Full-speed mammalian genetics: *in vivo* target validation in the drug discovery process

Alejandro Abuin, Kathleen H. Holt, Kenneth A. Platt, Arthur T. Sands and Brian P. Zambrowicz

The completion of the Human Genome Project has signaled the beginning of the post-genome era, with a corresponding shift in focus from the sequencing and identification of genes to the exploration of gene function. A rate-limiting step in deriving value from this gene sequence information is determining the potential pharmaceutical applications of genes and their encoded proteins. This validation step is crucial for focusing efforts and resources on only the most promising targets. Strategies using reverse mouse genetics provide excellent methods for validating potential targets and therapeutic proteins *in vivo* in a mammalian model system.

Alejandro Abuin
Kathleen H. Holt
Kenneth A. Platt
Arthur T. Sands
and Brian P. Zambrowicz*
Lexicon Genetics Incorporated, 4000 Research Forest Drive, The Woodlands, TX 77381, USA.
*e-mail: brian@lexgen.com

The pursuit of pharmaceutical targets has traditionally been predicated by a significant body of scientific literature. However, with the completion of the first draft of the human genome [1,2] comes an onslaught of new genes with little or no associated biology. Future therapeutic targets will be derived from this common pool of gene sequences, leading to intense competition to identify those genes with the greatest therapeutic potential. Those that can most rapidly and effectively integrate biological information into the target selection process will have an advantage. To manage the thousands of newly identified potential therapeutic targets, the pharmaceutical industry must make crucial decisions early in the drug discovery process. Before limited resources are expended, poor targets must be eliminated through rigorous biological assessment. Only those targets that demonstrate specific biology with therapeutic potential should be pursued, thus reducing the failure rate and increasing the overall efficiency of the drug discovery process [3–6].

Target validation has become an increasingly important component in drug discovery because poorly characterized genomic targets could clog drug discovery pipelines. Many methods are being used for target validation, producing data that varies widely in biological value. These methods include the bioinformatic categorization of a gene, analysis of expression levels or pattern in healthy versus diseased tissues, proteomics, cell-based assays, *in vivo* validation in an animal model and the ultimate validation of testing new chemical entities in human clinical trials. Because of the many tools being used to assign gene function, the definition of a 'validated' target can be highly subjective.

Here, we focus on the use of mouse genetics as a target validation tool before the initiation of small molecule drug discovery, therapeutic protein or antibody (Ab) programs. We define a 'validated' target as one whose *in vivo* function, as demonstrated in a mammalian model organism, indicates that its modulation might provide therapeutic potential. This *in vivo* mammalian target validation provides strong evidence that a particular gene is involved in the biology of interest and delivers the highest level of target validation before work in man.

The mouse as a tool for validating pharmaceutical targets

Genetically engineered mice have proven to be invaluable tools for the functional dissection of biological processes relevant to human disease, including immunology [7], cancer [8], neurobiology [9,10], cardiovascular biology [11], obesity [12] and many others. Knockout mice have been shown to model drug activity; phenotypes of mice deficient for specific pharmaceutical target proteins can resemble the human clinical phenotype caused by the corresponding antagonist drug. Examples of this type include mice deficient in the angiotensin-converting enzyme *(ACE)* [13] and cyclooxygenase-1 (*COX1*) genes [14]. These mouse knockout phenotypes resemble the effects on humans of ACE inhibitors (anti-hypertensive drugs) and non-steroidal anti-inflammatory drugs (NSAIDs). Conversely, knocking the gene out in the mouse can have an opposite phenotypic effect to that observed in humans after administration of an agonist drug to the corresponding protein target. Examples include the erythropoietin knockout [15], in which a consequence of the mutation is deficient red blood cell production, and the *GABA(A)-R-β3* knockout [16], in which the mutant mice show hyperactivity and hyper-responsiveness. Both these phenotypes are opposite to the effects of erythropoietin and benzodiazepine administration in humans. Growth hormone (GH) is an example of a protein that, when over-expressed in the mouse, results in a phenotype that mimics the action of a therapeutic protein in humans [17].

http://tibtech.trends.com 0167-7799/02/$ – see front matter PII: S0167-7799(01)01843-1

This use of mutant mouse phenotypes to help predict the effects of a small molecule drug or therapeutic protein can be extremely valuable to the pharmaceutical and biotechnology industries. For the examples described above, mouse genetics was used retrospectively to confirm the biological function of these targets after drugs were already available. In this context, the full power of mouse genetics will be realized when the predictive nature of mutant mouse phenotypes is applied early in the drug discovery process. This will lead to a better pool of targets and perhaps a corresponding increase in the success rate in the pharmaceutical industry.

Examples of drug targets that are being actively pursued by the pharmaceutical industry and that have been validated using the mouse early in the drug development process include cathepsin K, the melanocortin-3 and -4 receptors (*MC-3R* and *MC-4R*), and acetyl-CoA carboxylase 2 (*ACC2*). Cathepsin K is an osteoclast-specific cysteine protease that cleaves bone matrix proteins such as Type I and II collagen, osteonectin, and osteopontin [18]. This expression and enzymatic activity data suggested early on that cathepsin K might have an important role in bone degradation, and that chemical compounds that inhibit its activity might be useful in the treatment of osteoporosis [19]. Notably, cathepsin K knockout mice develop osteopetrosis as a result of impaired bone resorption [20], confirming the *in vivo* role of this protease in bone matrix degradation and its relevance as a potential osteoporosis drug target.

Disruption of the *MC-3R* and *MC-4R* genes has demonstrated their crucial roles in energy homeostasis – mutations in these genes lead to obesity and hyperinsulinemia in mice [21,22]. These results suggest that activation of these receptors might be a possible pharmaceutical treatment for obesity. Indeed, melanotan II, a melanocortin receptor agonist, increases metabolic rate and decreases food intake in wild-type mice but not in *MC-4R* mutant mice [23].

A striking example of a target validated using mouse genetics is the *ACC2* gene. Although the human *ACC2* gene had been identified several years ago [24], interest in ACC2 as a target for drug development was stimulated only recently after analysis of ACC2 function using a knockout mouse [25]. ACC2 mutant mice eat more than their wild-type littermates, yet burn more fat and store less fat in their adipocytes, making this enzyme a probable target for chemical antagonism in the treatment of obesity.

One example of a potential drug target that has been validated using mutant mice produced by gene trapping (reviewed below) is the puromycin-sensitive aminopeptidase (*PSA*) gene. As part of their phenotype, PSA deficient mice show an impaired pain response [26], which suggests that antagonists targeted to this enzyme might work as analgesics. A specific inhibitor of this protein has been developed [27]. It will be interesting to see whether the administration of this drug in mice will recapitulate the analgesic effect observed in the knockout animals.

The above examples demonstrate the power of mouse genetics for *in vivo* validation of potential drug targets. To apply mouse genetics to the validation and prioritization of the large number of novel drug targets derived from the sequencing of the human genome, high-throughput, large-scale mouse mutagenesis techniques will be required. Several methods are being used to perform large-scale genetic analysis in the mouse. These methods include gene targeting by homologous recombination, gene trapping and chemical mutagenesis using agents such as *N*-ethyl-*N*-nitrosurea (ENU).

Gene targeting using homologous recombination

The introduction of custom mutations in mice using homologous recombination between exogenous DNA and its genomic target sequences in embryonic stem (ES) cells is a sophisticated reverse genetic approach that was first described nearly two decades ago [28]. In recent years, the growing number of mutant mouse lines has led to an invaluable resource for physiological studies and yielded numerous models of inherited human disease.

Targeted mutagenesis offers the advantage of introducing precise and tractable alterations in any gene of interest. Examples of genetic alterations that can be produced by this approach include deletions to disrupt gene function, conditional alleles that allow for tissue-specific or temporal control of gene expression, point mutations, and targeted transgenesis, whereby any cDNA of interest can be introduced into a specific locus. In the case of standard null deletions, a reporter gene, such as β-galactosidase or green fluorescence protein (GFP), can be engineered into the construct to create a fusion transcript that functions as a readout of the gene's normal transcriptional activity. Because of the highly technical and time-consuming steps required for targeting vector design and construction, the entire process often takes many months of dedicated effort, thus limiting the total number of genes that can be examined using this process. Here, we discuss the latest technologies that afford significant gains in the efficiency of targeting vector engineering. These advances facilitate the industrialization of custom mutagenesis in mice for high-throughput analysis of gene function.

Gene targeting has been hampered by the fact that homologous recombination in mammalian cells is a rare event and is often highly variable from gene to gene. The use of isogenic DNA within targeting vectors and positive and negative selection strategies for increasing the probability of identifying targeted clones have greatly enhanced gene targeting

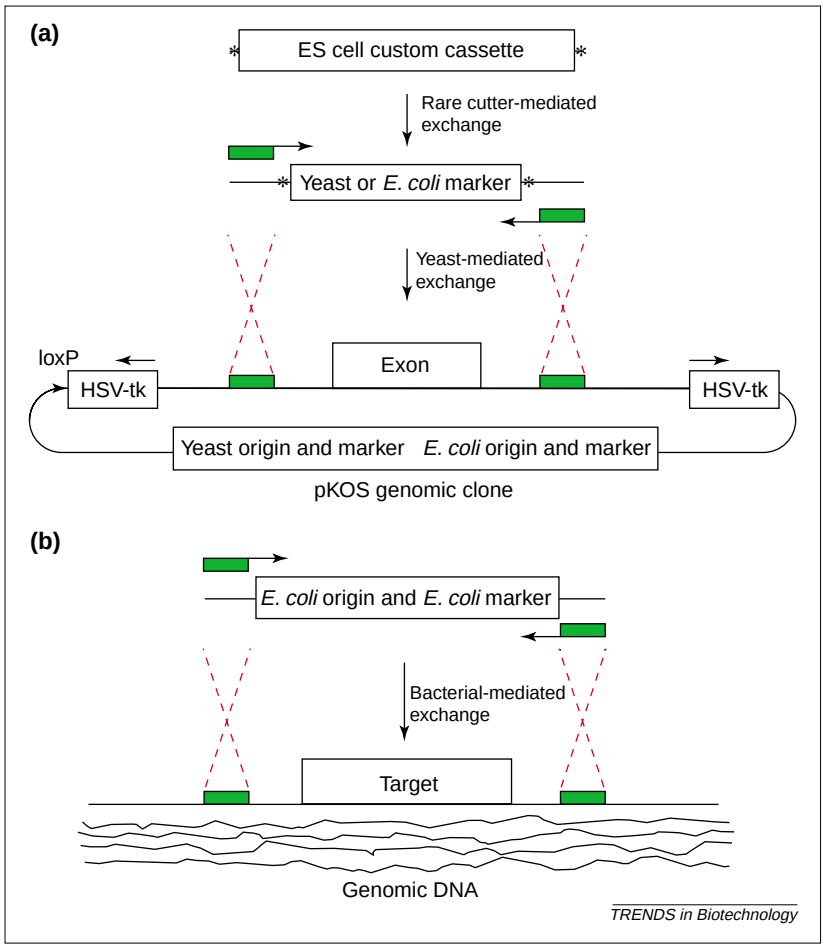

Fig. 1. Yeast and bacterial recombination systems facilitate target vector engineering. (a) The λKOS employs a murine 129 Sv/Ev^{brd} genomic library with negative selection cassettes flanking the genomic inserts (herpes simplex virus tyrosine kinase, *HSV-tk*)) [7]. Thirty to forty base pairs of homology to the target site in the genomic clone (green boxes) are appended onto the yeast or *Escherichia coli*-selectable marker (flanked by rare base-cutter restriction sites, indicated by asterisks) using PCR. The yeast-selectable marker and the pKOS genomic clone are co-transformed into yeast and plated onto selective media. The embryonic stem cell custom cassette is exchanged for the yeast-selectable marker by a one-directional cloning step using the restriction sites introduced by the yeast recombination step. (b) Cloning of large pieces of DNA for target vector construction using homologous recombination in *E. coli* can be accomplished using complex sources of target DNA, such as mouse genomic DNA. A linear cloning vector, consisting of an *E. coli* plasmid origin and an antibiotic drug-resistance marker, are modified using PCR or ligation to include >70 base pairs of homology to the target site in the genomic DNA (green boxes). The bacterial cloning vector and the total genomic DNA are co-transformed into an appropriate bacterial strain and plated onto selective media.

efficiency. However, homologous recombination remains a relatively rare event, making it very difficult to target some genes. With few developments showing promise for increasing the rate of homologous recombination in ES cells, the most recent technological improvements have focused on streamlining the DNA manipulations required for targeting vector construction.

A λ knockout shuttle (λKOS) system was recently described for rapid construction of targeting vectors [29]. This system uses a λ phage murine 129 Sv/Ev^{brd} genomic library containing negative selection cassettes flanking the genomic inserts (Fig. 1a). The library can be screened using PCR to clone the murine gene of interest, and the corresponding λ phage clone is converted to a high copy plasmid through CRE-mediated excision. Homologous recombination in yeast is used to introduce a yeast-selectable marker that is flanked by rare base-cutter restriction enzyme sites. A single directional cloning step is then used to exchange the yeast-selectable marker with the custom ES cell selection cassette. Similar strategies have focused on the use of bacterial recombination systems to engineer complex gene targeting vectors, including methods for rescuing regions containing the gene of interest from total mouse genomic DNA (Fig. 1b) [30]. These techniques circumvent the potentially complex cloning strategies required to flank the ES cell selection cassette with gene-specific arms of homology. In practice, these systems are highly efficient and targeting vectors can be produced rapidly.

Fortunately, knowledge of human and mouse genomic sequence greatly facilitates the design and construction of vectors for introducing targeted mutations in mouse ES cells. In the past, significant efforts were expended on defining exon–intron junctions, restriction mapping and sequencing of the large stretches of genomic DNA required for targeting vector engineering. These efforts have largely been replaced by bioinformatics-based sequence database searches. The completed mouse genome sequence will provide further efficiencies to the gene targeting process.

Gene targeting requires an initial selection of the gene(s) to be mutated, a biased process that might result in an inability to identify certain important genes. Because of the large number of potential target genes available, homologous recombination fits well with the target-driven approach being used throughout much of the biotechnology and pharmaceutical industries. This approach is biased towards the classes of genes that have traditionally been considered 'drugable' or amenable to modulation by small molecules. These 'drugable' families include G-protein-coupled receptors, nuclear hormone receptors, proteases, channels, transporters, kinases, phosphatases, phosphodiesterases and enzymes. Homologous recombination provides a strategy for focusing on genes that are good targets for small molecule drug discovery, enabling a more rapid translation of gene function discovery into therapeutic products.

High-throughput mutagenesis using gene trapping

Gene trapping is a high-throughput method of random mutagenesis in which the insertion of a DNA element into endogenous genes leads to their transcriptional disruption. Gene trapping elements can be endogenous in nature, such as transposable elements [31], or exogenous recombinant DNA constructs [32]. Gene trapping constructs are typically designed to lack an essential component, such as an enhancer [33], promoter [34] or polyadenylation signal [35], rendering them

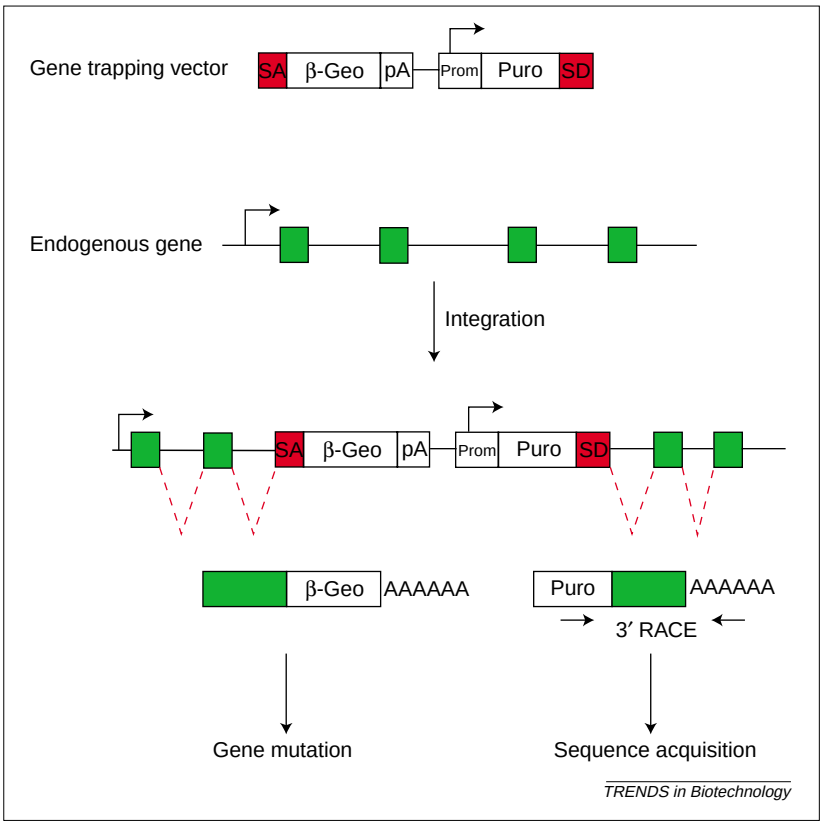

Fig. 2. Gene trapping; simultaneous mutation and identification of genes. A promoter-less β-Geo cassette is linked to a splice acceptor sequence (SA). β-Geo is a functional fusion between the β–galactosidase reporter gene and the selectable marker for Neomycin resistance. A promoter (prom) active in ES cells drives the expression of a polyA-less Puromycin selectable marker (puro) linked to a splice donor site (SD). Insertion of this construct into an intron of a transcriptionally active gene results in a selectable fusion transcript between the upstream exons and β-Geo. A sequence tag for the mutated gene can be obtained by sequencing the fusion transcipt between puro and the downstream exons.

transcriptionally active only when inserted into an endogenous gene. Trapping constructs most commonly used in ES cells carry a promoter-less reporter gene or selectable marker linked to a strong splice acceptor sequence. Insertion of this construct into an expressed gene results in a fusion transcript and mutation of the endogenous gene. More recently, constructs containing promoters directing the expression of exons or reporter genes lacking a polyadenylation sequence have been used to trap genes not expressed in ES cells and to automate the process of sequence acquisition (Fig. 2). Gene trapping constructs are usually delivered to the target cell by electroporation or via a retroviral construct. Unlike other random mutagenesis methods, such as chemical [36] or radiation-induced mutagenesis [37], gene trapping allows the rapid identification of the mutated gene because the known DNA sequence of the gene-trapping element can be used as an anchor point to obtain the sequence of the trapped locus by automated methods [38,39].

A variety of gene trapping strategies have been used in *in vitro* functional screens to select for mutations affecting specific cellular processes, such as glycosylation [40], tumor suppression [41] and lineage differentiation [42], or to pre-select for secreted or transmembrane proteins and genes involved in the regulation of neuronal wiring in the brain [39]. Although *in vitro* phenotype-driven screens are a powerful way of discovering the physiological functions of genes, their potential is limited by the amount and type of assays that can be implemented before the ES cells are used to generate mice. An alternative is to identify which genes have been trapped first and then generate mutant mice for the genes of interest. This can be achieved with the use of a gene-trapping vector that allows the simultaneous mutation and rapid sequence identification of the trapped gene [38].

We, as well as others, are using gene trapping approaches to identify and mutate large numbers of genes in mouse ES cells [34,38,43,44]. We have generated OmniBank™ [38], a repository of mutant mouse ES cell clones, each carrying a single gene trap mutation. The corresponding sequence tag for the mutated gene is catalogued for cross-reference in a computer database that enables a bioinformatics-driven phenotypic screen for gene function (Fig. 3). This resource contains more than 195 000 ES cell clones and their corresponding sequence tags grouped into more than 40 000 non-overlapping clusters. New clones are being added to the library at a rate of ~2000 per week, and the database contains mutations in >53% of all mammalian genes. This estimate is calculated by searching OmniBank for sequence tags with exact matches to a reference list of >5000 randomly chosen full-length mouse genes. The ability to quickly generate mutant mice from this collection of ES cell clones allows for the phenotypic analysis of large numbers of mutant mouse lines. We are generating an average of 40–50 distinct OmniBank gene trap mouse lines per month within sets of highly prioritized genes that have been pre-selected based on bioinformatics analyses of gene sequence. When bred to homozygosity, 70% of the ~300 mouse lines developed from this resource have been demonstrated to harbor gene trap mutations that result in loss of the endogenous transcript. The remaining 30% are hypomorphs or have normal levels of transcript for the trapped gene. New quality control measures allow identification and elimination of the majority of non-null stem cell lines before mouse production. This unique library provides an excellent direct approach for moving from gene sequence information to understanding gene function.

Gene trapping generates mutant ES cell clones that can be used for both hypothesis-driven screens that incorporate pre-screening of clones based on the sequence of the mutated gene and any associated data or pure phenotype-driven screens. The random and insertional nature of gene trapping technology, however, doesn't allow for the creation of precise mutations such as those created by gene targeting. Like other genome-wide mutagens, gene trap insertions do not always lead to a null mutation and not all genes will be good targets for trapping. Nevertheless, gene trapping provides the most high-throughput, cost-effective and productive method for large-scale bioinformatics-driven reverse genetics. This ability to maximize the

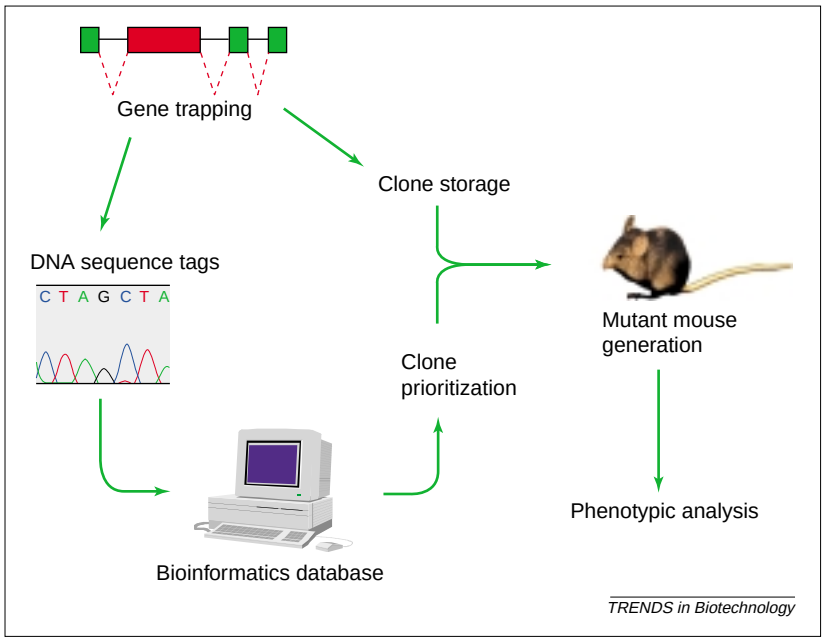

Fig. 3. OmniBank™ flow chart. Gene trapping in mouse embryonic stem (ES) cells is used to generate thousands of clones bearing mutations in individual genes. Mutant ES cell clones are cryo-preserved in duplicate. A sequence tag for the mutated gene is simultaneously generated and catalogued in a bioinformatics database. This database is used to identify sequence tags for the genes of interest and the corresponding ES cell clones are then used to generate mutant mice. The mice are put through general and directed phenotypic screens to identify pharmaceutically relevant genes.

number of genes that can be studied is essential in the race to harvest the best targets out of the genome. The more labor-intensive process of gene targeting offers unparalleled flexibility and specificity. Taken together, gene trapping and gene targeting offer complementary approaches for the analysis of mammalian gene function through ES cell technology.

Phenotypic screens using chemical mutagenesis

ENU is an efficient chemical mutagen and carcinogen that can be used to produce random mutations in the mouse germline. Although ENU mutagenesis has been used for decades, only recently has ENU mutagenesis been combined with systematic screening approaches to identify phenotypes with specific disease relevance [44,45]. In this method [46], the male germline is mutagenized using intraperitoneal injection of ENU. The mutagenized males are bred to wild-type females and the resulting F1 progeny are examined for dominant phenotypes (Fig. 4). Although most of the new phenotypes have been derived from dominant screens, there have been a small but growing number of recessive screens. For recessive screens, F1 progeny are bred to wild-type females, the resulting female F2 progeny are backcrossed to the mutagenized male and F3 progeny are scored for phenotypes. Mice with inherited phenotypes of interest are intercrossed with mice from a different strain and multiple offspring or meiosis are obtained for mapping the chromosomal position of the mutated gene.

The main advantage of the ENU approach is that it is unbiased and does not rely on preconceived ideas of gene function. The screens are a forward genetics approach based entirely on phenotype. This approach does not incorporate any gene pre-selection and could identify functionally relevant genes that would not be chosen using gene sequence or hypothesis-driven approaches. Another advantage is that ENU typically produces point mutations and the process can identify phenotypes that result from subtle mutations that would never be chosen and produced by gene targeting.

The major challenge of the ENU approach is the lack of prior knowledge of what genes have been mutated and the subsequent difficulty in identifying the point mutations responsible for any specific inherited phenotype. Although novel genes with novel biology can be identified, the results can sometimes be less satisfactory; the phenotype of interest might result from the mutation of a previously characterized gene or the mutation of a gene that is not 'drugable', making it more difficult to pursue for further drug development. To date, the large ENU efforts have resulted in the identification of very few genes, many of which have been previously known when mapped and identified by a candidate gene approach. It is hoped that the completion of the mouse genome sequencing project, microarray approaches and the combination of ENU with large chromosomal deletions will speed up the process of gene identification.

The ENU approach sets aside the massive amount of genomic information, including bioinformatics, expression analysis and proteomics data. Within the ENU paradigm, this valuable body of genomic data cannot be used to pre-select and prioritize genes to funnel through the drug discovery process or to focus phenotypic screens on relevant pathways or tissues. Genomic sequence information will, however, be useful to speed the mapping of point mutations back onto chromosomes so that a

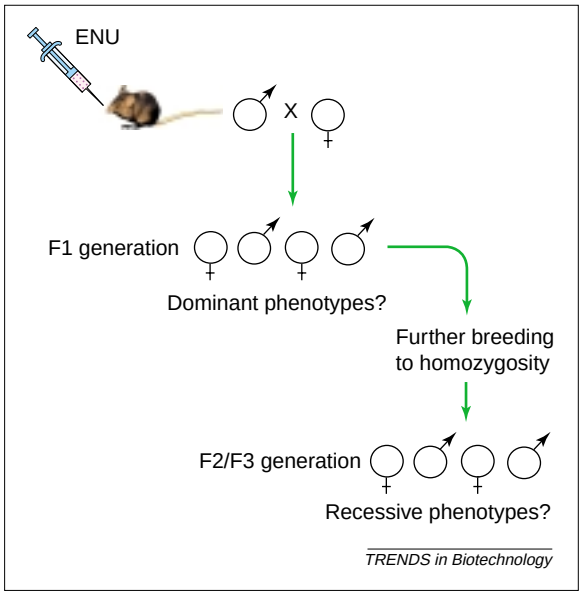

Fig. 4. *N*-ethyl-*N*-nitrosurea (ENU) mutagenesis screens. The germline of male mice is mutagenized by administration of ENU. Mutagenized males are bred to wild-type females and the F1 offspring is screened for phenotypes arising from dominant mutations. Further interbreeding steps can then be used to generate mice homozygous for the ENU-induced mutations, which are screened for recessive phenotypes.

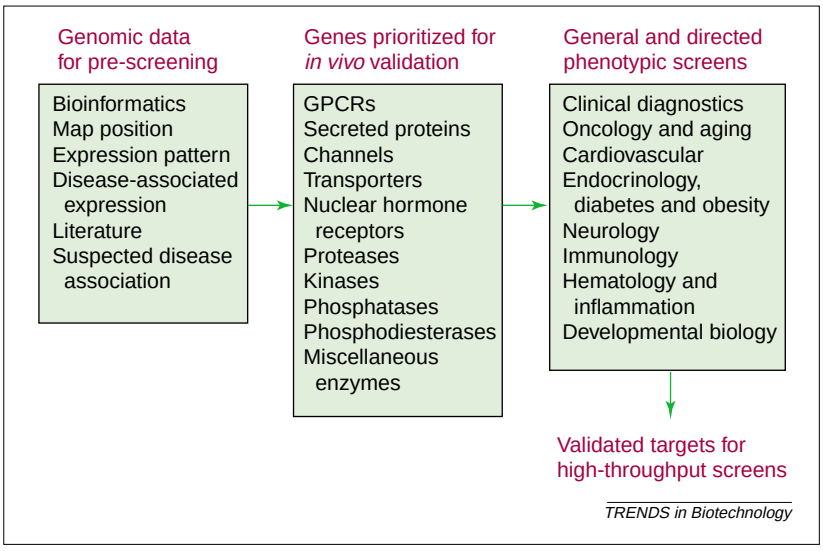

Fig. 5. This figure shows the combination of pre-screening and directed phenotypic screening for target validation. Genomics data is used to pre-select target genes to enrich the discovery pipeline. Prioritized targets are validated *in vivo* using reverse genetics approaches in the mouse. Mutant mice are put through general and directed phenotypic screens, resulting in validated targets for further drug development. Abbreviation: GPCRs, G-protein-coupled receptors.

candidate gene approach can then be undertaken to identify the mutated gene.

Despite the broad mutagenic activity of ENU, screens for dominant phenotypes can select for a small pool of genes. This has been observed with the isolation of multiple alleles of the same gene [e.g. *wheels* (*Whl*)] [45] both within and between the different ENU screening groups. Recessive screens will allow scanning of larger portions of the genome but will present formidable logistical challenges because of the sheer numbers of mice required to breed, maintain and map lines without knowledge of genotype.

Mouse genetics in the drug discovery pipeline

The technologies of gene targeting and gene trapping are currently being used on a large scale for validating potential drug targets. Despite the length of time required to complete any individual project, the volume of projects that can be performed in parallel in an industrialized setting facilitates the analysis of large numbers of genes. These two methods combine the ability to target any gene by homologous recombination with the high-throughput capabilities of gene trapping, maximizing both selectivity and capacity. Genes analyzed in this way are subject to a superior level of validation, including physiological function and potential disease indication, providing a robust pipeline of high-value targets. Hypothesis-driven gene targeting and gene trapping place biology and therapeutic potential at the forefront of the drug discovery process (Fig. 5). We are using mouse genetics to discern the function of 500–1000 genes per year and have a stated goal of determining the function of 5000 genes over the next five years. This will provide preliminary data on the *in vivo* function of virtually all members of the 'drugable' gene families. To derive value from mouse genetics, sophisticated phenotypic screens must be used to identify all pharmaceutically relevant biology. Screens must be continually re-evaluated and modified to maximize the information that can be obtained. This review is not meant to provide a detailed description of phenotypic analysis of knockout mice, as this is covered elsewhere [2,45,47]. Analysis of genetically altered mice has been modeled after human clinical evaluation, and has so far proven successful in extracting vital information about the physiological significance of gene function. This requires the deployment of a wide range of analytical equipment and assays, such as optic fundus angiography, computer-assisted tomography (CAT) scans, dual energy X-ray absorptiometry (DEXA), magnetic resonance imaging (MRI), complete blood counts, fluorescence-associated cell sorting (FACS) analysis, blood chemistry, urinalysis and pathology, as well as an overall evaluation of the animals for neurological and physiological integrity. In our experience, ~10% of gene knockouts lead to pharmaceutically relevant phenotypes, such as leanness, reduced cholesterol and/or triglycerides, or blunted inflammatory response. A preferred validated target will demonstrate very specific biology with therapeutic indications in a phenotypic background that is otherwise normal. The success of the genetic approach to target validation has been demonstrated by the identification of validated targets for all of our disease biology focus groups, including oncology, immunology and inflammation, cardiovascular biology, neurology, endocrinology and gastrointestinal biology. The scientific literature provides further support for *in vivo* validation with a growing list of targets characterized using mouse genetics [20–23,25].

Future prospects

Genetics has provided an impressive array of tools to define gene function in a variety of model organisms and is being used in increasing volume to discover and validate mammalian drug targets in mice. Targets with clear disease relevance are being identified regularly using mouse genetics, underscoring the value of this technology. Additional developments in mouse reverse genetic approaches, such as increasing the speed and efficiency of homologous recombination and saturating the mouse genome with gene trap mutagenic events, along with improvements in mouse phenotyping capabilities, will further increase an already formidable ability to discover mammalian gene function. The use of conditional and inducible or repressible systems will also have a significant impact on understanding gene function. The current throughput in mouse genetics makes it possible to position this high level of target validation upfront in the discovery process. This will enrich high-throughput screening with candidates of high pharmaceutical value and medical relevance. Such approaches can dramatically increase the efficiency of the drug discovery and development process in the post-genome era.

References

1 Lander, E.S. *et al.* (2001) Initial sequencing and analysis of the human genome. *Nature* 409, 860–921
2 Nolan, P.M. *et al.* (2000) A systematic, genome-wide, phenotype-driven mutagenesis programme for genetic function studies in the mouse. *Nat. Genet.* 4, 440–443
3 Harris, S. (2001) Transgenic knockouts as part of high-throughput, evidence-based target selection and validation strategies. *Drug Discov. Today* 6, 628–636
4 Syzmkowski, D.E. (2001) Too many targets, not enough target validation. *Drug Discov. Today* 6, 398–399
5 Boston Consulting Group . (2001) A revolution in R&D: the impact of genomics. *B.C.G. Focus* 1–15
6 Lehman-McKinsey, (2001) The impact of genomics on drug development. *Lehman-McKinsey Study*
7 Sato, M. *et al.* (2001) The interferon system and interferon regulatory factor transcription factors – studies from gene knockout mice. *Cytokine Growth Factor Rev.* 12, 133–142
8 Venkatachalam, S. and Donehower, L.A. (1998) Murine tumor suppressor models. *Mutat. Res.* 400, 391–407
9 Gingrich, N.A. and Hen, R. (2001) Dissecting the role of the serotonin system in neuropsychiatric disorders using knockout mice. Psychopharmacology 155, 1–10
10 Beck, B. (2001) KO's and organisation of peptidergic feeding behavior mechanisms. *Neurosci. Biobehav. Rev.* 25, 143–158
11 Fazio, S. and Linton, M.F. (2001) Mouse models of hyperlipidemia and atherosclerosis. *Front. Biosci.* 6, 515–525
12 Robinson, S.W. *et al.* (2000) Genetic models of obesity and energy balance in the mouse. *Annu. Rev. Genet.* 34, 687–745
13 Esther, CR, Jr. *et al.* (1996) Mice lacking angiotensin-converting enzyme have low blood pressure, renal pathology, and reduced male fertility. *Lab. Invest.* 74, 953–965
14 Langenbach, R. *et al.* (1995) Prostaglandin synthase 1 gene disruption in mice reduces arachidonic acid-induced inflammation and indomethacin-induced gastric ulceration. *Cell* 83, 483–492
15 Wu, C.S. *et al.* (1996) Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. *Cell* 83, 59–67
16 DeLorey, T.M. *et al.* (1998) Mice lacking the beta3 subunit of the GABA A receptor have the epilepsy phenotype and many of the behavioral characteristics of Angelman syndrome. *J. Neurosci.* 18, 8505–8514
17 Palmiter, R.D. *et al.* (1982) Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. *Nature* 300, 611–615
18 Lazner, F. *et al.* (1999) Osteopetrosis and osteoporosis: two sides of the same coin. *Hum. Mol. Genet.* 8, 1839–1846
19 Yamashita, D.S. *et al.* (2000) Cathepsin K and the design of inhibitors of cathepsin K. *Curr. Pharm. Des.* 6, 1–24
20 Saftig, P. *et al.* (1998) Impaired osteoclastic bone resorption leads to osteopetrosis in cathepsin-K-deficient mice. *Proc. Natl. Acad. Sci. U. S. A.* 95, 13453–13458
21 Huszar, D. *et al.* (1997) Targeted disruption of the melanocortin-4 receptor results in obesity in mice. *Cell* 88, 131–141
22 Chen, A.S. *et al.* (2000) Inactivation of the mouse melanocortin-3 receptor results in increased fat mass and reduced lean body mass. *Nat. Genet.* 26, 97–102
23 Chen, A.S. *et al.* (2000) Role of the melanocortin-4 receptor in metabolic rate and food intake in mice. *Transgenic Res.* 9, 145–154
24 Cronin, C.A. *et al.* (2001) The *lac* operator-repressor system is functional in the mouse. *Genes Dev.* 15, 1506–1517
25 Abu-Elheiga, L. *et al.* (2001) Continuous fatty acid oxidation and reduced fat storage in mice lacking Acetyl-CoA carboxylase 2. *Science* 291, 2613–2616
26 Osada, T. *et al.* (1999) Increased anxiety and impaired pain response in puromycin-sensitive aminopeptidase gene-deficient mice obtained by a mouse gene-trap method. *J. Neurosci.* 19, 6068–6078
27 Komoda, M. *et al.* (2001) Specific inhibitor of puromycin-sensitive aminopeptidase with a homophthalimide skeleton: identification of the target molecule and a structure–activity relationship study. *Bioorg. Med. Chem.* 9, 121–131
28 Venter, J.C. *et al.* (2001) The sequence of the human genome. *Science* 291, 1304–1351
29 Wattler, S. *et al.* (1999) Construction of gene targeting vectors from lambdaKOS genomic libraries. *BioTechniques* 26, 1150–1159
30 Angrand, P-O. *et al.* (1999) Simplified generation of targeting vectors using ET recombination. *Nucleic Acids Res.* 27, 16
31 Bellen, H.J. *et al.* (1989) P-element-mediated enhancer detection: a versatile method to study development in *Drosophila*. *Genes Dev.* 3, 1288–1300
32 Gossler, A. *et al.* (1989) Mouse embryonic stem cells and reporter constructs to detect developmentally regulated genes. *Science* 244, 463–465
33 O'Kane, C.J. and Gehring, W.J. (1987) Detection *in situ* of genomic regulatory elements in *Drosophila*. *Proc. Natl. Acad. Sci. U. S. A.* 84, 9123–9127
34 Hicks, G.G. *et al.* (1997) Functional genomics in mice by tagged sequence mutagenesis. *Nat. Genet.* 16, 338–344
35 Niwa, H. *et al.* (1993) An efficient gene-trap method using poly A trap vectors and characterization of gene-trap events. *J. Biochem.* 113, 343–349
36 Brown, S.D.M. and Peters, J. (1996) Combining mutagenesis and genomics in the mouse – closing the phenotype gap. *Trends Genet.* 12, 433–435
37 You, Y. *et al.* (1997) Chromosomal deletion complexes in mice by radiation of embryonic stem cells. *Nat. Genet.* 15, 285–288
38 Zambrowicz, B.P. *et al.* (1998) Disruption and sequence identification of 2,000 genes in mouse embryonic stem cells. *Nature* 392, 608–611
39 Leighton, P.A. *et al.* (2001) Defining brain wiring patterns and mechanisms through gene trapping in mice. *Nature* 410, 174–179
40 Hubbard, S.C. *et al.* (1994) Generation of Chinese hamster ovary cell glycosylation mutants by retroviral insertional mutagenesis. *J. Biol. Chem.* 269, 3717–3724
41 Li, L. and Cohen, S.N. (1996) *tsg101*: a novel tumor susceptibility gene isolated by controlled homozygous functional knockout of allelic loci in mammalian cells. *Cell* 85, 319–329
42 Gogos, J.A. *et al.* (1996) Gene trapping in differentiating cell lines: regulation of the lysosomal protease cathepsin-B in skeletal myoblast growth and fusion. *J. Cell Biol.* 134, 837–847
43 Skarnes, E. *et al.* (1992) A gene trap approach in mouse embryonic stem cells: the *lacZ* reporter is activated by splicing, reflects endogenous gene expression, and is mutagenic in mice. *Genes Dev.* 6, 903–918
44 Wiles, M.V. *et al.* (2000) Establishment of a gene-trap sequence tag library to generate mutant mice from embryonic stem cells. *Nat. Genet.* 24, 13–14
45 Hrabe de Angelis, M.H. *et al.* (2000) Genome-wide, large-scale production of mutant mice by ENU mutagenesis. *Nat Genet.* 4, 444–447
46 Rathkolb, B. *et al.* (2000) Large-scale *N*-ethyl-*N*-nitrosourea mutagenesis of mice — from phenotypes to genes. *Exp. Physiol.* 85, 635–644
47 Rogers, D. *et al.* (2001) SHIRPA, a protocol for behavioral assessment: validation for longitudinal study of neurological dysfunction in mice. *Neurosci. Lett.* 306, 89–92