Emerging high-throughput drug target validation technologies

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Identifying the right target for drug development is a critical bottleneck in the pharmaceutical and biotech industries. The genomics revolution has shifted the problem from a scarcity of targets to a surplus of putative drug targets. As the validity of a target cannot be simply inferred from correlative data, the key is confirmation of the causative role of a gene product in a particular disease. It should therefore be recognized that an effective therapeutic strategy requires an appropriate target validation technology to verify the right target.

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▼ Completion of the draft sequence of the human genome marked the beginning of the post-genomic era, and it is clear that the next steps involve the determination of gene- and, ultimately, protein-function. As drug development moves from being chemistry-driven to biology-driven, the key is understanding the biological function of the target. A recent study estimates that target validation currently represents approx. one-fifth of the cost and time involved in drug discovery and development [1]. Target validation represents a current bottleneck in the pharmaceutical industry; choosing the appropriate drug target early in the process provides a competitive advantage.

In this review, target validation is defined as confirmation of the functional or causative role of a gene product in disease and/or normal physiology of the organism, as measured by a relevant assay. The technologies described in this review are limited to those amenable to high-throughput formats in eukaryotic systems, including cell-based systems.

From genes to phenotype

Advances in molecular biology have created a clearer picture of how phenotype is determined (Fig. 1). A plethora of gene products can be made from only a few sets of genes via transcript splicing and post-translational modifications. Additional diversity is provided by the multi-functionality of proteins [2].

Different stages in the pathway can be targeted for development of the appropriate class of therapeutics, and requires the appropriate target validation technology (Fig. 2). For example, an antisense drug will be appropriate if the resulting phenotype from an antisense approach is the desired effect of the drug. Conversely, validating the function of a target at the protein level would be required for the development of therapeutics against a protein drug target.

Target validation strategies

The 'target-driven' approach starts from pre-characterized targets or correlative data providing circumstantial association with disease (Fig. 3a). The second strategy, or the 'discovery-driven' approach (Fig. 3b), resembles the classical genetic approach of finding genes directly relevant to function [3]; this has also been called 'inverse genomics' [4].

Target validation requires the following: (1) an inactivation or activation method, (2) a cellular system or small model-organism, and (3) a biological assay or readout that is relevant to and predictive of the disease. Although assays are the key drivers, they are often limited in throughput; however, recent advances are addressing these issues [5,6].

Genome-based target validation

The following technologies take advantage of the knowledge of the genome and the availability of cDNA clones. The general strategy is a 'gain-of-function' approach by overexpressing the genes and observing phenotypic changes attributed to the overexpression.

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Functional cloning with viruses

Viruses provide versatility in their ability to deliver genes efficiently in cell-culture and animal models, enabling thousands of genes to be delivered and screened. Retroviruses have been the workhorse for this approach and have been successfully used in the functional validation of several genes. However, in this technique, recovery of the target gene is difficult because of the use of replication-defective variants [7,8]. Viral vectors based on the Sindbis virus provide an efficient, rapid and alternative way of identifying poorly expressed genes [9]. The virus has wide host-range and a positive-strand RNA genome making it amenable for expression in mammalian cells. In addition, herpes simplex virus is another promising alternative and can facilitate the delivery of genes in difficult cell systems such as neurons and dendritic cells [10].

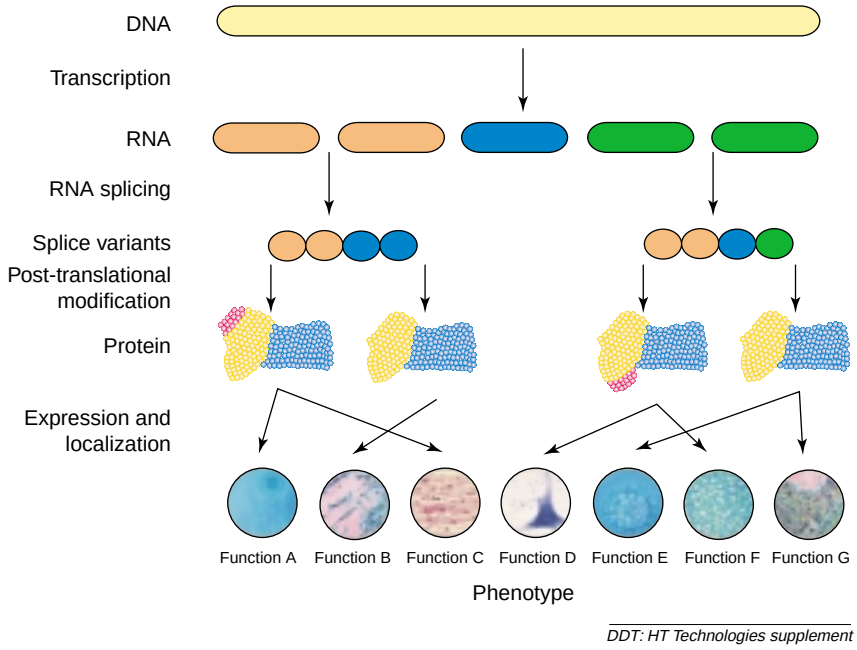

Figure 1. Functional complexity from a single coding element; the conversion of genetic information into a biological phenotype. One gene can encode for a plethora of different gene products generated by alternative splicing of gene transcripts and post-translational modifications. Further functional diversity is generated by protein-complex formation, and proteins that exhibit different functions depending on their cellular localization or the availability of a co-factor.

Functional cloning with the aid of robots

An interesting development that relies heavily on the automated transient transfection of individual cDNAs has been described [11]. A crucial component of the technology is a method for high-throughput preparation of vectors containing cDNA clones from bacteria to ensure nearly complete removal of lipopolysaccharide (LPS), thereby avoiding cytotoxic effects in the cell culture during transformation [12]. This approach has been successfully used to screen for apoptosis-inducing genes with a thoughput of 2000 transfections per hour.

One limitation of the technique is that the induced phenotype might not be replicated when using an antibody or small-molecule drug because these molecules act on the target via different mechanisms. Furthermore, the expressed protein must be fully characterized to ensure that other gene products or splice variants are not being expressed. However, results from rapid gain-of-function validation can be correlated with gene therapy or protein therapeutic strategies.

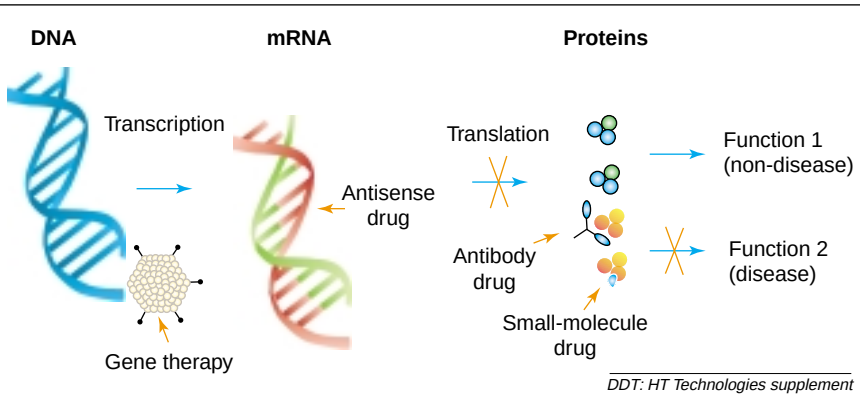

Figure 2. A good therapeutic strategy needs an appropriate target validation technology. The various steps in the gene-to-phenotype pathway provide different points for therapeutic intervention. In gene therapy approaches, viruses can be used to deliver genes into cells, and the phenotypic change attributed to the overexpression of a gene can be monitored. For antisense drugs, target validation by inactivation of a gene transcript is appropriate when the resulting phenotype is the desired effect of a drug. For the development of small-molecule and antibody therapeutics, however, it is necessary to validate the function of the target directly at the protein level.

Transcript-based target validation

Early approaches for target validation have been focused on transcript inactivation, and recent developments in the field, especially with the discovery of RNA interference (RNAi),

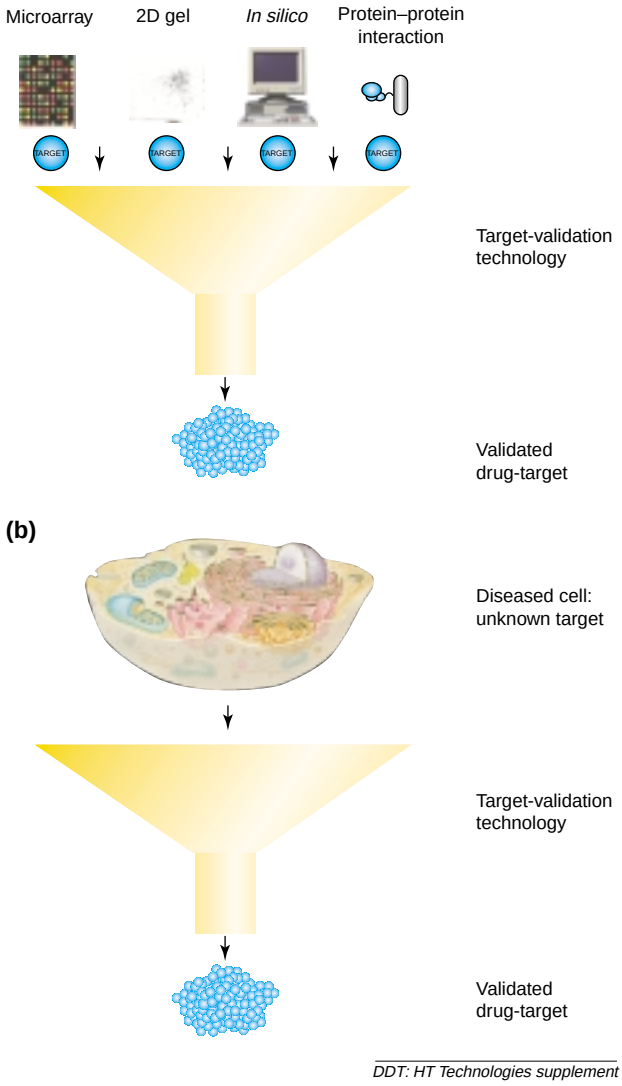

Figure 3. (a) Target-driven functional validation. Functional genomics technologies, such as differential display, expression proteomics, protein–protein interaction and *in silico* predictions, provide correlative data as to the relationship of a gene or protein to a particular physiological state. However, the causative role can be established by functional inactivation of the protein and monitoring of the resulting phenotype with a set of relevant biological assays. (b) Discovery-driven functional validation. This approach does not require previous knowledge of a target but is driven by the relevance of a set of functional assays for the disease of interest. Here, one starts with an induced phenotype, and continues with subsequent deconvolution of the genes or proteins responsible for the observed change in functionality.

reiterates mRNA as a favorite target for gene-function elucidation. The sequenced genomes of humans and several small model organisms have provided a framework for genome-wide efforts to design transcript-based inactivation.

Ribozymes

Ribozymes or catalytic RNA molecules have been used successfully to knock-down intracellular expression of a variety of viral and cellular targets. In the target-driven approach, a hairpin ribozyme binds to and cleaves a particular substrate; the nucleotide bases of its binding arms are created so as to be complementary to the 5′ and 3′ ends of a target sequence in the desired substrate RNA. The ribozyme is delivered to cells or expressed from a vector and the phenotypic change is scored.

In the discovery-driven approach, the nucleotide bases of the binding arms of a hairpin ribozyme are randomized, creating a library of thousands of ribozymes that can be selected for functionality [13,14]. The library is delivered as genes cloned in a viral vector for selection. After one round of selection, the selected ribozymes can be rescued and re-introduced into fresh cells to enrich for the desired ribozyme that is associated with a particular phenotype. Identification of the target is based on binding sequence complementarity with the selected ribozyme, followed by database query. The sequences are also used to clone the target gene, which is followed by targeting of additional sequences for ribozyme inactivation. This approach has been successfully used to identify a negative dominant regulator of BRCA1 [3], a gene involved in anchorage-independent cell growth [13], genes involved in TNF-α-mediated apoptosis [14], and a suppressor of fibroblast transformation [15].

Antisense

Antisense technologies involve oligonucleotides and modifications of oligonucleotides that are complementary to a portion of a target mRNA molecule. They are chemically modified to enhance entry into cells, increase resistance to nucleases, and enhance affinity for the complementary RNA strand, among other desirable properties [16,17]. Antisense molecules inhibit the ability of the mRNA to be read by translational or splicing machines, and so synthesis of the encoded proteins is blocked. Another mechanism involves the selective degradation of the hybrid molecule by an RNAase-H-dependent mechanism. Antisense molecules are widely used in the inactivation of transcripts in tissue culture and whole-animal models. A limitation of the technology is the lack of temporal resolution, although this could be overcome by a new generation of antisense molecules that can be activated by light [18]. However, even if an effect is observed in an animal model, it could still be necessary to modify the nucleotide for humans owing to possible sequence differences between the genes.

RNA interference

RNA interference is currently a popular method for target validation. It uses double-stranded RNA (dsRNA) rather than single-stranded antisense RNA as the interfering agent. In

the current model of RNAi action, dsRNA is introduced into the cell and digested into ~20-nucleotide short interfering RNAs (siRNAs) that are replicated by an RNA-dependent RNA polymerase. These bind to a nuclease complex to form the RNA-induced silencing complex (RISC), which subsequently cleaves the target mRNA.

RNA interference has been successfully applied in gene silencing experiments using nematodes and flies [19]. In nematodes, it is possible to perform 4000 RNAi experiments per week, and a complete genome can be functionally validated within five weeks. However, RNAi has shown a degree of nonspecificity in zebrafish knock-down experiments [20]. The initial success of RNAi in mammalian cells using shorter, synthetic, duplex RNAs (~20 base pairs) delivered by lipofection, led to excitement because it circumvented the stimulated generalized cellular responses through activation of dsRNA-dependent protein kinases [21]. Recent progress has been made in making the system work in mammalian cells with both the development of new vector systems that direct the stable expression of siRNAs [22], and the use of a human U6 small nuclear RNA (snRNA) promoter to drive nuclear expression of a single RNA transcript [23].

Transcript-based target validation technologies suppress the complete translation of a protein. The complete absence of a protein can have unexpected and undesirable physiological consequences because proteins often consist of multifunctional domains. Thus, results generated by these technologies cannot be simply extrapolated for the development of small-molecule or antibody therapeutics, which target only a specific structural and/or functional domain of a protein, while keeping the rest of the molecule functional. Moreover, if the target (e.g. a membrane protein) has a low turnover and is already expressed, an effect might not be observed. Although it is possible to wait for the complete turnover of the protein, once a shortage of a particular gene product is sensed, the cell might activate compensatory mechanisms, which are not readily detectable.

Transcript-based validation technologies are developing at a rapid rate owing to the availability of reagents and easy access to the technologies. Although there are certain problems surrounding the stability of antisense, RNAi, ribozymes, and their delivery into cells, these issues are being addressed. Recent positive results in late clinical trials have also renewed interest in antisense as a therapeutic. Currently, there is only one antisense anti-viral drug on the market [24]. In the future, the clinical success of antisense therapeutics will validate the approaches mentioned previously that target mRNA, and will lead to the rapid development of better antisense drugs.

Protein-based target validation

Proteins represent >90% of targets addressed by drugs on the market today [25], yet target validation at the protein level has not been as popular. With the number of proteins being orders-of-magnitude greater than the number of genes, the demand for protein-directed target validation is expected to accelerate in the near future.

Antibodies and intrabodies

Antibodies have been a rich source of specific agents for localizing proteins in cells and tissue by immunohistochemistry, and have a robust neutralizing ability to block protein function. Generation of antibodies has been significantly accelerated with the use of phage-display technology. For intracellular targets, antibodies must be delivered directly into the cells; this has been achieved primarly by micro-injection because cells do not readily take-up antibodies. However, micro-injection is not a high-throughput method, and new chemical reagents are being developed that efficiently deliver antibodies into the cell, thereby maintaining antibody functionality and cell viability [26].

Another approach is to develop antibodies that are expressed inside the cell, that is, intrabodies. This has been successfully demonstrated in several cases, including inactivation of viral infection. As antibodies are inherently unstable, new antibody frameworks are being engineered that are suitable for expression inside the highly reducing environment of the cytosol [27] (this environment is not suitable for the formation of disulfide bonds, which are required for the structural stability of antibodies). However, high-throughput applications could require the selection of an antibody by *in vivo* methods, such as the yeast two-hybrid approach. An additional limiting factor for the use of antibodies in target validation is the small fraction of neutralizing antibodies that are selected as being target-specific [28].

Aptamers

An alternative approach to the use of antibodies is aptamers: short oligonucleotides that can bind to a target with high-affinity and -specificity owing to their particular 3D structure. The generation of specific aptamers by SELEX (systematic evolution of ligands by exponential enrichment) [29] has been automated and is capable of generating large amounts of aptamer binders in just a few days [30]. However, aptamers preferentially bind to deep clefts on the protein surface, which limits their general application as functional inhibitors (e.g. of protein–protein interactions). Furthermore, the overall negative charge of nucleic acid aptamers might preclude selection of binders against negatively charged protein domains.

Aptamers have been successfully used to inhibit extracellular protein function and as 'intramers' to inactivate intracellular proteins [31,32]. Conceptually, in a discovery-driven approach, a viral vector or any genome-based delivery method can be used to deliver a library of aptamers to select for a particular

phenotype. The corresponding aptamer can then be deconvoluted from the sequence once recovered from the gene-delivery vehicle.

Peptides

Peptides represent another class of molecules that are used either as competitive inhibitors or to prevent target proteins from interacting with their protein partners in a cellular context. This can be rapidly achieved by selecting peptides that bind to target proteins either by phage display [33] or yeast two-hybrid technologies [34], or by using retroviral combinatorial peptide-libraries and screening them in appropriate functional assays [35]. Peptides are presented in a scaffold to facilitate the selection of more stable structures with higher affinity. In the discovery-driven approach, if a change in behavior of a specific cell occurs after transduction of the peptide library, it indicates that an interaction with a cellular target has been modified by a particular peptide, thereby implying a role in the disease of interest. Such an approach has been used for the selection of peptide motifs that confer resistance to Taxol-induced cell death, and was followed by the identification of the cellular target using a yeast two-hybrid approach [35]. Although this technology enables the direct selection of peptide leads, the identification of the actual target is technically difficult owing to both the poor affinity and, more importantly, the poor specificity of many peptides. In addition, it does not preclude the possibility that the peptide mimic is acting on several targets to result in the observed phenotype.

Chemical genetics

The concept of chemical genetics is based on the use of small molecules (rather than genetic mutations) to modulate protein function rapidly and conditionally, and to further elucidate biological function [36]. In a discovery-driven approach, phenotypic screens of structurally diverse and complex small-molecule libraries can provide individual probes of biological function, as well as information about the interaction properties of small molecules with biological systems. This method has been used to identify several interesting molecules and their corresponding targets [36].

Another target-directed chemical genetics approach involves the selection of bioactive small ligands directed against pre-characterized targets; this approach is capable of processing hundreds of targets per year [37]. The advantage of this method is that the small molecules that cause the desired biological effect immediately become a candidate drug-lead, simultaneously validating the target. However, both approaches could be limited by the specificity and bias of small-molecule libraries to a limited range of functional sites within a protein. Furthermore, in a discovery-driven approach, subsequent identification of the target using the small molecule as an affinity probe is technically challenging.

Large-scale protein expression and arrays

With the draft sequence of the human genome in hand, scientists are now beginning to clone and express all human genes systematically to determine their respective function [38]. A proof-of-concept was demonstrated using recombinational cloning to facilitate expression in multiple eukaryotic and prokaryotic systems [39]. A similar approach using yeast was used to screen for biochemical activities against an array of systematically cloned yeast genes, and led to the identification of previously unknown functions [40]. Recent reports describing a microchip format that displays thousands of functional proteins on its surface, indicate a promising approach to screen for protein function [41,42]. These systems will be highly suited to secreted proteins and soluble intracellular-proteins or -domains. Although arrays of membrane and organelle proteins is a challenging prospect, progress is being made [43].

Chromophore-assisted laser inactivation

Chromophore-assisted laser inactivation (CALI) is an efficient and direct method to determine the physiological function of proteins in cellular processes, with high spatial- and temporal-resolution. This technology uses a chromophore with special photochemical properties, linked to a ligand, such as an antibody, peptide or aptamer. Laser or incoherent light induces photochemical damage on the target protein. This technology has been applied to a wide variety of extracellular and intracellular proteins in several cell-based systems and small model organisms, and efficiently converts binding molecules to neutralizing molecules [44]. Recent developments have facilitated the use of CALI in high-throughput applications making it possible to validate hundreds of targets per year [45,46]. In a discovery-directed approach, ligands, such as antibodies, are systematically screened with CALI and the antibodies that lead to positive functional hits are used to immunoprecipitate the target protein. This is followed by subsequent identification using mass spectrometry and database searching (Xerion Pharmaceuticals; http://www.xerion-pharma.com) (unpublished results). However, the technology is limited in that an effect might not be observed if the synthesis of new proteins compensates for the inactivated proteins before the assay readout. However, an assay that measures an effect before newly synthesized proteins compensate for inactivated proteins, can alleviate this limitation.

Although protein-directed target validation methods are directly relevant for targets amenable to small molecules, antibodies or protein therapeutics, these methods should be further enhanced to increase throughput and accessibility equal to that

of genome- and transcript-based technologies. Protein-directed target validation will gain popularity as the technologies described here increase in throughput and accessibility. Once transcript- and genome-based strategies have completed genome-wide target validation efforts, the shift to protein-directed approaches will be imminent.

Concluding remarks

The problem for the pharmaceutical industry today is the shift from a paucity of targets to a plethora of pre-characterized targets. Choosing the right target becomes a critical success factor in the drug development process because it avoids costly mistakes further down the pipeline. Generating drug-leads has become a business commodity, and making leads against too many targets to avoid choosing a single wrong target is not economically feasible.

In the extreme case, a target is termed 'validated' only when a drug has been proven to work and is approved for safety after several years of clinical trials. Such retrospective definition will change in the future once technologies or a combination of technologies demonstrate high predictive value and increased success rates. The term 'target validation' has only been loosely defined; a better understanding of the physiological function of a target is required to develop an effective drug. Target validation is a continuous process, from identifying the function of the target to optimization of the lead compound. Therefore, the therapeutic molecule, whether small-molecule or antisense, is crucial in determining the appropriate target validation strategy and needs to be better appreciated.

Furthermore, the relevance of physiology is gaining popularity with the burgeoning field of systems biology. Before one can understand how a system works, one needs to understand the individual functions of the parts of that system, which mainly comprise proteins. Thus, the development of functional validation strategies – especially at the protein level – will continue to play an important role in the years to come.

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