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Supplemental Information

Lgr5-Positive Supporting Cells Generate New Hair Cells in the Postnatal Cochlea

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Inventory of Supplemental Information

Figure S1 shows Lgr5-Cre, Sox2-Cre, and Pou4f3-Cre lineage tagged ears (relates to Figure 1).

Figure S2 shows gentamicin damage in the cochlea (relates to Figure 1)

Table S1 shows quantification of reporter in Lgr5-Cre and Sox2-Cre mice (relates to Figure 1).

Supplemental Figures and Table

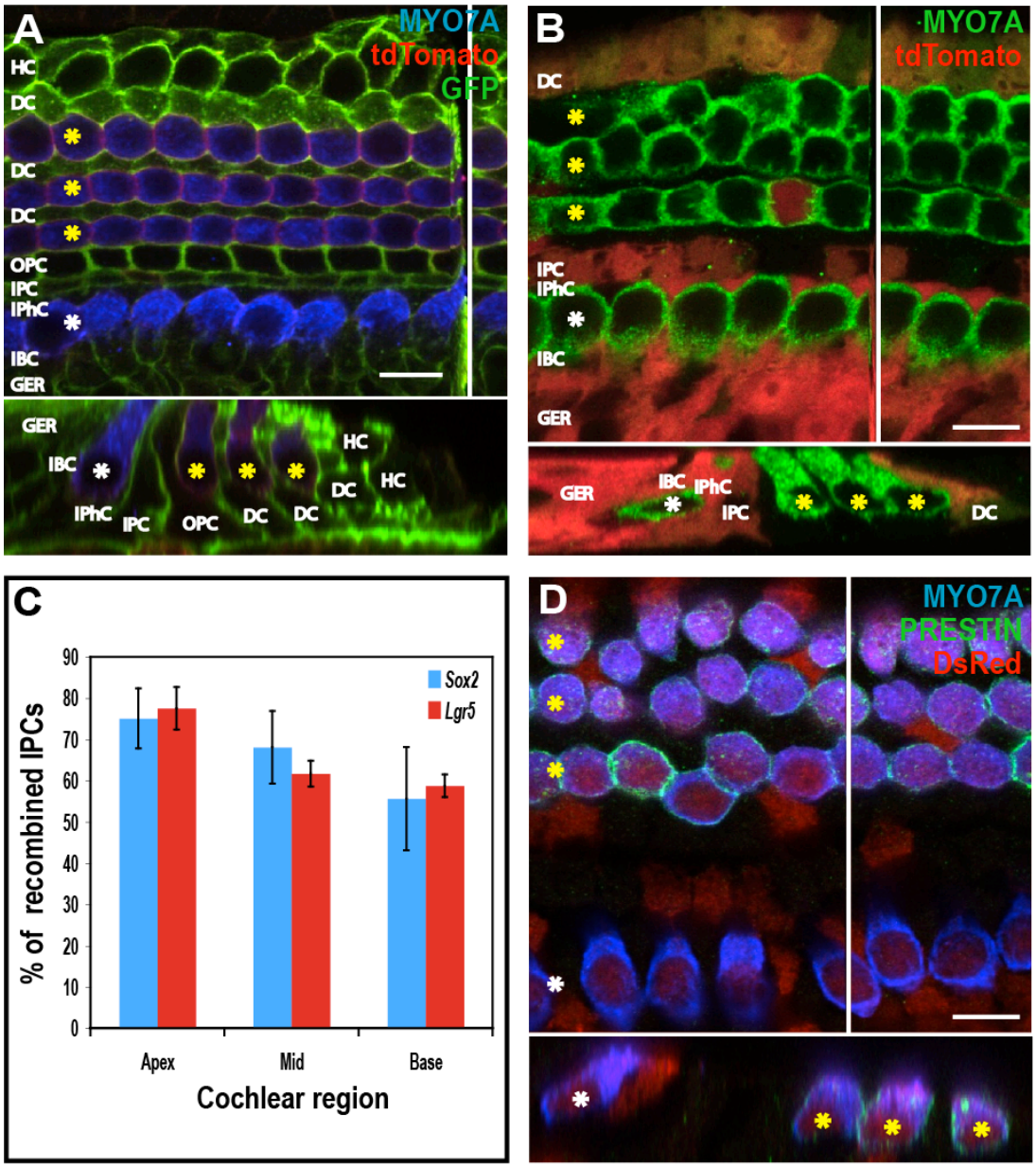

Figure S1. Reporter expression in *Sox2*, *Lgr5* and *Pou4f3* lineage tracing, related to Figure 1.

(A-B, D) In confocal slices and cross-sections from neonatal organ of Corti, OHCs are labeled with yellow asterisks and IHCs with white asterisks. A white vertical line indicates the location of the cross-section. The scale bar is 10 μm.

(A) The apex of a *Sox2-Cre-ER*/*CAG-tdTomato-EGFP* organ of Corti treated with tamoxifen at P1 shows reporter expression (stained for GFP, in green) in the Hensen's cell, Deiters' cells, inner and outer pillar cells, inner phalangeal cell, inner border cell, and the GER. **(B)** The apex of an *Lgr5-EGFP-IRES-Cre-ER*/*CAG-tdTomato* organ of Corti treated with tamoxifen at P0 and P1 exhibits reporter expression in the 3rd Deiters' cell, inner pillar cell, inner phalangeal cell, inner border cell, and the GER. **(C)** No significant difference in the mean percentage of inner pillar cells expressing the reporter in organ of Corti control cultures was identified between the *Sox2* (10 cultures from 2 experiments) and *Lgr5* (10 cultures from 2 experiments) lineage tracing markers. Error bars indicate SEM. **(D)** In an organ of Corti from a *Pou4f3-Cre*/*CAG-tdTomato* mouse, all IHCs and OHCs were reporter-positive (stained with a DsRed antibody to enhance the tdTomato signal). Some supporting cells were also reporter-positive.

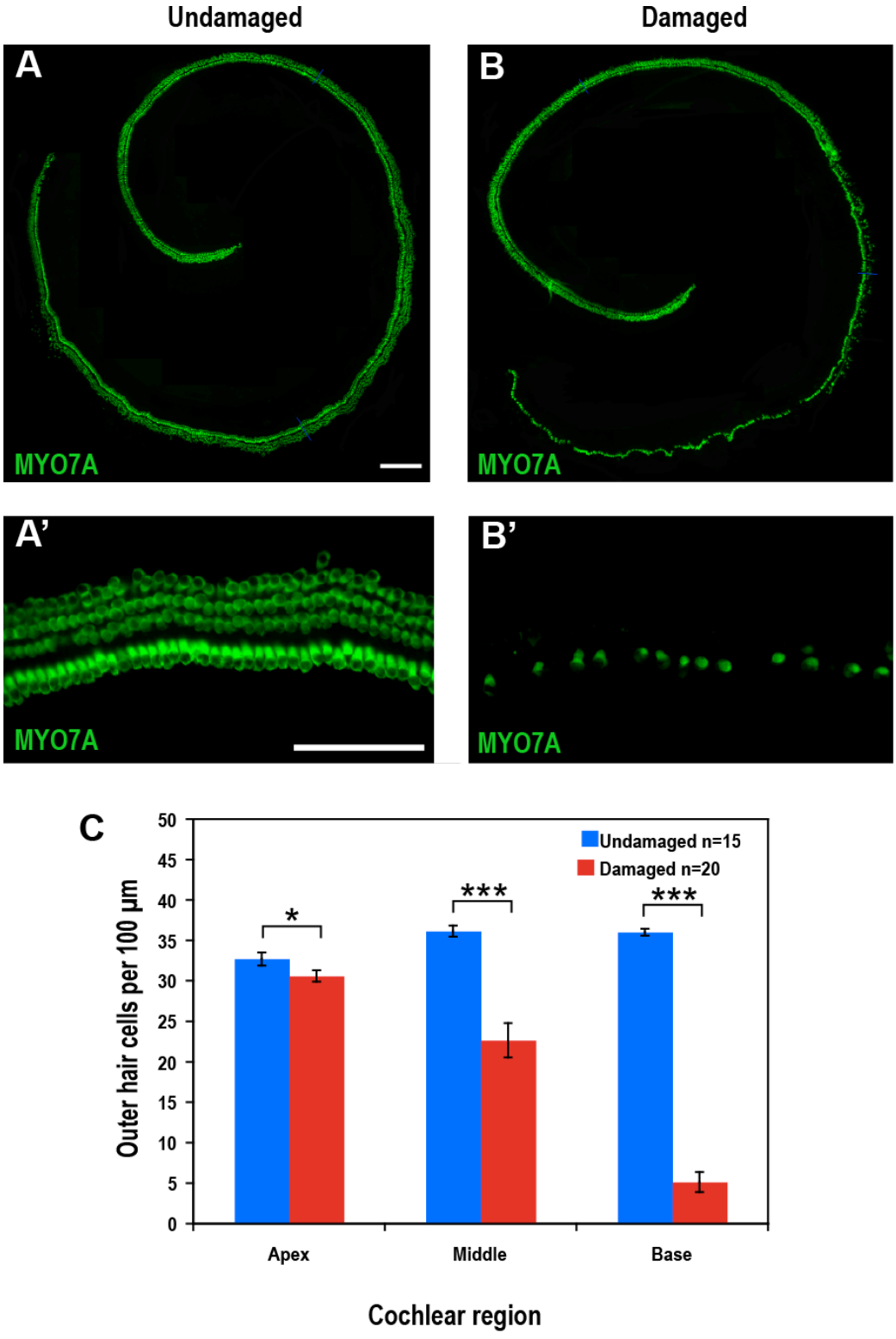

Figure S2. Gentamicin treatment causes hair cell damage, related to Figure 1.
(A-B) Neonatal organ of Corti explant cultures were stained for MYO7A to identify hair cells. The scale bar is 200 µm. Undamaged control (A) and gentamicin-damaged (B)

explants are shown. **(A'-B')** High power views of the organs of Corti from A and B are shown. The scale bar is 100 μm. **(C)** Quantification of mean OHC number per 100 μm (± SEM) for undamaged and damaged cultures shows that hair cells are significantly decreased following gentamicin treatment for all 3 cochlear regions. * $p < 0.05$, *** $p < 0.001$.

	***Sox2* (n=5)**	***Lgr5* (n=7)**
	Reporter+ OHCs	**Reporter+ OHCs**
Apex	54.47% ± 4.99%	19.80% ± 4%
Middle	2.43% ± 1.43%	1.95% ± 1.3%
Base	0.14% ± 0.06%	0.22% ± 0.18%

Table S1. Reporter expression in organ of Corti cultures, related to Figure 1.
Sox2 and *Lgr5* lineage-tagged organs of Corti showed reporter expression in some IHCs and OHCs, with the greatest frequency in the apex. The table shows the mean percentage of OHCs expressing the reporter in each cochlear region (± SEM). Reporter-positive hair cells arise from immature hair cells that express *Sox2* or *Lgr5* at the time of tamoxifen administration (P0 or P0 and P1).

Supplemental Data

Neonatal expression of *Sox2*. The apical region of an organ of Corti from a P6 *Sox2-Cre-ER/CAG-tdTomato-EGFP* mouse pup administered tamoxifen at P1 through its mother's milk showed expression of GFP in Deiters' cells, inner and outer pillar cells, inner border cells, inner phalangeal cells, Hensen's cells and the GER (Figure S1A). This pattern of recombination is similar to the SOX2 expression previously described (Oesterle et al., 2008). The mean *Sox2-Cre* recombination rate in the inner pillar cells was highest in the apex and lowest in the base (red bars in Figure S1C). *Sox2-Cre* recombination rates appeared relatively consistent across supporting cell types. Reporter expression was also noted in some IHCs and OHCs of control explant cultures. In 5 explants, the frequency of reporter-positive OHCs was greatest in the apex, infrequent in the middle region, and rare in the base (Table S1). Reporter expression in IHCs followed a similar pattern. Reporter-positive hair cells arise from immature hair cells that express *Sox2* at the time of tamoxifen administration (at P1).

Neonatal expression of *Lgr5*. Neonatal mouse expression of EGFP in the *Lgr5-EGFP-IRES-Cre-ER* mouse has previously been described in the inner border, inner pillar and 3rd Deiter's cells as well as the GER (Shi et al., 2012). Due to lower recombination efficiencies for *Lgr5-EGFP-IRES-Cre-ER* than for *Sox2-Cre-ER*, tamoxifen was administered through maternal milk at both P0 and P1. Reporter expression was observed in these double transgenic pups in the inner border, inner phalangeal, inner pillar and 3rd Deiters' cells, and the GER (Figure S1B). Mean recombination rates in inner pillar cells were similar to those seen for *Sox2* lineage tracing (blue bars in Figure S1C). In contrast, recombination rates for the 3rd Deiters' cells were considerably higher, with 86.4 ± 6.5% in the apex, 94.4 ± 2.8% in the middle, and 96.7 ± 1.5% in the base. Some reporter expression was noted in hair cells of untreated *Lgr5* lineage tracing explant cultures. In 7 explants, reporter-positive OHCs were observed most frequently in the apex, occasionally in the middle, and rarely in the base (Table S1). The red signal from the reporter was also evident in some IHCs, most frequently in the apex, and was thought to be due to continuing expression of *Lgr5* in some immature hair cells at P0 when tamoxifen is first delivered.

Neonatal expression of *Pou4f3*. *Pou4f3* is specifically expressed in the IHCs and OHCs by E15.5 (Xiang et al., 1997). Because the *Pou4f3* construct is not inducible, in double transgenic mice positive for both *Pou4f3-Cre* and a reporter, all native IHCs and OHCs should display reporter expression. In an organ of Corti from a *Pou4f3-Cre/CAG-tdTomato* mouse, all IHCs and OHCs were reporter-positive (Figure S1D).

In vitro treatment with gentamicin led to outer hair cell loss in the middle and basal regions. Organ of Corti explant cultures treated with 50 μM gentamicin overnight and examined 72 hours later showed significant OHC damage in the middle and basal regions, with limited damage in the apex (Figure S2). In some cultures, IHC loss was also observed, particularly in the basal region.

Supplemental References

Oesterle, E.C., Campbell, S., Taylor, R.R., Forge, A., and Hume, C.R. (2008). Sox2 and JAGGED1 expression in normal and drug-damaged adult mouse inner ear. J Assoc Res Otolaryngol *9*, 65-89.
Shi, F., Kempfle, J.S., and Edge, A.S. (2012). Wnt-responsive lgr5-expressing stem cells are hair cell progenitors in the cochlea. J Neurosci *32*, 9639-9648.
Xiang, M., Gan, L., Li, D., Chen, Z.Y., Zhou, L., O'Malley, B.W., Jr., Klein, W., and Nathans, J. (1997). Essential role of POU-domain factor Brn-3c in auditory and vestibular hair cell development. Proc Natl Acad Sci U S A *94*, 9445-9450.