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Conditional and inducible gene recombineering in the mouse inner ear

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Abstract

Genetically engineered mice have greatly improved our understanding of gene functions and disease mechanisms. Nevertheless, the traditional knock-out approach has limitations in the overall viability of mutants. The application of the *Cre/loxP* system in the inner ear can help bypass this difficulty by generation of conditional gene recombineering. However, to do so requires an expression system that allows ear-specific temporally inducible, gene abrogation of one or more of the increasingly available floxed genes. To date, three approaches have been successfully used to create murine inner ear-specific *Cre* lines: conventional transgenesis, BAC transgenesis, and gene knock-in. Unfortunately, timing of conditional *Cre* activity does not extend beyond the regulatory range of the gene controlling *Cre* expression. Rectification of this problem requires the generation of tamoxifen or tetracycline inducible systems in the inner ear. Examination of integrase expression at different loci will facilitate studies on the expression of exogenous transgenes. These genetic applications for the mouse genome will dramatically advance in vivo gene function studies.

Keywords

Recombineering; Recombinase; Integrase; Transgene; Inducible; Inner ear

1. Importance of developing recombineering mouse lines in inner ear

Genetically engineered mice have greatly improved our understanding of gene functions and disease mechanisms, thereby enhancing translational research (Gao et al., 2004; Liberman et al., 2004; Zuo, 2002). While of great value, genetically engineered mice also have limitations. Such limits include embryonic lethality, leaky expression, chromosomal insertional and positional variations, strain background effects, and biological redundancy. It is demonstrated that 15–20% of germline mutations result in embryonic lethal phenotypes (Zambrowicz et al., 2003). Some of these limitations relate to the use of ubiquitous promoters to create gain-of-function (GOF) transgenic mice as well as a wider expression pattern of a gene-of-interest (GOI) or the use of a gene's endogenous promoter to examine loss-of-function (LOF) by knock-out strategies or GOF and misexpression using knock-in techniques. Much needed, more restricted expression was achieved by incorporating gene promoter *cis* elements into transgenic construct design using mammalian and

nonmammalian recombination systems. These advanced systems include noneukaryotic recombinases for excision of targeted genes, inducibility using tetracycline or tamoxifen treatments, and, most recently, transposon technology (Albanese et al., 2002; Groth and Calos, 2004; Heine et al., 2005; Izsvak and Ivics, 2004; Liu et al., 2003; Ristevski, 2005; Tian et al., 2004). Inducible switches provide experimental and therapeutic avenues for both qualitative and quantitative regulation of gene expression and manipulation of gene networks or modules. Finely controlled gene expression circuitries should increase development of successful therapeutic interventions in future gene therapy and tissue engineering initiatives (Kramer et al., 2005). Despite this progress, temporal restriction of expression to a single organ or cell type by genetic engineering is only now being realized (Ficker et al., 2004; Gao et al., 2004; Powles et al., 2004). Presently, no transgenic model exists that permits complete control of spatial, temporal, and quantitative regulation of gene expression exclusively in the ear. Inner ear-directed expression is now possible (Ohyama and Groves, 2004), but expression in critical tissue(s) other than the inner ear can cause systemic defects that can inherently lead to misinterpretation of the resulting data. A universal system is needed for conditional alterations in inner ear expression of GOI to permit an analysis of inner ear function without additional systemic effects. The two major requirements for these constructs are that they should be: (a) topologically restricted to only the inner ear; and (b) conditionally inducible to control both the duration and level of expression.

Development of any given organism or organ requires the coordinated activation of hundreds of gene products, each tightly regulated in its spatio-temporal expression (Davidson et al., 2002; Levine and Davidson, 2005). Dissecting such networks of gene interaction has been possible thus far only in a few model organisms and organs, such as the developing vertebrate limb. Research in ear expression profiles suggests similar complexity for its development. For example, worldwide efforts to identify population isolates and pedigrees with hearing loss have currently identified 64 genes from 131 loci for auditory disorders. This approach, in combination with mouse strains carrying spontaneous, chemically induced, and genetically engineered mutations is currently being used to elucidate the biological basis for hearing and balance (Curtin et al., 2003; Gao et al., 2004; Rhodes et al., 2004; Washington et al., 2005; Zuo, 2002) and more recently to regulate hair cell formation as a therapeutic treatment for deafness (Izumikawa et al., 2005; Mantela et al., 2005; Raphael and Martin, 2005; Sage et al., 2005; Taylor and Forge, 2005). Despite these great strides, experimental genetic tools to manipulate inner ear gene interactions have limited faster progress as effects have to be studied basically one gene at a time, an approach that will not elucidate more complex inherited disorders. Unfortunately, even if a nonsyndromic or “normal” phenotype is observed, the impact of a mutation can have a much wider effect than originally thought. An example of genotype–phenotype relationship is found in the genes associated with Usher syndrome (Keats and Savas, 2004; Rhodes et al., 2004). Depending on the location and type of mutation within a gene and the severity of the defect, an asymptomatic, atypical, nonsyndromic, or syndromic phenotype can result (Liu et al., 1998; Self et al., 1998). Because of the wide spread expression of many genes, it may also be difficult to distinguish indirect and direct mutational effects on a single organ or tissue even in a nonsyndromic disease.

One of the limitations facing ear research is therefore the lack of an expression system that allows ear-specific, temporally inducible, gene abrogation of one or more of the increasingly available floxed genes or gene expression of a GOI. Even the most recent genetically engineered mouse lines (Gao et al., 2004; Ohyama and Groves, 2004) are not optimal to restrict effects of genetic manipulations only to the ear. Thus, a genetic-based system is needed by which the pathogenic or functional effect(s) of a mutation or expression of a GOI is isolated exclusively to the inner ear. If successful, such an expression system would allow

generation of viable mice that could also lead to novel physiological insights, thus enhancing the translational aspect of basic molecular research. Full assessment of gene function requires an ear and cell-type-restricted overexpression or misexpression of a GOI. Such GOF expression will not only complement the conditional LOF approach, but will also provide essential information that directly ties into translational research to replace mutated genes/gene products safely.

Ideally, interpretation of mutational effects on a single cell, tissue or organ type should reveal the precise mechanism(s) of the associated pathogenesis or function without additional systemic defects. We have established a number of criteria needed for a genetic-based system of functional analyses of genes in the inner ear. First and foremost, these genetic alterations should be exclusively restricted to the inner ear or specific cell types within the inner ear. Second, a common set of transgenes must be constructed that permit the excision, overexpression, knockdown, or misexpression of a normal or mutated gene. This will require that the expression in the nontargeted cells, tissues, and organs is completely unaltered; we will refer to this as a “deadbolt”. Third, an inducible system is needed that allows for specific, temporal expression activation of a GOI, regardless of the age; we will refer to this mechanism as the “doorlatch”. Fourth, a tightly controlled expression system is required for the quantitative control of GOI expression; we will refer to this as “unlocking”. Finally, this transgenic system should be “universally” adaptable to the current explosion in the number of ear relevant genes and permit the rapid integration of genetically altered GOIs as well as the gene-specific RNAi constructs and the use of the more global, natural microRNA integrated into these constructs.

2. Recombinases-fundamental reagents in genome tailoring

The recombinases are a large range of proteins split into two families, tyrosine recombinases and serine recombinases. Well over 100 members of these families have been found in all three biological kingdoms (Chen and Rice, 2003). Currently, there are a number of approaches for site-directed genome modification. These include the use of viral-induced, transposon-based and recombinase-mediated DNA integration systems (Coates et al., 2005; Gao et al., 2004). For the most part, the viral-induced and transposon-based are not site-specific and would not be appropriate for the current approach. Presently, two recombinases, Cre and Flp, are predominantly used as the basis of the genetically engineered mouse lines that facilitate a “deadbolt” system. Recently, a ϕ C31 integrase was used for insertion of a GOI (Belteki et al., 2003). Of the recombinase and integrase enzymes, Cre has a high site-selectivity and is highly efficient (Dong and Stuart, 2004).

2.1. Cre recombinase

The Cre-recombinase efficiently targets the *loxP* sequence and catalyzes the insertion, excision or inversion of DNA between two *loxP* sites (Fig. 1A). Cre recombinase is the 38-kDa product of the *Cre* gene of bacteriophage P1 and recognizes a 34-bp site within the P1 genome, called *loxP*. Cre efficiently catalyzes reciprocal conservative DNA recombination between pairs of *loxP* sites. The *loxP* site consists of two 13-bp inverted repeats flanking an 8-bp nonpalindromic core region that provides the *loxP* site with directionality. The Cre molecules are able to recombine between two *loxP* sites without the need for other cofactors. Postrecombination *loxP* sites are formed from two complementary halves of the prerecombination sites, and retain their function as target sites for Cre. The result of Cre-mediated recombination depends upon the location and orientation of the *loxP* sites in question. Cis configurations (*loxP* sites on the same DNA strand), can result in inversion of DNA segments when *loxP* sites are in opposite orientations, or excision when *loxP* sites are in the same orientation (Fig. 1). If the two *loxP* sites lie on different strands of DNA (trans-configuration), an insertion event will take place. However, since target excision is the most

favorable reaction, inserted genes will be quickly excised with continued Cre activity (Nagy, 2000).

Cre recombinase is therefore widely used as an important tool for the excision of specific sections of DNA flanked by two *loxP* sites ("floxed" DNA) (Gu et al., 1993; Nagy, 2000). The expression of a few Cre molecules per cell should be sufficient to excise *loxP* sites and any DNA they surround (Zuo, 2002). Mouse lines can be generated that express Cre under the control of a tissue- or cell-type-specific promoter. These lines can then have immense use in the creation of conditional knock-outs or transgenic lines. Lines containing a floxed gene of interest can be crossed with the Cre expressing line to generate conditional gene knock-outs on backcrossing with the floxed line. Alternatively, a floxed region can be added upstream from a transgene, preventing its expression. In cells expressing the Cre gene, this floxed section can be deleted, allowing expression of the downstream gene of interest and the creation of a conditional overexpressor.

2.2. Flp recombinase

Another site-specific recombinase, Flp recombinase from yeast, is available for engineering the mouse genome (Dymecki, 1996b). Similar to recombination between *loxP* by Cre, Flp recognizes *FRT* sites that result in similar genetic alterations including excision, inversion, and translocation of targeted DNA sequences. Flp recombinase also has a high site-selectivity, but is not as effective as Cre at 37 °C, because of reduced enzyme activity from the optimum 25–30 °C (Buchholz et al., 1996). This problem was addressed by production of a more efficient Flp recombinase (FLPe) activity (Voziyanov et al., 2003) and the use of the nuclear localization signal peptide or ligand binding domain of the estrogen receptor (Andreas et al., 2002; Hunter et al., 2005; Liu et al., 2003; Schaft et al., 2001). As of yet, there are no *Flp* lines reported for auditory research (Gao et al., 2004).

2.3. ϕ C31 integrase

Novel strategies have been developed to achieve site-specific insertion or cassette exchange since none of the known systems allowed efficient, selection-free identification of insertion or cassette exchange. The disadvantage of the Cre/*loxP* and Flp/*FRT* systems is that integration of DNA segments is highly unstable. Insertion events create two cis-recognition sites, which are immediate substrates for excision (Belteki et al., 2003). Although this problem may be overcome by the use of mutant recognition sites, alternative systems have been identified which allow for irreversible and highly efficient genomic integration or cassette exchange.

Recently, an integrase from Streptomyces phage C31 has been shown to function in mammalian embryonic stem cells and is compatible with germline transmission (Belteki et al., 2003). This enzyme catalyzes recombination between two nonidentical attachment sites, a 39 bp *attP* site and a 34 bp *attB* site, to form *attL* and *attR* sites (Fig. 1B), which are not target sites for integrase-mediated excision (Thorpe et al., 2000). No accessory cofactors are required for the integration reaction, therefore allowing it to take place in cells transfected with integrase and the required *attP* and *attB* containing substrates (Thorpe et al., 2000). Moreover, the integration reaction appears to be irreversible in the absence of any other cofactors, and nonpermissive pairs of sites such as *attP/attL* and *attB/attR* do not recombine (Thorpe and Smith, 1998). In nature, the excision reaction can take place, though it probably requires phage-encoded accessory factors, such as the Xis factor identified for the homologous serine integrases encoded by lactococcal phage TP901-1 (Breuner et al., 1999). Expression of integrase in hair cells or other cell types in the mouse cochlea could facilitate introducing exogenous gene expression in a cell-type-specific manner (Fig. 2B).

The upcoming mouse genome knock-out project aims, in part, to create conditional alleles of all genes (Austin et al., 2004). To take full advantage of this invaluable resource, the creation of inner ear-specific *Cre*, *Flp*, or ϕ C31 integrase lines will have to be synchronized with specific designs of large-scale conditional targeting strategies.

3. Analyzing Cre activities in the mouse inner ear

Testing *Cre* lines for their specificity and efficiency is always a key step in developing conditional gene targeting techniques. It is necessary to analyze the *Cre* expression pattern by crossing with a reporter line which can be easily analyzed. A good reporter line should be sensitive to detect and express reporter genes ubiquitously in all tissues. The most used reporter line is the universal *ROSA26* reporter (R26R) line which contains floxed multiple STOP codons followed by the *LacZ* cassette (Soriano, 1999). When *Cre* is expressed, it excises the stop codons and leaves one *loxP* site in the genome. Thus the *LacZ* will be expressed in the cells where *Cre* is expressed under the *ROSA26* promoter. It is also determined that in the inner ear there is little endogenous β galactosidase (β gal) activity present in the mouse cochlea although some bony structures around the cochlea could show positive signal in the R26R control mice or with prolonged incubation with the substrate (Li et al., 2004; Tian et al., 2004).

To characterize the *Cre* expression pattern, the *Cre* and R26R double positive mice should be analyzed using different approaches (Oberdick et al., 1994). The easiest and the most sensitive approach is Polymerase Chain Reaction (PCR). Since the STOP sequence between the two *loxP* sites is more than 3 kb, a standard PCR reaction using genomic DNA should not normally yield any positive product if using PCR primers flanking the two *loxP* sites. If any cell in the tissue has an observable PCR product, it indicates the presence of *Cre* activity and excision of the STOP sequence even just in a few cells in the tissue. Although PCR is extremely sensitive, it only provides a qualitative assessment without any cellular resolution to determine the extent of *Cre*-mediated excision of the floxed STOP sequence.

The second approach is X-gal staining. X-gal serves as a substrate for the *LacZ* gene product, β gal. The reaction products are round-shape blue dots present in the lysosomal compartments or in the nucleus if *LacZ* has a nuclear localization signal incorporated at the amino-terminus. R26R activity in association with *Atoh1-Cre*, *Foxg1-Cre*, *Pax2-Cre*, *Pres-Cre*, and *pPres-Cre* is associated with blue staining near the perinuclear region inner ear hair cells (Hebert and McConnell, 2000; Li et al., 2004; Matei et al., 2005; Ohyama and Groves, 2004; Tian et al., 2004). Another way to detect β gal activity is by *LacZ* immunohistochemistry. The *LacZ* antibody is able to recognize the β gal antigen in the cytoplasm of cochlear hair cells because of the lysosomal or nuclear compartment distribution (Kwon et al., 2001; Oberdick et al., 1994). The cellular staining pattern of immunohistochemistry should be quite similar to the X-gal reaction although minor discrepancies could be observed depending on the staining conditions.

Other important reporter lines that are similar to the R26R line include the *Z/AP* (Lobe et al., 1999) and *Z/EG* (Novak et al., 2000) mouse lines. Both of these lines use strong ubiquitous promoters, β actin and pCAGGS, respectively. The detection of human placenta alkaline phosphatase (hAP) and eGFP in the mouse tissues provides further independent information in detecting the *Cre* activity of excision at different loci.

4. Mouse models for inner ear function

The origins of genetically engineered experimental mouse models are rooted in the development of inbred mouse strains by mouse fanciers. In particular, many lines were developed based on coat color and behavioral traits. Fortunately, many of the lines with

behavioral anomalies provided a rich resource of spontaneous mutations involving defective inner ear genes. Subsequently, chemically induced mutations and genetically manipulated modifications, based on embryonic stem cell technologies, provided the two major sources of genetically altered mouse line variants. Use of these powerful tools provides a means to determine the function of genes and their products in studies of auditory and vestibular systems (Gao et al., 2004; Steel and Kros, 2001; Zuo, 2002). “Humanized” mutant mouse lines also furnish in vivo models to investigate the mechanism(s) of pathogenesis and to develop and test effective therapies. Spontaneous mutants, knock-outs, knock-ins, and more recently Bacterial Artificial Chromosome (BAC)-generated transgenesis have the distinct advantage of utilizing the native promoter of the GOI, thus retaining the normal expression patterns in fetal and postnatal mice. Conventional transgenic lines, in spite of their rapid production and general usefulness, are created by random integration in the genome resulting in variable expression patterns and are further marred by the effect from and upon flanking genes (Crusio, 2004). Flanking gene effects are also observed in knock-outs and knock-ins. Thus, the effects of linkage cannot be ignored especially if there is an effect on the flanking genes. Another fundamental complication of any induced-mutation experiment is epistatic interactions, where “background” effects can lead to misinterpretation of the data. Integration site effect will be minimized by selecting those founder lines with the appropriate inner ear expression pattern and the site of integration that are not near or within promoter regions nor indicative of gene disruption. So far, three methods are used to generate tissue-specific *Cre* lines: (1) traditional transgenesis method using tissue-specific promoter; (2) BAC transgenesis method; and (3) knock-in method using embryonic stem cells.

4.1. Traditional transgenesis method using tissue-specific promoter

Traditional transgenesis is the most common method for generating *Cre* lines in mice. Several traditional transgenic lines have been published for mouse hearing studies. They include *Atoh1-Cre* (Matei et al., 2005), *Prestin-Cre* (Li et al., 2004), and *ColA1-Cre* (Sage et al., 2005). *Atoh1-Cre* shows expression in the putative inner ear hair cell precursors as early as E11 and expression is maintained throughout adulthood (Matei et al., 2005). The *Atoh1-Cre* mice also have variable expression in supporting cells of the cochlear and vestibular endorgans. *Prestin-Cre* transgenic line, carrying a 9-kb genomic fragment encompassing the *prestin* regulatory element of intron 3, shows inner hair cell expression starting at P14 that is maintained through adulthood. At P50, the last row of outer hair cells also expresses Cre recombinase. This line also shows expression pattern in spiral ganglion cells and vestibular hair cells. *ColA1-Cre* is detected ubiquitously in E11.5 otocysts. The weakness of this conventional transgenic method is that only a limited number of tissue-specific promoters are well characterized. The lack of tissue-specific promoters will definitely cause the unexpected *Cre* expression pattern which will further hamper the analysis of gene functions. This of course limits the use of plasmids to generate tissue-specific *Cre* lines successfully.

4.2. BAC transgenesis

This approach takes advantage of BAC containing mouse genomic fragments to target gene expression in the mouse inner ear. The use of BAC technology has already been successfully applied in cochlear hair cell (Zuo et al., 1999). The advantages of this method are as follow: (1) Almost all mouse genes have detailed BAC clones identified due to the mouse genome sequencing project. All mapped BAC clones from the RPCI-22 and RPCI-23 mouse genomic libraries are currently available through BAC resources and Ensembl (Osoegawa et al., 2000); (2) These two BAC libraries contain genomic fragments ranging from 110 to 260 kb which should contain all the regulatory elements necessary to recapitulate the endogenous gene expression pattern for many genes. By introducing *Cre* into the BAC, *Cre*

expression is more likely to faithfully retain the “normal” endogenous gene expression pattern; and (3) BAC transgenes are not subject to strong position effects that can result in undesirable expression pattern. Thus, BACs are ideal recombinant tools for generating tissue-specific *Cre* expression in the mouse inner ear. Important drawbacks of this methodology are the additional *loxP* site in the BAC vector and the likelihood of other genes and promoter elements within these large genomic fragments. One *loxP* sequence is retained in the BAC cloning vector which could be transferred into the mouse genome and could result in multiple *loxP* sites existing in the mouse transgenic line. This could complicate the use of *Cre/loxP* strategy in these animals. These unwanted *loxP* sites are best removed before pronuclear injection.

The method of BAC modification has been improved recently (Branda and Dymecki, 2004). The first approach involves introduction of the shuttle vector into the BAC *E. coli* host (Gong et al., 2003; Yang et al., 1997). The cointegration step shows insertion of the entire shuttle vector into the BAC by homologous recombination while the unwanted BAC vector sequences are then eliminated through a second homologous recombination event. The efficiency of this method was reported to be 75% for cointegration and 63% for resolution (Gong et al., 2002). The alternative approach is to use the *RecE/ RecT* from *Rac* prophage or *Red α /Red β* from the *Red* operon of lambda phage in modifying the BACs (Muyrers et al., 1999; Zhang et al., 1998). The phage proteins can trigger homologous recombination with relatively short homologous arms. Red recombination has been employed to generate gene-targeting constructs for ES cells (Liu et al., 2003; Testa et al., 2003). Notably, both *RecA* and *ET/Red* cloning strategies give rise to high ratios of correct (homologous) to incorrect recombination. Recently, a highly efficient galactokinase (*galK*) positive/negative selection was developed for rapid manipulation of BACs for introduction of point mutations, deletions, and *loxP* or *FRT* sites (Warming et al., 2005). High-throughput BAC modification projects rely on these strategies for the production of BAC transgenic mice. *RecA*-mediated recombineering has been used for the insertion of reporter sequences to study gene expression in the central nervous system (Gong et al., 2003). The other modified *RecE/ RecT*-based approach has been applied in *VelociGene*, a high-throughput and automated method for generating reporter expression pattern from modified endogenous genes (Valenzuela et al., 2003).

The expression pattern of *Cre* lines generated with the BAC modification method is more likely to reflect the authentic endogenous gene expression pattern. Two strategies have been taken for BAC modification. One is to modify the ATG initiation site by inserting *Cre* after the ATG site. The other is to modify the sequences after the stop codon of the endogenous gene by inserting Internal Ribosome Entry Site (*IRES*) and *Cre* into the 3' noncoding region. In general, insertion of the *IRES* sequence before *Cre* will empirically increase *Cre* protein production by threefold. In the latter case, the transcript is transcribed under the endogenous gene promoter while *Cre* is translated under *IRES* mechanisms (Mountford and Smith, 1995; Zuo, 2002). So far, three *Cre* lines generated through BAC transgenesis have been used for hearing research. The first published BAC *Cre* line is *Otog-Cre* which expresses *Cre* molecules at E10 in the otic vesicle and at E18 in all cells of the gap junction epithelial network (Cohen-Salmon et al., 2002). The others include *Pax2-Cre* (Ohshima and Groves, 2004) and *Prestin-Cre* (Tian et al., 2004). *Pax2-Cre* shows a broad expression by postnatal day 0 (P0) in the mouse cochlea while BAC *Prestin-Cre* mice show detectable inner and outer hair cell expression starting at P6.

4.3. Knock-in method using embryonic stem cells

This method for generating cell-type-specific *Cre* line is to perform a knock-in which uses homologous recombination in ES cells to insert *Cre* into the endogenous locus. This

approach requires murine embryonic stem cell screening step, so it takes a relatively longer time and is not as efficient as the BAC modification method. The advantage of this approach is that the endogenous gene expression pattern is more likely to be obtained. Examples of these are *Foxg1-Cre* (Pirvola et al., 2002) and *Pax8-Cre* (Bouchard et al., 2004) mouse lines. *Foxg1-Cre* mice show whole otic epithelium expression pattern at E13.5. Cre activity in *Pax8-Cre* shows an early and broad expression in the cochlea similar to the native *Pax8* gene (Bouchard et al., 2004). However, even with this approach, a certain risk will remain, that expression variations will be observed in different mouse lines, as was shown for the *Foxg1-Cre* lines (Hebert and McConnell, 2000).

4.4. Common mouse strain background as a universal system

Because of the high efficiency for gene targeting, 129 ES cells are commonly used and most knock-out mice have a mixed 129 and C57B6 background. Both 129 and C57B6 strains are not ideal for hearing research, because of the onset of sensorineural hearing loss (mostly due to a hearing loss locus on each of chromosomes 5 and 10; Johnson and Zheng, 2002; Johnson et al., 2000) as early as 2–3 months of age in the C57B6 strain, and an early onset of age-related hearing loss in 129 strains. Moreover, hearing phenotypes in knock-out mice sometime vary significantly among individuals of different breeding pairs and generations in the mixed background. We therefore propose that gene-targeted mutant mice for hearing research should be created or transferred into a CBA/CaJ or FVB/N strain background.

CBA/CaJ is regarded as “the well hearing” and “gold standard” normal-hearing strain and has been used extensively as a control by hearing investigators (Erway et al., 1996; Johnson and Zheng, 2002; Johnson et al., 2000; Willott et al., 2003; Yoshida et al., 2000; Zheng et al., 1999). Furthermore, CBA/CaJ ES cells could be developed for hearing studies, although the CBA/CaJ strain is typically nonpermissive for gene targeting and germline transmission (www.thromb-x.com; Brook and Gardner, 1997; Schoonjans et al., 2003). For mutant mice in other strain backgrounds, one can transfer them into the desired CBA/CaJ background by standard successive backcrosses. The FVB/N strain could also be selected as the carrier for the transgenic constructs due to a normal hearing phenotype (http://www.jax.org/hmr/inbred96_2k1_deca.html), their prevalent use for transgenic production, availability of ES stem cells, large litter size, and excellent maternal behavior (Auerbach et al., 2003; Schoonjans et al., 2003; Taketo et al., 1991; Wang et al., 1993). The FVB/N does carry the *Pde6b^{rd1}* gene that causes early onset retinal degeneration (Chang et al., 2002). If this does present a problem, then a congenic line FVB.129P2-*Pde6b*^{+/J}, obtained commercially from Jackson Laboratories, can be used to replace the *rd1* with the wild-type allele (Gimenez et al., 2001).

5. Generating inducible *Cre* lines in the mouse inner ear and its applications

A protein of interest may have different functions during the lifetime of an individual, so, in some cases, the function of the protein may need to be studied at specific time points as well as in specific cell-types. The ability to inactivate a gene at a specific, controlled time point during development may greatly aid the understanding of gene function, and help distinguish pleiotropic functions of the gene. Use of site-specific recombinase systems has permitted the development of conditional alleles for activating and inactivating a gene in a “conditional” manner, resulting in spatially and temporally restricted expression patterns (Kwan, 2002). Conditional alleles can be a powerful tool in assessment of gene function in specific cells, cell lineages, tissues, and organs. *Cre/loxP* recombination system is widely used and now is combined with inducible transgenic constructs that are represented by

tamoxifen-mediated Cre-ER and Tet_{ON}/Tet_{OFF} systems (Hayashi and McMahon, 2002; Heine et al., 2005; Indra et al., 1999).

5.1. CreER^{T2}

One of the most efficient systems is the tamoxifen induction system. The system takes advantage of the nuclear localization capacity of the estrogen receptor–ligand binding domains when the ligand is present (Brocard et al., 1997) (Fig. 2). A mutated form of the ligand-binding domain of the estrogen receptor (ER) is fused to Cre recombinase. This mutated ER will not bind to the natural ligand, beta estradiol, and instead binds the synthetic ligands tamoxifen, or 4-hydroxy(OH) tamoxifen (Feil et al., 1996). In the absence of ligand, the Cre-ER fusion protein is located in the cytoplasm and bound to the heat-shock protein, Hsp90, hence Cre is inactive. On administration, Hsp90 is displaced by 4-OH-tamoxifen, which binds to the ER in its place. This exposes the ER's nuclear localization signal and causes translocation of the Cre-ER fusion protein to the nucleus, hence inducing Cre activity. The Cre recombinase is fused to the ER with a mutant ligand-binding domain (Cre-ER) which stabilizes the Cre recombinase in the cytoplasm. When tamoxifen is delivered, it specifically binds to the Cre-ER and Cre recombinase will move to the nucleus under the guide of nuclear localization signal where it executes its function. The published data show that it is possible to obtain inducibility of Cre-mediated excision, but in most cases only partial and a new and more sensitive derivative of ER (ERT2) has been developed to increase the efficiency of induction (Indra et al., 1999).

5.2. Tet_{ON}/Tet_{OFF} systems

The second method of inducing *Cre* expression in mouse tissues is the tetracycline-inducible system (Utomo et al., 1999). This system requires the cis- or trans-interaction of two transgenes derived from the controlling elements of the tetracycline resistance operon, *Tn10*, of *E. coli*. Two different tetracycline-controlled regulatory elements, a transactivator (tetR) and a reverse transactivator (rtetR), respectively provide off and on modes of *GOI* or *Cre* expression (Fig. 3). The C-terminal domain of the VP16 protein from the herpes simplex virus, essential for transcription of immediate early viral genes, was used to create hybrid transactivator proteins, rTA and rtTA (Gossen and Bujard, 1992; Gossen et al., 1995). The second transgene in this system has a tetracycline response element (TRE) required for rTA- and rtTA-specific binding which then permits a minimal CMV promoter to drive transcription of *GOI* or *Cre*. Tetracycline or doxycycline (dox) is given and provides a precise inducible, dose-dependent regulation of *GOI* or *Cre* expression. Since dox has very high affinity for rTA and good penetration of the blood-barrier, it should provide an ideal system to induce *GOI* or *Cre* expression in the mouse inner ear.

5.3. Conditional, tissue-specific, inducible system

A common misconception is that there are many genes that are ear-specific because of their unique function in the inner ear and the large number of nonsyndromic deafness loci and genes. There are, at best, only a few genes that appear to be exclusively expressed in the inner ear based on examination of the extensive EST databases, cDNA libraries, and the NCBI Gene Expression Omnibus (GEO) profiles and databases (Beisel et al., 2004). More sensitive imaging of gene expression can now be used to show all the expanded expression domains at the cellular, tissue, and temporal levels that were previously missed (Matei et al., 2005). Overcoming such known and unsuspected ancillary expression necessitates that a highly controlled and regimented design be established to restrict pathogenesis exclusively to the inner ear or specific cells within the inner ear. An ideal system design has combined a number of approaches to provide permanent and temporary barriers to restrict expression to the inner ear. Recombinase systems can serve as the basis for approach as “deadbolts” to

prevent excision or expression beyond the inner ear (Coates et al., 2005; Gao et al., 2004; Kolb et al., 2005). Both the bacteriophage P1-derived *Cre/loxP* and the yeast-derived *Flp/FRT* approaches can be used alone or in concert (Dymecki, 1996a; Fiering et al., 1993; Kwan, 2002; Muller, 1999; Schaft et al., 2001) (see Genesis 32(2) special issue “*Tissue Specific Expression of Cre Recombinase in Mice*”). Tissue restriction can be further mediated by using the addition of a third different promoter. For example, the *Pax2* and *Pou4f3* promoters could be used as the foundation for this system. A third promoter would be selected to target the *GOI* to a subset of inner ear cell type(s). We will provide an illustration of this system (see Fig. 4).

The major design element for restricted expression of the *GOI* is to identify two promoters which have a limited overlapping expression pattern that represents the targeted tissue or cell of interest. We have selected *Pax2* to drive *Cre* expression, since it is expressed at an early developmental stage in a number of tissues including the otic vesicle, and in the adult, expression is restricted to only the kidney and testis (Burton et al., 2004; Keller et al., 1994; Ohyama and Groves, 2004). *Pou4f3* expression is restricted to central and peripheral sensory neurons and sensory epithelium (Xiang et al., 1995). Thus, the overlap of *Pax2* and *Pou4f3* is restricted to inner ear hair cells (Fig. 4A). The second design feature is to insert a “deadbolt” to block expression of the *GOI* (Fig. 4B). A floxed reporter, such as *LacZ* or *eGFP*, inserted between the promoter and the *GOI* serves as the “deadbolt”. The promoter will drive reporter expression only, since the reporter now blocks expression of the adjacent downstream *GOI*. The *Pax2*-driven *Cre* recombinase will excise a floxed reporter [i.e., nuclear localization signal (*nls*)-*LacZ* immediately 3' (behind) the *Pou4f3* promoter elements], thus removing the “deadbolt” to permit expression of the *GOI*. This “deadbolt” will be excised in all cells expressing *Pax2*-driven *Cre* recombinase and their associated cell lineages. Another design component is to insert regulatory elements, “doorlatch”, between the promoter and the floxed reporter, thus permitting experimental regulation of when and how much of the *GOI* is expressed. The temporal and quantitative regulatory “doorlatch” is only induced by the presence of tetracycline or doxycycline when using the Tet_{ON} regulatory elements. Expression of the *Pou4f3*-driven *GOI* is promoted by administration of tetracycline, such that the “doorlatch” will be “unlocked” by the presence of tetracycline. However, the expression of the *GOI* is controlled by *Pou4f3* and the Tet_{ON} system, thereby this bicistronic promoter system will limit expression to only those *Pax2/ Pou4f3* overlapping areas where the floxed *LacZ* “deadbolt” is removed. Continued expression of the *nls-LacZ* reporter will be restricted to *Pou4f3* expressing cells not exposed to the *Pax2*-driven recombinase, thus permitting easy identification with a very sensitive reporter system. This also provides an internal control to verify the efficiency of excision of the floxed *nls-LacZ* in comparison to those regions having the intact *Pou4f3*-driven floxed *nls-LacZ*. Differential expression of a *GOI* in the inner ear can be mediated by switching out the *Pou4f3* promoter with a different gene promoter. Spiral ganglion cells can be conditionally targeted using the *Pou4f1* promoter (Gerrero et al., 1993; Xiang et al., 1995). The *Pou3f4* promoter could be used to target the expression of the *GOI* in the fibrocytes of the spiral ligament (Minowa et al., 1999; Phippard et al., 1999).

The goal of an inducible inner ear system is to provide a universal transgene system (i.e., “plug and play”) that will permit investigators to select the inner ear cell type and a *GOI* to examine the biological function and to establish translational mouse models for therapeutic studies for the inner ear. This system should be useful both for the spatial, temporal, and quantitative upregulation of *GOI* to study *GOF* and for the ear-targeted deletion of a single or multiple *GOIs* to dissect complex genetic interactions without comprised vitality. Most importantly, this approach should allow a combination in which a given native *GOI* is deleted and a mutated *GOI* is instead expressed. Such a “plug and play” approach is made possible by the security offered by our constructs which utilize a “deadbolt” and “doorlatch”

to regulate the spatiotemporal and quantitative expression of any gene of choice in the inner ear or specific inner ear cell types.

6. Summary

A spectrum of different *Cre* lines has already been generated by several hearing research groups for cochlear conditional gene targeting. Another application of these *Cre* lines will be generating cell-type-specific overexpressors when crossing with the mouse lines which contain exogenous transgene preceded by STOP sequences and a strong ubiquitous promoter. A joint effort by investigators to collect different *Cre* transgenic lines and generate more cell-type-specific *Cre* lines will speed up the process of dissecting the key components causing deafness when mutated. One of the largest *Cre* line database has been established in the following link (<http://www.mshri.on.ca/nagy/Cre-plan.htm>) (Nagy, 2000). Making good use of *Cre* and *Flp* lines will help better understand the mechanisms and ultimately may contribute to the cure for human deafness.

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Abbreviations

GOF	gain-of-function
GOI	gene-of-interest
LOF	loss-of-function
FLPe	efficient Flp recombinase
β gal	β galactosidase
PCR	polymerase chain reaction
IRES	internal ribosome entry site
BAC	Bacterial Artificial Chromosome
ER	estrogen receptor

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Fig. 1.

Integrase and recombinase recombination targets specific sites. (A) Cre-mediated recombination reactions. Recombination reactions are 1: Excision reaction between *cis loxP* sites in the same orientation; 2: Integration between *trans loxP* sites. Target excision is the more favorable reaction, hence inserted genes are quickly excised with continued Cre activity; and 3: Inversion reaction between *cis loxP* sites in opposite orientations. The brown triangles depict the *loxP* sites. (B) Phage ϕ C31 integrase catalyzes recombination between *attP* and *attB* sites to form *attL* and *attR* sites. This integration requires no accessory factors; however, the reverse excision reaction requires a Xis protein. This protein is not found in mammals, hence integration is essentially irreversible. Integration can therefore be used for stable cassette exchange (when two of each *attP* and *attB* sites are present as shown), as well as gene insertion into a host genome. (C) Comparison of three types of recombinase used in mammalian genome is depicted. Cre and Flp recombinases catalyze the recombination between two *loxP* sites and *FRT* sites, respectively. ϕ C31 integrase catalyzes the recombination between *attB* and *attP* sites (red and gray color dots) and inserts GOI into the genome while generating the new *attL* and *attR* sites which is an irreversible event.

Fig. 2.

Cre activity in the CreERT2 fusion protein is inducible by 4-OH-tamoxifen. In the absence of tamoxifen, CreER is bound to Hsp90 and located in the cytoplasm. Tamoxifen preferentially binds to the Estrogen Receptor (ER), displacing Hsp90 and inducing translocation of CreER to the nucleus, hence activating Cre.

Fig. 3.

Inducible regulation of GOI or Cre by tetracycline. (A) The Tet_{ON} system utilizes *rtetR* to regulate gene expression. In the absence of antibiotic, the rtTA protein is unable to bind to the TRE, but with the addition of tetracycline or doxycycline rtTA, it now promotes the expression of the GOI or Cre. (B) In the Tet_{OFF} system, the rTA is a normal-bound TRE and drives Cre or GOI transcription. When antibiotic is provided, the rTA is no longer able to bind to TRE, causing a cessation of transcription.

Fig. 4. Schematic representation of the overlapping embryonic (black) and postnatal (red) expression pattern of Pax2 and Pou4f3 promoters and the associated conditional, inducible expression constructs. (A) Note that GOI expression for this constructs is driven by a Tet_{ON} system that will be restricted to the embryonic and postnatal inner ear, an area of overlap (orange) for Pax2 (red) and Pou4f3 (yellow). (B) Regulation of the GOI is mediated by the absence or presence of Cre recombinase and the antibiotic (purple circle) tetracycline. The Pax2 promoter (red box) is used to drive Cre (gray box) expression. The loxP sites (triangle) are located 5' and 3' to the LacZ (dark blue box) gene. The Pou4f3 promoters in nonoverlapping expression areas (yellow box) and in overlapping with Pax2 expression are designated. The rtetR proteins (gray) in the absence tetracycline are unable to bind the Tet_{ON} regulatory elements (blue box) and the presence of antibiotics permits rtetR protein (colored) binding. Expression of the LacZ reporter or the GOI (green box) is indicated by the green arrow while blockage of expression is indicated by parallel vertical lines.