Atoh1 expression levels define the fate of rat cochlear nonsensory epithelial cells in vitro

WEN-WEI LUO*, JUAN-MEI YANG*, ZHAO HAN, YA-SHENG YUAN, HAI-BIN SHENG, XIANG LIU and FANG-LU CHI

Department of Otolaryngology-Head and Neck Surgery, Eye and ENT Hospital of Fudan University, Xuhui, Shanghai 200031, P.R. China

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Abstract. Atonal homolog 1 (Atoh1) is a basic helix-loop-helix transcription factor that is essential for inner ear hair cell differentiation. Previous studies have reported that Atoh1 gene transfer induces the production of ectopic hair cell-like cells (EHCLCs). In the present study, the effect of different Atoh1 expression levels and the duration of EHCLC formation on the lesser epithelial ridge (LER) of cochleae was examined using a human adenovirus serotype 5 (Ad5) vector encoding atoh1 and the reporter gene EGFP. Different Ad5-EGFP-atoh1/Ad5-EGFP virus titers were added to cultured cochlear explants and EHCLCs were detected in the LER at various time points. The results demonstrated that GFP alone did not induce EHCLCs. By contrast, Atoh1 expression induced EHCLCs as early as 2.5-5 days following EGFP-atoh1 infection in the LER and depending upon the viral titer, the number of EHCLCs increased with time. Higher Ad5-EGFP-atoh1 titers induced enhanced Atoh1 expression, resulting in an increase in EHCLCs. Lower Ad5-EGFP-atoh1 titers required more time for EHCLC formation and very low titers of Ad5-EGFP-atoh1 induced only weak Atoh1 expression and did not trigger EHCLC formation. In conclusion, the present study utilized an appropriate Ad5-EGFP-atoh1 titer range to induce Atoh1 expression and the subsequent production of EHCLCs. The results revealed that the Atoh1 expression level defined the fate of LER cells as either EHCLCs or nonsensory epithelial cells. This evidence may provide an important guideline for future studies into gene therapy strategies for the treatment of deafness.

Introduction

Hair cells (HCs) transform sound and balance signals into electrical impulses in the cochlear and vestibular end organs. By contrast to vertebrates that are able to spontaneously regenerate new hair and supporting cells (1,2), there is no effective way to stimulate their regeneration in mammals once hair cells have been damaged by noise, ototoxic drugs or aging, which hampers the treatment of sensorin, a neural hearing impairment that is caused by hair cell loss.

Atoh1 is a basic helix-loop-helix transcription factor that is crucial in hair cell formation (3,4). Knockout of atoh1 in mice results in the absence of differentiated hair cells and supporting cells, while Atoh1 overexpression in cultured explants or in vivo induces ectopic hair cell-like cell (EHCLC) formation (3,5-14).

Studies in a novel atoh1 ‘self-terminating’ mouse model have suggested that Atoh1 expression level and duration is crucial for inner and outer hair cell differentiation in vivo (15). Therefore, we aimed to investigate how Atoh1 affects EHCLC formation and whether Atoh1 expression defines the fate of LER cells as either ectopic, newly formed hair cells or nonsensory epithelial cells. In the present study, cultured explants were infected with several virus titers and EHCLC expression was detected in the LER at different time points. It was identified that the formation of EHCLCs was Atoh1 dependent, as no EHCLCs formed upon infection by GFP alone. Following LER infection with an appropriate titer (EGFP-atoh1) for Atoh1 expression (1.6x10⁹ PFU/ml), EHCLC production was detected as early as 2.5 days and the number of EHCLCs increased with time. Higher Ad5-EGFP-atoh1 titers induced increased Atoh1 expression and a larger quantity of hair cell-like cells appeared at earlier time points compared with lower titers. Lower Ad5-EGFP-atoh1 titers induced less Atoh1 expression and required a greater duration for EHCLC formation. Extremely low Ad5-EGFP-atoh1 titers induced only weak Atoh1 expression and no formation of EHCLCs. Therefore, Atoh1 expression levels define the fate of LER cells as either EHCLCs or nonsensory epithelial cells, and greater Atoh1 expression decreases the time required for EHCLC formation in the LER. These data define an appropriate Ad5-EGFP-atoh1 titer range for ectopic hair cell formation and which will act as an important guideline for future studies.
Materials and methods

Cultures of postnatal rat cochlea and atoh1 gene infection. This study was approved by the Institutional Animal Care and Animal Ethics Committee of Fudan University (Xuhui, Shanghai, China). One-day-old postnatal (P1) SD rats were used for the experiments and were purchased from Slaccas Experimental Animal Company (Xuhui, Shanghai, China). The rats were sacrificed by CO₂ asphyxiation. The cochlear explants cultured was prepared as described previously (11,12). The final concentrations of the Ad5-EGFP-atoh1 vector were 0.1x10⁸, 0.4x10⁸, 0.8x10⁸, 1.6x10⁸ and 2.4x10⁸ PFU/ml in serum-free DMEM/F12. The control group (Ad5-EGFP) included corresponding titers. The viruses used were as described previously (6,7,11,12).

Tissue preparation and immunofluorescence. The cochlear explants were fixed with 4% paraformaldehyde for 30 min and then treated with 0.1% Triton X-100 plus 10% donkey serum for 30 min. Following this, the explants were incubated with the following primary antibodies for 24 h at 4°C; myosin7A (1:100; Proteus Biosciences Inc., Ramona, CA, USA), myosin7A (1:200; Developmental Studies Hybridoma Bank, Iowa City, IA, USA), p27kip1 (1:100; Cell Signaling Technology, Inc., CA, USA) and Sox2 (1:300, Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA). The preparation was washed 3-5 times in PBS and then incubated with secondary antibodies for 2 h at 37°C in the dark. The secondary antibodies included donkey anti-mouse/rabbit Alexa Fluor 555 (1:1,000) and/or donkey anti-mouse/rabbit/goat (H+L) Alexa Fluor 647 (1:1,000; Molecular Probes, Invitrogen Life Technologies, Carlsbad, CA, USA). The specimens were visualized with a Zeiss LSM 510 confocal laser-scanning microscope (Carl Zeiss, Oberkochen, Germany) and only one image was captured by the microscope.

Cell counting and statistical analysis. Only cells at the LER region of the mid-basal turns were counted. Using random samples, the cells in 200 μm segments along the length of the cochlea were counted. Each group had at least five different cochlear explants and each explant was sampled at five areas. Ectopic hair cells were counted 3, 5, 7, 9 and 11 days post-infection. All the cell count was precisely performed by manually analyzing the confocal images. The values are expressed as the mean ± standard error and using a one-way ANOVA statistical test when appropriate. P<0.05 was considered to indicate a statistically significant difference.

Results

Ad5 vector transfection efficiency in the LER. The Ad5-EGFP/Ad5-EGFP-atoh1 transfection efficiency in the LER (outside of the outer hair cells) was determined by infection with different virus titers (Fig. 1). At a titer of 0.16x10⁷ PFU/ml, only 8±2% of LER cells were GFP positive with weak green fluorescence (Fig. 1J). At a titer of 0.4x10⁸ PFU/ml, 27±4% of LER cells were GFP positive with moderate green fluorescence (Fig. 1A and 1D and 1G). At 0.8x10⁸ PFU/ml, 91±7% of LER cells were GFP positive with moderate-to-strong green fluorescence (Fig. 1B and 1E and 1H). At 1.6x10⁹ PFU/ml, 94±9% of LER cells were GFP positive with strong green fluorescence (Fig. 1C and 1F and 1I). However, when 2.4x10⁹ PFU/ml was used, the cultured explants disintegrated (Fig. 1K). Higher viral infection efficiency was observed with increasing titer, because the transfection efficiency of 0.4x10⁸ PFU/ml was significantly higher than that of 0.16x10⁷ PFU/ml (n=5, P<0.05) and that of 0.8x10⁸ PFU/ml was significantly higher than that of 0.4x10⁹ PFU/ml (n=5, P<0.05), whereas the transfection efficiency of 1.6x10⁹ PFU/ml was similar to 0.8x10⁸ PFU/ml (n=5, P>0.05). However, the fluorescence intensity at 1.6x10⁹ PFU/ml
was higher than at 0.8x10^8 PFU/ml. Therefore, it was concluded that the most effective virus titer was 1.6x10^8 PFU/ml.

**Formation of new EHCLCs at the LER is Atoh1 dependent.** Following Ad-EGFP infection of the cultured explants (Fig. 2A), the LER cells presented robust EGFP fluorescence, however no myosin7A-positive cells were observed. The LER cells were unable to differentiate into hair cells. Following Ad5-EGFP-atoh1 infection, the LER was the target (Fig. 1B). Consistent with previous studies (11,14), Ad5-EGFP-atoh1 infection resulted in the induction of myosin7A-positive cells in the LER regions (Fig. 2B), suggesting that these newly formed hair cells were Atoh1 overexpression dependent. Many of the EGFP-positive cells were myosin7A negative, despite having been infected with Ad5-EGFP-atoh1. To determine whether the Atoh1 expression level or duration led to this phenomenon, different Ad5-EGFP-atoh1 titers were utilized, and the quantities and percentages of new hair cells were detected at different time points in the following study.

**EHCLC formation requires a certain Atoh1 expression level.** To address whether new EHCLC formation depends on Atoh1 expression, cultured explants were treated with Ad-EGFP-atoh1 at four different titers: 0.16x10^8, 0.4x10^8, 0.8x10^8 and 1.6x10^8 PFU/ml. The samples were fixed at five days following viral infection (DVI) and the numbers of EGFP-myosin7A double-positive cells and EGFP-positive cells were counted in the LER (Fig. 3). At 0.16x10^8 PFU/ml Ad5-EGFP-atoh1, no myosin7A-positive cells were detected, implying that low Atoh1 expression was unable to induce hair-cell-like cell formation (n=5). At 0.4x10^8 PFU/ml Ad5-EGFP-atoh1, there were 5±2 myosin7A-EGFP double-positive cells per 200 µm in the LER (4±3% of all EGFP-positive cells). At 0.8x10^8 PFU/ml Ad5-EGFP-atoh1, there were 22±4 myosin7A-EGFP double-positive cells per 200 µm in the LER (14±5% of all EGFP-positive cells). At 1.6x10^8 PFU/ml Ad5-EGFP-atoh1, there were 54±4 myosin7A-EGFP double-positive cells per 200 µm in LER (57±13% of all EGFP-positive cells; n=5). The number of EHCLCs in the higher virus titer groups was significantly greater compared with the lower virus titer groups (Fig. 2B). Furthermore, the LER to hair-cell-like cell conversion rate was significantly enhanced in the higher than in the lower virus titer group. These data demonstrate that increasing the virus titer increased Atoh1 expression and this subsequently increased the myosin7A-positive cell number in the LER. If Atoh1 expression was too low, few LER cells converted to hair-cell-like cells. Thus, EHCLCs production was dependent on specific Atoh1 expression levels.

**Higher Atoh1 expression reduces the duration of EHCLC formation in the LER.** The number of Atoh1-induced EHCLCs increased with time. When applied to cultured explants with 0.16x10^8 PFU/ml Ad5-EGFP-atoh1, no myosin-positive cells were detected even at 11 DVI. At 0.4x10^8 PFU/ml Ad5-EGFP-atoh1, 5±2 EHCLCs per
200 µm were detected as early as 5 DVI in the LER, which increased to 12±2 at 7 DVI, 11±2 at 9 DVI and 11±1 at 11 DVI (Fig. 4A and D). At 0.8x10^8 PFU/ml Ad5-EGFP-atoh1, 14±1 EHCLCs per 200 µm were detected as early as 3 DVI in the LER, which increased at 22±4 on 5 DVI, 29±7 at 7 DVI, 31±2 at 9 DVI and 31±1 at 11 DVI (Fig. 4B and D). At 1.6x10^8 PFU/ml Ad5-EGFP-atoh1, we detected myosin7A-positive cells as early as 60 h following atoh1 infection, 31±2 EHCLCs per 200 µm were detected at 3 DVI in the LER, which increased to 54±4 on 5 DVI, 70±5 on 7 DVI, 67±6 on 9 DVI and 67±2 on 11 DVI (Fig. 4C and D). Therefore, at a low titer (0.4x10^8 PFU/ml) with low Atoh1 expression, EHCLC formation required a longer time (5 days). At a higher titer (1.6x10^8 PFU/ml) with high Atoh1 expression, however, EHCLC formation required only 2.5 days. EHCLCs increased with time but remained constant from 7-11 days in all groups (Fig. 4D). In conclusion, the Atoh1 expression level critically affected the time required for EHCLC formation.

Atoh1 expression defines the fate of LER cells. The data indicated that the number of Atoh1-induced EHCLCs increased with time but this effect ceased at 7-11 days, regardless of the titer (Fig. 4D). Despite infection of cultured cochlear explants with Ad-EGFP-atoh1 at 1.6x10^8 PFU/ml, only ~71% of infected cells in the LER were able to transdifferentiate into hair cell-like cells. Following Ad-EGFP-atoh1 infection, a
number of the infected LER cells (EGFP positive) transformed to hair-cell-like cells (myosin7A positive) with an oblong or round shape, whereas other cells remained nonsensory epithelial cells (p27kip1) with a polygonal, flat shape (Fig. 5A and B). Furthermore, many myosin7A-positive cells clustered in the LER with sox2-positive cells surrounding them, indicating that hair-cell-like cells may induce supporting cell formation (Fig. 5C and D).

Discussion

The human Ad5 vector, encoding both Atoh1 and the reporter gene EGFP, is a useful tool for new hair cell production due to its high transfection efficiency, low level of target tissue damage and ease of control (6-12,14). The viral titration in the present study indicated that appropriate titers induce optimal Atoh1 expression. At titers >2.4x10^8 PFU/ml, cochlear cultured explants may be severely damaged. At titers <0.16x10^8 PFU/ml, although weak Atoh1 expression was observed, it was not sufficient to generate ectopic hair cell formation. Our data indicate that 0.4-1.6x10^8 PFU/ml Ad5-EGFP-atoh1 is an efficient and safe titer range for hair-cell-like cell formation in cultured cochlear explants.

At titers of 0.16-1.6x10^8 PFU/ml, higher infection efficiency and expression levels were observed (Fig. 1). GFP alone did not induce EHCLCs expression, whereas Ad5-EGFP-atoh1 did induce robust EHCLCs formation. Thus, EHCLCs formation in the LER was Atoh1 expression dependent. At titers <0.1x10^8 PFU/ml, although a number of weakly GFP-positive cells were identified, no myosin7A-positive cells were detected, even at 11 DVI. At titers of 0.4x10^8 PFU/ml Ad5-EGFP-atoh1, only ~4% of atoh1-infected cells converted to hair-cell-like cells by 5 DVI in the LER. At titers of 1.6x10^8 PFU/ml, ~54% of atoh1-infected cells converted to hair cell-like cells. These data suggest that hair cell formation requires a certain level of Atoh1, with higher expression inducing more hair cell-like cell formation.

At 0.4-1.6x10^8 PFU/ml Ad5-EGFP-atoh1, EHCLCs formation increased with time. At 0.4x10^8 PFU/ml, EHCLCs formation required 5 days. However, at 1.6x10^8 PFU/ml, ectopic hair cell formation only required 2.5 days. Thus, greater Atoh1 expression shortens the time required for EHCLC formation. Furthermore, the number of EHCLCs increased with time but then ceased increasing at 7 DVI for all titers. Even at 1.6x10^8 PFU/ml, only ~71% of Ad5-EGFP-atoh1-infected cells in the LER transdifferentiated into hair cell-like cells. The fate of the non-differentiated cells may help explain this phenomenon.

The data from the present study further revealed that hair cell formation requires a certain Atoh1 expression level; if it was too low, the LER did not convert into hair cell-like cells. A number of Ad5-EGFP-atoh1-infected LER cells (EGFP positive) had already converted to hair cell-like cells (myosin7A positive) with an oblong or round shape at 3 DVI, while the other LER cells remained nonsensory epithelial cells (p27kip1 positive) with a polygonal, flat shape (Fig. 5A and B). At 3 DVI, the majority of myosin7A-positive cells exhibited a strong green fluorescence and p27kip1-positive cells appeared to have weak or no green fluorescence. However, numerous myosin7A-positive cells were observed clustered in the LER with sox2-positive cells surrounding them (Fig. 5C and D), indicating that hair cell-like cells induce supporting cell formation, which has also been previously reported (10). Therefore, the majority of Ad5-EGFP-atoh1-infected LER
cells (with sufficient Atoh1 expression) converted into hair cells and induced the surrounding nonsensory epithelial cells to transform into supporting cells.

In the present study, an appropriate virus titer range for infecting cultured cochlear explants was examined, providing highly efficient infection and conversion rates but reducing the infection side effects. Atoh1 expression is critical to hair cell formation, as it defines the fate of LER cells as either hair cell-like cells or nonsensory epithelial cells. The present study provides an important guideline for future investigations to develop novel gene therapy strategies in the treatment of deafness.

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