RESEARCH ARTICLE

Cell proliferation during hair cell regeneration induced by $Math_1$ in vestibular epithelia *in vitro*

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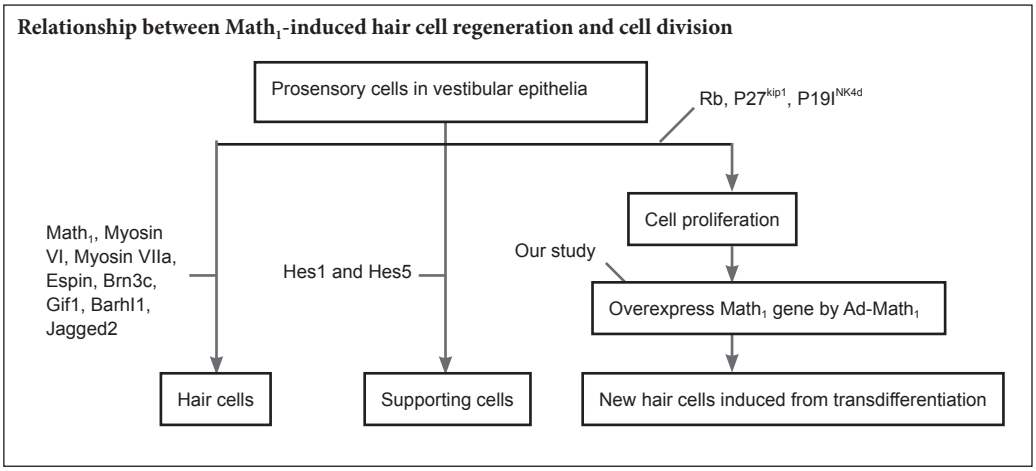
Graphical Abstract



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Abstract

Hair cell regeneration is the fundamental method of correcting hearing loss and balance disorders caused by hair cell damage or loss. How to promote hair cell regeneration is a hot focus in current research. In mammals, cochlear hair cells cannot be regenerated and few vestibular hair cells can be renewed through spontaneous regeneration. However, $Math_1$ gene transfer allows a few inner ear cells to be transformed into hair cells *in vitro* or *in vivo*. Hair cells can be renewed through two possible means in birds: supporting cell differentiation and transdifferentiation with or without cell division. Hair cell regeneration is strongly associated with cell proliferation. Therefore, this study explored the relationship between $Math_1$-induced vestibular hair cell regeneration and cell division in mammals. The mouse vestibule was isolated to harvest vestibular epithelial cells. Ad-$Math_1$-enhanced green fluorescent protein (EGFP) was used to track cell division during hair cell transformation. 5-Bromo-2′-deoxyuridine (BrdU) was added to track cell proliferation at various time points. Immunocytochemistry was utilized to determine cell differentiation and proliferation. Results demonstrated that when epithelial cells were in a higher proliferative stage, more of these cells differentiated into hair cells by $Math_1$ gene transfer. However, in the low proliferation stage, no BrdU-positive cells were seen after $Math_1$ gene transfer. Cell division always occurred before $Math_1$ transfection but not during or after $Math_1$ transfection, when cells were labeled with BrdU before and after Ad-$Math_1$-EGFP transfection. These results confirm that vestibular epithelial cells with high proliferative potential can differentiate into new hair cells by $Math_1$ gene transfer, but this process is independent of cell proliferation.

Key Words: *nerve regeneration; cell proliferation; cell division; $Math_1$; hair cells; hair cell renewal; supporting cell differentiation; vestibular cells; neural regeneration*

Introduction

Mammalian sensorineural hearing loss and some balance diseases are incurable. The most important reason for this is that cochlear hair cells cannot be regenerated, and only a few balance cells can be spontaneously regenerated. However, $Math_1$ over-expression-induced robust production of hair cells provides the potential to develop a strategy for functional hair cell regeneration (Zheng and Gao, 2000; Kawamoto et al., 2003; Staecker et al., 2007; Izumikawa et al., 2008; Han et al., 2010). Induced by $Math_1$, stem cells, cochlear and vestibular cells have the capacity to differentiate into new hair cells (Zheng and Gao, 2000; Kawamoto et al., 2003; Staecker et al., 2007; Gubbels et al., 2008; Huang et al., 2009; Han et al., 2010; Jeon et al., 2011). However, new hair cells cannot be regener-

ated using $Math_1$ gene transfer (Izumikawa et al., 2008). This then leads to the question: what types of cells have the potential to differentiate into new hair cells induced by $Math_1$? No confirmed factors have been reported to date.

In birds, hair cells can be spontaneously regenerated after injury. Two means of regeneration have been reported (Stone and Cotanche, 2007): the direct transdifferentiation of supporting cells into hair cells and supporting cell proliferation with the subsequent conversion into hair cells. In the mammalian embryonic stage, hair cells are generated by transdifferentiation with a lack of cell division and $Math_1$ playing a role in cell fate determination (Chen et al., 2002; Woods et al., 2004); After birth, *in vivo* cochlear hair cells could not be regenerated spontaneously, while *in vitro* supporting cells divided and transdifferentiated into hair cells after dissociation (White et al., 2006). Spontaneous regeneration or repair of balance hair cells with proliferation was observed *in vitro* and *in vivo* (Forge et al., 1993; Warchol et al., 1993). Previous studies showed that cells successfully differentiated into new hair cells by $Math_1$ had a high proliferative ability (Zheng and Gao, 2000; Gubbels et al., 2008; Huang et al., 2009; Han et al., 2010). The current study explored the relationship between $Math_1$ induced hair cell regeneration and cell division in mammals.

Materials and Methods

Animals

All animal experiments were performed under the guideline of the Ethics Committee Protocols of the Eye and Ear, Nose and Throat Hospital of Fudan University of China, and approved by the Chinese Science Academy Committee on Care and Use of Animals. The day when male specific-pathogen-free C57BL/6 mice (provided by Shanghai SLAC Laboratory Animal Co., Ltd., Shanghai, China; license No. SCXK (Hu) 2012-0002) were born was designated P0, the next day as P1, and P2, P3, and P4. P2–4 mice were used in this study.

Sample collection

A detailed protocol on dissecting vestibular end organs was previously reported (Huang et al., 2009). The dissection process was carried out in a sterile environment and samples were placed in chilled D-Hank's solution. Two fine forceps (0.1 mm at the point end; Dumont Biology, La Sagne, Switzerland), pairs of Vannas scissors and iris scissors, and stainless steel needles were used.

The heads of postnatal mice were removed and bisected through the midline. The brain tissue was removed with forceps. Utricle and cristae were harvested together, and attached to cover-slips pretreated with poly-L-lysine (Sigma, St. Louis, MO, USA). With the forceps, the otolithic membrane and nerve fibers at the back of the epithelia were removed before attachment. The utricle and cristae were attached to cover-slips with the hair cell side upwards. To obtain damaged utricles (Meyers and Corwin, 2007), stainless steel needles were pressed into utricles to form lesions in the hair cell epithelium, and cells within the lesion were removed with a sharp needle and forceps.

Culture and transfection of vestibular epithelia

Vestibular epithelia were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco) for the first 12–15 hours. DMEM/F12 medium supplemented with B27 was used in the following culture. Half of the medium was replaced with fresh culture medium every two days. The cultures were incubated in a 95% air, 5% CO_2-humidified environment at 37°C. Ad-$Math_1$-enhanced green fluorescent protein (EGFP) vectors (AD5-E1/E3-defected-$Math_1$/EGFP, PFU 1.0×10^{11}, Ad0112d, Beijing Sinogenemax Co., Beijing, China) or Ad-EGFP vectors (as controls) (AD-EGFP, PFU 1×10^{11}, Beijing Sinogenemax Co.) with a final concentration of 1×10^8/mL were added to the culture medium at 1 day *in vitro* (cultures were denoted as 0 day *in vitro* on the day of explantation) for 6–8 hours, and then the medium containing virus was replaced with fresh culture medium.

To track cell division during hair cell transformation, BrdU (Sigma) and Ad-$Math_1$-EGFP were added to the culture media at different time points (**Figure 1**), at a concentration of 10–15 μg/mL.

Immunocytochemistry

All samples were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 2 hours, permeabilized with 0.3% Triton X-100 in PBS for 30 minutes at room temperature, and then blocked with 5% normal goat serum for 30 minutes. BrdU samples were pretreated in 2 N HCl for 30 minutes at 37°C. Immunocytochemistry was performed using primary antibodies incubated for 12–48 hours at 4°C: rabbit polyclonal anti-Myosin VIIa antibody (1:100; Proteus BioSciences Inc., Ramona, CA, USA) followed by a secondary antibody, goat monoclonal anti-rabbit IgG conjugated to TRITC (1:400; Molecular Probes, Inc., Eugene, OR, USA) or Alexa Fluor 647 goat anti-rabbit IgG (A21245, diluted 1:150; Molecular Probes, Inc.) for 30 minutes at 37°C. Mouse anti-BrdU antibody (1:100; Sigma) followed by a secondary antibody, goat anti-mouse IgG conjugated to TRITC (1:400; Molecular Probes, Inc.) or Alexa Fluor 647 goat anti-mouse IgG (1:400; Molecular Probes, Inc.) for 30 minutes at 37°C. Hair bundles were stained with phalloidin-TRITC (1:400; Sigma) for 30 minutes at 37°C. The samples were examined and photographed using a Leica SP2 or SP5 confocal microscope (Wetzlar, German).

Results

Cells with different proliferative potentials had different capabilities to generate new hair cells induced by $Math_1$ gene transfer

Cells in the non-sensory region and mechanically damaged region of sensory epithelia had high proliferative potential and were induced into hair cells by $Math_1$ gene transfer

As discussed in a previous study (Huang et al., 2009), when vestibular epithelia were attached to culture dishes, cubic shaped cells spread and moved from the sensory region to the periphery, forming a non-sensory region. All of these

cells exhibited high cell proliferative potential as demonstrated by BrdU staining (**Figure 2A**) (BrdU was added as **Figure 1A**). Ad-$Math_1$-EGFP vectors were used in the culture media, and some of these non-sensory cells were transfected. Very few cells in the sensory region were transfected and differentiated into new hair cells as we previously reported (Huang et al., 2009). Some transfected cells differentiated into new hair cells, which were labeled by a hair cell specific protein, Myosin VIIa (**Figure 2B**). The ratio of new hair cells to transfected cells at 10 days *in vitro* was 69.5%. In the control group, Ad-EGFP vectors were used under the same conditions, and no new hair cells were found as previously reported (Huang et al., 2009).

When a hole or damage is made mechanically in the cultured postnatal mouse vestibular utricle, supporting cells around and in the damaged region spread and move to the center of the hole, and these cells have high proliferative capability (Meyers and Corwin, 2007). Our experiment indicated that when these cells in the damaged region were infected by Ad-$Math_1$-EGFP vectors, some became new hair cells at 3 and 10 days *in vitro* (**Figure 3**). The ratio of new hair cells to transfected cells at 10 days *in vitro* was 58.2%. In the control group, Ad-EGFP vectors were used under the same conditions, but no new hair cells were found.

Cells with low proliferative potential were induced by $Math_1$ gene and few were differentiated into new hair cells

After culturing for 7–10 days, there were very few proliferating supporting cells in the postnatal mice vestibular epithelial sheets. BrdU was used to detect dividing supporting cells at the beginning of the culture, and almost no BrdU-positive hair cells were found in the sensory region of the epithelia. When induced by $Math_1$ gene transfer, a small number of the cells differentiated into new hair cells (**Figure 4A**). The ratio of new hair cells to transfected cells was 9.4%. In the control group, Ad-EGFP vectors were used under the same conditions, but no new hair cells were seen (**Figure 4B**).

Cell division seldom occurred during or after new hair cell regeneration induced by $Math_1$

New hair cells induced by $Math_1$ gene transfer were visible in the non-sensory region as previously reported (Huang et al., 2009). To determine whether new hair cells in the non-sensory region originated from the proliferating cells in the non-sensory region, BrdU was added at the beginning of the culture according to protocol-1 (**Figure 1A**). New hair cells were labeled with BrdU in the non-sensory region of the samples treated with Ad-$Math_1$-EGFP (**Figure 5**). In other samples, to confirm cell division was required during new hair cell differentiation, BrdU was added after the administration of Ad-$Math_1$-EGFP according to protocol-2 (**Figure 1B**). Very few hair cells were labeled with BrdU in the non-sensory region of samples treated with Ad-$Math_1$-EGFP (**Figure 5**). These two experiments showed that cell division always occurred before $Math_1$ transfection but not during or after $Math_1$ transfection.

Discussion

Cells with high proliferative potential generated new cells induced by $Math_1$

Cells with high proliferative potential could generate hair cells by $Math_1$ gene transfer. However, the underlying mechanism remains unclear. During embryogenesis, $Math_1$ is expressed just before or during the timepoint when prosensory cells begin to leave the cell cycle, and these prosensory cells (target cells of $Math_1$) still have a high proliferative capability. RB1 and P27kip1 are thought to contribute to prosensory cells leaving the cell cycle (Chen and Segil, 1999; Löwenheim et al., 1999; Mantela et al., 2005; Liu and Zuo, 2008). When these genes are knocked out or knocked down, prosensory cells delay leaving the cell cycle and excessive new hair cells are formed. This might be because excessive hair cells remain in the cell cycle of prosensory cells for longer, resulting in greater numbers of new prosensory cells and the generation of new hair cells. Nevertheless, a lower number of proliferating prosensory cells results in fewer new hair cells. The Notch signaling pathway plays an important role in the differentiation of hair cells and supporting cells by mediating "lateral inhibition". Hes1 is one of the effectors that mediates this effect (Zheng et al., 2000; Zine et al., 2001); however, it contributes to the adequate proliferation of sensory precursor cells *via* the potential transcriptional downregulation of p27Kip1 (Murata et al., 2009). In $Hes1^{-/-}$ mice, prosensory cells with low proliferative potential for upregulated p27kip1 led to a low efficiency of hair cell differentiation despite normal $Math_1$ expression. Izumikawa et al. (2008) confirmed that Ad-$Math_1$-EGFP was administered into media tympanic scala after killing all hair cells and no differentiated cells were found despite the overexpression of $Math_1$. This study also demonstrated that the target cell is an important factor for $Math_1$ induced hair cell differentiation.

$Math_1$-induced hair cell regeneration *in vitro* was a process of trans-differentiation

In Mesh, the explanation for cell transdifferentiation is a naturally occurring phenomenon where terminally differentiated cells dedifferentiate to the point where they can switch cell lineage. The cells then differentiate into other cell types. Generally speaking, no cell division occurs during this process. In birds, hair cell regeneration occurs through two pathways: regeneration through supporting cell division and regeneration through direct transdifferentiation without cell division (Stone and Cotanche, 2007). During the embryonic stage, prosensory cells begin to differentiate into hair cells when these cells retreat from the cell cycle (Ruben, 1967; Chen et al., 2002). No cell division is found during hair cell differentiation, indicating cell transdifferentiation is the main process involved. BrdU is used to detect the cells that have experienced the S phase. In our study, BrdU was added after Ad-$Math_1$-EGFP was added to the culture medium, and cell transdifferentiation was expected to occur simultaneously. Most new hair cells were negative for BrdU, showing that new hair cell differentiation was independent of cell division. This was consistent with previously published results

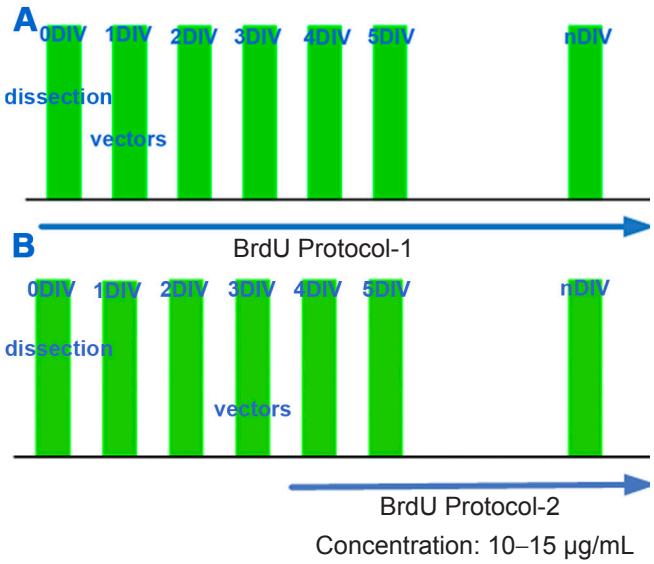

Figure 1 Protocol of vestibular epithelia labeling and transfection.
(A) BrdU protocol-1: BrdU was added at 0 DIV, and Ad-Math$_1$-EGFP at 1 DIV. (B) BrdU protocol-2: Ad-Math1-EGFP was added at 3 DIV and then BrdU at 4 DIV. Blue arrows indicate cultures with BrdU. DIV: Day *in vitro*; BrdU: 5-bromo-2′-deoxyuridine; EGFP: enhanced green fluorescent protein.

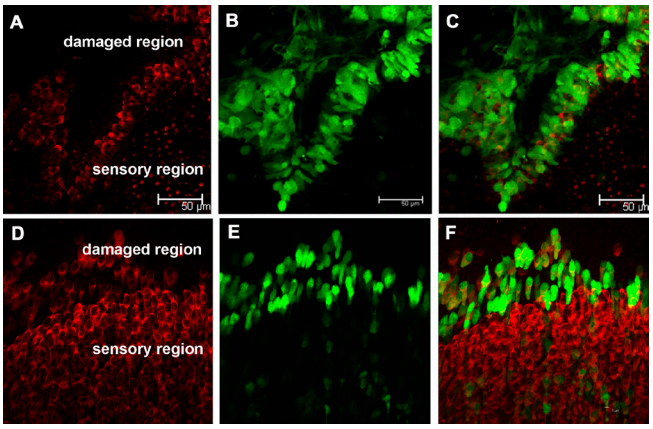

Figure 3 Cultured damaged utricle sensory epithelia transfected with Ad-Math$_1$-EGFP at different time points.
(A–C) At 3 days *in vitro*, transfected supporting cells and mesenchymal cells expressed EGFP (green, A), but original hair cells did not: hair cells stained by anti-Myosin VIIa antibody (red, TRITC stain, B). (C) Merged images of A and B. (D–F) At 11 days *in vitro*, new hair cells in the damaged region were stained with EGFP (green, D) and anti-Myosin VIIa (red, TRITC stain, E) antibody double staining (yellow). (F) Merged images of D and E. Scale bars: 50 μm. EGFP: Enhanced green fluorescent protein.

(White et al., 2006; Yang et al., 2013).

However, in this study, very few new hair cells were positive for BrdU under protocol-2 and new hair cells with two nuclei were also commonly found. Cell division might occur just before, during or after hair cell differentiation and thus explain these BrdU-positive new hair cells. Most new hair cells came from high proliferative cells, and this cell differentiation is independent of cell proliferation; therefore, cell division often starts before transdifferentiation. Images of many hair cells with two nuclei and new cell division were occasionally captured, but this was insufficient to determine whether division occurred after cell differentiation.

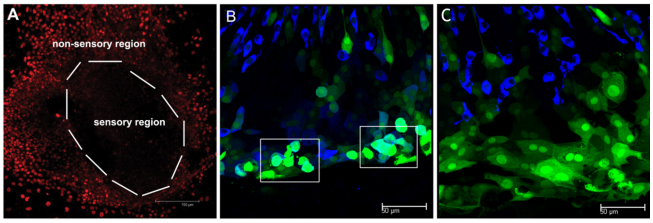

Figure 2 High proliferative cells and new hair cells in the non-sensory region are induced by Math$_1$.
(A) Cultured utricle at 5 days *in vitro*: More proliferating cells are visible in the non-sensory region compared with the sensory region; proliferated nuclei are labeled by BrdU (red, Apollo®567 stain). The white line shows the border of the sensory region (original hair cells and supporting cell region), and the non-sensory region. (B) Cultured utricle at 11 days *in vitro* treated by ad-Math$_1$-EGFP: in the non-sensory region, new hair cells are clustered in boxes, and stained by anti-Myosin VIIa antibody (blue, Cy5 stain). New hair cells with one or two cell nuclei are shown by white stars. (C) No new hair cells were labeled with Myosin VIIa or EGFP in the non-sensory region of cultured utricle treated with Ad-EGFP. Scale bars: 150 μm in A, 20 μm in B, C. BrdU: 5-Bromo-2′-deoxyuridine; EGFP: enhanced green fluorescent protein.

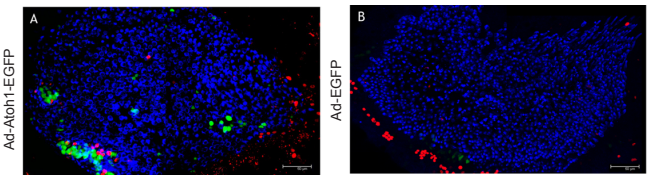

Figure 4 Proliferation of cultured utricle treated with Ad-Math$_1$-EGFP or Ad-EGFP at 10 days *in vitro*.
Hair cells were labeled by Myosin VIIa (blue, Cy5 stain); proliferating cell nuclei were stained by BrdU (red, Apollo®567 stain), and EGFP (green). (A) In the sensory region of the utricle, there were very few proliferating cells; EGFP- and Myosin VIIa-stained new hair cells were observed. (B) In the control group, cells were only induced by Ad-EGFP; there were no new hair cells stained with EGFP or Myosin VIIa. Scale bars: 50 μm. BrdU: 5-Bromo-2′-deoxyuridine; EGFP: enhanced green fluorescent protein.

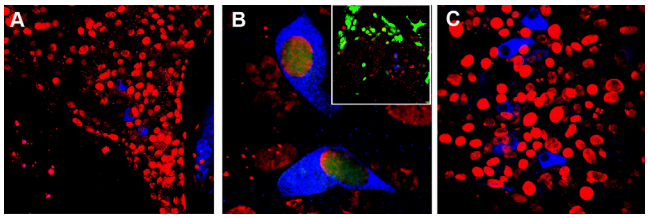

Figure 5 Cell division tracked by BrdU after new hair cell regeneration induced by Math$_1$ for 10 days.
(A) BrdU-positive new hair cells in the non-sensory region of cultured epithelia according to BrdU protocol-1. (B) Most new hair cells are negative for BrdU in the non-sensory region of cultured epithelia according to BrdU protocol-2. Hair cells were stained by Myosin VIIa (blue, Cy5 stain); proliferating cells were labeled by BrdU (red, Apollo®567 stain), and EGFP (green). (C) Magnified image of the box in B. Scale bars: 50 μm in A, C, 10 μm in B. BrdU: 5-Bromo-2′-deoxyuridine; EGFP: enhanced green fluorescent protein.

Conclusions

Proliferative stage of vestibular epithelial cells is associated

with their capability to differentiate into new hair cells by $Math_1$ gene transfer *in vitro*, but this regeneration is independent of cell proliferation.

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