DRUG DISCOVERY TODAY TARGETS

Biology calls the targets: combining RNAi and disease biology

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Target-based drug discovery starts with the identification of target genes and their respective protein products (associated with or controlling a disease-relevant phenotype) that, when inhibited or activated, ameliorate the associated disease. To identify disease-relevant genes, robust tools are needed to allow biology-driven target discovery and validation. Moreover, insight into the underlying biology of a disease is essential to model a disease *in vitro*. Key questions are: What are the disease hallmarks? What are, from a biological point of view, the best points for therapeutic intervention? How can scientists model these points *in vitro*? What is the desired target profile? The closer the cellular models resemble the disease situation, the better the target profile will be. The profile is the set of biological data needed to accept the target for drug discovery. In this review, a focused approach for target discovery and validation is presented. Arrayed adenoviral siRNA libraries and disease-based cellular models are used that generate high-quality and functionally validated targets.

Target discovery

Target-based drug discovery typically starts with the identification of genes and their respective proteins that are associated with, or control, a disease-relevant phenotype. When manipulated, they generate a window for therapeutic intervention. A prerequisite for success in the post-genomics era is the availability of robust tools that allow a target discovery and validation process based on disease-relevant, *in vitro*, cellular models. Heterotopic expression of wild-type or dominant-negative plasmids has provided some insights into biological functions of putative targets. Previously, this approach had limitations. Expression was usually poorly controlled and supraphysiological, creating the potential for expression artifacts. A generically applicable, robust gene-suppression tool that allows the knockdown of mammalian genes at an industrial scale (and at a genome-wide level) came with the discovery of RNA interference (RNAi).

RNAi

RNAi was first described in detail by Fire *et al.* in 1998 [1], beginning a new era in biology. In 2001, Tuschl and colleagues [2] identified double-stranded (ds) short interfering (si) RNA, 21–22 nucleotides in length, as the sequence-specific, active component of RNAi. This triggered the scientific and biotechnology communities to start developing RNAi tools for drug discovery research, including target discovery and validation, as well as siRNA-based therapeutics.

Expression of siRNA results in sequence-dependent degradation of the targeted mRNA and the subsequent reduction of endogenous levels of the encoded protein product. The overall decrease in protein level and activity is, among other variables, dependent on the level of RNA knockdown and the half-life of the protein. Before Tuschl and colleagues described siRNA in mammalian cells, RNAi was predominantly used for protein knockdown in *Drosophila* and

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Caenorhabditis elegans using dsRNA molecules 50–1000 base pairs in length [1,3]. In mammalian cells, long dsRNA was known to trigger an antiviral interferon response via the dimerization of dsRNA-dependent protein kinase (PKR) [4]. However, siRNAs do not necessarily elicit a full interferon response because such responses depend on the nature of the siRNA sequence used [5–7]. Research in the RNAi field has progressed at an impressive pace and the mechanistic understanding of the process has increased substantially. The site of cleavage on the mRNA strand in the siRNA–mRNA duplexed region has been determined and it was observed that limited mismatches, at specific positions in the siRNA–mRNA duplex, are tolerated [8–10]. The RNA-induced silencing complex (RISC), a well-conserved complex responsible for RNAi activity, has been characterized biochemically and multiple components of the RISC have been identified. Only one strand of the double-stranded siRNA duplex is incorporated into a functional siRNA–RISC complex, the other siRNA strand is degraded [11–14].

Naturally existing counterparts of siRNAs, small RNA species called micro RNAs (miRNAs), have been discovered. They play an important regulatory role in gene expression at the translational and transcriptional levels [15]. An exciting example of regulation by miRNAs is exemplified by *miR-375*, a miRNA (expressed in pancreatic islets) that regulates insulin secretion [16]. A better understanding of the mechanism of action of regulatory miRNAs will facilitate the development of more specific tools for elucidating biology through RNA regulation. Off-target effects of siRNAs can sometimes be explained by the undesired miRNA activity of a specific siRNA [17]. Therefore, improved understanding of the miRNA mechanism of action could also allow us to improve the design of siRNAs and to improve the design of experiments where siRNAs are used [18].

Design of siRNA sequences

Improvements in the design of functional siRNA sequences were important for further development of siRNA as a biological tool. Tuschl and colleagues [2] described the first rules for siRNA design. After extensive systematic analyses new rules were defined resulting in improved functional knockdown of designed sequences [19]. Important aspects of these rules are: the GC content of the siRNA should be 30–52%; at least three A or U nucleotides should fill positions 15–19 of the sense strand; specific nucleotides have specific positions (A at position 3, U at position 10, no G at position 13, no G at position 19, no C at position 19; A is preferred at position 19) [20]. These improved criteria enable scientists to design siRNAs with greater potency.

For siRNA duplexes (that are produced by chemical synthesis), off-target effects can be reduced by chemical modifications of the separate strands and by altering the relative stability of both termini of the siRNA duplex. Both techniques function by favouring the selective incorporation of the antisense strand into a RISC complex. As with any experiment, the choice of controls is important; scrambled controls, siRNAs against nonexpressed genes such as green fluorescent protein (GFP), or mutated siRNAs form suitable negative controls. Using these negative controls in addition to mock-transfected cells will provide sufficient information on nonspecific effects. It is currently not possible to completely exclude off-target effects by siRNAs or siRNAs that are produced by expression vectors. A good practical strategy is to generate data from multiple, independent siRNAs with different sequences, directed against the same target. Because it is very unlikely that different siRNAs against one specific target have the same off-target effect, this approach generates a high level of confidence that the observed biological effect is indeed caused by the suppression of the intended target. When dealing with general cellular phenotypes, such as cell growth or adhesion, this approach should be complemented with additional validation. This includes testing a dominant-negative mutant of the target or, if available, a small-molecule or antibody antagonist. The siRNA design algorithms use comprehensive BLAST analyses against the genome under investigation, to select specific sequences that have minimal or no chance of reacting with a different, unintended target (including the algorithm used for constructing an arrayed adenoviral siRNA library). For more general details on RNAi we refer to excellent reviews published in 2004 [21,22].

Delivery of RNAi to mammalian cells

Target-specific RNAi is introduced into mammalian cells by the transfection of synthetic siRNAs or by delivery through viral and/or non-viral expression vectors. When using vectors for delivery, a precursor in the form of a short hairpin RNA (shRNA) is expressed. After trimming the shRNA by Dicer, the functional equivalent of a siRNA is produced. The delivery method is one of the key challenges for using siRNA, especially the delivery to primary human cells. The development of viral delivery vectors has enabled a more efficient delivery of siRNA, into a wider spectrum of relevant cells, than was possible with transfection technologies. The benefits of using viral delivery tools are that delivery is robust, efficient and provides long-term expression (as a result of continuous expression from the shRNA expression cassette). Several groups have built siRNA libraries (viral or non-viral) to elucidate functions of genes in various biological processes. Several libraries have been constructed that focus on the drugable genome or that are genome-wide (both in synthetic and viral formats). The key to success is to combine any library with a biologically relevant readout in a cell type that is as close to the human physiology and pathology as possible. Rene Bernards and colleagues [23] at the Netherlands Cancer Institute have performed an oncology screen in a human cell line that identified novel components of the p53 pathway. For screens related to diseases other than cancer, the use of primary human cells is

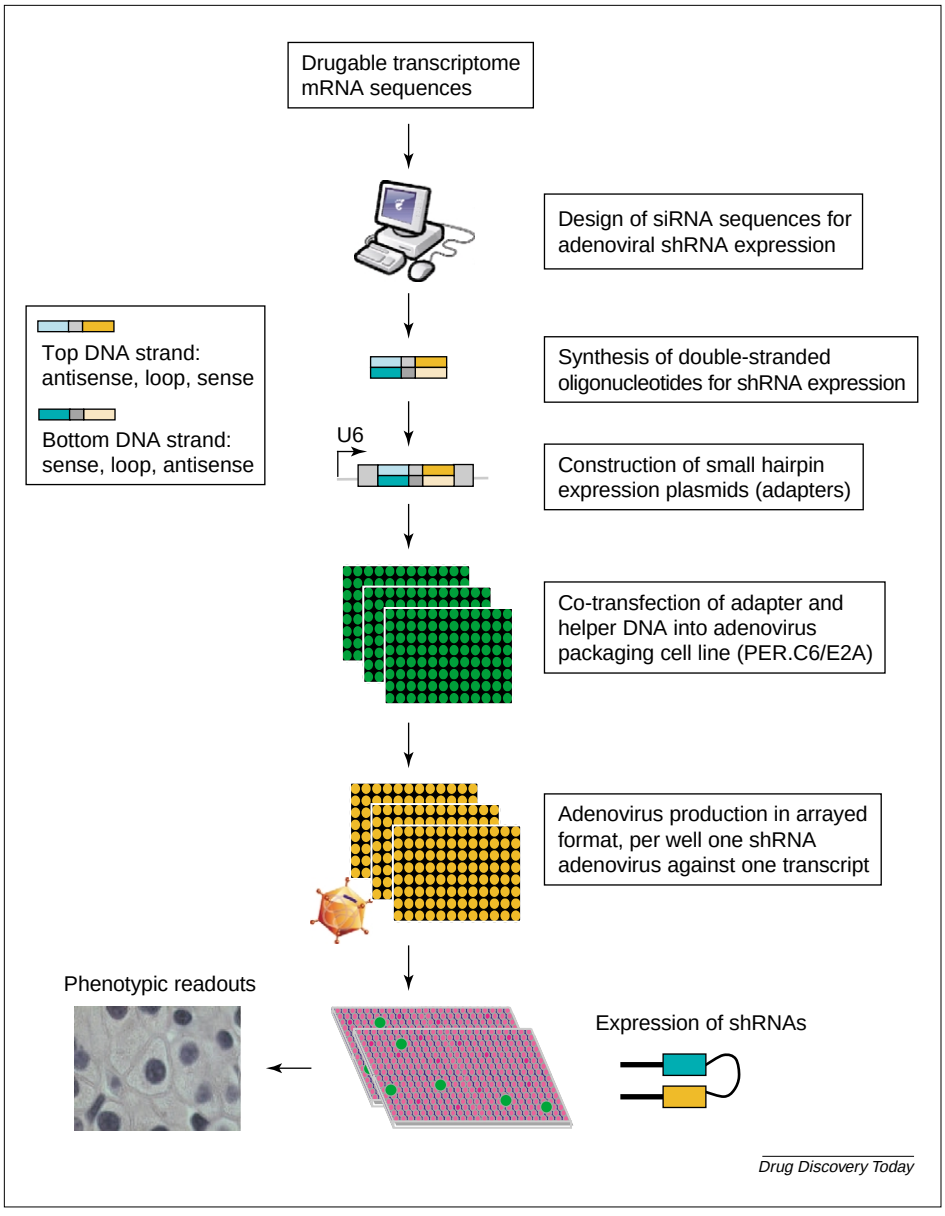

FIGURE 1

Construction of arrayed adenoviral siRNA library. Algorithms were used to design siRNA sequences directed against the drugable transcriptome. This sequence information is used to synthesize double-stranded DNA oligonucleotides that, when expressed upon adenoviral delivery, yield a small hairpin RNA (shRNA) that is processed to become a siRNA against a transcript. The whole process was scaled up using 96-well plates to allow the production of a library against the drugable genome encompassing ~4700 transcripts. For each transcript three independent adenoviral siRNA vectors were made. The total resulting adenoviral library consists of ~12,000 individual adenoviruses.

preferred to transformed cell lines. Adenoviral gene delivery is an excellent method to deliver siRNA to a variety of primary human cells and cell lines. We have used this robust delivery technology in our efforts to search for novel targets against major human diseases, in conjunction with high-throughput biology.

The arrayed adenoviral siRNA library, SilenceSelect™

Currently, when using RNAi for target discovery and validation, researchers use arrayed collections of synthetic siRNAs (either arrayed individually or as siRNA pools against the same transcript) that are transfected into the appropriate cellular models and assayed. Lentiviral shRNA collections are alternative, popular, viral siRNA delivery formats, successfully used either as pools or as individual arrays [24–26]. Based on the arrayed adenoviral cDNA knockin or gain-of-function platform [27], we built an arrayed adenoviral siRNA platform for biology-driven target discovery and validation called SilenceSelect™. The library consists of individual adenoviral siRNA vectors directed against one transcript and arrayed in individual wells. Finding a functional effect with an individual siRNA vector allows immediate identification of the target that is inhibited. Our adenovirus technology is based on the PER.C6 cell line that allows production of adenoviral vectors without any replication-competent or wild-type adenovirus contamination, an essential feature for large-scale applications in target discovery and validation [28]. Using PER.C6 allows safe, robust production of arrayed adenoviral libraries in microtitre plates. They carry shRNA expression constructs that, when expressed upon delivery into a cell, are efficiently processed into a siRNA targeting a specific mRNA [29].

The process of constructing an arrayed adenoviral siRNA library starts with the design of 19mer siRNA sequences against the genome of interest. This is followed by synthesizing oligonucleotides that are cloned into a U6-based shRNA expression cassette with an adenoviral adaptor plasmid in place of E1, an early set of genes essential for adenoviral replication (Figure 1). The adaptor plasmid is co-transfected into the PER.C6 packaging cell line [28] together with an E2A-deleted helper plasmid. PER.C6/E2A is a PER.C6 derivative and complements for E1 and E2A. E2A encodes a protein that controls expression of the late structural adenoviral genes. Efficient homologous recombination between adaptor and helper plasmids leads to a replication-competent DNA molecule and production of recombinant adenoviral particles. The resulting adenoviruses are replication-incompetent and can be used safely in exploratory biology programmes. The production of adenoviral particles by PER.C6 leads to cell lysis and the subsequent release of recombinant adenoviral particles with an average viral particle titre of 5×10^9 viral particles per ml. This unpurified format of arrayed adenoviruses is then used in target discovery and validation programmes.

Focus on the drugable genome

The use of RNAi within the pharma and biotech research communities is focused on the tractable target classes of the genome, usually those classes that are drugable through small molecule agonists or antagonists [30]. Whole-genome RNAi libraries are most commonly used in the academic research communities. Because Galádeno and its partners are focused on finding targets that can be progressed towards drugs, the arrayed adenoviral siRNA library is directed against the small-molecule drugable genome. The drugable genome consists of ~4700 transcripts corresponding to ~3700 gene loci. Every transcript is represented in the library by three adenoviral siRNAs directed against it to increase the chances for target suppression and to allow for multiple constructs to confirm target activity during screening. This arrayed adenoviral siRNA library forms the basic platform for our biology-driven discovery and validation of drug targets.

Starting a target discovery programme

Target discovery programmes have been built around specific therapeutic areas such as rheumatoid arthritis, osteoarthritis, asthma, osteoporosis and Alzheimer's disease. Other disease areas, such as cardiovascular disease, have been successfully addressed in partnerships with pharmaceutical companies. The design of cellular assays that model a disease appropriately is an obvious and essential element in setting up a disease-based target discovery and validation programme. A typical assay-development project starts by identifying a point of attack for a disease and involves a detailed analysis of the therapeutic possibilities and goals. It is crucial to identify the appropriate cell type(s) to use and a disease-relevant readout. One of our first experimental proofs of concept was obtained in a cellular model of asthma. Upon activation, primary human mast cells produce cytokines such as interleukin-13, a cytokine associated with asthma. A second example involves using activated synovial fibroblasts from patients with rheumatoid arthritis that secrete inflammatory cytokines and joint-degrading proteases. These two examples show phenotypes that significantly contribute to disease progression and can be designated 'bad' phenotypes. For these diseases the strategy is to develop molecules that inhibit cytokine secretion by activated mast cells or that inhibit inflammatory or matrix-degrading activities (secreted by rheumatoid arthritis synovial fibroblasts) to achieve a therapeutic benefit (i.e. a negative screen). The platform has also been used for the identification of targets that, when inhibited, stimulate a desired activity to achieve their therapeutic benefit (i.e. a positive screen). This was achieved for osteoporosis and osteoarthritis, where we screened for siRNA-mediated stimulation of anabolic pathways [31]. In the osteoporosis screen the objective was to identify targets that, when inhibited, would stimulate osteoblast differentiation towards osteoblasts and osteocytes, resulting in a net increase of bone synthesis and of bone mineral density. In the case of osteoarthritis we used collagen II, a marker for cartilage deposition and dedifferentiated human chondrocytes as the cell type of choice. We have searched for genes that, when inhibited, reverse the dedifferentiated phenotype of the chondrocytes back to cells that can deposit cartilage again.

It is worth mentioning cancer drug target discovery separately because it is often more challenging to find specific targets. One reason for this is that in oncology discovery programmes the cellular assays of choice are often based on general phenotypes, such as cell proliferation and apoptosis, and are run in tumour-derived cell lines. In such cases selectivity will often be too low and will not yield workable numbers of targets to take further. One way to avoid this is to screen multiple, preferably primary, tumour cells for inhibition of multiple, relevant phenotypes such as cell proliferation and apoptosis (preferably in a high-content setting to increase throughput). Selectivity can be increased by validating with more selective screens (often low throughput) such as anchorage-independence assays or reporter assays that allow scientists to link targets to known pathways (e.g. known tumour suppressor pathways). This approach allows the selection of broad-acting, non-cell-line specific targets applicable to a wide range of cancers or the selection of specific targets that are limited to certain types of cancers.

Not all diseases can be captured into an *in vitro* cellular assay model. For example, good cellular assays that provide a direct link to the disease are difficult to design for certain central nervous system (CNS) disorders, such as schizophrenia, and could make a programme difficult or impossible to set up, unless one settles for the second best choice of phenotypes. This increases the risk of identifying targets that are not relevant. In addition, including an *in vivo* target-validation model early in the target-validation process might be needed, resulting in a bias as the throughput of *in vivo* target validation is limited and researchers are not able to select targets based on disease-specific *in vitro* selection criteria.

Phenotypic readouts for screening an arrayed format

Assays compatible with the arrayed adenoviral platform are diverse and can be based on reporter- and fluorescent-based readouts, proliferation, differentiation, image-based assays using fluorescent dyes, immunochemical assays and/or multiplexed assays. The standard plate formats used are 96- and 384-wells, providing sufficient throughput for screening ~12,000 individual adenoviral siRNAs. The arrayed format and the nature of the adenoviral vector allows for a large number of human disease-relevant screens based on complex phenotypes or insensitive readouts. These phenotypic readouts are generally not possible when working with non-arrayed library formats. It is beyond the scope of this review to provide a complete overview but examples of array applications include

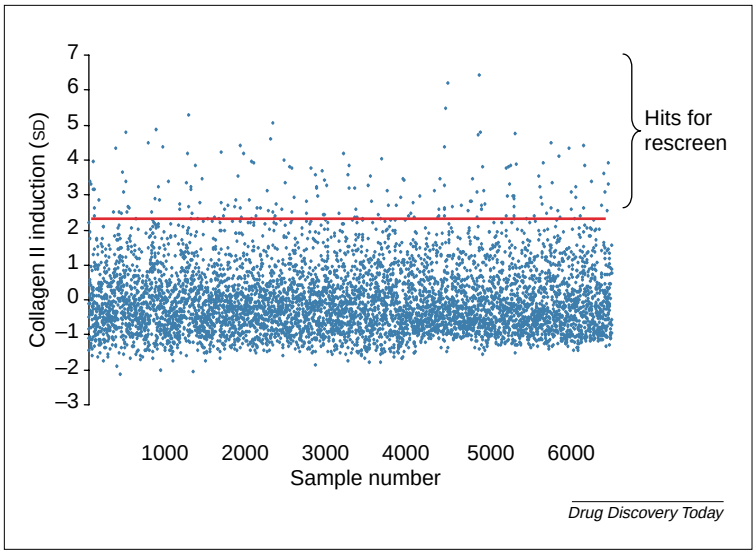

FIGURE 2

An example of primary adenoviral siRNA screening data. Primary human chondrocytes were seeded in 384-well plates, infected with the library. The expression of collagen II was measured by ELISA. The data were normalized to the average of all plates and hits were selected above a cut-off (red line) of 2.5 times the standard deviation (SD). All hits above this cut-off were selected for rescreening.

in vitro capillary formation from embedded endothelial cells (cancer, cardiovascular disease), differentiation of keratinocytes (psoriasis), adipocyte differentiation by measuring lipid droplet formation or adipokines (metabolic syndrome), cytokine production by airway smooth muscle cells (respiratory disease), mucus production by lung epithelial cells (respiratory disease), VCAM-1 expression in activated endothelial cells (inflammation) and neurotransmitter expression by neuronal cells (CNS disorders). The primary screening assays are automated and the process of screening is supported by an integrated laboratory information management system to ensure seamless project management, from project concept to target output. Using this platform, it is possible to screen and rescreen the arrayed adenoviral siRNA library in complex cellular assays in just three months.

Once an assay, its readout(s) and cell type have been chosen, the assay development phase can begin. In this phase, the conditions for adenoviral delivery are optimized using a marker gene assessing transfection efficiency, measuring the effect of adenoviral delivery on the cellular readout and the overall cellular viability. When available, adenoviral siRNAs against positive control genes are made and tested. All this is done at a range of virus to cell ratios or multiplicity of adenoviral infections (MOI) to identify the maximum dose possible without affecting cellular viability, basal readout levels (negative controls) and the maximal window for inhibition of activation of a disease phenotype, culminating in a robust screening protocol. During assay development one might find out that the actual window and robustness of the assays, as assessed with a control adenoviral siRNA, is unsatisfactory, which can result in abandoning the assay in question. A pilot screen is shown, in which a set of 1000 adenoviral siRNAs from the SilenceSelect™ library are tested to assess how the assay reacts to library viruses that cause a certain level of noise (Figure 2). This is caused by the slight variation in the functional MOI of the library-produced viruses. This noise level is typically within an acceptable range to allow identification of primary hits. Following the pilot screen the complete library is screened twice and hits are identified using a cut-off of the overall average ± 2–3 times the standard deviation. Depending on the quality of the assay, a lower or higher cut-off value is chosen to generate a hit rate of ~3–6%.

A critical path towards validated targets

Our focused approach towards biology-driven target discovery and validation is designed along a path of target acceptance criteria (Figure 3). A primary screen is followed by a series of secondary cellular assays to select targets with high disease relevance and to increase the biological confidence in the targets. The primary screen needs to ask the right biological question to avoid using cellular readouts that are too generic and that require extensive deconvolution of hits. The secondary assays can be high or low throughput and, like the primary screening assay, mostly employ primary tissue cultures to avoid physiologically irrelevant results when using cell lines. All target discovery and validation programmes include additional, generic target-validation steps. These involve testing tissue or cells from multiple donors to exclude donor-specific results and to verify that the target is indeed endogenously expressed in human tissue to exclude effects introduced by cell culture. To confirm on-target activity we design additional adenoviral siRNA constructs for those cases where only a single adenoviral siRNA has been identified in the primary screen. To date, 80–90% of the targets that are subjected to this kind of analysis have yielded two or more additional, positive siRNA constructs thus confirming on-target activity. This high percentage is perhaps not surprising as the probability to be off target after having gone through several selective biological assays is remote. Alternatives to this approach are cDNA add-back studies, preferably with silent mutations in the siRNA target sequence, before infection or delivery of a dominant-negative mutant cDNA of the identified target instead of additional adenoviral siRNAs [32]. Finally, confirmation that the target's mRNA or protein levels are suppressed and coincide with the inhibition of the various biological readouts is direct evidence for target specificity.

The final output of fully *in vitro* validated targets is determined and, for the most part, driven by biology to ensure that researchers end up with targets that answer all the biological questions asked during target discovery and validation. By contrast to expression profiling studies [e.g. microarrays, serial analysis of gene expression (SAGE), proteomics-based strategies] as the starting point for discovery, target discovery and validation using RNAi

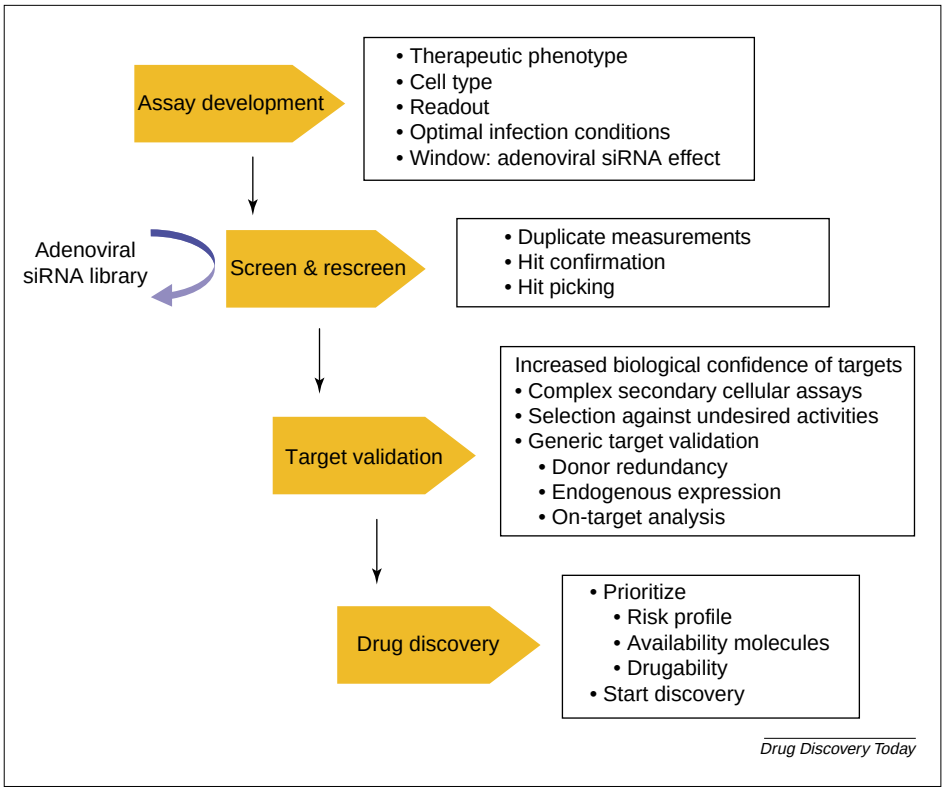

FIGURE 3

Critical path of target validation. Adenoviral siRNA hits that induce a desired biological effect upon screening are validated with a focused set of secondary cellular assays, specific for the disease under investigation, as well as generic assays including expression of targets in human tissue and cells, ensuring that the observed phenotypic effects are on target.

libraries is biology driven. We, therefore, avoid ending up with biased sets of targets that are merely associated with a disease and that call for significant biological validation. Depending on how selective the critical path of cellular assays is, attrition is not always high enough and final numbers range from 10 to 50 targets. Therefore, the targets need further prioritization. In this phase, ranking takes place along the lines of chemical tractability, the availability of agonists or antagonists for a particular target, endogenous expression in normal and disease tissues, and phenotypes in mouse transgenics and knockouts. These results are analyzed and a prioritized list of targets is the result, forming the basis of target selection for drug discovery.

In individual cases, additional experiments are performed to make a better judgment on a particular target or set of targets. For instance, if a specific antibody or small molecule is available against a target of interest, it is tested in our cellular models. In some cases, an existing inhibitor of a target can be taken directly into an animal model. Another example of additional validation is performing an informative pathway analysis using genes known to control the biology under scrutiny, providing links with existing pathway data and thereby increasing the level of confidence in a target. For example, expression levels and/or activities of known components can be measured in cells where the identified targets are knocked down. Another informative analysis is taking all targets identified in a programme and analyzing whether these are in the same or in parallel pathways by measuring their expression upon knockdown of each individual target. This allows linking the identified targets and visualizing a pathway.

Following the approach outlined above, we have run seven programmes in six different diseases so far. All yielded novel targets (covered by different drugable gene classes) as well as targets that were known from previous work by other researchers, validated either *in vitro* or *in vivo*. The identification of known components in a particular aspect of biology is important as it validates every specific approach.

Concluding remarks

Our approach has been applied to a range of diseases as described previously. The approach can be expanded to other types of targets and, therefore, drug discovery related questions. These include elucidating mechanisms of action of compounds when the pathway is unknown. For example, screening the adenoviral siRNA library in the presence of sub-optimal concentrations of an antagonist can identify targets linked to the pathways in which the compound is acting. The additive or synergistic effect of a siRNA will allow the identification of pathways linked to the drug, including parallel pathways. The applications of the target identification process could be broadened by adding classes other than the small-molecule drugable targets such as a full complement of the targets that are amenable to antibody therapeutics. The target discovery approach described here will also lend itself to the newly emerging commercial entities that are beginning to explore siRNA as direct therapeutics (either in viral format or as synthetic RNAs). Another possibility is to further increase the relevance of the cellular models and more closely mimic the complex situation *in vivo*. One can create genetic knockdown of specific targets and/or pathways to act as presensitizing mutations that could help to mimic the pathobiology seen in several diseases. In many diseases multiple cells and tissues interact and play a role in modulating the disease phenotype. This is the case in the joints of a patient with rheumatoid arthritis or the dermis and epidermis of a psoriatic patient. One challenge is to build multicellular systems wherein one or more components of the cellular interactions are functionally modified to elicit a process that is more similar to the disease state.

The biology-driven target discovery and validation described here has generated targets that are biologically validated and that hold great promise for future drug discovery programmes. It could indeed decrease overall attrition in the drug discovery process. In fact, several targets have been accepted for small molecule screening underscoring the confidence in targets that were identified through the biology-driven approach described in this review.

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