Research Article

A Sox10^{rtTA/+} Mouse Line Allows for Inducible Gene Expression in the Auditory and Balance Organs of the Inner Ear

BRADLEY J. WALTERS¹ AND JIAN ZUO¹

¹Department of Developmental Neurobiology, St. Jude Children's Research Hospital, 262 Danny Thomas Place, Memphis, TN 38105, USA

Received: 16 October 2014; Accepted: 2 April 2015; Online publication: 21 April 2015

ABSTRACT

Genetic mouse models provide invaluable tools for discerning gene function *in vivo*. Tetracycline-inducible systems (Tet-On/Off) provide temporal and cell-type specific control of gene expression, offering an alternative or even complementary approach to existing Cre/LoxP systems. Here we characterized a Sox10^{rtTA/+} knock-in mouse line which demonstrates inducible reverse tetracycline trans-activator (rtTA) activity and Tet-On transgene expression in the inner ear following induction with the tetracycline derivative doxycycline (Dox). These Sox10^{rtTA/+} mice do not exhibit any readily observable developmental or hearing phenotypes, and actively drive Tet-On transgene expression in Sox10 expressing cells in the inner ear. Sox10^{rtTA/+} activity was revealed by multiple Tet-On reporters to be nearly ubiquitous throughout the membranous labyrinth of the developing inner ear, and notably absent from hair cells, tympanic border cells, and ganglion neurons following postnatal Dox inductions. Interestingly, Dox-induced Sox10^{rtTA/+} activity declined with induction age, where Tet-On reporters became uninducible in adult cochlear epithelium. Co-administration of the loop diuretic furosemide was able to rescue Dox-induced reporter expression, though this method also caused significant cochlear hair cell loss. Surprisingly, Sox10^{rtTA/+} driven reporter expression in the cochlea persists for at least 54 days after cessation of neonatal induction, presumably due to the persistence of Dox within inner ear tissues. These findings highlight the utility of the Sox10^{rtTA/+} mouse line as a powerful tool

for functional genetic studies of the auditory and balance organs *in vivo*, but also reveal some important considerations that must be adequately controlled for in future studies that rely upon Tet-On/Off systems.

Keywords: cochlea, utricle, Lac-Z, reporter, Cre, Tet-On

INTRODUCTION

The tetracycline-responsive system for inducible expression of transgenes (Tet-On) is a powerful tool for *in vivo* or *in vitro* experiments that require temporally and spatially controlled gene expression (Gossen et al. 1995; Kistner et al. 1996; Mallo 2006; Sun et al. 2007). The Tet-On system consists of three components: a tetracycline-responsive promoter element (TRE), tetracycline or its derivative doxycycline (Dox), and the reverse tetracycline transactivator (rtTA). When all three elements are present, they form a complex at the TRE promoter that drives the expression of the gene of interest (GOI). However, in the absence of Dox, or in the absence of rtTA, no ectopic expression should occur. This mechanism thereby allows for acute and transient manipulation of GOI expression based upon the timing of Dox induction and its clearance from the tissue (Mallo 2006). Meanwhile, cell- or tissue-specific GOI expression is obtained through the use of a cell- or tissue-specific promoter to selectively drive the expression of the rtTA. While CreER and CrePR systems also offer cell-specific GOI expression, such expression is permanent and dependent upon inductions by Tamoxifen or RU486 which can have detrimental

Correspondence to: Jian Zuo · Department of Developmental Neurobiology · St. Jude Children's Research Hospital · 262 Danny Thomas Place, Memphis, TN 38105, USA. Telephone: (901)-595-3891; email: jian.zuo@stjude.org

side effects such as anti-progesterone and anti-glucocorticoid activity, infertility/sterility, and embryonic lethality (Danielian et al. 1998; Kellendonk et al. 1999; Motrich et al. 2007). In contrast, the Tet-On system uses tetracycline or Dox which lack these off target effects. Thus, Tet-On systems are particularly useful for embryonic and multi-generational studies, in addition to more traditional gene targeting experiments. Furthermore, the use of tetracycline family antibiotics to induce GOI expression allows for the combined use of Tet-On systems with CreER or CrePR models (Chiba et al. 2000; Robinson et al. 2013). In this way, one can manipulate the expression of multiple genes in the same cells or in different cell types within the same animal, either simultaneously or sequentially. Indeed, tetracycline-responsive systems have been successfully used in previous inner ear studies demonstrating their utility for auditory and balance research (Maison et al. 2007; Pan et al. 2010, 2013; Burger et al. 2011; Cox et al. 2014a, b). In these previously published models, Dox was effective in manipulating GOI expression in sensory and non-sensory embryonic progenitor cells, and in neonatal supporting cells (SCs) and hair cells (HCs) within the inner ear. However, there is little evidence for the use of Tet-On/Off systems at later postnatal and adult ages, and since tetracycline and Dox do not easily permeate the blood-labyrinth barrier (BLB) or the blood-brain barrier (Stupp et al. 1973; Michel et al. 1984), it remains unclear whether Dox-inducible systems can work well in the inner ear at or after P14 when the BLB is formed (Ehret 1976; Shnerson and Pujol 1981; Suzuki et al. 1998; Dai et al. 2006). Moreover, because previous analyses were performed shortly after Dox induction, it remains unclear how long GOI expression lasts in the inner ear after Dox treatment.

Here we describe the patterns of inducible transgene expression in the inner ear of a Sox10^{rtTA/+} knock-in mouse line which was generated via the insertion of an rtTA2^s-M2 construct into the Sox10 locus, replacing the open reading frame so as to be expressed in a pattern that recapitulates endogenous Sox10 (Ludwig et al. 2004). Indeed, Sox10^{rtTA/+}-driven expression of a nuclear localized Tet-On-LacZ (Furth et al. 1994) reporter in mouse embryonic spinal cord exhibited a large degree of overlap with endogenous Sox10 expression (Ludwig et al. 2004), suggesting that this line should be similarly effective in Sox10-expressing cells of the inner ear. Here, we used three independent Tet-On reporter lines to verify Sox10^{rtTA/+}-inducible transgene expression within the cochlea and utricle following Dox induction at various embryonic and postnatal ages. Sox10^{rtTA/+} mice exhibited Dox-inducible activity in numerous inner

ear cell types, in a pattern consistent with endogenous Sox10 expression (Watanabe et al. 2000; Wakaoka et al. 2013). Specifically, reporter expression was detected in numerous cell types in the membranous labyrinth and in the modiolus and the non-neuronal cells of the vestibular ganglia both embryonically and postnatally, but was largely absent from HCs, tympanic border mesenchymal cells, and ganglion neurons following postnatal induction. Interestingly, we found that Dox-induced Sox10^{rtTA/+} activity declined with the age of induction, being dramatically reduced at P13–P15 and undetectable at P27–P29, possibly due to the inability of Dox to cross the BLB. This limitation was largely overcome via co-administration of the loop diuretic furosemide which significantly improved Dox-induced reporter expression at P27–P29. However, this combination of Dox and Furosemide resulted in significant auditory HC loss. Surprisingly, we also found that Dox-induced reporter expression in the inner ear can persist for at least 54 days, long after neonatal induction, presumably due to the persistence of Dox within the tissues of the inner ear. Together, these data suggest that the Sox10^{rtTA/+} mouse line represents a useful tool for the manipulation of GOIs in the inner ear at embryonic, postnatal, and adult ages; however, some limitations of this technique are also illuminated, specifically the inability of Dox alone to induce expression in the adult organ of Corti, the loss of cochlear HCs in response to co-administration of Dox and furosemide, and the persistence of GOI expression which may limit the use of this method for transient genetic manipulation.

MATERIALS AND METHODS

Animal Models

All mice were housed and cared for at St. Jude Children's Research Hospital in accordance with IACUC guidelines and approved protocols. Sox10^{rtTA/+} mice (Ludwig et al. 2004) were obtained from the laboratory of Dr. Mikhail Pletnikov (Johns Hopkins University) with permission from Dr. Michael Wegner (Friedrich-Alexander-Universität) and subsequently re-derived at the St. Jude Children's Research Hospital by *in vitro* fertilization with C57BL/6 mice. Resulting Sox10^{rtTA/+} mice were bred with a Tet-On-LacZ mouse line that is available from the Jackson Laboratory (strain #002621), and further backcrossed to this strain (B6.SJL background) for at least three generations. Following these crosses, Sox10^{rtTA/+};Tet-On-LacZ^{+/ve} mice were characterized, and Sox10^{rtTA/+};Tet-On-LacZ^{+/ve} progeny were subsequently bred with either the Tet-On-mCherry (strain #014592), or the Tet-On-Cre (strain #006224) and Rosa26-loxP-stop-loxP-CAG-TdTomato (Rosa26^{tdTomato}, strain

#007908) reporter lines, also available from the Jackson Laboratory. Sox10^{rtTA/+} activity was induced in embryonic and postnatal mice using several different doses or combinations of Dox. Specifically, embryonic inductions were accomplished via provision of Teklad Dox Diet (Harlan) food pellets to the pregnant dam (*ad libitum*) at doses of 200 mg/kg or 2 g/kg from 17 to 21 days *post coitum* (corresponding to E17–P0 of the pups). Induction in postnatal mice was accomplished by one of two methods: (1) administration of Dox diet to the nursing dam at 2 g/kg (*ad libitum*) between P0 and P3, or (2) administration of Dox diet (2 g/kg) supplemented with a single intraperitoneal (i.p.) injection of Dox hyclate (Sigma Aldrich) given directly to the pups (100 mg/kg body weight, 10 mg/mL in 0.9 % sterile saline). This latter method was more effective than food alone for inducing reporter expression in cochlear supporting cells and was therefore used to induce reporter expression at multiple postnatal ages. For each time point, Dox diet was provided *ad libitum* for 48 h (P0–P2, P6–P8, P13–P15, P27–P29), with Dox injections given 24 h after the addition of the Dox food. Since previous data show that Sox10^{rtTA/+}-driven Tet-On-LacZ reporter activity reaches maximal levels by 24 h after initiation of Dox treatment and remains elevated for at least 3 days after cessation of Dox (Ludwig et al. 2004), mice were routinely euthanized at the time point at which the Dox diet was withdrawn (P0, P2, P8, P15, or P29) to reveal maximal expression. However, for one experiment, mice were allowed to survive to 5, 19, or 54 days following Dox treatment (P7, P21, or P56) to assess the transience of Tet-On reporter expression. In an attempt to successfully induce Sox10^{rtTA/+} activity in adults, one group of mice was provided Dox food (2 g/kg) *ad libitum* from P27 to P29 and injected at P28 with both Dox (100 mg/kg i.p.) and furosemide (400 mg/kg i.p.) which was administered 30 min after the Dox injection. These mice were euthanized and their temporal bones dissected out at P29.

Immunostaining

Temporal bones were fixed overnight in 2 % paraformaldehyde and then de-calcified in 0.12 M EDTA for 48 h. Cochleae and utricles were either dissected as whole mounts and immunostained free-floating, or the temporal bones were embedded in o.c.t. tissue tek (Fisher Scientific), frozen on dry ice, cut into 20- μ m-thick sections by cryostat, and immunostained on superfrost plus slides (Fisher Scientific). Whole mounts and sections were immunostained with the following primary antibodies: chicken anti-beta galactosidase (β -gal, 1:1000, Abcam: AB9361), rabbit anti-Sox2 (1:500, Millipore: AB5603), Alexa-647 conjugated rabbit anti-myosin VI (Myo6, 1:25

Proteus), rabbit anti-calbindin (1:500, Millipore: AB1778), and mouse anti-parvalbumin (1:1000, Sigma: P3088). Total numbers of Myo6-positive HCs were counted from six sample images from each cochlea corresponding to 300- μ m lengths along the tonotopic axis, and two sample areas of 10,000 μ m² (one central, one peripheral) from each utricle whole mount. Beta-galactosidase (β -gal)-positive cells were similarly counted from representative cochlear and utricular whole mount images and were recorded as percentages of Sox2-positive SCs or Myo6-positive HCs. Where comparisons to control genotypes were made, the control groups consisted of Dox-induced littermates that were Sox10^{+/+};Tet-On-LacZ⁺ and Sox10^{+/+};Tet-On-LacZ^{ve}.

Evoked Auditory Brainstem Recordings

Free-field, pure tone auditory brainstem recordings (ABRs) were recorded from mice following anesthesia by i.p. avertin (300–500 mg/kg body weight). Pure tone stimuli were presented at 4, 8, 12, 16, 22, and 32 kHz by a magnetic speaker placed 4 cm from the pinna. Measurements were made from 65-dB sound pressure level (SPL) down to 5-dB SPL by –5-dB increments. All stimuli presentation and recording were performed using an RZ-6 auditory workstation (Tucker Davis Technologies). Within 2 h following ABR recording, mice were euthanized by CO₂ and temporal bones fixed for analysis as described above.

Data Analysis

Cochlear lengths, areas of the utricular maculae, HC density measures, and body mass were compared using two-tailed, independent samples, Student's *t* tests. ABR thresholds were compared using a two-way repeated measures ANOVA with frequency as the within-subjects variable and genotype as the between-subjects variable. β -gal reporter expression in postnatal cochlear SCs was analyzed by two separate two-way repeated measures ANOVAs. The first ANOVA compared the percentages of reporter-positive SCs across age (between-subjects) and cochlear turn (within-subjects) and was followed by post hoc Tukey's honest significant difference (HSD) tests to make pairwise comparisons of the three ages of induction. Additionally, differences between apical and basal turns of the cochlea were compared at each level of induction age by Bonferroni corrected paired samples *t* tests (two-tailed). The second ANOVA compared percentages of reporter-positive SCs across age (between-subjects) and SC subtype (within-subjects) and was followed by Bonferroni corrected paired samples *t*-tests (two-tailed) to compare the SC subtypes at each level of induction age. To compare the HC density between Dox only and Dox plus furosemide induced samples,

two separate repeated measures ANOVAs (one for inner HCs, IHCs, and one for outer HCs, OHCs) were conducted using cochlear turn (apical vs. basal) as the within-subjects variable and induction method as the between-subjects variable. For the OHCs, Bonferroni corrected independent samples *t* tests (two-tailed) were conducted to compare HC density across induction method at each level of cochlear turn. Comparison of reporter-positive SCs between apical and basal turns of the cochleae in Dox plus furose-mide treated samples was accomplished by a two-tailed, paired samples *t* test. Finally, reporter expression following embryonic induction was compared by a two-tailed, independent samples *t* test with the dose of Dox as the independent variable. All error bars represent ± 1 standard error of the mean (S.E.M.), and all statistical analyses were conducted using SPSS 17 software (SPSS Inc.).

RESULTS

Sox10^{rtTA/+} Mice Do Not Exhibit Gross Morphological or Audiological Phenotypes

Dominant negative, monoallelic Sox10 mutations in humans are associated with Waardenburg syndrome which is characterized by abnormal pigmentation, intestinal aganglionosis (megacolon), and hearing loss (Pingault et al. 1998, 2013, 2014; Bondurand et al. 1999; Britsch et al. 2001; Paratore et al. 2002; Zhang et al. 2012; Watanabe et al. 2013; Jelena et al. 2014). While it has been shown that Sox10 heterozygous knockout or mutant mice can exhibit intestinal and pigmentation phenotypes similar to those seen in Waardenburg syndrome, the presence of these phenotypes appears to depend upon the genetic background (i.e., strain) of the mice (Southard-Smith et al. 1999a, b; Britsch et al. 2001; Cantrell et al. 2004). Additionally, whether Sox10 mutant mice exhibit any hearing impairment has yet to be described. To determine whether the Sox10^{rtTA/+} mice exhibit any audiological or gross morphological inner ear phenotypes in response to Sox10 heterozygosity, we compared a number of variables between uninduced Sox10^{rtTA/+} and uninduced Sox10^{+/+} mice at P28. Specifically, we measured body mass, ABR thresholds, cochlear length, utricular area, and HC density in both the cochleae and utricles. For all of these variables, we found no statistically significant differences between Sox10^{rtTA/+} mice and littermates that were homozygous wild-type at the Sox10 locus (Fig. 1). These results not only demonstrate that Sox10^{rtTA/+} mice can be useful for investigations and manipulation of GOIs in the inner ear, but also reinforce the hypothesis that the effect of Sox10 haploinsufficiency on hearing (and other Waardenburg-Shah

phenotypes) is dependent upon the context of genetic background.

Sox10^{rtTA/+}-Driven Reporter Expression is Widespread in the Inner Ear, Though Relatively Absent from HCs and Neurons After Postnatal Inductions

To ascertain whether Sox10^{rtTA/+} is able to drive GOI expression in inner ear tissues, we used multiple Tet-On reporter lines, namely, the Tet-On-LacZ (nuclear localized), Tet-On-mCherry (nuclear localized), and Tet-On-Cre; Rosa26^{tdTomato} mouse models. Indeed, the combined delivery of Dox via food and injection to neonatal Sox10^{rtTA/+} mice between P0 and P2 resulted in robust upregulation of all three of these reporters when analyzed at P2 (Fig. 2). Since a common issue with steroid receptor mediated Cre recombinase and Tet-On reporter systems is leakiness that can lead to transgene expression even in the absence of inducing agents (Wunderlich et al. 2001; Feil et al. 2009; Hosoda et al. 2011; Cox et al. 2014a, b), we also examined uninduced Sox10^{rtTA/+;Tet-On-LacZ^{ve}} mice as well as Sox10^{+/+;Tet-On-LacZ^{ve}} mice that were given Dox food and injections between P0 and P2, between P6 and P8, and between P13 and P15. We found no reporter expression in any of the uninduced Sox10^{rtTA/+;Tet-On-LacZ^{ve}} mice, nor in any of the Sox10^{+/+;Tet-On-LacZ^{ve}} mice that were given Dox food and injections between P6 and P8 or P13 and P15 (data not shown). However, following Dox food and injections between P0 and P2, we found a small number β -gal-positive cells in the modiolar region of the cochlea (Fig. 3), as well as in the non-neuronal cells of the vestibular ganglia, and in the stroma and SCs of the utricle (data not shown). These results were replicated using the Sox10^{+/+;Tet-On-Cre^{ve};TdT^{Tomato^{ve}}} mice, which showed similar patterns of leaky expression when induced at P0 and P2 (data not shown) but were completely devoid of any reporter expression following Dox treatment at P6–P8. While these results demonstrate a limited leakiness of the Tet-On-LacZ and Tet-On-Cre reporters used here, we recommend that all investigators independently validate any Tet-On transgene, and the appropriate dosing of Dox, before proceeding with experiments using the Sox10^{rtTA/+} line, or any other rtTA/Tet-On-n system in the inner ear. Similarly, for experiments to study gene function using these models, it may be important to use uninduced and rtTA-negative mice, in addition to mice lacking the Tet-On transgene as negative controls.

Further characterization of Sox10^{rtTA/+}-driven reporter activity after postnatal inductions revealed robust reporter expression in the SCs of the sensory regions (organ of Corti, utricle, cristae, and saccule), as well as in the lesser epithelial ridge, outer sulcus, stria vascularis, Reisner's membrane, interdental

FIG. 1. Sox10^{rtTA/+} mice do not exhibit any gross morphological or hearing phenotypes. **A** Body mass (in grams) of Sox10^{rtTA/+} and Sox10^{+/+} littermates at 4 weeks of age (P28) were nearly identical, being 15.10 ± 0.47 g for the Sox10^{rtTA/+} mice and 15.10 ± 0.49 g for the Sox10^{+/+} mice. **B** Similarly, there was no difference in the length of the cochlear epithelium when compared between Sox10^{rtTA/+} (6.033 ± 0.029 mm) and Sox10^{+/+} (6.023 ± 0.015 mm) littermates at P28. **C** There were also no differences in cochlear HC density in either inner HCs (IHCs) or outer HCs (OHCs), where Sox10^{rtTA/+} mice (dark gray) had an average of 11.8 ± 0.22 IHCs and 37.85 ± 0.87 OHCs per 100 μm of cochlear length and Sox10^{+/+} mice (light gray)

had an average of 11.7 ± 0.24 IHCs and 38.57 ± 0.91 OHCs per 100 μm of cochlear length. **D** Similarly, there was no difference in the surface area of utricular maculae when compared between Sox10^{rtTA/+} ($170,687 \pm 4604$ μm²) and Sox10^{+/+} mice ($171,969 \pm 2545$ μm²). **E** Nor was there any detectable difference in utricular HC density between Sox10^{rtTA/+} (221.8 ± 3.5 HCs/10,000 μm²) and Sox10^{+/+} (223.1 ± 2.4 HCs/10,000 μm²) samples. **F** Finally, there were no differences when we compared ABR thresholds between the Sox10^{rtTA/+} and Sox10^{+/+} genotypes at P28 ($F_{(1,7)} = 0.12$, $P = 0.74$). Error bars represent ± 1 S.E.M.

cells, inner sulcus cells, cells within the modiolus, and non-neuronal cells in the vestibular ganglia (Fig. 4). Counterstaining for HC and neuron-specific markers

such as Myo6, parvalbumin (Eybalin and Ripoll 1990; Dememes et al. 1993), calbindin (Liu and Davis 2014),

FIG. 2. Sox10^{rtTA/+} can induce reporter expression in the inner ears of three different mouse lines. Sox10^{rtTA/+} mice were bred with Tet-On-mCherry^{+/ve} (A), Tet-On-LacZ^{+/ve} (B), and Tet-On-Cre^{+/ve};Rosa26^{tdTom/+} (C) mouse lines. The resulting litters were induced with Dox food (2 g/kg) between P0 and P2 and an i.p. injection of Dox (100 mg/kg body weight) at P1 before being euthanized and the inner ears examined for reporter expression at P2. Endogenous mCherry (A, red) and tdTomato fluorescence (C, red), as well as immunostaining for beta-galactosidase (B, β-gal, green), revealed widespread reporter expression in the cochleae of Sox10^{rtTA/+} mice. Scale bars=100 μm.

FIG. 3. Leakiness of reporter transgenes is minimal in response to neonatal high dose Dox administration. Sox10^{+/+};Tet-On-LacZ^{+/ve} mice were given i.p. injections of Dox (100 mg/kg body weight) at P1, while nursing dams were provided with Dox food (2 g/kg, *ad libitum*) for the 48 h corresponding to P0–P2. Inner ear tissues were collected and fixed at P2. Immunostaining for beta galactosidase (β-gal, green) did not reveal any positive cells in the apical turns of the cochleae (A); however, a small number of nuclei (1.75 ± 1.03 per 320 μm of cochlear length) in the modiolar region of the basal turns were β-gal positive (B, arrows). We did not observe β-gal expression in any Sox2-positive cells (magenta) in either cochlear turn. Scale bars=20 μm.

and class III beta-tubulin (Tuj-1) (Barclay et al. 2011) suggested that reporter proteins (β -gal, mCherry, TdTomato) were absent from cochlear HCs and ganglia neurons after Dox induction between P0 and P2 and examination at P2 (β -gal shown in Fig. 4). Also, while reporter proteins were readily detected in cells of the stromal tissues directly beneath the SCs of the balance organs, we did not observe any reporter expression in the tympanic border mesenchymal cells that underlie the basilar membrane of the cochlea. In the balance organs, *Sox10^{rtTA/+}*-driven reporter expression was detectable in the majority of

SCs and in a small number of HCs (0.28 ± 0.23 % of HCs in the utricle at P2). In the vestibular ganglia (VG), reporter-positive cells were never seen to co-label with the neuronal markers calbindin, parvalbumin, or Tuj1 (Fig. 4). Rather, many, though not all, of the reporter-positive cells in both the modiolar region and in the vestibular ganglia were seen to co-label with the glial marker S100, suggesting that at least a portion of the reporter-positive cells were glia (Fig. 4). This pattern of expression largely overlaps with previous reports of Sox10 reporter expression in the inner ear (Watanabe et al. 2000;

FIG. 4. *Sox10^{rtTA/+}*-induced reporter expression is prevalent in the membranous labyrinth, and in non-neuronal cells of the modiolus, and vestibular ganglia. **A** Dox from P0 to P2 induced widespread beta galactosidase expression (β -gal, green) around the cochlear ducts and in the modiolus (Mod) of *Sox10^{rtTA/+};Tet-On-LacZ^{+/+}* mice. β -gal-positive nuclei are clearly visible in cells of the outer sulcus (OS), stria vascularis (SV), Reissner's membrane (RM), and modiolus (Mod), as well as in the interdental cells (IDCs), inner sulcus cells (IS), and supporting cells (SCs). β -gal expression was notably absent from the myosin VI (Myo6, red) positive inner HCs (IHC) and outer HCs (OHCs). Scale bar=50 μ m. **B, C** Higher-magnification images of the modiolar region containing the cochlear ganglion show that β -gal expression (green) does not overlap with the neuronal markers parvalbumin (Parv, blue, **B**) or calbindin (Calb, blue, **C**). Hoescht dye was used to counterstain nuclei (red); scale bars=50 μ m. **D** Sections from

the modiolar region that were immunostained for the pan-neuronal marker Tuj-1 (magenta) and β -gal (green) did not reveal any co-expression in any of the samples examined. Scale bar=20 μ m. **E** Dox from P0 to P2 also induced widespread β -gal expression (green) in and around the balance organs and in the non-neuronal cells of the vestibular ganglia of *Sox10^{rtTA/+};Tet-On-LacZ^{+/+}* mice. Reporter expression was robust in supporting cells but also occasionally seen in Myo6-positive HCs (red). Scale bar=100 μ m. **F** *Sox10rtTA*-driven β -gal expression (green) in both modiolar (not shown) and vestibular ganglia regions (shown) showed at least some overlap with S100 (magenta), a glial cell marker. Scale bar=20 μ m. **G** A higher magnification image of the boxed area from panel (**F**) more clearly shows the cells where β -gal (green) and S100 (magenta) are co-expressed (white). Scale bar=20 μ m.

Breuskin et al. 2009, 2010; Wakaoka et al. 2013) and suggests that Sox10^{rtTA/+} offers specificity in targeting a subset of cells in the inner ear that generally excludes HCs, tympanic border cells, and ganglia neurons. Together, these findings suggest that *Sox10* is transcriptionally active in many cochlear and vestibular cell types at neonatal ages and that the Sox10^{rtTA/+} mouse line provides a useful tool for the detection of Sox10 transcriptional activity as well as GOI manipulation in the inner ear.

Sox10^{rtTA/+}-Driven Gene Expression in Cochlear Supporting Cells is Dependent upon the Dose of Dox

We examined the reporter expression in Sox10^{rtTA/+};Tet-On-LacZ^{+/ve} mice following several different induction paradigms which allowed us to see the responsiveness of this system to varied doses of Dox. The overall pattern that emerged was that Sox10^{rtTA/+}-driven expression in the SCs of the organ of Corti and in the sensory epithelia of the balance organs require higher doses of Dox, while reporter expression outside of these areas, e.g., greater epithelial ridge (GER), inner sulcus, and modiolus, can be more easily induced by lower doses of Dox. For example, when looking in the cochlea specifically, Dox food alone (2 g/kg) provided from P0 to P5 successfully induced reporter expression in cells in the GER, inner sulcus, and modiolus but was insufficient to induce reporter expression in any cochlear SCs (Fig. 5). This lies in stark contrast to the robust reporter expression that is seen in the cochlear SCs of Sox10^{rtTA/+};Tet-On-LacZ^{+/ve} mice that were given a single i.p. injection of Dox (100 mg/kg body weight) at P1, in addition to the Dox food at P0–P5. Similarly, we induced Sox10^{rtTA/+} activity at embryonic ages (E17–P0) by providing Dox food to the pregnant dams at doses of either 200 mg/kg or 2000 mg/kg and saw a marked increase in reporter expression in the pillar and Deiters cells, though not the GER, at the higher dose (Fig. 5). Together, these data show that modulating the dose and/or route of administration of Dox can alter the expression pattern of Tet-On GOIs in the inner ear. Furthermore, this suggests that a refinement of cell specificity and/or efficiency may be achieved using the Sox10^{rtTA/+} model system to manipulate gene expression in more restricted populations of cells in the inner ear (e.g., non-sensory cells of the ganglia).

Sox10^{rtTA/+} Reporter Expression Declines with Age of Induction in Postnatal Mice but Can Be Partially Restored in Adults by Co-administration of the Loop-Diuretic Furosemide

Given that the expression of various genes in the inner ear can increase or decline with postnatal age

FIG. 5. Tet-On reporter expression is dose dependent in Sox10^{rtTA/+} mice. **A** β-gal expression is notably absent from the cochlear SCs (dashed lines) in Sox10^{rtTA/+};Tet-On-LacZ^{+/ve} pups taken at P6 from cages that were provided with Dox food at 2 g/kg for 6 days. **B** In contrast, Sox10^{rtTA/+};Tet-On-LacZ^{+/ve} pups that were in similarly fed cages but also received an i.p. injection of Dox (100 mg/kg body weight) at P1 showed robust β-gal reporter expression in cochlear SCs at P6. Scale bars for (A) and (B) = 50 μm. **C** Similarly, when pregnant dams were fed Dox food at 200 mg/kg for the days corresponding to E17–P0, β-gal expression was mosaic in the cochlear SCs (dashed lines) of the P0 pups. **D** However, increasing the concentration of the Dox feed to 2000 mg/kg resulted in a significant increase in the numbers of cochlear SCs that were β-gal positive in the P0, Sox10^{rtTA/+};Tet-On-LacZ^{+/ve} pups. Scale bars for (C) and (D) = 20 μm. **E** Using Sox2 and β-gal immunolabeling, we quantified the percentages of β-gal-positive pillar cells (PCs) and Deiters' cells (DCs) and compared them across the two different doses of Dox. For both cell types, there was a significant increase (****P* < 0.001) in the percent of β-gal-positive cells when the mice were fed Dox at 2000 mg/kg (dark gray bars) as compared to Dox at 200 mg/kg (striped bars). Error bars represent ±1 S.E.M.

(Walters and Zuo 2013) and vary across SC subtype (Bermingham-McDonogh et al. 2006; Chai et al. 2011; Liu et al. 2013) and/or along the tonotopic axis of the cochlea (Son et al. 2012), we analyzed reporter expression in the different SCs and in the apical and basal halves of cochleae from Sox10^{rtTA/+};Tet-On-LacZ^{+/ve} mice following Dox induction at several different postnatal induction ages (P0–P2, P6–P8, P13–P15, and P27–P29). Looking at β-gal expression immediately following Dox treatment (P2, P8, P15, and P29), we compared the percentages of β-gal-positive SCs across induction age and cochlear portion (apical turns vs. basal turns) (Fig. 6). The samples from P29 were excluded from statistical analysis because we did not detect any reporter-positive cells following that induction. Analysis of the remaining three induction groups (P2, P8, and P15) revealed a significant main effect of age

FIG. 6. The ability to induce Sox10^{rtTA/+}-driven reporter activity in the cochlea declines with age of Dox administration. **A, F** When Dox is administered to Sox10^{rtTA/+};Tet-On-LacZ^{+/ve} mice between P0 and P2, β-gal expression (green) is nearly ubiquitous in the nonsensory cells of the cochlear duct, including the Sox2-positive (magenta) SCs. **B, G** When an identical dose of Dox (a single i.p. injection at 100 mg/kg of body weight plus Dox food at 2 g/kg ad libitum) is administered between P6 and P8, the number of β-gal-positive cells in the cochlea is decreased, and the pattern of expression, particularly in the Sox2-positive SCs, is much more mosaic. **C, H** β-gal reporter expression declines even further when Dox is administered between P13 and P15, where only a small number of Sox2-positive SCs co-express β-gal. **D, I** From sample areas of the cochlea that were segregated by apical and basal half of the longitudinal axis of the cochlea, the numbers of β-gal-positive cells were counted as percentages of the Sox2-positive inner phalangeal cells (Iph), inner pillar cells (IPC), outer pillar cells (OPC), and Deiter's cells (DC). These data confirmed the declining reporter expression with age of induction, where the percentage of β-gal-positive SCs was significantly higher following the P0–P2 induction as compared to the P6–P8 induction (x vs. y , $P < 0.0001$), and the P13–P15 induction (x vs. z , $P < 0.0001$). Also, the percent of β-gal-positive SCs was significantly higher following the P6–P8 induction as compared to the P13–P15 induction (y vs. z , $P < 0.0001$). Additionally, reporter activity in the Sox10^{rtTA/+};Tet-On-LacZ^{+/ve} mice was higher in the apical turns of the

cochlea (dark gray bars) as compared to the basal turns (light gray bars) after the P0–P2 induction ($*P < 0.05$), and after the P6–P8 induction ($*P < 0.05$). Separating the data across the various SC subtypes revealed that the decline in reporter expression happens at different rates in the different cell types. In the Iph cells, there was no significant decline between the P0–P2 induction and the P6–P8 induction, but there was a significant drop in β-gal reporter expression between the P6–P8 induction and the P13–P15 induction. In contrast, IPCs showed a significant drop in β-gal reporter expression between the P0–P2 induction and the P6–P8 induction, which remained low after the P13–P15 induction. OPCs and DCs both exhibited a more gradual decline in reporter expression where the percentages of β-gal-positive cells were significantly less after P6–P8 than after P0–P2 and significantly less after P13–P15 than after P6–P8. All scale bars = 20 μm, error bars represent ± 1 S.E.M., $*P < 0.05$, $**P < 0.01$, $***P < 0.0001$. **E, J** Sox10 immunostaining (green) at P1 (**E**) and P58 (**J**) using identical settings on the confocal microscope suggest that the Sox10 locus remains transcriptionally active in the SCs and other cells lining the membranous labyrinth up until at least P58. This finding suggests that reduced reporter activity after P15 in the Sox10rtTA model is not due to a loss of transcriptional activity, but rather the impermeability of the blood labyrinth barrier to Dox. Sections were counterstained for the HC-specific protein myosin VIIa (Myo7a, red). Scale bars = 20 μm.

($F_{(1,9)} = 1011.65$, $P = 2.6 \times 10^{-11}$), a significant main effect of cochlear portion (Apex > Base, $F_{(1,9)} = 58.05$, $P = 3.3 \times 10^{-5}$), and a significant interaction between age and portion ($F_{(2,9)} = 21.06$, $P = 4.0 \times 10^{-4}$). Post hoc Tukey's HSD revealed significantly more reporter-positive SCs at P2 as compared to P8 ($P = 3.9 \times 10^{-7}$) and P15 ($P = 2.5 \times 10^{-8}$), and significantly more reporter-positive cells at P8 as compared to P15 ($P = 2.6 \times 10^{-8}$). Bonferroni corrected, two-tailed t tests suggested that the interaction between age and cochlear portion was driven by significantly more reporter-positive SCs in the apical turns compared to the basal turns at P2 ($t = 6.99$, $P = 0.018$) and P8 ($t = 6.86$, $P = 0.018$), but not at P15 ($t = -0.50$, $P = 1.00$). Similarly, when we compared the percent of reporter-positive SCs

across SC subtype (inner phalangeal (Iph), inner pillar (IPC), outer pillar (OPC), and Deiter's cells (DCs)) and across induction ages, this analysis revealed a main effect of induction age ($F_{(1,9)} = 749.27$, $P = 9.8 \times 10^{-11}$), a significant main effect of SC subtype ($F_{(3,27)} = 249.82$, $P = 8.5 \times 10^{-20}$), and a significant interaction between age and SC type ($F_{(6, 27)} = 107.75$, $P = 1.4 \times 10^{-17}$) (Fig. 6). Post hoc, Bonferroni corrected, two-tailed t tests revealed differential rates of declining reporter expression with induction age that were dependent upon the SC type. For Iph cells, there was no significant decrease in reporter expression between the P0–P2 and P6–P8 induction groups ($T_{(6)} = -0.28$, $P = 1.00$), but the reporter expression at P15 was significantly less than that at P2 ($T_{(6)} = 23.28$, $P = 4.8 \times 10^{-6}$) and at

P8 ($T_{(6)}=23.33$, $P=4.8\times 10^{-6}$). For the IPCs, however, the decline was more rapid, with the percent of β -gal-positive SCs being significantly lower at P8 than P2 ($T_{(6)}=20.12$, $P=1.2\times 10^{-5}$) and significantly lower at P15 than at P2 ($T_{(6)}=24.39$, $P=3.6\times 10^{-6}$), but no different between P8 and P15 ($T_{(6)}=-0.71$, $P=1.00$). In contrast, the OPCs and DCs exhibited a more gradual decline in reporter expression across induction age, where the percentages of β -gal-positive OPCs showed a significant decline between P2 and P8 ($T_{(6)}=6.59$, $P=0.012$) and another significant decline between P8 and P15 ($T_{(6)}=9.92$, $P=0.0012$). Similarly, the percent of β -gal-positive DCs declined significantly in the P6–P8 induction group as compared to the P0–P2 group ($T_{(6)}=10.76$, $P=4.8\times 10^{-4}$) but also declined significantly in the P13–P15 group as compared to the P6–P8 group ($T_{(6)}=34.93$, $P=4.8\times 10^{-7}$). Accordingly, the P13–P15 groups were also significantly lower than the P0–P2 groups for both OPCs ($T_{(6)}=21.28$, $P=1.2\times 10^{-5}$) and DCs ($T_{(6)}=43.40$, $P=1.2\times 10^{-7}$). These differential patterns of expression across the SC subtypes at each of the various induction ages suggest either that Sox10 is differentially expressed in SC subtypes or that the different SC subtypes respond differentially to Dox. This latter hypothesis could be due to either differential absorption of the Dox itself, or due to differences in the ability of Dox to access the Tet-On-LacZ transgene which may be inserted into a region that is differentially repressed across the SC types by DNA compaction, or the presence of histones or other DNA binding proteins. Together, these data suggest that Sox10^{rtTA/+} promotes robust and widespread expression of the Tet-On-lacZ reporter in the inner ear at early postnatal ages, when reporter expression is higher in the apical as compared to basal turns of the cochlea. However, an important finding is that effectiveness of Sox10^{rtTA/+} wanes and can vary across cochlear SC subtypes as the age of induction is increased.

We hypothesized that this age-dependent decline could be due to a decreased ability of Dox to cross the BLB as it matures and becomes more restrictive with age, rather than a result of the loss of Sox10 promoter activity. In support of this, immunostaining for Sox10 as late as P58 (Fig. 6) reveals readily detectable Sox10 protein expression, suggesting a maintenance of transcriptional activity. Furthermore, it has been previously shown that tetracycline is unable to cross the BLB in mature cochleae when delivered systemically, suggesting that the tetracycline derivative, Dox, may be similarly impermeant (Stupp et al. 1973). Moreover, the decline with age of Sox10^{rtTA/+} activity appears to be most dramatic at or after P14, correlating with the time at which the BLB and the

endolymphatic potential (EP) are established in rodent cochleae (Ehret 1976; Shnerson and Pujol 1981; Suzuki et al. 1998; Dai et al. 2006). Together, these data suggest that the decrease in Sox10^{rtTA/+}-driven reporter activity at later ages may be due to failure of Dox to cross the BLB. To directly test this hypothesis, we induced Sox10^{rtTA/+};Tet-On-LacZ⁺ mice between P27 and P29 with Dox but also added an injection of the loop diuretic furosemide, which, along with other loop diuretics, is well known to facilitate the passage of aminoglycoside antibiotics and other drugs across the BLB (Tran Ba Huy et al. 1983; Yamane et al. 1988; Ding et al. 2003; Higashiyama et al. 2003; Oesterle et al. 2008). Indeed, Sox10^{rtTA/+};Tet-On-LacZ⁺-driven β -gal expression can be largely restored in cochlear SCs by co-administration of furosemide with Dox (Fig. 7). However, this treatment resulted in a significant loss of cochlear HCs that was most prominent in the basal turn, but affected cell numbers in the apical half of the cochlea as well (Fig. 7). While this result was surprising because we did not observe any HC loss at any of the previous induction ages, nor after the administration of Dox alone between P27 and P29, previous reports do suggest that tetracycline, and minocycline, a derivative of tetracycline, can have ototoxic effects if they are provided in a manner or dose that allows entry into the cochlear duct (Stupp et al. 1973; Tachibana et al. 1973; Joy 1979). Despite the loss of cochlear HCs following this treatment, however, we did not observe any obvious loss of HCs in the utricles (which had an average of 237.57 ± 5.05 HCs per $10,000\ \mu\text{m}^2$), nor in the other balance organs (not counted) when examined at P29 following the Dox and furosemide treatment. However, we cannot rule out that HC loss might have occurred in the balance organs if the mice were allowed to survive for longer periods following the treatment. Together, these data suggest that the decline in Sox10^{rtTA/+} reporter expression on or after P29 is due, at least in part, to the inability of Dox to cross the BLB and that co-administration of furosemide with Dox treatment can improve rtTA-driven gene expression in adult inner ears, but at the expense of cochlear HC death.

Sox10^{rtTA/+}-Driven Gene Expression in the Inner Ear Is Persistent

Despite the declining ability of Sox10^{rtTA/+} to turn on Tet-On-LacZ reporter at later ages of induction, we found that Tet-On reporter expression following P0–P2 induction was surprisingly persistent. Several reports from other tissues tout transient GOI activation as one of the primary benefits of Tet-On systems as compared to other methods (Gossen et al. 1995; Mansuy et al. 1998; Knott et al. 2002; Mallo 2006; Liu

FIG. 7. Co-administration of furosemide rescues Dox-induced, Sox10^{rtTA/+}-driven reporter activity in the adult mouse cochlea. **A** A representative image from the middle turn of a P29 cochlea shows that β-gal reporter expression (green) is undetectable in any Sox2-positive SCs (magenta) or any other cells in the cochlear ducts of Sox10^{rtTA/+};Tet-On-LacZ^{+/ve} mice following *ad libitum* Dox food (2 g/kg) provided from P27 to P29 and a single i.p. injection of Dox (100 mg/kg of body weight) given at P28. **B, C** Adding a single i.p. injection of furosemide (Furos, 400 mg/kg of body weight) 30 min after the Dox injection at P28 rescues the β-gal reporter expression (green) in the Sox2-positive SCs (magenta) and other nonsensory cells in the apical (**B**) and basal (**C**) turns of the cochlea. **D** Parvalbumin (Parv) positive cochlear HC (white) appear normal in morphology and number in the Sox10^{rtTA/+};Tet-On-LacZ^{+/ve} mice when given Dox only from P27 to P29. **E, F** However, when Dox and furosemide are co-administered, some disorganization and HC loss are observed in the apical turns of the Sox10^{rtTA/+};Tet-On-LacZ^{+/ve} cochleae

(**E**), and the majority of outer HCs are lost in the basal turns (**F**). **G** Dox treatment alone (dark gray) between P27 and P29 did not result in any reporter-positive cells in the Sox10^{rtTA/+};Tet-On-LacZ^{+/ve} mice at P29 (N.D. = none detected). However, co-treatment with furosemide (striped bars) resulted in 15.01 ± 4.03 % of the Sox2-positive SCs in the apical turns being β-gal positive, which was significantly less (***P* < 0.01) than the 42.00 ± 5.25 % of the Sox2-positive SCs in the basal turns that were β-gal positive. **H** Combined Dox and furosemide treatment (Dox+Furos, striped bars) did not result in a significant loss of inner HCs (IHCs) in either the apical or the basal turns of the cochleae when compared to Dox only treatment (dark gray bars). **I** Combined Dox and furosemide treatment (striped bars) did, however, cause a significant loss of outer HCs (OHCs) in both the apical (**P* < 0.05) and the basal (****P* < 0.0001) cochlear turns, where the loss of these cells appeared to be more pronounced in the basal turns. Scale bars = 20 μm; error bars represent ± 1 S.E.M.

et al. 2011; Fan et al. 2012). However, under the current neonatal food plus injection paradigm (at P0–P2), β-gal protein was still detectable by immunofluorescence at P56 in Sox10^{rtTA/+};Tet-On-lacZ^{+/ve} mice, suggesting a persistence for β-gal protein of nearly 8 weeks from the end of Dox administration (Fig. 8). Since the half-life of β-gal mRNA in mammalian cells is approximately 5 min (Dickson et al. 1981), and the half-life of the β-gal protein is between 24 and 48 h

(Gonda et al. 1989; Smith et al. 1995), these results demonstrate that rtTA-driven gene expression may be particularly persistent in the inner ear and therefore may not offer the degree of transient GOI expression that is seen in other tissues. Indeed, this is consistent with a similar report of a transgenic Atoh1-rtTA mouse model that demonstrates persistent expression of both Tet-On-LacZ and Tet-On-mCherry reporters which are detectable in hair cells of the cochlea and

FIG. 8. β -gal reporter expression persists in the inner ears of Sox10^{rtTA/+};Tet-On-LacZ^{+ve} mice for nearly 8 weeks following neonatal Dox induction. **A** Numerous β -gal-positive cells (green) are still present at P7 in the cochlear ducts of Sox10^{rtTA/+};Tet-On-LacZ^{+ve} mice that were injected with Dox (100 mg/kg body weight) at P1 and housed in cages provided with Dox food (2 g/kg) between P0 and P2. Similarly, β -gal-positive cells are readily detectable in the cochlear ducts of Sox10^{rtTA/+};Tet-On-LacZ^{+ve} mice at P21 (**B**) and at P56 (**C**) following Dox inductions at P0–P2. **D** Additionally, we tested for the persistence of Tet-On-mCherry expression in Sox10^{rtTA/+} mice by inducing with a single i.p. injection (100 mg/kg) at P1 and aging the mice to P42. Despite the fact that this induction paradigm does not result in reporter-positive supporting cells in the organ of Corti (except in the apical hook), it does induce mCherry expression (red) in non-neuronal cells in the modiolar region that continue to express mCherry at P42. Parvalbumin staining (white) was used to label the HCs (lower right) and the type I neurons (upper left).

utricle for at least 6 weeks after cessation of Dox treatment (Cox et al. 2014a, b). Combined, the data from both the Sox10^{rtTA/+} and Atoh1-rtTA mouse models, and both the Tet-On-LacZ and Tet-On-mCherry reporters, suggest that Tet-On transgene expression can be remarkably persistent in the tissues of the inner ear and that the use of such Tet-On systems for transient expression should be considered cautiously and validated carefully.

DISCUSSION

Here we provide a detailed characterization of the Sox10^{rtTA/+} mouse line and its ability to induce Tet-On genes in the embryonic, postnatal, and adult inner ear. Initial characterization of Sox10^{rtTA/+} heterozygous mice revealed no gross morphological or audiological phenotypes, suggesting that this line may be useful for analysis of expression and hearing function following induction of Tet-On genes in vivo. However, it is important to note that phenotypes similar to those seen in Waardenburg syndrome have been noted in similarly designed Sox10^{LacZ/+} mice (Britsch et al. 2001), and the penetrance of such phenotypes in other Sox10 mouse models is known to depend upon background strain (Southard-Smith et al. 1999a, b; Cantrell et al. 2004). Therefore, while no hearing or balance phenotypes have been reported in any Sox10 haploinsufficient mouse lines to date,

we cannot rule out the possibility that breeding the Sox10^{rtTA/+} model onto another mouse background strain (e.g., C57Bl/6 J) (Cantrell et al. 2004) would result in inner ear or hearing loss phenotypes. We therefore suggest that inner ear researchers who plan to utilize this strain should backcross mice with the B6.SJL strain used here (i.e., Tet-On-LacZ), or, if using another strain, that they independently verify hearing or balance function prior to proceeding with genetic manipulations.

Using three independent reporter mouse lines, Tet-On-LacZ, Tet-On-mCherry, and Tet-On-Cre, we were able to show that Sox10^{rtTA/+}-driven reporter expression is widespread in the tissues of the inner ear. Specifically, Sox10^{rtTA/+}-driven reporter expression was largely ubiquitous in the tissues that comprise the membranous labyrinth during embryonic development, but was increasingly restricted and subsequently absent from HCs, tympanic border cells, and ganglia neurons following postnatal inductions. Additionally, we noted that reporter expression could be further restricted, so as to be significantly reduced or even absent from the cochlear SCs, using lower dose Dox inductions at embryonic and postnatal ages. Thus, this mouse line is a useful tool for manipulating gene expression within numerous cell types of the inner ear at various ages. Furthermore, its specificity can be manipulated to some extent by the dose and/or routes of administration of Dox.

One potential limitation of the use of rtTA/Tet-On-n systems in the inner ear is the potential inability of tetracycline or its derivatives to cross the BLB (Stupp et al. 1973). Indeed, the data presented here suggest that reporter activity in the cochlear duct declined with the age of Dox administration. This decline was significant when compared between neonatal inductions and inductions at P13–P15, a time when the BLB and EP are established (Ehret 1976; Shnerson and Pujol 1981; Suzuki et al. 1998; Dai et al. 2006). Similarly, reporter activity was absent from cochlear SCs following a P27–P29 Dox induction. However, co-administration of furosemide largely restored reporter expression in cochlear SCs, particularly in the basal turn. Since furosemide and other loop diuretics are well known to facilitate the transport of small molecules across the BLB (Tran Ba Huy et al. 1983; Yamane et al. 1988; Ding et al. 2003; Higashiyama et al. 2003; Oesterle et al. 2008), this data supports the hypothesis that Dox does not ordinarily cross the BLB and that either local delivery (Stupp et al. 1973) or the co-administration of a loop diuretic like furosemide may be required to induce Tet-On transgene expression in cells within the cochlear duct of adult mice. Still, the fact that Sox10 remains expressed in the adult inner ear (Watanabe et al. 2000) and the finding that co-treatment of Dox and

furosemide significantly improves Sox10^{rtTA/+}-driven reporter expression in adult inner ears suggest that the Sox10rtTA mouse model could be a useful tool for driving Tet-On gene expression in the adult inner ear sensory epithelia. It is important to note, however, that the combination of Dox and furosemide caused a large degree of cochlear HC loss. This is consistent with previous reports that suggest that tetracycline family antibiotics are ototoxic if they are able to cross the BLB and enter the perilymph (Stupp et al. 1973; Michel et al. 1984). This also suggests that Tet-On systems may not be very useful for studies of adult mouse cochleae if it is necessary to preserve auditory HCs and hearing. However, we did not observe any obvious loss of utricular HCs in this model, suggesting that this and other Tet-On models may still be useful for genetic manipulation in the adult balance organs. Additionally, it is possible that doses of Dox and/or furosemide may be titrated to achieve a level that is sufficient to induce transgene expression without being ototoxic; however, this will require further validation. Furthermore, newer, more sensitive versions of Tet-On transgenes can be developed to respond to lower doses of Dox (Fan et al. 2012), which may also potentially alleviate this limitation. Still, the Sox10rtTA model as presented, using Dox and furosemide at the doses described, can be useful for studies of gene function in adult cochlear SCs as well as in the adult utricular maculae where HC loss was not observed.

One of the most surprising findings reported here was that Sox10^{rtTA/+}-driven reporter expression persisted in the inner ear for at least 54 days following neonatal Dox treatment. This finding is surprising because one of the primary advantages in using the Tet-On system over other gene targeting approaches is that it should allow for the transient manipulation of transgene expression. Indeed, in other tissues, Tet-On reporter activity has been shown to dissipate within the first 2 to 3 weeks following withdrawal of Dox (Gossen et al. 1995; Mansuy et al. 1998; Knott et al. 2002; Mallo 2006; Liu et al. 2011; Fan et al. 2012). Similarly, the Sox10rtTA mouse line exhibits a significant downregulation of rtTA mRNA in the spinal cord within days following Dox withdrawal, although detectable levels of the transcript do persist for at least 12 days. Still, it is possible that Dox-mediated induction of Tet-On genes within the cochlear duct may not be as transient as what is seen in other tissues. For example, it has been shown that aminoglycoside antibiotics have a significantly longer half-life in the perilymph than in the circulation, heart, lung, or liver (Stupp et al. 1973). Thus, as the BLB becomes more restrictive, the cochlear duct may be trapping Dox and maintaining it at active concentrations for much longer periods of time. Indeed, a

similar persistence of Tet-On reporter expression was noted when using an Atoh1-rtTA transgenic mouse line in which both Tet-On-LacZ and Tet-On-mCherry expression persist for more than 6 weeks after Dox induction at P0–P3 (Cox et al. 2014b). Since other studies have shown the half-life for β -gal protein in the inner ear to be relatively short (Dickson et al. 1981; Gonda et al. 1989; Smith et al. 1995; Jan et al. 2013; Cox et al. 2014a), it is unlikely that β -gal protein itself is persisting in the absence of transcriptional activity. Rather, it is most likely that Dox is not cleared or inactivated from the inner ear as quickly as it is from the circulation, or from other tissues like the spinal cord. If this hypothesis is true, it may be possible to shorten the duration of Tet-On reporter expression in this or other rtTA models by altering the dose and duration of Dox administration. However, it is also important to note that our results with the Sox10^{rtTA/+} allele may differ from other rtTA and Tet-On mouse lines which may rely on different knock-in strategies, copy numbers, and types of promoters used. Also, mRNA stability, subcellular localization, and the half-life of the Tet-On protein being made could result in dramatically different patterns or duration of GOI expression. All of these factors suggest that individual use of the Sox10^{rtTA/+} allele and other rtTA mouse lines should be specifically validated for the Dox induction paradigm, tissue type, and Tet-On reporters to be used. Also, our finding of long-lasting Sox10^{rtTA/+}-driven reporter expression in the inner ear further implies that other inducible methods for presumed “transient” effects in the inner ear should be equally validated and the results be interpreted with caution.

Despite these concerns, however, the data here also demonstrate that Tet-On systems and the Sox10^{rtTA/+} model itself can still be very useful tools in the inner ear. Specifically, the Sox10^{rtTA/+} mice show robust reporter expression in many cell types of the inner ear when induced embryonically or neonatally, which allows for robust ectopic GOI expression at these time points. Also, reporter expression can be easily induced in the modiolus and non-neuronal cells of the vestibular ganglia and to a lesser extent in the SCs of the balance organs at adult ages, suggesting a utility for this model in adult ages as well. Furthermore, the combination of furosemide with Dox inductions allows for this model to be used to ectopically express GOIs in cochlear SCs, and while cochlear HC loss is an untoward side effect of the combined drug treatment, this model may still prove useful for studies where HCs and hearing function may not be necessary, or in studies aimed at promoting HC regeneration. Finally, the Sox10^{rtTA/+} and other rtTA models (Pan et al. 2010, 2013; Burger et al. 2011; Kelly et al. 2012; Cox et al. 2014b) hold great promise for the

study of development, function, and regeneration in the inner ear, all of which rely on the orchestration of multiple genes that need to be expressed or repressed in temporally and spatially controlled patterns. By combining the Sox10^{rtTA/+} or other rtTA models with existing knock-out, knock-in, or conditional Cre/loxP models, it will be possible to orchestrate such complex patterns of gene expression and recapitulate key developmental or mechanistic cellular events. In this way, we can broaden our understanding of such events and uncover the roles of genetic networks and synergistic interactions of key molecules in the development and function of the hearing and balance organs of the inner ear.

ACKNOWLEDGMENTS

The authors would like to thank Lingli Zhang and Mario Saucedo for assistance with genotyping and Dr. Brandon Cox for helpful comments in the preparation of this manuscript. This research was supported by funding from the National Institutes of Health (grants DC006471 (J.Z.), P30CA21765 (St. Jude)), American Lebanese Syrian Associated Charities (ALSAC) of St. Jude Children's Research Hospital, the Office of Naval Research (grants N000140911014, N000141210191, N000141210775 (J.Z.)), the National Organization for Hearing Research (NOHR) Foundation (B.W.), the Hearing Health Foundation (Emerging Research Grant, B.W.), and The Hartwell Foundation (Individual Biomedical Research Award, J.Z.).

Conflict of interest The authors declare that they have no conflict of interest.

REFERENCES

- BARCLAY M, RYAN AF, HOUSLEY GD (2011) Type I vs type II spiral ganglion neurons exhibit differential survival and neurogenesis during cochlear development. *Neural Dev* 6:33
- BERMINGHAM-McDONOGH O, OESTERLE EC, STONE JS, HUME CR, HUYNH HM, HAYASHI T (2006) Expression of Prox1 during mouse cochlear development. *J Comp Neurol* 496:172–186
- BONDURAND N, KULBRODT K, PINGAULT V, ENDERICH J, SAJUS M, TOMMERUP N, WARBURG M, HENNEKAM RC, READ AP, WEGNER M, GOOSSENS M (1999) A molecular analysis of the yemenite deaf-blind hypopigmentation syndrome: SOX10 dysfunction causes different neurocrisopathies. *Hum Mol Genet* 8:1785–1789
- BREUSKIN I, BODSON M, THELEN N, THIRY M, BORGS L, NGUYEN L, LEFEBVRE PP, MALGRANGE B (2009) Sox10 promotes the survival of cochlear progenitors during the establishment of the organ of Corti. *Dev Biol* 335:327–339
- BREUSKIN I, BODSON M, THELEN N, THIRY M, BORGS L, NGUYEN L, STOLT C, WEGNER M, LEFEBVRE PP, MALGRANGE B (2010) Glial but not neuronal development in the cochleo-vestibular ganglion requires Sox10. *J Neurochem* 114:1827–1839
- BRITSCH S, GOERICH DE, RIETHMACHER D, PEIRANO RI, ROSSNER M, NAVE KA, BIRCHMEIER C, WEGNER M (2001) The transcription factor Sox10 is a key regulator of peripheral glial development. *Genes Dev* 15:66–78
- BURGER A, KOESTERS R, SCHAFER BW, NIGGLI FK (2011) Generation of a novel rtTA transgenic mouse to induce time-controlled, tissue-specific alterations in Pax2-expressing cells. *Genesis* 49:797–802
- CANTRELL VA, OWENS SE, CHANDLER RL, AIREY DC, BRADLEY KM, SMITH JR, SOUTHARD-SMITH EM (2004) Interactions between Sox10 and EdnrB modulate penetrance and severity of aganglionosis in the Sox10Dom mouse model of Hirschsprung disease. *Hum Mol Genet* 13:2289–2301
- CHAI R, XIA A, WANG T, JAN TA, HAYASHI T, BIRMINGHAM-McDONOGH O, CHENG AG (2011) Dynamic expression of Lgr5, a Wnt target gene, in the developing and mature mouse cochlea. *J Assoc Res Otolaryngol* 12:455–469
- CHIBA H, CHAMON P, METZGER D (2000) F9 embryonal carcinoma cells engineered for tamoxifen-dependent Cre-mediated site-directed mutagenesis and doxycycline-inducible gene expression. *Exp Cell Res* 260:334–339
- COX BC, CHAI R, LENOIR A, LIU Z, ZHANG L, NGUYEN DH, CHALASANI K, STEIGELMAN KA, FANG J, CHENG AG, ZUO J (2014A) Spontaneous hair cell regeneration in the neonatal mouse cochlea in vivo. *Development* 141:816–829
- COX BC, DEARMAN JA, BRANCHECK J, ZINDY F, ROUSSEL MF, ZUO J (2014B) Generation of Atoh1-rtTA transgenic mice: a tool for inducible gene expression in hair cells of the inner ear. *Sci Rep* 4:6885
- DAI CF, MANGIARDI D, COTANCHE DA, STEYGER PS (2006) Uptake of fluorescent gentamicin by vertebrate sensory cells in vivo. *Hear Res* 213:64–78
- DANIELIAN PS, MUCCINO D, ROWITCH DH, MICHAEL SK, McMAHON AP (1998) Modification of gene activity in mouse embryos in utero by a tamoxifen-inducible form of Cre recombinase. *Curr Biol* 8:1323–1326
- DEMEMES D, EYBALIN M, RENARD N (1993) Cellular distribution of parvalbumin immunoreactivity in the peripheral vestibular system of three rodents. *Cell Tissue Res* 274:487–492
- DICKSON RC, SHEETZ RM, LACY LR (1981) Genetic regulation: yeast mutants constitutive for beta-galactosidase activity have an increased level of beta-galactosidase messenger ribonucleic acid. *Mol Cell Biol* 1:1048–1056
- DING D, McFADDEN SL, BROWNE RW, SALVI RJ (2003) Late dosing with ethacrynic acid can reduce gentamicin concentration in perilymph and protect cochlear hair cells. *Hear Res* 185:90–96
- EHRET G (1976) Development of absolute auditory thresholds in the house mouse (*Mus musculus*). *J Am Audiol Soc* 1:179–184
- EYBALIN M, RIPOLL C (1990) Immunolocalization of parvalbumin in two glutamatergic cell types of the guinea pig cochlea: inner hair cells and spiral ganglion neurons. *C R Acad Sci III* 310:639–644
- FAN X, PETIT M, GAMBOA M, HUANG M, DHAL S, DRUZIN ML, WU JC, CHEN-TSAI Y, NAYAK NR (2012) Transient, inducible, placenta-specific gene expression in mice. *Endocrinology* 153:5637–5644
- FEIL S, VALTCHEVA N, FEIL R (2009) Inducible Cre mice. *Methods Mol Biol* 530:343–363
- FURTH PA, ST ONGE L, BOGER H, GRUSS P, GOSSEN M, KISTNER A, BUJARD H, HENNIGHAUSEN L (1994) Temporal control of gene expression in transgenic mice by a tetracycline-responsive promoter. *Proc Natl Acad Sci U S A* 91:9302–9306
- GONDA DK, BAGHMAIR A, WUNNING I, TOBIAS JW, LANE WS, VARSHAVSKY A (1989) Universality and structure of the N-end rule. *J Biol Chem* 264:16700–16712
- GOSSEN M, FREUNDLIEB S, BENDER G, MULLER G, HILLEN W, BUJARD H (1995) Transcriptional activation by tetracyclines in mammalian cells. *Science* 268:1766–1769
- HIGASHIYAMA K, TAKEUCHI S, AZUMA H, SAWADA S, YAMAKAWA K, KAKIGI A, TAKEIDA T (2003) Bumetanide-induced enlargement of the intercellular space in the stria vascularis critically depends on Na⁺ transport. *Hear Res* 186:1–9
- HOSODA H, MIYAO T, UCHIDA S, SAKAI S, KIDA S (2011) Development of a tightly-regulated tetracycline-dependent transcriptional

- activator and repressor co-expression system for the strong induction of transgene expression. *Cytotechnology* 63:211–216
- JAN TA, CHAI R, SAYYID ZN, VAN AMERONGEN R, XIA A, WANG T, SINKKONEN ST, ZENG YA, LEVIN JR, HELLER S, NUSSE R, CHENG AG (2013) Tympanic border cells are Wnt-responsive and can act as progenitors for postnatal mouse cochlear cells. *Development* 140:1196–1206
- JELENA B, CHRISTINA L, ERIC V, FABIOLA QR (2014) Phenotypic variability in Waardenburg syndrome resulting from a 22q12.3-q13.1 microdeletion involving SOX10. *Am J Med Genet A* 164A:1512–1519
- JAY VA (1979) Minocycline and ototoxicity. *N Engl J Med* 301:1450–1451
- KELLENDONK C, TRONCHE F, CASANOVA E, ANLAG K, OPHERK C, SCHUTZ G (1999) Inducible site-specific recombination in the brain. *J Mol Biol* 285:175–182
- KELLY MC, CHANG Q, PAN A, LIN X, CHEN P (2012) Atoh1 directs the formation of sensory mosaics and induces cell proliferation in the postnatal mammalian cochlea in vivo. *J Neurosci* 32:6699–6710
- KISTNER A, GOSSEN M, ZIMMERMANN F, JERECIC J, ULLMER C, LUBBERT H, BUJARD H (1996) Doxycycline-mediated quantitative and tissue-specific control of gene expression in transgenic mice. *Proc Natl Acad Sci U S A* 93:10933–10938
- KNOTT A, GARKE K, URLINGER S, GUTHMANN J, MULLER Y, THELLMANN M, HILLEN W (2002) Tetracycline-dependent gene regulation: combinations of transregulators yield a variety of expression windows. *Biotechniques* 32:796, 798, 800 passim
- LIU W, DAVIS RL (2014) Calretinin and calbindin distribution patterns specify subpopulations of type I and type II spiral ganglion neurons in postnatal murine cochlea. *J Comp Neurol* 522:2299–2318
- LIU P, FU X, JOHNSON RL (2011) Efficient in vivo doxycycline and cre recombinase-mediated inducible transgene activation in the murine trabecular meshwork. *Invest Ophthalmol Vis Sci* 52:969–974
- LIU Z, LIU Z, WALTERS BJ, OWEN T, KOPAN R, ZUO J (2013) In vivo visualization of Notch1 proteolysis reveals the heterogeneity of Notch1 signaling activity in the mouse cochlea. *PLoS One* 8, e64903
- LUDWIG A, SCHLIERF B, SCHARDT A, NAVE KA, WEGNER M (2004) Sox10-rtTA mouse line for tetracycline-inducible expression of transgenes in neural crest cells and oligodendrocytes. *Genesis* 40:171–175
- MAISON SF, PARKER LL, YOUNG L, ADELMAN JP, ZUO J, LIBERMAN MC (2007) Overexpression of SK2 channels enhances efferent suppression of cochlear responses without enhancing noise resistance. *J Neurophysiol* 97:2930–2936
- MALLO M (2006) Controlled gene activation and inactivation in the mouse. *Front Biosci* 11:313–327
- MANSUY IM, WINDER DG, MOALLEM TM, OSMAN M, MAYFORD M, HAWKINS RD, KANDEL ER (1998) Inducible and reversible gene expression with the rtTA system for the study of memory. *Neuron* 21:257–265
- MICHEL G, MOSSER J, OLLE J (1984) Pharmacokinetics and tissue localization of doxycycline polyphosphate and doxycycline hydrochloride in the rat. *Eur J Drug Metab Pharmacokinet* 9:149–153
- MOTRICH RD, PONCE AA, RIVERO VE (2007) Effect of tamoxifen treatment on the semen quality and fertility of the male rat. *Fertil Steril* 88:452–461
- OESTERLE EC, CAMPBELL S, TAYLOR RR, FORGE A, HUME CR (2008) Sox2 and JAGGED1 expression in normal and drug-damaged adult mouse inner ear. *J Assoc Res Otolaryngol* 9:65–89
- PAN W, JIN Y, STANGER B, KIERNAN AE (2010) Notch signaling is required for the generation of hair cells and supporting cells in the mammalian inner ear. *Proc Natl Acad Sci U S A* 107:15798–15803
- PAN W, JIN Y, CHEN J, ROTTIER RJ, STEEL KP, KIERNAN AE (2013) Ectopic expression of activated notch or SOX2 reveals similar and unique roles in the development of the sensory cell progenitors in the mammalian inner ear. *J Neurosci* 33:16146–16157
- PARATORE C, EICHENBERGER C, SUTER U, SOMMER L (2002) Sox10 haploinsufficiency affects maintenance of progenitor cells in a mouse model of Hirschsprung disease. *Hum Mol Genet* 11:3075–3085
- PINGAULT V, BONDURAND N, KUHLEBRODT K, GOERICH DE, PREHU MO, PULITI A, HERBARTH B, HERMANS-BORGMEYER I, LEGIUS E, MATTHIJS G, AMIEL J, LYONNET S, CECCHERINI I, ROMEO G, SMITH JC, READ AP, WEGNER M, GOOSSENS M (1998) SOX10 mutations in patients with Waardenburg-Hirschsprung disease. *Nat Genet* 18:171–173
- PINGAULT V, BODEREAU V, BARAL V, MARCOS S, WATANABE Y, CHAOUI A, FOUVEAUT C, LEROY C, VERIER-MINE O, FRANCANNET C, DUPIN-DEGUINE D, ARCHAMBEAUD F, KURTZ FJ, YOUNG J, BERTHERAT J, MARLIN S, GOOSSENS M, HARDELIN JP, DODE C, BONDURAND N (2013) Loss-of-function mutations in SOX10 cause Kallmann syndrome with deafness. *Am J Hum Genet* 92:707–724
- PINGAULT V, PIERRE-LOUIS L, CHAOUI A, VERLOES A, SARRAZIN E, BRANDBERG G, BONDURAND N, ULDAHL P, MANOUVRIER-HANU S (2014) Phenotypic similarities and differences in patients with a p.Met112Ile mutation in SOX10. *Am J Med Genet A* 164A:2344–2350
- ROBINSON GL, ROBINSON JP, LASTWIKA KJ, HOLMEN SL, VANBROCKLIN MW (2013) Akt signaling accelerates tumor recurrence following ras inhibition in the context of ink4a/arf loss. *Genes Cancer* 4:476–485
- SHNERSON A, PUJOL R (1981) Age-related changes in the C57BL/6J mouse cochlea. I. Physiological findings. *Brain Res* 254:65–75
- SMITH RL, GELLER AI, ESCUDERO KW, WILCOX CL (1995) Long-term expression in sensory neurons in tissue culture from herpes simplex virus type 1 (HSV-1) promoters in an HSV-1-derived vector. *J Virol* 69:4593–4599
- SON EJ, WU L, YOON H, KIM S, CHOI JY, BOK J (2012) Developmental gene expression profiling along the tonotopic axis of the mouse cochlea. *PLoS One* 7, e40735
- SOUTHARD-SMITH EM, ANGRIST M, ELLISON JS, AGARWALA R, BAXEVANIS AD, CHAKRAVARTI A, PAVAN WJ (1999A) The Sox10(Dom) mouse: modeling the genetic variation of Waardenburg-Shah (WS4) syndrome. *Genome Res* 9:215–225
- SOUTHARD-SMITH EM, COLLINS JE, ELLISON JS, SMITH KJ, BAXEVANIS AD, TOUCHMAN JW, GREEN ED, DUNHAM I, PAVAN WJ (1999B) Comparative analyses of the Dominant megacolon-SOX10 genomic interval in mouse and human. *Mamm Genome* 10:744–749
- STUPP H, KUPPER K, LAGLER F, SOUS H, QUANTE M (1973) Inner ear concentrations and ototoxicity of different antibiotics in local and systemic application. *Audiology* 12:350–363
- SUN Y, CHEN X, XIAO D (2007) Tetracycline-inducible expression systems: new strategies and practices in the transgenic mouse modeling. *Acta Biochim Biophys Sin (Shanghai)* 39:235–246
- SUZUKI M, YAMASOBA T, KAGA K (1998) Development of the blood-labyrinth barrier in the rat. *Hear Res* 116:107–112
- TACHIBANA M, MACHINO M, TOYODA Y, SUZUKI M (1973) Morphologic effects of minocycline on the cochlea of the guinea pig. *Arch Klin Exp Ohren Nasen Kehlkopfheilkd* 204:163–174
- TRAN BA HUY P, MANUEL C, MEULEMANS A, STERKERS O, WASSEF M, AMIEL C (1983) Ethacrynic acid facilitates gentamicin entry into endolymph of the rat. *Hear Res* 11:191–202
- WAKAOKA T, MOTOHASHI T, HAYASHI H, KUZE B, AOKI M, MIZUTA K, KUNISADA T, ITO Y (2013) Tracing Sox10-expressing cells elucidates the dynamic development of the mouse inner ear. *Hear Res* 302:17–25
- WALTERS BJ, ZUO J (2013) Postnatal development, maturation and aging in the mouse cochlea and their effects on hair cell regeneration. *Hear Res* 297:68–83

- WATANABE K, TAKEDA K, KATORI Y, IKEDA K, OSHIMA T, YASUMOTO K, SATO H, TAKASAKA T, SHIBAHARA S (2000) Expression of the Sox10 gene during mouse inner ear development. *Brain Res Mol Brain Res* 84:141–145
- WATANABE Y, BRODERS-BONDON F, BARAL V, PAUL-GILLOTEAUX P, PINGAULT V, DUFOUR S, BONDURAND N (2013) Sox10 and Itgb1 interaction in enteric neural crest cell migration. *Dev Biol* 379:92–106
- WUNDERLICH FT, WILDNER H, RAJEWSKY K, EDENHOFFER F (2001) New variants of inducible Cre recombinase: a novel mutant of Cre-PR fusion protein exhibits enhanced sensitivity and an expanded range of inducibility. *Nucleic Acids Res* 29:E47
- YAMANE H, NAKAI Y, KONISHI K (1988) Furosemide-induced alteration of drug pathway to cochlea. *Acta Otolaryngol Suppl* 447:28–35
- ZHANG H, CHEN H, LUO H, AN J, SUN L, MEI L, HE C, JIANG L, JIANG W, XIA K, LI JD, FENG Y (2012) Functional analysis of Waardenburg syndrome-associated PAX3 and SOX10 mutations: report of a dominant-negative SOX10 mutation in Waardenburg syndrome type II. *Hum Genet* 131:491–503