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Manipulating the Mouse Genome Using Recombineering

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Abstract

Genetically engineered mouse models are indispensable for understanding the biological function of genes, understanding the genetic basis of human diseases and for preclinical testing of novel therapies. Generation of such mouse models has been possible because of our ability to manipulate the mouse genome. Recombineering is a highly efficient recombination-based method of genetic engineering that has revolutionized our ability to generate mouse models. Since recombineering technology is not dependent on the availability of restriction enzyme recognition sites, it allows us to modify the genome with great precision. It requires homology arms as short as 40 bases for recombination, which makes it relatively easy to generate targeting constructs to insert, change or delete either a single nucleotide or a DNA fragment several kb in size; insert selectable markers, reporter genes or add epitope tags to any gene of interest. In this review, we focus on the development of recombineering technology and its application in the generation of transgenic and knockout or knock-in mouse models. High throughput generation of gene targeting vectors, used to construct knockout alleles in mouse embryonic stem cells, is now feasible because of this technology. The challenge now is to use the “designer” mice to develop novel therapies to prevent, cure or effectively manage some of the most debilitating human diseases.

Keywords

Mouse models; Embryonic stem (ES) cell; knockout mice; gene targeting; recombineering; bacterial artificial chromosome (BAC); transgenic mice

Introduction

Animal models are routinely used to understand the etiology of human genetic disorders as well as to develop preclinical models to test the efficacy of novel therapies. Although, there are species-specific variations based on gene function studies, mouse is the most commonly used and reliable model system mainly because of its genetic and physiological closeness to humans. In addition, advancements in genome manipulation and embryonic stem (ES) cell technologies over the past three decades have made it possible to generate sophisticated transgenic, gene knock-in or knockout mouse models. It is now feasible to generate mutant mice in which inactivation, deletion or ectopic expression of any gene of interest can be spatially and temporally regulated.

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Historical landmarks in the development of genetically engineered mouse models

Development of methods to generate mice with targeted mutation in a gene of interest required the solution of two basic problems: how to target the desired mutation in mammalian cells and how to transfer the manipulated cells into the mouse germline. The solution to the first problem came in 1985, when Smithies et al. showed homologous recombination between an endogenous gene and an artificial targeting vector in mammalian cells [1]. Isolation of pluripotent ES cells from mouse blastocysts and their capacity to colonize the germ line of chimeric mice when injected into blastocysts solved the second problem [2–4]. These landmark achievements led to the generation of first genetically modified mouse in which the *Hypoxanthine Phosphoribosyl Transferase (HPRT)* locus was disrupted [5, 6]. However, isolation of correctly targeted ES cells was facilitated by the fact that only a single copy of this gene is present in an XY-ES cell and also *HPRT*-deficient cells can be positively selected in media containing 6-thioguanine. Subsequent use of a negative selection marker along with a positive selection marker in the targeting constructs allowed enrichment of cells undergoing homologous recombination, which made it possible to target even non-selectable gene [7, 8]. This approach has since been used to generate targeted mutation in hundreds of genes.

A major limitation in the generation of mouse models with desired mutation in the genome was its dependency on the use of conventional genetic engineering methods to generate complex targeting constructs. Often suitable restriction enzyme recognition sites are lacking or manipulation of large DNA fragments is required to generate appropriate targeting constructs, both of which limit the use of standard molecular biology techniques. Manipulation of the mouse genome has been greatly advanced by the availability of *E. coli* based cloning systems that allow construction of genomic libraries with large DNA inserts [9]. These constructs (Bacterial Artificial Chromosomes or BACs) are capable of maintaining inserts as large as 300 kb in size. Furthermore, the development of recombineering technology, a recombination-based method of genetic engineering, has offered exciting new opportunities to manipulate the genome. Here, we will discuss some of the recent advancements in recombineering technology and describe its application in the generation of innovative mouse models of human diseases and novel models to study gene function.

Genetic engineering using homologous recombination in yeast and bacteria

Homologous recombination based genome manipulation was first demonstrated in 1993 by Baudin et al. in yeast [10]. Yeast homologous recombination based method has been used to generate targeting vectors for mouse knockouts [11–13]. Although, homologous recombination in yeast is very efficient, use of yeast-based approaches for manipulation of mouse DNA has been minimal mainly due to generation of undesired genomic rearrangements.

Unlike yeast, *E. coli* degrades foreign linear DNA by the RecBCD exonuclease [14]. However, RecBCD exonuclease-deficient *E. coli* strain could overcome this hurdle and was used as one of the first *in vivo* cloning systems [15]. This *in vivo* cloning system has been used to clone PCR fragments into a plasmid vectors with homologous ends, introducing drug-selectable markers into *E. coli* chromosome and various other applications [15–19]. A major limitation of this system is its dependency on constitutively expressed recombination machinery, requirement of long homology arms and low frequency of recombinants [20].

Use of phage recombination machinery and development of recombineering

A more regulated phage-encoded recombination system has been developed in *E. coli* that allows direct manipulation of the bacterial chromosome as well as any DNA insert cloned in a plasmid or bacterial artificial chromosome (BAC) vectors [19, 21]. This technology is called recombineering and it uses the homologous recombination systems from bacteriophage in *E. coli* to manipulate DNA fragments *in vivo*. It is independent of the use of restriction enzymes and DNA ligase. This powerful system can be used to subclone as large as 80 kb fragment from bacterial artificial chromosomes (BAC) into standard plasmid vectors [22]. This technology has advantages over all other previous recombination based gene manipulation systems as discussed below.

Recombineering originated in 1998, when Dr. Francis Stewart's laboratory used the phage encoded recombination system for *in vivo* genetic manipulation using PCR amplified linear double-stranded DNA flanked by short (42 bp) homology arms [23]. This method, known as the ET cloning, was based on the functions of *recE* and *recT* proteins of λ prophage in *recBC* suppressor mutant of *E. coli*. The *recE* protein provides 5'–3' exonuclease activity and the *recT* protein binds to single-stranded DNA and promotes strand annealing [23]. Further, this method was also used in *recBC⁺* *E. coli* strains by utilizing the *gam* protein of bacteriophage lambda (λ) to suppress *recBCD* function. To allow the use of ET cloning system in any bacterial strain, a plasmid-based mobile system to express *gam* along with *recE* and *recT*, (pBAD-ET λ) was developed where *recE* expression is under the control of an inducible promoter. Expression of *recT* and *gam* genes are under the control of constitutive promoters [23].

Another very similar system that utilizes the λ -red phage homologous recombination machinery was first reported by Kenan Murphy and colleagues [24]. The Red recombination system of λ phage includes two genes: *exo* (*red α*) and *bet* (*red β*) in which *exo* is similar to *recE* and *bet* is like *recT*. The Red system works as efficiently as the ET system [25]. Subsequently, a replication defective λ -prophage based system was developed in which the expression of *exo*, *bet* and *gam* is under the control of their native regulatory elements [26]. In λ -phage genome, these genes are located next to each other in an operon (*p_L*) and their expression is coordinately controlled by both a repressor (CI) and transcription termination. Efficient expression of these genes from *p_L* promoter requires the removal of CI repressor as well as function of anti-transcription terminator N protein. The N protein, also expressed from *p_L* operon, prevents RNA polymerase termination of *p_L* transcripts. In the λ

prophage based system, the red recombination genes are present in single copy in the bacterial genome and their expression is tightly controlled by temperature sensitive λ CI857 repressor. This repressor is inactive at 42°C and turns on the promoter allowing coordinated expression of all three genes [26]. To facilitate BAC engineering this λ -prophage based system has been introduced into BAC host *E. coli* strain (DH10B) and has been efficiently used to engineer BACs [22, 27].

Due to incompatibility of some BACs to the defective λ -prophage system, a more versatile phage based systems (mini- λ and pSIM vectors) are now available that can be introduced to any *E. coli* strain [28, 29]. These mobile systems use endogenous Red recombination genes and regulatory elements. Mini- λ can integrate into the host genome but can be easily excised out via λ -attachment sites. The excised mini- λ can form circular DNA that can be easily purified also using standard plasmid purification protocol. The pSIM vectors are available with variety of resistance markers and require drug selection to be maintained into the host strains. They are based on low copy number plasmid and their replicon is temperature sensitive that allows easy curing of the plasmid after recombineering is complete.

A unique hybrid recombineering system uses both RecA and Red-mediated recombination systems. This strategy utilizes Red- recombination system to integrate a DNA sequence (“pop-in”) along with a selectable marker and *RecA* on a selectable cassette followed by removal of vector DNA sequence by RecA expressed from the cassette (“pop-out”) [30].

A new high-throughput recombineering system has been developed that can be carried out in a 96-well plate and can be applied to modify entire BAC library. This high-throughput recombineering is based on efficiently delivering the recombination machinery into entire BAC library using high titer lysate of a recombination deficient λ phage that carry a selectable marker to select the transduced clones. Once the phage is integrated into the genome it can be stably maintained into the host strain [31].

Applications of recombineering in mouse genome manipulation

Over the years, as the recombineering technology has become more efficient and user friendly, it has become an invaluable tool for genetic manipulation. Recombineering-based methods are being routinely used for mutagenesis and subcloning of genomic fragments cloned in BACs. Together, BACs and recombineering have provided novel approaches to study gene function and generate tools for genetic manipulation. BAC based transgenic mouse models have become a very useful and powerful transgenic model system. In 1997, King et al. were the first to use a BAC transgenic mouse to show the genetic complementation of mouse *clock* mutation [32]. Since then BAC transgenic mice have been used in several studies to complement mouse mutations [33–35]. This is due to the fact that the large insert size contains most regulatory elements required to recapitulate the endogenous gene expression. In addition, the large insert size eliminates the position effects resulting in variability in transgene expression observed in most cDNA-based transgene constructs. Recombineering-based BAC manipulation and use of BACs as transgenes have many different applications such as generation of reporter lines, expression of epitope-

tagged proteins, generation of humanized mouse model to study different human diseases or expression of site-specific recombinases to generate conditional knock-out mouse models. These applications are described in detail below.

1. Generation of reporter lines

The possibility that most *cis*-regulatory elements of any gene are present within a BAC clone has made it a useful tool to generate reporter mouse lines that accurately reflects endogenous gene expression [36]. Reporter genes such as *LacZ* (β -galactosidase), *GFP* (*Green Fluorescent Protein*) can be easily inserted in the BAC insert by recombineering [37–41]. Recently, recombineering technology has been used to physically link multiples genes together with their respective reporter genes [42]. The generation of multi-reporter BAC construct is based on recombineering-mediated BAC linking strategy (Figure 1). Linking of BACs is dependent on use of regions of homology, one of which is present in the vector backbone and the other is common to both genomic inserts. These homology regions flank the selectable marker that allows selection of desired linked clone after linking [43]. To generate a multi-reporter BAC construct Maye et al. first subcloned the genes of interest into BAC linking vectors followed by insertion of desired reporter genes into respective genes and then linked the different BACs containing reporter genes. Using this method, they successfully linked three genes together and generated a multi-reporter mouse line [42, 44].

2. Expression of epitope-tagged proteins

Finding a good antibody for a protein of choice is often a major hurdle in any proteomic research. This can be circumvented by the use of a tagged (FLAG, HA, c-myc etc) version of the protein. To express the tagged protein at physiological levels in mice, knock-in mouse models have been generated, where the tag is targeted to the endogenous gene by homologous recombination in embryonic stem cells. This is quite laborious and time consuming. Tagging the genes in a BAC clone with any desired tag can circumvent these problems. These BACs can be used to generate transgenic mice to express the tagged gene at physiological levels in tissues where it is normally expressed. Using recombineering, genes present in a BAC clone can be efficiently tagged with any epitope [45]. In 2008, Poser et al. developed an efficient, generic and high throughput approach of protein tagging and BAC transgenesis in mammalian cells. This approach can be used to study the localization, protein-protein and/or protein-DNA interactions of any protein [46].

3. Generation of Knockout mouse

A knockout mouse is a strain of mutant mouse in which the gene of interest is “knocked-out” or deleted or replaced by an exogenous DNA fragment such that the gene is rendered non-functional. Knockout mice have proven to be an invaluable tool to understand the biological function of mouse genes. Knockout mice have also been used to model various human diseases like cancer, obesity, heart disease, diabetes, arthritis, substance abuse, anxiety, aging and Parkinson disease [44, 47–54]. These models are also used for developing and testing new drugs [55–57].

Generation of knockout mice involves construction of a targeting vector that can be used to disrupt the gene of interest in ES cells. A typical targeting vector consists of two homology arms flanking a positive selection marker and a negative selection marker [*e.g. Thymidine Kinase (TK)* or *Diphtheria toxin (DT)*] next to one of the homology arms. The positive selection marker is inserted such that it replaces some of the key exons rendering the gene non-functional. Next, the targeting vector is electroporated into ES cells and by homologous recombination one allele of the target gene is replaced by the sequences cloned in the targeting vector. Correctly targeted ES cells are isolated by drug selection followed by confirmation by Southern analysis or PCR-based methods. These cells are then microinjected into blastocysts and implanted into pseudo-pregnant females to generate chimeras. Chimeras with ES cell contribution in their germline will transmit the mutant allele when bred to wild type mice. Offspring inheriting the mutant allele are heterozygous for the mutation and when these are intercrossed, homozygous offspring lacking any functional copy of the gene are obtained. If the gene is essential for viability, such mice die during embryogenesis. Phenotypic characterization of homozygous mice or embryos provides clues to the biological function of the targeted gene.

Construction of targeting vectors

An important and time consuming step in the generation of a knockout mouse model is the construction of gene targeting vectors that are used to delete or mutate genes by homologous recombination in mouse ES cells. Advances in recombineering technology and availability of BAC clones have greatly simplified the process, especially two key steps: a) retrieval or subcloning a large fragment of DNA (used as homology arms) from a BAC clone into a plasmid vector and b) insertion of a selectable marker into the retrieved fragment.

a) Retrieval of genomic DNA from a BAC clone—Retrieval or subcloning of a DNA fragment from a BAC clone into a plasmid DNA by recombineering requires a retrieval vector. The essential elements of a retrieval vector are a plasmid backbone sequence including an origin of replication and a selectable marker such as ampicillin or kanamycin resistance gene, flanked by 50 bases of homology to the 5' and 3' ends of DNA to be subcloned. The retrieval vector can be rapidly synthesized by PCR using two chimeric primers (5' 50 nucleotides of each primer is homologous to the two ends of DNA to be subcloned and 3' 20 nucleotides is homologous to the plasmid DNA) and the linear plasmid DNA as a template (Figure 2). Once electroporated into bacterial cells expressing the recombineering proteins, linear vector undergoes homologous recombination with the BAC DNA and by the process of gap repair circularizes the linear plasmid DNA allowing it to replicate in *E. coli* (Figure 2). Presence of an antibiotic resistance gene is used to select for the circular plasmid. The plasmid used to generate retrieval vector also contains a negative selection marker (*DT* or *TK* gene) [58].

b) Insertion of selectable markers—After the retrieval of the genomic DNA, the next step is the removal of functionally important exons and insertion of a selectable marker (genes that allows resistance to antibiotics like blasticidin, neomycin, hygromycin and functions in both, eukaryotic as well as prokaryotic cells). The selectable markers are under the control of both a prokaryotic (*e.g. Tn5, EM7*) as well as a eukaryotic promoter (*e.g.*

PGK). Such dual promoters allow the use of the same selectable marker in both bacterial (for recombineering) as well as mammalian cells (for gene targeting in ES cells). Such selectable markers can be inserted into the plasmid using a targeting construct containing selectable marker flanked by homologous region of target site. These constructs can be prepared by PCR using chimeric primers as described above.

4. Construction of conditional knockout vectors

A major limitation of the knockout mouse technology is that loss of function mutation in most functionally important genes results in embryonic lethality. This makes it difficult to analyze the function of the gene in adults, which is relevant to most human disease genes. This problem is circumvented by the development of conditional knockout approach where the deletion of the gene can be regulated: spatially as well as temporally. Tissue or cell specific deletion of any gene plays an important role in revealing the functions of genes essential for development and also to study the relationship between gene mutations and development of diseases in adult mice. Conditional deletion of a gene has been achieved by introducing bacteriophage and yeast-derived site-specific recombination (SSR) systems into the mouse. Bacteriophage P1 derived site specific recombinase “Cre” (causes recombination) and yeast derived recombinase “Flp” (Flippase) recognize 34 base pair DNA sequences *loxP* (locus of crossover in phage P1 or *FRT* (Flippase recognition target), respectively [59]. Depending on the orientation of the target sites with respect to one another, recombinases can excise, integrate or invert DNA sequences. Conditional deletion of a gene is achieved by flanking the critical exons of any gene by two *loxP* or *FRT* sites (conditional allele). Subsequent expression of the recombinases in a cell containing the conditional allele of a gene, either the entire gene or the critical exons can be deleted rendering the gene non-functional [60, 61]. Until recombineering technology became available, generation of conditional knockout vectors was considered a lengthy task. Using recombineering, conditional knockout targeting vectors can be rapidly generated by introducing *loxP* or *FRT* or both the sites along with the positive selection marker into the BAC DNA [58].

5. Transgenic mice expressing Cre or Flp recombinase

Conditional deletion of any gene relies on targeted expression of Cre or FLP recombinase in the cell/tissue of interest. In most cases the regulatory elements including the enhancer sequences that drive tissue specific expression of a gene are not well characterized. In the absence of such information, it is more convenient to use BAC-based transgenic mice to express Cre or Flp recombinase. This is conveniently achieved by inserting these recombinases by recombineering to the 5' or 3' ends of genes present in a BAC clone. These BAC clones can then be used to generate transgenic mice. This approach has greatly facilitated the generation of tissue specific Cre transgenic mouse lines. Several Cre lines for conditional expression of recombinases have been generated and some of them are listed in Table 1. Information on additional transgenic mouse lines expressing Cre can be obtained from The Jackson laboratory (<http://www.jax.org/search/Main.jsp?qt=cre+mice&x=0&y=0>) and also from CREATE (coordination of resources for conditional expression of mutated mouse alleles ; <http://www.creline.org/eucommtools>). To temporally regulate the expression

of Cre protein in cells, tamoxifen-inducible Cre-ER (estrogen receptor) fusion protein can be used [62]. This Cre-ER protein is functional only in the presence of tamoxifen, which can be injected into mice at the desired time.

6. High throughput knockout constructs

To allow generation of multiple knockouts in parallel, a high throughput largely automated approach was reported by Valenzuela *et al.* in 2003. This technology, named as VelociGene, uses the entire BAC insert for homologous recombination in mouse ES cells.

Recombineering is used to disrupt the gene of interest with a selection/reporter cassette in the BAC. The large size of the homology arms makes recombination extremely efficient. ES cell clones undergoing homologous recombination are screened either by quantitative PCR-based method to detect the loss of endogenous allele or by Fluorescent in-situ hybridization (FISH) using two different probes (one specific for endogenous allele and other for recombinant allele) [63].

Another high-throughput method to construct knockout mice has been developed where conventional gene targeting constructs are generated using the recombineering technology. The recombination machinery is transferred directly to the BAC containing *E. coli* using high titer λ lysogen and recombineering is carried out in 96-well format. Using this high throughput recombineering, authors could generate 94 constructs simultaneously [31]. More recently, similar approaches have been used to generate a conditional knockout resource for genome wide functional analysis of genes [64, 65].

7. Generation of hypomorphic alleles for functional dissection

For functional dissection of any gene, hypomorphic alleles, each with a subtle alteration in different functional domains, are very informative. Random mutagenesis approach using a chemical mutagen (*N*-ethyl-*N*-nitrosourea) has been efficiently used to create allelic series of *Smad2*, *Smad4* and *Kit ligand* (*Kitl*) in mouse ES cells [66–68]. The alterations generated by chemical mutagenesis are random. Recombineering allows generation of specific subtle alterations in any gene. This can be achieved either by seamless selection-counter selection method or by using single stranded (ss) oligonucleotides.

a) Selection-counter selection method

Seamless modification using selection-counter selection method uses two sequential steps (Figure 3). In the first step, a dual selection-counter selection cassette is inserted at the site of modification. In the next step, this cassette is replaced by a targeting cassette, which consists of the exogenous sequences including the mutation (point mutation, deletion or small insertions) that needs to be introduced. Recombinants are selected for loss of the counter-selectable marker based on the toxicity produced by counter selectable gene under specific conditions [69]. A number of selection-counter selection cassettes have been developed and they are listed in Table 2. To modify DNA containing significant repeating homologies use of a long homology arm (200 bp or more) is effective in seamless modification using counter selection recombineering [70].

One drawback of counter selection methods is that occasionally intra molecular recombination occurs resulting large genomic alterations. Recently, Bird et al. developed a method to reduce these unwanted events by expressing Red β alone during counter selection and using phosphothiotated oligonucleotides [71].

b) BAC modification using single-stranded (ss) DNA

Recombineering using single-stranded (ss) oligonucleotides is 10–100 fold more efficient than using double-stranded (ds) DNA [26, 72]. Oligonucleotide based recombineering has been successfully used to generate point mutations, insertions and deletions in mouse homolog of breast cancer susceptibility gene 2 (*BRCA2*) using short homology arms [73]. The recombinants were screened by “mismatch amplification mutation assay-PCR” (MAMA-PCR) [73, 74]. Because of the high rate of false positives, a two-step “hit and fix” method was developed. In the first step, 6–20 nucleotides surrounding the point of interest is replaced by an exogenous sequence. In the second step, that exogenous sequence is changed to the original sequence except the desired subtle change. In this method, recombinants can be screened by colony hybridization or PCR using the oligonucleotides designed from the region of change [75]. This approach has been used to introduce point mutations in human *BRCA1* and *BRCA2* genes cloned in a BAC vector to generate point mutations. These BACs were used to determine the functional significance of BRCA1/2 variants of unknown clinical significance using a mouse ES cell-based functional assay [76, 77].

8. Multiple alterations

A multipurpose allele of any gene can be constructed using sequential rounds of recombineering. Testa et al. generated a BAC targeting construct in which the authors introduced an N-terminal tag, a positive selectable marker and *loxP* in an intron to 5' of region to be deleted, another *loxP* site to the 3' of the region to be deleted followed by a reporter construct with an internal ribosome entry (IRES) site after the stop codon. In addition, cassette containing positive selection marker targeted into the intron contains a splice acceptor site that may generate a hypomorphic allele. After recombination into ES cells, this construct allows generation of three different alleles (tagged wild-type allele, conditional allele, and a tagged hypomorphic allele) in mice [78].

9. Humanized mouse models of human diseases

According to the published mouse genome sequence in 2002, 99% of mouse genes have human homolog. However, putative regulatory regions of the two genomes demonstrated lower level of conservation [79]. Using a strictly mouse genome based approach to validate human regulatory regions may not be very informative. Humanized mouse models in which human genes are introduced into mouse provide an approach to overcome this problem. Humanized mouse models can also be used to examine the physiological significance of single nucleotide changes identified in human genes. A humanized mouse model for *Breast Cancer Susceptibility Gene 1 (BRCA1)* revealed that a missense mutation in codon 64 that results in cysteine to glycine change, results in premature protein truncation because the single nucleotide change also generates a cryptic splice donor site resulting in aberrant splicing [80]. Similarly, humanized mouse models have been developed for Nijmegen

Breakage Syndrome, glaucoma and Parkinson's disease that allows the study of disease causing human mutations [81–83].

In these initial studies, human BACs were randomly integrated into the mouse genome. This made it essential to analyze multiple independent lines to rule out of the possibility that the BAC integration site did not influence the phenotype observed in transgenic mice. Also, often, instead of full-length BAC, fragments of the BAC DNA are inserted. To overcome these problems, a high throughput single copy site-specific method to target the BACs to the *Hprt* locus on X-chromosome using *Hprt* docking technology has been developed [84]. This approach was used to generate seven different alleles of human *Nuclear Receptor 2E1* (*NR2E1*) gene.

In recent years, several genetically engineered mouse models have been generated that have taken advantage of the BAC transgenic approach and are being used to understand the pathophysiology of various diseases. One of these is a novel mouse model of human α thalassemia [85]. In this model the mouse α globin regulatory domain was replaced with the human syntenic region by recombinase-mediated genomic replacement using modified BACs in mouse ES cells. The modified ES cells were used to generate mice that produce only human α globin chains. Multiple BAC transgenic mouse have also been developed to study basal ganglial neurodegenerative disorders, such as Parkinson's disease (PD) and Huntington's disease (HD). BAC-mediated over-expression of human mutant parkin-Q311X using dopamine transporter promoter or human Lrrk2 R1441G mutation in mice developed "hallmark features" of human PD patients [83, 86]. In another mouse model for PD, human BAC containing α -Synuclein (α -syn) was used to overexpress the Synuclein protein, the main component of Lewy bodies. These mice exhibited decreased anxiety-like behaviors, which may reflect non-motor symptoms of early PD [87]. By expressing full-length human mutant *Huntingtin* (*HTT*) gene with 97 glutamine repeats under the control of endogenous regulatory elements using BAC, transgenic mice was developed by Gray et al. in 2008. The behavioral and neuropathological phenotypes of the mice revealed that they are suitable for further investigation of HD pathogenesis and also for preclinical studies [88]. Zhou et al. introduced missense mutation (Tyr437His) into human Myocilin (*MYOC*) gene cloned in a BAC and used this mutant BAC to generate a mouse model of primary open-angle glaucoma. The pathological changes observed in the eye of transgenic mice reflected those observed in patients with glaucoma [89]. To study host pathogen interaction for human pathogens like *Neisseria gonorrhoeae*, a BAC transgenic mouse model has been developed that expresses human CEACAM1 (carcinoembryonic antigen related cell adhesion molecule-1). This model overcomes the limitation of studying interaction of *Neisseria gonorrhoeae* *in vivo*, since murine CEACAM1 does not bind to this human pathogen [90].

Limitations of recombineering

Although recombineering technology has many advantages over conventional cloning methods, there are certain limitations of this approach. A prerequisite for the use of recombineering based methods is the availability of the DNA sequence of the region to be manipulated. However, due to the availability of the sequence of most commonly used mouse strains, this is not a serious limitation. Because the targeting constructs for

recombineering are generated by PCR, occasionally undesired mutations can inadvertently be introduced into the genome. It is therefore recommended to confirm the sequence of the modified region to rule out the introduction of any unwanted mutation. Also, recombineering can be challenging to manipulate genomic targets that contain repetitive sequences because the presence of even short repetitive sequences in the homology arms can result in integration of the targeting constructs to random sites. Although not very effective, this can be overcome by increasing the length of homology arms. In spite of these limitations, recombineering technology continues to be a powerful tool that has allowed us to overcome numerous obstacles and made it possible to manipulate the mouse genome in ways that could only be imagined 10–15 years ago.

Concluding remarks

Since the sequencing of the human genome a decade ago, the challenge has been to develop innovative approaches for functional analysis of all the annotated genes. It was envisioned that mouse models would play an important role in this endeavor. This led to the development of ambitious projects including the establishment of the International Knockout Mouse Consortium (IKMC) in 2007. IKMC was established with the aim to generate mutation in all the protein-coding genes. Taking advantage of high throughput ES cell technologies including gene-trapping and recombineering-based method to generate gene targeting vectors, already targeted mutation in more than 17,400 unique genes have been generated in ES cells and 1,700 mouse lines carrying mostly conditional alleles of genes have been made available to the scientific community [65]. Such an achievement is a testament of the availability of tools and technologies that on one hand have oversimplified the process, yet on the other hand, made it possible to reach a level of complexity that allows us to alter the mouse genome as precisely as required in a relatively short time. Phenotypic characterization of these knockout mouse models, along with the conventional and BAC transgenic models will help us to understand the biological functions of the genes that remain uncharacterized so far. Several of these mouse models will be used in preclinical studies to test novel drugs designed to treat human diseases.

Recently, there has been an exponential growth of genome-wide association studies (GWAS) aimed at identifying loci associated with different human diseases [91–97]. Such studies have led to the identification several SNPs that are likely to be associated with different disorders in humans. The candidate variants associated with the diseases are present both in protein-coding regions and regulatory regions of genome. However, in most cases, the effect of the SNPs on gene function or disease predisposition remains elusive. Functional characterization of the SNPs using BAC-based humanized mouse models may provide clues to link the SNPs with the disease. It is expected that such efforts will be greatly aided by the recombineering technology.

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Figure 1. Strategy to link two BAC inserts together:

To link two BAC inserts together, the inserts are first subcloned using recombineering into two vectors (BAC linking vectors) containing different antibiotic resistance markers. Subcloned BAC inserts are then joined together using two homology regions (one is common to both BAC inserts and the other is from the antibiotic marker present in both the plasmid vectors used to subclone the BAC). The first BAC insert (BAC1) is maintained as circular DNA in recombineering competent cells. The vector for this BAC insert contains two antibiotic resistance markers (**Ab^a** and **Ab^b**) and a single copy origin of replication. The second BAC insert (BAC2) subcloned in a vector with two antibiotic resistance markers (**Ab^b** and **Ab^c**) is linearized and electroporated into the cells containing BAC1. Recombination between two BACs using the common homology region (**HR**) and **Ab^b** will result in a circular DNA that will have two BACs linked together. The linked BAC can be selected for resistance to antibiotic marker B and C (**Ab^b** and **Ab^c**) and sensitivity to antibiotic marker A (**Ab^a**). The BAC insert regions in the vectors are shown in gray.

Figure 2. Retrieval of BAC DNA into plasmid:

To retrieve a fragment of the BAC DNA insert into a plasmid, a targeting construct is generated by PCR using chimeric primers and linear plasmid DNA as template. Each PCR primer has 50 bases at its 5' end (gray) corresponding to the sequence of region of BAC to be retrieved to provide homology and approximately 20 bases at the 3' end from the plasmid sequence (black) to amplify the plasmid DNA. Homology regions for recombination are marked as "a" and "b". The homology region of plasmid sequence to the primers is from the flanking sequences between origin of replication (*Ori*) and antibiotic marker (*Amp*). The linear PCR product (retrieval vector) is then electroporated into recombineering competent cells containing the BAC. Recombination between the linear retrieval vector and the BAC

DNA results in the formation of a circular plasmid by gap repair that will have the desired sequence. Recombinant plasmids are selected for the marker present in plasmid (*Amp*).

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STEP 1: SELECTION **STEP 2: COUNTERSELECTION** **Figure 3. Seamless modification of BAC DNA:**

To modify a BAC insert by selection-counter selection method, first the selection/counter selection marker is amplified using chimeric primers (5' 50 bases of each primer has homology to the region to be targeted [dark gray] and 3' 20 bases of each primer has homology to the marker [light grey]) by PCR to generate targeting construct I. This construct is targeted to the target site by recombineering and selected for the positive selection marker. In the next step, a targeting construct containing desired modification (marked as filled star) along with homology arms is targeted to the cells containing recombinant DNA from the step 1. Correctly targeted clones are selected for the loss of the counter selection marker. Homology arms used are marked with "a" and "b".

Table 1.

Summary of BAC based Cre transgenic lines

Name of Cre line	Promoter used to express Cre	Genetic background of transgenic mice	Cells expressing Cre	References
<i>Ngn3-Cre</i>	<i>Neurogenin 3 (ngn3)</i>	CD1	Small intestinal enteroendocrine cells, significant fractions of goblet and Paneth cells in the intestine and a small number of duct and acinar cells in the pancreas	[98]
<i>BAC-Pcp2-IRES-Cre</i>	<i>Purkinje cell protein 2 (Pcp2)</i>	C57BL/6Ncr _ C3H/HeNcrMtv	Purkinje and retinal bipolar cell	[99]
<i>Pax2-IRES-cre</i>	<i>Pax2</i>	CD1	Inner ear, midbrain, cerebellum, olfactory bulb, kidney	[100]
<i>Prestin-IRES-Cre</i>	<i>Prestin</i>	FVB/NJ	Inner and outer ear hair cells, a subset of vestibular hair cells, spiral and vestibular ganglia in the inner ear, and a subset of cells in the testis, epididymis, and ear bone	[101]
<i>PR-BAC^{Cre}</i>	<i>Progesterone receptor (PR)</i>	Mixed background of FVB/N and C57BL6	Progesterone target tissues i.e., mammary gland, ovary, oviduct, uterus, pituitary gland.	[102]
<i>Sf1-Cre</i>	<i>Steroidogenic factor 1 (SF-1, officially designated Nr5a1)</i>	C57BL/6J	Somatic cells of the gonads, the adrenal cortex, the anterior pituitary, the spleen, and the ventromedial hypothalamic nucleus	[103]
<i>Foxl1-Cre</i>	<i>Foxl1</i>	B6/SJL hybrid	Gastrointestinal mesenchyme	[104]
<i>BAC-Dkk3-Cre</i>	<i>Dkk3</i>	C57BL/6J and C3H/HeJ hybrid	Retinal progenitors	[105]
<i>5-HT1A-Cre</i>	<i>5-HT1A receptor</i>	C57BL/6	Cerebral cortex, septum, hippocampus, dorsal raphe, thalamic, hypothalamic and amygdaloid nuclei, and spinal cord	[106]
<i>Pf4-Cre</i>	<i>Platelet factor 4 (Pf4), also called CXCL4</i>	C57BL/6	Megakaryocyte	[107]
<i>BAC-Col10-Cre</i>	<i>Col10a1</i>	FVB mice and FV/C56Bl F1 hybrids	Hypertrophic cartilage	[108]
<i>BAC-Ela-CreErT</i>	<i>Elastase gene</i>	Not available	Pancreatic acinar cells	[109]
<i>Pitx3-CreER(T2)</i>	<i>Pitx3</i>	FVB	Ocular lens and skeletal muscle	[110]
<i>Mitf-Cre</i>	<i>Microphthalmia-associated transcriptional factor (Mitf)</i>	CBA and C57/Bl6 hybrid	Melanocyte	[111]
<i>Cited1-CreER^{T2}</i>	<i>Cited1</i>	FVB	Cap mesenchyme	[112]
<i>Nkx2.1-Cre</i>	<i>Nkx2.1</i> (Homeodomain transcription factor)	C57BL/6	Brain, Thyroid, pituitary, lung	[113]
<i>Mcpt5-Cre</i>	<i>Mast cell protease (Mcp) 5</i>	C57BL/6	Mast cell	[114]
<i>GlcNAc6ST-2-Cre</i>	High endothelial venule (HEV) expressed sulfotransferase, N-acetylglucosamine-6-O-sulfotransferase 2 (<i>GlcNAc6ST-2</i>)	C57BL/6J and C3H/HeN hybrid	Colonic villi and in a small subset of cells in the brain, testis, stomach, small intestine, and lung	[115]
<i>Probasin-MerCreMe</i>	<i>Probasin</i>	C57BL/6	Prostate epithelium	[116]
<i>LC-1-hCD19-CreER(T2)</i>	Human <i>CD19</i> promoter	C57BL/6	B-lymphocyte	[117]
<i>Tbx18-Cre</i>	<i>Tbx18</i>	C57Bl/6 and CBA hybrids	Smooth muscle cells and stromal cells in lower urinary tract	[118]

Name of Cre line	Promoter used to express Cre	Genetic background of transgenic mice	Cells expressing Cre	References
<i>IL-7.Cre</i>	<i>IL-7</i>	FVB	IL-7 producing cells	[119]
<i>SMA-Cre-ER(T2)</i>	<i>Smooth muscle alpha actin (SMA)</i>	FVB/N	Vascular and visceral smooth muscle cells	[120]
<i>AdipoqCreER^{T2}</i>	<i>Adipoq</i>	C57BL/6	Adipose tissue	[121]
<i>Foxn4-Cre</i>	<i>Foxn4</i> (transcription factor)	C57BL/6 and CBA hybrids	Developing spinal cord	[122]
<i>Hsd17b1-iCreER(T)²</i>	<i>Hydroxysteroid (17-beta) dehydrogenase 1 (Hsd17b1)</i>	C57BL/6 and CBA hybrids	Ovarian granulosa cells	[123]
<i>TFF2-BAC-Cre(ERT2)</i>	<i>TFF2</i> promoter	Injected in C57BL/6 and CBA hybrids and maintained in C57BL/6	TFF2 expressing cells like gastric progenitor cells	[124]
<i>BAC-Vglut2::Cre</i>	<i>Vesicular glutamate transporter 2 (Vglut2)</i>	C57BL/6	Vglut2 positive cells in the spinal cord, thalamus, hypothalamus, superior colliculi, inferior colliculi and deep cerebellar nuclei together with nuclei in the midbrain and hindbrain	[125]
<i>GlyT2-Cre</i>	<i>Glycine transporter 2 (GlyT2)</i>	C57BL/6 and DBA/2J hybrids	Caudal regions of the central nervous system, i.e., brain stem and spinal cord	[126]
<i>Slco1c1-Cre</i>	<i>Solute carrier organic anion transporter 1c1 (SLCO1C1)</i>	Injected in C57BL/6 and DBA/2J hybrids and maintained in C57BL/6	Neurons of various brain structures, such as cortical layer 2/3 and the hippocampus	[127]
<i>AvCreERT2</i>	<i>Advillin</i>		Sensory ganglia	[128]
<i>SPO11-IRES-Cre</i>	<i>Sporulation protein 11 (Spo11)</i>	BalbC57	Meiotic germ cells	[129]
<i>minKCreER^{T2}</i>	<i>mink (KCNE1)</i>	CD1	Atrioventricular (AV) node, AV bundle, and bundle branches	[130]
<i>Neurog1-CreER(T2)</i>	<i>Neurog1</i>		Glutamatergic neurons	[131]
<i>MART-1::Cre</i>	<i>MART-1</i>	Injected in C57BL/6 and DBA/2J hybrids and maintained in C57BL/6	Pigment producing cells like melanocytes, retinal pigment epithelium	[132]
<i>DCX-CreER(T2)</i>	<i>Doublecortin (DCX)</i>	C57/B6L	Newborn neurons	[133]
<i>TgUPCreERT2</i>	<i>Uroplakins</i>	C57BL/6 and CBA hybrid	Urothelium	[134]
<i>Sox2-CreER</i>	<i>Sox2</i> (Transcription factor)	FVB	Central nervous system stem/progenitor cells	[135]
<i>Ikaros-Cre</i>	<i>Ikaros</i> (Transcription factor)	Not available	Retinal progenitor cells	[136]
<i>FoxP3-GFP-hCre</i>	<i>FoxP3</i>	Not available	Regulatory T cells	[137]
<i>Megsin-Cre</i>	<i>Megsin</i> (Serine protease inhibitor)	C57BL/6 and CBA hybrid	Skin, Forestomach, Esophagus	[138]
<i>Prrx11-CreER(T2)</i>	<i>Prrx11</i>	FVB/N	Primary somatosensory ganglia, weak expression in the spinal dorsal horn, mesencephalic trigeminal nucleus, principal sensory trigeminal nucleus, and spinal trigeminal nucleus	[139]
<i>Sox10-iCreER(T2)</i>	<i>Sox10</i> (Transcription factor)	C57BL/6	Neural crest cells, their derivatives and the oligodendrocyte lineage	[130, 140]

Table 2.

Different selection-counter selection cassettes used in generation of mutation by recombineering

Cassette	Selection	Counter-selection	References
<i>neo-sacB</i>	Resistance to kanamycin (<i>neo</i>)	Growth on Sucrose medium	[69]
<i>galK</i>	Growth on minimal media with galactose	Resistance to 2-deoxygalactose	[27]
<i>thyA</i>	Growth in the absence of thymine	Growth on trimethoprim and thymine	[141]
<i>neo-rpsL⁺</i>	Resistance to kanamycin	Resistance to streptomycin	[142]