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Fulfilling the promise: drug discovery in the post-genomic era

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The genomic era has brought with it a basic change in experimentation, enabling researchers to look more comprehensively at biological systems. The sequencing of the human genome coupled with advances in automation and parallelization technologies have afforded a fundamental transformation in the drug target discovery paradigm, towards systematic whole genome and proteome analyses. In conjunction with novel proteomic techniques, genome-wide annotation of function in cellular models is possible. Overlaying data derived from whole genome sequence, expression and functional analysis will facilitate the identification of causal genes in disease and significantly streamline the target validation process. Moreover, several parallel technological advances in small molecule screening have resulted in the development of expeditious and powerful platforms for elucidating inhibitors of protein or pathway function. Conversely, high-throughput and automated systems are currently being used to identify targets of orphan small molecules. The consolidation of these emerging functional genomics and drug discovery technologies promises to reap the fruits of the genomic revolution.

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▼ The recent completion of the human genomic sequence has ushered in the post-genomic era [1,2]. The ultimate promise of this effort is the elucidation of novel drug targets [3]. However, a defining challenge lies in identifying which of the many new sequences encode gene products likely to be therapeutic targets. Various methods and technologies are currently used to validate these genes as drug targets, ranging from hypothesis-driven studies of single genes in model physiological systems, to global scans for genes underlying disease processes.

Systematic alteration of single gene function in a relevant animal model represents a powerful approach for drug target validation. Despite its promise, however, this strategy is laborious, time-consuming and expensive, and thus realistically exacted on only a subset of genes. In comparison, genomic information, such as expression array and human sequence data, have made possible the identification of drug targets using bio-informatic algorithms (*in silico*). These methods are generally rapid, information-intensive and correlative with function and disease, but they frequently lack the physiological information necessary for robust target validation. As depicted in Fig. 1, these two approaches represent the extreme ends of the continuum of drug target identification and validation. Thus, the most relevant functional information is found at the apex of the pyramid, because alteration of specific gene function in model organisms represents the most direct evidence linking gene activity to disease. However, advantages of this methodology are offset by a significant reduction in throughput. Mining of genomic information represents a more comprehensive and scaleable approach, but the information derived implies only a circumstantial link to the disease. Several emerging functional genomic technologies attempt to ascribe gene function using cellular systems that mimic disease states. These cell-based strategies, represented by the body of the pyramid (Fig. 1), are surrogates of human disease that are amenable to high-throughput screening through automation and parallelization. They provide *in vitro* filters for targets identified through genomic methodologies to be rapidly triaged for validation in animal models. The evolution and application of these functional genomic methodologies are discussed below.

The aim of wide-scale functional genomics implementation is to expand the number of validated drug targets. In response, the urgency to identify corresponding pharmacological small molecule effectors in a rapid and robust manner will increase. However, these drug targets might not always be compatible with conventional schemes of ligand identification.

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In this review, we explore various novel biochemical and cellular approaches to identify small molecule modulators of non-tractable gene products. Furthermore, we discuss novel functional genomic techniques by which targets of orphan bioactive molecules, discovered in cellular and organismal systems, can be elucidated.

A new perspective

The initial sequence of the human genome, completed by the Human Genome Project and Celera (http://www.celera.com) [1,2], has deciphered the complex genetic code that directs the fate of the 100 trillion cells in the human body. Along with this genetic map comes the hope of elucidating the genetic architecture of disease. Although genetic information has enabled the identification of potential therapeutic targets on one level, it is clear that sequence information alone cannot resolve the complex patterns of gene activities, which ultimately dictate the physiology and pathology of an organism. Experimental approaches aimed to analyze gene function and the interactions of their products on a genome-wide scale are collectively termed 'functional genomics'. Although the availability of sequence data has marginally accelerated the pace of validated drug-target discovery, its existence has, more importantly, enabled a fundamental paradigm shift in functional genomics. This new biological perspective, analysis at the level of the genome, is anticipated to bring unequaled advances in the understanding of gene function and their causative role in disease.

A precedent of this new approach is the use of microarrays, or gene chips. Defined arrays of cDNAs or oligonucleotides printed on a glass surface, or 'chip', have enabled parallel monitoring of cellular transcription at the level of the genome. [4,5]. With a current capacity upwards of 54,000 gene clusters on a single chip (HG-U95; Affymetrix, http://www.affymetrix.com), data generated from genome sequencing projects in several organisms has provided the opportunity to build comprehensive maps of transcriptional regulation. Microarray technology continues to rapidly identify novel transcriptional cascades [6,7], biological processes [8,9] and disease markers [10–12]. Thus, this technology represents one of the first functional genomics platforms that exploit genome sequence data to analyze a biological process (gene transcription) on a gene-by-gene basis.

The primordial pool

Although microarray studies provided a pioneering approach to parallelized genome-level screens, initial functional genomic strategies for target identification relied on the interrogation of 'pooled' molecular libraries (see Table 1).

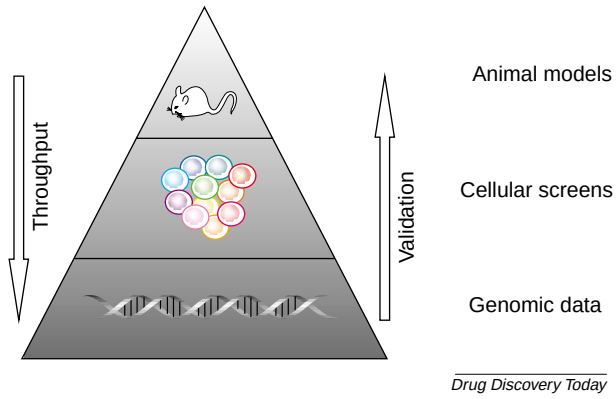

Figure 1. The hierarchy of target validation. This pyramid depicts the inversely proportional relationship between throughput and level of validation in experimental approaches to target identification. At the base of the pyramid lies genomic information, including sequence and microarray expression data. This type of data is attainable at a relatively rapid and robust scale, but, at best, provides a correlative relationship between a drug target and disease. The tip of the pyramid represents animal models that interrogate gene function (i.e. gene inactivation or transgenic). Although these types of studies provide the most direct link between gene function and disease, large-scale screening by this methodology is limited by monetary and time factors. Emerging cellular proteomics technologies, shown as the body of the pyramid, can provide a filter between genomic data and animal models. The genome-wide elucidation of gene functions in cell-based assays will aid in the identification of the most therapeutically relevant targets for a given disease. Thus, this would considerably curtail the number of genes requiring target validation in an animal model, .and provide a conduit by which genomic information can be rapidly translated into target validation.

For example, Fields and Song established a powerful methodology, the yeast two-hybrid system, to determine biochemical interaction between proteins through a genetic screen [13]. Gene libraries have also been used to elucidate the activities of encoded proteins or peptides by assaying for alterations in cellular phenotypes or a reporter (expression cloning) [14–16]. For example, Somia and colleagues identified Lfg, a suppressor of Fas-induced apoptosis, by transferring a library of retrovirally encoded cDNAs into mammalian cells such that each cell ectopically expresses a unique gene [17]. Incubation in the presence of Fas-ligand (to promote cell death) enabled the isolation of cells resistant to apoptosis and subsequent identification of genes that oppose Fas activity. A major advantage of expression cloning is that it can provide for elucidation of gene function within a normal mammalian cellular context of its encoded protein. This technique has enabled the identification of genes involved in apoptosis as well as other cellular processes ranging from cancer [18,19] to cell signaling [20].

Table 1. Pre-genomic screening approaches

Method	Detection (cellular system)	Refs
Phage display	*In vitro* protein–protein interaction (bacteriophage)	[27,28]
Two-hybrid	*In vivo* protein–protein interaction (yeast and mammalian)	[13,29]
Complementation cloning	Functional activity (yeast and mammalian)	[30,31]
Expression cloning	Functional activity (mammalian)	[16,17]
RDA	Transcriptional differences (mammalian)	[21]

These functional genomic systems were developed before the propagation of genome-driven platforms based on parallel and HTS. In general, these approaches relied on the interrogation of a 'pooled' molecular library through non-systematical selection schemes. These technologies represent pioneering methodologies that attempt to elucidate molecular function not reliant upon hypothesis-driven experimental design.

Functional genomic approaches used for the detection of differences in transcript levels, such as Representational Differential Analysis (RDA), rely on PCR-amplification of alternatively regulated genes [21]. This method is predicated on the preferential enrichment of a differentially expressed mRNA species, based on its size and relative abundance in a paired sample. This strategy has been successful for identifying several 'target genes' in *in vivo* cellular systems. Although this procedure does not require a pooled library, it is representative of the less systematic approaches to target identification prior to the genomic era.

Inherent shortcomings in these approaches towards screening of biochemical and genetic activities have encumbered rapid and comprehensive target identification. Because such tools generally include a competitive enrichment of molecules in a complex mixture through a selection schematic, they typically result in the selective identification of targets with significant activity over background. Because a gene library is inherently randomized and complex, the time required to definitively identify an effector gene is potentially significant. Finally, assays used in these experiments are not quantitative in nature and cannot be compared over multiple experiments. As a result of these shortcomings, 'pooled' functional genomic methodologies have had limited use in identifying a wide range of validated drug targets.

Parallel evolution

Systematic analyses using parallel investigation at multiple molecular levels provide an integrated approach to target discovery. In this context, microarrays provide one dimension to comprehensively quantitate transcriptional differences at a gene-by-gene level across the chip. Because microarray hybridizations directly determine expression levels, without complex selection procedures, results require no de-convolution, and can be compared across experiments to facilitate target identification using a larger data set. Despite the advantages of microarray technology over the pooled library approaches, the data derived from microarray studies provide only correlative information about gene function. Without further functional evidence, the ability of microarray-based technologies to identify drug targets is limited.

The use of arrayed molecules as an experimental approach has become more generalized. Currently, array strategies are also being used to determine activities and relationships at the level of the protein, which, in most cases, ultimately modulates physiological states. Several technologies that have sought to interrogate the activities of proteins at a genome-level are defining the burgeoning field of 'proteomics'. For instance, antibody arrays have been employed to investigate protein abundance [22], whereas high-throughput Immunoprecipitation-Mass Spectrometry (IP-MS) is being used to decipher *in vivo* protein–protein interactions at the level of the genome [23]. Recently, Ho *et al.* [24] and Gavin *et al.* [25] systemically elucidated biochemical interactions for the entire yeast proteome (complete set of proteins encoded by the yeast genome), resulting in the compilation of an elegant map of protein networks in *Saccharomyces cerevisiae*.

Cellular proteomic approaches

Cellular proteomic systems are increasingly being exploited to assess the activity of proteins. Ziauddin and Sabatini describe a methodology by which defined expression cDNAs are arrayed onto a glass slide, overlaid with a lawn of cells, and transfected using a lipid-based transfection reagent (reverse transfection) [26]. Protein activities corresponding to spotted cDNAs are evaluated by various phenotypic criteria applied at each discrete locus. For example, Ziauddin and Sabitini have used this methodology to screen a set of 192 cDNAs to identify genes whose protein products cause an increase in levels of phosphotyrosine within transfected cells. Their results yielded genes encoding five known tyrosine kinases as well as one with previously unknown function. Furthermore, their studies were extended to search for genes that cause apoptosis, changes in cell adhesion and cytoskeletal structure. This strategy is an example in which the new paradigm of parallel and systematic analysis has been applied to an established functional genomics technology: expression cloning.

Cellular proteomic approaches such as this promise to provide unparalleled advances towards annotation of gene function and the identification of novel drug targets. Genes can be systematically evaluated for their participation in signaling pathways as well as cellular processes and physiological phenotypes through overexpression. Assays can be done in arrayed format and across discrete loci, thereby expanding the range of possible cell-based functional screens. Furthermore, parallel analysis affords the identification of genes with less potent activities (i.e. slightly above background), and does not necessarily require a robust reporter system for success.

Industrialized cellular proteomics

Parallel efforts, termed Gene-by-Gene and Genome Functionalization by Arrayed cDNA Transduction (GFACT), are currently underway at the Functional Genomics Area (FGA) of Novartis Pharmaceuticals Corporation (http://www.novartis.com) [Labow, M., *et al.* pers. commun.] and the Genomics Institute of the Novartis Research Foundation (GNF), respectively. To facilitate this technology, individual clones from a pan-tissue human cDNA expression library were end-sequenced (Fig. 2a). Bioinformatic analysis was used to collapse the collection to amass full-length annotated cDNAs. This gene expression matrix was arrayed in 384-well format, such that each clone could be interrogated in parallel (Fig. 2b, step 1). Using a proprietary high-throughput, automation-based, transfection procedure, a gene matrix representing approximately 25% of the human genome can be screened in less than six hours.

Functional screens using this approach have been used to systematically examine genes that activate inflammation and cytokine production pathways. Briefly, a luciferase reporter containing binding sites for an immunomodulatory transcription factor is co-transfected en masse (Fig. 2b, step 2) with individual genes across the cDNA matrix. Luciferase activity is assayed in each individual well to determine cDNAs whose encoded proteins ectopically activate the pathway (Fig. 2b, step 3). Newly identified pathway members are then further investigated as potential anti-inflammatory targets. To date, putative novel functions for hundreds of genes involved in various signaling pathways, viral replication, cell cycle, apoptosis, cancer and differentiation have been assigned [Chanda *et al.*, unpublished data].

Several biotechnology companies have been incorporated to champion this emerging cellular proteomics tool. Kalypsys (http://www.kalypsys.com), Odyssey Pharmaceuticals (http://www.odysseypharm.com), Xantos AG (http://www.xantos.de) and Galapagos Genomics (http://www.galapagos-genomics.com) have established core and platform technologies to exploit the genome revolution by identifying gene functions and activities at the level of the genome. In these and other establishments, cellular phenotypic screening will also be greatly enhanced, as is the case with most functional genomic applications, by advances in detection technology. Novel high-throughput instrumentation will be required to fully exploit the diverse range of assays that are possible in cellular screening. Fluorescence imaging

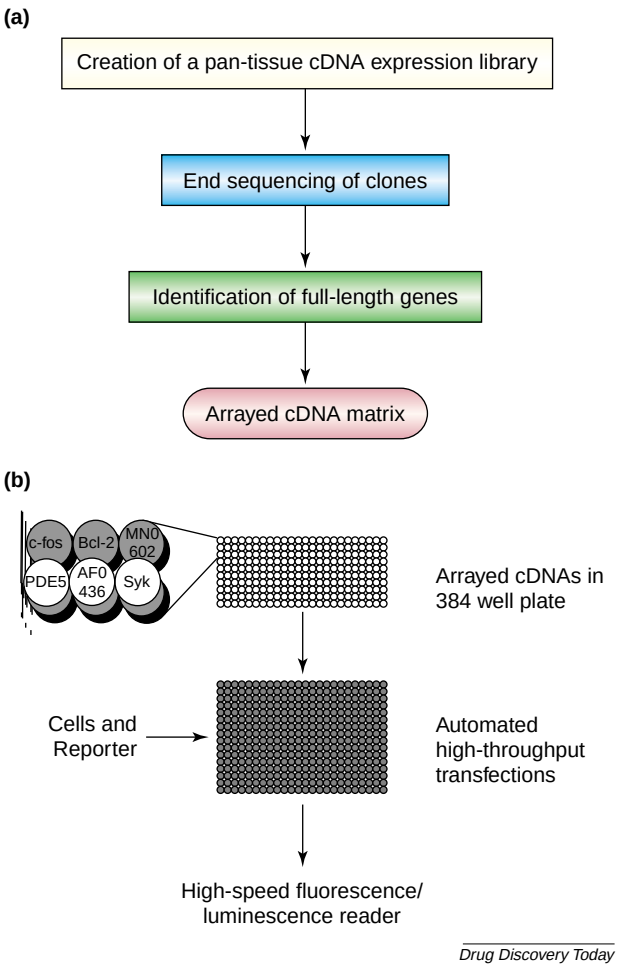

Figure 2. Genome functionalization by arrayed cDNA transduction (GFACT). **(a)** An annotated expression library ('matrix') is assembled from a pan-tissue human library, which is then end-sequenced and collapsed into a predicted full-length cDNA clone set. Each clone is arrayed into 384-well plate format **(b)** (step 1), such that each well contains a single addressable and unique gene. Using an automated high-throughput transfection protocol, the individual cDNAs and a fluorescence- or luminescence-encoding reporter gene construct are introduced into cells within the arrayed plates **(b)** (step 2). After an appropriate incubation time, cells are rapidly lysed (if necessary) and wells are interrogated by a high-speed fluorescence or luminescence plate reader **(b)** (step 3). Gene activity is correlated with relative fluorescence or luminescence output. The GFACT procedure for the transfection and activity measurement of 20,000 wells, discounting incubation time, requires approximately six hours.

technologies, such as those developed by Cellomics (http://www.cellomics.com), Q3DM (http://www.q3dm.com), Acumen (http://www.acumenbiosciences.com), Praelux (http://www.amershambiosciences.com) or Universal Imaging Corporation (http://www.image1.com), seek to expand the range of high-throughput phenotypic screens available to the research scientist.

Despite the forthcoming advances in robust, sensitive screening technologies, a significant challenge will lie ahead in development of validation strategies to rapidly identify those genes that will become the best drug targets. Some of these efforts will undoubtedly include the layering of data derived from high-throughput cell-based assays on existing genome sequence information and other functional genomics techniques, such as (microarray) expression profiling. For example, if a novel gene is found to induce uncontrolled proliferation in a cellular screen (cellular proteomic data), is in a druggable protein family (sequence data), and is preferentially expressed in prostate cancer (microarray data), it rapidly becomes a more logical target for therapeutic intervention. Moreover, established and intensive biochemical and genetic approaches, such as animal model studies, might still be required to validate potential drug targets. However, these labor-intensive studies will have a higher likelihood of success using targets that have been previously confirmed through genomic and cellular proteomic investigation. Companies that are able to properly balance the implementation of these emerging and established technologies hold the promise of discovering the next generation of therapeutic targets.

Target to small molecule: de-orphaning drug targets

The genomic era has brought with it a massive host of putative novel drug targets, potentially driving the number of targets into the thousands [2]. Using state-of-the-art informatics and sequence-based homology and structure prediction tools, a glut of new gene sequences belonging to the historically druggable protein classes have been gleaned [32]. With only minimal additional biological information, such proteins can be addressed with small molecules using conventional high-throughput screening methodologies including enzymatic or binding disruption assays. Conversely, there are instances when a classification is made, however, developing a useful screening assay is difficult, either because the target is an enzyme with no known or surrogate substrate, or a scaffold-binding protein with no known binding partners. Furthermore, many gene products cannot be classified, or do not fall into the druggable gene category, thus representing a challenge in the development of assays for pharmacological small molecule modulators. In such cases, a new challenge exists in finding chemical compound modifiers or ligands to de-orphanize novel drug targets.

Recently, several screening approaches have been developed to address the growing need to identify chemical ligands for novel, orphan, drug targets. These assays rely heavily on affinity methodologies. The premise is small molecule modulators of putative drug targets can be identified by panning through diverse sets of small molecules and selecting the highest affinity binders. Neogenesis Inc. and Evotec OAI use ALIS and CONA-HTS technologies, respectively, as automated ligand identification systems in which large diverse libraries are panned against a labeled protein target of interest [33–35]. Despite advances in identifying ligand binders for novel targets *in vitro*, the challenge still remains to establish relevant biological cellular and physiological systems in which to test these molecules for efficacy. Recently, Schreiber and others applied the use of high-density small molecule arrays of a 1,3 dioxane small molecule library to identify chemical ligands for the Ure2p transcriptional repressor in yeast [36]. Interestingly, the identified ligand, uretupamine, was capable of modulating Ure2p signaling function inside yeast cells. These approaches open the possibility of supplying any genomic-derived or orphan drug target with small molecule modulators, if not as the final product, at least as a template for chemical optimization for desired biological properties.

Small molecule to drug target: de-orphaning small molecules

The promise of genomics is the identification of novel drug targets. With few exceptions, information provided by genomics tools is insufficient to determine whether a gene corresponds to an optimal drug target. In the absence of viable drug targets for a particular disease, the pharmaceutical industry has been known to circumvent traditional target identification and search initially for efficacious small molecules in model systems of disease. Recent advances in cellular technologies enable one to interrogate disease-relevant pathways in cell systems for desirable chemical effectors. In fact, drugs such as penicillin or cyclosporin A were identified in relevant cellular assays prior to elucidation of their respective molecular targets [37,38]. The current challenge is to rapidly identify drug targets that have been pre-validated by the efficacy of their small molecule ligands.

Cell-free systems

Standard strategies to identify targets of small molecules involve affinity matrices with or without cation exchange high performance liquid chromatography, such as were

used to identify targets of the immunosuppressants, cyclosporin A and FK506, respectively [37,39]. Other examples using affinity chromatography include the identification of methionine aminopeptidase 2 as a target for fumigillin [40], the histone deacetylase for trapoxin [41], and an anti-apoptotic drug that targets glyceraldehyde-3-phosphate dehydrogenase [42]. Obstacles in these methods include identifying a linker-modified compound that retains biological activity, high levels of non-selective binding or competitively labeling a low abundance target in cells or extracts, and obtaining sufficient material after purification to identify the target's amino acid sequence identity.

Parallel, comprehensive scanning on a massive scale and automatable systems for small molecule de-convolution are coming to fruition. These informatic, affinity-based and genetically based technologies, which leverage automation, miniaturization, materials science and high-throughput methodologies, might bring forth the post-genomic era. Based on the premise that transcriptional profiles affected by specific gene alteration are comparable to profiles of small molecule 'perturbagens' of that gene, Hughes and Friend at Rosetta Inpharmatics (http://www.rii.com). used informatics to predict that the anaesthetic dyclonine targets Erg2p, the yeast homolog of the human Sigma receptor [43]. Comparing patterns of activity of 1000s of anti-proliferative small molecules against the NCI panel of tumor cell lines, the kenpaullones were predicted to target cyclin-dependent kinases by Sausville using the COMPARE algorithm [44]. Vast amounts of data brought about by high-throughput screening at NCI, and certainly from databases of pharmaceutical companies, are raising many exciting possibilities regarding experimental design and methods of predicting small molecule targets and mechanism of action.

In theory, spatial arrangement of proteins on a solid surface followed by hybridization of a known bioactive compound can facilitate small molecule target de-convolution. The patented SELDI ProteinChip® process that enables protein capture, purification, analysis and processing from complex biological mixtures directly on ProteinChip Array surfaces is one such system [45,46]. Schreiber employed a robot to place liquid protein samples on microscope slides at a density of 1,600 spots per square centimeter. Using these fabricated chips, researchers were able to demonstrate binding of a small molecule to its target on the chip [47]. At Yale University (http://www.yale.edu) the entire yeast proteome was printed on slides at high spatial density and screened for proteins that could interact with phospholipid molecules [48]. By this approach, novel calmodulin and phospholipid-interacting proteins were elucidated. Phage-encoded cDNA libraries combined with affinity chromatography enabled elucidation of FKBP12 as the FK506 small molecule target and F1 ATP-synthase as a target of a benzyl synthetic scaffold [49,50]. In this case, binding and function are equivocal, plus the method suffers from the need once again to affinity label the small molecule, which might abrogate its function, and thus, the relevance of binding.

Cellular systems

A new methodology termed 'chemical genetics' using mammalian cells as vehicles for small molecule target identification is advancing. In this incarnation of 'chemical genetics,' termed 'small molecule complementation,' the effect of a small molecule that inhibits a given signal transduction cascade can be competed out, or circumvented via overexpression of the molecular target of the small molecule. These *in cellulo* competition assays have been well-documented in the literature as a means to demonstrate interaction between a small molecule and its target protein. As an example, Gaynor and colleagues showed that IKK-beta was a target of aspirin and sodium salicylate as increasing levels of the kinase were able to compete out IKK-beta phosphorylation of IκBα in cells [51]. More recently, Lorens and others screened retroviral cDNA libraries for genes that could outcompete staurosporine's pro-apoptotic effect in NIH3T3 cells [52]. The GNF, FGA and Kalypsys Inc. are pioneering the industrialization of this approach, using arrayed cDNA expression libraries instead of pooled cDNA expression libraries as a means to establish a more sensitive and comprehensive functional genomic scan for targets of bioactive small molecules. The approach quickly narrows the number of potential targets from tens of thousands to just a few that can subsequently be assayed directly. In sum, the small molecule complementation approach provides an effective functional filter of the genome towards the elucidation of small molecule targets.

Conclusions

The completion of the human genome sequence is perhaps the most significant event in scientific history. The most practical implications of this endeavor will likely be manifested in the development of therapeutic molecules derived from the use of the genome data. However, there currently lies a formidable chasm between the analysis of genomic information and the discovery of pharmaceutical compounds. The genomic revolution has propelled a flurry of technological advances, which promises to culminate in a rapid and comprehensive target validation process for disease etiology. The generation of small molecule inhibitors is concurrently enabled by the introduction of novel methodologies, some of which are based on functional

genomic principles. The anticipated bridging of functional genomics-based target validation and high-throughput compound identification processes will define the pipeline for pharmacological exploitation of genome data.

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