A Review of Current Large-Scale Mouse Knockout Efforts

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Summary: After the successful completion of the human genome project (HGP), biological research in the post-genome era urgently needs an efficient approach for functional analysis of genes. Utilization of knockout mouse models has been powerful for elucidating the function of genes as well as finding new therapeutic interventions for human diseases. Gene trapping and gene targeting are two independent techniques for making knockout mice from embryonic stem (ES) cells. Gene trapping is high-throughput, random, and sequence-tagged while gene targeting enables the knockout of specific genes. It has been about 20 years since the first gene targeting and gene trapping mice were generated. In recent years, new tools have emerged for both gene targeting and gene trapping, and organizations have been formed to knock out genes in the mouse genome using either of the two methods. The knockout mouse project (KOMP) and the international gene trap consortium (IGTC) were initiated to create convenient resources for scientific research worldwide and knock out all the mouse genes. Organizers of KOMP regard it as important as the HGP. Gene targeting methods have changed from conventional gene targeting to high-throughput conditional gene targeting. The combined advantages of trapping and targeting elements are improving the gene trapping spectrum and gene targeting efficiency. As a newly-developed insertional mutation system, transposons have some advantages over retrovirus in trapping genes. Emergence of the international knockout mouse consortium (IKMP) is the beginning of a global collaboration to systematically knock out all the genes in the mouse genome for functional genomic research. genesis 48:73–85, 2010.
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INTRODUCTION

The past century has witnessed a boom in molecular genetics and one of the greatest projects—the human genome project (HGP) (Abramowicz, 2003; Johnson, 1987; Watson and Cook-Deegan, 1991). Accomplishment of this project provides us with the sequence information of our genome, which is now available to diagnose diseases at the level of genes (Burton and Stewart,

2003; Collins and Mansoura, 2001; Gottesman and Collins, 1994). Biological research has entered a new stage—the post-genome era, and the main work of this era is to decipher the function of each gene in our genome (Austin *et al.*, 2004; Eisenberg *et al.*, 2000). Comparative genomics has shown that the mouse and human genomes have high homology (Gregory *et al.*, 2002). Therefore, the mouse serves as a perfect model animal for functional genomic research of humans. The most efficient way to study the function of a gene is to make a knockout and observe the phenotype in the whole animal (Austin *et al.*, 2004; Brown and Hancock, 2006; Dinnyes and Szmolenszky, 2005). Knockout mice can also be used for mouse models of human diseases.

There are two main methods to make knockout mice: gene targeting and gene trapping. Gene targeting technology is based on successful ES cell culture and in vitro homologous recombination, and it is a good method for gene knockout and knock-in to introduce loss-of-function mutations in the mouse genome (Hogan and Lyons, 1988). The first mouse gene targeting experiment was completed in 1987 (Mansour *et al.*, 1988; Thomas and Capecchi, 1987). Gene targeting has accelerated the study of gene function during the last 20 years and generated crucial resources for biological research. In 2007 three scientists were awarded the Nobel Price for their research on the important technology of gene targeting (Mak, 2007).

Gene trapping mutagenesis was developed as an alternative to gene targeting technology. It is a high-throughput and random mutation technique (Abuin *et al.*, 2007; Gossler *et al.*, 1989; Kothary *et al.*, 1988). Though not as specific as gene targeting, a large number of mouse genes can be knocked out in a short period of time by trapping (Takeuchi, 1997; Zambrowicz and Friedrich, 1998).

The combination of gene trapping and gene targeting makes it possible to knock out all the mouse genes.

Improvements have also been made to overcome short-comings in the two techniques. In this review, I will describe the generally used methods for gene trapping and gene targeting as well as useful resources developed recently for mouse knockouts. Attention then will be given to newly-developed techniques in both fields, including the use of transposons as an insertional muta-tion strategy. Finally, I will focus on international efforts to knock out all the genes in the mouse genome.

Gene Trapping Versus Gene Targeting

Gene trapping. Gene trapping was originally devel-oped for the purpose of detecting the expression pat-terns of genes because expression of the reporter gene in trapping vector relies on the transcriptional regula-tory elements of endogenous genes near the insertion site (O'Kane and Gehring, 1987). Later it was found to be mutagenic when insertion occurred and some modifi-cations have been made to improve the mutational effi-ciency for disrupting genes (Gossler *et al*., 1989; Hicks *et al*., 1997; Skarnes *et al*., 1995). Insertion of the trap-ping vector is usually mediated by certain retroviruses (Amsterdam, 2003; Amsterdam *et al*., 1997; Qi *et al*., 2004). Trapping vectors usually have a unit that contains a selectable marker flanked by a splice acceptor (SA) and a polyA signal. When introduced into ES cells, the vec-tors randomly insert into the mouse genome. If the vec-tor inserts into an intron of a gene, SA works to generate fusion transcripts of the selection marker and the exons upstream of the endogenous gene, resulting in a trun-cated protein from the endogenous gene. Usually βgeo is used as the selection marker, and it is a fusion of β-ga-lactosidase (*lacZ*) and neomycin-resistance genes (*Neo*).

Now the widely used method is the polyA trap (Fig. 1a). Besides the trapping elements mentioned above, a PolyA trap vector has a second unit that contains a *PGK* promoter, a puromycin-resistance gene (*Puro*), and a splice donor (SD) signal. The polyA sequence in this sec-ond selection cassette is replaced by SD, and transcrip-tion depends on the endogenous polyA signal. SD func-tions to form a second fusion transcript of Puro and the exons downstream of the inserted site. In front of the SD signal are stop codons to prevent translation of exons downstream from the insertion site, which can be used to generate a sequence tag by $3'$ rapid amplification cDNA ends PCR ($3'$ RACE). The identity of the inserted gene can be found by blasting the sequence tag with known genes in the mouse genome (Zambrowicz *et al.,* 1998). The polyA vector can trap genes not expressed in ES cells, because the *PGK* promoter is active in ES cells (Niwa *et al*., 1993; Salminen *et al*., 1998). Thousands of *Neo*-resistant ES cell clones can be obtained in one trans-fection with the retrovirus, and the trapping vectors can integrate into different sites in these clones. As a result a large number of mutations can be obtained in only one experiment.

For more efficient gene trapping, improvements have been made to the original polyA trap vector (Fig. 1b). To disrupt genes completely, a stronger SA and polyA signal have replaced those in the old vector. Another alterna-tion is the introduction of the *GFP* gene in the vector, allowing easier screening of the trapped cells. One of the most important improvements is the introduction of *loxP* sites near each end of the vector. This helps to con-firm the functions of a gene if the phenotype can be restored after the vector is removed by Cre (Taniwaki *et al*., 2005). This method is called removable exon trap (RET) (Araki *et al*., 1999; Ishida and Leder, 1999; Mat-suda *et al*., 2004).

PolyA gene trap, as the second generation of gene trapping, is efficient for trapping genes not expressed in ES cells. However, recently it was found that the vector has bias toward the last intron of the gene, so the genes trapped sometimes remain partial functions. It has been shown that this phenomenon is caused by nonsense-mediated mRNA decay (NMD). To resolve this problem, RET gene trap vectors were modified by inserting inter-nal ribosomal entry sites (IRES) and three initiation co-dons in front of the SD sequence (Shigeoka *et al*., 2005). This is called UPATrap and a library based on this new vector was generated (http://bsw3.naist.jp/kawaichi/naistrap-e.html).

Gene targeting—Pop star in 2007. Gene targeting was accomplished on the basis of homologous recombi-nation and successful isolation of ES cells (Evans and Kaufman, 1981; Martin, 1981; Smithies *et al*., 1985). Twenty years after this technology was established, the Nobel Prize in Medicine for 2007 was awarded to Mario R. Capecchi, Sir Martin J. Evans, and Oliver Smithies for their discoveries of the principles for introducing spe-cific gene modifications in mice using ES cells.

Gene targeting is mainly used to knock out genes, but it can also be used to introduce other kinds of alterations (point mutations as in human diseases, Cre recombinase, other exogenous DNA, etc.) into the genome at specific locations. This is called knock-in. Conventional knock-out vectors contain a positive selectable marker (usually *Neo*) and a negative selectable marker (*TK*) (Mansour *et al*., 1988; Valenzuela *et al.,* 2003). This allows the replacement of specific genes with *Neo* through homolo-gous recombination between the targeting vector and the cognate sequence in the recipient ES cell genome af-ter the vector is transferred into these cells by electropo-ration. Only *Neo* resistant ES cells generated by homolo-gous recombination can grow under selection, and *TK* is used to eliminate random integration in the presence of gancyclovir (GANC). *DTA* (Diphtheria toxin A) is another negative selection marker that is used and was reported to allow more efficient enrichment of targeted clones (Capecchi, 1989). The *DTA* protein itself can kill ES cells, and no additional drug is needed in the me-dium. However, the use of *DTA* for selection may cause minimal toxicity due to transient expression prior to vec-tor integration (McCarrick *et al.,* 1993; Yagi *et al*., 1993). After positive–negative selection the ES cells are injected into mouse blastocysts to produce chimeras. By breed-ing these chimeras with wild-type mice, heterozygous

mice may be produced if the ES cells form the germline in the chimeras.

Conventional knockout can sometimes cause embryonic death if the gene knocked out plays essential roles in development. Conditional knockout, which relies on site-specific recombination systems, such as Cre-*loxP* or FLP-*Frt* were designed to circumvent this problem (Kuhn and Schwenk, 1997). Cre (or FLP) mediate the deletion of any sequence flanked by two *loxP* (or *Frt*) sites with high efficiency if these two sites are placed in the same orientation (Michael *et al.*, 1999). To construct a conditional knockout mouse, two mouse lines are needed: the floxed mice, which have the essential exons flanked by *loxP* (or *Frt*) and a Cre (or FLP) transgenic mouse. Cre recombinase is expressed in certain cell types or transiently expressed at certain developmental stages upon induction (Michael *et al.*, 1999). Crossing the floxed mouse with the Cre mouse allows the deletion of the *loxP*-flanked exons in specific tissues (tissue specific KO) and/or at specific time (stage specific KO). There is a collection of more than 500 Cre mouse lines expressing Cre recombinase in different tissues (Nagy and Mar, 2001; Nagy *et al.*, 2009) that are available for the generation of conditional knockouts. Researchers can search a Cre transgenic mouse line database (CreX-Mice) at Andras Nagy's laboratory (http://nagy.mshri.on.ca/cre/). Using this system, we can knock out genes in specific tissues and/or at a specific stage.

Polymerase chain reaction (PCR) has been widely used for making gene targeting constructs (conventional or conditional knockout). With the PCR method, the homologous arms are amplified by PCR using genomic DNA as template. The amplified arms are then subcloned into a plasmid containing *Neo* and *TK* as selectable markers at appropriate restriction sites. In recent years, an efficient method based on BAC modification has been used to retrieve conditional targeting constructs from *E. coli* containing BACs (Chan *et al.*, 2007; Copeland *et al.*, 2001; Liu *et al.*, 2003) (see Fig. 2). It relies on a recombineering system and shorter regions (200–500 bp) of homology to retrieve DNA fragments from BACs to plasmid (Lee *et al.*, 2001; Liu *et al.*, 2003). With the same mechanism, selection cassettes, which also contain shorter homologous regions (100–300 bp) are targeted into the retrieved DNA fragment. It has reduced the efforts to find the appropriate restriction sites for subcloning. This method can also be simplified to produce conventional knockout constructs.

IGTC and KOMP

IGTC—The international gene trap consortium. Gene trapping is high-throughput and in only two decades modified ES cell lines using this method have easily surpassed 650,000. A number of organizations have been generating trapping ES cell line resources (Table 1).

BayGenomics (http://baygenomics.ucsf.edu) was an NIH sponsored project on the research of functional genomics (Stryke *et al.*, 2003). It was the first government supported program to generate trapping ES cell lines for public use. This project was completed in July 2008, and had trapped 14,000 ES cell clones. Now all those trapped ES cell clones are distributed by mutant mouse regional resource centers (MMRRC) in the USA. Currently the minimum service fee for each ES cell line is $515 including handling and shipping, and it usually takes about 3–4 weeks to receive cells.

The Sanger Institute Gene Trap Resource (SIGTR, http://www.sanger.ac.uk/PostGenomics/genetrap/) was launched in April 2004, and it was mainly funded by the Welcome Trust in Europe. SIGTR used gene trapping vectors that were similar to those used by BayGenomics, and it has a collection of 11,800 gene trapping ES cell lines. Since March 2007 SIGTR has no longer distributed ES cell clones, and MMRRC in the USA has taken over this job.

Lexicon Genetics Incorporated (http://www.lexgen.com) is a company in Texas, USA, and it was the first private resource for gene trapping ES cell clones (Gao *et al.*, 2004). Its OmniBank owned the largest collection of gene-trapped ES cell lines from the 129/SvEv mouse strain, and it now has 270,000 trapped ES cell lines representing mutations in over 9,000 genes.

In 2005, a new gene trapping resource—the Texas Institute for Genomic Medicine (TIGM http://www.tigm.org/) was formed. TIGM is a joint research institute of Texas A&M University and the Texas A&M Health Science Center, and it was funded by the Texas Enterprise Fund. TIGM contracted Lexicon to create a comprehensive knockout mouse embryonic stem cell library (also called OmniBank II) containing 350,000 cell lines of the C57BL/6N mouse strain representing more than 10,000 unique genes (Hansen *et al.*, 2008). This project was completed in 2008 and now TIGM maintains the largest number of gene trapping cell clones of the C57BL/6N mouse strain. TIGM also has limited access to trapped ES cell lines from the 129/SvEv mouse strain in OmniBank.

The German Genetrap Consortium (GGTC http://tikus.gsf.de/) has a collection of more than 40,000 mutant ES cell lines in 2009. Using conditional gene trapping strategy GGTC assembled over 1,000 conditionally mutated genes before 2005 (Schnutgen *et al.*, 2005).

The Center for Modeling Human Disease Gene Trap Resource (CMHD http://www.cmhd.ca/genetrap) is a Canadian team for making gene trap mutants in mouse ES cells (Gao *et al.*, 2004). It has 11,463 gene trapped ES cell lines screened for in vitro reporter expression and the number of sequence tags is 19,723 (data on its website; January 10, 2008). The ES cell clones can be ordered through the Canadian Mouse Mutant Repository (CMMR).

In 2005, the International Gene Trap Consortium (IGTC http://www.genetrap.org) was established. IGTC tried to integrate all publicly available gene trap information (Araki *et al.*, 2009). Now IGTC has nine members among different countries, including those mentioned above except OmniBank of Lexicon Genetics (Roma
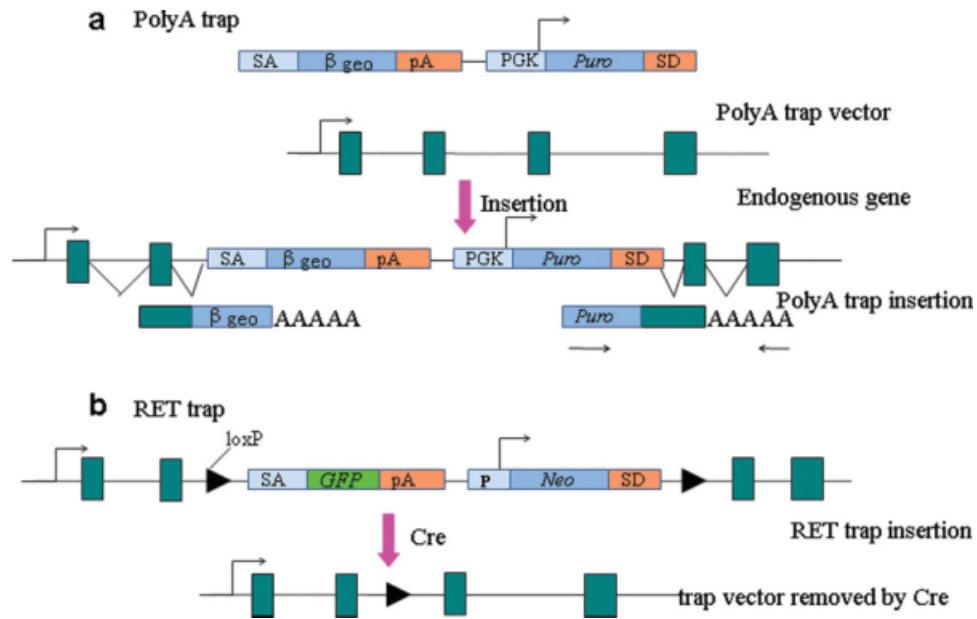
FIG. 1. (a) PolyA trap. There are two units in the retrovirus trapping vector. The first unit contains a splice acceptor (SA) sequence, a promoterless reporter/selection marker (βgeo) and a polyA (pA) signal. The second unit contains a PGK promoter, a second selection marker (Puro) and a splice donor (SD) signal. The vector can randomly insert in the genome of ES cells. With SA, the endogenous exons upstream form the first fusion transcript with βgeo and further transcription of this gene is terminated prematurely. With SD, Puro and the endogenous exons downstream are spliced together and this gives rise to a second fusion transcript. In front of the SD signal are stop codons to prevent further translation of this second fusion transcript. The two arrows represent primers used to generate sequence tags by 3′RACE. Modified from (Zambrowicz and Friedrich, 1998). (b) RET trap. The RET trapping vector contains GFP, which make it easier to report the occurrence of trapping. We may obtain the trapped mutation allele as a polyA trap, and the mouse carrying this mutation may have some phenotypic alternations. The most important improvement in RET trap is the introduction of the Cre-loxP system. The trapping vector is flanked by 2 loxP sites that can be removed by crossing the mutant mouse with another Cre transgenic line. The function of a gene may be confirmed after restoration of the phenotype. Modified from (Araki et al., 1999). Another kind of gene trapping vector UPATrap can be obtained by inserting IRES and three initiation codons into the RET trap vector.
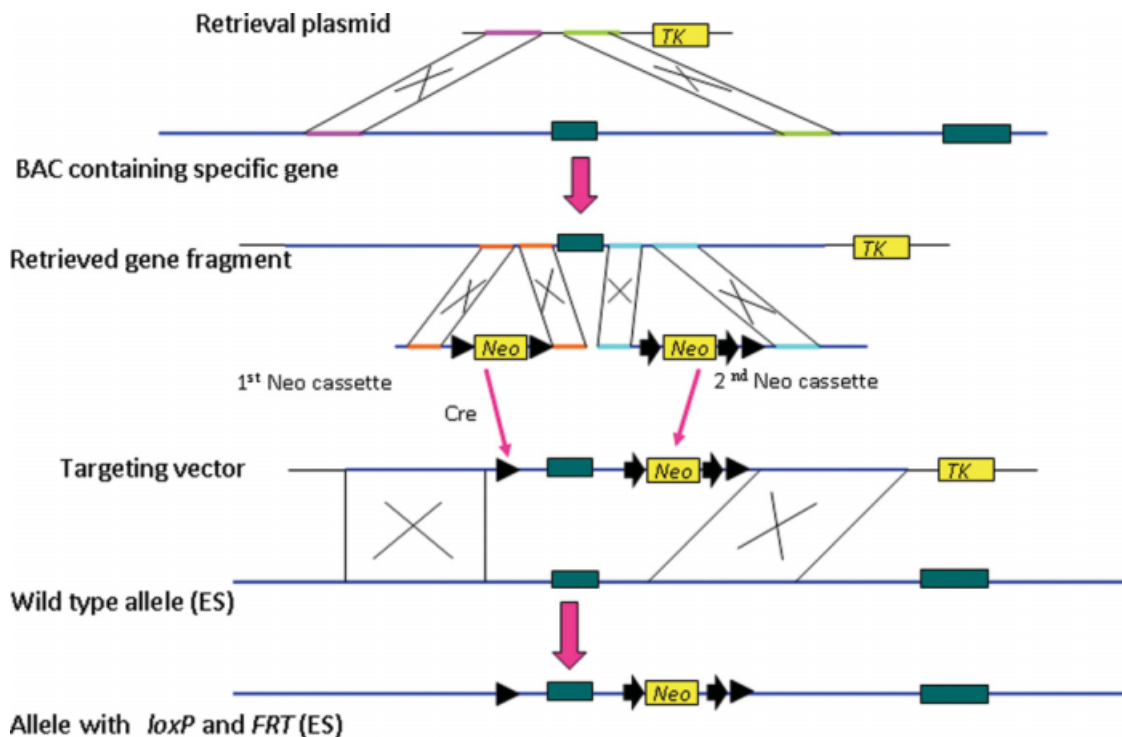
FIG. 2. Construction of a conditional gene targeting vector by retrieving and targeting in ES cells. First, the gene of interest is retrieved from BACs containing the DNA fragment into a retrieval plasmid with short homologous arms by recombineering in *E. coli*. Second, the first Neo cassette containing two loxP sites is introduced into the retrieved DNA fragment by recombineering. Then expression of Cre removes the selection gene, leaving only one loxP site. Third, the second Neo cassette containing Frt-Neo-Frt-loxP is targeted to the DNA fragment. Finally, the gene targeting vector is transferred by electroporation into ES cells, and ES cell clones with floxed exons generated through homologous recombination are selected. (Black arrow heads, loxP sites; arrows, Frt sites; thick lines with the same color represent DNA sequences with homology) Modified from (Liu *et al.*, 2003).

Table 1
International Knockout Mouse Resources

Knockout consortia		Knockout resources[a]
IGTC[b] (http://www.genetrap.org)	BayGenomics[c] (completed in 2008) (http://baygenomics.ucsf.edu)	14,000 trapped ES cell lines
	SIGTR[c] (2004–2007) (http://www.sanger.ac.uk/PostGenomics/genetrap/)	11,800 trapped ES cell lines
	GGTC (2005–2008, http://tikus.gsf.de/)	More than 40,000 trapped ES cell lines, including 1000 trapped conditionally
	CMHD[d] (http://www.cmhd.ca/genetrap)	11,463 trapped ES cell lines
	TIGM[e] (2005–2008, http://www.tigm.org/)	350,000 trapped ES cell lines representing 10,000 genes
IKMC	KOMP (http://www.knockoutmouse.org/)	3,500 genes targeted conventionally
		5,000 genes targeted conditionally
		250 knockout mice from Deltagen and Lexicon
	NorCOMM (http://www.norcomm.org/)	Aims to target 2,000 genes
	UNCOMM (http://www.eucomm.org/)	1,413 targeted cell lines and 2,572 targeting vectors using conditional methods
		4,565 cell lines trapped conditionally
Other Knockout Mouse Resources	Lexicon (http://www.lexgen.com)	270,000 trapped ES cell lines representing about 9,000 genes
	PBmice (http://www.idmshanghai.cn/PBmice/)	Aims to create 10,000 mutant mice covering 70% of the mouse genome
	MICER (http://www.sanger.ac.uk/PostGenomics/mousegenomics/)	93,960 insertional targeting vectors for making knockout and chromosome engineering
	CreXMice (http://nagy.mshri.on.ca/cre/)	500 Cre mouse lines

[a]Knockout resources: information collected before March, 2009.
[b]IGTC has nine members and has a total of more than 380,000 trapped ES cell lines; cell lines are distributed on a non-collaborative basis.
[c]ES cell lines are distributed by MMRRC.
[d]CMHD is part of NorCOMM.
[e]TIGM is also a member of IKMC.

et al., 2008). There is information on 380,000 gene trapped ES cell lines from different resources on the IGTC website. Cell lines are distributed on a noncollaborative basis for nominal handling and shipping fees.

KOMP—The knockout mouse project. The Knockout Mouse Project (KOMP http://www.knockoutmouse.org/) was first proposed at a meeting held in Banbury, Cold Spring Harbor in 2003. At that meeting, scientists in the field of molecular genetics talked about the next objective after the completion of human and mouse genome sequencing (Check, 2002; Marshall, 2002). They all agreed that attention should be turned to elucidating the function of mammalian genes, which would also give us a better understanding of human diseases. Knocking out a gene is the most powerful means to study its function and gain insight into human diseases. Thus, a genome-wide knockout mouse project would be as significant as the human genome project (Austin *et al.*, 2004). After the Banbury meeting, the NIH also planned its role in implementing KOMP. In March 2005, the NIH Planning Meeting for a Knockout Mouse Project was organized to produce a draft proposal for the concrete implementation of this project. Then as a beginning for this project, NIH announced the acquisition of 250 well-characterized knockout mice from Deltagen and Lexicon and made them available to researchers through publicly funded mouse repositories.

The main knockout manipulation of KOMP is performed by two centers: CSD (a collaborative team of the Children's Hospital Oakland Research Institute, the Wellcome Trust Sanger Institute and the University of California at Davis School of Veterinary Medicine) and Regeneron Pharmaceuticals. CSD aims to target 5,000 genes conditionally, and Regeneron Pharmaceuticals has the goal of creating knockouts representing 3,500 different genes using conventional gene targeting. Data coordination is by Jackson Laboratory as well as Children's Hospital Oakland Research Institute, and UC Davis serves as a repository center. UC Davis and the University of Missouri, Columbia also have taken the responsibility to contact the private owners of 300 knockout mice on a selected list and ask them to put their animals in a globally accessible repository.

A comparison of the two mutagenesis strategies. Gene trapping is high-throughput, but it is random and it is insufficient for knocking out every gene. Current data show that the number of gene trapped ES cell lines is much larger than the number of known mouse genes. By estimation, only 50–70% of all mouse genes have been trapped in the past. Some genes have been trapped more than once while some vectors inserted into noncoding regions (Abuin *et al.*, 2007; Roma *et al.*, 2007, 2008; Schnutgen *et al.*, 2008). Many experiments have shown the bias of trapping vectors and some "cold" genomic spots on the chromosomes have remained untrapped (Austin *et al.*, 2004; Schnutgen *et al.*, 2008; Shigeoka *et al.*, 2005). Sometimes alternative splicing occurs and results in low levels of wild-type transcripts (hypomorphic alleles). One analysis of OmniBank's non-

embryonic lethal mouse lines demonstrated that >96% of gene trapping led to complete absence of the wild-type message, with the remaining hypomorphic lines showing an average reduction in mRNA levels of 91.6% (Zambrowicz *et al*., 2003). Hypomorphic alleles can be useful for obtaining information about the protein or protein domain function (Lee *et al*., 2007; Stanford *et al*., 2001).

Gene targeting can knock out a specific gene, and it has been shown to be efficient for knocking out almost all the mouse genes (Austin *et al*., 2004; Barbaric *et al*., 2007; Brown and Hancock, 2006). However, it is time-consuming and requires high professional skills. Under the present conditions, neither gene targeting nor gene trapping can independently accomplish the project of knocking out all the mouse genes. Thus, there is an urgent demand for a combination of these technologies and resources.

Combination of Gene Trapping and Gene Targeting Elements

Generation of multipurpose knockout/conditional alleles by targeted trapping. Conventional knockout and conditional knockout are quite different in their manipulation. Conditional knockout leaves a gene intact in the ES cells and deletes exons in some tissues in the mice, while conventional knockout replaces crucial exons in ES cells. Recently scientists developed a new kind of gene targeting vector, which borrows some elements from the gene trapping vector (see Fig. 3). Using this vector, we can produce the gene trapping mutation, conventional targeted knockout, and conditional knockout in one targeting manipulation (Friedel *et al*., 2007). This method is called targeted trapping. The targeted trapping vector is similar to the conditional knockout vector except that the selection marker *Neo* was replaced by trapping elements that contain SA, βgeo, and a polyA signal. When the targeting vector is transferred into ES cells, homologous recombination leads to an insertional mutation that resembles the results of gene trapping. Since the selection cassette is flanked by two *Frt* sites, FLP recombinase in the ES cells enables the deletion of the trapping-oriented selection cassette and generates a conditional allele. Cre recombinase in ES cells leads to the deletion of the critical exons like that of the conventional knockout. Multipurpose mutations can be generated through one gene targeting manipulation.

However, targeted trapping can only be used to target genes that are active in ES cells because the βgeo selection marker is promoterless. CSD of KOMP and the European Conditional Mouse Mutagenesis Program (UNCOMM) have made some improvements to the vector. Another selectable marker under the control of a strong promoter that is active in ES cells was added downstream of the βgeo cassette. With this newly designed vector, we can target and trap any gene including those genes not expressed in ES cells (Friedel *et al*., 2007; Testa *et al*., 2004).
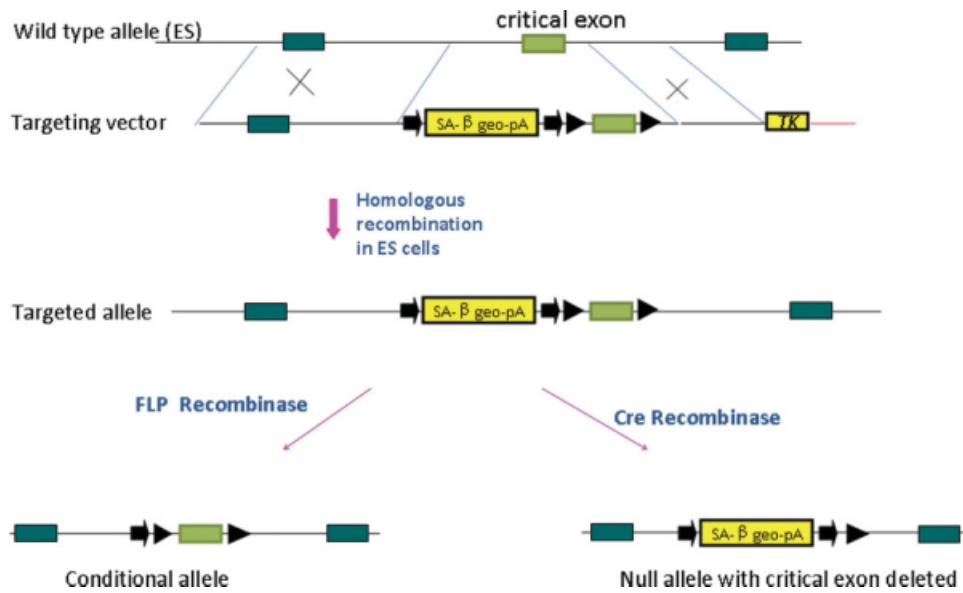
Conditional gene trapping. Conditional gene targeting circumvents the problem of embryonic lethal mutations, but the whole process is time-consuming, expensive, and technically challenging. Gene trapping is high-throughput, but it sometimes causes embryonic lethal. Recently, a new strategy called conditional gene trapping was developed (see Fig. 4). This method uses two pairs of heterotypic recombinase target sites to invert the DNA fragment between two homotypic target sites (Schnutgen, 2006; Schnutgen and Ghyselinck, 2007; Schnutgen *et al*., 2005).

Conditional gene trapping vectors contain a selection reporter cassette necessary for trapping and this cassette is flanked by four pairs of site-specific recombination sites in opposite orientations. *Frt* and *F3* are heterotypic target sites for FLP recombinase. *loxP* and *lox511* are heterotypic target sites for Cre recombinase. After transfection, the vector can insert into the intron of a gene similar to the trapping vectors. With SA, the upstream exons of the endogenous gene form a fusion transcript with the selectable marker, and the transcription of the downstream exons is terminated. This is an insertional mutation resembling that of gene trapping. We can make knockout mice using gene trapped ES cells, or change this mutated allele to a conditional allele at the level of ES cells. To make a conditional gene trapping allele, FLP recombinase is transiently expressed in ES cells. FLP acts to invert the reporter cassette of the vector through either *Frt* or *F3* sites (Fig. 4b). Since the SA is facing away from the direction of transcription, this allele is nonmutagenic. A mouse line carrying this conditional allele may be obtained using the above ES cells. By crossing these mice with another tissue specific Cre mouse, the trapping vector is reinverted through *lox P* or *lox5171*sites (Fig. 4d). The gene containing the trapping vector can be knocked out in some tissues (depending on Cre), for SA is in the same direction with transcription (Schnutgen, 2006).

Further improvements have been designed on the basis of conditional gene trapping by inserting the osteopontin enhancer elements (OPEs) between *Frt* and *F3* (upstream) to trap genes that are poorly expressed. OPEs provide transcription binding sites for transcriptional factors to activate the expression of the inserted genes (Schnutgen *et al.,* 2008).

Transposons as Insertional Tools in Knockout Mice

Recently, transposons have been used as a new tool to mutate mouse genes. Transposons like sleeping beauty (Dupuy *et al.,* 2001; Ivics and Izsvak, 2004; Yant *et al*., 2000) and *piggyBack* (*PB*) are mobile genetic elements that can excise from an original site and insert themselves into a new site in the genome through enzymes called transposases, and result in insertional mutations (Bestor, 2005; Carlson *et al*., 2005). *PB* was originally from the genome of the cabbage looper moth *Trichoplusia ni* and *PB*-derived vectors have been efficient in mu-

FIG. 3. Conditional targeted trapping used to generate multipurpose mutations. Transfer targeting vector into ES cells and select for homologous recombinants through expression of the βgeo marker. Homologous recombination leads to an insertional mutation that resembles gene trapping. FLP recombinase in the ES cells enables the deletion of a selection cassette including the trapping elements, to generate a conditional allele. The expression of Cre in the ES cells leads to the deletion of the critical exon like in a conventional knockout. Modified from (Friedel *et al*., 2007) and (Testa *et al*., 2004).
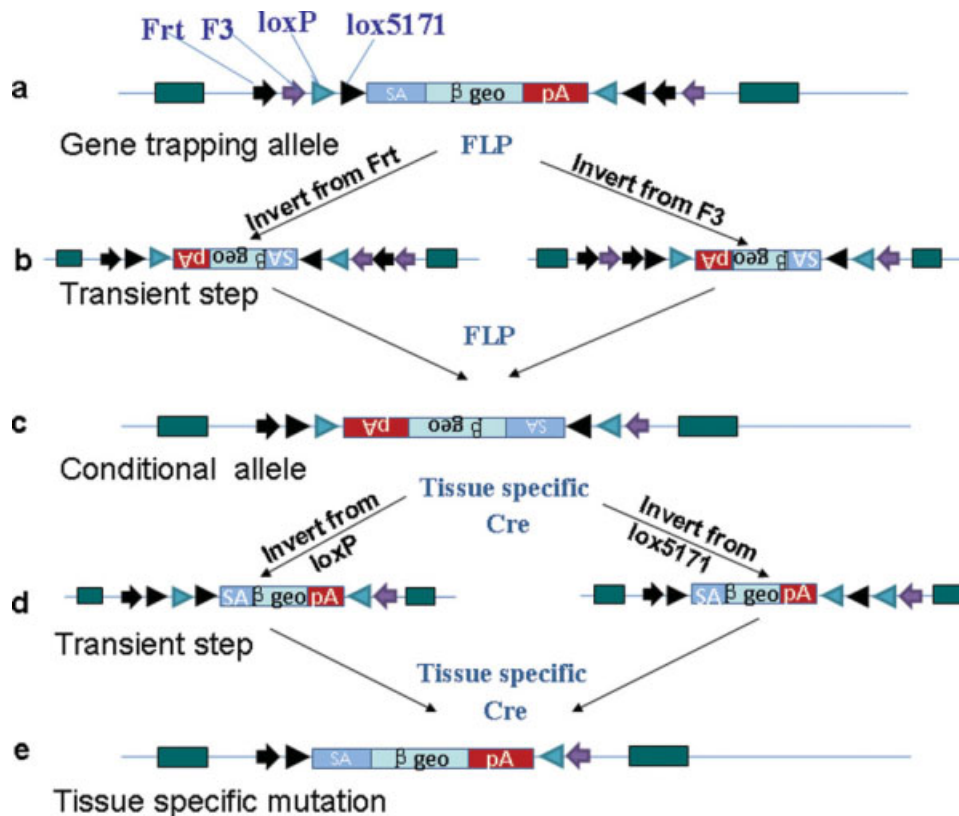
FIG. 4. Conditional gene trapping of UNCOMM. (**a**) Trapped allele after the insertion. The trapping vector inserts into the mouse genome and results in a mutation since the cassette is placed in the orientation necessary for trapping. (**b**) Transient step. The trapping vector is inverted by expressing FLP recombinase in ES cells. Since both Frt and F3 are target sequences for FLP, the cassette can be inverted from a pair of F3 or Frt. In result, a pair of homotypic site-specific recombination sites is placed in the same orientation. Recombination between these two homotypic sites excises one of the other heterotypic sites and the cassette is locked against re-inversion. (**c**) Conditional allele. The inversion restores gene function in ES cells because the SA signal is ineffective in producing a fusion transcript and the trapping vector is removed through RNA processing. (**d,e**) Transient step and trapped allele in specific tissues. By the same mechanism as the FLP-Frt/F3 system, the trapping vector is reinverted and results in a conditional mutation when bred with a tissue specific Cre mouse. Modified from (Schnutgen, 2006).
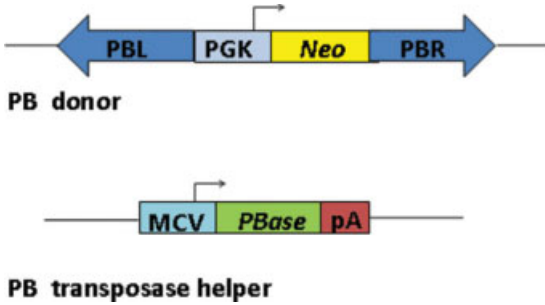
FIG. 5. Binary cotransfection system for insertional mutagenesis mediated by the *PiggBac* transposon. The transposable mutation system consists of two plasmids: a *PB* donor and a *PB* transposase helper. The *PB* donor has two repeated termini (PBL, left termini; PBR, right termini) required for transposition, and the *PB* helper contains the *PB* transposase gene under a ubiquitous promoter (*CMV*). In the presence of the helper plasmid, the donor plasmid can be inserted into the mouse genome with high efficiency. Modified from (Ding *et al*., 2005).

tagenesis of mammalian genomes. *PB* elements consist of 13 bp inverted terminal repeats (ITRs) and a transposase gene. To obtain stable mutations and eliminate retransposition, Ding *et al*. designed a "binary cotransfection system" (Ding *et al*., 2005). This system consists of a donor and a helper plasmid. The donor plasmid contains the *PB* elements in which the *PB* transposase was replaced with elements necessary for trapping mutations and obtaining sequence tags. The helper plasmid carries the *PB* transposase gene but lacks the two terminal sequences required for transposition (see Fig. 5). The two plasmids are cotransfected into ES cells. Transposons are excised from the donor plasmid and reinserted into the mouse genome by the transposase, which is encoded by the helper plasmid. Using this method, the Chinese Mammalian Functional Genome Project plans to create 10,000 mutant mice representing 70% of the mouse genome (Sun *et al.,* 2008). Resources of this kind are in the database of PBmice (http://www.idmshanghai.cn/PBmice/).

In comparison with other transposon systems, *PB* has high efficiency of transposition in the mouse genome. In addition, *PB* has no bias toward some sequences, and evidence demonstrates that *PB* tranposons are distributed evenly throughout the mouse genome (Sun *et al*., 2008). Recently another *PB*-based library was made by the Welcome Trust Sanger Institute. For higher mutation efficiency, the promoter was replaced by SA in the donor. Another improvement was the introduction of the Cre-*loxP* recombination system. They created 14,000 gene-trap clones and analysis revealed promising results: 8% of the trapped genes were not in OmniBank II (Wang *et al*., 2009).

The *PB* vector can be remobilized in the genome by *PB* transposase under induction. When *PB* transposase is transiently expressed, the *PB* vector is precisely-deleted from the insertion site and reintegrated into another site. This characteristic allows determination of the genotype/

phenotype correlation, and the reintegration can also create a new mutant allele in the genome (Sun *et al*., 2008). The *PB* transposon combined with Cre-*loxP* is also used to create large deletions and chromosome rearrangements, because *PB* transposons can insert the *loxP* sites into the mouse genome (Wu *et al*., 2007).

The application of *PB* transposons in mice expands the mutation coverage of traditional retroviral-based trapping systems because *PB* transposon has no bias toward any sequence and has a high efficiency of transposition in the mouse genome. Another important advantage of this system is the precise-excision and reinsertion into another site, which enables the production of mutagenic mice without repeating ES cell and blastocyst manipulation.

## Insertional Mutation Libraries to Facilitate Gene Targeting

The Mutagenic Insertion and Chromosome Engineering Resource (MICER, http://www.sanger.ac.uk/PostGenomics/mousegenomics/) at Sanger Institute has made it possible for us to create insertional mutations, large deletions, inversions, and duplications quickly and easily (Adams *et al*., 2004). Two libraries were constructed by ligating different genomic DNA fragments into two kinds of phage vectors. These two kinds of vectors contain different antibiotic genes, which are used for positive integration selection in mouse ES cells. Vectors can be inserted into the mouse genome through homologous recombination after being linearized or by making a gap in the genomic DNA fragment. Since the insertion usually leads to duplication of the homologous regions (see Fig. 6), it may be mutagenic because of a frameshift mutation (Zheng *et al*., 1999).

Why two libraries? They can be used to make large deletions or inversions. One library is called the 3′ *hprt* (hypoxanthine phosphoribosyl transferase) library while the other is the 5′ *hprt* library. Each introduces a *loxP* site into the genome, and two *loxP* sites can be used to generate a large deletion if they have the same orientation or they can be used to generate an inversion if they have opposite orientations (Yu and Bradley, 2001). The complete *hprt* resistance gene can be formed from the two *hprt* DNA fragments, and this is used for selection in ES cells (see Fig. 6). *Hprt* positive clones can be selected for in HAT (hypoxanthine, aminopterin and thymidine) medium (Szybalski, 1992). MICER provides information on the vector sequence and it has set up an index of 93,960 insertional targeting vectors, which are distributed by Geneservice (http://www.geneservice.co.uk/products/).

Other Methods for Functional Studies of Genes

As supplements to gene trapping and gene targeting, other genetic methods can also be used to study the functions of genes. ENU (*N*-ethyl-*N*-nitrosourea) is a chemical mutagen. Treatment of ES cells with ENU randomly causes point mutations in the genome (Vivian *et al*., 2002). An ES cell library, which consists of ES cell clones mutated by ENU, can be established. ENU libra-
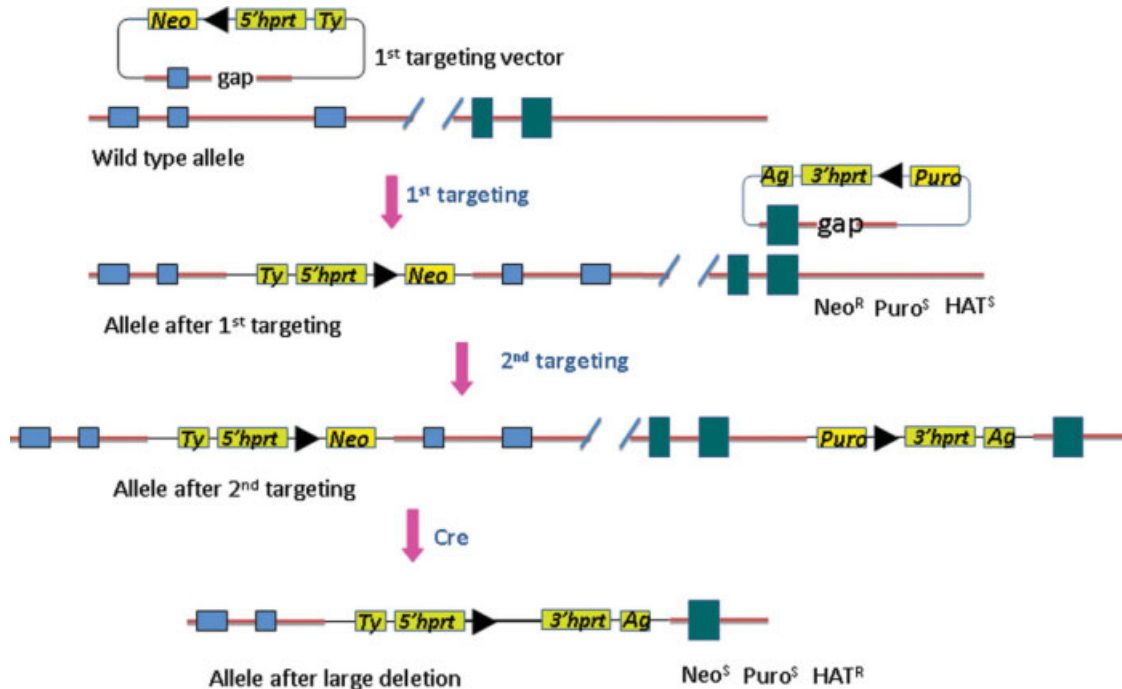
FIG. 6. Large deletions generated using MICER. This insertional mutagenic construct can be used to target ES cells after it is linearized or a small fragment is excised (to make a gap) in the genomic DNA fragment. The two targeting steps use two vectors, from the 5′hprt library and the 3′hprt library, to introduce the two loxP sites into the mouse genome. The 5′hprt fragment and the 3′hprt fragments must be in the same orientation for a deletion. The floxed DNA fragment is deleted by the expression of the Cre recombinase in ES cells and select for HAT resistant clones. Ty (Tyrosinase minigene with a color of gray on the albino background) and Ag (Agouti transgene under the control of the K14 promoter, yellow on the background of wide-type Agouti) are two different coat color marker genes for detecting the insertion of the vector in mice. Rectangles with different colors represent exons from different genes. Modified from (Adams *et al*., 2004; Zheng *et al*., 1999).

ries can provide an allelic series of silent, missense, nonsense, and splice site mutations to examine the effect of various mutations in a gene.

TILLING (Targeting Induced Local Lesions in Genomes) is a method that employs heteroduplex analysis to identify mutations in a specific gene (Henikoff *et al*., 2004; Moens *et al*., 2008). Certain genes are amplified by PCR using primers to specific genes and pooled genomic DNA from a number of chemical-mutated clones as a template. Wild-type and mutant sequences form heteroduplexes (mismatch sites, point mutation) after denaturation and renaturation of the amplified DNA. The identity of the heteroduplexes can be found by cleavage with an endonuclease and running the samples in a sequencing gel (Oleykowski *et al*., 1998). There has been a report on the combination of ENU mutagenesis and TILLING screenings to find mutations in a target gene (Glaser *et al*., 2005). This approach is especially useful for screening allelic series of mutations to examine the effect of various mutations in a gene (Henikoff *et al*., 2004; Moens *et al*., 2008).

How You Can Make Good Use of the Available Resources

With the valuable resources mentioned above, now it is much easier to make knockout mouse models, but sometimes it is puzzling for a beginner to know how to choose from these resources. For the gene you are interested in, you may first search existing gene trapped ES cell lines at the IGTC website, or targeted ES cell lines at www.komp.org/catalog.php (mentioned later). Other private resources like Lexicon can also be an option. Currently there are many more trapped ES cell lines available than targeted ES ones. And for each gene, there may be several trapped ES cell lines available. To make knockout mice, it is preferable to choose an ES cell line with the trapping vector inserted in a region upstream of the gene. If the target gene is expressed ubiquitously or highly expressed in some critical tissues like brain, liver, kidney etc., especially in an early developmental stage, you have to consider a conditional strategy (either conditional trapping or conditional targeting). In cases where it is hard to predict the chances of embryonic lethality, conditional targeted trapping may be a better choice, and with this we can produce conventional knockout and conditional knockout in one targeting manipulation (see Fig. 3).

Besides knocking out genes, some alleles can be used to detect the expression of the endogenous gene. For example, βgeo is a fusion of β-galactosidase (*lacZ*) and neomycin-resistance genes (*Neo*), which is used as selector/reporter in the polyA trap. *LacZ* staining in the mice can reflect the expression pattern of the gene trapped.

Worldwide Collaboration and Coordination

Since the establishment of the NIH KOMP, there has been a significant amount of effort around the world to organize mouse knockout projects (Table 1), such as the European Conditional Mouse Mutagenesis Program (EUCOMM) and the North American Conditional Mouse Mutagenesis project (NorCOMM).

EUCOMM (http://www.eucomm.org/) is funded by the European Union Framework 6 programme. The goal of EUCOMM is to generate a collection of 13,000 mutated ES cell lines using three conditional approaches (Grimm, 2006): conditional gene trapping, conditional gene targeting, and conditional targeted trapping (Friedel *et al*., 2005). EUCOMM conditional gene targeting vectors can be used to target any genes, including those not expressed in ES cells. As of 2009 there were 1,413 targeted ES cell lines, 2,572 targeting vectors, and 4,565 trapped ES cell lines (at the website of EUCOMM).

NorCOMM (http://www.norcomm.org/) is supported by a major grant from Genome Canada and contains two kinds of resources: random gene trapping mutagenesis and targeted knockouts. The gene trap resource is generated by three centers and two are IGTC members: ESDB (Mammalian Functional Genomics Centre) and CMHD. The third center is CMMR which restores and distributes the ES cells. NorCOMM aims to target ∼2,000 genes that have not been targeted or trapped before. ES cell lines are distributed by CMMR on a cost recovery basis.

Overlapping knockout efforts are a waste of time and resources (Grimm, 2006). Beyond this, the complexity and cost of knocking out all the mouse genes requires coordination and collaboration on a global level. Therefore, an international network of organizations—the International Knockout Mouse Consortium (IKMC, http://www.komp.org/ikmc/) was officially established just after the launch of the three knockout mouse projects mentioned above. A document stating the principles and strategies of IKMC was signed by the three organizations: NIH KOMP, UNCOMM, and NorCOMM. IKMC aims to minimize the overlaps, promote communication, share resources, and improve services (Collins *et al*., 2007b; Gondo, 2008). The first international meeting of IKMC was held in March, 2007. The US state-funded TIGM also joined IKMC during the meeting (Gondo, 2008). Vectors, ES cells, mice, and germplasm generated by all IKMC members can be browsed at the website www.komp.org/catalog.php. More organizations from different countries are expected to join in the near future. With all these efforts worldwide the number of knockout mouse resources is poised to increase dramatically over the next several years.

PERSPECTIVES

Producing knockout mice by trapping and targeting offers a powerful means to study the functions of genes in the context of a whole animal (Capecchi, 2001; Harris and Foord, 2000). In the postgenome era, mouse mutants with various phenotypes have served as critical research tools in understanding the function of mammalian genes (Takeuchi, 1997; Zambrowicz and Friedrich, 1998). By knocking out genes, the different functions of these genes have been annotated, including functions in development, metabolism (Kondo *et al*., 2006; Moreadith and Radford, 1997; Yen *et al*., 2006), the neural system (Buss *et al*., 2006; Russell, 2007; Walz *et al*., 2002; Wang *et al*., 2007), apoptosis (Altman *et al*., 2008; Wang *et al*., 2006) and cancer (Boominathan, 2007; Gerits *et al*., 2007; Kondo *et al*., 2006; Wu *et al*., 2008). As a high-throughput gene knockout strategy, gene trapping has other unique applications, such as finding new coding sequences, and reporting the expression level of a gene flanking the insertion site (Matsuda *et al*., 2004; Roma *et al*., 2007). Knocking out mouse genes has also opened up other areas of science especially in medical research. The mouse provides an excellent model for human diseases, and knockout mice can be used to reveal the mechanisms of disease and find new therapeutic targets (Hacking, 2008). In addition, deciphering the functions of genes in mice will also provide a tremendous opportunity for the pharmaceutical industry.

Scientists in the field of molecular genetics will clearly benefit from using the available reagents and taking advantage of recent advances in new methods. A tremendous amount of time, money, and effort will be saved. We can now make knockout mouse models much more efficient because for gene trapping, transposons and the osteopontin enhancer elements have been added to extend genomic coverage. Current high-throughput methods have also accelerated the generation of targeted mouse mutants. These methods include retrieving targeting vectors from BACs through recombineering, and using insertional targeting vectors from MICER libraries. Targeted trapping and conditional trapping take advantage of trapping and targeting to circumvent the shortcomings of a single approach. However, currently there is still some room for improvement in these resources and technologies. Achieving genome saturation depending only on trapping knockouts has not been accomplished, while high-throughput construction of targeted mice has still not been achieved (Abuin *et al*., 2007; Carlson *et al*., 2005; Stanford *et al*., 2001; Takeuchi, 1997), so a more efficient method for making targeted mice needs to be developed. Much of the attention on gene trapping is still focused on improving the technique, while functional annotation of the mutants is in its infancy.

The worldwide collaboration through IGTC has been successful and has helped researchers find appropriate products to some extent. At the same time other knockout projects were initiated and some progress has been made, especially the collaboration of KOMP, EUCOMM, NorEUCOMM, and TIGM to form IKMC (Collins *et al*., 2007a). Only by taking advantage of targeting and trapping can the goal of knocking out all the mouse genes for functional research be realized. This worldwide collaborative work will certainly bring great changes to the

postgenomic era and enhance the development of new therapies (Grimm, 2006).

Knockout mouse resources have been rapidly generated and a large number of knockouts have been analyzed. The knockout data including trapping and targeting is expanding rapidly because of the accumulation of information from ongoing projects worldwide. The timeline for the IKMC indicates that in 2010 the total number of mutated ES cell lines will reach 40,500 with 22,000 (about 14,000 unigenes) from trapping, 3,500 from targeting, and 15,000 from conditional targeting (Collins *et al*., 2007b). This total number is much larger than the total number of mouse genes. Duplication will be avoided as much as possible through the cooperation of the different organizations. It is promising to realize saturation mutagenesis in the mouse. In the near future, knockouts and collaborative efforts will finally help to decipher the function of each gene in the mammalian genome. This will be the largest international biological research endeavor and its successful completion will achieve another great milestone in genetics after the HGP.

ACKNOWLEDGMENTS

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