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RNA interference: gene silencing in the fast lane

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Abstract

Sequencing of whole genomes has provided new perspectives into the blueprints of diverse organisms. Knowing the sequences, however, does not always tell us much about the function of the genes that regulate development and homeostasis. RNA interference (RNAi) is becoming the method of choice for gene function analysis in cells and whole organisms. Here we review the approaches available to perform RNAi experiments in mammalian cells and in mice. We discuss usage of RNAi in cancer research and as a possible therapeutic tool for cancer treatment.

Keywords: RNAi; siRNA; RNase III; Gene silencing; Whole embryo culture

1. Introduction

Cancer cells arise from somatic cells due to alterations in the genetic program that circumvent normal maintenance and surveillance. Gene expression profiles and proteomic analyses of different cancers have shown a broad diversity of alterations, implying that many genes and many pathways may be involved in cellular transformation. To understand how genetic changes lead to various cancers, we first need to identify and understand the function of each individual player involved in the reprogramming process. Only with this knowledge will we be able to put each function into a context that can explain why and how altered genetic programs promote the uncontrolled growth of cells within an organism.

A powerful way to analyze gene function is to examine phenotypic changes after gene inactivation in cells or in whole organisms. In some cells or organisms, gene inactivation can be achieved in a directed manner, using homologous recombination, such as in *Escherichia coli* [1], *Saccharomyces cerevisiae* [2], the chicken DT40 cell line [3,4], or in mice through the use of engineered embryonic stem cells [5,6]. In cells or organisms where homologous recombination is not practical, randomly mutagenized organisms can be screened for lesions in a gene of interest. Unfortunately, these processes are time consuming and cost intensive. Given the number of genes in mammalian genomes, methods that accelerate gene function analyses are required to speed up discoveries in cancer research, and indeed in many different biological areas.

Antisense and ribozyme technologies are relatively straightforward techniques for gene function analyses [7,8]. However, while both techniques have been successfully used in some gene knock down experiments, they are not generally applicable due to the lack of specificity and incomplete efficiency. Recently, RNA interference (RNAi) has emerged as a powerful approach to silence genes in a variety of organisms. This technique uses gene specific double-stranded (ds) RNA to knock down gene expression at the level of messenger RNA [9]. Starting from the first paper by Fire et al. [10], RNAi has taken the scientific community by storm and has rapidly become a widely used method to test gene function in species as diverse as worms, flies, plants and mammals. This review summarizes methods for the effective synthesis of siRNAs and approaches for RNAi mediated gene silencing in mammalian cells. It focuses on the role of RNAi in cancer biology and mammalian developmental biology, with an outlook of the possible role of RNAi as a therapeutic tool.

2. RNAi as a functional genomic tool

Shortly after the original report of dsRNA mediated gene silencing in *Caenorhabditis elegans* [10] it was realized that this technology is particularly suitable for high-throughput functional genomic studies. Only 2.5 years after the initial report, two groups reported the functional analysis of almost all genes on *C. elegans* chromosomes I and III, respectively

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[11,12]. In fact, all 19,427 genes encoded by the *C. elegans* genome have since been analyzed [13] (A. Hyman, personal communication). The rapid analysis of gene function in *C. elegans* has been facilitated by the completion of the *C. elegans* genome sequence and through the ease of delivery of the dsRNA by soaking worms in dsRNA or by feeding worms *E. coli* that express dsRNA [14]. Although the delivery of dsRNA into other organisms may be more difficult, the use of RNAi as a functional genomic tool is not restricted to *C. elegans* and is particularly useful in organisms with completely sequenced genomes in plants [15], flies [16], fungi [17], and vertebrates [18].

3. How to trick the interferon response in mammalian cells

To mediate a gene specific RNAi effect in *C. elegans* or *Drosophila*, long dsRNA molecules can be used. The RNA is either injected into the animal or taken up through the digestive tract and is then processed in cells to silence the corresponding gene. The fact that dsRNA can have an effect on gene expression in mammalian cells has been known for a while. Long dsRNA triggers the interferon pathway in most mammalian cells by activating the dsRNA-activated protein kinase PKR [19,20]. Unfortunately, the PKR response initiates overall inhibition of gene expression and/or cell death. As a consequence, long dsRNA is not useful for specific gene silencing in most mammalian cells.

Biochemical analyses of the RNAi pathway revealed that long dsRNA is processed into approximately 20–25 bp fragments within cells by an RNase III like endoribonuclease called Dicer [21–24]. Detailed analyses of these processed RNAs, which are termed short interfering (si) RNA, revealed that they contain 2-nt 3′-end overhangs [25], the signature of all RNase III enzymes [26]. Interestingly, siRNA does not trigger a strong interferon response in mammalian cells, whilst efficiently and specifically acting as a silencing trigger for a corresponding gene [27]. This finding brought RNAi to mammalian cells and has enabled researchers to analyse gene function in mammalian cells. Currently, the exact length of dsRNA that triggers the interferon response is not known. However, the minimum length that induces interferon mediated cell death in many cell lines is certainly longer than 30 bp (F. Buchholz, unpublished data).

4. Generation of short interfering RNA

The first publications describing the use of siRNA to silence genes in mammalian cells used chemically synthesized RNA that was designed and annealed to form the typical structure of siRNA. Since then different methods to generate siRNA have been developed (summarized in Table 1). While the chemical synthesis of siRNA is still the most widely used method, it bears some disadvantages. First, it is currently the most expensive way to generate siRNA. Furthermore, due to the varying silencing efficiency of each possible siRNA that covers the target mRNA, different siRNAs have to be tested to identify one that works well. Even though rules have been defined that allow a prediction of the silencing efficacy of a designed siRNA [28], experimental tests that confirm these predictions are still required. A more cost effective way to generate siRNA is through in vitro transcription of either two complementary ssRNAs or through the generation of short hairpins that also work well in triggering an RNAi response. However, screening for effective molecules is also required for these methods. We refer to the references in Table 1 for a detailed description of these methods.

An alternative, and very cost effective way to generate siRNA is the enzymatic processing of siRNA molecules from long dsRNA with purified RNase III in vitro (Fig. 1) [29]. Recently, RNase III has become commercially available, eliminating the need to purify the enzyme in the laboratory. With this method the need to screen for effective siRNAs is eliminated, because the mixture of different siRNAs usually contains effective molecules. Furthermore, in comparison to other methods, oligos only have to be ordered once for all genes of interest. For all other methods different oligos have to be ordered for each gene. The IMAGE consortium collection of cDNA clones contains most genes expressed in a variety of organisms. For the human genome, for example, the German Resource Center for Genome Research has recently released a clone collection, that contains approximately 32,000 unique, sequence verified cDNA clones [30]. The vast majority of these clones are available

Table 1
Methods for generating siRNA

Method	Advantage	Disadvantage	Duration	References
Enzymatic digestion of long dsRNA	Cheap No screening for effective molecule	May cross-silence highly homologous genes	Transient	[29]
Chemical synthesis	Large quantities can be made Base modifications are possible	Expensive Requires screening for effective molecule	Transient	[27,59]
In vitro transcription	Cheap	Requires screening for effective molecule	Transient	[60]
In vitro transcription of hairpin	Cheap	Requires screening for effective molecule	Transient	[60,61]
Expression of hairpin from Pol III-promoter	No in vitro transcription required	Transfection of plasmid DNA is less effective Requires screening for effective molecule	Transient and stable	[60–65]

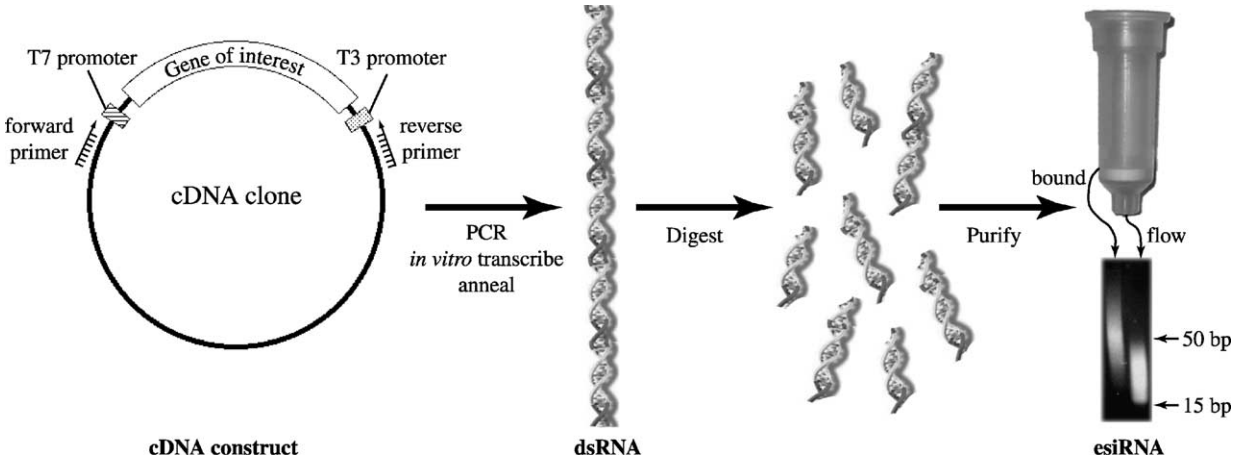

Fig. 1. Scheme for the preparation of endoribonuclease prepared siRNA. Important elements within the vector and the binding sites for the universal primers are depicted. An agarose gel from a typical esiRNA preparation is shown.

in constructs that harbor phage promoters and inserts that can be amplified using universal primers. The PCR product obtained with these primers can be directly used for in vitro transcription, followed by annealing of dsRNA and RNase III digestion. Endoribonuclease prepared short interfering (esi) RNA can be conveniently purified using standard spin columns and precipitation (see also Fig. 1). Because of its cost effectiveness and ease of production, this method is also very well suited for high throughput screens.

5. RNA interference in mammalian tissue culture cells

As quickly as the RNAi technology was adopted by the worm community, siRNA technology is being applied to mammalian tissue culture cells. Genes with very different functions have been successfully knocked down [18], indicating that siRNAs are widely applicable in mammalian cells (see also Table 2). HeLa cells are the most commonly used cell line, because it is easy to transfect and maintain. However, more and more groups have had success with cells that are difficult to transfect, such as primary cells [31–36]. For these cells, protocols that have been developed for plasmid transfection seem to work well for delivery of siRNA, including lipofection, non-cationic lipids, calcium phosphate transfection, and electroporation. It is important to test different transfection protocols for any given cell line, since the efficiency of knockdown is directly related to the delivery of siRNA into cells. In general, the transfection efficiency of siRNA seems to be higher than for plasmid DNA, independent of the transfection method and cell line

Table 2
RNAi in cancer research

Gene ontology	Origin of tumor	Silenced genes	Transfection method	References
Chromatin	Colon carcinoma	*DNMT1, 3A, 3B*	Lipofectin (Invitrogen)	[66]
	Hepatocellular carcinoma	*MBD2*	Oligofectamine (Invitrogen)	[67]
	Prostate carcinoma	*EZH2*	Oligofectamine	[37]
Apoptosis	Osteosarcoma	*53BP1*	Oligofectamine	[68,69]
	Cervical carcinoma	*DEDD*	TransIT-TKO (Mirus)	[70]
	Adenoviral transformed fibroblasts (E1A), lung carcinoma, osteosarcoma, colorectal carcinoma, breast carcinoma, cervical carcinoma	*Caspase-2, Apaf-1*	Oligofectamine	[71]
	Neuroblastoma	*p73*	Oligofectamine	[72]
	Osteosarcoma	*Fortilin, MCL1*	TransIT-TKO	[73]
Cell cycle	Retroviral transformed embryonal fibroblasts	$p21^{Cip1/Waf1}$	Oligofectamine	[35]
	Colon carcinoma	*KLF4*	DMRIE_C (Invitrogen)	[74]
	Osteosarcoma	*CP110*	Oligofectamine	[75]
	Adenovirus transformed kidney cells	*Spy1*	Calcium phosphate	[76]
Signal transduction	Prostate carcinoma	*p300*	Oligofectamine	[77]
	Bladder carcinoma	*SPK1, SPK2*	Oligofectamine	[78]
Tumor suppressor	Colon carcinoma	*DIP13*	Oligofectamine	[79]
Viral genes associated with cancer	Cervical carcinoma	*HPV E6, E7*	Oligofectamine	[80]
Oncogenic fusion proteins	Myeloid leukemia	*AML1/MTG8*	Electroporation	[81]
	Myeloid leukemia	*BCR-ABL*	Electroporation	[82]

used. Most likely this is due to the fact that plasmid DNA is larger and has to be delivered to the nucleus, whereas siRNA is small and is active in the cytoplasm. For many cell lines the efficiency of siRNA delivery with standard transfection methods is above 80%. Thus effects can be studied without using selection systems.

6. Silencing cancer associated genes

Gene function analyses in mammalian cancer cell lines have been rather difficult without RNAi technology. Most cell lines have a low targeting frequency for gene disruption by homologous recombination. This approach is further complicated by the fact that, because of a diploid genome, two rounds of targeting have to be conducted. In the case of an aneuploid cell line, a gene knockout through homologous recombination is virtually impossible. Antisense- and ribozyme approaches work for some genes, but these approaches are not reliable enough for systematic gene silencing in tissue culture cells. In contrast, RNAi offers an effective and convenient way to assay gene function in mammalian cancer cell lines. With this technology it should be possible to decipher genetic pathways that are required for uncontrolled proliferation, survival, metastasis, angiogenesis, etc. Interesting candidate genes could then be investigated as targets for therapeutical intervention. A handful of groups have already explored the usefulness of RNAi to study cancer-associated genes in tissue culture cells (Table 2). Genes belonging to different categories and different protein turnover rates were silenced. This list will certainly expand over the next few years and the knowledge gained will result in a better understanding of malignant cell growth, and lead to novel treatments.

7. Screening the genome for cancer associated genes

The sequenced human and mouse genomes have revealed that they contain 30,000–40,000 genes. One can assign some functional information to about 40% of these genes. For 60% there is no functional information, and even for many of the genes where we have some information, we still know very little about what they really do and how they interplay within gene networks. It took only 2.5 years to go from the first description of RNAi in *C. elegans* to large-scale functional genomic screens in this organism. It is only a question of time when the first genomic screen utilizing siRNAs in mammalian cells will be published. These screens will be a rich ground for new discovery, because many assays for gene function are available in tissue culture cells. The libraries of siRNAs simply have to be plugged into these assays to identify novel players in the pathways (see Fig. 2). Simple screens for inhibition of cell proliferation or apoptosis may be used to identify novel cancer associated genes. More elaborate screens may identify how these genes are

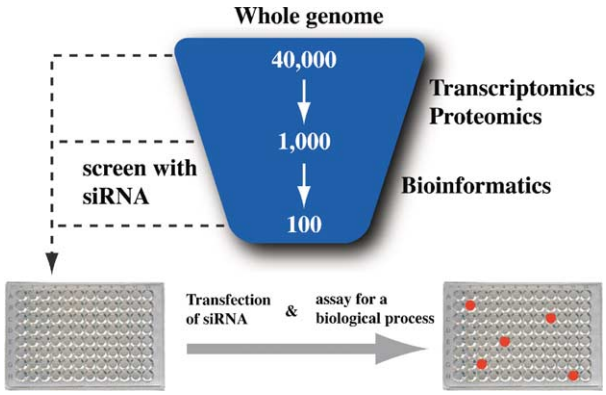

Fig. 2. Scheme for the identification of putative genes of biological processes using RNAi. RNAi in mammalian cells employing siRNAs can be used to screen whole genomes for putative genes. In order to reduce the number of siRNAs, this approach can be combined with other functional genomic approaches such as transcriptomics and proteomics, and bioinformatics.

associated to the malignant phenotype. Screening of large numbers of genes has the disadvantage that high throughput equipment is required to perform the screen. However, it is not always necessary to conduct whole genome screens. Instead, other functional genomic analysis methods, including gene expression profiling and proteomics, can be combined to reduce the pool of candidates (Fig. 2). Varambally et al. recently published an exciting example on this topic [37]. They used gene expression profiling on metastatic prostate cancers and identified 55 genes that were significantly up regulated. The polycomb group protein enhancer of zeste homolog 2 (EZH2) was at the top of the list of up regulated genes. To investigate whether this up regulation had biological meaning, the authors used RNAi to disrupt EZH2 expression in established prostate cancer derived cell lines. Interestingly, cell proliferation was greatly reduced in the cell lines investigated. This analysis identified *EZH2* as an important gene for diagnosis of metastatic prostate cancer. Even more interesting, *EZH2* may itself be a useful target for therapeutic intervention.

8. RNA interference in mice

While the screening in tissue culture cells is an important step to identify and test gene function and more specifically the function of cancer associated genes, they are not suitable to investigate gene function on the level of a whole organism. For these tests model organisms are required that allow physiological analysis of target genes. In *C. elegans*, dsRNA included in the food of the animal is taken up by the cells of the organism and disseminated [38]. Furthermore, in this animal the RNAi effect is systemic [10,39]. Delivering siRNA into cells in mammals will certainly be more difficult. For cancer research the mouse is a valuable model organism due to its relatively close evolutionary

relationship with humans, its short reproductive cycle and because its genome can be readily manipulated by molecular means [40–42]. Some success with delivering siRNA into adult mice has been reported [43,44] using the high pressure hydrodynamic transfection method [45,46]. Using this method efficient silencing of reporter genes were observed in some organs. Other groups have used viral delivery of siRNAs to infect cells within the animal [47], or generated transgenic animals that expressed hairpin siRNA directed against a reporter gene [48]. Another group reported silencing of a reporter gene in a grafted tumor cell line in nude mice [49].

Many cancer associated genes play an important role during normal embryonic development. The role these genes play during embryogenesis can give important clues for their role in cancer and can provide hints for therapeutic intervention. Both preimplantation embryos [50–52] and postimplantation embryos [53] have been investigated via RNAi. Studying mammalian development at postimplantation stages has the disadvantage that embryogenesis happens in utero and the embryo is difficult to access and visualize. Typically, embryos at different stages of development are dissected and compared. Recently, important advances have been made in whole embryo culture of mouse embryos [54]. These systems allow the observation of normal mouse development for up to 2 days in vitro. In addition, whole-embryo culture can conveniently be combined with various methods of introducing DNA into cells of the developing embryo, including electroporation (Fig. 3A) [55–57]. More importantly, siRNA can be efficiently delivered into cells of the developing mouse and specifically silence targeted genes (Fig. 3B) [53]. These assays allow fast and convenient silencing of genes in the developing mouse and should speed up functional analyses to identify genes that are implicated in tumor development and/or progression.

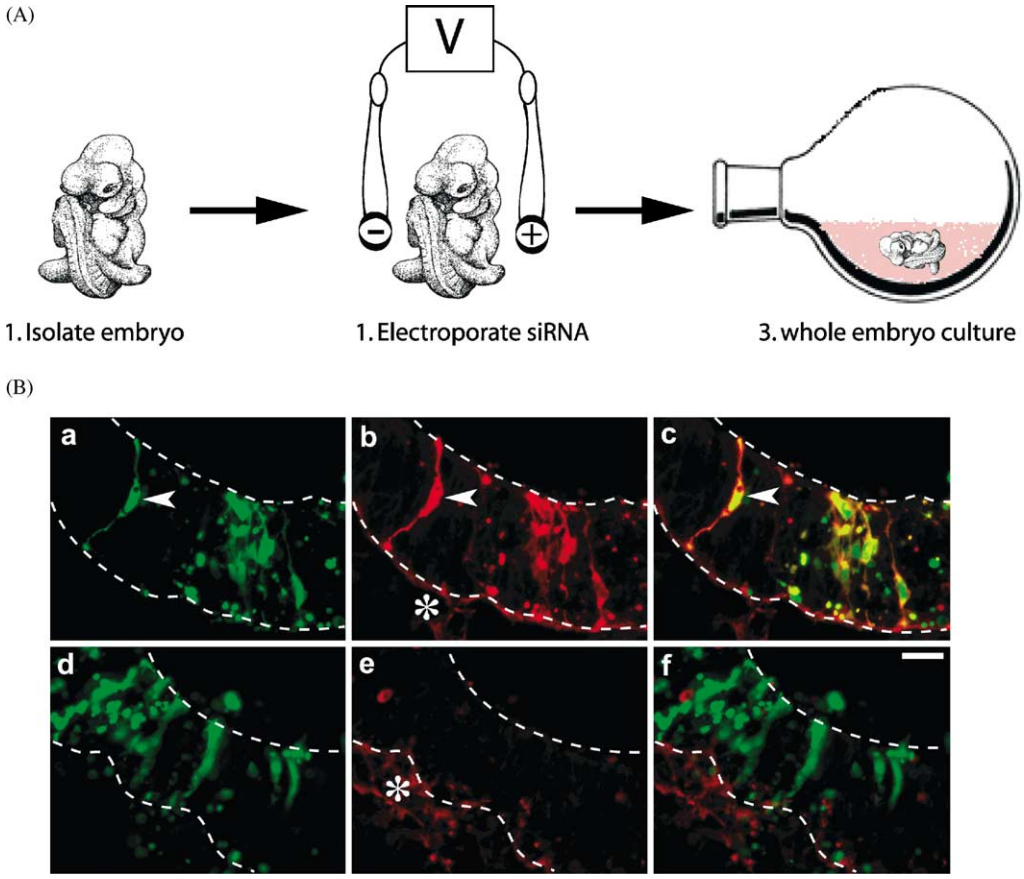

Fig. 3. RNAi in the postimplantation mouse embryo. (A) Cartoon illustrating the delivery of siRNA into the mouse embryo by electroporation, followed by whole embryo culture. (B) E10 mouse embryos were injected, into the lumen of the telencephalic neural tube, with the GFP-expressing plasmid pEGFP-N2 plus the βgal-expressing plasmid pSVpaXΔ, either without (a–c) or with (d–f) βgal-directed esiRNAs, followed by directed electroporation (lateral, cathode-right/anode-left orientation) and whole-embryo culture for 24 h. Horizontal cryosections through the left telencephalon were analyzed by double fluorescence for expression of GFP (green; a, d) and βgal immunoreactivity (red; b, e). Neuroepithelial cells expressing both GFP and βgal (arrowheads) appear yellow in the merge (c, f). Note the lack of βgal expression in neuroepithelial cells in the presence of βgal-directed esiRNAs. Upper and lower dashed lines indicate the lumenal (apical) surface and basal border of the neuroepithelium, respectively. Asterisks in (b, e) indicate the basal lamina and underlying mesenchymal cells, which cross-react with the secondary antibody used to detect βgal immunoreactivity. Scale bar in (f), 20 μm. Panel (B) from [53] "Copyright (2002) National Academy of Sciences, USA."

9. How to deliver?

It is undeniable that the use of siRNAs to silence specific genes will transform biology. This technology has a big impact on gene function analyses and in comparison with other functional genomic tools, like DNA micro arrays, generates phenotypes that directly show the relevance of the studied gene in the examined process. Most certainly, this technology will help to identify novel cancer associated genes.

It is more difficult to speculate which impact RNAi will have on the therapeutic site. The results obtained in mice are very promising. However, it is more than questionable whether high-pressure hydrodynamic transfection, or electroporation can be applied in humans. More promising may be the delivery of siRNAs via a lentivirus, adenovirus or other delivery vectors that are used in gene therapy. However, problems with integrating vectors have recently arisen from gene therapy trials [58]. Therefore, the biggest challenge for RNAi as a therapeutic tool will be a way to specifically deliver the effective molecules into cells in the body.

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