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Transgenic zebrafish expressing mutant human *RETGC-1* exhibit aberrant cone and rod morphology

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ABSTRACT

Cone-rod dystrophy 6 (CORD6) is an inherited blindness that presents with defective cone photoreceptor function in childhood, followed by loss of rod function. CORD6 results from mutations in *GUCY2D*, the human gene encoding retinal guanylate cyclase 1 (RETGC-1). RETGC-1 functions in phototransduction, synthesising cGMP to open ion channels in photoreceptor outer segments. As there is limited histopathological data on the CORD6 retina, our goal was to generate a CORD6 model by expressing mutant human *RETGC-1* in zebrafish cone photoreceptors and to investigate effects on retinal morphology and function. cDNAs encoding wildtype and mutant (E837D R838S) *RETGC-1* were cloned under the control of the cone-specific *gnat2* promoter and microinjected into zebrafish embryos to generate transgenic lines. *RETGC-1* mRNA expression in zebrafish eyes was confirmed by RT-PCR. Fluorescent microscopy analysed retinal morphology and visual behaviour was quantified by the optokinetic response (OKR). Stable transgenic lines expressing mutant or wildtype human *RETGC-1* in zebrafish eyes were generated. OKR assays of 5-day-old larvae did not uncover any deficits in visual behaviour. However, transgenic (E837D R838S) *RETGC-1* expression results in aberrant cone morphology and a reduced cone density. A reduction in the number of photoreceptor nuclei, the thickness of the outer nuclear layer and the labelling of rod outer segments, particularly in the central retina, was evident. Expression of mutant human *RETGC-1* leads to a retinal phenotype that includes aberrant photoreceptor morphology and a reduced number of photoreceptors. This phenotype likely explains the compromised visual function, characteristic of CORD6.

1. Introduction

Cone-rod dystrophies are a genetically heterogeneous group of retinopathies that have a combined prevalence of ~1 in 40,000 (Hamel, 2007). This heterogeneity arises, for example, from the array of genetic lesions in molecular components of the phototransduction cascade and visual cycle that result in dysfunctional photoreceptors. Cone-rod dystrophies are typified by a loss of cones with a subsequent loss of rods, causing dyschromatopsia and an initial loss in visual acuity, followed by nyctalopia and a decrease in peripheral vision (Gregory-Evans et al., 2000; Van Ghelue et al., 2000; Downes et al., 2001a,b). Electroretinogram analyses report dramatic reductions of the a-wave (photoreceptor-derived) and b-wave (bipolar cell-derived) profiles, and fundus examinations describe pigment deposits in the macula ("bull's-eye" maculopathy) and varying degrees of retinal atrophy in diseased retinas (Gregory-Evans et al., 2000).

Cone-rod dystrophy 6 (CORD6) is a severe autosomal dominant (ad) form of the disease, caused by mutations in the *GUCY2D-1* gene on chromosome 17p13.1, which encodes RETGC-1, a membrane-bound cGMP synthetase (Kelsell et al., 1998). In dark conditions, RETGC-1 activity opens some cGMP-gated ion channels in photoreceptor outer segments, allowing the influx of Ca^{2+} ions (Yau and Nakatani, 1985). RETGC-1 is tightly regulated by guanylate cyclase-activating proteins (GCAPs) in the photoreceptor outer segments, and by Ca^{2+}-dependent GCAPs (CD-GCAPs) at the photoreceptor-bipolar synapse and inner retinal neurons (Palczewski et al., 1994; Pozdnyakov et al., 1995; Duda and Koch, 2002; Krishnan et al., 2004). In the dark state, an ionic balance is maintained by the efflux of Na^+, Ca^{2+} and K^+ by ion pumps in the cell membrane,

Abbreviations: ad, autosomal dominant; CD-GCAP, calcium-dependent guanylate cyclase-activating protein; cGMP, cyclic guanosine monophosphate; CORD6, cone-rod dystrophy 6; dpf, days post fertilization; GCAP, guanylate cyclase-activating protein; gnat2, guanine nucleotide-binding protein G(t) subunit alpha-2; GUCY2D, guanylate cyclase 2D; INL, inner nuclear layer; OKR, optokinetic response; ONL, outer nuclear layer; PDE6, phosphodiesterase 6; retGC-1, retinal guanylate cyclase 1; ROS, rod outer segment.

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offsetting the influx of Ca^{2+} (Cervetto et al., 1989). In high (700 nM) intracellular Ca^{2+} conditions, GCAPs are inhibited from stimulating RETGC-1, thus cGMP is synthesised at a basal level (Dizhoor and Hurley, 1999). CD-GCAPs exert the opposite effect, increasing RETGC-1 activity (Duda et al., 1996; Sitaramayya, 2002). However, the initiation of phototransduction causes phosphodiesterase (PDE6) to hydrolyse cGMP more quickly than it can be synthesised by RETGC-1; this causes cGMP-gated channels to close, and as ion efflux pumps continue to function in the cell membrane, the intracellular level of Ca^{2+} drops and the cell hypopolarises, resulting in alterations in glutamate release at the photoreceptor ribbon synapse. The low (50 nM) Ca^{2+} conditions remove the inhibitory block on GCAPs, which stimulate RETGC-1 to increase production of cGMP, causing some cGMP-gated ion channels to reopen.

RETGC-1 functions as a homodimer, formed using a 36 amino acid sequence proposed to be the dimerisation domain (Tucker et al., 1999; Yu et al., 1999), though other data indicates that the catalytic domain of RETGC-1 may have an inherent dimerisation capability (Venkataraman et al., 1998). Several adCORD6 phenotypes are associated with mutations in this region, particularly residues 837–839, which *in vitro* are reported to strengthen RETGC-1 dimerisation leading to the active, dimerised RETGC-1 persisting during high Ca^{2+} conditions (Ramamurthy et al., 2001). This may lead to extra open cGMP-gated ion channels, regardless of light conditions, allowing an excess of Ca^{2+} ions to enter the photoreceptors. In rods, an excess of calcium is reported to cause apoptosis (Fox et al., 1999), and it has been proposed that this mechanism of action is responsible for the pathology of CORD6 (Ramamurthy et al., 2001). However, it has also been proposed that mutations in RETGC-1 lead to reduced catalytic activity *in vitro*, and that reduced cGMP synthesis causes the CORD6 disease phenotype (Duda et al., 1999, 2000).

CORD6 caused by RETGC-1 mutations has been well characterised in living patients, documenting "bulls-eye" maculopathy, photophobia, peripheral field loss, nyctalopia, RPE atrophy, and frequent and complete loss of electroretinographic response (Gregory-Evans et al., 2000; Van Ghelue et al., 2000; Downes et al., 2001a,b). The biochemical effects have been partly characterised *in vitro* (Tucker et al., 1997, 1999; Laura and Hurley, 1998; Ramamurthy et al., 2001). However, there is little data on the histopathology of CORD6 eyes, though recent advances in live imaging of eyes show photoreceptor abnormalities using optical coherence tomography (Kim et al., 2011). To establish an animal model of CORD6, we chose transgenic zebrafish (*Danio rerio*), small freshwater teleosts, whose diurnal colour vision and amenability to transgenesis have made them an attractive model for study of eye development and disease (Fini et al., 1997; Collery et al., 2006). We used the native zebrafish *gnat2* promoter (Kennedy et al., 2007) to drive cone-specific expression of either wildtype or mutant (E837D R838S) human RETGC-1. The highly efficient Tol2 transposase system was applied to make independent stable, transgenic lines expressing the human RETGC-1 variants (Kawakami et al., 2000). Adult zebrafish expressing mutant RETGC-1 show altered morphology of cones and rods and reduced labelling with photoreceptor-specific antibodies. Loss of photoreceptor nuclei is also evident. We propose that this zebrafish CORD6 model recapitulates aspects of the human disease phenotype, and that progressive deterioration in photoreceptor morphology underlies the visual deficits in human CORD6 sufferers.

2. Materials and methods

2.1. Transgenic constructs

Human *GUCY2D* cDNAs (wild type and E837D R838S variants) were subcloned downstream of the zebrafish cone transducin (alpha subunit) promoter (3.2*gnat2*) (Kennedy et al., 2007). The promoter-gene construct was then subcloned into the pT2KXIG vector for microinjection.

2.2. Generation of transgenic founder Zebrafish

25 ng/μl of the 3.2*gnat2-RETGC-1* transgenic constructs were co-injected with 25 ng/μl of *in vitro*-transcribed transposase mRNA into 1–2 cell embryos and surviving embryos raised to sexual maturity before outcrossing to isogenic wild type fish. Batches of embryos from outcrosses were taken at 5 dpf and DNA extracted for PCR using transgene-specific primers to identify transgenic founders. Siblings of transgenic embryos were raised to adulthood and individually identified using transgene-specific PCR performed on finclip-extracted DNA.

2.3. Tol2 excision assay

An assay for excision of the 3.2*gnat2-RETGC-1* transgenes was performed essentially as described (Kawakami et al., 1998). DNA was extracted from injected 1 dpf embryos with Tol2 plasmid carrying a transgenic construct along with transposase mRNA, and PCR was carried out using primers specific for the flanking regions of the Tol2 transposon system; in the event of excision of the transgenic construct from between these flanking regions, the primer binding sites are no longer separated by the intervening construct and are now only 200–300 bp apart. Generation of an amplicon of this size demonstrates that an excision event has taken place in the injected embryo following injection.

2.4. RT-PCR and real-time PCR analysis

Total RNA was extracted from adult transgenic fish eyes and from 5 dpf offspring of transgenic fish using TRIzol. cDNA was synthesised using Omniscript RT kit (QIAGEN) primed with random hexamers, and transgene-specific primers used in RT-PCR to demonstrate expression of *RETGC-1* mRNA. The same cDNA samples were used in Taqman quantitative real-time PCR to assay levels of *RETGC-1* expression using probe set Hs00164803_m1 (Applied Biosystems, Inc.), which were compared to expression levels in human retinal RNA samples (BioCat, Clontech).

2.5. Immunohistochemistry

Adult eyes were fixed overnight at 4 °C in 4% formaldehyde in PBS, then cryoprotected in a sucrose series before embedding in O.C.T. and freezing to −80 °C. Twelve micrometre sections were cut using a cryostat and thaw-mounted onto Superfrost Plus slides and rehydrated using PBS washes. Blocking was carried out using 2% (v/v) normal goat serum, 1% bovine serum albumin and 1% Triton X-100 in PBS for 1 h at room temperature. Red-green cone photoreceptors were labelled with zpr-1 antibody, while rod photoreceptors were labelled with zpr-3 antibody. These antibodies were generated as part of a panel of antibodies raised against homogenised zebrafish retina (Larison and Bremiller, 1990), and though the specific epitopes recognised by them are currently unknown, they are routinely used to label zebrafish photoreceptors. Anti-blue opsin and anti-UV opsin antibodies were a kind gift from Professor David Hyde, University of Notre Dame, Indiana. Primary antibodies were diluted 1:200 in blocking buffer overnight at 4 °C. Next day, slides were washed in PBST before incubation with cyanine-fluorescent secondary antibodies in darkness for 1 h at room temperature. After washing in PBS, sections were counterstained using 300 nM DAPI for 5 min in darkness, rinsed in PBS, and mounted in glycerol-based Vectashield.