**INTRODUCTION**

Loss of olfaction affects millions of people in the US and remains a therapeutic challenge (Doty et al., 1984; Hoffman et al., 2016; Murphy et al., 2002). Sensorineural causes, such as age-related decline, post-viral damage, head trauma, or genetic disorders affecting olfactory neuron function, are often permanent (Doty et al., 1984; Hoffman et al., 2016; Kern et al., 2000; McIntyre et al., 2012; Paik et al., 1992; Seiden, 2004; Wrobel and Leopold, 2004). Neurogenic exhaustion is a common feature of many acquired anosmias, in which the normal replacement of damaged or senescent olfactory sensory neurons (OSNs) from progenitor basal cells is overwhelmed (Holbrook et al., 2005). Regardless of the specific etiology, pathogenesis involving failures in neuronal maintenance, function, or renewal suggests that cell-based therapy to replace neurons may be broadly effective. The nasal olfactory epithelium (OE) houses bipolar OSNs (Figure 1) that detect odors at the epithelial surface and extend axons into the olfactory bulb. Due to their location in contact with the nasal airspace, OSNs are vulnerable to damage and cell death (Carr and Farbman, 1993). Tissue homeostasis is normally maintained by stem and progenitor cells in the basal layers of the epithelium, capable of generating new OSNs (Graziadei and Graziadei, 1979). In the mouse OE, neuropotent basal cells, specific markers for reserve and active stem cells, as well as aspects of their molecular regulation have been identified (Calof et al., 2001; Goldstein et al., 2015; Guillemot et al., 1993; Leung et al., 2007; Schwob et al., 2017; Tietjen et al., 2003). Globose basal cells (GBCs) are considered the principal pool of replicating stem cells that replenish OSNs, microvillar cells, sustentacular cells, and glands of the OE throughout life, whereas the horizontal basal cells represent a more quiescent reserve population (Fletcher et al., 2011; Huard et al., 1998; Leung et al., 2007). Single-cell transcriptional profiling has defined expression of the surface receptor c-Kit as a key feature of GBCs in the sensory lineage (Fletcher et al., 2017). In addition, cultured GBCs, puriﬁed using antibody to c-KIT, have been successfully expanded as de-differentiated basal cell islands (Goldstein et al., 2016). Thus, the c-KIT (+) population of olfactory stem cells has the potential for a cell-based therapy.

Olfactory stem cell engraftment has been attempted in rats and mice (Chen et al., 2004; Goldstein et al., 1998). Yet, recovery of function has been challenging to demonstrate because regeneration of host OE following experimental injury precludes measuring the contribution of exogenous stem cells. In principle, this hurdle could be overcome by using congenitally anosmic hosts rescued with wild-type stem cells. However, such anosmic mice survive weaning poorly or have other health concerns (Brunet et al., 1996; Lehman et al., 2008). To overcome
this limitation, we have developed a genetic, inducible model of hyposmia (IH) in which GBCs yield only non-functional OSNs. Specifically, the IH mouse (c-KitCreERT2/+; IFT88fl/fl), when induced, reconstitutes the OE with ciliopathic OSNs. Because olfactory receptor proteins and transduction events are normally localized to cilia, the ciliopathic neurons cannot detect odorants (Buck and Axel, 1991; McIntyre et al., 2012; Williams et al., 2014). We used this mouse model as a host for transplanting purified mouse c-KIT (+) GBCs, given their prior characterization as neuropotent stem cells (Fletcher et al., 2017; Goldstein et al., 2015). Here, we report the validation of the IH mouse model, the development of a simplified, robust cell engraftment paradigm, and demonstrate the efficacy of a cell-based therapy for olfactory loss. We show mature OSNs throughout the epithelium that are derived from engrafted cells and, importantly, demonstrate functional recovery via electrophysiology and behavioral assays.

**RESULTS**

**Purified Adult Olfactory c-KIT (+) GBCs Are Engraftment-Competent Cells**

Within the basal germinial layers of the OE, the c-KIT receptor is expressed on the surface of neuropotent GBCs (Figures 1A and 1B) (Fletcher et al., 2017; Goldstein et al., 2015; Goss et al., 2016). We have previously validated the isolation of live GBCs using immunoselection with antibody to c-KIT (Goldstein et al., 2016). To obtain large numbers of GBCs, we lesioned epithelium by injecting methimazole, which leads to cell death of mature OE and in vivo expansion of the GBC population (Bergman et al., 2002; Leung et al., 2007). By using available mice that express eGFP in all cells, we harvested and purified c-KIT (+) GBCs whose progeny could be traced as they colonized the regenerating epithelium. Cell engraftment was first tested by delivering cell suspension intranasally into
wild-type host mice (Figures 1C and 1D). We found that 5–10 μL droplets of purified GBCs could engraft by simple delivery to the nostrils of briefly anesthetized mice over a 20–30 min period, using small volumes to prevent aspiration. Flooding the nasal fossae with cell suspension, requiring tracheotomy as reported in prior assays (Chen et al., 2004; Goldstein et al., 1998; Jang et al., 2008), was found here to be unnecessary. Histologic examination of tissue 3 weeks following engraftment revealed engraftment-derived cell clusters throughout the OE (≥5 clusters/section, n = 6 mice), identifiable by eGFP expression (Figure 1E). We considered identification of a single group of one or more eGFP-bright cells in the OE to be a “cluster” and did not attempt to draw conclusions about clonality. While auto-fluorescence from lipofuscin or other pigments can be a concern, mice treated with vehicle (no cells) revealed no evidence of the bright eGFP signal. The presence of donor-derived OSNs was readily evident by their morphology, with somata in the middle layers of the pseudostratified OE and apical dendrites ending in dendritic knobs (Figure 1E). Moreover, sections through the olfactory bulb revealed the presence of eGFP-labeled axons in the olfactory nerve layers, which contain the fibers of OSNs projecting from the OE (Figures 1F and 1G). Labeled axons could be seen entering the glomerular layer, consistent with innervation by engraftment-derived OSNs. These local transplant studies confirm that the c-KIT (+) GBCs can engraft into the OE to produce OSNs.

Development of an Inducible Hyposmia Mouse Model

Existing syndromic or congenitally anosmic mice are undesirable transplant hosts because they have other systemic problems (i.e., the polycystic kidney disease model, termed ORPK mouse; Lehman et al., 2008) making studies using adult mice impossible, or they have severe problems with breeding or weaning. Moreover, the development of an experimentally induced loss of smell would more closely mirror the common human clinical conditions marked by acquired sensorineural anosmia or hyposmia, such as post-viral olfactory disorder or presbyosmia. We have developed a novel IH model based on producing ciliopathy selectively in OSNs regenerating after experimental lesion (Figure 2). We generated mice in which tamoxifen-inducible Cre-mediated excision of the intraflagellar transport protein IFT88 in the c-kit lineage results in reconstitution of the OE with neurons lacking normal cilia, incapable of odor transduction. The c-KItCreERT2/+ driver has been extensively validated to drive efficient recombination in the OSN lineage (Goldstein et al., 2015; Goss et al., 2016).

Initially, IH mice were assessed 3 weeks after induction (Figures 2A–2C). Sections from control mice displayed a normal thick layer of neuronal cilia at the apical epithelial surface, visualized by labeling with antibody to acetylated tubulin. In contrast, the regenerated OE of tamoxifen-treated IH c-KitCreERT2/+; IFT88fl/fl mice lacked the thick layer of neuronal cilia (Figures 2B and 2C). The presence of minimal, patchy label is consistent with efficient recombination and generally ciliopathic OE. Electro-olfactogram (EOG) was used to test for olfactory neuron function (Figures 2D and 2E). Since the olfactory receptor proteins and accompanying odor transduction apparatus are localized to OSN cilia, EOG, which detects field responses at the OE surface, can measure olfactory loss due to ciliopathy (McIntyre et al., 2012). Electrophysiologic responses to amyl acetate, a widely used odorant that activates a broad range of olfactory receptors on OSNs, were diminished in IH mice compared with controls by >50%, comparing mean peak amplitudes (Figure 2E). The diminished, but not completely absent, olfactory sensitivity would be expected in an inducible model and resembles many acquired anosmic/hyposmic conditions in humans. Finally, we examined the degree of ongoing cilia depletion in a cohort of mice maintained for 10 weeks on the tamoxifen protocol. As expected from an inducible system, cilia loss was incomplete, yet remained significantly reduced compared with controls (Figure 2F). Overall, histologic and electrophysiologic assessments confirm that our IH model provides a suitable host for testing of cell-based therapy for olfactory loss.

Cell-Treated Mice Recover Olfactory Function

We next asked whether the intranasal treatment of IH mice with wild-type eGFP-labeled c-KIT (+) GBCs would restore olfactory function. In initial experiments, IH mice received either cells (n = 4 mice) or vehicle (n = 5 mice); a control group received only methimazole, and neither tamoxifen nor cells, as a comparison for normal host OE reconstitution. Four weeks after engraftment, mice were euthanized for EOG testing of nasal tissue, as well as processing of tissue for histologic examination (Figure 3A).

Histologically, turbinate tissue was noted to contain large clusters of eGFP-labeled cells throughout the OE, morphologically consistent with engraftment-derived OSNs (Figures 3B–3F and S1). Quantification of engraftment-derived eGFP (+) cells revealed 368 ± 154 cells per coronal section, after applying Abercrombie correction (mean ± SEM, n = 4 host mice), reflecting robust engraftment overall. One mouse was found to have relatively poorer engraftment (Figure 3C) and was therefore excluded from further analyses. Tissue sections from engrafted mice were stained to detect cilia, using antibody to acetylated tubulin (Figures 3D and 3E). While patchy signal was identified across the OE of treated mice, there were clearly identifiable fields with robust cilia staining overlying eGFP (+) engraftment-derived neuronal somata and, at high magnification, their dendritic knobs (Figures 3D and 3E, box). Of interest,
adjacent regions lacking eGFP (+) cells also lacked acetylated tubulin labeling, consistent with host OSNs in our IH mice remaining largely ciliopathic (Figure 3D, asterisk; see also Figure S2).

Labeled fibers were seen in the olfactory nerve and glomerular layer of the olfactory bulb (Figure 3F). Individual eGFP-labeled axon fibers could be seen terminating within most glomeruli; many of which were also found to harbor tyrosine hydroxylase (+) periglomerular cells (Figure 3F), indicative of odor-induced activity (Baker, 1990; Baker et al., 1983). Widespread glomerular innervation is consistent with engraftment-derived neurons expressing a full complement of olfactory receptors, which also function as guidance cues directing axons to appropriate glomeruli (Mombaerts et al., 1996).

EOG testing demonstrated improved odor responses in the cell-treated group (Figure 3G). As expected, there were many fields in cell-treated IH mice with minimal or small-amplitude responses, suggesting that these areas lacked engraftment-derived OSNs. However, testing also revealed areas in nasal specimens from cell-treated mice that yielded robust odor responses. These findings fit with an interpretation that there are scattered clusters of engraftment-derived odor-responsive neurons present across the nasal mucosa. Overall analysis comparing amyl acetate responses in vehicle-treated (−2.73 ± 1.4 mV, mean ± SD) and cell-treated (−4.03 ± 1.4 mV) groups demonstrated improved EOG amplitudes following cellular therapy (p = 0.008).

Behavioral testing was also consistent with olfactory improvement in cell-treated mice. Experiments were repeated, preparing n = 10 mice in each group (controls, IH + vehicle, or IH + cell treatment) to permit behavior testing, since variability in mouse behavior assays are expected; 8–10 mice per group survived for analysis. Using a well-described odor behavior assay (Dewan et al., 2013), the time the mouse spent exploring a control versus an aversive odor was quantified (Figure 3H). Isopentylamine
IPA, an aversive odorant detected only via olfactory neurons, was used for testing (Dewan et al., 2013; Green et al., 2018). Both the control group and the cell-treated group (IH + cells) displayed aversive responses to IPA, while the IH group did not (p = 0.008). These results are consistent with engraftment-derived olfactory neurons providing sufficient input to the olfactory bulbs to drive behavior.

Taken together, our results demonstrate evidence for engraftment, production of odor-responsive OSNs, reinnervation of the olfactory bulbs, and recovery of an
olfactory behavior in cell-treated mice. The efficiency of engraftment, producing several hundred OE cells per section, appears adequate to reestablish meaningful olfactory input in this model.

**GBCs Expanded in Culture Remain Engraftment Competent**

For potential translational use of adult GBCs, expansion of purified cells in vitro will be necessary. Therefore, we also tested the engraftment potential of cells that have been expanded in culture (Figure 4A). Previously, we described a culture model in which purified c-KIT (+) GBCs were capable of expansion in culture by inducing de-differentiation into SOX2 (+) adherent islands (Goldstein et al., 2016). Here, we have found that these de-differentiated cells do not engraft when infused intranasally (and refer to these cultures as “non-engraftable”, Figure 4). Therefore, we modified the culture protocol to permit expansion and early differentiation, exposing cells for 12 h to RepSox, a specific inhibitor of the TGFβR1 receptor, rather than the continuous inhibitor exposure in our existing model. After 10 days, this culture condition yielded adherent islands, semi-adherent spheres, and process-bearing cellular outgrowth as the cultures expanded (Figure 4B). Comparing these modified cultures to our non-engraftable culture model, RT-qPCR demonstrated approximately a 10-fold increase in expression of olfactory neuron lineage genes Tubb3 and Ebf1 (n = 3 biological replicates, Figure 4C). Cultured GBCs prepared from eGFP-expressing mice using the modified protocol were tested by intranasal transplantation into wild-type hosts. As an assay for engraftment, following a 2-week recovery (Figure 4D), all mice were found to harbor engraftment-derived OE cells histologically. We found a low efficiency of engraftment with 11 ± 8.9 clusters per mouse (mean ± SD, n = 4 mice), nonetheless reflecting the presence of engraftment-competent cells in culture.

**Molecular Characterization of Engraftment-Competent Cultures**

We next sought to identify features that define engraftment competence in vitro and hypothesized that the transcriptional profile of these modified cultures should reflect lineage differentiation and the activation of relevant signaling pathways. We performed RNA sequencing (RNA-seq) on cells grown as engraftable cultures as well as cells grown in the standard de-differentiated culture model (non-engraftable) (Figure 4E). Using stringent analysis criteria, including a false discovery rate (FDR) <0.01, fold change of >2, and p < 0.01, approximately 800 genes were differentially expressed (Figure 4E). Considering the culture morphologies and the gene expression analysis, it is apparent that a mixture of cellular phenotypes emerge in the engraftable cultures. In accordance with these findings, Gene Ontology analysis (Table S2) for up- or downregulated genes included terms such as pluripotency signaling, cell differentiation, and nervous system development, as well as broader pathways such as differentiation, cell membrane, cell surface, and immune response. Nonetheless, focusing attention on upregulated genes in the neurogenic pathways enriched in the engraftable cultures (Figures 4F–4I), this subset includes neural progenitor markers such as Dcx (Duan et al., 2008; Jessberger et al., 2008), Wnt pathway/progenitor genes such as Lgr5 (Chen et al., 2014), and olfactory neuron transcriptional regulators such as Lhx2 and the Ebf family (Davis and Reed, 1996; Hirotta and Mombaerts, 2004). Finally, olfactory sensory neuronal genes, including Tubb3, Cd36, and Gfy, are
all highly enriched in the engraftable cultures (Kaneko-Goto et al., 2013; Lee et al., 2015; Xavier et al., 2016) (see also Figure S3 for culture immunocytochemistry). We conclude that purified c-KIT + cells cultured under appropriate conditions can give rise to a mixed population of renewing and differentiating cells, among which at least some cells are at an appropriate developmental stage for engraftment competence.

**DISCUSSION**

Cell-based therapy has potential for the treatment of olfactory sensory losses. Our results indicate that a purified murine adult olfactory stem cell treatment can rescue a mouse hyposmia model due to inducible ciliopathy. Treatment led to the development of engraftment-derived OSNs in the OE, the projection of their axons into the olfactory bulb of the brain, the development of improved electrophysiologic odor responses measured by EOG, and evidence for recovery at the behavioral level. Moreover, efforts to refine the culture conditions demonstrate that it is possible to promote the expansion of engraftment-competent purified adult GBCs.

A broad variety of clinical conditions are associated with hyposmia or anosmia, including genetic defects, trauma, aging, damage arising following viral upper respiratory infections, and conditions that are categorized as idiopathic. Available treatment options are lacking for all of these conditions. Whether the specific etiology causes OSN dysfunction, failures in maintenance of the OSN population, exhaustion within the basal cell neurogenic niche in the OE, or other poorly understood pathogenic etiologies, a cellular replacement therapy capable of restoring the OSN population holds promise for recovery of sensory function. Other therapeutic strategies, such as viral gene therapies, also hold potential for anosmia treatment, but these are usually tailored only to specific conditions, i.e., loss-of-function mutations (McIntyre et al., 2012). Accessing the OE at the olfactory cleft in humans requires only a simple, non-invasive nasal endoscopy, suggesting that the delivery of a local directed therapy is feasible. This accessibility contrasts with other sensory tissues, such as the cochlea, where delivery of a cell-based therapy has been reported in animal models of auditory neuropathy, but required meticulous invasive microsurgical access via the bony modiolus (Chen et al., 2012; Matsuoka et al., 2007).

Another consideration in a cell-based therapy for restoration of OSNs is the normal ongoing turnover within the OE. Would repeat treatments be necessary? The precise "normal" lifespan of specific OSNs is difficult to measure. OSNs are felt to live in the order of months (Carr and Farbman, 1993), but there is evidence for substantial variation, dependent on location within the nose in mice (Gaun et al., 2017). Also, there is clear evidence for activity-dependent mechanisms regulating OSN survival (Santoro and Dulaic, 2012; Zhao and Reed, 2001). We found that, among the eGFP-labeled engraftment-derived cells identified here, some cells are localized among the basal cell layers (e.g., Figure 4D). This is expected, in that the c-KIT (+) population, used in the current treatment approach, has been demonstrated to produce mitotically quiescent or label-retaining GBCs via clonal-resolution genetic fate mapping using the Brainbow2.1 Cre-reporter (Goss et al., 2016). The behavior of subsets of GBCs as label retaining or reserve populations has been described in detail (Jang et al., 2014). We have also performed serial engraftment experiments, providing direct evidence for the serial repopulating potential of basal cells, a classic definition of stemness (Figure S4).

The ability of new OSNs arising from stem cell engraftment in the OE to connect their axons to the olfactory bulb of the brain is required to deliver sensory input. We find here that eGFP (+) axon fibers are abundant in the olfactory nerve and glomerular layers of the bulbs, suggesting robust innervation from engraftment-derived OSNs. Because of the lifelong turnover and replacement of OSNs under normal conditions, it is apparent that a permissive environment for axon growth and guidance exists in this tissue (Barnett and Riddell, 2004; Graziai and Graziai, 1979; Verhaagen et al., 1990). The normal recovery from severe chemical lesion has been well studied in rats and mice, documenting appropriate axon regrowth to the bulbs and generally accurate re-establishment of olfactory receptor topology in the bulbs (Cheung et al., 2013; Iwema et al., 2004). An important finding from recent studies testing viral gene therapy to rescue anosmic mice indicated that successful infection and rescue of only a small percentage of olfactory neurons was sufficient to deliver olfactory input, reflected in behavior testing (Green et al., 2018). Therefore, the necessary efficiency of either a cellular or viral therapy for olfactory loss appears to be easily achievable, at least in animal models.

Other translational challenges for stem cell therapies include potential safety concerns and identification of appropriate cell sources. Using a defined adult stem cell population, we found no evidence of tumor growth or migration of cells intracranially, at least in the time frame of this study. Production of appropriate human cells remains another challenge. However, the recent report of an ability to expand purified adult murine GBCs in culture (Goldstein et al., 2016) should inform efforts to manipulate and propagate similar populations from human OE. It is important to contrast the cell populations used here from reports that have utilized mesenchymal cell populations from human nasal tissue (Delorme et al., 2010; Murrell...
et al., 2005; Nivet et al., 2011), highlighting the need for accurate nomenclature for adult “stem cells.” To date, there is no evidence that the nasal mesenchymal cells are in the OSN lineage. Importantly, the c-KIT (+) GBCs used here arise in the OE, not the mesenchyme, and are bona fide OSN stem cells (Goldstein et al., 2015; Goss et al., 2016). Further attention directed at cultivating human GBCs in vitro is required; alternately, reprogramming efforts to direct other somatic cells toward an olfactory progenitor phenotype may be of interest. Analysis of our transcriptional profiling from engraftment-competent cultures may inform these efforts, providing insights into the cellular phenotypes that are most relevant, and features such as cell surface proteins that correlate with engraftment competence.

In summary, there is a need for development of novel therapies for correction of sensory losses (Bermingham-McDonogh et al., 2012). We report here evidence for the ability to utilize a cell-based therapy for the treatment of sensorineural olfactory loss in an adult animal model. These findings provide proof of principle for an approach that has the potential to be of broad utility for a range of clinical conditions causing anosmia.

**EXPERIMENTAL PROCEDURES**

**Animals**

The Institutional Animal Care and Use Committee, University of Miami, approved all procedures. Mice were bred on a C57BL/6 background. Mouse strains included wild-type C57BL/6J (Jackson Lab, Bar Harbor, ME), c-KITCreERT2/+ provided by Dr. Dieter Saur, Technical University of Munich (Klein et al., 2013), IFT88floxed (stock no. 022409, Jackson Laboratory) possessing loxP sites flanking exons 4–6 of the IFT88 gene (Haycraft et al., 2007), and B6-eGFP (stock no. 003291, Jackson Laboratory), which expresses eGFP strongly in widespread tissues. The IH mouse was generated by backcrossing to create the c-KITCreERT2/+; IFT88floxed genotype.

Methimazole lesion was induced by giving a single dose of 75 μg/g body weight intraperitoneally for IH mice or 50 μg/g for B6-eGFP mice. Tamoxifen (Sigma, St. Louis) was dissolved in peanut oil and delivered at 75 mg/kg daily for 5 days and continued every 2–3 days in adult mice to induce recombination, starting 1 day following methimazole administration.

**Transplantation**

B6-eGFP donor mice were lesioned with methimazole 8 days prior to tissue collection. Cells from 3–4 mice were pooled for each host mouse engraftment. Host mice received methimazole 2 days prior to engraftment. Donor mice were decapitated following ketamine/xylazine anesthesia. Olfactory mucosa was collected from turbinates and septum, free of bone, and c-KIT (+) GBCs were purified based on immunomagnetic sorting, as described (Goldstein et al., 2016). Briefly, tissue was dissociated using collagenase 1 mg/mL, dispase 2 mg/mL with EDTA 1 mM for 20 min, followed by 2 min treatment with 0.0125% trypsin solution (Invitrogen). The reaction was stopped using fetal bovine serum (FBS), and cells were washed twice with Hank’s balanced salt solution (HBSS) and passed through a 70 μm strainer. The pellet was resuspended in 300 μL of buffer (HBSS containing 2% FBS and 1 mM EDTA). Cells were incubated with APC-anti-c-KIT diluted 1:20 (eBioscience no. 17-1171, San Diego, CA; RRID: AB_469430) for 15 min, followed by APC magnetic selection kit (STEMCELL Technologies, Vancouver, Canada) per instructions. Cells were resuspended in a solution of DMEM:F12 with HEPES (Invitrogen, Carlsbad, CA) containing 5% basement membrane extract (Geltrex, Invitrogen no. A14132), 5% FBS (Invitrogen), penicillin-streptomycin (Invitrogen), n-acetylcysteine (5 μg/mL; Sigma), Y27632 10 μM (STEMCELL Technologies), and held on ice. Host mice were lightly anesthetized, and cells were introduced to the naris via 5–10 μL droplets in 5 min intervals, alternating between the nostrils. Approximately 25 μL total volume was delivered per naris.

**Statistics**

Statistical comparisons were performed with Prism 7 (GraphPad), using the appropriate t test or ANOVA. p < 0.05 is considered significant. When feasible, blinded analysis was performed. For histologic cell counts from adjacent 10 μm cryosections (Figure 3), cell soma of 6 μm was estimated and Abercrombie correction was applied N = n × (T/(T + d)), where N = corrected count, n = cells counted per section, T = thickness of section, d = estimated cell soma diameter; each slide contained six adjacent sections, six slides per mouse. For EOG comparisons in Figure 3, testing indicated data were not normally distributed, therefore ANOVA with Kruskal-Wallis multiple comparison testing was used.

**Cell Culture**

To expand purified GBCs in vitro, we modified our previously detailed culture protocol (Goldstein et al., 2016) slightly. For “engraftable cultures,” cells were selected based on c-KIT expression as described above and were plated onto gelatin-coated culture dishes at approximately 10⁵ cells per well in NeuroCult NS-C Basal Medium, EFG 20 ng/mL, FGF2 10 ng/mL, heparin 2 μg/mL (all from STEMCELL Technologies), and penicillin-streptomycin (Invitrogen, Carlsbad, CA). RepSox (25 μM, STEMCELL Technologies), a TGF-β type 1 receptor (ALK5) inhibitor, was added along with Y27632 (10 μM, STEMCELL Technologies) overnight. Cultures were then maintained in base medium without inhibitors and were split 1:3 when 80% confluent, collecting both floating spheres and adherent cells. For “non-engraftable cultures,” additional cultures were prepared following our published protocol exactly, maintained in NS-C medium with SB431542 (10 μM) and BMP4 (10 ng/mL). For both culture preparations, ≥3 biological replicates were prepared using cells pooled from three mouse noses for each sample.

**Electro-olfactograms**

Air-phase EOGs were performed following established protocols (Cygnar et al., 2010). Details and reagents are provided in Supplemental Information.
Behavioral Assays
An olfactory avoidance assay was modified from published protocols (Dewan et al., 2013). Testing took place during the nocturnal phase. Freshly cleaned standard mouse housing cages without bedding were used as test arenas, divided into an odorized compartment with a curtain, covered with a plexiglass lid. Testing was performed in dim red light conditions, blocking visual cues or distractions. Mice were recorded using an infrared camera. The mice were habituated in the test arena for 3 min. Whatman 1 filter paper circles (2.5 cm diameter) in a 35 mm Petri dish were either impregnated with 20 μL of water as odorless control or with 10% IPA to test the ability to detect an aversive scent. The time spent investigating the odorized chamber was analyzed. Aversion index = timeodor – timewater. Control, IH mice, and cell-treated IH mice were tested 8–10 weeks post treatment (n = 8–10 mice per group), and data were analyzed by ANOVA with multiple comparisons.

RNA Sequencing
RNA-Seq Sample Preparation
Total RNA was prepared from olfactory cultures using column purification per protocol (Zymo Research Corp., Irvine, CA, USA). DNase I on-column digestion was performed. Samples were prepared in biological triplicates. Preparation and sequencing of RNA libraries was carried out in the John P. Hussman Institute for Human Genomics Center for Genome Technology (University of Miami Miller School of Medicine). Quality analysis was performed using an Agilent Bioanalyzer to confirm RNA integrity score >9. Using 500 ng of total RNA as input, the TruSeq Stranded Total RNA Library Prep Kit with Ribo-Zero (Illumina, San Diego, CA) was used to create ribosomal RNA-depleted sequencing libraries. Each sample had a unique barcode to allow for multiplexing and was sequencing to 35 million raw reads in a single-end 75 bp sequencing run on the Illumina NextSeq500.

RNA-Seq Bioinformatics Analysis
Raw sequence data were processed by the on-instrument Real Time Analysis software (v.2.4.11) to basecall files that were converted to de-multiplexed FASTQ files with the Illumina supplied scripts in the BCL2FASTQ software (v2.17). The quality of the reads was determined with FASTQC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) for per base sequence quality, duplication rates, and overrepresented k-mers. Illumina adapters were trimmed from the ends of the reads using the Trim Galore! package (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Reads were aligned to the mouse reference genome (mm10) with the STAR aligner (v2.5.0a) (Dobin et al., 2013). Gene count quantification for total RNA was performed using the GeneCounts function within STAR against the ENSEMBL v77 mouse transcript.gtf file.

Differential Expression and Pathway Analysis
Gene count data were input into edgeR software (Robinson et al., 2010) for differential expression analysis. Briefly, gene counts were normalized using the trimmed mean of M values (TMM) (Robinson and Oshlack, 2010) method to account for compositional difference between the libraries. Group differential expression was calculated using the generalized linear model likelihood ratio test (glmLRT) function implemented in edgeR. Genes meeting a nominally significant threshold (p < 0.05 and fold change ≥1.5) were input into The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 for pathway enrichment analysis (Huang da et al., 2009a, 2009b).

Imunochemistry and RT-qPCR
Details and reagents are provided in Supplemental Information.

ACCESSION NUMBERS
Sequence data generated from the RNA-sequencing experiments have been deposited in GEO under accession number GEO: GSE124443.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.stemcr.2019.05.001.

AUTHOR CONTRIBUTIONS
B.J.G., N.C., J.M.H., and S.K. designed the research; S.K., G.M.G., S.G., R.C., and B.J.G. performed experiments; S.K., G.M.G., S.G., N.C., R.C., and B.J.G. analyzed data; S.K. and B.J.G. wrote the manuscript; N.C., J.M.H., and B.J.G. edited the paper.

DECLARATION OF INTERESTS
J.M.H. discloses a financial interest with Longeveron.

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