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Applications of Gene Targeting in the Investigations of Human Airway Diseases

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Abstract

Gene targeting is a powerful tool to dissect gene's functions. Recent developments of recombineering and site-specific recombination have dramatically increased the efficacy of gene targeting. In last decade there has been a huge influx of data from gene targeting approaches to airway diseases. The approaches greatly help us to understand the mechanisms from signal transductions to immune response in airway diseases. Recent human association studies revealed many novel genes underlying complicated airway diseases. The next challenge will be the systematic analysis of the precise functions of these genes in the pathogenesis of the diseases. This review looks into the current development of gene targeting in mice in the investigations of airway diseases. It also provides a brief protocol for how to rapidly construct the targeting vectors with recombineering and site-specific recombination systems.

Keywords: Gene targeting; Recombineering; Respiratory diseases

Introduction

Human airway diseases are complex diseases underpinned by a broad range of environmental and genetic factors. Asthma and chronic obstructive pulmonary disease (COPD) are the most common airway diseases that are characterized by intermittent inflammation of the small airways of the lung with symptoms of wheeze and shortness of breath. The presence of inflammation can lead to irreversible airway scarring and intractable airflow limitation. Pulmonary endothelial cells, epithelial cells, bronchial smooth muscle cells and immune cells in lung actively participate in the response of airway inflammation. With the rapid development of recombineering and site specific recombination system in recent years, many genes have been targeted in mice for the research of the airway inflammatory response. The knockout (ko) mice provided valuable tools to study the mechanisms of airway diseases from signal transductions to immune responses. Recent human association studies for the complex airway diseases such as asthma and COPD revealed many novel genes that underlie the diseases. Understanding these genetic predispositions to airway diseases offers a means of developing new therapies and strategies for the prevention of disease. Gene targeting in mice will play important roles to dissect the novel gene's functions in human airway diseases.

Mouse Models for Human Airway Diseases

The mouse genome has 2.5 Gb and is 14% slightly smaller than the human genome. Over 90% of the mouse and human genomes can be partitioned into corresponding regions of conserved synteny. Both mouse and human genomes contain about 23,000-25,000 protein-coding genes. Approximately 80% of mouse genes have at least one identifiable orthologue in the human genome. Less than 1% of the mouse genes do not have any homologue currently detectable in the human genome [1]. The conserved gene sequence, structures and the extensive comparative genetic linkage map make the mouse the best model to identify human gene function and provide models of human diseases. The laboratory mice have been used to study human diseases throughout the last century. For a long time, the study was limited to a few visible spontaneous mutations such as agouti and obese [2]. Inbreeding mice can be used to map the disease traits. For airway diseases, genetic mapping in inbred strains can identify loci that are associated traits of asthma [3], acute lung injury [4], COPD and lung cancers [5]. Gene mapping requires two strains of mice in which the phenotypic trait or traits of interest are significantly different, for example, at opposite ends of the distribution. For dissecting the function of genes, many different methods have been developed to generate mutants in mouse genome at a higher rate, including gene-trapping [6], ENU mutagenesis [7] and gene targeting [8]. Since the gene targeting technology became a reality in ES cells, the mouse models have played prominent roles in the genomic and genetic approaches for human airway diseases.

Gene Targeting

Gene targeting in mice includes gene knock-in and gene knockout. A knock-in mouse model is produced when the normal mouse gene is replaced by a mutant version of the mouse gene using homologous recombination allowing study of the variant gene. A transgenic over expression model is when an allele of a human polymorphism is put into a mouse either with or without the mouse background.

A knockout mouse is a genetically engineered mouse in which one or more genes have been made inoperative and is the one of the most use in gene targeting. The disadvantage of gene knockout is that it can cause potential lethal or developmental effects and lead mouse death at the embryonic stage. To avoid this problem, a conditional knockout approach allows researchers to delete the gene of interest in a time- and space-dependent manner.

The technique of conditional gene expression applies site-specific DNA recombinase systems in mouse genome. Two members of the integrase family of site-specific recombinases are used at present to conditionally control gene expression by site-specific DNA recombination [9]. LoxP/Cre system is one of the most efficient and advanced tools for site-specific engineering in the mouse. The Cre recombinase is a 38-KDa protein encoded by bacteriophage P1 that recombines two 34-bp target sites on the genome without the need for

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any co-factor [10]. One of the other non-Cre recombianse is the Flp recombinases of *Saccharomyces cerevisiae*, which recombines sequence called FRT. The thermo-stable version Flpe has an *in vivo* performance compare to Cre [11]. FRT/Flpe system is widely used to remove the selection cassette after successful gene targeting. A typical conditional ko (cko) allele is made by inserting loxP sites into two introns of a gene or at the opposite ends of a gene. Expression of Cre recombinase of mice carrying cko allele catalyzes recombination between the loxP sites and inactivates the gene. By expressing Cre recombinase from a tissue-specific promoter, the gene can be inactivated in a cell-type specific fashion. The timing of Cre expression can also be controlled using inducible Cre expression systems.

Recombineering is a new form of chromosome engineering of homologues. With recombineering, it is possible to introduce selection cassette, FRT and loxP sites anywhere in the mouse genome [12]. The method is based upon efficient homologous recombination in *Escherichia coli* using recombination proteins provided from a defective temperature-sensitive λ prophage. This permits linear double strand DNA fragments to be inserted into DNA cloned on plasmids, BACs or PACs via homologous recombination. The λ recombination functions can be expressed from a plasmid or from a defective prophage integrated into the *E. coli* chromosome. In the defective prophage expression system, the recombination genes are expressed from the strong λ PL promoter, which is itself under the control of the temperature-sensitive λ cI857 repressor. Since the recombineering system discovery, it has greatly impacted on chromosome engineering for gene targeting [13].

The opportunities with mouse models are expanding as new and relevant techniques are being developed such as vectors which allow knock-in genes to be exchanged more readily–so called gene-swapping. A knockout/knock-in approach may be used to create mouse lines that express human allelic variants that can then be evaluated for their pharmacologic relevance. In these experiments the mouse gene is removed and replaced with human major and minor allelic variants. This technique of "zygotic injection" means that mouse lines carrying numerous human alleles could be a reality in the very near future [14].

The development of zinc finger nuclease (ZFN) system offers another means for genomic editing. ZFN has specifications by linking a DNA-binding domain of a versatile class of eukaryotic transcription factor zinc finger proteins (ZFPs) with the nuclease domain of the *FokI* restriction enzyme. Zinc finger domains can be engineered to target desired DNA sequences and this enables zinc-finger nucleases to target unique sequences within complex genomes [15]. ZFN driving targeting has been applied in mammalian cells [16], ES and iPS cells [17] and in plant cells [18].

Practical Protocol for Construct of cko Vector with Recombineering and Site-specific Recombinase Systems

Here, we provide a practical protocol in our lab for construct of cko that was modified from the original report [12]. Before construct the conditional ko vector, the genomic context of the target gene should be checked carefully. There should be not any SINEs, LINEs, LTR elements, DNA elements and small RNA elements in the targeting sequence. In order to avoid possible impaction of insertion on the conserved sequence, dog, cat, cow, human, mouse and rat homologous genomic DNA sequence will be aligned for the gene (http://www.ebi.ac.uk/Tools/clustalw/index.html). Two genomic sites that will be least conserved sequence for the insertion. The strategy of the first genomic point for inserting *FRT–PGK–EM7–NeobpA–FRT–lox*P fragment, and the second point for insertion of *lox*P is that, before the conditional knockout vector construction, Southern Blotting strategies should be worked out in wild type 129 ES cell DNA. Unique probes that lied outside the homology regions will be generated and southern blotting tests should be performed in both 5'arm and 3'arm for the wild type 129 ES cell mice DNA with appropriate restriction enzymes digestions.

Sub-cloning DNA via Recombineering

E. coli cell strains EL350 and EL250 will be used for recombineering. In both strains, PL operon encoding gam and the red recombination genes, *exo* and *bet*, is under tight control of the temperature sensitive l repressor (cI857). EL250 has an *Flpe* gene under the tight control of AraC and PBAD; while EL350 has a *Cre* gene under the same promoter. Upon addition of arabinose in the medium, expression of Cre and Flpe will be induced in the both strains [19]. Two PCR products will be generated for retrieval at both ends of the genomic DNA for subcloning. DNA template will be used 129 ES cell R1. Plasmid PL253 contains modified MC1-TK and this plasmid will be used to retrieve the BACs DNA for sub-cloning. The ampicillin resist positive clones will be selected and the correct inserts will be tested.

Targeting the *loxP* Site into the Second Genomic Point of Sub-cloned Plasmid DNA

PCR products for targeting the second site for insertion of *loxP* will be generated. Two PCR products will flank the second genome site. The PCR primers will be modified by specific resection enzyme for the ligation with DNA fragment that will be digested with *NotI* and *SalI*. The left PCR will be digested with *Not*I and *Eco*Rl, the right PCR will be digested with *Bam*Hl and *Sal*I, the plasmid PL452 (containing *loxP–PGK–EM7–NeobpA–loxP*) will be digested with *EcoRl* and *BamHl*. All four fragments will be ligated to pBSk vector. After transformation, the plasmid will be cut with *NotI* and *SalI*. The DNA fragment will be electroporated with subcloned DNA vector. The correct colonies will grow in kanamycin plate and ampicillin plate and this will be a floxed Neo vector. The one *loxP* site in the vector will be achieved by excising the *Neo* gene via Cre recombinase. The Cre expression will be induced in 0.1% arabinose in EL350 *E. coli* at a controlled temperature of 32°C. The colonies will only grow in ampicillin plate due to remove of the Neo selection marker. The correct vector with the insertion will be a floxed Neo-out vector.

Targeting the FRT–PGK–EM7–NeobpA–FRT–loxP Fragment into the First Genomic Point

After the construction of *loxP* site in the vector, the next step will insert the selection cassette and the other *loxP* into the first genome site. The Neo selection cassette fragment will be from Plasmid PL451that contains two *FRT* sites flanking Neo cassette and one *loxP* site (*FRT–PGK–EM7–NeobpA–FRT–loxP* fragment). The left PCR will be digested with *NotI* and *EcoRl*, the right PCR digested with *BamHl* and *SalI*, the plasmid PL451 will be digested with *EcoRl* and *BamHl*. All four fragments will be ligated to pBSk vector. The bands cutting from *Not*l and *Sal*l will be purified and will be used in recombineering with the suncloning vector that generated at last step. Correct colonies grow both on kanamycin plate and ampicillin plate. This vector will be a conditional ko vector.

Validation and Function Tests of cko Vector *in vitro*

The integration of vector was tested by different stages of building of the vector. With specific enzymes' digestions, the subcloning DNA

vector, the floxed Neo vector, the floxed Neo-out vector and conditional ko vector should show correct sizes under the digestions. In order to test the function of recombinases enzyme Cre and Flpe in the vector, the vectors could be tested in EL350 or EL250 in 0.1% arabinose at controlled temperature of 32°C.

The conditional ko vectors will be subsequently linearized and will be electroporated into R1 ES cells. Transformants will be selected for their G418 and ganciclovier resistance and after southern blotting test; right clone will be picked up.

The steps of construction of cko vector are summarised in (Figure 1). The detailed method for recombineering can be found in the original paper [12].

Applications of the Gene Targeting in Airway Diseases

There are many genes that have been targeted in the investigations of airway disease. This review listed some examples for the successful knockout works in mice for the research of the diseases. The airway inflammation is one of the most prominent features for airway diseases such as asthma, COPD. The development of airway inflammation depends not only on the immune response but also on an appropriate tissue response. The pathology associated with airway inflammation is thought to be mediated by TH2 cells with potentially important roles for the cytokines IL4, IL5 and IL13. Endothelial and epithelium cells play essential roles in inflammation by recruiting immune cells [20,21]. Caspase recruitment domain-containing protein 11 also known as CARD-containing MAGUK protein 1 (CARMA1) is essential for the development of allergic airway endothelial and epithelium cells. Mice with deletion in the *CARMA1* gene did not develop inflammation in a murine model of asthma. *CARMA1* ko mice did not develop airway eosinophilia, had no significant T cell recruitment into the airways. The *CARMA1* ko mice also had significantly decreased levels of Il4, Il5, and Il13, did not produce IgE, and did not develop airway hyper-responsiveness or mucus cell hypertrophy. The *CARMA1* ko mice are essential tools for investigating the NF-kB pathways of the MAPK kinases [22]. The p38 mitogen-activated protein kinase (MAPK) is also known to regulate many functions of airway inflammation. MAPK-activated kinase 2(MK2) is a serine/threonine kinase. *MK2* ko mice do not affect systemic Th2 immunity, but reduced endothelial permeability, as well as adhesion molecule and chemokine expression [23]. Another MAPK kinase *MAP3k8* ko mice produced low levels of TNF-α when exposed to lipopolysaccharide (LPS) and they are resistant to LPS/D-Galactosamine- induced pathology [24]. The co-stimulatory pathways in T cell activation play important roles in different phases of the generation of TH2 responses. The newly discovered pathway ICOS and its ligand B7RP-1was implicated in the development of TH2 effector activities [17]. *B7RP-1* ko mice were showed to resist the induction of inhalation tolerance but not required for the generation of Th2 response as it showed airway eosinophilic inflammation after OVA challenge and also significant expression of T1/ST2 on the T cells and production of Th2 cytokines Il4, Il5 and Il13 [25]. Reactive oxygen species (ROS) contribute to inflammation by damaging DNA, results in the activation of poly (ADP-ribose) polymerase-1 (PARP-1) and depletion of its substrate. Deletion of *Parp-1* prevented lung inflammation in a murine model of asthma [26]. Bcl6 is a potential sequence-specific transcriptional repressor and plays important roles in the development of T cell lineage [27]. *Bcl6* deficient mice develop a spontaneous and severe Th2-type inflammatory disease exhibit pronounced Th2 responses when challenged with an allergen [28] and Bcl6 controls the Th2 inflammatory activity by repressing GATA3 function [29]. GATA3 is a Th2-specific transcription factor and is also crucial for development of the T lineage and throughout thymus development. Using a conditional ko approach, Gata-3 was found to be required for optimal Th2 cytokine production *in vitro* and *in vivo* [30]. Loss of hypoxia-inducible transcription factor-1α (Hif-1α) also reduced eosinophil infiltration, goblet cell hyperplasia, levels of cytokines Il4,

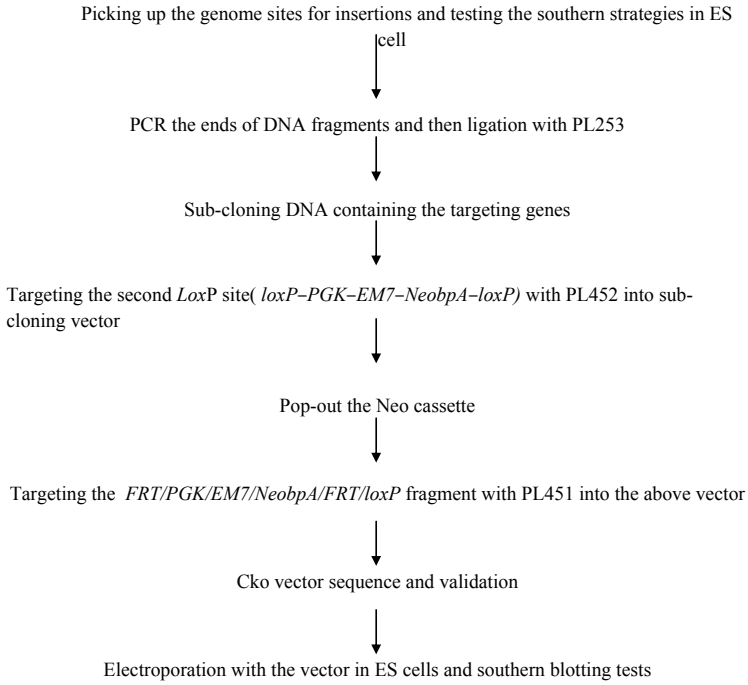

Figure 1: The steps of generating conditional knockout vector.

I55 and 13 in the mouse lung [31]. Pattern-recognition receptors (PRRs) are involved in pathophysiology of airway allergy, Interferon response factors IRF3 and IRF7 are transcription factors downstream of various PRRs. Airway allergy was strongly attenuated in *Irf3* ko mice, but knot in *Irf7* ko mice [32]. T-bet, a TH1-specific T-box transcription factor transactivates the *IFNγ* gene in TH1 cells and has the unique ability to redirect fully polarized TH2 cells into TH1 cells. T-bet ko mice demonstrated a physiological and inflammatory phenotype in murine airways similar to that created by allergen exposure in sensitized mice, in the absence of an induced inflammatory response [33].

Acute lung injury (ALI) and its severe form, acute respiratory distress syndrome, represent a clinical syndrome that results from numerous causes and is responsible for significant mortality. The most characteristic pathologic findings associated with ALI are alveolar oedema, caused by endothelial and epithelial injury, and the infiltration of inflammatory cells, especially neutrophils, into airspaces [34]. The endothelial form of the Ca^{2+}/calmodulin-dependent enzyme myosin light chain kinase *(MLCK) MLCK210* plays a critical role in the control of EC barrier function. *MLCK (MLCK210)* ko mice were protected against endotoxin-induced ALI and the lethal complications [35]. The transcription factor Nrf2 is essential inducible expressions of a group of detoxification and antioxidant enzymes. ALI induced by carrageenin was markedly exacerbated in *Nrf2* ko mice. Analysis of bronchoalveolar lavage fluids also revealed that the magnitude and the duration of acute inflammation, indicated by albumin concentration and the number of neutrophils, were significantly enhanced in *Nrf2* ko mice [36]. Respiratory distress syndrome (RDS) due to insufficient production of surfactant is a common and severe complication of preterm delivery. Loss of the hypoxia-inducible transcription factor-2α (HIF-2α) in mouse caused fatal RDS in neonatal mice due to insufficient surfactant production by alveolar type 2 cells [37].

Pulmonary fibrosis is the formation or development of excess fibrous connective tissue (fibrosis) in the lungs. The causes of fibrosis could be environmental and occupational pollutants, cigarette smoking. Inflammation is not believed as crucial to the aetiology. The abnormality of alveolar epithelium cells is a primary source of the disease development [15]. Tetraspanin CD151 and CD9 are membrane proteins which regulate cell activation, fusion, motility and signals. *Cd151* ko mice spontaneously exhibited age-related pulmonary fibrosis. Gene expression profiling revealed an enrichment of genes involved in connective tissues disorders in the lungs of *Cd151* ko mice, but not in *Cd9* ko mice [38]. Ca^{2+}-activated Cl^- channels (CaCCs) contribute to airway Cl^- and fluid secretion and were implication of the disease process of cystic fibrosis , but recent *mCLCA3* ko mice work didn't show any change in calcium-activated chloride conductance in murine airways. The results argue agonist a role of mCLCA3 in CLCA3-mediated Cl^- secretion in murine respiratory epithelia [39]. Table 1 listed the successful examples of mouse gene knockout for the research of airway diseases.

The Future Gene Targeting Work for the Airway Diseases

Recent positional cloning and GWAS studies in human have identified many novel genes that underlie airway diseases [40]. *ADAM33* [41], *PHF11* [42] *DPP10* [43] and *GPRA* [44] were positional

Genes	Synonyms	Chromosome Location	Function	Airway Disease Model	References	Ko mice Phenotypes
CARMA1	*Card11*	5	Protein biding	Airway inflammation	[22]	Ko mice did not develop airway inflammation, decrease il4, il5 and il13 levels in lungs
MK2	*MAPKAP kinase 2, Rps6kc1,Mapkapk2*	1	Kinases activities	Airway inflammation	[23]	Ko mice did not affect th2, reduce endothelial permeability
Map3k8	*c-COT, Cot, Cot/Tpl2, Tpl2, Tpl-2*	18	Kinases activities	Airway inflammation	[25]	Ko mice had Lower TNFα when exposed to lipopolysccharide
B7RP-1	*B7h, B7-H2, GL50, GL50-B, ICOS-L, LICOS*	10	Receptor binding	Airway inflammation	[17, 25]	Ko mice showed to resist to the induction of inhalation
Parp-1	*5830444G22Rik, Adprp, Adprt1, PARP, sPARP-1*	1	DNA binding	Airway inflammation	[26]	Ko mice were shown preventing lung inflammation
Bcl6	*Bcl5*	16	Chromatin binding	Airway inflammation	[28, 29]	Ko mice developed spontaneous severe Th2 type of inflammatory disease
Gata3	*Gata-3*	2	Th2 transcription factor	Airway inflammation	[30]	ko mice showed that Gata-3 was required for Th2 cytokine production
Hif1a	*bHLHe78, HIF1alpha, HIF-1alpha, MOP1*	12	Transcription factor	Airway inflammation	[31]	Ko mice showed reduced eosinophil infiltration, goblet cell hyperplasia, levels of Il4, Il5 and Il13
Irf3	*C920001K05Rik, IRF-3*	7	Transcription factor	Airway inflammation	[32]	Ko mice showed attenuation of airway allergy
T-bet	*Tbx21, Tblym, TBT1*	11	Transcription factor	Airway inflammation	[33]	Ko mice showed inflammatory phenotype in lung
MLCK210	*A930019C19Rik, Mlck, MLCK108, Mylk, telokin*	16	Kinases activities	Acute lung injury	[35]	Ko mice were protected against injury
Nrf2	*Nfe2l2*	2	Transcription factor	Acute lung injury	[36]	Ko mice showed remarkable acute lung injury induced by carrageenin
HIF-2a	*Epas1 bHLHe73, HLF, HRF, MOP2*	17	Transcription factor	Respiratory distress syndrome	[37]	Ko mice showed fatal RDS in neonatal stage
Cd151	*PETA-3, SFA-1, Tspan24*	7	Membrane protein	Pulmonary fibrosis	[38]	KO mice showed spontaneously pulmonary fibrosis

Table 1: The successful examples of gene knockout in mice for researches on airway diseases.

cloning genes underlie asthma while *SERPINE2* [45] for COPD. The first GWAS on asthma discovered that the *ORMDL3* locus was strongly associated with childhood disease [46]. The GWAS in 2010 the GABRIEL consortium reported the results of the biggest GWAS so far for identifying genes underlying asthma [47]. The GWAS replicated the association of SNPs in *ORMDL3* to childhood asthma and found novel associations within *IL1RL1/IL18R1*, *HLA-DQ*, *IL33*, *SMAD3*, and *IL2RB*. Other GWASs identified asthma associated genes *DENNDB1* [48], *PDED4* [49] and IgE associated genes IL4/IL13 [47], and *FCER1A* [47,50]. *GlCCI1* gene was found to have association with response to glucocorticoid therapy in asthma [51].

Some of these genes have already been successfully targeted. A recent mouse gene ko showed that Il33 worked as a crucial amplifier of innate immunity [52]. The induction of Il33 expression by environmental or endogenous triggers now suggests a wider role for the pathway during infection, inflammation and tissue damage [53]. *SMAD3* encodes SMAD (mothers against decapentaplegic homolog) family member 3. SMAD3 protein functions as a transcriptional modulator activated by TGFB. Mice lacking *Smad3* exhibit increased levels of pro-inflammatory cytokines in their lungs [54]. A conventional ko of *Smad3* was generated in 1998 and show accelerated wound healing and an impaired local inflammatory response [55]. But for positional clone gene ADAM33, *Adam33* ko mice showed normal allergen-induced airway hyperactivity, immunoglobulin E production, mucus metaplasia, and airway inflammation [56].

The next challenge for genomic and genetic approaches of airway diseases will focus on the newly identified susceptive genes; particularly for the genes their functions remain unknown. Gene targeting together with gene trapping, ENU mutagenesis and genome editing by ZFN in mice will play important roles to define their precise path-physiologic mechanisms of the airway diseases.

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