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REVIEW ARTICLE

CRISPR/Cas9 library screening for drug target discovery

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Abstract

CRISPR/Cas9-based tools have rapidly developed in recent years. These include CRISPR-based gene activation (CRISPRa) or inhibition (CRISPRi), for which there are libraries. CRISPR libraries for loss of function have been widely used to identify new biological mechanisms, such as drug resistance and cell survival signals. CRISPRa is highly useful in screening for gain of functions, and CRISPRi is a more powerful tool than RNA interference (RNAi) libraries in screening for loss of functions. Positive selection using a CRISPR library can detect survival cells with specific conditions, such as drug treatment, and it can easily clarify drug resistance mechanisms. Negative selection is capable of detecting dead or slow-growing cells efficiently, and it can identify survival-essential genes, which can be promising candidates for molecularly targeted drugs. In addition, negative selection can be applied for synthetic lethality interactions, where the perturbation of both genes simultaneously results in the loss of viability, but that of either gene alone does not affect viability. This mechanism is highly important to identifying the optimal combination of molecularly targeted drugs. Survival-co-essential genes in cancer cells can be identified using new methods, such as the paired guide RNA system and in combination with single-cell RNA sequencing techniques. These efficient methods can clarify interesting biological mechanisms and suggest candidates for molecularly targeted drugs. This review identifies what types of screenings were performed and suggests ideas for the next CRISPR screenings to develop new drugs.

Introduction

The clustered regularly interspaced short palindrome repeats (CRISPR)/Cas9 system is a new gene-editing technology that can induce double-strand breaks (DSBs), single-strand nicks, or anywhere guide ribonucleic acids (RNAs) can bind with the protospacer adjacent motif sequence. DSBs can induce cellular DNA repair, which then results in the mutations of targeted genes with non-homologous end joining or homology-directed repairs [1, 2].

Furthermore, adaptive applicants were invented with catalytically inactive Cas9 (dCas9) and other regulator co-factors, such as VP64 and KRAB. CRISPR-dCas9-VP64 (CRISPRa) can activate or increase gene expressions with transcription start site (TSS)-specific guide RNAs [3]. For the downregulation of targeted genes, CRISPR-dCas9-KRAB (CRISPRi) can interfere with or silence a transcription [4, 5]. Additional efficient modifications were developed to accelerate CRISPRa activity with other additional machineries, such as VPR, SAM (MS2/p65/HSF1), SunTag, and p300 [6–9]. The VPR, SAM, and SunTag systems were consistently superior to the previous VP64 standard in the activation of targeted genes [10].

Screenings with random mutagenesis are highly useful and widely used to identify specific genes to induce cell growth or death and to clarify the mechanisms of specific signaling pathways. Retroviral random mutagenesis was widely used to identify positive regulators, such as proto-oncogenes with random insertions of retroviruses and the activation of nearby genes through long-terminal repeat [11]. Retrovirus may not be integrated into and may not

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Table 1 Human CRISPR library

Name of library	Selection type	Screening method	Cell type	Suggested responsible genes	Ref.
Human GeCKO Knockout Pooled Library (GeCKO v1/GeCKO v2)	Positive	Vemurafenib	A375	*NF1, MED12, NF2, CUL3, TADA1, TADA2B*	[20, 38]
	Negative	Survival essential		Ribosomal structural	
	Positive	Ara-C	U937, MOLM13	*DCK, SLC29A*	[22]
	Positive	Hypoxia	K562	*VHL*	[39]
	Positive	Dengue virus Hepatitis C virus	Huh7.5.1, HAP1	*STT3A, STT3B* *RFK. FLAD1*	[40]
Human CRISPR Knockout Pooled Libraries Enriched sub-pools (kinase, nuclear, ribosomal, cell cycle)	Positive	6-TG	HL-60, KBM7	*MSH2, MSH6, MLH1, PMS2*	[20]
	Negative	Etoposide, Survival essential		*TOP2A, CDK6* Ribosomal, *BCR-ABL*	
Human Activity-Optimized CRISPR Knockout Library (LentiCRISPR v1/v2)		Survival essential	KBM7 (K562, Jiyoye, Raji)	RNA processing, Nuclear localization	[26]
	Negative	Synthetic lethal interaction with oncogenic RAS	NB4, PL-21, OCI-AML3, P31/FUJ, HEL, THP-1, EOL-1, MOLM13, MonoMac1, MV4;11, OCI-AML2, SKM-1, TF-1, OCI-AML5	*RCE1, ICMT, RAF1, SHOC2, PREX1*	[28]
Human CRISPR Knockout Pooled Library (Avana/Brunello)	Positive	Vemurafenib	A375	*NF1, MED12, NF2, CUL3, TADA1, TADA2B, ARIH2, KIRREL, MED23, PDCD10, PTEN, SUPT20H, TAF6L, TP53, UBE2F*	[41]
		6-TG	A375, HT29, HEK293T	*HPRT, NUDT5*	
	Negative Positive	Survival essential Type III secretion system-mediated cytotoxicity	HT29 HT29	161 genes Sulfation, SWI/SNF, WAVE complex, Collagen	[42]
Human Improved Genome-wide Knockout CRISPR Library	Negative	Oncogene related and "druggable" survival essential	MOLM13, HL-60, MV4-11, OCI-AML3, OCI-AML2	*KAT2A, SRPK1, CHEK1*	[25]
Toronto Knockout (TKO) CRISPR Library	Negative	Core essential or context dependent	GBM, RPE1, A375, HCT116, HeLa	mRNA splicing, protein folding or Oncogene-related genes	[24]
CombiGEM-CRISPR	Negative	Survival essential (paired)l	OVCAR8-ADR	*BRD4+KDM4C*, *BRD4+KDM6B*	[31]
CRISPR-base double knockout library (CDKO)	Negative	Survival essential (paired)	K562	*ATM+APEX1, MCL1+BCL2L1*	[32]
Human paired-guide RNA (pgRNA) Library for long non-coding RNAs (lncRNAs)	Positive Negative	Survival essential (paired)	Huh7.5	*LINC00176, LINC01087, LINC00882, LINC00883, AC004463.6, AC095067.1, HM13-AS1, RP11-128M1.1, RP11-439K3.1*	[33]
High-throughput screening of a CRISPR/Cas9 library	Positive	Anthrax toxin Diphtheria toxin	HeLa	*ANTXR1* *HBEGF*	[43]
Human genome-wide library v1	Positive	Nile Virus	HEK293T	*EMC2, EMC3, SEL1L, DERL2, UBE2G2, UBE2J1, HRD1*	[44]
Genome-wide library	Positive	Poliovirus	Cervix; HeLa Muscle; RD	*ST3GAL4*, *MGAT5*, *COG1*, *COG5*	[45]
Two plasmid human activity-optimized genome-wide library	Positive	HIV	Leukemia; CCRF-CEM	*CD4*, *CCR5*, *TPST2*, *ALCAM*	[46]

destroy both alleles of negative regulators, such as tumor suppressor genes (TSGs). This leads to the seldom identification of negative regulators using this method. Transposon mutagenesis is a much stronger mutation inducer and it is widely used to identify proto-oncogenes and TSGs, because the number of integrated transposons is much larger than that of retrovirus insertional mutagenesis. In addition, gene expressions can be terminated easily with splice

acceptors [12]. However, those two methods require many steps to identify the integration site and the factor responsible for screening phenotypes.

RNAi is a widely used and well-established method to downregulate specific target genes, and the short hairpin RNA (shRNA) library is a more sophisticated method to identify a candidate gene for loss-of-function screenings, although it cannot activate any gene expressions directly. Responsible targeted shRNA can be easily identified, especially when a barcode is encoded. In addition, shRNA libraries have a powerful screening method to identify candidates in a many fields of screenings [13–16].

Molecularly targeted drugs have been widely used for many kinds of cancers, including imatinib for *BCR-ABL* fusion in chronic myelogenous leukemia and some kinds of acute leukemia, vemurafenib for B-Raf in malignant melanoma, olaparib for poly-ADP ribose polymerase in BRCA-mutated ovarian cancers, and so on. However, not all cancers are curable, and even molecularly targeted drugs may have a problem with drug resistance. To overcome all kinds of cancers, the identification of molecular targets is important, as well as clarifying the mechanisms of drug resistance. Therefore, screenings with random mutagenesis, such as retroviruses, transposons, and shRNA libraries, have been applied to find new drug-targetable genes [17–19] and recently, CRISPR libraries were applied to screenings with genome-wide loss- or gain-of-functions. This review updated what kinds of screenings were conducted, which is helpful in designing new strategies in the next step.

CRISPR libraries for identifying drug targets

CRISPR libraries were first reported in 2013 by two different groups in *Science*, and they exhibited more efficient screening steps than those involved in shRNA libraries according to Shalem et al. [20] and Wang et al. [21] The former group used 64,800 gRNAs for 18,080 genes and the latter used 73,000 gRNAs composed from six categorized groups, such as “ribosomal protein,” “kinase,” “cell cycle,” “nuclear,” and others. Lately, at least 13 libraries for CRISPR knockout for humans are available (Table 1), and they are applied to not only drug screenings but also viral infection resistance. Three libraries for CRISPRa in humans and two for CRISPRi have also been established (Table 2). These libraries can easily induct human cells and they are applicable to clinical research. On the other hand, seven libraries for loss of function with a murine CRISPR library, two for murine CRISPRa, and one for CRISPRi are also available (Table 3). The murine CRISPR library has a great advantage *in vivo* model that could be applied to carcinogenesis or metastasis. The combination of a human model and a mouse model can bring new insights to eradicate cancers.

Mainly, there are two ways to use libraries to screen for target genes. One is positive selection, a conventional selection method that can collect growth-advantage-acquired populations by random mutagenesis. For example, cells can be exposed to an anti-cancer drug after random mutagenesis with a CRISPR library and then the drug-resistant population will be harvested for analyzing the sequence of gRNAs (Fig. 1a). By collecting genomic DNA and amplified guide RNAs or a barcode-containing region for sequencing, enriched guide RNAs will be identified as candidate genes for drug resistance.

Another method is negative/dropout screening, which can identify essential genes for survival under specific conditions. If one cell is carrying one guide RNA that is targeting a survival-essential gene, a knockout cell cannot grow. Induction of a pool of gRNA can make a pool of random mutants. After several optimal passages, a pool of guide RNAs of only survived cells will be harvested. By sequencing a pool of guide RNAs between initial status and survival status using next-generation sequencing, survival-essential candidate genes will be identified (Fig. 1b).

To obtain optimal screening candidates, maintaining the diversity of guide RNAs is important. To do so, lentiviral infection efficiency is also critical. Essentially, there are two systems of a CRSIPR/Cas9 library. One is an all-in-one plasmid system that includes guide RNAs and Cas9 with optimal promoters and selection markers (Fig. 1c). It has the advantage in induction of both guide RNAs and Cas9 in one cell at once. However, infection efficiency is lower because of the huge size of the all-in-one plasmid system, and this leads to the disadvantage of maintaining diversity. The second system is a two-plasmid system that has an advantage in infection efficiency, because plasmid contains only guide RNAs with optimal promoters. Once Cas9-expressing cells are established, it is relatively easy to maintain diversity (Fig. 1d). However, two transduction steps for Cas9 are needed, as well as concern for Cas9 activity. Two-plasmid systems can be easily modified. If a tetracycline-inducible Cas9 vector is used, condition-specific random knockout is possible.

CRISPR screening for drug resistance mechanisms

The reason cancer can be fatal is uncontrollable growth with drug resistance. To overcome drug resistance, a clarification of drug resistance mechanisms is important. The creation of artificial drug resistance cells was a widely used method to

Table 2 Human CRISPR library for activation and inhibition

Name of library	Type of CRISPR	Selection type	Screening method	Cell type	Suggested responsible genes	Ref.
Human CRISPR Activation Library (SAM library)	Activation	Positive	Vemurafenib	A375	*EGFR, PCDH7, ITGB5, ARHGFE1, BCAR3, GPR35, TFAP2C*	[7]
Human Genome-wide CRISPRa Libraries (v1/v2)	Activation	Negative	Survival essential	K562	Tumor suppressor, develop and differentiation	[23]
Human Genome-wide CRISPRi Libraries (v1/v2)	Inhibition	Positive	Ricin toxin		*SEC23B*, etc.	[29]
Human CRISPRi Non-coding Libraries (CRiNCL)	Inhibition	Negative	Survival essential	K562, U87, HeLa, HEK293T, MCF7, MDA-MB-231, iPS	499 loci	[33]
Human CRISPR lincRNA Activation Pooled Library	Activation	N/A	N/A	N/A	N/A	Unpublished

Table 3 Murine CRISPR library

Name of library	Type of CRISPR	Selection type	Cell type	Screening method	Suggested responsible genes	Ref.
Genome-wide Mouse Lentiviral CRISPR gRNA Library (Mouse Improved Genome-wide Knockout CRISPR Library v2)	Loss of function	Positive	Mouse ESC	6-TG Clostridium septicum alpha-toxin	MMR genes and *Hprt* GPI-anchor biosynthesis pathway	[47]
Broad GPP genome-wide (Asiago/Brie)	Loss of function	Positive	BV2	Interferon-gamma Norovirus	*Jak1, Stat1, Ifngr1, Ifngr2* *Cd300fl*	[41] [48]
Mouse CRISPR Knockout Pooled Library (GeCKO v2)	Loss of function Loss of function	N/A Positive	N/A NSCLC	N/A Metastasis *in vivo*	N/A *Nf2, Pten, Cdkn2a, Trim72, miR-345, miR-152*, etc.	[38] [49]
Two plasmid mouse activity-optimized genome-wide library	Loss of function	Negative	RAS-induced BaF3	Signal restriction	*Prex1*	[28]
Mouse CRISPR Activation Library	Activation	N/A	N/A	N/A	N/A	[38]
Mouse Genome-wide CRISPRa-v2 Libraries	Activation	N/A	N/A	N/A	N/A	[29]
Mouse Genome-wide CRISPRi-v2 Libraries	Inhibition	N/A	N/A	N/A	N/A	

clarify the mechanisms of drug resistance. Random mutations were widely used and CRISPR library easily apply to this type of screenings.

First, screening for loss of function with a CRISPR library was applied to explore the drug resistance mechanism of the BRAF protein kinase inhibitor, vemurafenib (PLX), in the melanoma cell line, A549. In this screening, *NF1*, *MED12, NF2*, *CUL3, TADA1*, and *TADA2B* were identified as PLX-resistant candidates. *NF1* and *MED12* were already reported, but interestingly, *NF2*, *CUL3, TADA1*, and *TADA2B* were not, suggesting a new drug resistance mechanism [20]. On the other hand, conventional anti-cancer drugs, such as 6-thioguianine (6-TG), etoposide, and cytarabine (Ara-C), were also tested. A DNA mismatch repair pathway (*MSH2*, *MSH6*, *MLH1*, and *PMS2*) was identified as a key molecule in 6-TG resistance, as previously reported [21]. *TOP2A* and *CDK6* were also identified in etoposide resistance screening. Etoposide poisons DNA topoisomerase IIA, and this identification of *TOP2A* is quite reasonable. However, the relationship between *CDK6* and etoposide is not well known and it might be helpful in understanding new drug resistance mechanisms in etoposide [21].

Cytarabine is widely used to treat cancers, such as leukemia. To clarify a new drug resistance mechanism of this conventional drug, a CRISPR library was applied to leukemia cell lines. At first, deoxycytidine kinase (DCK) was identified, as previously reported. To avoid the effects of gRNA on DCK, CRISPR library-resistant DCK with a silent mutation was induced in the targeted cell lines. After,

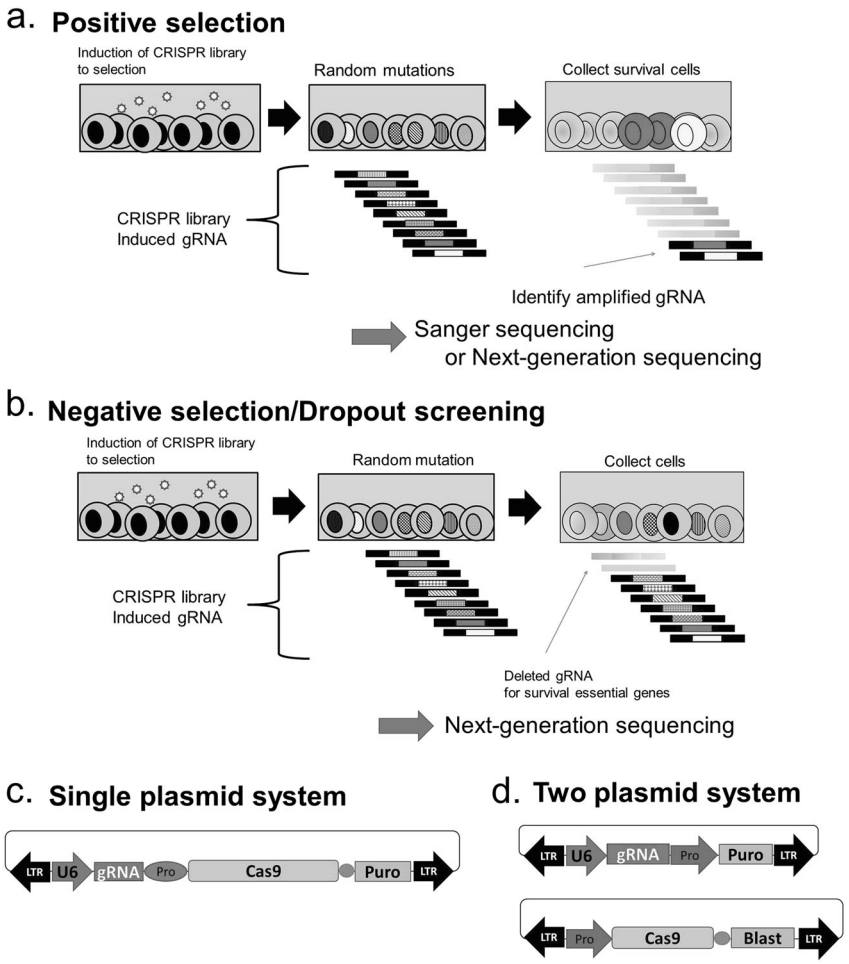

Fig. 1 CRISPR/Cas9 screenings. **a** Positive selection; collection of survival cells to reveal drug-resistant genes. **b** Negative selection; collection of survival cells and comparison a pool of gRNAs between initial state and survival state. **c** Single all-in-one plasmid system for CRISPR/Cas9. **d** Dual plasmid system (Pro promoter elements, Puro puromycin-resistant gene, Blast Blasticidin-resistant gene, "dot" means pT2 sequence)

the effect of gRNA on DCK was canceled, and SLC29A was identified as a second candidate, but it is also well known as a transporter of cytarabine into the cytoplasma [22]. Widely used drugs were well studied and strong factor like DCK could mask additional other factors.

CRISPR activation screenings of melanoma with positive selection by PLX were also performed. *EGFR*, *PCDH7*, *ITGB5*, *ARHGFE1*, *BCAR3*, *GPR35*, and *TFAP2C* were identified as PLX-resistant genes. The activation of those molecules might relate to the ERK pathway, resulting in PLX resistance. These candidates also suggested a new insight into a PLX-resistant pathway [8].

CRISPR inhibition overlaps potentially with RNA interference screenings and in fact, there were similar tendencies, but efficiency was higher [23].

CRISPR screening for cancer-essential genes to identify drug targets

CIRSPR library screening is capable of accurate negative selections. This screening can reveal what genes are essential for survival under specific conditions, such as cell type and oncogene status. At first, only genes essential for common survival were identified, such as ribosomal structural constituents [20]. The BCR-ABL of oncogenic fusion gene was also identified as a survival-essential gene in KBM7 cells that possess BCR-ABL [21]. This result suggests that negative selection is promising to identify a molecularly targeted drug. A comparison of different tissue types, such as brain (GBM), retina (RPE1), colon (HCT116), melanocyte (A375), and cervix (HeLa), revealed common essential genes, as well as mRNA splicing and protein folding in all kinds of tissues. Furthermore, cell type and activated signaling-specific survival-essential genes were also identified [24].

In an acute leukemic cell, specific survival genes were explored among MOLM-13, HL-60, MV-40, OCI-AML3, and OCI-AML2. In total, 492 survival-essential genes in acute myeloid leukemia (AML) were identified and 227 genes were druggable. Among them, the histone acetyltransferase gene (*KAT2A*) and spliceosome kinase gene (*SRPK1*) were considered attractive druggable targets under the leukemic state with MLL-AF9 translocation. This fusion

drives MOLM-13 cells. Indeed, a KAT2A inhibitor, MB-3, was strongly effective in MLOM-13 survival, and interestingly, this inhibitor is also effective for other primary AML cells with leading apoptosis [25].

To ensure the efficiency of screenings, a combination of multiple gene identification methods, such as retroviral random mutation with a splice acceptor, was more reliable. To identify cell survival-essential genes with this method, 330 unknown function genes from 1870 genes were identified. Those genes might involve RNA processing and nuclear localization. The pattern of a survival gene can reflect the cell type, and this result in the same types of cells using a similar signaling pathway for survival [26].

Some cancers already have a well-known oncogene, such as the RAS oncogene, a strong inducer of cell proliferation; however, no scientist has succeeded in creating RAS inhibitors because of the nature of RAS [27]. To overcome this problem, the identification of essential genes that target RAS synthetic lethal interactions is important. That is the perturbation of both genes simultaneously results in the loss of viability, but that of either gene alone does not affect viability. Therefore, CRISPR libraries were applied to six cell lines with a RAS mutation and six other wild-type cell lines with negative selection. Eventually, *RCE1* and *ICMT* were identified, and these two molecular genes were involved in an RAS synthetic lethal interaction. *RAF1*, encoding c-Raf, and *SHOC2* were also listed in this screening, and they are related to the MAPK pathway under the RAS signaling downstream. *PREX1* was also identified as a key survival-essential gene in all six cell lines. Interestingly, *PREX1* was able to activate MAPK pathway only in mutant RAS-driven AML cells [28].

CRISPR activation with negative selection was also performed for survival-essential genes in K562 cells. Interestingly, potential TSGs were identified, such as *TP73* of the p53-related protein. In addition, *CDKN1C (p57)* and *CDKN1A* (p21) of cell cycle inhibitors, *BAK1* and *BCL2L11* (BIM) of apoptosis factors, *ARID1A* of chromatin remodeling factors, CCAAT/enhancer-binding protein (*CEBP*), homeobox genes, Forkhead box genes, the Ikaros family of zinc-finger proteins, and the hematopoietic differentiation factor *SPL1* (PU.1) were also identified. These results were reasonable to explain how those molecules would prevent cell growth [23]. Horlbeck et al. [29] improved this library with minimal false positives and a non-specific toxicity.

CRISPR inhibition can knockdown targeted genes as shRNA. The screening of loss of function of long non-coding RNAs (lncRNAs) by CRISPR is difficult because in/del mutations cannot affect function without transcription. A new CRISPRi library for 16,401 loci of lncRNAs was tested with negative selection, and 499 lncRNA loci as survival-essential genes were identified [30].

Modified CRISPR screens: co-essentiality and non-coding RNAs

CRISPR screenings with negative selection can develop molecularly targeted drugs; however, not all survival pathways could be identified by this method. There might be a redundant pathway for cell survival, and the knockout of two or more genes would be needed for identification in this case.

To identify the combination of molecules, a two-wise barcoded CRISPR-Cas9 library in a single plasmid system was applied to the ovarian cell line, OVCAR8-ADR. Combinations of "*BRD4* + *KDM4C*" and "*BRD4* + *KDM6B*" were identified as survival combinational-essential genes [31]. Furthermore, Han et al. used a "CRISPR double knockout" system to find synergistic drug combinations. In total, 700 gRNA for about 207 druggable genes were combined to make 490,000 combinations. Finally, the combination "ATM inhibitor + APEX1 inhibitor" or "MCL1 inhibitor + BCL2L1 inhibitor" was validated [32]. In theory, the combination of molecularly targeted drugs could be more effective.

Long non-coding RNAs are difficult to knock out, as previously mentioned, so a paired-guide RNA library was applied to find growth-advantage genes and survival-essential genes. *LINC00176, LINC01087, LINC00882*, and *LINC00883* were identified in positive selection and *AC004463.6, AC095067.1, HM13-AS1, RP11-128M1.1*, and *RP11-439K3.1* were identified in negative selections [33]. Hopefully, non-coding RNA can also be a target of drugs in the future.

CRISPR screening with single-cell RNA sequencing technology

Single-cell genomics technologies are capable of characterizing new cell types and states, transitions from normal to disease, and response therapies in exactly a single cell [34]. An integrated method for a pooled CRISPR/Cas9 library, followed by massively parallel single-cell RNA-seq, can profile the perturbation and transcriptome of all targeted genes in the exact same cell. In addition, with conventional CRISPR screenings, M.O.I. should be adjusted to less than one to control the number of copies of guide RNA in a single cell. This means only a single loss of function can be investigated. However, this combination method can analyze multiple factors and their interactions. Indeed, Datlinger et al., Atray et al., and Diego et al. revealed cell-signaling modules with actual transcriptional profiles [35–37]. It would be more helpful to investigate complicated survival signals and the drug-resistant mechanisms [38].

Conclusions

CRISPR library screenings are more reliable mutation inducers than other previous methods, and they make it easy to address the responsible mutations. Therefore, it is highly important to design what can be revealed by those screenings using new strategies, including what has already been performed and what should be known. Positive/negative selection with CRISPR library might reveal the complicated mechanism of cell signaling and drug resistance. By using different cell types, different contexts, and new technologies, further ideas would give us more insight into discovering the Achilles heel of all cancers and overcoming all drug resistances.

Compliance with ethical standards

Conflict of interest D.A.L. is co-owner and advisor to NeoClone Biotechnology, Inc., B-MoGen Biotechnologies Inc., and Discovery Genomics, Inc. No resources or personnel from any company were involved in this research in any way. The remaining authors declare that they have no competing interests.

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