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Comparative high-throughput RNAi screening methodologies in *C. elegans* and mammalian cells

Review

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The discovery of RNAi in *Caenorhabditis elegans* has generated a paradigm shift in how research is performed. Targeted gene knockdown using high throughput screening approaches is becoming a routine feature of the scientific landscape, and researchers can now evaluate the function of each gene in the genome in a relatively short period of time. This review compares and contrasts high throughput screening methodologies in *C. elegans* and mammalian cells and highlights the breadth of applications of this technology.

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Introduction

First reported in the nematode model organism *Caenorhabditis elegans*, Fire and Mello showed that double stranded RNA induced potent and sequence-specific posttranscriptional gene silencing by degrading the target mRNA [1]. The discovery of RNA interference (RNAi) was fortuitously concurrent with the publication of the *C. elegans* genome sequence [2], shortly thereafter the *Drosophila* and human genomes were published [3–5]. The combination of RNAi and genome-wide sequence annotation enabled a radical paradigm-shift in how research can be done, leading to the application of high throughput analysis of gene function. The identification of most/all of the genes in an organism has allowed for the first time the question 'what does this gene do?' to be addressed in an unbiased genome scale approach, an area previously dominated by classic 'gene by gene' approaches to study biological processes. Genome-wide high-throughput RNAi screens rapidly began to populate the *C. elegans* literature [6–8], followed by Drosophila [9,10] and mammalian cell culture-based studies [11–13]. Importantly, RNAi has for the first time allowed detailed analysis of gene function in organisms unsuited to classical genetic studies, such as the stem cell model system planaria and some major parasites of importance to human health, such as *Trypanosoma brucei* [14]. Genome-wide screens have made significant advances in our knowledge and are now becoming a routine approach to understanding biological systems. Indeed, the term 'functional genomics' has been coined to describe our present era of analysis of individual genes on a genome scale.

This review will briefly describe the process of RNAi, and then focus on genome scale RNAi screening applications using the *C. elegans* organismal model system (*in vivo*) and mammalian cell culture based (*in vitro*) strategies. We will outline a range of applications and the infrastructure required to take advantage of these approaches.

The RNAi machine

RNA interference (RNAi) is an ancient pathway for protecting an organism's genome against viruses and mobile genetic elements such as transposons [15,16]. At the heart of RNAi are small non-coding RNAs that provide the sequence specificity for gene knockdown. A well defined mechanistic understanding of the RNAi pathway has been established in the decade that followed the discovery of RNAi. Double stranded RNAs (dsRNA) can be introduced into the cell by various mechanisms, however they are all processed down to a short RNA functional unit (~20–30 nucleotides), generally referred to as a short interfering RNA (siRNA). This processing is largely mediated by nuclear and cytoplasmic endoribonuclease RNase III family members (Fig. 1, for more detailed description see [17]). The execution of siRNA-mediated gene knockdown is directed by the RNA induced silencing complex (RISC), a large ribonucleoprotein complex that contains one strand of the siRNA molecule that is bound by an Argonaute protein and additional protein factors [18]. siRNA mediated degradation of the target mRNA generally requires 100% complementarity between the siRNA and mRNA. Another class of related small RNAs is the microRNAs (miRNAs) that are also bound by Argonaute proteins in RISC, which in animals only have partial complementarity to their target mRNAs and generally repress translation and mRNA stability. In an experimental context, small non-coding RNAs are designed to target specific mRNA molecules and can be generated via four basic approaches (Fig. 1).

Mammalian RNAi approaches require transient transfection of siRNA molecules that are chemically synthesised and can interact directly with the RISC complex. Short hairpin RNAs (shRNAs) are encoded in a viral vector either as pri-miRNAs or shRNAs and are processed by endogenous endoribonuclease RNAse III members Drosha and Dicer resulting in a 20–30 nucleotide siRNA (Fig. 1). An additional low throughput strategy is to chromosomally integrate transgenes that express shRNAs that are also processed by the endogenous RNAi machinery.

Unlike mammalian systems, long double stranded RNAs can be introduced into *C. elegans* without induction of an interferon response that is prevalent in mammalian cells. Long dsRNAs are processed in the cytoplasm resulting in the generation of siRNAs (Fig. 1). In *C. elegans* RNAi is particularly potent for two reasons. First, the primary siRNAs are amplified via the action of RNA-dependent RNA polymerases that lead to the generation of secondary siRNAs, which can degrade the same target mRNA [19,20]. Secondly, the RNAi is spread throughout the animal by the transport of siRNA molecules to adjacent cells via the action of specific transporters. Additional secondary siRNAs are generated in the recipient cells [21]. The combination of these factors allows for a systemic and heritable gene knockdown, a feature unique to *C. elegans* and plants.

C. elegans as a model organism for functional genomics

C. elegans is a non-pathogenic soil nematode that has made a remarkable contribution to understanding multicellular eukaryote biology over the past 30 years. With its high degree of conservation of genes and molecular pathways related to human disease,*C. elegans* is a model tool for ageing, neurobiology, cell migration, germline specific processes and diseases. Traditionally, classical genetics (forward genetics) was the principle means of studying gene function in *C. elegans*. Reverse genetics using RNAi takes advantage of our knowledge of the near complete gene complement of various organisms and allows for investigation of gene-specific function in all cell types simultaneously. RNAi often 'phenocopies' the genetic loss of function mutant; however, each gene is unique and the effectiveness of the knockdown depends on protein stability. One advantage of RNAi compared to genetic screens is that lethal genes can be assayed in a broad range of developmental stages. For example, a genetic mutant that results in embryonic lethality is difficult to examine in later life stages. However, by using RNAi to knock down gene expression later in development, it may be possible to unmask other functions of this gene. Another important advantage of RNAi is that the gene affecting a particular phenotype is already known, whereas in genetic screens once a mutant is discovered, the affected gene still needs to be identified, a process that can be very time consuming and does not permit a high throughput approach. Importantly, published *C. elegans* data are freely available from a highly curated and centralised database known as WormBase (http://www.wormbase.org). Here published RNAi screen data are annotated at the level of the individual gene and the specific assay, and for every gene the phenotype is recorded, whether it be a functional hit or not.

The advantages of screening in a whole organism include the maintenance of cell-to-cell communication, neuroendocrine

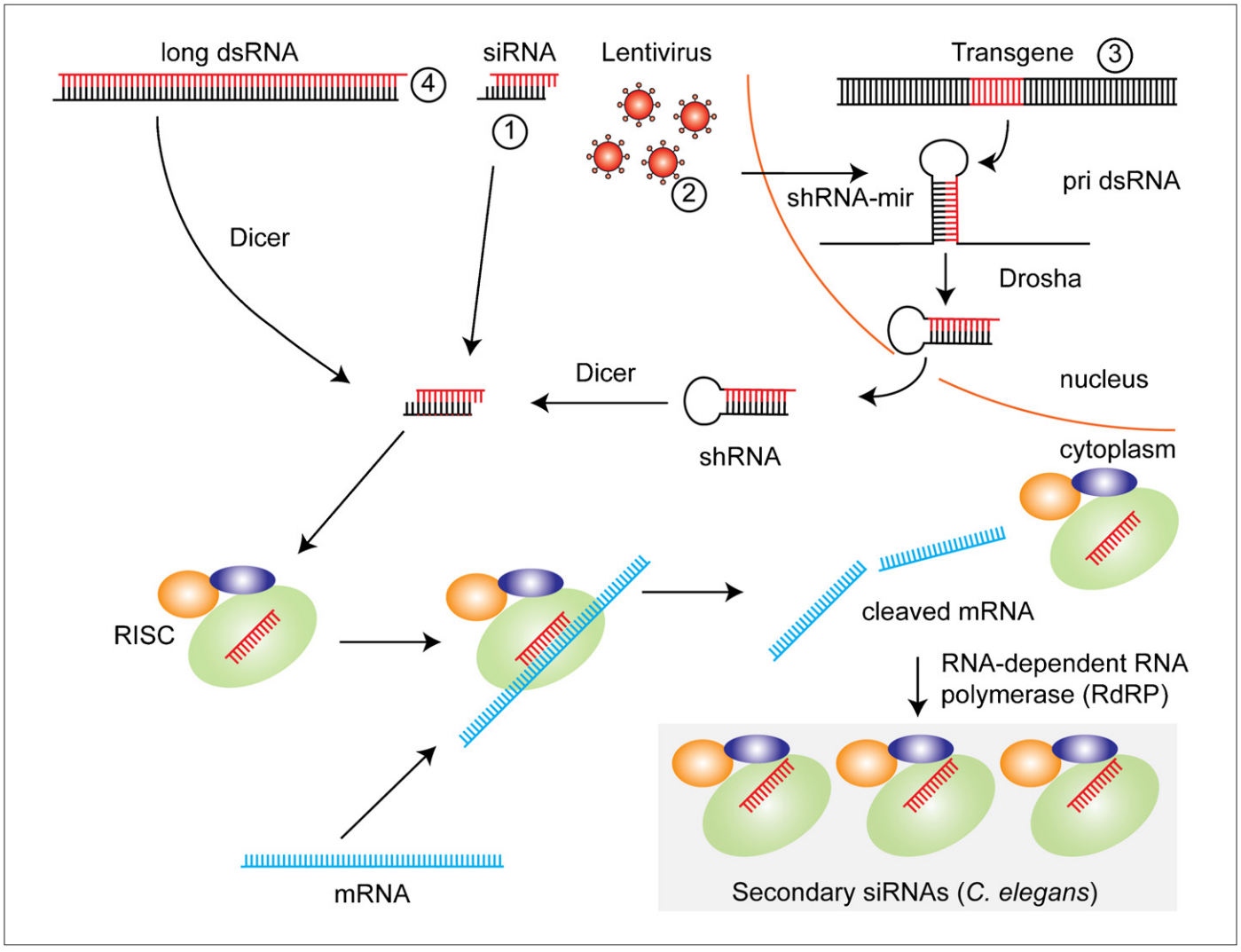

FIGURE 1

Summary of RNAs mediating RNAi. The 'functional unit' of RNAi is the ~20–30 nucleotide single stranded RNA (red) bound by the RISC complex which contains an argonaute protein (orange) and other co-factors (purple). The origin of the siRNAs can vary depending upon the organism and the experimental approach. (1) Most common in mammalian cell culture, synthetic siRNAs can be transfected into cells and one strand, the guide strand, becomes incorporated into RISC. (2) Virally encoded shRNA-mirs are first processed in the nucleolus by an RNase III enzyme called Drosha followed by export from the nucleus and further processing by Dicer. (3) Transgenes can encode shRNA-mirs, which are processed similarly to the virally delivered substrates. (4) Long double stranded RNAs are processed by the endoribonuclease RNase III family member Dicer into ~20–30 nucleotide siRNAs. In *C. elegans*, secondary siRNAs are generated by RNA-dependent RNA polymerases.

signalling and metabolism necessary to survive and reproduce. Genome-scale whole organismal RNAi screens in an arrayed plate format where a single known target is in each well/plate is restricted to *C. elegans* and the non-parasitic flatworm *Planaria* (Table 1). Genome-wide *Drosophila* RNAi lines are available, however they offer a much lower throughput, requiring genetic crossing of lines to evaluate a phenotype [22]. Investigating gene function in an organismal context has been shown to be an extremely powerful tool to extend our understanding the biology of mammalian cells [23].

Delivery of double stranded RNA into *C. elegans*

There are three methods to deliver dsRNA to *C. elegans*: microinjection, soaking and feeding, each with their advantages and limitations. Microinjection of dsRNA into the intestine of young adult worms provides the strongest level of knockdown, but requires a specialised microscope and injector, advanced technical skills and significant cost associated with *in vitro* synthesis of each dsRNA. Accordingly, throughput using this approach is vastly reduced compared to other methods and therefore there are relatively few microinjection based large-scale RNAi screens in *C. elegans* reported. This method is commonly used to verify functional phenotypes in a low throughput manner. An alternative to microinjection is to soak fourth stage larval worms in dsRNA for 24 h, after which the animal is moved to growth medium plates for phenotypic observation of the progeny [8]. The production of dsRNA is the same for microinjection and soaking but whilst this method also results in efficient knockdown of target genes, it requires considerably large volumes of dsRNA, thereby limiting its application for high throughput screening strategies. Soaking is more suitable for large numbers of worms within a single assay, offering a modest increase in throughput

TABLE 1

Organismal RNAi approaches

Organism	Approach	Life stage	Scale	Refs
C. elegans	Feeding	All	Genome	[7]
	Soaking	All	Subset of genome	[8]
	Microinjection	All	Genome	[32]
Drosophila	Microinjection	Embryos	Subset of genome	[73]
	RNAi transgenes	All	Individual genes	[74]
Planaria	Feeding	All	Subset of genome	[75]
Zebrafish	Microinjection of siRNAs	Embryo	Targeted subsets	[76]
Chicken	Retroviral delivery of shRNAs	Embryo and retina	Targeted subsets	[77,78]
Mouse	Microinjection of dsRNA or siRNA	Oocytes and early embryos	Individual genes	[79]

compared with microinjection. In contrast to microinjection and soaking, RNAi via feeding dsRNA to *C. elegans* via their *Escherichia coli* food source, either on agar plates or in liquid culture in 24-well or 96-well plate format offers an inexpensive, labour efficient means to facilitate gene knockdown. Feeding is the preferred method for high throughput genome-wide screening approaches as it permits large numbers of genes to be evaluated simultaneously due to the relative ease of growth and manipulation of the bacteria. Using this approach it is possible for two people to screen up to 1000–2000 genes per week. For a more detailed technical discussion see [24].

RNAi libraries and screen optimisation

Currently two bacterial 'feeding' RNAi libraries are available, known as the Ahringer and ORFeome libraries. These libraries are available from Open Biosystems and cover ~86% and ~55% of the approximately 20,000 protein coding genes in the *C. elegans* genome respectively [6,25,26] and together provide 94% gene coverage [27]. In both libraries the RNAi clone is housed in feeding vectors in bacterial strains, with the Ahringer library derived from genomic DNA containing the gene exons and the ORFeome library consisting of full-length cDNA open reading frame clones. The Ahringer library is arrayed in 384 well plate format that reduces freezer storage requirements but necessitates re-arraying to 96 well format for experimental use. The ORFeome collection is already arrayed in 96 well plates. Upon receipt of either library, it is crucial to make daughter plate copies for library longevity and as such, they represent a non-exhaustible resource. With the development of higher throughput sequencing technologies, the Ahringer library was recently sequence verified and 98.3% of the bacterial strains in the library were correctly annotated [28]. These results are freely available online via the CelRNAi database and provide an invaluable resource for groups that currently utilise the Ahringer library. Whilst the ORFeome library has not been verified to such an extent, it is general practice that any gene targets derived from a genome-wide screen be validated by sequencing before further investigation.

Typically, genome scale feeding screens use a standard set of conditions and therefore require less optimisation compared to mammalian RNAi screens. For feeding screens, bacteria containing a plasmid that expresses gene-specific dsRNA are grown in 96 well plates overnight and dsRNA production stimulated by the addition of the chemical Isopropyl β-D-1-thiogalactopyranoside (IPTG). After 1–4 h of induction, the bacteria are pelleted by centrifugation and subsequently resuspended in a *C. elegans* compatible liquid media such as M9 or S basal, or dispensed onto the surface of agar plates to form a lawn of bacteria. Worms at the desired developmental stage are then added to the well in a manual or automated fashion followed by incubation for 2–4 days at 15–25°C. Phenotypes are subsequently examined using various types of imaging applications (see below). In comparison to mammalian screens that analyse specific cellular features in a defined cell type (see below), *C. elegans* screens focus on organismal biology and are therefore generally less quantitative (Table 2). Screens are generally performed in duplicate or triplicate and stringency requires all replicates to be positive to be considered a hit.

RNAi screens in *C. elegans*

Developmental/morphological screens

The first genome scale high throughput RNAi screens were conducted in wild-type *C. elegans* and identified ~1700 genes that displayed loss-of-function phenotype(s) of which two-thirds had no previously described function [6,7] (Table 2). Analysis of these screens was limited to gross developmental or morphological abnormalities such as embryonic and larval lethality, sterility, and defects in movement, all phenotypes that could easily be scored under a dissecting stereo microscope. Such gross morphology screens have since been repeated using strains hypersensitive to RNAi such as *rrf-3* and have further expanded the number of genes associated with specific developmental or morphological phenotypes [29]. The very broad nature of the scored phenotypes provides limited detail about the specific processes underlying the defect. At the time, these screens were ground breaking and established the principles and methodology for conducting genome scale high throughput RNAi screens in *C. elegans* and other systems.RNAi screens to identify components of gene networks

Most biological pathways exist as an interconnected series of steps and complex genetic interactions. In some cases the absence of a single protein does not cause a phenotype; however, when additional components of the pathway are knocked down at the same time, synthetic phenotypes are revealed and can provide valuable information about gene networks not easily identified by other approaches. Two broad classes of genetic interaction screens can be used to identify gene regulatory networks. Suppressor screens start with a genetic mutant that displays a phenotype,

TABLE 2

Examples of RNAi screens in *C. elegans*

Scale	Genetic background	RNAi approach	Phenotype assayed	Refs
Chromosome 1	Wild-type	Feeding	Developmental/morphological	[6]
Subset	Wild-type	Soaking	Developmental/morphological	[8]
Subset of germline-enriched genes	Wild-type	Microinjection	Chromosome morphogenesis and nuclear organisation	[80]
Genome	Wild-type	Feeding	Developmental/morphological	[7]
Genome	*rrf-3**	Feeding	Developmental/morphological	[29]
Genome	Wild-type and insulin signalling mutants	Feeding	Fat metabolism	[81]
Genome	Wild-type	Feeding	Germ cell apoptosis	[31]
Genome	Wild-type containing reporter gene	Feeding	RNAi factors – derepression of reporter gene expression	[27]
Genome	Wild-type containing reporter gene	Feeding	Polyglutamine aggregation	[82]
Genome	Wild-type	Microinjection	Early embryogenesis – high-content assay	[32]
Genome	*lin-15b;eri-1**	Feeding	Longevity	[83]
Genome	*eri-1; let-7**	Feeding	miRNA co-factors	[30]
Genome	*nre-1; lin-15b** containing reporter gene	Feeding	Neuronal specification- ASEL neuron	[37]

*RNAi sensitive strains.

and an RNAi screen is conducted to identify genes that can reduce or eliminate the phenotype. These screens typically have low levels of false positives. Enhancer/synthetic screens use strains with either partial or complete (null) loss of function mutations, that by themselves do not display a strong phenotype, but identify genes that exacerbate or induce new phenotypes. For example, a synthetic RNAi screen designed to identify genes involved in the miRNA pathway used a weak allele of the miRNA gene *let-7*, which displays a very mild phenotype, and found 213 candidate genes that specifically induced a 'bursting' phenotype only in the *let-7* genetic background [30]. Post-screen genetic analysis reduced this number to 19 genes validated to function within the miRNA pathway. Whilst this example demonstrates the advantage of employing sensitised genetic backgrounds, it also illustrates the high false positive rate often associated with these types of screens, and the requirement for an additional assay to re-test the candidate screen hits. When conducting enhancer/synthetic screens it is important to screen the mutant and wild type worms simultaneously, to allow for identification of genes that only cause a defect in the mutant background.

Reporter gene based screens

For many phenotypes, simple visual inspection of RNAi treated worms is not sufficient to reveal any defect and in some cases specific stains/dyes can be used with live animals to uncover phenotypic changes. For example, in a genome wide RNAi screen to identify genes required for normal regulation of germ cell apoptosis, live worms were stained with the vital dye acridine orange. Knockdown of 21 genes reproducibly increased the levels of germ cell death, many of which share conservation with mammalian genes [31]. When a highly specific stain is available it can provide a robust and relatively simple method to identify genes required for very specific functions. However, the specificity of the stain is crucial, as non-specific staining will hamper interpretation and increase levels of false positives.

High content imaging screens

One of the first high content RNAi screens in *C. elegans* focused on identifying genes involved in early embryonic cell division [32]. In this study, dsRNA was injected into young adult hermaphrodites and the ability of the next generation of embryos to successfully complete the first cell division was followed using time-lapse differential interference contrast (DIC) microscopy of live embryos. Forty-five distinct defect categories were scored and clusters of genes and associated defects were generated. As will be discussed below, data management is a major issue with large-scale high content imaging – in this case ~40,000 movies of embryonic development were recorded and required the development of a customised laboratory management system.

Reporter gene-based screens

Highly specific screens can be conducted using strains containing fluorescently tagged proteins. Whilst this has been a common approach in mammalian *in vitro* experiments, its application in *C. elegans* has been less routine. Analysis of fluorescently tagged proteins in live multicellular organisms is more difficult compared to 2-dimensional mammalian cell based assays, and often requires extensive manual set-up and analysis. However, the ability to screen for very specific phenotypes makes these assays increasingly useful and the approach has been applied to a diverse range of biological questions, including transcriptional response to stress resistance [33], osmoprotective gene expression [34], germ cell function [35] and male gonadal differentiation [36].

C. elegans is widely used in neurobiology as it possesses a simple nervous system comprising only 302 neurons in the adult hermaphrodite, which makes it possible to evaluate gene function at a single neuron level. However, RNAi in neuronal cells has proven to be recalcitrant in wild-type animals. To overcome this limitation, many groups have used hypersensitive to RNAi strains coupled with fluorescent reporters that express in individual neurons, making it then a very powerful tool for investigating neuronal

development, degeneration and regeneration phenotypes. For example, a genome scale RNAi screen in the strain *nre-1;lin-15b* examined the development of an individual neuronal cell and identified 245 genes that affected this process [37].

Automation of C. elegans *screens*

The majority of *C. elegans* RNAi screens have relied upon manual workflows for both the screen set-up and phenotypic scoring. This requires a significant investment in time and can make scoring of fluorescent assays more subjective if based purely on visual inspection. A recent report of an automated high-content live animal drug screen using an ArrayScan high content microscope (Cellomics) has clearly demonstrated the potential of fluorescent reporter gene assays coupled with automated microscopy [38], and will probably prove highly useful to many RNAi screens. However, due to the current high cost of such instrumentation, adoption of this approach may be beyond the scope of most laboratories, and more likely to be accessed through shared core facilities. Another approach to automation is to use the Complex Object Parametric Analyzer and Sorter (COPAS), a worm flow cytometer that is capable of sorting worms on the basis of size and a range of fluorescent markers, such as GFP and RFP. A recent RNAi screen used this ability to sort worms on the basis of size and fluorescence and identified four genes that suppressed the growth defects normally associated with loss of the survival of motor neuron (SMN) protein [39]. Automation of *C. elegans* RNAi screens will become increasingly attractive as access to high-content live imaging and COPAS machines become common place and this will open up new and more complex phenotypes to be screened using RNAi.

Current limitations

In comparison to genetic screens that can identify loss and gain of function mutations, RNAi can only generate loss of function phenotypes, which may not always be as revealing as some gain of function phenotypes. Accepting that feeding RNAi generally results in less efficient gene knockdown compared with microinjection and soaking, it remains however, the most well suited to high throughput screens, and as such, the advantages in terms of speed and scalability outweigh this negative. Often it would be desirable to knockdown multiple genes simultaneously, however, the feeding approach generally does not work as effectively when two different bacteria expressing different dsRNAs are mixed together. There are reports that use of RNAi hypersensitive strains can overcome this to some degree, but in many cases these strains can display aberrant function in some tissues, especially in germ cells.

RNAi screening in cultured mammalian cells and cell lines

A major impediment to functional mammalian studies has been a lack of genetic resources that was rapidly overcome by the discovery that RNAi mechanisms were conserved in mammalian cells. Indeed, it was only several years after the discovery in *C. elegans* that the first application of RNAi in mammalian cells was published. Early approaches focused on the use of shRNAs with multiple constructs per gene target, each encoded in retroviral or lentiviral vector backbones [11,12]. The field advanced quickly and chemically synthesised siRNAs in arrayed 96 and 384 well format then became available. Rapid adoption of the technology resulted in the first genome wide publication using an arrayed format siRNA approach in 2007 [40]. Interestingly, whilst the mouse has served as a very strong model for human genetics, mammalian RNAi screens have largely focused on human cell lines and have been used to identify genes that regulate proliferation, cell survival, synthetic lethal responses to drugs, cell cycle, invasion and migration and host–pathogen interactions. Indeed, cancer biology is rather uniquely suited to RNAi screens through the extensive range of cell lines available, oncogenic dependency and drug sensitivity.

In contrast to the relative simplicity of RNAi screens in *C. elegans*, mammalian screens require greater levels of optimisation, rigorous statistical interpretation, and substantial robotic infrastructure and are generally more expensive to perform. Furthermore, the types of biological questions and approaches are quite different and the assays tend to be more quantitative. Both siRNA and shRNA approaches can be used to select for positive and negative phenotypes in individual experiments, or in the case of siRNA screens, within the same assay (e.g. accelerated and impaired cell migration [41]). Unlike the gross developmental phenotypes traditionally examined in the worm, in siRNA screens cell-based assays can measure very specific cellular perturbations where hundreds of individual cell features can be recorded in a single experiment [42]. Cells can be stained for multiple different features or stable lines can be created to express fluorescently tagged protein(s). These are analysed using high throughput, high content automated imaging, together with refined bioapplications and computer learning to identify specific cellular features. Imaging based screens are often coupled with other biochemical readouts, such as live dyes for viability, which can be evaluated using fluorescence in a high throughput plate reader. Other assays focus solely on fluorescent or luminescent biochemical readouts evaluated using a plate reader and include endpoint viability assays or modulation of expression of fluorescently tagged proteins. shRNA screens, particularly pooled viral screens, are generally not assayed at such a specific cellular level, but focus on holistic responses, such as drug resistance or cell survival.

Libraries

Long dsRNAs in mammalian cells can induce a strong interferon response; therefore, 21 nucleotide dsRNAs must be used to evade this viral defence mechanism [43]. Algorithms to predict effective siRNA sequences are constantly evolving and the number of human genes are continually being reviewed (NCBI RefSeq database). Depending on the company and the library version, the entire human and mouse genomes range from approximately 18,000–22,000 protein coding genes. Libraries are shipped lyophilised in 96 or 384 well format and must be very accurately rehydrated, diluted and aliquoted into multiple daughter plates and quite often re-arrayed into the preferred 384 well screening format. A large main frame robotic liquid handling infrastructure together with smaller automated cell dispensers and plate washers are necessary to undertake large screening efforts. All transfection, cell dispensing and media change steps are performed using these instruments under sterile biohazard containment conditions. To track this complex series of operations, a Laboratory Information

Management System (LIMS) is essential, and several International institutes have collectively invested in creating open source software [44]. The infrastructure requirements and associated costs are significant and preclude this from being achievable in a standard research laboratory, rather being restricted to core facilities or a small number of dedicated laboratories. shRNA viral pools of approximately 5000–10,000 constructs per pool, with an average of three to five constructs per gene are commercially available, however they come at a cost premium and are an exhaustible resource (Fig. 3c). Libraries can also be purchased as bacterial glycerol stocks arrayed in 96 well dishes, which offers the laboratory a renewable resource and significant flexibility in pooling strategies with the ability to create boutique collections such as collating all the members of a particular signalling pathway. Laboratories can prepare their own viral pools without any robotic infrastructure, however it is a considerable investment in time. Arrayed format shRNA screens (Fig. 3d) require robotic automation and are far less common due to the technical demands for produ-

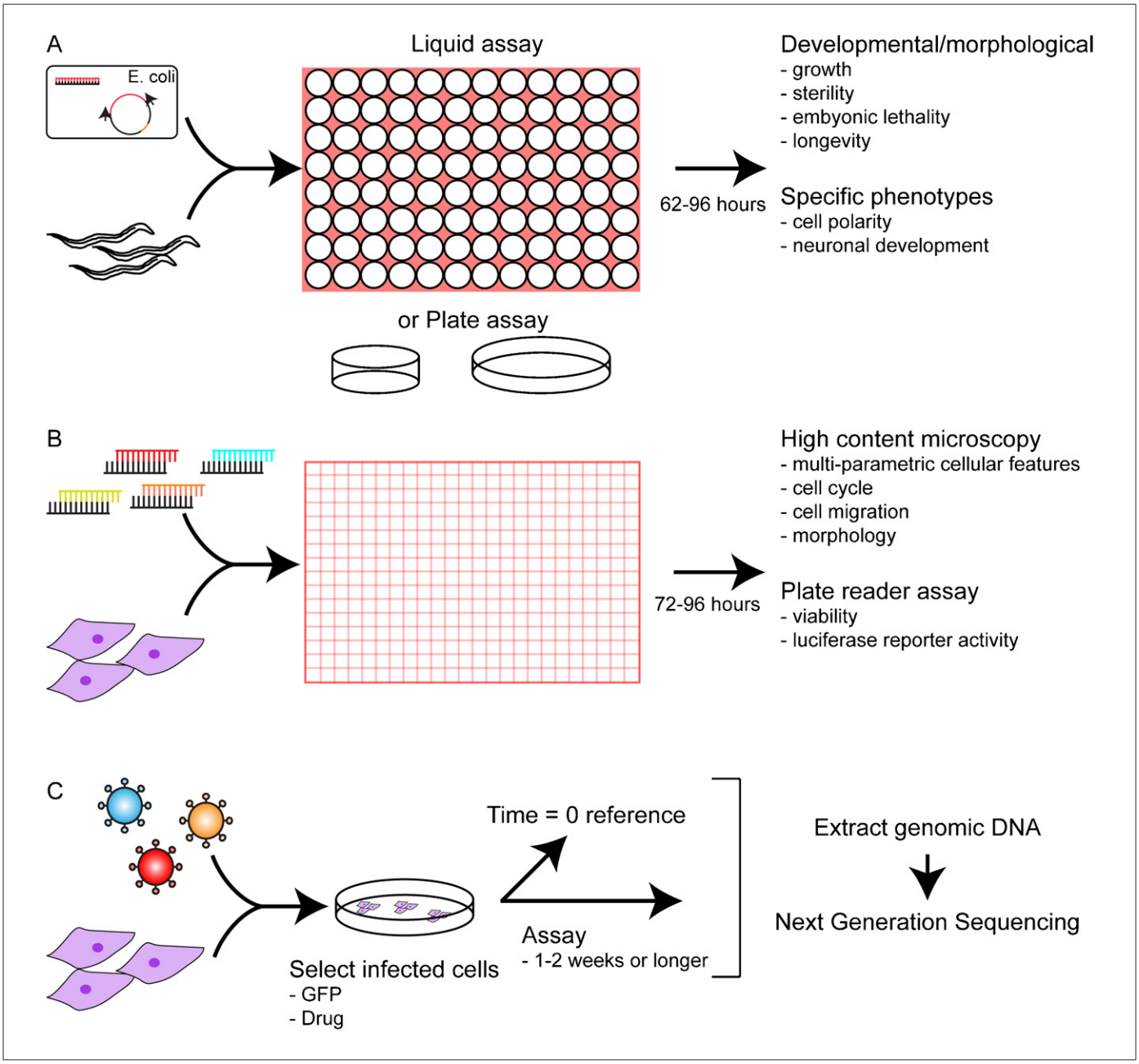

FIGURE 2

Comparative RNAi screening work flows. **(a)** In feeding RNAi, dsRNA is produced by bacteria and seeded into 96 well dishes or large format agarose plates and developmentally synchronised worms are added. Depending on the assay, worms are scored for phenotypic aberrations within 2–4 days. **(b)** Chemically synthesised siRNAs are transiently transfected into adherent cells in 384 well format and assayed 72–96 h post-transfection. **(c)** Lentiviral delivery of shRNA constructs in pooled format. Transduced cells are selected and expanded and then assayed for a defined period of time. Genomic DNA is extracted from reference (Time = 0) and assayed populations, and analysed by Next Generation Sequencing.

cing equivalent high titre virus in every well in high throughput [45].

RNAi delivery

dsRNA is most commonly delivered to mammalian cells via lipid-mediated transfection of chemically synthesised siRNA or viral infection of shRNA constructs. Although both siRNA and shRNA approaches knockdown their target genes to a similar extent they require different levels of processing once introduced into the cell (Fig. 1). The decision of which platform best suits the screen approach depends principally on the type of cells to be used and the type of assay. Selecting a cell line that reflects the biological question being asked is crucial to the success of a screen. The siRNA approach requires a transient transfection and is generally performed in established adherent cell lines with short-term assays completed within 72–96 h post-transfection (Fig. 2b). The shRNA approach permits long term, stable knockdown in cell lines (adherent and suspension), primary cells and slow dividing cells which broadens the assay possibilities beyond short term 2-dimensional tissue culture to assays that are several weeks long, and to complex 3-dimensional assays such as tumorigenesis assays and *in vivo* growth of cells transplanted into immunocompromised mice (Fig. 2c).

siRNA screens are typically performed in 384-well format with a single target in each well. Using Dharmacon's SMARTpool technology (Dharmacon RNAi Technologies; http://www.dharmacon.com), where four siRNAs targeting one gene are multiplexed in one well (Fig. 3a), a whole genome collection is approximately sixty 384 well plates. Other reagent vendors supply multiple individual siRNAs for each gene target arrayed singularly (Fig. 3b), which greatly increases the number of plates required to screen the genome. Screens are generally performed in duplicate or triplicate with a single biological replicate. Plates are screened on a weekly cycle and throughput is highly dependent on the type of assay being performed. Most laboratories have a standard pipeline for transfection development and miniaturisation to 384 well format and require strong and moderate positive controls and multiple negative controls (including lipid mock-transfection and non-targeting siRNAs). The optimisation time is very assay dependent, particularly when developing complex multi-feature imaging screens, but is crucial to the success of the screen. In general, once assay parameters and workflows are defined, the optimisation time far exceeds the time to conduct the actual screen. Statistical parameters for assessing robustness of controls from the perspective of dynamic range and variability are well established [46]. Data are generated on a weekly basis and analysed cumulatively to the point where a final ranked hit list is generated.

Assay development using the shRNA platform requires the researcher to evaluate the infectivity of their cell line, identify

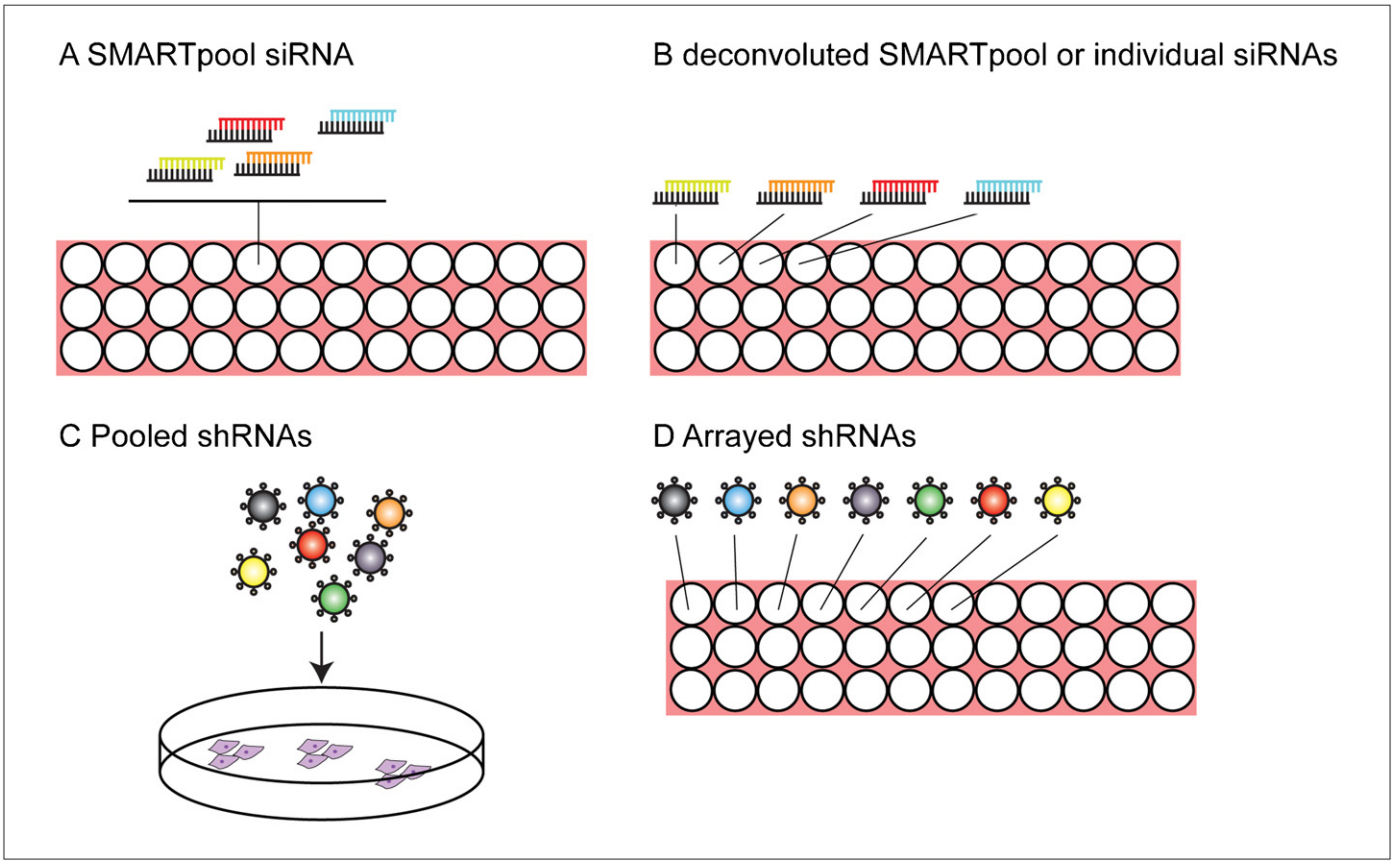

FIGURE 3

(a) SMARTpool siRNA reagents (Dharmacon RNAi Technologies) comprises four individual siRNA sequences directed against one gene target combined in the same well. **(b)** Validation of SMARTpool siRNA reagents requires deconvolution into the four individual constituent siRNAs that are then screened in individual wells. siRNA reagents from other vendors are provided in single array format as indicated. **(c)** shRNA pools can comprise any number of individual shRNA constructs (commonly for genome scale screens, 5000, 10,000 or more per pool), which are then transduced into a large cell population. **(d)** Individual shRNA constructs are arrayed in 96 well plate format with virus prepared in each well and cells are transduced in well format.

biologically relevant positive and negative shRNA constructs and determine the infectious viral dose they will screen with, aiming for 1 integration per cell. The shRNAs that contribute to the assay phenotype are identified by Next Generation Sequence analysis of genomic DNA extracted from the cell population present at the conclusion of the assay (Fig. 2c).

Validation strategies

C. elegans makes multiple siRNAs from every long dsRNA, which effectively ensures high level knockdown of a target gene. In mammalian cells, siRNAs are commonly screened using a pooled approach (e.g. SMARTpool) or at least three individual sequences. Using SMARTpool reagents, the most common validation method is to analyse the top 400 ranked hits (or more or less depending on researcher choice) by deconvolution of the SMARTpool into its constituent siRNAs or purchase additional new siRNA sequences and rescreen in the same assay (Fig. 3b). High confidence hits are defined as those in which three or four of the individual siRNAs recapitulate the SMARTpool phenotype [41]. Similar rules are applied when screening individual siRNA reagents from other vendors, such that multiple sequences must give a result within an acceptable cut off to be considered a hit. From a genome-wide screen, the total number of high confidence hits will depend on the statistical stringency and the assay type. Using a pooled shRNA screen approach and Next Generation Sequencing, shRNAs are statistically ranked as a measure of the frequency of the presence of the construct relative to the reference control (generally collected at T_0) (K. Jastrzebski and K. Simpson unpublished). The highest confidence hits are those for which multiple shRNAs to the same target are statistically enriched. If a single shRNA is highly ranked, then users will make virus individually for that construct and any other available constructs to the same target, and validate the phenotype using the same assay. The biological context of the assay will define what statistical and reproducibility parameters are applied to the analysis.

Applications

Mammalian RNAi screens have largely focused on the field of cancer biology with a wide diversity of applications. An extensive range of cell lines/disease states have been screened and approaches vary, ranging from whole genome, focused gene family collections such as the kinome (all kinases in the genome) or druggable genome (targets that may be amenable to small molecule drug design, represent about 40% of the genome) or custom targeted pathway collections (Table 3). RNAi screens have proven a powerful tool for identification of genes that (1) regulate drug resistance (primarily to chemotherapy agents) or sensitise cells to drug treatment, (2) activate cell cycle arrest or alter proliferation and (3) regulate DNA damage repair (reviewed by [47]).

The first published genome-scale siRNA screen identified 87 primary hits that were synthetic lethal interacting targets in response to treatment with the chemotherapeutic drug paclitaxel [40]. Synthetic lethal screens can also identify interactions with powerful oncogenes such as Ras [48]. Several recent kinome screens have identified drug responsive targets. For example, Arora *et al.* identified 55 primary targets that inhibited growth after cisplatin treatment in ovarian cells, and characterised CHK1 in detail leading to the identification of a small molecule inhibitor that sensitises cells to cisplatin treatment [49]. Diep *et al.* identified six validated targets that sensitise pancreatic cancer cells to erlotinib treatment [50].

Cell cycle regulation is a fundamental area of biology investigated using RNAi screening and includes a vast array of approaches. For example, Tsui *et al.* screened the whole genome to identify regulators of kinesin 5, which is required for mitotic

TABLE 3

Selected mammalian RNAi screens to provide an overview of the breadth of functional genomics applications, including the platform and specific cell line screened

Scale	Platform	Cell line	Screen assay phenotype	Refs
Genome	siRNA	NCI-H1155 – non-small cell lung carcinoma	Synthetic lethal paclitaxel-dependent assay	[40]
Genome	siRNA	U2OS – osteosarcoma	Host–pathogen *Trypanosoma cruzi*	[64]
Kinome	siRNA	34 breast cell lines normal to malignant	Breast carcinoma lethality	[54]
Kinome	siRNA	SCOV3 – ovarian	Mitotic progression in ovarian cancer	[52]
Genome	siRNA	U2OS – osteosarcoma	EGFP-FOXO1a reporter localisation	[84]
Genome	siRNA	H4 neuroblastoma	Autophagy using GFP-LC3 reporter	[85]
Genome	siRNA	C33A, HeLa, cervical carcinoma	Regulation of Papilloma virus	[86]
Genome	siRNA	HeLa cervical carcinoma	Kinesin-5 regulation	[51]
Genome	siRNA	HeLa cervical carcinoma	HIV infection	[57]
Phosphatome	shRNA	MCF10A-ERBB2 normal breast	Cell motility	[87]
Kinome and phosphatome	shRNA	HSC-3 – oral carcinoma	Growth inhibition	[88]
Kinome	siRNA	BxPC-3 Pancreatic	Drug resistance in human pancreatic cancer	[50]
Genome	siRNA	Human embryonic stem cells	Self-renewal and pluripotency	[67]
Kinome	siRNA	SKOV3 – ovarian	Cisplatin treatment of ovarian carcinoma cells	[49]
Custom collection	siRNA	MCF10A normal breast	Regulators of cell motility	[41]
Whole genome	shRNA	MCF10A normal breast	Regulation of cell invasion	[55]

spindle formation, using a small molecule inhibitor target to force cancer cells into apoptosis [51]. Performing live high content imaging over a series of days, they identified 15 validated targets and classified them into sub-groups of drug responsiveness, identifying known and novel regulators. Ahmed *et al*. performed a microscopy-based screen of the kinome in ovarian carcinoma cells and identified six novel high confidence candidates that modulate mitotic progression in combination with the drug paclitaxel [52].

Cell migration is not only a fundamental developmental process but is also crucial to cancer progression and there have been several screens that have used surrogate *in vitro* measurements to identify genes that positively or negatively modulate migration/invasion. Approaches include siRNA knockdown and quantitation of migration in epithelial cells using wound healing [41] and random migration approaches [53], in endothelial cells using sheet migration [54] and in epithelial cells using pooled shRNAs and quantitation using boyden chamber transwell migration [55].

Host–pathogen interaction studies have benefited enormously from RNAi screening technology, and researchers can now investigate responses to viral infection on a genome-scale using pathogens that are temporarily debilitating or can cause devastating loss of life and are an economic burden. Multiple screens investigating proteins involved in HIV infection have been performed [56–58] and others include Influenza A H1N1 [59,60], hepatitis C [61–63], *Trypanosoma cruzi* infection [64], *Salmonella typhimurium* [65] and west nile virus infection [66].

Validation and tertiary analysis in multiple cell lines using the same and/or different assays is common and has proven very powerful in a recent screen to identify genes required for stem cell self-renewal and pluripotency. Validation (deconvolution of all four SMARTpools) screens were performed in multiple cell lines, resulting in the identification of a key transcription factor required for the maintenance of human embryonic stem cell identity and pluripotency in somatic cells [67]. Taking a targeted approach in terms of library, a very broad approach in terms of the number of cell lines, and a highly encompassing approach in terms of integrating other genomic data (array CGH, microarray, next generation sequencing and mutation studies), Brough *et al*. [68] screened the kinome in 34 different breast carcinoma cell lines (ranging from non-transformed to highly malignant) to identify gene targets that caused cell death. By generating a viability profile for each cell line and using stringent statistical criteria, after the primary screen they focused on 20 cell lines to identify genetic dependencies and to classify into functional sub-groups. The ultimate goal of such research is to gain a better understanding of the complexity of cellular responses towards facilitating therapeutic strategies for personalised medicine.

Strengths and limitations

Off-target effects are the result of unintended genes being knocked down as a by-product of the action of an siRNA. Off-target effects have been a major problem in the mammalian field but this is diminishing as our knowledge of the RNAi pathway accumulates and manufacturers alter siRNA target sequences based on cumulative screening knowledge [69–71]. The validation steps outlined above are aimed at identifying off-target effects. It is challenging to predict the proportion of false positive and false negative hits in a screen, however, if the assay is robust, these should be largely eliminated through the validation process [72]. Protein stability hampers good knockdown, particularly in transient transfection approaches in which the protein half-life may be longer than the duration of the screen. It is important to remember that a screen is a tool, the assay is developed around several key candidates, and conditions manipulated as much as possible for the most robust outcome; however, when performed on a large scale we can expect to miss some targets.

Bioinformatics

Genome-wide screens generate significant data and the challenges are different for each screening platform. Nonetheless, in broad terms, each screening approach results in a rank order of gene targets, known as 'hits', which permits the screener to focus their secondary validation and subsequently define a high confidence hit list. For detailed information regarding analysis of mammalian siRNA screens see Birmingham *et al*. [46]. For shRNA screens, the development of Next Generation Sequencing pipelines has necessitated creating new analysis workflows that are still in their infancy. For siRNA screening, a significant amount of data can be obtained from one screen, particularly when performing multiparametric assays and all the data points from each library screen plate must be merged and analysed collectively via a normalisation strategy which covers daily and weekly variance. Once a high confidence hit list has been validated, a major challenge is to interpret the biological significance through integrating the data with other resources. These include genome-wide pathway analysis collated via commercial sources (e.g. GeneGo, Ingenuity) and open source databases (e.g. String, NIH DAVID), other screens with a similar focus or similar cell line, cancer-related resources (e.g. Oncomine, microarray data, next generation sequencing databases) and species-specific databases (e.g. WormBase (http://www.wormbase.org), FlyBase (http://www.flybase.org)).

Conclusion

In a little over a decade, RNAi has revolutionised our concept of how research is done and has proven itself to be a very powerful and highly adaptable technology. Its use will expand as assays become more refined and automation more accessible. Fundamental biological questions will continue to be addressed using *C. elegans* and the scope of screens will broaden and aid in unravelling the hits identified from mammalian screens. With the constant improvements in RNAi sequence prediction algorithms, RNAi screens are likely to become more efficient and on-target and will be a major importance in unravelling the mechanisms of disease. With the enormous global influx of data courtesy of Next Generation Sequencing, the concept of personalised medicine will provide an avenue for more customisable gene lists and focused screens.

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