Retinal Gene Therapy Using Adeno-Associated Viral Vectors: Multiple Applications for a Small Virus

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Early Days

MY INTEREST IN THE INTERACTION between light and biological molecules began well before my involvement with gene therapy for retinal degeneration (RD) when my PhD thesis reported the first intrinsic fluorescence of DNA bases under physiologic conditions (Daniels and Hauswirth, 1971). As a postdoctoral fellow at Johns Hopkins in S.-Y. Wang's lab, I continued this interest by studying the photochemistry of DNA (Hauswirth *et al.*, 1972; Khattak *et al.*, 1972; Hauswirth and Wang, 1973, 1977a,b). Seeing the "light," I moved to K.I. Berns' lab at Johns Hopkins to study the recently discovered, curious little virus called adeno-associated viral (AAV) vectors. We determined that replication of AAV's linear, single-stranded DNA began and ended at its termini (Hauswirth and Berns, 1977) and that there were multiple sequence arrangements at each end (Spear *et al.*, 1977). This led to the first proposal concerning AAV DNA replication through a self-priming mechanism (Berns *et al.*, 1979) and to the discovery that some AAV DNAs are packaged as internally deleted genomes (Hauswirth and Berns, 1979). Following Berns to the University of Florida, we showed that, in a natural AAV infection, the viral DNA genome integrated into chromosomes *in vitro* (Cheung *et al.*, 1980), a process later shown to be central to understanding AAV REP protein function and the basis for AAV latency during its life cycle (reviewed in Daya and Berns, 2008; Berns, 2013).

I then temporarily turned away from AAV biology to study mammalian mitochondrial DNA (mtDNA). In collaboration with P. Laipis (University of Florida), we constructed early physical (Laipis *et al.*, 1979) and genetic (Hauswirth *et al.*, 1980) maps of mammalian mtDNA and showed that mtDNA was maternally inherited (Hauswirth and Laipis, 1982), that mtDNA sequences could change rapidly (Olivo *et al.*, 1983), that sequence heterogeneity existed with a single animal tissue (Hauswirth *et al.*, 1984; Hauswirth and Clayton, 1985; Ghivizzani *et al.*, 1993), that some sequence heterogeneity was because of DNA replication slippage (Madsen *et al.*, 1993), and, in collaboration with D. Clayton (Stanford University), that the D-loop region contained an origin of replication and the start of heavy-strand transcription (Chang *et al.*, 1985). This laid the foundation for our understanding of mitochondrial disease genetics and led to my continued in-

terest in therapy for mitochondrial diseases of the retina as outlined below. It was during this period that N. Muzyczka, K. Berns, A. Srivastava, and R. Samulski developed AAV as a gene therapy vector at the University of Florida (Samulski *et al.*, 1982, 1983). However, one more connection needed to be established before this odyssey would lead to my eventual focus on retinal gene therapy.

Switching fields for a third final time, I began to study the fetal development of mammalian retinal photoreceptors (Timmers *et al.*, 1993, 1995; van Ginkel and Hauswirth, 1994; van Ginkel *et al.*, 1995). Realizing that genetic causes of retinal diseases, mostly photoreceptor genes, were beginning to be identified (Humphries *et al.*, 1992; Rosenfeld *et al.*, 1992) and that AAV vectors were being first developed down the hall (see above), with J. Flannery (University of Florida) we showed, essentially simultaneously with two other labs (Ali *et al.*, 1996; Bennett *et al.*, 1997), that subretinal AAV could efficiently transduce photoreceptors and the retinal pigment epithelium (RPE) (Flannery *et al.*, 1997). The way was now open for *in vivo* testing of AAV-mediated gene therapy in the retina.

Since these initial reports of retinal cell transduction by AAV vectors, we have shown in peer-reviewed studies that, if appropriately designed, AAV vectors can restore/prevent loss of retinal function, structure, and/or vision-elicited behavior in mouse, rat, dog, sheep, and nonhuman primate models of dominant, recessive, or X-linked forms of RD or color vision defects. The remainder of this review will briefly summarize a subset of these studies that seem best poised for clinical trial testing in the next few years. Our other published retinal gene therapy studies, which space limits preclude from being reviewed here, include successful treatments for retinal diseases/conditions in animal models because of mutations in *LRAT* (Batten *et al.*, 2005), *AIPL1* (Ramamurthy *et al.*, 2004; Ku *et al.*, 2011), *PDE6b* (Pang *et al.*, 2008; Deng *et al.*, 2013), *BBS4* (Simons *et al.*, 2011), *BEST1* (Guziewicz *et al.*, 2013), *GNAT2* (Deng *et al.*, 2009), *MFRP* (Dinculescu *et al.*, 2012), *RD3* (Molday *et al.*, 2013), *Myo7a* (Lopes *et al.*, 2013), *GCAP1* (Jiang *et al.*, 2013), whirlin (Zou *et al.*, 2011), or *Lpcat1* (Dai *et al.*, 2014).

Also not covered here are successful therapies to delay RD in animals using AAV-delivered cDNAs for the neurotrophins CNTF (Adamus *et al.*, 2003; Rhee *et al.*, 2007), GDNF (McGee Sanftner *et al.*, 2001), FGF-2 (Sapieha

et al., 2003), and BDNF (Martin *et al.*, 2003; Kwon *et al.*, 2007); the anti-apoptotic XIAP (Petrin *et al.*, 2003; Renwick *et al.*, 2006; Leonard *et al.*, 2007; Zadro-Lamoureux *et al.*, 2009; Yao *et al.*, 2011); the anti-oxidative stress agents SOD2 (Qi *et al.*, 2004, 2007a,b) and catalase (Guy *et al.*, 1998; Qi *et al.*, 2007c); the protein chaperone Grp78/Bip (Gorbatyuk *et al.*, 2010); and the stress response elements ERK1/2 (Dridi *et al.*, 2012), End2 (Bramall *et al.*, 2013), and Trkb (Cheng *et al.*, 2002), as well as an AAV-delivered optogenetic agent to provide light sensitivity to the retina when photoreceptors are absent (Doroudchi *et al.*, 2011) or expressing the anti-angiogenic factors PEDF (Mori *et al.*, 2002; Raisler *et al.*, 2002), sFLT (Pechan *et al.*, 2009; Lukason *et al.*, 2011) (currently in a clinical trial sponsored by Genzyme/Sanofi, NCT1024998), or p22phox siRNA (Li *et al.*, 2008) for treating animal models of vascular retinopathies. For a summary of this work, the reader is referred to a recent review (Boye *et al.*, 2013a).

Autosomal Dominant Rhodopsin RP

In view of the early identification of the dominant negative gain-of-function P23H mutation in the rhodopsin gene (*RHO*) as a common cause of autosomal dominant rhodopsin RP (ADRP), in a collaboration with A. Lewin (University Florida) and M. LaVail (University California, San Francisco), we tested whether an appropriately designed ribozyme (Drenser *et al.*, 1998) that would recognize and cleave the mutant transcript but not the normal transcript from the wild-type allele in transgenic P23H RHO rats could be therapeutic (Lewin *et al.*, 1998). Either a hammerhead or hairpin ribozyme under control of a mouse rhodopsin promoter in AAV2 significantly slowed rod cell loss, whereas inactive control ribozymes had no effect. This was the first demonstration that an AAV vector could be therapeutic for a dominant RD. We subsequently showed that wild-type RHO cDNA can also rescue rhodopsin ADRP RD in P23H RHO transgenic mice (Mao *et al.*, 2011). Using an AAV5 and a human RHO cDNA, the rate of RD was slowed in P23H mice at 6 months, with significant rod-mediated ERG improvements, suggesting that, in some ADRP cases, simply augmenting levels of wild-type rhodopsin can suppress mutant protein effects.

RPE65-Leber Congenital Amaurosis

RPE65 is primarily an RPE protein responsible for maintaining levels of photoreceptor *11-cis* retinaldehyde, the light-absorbing chromophore central to the initiation of the retinal light response. In collaboration with G. Acland, G. Aguirre, S. Jacobson, and J. Bennett (University Pennsylvania), we employed AAV2 with the CBA promoter driving canine RPE65 cDNA as the first proof of principle for gene therapy in a large animal model of RD (Acland *et al.*, 2001). Subretinal vector yielded significant improvements in visual function for at least 3 months, with a follow-up study showing 3-year persistence (Acland *et al.*, 2005). Lancelot, one of the originally treated dogs, retained improved visual function undiminished over his lifetime, more than 10 years after treatment (G. Acland and G. Aguirre, personal communication, 2011). Moreover, cortical responses determined by functional magnetic resonance imaging were also improved dramatically upon treatment (Aguirre *et al.*, 2007). The *rd12* mouse, a second RPE65 model of RPE65-Leber congenital amaurosis

(LCA2), also exhibits improved photoreceptor ERG responses and visually guided maze behavior after treatment, this time with an AAV5 vector (Pang *et al.*, 2006). We used this model to develop an *in vivo* bioassay for validating the potency of LCA2 vectors (Roman *et al.*, 2007) and for documenting stable vector function upon long-term storage of clinical trial material (Banin *et al.*, 2010). An important issue for any RD gene therapy is how advanced the disease can be and still potentially benefit from treatment. We found significant ERG gains in *rd12* mice receiving delayed treatment at 3 months of age, a model for midstage LCA2 (Li *et al.*, 2011). These studies have helped guide decisions regarding the ages and stages of human LCA2 patients that would most benefit from the LCA2 clinical trial.

LCA2 Gene Therapy Clinical Trial

Three clinical trials of RPE65 gene therapy for LCA2 have been independently initiated and interim outcomes published (NCT00481546, NCT00516477, and NCT00643747, clinicaltrials.gov). The longest published follow-up to date is 3 years. All employed an AAV2 vector carrying a normal human RPE65 cDNA delivered subretinally to the worse eye. In spite of small vector differences, it was concluded by all that subretinal AAV vector treatment elicits no vector-related adverse events or toxic immune responses. All trials also reported clinical measures of vision improvement, but to various levels of detail and significance.

Focusing on our Florida/Pennsylvania trial with A. Cideciyan and S. Jacobson, light sensitivity was quantified using a psychophysical full-field sensitivity test that avoided the vision fixation instability common in LCA2 patients (Cideciyan *et al.*, 2008; Hauswirth *et al.*, 2008). There were statistically significant improvements (10- to 10,000-fold) from baseline in all 15 patients. Areas of rod-mediated visual field improvement corresponded well to the surgical location of the subretinal vector bleb, demonstrating that therapy was limited to that retinal area receiving vector. Within this treated region, patients exhibited up to a 63,000-fold increase in light detection ability. For some, this improvement was all the functional gain possible given their baseline loss of photoreceptors.

Gains in both rod and cone function could be recorded in some patients and persisted for at least 1 year (Cideciyan *et al.*, 2009a). The usefulness of this improved function was quantified in a navigation test that showed significant improvement relative to baseline in five of six patients (Cideciyan *et al.*, 2008). Visual acuity was improved, but with mixed statistical significance, in nearly all patients in all three trials. However, in the context of retinal disease, particularly if foveal cones are affected, the location of highest acuity may move away from anatomical fovea into a nonfoveal region with better function. This was confirmed in a therapeutic context when some patients shifted their locus of vision fixation away from their baseline fovea into the extrafoveal treated area at various times after treatment (Cideciyan *et al.*, 2009b). Importantly, approximately half the patients experiencing a vector bleb within the fovea lost foveal thickness, presumably because of foveal cone loss assessed by optical coherence tomography analysis. We suggest that a patient's foveal cones may be particularly sensitive to subretinal vector-mediated foveal detachment, and that this site for vector

delivery should be cautiously approached in future trials. Finally, even though functional gains remain stable at 3 years, photoreceptor loss continued unabated within the treated area (Cideciyan *et al.*, 2013), and this remains to be fully understood.

Recessive GUCY2D LCA (LCA1)

Among genes causing early childhood LCA, guanylate cyclase-1 (*GC1*) is one of the most prevalent (den Hollander *et al.*, 2008). Moreover, unlike many genetic forms of LCA, even though function is unrecordable, rods and cones remain in the central retina (Pasadhika *et al.*, 2010; Jacobson, *et al.*, 2012). Thus, LCA1 is a potentially viable target for gene replacement therapy. In collaboration with S. Boye (University of Florida) and S. Jacobson (University of Pennsylvania), we therefore made two AAV5 vectors, one with the mouse *GC1* cDNA driven by the ubiquitous CBA promoter and the other with the rod/cone-specific human rhodopsin kinase (*hGRK1*) promoter, and tested them in the *GC1* knockout mouse. Both restored cone structure and ERG function significantly for at least 3 months (Boye *et al.*, 2010). Critically, cone-mediated navigation was also restored to wild-type levels. In a follow-up study, this robust therapeutic response was maintained for at least 11 months (Boye *et al.*, 2011), suggesting that therapy will persist for the animal's life.

A better phenotypic model for LCA1 is the *GC1/GC2* double-knockout mouse (Baehr *et al.*, 2007) because this animal exhibits loss of both rod and cone functions like LCA1 patients. Using the same AAV5 vector with the *hGRK1* promoter, both rod and cone ERG responses as well as rod- and cone-mediated visual behavior and cell structure were restored (Boye *et al.*, 2013b). Finally, we validated that the AAV5-*hGRK1* vector limited transgene expression in non-human primates to only rods and cones. These data form the basis for our current interest in taking LCA1 into a clinical trial supported by The Foundation Fighting Blindness and Genzyme/Sanofi.

MERTK-Associated Recessive RP

The mer receptor tyrosine kinase (MERTK), an RPE protein required for photoreceptor outer segment phagocytosis, when absent leads to RD. The RCS rat is a widely studied MERTK model in which progressive photoreceptor cell loss and ERG losses are evident. Using an AAV8(Y733F) vector, subretinal treatment preserved both retinal structure and ERG amplitudes for at least 1 year (Deng *et al.*, 2012). The safety of an AAV2 vector with the RPE-specific VMD2 promoter driving human MERTK expression was then validated in good laboratory practice-compliant rat studies (Conlon *et al.*, 2013) and a phase I clinical trial initiated in Saudi Arabia (F. Alkuraya and E. Abboud, personal communication, 2012).

X-Linked Juvenile Retinoschisis

X-linked juvenile retinoschisis (XLRs) is characterized by retinal cell layer splitting and photoreceptor ERG abnormalities that can lead to macular cell loss, vitreous hemorrhage, and/or retinal detachment. XLRs is caused by mutations in the retinoschisin (*RS1*) gene that encodes a retinal protein secreted from photoreceptors that associates with retinal cell surfaces. The *RS1* KO mouse recapitulates many

features of human XLRs, including retinal splitting and ERG abnormalities. With R. and L. Molday (University British Columbia) and B. Weber (University of Regensburg), subretinal AAV5 with a photoreceptor-specific mouse opsin promoter and the human *RS1* cDNA preserved retinal structure and ERG function for at least 13 months (Min *et al.*, 2005). Treatment of mice at 1/2, 1, or 2 months of age was therapeutic; however, no therapy was evident if treatment was delayed until 7 months (Janssen *et al.*, 2008), suggesting an age/stage beyond which gene replacement therapy may not be effective, thus emphasizing the need to carefully consider eligibility of XLRs patients for a clinical trial. The company AGTC is sponsoring the preclinical work needed for a clinical trial using this approach.

X-Linked Retinitis Pigmentosa

X-linked retinitis pigmentosa (XLRP), one of the most common causes of severe vision loss, is caused by mutations in the retinitis pigmentosa GTPase regulator (*RPGR*) gene and accounts for >70% of XLRP. With G. Aguirre, W. Beltran, S. Jacobson, and A. Cideciyan (University of Pennsylvania), subretinal AAV5 expressing the human *RPGR* cDNA under control of the *hGRK1* or *IRBP* promoter was tested in two different dog models of XLRP (Beltran *et al.*, 2012). Rod and cone ERG amplitudes were both improved over control retinas in three of four eyes. *In vivo* imaging confirmed preservation of photoreceptor nuclei and inner/outer segments, but only within vector-treated areas. Analysis of retinal sections showed normal photoreceptor structure and reversal of opsin mislocalization, again only in regions receiving vector. Areas of improved photoreceptor function also showed inner retinal correction of the bipolar cell dendrite retraction seen in untreated eyes. This success in a large animal model of XLRP using the human cDNA has prompted AGTC to sponsor the preclinical work needed to initiate an XLRP clinical trial.

Recessive Achromatopsia

Complete achromatopsia (ACHM) is associated with the absence of cone function. Patients have poor visual acuity (<20/100), discomfort in bright light, complete color blindness, and severely reduced or absent cone ERG responses. Mutations in genes encoding either the A3 or B3 subunits of the cone cyclic nucleotide gated ion channel (*CNGA3* or *CNGB3*) account 25% and 50% of ACHM, respectively, in the Western world, with several other phototransduction genes accounting for about 5% in aggregate, including the cone transducin alpha subunit, *GNAT2*. With an AAV5 vector and the human L/M opsin promoter driving expression of mouse *Gnat2* in the *Cpfl3* mouse model of ACHM, cone-mediated ERG amplitudes and visual acuity were restored to levels near those of age-matched, congenic wild-type mice (Alexander *et al.*, 2007). This was the first report of successful ACHM gene therapy. Subsequently, with A. Koraromy and G. Aguirre (University of Pennsylvania), we demonstrated a similar level of cone therapy in two lines of dogs modeling the B3 form of ACHM. Therapy persisted for at least 33 months, led to correction of cone outer segment protein mislocalization, and, critically, restored visually guided navigation in bright light that suppressed rod-mediated vision (Komaromy *et al.*, 2010). In the A3 ACHM

model, Cpf1 mouse, an AAV5 vector expressing mouse *CNGA3* in cones led to rescue of cone-mediated ERGs, restoration of normal visual acuities and contrast sensitivities, and normal expression and outer segment localization of both M- and S-opsins (Pang *et al.*, 2012). Therapy persisted for at least 5 months. Since macular cones are nonfunctional but still present in most ACHM patients (Genead *et al.*, 2011; J. Carroll, personal communication, 2014), the target cell for gene therapy is present. Based on these data, the preclinical studies of a B3 achromatopsia clinical trial are being sponsored by AGTC and the NIH/NEI. A parallel effort for the A3 form is also sponsored by AGTC in collaboration with E. Gootwine, R. Ofri, and E. Banin (Israel) and will involve a sheep model of A3 achromatopsia at the preclinical stage.

Nonhuman Primate Retinal Gene Therapy

X-linked human color blindness, the most common single-gene disorder of humans, is caused by the loss of the L- or M-opsin gene resulting in deficient red or green color sensitivity, respectively. As the only human retinal condition with a nonhuman primate counterpart (Mollon *et al.*, 1984), this unique model is critical for preclinical validation of cone-targeted gene therapy (Mancuso *et al.*, 2007). In collaboration with J. and M. Neitz (Med. College of Wisconsin, then University of Washington), we therefore employed an AAV5 vector with the human L/M opsin promoter driving human L-opsin cDNA expression to add red sensitivity to red-color-blind squirrel monkeys. Untreated animals were trained to perform a behavioral test that revealed the expected deficient red response (Mancuso *et al.*, 2006). However, upon subretinal vector, these animals exhibited near-normal red response thresholds after several months (Mancuso *et al.*, 2009). This enhanced red sensitivity persisted for at least 2 years and validates the concept that simple cone gene augmentation by AAV may be viable and safe in humans with a variety of cone diseases. A particularly interesting target is blue-cone monochromacy (BCM), in which both L- and M-opsin genes are not functional, because the vector used in this color-blind-monkey therapy could, in theory, be used without modification to restore red sensitivity in BCM foveal cones. Preclinical studies in preparation for a BCM clinical trial are being sponsored by the BCM Families Foundation.

Leber Hereditary Optic Neuropathy

Leber hereditary optic neuropathy (LHON) is a mitochondrial condition of retinal ganglion cells (RGCs) that leads to blindness in young adults. The most common genetic form, accounting for approximately half of LHON, is a G11778A transition in the mitochondrial subunit 4 of NADH dehydrogenase gene (*ND4*). RGC loss in 11778 ND4 LHON occurs only when nearly all mtDNA is mutated; therefore, in theory, only a minor fraction of normal ND4 need be supplied for therapy. The issue was how to deliver a therapeutic gene to the cytoplasmic, double-membrane mitochondrial organelle. With J. Guy (University of Florida, then University of Miami), we examined two alternatives. The first, allotropic expression involving recoding ND4 with cytoplasmic codons and adding a mitochondrial targeting signal to its N-terminus, was tested by vitreal delivery of mutant G11778A human ND4 cDNA in an AAV2 vector with the CBA promoter. In a normal mouse, we could create an LHON phenotype (Qi *et al.*, 2007d).

The safety of allotropic wild-type human ND4 gene delivery was then shown in mice with FLAG-tagged ND4 expressed via AAV2 (Guy *et al.*, 2009). The tagged ND4 protein was found within RGC mitochondria and did not result in any loss of RGCs. Thus, allotropic mitochondrial gene therapy is safe and feasible. This provided the experimental basis for an LHON gene therapy clinical trial sponsored by the NIH/NEI to be initiated soon (Koilkonda *et al.*, 2014). The second alternative for mitochondrial gene therapy is to deliver the mitochondrial gene directly into the affected organelle. By adding a mitochondrial targeting sequence to a surface-exposed AAV VP2 capsid protein, expression of wild-type human ND4 was seen in cells with the G11778A ND4 mutation and corrected the cell's defective ATP synthesis. Upon vitreal delivery to the mouse eye, mitochondrial levels of vectored human ND4 DNA reached 80% of the resident mouse ND4 homolog (Yu *et al.*, 2012). The delivered ND4 cDNAs remain episomal within the mitochondrion (Yu *et al.*, 2013). Thus, direct mtDNA delivery to the organelle is a feasible alternative to allotropic gene therapy for mitochondrial conditions.

Where Are We Now? Challenges and the Future

A safer delivery site for retinal AAV vectors

Although photoreceptors and the RPE are the primary target for genes causing RD as noted above, conventional placement of AAV vector in immediate proximity to these cells requires a subretinal injection that necessarily creates a local retinal detachment that has been shown in humans with RD to cause foveal cone loss within the detached area (Cideciyan *et al.*, 2008). The ability to transduce these cells without detaching the retina would require a vitreal delivery and penetration of vector through the internal limiting membrane and the entire inner retina before contacting photoreceptors, and then on past the photoreceptor layer if RPE cells are the target. A. Srivastava has shown that changing phosphorylatable AAV surface capsid amino acids, for example, tyrosines, to nonphosphorylatable residues, for example, phenylalanines, enhances transduction efficiency *in vitro* and *in vivo* (Zhong *et al.*, 2008a,b). We tested these vector variants in the mouse retina and found that they were not only more efficient at transducing photoreceptor/RPE cells upon subretinal delivery, but also capable of penetrating the retina from the vitreous to transduce these cells with surprising efficiency (Petr-Silva *et al.*, 2009, 2011). Testing this approach in RD animal models is underway. An alternative to such targeted capsid changes is to screen semirandom AAV capsid libraries for specific properties, that is, selective evolution (Dalkara *et al.*, 2013). A major issue with either approach is that the permeability of rodent and primate retinas are quite different. Thus, any AAV variant showing *in vivo* therapy from the vitreous in rodents will need validation in nonhuman primates before a clinical application would be feasible, and selection of such primate retina-penetrating AAV variants is underway.

Expanding the DNA capacity of AAV vectors for retinal disease

About 10–20% of cDNAs linked to RDs are too large for the ~4.7 kb limit of DNAs that can be efficiently encapsidated into wild-type AAV. In response, the dual-AAV-

vector approach has been developed so that one vector contains a promoter and a 5' segment of the cDNA, while the second vector contains a 3' segment and a poly-A addition sequence. Central overlapping sequences provide a target for homologous recombination once both vectors deliver their cargo DNA to the target cell nucleus. Various sequences have been engineered into this overlap region so that high-efficiency recombination followed by mRNA splicing occurs to yield an intact, full-length message. Such vectors are in development for Myo7a Usher 1D (Dyka *et al.*, 2014; Trapani *et al.*, 2014), ABCA4 recessive Stargardt disease (Trapani *et al.*, 2014), and CEP290 LCA, all RDs with causative cDNAs too large for a single AAV vector. Critically, mouse models exist for all three conditions, so pivotal *in vivo* tests of this technology should be forthcoming soon.

The future

Given the documented ability of appropriately designed AAV vectors to slow vision loss or restore vision function in a broad range of retinal degenerative diseases in a variety of animal models and species, and the current list of target retinal diseases that are in or soon to be in AAV vector gene therapy clinical trials, the near-term future for further validation of AAV for retinal gene therapy seems bright and portends even more validation of AAV vectors through clinical retinal applications within the decade.

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