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Biotechnology

EndoGenius Suppressor Assay

A novel tool for endogene inhibition

version 23.1

1. The importance of gene silencing and associated tools

The process of drug discovery and development of therapeutic approaches relies heavily on association of genotype with phenotype. One of the best ways to do this is to disrupt gene function and then analyze changes in phenotype. Using RNAi and CRISPR biological tools (Figure 1), researchers can study gene function by suppressing gene expression at the translational or genetic level, respectively (1).

Gene silencing is important in elucidating cellular processes. With the discovery of RNA interference (RNAi) in the 1990s, double-stranded RNAs of 18-22 nucleotides are used for targeted gene silencing at the post-transcriptional level (2, 3).

The primary function of native RNAi is to regulate gene expression. In some cases, RNAi is also responsible for resistance to virus or other pathogen infections (4).

siRNA Processing: The double strand RNA entering the cell is cleaved into smaller RNA fragments about 21 nucleotides long by an endonuclease, Dicer. These RNAs associate with the RNA-induced silencing complex (RISC), the antisense strand is separated from the sense strand and targeted to their complementary mRNAs (5) (Figure 1).

Figure 1. Popular gene silencing tools. This figure is created in BioRender.com

Following association of siRNA or miRNA with its targets, Argonaute, a protein in the RISC complex, cleaves mRNA and inhibits the expression of the protein encoded by the target gene. If the siRNA or miRNA sequences do not match the mRNA sequence perfectly, the mRNA is not fragmented, but translation is blocked because the RISC complex physically blocks the mRNA (5).

Once siRNAs to silence specific target genes are designed, they can be cloned into plasmid vectors and transfected into cells using synthetic siRNA, PCR products, or in vitro transcribed siRNAs. One advantage of RNAi is that mammalian cells naturally possess the endogenous mechanisms (Dicer and RISC) necessary for the process. This makes it relatively easy to carry out the experimental procedure. In the final step, gene silencing efficiency is usually determined by measuring mRNA transcript levels using methods such as quantitative RT-PCR, measuring protein levels using immunoblotting or immunofluorescence assays, or monitoring obvious phenotypic changes.

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On the other hand, in 2012, the Doudna and Charpentier research groups uncovered the potential use of RNA-guided Cas9 in programmed genome editing 25430774 (6). In 2013, Feng Zhang's group used the CRISPR system for the first time for genome editing in eukaryotic cells (human and mouse) (7). After that, CRISPR began to be used as a collaborative tool for editing the genomes of various organisms in research projects around the world.

The primary difference between RNAi and CRISPR is that RNAi reduces gene expression (knockdown) at the mRNA level, while CRISPR completely and permanently silencing (knockdown) the gene at the DNA level.

Both knockout and knockdown have their pros and cons.

Knockouts of essential genes are lethal and provide only partial information on gene function in studies in which the gene of interest plays a crucial role in the survival of the organism. In such cases, not completely silencing the gene may provide a better understanding of the gene effect on the phenotype (8).

Moreover, the reversible nature of knockdown may allow increase of protein expression to the normal levels in the same cells, making it possible to confirm the phenotypic effects.

More importantly, knockdown may be a safer option than permanent genome editing, as it is temporary (8). However, knockouts are effective in completely blocking protein expression and counteract the confounding effects of low protein expression levels remaining after knockout.

As CRISPR has become popular for its ease of genetic editing, variations in the method and new versions of CRISPR-related nucleases have also enabled researchers to use CRISPR for applications beyond gene knockouts (9). For example, CRISPRi allows silencing of genes without permanently disabling the gene. This is accomplished by using a dead Cas9 nuclease that physically blocks RNA polymerase and inhibits gene transcription, or by modulating gene regulators. Although the mechanism is different from knocking out genes, inhibition still occurs at the DNA level (10).

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2. Currently available gene silencing tools have certain drawbacks

Gene silencing via currently available tools has significant shortcomings;

- One of the major limitations of the RNAi silencing method is the high incidence of off-target effects. Silencing of unwanted RNA targets results in undesirable phenotypes and is therefore highly problematic for gene function screening experiments (26). Off-target effects in RNAi can be two types: sequence-independent and sequence-dependent. For example, numerous studies have shown that siRNAs trigger an interferon-activated pathway in certain cell types in a sequence independent manner, resulting in increased expression of interferon-regulated genes. In 2003, research showed for the first time that siRNA also targets sequences with limited complementarity. Even today, sequence-based off-target effects remain the most challenging issue in RNAi experiments (11).

- Mammalian systems have evolved a potent antiviral immune response to long double-stranded RNA. This includes the stimulation of interferons and inflammatory cytokines that dramatically alter gene expression and affect a variety of important cellular processes. In particular, siRNAs longer than 23 base pairs trigger strong immune responses that cause off-target effects and affect functional outputs (12). Certain siRNA sequence motifs, structures, delivery vehicles, and impurities in siRNA preparations can also stimulate immune responses (13).

- *Since siRNA-mediated effects rely on endogenous RNAi mechanisms, overloading the cell with siRNAs will occupy RNAi effector proteins that miRNAs need for gene expression regulation.* One study also reported that siRNA treatments can lead to significant off-target effects in cells, reporting upregulation of endogenous miRNA targets in a dose-dependent manner corresponding to the amount of siRNA used (14).

- In a genome-scale RNAi screening study, it was revealed that different siRNAs targeting the same gene cause different phenotypes in cells (15).

- Specific gene regions can be knocked out with the CRISPR system (16, 17). This leads to the complete disappearance of gene expression and does not allow the mimic of situations in which gene expression decreases. While optimizing siRNA design, concentrations, and chemical modifications has reduced some of the

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off-target effects of RNAi, a recent comparative study has shown that CRISPR has far fewer off-target effects than RNAi. *However, due to the off-target effects of the CRISPR method, additional genetic errors are likely to occur in the treated cells* (18).

- Although gene silencing with siRNAs, which is a frequently used gene silencing technique, performs relatively specific and effective gene silencing, this silencing is mostly at the post-transcriptional level and can achieve limited regulation at the transcriptional level (35).

- Besides, microRNAs with hundreds of targets are far from specifically gene silencing, and their use in gene therapy approaches is not realistic, and effective results have not been obtained in clinical studies.

Therefore, ***regulation of endogenous gene expression at transcription level with minimum off target effects*** *is a new and exciting approach in medical research.*

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3. How EndoGenius Suppressor Assay differs from currently available tools

EndoGenius Suppressor Assay utilizes oligonucleotides to targeted guidance of certain epigenetic regulators, which are already present within the cell, to the specific gene promoters to overexpress specific endogenes (Figure 2).

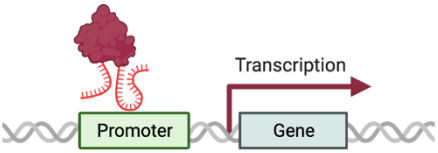

Figure 2. General mechanism of action of EndoGenius Suppressor Assay. This figure is created in BioRender.com

EndoGenius Suppressor Assay includes Control Mix, which is equivalent to control plasmids or non-targeting oligonucleotides, and the necessary carrier agent (Encapsulation Buffer), which is an optimized transfection reagent for effective delivery of Active Mix into the cells. Therefore, EndoGenius Suppressor Assay eliminates the need for purchasing additional controls and transfection reagents. EndoGenius Suppressor Assay is All-in-One solution.

Endogenous gene silencing via EndoGenius Suppressor Assay has significant **advantages**;

Delivery of only short oligonucleotide sequences compared to plasmids provide more effective transient overexpression of target genes. Fluorescent labeled Active Mix of EndoGenius Suppressor Assay can be delivered to cells with almost 100% efficiency (Figure 3).

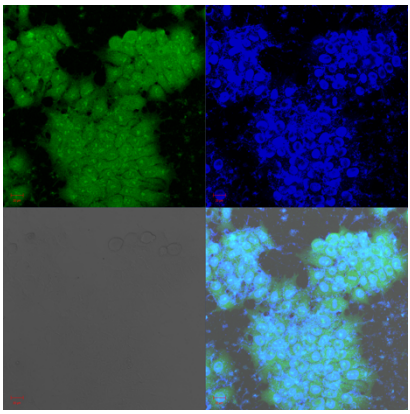

Figure 3. EndoGenius Suppressor Assay helps silencing of target gene with almost 100% efficiency.

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Directly regulating the expression of endogenous genes by targeting gene promoters offers several advantages compared with RNAi approaches to down-regulate gene expression (6). For downregulation of endogenous genes directly at the DNA level, efficiency is likely to increase as only two copies of DNA per cell need to be targeted compared to the thousands of mRNAs that are usually required to be targeted in RNAi approaches. CRISPRi system also necessitates utilization of large plasmids, technical experience and long optimization processes.

Utilization of EndoGenius Suppressor Assay allows inhibition of specific gene expression (Figure 4A) with minimal off-target effects (Figure 4B). It is quite easy to carry out an assay to see functional effects of suppressing an endogenous gene. For example, suppression of a specific oncogene results in significant decrease in viability (Figure 4C).

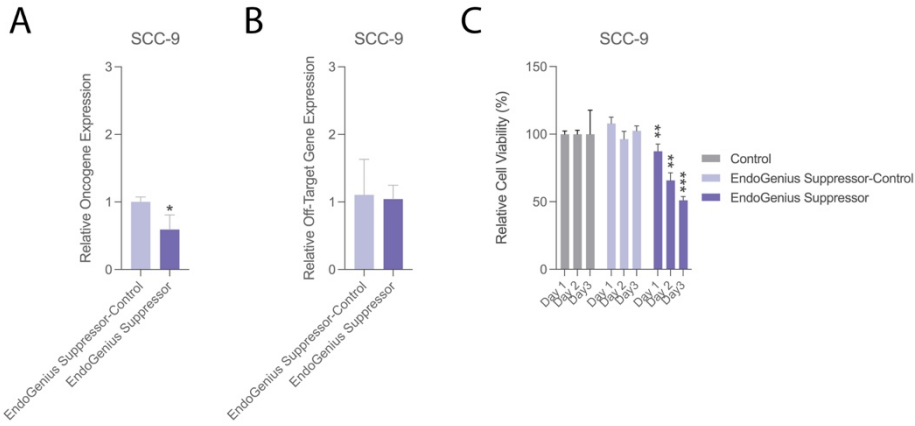

Figure 4. A. *EndoGenius* Suppressor specifically induce significant suppression of Oncogene 1, B. with no alteration in other genes. C. Suppression of Oncogene 1 results in significant decrease in cell viability.

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On the other hand, suppression of Tumor Suppressor Gene 1 using EndoGenius Suppressor Assay (Figure 5A), with no significant change in the expression of another tumor suppressor gene (Figure 5B), results in increased cell viability (Figure 5C).

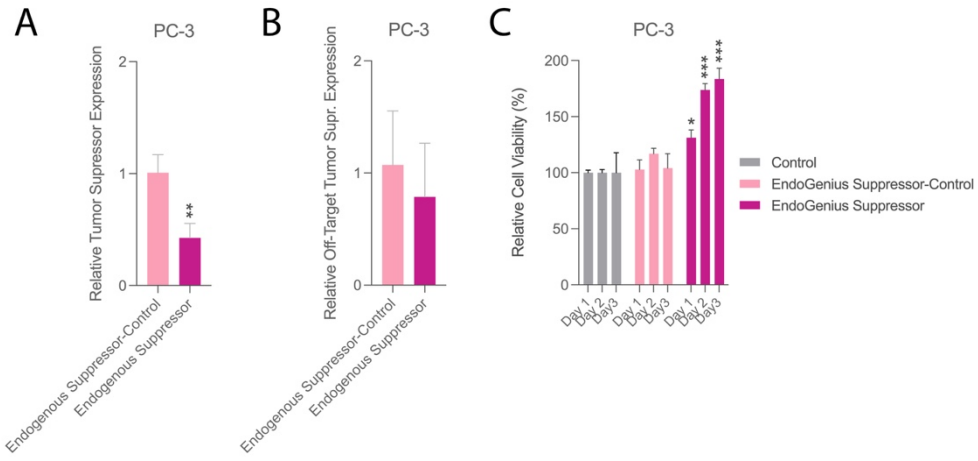

Figure 5. A. *EndoGenius* Suppressor specifically suppressed the Tumor Suppressor Gene 1, B. with no alteration in another tumor suppressor. C. Suppression of Tumor Suppressor 1 results in significant increase in cell viability.

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Conclusion

- √ EndoGenius Suppressor Assay helps overexpression of endogenes at transcription level and is an all-in-one assay, which necessitates no control plasmids, vectors, non-targeting oligos, vectors, transfection reagents.
- √ It provides delivery efficiency similar to viral transduction with no need for additional infrastructure for virus handling.
- √ Besides, it alters the expression of all splice variants that are expressed in that specific cell or tissue, which are under the control of same promoter.

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