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RESEARCH ARTICLE

SPECIAL ISSUE: *IN VITRO* MODELS OF HUMAN DISEASE
TO INFORM MECHANISM AND DRUG DISCOVERY

Human induced pluripotent stem cell-derived inner ear organoids reveal hair cell damage and plasticity after cisplatin and gentamicin exposure

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ABSTRACT

Ototoxicity is a leading cause of sensory deficits, including hearing loss and balance disorders. Predicting ototoxicity is challenging owing to translatability issues of animal models and limited access to human inner ear tissue. Known ototoxic drugs, such as cisplatin (a chemotherapeutic) and gentamicin (an aminoglycoside antibiotic), cause irreversible damage to sensory hair cells and neurons. Here, we establish human induced pluripotent stem cell (hiPSC)-derived inner ear organoids as an *in vitro* model for studying ototoxicity. Exposure to cisplatin and gentamicin led to hair cell and neuronal loss, disrupted organoid architecture and increased cell damage, including apoptosis, in a dose-dependent manner. Remarkably, prolonged culture of treated organoids showed re-emergence of otic vesicle structures with sensory hair cells and neurons. SOX10⁺ otic epithelial cells exhibited increased Ki-67 expression, indicating a potential for developmental plasticity. Our findings demonstrate the value of hiPSC-derived inner ear organoids as a platform for human ototoxicity modeling and provide a basis for testing otoprotective interventions, offering insights into the intrinsic plasticity of developing inner ear cells.

KEY WORDS: Inner ear, Organoids, Human induced pluripotent stem cells, Ototoxicity, Cisplatin, Gentamicin

INTRODUCTION

Inner ear diseases are highly prevalent sensory deficiencies that affect millions of people worldwide (WHO, 2021). Although the etiology of hearing and balance disorders is diverse, a considerable

proportion of these disorders is caused by the toxic effects of various chemical agents on the inner ear, a process defined as ototoxicity (Ganesan et al., 2018; Frymark et al., 2010). Two prominent categories of drugs commonly used in the clinic that cause ototoxicity are platinum-based chemotherapeutic agents and aminoglycoside antibiotics, from which cisplatin and gentamicin, respectively, are the most well-known compounds (Arslan et al., 1999).

Understanding the mechanisms of ototoxicity and developing preventative or therapeutic strategies requires effective research models. Rodent models – including mice, rabbits, rats and guinea pigs – have long been used in ototoxicity studies (Lin et al., 2021; Reynard and Thai-Van, 2023). These studies have elucidated key molecular pathways involved in ototoxic damage and have aided in the identification of potential interventions (Steyger, 2021; Reynard and Thai-Van, 2023). Gentamicin is vital for treating bacterial infections but carries the risk of damaging the inner ear. In organotypic explant cultures and animal models, gentamicin induces hair cell loss (Wei et al., 2005; Huth et al., 2011; Bai et al., 2023; Kucharava et al., 2019), entering through the mechanotransduction machinery of these sensory cells (Lee et al., 2013; Steyger, 2021; Huth et al., 2011; Bai et al., 2023; Park et al., 2017; Kucharava et al., 2019). Once inside, it disrupts mitochondrial function, leading to the release of cytochrome C, increased oxidative stress and activation of apoptotic pathways (Huth et al., 2011; Wei et al., 2005; Bai et al., 2023; Hu and Ma, 2021; Kucharava et al., 2019; Park et al., 2017; Qian et al., 2020). This damage extends beyond hair cells, resulting in the secondary degeneration of sensory neurons (Staecker et al., 1998), a process observed in both the cochlea and vestibular organs.

Cisplatin is one of the most potent chemotherapy drugs and, therefore, very effective against numerous malignant cancers, but the compound often induces hearing loss as a side effect (Lin et al., 2021; Reynard and Thai-Van, 2023; Ganesan et al., 2018). In organotypic explant and cell models to study cisplatin-induced ototoxicity, oxidative stress (Rybak et al., 2007; Hazlitt et al., 2018; Sheth et al., 2017; Umugire et al., 2023), inflammatory responses (Umugire et al., 2023; Hazlitt et al., 2018) and apoptotic pathways (Da-lian et al., 2009; Sheth et al., 2017; Hazlitt et al., 2018; Rybak et al., 2007; Abitbol et al., 2020; Li et al., 2016) are key factors leading to, among others, cochlear hair cell loss (Callejo et al., 2017; Rybak et al., 2007; Slaterry et al., 2014; Abitbol et al., 2020; Ding et al., 2011; Li et al., 2016), spiral ganglion damage (Rybak et al., 2007; Ding et al., 2011) and stria vascularis dysfunction (Abitbol et al., 2020; Lyu et al., 2023). The vestibular system is also affected by cisplatin, as shown by the loss of the sensory epithelium in the cristae ampullares and the utricular maculae (Callejo et al., 2017; Sergi et al., 2003; Sedó-Cabezón et al., 2014).

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The degenerating cells show features of cytoplasm vacuolization, nuclear fragmentation and cell necrosis (Sedó-Cabezón et al., 2014; Abitbol et al., 2020).

The preclinical models have provided invaluable information on the complex interactions between ototoxic agents and the inner ear's sensory epithelium and metabolic tissues. However, these models are not always fully translatable to human disease (Ohlemiller, 2019; Jeong et al., 2024; Blakley et al., 2008). These models differ in genetic or physiological characteristics, but there may also be variances in disease manifestation or drug responses. Recent developments in stem cell technology have given rise to innovative *in vitro* organ models, including inner ear organoids (IEOs) (Koehler et al., 2017; van der Valk et al., 2023). These three-dimensional (3D) culture systems can be derived from embryonic and human induced pluripotent stem cells (hiPSCs) and mimic the function and morphology of the human inner ear (van der Valk et al., 2021, 2023; Steinhart et al., 2023; Doda et al., 2023; Koehler et al., 2017; Moore et al., 2022). These miniaturized organ-like structures hold great promise for modeling human-specific responses to e.g. ototoxic agents, enabling researchers to study ototoxicity in a human (or even patient-derived) cellular model. Recently, IEOs have been used to investigate viral infections such as severe acute respiratory syndrome coronavirus 2 (SARS-CoV2; COVID-19) (Jeong et al., 2021) and to study genetic diseases, including CHARGE syndrome (caused by pathogenic variants in the *CHD7* gene) (Nie et al., 2022) and autosomal recessive deafness-8/10 (DFNB8/10; caused by variants in *TMPRSS3*) (Tang et al., 2019). Here, we expand the application of IEOs to include human ototoxicity studies.

In this study, we aimed to validate the hiPSC-derived IEO model in terms of the pathophysiological effects of the ototoxic agents cisplatin and gentamicin. To this end, IEOs containing sensory epithelium, hair cells and inner ear neurons were generated from hiPSCs in 3D culture. We used a vibratome setup to expose the inner ear structures to the ototoxic drugs. After exposure to cisplatin and gentamicin, we assessed human IEOs through morphological analysis, immunohistochemistry and quantitative PCR (qPCR) to evaluate drug-induced ototoxicity. We observed the expected effects of cisplatin and gentamicin, including hair cell loss, neuronal degeneration and apoptotic cell death. Strikingly, 1 week after drug exposure, hair cell re-emergence was observed, indicating a potential response of developmental plasticity. This work shows how IEOs are capable as serving as human *in vitro* models for ototoxicity studies.

RESULTS

Drug exposure in vibratome-sectioned IEOs

hiPSC-derived IEOs could provide a robust *in vitro* model for studying cisplatin- and gentamicin-induced ototoxicity. Given that the culture protocol produces a 3D cellular aggregate containing otic vesicles, vibratome slices were made to expose the inner ear cell types within the organoid in a consistent manner. Therefore, at day 75, IEOs were cut into 200 μm -thick slices using a vibratome to obtain access to the otic vesicles within (Fig. 1A–C). Post-sectioning, the slices were allowed to recover for 48 h before ototoxic treatments were applied. Vibratome-preserved CDH1⁺, SOX10⁺ otic epithelium containing MYO7A⁺ hair cells and surrounding TUBB3⁺ neurons remained present after this recovery period (Fig. 1D) (van der Valk et al., 2023, 2025). Occasional MYO7A–TUBB3 colocalization was observed, consistent with immature hair cell precursors that transiently express TUBB3 during development (Chacko et al., 2016; Sato et al., 2024). These results confirmed that vibratome sectioning preserves the structural and cellular integrity of the otic vesicles, enabling the continued use of the IEOs as a model for investigating ototoxicity.

Subsequent drug exposure experiments were performed using five conditions: a vehicle control [VHC; organoid maturation (OM) medium only], cisplatin at 30 μM and 100 μM , and gentamicin at 100 μM and 1000 μM . To assess the effect of these treatments, the organoid slices were cultured to allow for analysis of apoptosis, cell damage and structural integrity at 1, 3 and 7 days post-treatment.

Ototoxic compound treatment of IEOs leads to hair cell loss and structural damage

To evaluate the morphological effects of ototoxic compound treatment, vibratome sections exposed to cisplatin or gentamicin were processed and stained with Hematoxylin and Eosin (H&E). Morphological assessments were conducted on samples collected 1, 3 and 7 days post-treatment (Fig. S1). Evaluation criteria included the identification of hair cells and neurons based on their morphology, location and spatial proximity, as well as key structural integrity characteristics of the vesicle (see Table S1 for morphological criteria used in H&E interpretation). These features encompassed the presence of an intact lumen versus luminal collapse, evidence of stratification versus a monolayer indicative of cell loss, and the condition of the basal membrane (intact or disrupted).

Manual scoring (Fig. 2A) revealed a considerable lack of hair cells and neuron presence in samples treated with ototoxic compounds compared to vehicle controls. VHC-treated samples showed a clear presence of hair cells (indicated by hash tags in Fig. 2A') and neurons (indicated by asterisks in Fig. 2A'), classified according to the morphological criteria summarized in Table S1. In contrast, cisplatin 100 μM -treated vesicles showed complete cellular loss after 1 and 3 days of damage [day (D)78+1, D78+3] (Fig. 2A'). In these conditions, pyknotic nuclei, cell body damage and cellular debris were evident despite a persisting vesicle contour, consistent with widespread cell death caused by the high cisplatin concentration. This was also evident in samples treated with 1000 μM gentamicin, whereas those treated with lower gentamicin concentrations showed a lower degree of cell loss. The vesicle contour remained intact for both ototoxic compound-treated conditions, but widespread cell death and cell debris were observed, indicating tissue disruption.

Further analysis focused on the structural integrity of otic vesicles, including scoring for the presence or absence of a lumen (black stars in Fig. 2B), stratification (red arrowheads in Fig. 2B) and basal membrane integrity (green arrowheads in Fig. 2B). One day after treatment (D78+1), the 100 μM cisplatin and 1000 μM gentamicin conditions showed collapsed lumen, a lack of stratification with pycnotic nuclei visible within the vesicle and disrupted basal membranes (Fig. 2A', right; Fig. 2B', right). At 3 days post-treatment (D78+3), 100 μM cisplatin-treated otic vesicles showed continued collapse, loss of stratification and disrupted basal membranes, whereas other conditions showed milder effects (Fig. 2B', middle). Taken together, these results show that higher concentrations of ototoxic compounds lead to a higher degree of cellular damage, which peaks at 1 and 3 days after exposure for cisplatin and gentamicin, respectively.

Cisplatin or gentamicin treatment induces apoptosis and DNA damage in IEOs

To confirm the cell types affected by ototoxic compound treatment, morphological analysis of the otic vesicle was performed using markers for hair cells (MYO7A), neurons (TUBB3), and otic epithelium (SOX10) (Fig. 3A; Fig. S2). The otic vesicle was identified by SOX10⁺ epithelial cells delineating the outer border of the vesicle.

In VHC samples, hair cells were observed to be innervated by neurons within a stratified otic vesicle (Fig. 3A; Fig. S2). After

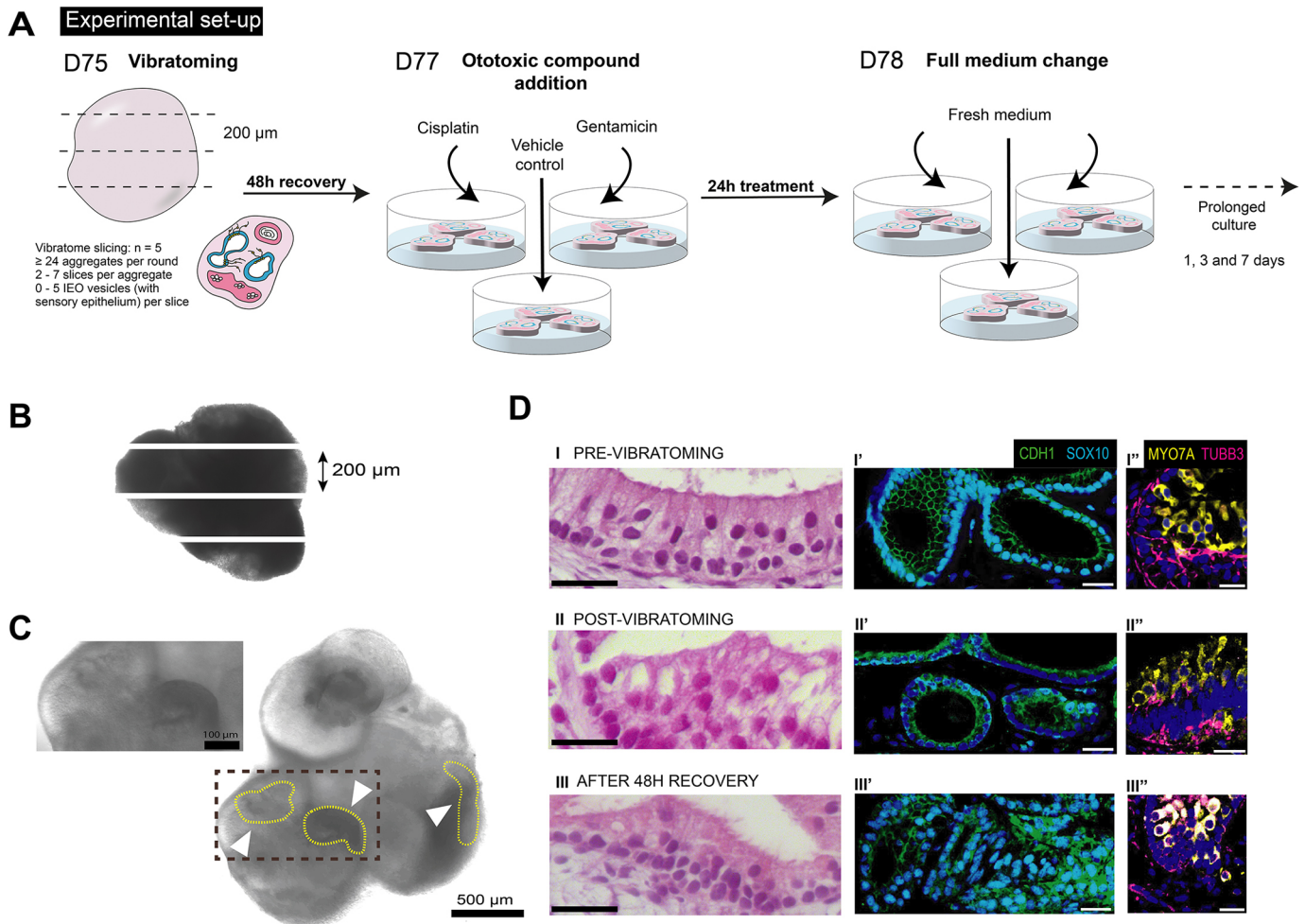


Fig. 1. Experimental set-up for ototoxicity study, demonstrating that vibratome sectioning preserves otic vesicles. (A) Schematic representation of experimental set-up. To expose target cells, inner ear organoids (IEOs) were sectioned with a vibratome at day (D)75, received treatment for 24 h at D77 and were fixed at 1, 3 or 7 days after treatment. (B) Side-view representation of an IEO sectioned into 200 µm-thick slices. (C) Top-view representation of a vibratome slice of an IEO, with arrowheads annotating the otic vesicles (inset). Scale bars: 100 µm and 500 µm. (D) Intact morphology of an otic vesicle without vibratome (I), directly after vibratome (II) and after 48 h recovery (III), showing Hematoxylin and Eosin (H&E) histological staining, followed by immunofluorescent staining of otic epithelium [CDH1⁺ (green)/SOX10⁺ (cyan)] and of otic vesicles containing hair cells (MYO7A⁺, yellow), neurons (TUBB3⁺, magenta) and cell nuclei (DAPI⁺, dark blue). In III, the apparent MYO7A–TUBB3 colocalization likely reflects immature hair cell precursors that transiently co-express neuronal β-III-tubulin during development. Scale bars: 25 µm.

cisplatin treatment (30 and 100 µM), scarcely any hair cells were found. Indication of loss of the neuronal integrity was observed 1 day post-treatment, while SOX10⁺ cells were still detectable in the remaining otic epithelium despite extensive cell damage (Fig. 3A; Fig. S2). Similarly, gentamicin treatment (100 and 1000 µM) resulted in comparable hair cell and neuronal fragmentation without seemingly compromising the integrity of the SOX10⁺ otic epithelium (Fig. 3A; Fig. S2).

Additional immunohistochemical analyses for cleaved caspase-3 (CC3) were performed to assess the level of damage caused by apoptosis. Cisplatin-treated otic vesicles displayed pronounced apoptotic activity, marked by CC3. Cisplatin was present throughout the organoid (Fig. 3B). Gentamicin-treated organoids similarly exhibited CC3 positivity, suggesting apoptosis specific to gentamicin exposure (Fig. 3C). To ensure that the antibiotic reagent normocin did not adversely affect the IEOs, and that it contained no gentamicin, we qualitatively examined control samples by immunofluorescent staining for gentamicin and visually assessed organoid morphology. These observations revealed no detectable gentamicin signal and no

visible signs of morphological change, suggesting that the impact of normocin on the IEOs was negligible (Fig. S3). Quantification of CC3⁺ cells, expressed as a percentage of total cells (DAPI⁺) within the IEOs, revealed a significant increase following cisplatin and gentamicin treatments (Fig. 3D). Longitudinal analysis showed a decreasing trend in CC3⁺ cells over time. Cytotoxicity, assessed by lactate dehydrogenase (LDH) assays, showed peak cell death at D3, followed by a decline by D7, with cisplatin exerting a broader cytotoxic effect across cell types compared to the otic-specific toxicity of gentamicin (Fig. 3E). These results correspond with the observed effects of cisplatin and gentamicin in morphological and immunohistochemical analyses.

Another type of damage that can occur after treatment with ototoxic compounds is DNA damage. Cisplatin can cause DNA damage directly, whereas DNA damage occurs as a secondary effect after reactive oxygen species production by gentamicin (Wu et al., 2002). To verify the presence of DNA damage, we performed RNA expression analysis of *H2AX*, a recognized DNA damage marker (Guthrie and Xu, 2013; Slattery et al., 2014; Benkafadar et al., 2016;

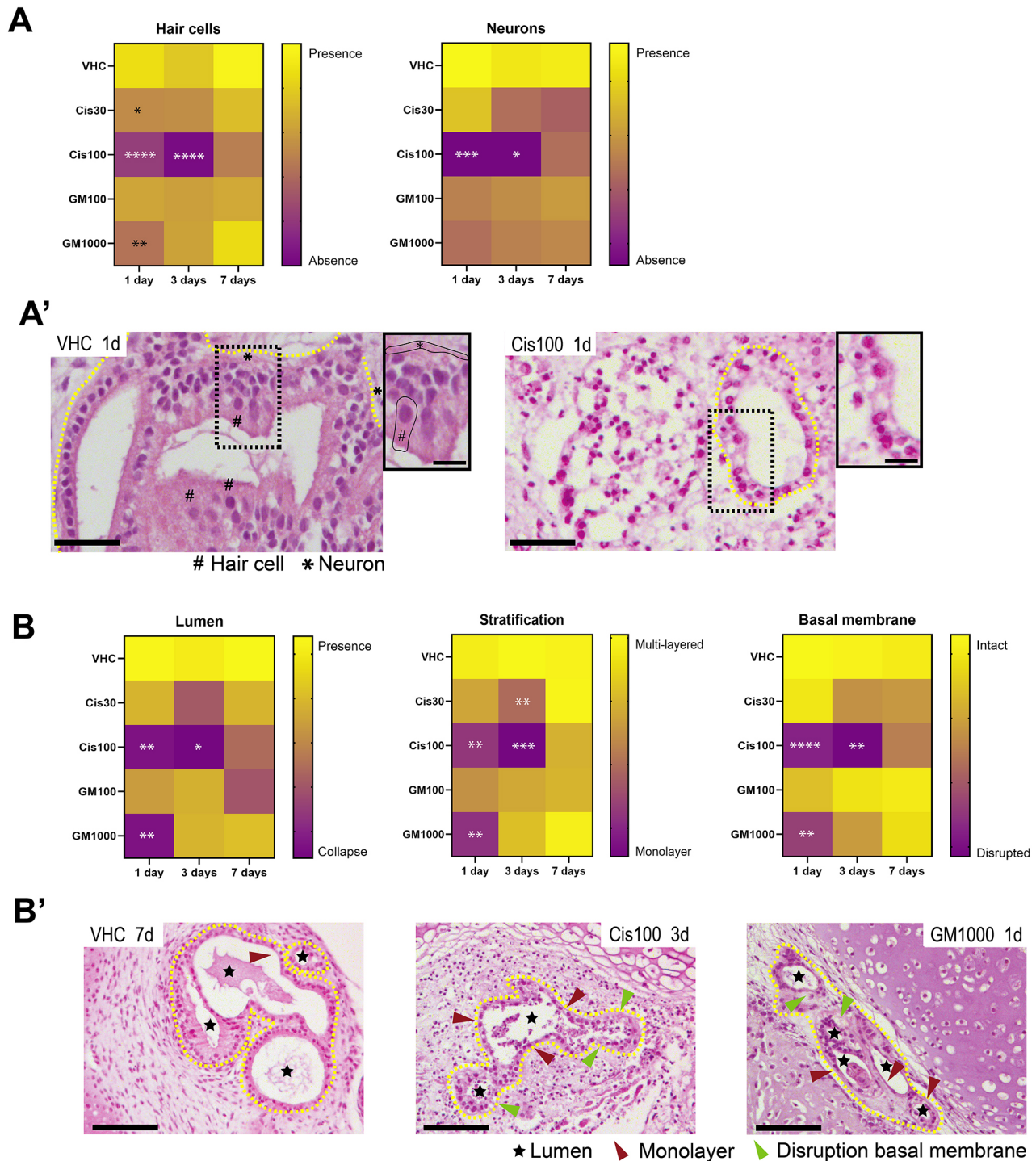


Fig. 2. Morphological analysis of IEO Sections showing ototoxicity-induced hair cell and neuron loss, along with compromised structural integrity. (A) Tables based on semi-quantitative scoring of the presence/absence of hair cells and neurons. Kruskal–Wallis test followed by Dunn’s multiple comparisons test [$*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$ compared to the vehicle control (VHC) from that timepoint]. Error bars represent s.d. ($n > 9$). Cis30, 30 μM cisplatin; Cis100, 100 μM cisplatin; GM100, 100 μM gentamycin; GM1000, 1000 μM gentamycin. (A') H&E examples of presence versus absence of hair cells (hash tags) and neurons (asterisks). Yellow dotted line outlines the otic vesicle. In 100 μM cisplatin-treated samples, annotated profiles frequently displayed pyknotic nuclei and cell body damage, consistent with widespread cytotoxicity despite persistence of the vesicle contour. Scale bars: 25 μm and 10 μm (insets). (B) Tables based on semi-quantitative scoring of the structural characteristics (lumen, stratification and basal membrane). Kruskal–Wallis test followed by Dunn’s multiple comparisons test [$*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$ compared to the VHC from that timepoint]. Error bars represent s.d. ($n > 9$). (B') Representative H&E images selected to illustrate key morphological changes observed after treatment, including collapse of the lumen (black stars), prevalence of monolayer stratification (e.g. epithelial simplification; red arrowheads) and disrupted basal membrane (green arrowheads). Yellow dotted line outlines the otic vesicle. These panels are chosen as examples. Scale bars: 100 μm .

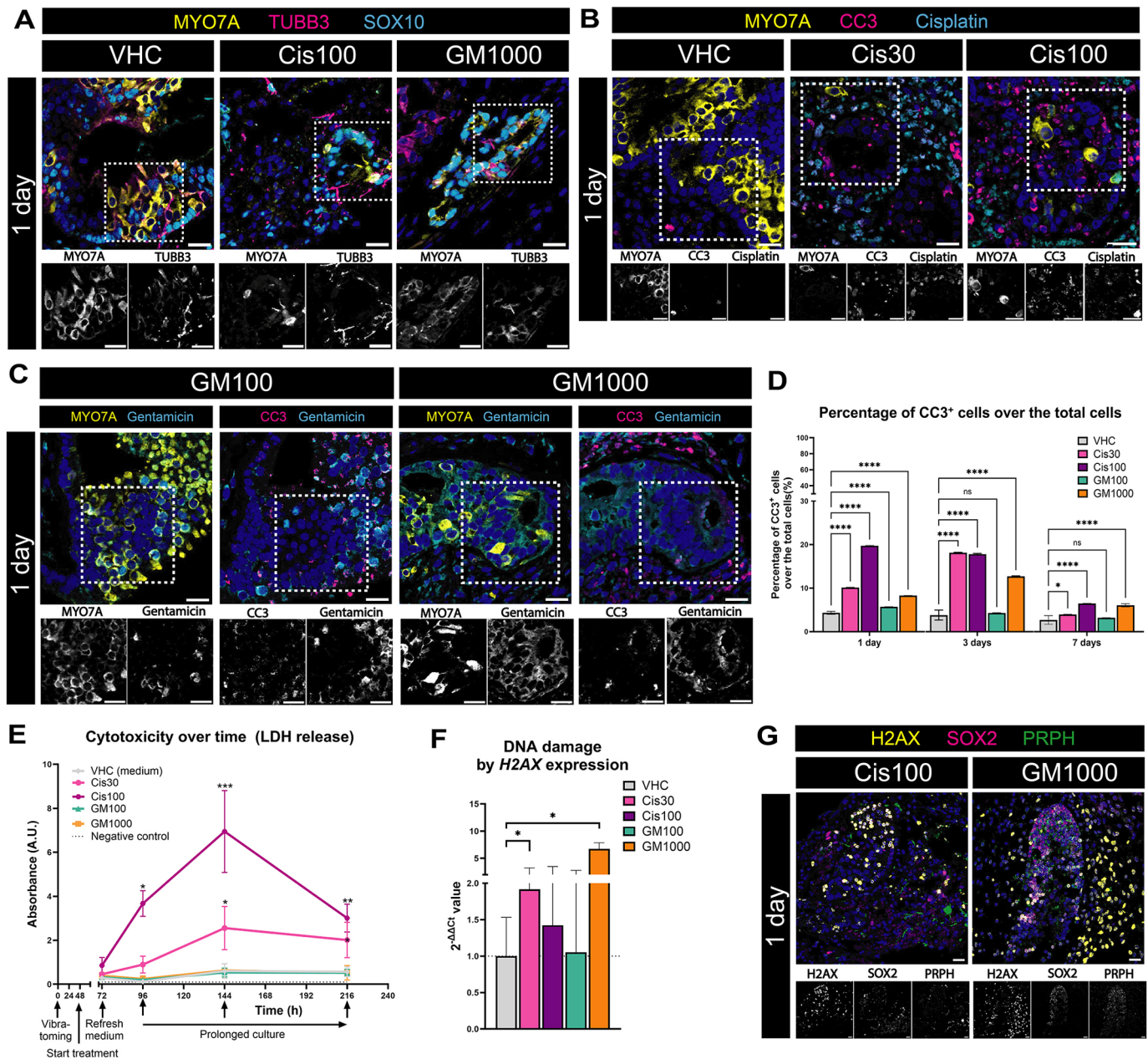


Fig. 3. Ototoxic compound treatment leads to cell damage and death as well as DNA damage. (A) Panels showing immunofluorescence (IF) staining of untreated (VHC) versus treated samples 1 day after treatment, containing hair cells (MYO7A⁺, yellow), neurons (TUBB3⁺, magenta), otic epithelium (SOX10⁺, cyan) and cell nuclei (DAPI⁺, dark blue). Images are from Fig. S2. Scale bars: 20 μ m. (B) Panels showing IF staining of VHC versus cisplatin-treated samples (30 and 100 μ M) at 1 day post-treatment, containing hair cells (MYO7A⁺, yellow), cleaved caspase-3 (CC3; magenta), cisplatin (cyan) and cell nuclei (DAPI⁺, dark blue). Scale bars: 20 μ m. (C) Panels showing IF staining of VHC versus gentamicin-treated samples (100 and 1000 μ M) at 1 day post-treatment, containing hair cells (MYO7A⁺, yellow), CC3 (magenta), gentamicin (cyan) and cell nuclei (DAPI⁺, dark blue). Scale bars: 20 μ m. (D) Percentage of CC3⁺ apoptotic cells in IEOs treated with cisplatin or gentamicin. The number of cells positive for CC3 was normalized to the total (DAPI⁺) number of cells. One-way ANOVA with multiple comparisons (ns, not significant; * $P \leq 0.05$, **** $P \leq 0.0001$). Data are represented as mean $\pm$ s.d. ($n=4$). (E) Cytotoxicity after cisplatin or gentamicin treatment as measured by release of LDH over time. Two-way ANOVA with Dunnett's multiple comparisons (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$). Error bars represent s.d. ($n=3$). (F) RNA expression levels of H2AX as measured by quantitative PCR (qPCR) normalized against the housekeeping gene (*RPS18*). Brown-Forsythe and Welch ANOVA with multiple comparisons. Data are represented as mean $\pm$ s.d. ($n=3$). * $P \leq 0.05$. (G) Panels showing IF staining of treated samples 1 day after treatment, containing neurons (PRPH⁺, green), SOX2 (magenta), DNA damage (H2AX⁺, yellow) and cell nuclei (DAPI⁺, dark blue). Scale bars: 20 μ m.

Redon et al., 2011; Umugire et al., 2023), which revealed a significant upregulation in cisplatin- and gentamicin-treated samples at 1 day post-treatment, thereby confirming that both agents compromise cellular DNA (Fig. 3F). In addition, we performed γ H2AX immunofluorescence staining (Fig. 3G), which revealed discrete nuclear staining within the otic vesicles of treated organoids,

consistent with localized sites of DNA damage. Co-staining with the neuronal marker peripherin (PRPH) suggested neurite fragmentation, although it remained inconclusive whether neurons themselves exhibited γ H2AX labeling. Together, these findings corroborate the transcriptional upregulation of H2AX and provide spatial confirmation of DNA damage in IEOs following ototoxic insult.

Proliferative responses and recovery in ototoxic compound-treated otic vesicles

Immunohistochemical analysis of IEO sections from 3 and 7 days post-treatment was performed to determine late effects of ototoxic compound treatment. Strikingly, at 7 days post-treatment, both cisplatin- and gentamicin-treated samples exhibited the reappearance of hair cells within a stratified otic vesicle containing a lumen, indicative of a partial recovery process (Fig. 4A). Despite structural similarities to vesicles observed in VHC conditions, disorganization in cellular orientation was apparent, with hair cells displaying a lack of uniform orientation.

To investigate the mechanisms underlying this reformation or recovery of the otic vesicle, we assessed cellular proliferation using the proliferation marker Ki-67 (also known as MKI67) (Fig. 4B,C). An increased presence of Ki-67⁺ nuclei was observed within the otic vesicles of samples treated with cisplatin or gentamicin compared to VHC samples. Notably, a majority of these Ki-67⁺ nuclei also expressed SOX10, a marker of otic epithelial cells, but not of hair cells. In some cases, Ki-67⁺ nuclei were found in cells that also expressed MYO7A, indicating a rare subset of triple-positive cells (Ki-67⁺, SOX10⁺, MYO7A⁺). Quantitative analysis confirmed significant upregulation of Ki-67 expression in SOX10⁺ nuclei within otic vesicles exposed to ototoxic compounds, compared to VHC conditions (Fig. 4E). This proliferative response was detectable at 3 days post-treatment and became more pronounced at 7 days post-treatment. SOX2 was included to further examine the origin of the newly proliferated otic cells (Fig. 4C). Quantitative analysis revealed the presence of triple-positive cells (Ki-67⁺, SOX2⁺, MYO7A⁺) in samples treated with ototoxic compounds, whereas such cells were largely absent in vehicle controls (Fig. 4F). Given the vestibular identity of the IEOs, the hair cells were assessed to correspond to type I or type II subtypes, using oncomodulin (OCM) as a marker of type I hair cells (Fig. 4D). At 7 days post-treatment, both hair cell types were detected in samples exposed to ototoxic compounds.

Because the otic vesicles treated with ototoxic compounds showed some form of recovery capacity, we hypothesized that a protective cellular process might be activated after drug exposure. Given that cisplatin and gentamicin are known to induce oxidative stress in otic cells (Tan and Song, 2023; Schacht et al., 2013), we examined the RNA expression of *SOD1*, which encodes the enzyme superoxide dismutase, a key mediator of oxidative stress mitigation (Campbell et al., 2011; Tan and Song, 2023; Li et al., 2022; Sheth et al., 2017). A significant upregulation of *SOD1* expression was observed at 3 days post-treatment, suggesting the activation of cellular repair and recovery mechanisms within the otic vesicle (Fig. 4G) (Kawamoto et al., 2004).

DISCUSSION

This study demonstrates that hiPSC-derived IEOs are a relevant *in vitro* model for assessing ototoxicity caused by cisplatin and gentamicin. IEOs offer a human-based alternative to animal models, providing a platform for therapeutic screening and treatment development for inner ear disease. Although IEOs do not consist of all inner ear cell types and require further optimization (van der Valk et al., 2023), they do contain the sensory hair cells with inner ear neurons necessary for studying ototoxicity. Here, we demonstrate that these organoids exhibit a response to cisplatin and gentamicin that closely resembles human ototoxicity, underscoring their relevance for investigating inner ear pathology and therapeutic interventions. We developed an *in vitro* exposure model, demonstrating that ototoxic treatment leads to significant morphological and immunohistochemical changes in IEOs,

including vesicle collapse, loss of stratification, basal membrane disruption, and absence of hair cells and neurons. These observations align with established animal models, in which cisplatin induces widespread cytotoxicity through DNA damage and oxidative stress affecting both hair cells and neurons (Abitbol et al., 2020; Waissbluth et al., 2022), whereas gentamicin primarily targets hair cells via mitochondrial dysfunction and reactive oxygen species production, with neuronal damage occurring secondarily owing to sensory cell loss and loss of trophic support (Lee et al., 2017; Tao and Segil, 2022; Dong et al., 2015), highlighting their value for studying drug-induced inner ear damage.

Our findings are consistent with these differential mechanisms. In cisplatin-treated IEOs, we observed pronounced and early apoptosis across multiple cell types. In contrast, gentamicin-treated organoids displayed apoptosis predominantly within hair cells, with neuronal loss appearing later. These results indicate that ototoxic drug exposure in IEOs primarily triggers apoptosis, as shown by a significant increase in CC3⁺ cells 1 day after treatment. In addition, elevated *H2AX* expression and γ H2AX nuclear staining within the otic vesicles confirm increased DNA damage following both treatments. Co-staining with the neuronal marker PRPH revealed signs of neurite fragmentation, although it remained unclear whether neurons themselves exhibited γ H2AX accumulation, suggesting that neuronal changes are secondary to hair cell loss or general tissue stress. A peak in LDH release 3 days post-treatment further indicates late-stage apoptosis. Together, these results confirm that IEOs can effectively model ototoxicity-induced cell death, making them a robust tool for pathophysiological studies.

Following initial loss of hair cells and neurons at 1 day post-treatment, these cells reappeared at 3 days post-treatment and were even more prominent at 7 days, suggesting potential recovery of the otic sensory epithelium. To elucidate this recovery mechanism, we assessed cell proliferation using Ki-67 and identified the cell types involved in hair cell regeneration. Ototoxic treatment significantly increased Ki-67⁺ cells within the SOX10⁺ otic epithelium and SOX2⁺ prosensory cells, with some of these cells also expressing MYO7A. We hypothesize that these triple-positive (Ki-67⁺, MYO7A⁺, and SOX10⁺ or SOX2⁺) cells arise from transdifferentiation, where a dividing SOX2⁺;SOX10⁺ epithelial prosensory cell generates one otic epithelial cell and one hair cell. As mature hair cells are SOX10⁺ and SOX2⁺ (Locher et al., 2013), they are distinguishable from newly formed hair cells. These findings provide evidence of cellular plasticity within the IEO model, reflecting a potential regenerative capacity. Injury likely triggers SOX2⁺;SOX10⁺ otic epithelial prosensory cells to re-enter the cell cycle and divide asymmetrically, producing one epithelial supporting cell that remains SOX2⁺;SOX10⁺, and one nascent hair cell that becomes MYO7A⁺ and retains SOX2.

The observation of hair cell re-emergence in IEOs may seem counterintuitive, as evidence for spontaneous regeneration in the human inner ear is limited. Although certain species, such as birds and amphibians, can regenerate inner ear hair cells after injury, this ability is largely absent in mammals (McGovern et al., 2019), including humans. Nevertheless, some evidence suggests residual regenerative potential or repair mechanisms. For example, supporting cells, which possess inherent plasticity, can differentiate into hair cell-like cells under specific *in vitro* conditions (Cruz et al., 2015; Golub et al., 2012; Kawamoto et al., 2009; Bramhall et al., 2014; Cox et al., 2014; Wang et al., 2015). In rodents, vestibular hair cell regeneration has been observed, particularly in the utricle and saccule, in which supporting cells can proliferate and transform into hair cells, although this capacity diminishes with age (Cruz et al., 2015; Golub et al., 2012; Kawamoto et al., 2009; Bramhall et al., 2014; Cox et al., 2014;

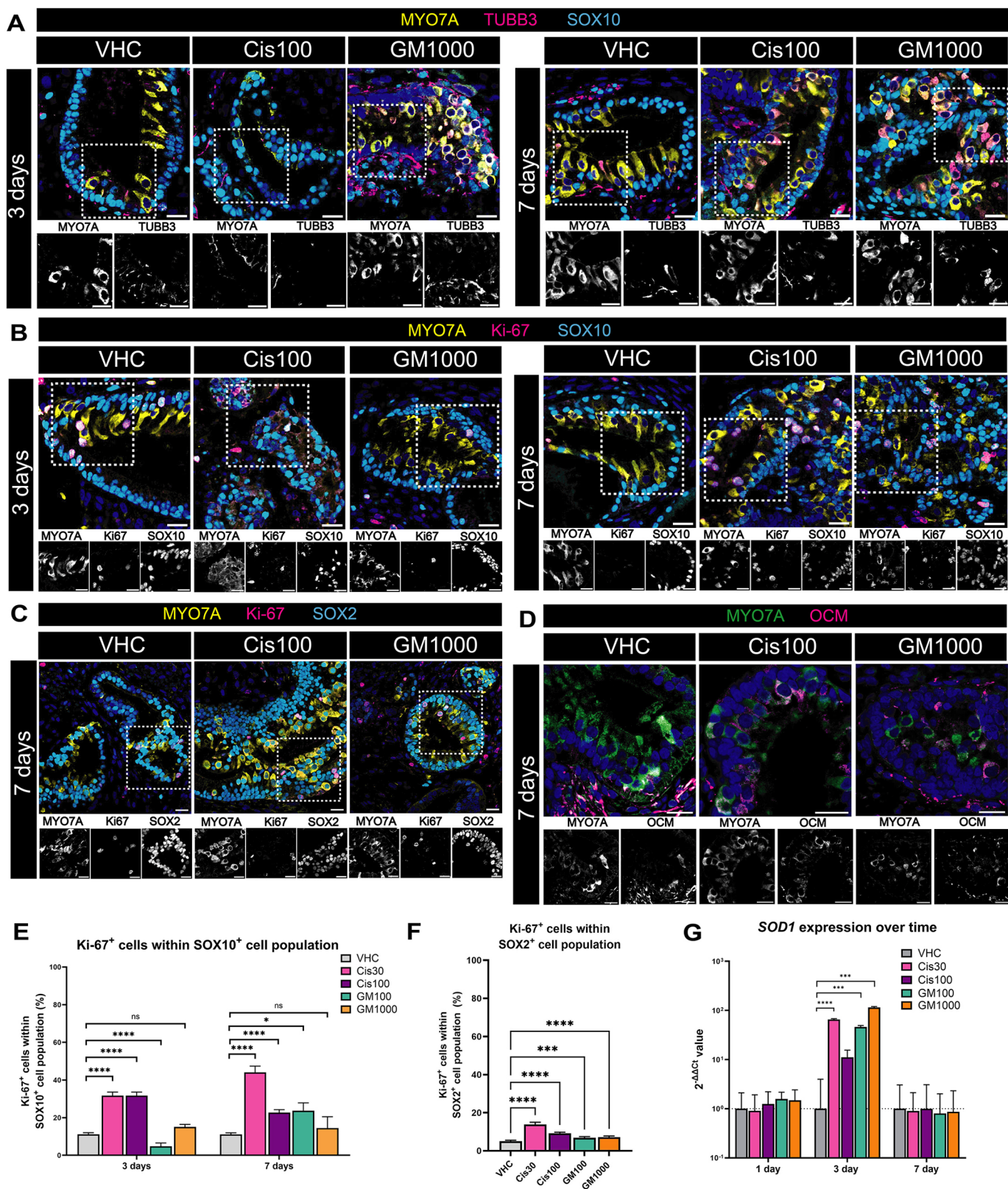


Fig. 4. See next page for legend.

Wang et al., 2015). In humans, supporting cells express markers of hair cell differentiation under certain conditions; however, this does not occur spontaneously (Taylor et al., 2015, 2018). Notably, mitotic activity has been observed in vestibular supporting cells of the adult human utricle, although it is insufficient for restoring function (Taylor et al., 2018). Human fetal cochlear cells showed a proliferative recovery process and subsequent specification to hair cell-like cells *in vitro* (Roccio et al., 2018). Considering these findings, it is critical

Fig. 4. Over time, the otic vesicle recovers with reappearance of hair cells, neurons and stratification. (A) Panels showing IF staining of untreated versus treated samples at 3 and 7 days post-treatment, containing hair cells (MYO7A⁺, yellow), neurons (TUBB3⁺, magenta), otic epithelium (SOX10⁺, cyan) and cell nuclei (DAPI⁺, dark blue). The 3 days GM1000 image and 7 days VHC, Cis100 and GM1000 images are from Fig. S2. Scale bars: 20 μ m. (B) Panels showing IF staining of VHC versus ototoxic compound-treated samples at 3 and 7 days post-treatment, containing hair cells (MYO7A⁺, yellow), Ki-67 (magenta), otic epithelium (SOX10⁺, cyan) and cell nuclei (DAPI⁺, dark blue). Scale bars: 20 μ m. (C) Panels showing IF staining of VHC versus ototoxic compound-treated samples at 7 days post-treatment, containing hair cells (MYO7A⁺, yellow), Ki-67 (magenta), sensory epithelium (SOX2⁺, cyan) and cell nuclei (DAPI⁺, dark blue). Scale bars: 20 μ m. (D) Panels showing IF staining of VHC versus ototoxic compound-treated samples at 7 days post-treatment, containing hair cells (MYO7A⁺, green), oncomodulin (OCM; magenta) and cell nuclei (DAPI⁺, dark blue). Scale bars: 20 μ m. (E) Percentage of Ki-67⁺ cells within SOX10⁺ epithelium. One-way ANOVA with multiple comparisons (ns, not significant; * $P \leq 0.05$, **** $P \leq 0.0001$). Data are represented as mean $\pm$ s.d. ($n=4$). (F) Percentage of Ki-67⁺ cells within SOX2⁺ population. One-way ANOVA with multiple comparisons (*** $P \leq 0.001$, **** $P \leq 0.0001$). Data are represented as mean $\pm$ s.d. ($n=8$). (G) RNA expression levels of *SOD1* as measured by qPCR normalized against housekeeping gene (*RPS18*). Brown–Forsythe and Welch ANOVA with multiple comparisons (*** $P \leq 0.001$, **** $P \leq 0.0001$). Data are represented as mean $\pm$ s.d. ($n=3$).

to emphasize that the IEO protocol used in this study predominantly resembles vestibular, not cochlear, tissue (van der Valk et al., 2023). This distinction is important, as it aligns with the regenerative capacity observed in the vestibular system of mammals, which has not been similarly demonstrated in the cochlear epithelium. Additionally, as these organoids exhibit a fetal-like developmental stage (Doda et al., 2023; van der Valk et al., 2023), their immature state may allow plasticity absent in fully mature tissue.

Although this model provides valuable insights into the ototoxic effects of cisplatin and gentamicin on human inner ear cells, it does not fully replicate clinical conditions (Lelli et al., 2009). Specifically, our model cannot mimic the systemic administration of ototoxic drugs typically used in clinical settings. Moreover, IEOs lack key cell types, such as metabolic and immune cells, which are critical for drug uptake and cellular interactions (Kros and Steyger, 2019; Steyger, 2021; Sheth et al., 2017; Li et al., 2023; Callejo et al., 2015; Anfuso et al., 2022). To further enhance the translatability of IEOs, the inner ear field can benefit from advances in other organoid models (Rogoz et al., 2015; Nozaki et al., 2016; Popova et al., 2021; Seo et al., 2022). Enhancing IEOs by incorporating immune cells could enable studies of repair mechanisms, as macrophage infiltration is known to protect cochlear and vestibular tissues after injury (Liu and Xu, 2024; Liu et al., 2019; Warchol, 2019; Miwa and Okano, 2022; Zhang et al., 2023). Moreover, integrating IEOs into organ-on-chip systems with endothelial cells and pericytes could model the blood–labyrinth barrier, better simulating drug administration routes (Sekulic et al., 2023a,b; Trune and Nguyen-Huynh, 2012). Such improvements would enhance the physiological relevance of IEOs, rendering them more suitable for studying human inner ear diseases.

In this study, we present a 3D human IEO model that accurately reflects drug-induced damage in the inner ear, offering a human-specific system for studying ototoxicity. This model allows for the assessment of drug-induced damage to human inner ear cells, facilitates the exploration of ototoxic mechanisms and offers a foundation for testing protective strategies. By providing a scalable, human-based platform, IEOs represent a significant step forward in ototoxicity research and therapy development.

MATERIALS AND METHODS

hiPSC culture

WTC-SOX2(mEGFP)-MYO7A(mRuby), a healthy hiPSC line, derived from the WTC-SOX2 line from the Allen Institute (Cell Line ID AICS-0074 cl.26) was generously gifted by Jingyuan Zhang and Karl R. Koehler (Boston Children's Hospital). The cell line contains an endogenous SOX2-mEGFP knock-in as described in the Allen Cell Atlas and Cellosaurus (CVCL_WM15). We did not utilize the reporter signal in this study.

Cells were cultured on six-wells plates coated with vitronectin recombinant human protein (A14700, Thermo Fisher Scientific, concentration 0.5 μ g/ml) and maintained with mTeSRTM Plus Basal Medium (85850, Stem Cell Technologies) with normocin (ANT-NR-1, Invivogen, concentration 100 μ g/ml). This medium was replenished every other day. The cells were passaged at ~80% confluency (every 4–5 days on average) as tiny clusters (three to five cells) using 0.5 mM EDTA (15575020, Gibco) in DPBS (14190144, Gibco).

Differentiation of hiPSCs into IEOs

IEO differentiation followed the protocols as published with minor alterations (Steinhart et al., 2023; Koehler et al., 2017; van der Valk et al., 2023, 2025). To summarize, hiPSCs were detached and dissociated using Accutase (Stempro Accutase Cell Dissociation Reagent, A1110501, Thermo Fisher Scientific) and collected as a single cell suspension in mTeSR medium containing Chroman 1 (HY-15392, MedChemExpress, concentration 50 nM), Emricasan (S7775, Selleckchem, concentration 5 μ M), Polyamine (P8483, Sigma-Aldrich, concentration 1 $\times$) and Trans-ISRIB (16258-5, Sanbio, concentration 700 nM) (CEPT) cocktail. Per well of a 96-wells U-bottom plate with ultra-low cell attachment surface (174925, Thermo Fisher Scientific), 2500 cells in 100 μ l were distributed. After centrifugation at 110 g for 6 min, cell aggregates were incubated at 37°C under 5% CO₂ for 48 h.

At differentiation day 0 (d0), all the cell aggregates were collected in 15 ml conical tubes, washed three times with Essential 6 medium (hereafter E6; A1516401, Gibco) and individually transferred to a new 96-well U-bottom with super-low cell attachment surface in 100 μ l E6 with normocin (100 μ g/ml), containing 2% Matrigel Growth Factor Reduced (hereafter, MG-GFR; 356231, Corning), 10 μ M SB431542 (04-0010-05, Reprocell), 4 ng/ml recombinant human FGF-basic (hereafter, bFGF; 100-18B, PeproTech) and optimal concentrations of 2.5 to 15 ng/ml recombinant human BMP-4 (314-BPE-010, R&D Systems). For optimal BMP-4 concentration, titrations were performed. On day 3, each well received 25 μ l E6, making the total volume 125 μ l with end concentration of 200 ng/ml LDN-193189 (04-0074-02, Reprocell) and 50 μ g/ml bFGF. On day 6, the total volume was increased to 200 μ l by addition of 75 μ l fresh E6 medium. On day 8, 100 μ l of the medium was exchanged for E6 containing 6 μ M CHIR99021 (hereafter, CHIR; 04-0004-02, Reprocell). On day 10, again 100 μ l medium was changed to E6 medium containing 3 μ M CHIR.

On day 12, the cell aggregates were transferred to a 24-well plate with super-low cell attachment surface (174930, Thermo Fisher Scientific) in OM medium containing 1% MG-GFR and 3 μ M CHIR and placed on an incubator orbital shaker. OM medium consists of a 1:1 mixture of advanced DMEM-F12 (12634010, Gibco) and Neurobasal Medium (10888022, Gibco), supplemented with 0.5 $\times$ B27 without Vitamin A (12587-010, Gibco), 0.5 $\times$ N2 supplement (17502-048, Gibco), 1 $\times$ GlutaMax (35050061, Gibco), 0.1 mM 2-mercaptoethanol (21-985-023, Thermo Fisher Scientific) and normocin. On day 15, half of the medium was changed to OM medium containing 1% MG-GFR and 3 μ M CHIR. Then, on day 18, a half-medium change was performed with OM only. On day 21, a full-medium change was performed with OM medium. Then, half of the medium was replenished every 2–3 days, with a full-medium change every 7 days. As the cell aggregates grew, the medium volume was gradually increased from 500 μ l at day 12 to 1 ml at day 45, to 1.5 ml on ~day 60 and later.

Vibratome sectioning and ototoxic compound addition

At D75, the cell aggregates underwent live sectioning using a Vibratome (Leica VT1200S). First, they were collected and embedded in 4% low-gelling temperature agarose (A0701-25G, Sigma-Aldrich). The vibratome was set to a speed of 0.2 mm/s, an amplitude of 1 mm and a slice thickness

of 200 μm . Slices were collected with a perforated spoon (10370-19, Fine Science Tools) to minimize tissue damage and kept at 37°C in OM medium.

After sectioning, slices were selected based on the presence of otic vesicles within the aggregate by light microscopy. Then, the slices were distributed randomly over the conditions and incubated in OM medium for 48 h at 37°C and 5% CO_2 . Next, two stock solutions were prepared with the ototoxic compounds diluted in OM medium: one with 1000 μM gentamicin sulfate salt (G3632, Sigma-Aldrich) in OM medium and one with 1000 μM cis-diamminedichloroplatinum (II) (cisplatin; 232120, Sigma-Aldrich) and diluted to the preferred concentrations (100 and 1000 μM for gentamicin and 30 and 100 μM for cisplatin, respectively). Concentrations were selected based on ranges commonly used in published *in vitro* ototoxicity models (including human utricle tissue) (Febles et al., 2022; Hu et al., 2020; Taylor et al., 2015, 2018). We tested two doses per drug, a lower dose to approximate indirect inner ear exposure *in vivo* and a higher dose to reliably induce ototoxic injury for robust phenotyping in the organoid system. Each condition received a full-medium change containing either gentamicin, cisplatin or no addition to the medium (VHC) and was incubated for 24 h, after which another full-medium change occurred. Samples were kept at 37°C and 5% CO_2 , and retrieved at either 1 day (D78+1), 3 days (D78+3) or 7 days (D78+7) after treatment.

Sliced aggregate processing, immunohistochemistry and image acquisition

The slices of the aggregates, collected after vibratome sectioning or ototoxic compound treatment, were retrieved in 4% paraformaldehyde overnight at 4°C, after which the samples were washed and kept in PBS until embedding. For immunohistochemistry, the samples were embedded in Histogel (HG-4000-012, Eprexia) and, after processing, embedded in paraffin. Sections of 5 μm were cut using a rotary microtome (HM355S, Thermo Fisher Scientific). For staining, sections were deparaffinized in xylene and rehydrated in a descending series of ethanol, after which the slides were rinsed with deionized water. For every ten slides, one slide was selected for staining with Hematoxylin (40859001, Klinipath) and Eosin (AB246823, Abcam). Subsequently, the slides were imaged using the Leica DM5500 fluorescent microscope. Morphological criteria used to interpret H&E-stained sections (e.g. features of hair cells, neurons, vesicle lumen and basal membrane, and signs of damage) are summarized in Table S1, including the semi-quantitative scoring criteria. This was done for three technical replicates, on at least six biological replicates ($n=6$).

Before immunostaining, antigen retrieval was performed in 10 mM sodium citrate buffer (pH 6.0; S1804-50G, Sigma-Aldrich) for 12 min at 97°C. Then, sections were rinsed with washing buffer consisting of 0.05% Tween-20 (H5152, Promega) in PBS and subsequently blocked for 30 min with blocking buffer consisting of PBS with 0.05% Tween-20 and 5% bovine serum albumin. The sections were incubated with primary antibodies (Table S2) of choice in blocking buffer, overnight at 4°C in a humidified chamber. After washing three times with washing buffer for 5 min and another round of blocking of 10 min, the sections were incubated with secondary antibodies (1:500) and 1:1000 4',6-diamidino-2-phenylindole (DAPI) at room temperature for 60 min. Hereafter, the sections were washed and mounted with ProLong™ Gold Antifade Mountant (P36934, Invitrogen). Subsequently, the slides were imaged using a Leica TCS SP8 upright confocal system with Leica objectives (20×/0.7 dry HC PL Apo, 40×/1.3 oil HC PL Apo CS2, 63×/1.4 oil HC PL Apo or 100×/1.3 oil HC PL Fluotar) and analyzed with LASX software (Leica, version 3.7.6.25997). All images represent z-stack optical sections acquired from paraffin-embedded samples.

Fiji image processing and quantification of DAPI⁺, CC3⁺, SOX2⁺ and Ki-67⁺ cells

Images were loaded to Fiji software 2.9.0 (Schindelin et al., 2012) and despeckled from noise. After splitting the separate channels, the background signal was filtered out by subtraction, and, subsequently, the intensity levels were normalized for the subtraction value. Each of the channels was merged to the appropriate coloring.

Quantification was performed using raw images. The channels were split and transformed into an 8-bit image, after which the threshold was set to filter out all non-specific background. Via the measure function in Fiji, the

DAPI⁺ nuclear diameter was measured, and set as the minimum threshold for counting the total amount of nuclei, as well as the CC3⁺ cells, to ensure counting only cells and not artefacts. The number of CC3⁺ cells was expressed as a percentage of the total number of DAPI⁺ nuclei within the otic vesicle domain, thereby normalizing apoptosis to the vesicle epithelial cell population rather than the entire section. SOX2 and Ki-67 are nuclear markers; therefore, a cell was considered positive when its nucleus showed staining. Quantification of these markers was performed using the same approach as for DAPI⁺ nuclei.

CellProfiler quantification of DAPI⁺, Ki-67⁺ and SOX10⁺ cells

Images were loaded into CellProfiler 4.2.8 (Carpenter et al., 2006). After splitting the separate channels, the DAPI⁺ nuclei were identified and counted. The signal was enhanced, and the intensity was rescaled in the Ki-67 and SOX10 channels. Hereafter, both the Ki-67⁺ and SOX10⁺ nuclei were identified, and objects were related to each other. Here, the overlap between both markers (as double positives) was calculated.

LDH cytotoxicity assay

At different time points, medium of the live vibratome slices was collected. For each condition, 100 μl medium was collected and transferred to a new 96-well plate (655-180, Greiner bio-one CELLSTAR). To each well, 100 μl reaction mixture was added. The reaction mixture contains the 2.17% (vol/vol) catalyst in the dye solution [Cytotoxicity Detection Kit (LDH), 11644793001, Roche]. The samples were incubated for 30 min at room temperature in the dark. For each well, absorbance was measured at 492 nm and 600 nm using a SpectraMax i3x (Molecular Devices). The data were analyzed by correcting for the negative controls and normalizing to the first timepoint. The cytotoxicity analysis was performed three times, with three replicates per condition and per timepoint.

RNA isolation

For the gene expression analysis, four to six samples per condition were combined for analysis. The slices were pelleted by centrifugation at 500 g for 1 min and resuspended in a 1:1 mixture of DNA/RNA protection reagent and nuclease-free water (Monarch Kit T2010, T2010S, New England Biolabs). To these samples, 1:10 Monarch Proteinase K reaction buffer (T2003, New England Biolabs) and 1:20 Monarch Proteinase K (T2001, New England Biolabs) was added after, which they were incubated at 55°C for 30 min. After brief vortexing and spinning down for 2 min at 16,000 g, the supernatant was transferred to a new tube, and RNA lysis buffer was added. gDNA was removed, and 96% ethanol was added to purify the RNA. Then, Monarch RNA priming buffer (T2013, New England Biolabs) was added, the column was washed twice, and the RNA was then dissolved in nuclease-free water. RNA concentration was measured using NanoDrop One (Thermo Fisher Scientific), and samples were stored at −80°C.

cDNA synthesis

For the generation of cDNA, a reaction mixture was made consisting of iScript reaction mix (iScript kit, 1708891, Bio-Rad), the iScript reverse transcriptase, nuclease-free water and the RNA template (500 ng), according to the iScript manual. Thermal cycling (Bio-Rad S1000 Thermal Cycler) occurred by priming for 5 min at 25°C, reverse transcription at 46°C for 20 min, reverse transcriptase inactivation for 1 min at 95°C and cooling at 4°C. The cDNA was stored at −80°C.

Quantitative reverse transcription PCR (qRT-PCR)

For gene expression analysis, the cDNA was diluted five times. Then, a master mix was prepared of forward primer and reverse primer (final concentration of 0.5 μM) and iQ™ SYBR® Green Supermix (1708880, Bio-Rad). A list of all primers is stated (Table S3). A real-time PCR system (Bio-Rad CFX Opus 384) thermal cycling program was set at 95°C for 3 min, followed by 60°C for 30 s and 72°C for 40 s, in a total of 40 cycles. Afterwards, the program set 55°C for 10 s and 95°C for 0.5 min, followed by 4°C. The CFX Maestro (Bio-Rad) software was used to analyze the data, including inter-plate calibration. Samples were measured in triplicates. From the raw data, the normalization happened via the calculation of Ct values and comparisons via the $2^{-\Delta\Delta\text{Ct}}$ approach.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism (version 10.2.3), with a *P*-value of ≤ 0.05 considered statistically significant for all analyses.

For the H&E semi-quantitative scoring, the statistical test was based on a three-level scoring method (see Table S1). A Kruskal–Wallis test followed by Dunn's multiple comparisons test was done with a confidence interval of 95% and $n > 9$ ($*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$ compared to the VHC from that specific timepoint).

For CC3⁺, Ki-67⁺ and DAPI⁺ cell quantification, an ordinary one-way ANOVA with multiple comparisons was used to compare VHC samples with those treated with ototoxic compounds. Data were obtained from four technical replicates, each with at least three biological replicates per condition.

The LDH cytotoxicity assay was analyzed using a two-way ANOVA with Dunnett's multiple comparisons test against VHC samples. Data were derived from three technical replicates, each with three biological replicates per condition.

Gene expression analysis by qPCR was performed using the Brown–Forsythe and Welch ANOVA with multiple comparisons to evaluate differences between VHC and drug-exposed samples. Data analysis included three technical replicates, each with three biological replicates per primer pair, per condition.

Declaration of generative AI in the writing process

During the preparation of this work, the authors used Chat Generative Pre-Trained Transformer (ChatGPT; OpenAI, San Francisco, CA, USA) to reword and rephrase text. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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Competing interests

K.R.K. is an inventor on patents relating to the IEO model. W.H.v.d.V., P.P.G.v.B. and H.L., with the Leiden University Medical Center, have filed a patent application related to the differentiation of the IEO model. The other authors declare no competing or financial interests.

Author contributions

Conceptualization: A.W.A.L., J.C.M.J.d.G., W.H.v.d.V., H.L.; Data curation: A.W.A.L., W.M.C.v.d.B., J.C.M.J.d.G.; Formal analysis: A.W.A.L., W.M.C.v.d.B., J.C.M.J.d.G.; Funding acquisition: P.P.G.v.B., W.H.v.d.V., H.L.; Investigation: A.W.A.L., W.M.C.v.d.B., E.F., J.C.M.J.d.G.; Methodology: A.W.A.L., W.M.C.v.d.B., K.R.K., J.C.M.J.d.G., W.H.v.d.V., H.L.; Project administration: A.W.A.L., P.P.G.v.B., W.H.v.d.V., H.L.; Resources: A.W.A.L., W.M.C.v.d.B., J.Z., K.R.K., J.C.M.J.d.G., W.H.v.d.V.; Software: A.W.A.L., W.M.C.v.d.B.; Supervision: W.M.C.v.d.B., P.P.G.v.B., W.H.v.d.V., H.L.; Validation: A.W.A.L., W.M.C.v.d.B., E.F., J.C.M.J.d.G., W.H.v.d.V., H.L.; Visualization: A.W.A.L., W.M.C.v.d.B.; Writing – original draft: A.W.A.L., W.M.C.v.d.B., W.H.v.d.V., H.L.; Writing – review & editing: A.W.A.L., W.M.C.v.d.B., J.Z., K.R.K., P.P.G.v.B., W.H.v.d.V., H.L.

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Data and resource availability

All relevant data and details of resources can be found within the article and its supplementary information.

Special Issue

This article is part of the special issue 'In Vitro Models of Human Disease to Inform Mechanism and Drug Discovery', edited by Vivian Li, Austin Smith and Joseph Wu. See related articles at <https://journals.biologists.com/dmm/collection/10529/Advanced-in-vitro-models>.

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