Choosing CRISPR-based screens in cancer

Vivien Marx

Many possibilities for parsing cancer emerge when labs combine gene editing and screens. And RNAi retains its spot in the menu of options.

Cancer cells are intensely scrutinized for vulnerabilities as labs seek genetic or epigenetic changes that propel tumor growth or drug resistance. Researchers want to understand cancer cell complexity by means that are less challenging and cheaper. Their options are expanding now that the CRISPR–Cas9 toolbox is getting "bigger and better," as Cem Kuscu and Mazhar Adli from the University of Virginia School of Medicine point out[1].

By engineering the machinery of CRISPR–Cas9 into a screening tool, scientists can modify sites by knockout at a genome-wide scale; other options include loss-of-function or gain-of-function screens that use transcriptional activation (CRISPRa), transcriptional repression (CRISPRi), base editing, directed mutagenesis, epigenetic editing, RNA interference (RNAi) or combinatorial methods[2].

An MIT–Harvard team, for example, built Combi-GEM-CRISPR for high-throughput combinations of genetic perturbations to explore, in parallel, how different gene networks or epigenetic regulators shape cancer cell phenotypes[3]. It might help make exploring drug combinations less challenging, say the method developers, who point to one screen at the National Cancer Institute (NCI). That screen took around two years, cost $4 million and involved 300,000 experiments with around 5,000 drug pairs in different combinations in each of the NCI-60 cancer cell lines, says Susan Holbeck from NCI's developmental therapeutics program.

As Martin Kampmann explains, CRISPR-based screens are a powerful way to systematically identify genes that control drug sensitivity and resistance in cancer cells. Kampmann is a researcher at the University of California at San Francisco (UCSF) and a member of the Chan Zuckerberg Biohub.

Taconic

Engineering the machinery of CRISPR–Cas9 into a screening tool presents many options.

For example, cancer cells can be drug-sensitive only when they have lost both gene A and gene B. Such synthetic-lethal genetic interactions are one type of cancer cell vulnerability that can be exploited in a targeted way. Cancers lacking certain *BRCA* genes are quite sensitive to poly(ADP-ribose) polymerase (PARP) inhibitors. Screening gives scientists data to map and quantify such complex genetic interactions, he says, and it offers hints about how to potentially preempt drug resistance.

These screens are a boon for academics and industrial drug developers because of the speedy way genetic changes and their biological consequences can be queried, says Johannes Zuber, a researcher at The Research Institute of Molecular Pathology in Vienna. RNAi has let labs suppress genes well, he says, but prior to CRISPR-based techniques it was laborious and sometimes even impossible to knock out one or many genes and get a true "null state" of a cell. CRISPR can be used to generate models that try to capture the complex mutational landscape of human tumors, and it can help to screen for therapeutic targets in new ways.

Classic CRISPR

'Classic' CRISPR–Cas9-based screens involve targeting wild-type Cas9 to genomic sites via guide RNAs (gRNAs). In the targeted genomic region, which might be a coding region, DNA damage and the ensuing repair processes lead to insertion or deletion mutations with a frame shift that can knock out the activity of the encoded protein, says Ji Luo, an NCI researcher who uses RNAi and CRISPR-based screening methods.

When cells are then exposed to one or several drugs, the surviving cells offer clues about drug-response genes. "But this approach fails whenever the phenotype of interest is more complex than just counting surviving cells," says Christoph Bock from the CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences in Vienna. When a lab wants to assess metastatic potential under certain conditions or see what happens when an important cancer pathway is upregulated, a classic CRISPR–Cas9 screen will not work. These insights might, however, be inferred from the cell's transcriptome.

False positives are another challenge with 'classic' CRISPR–Cas9-based screens in cancer research. CRISPR is an important knockout technique for work with aneuploid cancer cell lines, says Luo. But when a region in a cancer genome is amplified by, say, 100 copies, CRISPR–Cas9 will cut many or even all of the copies. This, he says, is not off-target but on-target cutting that cells might not survive.

UCSF researcher Jonathan Weissman points to the sensitivity of some cells to double-stranded cuts, such as those with the intact tumor-suppressor gene *p53* (*TP53*), which is mutated in many human cancers. A number of labs, he says, have run into issues with CRISPR-based screens in induced pluripotent stem cells, which this sensitivity might explain. In one screen, a *p53*-knockdown experiment rescued the toxicity. The result is perhaps not surprising, he says, given the role of *p53* "as a 'guard' of the genome."

CRISPRi avoids toxicity, says Kampmann, who was part of the team that developed this method, which included, among others, Weissman, Lei Qi and Wendell Lim at UCSF, and Jennifer Doudna of the University of California, Berkeley[4].

CRISPRi

CRISPRi represses transcription without introducing DNA breaks, thereby avoiding DNA-damage-associated toxicity, says Luo. With CRISPRi, enzymatically dead Cas9 (dCas9) binds to DNA. Expression is silenced when dCas9 is fused to a transcriptional repressor domain, and a customized gRNA is used to target a promoter or the transcription start site of a gene. Gene expression can also be silenced by epigenetic means by fusion of dCas9 to an enzyme that introduces repressive histone marks.

CRISPRi can be deployed for high-throughput querying of gene function genome-wide. The developers used dCas9 and the Krüppel-associated box (KRAB) domain to create fusion proteins to repress transcription in coding and noncoding genes. They note that the level of transcriptional repression that can be achieved with CRISPRi in mammalian cells will vary from gene to gene. And the number of possible gene-target sites for CRISPRi can be limited because of the protospacer-adjacent motif (PAM), the short DNA sequence that helps target dCas9 to the genomic site of interest.

The off-target effects are much reduced with CRISPRi compared to RNAi, says Kampmann. One similarity between the two methods is that gene expression might not be completely eliminated. CRISPRi can create hypermorphs instead of nulls, says Luo, which may or may not be desired in a given experiment.

UCSF

CRISPRi can avoid the toxicity often associated with CRISPR-based screens, says Martin Kampmann.

Gene repression can help cancer researchers study the "addiction" of some cancer cells to certain genes, says Zuber. They might try dialing down the expression of those genes, which can be detrimental—even deadly—to cancer cells while leaving healthy cells unscathed. Both RNAi and CRISPRi are possible tools to explore if tumor growth is halted by the tuning of cancer cell gene expression to low levels. "We haven't used CRISPRi a whole lot," he says. He wonders whether efficiencies in labs might vary because cell types have different nucleosome positioning or transcriptional start sites.

Weissman acknowledges that it has been more challenging to establish CRISPRi in some cells than in others. He says that in his lab, CRISPRi works in a wide variety of primary cells and transformed cells, and the team has been able "to get it to work in pretty much every cell we have set our mind to." He and his colleagues would like to follow up on situations in which cells express dCas9–KRAB in an intact form but do not yield good CRISPRi. "We have not seen this ourselves but it would be quite interesting to direct such a case," he says. He and his team offer users a range of tips. For example, for CRISPRi it is helpful to incorporate a ubiquitous chromatin opening frame upstream of the promoter to prevent silencing of the lentiviral dCas9 constructs.

CRISPRa

CRISPRa is gene activation by a dCas9 fusion protein linked to one or several transcription activator domains. It is, says Zuber, one screening tool that has been missing in mammalian genetics. The method offers a new way to do genome-wide gain-of-function experiments and complements loss-of-function screens, says Luo. There are several "flavors" of CRISPRa, which all can drive gene expression to "supra-physiological" levels when the complex is targeted to a gene's promoter region.

CRISPRa can help shed light on drug-resistance mechanisms, which can be due to overexpression of the drug's target, says Luo. Or genes that drive alternate pathways might be overexpressed. Drug resistance can also occur because a point mutation in the target stops a drug from binding to its target, or when the loss of a so-called pro-death gene renders the cell resistant to the drug's cell-killing effect. Labs might find it helpful to combine methods, says Luo: CRISPR–Cas9 or CRISPRi for loss-of-function approaches, CRISPRa for overexpression mechanisms, and other techniques that generate point mutations in virtually any gene.

A number of large-scale libraries for CRISPRi and CRISPRa exist and can be applied to loss- and gain-of-function genomic screens in mouse and human cells. Stanford University researcher Michael Bassik calls CRISPRi generally "super-useful for genome-wide screens," and recommends that new users look for the most updated libraries for both CRISPRa and CRISPRi. The updated CRISPRi libraries on Addgene appear to be more efficient and have more specific repression, he says.

Point mutations

Some screens can harness targeted edits at the single-base level. David Liu and his team at Harvard University built a base editor by fusing dCas9 to a cytidine deaminase[5]. The engineered fusion, when paired with a gRNA, finds cytosine in a targeted genomic region and deaminates cytosine into uracil, which then binds like thymine, thereby facilitating a C-to-T or G-to-A base substitution.

Following on this work, the team expanded the approach and, for example, built additional C-to-T base editors that can be used as modular toolsets that have a controlled editing-window width. By controlling the size of the base-editing 'spotlight' or genomic-activity window, "one can more precisely target just one C even in a stretch of multiple neighboring Cs," says Liu.

What makes the base editors fairly efficient, clean and permanent in cells, says Liu, is the inclusion of an inhibitor of cellular base-excision repair, such as a uracil glycosylase inhibitor. Without it, the C-to-U conversion would be destroyed and the results would be muddied, he says. The base conversion would mainly revert to C,

but a change to T, G or A might also occur. A certain type of Cas9 nickase activity is also required. The scientists mutated dCas9 to trick the cell's mismatch-repair machinery into fixing the G-containing strand of the C-to-G base pair. That, he says, greatly increases the base-editing efficiency.

The team tested base editing, for example, in a human breast cancer cell line with a mutation in the *p53* gene. They were able to correct this mutation in 3.3–7.6% of cells. "While we don't dwell too much on efficiency numbers for *in vitro* research studies, because they can differ greatly based on cell delivery efficiency and from target to target, we routinely observe base-editing efficiencies in the 20–70% range for cells that are transfectable," says Liu. "We have also recently achieved base editing *in vivo* in exciting, unpublished experiments."

Liu's work shows "pretty remarkable precision," says Bassik, in that it leads to a precise single-nucleotide change. Bassik, also motivated to avoid Cas9's typical and "somewhat unpredictable" insertions and deletions (indels), has taken a different approach to achieve diverse, localized point mutations. His method and another, separate, one developed by researchers at Shanghai Jiao Tong University School of Medicine apply targeted mutagenesis by using B-cell-specific activation-induced cytidine deaminase (AID) enzyme, which is involved in somatic hypermutation and leads to a diverse set of antibodies in the body[6,7]. The teams use AID in slightly different ways with approaches that, as Kuscu and Adli note, establish CRISPR–AID as an "efficient tool for saturating mutagenesis."

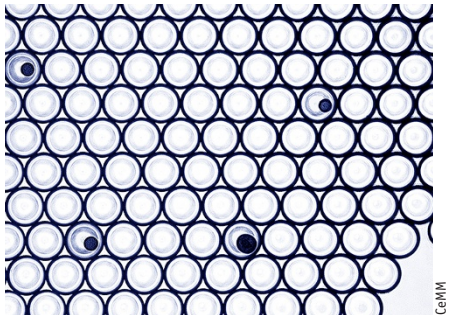
Bassik had been working independently on methods similar to Liu's but then switched to a directed mutagenesis approach with a hyperactive variant of AID and an approach he named CRISPR-X. The method mutagenizes locally without creating indels and leads to a mutation window of 100 base pairs (bp) around a given gRNA. To tile a gene, multiple gRNAs can be used, he says. The method directly mutagenizes the native, endogenous protein without the traditional modes of protein overexpression and PCR-based mutagenesis. Zuber finds both CRISPR-X and base editing promising methods as ways to generate genetic variants.

An alternative way to make mutants is to introduce a library of oligos into a site in the middle of a gene, says Bassik. But researchers need a new cut and a new library for each intended mutation, so this approach is a major undertaking. With CRISPR-X, a lab obtains many possible mutations within a 100-bp window. Gaelen Hess, a postdoctoral fellow in his lab, engineered the particular "hyperactive" AID variant, and colleagues at Stanford helped with the challenging issue of quantifying the mutation spectrum to determine how large the cell population needs to be in order to represent the desired mutation space. Among other projects, Bassik plans to use CRISPR-X to model a tumor changing as it might in the body of someone with cancer. The screen 'asks' for resistant mutations to arise within days or a few weeks, he says, and sequencing can reveal the mutations more fully.

Single-cell approaches

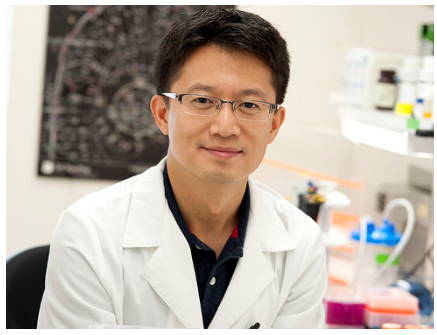
CRISPR-droplet sequencing, or CROP-seq, involves pooled CRISPR screens and a single-cell readout based on RNA sequencing. The method can be matched up well with base-editing techniques, says CROP-seq developer Bock, such as for discerning somatic mutations driving a cancer from passenger mutations. With base editing, researchers could introduce a large number of mutations into a pool of cells, and then use CROP-seq to connect transcriptome signatures to the gRNAs controlling the base editing. A lab could then computationally annotate each cell and each gRNA or target gene according to the observed phenotypic effect, such as a proliferation or drug resistance. It's an "ultra-scalable way of experimentally assessing the functional impact of cancer mutations," he says.

When presenting the method, Bock used CRISPR libraries with over 100 gRNAs, but that number is not the limit, he says. gRNA libraries for more than 1,000 genes are both feasible and increasingly affordable. The cost of single-cell RNA-seq is the main limitation. He plans to use the approach to parse epigenetic heterogeneity in cancer, which might help to explain why childhood cancers and leukemia differ in their clinical course despite little genetic heterogeneity in the cancer genomes.



CeMM

CROP-seq pooled CRISPR screens with a single-cell RNA-seq readout. Here, droplets containing beads and lysed cells are ready for single-cell sequencing.



NCI

Drug resistance can have many causes. Labs can combine methods, says Ji Luo.

Cancer geneticists tend to question the notion that epigenetic changes can drive cancer, says Bock. With a new grant, he wants to combine CROP-seq and CRISPR-based epigenome programming in leukemia to assess the functional consequences of targeted epigenetic alterations in high throughput. Another experiment he envisions is to erase a cancer cell's epigenetic alterations such that it loses its malignancy. The approach could offer hints about exploitable "epigenetic vulnerabilities" in cancer cells.

Weissman and his lab are also exploring targeted epigenetic and reprogramming approaches. They want to develop tools to be used on any locus and be able to control the timing and magnitude of the alterations. The epigenetic controls will be inducible, for example, with light or drugs.

In vivo

An *in vivo* screen has many strengths compared to an *in vitro* screen, says Luo, but such screens are technically challenging. "But hard technical problems can be overcome with better technologies," he says. For example, the creation of Cas9 mice could facilitate the development of more sophisticated *in vivo* screens, says Luo. These animals express Cas9 protein in the germline.

For now, labs tend to explore cancer biology questions by taking their favorite targets from *in vitro* screens and testing them *in vivo*, says Zuber. A genome-wide screen involving a library of 100,000 gRNAs would require millions of transplanted cells to form a tumor *in vivo*. It is more realistic and promising, he says, to use smaller

libraries to, for example, look at hundreds of candidates.

Working with results from *in vitro* screens with *in vivo* models is daily routine for John Couse, a former NIH researcher who directs Taconic's design and development of genetically engineered mouse models. He uses CRISPR–Cas9, RNAi and traditional mutagenesis approaches. Some of his clients are academics, but mainly it is commercial drug developers who come to him with targets of interest from in-house *in vitro* screens. In cancer research, these might be several or as many as 40 genes that a client seeks to study in a genetically engineered model, says Couse, to look at signaling pathways, tumor suppressor genes or oncogenes.

"It's hard to study tumor biology *in vitro*," says Couse. But *in vivo* work is neither high-throughput nor as low-cost as work in cells. "It takes six months to a year to generate an animal," he says. Taconic is exploring how one might begin *in vivo* modeling before *in vitro* screens are completed. "It requires some shared risk on our part," he says, and it moves his company into the role of research partner rather than traditional contract research organization. He and his team keep tabs on emerging techniques such as CRISPRa, CRISPRi and base editing; to date they have not applied them to *in vivo* models in his projects. He believes the methods will perhaps become routine. His team uses RNAi, but it is not as popular as it once was. The company has generated around 150 models with RNAi for drug discovery clients; he does not disclose the number of CRISPR-based animal models.

Taconic

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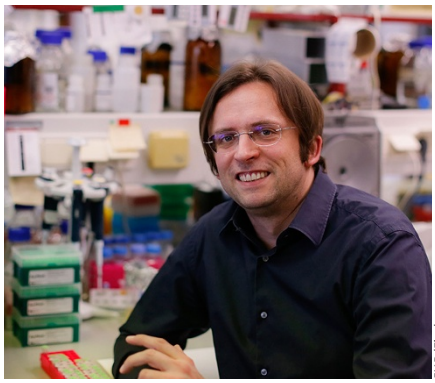
If money were no object, Taconic clients would probably ask for a spectrum of models around the genes of interest, so they could see what happens when a gene is always off, sometimes off, off at particular times in development. It has not been possible to study the interaction of non-coding RNAs *in vivo*, but CRISPR brings that possibility closer, too, says Couse. For now CRISPR is used in "relatively conventional approaches" to manipulate the genome, especially around point mutations. Many cancers are directly related to point mutations in oncogenes and tumor suppressor genes, he says.

His projects might involve the generation of a mouse lacking two genes, with the option of conditionally repressing or activating a third gene to observe a genetic interaction. "That's where we can really start to consult," says Couse. He advises clients on strategy selection. A client might wish to halt the expression of one or several genes in all tissues or in just one. A gene might also be knocked out when the animal enters a certain developmental stage or is exposed to a drug. All of these models are geared toward studying which genes play a role in establishing a cancer or are crucial to disease progression.

When CRISPR is applied *in vivo*, there are three main ways to handle off-target effects, says Couse: good gRNA design; monitoring and sequencing potential sites for off-target effects; breeding out off-targets. This applies only to mice, he notes; Taconic does no human gene-editing projects.

So far, RNAi has not worked as well as hoped *in vivo*, and with CRISPR, *in vivo* experiments are just emerging. "In the models we're still kind of stuck with genes that are on or they're off or they're changed 100%," says Couse. "And that's not how biology works." With *in vivo* RNAi, the animal must be treated with a drug to induce the interference, which brings in the confounding factor of drug pharmacology. Any *in vivo* tools have their limits, he says, which scientists should factor in.

CRISPR-based techniques continue to accelerate cancer research, says Zuber. Almost every six months major advances occur, and researchers can home in on cellular pathways and genes of interest with increasing precision. CRISPR-based engineering will change the way mouse models can be built for human cancer research, he says, and avoid issues that make xenografts less than ideal for modeling tumors.



IMP/Fischer

CRISPR and RNAi-based screens can help researchers study the complex mutational landscape of human tumors, says Johannes Zuber.

Generally speaking, pharmaceutical researchers screen in cell lines, but comprehensive genetic validation after that step has been challenging, says Zuber. New tools are changing this, which might alter the approximately 90% failure rate of cancer drug candidates. With CRISPR- and RNAi-based techniques labs might more readily identify and validate new candidate targets in much greater depth, he says, and generate animal models that better reflect the genetic complexity of human tumors.

Prior to his research career, Zuber was an oncologist for five years. He wants research advances to help people with cancer. That is a responsibility for academic researchers, especially now that they have tools in hand that let them gain "genetic confidence" in targets. These advances are not just a responsibility for pharma, he says; "after all, academic research is paid by society as well."

Vivien Marx is technology editor for *Nature Methods* (v.marx@us.nature.com).

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