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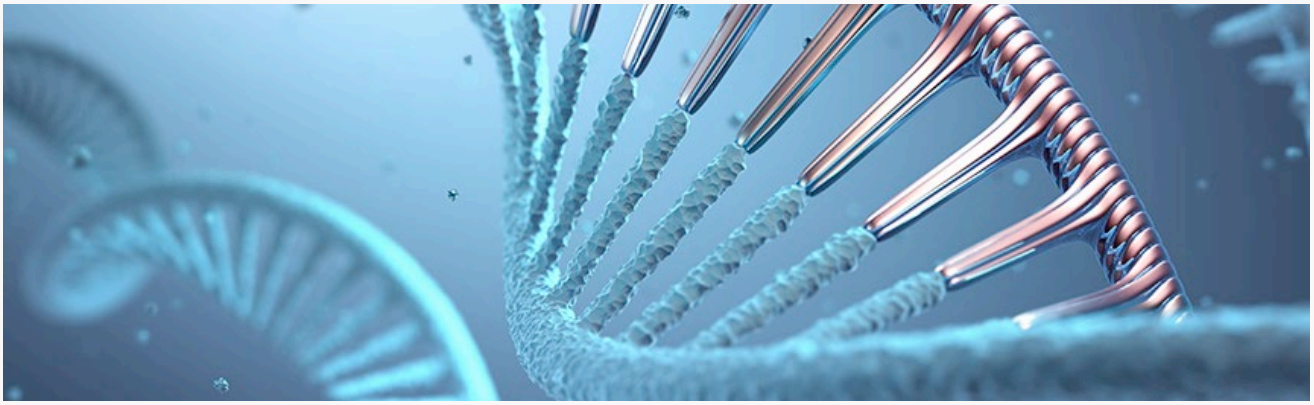


THINKSTOCK

TOOLS & TECHNIQUES

# SYNTHETIC EXPRESSIONS

By Karen Tkach Tuzman, Associate Editor

Bringing synthetic biology to transcriptional regulation could catalyze a new generation of gene therapies that are more tunable, disease-specific and easy to deliver than the products dominating the field.

The new constructs include synthetic promoters that can optimize expression of vector-encoded genes, and synthetic transcription factors that can activate silent regions of a patient's own genome.

Gene therapies typically employ promoters that are either constitutively active or tissue-specific to drive the expression of genes encoded in delivery vectors. The trade-off is that constitutively active promoters induce higher gene expression, but are more prone to off-target effects.

Synthetic promoters offer the chance to bridge the gap by combining optimized sequences from different natural promoters to concentrate potent expression in disease-relevant cell types or tissues. The technology, which first took off in agricultural engineering, is now gaining traction for biomedical applications (see "Synthetic Synthesis").

"These are synthetic promoters in that they're DNA sequences that don't exist in nature, but they're comprised of natural sequences that guide cell type-specific gene expression," said Michael Roberts, founder and CSO of Synpromics Ltd., a synthetic biology company with a platform of synthetic promoters. "They prevent gene expression where you don't want it, where it would have a toxic effect or induce an immune response."

Synthetic transcription factors could solve the second major challenge of gene therapy vectors: their limited carrying capacity, which prevents them from delivering large genes like dystrophin.

The technology, which is less well-established than that of synthetic promoters, aims to boost expression of large genes without cramming them into vectors, by recruiting transcriptional activators to genomic target sequences.

"With synthetic transcription factors, you're trying to work with the genome as it is, flaws and all," said Aseem Ansari, a professor of biochemistry at University of Wisconsin-Madison, whose lab works on both synthetic transcription factors and promoters.

Although the use of synthetic biology in this way is growing, most companies in the space are developing therapeutics using orthogonal synthetic promoters and transcription factors derived from yeast or bacteria, which are thought to minimally interfere with mammalian gene expression.

So far, a smaller set of players have disclosed synthetic technologies whose therapeutic effects depend on mimicking or interacting with components of the human genome.

# SYNTHETIC SYNTHESIS

Synthetic promoters and transcription factors could advance gene therapies by enabling controlled expression of exogenous or endogenous genes. Design strategies include combining natural cell-specific promoter sequences to create new promoters, and co-opting CRISPR machinery or pharmacological DNA binders to generate transcription factors.

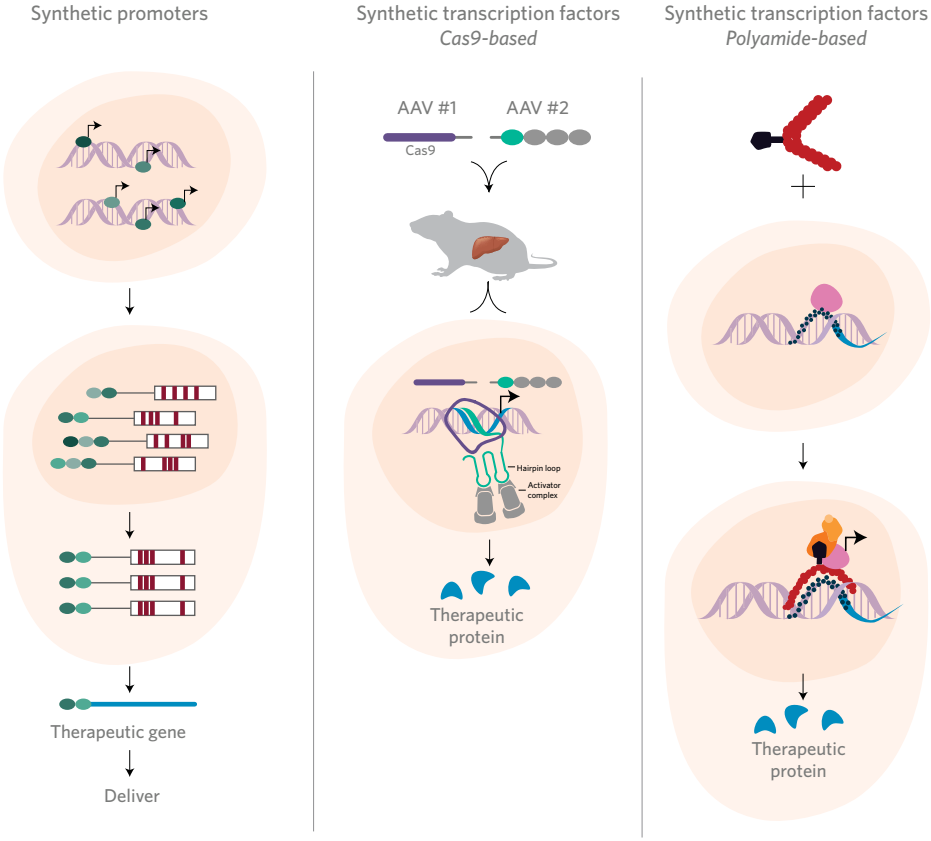
**Synthetic promoters. Synpromics Ltd.**'s PromPT platform uses bioinformatics to find promoters **(green)** that are activated in a cell type of interest. The platform generates combinations of the natural promoter sequences, clones them into constructs containing DNA barcodes **(striped bars)**, and transfects the barcoded library into the relevant cell type. The strongest promoter combinations are identified by finding the barcodes that are most abundantly transcribed. Top hits are used to drive cell type-specific expression of a therapeutic gene.

**Cas9-based synthetic transcription factors.** A group from the **Salk Institute for Biological Studies** developed a synthetic transcription factor system that can be delivered in two adeno-associated viral (AAV) vectors to drive gene expression *in vivo*. One vector encoded the nuclease Cas9 **(purple)**; the second encoded a "dead" guide RNA, attached to a hairpin aptamer **(green)** that doesn't trigger Cas9 enzymatic activity, as well as an engineered transcriptional activator complex **(gray).** The role of the Cas9 and the guide RNA is to find the relevant promoter sequence on the genome and recruit the engineered transcriptional machinery provided by the second vector.

*In vivo*, the vectors home to the liver, induce transcription and drive expression of the desired protein. The Salk team demonstrated the system can yield disease-modifying liver levels of therapeutic proteins in mouse models of acute kidney injury (AKI), Type I diabetes and Duchenne muscular dystrophy (DMD).

**Polyamide-based synthetic transcription factors.** A group from the **University of Wisconsin-Madison** developed a synthetic transcription factor that links the BRD4 ligand JQ1 **(black)** to a polyamide compound **(red)** that has high affinity for the GAA repeats **(dark circles)** in the FXN gene that underlie the pathogenesis of Friedreich's ataxia. GAA repeats stall RNA polymerase II **(pink)**, preventing FXN expression and causing disease symptoms. In patient-derived cells, the synthetic transcription factor recruited endogenous transcriptional activators **(orange)** to the FXN promoter and induced expression of the therapeutic protein.

BRD4 - Bromodomain containing 4; Cas9 - CRISPR-associated protein 9; FXN (FRDA) - Frataxin



## GETTING PROMOTED

Synthetic promoters have been around since the 1990s, but the rise of next-generation sequencing (NGS) technologies has fueled the identification of promoter and enhancer components that provided the basis for the technology's advancement, according to Synpromics' Roberts.

"Since 2011, when we were patenting this idea, the genomic space has really expanded dramatically, and we've been able to expand our technology dramatically too," Roberts told BioCentury.

Synpromics, which first focused on agricultural and industrial biology, began developing synthetic promoters for cell and gene therapies about three years ago. Now a leader in that space, the company has 26 commercial biomedical programs partnered with 18 companies or universities, including five disclosed gene therapy collaborations. All of its programs are in preclinical development (see "Promoting Partnerships").

At least one other company, Senti Biosciences Inc., has IP covering synthetic promoters for cell-type specific expression of genetic constructs *in vivo*. Senti was co-founded by Timothy Lu, an associate professor at the Massachusetts Institute of Technology (MIT), who has published on the use of synthetic promoters in cancer to drive tumor-specific expression of immunogenic proteins. Lu declined to comment on Senti's plans for the technology.

So far, Synpromics and its partners have primarily developed synthetic promoters for liver, muscle and eye cells. In November, the company announced a collaboration with University College London scientists to develop promoters specific for different neuronal cell populations.

Using its screening system, Synpromics has been developing "a rubric for generation of synthetic promoters," said Roberts. "We're now at the stage where we're building synthetic promoters in a more rational fashion."

The company's PromPT platform uses bioinformatics to identify natural promoters and enhancers that serve as building blocks for synthetic promoters that are cell type-specific or stimulus-responsive. A wet lab component then generates a barcoded

## PROMOTING PARTNERSHIPS

**Synpromics Ltd.** is engaged in 18 external partnerships involving its synthetic promoter technology. Below are eight collaborations for which the partners have disclosed the project's goals. The company has not disclosed project goals with its 10 other partners: **BioMarin Pharmaceutical Inc.** (NASDAQ:BMRN), **Homology Medicines Inc.**, **Lonza Group Ltd.** (SIX:LONN), four undisclosed companies, **Queen's University Belfast**, **University of Edinburgh** and **Royal Holloway**, **University of London**. *Source: Company presentation, company websites; BCIQ: BioCentury Online Intelligence*

| YEAR | PARTNER | PURPOSE |
|---|---|---|
| 2015 | **Applied Genetic Technologies Corp.** (NASDAQ:AGTC) | Develop synthetic promoters specific for multiple cell types for use in development of gene therapy candidates against multiple targets |
| 2015; 2016 | **Adverum Biotechnologies Inc.** (NASDAQ:ADVM) | Develop promoters for the treatment of eye diseases; develop promoters that specifically regulate gene expression in certain types of liver cells |
| 2015; 2016 | **uniQure N.V.** (NASDAQ:QURE) | Develop synthetic promoters with up-regulated liver cell-specific activity suitable for gene expression using an adeno-associated viral (AAV) vector; develop ultra-small synthetic promoters that show high levels of liver cell-specific activity |
| 2016 | **Cell and Gene Therapy Catapult** | Create stable producer cell lines for high-titer and large-scale manufacturing of industry-relevant viral vectors, including retroviruses and AAVs |
| 2016 | **Sartorius AG** (Xetra:SRT) | Evaluate and test synthetic promoters with Sartorius' biopharmaceutical manufacturing platform to reduce timelines and increase yields in the development of stable cell lines for the production of biopharmaceuticals |
| 2017 | GE Healthcare unit of **General Electric Co.** (NYSE:GE) | Develop a barcoded library of synthetic promoters for screening on GE Healthcare's biopharmaceutical manufacturing platform to increase the yield of biopharmaceuticals, including proteins that are difficult to manufacture |
| 2017 | **Solid Biosciences LLC** | Synpromics will provide Solid Biosciences access to a set of muscle-selective promoter candidates to enhance the latter's investigational gene therapy candidates for Duchenne muscular dystrophy (DMD) |
| 2017 | **University College London** | Generate synthetic gene promoters to control therapeutic gene expression in different subpopulations of neurons, and develop a gene therapy for Parkinson's disease |

library of random combinations of the building blocks, and looks for combinations that promote gene expression in the desired cell type or treatment context.

"The barcode is in the mRNA, and you do RNAseq to find out how many times that barcode appears. That gives us an indirect readout of the strength of the promoter," said Roberts.

Roberts said the platform allows tunable gene expression either by generating inducible promoters whose strength is dependent on the concentration of a pharmacological agent, or by producing a range of cell type-specific promoters of varying strengths.

> "We're now at the stage where we're building synthetic promoters in a more rational fashion."
>
> Michael Roberts, Synpromics

"We can create promoters with a 50-fold dynamic range of expression, where the strongest promoter is about 50-times more active than the weakest promoter, which is still more active than a typical biological promoter like CMV," he said. Roberts added that synthetic promoters also have a play in gene editing, where they can enable transient or cell-type specific nuclease expression.

The company is also using synthetic promoters to create stable cell lines for efficient and scalable manufacturing of adeno-associated viruses (AAVs) and lentiviruses. "Manufacturing is a huge bottleneck in gene therapy, particularly with AAV because there are so many clinical trials going on and very few manufacturers," he said.

### *IN VIVO* TURN-ON

For synthetic transcription factors, one emerging strategy is to retool Cas9 and complementary guide RNA (gRNA), the key elements of CRISPR-based gene editing, to target endogenous promoter sequences and turn on gene expression.

CRISPR pioneer Feng Zhang, a core institute member of the Broad Institute of MIT and Harvard and co-founder of Editas Medicine Inc., was an early mover with this approach. Zhang developed a synthetic Cas9-based gene activation system, dubbed Synergistic Activation Mediator (SAM), and used it in a cell culture screen on a library of over 70,000 gRNAs to look for genes whose up-regulation induced resistance to BRAF inhibitors. Zhang did not respond to requests to comment.

Juan Carlos Izpisua Belmonte, a professor in the Gene Expression Laboratory at the Salk Institute for Biological Studies, has modified the system's components to induce higher levels of target gene activation, and fit inside single or dual AAV vectors, making it more feasible to use the technology *in vivo*.

"A distinguishing characteristic of our technology is that, in principle, size is not a limiting factor," Belmonte told BioCentury.

In a paper published last month in *Cell,* his group argued Zhang's original system hasn't been shown to trigger physiologically relevant phenotypic changes in adult mammals and is too large to fit in commonly used viral vectors.

Zhang's system used full-length gRNAs and catalytically dead Cas9 to activate gene expression by recruiting activators without inducing double-stranded breaks. The gRNA was linked to a hairpin aptamer, which bound a synthetic complex that included the transcriptional activators HSF1 and RELA; in addition, the dead Cas9 was fused to a viral transcriptional activator known as VP64.

In subsequent versions of the system, Zhang's team used shortened "dead" gRNAs and wild-type Cas9; his team has patented the approach.

Belmonte's team used the "dead" guide RNA strategy and increased the system's potency by optimizing the gRNA aptamer and transcription factor complex. That enabled them to shrink the system by leaving out VP64, and induce strong target gene expression *in vivo*.

> "With synthetic transcription factors, you're trying to work with the genome as it is, flaws and all."
>
> Aseem Ansari, University of Wisconsin

In adult mice expressing a Cas9 transgene, *in vivo* delivery of Belmonte's system in a single AAV serotype 9 (AAV9) vector activated liver expression of therapeutic genes. In Cas9 transgenic models of acute kidney injury (AKI), Type I diabetes and Duchenne muscular dystrophy (DMD), the system reduced disease symptoms by activating liver expression of IL-10 or klotho, PDX1, and klotho or UTRN, respectively.

Those experiments relied on transgenic animals engineered to express Cas9. To show the system can work in a more representative model, the team performed an experiment in standard DMD mice using one AAV to deliver Cas9 and a second AAV to deliver the guide RNA aptamer and transcriptional activator constructs. Neonatal treatment with the two-vector system reduced DMD symptoms after birth by activating the muscle regulators FST or UTRN.

Belmonte said there's still a long road from here to the clinic for the synthetic transcription factor system, which will require testing whether the system triggers host immune responses in mice or large animals.

Salk is filing a patent application on the technology.

## GOING VECTOR-FREE

An alternative strategy is to build pharmacological triggers into the design of synthetic transcription factors, avoiding the need for gene expression vectors all together.

In a December *Science* study, Wisconsin's Ansari showcased how the concept could work in Friedreich's ataxia, by reactivating RNA polymerase after it halts at the GAA repeats of the FXN gene that underly the disease. But he believes the strategy could extend to multiple sequences and diseases, in particular other cases where sequence repeats drive disease.

Ansari's team developed a synthetic transcription elongation factor that links a polyamide compound with high affinity for large numbers of GAA repeats with the tool compound JQ1, which binds the epigenetic regulator BRD4.

The conjugate recruited BRD4 to GAA repeats in the first intron of FXN; in turn, BRD4 recruited transcriptional activators that activated the paused RNA polymerase and turned on FXN expression, overcoming the repressive effects of the histone marks on GAA repeats.

Ansari noted that while free JQ1 inhibits BRD4 histone binding at high doses, the molecule does not interfere with BRD4 recruitment of transcriptional activators. Moreover, the polyamide-tethered molecule can be delivered at low doses, which don't perturb global BRD4 activity.

"We provide the molecule at concentrations low enough that the only places JQ1 lingers is on the sites where the polyamide is tethering it," he said.

In a patient-derived cell line, the molecule selectively bound the GAA sequences in the FXN promoter and restored FXN expression to normal levels. The compound did not up-regulate FXN expression in a cell line derived from the patient's healthy sibling, which had fewer than 30 GAA repeats, indicating its specificity for pathological sequences.

Transcriptomic and cell-based studies showed the compound minimally perturbed global gene expression, and selectively induced gene expression at GAA sites with paused RNA polymerases.

"The molecule goes to many places with GAA repeats, and we rank ordered them," said Ansari. "It goes to 250 things, why is it only affecting one gene? The reason is that only that gene has a polymerase that is stuck."

He added: "Even CRISPR has a broader off-target effect than that."

Ansari's group is optimizing the molecule for therapeutic use. Wisconsin Alumni Research Foundation has filed patent applications covering the synthetic transcription elongation factor technology.

Synpromics' Roberts thinks Ansari's technology is an "excellent way in which to influence endogenous regulation of the

genome," and agrees it could have applications in other diseases where transcription is paused.

Still, he said, the technology will face many of the standard limitations of gene therapy. "As with all gene-based therapies, getting sufficient delivery of the synthetic transcription elongation factors to the target cell type in order to mediate a therapeutic response would be somewhat of a challenge."

> "A distinguishing characteristic of our technology is that, in principle, size is not a limiting factor."
>
> Juan Carlos Izpisua Belmonte, Salk Institute

He believes synthetic promoters have a wider range of applications than synthetic transcription factors, but thinks there could be cases where combining the two technologies will produce synergistic effects.

Ansari said synthetic promoters are more tunable but can only be used with artificial gene sequences. In contrast, synthetic transcription factors can activate expression of both endogenous genomic sequences and exogenous gene constructs, and genetic elements beyond the transcription start site. "The power of synthetic transcription factors is they have a broader palate."

## COMPANIES AND INSTITUTIONS MENTIONED

**Broad Institute of MIT and Harvard**, Cambridge, Mass.
**Editas Medicine Inc.** (NASDAQ: EDIT), Cambridge, Mass.
**Massachusetts Institute of Technology** (MIT), Cambridge, Mass.
**Salk Institute for Biological Studies**, La Jolla, Calif.
**Senti Biosciences Inc.**, South San Francisco, Calif.
**Synpromics Ltd.**, Edinburgh, U.K.
**University College London**, London, U.K.
**University of Wisconsin-Madison**, Madison, Wis.
**Wisconsin Alumni Research Foundation**, Madison, Wis.

## TARGETS

**BRD4** - Bromodomain containing 4
**Cas9** - CRISPR-associated protein 9
**FST** - Follistatin
**FXN** (FRDA) - Frataxin
**HSF1** - Heat shock transcription factor 1
**IL-10** - Interleukin-10
**PDX1** (IPF1) - Pancreatic and duodenal homeobox 1
**RELA** (p65) - v-rel reticuloendotheliosis viral oncogene homolog A
**UTRN** - Utrophin

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