



Letter to the editor

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Dear Editors,

We are writing to share our observations regarding the recently published paper by Mohammad Faraz Zafeer et al., titled: “Human organoids for rapid validation of gene variants linked to cochlear malformations”, published on January 9, 2025. We greatly appreciate the authors’ dedication to advancing inner ear organoid (IEO) research and their innovative approach to investigating cochlear malformations. However, we would like to raise a few points for consideration that might help further refine and validate the findings.

The impact of the work could be enhanced by clarifying whether the differentiation of the patient-specific knock-in and knock-out lines leads to formation of organoids with true inner ear identity. While the manuscript presents evidence of proper differentiation for control lines (Fig. S10), similar evidence is not provided for the pathogenic lines. Additionally, morphology of differentiated control lines is not consistent when comparing Fig. 4 and Fig. S9 to Fig. S10 and what is known in the literature. This raises the question whether proper ectodermal specification, followed by otic placode to vesicle induction and subsequent inner ear organoid maturation is achieved in the experimental conditions shown.

Otic vesicles are considered hallmarks of proper early differentiation in IEOs (Koehler et al. 2017; Ueda et al. 2022; Doda et al. 2023; Moore et al. 2023; Steinhart et al. 2023; van der Valk et al. 2023). While the H&E images provided (Fig. 3, Fig. S5 and Fig. S8) show structures with a lumen, these structures lack the typical morphology of IEOs. Instead, the observed structures potentially resemble

those in a neural-ectoderm differentiation trajectory, indicated by the multiple cell layers surrounding the lumen. All three genes of which variants are studied, being FGF3, GREB1L and TBXIP1, also play a role in development of neural ectoderm(-derived tissues) and could potentially be another explanation for the observed phenotypes.

In a similar line, while indicative of early otic development, the presented staining patterns of PAX2 and PAX8 appear throughout the imaged areas (Fig. 3E) and seem to be less spatially confined than typically observed in well-differentiated IEOs. In otic vesicles, the staining would be expected to concentrate within the vesicle rather than extend into the surrounding tissue and be limited to 2–3 cell layers. The observed broader distribution may suggest incomplete or suboptimal differentiation, possibly leading to the formation of neural rosettes. More mature IEOs normally consist of a more layered appearance containing supporting cells with differentiating hair cells closer to the lumen (usually 2–3 cell layers). The nuclei of these early hair cells are located towards the basolateral side of the cell body, while most of the cytoplasm is located on the apical side. Additionally, immunohistochemistry for MYO7A (Fig. 4) should show the characteristic organization and defined contours typically associated with hair cells, as is demonstrated in Fig. S10 for control line differentiations and as presented here for reference (Fig. 1). Staining for SOX10 in addition to MYO7A, might help to confirm the identity of otic-vesicle like structures. Confirmation of properly guided differentiation to achieve otic vesicle confirmation in all cell lines used is required to confirm the otic identity of the organoid created and draw further experimental conclusions.

Based on these observations, we hypothesize that the structures generated may result either from the pathogenicity of the variants or from suboptimal BMP-4 levels during early differentiation stages. BMP-4 is crucial for induction of surface ectoderm and subsequent otic placode formation, which is essential for proper vestibular or cochlear organoid development (Doda et al. 2023; van der Valk et al. 2023). To address this, it would be helpful to confirm whether a titration of BMP-4 (0–40 ng/ml) was performed to determine the

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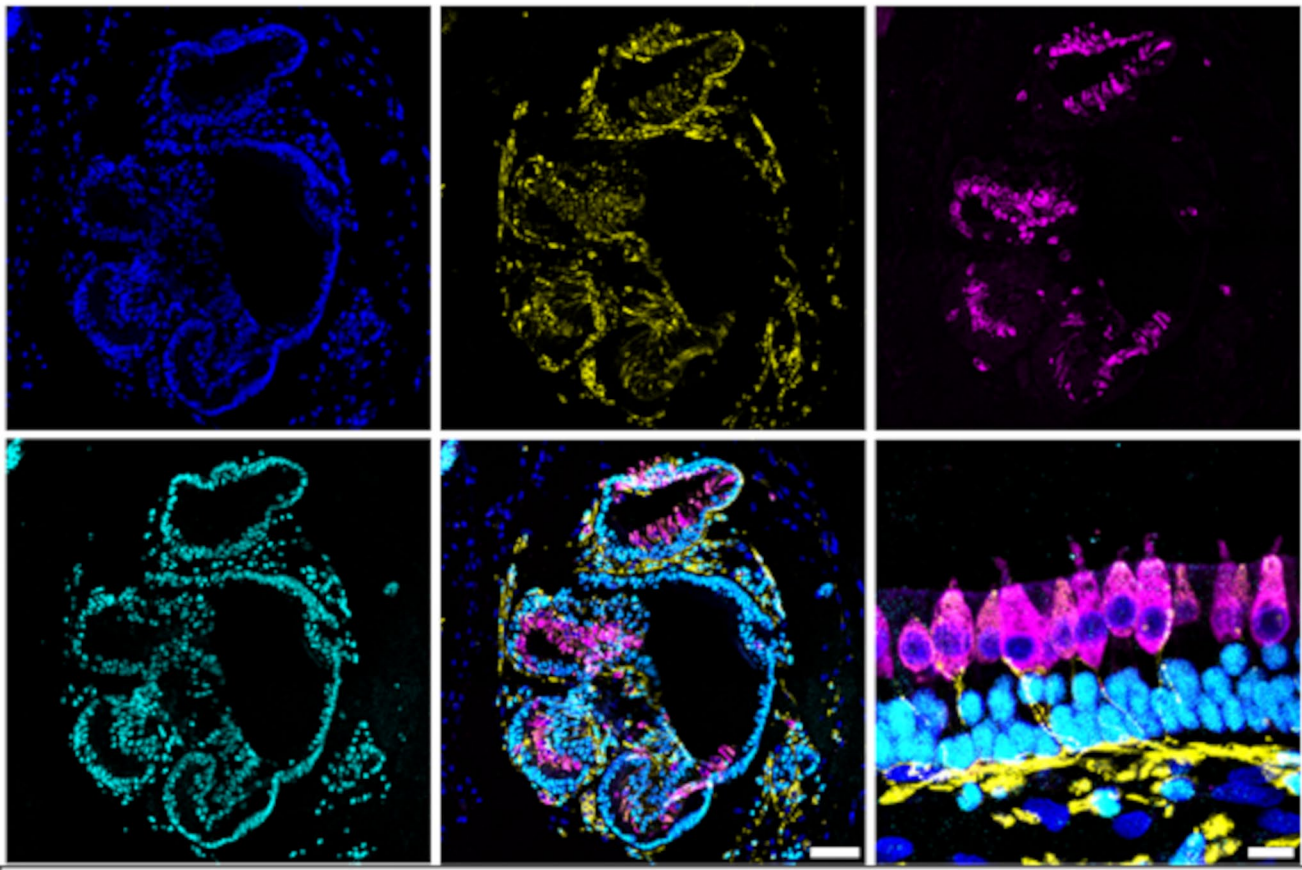


Fig. 1 Inner ear organoids immunostained for DAPI (blue), TUBB3 (yellow), MYO7A (magenta), and SOX10 (cyan). The image shows multiple vesicle-like structures lined by a SOX10-positive epithelium, containing MYO7A-positive hair cells and TUBB3-positive neuronal innervation. A higher magnification (lower right panel) highlights the distinctive MYO7A staining pattern of cultured hair cells, with their

stereocilia protruding into the lumen. The SOX10-negative nuclei of the hair cells are clearly visible and are easily distinguishable from the SOX10-positive nuclei of the supporting cells in the basal layers. Additionally, SOX10-positive cells located outside the epithelial layer, closely associated with neuronal structures, are presumed to be glial cells. Scale bars: 50 μm (overview) and 10 μm (higher magnification)

optimal concentration for differentiation. In our experience, BMP-4 activity differs between vendors, lots and sometimes cell lines. This difficulty presents a limitation in the field and makes standardization more challenging. Additionally, including appropriate immunohistochemistry controls, such as isotype controls, could further validate the specificity of the observed staining. Finally, confirming that surface ectoderm-derived structures, as described in the original protocol by Koehler et al. (2017), are generated instead of neural ectodermal structures would provide stronger evidence for the otic identity of all control and pathogenic cell line-derived organoids.

We hope these suggestions are taken in the collaborative spirit in which they are offered. Addressing these points would strengthen the robustness of the findings and provide greater clarity regarding the developmental nature of the generated organoids. The authors have presented an interesting concept for the study of gene variants potentially involved in cochlear malformations. We look forward to

seeing how these exciting advancements continue to evolve and contribute to the field.

Author contributions All authors extensively discussed the original manuscript. W.V. wrote the draft of the letter, while W.B., E.F., and H.L. prepared the figure. All authors reviewed and approved the final version of the manuscript.

Data availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

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