JARO 13: 295–322 (2012)
DOI: 10.1007/s10162-012-0324-5
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JARO
Journal of the Association for Research in Otolaryngology

Invited Review

Conditional Gene Expression in the Mouse Inner Ear Using Cre-*loxP*

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Received: 5 December 2011; Accepted: 19 March 2012; Online publication: 24 April 2012

ABSTRACT

In recent years, there has been significant progress in the use of Cre-*loxP* technology for conditional gene expression in the inner ear. Here, we introduce the basic concepts of this powerful technology, emphasizing the differences between Cre and CreER. We describe the creation and Cre expression pattern of each Cre and CreER mouse line that has been reported to have expression in auditory and vestibular organs. We compare the Cre expression patterns between Atoh1-CreER^{TM} and Atoh1-CreER^{T2} and report a new line, Fgfr3-iCreER^{T2}, which displays inducible Cre activity in cochlear supporting cells. We also explain how results can vary when transgenic vs. knock-in Cre/CreER alleles are used to alter gene expression. We discuss practical issues that arise when using the Cre-*loxP* system, such as the use of proper controls, Cre efficiency, reporter expression efficiency, and Cre leakiness. Finally, we introduce other methods for conditional gene expression, including Flp recombinase and the tetracycline-inducible system, which can be combined with Cre-*loxP* mouse models to investigate conditional expression of more than one gene.

Keywords: cochlea, conditional gene deletion CreER, Cre efficiency, Cre recombinase, ectopic gene expression, Flp recombinase, knock-in, LoxP, reporter lines, Tet-on, Tet-off, transgenic, utricle, vestibular

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Abbreviations: ABR – Auditory brainstem response; BAC – Bacterial artificial chromosome; βgal – Beta-galactosidase; BMP – Bone morphogenetic protein; BDNF – Brain-derived neurotrophic factor; CFP – Cyan fluorescent protein; Col1A1 – Alpha (1) collagen promoter; Col2A1 – Type II collagen promoter; DTA – Diphtheria toxin fragment A; E – Embryonic day; eGFP – Enhanced green fluorescent protein; ES – Embryonic stem cells; GER – Greater epithelial ridge; GFAP – Glial fibrillary acidic protein; GFP – Green fluorescent protein; GOI – Gene of interest; Hsp90 – Heat shock protein 90; iCsp3 – Drug-inducible dimerizable caspase 3; IRES – Internal ribosome entry site; KOMP – NIH knockout mouse project; LER – Lesser epithelial ridge; myo7a – Myosin VIIa; NICD – Notch intracellular domain; P – Postnatal day; PAC – Phage artificial chromosome; Pkd1 – Polycystic kidney disease 1; Rb – Retinoblastoma protein; RFP – Red fluorescent protein; rtTA – Reverse tetracycline transactivator; SHH – Sonic hedgehog; TRE – Tetracycline responsive element; tTA – Tetracycline transactivator

INTRODUCTION

The use of knockout or germline deletion mice has been extremely useful in the past few decades to investigate the role of specific genes in tissues or organs, including the inner ear; however, this approach deletes the gene of interest (GOI) from every cell in the body throughout the life of the mouse and ~15–20 % of germline deletions result in embryonic lethality (Zambrowicz et al. 2003). In addition, germline deletion models identify only the earliest functions of

the GOI, with postnatal gene functions often masked by embryonic effects. Germline gene inactivation can also cause pleiotropic effects, where the deletion of a single gene influences multiple phenotypes, again preventing the determination of an individual GOI's function in a particular organ. Many genes play different roles in various cell types as well as different roles during embryonic development compared to postnatal ages; thus, cell type specificity and temporal control of gene expression (known as conditional gene expression) is needed to fully understand their function. Mouse models that allow conditional gene expression permit the discovery and dissection of GOI functions in a manner that is specific to a chosen cell type and throughout the life of the mouse.

The most common method to alter gene expression in a conditional manner is the use of the Cre-*loxP* system (Kwan 2002). Here, we discuss the basics of Cre-*loxP* technology including the differences between Cre and CreER and the generation of knock-in, conventional transgenic, and bacterial artificial chromosome (BAC) transgenic lines. We list each Cre/CreER mouse strain that has been published in the inner ear, organized by when Cre expression occurs (separating embryonic and postnatal ages) and where Cre expression occurs (separating auditory, vestibular, ganglionic, and non-sensory regions). We describe in detail how each mouse line was created, its Cre expression pattern, and the relevant biological discoveries that have been made using each conditional allele. The last section of the review discusses practical issues that arise when using the Cre-*loxP* system, such as the use of proper controls, Cre efficiency, reporter expression efficiency, and Cre leakiness. We also discuss applications for fate mapping and mosaic Cre expression patterns. Finally, we present the basics of two other methods to control gene expression in a conditional manner—Flp recombinase and the tetracycline-inducible system. This review serves as a thorough introduction to conditional gene deletion and its use in inner ear research as well as a compilation of current information for researchers who routinely use conditional mouse models.

THE CRE-*LOXP* SYSTEM

Cre is an enzyme originally from P1 bacteriophage that acts as a site-specific recombinase, recognizing a short sequence of DNA called a *loxP* site. A *loxP* site is a consensus 34-bp DNA sequence that is not present in the mouse genome and has directionality. Cre-mediated recombination of genes flanked by *loxP* sites (also called a floxed sequence) can result in the excision, inversion, or translocation of DNA depending upon the location and orientation of the *loxP* sites. The most common use of Cre recombinase is to excise or delete the floxed DNA sequence which occurs when two *loxP* sites are on the same strand of DNA and are in the same orientation (Nagy 2000). Numerous studies have shown that Cre-mediated recombination can occur in a variety of cell types (Sauer et al. 1989; Kuhn et al. 1995; Kellendonk et al. 1999; Feltri et al. 1999; Nagy et al. 2000) and that only a few Cre molecules per cell are needed to excise the floxed DNA (Nagy 2000). It is important to note that Cre-mediated recombination is a permanent deletion of the floxed DNA, and if cell division occurs after Cre-mediated recombination, all daughter cells will inherit this gene deletion.

To allow for cell type-specific control of gene deletions, mouse models have been created where Cre expression is controlled by a cell type-specific promoter. These lines can then be crossed with mouse lines containing a relevant part of a GOI that is surrounded by *loxP* sites in the genome to generate conditional gene deletion (Fig. 1A). The overexpression or ectopic expression of a gene can also be induced using the Cre-*loxP* system. In this case, a construct containing a promoter and a floxed "stop" sequence upstream of a GOI (i.e., promoter-*loxP*-stop-*loxP*-GOI) is inserted into the genome; thus, only Cre^+ cells are able to remove the "stop" sequence and overexpress or ectopically express the GOI (Fig. 1B; Zuo 2002; Gao et al. 2004). This strategy is also used for reporter alleles so that lacZ, green fluorescent protein (GFP), or other fluorescent molecules are expressed in Cre^+ cells only when the floxed "stop" sequence is removed.

To gain temporal control of gene expression, the ligand-binding domain of an engineered steroid hormone receptor is fused to the Cre enzyme and the Cre molecules are sequestered in the cytoplasm by heat shock protein 90 (Hsp90), keeping Cre inactive. The translocation of the Cre–hormone receptor fusion protein to the nucleus, which allows Cre to become active, only occurs in a ligand-dependent (or inducible) manner (Fig. 1C; Feil et al. 1996; Hayashi and McMahon 2002). To prevent Cre activation by endogenous steroid hormones, the engineered steroid hormone receptor contains specific point mutations that make it insensitive to endogenous steroid hormones while retaining binding affinity for synthetic analogues. The most common use of this strategy is CreER alleles, where an altered ligand-binding domain of the estrogen receptor is fused to Cre and mediates tamoxifen-dependent translocation to the nucleus, but is insensitive to endogenous estradiol (Hayashi and McMahon 2002). Therefore, only exposure to tamoxifen will induce Cre activity allowing temporal control of gene expression. Note that tamoxifen is a prodrug that is hydrolyzed by the liver

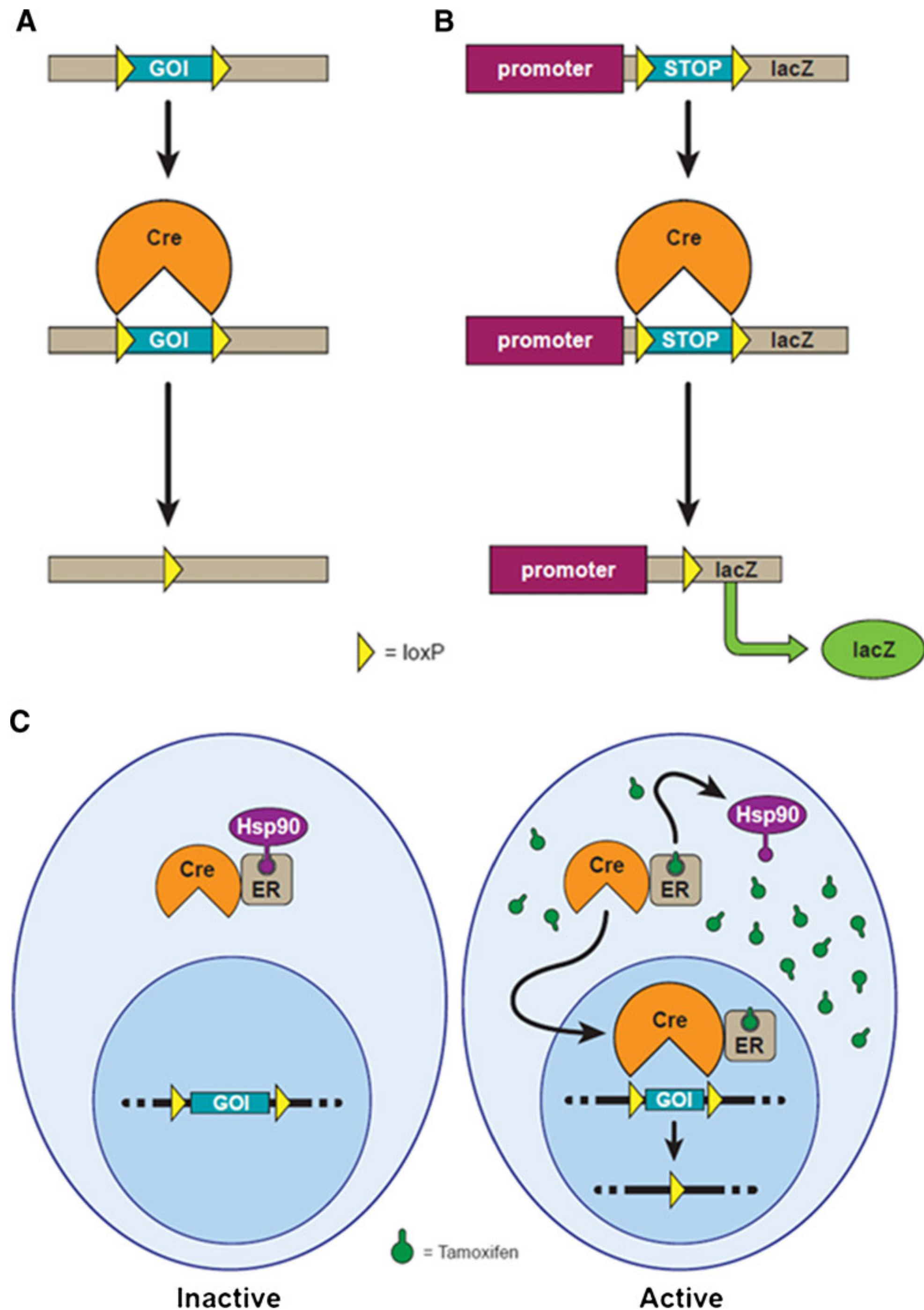

FIG. 1. Cre-mediated excision and the mechanism of inducible CreER. **A** Diagram of Cre-mediated deletion of a GOI flanked by *loxP* sites. **B** Diagram of a reporter allele where Cre-mediated deletion of a floxed "stop" sequence results in the expression of lacZ. **C** Diagram of inducible CreER-mediated excision. In the absence of tamoxifen, CreER is sequestered in the cytoplasm by Hsp90. In the presence of tamoxifen, CreER is translocated to the nucleus where it recognizes *loxP* sequences and cleaves the DNA.

to its active form, 4-hydroxy-tamoxifen (Furr and Jordan 1984); thus, in vitro induction of CreER requires the use of 4-hydroxy-tamoxifen instead of tamoxifen to activate CreER. Two mutations in the estrogen receptor are commonly used to generate CreER alleles. CreER^{TM} (also called CreER^{T}) contains a single-point mutation, G521R, in the ligand-binding domain of the estrogen receptor, whereas CreER^{T2} contains a triple mutation (G400V, M543A, and L544A) in this region (Feil et al. 1997; Indra et al. 1999). The triple mutation results in a four to tenfold increase in the sensitivity of CreER^{T2} to 4-hydroxy-tamoxifen compared to CreER^{TM}; thus, in theory, less tamoxifen is needed to induce Cre activity for CreER^{T2} alleles

(Danielian et al. 1998; Indra et al. 1999; Li et al. 2000). It is important to remember that many other factors may affect Cre activity, such as the activity of the promoter driving Cre expression at the time of induction, so the dose of tamoxifen needed to get optimal results for each CreER line needs to be empirically tested with a reporter line. Other inducible Cre alleles, such as CrePR where the ligand-binding domain of the progesterone receptor is fused to Cre, also exist, but are used less frequently (Wunderlich et al. 2001).

GENERATION OF Cre/CreER LINES

Cre/CreER lines are generated by inserting the Cre coding sequence into the endogenous coding region of a gene to "highjack" the promoter of interest (i.e., knock-in) or by creating a transgene by randomly inserting into the genome a fragment of DNA that contains the promoter of interest (and/or enhancer regions) followed by the Cre coding sequence. Conventional transgenic lines use minimal promoter regions (<10 kb), while BAC transgenic lines contain large fragments of DNA (>100 kb). There are pros and cons for each of these methods that affect the expression pattern and expression level of Cre/CreER.

Knock-in alleles use homologous recombination in embryonic stem (ES) cells to insert the Cre coding sequence into the endogenous locus of the GOI. This technique is expensive and time-consuming as hundreds of ES cells need to be screened to find the handful where homologous recombination occurred. However, the knock-in approach is advantageous because it is more likely that the endogenous gene expression pattern will be reproduced by the Cre expression pattern (Tian et al. 2006). If the Cre coding sequence replaces or disrupts the endogenous gene sequence, knock-in alleles are null alleles; thus, only heterozygotes can be used for conditional gene expression. In this case, it is also important to consider that a gene may be haploinsufficient, generating a phenotype when only one allele is present and complicating the conclusions made from the deletion of a GOI. When using such knock-in alleles, it is best to compare results to controls that use the same Cre allele without the floxed GOI to test for potential haploinsufficiency phenotypes.

To prevent a knock-in allele from generating a null allele, an internal ribosome entry site (IRES) can be used to drive Cre expression. The translation of mRNA is generally dependent upon 5′ cap-mediated translation; however, the addition of IRES promotes an internal initiation of translation, allowing a second protein, such as Cre, to be made (Mountford and Smith 1995; Martinez-Salas 1999). In most cases, the IRES-Cre construct is added to the 3′-end of the GOI after the stop codon. It is important to note that the efficiency of IRES-mediated translation varies according to cell type and strain background (Kazadi et al. 2008).

The generation of transgenic lines is much faster and cheaper than knock-in alleles, but is dependent on having a well-defined promoter region. Even then, other regulatory elements outside the promoter region may be needed to recapitulate the endogenous gene expression pattern; thus, many transgenic lines have unexpected Cre expression patterns. In addition, transgenes are randomly inserted into the genome, which can cause variation in Cre expression due to the effects of enhancers or repressors in the local genomic region (called positional effects). Thus, multiple founders need to be analyzed for each transgenic line created (Zuo 2002; Tian et al. 2006). The addition of insulators (i.e., an intron region of the chicken beta-globin gene) in front of the transgenic promoter may lessen these positional effects (Burgess-Beusse et al. 2002). Sometimes the insertion of a transgene into the genome can inactivate an endogenous gene, so it is best to use transgenic Cre lines as heterozygotes. Since conventional transgenes are relatively small, multiple copies of the transgene are commonly inserted into the genome at the same site or at multiple insertion sites. This can increase the level of Cre expression and thus affect the pattern of Cre expression. In addition, multiple transgene copies can be reduced after successive generations and cause genetic drift of Cre expression.

In contrast, BAC transgenic lines contain very large segments of DNA that should include all regulatory elements necessary to recapitulate the endogenous gene expression pattern driven by the promoter of interest. Although BAC transgenes are not subject to strong positional effects, which often occur in conventional transgenic lines, they are well known to rearrange or break apart when integrated into the host genome. In fact, rearrangements can even occur in offspring after germline transmission is obtained; thus, Cre expression patterns may be different between founder lines and successive generations. BAC transgene insertion into the genome can also inactivate endogenous genes, so it is also wise to use these alleles as heterozygotes. It is also important to note that BAC vectors may contain additional *loxP* sites. If just one *loxP* sequence is retained in the BAC cloning vector, it can be transferred into the mouse genome and can affect the outcome of Cre-mediated excision. In addition, the large size of BAC transgenes increases the likelihood that other genes and/or promoter elements are within these transgenes (Tian et al. 2006; Yu and Zuo 2009).

REPORTER LINES

To determine the pattern of Cre activity and to quantify the percentage of Cre^+ cells, reporter mouse

lines are used. The most commonly used line is the ROSA26^{LacZ} reporter (also called ROSA26R or R26R), where *lacZ*, encoding β-galactosidase (βgal), is expressed in Cre^{+} cells after the removal of the floxed "stop" sequence (Soriano 1999). βgal expression can only be visualized in the presence of its substrate, X-gal, or using βgal antibodies. Many other reporter lines exist that use the *ROSA26* locus or the CAG promoter (a combination of the CMV enhancer with the chicken β-actin promoter) such as ROSA26^{eGFP} (Giel-Moloney et al. 2007), ROSA26^{eYFP} (Srinivas et al. 2001), CAG^{lacZ} (Akagi et al. 1997), and CAG^{eGFP} (Kawamoto et al. 2000). In theory, a reporter line driven by the *ROSA26* locus or a CAG promoter is ubiquitously expressed, and recent work has demonstrated that the CAG promoter is eight to tenfold stronger than the *ROSA26* promoter (Nyabi et al. 2009; Chen et al. 2011).

xA more complex reporter line that is commonly used is Z/EG. Here, the CAG promoter is used to express *lacZ* in all cells until Cre-mediated recombination deletes the floxed *lacZ* gene, allowing the expression of a second reporter, enhanced GFP (eGFP) (Novak et al. 2000). Since eGFP has endogenous fluorescence, Cre activity can be monitored in live samples and Cre^{+} live cells can be isolated using FACS. Other reporter lines that use the same strategy are the Z/AP line, where human alkaline phosphatase is expressed after the deletion of the floxed *lacZ* sequence (Lobe et al. 1999), and the mT/mG line, where membrane-targeted GFP is expressed after the deletion of a floxed membrane-targeted tdTomato (Muzumdar et al. 2007).

Imaging the fluorescent reporter molecule does not reflect the current expression of Cre, but instead provides a history of Cre expression since the progeny of Cre^{+} cells will permanently express the reporter regardless of whether they currently express Cre. In addition, it is important to consider that it takes time for the Cre enzyme to be translated and translocated into the nucleus for recombination to occur (Nagy 2000). It takes additional time for the reporter protein to be synthesized at a level high enough for detection. Several groups have estimated this to take approximately 12 h by comparing the expression of the cell type-specific promoter driving Cre with reporter expression (Bouchard et al. 2004; Matei et al. 2005). The delay for inducible CreER alleles occurs for another reason. After injection, it takes time for tamoxifen to enter the blood stream, be hydrolyzed to its active form, reach cells in the inner ear, and activate the CreER molecule for translocation to the nucleus. Cre-mediated recombination has been detected at the level of the genome 24–30 h after tamoxifen injection (Weber et al. 2008); however, detection using different reporter lines may vary depending on the strength of the fluorescent reporter molecule (Madisen et al. 2010). For this reason, most labs wait 5–10 days after tamoxifen injection to analyze reporter samples.

Cre/CreER LINES FOR THE INNER EAR

In recent years, there has been a significant expansion of Cre and CreER mouse lines that are useful for inner ear research. We will continue to have additional strains in the future due to the NIH Neuroscience Blueprint Cre-Driver Network whose goal is to provide the Neuroscience Community with mouse strains suitable for the cell type-specific perturbation of gene function in the nervous system (http://www.credrivermice.org/index). In addition, the NIH Knockout Mouse Project (KOMP) and the European Conditional Mouse Mutant Program have initiated plans to create floxed alleles for every gene in the mouse genome (Austin et al. 2004; Auwerx et al. 2004). The combination of these resources offers the unique opportunity to study the role of any GOI in a particular cell type of the inner ear at a particular age. Here, we discuss conditional gene expression relevant to research in the inner ear with a review of Cre and CreER mouse lines that have Cre activity in specific cell types (Tables 1 and 2), as well as a discussion of practical issues that arise when using conditional gene expression. In the following section, several Cre/CreER alleles are presented more than once as their Cre expression pattern has been described in multiple inner ear cell types and at different stages of development. It is important to note that the majority of the Cre/CreER alleles that we discuss have Cre expression in other tissues and organs in addition to the inner ear.

Cre/CreER LINES FOR THE DEVELOPING OTIC VESICLE AND OTOCYST

This section will summarize mouse lines with Cre expression occurring in the developing structures of the inner ear during early embryogenesis. While reporter activity persists to postnatal ages, the onset of Cre expression began embryonically.

Foxg1-Cre is a knock-in mouse line that is a modified version of the Foxg1-lacZ mouse (Xuan et al. 1995) where the Cre coding sequence was inserted into the Foxg1 locus and disrupts endogenous Foxg1 expression. Using ROSA26^{LacZ} reporter mice (Hebert and McConnell 2000) and Z/AP reporter mice (Pirvola et al. 2002), Cre^{+} cells were first detected at embryonic day (E) 8.5 in the otic placode. By E13.5, reporter expression was found throughout the otic

TABLE 1

Summary of Cre lines described in the review

Conditional allele	*Type*	*Expression pattern*	*Level of expression*	*Original citation*	*Source*
Atoh1-Cre	Knock-in	E13.5: vestibular sensory regions; E14.5: basal turn of the cochlea; P0: HCs and SCs in cochlea and vestibular organs	Cochlear and vestibular HCs (90 %), cochlear SCs (60 %), utricle SCs (6 %), saccule SCs (15 %), cristae SCs (42 %)	Yang et al. (2010a)	Not commercially available
Atoh1-Cre	Transgenic	E11: otocyst; E18.5: HCs and SCs in cochlea and vestibular organs, spiral and vestibular ganglion neurons	Cre expression not quantified	Matei et al. (2005)	Jax stock #11104
CAG-Cre	Transgenic	Ubiquitous expression	Cre expression not quantified	Sakai and Miyazaki (1997)	Not commercially available
Col1A1-Cre	Transgenic	Not characterized with a reporter line		Liu et al. (2004)	MMRRC stock #15398-UCD
Col2A1-Cre	Transgenic	E10.5: non-sensory regions of the otocyst and mesenchymal cells	Cre expression not quantified	Ovchinnikov et al. (2000)	Jax stock #3554
Fgf16-Cre	Knock-in	E10.5: otic vesicle; P1: semicircular canal cristae, stria vascularis, cochlear spiral prominence epithelium	Cre expression not quantified	Hatch et al. (2009)	Not commercially available
Foxg1-Cre	Knock-in	E8.5: otic placode; E13.5: cochlea, vestibular organs, spiral and vestibular ganglia	Cre expression not quantified	Hebert and McConnell (2000)	Jax stock #4337
hGFAP-Cre	Transgenic	E13.5: utricle, cristae, non-sensory cells around vestibular organs, postnatal cochlear SCs	Cre expression not quantified	Zhuo et al. (2001)	Jax stock #4600
Gfi1-Cre	Knock-in	E13.5: vestibular HCs; E15.5: cochlear HCs in basal turn; E18.5: cochlear and vestibular HCs	Cochlear HCs (93 %), HCs in utricle, saccule, and cristae (90 %)	Yang et al. (2010b)	Not commercially available
Hoxb2-r4-Cre	Transgenic	E8.5: otic placode; P0: cochlea, vestibular organs, spiral and vestibular ganglia	Cre expression not quantified	Szeto et al. (2009)	Not commercially available
Otog-Cre	Transgenic	Not characterized with a reporter line		Cohen-Salmon et al. (2002)	Not commercially available
Pax2-Cre	BAC transgenic	E8.5: otic placode; P0: cochlea, vestibular organs, spiral and vestibular ganglia	Cre expression not quantified	Ohyama and Groves (2004)	MMRRC stock #10569-UNC
Pax3-Cre	Knock-in	E9: otic vesicle and developing spiral and vestibular ganglion; E11.5 to E17.5: cochlea, utricle and saccule	Cre expression not quantified	Engleka et al. (2005)	Jax stock #5549
Pax8-Cre	Knock-in	E8.5: otic placode; E16.5: epithelial components of the inner ear and spiral and vestibular ganglia	Cre expression not quantified	Bouchard et al. (2002)	EMMA stock #EM: 00141
Pou3f4-Cre	Transgenic	E14.5: otic mesenchyme, adult: temporal bone, spiral ligament, spiral limbus, tympanic border cells, Reissner's membrane, mesenchymal cells, non-sensory cells in utricle	Cre expression not quantified	Ahn et al. (2009)	Not commercially available
Pou4f3-Cre	Transgenic	E12.5: utricle sensory epithelium; E13.5: zone of non-proliferating cells in cochlea; P6: cochlear HCs and SCs, vestibular HCs and stroma	Cre expression not quantified	Sage et al. (2006)	Not commercially available

Prestin-Cre	BAC transgenic	P6: cochlear and vestibular HCs, spiral ganglia region	Cre expression not quantified	Tian et al. (2004)	Not commercially available
Prestin-Cre	Transgenic	P14: cochlear inner HCs, vestibular HCs, spiral ganglia region; P50: last row of cochlear outer HCs	Cre expression not quantified	Li et al. (2004)	Not commercially available
Prox1-eGFP/Cre	Knock-in	P23: cochlear HCs, pillar cells, Deiters' cells, GER and LER	Outer HCs (30 %), inner HCs (4 %), pillar cells and Deiters' cells (almost 100 %)	Liu et al. (2012)	Not commercially available
SHH-EGFP/Cre	Knock-in	P0: spiral ganglion neurons, peripheral nerve fibers surrounding HCs and SCs	Spiral ganglion neurons (100 %)	Harfe et al. (2004)	Jax stock #5622
Sox2-Cre	Transgenic	Ubiquitous expression	Cre expression not quantified	Hayashi et al. (2002)	Jax stock #4783
Wnt1-Cre	Transgenic	E9: otic vesicle, developing spiral and vestibular ganglion; E11.5 to E17.5: cochlea, utricle and saccule	Cre expression not quantified	Danielian et al. (1998)	Jax stock #3829

epithelium, including the cochlea, vestibular organs, and both spiral and vestibular ganglia, while the surrounding mesenchyme was Cre-negative. Foxg1-Cre has been used in numerous studies to investigate the deletion or overexpression of various genes (Pirvola et al. 2002; Arnold et al. 2006; Zelarayan et al. 2007; Barrionuevo et al. 2008; Jones et al. 2008; Rickheit et al. 2008; Grimsley-Myers et al. 2009; Schultz et al. 2009; Wang et al. 2009; Yamamoto et al. 2009; Deng et al. 2010; Freyer and Morrow 2010; Haugas et al. 2010; Hurd et al. 2010; Hwang et al. 2010; Sipe and Lu 2011). We highlight a few studies here. Yamamoto et al. (2011) challenged the established dogma that Notch signaling is required to specify cochlear sensory progenitor cells using Foxg1-Cre-mediated deletion of RBP-J. Three other papers used Foxg1-Cre to delete Notch receptors (Delta1, Jagged1, and Notch1) to demonstrate a role for Notch signaling in lateral induction and lateral inhibition in the inner ear (Kiernan et al. 2005, 2006; Brooker et al. 2006). Foxg1-Cre was also used to ectopically express the Notch intracellular domain (NICD) which showed that Notch signaling is sufficient to generate ectopic sensory patches (Hartman et al. 2010; Pan et al. 2010). The role of N-myc in the development of the otic vesicle and its control over proliferation in the otic epithelium were discovered using both Foxg1-Cre and Pax2-Cre lines (Dominguez-Frutos et al. 2011; Kopecky et al. 2011). Comparison of the germline knockout of Sonic hedgehog (SHH) with Foxg1-Cre-mediated deletion of Smoothened demonstrated SHH's direct role in the formation of ventral otic structures (cochlea and saccule) and SHH's indirect role in the formation of dorsal structures (utricle, semicircular canal cristae, and the endolymphatic duct). Conditional deletion of Smoothened also revealed that SHH signaling regulates the proliferation of neurogenic progenitors which give rise to spiral and vestibular ganglion neurons (Brown and Epstein 2011). Fgf8's role in regulating the development of cochlear pillar cells was also discovered using the Foxg1-Cre allele (Jacques et al. 2007). Heterozygous Foxg1-Cre mice have only one copy of the Foxg1 gene (Hebert and McConnell 2000), which has been reported to cause haploinsufficiency phenotypes that include proliferation in other organs (Shen et al. 2006; Eagleson et al. 2007; Siegenthaler et al. 2008). However, no change in proliferation in the inner ear has been reported in several papers where proper controls of Foxg1-Cre mice (without the floxed allele) were used (Yamamoto et al. 2009, 2011; Hartman et al. 2010; Brown and Epstein 2011).

The **Pax2-Cre** transgenic allele was created using a BAC that contains a 101-kb region 5′ to the mouse Pax2 gene as well as 20 kb of the Pax2 coding region, which includes the first three exons of the Pax2 gene.

TABLE 2

Summary of CreER lines described in the review

Conditional allele	*Type*	*Tamoxifen induction*	*Expression pattern*	*Original citation*	*Source*
Atoh1-CreER^{TM}	Transgenic	E16 only	Cochlear HCs (not quantified)	Chow et al. (2006)	MMRRC stock #29581-UNC
		P0 only	Cochlear inner HCs (40 %), outer HCs (50 %), utricle and saccule HCs (40 %), cristae HCs (10 %)		
		P0 and P1	Cochlear inner HCs (80 %), outer HCs (90 %), utricle and saccule HCs (60 %), cristae HCs (15 %)		
		P0, P1, and P2	Cochlear inner HCs (80 %), outer HCs (90 %), utricle and saccule HCs (60 %), cristae HCs (20 %)		
Atoh1-CreER^{T2}	Transgenic	E12.75	Cochlear inner HCs (40 %), outer HCs (44 %)	Machold and Fishell (2005)	Jax stock #7684
		E12.75 and E13.75	Cochlear inner HCs (90 %), outer HCs (97 %)		
		P0 and P1	Cochlear HCs (10–20 % depending on turn), utricle HCs (not quantified)		
CAG-CreER^{TM}	Transgenic	Varies	Ubiquitous expression	Hayashi and McMahon (2002)	Jax stock #4453
Fgfr3-iCreER^{T2}	PAC transgenic	P0 and P1	Pillar and Deiters' cells (100 %), cochlear outer HCs (25–75 % depending on turn), Hensen or Claudius cells (not quantified), spiral lamina	Rivers et al. (2008)	Not commercially available
		P2 and P3	Pillar and Deiters' cells (100 %), cochlear outer HCs (>30 % depending on turn), Hensen or Claudius cells (not quantified), spiral lamina		
		P6 and P7	Pillar and Deiters' cells (100 %), cochlear outer HCs (>20 % depending on turn), Hensen or Claudius cells (not quantified), spiral lamina		
		P12 and P13 or P30	Pillar and Deiters' cells (100 %), Hensen or Claudius cells (not quantified), spiral lamina		
Ngn1-**CreER^{T2}**	BAC transgenic	E8.5 to E13.5	HCs, SCs and non-sensory epithelium of vestibular organs, spiral and vestibular ganglia, GER (no quantifications were done)	Raft et al. (2007)	Jax stock #8529
		E8.5 only	Vestibular ganglion neurons (not quantified)		
		E12.5 only	Spiral ganglion neurons (not quantified)		
Plp-CreER^{T2}	Transgenic	P0–P7, P3–P9, or P10-P16	Inner phalangeal cells, Schwann cells in the spiral lamina, vestibular SCs, vestibular Schwann cells and satellite cells (no quantifications were done)	Doerflinger et al., (2003)	Jax stock #5975
		P0 and P1	Inner phalangeal cells (50–80 % depending on turn), pillar and Deiters' cells (5–10 %), utricle HCs and SCs (not quantified)		
Prestin-CreER^{T2}	Knock-in	P0, P1, and P2	Cochlear outer HCs (15–60 % depending on turn)	Fang et al. (2012)	Not commercially available
		P2 and older	Cochlear outer HCs (100 %)		
Prox1-CreER^{T2}	Knock-in	E16	Deiters' cells (72 %), outer pillar cells (18 %), inner pillar cells (3 %), cochlear outer HCs (7 %)	Srinivasan et al. (2007)	Not commercially available
		P0 and P1	Pillar cells (5 %), Deiters' cells (5–10 %)		
ROSA26-CreER	Knock-in	Varies	Ubiquitous expression	Vooijs et al. (2001)	Not commercially available
ROSA26-CreER	Knock-in	Varies	Ubiquitous expression	Badea et al. (2003)	Jax stock #4847

An IRES followed by the Cre coding sequence was inserted into exon 2 of this Pax2 sequence. Cre expression patterns were characterized with ROSA26-^{LacZ} and Z/EG reporter mice and first detected at E8.5 in the otic placode. By postnatal day (P) 0, most cells in the cochlea and vestibular organs were Cre^{+}, including cells in the organ of Corti, stria vascularis, Reissner's membrane, sensory epithelia of the vestibular organs, and both the spiral and vestibular ganglia (Ohyama and Groves 2004). Pax2-Cre has also been used extensively (Arnold et al. 2006; Rocha-Sanchez et al. 2007; Doetzlhofer et al. 2009; Grimsley-Myers et al. 2009; Soukup et al. 2009; Wang et al. 2009; Abraira et al. 2010; Fritzsch et al. 2010; Jahan et al. 2010a; Basch et al. 2011; Dominguez-Frutos et al. 2011; Kopecky et al. 2011; Pan et al. 2011). Neurod1 deletion using Pax2-Cre disrupted the basal-to-apical gradient of hair cell (HC) differentiation and produced ectopic HCs in the spiral and vestibular ganglia regions. These results suggest an antagonistic relationship between Neurod1 and Atoh1 during inner ear development (Jahan et al. 2010b). Pax2-Cre was also used to demonstrate that bone morphogenetic protein (BMP) signaling promotes the formation of the abneural side of the cochlea (i.e., organ of Corti and lesser epithelial ridge (LER)) while repressing the formation of the neural side (i.e., greater epithelial ridge (GER); Ohyama et al. 2010). Pax2-Cre-mediated deletion and activation of canonical Wnt signaling demonstrated Wnt's role in mediating the otic placode–cranial epidermis fate decision by promoting an otic placode fate (Ohyama et al. 2006). The Pax2-Cre allele was also used to show the interaction between Notch signaling and Wnt signaling in the otic placode (Jayasena et al. 2008).

Pax3-Cre is a knock-in mouse line where the Cre coding sequence was inserted into the first exon of the *Pax3* gene, creating a null allele of Pax3. Homozygous Pax3-Cre mice die embryonically (Engleka et al. 2005). Cre expression was characterized in the developing otic epithelium using the RCE:loxP reporter where the CAG promoter was inserted after the *ROSA26* locus followed by a floxed "stop" sequence and eGFP (Sousa et al. 2009). Cre^{+} cells were first detected at E9 in the prosensory epithelium of the otic vesicle, as well as within the developing spiral and vestibular ganglion. In later stages of development (E11.5 to E17.5), Pax3-Cre activity is present in HCs and supporting cells (SCs) of the cochlea, utricle, and saccule. There was also Cre activity detected in the cells of the GER, stria vascularis, endolymphatic duct, and periotic mesenchyme. No Cre activity was detected in the semicircular canals cristae at any age. This use of Pax3-Cre (together with Wnt1-Cre described below) provided the novel and unexpected finding that embryonic neural tube cells contribute to the formation of the otic vesicle and can develop into both sensory and non-sensory cells (Freyer et al. 2011).

The transgenic **Wnt1-Cre** line uses the Wnt1 enhancer to drive the expression of Cre (Danielian et al. 1998). Using the RCE:loxP reporter, Cre expression was very similar to the Pax3-Cre line, but with fewer Cre^{+} cells in each of the locations (Freyer et al. 2011).

Pax8-Cre is a knock-in mouse line where the Cre coding sequence replaced exon 3 of the *Pax8* gene, creating a null allele of Pax8 (Bouchard et al. 2002). Using the Z/AP reporter line, Cre activity was first detected at E8.5 in the otic placode, and by E10.5, most cells in the otic vesicle were Cre^{+}. At E16.5, most epithelial components of the inner ear had Cre activity, as well as the spiral and vestibular ganglia; however, reporter expression in the semicircular canal cristae was patchy (Bouchard et al. 2004).

The transgenic **Hoxb2-r4-Cre** line uses the Hoxb2 r4 enhancer, a 1.4-kb fragment of the 5′ region of the Hoxb2 gene, to drive the expression of Cre. Using the ROSA26^{LacZ} reporter line, βgal expression was first detected in the pre-otic field at the five-somite stage (~E8.5); thus, when the otic placode and otic vesicle develop, these cells are Cre^{+} as well. Accordingly, at P0, most cells are Cre^{+} in the cochlea and vestibular organs, as well as in the spiral and vestibular ganglia, stria vascularis, and Reissner's membrane (Szeto et al. 2009).

There are several Cre lines that use a collagen promoter to drive the expression of Cre recombinase. **Col1A1-Cre** is a transgenic line that uses a 3.6-kb fragment of the rat alpha1(I) collagen (Col1A1) promoter (Liu et al. 2004). This line was not characterized in the developing otocyst using a reporter allele; however, endogenous *Col1A1* was detected by in situ hybridization at E11.5 with ubiquitous expression in the otocyst. Col1A1-Cre was used to delete the retinoblastoma protein (Rb) in the developing inner ear, which resulted in an increase in HCs and SCs in both the cochlea and utricle. Rb-null HCs had stereocilia bundles, were innervated, and continued to divide at late embryonic ages. Thus, cell fate determination and differentiation into HCs and SCs does not require the presence of Rb and can occur even while cells are proliferating (Sage et al. 2005). There is a second Col1A1-Cre allele that uses a truncated 2.3-kb fragment of the rat *Col1A1* promoter (Liu et al. 2004), but this line has not been described in the inner ear.

The type II collagen promoter (Col2A1) has also been used to generate a transgenic Cre mouse line. **Col2A1-Cre** uses 3 kb of the *Col2A1* promoter, the first exon of *Col2A1*, with a mutated initiation codon, and a 3.02-kb fragment of intron 1 followed by IRES to

drive the expression of Cre (Ovchinnikov et al. 2000). Using the ROSA26^{LacZ} reporter line, βgal expression was first detected at E10.5 as small clusters of cells in the non-sensory regions of the otocyst, as well as in surrounding mesenchymal cells. Col2A1-Cre has been used to ectopically express the NICD in a transient manner by combining it with the tetracycline-inducible system. The expression of NICD induced ectopic HCs and SCs in the non-sensory regions of the cochlea and vestibular system, demonstrating that Notch signaling is sufficient for the initiation of sensory cell fate in the developing inner ear (Pan et al. 2010).

Otogelin-Cre or **Otog-Cre** is a transgenic BAC mouse line where Cre is under the control of the murine *Otog* promoter. A reporter line was not used to characterize Otog-Cre; however, endogenous Otog expression was detected at E10 in the otic vesicle and at E18 in the non-sensory cells of the sensory epithelium of the cochlea and vestibular organs. Otog-Cre-mediated deletion of connexin26 resulted in normal development of the inner ear, but by hearing onset, progressive cell death was described beginning with inner phalangeal and border cells and continuing to outer HCs and other SC subtypes. Inner HCs remained intact and the vestibular system was not affected. These results demonstrate that connexin26 and the gap junction network are required for the survival of cochlear HCs and SCs and, thus, auditory function (Cohen-Salmon et al. 2002).

The BAC transgenic line, **Neurogenin1-CreER^{T2}** (also called Ngn1-CreER^{T2} or Neurog1-CreER^{T2}), contains 113 kb of the 5′ sequence, the *Ngn1* coding sequence, and 71 kb of the 3′ sequence. The CreER^{T2} coding sequence replaced the *Ngn1* coding sequence in the BAC. Using the Z/EG reporter line and tamoxifen (1 mg/40 g) given twice daily by gavage from E8.5 to E13.5, analysis at E14.5 revealed many Cre^{+} HCs and SCs in the utricle and saccule, while only a few Cre^{+} SCs were found in the cristae. There were also many Cre^{+} cells in the surrounding non-sensory epithelium of the utricle and saccule as well as in the spiral and vestibular ganglia. In the cochlea, Cre^{+} cells were found only in the GER. Similar results were described when tamoxifen was given at E8.5 and E9.5. These results indicate that the sensory regions of the utricle and saccule, but not the cristae and cochlea, are derived from the neurogenic region of the otocyst (Raft et al. 2007). More fate mapping studies using the Ngn1-CreER^{T2} line were conducted to determine the specification of vestibular and spiral ganglion neurons. Early tamoxifen injections (E8.5) primarily labeled vestibular ganglion neurons, while tamoxifen given at E12.5 primarily labeled spiral ganglion neurons; thus, Ngn1^{+} precursor cells change with age to generate these two cell populations (Koundakjian et al. 2007). Ngn1-CreER^{T2}-mediated deletion of ephrin-B2 with tamoxifen given by gavage at E9.5–E10.5 demonstrated ephrin-B2's role in the fasciculation of spiral ganglion neurons (Coate et al. 2012). There is also a non-inducible Ngn1-Cre allele that used the same BAC construct, but it has only been described in the brain (Lundell et al. 2009).

Fgf16-Cre is a knock-in mouse line with an IRES-Cre cassette inserted into the first coding exon of Fgf16. Even though this line is a null allele, the inner ears of homozygotes are structurally and functionally normal. Using the ROSA26^{LacZ} reporter line, βgal expression was first apparent in the otic vesicle at E10.5. By P1, Cre^{+} cells were detected in the sensory and non-sensory regions of the semicircular canal cristae, at the base of the stria vascularis, and in the cochlear spiral prominence epithelium (Hatch et al. 2009).

Cre/CreER LINES FOR THE DEVELOPING ORGAN OF CORTI

This section will focus on Cre lines with expression occurring in the developing sensory epithelium of the cochlea (Fig. 2) during late embryogenesis, when prosensory cells are in the process of committing to either a HC or SC fate.

Pou4f3-Cre is a transgenic allele which uses a 9-kb region 5′ to the *Pou4f3* gene as the promoter to drive Cre expression. Using the ROSA26^{LacZ} reporter, βgal expression was first detected at E13.5 in the zone of non-proliferating cells of the developing cochlea and by P6 in both HCs and SCs. Quantification of Cre^{+} cells was not reported (Sage et al. 2006). Pou4f3-Cre was used to delete Rb, which extended HC and SC proliferation to neonatal ages while preserving the initial development of mechanoelectrical transduction; however, degeneration occurred in the adult organ of Corti that led to severe hearing loss. These experiments suggest that transient regulation of Rb might be a strategy to achieve cochlear HC regeneration (Sage et al. 2006). Pou4f3-Cre-mediated deletion of Dicer1 caused malformations in HC stereocilia bundles, degeneration of HCs, and subsequent hearing loss. Interestingly, vestibular HCs were less effected and only a mild vestibular phenotype was detected (Friedman et al. 2009) The Pou4f3-Cre line has also been used to ablate HCs in a mosaic but reproducible manner by the expression of a drug-inducible, dimerizable caspase 3 (iCsp3) that leads to hearing loss after a week of drug administration. In the cochlea, ~60 % of HCs expressed iCsp3 (Fujioka et al. 2011).

There are two Atoh1-Cre lines, a knock-in allele (Yang et al. 2010a) and a transgenic allele (Matei et al.

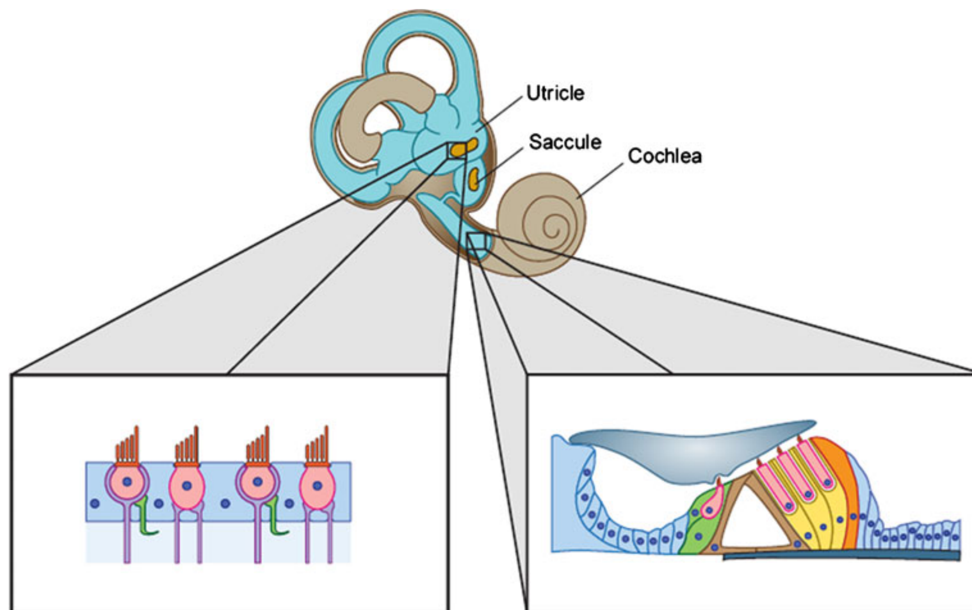

FIG. 2. Diagram of the inner ear and cell types in the sensory epithelium of the cochlea and utricle. In the magnified image of the utricle, HCs are in *pink* and SCs are in *blue*. The magnified image of the cochlea focuses on the organ of Corti, with HCs in *pink*. *Green* SCs *underneath* the inner HC are inner phalangeal cells/border cells. *Brown* SCs *between* the inner and outer HCs are pillar cells. *Yellow* SCs *underneath* the three outer HCs are Deiters' cells. The *orange* SC *lateral to* the outer HCs is a Hensen cell.

2005). The **Atoh1-Cre knock-in** line replaced the entire Atoh1 coding sequence with the Cre coding sequence. Homozygous Atoh1-Cre mice die soon after birth, whereas heterozygous Atoh1-Cre mice are viable and display no visible defects. Using the ROSA26^{LacZ} reporter, βgal expression was first detected in the basal turn of the cochlea at E14.5 and progressed to middle and apical turns over the next few days. By P0, βgal expression was observed in all turns of the cochlea in both HCs and SC, including Deiters' cells, pillar cells, and inner phalangeal cells. Approximately 90 % of HCs and 60 % of SCs were Cre^{+}. These findings prompted the conclusion that Atoh1 is expressed in prosensory progenitor cells before HC and SC fates are specified (Yang et al. 2010a). The **Atoh1-Cre transgenic** line used the same Atoh1 enhancer fragment used to create the Atoh1-eGFP mouse (Chen et al. 2002) and was also characterized with the ROSA26^{LacZ} reporter line. In contrast to the Atoh1-Cre knock-in line, βgal expression was first detected in the Atoh1-Cre transgenic line at E11 in areas that correspond to the future sensory epithelium. At E18.5, almost all cochlear HCs expressed βgal, except for those in the most apical tip. The Atoh1-Cre transgenic line also had Cre expression in SCs. Quantification of Cre^{+} cells was not reported (Matei et al. 2005). The Atoh1-Cre transgenic line has been used to delete Dicer1, showing the important roles of microRNAs in cochlear gene expression profiles and maintaining the apex to base gradient of gene expression. This model also demonstrated that microRNAs are required for HC survival (Weston et al. 2011). Transgenic Atoh1-Cre-mediated deletion of beta or gamma actin isoforms showed that only one of these genes is needed for the normal development of HC stereocilia bundles, while each gene plays a different role in the maintenance of stereocilia during aging (Perrin et al. 2010). Comparison of Atoh1-Cre-mediated Neuro1d deletion with Pax2-Cre-mediated Neuro1d deletion demonstrated that Neurod1's roles in HC differentiation and maturation are relevant only at very early embryonic ages (Jahan et al. 2010b).

There are also two inducible Cre alleles which use the Atoh1 enhancer to drive the expression of CreER: **Atoh1-CreER^{TM}** (Chow et al. 2006) and **Atoh1-CreER^{T2}** (Machold and Fishell 2005). The transgenic **Atoh1-CreER^{TM}** allele was induced with tamoxifen (100 μg/g, IP) at E16 and analyzed at E19 using a Rosa26-loxP-stop-loxP-NICD-IRES-eGFP line (which expresses both NICD and eGFP after Cre-mediated excision of the floxed stop sequence). eGFP expression was only detected in HCs; however, quantification was not performed. The eGFP^{+} HCs in this model also had ectopic expression of NICD, but appeared normal (Liu et al. 2012b). The transgenic **Atoh1-CreER^{T2}** line was characterized with the ROSA26^{eYFP} reporter and embryonic tamoxifen induction (the tamoxifen dose and route of administration were not specified). After a single tamoxifen dose at E12.75, eYFP expression was detected in 40 % of inner HCs and 44 % of outer HCs at E18.5. These percentages increased to 90 % of inner HCs and 97 % of outer HCs when a second tamoxifen dose was given at

E13.75. Cre activity was not observed in SCs with either induction paradigm. The Atoh1-CreER^{T2} allele was used to delete Eya1 and Six1, demonstrating that these genes are required for HC development and Atoh1 expression (Ahmed et al. 2012).

Gfi1-Cre is a knock-in allele where the Cre coding sequence replaced the endogenous Gfi1 gene; thus, homozygous Gfi1-Cre mice cannot be used because of their severe phenotype where HCs do not form. This line was characterized with the ROSA26^{LacZ} reporter where βgal expression was first seen in inner HCs of the basal turn at E15.5 that progressed to outer HCs and other turns of the cochlea over the following days. By E18.5, nearly all HCs (~93 %) in the entire cochlea were labeled by βgal. No other cell types in the cochlea were labeled (Yang et al. 2010b). Gfi1-Cre-mediated deletion of BMP2 demonstrated that this gene is not required for normal HC formation or hearing ability (Hwang et al. 2010).

The **Prox1-CreER^{T2}** knock-in allele was created by inserting IRES-CreER^{T2} into the mouse *Prox1* locus (Srinivasan et al. 2007). Heterozygous Prox1-CreER^{T2} mice have normal Prox1 expression, normal morphology of the organ of Corti, and normal hearing. Homozygous mice were not used since the Cre coding sequence is followed by a Neo-cassette which may affect the expression levels of Prox1 (Yu et al. 2010). The Cre expression pattern was characterized using the ROSA26^{eYFP} reporter line and tamoxifen (100 μg/g, IP) given at E16. When the cochlea was analyzed at E19, Cre expression was detected in ~72 % of Deiters' cells, ~18 % outer pillar cells, ~3 % inner pillar cells, and ~7 % of outer HCs. No Cre^{+} inner HCs were found (Liu et al. 2012b).

Cre/CreER LINES FOR THE DEVELOPING VESTIBULAR ORGANS

This section will focus on Cre lines with expression occurring in the developing sensory epithelium of vestibular organs: the utricle, saccule, and semicircular canal cristae (Fig. 2).

Cre activity from the transgenic **Pou4f3-Cre** mouse line was first seen using the ROSA26^{LacZ} reporter in the sensory epithelium of the utricle at E12.5. By E13.5, βgal expression was detected in vestibular HCs. At P6, the only βgal^{+} cells in the sensory epithelium were HCs, but there were also βgal^{+} cells in the stroma (containing nerve fibers) beneath the epithelium. Quantification of Cre^{+} cells was not reported. Pou4f3-Cre-mediated deletion of Rb was also studied in the utricle where HCs continued to divide until 6 weeks of age and partial vestibular function was maintained at 6 months. Vestibular Rb-null HCs died at a slow rate, which contrasts with the rapid cell death seen in cochlear HCs when Rb was deleted using the same Cre line. Thus, Rb plays different roles in the survival of these two types of HCs (Sage et al. 2006).

ROSA26^{LacZ} reporter expression was also detected in the vestibular system with the two Atoh1-Cre lines. Cre^{+} cells in the **Atoh1-Cre knock-in** allele were first detected in vestibular sensory regions at E13.5 and increased to P0 where both HCs and SCs in the utricle, saccule, and cristae expressed βgal. Specifically, there were ~90 % Cre^{+} HCs in each of these organs, and the number of Cre^{+} SCs varied from 6 % in the utricle to 15 % in the saccule and 42 % in the cristae. These data also suggest that, as in the cochlea, Atoh1 is expressed in vestibular progenitor cells before HC and SC fates are specified (Yang et al. 2010a). Cre activity in vestibular organs of the **Atoh1-Cre transgenic** line was detected at E11 and by E18.5 was found in vestibular HCs and SCs. Quantification of Cre^{+} cells was not reported (Matei et al. 2005). The **Atoh1-CreER^{TM}** and **Atoh1-CreER^{T2}** alleles were not characterized in the embryonic vestibular system.

The knock-in **Gfi1-Cre** allele also has Cre expression in vestibular HCs beginning at E13.5 and progressively increasing to P0, where ~90 % of HCs in the saccule, utricle, and cristae are βgal^{+}. No vestibular SCs were Cre^{+} (Yang et al. 2010b).

Cre/CreER LINES FOR THE POSTNATAL ORGAN OF CORTI

This section will focus on Cre lines with expression occurring in the sensory epithelium of the cochlea after birth.

The transgenic **Atoh1-CreER^{TM}** line also has Cre activity in the postnatal cochlea After tamoxifen injection(s) (3 mg/40 g, IP) at birth, the only Cre^{+} cells found in the organ of Corti were HCs (as revealed using the ROSA26^{LacZ} reporter line). One tamoxifen injection at P0 resulted in 40 % Cre^{+} inner HCs and 50 % Cre^{+} outer HCs. These percentages increased to 80 % Cre^{+} inner HCs and 90 % Cre^{+} outer HCs when tamoxifen was given once at P0 and again 24 h later at P1. No further increase was seen with a third injection given at P2 (Chow et al. 2006; Weber et al. 2008). In contrast to the results obtained with embryonic Rb deletion using the Col1A1-Cre (Sage et al. 2005) and Pou4f3-Cre lines (Sage et al. 2006), deletion of Rb in neonatal HCs using Atoh1-CreER^{TM} produced S phase reentry followed by cell death. Thus, Rb plays an age-dependent role in HC proliferation (Weber et al. 2008). Atoh1-CreER^{TM}-mediated deletion of Pkd1 ruled out the involvement of this protein as the major component in the mechanoelectrical transduction channel complex or in planar cell polarity mechanisms, but demonstrated

its requirement for normal stereocilia number and structure (Steigelman et al. 2011). This line has also been used to drive the expression of DTA as a method of damaging HCs in the neonatal cochlea, in vivo (Cox et al. 2010). Ectopic NICD expression in neonatal HCs using the Atoh1-CreER^{TM} allele resulted in the reactivation of Sox2 in inner and outer HCs and of Prox1 only in outer HCs. Despite the expression of NICD, Sox2, and Prox1, HCs continued to develop normally, demonstrating that once a HC fate is committed activation of the Notch pathway will not impact their development (Liu et al. 2012b).

We also investigated the Cre expression pattern of **Atoh1-CreER^{T2}** mice (Machold and Fishell 2005) with tamoxifen induction after birth. We bred this line with the ROSA26^{eYFP} reporter and gave tamoxifen injections (3 mg/40 g, IP) once a day at P0 and P1. Analysis at P6 revealed only Cre^{+} HCs, but a much lower number than what was observed with the Atoh1-CreER^{TM} line under the identical conditions. We found ~20 % Cre^{+} inner and outer HCs in the apical turn of the cochlea and <10 % in middle and basal turns (Fig. 3). Both Atoh1-CreER strains are transgenics; thus, positional effects and the copy number of the transgene likely play a role in the difference of Cre activity observed.

There are three mouse strains that use some form of the *prestin* locus to drive the expression of Cre or CreER: Prestin-Cre (transgenic), Prestin-Cre (BAC transgenic), and Prestin-CreER^{T2}. The **Prestin-Cre (transgenic)** allele uses a 9-kb fragment of mouse *prestin*, which contains the putative thyroid hormone-responsive element, the first coding exon (exon 3), and part of intron 3, but not the promoter region upstream of exon 1, to drive the expression of Cre. Using the ROSA26^{LacZ} reporter line, Cre activity was first detected at P14 in inner HCs, and this increased so that by P135 most inner HCs were Cre^{+}. In addition, faint βgal expression was observed in the last row of outer HCs in the apical and middle turns of the cochlea starting at P50 (Li et al. 2004). However, analysis of successive generations of this line with the ROSA26^{eYFP} and Ai6 (where deletion of the floxed "stop" sequence drives the expression of ZsGreen; Madisen et al. 2010) reporter lines did not reveal detectable reporter signals in adult HCs, likely due to genetic drift of transgenic expression (Dearman and Zuo, unpublished).

The **Prestin-Cre (BAC transgenic)** mouse line used a modified BAC containing ~150 kb of genomic DNA which includes the coding region of *prestin* (~50 kb) followed by an IRES-Cre cassette after the stop codon in exon 20. This line has only a single copy of the transgenic BAC; however, it was not determined how much of the *prestin* promoter region is contained within the BAC, which may be the reason why Cre expression is different from endogenous prestin expression. When this line was characterized with the ROSA26^{LacZ} reporter line, βgal expression was first detected at P6 in the majority of outer HCs in the middle and basal turns of the cochlea, while only a few outer HCs in the apical turn were Cre^{+}. Some inner HCs also had βgal expression, and this expression level increased between P6 and P60 (Tian et al. 2004). Conditional deletion of the adherence junction protein, vezatin, using the Prestin-Cre (BAC transgenic) line showed no effects on hearing development or function at early ages, but an increased sensitivity to noise and age-related hearing loss. These findings highlight the role of HC–SC junctions in hearing (Bahloul et al. 2009). Deletion of thyroid hormone receptor beta using this Cre line clarified that malformation of the tectorial membrane (cochlear cause) and not delayed BK channel expression in HCs (retro-cochlear cause) produced hearing loss in the absence of this receptor (Winter et al. 2009).

Most recently, **Prestin-CreER^{T2}** was generated using a knock-in method which resulted in a Cre expression pattern that more accurately recapitulates the endogenous expression of prestin. IRES-CreER^{T2} was inserted into the *prestin* locus after the stop codon in exon 20. Unlike most knock-in alleles, endogenous prestin was not affected and homozygous Prestin-CreER^{T2} mice have normal hearing, as tested by auditory brainstem response (ABR). Using the CAG^{eGFP} and Ai6 (where deletion of the floxed "stop" sequence drives expression of ZsGreen; Madisen et al. 2010) reporters, Cre expression patterns were investigated after different tamoxifen induction paradigms. Cre activity was very specific to the outer HCs of the cochlea with all induction paradigms. Early tamoxifen injections (3 mg/40 g, IP) once a day at P0, P1, and P2 produced a gradient of Cre^{+} outer HCs with 60 % in the base, 35 % in the middle, and 15 % in the apex. In contrast, tamoxifen injections given once a day for 2 days beginning at P2 or any time after resulted in close to a 100 % of Cre^{+} outer HCs throughout the length of the cochlea (Fang et al. 2012).

The **Prox1-CreER^{T2}** knock-in allele (Srinivasan et al. 2007) also has Cre expression in the postnatal cochlea. Using several reporter lines and tamoxifen (3 mg/40 g, IP) injections given once a day at P0 and P1, only pillar cells and Deiters' cells were Cre^{+} in the organ of Corti. Specifically, 5 % of pillar cells were Cre^{+} throughout the length of the cochlea, and the percentage of Cre^{+} Deiters' cells varied among turns with 10 % in the apex and 5 % in the middle and base. Deletion of Rb using the Prox1-CreER^{T2} allele resulted in cell cycle reentry of both pillar cells and Deiters' cells, while only pillar cells were able to complete the cell cycle and increase in number. This

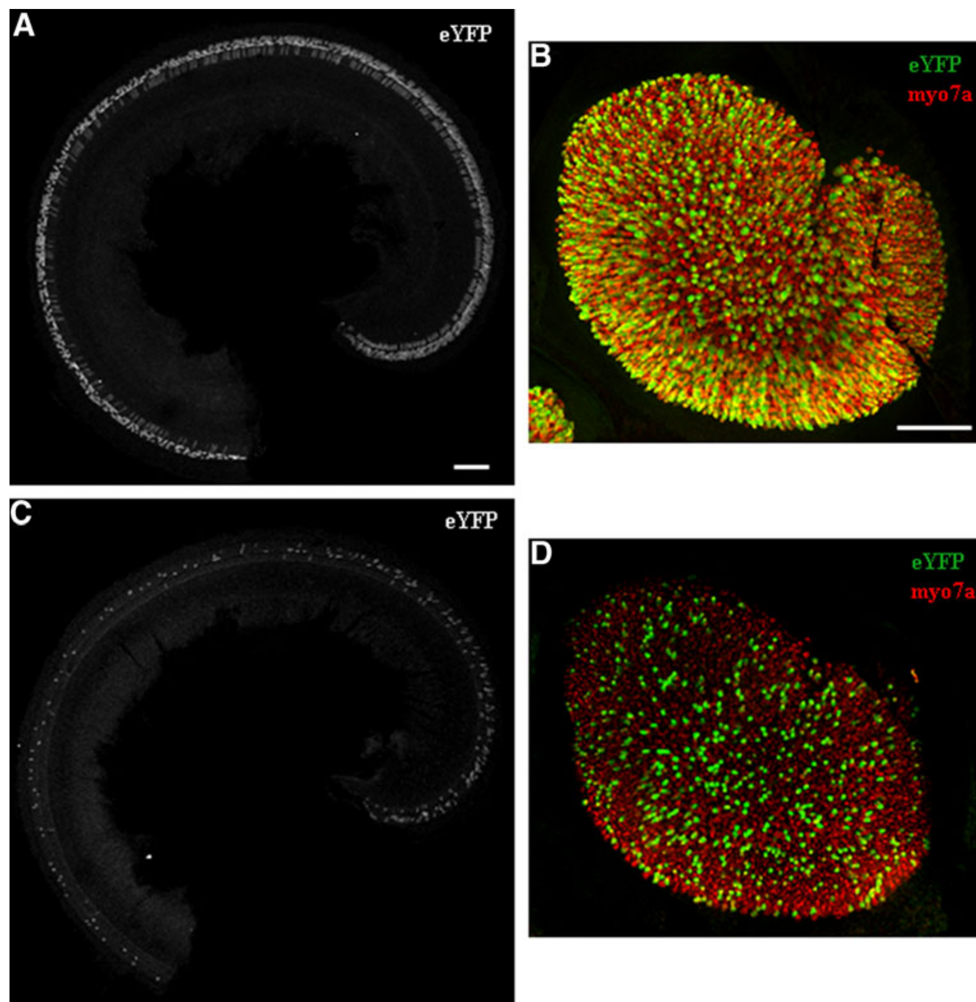

FIG. 3. Comparison of the transgenic lines, Atoh1-CreER^{TM} and Atoh1-CreER^{T2}. Representative confocal projection images of the apical turn of the cochlea (**A**) and utricle (**B**) from Atoh1-CreER^{TM}; ROSA26^{eYFP} mice, induced with tamoxifen at P0 and P1. Representative confocal projection images of the apical turn of the cochlea (**C**) and utricle (**D**) from Atoh1-CreER^{T2};ROSA26^{eYFP} mice, induced with tamoxifen at P0 and P1. In **A** and **C**, eYFP is in *white*. In **B** and **D**, eYFP is in *green* and the HC marker, myosin VIIa (*myo7a*), is in *red*. *Scale bars*, 100 μm.

finding highlights the heterogeneity in the role of Rb between these two SC subtypes (Yu et al. 2010).

There is a second allele which uses the Prox1 locus to drive Cre expression. **Prox1-eGFP/Cre** is a knock-in line where an eGFP/Cre fusion protein was inserted downstream of the Prox1 translation start site (Srinivasan et al. 2010). This allele was recently characterized in the cochlea using the ROSA26^{eYFP} reporter line. At P23, Cre expression was detected in almost all pillar cells and Deiters' cells, ~30 % outer HCs, and ~4 % inner HCs. There was also Cre activity detected in the GER and LER (Liu et al. 2012b).

Fgfr3-iCreER^{T2} is a phage artificial chromosome (PAC) transgenic mouse line that expresses a "codon-improved" version of Cre (iCre) driven by the Fgfr3 promoter. Specifically, iCreER^{T2} was inserted into the first exon of the Fgfr3 gene in the PAC allele (Rivers et al. 2008; Young et al. 2010). Using the Ai14 reporter line (where deletion of the floxed "stop" sequence drives expression of tdTomato; Madisen et al. 2010), we tested tamoxifen induction at several ages where the majority of Cre^{+} cells in the cochlea were pillar cells and Deiters' cells. After tamoxifen (3 mg/40 g, IP) injection once a day at P0 and P1, tdTomato expression was detected in ~100 % of pillar and Deiters' cells, 25–75 % of outer HCs depending on the turn of the cochlea, and a small fraction of Hensen or Claudius cells (Fig. 4A–C). When we gave tamoxifen (3 mg/40 g, IP) once a day at P2 and P3 (Fig. 4D) or at P6 and P7 (Fig. 4E), the percentage of Cre^{+} outer HCs decreased to <30 %, while Cre^{+} pillar and Deiters' cells remained at 100 %. There were still some Cre^{+} Hensen or Claudius cells with both induction paradigms. When we gave tamoxifen (3 mg/40 g, IP) once a day at P12 and P13 (Fig. 4F) or one injection of tamoxifen (9 mg/40 g, IP) at P30

(data not shown), we no longer observed Cre+ outer HCs, while Cre+ pillar and Deiters' cells remained at 100 %. A low percentage of Cre+ Hensen and Claudius cells also remained. Cre+ inner HCs were never found with any induction paradigm. Cre+ cells were also found in the spiral lamina (data not shown).

Plp-CreER^{T2} is a transgenic mouse line that uses 2.4 kb of the 5′ region, exon 1, and intron 1 of the mouse proteolipid protein 1 gene to drive CreER expression (Doerflinger et al. 2003). Several tamoxifen induction paradigms have been characterized with either the ROSA26^{LacZ} or ROSA26^{eYFP} reporter lines. Tamoxifen (33 mg/kg, IP) given once a day from P0 to P7, P3 to P9, or P10 to P16 results in Cre+ inner phalangeal cells in the organ of Corti and Cre+ Schwann cells in the spiral lamina. The amount of Cre+ cells was not quantified (Gomez-Casati et al. 2010). We also characterized this line using the Ai14 reporter line (where deletion of the floxed "stop" sequence drives expression of tdTomato; Madisen et al. 2010). Tamoxifen (3 mg/40 g, IP) given once a day at P0 and P1 results in 50 % tdTomato+ inner phalangeal cells in the apical turn and 80 % tdTomato+ inner phalangeal cells in the middle and basal turns of the cochlea. We also found 5–10 % Tomato+ pillar and Deiters' cells. No Cre+ HCs were found (Fig. 5A–C). We also found a large number of Cre+ Schwann cells in the spiral lamina (data not shown). There was no effect on morphology or cellular organization of the inner ear when BDNF was deleted using the Plp-CreER^{T2} allele and tamoxifen injections were given once a day from P5 to P11 (Gomez-Casati et al. 2010).

There are at least eight lines that use either the mouse or human glial fibrillary acidic protein (GFAP) promoter to drive the expression of Cre or CreER, of which only one allele has been used in the inner ear. The transgenic **hGFAP-Cre** line used 2.2 kb of the 5′ region from the human GFAP gene with the *Gfa2* promoter to drive the expression of Cre (Zhuo et al. 2001). Using the mT/mG and ROSA26^{eYFP} reporter lines, Cre expression was detected in some SCs postnatally, but the amount was not quantified (Hartman et al. 2010).

Cre/CreER LINES FOR THE POSTNATAL VESTIBULAR SYSTEM

Several of the alleles that have Cre activity in the postnatal cochlea are also expressed in vestibular organs; however, less is known. This section will focus on alleles where Cre activity occurs in the sensory epithelia of vestibular organs after birth.

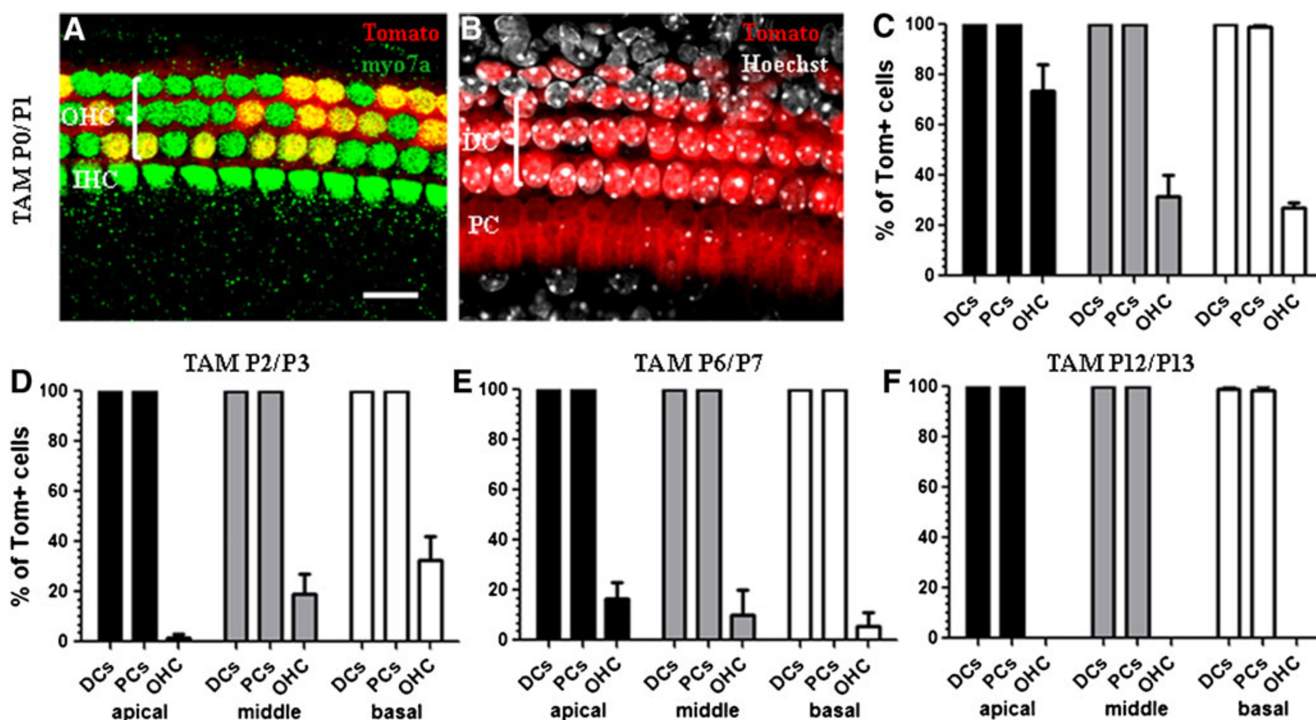

FIG. 4. Fgfr3-iCreER^{T2} expression pattern in the cochlea. Representative confocal images of the organ of Corti from Fgfr3-iCreER^{T2};Ai14 mice, induced with tamoxifen at P0 and P1. **A** Slice image taken at the HC layer with tdTomato expression (*red*) and HCs labeled by myosin VIIA (*myo7a*, *green*). **B** Slice image at the SC nuclear layer with tdTomato expression (*red*) and SC nuclei labeled by Hoechst (*white*). *Scale bar*, 10 μm. Quantification of tdTomato+ cells expressed as a percentage of total cells by type after tamoxifen induction at P0/P1 (**C**), P2/P3 (**D**), P6/P7 (**E**), and P12/P13 (**F**). *DCs* Deiters' cells, *PCs* pillar cells, *OHC* outer hair cells.

The transgenic **Atoh1-CreER^{TM}** allele also has Cre expression in vestibular HCs when tamoxifen is given at birth. Again, the percentage of Cre^{+} HCs increased with multiple tamoxifen injections (3 mg/40 g, IP). One tamoxifen injection at P0 resulted in 40 % Cre^{+} HCs in the utricle and saccule, but only 10 % Cre^{+} HCs in the cristae. With tamoxifen at P0 and P1, 55–60 % of HCs in the utricle and saccule were Cre^{+} and Cre^{+} HCs in the cristae increased slightly to 15 %. No increase in Cre^{+} HCs in the utricle and saccule was seen with a third injection given at P2, but it did increase Cre^{+} HCs in the cristae to 20 % (Chow et al. 2006).

We also found Cre activity in the utricle of the transgenic **Atoh1-CreER^{T2}** allele using the ROSA26^{eYFP} reporter line. Similar to the cochlea, Cre activity was specific to HCs, but there were fewer Cre^{+} HCs in the utricle after tamoxifen injection at P0 and P1 (Fig. 3). We did not characterize the other vestibular organs.

Using the ROSA26^{LacZ} reporter line, Cre activity was detected in vestibular HCs in both the **Prestin-Cre (transgenic)** line (Li et al. 2004) and the **Prestin-Cre (BAC transgenic)** allele (Tian et al. 2004). No Cre^{+} cells were found in vestibular organs using the **Prestin-CreER^{T2}** knock-in allele (Fang et al. 2012).

After tamoxifen (33 mg/kg, IP) injection once a day from P0 to P7, P3 to P9, or P10 to P16, the transgenic **Plp-CreER^{T2}** line showed Cre activity in most SCs in the utricle and saccule, while only some SCs were Cre^{+} in the cristae. Cre activity was also observed in vestibular Schwann cells and satellite cells (Gomez-Casati et al. 2010). We also characterized this line using the ROSA26^{eYFP} reporter line. When tamoxifen (3 mg/40 g, IP) was given once each at P0 and P1, both Cre^{+} HCs and SCs were found in the utricle (Fig. 5D–G). Other vestibular organs were not characterized.

Cre activity in the vestibular system has only been described in one of the transgenic **hGFAP-Cre** lines (Zhuo et al. 2001). Reporter expression was first detected at E13.5 in cells within the utricle and cristae as well as in non-sensory cells in the regions around the vestibular organs. Ectopic expression of NICD using the hGFAP-Cre line resulted in ectopic sensory patches that contained both HCs and SCs. Interestingly, ectopic HCs were not Cre^{+}, which suggests that

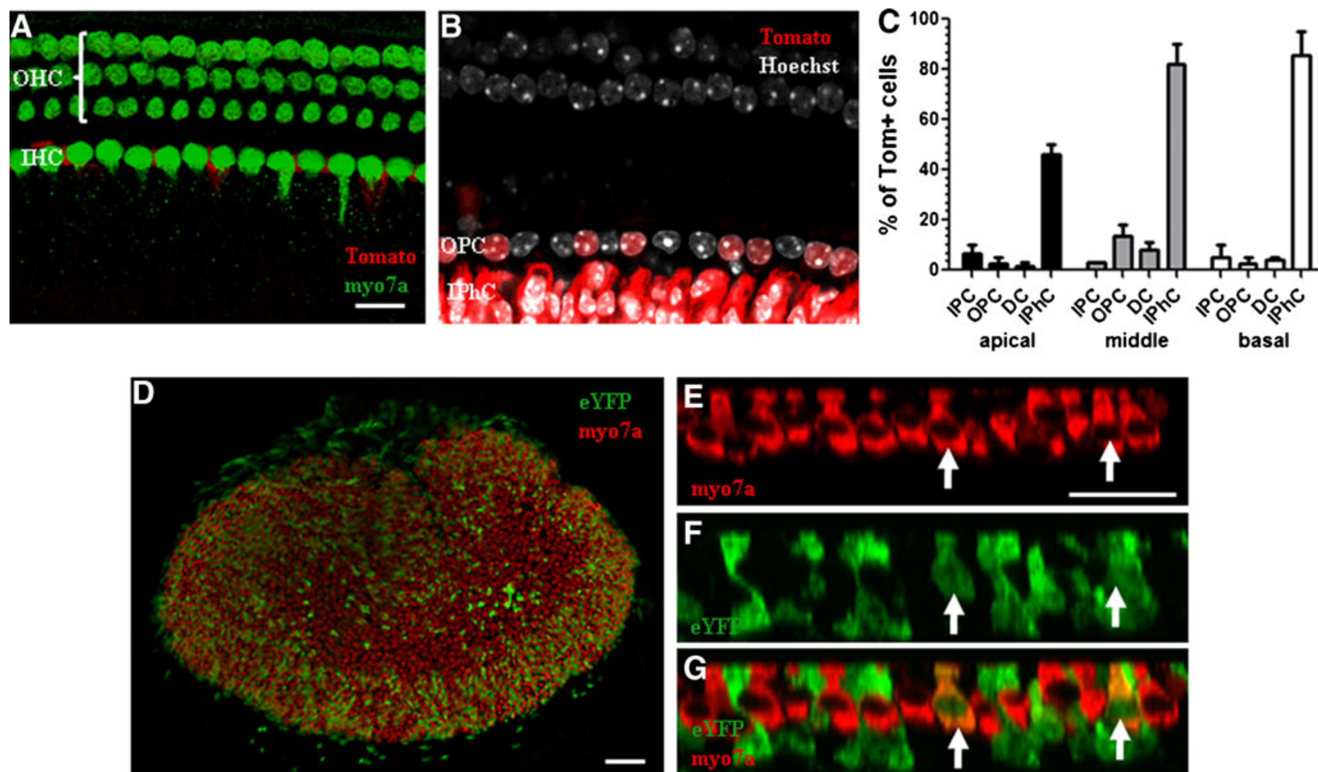

FIG. 5. Plp-CreER^{T2} expression pattern in the cochlea and utricle. Representative confocal images of the organ of Corti from Plp-CreER^{T2};Ai14 mice, induced with tamoxifen at P0 and P1. **A** Slice image taken at the HC layer with tdTomato expression (*red*) and HCs labeled by myosin VIIA (*myo7a*, *green*). **B** Slice image at the SC nuclear layer with tdTomato expression (*red*) and SC nuclei labeled by Hoechst (*white*). *Scale bar*, 20 μm. **C** Quantification of tdTomato^{+} cells expressed as a percentage of total cells by type. *IPC* inner pillar cells, *OPC* outer pillar cells, *DC* Deiters' cells, *IPhC* inner phalangeal cells. **D**–**G** Representative confocal images of the utricle from Plp-CreER^{T2}; ROSA26^{eYFP} mice, induced with tamoxifen at P0 and P1. **E**–**G** Artificial slice images of HCs labeled by myosin VIIA (*myo7A*, *red*) and eYFP expression (*green*). Cre activity is expressed in both HCs (*arrows*) and SCs. *Scale bars*, 50 μm (**D**) and 20 μm (**E**–**G**).

they were induced by neighboring NICD+ cells. This finding is consistent with the known role of Notch in lateral inhibition. In addition, there was a marked decrease in the number of HCs found in the ectopic sensory patches of adult mice where NICD remained permanently expressed (Hartman et al. 2010).

Cre/CreER LINES FOR THE SPIRAL AND VESTIBULAR GANGLIA

Similar to the vestibular organs, the spiral and vestibular ganglia regions are less studied in the reviewed Cre alleles. Here, we list what is known about Cre activity in these regions; however, with the exception of the SHH-eGFP/Cre line, very little detail has been reported.

The **Atoh1-Cre transgenic** line showed Cre activity in both the spiral ganglion and vestibular ganglion neurons (Matei et al. 2005); however, this was not detected in the **Atoh1-Cre knock-in** allele (Yang et al. 2010a).

Cre activity was detected in the majority of cells in the spiral ganglia region in the **Prestin-Cre (transgenic)** line (Li et al. 2004), while only a small fraction were Cre+ in the **Prestin-Cre (BAC transgenic)** allele (Tian et al. 2004). In addition, the **Prestin-Cre (BAC transgenic)** allele had Cre activity in the vestibular ganglia (Tian et al. 2004). No Cre+ cells were found in these neuronal regions using the **Prestin-CreER^{T2}** allele (Fang et al. 2012).

The **SHH-eGFP/Cre** knock-in allele was created by inserting an eGFP/Cre fusion protein into the SHH locus, replacing the first 12 amino acids of SHH and creating a null allele. Heterozygous mice exhibited no noticeable phenotypes (Harfe et al. 2004). The expression of eGFP was used to detect SHH expression in spiral ganglion neurons between E13.5 and E17.5. All spiral ganglion neurons expressed eGFP at E13.5, followed by the loss of eGFP expression progressing from the base to the apex. Using the ROSA26^{eYFP} reporter line, Cre expression at P0 was detected in almost 100 % of spiral ganglion neurons and in some peripheral nerve fibers surrounding HCs and SCs. Surrounding glial cells were Cre-negative. Vestibular ganglia were not investigated. The basal-to-apical decline in SHH-eGFP expression in spiral ganglion neurons occurs at a similar time as the basal-to-apical gradient of cochlear HC differentiation, which suggests that SHH signaling may inhibit HC differentiation (Liu et al. 2010).

Cre/CreER LINES IN NON-SENSORY REGIONS OF THE INNER EAR

Another Cre line from the POU domain family of transcription factors, **Pou3f4-Cre**, has a very different Cre expression pattern compared to the Pou4f3-Cre allele. The Pou3f4 enhancer was fused to the herpes thymidine kinase promoter to drive Cre expression in the Pou3f4-Cre transgenic allele. When analyzed with the ROSA26^{LacZ} reporter, βgal expression was detected in the otic mesenchyme as early as E14.5. In adult mice, Cre+ cells were found in structures derived from otic mesenchyme, including the temporal bone, spiral ligament, spiral limbus, tympanic border cells, Reissner's membrane, and mesenchymal cells that lie underneath the sensory epithelia of the cochlea and vestibular organs. The non-sensory epithelium of the utricle was also Cre+, as well as a few cells in the spiral ganglion region. Cre activity was not detected in structures derived from the embryonic otic epithelium, such as the organ of Corti, the sensory epithelia of the vestibular organs, and the stria vascularis (Ahn et al. 2009).

Cre/CreER LINES WITH UBIQUITOUS EXPRESSION

There are two knock-in alleles that use the *ROSA26* locus to drive the expression of CreER^{TM}. Both have ubiquitous expression in all tissues of the body; however, the number of Cre+ cells varies depending on the tissue and tamoxifen induction paradigm used. Deletion of connexin26 using the **ROSA26-CreER^{TM}** allele (Vooijs et al. 2001) and tamoxifen injection at E19 caused outer HC and SC death beginning at the onset of hearing (P14). In addition, the tunnel of Corti and spaces of Nuel never opened. These results demonstrate that connexin26 plays a role in the postnatal maturation and survival of cells in the organ of Corti (Sun et al. 2009; Wang et al. 2009). The second **ROSA26-CreER^{TM}** allele (Badea et al. 2003) was used to delete Kif3a, a ciliary protein, at E10.5. The HCs in Kif3a conditional knockout mice had stereocilia bundles with planar cell polarity defects that were similar to a mutant mouse model of another ciliary protein, Ift88 (also called Polaris). Together, these results demonstrate the requirement of ciliary genes for the regulation of planar cell polarity in HC stereocilia bundles (Jones et al. 2008).

The CAG promoter has also been used to generate mouse lines with ubiquitous expression of Cre/CreER. The transgenic **CAG-Cre** allele generates Cre-mediated recombination before the two-cell stage of the embryo (Sakai and Miyazaki 1997). The transgenic **CAG-CreER^{TM}** allele is likely more useful since Cre expression can be induced at any age. There is a tamoxifen dose-dependent level of Cre recombination in this line, as well as a low percentage of cells that undergo spontaneous Cre-mediated recombination in the absence of tamoxifen (called

"leakiness"; Hayashi and McMahon 2002; Oesterle et al. 2011). Ectopic expression of NICD induced by CAG-CreER^{TM} at E10.5 resulted in the formation of ectopic HC/SC patches in the non-sensory regions of both the cochlea and utricle; however, when tamoxifen was given at E13, ectopic HC/SC patches were only found in utricular non-sensory regions (Liu et al. 2012a). Similarly, ectopic expression of NICD induced by CAG-CreER^{TM} in cultured cochlear explants at E13.5 did not result in the formation of ectopic HCs or SCs (Basch et al. 2011). When tamoxifen was given once a day at P0 and P1, no ectopic HCs were observed in either the cochlea or utricle (Liu et al. 2012a). Taken together, these results suggest that the ability of Notch signaling to produce HCs and SCs is age-dependent and differs between the cochlea and vestibular system. CAG-CreER^{TM}-mediated deletion of the transcription factor, Tbx1, with tamoxifen injections at E10.5 and E11.5 resulted in a smaller utricle and saccule organs and a "cochlea" that lacked the spiral shape, but still had some HCs while the semicircular canal cristae failed to form. When Tbx1 deletion occurred with a later tamoxifen induction (E14.5), the cochlea appeared normal and the semicircular canal cristae were underdeveloped (Xu et al. 2007). This CreER line was also used to delete the cell cycle inhibitor, p27^{Kip1}, with tamoxifen induction at P3 that resulted in the proliferation of cochlear SCs. SCs also proliferated when tamoxifen was administered at 6 weeks of age, but to a lesser extent. These findings demonstrate that p27^{Kip1} is required to maintain the quiescence of postnatal SCs and that dependence on p27^{Kip1} declines with age (Oesterle et al. 2011).

The transgenic **Sox2-Cre** allele is another ubiquitously expressed Cre line that uses a 12.5-kb enhancer/promoter element from *Sox2* to drive the expression of Cre. Using the ROSA26^{LacZ} reporter, βgal expression was observed at E7.5 in all three germ layers of the mouse embryo as well as in epiblast-derived extra-embryonic membranes (Hayashi et al. 2002).

POTENTIALLY USEFUL Cre/CreER LINES FOR INNER EAR RESEARCH

There are hundreds of Cre/CreER lines that have been made for research in various organs of the body and may also be useful for studies in the inner ear (Table 3). For example, the knock-in **Shh-CreER^{T2}** allele (Harfe et al. 2004), the transgenic **Sox2-CreER** line (Favaro et al. 2009; Arnold et al. 2011), and the knock-in **Lgr5-eGFP-IRES-CreER^{T2}** line (Barker et al. 2007) are all driven by promoters with known expression in the inner ear. In addition, the NIH Neuroscience Blueprint Cre-Driver Network (http://www.credrivermice.org/index) has generated many new Cre/CreER alleles that may be useful for research in the inner ear (i.e., **Otoferlin-Cre**, http://www.informatics.jax.org/javawi2/servlet/WIFetch?page=alleleDetail&key=667536; **Otoferlin-CreER^{T2}**, http://www.informatics.jax.org/javawi2/servlet/WIFetch?page=alleleDetail&key=667535; and **Calbindin2-CreER^{T2}** (calbindin2 is also known as calretinin), http://www.informatics.jax.org/searches/accession_report.cgi?id=MGI:4880758).

PRACTICAL ISSUES RELATED TO CONDITIONAL GENE EXPRESSION

Nature of conditional alleles

In general, the Cre expression pattern is the most important factor when deciding which Cre/CreER allele to use in your research. It is also important to consider the type of conditional allele (knock-in, transgenic, or BAC transgenic). Comparison of the three strains that use the *prestin* promoter to drive Cre expression can illustrate the differences between the different types of conditional alleles. The Prestin-Cre (transgenic) allele and Prestin-Cre (BAC transgenic) allele both show Cre activity in cell types where the endogenous prestin protein is not detected, such as inner HCs, vestibular HCs, and spiral ganglion neurons (Li et al. 2004; Tian et al. 2004). However, the knock-in Prestin-CreER^{T2} line has Cre activity that is very specific to the outer HCs of the cochlea, where endogenous prestin is found (Fang et al. 2012). The most likely explanation is that the regulatory elements which regulate the prestin promoter are quite far from the promoter region and thus are not part of either transgene. In addition, unlike most knock-in alleles, the Prestin-CreER^{T2} line is not a null allele of prestin. Instead, it uses IRES to drive the expression of CreER^{T2}; thus, endogenous prestin is still made and homozygous Prestin-CreER^{T2} mice have normal expression of prestin (Fang et al. 2012).

Comparison of the two Atoh1-Cre alleles provides another example that illustrates the difference between knock-in and transgenic lines. Cre expression from the knock-in Atoh1-Cre line was first detected in the basal turn of the cochlea at E14.5 which corresponds with the Atoh1 in situ results that were first detected in the basal turn at E13.5 (Yang et al. 2010a). In contrast, Cre expression from the transgenic Atoh1-Cre allele was detected at E11 in the developing sensory epithelium (Matei et al. 2005). The two transgenic Atoh1-CreER alleles also have varying Cre expression patterns. With the same tamoxifen induction paradigm and reporter line, the Atoh1-CreER^{TM} allele has Cre activity in 80–90 % of HCs (Chow et al. 2006; Weber et al. 2008), while the Atoh1-CreER^{T2}

TABLE 3

Potentially useful Cre/CreER lines for inner ear research

Conditional allele	*Type*	*Original citation*	*Source*
Atoh1-CrePR	Knock-in	Rose et al. (2009)	Jax stock #13594
Calretinin-CreER	Knock-in	Taniguchi et al. (2011)	Jax stock #13730
Calretinin-IRES-Cre	Knock-in	Taniguchi et al. (2011)	Jax stock #10774
Dlx5-CreER	Knock-in	Taniguchi et al. (2011)	Jax stock #10705
Gli1-CreER	Knock-in	(Ahn et al. 2004)	Jax stock #7913
Hes1-CreER	Knock-in	Kopinke et al. (2011)	Not commercially available
Id2-CreER	Knock-in	Rawlins et al. (2009)	Jax stock #16222
Isl1-IRES-Cre	Knock-in	Srinivas et al. (2001)	Not commercially available
Isl1-mER-Cre-mER	Knock-in	Laugwitz et al. (2005)	Not commercially available
Lgr5-eGFP-IRES-CreER^{T2}	Knock-in	Barker et al. (2007)	Jax stock #8875
Notch1-Cre	Knock-in	Vooijs et al. (2007)	Jax stock #6953
Otoferlin-Cre	Knock-in	N/A	MMRRC stock #032781-MU
Otoferlin-CreER	Knock-in	N/A	MMRRC stock #032782-MU
Parvalbumin-2A-Cre	Knock-in	Madisen et al. (2010)	Jax stock #12358
Parvalbumin-CreER	Knock-in	Taniguchi et al. (2011)	Jax stock #10777
Shh-CreER^{T2}	Knock-in	Harfe et al. (2004)	Jax stock #5623
Sox2-CreER	Knock-in	Arnold et al. (2011)	Not commercially available
Sox2-CreER	Transgenic	Favaro et al. (2009)	Not commercially available
Sox9-CreER	Transgenic	Kopp et al. (2011)	Not commercially available

line has Cre activity in 20 % or less of HCs depending on the turn of the cochlea (Fig. 3). Given that both lines use the Atoh1 enhancer to drive Cre expression, it is tempting to think of them as equal and assume that the Atoh1-CreER^{T2} line would be better since CreER^{T2} has a higher affinity for tamoxifen. However, these strains are transgenics; thus, positional effects and the copy number of the transgene likely play a role in the difference of Cre activity observed. Therefore, it is very important to empirically test each Cre line with reporter lines to correctly interpret the effects of specific gene manipulations.

Cre efficiency

There are many other factors to consider when making conclusions from data obtained using Cre/CreER alleles, such as Cre efficiency, reporter expression efficiency, and Cre leakiness. The ability of the Cre recombinase enzyme to excise DNA between *loxP* sites is referred to as Cre efficiency or recombination frequency. Both cellular Cre and nuclear CreER levels and the number of *loxP* sites in the genome can be important for Cre efficiency. Even though only a few Cre molecules per cell are needed to excise floxed DNA (Nagy 2000), the expression level of the promoter driving Cre can affect Cre efficiency (Araki et al. 1997). In addition, Cre recombination efficiency can be affected by the location of *loxP* sites within the GOI and the distance between *loxP* sites (Zheng et al. 2000; Vooijs et al. 2001). For CreER alleles, the dose and timing of tamoxifen injections can affect the level of CreER activity (Vooijs et al. 2001). When CreER levels are relatively low, Cre efficiency can be variable or mosaic for more than one floxed locus. To illustrate this point, we bred the Prox1-CreER^{T2} allele with both the CAG^{eGFP} and ROSA26^{lacZ} reporter lines. In the cochlea of Prox1-CreER^{T2};CAG^{eGFP};ROSA26^{lacZ} mice, the majority of cells are either GFP^{+} or βgal^{+}, while only a few cells express both reporter molecules (Fig. 6). Thus, the majority of cells in this model only have enough Cre activity to excise one floxed allele and only a few cells display Cre activity for both floxed alleles. This may have significant implications when using two independent floxed genes (i.e., one floxed GOI and one reporter for tracing purposes). Moreover, it is well known that homozygous floxed alleles of the same gene may exhibit differential excision efficiency; therefore, it is highly recommended that instead of using homozygous floxed alleles, a deleted (delta) allele and a floxed allele of the same gene should be used to ensure complete deletion when a weak Cre line is used, such as Prox1-CreER^{T2}.

Another application of a Cre line with low efficiency is to generate a mosaic of Cre^{+} and Cre-negative cells in the same tissue. Mosaic Cre expression patterns can also be created using an inducible CreER allele by giving lower doses of tamoxifen to activate CreER in fewer cells. The use of mosaic Cre expression to delete a GOI provides the opportunity to determine whether the effects of GOI inactivation are cell autonomous or non-cell autonomous (Weber et al. 2008; Yu et al. 2010).

Reporter expression efficiency

Reporter expression efficiency is dependent upon Cre efficiency, but also relies on the expression level of the

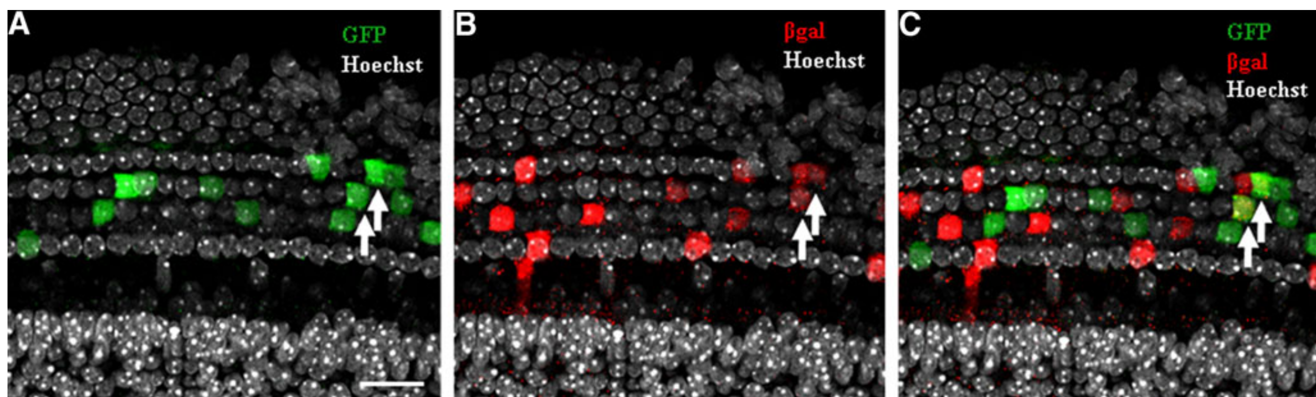

FIG. 6. Illustration of Cre efficiency using a low efficiency Cre and two reporter lines. **A–C** Representative confocal images of the organ of Corti of Prox1-CreER^{T2};CAG^{eGFP};ROSA26^{lacZ} mice, induced with tamoxifen at P0 and P1. Many cells expressed either GFP (*green*) (**A**) or lacZ (βgal antibody labeling in *red*) (**B**), while only two cells were both GFP^{+} and lacZ^{+} (*arrows*). Nuclei are labeled with Hoechst in *white*. *Scale bar*, 20 μm.

promoter driving the reporter gene and the detection sensitivity of the reporter molecule. A good reporter line should express reporter genes ubiquitously in all tissues, and the reporter molecule should have a high level of sensitivity. The *ROSA26* locus was thought to be ubiquitously expressed in every cell of the mouse; however, recent evidence suggests that it is down-regulated in the adult. Thus, cells with no detectable reporter expression may still have Cre activity. To address this issue, new Cre reporter lines were created where the *ROSA26* locus was modified by the insertion of a construct containing the CAG promoter followed by a floxed "stop" sequence and the brightest fluorescent proteins available, ZsGreen or tdTomato. When directly compared to the ROSA26^{eYFP} reporter line using several different Cre lines in the brain, these reporter lines, termed Ai6 for ZsGreen and Ai9 or Ai14 for tdTomato, had substantially stronger fluorescence and labeled more Cre^{+} cells than expected from previous reports (Madisen et al. 2010).

We performed similar studies in the utricle using Plp-CreER^{T2};ROSA26^{eYFP} mice compared to Plp-CreER^{T2};Ai14 mice. After tamoxifen (3 mg/40 g, IP) induction at P0 and P1, we found a much larger number of Cre^{+} HCs and SCs using the Ai14 reporter compared to the eYFP reporter (Fig. 7). Since most of the Cre/CreER alleles described in this review were not characterized with one of these new Ai reporter lines, it is likely that current reports are an underestimate of their Cre activity. It is also worth noting that the ZsGreen fluorescence in the Ai6 line has uneven distribution at adult ages with punctate staining in the cytoplasm that affects the quality of the image.

Similar efficiency issues apply when deleting the floxed region in a GOI, and one should not assume that the percentage of reporter^{+} cells will be the same as the percentage of GOI-deleted cells. If possible, it is best to perform immunostaining of the GOI to confirm the extent of the deletion. In addition, when using inducible alleles to delete genes postnatally, it is important to consider the protein half-life of the GOI since it may be very stable in a mature cell (Hayashi and McMahon 2002). Cre-mediated excision occurs at the level of the genome; thus, it takes time for the protein that was made prior to tamoxifen induction to be degraded to see a phenotype caused by deletion of the GOI.

Cre leakage

Another important issue to consider with inducible Cre lines is "leakage." It is possible that some CreER molecules are translocated to the nucleus in the absence of tamoxifen. Although Cre "leakage" effect is uncommon, it needs to be empirically tested for each CreER line (Zuo 2002). This can be easily done with controls using a reporter allele without tamoxifen injection. In fact, the CAG-CreER^{TM} allele has been reported to have a low level of Cre leakiness (Hayashi and McMahon 2002; Oesterle et al. 2011).

Strain background

Strain background effects also need to be considered when making conclusions. Due to high efficiency for gene targeting, 129 ES cells are commonly used to generate knock-in alleles and offspring after germline transmission are often mixed with the C57Bl/6 background. Both of these strains are not ideal for hearing research due to the *Ahl* loci which causes early onset of age-related hearing loss (Johnson and Zheng 2002; Ohlemiller 2004); however, these strains have normal hearing before 2 months of age (Zheng et al. 1999). If animals older than 2 months are needed for a study, it would be best to back-cross the desired lines to the CBA/CaJ strain, which is the "gold

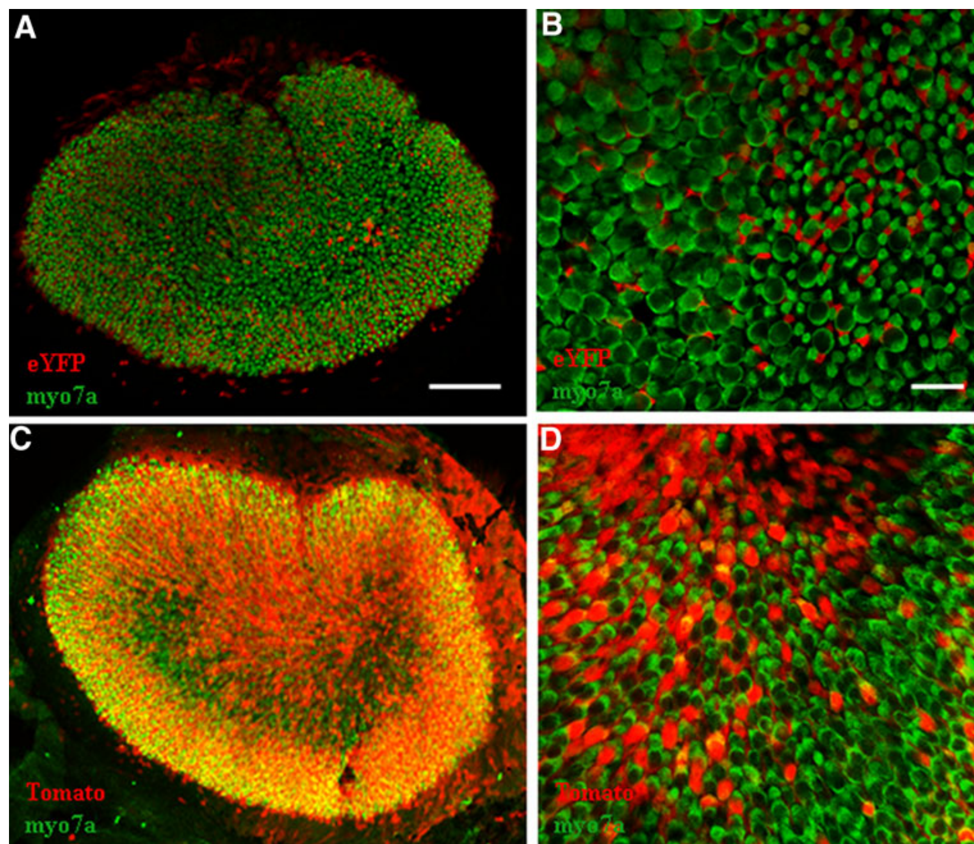

FIG. 7. Comparison of the ROSA26^{eYFP} and the Ai14 reporter lines in the utricle. Representative confocal images of the utricle of Plp-CreER^{T2};ROSA26^{eYFP} mice (**A**, **B**) and Plp-CreER^{T2};Ai14 mice (**C**, **D**) both induced with tamoxifen at P0 and P1. HCs labeled by myosin VIIA (*myo7a*, *green*), while YFP and tdTomato are in *red*. *Scale bars*, 100 μm (**A**, **C**) and 20 μm (**B**, **D**).

standard" for auditory research (Erway et al. 1996; Zheng et al. 1999; Johnson et al. 2000; Yoshida et al. 2000; Johnson and Zheng 2002; Willott et al. 2003). The recent KOMP effort was designed to create null and floxed alleles for every gene in the genome using the C57Bl/6 background because it is the most widely used strain and the complete genome sequence is available (Austin et al. 2004). Thus, strain effects with C57Bl/6 in hearing research should be taken into consideration when taking advantage of these valuable resources from KOMP or other consortiums. In addition, strain background can have an effect on Cre efficiency and the pattern of Cre expression (Hebert and McConnell 2000).

Toxicity

The scientific community has recently expressed concern that the expression of Cre might have toxic effects to cells, which is unrelated to the deletion of the GOI. The most appropriate control to measure potential Cre toxicity is to use the same Cre allele without the floxed GOI (Editorial 2007). There is also concern that tamoxifen treatment may have toxic effects. While tamoxifen does impair reproductive development (Gill-Sharma et al. 2001; Mehasseb et al. 2009), no effects on hearing sensitivity, as measured by ABR, have been reported (Li et al. 2004; Tian et al. 2004; Chow et al. 2006; Yu et al. 2010).

Fate mapping

Fate mapping (or lineage tracing) takes advantage of the permanent gene deletion caused by Cre-mediated recombination. Immunostaining of the Cre protein cannot be used for fate mapping because the Cre enzyme is only expressed while the cell type-specific promoter is active. The use of cell type-specific Cre/CreER alleles in combination with a reporter allele will permanently label Cre^{+} cells regardless of whether they change cell fate or if the promoter driving Cre is downregulated. Therefore, daughter cells (progeny) from Cre^{+} cells will continue to have the reporter label since the deletion of the floxed DNA sequence occurs at the level of the genome and is passed on (Nagy 2000). This concept is advantageous when trying to investigate the cell of origin for a structure, tumor, or regenerated cell population since cell-specific markers may be turned off with time or with cell fate changes. Many use the terms fate mapping

and lineage tracing interchangeably, and while the basic concepts hold true for both, lineage tracing is fate mapping performed at the single-cell level. It is also important to note that if the goal is to determine the cell of origin during development, it is best to use a knock-in Cre/CreER line so that the Cre expression pattern faithfully recapitulates endogenous expression of the promoter driving Cre/CreER. However, if the goal is to trace cells when there is a cell fate change (i.e., after ectopic expression NICD or in HC regeneration studies), a knock-in Cre/CreER line is not required. Control samples of the same age without the gene manipulation or drug treatment can be used to show what cell types normally have Cre expression. For example, the Ngn1-CreER^{T2} line was used to trace the progeny of Ngn1-expressing cells after the sensory epithelia were formed (Raft et al. 2007), and the Atoh1-Cre (knock-in) line was used to conclude that Atoh1 expression occurs in the common progenitor cells that give rise to both HCs and SCs (Yang et al. 2010a).

Complex uses of Cre technology

As discussed previously, Cre-mediated recombination will excise DNA between floxed regions when two *loxP* sites are on the same strand of DNA and are in the same orientation. Mutations in the *loxP* sequence have been created to generate incompatible or mismatched *loxP* sites where the efficiency of recombination is reduced (Langer et al. 2002). This concept was used with *loxP* and *loxH* to generate a mosaic expression of iCsp3 (Fujioka et al. 2011). In addition, Brainbow 1.0 reporter lines use two *loxP* sites and two mutated *loxP* sites, allowing two possible recombination outcomes to occur in equal proportion. Brainbow 1.0 cells are red and in the presence of Cre become either yellow or cyan, depending on which segment of DNA is deleted. Brainbow 1.1 lines added to this complexity with an additional set of mutated *loxP* sites and a third reporter gene. Additional colors are possible when more than one copy of the Brainbow transgene is inserted into the genome because each construct recombines independently, allowing more than one fluorescent protein to be expressed in a single cell (Livet et al. 2007).

Cre-mediated recombination can also result in the inversion of DNA between *loxP* sites when the *loxP* sites are in opposite directions (i.e., *loxP* sites face either other). Brainbow 2.0 utilizes this concept with a construct where red fluorescent protein (RFP) is in the sense direction, cyan fluorescent protein (CFP) is in the antisense orientation, and both are surrounded by *loxP* sites facing each other. Thus, RFP is normally expressed, and when Cre-mediated recombination occurs, cells stop making RFP and begin making CFP. Brainbow 2.1 mice have two invertible DNA segments positioned in tandem to generate three different possible recombination outcomes which occur stochastically. Both Brainbow transgenic lines produce neurons in a variety of colors, which allow tracing of neuronal processes at the individual level and visualization of their synaptic interactions (Livet et al. 2007). These lines are driven by the Thy1 promoter and may be useful for neuronal tracing in the inner ear; however, none of the Brainbow lines had reporter expression in cochlear HCs or SCs when appropriate Cre lines (Atoh1-CreER^{TM} and Prox1-CreER^{T2}) were used (Fang et al. 2009).

Recently, a knock-in R26R-Confetti mouse line was generated and first used in the intestine. Instead of the Thy1 promoter, the Brainbow 2.1 cassette was inserted into the *ROSA26* locus with the addition of a strong CAG promoter and a floxed "stop" codon. Since there are five *loxP* sites, there are ten recombination outcomes, six of which include the "stop" codon and four of which produce a fluorescent protein (CFP, GFP, YFP, or RFP; Snippert et al. 2010). Since the *ROSA26* locus is known to be active in the inner ear, this line is expected to give reporter expression in various cell types, including HCs and SCs.

The Brainbow and R26-Confetti mouse lines offer advantages over single-color reporter lines because tracing at the single-cell level (lineage tracing) can be performed when CreER lines are used. This was recently done in the intestine where Lgr5^{+} cells were labeled with the R26-Confetti mouse. Soon after tamoxifen induction, cells of various colors were observed due to the random recombination of the R26-Confetti allele; however, after several weeks, each intestinal crypt was labeled by a single color, demonstrating that it was populated by a single Lgr5^{+} cell (Snippert et al. 2010). Similar studies could be performed in the inner ear. For example, prosensory progenitor cells could be labeled with the R26-Confetti line to show how many HCs and SCs are derived from a single progenitor cell. In addition, the fluorescent proteins in Brainbow mice are also present in neuronal processes and synapses; thus, this line could be used with a spiral/vestibular ganglion CreER allele to visualize the innervation pattern of a single neuron.

OTHER METHODS FOR CONDITIONAL GENE EXPRESSION

In addition to Cre recombinase, Flp recombinase from yeast has been used to generate conditional alleles in mice. Similar to recombination between *loxP* sites by Cre, Flp recognizes *FRT* sites and can excise, invert, or translocate the fragment of DNA between *FRT* sites in a permanent manner (Dymecki 1996). To

optimize Flp recombinase activity, a more efficient Flpe enzyme was made that has higher activity at physiological temperatures (Buchholz et al. 1998). Flpe was further modified with the addition of a nuclear localization sequence which improved its performance in mammalian cells (Schaft et al. 2001). In 2007, a codon-optimized version of Flpe, termed Flpo, was generated. In mammalian cells, Flpo is reported to have five times more activity than Flpe (Raymond and Soriano 2007; Kranz et al. 2010). Currently, no Flp mouse lines have been reported with specific activity in the inner ear, although several attempts were made to express Flp specifically in cochlear HCs (Dearman and Zuo 2010). We are also not aware of any inducible Flp mouse lines.

To transiently alter gene expression, the tetracycline-inducible system is used to either repress or activate gene transcription. The tetracycline transactivator (tTA) is a fusion protein that combines the tet repressor-binding domain and an activation domain of VP16, a transcription factor from herpes simplex virus (Gossen and Bujard 1992). It binds to and activates the promoter of a second transgene which contains a tetracycline response element (TRE). When tetracycline or doxycycline is given, it binds to tTA and prevents tTA from binding to the TRE sequence, therefore blocking transcription (also called Tet-off system). Mutations in four amino acids of the tet repressor-binding domain of tTA generate a reverse tetracycline transactivator (rtTA) which works in the opposite manner to activate transcription in the presence of tetracycline or doxycycline (also called Tet-on system; Gossen et al. 1995). Both tTA and rtTA have been shown to work in transgenic mice, where doxycycline is given in the food and/or drinking water (Kistner et al. 1996). The cell type specificity comes from the use of cell type-specific promoters to express the tTA or rtTA protein. An important hallmark of the Tet-on/off systems is that the expression of the GOI can be transiently activated or inactivated only when doxycycline is present. When doxycycline is withdrawn, expression of the GOI will be reversed. Doxycycline will effectively cross the blood–labyrinth barrier, as demonstrated by the use of the Tet-on system in the cochlea to study ectopic expression of NICD (Pan et al. 2010). For gene functional studies in the inner ear, it often becomes essential, albeit challenging, to combine two independent methods (i.e., Cre-*loxP* and Tet-on/off) in a single mouse.

CONCLUDING REMARKS

Many significant advances have been made in the inner ear using conditional gene expression and Cre-*loxP* technology. In addition to fate mapping with reporter lines to determine the cell origin of various inner ear cell types, temporal control of gene expression has led to the discovery of age-dependent changes in the function of a particular gene (i.e., Rb and Notch signaling). Here, we summarize 32 Cre/CreER lines with expression in the developing or postnatal inner ear to provide a resource for auditory and vestibular researchers. For postnatal studies, there are many good alleles to study specific cell types in the cochlea; however, this is not the case for vestibular organs. Better characterization, as well as more Cre/CreER lines, is needed. In addition, the majority of the Cre/CreER lines we summarized were characterized using the ROSA26^{LacZ} reporter, which likely underestimated their Cre activity. It would be wise to repeat Cre expression pattern analysis in future studies using one of the Ai reporter lines.

Since conditional gene expression is a growing field and a young one for inner ear research, we have also provided a table of 19 Cre/CreER alleles that may be useful in auditory and vestibular organs, but have not been characterized yet. The NIH Neuroscience Blueprint Cre-Driver Network will continue to create additional strains in the future. We anticipate that Flp and tetracycline-inducible strains expressed in specific cell types of the inner ear will also be created in the future and can be combined with the Cre-*loxP* system for more complex approaches. In summary, conditional gene expression offers much opportunity to expand our knowledge of the inner ear and build upon the findings obtained using germline knockout mice.

ACKNOWLEDGMENTS

We thank Betsy Williford in the Biomedical Communications Department of St. Jude Children's Research Hospital for her assistance in creating the artwork for figures. This work was supported in part by grants from the National Institute of Health (DC006471 (J.Z.), DC008800 (J.Z.), DC010310 (B.C.C.) and CA21765); the Office of Naval Research (N000140911014); the Sir Henry Wellcome Trust fellowship (M.M.M.L.); and the American Lebanese Syrian Associated Charities (ALSAC) of St. Jude Children's Research Hospital. J. Zuo is a recipient of The Hartwell Individual Biomedical Research Award.

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