In vivo RNAi: Today and Tomorrow

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SUMMARY

RNA interference (RNAi) provides a powerful reverse genetics approach to analyze gene functions both in tissue culture and in vivo. Because of its widespread applicability and effectiveness it has become an essential part of the tool box kits of model organisms such as *Caenorhabditis elegans, Drosophila,* and the mouse. In addition, the use of RNAi in animals in which genetic tools are either poorly developed or nonexistent enables a myriad of fundamental questions to be asked. Here, we review the methods and applications of in vivo RNAi to characterize gene functions in model organisms and discuss their impact to the study of developmental as well as evolutionary questions. Further, we discuss the applications of RNAi technologies to crop improvement, pest control and RNAi therapeutics, thus providing an appreciation of the potential for phenomenal applications of RNAi to agriculture and medicine.

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1 INTRODUCTION

Genetic screening is one of the most powerful methods available for gaining insights into complex biological processes. Indeed, much of what we have learned from model organisms such as *Saccharomyces cerevisiae*, *Caenorhabditis elegans,* and *Drosophila melanogaster* can be traced back to genetic screens designed to identify sets of mutations that perturb specific processes. For example, screens in yeast have led to the identification of key regulators of the cell cycle (Hartwell et al. 1974); screens in *C. elegans* have identified the genetic regulation of programmed cell death (Horvitz et al. 1999); and screens for mutations that cause embryonic lethality in *Drosophila* have elucidated the logic of body patterning in a multicellular organism (Nusslein-Volhard and Wieschaus 1980; St Johnston and Nusslein-Volhard, 1992).

Over the years many improvements and tools for genetic manipulation have become available, and as a result there now exist powerful "tool-boxes" for each model organism (Nagy et al. 2003; Venken and Bellen 2005; Kaletta and Hengartner 2006). Sophistication in approaches and tools facilitates the ease of genetic screening as well as the identification of genetic alteration(s) and requisite follow-up analyses of mutant phenotypes. For example, innovations such as mosaic analysis and tissue-specific expression of transgenes have allowed researchers to study gene function in a wider variety of tissues, stages, and contexts.

Soon after the initial discovery by Fire and Mello (Fire et al. 1998) that double-stranded RNAs (dsRNAs) can be used to knockdown the activity of individual genes, many RNA interference (RNAi)-based methods were (and continue to be) added to the tool-boxes of various organisms. These methods have truly revolutionized the field of functional genomics because of their relative ease, and most significantly, because RNAi, unlike more traditional genetic screening methods, provides a powerful reverse genetic approach, especially for organisms in which genetics is difficult, as is the case with mammalian systems. Importantly, the power of RNAi-based methods for genetic analyses became fully realized when the genome sequences of various organisms were completed (*C. elegans* Sequencing Consortium, 1998; Adams et al. 2000; Venter et al. 2001; Waterston et al. 2002; The Rat Genome Sequencing Project Consortium, 2004). Thus, the identification of all genes in the *C. elegans, Drosophila*, mouse, rat, and human genomes has led to the construction of numerous genome-wide RNAi resources, allowing reverse genetic screens either in tissue culture or in vivo. Today, genome-wide RNAi screening is possible in vivo in *C. elegans*, in tissue culture cells and in vivo in *Drosophila*, and in cell lines from mice, rats, and humans.

RNAi is a well-established tool for studies in tissue culture and, following the first genome-wide RNAi screen performed in *Drosophila* cells (Boutros et al. 2004), RNAi high-throughput screening (HTS) has become routine both in *Drosophila* and mammalian cells. Cell-based screening has been extensively reviewed in the past (Echeverri and Perrimon 2006; Perrimon and Mathey–Prevot, 2007; Boutros and Ahringer 2008; Mohr et al. 2011). In this review, we focus on in vivo methods and applications of RNAi. In most organisms, methods for in vivo RNAi are still in development and we discuss the state of the field, what has been learned so far, and future development. In particular, we describe the application of in vivo RNAi to characterize the function of pleiotropic genes and discuss its impact for the study of organisms for which genetic tools are either nonexistent or poorly developed.

2 RNAi REAGENTS FOR IN VIVO SCREENING

Four different types of RNAi reagents are used for in vivo studies: synthetic siRNAs, small hairpin RNAs (shRNAs), small hairpin microRNAs (shmiRNAs), and long dsRNAs (reviews by Echeverri and Perrimon 2006; Lee and Kumar 2009) (Fig. 1).

Synthetic siRNAs are small RNA duplexes composed of 19 complementary base pairs (bps) and 2-nucleotide 3′ overhangs. They are transfected into cells or injected into animals. On entering cells one strand of the siRNA duplex is incorporated into the multi-subunit ribonucleoprotein complex (RISC) and directs RISC to the target mRNA by complementary base-pairing, resulting in mRNA degradation. The effects of the siRNAs are transient, especially in actively dividing cells.

In contrast, shRNA and shmiRNA-synthesizing vectors allow for controlled or continuous expression of small transcripts in the cell that contain both the sense and antisense strand complementary to the selected mRNA target. They are either transfected into cells as plasmid DNAs or delivered using viral particles, and are maintained as extra-chromosomal copies or stably integrated in the genome as transgenes. The 50–70 bps single-stranded RNA transcripts fold back to form a stem-loop structure. ShRNAs are processed in the cytoplasm by the ribonuclease Dicer to generate siRNAs. ShmiRNAs are a variation of shRNAs in that sequences for the silencing trigger are embedded in an endogenous miRNA expression cassette. ShmiRNAs therefore exploit the endogenous microRNA pathway for the biogenesis and subsequent loading of siRNAs into RISC, and are usually more effective in knocking down target mRNAs than shRNAs.

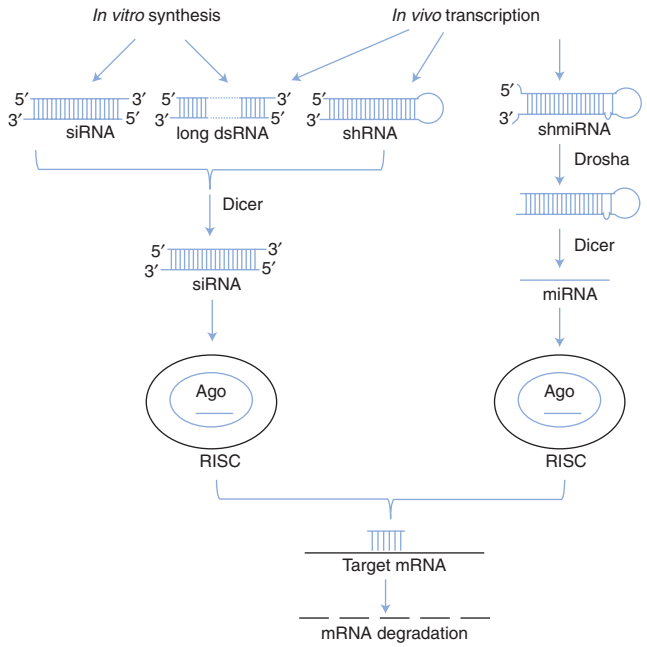


Figure 1. SiRNAs, shRNAs, shmiRNAs, and long dsRNAs pathways.

For use as RNAi reagents, long dsRNAs are usually 200–500 nucleotides (nts) in length. They can be injected into animals and in many cases, into eggs; delivered via bacteria (see sections on *C. elegans* and Planaria later); expressed as transgenes (see sections on *C. elegans, Drosophila* and mouse); or delivered into cultured cells by transfection or bathing (*Drosophila*). With the exception of the esiRNA (endoribonuclease-prepared siRNAs) method (Yang et al. 2002), whereby long dsRNAs are used to produce a pool of small, diced siRNAs that is then transfected into cells, long dsRNAs are not used in mammalian systems as they trigger an unwanted interferon response that can mask gene-specific effects.

3 IN VIVO RNAi SCREENING IN *C. ELEGANS*

The discovery that a dsRNA introduced into the nematode *C. elegans* is able to degrade a specific mRNA (Fire et al. 1998) marked the beginning of the revolution in in vivo RNAi. Importantly, RNAi in *C. elegans* is both systemic and transitive. First, injection, or expression, of a dsRNA into one tissue can lead to gene silencing in other tissues (Fire et al. 1998; Winston et al. 2002). Genetic analysis of this systemic effect has identified a number of genes involved in the phenomenon, including the multispan transmembrane protein SID-1, which is sufficient to confer cellular uptake of dsRNA to cells (Feinberg and Hunter 2003). Second, RNAi in *C. elegans* is transitive, whereby an RNA-dependent RNA polymerase (RdRP) is involved in an amplification step of RNAi and as a result, siRNAs that are derived from regions upstream of the original dsRNA sequences are produced (Alder et al. 2003).

The relative ease of methods required to perform RNAi experiments in *C. elegans* makes this genetically amenable model organism a logical choice for the development of technologies to study gene function on a genome-wide scale (review by Sugimoto et al. 2004). DsRNAs can be introduced into the nematode by simply soaking the animal in a solution of dsRNA, by feeding the worms bacteria that express long dsRNAs, by injection of dsRNA, or by generating transgenic hairpin-expressing animals.

Many genome-wide RNAi screens have been performed in the past 10 yr in *C. elegans* to interrogate a large variety of biological questions in developmental biology, cell signaling, aging, metabolic regulation, and neurodegenerative diseases, to name a few. These screens have been performed either in a wild-type strain or specific mutant backgrounds and either by injection or by feeding (see reviews by Kaletta and Hengartner 2006; Boutros and Ahringer 2008). In a landmark study based on injection into eggs, Sönnichsen et al. (2005) performed a genome-wide screen to identify all genes required for the first two rounds of cell division by examining embryonic phenotypes using time-lapse microscopy. However, the method of choice is large-scale RNAi screening by feeding worms bacteria that produce dsRNAs (Timmons and Fire 1998) because first, the method is less tedious by far, and second, RNAi libraries in bacteria that cover most of the 20,000 *C. elegans* genes are available (Fraser et al. 2000; Kim et al. 2005; Boutros and Ahringer 2008). For example, Ashrafi et al. (2003) screened 16,757 genes for their roles in fat storage in living worms using Nile Red staining of tissue lipids. They isolated 305 genes that when knocked down, lead to reduced body fat and 112 genes that lead to increased fat storage, representing a core set of fat regulatory genes as well as pathway-specific fat regulators.

Importantly, RNAi screening in *C. elegans* can easily be performed in various combinations, either in mutant backgrounds or by using multiple RNAs, to identify synthetic phenotypes. Such screens are a powerful means to gain an understanding of the structure of signaling networks, disease susceptibility, and identification of new drug targets. (Lehner et al. 2006), for example, systematically tested approximately 65,000 pairs of genes for their abilities to interact genetically and identified 350 genetic interactions between components of the EGF/Ras, Notch, and Wnt pathways.

Finally, an important issue with large-scale RNAi screening (also discussed later) is the rate of false positive and negative results associated with the method. False positives that occur when novel unexpected phenotypes

are associated with RNAi lines appear to be a minor contributor to false discovery in *C. elegans* (Sönnichsen et al. 2005). False negatives on the other hand, because of the variability of knockdown associated with the feeding techniques, can be more of an issue (depending on the screen) and may account for the 10%–30% variability observed between screens even if they are performed in the same laboratory (Simmer et al. 2003).

4 IN VIVO RNAi SCREENING IN *DROSOPHILA*

In *Drosophila*, feeding methods for RNAi delivery, as in *C. elegans*, do not appear to work; however, RNAi reagents can be delivered either by injection into precellular blastoderm embryos or as transgenes. Importantly, although there have been reports that systemic and transitive RNAi may occur in *Drosophila* (Saleh et al. 2009; Lipardi and Patterson 2009), this does not appear to occur when the dsRNA is produced from a transgene (Roignant et al. 2003).

Injection of dsRNAs as short as 200 bps and as long as 2000 bps, as well as short 21–22 nts siRNAs injected into embryos, have been shown to have potent interfering activities (Kennerdell and Carthew 1998; Misquitta and Paterson 1999; Williams and Rubin 2002; Misquitta et al. 2008). This approach has been used, for example, to clarify the role of the MyoD-related gene *nautilus* in embryonic somatic muscle formation (Misquitta and Paterson, 1999), and the roles of both the Frizzled1 and Frizzled2 receptors in Wingless signaling (Kennerdell and Carthew 1998). RNAi injection has been used systematically to screen more than 5000 genes for cardiogenic and embryonic nervous system phenotypes. For the heart screen, dsRNA-injected embryos that carry the *D-mef2-lacZ* transgene to detect cardiac cells were examined. For the nervous system screen, embryos were stained using the 22C10 antibody that detects the entire peripheral nervous system and a subset of central nervous system neurons. This approach led to the identification of many new genes involved in either heart or neural development (Kim et al. 2004) (http://flyembryo.nhlbi.nih.gov/).

RNAi by injection has somewhat limited applications as this approach is restricted to studies of gene function during embryonic development and maternally loaded proteins may mask embryonic phenotypes. Transgenic RNAi, on the other hand, has been widely used to study gene function in somatic tissues. Importantly, and unlike in *C. elegans*, in *Drosophila* RNAi is cell-autonomous, and because of this, targeted expression of RNAi constructs using the Gal4/UAS system (Brand and Perrimon 1993) can be used for cell- or tissue-specific interrogation of gene function. Indeed, this approach has been used extensively (Fig. 2). To date, transgenic RNAi lines have been shown to be potent in all somatic tissues, including neurons and muscles. However, for unknown reasons they do not appear to be effective in the female germ line.

Several groups, working independently, have developed vectors that have been used to generate transgenic RNAi fly strains. The initial vectors were based on transgenes having an inverted–repeat configuration, driven from either a single promoter or symmetrically transcribed from opposing promoters (Lam and Thummel 2000; Fortier and Belote 2000; Martinek and Young 2000; Kennerdell and Carthew 2000; Giordano et al. 2002). Because these vectors generated variable RNAi silencing effects, a number of modifications were introduced based on the observation in plants that intron-spliced hairpin RNAs are more efficient at gene silencing than the hairpin loop RNA (Smith et al. 2000). Thus, a number of groups designed vectors that include intron sequences from genes such as *mub* (Reichhart et al. 2002), *white* (Lee and Carthew 2003), *Ret* (Pili-Floury et al. 2004), or *fushi-tarazu* (*ftz*) (Kondo et al. 2006), as well as genomic/cDNA hybrids (Kalidas and Smith 2002). Additionally, the position of the *ftz* intron within the construct, e.g., located to the end of the hairpin structure, was tested (Fig. 3) (Dietzl et al. 2007). Altogether, these intron-containing vectors gave more robust RNAi phenotypes than the inverted–repeat configuration, most likely because of the enhanced formation of duplex dsRNAs following the splicing event and/or enhanced export of the processed mRNAs from the nucleus. Finally, in addition to the RNAi vectors that generate long dsRNAs, small hairpin microRNA-based (shmiRNA) RNAi constructs that generate a single siRNA have been shown to work as *Drosophila* transgenes (Chen et al. 2007; Haley et al. 2008). Although these vectors appear to work effectively in the soma, their overall effectiveness, especially compared with long dsRNAs, has not been tested systematically.

A major source of variability between these first generation vectors is caused by the method of transgenesis used, in which the constructs are integrated into the genome at random positions using P-element based transformation. Indeed, Dietzl et al. (2007) estimated that only 60% of their RNAi lines are effective with this most likely because of position effects associated with a large number of the random insertions. To solve this source of variability, a series of vectors, the "VALIUM" series, were generated. These rely on the phiC31-mediated site-specific integration approach (Groth et al. 2004) and the RNAi constructs were strategically integrated into sites in the genome that had been preselected for optimal expression (Ni et al. 2008; Ni et al. 2009). Specifically, a number of genomic sites were identified for which high levels of induced, Gal4-driven gene expression is observed, and importantly, low basal

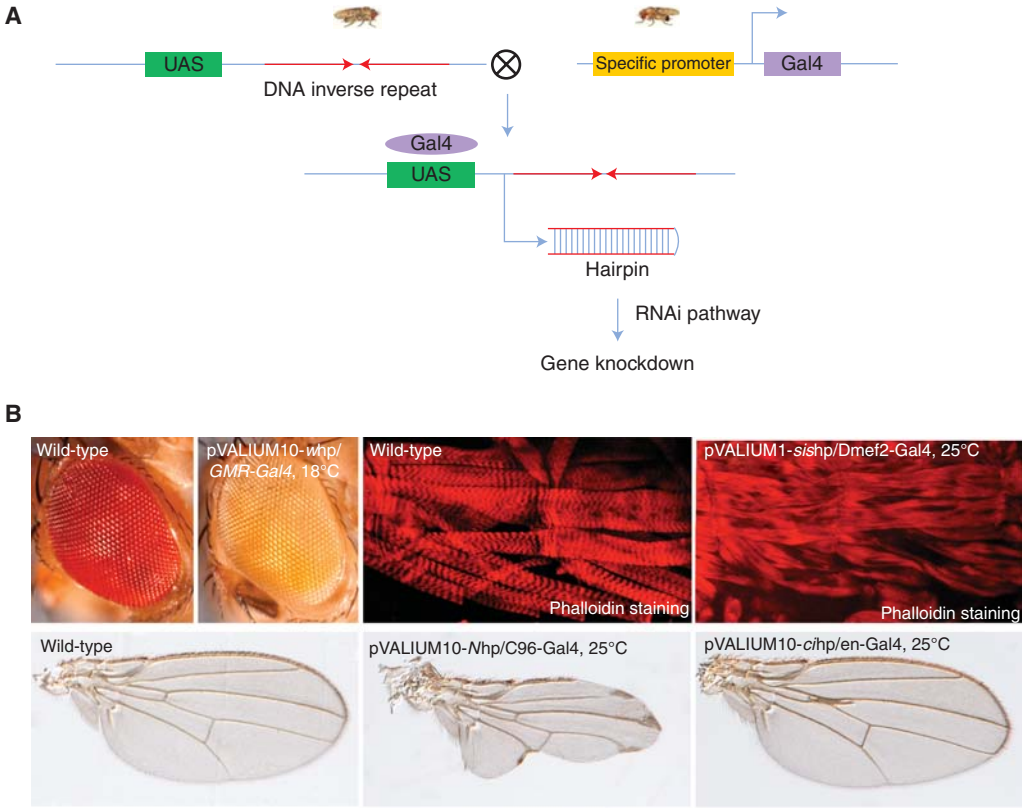


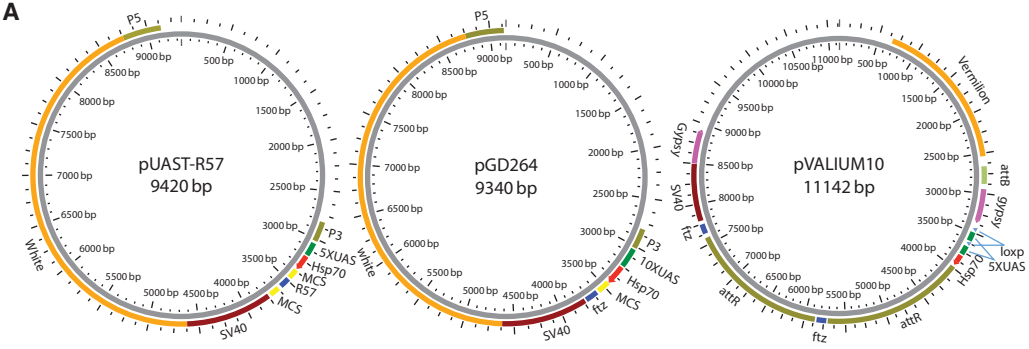
Figure 2. Transgenic RNAi in *Drosophila*. (*A*) Tissue expression of the transgenic RNAi construct is achieved following a cross between a UAS-hairpin and a Gal4 driver line. The main advantage of this method, in addition to its relatively simple design and fast execution time, is that it allows spatial and temporal control of the knockdown construct, which is essential for characterizing genes with pleiotropic functions. As thousands of Gal4 lines are available, appropriate Gal4 drivers are basically available for most questions to be addressed in the intact animal. (*B*) Examples of tissue specific RNAi phenotypes generated in the eye (knockdown of the *white* gene in the eye using the GMR-Gal4 driver), muscle (knockdown of the *sallimus* (*sis*) gene in the eye using the Dmef2-Gal4 driver), and wings (knockdown of the *Notch (N)* gene and *cubitus interruptus* (ci) genes in the wing using the C96-Gal4 and en-Gal4 drivers, respectively).

levels are seen in the absence of the Gal4 driver (Markstein et al. 2008). Furthermore, a series of related VALIUM vectors were built and tested for their ability to produce optimal RNAi effects. From these analyses, one optimal vector, VALIUM10, proved excellent for somatic RNAi (Figs. 2, 3) (Ni et al. 2009).

In flies, transgenic RNAi is particularly applicable to studies relevant to human biology such as cancer and metastasis, inflammation and wound healing, metabolic disorders, immunity, aging, and central nervous system disorders. This is exemplified by the hundreds of fly lines generated by individual laboratories, which can be identified either from the published literature or in the *Drosophila* database Flybase (http://flybase.bio.indiana.edu/). Building on the proven strength of transgenic RNAi, three independent efforts have already generated large-scale resources, such that RNAi lines that cover most of the *Drosophila* 13,929 protein-encoding genes (Tweedie et al. 2009) are now available (Fig. 3).

Two recently published large-scale screens, both using the Dietzl et al. (2007) library, illustrate that in flies complex developmental processes can be dissected on a genome-wide level using transgenic RNAi. First, Mummery-Widmer et al. (2009) screened for novel components of the Notch pathway by examining the effect of RNAi lines on external sensory organ development. In particular, they identified six new genes involved in asymmetric cell division and 23 novel genes regulating Notch signaling. Among the many interesting genes identified as Notch regulators were genes involved in nuclear import and the COP9 signallosome. In the second whole-genome study, Cronin et al. (2009) screened the RNAi lines for their ability to be resistant or susceptible to the ingestion of pathogenic Gram-negative bacteria *Serratia marcescens*. The initial

A



B

Collection	Japan	Vienna	TRiP
Vector	pUAST-R57	pGD265	pVALIUM10
# of genes targeted	6000	13327	3000
# of lines	11000	15072	3000
Website	http://www.shigen.nig.ac.jp/fly/nigfly/about/aboutRunai.jsp	http://stockcenter.vdrc.at/control/main	http://flyrnai.org/TRiP-Home.html

Figure 3. Structure of the vectors (*A*) and transgenic RNAi resources (*B*) available in *Drosophila*.

screen identified 95 resistant and 790 susceptible candidates that were subsequently analyzed using different Gal4 drivers to determine the site of action of the identified genes. A number of genes involved in intracellular processes, the immune system, the stress response, as well as genes associated with stem cell proliferation, growth, and cell death were shown to be required in the gut. Others, involved in phagocytosis and the stress response, were required in macrophages. Building on these observations, the authors characterized a requirement for the JAK/STAT pathway in response to intestinal *Serratia* infection.

An important issue with regard to *Drosophila* RNAi screens in tissue culture concerns false positives that occur from sequence specific off-target effects (OTEs) (Kulkarni et al. 2006; Ma et al. 2006; review by Perrimon and Mathey-Prevot 2007). OTEs can be avoided by selecting sequences that do not contain 19 nts or longer cross-hybridizing stretches to other genes or tri-nucleotide CAN (CA[AGCT]) repeats. In this regard, a number of software tools are available for identifying the most common off-target sequences so that they can be excluded from RNAi constructs. These include E-RNAi from the German Cancer Research Center (http://www.dkfz.de/signaling2/ernai/; Arziman et al. 2005) and SnapDragon from the *Drosophila* RNAi Screening Center (http://www.flyrnai.org/cgi-bin/RNAi_find_primers.pl; Flockhart et al. 2006). In vivo, although it is difficult to fully evaluate the rates of false positives and negatives in general, as it depends on the reagents used, the specific Gal4 driver used, and the temperature at which the flies are screened, the consensus is that OTEs appear negligible if sequences that avoid potentially problematic sequences are used (Dietzl et al. 2007; Ni et al. 2008; Ni et al. 2009). The rate of false negatives, however, in the fly screens, as discussed in the case of *C. elegans*, may be more of an issue. For example, Dietzl et al. (2007) estimate that 40% of their RNAi lines may not generate phenotypes because of low transgene expression. In some cases the effectiveness of individual RNAi lines can be improved by co-expressing Dicer2 (Dietzl et al. 2007) but in general, the newer generation of RNAi lines based on targeted insertion (Ni et al. 2009) are more likely to significantly decrease the overall rate of false negatives in RNAi screens.

Although false positives associated with transgenic RNAi appear to be less of an issue than RNAi in tissue culture, results derived from a single fly line still have to be taken with caution. To validate a transgenic RNAi phenotype, two simple follow-up experiments can be performed. First, the result can be confirmed with a second independent line, which becomes easier as more transgenic RNAi lines are generated. Second, and most conclusive, the RNAi induced phenotype can be rescued via expression of a transcript that can confer gene activity but evades the RNAi treatment, such as by having a divergent nucleotide sequence or exogenous 3′UTR (Stielow et al. 2008). Using genomic DNA of closely related species, Kondo et al. (2009)

have established a cross-species RNAi rescue method useful to rescue RNAi phenotypes. This straightforward and reliable method, based on genomic DNA fragments contained in fosmids, can be used to quickly build the construct needed to generate the transgenic flies harboring genomic DNA of a sibling *Drosophila* species that can confer activity but avoid knockdown.

5 APPLICATIONS OF IN VIVO RNAi TO THE DEVELOPMENTAL BIOLOGY OF EMERGING SYSTEMS AND EVO-DEVO

The application of RNAi to non-model and/or emerging model organisms in which few, if any, genetic tools are available is providing biologists a powerful means to characterize the roles of specific genes throughout development and evolution. As transformation methods are available in only a handful of organisms, RNAi is being delivered to emerging models either by feeding or following injection of RNAi reagents.

A large-scale RNAi screen for gene function has been performed in the planarian, *Schmidtea mediterranea*, an organism not previously accessible to extensive genetic manipulation. As in *C. elegans*, RNAi is delivered to a Planarian by feeding. In a landmark study, Reddien et al. (2005) screened 1065 genes, 5%–7% of the total, and described phenotypes associated with 240 of them. Many of these showed specific defects in regeneration, and in particular, defects were observed during stem cell/neoblast proliferation in amputated animals. Many biological insights are emerging from this work; for example, a recent study implicates the Wnt/beta-catenin pathway in antero-posterior polarity of the blastema during regeneration (Petersen and Reddien 2008).

RNAi is being used to address evo-devo questions in jellyfish, wasps, beetles, crickets, spiders, etc. For example, in the long germ band parasitic wasp *Nasonia vitripennis*, injection of pupae with dsRNAs (Lynch and Desplan 2006) has been used to examine the logic of antero-posterior patterning, and in particular mechanisms that differ from the short germ band patterning of *Drosophila*. Further, these in vivo RNAi studies have clarified the ancestral roles of the *bicoid* and *caudal* genes as patterning organizers, and helped to elucidate how these functions have evolved in higher dipterans such as *Drosophila* (Olesnicky et al. 2006; Brent et al. 2007).

6 IN VIVO RNAi IN VERTEBRATE MODELS

RNAi-based methods are now a common tool for gene perturbation in mammalian tissue culture cells (mouse, rat, monkey, and human). Beyond cell culture screens (see recent review by Mohr et al. 2011), RNAi is being used in a number of in vivo studies in which the RNAi reagents are delivered topically and directed to specific tissues or organs, such as the retina, brain, or muscles; using ex vivo, for example in hematopoietic cells; or delivered as transgenes (Sandy et al. 2005).

RNAi can be achieved locally by delivering synthetic siRNAs or using shRNAs delivered via viral particles or following transfection of plasmid DNAs. Many vectors have been built, based on either shRNA or shmiRNA designs, to optimize the level of expression of the RNAi reagent. Furthermore, much effort has been devoted to the development of methods for conditional RNAi that include irreversible and reversible approaches (Sandy et al. 2005; Lee and Kumar 2009). For example, a number of vectors based on the Cre/loxP and FLP-FRT systems have been used to induce RNAi in an irreversible way. In addition, Tet-, Ecdysone-, LacR, HIV-1 tat-, and HIV-1 LTR-, based systems have been explored for reversible conditional RNAi systems. In addition, vectors have been built for generating transgenic animals that can be either inserted into the genome at random or at targeted sites to ensure expression. To date, most of the published studies are still at the proof of principle stage (Sandy et al. 2005; Lee and Kumar 2009). Importantly, more studies are needed to evaluate technical aspects of RNAi effects, such as the level of knockdown in various cell types, variability because of the insertion site, potential epigenetic silencing of the construct, etc. Regardless of these limitations, from intense ongoing efforts will emerge many exciting applications for RNAi-based methods in the coming years (see review by Lee and Kumar 2009).

To date, and unlike the mouse, RNAi in *Xenopus* and zebra fish has not had a great impact, due in part to mixed results on the efficacy of some of the RNAi reagents, the prevalent use of the well-established method of antisense oligonucleotide morpholinos, and the lack of effective methods for controlled gene expression. In *Xenopus*, injection of siRNAs or long dsRNAs into oocytes and early blastomeres appears to work well (Zhou et al. 2002; Nakano et al. 2002), and gene silencing via transgenesis has been shown, although some difficulties have been observed in the silencing of genes at later stages of development (Li and Rohrer 2006). Similarly, in zebra fish, although a few studies have shown that dsRNAs, shRNAs and siRNAs can be effective for gene knockdown, a number of studies report that unexplained morphological abnormalities can be associated with RNAi-injected embryos (Wargelius et al. 1999; Skromne and Prince 2008). Altogether, it is not clear to what extent, at least in the absence of major technical advances, RNAi-based methods will become mainstream in fish or *Xenopus*.

Finally, in chick embryos, electroporation of siRNAs, as well as delivery using Replication Competent Avian Splice (RCAS) retroviruses to introduce hairpins intotissues, have been used successfully (Harpavat and Cepko, 2006). The RCAS approach is of particular interest as it is long lasting and transmissible because infected cells release more virus that spread to neighboring cells.

7 IN VIVO RNAi IN PLANTS: APPLICATIONS TOWARD CROP IMPROVEMENT

In plants, as in *C. elegans*, RNAi is both systemic and heritable. The siRNAs move between cells through channels in cell walls, thus enabling communication and transport throughout the plant. In addition, methylation of promoters targeted by RNAi confers heritability, as the new methylation pattern is copied in each new generation of the cell (Jones et al. 2001). Interestingly, in plants, endogenously encoded miRNAs rather than inhibiting translation are nearly or perfectly complementary to their target genes and induce mRNA cleavage by interaction with RISC.

The focus of in vivo RNAi applications in plants is directed toward the improvement of plant productivity and/or nutritional value (see reviews by Kusaba, 2004; Tang et al. 2007; Hebert et al. 2008). Among the exciting applications in which RNAi could have a major impact in agriculture is the improvement of essential food crops such as corn and rice. In addition, RNAi could be used to engineer food plants rendering them rich in dietary protein; for example, lowering the levels of natural plant toxins in cotton seeds could make this abundant plant appropriate for human consumption. Although we are still far from seeing RNAi-modified plant products in agriculture, especially considering the controversies and concerns surrounding growing genetically modified plants for human consumption, a number of successful applications have already emerged, particularly the ability of RNAi to confer resistance to common plant viruses (Zadeh and Foster 2004) and fortification of plants such as tomatoes with dietary antioxidants (Niggeweg et al. 2004).

8 IN VIVO RNAi APPLICATIONS TOWARD VIRUS AND PEST CONTROL

RNAi may have important agricultural applications as illustrated by ongoing attempts to use RNAi approaches to remedy the colony collapse disorder (CCD) in European honeybees. In recent years, millions of beehives have disappeared, most likely because of the spread through bee colonies of a lethal virus, the Israeli acute paralysis virus (IAPV). The current working hypothesis is that IAPV infection, together with poor nutrition and exposure to pesticides, weakens bee colonies to the extent that they simply disappear. One RNAi-based strategy being explored to fight IAPV infection is to feed bees siRNAs targeting specific IAPV sequences such that, following viral entry into bee cells, translation of viral proteins is blocked (Cox-Foster and vanEngelsdorp 2009).

RNAi is also becoming an important tool to combat insect pests, in particular *Anopheles gambia*, the vector for Plasmodium, the protozoan responsible for malaria. RNAi reagents are being used to dissect host-pathogen interactions and have already provided fundamental insight into the insect defense mechanisms to control the protozoan, such as the identification of the pattern-recognition receptor TEP1 in host defense (Blandin et al. 2004).

Methods to disseminate RNAi expressing transgenes that may confer resistance to a pathogen within a population are also being explored. One of the strategies being considered for the control of pathogen-laden pests is to rapidly convert a pathogen-bearing insect population to a genetically modified population that is resistant to the pathogen. For example, if wild mosquito populations could be replaced with malaria or dengue-resistant ones, this alone may provide an effective means to control these devastating diseases. The challenge with such an approach is to develop a method for rapid replacement of the wild population. In one clever demonstration, Chen et al. (2007) reported an RNAi-based method in *Drosophila* that achieves the selfish drive of a genetic element into a population. Although the approach is still at an early stage in development, the results of this study show the feasibility of RNAi-based population replacement.

9 IN VIVO RNAi APPLICATIONS IN MEDICINE

RNAi has the potential to offer more specificity and flexibility than traditional drugs to silence gene expression. In addition, because any protein that causes or contributes to a disease is susceptible to RNAi, previous disease targets considered "undruggable" are now accessible. Not surprisingly, RNAi has become a major focus for biotechnology and pharmaceutical companies, which are now in the early stages of developing RNAi therapeutics, mostly based on siRNAs, to target viral infection, cancer, hypercholesterolemia, cardiovascular disease, macular degeneration, and neurodegenerative diseases (Sah et al. 2006).

Critical issues with RNAi as a therapeutic are delivery, specificity and stability of the RNAi reagents. Delivery is currently considered the biggest hurdle as the introduction of siRNAs systemically into body fluids can result in their degradation, off-target effects, and immune detection and subsequent reactions (see for example Zimmermann et al. 2006). Thus, many efforts are focused on developing ways

to modify an RNAi or attach them to delivery agents that will protect them until they reach their therapeutic destinations. These include delivery as particles or complexes using lipid nanoparticles that encapsulate the siRNA or combining siRNA molecules with peptide-based polymers. Additional advances in therapeutic applications are likely to come from chemical modifications or other approaches to improving the specificity and potency of RNAi reagents.

10 CONCLUDING REMARKS

RNAi-based methods are providing unprecedented tools useful to address fundamental questions in the biology of living organisms. As exemplified by in vivo screens in *C. elegans* and *Drosophila* as reviewed here, these tools are enhancing and/or replacing more classical genetic approaches and manipulations. Further, as most organisms possess the cellular machinery for RNAi, this near-universal approach makes loss-of-function studies approachable in organisms in which genetic tools do not exist. Finally, with the growing appreciation for the fundamental potential of RNAi and a burgeoning collection of RNAi technologies and reagents, the diversity in in vivo applications to biology, medicine, and agriculture is seemingly limitless.

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