Knockdown of Foxg1 in supporting cells increases the trans-differentiation of supporting cells into hair cells in the neonatal mouse cochlea

Shasha Zhang1 · Yuan Zhang1 · Ying Dong1 · Lingna Guo1 · Zhong Zhang1 · Buwei Shao1 · Jieyu Qi1 · Han Zhou5 · Weijie Zhu1 · Xiaqian Yan1 · Guodong Hong1 · Liyan Zhang5 · Xiaoli Zhang5 · Mingliang Tang1 · Chunjie Zhao1 · Xia Gao5 · Renjie Chai1,2,3,4,5,6

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Abstract
Foxg1 is one of the forkhead box genes that are involved in morphogenesis, cell fate determination, and proliferation, and Foxg1 was previously reported to be required for morphogenesis of the mammalian inner ear. However, Foxg1 knock-out mice die at birth, and thus the role of Foxg1 in regulating hair cell (HC) regeneration after birth remains unclear. Here we used Sox2CreER/+ Foxg1loxP/loxP mice and Lgr5-EGFPcreER/+ Foxg1loxP/loxP mice to conditionally knock down Foxg1 specifically in Sox2+ SCs and Lgr5+ progenitors, respectively, in neonatal mice. We found that Foxg1 conditional knockdown (cKD) in Sox2+ SCs and Lgr5+ progenitors at postnatal day (P)1 both led to large numbers of extra HCs, especially extra inner HCs (IHCs) at P7, and these extra IHCs with normal hair bundles and synapses could survive at least to P30. The EdU assay failed to detect any EdU+ SCs, while the SC number was significantly decreased in Foxg1 cKD mice, and lineage tracing data showed that much more tdTomato+ HCs originated from Sox2+ SCs in Foxg1 cKD mice compared to the control mice. Moreover, the sphere-forming assay showed that Foxg1 cKD in Lgr5+ progenitors did not significantly change their sphere-forming ability. All these results suggest that Foxg1 cKD promotes HC regeneration and leads to large numbers of extra HCs probably by inducing direct trans-differentiation of SCs and progenitors to HCs. Real-time qPCR showed that cell cycle and Notch signaling pathways were significantly down-regulated in Foxg1 cKD mice cochlear SCs. Together, this study provides new evidence for the role of Foxg1 in regulating HC regeneration from SCs and progenitors in the neonatal mouse cochlea.

Keywords Foxg1 · Hair cells · Supporting cells · Progenitors · Proliferation · Trans-differentiation
**Introduction**

The loss of hair cells (HCs) is the main cause of sensorineural hearing loss, which is one of the most common health problems around the world. HC loss is irreversible in adult mammals, whereas HCs can be regenerated from supporting cells (SCs) in the inner ear of birds and fish [1]. Recent studies have shown that in newborn mice, HCs can also be regenerated from SCs, especially from a subset of Lgr5+ progenitor cells [2–8]. However, this regenerative ability is quickly lost as the mice age [2–4, 9, 10].

Recent studies have shown that several signaling pathways play important roles in HC regeneration by inducing the proliferation and differentiation of SCs and Lgr5+ progenitors. The up-regulation of canonical Wnt signaling induces the proliferation of sensory precursors in the postnatal mouse cochlea [3, 4, 11–17], while Notch inhibition induces mitotic generation of HCs in the mammalian cochlea via activation of the Wnt pathway [12, 13]. Inhibition induces mitotic generation of HCs in the postnatal mouse cochlea [3, 4, 11–17], while Notch signaling induces the proliferation of sensory precursors in progenitors. The up-regulation of canonical Wnt signaling plays important roles in HC regeneration by inducing the proliferation and differentiation of SCs and Lgr5+ progenitors. Our data suggest that Foxg1 cKD in cochlear SCs and progenitors probably promote the direct trans-differentiation of SCs and Lgr5+ progenitors into HCs, but it does not significantly change the proliferation ability of SCs and Lgr5+ progenitors in neonatal mouse cochlea.

**Materials and methods**

**Animals**

Lgr5-EGFP-IRES-CreERT2 mice (Stock #008875, Jackson Laboratory) [4, 70, 71], Sox2-CreER mice (Stock #017593, Jackson Laboratory) [14], and Rosa26-tdTomato reporter mice (Stock #007914, Jackson Laboratory) [4, 72] of both sexes were used in the experiments. The mouse breeding strategy is shown in Fig. S1. The Foxg1-floxp mice were a gift from Prof. Chunjie Zhao from Southeast University [24]. Sox9-ires-CreER mice were a gift from Prof. Fengchao Wang from the National Institute of Biological Sciences (NIBS), Beijing [73]. We performed all animal procedures according to protocols that were approved by the Animal Care and Use Committee of Southeast University and that were consistent with the National Institute of Health’s Guide for the Care and Use of Laboratory Animals. We made all efforts to minimize the number of animals used and to prevent their suffering.

**Genotyping PCR**

Transgenic mice were genotyped using genomic DNA, which was extracted from mice tail tips by adding 180 µl...
50 mM NaOH, incubating at 98 °C for 1 h, and then adding 20 μl 1 M Tris–HCl pH 7.0. The genotyping primers were used as follows: Lgr5: (F) CTG CTC TCT GCT CCC AGT CT; wild type (R) 5’-ATA CCC CAT CCC TTT TGA GC-3’; mutant (R) 5’-GAA CTT CAG GTG CTT CG-3’. tdTomato: wild type (F) 5’-AGG GAG GTA GCA GTG GAG T-3’; wild type (R) 5’-CCG AAA ATC TGT GGG AAG TC-3’; mutant (F) 5’-GCC ATT AAA GCA GGC TAT C-3’; mutant (R) 5’-CTG TTC CTG TCT GGC ATG-3’. Sox2: wild type (F) 5’-CTA GGC CAC AGA ATT GAA AGA TCT-3’; wild type (R) 5’-GTA GGG GGA AAT TCTA GCA TCA TCC-3’; mutant (F) 5’-GCG TGC TGG CAA AAA CTA TC-3’; mutant (R) 5’-GTG AAA CAG CAT TGC TGT CAC TT-3’. Foxg1: wild type (F) 5’-ATA GAG ATT TGC TGA GTT GGA-3’; mutant (F) 5’-GCA TCG CAT TGT CTT AGG TG-3’; mutant (R) 5’-TTA GGC CAC AGA ATT GAA AGA TCT-3’; Sox9: (F) 5’-GCC TGC ATT ACC GGT TAG GCC TGC ATT-3’. The extracted genomic DNA and primers were used in the following PCR system to genotype the mice: genomic DNA 3 μl, primer mix 2 μl, 2 × PCR mix (Thermo) 10 μl, and H2O were added to a total volume of 20 μl. PCR conditions were an initial denaturing step of 3 min at 95 °C followed by 38 cycles of 30 s denaturation at 95 °C, 30 s annealing at 60 °C, and 30 s extension at 72 °C.

**In vivo cKD of Foxg1 in Sox2+ SCs, Sox9+ SCs, and Lgr5+ progenitors in the mouse cochlea**

Sox2<sup>CreER/</sup>+/Foxg1<sup>loxp/loxp</sup> mice, Sox9<sup>CreER/</sup>+/Foxg1<sup>loxp/loxp</sup> mice, and Lgr5<sup>-EGFPCreER/</sup>+/Foxg1<sup>loxp/loxp</sup> mice were bred to conditionally knockdown Foxg1 in Sox2+ SCs, Sox9+ SCs, and Lgr5+ progenitors, respectively. To activate the Cre enzyme, postnatal day (P)1 or P3 mice were injected with tamoxifen (Sigma) intraperitoneally (I.P.) (1.5 mg/25 g Cre enzyme, postnatal day (P)1 or P3 mice were injected subcutaneously (SCs, and Lgr5+ progenitors, respectively. To activate the Cre enzyme, Sox2<sup>CreER/+</sup> Foxg1<sup>loxp/loxp</sup> Rosa26-tdTomato mice to get Sox2<sup>CreER/+</sup> Foxg1<sup>loxp/loxp</sup> Rosa26-tdTomato triple-positive mice and to lineage trace Sox2+ SCs in the cochlea. To activate the Cre enzyme, Sox2<sup>CreER/+</sup> Foxg1<sup>loxp/loxp</sup> Rosa26-tdTomato triple-positive mice were I.P. injected with tamoxifen at P3. Mice were sacrificed at P9, and the cochleae were examined. Sox2<sup>CreER/+</sup> Rosa26-tdTomato mice were used as controls, and were injected with the same dose of tamoxifen.

**Immunostaining and image acquisition**

For neonatal mice (P0–P7), the cochleae were dissected with sharp forceps (WPI) in cold HBSS and then fixed in 4% paraformaldehyde for 1 h at room temperature (RT). For mice older than P7, cochleae were fixed in 4% paraformaldehyde for 1 h, decalcified with 0.5 M EDTA for 1–3 days (depending on the age of the mice), both at RT, and then dissected in HBSS. The cochleae were washed with PBS and then blocked with blocking solution (5% donkey serum, 0.5% Triton X100, 0.02% sodium azide, and 1% bovine serum albumin in pH 7.4 PBS) for 1 h at RT. The cochleae were then incubated with primary antibodies diluted in PBT1 (2.5% donkey serum, 0.1% Triton X100, 0.02% sodium azide, and 1% bovine serum albumin in pH 7.4 PBS) for 4 h at RT. The cochleae were then incubated with secondary antibodies conjugated secondary antibody (Invitrogen) or phalloidin (Invitrogen), both diluted 1:400 in PBT2 (0.1% Triton X100 and 1% bovine serum albumin in pH 7.4 PBS), for 1 h at RT. After another three times of washing, the cochleae were mounted in antifade fluorescence mounting medium (DAKO). The primary antibodies used were anti-Myosin7a (myo7a; Proteus Bioscience, #25-6790; DSHB, #138-1; both 1:1000 dilution in PBT1), anti-Sox2 (Santa Cruz Biotechnology, #17320, 1:400 dilution in PBT1), anti-Foxg1 (Abcam, #ab18259, 1:400 dilution in PBT1), anti-Ctbp2 (BD Biosciences, #612044, 1:400 dilution in PBT1), anti-PSD95 (Millipore, #MAB1596, 1:400 dilution in PBT1), and anti-Tuj1 (Neuromics, # MO15013, 1:400 dilution in PBT1). The Click-it EdU imaging kit (Invitrogen) was used after blocking to label proliferating cells. For FM1-43 staining, the cochleae were dissected, incubated with 4 µM FM1-43 (Thermo) at RT for 30 s, and then washed with PBS.

**Auditory brainstem response (ABR) test**

P30 mice were I.P. injected with 0.01 g/ml pentobarbital sodium (100 mg/kg body weight) to achieve deep anesthesia, and a TDT System III workstation running SigGen32 software (Tucker-Davis Technologies) was used to test mice for closed-field ABR thresholds as previously described [75]. The ABR test was performed in a soundproof room, and three fine needle electrodes were inserted in the mice at the cranial vertex, underneath the tested ear, and at the back near the tail. ABR tone pips of 4 kHz, 8 kHz, 12 kHz, 16 kHz, 24 kHz, and 32 kHz were generated. Auditory thresholds were determined by decreasing the sound intensities from 90 dB in 10 dB steps until the lowest sound intensity at which the first wave could be recognized. The ABR data were analyzed using GraphPad Prism 6 software.

**In vivo lineage tracing of Sox2+ SCs in the cochlea**

Sox2<sup>CreER/+</sup> Foxg1<sup>loxp/loxp</sup> Rosa26-tdTomato mice to get Sox2<sup>CreER/+</sup> Foxg1<sup>loxp/loxp</sup> Rosa26-tdTomato triple-positive mice and to lineage trace Sox2+ SCs in the cochlea. To activate the Cre enzyme, Sox2<sup>CreER/+</sup> Foxg1<sup>loxp/loxp</sup> Rosa26-tdTomato triple-positive mice were I.P. injected with tamoxifen at P3. Mice were sacrificed at P9, and the cochleae were examined. Sox2<sup>CreER/+</sup> Rosa26-tdTomato mice were used as controls, and were injected with the same dose of tamoxifen.
**Fig. 1** Foxg1 cKD in Sox2+SCs results in increased HC number and decreased SC number. 

*a* Tamoxifen was I.P. injected into P1 Sox2CreER/+ Foxg1loxPloxP Rosa26-tdTomato mice to knock down Foxg1 in Sox2+ SCs, and the mice were sacrificed at P3 for FAC sorting of Sox2+ SCs for real-time qPCR. 

*b* FAC sorting data for Sox2+ SCs. c Quantification of Foxg1 mRNA expression based on four independent qPCR experiments. 

***p < 0.001. d Tamoxifen was I.P. injected into P1 Sox2CreER/+ Foxg1loxPloxP mice to knockdown Foxg1 in Sox2+SCs, and the mice were sacrificed at P7. e-i Extra IHCs (arrows) and OHCs (square brackets) are seen in the apical (Apex), middle (Middle), and basal (Base) turns of P7 Sox2CreER/+ Foxg1loxPloxP mice cochlea (e). Statistical analysis of the extra IHCs is shown in (f). The HC layer (g) and SC layer (i) are also shown. 3D reconstruction of extra HCs (white and yellow arrows) is shown in (h). Scale bar, 20 µm. Sox2 CreER/+ and Foxg1 loxp/loxp mice were used as controls. Myo7a and Sox2 were used as HC and SC markers, respectively. (j, k) Quantification of the total IHCs, total OHCs, total SCs (j), and different kinds of SCs (k) per 100 µm cochlea length. The n refers to the number of mice. *p < 0.05, **p < 0.01, ***p < 0.001. DC Deiter’s cell. OPC outer pillar cell. IPC inner pillar cell. IPhC inner phalangeal cell. IBC inner border cell

TUNEL Kit (Roche) was used to detect apoptotic cells in cochlea of P7 Foxg1 cKD mice and control mice according to the manufacturer’s instructions. For image acquisition, all images were scanned with a Zeiss microscope (LSM 710) with the same hardware settings, including laser intensity, gain, etc., to enable a direct comparison between treatment conditions. Because SCs are not always in the same layer, we performed Z projection with ImageJ software to catch all of the SCs for some of the SC layer images, including Figs. 1i and 4b. Also, because the nucleus of extra inner HCs (IHCs) were not in the same layer with the nucleus of regular IHCs as shown in the cross-section image in Figs. 1g, 3c, 6c and e, Fig. S5C and Fig. S7E, we performed Z projection with ImageJ software to catch the Ctbp2 and PSD95 staining images in Fig. 6c and Fig. S6A. The other images were all single confocal planes. To better show the location of the extra IHCs, we also performed 3D reconstruction with the Zeiss software in Figs. 1h and Fig. 6c.

**Data quantification**

For most of the data quantification, such as total IHC number, total outer HC (OHC) number and total SC number, we randomly took one or two 20× or 40× low-magnification confocal images of the cochlea in each turn as representative images. The cochleae were all in the center of the image (320 µm or 160 µm cochlear length per image). We counted the number of total IHCs, OHCs or SCs in the image, averaged the results of two images for each turn and presented the data as per 100 µm. For SC quantification, we counted three rows of Deiter’s cells (DCs), inner pillar cells (IPCs) and outer pillar cells (OPCs). If the counting object was relatively rare and the randomly taken images would not represent the true counting result, such as EdU+ SC number and tdTomato+ HC number, we quantified the whole cochlea and present the data as per turn or per cochlea. For all experiments, the treatment conditions were blinded to the analyst. At least three mice were used for quantification, and only one ear of each mouse was analyzed.

**Sphere-forming assay**

Lgr5-EGFPCreER/+ Foxg1loxPloxP mice and Lgr5-EGFPCreER/+ control mice were I.P. injected with tamoxifen at P1 and sacrificed at P3. The cochleae were dissected at P3 and then digested with trypsin into single cells for FAC sorting of Lgr5+ cells. The sorted Lgr5+ cells from Foxg1 cKD mice and control mice were separately cultured at a density of 2 cells/µl in Costar ultra-low attachment dishes for 5 days in DMEM/F12 medium supplemented with N2 (1:100 dilution, Invitrogen), B27 (1:50 dilution, Invitrogen), heparin sulfate (50 ng/ml, Sigma), and the growth factors bFGF (10 ng/ml, Sigma), EGF (20 ng/ml, Sigma), and IGF-1 (50 ng/ml, Sigma). Spheres were then digested with trypsin into single cells and cultured in the same way for the next generation. Images of all the spheres in each well of each generation were taken with a Zeiss microscope (HAL 100) at the end of the culture, and the sphere numbers and diameters were quantified.

**Scanning electron microscopy (SEM)**

As previously described [45], the cochlea were dissected, postfixed in 0.5% OsO4, dehydrated in ethanol, dried, and then coated with gold. A scanning electron microscope (FEI Quanta 200) operating at 15 kV was used to take images of the hair bundles.

**RNA extraction and real-time qPCR**

Sox2CreER/+ Foxg1loxPloxP Rosa26-tdTomato mice and Sox2CreER/+ Rosa26-tdTomato mice were I.P. injected with tamoxifen at P1 and then sacrificed at P3. The cochleae were dissected and then digested with trypsin into single cells for FAC sorting of Sox2+ SCs. Approximately, 50,000 cochlear Sox2+ SCs from Foxg1 cKD mice and control mice were used to extract total RNA with the GenElute™ Single Cell RNA Purification Kit (Sigma, #RN3000). RNA was reverse transcribed into cDNA, and real-time quantitative polymerase chain reaction (real-time qPCR) was performed using the FastStart Universal SYBR Green Master (ROX) kit (Roche) on a Bio-Rad C1000 Touch thermal cycler to quantify the gene expression levels. Real-time qPCR conditions were an initial denaturing step of 15 s at 95°C followed by 40 cycles of 15 s denaturation at 95°C, 60 s annealing at 60°C, and 20 s extension at 72°C. Gapdh was used as the reference endogenous gene, and gene expression was quantified using
the ΔΔCT method as follows: after obtaining the CT value of the gene expression, we normalized these data to the Gapdh CT value (ΔΔCT) to eliminate the sample differences (e.g., the small differences in cell number and so on). We next normalized the data to the control group data (∆∆CT) to compare the group differences, after which we calculated the 2^−ΔΔCT value to quantify the fold difference between the control group and Foxg1 cKD group. The real-time qPCR primers are shown in Supplementary Table 1.

Statistical analysis

For each experimental condition, at least three independent experiments were performed, and the “n” in the figures refers to the number of mice, cell culture wells, or real-time qPCR experimental repetitions as illustrated in the figure legends. Data were analyzed using GraphPad Prism 6 software and are presented as means ± standard errors of the means. Two-tailed, unpaired Student’s t tests were used to determine statistical significance when comparing two groups, and two-way ANOVA followed by a Bonferroni post-test was used when comparing more than two groups. A value of p < 0.05 was considered statistically significant.

Results

Foxg1 cKD in neonatal mouse cochlear SCs led to significantly greater numbers of HCs and fewer SCs

Foxg1 plays important roles in brain and eye development, especially in neuron differentiation, and Foxg1 knock-out leads to inner ear malformation and multiple rows of HCs during embryonic development [44, 45], as well as the loss of clear OHC/IHC distinctions and reduced p75+ IPCs [29]. We speculated that Foxg1 might play an important role in HC regeneration; however, Foxg1 knock-out mice die at birth, and thus the role of Foxg1 in HC regeneration after birth remains unclear. To investigate the role of Foxg1 in SCs, tamoxifen was I.P. injected into P1 Sox2CreER/+ Foxg1loxP/loxP mice to induce the Cre enzyme activity and thus conditionally knock down Foxg1 in Sox2+ SCs (Fig. 1a and d). The Foxg1loxP/loxP mice and Sox2CreER/+ mice were used as controls. Foxg1 was successfully down-regulated in the cochlear SCs of Sox2CreER/+ Foxg1loxP/loxP mice (Fig. 1a–c and Fig. S2). P7 Foxg1 cKD mice were sacrificed to find numerous extra IHCs in the apical, middle, and basal turns, and four rows of OHCs were also found in the apical turns (Fig. 1e, g and h). Although extra IHCs could also be seen in the cochlea of Sox2CreER/+ control mice due to Sox2 haploinsufficiency as reported recently [76, 77], statistical analysis showed that there were significantly more extra IHCs in the Foxg1 cKD mice compared to the controls (Fig. 1f). We statistically analyzed the number of total HCs, OHCs, and SCs per 100 μm cochlea length, and found significantly more IHCs in the cochleae of Foxg1 cKD mice compared to Sox2CreER/+ control mice, and the number of extra IHCs decreased from the apical turns to the basal turns (Fig. 1f and j, Table S2). We also found four rows of OHCs in some parts of the apical turns of Foxg1 cKD mice cochleae, and the statistical analysis showed a significant increase of apical OHC number (Fig. 1f). As previously reported [29, 30], we also quantified the numbers of various cell types of SCs and found that the numbers of IPCs and OPCs were significantly decreased in the apical and middle turns of Foxg1 cKD mice cochleae, respectively (Fig. 1i, j and k), which suggest that the extra HCs might be generated by direct trans-differentiation of SCs.

To further verify the role of Foxg1 in SCs, we also bred Sox9CreER/+ Foxg1loxP/loxP mice to conditionally knock down Foxg1 in Sox9+ SCs. Similar to previous reports [78], we found that Sox9 is expressed in SCs of the cochlea (Fig. S3A). And Sox9CreER/+ Foxg1loxP/loxP mice cochleae showed more extra HCs in the apical turns compared to Sox9CreER/+ control mice (Fig. S3A–C, Table S4), which is consistent with the phenotype of Sox2CreER/+ Foxg1loxP/loxP mice. However, there were not significantly more extra HCs in the middle and basal turns of Sox9CreER/+ Foxg1loxP/loxP mouse cochleae compared to Sox9CreER/+ control mice, which might be due to the relative low Cre efficiency in Sox9-CreER mice.

To determine the initial Cre induction ratio and the initial tdTomato labeling of Sox2CreER/+ Rosa26-tdTomato mice, Sox9CreER/+ Rosa26-tdTomato mice, and Lgr5-EGFPcreER/+ Rosa26-tdTomato mice, we measured how many HCs were labeled by tdTomato when tamoxifen was injected at P1 or P3 and the mice were sacrificed 48 h later at P3 or P5, respectively, in which tdTomato only labeled the original Sox2+, Lgr5+ and Sox9+ cells at P1 or P3 but not the subsequently generated HCs. For Sox2CreER/+ Rosa26-tdTomato mice, at P3 only some of the HCs in the apex tip and part of the apex were labeled by tdTomato (Fig. S7A, the yellow bracket in ① and the white bracket in ②), and in the rest of the apical turns and all of the middle and basal turns. Only very few HCs were labeled by tdTomato (as indicated by white arrowheads). Fig. S7B shows the higher magnification of the apex tip, apex, middle, and base of the P3 cochlea. At P5, only a small number of HCs in the apex tip (the white bracketed region) was labeled by tdTomato, and we found very few tdTomato+ HCs (white arrows) in the apical, middle, and basal turns (Fig. S7C and D). Thus, only a small part of the apical HCs in Sox2CreER/+ Rosa26-tdTomato mice cochleae was originally labeled by tdTomato when tamoxifen was injected at P1 and P3. More importantly, in all the experiments we used Sox2CreER/+ Rosa26-tdTomato mice as the controls. Sox2CreER/+ Foxg1loxP/loxP Rosa26-tdTomato
mice were compared with Sox2^{CreER/+} Rosa26-tdTomato controls, and the originally labeled tdTomato+ HCs also appeared in the controls, thus the increased number of tdTomato+ HCs was only because of Foxg1 cKD in SCs. For Lgr5-EGFP^{CreER/+} and Sox9^{CreER/+} mice, we also injected tamoxifen at P1 and sacrificed the mice at P3. Lgr5-EGFP^{CreER/+} Rosa26-tdTomato results are shown in Fig. S7E, and Sox9^{CreER/+} Rosa26-tdTomato results are shown in Fig.S3A. We did not find any tdTomato+ HCs in either of these mice, and the tdTomato+ cells were restricted to Lgr5+ progenitors and Sox9+ SCs.

The extra IHCs survived at least to P30

It was previously reported that some of the newly regenerated HCs will progressively die [10]. Thus, we also analyzed the survival of the extra IHCs in Foxg1 cKD mice cochleae and found that in P7, P14, and P30 mice the extra IHCs still existed in the cKD cochlea (Fig. 2a–c). The statistical analysis showed that the number of extra IHCs was not significantly changed from P7 to P30 (Fig. 2d and e), which suggests that the extra IHCs could survive at least to P30. We also observed that P30 Foxg1 cKD mice were significantly smaller than the control mice (Fig. S4A and B). The ABR test showed that the low-frequency (4 kHz and 8 kHz) hearing thresholds of the Foxg1 cKD mice were significantly increased (Fig. S4C), which might due to the extra IHCs in the apical turns.

To verify whether the extra HCs can be generated at a later age, we injected mice with tamoxifen at P7 and sacrificed them at P14 and analyzed the HCs. We found very few extra HCs at P14 in both Sox2^{CreER/+} Foxg1^{lox/lox} mice and Sox2^{CreER/+} control mice, and there was no significant difference in HC number between the two groups (Fig. S5A and B). This is consistent with previous reports that SCs rapidly lose the ability to regenerate HCs after P7 [2–4, 9], and suggests that the majority of the extra HCs existing at P30 are mainly generated before P7.

**Foxg1 cKD in neonatal mouse cochlear Lgr5+ progenitors led to significantly more IHCs that could survive at least to P30**

To determine whether Foxg1 cKD in Lgr5+ progenitors also leads to extra HCs, we crossed Lgr5-EGFP-CreERT2 mice with Foxg1^{floxp} mice to generate Lgr5-EGFP^{CreERT2/+} Foxg1^{lox/lox} double-positive mice. To activate the Cre enzyme, tamoxifen was I.P. injected into P1 mice, and the cochleae were dissected at P7, P14, and P30 (Fig. 3a). Lgr5-EGFP^{CreERT2/+} mice and Foxg1^{lox/lox} mice were used as the controls. A significant number of extra IHCs were also found in P7 Lgr5-EGFP^{CreERT2/+} Foxg1^{lox/lox} double-positive mice compared to the control mice and the number of extra IHCs decreased from apical turns to basal turns (Fig. 3b–d). We also quantified the number of total IHCs, OHCs, and SCs per 100 μm cochlea length at P7 and found significantly more IHCs in the cochlea of Foxg1 cKD mice compared to the Lgr5^{CreERT2/+} control mice (Fig. 3d). However, the statistical analysis showed no significant increase of OHC number and no significant decrease of SC number (Fig. 3d, Table S3). Significant more extra IHCs were still found in both P14 and P30 Foxg1 cKD mice compared to the control mice (Fig. 3e–g). These results suggest that Foxg1 cKD in Lgr5+ progenitors also induces the generation of extra IHCs in neonatal mouse cochlea.

**Foxg1 cKD in neonatal mouse cochlear SCs and progenitors did not significantly change their proliferation ability in vivo and in vitro**

The generation of extra HCs might be the result of mitotic HC generation, direct trans-differentiation of SCs to HCs, or both. To determine the mechanism behind the generation of extra HCs in Foxg1 cKD mice, we I.P. injected tamoxifen to Sox2^{CreER/+} Foxg1^{lox/lox} double-negative mice at P1, and then I.P. injected EdU (50 mg/kg body weight) from P3 to P5 to mark proliferating cells (Fig. 4a). Mice were sacrificed at P7, and EdU was detected using the Click-it EdU imaging kit. However, we failed to detect any EdU+/Sox2+ SCs in any of the three Sox2^{CreER/+} Foxg1^{lox/lox} mice cochleae (Fig. 4b), indicating that the new HCs might not be generated by mitotic generation. We used both Sox2^{CreER/+} mice and Foxg1^{lox/lox} mice as the controls and treated it the same way. We did not find any EdU+ SCs in any of the three Foxg1^{lox/lox} mice or in two of the three Sox2^{CreER/+} mice. We only found a few EdU+ SCs in the third Sox2^{CreER/+} mouse. However, the statistical analysis showed no significant difference (Fig. 4b and c).

To verify whether Foxg1 cKD will lead to apoptosis in SCs, we performed a TUNEL assay to measure apoptosis of SCs in Foxg1 cKD mice cochleae. At P7, we did not detect any TUNEL+ cells among the IPCs, OPCs, or three rows of DCs in either Foxg1 cKD mice or Sox2^{CreER/+} control mice, while only a few TUNEL+ cells were found among the Hensen cells in both mice (Fig. S5C). The quantification results showed no significant difference in the number of TUNEL+ Hensen cells between Foxg1 cKD mice (8.5 ± 2.5 per 100 μm) and Sox2^{CreER/+} control mice (9.5 ± 1.5 per 100 μm). This suggests that the decrease of cochlear SC number in Foxg1 cKD mice probably is not caused by apoptosis of SCs.

To further evaluate the effect of Foxg1 in regulating the proliferation and sphere-forming ability of Lgr5+ progenitors, Lgr5-EGFP^{CreERT2/+} Foxg1^{lox/lox} mice and Lgr5-EGFP^{CreERT2/+} control mice were I.P. injected with tamoxifen at P1 and sacrificed at P3. The cochleae were dissected and then...
Fig. 2 The extra IHCs could survive to P30. a Tamoxifen was I.P. injected at P1, and the mice were sacrificed at P7, P14, and P30. b, c Extra IHCs (arrows) and OHCs (square brackets) are seen in the apical (Apex), middle (Middle), and basal (Base) turns of P7 Sox2CreER/+ Foxg1loxp/loxp mice cochleae. Sox2CreER/+ mice were used as controls. Myo7a was used as the HC marker. Scale bar, 50 µm. (d, e) Quantification of the total IHCs and OHCs per 100 µm cochlea length at P14 and P30 (d) and the comparison between the three ages in control and Foxg1 cKD mice (e). The n refers to the number of mice. *p<0.05, **p<0.01, ***p<0.001, n.s. not significant.
digested with trypsin for FAC sorting of Lgr5+ progenitors. The sorted Lgr5+ progenitors were then cultured in vitro to form spheres which were then passaged for three generations (Fig. 4d). The sphere-forming assays showed that Lgr5+ progenitors of Foxg1 cKD mice showed no significant differences in either sphere number or sphere diameter of all three generations compared to the control mice (Fig. 4d–f), suggesting that Foxg1 cKD in Lgr5+ progenitors does not significantly affect the proliferation and sphere-forming ability of Lgr5+ progenitors.

**The extra HCs in Foxg1 cKD mouse cochlea originated from Sox2+ SCs in the neonatal mouse cochlea**

Next, we crossed Sox2 CreER/+ Foxg1 loxp/loxp double-positive mice with Foxg1 loxp/loxp Rosa26-tdTomato mice to generate Sox2 CreER/+ Foxg1 loxp/loxp Rosa26-tdTomato triple-positive Foxg1 cKD mice to lineage trace Sox2+ SCs (Fig. 5a). Sox2 CreER/+ Rosa26-tdTomato mice were used as the controls. We found significantly more tdTomato+ IHCs and OHCs in the apical and middle turns of triple-positive mice cochleae than that of control mice cochleae (Fig. 5b–d). These results suggest that the extra HCs in Foxg1 cKD mice cochlea originate from Sox2+ SCs and Foxg1 cKD increase the HC regeneration and SC differentiation. Considering all the results we showed above, Foxg1 cKD promotes HC regeneration and leads to large numbers of extra HCs probably by mainly inducing direct trans-differentiation rather than mitotic HC generation.

**The extra IHCs had normal hair bundles, synapse number, FM1-43+ mechanotransduction (MET) channels, and innervation**

To confirm whether the newly formed extra HCs in Foxg1 cKD mice cochlea have normal HC characteristics, we investigated the hair bundles and the synapse number of the extra HCs. We used phalloidin to stain the hair bundles and found that the extra HCs had normal hair bundles (Fig. 6a). SEM also showed normal hair bundles of the extra HCs (Fig. 6b). Next, we used Ctbp2 to stain the synapses of the IHCs and found that the extra HCs also had normal synapse number, similar to the control IHCs (Fig. 6c and d). We also used FM1-43 to verify whether the extra HCs have functional MET channels, and found that the extra HCs were all FM1-43+, just like the normal IHCs (Fig. 6e), suggesting that the extra HCs also have the ability to uptake FM1-43 dye and have functional MET channels. Moreover, to directly show the innervation of the extra IHCs with spiral ganglion neurons, we used Ctbp2 and PSD95 to label the pre- and post-synapse, respectively, and found that normal IHCs and extra IHCs had similar numbers of innervated synapses (Fig. S6A and B). We also used Tuj1 to label the axons that innervate the IHCs and found that all the extra IHCs had neuronal axons branching to them (Fig. S6C). Together, these results suggest that the extra IHCs have normal IHC functions as we investigated.

**Characterization of gene expression changes in Foxg1 cKD mouse cochlear SCs by real-time qPCR**

To determine the mechanism through which Foxg1 is involved in HC regeneration, we used Sox2 CreER/+ Foxg1 loxp/loxp Rosa26-tdTomato triple-positive mice and Sox2 CreER/+ Rosa26-tdTomato control mice to isolate the tdTomato+/Sox2+ SCs by flow cytometry, and the mRNA was then extracted for real-time qPCR to quantify the related gene expression level (Fig. 1a and b). As expected, Foxg1 was down-regulated in Foxg1 cKD SCs (Fig. 1c). The mRNA expression of Atoh1 and Gfi1, two transcription factors that regulate HC generation, was up-regulated (Fig. 7a), which is consistent with our experimental results. However, the mRNA expression of the other important factors Pou4f3, Neurog1, and Sox2 did not change.

Considering that Foxg1 has been reported to regulate genes involved in the TGFβ, Notch, and Wnt signaling pathways as well as some cell cycle genes [35–43, 79], we analyzed these pathways by real-time qPCR. To determine the effect of Foxg1 cKD on proliferation-related signaling pathways, we quantified the mRNA expression levels of some important cell cycling genes and Wnt signaling genes. We found that the cell cycle repressors Cdkn1a, Cdkn1c, Cdkn2a, and Gadd45 were all up-regulated and cell cycle-dependent kinase Cdk2 was down-regulated in Foxg1 cKD SCs (Fig. 7b). The expression of most Wnt signaling pathway genes did not change significantly, while only β-catenin (Ctnnb1) and Gsk3β were down-regulated (Fig. 7c). Our results presented above showed that Foxg1 cKD might lead to extra HCs by promoting the direct trans-differentiation of SCs. Thus, we checked two cell differentiation-related pathways, the Notch and TGFβ signaling pathways. We found that many genes of the Notch signaling pathway, such as Notch 1-3, Hes1, Hes5, Jag2, and Hey1, were significantly down-regulated (Fig. 7d). The Notch-related transcription factors, Tle1 and Tle2, were also down-regulated (Fig. 7d). However, the expression of most TGFβ signaling pathway genes did not change significantly, while only Tgfb1 and Smad3 were down-regulated (Fig. 7e). All these results suggest that Foxg1 cKD in SCs probably leads to the generation of new HCs mainly through down-regulation of the cell cycle pathway and the Notch signaling pathway.
Discussion

It is known that a limited number of HCs can be regenerated in newborn mice from SCs and inner ear progenitor cells, and several studies have shown that many important signaling pathways are involved in HC regeneration, such as Wnt, Notch, and Shh [7, 8, 11–14, 80–84]. Many other genes and related pathways have also been shown to play important roles in HC regeneration [85, 86], and these pathways might have crosstalk with each other to affect the proliferation and differentiation of SCs and Lgr5+ progenitors [13, 14]. Foxg1, one of the FOX protein family members, plays important roles in brain, eye, and ear development [24, 35, 36, 44, 45, 55, 57, 60, 64, 66]. In the inner ear, previous studies showed that during embryonic development Foxg1 knock-out mice have shortened cochleae with multiple extra rows of HCs [44, 45]. However, Foxg1-null mice show hypoplasia of the telencephalon, abnormal eye and ear development, and die soon after birth [44, 45, 55], thus the role of Foxg1 in HC regeneration in the postnatal mouse cochlea is still unclear. In this study, we found that Foxg1 cKD in both Sox2+ SCs and Lgr5+ progenitors led to significant numbers of extra HCs, especially extra IHCs that could survive at least to P30. The extra IHCs had normal hair bundles and synapses. Moreover, Foxg1 cKD failed to induce the proliferation of SCs, and lineage tracing data showed that more tdTomato+ HCs originated from Sox2+ SCs in the cKD mouse cochlea, and thus the new extra HCs were most likely generated by direct trans-differentiation of SCs. Real-time qPCR data showed that cell cycle genes and the Notch signaling pathway might be involved in this process.

The role of Foxg1 has been characterized mainly in forebrain development [58–61], and the absence of Foxg1 leads to structural defects of both the dorsal and ventral telencephalon due to reduced proliferation and premature differentiation of neuroepithelial cells [55]. In cortical progenitor cells, Foxg1 promotes self-renewal of neural precursors and inhibits neuronal differentiation [55, 59, 62, 63]. The dynamic expression of Foxg1 during cortical development is essential for the proper assembly of the cerebral cortex, and Foxg1 is down-regulated when progenitors undergo neuronal differentiation and up-regulated when differentiating neurons integrate into the cortical plate [64]. In postnatal mice, Foxg1 also plays important roles in maintaining the hippocampal dentate gyrus progenitor pool, and the lack of Foxg1 promotes both gliogenesis and neurogenesis [24]. The results of these studies are consistent with our findings that Foxg1 cKD increased the differentiation of SCs and led to the generation of extra HCs. However, we did not observe any significant differences in sphere number or sphere diameter in Foxg1 cKD Lgr5+ progenitors, suggesting that Foxg1 might have no significant effects in regulating the proliferation of Lgr5+ progenitors in the postnatal mouse cochlea. In one of the three Sox2CreER/+ mice, we could find a few EdU+ SCs, while we could not find any EdU+ SCs in any of the three Sox2CreER/+ Foxg1lox/lox mouse, three Foxg1lox/lox mice or the other two Sox2CreER/+ mice. Though the statistical analysis showed no significant difference, we suspect that Foxg1 cKD might slightly decrease the proliferation of neonatal mouse cochlear SCs.

Pauley et al. reported the embryonic phenotype of the Foxg1-null mouse cochlea in which they showed that Foxg1-null mice have shortened cochleae and multiple rows of extra HCs and SCs [45]. Our results showing that Foxg1 cKD in both SCs and Lgr5+ progenitors results in significantly more HCs in neonatal mice cochleae which are consistent with their results in embryonic mice. They also suspected that Notch signaling might be involved in this process, and this hypothesis is supported by our results. However, they found multiple rows of SCs, while we found that Foxg1 cKD in SCs led to decreased numbers of SCs. This might be because Foxg1 plays different roles during different development stages.

One recent report showed that Sox2 haploinsufficiency (Sox2-CreER, Sox2-EGFP, in which one allele of the Sox2 gene is replaced by CreER or EGFP such that Sox2 is expressed at only half of the normal expression level) also increases the IHC number in vivo [76, 77]. Thus in our study, we also used Sox2CreER/+ mice as the control to avoid overestimating the effect of cKD of Foxg1. The statistical analysis showed that although Sox2CreER/+ mice also had some extra HCs, Sox2CreER/+ Foxg1lox/loxoxoxox mice had significantly more extra HCs than Sox2CreER/+ mice (Fig. 1e–g). Moreover, there were significantly more newly generated HCs (Myo7a+ and tdTomato+ cells) in Sox2CreER/+ Foxg1lox/loxoxoxox mouse than in Sox2CreER/+ Rosa26-ttdTomato mice (Fig. 5). To verify this finding, we used two other CreER lines—Lgr5-EGFPCreER/+ mice and Sox9CreER/+ mice. In one experiment, we used Lgr5-EGFP-CreER/+ mice as the control and found that Lgr5-EGFP-CreER/+ Foxg1lox/loxoxoxox mice had many more extra IHCs in the apical...
and middle turns compared with Lgr5-EGFP CreER/+ mice (Fig. 3b–d). In the other experiment, we used Sox9CreER/+ Foxg1loxp/loxp mice to further verify the effects of Foxg1 in SCs, and we found that these mice also had many more extra HCs in the apical turns compared with Sox9CreER/+ control mice (Fig. S3B and C). These results all suggest that cKD of Foxg1 in SCs leads to the extra HCs. However, when we quantified the SC number in Lgr5-EGFP CreER/+ Foxg1loxp/loxp mice and Sox9CreER/+ Foxg1loxp/loxp mice, we found that the average number of apical SCs was smaller in Lgr5-EGFP CreER/+ Foxg1loxp/loxp mice than that in Lgr5-EGFP CreER/+ control mice (70.78 ± 2.76 and 71.46 ± 1.55 per 100 μm, Fig. 4)

**Fig. 4** The proliferation of Sox2+ SCs and Lgr5+ progenitors has no change in Foxg1 cKD mice. **a** EdU (50 mg/kg body weight) was injected at P3, P4, and P5 to label proliferating cells. **b** EdU was stained (blue) in Sox2CreER/+ Foxg1loxp/loxp, Foxg1loxp/loxp, and Sox2CreER/+ mice. Myo7a and Sox2 were used as HC and SC markers, respectively. Scale bar, 20 μm. **c** Quantification of EdU+ SCs per cochlea. n = 3 mice per group. n.s. not significant. **d** Tamoxifen was injected into Lgr5-EGFP CreER/+ Foxg1loxp/loxp mice to conditionally knockdown Foxg1 in Lgr5+ progenitors. After 2 days, Lgr5+ progenitors were isolated by FAC sorting and cultured in vitro for 5 days to form spheres. **e** Spheres formed by Lgr5+ progenitors from Lgr5-EGFP CreER/+ Foxg1loxp/loxp and Lgr5-EGFP CreER/+ mice. Scale bar, 50 μm. **f** Quantification of sphere number per well and sphere diameter of each passage. At least three wells of spheres were quantified. n.s. not significant.
Knockdown of Foxg1 in supporting cells increases the trans-differentiation of supporting... respectively, Table S3), and the average number of apical SCs was smaller in Sox9CreER/+ Foxg1loxp/loxp mice than that in Sox9CreER/+ control mice (71.13 ± 1.02 and 71.67 ± 2.66 per 100 µm, respectively, Table S4), but these differences were not statistically significant. This might because the Cre efficiency of Lgr5-EGFPCreER/+ and Sox9CreER/+ is not as high as Sox2CreER/+ mouse, which was demonstrated by the greater number of extra HCs in Sox2CreER/+ Foxg1loxp/loxp mice than that in Lgr5-EGFPCreER/+ Foxg1loxp/loxp and Sox9CreER/+ Foxg1loxp/loxp mice. Thus, the decreased SC numbers of Lgr5-EGFPCreER/+ Foxg1loxp/loxp and Sox9CreER/+ Foxg1loxp/loxp mice were also much lower than that of Sox2CreER/+ Foxg1loxp/loxp mice, and the decreased SC number of Lgr5-EGFPCreER/+ Foxg1loxp/loxp mice and Sox9CreER/+ Foxg1loxp/loxp mice was too few to result in the total SC number significantly decreased in Lgr5-EGFPCreER/+ Foxg1loxp/loxp and Sox9CreER/+ Foxg1loxp/loxp mice.

During embryonic development, Foxg1 plays important roles in neurogenesis through crosstalk with many other signaling pathways that also regulate neuronal progenitor...
proliferation and neuronal differentiation [38, 39, 42, 43, 87]. Foxg1 represses TGFβ-induced neuronal differentiation and associates with the FoxO/Smad complex to regulate cell cycle progression in early developmental stages [40, 42, 43]. Foxg1 is also involved in the regulation of progenitor cell differentiation in the telencephalon by interacting with the Notch signaling pathway factors Hes1 and Groucho/TLE [39]. Foxg1 coordinates the activity of the Shh pathway and Wnt/β-catenin pathway by acting as a downstream effector of the Shh pathway and as a direct transcriptional repressor of Wnt ligands [37]. Foxg1 was also reported to suppress the Wnt/β-catenin pathway to restrict tissue development [35, 36] and to directly repress the cell cycle repressor Cdkn1a [40, 42, 79]. In addition, altered cellular interactions change the detailed mosaic pattern of the organ of Corti, which was recently demonstrated in a model of Atoh1 replacement with Neurog1 [31].

Because the TGFβ, Notch, and Wnt pathways and some cell cycle repressors were reported to have a crosstalk with Foxg1, we analyzed these pathways by real-time qPCR. We found that the most obvious expression changes were among genes in the Notch pathway and genes of cell cycle repressors, while the expression of most genes in the TGFβ and Wnt pathways was not significantly altered by cKD of

**Fig. 6** Hair bundle, synapse, and FM1-43 staining of the extra IHCs. 

- **A** Phalloidin was used to stain the hair bundles of the HCs. Extra IHCs are indicated by arrows. Scale bar, 20 µm.
- **B** Hair bundles of the extra IHCs by SEM. Scale bar, 5 µm.
- **C** Ctbp2 was used to stain synapses (dotted staining) of IHCs. Each IHC and its Ctbp2+ synapses are indicated by dotted white circles. Extra IHCs (white arrows) and normal IHCs (yellow arrows) are shown in both confocal images and 3D reconstructions.
- **D** Quantification of the synapse number of IHCs. n=5 mice per group. n.s. not significant.
- **E** FM1-43 dye was up taken by extra IHCs (white arrows). Scale bar, 5 µm.
Knockdown of Foxg1 in supporting cells increases the trans-differentiation of supporting cells. Many previous studies have suggested that Notch is a very important pathway involved in HC regeneration [5, 8, 12–14, 19, 24, 74, 84, 88], and down-regulation of the Notch signaling pathway in the Foxg1 cKD SCs might be one of the important mechanisms leading to the phenotype of extra HCs. Hes1, Hes5, and Hey1 are three of the important Notch downstream transcription factors, and knock-out of Hes1, Hes5, and Hey1 in the inner ear also results in extra HCs [89–92], which is consistent with our results, and thus down-regulation of Hes1, Hes5, and Hey1 by Foxg1 cKD might contribute to the phenotype of extra HCs. Also, Hes and Hey were reported to regulate HC differentiation by regulating the Atoh1 promoter [93, 94], which is also consistent with Atoh1 up-regulation in Foxg1 cKD mice cochleae (Fig. 7a). One recent work demonstrated that replacement of one allele of Atoh1 by Neurog1 combined with a self-terminating second Atoh1 allele rescued most IHCs and some OHCs as compared with the massive loss of IHCs in the Atoh1-Cre; Atoh1<sup>fl/fl</sup> mouse [31]. However, we did not find any expression changes of Neurog1 in Foxg1 cKD SCs (Fig. 7a), which suggests that the phenotype of Foxg1 cKD in SCs might not involve Neurog1. The lateral inhibition of Notch receptors plays important roles in inner ear development and HC regeneration [21, 23–25], and three of the Notch receptors, Notch1–3, were down-regulated in Foxg1 cKD SCs. TLEs are involved in the gene regulatory functions of a variety of signaling pathways, including Notch and Wnt signaling [22, 95]. Groucho/TLE1 inhibits neuron differentiation [96], and Foxg1 is involved in the regulation of progenitor cell differentiation in the telencephalon by interacting with Groucho/TLE and Hes [39, 79, 97]. Tle1 and Tle2 were both down-regulated by Foxg1 cKD (Fig. 7d), which suggests that TLEs might play important roles in HC regeneration. Jag2, one of the Notch ligands, was also down-regulated by Foxg1 cKD, and null mutation of the Jag2 gene was reported to cause supernumerary HC differentiation in the cochlea [98, 99], which is consistent with our results.
with our results. The cell cycle repressor Cdkn1a, which is a downstream target of Foxg1, was up-regulated in Foxg1 cKD SCs. The other cell cycle repressors Cdkn1c, Cdkn2a, and Gadd45g were also up-regulated, while cell cycle-dependent kinase Cdk2 was down-regulated. These results suggest that cell cycle pathway is repressed to some extent in Foxg1 cKD SCs. However, we did not observe any significant decrease of the proliferative ability of Foxg1 cKD SCs or Lgr5+ progenitors (Fig. 4), which might be due to the overall combined effects of other genes.

In summary, we specifically knocked down Foxg1 in Sox2+ SCs and Lgr5+ progenitors of neonatal mice cochlea and found that this resulted in significantly more HCs. Because we found reduced numbers of SCs and no obviously proliferating SCs, and because we lineage traced more tdTomato+ HCs after cKD of Foxg1, we hypothesize that Foxg1 cKD probably leads to the generation of extra HCs through direct trans-differentiation of SCs and progenitors into HCs. In addition, the real-time qPCR results showed that some cell cycle repressors were up-regulated, while genes involved in the Notch signaling pathway were significantly down-regulated in Foxg1 cKD SCs, which might contribute to the generation of extra HCs in Foxg1 cKD mice cochlea.

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Author contributions SZ and RC conceived and designed the experiments. SZ, YZ, LD, LG, ZZ, BS, JQ, HZ, WZ, XY, GH, and LZ performed the experiments. SZ, XZ, MT, CZ, XG, and RC analyzed the data. SZ and RC wrote the paper. All authors read and approved the final manuscript.

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Compliance with ethical standards

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

Ethical approval We performed all animal procedures according to protocols that were approved by the Animal Care and Use Committee of Southeast University and that were consistent with the National Institute of Health’s Guide for the Care and Use of Laboratory Animals. We made all efforts to minimize the number of animals used and to prevent their suffering.

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