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Functional genomics using high-throughput RNA interference

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RNA interference (RNAi) describes the post-transcriptional silencing of gene expression that occurs in response to the introduction of double-stranded RNA into cells. Application of RNAi in experimental systems has provided a great leap forward in the elucidation of gene function. To facilitate large-scale functional genomics studies using RNAi, several high throughput approaches have been developed based on microarray or microwell assays. Recent establishment of large libraries of RNAi reagents combined with a variety of detection assays further opens the door for genome-wide screens of gene function in mammalian cells.

The completion of human and mouse genome projects has resulted in vast amounts of sequence information and the identification of thousands of open reading frames. For most of these genes, functional information about their protein products remains unknown. High throughput approaches in functional protein analysis are required to acquire this information, thus stimulating the development of novel technologies.

DNA microarrays have become a well-established research tool in modern genetics. This technique is based on the arrangement of hundreds or thousands of DNA or oligonucleotide sequences on a solid support, such as glass or nylon, so that each DNA spot can be identified by its coordinates. Microarrays have been used primarly in high throughput gene expression profiling studies, in which the expression of thousands of genes can be simultaneously analysed. These studies, however, do not provide any information about the expression of proteins. During their biosynthesis, many proteins are post-translationally modified by acetylation, glycosylation, phosphorylation or cleavage. Hence, the functional effects exerted by the expression of any single gene are often multilateral. Because virtually all targets for drug development are proteins, the development of high throughput methodologies for their analysis is in great demand.

A recently developed transfected-cell array (TCA) technique is being seen as a breakthrough for high throughput functional genomics in cell biology [1–3]. Full-length open reading frames of genes cloned in expression vectors are printed at a high density on a glass slide along with a lipid transfection reagent. The microarray is subsequently covered with a layer of cells. Cells growing on top of the DNA spots are transfected, resulting in the expression of specific proteins in spatially distinctive groups of cells (Figure 1). The phenotypic effects of this 'reverse transfection' of hundreds or thousands of gene products can be detected using specific cell-based bioassays.

As each cell cluster expresses a particular protein and cell clusters are spatially separated, the TCA can be considered as a particular type of protein microarray. The TCA applies eukaryotic cells, allowing for post-translational modification (e.g. glycosylation) of expressed proteins. Utilization of different cell lines provides the opportunity to screen for protein functions where cell-type-dependent post-translational modifications and protein–protein interactions are important. Hence, investigation of protein function within the context of the living cell represents an

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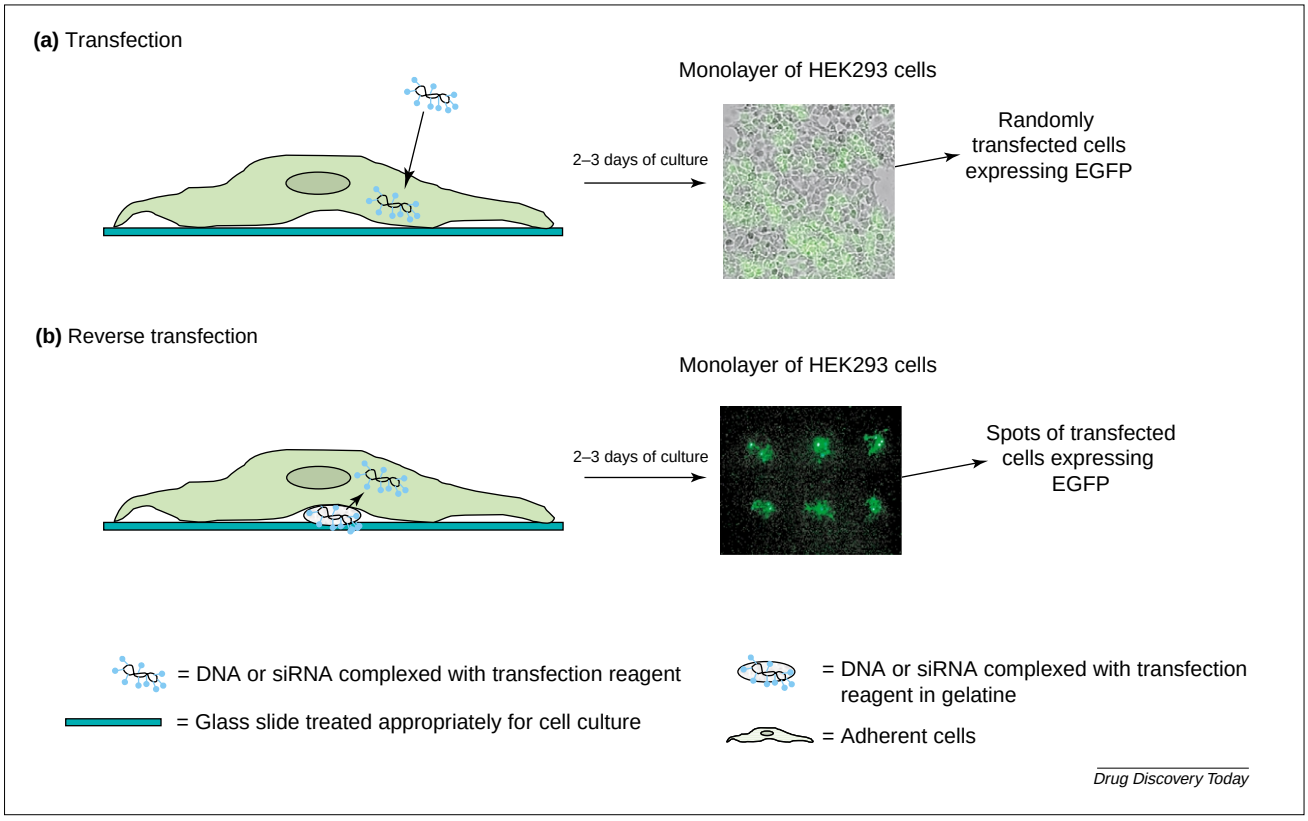

FIGURE 1

Principle of reverse transfection. In the course of normal transfection **(a)**, the cells are randomly transfected with a nucleic acid entering the cell from the surrounding solution. The principle of the cell arrays is based on the reverse transfection process **(b)**, whereby nucleic acid enters the recipient cell from the solid support. Thus, spatial separation of the transfection events can be achieved in the same experiment.

attractive alternative in genome-wide functional studies, especially for RNA interference (RNAi) approaches.

RNAi in non-mammalian cells

Two of the most common ways to investigate gene function in the context of the entire organism are gain-of-function and loss-of-function studies using trangenic and knockout animal technology, respectively [4]. However, both approaches have drawbacks. One is the enormous cost and time required for generating genetically modified animals. Moreover, the majority of genetically modified animals do not display a distinct change in phenotype that would allow for its unambiguous linkage to the mutated gene [5]. Taking these into account, there is a clear need for the identification of candiate genes before endeavouring on *in vivo* studies.

RNAi was first described in nematodes [6] and has quickly become the fastest expanding field in molecular biology. The mechanism of action of RNAi is based on sequence-specific interactions between small interfering RNAs (siRNAs) and mRNA molecules (Figure 2). These interactions trigger several enzymatic reactions that result in the degradation of targeted mRNA molecules.

Naturally occurring RNAi silencing is involved in the response to viruses and transposable elements as well as gene regulation and heterochromatin remodelling. In plants, virus infection induces a RNAi response, which targets viral mRNAs for degradation [7]. In *Caenorhabditis elegans*, RNAi has been proposed as a protective mechanism in the maintenance of genome stability against endogenous transposon activity [8]. Double-stranded RNA can also induce methylation of endogenous sequences homologous to viroids in infected plants [9]. Similarly, sequence homology of dsRNA to certain promoter sequences leads to the methylation of the latter [10]. Furthermore, RNAi-related proteins, such as piwi, are required for transgene silencing at transcriptional and post-transcriptional levels in *Drosophila* [11]. Naturally occuring small (22 nt) non-coding RNAs known as microRNAs (miRNAs) have been discovered in animals, including mammals, and plants. miRNAs regulate gene expression through two modes of action. In plants, miRNA binds complementary mRNA, leading to its distruction through the RNAi machinery [12]. In contrast, animal miRNAs do not require perfect complementarity to their mRNA targets and the mechanism of action is related to inhibition of protein synthesis [13]. It is suggested that animal genomes contain several hundred miRNA genes, which are largely regulated by their own promoters [14]. There are still limited data concerning the function of miRNAs. In *C. elegans*, miRNAs regulate expression of nuclear proteins and transcriptional factors [15]. In *Drosophila*, miRNAs are involved in regulation of

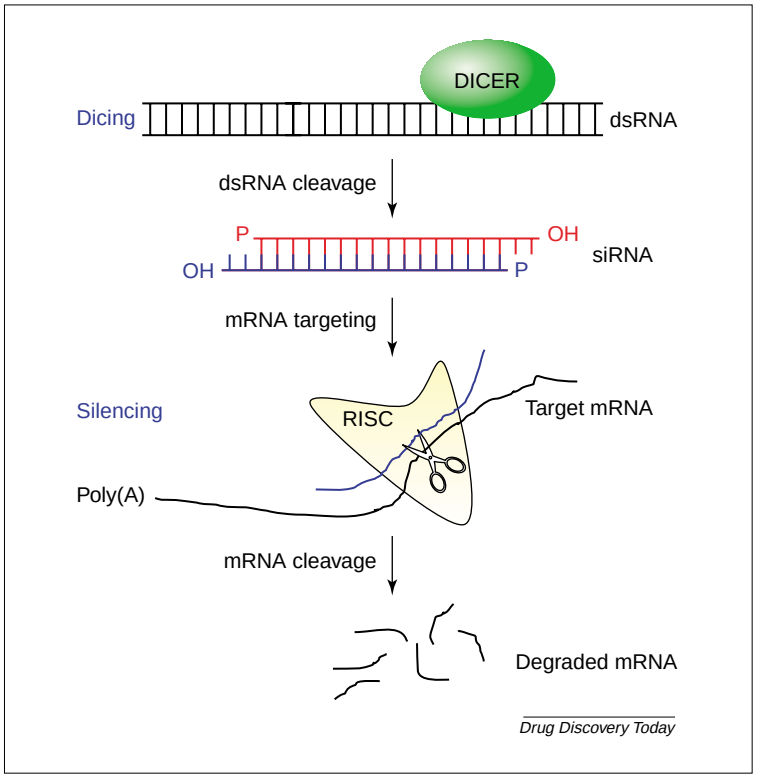

FIGURE 2

RNAi silencing mechanism. Long double-stranded RNA (dsRNA) is processed to short interfering RNA (siRNA) duplexes by the enzyme Dicer, which exhibits RNase-III-like activity. A single strand of the siRNA is incorporated into the group of cytoplasmic proteins to form a RNA-induced silencing complex (RISC). Activated RISC, guided by the antisense siRNA strand, performs endonucleolytic cleavage of target mRNA. Thus produced mRNA fragments are rapidly degraded by cytoplasmic nucleases.

apoptosis and fat metabolism [16]. Finally, prediction studies towards identification of the mRNA targets for mammalian miRNAs suggest a broad range of molecular functions such as regulation of transcription, signal transduction or protein and nucleotide binding [17].

Although discovered as a natural process, RNAi quickly proved to be an excellent way for efficient gene silencing [18]. Initially, silencing studies were performed in lower eukaryotes such as *C. elegans* [5], or *Drosophila melanogaster* [19,20] using long dsRNA. Silencing of single genes was soon followed by high throughput approaches, mostly in *C. elegans*. Taking advantage of fast and inexpensive ways of dsRNA transfer by soaking the worms in dsRNA solution [21] or feeding them with bacteria expressing dsRNA, many cellular functions were analysed, including fat storage [22] and protection against DNA mutations [23]. Because the silencing effect of RNAi in worms is transmitted to offspring [24], the physiological impact of gene knockdown can be observed throughout the development of the animal. Moreover, the RNAi response is systemic in character so that silencing of gene expression is observed in tissues distally located from the place of dsRNA delivery [25]. Several recent studies on *C. elegans* showed clearly that the RNAi approach can generate dozens of phenotypes, leading to the discovery of new genes and their functional characterization [26,27]. For example, Kamath *et al.* [26] performed RNAi analysis of 86% of the predicted 19 427 genes in the *C. elegans* genome and identified 1722 mutant phenotypes out of which two-thirds were not previously accociated with a phenotype. Another example of genome-wide RNAi application comes from the studies of Lettre *et al.* [27], who aimed to identify genes affecting apoptosis in *C. elegans*; 21 genes stimulating apoptosis have so far been identified. Because apoptotic pathways are conserved, it has been suggested that many of these genes are involved in maintenance of mammalian genome stability, p53 activation and regulation of fertility. The *Drosophila* genome has also been subjected to RNAi analysis. Boutros *et al.* [28] investigated growth and viability of *Drosophila* cells using a library of 19 470 dsRNAs. They targeted 91% of predicted *Drosophila* genes and found 438 dsRNAs identifying essential genes. In particular, a homolog of mammalian acute myeloid leukemia gene (*AML1*) was characterised for its role in cell survival [28]. Lum *et al.* used an RNAi approach in their study on signaling responses to the Hedgehog protein [29]. Systematic screening of all kinases and phosphatases resulted in identification of a cell-surface protein (Dally-like protein) required for Hedgehog signal reception and a tumor suppressor casein kinase 1α.

siRNAs allow gene silencing in mammalian cells

Initially, application of RNAi in mammalian cells was hampered by the fact that dsRNA molecules longer than 30 nt trigger an interferon response [30], which ultimately leads to cell death. By contrast, 21 nt siRNAs do not stimulate the interferon response [31,32] and can be delivered into cells either as chemically synthesized molecules [33] or by transfection of plasmids expressing siRNA [34]. A wide range of mammalian genes involved in apoptosis [35,36], the cell cycle [37,38] and signal transduction [39,40] have been successfully knocked down using this technique. Chemically synthesized siRNAs are easy to transfect and a high intracellular concentration of siRNA molecules can be achieved. High synthesis costs and a need for silencing efficiency evaluation, however, hinder their wide application in high throughput studies (Box I).

Gene silencing using direct transfection of siRNA is transient and is limited by the rate of cell division. This is because mammalian cells, unlike *C. elegans*, are not able to amplify RNAi. To overcome this obstacle and to increase the cost effectiveness of RNAi studies, vector-based systems for siRNA delivery have been developed [34,43–45]. Cells can be stably transfected with plasmid-expressing short hairpin RNA (shRNA) for long-term sustainment of silencing. Moreover, shRNA plasmids can carry drug resistance markers, making the cells suitable for selection. These vectors contain a RNA polymerase III promoter that drives the synthesis of shRNAs. The shRNAs are then processed intracellularly into siRNA-like molecules [46]. Alternatively, two tandem polymerase III promoters have been used to

BOX 1

Target specificity of siRNA molecules

In contrast to the long dsRNAs used in non-mammalian cells, siRNA molecules have to be optimised to efficiently knockdown a particular mRNA, as the effectiveness of gene silencing depends upon the localisation of the targeted mRNA region. Despite well-developed sequence design algorithms, each target mRNA must be verified for an efficient siRNA, as the silencing potential of siRNAs to different gene regions can differ dramatically. Consequently, genome-wide approaches based on chemically synthesized siRNAs are very expensive. A cost-effective alternative has recently been developed whereby siRNA molecules are generated from long dsRNA by enzymatic processing using purified RNase III from *Escherichia coli* [41,42]. Partial digestion of dsRNA by RNase III results in production of endoribonuclease-prepared siRNA (esiRNA) of 20–25 bp in length, which efficiently mediates RNAi in cultured mammalian cells. The esiRNA prepared from a single dsRNA can target multiple sites within mRNA, thus increasing effectiveness of gene silencing. Interestingly, shorter siRNAs (12–15 bp) arising as a result of more extensive RNase digestion are not able to induce an RNAi response in mammalian cells [41].

obtain sense and antisense siRNA sequences from expression constructs (convergent transcription). In addition to plasmid-based systems, PCR-derived and enzyme-generated siRNA expression cassettes based on single- or dual-promoter systems can be used to efficiently suppress gene activity [47].

The silencing effect can be further prolonged using retroviral or lentiviral RNA expression vectors [48–50]. Viral-based RNA delivery systems are chosen for the transfection of primary cells [51]. Primary cells are difficult to transfect, but they represent the most interesting target for gene silencing because their physiology mirrors the clinical or physiological condition of tissues or organs from which they have been derived. Transfection by electroporation has recently been proposed as an alternative to virus-based RNAi delivery [52,53].

Finally, the development of cell- and tissue-specific RNAi expression constructs will lead to greater versatility and more refined applications of this technology. Although most of the vector-based RNAi systems were used for silencing a limited number of genes, large siRNA expression libraries in high throughput RNAi screens allow for the identification of new genes involved in different cellular processes. Indeed, large siRNA expression libraries have already been used in cell-based RNAi screens and have generated important results (summarized in Table 1).

Although ~3000 disease-modifying genes are supposed to be present in the human genome, only ~500 are subject to pharmaceutical investigation [54]. Therefore, the adaptation of genome-wide loss-of-function screens using siRNAs for the interrogation of complex phenotypes promises to lead to the development of novel targets and consequently novel therapeutic strategies. However, there are still some limitations of this technology that have to be overcome [55]. It has been observed that gene silencing by siRNA is not entirely sequence specific [56,57]. False-positive results can arise owing to sequence-independent off-target effects resulting from an interferon response induced by some siRNAs but more prominently by shRNAs [58,59]. Alternatively, sequence-specific off-target responses can be caused by crossreaction of the siRNA with related sequences or by interference with transcription [60]. As most off-target effects are likely to be concentration dependent, much effort is being put into the development of better methods for introducing siRNA into cells to reduce both the amount of siRNA necessary for transfection and the cellular toxicity of transfection reagents.

False-negative results can be expected depending upon the cell type and gene of interest, possibly owing to redundancy of target genes whereby losing the function of one gene is compensated for by products of functionally related genes. More frequently, however, inefficient knockdown of the RNA of interest and/or a long half-life of the analysed protein will hamper the identification of important gene functions. Consequently, to obtain better gene silencing, the prediction and validation of the best targeted region within the gene has to be further improved.

siRNA transfection was found to be more efficient compared with that of plasmid DNA expressing shRNA in both conventional [61] and reverse transfection assays (unpublished observations). However, it was demonstrated at least in one study that chemically synthesized siRNA- and vector-based-shRNA-mediated silencing could be obtained with comparable efficiencies in microarray-based cell transfections [62].

Technological platforms for RNAi

At present, most large-scale RNAi-based genetic screens of cells in culture have used 6-, 12-, 24-, 96- or (in some cases) even 384-well plate formats. These high throughput assays have allowed for the selection of siRNAs that effectively reduce mRNA and protein levels [63] but, more importantly, they have the potential to identify key factors that play a role in biological processes. However, until now most of these RNAi screens have been limited to selected subsets of genes for particular protein families.

As large libraries of shRNAs become available (see Table 1) and companies start to offer comprehensive sets of chemically synthesized siRNAs, there is a growing need to take high throughput RNAi screening for mammalian cells beyond the microwell format. In order to study multiple cellular processes, protein detection technologies have to be adopted by large-scale screening platforms, with analogy to the analysis of gene expression using DNA microarrays.

Reverse TCAs are a suitable platform for high throughput cellular assays using RNAi and are, in many aspects, superior to the microwell-based approaches (see Table 2). Several groups, including the author's laboratory, have focused on adapting the TCA platform for the purpose of large-scale

TABLE 1

Published large-scale RNAi libraries

Delivery system	Description	Application	Number of targeted genes	Number of siRNAs/ clones	Refs
In vitro synthesized siRNA	Chemically synthesized siRNA library	Identification of modulators of TRAIL-induced apoptosis	510	510	[75]
	Chemically synthesized siRNA library	Identification of negative regulators of the phosphoinositide 3-kinase pathway	30	148	[76]
EsiRNA	*Escherichia coli* RNase III-mediated cleavage of dsRNA into esiRNA	Inhibition of hepatitis C virus RNA replication by esiRNAs	4	26	[41,77]
Plasmid vectors					
Pdual	A dual promoter siRNA expression system that allows the facile construction of siRNA expression libraries using a single-step PCR protocol	Identification of genes involved in NF-κB signaling	>8000 human genes	>16 000	[78]
REGS	Restriction enzyme-generated siRNA (REGS) system for building unique siRNA vectors from cDNAs	Silencing of Oct-3/4 and MyoD	>4x10^4	>4x10^5	[79]
ShRNAi	Development of tandem-type and hairpin-type siRNA expression vectors in piGENE hU6 vector to generate an siRNA expression library	Identification of genes involved in apoptosis	>384	>384	[80]
PHippy	A convergent opposing siRNA expression system using 'HI inverted U6 promoter plasmid' and PCR-amplified target sequences	Silencing of PGL3 luciferase and low-density lipoprotein receptor-related protein 6	>120	120	[81]
Expression Arrest™ [a]	A large-scale library of RNAi-inducing shRNA expression vectors targeting human and mouse genes	Identification of genes involved in proteasome function	>15 000	>28 000	[45]
PSUPER	Collection of 50 shRNA-expressing constructs against de-ubiquinating human enzymes	Discovery of the inhibitory effect of cylindromatosis tumour suppressor gene (*CYLD*) on NF-κB gene expression	50	50	[82]
Virus vectors					
EPRIL	Enzymatic production of RNAi library (EPRIL) by which cDNAs are converted through a sequence of enzymatic treatments (PCR, DNase I digestion) into an RNAi library	Isolation of gene (*IP3R*)-specific shRNA clones	>240	>240	[42]
SPEED	SiRNA Produced by Enzymatic Engineering of DNA (SPEED), a retroviral shRNA library derived from double-stranded cDNA library	Isolation of gene (*CD45*, *CD53*)-specific shRNA clones	3x10^6	3x10^6	[83]
Nki library (pRetroSuper)	A human shRNA library in the pRetroSuper vector, including a barcode system for the rapid identification of individual siRNA clones associated with a specific phenotype	Identification of new components of the p53 pathway	7914	23 742	[66]

[a]Library consists of sequence-verified shRNA expression cassettes contained within multi-functional vectors. NF-κB, nuclear factor-κB; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

loss-of-function studies [62,64,65]. RNAi cell arrays are generated by printing different siRNAs in gelatin solution onto a modified glass surface. The array can be either stored or immediately treated with transfection reagent and covered with a monolayer of recipient cells. Transfection efficiency can be monitored using fluorochrome (e.g. rhodamine) conjugated siRNAs [64] or by cotransfection with reporter plasmids [65].

Although conceptually similar to array-based screens in multi-well format, the TCA method significantly reduces the costs for high throughput loss-of-function screens, as it requires less reagents and cells per assay. Furthermore, the method uses technologies already well-developed for the generation (printing robots) and analysis (array scanners) of DNA arrays, making the technology widely accessible.

High throughput RNAi screening can be performed in array format or using pooled siRNA libraries. In the former, each siRNA sequence is tested individually using microwell- or cell array-based assays. Alternatively, by pooling small numbers of RNAi reagents, the effect of silencing mulitple genes in one cell can be interrogated; thus a reduced number of assays has to be performed to screen the whole library. This strategy has already been applied with vector-based libraries. It is particularly useful in combination with

TABLE 2

Comparison of microwell plate- and microarray-based RNAi platforms

96-microwell plate	Cell array
Advantages	
Possibility of flow-cytometric analysis	Low reagent consumption (reagents necessary for single-well transfection sufficient for up to 500 reverse transfections)
Possibility of transfection by electroporation (primary cells)	Cost-effective system for silencing with chemically synthesized siRNAs
Suitable for non-adhesive cells	Higher throughput (up to 10 000 samples) in a single experiment
Cost-effective for smaller sample sets	Possibility of long-term storage of printed microarrays
Well-established functional assays for microwell plate format	Suitable for analysis on a single cell level (e.g. subcellular protein distribution)
Disadvantages	
Higher consumption of reagents	Limited to adhesive cells
Higher number of cells per sample required	Not suitable for screening of protein targets with a long turn-over (over 72 h)
High throughput experiments require expensive automation of dispensing processes	Transfection limited to lipid-based approaches
Detection of the collective signal from many cells	Low number of targeted cells per sample hampers advanced statistical analysis

selective screens, whereby loss of function leads to the selective survival or death of cells [66]. In the case of pooled siRNA libraries, individual shRNA vectors can be identified, linking the vector sequence with a unique DNA barcode. To track the fate of the individual shRNAs in transfected cells, barcodes are detected through hybridisation with microarrays containing oligonucleotide probes complementary to particular barcodes [45,66].

Targeted genes are either exogenous genes, introduced by transient or stable transfection of expression plasmids, or endogenous genes. Targeting of exogenous genes allows for the evaluation of the silencing capacity of different siRNAs specific for the same target gene, and can be used as a validation platform for functional siRNA selection [62,67]. Functional validation of potential siRNA molecules is still necessary despite several algorithms that exist for sequence design [46]. Indeed, for most algorithms, it is estimated that only one out of two, or even one out of five, designed siRNAs will efficiently knockdown a target gene [67]. When using cell arrays, large numbers of individual siRNAs can be validated so there is no need to pool siRNAs corresponding to the particular gene target or to introduce sequence identifiers such as molecular barcodes.

Targeting of endogenous genes by reverse transfection of siRNA molecules opens the possibility of parallel examination of many genes that regulate, or are supposed to regulate, particular cellular processes such as apoptosis. Gene silencing can be monitored on cell arrays by specific fluorescent monoclonal antibodies. On the array, clusters of cells that are negative will appear as 'holes' in a monolayer of cells that otherwise express the protein [64]. The power of cell arrays, however, lies in the ability to analyse downstream cellular processes that are affected by gene silencing, either separately or in combination through siRNA multiplexing. For example, specific antibodies can be used to detect changes in the phosphorylation state of cell membrane bound receptors or transcription factors [68]. This allows for the identification of essential or novel cell signaling molecules using genome-wide siRNA libraries, and is of particular importance when protein tyrosine phosphatases [69] and kinases [70] are considered as drug targets for human diseases such as diabetes, cancer and autoimmunity. Alternatively, gene silencing can induce cellular processes such as apoptosis or changes in intercellular adhesion that can be monitored by specific reagents (e.g. Annexin V) or analysed through morphological changes of transfected cells, respectively [1]. For the identification of genes regulating apoptosis, reverse transfection of siRNAs can be combined with administration of prodrug, which is a precursor converted to drug by endogenous activity (e.g. doxorubicin, tumour necrosis factor-α). Several specific siRNAs efficiently sensitize cancer cells for proapoptotic stimuli [71,72], which can help develop novel therapeutic strategies. The possibility of combining microwell assays with cell arrays by printing small arrays in microwells is particularly attractive. This allows for the simultaneous screening of compounds on multiple genes per microwell, significantly reducing the cost and need for manipulation of assays.

Concluding remarks

One of the possible applications using RNAi cell arrays is the identification of transcription factors acting on target genes. Predicted promoter regions [73] can be cloned in vectors upstream of reporter genes, and cell lines stably expressing the reporter construct can be subjected to genome-wide siRNA arrays.

Similarly, cell lines derived from gene-targeted mice can be used. Gene trapping is a high-throughput approach that is used to introduce insertional mutations across the genome, resulting in a collection of targeted mice. Gene trap vectors simultaneously mutate and report the expression of the endogenous gene at the site of insertion [74] and can therefore be used to analyse cell- and tissue-specific regulation of gene expression. RNAi screens using both stable transfected cell lines (minimal promoter constructs) and gene-trapped cell lines (endogeneous regulated expression) could lead to the identification of transcription or signal transduction factors involved in gene-specific expression activity.

Despite its short history, RNAi has already profoundly changed the landscape of functional gene analysis. It has greatly contributed to the characterization of gene products and allowed cost-effective large-scale loss-of-function studies on a genome-wide level. Combination of microarray

technology and cellular biology methods should in the future provide additional impetus toward the development of robust RNAi-based screening methods and consequently toward efficient drug target selection and evaluation.

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