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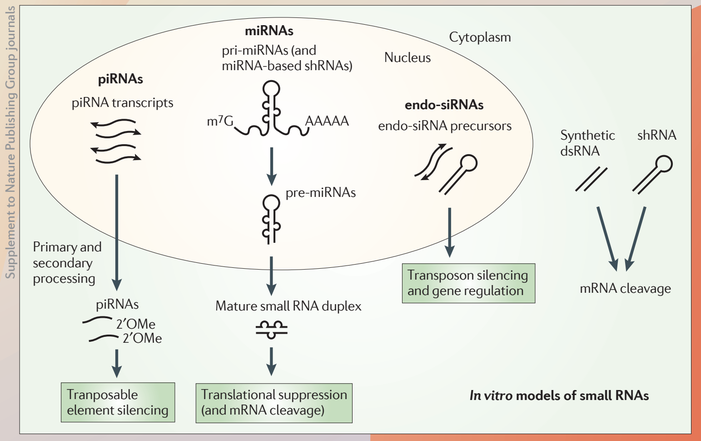
GENETICS

Tools for studying and using small RNAs: from pathways to functions to therapies

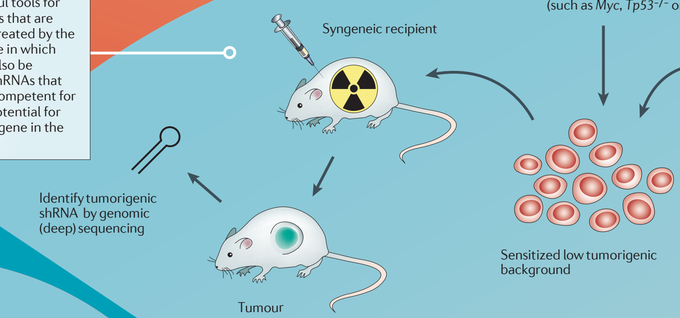
Kenneth Chang and Gregory J. Hannon

During the past decade, small RNAs have emerged as crucial regulators of gene expression and genome function, having roles in almost every aspect of biology[1]. Many small RNAs act through RNA interference (RNAi)-related mechanisms, which involve programming the RNA-induced silencing complex (RISC) to recognize and repress targets. One class of small RNA, the microRNAs (miRNAs), naturally regulates programmes of gene expression. Altered miRNA function contributes to human disease, and manipulation of specific miRNAs is now being pursued as a novel therapeutic modality. Small RNAs have also been adapted for use as tools based on reprogramming the RNAi machinery to silence specific coding or non-coding RNAs. These tools have been exploited to investigate gene function in cultured cells and in living animals. Genome-scale collections of silencing triggers permit phenotype-based genetic screens to be carried out easily in organisms in which they were previously difficult or impossible. Such strategies are being used to discover and validate new therapeutic targets, and small RNAs themselves may offer a mechanism for inhibiting targets that are currently viewed as 'undruggable'.

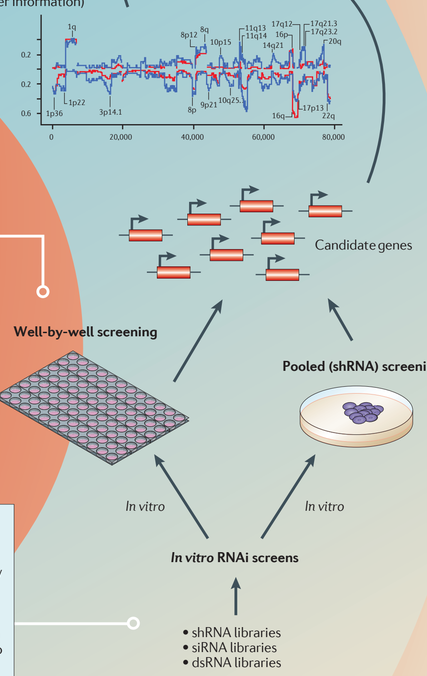


Small RNA biogenesis
Currently, our understanding of small RNA biogenesis is most complete for small RNAs that exert their effects through RISC. In animals, three main classes of such small RNAs are recognized: small interfering RNAs (siRNAs), PIWI-interacting RNAs (piRNAs), and miRNAs[1]. siRNAs are derived from double-stranded precursors that are processed by the RNase III family enzyme, Dicer. In mammals, endogenous siRNAs are most abundant in germ cells, but in invertebrates they are more widespread. miRNA precursors contain short hairpin segments that contain the mature miRNA sequence. These precursors are processed through the serial action of two double-stranded RNAses (dsRNAses), Drosha and Dicer. piRNAs are mainly expressed in germ cells, in which they guard against the activity of transposons. Their biogenesis remains to be fully understood.

***In vivo* approaches**
Short hairpin RNAs (shRNAs) are particularly powerful tools for probing gene function in transgenic animals. Animals that are mosaic for knockdown of a gene of interest can be created by the transplantation of shRNA-modified stem cells[23]. Mice in which knockdown is achieved throughout the animal can also be produced[24]. This can be achieved using optimized shRNAs that are integrated into a specific genomic locus that is competent for expression in most tissues. This strategy offers the potential for tissue-specific or regulated repression of nearly any gene in the tissue or cell type of interest.



Small RNA tools for screening
Small RNA tools have revolutionized the ability to scan genomes for proteins that have an effect on a specified phenotype. Libraries of long dsRNAs have been widely used in worms and *Drosophila melanogaster* cells, and collections of transgenic flies expressing dsRNA triggers are readily available[17–19]. In mammals, both genome-wide and subgenomic, focused libraries of synthetic siRNAs and shRNA expression constructs are widely used[20–22]. Screening can be carried out using each trigger individually, often using high-content measurement methods, or in multiplexed formats, which often use a selective pressure to detect an impact on a specific process.

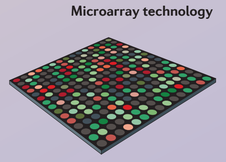


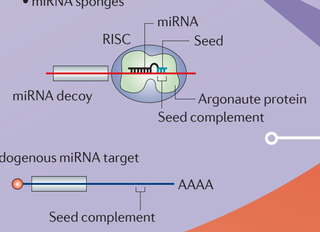
Types of small RNA tools
The repressive potential of small RNA pathways can be reoriented towards selected cellular genes, enabling studies of gene function. In mammals, two types of RNAi triggers are widely used. siRNAs are transiently delivered to cells, usually in culture, and enter RISC without the need for further processing[11]. shRNAs must be expressed from a transiently delivered or genomically integrated vector and must be processed by Drosha and Dicer or, in some cases, Dicer alone[12–14]. siRNAs are simple to use but silence their targets only transiently, limited by the persistence of the chemically synthesized trigger. shRNAs offer more flexibility; integrated constructs can provide essentially permanent repression and the shRNAs themselves can be expressed from regulated promoters, enabling tuning of repression levels and examination of reversible effects[15,16].

Small RNAs in therapy
Both single-gene and genome-wide approaches using small RNAs have great potential for the discovery of new therapeutic targets for a variety of diseases. Some of these targets will lie within protein classes that are amenable to conventional drug discovery. Others will be among protein classes generally considered 'undruggable'. In these cases, small RNAs themselves offer a novel therapeutic modality, although delivery of small RNAs to target cells *in vivo* has proved challenging. Many groups are also pursuing the use of gene therapy approaches to express shRNAs in specific disease contexts.

Targeting small RNAs for therapeutics
Alterations in miRNAs themselves, or in their binding sites on crucial targets, have been associated with human disease. This presents an opportunity to agonize or antagonize specific miRNAs for therapeutic benefit. Several strategies have been developed to deliver antagomirs *in vivo*. These approaches have been validated in preclinical models, including rodents and primates, and some miRNA antagonists have entered human clinical trials[25].

Small RNA discovery
Small RNA discovery has been revolutionized by next-generation sequencing. Following ligation of specific linkers to small RNAs, cDNAs can be produced, which are ideally suited to sequencing using short-read platforms. Such approaches have expanded the catalogue of small RNAs that are thought to act through RISC and have enabled the discovery of novel small RNA classes. Deep sequencing has also proved useful for monitoring the expression of annotated small RNAs, but alternative strategies, such as microarrays and quantitative PCR (qPCR), also provide economical alternatives. Databases now offer online catalogues of known small RNAs[2].





TOOLS FOR STUDYING SMALL RNAs

FUNCTION

Small RNA function
Understanding the function of small RNAs often begins with studying their expression patterns. The identification of potential targets relies on the assumption that the small RNA and the target must share some sequence complementarity. Computational algorithms predict miRNA targets on the basis of the presumed character of miRNA–mRNA interactions and the conservation of their binding sites[3,4]. Several biochemical methods of target identification have also been developed, such as crosslinking immunoprecipitation (CLIP)[5] and tandem affinity purification of miRNA target mRNAs (TAP-Tar)[6]. Functional studies of small RNAs can rely on conventional genetic strategies, such as knockouts. Overexpression of miRNA and the use of miRNA antagonists are also popular approaches. Antagomirs are chemically synthesized miRNA-complementary oligonucleotides with modified chemical backbones that act as antisense inhibitors of miRNA function[7–9]. Genetically encoded 'decoys' called miRNA sponges that contain miRNA binding sites function similarly to antagomirs by titrating the miRNA away from its natural targets[10]. Model organisms, such as *Caenorhabditis elegans*, *Drosophila melanogaster* and zebrafish, have also proved to be key for understanding the function of conserved small RNAs.

Abbreviations
m7G, 7-methylguanosine cap; 2′OMe, 2′-*O*-methyl; MSCV, murine stem cell virus.

Affiliations
Kenneth Chang and Gregory J. Hannon are at Cold Spring Harbor Laboratory, Watson School of Biological Sciences, 1 Bungtown Road, Cold Spring Harbor, New York 11724, USA.

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