What are the advantages of using CRISPR activatory compared to classical gene overexpression?

Author: Thomas Childs

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UCL Queen Square Institute of Neurology

Contents

1. Abbreviations

AAV	Adeno-associated virus
cDNA	Complementary deoxyribonucleic acid
CRISPR	Clustered regularly interspaced short palindromic repeats
CRISPRa	CRISPR activation
crRNA	CRISPR ribonucleic acid
dCas9	Endonuclease dead Cas9
mRNA	Messenger RNA
pre-crRNA	Precursor CRISPR RNA
pre-mRNA	Precursor messenger RNA
sgRNA	Single guide RNA
TSS	Transcription start site

2. Introduction

The ability to artificially overexpress genes is exceedingly useful for scientific research and the development of new therapeutics. Perhaps the most important application will be in the development of new gene therapies to treat human disease. The classical approach to gene overexpression has been indispensable, but there are a number of limitations that new emergent techniques overcome. Clustered regularly interspaced short palindromic repeats activation (CRISPRa), a modification of the CRISPR/Cas system, is one such technique. This essay will highlight the advantages of CRISPRa compared to classical gene overexpression. As neurological diseases have proved to be particularly challenging to treat, the question will be addressed in the context of neurological gene therapy, as this will arguably be the most impactful application of these techniques.

3. Methods

A broad search was carried out on PubMed (US National Library of Medicine) in January 2020 using a combination of the terms: CRISPR, CRISPRa, dCas9, gene overexpression, transgene activation, transgene expression, transcriptional programming, gene therapy and overexpression (Fig 1.).

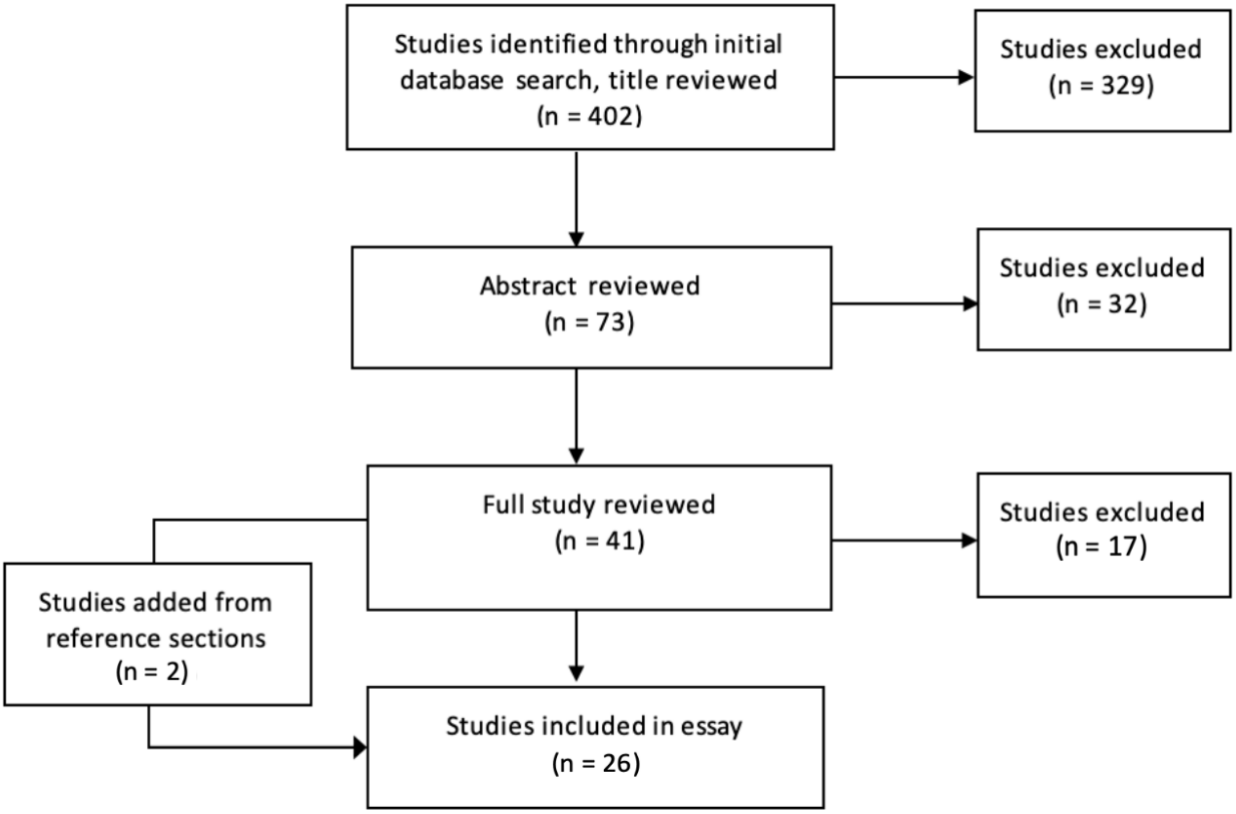

Figure 1. Flow diagram of literature selection process
Studies were excluded if they did not contain information on CRISPR, CRISPRa, or classical gene overexpression; if they were not available in English; or were published before 2000. References of selected papers were analysed to identify key studies not obtained from the initial searches.

4. Classical gene overexpression

The classical approach to gene overexpression is to deliver an exogenous complementary deoxyribonucleic acid (cDNA) sequence to target cells. For most neurological diseases, the targets of gene therapy will be neurons. Due to the elaborate architecture of the brain, the exogenous gene must be delivered to neurons *in vivo.* Viruses have evolved to efficiently transfer their genes into host cells, making them desirable vectors for *in vivo* gene delivery (Kay *et al.* 2001). While non-viral vectors are available, their efficiency is poor (Elsabahy *et al.* 2011). Therefore, most preclinical studies using gene therapy for neurological diseases use a viral vector for gene delivery (Simonato *et al.* 2013). The exogenous gene can persist in the host cell, and expression of the gene can compensate for the disease-causing mutation. For example, expression of a wild-type gene could compensate for a loss-of-function mutation, or the exogenous gene could counteract a gain-of-function mutation.

5. CRISPRa

The CRISPR/Cas system is a bacterial and archaeal immune system that defends against invading viral and plasmid nucleic acids (Wiedenheft *et al.* 2012). Short fragments of foreign genetic material can be incorporated into the CRISPR element of the host's genome. Transcription of the CRISPR loci produces precursor CRISPR ribonucleic acids (pre-crRNAs), which are processed to short crRNAs that associate with Cas endonucleases (Deltcheva *et al.* 2011; Gesner *et al.* 2011). Complementarity of the crRNA with the foreign nucleic acid facilitates base-pairing between them, and the associated Cas endonuclease then cleaves the invading nucleic acid, conferring immunity against the invader (Brouns *et al.* 2008; Marraffini and Sontheimer 2008; Hale *et al.* 2009). The type II CRISPR/Cas system, with its signature Cas9 endonuclease, has been exploited for genetic engineering. A single-guide RNA (sgRNA) can be designed to target Cas9 to a DNA sequence of choice, and the system delivered to a host *in vivo* using a viral vector (Jinek *et al.* 2012). Furthermore, endonuclease dead Cas9 (dCas9) can be fused to a transcriptional activator, and the sgRNA engineered to direct the fusion protein to a DNA sequence upstream of the transcriptional start site (TSS) of a target gene (Maeder *et al.* 2013; Mali *et al.* 2013; Chavez *et al.* 2015; Dong *et al.* 2018). The activation domain will activate transcription of the target gene, hence the technique is termed CRISPR activation. Increasing expression of a wild-type gene can then compensate for disease-causing mutations.

6. Advantages of CRISPRa

6.1 Large gene transcripts

Both CRISPRa and classical gene overexpression rely on viral vectors for *in vivo* delivery of the gene therapy. While recent advances have improved the delivery of viral vectors to the brain (Chan *et al.* 2017), the limited capacity of viruses remains a problem. Adeno-associated virus (AAV), the most promising neurological gene therapy vector, has a transgene capacity of about 4.5 kb (Simonato *et al.* 2013; Choudhury *et al.* 2017). This presents as a severe limitation for classical gene overexpression, as the efficient delivery of genes with large cDNA sequences in a single vector is not possible. Trapani *et al* (2014) used dual AAV vectors, each containing a fragment of the transgene, to deliver genes that exceed the capacity of a single vector. While they demonstrated successful transduction of transgenes both *in vitro* and *in vivo*, the transduction efficiency was lower than with single normal sized vectors. It is also worth noting that *in vivo* transduction was carried out in mouse and pig retinal tissue. The efficiency of dual vectors may be influenced by tissue/cell type, potentially due to differences in DNA repair pathways (Chamberlain *et al.* 2016). It is therefore unknown how well this technique would work in human neurons. There is no limitation on the size of gene that can be overexpressed with CRISPRa, as it simply increases the expression of a gene that is already present.

6.2 Endogenous gene activation

The classical activation approach involves overexpressing an exogenous gene from an exogenous promoter. A disadvantage of this method is that it subverts the natural mechanisms that regulate genomic gene expression. While different regulatory elements can be included in the transgenic construct to modulate the level of expression (Deverman *et al.* 2018), such as promoters of different strengths, these elements are constitutive and expression cannot be modified after vector administration. There is a risk that cells transduced by multiple viral particles could receive multiple copies of the gene, or that the exogenous promoter may produce ectopic expression. As these therapies are permanent, the risk of pathogenic overexpression is a serious concern.

CRISPRa simply increases the expression of an endogenous gene by activating its endogenous promoter. This method of gene expression is more physiological, preserves the natural gene regulation mechanisms, and is consequently less prone to gene overexpression side effects (Eyquem *et al.* 2017). Furthermore, CRISPRa constructs can be engineered to modulate the level of expression. Cheng *et al.* (2013) demonstrated that increasing the number of VP16 transcriptional activation domains fused to dCas9 can increase the level of gene activation. They also showed that sgRNAs complementary to different regions upstream of the TSS induce activation with different strengths, and co-transfecting multiple sgRNAs leads to greater activation than single sgRNAs. Mali *et al* (2013) produced similar results while targeting different genes, highlighting the reliability of the results. While targeting the sgRNA to different regions upstream of the TSS is a useful way to modulate activity, relying on multiple sgRNAs

limits the scalability of the technique. Second generation CRISPRa systems somewhat overcome this limitation by fusing multiple *different* activation domains to improve activation efficiency (Chavez *et al.* 2015; Konermann *et al.* 2015). The increased size of these fusion proteins, however, limits the applicability to *in vivo* gene therapy, as many exceed the capacity of AAV delivery vectors. Despite this limitation, the flexibility of CRISPRa permits the level of induced activation to be finely controlled, allowing increased expression of an endogenous gene within a physiological range while preserving natural regulation mechanisms.

6.3 Splice variants and biogenesis mechanisms

The biogenesis of a functional protein from genomic DNA contains a number of steps that can regulate expression or increase the diversity of possible protein products. Alternative splicing of precursor messenger RNA (pre-mRNA) introns leads to a range of splice variants from the same gene, and this has important implications. Alternative splice variants can regulate transcript levels (Lewis *et al.* 2003) or add new elements to the final protein to modulate localisation, binding properties, function and stability (Stamm *et al.* 2005). The overexpressed construct in the classical approach is cDNA. cDNA is produced via reverse transcription of mature mRNA, and consequently lacks introns and the natural biogenesis mechanisms that mediate genomic DNA expression. The classical approach to gene overexpression therefore lacks alternative splicing and the aforementioned benefits. As CRISPRa simply activates the natural expression mechanisms, introns will be present in the pre-mRNA transcript and alternative splicing can function to regulate expression and increase protein diversity in the normal physiological manner.

6.4 Multi-gene activation

The ability to activate multiple genes simultaneously will enable the delivery of a multi-dimensional gene therapy. The classical approach has poor utility for multi-gene activation. Each additional gene to be overexpressed must be delivered to the patient's cells. The small capacity of AAVs necessitates the use of multiple vectors to carry all the genes, severely limiting the scalability of the technique. Since it is the sgRNA that targets the dCas9-activator to the target DNA sequence in CRISPRa, multiple sgRNAs can be designed that are complementary to regions upstream of many different genes (Cheng *et al.* 2013; Konermann *et al.* 2015; Zhou *et al.* 2018). As sgRNAs are less than 100 nucleotides long, multiple can be packaged into a single vector, allowing the system to easily scale for multi-gene activation. Cheng *et al.* demonstrated that human HEK293T cells can be co-transfected with multiple sgRNAs targeting different genes to successfully activate multiple endogenous genes. Furthermore, administering different ratios of sgRNAs resulted in different ratios of gene activation. However, these experiments were carried out via transfection of cultured HEK cells, not viral transduction of a living organism, and hence the generalisability to human gene therapy is limited. While Zhou *et al.* successfully activated multiple genes in the brains of live mice, they first generated mice transgenic for the large dCas9-activator construct via transposon delivery to zygotes. Viral delivery of this large construct into mature human brains would be a challenge for human multiplex gene therapies.

7. Conclusion

While the classical approach to gene overexpression has paved the way for advances in gene therapy, it has many limitations which have been overcome by CRISPRa. Activating large and multiple endogenous genes via natural biogenesis mechanisms that preserve alternative splicing and gene regulation is only possible with this newer technology. Moving forward, it is important to remain vigilant of the possibility of off-target binding of sgRNAs and dCas9 (Wu *et al.* 2014). Ensuring sgRNAs are designed with high specificity will be essential to human applications of CRISPRa therapy.

8. References

Brouns, S.J., Jore, M.M., Lundgren, M., Westra, E.R., Slijkhuis, R.J., Snijders, A.P., Dickman, M.J., Makarova, K.S., Koonin, E.V. and van der Oost, J. (2008) 'Small CRISPR RNAs guide antiviral defense in prokaryotes', *Science*, 321(5891), 960-4, available: http://dx.doi.org/10.1126/science.1159689.

Chamberlain, K., Riyad, J.M. and Weber, T. (2016) 'Expressing Transgenes That Exceed the Packaging Capacity of Adeno-Associated Virus Capsids', *Human Gene Therapy Methods*, 27(1), 1-12, available: http://dx.doi.org/10.1089/hgtb.2015.140.

Chan, K.Y., Jang, M.J., Yoo, B.B., Greenbaum, A., Ravi, N., Wu, W.-L., Sánchez-Guardado, L., Lois, C., Mazmanian, S.K., Deverman, B.E. and Gradinaru, V. (2017) 'Engineered AAVs for efficient noninvasive gene delivery to the central and peripheral nervous systems', *Nature Neuroscience*, 20(8), 1172-1179, available: http://dx.doi.org/10.1038/nn.4593.

Chavez, A., Scheiman, J., Vora, S., Pruitt, B.W., Tuttle, M., P R Iyer, E., Lin, S., Kiani, S., Guzman, C.D., Wiegand, D.J., Ter-Ovanesyan, D., Braff, J.L., Davidsohn, N., Housden, B.E., Perrimon, N., Weiss, R., Aach, J., Collins, J.J. and Church, G.M. (2015) 'Highly efficient Cas9-mediated transcriptional programming', *Nature Methods*, 12(4), 326-328, available: http://dx.doi.org/10.1038/nmeth.3312.

Cheng, A.W., Wang, H., Yang, H., Shi, L., Katz, Y., Theunissen, T.W., Rangarajan, S., Shivalila, C.S., Dadon, D.B. and Jaenisch, R. (2013) 'Multiplexed activation of endogenous genes by CRISPR-on, an RNA-guided transcriptional activator system', *Cell Research*, 23(10), 1163-1171, available: http://dx.doi.org/10.1038/cr.2013.122.

Choudhury, S.R., Hudry, E., Maguire, C.A., Sena-Esteves, M., Breakefield, X.O. and Grandi, P. (2017) 'Viral vectors for therapy of neurologic diseases', *Neuropharmacology*, 120, 63-80, available: http://dx.doi.org/10.1016/j.neuropharm.2016.02.013.

Deltcheva, E., Chylinski, K., Sharma, C.M., Gonzales, K., Chao, Y., Pirzada, Z.A., Eckert, M.R., Vogel, J. and Charpentier, E. (2011) 'CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III', *Nature*, 471(7340), 602-607, available: http://dx.doi.org/10.1038/nature09886.

Deverman, B.E., Ravina, B.M., Bankiewicz, K.S., Paul, S.M. and Sah, D.W.Y. (2018) 'Gene therapy for neurological disorders: progress and prospects', *Nat Rev Drug Discov*, 17(9), 641-659, available: http://dx.doi.org/10.1038/nrd.2018.110.

Dong, C., Fontana, J., Patel, A., Carothers, J.M. and Zalatan, J.G. (2018) 'Synthetic CRISPR-Cas gene activators for transcriptional reprogramming in bacteria', *Nature Communications*, 9(1), available: http://dx.doi.org/10.1038/s41467-018-04901-6.

Elsabahy, M., Nazarali, A. and Foldvari, M. (2011) 'Non-viral nucleic acid delivery: key challenges and future directions', *Curr Drug Deliv*, 8(3), 235-44, available: http://dx.doi.org/10.2174/156720111795256174.

Eyquem, J., Mansilla-Soto, J., Giavridis, T., van der Stegen, S.J., Hamieh, M., Cunanan, K.M., Odak, A., Gonen, M. and Sadelain, M. (2017) 'Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection', *Nature*, 543(7643), 113-117, available: http://dx.doi.org/10.1038/nature21405.

Gesner, E.M., Schellenberg, M.J., Garside, E.L., George, M.M. and Macmillan, A.M. (2011) 'Recognition and maturation of effector RNAs in a CRISPR interference pathway', *Nature Structural & Molecular Biology*, 18(6), 688-692, available: http://dx.doi.org/10.1038/nsmb.2042.

Hale, C.R., Zhao, P., Olson, S., Duff, M.O., Graveley, B.R., Wells, L., Terns, R.M. and Terns, M.P. (2009) 'RNA-guided RNA cleavage by a CRISPR RNA-Cas protein complex', *Cell*, 139(5), 945-956, available: http://dx.doi.org/10.1016/j.cell.2009.07.040.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A. and Charpentier, E. (2012) 'A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity', *Science*, 337(6096), 816-821, available: http://dx.doi.org/10.1126/science.1225829.

Kay, M.A., Glorioso, J.C. and Naldini, L. (2001) 'Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics', *Nature Medicine*, 7(1), 33-40, available: http://dx.doi.org/10.1038/83324.

Konermann, S., Brigham, M.D., Trevino, A.E., Joung, J., Abudayyeh, O.O., Barcena, C., Hsu, P.D., Habib, N., Gootenberg, J.S., Nishimasu, H., Nureki, O. and Zhang, F. (2015) 'Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex', *Nature*, 517(7536), 583-588, available: http://dx.doi.org/10.1038/nature14136.

Lewis, B.P., Green, R.E. and Brenner, S.E. (2003) 'Evidence for the widespread coupling of alternative splicing and nonsense-mediated mRNA decay in humans', *Proceedings of the National Academy of Sciences*, 100(1), 189, available: http://dx.doi.org/10.1073/pnas.0136770100.

Maeder, M.L., Linder, S.J., Cascio, V.M., Fu, Y., Ho, Q.H. and Joung, J.K. (2013) 'CRISPR RNA-guided activation of endogenous human genes', *Nat Methods*, 10(10), 977-9, available: http://dx.doi.org/10.1038/nmeth.2598.

Mali, P., Aach, J., Stranges, P.B., Esvelt, K.M., Moosburner, M., Kosuri, S., Yang, L. and Church, G.M. (2013) 'CAS9 transcriptional activators for target specificity screening and paired nickases

for cooperative genome engineering', *Nature Biotechnology*, 31(9), 833-838, available: http://dx.doi.org/10.1038/nbt.2675.

Marraffini, L.A. and Sontheimer, E.J. (2008) 'CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA', *Science*, 322(5909), 1843-5, available: http://dx.doi.org/10.1126/science.1165771.

Simonato, M., Bennett, J., Boulis, N.M., Castro, M.G., Fink, D.J., Goins, W.F., Gray, S.J., Lowenstein, P.R., Vandenberghe, L.H., Wilson, T.J., Wolfe, J.H. and Glorioso, J.C. (2013) 'Progress in gene therapy for neurological disorders', *Nature Reviews Neurology*, 9(5), 277-291, available: http://dx.doi.org/10.1038/nrneurol.2013.56.

Stamm, S., Ben-Ari, S., Rafalska, I., Tang, Y., Zhang, Z., Toiber, D., Thanaraj, T.A. and Soreq, H. (2005) 'Function of alternative splicing', *Gene*, 344, 1-20, available: http://dx.doi.org/10.1016/j.gene.2004.10.022.

Trapani, I., Colella, P., Sommella, A., Iodice, C., Cesi, G., Simone, S., Marrocco, E., Rossi, S., Giunti, M., Palfi, A., Farrar, G.J., Polishchuk, R. and Auricchio, A. (2014) 'Effective delivery of large genes to the retina by dual AAV vectors', *EMBO Molecular Medicine*, 6(2), 194-211, available: http://dx.doi.org/10.1002/emmm.201302948.

Wiedenheft, B., Sternberg, S.H. and Doudna, J.A. (2012) 'RNA-guided genetic silencing systems in bacteria and archaea', *Nature*, 482(7385), 331-338, available: http://dx.doi.org/10.1038/nature10886.

Wu, X., Scott, D.A., Kriz, A.J., Chiu, A.C., Hsu, P.D., Dadon, D.B., Cheng, A.W., Trevino, A.E., Konermann, S., Chen, S., Jaenisch, R., Zhang, F. and Sharp, P.A. (2014) 'Genome-wide binding of the CRISPR endonuclease Cas9 in mammalian cells', *Nature Biotechnology*, 32(7), 670-676, available: http://dx.doi.org/10.1038/nbt.2889.

Zhou, H., Liu, J., Zhou, C., Gao, N., Rao, Z., Li, H., Hu, X., Li, C., Yao, X., Shen, X., Sun, Y., Wei, Y., Liu, F., Ying, W., Zhang, J., Tang, C., Zhang, X., Xu, H., Shi, L., Cheng, L., Huang, P. and Yang, H. (2018) 'In vivo simultaneous transcriptional activation of multiple genes in the brain using CRISPR–dCas9-activator transgenic mice', *Nature Neuroscience*, 21(3), 440-446, available: http://dx.doi.org/10.1038/s41593-017-0060-6.