

Award Number: W81XWH-12-1-0492

TITLE: A Stem Cell-Seeded Nanofibrous Scaffold for Auditory Nerve Replacement

PRINCIPAL INVESTIGATOR: Dr. Robert Duncan

CONTRACTING ORGANIZATION: Regents of the University of Michigan  
Ann Arbor, MI 48109

REPORT DATE: October 2014

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

<b>REPORT DOCUMENTATION PAGE</b>			<i>Form Approved</i> <i>OMB No. 0704-0188</i>		
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. <b>PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.</b>					
<b>1. REPORT DATE</b> October 2014		<b>2. REPORT TYPE</b> Annual		<b>3. DATES COVERED</b> 30 Sept2013 - 29 Sept2014	
<b>4. TITLE AND SUBTITLE</b>  A Stem Cell-Seeded Nanofibrous Scaffold for Auditory Nerve Replacement			<b>5a. CONTRACT NUMBER</b>		
			<b>5b. GRANT NUMBER</b> W81XWH-12-1-0492		
			<b>5c. PROGRAM ELEMENT NUMBER</b>		
Dr. Robert Duncan, PhD  E-Mail: <a href="mailto:rkduncan@umich.edu">rkduncan@umich.edu</a>			<b>5d. PROJECT NUMBER</b>		
			<b>5e. TASK NUMBER</b>		
			<b>5f. WORK UNIT NUMBER</b>		
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b>  Regents of The University of Michigan 1058 Wolverine Tower 3003 South State Street Ann Arbor, Michigan 48109-1274			<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>		
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			<b>10. SPONSOR/MONITOR'S ACRONYM(S)</b>		
			<b>11. SPONSOR/MONITOR'S REPORT NUMBER(S)</b>		
<b>12. DISTRIBUTION / AVAILABILITY STATEMENT</b> Approved for Public Release; Distribution Unlimited					
<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b> The chief aim of our study is restoration of hearing by regeneration of peripheral auditory neurons. The study takes a systematic approach in three objectives aiming to push human stem cells toward an auditory neural fate, embed the cells on a functionalized scaffold, and implant the device in a deafened animal model. In the first year of the project grant, we have addressed three key tasks: (1) derivation of sensory neurons from human pluripotent stem cells (hPSCs), (2) development of implantable nanofibrous substrates, and (3) optimization of the deafness model. In contrast to prior experiments in mouse embryonic stem cells, generation of sensory neurons simply by overexpression of neurogenin-1 in human embryonic stem cells was inefficient. As a result, we have established a small molecule programming strategy for producing sensory glutamatergic neurons that express otic transcriptional programs. These methods will be combined with neurogenin-1 overexpression to optimize derivation of auditory-like neurons. In addition, we developed novel methods for coiling a nanofiber scaffold to maintain the auditory nerve topology and establishing ouabain as an effective chemical tool for destroying endogenous auditory neurons in guinea pigs.					
<b>15. SUBJECT TERMS</b> Human stem cells, neurodifferentiation, deafness model, nanofiber					
<b>16. SECURITY CLASSIFICATION OF:</b>			<b>17. LIMITATION OF ABSTRACT</b>	<b>18. NUMBER OF PAGES</b>	<b>19a. NAME OF RESPONSIBLE PERSON</b> USAMRMC
<b>a. REPORT</b> U	<b>b. ABSTRACT</b> U	<b>c. THIS PAGE</b> U			UU

## Table of Contents

	<u>Page</u>
Cover Form	1
Report Documentation Page	2
Table of Contents	3
1. Introduction	4
2. Keywords	4
3. Accomplishments	4 - 8
4. Reportable Outcomes	8 - 9
5. Conclusion	9
6. References	9 - 10
7. Supporting Data	11 - 17
8. Participants & Other Collaborating Organizations	18 - 20

## **INTRODUCTION:**

An estimated 300 million individuals world-wide suffer some form of hearing loss, and military personnel exhibit auditory deficits at an exceptionally disproportionate level compared with the lay population. In fact, the ear is the organ most affected by concussive or penetrating injuries to the head. The devastating impact of widespread hearing loss on practical, social, and economic levels drives our interest in devising ways to restore hearing completely. Complete restoration of hearing is a particularly challenging task. In this project, we explore a systematic approach for developing a next-generation auditory prosthesis by replacing damaged auditory nerve with stem cell-derived neurons, grown on a nanofiber substrate, and electrically stimulated. We have divided our research goals into three primary objections. Objective 1 seeks to recapitulate the physiology of auditory neurons using human pluripotent stem cells. In Objective 2, we will develop a mat of aligned nanofibers to guide the arrangement of stem cell-derived neurons to mimic the arrangement as well as the physiology of the auditory ganglion. We also develop the means to electrically stimulate derived neurons on this nanofiber substrate. And finally, in Objective 3, we will implant a prototype device in an animal model of neural hearing loss, examining the capacity for stem cell-derived neurons to functionally integrate with the auditory brainstem.

## **KEYWORDS:**

auditory neurons, human pluripotent stem cells, nanofibrous substrate, neural hearing loss

EBs - embryoid bodies

hPSCs - pushing human pluripotent stem cells

IAM - internal auditory meatus

PCL - polycaprolactone

PEG - polyethylene glycol

Pur - purmorphamine

## **ACCOMPLISHMENTS:**

### **A. KEY RESEARCH ACCOMPLISHMENTS**

Tasks and deliverables are organized according to the major Objectives in the proposal. Progress on Year 1 tasks is bulleted along with any challenges and recommendations. In some cases, tasks originally scheduled for Years 2-4 were initiated early, and progress is bulleted accordingly. All other tasks are listed as “pending”.

Task 1. IACUC review of animal care and use application. The application is under development and will be submitted prior to any award. We estimate some time for modification of this protocol based on feedback from reviewers

- Complete

### **Objective 1: Evaluate the combination of genetic and neurotrophic cues on the differentiation of adult hiPSCs toward an auditory nerve-like phenotype**

Task 2-5. Gene expression analysis using RNA sequencing to compare the transcriptomes of induced cells and native auditory nerve, upregulation of neurotrophic factor receptors and BDNF/NT3-effects on electrical properties.

- Our goal in Objective 1 is to establish a reliable, efficient method for pushing human pluripotent stem cells (hPSCs) to an auditory-nerve fate. Indeed, this is the underpinning of all remaining tasks. We began our efforts with human embryonic stem cells (H7 line from WiCell) because these are far simpler

to maintain in culture than hiPSCs. We have two working methods to push cells toward a neuronal phenotype with markers that indicate overlap with an auditory sensorineural phenotype (Six1, Pax2 expression). These methods are being replicated in an additional ESC line (H9) and two hiPSC lines.

- Both induction methods also produce cells with markers of other neural lineages so the degree of specificity for an auditory phenotype is unclear. We are continuing to pursue overexpression of *Neur1* by Adenovirus and now by an inducible Lentivirus after predifferentiating to a sensory placode phenotype, marked by Pax2, *Eya1*, and Six1 expression. We will continue this in Year 3.
- Method 1: Stepwise programming with small molecule morphogens according to Kim et al. (2011), as reported in Year 1 Progress Report. Briefly, embryoid bodies (EBs) are generated by spin-aggregation in Aggrewell-800 micropatterned plates (or Sylgard alternatives, see Year 1 report) and pushed toward a neuroectodermal fate using the SMAD-inhibitors *noggin* and *dorsomorphin*. On day 2, EBs were plated onto Matrigel and incubated in DMEM/F12 with or without combinations of the morphogens retinoic acid (RA) and sonic-hedgehog agonist *purmorphamine* (Pur). Though both are required for otic patterning, little is known about how each influences the expression of various proneural basic-helix-loop-helix (bHLH) transcription factors and their downstream placode programs. After formation of rosettes and supplementation by FGF-2, neural precursors are isolated and plated on Matrigel in the presence of Neurobasal with N2 and B27. Cells cultured under terminal differentiation conditions for 6 weeks express mature neuronal markers (e.g. MAP2), appear to be glutamatergic (e.g. vGlut1-positive), and make synaptophysin-positive contacts with one another indicating synaptogenesis in vitro (Summarized in Figure 1). We have confirmed that these cells are electrically responsive at the 6 week time point. In addition, they express Pax2 and Six1 during the rosette phase of induction, indicating a possible otic placode-like fate. Neurons produced by *Noggin/Dorsomorphin* upregulate *TrkB* and *TrkC* receptors, which are canonical receptors for the neurotrophins BDNF and NT3 under investigation here (Figure 2). BDNF and NT3 enhance neuron production, survival, and neurite elongation. The electrophysiology study of BDNF/NT3 effects on sensory neurons is underway. In Year 3, we will complete this study on sensory produced by Method 1 and determine whether these effects are replicated by BDNF/NT3 on neurons produced by Method 2.
- However, they also express Pax6 which is associated with other placodes, CNS neurons, and those derived from the neural crest. As a result, we have also begun to pursue another induction protocol that addresses the potential role of BMPs in patterning and speeds the protocol by avoiding the embryoid body formation step (Method 2).
- Method 2: A recent report showed induction of preplacodal cells expressing the otic marker Six1 by differentiating adherent cells in a simple defined medium (Leung et al., 2013). These authors showed a dependence on BMP activity and formation of placodal cells vs cells from neural crest or epidermis. BMPs are essential for the early lineage choice in the ectoderm. We initially tried to replicate their protocol but were unable to reproduce the Six1/otic marker expression. Dr. Hackelberg then sought to determine whether basal levels of BMP4 activity or lack thereof might contribute to differences between our cultures and those from Leung et al. She examined the effect of BMP4, BMP blocker LDN212854, and the BMP/SMAD inhibitor *noggin*. Quantitative PCR results indicated that moderate BMP inhibition with 10  $\mu$ M LDN212854 produced cultures with the optimal expression of 3 otic markers (*GATA3*, *SIX1*, *PAX2*) (see heat map in Figure 3). These results were also dependent on cell seeding density indicating that autocrine influence is a major factor in induction programs, and arguing for systematic

stepwise experiments such as these. We are pursuing this induction program in parallel with Method 1 to identify those cells with the most auditory-like phenotype.

- EB deliverable: Update from Year 1. In transitioning our studies toward a stepwise protocol in induction Method 1, it was necessary to form embryoid bodies. Generation of EBs is notoriously associated with culture heterogeneities because of differences in EB size and shape. In the Year 1 Progress report, we demonstrated a new method to spin-aggregate in custom designed PDMS (Sylgard) microwells. We have continued using this approach for EB formation and have investigated whether coatings could alter fate specification. Early results reported in the Year 1 document showed no bias toward the major germ lines using basic early markers of mesoderm, endoderm, ectoderm. Interestingly, pushing this system further to determine whether these methods might have impact on other developmental genes like Pax2 and Pax6 has showed a bias on gene transcription. We are following up on this observation to determine the reproducibility at the qPCR level and effect at the protein level with immunohistochemistry. Preliminary data suggests an upregulation of Pax2 when PDMS inserts are coated with polyethylene glycol (PEG), which could enhance our specification of otic progenitors. It will be important to determine whether other otic genes are modulated in a similar way. Since PEG would reduce cell adhesion, we can only speculate that PEG-related effects on gene transcription are related to the size, shape, and mechanics of EB formation. If replicated, mechanism could be tested using chemical modulators of cell adhesion or PDMS substrates with different stiffnesses.

**Objective 2: Design a first-generation electrical interface for stimulation of stem cell-derived neurons grown on a nanofiber substrate. All tasks rely solely on cell lines.**

Task 6. Minimize thickness of nanofiber mat to maximize flexibility

- In vitro studies of cells on 2D nanofiber mats have progressed. We have optimized adherence on aligned and unaligned polycaprolactone (PCL) fiber mats from our collaborators and using commercial products from Nanofiber Solutions (Figure 4). We are introducing neural progenitor cells (NPCs) to these mats since we have decided to do this in the in vivo approach in Objective 3 (rather than introduce ESCs/iPSCs and differentiate from the beginning). This was required due to the stepwise differentiation programs requiring EB formation and/or adherent colony cultures of neural rosettes, which would not be possible on a nanofiber mat.
- Preliminary results show that these cells are excitable. We are advancing now to electrical recordings.

Tasks 7-12. Design, implement, and examine efficacy of electrical interface to nanofiber substrate

- Pending tasks

**Objective 3: Optimize the integration of stem cell-derived neurons following in vivo transplantation of the seeded nanofibrous scaffold**

**Task 13. Group 1: Pilot deafening. Confirm efficacy of  $\beta$ -bungarotoxin in guinea pig and time point of nerve death. 10 guinea pigs will be required, including an expected failure rate of 30% from morbidity and mortality**

- See Year 1 report; ouabain treatment produces the desirable neuron loss in guinea pigs.

**Task 14. Group 2: Pilot implantation. Confirm positional stability and integrity of implant over time. 15 guinea pigs will be required, including an expected failure rate of 30% from morbidity and mortality. No hiPSCs involved**

- While advancing the challenging experiments in Objectives 1 and 2, we have spent considerable attention on Objective 3 tasks in preparation for in vivo experiments in Years 3 and 4. We are now ready to pilot these experiments.
- We are continuing to optimize the surgical approach and define the implant design constraints. We now believe we can implant conduit scaffold 0.5-0.7 mm in diameter and 2-2.5 mm long to pass through the cochleostomy and reach the internal auditory meatus (IAM), which is the passageway linking the auditory nerve ganglion with the auditory brainstem. In the Year 1 Progress report, we showed a novel technique to form a self-rolling conduit with concentric layers of nanofiber films. These have proven too compliant for implantation. We are solving this issue by constructing a hollow PCL tube with a novel repeated dipping/leaching approach, then loading these with (1) bundled nanofibers (Figure 5) or (2) the self-rolled conduit (Figure 6).
- See Year 1 Report for methods/results on electrospinning the nanofibers and forming the self-rolling conduit.
- PCL tube formation: Polycaprolactone was dissolved in chloroform solvent at a concentration of 15% (w/v). A high torque industrial stirring motor (Caframo Ltd., Warton, Ontario, Canada) was set at a rotational velocity of 100 RPM in order to rotate a 27.5 gauge syringe tip. The PCL solution was loaded into a 3 ml syringe tip and mounted onto a KDS 100 (KD Scientific, Holliston, MA) syringe pump set at dispensing rate of 0.05 ml/hr. The syringe pump was placed on linear stage programmed to move to a distance of 15 mm, hold the position for 10 seconds and return to its original position repeating the process every 90 seconds for a total time of 10 minutes. The tip of the syringe dispensing the PCL solution was placed at distance 5 mm away from the rotating syringe tip such that their heights were level facing tip-to-tip. As the linear stage drove the syringe to a distance of 15 mm, the rotating syringe tip entered the tip of the dispensing syringe coating itself with polymer (Figure 5A). Rotation allowed the tip to be coated evenly in PCL polymer. After 10 minutes of coating, the PCL-coated syringe tip was allowed to dry for a period of 15 minutes. After the syringe coatings were completely dry, excess polymer was cut from the tip of the coated syringe. Needle tip forceps were used to remove the newly formed hollow PCL tube from the syringe tip, revealing a hollow tube <1mm in diameter (Figure 5B). The hollow tube PCL conduit was then placed onto a different 27.5 gauge syringe tip connected to the end of low pressure vacuum lead. The low pressure vacuum was used to pull the PCL fiber bundles previously collected from the electrospinning process through the conduit (Figure 5C). Next, the conduits are oxygen plasma treated to increase hydrophilicity. These are placed in a microfluidic mold that allows slow infiltration of NPCs (Figure 5D). This method has allowed us to seed conduits with about 1,000 cells (compared to <100 when using capillary action alone to infiltrate the conduits) (Figure 5E).
- PCL tubes with self-rolled conduits: We continue to pursue use of the self-rolled nanofiber mats because these conduits have a concentrically wrapped wall that complements the spiraled interior of the cochlea (Figure 6A) and provides a physical barrier to limit cell migration (Figure 6B). The walls of the rolled conduits are thin and weak leaving them vulnerable to collapsing during the process of implantation. The bundled conduits have hard exterior walls that have withstood the load forces associated with

insertion of the conduit during practice surgeries. Our future objectives are therefore to insert the concentrically rolled conduits inside of an empty bundled conduit preserving the desired internal morphology and providing protection necessary for implantation. Preliminary experiments are encouraging (Figure 6C).

- Surgical approach: We used guinea pig temporal bones to develop the surgical approach for implantation within the internal auditory meatus (IAM). In Figure 7A, we show the lateral wall of an intact guinea pig bone with a cochleostomy and green-stained bundled-conduit. From the dural side, the conduit can be seen extending through the most medial aspect of the IAM (Figure 7B). We have replicated this in the live guinea pig but placement into the IAM has been challenging due to a small bend in this passageway. Placement is certainly adequate for us to begin initial phases of in vivo work to determine health of implanted cells on the inserted scaffold, inflammatory reactions, scarring, and neurogenesis in the implanted NPCs. We will continue to optimize the approach angle and drilling techniques to maximize placement within the IAM.

### **Tasks 15 through 18 examine the functional integration of hiPSC-derived neurons following implantation of the neuralized nanofibers.**

- Pending tasks for Years 2-4

## **B. REPORTABLE OUTCOMES**

### **Abstracts**

1. Duncan RK, Liu L, Schaefer S, Decker M (2014) Serum-free and feeder-free derivation of human neural progenitors with fasciculated architectures. Abstracts of the 37th MidWinter Meeting of the Association for Research in Otolaryngology, 37:388.
2. Schaefer S, Varma S, Duncan RK (2014) Micropatterned silicone substrates for affordable and reproducible embryoid body formation. Abstracts of the 37th MidWinter Meeting of the Association for Research in Otolaryngology, 37:391.
3. Hackelberg S., Rastogi A, Tuck SJ, White C, Liu L, Prieskorn D, Corey JM, Miller, JM, Duncan RK (2015) Nanofibrous scaffolds for the integration of human embryonic stem cells into the cochlea. Abstracts of the 38<sup>th</sup> MidWinter Meeting of the Association for Research in Otolaryngology, accepted.

### **Development of cell lines**

Human pluripotent stem cell lines stably expressing Neurog1, either constitutively or inducible, are currently being developed.

### **Databases**

We have generated a normative database of the number spiral ganglion neurons in the normal and ouabain treated guinea pig. These normative data will form the reference for determining the extent of new neural growth in the implanted deafened ear.

## **Research opportunities applied for based on experience supported by this award**

Experience in nanofiber scaffold design and stem cell differentiation garnered during this project year has facilitated an application by our co-investigator Dr. Joseph Corey to the VA BLRD entitled “Accelerating the growth of motor axons using mechanical tension.” A revised application is being resubmitted and new proposals on cell seeding nanofiber scaffolds are being developed. There is no scientific overlap with the current award.

## **C. CONCLUSION**

The research completed to date shows that simple overexpression of Neurog1 is a less efficient method for generating stem cell-derived neurons using hESCs compared with prior work in mESCs. This could reflect basic differences between mouse and human or simple differences in two separate stem cell lines. Regardless, a more robust method for generating auditory nerve-like cells is required, particularly one that can be easily translated to a variety of human pluripotent stem cell lines. We have two viable methods and seek to streamline the stepwise protocol to enhance the speed of differentiation while creating neurons with molecular and physiological phenotypes indicative of auditory neurons.

Our advanced studies on nanofiber scaffold design have provided a unique and novel method for self-rolling nanofibrous substrates. Now, we show improved methods for increasing these stiffness of these substrates with a PCL tube and novel methods for loading the scaffolds with NPCs. Cells are able to survive the process and adhere to the scaffold. Preliminary data shows good alignment of axonal projections along the nanofibers inside the conduits. With improvements in the implantation approach, we are now ready to enter the in vivo phase with cell-seeded conduits, paving the way for a viable auditory nerve graft.

## **D. REFERENCES**

- Cho YB, Cho HH, Jang S, Jeong HS, Park JS, (2011), Transplantation of neural differentiated human mesenchymal stem cells into the cochlea of an auditory-neuropathy guinea pig model. *J Korean Med Sci.*, 26:492-498. PMID: PMC3069567.
- Dahlmann J, Kensah G, Kempf H, Skvorc D, Gawol A, Elliott DA, Drager G, Zweigerdt R, Martin U, Gruh I, (2013), The use of agarose microwells for scalable embryoid body formation and cardiac differentiation of human and murine pluripotent stem cells. *Biomaterials* 34:2463-2471.
- Hashino E, Fritsch MH (eds.), (2011), Embryonic stem cell-derived neurons for inner ear therapy: InTech.
- Hester ME, Murtha MJ, Song S, Rao M, Miranda CJ, Meyer K, Tian J, Boulting G, Schaffer DV, Zhu MX, Pfaff SL, Gage FH, Kaspar BK, (2011), Rapid and efficient generation of functional motor neurons from human pluripotent stem cells using gene delivered transcription factor codes. *Mol Ther* 19:1905-1912. PMID: PMC3188742.
- Hill GW, 3rd, Purcell EK, Liu L, Velkey JM, Altschuler RA, Duncan RK, (2012), Netrin-1-mediated axon guidance in mouse embryonic stem cells overexpressing neurogenin-1. *Stem Cells Dev* 21:2827-2837. PMID: PMC3464074.
- Kim JE, O'Sullivan ML, Sanchez CA, Hwang M, Israel MA, Brennand K, Deerinck TJ, Goldstein LS, Gage FH, Ellisman MH, Ghosh A, (2011), Investigating synapse formation and function using human pluripotent stem cell-derived neurons. *Proc Natl Acad Sci U S A* 108:3005-3010. PMID: PMC3041068.

Leung AW, Morest DK, Li JYH, (2013), Differential BMP signaling controls formation and differentiation of multipotent preplacodal ectoderm progenitors from human embryonic stem cells. *Dev Biol* 379:208-220. PMID: PMC3712508.

Ma Q, Anderson DJ, Fritsch B, (2000), Neurogenin 1 null mutant ears develop fewer, morphologically normal hair cells in smaller sensory epithelia devoid of innervation. *J Assoc Res Otolaryngol* 1:129-143. PMID: PMC2504536.

Norrman K, Fischer Y, Bonnamy B, Wolfhagen Sand F, Ravassard P, Semb H, (2010), Quantitative comparison of constitutive promoters in human ES cells. *PLoS One* 5:e12413. PMID: PMC2928720.

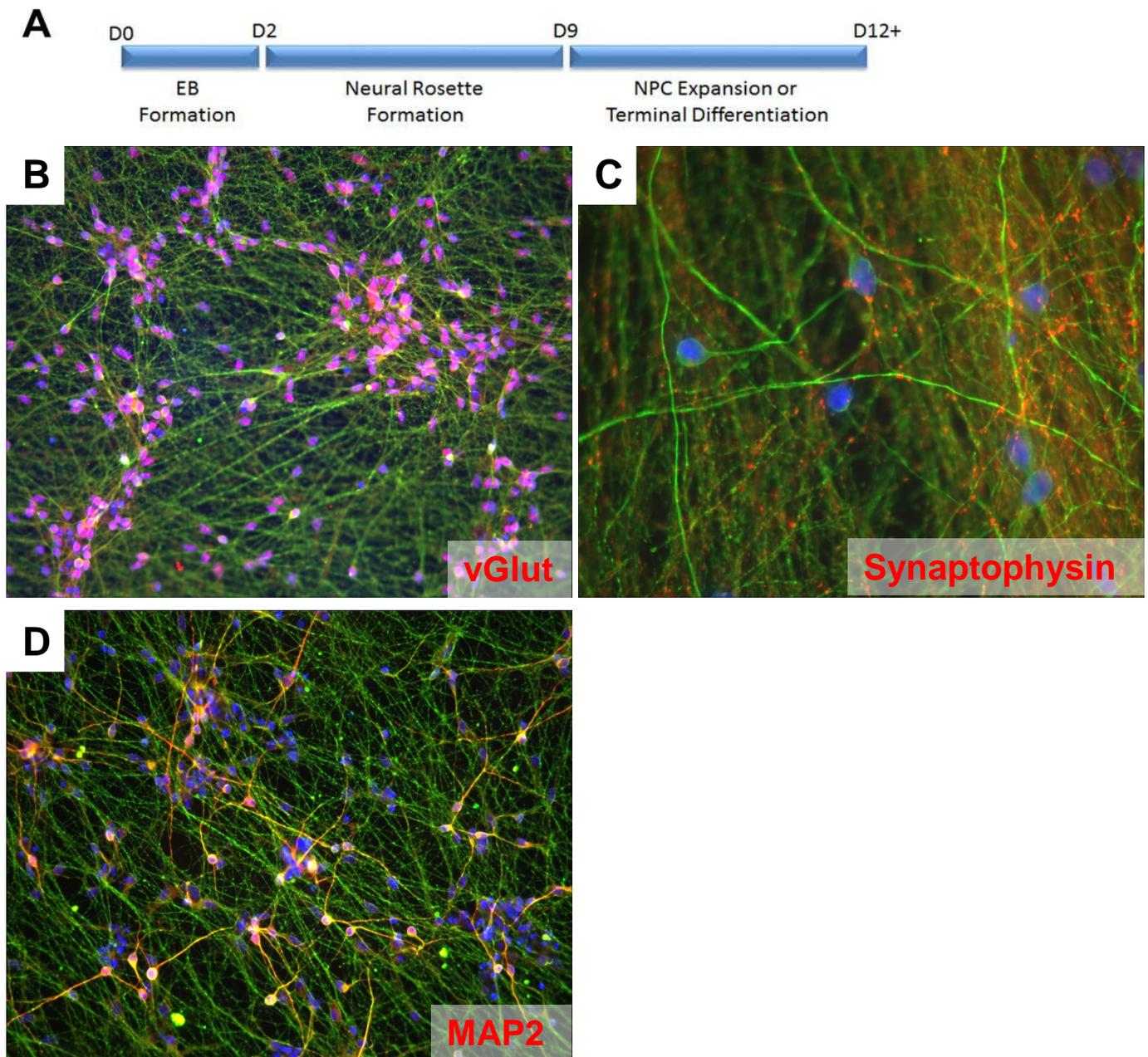
Purcell EK, Naim Y, Yang A, Leach MK, Velkey JM, Duncan RK, Corey JM, (2012), Combining topographical and genetic cues to promote neuronal fate specification in stem cells. *Biomacromolecules* 13:3427-3438. PMID: PMC3992984.

Purcell EK, Yang A, Liu L, Velkey JM, Morales MM, Duncan RK, (2013), BDNF profoundly and specifically increases KCNQ4 expression in neurons derived from embryonic stem cells. *Stem Cell Res* 10:29-35. PMID: PMC3525722.

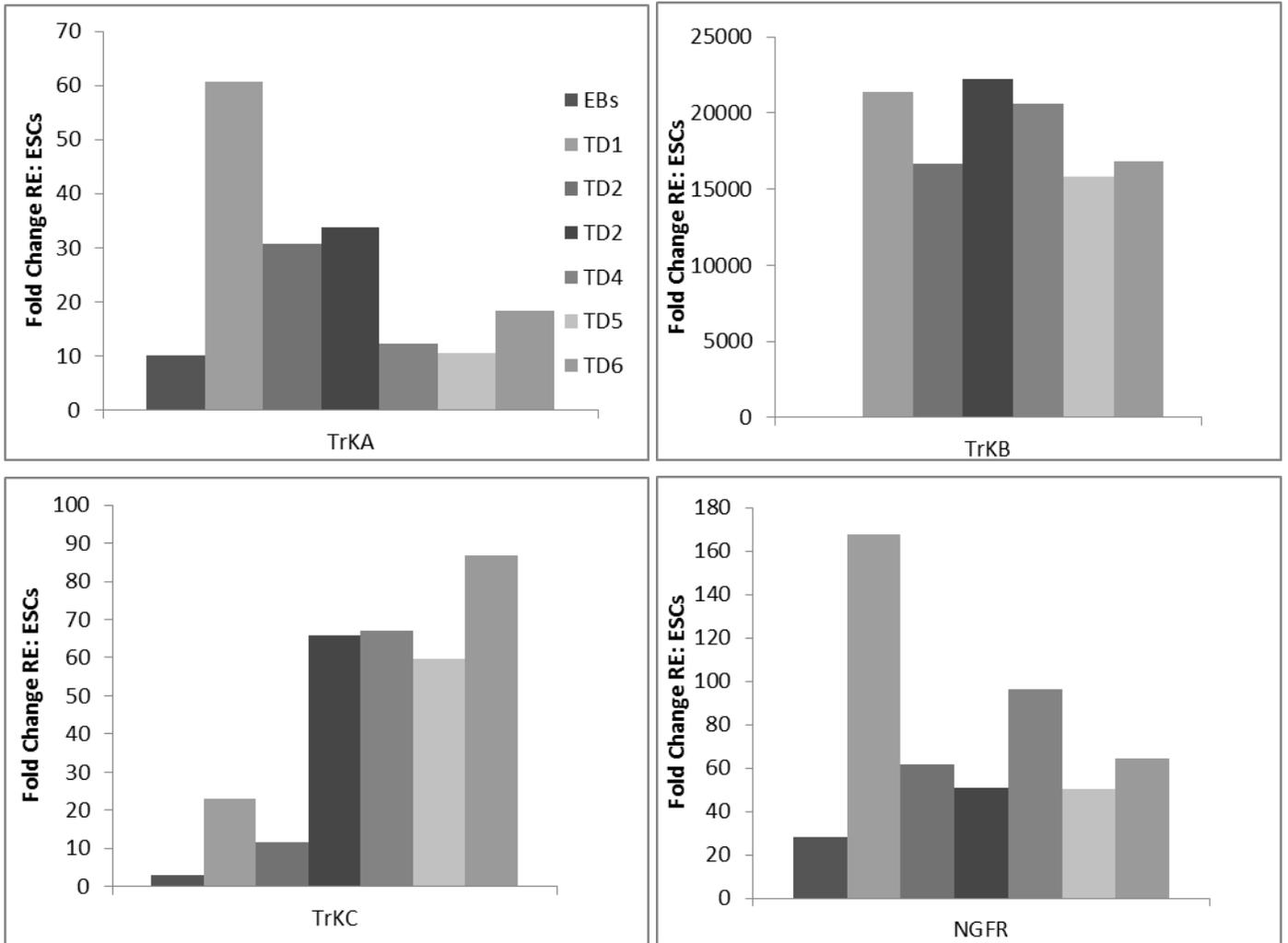
Schlosser G, (2006), Induction and specification of cranial placodes. *Dev Biol* 294:303-351.

Tong M, Hernandez JL, Purcell EK, Altschuler RA, Duncan RK, (2010), The intrinsic electrophysiological properties of neurons derived from mouse embryonic stem cells overexpressing neurogenin-1. *Am J Physiol Cell Physiol* 299:C1335-1344. PMID: PMC3006329.

## SUPPORTING DATA



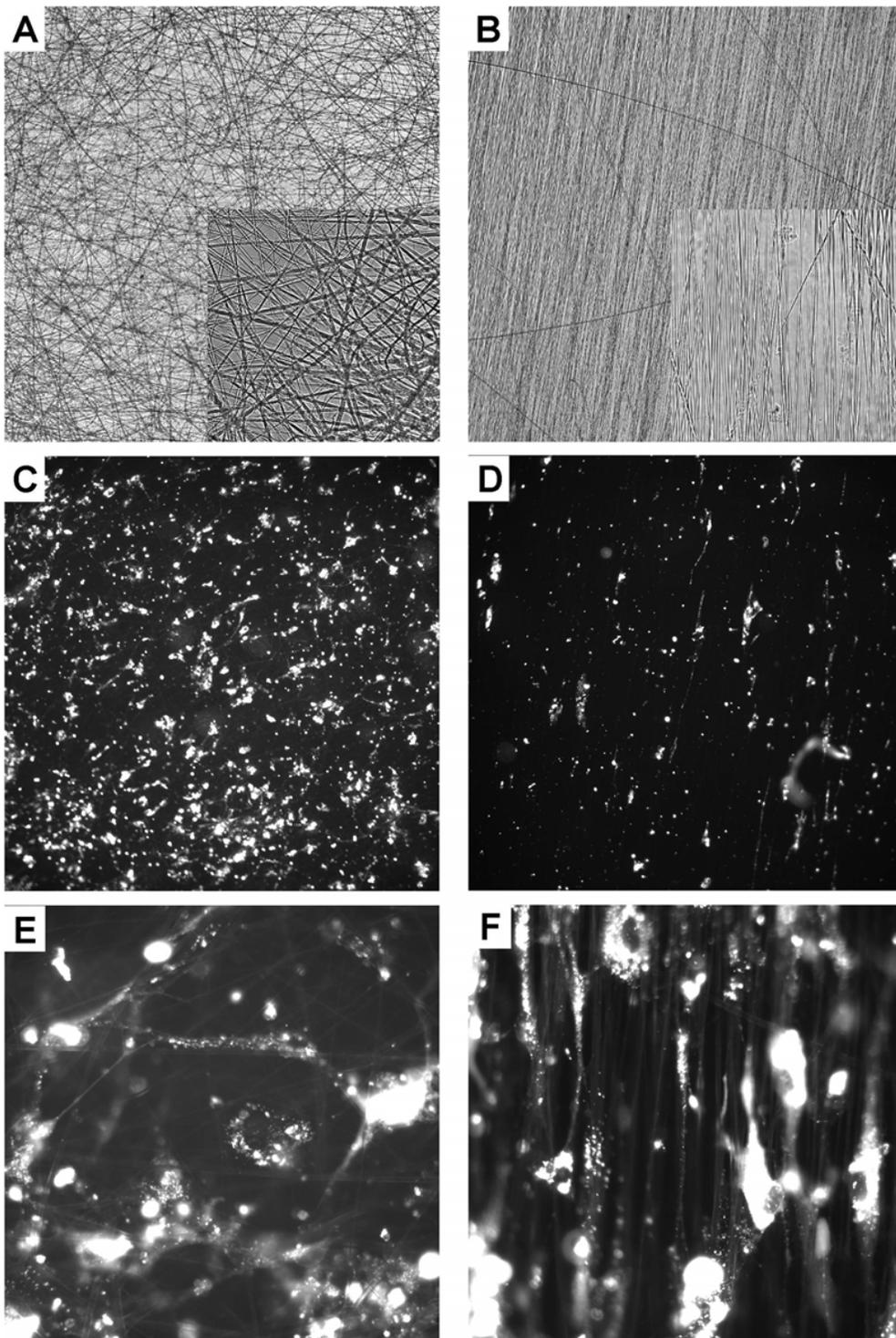
**Figure 1. Maturation of hESC-derived neurons using a stepwise protocol.** (A) Stepwise protocol, forming embryoid bodies (EBs) in the presence of noggin/dorsomorphin, forming rosettes in the presence of retinoic acid and purmorphamine, and terminal differentiating in a proneural media after brief exposure to FGF2. After six weeks of terminal differentiation, neurons express vGlut1 (B), form synaptophysin positive contacts with one another (C), and express the dendritic marker MAP2 (D).



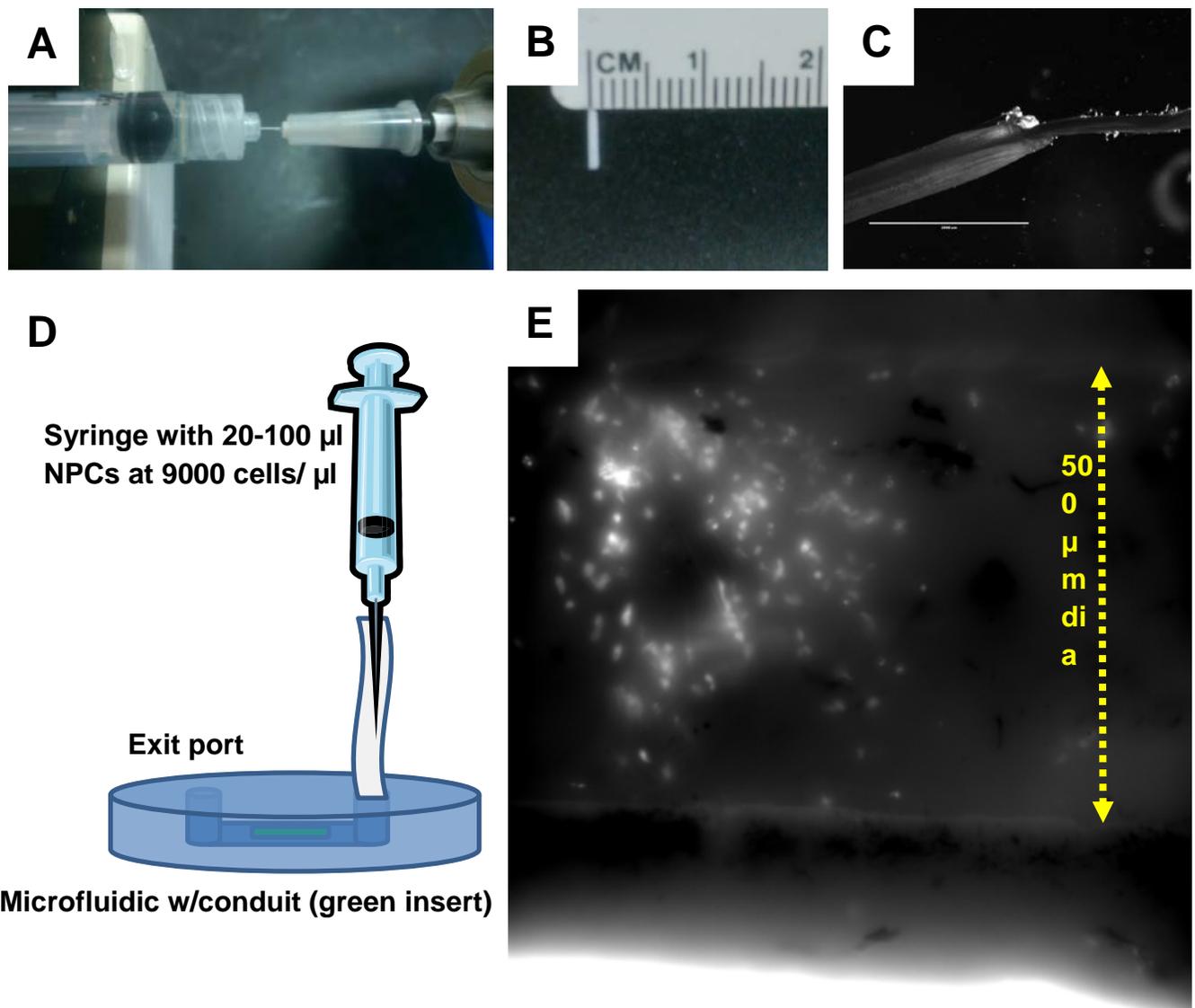
**Figure 2. Quantitative PCR for neurotrophic factor receptors in hESC-induced neurons using Method 1 and undergoing terminal differentiation (TD) for up to 6 weeks (TD6) compared to expression levels in embryoid bodies (EBs) and plotted as fold change relative to levels in human ESCs.** TrkB shows the highest change in expression due to neural induction and TrkC shows increasing expression levels during terminal differentiation, whereas TrkA and NGFR (p75<sup>ntr</sup>) show decreasing expression levels during terminal differentiation. TrkB and TrkC are canonical receptors for BDNF and NT3, the major neurotrophic receptors involved in auditory nerve development.



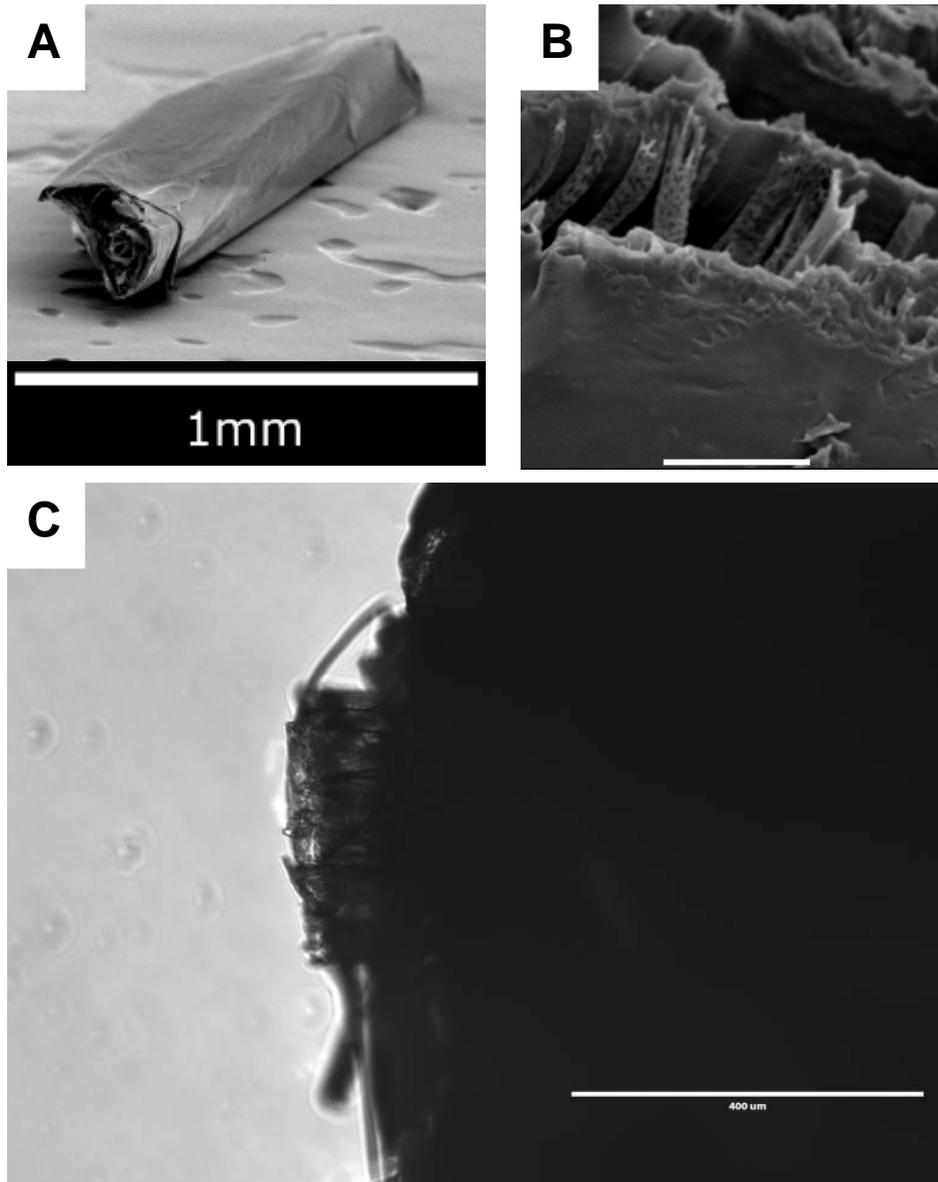
**Figure 3: Otic preplacode differentiation protocol.** (A) Cartoon of the general differentiation scheme. hESCs are dissociated and plated as single cells at D-2. At D0, medium is changed from mTeSR to a serum-free differentiation medium. From this time point on medium is changed every second day. To test for the influence of BMP signaling on the expression of genes characteristic for otic preplacode differentiation, the medium was supplemented either with a BMP agonist (BMP4) or inhibitors (human recombinant Noggin or LDN212854). After 8 days of differentiation cells were harvested for RT-PCR. (B) Influence of BMP signaling on expression of otic preplacode genes. The relative expression of individual genes following activation or inhibition of BMP signaling is shown in grayscales. GATA3, SIX1 and PAX2 represent markers for otic preplacode differentiation. ID1 transcripts 1 and 2 represent BMP downstream signaling. To test the influence of cell density, hESCs were seeded at a low ( $2.5 \times 10^3$ ) and high density ( $2 \times 10^4$ ). Concentrations are given as ng/ml for BMP4 and Noggin and  $\mu\text{M}$  for LDN212854.



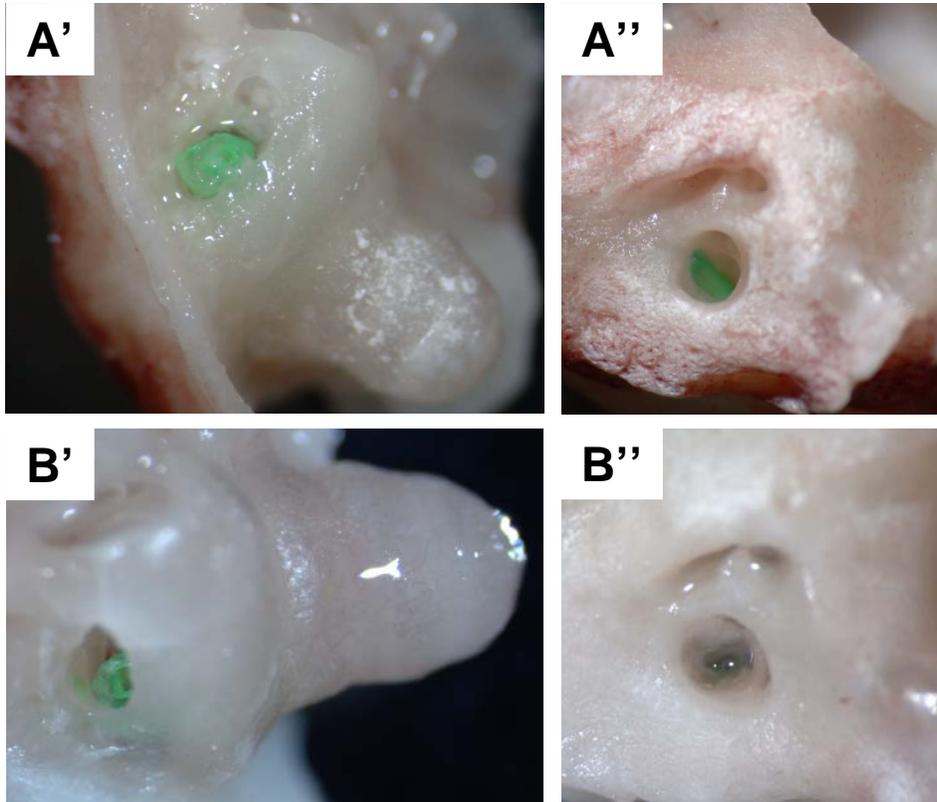
**Figure 4. Growth of hESC derived Neurons on unaligned and aligned Nanofibers.** (A) and (B): Assembly of unaligned and aligned nanofibers (Nanofiber Solutions™, average fiber diameter 700 nm) is shown in 10x and 60x (inset) magnification. (C) – (F) hESC derived Neurons live stained with CM-Dil (Invitrogen V-22888) were grown on unaligned (C and E) and aligned (D and F) Matrigel® coated nanofibers for 19 days and visualised using epifluorescence imaging. Neurons grown on aligned nanofibers show directed growth corresponding to fiber orientation. Magnification is 10x for (C) and (D) and 60x for (E) and (F).



**Figure 5. Formation and cell-seeding a bundled nanofiber conduit in a hollow PCL tube.** (A) Apparatus for repeated dipping/leaching of PCL to form a stiff hollow tube. (B) Use of a 27.5G needle tip allows formation of tubes with outer diameters between 0.7 and 1.0 mm. (C) Nanofiber mats are drawn into the hollow tube with suction. (D) A PDMS microfluidic chamber is used to infiltrate the scaffold with neural progenitor cells (NPCs). (E) A cell-seeded scaffold was cultured for 24 hours and then stained for Hoechst to identify adherent cells. The 2 mm long scaffold with 0.5 mm inner diameter was bifurcated and imaged with epifluorescence. In this approximately 0.5 x 0.5 mm view, hundreds of NPCs were introduced. Using this method, we can infiltrate scaffolds with ~1,000 cells. We are investigating alternative methods for loading in order to reach 5,000 to 10,000 cells. Since we are introducing NPCs, there is some expectation that cells may continue to divide for several days, increasing cell numbers further.



**Figure 6. Self-rolled nanofiber conduit construction.** (A) SEM of self-rolled nanofiber mat on P4VP. (B) High mag images of nanofibers inside the layers of a rolled conduit (scale: 10 microns). Dehydration and rolling processes causes some collapse of the rolled substrate and produces a fragile scaffold that is too compliant for implantation. Therefore, we will place the rolled conduits inside the hollow PCL tube in Figure 5. Initial experiments showing proper placement are encouraging (C; scale: 400 microns). This structure gives the benefits of a highlight aligned fiber mat in a coiled arrangement with the added stiffness of the PCL tube to aid implantation.



**Figure 7. Implantation of bundled nanofiber scaffold.** Dye labeled scaffolds approximately 500 to 700  $\mu\text{m}$  in diameter were loaded with PCL nanofibers and implanted in (A) excised temporal bones or (B) live animals and removed to confirm placement. The cochleostomy (A') allows placement into the internal auditory meatus (IAM) (A'' shown from dural side). Placed in live animals and evaluated post excision of the temporal bone, the scaffold can again be seen at the cochleostomy (B') and inside the IAM (B''). But a 25 to 45° turn in the IAM must be overcome to get sufficient penetration.

## PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	Robert Keith Duncan
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0002-8949-8727
Nearest person month worked:	8
Contribution to Project:	Dr. Duncan is an Associate Professor of Otolaryngology in the Kresge Hearing Research Institute. He is a physiologist with expertise in ion channel structure and function. As Principal Investigator, Dr. Duncan will supervise all aspects of the proposed studies including procedural design, data analysis and interpretation, and determination of new directions. He will prepare peer-reviewed manuscripts and present the results at national meetings. Dr. Duncan will also actively participate in collection of electrophysiology data. He will coordinate experiments with collaborating laboratories (Drs. Corey, Miller, and Shore) and core facilities (Drs. Lyons and Cavalcoli). Dr. Duncan will ensure close interaction of the investigating team through weekly project meetings.
Funding Support:	Award Ended: R21 DC011631, Principal Investigator: Raphael, Title: Integrating Cells into the Auditory Epithelium of Deaf Ears

Name:	Dr. Joseph Corey, M.D., Ph.D.
Project Role:	Co-Investigator
Nearest person month worked:	2
Contribution to Project:	Dr. Corey is an expert in using patterned substrates to guide the outgrowth of primary neurons in culture. He will supervise the construction of aligned nanofiber substrates and the covalent linkage of bioactive molecules, if required. Dr. Corey has a joint UM-VA appointment. A memorandum of understanding is on file. All effort will come from Dr. Corey's UM appointment.
Funding Support:	Award Ended: Sponsor: VA RR&D Merit Review, PI: Corey JM Award Period: 04/01/2010 – 09/30/2014 Title: Surface Modified Nanofibers for Nerve Regeneration

Name:	Dr. Josef Miller, Ph.D.
Project Role:	Co-Investigator
Nearest person month worked:	1
Contribution to Project:	Dr. Miller is an expert in cochlear physiology, drug delivery, and the prevention of hearing loss. His laboratory has extensive expertise in surgical approaches to place electrode arrays and perfusion devices into the cochlear duct. He will directly supervise Ms. Prieskorn, a member of his laboratory staff, in placement of the nanofiber array in guinea pigs, installation of a stimulating electrode, and measurement of electrically evoked auditory brainstem responses.

Funding Support:	<p>New Award:  R44 DC00847, PI: Pierstorff, Award Period: 06/09/14 – 05/31/15  Effort: 0.0 calendar months  Sponsor: NIH/Subaward from O’Ray Pharma, Inc.  Award Amount: \$116,526 DC year 1  Title: Sustained Release Inner Ear Delivery of Corticosteroid  Role: Co-Investigator (PI Subaward)</p> <p>Ended Awards:  W81XWH-11-1-0414, PI Altschuler,  Sponsor: Department of Defense – Army  Award Period: 07/01/11 – 07/31/14  Title: Prevention and Treatment of Noise-Induced Tinnitus  Role: Co-Investigator</p> <p>U01 DC008423, PI: Miller, Award Period: 02/01/08 – 01/31/14  Sponsor: NIH/NIDCD  Title: Micronutrient Intervention to Reduce Noise-Induced Hearing Loss</p>
------------------	--

Name:	Dr. Susan Shore, Ph.D.
Project Role:	Co-Investigator
Nearest person month worked:	10
Contribution to Project:	Dr. Shore is an expert in the physiology of the auditory brainstem. She has significant expertise in tract tracing, preparation of cochlear nucleus slices, and multielectrode recordings of electrically and acoustically evoked activity in the cochlear nucleus. Dr. Shore will assist in evaluating integration of hiPS-derived neurons in cochlear nucleus explants and <i>in vivo</i> preparations. She will also guide electrophysiology experiments, recording CN responses from electrical stimulation of the hiPS-seeded nanofiber array implanted into guinea pigs.
Funding Support:	<p>New Awards:  T32 DC000011, PI: Shore, Award Period: 07/01/14 – 06/30/19  Sponsor: NIH/NIDCD, Effort: 0.60 calendar months  Award Amount: \$337,962 DC Year 1  Title: Sensory Mechanisms and Disorders</p> <p>PI: Shore, Award Period: 07/01/14 – 06/30/15  Sponsor: UM Coulter Translational Research Partnership Program  Effort: 1.2 calendar months  Award Amount: \$140,327 year  Title: Combined Auditory-Somatosensory Stimulation to Alleviate Tinnitus</p> <p>Ended Awards:  W81XWH-11-1-0414, PI Altschuler,  Sponsor: Department of Defense – Army  Award Period: 07/01/11 – 07/31/14  Title: Prevention and Treatment of Noise-Induced Tinnitus  Role: Co-Investigator</p>

Name:	Liqian Liu
Project Role:	Research Laboratory Tech Sr.
Researcher Identifier (e.g. ORCID ID):	Not Applicable
Nearest person month worked:	13
Contribution to Project:	Ms. Liu is an exceptional molecular biologist with expertise in cell culture, stem cell differentiation, quantification of gene expression, preparation of cloned gene products, and protein analysis (Western blot, co-immunoprecipitation, sucrose gradient fractionation). Ms. Liu will be responsible for maintaining hiPS cell stocks, active cultures, differentiation into neural cells, and cell sorting. She will also prepare RNA for gene expression studies and perform histology experiments as needed. Ms. Liu manages animal colonies, reagent inventory, and all purchasing.
Funding Support:	NA

Name:	Sandra Hackelberg
Project Role:	Post-Doctoral Fellow
Nearest person month worked:	10
Contribution to Project:	Dr. Hackelberg will be responsible for designing, conducting, and analyzing electrophysiology experiments. Sandra will work with Ms. Prieskorn to implant guinea pigs and perform all electrophysiological / histological experiments. She also will be responsible for manuscript preparation and scientific communication at national meetings.
Funding Support:	NA

Name:	Diane Prieskorn
Project Role:	Admin Specialist Senior Health
Researcher Identifier (e.g. ORCID ID):	Not Applicable
Nearest person month worked:	3
Contribution to Project:	Ms. Prieskorn is an expert in surgical approaches to the guinea pig cochlea, including chronic placement of electrical arrays and drug delivery apparatus'. She will work closely with Drs. Miller and Purcell to place nanofiber arrays into the internal auditory meatus and evaluate functional integration with electrically evoked auditory brainstem responses.
Funding Support:	NA