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Gene Therapy for C1 Esterase Inhibitor Deficiency in a Murine Model of Hereditary Angioedema

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Abstract

Background.—Hereditary angioedema (HAE) is a life-threatening, autosomal dominant disorder characterized by unpredictable, episodic swelling of the face, upper airway, oropharynx, extremities, genitalia and gastrointestinal tract. Almost all cases of HAE are caused by mutations in the *SERPING1* gene resulting in a deficiency in functional plasma C1 esterase inhibitor (C1EI), a serine protease inhibitor that normally inhibits proteases in the contact, complement, and fibrinolytic systems. Current treatment of HAE includes long-term prophylaxis with attenuated androgens or human plasma-derived C1EI, and management of acute attacks with human plasma-derived or recombinant C1EI, bradykinin and kallikrein inhibitors, each of which require repeated administration. As an approach to effectively treat HAE with a single treatment, we hypothesized that a one-time intravenous administration of an adeno-associated virus (AAV) gene transfer vector expressing the genetic sequence of the normal human C1 esterase-inhibitor (AAVrh. 10hC1EI) would provide sustained circulating C1EI levels sufficient to prevent angioedema episodes.

Methods.—To study the efficacy of AAVrh.10hC1EI, we used CRISPR/Cas9 technology to create a heterozygote C1EI deficient mouse model (S63+/−) that shares characteristics associated with HAE in humans including decreased plasma C1EI and C4 levels. Phenotypically, these mice have increased vascular permeability of skin and internal organs.

Results.—Systemic administration of AAVrh.10hC1EI to the S63+/− mice resulted in sustained human C1EI activity levels above the predicted therapeutic levels and correction of the vascular leak in skin and internal organs.

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Conflicts statement. Cornell University has licensed the patent disclosure relating to gene therapy for C1EI deficiency to Adverum Biotechnologies. RGC is a shareholder and a consultant to Adverum. OP, MC and RGC are inventors on the patent disclosure. TQ, ASW, ARR, DS and SMK have no conflicts

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Conclusion.—A single treatment with AAVrh.10hC1EI has the potential to provide long term protection from angioedema attacks in affected individuals.

Keywords

C1 esterase inhibitor; complement; gene therapy; hereditary angioedema; vascular permeability

Introduction

Hereditary angioedema (HAE) is an autosomal dominant disorder characterized by episodic attacks of swelling of the face, extremities, genitalia, gastrointestinal tract and upper airways[1–3]. These attacks account for >20,000 emergency department visits annually in the US, often result in hospitalization, vary in frequency and severity, are unpredictable, and can last for up to 5 days[2,4]. Upper respiratory tract edema can result in laryngeal swelling and death by asphyxiation[5,6]. Over 50% of individuals with HAE have at least one laryngeal attack in their lifetime, and death by asphyxiation can occur in >25% of those affected[1,3,6]. Abdominal attacks secondary to edema of the walls of the gastrointestinal tract are accompanied by pain, nausea, vomiting, and diarrhea and are frequently misdiagnosed resulting in unnecessary surgery, delay in diagnosis, and narcotic dependence[7–10]. Disfiguring cutaneous attacks are associated with social stigma and depression requiring psychotropic or antidepressant medication and swelling may lead to impaired quality of life[7,11].

HAE is caused by mutations in the *SERPING1* gene, resulting in low levels of functional C1 esterase inhibitor (C1EI or C1INH), the largest member of the serine protease inhibitor (SERPIN) superfamily[12–14]. C1EI is produced primarily by the liver and secreted into blood where it functions as the major inhibitor of C1r, C1s, mannose binding lectin-associated serine protease MASP-1, MASP-2, factor XII and kallikrein in the contact system, factor XI and thrombin in the coagulation system, and tissue plasminogen-activator (tPA) and plasmin in the fibrinolytic system[15–18]. Low functional C1EI levels cause increased activation of C1, with consequent reduction in C2 and C4[16,18,19], and increased formation of kallikrein, leading to accumulation of bradykinin which triggers episodes of increased vascular permeability[12,18,19]. Over 200 different mutations in the *SERPING1* gene have been reported worldwide[6,20]. HAE type I mutations (85% of cases) result in either a truncated or a misfolded protein that cannot be secreted. HAE type II (15% of cases) originates from mutations in the protein active site or nearby amino acids resulting in inactivity of the enzyme[3,18,19].

Treatment strategies for HAE are targeted to treating acute attacks with on demand therapy or preventing attacks with prophylactic therapy[21,22]. Acute HAE attacks can be managed with early therapeutic intervention by administration of plasma derived C1INH (Berinert®), recombinant C1INH (Ruconest®), kallikrein inhibitor (Kalbitor®), or inhibiton of the bradykinin receptor (Firazyr®)[23–26]. However, long-term prophylaxis is required to sustain a normal quality of life in individuals with frequent and/or severe episodes of angioedema. Approved prophylactic therapy includes plasma derived C1INH (Cinryze®, Haegarda®), synthetic attenuated androgens (Danatrol®, Winstrol®) and antifibrinolytics[23–29].

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Administration of plasma derived or recombinant C1INH is generally well-tolerated and is effective at reducing the incidence, severity and duration of HAE attacks, however these therapies are complicated by a high economic burden and the need for repeated administration carries a risk of limited compliance[7,21,30–34]. Although effective at reducing the frequency and severity of HAE attacks in many patients, chronic androgen use is associated with numerous side effects, which often leads to its discontinuation or patient noncompliance[35,36].

As a strategy to correct the consequences of the genetic defect with a one-time therapy, we hypothesized that a single intravenous administration of an adeno-associated virus coding for the normal, human C1 esterase inhibitor (hC1EI) would provide sustained, long-term therapeutic levels to protect from the unpredictable and debilitating attacks of angioedema. To evaluate this hypothesis, we created a C1EI deficient mouse model that mimics the clinical and molecular phenotype of HAE. After a single administration of a serotype rh.10 AAV vector coding for human C1EI (AAVrh.10hC1EI), there was persistent expression of hC1EI, resulting in long-term protection against the clinical phenotype.

Methods

Generation of a C1EI Deficient Murine Model

Clustered regularly interspaced short palindromic repeats (CRISPR) technology was used to generate a heterozygote C1EI mouse model utilizing the approach of Romanienko et. al[37] (Mouse Genetics Core, Memorial Sloan-Kettering Cancer Center). The guide RNA GGCAGTACTGTAGGCTCTGG (gRNA51) in conjunction with CRISPR associated protein 9 (Cas9) mRNA were co-injected into the pronucleus of mouse zygotes (10 ng/μl) using conventional techniques[38]. Founder mice were first examined using a T7 endonuclease I digestion assay to detect nucleotide changes in the target region of the mouse genome. For this purpose, polymerase chain reaction (PCR) products of the target locus were synthesized using the following primers: SERPA – GCTTCTTGAACCACAGGATAGAGC, SERPB - CAGAAGGGTTCAGTAGTAGCCTG.

Of the 88 founder mice examined, 43 (49%) had indels based on cleavage by T7 endonuclease, suggesting a deletion and/or insertion. A subset of progeny mice was further examined for deletions by sequencing PCR amplicons of the mutated *SERPING1* locus. Deletions ranging from 10 to 45 nucleotides were observed in 4 individual mouse lines. One *SERPING1* knockout mouse chosen for further analysis (S63) had a deletion that created a frameshift, introducing an early stop codon in exon 3 (Figure 1A). S63 mice were bred with C57Bl/6J albino mice (Jackson Laboratory, Bar Harbor, ME). All mice were housed in microisolator cages and maintained according to standard guidelines.

Genotyping of the S63 SERPING1 mice was carried out using tail tissue. Samples were lysed in 65 μl of 25 mM NaOH, 0.2mM ethylenediaminetetraacetic acid (EDTA) at 95°C for 45 min, then neutralized with 65 μl of 40 mM Tris-HCl. PCR was used to assess exon 3 of the *SERPING1* gene (forward primer - GTTATTGTGATGGCTACACTGG, reverse primer - GATCCACTGGAGGCTCAAG). The PCR reaction mix (10 μl) contained 0.1 μg DNA polymerase (Clontech, Mount View, CA), 5 pmol forward and reverse primer pairs and 2

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mM Nucleotide Mix (Thermo Fisher, Grand Island, NY). The standard PCR conditions included: denaturation (94 °C, 15 sec), annealing (64 °C, 30 sec), and extension (72 °C, 90 sec), repeated 35 times. PCR products were loaded onto a 4% agarose gel and run with 1X TAE. Amplification of DNA from wild-type mice result in a single band of 120 base pairs (bp). To detect the mutation introduced by CRISPR-Cas9, genomic DNA was extracted from tail biopsies, amplified by *SERPING1* exon 3 flanking primers, and sequenced. Analysis revealed a deletion of ten nucleotides, creating a frameshift that introduced an early stop codon. The resultant protein was truncated at 43 amino acids in length. The genotype of the C1EI mice was verified by PCR amplification using the primers indicated above. DNA from the S63+/– mice genotyping appeared as two bands of 110 bp and 120 base pairs and wild-type S63+/+ mice appeared as a single band of 120 base pairs (Figure 1B).

Characterization of the S63+/– Murine Model

Murine C1EI (mC1EI) and murine C4 (mC4) were evaluated in mouse sera by ELISA according to manufacturer's instructions (Biomatik, Wilmington, DE). To assess the vascular leak phenotype, Evans blue dye (Sigma-Aldrich., St. Louis, Missouri; 30 mg/kg in 100 µl PBS) was injected into the tail vein of 6 to 8 wk-old mice. Photographs of the rear paws and snout were taken 30 min after Evans blue dye injection.

AAV Vectors

The AAVrh.10hC1EI vector is comprised of the nonhuman primate-derived rh.10 capsid pseudotyped with AAV2 inverted terminal repeats, surrounding the hC1EI expression cassette. The expression cassette consists of the cytomegalovirus (CMV) enhancer chicken–β-actin promoter (CAG promoter), the human *C1EI* coding sequence, and rabbit β-globin polyadenylation signal (Supplemental Figure 1A). The h*C1EI* cDNA sequence was optimized for increased mRNA stability and to reduce the possibility of *trans*-inhibition by the mutant mRNA using human-biased codons and removal of mRNA instability elements, low (<30%) or high (>80%) GC regions, translation initiation sequences within the coding region and potential splicing signals. The optimized h*C1EI* cDNA was synthetized with an optimal Kozak consensus and cloned into the pAAV plasmid under control of the CAG promoter. The AAVrh.10hC1EI vector was produced by co-transfection of the pAAV plasmid together with a plasmid carrying the AAV Rep proteins derived from AAV2 (needed for vector replication), the AAVrh.10 viral structural (Cap) proteins VP1, 2 and 3 (which define the serotype of the produced rh.10 AAV vector) and the adenovirus helper functions of E2, E4 and VA RNA into human embryonic kidney 293T cells (HEK 293T; American Type Culture Collection). The AAVrh.10hC1EI vector was purified by iodixanol gradient and QHP anion exchange chromatography as previously described[39]. Vector genome titers were determined by quantitative TaqMan real-time PCR analysis. A vector coding for human α1-antitrypsin (AAVrh.10hα1AT), which is the same construct as described above with the h*C1EI* cDNA replaced by h*α1AT* cDNA, was used as a control for the *in vivo* expression studies[40].

In Vivo Expression of AAVrh.10hC1EI in S63 Heterozygous (S63 +/–) Mice

All animal studies were conducted under protocols reviewed and approved by the Weill Cornell Institutional Animal Care and Use Committee. To assess AAVrh.10hC1EI-directed

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expression of hC1EI protein *in vivo*, S63+/– mice, age 6 to 8 wk, were injected (intravenously in 100 μl) with a one-time dose of AAVrh.10hC1EI (10^{11} gc), or AAVrh.10hα1AT at 10^{11} gc (control vector), or 100 μl PBS as a negative control. Activity of hC1EI was measured in serum at 0 wk, and at multiple time points over the course of 24 wk by a chromogenic activity assay that measured the activity of the protein based on its ability to inhibit its natural substrate, C1 esterase (TECHNOCHROM® C1-INH [CE], DiaPharma Group, West Chester, OH). The activity assay was conducted per the manufacturer's protocol, and results expressed as a unit of function (U/ml). Native mouse C1EI was not detected by the activity assay.

Prevention of Vascular Leak

To demonstrate that AAVrh.10hC1EI therapy reversed increased vascular permeability observed in heterozygous S63+/– mice, Evans blue dye was injected intravenously and extravasation of dye from the vasculature was evaluated by photographing of rear paws and snouts with images taken 30 min after administration in both vector treated and non-treated mice at 2, 6, and 24 wk after therapy. At each time point, a subset of mice were euthanized, and rear paws, heart, lungs, spleen, small intestine, and kidneys were removed, blotted dry, and weighed. Evans blue dye was extracted from organs with 1 ml of formamide overnight at 55°C and measured spectrophotometrically at 600 nm[41,42]. The absorbance was measured three times for each organ and the average value was determined with formamide used as a blank. The amount of dye recovered was calculated by extrapolating from a standard curve prepared with different concentrations of Evans blue dye solution in 1 ml of formamide. Vascular permeability was quantified spectrophotometrically by measuring the amount of dye per mg of tissue.

Statistics

All data are presented as means ± standard error of the mean (SEM) unless otherwise stated; the "n" value for each group is stated in the figure or figure legend. Differences between groups were analyzed using an unpaired two-tailed Student's t test. p values <0.05 were considered significant for all comparisons.

Results

C1EI Deficient Murine Model Phenotype

The heterozygous C1EI deficient S63+/– mice appeared normal at birth, and subsequently developed and bred normally with no differences in litter size. The serum level of murine C1EI in the S63+/– female and male mice was significantly less when compared with wild-type controls, ($p<0.001$; males and $p<0.02$; females, Figure 1C). Serum levels of C4 in the S63+/– mice were significantly lower than levels measured in wild-type controls ($p<0.001$, males; $p<0.004$, females, Figure 1D). The S63+/– mice did not develop spontaneous episodes of swelling. However, the S63+/– mice exhibited increased vascular permeability as evidenced by the extravasation of Evans blue dye in the paws and snout, far greater than that observed in wild-type controls (Figure 1E).

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AAVrh.10hC1EI-mediated Therapy

In vitro assessment demonstrated that the plasmids used to generate AAVrh.10hC1EI produced the AAVrh.10 capsid proteins and the human *C1EI* transgene (Supplemental Figure 1B, C). *In vivo* expression of hC1EI after AAVrh.10hC1EI gene transfer was demonstrated in S63+/− mice after a single intravenous administration of the vector (10^{11} gc; Figure 2). Human C1EI activity was observed in S63+/− mice for at least 24 wk, the last time point evaluated. As is typically observed in mice treated with experimental AAV vectors, the male mice had higher levels of the expressed protein compared to the female mice[40,43–45]. No hC1EI activity was detected in serum from control mice that received intravenous AAVrh.10hα1AT or PBS administration (Figure 2).

Vascular permeability of vector treated and untreated S63+/− and wild-type control mice was assessed using Evans blue dye at 2, 6 and 24 wk post-administration of AAVrh.10hC1EI (10^{11} gc). Untreated S63+/− mice visually exhibited greater extravasation of dye in their rear paws and snouts compared to wild-type controls (C57Bl/6J mice; Figure 3). In contrast, over 24 wk, the AAVrh.10hC1EI treated group displayed phenotypic results that were similar to the wild-type female and male controls (Figure 3). The observed phenotype was validated by quantitative spectrophotometric analysis of extracted dye from rear-paws (Figure 4A). The untreated S63+/− mice had significantly increased dye in the kidneys, intestines, lungs, spleen and heart compared to the wild-type controls ($p<0.01$, Figure 4B–F). In contrast, both male and female AAVrh.10hC1EI vector-treated mice had levels of dye extravasation comparable to the untreated wild-type controls. Comparisons of the S63+/− treated *vs.* untreated male and female mice rear paws and internal organs were significant ($p<0.05$) with the following exceptions; lungs (females, 2 and 24 wk), spleen (males, 2 and 6 wk) and intestines (female, 24 wk; Figure 4).

Discussion

Hereditary angioedema (HAE), is a rare autosomal dominant disorder resulting from mutations in the C1-inhibitor gene (*SERPING1*)[12–14,18]. The disorder afflicts 1 in 10,000 to 1 in 50,000 persons and has been reported in all races without gender predominance[3,46]. Affected individuals face a number of challenges including living with the uncertainty of when the next attack will present, the variability of the disease and a lack of identifiable triggers[2,8,47]. The goals of treatment for HAE are focused on life-saving efforts, slowing the progression and severity of attacks, reducing the number of future attacks, and their impact on quality of life[3,19,21,48,49]. Current therapies with plasma derived and recombinant C1INH therapies are well tolerated, however, the main challenge is that the protection provided by a single administration of these products is short, estimated at 3–4 days, requiring repeated drug administration to maintain persistent efficacy[21,48]. Other limitations of current angioedema therapies, in addition to the inconvenience of repeated administration, include product side-effects, safety profiles, and the high cost of most of the available therapies[7,8,21,32–34,50].

To circumvent the requirement for frequent C1EI administration, we have developed a novel AAV-mediated gene therapy strategy to mediate persistent therapeutic serum levels of C1EI with a single administration of a gene transfer vector coding for human *C1EI* in a novel

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murine model of HAE that mimics the human disease. The data demonstrates that AAV-mediated C1EI therapy markedly reduces the vascular permeability phenotype in these mice.

In the present study, we established and characterized a mouse model that mimics hereditary angioedema at the molecular level. In particular, mice had markedly decreased circulating levels of C1EI and C4. Intra-strain gender-dependent differences were detected in C4 and C1EI levels, but overall, as in humans, C1EI levels in most mice were approximately 50% of normal and C4 levels were overall decreased. On average, C4 levels in the female S63+/− mice, did not drop below 50% of wild type controls, but there were no differences in the vascular permeability data between male and female S63+/− mice. Although there are no intergender differences in C4 levels in humans with HAE, the mouse data suggests dysregulated activation of the complement system, akin to the human disease. S63+/− mice did not have spontaneous or triggered episodes of swelling involving the skin or organs as in the human disease, but mice displayed increased vascular permeability, as demonstrated by analysis of the extravasation of intravenously injected Evans blue dye in multiple organs. Although there is a previously described C1INH-deficient mouse[41,51], we developed the S63+/− mouse because the previously described mouse is owned by Lexicon Pharmaceuticals (The Woodlands, TX) and is not freely available to the academic community[41,51].

Our study demonstrates that a one-time therapy with AAVrh.10hC1EI provided sufficient human C1EI systemically, in both male and female mice, to abrogate the increased vascular permeability phenotype associated with HAE. With the goal of translating this therapy to human clinical trials, human C1 esterase inhibitor was used. Given the high homology between murine and human SERPING1 at the protein level (78% identity), the murine studies used human C1 esterase inhibitor, instead of mouse C1 esterase inhibitor, as we hypothesized that the human therapy would successfully reverse the mouse phenotype[52]. Therapy with AAVrh.10hC1EI was effective at protecting against increased vascular permeability in the paws, snout, and internal organs of these mice. Although the C1EI deficient mice did not display any obvious spontaneous clinical phenotypic abnormalities, after intravenous injection of Evans blue dye, which binds to serum albumin, the mice displayed increased vascular permeability compared with wild-type controls. Shortly after Evans blue dye injection, the rear paws, snout, and internal organs in C1EI deficient mice turned blue; whereas, wild-type control mice turned only slightly blue, as observed both clinically and by spectrophotometric measurement of the extracted dye. A one-time dose of AAVrh.10hC1EI reversed the increased vascular permeability after Evans blue dye injection up to 24 wk after therapy, the last time point evaluated. While the current study is limited to 24 weeks, AAV-based gene therapies in our lab and others have demonstrated long term expression measured in years and limited only by the longest time point evaluated[53,54,55]. This study establishes that a gene therapy expressing C1EI provides protection from increased vascular permeability in the mouse model, but translation to drug development requires formal safety and toxicology studies that will inform the viability of this approach with respect to dose and associated drug safety.

The protection against increased vascular permeability coupled to the long term gene expression studies suggests that AAVrh.10hC1EI has a long-lasting protective effect and

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indicates that protection can be achieved against HAE attacks by a single dose, an advantage of the delivery system. Thus, in a clinical setting, AAVrh.10hC1EI therapy could offer long-term protection to individuals affected by HAE following a single administration of the vector.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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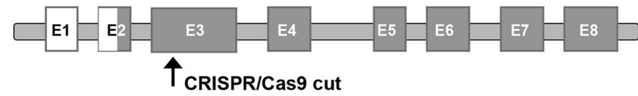
A. **Murine *SERPING1* gene**
Coding exons E2 to E8

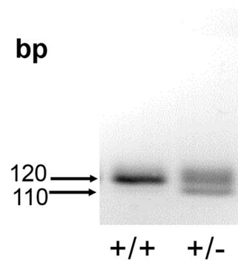


Exon 3 deletion

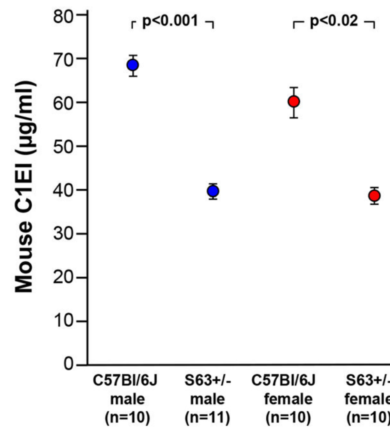
Wild-type AAAAGCAGAGAGAGCTTCCCTGAAAGAGATGACTCCTGGAGTCCCCCAGAGCCTACAGTACT

Mutant S63 AAAAGCAGAGAGAGCTTCCCTGAAAGAGATGACTCCTGGAG--------------------CCTACAGTACT

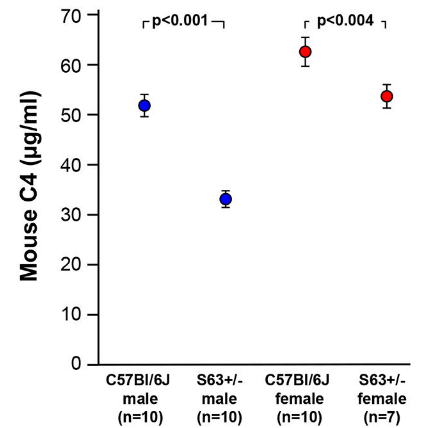
B. Genotype analysis



C. Mouse C1EI



D. Mouse C4



E. Vascular permeability

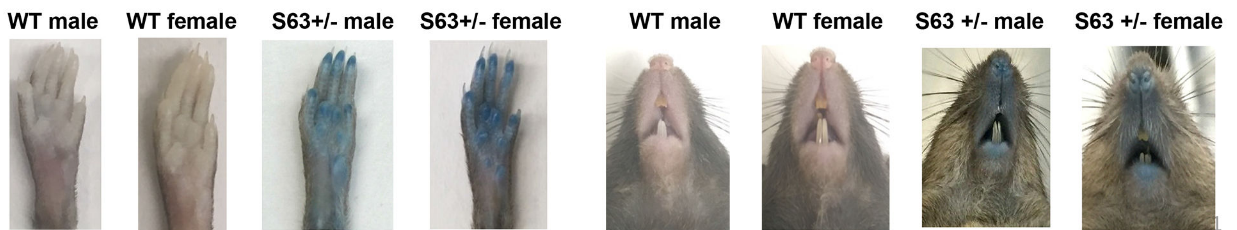


Figure 1.

Characterization of the S63+/– mouse. **A**. CRISPR/Cas9 deleted sequences in exon 3 of the murine *SERPING1* gene. **B**. PCR assessment of exon 3 of wild-type and S63 mice. **C**. Heterozygote S63+/– and wild-type serum C1EI levels as measured by ELISA. **D**. Heterozygote S63+/– and wild-type serum C4 levels as measured by ELISA. **E.** Increased vascular permeability in S63+/– mutant mice 30 min after Evans blue dye injection. Shown are photographs of wild-type (WT) and S63+/– males and female rear paws and snouts.

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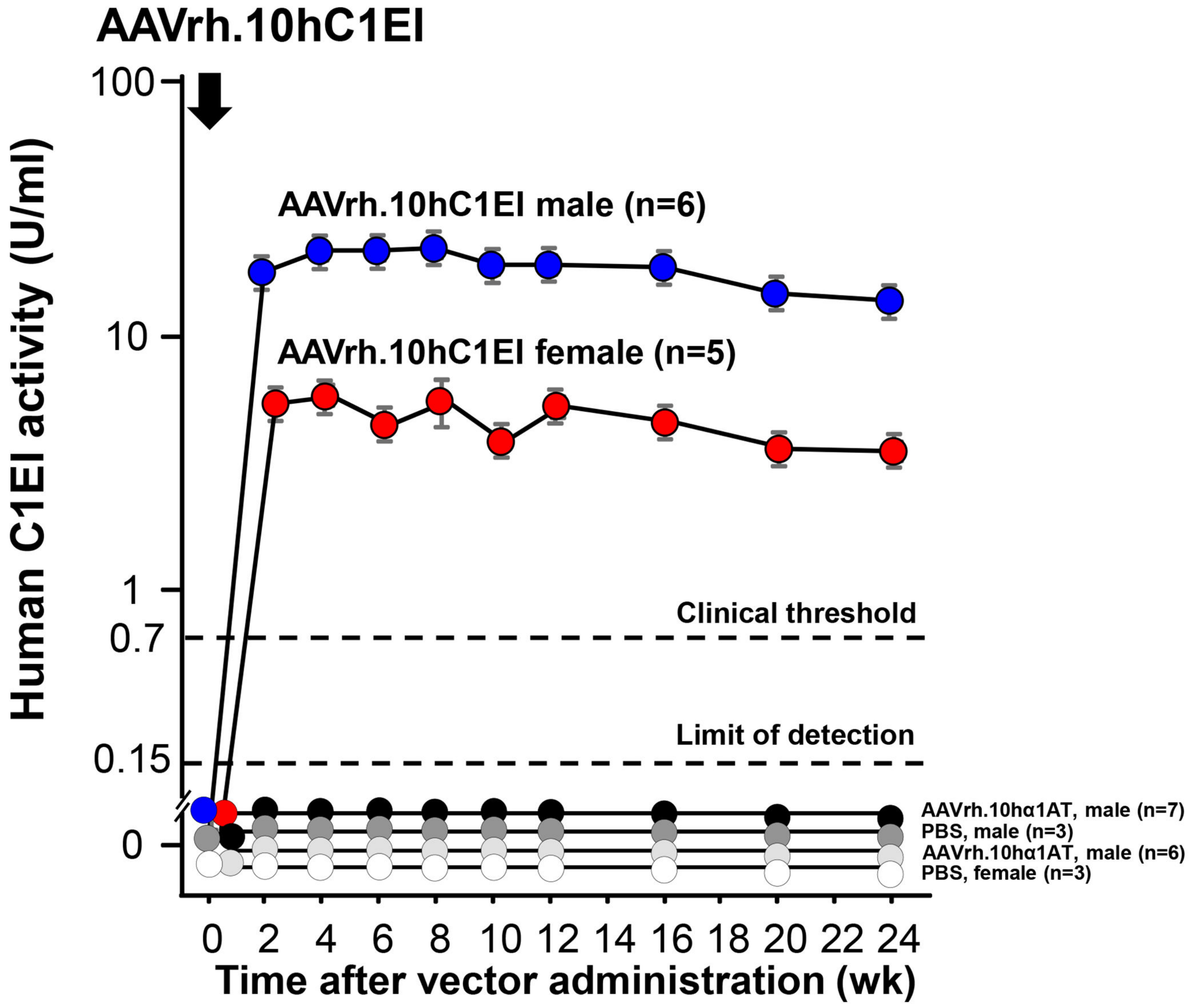

Figure 2.

AAVrh.10hC1EI-mediated persistent expression of human C1EI levels over time, following a single intravenous administration (10^{11} gc) to S63+/− mice. AAVrh.10hα1AT (10^{11} gc) and PBS were controls. Values are presented as means ± SEM. Numbers of mice in each treatment cohort are shown in the labels. The clinical threshold for C1EI is shown (0.7 U/ml) by the upper black dashed line. Limit of detection for the assay (0.15 U/ml) is shown by the lower black dashed line.

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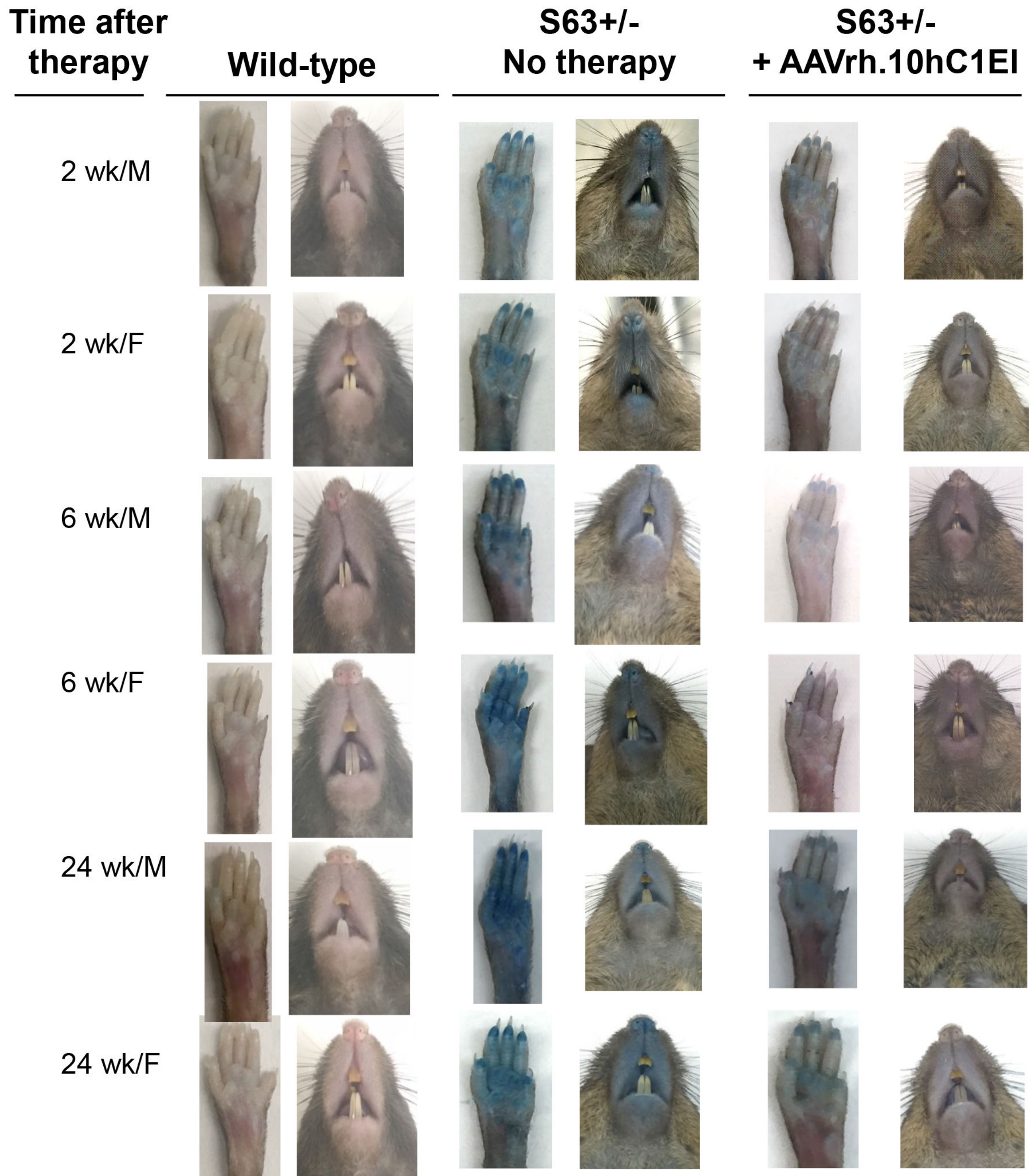

Figure 3.
AAVrh.10hC1EI correction of vascular permeability in S63+/− mice. Shown is assessment of S63+/− mice 2, 6 and 24 wk after treatment with AAVrh.10hC1EI (10^{11} gc) or PBS. Extravasation of dye in rear paws and snouts of S63+/− untreated and S63+/− treated mice 30 min after Evans blue dye administration. Age-matched C57BL/6J mice were used as wild-type controls. Shown are data for males and females.

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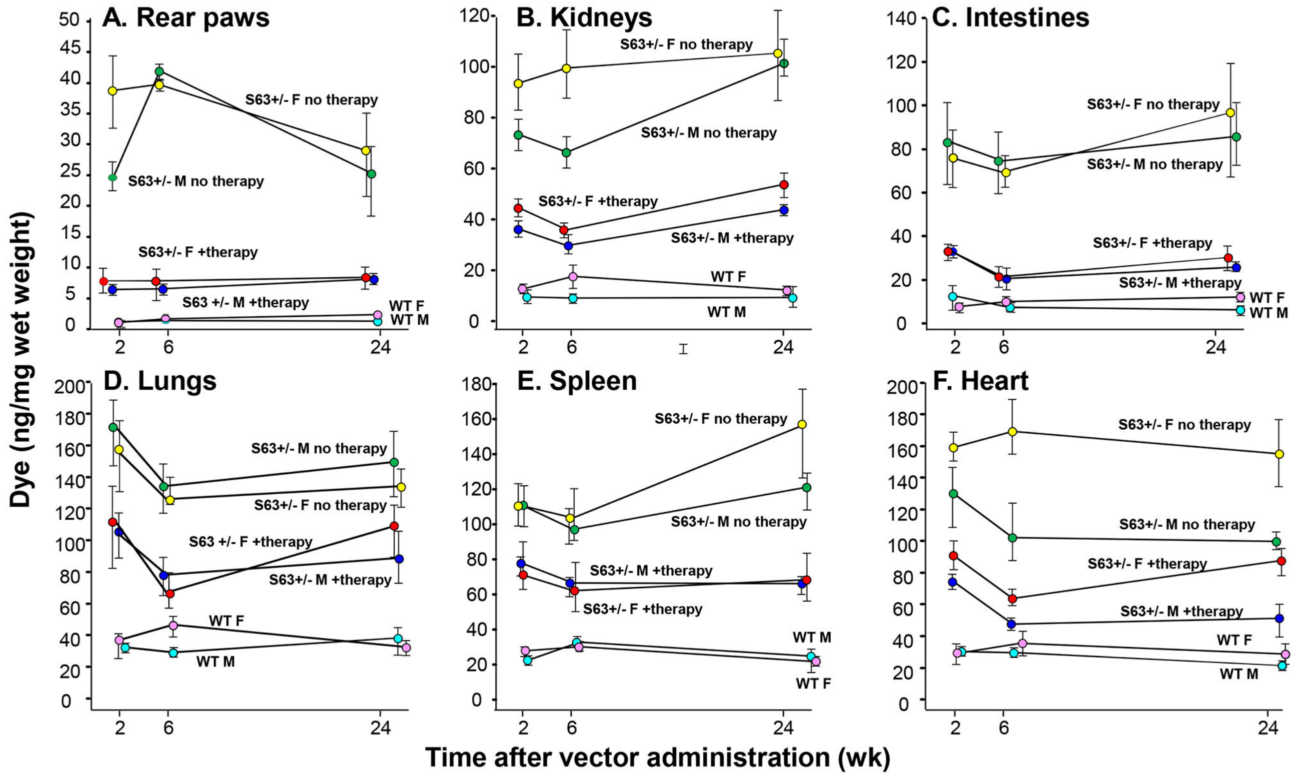

Evans Blue Dye (ng/mg organ) in heterozygous (S63 +/-) treated *vs* untreated male and female mice

Time point (wk)	Organ	p value Males	p value Females	Time point (wk)	Organ	p value Males	p value Females	Time point (wk)	Organ	p value Males	p value Females
2	Hind paws	<0.0001	<0.0001	6	Hind paws	<0.0001	<0.0001	24	Hind paws	<0.004	<0.002
	Lungs	<0.04	>0.1		Lungs	<0.02	<0.04		Lungs	>0.08	>0.2
	Kidneys	<0.0001	<0.0001		Kidneys	<0.0002	<0.0001		Kidneys	<0.004	<0.003
	Intestines	<0.04	<0.009		Intestines	<0.03	<0.006		Intestines	<0.02	>0.06
	Spleen	>0.1	<0.02		Spleen	>0.1	<0.05		Spleen	<0.02	<0.04
	Heart	<0.03	<0.002		Heart	<0.05	<0.004		Heart	<0.02	<0.05

Figure 4.

Spectrophotometric analysis of vascular permeability of various organs of S63+/− mice following AAVrh.10hC1EI therapy. Shown is data over time for S63+/− mice and wild-type controls. Evans blue dye was administered intravenously and tissues sampled following necropsy at 30 min. Whole tissue was incubated with 1 ml formamide to extract the extravasated Evans blue dye. Optical density was measured at 600 nm and the measurements converted into ng dye extravasated per mg tissue. **A**. Rear paws; **B.** kidneys; **C.** small intestines; **D.** lung; **E.** spleen; and **F.** heart. p values between treated and untreated S63+/− mice are presented in the table for all time-points. n=3–5 mice/group at 2, 6, and 24 wk time-points.