



# Some Ototoxic Drugs Destroy Cochlear Support Cells Before Damaging Sensory Hair Cells

Dalian Ding<sup>1,2</sup> · Jianhui Zhang<sup>2</sup> · Haiyan Jiang<sup>1</sup> · Weijun Xuan<sup>3</sup> · Weidong Qi<sup>4</sup> · Richard Salvi<sup>1,5</sup>

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## Abstract

A wide variety of ototoxic drugs are capable of damaging the sensory hair cells in the mammalian cochlea resulting in permanent hearing loss. However, the toxic properties of these drugs suggest that some could potentially damage cochlear support cells as well. To test the hypothesis, we treated postnatal day three rat cochlear cultures with toxic doses of gentamicin, cisplatin, mefloquine, and cadmium. Gentamicin primarily destroyed the hair cells and disrupted the intercellular connection with the surrounding support cells. Gentamicin-induced hair cell death was initiated through the caspase-9 intrinsic apoptotic pathway followed by activation of downstream executioner caspase-3. In contrast, cisplatin, mefloquine, and cadmium initially damaged the support cells and only later damaged the hair cells. Support cell death was initiated through the caspase-8 extrinsic apoptotic pathway followed later by downstream activation of caspase-3. Cisplatin, mefloquine, and cadmium significantly reduced the expression of actin and laminin, in the extracellular matrix, leading to significant disarray of the sensory epithelium.

**Keywords** Hair cells · Support cells · Cochlea · Apoptosis · Necrosis · Ototoxicity · Caspase

## Abbreviations

Cd	Cadmium
CIS	Cisplatin
DC	Deiters cell
ECM	Extracellular matrix
GM	Gentamycin
HC	Hensen cells
IHC	Inner hair cells
IS	Inner sulcus
ISC	Inner sulcus cells

MEF	Mefloquine
OHC	Outer hair cells
OS	Outer sulcus
PC	Pillar cell
PBS	Phosphate buffered saline
SC	Support cells

## Introduction

The cochlea has an exquisite architecture epitomized by three parallel rows of outer hair cells (OHC) and one row of inner hair cells (IHC) surrounded by a diverse array of support cells (SC). This highly organized matrix of hair cells and SC forms the organ of Corti, which spirals around the modiolus from the base to the apex of the cochlea. The well-defined cellular architecture of the organ of Corti is disrupted to varying degrees when the sensory hair cells are destroyed by ototoxic drugs and other ototraumatic agents. Because most ototoxic drugs damage the sensory hair cells resulting in permanent hearing loss, the sensory cells are generally considered the most vulnerable and important structures in the cochlea. Consequently, most studies have focused on understanding the mechanisms that result in hair cell death while paying less attention to SC which contribute to the structural and

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Dalian Ding and Jianhui Zhang contributed equally to this work.

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✉ Richard Salvi  
salvi@buffalo.edu

<sup>1</sup> Center for Hearing and Deafness, University at Buffalo, 137 Cary Hall, Buffalo, NY 14214, USA

<sup>2</sup> Department of Otolaryngology Head and Neck Surgery, The Third People's Hospital of Chengdu, Chengdu, China

<sup>3</sup> Department of Otolaryngology, Ren-Ai Hospital, Guangxi University of Chinese Medicine, Nanning, China

<sup>4</sup> Department of Otolaryngology Head and Neck Surgery, Huashan Hospital, Fudan University, Shanghai, China

<sup>5</sup> Department of Audiology and Speech-Language Pathology, Asia University, Taiwan, China

functional integrity of the cochlear sensory epithelium. In recent years, there has been a growing interest in the roles that SC play in the development and maintenance of the cochlea and the extent to which ototoxic drugs damage the SC.

Because cochlear hair cells do not regenerate, there is considerable interest in understanding the biological mechanisms that lead to cell death because this could aid in the development of new therapies to promote the survival of both hair cells and SC. Cochlear hair cells and SC can degenerate in different ways such as through necrosis, apoptosis, or anoikis (Alam et al. 2000; He et al. 2017; Hou et al. 2019; Menardo et al. 2012; Nakagawa et al. 1998; Nicotera et al. 1999; Zhang et al. 2018). Necrosis is an energy-independent uncontrolled form of cell death that leads to karyolysis, cell swelling, and the eventual rupture of the cell that leads to the release of the cell's contents into the surrounding extracellular space (Elmore 2007). This spillage can disrupt the extracellular matrix and cell-cell interconnections which are critically important for reestablishing cochlear homeostasis (Heaney et al. 2002; Milner and Campbell 2003; Pietrogrande et al. 2018; Raphael 2002). Apoptosis, by contrast, is a highly orchestrated form of programmed cell death that leads to the removal of damaged or unwanted cells with minimal leakage of the cell's contents into the surrounding environment (D'Arcy 2019; Elmore 2007). Morphologically, apoptosis is characterized by cell shrinkage, membrane blebbing, nuclear condensation, and the eventual breakdown of the cell into apoptotic fragments (Kerr et al. 1972). Apoptotic cell death also disrupts intercellular connections and the extracellular environment (Boudreau et al. 1995; Bozzo et al. 2006; Stupack et al. 2001). Apoptosis is triggered by initiator caspase-8 and/or caspase-9. Initiator caspase-9 is a member of the intrinsic cell death pathway activated by intracellular signals that target mitochondria. Mitochondrial dysfunction leads to the opening of the mitochondrial transition pore, loss of mitochondrial membrane potential, and release of cytochrome c. Initiator caspase-8, a member of the extrinsic cell death pathway, is activated by death receptors located on the cell's membrane such as TNF $\alpha$ /TNFR1. Activation of caspase-8 and/or caspase-9 results in the activation of downstream executioner caspase-3 and eventual breakdown of the cell into apoptotic bodies released into the extracellular space.

The hair cells and SC are anchored to one another through the extracellular matrix (ECM) and a variety of cell-cell interconnections (D'Arcangelo et al. 1975; Raphael and Altschuler 2003; Richardson et al. 1987). The ECM and intercellular contacts play important roles in growth, differentiation, survival, and cell death (Chiarugi and Giannoni 2008; Setz et al. 2011; Shanmugathasan and Jothy 2000). When the extracellular matrix and intercellular contacts are disrupted, cells become detached from one another resulting in disorganization of the cochlear sensory epithelium. Cellular detachment triggers a unique form of programmed cell death referred to as

anoikis (Frisch 2000; Frisch and Sreaton 2001; Hammar et al. 2004; Miyazaki et al. 2004). Failure of detached cells to undergo anoikis can lead to tumor metastasis (Chun et al. 2013; Taddei et al. 2012).

In the current study, we set out to characterize several aspects of cell death that occurs in hair cells and SC in postnatal cochlear organotypic cultures treated with four different ototoxic compounds: gentamycin (GM), an antibiotic; cisplatin (CIS), an antineoplastic agent; mefloquine (MEF), an antimalarial; and cadmium (Cd), a neurotoxic metal. These ototoxic compounds are known to damage the hair cells, but the extent to which they damage SC is poorly understood. It is also unclear if ototoxic damage to SC occurs before or after the hair cells are damaged. Because these ototoxic compounds likely disrupt the extracellular matrix (ECM) and intercellular connections between hair cells and SC, laminin labeling was used to assess the effects of these ototoxic drugs on the ECM (Hamill et al. 2009).

## Methods

**Subjects** All experiments were performed with postnatal day-3 Sprague-Dawley rat pups (Charles River, Wilmington, MA).

**Animal Subject Approval** This project was approved by the University at Buffalo Institutional Animal Care and Use Committee and carried out in accordance with NIH guidelines.

**Cochlear Organotypic Cultures** Our procedures for preparing postnatal cochlear organotypic cultures have been described in detail in previous publications (Deng et al. 2013; Ding et al. 2011; Ding et al. 2013b; Qi et al. 2008; Zhang et al. 2018). Briefly, the cochlear basilar membrane was carefully dissected out from the temporal bone and placed on top of a drop of rat collagen gel in serum-free medium. Cochlear explants were cultured in standard serum-free medium overnight and then treated with one of four ototoxic drugs to assess the time course of cell death and the characteristics of the cell death pathway. The number of cochlear culture explants used for each experimental condition (e.g., drug, dose, duration, staining method) was  $n \geq 6$ . For consistency, photomicrographs are shown for the upper basal turn. The location and numbers of samples evaluated for each condition are indicated in each figure legend.

**Ototoxic Drugs** Cochlear cell death was evaluated with four ototoxic compounds using drug doses and exposure durations known to cause hair cell loss. Some explants were treated with 0.25 mM gentamycin (GM, Sigma # G-6896) for 24 h to examine the cell death pathways associated with this aminoglycoside antibiotic. Others were treated with 50  $\mu$ M cisplatin

(CIS, Aldrich #479306) for 24 h or 48 h to identify the programmed cell death pathways associated with this widely used anticancer platinum drug. Another group was treated with 50  $\mu$ M mefloquine (MEF, Sigma #M2319) for 12 h or 24 h to examine programmed cell death with an antimalarial drug with ototoxic properties. Finally, some cultures were treated with 100  $\mu$ M cadmium chloride (Cd, Sigma #655198) for 24 h or 48 h, an ototoxic heavy metal.

**F-actin and Nuclear Staining** At the end of the experiment, the cochlear explants were fixed with 10% formalin in 0.1 M phosphate-buffered saline (PBS). To label F-actin, which is heavily expressed in the cuticular plate and stereocilia of hair cells, some samples were labeled with Acti-stain 488 phalloidin (1:100, Cat. # PHDG1-A, Cytoskeleton, Inc.) or Acti-stain phalloidin 555 (Cat. # PHDH1A, Cytoskeleton). Nuclei were stained with To-Pro-3 (Life Technologies, #T3605).

**Immunolabeling** To identify sensory hair cells, samples were labeled with a rabbit antibody against myosin VI (1:100, Sigma M5187). To recognize SC, samples were labeled with a goat anti-SOX2 monoclonal antibody (1:100, Santa Cruz Biotechnology sc-365823). To assess the extracellular matrix, cultures were labeled with a rabbit anti-laminin antibody (1:100, Abcam; Ab11575). Following fixation, some explants were incubated with one or more primary antibodies (myosin VI, SOX2 and/or laminin) in 1% Triton X-100 and 5% donkey serum in 0.1 M PBS for 24 h. After rinsing 3X with PBS, specimens were incubated with TRITC conjugated donkey anti-rabbit secondary antibody for 2 h (1:200, Jackson ImmunoResearch, #79266) to visualize the myosin VI in hair cells, or Alexa Fluor 488 conjugated donkey anti-goat secondary antibody for 2 h (1:200, Invitrogen 474685) to label SOX2 in SC, and Alexa Fluor 488 conjugated donkey anti-rabbit secondary antibody for 2 h (1:200, Abcam ab150073) to show the laminin in ECM, respectively.

**Caspases** Ototoxic activation of caspase-3, -8, and -9 was carried out using procedures described in our earlier publications (Deng et al. 2013; Ding et al. 2007, 2013a; Ding and Salvi 2010; Dong et al. 2014; Yu et al. 2015; Zhang et al. 2018). To determine if the intrinsic and/or extrinsic initiator caspase pathways were activated by the ototoxic treatments, samples were evaluated with CaspGLOW Red Active caspase-8 and caspase-9 staining kits (BioVision K188, K118). Ototoxic activation of the downstream executioner pathway was evaluated using a caspase-3 staining kit (BioVision K183). Briefly, the cochlear explants were incubated with CaspGLOW Red Active caspase-8, or CaspGLOW Red Active caspase-9, or CaspGLOW Fluorescein Active caspase-3 for 1 h at the end

of experiment. Afterwards, specimens were fixed with 10% formalin in PBS for 3 h.

**Microscopy** As described previously, the surface preparations of the cochlear sensory epithelium were mounted in glycerin on glass slides and evaluated with a confocal microscope (Zeiss LSM-510) (Zhang et al. 2018) using appropriate filters to detect green fluorescence (excitation 488 nm, emission 519 nm), red fluorescence (excitation 550 nm, emission 570 nm), and blue/purple fluorescence (excitation 642 nm, emission 661 nm) respectively. Collected confocal images were further processed with Zeiss LSM Image Examiner and post-processed with Adobe Photoshop software.

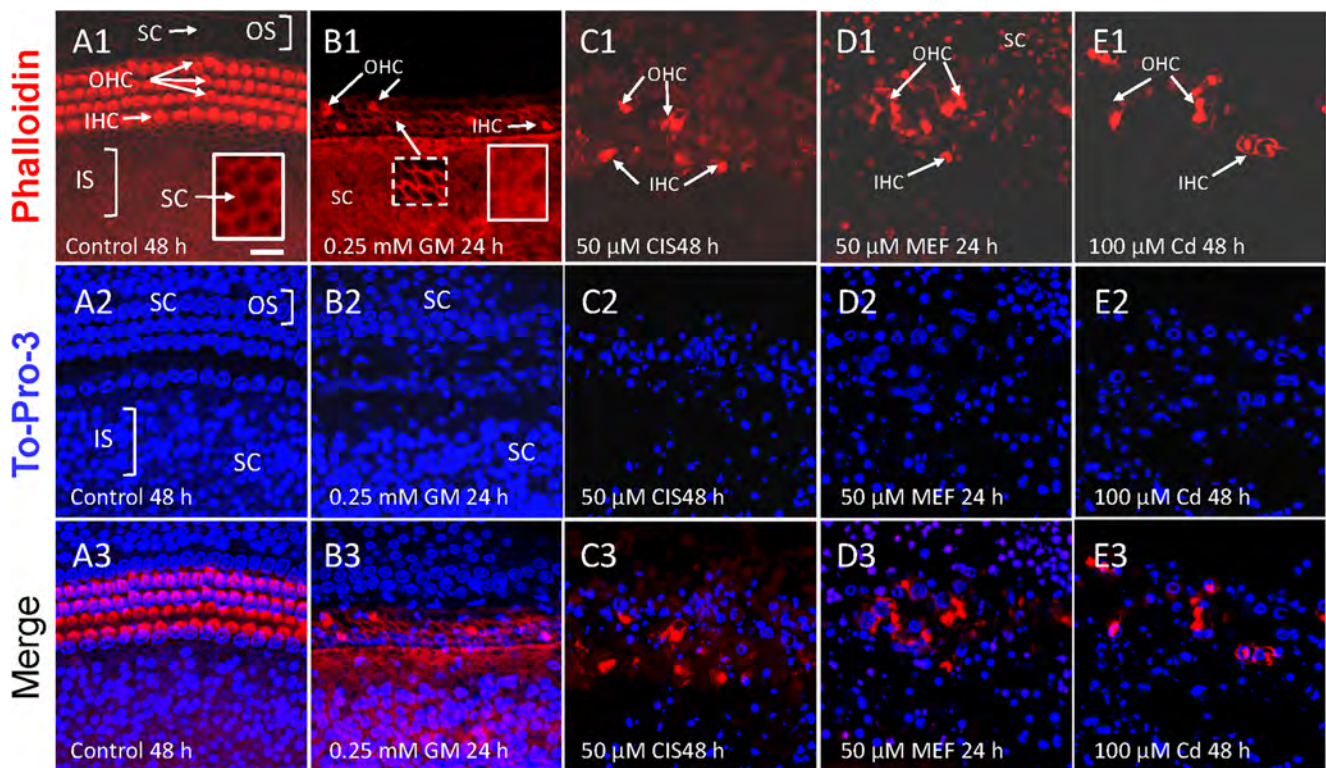
## Results

### Hair Cell and Support Cell Degeneration and Disarray

Cochlear organotypic cultures were labeled with fluorescently labeled phalloidin in order to visualize the surface architecture of the cochlear sensory epithelium. Actin is abundantly expressed in the hair cell stereocilia and cuticular plate and to a lesser extent in the borders of the SC. The surface architecture of a normal cochlea maintained in culture medium for 48 h is shown in Fig. 1A1; this representative sample shows robust actin labeling of the stereocilia and cuticular plate in the three orderly parallel rows of OHC and a single row of IHC (Fig. 1). Less intense but distinct actin labeling was also present along the borders of the hexagonal boundaries of the SC in the inner sulcus (IS) and outer sulcus (OS) (Fig. 1 inset). Figure 1A2 shows To-Pro-3 labeling of nuclei in the SC and hair cells in deeper layers of the organ of Corti; Fig. 1A3 shows the merge of panels A1 and A2.

When cochlear cultures were treated for 24 h with 0.25 mM GM, many OHC and IHC were destroyed and only a few were still present (Fig. 1B1). In the place formerly occupied by a missing hair cell, there was a crisp hexagonal band of phalloidin (Fig. 1B1, inset with dashed border). The hair cell lesion reduced the radial width from the row of IHC to the third row of OHC (Fig. 1A1 versus 1B1). The IS region was characterized by diffuse actin labeling of SC; the SC borders were blurry and diffuse labeling was present over the surface of the SC. Figure 1B2 shows the To-Pro-3 labeling of nuclei in deeper layers throughout the organ of Corti; Fig. 1B3 shows the merge of panels B1 and B2.

The surface architecture of the sensory epithelium was altered in a distinctly different manner when cultures were treated with 50  $\mu$ M of CIS for 48 h. CIS caused considerable OHC and IHC loss. The vestiges of the remaining hair cells were often shrunken and distorted and the radial distance between IHC and the outermost row OHC seemed to have increased (Fig. 1C1). Notably, CIS largely eliminated the phalloidin labeling of the SC in the IS and OS regions. The paucity of



**Fig. 1** Representative photomicrographs illustrating the surface architecture of cochlear organotypic cultures approximately 40% of the distance from the base of the cochlea. Samples labeled with Acti-stain phalloidin 555 (top row) which preferentially labels the actin in the stereocilia and cuticular plate of the three rows of outer hair cells (OHC), one row of inner hair cells (IHC) and the hexagonal borders of the supporting cells (SC) in the inner sulcus (IC) and outer sulcus (OS) region of the epithelium. Nuclei stained with To-Pro-3 (blue, middle row). Merge of phalloidin and To-Pro-3 in bottom row. (A1) Control specimens maintained in culture for 48 h. Note intense labeling of the apical pole of three orderly rows of OHC and single row of IHC and actin labeling of borders of SC in the inner sulcus (IS) and outer sulcus (OS) regions. Inset shows higher magnification view of the hexagonal borders of SC. Note thick actin border surrounding the SC and minimal actin labeling within the border. Scale bar 20  $\mu\text{m}$ . (A2) Note To-Pro-3 of hair cells and support cells in deeper layer of organ of Corti. (A3) Merge of A1 and A2. (B1) Cochlear culture treated with 0.25 mM gentamycin (GM) for 24 h. Note massive loss of stereocilia and cuticular plate of OHC and IHC; missing hair cells surrounded by circumferential ring of actin arranged in orderly

rows corresponding to the missing hair cells (inset with dashed border). Inset with solid border shows higher magnification view of apical surface of SC. Note weak labeling of actin borders of SC and diffuse labeling inside the SC border. (B2) Note To-Pro-3 labeled nuclei of SC. (B3) Merge of B1 and B2. (C1) Cochlear culture treated with 50  $\mu\text{M}$  of cisplatin (CIS) for 48 h. Note massive loss of OHC and IHC, disorganized hair cell rows and minimal actin labeling in the SC regions. Cuticular plate of remaining hair cells shrunken or fragmented. (C2) Note paucity of To-Pro-3 labeled nuclei throughout organ of Corti. (C3) Merge of C1 and C2. (D1) Cochlear culture treated with 50  $\mu\text{M}$  mefloquine (MEF) for 24 h. Many OHC and IHC missing; cuticular plate of remaining hair cells shrunken or fragmented and hair cell rows in disarray. Note minimal actin labeling in SC regions. (D2) Note paucity of To-Pro-3 labeled nuclei throughout organ of Corti. (D3) Merge of D1 and D2. (E1) Cochlear culture treated with 100  $\mu\text{M}$  of cadmium (Cd) for 48 h. Hair cell rows in moderate disarray; many OHC and IHC missing. Minimal actin labeling in SC regions. (E2) Note lack of To-Pro-3 nuclear labeling throughout organ of Corti. (E3) Merge of E1 and E2

To-Pro-3 labeled nuclei in the SC and hair cell regions is clearly evident in Fig. 1C2 and the merge of panel C1 and C2 in Fig. 1C3.

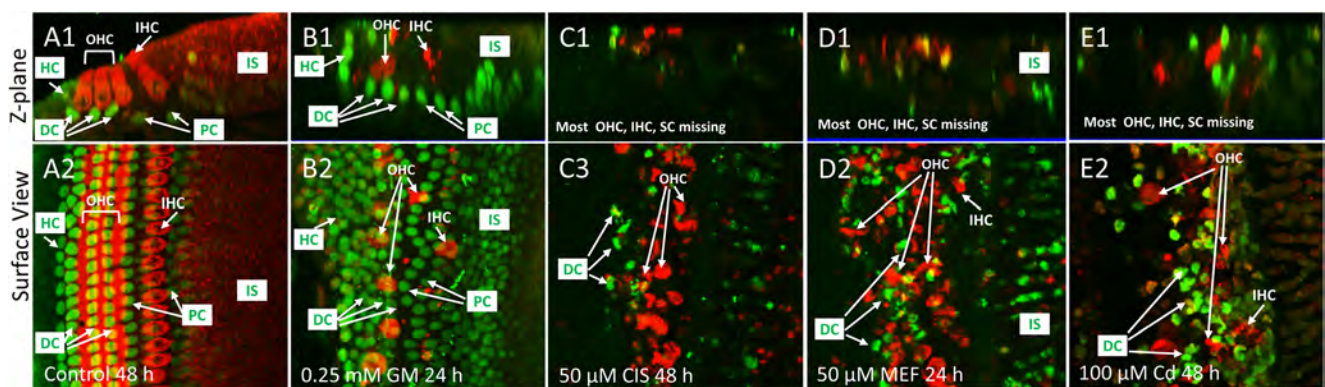
Treatment with MEF for 24 h disrupted the surface architecture of the sensory epithelium in a manner similar to CIS (Fig. 1D1). Many OHC and IHC were missing and remnants of the residual hair cells were shrunken and distorted. There was nearly complete absence of phalloidin labeling of SC and the radial distance between IHC and the outermost OHC appeared larger than normal. Figure 1D2 shows the dearth of To-Pro-3 labeled nuclei in the SC and hair cell region in deeper layers of the organ of Corti; Fig. 1D3 shows the merge of panel D1 and D2.

Cd treatment for 48 h destroyed many IHC and OHC (Fig. 1E1). The vestiges of the remaining hair cells were shrunken and the hair cell rows were disorganized. Cd largely eliminated phalloidin labeling from SC, much like CIS and MEF. Figure 1E2 shows the To-Pro-3 labeling of nuclei in the deeper layers of the organ of Corti; there was a paucity of nuclei in the SC and hair cell regions. Figure 1E3 shows the merge of panel E1 and E2. Taken together, these results suggest that CIS, MEF, and Cd ototoxicity largely abolished the expression of actin, an important structural protein present in most SC whereas GM seemed to alter the distribution and enhance the expression of actin in certain regions.

**Ototoxic Destruction of SC** Most ototoxicity studies focus on damage to the sensory hair cells, but the preceding results suggest that these drugs might be toxic to SC. To determine the extent to which these four ototoxic drugs damaged the SC and hair cells, cochlear cultures were labeled with antibodies against myosin VI to visualize the hair cells and SOX2 to assess the SC. The surface view (Fig. 2A2) and Z-plane images (Fig. 2A1) illustrate the location of the SC surrounding the hair cells in a normal control cochlea maintained in culture medium for 48 h. Myosin VI (red) was present throughout the cytoplasm of the three parallel rows of OHC and one row of IHC. SOX2 (green) labeling filled the Hensen cells (HC) alongside the third row of OHC and the Deiters cells (DC) beneath the three rows of OHC; moderate labeling was also present in the pillar cells (PC) and some SC in the IS region. Treatment with 0.25 mM GM for 24 h resulted in significant loss of OHC and IHC (Fig. 2). Myosin-labeled remnants of damaged hair cells were present within the sensory epithelium surrounded by rows of HC, DC, PC (Fig. 2B1), and SC in the IS region (Fig. 2B2). Treatment with 50  $\mu$ M CIS for 48 h not only damaged or destroyed most IHC and OHC labeled with myosin VI, but also almost completely eliminated the SOX2 labeling previously expressed in HC, DC, PC, and other SC in the IS region (Fig. 2C1–2). Similarly, treatment with 50  $\mu$ M MEF for 24 h destroyed most myosin VI labeled hair cells and SOX2 labeled HC, DC, PC, and other SC in the IS region (Fig. 2D1–2). Treatment with 100  $\mu$ M Cd for 48 h also destroyed or damaged most myosin VI labeled hair cells and eliminated many of the SOX2 labeled SC cells throughout the sensory epithelium (Fig. 2E1–2).

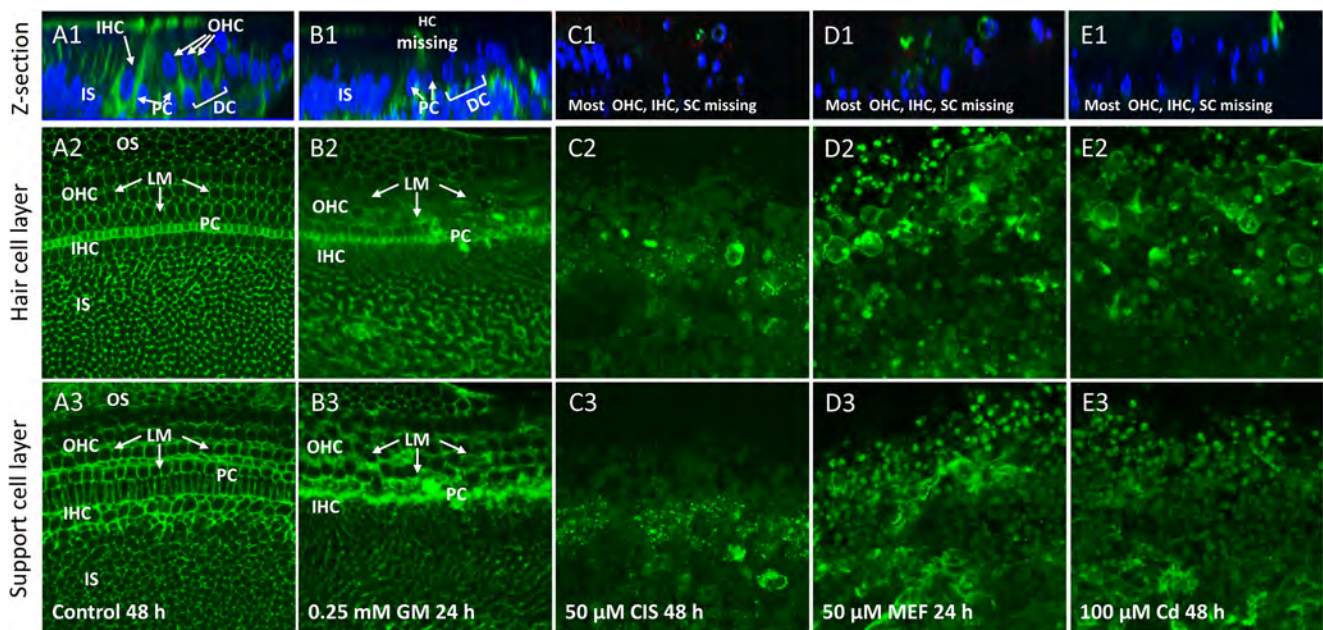
**Ototoxic Damage to Extracellular Matrix** Laminin, present in the ECM, is involved in cell adhesion and regulating tissue

architecture. To determine the extent to which the ECM was disrupted, cochlear organotypic cultures were labeled with an antibody against laminin and cell nuclei were labeled with To-Pro-3. In radial sections of normal cochlear explants (Fig. 3A1), laminin labeling was most evident in the upper layer of the epithelium above the nuclei of the hair cells and SC (Fig. 3A1). Laminin-only-labeled cross sections (To-Pro-3 labeling removed) of cochlear surface preparations were taken at the level of the hair cells or SC. In cross sections in the focal plane of the hair cells (Fig. 3A2), highly organized circumferential rings of laminin were evident in the OS, OHC, and IHC regions. A strong band of laminin was also present in the PC region, whereas regularly spaced patches of laminin labeling occupied the IS area. In confocal images through the SC layer (Fig. 3A3), circumferential rings of laminin were present throughout. Treatment with 0.25 mM GM for 24 h resulted in the loss of many hair cell nuclei, but the nuclei of SC were still present throughout the deeper layers of the epithelium (Fig. 3B1). Confocal images taken through the hair cell layer (Fig. 3B2) of the epithelium revealed blurred, laminin labeling mainly in the OHC, IHC, and PC regions. Images taken in the deeper SC layer (Fig. 3B3) revealed thickened circumferential rings of laminin mainly in the OHC, IHC, and PC regions. When cultures were treated with 50  $\mu$ M of CIS for 48 h, there was a large reduction in the number of nuclei seen in radial sections throughout the epithelium (Fig. 3C1) and almost complete loss of laminin labeling. There was a large reduction and considerable disarray in laminin staining in cross sections taken through the hair cell layer (Fig. 3C2) and SC layer (Fig. 3C3). In cultures treated with 50  $\mu$ M of MEF for 24 h, there was a major loss of hair cell nuclei and SC nuclei and a loss of laminin labeling in radial cross sections of the sensory epithelium (Fig. 3D1). Cross sections taken through the hair



**Fig. 2** Representative photomicrographs ( $n \geq 6$  replicates per condition) from the upper basal turn of cochlear organotypic cultures labeled with myosin VI (red) and SOX2 (green). Representative Z-plane image (A1) and surface view of control cochlear cultured for 48 h. Cytoplasm of three rows of outer hair cells (OHC) and one row of inner hair cells (IHC) labeled with myosin VI. SOX2 expressed in Hensen cells (HC), Deiters cells (DC), pillar cells (PC), and SC in inner sulcus cells (IS) region. (B1–2) Treatment with 0.25 mM GM for 24 h resulted in loss of most myosin

VI expressing OHC and IHC; however, SOX2 labeling was still present in HC, DC, PC, and other SC in the IS region. (C1–2) Many myosin VI labeled OHC and IHC and SOX2 labeled SC missing after 48 h treatment with 50  $\mu$ M CIS. (D1–2) Treatment with 50  $\mu$ M MEF resulted in significant loss of myosin VI labeled OHC and IHC and SOX2 labeled SC. (E1–2) Many myosin VI labeled OHC and IHC and SOX2 labeled SC missing after treatment with 100  $\mu$ M Cd



**Fig. 3** Cochlear organotypic cultures ( $n \geq 6$  replicates per condition) from the upper basal turn labeled with an antibody against laminin (LM, green, all rows) and/or with To-Pro-3 (blue, top row only) which labels nuclei. Top row: Representative Z-plane images showing the location of laminin and cell nuclei. Middle row and bottom row only show laminin-labeling of optical sections of surface preparations. Middle row shows sections taken at the level of the hair cells; bottom row shows section from the SC layer. (A1) Z-plane image of control cochlea cultured for 48 h. Strong laminin labeling near and below the surface; nuclear labeling mainly located below the surface in regions occupied by inner hair cells (IHC), outer hair cells (OHC), pillar cells (PC), Deiters cells (DC), and other SC in the inner sulcus (IS) region. (A2) Circumferential rings of LM in OHC

and IHC region and OS region. Strong labeling of laminin in band of PC. Many patches of laminin in IS region. (A3). Circumferential rings of laminin throughout the SC layer. (B1–2) Treatment with 0.25 mM GM for 24 h resulted in blurring of circumferential rings of laminin in IHC, OHC, and PC regions. (B3) Thickening of circumferential rings of laminin mainly in OHC, IHC, and PC regions. (C1–3) Treatment with 50  $\mu$ M CIS for 48 h reduced the number of cell nuclei; laminin labeling also greatly reduced. (D1–3) Treatment with 50  $\mu$ M MEF reduced the number of nuclei; laminin labeling in hair cell layer and SC layers reduced and disorganized. (E1–3) Treatment with 100  $\mu$ M Cd eliminates nuclei; laminin labeling in hair cell and SC layers reduced and greatly disorganized

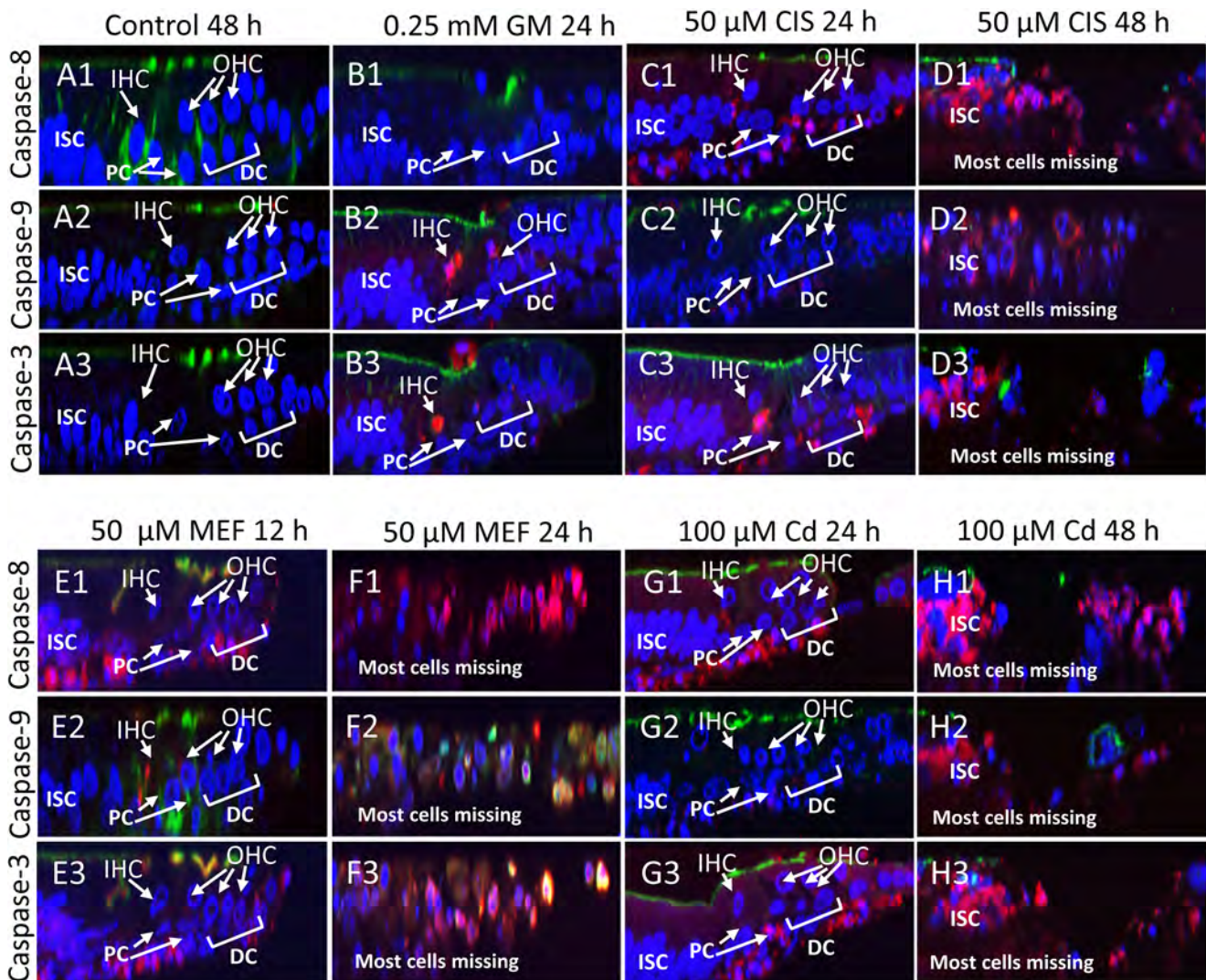
cell layer (Fig. 3D2) and SC layer (Fig. 3D3) revealed a significant reduction and considerable disarray in laminin labeling. When cultures were treated with 100  $\mu$ M of Cd for 48 h, there was major loss of hair cell nuclei and reduction of laminin labeling in radial cross sections of the sensory epithelium (Fig. 3E1). Cross sections through the hair cell and SC layers revealed significant loss and disarray in laminin labeling.

The preceding results indicate that GM primarily damages the hair cells and has only a minor influence on the SC and the expression of laminin in the ECM. On the other hand, CIS, MEF, and Cd not only caused significant hair cell damage, but also triggered a massive loss of SC and laminin labeling in the ECM which resulted in considerable disorganization of the sensory epithelium.

**Caspase-Mediated Ototoxicity** Because the structural damage induced by GM differed from that evoked by the other three ototoxins, we expected that there would be major differences in the caspase-mediated cell death pathway triggered by GM versus the other three ototoxins. To test this hypothesis, cell permeable carboxy-fluorescein probes were used to evaluate activation of initiator caspase-8 of the extrinsic apoptotic pathway, initiator caspase-9 of the intrinsic apoptotic pathway and

downstream executioner caspase-3. (Note: for consistency in caspase image presentation, CaspGLOW active fluorescein, normally green, was presented as red in the confocal images). Assessments were made at an early and a late time point to determine if the processes initiating cell death were triggered by the extrinsic pathway or intrinsic caspase pathway. Cochlear cultures were co-labeled with To-Pro-3 to identify nuclei and with fluorescently labeled phalloidin to visualize actin. As expected, in control cochlea cultured for 48 h, there was no evidence of activated caspase-8, caspase-9, or caspase-3 in Z-plane images of the sensory epithelium (Fig. 4A1–3). In cochlear cultures treated with 0.25 mM GM for only 12 h, initiator caspase-9 could be seen in the IHC and OHC region along with less intense staining of downstream executioner caspase-3. There was no evidence of caspase-3, caspase-8, or caspase-9 activation or cell nuclear loss in SC regions, consistent with the SOX2 labeling in Fig. 2. These results suggest that GM-induced hair cell loss is initiated through the intrinsic caspase-8 mitochondrial pathway.

A much different picture emerged when cochlear cultures were treated with 50  $\mu$ M CIS for only 24 h. Under these conditions, labeling of initiator caspase-8 of the extrinsic



**Fig. 4** Caspase-mediated cell death in cochlear cultures treated with four different ototoxic drugs. Representative photomicrographs ( $n \geq 6$  replicates per condition) from the upper basal turn. Z-plane sections of cochlear cultures labeled with Acti-stain 488 phalloidin (green) to label actin, To-Pro-3 (blue) to label nuclei and CaspGLOW Fluorescein Active caspase-3 (red color), and CaspGLOW Red caspase-8 (red color) and CaspGLOW Red Active caspase-9 (red color) to label activated caspases. (A1–3) Control cochlea cultured for 48 h. Actin (green) expressed in stereocilia above inner hair cell (IHC) and outer hair cell (OHC) nuclei. Note absence of activated caspase-3 (A3), caspase-8 (A1), and caspase-9 (A2). (B1–3) Cochlear cultures treated with 0.25 mM GM for 24 h. (B1) Nuclei (blue) present in pillar cells (PC), Deiters cells (DC), and inner sulcus cells (ISC), but missing from region normally occupied by IHC and OHC. Note absence of extrinsic caspase-8 labeling. (B2) Activated caspase-9 present in region normally occupied by IHC and OHC. (B3) Executioner caspase-3 present in region normally occupied by IHC and OHC. (C1–3) Cochlear cultures treated with 50  $\mu$ M CIS for 24 h. (C1)

pathway was observed in the SC (DC, PC, ISC) located underneath the hair cells (Fig. 4C1). Executioner caspase-3 labeling was also observed in the same supporting cell region (Fig. 4C3); there was no evidence of initiator caspase-9 at this early time point. However, when cochleae were cultured with the same dose of CIS

IHC and OHC nuclei still present 24 h after CIS treatment. Note activated caspase-8 around the DC, PC, and SC at lower edge of the epithelium. (C2) Note absence of activated caspase-9. (C3) Note activated caspase-3 around PC, DC, and lower edge of epithelium. (D1–3) Cochlea cultured with 50  $\mu$ M of CIS for 48 h. (D1–3) Most hair cell and SC missing. Note presence of activated caspase-8, caspase-9, and caspase-3. (E1–3) Cochlea cultured with 50  $\mu$ M of MEF for 12 h. Note moderate reduction in hair cell and SC nuclei and strong activation of caspase-8 (E1) and caspase-3 in the region around and below the DC, PC, and ISC. Caspase-9 largely absent. (F1–3) Cochlea cultured with 50  $\mu$ M of MEF for 24 h. Note massive loss of hair cell and SC nuclei and strong labeling of activated caspase-8 and caspase-3. (G1–3) Cochlea cultured with 100  $\mu$ M of Cd for 24 h. Note moderate loss of hair cell and SC nuclei. Strong labeling of activated caspase-8 and caspase-3 beneath the PC and DC. Note absence of caspase-9. (H1–H3) Cochlea cultured with 100  $\mu$ M of Cd for 48 h. Most hair cell and supporting cell nuclei missing. Strong labeling of activated caspase-8, caspase-9 and caspase-3 in SC

for 48 h, all three caspases appeared (Fig. 4D1–3), but the labeling was stronger and more widespread for caspase-8 than caspase-9. These results suggest that CIS-induced cell death is triggered by death receptors located on the SC in DC, PC, HC, and ISC; only later did the hair cells begin to die off.

A similar picture emerged when cochlear cultures were treated with 50  $\mu\text{M}$  of MEF for only 12 h. During the early stage of damage, initiator caspase-8 of the extrinsic pathway only appeared in SC (DC, PC, ISC) below the hair cell layer (Fig. 4E1); less intense caspase-3 labeling also appeared in this SC region at this time (Fig. 4E3). There was very little labeling of initiator caspase-9 of the intrinsic pathway (Fig. 4E2). When cochlear cultures were treated with the same dose of MEF for 24 h, there was extensive loss cell nuclei throughout the sensory epithelium. This was accompanied by strong labeling of initiator caspase-8 and executioner caspase-3 (Fig. 4F3) and less intense labeling of initiator caspase-9 in the intrinsic cell death pathway (Fig. 4F2). Thus, MEF-induced ototoxicity appears to begin by activating death receptors on the SC leading to activation of initiator caspase-8 and then downstream activation of caspase-3.

The cell death pathway elicited with Cd was similar to that for CIS and MEF. When cochlear cultures were treated with 100  $\mu\text{M}$  for 24 h, strong initiator caspase-8 labeling was evident in the SC beneath the hair cells (Fig. 4G1); executioner caspase-3 also appeared in this region (Fig. 4G3). However, there was little evidence of initiator caspase-9 (Fig. 4G2). However, when cochleae were cultured for 48 h with the same dose of Cd, there was robust labeling of caspase-8, caspase-9, and caspase-3 and many missing cell nuclei (Fig. 4H1–3). Thus, Cd-ototoxicity also appears to be triggered by membrane damage to SC which leads to the activation of caspase-8 followed by executioner caspase-3.

## Discussion

The location, temporal progression, and apoptotic cell death programs initiated by CIS, MEF, and Cd were strikingly different from those provoked by GM. GM primarily damaged the hair cells and caused mild to moderate disruption to the SC and ECM in the vicinity of the lesion (Figs. 1, 2, and 3). In the vicinity of the hair cell lesion, there was increased expression/thickening of laminin, a component of the ECM suggestive of a homeostatic repair process. GM-induced hair cell death was initiated through the intrinsic mitochondrial pathway, as evidenced by activation of caspase-9, followed by executioner caspase-3 (Fig. 4B1–2). However, there was no evidence of extrinsic caspase-8 activation at the time when these other caspases were activated (Fig. 4B3). In contrast, CIS, MEF, and Cd treatments caused massive loss of both hair cells and SC and major disruption of laminin, a component of the ECM (Figs. 1, 2, and 3). With all three of these ototoxins, the earliest signs of cell death appeared in the SC mainly beneath the hair cells. In the early stages of apoptosis, there were clear signs of initiator caspase-8 activation (Fig. 4C1, F1, and G1) and downstream executioner caspase-3 activation, but little evidence of caspase-9. Only later as the lesion evolved and expanded across the epithelium did initiator caspase-9 appear (Fig. 4D2, F2, G2).

**When SC Death Comes First** Our results indicate that CIS, MEF, and Cd damage begins in the SC prior to damaging the hair cells, unlike GM which mainly targets the hair cell (Figs. 1 and 2). The early damage to SC from CIS, MEF, and Cd was associated with activation of the extrinsic caspase-8 pathway. Cisplatin, which generates damaging reactive oxygen species (Clerici et al. 1996; Gonzalez-Garcia et al. 2010), can initiate caspase-8-mediated apoptosis by acting on the Fas-receptor associated with the FADD adaptor protein (Fas-associated protein with death domain) (Huang et al. 2010, 2003; Micheau et al. 1999). Early activation of caspase-8 has also been seen in the adult cochlea with ethacrynic acid/cisplatin-induced hearing loss (Ding et al. 2007). Mefloquine also caused widespread activation of caspase-8 in vestibular organ culture in both SC and hair cells (Yu et al. 2011). MEF also activated caspase-9, but the labeling was less intense and less widespread consistent with our results. Others have reported that Cd-induced apoptosis in cultured kidney podocytes is initiated through the Fas-FADD caspase-8 mediated pathway (Eichler et al. 2006) consistent with our results. However, in proliferating HEI-OC1 cells derived from the organ of Corti, Cd induced greater activation of caspase-9 than caspase-8 (Kim et al. 2008). This difference could be due to the fact that HEI-OC1 cells are proliferating cells lacking the differentiated features of the cells in our cochlear explants.

**Extracellular Matrix** Laminin, a secreted glycoprotein, is incorporated into the ECM where it participates in cell adhesion, differentiation, and cell migration (Chuang et al. 2014; Davies 2011; Ishii et al. 1992; Racz et al. 2014; Tsuprun and Santi 1999; Zhang et al. 2018). GM destroyed the hair cells and altered the general profile of the ECM (Fig. 3B1–3) and the pattern of actin labeling mainly in the region of hair cell loss (Fig. 1B1); however, the ECM remained largely intact (Fig. 3B1–3). These changes are likely related to cell migration, wound healing, structural repair, and re-establishment of cell-cell contacts in the organ of Corti (Anttonen et al. 2014; Collado et al. 2011; Hordichok and Steyger 2007; Hultcrantz and Li 1995; Raphael and Altschuler 1991). Maintenance of the ECM and cell-cell contacts likely minimizes the activation of the caspase-8 cell death pathway during GM ototoxicity. Conversely, treatments with CIS, MEF, and Cd resulted in significant disruption of the ECM as reflected in the loss of laminin labeling around both hair cells and SC (Fig. 3C–E). Loss of laminin labeling and disruption of the ECM may serve as a trigger for caspase-8-mediated cell death reminiscent of paraquat-induced cell death and anoikis previously observed in the cochlea (Zhang et al. 2018).

**Synopsis** In cochlear organotypic cultures, GM triggered the intrinsic caspase-9 cell death pathway in the hair cells resulting in loss of laminin labeling and mild disorganization of the organ of Corti in the vicinity of the missing hair cells.

CIS, MEF, and Cd by contrast initially triggered the extrinsic caspase-8 cell death pathway in the SC located beneath the hair cells resulting in massive loss of SC that preceded loss of the sensory hair cells.

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## Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflict of interest.

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