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Current Progress in Generation of Genetically Modified Mice

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Manipulation of the mouse genome by activating or inactivating the gene has contributed to the understanding of the function of the gene in the subset of cells during embryonic development or postnatal period of life. Most of all, gene targeting, which largely depends on the availability of mouse embryonic stem (ES) cells, is the milestone of development of animal models for human disease. Recombinase-mediated genome modification (Cre-LoxP and Flp-Frt etc) and the ligand-dependent regulation system, more accurate and elaborate manipulation tools, have been successfully developed and applied to dissect the mechanisms governing complex biological processes and to understand the role of protein in temporal-and spatial aspects of development. As technologies concerning refined manipulation of mouse genome are developed, they are expected to open new opportunities to better understand the diverse *in vivo* functions of genes.

Key words – Embryonic stem cell, gene targeting, genetically modified mice, site-specific recombinase

Introduction

The Human Genome Project provides genetic blueprints of humans and precise information of human gene structure and variations, making it possible to study and understand the molecular mechanisms of normal development, as well as those responsible for pathogenesis [31]. Since a mouse has anatomic, physiologic, and genetic similarities to humans, it is an excellent experimental model for studying and defining human gene function. a mouse is also a popular model because its relatively short life cycle and available genetic resources, enable researchers to easily manipulate the mouse genome with use of molecular tools by eliminating (gene knock-out) or over-expressing genes in the entire animal, or in specific tissue with a spatial- or temporal-specific manner [31]. use of the mouse as a model for human disease; can be emphasized by the fact that more than 100 mouse models of human diseases in which the homologous genes have been shown to be mutated in both human and mice, were generated by identification of spontaneous, radiation= or chemically-induced mutants [2]. These indicate usefulness of the mouse as a human disease model. In this review, basic principles of genome modification will be described, as well as the introduction and discussion of elaborate approaches as tools to assess the protein function at given subsets of cells within given times of life.

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Genetically modification of mouse genome

Gene targeting

Genetically modified mice can be made by direct pronuclear injection of transgenic DNA into fertilized eggs [15], or injection of genetically modified mouse ES cells into the blastocyst. (The fate of directly-injected DNA is random integration into genome. Transgene expression is relying on the region in which transgene is integrated and copy numbers of transgene. On the contrary, gene targeting takes advantages of embryonic stem (ES) cells, which have higher frequencies of homologous recombination by which the genomic DNA fragment introduced into ES cells can pair together and recombine with the endogenous homologous sequence. These genetically-modified ES cells are being injected into the blastocyst [31].

Procedures for gene targeted mice

Once the targeting vector is prepared with homologous arms and selectable markers, DNA is introduced into ES cells by electroporation, followed by drug selection depending on the selectable marker. After selecting in the presence of appropriate antibiotics to remove any cells that have not stably integrated foreign DNA into their genome, the surviving ES cell clones are then picked and screened

by PCR and/or Southern hybridization to confirm homologous recombination. They are then subject to injection into blastocysts and transferred to the uterus of a pseudo-pregnant mother. Examination of coat color of the pups is applied to detect chimera. Male mice showing high degrees of chimerism are selected to mate with wild type female mice to confirm the germline transmission of targeted alleles in the F1 generation (Fig. 1).

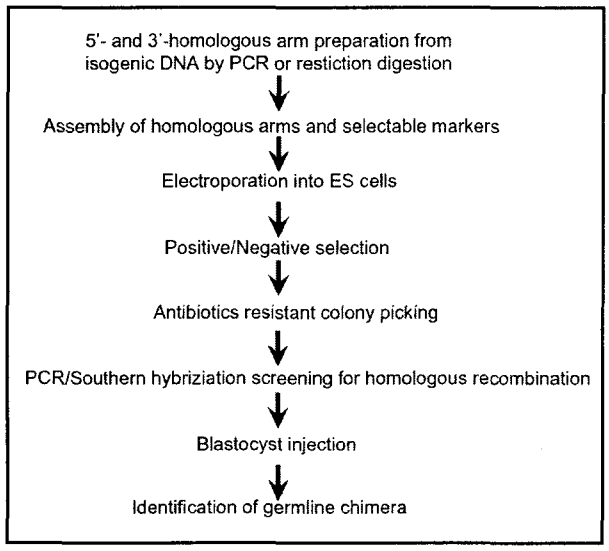

Fig. 1. Outline for genetic manipulation of the mouse genome using homologous recombination.

Generating "knock-out" mice carrying the null allele

The knock-out approach (making null alleles) by gene-targeting is the most commonly used tool;as a loss of function study to examine the physiological role of genes. A targeting vector is designed to recombine with and introduce mutation into a specific chromosomal locus. The minimal components of a targeting vector are 5'- and 3'-arms which have homologous DNA sequences to specific chromosomal locus to be modified, and positive/negative selection cassettes in plasmid vector backbone (Fig. 2A). The replacement and insertion vectors can be used for gene-targeting in animal cells. The fundamental and key factors to be considered for construction of replacement vectors are homologous sequences to target locus (5-8 kb) from isogenic DNA, positive (ex: NeoR, HygroR and PuroR)/negative selection markers (thymidine kinase and diphtheria toxin-A subunit), and plasmid backbones with linearization sites outside of the homologous sequences. After recombination occurs, the final product is a replacement of the chromosomal homology with all components

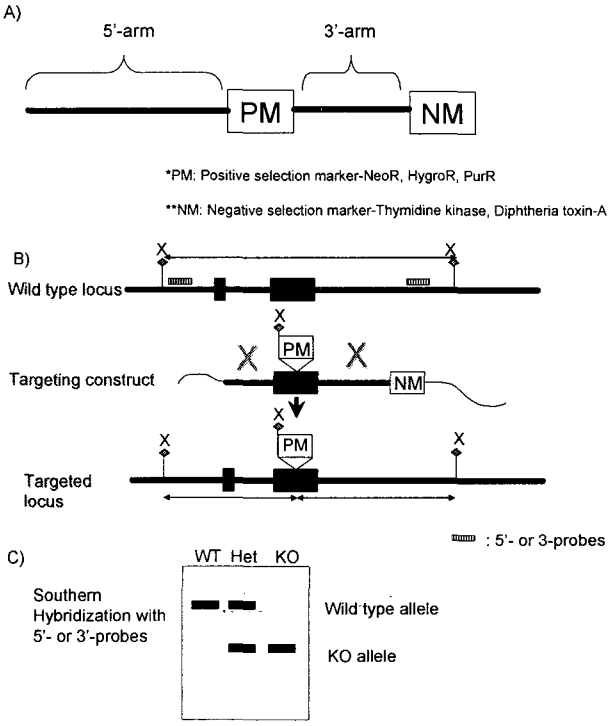

Fig. 2. Homologous recombination for gene knock-out of mose gene. A) Components for gene targeting vector. B) Homologous recombination between targeting construct and wild type homologous sequences and post-recombination product. C) Detection of homologous recombinated allele by Southern hybridization.

of the vector flanked on both sides by homologous sequences (Fig. 2B) [6,14]. The basic elements of an insertion vector are the same as those in a replacement vector. However, the major difference between the two vector types is that the linearization site of an insertion vector is made within the homologous sequences.

Generating "knock-in" mice

Knock-in experiments are used to insert a transgene, such as a cDNA or a reporter construct (LacZ or florescence proteins) contained in a targeting vector, thus utilizing the regulatory elements for transcriptional control of an endogenous gene. The knock-in approaches using reporter genes, such as LacZ or GFP, can applied to monitor the expression pattern of the gene during embryonic development and in adult mice, and to construct fate maps of precursor cells from the early stages of development or differentiation. For example, LacZ has been inserted into the Pax3 locus to examine the role of Pax3 in the neuroepithelium and somites [22]. GFP transgene was introduced to make fusion protein with FoxP3 protein [12] to

assess the function FoxP3 transcription factor, which is indispensable for regulatory T cell development, and to study the ontogeny of it. On the other hand, CD4-CD8 domain swapped mice were successfully made to elucidate the role of the signal strength of costimulatory molecules on $CD4^+CD8^+$double positive differentiation to single positive T cells [9]. As shown in Fig. 3, such knock-in vectors are essentially replacement targeting vectors containing the transgene and a positive selectable marker, and are designed such that after homologous recombination, the transgene is under the control of the endogenous regulatory elements of the locus for transcription. This method is based on the production of a fusion protein between the endogenous and knocked in products. However, when there are concerns regarding to fusion protein (for example, secreted proteins), the transgene can be placed into a 5'-untranslated region (5'-UTR) where the endogenous translational start site is located (although there is concern for occurrence of alternative splicing to transgene),or an internal ribosome entry site (IRES) element [26] can be placed either in a 5'-UTR or 3'-UTR to generate a bi-cistronic mRNA [19,20].

Site-Specific Recombination

Cre-LoxP and Flp-Frt system

The simplest site-specific recombination systems are those composed of a recombinase enzyme and its target sequence. These systems allow for the deletion, insertion, inversion, or translocation of specific regions of DNA. The most commonly used recombinases, integrase superfamily, for genetic modification of the genomes of a fruit fly, zebra fish, frog and plants, as well as a mouse, are the Cre-LoxP system from the bacteriophage P1 and the Flp-Frt system from the budding yeast *S. cerevisiae* [8,23,25,29,33].

Cre recomibinase, 38 kDa protein with 343 amino acids (a.a.) working as a tetrameric complex with no need for co-factors, recognizes and catalyze the site-specific recombination between DNA sequences named LoxP (locus of cross-over (x) in P1) of 34 base pairs (bp) with two 13 bp palindromic sequences flanking by 8 bp non-symmetrical central sequences on which the orientation of the LoxP element is dependent (Fig. 4A) [30]. Cre can recombine two LoxP sites if they are located both in the same DNA strand or in different DNA strands. If two LoxP sites are placed in orientation, the reaction will be the excision of the DNA fragment flanked by the LoxP sequences that form a circular DNA, and a LoxP site will remain in each one of the DNA sequences (Fig. 4B). On the contrary, if two LoxP sites have opposing orientations, the outcome of recombination will be the inversion of the DNA fragment flanked by LoxP sites (Fig. 4C).

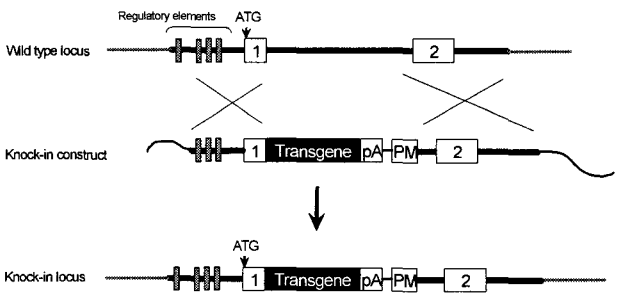

Fig. 3. Generation of knock-in allele carrying mice.

The 2-micron yeast plasmid Flp recombinase is a 45 kDa protein with 424 a.a. which recognize and catalyze the site-specific recombination, as Cre recombinase does, between 34bp sequences named Frt (Flp Recombination Target) [32]. The mechanism of this recombinase action is similar to that of Cre recombinase as stated above. In spite of the sequence differences between both LoxP and Frt, both systems are organized in the same way with two inverted repeats flanking non-symmetrical sequences.

Although recombination activity of Cre *in vitro* and *in vivo* is more effective than that of Flp(198), mutant Flps were generated through *in vitro* mutagenesis, such as enhanced Flp (FLPe), having improved recombinase activity

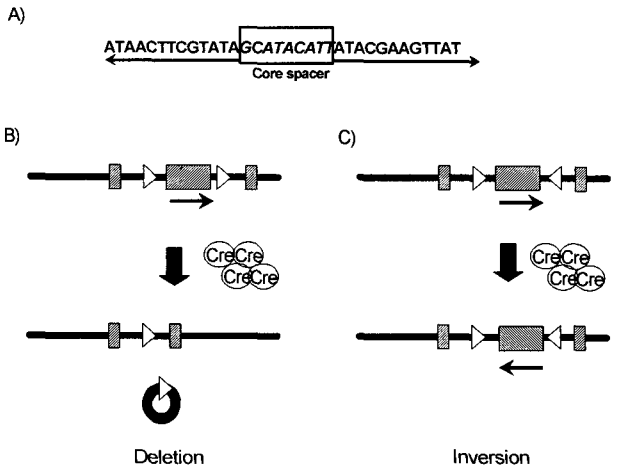

Fig. 4. Site-specific modification of mouse genome using Cre-recombinase. A) LoxP sequences recognized by Cre recombinase. B) Deletion mediated by Cre-recombinase. C) Inversion mediated by Cre-recombinase.

over wild-type Flp. Indeed, deleter mice which carried targeted FLPe in the ROSA26 locus by gene targeting, termed the FLPeR ("flipper") strain [10], show that Flpe recombines as effective as Cre-LoxP [28].

The main methodology for tissue-specific Cre-mediated excision is the use of established transgenic lines expressing Cre under the control of a promoter with the required specificity. For example, to examine the function of DNA polymerase b in T lymphocytes which is expressed ubiquitously and embryonic lethal when ablated from the germ line, mice conditional for this gene were generated and mated with Cre transgenic mice expressing Cre recombinase specifically in T lymphocytes lineage under the control of T lymphocyte specific *lck* promoter (Fig. 5) [13]. There are numerous examples of the use of Cre-expressing mice [3] to be applied to conditional gene modification.

Recently, to overcome the limited availability of Cre transgenic mice, recombinant Cre fusion proteins with peptides which are able to penetrate cell membrane, have been developed to promote the uptake the Cre fusion protein. Hydrophobic peptides from the Kaposi fibroblast growth factor (FGF-4), or basic peptides derived from HIV-TAT [18,25],have been used to generate recombinant Cre fusion protein to facilitate the transfer of Cre into a variety of cultured cell types and even in specific tissues after intraperitoneal administration. This novel form of Cre, which is able to penetrate into cells, will give new opportunities for genetic manipulation of cells both *in vitro* and *in vivo* [5].

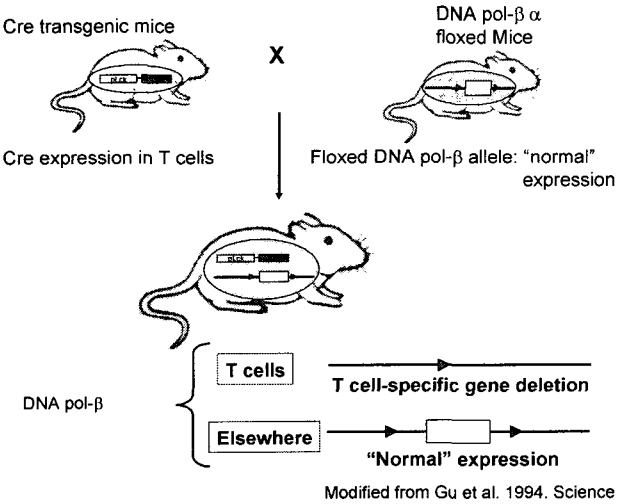

Fig. 5. Generation of T cell specific modification of DNA polymerase-b gene in mice.

Temporal- and tissue-specific expression of Cre

To generate a mouse model in which conditional activation or inactivation of gene expression regulated more accurately by a temporal-, spatial-, or tissue-specific manner, chemically inducible forms of Cre have also been developed by the fusion of Cre recombinase with the ligand-binding domain of a mutated human estrogen receptor (ERa), giving rise to Cre-ER^{T} or Cre-ER^{T2} (Fig. 6A). Translocation of Cre-ER^{T}or ER^{T2} is regulated by administration of tamoxifen, or its derivative 4-hydroxytamoxifen (4-OHT) [4,11,16], resulting in a Cre-recombinase-mediated recombination after nuclear translocation (Fig. 6B). Accordingly, recombination mediated by Cre relies on 4-OHT administration in mice bearing a Cre-ER^{T} or Cre-ER^{T2} transgene. This approach has been used to selectively ablate expression of the retinoid X receptor (RXR) in adult mice keratinocytes, by putting expression of these recombinases under control of the bovine keratin-5 promoter [21]. In combination with the knock-in approach, cDNA of Cre-ER^{T2} was inserted in 5'UTR of the first exon of *Gli-1* gene, one of the sonic hedgehog (*shh*) targets, to mark the cells responding to *Shh* signaling in limb pattering in developing embryo [1].

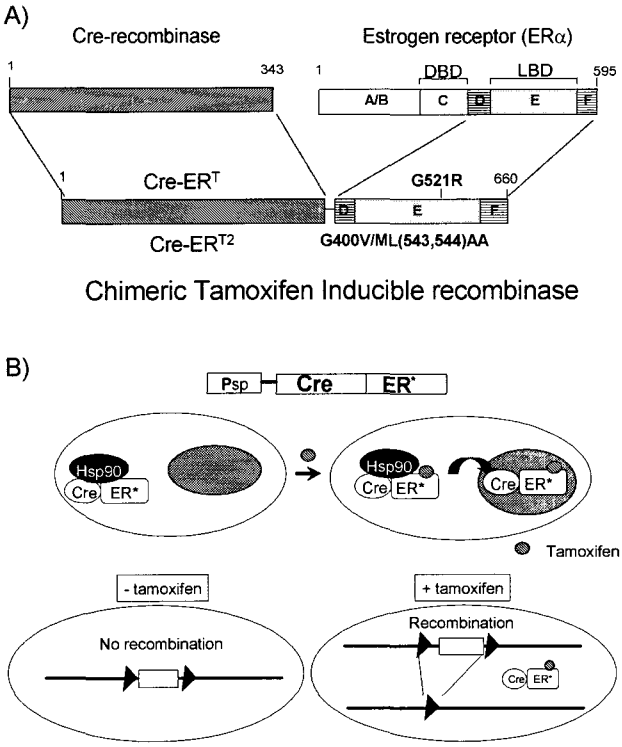

Fig. 6. Genome manipulation using ligand-inducible activation system. A) Schematic representation of Cr-ER mutants. B) Tamoxifen-inducible recombination mediated by nuclear translocated Cre-ER fusion protein.

Conclusion Remarks

Genetic modification of mouse genome has been achieved through transgenesis with random integration in genome or gene-targeting, which takes advantages of the properties of mouse ES cells. These genetically modified mice yielded remarkable advances in understanding the mechanism governing the developmental processes and elucidate genetic and/or physiological interactions between pathways important in the pathophysiology of disease. Despite crucial contributions of knock-out mice, by the nature of this approach, the lack of protein that is indispensable in embryogenesis can lead to early lethality, therefore hampering analysis of possible functions at subsequent or late stages [24]. Moreover, most genes function in distinct cell types during development and postnatal stages [19]. Therefore, the development of more elaborate molecular biology tools, such as Cre-LoxP or Flp-Frt system, has opened up new opportunities to inactivate or activate the gene expression in tissue-; or development- specific manner, depending on the availability of the proper Cre transgenic mice line which are supposed to express Cre recombinase under the control of a specific promoter and/or enhancer. As site-specific recombinase expression is largely relying on promoter elements for transcriptional control, in some instance, spatial- and temporal-specific recombination may not be achieved for specific modification of genome to determine the function of a given protein in a defined subset of cells at any given time during the entire life of an animal. To control the genetic modification more accurately, fusion protein of Cre with ligand binding domain of human ERa was developed, in which system Cre recombinase-mediated recombination depends on the nuclear translocation by ligand administration.

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초록 : 유전자 조작 마우스 개발의 최신 연구 동향

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생쥐 유전자를 과발현 시키거나 제거하는 유전자 조작 기술의 발달은 배 발생 단계나 출생 후 특정한 세포에서의 특정 단계에서의 유전자 기능을 이해하는 많은 기여를 하고 있다. 특히, 높은 상동 재조합 활성을 가지는 생쥐 배 줄기세포를 이용한 유전자 적중 기법은 인간 질환을 이해하는데 필수적인 동물 모델 개발에 중요한 기여를 하였다. 최근에는 Cre과 Flp와 같은 염기서열 특이적 재조합 효소와 라이겐드에 의한 조절 시스템의 도입으로 좀 더 정확하고 정교한 유전자 발현 조절을 위해 개발되어 복잡한 생명현상을 지배하는 메카니즘과 시간과 공간에서 작동하는 유전자의 기능을 이해하는데 많은 기여를 하고 있다. 마우스 게놈을 세밀하게 조작할 수 있는 새로운 분자생물학적 도구의 적용으로 *in vivo* 상에서 유전자의 다양한 기능을 좀 더 정확하게 이해할 수 있는 기회가 열릴 것으로 기대된다.