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GENE EXPRESSION SILENCING METHODS IN IN VITRO MODELS

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ABSTRACT

Gene expression silencing is one of the key tools in molecular biology and is widely applied in in vitro studies to investigate gene function and the mechanisms regulating cellular processes. The dynamic development of molecular techniques has resulted in the availability of numerous strategies that enable either transient or permanent reduction of gene activity at different stages of gene expression. The aim of this work is to review and compare gene expression silencing methods used in in vitro models, with particular emphasis on their mechanisms of action, efficiency, durability of the effect, and experimental limitations.

The article discusses classical approaches based on RNA interference, including the use of siRNA, shRNA, and microRNA modulation, as well as strategies employing antisense oligonucleotides. Special attention is given to technologies based on the CRISPR system, including CRISPRi as a tool for reversible transcriptional repression and CRISPR–Cas9, which enables permanent disruption of gene function. Issues related to the delivery of molecular tools into cells, validation of silencing efficiency, and the importance of appropriate experimental controls are also addressed.

Analysis of the literature indicates that the choice of an appropriate method should be tailored to the research objective, the characteristics of the gene under investigation, and the planned duration of the experiment. Increasingly, a complementary approach—combining different gene silencing strategies is recommended, as it enhances the reliability of results obtained in in vitro studies.

KEYWORDS

Gene Silencing, RNA Interference, CRISPRi, Antisense Oligonucleotides

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Concept and significance of gene expression silencing

Gene expression silencing is one of the fundamental tools in molecular biology, enabling the investigation of gene function through the deliberate reduction of gene activity [1]. This term encompasses a variety of methods that lead to a decrease in the amount of mRNA or protein produced from a given gene without permanently altering the DNA sequence [2]. Depending on the selected approach, the silencing effect may be achieved at the initiation of transcription or at later stages—for example, by influencing mRNA stability or the efficiency of translation into protein [3]. In some cases, it is difficult to clearly separate these processes due to their partial overlap; however, in practice, the specific point of intervention largely determines the nature and magnitude of gene silencing [4].

In studies employing *in vitro* models, gene silencing plays a particularly important role [5], as it allows for the assessment of individual gene functions under relatively well-controlled conditions [6]. Cell-based models make it possible to examine the consequences of reduced gene expression, which may affect a wide range of biological processes, such as cell proliferation, differentiation, and survival [7]. It should also be noted that, in contrast to complete gene knockout, gene silencing typically results in a partial and reversible effect [8]. This feature is especially important when studying genes that are essential for normal cellular function [9].

An additional advantage of gene silencing methods is their ability to facilitate analysis of the relationship between gene expression levels and cellular responses [8]. This enables the investigation of dose-dependent effects of gene products [10]. Consequently, these strategies are widely applied in both basic and applied research, including the validation of potential therapeutic targets and high-throughput screening studies [11]. Nevertheless, accurate interpretation of the results requires a thorough understanding of the mechanisms underlying each method, as well as consideration of their limitations, such as variable efficiency and the potential occurrence of off-target effects [12].

Methodology

This work constitutes a literature review of methods for gene expression silencing in *in vitro* models. The analysis focuses on scientific publications available in the PubMed database, including both review articles and original research studies relevant to the topic. The selection of literature was based on the relevance of the publications to issues related to RNA interference, antisense oligonucleotides, and CRISPR technologies applied in *in vitro* research.

The review includes studies describing the mechanisms of action of individual methods, their experimental applications, advantages, and limitations, with particular emphasis on efficiency, durability of the effect, and aspects of result validation. The objective of the literature analysis was to provide a concise synthesis of the current state of knowledge and to compare available gene expression silencing strategies in terms of their practical application in molecular biology research.

Methods of gene expression silencing at the RNA level – RNA interference (RNAi)

RNA interference (RNAi) is one of the most widely used methods for gene expression silencing in *in vitro* studies [7]. It operates through short RNA molecules which, upon introduction into the cell, engage the endogenous regulatory pathway, leading to a specific reduction in the level of the target transcript [13]. A central component of this process is the RNA-induced silencing complex (RISC), which recognizes sequences complementary to the target mRNA and initiates either its degradation or the inhibition of translation [14].

The simplest application of RNAi involves small interfering RNAs (siRNAs), which are extensively used in short-term experiments [15]. siRNAs enable rapid and efficient downregulation of specific gene expression, making them a valuable tool for functional analyses and preliminary validation of research hypotheses [16]. However, this approach is limited by the transient nature of the effect and the strong dependence of silencing efficiency on transfection efficacy and the intracellular stability of siRNA molecules [12].

More prolonged gene silencing can be achieved using short hairpin RNAs (shRNAs), which are processed into their active form following expression within the cell [17]. shRNAs are typically delivered via plasmid or viral vectors, allowing stable expression and long-term modulation of target mRNA levels [7]. This strategy is particularly useful in studies requiring extended observation periods, although it entails greater technical complexity and necessitates careful experimental control [7].

RNAi-based approaches also include modulation of microRNA activity through the use of microRNA mimics or inhibitors [4]. This strategy enables the manipulation of entire regulatory networks, which may yield valuable biological insights but also complicates the interpretation of observed effects [3]. Despite the broad applicability of RNAi, these methods carry a risk of off-target effects and activation of cellular responses to exogenous RNA, underscoring the importance of appropriate controls and independent validation of results [12].

The limitations of RNA interference–based methods, such as variable durability of the silencing effect and the risk of nonspecific interactions, have contributed to the development of alternative gene silencing strategies [12]. These include approaches based on antisense oligonucleotides as well as CRISPR-based technologies [18].

Antisense oligonucleotides

Antisense oligonucleotides (ASOs) constitute a distinct class of tools used for gene expression silencing through direct interaction with target RNA molecules [19]. These single-stranded nucleic acid sequences are designed to bind complementarily to specific regions of a transcript, leading to inhibition of its function or its degradation. Unlike classical RNA interference–based approaches, ASOs do not require the endogenous RNA-induced silencing complex (RISC) and can exert their effects in both the cytoplasm and the nucleus [20].

One of the best-characterized mechanisms of ASO action is RNase H–dependent mRNA degradation [19]. In this process, the enzyme recognizes DNA–RNA hybrids and initiates transcript cleavage [21]. Oligonucleotides designed for this purpose, known as gapmers, are widely used in *in vitro* studies due to their high efficiency and ability to target nuclear transcripts [20]. Another strategy involves ASOs that modulate pre-mRNA splicing [22]. These molecules function by blocking splice sites or regulatory splicing elements, resulting in altered mRNA structure and often leading to the production of non-functional protein products [23].

The use of antisense oligonucleotides offers several important advantages, including high sequence specificity and the ability to precisely interfere with RNA processing [19]. However, the effectiveness of ASOs in *in vitro* models largely depends on their chemical modifications, which influence molecular stability, cellular uptake, and potential toxicity [21]. Consequently, ASO-based approaches require careful optimization of experimental conditions and thorough validation of the obtained results [18].

Although antisense oligonucleotides are characterized by high specificity and broad applicability, the demand for more stable and precise regulation of gene expression has contributed to the development of technologies based on the CRISPR system [1]. These innovative approaches enable targeted control of gene activity at the transcriptional level [24].

CRISPR Technologies in Gene Expression Silencing

The introduction of CRISPR technologies has revolutionized our approach to the regulation of gene expression in *in vitro* models [25]. These tools now enable precise and targeted inhibition of gene activity without the need for permanent genomic modification [26]. In the context of gene expression silencing, the use of a catalytically inactive form of the Cas9 nuclease (dCas9) is of key importance [24]. This variant retains the ability to bind specific DNA sequences but does not induce double-strand breaks [27].

The most commonly employed strategy is CRISPR interference (CRISPRi), which involves the fusion of dCas9 with repressor domains such as KRAB [24]. Guided by a carefully designed guide RNA, this complex binds to the promoter or regulatory regions of a gene, leading to inhibition of transcription initiation and alterations in the local chromatin structure [26]. The resulting effect is characterized by relative stability and reversibility, making CRISPRi a highly useful tool for functional studies and the analysis of non-coding genes [27].

An extension of the classical CRISPRi approach includes epigenetic editing techniques, in which dCas9 is fused to chromatin-modifying enzymes, such as DNA methyltransferases or histone demethylases [28]. These methods enable more durable modulation of gene activity through the introduction of targeted epigenetic changes [29]. However, interpretation of the observed effects may be more complex due to the long-term nature of these modifications [30].

Despite the numerous advantages of CRISPR technologies, their application to gene expression silencing requires careful design of guide RNAs and optimization of delivery conditions to cells [31]. The

efficiency of silencing may be limited by chromatin accessibility at the target site, and although the risk of off-target effects is lower than that associated with RNAi-based methods, the use of appropriate experimental controls remains essential [32,8].

Although dCas9-based technologies allow for controlled and reversible transcriptional repression, many *in vitro* studies still rely on the CRISPR–Cas9 system, which results in permanent gene knockout. This approach enables the assessment of long-term consequences of gene loss [8,33].

CRISPR–Cas9 as a Method for Functional Gene Knockout

The CRISPR–Cas9 system is widely used in *in vitro* studies as a tool for the permanent ablation of gene function through the introduction of targeted DNA damage. Its mechanism of action is based on the induction of double-strand breaks at the target locus, which are subsequently repaired predominantly via the non-homologous end joining (NHEJ) pathway. This process frequently results in insertions or deletions, leading to frameshift mutations and loss of gene functionality [33,34,35].

The application of CRISPR–Cas9 enables the investigation of long-term effects resulting from complete loss of gene activity, which is particularly important for genes in which partial silencing does not produce clear phenotypic changes [8,33]. However, it should be noted that complete absence of a gene product may trigger compensatory mechanisms or selection of cellular subpopulations with altered expression profiles, factors that must be taken into account when interpreting the obtained results [36].

In *in vitro* studies employing CRISPR–Cas9, one of the key challenges is the mosaicism of edited cell populations, which arises from the heterogeneity of introduced mutations [37]. In practice, this often necessitates the isolation and characterization of individual clones, thereby extending the duration and increasing the complexity of experiments [38]. Additionally, the risk of off-target effects and activation of DNA damage responses underscores the need for appropriate controls and multi-level validation of the obtained results [39].

Regardless of the chosen strategy for gene silencing or knockout, the effectiveness of the resulting outcome in *in vitro* models largely depends on the method used to deliver the appropriate molecular tools into cells [18].

Methods for Delivering Gene-Silencing Tools to Cells *In Vitro*

The efficiency of gene expression silencing in *in vitro* models largely depends on how effectively the appropriate molecules are delivered into cells. Even the most carefully designed molecular tool may fail to produce the expected outcomes if its transfer into cells is insufficient or induces excessive cellular stress. Therefore, the choice of a delivery method represents a critical element in the planning of gene-silencing experiments [18,40].

The most commonly used approach in *in vitro* studies is chemical transfection, which relies on lipids or cationic polymers to form complexes with nucleic acids [41]. This technique is relatively simple and well tolerated by many cell lines; however, its efficiency may be limited in primary cells or in cells with low proliferative capacity [42]. An alternative strategy is electroporation, including nucleofection-based techniques, which allow direct delivery of genetic material into the cell interior, albeit at the cost of increased cytotoxicity and the need for precise optimization of experimental parameters [43].

In studies requiring long-term gene expression silencing, viral vectors—particularly lentiviruses—are frequently employed. These vectors offer high transduction efficiency and stable expression of the delivered sequences, which is essential for long-term experiments. Nevertheless, the use of viral vectors is associated with greater technical complexity and the risk of genomic integration of the delivered genetic material [44,45].

The selection of an appropriate delivery method should take into account the cell type, the characteristics of the gene-silencing tool employed, and the intended duration of the experiment [42]. Optimization of transfection or transduction conditions is essential to obtain reproducible and reliable results in *in vitro* studies [41,18].

Regardless of the chosen delivery strategy, rigorous assessment of the extent and consequences of silencing of the target gene is crucial for the reliability and interpretability of the obtained results [7].

Validation of Gene Silencing Efficiency

Assessment of the efficiency of gene expression silencing is a critical step in studies employing *in vitro* models and is essential for the correct interpretation of experimental results [7]. Given that gene expression is a multistep process, validation should include analyses at both the transcript and protein levels [8]. It is important to note that changes in mRNA abundance do not always directly reflect protein levels within the cell [46].

The primary method for quantitative evaluation of mRNA levels is RT–qPCR, which allows precise determination of the extent to which expression of the target gene is reduced [47]. In broader-scale studies, RNA sequencing (RNA-seq) is increasingly employed, as it enables comprehensive analysis of global gene expression changes and identification of potential off-target effects associated with the applied silencing strategy [48]. Protein-level assessment is typically performed using immunochemical techniques such as Western blotting, flow cytometry, or immunofluorescence [49].

Molecular analyses should be complemented by functional validation, which includes evaluation of phenotypic changes, enzymatic activity, and cellular responses to specific stimuli [8]. A key aspect of reliable validation is the use of appropriate experimental controls, including control sequences and independent strategies for silencing the same gene [11]. Such an approach reduces the risk of misinterpretation and increases the robustness of conclusions drawn from *in vitro* studies [7].

The diversity of available gene expression silencing strategies and their distinct mechanisms of action underscore the importance of directly comparing these methods in terms of efficiency, durability of the effect, and experimental limitations [1,8].

Comparison of Gene Expression Silencing Methods

Available gene expression silencing methods differ substantially in their mechanisms of action and in the nature of the effects they produce, which directly influences their suitability for specific experimental applications [8]. Strategies based on RNA interference and antisense oligonucleotides primarily reduce mRNA levels, enabling rapid and typically reversible gene silencing [16,19]. These approaches are particularly useful in short-term studies; however, their effectiveness may be limited by variability in transfection efficiency and by the risk of off-target effects [7,12].

CRISPR-based technologies provide markedly greater control over gene regulation [25]. CRISPR interference (CRISPRi) enables stable transcriptional repression without permanent modification of the genome, making it an attractive alternative to conventional RNAi-based approaches [8,24]. In contrast, the use of CRISPR–Cas9 results in permanent gene knockout, allowing investigation of the long-term consequences of gene loss. This strategy, however, is associated with the need for clonal selection and the potential activation of compensatory mechanisms [34].

The choice of an optimal gene silencing method in *in vitro* models should be guided by the research objective, the characteristics of the target gene, and the intended duration of the experiment [8]. In current research practice, complementary approaches that combine multiple silencing strategies are increasingly employed to corroborate findings and enhance the reliability of the resulting conclusions [1,11].

Conclusions

Gene expression silencing methods constitute one of the key research tools used in *in vitro* models to investigate gene function and the mechanisms regulating cellular processes. A review of the literature indicates that the available strategies differ substantially in their mode of action, durability of the effect, and degree of cellular perturbation, which directly affects their suitability for different experimental applications.

Classical approaches based on RNA interference and antisense oligonucleotides enable rapid and generally reversible reduction of gene expression levels. As a result, they are particularly useful in short-term studies and for preliminary validation of gene function. However, these techniques may face several limitations, including off-target effects, variable efficacy, and strong dependence on the efficiency of molecule delivery into cells.

The development of CRISPR technologies has opened new avenues for the regulation of gene expression in *in vitro* models. The use of CRISPR interference (CRISPRi) allows for precise and relatively stable transcriptional repression without introducing permanent changes to the genome. In contrast, the CRISPR–Cas9 system enables permanent gene knockout, facilitating the investigation of long-term consequences of gene loss. The choice between these approaches should be guided by the study objective, the characteristics of the target gene, and the planned observation period.

Based on the reviewed literature, effective gene expression silencing in *in vitro* models requires not only the selection of an appropriate method but also careful optimization of experimental conditions and rigorous validation of the obtained results. Combining multiple silencing strategies and confirming outcomes at several levels of analysis increases the reliability of conclusions and currently represents a recommended approach in functional studies.

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