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Poly (ethylene-co-vinyl alcohol) is a suitable substrate for human olfactory neuroepithelial cell differentiation *in vitro* through a defined regulatory pathway

Sheng-Tien Li [a], Tai-Horng Young [a,*,1], Tsung-Wei Huang [b,c,d,*,1]

[a] Institute of Biomedical Engineering, College of Medicine and College of Engineering, National Taiwan University, No. 1 Sec. 1 Jen-Ai Road, Taipei 100, Taiwan
[b] Department of Electrical Engineering, College of Electrical and Communication Engineering, Yuan Ze University, Taoyuan, Taiwan
[c] Department of Otolaryngology, Far Eastern Memorial Hospital, Taipei, Taiwan
[d] Department of Health Care Administration, Oriental Institute of Technology, Taipei, Taiwan

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ABSTRACT

Olfactory dysfunction significantly influences patients' life quality, but currently has no adequate treatment. Poly (ethylene-co-vinyl alcohol) (EVAL) mediates cell adhesion, growth and modulates differentiation of neural stem cells. However, whether EVAL is a suitable substrate to establish an *in vitro* culture system that can promote development and differentiation of human olfactory neuroepithelial cells (HONCs) remains unexplored. This study isolates and cultures HONCs on controls and EVAL films for 21 days. The effects of treatment are assessed using immunocytochemistry, microarray analysis, quantitative PCR, ELISA and western blots following culturing. Most of the cell morphology on controls is epithelial and expresses markers of sustentacular cells (SCs), *cadherin-1* and *cytokeratin18*, whereas the main population on EVAL presents as morphology with extended thin processes and possesses markers of mature olfactory sensory neurons (OSNs), *olfactory marker protein* (*OMP*). Microarray analyses reveal *neuropeptide Y* (*NPY*) and *amphiregulin* (*AREG*) are the two important regulating factors on EVAL films. HONCs cultured on EVAL films enhance the development of mature OSNs through *NPY* signaling, and significantly decrease the growth of SCs by blocking epidermal growth factor receptor (*EGFR*) activation. EVAL is a potential biomaterial to serve as an ideal substrate for treating olfactory dysfunction in the future.

Statement of Significance

Olfaction not only contributes to enjoyments of food, but provides a clue to escape from dangerous environmental hazards. However, loss of smell is commonly progressive and there is no good prognostic approach for olfactory dysfunction. Here, we use poly (ethylene-co-vinyl alcohol) (EVAL) to establish an *in vitro* culture system that promotes development and differentiation of human olfactory neuroepithelial cells. We show that EVAL not only enhances the development of mature olfactory sensory neurons through *neuronpeptide Y* signaling, but significantly protects the olfactory neuroepithelium from metaplasia by inhibiting *EGFR* activation. Therefore, EVAL is a potential biomaterial to serve as an ideal substrate for treating olfactory dysfunction in the future.

1. Introduction

Olfactory dysfunction debilitates our ability to identify nearby environmental hazards and lowers quality of life by reducing appetite and sexual ability [1]. The incidence of olfactory dysfunction has continuously increased from 3% to 20% in the past two decades [2–5]. This growth may imply that patients are increasingly concerned about quality of life, because this defect is commonly

* Corresponding authors at: Institute of Biomedical Engineering, College of Medicine and College of Engineering, National Taiwan University, No. 1 Sec. 1 Jen-Ai Road, Taipei 100, Taiwan (T-H. Young) and Department of Otolaryngology, Far Eastern Memorial Hospital, 21, Section 2, Nan-Ya South Road, Pan Chiao 220, Taipei, Taiwan (T-W. Huang).

E-mail addresses: thyoung@ntu.edu.tw (T.-H. Young), huangtw@ntu.edu.tw (T.-W. Huang).

[1] These authors contributed equally.

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progressive and may go unrecognized. The etiology results from sinonasal inflammation, head trauma, viral infection and atrophy of the olfactory neuroepithelium as age increases. Additionally, it is thought to affect 90% of neurodegenerative patients as an early marker [6]. However, clinical practice still has no adequate approach showing a good prognosis in olfactory dysfunction [7, 8]. Consequently, new modalities for patients with olfactory loss need to be developed.

In the olfactory system, the olfactory neuroepithelium lines the surface of the nasal septum, and operates the first relay point of odor sensation. This neuroepithelium is also an attractive model to study neurogenesis due to its extracranial location and regenerative capacity. The olfactory neuroepithelium has various cell lineages, such as basal cells, ensheathing cells (which express *S100β*), sustentacular cells (SCs) (which express *cadherin-1* (*CDH1*) and *cytokeratin18* (*CK18*)), and olfactory sensory neurons (OSNs) (which express *olfactory marker protein* (*OMP*)) [9,10]. Additionally, the application of olfactory neuroepithelial cells for transplantation has attracted attention in the treatment of many neurological disorders, including multiple sclerosis, spinal cord injury and even stroke, but is rarely applied as a treatment for olfactory dysfunction [11–13]. Therefore, a therapeutic strategy is to regenerate the olfactory neuroepithelium by using autologous olfactory neuroepithelial cells to repair damage.

Among culture conditions, biomaterials play a crucial role in mediating cellular morphology, proliferation and differentiation. Our previous reports found that poly (ethylene-co-vinyl alcohol) (EVAL), which contains both hydrophilic vinyl alcohol segments and hydrophobic ethylene segments, exhibited distinct cell adhesion and growth, compared to those on normal culture plates [14,15]. In particular, EVAL can modulate differentiation of neural stem cells [16]. However, researchers have not yet explored whether EVAL can promote growth of human olfactory neuroepithelial cells (HONCs) and maturation of OSNs.

Additionally, two genes in the category of neuron projection development of Gene Ontology, *neuropeptide Y (NPY)* and *amphiregulin (AREG)*, might mediate development of the olfactory system. Previous reports indicate *NPY* is an orexigenic peptide that regulates feeding behavior. The olfactory system becomes more active and sensitive when *NPY* elicits hunger responses, because OSNs are responsive to *NPY* during starvation [17]. Conversely, *amphiregulin (AREG)*, one of the agonists of *epidermal growth factor receptor* (*EGFR*), is involved in cell growth, proinflammatory disease and chronic respiratory disease. This factor provokes hyperplasia of airway epithelial cells through increasing *EGFR* phosphorylation in normal airway epithelial cells [18,19]. However, no evidence demonstrates the roles of *NPY* and *AREG* in the differentiation of human olfactory neuroepithelium. This study aims to establish an *in vitro* culture system with EVAL that can promote development and differentiation of HONCs, and to analyze whether the lineage relationship between substrate and responsive cells is through a defined regulatory pathway.

2. Materials and methods

2.1. Cell preparation and culture

HONCs were harvested from adult olfactory neuroepithelium during endoscopic sinus surgery, which was approved by an institutional review board (105104-F). All patients gave informed consent to the harvesting. Biopsy specimens were transferred into Hank's balanced salt solution (HBSS), minced finely and then digested with 0.125% Trypsin/EDTA for 30 min at 37 °C. The pellets were then gathered by centrifuge, and resuspended in the iscove's modified dulbecco's media (IMDM; Invitrogen, CA, USA) with 10% fetal bovine serum and 1% penicillin/streptomycin. Finally, they were seeded to six-wells coated with laminin-co-fibronectin or EVAL films for 21 days, based on the previous report, and incubated in a humidified atmosphere containing 5% CO_2 and 95% air [20]. The substrate with laminin-co-fibronectin was adopted as a control. Cell morphology was observed under an inverse phase contrast microscope (TS-100, Nikon, Tokyo, Japan) and scanning electron microscope (SEM) (S-4800; Hitachi, Tokyo, Japan). Further, the percentage of spread area (cell spread area per view),

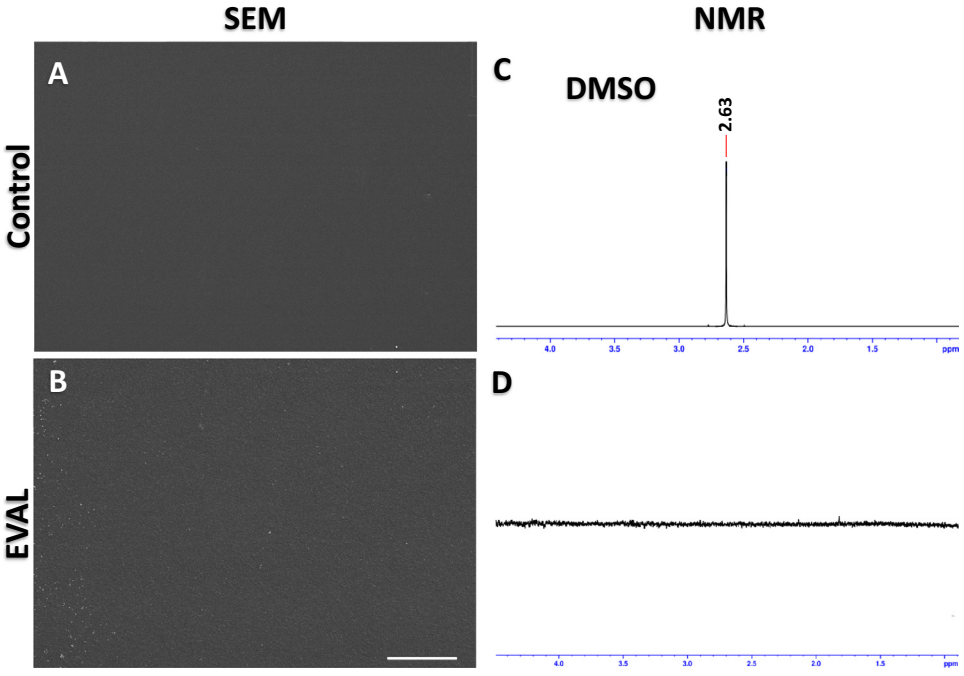

Fig. 1. A-B: SEM images of surfaces on control (A) and EVAL-coated plates (B). C-D: The ^{1}H NMR of DMSO (C) and immersed EVAL-coated plates in deuterium oxide (D). Scale Bar = 100 μm.