The Application of High Throughput siRNA Screening Technology to Study Host-Pathogen Interactions

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Abstract: Recent advances in high throughput screening technologies have accelerated the identification and characterization of potential factors involved in host-virus interactions, facilitating early detection and diagnosis of diseases, as well as providing promising drug targets. The last decade has seen a plethora of successful examples of high throughput screening approaches, especially siRNA screening. With support from protein interaction studies, mRNA expression profiling, and bioinformatics, siRNA screening has also been successfully utilized to identify host factors required for a number of viruses including HIV, West Nile virus and H1N1 virus. Such studies have raised the awareness of virologists, and have opened a new chapter of global analysis of host-pathogen interactions. However, to play a more defining role in prognostics, diagnostics and therapeutics for virus diseases, acknowledged drawbacks, including false positives and negatives, inherent in this technology, must be successfully addressed.

Keywords: Drug target, host factor, pathogen, siRNA, virus.

INTRODUCTION

Throughout history, virus has posed a major threat to civilization, and must rank as one of the major killers of human beings. It is virtually impossible to accurately determine how many people will get sick or die from all kinds of virus infections every year, yet, we can speculate on the number from some specific examples. Across the world, more than 2 billion people have been infected with human hepatitis B virus (HBV) and about 350 million remain chronically infected [1-11]. Unfortunately, HBV infection plays an important role in the pathogenesis and development of hepatocellular carcinoma (HCC) [12, 13]. Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide, particularly in China, but still remains one of the more difficult to treat. Last year, the the worldwide H1N1 flu virus pandemic caused both personnel and economic suffering in North America, Asia and Europe [14-23]. In particular, the increasing reliance on global air travel aids the rapid spread of such diseases. Perhaps an even more serious threat is posed by the human immunodeficiency virus (HIV), a lentivirus (a member of the retrovirus family) that causes acquired immunodeficiency syndrome (AIDS) [24, 25]. This currently infects about 0.6% of the world's population [27-35], and killed more than 25 million people between 1986 and 2005 [26]. In 2009 it was estimated that 33.3 million people were living with HIV/AIDS with 2.6 million newly infected cases and 1.8 million deaths, leaving 16.6 million children orphaned [http://www.avert.org/worldstats.htm]. It is therefore urgent for virologists and medical scientists to expedite new ways to dissect the molecular mechanisms of virus infection and find new drugs and approaches for improved treatment.

It is widely acknowledged that viruses has a relatively limited size of genome, ranging in size from approximately 3,200 nucleotides (e.g. Hepadnaviruses) to approximately 1.2 million base pairs (Mbp, Mimivirus). The number of proteins they encode is therefore relatively small. It is now realized that many viruses have to manipulate the host cellular machinery and metabolism to favor their life cycle. HIV, for instance, encodes only 15 proteins [36], and thus must exploit multiple host cell functions for successful infection [36]. West Nile Virus (WNV), a flavivirus which encodes 10 proteins, has also been demonstrated to manipulate host proteins to facilitate infection [37]. The bulk of the evidence has confirmed that many steps in the viral life cycle, including intracellular trafficking, gene expression, replication and virion assembly, depend on interactions with specific host cell gene products [38-43]. Although the identity of many of these host molecules is still unknown, emerging data indicate that their identification and characterization can provide new insights into the detailed life cycle of the virus and reveal potentially valuable targets for prophylactic and therapeutic intervention. Targeting host proteins as antiviral targets may provide a novel and effective approach to treatment. An inhibitor that specifically targets host proteins will not only overcome the genetic heterogeneity within a virus strain, but will also potentially minimize the selection of drug-resistant mutants due to the lower rate of mutants of host genes compared to the virus [44]. Research on potential host factors involved in interactions host-virus interactions will accelerate the development of such new classes of antiviral drugs.

Recently, the innovative idea of genome-wide screening has brought new impetus to the study of host-pathogen interactions. Emerging high throughput RNAi screening

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1875-5402/12 $58.00+.00

technology has played a pivotal role in the identification of host proteins involved in virus infection since it is an unbiased, large-scale screening methodology. Its potential is evidenced by the large number of which have identified thousands of host factors involved during infection with HIV, HCV, H1N1, Mycobacterium tuberculosis and PIV5 [44-52]. This revolutionary technology has paved way for the development of many new, effective tailor-made drugs, and has opened a new chapter of research on host-pathogen interactions.

In this review, we will overview siRNA screening technology and address its current limitations and possible methods of overcoming them.

siRNA SCREENING TECHNOLOGY AND ITS APPLICATION

RNAi was first discovered in *C. elegans* when Fire and colleagues [47] observed that the introduction of dsRNA was an effective method for silencing gene expression. The molecular mechanisms underlying RNAi, which have been summarized in recent publications [53, 54] are shown in Fig. (**1**). Briefly, the RNA is introduced into the cytoplasm *via* the plasma membrane and cleaved into short fragments by the enzyme dicer. These associate with the RNA silencing complex (RISC). After strand separation, RISC binds to the mRNA which is targeted by the single RNA strand within the complex and cleaves the mRNA. RNAi has been shown to work in a similar manner in every metazoan and the technology has been use to generate a wide variety of loss-of-function phenotypes. The first large-scale RNAi screening was pioneered in *C. elegans* in 2000 [55, 56]. This elegantly demonstrated how biologically significant insights can be gained using a variety of phenotype-based assays. Since then, further development and refinement have gradually optimized this method.

This technique is now becoming widely used to identify host factors involved in virus infection. The methodology is quite simple: by individually degrading mRNAs of host genes, thus silencing the expression of them, it is possible to screen out host factors indispensable for virus infection [57]. In *C. elegans*, long dsRNA are injected directly into worms to perform RNAi screening, while in *Drosophila* and mammals, dsRNA or siRNA are usually used to treat cells. With the establishment and development of siRNA libraries, high throughput siRNA screening has accelerated the identification of host factors operational during HIV, HCV, influenza A and *Plasmodium* Sporozoite infection [38, 44-46, 58-61].

COMPARISON WITH CLASSICAL FORWARD GENETICS

Strictly speaking, siRNA screening represents a powerful forward genetics approach to dissect virus-host cell interactions [38, 46, 62]. To get a full appreciation of high throughput RNAi screening technology, we must turn first to forward genetics. By definition, in forward genetics, one begins with the phenotype of unknown origin and ends with the identification of the mutated gene. This involves no preconceptions. Hypotheses are the outcome of genetic investigations rather than the starting point [63]. Therefore, as a forward genetics approach, siRNA screening has the innate ability to identify specific roles for genes and biological processes free of *a priori* hypotheses regarding their function.

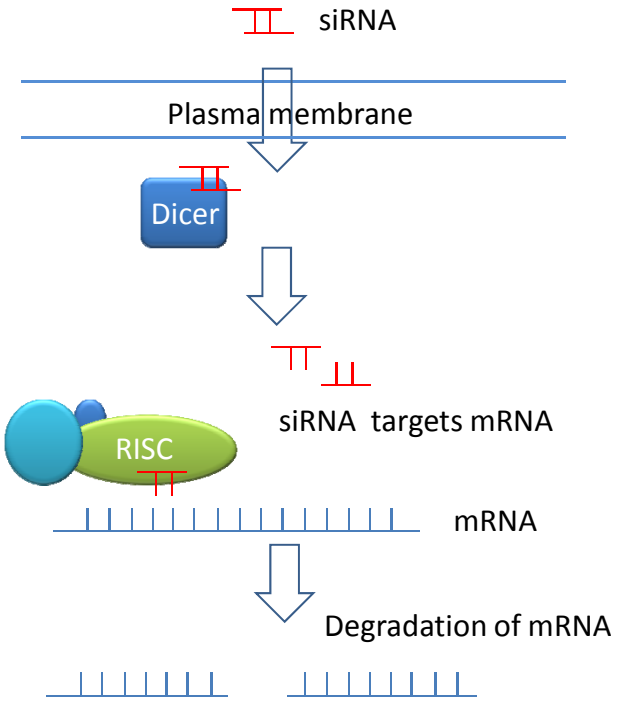

Fig. (1). Biochemical principles of siRNA silencing. Initially, siRNA is introduced into the cell *via* the plasma membrane. Then, siRNA associate with the RNA Induced Silencing Complex (RISC). After strand separation, the antisense strand of siRNA binds to its complementary/target mRNA. Finally, nucleases within the RISC degrade the targeted mRNA.

Yet, genome-wide siRNA screening, as a newly emerging technology, is distinct from classical forward genetics. By classical forward genetics, Poltorak and colleague [64] first revealed that mammalian Toll-like receptors (TLRs) acted as specific sensors of microbial molecules and were required for pathogen recognition and response to infection. This was the first successful example of the application of forward genetics to reveal specific host-pathogen interactions.

Compared with genome-wide siRNA screening, classical forward genetics is relatively simpler. Classical forward genetics begin with an existing biological phenomenon, then after treatment with mutagens, a specific phenotype can be obtained. Subsequent positional cloning will identify the specific mutation involved. This in turn leads to hypotheses concerning the biological function of this gene. Further experiments can ultimately shed light on the underlying mechanism behind the biological phenomenon [63].

In addition, classical forward genetics deals with *in vivo* phenotypes, which are more tightly associated to the whole body situation. In sharp contrast, high throughput RNAi screening only can only be applied to cell-based assay systems, which may give rise to artificial results due to, for example, off target effects or incomplete knockdown of a target gene. Verification that the RNA-mediated knockdown

of the target gene is responsible for the phenotype should be carefully undertaken [Kassner]. A second major limitation is that the cell type (and hence the research model) is restricted to those that are readily transfectable (e.g. 293T, Hela cells, Drosophila or *C. elegans*) [65]. Additionally, data analysis is much easier for classical forward genetics experiments. High throughput RNAi screening can generate a large amounts of data, requiring subsequent validation, placing heavy demands on resources [39].

Whlist the task of identifying the host factors involved in virus infection is urgently required, classical forward genetics approaches will be relatively time-consuming bearing in mind the size of the human genome currently estimated to be approximately 25,000 genes. This goal may take several decades to achieve with classical forward genetic screening methods using current technologies. Thus, in spite of its aforementioned merits, it may not be wise to rely solely on classical forward genetics.

MAIN LIMITATIONS OF siRNA SCREENING - FALSE POSITIVES AND NEGATIVES

Combined with the rapid development in our understanding of the underlying silencing pathways, the explosive speed at which siRNA screening technology has been adopted has diverted the attention of some scientists to stay conscious of its limitations [66]. This has resulted in a surprising lack of correlation between what appear to be similar screens. Major concerns include false positives, arising from off-target effects (OTE), where the promiscuous actions of the siRNA gives rise to the observed phenotype due to the depletion of a second unintended target, as well as false negatives stemming partly from inefficient targeting or nonspecific toxicity [refs].

Echeverri and coworkers classified OTE into two groups: sequence-dependent off-target effects and sequence-independent off-target effects [66]. They assumed that sequence-dependent off-target effects arise from the relatively high tolerance for mismatches between the siRNA guide strand, the ultimate targeting molecule, and the complementary target mRNA sequence which has several bases known as the guide strand's 'seed region', outside the short 'core' targeting region [66]. Some improved experimental designs such as alternative RNA backbone chemistries, structural variants and advanced design algorithms have been utilized to reduce the occurrence of these effects. For instance, as the efficacy of siRNAs against HCV is influenced by structure and target site accessibility, recent research by Sagan and colleagues [67] demonstrated that viral RNA microarrays (VRMs) and an HCV viral RNA-coated magnetic bead-based (VRB) assay could improve efficiency. However, it is currently impossible to rule out off-target effects of siRNA screening technology through experimental design alone.

Therefore, two main methods, rescue and redundancy, have been proposed to validate screening data arising from RNAi methodology [66] (Fig. **2**). Rescue experiments are undoubtedly the optimium control. However, they are technically difficult and are not suited to large scale application. When redundancy is used for verification, reports of siRNA-induced phenotypes should be controlled using at least 2 distinct siRNA sequences, and ideally confirmed through rescue experiments [66].

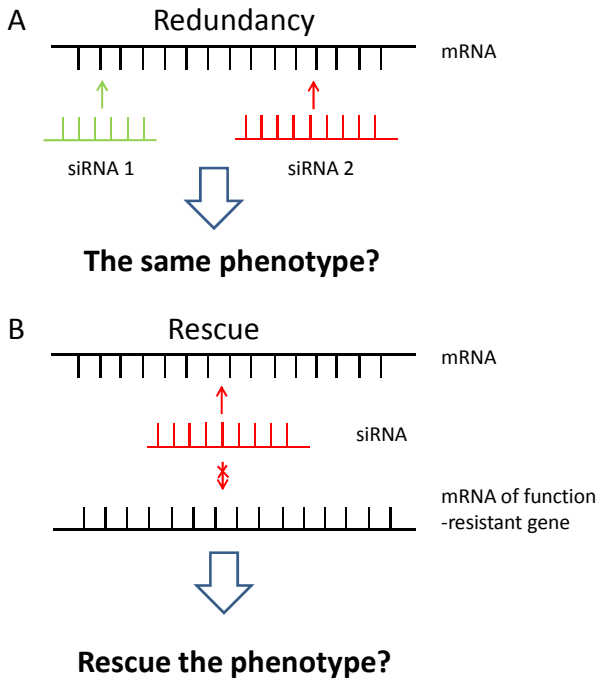

Fig. (2). Rescue and redundancy approaches to minimize sequence-dependent off-target effects. A. Redundancy: this tests whether different siRNA targeting the same mRNA strand can lead to the same phenomenon. **B**. Rescue: this tests whether a resistant version of gene can rescue the siRNA-induced phenotype.

When it comes to minimizing false negatives, on the one hand, the toxicity associated with each pair of siRNAs should be measured by assaying cell viability to optimise the dose that should be transfected. False negatives can be minimized by retesting primary screening results, especially when in published work, the related genes have been indicated to function in certain pathways. Unfortunately it is not yet possible to predict whether the use of additional RNAi or changing parameters such as cell culture or viral isolate will work without conducting the appropriate experiments [68].

In conclusion, due to the very significant false-negative and false-positive rates inherent to high throughput siRNA screening, no single screen can be expected to yield a truly comprehensive map of all of the host genes that are involved in a particular process. We will now discuss alternative approaches to further improve the technology.

APPROACHES TO OPTIMIZE siRNA SCREENING

Relying on siRNA experiments in the absence of any other supporting data from non-siRNA approaches is clearly a risk. Scientists must integrate raw data from siRNA screening experiments with information from external databases such as Gene Ontology (GO), Prot-Prot interaction, expression profile databases, domain structure (InterPro) or RNAi phenotype.

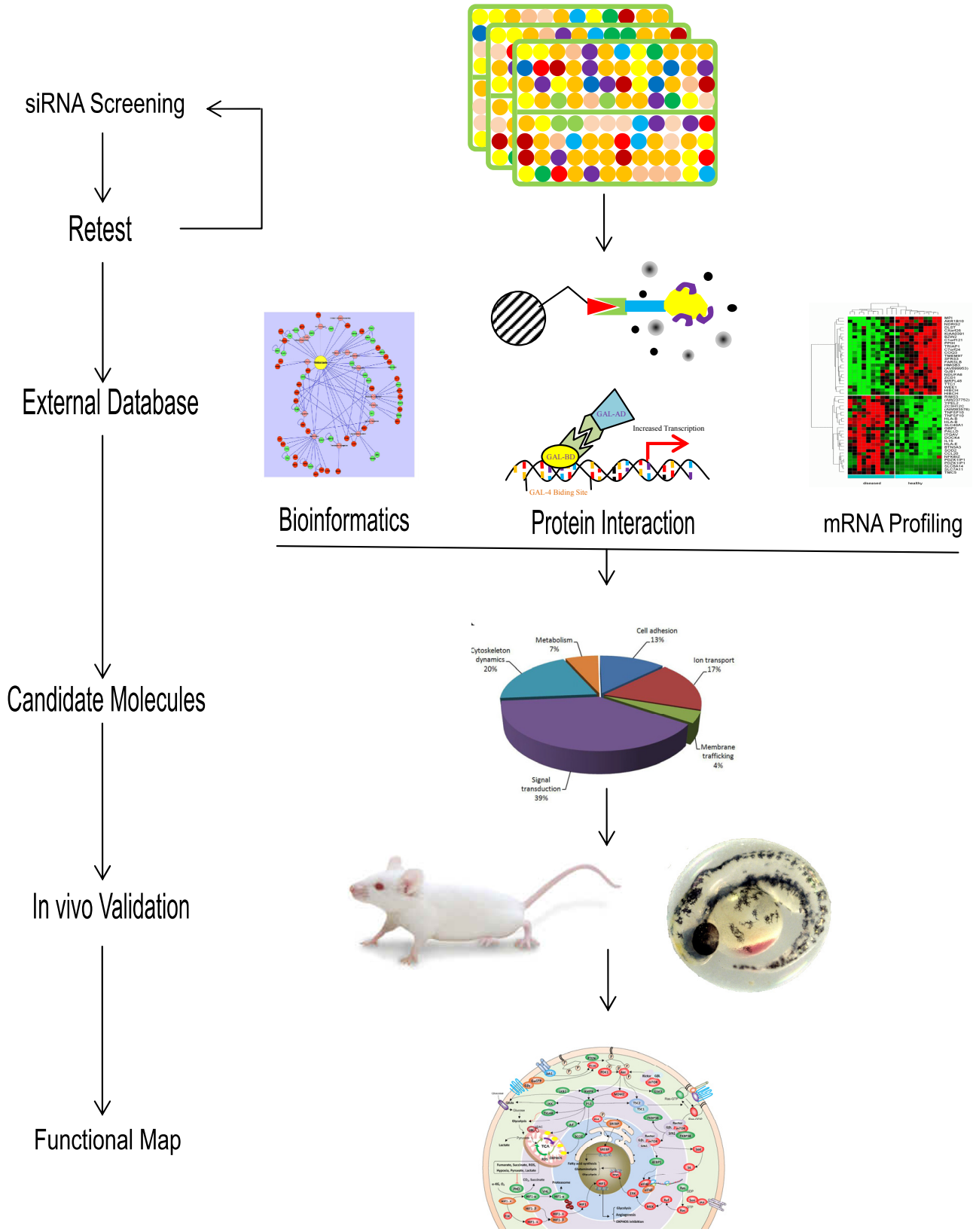

Fig. (3). A systems biology approach using high throughput siRNA screening to identify host-pathogen interactions. Initially genome-wide siRNA screen results should be retested. Then, combined with external datasets from mRNA profiling, bioinformatics and protein interaction studies, candidate genes can be identified. Further *in vivo* experiments will provide validation to delineate a functional map addressing the function of genes involved in host-pathogen interactions.

A newly emerging method is the use of bioinformatics tools for detailed interrogation of raw data. Li and colleagues in their genome-wide genetic screen for host factors required for hepatitis C virus propagation integrated the HCV host factor database, and revealed statistically enriched complexes and pathways [69]. To confirm new connections and minimize bias, they performed bioinformatics analysis after the selection of their validated gene set. Bushman and colleagues introduced the MCODE software, which identifies highly interactive regios (clusters) in a network () to predict potential signaling pathways in host factors identified by siRNA screening [70]. This is an excellent example of the successful interface of biology and computer software. It therefore seems reasonable to predict that bioinformatics will play an increasingly important role in analyzing the results of high throughput siRNA screening. The goal of finding new drugs and treatment cannot be accomplished without a whole and vivid picture of host-pathogen interactions. Taking into account the enormous amount of information that can be generated in high throughput siRNA screening, comprehensive interpretation of that data will benefit from even more advanced bioinformatics tools.

If we adopt an approach which does not only rely on identifying host factors based solely on ranking siRNA activities, we will be able to more effectively mine the genetic data sets to identify those factors most likely to be immediate regulators of virus infection, and establish a network of host-pathogen interactions that coordinate virus infection. By developing additional selection criteria including protein interactions, mRNA expression, and gene ontology, König and his colleague [71] identified a number of host genes that coordinate HIV-1 infection which were quite different from those published before, although this, of course, may also be due to the use of independent RNAi libraries.

The establishment of human siRNA library poses a major obstacle for researchers in this field since it is technically difficult, time consuming and expensive. To solve this problem, Hao and colleagues [72] turned to Drosophila, one of the major model organisms that have been used for genetic screens to study a variety of cellular and developmental processes. They demonstrated that genome-wide RNAi screening analysis can be easily facilitated in a well-developed model systems such as *Drosophila*, whose genome contains only approximate 14,000 genes that can nearly all be specifically targeted for high efficiency mRNA depletion using siRNA libraries. Due to its powerful genetics and conservation with vertebrates, *Drosophila* has enabled numerous important contributions to mammalian cell biology [73-76]. Thus, in principle, *Drosophila* RNAi studies could accelerate identification of host interactions essential for viral replication in other systems. This strategy could be applied to the identification of novel host factors involved in the replication of other viruses, providing at least a component of their replication cycle is supported by Drosophila cells.

To identify host factors involved in the viral life cycle [38], researchers have applied an innovative, two-part screening protocol to detect proteins needed for the complete viral life cycle, from viral-host receptor binding to the completion of a second round of viral infection. Part one of the study consisted of infecting siRNA–transfected cells with one strain of HIV and staining for a reporter gene product 48 hours later. This screen detects host proteins essential for viral entry, but is less sensitive for factors affecting viral assembly and budding. To identify late-acting factors, they incubated culture supernatants from the initial study with fresh reporter cells and assayed for Tat-dependent reporter gene expression after 24 hours. A similar successful application of this approach was used in a genome-wide genetic screen for host factors required for hepatitis C virus propagation [69].

When using high throughput assays, it is fundamentally important to exclude the results caused by assays noise to ensure low variability. Screening tens of thousands of samples requires the use of statistical tools developed for significant assay optimization like Bonferroni correction or False Discovery Rateto ensure data is statistically significant [68].

CONCLUSIONS

High throughput siRNA screening approach allows a rapid, unbiased and large-scale identification of virus host cofactors independent of any preconceived models of the virus life cycle or any assumptions about gene functions. Despite its powerful potential, we must also appreciate the current limitations of the method. Without a full understanding of these we will be unable be optimally deploying our limited resources to win the battle against viruses.

Firstly, to ensure a low rate of false positives and false negatives, assessing the overlap with genes identified in published peer-viewed studies is a sound choice. Then, the development of suitable cell-based assays for siRNA screening and miniaturization of assay formats will be the main challenges confronting us. Moreover, a comprehensive phenotype database that integrates siRNA screening, expression profiling and bioinformatics will be a powerful and valuable resource. Finally, research into improved methods of siRNA screening will ultimately yield even more powerful tools.

As shown in Fig. (**3**), if siRNA screening can be incorporated into a systems biology approach using other complimentary methods to shed light on the host-pathogen interactions, an even brighter future for the use of siRNA screening lies around the corner.

ACKNOWLEDGEMENTS

This work was supported by grants from the National 973 Basic Research Program of China (2011CB910703, 2012CB 518900).

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Received: February 25, 2011 **Revised: March 30, 2011** **Accepted: April 6, 2011**