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Use of the CRISPR/Cas9-Based Epigenetic Gene Activation System *in Vivo*: a New Potential Therapeutic Modality

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Epigenetic marks have a high degree of cellular-specificity and cause heritable changes in gene expression and cell phenotype in response to developmental and environmental cues, without directly altering the DNA sequence (1). The discovery of novel, modifiable epigenetic targets has led to the emergence of molecular-based epigenetic therapy, for example in the field of hepatocellular carcinoma; multiple clinical trials are ongoing to test the efficacy of histone deacetylase inhibitors and DNA methyltransferase inhibitors (2).. Concerns remain, however, that emerging epigenetic therapies targeting histone and DNA modifying activities (1, 2) ubiquitously alter epigenetic marks, and are thus prone to off-target effects. On the other hand, the discovery of the CRISPR/Cas9 genome editing system derived from prokaryotic adaptive immune systems, has led to the development of tools for rapid and efficient RNA-based, sequence-specific genome editing (3). Although clinical application of gene therapy in liver disease has proven challenging as sustained expression of the transgene is limited by the episomal nature of the vector and the patient immune responses (4), there has been a growing excitement over the last decade that CRISPR-mediated epigenome editing may be able to overcome some of these hurdles and be amenable to therapeutic applications [as reviewed in detail in (5)].

A recent publication by Liao HK, et al. entitled "In Vivo Target Gene Activation via CRISPR/Cas9-Mediated Trans-epigenetic Modulation" in Cell, 2017. 171(7): p. 1495–1507 e15 (6), defines a new application of an optimized CRISPR/Cas9 system for *in vivo* activation of target genes, with the aim of developing specific epigenetic therapy without off-target effects. The authors identified a combination of co-transcriptional activators and single guide RNA (sgRNA)s that fit within a single adeno associated virus (AAV) vector and induce high levels of transcriptional gene activation (TGA) when injected into Cas9-

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expressing mice. Liao et al. used this technology to ameliorate pathologic phenotypes in mouse models of diabetes, muscular dystrophy and acute kidney disease. In an effort to expand the application of this system to wild type mice and eventually to human who do not express Cas9, the authors also generated a dual-AAV system and showed that co-injection of AAV-Cas9 with an AAV-gRNA that targeted the *utrophin* gene, improved the muscular dystrophy phenotype of Duchenne muscular dystrophy (DMD) mdx mice.

The authors adapted the synergistic activation mediator (SAM) system that relies on an engineered hairpin aptamer containing two MS2 domains, which recruit the MS2:P65:HSF1 (MPH) transcriptional activation complex to the target locus. The authors used short sgRNAs [14–15 base pairs (bp) rather than 20 bp] to guide wild-type Cas9 to the target locus. These sgRNAs prevent Cas9 from creating DNA strand breaks (DSB) and are therefore called dead sgRNAs (dgRNAs). TGA is most effective when sgRNAs target sequences between –100 and +50 bp of the transcriptional start site. The authors targeted the mouse *follistatin* (*Fst*) gene, since *Fst* overexpression increases muscle mass, and delivered AAV-dgFst-T2-MPH via intramuscular injection into the hind-limb of Cas9-expressing mice, resulting in increased muscle mass and strength. Then, the authors demonstrated that the CRISPR/Cas9 TGA system induced the expression of functional Klotho/IL-10 protein, since both proteins are known to reduce kidney injury, and demonstrated a protective effect in a mouse model of acute kidney injury. Furthermore, the authors employed their CRISPR/Cas9 TGA system to induce *in vivo* trans-differentiation of liver cells into insulin-producing cells. The authors overexpressed pancreatic and duodenal homeobox gene 1 (*Pdx1*) in liver cells with the goal of generating insulin secreting cells to treat a mouse model of type I diabetes. Injection of AAV-dgPdx1-MPH in Cas9 mice resulted in hepatocyte upregulation of insulin 1 (*Ins1*), insulin 2 (*Ins2*), and proprotein convertase subtilisin/kexin type 1 (*Pcsk1*). Chromatin-immunoprecipitation (ChIP)-PCR in liver samples from mice injected with AAV-dgPdx1-MPH showed enrichment of the activating H3K4me3 and H3K27ac epigenetic marks at the *Pdx1* locus, consistent with transcriptionally active genes. These patterns of epigenetic modification are similar to those observed in tissues naturally expressing *Pdx1*, such as cells of the small intestine. Hence, the CRISPR/Cas9 TGA system transcriptionally activated a gene that is normally silent in liver via the recruitment of activators and subsequent remodeling of histone marks, suggesting that the system can be exploited for cell fate engineering.

The new system consisting of the modified CRISPR/Cas9 machinery and co-transcriptional complex has many strengths, as it provides a targeted epigenetic approach with multiple potential therapeutic applications including: (1) rescuing levels of endogenous gene expression, (2) activating silent genes, (3) compensating for genetic defects, and (4) altering cell fate by trans differentiation. More importantly, the system generates no detectable DSB (although more comprehensive approaches with whole genome sequencing will be needed to validate a lack of DSBs), which eliminates the risk of unintended mutations in the target genome. Potential off-target gene activation events will also need to be addressed. Furthermore the system provides a new opportunity to modulate diseases caused by large genes like Duchene muscular dystrophy, where gene delivery by AAV is impossible due to its restricted carrying capacity (assuming another endogenous gene can compensate for the defective one). The limitations of this system, as recognized by the authors, include the need

for longer term studies to assess the sustained efficacy of the AAV-CRISPR/Cas9 TGA system, and the host immune response to the system. Given that the AAV-CRISPR/Cas9 TGA system is inducing changes to the chromatin, it will be of interest to determine whether the remodeled chromatin becomes stable and heritable through cell division, even in the absence of the transgenic activator, which would greatly enhance the power of the system. The system requires further improvements in specificity by integrating the use of tissue-specific promoters and AAV serotypes with specific tissue tropism, since local injection was more efficacious than systemic administration. Other potential limitations of widespread application relate to chromatin accessibility depending on the local chromatin environment, and the specificity, fidelity, and delivery of CRISPR systems repurposed for epigenome editing, since Cas9 clearly has preferred sites of activity in the genome and did not evolve to contend with eukaryotic chromatin. (5, 7) Additional limitations include: the technical challenge of needing to transduce a single human hepatocyte with two AAV vectors, the applicability of this system in heterozygous disease where targeting a specific allele is not feasible, and the unknown consequences of overexpressing a single gene on the cell homeostasis. Finally, to fully realize the potential of epigenome editing methods like this, a system that permits targeted gene repression will also be needed. Much effort is underway to develop such systems, although as with gene activation, issues surrounding the extent and stability of repression of targeted loci need to be dealt with (8).

In the context of human liver diseases, the ability to specifically target gene activation or repression to one or a small group of genes holds great promise for treating a number of conditions (Table. 1). These conditions can be targeted by: 1) silencing the pathogenic gene, an example being alpha-1 antitrypsin (AAT) deficiency, where silencing of the mutant Z allele in a homozygous status could prevent progression of liver disease; 2) activation of a silent gene, for example in females with ornithine transcarbamylase (OTC) deficiency, where transcriptional activation of the normal OTC allele on the lyonized X chromosome in the liver would provide sufficient enzyme to maintain a normal phenotype; 3) rescuing levels of endogenous gene expression, an example being patients with cystic fibrosis (CF) due residual CF transmembrane regulator (CFTR) function mutation; and 4) compensating for a genetic defect, where hemochromatosis serves as a good example wherein overexpression of the iron regulatory protein hepcidin through transcriptional activation of the *HAMP* gene, would reduce the iron accumulation observed in HFE-deficient patients. In summary, the system developed by Liao et al., provides a valuable new tool for biomedical researchers. With further validation and refinement, the AAV-CRISPR/Cas9 TGA system offers potential therapeutic application to a wide variety of human liver diseases, and holds promise for the future of epigenetic therapy and personalized medicine.

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List of abbreviations

CRISPR	Clustered regularly interspaced short palindromic repeats
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DSBs	DNA strand breaks
TGA	target gene activation
sgRNA	single guide RNA
dgRNAs	dead sgRNAs
AAV	adeno-associated virus
MPH	MS2:P65:HSF1
bp	base pairs
SAM	synergistic activation mediator
Fst	follistatin
Il10	interleukin 10
Pdx1	pancreatic and duodenal homeobox gene 1
Ins1	insulin 1
Ins2	insulin 2
Pcsk1	proprotein convertase subtilisin/kexin type 1
ChIP	chromatin-immunoprecipitation
STZ	streptozotocin
DMD	Duchenne muscular dystrophy
AAT	alpha one antitrypsin
OTC	ornithine transcarbamylase deficiency

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Table 1

Potential applications of the AAV-CRISPR/Cas9 gene regulation system in human liver diseases.

Disease	Gene affected/ applicable scenario	Transmission	Strategy
AAT deficiency	<i>SERPINA1</i> / Z allele	Codominant	Silencing the mutant AAT-Z allele
OTC deficiency	<i>OTC</i> / X inactivation	X-linked	Activation of silent gene
CF	<i>CFTR</i> / residual function mutation	Autosomal recessive	Rescuing endogenous CFTR level
Hemochromatosis	<i>HFE</i> / C282Y mutation	Autosomal recessive	Compensatory activation of HAMP gene to overexpress hepcidin

Alpha one antitrypsin (AAT), Ornithine transcarbamylase (OTC), cystic fibrosis (CF), CF transmembrane regulator (CFTR)