Host gene targets for novel influenza therapies elucidated by high-throughput RNA interference screens

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ABSTRACT **Influenza virus encodes only 11 viral proteins but replicates in a broad range of avian and mammalian species by exploiting host cell functions. Genome-wide RNA interference (RNAi) has proven to be a powerful tool for identifying the host molecules that participate in each step of virus replication. Meta-analysis of findings from genome-wide RNAi screens has shown influenza virus to be dependent on functional nodes in host cell pathways, requiring a wide variety of molecules and cellular proteins for replication. Because rapid evolution of the influenza A viruses persistently complicates the effectiveness of vaccines and therapeutics, a further understanding of the complex host cell pathways coopted by influenza virus for replication may provide new targets and strategies for antiviral therapy. RNAi genome screening technologies together with bioinformatics can provide the ability to rapidly identify specific host factors involved in resistance and susceptibility to influenza virus, allowing for novel disease intervention strategies.—Meliopoulos, V. A., Andersen, L. E., Birrer, K. F., Simpson, K. J., Lowenthal, J. W., Bean, A. G. D., Stambas, J., Stewart, C. R., Tompkins, S. M., van Beusechem, V. W., Fraser, I., Mhlanga, M., Barichievy, S., Smith, Q., Leake, D., Karpilow, J., Buck, A., Jona, G., Tripp, R. A. Host gene targets for novel influenza therapies elucidated by high-throughput RNA interference screens. *FASEB J.* 26, 1372–1386 (2012). www.fasebj.org**

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Many emerging infectious diseases (EIDs) in humans are zoonotic: deriving from animals or animal products. EIDs are broadly defined to include new agents, existing yet previously undetected agents, the reemergence of known agents, and/or expansion of a known agent into a new geographic range (1). Influenza viruses are well-documented examples of EIDs. In 2009, the H1N1 pandemic virus erupted from the swine population and quickly spread to over 200 countries, overtaking preexisting H1N1 and H3N2 viruses in the human population and becoming the dominant circulating strain (2). Similarly, outbreaks of highly patho-

Abbreviations: BSL, biosafety level; CMV, cytomegalovirus; dsRNA, double-stranded RNA; EGFR, epidermal growth factor receptor; EID, emerging infectious disease; ERK, extracellular regulated kinase; HA, hemagglutinin; MAPK, mitogen-activated protein kinase; MIARE, minimum information about an RNAi experiment; miRNA, microRNA; NF-κB, nuclear factor-κB; NGS, next-generation sequencing; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; RISC, RNA-induced silencing complex; RNAi, RNA interference; RNP, ribonucleoprotein; RTK, receptor tyrosine kinase; shRNA, short hairpin RNA; siRNA, small interfering RNA; TLR, Toll-like receptor; Y2H, yeast 2-hybrid.

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genic H5N1 influenza viruses continue to emerge and exhibit up to 60% mortality rates in humans (3). These outbreaks highlight the persistent and devastating nature of influenza infections, and increase the risk of new pandemics.

Despite scientific advancements over the past 3 decades, EIDs continue to inflict substantial social and economic costs. For example, influenza is a leading cause of morbidity and mortality in the world (4) with seasonal viruses affecting up to 15% of the human population, causing severe illness in 3–5 million people and fatality of ~500,000 individuals/yr (5). Coupled with this is the economic burden that is associated with widespread influenza infection. In the United States alone, financial losses, resulting from seasonal influenza infection are estimated to exceed $87 billion annually (6).

The ability of influenza viruses to continuously mutate has made control strategies based on vaccination difficult. Simultaneously, resistance to current antivirals is increasing. For these reasons, new strategies to combat EIDs such as influenza are required, and an expanded knowledge of host-virus interactions is a crucial first step. Despite the complexity of influenza biology, viruses of this class contain only 8 gene segments (7) and therefore lack the full complement of proteins required to produce infectious virus. As such, influenza, like all viruses, must coopt a wide array of host proteins, noncoding RNAs, and cellular processes (for example, transport vesicles) to generate infectious viral particles. The replicative cycle of orthomyxoviruses can be divided into early, middle, and late stages (8). Early stages involve events linked to virus binding *via* hemagglutinin (HA) to sialic acid moieties found on the host cell membrane and internalization of the viral particles (9). Midstage events include the expression and translation of viral genes (10), while late events comprise virus protein trafficking to the cell membrane, virion assembly, and budding from the cell (11, 12). Understanding the host contribution to viral replication and immune evasion is essential for discovering new therapeutic strategies. Genome screening technologies that use RNA interference (RNAi), together with bioinformatics, provide the ability to rapidly identify the complement of essential host functions and pathways that are essential to the virus.

THE RNAi PATHWAY

RNAi is a natural, sequence-specific post-transcriptional gene silencing pathway (**Fig. 1**) present in most eukaryotes (13, 14). Components of the RNAi pathway serve multiple roles that can vary in different organisms. On the one hand, elements of the pathway participate in the innate immune response by recogniz-

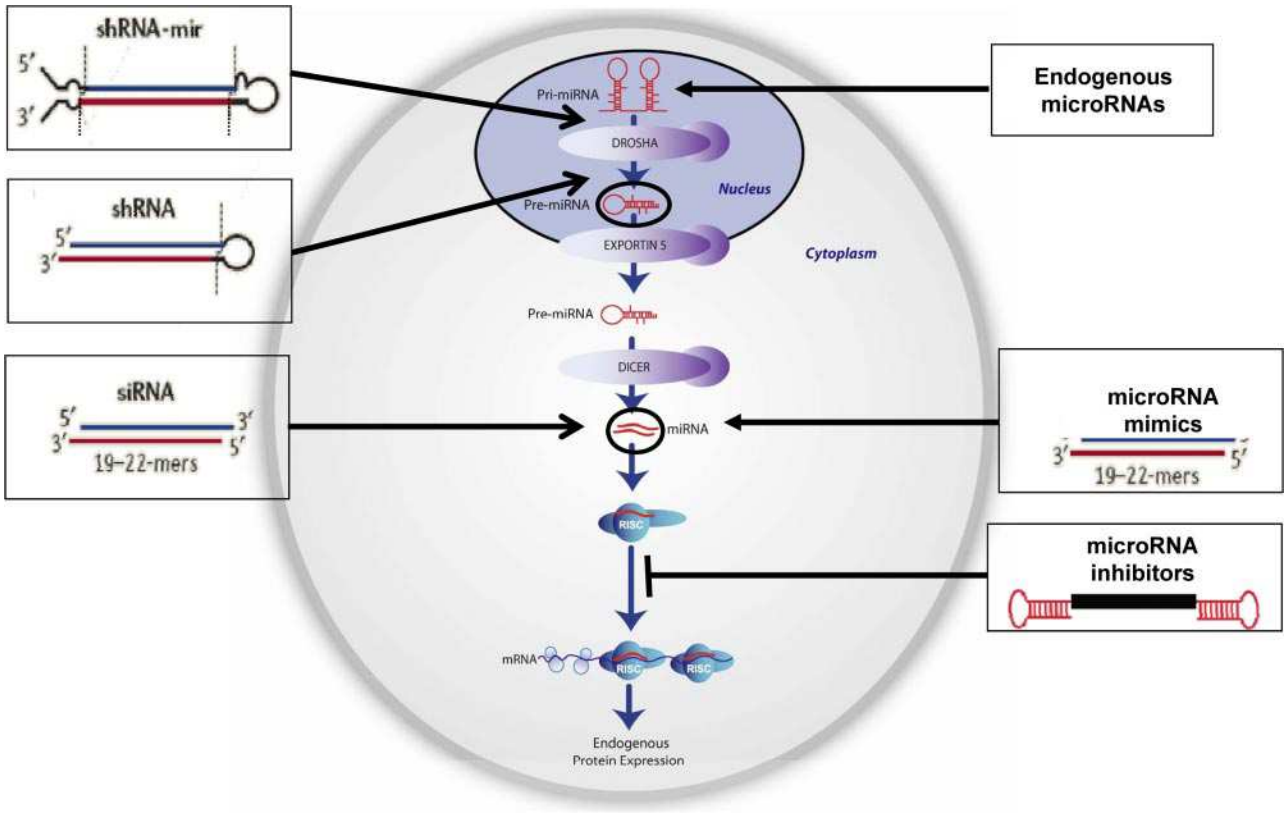

Figure 1. RNAi pathway. Endogenous miRNAs are transcribed as Pri-miRNAs that are subsequently processed by Drosha and Dicer to generate mature miRNAs. The guide strand is then incorporated into the RNA-induced silencing complex (RISC) to facilitate gene silencing. Reagents that have been developed for screening include expressed constructs (shRNA and shRNA-miR), as well as synthetic constructs: siRNA, miRNA mimics, and miRNA inhibitors.

ing and degrading long double-stranded RNAs (dsRNAs) typically associated with viral infection (15–17). Simultaneously, the RNAi pathway has a recognized role in regulating host gene expression related to a myriad of cell functions by guiding the maturation and transcript targeting capabilities of a unique class of noncoding RNAs called microRNAs (miRNAs; refs. 18, 19). This aspect of RNAi is particularly intriguing given that some viruses, in particular herpesviruses, encode miRNAs that regulate host gene expression and, therefore, have a critical role in the dynamics of host-pathogen interactions (20–22).

Outside the important role in gene modulation, the RNAi pathway is now recognized as a preeminent means for studying gene function (23, 24). In this regard, researchers have created entire libraries of gene-specific targeting reagents that silence individual gene function by entering the pathway at one of two distinct positions. In one instance, synthetic dsRNA reagents referred to as small interfering RNAs (siRNAs) have been generated. When introduced into cells, siRNAs associate with the RNA-induced silencing complex (RISC), where the siRNA guide strand anneals to its complementary target mRNA (25, 26) and facilitates cleavage by Argonaute 2, a core endonuclease of RISC (Fig. 1). In an alternative approach, researchers have developed collections of gene-targeting short hairpin RNAs (shRNAs) that can be generated from plasmid or viral expression constructs (27–29). Like their endogenous miRNA counterparts, shRNAs are expressed in the nucleus and are predicted to associate with RISC only after being processed by two separate endonucleases, Drosha and Dicer (Fig. 1 and refs. 18, 19).

GENERAL WORKFLOW OF HOST-PATHOGEN SCREENING USING RNAi TECHNOLOGY

The complexity of RNAi screens for host-pathogen interactions requires thoughtful consideration of the silencing reagent, the cell line, and virus to be used. RNAi screening typically begins with the plating of the target cells and introduction of the silencing reagent. For siRNA-based studies, screens are generally performed in an arrayed format where individual genes are targeted with anywhere from 1 to 4 siRNAs/well. In most cases, pooled reagents are preferred, as they provide a cumulative silencing effect that greatly increases the efficacy of gene knockdown. Synthetic silencing reagents can be introduced *via* forward or reverse transfection protocols (30–33), which, in many cell types, lead to efficient intracellular delivery of the siRNA. For cases in which shRNAs are used and expressed from a viral construct, one of two approaches can be employed. In the first, shRNA workflows can parallel siRNA screening processes by adopting a 1-gene-1-well arrayed platform. Alternatively, shRNAs screens often make use of pools of viral particles that contain expression constructs targeting hundreds to thousands of genes. In this scenario, cell plating is followed by virus transduction at low multiplicities of infection, thus ensuring that each infected cell receives only one viral particle. Drug selection follows transduction to eliminate cells that do not carry a gene-targeting construct (27–29). Pooled shRNA screens are best suited to output assays that permit selection or outgrowth of cells that exhibit the phenotype of interest, where specific shRNAs, which influence the phenotype (and are consequently enriched or depleted from the population) can be identified by either microarray or next-generation sequencing (NGS). For an overview of the comparative workflows for siRNA *vs.* shRNA, see **Table 1**.

Regardless of the type of silencing reagent employed, the cells are subsequently cultured for a period of time that allows for gene knockdown and accounts for any unique attributes of the screen. For instance, if the intent of the screen is to study viral uptake, infection may be delayed to avoid lipid-mediated siRNA transfection affecting the ability of the virus to bind the cell. Cells are then infected with the virus of choice, cultured for an additional period of time, and assayed by one or more techniques (*e.g.*, transcriptional reporters, viral titers) to determine the effect of gene knockdown on various aspects of the viral life cycle. It is worth noting that different silencing reagents permit different types of assays to be incorporated in the workflow. Although the period of gene silencing provided by siRNA can vary with the gene and the cell type being studied, synthetic siRNAs generally provide 3–7 d of gene knockdown (34, 35). As such, these reagents are compatible with assays that are performed within this brief window. In contrast, shRNAs expression constructs, delivered by a lentivirus that stably integrates into the host genome permit assays that extend beyond the transient (siRNA) window to be performed.

RNAi screens that utilize siRNA technology are greatly aided by automation, in which extreme accuracy, reproducibility, and, to some extent, speed are necessary for managing the siRNA library resource, dispensing and aspirating cells and medium, and performing lipid-mediated transfection. For screens that involve infectious reagents, liquid handling platforms that are modular are attractive, as they facilitate the separation of steps that require high-level biocontainment from those that are common to all RNAi screens.

Both screening processes are iterative, and full genome-wide screens can take anywhere from weeks to months, depending on the assay complexity, the level of automation, the particulars of the pathogen being investigated, and the logistics used to divide and screen the gene-silencing collection. With regard to hit identification, a term used to identify genes that when silenced have a significant effect on the function being assayed, a number of bioinformatic approaches have been described. In one instance, a Z' factor is calculated to determine how distinct the positive and negative controls are from each other; subsequently, a Z score, which is a method of normalization to the mean value of the samples on each plate, is commonly used to

TABLE 1. *Comparison of screen stages for shRNA and siRNA screens*

Screen stage	shRNA	siRNA
1. Cell-based optimization	Define MOI for cell line	Establish transfection conditions
	Identify +ve, −ve controls	Identify +ve, −ve controls
	Make virus for individual shRNAs	siGLO and nontargeting control
2. Assay development	Establish screen phenotype	Define assay parameters
	Verify phenotype of controls in dilution of pools	Robustness, *Z′* factor
		Develop automation
		Identify analysis rules
3. Screen	Transduce cells with library pool	Primary SMARTpool screen
	Select with GFP/puromycin	Duplicate or triplicate technical replicates
	Assay: select reference population	Analysis ongoing
	Freeze cells, extract genomic DNA	
	Amplify gDNA and NGS analysis	
4. Bioinformatics	Process NGS data	Statistical analysis
	Statistically rank shRNAs	Define hit list
5. Validation	Identify individual shRNA hits	Secondary validation screen
	Make virus for all constructs per target	Deconvolute SMARTpools
	Rescreen using same assay	Same assay or different assay
	Verify knockdown	Additional cell lines
6. Bioinformatics	Pathway analysis	Pathway analysis
	Data mining	Data mining
		Tertiary analysis, more cell lines, different assays

shRNA and siRNA screen stages broken into chronological order. Stages can take different times depending on the assay and quantitation method. Bioinformatics analysis is an ongoing effort that intervenes in the screen process at several points. Central to each screening platform is identification of robust positive (+ve) and negative controls (−ve). For the siRNA platform, siGLO, a fluorescent reporter, is used to indicate transfection efficiency.

rank genes (36). Candidate genes are then categorized for defining features before being validated. A statistical enrichment of targets relative to a reference population is then ranked for validation. Many parameters may be captured in a screen, and these are weighted on the importance to the principle screen phenotype and classified into distinct bins to refine the gene list prior to validation. For example, for an shRNA screen, following NGS analysis, data are subjected to a statistical enrichment of targets relative to a reference population, which results in a ranked list to identify targets for validation.

siRNAS AND shRNAS

siRNA libraries have been constructed with a variety of designs, and while these reagents have been used in a range of studies and cell types, it is important to consider which of these tools is best suited for host-pathogen screens. One potentially relevant consideration centers on activation of cellular immunity pathways. It is well documented that viral infection is monitored by pathogen recognition receptors (PRRs) that trigger an innate immune response upon identifying an evolutionarily conserved pathogen-associated molecular pattern (PAMP) (37). For example, recognition of influenza single-stranded RNA genome by the endosomal PRR, Toll-like receptor 7 (TLR7), leads to a strong antiviral cytokine response (38–40). Previous studies have also shown that transfection of short dsRNA (such as siRNA) can also perturb cellular immunity. For example, it has been demonstrated that siRNAs longer than ~23–25 bp strongly up-regulate interferon (IFN)-stimulated genes in HeLa cells (41). Other groups have shown that siRNAs can activate a type I interferon response, particularly through TLR7 and TLR8 (42, 43). Thus, if both siRNA and virus can independently stimulate TLR pathways, the question arises of how one can effectively screen for host genes that play a role in virus replication. One solution may lie in reagent selection. For example, while most siRNAs are designed to have complete complementarity to the target transcript, one design variable is duplex length. Previous studies have shown that in some cell types, short (19 bp) siRNAs effectively silence genes but are weaker activators of TLR signaling pathways (44). As such, under certain circumstances, these reagents can minimize the immune stimulatory effects that would otherwise complicate RNAi screens involving pathogens. In addition, several groups have demonstrated that modification of particular nucleotides with defined chemical groups (*e.g.*, 2′-*O*-methyl,2′OMe) can greatly reduce the immunostimulatory effects of siRNA (45–47). Such modifications, which can act in either *cis* or *trans*, limit TLR7/8 activation and thereby prevent the triggering of downstream interferon and cytokine pathways.

Separately, another noteworthy caveat of all RNAi screening is siRNA specificity. Extensive studies have shown that in addition to targeting the mRNA of interest, siRNAs can act in a similar fashion to miRNAs and attenuate the translation of dozens of genes (48, 49). False positives (referred to as "off-targets") induced by this mechanism are frequently mediated by

the seed region of the siRNA (nt 2–8), and may result in a 2- to 4-fold down-regulation of unintended targets (49). Notably, while off-targets are generally thought to be genes encoded by the cell, in the case of RNAi screens designed to identify host-pathogen interactions, off-targets can also include genes encoded by the pathogen.

Multiple approaches have been developed to minimize the effects of off-targets, including chemical modification of the siRNA duplex to reduce seed-mediated gene silencing. For example, 2′-*O*-methyl modification of the second nucleotide of the siRNA antisense strand can greatly reduce seed-mediated off-target effects (50). Use of these reagents during primary screening can minimize the number of false positives and thereby reduce the time and costs associated with downstream validation. Alternatively, in cases where pools of siRNA were used in the primary screen, deconvolution of the pools and demonstration that multiple individual siRNAs (having different seed sequences) give the same phenotype increases the level of confidence that the phenotype is related to the knockdown of the targeted gene. Lastly, bioinformatics can be effectively used to identify off-target effects. Cross-checking the seed sequence of siRNAs that induce a phenotype with the seeds of known miRNAs (host and pathogen) can often identify instances in which one or more siRNAs are mimicking a miRNA. Although these hits should not be excluded from further study (due to potentially interesting host miRNA-pathogen interactions), they may indicate that the observed phenotypes are the result of events unrelated to the knockdown of the intended target gene.

Similar considerations may be relevant when performing screens with shRNA expression cassettes. In most cases, shRNAs are generated from DNA-based expression constructs, and the predominant collections of shRNA libraries use either lentiviral or retroviral delivery systems to facilitate the entry and integration of the cassette into the host genome (27, 28). Previous genome profiling studies of lentiviral (HIV)-infected $CD4^+$ T cells have shown that infection leads to differential expression of multiple gene categories, including those related to complement activation, actin filaments, and proteasome cores (51). Thus, although the current shRNA platforms are essential for performing host-pathogen studies in cases where the cell type of choice is refractory to lipid-mediated transfection or an extended period of gene knockdown is required, one must consider the potential contributions that the delivery platform has on the outcome of host-pathogen screens.

miRNAS

miRNAs have emerged as essential regulators of eukaryotic gene expression (52–55). Mature miRNA sequences have been found in >150 species, including viruses, with >1000 identified in humans (52, 54, 56, 57). Recent studies have identified both host and viral encoded miRNAs as critical elements regulating virus replication. For example, human miR-122 has been identified as an essential component affecting the biology of hepatitis C virus replication (58). Interestingly, both procytomegalovirus (pro-CMV) and anti-CMV miRNAs have been identified as encoded by the mouse genome (59). These and other studies emphasize the need for miRNA screening to accompany siRNA/shRNA screens that target protein-coding genes.

Identifying host-encoded miRNAs that are relevant to viral infection generally involves a two-pronged approach. Synthetic miRNAs mimics (or equivalent sets of reagents expressed from *e.g.*, a plasmid or viral-based vector) can be used to increase the cellular concentration of any given miRNA. In contrast, miRNA inhibitors are designed against mature miRNAs and can act as artificial targets to prevent the endogenous miRNA from interacting with its natural targets (60). A library of mimics and inhibitors was recently used to identify miRNAs that play a role in diverse herpes virus infections [murine CMV (MCMV), murine γ herpes virus-68 (MHV-68), and herpes simplex virus-1 (HSV-1); ref. 59]. By evaluating the phenotypic effect induced by mimics and corresponding inhibitors, the study was able to identify 4 antiviral and 3 proviral miRNAs that acted across diverse β-herpes viruses. Further analysis implicated the miRNAs in a variety of host signaling networks, including extracellular regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/AKT signaling, among others (59). Because these pathways are also implicated in influenza infection (61, 62), these findings suggest that miRNAs might have broad therapeutic potential in prospective disease intervention strategies.

CELL TYPES

As the scope of many screens is translational, the cell type chosen often has qualities consistent with evaluating druggable, pharmacokinetic, and cytotoxicity properties. As far as is practical, researchers try to use cell types that are most representative of their biology of interest (for example a lung cell line to investigate influenza infection), but full genome-wide RNAi screens with primary cells is often impractical. For this reason, most screens utilize immortalized cell lines. In general, these cells are amenable to the standard transfection/transduction procedures used in RNAi screening, but it is recognized that cell lines maintained in culture for long periods of time have significantly altered genomes, epigenomes, and transcriptomes. In truth, these differences may be one of the largest contributors to screen-to-screen variation observed across host-pathogen studies. For this reason, putative hits identified in the primary screen are often validated using counterscreens that employ alternative cell types, related viruses, or siRNAs (or shRNAs) targeting different seed sites on the same gene, or small-molecule

inhibitors known to target the gene of interest. This iterative process is designed to identify candidate genes for *in vivo* testing and (ultimately) clinical studies.

Finally, it is worth noting that the quality assurance or quality control of cell lines is particularly important in host-pathogen screening. It is recognized that cell lines derived from a range of sources can be contaminated with pathogens, such as mycoplasma. Given the focus of host-pathogen screens and the possibility that contaminant pathogens can augment the cellular physiology, frequent testing of cell cultures over the course of screening and validation is necessary to minimize the possibility that underlying contamination alters screen outputs.

VIRUSES

Viruses grow rapidly and are capable of accumulating mutations (*e.g.*, point mutations, deletions) in very short period of time. While DNA viruses are generally more genetically stable than RNA viruses (due to the cellular error correction mechanism for DNA), mutations in either viral class can result in defective progeny [referred to as defective interfering (DI) particles] that lack the necessary complement of genes for infection and replication. As these features can dramatically alter the output and interpretation of an RNAi screen, studies designed to identify host-pathogen interactions should use low-passage viral particles from recently isolated stocks.

In one scenario, the virus under investigation may be of serious concern to human and/or animal health, and is therefore subject to high-level biosafety containment. In this case, the virus may require enhanced containment, such as biosafety level (BSL)-3 or BSL-4 that uses biosafety cabinets, isolators, and personal protection equipment, including HEPA-filtered exhaust air. To minimize the impact of these restrictions on assay workflow, RNAi screens that utilize high-level pathogens limit the amount of work performed at the highest level of containment by performing siRNA transfections and cell handling in regular BSL-2 laboratories, and moving cells to high biocontainment for the infection phases only. Once the infection period is complete, protocols allow for virus-infected plates to be decontaminated for removal, allowing functional readouts to be performed outside containment. Such an approach has been taken to screen chemical compound libraries for inhibitors of Hendra virus (63).

In the absence of biocontainment facilities, one way to study high-risk viruses at lower biosafety levels, *e.g.*, BSL-2 is to use pseudotype viruses. These recombinant model viruses are constructed by replacing the native envelope glycoprotein of a BSL-2 level virus (*e.g.*, vesicular stomatitis virus) with the envelope glycoprotein of the high-risk virus of interest (*e.g.*, Ebola). Pseudotype viruses mimic the viral entry process of the original virus and are competent for a single cycle of infection. The shortcomings associated with using pseudoviral particles are that not all envelope proteins can be incorporated in the carrier virus and their value in investigating postentry processes is limited. As such, researchers can gain valuable information regarding host-encoded viral receptors and endocytic pathways that facilitate viral entry, but subsequent steps in replication may be masked. Such viruses have been used in vaccine development for highly pathogenic H5N1 influenza virus, Ebola, and Lassa hemorrhagic fever virus as means to work around biosafety issues in working with wild-type viruses (64–66).

META-ANALYSIS OF RNAi SCREENS FOR INFLUENZA VIRUS

To date, 6 RNAi screens incorporating variable methodologies and endpoints have been performed to detect host contributions to influenza replication. Some screens focused on early replication events only (31, 33), while others included both early and late events (30, 32, 67). Furthermore, each screen examined different subsets of host genes and utilized unrelated cell models ranging from permissive cells (32, 33) to cells that influenza does not naturally infect (30, 31, 67). The 6 screens generated a list of potentially relevant host genes that represented ~2% of the screened genes. An exception was Shapira *et al.* (67), which focused on genes previously implicated in a yeast 2-hybrid (Y2H) study, and therefore observed a significantly higher (35%) hit rate. As has been observed in several host-pathogen RNAi screens, the candidates identified across all of the screens had little overlap. Overall, only 3 genes were independently validated in 4 of the 6 screens, 9 genes were validated in 3 of 6 screens, and 86 genes were validated across 2 screens (**Table 2**). The absence of overlap in influenza screen hit lists suggests that the identity of specific cellular factors involved in the response to an influenza virus infection is context dependent and influenced by experimental factors that vary from screen to screen. This is supported by the observation that hit lists identified by Karlas *et al.* (32) and Konig *et al.* (33), both of which used A549 cells and WSN/33 virus, show the highest degree of overlap. Given that other factors, including differences in sources of media and serum, variability in the chromosomal insertion position of reporter constructs, and inconsistency in the silencing efficiency of different siRNAs collections, may all contribute to the observed disparities, a more global approach to screen analysis is required. In the context of host-pathogen RNAi screens, this has generally come in the form of meta-analysis that includes in-depth literature review and programs such as ingenuity pathway analysis (68) and others that can identify significant networks, top functions and canonical pathways associated with the different gene hits uncovered by the screen and provide biological insight into the interactions between genes, proteins, chemicals, pathways, cellular phenotypes, and disease processes (69–76). The ob-

TABLE 2. *Results from host-pathogen RNAi screens*

Screen	Cell line	Influenza virus	Readout	Genes screened	Validated hits	Validation	Reference
siRNA screen (Ambion, Austin, TX, USA)	*Drosophila* D-Mel2	Recombinant A/WSN/33	Luciferase activity	13,071	121 (110 ↓; 11 ↑)	Decreased luciferase expression in 2 replicates, inhibiting ≥mean ± 2.5 SD in ≥1 replicate, and phenotype consistent when targeted with an alternate dsRNA amplicon	31
siRNA screen (Dharmacon, Lafayette, CO, USA)	Human U2OS	A/PR/8/34 H1N1	HA immunostain	17,877	260 (250 ↓; 10 ↑)	Rescreen with individual siRNAs from pool	30
Virus-host direct interactions (Y2H), transcriptional responses (microarray), and pathway association (IPA)	Human HBEC	A/PR/8/34, ΔNS1,[a] or vRNA	Infectious virus or IFNβ production	1,745	616	siRNA to candidate gene affected the phenotype in ≥1 of 3 functional assays	67
siRNA screen (Qiagen, Valencia, CA, USA; Invitrogen, Carlsbad, USA; IDT, Coralville, IA, USA)	Human A549	Recombinant A/WSN/33, SOIV A/NL/602/09	Luciferase activity	19,628	295 (295 WSN, 12 SOIV)	≥2 unique siRNAs to candidate gene reduced viral infection ≥35%	33
siRNA screen (Qiagen)	Human A549	A/WSN/33, SOIV A/Hamburg/04, HPAI A/VN/1203/04	Infectious virus quantified using a 293T cell reporter system and NP immunostain; viral replication measured by titrating A549 supernatant on MDCK cells	22,843	168 (119 WSN, 121 SOIV, 6 HPAI)	≥2 unique siRNAs to candidate gene decreased virus replication >5-fold	32
siRNA screen (Dharmacon)	Human A549	A/WSN/33	Amount of infectious virus, NP expression, M gene levels	1,201	28 (25 ↓; 3 ↑)	Phenotype is emulated using a novel siRNA targeting a different seed region of the hit gene	Unpublished results

↓, hits that decreased virus replication; ↑, hits that increased virus replication; HA, hemagglutinin; NA, neuraminidase; VSV-G, vesicular stomatitis virus glycoprotein G; eGFP, enhanced green fluorescence protein; HBEC, human bronchial epithelial cells; NP, influenza virus nucleoprotein; M, influenza virus matrix protein; Y2H, yeast 2-hybrid; IPA, ingenuity pathway analysis; vRNA, viral RNA; IFN, interferon; SOIV, swine-origin influenza virus; HPAI, highly pathogenic avian-origin influenza virus; MDCK, Madin-Darby canine kidney. [a]ΔNS1, PR8 virus lacking the nonstructural gene.

served pathway fidelity from such analysis suggests a conserved set of core processes that are robust to experimental variability and may be coopted during influenza virus infection and replication in a mammalian cell. Examples of a subset of processes identified by these means include the following host cell signaling pathways:

RECEPTOR TYROSINE KINASE (RTK) SIGNALING

Within the host cell, a major mechanism that transmits extracellular signals to intracellular signaling is the engagement of RTKs (**Fig. 2**). Among the family of RTKs are the group of epidermal growth factor receptors (EGFRs), consisting of four members (EGF, ErbB2, ErbB3, and ErbB4 receptors; ref. 77). ErbB expression has been associated with a multitude of cellular functions and responses, including proliferation, cell migration, differentiation, and apoptosis (78–81). Cellular endocytosis of influenza coopts pathways used by EGFRs, resulting in protein ubiquitinylation and sorting into the vacuolar pathway (82). In addition, influenza virus particles are sorted into the same population of late endosomes as EGFRs (83). Specific inhibition of tyrosine kinases by small-molecule inhibitors, as well as specific EGFR inhibition *via* RNAi, reduces virus uptake and subsequent virus titers (84). Furthermore, attachment of influenza virus to the host cell causes clustering of plasma membrane lipids, similar to that seen following EGF stimulation. Therefore, on influenza virus binding to host cell sialic acids, it is able to cluster and activate EGFRs and other RTKs to form a lipid raft-based signaling platform (84). This leads to receptor-mediated signaling events, which enhance influenza virus uptake and subsequent viral replication. It is thought that this activation is not mediated by viral engagement of a particular receptor kinase but is a more general phenomenon that affects several RTKs (84). This is additionally supported by results of a recent siRNA screening study, which identified the involvement of fibroblast growth factor receptors FGFR 1, 2, and 4 as RTKs in the very early steps of viral infection (33). Therefore, influenza virus entry accompanied by down-regulation of signaling receptors promotes coendocytosis of the virus into the host cell.

PROTEIN KINASE C (PKC) SIGNALING

PKC belongs to large family of serine/threonine kinases involved in a multitude of physiological processes (85). PKC plays an integral role in sodium ion transport, important for maintaining the low pH in the endosome (86–89). PKC has also been shown to be critical for the entry of enveloped viruses *via* receptor-mediated endocytosis (90). Upon influenza virus infection, the HA rapidly activates PKC (87, 90), and it has been shown that a specific inhibitor of PKC prevents influenza virus replication by inhibiting the entry of the virus. Similarly, influenza virus replication has also been reported in cells expressing a phosphorylation-deficient form of PKC (91).

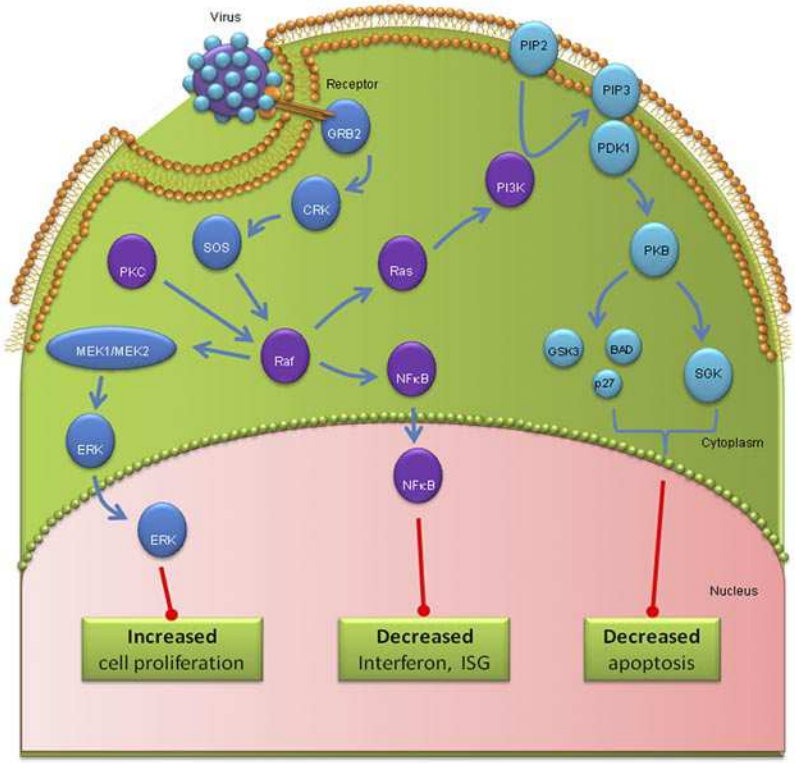

Figure 2. Host signaling factors. On binding to host cell sialic acids, influenza virus activates RTKs to form lipid raft-based signaling platforms that enhance influenza uptake and cell proliferation. HA-mediated activation of protein kinase C (PKC) also aids viral entry and cellular proliferation. RTKs activate phosphatidylinositol 3-kinase (PI3K) signaling, inhibiting cellular apoptosis. The Raf/MEK/ERK cascade downstream of RTKs is required for efficient nuclear export of viral ribonucleoproteins. Viral manipulation of NF-κB inhibits antiviral type I interferon production and resultant interferon-stimulated gene (ISG) production.

PI3K SIGNALING

The family of PI3Ks regulates various cellular events, such as cell metabolism, proliferation, and survival (92, 93). The major function of the PI3K is to phosphorylate membrane phospholipids. On PI3K activation, phosphatidylinositol-3,4,5-triphosphate (PIP3) is generated by phosphorylation of phosphatidylinositol-4,5-bisphosphate, which functions as a second messenger through interaction with pleckstrin homology domain-containing proteins such as Akt/PKB and phosphoinositide-dependent kinase (PDK)-1 (92). Cells treated with inhibitors of PI3K or PIP3 show significantly decreased influenza virus titers (94), suggesting that PI3K performs influenza-supportive functions.

Raf/MEK/ERK SIGNALING

Infection with influenza virus leads to activation of a variety of different MAPK cascades (95–98). They are activated by a variety of extracellular stimuli, such as growths factors, cytokines, and environmental stress factors like osmotic stress or ultraviolet light. Downstream substrates for MAPKs are transcription factors (*e.g.*, ATF2, ELK-1, or c-Jun), and other protein kinases, such as the MAP kinase-activated protein kinases MK2 and MK3. Thereby, MAPK pathways regulate a variety of cellular responses such as gene expression, proliferation, differentiation, apoptosis, and immune responses (62, 99, 100).

Influenza ribonucleoprotein (RNP) formation and nuclear export are important steps in the life cycle of influenza virus, and data indicate that Raf/MEK/ERK cascade is required for an efficient nuclear RNP export, as indicated by several studies (98, 101). Inhibition of Raf signaling results in nuclear retention of viral RNP and the concomitant inhibition of virus production (98). Influenza virus HA membrane accumulation and its tight association with lipid raft domain trigger the activation of MAPK cascades *via* PKC-α activation and RNP export (102). HA membrane accumulation is enhanced by the higher polymerase activity of influenza virus, resulting in up-regulation of the MAPK cascade and more efficient nuclear RNP-export, along with virus production (103). In addition, p38 MAPK and JNK have been shown to regulate the expression of proinflammatory cytokines in influenza virus-infected cells (95, 104–106).

NUCLEAR FACTOR κB (NF-κB) SIGNALING

An important influenza virus-induced signaling mediator is the transcription factor NF-κB. This factor regulates expression of a variety of antiviral cytokines, including IFN-β, which is the initiator of a strong type I IFN defense program (107). Although NF-κB is generally regarded as a central factor in the innate immune defense (108), two independent studies demonstrated that replication of influenza viruses were impaired rather than enhanced in cells where this pathway was blocked (109, 110). NF-κB acts *via* induction of proapoptotic factors, such as TNF-related apoptosis-inducing ligand (TRAIL) or FasL (109), and subsequent activation of caspases (111). This results in an enhanced export of viral RNPs from the nucleus, presumably by specific cleavage of nuclear pore proteins, resulting in an enhanced diffusion of the RNP through the pores (112, 113). Lastly, NF-κB differentially regulates viral RNA synthesis (10). Each of these mechanisms may contribute to a different extent to the enhancing effect of NF-κB on virus propagation, thereby identifying the factor as a potential target for antiviral intervention. Besides the direct antiviral action, NF-κB inhibition may also indirectly influence and, in fact, exacerbate the pathogenesis of influenza virus, since the majority of cytokines/chemokines induced during infection with highly pathogenic viruses are regulated by NF-κB (107).

To summarize, the host cell pathway overlap identified among RNAi screens for influenza virus (Table 2) indicates that core cell signaling processes are coopted by influenza virus. Variability in specific gene hit lists from different screens is likely explained by variations in the efficacy of knockdown of specific gene targets by different RNAi libraries, variation in the timing of individual screen assays, and other factors related to the assays.

CHALLENGES OF RNAi SCREENING

While RNAi screening has demonstrated enormous potential in improving our understanding of host-pathogen interactions, several challenges remain. As just described, one issue focuses on the lack of overlap associated with hits identified across different screens. Differences in viral subtypes, host cells, assay types, and reagents may be responsible for some of the differences, but, to date, there has been no definitive study to address these discrepancies. Several practices could offset the challenges associated with disparate hit lists. One would be to follow the direction of researchers in the microarray field who have developed a consistent set of minimum information standards complemented with a centralized, publicly accessible portal for data submission and review (114, 115). Minimum information about an RNAi experiment (MIARE; http://miare.sourceforge.net/) is a standards concept for RNAi screening that advocates all published screening data include extensive experimental details regarding target cell sources, delivery methods, assay design, plate layout, reagent composition, controls, and metrics of data analysis (116). In 2008, a database was established for submission of MIARE-compliant RNAi screens. While the ongoing development of the MIARE standards and the codevelopment of the PubMed database for RNAi screens represents important progress, community-wide adoption of MIARE standards and submission

policies has not yet been achieved. A revitalized collaborative effort by academic, government, and industrial partners would greatly accelerate the acceptance and implementation of MIARE and thereby facilitate the community's ability to address questions regarding screen reproducibility.

A second practice that could address the issue of disparate hit lists pertains to efforts in hit validation. In cases in which siRNA pools are used in the primary screen, deconvolution of the pool and identification of instances in which ≥2 individual siRNAs from a single pool induce the same phenotype have been used in validation (30). In other cases, validation employs the use of completely different sets of siRNAs targeting a different seed site on the same gene (32, 33), the use shRNAs (28), multiple screening assays (67), multiple viral strains (32, 33, 67), bioinformatics (29), and completely different gene-silencing technologies, for example small molecules (117). Whether hits validated by each of these procedures should be considered equal is open to debate; however, adopting a standard validation method may bring greater parity between screens.

Related to techniques employed for hit validation are the criteria used to identify a primary hit. Most screens to date have defined a hit based on a plate mean, or standard deviation from the mean, or relative to a negative control (118). However, in a recent study where 15 separate parameters were used to compile a hit signature, there was no improvement in the concordance with previous screens or increase in validation of the hits during secondary experiments (119, 120).

COMPARING RNAi SCREENING TO ANALOGOUS TECHNOLOGIES

Because a major goal of RNAi host-pathogen screens is to identify host genes and pathways that facilitate viral replication, it is important to note other analogous technologies that have been used to address this question, such as compound screens, microarrays, and NGS and Y2H screens.

Compound library screens

Small-molecule inhibitor and drug compound library screens involve high-throughput analysis of potential pharmacological agents that modulate biological pathways and sometimes specific host genes. A major advantage of compound library screens is the large number of candidate compounds, sometimes in the millions, which come from bioactive, commercial, academic and natural extract libraries, which can be screened for antiviral potential, often without the need for delivery reagents. Although a major limitation is often the lack of information that compound screens provide on the cellular target and mechanisms linked to antiviral activity, advances in screening technology platforms can compensate. For example, high-content analysis methods that use images of living cells as the basic unit for molecule discovery can track, quantitate, and provide qualitative information of the proteins of interest present in the cells using fluorescent tags, such as the green fluorescent protein, or by fluorescent antibodies. Image analysis is then used to measure changes in properties of the cells caused by treatment with candidate compounds, such as chemical inhibitors or RNA interference. However, it is important to note that compound library screens are often complicated by the need to perform analyses at multiple compound concentrations to address toxicity concerns, as cell death could be mistakenly identified as efficacy. Other problems associated with compound and small-molecule library screens include poor solubility of drugs, poor and/or variable cellular uptake, and drug specificity issues (121). Despite these concerns, a number of compound library screens have been conducted on Madin-Darby canine kidney (MDCK) cells to identify influenza inhibitors, and these results have led to follow-up validation studies in human cells (121, 122).

Microarrays and NGS

Microarrays have been used extensively to study host pathways implicated in virus life cycles and to inform our understanding of viral pathogenesis (123). In the first reported microarray study of host-influenza virus interaction, the expression levels of over 4600 genes were measured in HeLa cells in response to live or heat-inactivated influenza A/WSN/33 (H1N1) exposure (124). Of the 329 differentially expressed genes identified within 8 h of influenza infection, the majority were down-regulated and could be classified into 5 main groups—protein synthesis, cytokine signaling, ubiquitin pathway factors, mRNA processing, export proteins, and transcription factors. Broad comparisons of the gene families and pathways show a reasonable level of complementarity with those identified more recently by RNAi screening. Experiments performed using microarray technology have identified a number of host pathways that are impacted by a range of viruses, including HIV (125), human papillomavirus (126), and herpes simplex virus (127), and by treatments with IFNs -α, -β, and -γ (128). These studies have provided valuable information regarding host pathways and have provided new candidates for antiviral therapies.

Despite the large amount of microarray data and infrastructure available to researchers, it appears that NGS may supersede this technology due mainly to improvements in transcriptome coverage, sensitivity, and resolution. Since microarrays are limited by the genome coverage of their probe sets, NGS has the advantage of identifying expression changes of poorly characterized transcripts, particularly those from the small noncoding RNA families. NGS-generated genome-wide expression profiles of host cells are emerging for a number of viruses, including *Mimivirus* (129), vaccinia virus (130), and HIV (131). These studies provide a global view of how the host genome is

impacted by viral infection, in addition to changes in virus gene expression levels during infection. NGS analyses of host cells in response to influenza infection are eagerly anticipated. An important distinction is the information based on loss-of-function resulting from RNAi-mediated gene silencing. Microarray and sequencing approaches, while providing detailed readouts on fold changes in gene expression, may not determine the importance of specific genes in biological processes, and may ignore important genes and pathways if low level changes in expression are observed following infection.

Y2H screens

Y2H technology allows screening of protein-protein and protein-DNA interactions (132). Y2H screens have identified a protein-protein "interactome" network for Epstein-Barr virus (133), SARS-like coronavirus (134), and hepatitis C virus (135) infections. A comprehensive study has recently been conducted in which intraviral protein-protein interactions were analyzed by Y2H technology for 10 influenza virus (A/PR/8/34) proteins, in addition to interactions between these viral proteins with 12,000 human proteins from the Human ORFeome (67). While the influenza NS1 gene segment is known to interact with host pathways to disrupt antiviral actions (reviewed in ref. 17), numerous novel contacts of host proteins with PB1, PB2, and NP influenza gene segments were identified. This study elegantly combined Y2H and RNAi technology to identify host proteins and pathways involved in physical recognition of influenza proteins and the resultant pathways mediating antiviral responses.

CONCLUSIONS

The importance of influenza virus to global human and animal health, coupled with the current limitations associated with vaccine production and antiviral therapeutic pipelines, underscores the need for novel solutions to accelerate the development of virus intervention strategies. Genome-wide RNAi screening is an emerging technology with the power to detail host-virus interactions, furthering our understanding of virus pathogenesis and thereby driving the development of next-generation antivirals. On the basis of the findings from the meta-analyses of influenza screens (31, 67, 136–138), it is likely that influenza virus principally coopts host genes late during virus replication. Succinctly, influenza virus carries all necessary proteins to infect a cell and deliver its vRNP into the cell nucleus. However, viral proteins are synthesized in the cytoplasm; thus, the virus must use host proteins and pathways to import NP and RNA polymerases back into the nucleus to form new vRNP. Likewise, the assembly of influenza components occurs at the plasma membrane, requiring host mechanisms to move new vRNP from the nucleus into the cytoplasm, and then traffic the viral protein components toward the membrane for assembly and packaging of new virions. This hypothesis is consistent with the subtle differences that have been observed in viral genomic expression relative to the huge differences observed in high-content analysis and in virus replication (33, 136). In addition, in the meta-analyses studies (31, 67, 136–138), host factors linked with regulating cell death are affected by influenza virus, a feature likely required to facilitate replication.

Given the scale of information generated by RNAi screens, it is critically important and beneficial to collaborate in this research arena. There are admirable examples of how scientific consortia and centers composed of dispersed networks of laboratories have successfully worked together to achieve significant goals. The U.S. National Institute of Allergy and Infectious Diseases Centers for Excellence in Influenza Research and Surveillance and the Human Genome Project provide two examples where intervention strategies are tackled by academic, government, and industrial entities worldwide and represent good examples as to how disparate entities can work in partnership toward a common goal. Similarly, the efforts by organizations such as the RNAi Global Initiative are designed to tackle the challenges represented by EIDs, such as influenza, to benefit human health. It will be vital to the success of this emerging field that such collaborative initiatives promote the development of data standards and best practices that facilitate the comparison of data from complementary screens. This should lead to a better understanding of the factors that most strongly influence screen reproducibility and data quality, and ultimately improve the potential of RNAi technology to identify optimal antiviral-drug candidates. FJ

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