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Scalable multiplex CRISPR knockouts and a new knock-in research method to support personalized medicine discoveries

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Scalable multiplex CRISPR knockouts and a new knock-in research method to support personalized medicine discoveries

By Anand Narayanan, PhD, Yale School of Medicine and Branimir Bugarija, PhD, Commercial and Technical Manager, CRISPR, Integrated DNA Technologies »

The Silent Killer

*"I'd never really thought about blood pressure before but at a check-up, my doctor told me it was too high – at 160/90 mmHg. He prescribed beta-blockers but they made me feel like a zombie, so I stopped taking them. I was only 36," said Mike. **
"Three years later, I started feeling increasingly nauseous until it got so bad, I went to the hospital. The doctor taking my blood pressure was horrified – it was 210/120 mmHg! I'd had three brain hemorrhages (a type of stroke) because of the high blood pressure (hypertension) and because the hypertension had been long-term, my kidneys were functioning at only 60%. Nowadays, I take five types of blood pressure pills. I am slimmer and fitter, so my pills are more effective. The doctors still don't know what caused my high blood pressure, but with the help of medication, my blood pressure is back to a healthy level and my kidneys have improved, though they will never return to 100%. I was very lucky."

Introduction

Hypertension is a serious medical condition that increases the risks of heart, brain, kidney and other diseases.¹ It puts one at risk for heart disease and stroke, the leading causes of death in the US.² Worldwide, an estimated 1.28 billion people between the ages of 30 to 79 years old have hypertension, with nearly half (~46%) of them not knowing that they have it.¹ To tackle the rising prevalence of this "silent killer," (see inset, *The Silent Killer*) many nations are adopting the dual approach of prevention and treatment.³ Hypertension was previously untreatable, but tremendous progress has been in the past 50 years.⁴ Several drugs have been developed to help individuals manage their condition when a lifestyle modification proves to be insufficient or when drug therapies are needed to treat a more advanced disease.⁵⁻⁷ Research is underway to further the elucidation of the disease mechanisms driving hypertension and thereby develop precision medicines to improve the treatment of hypertension.⁸

The last 40 years of research has unveiled that hypertension is a complex disease that arises due to a variety of interacting factors.^{8,9} In rare cases, hypertension can be attributed to a single cause – e.g., a genetic mutation or neuroendocrine tumor. Most cases, however, are the result of an interplay of multiple racial/ethnic and genetic variants and pleiotropic effects,^{10,11} compounded by environmental and lifestyle factors.^{8,9} To investigate and understand this interplay in patients, scientists have developed human disease models by generating the same mutations in cell culture or animal models; by recreating the patient's variants in laboratory models, researchers can study the disease in its complexity and eventually develop a treatment. One of the latest

technologies for recapitulating human mutations in *in vitro* and *in vivo* models is the gene editing tool, CRISPR/Cas9.¹²

Efficient generation of multiple transgenic models using multiplex CRISPR/Cas9 technology

CRISPR/Cas9 has been traditionally used to edit genes at specific locations on the genome (known as loci). This one-gene knockout approach is useful for targeting genes that exists in one copy in the genome, but there are circumstances in which this approach may fall short. As one of us (AN) has worked with multiple research teams at Yale School of Medicine and led projects to

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advance the CRISPR/Cas9 gene editing technology, we thought to use our experience with non-local gene duplication events to create multiple copies of a single microRNA*** (miRNA) gene on separate chromosomes to develop hypertension model systems. Hence, to succeed in abrogating the activity of an entire miRNA family, all the copies must be mutated simultaneously. To achieve comprehensive targeting of genes in multiple loci, the team developed a "multiplexed"

CRISPR/Cas9 method, where multiple loci can be mutated simultaneously. This method has resulted in the abrogation of the activity of multiple members of the miRNA family in different loci with minimum off-targets.

Further, these mutations are heritable in the modified cell lines, and this has enabled the functional analysis of miRNA gene families in biological processes.¹³ This approach is not only more efficient than the one-at-a-time method but is also scalable; hence, we can mutate numerous loci and thus enable the rapid generation of multiple full loss-of-function models. Additionally, the approach can create gene knockouts that exist in single copy. In a recent article, we used this approach to create a gene-knockout zebrafish model, which consequently revealed an elusive downstream target that further led to the identification of a regulatory axis implicated in the developmental and disease pathways of human skeletal muscle.¹⁴

In genome editing, it is often easier to tear down than to build up

As noted in the preceding paragraphs, employing the multiplex CRISPR/Cas9 strategy allows multiple loss-of-function models to be efficiently generated. But what about CRISPR knock-ins, which are vital for generating single nucleotide variants (SNVs) in cells, especially for creating cell-based models of human diseases?

With knockouts, the CRISPR/Cas9 technology is aimed at the locus of the target gene where the Cas9 enzyme cuts the double-stranded DNA. Those cuts are, in fact, double-stranded breaks, which are then healed using the cell's main DNA-repair mechanism for joining the cut ends back together. This healing mechanism, known as non-homologous end joining (NHEJ), is the preferred mechanism of the cell because it can be activated at any time. That availability and consequent repair speed are achieved at the cost of the fidelity of the repaired DNA, meaning that the repaired DNA often incorporates extra insertions or deletions of DNA (indels) (see Figure 1).

This low-fidelity repair works well for generating loss-of-function models in CRISPR knockout experiments, because it compounds the disruption of the gene. Conversely, for gene knock-ins, this lack of fidelity presents a problem because the genes need to be precisely edited, without indels. Not only is this vital for generating SNVs; fidelity is also important for the development of most gene therapies, which require making accurate edits to human genomes.

So, how can the error-prone NHEJ repair pathway be overcome or circumvented for knock-ins? The answer is to induce the cell to use its other DNA-repair mechanism, known as ¹⁵

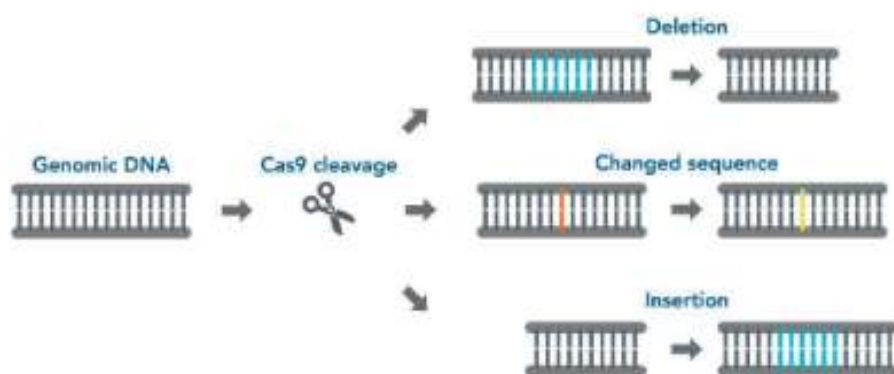


Figure 1: Possible outcomes after genomic DNA cleavage with CRISPR/Cas9
Cellular DNA repair pathways such as NHEJ can lead to deletions, changed sequences, and small insertions.²²

homology-directed repair (HDR). HDR uses a DNA template to make sure the repair is precise, without any indels (see Figure 2).²⁵ Despite having a slower repair speed than NHEJ, HDR is generally the preferred pathway for CRISPR knock-in experiments. Methods to promote HDR over NHEJ have been developed for use in knock-in experiments where precisely edited genes are required.^{15, 28}

Designing and executing projects

Being able to knock-in genes using methods such as CRISPR is increasingly important for precision medicine development. Optimizing the various interdependent elements of a CRISPR knock-in experiment, however, can be challenging. Tools that

simplify the process, from design to execution of experiments, are rapidly becoming available.

For the knock-in projects, one of us (AN) has worked extensively with Integrated DNA Technologies’ (IDT) CRISPR/Functional Genomics team on CRISPR/Cas9 based knock-ins. IDT’s Alt-R™ HDR Design Tool is a customizable design tool that allows design of guide RNA sequences. The platform also allows selection of donor DNA to target and incorporate desired modifications in the genome in multiple model organisms.

IDT offers optimized transfection and electroporation protocols for ribonucleoprotein (RNP) delivery, supplementary reagents to bias the cell machinery’s repair pathway to promote HDR over NHEJ, electroporation enhancers, and buffers

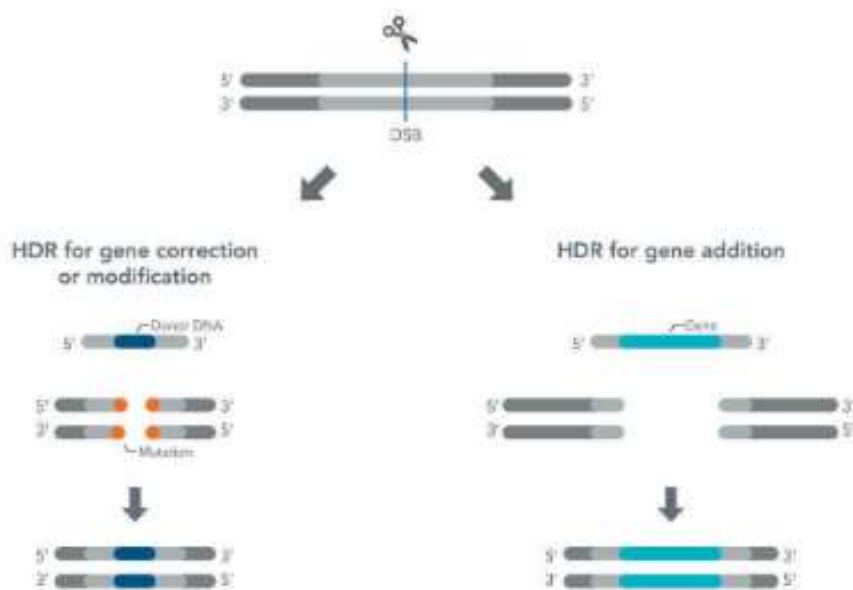


Figure 2: HDR can incorporate new sequences into genomic DNA
Small sequences can be changed for purposes of gene correction (left), and large sequences such as whole genes can be added (right).²²

(Figures 3 and 4). The design tool, reagents, and technical expertise of internal scientists, coupled with the service of IDT’s sales team, ensures continued support at all stages of CRISPR/Cas9 based knock-in projects.

Characterizing variants of unknown significance in research for informing the development of precision medicine

Much of the genetic architecture of blood pressure has been discovered using genome-wide association studies (GWAS), which involve scanning hundreds of thousands of common genetic variants across the genomes of numerous people to find the variants that are statistically associated with a disease or specific characteristic.^{29,30} Genetic variants associated (or not) with the particular disease or trait (characteristic) of interest may then be classified as pathogenic or benign, respectively.

Those that are pathogenic can be investigated further, for example, in functional studies to enhance the characterization of the gene and the role it plays in the disease process. Functional studies are often performed using cellular and animal models of disease as an important step in elucidating and validating findings before progressing to clinical evaluations. Variants that prove useful in these evaluations can go

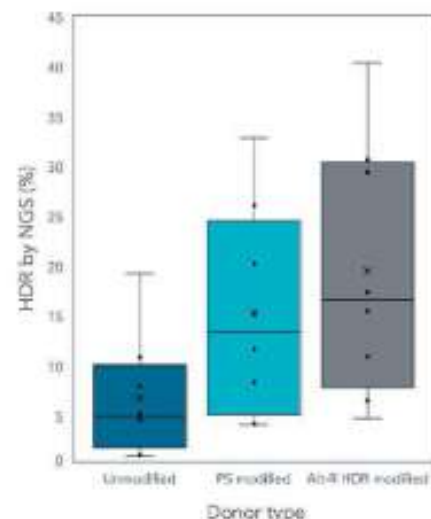


Figure 3: Alt-R HDR Donor Oligos Improve HDR efficiency

The HDR rates from experiments using 3 different donor options were compared (N=8). Donor oligos with Alt-R HDR modifiers showed increased HDR rates compared to other formats and gave the highest HDR rates. IDT’s Alt-R™ HDR Design Tool is a customizable design tool that allows design of guide RNA sequences.

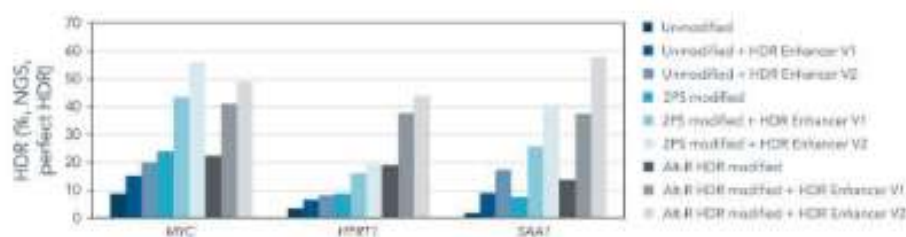


Figure 4: Alt-R HDR modified donors and Alt-R HDR Enhancer led to higher HDR rates

IDT investigated whether combining Alt-R HDR Enhancer with Alt-R modifications of HDR donor oligos would improve HDR rates further. The data demonstrated that this works well, leading to higher HDR rates. This histogram depicts the amount of HDR achieved with CRISPR ribonucleic protein complexes comprised of Alt-R S.p. HiFi Cas9 Nuclease V3 and Alt-R CRISPR-Cas9 crRNA (crRNA) and trans-activating crRNA (tracrRNA), targeting three genomic loci (MYC, HPRT1, and SAA1) along with single-stranded Alt-R HDR donor oligos delivered to HeLa cells by electroporation with Alt-R Cas9 Electroporation Enhancer using the 4D-Nucleofector™ System (Lonza). Unmodified, PS modified, or Alt-R HDR modified donor templates were used (N=1).¹⁷

on to clinical settings to inform diagnoses and/or the selection of therapies for precision medicine applications.^{89,90}

Certain variants, however, cannot be clearly classified as either pathogenic or benign. These variants of unknown or uncertain significance (VUS) pose a problem because so little is known about them. Not only does this lack of information mean that VUS cannot be used to inform clinical decisions but also that the lack can be a key bottleneck when VUS comprise a large proportion of the total number of variants identified in a research study.¹⁹ That is why the scientific community is now looking for ways to accurately classify and characterize VUS.²⁰

One crucial step in the process is the identification of the right cell line to use, as the cell line must be appropriate for observing the behavior and function of the VUS in comparison with known variants. This selection process can be applied to investigate a variety of VUS. While the overall workflow is streamlined, the steps still need to be adapted and optimized for each VUS being investigated.

By using the appropriate cell line for each variant and combining CRISPR knock-ins using IDT technology with next generation sequencing

(NGS) and single-cell sequencing, the Yale team has created research methods that offer functional insights and potential classification of VUS. These technologies may allow avenues for therapeutic intervention in precision medicine.

One research step further toward the overarching goal that is precision medicine

In collaboration with physician scientists, the Yale team is studying human VUS in three diseases: autosomal dominant tubulo-interstitial kidney disease (ADTKD), cerebral palsy, and hypertension. This research method has helped to gain insights into some VUS in terms of whether they lean toward being pathogenic or benign. The workflow is flexible and can be optimized for each VUS to achieve maximum results. In addition, it is expected that the right cell models can be created with the selection of the appropriate cell lines and assays to functionally characterize the VUS. Valuable findings in cell models would then be translated to, and validated in, animal models. This research is in progress and the team is looking to publish the results shortly. We look to the development of these innovative methods to enable new precision medicine discoveries. [\[Link\]](#)

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Footnotes

- *Not patient's real name quite adapted from true patient story.²¹
- **Pleiotropy is when a single gene influences two or more seemingly unrelated characteristics.
- ***microRNAs are a family of RNA species that are implicated in gene regulation and expression.

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Dr. Anand Narayanan

Anand is an Associate Research Scientist at Yale School of Medicine, where he focuses his research on gene editing, functional genomics, assay development, next generation sequencing, personalized medicine, and cell line engineering. He earned his PhD in molecular, cell and developmental biology from Texas A&M University, College Station, TX.



Dr. Branimir Bugarija

Branimir is a commercial and technical manager, CRISPR/Functional Genomics at Integrated DNA Technologies. He works closely with researchers to design and execute experiments, and collaborates with sales, product management, and R&D to develop new tools for improving molecular biology research. He earned his PhD in molecular biology from the University of Chicago and his Bachelor of Science in biology from University of Pittsburgh.