MicroRNA Signature and Cellular Characterization of Undifferentiated and Differentiated House Ear Institute-Organ of Corti 1 (HEI-OC1) Cells

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ABSTRACT

MicroRNAs (miRNAs) regulate gene expressions and control a wide variety of cellular functions. House Ear Institute-Organ of Corti 1 (HEI-OC1) cells are widely used to screen ototoxic drugs and investigate cellular and genetic alterations in response to various conditions. HEI-OC1 cells are almost exclusively studied under permissive conditions that promote cell replication at the expense of differentiation. Many researchers suggest that permissive culture condition findings are relevant to understanding human hearing disorders. The mature human cochlea however consists of differentiated cells and lacks proliferative capacity. This study therefore aimed to compare the miRNA profiles and cellular characteristics of HEI-OC1 cells cultured under permissive (P-HEI-OC1) and non-permissive (NP-HEI-OC1) conditions. A significant increase in the level of expression of tubulin β1 class VI (Tubb1), e-cadherin (Cdh1), espin (Espn), and SRY (sex determining region Y)-box2 (Sox2) mRNAs was identified in non-permissive cells compared with permissive cells ($P < 0.05$, Kruskal–Wallis H test, 2-sided). miR-200 family, miR-34b/c, and miR-449a/b functionally related cluster miRNAs, rodent-specific maternally imprinted gene Sfmbt2 intron 10th cluster miRNAs (-466a/-467a), and miR-17 family were significantly ($P < 0.05$, Welch’s $t$-test, 2-tailed) differentially expressed in non-permissive cells when compared with permissive cells. Putative target genes were significantly predominantly enriched in mitogen-activated protein kinase (MAPK), epidermal growth factor family of receptor tyrosine kinases (ErbB), and Ras signaling pathways in non-permissive cells compared with permissive cells. This distinct miRNA signature of differentiated HEI-OC1 cells could help in understanding miRNA-mediated cellular responses in the adult cochlea.

Keywords: HEI-OC1 cells, Undifferentiated, Differentiated, miRNA profile, Gene expression

INTRODUCTION

MicroRNAs (miRNAs) are small 18–24-nucleotide non-coding single-stranded RNAs that repress or degrade messenger RNAs (mRNAs) by binding to their complementary sequences at the 3′-untranslated region (3′UTR) (Filipowicz et al. 2008; Obergosterer et al. 2006). miRNAs play critical roles in various kinds of biological processes, such as cellular development, differentiation, metabolism, proliferation, migration, and apoptosis (Pasquinelli 2012; Pelaez and Carthew 2012; Kim et al. 2009), and their altered expression is associated with many human pathologies (Calin and Croce 2006; Esquela-Kerscher and Slack 2006; van Rooij et al. 2006).
MiRNAs play a fundamental role in the regulation of gene expression in the inner ear and associated structures (Mahmoudian-Sani et al. 2017). They are crucial for inner ear development and are involved in the morphogenesis and neurosensory processes that lead to a functional auditory organ (Rudnicki and Avraham 2012). The coordinated expression of miR-183 family members (miR-183, miR-96, and miR-182) has been demonstrated to be particularly important in the development of the sensory cells of the inner ear of mice and other vertebrates (Weston et al. 2006; Sacheli et al. 2009; Li et al. 2010; Friedman et al. 2009). Recent studies show that two single-base mutations in the seed region of miR-96 result in autosomal dominant, progressive hearing loss in both humans and mice (Solda et al. 2012; Mencia et al. 2009; Lewis et al. 2009). This mutation alters the function of miR-96 and their consequent gene expression profile in the mouse organ of Corti such as oncomodulin (Ocm), prestin (Slc26a5), and growth factor independent 1 (Gfi1) which have been known to result in deafness and hair cell degeneration (Lewis et al. 2016). These findings demonstrate the importance of miRNA-mediated gene regulation in the cochlea.

House Ear Institute-Organ of Corti 1 (HEI-OC1) cells are one of the few auditory cell lines widely used for research purposes. These cells were derived from the auditory organ of the transgenic mouse Immortomouse™, which harbors a temperature-sensitive mutant of the SV40 large T antigen gene under the control of an interferon-gamma-inducible promoter element (Jat et al. 1991; Kalinec et al. 2003). Incubation of Immortomouse™-derived HEI-OC1 cells at permissive conditions (33 °C, 10 % CO2) induces immortalizing conditions (NP-HEI-OC1 cells) than those under permissive culture conditions with that of HEI-OC1 cells maintained under permissive conditions. The identity of putative and validated target genes of miRNAs found to be differentially expressed under non-permissive conditions was sought using gene functional analysis. Cellular characterization studies were undertaken to document differences in the morphology, protein, and gene expression of HEI-OC1 cells under permissive and non-permissive conditions.

**METHODOLOGY**

This study was approved by the Biosafety Committee of the University of British Columbia, Vancouver, Canada.

**Cell Culture**

HEI-OC1 cells (kindly provided by Dr. F. Kalinec), derived from the transgenic mouse postnatal organ of Corti, were used to investigate their miRNA expression profiles during proliferation and differentiation. HEI-OC1 cells were cultured under permissive and non-permissive culture conditions as recommended by Kalinec et al. (2016b) to promote proliferation and differentiation, respectively. All cultures were grown in T25 flasks (Nunc™ Non-treated) in Dulbecco’s Modified Eagle’s Medium (DMEM), containing 10 % fetal bovine serum (FBS) without supplements and antibiotics in a humidified incubator. Cell morphology was captured with a phase-contrast Zeiss Axio Vert.A1 inverted microscope.

Permissive cultures (P-HEI-OC1 cells) were incubated at 33 °C and 10 % CO2 as recommended (Kalinec et al. 2016b). The growth medium was replaced every 2 days. The cells were harvested for experiments once the cultures achieved 100 % confluence usually after 5–7 days of incubation.

Non-permissive cultures were obtained by initially incubating HEI-OC1 cells under permissive conditions until they reach 80–100 % confluence. They were then moved to previously described non-permissive conditions: 39 °C and 5 % CO2 to promote cell differentiation (Kalinec et al. 2016b). The cells were maintained over 2 incubation periods: 1 week (NP1-) and 2 week (NP2-). HEI-OC1 cells under non-permissive culture conditions (NP-HEI-OC1 cells) changed cellular morphologies and started dying as previously described (Kalinec et al. 2003; Devarajan et al. 2002). To minimize the effects of toxins released by dead cells, the growth medium was fully replaced daily and the cultures were harvested after 1-week and 2-week incubation periods, respectively, for further study.
miRNA Profiling of HEI-OC1 Cells Maintained at Permissive and Non-permissive Conditions

miRNA Extraction from HEI-OC1 Cells

Cells were washed with Dulbecco’s phosphate-buffered saline (DPBS) buffer and trypsinized with 0.25 % trypsin-EDTA and incubated at 37 °C for 5 min. Trypsinization was stopped by adding 9 ml of DMEM medium, and the pooled suspension was centrifuged at 1500 rpm for 10 min to obtain the cell pellets for subsequent RNA extractions. miRNA was extracted from the cell pellets using miRNasy easy kit (Qiagen) as per manufacturer’s protocol. Extracted miRNAs were quantified in a BioTek (EPOCH) microplate spectrophotometer using Gen5 software.

Reverse Transcription (RT) and Pre-amplification

RT was performed with TaqMan miRNA RT kit (Applied Biosystems) as previously described (Nunez et al. 2020) with slight modifications. Briefly, a RT reaction mixture consisting of 0.8 μl megaplex RT primers (Rodent Pools A+B), 0.2 μl 100 mM dNTPs (with dTTP), 1.5 μl multi-scribe reverse transcriptase (50U/μl), 0.8 μl 10X RT buffer, 0.9 μl MgCl2 (25 mM), 0.1 μl RNase inhibitor (20U/μl), 350 ng RNA template, and nuclease-free water to a final volume of 25.0 μl was prepared. RT reaction was carried out on a BioRad T100™ thermal cycler according to the manufacturer’s recommended thermal cycling conditions.

Pre-amplification of the cDNA product after RT was performed using 12.5 μl TaqMan preAmp master mix (2X), 2.5 μl megaplex preAmp primers (Rodent Pools A+B) (10X) and nuclease-free water to a final volume of 7.5 μl was prepared. RT reaction was carried out on a BioRad T100™ thermal cycler according to the manufacturer’s recommended thermal cycling conditions.

TaqMan Low-Density Array (TLDA)

The miRNA profiling of 768 miRNAs was performed with TLDA cards (Rodent Pools A+B Cards Set v3.0). To prepare the real-time PCR reaction mix, 9 μl of diluted pre-amplification product (1:4), 450 μl of TaqMan™ universal PCR master mix (no AmpErase™ UNG) (2X), and 441 μl of nuclease-free water were added to a final volume of 900 μl. One hundred microliters of the PCR reaction mix was loaded onto each row of the 384-well TLDA cards (A or B), centrifuged for 1–2 min at 1200 rpm, sealed carefully and run in a ViiA™ 7 Real-Time PCR System at recommended settings and cycling conditions. HEI-OC1 cells were grown twice under each set of culture conditions (P-, NP1-, and NP2- HEI-OC1 cells), and TLDA assays were repeated on cells drawn separately from the duplicated cell cultures. Relative miRNA levels were calculated using the comparative threshold cycle (Ct) method (ΔΔCt) normalized to a global mean value and at a cut off Ct level < 35.0.

The TLDA cards tested for 596 Mus musculus miRNAs, 78 Rattus norvegicus miRNAs, 76 Homo sapiens miRNAs, and 18 controls. All non-mouse species’ differentially expressed miRNAs (DEMs) were searched to determine if they shared the same conserved sequences as mouse miRNAs using miRBase database (Release 22.1, http://mirbase.org). Non-mouse DEMs that were homologous to mouse miRNAs were included and non-homologues were excluded from analysis. In addition, DEMs that are not defined as miRNAs currently by the miRBase database (dead entries) were also excluded.

Prediction of Putative and Validated Target Genes and Their Functional Enrichment Analysis

The putative and validated target genes of DEMs were obtained using miRWalk3.0 database with filters miRDB and miRTarBase, respectively, at a binding probability of 1.0 within the 3-UTR region (Sticht et al. 2018). The DAVID Bioinformatics Resources 6.8 NIAID/NIH functional annotation tool (da Huang da et al. 2009a; da Huang et al. 2009b) was used to determine if the identified target genes were statistically significantly (at a cut off adjusted P value <0.05) associated with functional terms: Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology Biological Process (GOBP).

Cellular Characterization of Permissive and Non-permissive HEI-OC1 Cells

Gene Expression

Eighteen target genes which have been reported in inner ear studies or indicated from our prior miRNA findings in HEI-OC1 cells (Wijesinghe et al. 2021a, b), namely, atonal bHLH transcription factor 1 (Atoh1) (Hongmiao et al. 2014), POU domain, class 4, transcription factor 3 ( Pou4f3) (Hertzano et al. 2004), espin (Espn) (Zheng et al. 2014), myosin 7a (Myo7a) (Hasson et al. 1995, 1997), prestin (Sh26a5) (Park et al. 2016), SRY (sex determining region Y)-box2 (Sox2) (Kiernan et al. 2005; Hume et al. 2007; Kempfle et al. 2016), paired box 2 (Pax2) (Christophorou et al. 2010), cyclin-dependent kinase inhibitor 1b (Cdkn1b or p27Kip1) (Chen and Segil 1999), tubulin B1 class VI (Tubb1) and tubulin β3 class III (Tubb3) (Hallworth and Ludueña 2000; Hallworth et al. 2000; Jensen-Smith et al. 2003), nestin (Nes) (Watanabe et al. 2012; Lou et al. 2014), cytokertatin 18 (Krt18) (Cyr et al. 2000; Wijesinghe et al. 2021a, b) and vimentin (Vim) (Yamasoba and Kondo 2006), sci-like with four mbt domains 2 (Sfmtb2), zinc finger E-box-binding homeobox 1 (Zeb1), zinc finger E-Box binding homeobox 2 (Zeb2), c-cadherin (Cdh1), and tubulin β3 class I (Tubb5) were used. Two endogenous controls, glycerolaldehyde-3-phosphate dehydrogenase (Gapdh) and hypoxanthine guanine phosphoribosyl transferase 1 (Hprt1)
were tested for data normalization. Primer3Plus software (Untergasser et al. 2007) was used to design forward and reverse primers (Table 1).

RNA (RNase® mini kit, QIAGEN) was extracted from P-, NP1-, and NP2- HEI-OC1 cell pellets which were dissolved in 350 μl RLT buffer containing 0.01 % 14.3 M β-mercaptoethanol according to the manufacturer’s protocol. The quantity and quality of extracted RNA were determined prior to cDNA preparation. cDNA synthesis was performed with SuperScript™ VILO™ cDNA synthesis kit (Invitrogen) as per manufacturer’s protocol in a BioRadT100™ thermal cycler. Synthesized cDNAs were then diluted to a concentration of 5 ng/μl.

In brief, the RT-qPCR reaction mix per well consisted of 1 μl of HyPure™ molecular biology grade water, 5 μl SYBR select master mix at the manufacturer’s supplied concentration, 1 μl of each forward and reverse primers (10 μM), and 2 μl of diluted cDNA (5 ng/μl). After the reaction mix was added to the wells, the plate was centrifuged for a few seconds in a Mini PCR Plate Spinner. RT-qPCR with an initial denaturing step of 95 °C for 10 min and followed by 40 amplification cycles of 15 s at 95 °C and 1 min at 60 °C duration was undertaken on a Quant Studio™ 3 Real-Time PCR system (Applied Biosystems). All target gene tests were repeated a minimum of three times on each sample. Relative mRNA levels were determined using the comparative cycle threshold method at a cut-off Ct < 40.0. Gapdh was used as a reference gene for data normalization. The relative mRNA level was expressed as the mRNA copies of the gene of interest per 1000 copies of Gapdh mRNA [2−∆∆Ct/1000 = 1000/2(∆Ctavg. target gene Ct – avg. reference gene Ct)] (Schmittgen and Livak 2008; Huang et al. 2014).

mRNA-miRNA Interactions

Since the tested target genes were primarily selected from previous inner ear studies, it is worthwhile to predict their biological target miRNAs to determine the mRNA-miRNA interactions. Therefore, target genes used for the cellular characterization of P-, NP1-, and NP2- HEI-OC1 cells were then searched for their biological target miRNAs by searching for the presence of conserved sequences (8mer and 7mer) that match the seed region of each miRNA with TargetScanMouse version 7.2 (Agarwal et al. 2015). miRNAs that shared poorly conserved sequences were excluded. mRNA-miRNA interactions are illustrated using Cytoscape version 3.7.1 (Shannon et al. 2003).

Fluorescence Immunocytochemistry (ICC)

HEI-OC1 cells were grown in 8-well chamber slides (Lab Tek, Permanox TC Surface, Treated) under varying culture conditions as described above. The immunocytochemical characteristics of the HEI-OC1 cells under permissive and non-permissive culture conditions were determined using 6 selected protein markers,

| Table 1 | Primer sequences used to amplify the target genes in HEI-OC1 cells |
|---------|---------------------------------------------------------------|
| Genes (Mus musculus) | Primers | Sequences 5′-3′ |
| Atoh1 | Forward | ACATCTCCGATCCCAAGAG |
| Reverse | ACAACGATCCACCAAGCA |
| Tubb1 | Forward | GCTGCTGTCCTATTCAGAACA |
| Reverse | GCTCAGAGACCCCTGACTGA |
| Tubb3 | Forward | TGAGGCCCTCTTCACGGTTG |
| Reverse | CGCCAGCAGTCTCAGATGA |
| Tubb5 | Forward | TTAGCTGACCCACTCAGT |
| Reverse | AGACAGGGTGCGATTGAGG |
| Cdh1 | Forward | CAAGAGACCCCTTTTTCG |
| Reverse | GACTGTTCTTTTCCCCTG |
| Espn | Forward | GGGAGGATAGGAGAGAAG |
| Reverse | CCAAACGCGTATAAGGGTC |
| Myo7a | Forward | CAACATGAAACGCAACCC |
| Reverse | CCAAAAGCGTGATTGTC |
| Nes | Forward | CAGAACTCCATAGGAACTC |
| Reverse | ACCGAGCCTTTTCGGT |
| p27Kip1 | Forward | TGGAGGCGGCTGGAACAG |
| Reverse | GCTCAGAGACCCCTGACTGA |
| Pax2 | Forward | TCCAGTGTCCTATCCATCA |
| Reverse | GGGAGGAGGCGGGCTGGAACAG |
| Pou4f3 | Forward | GTTCAGGGTGGTGGAGTGCA |
| Reverse | TCATGTTTGTGGCCCGACAG |
| Slc26a5 | Forward | ACATGTTGGAAGTGGTTG |
| Reverse | CCATGCATTATTGGAGAAGT |
| Slmbt2 | Forward | GCATCCTCCAAAAGCAAGAG |
| Reverse | GAGAGATCTTTGCCAGAAG |
| Sox2 | Forward | AAAGGTTCAGGCTGGGTTT |
| Reverse | AGAACAGAAACGCTCTT |
| Krt18 | Forward | AGACTTGGTTGGTGGACAC |
| Reverse | ATCGGGCCTATCAGAGAA |
| Vim | Forward | GCAGCGGTCTATTTCCTCATC |
| Reverse | GTAGGTTGGCGGGCTGTA |
| Zeb1 | Forward | GGGGATCTACACATTTT |
| Reverse | AAGGGCCTGGAACCAAC |
| Zeb2 | Forward | GCACAGGCCTTATGCTGTA |
| Reverse | CCCTGTCTTCTGCTGGAG |
| Gapdh | Forward | CCAACAGCACTCCACTC |
| Reverse | AAGGCAGAAATGAGCTT |
| Hprt1 | Forward | GCCCACAAATTGTACCGGT |
| Reverse | TTGGGCTCACTTCAGGCTT |

TABLE 1

Prime sequences used to amplify the target genes in HEI-OC1 cells

| Genes (Mus musculus) | Primers | Sequences 5′-3′ |
|----------------------|---------|----------------|
| Atoh1                | Forward | ACATCTCCGATCCCAAGAG |
| Reverse              | ACAACGATCCACCAAGCA |
| Tubb1                | Forward | GCTGCTGTCCTATTCAGAACA |
| Reverse              | GCTCAGAGACCCCTGACTGA |
| Tubb3                | Forward | TGAGGCCCTCTTCACGGTTG |
| Reverse              | CGCCAGCAGTCTCAGATGA |
| Tubb5                | Forward | TTAGCTGACCCACTCAGT |
| Reverse              | AGACAGGGTGCGATTGAGG |
| Cdh1                 | Forward | CAAGAGACCCCTTTTTCG |
| Reverse              | GACTGTTCTTTTCCCCTG |
| Espn                 | Forward | GGGAGGATAGGAGAGAAG |
| Reverse              | CCAAACGCGTATAAGGGTC |
| Myo7a                | Forward | CAACATGAAACGCAACCC |
| Reverse              | CCAAAAGCGTGATTGTC |
| Nes                  | Forward | CAGAACTCCATAGGAACTC |
| Reverse              | ACCGAGCCTTTTCGGT |
| p27Kip1              | Forward | TGGAGGCGGCTGGAACAG |
| Reverse              | GCTCAGAGACCCCTGACTGA |
| Pax2                 | Forward | TCCAGTGTCCTATCCATCA |
| Reverse              | GGGAGGAGGCGGGCTGGAACAG |
| Pou4f3               | Forward | GTTCAGGGTGGTGGAGTGCA |
| Reverse              | TCATGTTTGTGGCCCGACAG |
| Slc26a5              | Forward | ACATGTTGGAAGTGGTTG |
| Reverse              | CCATGCATTATTGGAGAAGT |
| Slmbt2               | Forward | GCATCCTCCAAAAGCAAGAG |
| Reverse              | GAGAGATCTTTGCCAGAAG |
| Sox2                 | Forward | AAAGGTTCAGGCTGGGTTT |
| Reverse              | AGAACAGAAACGCTCTT |
| Krt18                | Forward | AGACTTGGTTGGTGGACAC |
| Reverse              | ATCGGGCCTATCAGAGAA |
| Vim                  | Forward | GCAGCGGTCTATTTCCTCATC |
| Reverse              | GTAGGTTGGCGGGCTGTA |
| Zeb1                 | Forward | GGGGATCTACACATTTT |
| Reverse              | AAGGGCCTGGAACCAAC |
| Zeb2                 | Forward | GCACAGGCCTTATGCTGTA |
| Reverse              | CCCTGTCTTCTGCTGGAG |
| Gapdh                | Forward | CCAACAGCACTCCACTC |
| Reverse              | AAGGCAGAAATGAGCTT |
| Hprt1                | Forward | GCCCACAAATTGTACCGGT |
| Reverse              | TTGGGCTCACTTCAGGCTT |
namely, inner (myosin 7a) and outer (prestin) hair cell markers, stem/progenitor cell markers Sox2 and nestin, and epithelial-mesenchymal transition markers (EMT) e-cadherin and vimentin.

HEI-OC1 cells that reached ≥80 % confluence were used for fluorescence ICC. Culture medium was removed, and the cells were washed for 1 min in DPBS 3 times. The cells were then fixed in 4 % paraformaldehyde for 15 min, followed by permeabilization in 0.1 % Triton-X 100 for 15 min. These cells were washed for 1 min in DPBS, 3 times. Thereafter, the cells were blocked using 3 % bovine serum albumin (BSA) at room temperature for 30 min prior to incubation at 4 °C overnight with primary antibodies [myosin 7a 1:100 dilution (rabbit polyclonal- ab3481, ABCAM); prestin 1:100 dilution (goat polyclonal- SC22692, Santa Cruz Biotechnology); nestin, neural stem cell marker 1:100 dilution (rabbit polyclonal- ab92391, ABCAM); Sox2 1:100 dilution (rabbit polyclonal- ab97959 ABCAM); e-cadherin 1:100 (rabbit polyclonal- ab15148), and vimentin 1:200 dilution (rabbit polyclonal- PA5-27,231, Invitrogen)] dissolved in 3 % BSA. The following day, primary antibodies were drained, and the chamber slides washed for 1 min in DPBS 3 times. Then, the cells were incubated at room temperature with secondary antibodies in the dark [donkey anti-rabbit Alexa Fluor®488 1:500 dilution (A21206, Invitrogen) and donkey anti-goat Alexa Fluor®488 1:500 dilution (A11055, Invitrogen)], respectively, to the primary antibodies for 1 h in a shaker. After incubation, secondary antibodies were drained, and the chamber slides washed for 1 min in DPBS 3 times. The cells were then mounted with ProLong™ Gold Antifade Mountant with DAPI (P36931, Invitrogen). Images were captured using a Zeiss Axio Vert.A1 Inverted Microscope. Immunofluorescence staining was performed in duplicate for each P-, NP1-, and NP2- HEI-OC1 cell cultures, respectively.

The number of immunofluorescence-positive cells per 400 × magnification field was recorded to determine the protein expressions semi quantitatively. Viable (DAPI positive nuclei) cells were relatively sparse and unevenly distributed under non-permissive culture conditions. Thus, areas in each culture with high numbers of DAPI-positive nuclei were selected for counting the number of antibody positively and negatively stained cells. P-HEI-OC1 cultures contained high levels of evenly distributed viable cells making selection of ideal high-power fields for incubation, respectively. c and f NP2-HEI-OC1 cells at 16th and 21st day of incubation, respectively. Images were captured with phase-contrast microscopy (scale bars are indicated).
study straightforward. Counts from 5 non-overlapping fields were recorded in all culture conditions and the average counts determined.

Statistical Analysis

Normalized mean Ct values of miRNAs expressed in P-, NP1-, and NP2- HEI-OC1 cell cultures (NP1 vs. P-, NP2 vs. P-, and NP2 vs. NP1-) were statistically compared using Welch’s t-test (2-tailed). P values of all tested miRNAs were subjected to Benjamini–Hochberg correction (false discovery rate, 50 %) at a significance level of P<0.05 (McDonald 2014; Benjamini and Hochberg 1995) for each inter-group comparison. DEMs were then defined as those that demonstrated a statistically significant intergroup fold difference (2^−∆∆Ct) > 2.0 (upregulated) and/or <0.5 (downregulated).

For the gene expression, normalized mean Ct values and for protein expression, the proportions of antibody positively stained cells were compared across P-, NP1-, and NP2-HEI-OC1 cell cultures using non-parametric Kruskal–Wallis H test (2-sided), followed by Dunn’s post hoc test at a Bonferroni-adjusted significance level of P<0.05 for multiple tests.

Welch’s t-test for miRNA expression and non-parametric Kruskal–Wallis H test for gene and protein expression were applied as the standard deviations were different for some tested miRNAs, genes, and proteins in permissive and non-permissive cultures. SPSS version 25.0 (IBM Corp., Armonk, New York) and GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA) were used for statistical analysis and to generate graphs.

RESULTS

Cell Culture Morphology

Culturing HEI-OC1 cells under permissive conditions facilitated proliferation, while non-permissive conditions promoted differentiation. Permissive condition cultures demonstrated small cell size and increased cell numbers (Fig. 1a and d), in keeping with a high proliferative phase. The morphology changed from spindle-shaped to cobblestone-shaped cells when the cells were transitioned from permissive to non-permissive conditions as illustrated (Fig. 1d and b, respectively). The number of cells decreased, individual cell size increased, and nuclear clumping and debris accumulation increased consistent with more cell death at 2 weeks’ incubation under non-permissive conditions (Fig. 1e, c, and f).

DEMs in HEI-OC1 Cells Maintained at Permissive and Non-permissive Culture Conditions

In P-HEI-OC1 cells, 402 out of 768 miRNAs tested were expressed at a mean Ct cut off level < 35.0 (Fig. 2a). Similarly, 413 and 361 miRNAs were expressed in NP1- and NP2- HEI-OC1 cells, respectively (Fig. 2a). In all three
cultures, 346 miRNAs were detected (Suppl. Tables 1 and 2).

Thirty, 28, and 21 DEMs were identified in NP1- versus (vs) P-, NP2- vs. P-, and NP2- vs. NP1- HEI-OC1 cells, respectively (Fig. 2b). All up- and downregulated DEMs are summarized (Tables 2, 3, and 4, respectively). Three miRNAs miR-1948-3p, -20a-3p, and -486a-5p were differentially expressed in all three comparisons, and among them, miR-20a-3p was commonly downregulated. Seven miRNAs hsa-miR-93-3p, hsa-miR-214-3p, -miR-186-3p, -1971, -2134, -222-3p, and -34c-3p were consistently differentially expressed in non-permissive cells compared with permissive cells (NP1- and NP2- HEI-OC1 cells). DEMs with twofold intergroup difference at \( P < 0.05 \); FDR corrected by Benjamini–Hochberg procedure are presented. Non-mouse species DEMs (rno-miR-350/-99a-3p/ and hsa-miR-l48b-5p) which were not homologues of mouse miRNAs are excluded (FC, fold change; \( P^* \), actual \( P \) value).

### Table 2

| DEMs upregulated (n=21) | FC (>2.0) | \( P^* \) value (<0.05) | DEMs downregulated (n=09) | FC (<0.5) | \( P^* \) value (<0.05) |
|------------------------|-----------|-----------------------|---------------------------|-----------|-----------------------|
| miR-146b-5p            | 10.67     | 0.0005                | miR-2134                  | 0.03      | 0.0032                |
| hsa-miR-93-3p          | 3.66      | 0.0006                | miR-466 k                 | 0.19      | 0.0133                |
| miR-872-3p             | 4.97      | 0.0032                | miR-20a-3p                | 0.23      | 0.0135                |
| miR-200c-3p            | 14.06     | 0.0033                | miR-712-5p                | 0.03      | 0.0194                |
| miR-877-3p             | 14.05     | 0.0041                | miR-466a-3p               | 0.16      | 0.0238                |
| miR-133a-3p            | 19.24     | 0.0052                | miR-1971                  | 0.11      | 0.0257                |
| miR-1948-3p            | 17.89     | 0.0057                | miR-1903                  | 0.04      | 0.0267                |
| miR-339-3p             | 4.20      | 0.0074                | miR-701-5p                | 0.15      | 0.0288                |
| miR-574-3p             | 1.07      | 0.011                 | miR-186-3p                | 0.24      | 0.0292                |
| miR-301b-3p            | 3.33      | 0.0123                |                           |           |                       |
| miR-193                | 13.47     | 0.0124                |                           |           |                       |
| miR-129-2-3p           | 4.80      | 0.0156                |                           |           |                       |
| miR-146a-5p            | 11.48     | 0.016                 |                           |           |                       |
| miR-34c-3p             | 65.36     | 0.0176                |                           |           |                       |
| hsa-miR-200c-3p        | 51.03     | 0.0191                |                           |           |                       |
| hsa-miR-214-3p         | 7.63      | 0.0192                |                           |           |                       |
| miR-199a-3p            | 3.65      | 0.0235                |                           |           |                       |
| miR-222-3p             | 9.46      | 0.025                 |                           |           |                       |
| miR-486a-5p            | 3.74      | 0.0256                |                           |           |                       |
| miR-449a-5p            | 9.23      | 0.0298                |                           |           |                       |
| miR-547-3p             | 8.75      | 0.0307                |                           |           |                       |

miR-340 (-3p/-5p) was consistently downregulated in NP2-HEI-OC1 cells. Rodent-specific maternally imprinted gene Sfmbt2 intron 10th cluster miRNAs miR-466a-3p and -467a-5p were downregulated in NP1- and NP2- HEI-OC1 cells, respectively, compared with P-HEI-OC1 cells.

DEMs with twofold intergroup difference at \( P < 0.05 \); FDR corrected by Benjamini–Hochberg procedure are presented. Non-mouse species DEMs (rno-miR-350/-146b-5p/ and hsa-miR-l48b-5p) which were not homologues of mouse miRNAs are excluded (FC, fold change; \( P^* \), actual \( P \) value).

DEMs with twofold intergroup difference at \( P < 0.05 \); FDR corrected by Benjamini–Hochberg procedure are presented. Non-mouse species DEMs (rno-miR-350/-99a-3p/ and -l48b-5p) which were not homologues of mouse miRNAs are excluded (FC, fold change; \( P^* \), actual \( P \) value).
Putative Target Genes’ Enriched Significant Functional Annotations for DEMs in Non-permissive HEI-OC1 Cells

There were 2211, 1876, and 1232 putative target genes identified for DEMs in NP⁴- compared with P-HEI-OC1 cells, NP⁵- compared with P-HEI-OC1 cells, and NP⁵- compared with NP⁴-HEI-OC1 cells, respectively. Of these putative genes, 786, 674, and 431 were recognized by KEGG pathways (Fig. 3), and 1955, 1661, and 1086 of these genes by GOBP terms (Fig. 4), respectively. Excluding cancers, mitogen-activated protein kinase (MAPK), epidermal growth factor family of receptor tyrosine kinases (ErbB), and Ras were predominantly enriched KEGG pathways in non-permissive cells when compared with permissive cells ($P < 0.0001$) (Fig. 3a and b, respectively). GOBP terms: rhythmic process, multicellular organism development, and organ morphogenesis were predominantly enriched in NP⁵-HEI-OC1 cells when compared with NP¹-HEI-OC1 cells ($P < 0.00001$) (Fig. 4c).

Validated Target Genes’ Enriched Significant Functional Annotations for DEMs in Non-permissive HEI-OC1 Cells

There were 333, 207, and 125 validated target genes identified for DEMs in NP⁴- vs. P-, NP⁵- vs. P-, and NP⁵- vs. NP⁴- HEI-OC1 cells, respectively. Of these validated genes, 134, 80, and 45 were recognized by KEGG pathways, and 301, 185, and 112 of these genes by GOBP terms, respectively (Fig. 5). Carbohydrate digestion and absorption, glutamatergic synapse, and mechanistic or mammalian target of rapamycin (mTOR) signaling were predominantly enriched KEGG pathways in NP⁴-HEI-OC1 cells when compared with NP¹-HEI-OC1 cells ($P < 0.00001$) (Fig. 5a). Signaling pathways regulating pluripotency of stem cells was the only significantly enriched KEGG pathway in NP⁵-HEI-OC1 cells when compared with P-HEI-OC1 cells ($P = 0.01$). Excluding transcription and regulation of transcription, the GOBP term nervous system development was

### TABLE 3

Significantly differentially expressed miRNAs in NP²-HEI-OC1 cells compared with P-HEI-OC1 cells

| DEMs upregulated (n=10) | FC ($> 2.0$) | $P$ value ($< 0.05$) | DEMs downregulated (n=18) | FC ($< 0.5$) | $P$ value ($< 0.05$) |
|------------------------|-------------|----------------------|---------------------------|-------------|----------------------|
| miR-1948-3p            | 4.25        | 0.009                | miR-20a-3p                | 0.07        | 0.0041               |
| miR-449b               | 4.84        | 0.0094               | hsa-miR-340-5p            | 0.07        | 0.0074               |
| hsa-miR-425-5p         | 16.22       | 0.0117               | miR-486a-5p               | 0.14        | 0.0078               |
| hsa-miR-214-3p         | 6.42        | 0.0121               | miR-2134                  | 0.06        | 0.0096               |
| rno-miR-664-3p         | 32.20       | 0.0202               | miR-340-3p                | 0.05        | 0.0204               |
| miR-34c-3p             | 17.97       | 0.0203               | miR-1971                  | 0.09        | 0.0213               |
| hsa-miR-200c-3p        | 44.58       | 0.021                | hsa-miR-33a-3p            | 0.05        | 0.0213               |
| miR-674-3p             | 3.51        | 0.0363               | miR-467a-5p               | 0.03        | 0.0237               |
| miR-222-3p             | 4.15        | 0.0449               | hsa-miR-196a-5p           | 0.29        | 0.0269               |
| miR-1943-5p            | 2.96        | 0.0476               | miR-24-2-5p               | 0.42        | 0.027               |
| miR-322-3p             | 0.17        | 0.0274               | miR-199a-5p               | 0.20        | 0.0304               |
| miR-322-5p             | 0.11        | 0.0307               | miR-322-5p                | 0.29        | 0.0369               |
| hsa-miR-106b-3p        | 0.29        | 0.0412               | miR-17-5p                 | 0.13        | 0.041               |
| miR-186-3p             | 0.03        | 0.0431               | miR-340-5p                | 0.03        | 0.0431               |
| miR-99a-5p             | 0.11        | 0.0434               | hsa-miR-106b-3p           | 0.29        | 0.0369               |

Excluding transcription and regulation of transcription, the GOBP terms dendrite morphogenesis, phosphorylation, protein phosphorylation, and nervous system development were predominantly enriched in non-permissive cells when compared with permissive cells ($P < 0.01$) (Fig. 4a and b, respectively). GOBP terms: rhythmic process, multicellular organism development, and organ morphogenesis were predominantly enriched in NP⁵-HEI-OC1 cells when compared with NP¹-HEI-OC1 cells ($P < 0.00001$) (Fig. 4c).
DEMs in NP2-HEI-OC1 cells when compared with significantly enriched for validated target gens of None of the KEGG pathways and GOBP terms was NP1-HEI-OC1 cells (P < 0.05, Bonferroni corrected Dunn’s test) when compared with NP1- and/or P- HEI-OC1 cells. Kitl8 expression was significantly increased (P < 0.01, Bonferroni corrected Dunn’s test) in NP2-HEI-OC1 cells when compared with P-HEI-OC1 cells. Zeb2 expression level was comparable across all three cultures. Sfnb2 expression was comparable between P- and NP1- HEI-OC1 cells, whereas it was not detected in NP2-HEI-OC1 cells at Ct < 40.0. Slc26a5 was relatively poorly expressed in all three cultures.

These target genes were primarily selected from the literature. Therefore, we searched their biological target miRNAs using TargetScanMouse version 7.2 (Fig. 9). Most interestingly, Atoh1 and Sfnb2 showed conserved sites that match the seed regions of miR-34a-5p/-34b-5p/-34c-5p/-449a-5p, 449b, and 449c-5p. Likewise, miR-200c-3p and -200b-3p target Sox2, Tubb3, Tubb5, Zeb1, and Zeb2. miR-30 family miRNAs -30a-5p/-30b-5p/30c-5p/-30d-5p and -30e-5p target Espn, Vim, and Zeb2. miR-196a-3p and -196b-5p target Slc26a5 and p27kip1 (Cdkn1b), miR-301a-3p and -301b-3p target Zeb1 and Zeb2, and miR-222-3p targets Zeb2 and p27kip1. Tubb1, Nes, and Kitl8 did not show conserved sites that match the seed regions of any miRNAs.

Fluorescence Immunocytochemistry (ICC) on HEI-OC1 Cells

Six protein markers myosin 7a, prestin, Sox2, nestin, e-cadherin, and vimentin were used to characterize HEI-OC1 cells maintained under permissive and non-permissive conditions, and the proportion of antibody-positive cells were analyzed using Kruskal–Wallis H test followed by Bonferroni-corrected Dunn’s post hoc test (Table 6) and presented for all three cultures (Figs. 10–12). Myosin 7a-positive apical projections were dispersed in P-HEI-OC1 cells (Fig. 10a), whereas myosin 7a-positive apical projections were densely packed in NP- and NP2- HEI-OC1 cells, respectively (Fig. 10b and c). Plasma membrane localization of prestin was identified in P-HEI-OC1 cells (Fig. 10d); however, signal was much stronger and stable in NP1-HEI-OC1 cells (Fig. 10e). Prestin expression was slightly unstable in NP3-HEI-OC1 cells (Fig. 10f) because of increasing cell’s vulnerability to multiple washing steps in the immunostaining procedures. The proportion of myosin 7a (P = 0.018, Kruskal–Wallis H test) and prestin (P = 0.009, Kruskal–Wallis H test)–positive cells differed significantly across P-, NP1-, and NP2- HEI-OC1 cell cultures (Table 6). The proportion of myosin 7a-positive cells was significantly reduced in NP2- compared to NP1-HEI-OC1 cells (P = 0.006, Bonferroni-corrected Dunn’s test) (Fig. 10g), whereas the proportion of prestin-positive cells was significantly decreased in P- compared

### Table 4

Significantly differentially expressed miRNAs in NP1-HEI-OC1 cells compared with NP1-HEI-OC1 cells

| DEMs downregulated | FC (<0.5) | P value (<0.05) |
|--------------------|----------|----------------|
| miR-34b-5p         | 0.11     | 0.0013         |
| miR-1948-3p        | 0.24     | 0.0028         |
| miR-449a-5p        | 0.06     | 0.0066         |
| miR-24-2-5p        | 0.42     | 0.007          |
| miR-140-5p         | 0.08     | 0.008          |
| miR-130a-3p        | 0.25     | 0.0092         |
| hsa-miR-744-3p     | 0.33     | 0.0101         |
| let-7d-3p          | 0.05     | 0.015          |
| hsa-miR-340-5p     | 0.13     | 0.0157         |
| miR-20a-3p         | 0.30     | 0.0162         |
| miR-486a-5p        | 0.04     | 0.0169         |
| miR-340-3p         | 0.05     | 0.0208         |
| miR-301a-3p        | 0.18     | 0.022          |
| miR-301b-3p        | 0.15     | 0.0236         |
| hsa-miR-140-3p     | 0.10     | 0.0266         |
| hsa-miR-33a-3p     | 0.18     | 0.0318         |
| miR-345-5p         | 0.49     | 0.0341         |
| miR-106a-5p        | 0.33     | 0.0354         |
| miR-199a-5p        | 0.20     | 0.0402         |
| miR-484            | 0.20     | 0.042          |
| hsa-miR-423-3p     | 0.49     | 0.0433         |

predominantly enriched in NP1-HEI-OC1 cells when compared with P-HEI-OC1 cells (P < 0.05) (Fig. 5b). None of the KEGG pathways and GOBP terms was significantly enriched for validated target gens of DEMs in NP2-HEI-OC1 cells when compared with NP1-HEI-OC1 cells (P > 0.05).

Relative Gene Expressions in Permissive and Non-permissive HEI-OC1 Cells

The relative mRNA levels of 18 target genes tested in P-, NP1-, and NP2- HEI-OC1 cells are illustrated using mRNA copies of gene of interest per 1000 Gapdh mRNA (Fig. 6). Box and Whisker plots are presented for target genes’ Ct values normalized to Gapdh across these cultures (Figs. 7 and 8), and their P values (Kruskal–Wallis H test and Dunn’s post hoc, 2-sided) are summarized (Table 5). Tubb1, Cdh1, Espn, and Sox2 mRNA levels were significantly elevated in non-permissive compared with permissive HEI-OC1 cells (P < 0.05, Bonferroni corrected Dunn’s test). Atoh1, Tubb3, Myo7a, Pou4f3, and Zeb1 expressions were significantly reduced in P-HEI-OC1 cells when compared with NP1-HEI-OC1 cells (P < 0.01, Bonferroni corrected Dunn’s test). Tubb5, Pax2, p27kip1, and Vim expressions were significantly decreased in NP2-HEI-OC1 cells (P < 0.01, Bonferroni corrected Dunn’s test) when compared with NP1- and/or P- HEI-OC1 cells.
Sox2 expression was detected in both P- and NP1-HEI-OC1 cells (Fig. 11a and b, respectively); on the other hand, Sox2 expression was diminished or disappeared in the nuclei of NP2-HEI-OC1 cells (Fig. 11c). NP1-HEI-OC1 cells contained both Sox2-positive and Sox2-negative cells. Sox2-negative cells could be considered as differentiated cells like Sox2-negative cells identified in NP2-HEI-OC1 cells. Between P- and NP1-HEI-OC1 cells, Sox2 was overexpressed or the signals were strong in NP1-HEI-OC1 cells, indicating its importance.

**Fig. 3** Significantly enriched KEGG pathways for putative target genes of DEMs in non-permissive HEI-OC1 cells. 

- **a** NP1- compared with P-HEI-OC1 cells.
- **b** NP2- compared with P-HEI-OC1 cells.
- **c** NP2- compared with NP1-HEI-OC1 cells. Significantly enriched top 15 KEGG pathways are presented excluding cancers. The number of genes involved in each pathway is labelled in each panel and the legend keys are presented at descending corrected P values (cut-off P < 0.05).
during hair cell differentiation. Nestin expression was strong in the nuclei of both P- and NP⁺- HEI-OC1 cells (Fig. 11d and e, respectively), whereas nestin expression was predominantly detected in the cytoplasm of differentiated NP⁻-HEI-OC1 cells (Fig. 11f). Sox2 expression was significantly decreased in NP⁻- compared with NP⁺- HEI-OC1 cell cultures ($P = 0.001$, Bonferroni-corrected Dunn’s test), whereas nestin expression was comparable across P-, NP⁺-, and NP⁻- HEI-OC1 cell cultures ($P = 0.96$, Kruskal–Wallis H test) (Table 6). The proportion of Sox2- and nestin-positive cells with respect to total number of DAPI-stained nuclei is presented (Fig. 11g and h).

Vimentin, a mesenchymal cell marker, was comparably expressed in all three HEI-OC1 cell cultures (Fig. 12a–c, respectively), and its semi quantification was not significantly different ($P = 0.077$, Kruskal–Wallis H test) among these cells (Fig. 12g). E-cadherin is an epithelial cell marker, involved in cell–cell adhesion. E-cadherin expression was comparatively weak in P-HEI-OC1 cell cultures (Fig. 12d). Due to increasing cell death and vulnerability to staining steps, remnants of e-cadherin protein were identified in non-permissive cells (Fig. 12e and f, respectively), and therefore, protein semi quantification was not carried out.

**Fig. 4** Significantly enriched GOBP terms of putative target genes of DEMs in non-permissive HEI-OC1 cells. a NP⁺- compared with P-HEI-OC1 cells. b NP⁻- compared with P-HEI-OC1 cells. c NP⁻- compared with NP⁺-HEI-OC1 cells. Significantly enriched top 15 GOBP terms are presented. The number of genes involved in each pathway is labelled in each panel and the legend keys are presented at descending corrected $P$ values (cut-off $P<0.05$)
Fig. 5  Significantly enriched functional annotations for validated target genes of DEMs in non-permissive HEI-OC1 cells. A KEGG pathways in NP1- compared with P-HEI-OC1 cells. B GOBP terms in NP1- compared with P-HEI-OC1 cells. The number of genes involved in each pathway is labelled in each panel, and the legend keys are presented at descending corrected P values (cut off P<0.05).

Fig. 6  Relative mRNA levels of target genes expressed in P-, NP1-, NP2-HEI-OC1 cells. Genes encoding for Atoh1, Tubb3, Tubb5, Cdh1, Espn, Myo7a, Nes, p27kip1, Pax2, Pou4f3, Slk26a5, Slmbt2, Sox2, Krt18, Vim, Zeb1, and Zeb2 are presented using mRNA copies gene of interest per 1000 Gapdh mRNA in log2. Bar graph was generated with GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA).
DISCUSSION

Inner ear tissue differentiation and maintenance are regulated and controlled by conserved sets of cell-specific miRNAs (Friedman et al. 2009). Here, we demonstrated differences in the miRNA signature of undifferentiated and differentiated HEI-OC1 cells.

Putative target genes of the DEMs in non-permissive HEI-OC1 cells revealed that MAPK, ErbB, and Ras signaling pathways were the predominantly significantly enriched KEGG pathways in differentiated HEI-OC1 cells (Fig. 3a and b). ErbBs are widely expressed in varying degrees in overlapping populations of sensory and non-sensory cells within the neonatal and adult inner ear (Hume et al. 2003). Hume et al. (2003) suggest that the expression of the ErbBs in supporting cells, hair cells, and non-sensory cells are potentially involved in the regulation of multiple processes including survival, synaptic maintenance, and cochlear homeostasis, in addition to a role in proliferation. In this current study, miRNA signature of non-permissive HEI-OC1 cells confirmed the functional enrichment of ErbB signaling pathway when compared with permissive HEI-OC1 cells. In addition to ErbB, Ras and MAPK signaling pathways were also enriched in non-permissive cells. Ras/MAPK pathway is essential in the regulation of cell cycle, differentiation, growth, and cell senescence, all of which are critical to normal growth and development (Tidyman and Rauen 2009). Haque et al. (2016), for the first time, showed that mitogen-activated protein 3 kinase 4 (MEKK4) signaling is highly regulated during inner ear development and is critical to normal cytoarchitecture and function as deficient mice exhibit a significant reduction of hair cells and hearing loss. Meanwhile, FoxO signaling was predominantly enriched in NP2-HEI-OC1 cells when compared with NP1-HEI-OC1 cells (Fig. 3c), suggesting the activation of cellular physiological events such as apoptosis with increasing incubation period.

DEMs miR-200-3p and miR-34c-3p were significantly and consistently upregulated in non-permissive cultures. The miR-200 family has been shown to inhibit epithelial to mesenchymal transition, by maintaining the epithelial phenotype through direct targeting of transcriptional repressors of e-cadherin (Cdh1), Zeb1, and Zeb2 (Korpali and Kang 2008). Cdh1 expression was significantly elevated in both NP1- and NP2- HEI-OC1 cells compared with P-HEI-OC1 cells (P≤0.001 and P=0.01 respectively, Bonferroni-corrected Dunn’s test)
Sox2 is also targeted by miR-200c-3p (Fig. 9). Sox2, an important transcription factor, plays multiple roles, most prominently in cellular reprogramming and stem cell pluripotency. In addition, Sox2 is considered as a marker of the prosensory domain in the developing cochlea from which the cochlear and vestibular epithelia develop (Kiernan et al. 2005; Hume et al. 2007). Kempfle et al. (2016) reported that Sox2 is required in the cochlea to both expand progenitor cells and initiate their differentiation into hair cells. This is supported by our study where fluorescence signals obtained for Sox2-positive cells were very strong in NP1- compared with P- HEI-OC1 cells (Fig. 11a and b). Furthermore, Sox2 expression was significantly elevated in both NP1- and NP2- HEI-OC1 cells (P=0.002 and P=0.001, respectively, Bonferroni-corrected Dunn’s test) compared with P-HEI-OC1 cells (Table 5), whilst Sox2 protein expression was significantly (P=0.001, Bonferroni-corrected Dunn’s test) decreased in NP2- compared with NP1- HEI-OC1 cells (Table 6). Low levels of Sox2 protein expression despite high levels of Sox2 gene expression in the presence of miR-200c-3p upregulation in NP2-HEI-OC1 cells is consistent with miR-200c-3p’s inhibition of Sox2 protein synthesis by translational repression. It is likely that the effect of miR-200c-3p upregulation is sub-maximal in NP1-HEI-OC1 cell
cultures which have not fully transitioned to a predominant differentiated cell culture as reflected by the presence of a mix of Sox2 antibody–positive and Sox2 antibody–negative cells.

Moreover, Tubb3 and Tubb5 are targets of miR-200c-3p (Fig. 9) based on their seed region homology. Microtubules are elaborated in a specific temporal pattern in the development of gerbil post-mitotic organ of Corti (Hallworth and Ludueña 2000; Hallworth et al. 2000). Jensen-Smith et al. (2003) describe that in the adult organ of Corti, each of the five major cell types synthesize a different subset of tubulin isotypes. To be specific, inner hair cells synthesize only Tubb1 and 2, while outer hair cells make Tubb1 and 4. Only Tubb2 and 4 are found in both inner and outer pillar cells, while Tubb1, 2, and 4 are present in Deiters cells, and Tubb1, 2, and 3 are found in organ of Corti dendrites. Tubb3 is commonly referred as the “neuron-specific” tubulin; however, Stone and Rubel (2000) reported Tubb3 in both mature and regenerating chick cochlea hair cells. We tested 3 tubulin isotypes: genes Tubb1, 3, and 5 in P-, NP1-, and NP2- HEI-OC1 cells. Tubb1 expression was significantly elevated in both NP1- and NP2-HEI-OC1 cells (P<0.001 and P=0.003, respectively, Bonferroni-corrected Dunn’s test) compared with P- HEI-OC1 cells (Table 5), suggesting the presence of mature hair cells and/or organ of Corti dendrites in non-permissive cultures. Tubb3 expression which is a mature neuron marker was elevated in NP1- but not NP2- when statistically compared with P-HEI-OC1 cells (Table 5). This is consistent with putative and validated genes targeted by DEMs in NP1- HEI-OC1 cells being predominantly overexpressed in biological processes: dendrite morphogenesis and nervous system development, respectively (Figs. 4a and 5b). Tubb5 a target of miR-200c-3p a DEM in non-permissive cells was significantly reduced in NP2- compared with NP1- and P-HEI-OC1 cells (P=0.002 and P<0.001, respectively, Bonferroni-corrected Dunn’s test) (Table 5). Reduced level of Tubb5 expression in NP2-HEI-OC1 cells suggests the inhibitory role of upregulated miR-200c-3p in differentiated HEI-OC1 cells possibly via mRNA

| Target genes | Normalized mean Ct (± SD) | Kruskal–Wallis H test NP1- vs. P- | NP2- vs. P- | NP2- vs. NP1- |
|--------------|--------------------------|-------------------------------|----------------|----------------|
|              | P-             | NP1-            | NP2-            |
| Atoh1        | 15.12 (0.93)  | 6.85 (0.74)  | 7.25 (0.25)  | 0.013         | 0.005**         | 0.066         | 0.834         |
| Tubb1        | 14.2 (0.26)   | 6.27 (0.62)  | 6.72 (0.98)  | <0.001        | <0.001***       | 0.003**       | 0.342         |
| Tubb3        | 8.07 (0.15)   | 4.51 (0.54)  | 6.3 (1.03)   | <0.001        | <0.001***       | 0.036         | 0.056         |
| Tubb5        | -0.40 (0.39)  | -0.91 (2.35) | 4.58 (3.26)  | <0.001        | 0.423           | 0.002**       | <0.001**      |
| Cdh1         | 13.6 (0.25)   | 5.91 (1.12)  | 7.4 (0.81)   | <0.001        | <0.001***       | 0.010*        | 0.086         |
| Espn         | 12.81 (0.45)  | 8.37 (2.69)  | 9.19 (0.01)  | 0.004         | 0.002**         | 0.015*        | 0.642         |
| Myo7a        | 13.07 (0.81)  | 8.13 (0.7)   | 12.25 (0.22) | 0.001         | <0.001***       | 0.509         | 0.105         |
| Pou4f3       | 14.61 (0.49)  | 6.68 (1.18)  | 7.3 (0.91)   | 0.003         | 0.002**         | 0.025         | 0.566         |
| Slc26a5      | n/d           | n/d            | n/d            | n/a           | n/a             | n/a           | n/a           |
| Sox2         | 16.07 (0.59)  | 7.48 (1.54)  | 6.63 (0.32)  | 0.001         | 0.002**         | 0.001**       | 0.570         |
| p27kip1      | 5.98 (0.57)   | 1.91 (1.23)  | 8.46 (0.47)  | 0.007         | 0.117           | 0.117         | 0.002**       |
| Ptx2         | 5.42 (0.83)   | 2.84 (1.48)  | 10.8 (2.23)  | <0.001        | 0.029           | 0.104         | <0.001***     |
| Krt18        | 18.47 (0.25)  | 14.97 (0.42) | 13.06 (0.75) | 0.022         | 0.167           | 0.007**       | 0.070         |
| Nes          | 4.58 (0.42)   | 2.12 (0.99)  | 6.24 (1.21)  | <0.001        | 0.019           | 0.114         | <0.001***     |
| Sfrs1        | 9.73 (0.21)   | 8.87 (1.56)  | n/d           | 0.099         | n/a             | n/a           | n/a           |
| Vim          | -0.70 (1.4)   | -2.47 (1.72) | 3.26 (1.31)  | <0.001        | 0.070           | 0.007**       | <0.001***     |
| Zeb1         | 7.02 (0.48)   | 5.11 (0.83)  | 6.08 (1.81)  | 0.001         | <0.001***       | 0.026         | 0.147         |
| Zeb2         | 5.84 (0.37)   | 3.77 (1.85)  | 5.94 (0.4)   | 0.030         | 0.019           | 0.735         | 0.044         |

Normalized mean Ct values were compared using non-parametric Kruskal–Wallis H test (2-sided) followed by Dunn’s post hoc test. Significantly different inter-group differences in normalized mean Ct values expressed at a Bonferroni adjusted for multiple comparisons significance level of P<0.05 indicated as <0.05*, <0.01**, <0.001***

CT cycle threshold, SD standard deviation, actual P value (P*), not determined (n/d), not applicable (n/a)
In addition, \textit{Espn}, which is required for the growth of hair cell stereocilia (Zheng et al. 2014), was significantly increased in NP1- and NP2- compared with P-HEI-OC1 cells ($P = 0.002$ and $P = 0.015$, respectively, Bonferroni-corrected Dunn’s test). miR-30 family
miRNAs and miR-384-5p target \textit{Espn} (Fig. 9). Though it is not statistically significant, miR-384-5p expression was downregulated (FC $\leq 0.1$) in non-permissive cells (Suppl. Table 2).

The miR-34 (a/b/c) and miR-449 (a/b/c) families are two functionally related miRNA clusters (Bao et al. 2012; Lize et al. 2011, 2010). Simultaneous inactivation of miR-34b/c and miR-449 is reported to disrupt their target genes involved in cell fate control, brain development, and microtubule dynamics in mice (Wu et al. 2014). In our study, miR-34c-3p and -449a-5p, and miR-34c-3p miR-449b were significantly upregulated in NP1- and NP2- HEI-OC1 cells (Tables 2 and 3). This observation suggests the possible role of upregulated miR-34 and miR-449 family members in microtubule dynamics and ciliogenesis of differentiated HEI-OC1 cells. However, a search of TargetScan failed to identify Tubb1, Tubb3, Tubb5, Zeb1, and Zeb2 with miR-34a-5p/-b-5p/-c-5p/-449a-5p, -449b, and -449c-5p were found to target Atoh1 with miR-126a-5p and -448-3p (dark grey); Smibt2 and Zeb2 with miR-92a-3p/-92b-3p/-344d-3p/-344e-3p/-25-3p/-32-5p/-137-3p/-363-3p/-367-3p and -410-3p (light purple); Zeb1 and Zeb 2 with miR-200a-3p/-34a/-34b/-34c/-449a-5p, 449b and 449c-5p (light grey); Pax2 and Pou4f3 with miR-196a-5p and miR-196b-5p (light red); Pax2, Sox2, and Zeb2 with miR-218-5p (dark pink); Pax2, Sox2, and Zeb2 with miR-129-5p (dark green); Atoh1, e-cadherin (Cdh1), and Smibt2 with miR-330-3p.1 (gold-enrold); Tubb5, Zeb1 and Zeb2 with miR-369-3p (olive); Smibt2, Vim and Zeb2 with miR-138-5p (purple); p27\(^{\text{Kip1}}\) and Zeb2 with miR-377-3p/-222-3p and -221-3p (light grey); Pax2 and Pou4f3 with miR-126a-5p and -448-3p (dark grey); Smibt2 and Zeb2 with miR-92a-3p/-92b-3p/-344d-3p/-344e-3p/-25-3p/-32-5p/-137-3p/-363-3p/-367-3p and -410-3p (light purple); Zeb1 and Zeb 2 with miR-200a-3p/-183-5p/-183-5p.2/-101a-3p.1/-101a-3p.2/-101b-3p.1/-101b-3p.2/-130a-3p/-130b-3p/-301a-3p/-301b-3p/-139-5p/-141-3p/-142a.3p.1/-144-3p/-205-5p and -6715-5p (light pink); Vim and Zeb2 with miR-320-3p (maroon); Tubb5 and p27\(^{\text{Kip1}}\) with miR-455-5p (light blue); Smibt2 and Zeb1 with miR-128-3p (dark blue); Pax2 and Zeb2 with miR-153-3p (lavender); and Slc26a5 and Zeb2 with miR-499-5p (dark red) were shared conserved sequences.

Source nodes were genes (turquoise, rectangle) and the target nodes were miRNAs (different colors, ellipse).
in NP1-HEI-OC1 cells (P = 0.005, Bonferroni-corrected Dunn’s test) compared with P-HEI-OC1 cells (Table 5).

The increased level of Atoh1 expression with a parallel increase of miR-34c-3p/-449a-5p in NP1-HEI-OC1 cells (Table 1) is consistent with the miR-34/449 family’s role in promoting epithelial cell differentiation (Otto et al. 2017). However, the mechanism by which miR-34/-449 family achieves this effect in HEI-OC1 cells needs further investigation.

Several of the miRNAs implicated in mouse 3′UTR evolution derive from a single rapidly expanded rodent-specific miRNA cluster located in the intron of Sfmbt2, a maternally imprinted polycomb gene. These miRNAs are expressed in both embryonic stem cells and the placenta (Zheng et al. 2011). miR-297 s, miR-466 s, miR-467 s, and miR-669 s fall into the Sfmbt2 miRNA cluster in the 10th intron of chromosome 2, based on sequence similarity (Zheng et al. 2011). miR-467a an abundant member of the Sfmbt2 cluster promotes cell proliferation, and the remaining members of this cluster are enriched in pathways that regulate cellular growth (Zheng et al. 2011). In our study, Sfmbt2 expression was found comparable between P- and NP1-HEI-OC1 cells, whereas it was not determined in NP2-HEI-OC1 cells (Fig. 8). Significantly downregulated miR-466a-3p in NP1-HEI-OC1 cells and miR-467a-3p in NP2-HEI-OC1 cells suggest the changes in cellular growth and lack of proliferation, respectively. As like Atoh1, Sfmbt2 showed conserved sequences that match the seed regions of miR-34 and miR-449 family miRNAs. Therefore, we propose that the coordinated regulation of Atoh1, Sfmbt2, and miR-34/-449 family miRNAs could play a vital role in HEI-OC1 cell proliferation and differentiation. In addition, miR-17 family miRNAs -17-5p and -20a-3p and its paralogous -106a-5p were significantly downregulated in NP1-HEI-OC1 cells (Tables 3 and 4). Downregulation of these miRNAs has been reported to be associated with ageing and senescence (Hackl et al. 2010).

| Target protein | Mean (± SD) | P- | NP1. | NP2. |
|---------------|------------|----|------|------|
| Myosin 7a     | 0.42 (0.06)| 0.58 (0.25)| 0.27 (0.12)|
| Prestin       | 0.05 (0.05)| 0.41 (0.21)| 0.39 (0.24)|
| Sox2          | 0.19 (0.08)| 0.56 (0.27)| 0.00   |
| Nestin        | 0.84 (0.15)| 0.86 (0.14)| 0.79 (0.22)|
| Vimentin      | 0.88 (0.16)| 0.90 (0.22)| 0.67 (0.23)|

SD standard deviation, actual P value (P), not applicable (n/a)
HEI-OC1 cells compared to permissive HEI-OC1 cells (Fig. 10d-f) reflecting a significantly higher level of prestin protein in both NP1- and NP2-HEI-OC1 cells, respectively. It is notable that miR-196a-5p and miR-322-5p that target Slc26a5 are differentially downregulated in NP2-HEI-OC1 cells which is consistent with a reduction in miRNA inhibition of prestin gene function and can partly explain the relative abundance of prestin in differentiated HEI-OC1 cells. Downregulation of these miRNAs may not be measurably significant in NP1-HEI-OC1 cells which have not fully transitioned.

Kalinec et al. (2016b) consider 2 weeks under non-permissive conditions the minimum time to achieve cultures of predominantly differentiated HEI-OC1 cells and that under that time there will still be a high level of undifferentiated cells. It is also important to note that miR-196a/b targets Slc26a5 and p27kip1 (Fig. 9) and their interactions in HEI-OC1 cells require further investigation.

There are some limitations to this study. The number of viable cells reduced considerably under non-permissive conditions, necessitating the adoption of
a semi-quantitative immunofluorescence approach to determine the protein expression levels under different culture conditions. In addition, the treated surfaces of the chamber slides (Lab Tek, Permanox TC Surface) used for fluorescence staining could have induced hair cell differentiation, resulting in inconsistencies between the gene and protein expression findings (Wijesinghe et al. 2021a, b; Liu et al. 2016). COVID-19 pandemic restrictions on lab access and reagents/laboratory supplies prevented PCR prestin optimization with multiple primer sets. In future work, we aim to explore the impact of the DEM changes on protein expressions under permissive and non-permissive culture conditions.

Despite these limitations, the distinct miRNA signature of differentiated HEI-OC1 cells could help in understanding miRNA-mediated cellular responses in the adult cochlea. Our findings suggest the potential mRNA-miRNA interactions that could be used in future inner ear hair cell regeneration and therapeutic studies.
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Author Contribution DAN and PW generated the idea; DAN secured grant funding; DAN, PW, and CG designed the study; PW performed the miRNA assays and cell culture experiments; PW and CG performed RNA and bioinformatic analyses; PW drafted the paper; DAN and CG critically revised the paper.

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Availability of Data and Material Data transparency will be maintained if required.

Fig. 12 Immunofluorescence staining of EMT markers in permissive and non-permissive HEI-OC1 cells. a, b, and c Vimentin, a mesenchymal cell marker identified in P-, NP1-, and NP2-HEI-OC1 cells, respectively. d, e, and f Weak signals obtained for cell–cell adhesion marker e-cadherin in P-, NP1-, and NP2-HEI-OC1 cells, respectively. g Proportion of vimentin-positive cells in P-, NP1-, and NP2-HEI-OC1 cells, respectively (error bars indicate standard deviation). DAPI was used to stain the nuclei. Phase contrast microscopic images are presented with scale bar. Proportions of antibody-stained cells (positive) were compared using non-parametric Kruskal–Wallis H test (2-sided) followed by Dunn’s post hoc test. Significant inter-group differences in the proportion of positive cells expressed at a Bonferroni adjusted for multiple comparisons significance level of \( P < 0.05 \) indicated as < 0.05*, < 0.01**, and < 0.001***.

Ethics Approval Biosafety Committee of the University of British Columbia, Vancouver, Canada.

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