RNA interference

Functional Genomics in Mammalian Cells by RNAi: Opportunities and Challenges

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Recent technological advances in biological sciences have extended the repertoire of experimentation towards systematic analysis of gene functions. The sequencing of whole genomes has provided scientists with the blueprint of life, and whole genome gene expression array analyses provide a general view of the expression states of cells or tissues. A relatively recent technological addition for systematic analyses has been the discovery of RNAi and its scaling to whole genome analyses. Here, I discuss recent advances that have been made in RNAi screening in mammalian cells and present new possibilities and challenges that this technology offers.

Key Words: RNAi, siRNA, shRNA, esiRNA, off-target-effect, screening

RNAi works by reducing the production of a protein through posttranscriptional gene silencing[1], when double-stranded RNA molecules are cleaved by the endoribonuclease Dicer to form short interfering (si)RNAs. These siRNAs are recognized by the RNA-induced silencing complex (RISC), which searches complementary mRNA molecules and targets them for degradation[2]. Consequently, the reduction of specific mRNAs leads to the reduction of the corresponding protein(s). The major merit of RNAi is its ease and speed of use to perform loss-of-function experiments (Fig. 1). In model organisms like *C.elegans* and *Drosophila*, long double stranded (ds)RNAs that perfectly match a certain transcript can be easily brought into cells via soaking, or feeding, to result in the depletion of the protein(s) of interest. RNAi in mammalian cells is slightly more complicated because

- short dsRNAs (~ 20-30 base pairs long) have to be used to avoid an interferon response elicited by long dsRNA in most mammalian cells and
- the silencing trigger molecules have to be transfected into most cells using transfection reagents.

However, different technologies have been developed to allow the efficient use of RNAi in mammalian cells, which makes this technology a very powerful approach to study gene function in mammalian cells.

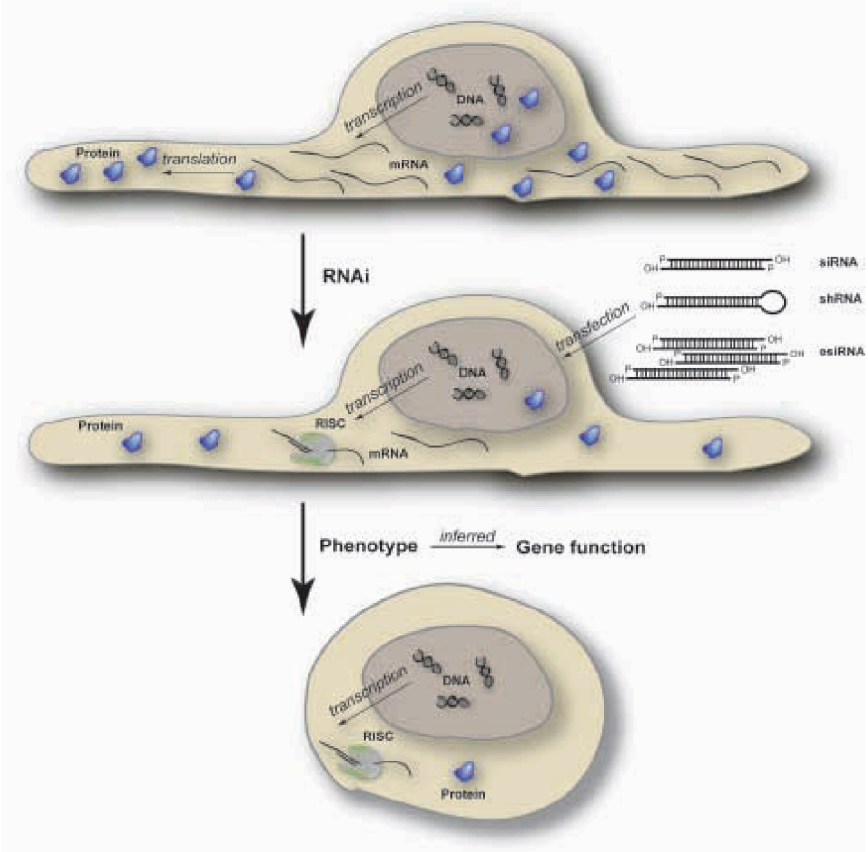

Fig. 1: Schematic presentation of RNAi in a mammalian cell. Critical steps in the silencing process are illustrated.

Which Silencing Trigger to use

The first molecules that were used to trigger RNAi in mammalian cells, without inducing the interferon response, were chemically synthesized siRNAs[3]. Shortly after this discovery, Brummelkamp and colleagues showed that expressed short-hairpin (sh)RNAs can also be used to trigger RNAi in mammalian cells[4]. Chemically synthesized siRNAs are relatively expensive and the costs prohibit the use in large numbers for most laboratories. shRNAs require a higher up-front investment, as DNA oligonucleotides have to be cloned into constructs and verified to confirm the correct sequence. After this initial investment, this resource is, in principle, renewable because the constructs can be propagated in bacteria. For both methods it is important to choose the right sequence, as some sequences work better to trigger RNAi than others. Recent studies have shed light on some aspects of what makes a good silencing trigger. Criteria for an effective design have been derived and can be summarized as: a low internal stability at the 5′ antisense strand and a high internal stability at the 5′ sense strand, a G/C content between 30-50%; internal repeats and palindromes should be avoided, positions 3 and 19 are preferentially an A; Position 10 is preferentially an U; a G at position 13 and a G or C at position 19 should be avoided; close homology to other transcripts should be avoided to reduce possible cross-silencing (see also specificity and off-target effects below). However, we still don't understand every aspect of the RNAi process and, therefore, it is still advisable to test the efficiency of each silencing trigger.

An alternative way to generate effective and specific silencing triggers is the pro-

duction of endoribonuclease prepared short interfering (esi)RNA[5]. For this method, long double stranded RNA is digested with an endoribonuclease, like *E. coli* RNaseIII or purified Dicer, *in vitro*. The pool of siRNAs is then purified to yield ready-to-transfect esiRNA. This method has the advantage of being very cost effective, as the reagents required to produce esiRNA are relatively inexpensive. The method also has the advantage that laborious tests of the silencing efficiencies are not necessary, because the pool of different siRNAs typically contains effective silencers. Because of these facts, esiRNA is also an excellent source for the generation of libraries to target a large number of different genes[6].

Large-scale Screens

Large-scale RNAi screens are useful for many aspects of biology and medicine. First, genome wide RNAi screens in mammalian cells promise an important leap forward in drug target discovery. Cell-based assays exist for many human diseases, that could be adapted to allow a genome wide RNAi screen to identify target genes for a possible therapy (for instance, novel cancer therapy targets). Second, the systematic analyses of gene function in cells may provide novel insights into biological pathways and interactions. Furthermore, the comparative analyses of two different assays (phenotypic profiling) may allow predictions of genetic interaction of different pathways. These analyses may be very important to fuel the relatively new area of systems biology.

The different properties of the developed RNAi reagents allow different types of screens to be carried out[6-9]. In general one can divide these types into two categories: Gene-by-gene screens and positive selection screens (Fig. 2). In gene-by-gene screens, each assay point corresponds to silencing of a single transcript, with a phenotype being assayed. In the positive selection assay, a panel of different transcripts is silenced at the same time in a mixed population of cells. Here, the screen is designed in such a way, that only cells that acquire a certain phenotype will survive. These cells are expanded and the RNAi sequence that resulted in the effect is subsequently identified. By nature of positive selection screens, this approach is not practical to analyze essential genes. The gene-by-gene screen can be carried out with all RNAi resources, although the shRNA libraries have the disadvantage that plasmid transfection is typically less efficient than siRNA transfection and that it is difficult to obtain a normalized virus-based shRNA library. On the other hand, positive selection screens are only practical with shRNA libraries.

Both types of screens have advantages and disadvantages: A major advantage of the selective screen is the ability to pool large numbers of RNAi vectors together, and therefore to reduce the complexity and infrastructure necessary to perform a screen. In addition, long-term expression of the shRNAs allows monitoring of the depletion of proteins with a very long half-life or to monitor the effects after long term depletion of the protein. This is not possible with the transient knockdowns using siRNA- or esiRNA libraries.

A disadvantage of the shRNA libraries is the stability of the library. The shRNA libraries are typically maintained in bacteria, which tend to recombine out the hairpin sequences. Therefore, shRNA libraries may loose complexity after serial replating.

Gene-by-gene screens have the advantage that they are more versatile, and can be adapted to investigate a wide range of biological phenomena. SiRNA- or esiRNA- libraries appear to be more useful for many of these screens, because the dsRNA molecules are small and only have to reach the cytoplasm of the cell. In contrast, the large plasmid expression-based shRNA libraries have to enter the nucleus in order to deliver the silencing trigger. The high cost of chemically synthesized siRNA libraries will have to come down to make this resource useful for academic research. However, the recent development of esiRNA based libraries

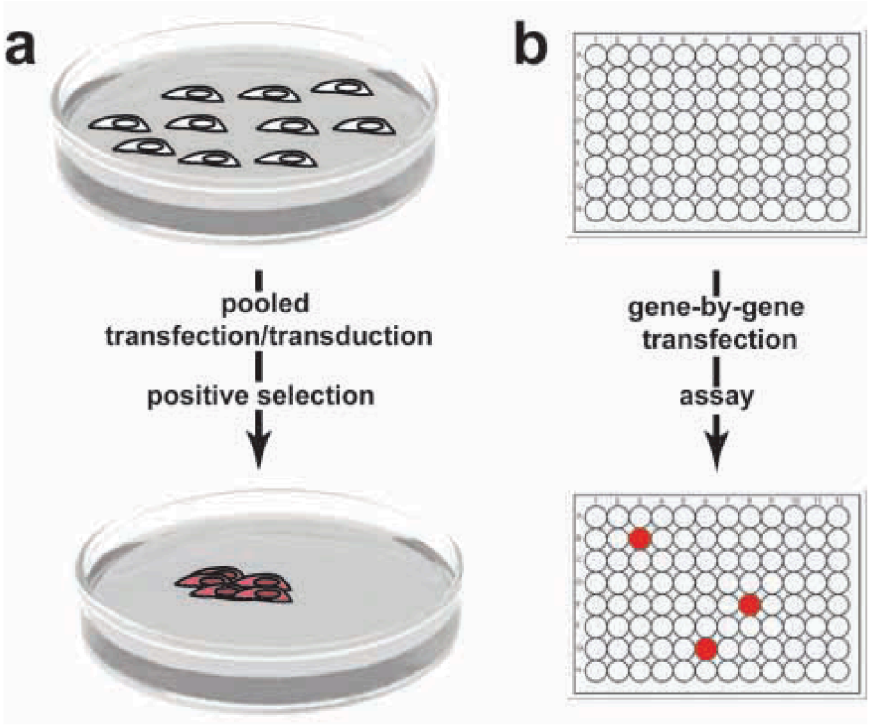

Fig. 2: Strategies of RNAi screens in mammalian cells. The strategy of pooling of DNA-based libraries and transfection or infection in bulk into cells is illustrated in a). The gene-by-gene approach where each silencing trigger is individually transfected in an arrayed format is shown in b).

offer to bring large-scale RNAi screens to an affordable cost for academic research.

Specificity and off-target effects

The possibilities of RNAi screens in mammalian cells have created a lot of excitement and expectation as to what RNAi can deliver for biology and medicine. However, RNAi is not without problems and challenges and caution should be taken to avoid a hangover after the initial euphoria. Other promising approaches had a similar positive tune at the beginning, which was followed by a certain amount of disenchantment (e.g. antisense and ribozyme approaches). A detailed understanding of the mechanism of RNAi is required to design the best experiments. Recent reports have, for instance, reported RNAi-mediated chromatin modifications through DNA methylation[10-11], and translational inhibition of gene expression by siRNAs[12]. Therefore, it is important not to rule out the possibility that RNAi mediated through DNA methylation and/or translational repression may also be playing a role in the observed phenotypes.

A significant challenge for RNAi screens is the specificity of each knockdown. Several groups have reported altered expression of other than the intended target after transfection of a silencing trigger[13,14]. These effects are commonly referred to as off-target effects. An important criterion to minimize off-target effects is to use an optimal dose of the silencing trigger within the cell. It is known that overloading a cell with a silencing trigger increases off-target effects. Therefore, it is important to use no more siRNA agent than necessary to obtain a specific phenotype. This problem is more difficult to solve for expression-based shRNA constructs, as the promoter used to drive the expression of the hairpin determines the amount of shRNA present in the cell. In contrast, the concentration of siRNAs or esiRNAs in cells can simply be adjusted by varying the amount used to transfect the cells.

Another aspect on how to minimize off-target effects is the design of the silencing trigger. The sequence should be chosen such that a minimum of homology is shared with other transcripts. However, it may be difficult to design specific enough siRNAs for each transcript as one study reported off-target effects with as little as 11 contiguous bases of homology[13]. One way to avoid prominent off-target effects is the pooling of different siRNAs targeting the same transcript (Fig. 3). Because each single siRNA targets the same intended transcript, but has a different characteristic off-target signature, the overall off-target effects are diluted out. Because esiRNAs are a pool of different siRNAs targeting the same transcript, they should have a lower risk of generating off-target effects.

How to Verify an RNAi Phenotype?

Given the significant concerns about the specificity of RNAi-mediated repression, it is important to verify the results obtained rigorously. For a start, the observed phenotype should be confirmed in a secondary assay. Ideally, this assay should have a different readout as the primary assay. Second, an independent silencer targeting the same transcript should be used and the identical phenotype should be observed. Here it is important to correlate the observed phenotype with the effectiveness of suppression, because only siRNAs with a similar silencing efficiency are likely to present the same phenotype. Ideally, this comparison should be done at the protein level, because it has been reported that siRNAs that have the same silencing efficiency at mRNA levels show differences at protein levels. Because ultimately, a phenotype correlates to suppression at the protein level, a comparison only at mRNA level could be misleading. Ultimately, the best experiment to demonstrate a phenotype triggered by RNAi is to perform a rescue experiment. This can be achieved by introducing silent mutations into a cDNA construct that is introduced into the cells[15]. When these cells are transfected with siRNAs silencing the endogenous gene, the phenotype should not be visible. Similarly, the siRNAs could be chosen to target a sequence in the 3'-UTR of the endogenous transcript. Again, the expression of a cDNA construct utilizing a different 3-UTR should then result in a rescue of the phenotype. Unfortunately, these approa-

Fig. 3: Comparison of off-target effect with a single siRNA versus a pool of different siRNAs. Note that each siRNA has a different off-target signature (depicted in different colors). Larger and bold characters illustrate that the proportional off-target effects with the single siRNA are higher.

ches have a major caveat in that the expression of the cDNA constructs will typically lead to expression levels that differ from the expression level of the endogenous gene. In addition, the cDNA constructs will only allow the expression of one transcript of the gene. However, many mammalian genes are alternatively spliced and therefore only a fraction of the transcripts coming from one gene will be reintroduced into the cells with a cDNA. Because of these limitations cDNA rescue approaches don't seem to offer a generally applicable solution for RNAi rescue experiments. Approaches are required that allow physiological expression and alternative splicing of RNAi refractory genes in mammalian cells. One way to solve this problem may be the use of large genomic fragments that allow physiological expression and alternative splicing.

Perspective

In a very short time RNAi has emerged as a remarkable new pathway of gene regulation and as a powerful tool to study gene function in mammalian cells. Taking the precautions mentioned above, and further dissecting the molecular mechanisms underlying RNAi, will further expand our knowledge on biological processes and make RNAi more useful as a tool for systematic analyses of gene function(s). In the near future we will witness a wealth of new data resulting from whole genome screens. Importantly, mammalian cell lines are an excellent source to decipher many aspects of mammalian biology because many cell-based assays already exist to be plugged into whole genome RNAi screens. Especially, the use of reporter cell lines will be useful and their detailed analyses via time-lapse microscopy will create detailed information on the phenotypes. These cellular-genomics approaches will allow phenotypic profiling of cellular processes and will, therefore, add another dimension to the existing genomic approaches. These data sets will also create a new challenge for bioinformatics, because sophisticated algorithms will be required to streamline data analyses. Furthermore, RNAi holds the potential of revolutionizing the treatment of human disease. If precise delivery protocols can be established to target specific cells, RNAi may be the method of choice to regulate malfunctioning cells in the body. Only time will tell if RNAi will stand up to these high expectations.

Acknowledgements

I thank Judith Nicholls for critical reading of this manuscript.

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