Bestrophin 1 – Phenotypes and Functional Aspects in Bestrophinopathies

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ABSTRACT

This is to review the current state of knowledge on the functional and clinical aspects of bestrophin 1, a prominent member of a family of proteins involved in the control and properties of the light peak of the EOG. Initially human bestrophin 1 gene (BEST1) mutations were identified to underlie Best vitelliform macular dystrophy (VMD), a dominantly inherited, juvenile-onset form of macular degeneration. In the recent past the phenotypical spectrum of retinal disorders associated with BEST1 mutations has been extended and the term bestrophinopathies was coined. The physiological role of bestrophin 1 is still not completely understood but has been linked to the generation of a transepithelial chloride current by controlling voltage-dependent calcium channels (VDCC). Dysfunction of bestrophin 1 may result in abnormal ion and fluid transport by the retinal pigment epithelium (RPE) disturbing and even disrupting direct interactions between the RPE and the photoreceptors.

Keywords: Best disease, bestrophinopathies, bestrophin 1, electrooculogram, light peak, retinal pigment epithelium

INTRODUCTION

Best vitelliform macular dystrophy (VMD, OMIM 153700) is a disease whose phenotype seems easily recognizable. The conventional features of vitelliform macular lesions and the reduced light peak in the electrooculogram (EOG) are highly indicative of VMD. In 1905 Friedrich Best described the basic morphological features of VMD for the first time.¹ However, a deeper insight into the genetics and biochemistry of the underlying protein bestrophin 1 and the development of novel ophthalmological examination techniques raised more questions than answers on the etiology of the disease in recent years. In addition to the autosomal dominant pattern of inheritance in conventional VMD incomplete transmission of the affection, i.e. reduced penetrance, may occur. Other phenotypes assigned to specific mutations in BEST1 like multifocal vitelliform lesions outside the fovea but with cystoid changes reminiscent of juvenile retinoschisis in autosomal recessive bestrophinopathy (ARB, OMIM 611809) or peripheral degenerations in autosomal dominant vitreoretino-choroidopathy (ADVIRC, VRCP, OMIM 193220) broadened the phenotypic spectrum without good clues as to the underlying pathomechanisms.

In this review we take the reader from the ophthalmoscope to the retinal pigment epithelium (RPE) to shed some light on the phenotypic variation reported so far in bestrophinopathies and to summarize the knowledge on bestrophin 1 in the eye.

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Discovery and Description of the BEST1 Gene and its Nomenclature

Friedrich Best described a juvenile vitelliform macular dystrophy (VMD) for the first time in a family from Giessen in 1905. It took until the 1970s before the first attempts were made to identify the gene underlying the disease in a Swedish family. The locus for VMD was determined by linkage analysis to localize in the pericentric region of chromosome 11 (11q13). In 1998 the human BEST1 gene was cloned. The gene was initially named VMD2 (Vitelliform Macular Dystrophy type 2). It consists of 11 exons and spans 11.5 kb of genomic DNA.

The promoter of BEST1 covers bases –253 to +38. The promoter was sufficient to direct RPE-specific expression. Two positive regulatory elements, −585 to −541 bp for high level expression and −56 to −42 bp for low level expression, were identified. The regulatory elements resemble conserved E-boxes (E-box 1 and 2). E-box 1 is required for directing RPE-specific expression of BEST1 by SOX9.

Exons 2–11 are coding for a transmembrane protein consisting of 585 amino acids (aa) named bestrophin 1. Immunohistochemistry showed bestrophin 1 to be localized at the basolateral membrane of RPE cells. Strauß and colleagues identified bestrophin 1 in the fraction of cytosolic membrane proteins after differential centrifugation of freshly isolated porcine RPE cells. Immunohistochemistry co-localised bestrophin with ER markers like stromal interaction molecule 1 (STIM1) in the cytoplasm of HEK293 cells. Finally subcellular compartimentalization studies in RPE cells derived from human-induced Pluripotent Stem Cells (hiPSC-RPE) confirmed bestrophin 1 to co-partition with STIM1. A colocalization of bestrophin 1 and STIM1 in the ER is not necessarily contradictory with the basolateral localization of bestrophin 1 since STIM1, a calcium sensor in the ER, is able to induce a translocation of ER membranes towards the plasma membrane. The implications of these data will be discussed later in relation to bestrophin 1 function.

Bestrophin genes have been identified throughout the whole animal kingdom and even in plants, fungi, and bacteria distant homologues were found. The human genome encodes for four bestrophin-like proteins (paralogs). Studies on the phylogenetic relationship revealed that all vertebrate bestrophins identified so far belong to one of four branches of the phylogenetic tree. All other mammals have three or four bestrophin paralogs. In mice three paralogs and one pseudogene were identified. Two systems of nomenclature for mammalian bestrophins exist from history: BEST-1, -2, -3, and -4 and VMD2, VMD2L1 (VMD2-like protein 1), VMD2L2 and VMD2L3. The Human Gene Nomenclature Committee (HUGO, http://www.genenames.org/cgi-bin/hcop.pl) recommends usage of the BEST nomenclature which shall be used further on.

Bestrophinopathies – The Phenotypical Spectrum of Mutations in BEST1

Bestrophinopathies represent a young term summarizing the heterogeneous phenotypes of a series of degenerative eye disorders caused by mutations in the human BEST1 gene. Until now, according to the clinical manifestations and modes of inheritance, bestrophinopathies have been classified into four major categories: autosomal dominant juvenile vitelliform macular dystrophy (VMD) or Best disease, autosomal dominant vitreoretinchoroidopathy (VRCP), autosomal dominant retinitis pigmentosa (RP)-like retinal degeneration, and autosomal recessive bestrophinopathy (ARB).

Best Vitelliform Macular Dystrophy – Best Disease (VMD)

The typical diagnostic criteria of VMD include a positive family history with an autosomal dominant mode of inheritance, presence of a vitelliform lesion in the macular area on ophthalmoscopy (Figure 1), and a moderately to severely decreased light peak of the EOG (Table 1). Gass described five stages: subclinical stage: also named pre-vitelliform stage, vitelliform stage, pseudohypopyon stage, vitelliruptive (scrambled-egg) stage, and atrophic stage presenting with a retinal scar. Disease progression is individual. Onset may be as early as during the first decade of life. Affected individuals from the same family may pass through consecutive stages with slower progression or reach manifestation of the disease at a later age. Even though VMD is a genetic disorder affecting both eyes, independent progression of the disease in each eye is common and unilateral affection may last over a longer period. Progression through all stages will eventually occur bilaterally but may take a long time. Legal blindness is reached only rarely and visual acuity or disease stage do not correlate with age (Figure 2A, B), although an advanced disease stage is associated with visual loss (Figure 2C). A regular follow-up scheme is therefore recommended even though the disease may remain stationary.

In VMD, heterozygous mutations in the BEST1 gene have been identified. Incomplete penetrance is common leading to healthy carriers in 29–60% of families based on subjective visual function. A deeper examination of published families revealed an unremarkable fundus examination or an unaltered EOG response in about 15% of mutation carriers only (Table 2). Interestingly, 8% of patients presented with either an unremarkable fundus examination or
FIGURE 1. Imaging of Best disease stages. (A) Vitelliform stage: The fundus photography of the right eye of an 11-year-old patient (mutation: c.1120dupG (p.E374Gfs*27)) reveals a typical yellow lesion in the fovea. Fundus autofluorescence (FAF) recording of a 50° field showed a corresponding region with strongly increased FAF in the fovea. The 30° field FAF recording locates the Spectral-Domain (SD) OCT scan. The scan correlates the foveal lesion (black arrow) with a central domed structure which displaced all layers above the RPE. (B) Vitelliruptive stage: The fundus photography of the left eye of a 15-year-old patient (mutation: p.Q96R)
unaltered EOG response in at least one eye. These could be identified by examination of the fellow eye (Table 2). Thus systematic clinical examination and molecular genetic analysis of direct relatives or siblings of sporadic cases is recommended to reveal asymptomatic carriers and to confirm the mode of inheritance for improved counseling.

VMD is characterized by the presence of yellowish lesions in the macular area. The eventually occurring decline of central vision at later stages of disease is related to a changing morphology of the lesions. Histological studies revealed cavities between the RPE and the photoreceptor layer filled with yet uncharacterized material.25–30 Interestingly, these cavities were neither positive for lipophilic substances as shown by SUDAN Black or periodic acid Schiff-base (PAS) staining nor did they show increased fundus autofluorescence (FAF).28 Positive PAS and SUDAN Black staining localized in the RPE which presented as a place of lipofuscin accumulation in all studies so far.28–34 This led O’Gorman and co-authors to draw the conclusion that VMD is a disease of an inability of the RPE to correctly phagocytose photoreceptor outer segments (POS).28

### TABLE 1. Characteristics of Bestrophinopathies.

| Disease | VMD | VRCP | RP50 | adMVD | ARB |
|---------|-----|------|------|-------|-----|
| Inheritance | ad | ad | ad | ad | ar |
| Age at onset | Childhood – seniority | Childhood – 4th decade | Adulthood | Adulthood – seniority | Childhood |
| Visual Acuity (Median) | 1.0–0.1 (0.5) | 1.0–0.0 (0.67) | 0.8–0.05 (0.8) | 1.0–0.05 (0.67) | 1.0–0.1 (0.8) |
| Fundus | Vitelliform macular lesion that develops a fibrotic scar on progression | Peripheral pigmentation with bone spicules, progressing towards central retina | Peripheral pigmentation with bone spicules, progressing towards central retina | Multiple yellow lesions in the mid-periphery, vitelliform macular lesion and atrophy, drusen, CNV, macular lesion like VMD | Yellow lesions along the vascular arcades and sparing the macula, in the macula small white spots |
| FAF | FAF covering the macular area that is reduced to irregular FAF spots on progression to the fibrotic stage | nda | nda | Multifocal spots inside and outside the macula | Multifocal spots outside the macula |
| OCT | Serous lesion in macula and severe thickening of the RPE-POS layer, stratification disintegrates upon progression | nda | nda | Macular lesion resembles VMD | Macular lesion resembles VMD, INL with hyporeflective areas resembling RS1 |
| EOG | Arden ratio ↓ | Arden ratio ↓ | nda | Arden ratio ↓ | Arden ratio ↓ |
| ERG | Normal, later ↓ | Scotopic ↓ | nda | Scotopic ↓ | Scotopic ↓ |
| mfERG | ↓ | Photopic ↓ | nda | Photopic ↓ | ↓ |

VMD, Best vitelliform macular dystrophy; VRCP, Vitreoretinochoroidopathy; RP50, concentric Retinitis pigmentosa-like retinal dystrophy; adMVD, autosomal dominant multifocal vitelliform dystrophy; ARB, autosomal recessive Bestrophinopathy; RS1, X-linked retinoschisis; ad, autosomal dominant; ar, autosomal recessive; FAF, fundus autofluorescence; nda, no data available; CNV, choroidal neovascularization; ↓, reduced; EOG, electrooculogram; ERG, electroretinogram; mfERG, multifocal ERG; OCT, optical coherence tomography, RPE-POS, layer corresponding to photoreceptor outer segments and retinal pigment epithelium.
Optical coherence tomography (OCT) very well depicts the histo-morphological findings. FAF imaging localizes the lesions to corresponding areas of increased fluorescent material in the histological studies. Therefore FAF is a helpful tool to facilitate early diagnosis of VMD and assists differentiation of VMD from phenocopies. FAF imaging is able to visualize small amounts of vitelliform material by increased FAF even in the early vitelliform stage. Scars and atrophic regions in advanced VMD lesions are characterized by decreased FAF. Spectral domain OCT (SD-OCT) meanwhile provides detailed insight into retinal stratification. Cavities within the layers of the retina are well depicted and assist differentiation from drusen (Figure 1). SD-OCT not only provides better resolution of the retinal layers by an increased amount of recorded data per time but also uses a slightly different recording wavelength (840 nm) compared to time domain (TD) OCT (820 nm). The altered recording wavelength results in detection of additional hyperreflective structures in the cavities (Figure 3). SD-OCT supports the notion of thickening of the RPE and that the detachment occurs between the RPE and the POS layer compared to patients with RPE detachment (Figures 1 and 4).

Studies on hiPSC-RPE derived from VMD patients supported an increase in accumulation of autofluorescent material upon long-term feeding of the cultured cells with photoreceptor outer segments (POS). In addition a decreased net fluid transport across the cell monolayer leading to fluid-filled domes above the hiPSC-RPE culture could be shown. Therefore, the cavities above the macula identified in histological studies and OCT should be regarded as edemas and the characteristic FAF can be assigned to accumulation of autofluorescent material in the RPE. Edemas caused by a decreased net fluid transport over the RPE also explain the observation that the outer segment (OS) layer thickness measured by SD-OCT increases in patients carrying VMD mutations during illumination.

As indicated in the overview of BEST1 mutations on the Retina International website (http://www.retina-international.com/sci-news/best-1-mutation/) 225 mutations associated with bestrophinopathies have been identified to date in the BEST1 gene. Seventy percent of these mutations cause VMD and associated late onset forms. Seventy three percent of mutations were missense mutations located in different domains of bestrophin 1. The majority of these missense mutations were distributed in the N-terminal half of the protein, where the highest evolutionary conservation exists (Figure 5). Other mutations including frameshifts, splice errors, and in-frame deletions have been reported in VMD. A summary of functional tests on BEST1 mutations is given in Supplementary Table S1 (available online only).
Peripheral Pigmentary Retinopathy – Vitreoretinochoodopathy (VRCP) vs concentric Retinitis pigmentosa (RP50)

Kaufman and colleagues\textsuperscript{37} and Blair and colleagues\textsuperscript{38} separately described two independent families affected with a novel fundus dystrophy termed autosomal dominant vitreoretinochoodopathy (ADVIRC, OMIM 193220, official acronym VRCP). The reports on VRCP remained rare.\textsuperscript{37–46} The main characteristic of VRCP is a peripheral chorioretinal pigmentary disorder usually present over 360° between a discrete posterior boundary in the equatorial region and the ora serrata. Patients may experience first symptoms as early as during the second decade of life but the disease also presents with late onset and reduced penetrance.\textsuperscript{38,39} During the early stages the region between the macula and the...

FIGURE 3. Comparison of time domain and spectral domain OCT recordings in Best disease at vitelliform stage. (A) Fundus photography, fundus autofluorescence (FAF), and spectral domain (SD)-OCT scan from a 15-year-old patient (\textit{BEST1}: p.Q96R (c.287A>G)). The SD-OCT scan is also given in pseudo-color for comparison to the time-domain (TD)-OCT scan in (B). (B) Fundus photography, FAF, and TD-OCT scan were recorded in a 7-year-old patient (Mutation: c.969delTC (p.I295del)). Vertical (v) and horizontal (h) B-scans are presented. Resolution of SD-OCT is not simply better than TD-OCT but also reveals additional hyperreflective structures within the hyporeflective area given in TD-OCT.

FIGURE 4. Comparison of SD-OCT recordings from patients with VMD and RPE detachment. (A) SD-OCT scan from Figure 1(A) of a patient with \textit{BEST1} mutation 1120dupG (p.E374Gfs*27). (B) SD-OCT scan of a 45-year-old female patient with RPE detachment. Note the remaining RPE-layer in the VMD patient compared to the thin line of Bruch’s membrane in the patient with RPE detachment (black arrows).
Equatorial border appears normal but the whole retina is affected in later stages (Figure 6A, B). In the affected area, yellow-white dots occur frequently and are observed later also in the posterior pole. The yellow-white dots are located at the level of the internal limiting membrane or in the retina, but not in the retinal pigment epithelium. A variety of retinal blood vessel lesions appear in VRCP. All blood vessel lesions can cause breakdown of the blood-retinal barrier and formation of neovascularization throughout the retina. Vitreous degeneration is commonly seen. The EOG is pathological in the affected individuals, but normal EOG may occur. The Ganzfeld electroretinogram (ERG) provides normal responses in amplitude and implicit times until the disease reaches later stages with general affection of the retina. Many clinical observations from families showed a slowly progressive vitreoretinal degeneration and good visual prognosis. Anyway, visual activity can decrease significantly when vitreous haemorrhage or macular oedema occur.

A histopathological study on an eye of an 88-year-old donor with VRCP revealed focal atrophy of the RPE. Altered RPE cells surrounding retinal blood vessels lined the internal limiting membrane. Along the equator multifocal loss of photoreceptor cells was recognized. Cellular debris, condensed vitreous, and layers of Müller cells formed a preretinal membrane.

Moreover, ocular developmental abnormalities such as nanophthalmos, microcornea, shallow anterior chamber, presenile cataract, and closed angle glaucoma were reported to accompany VRCP. Currently four BEST1 mutations have been identified in VRCP patients (see Table 3). Even though these mutations are missense mutations all of them were characterized to affect splicing causing exon skipping and exon duplication.

Recently five families from a RP screening panel were reported as concentric retinitis pigmentosa-like retinal dystrophy (RP50, OMIM 613194). The patients carried missense mutations in BEST1 causing a severe form of retinal dystrophy with pigmentary changes in the periphery and a demarcation line between severe
peripheral pigmentation and normal-appearing central retina. Ganzfeld ERGs were reduced for both cone and rod responses in patients with progressed disease stage. No EOG recordings which may have revealed reductions of the light peak indicating an involvement of BEST1 were performed. The fundus appearance of these patients was comparable with VRCP patients (Figure 6) but vitreous changes or developmental changes of the anterior pole of the eye were not recognized. The underlying mutations were identified in the heterozygous state (Table 3) in 4/5 of the patients and segregated in an autosomal dominant manner.

The patients from the fifth family were homozygous for p.L140V and presented with light ARB-like fundus changes and pigment spots. The mode of inheritance was compatible with pseudo dominance and an autosomal recessive mode of inheritance.

The mutations reported in the concentric RP patients (p.I205T, p.Y227C, and p.D228N) may affect the activity of splice enhancers as predicted by the Human Splicing Finder website (V2.4.1, http://www.umd.be/HSF/) and follow the predictions for VRCP mutations which were expected to abolish pre-mRNA splicing even though their effect could not be quantified in vivo. The Human Splicing Finder website also predicted aberrant splicing for p.L140V. Aberrant splicing was previously reported in ARB but was not quantified as given for the VRCP-causing mutations. Transient transfection into MDCKII and HEK293 cells did not reveal a common functional effect. p.D228N and p.L140V mutants expressed in MDCKII cells localized the gene product in the cytoplasm while p.I205T behaved wildtype-like but produced reduced whole cell currents in patch clamp recordings from transiently transfected HEK293 cells like p.L140V (Supplementary Table S1). These studies did not allow a prediction of splicing quality since the vectors were made from cDNA. Investigations on the effect on aberrant splicing may provide more conclusive details to understand the functional differences between p.I205T, p.D228N, and p.L140V. Therefore, the data suggested that family five had to be considered an ARB family. The prerequisites for a recessive bestrophinopathy will be discussed later.

A phenotype presenting with peripheral pigmen-
tary changes of the retina either RP-like or like VRCP
is in agreement with the RPE-wide expression of BEST1. Since the function of BEST1 is not finally understood and therefore the effects of mutations cannot be tested conclusively to explain the pathogenic effects of mutations like p.Y227C, it may be advisable to name the resulting disease as pigmented retinopathy by addressing the common morphological features to discern the disease from other forms of bestrophinopathies and RP.

**Autosomal Dominant Multifocal Vitelliform Dystrophy**

As indicated above the common phenotype of BEST1 mutation is a vitelliform lesion in the
macular area that becomes fibrotic on progression of VMD (Table 1). Some carriers of BEST1 mutations develop clinical signs quite late in life while their affected relatives already went through most of the disease stages at a much younger age. This is not unexpected in incomplete penetrance but is remarkable when patients with late onset develop a multifocal distribution of yellowish lesions instead of the expected monofocal vitelliform lesion. Autosomal dominant multifocal vitelliform macular dystrophy is characterized by a central vitelliform lesion accompanied by numerous eccentric lesions. The peripheral lesions are usually located superonasal to the optic disc and adjacent to the temporal vascular arcades. The lesions vary in size, number and appearance. The peripheral lesions generally paralleled the development of the macular lesion in most of the cases. The EOG findings in those patients were abnormal although even normal Arden ratios could occur. Ganzfeld ERG typically revealed normal cone and rod responses. Mutations in BEST1 associated with multifocal vitelliform dystrophy were mostly missense mutations. Seventy-five percent of these mutations were also identified in patients with typical VMD or adult onset vitelliform macular degeneration (AVMD, OMIM 608161). Dominantly inherited multifocal lesions were present in 5% of the reported BEST1 mutations but the late onset of the autosomal dominant multifocal forms and the co-occurrence with VMD patients in the same family would indicate that autosomal dominant multifocal forms are not a distinct genetic entity. This notion is supported by the classification in OMIM (http://omim.org/) which includes autosomal dominant multifocal vitelliform dystrophy as part of VMD (OMIM 153700).

Autosomal Recessive Bestrophinopathy (ARB)
In 2006 Schatz and co-authors reported ARB (OMIM 611809) for the first time in a Danish family. The condition was caused by compound heterozygous BEST1 mutations. Since BEST1 mutations were thought to segregate only in an autosomal dominant manner, Schatz interpreted this condition as a modifier effect of the first onto the second mutation. Later in 2008 Burgess and colleagues coined the term ARB for the first time. The retinal phenotype associated with compound heterozygous or homozygous variations in BEST1 presented with different clinical characteristics including extrafoveal and extramacular subretinal deposits as already described in multifocal vitelliform dystrophy (Table 1). The macular lesions exhibited some subretinal fibrosis inferior to the fovea, instead of being centered on the macula. Numerous extramacular lesions (punctate flecks) usually appear round in shape, scattered along the temporal vascular arcades as dots of white or yellow color and may coalesce over time (see Figure 7). Macular edema and subretinal fluid was usually detected on OCT imaging, while no patients were ever noted to have autofluorescent yellow vitelliform lesions covering the whole macula like in VMD. In addition to a serious reduction in the EOG light rise similar to that associated with VMD and VRCP, abnormal Ganzfeld ERG and significant reduction in the pattern ERG were detected.

ARB is a rare condition with several reports of single cases and few families from separate facilities only. Molecular analysis revealed several mutations in BEST1 alleles including 22 missense mutations, four nonsense mutations, ten deletions, two duplications, three splice site mutations, and one mutation in the promoter region of BEST1 (Table 3).

Recently, Pomares and colleagues approached this issue by determining the rate of nonsense-mediated decay (NMD) a quality control mechanism associated with preterm translation stops due to nonsense mutations or frameshift mutations. The authors presented the absence of BEST1 messenger RNA in RNA-preparations from fresh blood lymphocytes of an ARB patient. This way the authors supported ARB as the null phenotype of BEST1 mutations and ruled out haploinsufficiency as the underlying mechanism in these mutations. Whether illegitimate expression of BEST1 in preparations of mRNA from fresh peripheral blood lymphocytes is suitable to solve this delicate question may be considered contradictory. The low expression of BEST1 is lost after immortalization of lymphocytes in culture and therefore timing of RNA preparation is critical to obtain useful RNA samples.

An approach to test the hypothesis of NMD may involve the autosomal recessive cmr dog models. mRNA from RPE is easily accessible in these dogs and the data on mRNA stability should be comparable to human ARB patients since the dogs develop a comparable phenotype (see below). In contrast Best1 knock-out mice did not develop morphological changes although an ATP-dependent increase in intracellular stored calcium is reported. Whether autosomal recessive mutations exert different effects on intracellular calcium stores in dogs and men compared to knock-out mice or whether increased intracellular stored calcium is tolerated differently in these species requires a deeper insight into the function of bestrophin 1.

Several ARB-related mutations were identified in codons that harbour VMD mutations and partially produce identical amino acid exchanges. Explaining the functional basis of this observation...
will considerably help understanding the disease mechanism of ARB and VMD.

**Glaucoma**

Recent reports on the association of VMD and angle-closure glaucoma (ACG) alerted the VMD community. Some patients were reported with juvenile onset glaucoma but the majority of patients were diagnosed in their fourth and fifth decade. Among 357 individuals published with disease-causing mutations only 21 have been reported with glaucoma at a median age of 38 years. ACG was most frequently reported but also pupil-block glaucoma was diagnosed. The patients presented as sporadic cases of VMD, ARB, or VRCP but no common correlation of ACG and bestrophinopathies was reported. Recently shallow posterior chambers were reported in five of ten ARB patients aged 36–60 years. Only one patient (age 60 y) had physiologically increased intraocular pressure (IOP). These patients were treated precautionary with YAG laser iridotomy and one additional patient developed an increased IOP after treatment. Therefore, coincidence of glaucoma and bestrophinopathies is more likely than association.

**Animal Models of Bestrophinopathies and Novel Options in Treatment**

Animal models carrying Best1 mutations are rare. Mouse knock-out models of Best1 do not exhibit a corresponding phenotype and therefore, are useless in studying morphology and effects of treatment in bestrophinopathies. Knock-out mice on the other hand provide useful electrophysiological data. Knock-in mice (p.W93C) resembled the features of human disease with serous retinal detachments in mice carrying the mutation in the homozygous and heterozygous state. The detachments in the knock-in mice localized in the central region of the fundus temporal to the optic disc. The location is corresponding to the human macula. Like in human disease, reduced penetrance was observed by detecting retinal detachments in only 40% of mice carrying the mutation. Photoreceptor outer segments (OS) contained vacuoles from disrupted discs. Vacuoles in the photoreceptor inner segments (IS) corresponded to disrupted mitochondria. OS and IS appeared elongated. Development of a debris zone between RPE and retina-containing shed OS, lipofuscin granules, and stacked RPE microvilli indicated impaired phagocytosis of OS leading to the accumulation of autofluorescence with age. Serous detachments resolved only...
late in life but atrophic stages like in human disease were not observed.\textsuperscript{69} The histological features corresponded well to the observations in human disease.\textsuperscript{29–33} The absent atrophic stages may be attributed to the morphology and function of the human macula which has no equivalent in mice.

Flash ERG in knock-in mice revealed normal a-waves and b-waves throughout life.\textsuperscript{69} To investigate functional features corresponding to the human EOG directly coupled (dc)-ERG\textsuperscript{73} was applied in knock-in mice measuring long-term light evoked ion flux of the retina and RPE.\textsuperscript{69} The c-wave amplitude (corresponding to an increase of subretinal potassium concentration), the fast oscillation (FO, corresponding to delayed hyperpolarization of the basal membrane of the RPE) and the maximum amplitude of the transepithelial potential (TEP) over the basal RPE membrane (corresponding to the light peak (LP) of the human EOG) peak at 2 cd/cm\textsuperscript{2} in a luminance-dependent function in wild type mice. In homozygous knock-in mice the peaks of the c-wave and FO were shifted towards higher illumination and increased. In heterozygous knock-in mice the peaks were reduced compared to wild type mice with a delayed decline of the c-wave to base line at higher illumination. The light peak in homozygous and heterozygous knock-in mice was constant between $-1 \log$ cd/m\textsuperscript{2} and 1 $\log$ cd/m\textsuperscript{2} illumination and never exceeded the peak maximum of the wild type. The decline function towards base line over 2 cd/cm\textsuperscript{2} was shifted towards higher illumination in homozygous and heterozygous knock-in mice.\textsuperscript{69} This behavior is different from knock-out mice that followed wild type amplitudes with only slight deviations in amplitude and a shift towards higher illumination.\textsuperscript{68} These data indicate a function of bestrophin 1 in modulating the TEP by sensing either a “light peak substance” (LPS) hypothesized to initiate the underlying ion flux or by sensing the flow of a reporter ion responding to the light-induced ion flux in the retina and RPE. ATP was one of the first LPS’ considered to initiate the TEP and ion flux over the RPE.\textsuperscript{74} Intracellular calcium concentration rises upon application of ATP to the cell culture medium in excised wild type mouse RPE.\textsuperscript{69} The knock-in mice RPE did not respond to the application of external ATP by increasing intracellular calcium concentrations while knock-out mice RPE almost doubled the concentration of intracellular calcium.\textsuperscript{69} This observation supports a function of bestrophin 1 towards the control of intracellular calcium concentration upon ATP induction in the RPE that is abolished by mutations like p.W93C in a dominant negative manner.

Finally, chloride conductance in RPE cells tested against varying concentrations of calcium was equal to wild type in knock-in and knock-out mice\textsuperscript{68,69} thus excluding a possible function of bestrophin 1 as a chloride channel and in direct control of calcium-activated chloride channels.

Expression of Best1 mutations (p.W93C, p.R218C) by adenovirus-mediated gene transfer in rat models revealed no mislocalization of the mutant gene product in the RPE nor could the authors observe any morphological pathology.\textsuperscript{75} ERG responses were reduced in vector (mutant and control) treated rats compared to untreated rats. The RPE-generated components of the dc-ERG (c-wave and FO) were increased to an upper limit in WT-injected rats. The LP was reduced in rats treated with mutant Best1.\textsuperscript{75} This indicates the involvement of bestrophin 1 in the generation of the LP but also raised the question whether the functional effect is involved in the generation of the clinical phenotype or is just accompanying the molecular cause.

The first useful animal model of bestrophinopathies was the canine multifocal retinopathy (cmr). To date three naturally occurring Best1 mutations have been described in cmr in eight dog breeds (Dogue de Bordeaux, English mastiff, Italian cane corso, Coton de Tulear, Lapponian herder, Australian shepherd, Great Pyrenees, Bullmastiff).\textsuperscript{34,76–78} cmr is inherited in an autosomal recessive manner and presents comparable to ARB. The RPE is described in these models as hypertrophied and increased autofluorescence is present within the RPE in tan brown and fluorescent inclusions.\textsuperscript{34}

Recently a treatment trial was initiated using AAV-Best1 constructs in a substitution therapy.\textsuperscript{79} Preliminary results were promising but final data have not yet been published. Successful treatment of the canine model provides a good starting point for treatment trials in human ARB patients.

The phenotypic differences between the knock-out mice, and cmr dogs and ARB indicate differences between either the function of bestrophin 1 in the RPE of mice, dogs, and man or differences on the function of the RPE in these species. A question closely linked to the function of bestrophin 1.

**Bestrophin – A Player between Chloride and Calcium**

A total of 262 mutations have been identified within BEST1 and 86% of these have been associated with VMD and other retinopathies (http://www.retinainternational.org/sci-news/best1mut.htm). Unfortunately, the exact function of bestrophin 1 still remains unclear. A summary of experimental evaluations of heterologously expressed mutant bestrophin 1 is given in Supplementary Table S1. The data describe different aspects of dysfunction at the cellular level but are unable to explain the clinical heterogeneity seen in bestrophinopathies.
Human bestrophin 1 is predominantly expressed in the RPE.\textsuperscript{10} The RPE plays essential roles in retinal homeostasis including control of the ion-concentration and the composition of the fluid surrounding the photoreceptor outer segments (POS),\textsuperscript{80} regeneration of the visual pigment, and phagocytosis of shed photoreceptor discs.\textsuperscript{72,81,82} Ion haemostasis affects most of these processes and therefore bestrophin 1 is involved directly or indirectly in the maintenance of the retina. Understanding the function of bestrophin 1 and the mechanisms by which mutations in human bestrophin 1 lead to VMD is therefore crucial for the development of useful therapies and correct determination of the dosage of the therapeutics.

The expression pattern of \textit{BEST1} differs between human and mouse. Human \textit{BEST1} is predominantly expressed in the RPE. In addition expression in airway epithelial tissue and several human cell lines was reported.\textsuperscript{83–86} Murine \textit{Best1} expression could be shown on tissue preparations from colon, kidney, trachea and brain in addition to RPE.\textsuperscript{5,85,87} Interestingly the expression of the gene outside of the RPE is not related to any pathology apart from the eye neither in \textit{Best1} knock-out nor knock-in mice nor in bestrophinopathy patients. This observation contradicts recent reports on the influence of bestrophin 1 on glutamate release in astrocytes which should create a considerable dysfunction.\textsuperscript{87–90} The extracellular data provide some hint towards the function of bestrophin 1 as a component of calcium-dependent signal chains controlling a tissue-specific flow of molecules. The RPE-specific signal chain may be more dependent on bestrophin 1 or the molecule flow controlled by bestrophin 1 may be more crucial for RPE function than in other tissues like lung or brain.

\textbf{Intracellular Localization}

The first reports on bestrophin 1 function localized the protein at the basal plasma membrane of the RPE.\textsuperscript{10} Further studies approached the intracellular localization in cell lines (HEK293,\textsuperscript{91–95} MDCKII,\textsuperscript{17,50,96–98} human fetal RPE (hfRPE)\textsuperscript{13,98} upon heterologous expression of wildtype and mutant bestrophin 1 and under intrinsic expression of wildtype and mutant bestrophin 1 in hiPSC-RPE\textsuperscript{13}) (Supplementary Table S1). Bestrophin 1 expression in HEK293 cells remained within the cytoplasm\textsuperscript{94} but could translocate to the plasma membrane upon interaction with Ca\textsubscript{1,3} subunits.\textsuperscript{95} MDCKII, hfRPE, and hiPSC-RPE cell lines polarized on transwell filters localized bestrophin 1 in or near the plasma membrane that had direct contact to the plasma membrane of neighboring cells. Even though reported otherwise a localization of bestrophin 1 at the basal side of the polarized cells could be confirmed in hiPSC-RPE cells only.\textsuperscript{13,17,50,96–98} Whether this is caused by the labels co-expressed in heterologously expressed bestrophin 1 constructs or by effects from overexpression needs to be investigated. Some mutations of bestrophin 1 (see Supplementary Table S1) did not correctly localize to the plasma membrane but were dispersed throughout the cytoplasm when heterologously expressed in polarized MDCKII cell cultures.\textsuperscript{50,97,98} This was independent of co-expression with wildtype bestrophin 1 for some mutations.\textsuperscript{96} At co-expression with intrinsically expressed bestrophin 1 in hfRPE cells some mutations behave differently compared to transient transfection in MDCKII cells.\textsuperscript{97} Whether this is an effect of heterologous vs. intrinsic expression or specific to the mutations tested requires further investigations on the nature of interaction of mutant and wildtype protein.

\textbf{Structural Aspects of Bestrophin 1}

Throughout the phyla hydrophy analysis and transmembrane domain (TMD) prediction algorithms found six potential transmembrane domains of which TMD2, TMD5 and TMD6 are very highly conserved. Bestrophins can be divided into a conserved N-terminal domain that includes the six transmembrane regions, and a C-terminal region that is highly variable in length and sequence within different species. The border between N- and C-terminal domain was placed at position 364 in human bestrophin 1.\textsuperscript{20}

Two models have been discussed as to the tertiary structure of bestrophin 1. The first model was published by Tsunenari and colleagues in 2003. Six hydrophobic domains were identified by hydrophy plot of which TMD1, TMD2, TMD4, and TMD6 were predicted to traverse the membrane while TMD5 might be a re-entrant loop (Figure 5).\textsuperscript{20}

Milenkovic and co-authors re-examined the topology of bestrophin 1 by testing isolated transmembrane domain constructs in the Lep expression system in reticulocyte lysate with canine pancreatic microsomes.\textsuperscript{17} From these results the authors concluded that only TMD1, TMD2, TMD5, and TMD6 transverse the membrane.\textsuperscript{17} In direct comparison the two models presented are contradictory. The methods applied by both groups have limitations from the artificial constructs that may fail to fold or enter the plasma membrane in a non-physiological way. However, both models share the prediction that the highly conserved N-terminal part consisting of the first 350 amino acids contains all predicted TMDs.\textsuperscript{17,20}

Bestrophin 1 was initially predicted to be an oligomeric calcium-activated chloride channel (CACC).\textsuperscript{19} Therefore, the composition of higher order complexes including bestrophin 1 was of interest. Sun and colleagues\textsuperscript{19} investigated the oligomerization of bestrophin 1 in forming the pore of a putative CACC. Complex formation of bestrophin 1 with other ion channel subunits was also implicated by bestrophin 1-mediated modulation of the light
peak. Whether these complexes include bestrophin 1 monomers or oligomers is yet to be defined. Quality and quantity of oligomerization may depend on mutations involved in VMD. Either formation of heterooligomers of mutant and wild type bestrophin 1 or a preference to form homooligomers of either wild type or mutant monomers will have an impact on the activity of the oligomeric complexes. In this regard oligomerization quality may explain reduced penetrance in VMD through mutation-dependent formation of the oligomers. Sun and co-authors\(^1\) reported that bestrophin 1 forms tetramers or pentamers when overexpressed in HEK293 cells and that C.\(\text{elegans}\) and Drosophila bestrophins associated at a much lower efficiency with human bestrophin 1 leading to the conclusion that bestrophin 1 forms species-specific homooligomers.\(^1\) Four bestrophin paralogues exist in men raising the question whether species-specific formation of bestrophin heterooligomers, e.g. human bestrophin 1 with human bestrophin 2, may modulate bestrophin 1 activity. Co-expression and immunoprecipitation predicted interaction of human bestrophin 1 with human bestrophin 2 and 4.\(^{19,94}\) In a heterologous expression system the transfected genes are not expressed under physiological control. Thus HEK293 cells do not translocate bestrophin 1, 2 and 4 into the cell membrane and aggregation of a major fraction of protein in the ER could be observed.\(^{94,96}\) Therefore, the number of monomers and the composition of the complexes might be regarded as an artefact of overexpression. To circumvent artefacts by in vivo-like conditions Stanton and colleagues\(^{18}\) extracted bestrophin 1 from porcine RPE. The authors applied a combination of gel exclusion chromatography and velocity sedimentation. The mass of the eluted complex was calculated at approx. 138 kDa. This is about twice the size of the bestrophin monomer (68 kDa) leading to the conclusion that bestrophin 1 acts as a homodimer in porcine RPE.\(^{18}\) Compared to overexpression in HEK293 cells the detergent and ionic conditions used in exclusion chromatography and velocity sedimentation of endogenous bestrophin 1 might have disrupted higher order structures of bestrophin 1 predicting a number of monomers in the complex that is too low. Since bestrophin 1 is expected to interact with ion channel subunits purification by immunoprecipitation should also precipitate complexes of bestrophin 1 and interacting channel subunits. Therefore, either the ion channel subunit interacting with bestrophin 1 has a size of \(~70\) kDa and bestrophin 1 is not forming oligomers or bestrophin 1 forms homodimers and the interaction of ion channel subunits with these homodimers is weak and lost during chromatography and sedimentation. Johnson and colleagues\(^{97}\) studied the interaction of mutant (p.V9M, p.W93C, p.R218C) and wildtype bestrophin 1 molecules by co-immunoprecipitation and fluorescence resonance energy transfer (FRET) upon heterologous expression of c-myc-tagged bestrophin 1 and fusion proteins of bestrophin 1 and yellow and cyan fluorescent protein (YFP and CFP respectively). Their results confirmed the interaction of bestrophin 1 monomers by both methods for wildtype homomers and mutant-wildtype heteromers. The data obtained by FRET supported a co-localization of wildtype monomers in the cell periphery adjacent to the cell membrane. Co-localization of mutant and wildtype heteromers followed wildtype localization for p.W93C and p.R218C while p.V9M containing heteromers were spread throughout the cytoplasm. The data did not allow a quantitation of interacting monomers nor could a conclusion on the number of interacting monomers in heteromeric complexes be drawn. Therefore higher order complexes formed by bestrophin 1 must exist to provide the predicted functions but the composition of these complexes is still under investigation.

**Bestrophin 1 function in the RPE**

As mentioned above, bestrophin 1 has been localized at the basolateral plasma membrane of the RPE and it was hypothesized that bestrophin 1 acts as a CACC.\(^{10}\) This notion was based on the diminished LP of the EOG in VMD patients with the argument that the LP was caused by a chloride conductance across the basolateral membrane of the RPE. The chloride conductance was postulated to be calcium-sensitive.\(^{99}\) Sun and co-authors\(^{19}\) reported the induction of chloride conductances in HEK293 cells in whole cell recordings upon transfection with \(h\)BEST1 and other members of the bestrophin family. The chloride conductances were absent in HEK293 cells transfected with \(h\)BEST1 carrying VMD associated mutations.\(^{19}\) Tsunenari and colleagues\(^20\) continued in this notion and predicted p.Cys69 as a Cystein residue involved in the channel pore by whole cell recordings using cystein-directed inhibitors and mutagenesis of cystein residues in bestrophin 1. The currents elicited by wild type bestrophin and mutants of cystein residues \(\text{in vivo}\) deserve further investigations since mutations in only two of the five cystein residues (see Figure 5) were naturally occurring in men. Interestingly, p.Cys69Trp is regarded as a SNP (rs62641692, \text{http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=7439}) but p.Cys221 carries two mutations involved in VMD (p.Cys221Trp\(^{100}\) and p.Cys221Phe\(^{58}\)) indicating a more important contribution of p.Cys221 to bestrophin 1 function \(\text{in vivo}\) and contradicting the proposed function of the cystein residues in bestrophin 1.

The function as a CACC was first challenged when Marmorstein and colleagues reported on a knockout mouse (see above).\(^{69}\) The data from the mouse model indicate an influence upon other ion channels in the RPE and bestrophin 1-expressing tissue possibly by
intracellular calcium stores. Several studies have been conducted to identify proteins interacting with bestrophin 1 to establish the chloride flow over the basal membrane. Thus a voltage-dependent calcium channel (VDCC) Cα1,3 (CACNA1D) and specifically its β₁-subunit (CACNB4) and the calcium sensor of the ER STIM1 were reported as interacting proteins identified by immunohistochemical methods and co-immunoprecipitation. Overexpressed CACNA1D has been localized to the plasma membrane and cytosol in HEK293 upon co-expression with bestrophin 1 and CACNB4. In addition the physiological localization of CACNA1D could be shown in the basolateral plasma membrane of the RPE in porcine cryosections as well as inside the RPE cells. The interaction of bestrophin 1 with CACNB4 was dependent on a proline-rich motif in the bestrophin part following the last TMD (330PXXF334). Other β-subunits were also able to interact with bestrophin 1 but did not cause a comparable effect on membrane localization possibly due to missing interaction with CACNA1D. STIM1 is localized in the ER membrane where it oligomerizes upon reduced calcium concentration in the ER calcium stores. Upon oligomerization the complex translocates to the plasma membrane to activate ORAI1 a store-operated calcium release-activated calcium (CRAC) channel proposed to replenish intracellular calcium stores. In this regard Cα1,3 may also serve to replenish intracellular calcium stores under the control of bestrophin 1.

To solve the question, whether bestrophin 1 is a channel, patch clamp and electrophysiological recordings in the presence of bestrophin 1 were applied. These recordings clearly state that ATP releases a chloride current on the basal side of the RPE and from cells expressing bestrophin 1. This current is abolished or reduced in the absence of bestrophin 1 and increased upon expression of mutant BEST1. Chloride currents in the RPE may be elicited by anoctamin 1 which produces a chloride current out of the cell and is opened by increasing intracellular calcium concentrations. The link from ATP to anoctamin 1 can be drawn independently from bestrophin 1 by the activation of purinergic receptor P2Y₂. P2Y₂ is linked to a rise in intracellular inositol trisphosphate (IP₃). IP₃ activates the IP₃-receptor (IP₃R) in the ER membrane which causes a rise in the intracellular calcium concentration. This pathway is independent of bestrophin 1 and in line with the data obtained from knock-out mice that do not express bestrophin 1. Bestrophin 1 fits into this model as a blocker of downstream calcium channels like Cα₁,3 which was identified as the principle pore forming unit VDCC involved in stimulating the LP upon direct interaction with bestrophin 1. Another candidate would be ORAI1 which is controlled by a calcium sensor like STIM1 that co-partitioned with bestrophin 1. This model supports a direct effect of BEST1 missense mutations which strongly hinder the activation of the calcium channels and explains the increased LP in knock-out mice by a loss of inhibition of the calcium channels. Whether this is obtained by a direct interaction and of what nature this interaction is needs to be confirmed.

In addition the model can be extended upon the observation that disease-causing mutations in BEST1 abolish HCO₃⁻ conductance. Supposing that the HCO₃⁻-channel is also sensitive to the intracellular calcium concentration BEST1 mutations cause abnormal HCO₃⁻ transport in the RPE. The retina is one of the metabolically most active tissues in the body and so it produces very large amounts of CO₂. Increased CO₂ lowers the intracellular pH which finally inhibits photoreceptor function by preventing the engulfment of POS, influences the maturation and acidification of lysosomes, and blocks the delivery of lysosomal enzymes. Therefore, removal of HCO₃⁻ from the subretinal space is a critical step for photoreceptor function. Furthermore, altered phagocytosis and phagolysosomal function are hypothesized to increase the formation of A2E and other lipofuscin components. Lipofuscin accumulates in VMD and was reported for hiPSC-RPE as a long-term effect of POS feeding. Lipofuscin accumulation further coincides with calcium overload after POS phagocytosis. Impaired HCO₃⁻-transport is therefore a reasonable dysfunction caused by BEST1 mutations in VMD.

**Regulation**

**Regulation by Calcium.** The C-terminus of human bestrophin 1 contains a cluster of five contiguous acidic amino acids immediately following the last TMD preceding an EF-hand binding motif and a further regulatory domain consisting of acidic amino acids terminal to the EF hand essential for calcium sensing (Figure 5). Bestrophin 1 is activated by calcium and ATP accelerates this activation. The underlying mechanism remains unknown. Even more indirect mechanisms are discussed to activate the channel that might involve phosphorylation or other enzymatic processes. The acidic domain of bestrophin 1 is a very important region in channel gating and sensitive to mutations thus leading to blocked channels. The EF hand binding motif coordinates calcium binding and mutations within this structure reduce calcium-sensitivity significantly. The calcium sensing domain may be considered a feedback mechanism to control the activity of the VDCC.

**Regulation by N-C-terminal Interaction.** Mutations in the C-terminus and in the N-terminus reduce channel activation especially at residues R25 and K30 which are well conserved among mammalian bestrophins. Qu and colleagues demonstrated a N-C-terminal
interaction in human bestrophin 1 which is calcium-independent and was abolished by disease-causing mutations. The authors reported that wild type and mutant subunits were able to co-assemble inducing chloride currents.

**Regulation by Phosphorylation.** Phosphorylation is one of the key concepts for stimulation of protein activity. Human bestrophin 1 carries a protein kinase C (PKC) phosphorylation site (Ser358) that is important for sustained chloride flow. p21-activated protein kinase (PAK2) could be shown to phosphorylate bestrophin 1 accompanied by enhanced flow of chloride and potassium over the basolateral membrane of the RPE. The corresponding activity was recently discussed for Ca\(^{2+}\)/calmodulin dependent kinase II (CaMKII) in Drosophila bestrophin 1. This process is reversed in human by protein phosphatase 2A (PP2A) which interacts with bestrophin. Dephosphorylation of bestrophin 1 by PP2A therefore controls VDCC and later via intracellular calcium the flow of chloride and potassium via anoctamin 1 and SK4.

**CONCLUSION**

Bestrophinopathies summarize the various phenotypes caused by mutations of the BEST1 gene. Bestrophin 1, the gene product of BEST1, is expressed throughout the RPE but mutations preferentially cause a dominant phenotype (VMD) with morphological changes restricted to the macula with a functional impact on the whole RPE as can be seen from the EOG. Even though extensive studies on wildtype and mutant bestrophin 1 elucidated single aspects on partner proteins and cellular processes linked to bestrophin 1, its function and therefore the cause of the diverse phenotypical presentation of bestrophinopathies are still unsolved. Phenotypes involving the whole retina were correlated with null mutations and splice site mutations but a rationale to classify the known and novel mutations could not be drawn. Studies on dog models of multifocal retinopathy provide some hope for the patients since preliminary results from these animal models have indicated that treatment by gene substitution therapy may be an option in autosomal recessive forms.

**DECLARATION OF INTEREST**

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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