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# Stem Cells and the Bird Cochlea—Where Is Everybody?

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In sharp contrast to the adult mammalian cochlea, which lacks regenerative ability, the mature avian cochlea, or basilar papilla (BP) is capable of complete recovery from hearing loss after damage. Avian sensory hair cell regeneration relies on rousing quiescent supporting cells to proliferate or transdifferentiate after hair cell death. Unlike mammalian cochlear supporting cells, which have clearly defined subtypes, avian BP supporting cells are deceptively indistinguishable and molecular markers have yet to be identified. Despite the importance of supporting cells as the putative stem cells in avian regeneration, it is unknown whether all supporting cells possess equal capability to give rise to a hair cell or if a specialized subpopulation exists. In this perspective, we reinvigorate the concept of a stem cell in the BP, and form comparisons to other regenerating tissues that show cell-cycle reentry after damage. Special emphasis is given to the structure of the BP and how anatomy informs both the potential, intrinsic heterogeneity of the supporting cell layer as well as the choice between mitotic and nonmitotic regenerative strategies.

## HOW THE AVIAN COCHLEA BECAME A MODEL FOR HAIR CELL REGENERATION

The concept of hair cell regeneration in the nonmammalian cochlea came late to the party with respect to other nonmammalian regenerative models (e.g., amphibian tail, lens, or limb regeneration). This tardiness is largely the result of the lack of accessibility of hearing organs, contributing to technical difficulty in both dissection and manipulation. Traumatizing cochlear hair cells with aminoglycoside antibiotics or loud noise exposure was first shown in the 1940s and 1950s. Acoustic trauma was found to damage the guinea pig organ of Corti (Lurie et al. 1944) and streptomycin was shown to be ototoxic in felines (Hawkins and Lurie 1952). Damage paradigms became useful in identifying intrinsic properties of specific hair cell subtypes like, for example, those of the outer hair cells that were revealed following their death induced by kanamycin in the base of the guinea pig cochlea (Dallos et al. 1972). Furthermore, researchers were able to assess whether recordings from sensory ganglia in a specific tonotopic location were altered when the corresponding

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hair cells were damaged (Robertson and Johnstone 1979).

The avian hearing organ, the basilar papilla (BP), was initially not appreciated as a suitable comparative model for the mammalian organ of Corti. Early anatomical papers described the avian BP as an unorganized, homogenous mass of cells (Retzius 1884; Held 1926). Furthermore, the nucleus isthmi of birds, which projects to the visual system, was believed to be the auditory center, resulting in years of frustrating attempts to construct homology with the mammalian cochlear nucleus (Boord 1969). Because of this apparent lack of evolutionary conservation, it is plausible that the avian auditory system was deemed too archaic for comparative studies. This all changed when ultrastructural imaging showed that the avian cochlea was indeed quite similar to the mammalian organ of Corti, containing short and tall hair cells, corresponding to mammalian outer and inner hair cells, respectively, and resemblances in innervation patterns (Vinnikov et al. 1965; Boord 1969; Rosenhall 1971; Takasaka and Smith 1971). Once accepted as a homologous hearing organ (reviewed in Basch et al. 2016), the BP was further championed to have advantages much akin to the early avian embryo itself—morphological linearity. That is, the avian cochlea, being uncoiled, is more amenable to reconstruction by serial sectioning, compared with the spiraled mammalian organ of Corti (Rubel and Ryals 1982).

Initial experiments leveraging an acoustical damage paradigm led to the demonstration that birds could regenerate lost hair cells, resulting in functional recovery of hearing. Here, the focus was tonotopy, whereby researchers applied a pure tone noise exposure at various frequencies to assess the vulnerability of hair cells to various frequencies from the apex (distal end) to the base ( proximal end) of the BP, allowing conclusions to be made about their inherent tuning (Rubel and Ryals 1982; Lippe and Rubel 1983). In the wake of these studies, the occurrence of new hair cells with small hair bundles in sound-lesioned areas was reported (Cotanche 1987). Tritium-labeled ($H^3$) thymidine and autoradiography was subsequently used to show the mitotic production of new hair cells in the BP after acoustic trauma (Corwin and Cotanche 1988; Ryals and Rubel 1988). The ramifications of these findings for the field were particularly exciting because they inspired a long-lasting (and still-continuing) quest to find biological cures for hearing loss (reviewed in Rubel et al. 2013).

## MITOTIC AND NONMITOTIC REGENERATION IN THE AVIAN COCHLEA

The cellular organization of the chicken BP sensory epithelium becomes evident in cross sections of the cochlear duct (Fig. 1). Clearly visible is a neural-to-abneural gradient of distinct hair cell morphologies and innervation (Fischer et al. 1992). For the purpose of this discussion, we will refer to "neural" and "abneural" as distinct segments of the sensory epithelium where tall and short hair cells are located, respectively (Fig. 1B). Supporting cells belonging to the neural segment show rounder and sparsely packed nuclei compared with those in the abneural segment where the supporting cell nuclei are oval and more tightly packed (Figs. 1B and 2A). The ratio of supporting cells to hair cells on the abneural side is also much higher (Fig. 2B), as previously reported for embryonic stages (Goodyear and Richardson 1997). These differences are, in part, constrained by the larger volume of the sensory epithelium in the neural segment, in which cells can "spread out" from the basement membrane to the apical surface. Whether all supporting cells span the entire distance of the epithelium to surround hair cells apically has not been determined because demarcating supporting cell plasma membranes in the BP have posed technical challenges. Also noticeable is a group of supporting cells whose nuclei line up like "pearls on a string" along the basement membrane juxtaposed to the habenula perforata on the neural limbus; these cells appear distinctively different based on the ordered arrangement of their nuclei (Fig. 1B).

The observed robust regeneration of hair cells prompted the conclusion that the chicken BP harbors somatic stem cells (Corwin and Cotanche 1988; Ryals and Rubel 1988). Circumstantial evidence suggests that hair cell regeneration in the BP indeed is a stem cell–driven

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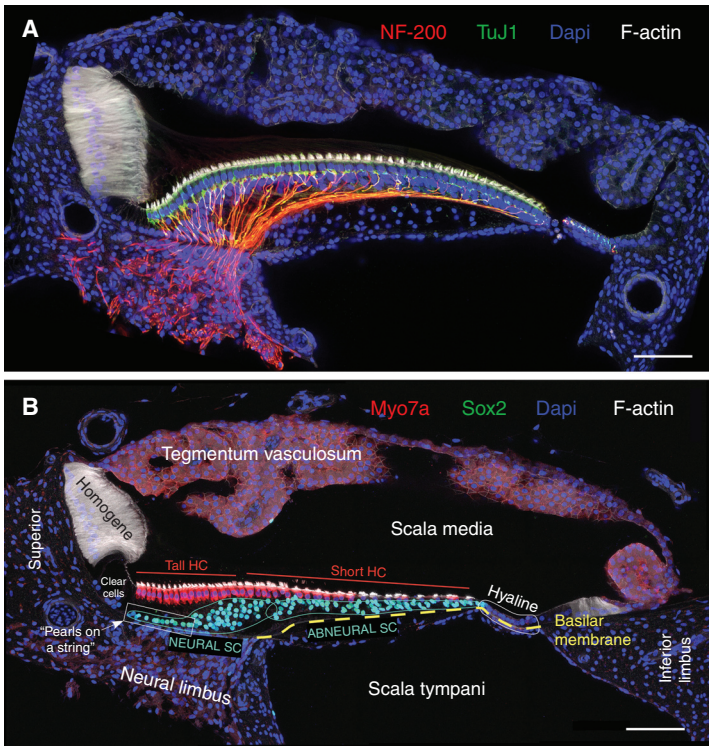


**Figure 1.** Cross-sectional anatomy of the chicken basilar papilla (BP). Shown are transverse vibratome sections taken from the middle tonotopic region of the chicken BP, roughly equidistant from proximal and distal ends. Embryonic day 20 (*A*) and posthatch day 8 (*B*) were immunostained for (*A*) neurofilament-200 (NF-200, Sigma) and tubulin β III (TuJ1, EMD Millipore) or (*B*) myosin-VIIa (Myo7a, Proteus) and Sox2 (Santa Cruz Biotech). Phalloidin labels filamentous (F)-actin and Dapi stains nuclei. Sections were imaged with a Zeiss LSM880 confocal microscope at 40× magnification using Zen Black acquisition software. SC, Supporting cell; HC, hair cell. Scale bars, 50 μm.

process, albeit with an interesting twist: New hair cells can arise from either asymmetrically dividing supporting cells, or via phenotypic conversion (also known as "direct transdifferentiation") from underlying supporting cells (Adler and Raphael 1996; Stone and Rubel 2000; Roberson et al. 2004; Cafaro et al. 2007). The latter process was initially discovered in the frog saccule and was based on the observation of intermediate phenotypes between supporting cells and hair cells (Adler et al. 1997; Steyger et al. 1997); transdifferentiation was later verified with mitotic inhibitors (Baird et al. 1996; Baird et al. 2000). Although mitotic and nonmitotic strategies occur largely in parallel, the abneural region of the BP preferentially engages in nonmitotic, direct transdifferentiation—only about one-third of newly generated abneural hair cells incorporate continuously supplied thymidine analog, bromodeoxyuridine (BrdU) when analyzed at 6 days post-gentamicin (Cafaro et al. 2007). This is compared with three-fourths of new hair cells incorporating thymidine analog, 5-ethynyl-2′-deoxyuridine (EdU) on the neural side (Cafaro et al. 2007). Given that the time course of regeneration is on the order of months, these studies still represent only an early snapshot of regeneration, before the full complement of hair cells is restored.

Although relatively more hair cells are regenerated by transdifferentiation in the abneural segment, a corresponding increase in dividing abneural supporting cells is not observed (Cafaro et al. 2007). This leads to the intriguing

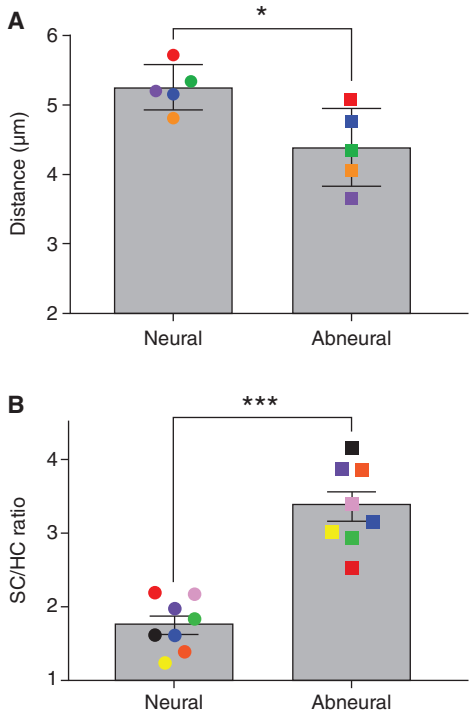


**Figure 2.** Abneural supporting cells (SCs) in the chicken basilar papilla (BP) are more tightly packed (*A*) and more numerous than hair cells (HCs) (*B*) compared with the neural side. Transverse vibratome sections taken from the middle tonotopic region of the chicken BP (posthatch day 8–10) were immunostained for myosin-VIIa (Proteus) and Sox2 (Santa Cruz Biotech). Sections were imaged as in Figure 1. Using syGlass software (see syglass.io), SC and HC nuclei number and location were recorded, standardizing voxel size and Z-depth across sections. Counts and XYZ coordinates were exported. Distance measurements (*A*) between the nearest neighboring SC nuclei was conducted in MATLAB vR2017b. Data visualization and statistics (paired *t*-test) were performed in GraphPad Prism v7. Each data point of the same color represents one chicken. Error bars, SEM. $^{*}p \leq 0.05$, $^{***}p \leq 0.001$.

question of whether supporting cells are ultimately being replenished. It is conceivable that transdifferentiation happens from a surplus pool of supporting cells, but that the sustainability of this pool might be limited. The tighter packing of supporting cells and increased supporting cell-to-hair-cell ratio on the abneural side (Fig. 2) suggests that perhaps "spare" cells are in reserve for when damage occurs. Supporting cell division might only occur after repeated hair cell loss when cell density decreases below a critical threshold. Alternatively, supporting cells are being replenished but the dynamics of this process are too slow to be unraveled even with long-term infusion of thymidine analogs. Experimental evidence using incorporation of EdU after hair cell loss in the chicken utricle supports the notion that supporting cells that transdifferentiate into new hair cells in vestibular sensory epithelia are being replenished via symmetric supporting cell divisions (Scheibinger et al. 2018). Finally, it is possible that there are new mitotically generated supporting cells migrating into the abneural region from the neural side, but this may only happen when supporting cell density on the abneural side reaches a low threshold. It remains an open question whether supporting cells that transdifferentiate into new hair cells in the abneural segment of the BP are being replenished in the first place and, if so, by which mechanism.

## THE ABSTRACT CONCEPT OF SOMATIC STEM CELLS AND MITOTIC HAIR CELL REGENERATION

The concept of somatic stem cells that define the regenerative capacity of a particular organ is complex because tissues differ widely in their need for new cells and in their response to injury. For example, the skin or the intestine require sustained cell production as well as rapid regenerative responses. In other tissues, such as the lung, reentry into the cell cycle only occurs in response to damage. Defining specific criteria that unequivocally signify a phenotypic "stem cell" is difficult, and likely irrelevant. Hallmark features of, for example, epidermal, hair follicle, and feather stem cells (Potten and Morris 1988; Cotsarelis et al. 1990; Yue et al. 2005), include self-renewal, label retention (e.g., quiescence), and expression of specific "stem" markers. These criteria have been relaxed based on findings such as the discovery of multiple cell types that show stem cell characteristics within the same tissue such as the intestinal crypt. Here, slow-cycling cells coexist with actively dividing cells (re-

viewed in Grompe 2012). Such specialized arrangements most likely exist for many adult tissues that require constant production of new cells in the undamaged state as well as the need for a repair response after injury.

The avian BP does not proliferate during normal homeostasis but shows a rapid regenerative response involving S-phase reentry and cell division following hair cell loss or damage (Corwin and Cotanche 1988; Ryals and Rubel 1988; Raphael 1992; Stone and Cotanche 1994; Hashino et al. 1995; Cafaro et al. 2007). Classical label-retaining (e.g., slow-cycling or quiescent) cells have not been reported in this organ, nor has a stem cell niche been identified. One possibility is that many, if not all, supporting cells, rather than a specialized population, are able to reenter the cell cycle to produce new hair cells and new supporting cells. This situation is not unusual as many of the internal organs of the body possess "facultative" stem cells—cells that are ordinarily unipotent and terminally differentiated, but under specific circumstances, such as post-injury, reenter the cell cycle. For example, replenishment of adult β cells after partial pancreatectomy proved to be mostly stochastic and each pancreatic cell was found to possess equal capacity to divide (Dor et al. 2004; Teta et al. 2007). Because supporting cells of the chicken BP give an initial impression of homogeneity in which no distinguishing markers have been identified (yet), the stochastic, facultative stem cell as a workhorse of regeneration is a valid hypothesis in the BP.

On the other hand, because different regions in the chicken BP harbor supporting cells with different morphologies (Fig. 1B), one might argue that heterogeneity is observable. Distinct supporting cell types are likely defined or influenced spatially within the BP in specific relation to apical/basal, neural/abneural, and tonotopic location. With respect to stemness, though, it is particularly interesting to consider the environment sensed by supporting cells with "pearls-on-a-string" nuclei lined up close to the basement membrane on the neural side of the BP (Fig. 1B). In lung epithelia, signaling from the underlying stromal layer define the 1% of Wnt-responsive type II alveolar cells that serve as facultative stem cells (Barkauskas et al. 2013; Desai et al. 2014; Ouadah 2018). Much like the supporting cells of the chicken cochlea, type II alveolar cells were once thought to be a homogeneous population with very low turnover. Recently, not only was it found that stem cells constitute only 1% of type II alveolar cells, but also that they express *Axin2,* a marker for active Wnt signaling, and are very close in proximity to a *Wnt5a*-source located in the stromal layer (Ouadah 2018). One could draw an analogy in the chicken; indeed, the group of supporting cells that hug the basement membrane on top of the neural limbus (Fig. 1B) is a potential candidate cell population to receive signals that promote S-phase reentry after damage.

Research to identify factors involved in cell-cycle reentry in the avian ear have mainly been conducted with cultured tissue. Although the use of cultured tissue does not preclude the results from being applicable to the in vivo situation, additional experiments are nevertheless required to assess the true significance of the pathways identified. For example, epidermal growth factor receptor (EGFR) signaling has been reported as essential for cell-cycle reentry; insulin and insulin-like growth factor (IGF)-1 as promoting, and fibroblast growth factor (FGF)-2 as inhibitory for cell-cycle reentry in the avian BP (Oesterle et al. 1997, 2000; White et al. 2012). Moreover, WNT signaling, particularly WNT4, as well as transforming growth factor (TGF)-β and JUN family transcription factors were identified in a knockdown small interfering RNA (siRNA) screen as being essential for cell proliferation in avian inner ear sensory epithelia. In contrast, inhibition of γ-secretase or ADAM10 led to increased proliferation, particularly in the utricle (Alvarado et al. 2011; Warchol et al. 2017).

No clear picture has yet emerged about the order of events that ultimately regulate hair cell regeneration, despite large-scale efforts using cultured sensory epithelia and systematic testing for pathways, informed by transcriptomic analyses of regenerating sensory epithelia (Hawkins et al. 2007; Alvarado et al. 2011; Ku et al. 2014). Single-cell trajectory analysis (Durruthy-Durruthy and Heller 2015; Ellwanger et al. 2018) should provide more refined insights into

solving this challenging problem. Of particular importance will be the identification of triggers that initiate cell-cycle reentry of supporting cells, as well as coordinating mechanisms that ensure a response that neither undershoots nor overshoots cell numbers and ratios. Quantitative assessments of this process in regenerating chicken utricles in vivo suggest that even after considerable hair cell loss, the number of cells in the regenerating sensory epithelium during the dynamic proliferative phase remains well controlled (Scheibinger et al. 2018).

## CHOICE BETWEEN TWO REGENERATIVE MODES

Whether differential "regeneration modes" in the BP are influenced by cell-intrinsic properties of specific supporting cell subtypes or rather by mechanical forces generated by the gross anatomy of the cochlea remains an open question. The recent finding that cell-cycle regulation in the embryonic mouse utricle is limited by the stiffness of the elastic boundary of the tissue, as manipulated in culture (Gnedeva et al. 2017), suggests that radial stiffness of the basilar membrane might similarly limit the capacity of abneural supporting cells to reenter the cell cycle. In the mouse utricle, Hippo controls supporting cell proliferation through signaling mediated by elastic force-dependent Yap nuclear translocation (Gnedeva et al. 2017). Ototoxic injury of the chicken utricle similarly leads to Yap nuclear translocation in supporting cells (Warchol et al. 2018), suggesting that the Hippo pathway is active in the regenerating chicken inner ear.

Anatomically, the neural region of the chicken BP is a natural extension of the neural limbus, whereas the abneural region is located on the free basilar membrane that connects via a group of cells called the hyaline cells to the inferior limbus, or lateral cartilaginous plate (Fig. 1) (Manley 1990). Chicken hyaline cells are efferently innervated (Fig. 3) (Odinokova and Prokof'eva 1975; Keppler et al. 1994; Frisancho et al. 1997) and harbor obliquely angled actin filaments in their basal region that are generally radially oriented in a direction toward the abneural edge of the short hair cells (Cotanche et al. 1992). These cells possess smooth muscle myosin and functional muscarinic acetylcholine receptors that stimulate the release of intracellular $Ca^{2+}$ in response to activation by acetylcholine, which potentially contributes to concave bending of the basilar membrane (Cotanche et al. 1992; Lippe et al. 2002). These specializations support a hypothesis initially raised by Drenckhahn and colleagues (1991) based on similar observations in the caiman cochlea, which suggested that hyaline cells might be contractile and involved in regulating radial tension on the basilar membrane. This tension, in combination with tighter supporting cell packing in the abneural region (Fig. 2A), could preclude nuclear YAP translocation and thereby prevents supporting cells from reentering the cell cycle. The Hippo pathway is, therefore, a reasonable candidate for a signal that suppresses the cell cycle in the abneural region of the BP. Whether the radial force and tension in this region changes after hair cell ejection remains an open question.

Each regenerative mode is likely to exploit distinct molecular mechanisms. If a supporting

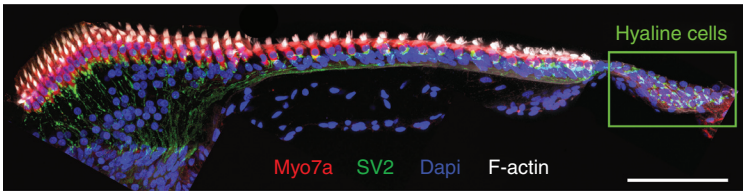


**Figure 3.** Chicken basilar papilla (BP) hyaline cells are innervated. Shown is a transverse vibratome section taken from the middle tonotopic region of the posthatch day 7 chicken BP immunostained for synaptic vesicles (SV2, Developmental Studies Hybridoma Bank) and myosin-VIIa (Myo7a, Proteus). Phalloidin labels F-actin and Dapi stains nuclei. Sections were imaged as in Figure 1. Scale bar, 50 μm.

cell chooses to enter the cell cycle, has it regressed to a more pluripotent state compared with a cell that clandestinely morphed into a hair cell without dividing? As a supporting cell transdifferentiates, does it also regress or effectively "dedifferentiate" or does it transition through an unnatural intermediate to become a hair cell? The appearance of *Atoh1,* which is essential for hair cell differentiation (Bermingham et al. 1999), is likely to be a major player in a supporting cell's trajectory toward hair cell fate (Ellwanger et al. 2018). However, it is unclear whether the events leading up to *Atoh1* expression during regeneration are simply a recapitulation of developmental processes. Discriminating between signaling pathways required for the initial trigger of regeneration that ultimately lead to S-phase reentry or transdifferentiation are likely to be different compared with those used during the normal generation of hair cells. Understanding early responses of supporting cells that are triggered by hair cell loss, as well as classifying latent cellular subtypes, will likely be an important line of experimentation moving forward.

## CONCLUDING REMARKS

Uncovering where stem cells are hiding within the chicken BP will require a progressive viewpoint of what defines a stem cell. As more tissues are studied that defy the classical stem cell paradigm, and as research uses more refined genetic tools, additional features of stem cells emerge. This is particularly relevant in the context of damage/injury whereby cells adopt creative methods for replenishment. The existence of facultative stem cells provides new insight into how seemingly unipotent and terminally differentiated cells are suddenly "reawakened" to reenter the cell cycle under duress. Whether phenotypic conversion (also known as direct transdifferentiation) also relies on a similar rekindling of multipotency or "dedifferentiation" toward developmental origin is an open question. Definitive experimental evidence is needed to clarify which supporting cells in the BP have the ability to undergo mitotic hair cell regeneration. It is currently unresolved whether all supporting cells are able to initiate a regenerative program and are therefore per se facultative somatic stem cells, or whether regeneration can only be performed by specialized and dedicated cochlear stem cells.

To find stem cells, which will assuredly reside within the supporting cell layer, extending beyond hair-cell-centric vantage points (whole-mount, top-down imaging) is important. Viewing the cochlea in a transverse section (Fig. 1) provides additional information about supporting cells, revealing cytomorphological heterogeneity that is likely imposed by the greater BP anatomy. Whether descriptive morphological observations correlate with regenerative potential and molecular heterogeneity is unknown. To investigate this relationship, single-cell transcriptomic analysis of the undamaged BP will be useful to identify inherent differences. Nevertheless, transcriptomic variations between supporting cell subpopulations might be difficult to detect if all are poised to respond but require an external signal from the underlying stromal layer or persistent elastic force potentially generated from the hyaline cells. Similarly, supporting cells might change their global epigenetic signature during regeneration rather than altering a specific, genetic pathway. Characterizing the possible reopening of chromatin during regeneration will be an important experimental direction, especially to identify early events. Likewise, it will be crucial to dissect cell–cell signaling at the onset during the progression and at the termination of the distinct regenerative modes. Identification at the cellular level of who is talking to whom, when, and what about is going to be the challenge when applying novel technologies such as single-cell trajectory reconstruction and in situ validation with multiprobe messenger RNA (mRNA)-detection methods (Wang et al. 2012; Nagendran et al. 2018). Ultimately, we argue that, given these technological innovations, the time has come for researchers to unravel the mechanisms of hair cell regeneration in the avian cochlea.

In the neonatal mouse organ of Corti, supporting cells are able to reenter the cell cycle and produce new hair cells in vitro and in vivo (White et al. 2006; Oshima et al. 2007; Sinkkonen et al. 2011; Shi et al. 2012, 2013; Cox et al.

2014; Li et al. 2015; Wu et al. 2016; McLean et al. 2017). This ability is lost, however, between the second and third postnatal week (White et al. 2006; Oshima et al. 2007). It is debatable whether the regenerative ability of some neonatal supporting cells is reflective of a "rudimentary" stemness or a remnant of developmental capacity that ceases with maturation of the organ of Corti. The crucial question for instigating hair cell regeneration in the mammalian cochlea, however, is whether the regenerative capability of supporting cells is fundamentally lost, irrevocably, in the mature organ of Corti. Or alternatively (and optimistically), the lack of regenerative capacity in mature mammalian cochlear supporting cells is not intrinsic or indelible, and these cells can be engaged in a regenerative program given the proper extrinsic signals. Work that aims to unravel the mechanisms of hair cell regeneration in nonmammalian vertebrates is critical in this respect because it can provide important clues about the sequence of signaling events that orchestrate the regenerative response.

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