RNAi vs CRISPR for Genomic Interrogation

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December 16, 2016

RNAI CRISPR

High-throughput functional genomics studies have helped researchers study the genome in a detailed, unbiased manner. Large-scale RNA interference (RNAi) and CRISPR screens have been used to test the effect of gene knockdown and knockout on cellular phenotype. As these two technologies have developed in parallel, often using similar introduction and delivery methods (e.g. lentivirus), they each have demonstrated their advantages and disadvantages in genomic interrogation.

RNA INTERFERENCE AND CRISPR GENOME EDITING		
TECHNIQUE	**GENETIC EFFECT**	**EXPERIMENTAL USE**
RNAi	• Transient post-transcriptional expression inhibition in cytoplasm • No genomic modification • Moderate phenotypic outcome (knockdown)	• Modulated gene knockdown mimicking drug phenotype • Essential gene search
CRISPR	• Permanent pre-transcriptional genomic mutation • Indel mutations or precise modification of genome • Robust phenotypic outcome (knockout) leading to true loss of function	• Complete gene knockout for clear phenotypic outcome • Mapping/pinpointed targeting of noncoding genome • Essential gene search

Table 1. *RNAi and CRISPR libraries can each be useful for interrogating the genome. Choosing one or the other will depend on the goals of the project.*

The utility of RNAi and CRISPR depends on on the investigator's experimental intent (Table 1). Neither process has eclipsed the other in terms of validity. CRISPR, for example, has a wide variety of applications for knocking down or knocking out expression across the whole genome. On the other hand, RNAi offers a relatively simple process for inhibiting gene expression and measuring phenotypic outcome. This piece will break down the intrinsic differences and similarities between these tools with the aim of helping investigators decide what approach works best for their project.

To Meet the Need for Genetic Interrogation, RNAi and CRISPR Emerge

In 1998, Craig Mello and Andrew Fire helped revolutionize functional genetics with their paper on RNAi (Fig. 1a). The ability to directly inhibit the expression of specific genes offers unprecedented access into the purpose and nature of the coding genome. RNAi had direct implications both in basic science and in the clinic with broad application across eukaryotic systems, especially in animals.

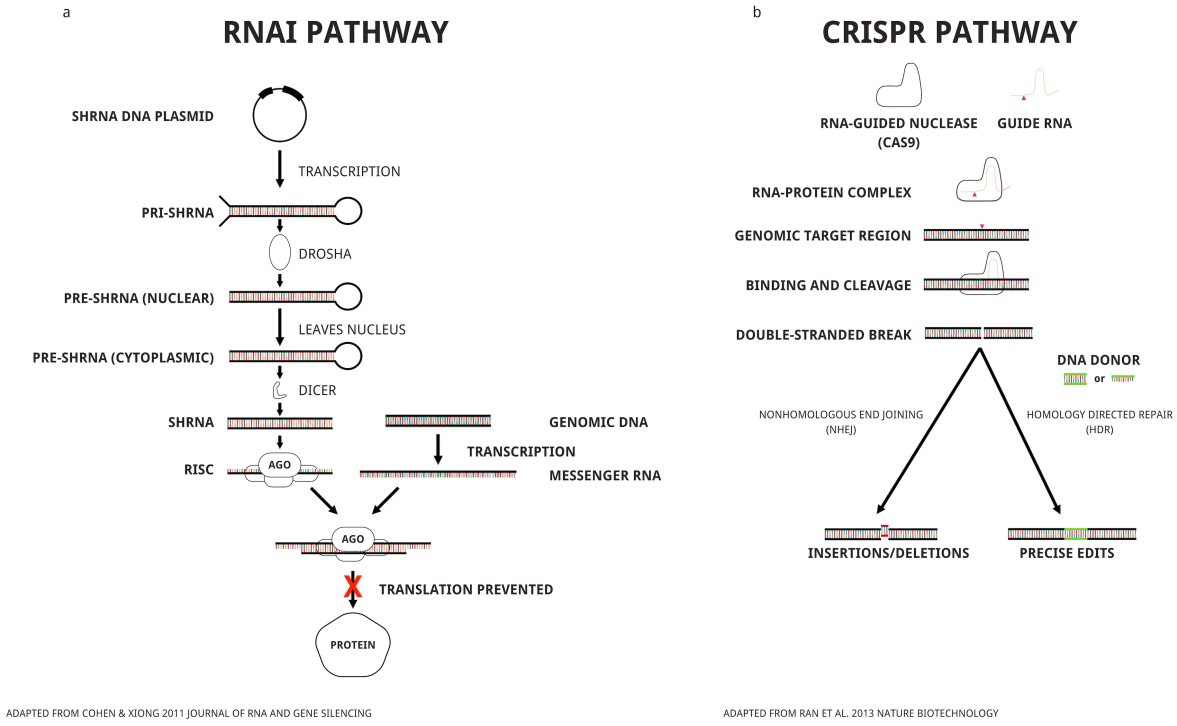

Figure 1. *a) The RNAi pathway begins by transcribing a pri-shRNA. This is then processed by Drosha into pre-shRNA, exported to the cytoplasm and further processed by Dicer. Next, the final antisense RNA forms an RNA-induced silencing complex with argonaute and other proteins. RISC then inhibits translation of a complementary messenger RNA (Cohen & Xiong 2011*). b) In the CRISPR pathway, an sgRNA with a unique protospacer element complexes with a CRISPR nuclease (e.g. SpCas9). The complex then locates a complementary target sequence of genomic DNA and induces a

double-stranded break. This break is repaired via endogenous repair pathways (*Ran et al. 2013).*

In 2012 and 2013, Jennifer Doudna, Emmanuelle Charpentier and Feng Zhang presented CRISPR as another key tool for genetic interrogation through genome engineering (Fig. 1b). Like RNAi, CRISPR was a modular technique that opened new doors for functional validation. Since then, many researchers have begun to migrate from RNAi-based studies to CRISPR.

The Simplicity of RNA Interference

Advantages of RNAi

RNA interference offers several key advantages over CRISPR. Perhaps most obviously, gene inhibition via RNAi is a transient effect. The shRNA permanently degrades messenger RNAs (mRNAs), but unlike in CRISPR, the genome remains unaltered. This means that after the shRNA is cleared from the cell, the gene should resume expression. RNAi transience contrasts with CRISPR nucleases which irreversibly edit the DNA of the cell itself (Boettcher & McManus 2016). While the irreversibility of CRISPR can be somewhat mitigated using precise homologous recombination to restore the gene to its wild-type sequence, this is a low-efficiency and often low-throughput process.

The importance of reversible gene knockdown is predicated on the desired outcome of genetic interrogation. For example, a study by Gupta et al. in 2003 developed an inducible shRNA system targeting *p53*. They found that upon induction, the RNAi pathway inhibited p53 translation. This led to changes in cell morphology and the inability to manage the cell cycle in the presence of γ-irradiation. When the inducer was removed, normal phenotypes (morphology, protein production, cell cycle management) were rescued. This offered confidence that the changes induced by p53-targeting shRNA was the direct cause of the observed phenotypic effect (Gupta et al. 2003). Completing this same experiment with traditional CRISPR gene knockout would have been limited by the permanence of CRISPR DNA editing.

Additionally, RNAi offers the possibility of gene knockdown instead of gene knockout. This can be a useful tool in the context of a dose-dependent drug discovery investigation where full knockout would generate a severe phenotype (Boettcher & McManus 2016). In the study by Gupta et al., they found that their inducible system was dose-dependent. They successfully modulated phenotype (and p53 expression) by applying an increased or decreased level of inducer. The group pointed out that knockdown can be a significant advantage in studies of essential genes where knockout would induce cell death (Gupta et al. 2003).

Moreover, RNAi targets gene transcripts. This means that regardless of how many copies of a gene are present in the model cell line or organism, all transcripts will see some knockdown effect. Similarly, because RNAi deals with mRNA, there is no concern about the chromatin structure of DNA — a factor which can impact the efficacy of CRISPR experiments (Boettcher & McManus 2016).

In animal cells, RNAi can also be faster and easier to use than CRISPR depending on experimental context. This is because animal cells already contain the processing machinery (e.g. drosha, RISC components) necessary for RNAi. Therefore, no extra time or labor needs to be spent delivering an exogenous nuclease (e.g. Cas9) or other components into the cell (Boettcher & McManus 2016). Further, because RNAi targets transcripts regardless of ploidy, cells can be harvested rapidly following shRNA treatment. With CRISPR, it may be necessary to validate homozygous knockouts within the subpopulation via sequencing (Boettcher & McManus 2016).

Disadvantages of RNAi

Unfortunately, RNA interference remains an imperfect tool. Notably, the off-target effects of RNAi are substantial. This can occur in two ways: sequence-dependent and sequence-independent. The sequence-dependent off-target effect occurs because shRNAs have a mismatch tolerance when seeking out complementary messenger RNA sequences. Mismatch tolerance is also present in CRISPR, albeit to a lesser, more algorithmically predictable degree (Housden & Perrimon 2016) that is further limited by the availability of protospacer adjacent motifs (PAMs) (Boettcher & McManus 2016).

The RNAi pathway can affect other genes in a sequence-independent manner by flooding the endogenous microRNA (miRNA) pathway of the cell. The cell naturally uses miRNAs to regulate gene expression; interfering with this process can therefore prevent the cell from normal modulation of genetic transcripts (Boettcher & McManus 2016). Conversely, CRISPR does not rely on endogenous regulatory pathways to induce mutations and therefore does not generate sequence-agnostic off-target effects. Once again, this makes the specificity of CRISPR systems more predictable.

Another major issue with RNAi is that although animal cells contain the requisite siRNA machinery, many other eukaryotic and prokaryotic species do not. Therefore, the advantage of RNAi not requiring exogenous machinery diminishes. Editing genes in bacteria, for example, is more difficult with RNAi than CRISPR. It should be noted, of course, that CRISPR also faces some roadblocks in that field.

The Flexibility of CRISPR

Advantages of CRISPR

One of the most striking features of CRISPR is that it often offers more robust phenotypes than RNAi. This is due to the fact that CRISPR induces full gene knockout. In RNAi, genes are knocked down, but there is usually some residual expression that can obfuscate obvious phenotypic observations in cell structure, viability or proliferation. CRISPR-edited cells can also be selected for and grown as their own separate knockout cell line for a given gene with no chance of those cells resuming gene expression.

The result of stronger, more consistent phenotypic readout is that CRISPR is either comparable to or more sensitive than RNAi-based screens (Shalem et al. 2014, Housden & Perrimon 2016). Following from this, CRISPR screens also tend to find more true positive genes than RNAi in essentiality studies (Wang et al. 2015). This may be because

RNAi generates a hypomorphic effect which isn't severe enough to induce dropout (Housden & Perrimon 2016). It should be noted, that CRISPR doesn't always generate cell knockout, either; in-frame mutations, non-deleterious edits to coding DNA or incomplete targeting of polyploid cells may still yield functional protein expression (Housden & Perrimon 2016).

Another significant advantage of CRISPR is that it ostensibly gives researchers the tools to study the whole genome. In contrast to RNAi, CRISPR isn't limited to transcript inhibition. In fact, it doesn't need to target protein coding regions of DNA at all. CRISPR can be used to target noncoding regions (98% of the human genome) and elucidate their regulatory function (Shalem et al. 2014), compensating for a blind spot that RNA interference research cannot address. Precisely editing noncoding regions may also offer modulated therapeutic effects that mimic the effects of gene knockdown. Along these same lines, CRISPR can be used to investigate nuclear targets like long noncoding RNAs (lncRNAs) (Zhu et al. 2016). Meanwhile, RNAi is restricted to cytoplasmic RNA (e.g. mRNA) (Boettcher & McManus 2016).

Disadvantages of CRISPR

Despite these benefits, CRISPR also has limitations. RNA-guided endonucleases (RGENs) can only target regions that contain specific PAMs. Specifically, the most widely used CRISPR nuclease ortholog, *Streptococcus pyogenes* Cas9 (SpCas9), must recognize NGG sites in order to bind and cleave genomic DNA. This presents a problem for studying AT-rich regions. Dependence on PAM sites further prevents the investigator from fine-tuning CRISPR target regions to precisely map the genome. Some investigators have suggested using multiple Cas9 orthologs (e.g. NmCas9) or species (e.g. Cpf1) that recognize other PAMs to more completely cover the genome. Nevertheless, PAM-limited targeting remains a problem that RNAi, which only focuses on transcripts, does not have to contend with.

RNAI VERSUS CRISPR

TECHNIQUE	ADVANTAGE	DISADVANTAGE
RNAi	• Rapid (no need for exogenous nuclease) • Can target any transcript regardless of ploidy • Not inhibited by 3D DNA/chromatin context • Knockdown effect predictable, no genome mutations involved • Transient knockdown of essential genes can keep cell alive for study	• Limited to animal models • Cannot target noncoding regions of the genome • Only accesses cytoplasmic RNA • Not always as good at finding essential genes • Library complexity impacts performance • High off-target effect based on mismatch tolerance and interference with endogenous miRNA pathways
CRISPR	• Can be used in non-eukaryotic cells • Can target noncoding elements of DNA • Can target nuclear RNA elements • Can be better at finding essential genes • Library complexity may not impact performance • Mismatch tolerance throughout genome, but off-target can be mitigated with modified sgRNAs and RGENs	• Comparatively slower (requires exogenous nuclease) • Relies on presence of appropriate PAM sites • Can be stymied by polyploidy • Can be inhibited by structure of chromatin • Unpredictable mutations • Knockout of essential genes leads to death, no room intermediate study

Table 2. *A table broadly summarizing the advantages and disadvantages of CRISPR and RNAi.*

Best Practices Moving Forward

Using CRISPR and RNAi In Concert

The competition between RNAi and CRISPR belies the cooperative potential of the two techniques. Comparison studies have shown that while there are differences between these approaches, there is a distinct lack of crossover in their use. For example, CRISPR is an excellent tool for studying phenotypes associated with complete gene knockout because it can introduce null mutations. Meanwhile, RNAi offers post-transcriptional inhibition which allows more moderate, less permanent outcomes (Housden & Perrimon 2016).

Even within the same type of investigation, the two methods can be complementary. For example, Morgens et al. found in their search for essential genes that CRISPR screens identified genes that RNAi screens did not, and vice versa. This can be due to a number of factors (Fig. 2). For example, the genes found using CRISPR only may be necessary shRNA machinery and are therefore undetectable with RNAi. Gene knockout by CRISPR may offer a more dramatic (cell death) phenotype than gene knockdown, thereby capturing essential genes that RNAi misses. Conversely, RNAi-only essential genes may be composed of polyploid genes less easily and less completely targeted by CRISPR (Housden et al. 2016).

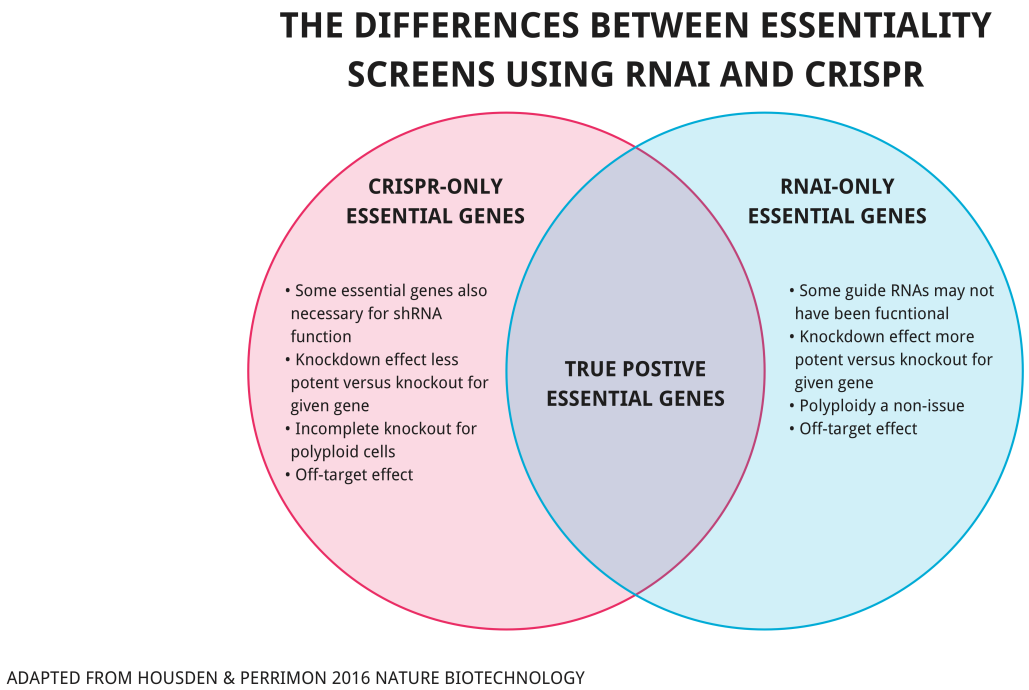

Figure 2. *Essential gene studies performed with RNAi and CRISPR libraries yield different gene lists. Often CRISPR finds more, but other reasons for lack of overlap between these techniques are briefly listed in this Venn diagram.*

For the genes that both libraries identified, the investigators can be more certain that they are true positive essential genes. Overlapping these lists can help negate the off-target effects of both RNAi and CRISPR as two independent assays yielded the same result. In concert, all three categories can be useful in generating a truly robust list of essential genes for further study (Housden et al. 2016). Indeed, Deans et al. 2015 found that using RNAi and CRISPR together produced more robust gene lists because neither approach captured all truly essential genes independently.

CRISPR Interference Takes Shape

Another development in genetic interrogation is the advent of CRISPR interference, or CRISPRi (Gilbert et al. 2013). CRISPRi uses a catalytically inactive Cas9 (dCas9) to achieve steric inhibition of gene expression. dCas9 may also be fused to a repressor such as KRAB. Regardless, the phenotypic outcome of CRISPRi is transient gene repression without long-term effects to the genome. This technique is therefore more comparable to RNAi.

Nevertheless, there are still important differences. CRISPRi remains dependent on the presence of PAM sites and still requires the investigator to introduce exogenous machinery (RGENs) into the cell or organism. The technique also relies on transcription start site (TSS) data which may not be available of a given model organism; an investigator would not need this data to perform an RNAi experiment.

Still, CRISPRi has been shown to yield lower off-target effects and higher gene inhibition efficiency than RNAi and may therefore represent a truer replacement for that technique (Boettcher & McManus 2016). In a recent study, CRISPRi was also successfully used in a large-scale library to investigate lncRNAs in the noncoding genome (Liu et al. 2017). This offered a different approach to the aforementioned tiling paper from Zhu et al. where lncRNAs were explored using traditional CRISPR editing. The work by Liu et al. suggests even broader utility for CRISPRi than RNAi while retaining similar experimental outcomes.

RNA-Targeting CRISPR Opens Up New Possibilities

Two new CRISPR-based approaches could also compete with RNAi (Nelles et al. 2016, Abudayyeh et al. 2016). These methods do not rely on genomic DNA structure or sequence information (e.g. TSS). In Nelles et al., the authors used catalytically inactive RNA-targeting Cas9 (RCas9) to directly bind to messenger RNA. For now, although this novel technique allows RNA tracking *in situ*, RCas9 does not seem to impact translation. Conversely, Abudayyeh et al. use an alternative nuclease, C2c2, to target and cleave single stranded RNA (similar to the RISC complex).

It is important to note that RCas9 wasn't designed to inhibit RNA translation and that C2c2 hasn't been widely adopted. Yet both systems can target transcripts and not the genome, avoiding the pitfalls of polyploidy seen in CRISPR and CRISPRi experiments. These studies suggest that we may see future systems emerge that closely mimic the advantages of RNAi while taking advantage of the benefits of CRISPR biology.

Sources

Abudayyeh OO, Gootenberg JS, Konermann S et al. C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector. Science. 2016 Aug 5;353(6299):aaf5573. doi: 10.1126/science.aaf5573. PubMed PMID: 27256883.

Boettcher M, McManus MT. Choosing the Right Tool for the Job: RNAi, TALEN, or CRISPR. Mol Cell. 2015 May 21;58(4):575-85. doi: 10.1016/j.molcel.2015.04.028. Review. PubMed PMID: 26000843.

Fire A, Xu S, Montgomery MK et al. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature. 1998 Feb 19;391(6669):806-11. PubMed PMID: 9486653.

Gilbert LA, Larson MH, Morsut L et al. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. Cell. 2013 Jul 18;154(2):442-51. doi: 10.1016/j.cell.2013.06.044. PubMed PMID: 23849981.

Gupta S, Schoer RA, Egan JE et al. Inducible, reversible, and stable RNA interference in mammalian cells. Proc Natl Acad Sci U S A. 2004 Feb 17;101(7):1927-32. PubMed PMID: 14762164.

Housden BE, Perrimon N. Comparing CRISPR and RNAi-based screening technologies. Nat Biotechnol. 2016 Jun 9;34(6):621-3. doi: 10.1038/nbt.3599. PubMed PMID: 27281421.

Jinek M, Chylinski K, Fonfara I et al. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science. 2012 Aug 17;337(6096):816-21. doi: 10.1126/science.1225829. PubMed PMID: 22745249.

Liu SJ, Horlbeck MA, Cho SW et al. CRISPRi-based genome-scale identification of functional long noncoding RNA loci in human cells. Science. 2016 Dec doi: 10.1126/science.aah7111

Nelles DA, Fang MY, O'Connell MR et al. Programmable RNA Tracking in Live Cells with CRISPR/Cas9. Cell. 2016 Apr 7;165(2):488-96. doi: 10.1016/j.cell.2016.02.054. PubMed PMID: 26997482.

Ran FA, Hsu PD, Wright J et al. Genome engineering using the CRISPR-Cas9 system. Nat Protoc. 2013 Nov;8(11):2281-308. doi: 10.1038/nprot.2013.143. PubMed PMID: 24157548.

Shalem O, Sanjana NE, Hartenian E et al. Genome-scale CRISPR-Cas9 knockout screening in human cells. Science. 2014 Jan 3;343(6166):84-7. doi: 10.1126/science.1247005. PubMed PMID: 24336571.

Zhu S, Li W, Liu J et al. Genome-scale deletion screening of human long non-coding RNAs using a paired-guide RNA CRISPR-Cas9 library. Nat Biotechnol. 2016 Oct 31. doi: 10.1038/nbt.3715. [Epub ahead of print] PubMed PMID: 27798563.

Søren Hough and Leigh Brody contributed to this Resource.