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What have RNAi screens taught us about viral-host interactions?

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Abstract

The blossoming of genomic technologies and miniaturization has opened up the field of genomic scale cell-based screening to the study of viral-host interactions. RNAi technology, while still at its infancy, is being used to identify cellular factors required for various viral infections. This has led to the discovery of hundreds of new factors, and has increased our knowledge of the host factors that impact viral infection and highlighted the cellular pathways at play.

Introduction

Viral pathogens are amazing. Despite the small size of their genomes, they can reprogram cells to promote viral replication via the subversion of a plethora of cellular factors. Our understanding of how viruses exploit cellular genes has not only contributed to our knowledge of pathogenesis, but has also revealed much of the inner workings of the cell. Viruses have taught us about transcription factors, capping, splicing, mRNA transport, translation, and more. The rapid evolution of viruses makes it particularly difficult to treat infections. While broad-spectrum antibiotics have been relatively easy to identify and have proven efficacious against bacterial pathogens, broad-spectrum antivirals have been elusive. Historically, the development of antiviral therapeutics has focused on essential viral proteins as targets. A priori, this seems like the simplest strategy as it provides a unique, infection-specific target. However, the high mutation rate of many viruses, in particular RNA viruses, has made this task difficult, requiring an arsenal of drugs against multiple targets to achieve viral suppression.

More recently, with the advent of cell-based screening technologies, it has become possible to screen for inhibitors of viral infection during a bona fide infection cycle, rather than in simplified in vitro systems. Cell-based screening has allowed the identification of small molecule inhibitors of cellular targets required for infection. Advances in genomic technologies and RNA interference (RNAi) methodologies has allowed for the development of high-throughput genome-scale RNAi screens for cellular factors that, when depleted, impact infection. This, at least in principle, allows for the identification of all genes in a given host cell that either promote or inhibit infection. This systems approach has enormous potential for the discovery of both new therapeutic targets, and a better understanding of virus-host interactions. In the last year, a number of genome-scale RNAi screens were performed against human viral pathogens. This has led to the identification of a large number of new host factors that influence viral replication. This work has greatly expanded our knowledge of the interface between each virus and its host, and will undoubtedly lead to continued insight into the cell biological roles of many of these genes. However, as with any new technology, there are a

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number of important issues that have arisen, most strikingly the surprising lack of concordance between the results of seemingly similar screens. This review will discuss the recent findings, highlighting particular findings and suggest guidelines to improve future screens.

HIV

Three siRNA screens were published in the last year with the goal of identifying the complete set of cellular genes required for HIV infection (Table 1) [1–3].

The first, by Brass *et al.*, was performed in HeLa cells transduced with the receptors CD4 and CCR5 as well as a β -galactosidase (β -gal) reporter gene responsive to the HIV transactivator tat [3]. Transfection of these cells with a genome-wide library of siRNAs (4 siRNAs/pool) for 72 hours was followed by infection with replication-competent HIV IIIB isolate. 48 hours post-infection, the cells were processed for immunofluorescence and stained for the viral antigen p24. This assay is sensitive to all of the steps leading to p24 translation including: entry, uncoating, reverse transcription, nuclear import, integration, transcription and translation. As a second measure of productive infection, the authors removed the cultured supernatant which contained released virus, infected fresh cells, and measured the levels of tat-mediated β -gal as an indirect measure of virus release. From this dual screen, performed in duplicate, the authors identified 273 genes that impacted either the percentage of p24 positive cells or beta-gal production by at least 2 standard deviations (2–3-fold reduction). Of these, 101 genes that decreased p24 were supported by multiple siRNAs as were 31 genes that impacted β -gal production. These genes fall into classes spanning many aspects of cell biology. In particular, a number of components of the nuclear pore, the transcriptional Mediator complex as well as genes involved in vesicular trafficking were identified. Characterization of three candidates (Rab6, TNPO3 and Med28) from these overrepresented groups was performed. Rab6, a GTPase required for Golgi retrograde transport, is seemingly required for viral fusion as it is dispensable for infection of pseudotyped virus. TNPO3, a nuclear import factor, is required between reverse transcription and integration suggesting that it plays a role in nuclear import. Lastly, the authors found that Med28, an integral component of the Mediator, a well-known transcription cofactor, is required for HIV-1 gene expression, but not expression of other closely related retroviruses suggesting a specific dependence on this complex.

The second screen, published by König *et al.*, was performed in 293T cells and screened using a VSV-G pseudotyped replication-incompetent HIV-1 virus that expresses a luciferase reporter [2]. Thus, only the steps downstream of HIV-1 entry including reverse transcription, integration and transcription were queried. Cells were transfected with a genome-wide library (6 siRNAs/gene, 3 wells/gene) for 48 hours followed by infection with the HIV-1 luciferase reporter virions and assayed for luciferase expression 24 hours later. This assay protocol was repeated with pseudotyped Moloney murine leukemia virus (MLV), adeno-associated virus, and cell viability. Using a complicated set of criteria, starting with 2400 genes, the authors identified 295 genes supported by multiple siRNAs that decreased luciferase by 45%, had minimal toxicity, and were supported by independent criteria including expression profiling, gene ontology, yeast two hybrid, etc. Interestingly, 80% of the genes that impact HIV-1 also impact MLV replication, suggesting that these steps are highly conserved between retroviruses. Next, identified genes were re-confirmed, and found to include the ubiquitin proteasome system, DNA repair, and amino acid ligase activity. The authors performed secondary analysis on those genes that did not impact VSV G entry to identify which step in the HIV lifecycle was dependent upon the candidate gene function. 44 genes were found that impacted reverse transcription, of which 12 are likely directly involved in either uncoating or early steps in the process and have domains consistent with nucleic acid binding, while 23 genes affected the kinetics of DNA production and the rest were difficult to classify. In addition, the authors

identified 6 genes that facilitate nuclear import, amongst which were a number of components of the nuclear pore, and 9 genes required for integration of the HIV-1 DNA genome.

The third screen, by Zhou *et al* was performed in the same HeLa cells used by Brass *et al* [1]. The cells were transfected with a genome-wide library (3 siRNAs/pool) and were subsequently infected with replication competent HXB2 HIV-1 24 hours later. At 48 hours post-infection the production of tat-dependent β -gal was measured to assess the first round of virus infection and tat activation. Subsequently, β -gal expression was measured at 96 hours post-infection to assay an additional round of virus spread. 931 pools, identified by effects on β -gal but not cell viability, were screened for their effect on viral shedding, leading to 390 genes that inhibited in this independent assay. Of these, 311 are expressed appropriately, and 291 were screened against an additional siRNA pool. This led to the identification of 205 genes that reproducibly inhibited HIV-mediated β -gal production while one gene, GM2A, enhanced activity by >2-fold. 75 genes that inhibited β -gal were found to be required for HIV Tat-mediated transactivation of the LTR promoter (<60% of WT) in an independent assay, and included components of the Mediator complex (7 genes), also identified in the Brass *et al* screen. Another set of genes that were over-represented, and validated by cDNA rescue, were involved in Akt/NF κ B signaling, although the step in the lifecycle was not addressed. Lastly, the authors identified 9 genes that had druggable domains and were able to cDNA rescue 4 of them, suggesting that they play a bona fide role in HIV replication. Further analysis demonstrated that BMP2K (kinase) and NEIL3 (DNA repair) were required for reverse transcription as was SERPINB6 (protease inhibitor), to a lesser extent.

A priori, one would have thought that these screens would have identified a large number of overlapping genes. However, there was minimal overlap (<7% for any pairwise combination) which is somewhat remarkable. Some of the differences are likely due to the different conditions tested by the three assays (cell type, virus isolate, and assay end point discrepancies), while other differences in outcome are likely due to technical issues such as off-target effects and filtering parameters. Taken together, while many of the genes identified in only one screen may ultimately be false positives, many others will eventually be found to play essential roles in HIV replication, making these data sets of enormous interest. In support of this contention, meta-analysis of these gene sets suggests that, while the individual genes identified may not be reproduced between screens, each screen identified genes belonging to some of the same cellular pathways [4]. For example, all three screens were enriched for: Nuclear Pore/Transport, DNA repair, Ubiquitin associated factors, Mediator complex, RNA binding proteins, GTP binding factors, and Helicases. Thus, these screens seemed to have uncovered common cellular pathways affecting HIV replication, even as they lack concordance on individual genes' essentiality.

A complementary approach to RNAi loss-of-function screening is to perform arrayed cDNA screening to identify gain-of-function activities. A screen by Nguyen *et al* used the same HeLa cell line as Brass and Zhou, and co-transfected a library of 15,000 human cDNAs along with a tat-responsive luciferase reporter [5]. 24 hours post-transfection, the cells were infected with replication competent HIV-1 (HIV-IIIB, used by Brass *et al*) and were assayed for luciferase activity three days post-infection. The authors identified 315 genes which, when over-expressed, increased tat-mediated luciferase production. Genes involved in mitochondrial biology, transcription, translation, and anti-apoptotic functions were over-represented. 3 of 6 of the top candidates were found to be required for replication when siRNAs were tested, validating this approach.

HCV

A number of screens have been performed recently to identify cellular factors that impact replication of HCV or subgenomic replicons (Table 2). Replicons are more broadly used because it is still technically difficult to work with infectious virus, although hopefully, this will be overcome in the near future.

Ng, *et al* screened 4,000 druggable genes (4 siRNAs/pool) using Huh7 cells that stably maintain a HCV (genotype 1b N strain) subgenomic replicon expressing HCV NS3-NS5B and the reporter secreted alkaline phosphatase (SEAP). Four days post transfection, SEAP activity was measured in the supernatants and a toxicity assay (MTT) was performed on the cell monolayer. The 200 top candidates were re-screened using a quantitative RT-PCR assay against HCV RNA and a cellular gene for toxicity evaluation, followed by individual siRNA testing. Nine genes passed these tests and were found to also inhibit the replication of a HCV 1a replicon and a monocistronic replicon. Four of these genes are members of the TNF/LT signaling pathway making this pathway a target for anti-HCV therapeutics.

A genome-wide siRNA screen was performed in pools (4 siRNAs/gene) by Tai *et al* using a similar system as Ng *et al* except that instead of SEAP, luciferase was used as the reporter, and cellular ATP levels were monitored as a measure of viability 3 days post transfection. The authors identified 236 genes, of which 24 targeting ribosomal proteins were not further evaluated. Of the remainder, 96 were supported by multiple siRNAs and fell into a number of over-represented categories including vesicular sorting, trafficking, and biogenesis. Five of the six top candidates were reconfirmed using a different replicon system (OR6). In addition, PI4KA, a ubiquitous kinase present in intracellular compartments, the retrograde COP1 coatamer, and hepcidin, a peptide hormone that controls iron transport, are required for replication of OR6 and of JFH1 genotype 2a virus. The precise requirements for these genes are unclear, but PI4KA is likely required to establish correct replication foci. Despite the similarities in the assay and reagents used, none of the 9 genes identified by Ng *et al* were identified by Tai *et al*.

In addition to these larger screens, three small scale screens were also performed. Supekova *et al* screened the human kinome (510 genes) for their role in HCV replicon replication using a similar assay to Tai *et al* [6]. Secondary validation identified 3 kinases as required for HCV replicon propagation: CSK, JAK1 and VRK1. While these genes were present in the screens described above, they were not identified as hits. Randall *et al* screened a set of 62 candidate genes that were chosen because they are interactors of HCV RNA or proteins. Individual siRNAs (2 per gene) were transfected into Huh7.5 cells followed by infection with HCV FL-J6/JFH genotype 2a virus at 24, 48 or 72 hours post transfection, and the supernatants were assayed at 48 hours post infection by quantitative RT-PCR, and cell viability was also measured. The authors identified 26 genes that reduced virus production by >3-fold, and of these, 14 also decreased HCV RNA by >3-fold. The strongest hit, DDX3X, was also identified in HIV and Brome Mosaic Virus screens, suggesting that it may play a fundamental role in RNA virus replication [1,3,7]. Lastly, Randall and colleagues performed another small scale screen of 140 genes involved in vesicular trafficking using a similar assay as Randall *et al* [8]. They identified 7 genes that were supported by multiple siRNAs, and whose knockdown inhibited both HCV JFH viral replication and replicon replication. This included PI4KA previously identified by Tai *et al*.

West Nile and Dengue

Krishnan *et al* screened the human genome for factors required for early steps in the West Nile virus lifecycle using an imaging based screening strategy that monitored the production of the viral envelope, E (Table 3) [9]. HeLa cells were transfected with a genome-wide library (4

siRNAs/pool) and 3 days later infected with WNV LinI for 24 hrs. The authors identified 283 genes that were required for replication and 22 genes that, when lost, led to increased levels of WNV infection, and both groups were supported by multiple siRNAs. These genes fell into a number of categories including intracellular trafficking, transporters, and the ERAD (Endoplasmic Reticulum Associated Protein Degradation) pathway. Follow-up of one gene, CBLL1, demonstrated a likely role in entry and ERAD genes post-entry. The authors then went on to test the role of these candidate factors in the replication of the related virus, Dengue. All of the candidates identified as antiviral in the WNV screen also impacted Dengue infection, while 36% of the factors required for WNV were also required for Dengue infection, including previously implicated genes such as the vATPase, CBLL1 and many of the ERAD genes. This is quite provocative and may eventually lead to the discovery of pan-anti-flaviviral therapeutic targets, and further suggests that the host's intrinsic innate responses to these viruses may be similar.

In addition, a genome-wide screen for cellular factors required for Dengue virus by Sessions *et al* used the genetically tractable *Drosophila* system to identify cellular factors required for Dengue infection (Table 3) [10]. Sessions *et al* took advantage of the robust RNAi technology in *Drosophila*. Use of this technology for the exploration of virus-host interactions had been pioneered by Cherry *et al*, who first screened for factors required for an insect picornavirus, identifying many factors that were also required in human cells for poliovirus replication [11,12]. Screening in insect cells for an arthropod-borne pathogen allows one to potentially identify factors required in the insect host as well as vertebrate host. To allow for robust infection, Sessions *et al* selected for Dengue 2 variants that had increased permissivity on *Drosophila* S2 cells. Image based screening identified 116 genes that inhibited infectivity by ≥ 1.5 -fold. Of these, 1 out of 4 genes that were tested in mosquitoes impacted infection of the natural host (lola (AAEL009212)). Moreover, siRNA pools targeting the 82 human homologs of these 116 factors revealed that 7 vATPase components and 16 other genes impacted Dengue 2 infection of Huh-7 cells, with multiple siRNAs showing ≥ 1.5 -fold effects. Nineteen other genes also inhibited Dengue 2, but were only represented by a single active siRNA. Comparison of this gene set with the West Nile screen revealed little overlap, only sec61 and the vATPase were common.

Influenza

A genome-wide screen for cellular factors required for influenza replication by Hao *et al* also took advantage of the fact that RNAi technology is quite robust in *Drosophila* cells (Table 3) [13]. While wild type influenza was non-infectious in this system, the authors modified the virus by replacing the flu envelope with VSV G and a reporter (GFP or luciferase) replaced NA. Using this pseudotyped replication incompetent virus, the authors were able to query VSV G-dependent entry, influenza uncoating, nuclear import, RNA transcription and translation. The authors identified and validated 110 factors that, when lost, led to a decrease in luciferase levels. Three highly conserved genes were tested for their role in human cells. ATP6V0D1 encodes a subunit of the vATPase required for acidification, COX6A1, a subunit of cytochrome c oxidase involved in mitochondrial electron transport, and NXF1, a nuclear RNA export factor. Further support of the specificity and requirement for these factors was revealed when it was shown that these genes are required for efficient infection of bona fide influenza strains and not VSV or vaccinia in 293 cells.

Screening Validation

While RNAi technology allows for large-scale loss-of-function assays to be performed, there are a significant number of caveats associated both with the technology itself, as well as high throughput assay development. Screening tens of thousands of wells requires significant assay

optimization. Miniaturization of assays (384 well format is common) requires low variability and robust positive and negative controls. The tighter the assay the smaller the effect of a perturbation becomes statistically significant. Statistical tools developed for microarray analysis, including correction for multiple hypotheses testing (Bonferroni correction or False Discovery Rate) should be applied to screening results. This would reduce the reporting of spurious results, caused by assay noise, that are unlikely to reproduce. Moreover, biological significance should also be taken into consideration (Is a less than a 2-fold effect meaningful?).

Once a screen has been conducted and hits identified, the next important consideration in RNAi screens is whether siRNAs are working on- or off-target [14]. Screening results are contaminated with false positives and conspicuously lack known players (false negatives). The limitations of the RNAi technology need to be acknowledged and mitigated. False positives are significantly reduced in data sets supported by multiple independent siRNA sequences against the same gene. At a minimum two independent RNAi reagents should phenocopy the original results in a secondary validation screen. Orthogonal assays (i.e. non RNAi-based methods for siRNA screens) need to be applied systematically to primary screen hits. For example, dominant negatives, small molecule inhibitors or rescue with an RNAi-resistant cDNA would demonstrate that the indicated gene is indeed required. It is only by broadly testing other modulators of the biology, and reporting the fractional validation rate, that generalizable conclusions can be made about the gene lists published with genomic screens. Another useful method to remove candidates that may not be direct or relevant is to perform a related but nonidentical assay (e.g. a different cell line, or viral isolate, or different read-out, etc.). Small changes in the technical aspects of the assay can remove genes that may be affecting the assay technology rather than the biology of interest.

False negatives can be minimized by using hypothesis-driven follow-up of primary screening results. For example, if certain cellular pathways are found to be enriched in primary screening results--and in other authors' published work--negative screening results for other pathway members should be validated with additional siRNA sequences to ensure that reagent inefficiency is not responsible for the lack of effect.

Of course, assay optimization, the efficacy of the genomic libraries (RNAi knockdown or cDNA expression levels), and secondary validation of screen results determine the validity and value of the candidate gene-list. Many publications implicate a relatively large list of genes in the primary screen results but only discuss validation of a small fraction of genes. Without reporting the rate of failure of primary hits to confirm in secondary screens, others in the field are left to wonder about the support for unconfirmed results. With that said, other parameters including the choice of cell type, viral isolate, and read-out also play a fundamental role in determining the quality and relevance of the candidate gene-set. To increase the utility of these candidate genes, standardized validation protocols and statistical tests are necessary. In the meantime, further study of each of the candidates identified is required before we can be certain that they are indeed important for viral replication.

Future Directions

This is an exciting time for research on virus-host interactions. CCR5 inhibitors which block HIV entry are now on the market demonstrating the utility of this quest for cellular factors required for viral infection as novel drug targets [15]. As this methodology improves and becomes more broadly accessible, screens for all viruses in multiple relevant cell-types will be performed. In addition, screens assaying specific steps in the virus life cycle will likely improve our understanding of viral biology. These new approaches will undoubtedly lead to new therapeutic targets. For example, an inhibitor of DDX3X, identified in siRNA screens listed above, is a cellular gene required for HIV and HCV replication. Initial studies have shown

that it is effective in primary cells against HIV and has no toxicity in mice [16]. Thus, a systems approach is starting to show the promise of querying virus-host interactions in a comprehensive manner.

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Table 1

Ref.	HIV siRNA screens		arrayed?	Time (h)	virus	time	readout	steps?	viability?	VALIDATION
	Cell line	siRNA library								
Brass, et al.	HELA-CD4, CCR5 Tat-B-gal	genome-wide	4siRNA/pool 2siRNA/pool	72	HIV-1 IIB (- accessory genes) VSV-G pseudotyped	Part I: 48 Part II: sup on new cells 48	Part I: p24 IF Part II: B-gal	Part I: entry, uncoating, RT, nuclear import, integration, txn, tln Part II: Virion assembly and release	cell number	>1siRNA 101 part I 31 part II
Konig, et al.	293T	genome-wide	6 siRNA/gene	48	HIV luciferase	24	Luciferase	uncoating, RT, nuclear import, integration, txn, tln	alamar blue	>1siRNA 295
Zhou, et al.	HELA-CD4, CCR5 Tat-B-gal	genome-wide	3siRNA/pool	24	HIV-1 HXB2	Part I: 48 Part II: 96	Part I and II: Tat-b-gal	Part I: entry, uncoating, RT, nuclear import, integration, txn, tln Part II: viral assembly and spread	ATP	second pool 224

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Table 2

HCV siRNA screens													Cherry
Ref.	Cell line	siRNA library	arrayed?	Time (d)	virus	Time (d)	readout	steps?	viability ?	assay	secondary?	validate	candidates
Tai et al.	Huh-7/Rep-Feo	genome-wide	4siRNAs/pool	3	subgenomic 1b HCV replicon		luciferase reporter	RNA replication, translation	yes	replicon RNA levels		>1 siRNA	96
Ng et al.	Huh-7/EN5-3	druggable (4K)	4siRNAs/pool	4	subgenomic 1b N HCV replicon		SEAP reporter	RNA replication, translation	yes	replicon RNA levels		>1 siRNA	9
Supekova et al.	Huh-7/pFK-1389ne o/luc	kinome (510)	1 siRNA		subgenomic 1b (con1) HCV replicon		luciferase reporter	RNA replication, translation	yes	replicon RNA levels		>1 siRNA	3
Randall et al	Huh-7.5	62 genes	2 singles/gene	1,2,3	HCV JFH virus	2	supernatant RT-qPCR	RNA replication, translation assembly release	yes	viral RNA levels	replicon RNA levels		26
Berger et al	Huh-7.5	140 genes	4siRNAs/pool	3	HCV JFH virus	2	supernatant RT-qPCR	RNA replication, translation assembly release	yes	viral RNA levels	replicon RNA levels	>1 siRNA	7

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Table 3

Other genome-wide viral siRNA screens												
Virus	Ref.	Cell line	siRNA library	arrayed?	Time (h)	virus	Time (h)	readout	steps?	viability?	VALIDATION	filtered set
West Nile	Krishnan, et al.	HeLa	genome-wide	4siRNA/Pool	72	LIN I	24	anti-E, IF	entry, translation	cell number	>1 siRNA	283 down, 22 up
Dengue	Sessions, et al.	Drosophila, S2	genome-wide	single dsRNA	48	DEN2-selected	72	anti-E, IF	entry, translation	cell number	>1 dsRNA	Dengue (100% up, 36% down) 1 gene (mosquito) 23 genes >1 siRNA (human)
Influenza	Hao, et al	Drosophila, DLI	genome-wide	single dsRNA	48	VSV-G pseudotyped Flu, Luciferase reporter	24	Luciferase reporter	VSV-G entry, Uncoating, Nuclear import, Transcription, translation	ATP	>1 dsRNA	110 3 genes (human)
DCV	Cherry et al.	Drosophila, 32	genome-wide	single dsRNA	72	DCVc	24	anti-capsid, IF	entry, RNA replication, translation	cell number	>1 dsRNA	110 two classes in human cells with poliovirus

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