HHS Public Access

Author manuscript

Neuroscience. Author manuscript; available in PMC 2017 March 01.

Published in final edited form as:

Neuroscience. 2016 March 1; 316: 311–320. doi:10.1016/j.neuroscience.2015.12.050.

TAUROURSODEOXYCHOLIC ACID PREVENTS HEARING LOSS AND HAIR CELL DEATH IN *Cdh23*$^{erl/erl}$ MICE

J. HU[a,b], **M. XU**[a], **J. YUAN**[b], **B. LI**[c], **S. Entenman**[b], **H. YU**[b], and **Q.Y. ZHENG**[a,b,c,*]

[a]Department of Otorhinolaryngology-Head & Neck Surgery, Second Affiliated Hospital, Xi'an Jiaotong University School of Medicine, 157 Xiwu Road, Xi'an 710014, Shaanxi, PR China

[b]Department of Otolaryngology-Head & Neck Surgery, Case Western Reserve University, 11100 Euclid Avenue, Cleveland, OH 44106, USA

[c]Transformative Otology and Neuroscience Center, Binzhou Medical University, 346 Guanhai Road, Yantai 264003, Shandong, PR China

Abstract

Sensorineural hearing loss has long been the subject of experimental and clinical research for many years. The recently identified novel mutation of the *Cdh23* gene, *Cdh23*$^{erl/erl}$, was proven to be a mouse model of human autosomal recessive nonsyndromic deafness (DFNB12). Tauroursodeoxycholic acid (TUDCA), a taurine-conjugated bile acid, has been used in experimental research and clinical applications related to liver disease, diabetes, neurodegenerative diseases, and other diseases associated with apoptosis. Because hair cell apoptosis was implied to be the cellular mechanism leading to hearing loss in *Cdh23*$^{erl/erl}$ mice (*erl* mice), this study investigated TUDCA's otoprotective effects in *erl* mice: preventing hearing impairment and protecting against hair cell death. Our results showed that systemic treatment with TUDCA significantly alleviated hearing loss and suppressed hair cell death in *erl* mice. Additionally, TUDCA inhibited apoptotic genes and caspase-3 activation in *erl* mouse cochleae. The data suggest that TUDCA could be a potential therapeutic agent for human DFNB12.

Keywords

Cdh23; apoptosis; hearing loss; tauroursodeoxycholic acid (TUDCA); otoprotection

[*]Corresponding author: Qing Yin Zheng, Tel: +1 216-844-3441; Fax: +1 216-844-7268. qyz@case.edu.

Author Contributions:

Q. Y. ZHENG, study design, data analysis, final approval, and accountability for all aspects of the work; J. HU, study design, study performance, data analysis, drafting, final approval, and accountability for all aspects of the work; M. XU, study design, data analysis, and final approval; J. YUAN, B. LI, S. Entenman, and H. YU, study performance.

INTRODUCTION

Hearing loss is one of the most common sensory impairments in humans, affecting about 1.3 billion people worldwide (Vos et al., 2013). At least 50% of deafness presenting before speech development is genetic, and approximately 70% of hereditary deafness is nonsyndromic (no other anomaly exists) (Smith et al., 2014). The different gene loci for autosomal recessive nonsyndromic deafness are presented by DFNB (Genetic Evaluation of Congenital Hearing Loss Expert Panel, 2002). More than 70 DFNB loci have been identified (Smith et al., 2014). In previous research studies of mouse models of DFNB, the mice were either deaf at birth or had very late-onset deafness with slow progression. In our recent study, a novel point mutation (T208C) of the *Cadherin23* (*Cdh23*) gene in mice was identified and named *erl* (Han et al., 2012). The $Cdh23^{erl/erl}$ mutant mice (*erl* mice) showed progressive hearing loss beginning at postnatal day (P) 27 and developed prolonged deafness by P100. This mutation is considered to be a novel mouse model for DFNB12. Because of the time interval from hearing loss initiation to total deafness, the *erl* mice are an ideal tool for testing otoprotective drugs and screening potential therapies. We demonstrated that hair cell apoptosis was the pathological mechanism through which *erl* mutation led to hearing loss. Furthermore, treatment with erythropoietin and the apoptosis inhibitor Z-VAD-FMK could significantly preserve cochlear hair cells and prevent hearing loss in *erl* mutants (Han et al., 2012, Han et al., 2013a).

Tauroursodeoxycholic acid (TUDCA) is a taurine-conjugated bile acid derived from ursodeoxycholic acid (UDCA). Under natural physiological conditions, UDCA is present at a low concentration in human bile. For the past thousand years, UDCA has been isolated from dried black bear gallbladders and used in the treatment of several illnesses in traditional Chinese medicine (Beuers, 2006). Nowadays, TUDCA has been chemically synthesized and is widely used in clinical and experimental research to treat liver disease, diabetes, and neurodegenerative diseases (Momose et al., 1997, Keene et al., 2002, Rodrigues et al., 2003, Green and Kroemer, 2004, Ozcan et al., 2006, Kars et al., 2010, Ceylan-Isik et al., 2011, Laukens et al., 2014). Previous research revealed that TUDCA functioned by modulating the apoptotic threshold in various cell types (Rodrigues et al., 2003, Amaral et al., 2009, Ramalho et al., 2013). Given TUDCA's cell-protective effects in disorders associated with apoptosis and its clinical safety, we measured its hearing protective effects using *erl* mice as an animal model for screening new otoprotective drugs.

In the present study, we found that TUDCA showed protective effects against hearing loss and hair cell apoptosis in *erl* mice. To the best of our knowledge, this is the first *in vivo* study about TUDCA's otoprotective effects in the mouse model of *Cdh23* mutations. These data suggest that TUDCA is a potential therapeutic agent for human DFNB12.

EXPERIMENTAL PROCEDURES

Mice and treatment

All experiments were approved by the Animal Research Committee of the Case Western Reserve University School of Medicine (protocol R01DC009246). All mice were housed in the same environment, and received treatments by intraperitoneal injection. The *erl* mutant

mouse model was developed from the C57BL/6J (B6) genetic background, which was homozygous for *Cdh23*^{ahl} mutation (Han et al., 2012, Han et al., 2013a). Thus we chose B6 mice as controls to test whether TUDCA had any toxic effect in the ear. A total of 14 B6 mice and 85 *erl* mice were used in this study. The B6 mice were randomized into two groups with both genders: a TUDCA-treated group (treated with TUDCA, 100 mg/kg, EMD Chemicals Inc. Catalog No. 580549, diluted in 1×PBS, phosphate-buffered saline), and an untreated group. The *erl* mice were randomized into three groups with both genders: a test group (treated with the same dosage of TUDCA), a vehicle group (treated with an equal volume of PBS), and a control group (untreated). All treatments started on P7, with subsequent injections given every other day for the first eight weeks. The injections were then continued once weekly for the duration of the experiments. The starting time point was selected to prevent caspase upregulation, which was detected at P14 in untreated *erl* mice in our previous work. The TUDCA dosage was selected from preliminary experiments that showed it to be a good balance between safety and effectiveness, and with reference to previous reports (Rodrigues et al., 2003, Drack et al., 2012).

Auditory-evoked brainstem response (ABR) and distortion product oto-acoustic emission (DPOAE) testing

A computer-aided evoked potential system (Intelligent Hearing Systems, the Smart-EP software) was used in ABR testing, as previously described (Zheng et al., 1999). Mice were anesthetized, and the body temperature was maintained at 37°C. Subdermal needle electrodes were used; the recording electrode was inserted at the vertex of the skull, the ground electrode was inserted in the apex of the nose, and the reference electrodes were fixed near each ear. Clicks and tone bursts of 8 kHz, 16 kHz, and 32 kHz were channeled through an inserted earphone. ABR thresholds were identified as the lowest stimulus (sound pressure level, SPL) at which clear and repeatable ABR waveforms could be recognized. As previously reported, mice showing ABR thresholds above 55 dB SPL (for the click stimulus), 40 dB SPL (for 8 kHz tone bursts), 35 dB SPL (for 16 kHz), or 60 dB SPL (for 32 kHz) were considered to be hearing impaired (Han et al., 2013a).

The Intelligent Hearing System (Smart EP 3.30 Software) was used in DPOAE measurements. The test was conducted for pure tones at frequencies ranging from 4.4 to 20.3 kHz. Frequencies were acquired with an F2:F1 ratio of 1.22 and with primary stimulus of 65/55 dB SPL. The test model started from the lowest frequency and increased to the highest. The distortion product (DP) level (2F1–F2) of DPOAE amplitudes (in dB SPL) was extracted from the averaged spectra along with the noise floor.

Surface preparation and hair cell counting

The surface preparation was performed by a modified method as described previously (Han et al., 2012). Briefly, the mice were euthanized, and the temporal bones were collected. The surface preparations were performed using a modified protocol. The temporal bones were fixed in 4% paraformaldehyde overnight. The organ of Corti was then carefully micro-dissected out and was cut into three separate segments: apical, middle, and basal turn. The surface preparations were permeabilized in 0.2% Triton X-100, stained for F-actin with Alexa Fluor 568 phalloidin (Invitrogen), and finally observed with a fluorescence

microscope (Leica). Hair cells were counted as present if cell bodies and V-shaped hair bundles were intact. The outer hair cells (OHCs) were counted in three discontinuous microscope views (×40 magnification) of each segment, and the average percentage of missing cells was obtained.

Scanning electron microscope (SEM)

The SEM analysis was performed as previously described (Furness et al., 2013). Briefly, after cardiac perfusion with 1% PBS and then with 2.5% glutaraldehyde, the entire cochleae was dissected. The bony capsule, spiral ligament, and Reissner's membrane were carefully removed from the apical turn to the basal turn so as to expose the whole organ of Corti. Afterward, the specimens were incubated in 1% osmium tetroxide (O) three times for one hour each, and in 1% thiocarbohydrazide (T) twice for one hour each (the OTOTO technique). The specimens were dehydrated in a gradient ethanol series, critical point dried using carbon dioxide (CO_2), and finally coated in gold and then palladium. The samples were then viewed under a high-resolution SEM (Helios Nano Lab 650).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining

Apoptotic hair cells were detected by TUNEL assay according to the manufacturer's protocol. Briefly, the inner ears were fixed in 4% paraformaldehyde overnight. The organ of Corti was microdissected, as described above. The surface preparations were permeabilized in 0.2% Triton X-100. The specimens were incubated with the TUNEL Kit (In Situ Cell Death Detection Kit, Fluorescein, Roche) at 37°C for one hour in a humidity chamber and then counterstained with DAPI for 15 minutes at room temperature. The samples were then viewed under a fluorescence microscope (Leica). The numbers of TUNEL-positive cells were counted from three random microscope views (×40 magnification) from the middle and basal cochlear turns for each animal, and the percentage of apoptotic cells was calculated.

Real-time quantitative PCR

The inner ears were quickly isolated, and cochleae and vestibules were quickly separated. TRIzol® (Invitrogen) was used for total RNA extraction, according to the standard protocol. The cDNA was synthesized with 1 μg of total RNA using the Primer Script RT Reagent Kit (Invitrogen) with random hexamers in a 10 μL reaction volume. The cDNAs were mixed with the reagent of SYBR Green/ROX Master Mix (Bioscience). Primer sequences used in the current study are listed in Table 1. Samples were then analyzed in a 96-well plate using the ABI 7300 Sequence Detection System. The PCR thermal cycling conditions were as follows: 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for one minute, and finally a dissociation curve of 95°C for 15 seconds, 60°C for one minute, 95°C for 15 seconds, and 60°C for 15 seconds was added. The gene expression was calculated relative to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase and then analyzed using the $2^{-\Delta\Delta CT}$ method.

Western Blotting

The inner ears were isolated, and the cochleae and vestibules were immediately separated. The cochleae were lysed using ice-cold RIPA buffer. Then equal amounts of proteins were subjected to SDS-PAGE and transferred onto the PVDF membrane. The PVDF membrane was blocked for 1 hour in 5% ECL prime blocking agent and incubated overnight at 4°C with 1:1000 diluted primary antibodies: anti-cleaved caspase-3 (Cell Signaling, 9661), and anti-β-actin (Santa Cruz, sc-130657). After washing with TBST, the membrane was incubated in 1:5000 diluted secondary antibodies (Santa Cruz, sc-2054). The protein bands were visualized using the chemiluminescence-emanating ChemiDoc™ MPImaging System (Bio-Rad).

Statistical analysis

Data are presented as mean ± standard error of the mean (s.e.m). The analysis was performed using the SPSS 18.0 software. Statistical significance was tested with a one-way ANOVA test or Student's t-test. *P* values less than 0.05 were considered significant.

RESULTS

TUDCA prevents hearing loss in *erl* mice

Our previous studies have demonstrated that *erl* mice showed progressive postnatal onset hearing loss more often than B6 mice. In this study, the age-matched B6 mice and *erl* mice with or without TUDCA treatment underwent ABR test at 4 weeks (Fig. 1a). The results show that the TUDCA-treated B6 mice exhibited a similar hearing level to untreated B6 mice, suggesting that there was no toxic effect in the ears. Compared with untreated B6 mice, the untreated *erl* mice exhibited significant hearing loss at 4 weeks, which was consistent with our previous finding (Han et al., 2012). Moreover, the hearing loss in *erl* mice could be alleviated by TUDCA treatment at 4 weeks. In follow-up studies, the TUDCA-treated *erl* mice showed lower ABR thresholds compared with the untreated and PBS-treated *erl* mice up to 12 weeks of age. Significantly lower ABR thresholds were found in the TUDCA group than in the PBS group or the untreated group for click and tone burst (8 kHz, 16 kHz, and 32 kHz) stimuli during the experimental period (Fig. 1b–e). No significant difference was found in the ABR thresholds between the PBS group and the untreated group at any time. The otoprotective effects were measurable from 4 weeks of age for high-frequency stimuli (16 kHz and 32 kHz tone bursts, Fig. 1a, d, e) and became significant from 6 weeks for low-frequency stimuli (clicks and 8 kHz tone bursts, Fig. 1b and c). Additionally, the hearing improvement of TUDCA was found to be similar or even better than Z-VAD-FMK and erythropoietin from our previous publications (Han et al., 2012, Han et al., 2013a). The ABR threshold shift (calculated by untreated ABR thresholds minus the thresholds in each of TUDCA, Z-VAD-FMK, and erythropoietin treated studies) in TUDCA treatment was comparable to erythropoietin and Z-VAD-FMK at 4, 6, and 8 weeks (data not shown). However, TUDCA exhibited higher ABR threshold shift for 8k Hz stimuli than Z-VAD-FMK treatment at 12 weeks (Fig. 1f), indicating better hearing improvement at lower frequency in older *erl* mice (data not available at 12 weeks for erythropoietin). Therefore, TUDCA's otoprotective effect was consistent with those conferred by erythropoietin and Z-VAD-FMK in our previous work.

The otoprotective effects of TUDCA were further confirmed by higher DP levels in DPOAE tests. The DP level (in dB SPL) was extracted from the averaged spectra along with the noise floor (Fig. 2a). The TUDCA group exhibited much higher DP values than did the PBS group or the untreated group at different time points. No meaningful difference was found between the PBS group and the untreated group. At early ages (6 weeks and 8 weeks), the DP-improvement by TUDCA was mainly exhibited at high frequencies (Fig. 2b, c); while at a late age (12 weeks), the TUDCA-treated mice exhibited relatively higher DP value at middle- and low- frequencies (Fig. 2d). Fig. 2a shows that at 12 weeks, the TUDCA-treated mice exhibited DPOAE amplitudes much higher above the noise floor than the PBS-treated mice did.

TUDCA protects against hair cell loss in *erl* mice

TUDCA significantly alleviated OHC loss in *erl* mice, as detected by surface preparation and OHC counting. At the age of 10 weeks, mice treated with TUDCA demonstrated minimal OHC loss across the full length of the organ of Corti. On the contrary, the PBS-treated mice exhibited obvious OHC loss in the basal and middle turns and a small amount of cell loss in the apical turn of their cochleae (Fig. 3a). A quantitative study was performed by OHC counting from three mice in each group. The data showed that the mean percentage of OHC loss in the TUDCA group was appreciably less than that of the PBS group (Fig. 3b).

Furthermore, the morphometric and subcellular structures detected by SEM affirmed TUDCA's hair cell protective effects. At 9 weeks of age, the PBS-treated mice lose almost all OHC with no detectable subcellular structure of hair bundles in the basal turn, and displayed significant cell body and stereocilia loss in the apical turns (Fig. 4a). On the contrary, the TUDCA-treated mice showed minimal hair cell loss even in the basal turn (Fig. 4b). The high-magnification images in PBS-treated mice revealed a disordered arrangement and an evident loss of stereocilia, but the images in TUDCA-treated mice showed well-arranged rows with minimal loss of stereocilia (Fig. 4a′ and b′).

TUDCA inhibits hair cell apoptosis in *erl* mice

In agreement with the morphological measurement, surface preparation with TUNEL staining confirmed TUDCA's otoprotective effects in *erl* mice cochleae. We performed TUNEL staining in untreated *erl* mice at multi-time points. We found that the TUNEL-positive OHCs were very few at an early age (4 weeks); however, because of the OHC death and cell missing in the organ of Corti, it was difficult to observe and count the percentage of TUNEL-positive OHCs at late age (12 weeks). The most distinct TUNEL-staining images were observed at around 7–8 weeks of ages. Thus we chose this time point to perform TUNEL staining in the PBS-treated and TUDCA-treated *erl* mice. At 7 weeks, in the basal turns of cochleae, the PBS group exhibited more TUNEL-positive OHCs in the organ of Corti, and the TUNEL-positive cells were mainly localized in the outer rows of OHCs. On the contrary, the TUDCA group exhibited only a few TUNEL-positive OHCs (Fig. 5a). A quantitative study showed that the mean percentage of TUNEL-positive cells in the TUDCA-treated mice was significantly lower than that of the PBS-treated mice (Fig. 5b).

TUDCA suppresses apoptosis-related genes and caspase-3 activation in *erl* mice

To further determine the mechanism through which TUDCA protects against hair cell death, we assessed the apoptosis-related genes in the PBS and TUDCA groups. Our previous research revealed that at mRNA level, apoptosis-related genes are upregulated in *erl* mice cochleae at 2 weeks and 2 months. In follow-up studies, we found that these apoptosis-related genes were upregulated in *erl* mouse cochleae at 2 weeks, 1 month (P30), and 2 months. Because the *erl* mutant mice showed hearing impairment beginning at around 1 month, we chose P30 as the time point for mRNA tests in this study. The results showed that some of the apoptosis-related genes are down-regulated by TUDCA treatment (Fig. 6a). At P30, a significant difference between the PBS and TUDCA groups was found in caspase-3 and caspase-9 mRNA expression. However, there was no significant difference between the PBS and TUDCA groups in terms of the mRNA levels of the caspase-8 or caspase-12 genes. Also, the mRNA level of transforming growth factor (TNF-α), which was shown to be upregulated in *erl* mouse cochleae in previous work, demonstrated no significant difference between the PBS and TUDCA groups (Fig. 6a). We further tested the cleaved caspase-3 protein in the PBS and TUDCA groups at P30. Western blot showed that the cleaved caspase-3 was much less in the TUDCA-treated cochleae than in the PBS-treated cochleae (Fig. 6b).

DISCUSSION

As a Food and Drug Administration (FDA) approved medication, TUDCA has already been used in experimental research and clinical applications related to liver disease and diabetes without significant side effects (Beuers, 2006, Kars et al., 2010). More specifically, TUDCA was recently used to protect visual function in animal models of neurodegenerative diseases, including retinitis pigmentosa and retinal detachment (Mantopoulos et al., 2011, Han et al., 2013b). Therefore, it is reasonable to presume TUDCA to be a potential therapeutic drug in the treatment of sensorineural hearing loss. In the present study, we reported that TUDCA treatment in *erl* mice led to a marked alleviation of hearing impairment and an evident reduction in hair cell apoptosis and degeneration. This study extends our previous research on the animal model of postnatal onset hearing loss and suggests that TUDCA might be a viable therapeutic agent for the treatment of human DFNB12. This study can serve as a starting point for gathering evidence about the underlying mechanism of the molecular events through which TUDCA prevents hearing loss in *Cdh23* gene mutations. With respect to the dosage, in a previous research on the mouse model of retinitis pigmentosa, mice with high-dose TUDCA (500 mg/kg) treatment showed negative impact on the body weight of developing mice (Drack et al., 2012). In our preliminary study, we also observed reduced body weight with TUDCA at 200 mg/kg starting at P7. Moreover, we found that TUDCA at 100 mg/kg could protect against hearing loss with no evident toxic effects, thus found a good balance between safety and effectiveness. Follow-up studies on a wider range of TUDCA dosages will be pursued to establish the optimal dosage and toxicity profile for potential translational use in clinical studies.

As a novel mutation of the *Cdh23* gene, the *erl* mutation leads to the amino-acid substitution of serine to proline (S70P) in the first ectodomain of the CDH23 protein. The CDH23

protein is localized in the upper part of the tip-link in cochlear hair cells and plays a key role in the mechanisms of hearing generation and sensorineural deafness (Siemens et al., 2004, Kazmierczak et al., 2007, Wu et al., 2012, Fettiplace and Kim, 2014, Géléoc and Holt, 2014). In humans, DFNB12 and age-related hearing loss are associated with missense mutations of *Cdh23*, whereas Usher syndrome type 1D (USH1D, characterized by deaf-blindness simultaneous with vestibular dysfunction) is associated with nonsense mutations of *Cdh23* (Bolz et al., 2001, Bork et al., 2001, Astuto et al., 2002, Miyagawa et al., 2012). In mice, null mutations of *Cdh23* lead to the *waltzer* phenotype, which is characterized by hearing loss and vestibular dysfunction (Di Palma et al., 2001, Wilson et al., 2001). On the other hand, missense mutations of *Cdh23* cause congenital deafness or progressive hearing loss without vestibular dysfunction, and are therefore regarded as animal models of DFNB12 (Schwander et al., 2009, Manji et al., 2011, Miyasaka et al., 2012). The *erl* mutant mice showed progressive hearing loss without vestibular dysfunction and proved to be a mouse model for DFNB12.

The hearing impairment in *erl* mice starts at P27 and develops to complete deafness at P100 (Han et al., 2012). In this study, systemic TUDCA treatment remarkably alleviated hearing loss in *erl* mice up to 12 weeks, as detected by ABR and DPOAE tests (Fig. 1 and 2). In ABR tests, the otoprotective effects occur earlier for high-frequency stimuli (starting at 4 weeks) than for low-frequency stimuli (starting at 6 weeks). In DPOAE tests, the DP-improvement was exhibited at high frequencies at an early age (6 weeks) and at low frequencies at a late age (12 weeks). The OHC loss in *erl* mice becomes notable from 2 months of age and is mainly localized in the basal and middle turns of cochleae, as revealed by cytocochleograms (Han et al., 2012). In the current study, the histological examinations by surface preparation displayed a lower percentage of OHC loss in the basal and middle turns of cochleae in TUDCA-treated *erl* mice (Fig. 3). The PBS-treated mice exhibited almost all OHC loss and no detectable subcellular structure of hair bundles in the basal turn by SEM. However, in the apical turns, the PBS-treated mice showed significant OHC loss and detectable stereocilia loss (Fig. 4a and a′). On the contrary, the TUDCA-treated mice showed minimal hair cell loss and well-arranged rows of stereocilia, even in the basal turns (Fig. 4b and b′). Considering the basal to apical gradient of the cochlea, corresponding to high to low sound frequencies respectively (Müller et al., 2005, Lentz et al., 2013), these results provided a pronounced correlation between anatomical observations and functional examinations. Moreover, TUDCA treatment exhibited comparable or even better hearing improvement (confirmed by ABR threshold shift) than erythropoietin and Z-VAD-FMK treatment in our previous work (Han et al., 2012, Han et al., 2013a). The data provide further evidence that anti-apoptosis is an effective therapeutic mechanism against *erl* mouse hearing loss.

DNA fragmentation detected by TUNEL staining was further evidence of hair cell preservation in TUDCA-treated *erl* mice at 7 weeks. In agreement with research in retinal neural cells (Rodrigues et al., 2003, Gaspar et al., 2013), TUDCA significantly prevented an increase in apoptosis in *erl* mouse cochleae, especially the OHCs in the outer rows (Fig. 5a). TUDCA's anti-apoptotic effect was considered to be associated with caspase-3 activation and apoptotic threshold reduction (Ramalho et al., 2008, Ramalho et al., 2013). Our previous

study showed that both extrinsic and intrinsic apoptotic pathways were activated in *erl* mutants (Han et al., 2012). The mRNA levels of TNF-α, caspase-3, caspase-8, caspase-9, and caspase-12 were significantly upregulated, suggesting apoptosis activation in *erl* mouse's inner ears (Han et al., 2012). In the present study, we found that TUDCA significantly suppressed the increased mRNA levels for caspase-3 and caspase-9 but not for TNF-α, caspase-8, or caspase-12 in *erl* mice (Fig. 6a). As the activated, downstream and effector caspase of apoptosis pathway, the activated caspase-3 (cleaved caspase-3) was evident in the cytoplasm of OHCs in *erl* mice (Han et al., 2012). In the present study, the cleaved caspase-3 protein extracted from the *erl* mouse cochleae in the TUDCA group was significantly less than that in the PBS group detected by Western blot (Fig. 6b), indicating that the caspase-3 activation in *erl* mouse cochleae was suppressed by TUDCA. These findings suggest that TUDCA could partly inhibit the mitochondria- mediated intrinsic apoptosis in *erl* mice. Further experiments are warranted to determine the specific targets of TUDCA's otoprotective effects.

As an animal model of DFNB12, *erl* mice with TUDCA treatment showed significant hearing restoration and hair cell preservation. Given the safety profiles in humans, our results extended the clinical indication of the anti-apoptotic effects of TUDCA treatment, highlighting its application as a therapeutic drug in the treatment of DFNB12. In addition, it is assumed that early treatment with TUDCA might protect against the blindness and deafness in human Usher syndrome, which shares the same gene mutation as DFNB12.

CONCLUSION

The results of the current study demonstrate that systemic treatment with TUDCA could prevent hearing impairment by inhibiting hair cell death in *erl* mice. Further experiments are needed to determine the specific molecular mechanisms for TUDCA's otoprotective effects. More importantly, as an FDA-approved drug, clinical trials are necessary to confirm TUDCA's protective efficacy in human DFNB12 and in other types of inherited sensorineural hearing loss caused by *Cdh23* gene mutations, such as USH1D.

Acknowledgments

This study was supported by the China Scholarship Council (No. 201306280095), the National Institutes of Health (R01DC015111), the Foundation of Taishan Scholar (tshw20110515), the Natural Science Foundation of Shandong Province (ZR2012HZ004), and the National Natural Science Foundation of China (81271085, 81530030 and 81400467).

Abbreviations

ABR	auditory-evoked brainstem response
Cdh23	*Cadherin23*
DFNB	autosomal recessive nonsyndromic deafness
DPOAE	distortion product oto-acoustic emission
FDA	Food and Drug Administration

OHC	outer hair cell
PBS	phosphate-buffered saline
SEM	scanning electron microscope
s.e.m	standard error of the mean
SPL	sound pressure level
TNF	transforming growth factor
TUDCA	tauroursodeoxycholic acid
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
UDCA	ursodeoxycholic acid
USH1D	Usher syndrome type 1D

References

Amaral JD, Viana RJ, Ramalho RM, Steer CJ, Rodrigues CM. Bile acids: regulation of apoptosis by ursodeoxycholic acid. J Lipid Res. 2009; 50:1721–1734. [PubMed: 19417220]

Astuto L, Bork J, Weston M, Askew J, Fields R, Orten D, Ohliger S, Riazuddin S, Morell R, Khan S. CDH23 Mutation and Phenotype Heterogeneity: A Profile of 107 Diverse Families with Usher Syndrome and Nonsyndromic Deafness. The American Journal of Human Genetics. 2002; 71:262–275. [PubMed: 12075507]

Beuers U. Drug insight: Mechanisms and sites of action of ursodeoxycholic acid in cholestasis. Nature clinical practice. Gastroenterology & hepatology. 2006; 3:318–328. [PubMed: 16741551]

Bolz H, von Brederlow B, Ramirez A, Bryda EC, Kutsche K, Nothwang HG, Seeliger M, del CSCM, Vila MC, Molina OP, Gal A, Kubisch C. Mutation of CDH23, encoding a new member of the cadherin gene family, causes Usher syndrome type 1D. Nat Genet. 2001; 27:108–112. [PubMed: 11138009]

Bork JM, Peters LM, Riazuddin S, Bernstein SL, Ahmed ZM, Ness SL, Polomeno R, Ramesh A, Schloss M, Srisailpathy CR, Wayne S, Bellman S, Desmukh D, Ahmed Z, Khan SN, Kaloustian VM, Li XC, Lalwani A, Riazuddin S, Bitner-Glindzicz M, Nance WE, Liu XZ, Wistow G, Smith RJ, Griffith AJ, Wilcox ER, Friedman TB, Morell RJ. Usher syndrome 1D and nonsyndromic autosomal recessive deafness DFNB12 are caused by allelic mutations of the novel cadherin-like gene CDH23. American journal of human genetics. 2001; 68:26–37. [PubMed: 11090341]

Ceylan-Isik AF, Sreejayan N, Ren J. Endoplasmic reticulum chaperon tauroursodeoxycholic acid alleviates obesity-induced myocardial contractile dysfunction. J Mol Cell Cardiol. 2011; 50:107–116. [PubMed: 21035453]

Noben-Trauth K. Mutations in Cdh23, encoding a new type of cadherin, cause stereocilia disorganization in waltzer, the mouse model for Usher syndrome type 1D. Nature genetics. 2001; 27:103–107. [PubMed: 11138008]

Drack AV, Dumitrescu AV, Bhattarai S, Gratie D, Stone EM, Mullins R, Sheffield VC. TUDCA slows retinal degeneration in two different mouse models of retinitis pigmentosa and prevents obesity in Bardet-Biedl syndrome type 1 mice. Investigative ophthalmology & visual science. 2012; 53:100–106. [PubMed: 22110077]

Fettiplace R, Kim KX. The physiology of mechanoelectrical transduction channels in hearing. Physiol Rev. 2014; 94:951–986. [PubMed: 24987009]

Furness DN, Johnson SL, Manor U, Rüttiger L, Tocchetti A, Offenhauser N, Olt J, Goodyear RJ, Vijayakumar S, Dai Y. Progressive hearing loss and gradual deterioration of sensory hair bundles in the ears of mice lacking the actin-binding protein Eps8L2. Proceedings of the National Academy of Sciences. 2013; 110:13898–13903.

Gaspar JM, Martins A, Cruz R, Rodrigues CM, Ambrosio AF, Santiago AR. Tauroursodeoxycholic acid protects retinal neural cells from cell death induced by prolonged exposure to elevated glucose. Neuroscience. 2013; 253:380–388. [PubMed: 24012838]

Genetic Evaluation of Congenital Hearing Loss Expert Panel. Genetics evaluation guidelines for the etiologic diagnosis of congenital hearing loss. Genetics in Medicine. 2002; 4:162. [PubMed: 12180152]

Géléoc GS, Holt JR. Sound strategies for hearing restoration. Science. 2014; 344:1241062. [PubMed: 24812404]

Green DR, Kroemer G. The pathophysiology of mitochondrial cell death. Science. 2004; 305:626–629. [PubMed: 15286356]

Han F, Yu H, Tian C, Chen HE, Benedict-Alderfer C, Zheng Y, Wang Q, Han X, Zheng QY. A new mouse mutant of the Cdh23 gene with early-onset hearing loss facilitates evaluation of otoprotection drugs. Pharmacogenomics J. 2012; 12:30–44. [PubMed: 20644563]

Han F, Yu H, Zheng T, Ma X, Zhao X, Li P, Le L, Su Y, Zheng QY. Otoprotective effects of erythropoietin on Cdh23erl/erl mice. Neuroscience. 2013a; 237:1–6. [PubMed: 23384607]

Han J, Dinculescu A, Dai X, Du W, Smith WC, Pang J. Review: the history and role of naturally occurring mouse models with Pde6b mutations. Mol Vis. 2013b; 19:2579–2589. [PubMed: 24367157]

Kars M, Yang L, Gregor MF, Mohammed BS, Pietka TA, Finck BN, Patterson BW, Horton JD, Mittendorfer B, Hotamisligil GS, Klein S. Tauroursodeoxycholic Acid may improve liver and muscle but not adipose tissue insulin sensitivity in obese men and women. Diabetes. 2010; 59:1899–1905. [PubMed: 20522594]

Kazmierczak P, Sakaguchi H, Tokita J, Wilson-Kubalek EM, Milligan RA, Muller U, Kachar B. Cadherin 23 and protocadherin 15 interact to form tip-link filaments in sensory hair cells. Nature. 2007; 449:87–91. [PubMed: 17805295]

Keene CD, Rodrigues CM, Eich T, Chhabra MS, Steer CJ, Low WC. Tauroursodeoxycholic acid, a bile acid, is neuroprotective in a transgenic animal model of Huntington's disease. Proceedings of the National Academy of Sciences of the United States of America. 2002; 99:10671–10676. [PubMed: 12149470]

Laukens D, Devisscher L, Van den Bossche L, Hindryckx P, Vandenbroucke RE, Vandewynckel YP, Cuvelier C, Brinkman BM, Libert C, Vandenabeele P, De Vos M. Tauroursodeoxycholic acid inhibits experimental colitis by preventing early intestinal epithelial cell death. Laboratory investigation; a journal of technical methods and pathology. 2014; 94:1419–1430.

Lentz JJ, Jodelka FM, Hinrich AJ, McCaffrey KE, Farris HE, Spalitta MJ, Bazan NG, Duelli DM, Rigo F, Hastings ML. Rescue of hearing and vestibular function by antisense oligonucleotides in a mouse model of human deafness. Nature medicine. 2013; 19:345–350.

Manji SS, Miller KA, Williams LH, Andreasen L, Siboe M, Rose E, Bahlo M, Kuiper M, Dahl HH. An ENU-induced mutation of Cdh23 causes congenital hearing loss, but no vestibular dysfunction, in mice. Am J Pathol. 2011; 179:903–914. [PubMed: 21689626]

Mantopoulos D, Murakami Y, Comander J, Thanos A, Roh M, Miller JW, Vavvas DG. Tauroursodeoxycholic acid (TUDCA) protects photoreceptors from cell death after experimental retinal detachment. PLoS One. 2011; 6:e24245. [PubMed: 21961034]

Miyagawa M, Nishio S-y, Usami S-i. Prevalence and clinical features of hearing loss patients with CDH23 mutations: a large cohort study. Plos one. 2012; 7:e40366. [PubMed: 22899989]

Miyasaka Y, Suzuki S, Ohshiba Y, Watanabe K, Sagara Y, Yasuda SP, Matsuoka K, Shitara H, Yonekawa H, Kominami R. Compound Heterozygosity of the Functionally Null Cdh23 (v-ngt) and Hypomorphic Cdh23 (ahl) Alleles Leads to Early-onset Progressive Hearing Loss in Mice. Experimental animals/Japanese Association for Laboratory Animal Science. 2012; 62:333–346. [PubMed: 24172198]

Momose T, Tsubaki T, Iida T, Nambara T. An improved synthesis of taurine- and glycine-conjugated bile acids. Lipids. 1997; 32:775–778. [PubMed: 9252967]

Müller M, Hünerbein Kv, Hoidis S, Smolders JW. A physiological place–frequency map of the cochlea in the CBA/J mouse. Hearing research. 2005; 202:63–73. [PubMed: 15811700]

Ozcan U, Yilmaz E, Ozcan L, Furuhashi M, Vaillancourt E, Smith RO, Gorgun CZ, Hotamisligil GS. Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. Science. 2006; 313:1137–1140. [PubMed: 16931765]

Ramalho RM, Nunes AF, Dias RB, Amaral JD, Lo AC, D'Hooge R, Sebastião AM, Rodrigues CM. Tauroursodeoxycholic acid suppresses amyloid β-induced synaptic toxicity in vitro and in APP/PS1 mice. Neurobiology of aging. 2013; 34:551–561. [PubMed: 22621777]

Ramalho RM, Viana RJ, Castro RE, Steer CJ, Low WC, Rodrigues CM. Apoptosis in transgenic mice expressing the P301L mutated form of human tau. Mol Med. 2008; 14:309–317. [PubMed: 18368144]

Rodrigues CM, Sola S, Nan Z, Castro RE, Ribeiro PS, Low WC, Steer CJ. Tauroursodeoxycholic acid reduces apoptosis and protects against neurological injury after acute hemorrhagic stroke in rats. Proceedings of the National Academy of Sciences of the United States of America. 2003; 100:6087–6092. [PubMed: 12721362]

Schwander M, Xiong W, Tokita J, Lelli A, Elledge HM, Kazmierczak P, Sczaniecka A, Kolatkar A, Wiltshire T, Kuhn P, Holt JR, Kachar B, Tarantino L, Muller U. A mouse model for nonsyndromic deafness (DFNB12) links hearing loss to defects in tip links of mechanosensory hair cells. Proceedings of the National Academy of Sciences of the United States of America. 2009; 106:5252–5257. [PubMed: 19270079]

Siemens J, Lillo C, Dumont RA, Reynolds A, Williams DS, Gillespie PG, Müller U. Cadherin 23 is a component of the tip link in hair-cell stereocilia. Nature. 2004; 428:950–955. [PubMed: 15057245]

Smith RJ, Shearer AE, Hildebrand MS, Van Camp G. Deafness and hereditary hearing loss overview. 2014

Vos T, Flaxman AD, Naghavi M, Lozano R, Michaud C, Ezzati M, Shibuya K, Salomon JA, Abdalla S, Aboyans V. Years lived with disability (YLDs) for 1160 sequelae of 289 diseases and injuries 1990–2010: a systematic analysis for the Global Burden of Disease Study 2010. The Lancet. 2013; 380:2163–2196.

Wilson SM, Householder DB, Coppola V, Tessarollo L, Fritzsch B, Lee EC, Goss D, Carlson GA, Copeland NG, Jenkins NA. Mutations in Cdh23 cause nonsyndromic hearing loss in waltzer mice. Genomics. 2001; 74:228–233. [PubMed: 11386759]

Wu L, Pan L, Zhang C, Zhang M. Large protein assemblies formed by multivalent interactions between cadherin23 and harmonin suggest a stable anchorage structure at the tip link of stereocilia. The Journal of biological chemistry. 2012; 287:33460–33471. [PubMed: 22879593]

Zheng QY, Johnson KR, Erway LC. Assessment of hearing in 80 inbred strains of mice by ABR threshold analyses. Hearing research. 1999; 130:94–107. [PubMed: 10320101]

Highlights

- *Cdh23*$^{erl/erl}$ mutant mouse is an established model of genetic hearing loss.
- Tauroursodeoxycholic acid (TUDCA) has been used in diseases related to apoptosis.
- We studied otoprotective effects and mechanisms of TUDCA in *Cdh23*$^{erl/erl}$ mice.
- TUDCA protects against hearing loss and hair cell death in *Cdh23*$^{erl/erl}$ mice.
- TUDCA partly inhibits the intrinsic apoptosis in *Cdh23*$^{erl/erl}$ mouse cochleae.

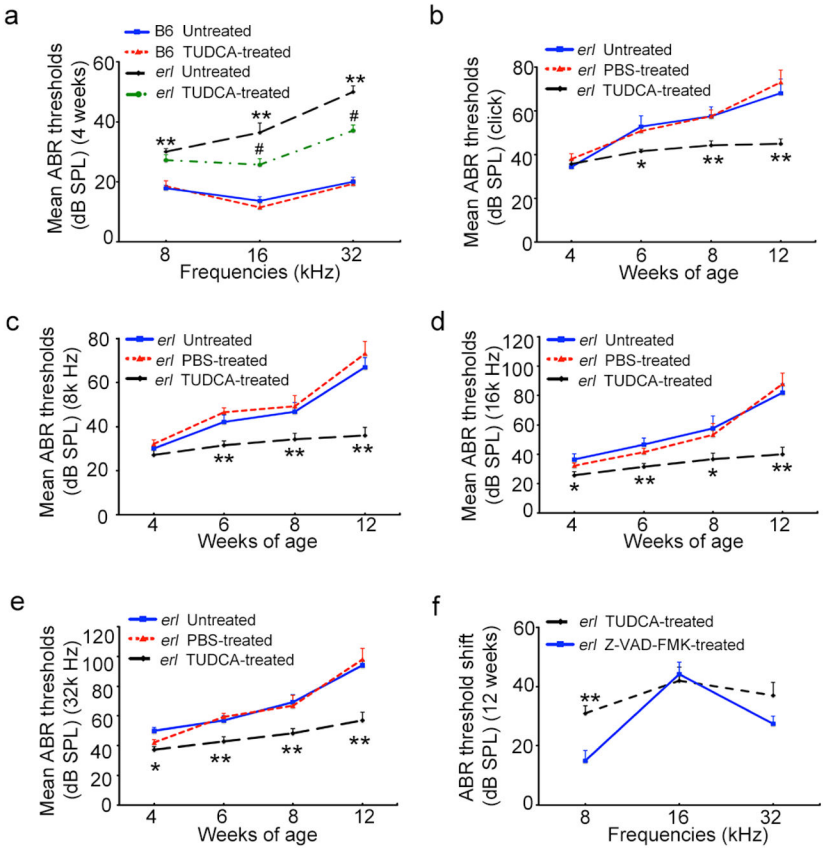

Fig. 1.

TUDCA decreases ABR thresholds in *erl* mice. (a) ABR thresholds in B6 and *erl* mice with or without TUDCA treatment at 4 weeks of age at stimuli of 8 kHz, 16 kHz, and 32 kHz tone bursts. The *erl* mutant mice exhibited higher ABR thresholds than B6 mice, and could be alleviated by TUDCA. The TUDCA-treated B6 mice exhibited similar hearing levels with untreated B6 mice. Data are presented as the mean ± s.e.m (n = 7 mice per group). $^{**}P < 0.01$ from untreated B6 mice, $^{\#}P < 0.05$ from TUDCA-treated *erl* mice, student t-test. (b–e) ABR thresholds were obtained at 4, 6, 8, and 12 weeks (W) of mouse age for various stimuli: clicks (b) and tone bursts of 8 kHz (c), 16 kHz (d), and 32 kHz (e). ABR thresholds in the TUDCA group were significantly lower than those in the PBS group or the untreated group. No significant difference was found between the untreated and PBS groups. Data are presented as the mean ± s.e.m (n = 7 mice at 4 and 6 weeks per group; n = 5 mice at 8 and 12 weeks per group). $^{**}P < 0.01$, $^{*}P < 0.05$, one-way ANOVA test. (f) TUDCA exhibited similar or even better hearing improvement compared to Z-VAD-FMK at 12 weeks. ABR threshold shifts were calculated by untreated ABR thresholds minus the thresholds in each of TUDCA and Z-VAD-FMK treated studies. Data are presented as the mean ± s.e.m. (n = 5 mice in TUDCA group; n = 6 mice in Z-VAD-FMK group). $^{*}P < 0.05$, student t-test.

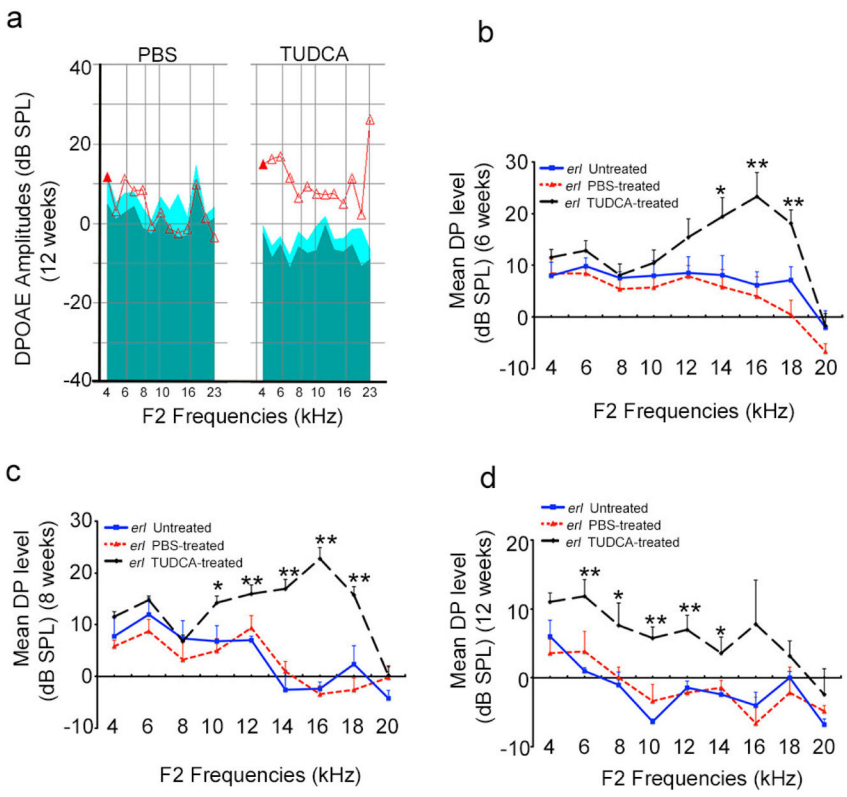

Fig. 2.

TUDCA increases DP levels in *erl* mice. (a) The TUDCA-treated mouse presented DPOAE amplitudes much higher above the noise floor than the PBS-treated mouse at 12 weeks of age. (b–d) The DPOAE tests were performed at 6, 8, and 12 weeks of age, and the DP (2F1–F2) levels at different F2 frequencies were compared across the three groups. The DP value in the TUDCA group was significantly higher than those of the PBS and untreated groups. No meaningful difference was found between the PBS and untreated groups. Data are presented as the mean ± s.e.m (n = 7 mice at 6 weeks per group; n = 5 mice at 8 and 12 weeks per group). $^{**}P < 0.01$, $^{*}P < 0.05$, one-way ANOVA test.

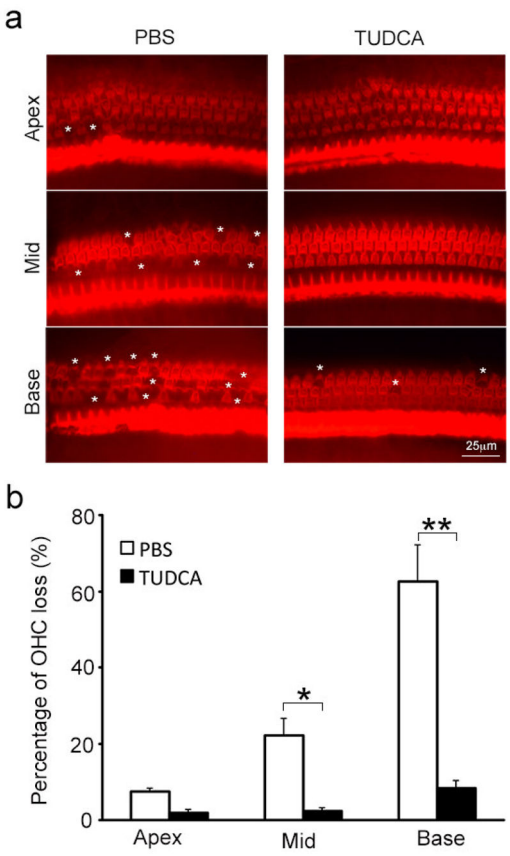

Fig. 3.

TUDCA prevents OHC loss in *erl* mice at 10 weeks of age. (a) The whole-mount preparations from the apex, middle, and basal turns of the cochleae were stained for F-actin. Obvious OHC loss was observed in the basal and middle turns, and a small amount of OHC loss was found in the apical turn in the PBS-treated mouse. Very minimal OHC loss was found in the TUDCA-treated mouse. White stars indicate areas of OHC loss. (b) The mean percentages of OHC loss are shown for each turn of the cochleae in the PBS and TUDCA groups at 10 weeks of age. Compared to the TUDCA group, the PBS group exhibited a higher percentage of OHC loss in the middle and basal turns. Data are presented as the mean ± s.e.m (n = 3 mice per group). $^{**}P < 0.01$, $^{*}P < 0.05$, student t-test.

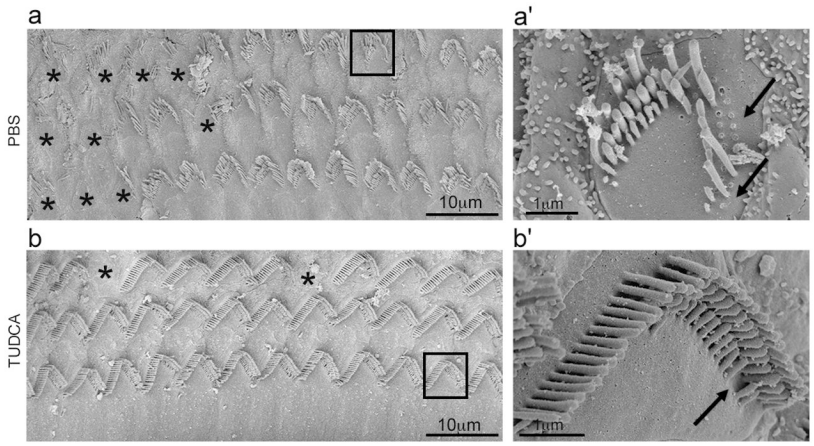

Fig. 4.

OHC morphology and subcellular structures detected by SEM at 9 weeks of age. The PBS-treated mouse showed greater OHC and stereocilia loss in the apical turn (a), while the TUDCA-treated mouse showed very minimal OHC loss even in the basal turn (b). The high-magnification images showed single-OHC subcellular structures in the square boxes in (a) and (b). The PBS-treated mouse exhibited evident disruption and several lack of stereocilia (a′). The TUDCA-treated mouse showed neatly arranged rows and occasional loss of stereocilia (b′). Stars and arrows indicate stereocilia loss.

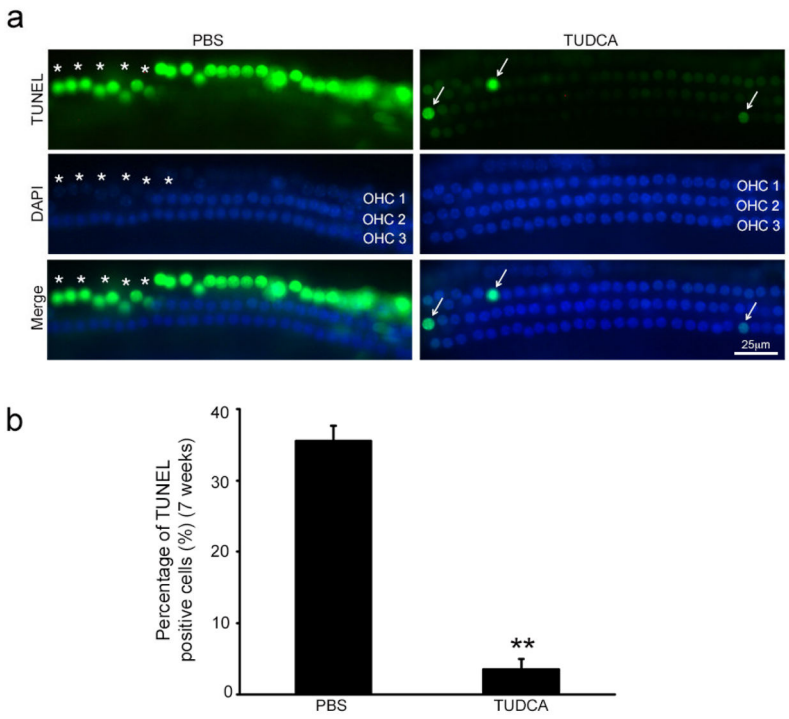

Fig. 5.

TUDCA reduces OHC apoptosis in *erl* mice at 7 weeks of age. (a) Apoptotic cells were seen as green-signal-stained (TUNEL-positive) nuclei in the basal turns of cochleae from different groups. A greater number of TUNEL-positive OHCs were observed in the PBS-treated mouse, and the TUNEL-positive cells were mainly localized in the outer rows of OHCs. Some OHCs were dead and lost from the cochleae, and these cells were neither TUNEL nor DAPI stained. The stars indicate OHC loss in the PBS-treated mouse. A very few TUNEL-positive OHCs were seen in the TUDCA-treated mouse. The arrows indicate TUNEL-positive OHCs in different rows. (b) The mean percentage of TUNEL-positive OHCs in the PBS-treated mice was significantly higher than it was in the TUDCA-treated mice. Data are presented as the mean ± s.e.m (n = 3 mice per group). $^{**}P < 0.01$, student t-test.

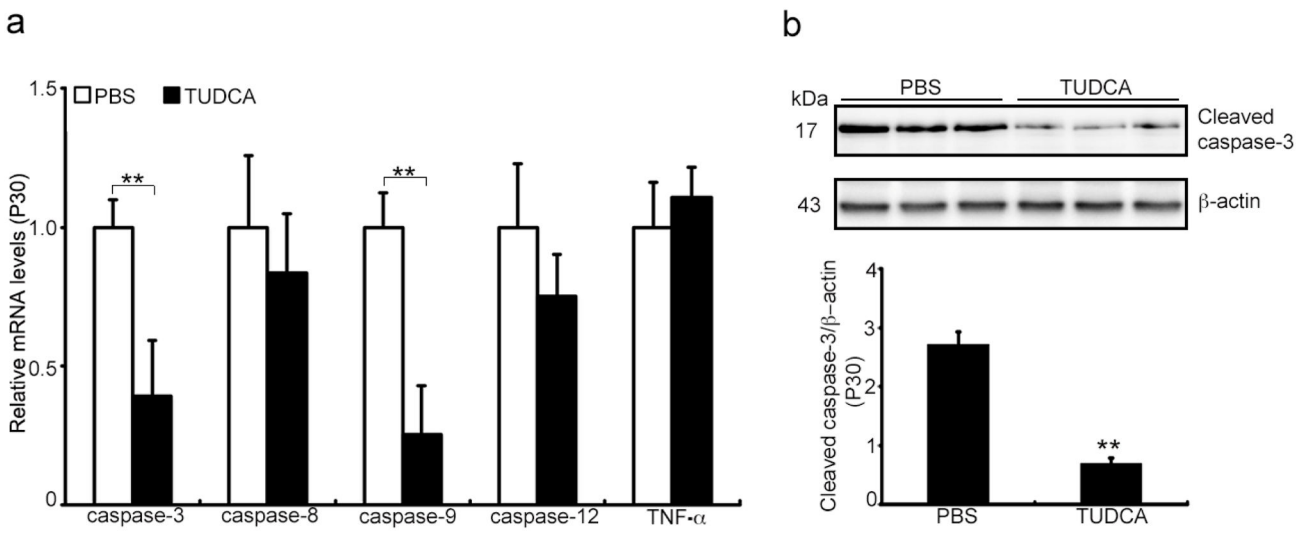

Fig. 6.

TUDCA suppresses apoptosis-related genes and caspase-3 activation at P30. (a) The caspase-3 and caspase-9 mRNA levels were markedly lower in the TUDCA group than in the PBS group at P30. No significant difference was found in the mRNA levels of caspase-8, caspase-12, or TNF-αmRNA levels. Data are presented as the mean ± s.e.m (n = 3 mice per group). $^{*}P < 0.05$, student t-test. (b) The cleaved caspase-3 protein levels in the cochleae from the TUDCA-treated mice were significantly lower than those from the PBS-treated mice at P30. Data are presented as the mean ± s.e.m (n = 3 mice per group). $^{**}P < 0.01$, student t-test.

Table 1

Primer Sequences

Primers	Sequences
Caspase-3 F	ATGGAGAACAACAAAACCTCAGT
Caspase-3 R	TTGCTCCCATGTATGGTCTTTAC
Caspase-8 F	ATGGCGGAACTGTGTGACTCG
Caspase-8 R	GTCACCGTGGGATAGGATACAGCA
Caspase-9 F	CCTAGTGAGCGAGCTGCAAG
Caspase-9 R	ACCGCTTTGCAAGAGTGAAG
Caspase-12 F	AGACAGAGTTAATGCAGTTTGCT
Caspase-12 R	TTCACCCCACAGATTCCTTCC
TNF-α F	CCACCACGCTCTTCTGTCTAC
TNF-α R	CCTTGAAGAGAACCTGGGAGT
Gapdh F	AGGTCGGTGTGAACGGATTTG
Gapdh R	TGTAGACCATGTAGTTGAGGTCA