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Single-cell sequencing: a powerful technique to understand the pathophysiology of auditory disorders

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Deciphering the inner ear transcriptome has allowed major breakthroughs in therapeutic development for auditory disorders. Single-cell sequencing (SCS) technologies, through the analysis of cellular heterogeneity and the identification of novel gene markers, can significantly enhance our understanding about auditory disorders. The application of SCS has allowed the identification of gene targets specific to the inner ear cellular subtypes such as supporting cells, hair cells, stria vascularis cells, and spiral ganglion neuron cells. SCS has provided new insights into the molecular mechanisms underlying the pathophysiology of auditory disorders such as noise-trauma induced hearing loss, ototoxicity, sudden sensorineural hearing loss (SSNHL), vestibular schwannomas and acute otitis media, leading to the discovery of novel therapeutic targets. The objective of this perspective article is to discuss recent advancements regarding the utilization of SCS for inner ear research. With global initiatives striving to overcome the increasing burden of hearing loss, the SCS technique holds a great potential in revolutionizing the management of auditory disorders, paving the way to develop effective treatment modalities.

KEYWORDS

auditory disorders, single cell sequencing, inner ear, molecular mechanisms, cellular diversity, cochlea

Introduction

Single-cell sequencing (SCS) has emerged as a powerful technique leading to key discoveries in the medical field (Allen et al., 2023; Krämer et al., 2023; Yu et al., 2023; Zhang et al., 2023). Previous studies focused on analyzing bulk tissue samples and determined the average expression of gene transcripts (Pijuan-Sala et al., 2019). SCS analyzes the transcriptome of individual cells and can help in understanding the specific function of a single cell (Chang et al., 2023). Understanding tissue heterogeneity has proven impactful in cancer research, where tumor heterogeneity and rare cell types have allowed a better understanding of the disease (Pyle and Hoa, 2020; van der Leun et al., 2020; Lei et al., 2021; Liu et al., 2023; Xia et al., 2023).

Besides cancer, auditory research has also been greatly impacted by SCS. There has been an increased interest in the auditory community to study inner ear tissue heterogeneity, auditory disorders and their underlying molecular mechanisms using SCS (Ranum et al., 2019; Pyle and Hoa, 2020; Petitpré et al., 2022). SCS has allowed the identification of unknown cell subtypes in the inner ear and novel cellular markers through the study of cellular heterogeneity (Yizhar-Barnea and Avraham, 2017).

The aim of this brief perspective article is to discuss the recent advancements regarding the utilization of SCS for inner ear research (Table 1). SCS holds a great potential to better understand the function of the heterogenous cell population in the inner ear as well as to decipher the molecular mechanisms underlying auditory disorders, thus paving the way to develop novel therapeutic modalities.

SCS in inner ear research

Since the launch of the human genome project in the year 2000 and the development of next generation sequencing (NGS) technologies, the tools available to study the genetic basis of disease pathophysiology have continued to transform. NGS offers high-throughput sequencing, but requires an important pool of cells and provides bulk sample gene expression (van Dijk et al., 2014). However, transcript expression depends on gene expression level and cell populations. To address this issue, the development of SCS has allowed genomic, epigenomic, and whole transcriptome information to be obtained from single cells (Wu et al., 2021). Using this revolutionary technique, novel discoveries in tissue heterogeneity and biology at the single-cell level have been achieved.

First described by Tang et al. (2009), who used individual blastomeres, SCS involves cell isolation, capture, and lysis followed by nucleic acid extraction, amplification, and sequencing using NGS (Figure 1; Wu et al., 2021). A recent study compared the different techniques used in cell type-specific expression analysis of the inner ear. It was concluded that a combined approach using fluorescent-activated cell sorting (FACS) and SCS was best to overcome the specific challenges of inner ear single-cell transcriptome analysis (Hertzano et al., 2021).

To date, SCS of the inner ear has been performed primarily on animal models, especially mice, focusing on inner ear development and hair cell regeneration (Durruthy-Durruthy and Heller, 2015; Pyle and Hoa, 2020; Qian et al., 2022). Given the global burden of hearing loss, SCS can be used to identify the different signaling cascades involved in the auditory pathway. Through the application of SCS, differential expression of transcript isoforms of deafness genes can be analyzed in single inner ear hair cells (HCs). Identifying these isoforms is a powerful tool for gene therapy development, allowing cell-type specificity and limiting off-target effects. A study analyzed 12 deafness genes and led to the identification of 20 highly conserved exons as therapeutic targets (Ranum et al., 2019). As cochlear tissue is underrepresented in annotation databases, these studies are essential to support targeted and personalized treatment of auditory disorders.

Inner ear: a complex sensory structure with rich cellular diversity

Advances in SCS have made unprecedented strides in understanding the genetic etiology of auditory disorders. Several genes involved in the auditory pathways have been identified using SCS. The organ of Corti (OC), composed of HCs and spiral ganglion neurons (SGNs), has been largely investigated for its role in neural sound transmission. Conversely, the stria vascularis (SV), well-known for its role in providing the necessary blood supply to the OC, has been found to play an important sensory role using SCS technique. The SV is composed of three main cell types: marginal cells, intermediate cells, and basal cells, along with other cell types such as spindle cells and macrophages. Single-cell RNA sequencing has led to the discovery of several genes in the SV implicated in hearing that were challenging to identify previously. These genes include *Abcg1* and *Heyl* in marginal cells, *P2rx2* and *Kcnj16* in spindle cells, *Nrp2* and *Kcnj13* in intermediate cells, and *Sox8* and *Nr2f2* in basal cells (Korrapati et al., 2019). The information derived from the identification of genes implicated in sensorineural hearing loss (SNHL) using SCS can be used to develop cell-targeted therapeutic strategies.

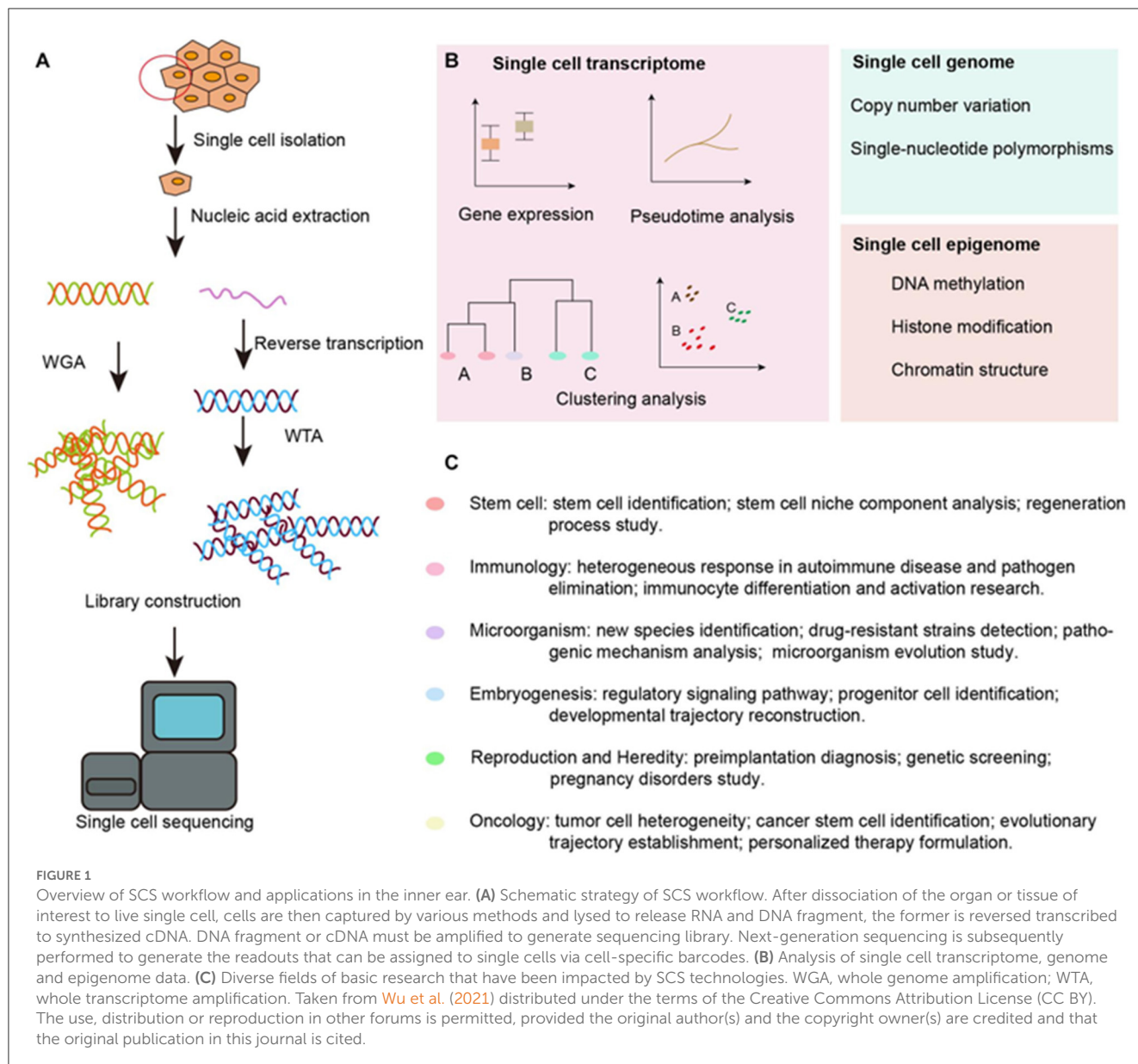
Single-cell RNA sequencing has also been used to analyze the sensory epithelial cells of the inner ear leading to the identification of several new genes. These genes include *Rasd2*, *Anxa4*, and *Pcp4* on HCs and *Mia1* and *Cdh4* on medial supporting cells and *Cntn1* within lateral supporting cells (Burns et al., 2015). In the inner ear, HCs and supporting cells are both found in epithelial tissues involved in balance and hearing. Damaged or dysfunctional genes in these cells may contribute to SNHL and/or vestibular pathologies. Research into the regeneration of HCs and supporting cells in the cochlea may show promise for patients with SNHL due to a variety of factors including loud noise exposure, chemotherapy, and aging. Specifically, researchers have investigated the potential of using supporting cells to act as precursors for generating HCs (Walters et al., 2017). The expression of genes associated with epithelial tissue in the cochlea identified through SCS can be manipulated to attenuate hearing loss. For instance, Walters et al. discovered that deleting the *p27* gene while upregulating the expression of the *Atoh1* gene in adult mouse cochleae triggered the conversion of supporting cells to HCs following damage to HCs resulting from loud noise. Similarly, upregulation of *Pou4f3* or *Gata3* with *Atoh1* bolstered the transformation of supporting cells to HCs in adult mice (Walters et al., 2017). These findings demonstrate the potential for gene therapy to be an important tool in attenuating SNHL.

SGNs are neural cells that transmit information from HCs of the cochlea to the central cochlear nucleus in the brainstem (Shrestha et al., 2018). Type I SGNs make up the majority of SGNs and innervate inner HCs (IHCs) while Type II SGNs innervate OHCs. Type I SGNs function primarily to transmit sound information to the brain while Type II SGNs play a role in pain sensing and damage perception; these differences in function are mainly due to differences in structure and molecular signaling (Liu et al., 2015). Outer hair cells (OHCs) amplify sounds that enter the cochlea. These amplified sound-induced vibrations are transmitted

TABLE 1 A summary of application of single cell sequencing (SCS) in auditory disorders.

Application	Cell-types studied	Genes/pathways identified	References
Genetic etiology	Stria vascularis (SV) Spiral Ganglion Neurons (SGNs) Inner ear sensory epithelial cells	<i>Abcg1, Heyl, P2rx2, Kcnj16, Nrp2, Kcnj13, Sox8, and Nr2f2</i> <i>Pou4f1, Lygd1, Calb2, and Ntng1</i> <i>Rasd2, Anxa4, Pcp4, Mia1, Cdh4, and Cntn1</i>	Burns et al., 2015; Shrestha et al., 2018; Korrapati et al., 2019
Ototoxicity	OHCs	Cisplatin causes high-frequency hearing loss due to accumulation in the basal turn	Prayuenyong et al., 2021
	SV	Cisplatin enters blood-labyrinthine barrier using CTR1 and OCT2	Wang et al., 2023
	SV	Aminoglycosides enter blood-labyrinthine barrier using TRPV1 and TRPV4	Steyger, 2021
	SV	Highest accumulation of cisplatin is present in the SV and is still present up to 18 months after treatment	Breglio et al., 2017
	HCs Auditory HEI-OC1 cell line	Sorcin is decreased with increasing levels of cisplatin Without sorcin, there is increased caspase-3 and Bax and decreased Bcl-2 Decreased sorcin leads to activation of ERK1/2, which is implicated in apoptosis	Wang et al., 2021
Noise trauma	OHCs Support cells	Increase in apoptotic genes (<i>Jun, Bcl10, and Tgfb2</i>) Increase in immune-related genes (<i>Ifr7, Ccl5, and Tlr3</i>) Increase in genes for mRNA metabolic processes (<i>Sfpq, Hnrmpu, and Snrmp48</i>) Increase in genes for nerve impulses (<i>Grm3, Asic2, and Cacna1e</i>)	Milon et al., 2021
	Type 1A SGNs	Promoters were highly active with transcription factors <i>Atf3, Atf4, and Atf5</i> , which upregulate pro-survival genes, <i>Fgf21, Cntfr, Hspa5, and Gas6</i>	Milon et al., 2021
	LW	Downregulation of potassium ion transport genes	Milon et al., 2021
Sudden sensorineural hearing loss	SV	Increased expression of <i>Sod1, Gpx3, Mif, Gjb2, Nr3c1, Clock, Gpx1, Sod2, and Nr3c2</i>	Nelson et al., 2021
	Pillar cells Deiters cells	Increased expression of <i>ApoE, Gjb2, and Pon2</i>	Nelson et al., 2021
	IHCs OHCs Deiters cells	Increased expression of <i>Sod1, Gpx1, Mif, Gjb2, Nr3c1, Nr3c2, and Gpx3</i>	Nelson et al., 2021
Vestibular schwannoma	Schwann cells Myeloid cells Fibroblasts Lymphocytes Endothelial cells	Hematopoiesis MAPK pathway Chemokine activity Myelination (MT-ATP6, ILI27, and NDMAP) Proteasome pathway (FBXW7) Apoptosis (TEAD4) pathway (FBXW7) Apoptosis (TEAD4) Angiogenesis (PKNOX1) CLEC9A, TXNIP, and AREG CLEC9A, TXNIP, and AREG (dendritic cells), NF-KB and PI3K (monocytes) CXCL signaling pathway ANXA1 (calcium-dependent phospholipid binding protein) CXCL signaling pathway PKNOX1 (angiogenesis)	Yidian et al., 2022
Acute otitis media	Macrophages Neutrophils Fibroblasts Epithelial cells T and B immune cells Dendritic cells Endothelial cells	<i>mrc1</i> Decreased interaction of expression of CSF1 and IL34 with CSF1-receptor CCL3-CCR5 and CXCL1-CXCR2 IL1β-IL1R2 IGF1-IGF1R CCL3-CCR5 and IGF1-IGF1R CCL3-CCR5 and CXCL1-CXCR2 IL1β-IL1R2 CSF1 and IL34 with CSF1-receptor IGF1-IGF1 receptor IGF1-IGF1 receptor CSF1 and IL34 with CSF1-receptor CCL3-CCR5 and CXCL1-CXCR2 IGF1-IGF1 receptor CCL3-CCR5 and CXCL1-CXCR2	Rao et al., 2021

SV, stria vascularis; SGNs, spiral ganglion neurons; HCs, hair cells; OHCs, outer hair cells; IHCs, inner hair cells; LW, lateral wall.



to IHCs, which trigger the opening of transduction channels at the apical surfaces of IHCs, thus causing the excitation of SGNs (Sun et al., 2018). SCS has been used to identify 3 subtypes of Type I SGNs: subtypes Ia, Ib, and Ic (Shrestha et al., 2018). These subtypes differ by their expression of multiple genes, such as *Pou4f1*, *Lypd1*, *Calb2*, and *Ntn1*, that affect neuronal differentiation and function. For instance, many of these genes encode proteins related to calcium and potassium channel proteins in the plasma membranes of Type I SGNs. Variation in gene expression among the Type I SGN subtypes correlates with differences in expression of certain proteins, most notably membrane proteins, which can result in differences in physiology and synaptic transmission among SGN subtypes (Shrestha et al., 2018). Using SCS, it may be possible to identify different gene isoforms expressed in SGN cellular subtypes so as to specifically address the cells with pathologic ionic homeostasis through cell-specific gene targeting.

Understanding molecular mechanisms underlying auditory disorders

Ototoxicity

SCS is now being increasingly used to better understand the molecular mechanisms underlying drug-induced ototoxicity such as due to cisplatin or aminoglycosides. Cisplatin is a chemotherapeutic drug that is used in the treatment of many malignant tumors. However, it has been found that cisplatin accumulates preferentially in the basal turn of the cochlea, causing loss of OHCs in the OC, leading to high-frequency hearing loss (Prayuenyong et al., 2021). Cisplatin enters the inner ear by crossing the blood-labyrinthine barrier specifically, entering the SV marginal cells, using CTR1 and OCT2 (Wang et al., 2023). This damages the Na⁺/K⁺/2Cl⁻ channel, which is integral

in maintaining a healthy endolymph. Aminoglycosides, broad-spectrum antibiotics, are also known to cause ototoxicity in a similar manner but are thought to utilize TRPV1 and TRPV4 (Steyger, 2021). A pharmacokinetic study demonstrated that cisplatin continues to accumulate in the inner ear following each dose, and the cells show limited ability to eliminate it. The highest level of accumulation was identified in the SV. Additionally, they analyzed postmortem cochleae from patients who were treated with cisplatin and identified that the drug was still present up to 18 months after treatment (Breglio et al., 2017).

Researchers have used SCS to discover the role of soluble resistance-related calcium binding protein (sorcin) in cisplatin-induced ototoxicity (Wang et al., 2021). Sorcin is a calcium-binding protein that is known for its involvement in calcium homeostasis, apoptosis, and tumor progression (Shabnam et al., 2018). In particular, it can reduce apoptosis by regulating apoptotic proteins, including Bax, Bcl-2, and caspase-3, and is involved in signaling pathways, including ERK, MAPK/ERK, STAT3, and P13K/Akt (Shabnam et al., 2018). *Sorcin* is expressed in cochlear HCs and auditory HEI-OC-1 cells (Wang et al., 2021). It has been shown that with increasing levels of cisplatin, *sorcin* decreased, and the damage was most pronounced in the basal turn of the cochlea (Wang et al., 2021). They also found that when *sorcin* was knocked out using siRNA, there was a higher level of pro-apoptotic proteins, such as caspase-3 and Bax, and a decreased amount of anti-apoptotic proteins, such as Bcl-2, in response to cisplatin exposure (Wang et al., 2021). Additionally, higher levels of reactive oxygen species (ROS) and matrix metalloproteases (MMP) were observed, indicating mitochondrial dysfunction (Wang et al., 2021). There was activation of the ERK1/2 signaling pathway, previously implicated in apoptosis of HCs following knockdown of *sorcin*. SCS can be utilized to further investigate the molecular mechanisms underlying the otoprotective effects of *sorcin* and its cell-specific expression following cisplatin exposure paving the way for developing treatment modalities to prevent hearing loss.

Noise trauma

SCS is also being utilized to study how noise affects the cochlear sensory compartment and leads to SNHL. Milon et al. (2021) sequenced cell-type specific immunoprecipitated RNA (RiboTag-IP) to isolate cells along with SCS to study specific transcriptional cell responses to stress (Milon et al., 2021). Mice were exposed to 8 to 16 kHz noise bands at 105 dB for 2 h, causing a permanent threshold shift across all frequencies. OHCs and support cells were analyzed and a difference in 1,947 and 1,796 expressed genes, respectively, compared to controls was observed. There was an increase in genes implicated in apoptosis (*Jun*, *Bcl10*, and *Tgfb2*) as well as immune-related genes (*Ifr7*, *Ccl5*, and *Tlr3*). Additionally, some were specific for OHCs, which included genes that function in mRNA metabolic processes (*Sfpq*, *Hnrnpu*, and *Snrmp48*), and some were specific for supporting cells, including genes for the transmission of nerve impulses (*Grm3*, *Asic2*, and *Cacna1e*). It was also observed that type 1A SGN elicit a strong transcriptional response to noise. The promoters of genes expressed by these

cells were highly interactive with transcription factors from the activating transcription factor (ATF) family, specifically *Atf3*, *Atf4*, and *Atf5*. These factors may be protective after noise exposure because they upregulate pro-survival genes, such as *Fgf21*, *Cntfr*, *Hspa5*, and *Gas6*. Next, they studied lateral wall (LW). A total of 408 differentially expressed genes were downregulated in these cells. There was a downregulation of potassium ion transport genes suggesting decreased functioning of LW cells in transporting potassium into the endolymph following noise exposure (Milon et al., 2021). While there are cell-type specific responses to noise trauma, the cochlea was also found to mount a response that was shared amongst the cell types. About 36 genes, including *Stat3* and *Irf7*, were found to be upregulated across all cell types and were specifically responsive to transcription factors IRF7 and STAT3 (Milon et al., 2021). Using this gene expression data, metformin was identified as the top candidate for potential pharmacologic therapy for noise-induced hearing loss (NIHL). These findings suggest the potential of SCS to identify novel therapeutic agents based on cell-specific gene expression following auditory insults such as noise exposure.

Sudden sensorineural hearing loss

Sudden sensorineural hearing loss (SSNHL) is defined as a hearing loss of at least 30 dB that spans a minimum of three contiguous audiometric frequencies and develops within 72 h (Nelson et al., 2021). SCS holds great potential to understand the genetic etiology of SSNHL. However, so far only one study has used this technique. A study reviewed SCS data that had been performed on particular cell types within the cochlea, including the SV, Pillar cells and Deiter cells, as well as IHCs and OHCs, to determine if there were certain genes implicated in SSNHL (Nelson et al., 2021). It was observed that the SV showed increased expression of *Sod1*, *Gpx3*, *Mif*, *Gjb2*, *Nr3c1*, *Clock*, *Gpx1*, *Sod2*, and *Nr3c2*. Pillar and Deiters cells show increased expression of *ApoE*, *Gjb2*, and *Pon2*. Finally, IHCs, OHCs, and Deiters cells show increased expression of *Sod1*, *Gpx1*, *Mif*, *Gjb2*, *Nr3c1*, *Nr3c2*, and *Gpx3*. Superoxide dismutase (*Sod1/2*) and macrophage migration inhibitory factor (*Mif*) may be particularly important and have been seen to be implicated in other types of hearing loss as well, including noise-induced and age-related (Nelson et al., 2021). Additionally, they evaluated the different expression of glutathione peroxidase isoforms. Notably, in the SV, *Gpx3* was expressed at a higher rate than *Gpx1* in the marginal, intermediate, and basal cells. This is in contrast to other cells in the OC, which preferentially expressed *Gpx1* (Nelson et al., 2021). An important limitation of this study was that they used a previously published mouse whole transcriptome dataset with poor resolution, impeding any definitive conclusion concerning gene expression patterns. Additionally, candidate genes were identified based on previously identified pathways, such as inflammation or oxidative stress. Finally, the data addressed was purely observational, and the functional significance of the identified genes was not determined (Nelson et al., 2021). However, results suggest that utilizing SCS methods can enhance our understanding regarding the underlying molecular mechanisms involved in SSNHL.

Vestibular schwannomas

Vestibular schwannomas (VS) are benign tumors arising from the vestibulocochlear nerve's Schwann cells and are the most common neoplastic cause of hearing loss. Current treatment options comprise surgery or radiotherapy, and monoclonal antibody angiogenesis inhibitors for VS associated to neurofibromatosis type 2 (NF2). Although bulk analysis of VS tissue has been performed (de Vries et al., 2019; Huang et al., 2019), no information on the cellular microenvironment is available. By applying SCS to VS tissue samples, the different cell types and factors in these tumors as well as candidate biomarkers of VS can be identified leading to the discovery of potential drug targets. A study performed SCS on three human tissue samples of sporadic VS and was able to identify six cell types involved in the tumor microenvironment: Schwann cells, myeloid cells, fibroblasts, lymphocytes (B and T cells), and endothelial cells (Yidian et al., 2022). Six Schwann cell subgroups were identified based on antigen presenting genes and HLA phenotypes. A particular subgroup SC-C3 was enriched in hematopoiesis and in the MAPK signaling pathway, typically activated after peripheral nerve injury, and initiating cell differentiation. Cellular subgroup SC-C4 was correlated to chemokine activation and SC-C5 to myelination, suggesting the high heterogeneity of the expression profiles and function of this cell type in VS. Analysis of transcription factors revealed SC-C5 expressed TEAD4, involved in the Hippo signaling pathway which induces apoptosis and limits proliferation (Yidian et al., 2022). Analysis of the myeloid cell population, and more particularly a subtype of monocytes, activated PI3K a transduction pathway involved in chemotherapy resistance. Through SCS, cellular interactions were also studied, and allowed to identify specific cellular links, especially those between fibroblasts and immune cells. Two different fibroblast clusters were found to be mainly involved in the CXCL signaling pathway, promoting angiogenesis in tumor tissue (Yidian et al., 2022). The application of SCS in sporadic VS revealed fundamental characteristics on cellular heterogeneity, microenvironment, and molecular pathways, paving the way for specific drug development.

Acute otitis media

Acute otitis media (AOM) refers to middle ear infection leading to inflammation and is a global healthcare burden. Studies on middle ear mucosa (MEM) have been performed using flow cytometry, bulk DNA analysis (Forséni Flodin, 2001; Jecker et al., 2001), and more recently SCS (Ryan et al., 2020). An animal study focusing on MEM, used SCS to clarify the alterations induced by inflammation and multicellular interactions resulting in the identification of new cell subtypes (Rao et al., 2021). Eight major cell subtypes were identified by SCS. Macrophage subtype M2 specifically expressed *mrc1*, and communicated with all other cellular subtypes, thus playing a key role in the intercellular crosstalk during inflammation. Colony-stimulating factor 1 (CSF1) and IL-34 interaction with CSF1-receptor were both increased in the M2 subpopulation of inflamed and normal mucosa, suggesting

the important role of these macrophages in the middle ear. This study identified dual-feature cells and described their specific roles by comparing SCS data to the Gene Ontology database (Rao et al., 2021). Key discoveries such as the crosstalk through IGF-1 between macrophages and the dual-feature epithelial-phagocytic cells were described, such as the increase in IGF1 in normal mucosa and its decrease AOM. SCS holds a great potential in elucidating precise molecular pathways underlying pathophysiology of AOM that needs to be harnessed in future studies.

Conclusion and future directions

The advent of SCS has significantly enhanced our understanding of inner ear cellular heterogeneity as well as the genes and molecular pathways involved in hearing loss. Studies using SCS have identified novel marker genes involved in auditory disorders such as ototoxicity, NIHL, and SSHNL, thus providing the key targets for gene therapy. Nevertheless, the limits of viral transfections, their efficacy on the human ear, and the acceptability of genetic treatment by patients, will determine the clinical translation of gene therapy.

Although SCS has revolutionized genetic research, current methods still need some improvement. The cell isolation for SCS is challenging, as the inner ear lies nested at the base of the skull in the robust petrous part of the temporal bone, making it difficult to access and reach the desired cell types. In addition, cells are scarce, with hair cells representing <3% of an intact sensory epithelium, as well as difficult to dissect from the labyrinthine structures. The cell dissociation of SCS can also alter the gene expression profiles. The other important limitation of SCS is that it requires a large number of intact viable cells to correctly profile auditory disorders. However, current techniques impact the spatial resolution necessary to identify cells and their function (Ranum et al., 2019). With the concurrent development of spatial transcriptomics, linking gene function to the anatomical location of cells, it is feasible to overcome some of these challenges (Srivatsan et al., 2021; Vahid et al., 2023). Given the overwhelming amount of data collected through SCS, data storage and data analysis software development is another major limiting factor for this technology. Efforts aimed toward creating and continuously updating universal database platforms will determine the optimal use of the entirety of the complex information discovered through SCS.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

RM and AAE declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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