Functional Features of Trans-Differentiated Hair Cells Mediated by *Atoh1* Reveals a Primordial Mechanism

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Evolution has transformed a simple ear with few vestibular maculae into a complex three-dimensional structure consisting of nine distinct endorgans. It is debatable whether the sensory epithelia underwent progressive segregation or emerged from distinct sensory patches. To address these uncertainties we examined the morphological and functional phenotype of trans-differentiated rat hair cells to reveal their primitive or endorgan-specific origins. Additionally, it is uncertain how *Atoh1*-mediated trans-differentiated hair cells trigger the processes that establish their neural ranking from the vestibulocochlear ganglia. We have demonstrated that the morphology and functional expression of ionic currents in trans-differentiated hair cells resemble those of “ancestral” hair cells, even at the lesser epithelia ridge aspects of the cochlea. The structures of stereociliary bundles of trans-differentiated hair cells were in keeping with cells in the vestibule. Functionally, the transient expression of Na^+ and I_h currents initiates and promotes evoked spikes. Additionally, Ca^{2+} current was expressed and underwent developmental changes. These events correlate well with the innervation of ectopic hair cells. New “born” hair cells at the abneural aspects of the cochlea are innervated by spiral ganglion neurons, presumably under the tropic influence of chemoattractants. The disappearance of inward currents coincides well with the attenuation of evoked electrical activity, remarkably recapitulating the development of hair cells. Ectopic hair cells underwent stepwise changes in the magnitude and kinetics of transducer currents. We propose that *Atoh1* mediates trans-differentiation of morphological and functional “ancestral” hair cells that are likely to undergo diversification in an endorgan-specific manner.

Introduction

The mammalian inner ear consists of nine distinct sensory patches, serving as endorgans for our sense of hearing and balance. The complex three-dimensional inner ear sensory structures are purported to have evolved from a simple two-canal and one-macula communis from ancestral vertebrates (Fritzsche and Beisel, 2001; Fritzsche et al., 2002). Whereas it was asserted earlier that the sensory patches developed through parallel evolution followed by progressive segregation, recent molecular evidence suggests that the functionally variant sensory epithelia evolved from multiple divisions of a single primordium (Fritzsche, 1998; Fritzsche et al., 1999, 2002; Fritzsche and Beisel, 2001). Subsequent structural diversification might have resulted in the detection of previously untapped mechanical stimulation (Morsli et al., 1998; Fariñas et al., 2001). Moreover, within the molecular domain, a

boundary model in which sensory patches in the inner ear evolved from discrete organs have been invoked (Wu and Oh, 1996; Brigande et al., 2000; Fekete and Wu, 2002). Thus, the uncertainties of the etiology of the inner ear sensory epithelia abound.

A mammalian homolog of the *Drosophila* atonal gene, *Atoh1* is a positive regulator for the differentiation of inner ear hair cells (Bermingham et al., 1999). Null deletion of the gene in mice does not alter the development of auditory and vestibular sensory primordial and supporting cells, but their differentiation into hair cells is abolished (Chen et al., 2002). Indeed, *Atoh1* is not only required but is also sufficient for the production of hair cells (Zheng and Gao, 2000), and overexpression of the gene in the matured inner ear of guinea pigs resulted in induction of new hair cells and improvement in hearing (Kawamoto et al., 2003; Izumikawa et al., 2005). The expression of *Atoh1* occurs early in development in the fish lateral line (Sarrazin et al., 2006) and in the chicken and mouse inner ears (Chen et al., 2002; Cafaro et al., 2007; Stone and Cotanche, 2007) in a subpopulation of cells within the sensory primordial that differentiate solely into hair cells. Thus, we surmised that the induction of ectopic hair cells by *Atoh1* may recapitulate the early ancestral development of these sensory cells. However, the process whereby *Atoh1*-mediated newborn hair cells acquire their functionality and neuronal connectivity is unknown.

Here, in the rat cochlea, we have shown that ectopic hair cells induced by *Atoh1* in the lesser epithelia ridge (LER) assumed hair

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cell phenotype and received innervation from spiral ganglion neurons following a period of transient expression of Na^+ and inward rectifier (I_h) currents, promoting membrane oscillations and evoked spikes in a manner reminiscent of developing and regenerating hair cells (Levic et al., 2007). These inwardly directed currents are suppressed as the electrical properties of “newborn” hair cells transition to maturity. Ectopic hair cells underwent stepwise changes in the magnitude and kinetics of transducer currents. We assert that *Atoh1* mediates the trans-differentiation of morphological and functional “ancestral” hair cells that are likely to undergo further diversification in an endorgan-specific manner.

Materials and Methods

Postnatal rats' cochleae cultures and *Atoh1* gene infection. This study was approved by the Animal Ethics Committee of the University of California, Davis. Two-day-old postnatal SD (P2) male and female rats were used for the experiments. Animals were killed by carbon dioxide inhalation, their skin was disinfected with 75% ethanol, the animals were then decapitated, and the cochleae were dissected out. The basilar membrane and the associated organ of Corti were carefully stripped from the modiolus and were subsequently cut into three equal segments. Similarly, the utricle was isolated as described previously (Dou et al., 2004). These segments were then explanted onto poly-L-lysine-coated glass coverslips with Dulbecco's Modified Eagle Medium: nutrient mixture F-12 (DMEM/F12, Invitrogen) medium containing 5% fetal bovine serum (FBS, Invitrogen). On day two after explant, the medium was replaced with serum-free DMEM/F12 supplemented with B27 (Invitrogen), and the culture medium was replaced every other day. Ad5-EGFP-*Atoh1* was co-incubated with cochlear and utricular explants on day two for 20 h to overexpress *Atoh1* in the cochlear segments, and the control group was infected with Ad5-EGFP (Huang et al., 2009). The final concentration of the Ad5 vector was 1.0×10^8 PFU/ml in a serum-free DMEM/F12 medium. The culture dishes were kept in an incubator at 37°C with 5% CO_2 in a humid environment.

Tissue preparations and immunofluorescence. The cochlear explants were washed with 0.01 M PBS and then fixed in 4% paraformaldehyde in PBS. The preparation was treated with 0.1% Triton X-100 in PBS for 30 min at room temperature. Subsequently, the preparation was treated with 10% goat serum in PBS for ~30 min to block nonspecific binding and the specimens were then incubated with the following primary antibodies for 24 h at 4°C: myosin VIIA (Rabbit; 25–6790; Proteus Biosciences, 1:100), myosin VIIA (Mouse; MYO7A 138–1; DSHB, 1:100), TUJ1 (1:600) plasma membrane Ca^{2+} -ATPase 2, and PMCA2 (Rabbit; PA1915; Thermo Scientific, 1:200). The specimen was washed 3–5 times in PBS and then incubated with secondary antibodies for 50 min at 37°C in the dark. The secondary antibodies included goat anti-mouse Alexa Fluor 647 (1:400), goat anti-rabbit IgG (H+L) Alexa Fluor 647 (1:400), goat anti-mouse IgG (H+L) Alexa Fluor 555 (1:1000), and goat anti-rabbit IgG (H+L) Alexa Fluor 555 (1:1000) (Invitrogen). Specimens were viewed under a Zeiss LSM 510 confocal laser-scanning microscope (Carl Zeiss). Imaris software was used to construct 3D images.

Electrical recordings. Action potentials were amplified (100×), filtered (bandpass 2–5 kHz), and digitized at 5–50 kHz. Ionic currents were recorded in a whole-cell voltage-clamp configuration, using 2–3 M Ω resistance pipettes. The patch pipettes were pulled from glass capillaries (Sutter Instruments). Membrane voltages and currents were amplified with an Axopatch 200B amplifier (Molecular Devices) and filtered at a frequency of 2–5 kHz through a low-pass Bessel filter. The data were digitized using an analog-to-digital converter (Digidata 1200; Molecular Devices). The sampling frequency was determined by the protocols used. No online leak current subtraction was made, and as such only recordings with holding currents <20 pA were accepted for analyses. The liquid junction potentials were measured (3.1 ± 0.5 mV, $n = 84$) and corrected online. The capacitive transients were used to estimate the capacitance of the cell as an indirect measure of cell size. Membrane capacitance was calculated by dividing the area under the transient current in response to a voltage step as described previously (Levic et al., 2007). The capacitive

decay was fitted with a single exponential curve to determine the membrane time constant. Series resistance was estimated from the membrane time constant, given its capacitance. This study included ~1083 cells with a series resistance (R_s) within a 5–10 M Ω range. A series resistance compensation of ~60–90% was used to reduce potential voltage error artifacts. The seal resistances ranged from 5 to 20 G Ω .

To record K^+ currents and action potentials, the pipette solution contained the following (in mM): 120 KCl, 2 MgCl₂, 0.1 CaCl₂, 1 EGTA, 5 K₂ATP, 0.1 Na₂GTP, and 10 HEPES-KOH (pH 7.4, ~290 mmos). The external solution contained the following (in mM): 130 NaCl, 5.8 KCl, 0.9 MgCl₂, 1.3 CaCl₂, 5.6 D-glucose, and 10 HEPES-NaOH (pH 7.4, ~290 mmos). Transduction currents were recorded from Ad5-EGFP-*Atoh1*-infected hair cells in the intact sensory epithelia of newly formed hair cells at the LER using an upright microscope. Hair cells were held at a holding potential of –80 mV with the tight-seal, whole-cell voltage-clamp configuration. Bundle displacement used a stiff probe stimulator as described previously (Yamoah and Gillespie, 1996; Yamoah et al., 1998b; Waguespack et al., 2007). In most cases, mechanical stimuli had durations of 100–250 ms with an interstimulus interval of 2–4 s. The recording electrode solution contained the following (in mM): 100 CsCl, 3 MgCl₂, 2 Na₂ATP, 1 EGTA, and 5 HEPES. CsOH was used to adjust the final pH to 7.3. The external solution contained 1.5 mM CaCl₂ and was oxygenated at room temperature.

To record Ca^{2+} and Na^+ currents (I_{Ca} and I_{Na}), the pipette solution contained the following (in mM): 60 CsCl, 60 N-methyl-D-glucamine chloride (NMGCl), 5 Na₂ATP, 0.1 Na₂GTP, 5 EGTA, and 10 HEPES-CsOH, pH 7.3. The external solution for recording I_{Ca} contained the following (in mM): 105 NMGCl, 2.8 KCl, 1 MgCl₂, 35 TEACl, 5 CaCl₂, 10 D-glucose, and 10 HEPES-NaOH, pH 7.4. The external solution for recording I_{Na} contained the following (in mM): 80 NaCl, 35 NMGCl, 2.8 KCl, 1 MgCl₂, 25 TEACl, 0.1 CaCl₂, 10 D-glucose, and 10 HEPES-NaOH (pH 7.4).

Data analysis. Data were analyzed using pClamp8 (Molecular Devices), Origin7.0 (Microcal Software). Differences between groups were tested using Student's *t* tests and the null hypothesis was rejected when the *p* value was <0.05. The *n* reported reflect the number of cells.

Results

Ad5-EGFP-*Atoh1*-infected cells exhibit characteristic features of developing and regenerating hair cells

Primordial rhythmic electrical activity that is manifested as spontaneous and evoked action potentials is a functional signature of developing and regenerating hair cells (Marcotti and Kros, 1999; Marcotti et al., 2003a,b; Levic et al., 2007). The importance of spike activity in developing hair cells is underpinned by the evidence that it ceases upon hair cell maturation. Although the functional significance of spike activity is not fully understood in detail, the ensuing Ca^{2+} -mediated release of neurotrophins may guide neuronal outgrowth, facilitating synaptogenesis and establishing the neuronal niche of newly differentiated hair cells (Chabbert et al., 2003; Eatock and Hurley, 2003; Levic et al., 2007). To establish whether spike activity is indeed required for the transformation of differentiating cells, we monitored the membrane potential of Ad5-EGFP-*Atoh1*-infected cells. Shown in Figure 1A are evoked spikes recorded in a 2.5-d-old infected ectopic hair cell at the LER. In contrast to Ad5-EGFP-infected cells, the apparent mean resting membrane potential (V_m) of Ad5-EGFP-*Atoh1*-infected cells was -47 ± 4 mV ($n = 68$) compared with -69 ± 7 mV ($n = 78$), $p < 0.01$. Ad5-EGFP-infected cells were resistant to evoked spikes. As shown in the inset (Fig. 1A), we were unable to elicit action potentials after injecting 500 pA of current. Moreover, injection of negative current in Ad5-EGFP-*Atoh1*-infected cells generated rebound spikes as shown in Figure 1B. To determine the underlying ionic current responsible for the evoked spikes, we applied an external solution containing the Na^+ current blocker tetrodotoxin (TTX; 200 nM). The

Figure 1. Evoked action potentials from newly formed hair cells at the LER aspects of the cochlea. Membrane action potentials were evoked by current injection 2.5-DPI. **A**, Current-clamp recordings of a 2.5-DPI cell at the LER stimulated with a 20, 30, 40, and 50 pA current step. As indicated, 20 pA was not sufficient to elicit a spike. However, a current magnitude equal to and more than 30 pA was adequate to evoke all-or-none action potentials. In contrast, as shown in the inset, injection of a current magnitude as large as 500 pA did not evoke action potentials in Ad5-EGFP-infected cells. **B**, In a different cell, at 5-DPI Ad5-EGFP-Atoh1, application of a brief ~ 5 ms injection of negative current (-90 , -70 , and -50 pA) resulted with rebound action potentials at the termination of the pulses. The corresponding traces and the injected currents are shown. **C**, To determine the underlying current for the upstroke phase of these evoked spikes, we recorded control spikes with a 60-pA-injected current (trace shown in gray). Upon application of bath solution containing 200 nM TTX, the evoked spike was abolished (shown in the bold black trace). Recordings were obtained from a 5-DPI cell. **D**, Shown is a histogram of the summary data of different concentrations of TTX on the evoked spike amplitude generated from 5-DPI cells at the greater epithelia ridge ($n = 9$ infected cells). The effect of TTX was dose-dependent. **E**, Shown is a rare recording of spontaneous action potentials in a 7-DPI cell. Of 280 trans-differentiated cells at the LER aspect of the cochlea that were sampled under current-clamp configuration, evoked spikes occurred as single events in 243 cells (**A–C**), and 31 cells showed membrane oscillations ranging between -50 and -39 mV but were defiant to evoked spiking activity. Only three cells were spontaneously active (age of newly formed hair cells were 5-, 7-, and 10-DPI).

evoked spike was attenuated by TTX (Fig. 1C). Meanwhile, the effect of TTX on evoked spike amplitude was dose-dependent (Fig. 1D). Of 280 trans-differentiated cells that were examined at the LER, 243 cells exhibited evoked spikes, and 31 cells showed rhythmic membrane oscillations ranging from -50 to -39 mV. The remaining three cells exhibited spontaneous action potentials as depicted in Figure 1E.

The underlying inward currents for evoked and spontaneous action potentials in Ad5-EGFP-Atoh1-infected cells

We examined Na^+ currents from Ad5-EGFP-Atoh1-infected cells at the lesser epithelia ridge of the cochlea by suppressing outward K^+ and inward Ca^{2+} currents. In contrast to supporting and Ad5-EGFP-infected cells, two days post Ad5-EGFP-Atoh1 infection (2-DPI), a rapidly activating inward Na^+ current was visible. Figure 2A shows traces of the Na^+ current from the earliest time point examined, 2-DPI to 20-DPI. The magnitude of the Na^+ current increased with time and then gradually declined.

By 20-DPI the size of the current had dwindled by ~ 8 -fold with peak current density at 3-DPI. Figure 2B summarizes the current density-voltage relationship at different stages of post Ad5-EGFP-Atoh1 infection. There was an ~ 6 -fold increase in the current density between 2- and 3-DPI. Meanwhile, the magnitude of the transient Na^+ current showed marked decline ~ 10 -DPI and beyond. The half-activation voltage ($V_{1/2}$) was shifted to the left by ~ 8 mV for currents recorded at 2-DPI compared with 3-DPI (Fig. 2C). Moreover, by 10-DPI the activation shifted rightward by ~ 7 mV compared with the initial value at day two. Analyses of the voltage dependence of activation revealed that the half-voltage inactivation, $V_{1/2}$ (in mV), of the steady-state inactivation curve for currents at 2-, 3-, and 10-DPI were ~ -56 , -71 , and -59 mV, respectively (Fig. 2D). Window currents were apparent from the crossovers of the activation and inactivation curves for currents at 2- and 5-DPI, suggesting that the transient Na^+ currents were partially activated at rest. At and beyond 10-DPI, the activation and inactivation properties of the Na^+ cur-

Figure 2. Transient expression of TTX-sensitive Na^+ current in newly formed hair cells at the LER of the cochlea. Ad5-EGFP-Atoh1-infected cells at the LER of the cochlea express voltage-dependent Na^+ currents but not EGFP alone-infected cells. **A**, EGFP-infected cells were held at a holding potential of -110 mV and stepped to depolarizing voltage steps from -90 to 50 mV, using ΔV of 5 mV, as shown. The bath and pipette solutions consisted of components (see Materials and Methods) that suppressed inward Ca^{2+} and outward K^+ currents. Under these conditions, no active current was recorded in EGFP-infected cells. The example shown (left) was recorded from a 7-d-old LER cochlea post Ad5-EGFP-infection ($n = 7$). In contrast, Ad5-EGFP-Atoh1-infected cells expressed voltage-dependent Na^+ current, appearing from 1-DPI and peaking at 3-DPI and declining from 5-DPI to 20-DPI, as shown with exemplary traces from 2-DPI to 20-DPI. **B**, The current-density-voltage (I - V) relationships of Na^+ current in EGFP-infected cells 7-DPI ($n = 11$) and EGFP-Atoh1-infected newly formed hair cells 2-DPI ($n = 9$), 3-DPI ($n = 11$), and 10-DPI ($n = 6$) show the transient expression pattern. **C**, Using the predicted reversal potential (E) of Na^+ (E_{Na}), ~ 53 mV, we determined the driving force of the current ($V - E_{\text{Na}}$) and calculated the conductance (g) at a given voltage (V). The resulting ratios of the conductance were plotted and fitted to a Boltzmann function in the form: $g/g_{\text{max}} = [1 + \exp((V_{1/2} - V)/k_m)]^{-1}$, where $V_{1/2}$ is the half-activation voltage (mV) and k_m is a slope factor in (mV); $V_{1/2}$ activation at 2-DPI was -34 ± 2 mV and $k_m = 3.4 \pm 0.9$ ($n = 7$); $V_{1/2}$ activation at 3-DPI was -42 ± 2 mV, $k_m = 4.0 \pm 1.8$ ($n = 5$); and finally the $V_{1/2}$ activation at 10-DPI was -27 ± 1 mV, $k_m = 6.0 \pm 0.7$ ($n = 6$). **D**, Using the classical steady-state inactivation protocol, long-duration prepulses, ~ 20 ms, were used to activate and inactivate the Na^+ current and the voltage dependence of inactivation was tested at a step potential of -10 mV. In the inset, we show a family of traces generated from the steady-state inactivation protocol. The ensuing steady-state voltage-dependent inactivations were fitted with a Boltzmann function in the form: $g/g_{\text{max}} = [1 + \exp((V - V_{1/2})/k_h)]^{-1}$, where $V_{1/2}$ is the half-inactivation voltage (mV) and k_h is the slope factor (mV). $V_{1/2}$ inactivation at 2-DPI was -56.0 ± 1.2 mV, $k_h = 8.4 \pm 1.1$ ($n = 7$); the $V_{1/2}$ of inactivation at 3-DPI was -71.2 ± 2.5 mV, $k_h = 8.4 \pm 1.3$ ($n = 5$); and the $V_{1/2}$ of inactivation at 10-DPI after Atoh1 infection was -59.2 ± 0.5 mV, $k_h = 8.5 \pm 0.3$ ($n = 5$). **E**, The dose-response effects of TTX on the Na^+ current were generated using a logistic function: $[A_1 - A_2/(1 + X/X_0)^p] + A_2$. Here are the initial and final current ratios, respectively. X is the concentration of TTX, X_0 is the half-blocking concentration (IC_{50}), and p is the Hill coefficient. The data were generated using 5-DPI cells ($n = 7$) after Atoh1 infection. The inset represents an example of a control trace (in black) on the effects of 100 nM TTX (in blue) on the Na^+ current. The IC_{50} was 49 nM. **F**, The voltage dependence of the kinetics of inactivation of the Na^+ currents is shown as time constant (τ) versus voltage. **G**, Time-dependent inactivation of Na^+ current. Cells were held at different membrane voltages (-110 , -90 , and -70 mV), and the current was activated using a prepulse with a duration to attain complete inactivation followed by a test pulse at different time intervals to determine the time dependence of recovery from inactivation. The cells were from the newly formed hair cells 5-DPI. The time constants (τ) of recovery from inactivation were fitted with two τ values as follow; at -110 mV, $\tau_1 = 2.3 \pm 0.2$ ms and $\tau_2 = 14.4 \pm 1.9$ ms ($n = 7$); at -90 mV, $\tau_1 = 3.8 \pm 0.3$ ms and $\tau_2 = 8.2 \pm 1.1$ ms ($n = 6$); and at -70 mV, one τ sufficed to fit the data at $\tau = 10.4 \pm 0.4$ ms ($n = 5$).

rent fell outside of the dynamic range of the membrane potentials of Ad5-EGFP-Atoh1-infected cells. We determined the sensitivity of the Na^+ current to TTX using currents measured at 5-DPI. Shown in the inset in Figure 2E are control traces and the inhibitory effects of TTX. The half-blocking concentration determined from the data was ~ 50 nM with a Hill coefficient of ~ 1 . Only a small component ($< 5\%$) of the Na^+ current appeared insensitive to TTX. As expected from a voltage-dependent current, the time constants of inactivation were faster with increasing depolarization (Fig. 2F). Additionally, we examined the time dependence of recovery from inactivation using holding voltages -110 ,

-90 , and 70 mV (Fig. 2G). The time constants (τ) of recovery from inactivation were fitted with two τ s as follow; at -110 mV, $\tau_1 = 2.3 \pm 0.2$ ms and $\tau_2 = 14.4 \pm 1.9$ ms ($n = 7$); at -90 mV, $\tau_1 = 3.8 \pm 0.3$ ms and $\tau_2 = 8.2 \pm 1.1$ ms ($n = 6$); and at -70 mV, one τ sufficed to fit the data $\tau = 10.4 \pm 0.4$ ms ($n = 5$).

We recorded Ca^{2+} currents from Ad5-EGFP alone- and Ad5-EGFP-Atoh1-infected cells at the LER using 5 mM external Ca^{2+} . NMG $^+$ was used to replace Na^+ ions and we examined the longitudinal expression of inward Ca^{2+} currents. Cells were held at -80 mV and stepped to depolarizing voltage steps with increments of 10 mV. No inward Ca^{2+} currents were apparent in

Figure 3. Calcium current of newly formed hair cells at the LER of the cochlea. To record Ca^{2+} currents, outward K^{+} currents were suppressed and inward Na^{+} current was eliminated using ion substitution (see Materials and Methods). Cells were held at a holding potential of -80 mV and the step voltages ranged from -70 to 40 mV with intervals of 10 mV. **A**, Supporting cells at the LER 7 d postinfection with Ad5-EGFP alone did not express any inward Ca^{2+} current as shown (left traces) and summarized in **B**. However, 3 d post Ad5-EGFP-Atoh1 infection, 3-DPI cells began to express a small inward Ca^{2+} current and the amplitude increased with time from 5-DPI to 10-DPI, but declined at 15-DPI as illustrated. **B**, The current-voltage (I - V) relationships expressed in the form of current-density against voltage show the changes in Ca^{2+} current-densities post EGFP-Atoh1 infection in newly formed hair cells at the LER (3-DPI, $n = 7$; 5-DPI, $n = 7$; 10-DPI, $n = 7$; and 15-DPI, $n = 8$). **C**, For Ca^{2+} currents we used the apparent reversal potential (E) of Ca^{2+} (E_{Ca} , ~ 71 mV, to determine the driving force of the current ($V - E_{\text{Ca}}$) and calculated the conductance (g) at a given voltage (V). The resulting ratios of the conductance were plotted and fitted to a Boltzmann function in the form; $g/g_{\text{max}} = [1 + \exp((V_{1/2} - V)/k_m)]^{-1}$, where $V_{1/2}$ is the half-activation voltage (mV) and k_m is a slope factor (mV), $V_{1/2}$ activation at 5-DPI = -29 ± 4 mV, and $k_m = 8.8 \pm 0.4$ ($n = 7$). As shown in the inset, application of the dihydropyridine agonist bay K8644 ($5 \mu\text{M}$) resulted in at least a 2.5-fold increase in the current amplitude. The steady-activated activation curve shifted to the left with $V_{1/2}$ activation at 5-DPI = -43 ± 3 mV, $k_m = 9.1 \pm 0.6$ ($n = 7$). **D**, Shown are the control traces and the remaining traces after application of $5 \mu\text{M}$ nifedipine. The transient current remained insensitive to nifedipine. The dose-response effects of nifedipine are shown in the histogram ($n = 5$).

Ad5-EGFP-infected cells from 1- to 20-DPI (the example shown was recorded at day seven after infection; also see summary data in Fig. 3B). However, in Ad5-EGFP-Atoh1-infected cells, a transient Ca^{2+} current was visible by 3-DPI (Fig. 3A) and the magnitude of the Ca^{2+} current increased by ~ 16 -fold by 10-DPI, until it was downregulated at 15-DPI (Fig. 3A,B). Consistent with the expression of L-type Ca^{2+} currents in hair cells (Rodriguez-Contreras and Yamoah, 2001; Knirsch et al., 2007; Levic et al., 2007; Zampini et al., 2010), the sustained and predominant component of the current was sensitive to the dihydropyridine (DHP) agonist bay K8644 (Fig. 3C,D). As shown in the inset, application of bay K8644 ($5 \mu\text{M}$) resulted in at least a 2.5-fold increase in the current amplitude. The steady-state activation curve shifted to the left with $V_{1/2}$ of activation at 5-DPI from control, -29 ± 4 mV to bay K8644, -43 ± 3 mV ($p < 0.01$; $n = 7$). The reverse experiments were conducted using the DHP antagonist nifedipine, which blocked the sustained Ca^{2+} current (Fig. 3D). Moreover, the transient Ca^{2+} current component remained insensitive to $5 \mu\text{M}$ nifedipine. The dose-response effect

of nifedipine on the sustained Ca^{2+} current is shown in the histogram (Fig. 3D). In accord with previous studies, a transient Ca^{2+} current was eminent in developing hair cells (Levic et al., 2007).

Whereas inward rectifier $\text{K}^{+}/\text{Na}^{+}$ -permeable conductances promote evoked spike activity and spontaneous action potentials (SAPs) as seen in photoreceptors and vestibular hair cells (Holt and Eatock, 1995; Yamoah et al., 1998a), K^{+} -selective inward rectifier conductances tend to clamp the membrane potential in the vicinity of the reversal potential of K^{+} , making cells quiescent (Yamoah et al., 1998a). The inward rectifier current in Ad5-EGFP-Atoh1-infected cells debuted at 2-DPI, reaching maximum amplitude at 5-DPI, and gradually plummeting at 15-DPI and beyond (Fig. 4A,B). Shown are the current-density voltage relationships at 5-, 10-, and 15-DPI (Fig. 4B). The permeation properties of the inward rectifier current were assessed by altering the bath $[\text{Na}^{+}]$. An increase in bath $[\text{Na}^{+}]$ shifted the reversal potential to more positive voltages, whereas the reverse was the case when $[\text{Na}^{+}]$ was reduced (Fig. 4C). The reversal potential of

Figure 4. Time-dependent inward rectifier current (I_h) of newly formed hair cells appears early, and decreases and disappears at later stages. **A**, Ad5-EGFP-Atoh1-infected cells were held at -30 mV and stepped to positive and negative voltages relative to the holding voltage ($\Delta V = 10$ mV). The I_h debuted at 2-DPI and the magnitude peaked at 5-DPI, as shown. However, by 15-DPI the magnitude of the inward rectifier current decreased drastically. **B**, The current-density and voltage relationship curve of I_h in Ad5-EGFP-infected cells and Ad5-EGFP-Atoh1-mediated newly formed hair cells at 5-DPI ($n = 8$), 10-DPI ($n = 18$), and 15-DPI ($n = 7$). **C**, Changes in $[Na^+]_{ext}$ altered the apparent reversal potential of I_h . The apparent reversal potential was calculated from the regression line generated from instantaneous current voltage relationships. Alteration of the apparent reversal potential (E_{rev}) by changing $[Na^+]_{ext}$ suggested the I_h channel is permeable to Na^+ , which is consistent with previous studies (Yamoah et al., 1998a). Shown are representative plots of the instantaneous I - V relationship generated from tail current records with varying $[Na^+]_{ext}$. The $[Na^+]_{ext}$, the corresponding E_{rev-Ih} , and the conductances were 60 mM ($\bullet$), -44.7 mV, 1.1 nS; 70 mM ($\circ$), -36.6 mV, 1.2 nS; 90 mM ($\blacksquare$), -33.7 mV, 1.3 nS. By way of the Goldman-Hodgkin-Katz equation, the calculated permeability ratio of K^+ versus Na^+ (P_K/P_{Na}) = 0.8 for I_h in newly formed hair cells. **D**, Steady-state voltage-dependent activation curves of I_h in newly formed hair cells 5-DPI ($n = 8$), 10-DPI ($n = 7$), and 15-DPI ($n = 5$) after EGFP Atoh1 infection. Using the E_{rev} of the current, we determined the driving force of the current ($V - E_{rev-Ih}$) and calculated the conductance (g) at a given voltage (V). The resulting ratios of the conductance were plotted and fitted to a Boltzmann function in the form: $g/g_{max} = [1 + \exp((V_{1/2} - V)/k_m)]^{-1}$, where $V_{1/2}$ is the half-activation voltage (mV) and k_m is a slope factor (mV). At 5-DPI, the $V_{1/2}$ activation = -95.2 ± 1.4 mV, $k_m = 15.3 \pm 1.7$ (n = 7); at 10-DPI, $V_{1/2}$ activation = -87.2 ± 0.6 mV, $k_m = 16.5 \pm 0.8$ (n = 7); and at 15-DPI, $V_{1/2}$ activation = -94.5 ± 1.2 mV, $k_m = 14.0 \pm 1.4$ (n = 5). **E**, A family of control I_h traces (left top) was recorded from 7-DPI cells and the effects of 0.5 mM $BaCl_2$ in the external solution were examined. Ba^{2+} had no effect on I_h (middle). On right, we showed the current traces after washout. Using the same cell, we applied 0.5 mM Cs^+ , which blocked I_h (bottom left) reversibly (bottom right). **F**, A summary of the current-density voltage relationship on the effects of Ba^{2+} ($\square$) and Cs^+ ($\circ$) as compared with control ($\bullet$). **G**, Shown is a bar graph summarizing the total number of cells we sampled and the distribution of expression of I_h and the time-dependent changes. At 5-DPI, 100% of the cells expressed I_h ($n = 32$) and by 10-DPI $\sim 51\%$ of the hair cells expressed I_h ($n = 27$). Finally, by 15-DPI only $\sim 17\%$ of the newly formed hair cells expressed I_h ($n = 27$).

the hyperpolarization-activated current (I_h) as determined from instantaneous current-voltage relationship using 70 mM bath $[Na^+]_{ext}$ was -37 ± 6 mV ($n = 11$) at 5-DPI. The results were consistent with a K^+/Na^+ -permeable conductance. Using the Goldman-Hodgkin-Katz equation (Hille, 1978), we determined the permeability ratio of K^+ versus Na^+ (P_K/P_{Na}) to be ~ 0.8 . The steady-state activation curves were fitted according to the Boltzmann function (Fig. 4D). At 5-DPI, the $V_{1/2}$ activation = -95.2 ± 1.4 mV, $k_m = 15.3 \pm 1.7$ (n = 7); at 10-DPI, $V_{1/2}$ activation = -87.2 ± 0.6 mV, $k_m = 16.5 \pm 0.8$ (n = 7); and at day 15-DPI, $V_{1/2}$ activation = -94.5 ± 1.2 mV, $k_m = 14.0 \pm 1.4$ (n = 5). As illustrated in Figure 4E, the current was insensitive to external Ba^{2+} but was blocked reversibly by external Cs^+ , consistent with I_h (Yamoah et al., 1998a). The current-density volt-

age relationships summarize the effects of Ba^{2+} and Cs^+ on I_h (Fig. 4F). The total number of cells that were sampled for I_h and the time-dependent changes portray a current that was fleeting in nature. At 5-DPI, 100% of the cells expressed I_h ($n = 32$) and by 10-DPI, $\sim 51\%$ of newly formed hair cells expressed I_h ($n = 27$). Finally, by 15-DPI only $\sim 17\%$ of newly formed hair cells expressed I_h ($n = 27$; Fig. 4G).

Outward currents in Ad5-EGFP-Atoh1-infected cells

Macroscopic outward K^+ currents were recorded from Ad5-EGFP-Atoh1- and Ad5-EGFP-infected cells. Figure 5A (left) shows outward currents evoked from a holding potential of -90 mV from Ad5-EGFP-infected cells, 7-DPI. EGFP alone-infected and supporting cells are endowed with a small and sustained K^+

Figure 5. Outward K⁺ current of newly formed hair cells at the LER. **A**, Cells were held at a holding potential of -90 mV and stepped to voltage ranges from -90 to 60 mV using ΔV of 10 mV. Supporting cells infected with Ad5-EGFP alone expressed a relatively small and sustained outward K⁺ current for day 5 ($n = 4$), 7 ($n = 10$), and 10 ($n = 3$). Shown is an example of the protocol used to generate the family of current traces. The representative traces (left) were recorded from a day 7 cell post Ad5-EGFP infection (7-DPI). Shown in the middle are current traces recordings from a day 5 cell post Ad5-EGFP-Atoh1 infection (5-DPI). The newly formed hair cells began to express a transient outward K⁺ current, which was generated using the same protocol as described above. Subsequently at 10-DPI and 15-DPI, newly formed hair cells predominantly expressed a sustained outward K⁺ current. **B**, A summary of the peak current-density voltage relationship of K⁺ currents recorded from Ad5-EGFP alone-infected cells 7-DPI (□, $n = 10$). Also, we show data from Ad5-EGFP-Atoh1 after infected cells 5-DPI (■, $n = 15$), 10-DPI (○, $n = 17$), and 15-DPI (●, $n = 21$). **C**, Shown the changes of transient and sustained components at different stages after Atoh1 infection. To record the transient current, cells were held at -90 mV and stepped to -20 mV. The sustained current was recorded from a holding potential of -30 mV and stepped to 0 mV. Holding cells at -30 mV was sufficient to remove the transient current by way of inactivation. The histogram is a summary of the changes in the transient and sustained K⁺ currents in newly formed hair cells. **D**, Shown is a bar graph depicting the percentages of cells that expressed transient and sustained components of K⁺ currents in newly formed hair cells. At day 5, ~75% of newly formed hair cells showed the transient current, and the remaining 25% had a sustained component ($n = 12$). By day 10, ~11% of newly formed hair cells expressed the transient current, and the remaining ~89% expressed the sustained current ($n = 27$). Finally, by day 15 only a small fraction, ~5%, of newly formed hair cells expressed K⁺ current with a transient component ($n = 17$).

current. In contrast, 2-DPI of Ad5-EGFP-Atoh1 a transient outward K⁺ current emerged, which increased in magnitude until ~day 5 (Fig. 5A, middle). Subsequently, a sustained outward K⁺ current surfaced, reaching maximum amplitude at 15-DPI. The current density of the outward K⁺ current increased by at least 2.5-fold from 5-DPI to 15-DPI and transitioned from a mainly transient to sustained component (Fig. 5A). Analyses of the early transient K⁺ currents portray a current that was exquisitely sensitive to the holding voltage (Fig. 6A) and was blocked by 2.5 mM 4-aminopyridine (4-AP) (data not shown), meeting the criteria as I_A (Yamoah, 1997). The current density-voltage relationships of the transient (I_A) and sustained currents at 5-DPI and 15-DPI are shown (Fig. 5B). We examined the transition between the transient and sustained K⁺ current (Fig. 5C). Additionally, percentages of cells that expressed the transient and sustained components of K⁺ currents in newly formed hair cells were assessed outlining dynamic changes in current transition. At 5-DPI, ~75% of newly formed hair cells showed the transient current, and the remaining 25% had a sustained component ($n = 12$). By 10-DPI, ~11% of newly formed hair cells expressed the transient current, and the remaining ~89% expressed the sustained current ($n = 27$). Finally, by 15-DPI only ~5% of newly formed hair cells expressed K⁺ current with a transient component ($n = 17$).

The steady-state activation of the transient current was generated from standard tail currents and fitted with a Boltzmann

function (Fig. 6A,B). The voltage-dependent activation of the transient current is described with a half-activation of 15.8 ± 1.9 mV and a slope factor of 15.7 ± 1.4 mV ($n = 6$). Likewise, the steady-state inactivation was investigated by the application of ~3 s conditioning pulses to potentials between -90 and 60 mV, followed by a test pulse of 10 mV (Fig. 6B, see inset for example of current traces). The non-inactivating component elicited at the test pulse was subtracted from the total current to generate the inactivation curve (Fig. 6B). I_A was half-inactivated at -16 ± 0.3 mV, and the curve had a slope factor of 8.2 ± 0.3 mV ($n = 9$). At 10-DPI and 15-DPI, when ~90–95% of the outward K⁺ current was derived from the sustained current, we examined the steady-state activation properties (Fig. 6C,D). The half-maximal activation voltages and slope factors were as follows: 10-DPI = -15.0 ± 1.4 mV and 11.1 ± 1.0 mV ($n = 9$) and 15-DPI = -4.7 ± 0.4 mV and 10.8 ± 0.1 mV ($n = 6$).

Mechano-electrical transducer currents in Ad5-EGFP-Atoh1-infected cells

To obtain direct evidence for mechano-electrical transduction in Ad5-EGFP-Atoh1-infected cells, we recorded the transducer currents from 7-DPI hair cells using the whole-cell patch-clamp technique. Cells were held at -80 mV. Hair bundles were displaced with a stiff-probe (pulled glass electrode) that was attached to a piezoelectric stack actuator (Physik Instrument). We

Figure 6. Steady-state activation and inactivation of transient and sustained outward K⁺ currents in newly formed hair cells mediated by Ad5-EGFP-Atoh1 infection. **A**, We examined the transient component of the outward K⁺ current by recording 5-DPI cells. Cells were held at a -90 mV holding potential and stepped to varying voltages ranging from -90 to 60 mV (left). The same cells were held at a holding potential of -30 mV and stepped to similar varying voltages (middle). The difference current traces (current at -90 mV minus current at -30 mV holding potentials) shown in gray (right). **B**, The voltage dependence of activation and inactivation of the transient current were examined as described in Figure 2. We used the apparent reversal potential of the K⁺ current ($E_K = -86$ mV) determined from instantaneous current-voltage relations (data not shown). Shown in the inset on the right are representative current traces used to generate the steady-state inactivation curve. The Boltzmann function fits of the activation and inactivation curves were; $V_{1/2}$ activation = 15.8 ± 1.9 mV, $k = 15.8 \pm 1.4$ (n = 6); $V_{1/2}$ inactivation = -16.1 ± 0.3 mV, $k = 8.2 \pm 0.3$ (n = 9). **C**, Current traces recorded from a holding potential of -90 mV and stepped to varying potentials using ΔV of 10 mV in 10-DPI cells. A substantial component of the current represented the sustained K⁺ current. **D**, The steady-state activation curves for hair cells 10-DPI and 15-DPI were; $V_{1/2}$ activation (10-DPI) = -15.0 ± 1.4 mV, $k = 11.1 \pm 1.0$ (n = 9); and $V_{1/2}$ activation (15-DPI) = -4.7 ± 0.4 mV, $k = 10.8 \pm 0.4$ (n = 6).

could not assess the exact time of first appearance of transducer currents because before 6-DPI the hair bundles were too stunted to be stimulated mechanically with a glass electrode.

The maximum currents at 7-DPI and 15-DPI for cells at the apical aspects of the LER of the cochlea were as follows: 76 ± 24 pA (n = 12) and 193 ± 38 pA (n = 12) (Fig. 7A), respectively. A one-state Boltzmann fit to the displacement open channel probability (D-P_o) curves showed a half-maximum displacement ($X_{1/2}$) at 7-DPI and 15-DPI in ectopic hair cells at the apical LER to be 0.61 ± 0.06 and 0.51 ± 0.08 μ m (n = 7; Fig. 7B). Atoh1-mediated trans-differentiated hair cells at the basal aspects of the LER expressed transducer currents with magnitudes similar to cells at the apical region at 7-DPI [maximum current for apical LER cells = 76 ± 24 pA (n = 12) and basal hair cells = 89 ± 39 pA (n = 11; p = 0.1; Fig. 7C)]. Moreover, by 15-DPI the magnitude of the transducer current of basal LER cells increased by ~2-fold compared with cells at the apical aspects (Fig. 7C). Leftward shifts in the displacement-response relationship as cell matured from 7-DPI to 15-DPI were common trends (Fig. 7D, F, H). This is also illustrated in the summary data showing changes in $X_{1/2}$ from 7 to 15-DPI (Fig. 7I). A progressive increase in the

transducer current magnitude and changes in the kinetics of adaptation from 7-DPI to 15-DPI in Atoh1-mediated ectopic hair cells in the utricle were consistent with alterations described in cells at the LER (Fig. 7E, G). However, for trans-differentiated cells in the maculae, of 38 cells, 25 had enhanced slow kinetics of adaptation (Fig. 7G, J).

Development of hair bundles and expression of hair bundle proteins in Ad5-EGFP-Atoh1-infected cells

To determine whether the appearance of stereociliary bundles matched well with the observed transducer current, we prepared the cochlea and utricle at 3-DPI and 6-DPI of Ad5-EGFP-Atoh1 for scanning electron microscopy to assess hair bundle development and morphology. In accordance with previous studies (Cotanche and Corwin, 1991), preambles to the appearance of hair bundles were staged with increased microvilli followed by a staircase configuration of stereociliary bundles and systematic pruning of the microvilli on the apical aspects of trans-differentiated hair cells (Fig. 8A–H). By 6-DPI, stereociliary bundles were found predominantly on the apical surface of ectopic cochlear and utricular hair cells. A prominent feature of the newly differ-

Figure 7. Mechano-electrical transducer (MET) current of newly formed hair cell-like cells. Time-dependent changes in MET current magnitude and properties. Newly differentiated hair cells were held at a holding potential of -80 mV via a patch electrode containing K^+ channel blockers (see Materials and Methods). A second glass electrode was used to displace hair bundles in steps of ~ 0.1 μm from -0.15 to 1.0 μm . **A**, Examples of MET current traces recorded from newly formed hair cells at the LER at the apical aspects of the cochlea 7-DPI (left). The right panel shows similar MET current traces recorded from newly formed hair cells 15-DPI. The magnitude of the current was increased by at least twofold from 7-DPI to 15-DPI. The maximum MET current for ectopic hair cells at the apical aspects of the LER at 7-DPI was 76 ± 24 pA ($n = 12$) and 15-DPI was 193 ± 38 pA ($n = 12$; $p < 0.01$). **B**, The displacement-response (D-R) relationships shown as normalized current against displacement were fitted to a single Boltzmann function and the best fits for the half-maximum displacement ($X_{1/2}$) at 7-DPI were 0.61 ± 0.06 μm and at 15-DPI 0.51 ± 0.08 μm ($n = 7$). The slope factors (in μm^{-1}) were 0.082 ± 0.011 and 0.084 ± 0.020 ($n = 7$) at 7-DPI and 15-DPI, respectively. **C**, MET currents from ectopic hair cells at the basal aspects of the LER at 7-DPI and 15-DPI. The magnitude of the maximum current for ectopic hair cells at the basal aspects of the LER at 7-DPI was 89 ± 39 pA ($n = 11$) and at 15-DPI was 485 ± 98 pA ($n = 11$; $p < 0.01$). **D**, For hair cells at the basal LER, the $X_{1/2}$ values at 7-DPI were 0.56 ± 0.08 μm and at 15-DPI 0.35 ± 0.05 μm ($n = 8$). The slope factors (in μm^{-1}) were 0.05 ± 0.02 and 0.10 ± 0.01 ($n = 8$) at 7-DPI and 15-DPI, respectively. **E**, Representative MET current traces generated from Atoh1-mediated trans-differentiated hair cells outside the utricular maculae. The left traces were from 7-DPI cells and the right traces are from 15-DPI cells. The MET current magnitude increased by ~ 3 -fold from 7-DPI to 15-DPI [maximum current at 7-DPI = 195 ± 48 pA and at 15-DPI = 656 ± 106 pA ($n = 7$)]. **F**, At 7-DPI, the $X_{1/2}$ values were 0.44 ± 0.05 μm and at 15-DPI 0.33 ± 0.02 μm ($n = 7$). The slope factors (in μm^{-1}) were 0.06 ± 0.01 and 0.09 ± 0.04 ($n = 7$) at 7-DPI and 15-DPI, respectively. **G**, In contrast to ectopic hair cells, Atoh1-mediated newly differentiated cells in the utricular maculae expressed MET currents that were ~ 2.5 -fold (475 ± 61 pA, $n = 10$) larger in magnitude than ectopic hair cells by 7-DPI. The size of the MET current increased further by ~ 1.5 -fold by 15-DPI (742 ± 79 pA, $n = 10$). **H**, For newly differentiated hair cells in the utricular maculae, the $X_{1/2}$ values at 7-DPI were 0.37 ± 0.04 μm and at 15-DPI 0.26 ± 0.06 μm ($n = 10$). The slope factors (in μm^{-1}) were 0.06 ± 0.01 and 0.13 ± 0.04 ($n = 10$) at 7-DPI and 15-DPI, respectively. **I**, Summary data of changes in $X_{1/2}$ from 7-DPI to 15-DPI (apical LER cells (●), basal LER cells (○), utricular ectopic cells (■), Atoh1-mediated hair cells in utricular maculae (□); $n = 7$; error bars represent SEM). **J**, The profile of adaptation were best fitted with two-exponential time constants (time constants of adaptation, τ_{adap}), fast and slow τ_{adap} . Shown in the inset is an example of MET currents decay, which was fitted with two τ_{adap} (fitting curve in green; $r^2 = 0.85$). Summary data of fast τ_{adap} from 7-DPI to 15-DPI [apical LER cells (●), basal LER cells (○), utricular ectopic cells (■), Atoh1-mediated hair cells in utricular maculae (□); $n = 6$]. Symbols for slow τ_{adap} are as follows: apical LER cells (▲), basal LER cells (△), utricular ectopic cells (◆), Atoh1-mediated hair cells in utricular maculae (224); $n = 6$.

entiated hair cells was the morphology of hair bundles. Kinocilia were present in $\sim 60\%$ of the hair bundles. Invariably, none of the ectopic cells at the lesser epithelia ridge ever assumed the characteristic features of inner and/or outer hair cell bundle structures. Instead, of 289 cells that were counted in different preparations, all bore a striking resemblance to vestibular hair cell bundles. Several components of the transducer apparatus have been identified. These apparatuses are expressed at distinct stages of development (Si et al., 2003). We examined whether two of these proteins, i.e., myosin VIIA and plasma membrane Ca^{2+} ATPase isoform 2 (PMCA2), are expressed in newly trans-differentiated hair cells in the utricle at day three post Ad5-EGFP-Atoh1 infec-

tion. As it was evident in Ad5-EGFP-Atoh1-infected cells, there were marked expressions of myosin VIIA and PMCA2 (Fig. 9A–N). The expression of PMCA2 was consolidated mainly at the apical aspects of newly differentiated hair cells, raising the possibility that these cells may have been equipped with transducer current machinery earlier than it was practical to record the currents as demonstrated in Figure 7, albeit smaller in magnitude.

Innervations of Ad5-EGFP-Atoh1-infected cells

We have demonstrated that Ad5-EGFP-Atoh1-infected cells differentiate into hair cells equipped with transducer current machinery. Additionally, the newly differentiated cells undergo

Figure 8. Developmental changes of hair bundle morphology in newly formed hair cells at the LER and the utricle. Shown are hair bundle morphology of native hair cells and newly formed hair cells induced by Atoh1 in the cochlea and utricle. **A**, Scanning electron micrograph of the apical aspects of a native outer hair cell from the apical turn of the cochlea. **B**, At 3-DPI, there was an increased appearance of microvilli on the apical aspects of the newly formed hair cell-like cells at the LER, with a single long kinocilium-like bundle (white arrows). **C**, By 4-DPI, there were scattered stereocilia-like bundles at the surface of the newly formed hair cell-like cells in the LER. **D**, Hair bundles attained their organized stair case shape by 6-DPI. Note that in contrast to native cochlear hair bundles, newly formed hair bundles at the LER do not have the typical V-shape morphology. **E–H**, Comparison of bundles of native hair cells and ectopic hair cells induced by Atoh1 in the utricle. **E**, Typical native hair cells from the utricle. **F**, By 3-DPI, there were increased stereocilia-like bundles at the surface of the newly formed hair cell-like cells, with an associated long kinocilium. **G**, Finally, by 6-DPI, the stereocilia of the newly formed hair cells become well organized in a staircase morphology. **H**, Shown is a magnified view of a hair bundle of one of the newly formed hair cell-like cells from **G**.

ionic conductance transformations that promote a transition from the signature phenotype of evoked action potentials in developing and regenerating hair cells to mature quiescent cells (Marcotti et al., 2003a; Housley et al., 2006; Levic et al., 2007). Cessation of this rhythmic membrane activity dovetails well with the establishment of synapses (Pienkowski and Harrison, 2005; Housley et al., 2006). We examined whether ectopic hair cells also received innervation from spiral ganglion neurons, testing the correlative hypothesis that rhythmic membrane oscillations of the newly differentiated hair cells are preambles to synaptogenesis (Eatock and Hurley, 2003). The LER of the developing cochlea is abneural to projections from the spiral ganglion neurons. Surprisingly, as demonstrated in Figure 10, following differentiation of new hair cells at the LER, we identified neurite outgrowths that traverse $\sim 200 \mu\text{m}$ distance of the previously abneural aspects of the cochlea to make contacts with new hair cells. Thus, Ad5-EGFP-Atoh1 infection-mediated trans-differentiation may be equipped with the intrinsic and extrinsic modalities to form bonafide hair cells.

Discussion

The past decade has witnessed several discoveries about the early events in evolution and development that transformed two simple patches of the inner ear sensory epithelia to a complex nine-patched 3D structure. Traces of these processes are increasingly becoming apparent in some details, e.g., the identification of endorgan-specific rudimentary genes such as *hHLH*, *Wn*, and *Otx* (Morsli et al., 1999; Reichert and Simeone, 1999; Fritzsche et al., 2001; Fekete and Wu, 2002), and cell-fate acquisition genes such as *Atoh1* for hair cells and *neurogenin 1* (*ngn-1*) as a proneuronal gene (Bermingham et al., 1999; Ma et al., 2000). In contrast, the ensuing specialization of endorgan-specific hair cells and whether they evolved from discrete organs as opposed to a progressive segregation from a single rudimentary organ have remained largely under-studied.

The major finding of this study was the observation that Atoh1-mediated trans-differentiation of supporting cells into hair cells at the LER of the cochlea assumes a morphological phenotype that is in keeping with the gross structure of “ancestral” hair cells that have features similar to vestibular hair cells. In

contrast to developing and regenerating inner hair cells in mice and the chicken basilar papilla (Marcotti et al., 2003b; Levic et al., 2007), which showed robust spontaneous and evoked spike activity, the developing rat utricular hair cells have not been shown to exhibit spontaneous action potentials and are resistant to multiple spikes induced by depolarization or as “anode-break” responses to membrane hyperpolarization (Chabbert et al., 2003; Géléoc et al., 2004; Wooltorton et al., 2007). Of 280 trans-differentiated cells at the lesser epithelia ridge aspects of the cochlea that were sampled under current-clamp configuration, 243 cells showed evoked spikes that occurred as single events, and 31 cells showed membrane oscillations ranging between -50 to -39 mV but were defiant to evoked spiking activity. The remaining three cells exhibited SAPs, as illustrated (Fig. 1E). Additionally, in stark contrast to immature cochlea/basilar papillar hair cells of mice and chickens, which express marked Ca^{2+} -dependent spikes (Marcotti et al., 2003a; Levic et al., 2007), application of TTX and replacement of external Na^{+} with monovalent cations virtually attenuated evoked action potentials in ectopic Atoh1-mediated hair cells at the lesser epithelia ridge, which is similar to findings from immature utricular hair cells (Chabbert et al., 2003; Géléoc et al., 2004). These findings are also consistent with Na^{+} currents reported for Atoh1-induced hair cells produced in the cochlea by *in utero* gene transfer (Gubbels et al., 2008). Thus, the functional characteristics of Atoh1-mediated trans-differentiated hair cells were reminiscent of the developing vestibular hair cells.

The underlying Na^{+} currents (I_{Na}) control the upstroke phase and frequency of action potentials in immature hair cells in the cochlea and basilar papilla (Marcotti et al., 2003a; Levic et al., 2007) and initiate spike activity in the utricle (Géléoc et al., 2004; Wooltorton et al., 2007). The fleeting expression of I_{Na} is a shared phenotype with Atoh1-mediated hair cells. The canonical description of Na^{+} currents in immature hair cells falls under three distinct biophysical and pharmacological properties, namely, (1) TTX-resistant Na^{+} currents with a $V_{1/2}$ of voltage-dependent inactivation ($V_{1/2i}$) of ~ -90 mV (Oliver et al., 1997; Géléoc et al., 2004; Wooltorton et al., 2007), (2) TTX-sensitive Na^{+} currents with a $V_{1/2i}$ of ~ -80 to -70 mV (Chabbert et al., 2003; Marcotti

Figure 9. Newly formed hair cells in the sensory epithelium of the utricle and ectopic hair cells induced by Atoh1. Six days after Ad5-EGFP-Atoh1 infection, newly formed hair cell-like cells emerged as ectopic hair cells in the utricle. **A**, Shown is EGFP Atoh1 expression in the sensory (large left square) and ectopic (small right square) regions in the utricle (in green). **B**, The plasma membrane Ca^{2+} ATPase isoform 2 (PMCA2) is expressed in hair bundles (Yamoah et al., 1998b). PMCA2 is expressed in the bundles of native (large left square) and ectopic hair cells (small right square) in the utricle (in red). **C**, Shows a merged image of **A** and **B** of newly formed hair cells. **D**, Photomicrographs of a magnified view of the sensory region (large left square) of the utricle from **A–C**. **E**, Shown is the labeling of myosin VIIA expression in hair cells (blue). **F–G**, PMCA2 labeling (red) and merged image, respectively. **H–J**, Magnified views of the ectopic region of the utricle from the small right square in **A–C**; EGFP is in green, myosin VIIA-positive cells are in blue, and PMCA2 is in red. **K**, Shown is the merged image of **H–J**. **L–M**, Magnified views of ectopic hair cells showing labeling of myosin VIIA, PMCA2, and EGFP. **N**, A 3D reconstructed image of ectopic hair cells (myosin VIIA, blue; PMCA2, red). Scale bars: **A–C**, 100 μm ; **D–K**, 20 μm ; **L–M**, 10 μm .

et al., 2003b; Wooltorton et al., 2007), and finally (3) TTX-sensitive Na^+ current with a $V_{1/2I}$ of ~ -95 mV (Evans and Fuchs, 1987; Witt et al., 1994; Masetto et al., 2003). Although it has been suggested that the recorded $V_{1/2I}$ raise the likelihood that a substantial population of Na^+ channels in hair cells are physiologically silent at the recorded resting membrane potentials (-50 to -70 mV) (Witt et al., 1994), it is conceivable that the *in vivo* resting membrane potentials are more negative than was documented in hair cells of the goldfish saccule (Sugihara and Furukawa, 1989). The voltage dependence of activation and inactivation of Na^+ currents are labile depending on the extent of the intracellular subunit phosphorylation (Chen et al., 2006; Chen et al., 2008),

auxiliary subunit modulations (Ko et al., 2005; Maltsev et al., 2009), extracellular glycosylation (Tyrrell et al., 2001; Stocker and Bennett, 2006), and Ca^{2+} /Calmodulin modulation (Wingo et al., 2004; Biswas et al., 2009). For example, $V_{1/2I}$ of Na^+ channels are exquisitely sensitive to available intracellular Ca^{2+} concentrations, shifting to extreme negative voltages in apparent 0 mM intracellular Ca^{2+} concentration ($V_{1/2I} = \sim -100$ mV) (Wingo et al., 2004). Thus, it is plausible to speculate that the diverse $V_{1/2I}$ of Na^+ currents, which have been reported for immature hair cells, are reflections of the extent of Ca^{2+} chelation of patch-pipette solutions used in different studies. Virtual attenuation of evoked APs upon substitution of Na^+ ions, without a sub-

Figure 10. Newly formed hair cells at the LER induced by Atoh1 are innervated by native spiral ganglion neurons in the cochlea. Examination of newly formed ectopic hair cells 7-DPI shows that ectopic hair cells are innervated by spiral ganglion neurons (SGNs). **A**, EGFP-Atoh1 overexpression in cells at the LER is in green. **B**, Shown are myosin VIIA-positive cells at both the sensory epithelia region (bottom; outlined with a white bracket) and at the LER (top cells) in red. **C**, Radial fibers of SGNs that project to the sensory and ectopic epithelia regions in blue. **D**, Shown is a merged image of radial fibers of SGNs that project to EGFP-Atoh1-myosin VIIA-positive cells. **E–H**, A 3D reconstruction of radial fibers of SGNs projecting to EGFP-Atoh1-myosin VIIA-positive cells (face-up view). **I–L**, Face down view of 3D reconstruction of radial fibers SGNs projecting to EGFP-Atoh1-myosin VIIA-positive cells. Scale bars: **A–D**, 20 μ m; **E–M**, 30 μ m. White bracket: sensory region.

stantial change in membrane surface charge, is strong evidence for the contribution of Na^+ currents in the initiation of APs in this report. Moreover, the steady-state voltage dependence gleaned from Atoh1-mediated hair cells suggests that $\sim 30\%$ of the Na^+ current is available for activation at rest (-60 to -45 mV), which may suffice to initiate APs.

Another inward current which may promote membrane depolarization and oscillations in immature hair cells would be the K^+/Na^+ -permeable inward rectifier current. Previous reports have identified inward rectifier currents in a variety of developing and adult hair cells from auditory and vestibular endorgans (Ohmori, 1984; Fuchs and Evans, 1990; Masetto et al., 1994; Holt and Eatock, 1995; Marcotti et al., 1999; Géléc et al., 2004). Inward rectifier currents vary from those described as K^+ -selective I_{K1} to K^+/Na^+ -permeable I_{h} . Invariably, the inward rectifier currents are developmentally regulated, appearing transiently during development as seen in inner hair cells (Marcotti et al., 1999) or undergoing apparent changes in their selectivity, such that the estimated reversal potentials of the current transition from ~ -35 mV at E14 to ~ -53 mV at P0 in the developing mouse utricular hair cells (Géléc et al., 2004). Because the recorded resting membrane potentials of hair cells are usually more positive (-50 to -70 mV) than the reversal potential of K^+ (~ -80 mV), the monovalent cation-selective current may contribute substantially in dictating the resting membrane potential of developing hair cells (Holt and Eatock, 1995), promoting membrane depolarization in developing and Atoh1-mediated transdifferentiated hair cells. The acquisition of transducer currents in the developing and regenerating cochlea/basilar papilla and utricle have been studied extensively. Whereas transducer currents in the utricle are expressed in an all-or-none fashion (Géléc and Holt, 2003), developing and regenerating hair cells in the cochlea and basilar papilla acquire transducer currents in a stepwise man-

ner (Si et al., 2003; Waguespack et al., 2007). In Ad5-EGFP-Atoh1-infected cells, the time constant of adaptation appeared to increase in contrast to previous reports. The apparent differences may ensue from the following: (1) *in vitro* trans-differentiation conditions and (2) immature bundle geometry at the recording time. However, it is very unlikely that it is a function of stimulus method since recording from mature cells from the utricle and cochlea yielded data that were in keeping with previous studies (data not shown).

The acquisition of voltage-dependent outward conductances in newly differentiated hair cells under the induction of Atoh1 at the lesser epithelia ridge also dovetailed well with developing vestibular and primordial auditory hair cells. In mammalian auditory hair cells, a delayed rectifier K^+ current (I_{K}) debuts at E14.5 at the basal and at E15.5 at the apical cochlea, increasing in magnitude until the onset of hearing (Marcotti et al., 2003a; Helyer et al., 2005; Housley et al., 2006). Other outward K^+ conductances such as Ca^{2+} -activated K^+ and KCNQ4 currents are unveiled at, or near, the onset of hearing (Kros et al., 1998; Marcotti et al., 2003a, 2004). In stark contrast to developing mammalian auditory hair cells, developing rat utricular and chicken vestibular hair cells expressed a transient outward conductance that is sensitive to 4-AP, which transitioned later into a sustained outward conductance (Sokolowski et al., 1993; Lennan et al., 1999). Indeed, hair cells derived from embryonic and induced pluripotent stem cells expressed a rapidly activating K^+ current that resembles I_{A} (Oshima et al., 2010). Thus, the manner in which Atoh1-mediated ectopic hair cells develop mirrors the developmental changes reported from developing vestibular hair cells.

Patterned membrane potential fluctuations in developing sensory neurons play an instructive role in mediating the release of tropic and trophic factors to promote directional outgrowth, survival of neurons, as well as refinement of synapses (Hemond and Morest, 1992; Katz and Shatz, 1996; Zhang and Poo, 2001;

Chabbert et al., 2003; Blankenship and Feller, 2010). The structure of different firing/spontaneous/evoked patterns encodes information that may translate into variable gene expression that contributes to proper functional development (Fields and Itoh, 1996; Katz and Shatz, 1996; Tao et al., 2002; Johnson et al., 2007). Moreover, developing and regenerating hair cells undergo spontaneous and evoked membrane fluctuations that are thought to enhance neurotrophin (Chabbert et al., 2003) and neurotransmitter (Marcotti et al., 2003b) release to serve as chemoattractants for directional neural outgrowths and affect activity-dependent regulation of postsynaptic neurites (Fekete and Campero, 2007). The observed directed outgrowth of neurites toward newborn hair cells correlates well with the concept that ectopic hair cells release chemoattractants. These findings are in keeping with previous studies suggesting that ectopic hair cells may emit chemoattractants for directional migration of spiral ganglion neuron innervation at the neural aspects of the cochlea (Zheng and Gao, 2000; Gubbels et al., 2008). Additionally, after inducing the expression of Wnt/ β -catenin signaling in the chicken cochlear duct, ectopic vestibular sensory cells received innervation from spiral ganglion neurons (Stevens et al., 2003), promoting the view that ectopic sensory patches are the hub for chemotropins. In contrast to studies that inferred that the developing hair cells may be sufficient to attract projecting neurites, several loss-of-function studies of *Pou4f3* and *Atoh1* knock-out mice have suggested that hair cells are not necessary for innervation of sensory primordia (Xiang et al., 2003; Fritzsche et al., 2005). Compensatory changes associated with loss-of-function experiments may underlie the apparent differences in the later findings. Nonetheless, innervation of ectopic hair cells at a considerable distance away, at the abneural aspect of the cochlea, as documented in this report, is strong evidence to support the notion that trans-differentiated hair cells may produce chemotropins to confer neurite pathfinding mechanisms.

The implications of these findings raise the likelihood that subsequent to *Atoh1*-mediated trans-differentiation of hair cells, endorgan-specific genes may be required to affect further morphological and functional diversification. Additionally, this study furnishes important morphological and functional features that exist in *Atoh1*-mediated trans-differentiated hair cells and developing and regenerating hair cells (Levic et al., 2007). Finally, the findings established by this study not only deepen our mechanistic understanding of the importance of primordial rhythmic electrical activity in developing and regenerating hair cells, but also bring to light previously unidentified, yet significant, common features of the trans-differentiating hair cells that may be relevant for maturation.

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