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Supplemental Information

Enhanced Inner-Ear Organoid Formation from Mouse Embryonic Stem Cells by Photobiomodulation

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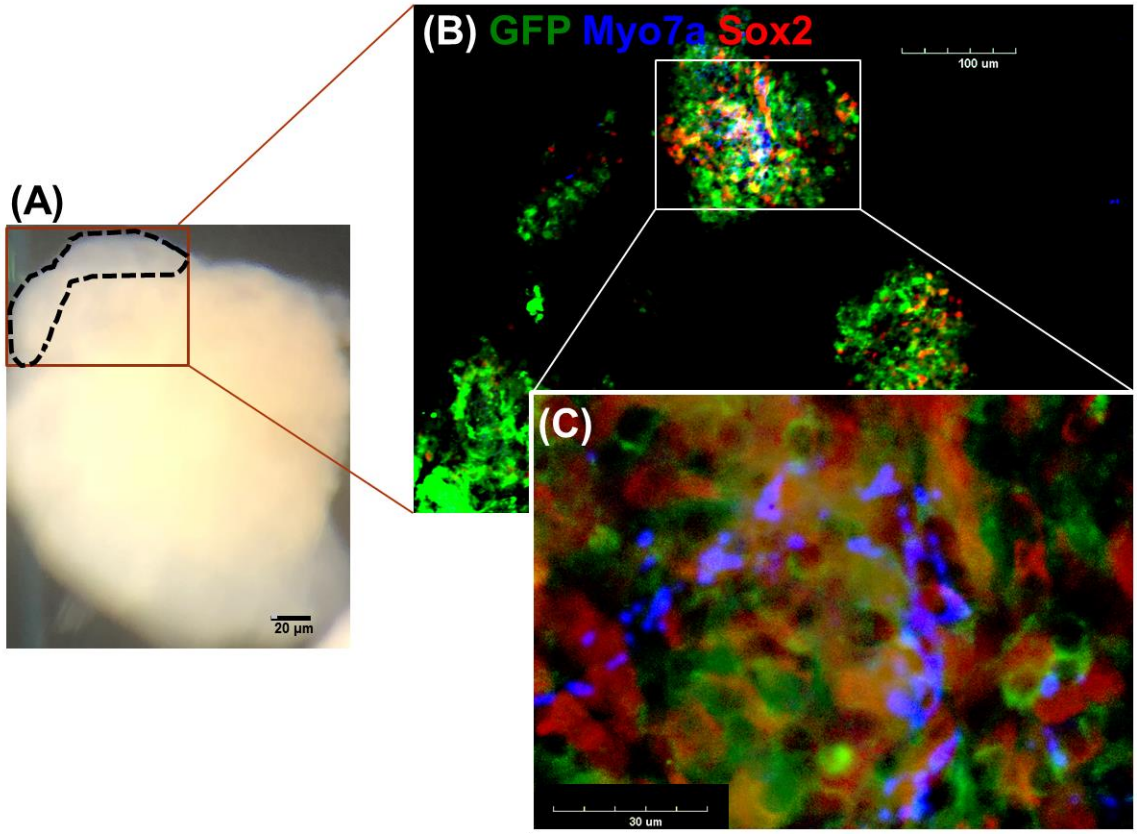
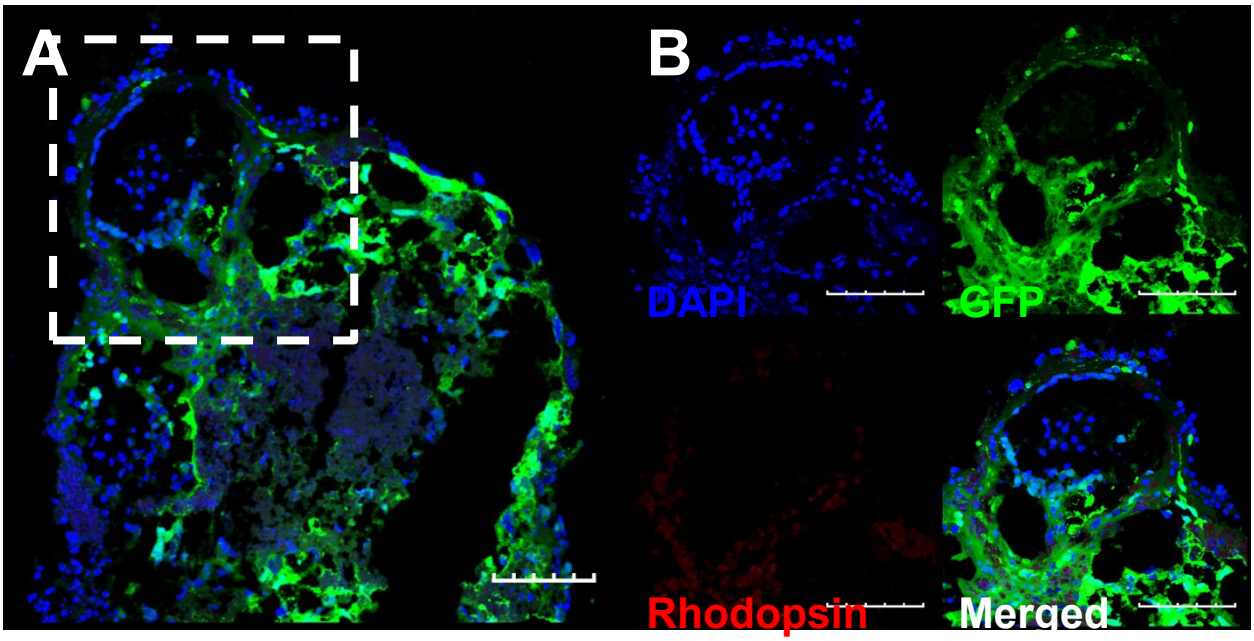
SUPPLEMENTARY FIGURES



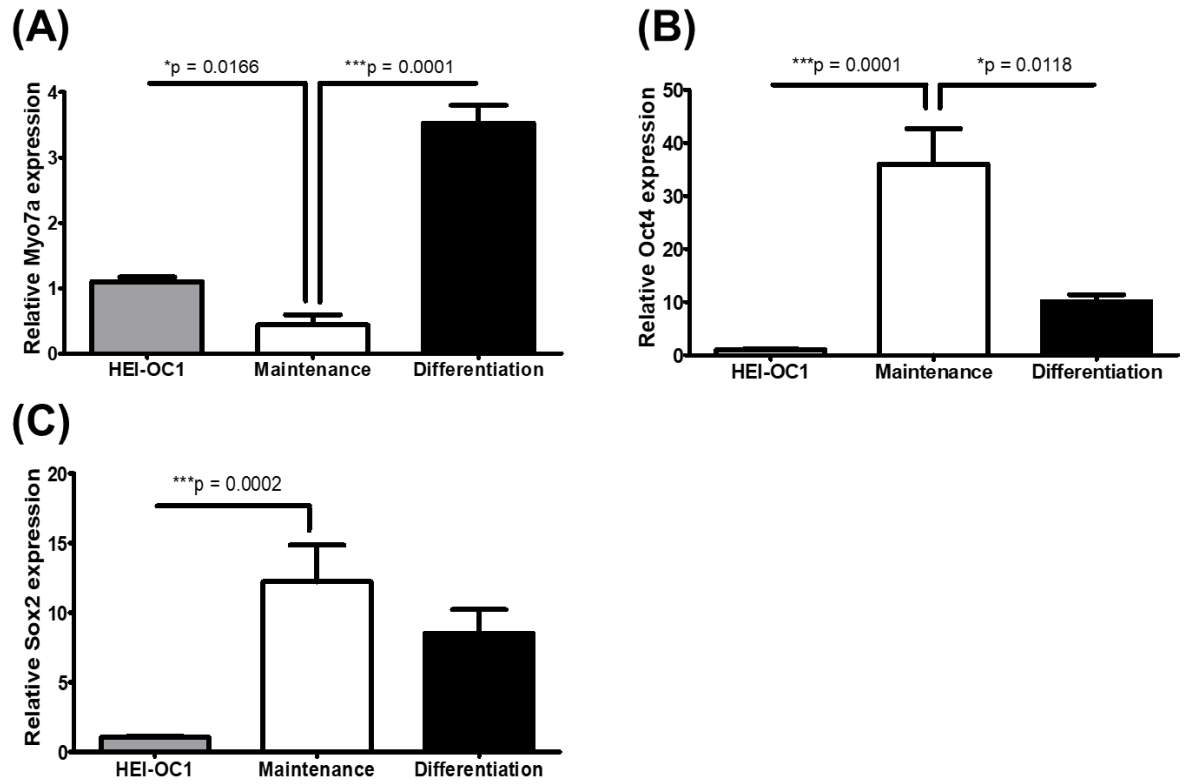
Supplementary Figure 1. Observation of MyosinVIIa positive cells in the organoid (mounted after dissection)

At 18 day of differentiation, tissues were fixed and prepared for the epifluorescence analysis. Image (A) is showing the organoids at the time point of histologic analysis. Tissues within the broken line of Image (A) is dissected to expose the inner surface of organoid and then mounted with the inner surface on top of slide glass. In the epifluorescence analysis of the tissue, Myosin VIIa positive cells (blue) and Sox2 positive cells (red) inside the organoid was observed at the lower (B) and higher magnification (C). Scale bars in each image are different size, size are marked in each separate images.



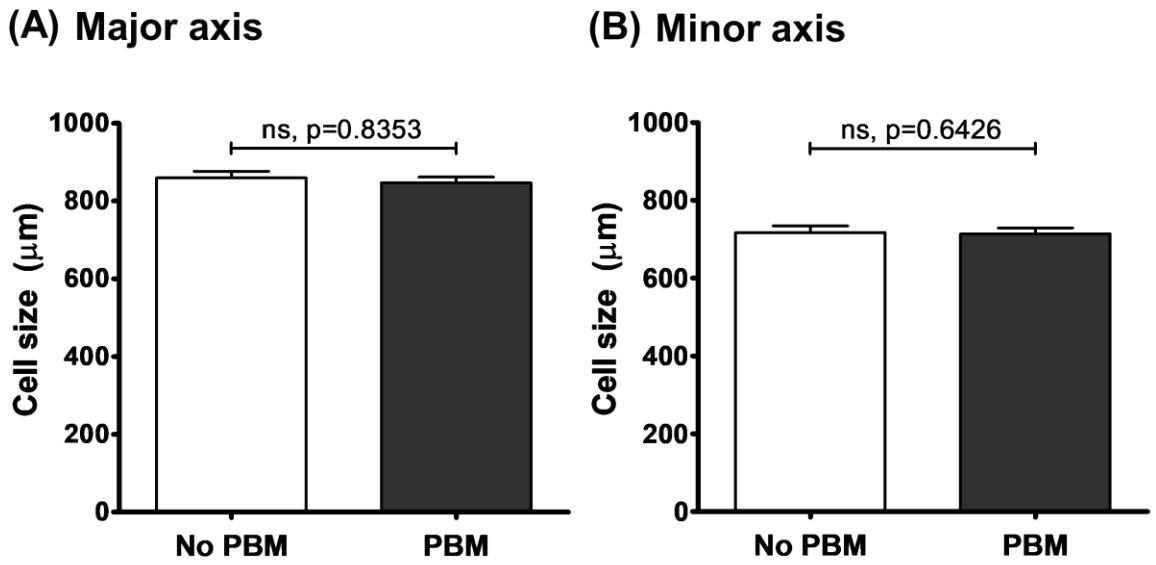
Supplementary Figure 2. Epifluorescence analysis of organoid showing Rhodopsin positive cell

Rhodopsin staining was done to exclude the possibility of PBM inducing ESCs differentiating into other Myo7a positive expressing cells such as the photoreceptor progenitors in the optic cup. (A) Low magnification showing the EB with organoids and (B) high magnification images (dotted rectangle in low magnification) showing the absence of possible photoreceptor progenitors. Scale bars indicate 100µm.



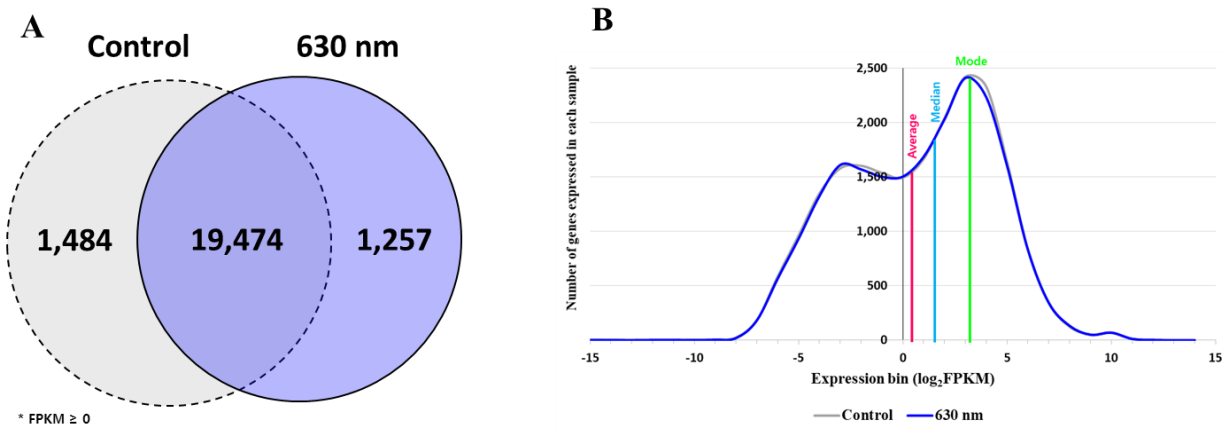
Supplementary. Figure 3. Detection of Myosin VIIa, Oct4 and Sox2 with real-time polymerase chain reaction (RT PCR)

Using the RT-PCR test, DNA expression of markers of stem cell, hair cell was observed. Statistically higher ratio of Myosin VIIa DNA expression was observed in both HEI-OC1 cell (Gray; auditory cell line) and differentiated (Black; day 18) organoids compared to maintenance (prior to differentiation process; white) (A). Statistically higher ratio of Oct4 DNA expression compared to the two other cell conditions was observed in maintenance (B). Significant higher rate of Sox2 DNA expression compared to HEI-OC1 cell was observed in maintenance stem cells (C). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.



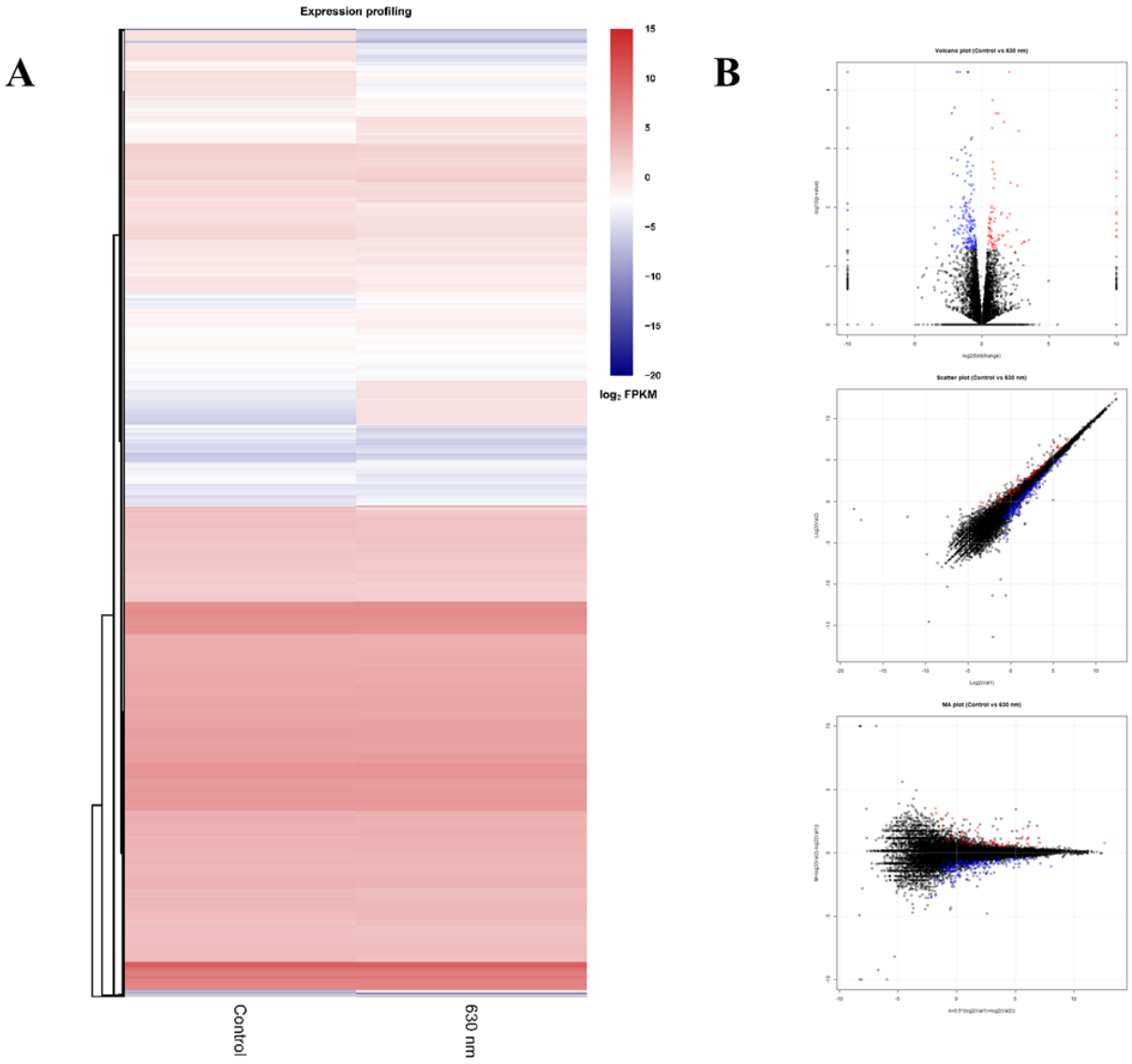
Supplementary. Figure 4. Diameters of EBs at 14 days after initiation of otic differentiation

Major axis (longest diameter, A) and minor axis (shortest diameter, B) were respectively measured in both PBM and no- PBM group. Both major and minor axis were not significantly different between PBM and no-PBM group (Major axis: two tailed Man Whitney test: U = 433.5 p = 0.8353; Minor axis: two tailed Man Whitney test: U = 416.5 p = 0.8353).



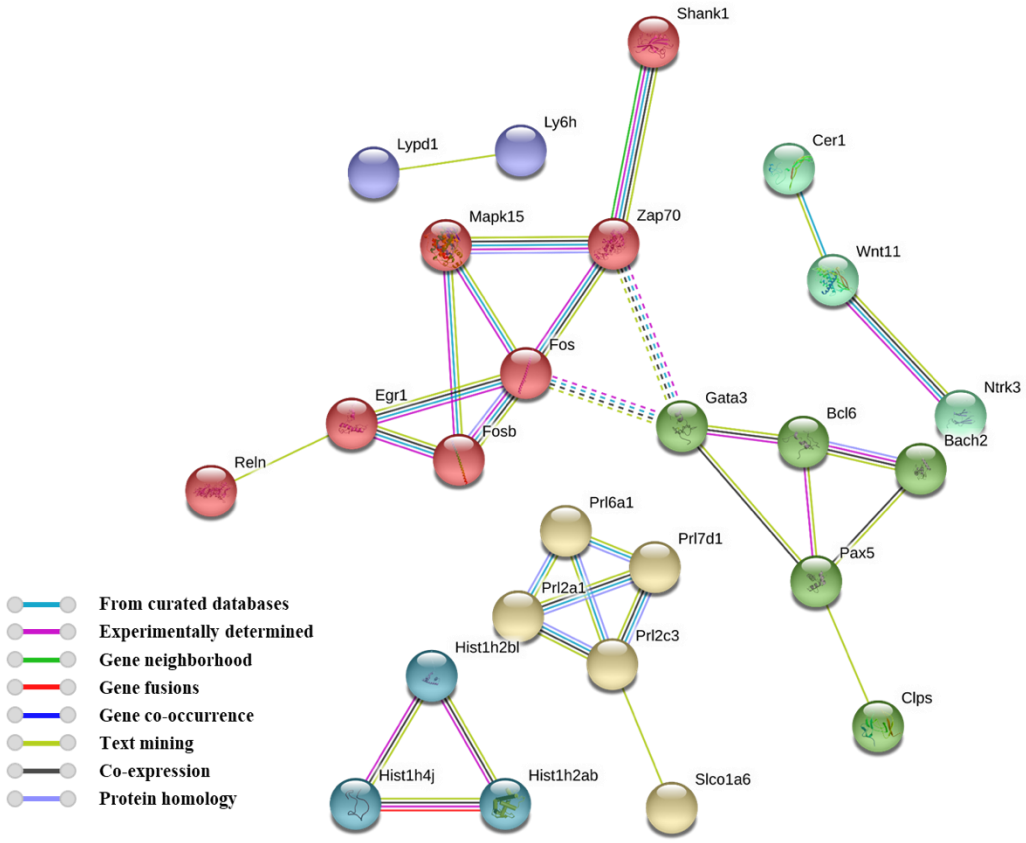
Supplementary Figure 5. Bioinformatic analysis for gene expression profile

(A) Summary of total expressed genes in two cases of inner ear hair cell-like cell (Control and 630 nm). The number of unique and shared genes from two cases of EB represented in the Venn diagram. A total of 19,474 genes are commonly expressed in all cases. (B) The comparison diagram of gene expression level. Distribution of FPKM values for the expressed genes in Control and 630 nm represented with grey and blue curve, respectively. An average, median, and mode value of FPKM are indicated by green, sky, pink lines, respectively. The distribution density was similar for two samples.



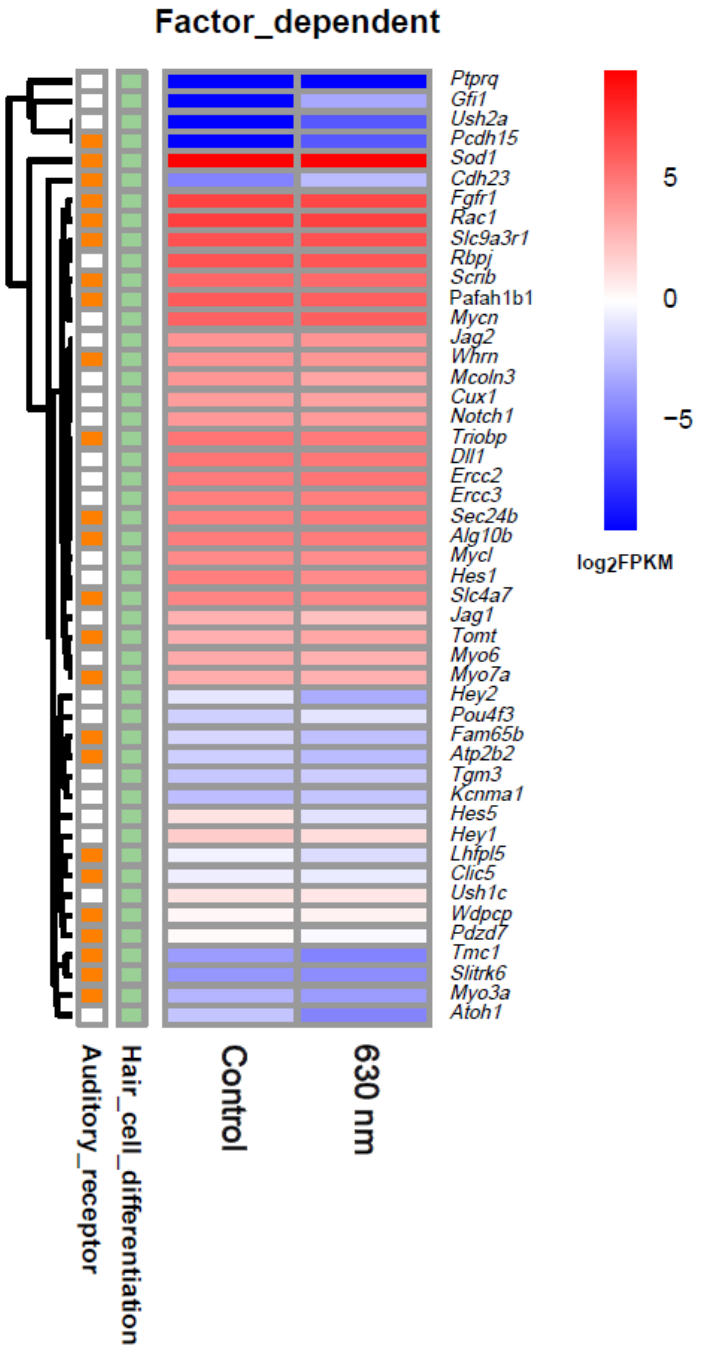
Supplementary Figure 6. Hierarchical clustering analysis of all expressed genes (22,215) and statistical comparison of DEGs

(A) (B) The FPKM volcano, scatter, and MA plots constructed by pairwise comparison between Control and 630 nm. Red and blue dots in this statistical analysis represent the statistically significant up- and down-regulated DEGs. The middle line in black dots indicated no difference at the mean expression values between samples. The number of down-regulate DEGs were higher than up-regulate DEGs in PBM-irradiated EBs.



Supplementary Figure 7. Protein–protein interaction network based on DEGs

The network containing 25 out of 43 identified proteins was mapped by the STRING: functional protein association networks analysis based on various evidence. Each evidences are indicated by different line colors as shown in the legend. In the evidence view, nodes and edged indicated genes and the interaction between the nodes. As shown, most of the proteins in this network were found to be closely related to each other.



Supplementary Figure 8. Gene expression profiling of reference gene related with hair cell sensory system

Gene expression profiling of the auditory receptor and hair cell differentiation-related genes obtained from the Amigo2 database was performed. Log_2-transformed FPKM values were used for creating the heatmap. As shown in the figure, hair cell sensory system-related genes showed similar pattern of transcriptional expression. A detailed gene expression value for these genes was annotated at Supplementary Table 4

Supplementary Table 1. Summary statistics for RNA sequencing data

Case	Raw data	After filtering (%)	Mapped reads (%)	Uniquely mapped reads (%)	Ensembl 77 (40,993 coding genes) Mus musculus	
					Expressed genes (FPKM > 0)	Unexpressed genes
Con	58,667,412	56,952,536 (97.1%)	53,233,180 (93.5%)	50,054,468 (87.9%)	20,958	20,035
630 nm	55,266,616	53,390,754 (96.6%)	49,641,737 (93%)	46,558,351 (87.2%)	20,731	20,262

Supplementary Table 2. Expression level of 155 DEGs (attached as separated excel file)

Supplementary Table 3. Gene ontology analysis for DEGs using DAVID functional annotation tool v6.8 (https://david-d.ncifcrf.gov)

	Category	Term	Count	*P*-Value	Genes
Up regulated	Biological Process	GO:0006366~transcription from RNA polymerase II promoter	4	0.0002	EGR1, FOS, NMI, FOSB
		GO:0051412~response to corticosterone	2	0.0184	FOS, FOSB
		GO:0032570~response to progesterone	2	0.0262	FOS, FOSB
		GO:0007616~long-term memory	2	0.0297	EGR1, SHANK1
		GO:0032870~cellular response to hormone stimulus	2	0.0408	FOS, FOSB
		GO:0051591~response to cAMP	2	0.0442	FOS, FOSB
		GO:0071277~cellular response to calcium ion	2	0.0459	FOS, FOSB
		GO:0035914~skeletal muscle cell differentiation	2	0.0476	EGR1, FOS
	Cellular Component	GO:0005654~nucleoplasm	6	0.0119	EGR1, TEX12, FOS, NMI, HIST1H2BL, HIST1H4J
	Molecular Function	GO:0003690~double-stranded DNA binding	3	0.0059	EGR1, FOS, FOSB
		GO:0003677~DNA binding	6	0.0160	HIST1H2AB, EGR1, FOS, HIST1H2BL, FOSB, HIST1H4J
		GO:0001077~transcriptional activator activity, RNA polymerase II core promoter proximal region sequence-specific binding	3	0.0219	EGR1, FOS, FOSB
		GO:0007399~nervous system development	7	0.0020	NTRK3, CER1, FEZF1, APOB, HES5, GATA3, PAX5

Down regulated	Biological Process	GO:0010628~positive regulation of gene expression	7	0.0026	NTRK3, APOB, GATA3, WNT11, SOX17, CTSH, QK
		GO:0006805~xenobiotic metabolic process	3	0.0031	CYP1B1, CYP26A1, SLCO1A6
		GO:0006508~proteolysis	8	0.0040	NRIP3, AGBL3, CTSJ, RELN, PGA5, TMPRSS12, CTSH, PRSS12
		GO:0001764~neuron migration	4	0.0093	NTRK3, FEZF1, GATA3, RELN
		GO:0000122~negative regulation of transcription from RNA polymerase II promoter	8	0.0131	FEZF1, BACH2, HES5, GATA3, MAPK15, PAX5, BCL6, SOX17
		GO:0072086~specification of loop of Henle identity	2	0.0139	HES5, IRX2
		GO:0045893~positive regulation of transcription, DNA-templated	7	0.0149	FEZF1, HES5, GATA3, PAX5, LHX5, WNT11, SOX17
		GO:0030178~negative regulation of Wnt signaling pathway	3	0.0163	CER1, CXXC4, SOX17
		GO:0045061~thymic T cell selection	2	0.0173	GATA3, ZAP70
		GO:0071300~cellular response to retinoic acid	3	0.0216	NTRK3, LTK, CYP26A1
		GO:0006355~regulation of transcription, DNA-templated	15	0.0231	BACH2, IRX2, PAX5, FOXO6, FEZF1, HES5, GATA3, ZFP300, LHX5, BCL6, GM12258, SOX17, SP8, FOXD4, NHLH1
		GO:0095500~acetylcholine receptor signaling pathway	2	0.0241	LYPD1, LY6H
		GO:0007275~multicellular organism development	9	0.0258	NTRK3, FEZF1, HES5, PAQR7, PAX5, WNT11, RELN, NHLH1, QK
		GO:0031638~zymogen activation	2	0.0343	CTSH, PRSS12
		GO:0042074~cell migration involved in gastrulation	2	0.0377	CER1, SOX17
		GO:0008285~negative regulation of cell proliferation	5	0.0448	CER1, FEZF1, CYP1B1, GATA3, BCL6
		GO:0007169~transmembrane receptor protein tyrosine kinase signaling pathway	3	0.0476	NTRK3, LTK, ZAP70
		GO:0001775~cell activation	2	0.0477	LYPD1, GATA3
		GO:0045666~positive regulation of neuron differentiation	3	0.0487	RNF112, FEZF1, BCL6
	Cellular Component	GO:0005576~extracellular region	14	0.0033	CER1, LYPD1, PRL6A1, PRL7D1, PRL2A1, PRL2C3, APOB, CLPS, CHIL1, RELN, STC1, WNT11, PGA5, PRSS12
		GO:0005615~extracellular space	10	0.0494	PRL2C3, CER1, APOB, CTSJ, CHIL1, WNT11, STC1, RELN, PRL7D1, CTSH
		GO:0005179~hormone activity	5	0.0008	PRL2C3, PRL6A1, STC1, PRL7D1, PRL2A1

Molecular Function	GO:0043565~sequence-specific DNA binding	8	0.0083	BACH2, GATA3, IRX2, LHX5, BCL6, SOX17, FOXD4, FOXO6
	GO:0008233~peptidase activity	7	0.0115	AGBL3, CTSJ, RELN, PGA5, TMPRSS12, CTSH, PRSS12
	GO:0003700~transcription factor activity, sequence-specific DNA binding	9	0.0150	BACH2, ZFP300, GATA3, PAX5, BCL6, GM12258, SOX17, FOXD4, FOXO6
	GO:0003677~DNA binding	14	0.0153	BACH2, IRX2, PAX5, CXXC4, FOXO6, FEZF1, HES5, GATA3, LHX5, BCL6, SOX17, SP8, FOXD4, NHLH1
	GO:0030550~acetylcholine receptor inhibitor activity	2	0.0218	LYPD1, LY6H
	GO:0000981~RNA polymerase II transcription factor activity, sequence-specific DNA binding	4	0.0261	SOX17, FOXD4, NHLH1, FOXO6
	GO:0033130~acetylcholine receptor binding	2	0.0432	LYPD1, LY6H

Supplementary Table 4. Hair cell associated processes (attached as separated excel file)

Table S5. Antibodies and stains used for cell identification

Antibody	Host	Supplier	Cat. No.	Dilution
Brn3c	Mouse	Santa Cruz	SC81980	1:50
Calcium Green-1 AM		Invitrogen	C3011MP	4 µM
E-cadherin	Mouse	BD Bioscience	610181	1:200
FM1-43 (SynaptoGreen C4)		Biotium	70022	5 µM
H_2DCFDA		Invitrogen	D399	20 µM
Laminin-β1	Rabbit	Abcam	AB109293	1:50
Myosin VIIa	Rabbit	Proteus Bioscience	25-6790	1:100
Pax8	Rabbit	Abcam	AB97477	1:100
Sox1	Goat	R&D Systems	AF3369	1:100
Sox2	Mouse	BD Pharmigen	561489	1:100
Tubb3	Mouse	Biolegend	801201	1:500