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Cell-based genomic screening: elucidating virus-host interactions

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Brief summary

Viruses rely on host cell machinery for successful infection, while at the same time evading the host immune response. Characterization of these processes has revealed insights both into fundamental cellular processes as well as the nuances of viral replication. The recent advent of cell-based screening coupled with RNAi technology, has greatly facilitated studies focused on characterizing the virus-host interface and has expanded our understanding of cellular factors that impact viral infection. These findings have led to the discovery of potential therapeutic targets, but there is certainly more to be discovered. In this article we will review the recent progress in this arena and discuss the challenges and future of this emerging field.

Introduction

Viruses are obligate intracellular pathogens with a limited coding capacity and thus require host cell factors for their replication. Understanding how viruses exploit cellular machinery has greatly contributed to our knowledge of fundamental principles of cell biology including transcription factors, DNA replication, mRNA capping, RNA splicing, mRNA transport, vesicular trafficking and translation. This is because viral proteins interact with and regulate their host cell environment to facilitate replication using sophisticated strategies that interface with normal biological processes. The identification of host cell factors has been difficult in large part due to the lack of systematic methods for their identification. Recent technological breakthroughs have allowed for the explosion of new cell-based screening approaches to discover cellular factors involved in viral infection. These include affordable instrumentation for processing in high density microtiter plates (e.g. 384 well), coupled with sensitive readers and off-the-shelf analysis and informatics pipelines. Furthermore, genome sequencing coupled with accurate annotations have allowed for the development of new tools for genomic perturbations. Indeed, the discovery and development of robust RNA interference (RNAi) methodologies has opened the door to systematic loss-of-function screening. Additional robust and affordable unbiased screening methods including the identification of protein-protein interactions (yeast two hybrid, shotgun proteomics) and transcriptomics (microarrays, RNA-seq) can be coupled with cell-based screening technologies to allow for the rapid and systematic discovery of cellular genes that impact viral infection.

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Previous reviews have focused on the description of particular RNAi screens targeting viral pathogens [1–6] and thus we will focus this review on recent advances in the field of high throughput cell based screens, outlining some of the technological limitations inherent in the system, and suggest some of the alternate approaches which can complement RNAi screens. Furthermore, we will discuss where the field can continue to make important contributions not only in understanding virus biology but also in identifying novel drug targets against virus infections.

Cell-based screening: Genetic versus chemical screening

The vast majority of antiviral therapeutics on the market inhibit a viral protein with catalytic activity. This is in large part due to the fact that viruses encode essential enzymes distinct from cellular genes making them amenable to specific therapeutic targeting. By applying high-throughput small molecule screening technologies, target-based enzymatic assays identify specific drugs that inhibit these essential viral proteins. The growth of these small molecule libraries (>2M compounds) in the pharmaceutical industry has led to the development of robust screening technologies with decreasing cost. While these target-based biochemical assays were the choice du jour for many years, it has become clear that for many complex diseases, the best target protein for therapeutic intervention is unknown. Compound screening using cell-based assays readily provides tool compounds, but the path to a therapeutic or even the target is difficult. For studies with viruses, that have a limited coding capacity, it can be reasonably straightforward to determine if a given compound targets a viral protein. But if the compound does not function in this manner, the identification of the cellular target is less straightforward. With that said, recent studies have successfully used this strategy to discover important new targets. Using a small molecule library, REDD1 as a factor restricting influenza virus and vesicular stomatitis virus (VSV) infection, nieman pick C1 (NPC1) as a cellular receptor for Ebola virus and Protein kinase C ϵ (PKC ϵ) as a factor required for Rift valley fever virus infection were identified [7–9]. Although the development of therapeutics using small molecule-based screening has been successful, reverse genetic screens overcome many of the limitations of small molecule screening as the gene-of-interest is revealed directly by the sequence of the perturbant in the well.

Loss of function vs gain of function

There are two basic approaches that can be applied in genetic screens. First, there are gain-of-function strategies where ectopic expression of cDNAs can probe gene function. While these tools initially relied on shotgun cloned cDNA libraries, the recent development of fully sequenced, full-length, arrayed cDNA libraries (e.g. MGC collection) has expanded the utility of these approaches. Second, the recent advent of RNAi methodologies has allowed for robust loss-of-function screening. There are a number of RNAi tools that can be used for this: small interfering RNAs (siRNAs), short hairpin RNAs (shRNAs) and long double-stranded RNAs (dsRNAs) which are commercially available and the most common reagents. Long dsRNAs are widely used in insect systems providing robust knock down and low off-target effects (reviewed in [10–13]. Unfortunately, due to the induction of type I interferon responses, long dsRNAs cannot be used in vertebrate systems. Rather, siRNAs and shRNAs which knockdown genes in a sequence specific manner are used in mammalian systems (reviewed in [14,15]).

Genome-wide RNAi screen vs targeted small-scale screens

Genome-wide screening provides us with the most comprehensive and unbiased view of the cellular factors that impact viral infection. However, while these genome-wide screens are the most comprehensive, there are some cases in which more targeted small-scale screens

can provide powerful insights. Firstly, genome-wide screens are costly, difficult to execute, and thus are typically performed only in duplicate using simple assays (typically add and read). In contrast, small-scale screens can provide insight into gene families (eg druggable genes, kinases) or focus on a particular biology (eg vesicular trafficking, lipid synthesis). Furthermore, they can be performed more robustly with more complex assays. To date, a number of small scale screens have been performed to identify candidates regulating diverse viruses. To identify possible druggable targets for HCV, siRNA screens were conducted using a human kinase library, which identified factors required for entry (e.g., EGFR, EPHA2) and those involved in replication (e.g. PI4KIIIalpha) [16,17]. Genome-wide RNAi screening by Tai et al. (using a sub-genomic replicon) and Brass et al. (using infectious HCV) also identified PI4KIIIalpha as an essential component in HCV replication but failed to identify EGFR and EPHA2[18,19]. Similarly, using a human druggable genome RNAi library, host factors involved in enterovirus (poliovirus, PV and coxsackie virus B, CVB) and Borna disease virus infection were identified revealing novel druggable candidates such as adenylate cyclases, LMTK2 (for PV and CVB), and ADAM17, cathepsin L (for BDV) [20,21]. In contrast, other groups have applied RNAi screening targeting specific cellular pathways which have led to the discovery of new genes involved in infection including Ars2 as antiviral against multiple RNA viruses [22–26]. A targeted membrane trafficking gene library revealed 7 genes including PI4KIIIalpha identified by others in larger screens as important for hepatitis C virus (HCV) replication [16,18,19,27]. Also, screening a sub-library against genes associated with pathways that have been shown to be critical for replication of other viruses revealed the role of fatty acid biosynthesis (FASN and ACACA), ER stress (ATF6, XBP1) and actin polymerization (CDC42 and DIAPH1) in dengue virus infection [28].

General workflow for arrayed cell-based screens

For a detailed description of the general protocol for RNAi screening, please refer to other more comprehensive reviews on this topic [29–33]. The general workflow for arrayed cell based screening is briefly outlined in Figure 1A. There are a variety of reagents available for RNAi screening, and these should be selected as appropriate for the cell type chosen and assay length. Long dsRNAs are potent and are routinely used in insect cell screens. siRNAs are potent, but require transfection, and are transient necessitating a short assay window. In contrast, shRNAs are delivered either in plasmid form or packaged into viruses (lenti- or retroviral) and can be delivered for long-term knock-down if the cells are selected for antibiotic resistance. In all of these cases, post-transduction, the cells must be challenged with the appropriate virus (or viral component) and an end-point must be chosen for assay read-out, typically using a plate-based whole-well read-out (eg luciferase) or image-based high-content read-out (eg. viral antigen production).

There are a number of important considerations in performing and optimizing cell-based screening. Once the virus of interest is identified, the appropriate cell-type must be chosen. Because these assays require robust differences, easily transfectable cell lines with stable properties are most readily adapted (e.g. HeLa, 293T, U2OS); however, since infected cells in vivo are more complex and specialized, attempts to screen in more relevant cell types are part of the future of screening. Indeed, a recent screen in polarized endothelial cells revealed novel aspects of enterovirus infection [20]. One important question that must be addressed prior to selecting the appropriate cell type is the biology that one is interested in. For example, is the screen meant to identify genes that impact a particular step in the lifecycle? If one can focus the assay on the biology interested in, then it's more likely to reveal genes of interest. Generally, investigators have relied on the use of virally-expressed reporters because they are the most robust readouts; however, many viruses cannot be readily reverse engineered, necessitating the use of antibodies or more indirect read-outs. Normalization of

cell number is an important consideration since many perturbants will impact the health of the cells. Indeed, the optimization of the screening assay will in part dictate the biology queried. The statistical criteria used both to optimize the assay ($Z' > 0.5$ is standard) and to identify 'hits' is also an important consideration. A positive control which blocks infection can be used to optimize the screen for identifying required factors, while a positive control that leads to increased infection can be used to identify factors restricting infection. Further, different analysis algorithms will impact hit detection; in depth description of statistical analyses used in the RNAi screen can be found in [34]. Optimally, screens should have a low false negative and false positive rate. This is difficult to achieve, and the choice of cut-offs is largely based on decisions which lead to either a higher false negative or higher false positive rate. In order to minimize false negatives, more false positives may be tolerated in primary screens, but this increases the work and costs in the confirmation stage. Thus, a thorough, large and expensive hit triage plan is in many cases the mark of a good screen. Nevertheless, these choices contribute to the low overlap between seemingly similar screens [35–37]. Unfortunately, there is no absolute guideline to follow since these choices are somewhat subjective. However, combining statistical criteria with biological criteria (e.g. robust Z score with $p < 0.05$ and a 2-fold cutoff) should help to guide data analysis to identify a reasonable primary gene list.

Performing the screen itself is of course just the beginning. As with any high-throughput platform, validation of the resulting gene list is essential. Ideally, for the secondary validation of RNAi screens, at a minimum more than one RNAi reagent should show a consistent phenotype. Furthermore, the use of an orthogonal assay in an independent cell type will remove much of the noise in these gene lists. Of course, the high confidence gene set should be used to generate hypotheses which are further explored, eventually leading to new mechanistic insight into virus-host interactions.

Cell-based screens and viruses

The initial genome-wide RNAi screens largely used viral infection as a read-out and focused on the identification of cellular factors required for infection [18,19,38–51]. These are listed in Table 1. A number of recent reviews have discussed these screens [3–6,35]. Surprisingly, in total, all of these screens report only 52 restriction factors. This may be partly due to the assay format used for these screens. For example, in the screen by Panda et al., the average percent infection was 60%, skewing the assay for discovery of factors that facilitate infection [45]. With that said, there have been some cell-intrinsic factors identified in genome-wide RNAi screens including IFITM1-3 which restricts influenza [42], and MCT4 which restricts WNV [51], suggesting that in fact restriction factors do exist but the assays have to be tuned to identify them. Subsequently, the importance of IFITM3 in vivo was demonstrated in mice where the IFITM3 mutant mice showed increased virus replication [52]. IFITM3 was also shown to be responsible for protection against influenza in humans as a single nucleotide polymorphism in IFITM3 allele impairs its ability to restrict influenza virus [52]. Since typical RNAi screens rely on depletion of the basal transcriptome and many antiviral factors are induced by viral infection, it may be difficult to identify such genes using simple RNAi approaches. Rather, ectopic expression may more robustly identify restriction factors. Early studies using cDNA libraries identified restriction factors including Zinc finger protein ZAP as a restriction factor against retroviruses [53]. And recently two groups took advantage of the fact that Interferon stimulated genes (ISGs) are likely antiviral effectors and screened ectopic expression of ISGs for those that restrict virus infection [54,55]. Collectively these studies, identified many novel antiviral regulators, and perhaps surprisingly, Schoggins et al., identified six ISGs that facilitate virus infection suggesting complex biological functions of type I interferons [54].

New technologies are also providing additional methods to identify host factors that are required for infection. Indeed pooled library screening has become more robust with the advent of high-throughput sequencing. The workflow for these screens is depicted in Figure 1B. Although the pooled shRNA screens are in general lengthier compared to siRNA screens, they offer durable gene knockdown and can be used in hard to transfect cells. Furthermore, insertional mutagenesis in populations and then challenging with viruses followed by high-throughput sequencing of insertion sites can reveal interrupted genes that likely confer resistance expanding our knowledge of host factor required for virus-induced cytotoxicity. While historically, this has been difficult due to the diploid redundancy of genomes, the development of a haploid cell system has been pioneered to identify factors required for influenza virus [56] and more recently, NPC1 as a cellular receptor for Ebola virus [57]. Another technical development is of random homozygous gene perturbation (RHZP) that can deplete both copies of genes in a diploid genome, this system was used to identify genes required for a number of viruses including influenza and HIV [58–63]. Using this system, Murray et al., identified Rab9 as a factor required for infection of several enveloped viruses such as Marburg virus, measles virus, HIV and Ebola virus [59]. Furthermore, a number of cytoskeletal associated proteins, insulin growth factor II (IGF-II) pathway and a putative cell surface protein OL-16 were identified as factors required for reovirus infection [61,63].

Comparative phenotype profiling

To gain additional insight into these host factor dependencies, an effort has been made to determine the spectrum of viruses within a family and between families that hijack a given cellular factor or process. This analysis is complicated by the fact that there is seemingly only a modest overlap between RNAi screens against the same virus (reviewed in [4–6,36]; however, this has been overcome in some cases when the comparisons are made within the same group. Coyne et al. performed a comparative screen between poliovirus and coxsackievirus B (CVB) infections of polarized endothelial cells and found more than 70% overlap between the host factor dependency [20]. Panda et al., compared their validated gene set and found that 35% of the validated genes that promote VSV infection are also required by lymphocytic choriomeningitis virus (LCMV) and human parainfluenza virus type 3 (HPIV3) [45]. Furthermore, there was high overlap among flavivirus dependencies [51]. But perhaps the most surprising finding is that the COPI complex has been identified and validated as required for many of the viruses tested including influenza, VSV, LCMV, HPIV3, HCV, Drosophila C virus (DCV) and poliovirus [18,42–45,64].

The future: more sophisticated assays and phenotypes

The initial virus RNAi screens largely relied on simple read-outs focusing on either a simple reporter or antigen expressed during viral replication, thereby monitoring many steps in the viral life cycle simultaneously. However, as the technology becomes more robust, the assays and the complex cell biological interactions between viruses and hosts will be more thoroughly explored. In fact some recent genome-wide RNAi screens set the foundation to reveal complex phenotypes. A high-content screen by Orvedahl et al., identified cellular genes which regulate Sindbis induced virophagy but not cellular autophagy by looking at co-localization between SINV capsid protein and an autophagic marker [65]. Another, approach involved a synthetic lethal RNAi screen wherein the authors screened for genes whose depletion in multiple cancer cell lines led to increased virus-induced cytotoxicity by a rhabdovirus maraba virus, identifying the ER stress response [66]. Zhao et al. performed a genome-wide siRNA screen in the presence of interferon, scoring genes that when depleted led to increased infection, and validated 93 genes including several new genes that are required for this antiviral pathway [67].

The future: combining cell-based screens with other 'omics approaches

The explosion of genomic technology has opened the door to more comprehensive mapping of interactions. Functional genomics including protein-protein interactions and transcriptional profiling can be combined with cell-based screening strategies to elucidate the 'systems biology' of virus-host interactions. In a recent article Shapira, et al., combined yeast two hybrid analysis and gene expression profiling to generate a network of host proteins interacting with influenza virus proteins [68] which generated a directed gene set which they used for RNAi screening and led to the discovery of WNT signaling playing a regulatory role in influenza infection. Likewise, König et al., analyzed gene expression profiles and yeast two-hybrid analysis and integrated these results with the results obtained in their genome-wide RNAi screen and developed comprehensive map of regulators of HIV infection [39]. Thus, proteomics can be used to generate a candidate set to be tested with RNAi screening. This approach has been applied to discover host factors incorporated into Ebola virions that play functional roles in viral infection [69].

Another increasingly used strategy takes advantage of the NCI60 cell line panel for which there is available microarray data. By correlating gene expression patterns with viral infectivity, genes predictive for Adeno associated virus 5 infection and ebola virus infection were tested using RNAi, leading to the identification of novel genes important for virus infection [70,71].

Challenges and the future of cell-based screening in viral-host discovery

While the last decade has certainly seen an explosion in the use of cell-based genomic screening, there are certainly areas in need of improvement. First and foremost is the need to increase the robustness and validation of gene lists from cell-based screens. This would be aided by more potent and selective RNAi reagents, since the technology is fraught with weak silencing and off-target effects [72–74]. Second, more sophisticated and user-friendly statistical models that take into account the non-normal distribution of data, and the need to superimpose prior biological knowledge on the data will improve the results. Third, as the instrumentation improves and more diverse add-and-read assays are developed, this will facilitate more complex assays, probing individual steps in the viral lifecycle, and important aspects of innate antiviral restriction. Furthermore, as genomic screening methodology has become more standardized, defined requirements for publication is needed. The MIARE (Minimum Information About an RNAi Experiment) and MIACA (Minimum Information about a cellular assay) guidelines are being recognized as information standards. Collaborative efforts between different agencies and laboratories will help to streamline data presentation and validation methods in order to establish best practices for data analysis from complementary screens. Indeed, the lack of public repositories for this primary and secondary data has made the comparative analysis of these data sets difficult. Model organisms including *C. elegans* and *Drosophila* have already generated such databases and this should be a priority going forward (e.g. GenomeRNAi and NCBI pubchem database). However, even if the primary data sets are more robust and available on the web, criteria for validation of primary screen data should also be established. Many use the criteria of 2 independent RNAi reagents as the gold standard. While this is certainly a minimum, we would suggest that validation requires an orthogonal assay and independent cell line, a condition that will greatly improve the robustness of screen-derived gene lists. Indeed, identification of mechanism-of-action should be the goal of these studies. Altogether, no single approach can reveal the 'system' at play and therefore, the integration of different 'omics' technologies will allow us to have an unprecedented insight into virus-host interactions. While many challenges remain, improved methodology combined with

advancing technology promise to yield significant breakthroughs in our understanding of virus-host interactions.

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Highlight

- Various cell based approaches to uncover virus-host interactions are reviewed.
- Recent discoveries of some genes and pathways using different cell based screening approaches discussed.
- Comparisons of strengths and weakness of some of the cell-based screening strategies were assessed.
- Integration of multiple high-throughput 'omics' approaches may open new avenues.

Fig. 1.
General workflow for arrayed cell based screening.

Table 1

Virus	RNAi library (# genes) (Company)	Cell Type	Duration of RNAi	Infection length	Assay read out	Statistical analysis	Primary list	Type of secondary validation	Validated	Reference
HIV-HIB	4siRNA/gene (21121 genes) Dharmacon	TZM-bl HeLa cells expressing CD4, CCR5	72h	48h part1 24h part2	IF and HCS for part1 and Beta-gal, plate reader for part2	Z score	386 VSF	Deconvolution of primary list	273 VSF (1 siRNA)	Brass et al
VSV-G pseudotype HIV	6 siRNA/gene and 2siRNA per well (19628 genes) Qiagen, Ambion	HEK 293T	48h	24h	Luciferase, plate reader	Redundant siRNA activity, ontology	800 VSF	Deconvolution, Decision matrix based on microarray, protein interaction	295 VSF	Konig et al.
HIV-HXB2	3 siRNA/gene (19709 genes) In house	P4/R5 HeLa derived	24h	48h	Beta-gal, plate reader	Strictly standardized mean difference	390 VSF	Deconvolution	232 Total 205 VSF 1 VRF	Zhou et al.
HIV-NL4-3	shRNA (54509 transcript and EST) System Biosciences	Jurkat	3 weeks	4 weeks	Microarray based barcoding detection	>2 fold enrichment over background			252 VSF	Yeung et al.
HCV Genotype 1b replicon	4 siRNA/gene (21094 genes) Dharmacon	Huh7/Rep/Feo replicon cell line	72h		Luciferase, plate reader	Z score	236 VSF	Deconvolution	96 VSF	Tai et al.
HCV JFH-1	Pool of 4 siRNA (19470 siRNA) Dharmacon	Huh 7.5.1	72h	48h part1 48 h part2	IF, HCS for part1 and 2	Plate mean based comparison	521 total 407 VSF and 114 VRF)	Deconvolution	262 Total 237 VSF 25 VRF	Li et al.
WNV	Pool of 4 siRNA (21,121 genes) Dharmacon	HeLa	72h	24h	IF, HCS	Z-Score		Deconvolution	305 Total 283 VSF 22 VRF	Krishnan et al
VSV	2siRNA per well, 2 wells per gene (22909 genes)Qiagen	HeLa	52h	18h	GFP, HCS	Sum rank	173 VSF	siRNA from Dharmacon, 3 independent siRNA scored as hit	72 VSF	Panda et al.
Influenza PR8	Pool of 4 siRNA (17877 genes) Dharmacon	U2OS	72h	12h	IF, HCS for part1	Plate mean	334 total 312 VSF 22 VRF	Deconvolution	133 Total 129 VSF 4 VRF	Brass et al.
Influenza WSN	6 siRNA/gene and 2siRNA per well (19628 genes) Qiagen, Invitrogen, IDT	A549	48h	12,24,36h	Luciferase, plate reader	Redundant siRNA activity, ontology	624 VSF	Deconvolution	295 VSF	Konig et al.
Influenza WSN	4 siRNA per gene (23000 genes) Qiagen	A549	48h	24h part1 16h part2	IF, HCS for part1 and Luciferase, plate reader for part2	Z score	287 VSF	Deconvolution	168 VSF	Karlas et al.

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Virus	RNAi library (# genes) (Company)	Cell Type	Duration of RNAi	Infection length	Assay read out	Statistical analysis	Primary list	Type of secondary validation	Validated	Reference
Papilloma virus	4 siRNA per gene (21121 genes) Dharmacon	C33A cells expressing BPV E2 and IL2α	72h		Luciferase, plate reader	Z score	511 VSF	Deconvolution	311 1 siRNA and 130 2 siRNAs	Smith et al.
SV40	200000 shRNA, (47400 human transcripts) System Biosciences	HeLa E6 cells	2d,5d,14d	At 14d post shRNA transduction	Cell proliferation, visual selection		Not reported		Not reported	Goodwin et al.
Influenza VSVG pseudotype	dsRNA (13071 genes) Ambion	Drosophila DL1	48h	24h	Luciferase, plate reader	Plate mean	176 VSF	Independent dsRNA	110 VSF	Hao et al.
DENV Drosophila adapted Dengue-2	22632 dsRNA DRSC	Drosophila D.mel 2 cells	48h	72h	IF, HCS	Sum rank	218 VSF	Independent dsRNA	116 VSF	Sessions et al.
DCV	21000 dsRNA DRSC	Drosophila S2	72h	24h	IF, HCS	Visual difference	210 VSF	Independent dsRNA	112 VSF	Cherry et al.

Abbreviations: Drosophila C virus, DCV; Dengue virus, DENV; Vesicular stomatitis virus, VSV; Hepatitis C virus, HCV; West Nile virus, WNV; HIV, Human immunodeficiency virus, HIV; Simian virus 40, SV40; Virus susceptibility factor, VSF; Virus resistance factor, VRF; Immunofluorescence staining, IF; high content screening, HCS; Drosophila RNAi Screening Center, DRSC.