

Manipulating the Mouse Genome Using Recombineering

Kajal Biswas and Shyam K Sharan*

Mouse Cancer Genetics Program, Center for Cancer Research, National Cancer Institute at Frederick, Frederick, Maryland 21702, USA

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 or 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 to the generation of genetically engineered mouse models. High-throughput generation of gene targeting vectors, used to construct use these "designer" mice to develop novel therapies to prevent, cure, or effectively manage some 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 speciesspecific variations based on gene function studies, the mouse is the most reliable and commonly used model system, mainly because of its genetic and physiological similarity 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 genetically engineered 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.

Historical landmarks in the development of genetically engineered mouse models

Development of methods to generate mice with a 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, which are able 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 the 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 in this case by the fact that only a single copy of this gene is present in an XY-ES cell and that 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 genes [7,8]. This approach has since been used to generate targeted mutations in hundreds of genes.

A major limitation to the generation of mouse models with desired mutations in their genomes was a 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 Escherichia coli-based cloning systems that allow construction of genomic libraries with large DNA inserts [9]. These constructs, called bacterial artificial chromosomes (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 yeast by Baudin et al. [10]. The yeast homologous recombination-based method has subsequently been used to generate targeting vectors for mouse knockouts [11-13]. Although homologous recombination in yeast is very efficient, use of yeast-based approaches

*Corresponding author: Shyam K Sharan, Building 560, Room 32-31C, 1050 Boyles Street, National Cancer Institute at Frederick, Frederick, MD 21702, USA, Tel: (301) 846-5140; Fax: (301) 846-7017; E-mail: sharans@mail.nih.gov

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for manipulation of mouse DNA has been minimal, mainly due to generation of undesired genomic rearrangements.

Unlike yeast, *E. coli* degrades foreign linear DNA with its RecBCD exonuclease [14]. Consequently, any genetic manipulation by homologous recombination using a linear targeting construct is not feasible in *E. coli*. However, development of a RecBCD exonuclease-deficient *E. coli* strain overcame this hurdle and was used as one of the first *in vivo* cloning systems [15]. This system has been used to clone PCR fragments into plasmid vectors with homologous ends, introducing drug-selectable markers into the *E. coli* chromosome, and for various other applications [15-19]. The major limitations of this system are that it depends on constitutively expressed recombination machinery, requires long homology arms, and produces a 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 into a plasmid or BAC vector [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 does not require the use of restriction enzymes and DNA ligase. This powerful system can be used to subclone a fragment as large as 80 kb from BACs 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 a 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 ET cloning, is based on the functions of the recE and recT proteins of the Rac prophage in an E. coli recBC suppressor mutant. The recE protein provides 5' to 3' exonuclease activity, and the recT protein binds to single-stranded (ss) DNA and promotes strand annealing [23]. Further, this method was also used in recBC⁺ E. coli strains by utilizing the gam protein of λ) bacteriophagetosuppress lambda (recBCD function. To allow the use of the ET cloning system in any bacterial strain, a plasmid-based mobile system that expresses gam along with recE and recT (called pBAD-ETA) was developed, with *recE* expression under the control of an inducible promoter and expression of the *recT* and *gam* genes under the control of constitutive promoters [23].

Another, similar, system, which utilizes the λ Red phage homologous recombination machinery, was first reported by Kenan Murphy and colleagues [24]. The Red recombination λ phage system of includes two genes: *exo* (*red* α) and *bet* (*red* β), in which *exo* is similar to *recE* and bet is analogous to *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 the under the control of their native regulatory elements [26]. In the λ phage genome, these genes are located next to each other in an operon (p_L), and their expression is coordinately controlled by a repressor (CI) and transcription termination. Efficient expression of these genes from the p_L promoter requires the removal of the CI repressor as well as the function of the anti-transcription terminator N protein. The N protein, also expressed from the p_L operon, prevents RNA polymerase termination of p_I transcripts λ . In the prophage-based system, the Red recombination genes are present in single copies in the bacterial genome, and their expression is tightly controlled by the temperature-sensitive λ CI857 repressor. This repressor is inactivated at 42°C, which 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 a BAC host *E. coli* strain (DH10B) and has been efficiently used to engineer BACs [22,27].

Due to incompatibility between some BACs and the defective λ prophage system, more versatile phage-based systems (mini- λ and pSIM vectors) are now available that can be introduced into 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 λ be easily excised via -attachment sites. Also, the excised mini- λ can form circular DNA that can be easily purified using standard plasmid purification protocols. The pSIM vectors are available with a variety of resistance markers and require drug selection to be maintained in the host strains. They are based on low-copy-number plasmids, and their replicons are temperature sensitive, which allows easy curing of the plasmids after recombineering is complete.

A unique hybrid recombineering system uses both RecA and Red-mediated recombination. This strategy utilizes the Red system to integrate a DNA sequence ("pop-in") using a selectable marker. In the next step, *RecA*, which is inserted along with the selectable marker in the first step, is used for the removal of the vector DNA sequence ("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 an entire BAC library. This high-throughput recombineering is based on efficiently delivering the recombination machinery into an entire BAC library using a high-titer lysate of a recombination-deficient λ phage that carries a selectable marker to select the transduced clones. Once the phage is integrated into the genome, it can be stably maintained in the host strain [31].

Applications of recombineering in mouse genome manipulation

Over the years, as 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. [32] were the first to use a BAC transgenic mouse to show the genetic complementation of the mouse Clock mutation. Since then, BAC transgenic mice have been used in several studies to complement mouse mutations [33-35]. This system works well due to the fact that the large insert size contains most of the regulatory elements required to recapitulate the endogenous gene expression. In addition, the large insert size eliminates the position effects that typically result in the transgene expression variability observed in most cDNA-based transgene constructs.

By using recombineering, any selectable marker can be easily inserted into a BAC clone simply by generating a targeting construct containing the selectable gene flanked by the homology arms (40-80 bases each) containing sequences from the region where the marker

needs to be targeted. Such targeting constructs can be rapidly generated by PCR using chimeric primers that have 20 bases complementary to the selectable gene and 40-80 bases corresponding to the region of homology. In the presence of the recombineering proteins, the selectable gene can be inserted at the target site and recombinant clones can be selected for the presence of the selectable maker. The same approach can also be used to insert any non-selectable DNA fragment. In such cases a selectable marker, like any antibiotic resistance gene is included next to the non-selectable gene in the targeting construct. The selectable marker can be subsequently removed from the BAC by flanking it with two *loxP* or *FRT* sites. The selectable marker can be excised in the presence of Cre or Flp recombinase. For seamless insertion of a non-selectable marker, a two-step approach of selection/ counter-selection can be used as described below in subsection 7 (Generation of hypomorphic alleles for functional dissection). These approaches have overly simplified generation of BAC transgene constructs that can be used for 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 knockout mouse models. These applications are described in detail below.

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 reflect endogenous gene expression [36]. Reporter genes, such as *LacZ* (β -galactosidase) and *GFP* (green fluorescent protein) can be easily inserted in the BAC by recombineering [37-41]. Recently, recombineering technology has

been used to physically link together multiple genes, along with their respective reporter genes [42]. The generation of a multi-reporter BAC construct is based on recombineering-mediated BAC linking strategy (Figure 1). Linking of BACs is dependent on the use of regions of homology, one of which is present in the vector backbone and the other common to both genomic inserts. These homology regions flank the selectable marker that allows the selection of a desired linked clone after linking [43]. To generate a multi-reporter BAC construct, Maye et al. [42] first subcloned genes of interest into BAC linking vectors, inserted the desired reporter genes into respective genes, and then linked the different BACs containing the reporter genes. Using this method, they successfully linked three genes and generated a multi-reporter mouse line [44].

Expression of Epitope-Tagged Proteins

Finding a good antibody against 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, knockin mouse models have been generated in which the tag is targeted to the endogenous gene by homologous recombination in ES cells. This process, however, 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 that 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. [46] developed an efficient, generic, and high-throughput approach for protein tagging and BAC transgenesis in mammalian cells. This



(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 (Aba and Abb) and a single copy origin of replication. The second BAC insert (BAC2) subcloned in a vector with two antibiotic resistance markers (Aba and Abc) is linearized and electroporated into the cells containing BAC1. Recombination between two BACs using the common homology region (HR) and Abb 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 (Abb and Abc) and sensitivity to antibiotic marker A (Aba). The BAC insert regions in the vectors are shown in gray.

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approach can be used to study the localization, protein-protein, and/ or protein-DNA interactions of any protein.

Generation of Knockout Mouse

A knockout mouse is a mutant mouse strain in which the gene of interest is knocked out by deletion or by replacement with an exogenous DNA fragment, rendering the gene nonfunctional. Knockout mice have proven to be an invaluable tool for understanding the biological functions of mouse genes. Knockout mice have also been used to model various human diseases, such as cancer, obesity, heart disease, diabetes, arthritis, substance abuse, anxiety, aging, and Parkinson's disease (PD) [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 nonfunctional. 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 into the targeting vector. Correctly targeted ES cells are isolated by drug selection, confirmed by Southern analysis or PCR-based methods, and then microinjected into blastocysts and implanted into pseudo-pregnant females to generate chimeras. Chimeras with the 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 increased 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.

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 that includes an origin of replication and a selectable marker, such as an ampicillin or kanamycin resistance gene, flanked by 50 bases homologous to the 5'and 3'ends of the DNA to be subcloned. The retrieval vector can be rapidly synthesized by PCR using two chimeric primers (at the 5' end, 50 nucleotides of each primer are homologous to the two ends of the DNA to be subcloned and, at the 3' end, 20 nucleotides are homologous to the plasmid DNA) and the linear plasmid DNA as a template (Figure 2). Once electroporated into bacterial cells expressing the recombineering proteins, the 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*



meric primers

Amp

PCR

Amp

genomic DNA, the next step is the removal of functionally important exons and insertion of a selectable marker (genes that allow resistance to antibiotics such as blasticidin, neomycin, or hygromycin, and function in both eukaryotic and prokaryotic cells). The selectable makers are under the control of a prokaryotic (e.g. Tn5, EM7) and a eukaryotic (e.g. *PGK*) promoter. Such dual promoters allow the use of the same selectable maker in both bacterial (for recombineering) and mammalian cells (for gene targeting in ES cells). These selectable markers can be inserted into the plasmid using a targeting construct containing a selectable marker flanked by regions homologous to the target site. These constructs can be prepared by PCR using chimeric primers, as described above.

Construction of Conditional Knockout Vectors

A major limitation of the knockout mouse technology is that a lossof-function mutation in most functionally important genes results in embryonic lethality. This makes it difficult to analyze the function of the gene in adult mice, which is relevant to most human disease genes. This problem is circumvented by the development of the conditional knockout approach, in which 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 for studying the relationship between

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gene mutations and disease development in adult mice. Conditional gene deletions have been achieved by introducing bacteriophageand 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 the 34-base-pair DNA sequences for *loxP* (locus of crossover in phage P1) and *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 any gene is achieved by flanking the critical exons of the gene with two *loxP* or *FRT* sites (creating a conditional allele). Subsequent expression of the recombinases in a cell containing the conditional allele of a gene allows either the entire gene or the critical exons to be deleted, rendering the gene nonfunctional [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) sites, along with a positive selection marker, into the BAC DNA [58].

Transgenic Mice Expressing Cre or Flp Recombinase

Conditional deletion of any gene relies on the targeted expression of Cre or FLP recombinase in the cell or 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 BACbased transgenic mice to express Cre or Flp recombinase. This is conveniently achieved by using recombineering to insert the Cre or Flp cDNA after the start codon of a gene cloned in a BAC vector. This ensures that the recombinase is expressed under the control of the

regulatory elements of this gene, which is expressed in the tissue or cell type(s) where the conditional allele has to be deleted. The Cre or Flp containing BAC clones can then be used to generate transgenic mice. This approach has greatly facilitated the generation of tissuespecific 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 that express Cre can be obtained from The Jackson Laboratory (http:// www.jax.org/search/Main.jsp?qt=cre+mice&x=0&y=0), as well as from the CREATE (coordination of resources for conditional expression of mutated mouse alleles) consortium (http://www.creline.org/ eucommtools). To temporally regulate the expression of Cre protein in cells, a tamoxifen-inducible Cre-estrogen receptor (ER) 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.

High-Throughput Knockout Constructs

To allow generation of multiple knockouts in parallel, a highthroughput, largely automated approach was reported by Valenzuela et al. [63] in 2003. This technology, named 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 PCRbased methods to detect the loss of endogenous allele or by fluorescent *in situ* hybridization using two different probes (one specific for the endogenous allele and other for the recombinant allele).

Another high-throughput method for constructing knockout mice



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Name of Cre line	Promoter used to express Cre	Genetic background of transgenic mice	Cells expressing Cre	References
Ngn3-Cre	Neurogenin 3 (ngn3)	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]
BAC-Pcp2-IRES-Cre Pax2-IRES-cre	Purkinjie cell protein 2 (Pcp2) Pax2	C57BL/6NCr _ C3H/HeNCrMtv CD1	Purkinjie and retinal bipolar cell Inner ear, midbrain,cerebellum, olfactory bulb, kidney	[99] [100]
Prestin-IRES-Cre	Prestin	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]
PR-BAC ^{iCre}	Progestin receptor (PR)	Mixed background of FVB/N and C57BL6	Progestin target tissues i.e., mammary gland, ovary, oviduct, uterus, pituitary gland.	[102]
Sf1-Cre Foxl1-Cre	Steroidogenic factor 1 (SF-1,officially designated Nr5a1) Foxl1	C57BL/6J B6/SJL hybrid	Somatic cells of the gonads, the adrenal cortex, the anterior pituitary, the spleen, and the ventromedial hypothalamic nucleus Gastrointestinal mesenchyme	[103] [104]
BAC-Dkk3-Cre	Dkk3	C57BL/6J and C3H/HeJ hybrid	Retinal progenitors	[105]
5-HT1A-Cre	5-HT1A receptor	C57BL/6	Cerebral cortex, septum, hippocampus, dorsal ra- phe, thalamic, hypothalamic and amygdaloid nuclei, and spinal cord	[106]
Pf4-Cre	Platelet factor 4 (Pf4), also called CXCL4	C57BL/6	Megakaryocyte	[107]
BAC-Col10-Cre	Col10a1	FVB mice and FV/C56BI F1 hybrids	Hypertrophic cartilage	[108]
BAC-Ela-CreErT	Elastase gene	Not available	Pancreatic acinar cells	[109]
Pitx3-CreER(T2)	Pitx3	FVB	Ocular lens and skeletal muscle	[110]
Mitf-Cre	Microphthalmia-associated transcriptional factor (Mitf)	CBA and C57/BI6 hybrid	Melanocyte	[111]
Cited1-CreERT2	Cited1	FVB	Cap mesenchyme	[112]
Nkx2.1-Cre	Nkx2.1 (Homeodomain transcription factor)	C57BL/6	Brain, Thyroid, pituitary, lung	[113]
Mcpt5-Cre GlcNAc6ST-2-Cre	Mast cell protease (Mcpt) 5 High endothelial venule (HEV) expressed sulfotransferase, N- acetylglucosamine- 6-O-sulfotransferase 2 (GlcNAc6ST-2)	C57BL/6 C57BL/6J and C3H/HeN hybrid	Mast cell Colonic villi and in a small subset of cells in the brain, testis, stomach, small intestine, and lung	[114] [115]
Probasin-MerCreMe	Probasin	C57BL/6	Prostate epithelium	[116]
LC-1-hCD19-CreER(T2)	Human CD19 promoter	C57BL/6	B-lymphocyte	[117]
Tbx18-Cre	Tbx18	C57BI/6 and CBA hybrids	Smooth muscle cells and stromal cells in lower urinary tract	[118]
IL-7.Cre	IL-7	FVB	IL-7 producing cells	[119]
SMA-Cre-ER(T2)	Smooth muscle alpha actin (SMA)	FVB/N	Vascular and visceral smooth muscle cells	[120]
AdipogCreERT2	Adipog	C57BL/6	Adipose tissue	[121]
Foxn4-Cre	Eoxn4 (transcription factor)	C57BL/6 and CBA bybrids	Developing spinal cord	[122]
Hsd17b1-iCreER(T) ²	Hydroxysteroid (17-beta) dehydrogenase 1 (Hsd17b1)	C57BL/6 and CBA hybrids	Ovarian granulosa cells	[123]
TFF2-BAC-Cre(ERT2)	TFF2 promoter	Injected in C57BL/6 and CBA hybrids and maintained in C57BL/6	TFF2 expressing cells like gastric progenitor cells	[124]
BAC-Vglut2::Cre	Vesicular glutamate transporter 2 (Vglut2)	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]
GlyT2-Cre	Glycine transporter 2 (GlyT2)	C57BL/6 and DBA/2J hybrids	Caudal regions of the central nervous system, i.e., brain stem and spinal cord	[126]
Slco1c1-Cre AvCreERT2	Solute carrier organic anion transporter 1c1 (SLCO1C1) Advillin	Injected in C57BL/6 and DBA/2J hybrids and maintained in C57BL/6	Neurones of various brain structures, such as cortical layer 2/3 and the hippocampus Sensory ganglia	[127] [128]
SPO11-IRES-Cre	Sporulation protein 11 (Spo11)	BalbC57	Meiotic germ cells	[129]
minKCreER ^{T2}	mink (KCNE1)	CD1	Atrioventricular (AV) node, AV bundle, and bundle branches	[130]
Neurog1-CreER(T2)	Neurog1		Glutamatergic neurons	[131]

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				[400]
MART-1::Cre	MAR1-1	DBA/2J hybrids and maintained in C57BL/6	retinal pigment epithelium	[132]
DCX-CreER(T2)		C57/B6L	Newborn neurons	[133]
TgUPCreERT2	Uroplakins	C57BI/6 and CBA hybrid	Urothelium	[134]
Sox2-CreER	Sox2 (Transcription factor)	FVB	Central nervous system stem/progenitor cells	[135]
Ikaros-Cre	Ikaros (Transcription factor)	Not available	Retinal progenitor cells	[136]
FoxP3-GFP-hCre	FoxP3	Not available	Regulatory T cells	[137]
Megsin-Cre	Megsin (Serine protease inhibitor)	C57BI/6 and CBA hybrid	Skin, Forestomach, Esophagus	[138]
Prrxl1-CreER (T2)	Prrxl1	FVB/N	Primary somatosensory ganglia, weak expression in the spinal dorsal horn, mesencephalic trigeminal nucleus, principal sensory trigeminal nucleus, and spinal trigeminal nucleus	[139]
Sox10-iCreER (T2)	Sox10 (Transcription factor)	C57BL/6	Neural crest cells, their derivatives and the	[130,140]

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Casssette	Selection	Counter-selection	References
neo-sacB	Resistance to kanamycin (neo)	Growth on Sucrose medium	[69]
galK	Growth on minimal media with galactose	Resistance to 2- deoxygalactose	[27]
ThyA	Growth in the absence of thymine	Growth on trimethopterin and thymine	[141]
neo-rpsL⁺	Resistance tokanamycin	Resistance to streptomycin	[142]

 Table 2: Different selection-counter selection cassettes used in generation of mutation by recombineering.

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

Generation of Hypomorphic Alleles for Functional Dissection

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

Selection/counter-selection method

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

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

BAC modification using ss DNA

Recombineering using 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 the mouse homolog of breast cancer susceptibility gene 2 (BRCA2) using short homology arms [73]. The recombinants were screened by mismatch amplification mutation assay-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 are replaced with an exogenous sequence. In the second step, that exogenous sequence is changed back to the original sequence, except for the desired subtle change. With this method, recombinants can be screened by colony hybridization or PCR using oligonucleotides complementary to the altered region [75]. This approach has been used to introduce point mutations in human BRCA1 and BRCA2 genes cloned into a BAC vector. 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].

Multiple Alterations

A multipurpose allele of any gene can be constructed using sequential rounds of recombineering. Testa et al. [78] generated a BAC-based gene targeting construct in which the authors introduced an N-terminal tag, a positive selectable marker, and *loxP* into an intron at the 5' end of the region to be deleted, then another *loxP* site to the 3' end of the region to be deleted, followed by a reporter construct with an internal ribosome entry site (IRES) after the stop codon. In addition, the cassette containing the positive selection marker targeted to 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 (a tagged wild-type allele, a conditional allele, and a tagged hypomorphic allele) in mice.

Humanized Mouse Models of Human Diseases

According to the mouse genome sequence published in 2002, 99% of mouse genes have human homologs. However, the putative regulatory regions of the two genomes demonstrated a lower level of conservation [79]. Thus, 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 mice, provide an approach that can 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 a cysteine-to-glycine change causes 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 PD, allowing 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, BAC fragments are often inserted instead of full-length BAC DNA. To overcome these problems, a high-throughput, single-copy, site-specific method to target the BACs to the *Hprt* locus on the X-chromosome using *Hprt* docking technology has been developed [84]. This approach was used to generate seven different alleles of the human nuclear receptor 2E1 (*NR2E1*) gene.

In recent years, several genetically engineered mouse models have been generated using the BAC transgenic approach, and these models are now 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 a-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 a-globin chains. Multiple BAC transgenic mouse models have also been developed to study basal ganglia neurodegenerative disorders, such as PD and Huntington's disease (HD). BAC-mediated overexpression of human mutant parkin-Q311X (using a dopamine transporter promoter or a human Lrrk2 R1441G mutation) in mice caused them to develop the hallmark features of human PD patients [83,86]. In another mouse PD model, a human BAC containing α -synuclein (α -syn) was used to overexpress the a-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 a full-length human mutant huntingtin (HTT) gene, with 97 glutamine repeats under the control of endogenous regulatory elements using a BAC, transgenic mice were developed by Gray et al. [88]. The behavioral and neuropathological phenotypes of the mice revealed that they are suitable for further investigation of HD pathogenesis and also for preclinical studies. Zhou et al. [89] introduced a missense mutation (Tyr437His) into a human myocilin (MYOC) gene cloned into a BAC and used this mutant BAC to generate a mouse model of primary open-angle glaucoma. The pathological changes observed in the eyes of the transgenic mice reflected those observed in patients with glaucoma. To study host-pathogen interactions for human pathogens such as Neisseria gonorrhoeae, a BAC transgenic mouse model has been developed that expresses human carcinoembryonic antigen related cell adhesion molecule-1 (CEACAM1). This model overcomes the limitation of studying host-pathogen interactions of *N. 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 to 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, since the sequences of most commonly used mouse strains are available, this is not a serious limitation. Also, because the targeting constructs for recombineering are generated by PCR, occasionally undesired mutations can inadvertently be introduced into the genome. It is therefore recommended that the sequence of the modified region be confirmed to rule out the introduction of any unwanted mutation. Additionally, recombineering can be challenging when manipulating 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. This can be overcome, to a limited degree, by increasing the length of the homology arms. In spite of these limitations, recombineering technology continues to be a powerful tool that has allowed us to overcome numerous obstacles and has 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 of generating mutations in all protein-coding genes. Taking advantage of high-throughput ES cell technologies, including gene-trapping and recombineering-based methods to generate gene-targeting vectors, targeted mutations in more than 17,400 unique genes have been generated in ES cells, and 1,700 mouse lines carrying mostly conditional gene alleles have been made available to the scientific community [65]. Such an achievement is a testament to the availability of tools and technologies that, on one hand, have oversimplified the process, yet on the other hand, have 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 single-nucleotide polymorphisms (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 and regulatory regions of the 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 recombineering technology (Tables 1 and 2).

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