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CRISPR-Cas9 knockout screening for functional genomics

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Large-scale genetic screening offers a critical tool to dissect gene function and pathways underlying biological processes and human disease. Despite the success of genome-wide genetic screens in microorganisms such as yeast, the creation of genome-scale homozygous mutant libraries for metazoans with diploid genomes is time and cost consuming and low throughput. RNA interference (RNAi) has been a predominant solution for functional genomics during the past years since a double strand RNA can inactivate gene function by causing sequence-specific degradation of mRNA. However, the RNAi approach has intrinsic limitations because the suppression of target gene expression at the posttranscriptional level is often insufficient to recapitulate the knockout phenotype and it can have confounding off-target effects to other mRNA [1]. An efficient, high-throughput and loss-of-function technique is still highly demanding across species. Dr. Wei WenSheng's laboratory from the Peking University reported a large-scale knockout screen using a CRISPR-Cas9 library in human cells [2], and their work along with three other recent studies provides the hallmark for a new era of functional genomics [3−5] (Table 1).

The clustered regularly interspaced short palindromic repeat (CRISPR)-Cas9 system derived from *Streptococcus pyogenes* has emerged as a powerful tool to introduce double-strand breaks (DSBs) at the target genomic loci in a variety of model organisms [1]. This two-component scheme consists of a single-guide RNA (sgRNA) and a DNA endonuclease Cas9 protein. Binding of sgRNA to the target sequence via Watson-Crick base pairing dictates Cas9 to the specific region in the genome for DNA cleavage. Distinct from other programmable endonucleases, such as zinc-finger nucleases and transcription activator-like effector nucleases (TALENs), the targeting specificity of CRISPR-Cas9 is conferred by a 20-base pair (bp) sequence of the sgRNA, offering a greater ease to construct knockout reagents at the genome scale.

Dr Wei's group designed a focused CRISPR-Cas9-based lentiviral library for large-scale knockout screens in human cells and identified the host genes essential for the intoxication of cells by anthrax and diphtheria toxins. Wei and colleagues first constructed cell lines with a constitutive expression of Cas9 endonuclease and OCT1, which was reported to enhance U6 promoter activity to express sgRNA. After selecting the cell clones with the highest efficiency of generating indel mutations, sgRNAs were delivered into these cell lines via lentiviral infection. Library screening was then performed by toxin treatment for three rounds and the positive hits were identified by PCR and high-throughput sequencing analysis. The toxin resistant screens respectively uncovered 19 candidate genes for diphtheria and chimaeric anthrax resistance and 15 genes for diphtheria toxin tolerance from a total of 291 genes. Among these candidate genes, the anthrax toxin receptor and the diphtheria toxin receptor are top-ranking in the list of the positive hits, and they further validated other candidates by functional analysis.

Three parallel genome-wide CRISPR-Cas9 knockout screens have been recently reported in mouse or human cell lines [3−5] (Table 1): Yusa lab screened mouse embryonic stem cell lines with a lentiviral library for resistance to *Clostridium* septicum alpha-toxin or 6-thioguanine; Lander

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Table 1 Summary of functional genomic screens using the CRISPR-Cas9 system

Screen aims	Cell lines	Cas9	sgRNA library	Target genes	Results	Ref
Resistance to alpha-toxin or 6-thioguanine	ESCs	Constitutive expression; integrants	87897	19150	27 known & 4 novel hits	[3]
Resistance to 6-thioguanine or etoposide; cell proliferation	KBM7 HL60	Constant or Inducible; integrants	73151	7114	Many	[4]
Cell Viability; resistance to vemurafenib	HUES62 A375	lentiviral vector	64751	18080	Many; 2 known & 4 novel hits	[5]
Resistance to anthrax or diphtheria toxins	HeLa	Constitutive expression; integrants	869	291	19 or 15 hits	[2]

lab performed resistant screens for 6-thioguanine or for the DNA topoisomerase II poison etoposide as well as a negative selection screen for essential genes; and Zhang lab screened for genes essential for cell viability or for the resistance to vemurafenib, a therapeutic RAF inhibitor. All of these screens have successfully identified a few interesting candidate genes, which pave new avenues for understanding the corresponding biomedical processes. Although the scale of Wei's screen is smaller than these three screens, it yielded the higher positive hit ratio than others because the screen was based on a focused library, particularly beneficial for knowledge-based studies.

Off-target mutations have been considered as a major technique caveat to the broad application of the CRISPR-Cas9 system. However, the true and false-positive hits from knockout screens should be easily discriminated by the subsequent confirmatory analysis. Nevertheless, off-target effects were carefully assessed by extensive deep sequencing in these studies; and their results demonstrated that off-target cleavages were either located in the non-coding regions or generated limited loss-of-function mutations.

The CRISPR-Cas9 system offers a cost-effective, highly versatile and scalable approach to produce knockouts for functional genomic studies in mammalian cells. We expect that genome-wide screens using the CRISPR-Cas9 library will be widely applied to empower the discovery of gene functions in numerous biological processes across species. In addition to the loss-of-function screens for understanding the coding sequence, the CRISPR-Cas9 can target regulatory elements such as promoters and enhancers in the entire genome, facilitating functional analysis of these components in the genome. Taken together, the CRISPR-Cas9 knockout screens hold great promise for function genomics in the coming decade.

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