Stanniocalcin-1: A Model for Evaluating Rescue of Photoreceptor Degeneration in the Rhodopsin Transgenic Pig

Patrick D. Bradley, MD, MPH, Gavin W. Roddy, MD, PhD, Wankun Xie, MD, PhD, Robert H. Rosa, Jr., MD

**Purpose:** To assess the histologic rescue of photoreceptors with stanniocalcin-1 (STC-1) protein therapy in an in vivo porcine model of retinal degeneration. To assess the functional therapeutic efficacy of stanniocalcin-1 (STC-1) protein therapy on the rescue of photoreceptors in an in vivo porcine model of retinal degeneration. To test the hypotheses that STC-1 improves the expression of photoreceptor-specific gene transcripts, increases the expression of UCP-2, and decreases the levels of two protein adducts generated by reactive oxygen species in the retina.

**Methods/Design:** Experimental animal protocol evaluating potential rescue of photoreceptor degeneration in the rhodopsin transgenic pig via intravitreal injection of stanniocalcin-1.

**Main Outcome Measures:** Electroretinography, Optical Coherence Tomography, Histological Analysis, Fundus Autofluorescence, Real-Time Reverse Transcriptase PCR, Microarray-Assay, ELISAs for markers of oxidative damage.

**Conclusion:** Intravitreal injection of STC-1 induced rescue of photoreceptors in two well described rat models of retinal degeneration. The rhodopsin transgenic pig is a higher order vertebrate model of retinal degeneration that is a good model for human disease. If photoreceptor rescue can be demonstrated in the pig model of retinal degeneration, FDA trials involving human experimentation for toxicity and safety could likely be pursued. If proven a safe and effective therapy for retinal degeneration, STC-1 would provide a tremendous clinical benefit to the large number of individual patients suffering from retinal degeneration, including nonexudative age-related macular degeneration and retinitis pigmentosa.

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Age-related macular degeneration (AMD) is the leading cause of severe vision loss in people over 50 years of age in the United States. It has been estimated that nearly 15 million North Americans have the nonexudative form of AMD. This number represents nearly 90 percent of all patients diagnosed with AMD. As the population of the United States ages, this number will likely continue to increase. Oxidative stress and photoreceptor apoptosis are significant features of several forms of retinal degeneration (RD) including nonexudative AMD and retinitis pigmentosa. Currently there are no effective therapies for these forms of retinal degeneration.

The photoreceptors and retinal pigment epithelium (RPE) are sensitive to oxidative stress and apoptosis as a result of their proximity to the choroid and its associated vascular
supply. The oxygen content of the choroid approaches the level of the arterial supply. In addition, the RPE has an extensive exposure to the UV radiation associated with sunlight which creates reactive oxygen species (ROS). Finally, there is a high concentration of polyunsaturated fatty acids in the anatomical region which are oxidized to toxic products which induce inflammation and can trigger the complement cascade.

Roddy et al previously demonstrated that mesenchymal stem cells reduce apoptosis by being activated to secrete stanniocalcin-1 (STC-1). STC-1 is a multifunctional protein that reduces oxidative stress by upregulating mitochondrial uncoupling protein-2 (UCP-2). UCP-2 uncouples oxidative phosphorylation and thereby provides an increased flow of electrons to reduce ROS. Therefore, one significant effect of STC-1 is probably to decrease apoptosis by reducing oxidative stress.

Roddy et al demonstrated that intravitreal injection of STC-1 can rescue photoreceptors in 2 distinct models of retinal degeneration. The S334ter-3 rhodopsin transgenic rat represents a rapid model of both rod and cone degeneration. When injected with STC-1, the rhodopsin transgenic rat demonstrated a decreased loss of photoreceptor nuclei and transcripts and resulted in measurable retinal function where none is typically present in this rapid model of degeneration. In addition, Roddy et al tested STC-1 in the Royal College of Surgeons (RCS) rat characterized by a slower photoreceptor degeneration. Intravitreal STC-1 reduced the number of pyknotic nuclei in photoreceptors, delayed the loss of photoreceptor transcripts, and demonstrated improved function of rod photoreceptors. Furthermore, the RCS rat eyes injected with STC-1 were shown to have upregulated UCP-2 and decreased levels of two protein adducts generated by reactive oxygen species (ROS).

Due to the findings of Roddy et al and their success in demonstrating the histologic and physiologic rescue of photoreceptors in the two rat models of degeneration after intravitreal injection of STC-1, the next phase of development of a therapeutic model will be to determine the ability of STC-1 to protect photoreceptors in a higher order vertebrate. In addition, rescue needs to be evaluated over a longer period of time in a slower model of retinal degeneration.

The rhodopsin transgenic pig is a well described model of retinal degeneration in the literature. Petters et al created transgenic pigs that express a mutated rhodopsin gene (Pro347Leu). The normal pig eye is similar to that of the human in size and percentage of photoreceptors. In the initial report on the rhodopsin transgenic pigs by Peters et al, it was argued that these animals represent a useful animal model for retinitis pigmentosa in humans. Like RP patients with the same mutation, these pigs have both early and severe rod loss; initially, their cones are relatively spared, but the surviving cones slowly degenerate. By age 20 months, the cone electroretinogram is markedly abnormal. Given the strong similarities in phenotype to that of human RP patients, these transgenic pigs have been recognized as a large animal model for the study of cone degeneration and have been used for preclinical treatment trials. Therefore, since RP is a form of retinal degeneration that is associated with apoptosis, we propose to perform an STC-1 animal protocol in the rhodopsin transgenic pig based on our previous research. The funding and research team for this new protocol have been acquired. An optimization protocol has been performed on a series of domestic pigs in preparation for the
experimentation. Consistent and reproducible ERGs were obtained in domestic pigs. The research team is now in the process of acquiring the experimental model.

If STC-1 is successful in demonstrating rescue in the pig model of retinal degeneration, it represents a promising new therapy for retinal degeneration in humans which involve apoptosis and oxidative stress, including retinitis pigmentosa and the nonexudative form of AMD. If successful in the pig model of retinal degeneration, the next phase in the therapeutic developmental process would be early phase clinical trials evaluating the safety and efficacy in human subjects.

Methods

The experimental animal use protocol has been approved by the Institutional Animal Care and Use Committees of Texas A&M Health Science Center.

Recombinant human STC-1 used in this study (BioVender Research and Diagnostic Products) will be used according to the manufacturer’s instructions. Distilled water will be added to a vial of STC-1 that was lyophylized in 20 mmol/l Tris buffer, 20 mmol/l NaCl to yield a final solution of 0.5 mg/ml suspended in Hank’s balanced salt solution at a concentration of 20,000 cells/μl. The pigs will be sedated with telazol 4mg/kg i.m. and anesthetized with isoflurane after endotracheal intubation for the entire length of the procedure. Tropicamide 1% and phenylephrine 2.5% will be placed in both eyes. Retinal fundus photography will be performed with the RetCam II (Clarity Medical Systems) pre- and post-injection.

Electroretinography will be performed (see ERG methods) pre- and post-injection. The animal subject will be prepped with topical betadine 5%. With sterile technique, an intravitreal injection of STC-1 protein (25 micrograms) in 50 μl HBSS will be then performed (entry site 1 mm posterior to the limbus) in the right eye with a 30-gauge needle attached to a Hamilton syringe. The left eye will be injected with vehicle control (HBSS) and serve as an internal control to assess the rescue of retinal degeneration in a given animal subject. The animal subjects will be recovered from anesthesia with routine post-operative care and followed until their assigned timepoint for morphologic, functional (ERG), and molecular/biologic analyses. The eyes will be enucleated at the assigned timepoints under anesthesia, and the animal subjects will then be euthanized.

Electroretinography: Full-field scotopic and photopic electroretinograms (ERG) of the pigs will be generated using the ESPION system (Diagnosys LLC). After sedation with a telazol 4mg/kg i.m. and induction with isoflurane anesthesia, two subdermal electrodes (Grass F-E7, Astro-Med, Inc, West Warwick, RI) will be placed subcutaneously at the temporal canthus and the rostrum, respectively. An ERG jet contact lens monopolar electrode (Universal SA, La Chaux-de-Fons, Switzerland) will be placed on the cornea with lubricant gel (0.3% hypromellose, Novartis) as the active electrode. Full-field ERGs will be recorded with the ESPION System (Diagnosys LLC). Three ERG responses will be elicited: rod b-wave to dim blue flashes (-1.9 log scot cd.s.m², dark-adapted for >2hr); a cone ERG at 1-Hz (2.8 cd.s.m² white flashes on a 25 cd.m² white background); and cone ERGs at 29-Hz (2.8 cd.s.m² white flashes on a 6 cd.m² white background). ERG responses to 2 blue (2.9 and 0.6 log scot cd.s.m²) and 1 red stimulus (1.9 log cd.s.m²) will be recorded in the dark-adapted state. The leading edge of these responses (initial 5-10 ms
depending on stimuli) is expected to have rod and cone contributions. The saturated rod response will be isolated by digital substraction of the cone component (Petters et al. 1997). ERG may be performed at baseline prior to intravitreal injection and at the assigned timepoints for assessment of rescue of retinal degeneration.

**Retinal Fundus Photography.** The RetCam II (Clarity Medical Systems) may be utilized to document the clinical status and appearance of the retinal fundus of the animal subjects under anesthesia immediately pre- and post-injection and at the assigned timepoint for assessment of rescue of retinal degeneration prior to enucleation.

**Optical Coherence Tomography.** Spectral Domain Optical Coherence Tomography may be utilized in to document the thickness and microarchitecture of the retina of the animal subjects under anesthesia immediately pre- and post-injection and at the assigned timepoint for assessment of rescue of retinal degeneration prior to enucleation. This technology is noninvasive and performed with instrumentation similar to that used for retinal fundus photography (Heidelberg Spectralis).

**Fundus Autofluorescence.** Fundus Autofluorescence technology may be utilized for monitoring the effects aimed at lowering lipofuscin accumulation in the retinal pigment epithelium in the animal subjects under anesthesia immediately pre- and post-injection and at the assigned timepoint for assessment of rescue of retinal degeneration prior to enucleation. This technology is noninvasive and performed with instrumentation similar to that used for retinal fundus photography (Heidelberg Spectralis).

**Microarray assays.** RNA from each sample will be applied for microarrays according to manufacturer’s directions. Briefly, poly-A RNA controls will be added into each sample to provide exogenous positive controls to monitor the eukaryotic target labeling process. T7 oligo(dT) primer will be used to generate first strand cDNA followed by second strand cDNA synthesis. To generate biotin modified aRNA, in vitro transcription will be performed followed by purification and quantification of labeled aRNA. A total of 15 μg of aRNA will be fragmented and hybridized onto pig arrays followed by array washing and staining. Arrays will be scanned with with GeneChip Scanner and images will be checked for quality. Data will be normalized using robust multi-array algorithm and gene level analysis will be performed. To obtain up- and downregulated genes, STC-1-treated samples will be compared with phosphate-buffered saline-treated samples within each model and expression level changes of at least 1.5-fold will be considered significant.

**Real time RT-PCR assays.** For RNA extraction, retinas will be isolated by surgical excision, immediately placed in RNA isolation reagent and frozen at −80 °C. The samples were rapidly thawed and homogenized on ice, and total RNA was extracted. cDNA will be generated by reverse transcription using 1 μg total RNA. Real-time amplification will be performed using TaqMan Universal PCR Master Mix. Values will be normalized to 18s RNA and expressed as a fold change compared to the fellow eye.

**Histological analysis.** Tissue processing and histological analysis will be performed. Following euthanasia by overdose of carbon dioxide, eyes will be enucleated and immediately fixed in a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde. Eyes will be embedded in epoxy resin, and 1-μm thick sections will be made along the vertical meridian. To quantify photoreceptor loss, a mean ONL thickness will be obtained by taking
an average of a total of 54 measurements from the superior and inferior hemispheres (27 per hemisphere) using the Bioquant Morphometry System. Pyknotic index will determined by counting numbers of pyknotic photoreceptor nuclei as a percent of total photoreceptor nuclei.

Statistics: Paired, two-tailed Student’s t-tests will be used to compare treated and control eyes from the same pig in all experiments.

Conclusions

Roddy et al demonstrated that intravitreal injection of STC-1 induced rescue of photoreceptors in two well described rat models of retinal degeneration\(^1\). In addition, they have shown that at least one contributing mechanism is via upregulation of UP-2 and subsequent decrease in oxidative stress and apoptosis\(^1,4,5\). The rhodopsin transgenic pig is a higher order vertebrate genetically engineered by Petters et al in order to establish a model of retinal degeneration which simulates human disease\(^6\). If photoreceptor rescue can be demonstrated in the rhodopsin transgenic pig, FDA trials involving human experimentation for toxicity and safety could likely be pursued as the next step in the development of a therapeutic model\(^1\). If proven a safe and effective therapy for retinal degeneration, STC-1 would provide a tremendous clinical benefit to the large number of individual patients suffering from retinal degeneration, including nonexudative age-related macular degeneration and retinitis pigmentosa for which there is currently no effective treatment\(^1\).

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The previous research and publications describing the use of STC-1 in the rat model of retinal degeneration by Gavin W. Roddy, MD, PhD and Robert H. Rosa, Jr., MD are the basis of this review and the experimental protocol described herein.

References


