

Genome-wide screening using RNA interference to study host factors in viral replication and pathogenesis

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Abstract

With the recent development of short interfering RNA and short hairpin RNA expression libraries, RNA interference (RNAi) technology has been extensively employed to identify genes involved in diverse cellular processes, such as signal transduction, cell cycle, cancer biology and host–pathogen interactions. In the field of viral infection, this approach has already identified hundreds of new genes not previously known to be important for various virus lifecycles. In this brief review, we focus on recent studies performed using genome-wide RNAi-based screens in mammalian cells for the identification of essential host factors for viral infection and pathogenesis.

Keywords: siRNA, shRNA, HIV, genome-wide screening, virus replication

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Introduction

Viruses are intracellular parasites that exploit host cell machineries in every step of their lifecycles.^{1,2} In recent years, it has become clear that human cells conserve the RNA interference (RNAi) machinery found across plant and animal kingdoms.³ The RNA-dependent gene-silencing process was initially described for the nematode *Caenorhabditis elegans* wherein double-stranded RNAs (dsRNAs) can trigger the cleavage and subsequent degradation of sequence homologous mRNA transcripts.⁴ Indeed, increasing amounts of experimental findings are consistent with the RNAi mechanism being employed by human cells to restrict viral infections.^{5–10} Accordingly, viruses have evolved RNAi suppressors,^{11–18} which may serve to change the small non-coding RNA profile in virus-infected host cells.^{19–21}

The conservation of RNAi-machinery in human cells has allowed for the development of RNAi-based, genome-wide screening techniques to identify and investigate the cellular factors involved in viral replication and pathogenesis. Originally developed in *C. elegans* and *Drosophila* using long dsRNA,^{22–25} large-scale RNAi screens have been successfully applied using either synthetic short interfering RNAs (siRNAs) or vector-expressed short hairpin RNAs (shRNAs) for mammalian cells.^{26–31} Genome-scale RNAi screens in mammalian cells have been performed for studies in cancer biology and host–pathogen interactions.^{32–44}

Here, we review in brief the extant experience of RNAi-based screens using siRNAs and shRNAs to study the cell factors used by various viruses for replication in host cells.

siRNA and shRNA methods for RNAi-based genome-wide screens

Two methodological approaches have been applied commonly for RNAi-based genome screenings – large-scale transfections using a synthetic siRNA library or viral transduction of an shRNA library into cells (Figure 1). These two methods are typically performed using two different formats. The application of siRNA libraries is usually performed in a cell-based array (commonly in 96- or 384-well microtiter plates), in which individual genes are silenced in each well. On the other hand, the shRNA library is applied in a pooled format, where the entire library is introduced into cells and a subsequent selection and/or screening readout is employed in bulk. Both of these approaches have been successfully used to study different biological processes, and each has advantages and disadvantages.

In the arrayed format, the siRNAs are transfected into cells, and the detection of the assay is monitored by individual readouts from each well (e.g. colorimetric, fluorescence or luminescence plate reading). The advantage of this assay is that the plate format facilitates high throughput

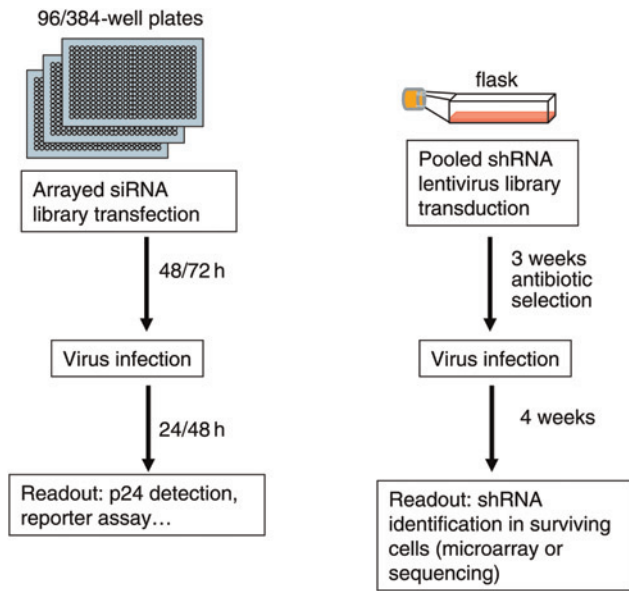


Figure 1 Schematics of the two different assay formats used in RNA interference-based genome-wide screens. The arrayed format used with short interfering RNA (siRNA) libraries (left) and the pooled format used with short hairpin RNA (shRNA) libraries (right) are represented. The timing of the specific steps is indicated

manipulations, and that the identity of the gene candidate is directly given by the position on the plate. Moreover, several parameters can be evaluated for each well or targeted gene. However, when a large number of genes are targeted in a genome-scale analysis, numerous plates must be screened in a 'labor-intensive' manner which would require the use of robotic machines. Also, because the siRNA-based screenings are transfection-based, this approach can be applied best to cells that are easily transfected, and thus the approach may not be useful for studies that require primary cells which are often difficult to transfect.

In a separate approach, retroviral vectors expressing shRNAs packaged into lentiviral particles are used to constitute an shRNA library that can be transduced into cells in a pooled format.^{27,28} These particles can be employed to stably transduce target cells, and the cells can be enriched by the selection of a co-transduced drug marker. shRNA-transduced cells can then be infected by virus, and the resulting outcome can be phenotypically characterized. For example, in a recent assay, cells that survived lytic infection by human immunodeficiency virus type 1 (HIV-1) represented those that had received an shRNA which had knocked down a cellular protein needed for intracellular HIV-1 replication.³⁴ Alternatively, depending on how the assay is set up, infected cells could be isolated using fluorescence-activated cell sorter (FACS) or other readouts. Thus, in an shRNA screening focused on kinases and phosphatases genes, Rato *et al.*⁴⁵ used FACS to eliminate HIV-infected cells and selected for cells which were resistant to HIV. In this way, they were able to implicate specific cellular kinases and phosphatases that act in the HIV-1 lifecycle. In the shRNA-library transduction approach, the shRNAs resident in the selected cells can then be amplified by polymerase chain reaction followed by sequencing or microarray hybridization for direct identification. An advantage of the

shRNA-transduction approach using lentiviral particles instead of transfection is that transduction can introduce genetic material into a much larger range of dividing and non-dividing cells. Hence, this method is amenable for studying primary cells that may otherwise be refractory to transfection. Pooled shRNA screenings are also more easily conducted by most laboratories because this type of screening can generally be successfully executed without the need for expensive robotic tools.

RNAi-based genome-wide screens applied to host-virus interactions studies

Genomewide screenings in mammalian cells have been used for HIV,^{34,39,42,43} influenza viruses,^{32,33,38} West Nile virus⁴¹ and hepatitis C virus,^{35,37} among others. We discuss in brief some of these screenings below.

HIV

In the literature, there are currently four studies of genome-wide screenings for host cell factors that are important for HIV-1 gene expression and replication (Table 1).^{34,39,42,43} Out of the four HIV screens, three were performed using siRNAs.^{39,42,43} Two of these used siRNA transfection into HeLa cells engineered to express the viral receptor CD4 followed by infection with virus.^{39,43} Thus, Brass *et al.*⁴³ investigated early and late steps of the HIV lifecycle (from viral entry to virus budding and infectivity), and identified 273 genes out of a total of 20,000 mRNAs that were knocked down. By comparison, Zhou *et al.*³⁹ used a similar strategy and found 232 candidate genes. Surprisingly, between the two studies only 15 genes overlapped. A third study by König *et al.*⁴² focused only on the early steps of the HIV lifecycle, from uncoating to protein synthesis. This system differed from the previous two screenings in its use of 293T cells instead of HeLa, and in the use of a virus pseudotyped with vesicular stomatitis virus G protein. Not surprisingly, of the 295 genes identified, very limited overlap was observed between this work,⁴² and the two other studies.^{39,43}

The lack of overlap between these three screenings illustrates that in different settings different genes may be limiting for HIV-1 replication. The issue then becomes how does one interpret meaningfully these divergent findings.^{46,47} Bushman *et al.*⁴⁸ attempted a meta-analysis to integrate the three siRNAs screens together with data obtained from other methods. The analyses showed that variations between replicates, between time points and between filtering thresholds all likely contributed to the differences observed between the siRNA screens. A take-home message appears to be that the various studies converge more at identifying relevant cellular pathways rather than at identifying the exact same genes within a commonly identified pathway.⁴⁶

A fourth HIV study was performed by Yeung *et al.*³⁴ using an shRNA library transduced into cells in a pooled format. The shRNA screening employed transduction instead of transfection; thus the investigators could use suspension Jurkat human T-cells which is a more physiological

Table 1 Genome-wide RNA interference-based screens for viral host factors in mammalian cells

Virus	Cells	Assay readout	Candidate genes/genes tested (reagent)	References
HIV				
HIV-1 (IIIB)	HeLa (CD4 + , BGal reporter)	p24; reporter activation	273/21,121 (siRNA)	43
HIV-1 (HXB2)	HeLa (CD4 + , BGal reporter)	Reporter activation	232/19,709 (siRNA)	39
HIV-1 Luc vector, VSV-G pseudotyped	293T	Luc reporter	295/19,628 (siRNA)	42
HIV-1 (NL4.3)	Jurkat	Cell survival	252/54,509 (shRNA)	34
Influenza				
A/Puerto Rico/8/34 (H1N1; PR8)	Osteosarcoma cells (U2OS)	HA expression	133/17,877 (siRNA)	38
Recombinant WSN (HA-Luc)	Human lung cells (A549)	Luciferase activity	295/19,628 (siRNA)	32
A/WSN/33 (H1N1)	Human lung cells (A549)	NP staining; Luc reporter	287/22,843 (siRNA)	33
HCV				
HCV (1b Luc replicon)	Huh7/Rep-Feo	Luc reporter	96/21,094 (siRNA)	35
HCV (JFH-1 genotype 2a)	Human hepatocellular cell line (Huh 7.5.1)	Core 6G7 protein staining	262/19,470 (siRNA)	37
WNV				
WNV (2471)	HeLa	Viral E-protein staining	305 (WNV)/21,121 (siRNA)	41
DENV (NGC)			(124 shared with DENV)	

DENV, dengue virus; HA, hemagglutinin; VSV-G, vesicular stomatitis virus G protein; WNV, West Nile virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; NP, nucleoprotein; siRNA, short interfering RNA

target cell model for HIV-infection than the adherent HeLa and 293T cells used in the three siRNA screens. The shRNA approach also allows for stable durable knockdown of cellular mRNAs over an extended time period. Thus, indirect apparent effects on viral replication due to cytotoxicity from the knockdown of certain genes are less likely to emerge as false-positive candidates in the shRNA- than the siRNA-screening. In the approach performed by Yeung *et al.*, cells were transduced with an shRNA library that targeted 54,509 human transcripts, and then the cells were selected over three weeks with puromycin for those that had stably taken up an shRNA. (Each shRNA in the library was marked with a puromycin-resistance gene.) At this stage, the analysis of puromycin-selected cells by Yeung *et al.* showed that only shRNA clones targeting 9357 genes out of the starting 54,509 were still represented in the surviving cells. This result suggested that only 18.2% of the cell's total transcripts can be tolerably knocked down without affecting Jurkat cell viability in tissue culture. A corollary of this finding is that the extended knockdown of 82% of cellular transcripts is cytotoxic to cells in tissue culture. The puromycin-resistant cells were then infected for an additional four weeks with HIV-1, and cells that survived lytic infection by the virus were analyzed for their shRNA content. The microarray hybridization results showed that shRNAs targeting 252 distinct transcripts were enriched between 2- and 22-fold in the survivor cells. These transcripts represent candidate cellular factors that contribute to HIV-1 replication in Jurkat cells. Of course, further detailed functional validations are needed before one can understand how each gene affects HIV-1 propagation in Jurkat cells. It should be noted that because their approach differed in both duration of selection and cell type which was employed, not unexpectedly, Yeung *et al.* observed only minimal overlap with genes identified in the other three siRNA studies.^{39,42,43} Nevertheless, there was significant concordance in the pathways identified as important by Yeung *et al.*³⁴ and those

identified earlier.⁴⁶ By combining the results of these four studies, 40 genes were found common to at least two of the four screens (Table 2).

Influenza

To date, three independent genome-wide RNAi screens in mammalian cells have been published on identifying host factors important for influenza virus replication. Two of these studies were performed using the same human lung cell line A549,^{32,33} and the third one used osteosarcoma cells.³⁸ Whereas the study of Karlas *et al.* covered the entire viral replication cycle, from viral entry to budding, the other two studies were limited to the early and middle stages of the viral lifecycle, from virus entry to protein translation. By targeting more than 17,000 genes, they identified 120,³⁸ 295³² and 287³³ human genes involved in influenza virus replication. As in the case of HIV, only minimal overlap was observed in the lists of candidates from these three studies, with a maximum of 32 overlapping genes between the screens conducted by Konig *et al.* and Karlas *et al.* and a total of 49 genes found in at least two of the three screens (Table 2).

As was done for the HIV-1 analyses, Watanabe *et al.*⁴⁹ combined the results of these three influenza virus studies in a meta-analysis with data obtained from other methods, and finally arrived at a list of 128 human genes common to at least two genome-wide screens. Bioinformatics analysis of these genes revealed that they formed clusters of several proteins involved in similar host cellular functions important for the influenza virus lifecycle, such as endocytosis, nuclear transport and translation initiation.

Other viruses

Genome-wide siRNA-based screenings have been used in the study of two other viruses, West Nile virus (WNV) and hepatitis C virus (HCV). Krishnan *et al.*⁴¹ recently

Table 2 List of genes detected in at least two of the genome-wide RNA interference-based screenings listed in Table 1 for HIV, influenza and HCV

HIV			Influenza		HCV
ADRBK1	MED28	AHCYL1	JUN	RPS16	ARCN1
AKT1	MED4	AIG1	KIAA0652	RUNX1	CEBPD
ANAPC2	MED6	ARCN1	KPNB1	SF3A1	COPA
ANKRD30A2	MED7	ATP6AP1	LRP1B	SF3B1	COPB1
CAV2	MID11P1	ATP6AP2	LY6G6C	SF3B14	CTSF
CCNT1	MRE11A	ATP6V0B	MAP2K3	SNRP70	DLK1
CD4	NFKB1	ATP6V0C	MDM2	TNK2	DLX6
CH5T1	NUP153	ATP6V0D1	NHP2L1	TRIM28	HAS1
CTDP1	NUP98	ATP6V1A	NUP98	WDR18	IDS
CXCR4	RAB11	ATP6V1B2	NXF1		ITGA7
DDX3X	RAB28	BZRAP1	OPRS1		NOP5/NOP58
DMXL1	RANBP2	CD81	PHF2		NUAK2
EPAS1	RELA	CLK1	PIP5K1C		PI4KA
HMCN2	RGPD8	COPA	PLK3		RABEPK
IBTK	RNF26	COPB2	PPP1R14D		TRIM62
IDH1	RPL3	COPG	PRPF8		
JAK1	TCEB3	FKBP8	PTPRN		
MAP4	TNPO3	IGSF1	PTS		
MED14	TRIM55	IKBKE	RACGAP1		
MED19	WNK1	IL17RA	RPS10		

In bold are the genes detected in more than two screens of the same virus. In red are the genes common between two different viruses
HIV, human immunodeficiency virus; HCV, hepatitis C virus

identified 305 host factors that influence infection by WNV, with 283 factors facilitating and 22 antagonizing virus infection. Interestingly, the study also revealed that the silencing of 36% of the host factors used by WNV also reduced infection by dengue virus, a related flavivirus.

Two studies concerning HCV were recently published (Table 1). Tail *et al.*³⁵ reported 96 genes candidates for HCV virus replication using a subgenomic replicon system. However, the replicon system was not able to address the full HCV lifecycle. Using a different system recapitulating the complete lifecycle of the virus, Li *et al.*³⁷ identified 262 host factors important for HCV replication. Over 30 of the genes were previously implicated in the replication of HCV or WNV (a related flavivirus) using functional genomic studies. A comparison of Li *et al.* with the work of Tail *et al.*³⁵ performed with a HCV replicon system showed an overlap of 15 genes out of 96 identified (Table 2). Interestingly, when compared with the host factors identified for HIV, the analysis revealed 10 common factors needed for the replication of both HIV and HCV. This is of particular interest considering that an estimated 25–30% of HIV-infected individuals are chronically co-infected with HCV.⁵⁰

Conclusion

Genome-wide RNAi-based screening represents a powerful new approach for the identification of host factors involved in the virus lifecycle. To date, at least several hundreds of potential candidate genes have been identified in the few screenings discussed here for the various viruses. Moreover, considering that there was only limited concordance between the different screenings, this suggests that viral infection in varying settings may be limited by different factors. Thus, the picture for 'required' cellular factors for virus replication in human cells may be quite complex. Accordingly, future screenings will likely identify several hundreds more candidates, each specific for divergent scenarios. An ultimate goal is for investigators to carefully

validate the mechanism whereby each identified candidate assists/detracts viral infection.

By comparing the list of factors identified for different types of viruses, we appreciate only a few common factors (Table 2). This is not surprising considering the poor overlap observed between independent screens conducted for the same virus. However, if data-sets for each virus could be accrued that analyze different cell types and different settings of infection and if more studies could be extended to additional viruses, then the ever larger list of genes could reveal informative patterns to tell which are factors common to many viruses and which are factors specific to individual viruses (under defined conditions).

The study by Yeung *et al.*³⁴ for HIV-1 is the first genome-wide screen for host factors involved in virus infection using shRNA vectors in a pooled format. This method seems promising because it broadens the amenable range of cells for functional analyses that could otherwise be limited only to cells that are easily transfectible by siRNAs. Thus, shRNA-based lentiviral transductions could potentially be performed in physiologically relevant primary target cells. Moreover, the transduction approach offers numerous versatilities such as the use of a shRNA library under inducible expression and the use of cell sorting after selection of transduced cells for phenotypical readouts.

By accelerating the discovery of new host factors important for the virus replication, genome-wide RNAi-based screens will undoubtedly reveal new therapeutic targets. The next few years promise to be exciting and productive.

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