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On the in vivo origin of human nasal mesenchymal stem cell cultures

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Abstract

Objectives: Mesenchymal stem cells (MSCs), classically expanded in culture from bone marrow, are of broad interest to the regenerative medicine community. Human nasal turbinate mesenchymal-like stem cell cultures have also been described, defined by an in vitro phenotype similar to bone marrow MSCs. Nonetheless, the identity in vivo of the cells that give rise to nasal MSC-like cultures remains unclear, and these cells are often suggested to be related to olfactory lineages. Here, we sought to define the in vivo phenotype of human nasal MSC-like cells.

Methods: Human turbinate tissue samples were used for RNA and immunohistochemical analysis. We also analyzed a recently published single cell RNA-sequencing dataset from adult human olfactory and respiratory mucosa samples from our lab, to focus on cell populations expressing MSC markers. Immunochemistry was performed to stain turbinate sections and nasal MSC cultures for selected markers.

Results: While there is no single MSC-specific gene, we identified a human nasal mucosal cell population in vivo that uniquely expressed transcripts characteristic of typical MSC cultures, including ENG (CD105), NES, and CD34, and lacked expression of other transcripts associated with surface epithelia. The expression of transcription factors such as SOX17, EBF1, and FOXP1 suggests cells in the MSC-like cluster maintain an ability to direct cell fate, consistent with the behavior of nasal MSC-like cells in vitro. SOX17 was found to be uniformly expressed by nasal MSC cultures, consistent with the in vivo data. Immunohistochemistry of human nasal tissue samples indicated that ENG, CD34, and SOX17 expression localized selectively to cells surrounding blood vessels in the lamina propria.

Conclusion: Our findings provide evidence that the in vivo origin of nasal MSC-like cultures is likely a vascular or pericyte population, rather than cells related to the olfactory neuronal lineage.

Level of evidence: NA.

1 | INTRODUCTION

An ability to cultivate cells in culture that exhibit self-renewal and a capacity for lineage-specific differentiation, hallmarks of stemness, has led to interest in the use of stem cell cultures as cell-based treatment strategies for a range of diseases. Stem cell culture models include (a) embryonic pluripotent stem cells, (b) genetically or chemically reprogrammed somatic cells, termed induced pluripotent stem cells (iPSCs), or (c) tissue-specific stem cells that support cellular renewal or homeostasis in adult tissue niches[1-3]. The best-characterized adult tissue-specific stem cells are bone marrow-derived hematopoietic stem cells, which can produce the range of blood cell lineages and have been utilized clinically for many years in bone marrow transplants.[4] An additional bone marrow-derived stem cell population, termed bone marrow mesenchymal stem cells (MSCs), is thought to function in support of the hematopoietic stem cell niche.[5-7] MSCs are typically described as cells capable of growth on tissue-culture plastic with a capacity to form various mesodermal phenotypes, expressing surface glycoproteins such as CD105 (Endoglin), and lacking expression of lineage-specific markers. In recent years, using these general criteria, MSC-like cells have been propagated in culture from many adult tissues, including the human nasal mucosa.[8-11]

In the nasal cavity, specific mucosal progenitor cell pools can be distinguished, including: (a) the neurogenic basal stem cells within the olfactory neuroepithelium, restricted to the olfactory cleft[12-14]; (b) respiratory epithelial basal cells that homeostatically replenish the secretory and ciliated cells; (c) submucosal glands which may also house a reserve epithelial stem cell population, especially in the lower airways[15]; and (d) stromal cells situated deep to the olfactory or respiratory surface epithelium, housing an undefined population that appears to give rise to MSC-like cells in vitro.[10,16] The basal cells of the rodent adult olfactory neuroepithelium have been well-characterized, and include stem cells capable of producing olfactory neurons and sustentacular cells, under conditions of normal turnover or in response to experimentally-induced injury.[13,14,17] Recent data using single cell analysis techniques have identified similar progenitor cell populations from adult *human* olfactory mucosa.[18] However, human nasal MSC-like cells have been described only from cell culture models, based on their ability to be easily grown in vitro from turbinate or nasal septal tissue. Characterization of nasal MSC-like cultures typically reveals expression of markers common to bone marrow MSCs, and an ability to give rise to bone, cartilage, or adipocyte lineages, and possible neuronal-like phenotypes in vitro.[8,19] Given the highly regenerative nature of adult olfactory neuroepithelium, there has been frequent speculation that nasal MSC-like cultures may arise from cells of olfactory lineages, and enthusiasm for the use of nasal

MSCs therapeutically.[20,21] Nonetheless, the identity of the cells within adult human nasal mucosa that are the in vivo correlate of the MSC-like cell cultures has remained poorly understood.

In an effort to identify the in vivo phenotype of adult human nasal MSCs, we utilized newly available single cell transcriptional profiling data along with immunochemical studies. Our lab has recently analyzed adult human nasal mucosa biopsies using single cell RNA-sequencing (scRNA-seq), providing datasets enriched for respiratory and olfactory mucosa.[18] Here, we have focused attention to cell populations expressing known MSC-like markers, and combined this analysis with immunochemistry using nasal MSC cultures and histologic sections to localize in vivo the cells expressing an MSC-like phenotype.

2 | METHODS

2.1 | Human nasal tissue biopsy

Human nasal mucosa samples (n = 6, age 37-75 years) were obtained with patient consent and with protocol approval by the Institutional Review Board through the Human Subjects Research Office at the University of Miami, as described previously.[10,18] Nasal tissue was obtained from adult patients undergoing transnasal endoscopic surgery to access the pituitary or skull base. Once the mucosa was carefully excised from nasal septum, superior turbinate, or middle turbinate, samples were held on ice in Hank's Balanced Salt Solution. Samples were dissected to remove bone and deep stroma, while preserving the epithelium and underlying lamina propria. Portions of the specimen were processed to use for histology or cell culture; datasets from biopsy samples for RNA analysis were previously described.[18]

2.1.1 | Single-cell RNA sequencing analysis

Data from our previously published scRNA-seq datasets[18] obtained from adult human nasal mucosal samples were re-analyzed here (GEO accession: GSE139522). For the individual sample analysis (Figures 1 and 2), raw data processing was performed using Cellranger (2.1.1). Seurat (v2.3.4) was utilized for filtering to retain cells that had greater than 400 UMIs, expressed between 100 to 8000 genes inclusive, and mitochondrial content less than 10%. Data were normalized using the "LogNormalize" method and using a scale factor of 10 000. Using Seurat's Scale. Data () function and "vars.to.regress" option UMI's and percent mitochondrial content were used to regress out unwanted sources of variation. The number of variably expressed genes was calculated using the following criteria: normalized expression between
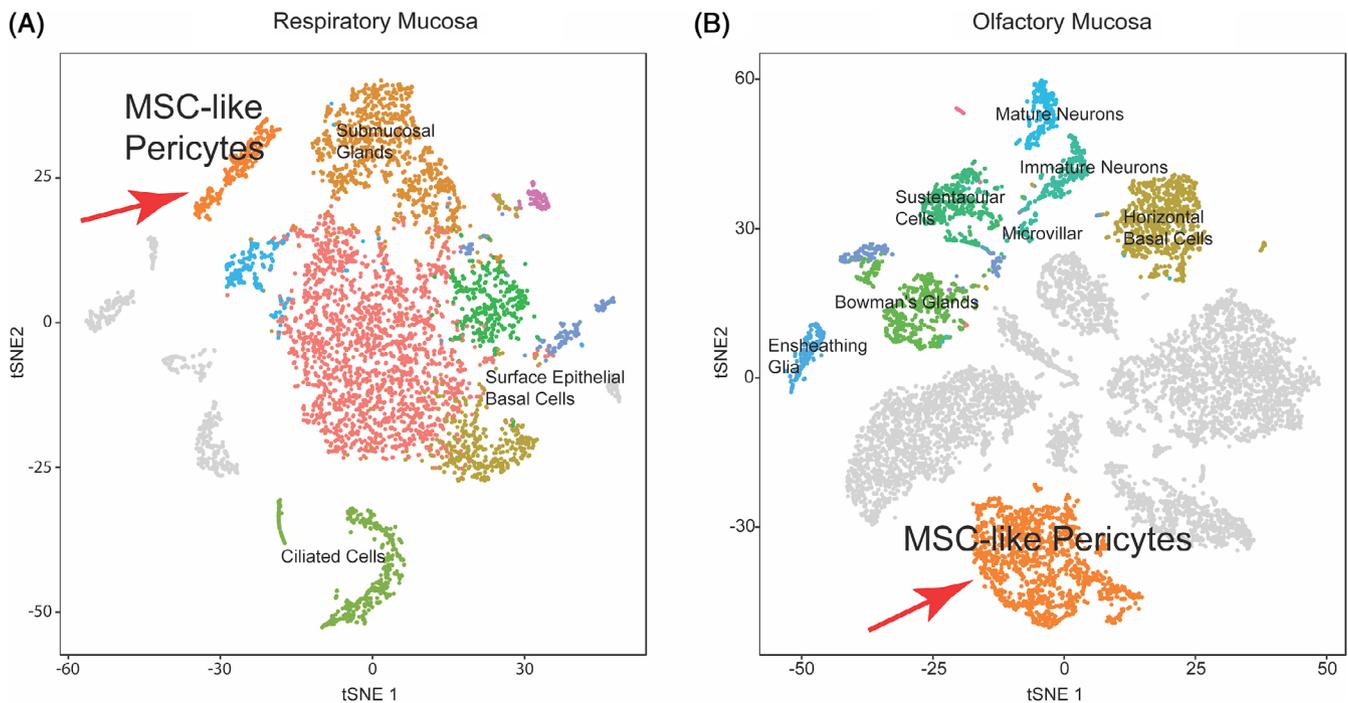
FIGURE 1 Cell populations isolated from human olfactory and respiratory mucosa. scRNA-seq data from two biopsy samples of adult human turbinate tissue, individually visualized by t-distributed stochastic neighbor embedding (tSNE) plots; cells are clustered based on feature similarities. The phenotypes of key cell clusters are indicated, based on gene expression profiles. A, Respiratory mucosa (*n* = 1; 5650 cells) and B, olfactory mucosa (*n* = 1; 11 188 cells); note differing surface epithelial populations in each tissue sample. Red arrow points to cluster of MSC-like pericytes common to both biopsies

0.125 and 3, and a quantile- normalized variance exceeding 0.5. To reduce dimensionality of this dataset, principle component analysis (PCA) was performed, and the first 30 principle components further summarized using t-distributed stochastic neighbor embedding (tSNE) dimensionality reduction.[22] The RunTSNE() wrapper function was used with the Barnes-Hut implementation of the "Rtsne" package (0.15). The Seurat (v3.1.5) UpdateSeuratObject() and DotPlot() functions were utilized to generate the plots from the Seurat v2 dataset generated above. For the aggregate sample analysis (Figure 3), raw data processing was performed using Cellranger (3.0.2). Analysis in Seurat (v3.0.0)[23] was performed as described previously[18] in R (3.5.2), to specifically investigate cells expressing MSC-like marker genes. Briefly, using the top 30 principle components, data were visualized by tSNE.[22] Cluster metadata was obtained[18] to annotate cell subpopulations, using analysis of transcript enrichment and comparison to known cell markers to assign phenotypes to cell clusters. Gene ontology (GO) analysis was performed here to identify biological processes enriched within distinct clusters of cells, using ToppGene.[24,25] The DotPlot function in Seurat (v3.1.5) was used to visualize selected transcript expression among clusters.

2.1.2 | Immunochemical staining

Nasal tissue samples used for histology were fixed within 1 hour of harvest in 4% paraformaldehyde in phosphate buffer for 3 hours at room temperature, rinsed in phosphate-buffered saline (PBS), cryoprotected in 30% sucrose/250 mM EDTA-PBS solution, then frozen in optimal cutting temperature medium (O.C.T., VWR, Radnor, PA), and cryosectioned at 10 μm on a Leica CM1850 cryostat. Tissue sections were mounted onto Superfrost Plus glass slides (VWR) and stored at −20°C. Tissue sections were treated with an ethanol gradient (70%, 95%, 100%, 95%, 70%), rinsed in PBS, and incubated in blocking solution (5% bovine serum albumin, 4% non-fat dry milk, and 0.01% Triton X-100) for 30 minutes at room temperature. Primary antibodies diluted in the blocking buffer were incubated overnight on tissue sections in a humidified chamber at 4°C. Species-specific fluorescent-conjugated secondary antibodies were used for detection by incubating with tissue sections for 45 minutes at room temperature. All tissue sections were counter-stained with 4,6-diamidino-2-phenylindole (DAPI). Images were obtained using a Leica DMi8 microscope and images were processed using ImageJ. See Table 1 for list of antibodies and dilutions.

For immunocytochemical staining of cultured cells, cultures were washed with PBS and fixed in 4% paraformaldehyde for 10 minutes at room temperature. Fixed cells were washed with PBS and incubated in blocking buffer for 30 minutes at room temperature. Primary antibodies and secondary antibodies were applied as described above, followed by imaging as described.

2.1.3 | Olfactory mucosa cell cultures

MSC-like cultures were maintained per previously published protocol.[10] Briefly, cells below passage 8 were grown on tissue-culture
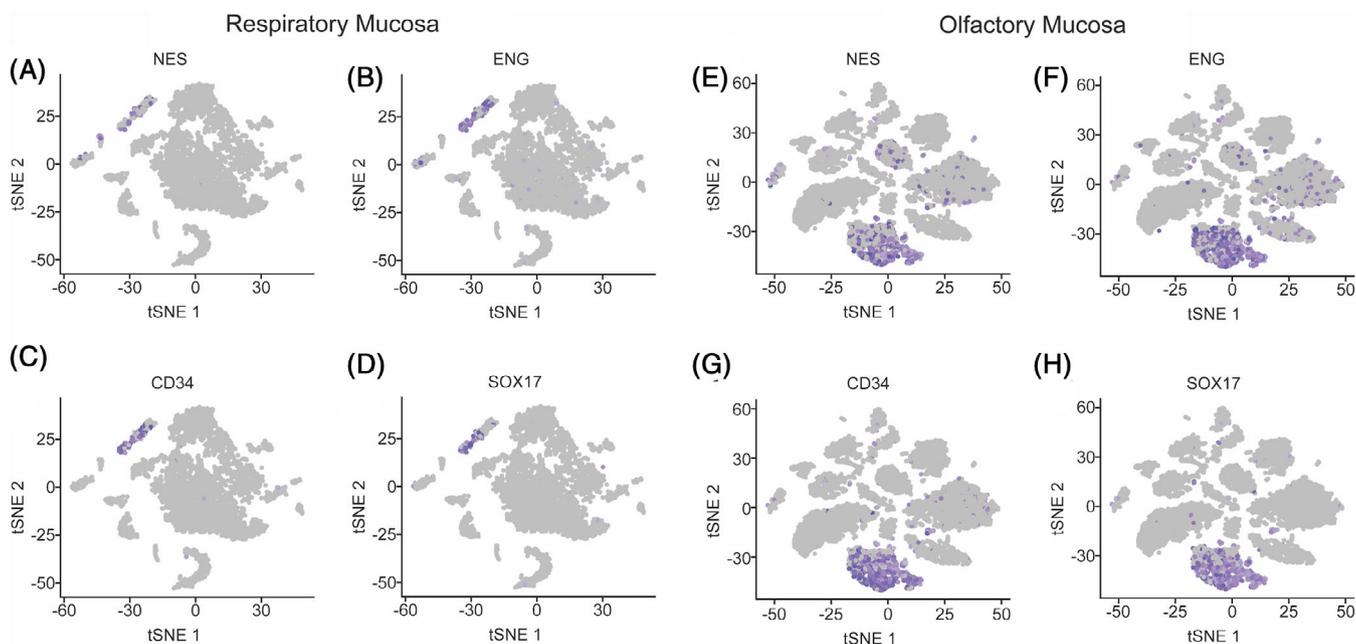
FIGURE 2 Gene expression profiles of respiratory and olfactory mucosa samples. For two individual biopsy samples, feature plots visualize gene expression (purple) of NES, A and E, ENG, B and F, CD34, C and G, and SOX17, D and H, in respiratory and olfactory mucosa. The cell clusters enriched for expression of these transcripts correspond to pericyte/MSC-like cell clusters in Figure 1 tSNE plots

treated plastic flasks in DMEM with 10% fetal bovine serum (Gibco) and penicillin-streptomycin supplement (Invitrogen). To increase homogeneity of cultures, cells used here were also treated by immunomagnetic positive selection using antibody to CD271 per manufacturer's protocol (#18659, Stemcell Tech), as described.[26] The CD271+ fraction was then expanded, characterized by flow cytometry as described,[10] and cryopreserved as nasal MSCs for the experiments described here.

3 | RESULTS

Datasets from scRNA-seq from human nasal mucosa[18] (n = 4 subjects) were utilized here. Graph based cell clustering, based on feature similarities using the top 30 principle components, was visualized in two dimensions via tSNE plot (Figure 1). Cell cluster phenotypes were annotated previously using GO and pathway analysis, as described.[18] Individual samples were comprised of cell types found in respiratory mucosa or olfactory mucosa. One respiratory-enriched sample and one olfactory-enriched sample are visualized in Figure 1. Respiratory mucosa included basal cells, secretory/goblet cells and ciliated cells (Figure 1A), while olfactory mucosa contained olfactory sensory neurons, basal cells, and sustentacular cells (Figure 1B). A variety of leukocytes were present, not annotated further on these plots, for simplicity. All biopsies also included lamina propria cell populations, as expected.

Focusing attention to the expression of genes typically expressed by MSCs, we identified a MSC-like population present in each of the nasal tissue samples, defined by the co-expression of Endoglin

(ENG/CD105), and NES (Figure 2A,B,E,F).[7] The MSC markers localized uniquely to the cluster previously annotated as pericytes, a population expressing vascular-related genes. These MSC-like clusters also expressed CD34 (Figure 2C,G), a surface glycoprotein associated with MSC-like adventitial pericytes,[27] and weakly present on hematopoietic progenitors. In addition, the transcription factor SOX17 was strongly expressed in the pericyte/MSC-like clusters (Figure 2D,H). SOX17 is a transcription factor expressed in endoderm differentiation during development, and is important for arterial specification. In adults, SOX17 maintains arterial identity in mature vasculature.[28] None of the markers used to define the MSC-like cells, including SOX17, were expressed by epithelial cells in either respiratory or olfactory samples, suggesting the MSC population resides in the underlying lamina propria.

We further analyzed the single cell data using integrated respiratory and olfactory samples from 4 biopsies, and re-projected the combined data as a single tSNE plot (Figure 3A). In the combined plot, the MSC-like cells clustered together as a single population, as marked in the plot (red arrow). A DotPlot was utilized to visualize expression of genes of interest across all populations in the dataset (Figure 3B). By DotPlot, LEPR, CD34, ENG, and SOX17 are highly specific to the cluster previously annotated as pericytes. Other genes, such as transcription factors EBF1 (Early B-Cell Factor 1) and FOXP1 expressed by cells within the pericyte cluster, are expressed more broadly. While EBF1 is also expressed in olfactory neurons, it is not an olfactory-specific gene, having important functions in hematopoietic cells to orchestrate B cell differentiation.[29,30] We further visualized genes of interest overlaid onto the tSNE plot (Figure 3C), showing their enrichment in the pericyte cluster. The contribution of cells from individual
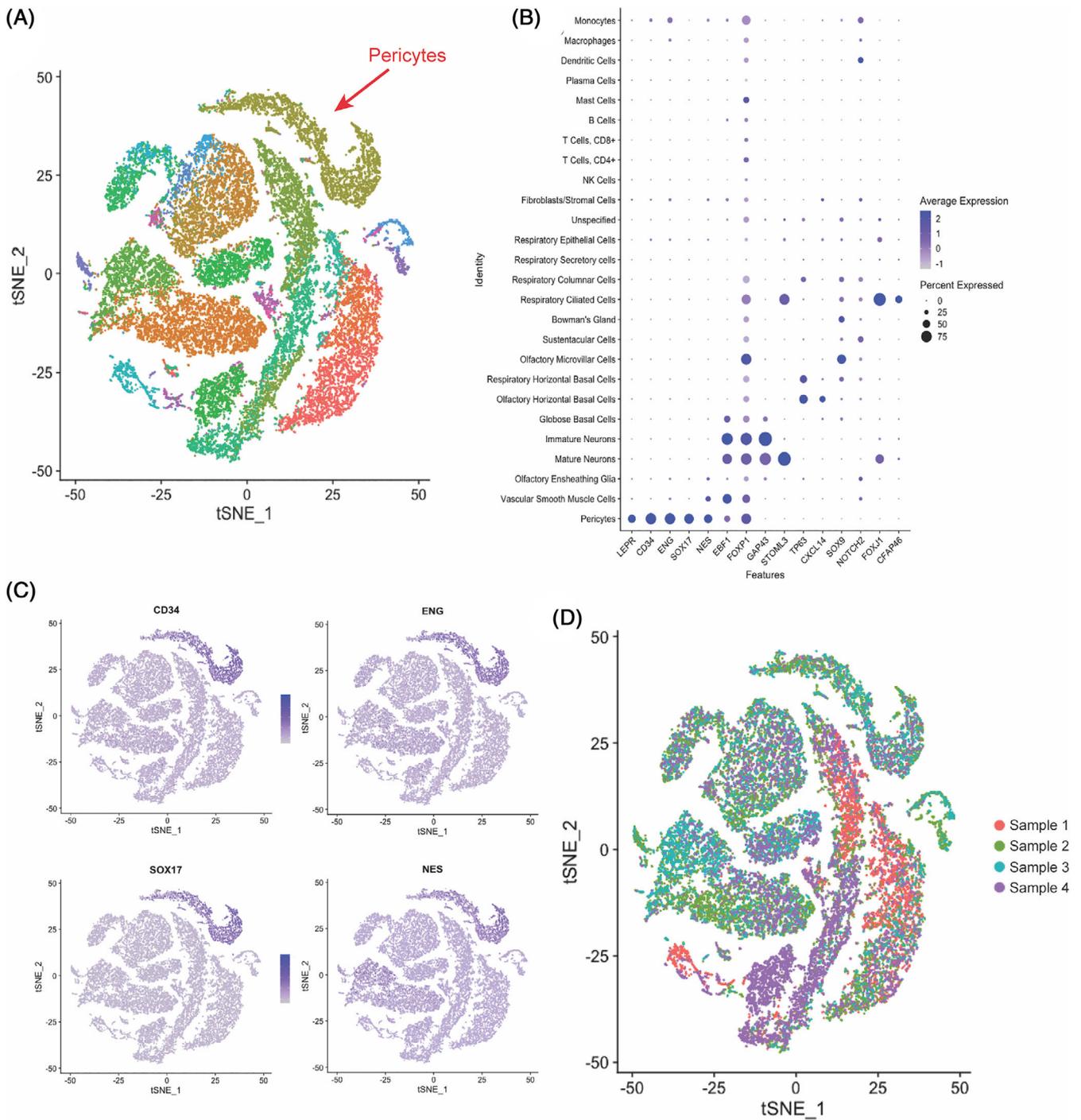
FIGURE 3 Aggregate analysis of nasal tissue biopsies. Data from the respiratory and olfactory mucosa scRNA-seq analysis were combined to create a single aggregate dataset. The plots depict annotated clusters from 28 726 combined olfactory and respiratory mucosal cells, n = 4 patients. A, Integrated dataset, projected in a single new tSNE plot (4 biopsy samples combined). Red arrow indicates the pericyte/MSC-like populations, clustering together. B, DotPlot analysis visualizes gene expression within clusters (rows) as well as the proportion of cells (dot size) in a cluster expressing gene of interest (column labels); color reflects average expression level. The red arrow points to the pericyte/MSC-like cluster. Note that MSC-like marker genes are only expressed within the pericyte cell cluster. Other selected cell type-specific marker genes are provided for reference. C, FeaturePlots visualizing the distribution of gene expression across tSNE plots for indicated genes. D, tSNE plot depicting the contribution of cells from individual samples by color, as indicated in legend (5538-11 184 cells per sample, n = 4 samples); cells from all four biopsies are localized within the pericyte cell cluster

biopsies, by color, was also plotted (Figure 3D), confirming that cells from each sample contribute to the pericyte cluster, as expected. It is important to consider that further sub-clustering of cell populations

may be possible, adjusting numbers of principle components or resolution parameters in the analysis algorithms. However, we show here that the cells expressing the pericyte/nasal MSC-like genes are

TABLE 1 Antibodies used for immunofluorescence

Reagent	Source	Identifier	Dilution
Mouse anti-CD105	Bio-Rad	Cat# MCA1557T; AB_1100559	1:50
Mouse anti-CD34	Bio-Rad	Cat# MCA1578; AB_1125259	1:50
Rabbit anti-SOX17	Cell Signaling Technologies	Cat# 81778; AB_2650582	1:200
Mouse anti-TuJ1	BioLegend	Cat# 801201; AB_2313773	1:500
AlexaFluor 594 Goat anti-Mouse	Jackson ImmunoResearch	Cat# 115-585-146; AB_2338881	1:100
AlexaFluor 488 Donkey anti-Mouse	Jackson ImmunoResearch	Cat# 715-545-150; AB_2340846	1:100
AlexaFluor 594 Goat anti-Rabbit	Jackson ImmunoResearch	Cat# 111-585-144; AB_2307325	1:100
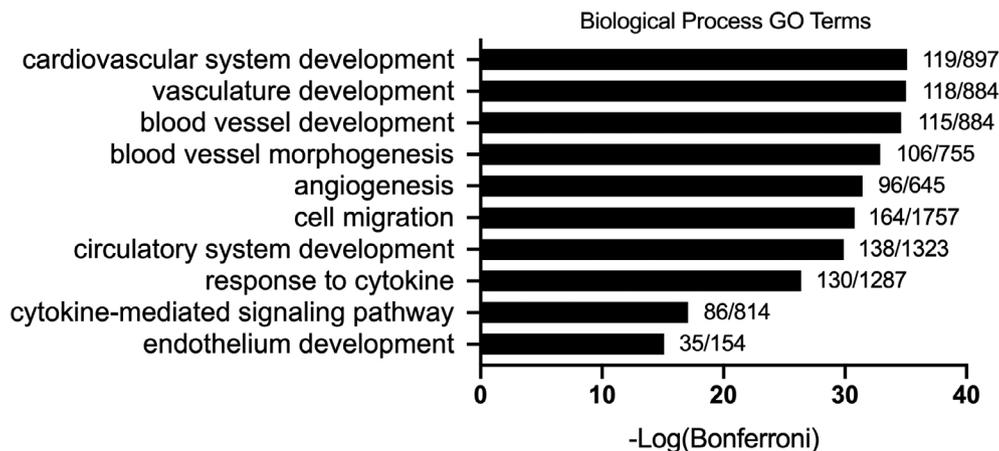
FIGURE 4 Gene ontology (GO) analysis of highly enriched genes within MSC-like pericyte cluster. A total of 702 genes were enriched in the MSC-like pericyte cluster. Of those, 691 were recognized by the GO algorithm and were inputted for subsequent analysis. A selection of biological process GO terms are graphed in decreasing order of significance, −Log(Bonferroni). Numbers to the right of each bar represent the number of genes annotated to the GO term from the input out of the total number of genes present in the given annotation

clustered together. In addition, the analysis yielded a list of >500 genes that were enriched in the pericyte/MSC-like cell cluster; in a GO analysis to identify biological processes associated with this population, top terms included "cardiovascular development," "vascular development," "blood vessel development," and "angiogenesis" (Figure 4), consistent with a lamina propria vasculature-associated phenotype.

Immunohistochemistry was performed to validate scRNA-seq findings and to localize expression of MSC makers in vivo. Human nasal tissue sections were stained using antibodies directed against ENG and CD34 (Figure 5A,B). ENG+ and CD34+ labeling was located within and surrounding blood vessels in the lamina propria, consistent with the RNA analysis identifying expression of these genes in the pericyte population. Staining with anti-SOX17 (Figure 5C) revealed that SOX17+ cells are also distributed within the lamina propria vasculature. Antibody TuJ1 against neuron-specific β-tubulin confirmed the presence of olfactory neurons on adjacent sections (not shown). SOX17+ nuclei could be seen clearly scattered in the lamina propria, and on close inspection surround blood vessel lumens (Figure 5D). SOX17+ cells were absent from the overlying olfactory neuroepithelium.

Human nasal MSC-like cultures were also analyzed for SOX17 expression. Low passage nasal MSCs, characterized as described previously,[10] were fixed and processed for immunocytochemistry

(Figure 5E). In agreement with our scRNA-seq analysis and immunohistochemistry results, nasal MSC-like cultures strongly expressed SOX17 protein in their nuclei. The expression of SOX17 in nasal MSC-like cells in vitro suggests these cultures arise from a pericyte or vascular cell in human turbinate lamina propria tissue, rather than from cells associated with olfactory neuroepithelium populations.

4 | DISCUSSION

In the present study, we show that a population of mucosal vascular pericytes is the most likely cellular origin of human nasal MSC-like cultures. We identified the co-expression of MSC glycoproteins (ENG), a bone marrow pericyte marker (LEPR), and a pericyte/endothelial-specific transcription factor (SOX17) by a unique population of nasal mucosal cells isolated from adult humans. In histologic sections, expression of these markers localized to peri-vascular regions in the lamina propria, completely distinct from the surface epithelium. Nasal MSC-like cultures propagated from human turbinate tissue using standard protocols were also found to continue to express SOX17 uniformly in vitro. Together, the data suggest strongly that the in vivo origin of nasal MSC-like cultures is likely to be a pericyte population.
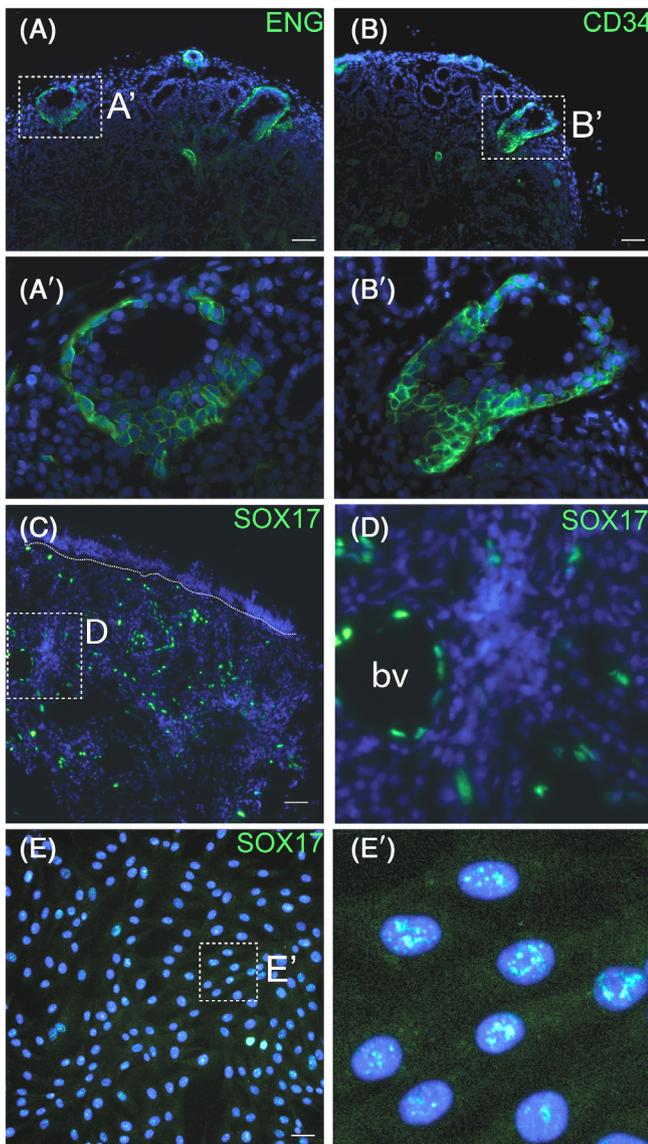
FIGURE 5   Immunofluorescence staining of nasal tissue biopsies and nasal MSC cultures. Human nasal turbinate tissue probed with antibodies against A, ENG; B, CD34; and C, D, SOX17. E, Human nasal MSC-like cultures stained for SOX17. Nuclei are counterstained with DAPI; bv, blood vessel, scale bars = 50 μm in panels A-C, 20 μm in panel E

It is important to consider proper terminology when referring to human nasal culture systems, as the MSC-like cell phenotype is quite distinct from the epithelial basal cells of the respiratory surface epithelium or of the olfactory neuroepithelium, which express keratins and/or distinct transcription factor programs including TP63, SOX2, or members of the neurogenic basic helix-loop-helix families such as NEUROG1 or HES6.[12,15,17] Furthermore, understanding the biological origins and in vivo lineage capacities of cell culture models has important implications regarding downstream assays or applications. For instance, nasal cell cultures have been used to investigate epigenetic changes in neuronal-like cells, or in models of neural cell-based therapies.[20,31,32] An accurate understanding of the culture model cell phenotypes is necessary to inform downstream experiments.

In our analysis, the transcription factor SOX17 was exclusively expressed within the pericyte/MSC-like cell clusters. Given that transcription factors confer and maintain cell identity, they are reliable biomarkers for classifying cell phenotypes. Immunohistochemically, SOX17 + cells in nasal tissue were present only within the lamina propria, underneath the surface epithelium and generally near blood vessels. This finding is consistent with the known function of SOX17 as a transcription factor important for regulating differentiation of endodermal organs in development, as well as the differentiation, maturation, and maintenance of arterial vasculature in adults.[28,33] Other markers such as CD34 and ENG were also only identified in the lamina propria, particularly around blood vessels, with no detectable expression in surface epithelium. These findings, in combination with bioinformatic pathway analysis of gene expression sequencing data, identify the population as vascular pericytes. In other tissues, pericytes have been reported to hold stem-like properties and to be a prominent source of multipotent MSC-like cultures, consistent with the findings reported here.[6,34-40]

Under certain culture conditions, pericyte-derived MSC-like cultures are capable of generating cells with neurite-like outgrowths expressing neuron-specific β-tubulin, recognized by antibody TuJ1.[34,37] However, in the absence of comprehensive characterization, the term "neuron-like cells" is favored for MSC-derived phenotypes, as the in vitro generation of functional neuronal cells from such cultures remains controversial. The endoscopic accessibility of turbinate tissue, and reliable cell culture protocols for propagation of nasal MSC-like cells has, nonetheless, made them a subject of considerable interest for potential cell-based therapies.

5 | CONCLUSION

We report that a vascular pericyte population residing within the lamina propria of human nasal mucosa is the likely source of nasal MSC-like cultures. Although variations on the term "olfactory stem cell" are frequently used to describe MSC-like cultures derived from nasal mucosal tissue, no evidence exists to suggest that vascular pericytes are in the olfactory neuron lineage in vivo. These findings have implications for studies utilizing nasal MSC-like culture models.

CONFLICT OF INTEREST
The authors declare no potential conflict of interest.

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