Computational Synthetic Biology EC552, Boston University

Literature Search for Laboratory Research Method and Protocol for Gene Therapy

Received 22 April 2022,
Accepted 22 April 2022

DOI: 000.00000

Jinsaan Yoon, Niky Popov, Stephen Sweet

The following is a condensed report of the paper [Riedmayr, L.M., Hinrichsmeyer, K.S., Karguth, N. *et al.* dCas9-VPR-mediated transcriptional activation of functionally equivalent genes for gene therapy. *Nat Protoc* **17,** 781–818 (2022). https://doi.org/10.1038/s41596-021-00666-3] in which they investigate the gene therapy approach for inherited disorders caused by mutations in such genes is to transcriptionally activate the appropriate counterpart(s) to compensate for the missing gene function. We provide information from the paper regarding the protocl being investigated, the importance of the protocol, the reagents and corresponding volumes of the protocol, and the description of the experimental protocol.

The protocol being investigated

CRISPR–Cas9 is a powerful endonuclease-based DNA-editing system and represents a popular tool for targeted genome editing. By fusion of an endonuclease-deficient ('dead') version of Cas9 (dCas9) to transcriptional activator domains and/or by modifying the sgRNA scaffold4 it can be repurposed to activate the expression of specific genes. In this context, the Cas9-sgRNA complex is used as a shuttle to guide the activator domains to specific promoter regions and drive the expression of a target gene. Several dCas9-based transactivation modules (commonly referred to as CRISPRa) have been developed so far. The first generation was created by fusing dCas9 to synthetic VP64 or p65 activation domains, yielding rather low levels of gene expression. This system was later improved by combining the three activator domains VP64, p65 and Rta and adding them to dCas9 to create a tripartite transactivation module (dCas9-VPR). Another effective strategy is to incorporate MS2 stem loops into the sgRNA scaffold to recruit different activation domains. A previous evaluation of various dCas9-based transactivation modules has shown that dCas9-VPR, together with two other CRISPRa modules referred to as dCas9-SunTag14 and dCas9-SAM15, results in the highest transactivation efficiency across various cell types and species. The authors of the paper provided proof of principle for a successful and safe gene therapy for inherited blindness in mice using the dCas9-VPR transactivation module.

Importance of Protocol

In principle, the gene therapy concept described herein can be used for all hereditary diseases caused by mutations in genes for which functional equivalents exist. One group of diseases particularly suitable for such a treatment are inherited retinal dystrophies (IRDs). Our duplex retina contains specialized rod and cone photoreceptors equipped with a similar, but distinct, set of genes. Many genes associated with IRDs encode structural components or members of the phototransduction cascade of rod and cone photoreceptors (e.g., the visual pigments (opsins)). Such key components are often expressed in just one of the two photoreceptor subtypes but could in principle perform a similar function in the other subtype as well. For example, the visual pigment rhodopsin is exclusively expressed in rods, while most mammals including mice express two other opsin types in cones (i.e., the medium wavelength–sensitive M-opsin and the short wavelength–sensitive S-opsin). Interestingly, studies on mouse models demonstrated that rhodopsin and cone opsins are functionally equivalent. In our recent work, we applied the concept of transactivation gene therapy by activating the cone photoreceptor-specific M-opsin gene (Opn1mw) in the rod photoreceptors of a rhodopsin-deficient mouse model and were able to ameliorate the disease phenotype without adverse effects. The same concept could be applied to other IRD-linked genes that possess functionally equivalent partners. To demonstrate the general therapeutic potential of this method for this type of inherited disease, we specifically searched for potential gene activation candidates for a total of 257 known IRD-associated genes. Proteins meeting the following criteria were considered potential gene activation candidates: (i) ≥30% amino acid sequence identity to the affected protein, (ii) a similar length compared to the affected protein (deviation <20%) and (iii) a conserved domain structure compared to the affected protein. Using these criteria, we found potential functional equivalents for 117 IRD-linked genes (45.53%), which therefore represent promising targets for transactivation gene therapy. Other CRISPRa modules have been successfully used in previous work to treat diseases such as diabetes mellitus, muscular dystrophy or acute kidney injury. The CRISPRa modules used in these studies include slightly modified variants of the SpdCas9-SAM and Staphylococcus aureus (Sa)dCas9-VP64 expression cassettes. The results of the paper show CRISPRa to be a readily applicable strategy that can be used to treat various diseases.

Reagents and Corresponding Volume

Table 1 List of Reagents and its Volume

Reagents	Volume
1.0.5 M EDTA solution	100mL
2. 1 M MgCl2 solution	100mL
3. 10% SDS solution	100mL
4. 2.5 M KCL solution	100mL
5. 2.5 M CaCl2 solution	100mL
6. 2.5 NaCl solution	1000mL
7. 5 M NaCl solution	100mL
8. 5M Tris solution	100mL
9. 0.014% Tween/PBS-MK solution	500mL
10. 70% ethanol	~
11. 1.01 Phosphate Buffer	2000mL
12. 5 µg/µl Hoechst solution	100mL
13. LB(+) medium	1000mL
14. MP1 resuspension buffer.	1000mL
15. MP2 lysis buffer.	1000mL
16. MP3 neutralization buffer.	1000mL
17. RLT Plus buffer + β-mercaptoethanol	(10 µl/ml)
18. TBE electrophoresis buffer	5000mL
19. Triton X-100 lysis buffer	500mL
20. 30% (wt/vol) sucrose solution	500mL
21. Trypsin 0.5%/EDTA 0.2% (wt/vol).	500mL
22. Poly-L-lysine solution	50ml
23. 40% (wt/vol) polyethylene glycol (PEG) solution	200ml

Experimental protocol

Here, we describe in detail how to design transactivation gene therapies using dCas9-VPR. We provide a protocol on how to design sgRNA arrays, evaluate their transactivation efficiency in vitro and produce rAAVs expressing all components necessary for transactivation in vivo. Moreover, we describe how to evaluate the functionality of the transactivation-based gene therapy in an appropriate animal model. We exemplify the approach by focusing on retinal gene therapy for IRDs; however, the protocol can be adapted and applied to target disorders affecting other body systems such as the heart, skeletal muscles or the brain

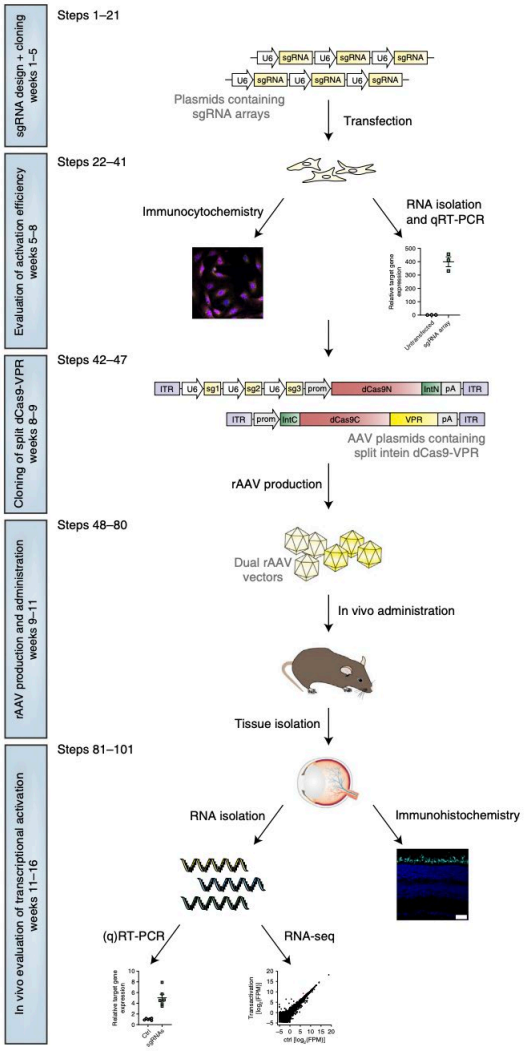

Fig. 1 Timeline and workflow of experiments

Protocol for Design and selection of sgRNAs for transactivation

The basic process of designing sgRNAs suitable for transactivation is consistent for all Cas9-based transactivation modules. First, the sgRNA target region needs to be located upstream of the transcriptional start site (TSS) of the chosen functionally equivalent gene. We recommend a maximum distance of 500 bp to the TSS. For CRISPRa, sgRNAs targeting areas further upstream resulted in lower transactivation efficiencies. Moreover, it has been shown that sgRNAs targeting regions closer to the TSS are more likely to result in high transactivation efficiencies. Second, the target sequence needs to be situated next to a protospacer adjacent motif (PAM) sequence, which varies depending on the Cas9 orthologue to be used. In this protocol, we use the Streptococcus pyogenes Cas9 characterized by the 'NGG' PAM motif. Third, suitable sgRNAs should have a high on-target and a low off-target activity, which can be evaluated using different software such as CRISPOR (http://crispor.tefor.net). We also suggest considering using more than one sgRNA for the transactivation of a single gene to ensure adequate expression levels. In the dual rAAV vectors containing the dCas9-VPR split intein expression cassettes used in this protocol, up to three sgRNAs

including promoter and termination signal can be packaged. Nevertheless, we suggest designing and testing several sgRNA expression cassettes containing one to three sgRNAs and comparing their transactivation efficiencies in vitro. For a control, we highly recommend including at least one nontargeting sgRNA. This can be used as a reference for the in vivo experiments assessing the gene expression and potential adverse effects upon transactivation.

Protocol for In vitro evaluation of sgRNA efficiency for transcriptional activation by quantitative reverse-transcription PCR (qRT-PCR) and immunocytochemistry

Before testing the designed sgRNAs in vivo, their activation efficiency should be evaluated in vitro. Several important points must be considered when choosing the right cell line for this purpose. If one aims at transactivating the functional equivalent of an IRD-causing gene in a mouse model, we recommend using the 661W cell line originating from a murine retinoblastoma and expressing cone photoreceptor markers. However, if your target gene is already expressed in this cell line at medium or high levels, an additional increase in gene expression caused by transactivation might be challenging. In this case, we recommend using mouse embryonic fibroblasts (MEFs) or other mouse-derived immortalized cell lines. Here, we describe how to evaluate the transactivation efficiency of sgRNA cassettes via qRT-PCR. If all tested sgRNA cassettes consisting of an sgRNA triplet result in mediocre or low transactivation, consider testing the individual sgRNAs in rearranged triplets in a combinatorial manner. Moreover, each sgRNA can also be tested individually, and the top three candidates can subsequently be picked for the final sgRNA triple cassette. Depending on the cell type and transactivated gene, a potential overexpression of the target protein in vitro might be harmful to the cells. Such cases might require fine-tuning of the transactivation efficiency. However, it is important to consider that the expression levels obtained in vitro and in vivo may differ. The in vivo expression is typically lower because of lower (co-)transduction of dual rAAVs and/or the additional reconstitution step of dCas9-VPR (see ‘Anticipated results’). Therefore, we advise studying cell toxicity only in vivo. If required, lower expression levels of the target gene can be obtained by testing other sgRNAs or reducing their overall number or by methods that yield lower dCas9-VPR expression (e.g., using weaker promoters). Finally, we highly recommend additionally confirming that the transactivation results in a successful expression and, if possible, the proper function of the protein encoded by the target gene. Protein expression can be addressed via immunocytochemistry, western blotting, quantitative proteomics59 or other appropriate techniques. Here, we describe a detailed protocol for protein detection using immunocytochemistry. Functional assays are more demanding and require an exact knowledge of the function of the corresponding protein. Depending on the protein class, functional analyses can be performed in living or fixed cells, in isolated cellular compartments or cellular fragments by using several techniques (e.g., patch clamp analysis for ion channels, imaging-based assays for members of the signaling cascades (e.g., ratiometric or intensiometric Ca2+ imaging60, biosensors61 and florescence resonance energy transfer62), ligand binding assays and a plethora of other techniques that have been developed to address the functionality of specific proteins).

Application of transactivation gene therapy in vivo

Before transactivation-based gene therapy can be applied in vivo, various factors must be taken into account. First, it is important to choose an adequate promoter for a tissue-specific transactivation of the target gene, because its unspecific expression might lead to harmful effects in other cell types. In this context, promoter length must also be considered, because longer promoters in combination with dCas9-VPR, sgRNAs, split inteins and other elements of the expression cassette could exceed the genomic DNA payload of the rAAV vectors. When using the plasmids provided by our laboratory on Addgene (plasmid nos. 165450 and 166692) for rAAV production, the length of the promoter sequence should not exceed 500 bp, to remain within the packaging limit. If your target disease requires the transactivation of a cone-specific gene in rod photoreceptors, you may use the short human rhodopsin promoter contained in the provided plasmids. For ubiquitous expression, the core EF1α promoter (212 bp) may be used. Second, it is important to choose an appropriate AAV serotype for an optimal transduction of the target tissue. For this purpose, you should also consider using pseudotyped or specifically engineered capsid variants characterized by an increased transduction efficiency. When targeting the retina, we recommend using the AAV8 Y733F or the AAV7m8 capsid variant. Third, an appropriate route of administration must be selected according to the target tissue and the selected AAV serotype. The following protocol describes an administration of rAAV vectors to the eye designed for the treatment of IRDs (i.e., via subretinal or intravitreal injection). If you want to target other tissues (e.g., the liver, the heart or skeletal muscles), consider applying the rAAVs systemically via a tail vein injection or locally (e.g., via intramuscular injection). To minimize variability within in vivo experiments, consider using animals only of the same sex. This is particularly important if the gene to be transactivated is located on the X chromosome, because this might result in lower transactivation efficiencies in males than in females. Furthermore, using age-matched animals can be advantageous, especially when testing transactivation gene therapy in a disease model characterized by rapid degeneration. Optimally, all animals should be injected at the exact same age and, if possible, during a similar time of the day. Although the likelihood of off-target effects in dCas9-VPR–mediated gene activation is very low, it is highly advisable to analyze such effects in all in vivo experiments aiming for a future clinical application. Because a catalytically deficient Cas9 is used for this CRISPRa approach, conventional off-target effects (i.e., permanent changes in nontargeted genomic sequences) are not expected to occur. Instead, unspecific binding of dCas9-VPR in off-target regions might result in an up- or downregulation of nontargeted transcripts. This can be examined via RNA-seq experiments. For this purpose, a control tissue injected with rAAVs containing a nontargeting sgRNA and the split dCas9-VPR or a noninjected control tissue is required. Ideally, only the transactivated gene should be expressed at significantly higher levels in the tissue injected with targeting sgRNAs compared with the injected or noninjected control tissue. However, when comparing rAAV-injected to noninjected control tissue, the gene expression might be altered as a response to the rAAV injection rather than the transactivation gene therapy. Therefore, we recommend using the injected control rather than the noninjected tissue as a control for this experiment. In some cases, the promoter

region of another gene might be present on the opposite strand and in close proximity to the target region. If so, the expression of this gene also needs to be investigated more comprehensively (e.g., using next-generation sequencing techniques). With some modification, the protocol described herein could be applied to other model organisms (e.g., dog or pig) for ocular or non-ocular diseases. Higher titers and larger volumes of rAAVs might be required for these experiments. Notably, the initial proof-of-principle experiments for the transactivation of a functionally equivalent gene in the target cells can also be performed in wild-type animals. However, in wild-type animals, only the transactivation efficiency can be investigated at the RNA and protein level. To evaluate potential therapeutic effects of the CRISPRa approach, model organisms for the respective diseases are indispensable.

Conflicts of interest

"There are no conflicts to declare".

Notes and references

1 Riedmayr, L.M., Hinrichsmeyer, K.S., Karguth, N. *et al.* dCas9-VPR-mediated transcriptional activation of functionally equivalent genes for gene therapy. *Nat Protoc* **17,** 781–818 (2022). https://doi.org/10.1038/s41596-021-00666-3