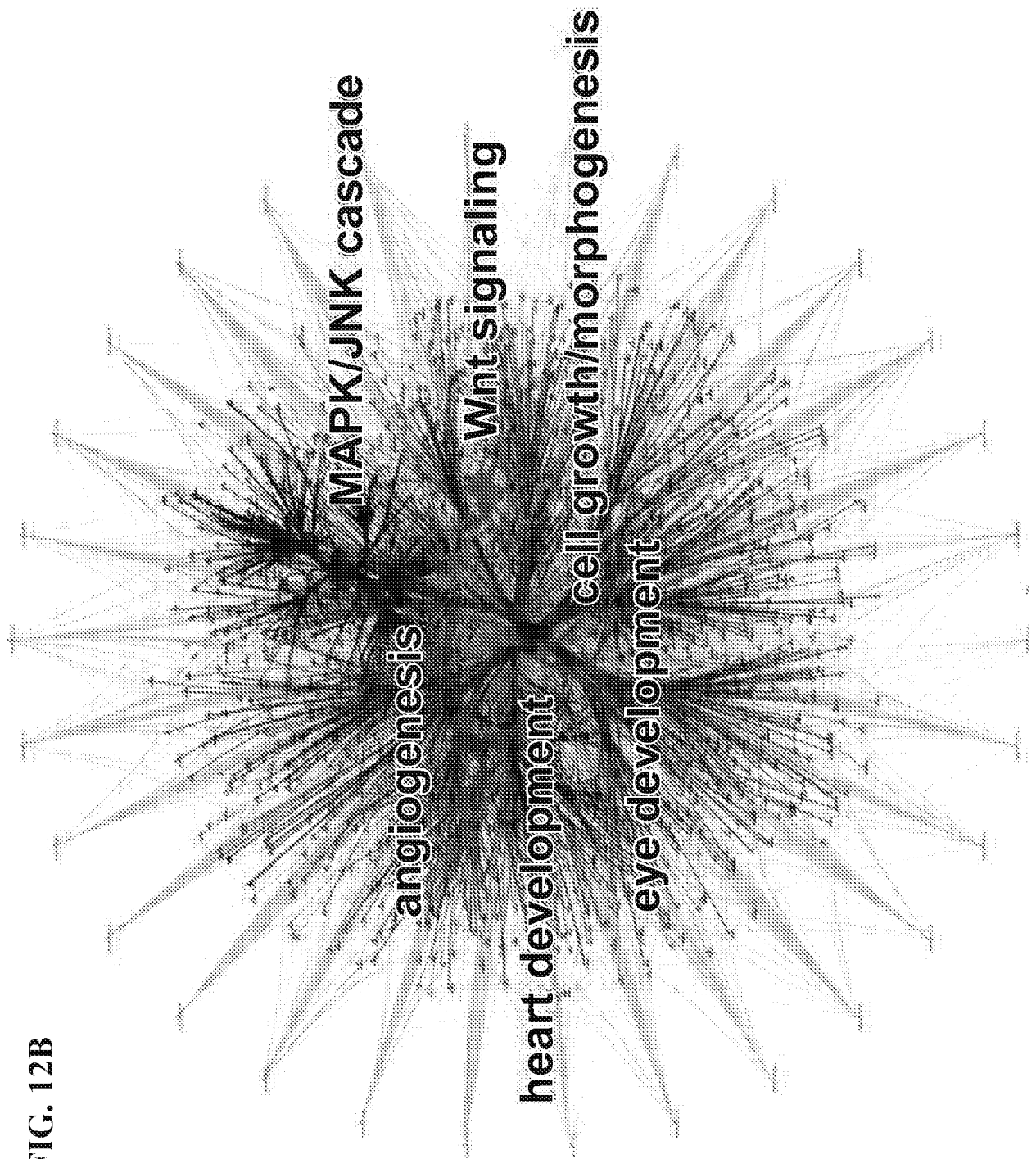


FIG. 12A

FIG. 12B



miR-204

miR-184

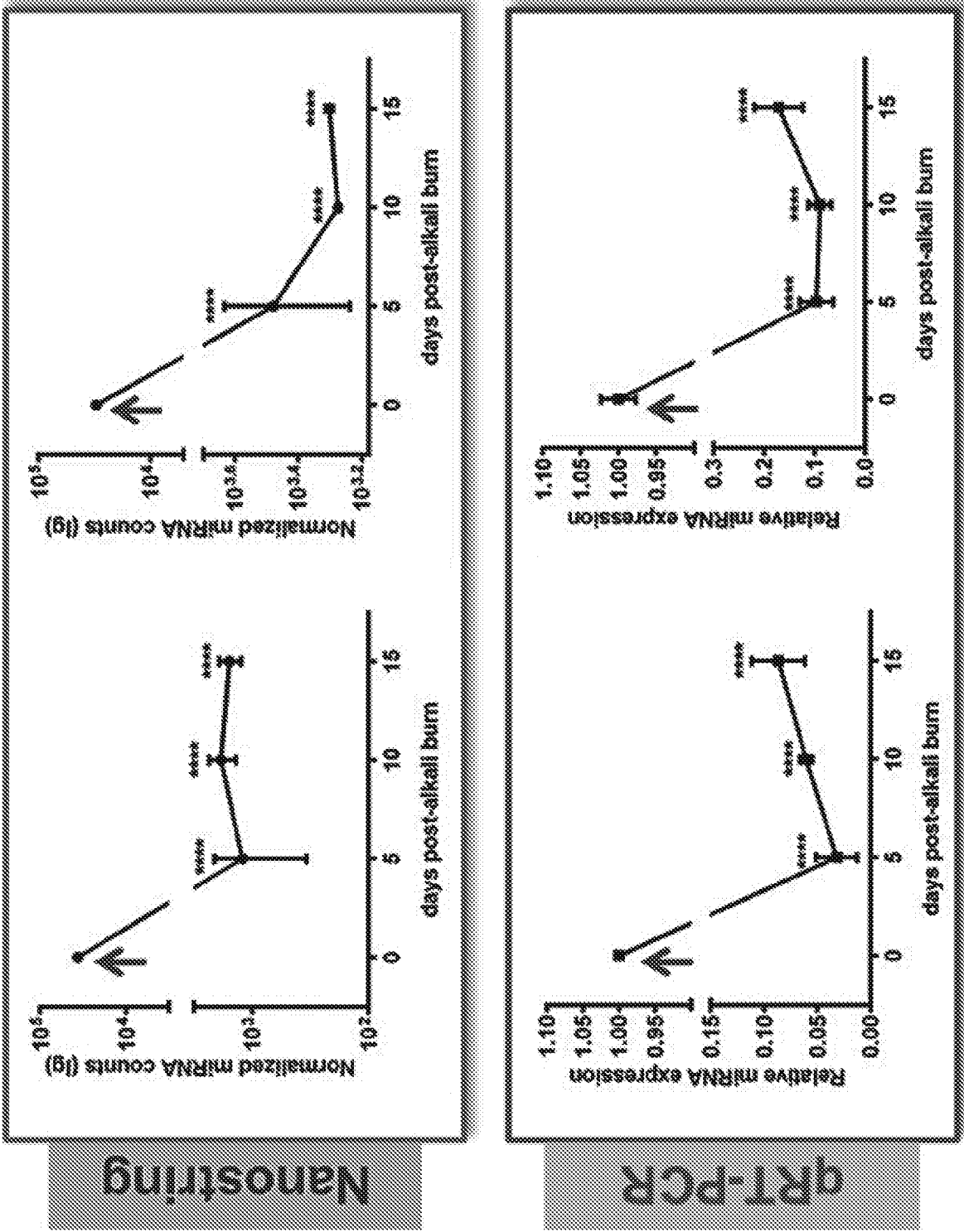
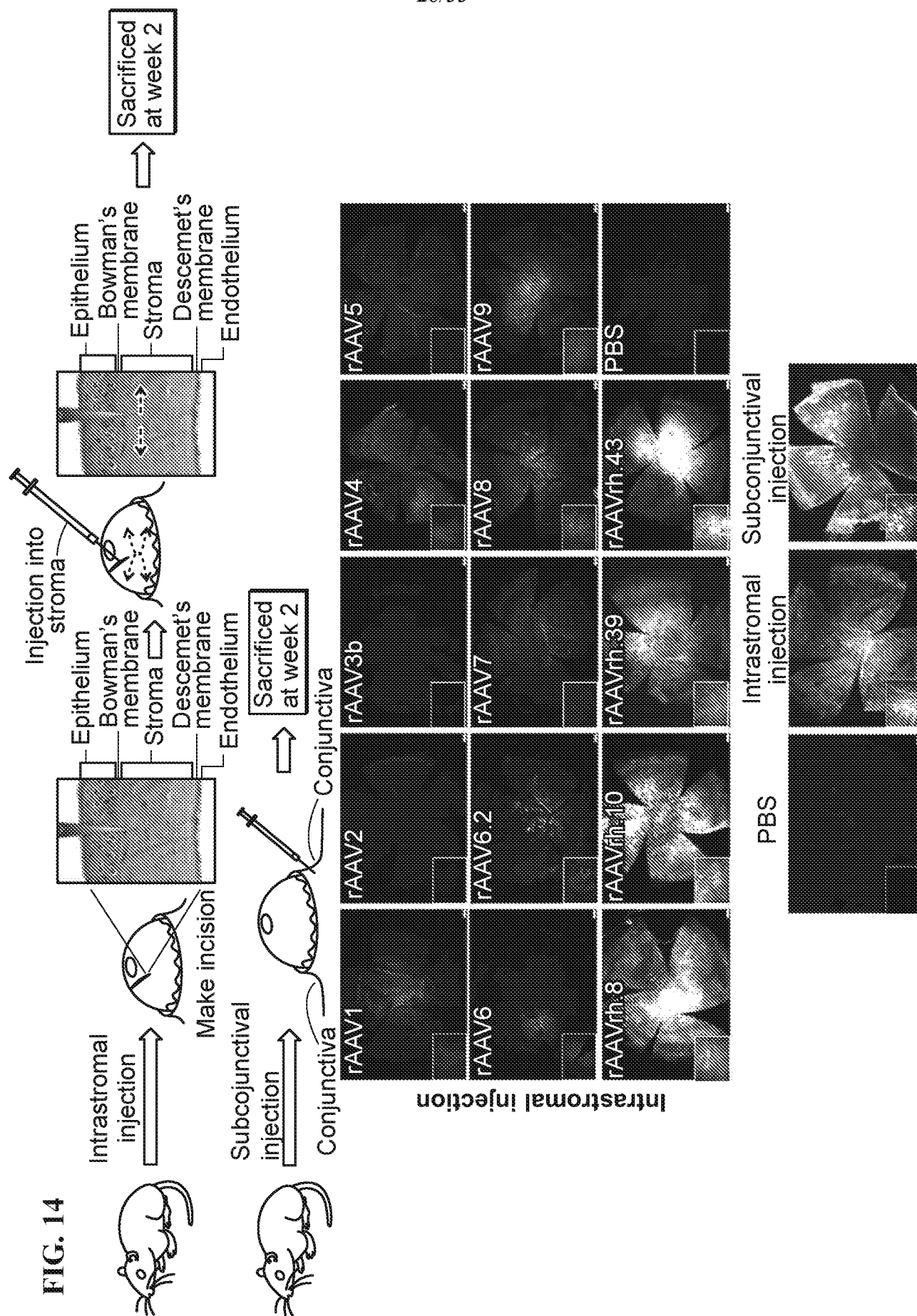


FIG. 13

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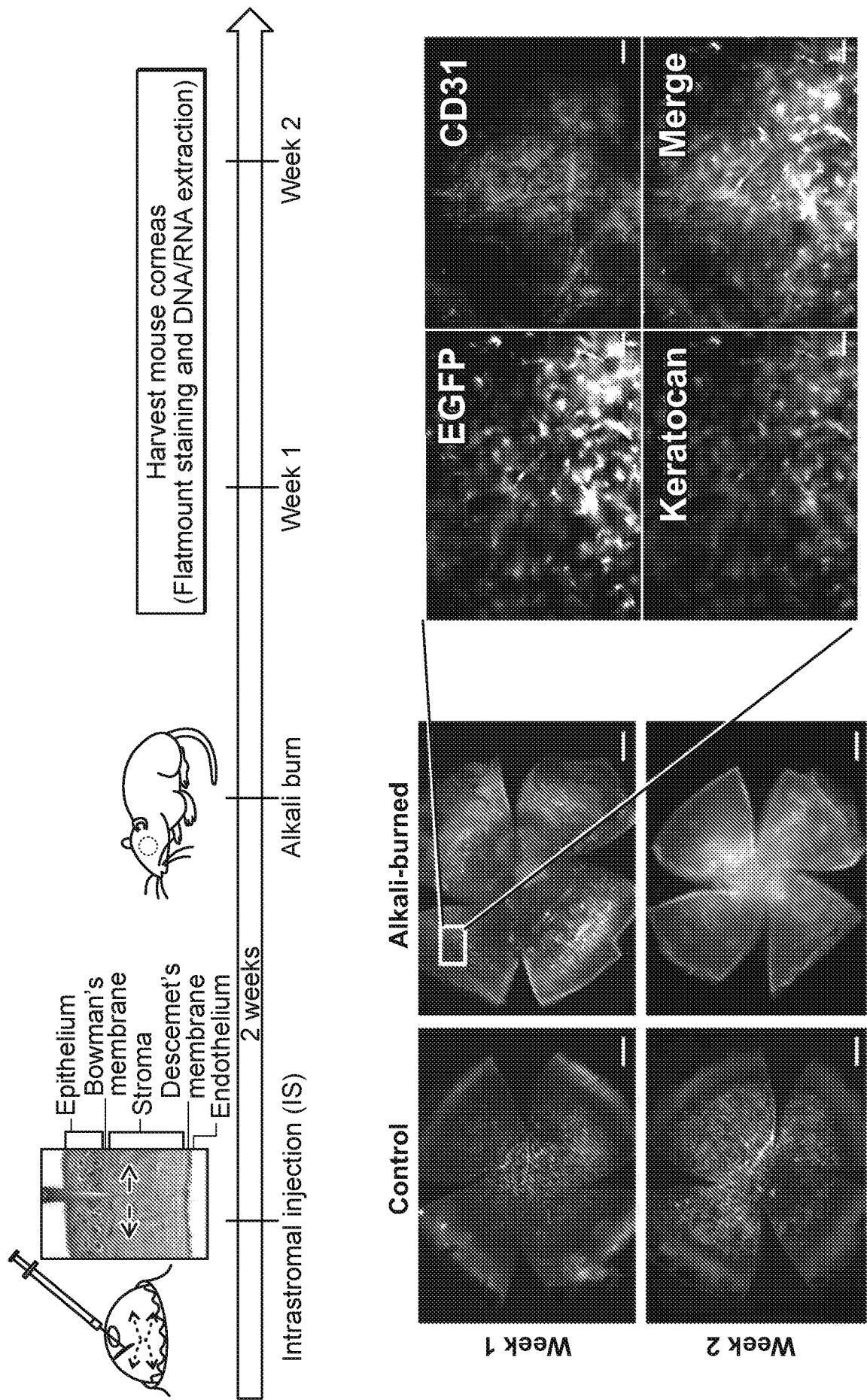
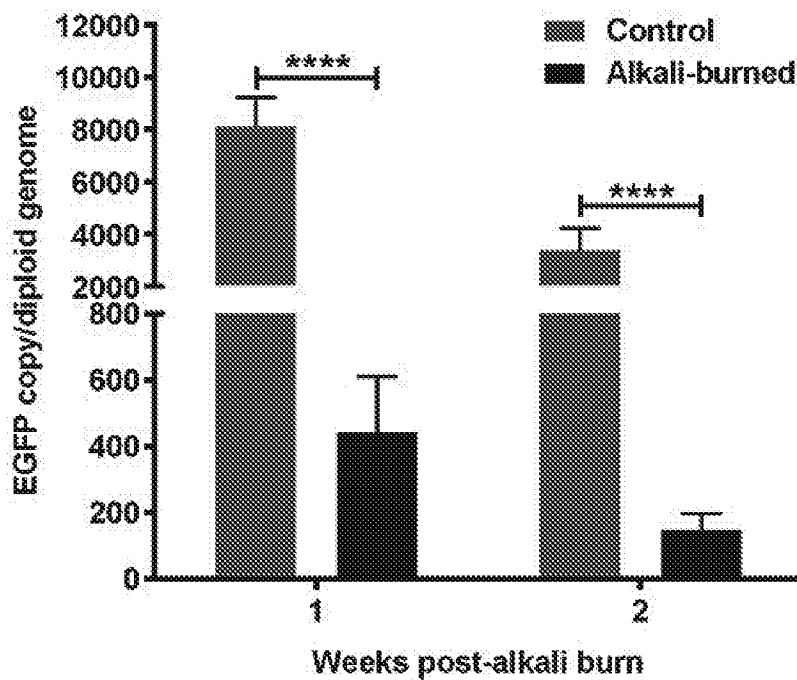
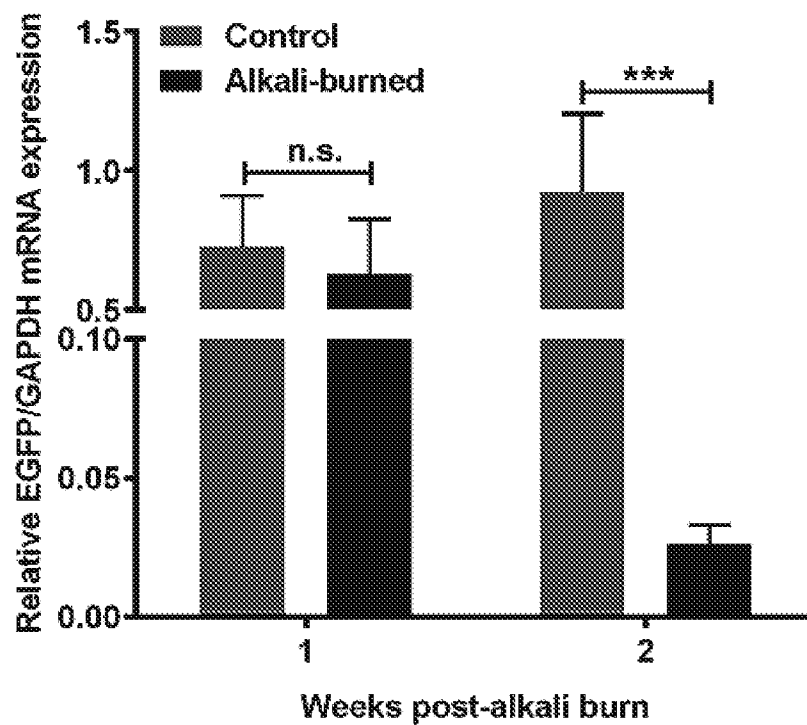


FIG. 15

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Genomic copy**mRNA expression****FIG. 16**

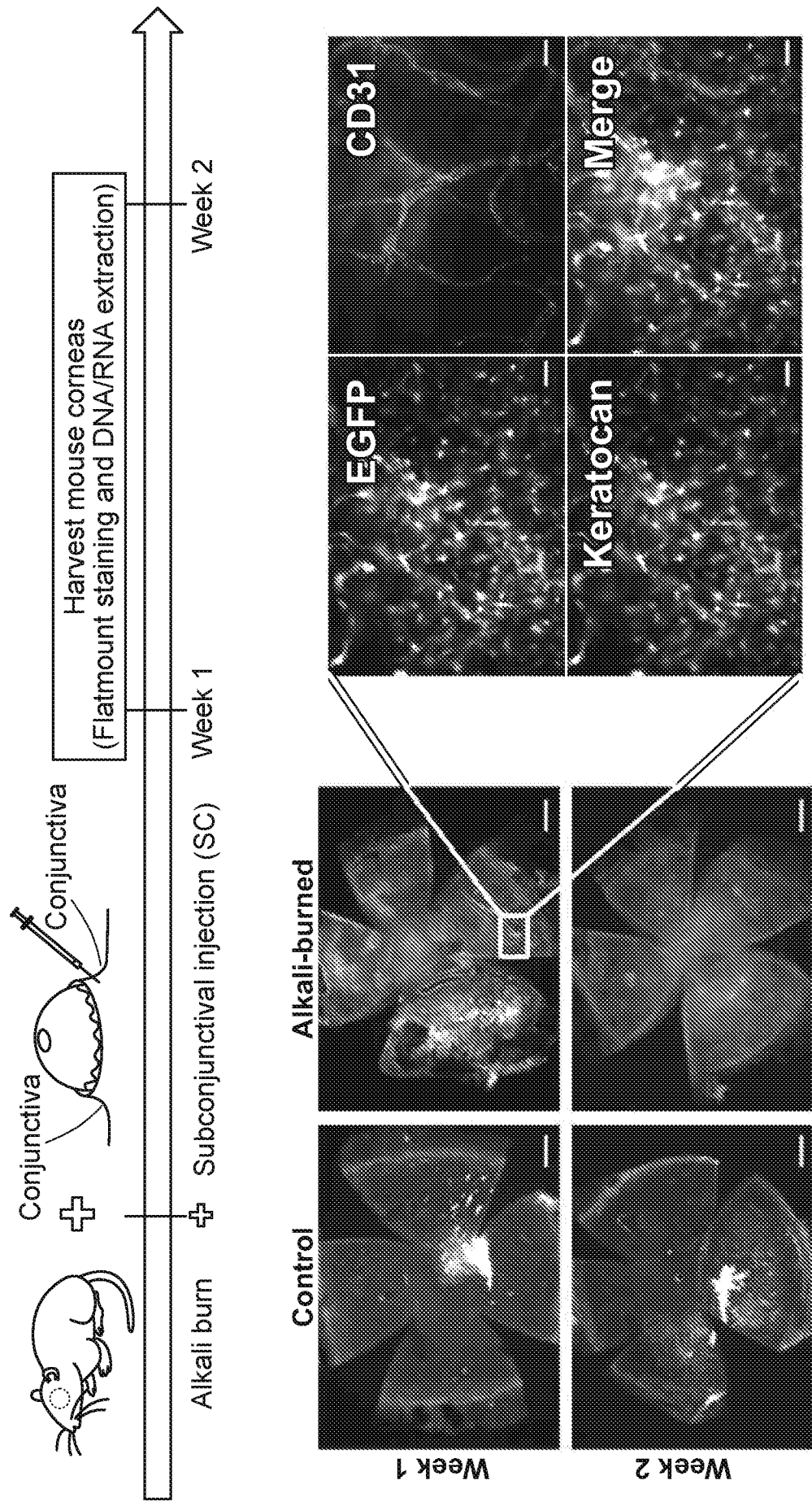


FIG. 17

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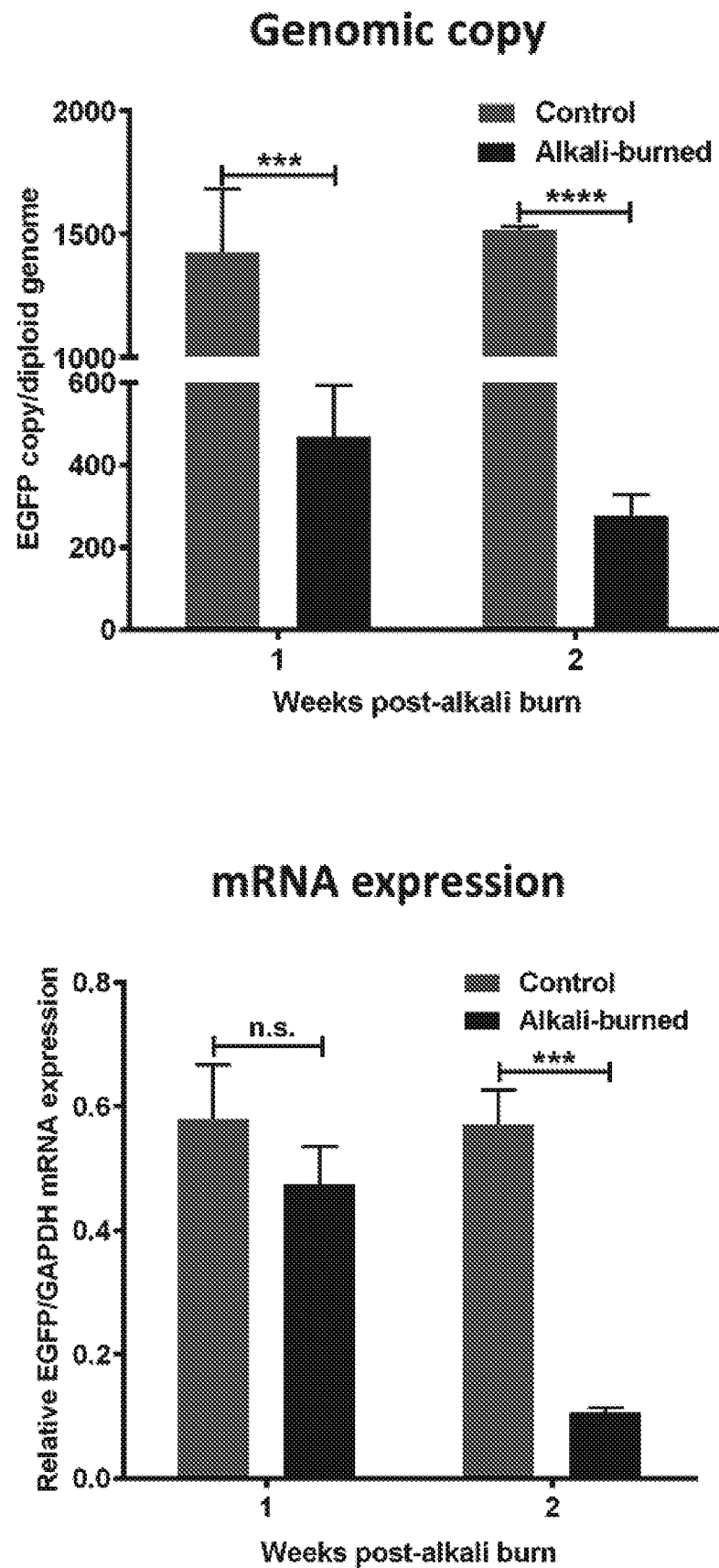


FIG. 18

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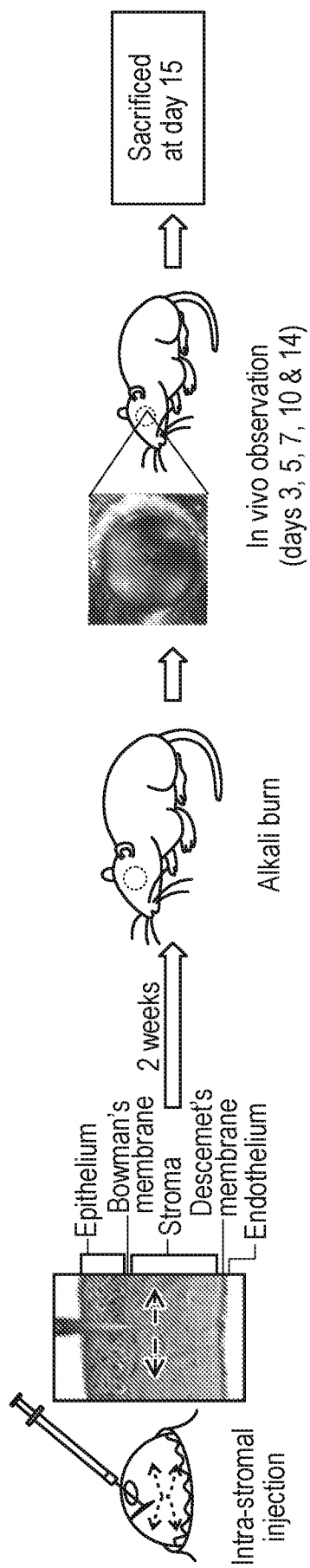
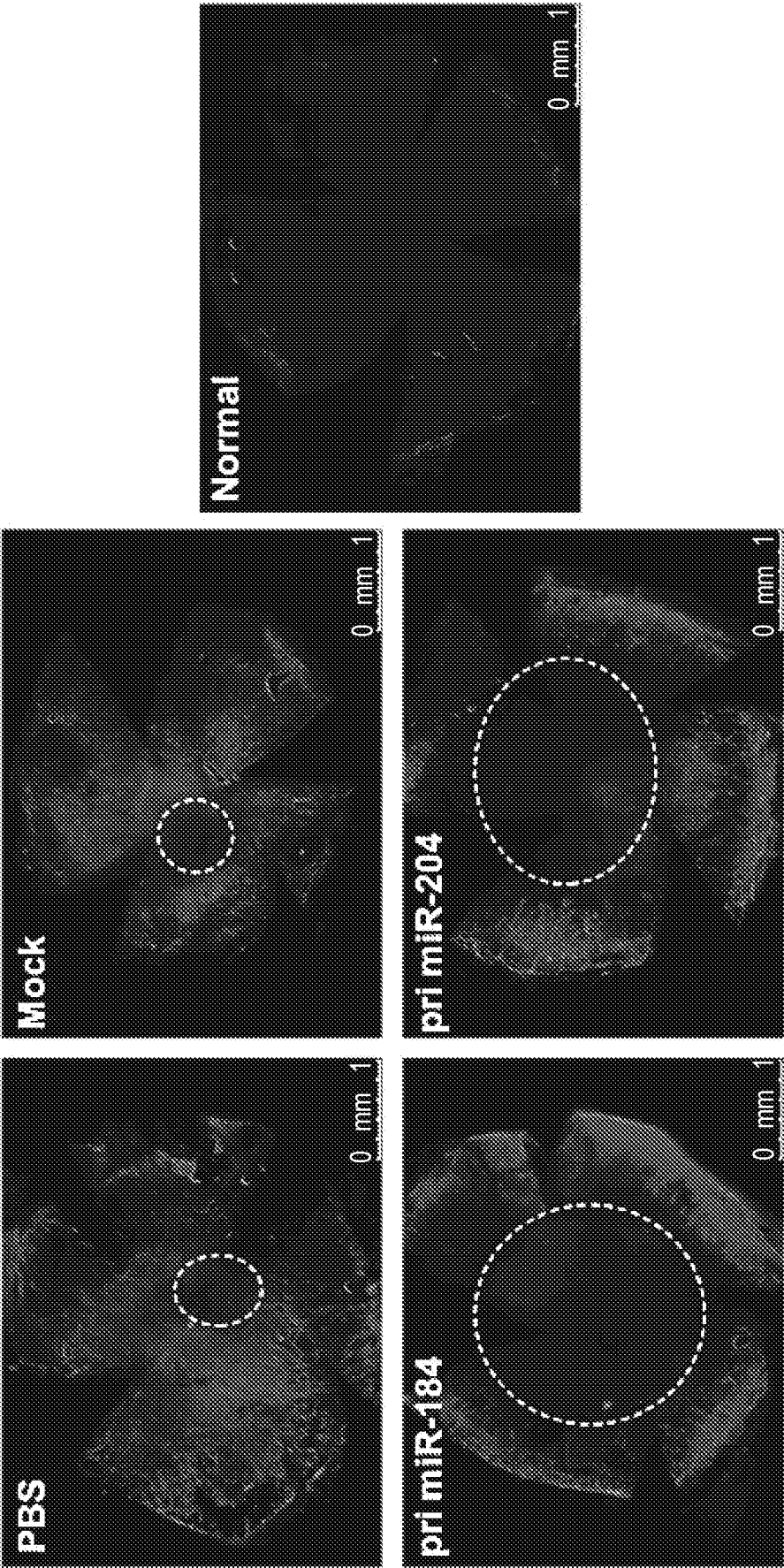


FIG. 19A



dash circle: avascular area

FIG. 19B

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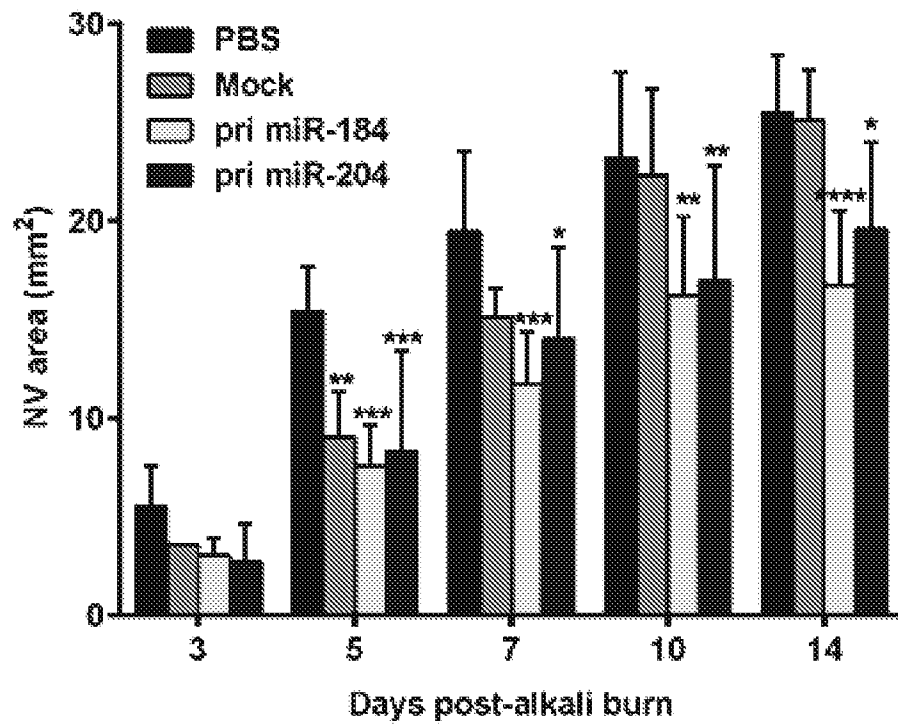
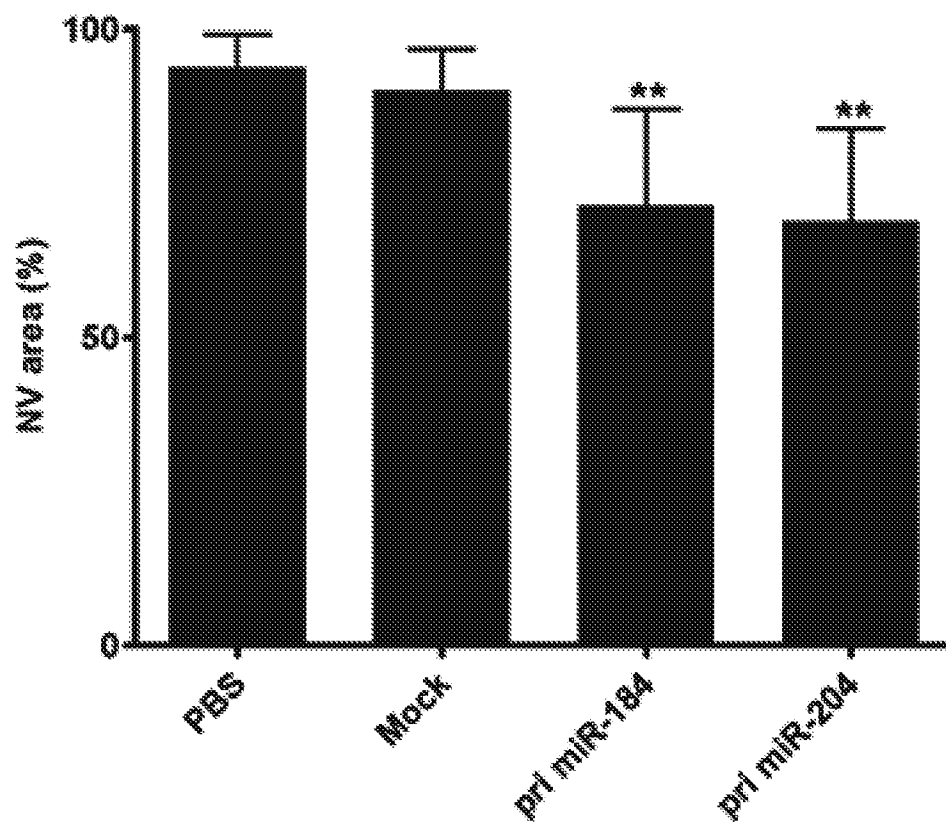
in vivo

FIG. 19C

flatmount

*: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001

FIG. 19D

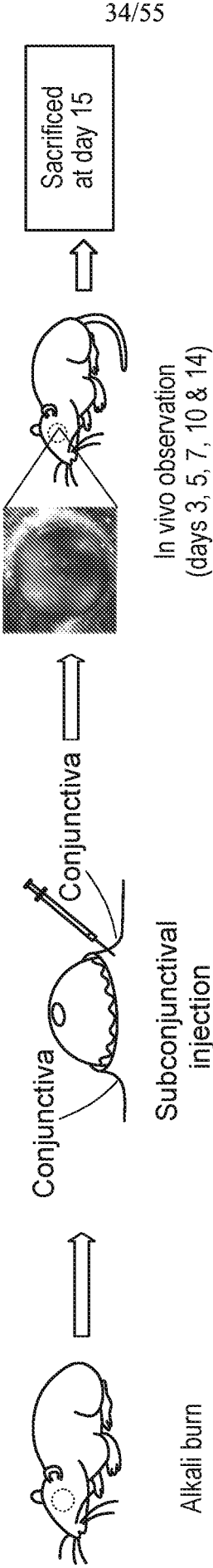


FIG. 20A

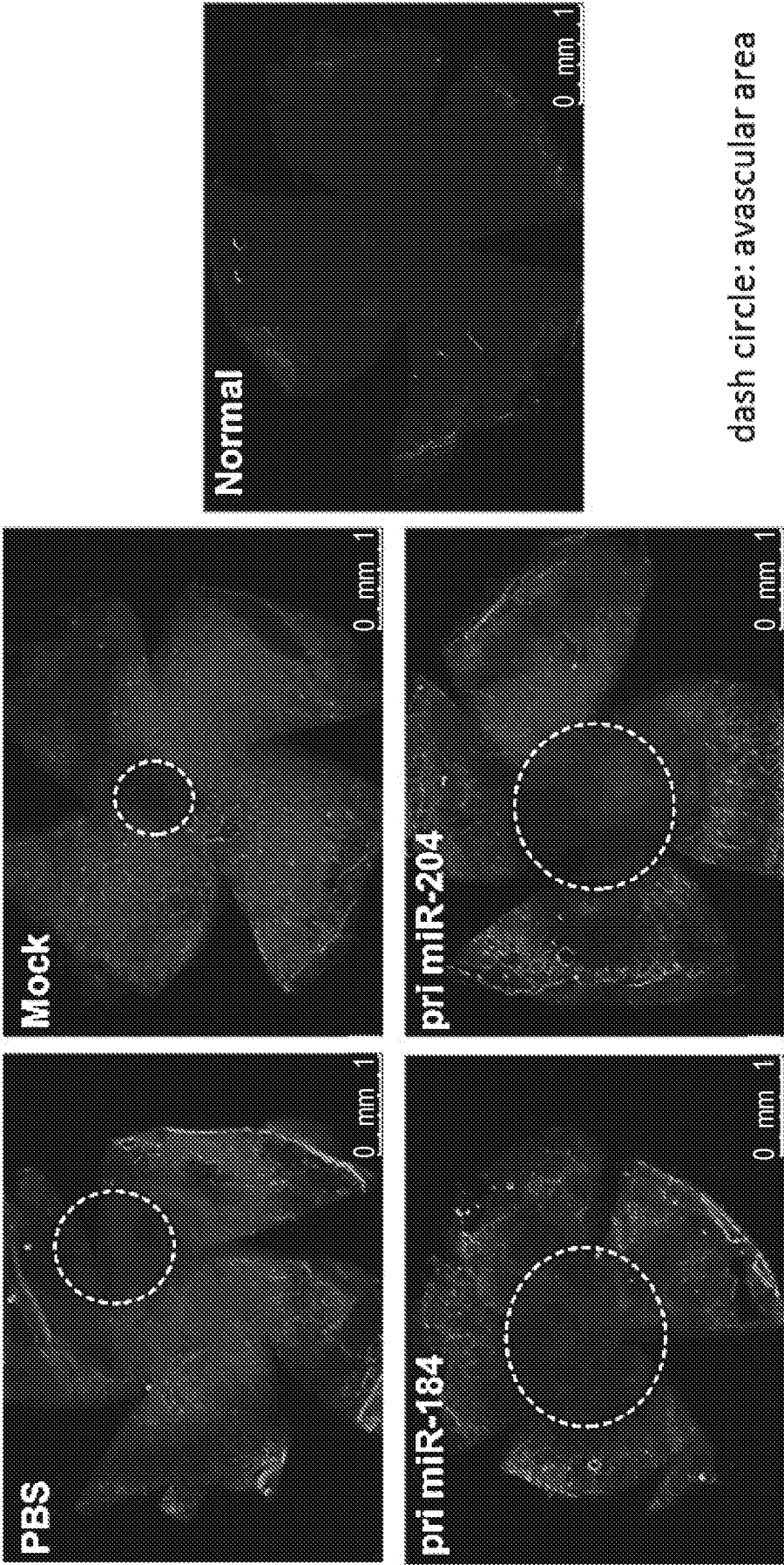
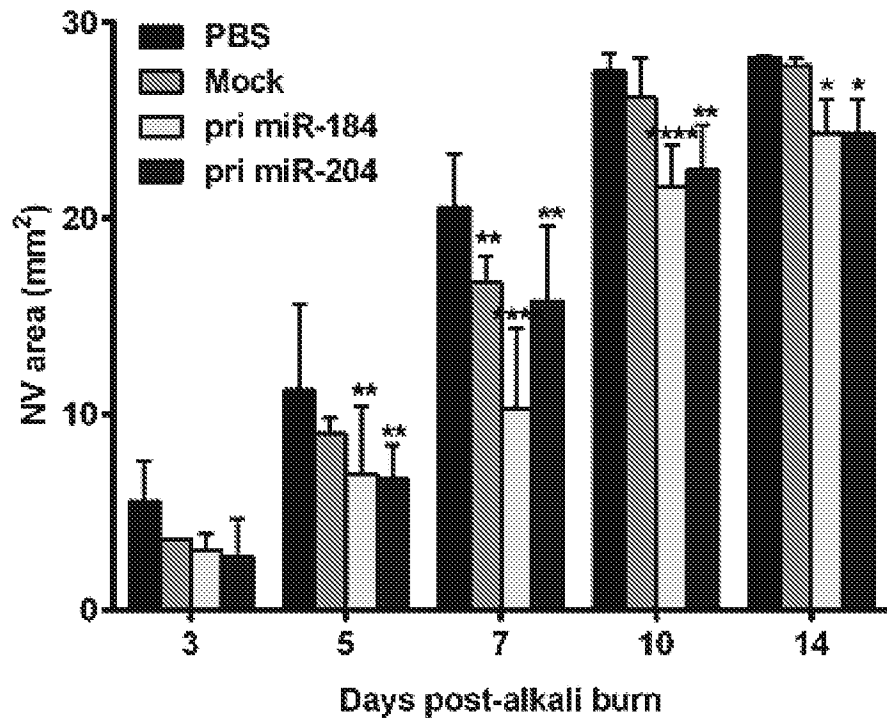
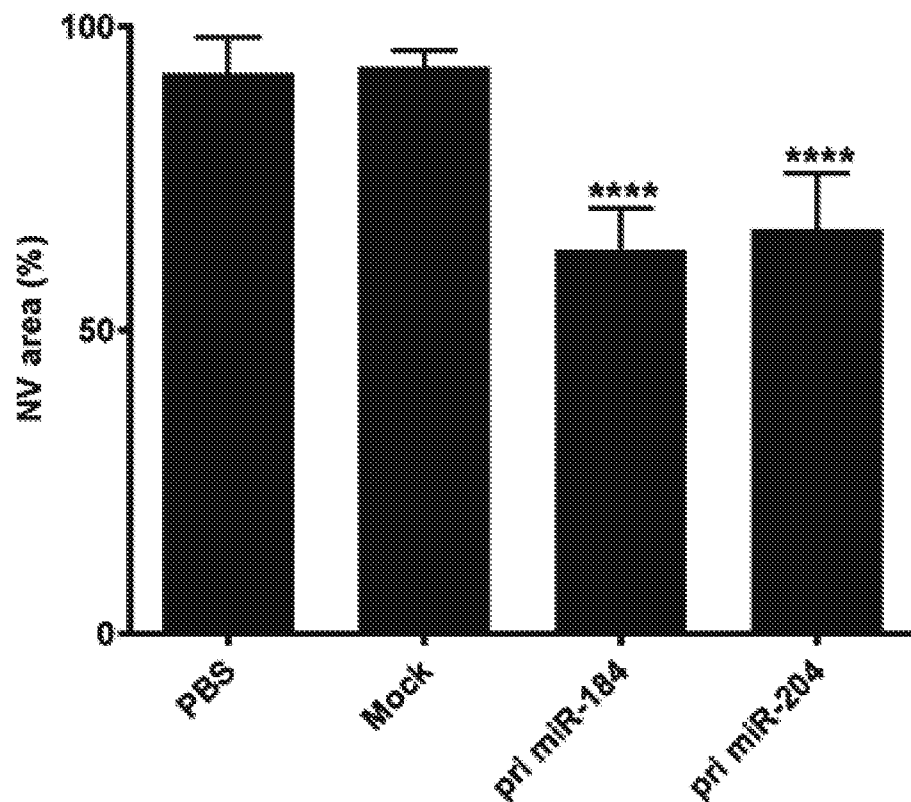


FIG. 20B

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in vivo**FIG. 20C****flatmount**

*: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001

FIG. 20D

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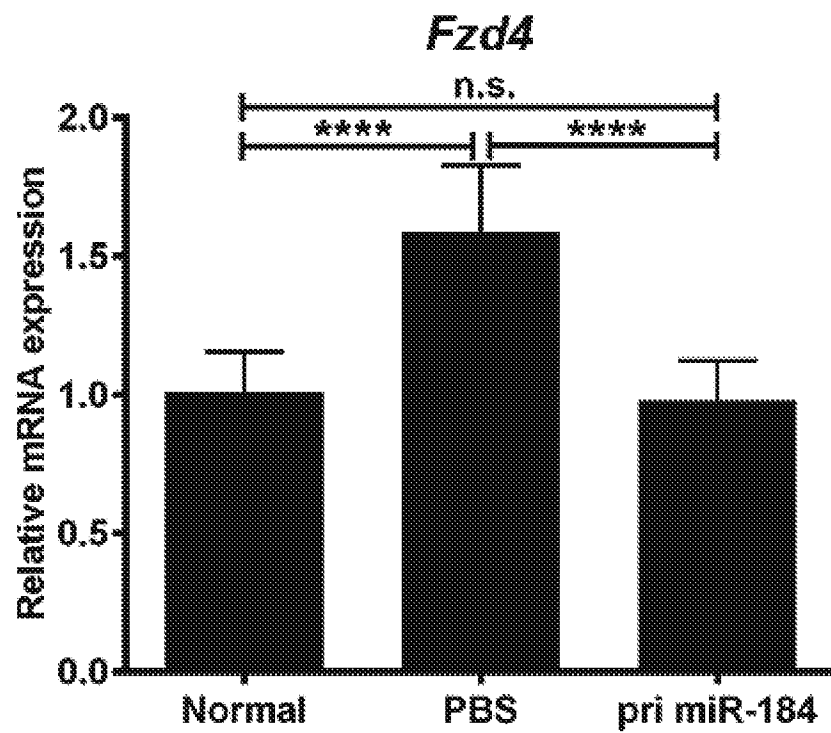
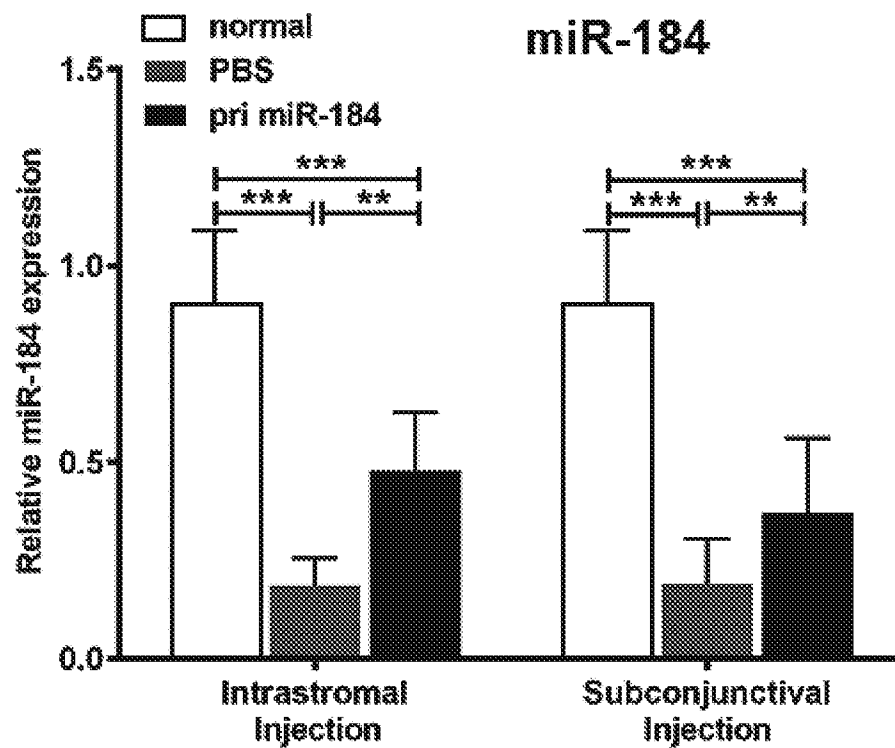


FIG. 21

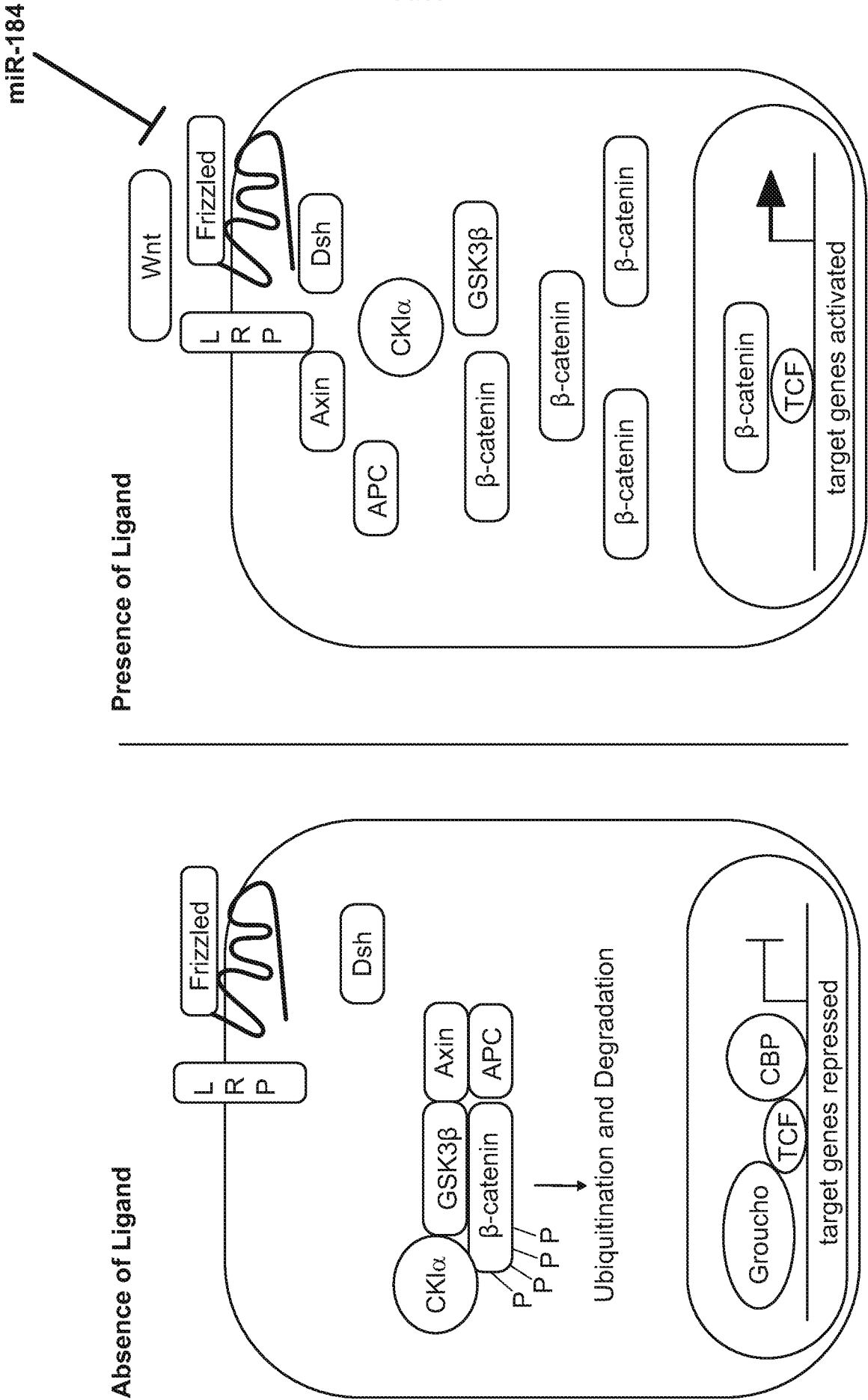


FIG. 21 (Continued)

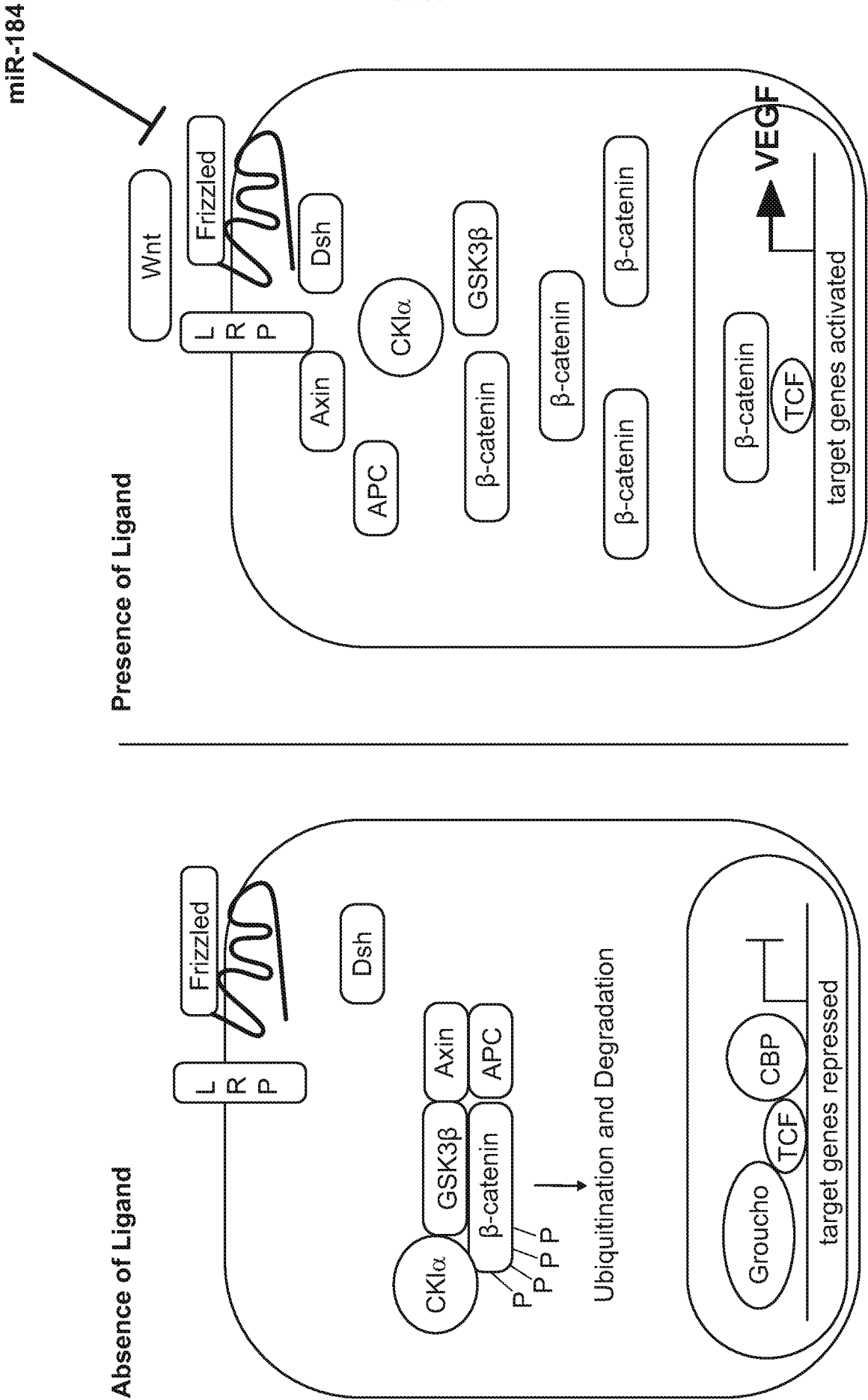


FIG. 22

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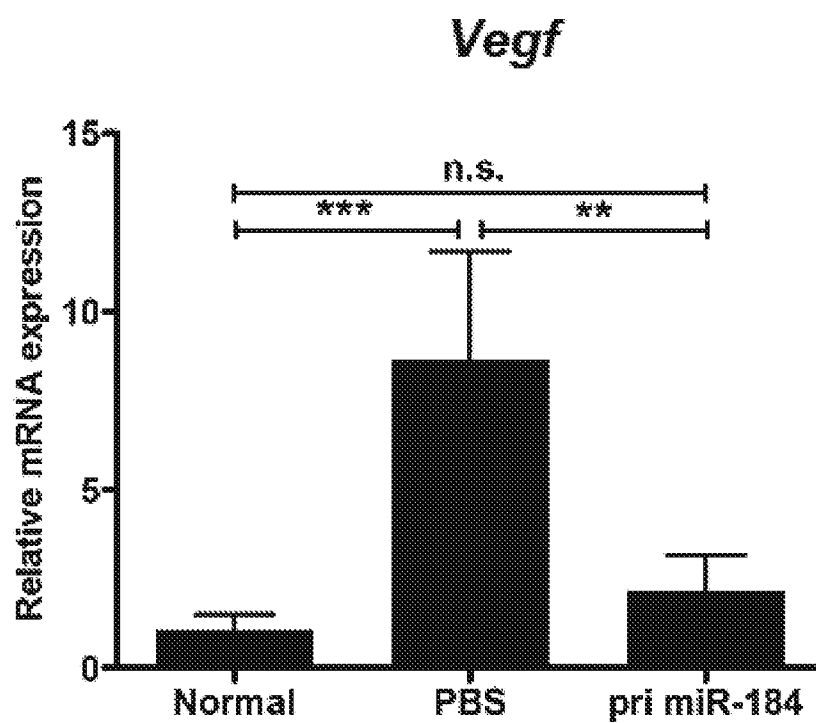
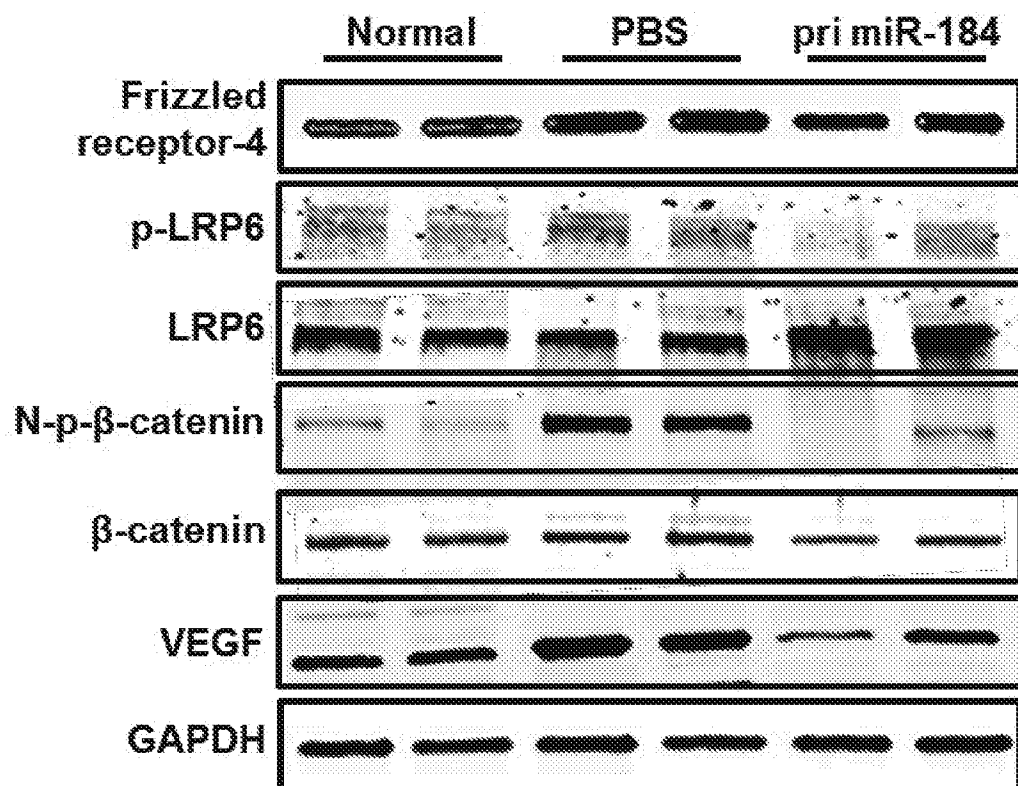


FIG. 22 (Continued)

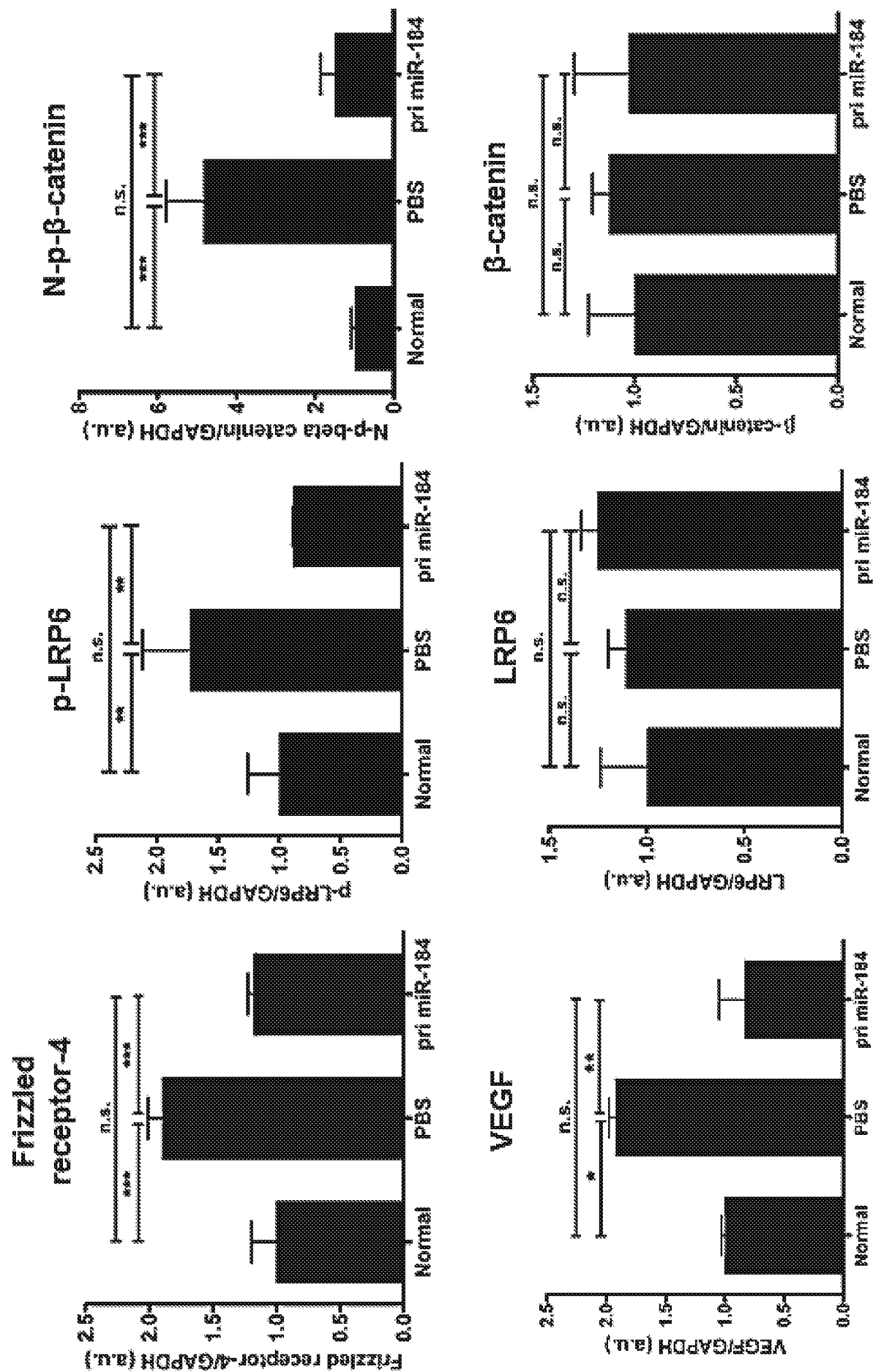


FIG. 22 (Continued)

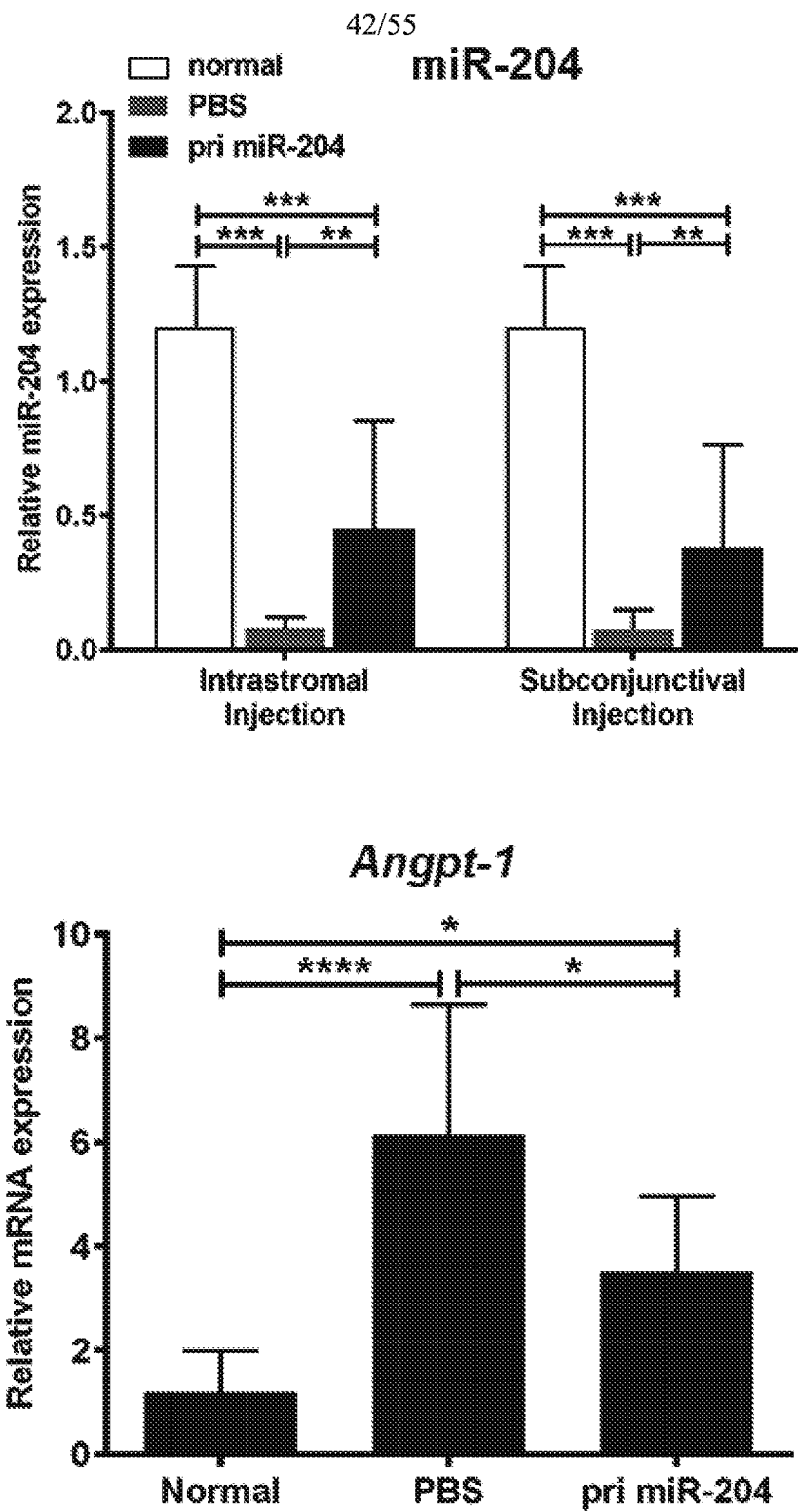


FIG. 23

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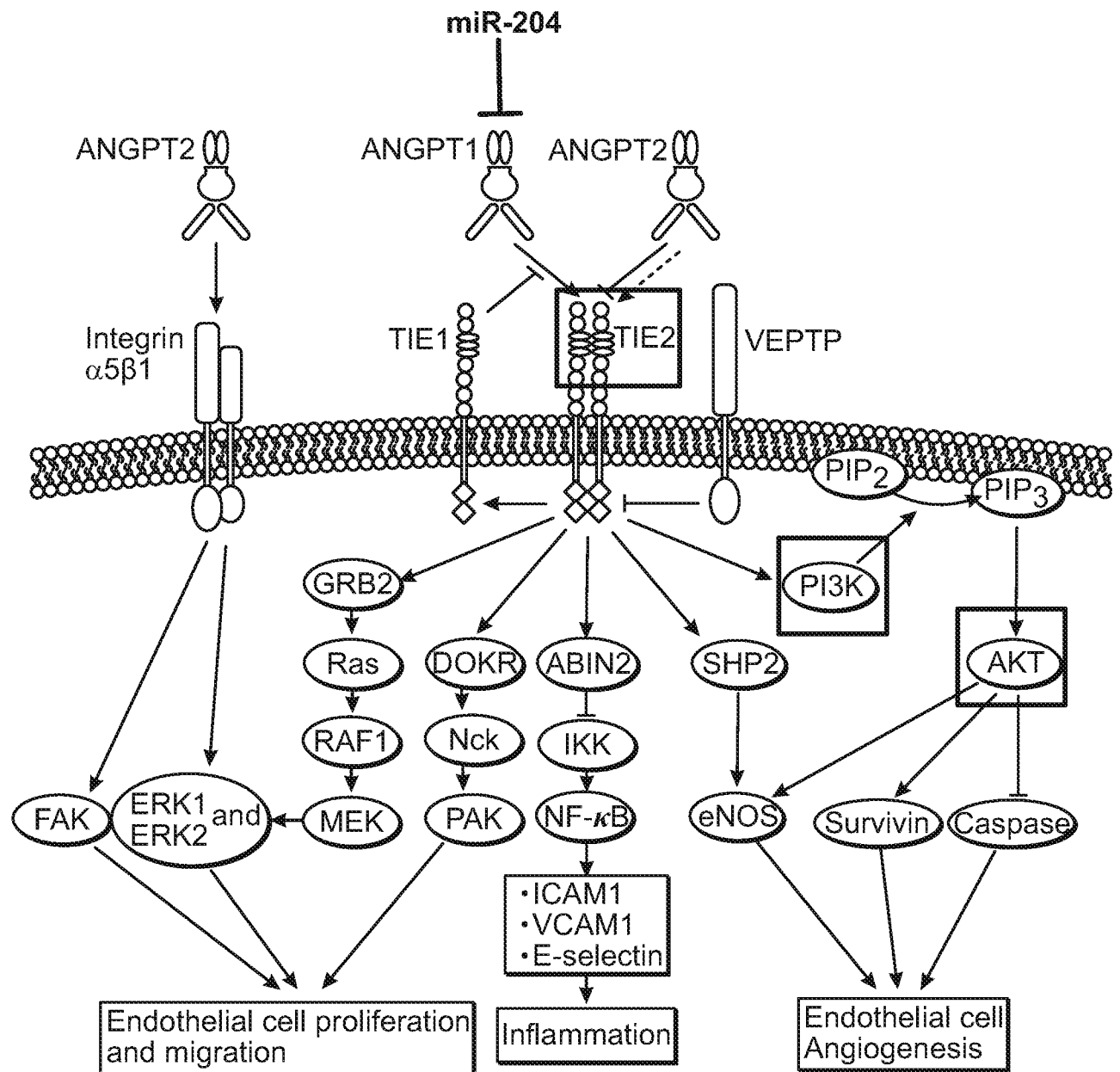


FIG. 23 (Continued)

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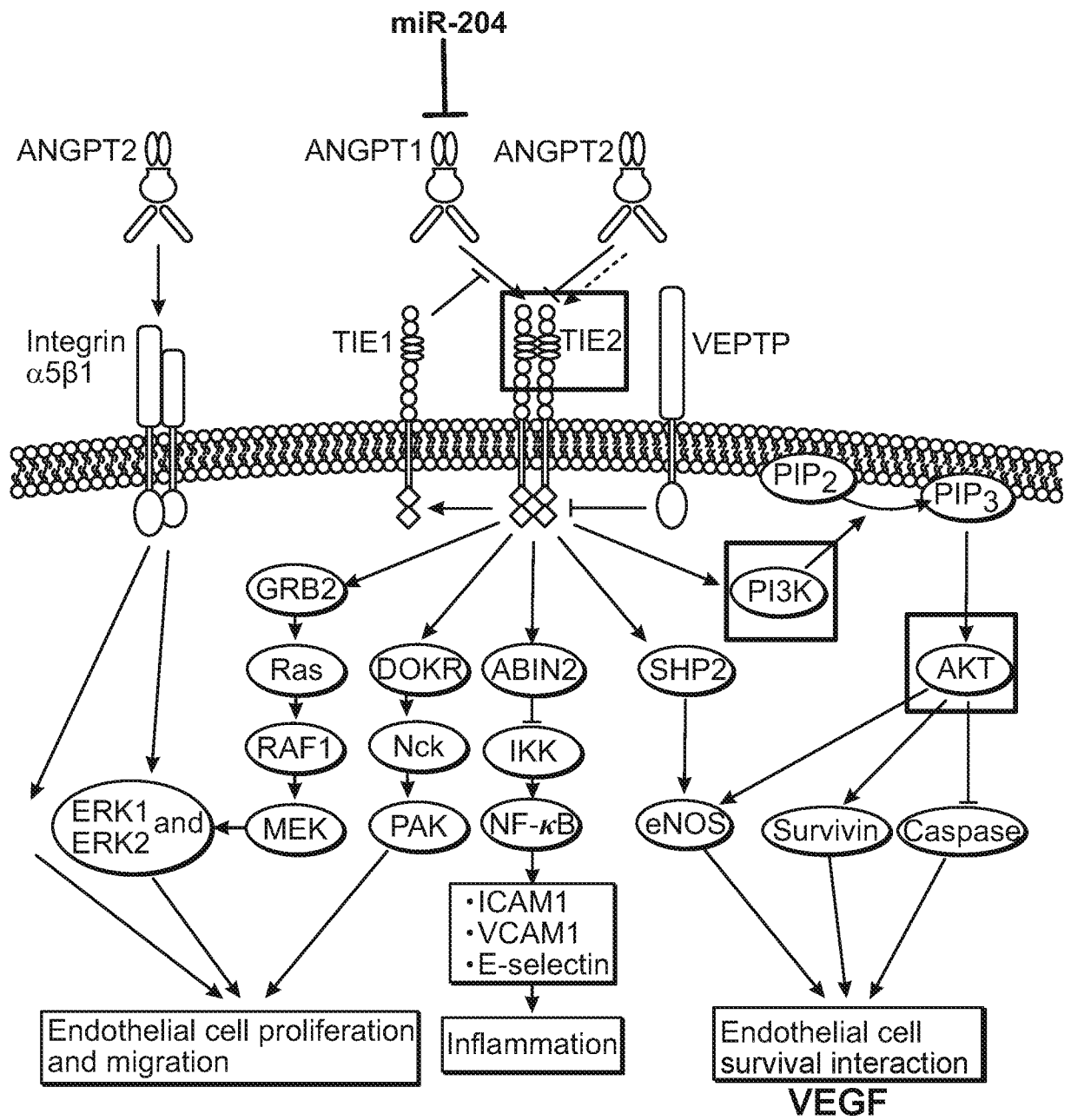


FIG. 24

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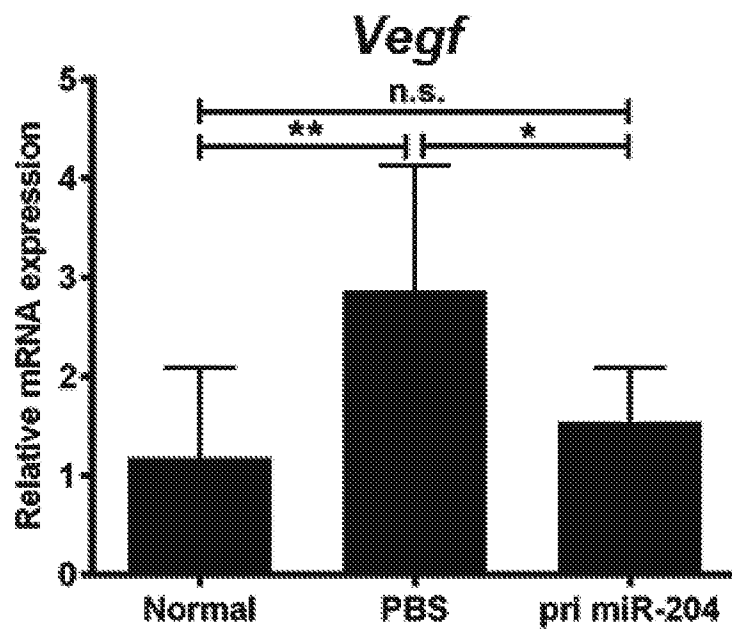
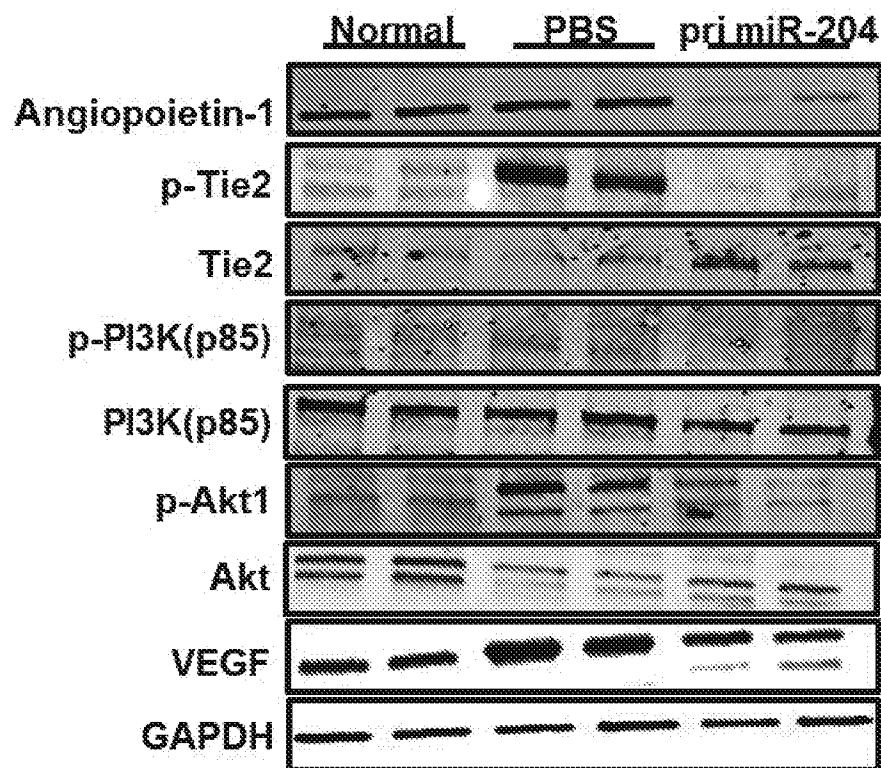


FIG. 24 (Continued)

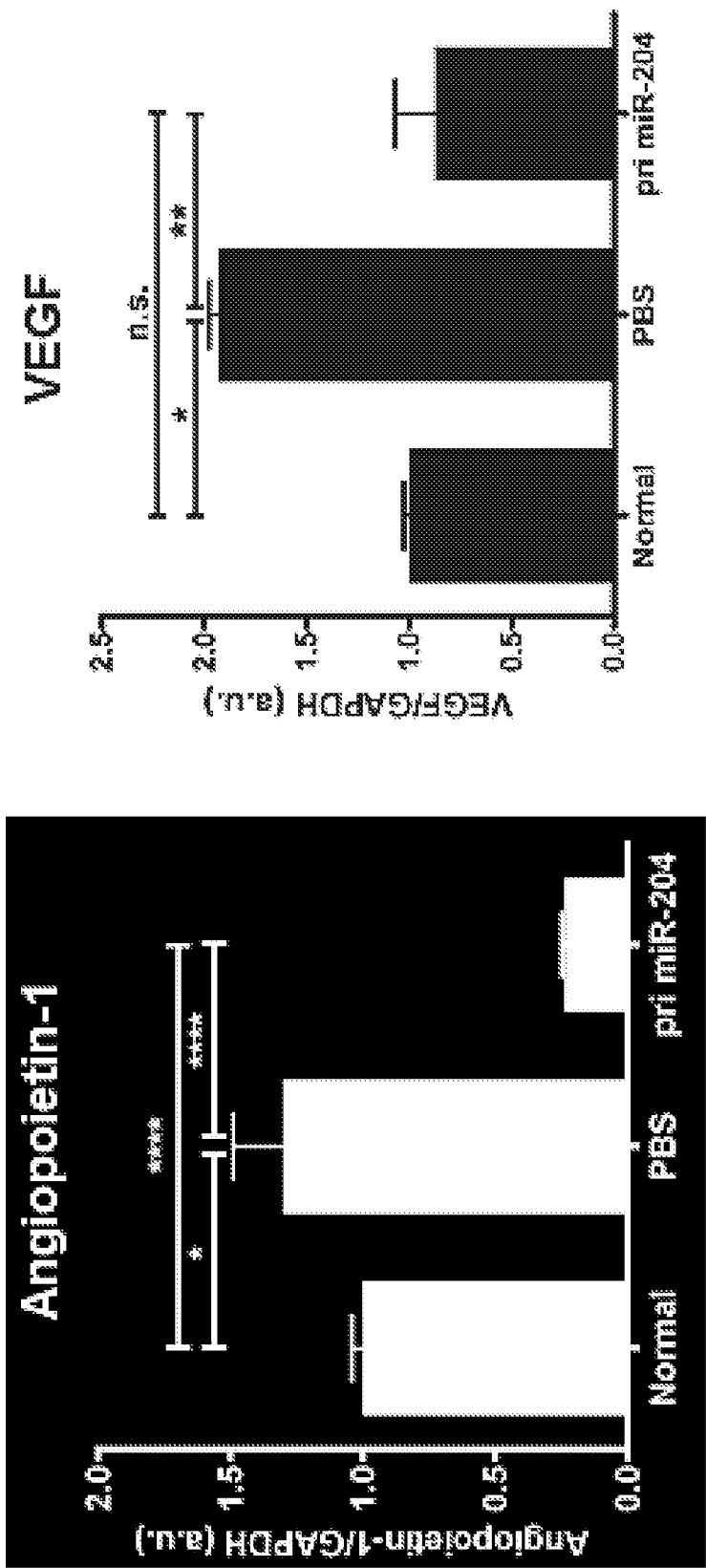


FIG. 24 (Continued)

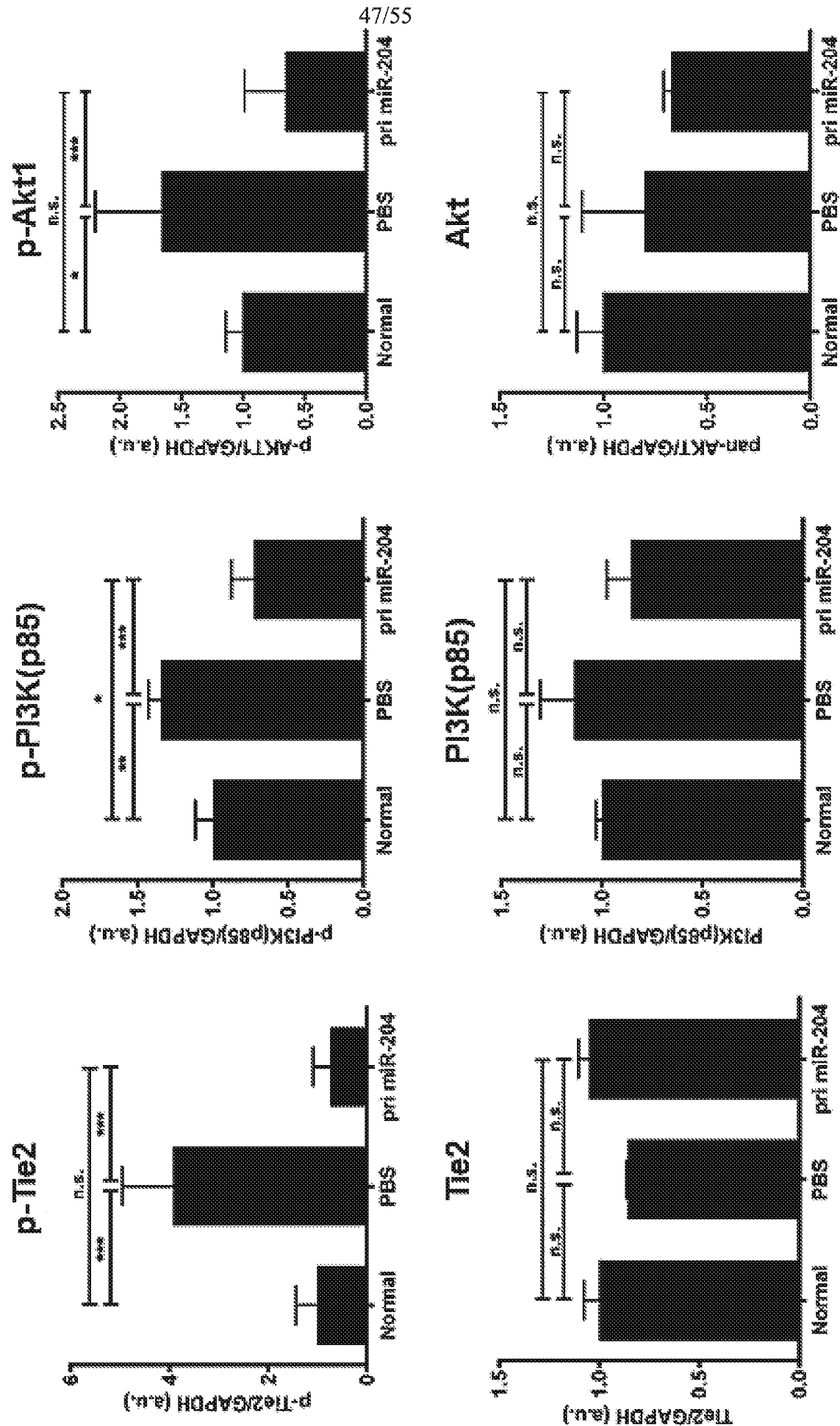


FIG. 24 (Continued)

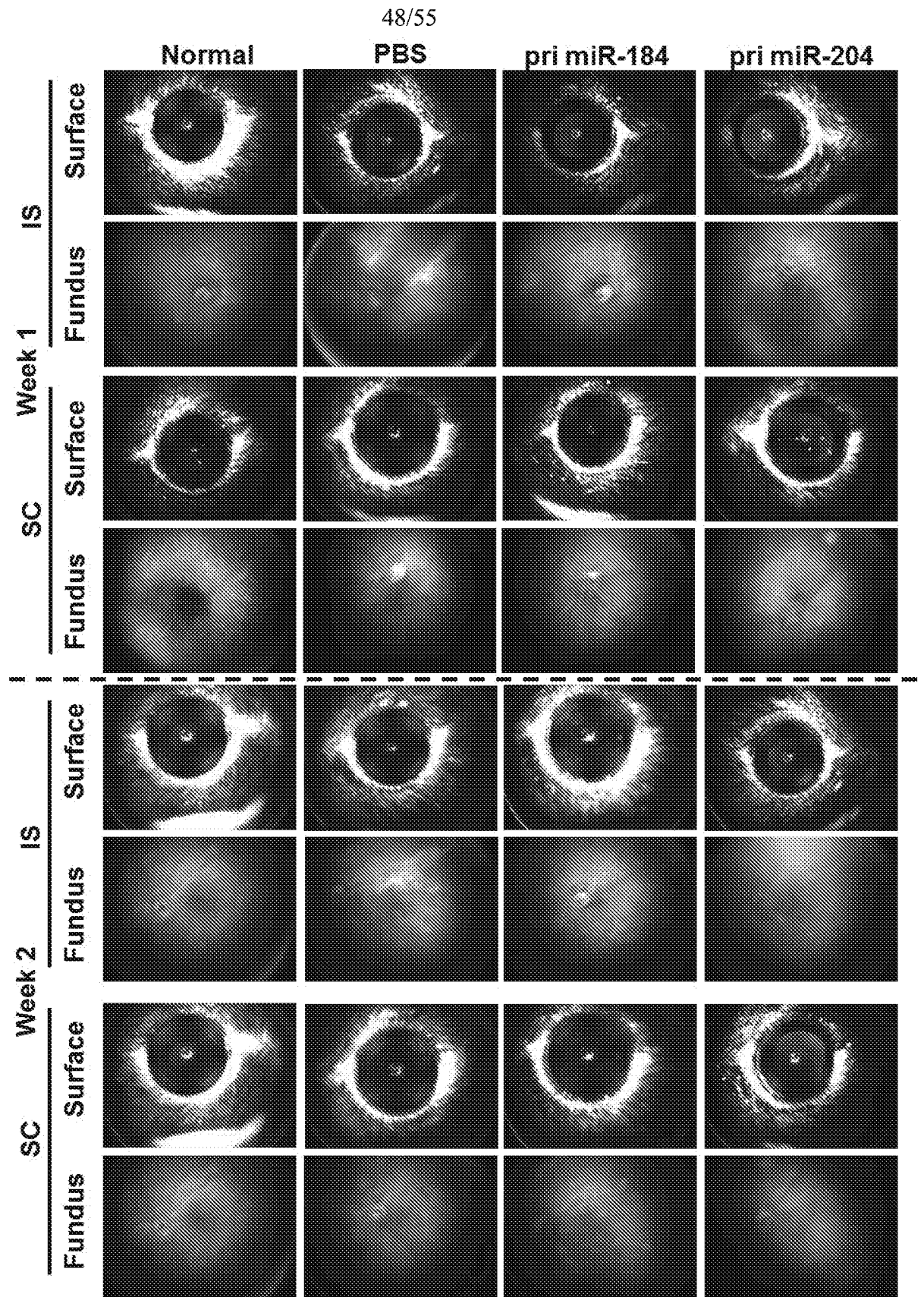


FIG. 25A

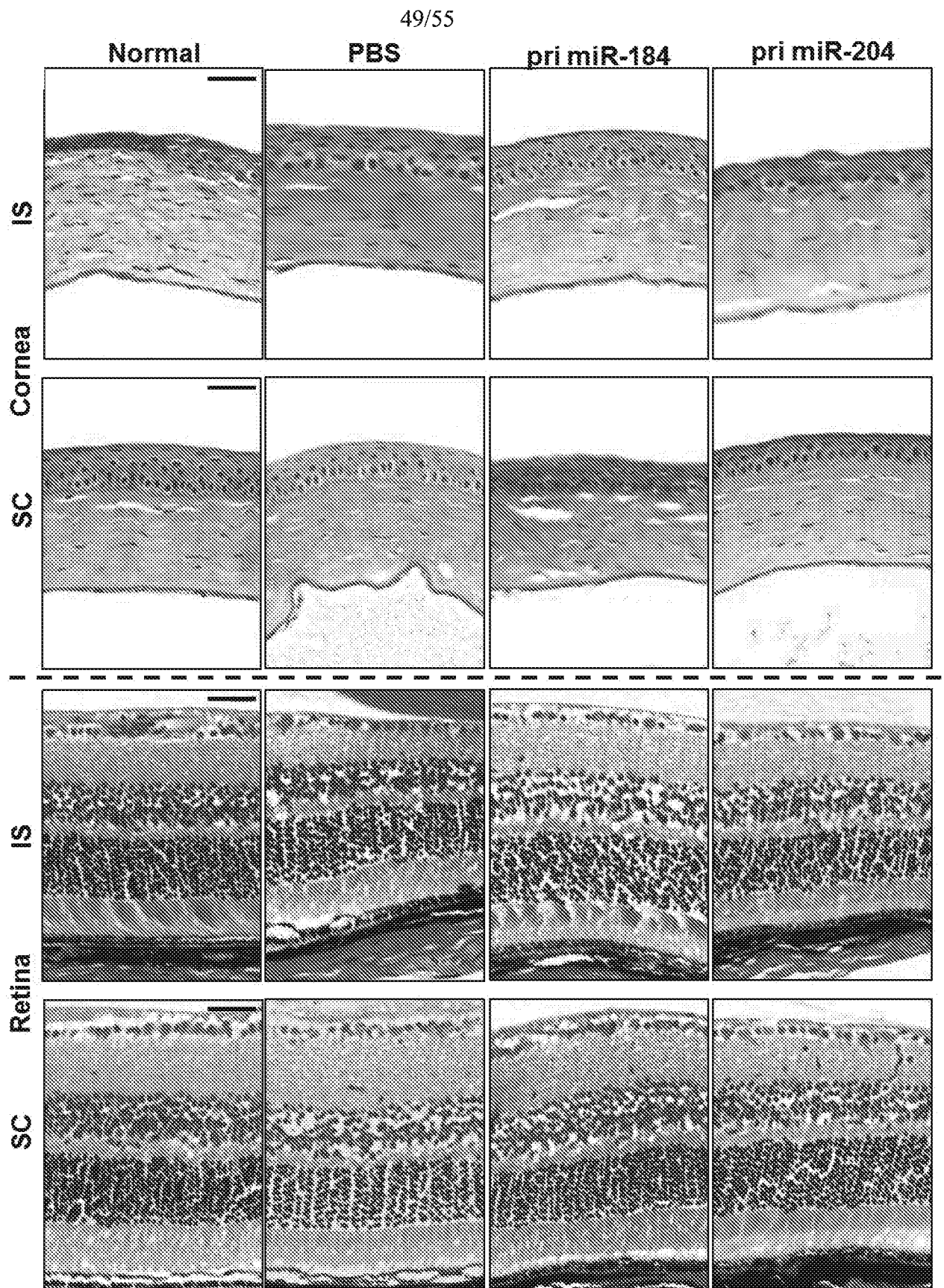


FIG. 25B

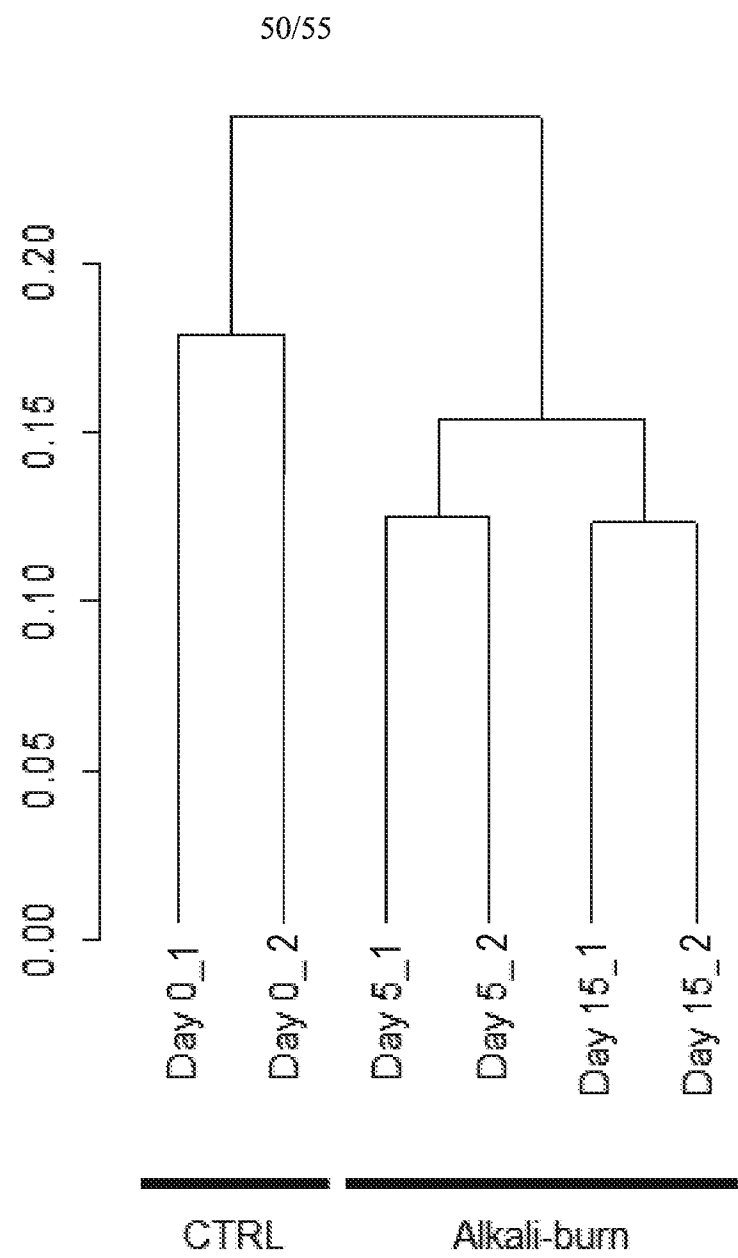


FIG. 26A

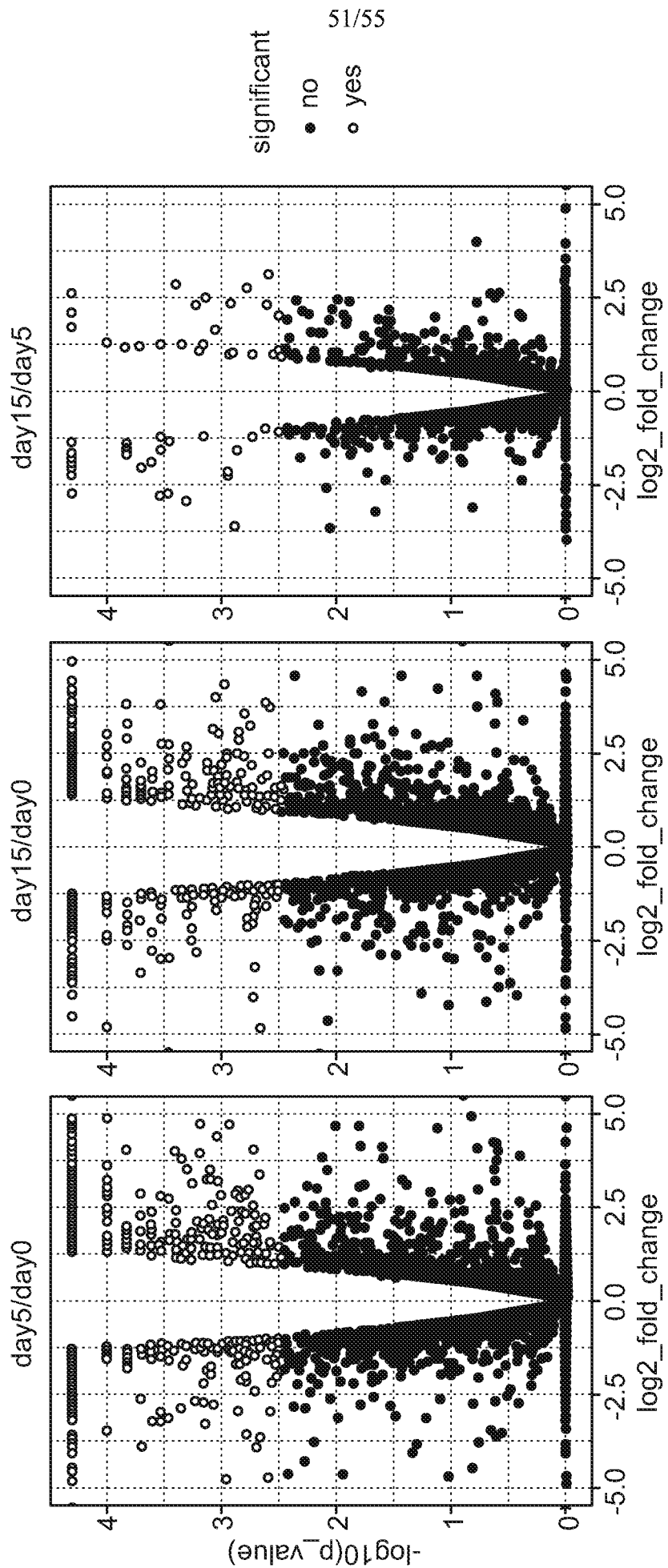


FIG. 26B

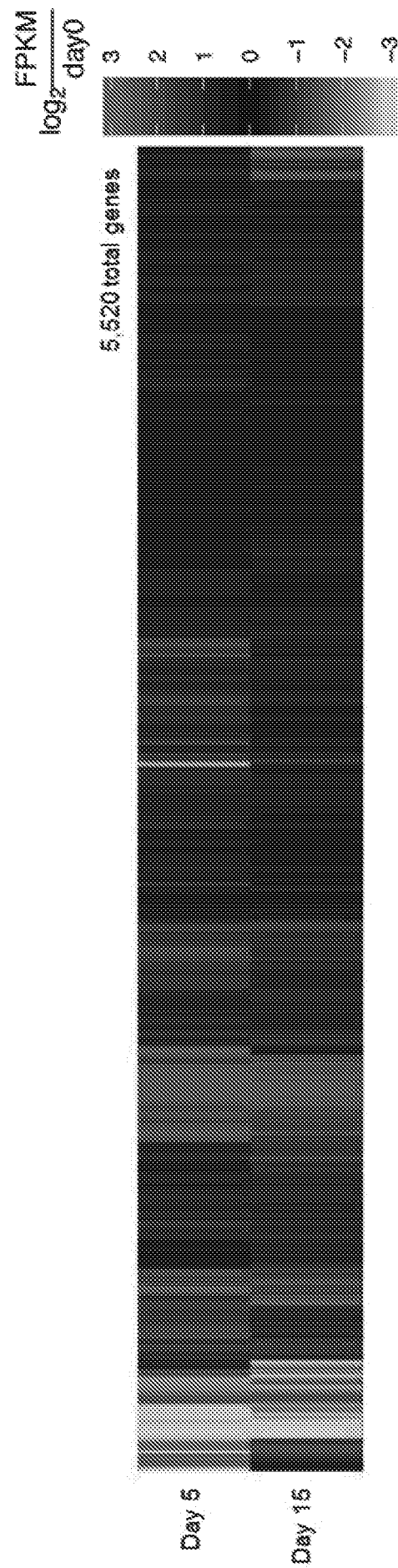


FIG. 26C

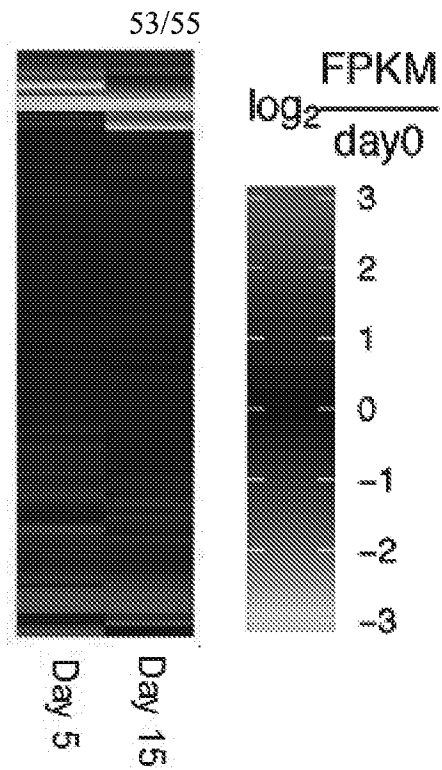


FIG. 27A

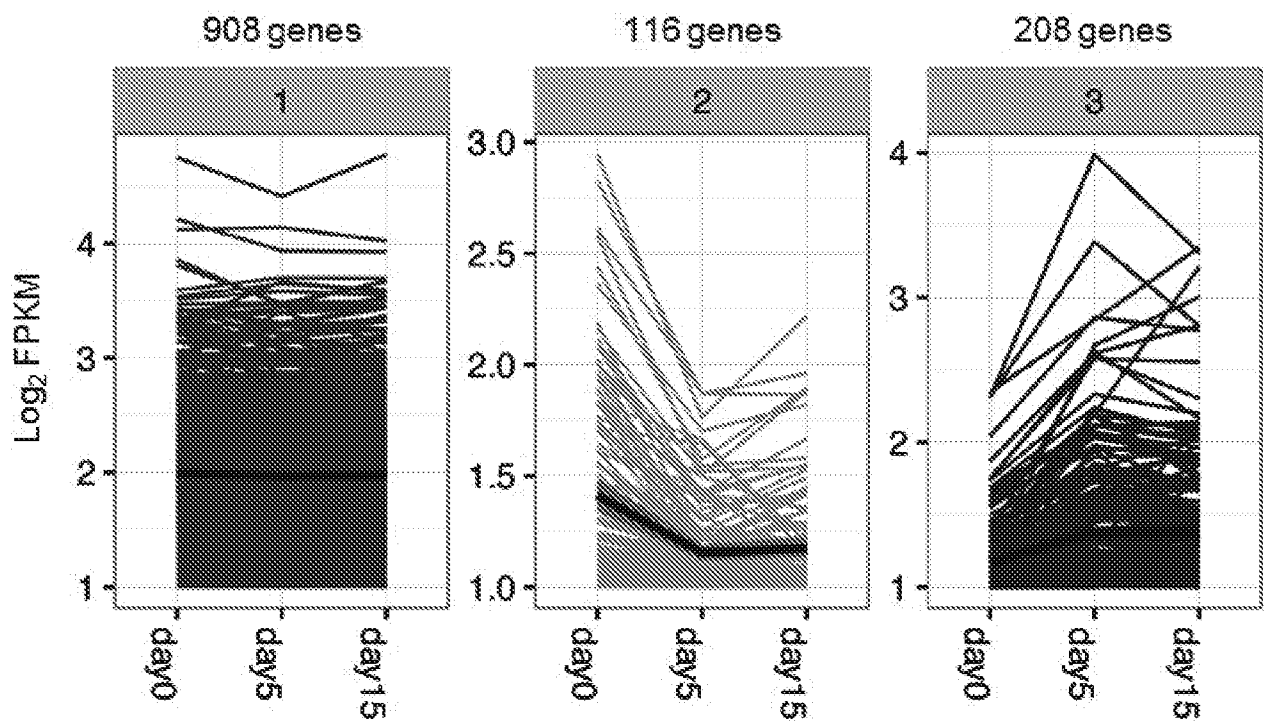


FIG. 27B

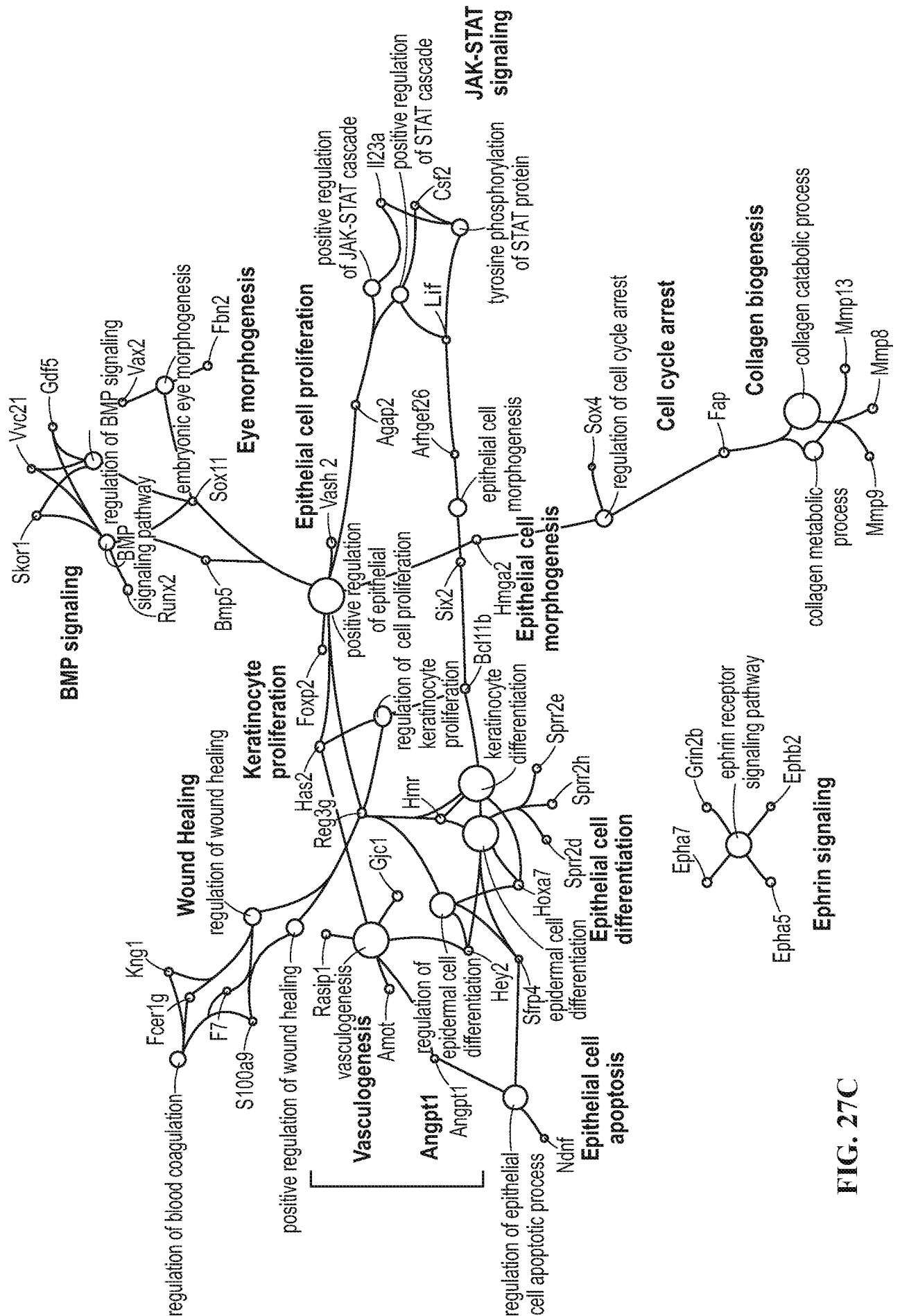


FIG. 27C

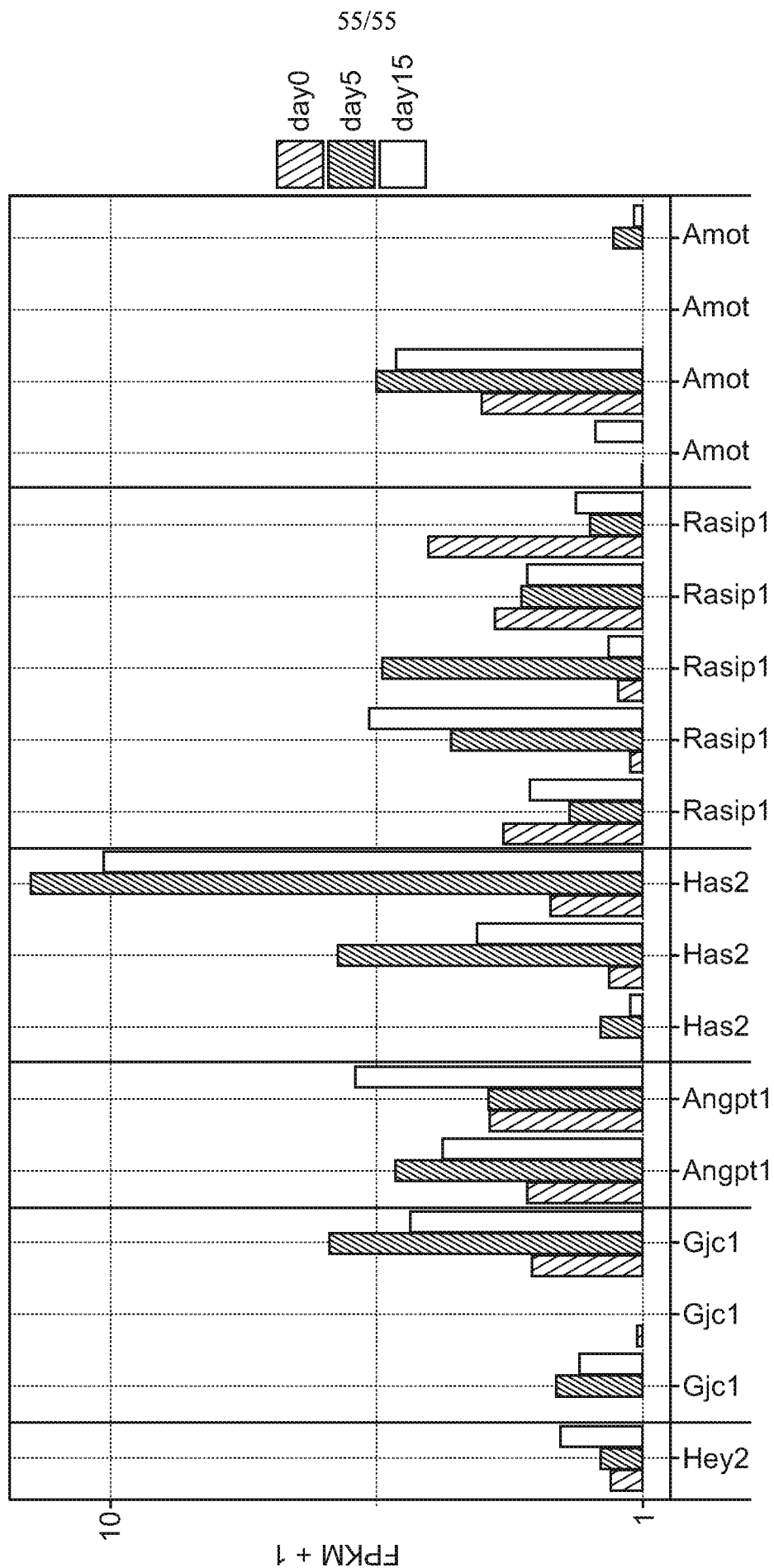


FIG. 28

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/17469

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 48/00, A61P 27/02, C12N 15/63, C12N 15/86 (2017.01)

CPC - C12N 5/0621, C12N 15/63, C12N 15/86, A61K 9/0048, C12N 7/00, C12N 2750/14121

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2012/0077870 A1 (BLANKS et al.) 29 March 2012 (29.03.2012) para [0016], [0060], [0062], [0065], [0069], [0090], [0107], [0126]	1, 19, 37-39, 55-57, 73, 86-88 ----- 2, 20, 74
Y	US 2005/0255089 A1 (CHIORINI et al.) 17 November 2005 (17.11.2005) para [0020]; SEQ ID NO: 4	2, 20, 74

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

20 May 2017

Date of mailing of the international search report

15 JUN 2017

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/17469

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 4-18, 22-36, 40-54, 58-72, 76-85, 89-98
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

-----please see extra sheet-----

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-2, 19-20, 37-39, 55-57, 73-74, 86-88, limited to SEQ ID NO: 7

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/17469

Continuation of: Box No. III Observations where unity of invention is lacking

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I+: Claims 1-3, 19-21, 37-39, 55-57, 73-75, 86-88, drawn to methods for delivering a transgene to ocular tissue or treating a disease by administering a recombinant adeno-associated virus, and to compositions comprising said recombinant adeno-associated virus. The methods and recombinant adeno-associated virus will be searched to the extent that the recombinant adeno-associated virus (rAAV) encompasses a capsid protein that comprises an amino acid sequence with at least 70% identity to SEQ ID NO: 7. It is believed that claims 1-2, 19-20, 37-39, 55-57, 73-74, 86-88, limited to SEQ ID NO: 7, encompass this first named invention, and thus these claims will be searched without fee to the extent that they encompass an rAAV having a capsid protein sequence with at least 70% identity to SEQ ID NO: 7. Additional capsid protein sequences will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected capsid protein sequence(s). Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched. An exemplary election would be an rAAV that encompasses a capsid protein that comprises an amino acid sequence with identity to SEQ ID NO: 8, i.e. claims 1-2, 19-20, 37-39, 55-57, 73-74, 86-88, limited to SEQ ID NO:8.

The inventions listed as Groups I+ do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features

The technical feature of each of the inventions listed as Group I+ is the specific capsid protein sequence recited therein. Each invention requires a capsid protein sequence, not required by any of the other inventions

Common Technical Features

The features shared by the inventions listed as Groups I+ are the methods of claims 1, 19, 37, 55, and the compositions of claims 73 and 86.

However, these shared technical features do not represent a contribution over prior art, because the shared technical features are taught by US 2012/0077870 A1 to Blanks et al. (hereinafter 'Blanks').

Blanks discloses [claim 1] a method for delivering a transgene to ocular tissue (para [0062] "a method of prevention and/or treatment of ocular neovascularization as well as diseases associated with ocular neovascularization, via the delivery of the expression vector of the subject invention using gene therapy. In one embodiment, the method comprises administering, into a target ocular tissue of the subject, an expression vector comprising a transgene"), the method comprising: administering to ocular tissue of a subject an effective amount of rAAV (para [0062] "administering, into a target ocular tissue of the subject, an expression vector comprising a transgene"; para [0060] "Preferred embodiments of the vectors are recombinant adeno-associated virus (rAAV) vectors"; para [0107] "The expression vectors and therapeutic composition comprising the expression vectors is administered in an effective amount"), wherein the rAAV comprises (i) a capsid protein having a serotype selected from the group consisting of AAV5, AAV6, AAV6.2, AAV7, AAV8, AAV9, AAVrh.10, AAVrh.39, and AAVrh.43 (para [0060] "the AAV vectors are selected from . . . AAV5, AAV6, or AAV7"), and (ii) a nucleic acid comprising a promoter operably linked to a transgene (para [0062] "the transgene is operably linked to an ocular-specific promoter . . . wherein the ocular-specific promoter selectively drives gene expression in the ocular tissue associated with neovascularization").

Blanks discloses [claim 19] a method for treating an ocular disease (para [0016] "methods for effective treatment of pathological ocular neovascularization using gene therapy"), the method comprising: administering to a subject having or suspected of having an ocular disease an effective amount of rAAV (para [0062] "administering, into a target ocular tissue of the subject, an expression vector comprising a transgene"; para [0060] "Preferred embodiments of the vectors are recombinant adeno-associated virus (rAAV) vectors"; para [0065] "In one embodiment, the subject or patient has pathological ocular neovascularization"), wherein the rAAV comprises

(i) a capsid protein having a serotype selected from the group consisting of AAV5, AAV6, AAV6.2, AAV7, AAV8, AAV9, AAVrh.10, AAVrh.39, and AAVrh.43 (para [0060] "the AAV vectors are selected from . . . AAV5, AAV6, or AAV7"), and (ii) a nucleic acid comprising a promoter operably linked to a transgene (para [0062] "the transgene is operably linked to an ocular-specific promoter . . . wherein the ocular-specific promoter selectively drives gene expression in the ocular tissue associated with neovascularization").

-----please see next extra sheet-----

Continuation of: Box No. III Observations where unity of invention is lacking

Blanks discloses [claim 37] a method for delivering a transgene to ocular tissue (para [0062] "delivery of the expression vector of the subject invention using gene therapy. In one embodiment, the method comprises administering, into a target ocular tissue of the subject, an expression vector comprising a transgene"), the method comprising:

administering to ocular tissue of a subject an effective amount of rAAV (para [0062] "administering, into a target ocular tissue of the subject, an expression vector comprising a transgene"; para [0060] "Preferred embodiments of the vectors are recombinant adeno-associated virus (rAAV) vectors"; para [0107] "The expression vectors and therapeutic composition comprising the expression vectors is administered in an effective amount"), wherein the rAAV comprises

(i) a capsid protein (para [0060] "the AAV vectors are selected from . . . AAV5, AAV6, or AAV7", which are capsid protein serotypes), and

(ii) a nucleic acid comprising a promoter operably linked to a transgene (para [0062] "the transgene is operably linked to an ocular-specific promoter . . . wherein the ocular-specific promoter selectively drives gene expression in the ocular tissue associated with neovascularization"), wherein the transgene encodes a gene associated with an ocular disease (para [0090] "a sequence encoding a nucleic acid encoding a therapeutic transgene (such as an angiogenesis inhibitor)"; para [0126] "In one embodiment, the composition is used to treat neovascular ocular disease by localized (for example, in ocular tissue) concurrent administration with other medicaments that act to block angiogenesis").

Blanks discloses [claim 55] a method for treating a disease (para [0016] "methods for effective treatment of pathological ocular neovascularization using gene therapy"), the method comprising:

administering to ocular tissue of a subject an effective amount of rAAV (para [0062] "administering, into a target ocular tissue of the subject, an expression vector comprising a transgene"; para [0060] "Preferred embodiments of the vectors are recombinant adeno-associated virus (rAAV) vectors"; para [0107] "The expression vectors and therapeutic composition comprising the expression vectors is administered in an effective amount"), wherein the rAAV comprises

(i) a capsid protein (para [0060] "the AAV vectors are selected from . . . AAV5, AAV6, or AAV7", which are capsid protein serotypes), and

(ii) a nucleic acid comprising a promoter operably linked to a transgene (para [0062] "the transgene is operably linked to an ocular-specific promoter . . . wherein the ocular-specific promoter selectively drives gene expression in the ocular tissue associated with neovascularization"), wherein the transgene encodes a gene associated with an ocular disease (para [0090] "a sequence encoding a nucleic acid encoding a therapeutic transgene (such as an angiogenesis inhibitor)"; para [0126] "In one embodiment, the composition is used to treat neovascular ocular disease by localized (for example, in ocular tissue) concurrent administration with other medicaments that act to block angiogenesis").

Blanks discloses [claim 73] a recombinant adeno-associated virus (para [0060] "Preferred embodiments of the vectors are recombinant adeno-associated virus (rAAV) vectors") comprising:

(i) a capsid protein having a serotype selected from the group consisting of AAV5, AAV6, AAV6.2, AAV7, AAV8, AAV9, AAVrh.10, AAVrh.39, and AAVrh.43 (para [0060] "In certain specific embodiment, the AAV vectors are selected from . . . AAV5, AAV6, or AAV7"); and,

(ii) a nucleic acid comprising a promoter operably linked to a transgene (para [0062] "the transgene is operably linked to an ocular-specific promoter . . . wherein the ocular-specific promoter selectively drives gene expression in the ocular tissue associated with neovascularization").

Blanks discloses [claim 86] a recombinant adeno-associated virus (rAAV) (para [0060] "Preferred embodiments of the vectors are recombinant adeno-associated virus (rAAV) vectors") comprising:

(i) a capsid protein (para [0060] "the AAV vectors are selected from . . . AAV5, AAV6, or AAV7", which are capsid protein serotypes); and,

(ii) a nucleic acid comprising a promoter operably linked to a transgene (para [0062] "the transgene is operably linked to an ocular-specific promoter . . . wherein the ocular-specific promoter selectively drives gene expression in the ocular tissue associated with neovascularization"), wherein the transgene encodes a gene associated with an ocular disease (para [0090] "a sequence encoding a nucleic acid encoding a therapeutic transgene (such as an angiogenesis inhibitor)"; para [0126] "In one embodiment, the composition is used to treat neovascular ocular disease by localized (for example, in ocular tissue) concurrent administration with other medicaments that act to block angiogenesis").

As the technical features were known in the art at the time of the invention, they cannot be considered special technical features that would otherwise unify the groups.

The inventions listed as Groups I+ therefore lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.