Expert Opinion

High-content siRNA screening for target identification and validation

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Background: Automated microscopy and image analysis have progressed tremendously over the past 10 years and opened up a new era of high-content screening. In parallel, RNA interference (RNAi) has revolutionized the functional analysis of genes. *Objective*: The focus of this review is screening of RNAi libraries, and in particular in screening of short interfering RNA (siRNA) libraries for target identification and validation in mammalian cell systems. *Methods*: Recent literature of high-content siRNA screening in oncology, in intracellular trafficking and infection biology, and in neurobiology is reviewed and placed in the context of a discussion on hit verification. *Results/conclusion*: Various methods have been established for the application of RNAi in mammalian cells also, which allows efficient and reproducible silencing of individual genes to gain functional information on each individual gene. Complex multi-parametric cell-based assays, combined with both technologies, provide an extraordinary valuable tool to help understand biological and pathological processes in a systematic way and discover new targets for pharmaceutical exploitation.

Keywords: automated microscopy, functional genomics, high-content, high-throughput, image analysis, multi-parametric assay, RNA interference, RNAi, screening, short interfering RNA, siRNA, systems biology, target identification, target validation

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1. Introduction

High-content screening (HCS) has been defined as multiplexed functional screening based on imaging multiple markers (e.g., nuclei, mitochondria) in the physiological context of intact cells by the extraction of multicolor fluorescence information [1]. The major technological components consist of fluorescent reagents and proteins, automated liquid handling devices, automated imaging systems, sophisticated image analysis software and data management systems.

1.1 Components for HCS

Cell-based assays applied in HCS exhibit multiple-color staining and allow simultaneous qualitative and quantitative extraction of various parameters from each individual cell, both at the level of target staining intensities and phenotypic pattern recognition. A major breakthrough was the discovery of the green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* and other fluorescent proteins that can be fused to any desired target or marker protein and the consequent innovation in this area provided a broad variety of fluorescent proteins covering the whole light spectrum [2,3]. Conveniently, stable cell lines are widely used [4] and are also available commercially. Further, a broad diversity of reagents is available for staining, such as labeled target- or modification-specific antibodies, fluorescent probes and substrates, or environmental indicators

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(Ca^{2+}, membrane potential, pH etc.). The number of ready-to-use assay kits provided by vendors is continuously growing. A rapidly increasing number of HCS assays that have been proven in screening projects, have been published, such as assays for analyzing mitotic spindle formation [5], cell proliferation [6,7], apoptosis [8], migration [9,10], nuclear translocation (e.g., GPCR–GFP [11], NFAT–GFP [12], or FOXO1–GFP [13]), inflammation [14,15], Wnt signaling for activation of β-catenin [16], EGFR signaling [17], function of gap junctions [18], or neurite outgrowth [19].

However, important factors that limit the maximal exploitation of this technology are the prolonged assay development times and the unavailability of robust cellular assays for screening. At present, academic research groups contribute significantly with innovative and uncertain assay development projects, for example, in primary cells or differentiation models. These assays are complex and very demanding but are of high physiological relevance. Very recent examples describe assays using primary neuronal cells [20], high-throughput assays for phagocytosis, phagosome maturation and bacterial invasion [21], or osteogenic differentiation of human mesenchymal stem cells [22].

A significant task of assay development for HCS is to provide a correlating automated image analysis solution. Most image acquisition systems are delivered with an image analysis software package that offers ready-to-apply scripts for several standard assays. Nevertheless, many of the protocols have to be adapted to particular cell lines or assay conditions. Additional commercial image analysis systems are available that are not linked to a particular imaging platform. Academic groups developed and published their own solutions, such as the open-source image analysis platform CellProfiler [23-25]. Catalogues of subcellular phenotypes have been described and are automatically recognized by a machine-learning based classification method [26]. Kinetic HCS, along with correlating instrument and image analysis development, has been described for reporting chromosome segregation and structure by time-lapse microscopy over several consecutive days [27].

Automated microscopes are capable of automatic focusing, multichannel image capturing and handling microtiter plates. Robotic systems are available for liquid handling and cell processing. An industrial infrastructure for target identification by RNA interference (RNAi) has been described first by Xin and co-workers [28], whereas instrumentation and workflows for HCS have been published in special issues of *Methods in Molecular Biology* and *Methods in Enzymology* [29,30]. A detailed overview on the components for HCS, particularly considering the needs for large-scale short interfering RNA (siRNA) delivery, high-content siRNA screening and hit verification strategies, has been given recently [31].

1.2 Application of HCS in research and development

In the beginning, HCS was mainly used by pharmaceutical and biotech companies to characterize new chemical entities against selected targets in the cellular context, and to derive toxicity profiles. Although the technology not only gives information on morphology, phenotypes and genotypes but also provides functional data, it has been established as a powerful tool in modern (academic) research and drug discovery. Today, high-content analysis, as well as HCS, is applied throughout the pharmaceutical research and development process, such as pathway analysis, target identification and validation, primary and secondary screening, studying mode-of-action, supporting the hit-to-lead process, identification of biomarkers, determining various toxicities in a wide range of *in vitro* models, assessing genotoxic risks [32,33]. More recently, the tracking of kinetic cellular processes in living cells was explored to support basic research and also pharmaceutical R&D. Besides the private sector, screening in academic institutions has been established during the past 10 years, and in particular several academic HCS units have been set up recently. Although the primary HCS literature is still rare, a considerable number of review articles summarizing strategies, progress and developments in HCS have been already published [31-34]. The original published literature on screening of high-content chemical libraries has been reviewed very recently [35]; therefore, the focus of this review is screening of RNAi libraries, and in particular screening of siRNA libraries for target identification and validation in mammalian cell systems. In Figure 1 a typical workflow of an siRNA screening project is illustrated.

1.3 RNAi applied to functional genomics

RNAi is a natural process to specifically silence individual genes and has been discovered in the model organism *Caenorhabditis elegans* by Craig Mello and Andrew Fire [36]. RNAi has been revolutionizing the functional analysis of genes [37,38] not only in model organisms, where several impressive genome-wide screens were performed in *C. elegans* [39,40], but also in whole organisms or *Drosophila* cell lines [41]. The elaborated and applied methods have been well documented elsewhere [42-45]. The major breakthrough to enable wider applications were achieved by demonstrating that chemically synthesized 21-meric siRNAs, the effector molecules in the RNAi process, induce RNAi in human and other mammalian cells [46] by circumventing their natural defense mechanism against long double stranded RNA. Since then various methods have been established for application in mammalian cells, such as chemically synthesized siRNAs [46], short hairpin siRNAs (shRNAs) expressed from vectors such as plasmids or viruses [47,48], and endoribonuclease-prepared siRNAs (esiRNA) which are produced by *in vitro* digesting double stranded long RNA (Dicer or RNaseIII [49,50]). These methods allow efficient and reproducible silencing of individual genes and provide an opportunity to gather functional information on each individual desired gene. Algorithms have been developed to predict siRNA molecules

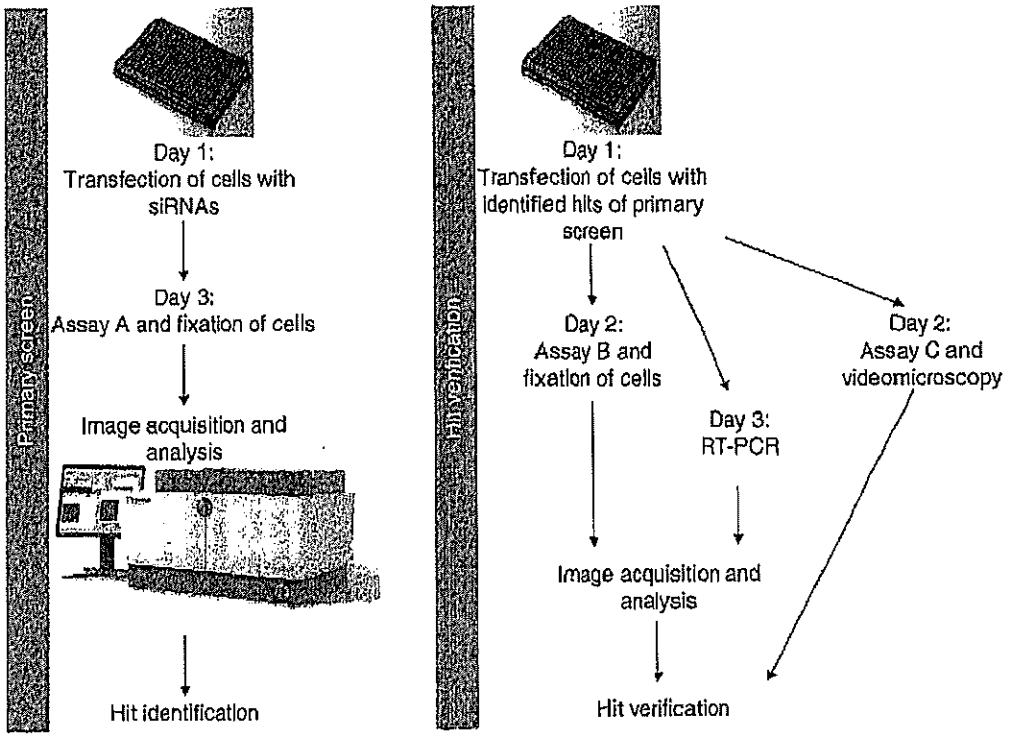

Figure 1. A general overview sketch of an RNAi screening campaign.
RNAi: RNA interference; RT-PCR: Reverse transcription polymerase chain reaction; siRNA: Short interfering RNA.

that exhibit a high probability to be functional [51]. Such siRNAs are commercially available against all predicted genes of the human, mouse and rat genomes.

2. siRNA screening in mammalian cells

Large libraries have been assembled targeting individual target classes of interest (i.e., the 'druggable' genome [52] or the whole genome) and are applied to systematic analysis of the genome towards involvement of the individual genes in the assayed cellular processes. The majority of published siRNA screens in mammalian cells apply reporter gene assays or other homogeneous cell-based assays. In particular, pharmaceutical research labs have access to large-scale siRNA libraries and an automated infrastructure, and published several cell-based RNAi screens using homogeneous read-out (e.g., to search for new targets in oncology [53,54], hepatitis C virus replication [55], or Alzheimer's disease [56]). More recently, multi-parametric assays, based on automated microscopy, accelerated in numbers of publications.

In one of the first RNAi screens performed in mammalian cells, over 5,000 pools of esiRNAs were transfected into a human cervical carcinoma cell line [57]. Although the primary screen was analyzed using a homogeneous proliferation assay, it should be noted here because all 275 'toxic' hits were analyzed subsequently using time-lapse video microscopy. The first published primary high-content screen that applied a chemically synthesized siRNA library targeting all human kinases demonstrated an impressive complex network of positive and negative regulation of two distinct endocytosis pathways, those that are also used by viruses to infect host cells [58]. The hit definition and verification strategies are summarized in Table 1.

2.1 High-content siRNA screening in oncology

At present, the most popular field of application for high-content siRNA screens to systematically screen for new targets along with subsequent validation is in the field of oncology. Multi-parametric assays have been developed for proliferation, cell cycle and signal transduction pathway analysis, apoptosis, migration or invasion, and for use in screens. In addition, chemosensitizer screens have been explored to identify targets that would potentiate the activity of existing chemotherapeutics [56,59].

The first oncology relevant siRNA screen performed in mammalian cells was published in December 2004 [57]. As mentioned earlier, over 5,000 pools of esiRNAs were transfected into HeLa cells. The screening strategy applied was straightforward: the primary screen was kept simple and inexpensive, the antiproliferative effects of target knockdown was measured by a homogeneous proliferation assay based on water-soluble tetrazolium (WST-1). Subsequently, all 275 'toxic' hits were analyzed in a secondary screen applying time-lapse video microscopy to observe proliferation of cells for 72 h. Strikingly, one target accelerated proliferation upon depletion. The remaining hits were grouped into three categories: mitotic arrest, cell death upon entry into mitosis and aberrant cytokinesis. The majority of depleted

Table 1. Hit definition and verification strategies used in recent siRNA screening publications.

Primary screening				Hit verification		Ref.
Assay	**Library**	**Hit definition**	**Number of hits, results**	**Assay**	**Results**	
Cell viability using WST-1 (Roche Diagnostics)	5,305 esiRNAs	Normalized value greater than 1.645 SD or less than -1.645 SD obtained at least twice	275	Video microscopy for spatial and temporal visualization of mitosis and cytokinesis	37 hits	[57]
SV40 and VSV infection to monitor clathrin- and caveolae/ raft-mediated endocytosis	siRNAs targeting the human kinome (590 kinases, 3 siRNAs/gene)	Inhibition: RII ≤ 0.33 Enhancement: RII ≥ 3 Involved in both clathrin- and caveolae-mediated endocytosis pathways: both RIIs < 0.4 or > 2.50	208 (92 kinases scored in VSV infection, 80 in SV40 infection, and 36 affecting both VSV and SV40 infection)	i) Rescreening of 50 kinases with independent siRNA sequences resulted in the same phenotype in 48/50 (SV40) and 50/50 cases (VSV) ii) Transferrin uptake, LDL uptake, cholera toxin B uptake; anti-EEA1 or -LAMP1 antibody staining; distribution and morphology of caveolin-1-GFP	Network of positive and negative regulation of two distinct endocytosis pathways (two-step cluster analysis)	[58]
DNA content analysis with propidium iodide staining	Genome-scale human esiRNA library targeting 17,828 genes	The DNA content histograms obtained were analyzed and scored as a hit when a distance with a highly significant difference (more than 3 SD) from that of eight negative controls was observed	2,146	i) Correlation of those hits to expression status in HeLa cells ii) Resynthesis of esiRNAs for the genes and repetition of DNA content analysis iii) Synthesis of non-overlapping esiRNA for the genes and repetition of DNA content analysis iv) Nine-parameter fingerprint	1,726 1,351 743 217 known genes associated with cell cycle 252 previously uncharacterized genes 882 known genes not associated with cell cycle yet	[60]
Cell cycle, cell size, and proliferation based on DAPI staining	48,746 siRNAs targeting 24,373 genes plus 10,000 siRNAs targeting 5,000 genes of the 'druggable' genome	Alteration of cell cycle progression in two replicates Cells with σ-value (the number of SD from the plate mean of the middle 50% of the population) for any descriptor of ≥ 4 were manually inspected and the ones with strong phenotypic defects are reported	1,152	Anti-γ-tubulin antibody, anti-α-tubulin antibody and anti-phosphohistone-H3 staining (MI)	Phase-specific networks	[61]

β-Gal: β-Galactosidase; DAPI: 4,6-Diamidino-2-phenylindole; DHC: Dynein heavy chain; esiRNA: Endoribonuclease-prepared siRNA; GFP: Green fluorescent protein; MI: Mitotic index; RII: Relative infection index (the ratio of infected siRNA treated cells to infected control treated cells); RSV: Respiratory syncytial virus; RT–PCR: Polymerase chain reaction after reverse transcription of RNA; SD: Standard deviation; shRNA: Short hairpin siRNA; siRNA: Short interfering RNA; SV40: Simian virus 40; VSV: Vesicular stomatitis virus; WST-1: Water-soluble tetrazolium (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate).

Table 1. Hit definition and verification strategies used in recent siRNA screening publications (continued).

Primary screening				Hit verification		Ref.
Assay	**Library**	**Hit definition**	**Number of hits, results**	**Assay**	**Results**	
RSV infection	Pools of 4 siRNAs targeting 79 genes involved in actin polymerization, cytoskeleton rearrangement, endocytosis and vesicle/cargo movement	Setting the threshold of significance of the screen to two times the average SD of all the infection data	Clathrin-mediated endocytosis was discovered to be involved in RSV infection	Individual siRNAs from each pool applied to primary assay, western blot analysis, expression of dominant-negative mutants	5 hits: 3 out of 4 siRNAs were inducing the phenotype 11 hits: 2 out of 4 siRNAs to score positive	[66]
HIV infection	21,121 pools of four siRNAs per gene	Decrease the percentage of p24-positive cells or β-Gal activity by ≥ 2 SD from the plate mean and siRNAs did not decrease viable cells by > 2 SD	386	Individual siRNAs from each pool applied to primary assay	Identification of 273 HIV-dependency factors with at least one siRNA per target to score	[67]
Intracellular bacterial growth	Pooled siRNAs targeting 779 kinases	For intracellular growth of *Salmonella typhimurium*, H-89-treated samples were taken as a reference. Median (triplicate) GFP fluorescence per infected cell was compared between siRNA transfected and H-89-treated MCF7 cells and plotted in a heat map or as a bar diagram	Downregulation of 11 kinases and 3 kinase-associated proteins reduced intracellular *S. typhimurium* growth	Multiple individual shRNA sequences per target were tested for their effect on intracellular growth of Lux-*S. Typhimurium*, *in vitro* kinase, GTPase assays	A hit was considered validated when confirmed by at least two independent siRNAs The kinases identified clustered in one network around AKT1	[68]
Secretory protein transport from the endoplasmic reticulum to the cell surface	92 siRNAs targeting 37 putative membrane traffic proteins	Less than 15 'quantifiable' cells were not considered valid and were excluded Deviation from the negative controls of 2 SD units was used for hit detection Single-cell quantification	7	Five replicate experiments at different times RT–PCR	RNAi screening platform to identify secretion machinery in mammalian cells	[69]

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Table 1. Hit definition and verification strategies used in recent siRNA screening publications (continued).

Primary screening				Hit verification		Ref.
Assay	Library	Hit definition	Number of hits, results	Assay	Results	
Synapse density determined by counting co-localization of synapsin I and PSD-95 dots present along the dendrites of GFP-expressing neurons	160 genes comprising 105 unique pools of diced siRNAs targeting 1 – 4 genes	A pool was considered 'positive' if it was significantly different ($p < 0.05$) from control by a two-sample t test	4	Positive pools were rescreened, and those that were positive by the above criteria in two or more independent experiments were analyzed further Per target, three pSUPER shRNA expressing plasmid vectors were transfected in pools Test in heterologous cells Deconvolution of pools Rescue experiments	Cadherin-13 and cadherin-11 were identified to regulate synapse development by different mechanisms	[71]
Neurite outgrowth	siRNAs targeting 85 tyrosine kinases	Genes yielding p-values less than 0.05 in two runs were identified	9	Morphological analysis of twinfilin-2-overexpressing cells (PTK9L) and validation in primary cells of rat cortex origin	The actin-binding PTK9L is involved in neurite outgrowth	[72]
Neurite outgrowth and retraction	siRNAs targeting the human kinome (750 genes, 3 siRNAs/gene)	A hit was determined when at least two siRNAs per target scored in two runs	Neurite outgrowth: 59 kinases are identified as positive regulators, 66 kinases as inhibitors Neurite retraction was inhibited by 79 kinases	RT–PCR Validation in primary neurons and demonstration of orthologue genes in *Drosophila* retinal degeneration	Identification of a large group of kinases with previously unknown function or kinases not previously implicated in regulation of axonal growth/maintenance	[73]

β-Gal: β-Galactosidase; DAPI: 4,6-Diamidino-2-phenylindole; DHC: Dynein heavy chain; esiRNA: Endoribonuclease-prepared siRNA; GFP: Green fluorescent protein; MI: Mitotic index; RII: Relative infection index (the ratio of infected siRNA treated cells to infected control treated cells); RSV: Respiratory syncytial virus; RT–PCR: Polymerase chain reaction after reverse transcription of RNA; SD: Standard deviation; shRNA: Short hairpin siRNA; siRNA: Short interfering RNA; SV40: Simian virus 40; VSV: Vesicular stomatitis virus; WST-1: Water-soluble tetrazolium (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate).

targets that lead to mitotic arrest subsequently induced cell death. Of the 12 genes whose depletion caused aberrant cytokinesis, 10 also displayed mitotic arrest. Among the identified genes APC/C subunits CDC16 and CDC27 are already characterized ones along with receptor proteins, splicing factors, translational factors, and seven unknown targets. Interestingly, 23 genes were never related to cell division. The still limited knowledge on gene functions associated even with basic principles such as cell division was shown on the one hand and, on the other hand, the opportunity of identifying new disease-relevant targets, by systematically screening the genome in mammalian cells using the RNAi technology, was demonstrated.

Subsequently, the same group ran a genome-wide phenotypic primary screen applying a library of esiRNAs targeting nearly 18,000 genes and staining HeLa cells using propidium iodide 72 h after transfection [60]. DNA content analysis was performed by laser scanning cytometry. A total of 2,146 genes were identified that altered cell cycle progression or ploidy upon depletion by RNAi. Gene expression for the primary hits was verified by RT–PCR (polymerase chain reaction after reverse transcription of RNA). Next secondary esiRNA molecules that were non-overlapping with the first set were synthesized and 1,726 hits were subjected to secondary screening. DNA content analysis was repeated but further cell size was measured by flow cytometry and a mitotic index determined by microscopy. For 1,351 genes, nine-parameter fingerprints were determined. Results were compared with genome-wide screens in *C. elegans* and *Drosophila* and also with results of the siRNA screen in U2-OS cells, described below [61]. The 68 genes that played a role in mitosis or cytokinesis were further analyzed by video microscopy for higher resolution to describe more accurately the phenotypical alterations.

The mitotic index assay is widely used with three colors to stain the nucleus (DNA), phosphorylated histone H3 as a marker for mitosis, and actin to detect cytoplasm and cell shape. This assay exists in several variations and has been applied in various studies.

A small screen focused on the microtubulin-based motor proteins was performed to analyze the involvement of the motor proteins in mitosis and cytokinesis [62]. For all 12 hits downregulation of the correlating mRNA by the transfected esiRNAs was confirmed. Subsequently, four-marker immunofluorescent analysis, time-lapse microscopy and genetic rescue experiments were performed to confirm the hits and to determine mode-of-action of the hits.

On a far larger scale, also using the mitotic index assay, a lentiviral vector based shRNA library, consisting of ~ 5,000 shRNAs targeting 1,000 genes was screened by infecting the colon cancer cell line HT29 [63]. The library included constructs targeting nearly 500 kinases and 180 phosphatases, which represent primary target classes in modern drug discovery. The average mitotic index was determined at 5.1, which means that 5.1% of the total cells in the population were in mitosis and, therefore, phosphorylated H3 positive at the same time. With known controls, such as shRNAs targeting CDC2/CDK1 or CDK2, mitotic indexes of 9.7 or 35 were detected, respectively, whereas by targeting aurora B, a low mitotic index was induced. Assaying a different cell line confirmed the data. Further parameters such as changes in cell or nuclear morphology were measured, which allowed grouping of the targets whose depletion caused similar phenotypes. Subsequently, the phenotypes were confirmed in a second cell line, a cell cycle analysis was performed using FACS, and in parallel the induction of apoptosis was quantified.

A genome-scale functional screen for human cell cycle regulators was performed by automated single-cell fluorescent microscopy by simply analyzing DAPI (4,6-diamidino-2-phenylindole) stained nuclei, their DNA staining intensity and their nuclear shape [61]. For primary screening, the two siRNAs targeting the same gene were pooled and U2-OS cells were reverse transfected in duplicates in 384-well microtiter plates. At 10× magnification, 20 images per well were acquired and automated image analysis discriminated between cells in G1, S or G2/M cell cycle phases and several other parameters. For 57 selected genes, which were not described in the context of cell cycle function siRNAs yet, were resynthesized. All the pooled siRNAs reconfirmed the primary screening data. In the next step, individual siRNAs (five siRNAs/target) were tested against 24 of these genes, and for each of the 19 genes at least two individual siRNAs induced identical phenotypes. For 18 of the 19 genes, correlating mRNA depletion was verified by RT–PCR.

Oscillation of gene expression during the cell cycle is an important mechanism of regulation. Therefore, the functional data of the primary screen in U2-OS cells was compared with a periodic cell cycle mRNA expression data set of HeLa cells. In 44 genes it was identified that depletion caused cell cycle arrest in the same phase that they exhibited maximum expression.

Hits of the primary screen were clustered in eight phenotypic categories based on cell cycle phase, nuclear area and nuclear morphology. In particular, the group of G2/M arrest phenotypes correlated with severe cell loss, which might indicate that this group of genes contains good targets for developing anticancer drugs such as CDK1, Plk-1, Kif11, or aurora kinases. Hits were classified by protein function. Not surprising is the high prevalence of kinases and phosphatases, as cell cycle progression is regulated mainly by phosphorylation. Moreover, the data were mapped to a knowledge-based database. For example, the analysis of G1 genes identified receptor tyrosine kinase, ras-mediated and integrin signaling components activating the CDK4/6-cyclin/pRb/E2F-pathway. In one illustrated secondary assay [61] staining the cells with DAPI, α-tubulin and γ-tubulin defects in mitosis and cytokinesis were

visualized. The published material provides a rich pool for comparing own target discovery data and will assist in prioritizing new targets for cancer drug development.

For cell migration, a high-throughput assay was developed [10] and has been used in chemical screens [9]. This assay is also called a 'wound healing assay', as with 96- or 384-pin tool devices cells are scratched off an adherent monolayer and the closure of the wound can be easily detected by microscopy, and for large-scale screens automatically quantified by image analysis programs. A library of 11,000 individual siRNAs targeting 5,234 human genes was probed for genes promoting tumor cell migration in such an assay [64]. Transfection of the siRNA library into SKOV-3 cells was performed in duplicate in 384-well microtiter cell culture plates. After 48 h, ~ 3 mm long scratches were inserted into the confluent cell layer by pipette tips mounted on an automated 384-needle device. The plates were incubated again to allow wound closure for another 12 h. The duplicate plates were split and one set was assayed for cell viability. The other set was fixed and DAPI stained for automated microscopy at low magnification (4×) covering the whole scratched area on one image. Automated image analysis based on a qualitative migration scoring algorithm revealed 523 siRNAs that inhibited 'wound healing', which means that inhibited cell migration closes the gaps. Interestingly, both siRNAs per target showed identical phenotypes for only 22 genes. Owing to the significant potential for off-target effects, only those 22 genes were followed-up. The correlating siRNAs were resynthesized and tested again in the migration assay and one replicate was used for quantitative RT–PCR. For 17 genes, the phenotype was confirmed with both siRNAs but only for four genes did the RT–PCR data and the antimigratory phenotype match. Afterwards, migration was tested in four more cell lines, resulting in two genes validated in all cell lines, whereas the siRNAs against the two remaining genes were active only in two cell lines. For one gene, knock-down was demonstrated to inhibit invasion in a matrigel Boyden chamber assay.

2.2 High-content siRNA screening in intracellular trafficking and infection biology

Another basic cellular process that has been analyzed systematically by siRNA screens is endocytosis. The first published analysis [58] described a trend-setting screening strategy. For the primary screens easy, robust and low-cost yes/no-typed assays were applied. Two viruses, simian virus 40 (SV40) and vesicular stomatitis virus (VSV), were used, which hijack the two distinct clathrin- and caveolae/raft-mediated endocytic pathways. Cell nuclei were stained by DAPI whereas infected cells were marked by a second color and counted automatically. Three days after transfection, cells were infected at a low multiplicity of infection (0.1) that resulted in ~ 10% infected cells in the control wells. Approximately 3,000 cells were analyzed. For the secondary screens, more complex independent assays at a higher resolution and lower throughput were chosen that allowed mechanistic studies and dissection of the pathways. To keep the assay development times to a minimum, only two-colored assays were chosen but seven independent assays were developed. The viruses were replaced by other fluorescently labeled cargoes such as transferrin, LDL or the cholera toxin. Moreover, endosomal structures, such as early endosomes, late endosomes or caveosomes, were immunostained. Finally, a cell viability and apoptosis assay was added. Analysis of the images was done manually, and phenotypes were divided into classes by eye.

Approximately 200 kinases were discovered to be involved in endocytosis whereas roughly a quarter of the targets were poorly characterized or new [58]. Rescreening the whole kinome library in the transferrin secondary assay based on an independent test principle verified 72% (92/128) of the VSV primary hits playing a role in clathrin-mediated endocytosis. For selected hits of the SV40 primary screen, 87% (34/39) was confirmed to regulate caveolae trafficking. One of the most important results was that only a very few kinases regulated both pathways. The vast majority of kinases activated, or inhibited, one of the pathways specifically although a high fraction of the human kinome seemed to be involved in regulating endocytic processes; an impressive complex network of regulation was illustrated [58].

Related screens have been published for iron uptake through endocytosis [65], respiratory syncytial virus (RSV) infection [66], HIV infection [67], intracellular bacterial growth [68] and secretory protein transport from the endoplasmic reticulum to the cell surface [69]. Galvez *et al.* [65] surveyed the human signaling proteome for regulators that increase or decrease transferrin uptake and recycling of transferrin and its receptor. Fluorescent transferrin uptake was measured in duplicate 60 h after transfection and after 30 min of transferrin incubation. Primary hits were assayed again at a higher concentration of esiRNAs. Of the primary hits 154 (84%) were confirmed and showed similar or stronger phenotypes. New batches were synthesized, rescreened, and 80% of the hits were confirmed. A second set of esiRNAs generated against different sites of the target mRNAs were less successful, only 21 or 71 esiRNAs scored. It is not understood yet why the reconfirmation rate was so low. Off-target effects may be considered but should be not the main reason. For some of the 21 high confidence hits, synthetic siRNAs were purchased and particularly the pI3K-mTOR pathway was analyzed, which was identified as the primary signaling module controlling iron uptake.

In a mini-screen targeting 79 genes known to be involved in actin polymerization, cytoskeleton rearrangement, endocytosis and vesicle/cargo movement impact of protein knock-down on cellular infection by a GFP-recombinant respiratory syncytial virus was analyzed [66]. Surprisingly, clathrin-mediated endocytosis was discovered to be involved in RSV infection. To confirm the results, the pools of four siRNAs per target used for the primary screen were

deconvoluted and individual siRNAs were applied to the same assay. Genes targeted by two or more siRNAs resulting in a significant reduction in viral infection were considered to be real. No hit revealed four active siRNAs. For 5 hits, 3 out of 4 siRNAs and for 11 hits, 2 out of 4 siRNAs induced the phentotype scoring positive. The number of 1 out of 4 events was not mentioned. The second line of confirmation was performed by western blot analysis to verify protein depletion upon siRNA treatments. The expression of dominant-negative mutants confirmed the role of clathrin-mediated endocytosis in RSV infection.

Most recently, in a complex screening strategy, host targets were identified that are necessary for HIV infection and subsequent processes of the viral life cycle such as viral assembly and budding [67]. The first genome-wide screen using pooled siRNAs detected the HIV *gag* gene product p24 upon successful infection, whereas the supernatant of infected cells was tested in Tat-dependent reporter gene expression. Then the pools were deconvoluted and the four siRNAs per target were rescreened individually. Approximately 71% of the hits were confirmed with at least one siRNA per target to score.

Another application-oriented kinome-wide siRNA screen revealed regulators of intracellular bacterial growth, particularly of *Salmonella typhimurium* and *Mycobacterium tuberculosis* [68]. Cells were transfected with pooled siRNAs and later infected with luciferase or GFP expressing bacteria. Mean fluorescence per cell was measured to quantify intracellular growth of the bacteria.

Simpson *et al.* [69] focused on technology development rather than on publishing screening data. However, in a pilot screen of 92 siRNAs targeting 37 putative membrane traffic proteins, 7 were identified to be important for delivery of the secretion marker, a temperature sensitive form of the viral transmembrane protein tsO45G, to the cell surface. Automated image analysis was used to quantify and calculate the ratio of cell surface located versus cytosolic CFP-labeled secretion marker. A platform has been established consisting of a well-characterized assay, a solid-phase reverse transfection technology suitable for high-throughput screening [26,70], automated image acquisition and an image analysis software solution, to implement large-scale screening projects.

2.3 High-content siRNA screening in neurobiology

A conceptually well thought HCS project discovered genes required for synapse formation and maintenance [71], and a valuable screening and hit verification strategy was described Gene expression chip experiments were evaluated in which genes were up- or downregulated during synapse development. Of these, ~ 160 genes were selected and a focused esiRNA library was produced. Individual pools of esiRNAs were used for transfection, or two or three targets were silenced simultaneously after transfection into cultured hippocampal neurons. A three-colored assay was performed and synapse density determined by counting co-localization of synapsin I and PSD-95 dots present along the dendrites of GFP-expressing neurons. Very few hits were reproducible and subsequently verified by independent RNAi technology:

In a small-scale screen searching for genes involved in neurite outgrowth, 85 tyrosine kinases were analyzed by siRNAs [72]. Differentiation was induced in the neuronal cell line SH-SY5Y and an index for neurite outgrowth was calculated as a ratio of cell surface area to nuclear area after two-color image analysis. siRNAs against nine tyrosine kinases inhibited neurite extension compared with negative control siRNAs significantly and reproducibly.

A kinome-wide siRNA screen revealed new kinases involved in neurite outgrowth and retraction [73]. Human neuroblastoma SH-SY5Y cells were transfected with three siRNAs per target individually. After 48 h incubation, cells were stained with the neuronal-specific anti-βIII tubulin antibody, and the average neurite length and other parameters were determined using a commercial image analysis package. For 13 kinases, three siRNAs per target reduced neurite outgrowth, whereas for 15 other kinases only two siRNAs induced the phenotype. On-target effects of the siRNAs were determined by quantitative RT–PCR.

3. Conclusion

Sophisticated image acquisition and image analysis systems have been developed and proven in large-scale HCSs. Large molecular libraries of RNAi-inducing reagents are available. Consequently, several high-content siRNA screening projects have been published in this new discipline during the past 3 years. Extensive hit verification strategies have been followed to confirm and validate the primary derived hits. The major problems are the technical limitations of applying current siRNA design. Owing to the unspecific side effects of many molecules, in some of the published projects a remarkable decrease in the number of hits during the verification and validation process was observed. Therefore, siRNA technology has to be further improved and appropriate screening and hit verification strategies have to be developed.

4. Expert opinion

The merger of microscopy-based HCS and the gene silencing RNAi technology provides a new tool for target identification and validation. Automated imaging platforms have reached a stage of maturity that allows high-throughput applications. Automated image analysis is still lagging behind and has not reached the same sophistication as the imaging instruments. Particularly at high resolution, complex patterns and kinetic movements, automated image analysis is still in its infancy. Few of the numerous screening projects have been evaluated by eye rather than using an unbiased automated image analysis software. The major reasons are the very limited availability of trained image analysts and the high complexity of developing own algorithms. Even applying one of the more sophisticated image analysis software

solutions is not trivial and is therefore reserved to specialists rather than being managed by general research assistants or students who join the lab short-term. Moreover, the time required to develop an image analysis solution is frequently underestimated. The problem should be considered early on during any high-content assay development process.

A huge variety of assays in cell biology are published and are on the shelves in academic research groups. However, the vast majority is not robust enough for screening, and the transition of a bench-scale individual experiment from an academic research group into a screenable assay is a major challenge; success rates are sometimes disappointing. Nevertheless, more complex and physiologically more relevant assays are needed, not only to support the pharmaceutical R&D process completely from target discovery through to drug development but also to serve the academic research efforts to understand biological processes and their pathological degeneration in a systematic and more integral approach, as is the aim in all 'omics' research areas and systems biology. The major directions of current development are approaches to use primary and stem cells or organ systems. In addition, the hope is on kinetic assays that allow extraction of even more complex information and are a valuable tool to understand mode-of-action at temporal resolution. Again, image analysis will be the major hurdle.

Large collections of RNAi-inducing reagents, very diverse in their technology, are available. Consequently, a large number of siRNA screening projects have been published during recent years. Strikingly, siRNA molecules are obviously not as specific as was thought for a long time. Off-target effects have been described and the reasons for those unwanted activities have been discovered [74]. Although this knowledge has probably been incorporated in all modern siRNA design algorithms, and experimental consequences of these effects have been drawn, to lower the risk by, for example, minimizing siRNA concentration, it is still hard to find a second siRNA molecule. Although at low resolution, or low complexity, there is a relatively high chance of identifying two or more siRNAs per target that demonstrate phenotype induction, there remains a significant risk of reporting false positives particularly when analyzed end points can be affected by different mechanisms (e.g., apoptosis or proliferation). On the other hand, in high-resolution truly multi-parametric assays, it is harder to find two or more siRNAs exhibiting the same signature across the whole range of parameters, but there is confidence that the phenotype induction is correlated with the on-target effect of the siRNA molecules. Those observed limitations initiated a major worldwide discussion on keeping minimal standards and developing strategies to lower the risk, improve the success rate and, particularly, the quality and reliability of research results [30,75-77]. Pooling a limited number (three to four) of chemically synthesized siRNAs or using pools of hundreds of individual esiRNA molecules derived from enzymatic synthesis to reduce concentration of individual molecules might be one approach to reduce the risk. Chemical modifications of siRNAs or immediate screening with a high number of individual siRNA molecules (5 – 10 siRNAs/target) in parallel at low nanomolar or even subnanomolar concentrations are other valuable approaches. The siRNA technology and the techniques of transfecting cells with siRNAs have definitively to be improved further, and technological comparisons on a larger scale, and at sufficient resolution at the functional level that HCS provides, are required. Minimal standards for siRNA screening should be applied in all labs, otherwise the research 'market' will be flooded with masses of data no one can trust.

Given the imponderability of the technologies applied during primary screening, it is vital to prove and verify the hits. Verification of the target expression as well as its down-regulation on mRNA and/or protein level by the siRNA molecules is necessary. Functional verification has to be achieved by applying further independent siRNAs, other RNAi molecule types or classical antisense oligonucleotides, and assays of independent test principles or heterologous cell systems. Genetic rescue experiments using dominant-negative and/or RNAi-resistant target variants represent a convincing strategy to demonstrate specificity of the observed phenotypic alteration induced by the RNAi-inducing molecule.

In the end, RNAi is only one but at present the most dynamic and promising, technology in functional genomics and target validation. Other tools remain valid and will further support functional gene analysis. The identification of protein (or mRNA) expression in normal versus diseased tissue or individual cell types and the measurement of protein levels in clinical samples will further determine the relationship of a new target to progression of the disease. Functional antibodies, knockout or other transgenic mouse models will provide further insight on functions of the target. Although all these tools will support the progress during the validation process, only the identification of a modulator and its successful clinical application will finally prove the approach and validate the target.

Declaration of interest

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