Perspectives

Functional Genomic Analysis of Cell Division by Endoribonuclease-Prepared siRNAs

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KEY WORDS
RNAi, gene silencing, screening, off-target effects, mitosis, siRNA, esiRNA, shRNA

ABBREVIATIONS
RNAi RNA interference
siRNA short interfering RNA
shRNA short hairpin RNA
esiRNA endoribonuclease-prepared siRNA
dsRNA double-stranded RNA
RISC RNA-Induced Silencing Complex
GFP Green Fluorescent Protein
APC Anaphase-Promoting Complex

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ABSTRACT

The emergence of RNA interference (RNAi) technology has revolutionized functional genomic analyses in cell biology, including the study of cell division. In particular, the introduction of short interfering RNAs (siRNAs) and short hairpin RNAs (shRNAs) has facilitated loss-of-function studies in mammalian cell lines. We have pioneered the rapid and cost-efficient generation of libraries of endoribonuclease-prepared short interfering RNAs (esiRNAs) for large-scale genetic screens in mammalian tissue culture cells. Our first pilot screen of 5305 genes in human Hela cells identified novel genes required for cell division, establishing esiRNA as a sophisticated method for loss-of-function screens. Future genome-wide studies with esiRNA, using image-based assays and video microscopy, will help provide novel insights on the molecular mechanisms that govern cell division in mammalian cells.

Cell division in eukaryotes requires proper chromosome duplication, condensation, segregation, spindle assembly and cytokinesis. These processes have been extensively studied over many years in different model organisms. The completion of the human genome, and the genomes of key model organisms, has provided the opportunity to identify many genes involved in cell division. However, there are probably many genes, which contribute to the division of a cell that are still waiting to be discovered. The identification of these genes requires efficient tools for the analysis of gene function. In particular, large-scale loss-of-function screens can speed up the discovery of genes involved in cell division. For a long time such studies have been limited to yeast, in which gene inactivation can be easily achieved in a large scale. While these studies have certainly provided fundamental insights, the use of yeast imposes limitations for the understanding of cell division and cell cycle progression in multicellular eukaryotes, e.g., for disease-associated aberrations of these processes, such as cancer.

Recently, RNAi has emerged as a powerful tool for loss-of-function studies in a variety of eukaryotes. RNAi is an evolutionarily conserved defence mechanism, in which genes are specifically silenced through degradation of mRNAs mediated by homologous double-stranded (ds)RNA molecules. The analysis of the processing of dsRNA in RNAi revealed that long dsRNA is digested by the RNase III-like enzyme Dicer. The resulting 21–23 nt long dsRNAs, or siRNAs, trigger specific degradation of mRNAs with a complementary sequence in the RNA-induced silencing complex (RISC). In this complex, the antisense strand of a short dsRNA is used to recognize complementary mRNAs, which are then degraded by endonucleolytic cleavage. The use of RNAi to study gene functions by specific gene silencing was first introduced for invertebrates such as C. elegans. This was done by introducing long dsRNA into the cells of these organisms. Genome-wide RNAi screens using this approach have identified novel genes for diverse cellular processes, including cell division, in worm and fruitfly.

Because long dsRNA causes a strong, nonspecific interferon response in many vertebrates, siRNAs have to be used for specific gene silencing in these organisms. siRNAs are either chemically synthesized or generated by in vitro and in vivo transcription. Alternatively, shRNAs can be used, which are intracellularly processed into siRNAs and have also proven to be efficient mediators of RNAi. The use of siRNAs and shRNAs has provided very useful tools for functional genomic studies in mammalian cell lines.

Two drawbacks of chemically synthesized siRNAs that may hamper their use for large-scale screens are the variable inhibitory abilities of different siRNAs and the cost-intensive synthesis. Consequently, each siRNA must be screened for its silencing efficiency, or more than one siRNA per gene has to be used to ensure efficient gene silencing. The use of shRNA or siRNA expression libraries, which are in principle unlimited...
resources, may overcome the cost problem of using chemically synthesized siRNAs for academic research. In addition, the ability of these approaches to generate stable knockdowns may be useful for positive selection screens and for long-term analyses of knockdown phenotypes. However, there are also some disadvantages for expression vectors in gene-by-gene screens. First, the transfection of plasmid DNA is in general less efficient and more toxic than for siRNAs. Second, the alternative use of virus infection in a large scale may be problematic because of differences in virus titers. Third, in contrast to siRNAs, the concentration of intracellularly expressed dsRNAs cannot be closely controlled.

Yang et al. have pioneered an approach to circumvent these problems by preparing siRNA through digestion of long dsRNA with a Dicer-like enzyme in vitro (Fig. 1a). This process generates a great variety of siRNAs able to interact with multiple sites of the target mRNA. By using bacterial RNase III, which can be easily expressed in E. coli and purified, a heterogeneous siRNA population of a gene is generated by limited digestion of long dsRNA in vitro. The resulting esiRNAs have proven to knock down genes efficiently in cell culture and in the developing mouse. Because esiRNAs can target multiple sites, screens for effective molecules are not necessary. In addition, the pooling of different siRNAs inherent to the esiRNA approach may ensure a higher specificity of RNAi phenotypes (Fig. 1B). Recent publications reported off-target effects for individual siRNAs, that in addition to the targeted genes, led to changes in the expression of other genes on both mRNA and protein levels challenging the specificity of RNAi in loss-of-function studies. Because these effects apparently require a relatively low level of sequence similarity to the off-target mRNA, it can be assumed that most siRNAs have an individual cross-silencing signature. Pooling of siRNAs has been suggested to reduce off-target effects while maintaining efficient silencing of the specific target gene. Especially, esiRNAs representing hundreds of different siRNAs may exhibit a reduced risk for cross-silencing as long as highly conserved mRNA regions are avoided as target sites. For these reasons and due to the simplicity, cost-effectiveness and time-efficiency of the esiRNA synthesis this approach appears to be a versatile method for large-scale screens in mammalian cells.

We have recently used esiRNA for large scale analysis of genes required for cell division in human cells. For this pilot study, we generated an esiRNA library from a cDNA clone collection representing 15,497 human genes and screened 5305 esiRNAs from this set. Assuming that many genes essential for cell division would result in reduced viability, we used a metabolic assay as a rapid initial screen. For instance, the visualization of chromosomes by the use of fluorescent reporter cell lines, e.g., for chromosomes, spindle and centrosomes, will certainly enable a more detailed analysis of RNAi phenotypes. The rapid cell viability assay we used in the pilot screen efficiently detected pronounced mitotic defects leading to cell death or a reduction in cell growth. For future studies, imaged-based primary assays will certainly further facilitate the identification of cell division phenotypes. Such visual screens require automated microscopes and quantitative analyses using computerized software. Several systems for automated image-based assays have become available in recent years, which will enable genome-wide screens with visual outputs. Positives from such primary visual screens can be further analyzed by time-lapse video microscopy. The use of phase-contrast video microscopy in our pilot screen allowed a detailed description of cell division phenotypes. The combination of video microscopy with fluorescent reporter cell lines, e.g., for chromosomes, spindle and centrosomes, will certainly enable a more detailed analysis of RNAi phenotypes. We have already performed fluorescent time-lapse microscopy for some of the genes that we identified in our pilot screen. For instance, the visualization of chromosomes by the use of a Histone-GFP (H2B-GFP) cell line allowed a more detailed characterization of mitotic phenotypes. The knockdowns of the spliceosome protein SNW1, the anaphase-promoting complex (APC) subunit CDC16 and the valosin-containing protein (VCP or p97), resulted in mitotic arrest phenotypes as apparent by the increased number of rounded-up cells in the phase contrast images (Fig. 2). However, the specific stage of arrest in mitosis could not be

Figure 1. Principle and advantages of esiRNA technology. (A) Schematic presentation of esiRNA synthesis. (B) Comparison of off-target effects between esiRNA and a single siRNA. Note that each siRNA may contain a different cross-silencing signature (depicted by different letters). Larger letters for a single siRNA indicate a potentially higher risk for off-target effects.

esiRNA as a versatile method for RNAi screens in mammalian tissue culture cells. The remaining ~10,000 esiRNAs from our library that were not included in our first screen are currently being used in the same way to identify additional genes required for cell division.

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The rapid cell viability assay we used in the pilot screen efficiently detected pronounced mitotic defects leading to cell death or a reduction in cell growth. For future studies, imaged-based primary assays will certainly further facilitate the identification of cell division phenotypes. Such visual screens require automated microscopes and quantitative analyses using computerized software. Several systems for automated image-based assays have become available in recent years, which will enable genome-wide screens with visual outputs. Positives from such primary visual screens can be further analyzed by time-lapse video microscopy. The use of phase-contrast video microscopy in our pilot screen allowed a detailed description of cell division phenotypes. The combination of video microscopy with fluorescent reporter cell lines, e.g., for chromosomes, spindle and centrosomes, will certainly enable a more detailed analysis of RNAi phenotypes. We have already performed fluorescent time-lapse microscopy for some of the genes that we identified in our pilot screen. For instance, the visualization of chromosomes by the use of a Histone-GFP (H2B-GFP) cell line allowed a more detailed characterization of mitotic phenotypes. The knockdowns of the spliceosome protein SNW1, the anaphase-promoting complex (APC) subunit CDC16 and the valosin-containing protein (VCP or p97), resulted in mitotic arrest phenotypes as apparent by the increased number of rounded-up cells in the phase contrast images (Fig. 2). However, the specific stage of arrest in mitosis could not be
determined with this assay. In contrast, the analysis of movies of Histone-GFP cells revealed profound differences between the three phenotypes (movies are available at www.mpi-cbg.de/esiRNA): The depletion of \textit{SNW1} led to an impaired alignment of chromosomes resulting in a prometaphase arrest. Some of the arrested cells completed mitosis with the formation of micronuclei. Silencing of the APC subunit \textit{CDC16} caused a metaphase arrest consistent with the function of the APC in the transition from metaphase to anaphase. The knockdown of \textit{VCP} resulted in metaphase formation followed by several unsuccessful attempts of the cell to move the chromosomes to the poles, suggesting that \textit{VCP} is required for chromosome segregation. These examples highlight the potential of fluorescent reporter cell lines for future screens.

The prediction algorithms for the design of efficient siRNAs have improved with the growing mechanistic understanding of RNAi.\textsuperscript{24,25} Likewise, we aim to further improve the quality of esiRNA. Although >90% of esiRNAs generated from cDNA clones exhibit a silencing efficiency >70% at the mRNA level, the use of predesigned esiRNA can further improve the overall silencing efficiency (unpublished data). This can be achieved by in silico analysis of the mRNA sequence using state-of-the-art design criteria for siRNA implemented in the software DEQOR.\textsuperscript{26} We have already performed an analysis of all human mRNAs of known and predicted protein-coding genes in order to select the most specific and efficient region of mRNA for esiRNA production. Based on this analysis we have designed T7 tagged PCR primers, amplifying regions with a length of 400–600 bp for in vitro transcription and RNase III digestion. Currently, the generation of the ~20,000 PCR products is underway, and the genome-wide human esiRNA library will be completed by the middle of 2005. The PCR template library can be easily reamplified with one universal T7 primer, and will be made available as an unlimited resource to the scientific community.

In summary, esiRNA library technology is an efficient and specific screening tool. The use of esiRNAs in large-scale visual screens will certainly uncover genes associated with cell division, which will be starting points for novel insights into this fundamental biological process.

**References**


