

Original Research

Evaluation of the Expansion and Neuronal Differentiation Potency of Cultured Olfactory Epithelium Progenitor Cells from a Rat Model of Depression

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Abstract

Background: Olfactory impairment has been reported in patients with depression and in rodent models of depression. Olfactory epithelium (OE) is the only peripheral neural tissue connected to the brain that has the potential for self-renewal. We hypothesized the olfactory deficit during depression may be related to the dysfunction of OE progenitor cells. The aim of the present study was therefore to evaluate the expansion and neuronal differentiation potency of cultured OE progenitor cells obtained from a rat model of depression.

Methods: Rats were exposed to chronic unpredictable mild stress procedures to establish a depressive-like state. Depressive-like behavior and olfactory sensing function were then evaluated and compared with control rats. Primary OE progenitor cells were cultured *in vitro*. The proliferation potency and survival of OE progenitor cells were assessed by 5-Ethynyl-2'-deoxyuridine staining and Cell Counting Kit-8 (CCK8), respectively, while cellular apoptosis was measured by flow cytometry. The neuronal differentiation potency of OE progenitor cells was evaluated by measurement of the protein and mRNA level of β -3 *tubulin*, a marker of neural cells. mRNA expression associated with neural stemness was examined by quantitative reverse transcription polymerase chain reaction (RT-PCR).

Results: Depressive-like rats showed decreased olfactory function. OE progenitor cells from depressive-like rats showed reduced cell proliferation/survival and neuronal differentiation potency. Moreover, OE progenitor cells from depressive-like rats showed decreased expression of mRNA related to neural stemness. **Conclusions:** These results indicate the impaired function of OE progenitor cells may contribute to the olfactory deficit observed during depression. The OE may therefore provide a window for the study of depression.

Keywords: depression; olfactory epithelium; progenitor cells; cell expansion; neuronal differentiation

1. Introduction

Depression is a debilitating disorder with high morbidity and mortality. Despite considerable research, the mechanisms underlying depression remain unclear. The lack of access to living human brains is a major barrier to research in this field. While autopsy studies can be informative, autopsy brains are a scarce resource and are difficult to obtain. Moreover, results obtained from autopsy brain tissue are biased due to the effects of disease course and drug therapy. Hence, there is an urgent need for alternative ways to study depression. Olfactory epithelium (OE) is located in the nasal cavity and is a unique neural tissue comprised of abundant progenitor cells and olfactory sensory neurons (OSN) that regenerate throughout life [1]. Although OE is located in the peripheral part of the olfactory pathway, OSN can communicate with neurons in the olfactory bulb via axons that extend through the cribriform plate. Thus, molecular anomalies in the brain are always reflected in the olfactory pathway [2]. Moreover, OE can be readily obtained with minimal trauma. OE and OE-derived progenitor cells are therefore emerging as promising resources

for the investigation of neuropsychiatric diseases. Aggravation of α -synuclein in the brain is a characteristic pathologic change observed in Parkinson's disease (PD) and multiple system atrophy (MSA). Recent work indicates these pathological changes also exist in the OE of patients with PD or MSA, thus making OE a promising source of biomarkers for the diagnosis of these diseases [3]. Reduced primary cilia formation is observed in OE-derived progenitor cells from schizophrenia patients, suggesting the potential use of patient-derived cell models for the diagnosis of neuropsychiatric conditions [4]. In addition, transplantation of OE-derived progenitor cells shows beneficial effects in a mouse model of Alzheimer's disease due to their potential for self-renewal [5].

Recent clinical evidence has shown that patients suffering from depression have difficulty in identifying odors [6,7]. Moreover, olfactory performance is sensitive to antidepressant therapy, with improved olfactory scores in depressed patients being associated with the remission of clinical symptoms [8]. Another study reported that olfactory dysfunction implies a longer duration and course of de-



pression, even though olfactory sensory function did not correlate positively with the severity of depression [9]. A neuroimaging study found the olfactory bulb had reduced volume in depressed patients compared to normal controls [10]. This clinical evidence for olfactory dysfunction may therefore provide new opportunities to investigate depression. However, the cause of olfactory dysfunction in depressed patients remains largely unclear. The hippocampus plays a pivotal role in stress regulation and cognitive processes, and altered neurogenic activity in the hippocampus has been associated with depression [11]. A postmortem study reported that depressed patients showed a decreased level of NeuN+ neurons in the hippocampus [12]. Enhancement of neurogenesis in the hippocampus may therefore protect against depression [13]. These studies suggest that olfactory sensing dysfunction during depression may be due to decreased neurogenesis of OE. Although peripheral neural tissue is exposed to environmental toxins, OE is capable of self-healing via increased proliferation and neuronal differentiation of progenitor cells. Poor quality of OE progenitor cells could therefore contribute to olfactory dysfunction in depression.

In the present study we evaluated olfactory function in the chronic unpredicted mild stress (CUMS)-induced rat model of depression. Primary OE progenitor cells were cultured and their proliferation, survival and neuronal differentiation potency was examined. We hypothesized that olfactory dysfunction during depression may be associated with decreased expansion and neuronal differentiation of OE progenitor cells.

2. Materials and Methods

2.1 Animals and Treatment

All animal procedures were approved by the Ethics Committee of The Affiliated Nanhua Hospital. Male Sprague-Dawley (SD) rats were purchased from Hunan SJA Laboratory Animal Co., Ltd (Changsha, Hunan, China) aged 13–14 weeks (240–270 g) were maintained at 22 °C in a 12-hour light/dark cycle with free access to food and water, except when carrying out the CUMS procedure. Animals were randomly assigned to CUMS or control groups. The experimental groups were kept in isolation. After 48 hours of adaptation, rats were subjected to CUMS procedures consisting of a variety of unpredictable mild stressors. These included swimming in cold water (4 °C) or hot water (45 °C) for five minutes, cage tilting for 24 hours, water deprivation for 24 hours, fasting for 48 hours, shaking for 15 minutes, wet bedding for 24 hours, tail nip for two minutes, and inversion of the light/dark cycle. Subjects received one of these stressors at random each day for 21 consecutive days. The control group was given usual daily care and kept in groups. After the CUMS procedures, rats were subjected to behavioral tests as described below. Rats were then sacrificed by decapitation and the olfactory mucosa was collected.

2.2 Behavioral Tests

Behavioral tests were performed after the CUMS procedure to evaluate depressive-like behavior and olfactory function. Each experiment was repeated three times and the average result recorded.

For the sucrose preference test, rats were first adapted to sucrose water before the test. Briefly, a bottle of 1% sucrose water and a bottle of purified water were given to each subject. Bottles were randomly fixed on each cage's right or left side. The position of the two bottles was swapped 12 hours later. The sucrose preference test was carried out after 24 hours of adaptation. This was performed for 3 hours. The sucrose preference was calculated as: sucrose water consumption/total water consumption.

For the forced swimming test, rats were placed in a beaker filled with water at 25 ± 1 °C and the depth was set to 20 centimeters. After 6 minutes of adaptation, the immobility time was recorded during 4 minutes.

For the open field test, rats were placed in a square container (100 × 100 cm). Following adaptation, the number standing upright was recorded during 4 minutes.

The buried food-seeking test was used to assess olfactory function as previously described [14]. Briefly, each subject was food-deprived for 24 hours, then placed at the center of the test cage to search for a 2 g regular food chow pellet buried 8 centimeters beneath the bedding in a randomly chosen corner of the cage. The latency time, defined as the time between placing the rat into the cage and grasping the buried food with its forepaws and/or teeth, was recorded. If animals could not find the food pellet within 600 s, the test was terminated and a score of 600 s was given.

2.3 Hematoxylin-Eosin Staining

Following deparaffinization and rehydration, tissue slices were stained with hematoxylin for 5 minutes and then immersed five times in 1% acid ethanol. After several washes, slices were stained with eosin for 3 minutes and then dehydrated with graded alcohol and washed in xylene. Images were captured using a fluorescence microscope (Olympus, Tokyo, Japan).

2.4 Culture of OE Progenitor Cells

OE was dissected from the olfactory mucosa under a dissecting microscope (SZX7, Olympus, Tokyo, Japan), cut into small pieces, digested in 0.125% Trypsin-ethylenediaminetetraacetic acid (EDTA) (Gibco, Anaheim, CA, USA) for 30 minutes, then centrifuged at 1400 rpm for 5 minutes and the cell pellet collected. To generate neurospheres, cells were resuspended and cultured in Dulbecco's Modified Eagle's Medium/Ham's F 12 nutrient medium (DMEM/F12) supplemented with 50 ng/mL Epidermal Growth Factor (EGF) (PROSPEC, cyt-217, Rehovot, Israel), 50 ng/mL Recombinant Fibroblast Growth Factor 2 (FGF2) (PROSPEC, cyt-218), 1% ITS-X (Gibco, 51500056, USA), 2% B27 (Gibco, Anaheim, CA, USA),

Table 1. The primer sequences used.

Target gene	Forward primer sequence	Reverse primer sequence
<i>β-3 tubulin</i>	CAACTATGTGGGGGACTCGG	TGGCTCTGGGCACATACTTG
<i>NES</i>	GTGACCCTTGGGTTAGAGGC	CTGGCAAAATGCCTTGGGTC
<i>SOX2</i>	ACATGGCCCAGCACTACCAGA	ATCTCTCCCCTTCTCCAGTTCG
<i>OLIG2</i>	TGGGGACGCTGTTTTAGCA	CAGAACACCCCCTCCCAAAT
<i>GAPDH</i>	ACAGCAACAGGGTGGTGGAC	TTTGAGGGTGCAGCGAACTT

and 1% streptomycin/penicillin. The culture medium was changed every 4 days. For the purification of OE progenitor cells, the primary neurosphere was dissociated and the cells cultured again to generate secondary neurospheres. The fourth-generation neurospheres were collected for subsequent experiments. A commercially available Quantitative PCR mycoplasma detection kit was used for mycoplasma testing (Thermo Fisher, #4460623, Fisher, Waltham, MA, USA), no mycoplasma infection was found in the indicated cells. Nestin (a marker for neural stem cells) immunofluorescence staining was used for authentication of progenitor cells.

2.5 Immunofluorescence Staining

Purified progenitor cells dissociated from fourth-generation neurospheres were seeded onto poly-L-lysine-coated coverslips. Cells were fixed and washed, blocked for 60 minutes at room temperature, and incubated with primary antibody against nestin (1:1000; #73349, Cell Signaling Technology, Danvers, MA, USA). The samples were then incubated with a fluorescent secondary antibody (1:1000; A23220, Abbkine, Wuhan, Hubei, China) for two hours in the dark at room temperature. Finally, samples were counterstained with 4',6-diamidino-2-phenylindole (DAPI, D9542, Sigma, MO, USA) and a fluorescence microscope (Olympus, Tokyo, Japan) was used to analyze the cells. Images were processed using Image-Pro plus software (V6.0, Media Cybernetics, Inc., Baltimore, MD, USA).

2.6 EdU Staining

OE progenitor cells were labeled with 5-Ethynyl-2'-Deoxyuridine (EdU) solution (Servicebio, GDP1023, Wuhan, Hubei, China) for four hours and then fixed with paraformaldehyde. After washing, cells were permeabilized with 0.5% Triton-X and then incubated with Apollo solution. DNA was revealed by staining with DAPI. Preparations were analyzed using a fluorescence microscope (Olympus, Tokyo, Japan).

2.7 Cell Viability

Cells were seeded onto a 96-well plate at 1×10^4 cells per well. Before testing, cells were incubated for two hours at 37 °C with 10% buffer from the Cell Counting kit-8 (C0039, Beyotime, Beijing, China). A microplate reader (Varioskan LUX, Thermo Fisher, Waltham, MA, USA) was used to analyze the absorbance in each well at 450 nm.

2.8 Cell Apoptosis

Cells were stained with PI (AP101, Multi Sciences, Hangzhou, Zhejiang, China) and Annexin (AP101, Multi Sciences) as per the manufacturer's instructions. Cell apoptosis was then determined by flow cytometry methods.

2.9 Quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA from OE progenitor cells was extracted using TRIZOL reagent (Sigma-Aldrich, St. Louis, MO, USA). cDNA was synthesized using a commercially available kit (Takara, Kusatsu, Japan). The expression of mRNA was examined using the SYBR Premix EX Taq I kit (Takara, Japan) on a 7300 Plus Real-Time polymerase chain reaction (PCR) System (Thermo Fisher, Waltham, MA, USA). Thermocycling conditions were as follows: an initial 95 °C step for 30 s, followed by 40 cycles at 95 °C for 5 s, and 60 °C for 60 s. *GAPDH* was used as the internal reference. The primer sequences used are shown in Table 1.

2.10 Western Blotting

Cells were homogenized using Radioimmunoprecipitation assay buffer (RIPA) buffer containing Phenylmethanesulfonyl fluoride (PMSF). Proteins were subjected to 10% Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) and transferred onto Polyvinylidene Fluoride (PVDF) membranes. After blocking for one hour, the membrane was incubated separately with primary antibodies against β -3 tubulin (1:1000; #5568, Cell Signaling Technology, Danvers, MA, USA) or β -actin (1:1000; #4970, Cell Signaling Technology). The membranes were then incubated with peroxidase-conjugated secondary antibody. The antigen-antibody-peroxidase complexes were detected with an enhanced chemiluminescence imaging kit (Millipore, Burlington, MA, USA), and the complexes visualized by a chemiluminescence imaging system (Biorad, Hercules, CA, USA).

2.11 Statistics

All data were expressed as the mean \pm Standard Error of Measurement (SEM). SPSS 22 (IBM Corp., Armonk, NY, USA) was used for the statistical analysis. Experiments with two groups were compared by independent-sample *t*-test. The significance level was set at $p < 0.05$.

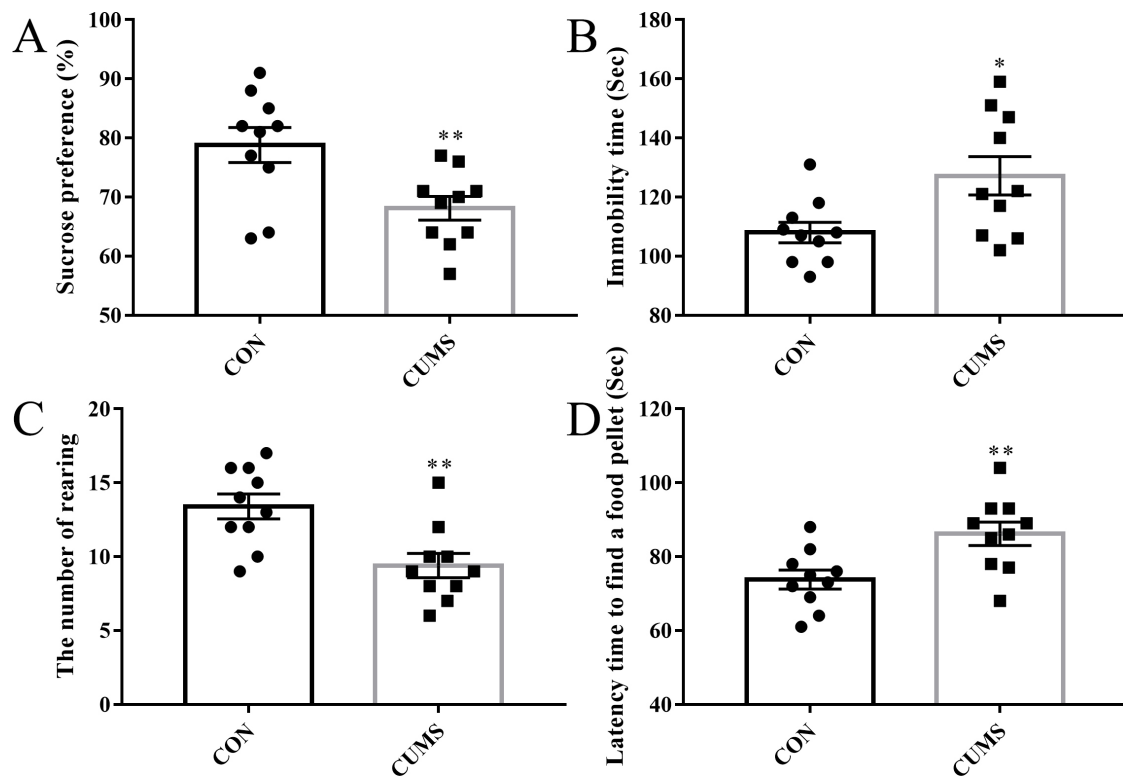


Fig. 1. Evaluation of depressive-like behavior and olfactory sensing function. (A) Sucrose preference test. (B) Immobility time recorded in the forced swimming test. (C) The number of rearing in the open field test. (D) Latency time to find food in the buried food-seeking test. $n = 10$ per group. $*p < 0.05$, $**p < 0.01$ compared to the control group. CON, control group; CUMS, chronic unpredictable mild stress.

3. Results

3.1 Depressive-Like Behavior Tests and Buried Food-Seeking Test

A series of tests for depressive-like behavior was performed to evaluate the rat model of depression. As shown in Fig. 1, rats subjected to CUMS procedures exhibited reduced glucose consumption ($p = 0.008$; Fig. 1A), increased immobility ($p = 0.017$; Fig. 1B), and decreased body rearing ($p = 0.003$; Fig. 1C) compared to the control group. Moreover, depressive-like rats showed poorer olfactory function, as demonstrated by the buried food-seeking test ($p = 0.007$; Fig. 1D). These results indicate the state of depression was correlated with reduced olfactory performance in a rat model.

3.2 Identification of OE Progenitor Cells

OE was isolated from olfactory mucosa, which is composed of the upper OE and the lower lamina propria (Fig. 2A). Neurosphere formation (an aggregated form of neural progenitor cells) is a key feature of neural progenitor cells. OE biopsies were collected for generating neurospheres, which emerged after culturing for 4 days in sphere formation medium (Fig. 2B). For the purification of progenitor cells, primary neurospheres were dissociated and cultured to generate secondary neurospheres.

Fourth-generation neurospheres were then collected for the following experiment. Purified progenitor cells derived from fourth-generation neurospheres stained positively for Nestin (a marker of neural progenitor cells) using immunofluorescence (Fig. 2C). These results demonstrate the successful establishment of an *in vitro* model of OE progenitor cells.

3.3 Cultured OE Progenitor Cells from Rats Exposed to CUMS Show Decreased Cell Proliferation and Survival

EdU labeling was used to evaluate the proliferative potential of purified OE progenitor cells (Fig. 3A). Compared to controls, OE progenitor cells derived from depressive-like rats showed reduced proliferative potential ($p < 0.001$; Fig. 3B). Cell apoptosis was examined by flow cytometry (Fig. 3C). OE progenitor cells derived from rats exposed to CUMS showed increased apoptosis ($p = 0.002$; Fig. 3D). Furthermore, OE progenitor cells from depressive-like rats showed decreased cell viability, as measured by Cell Counting Kit-8 (CCK8) ($p = 0.012$; Fig. 3E). These results suggest that OE progenitor cells from depressive-like rats have reduced ability for expansion.

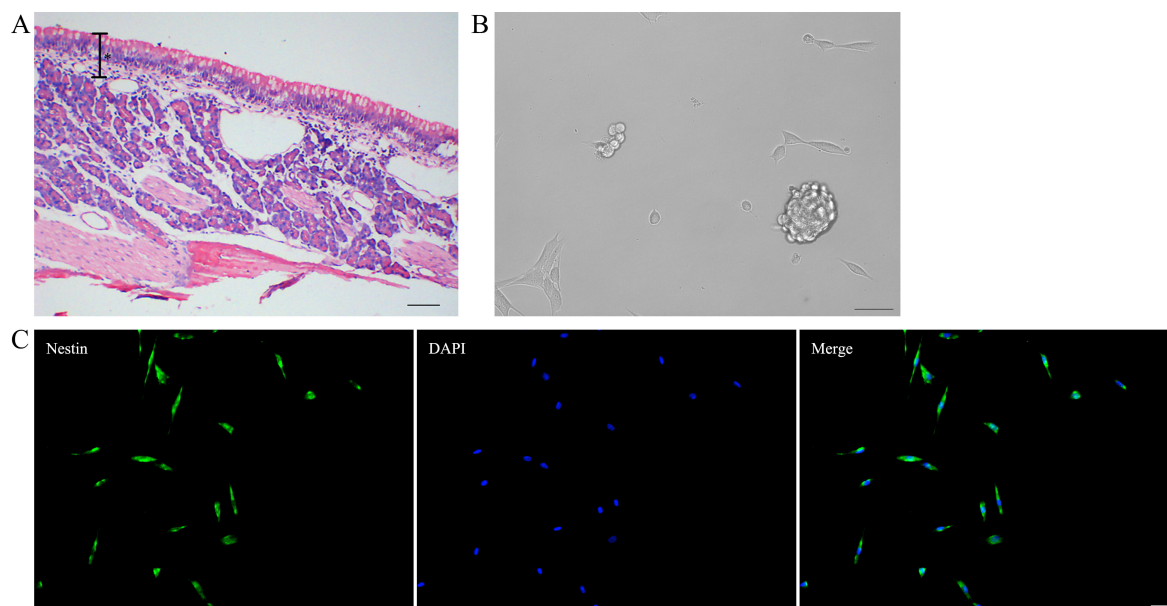


Fig. 2. Identification of olfactory epithelium (OE) progenitor cells. (A) HE staining of the olfactory mucosa. * indicates the OE portion of olfactory mucosa. Scale bar is 100 μ m. (B) Neurosphere formation. Scale bar is 100 μ m. (C) Purified progenitor cells derived from fourth-generation neurospheres express nestin. Scale bar is 50 μ m. HE, hematoxylin-eosin; DAPI, 4',6-diamidino-2-phenylindole.

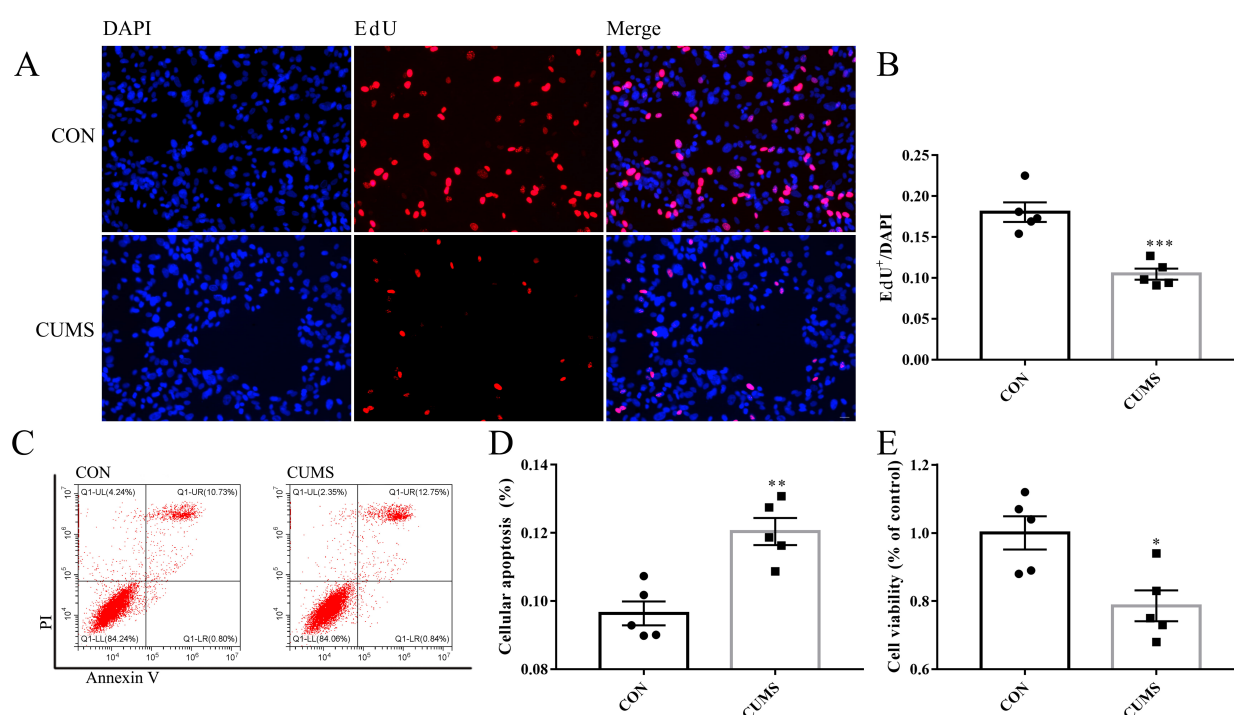


Fig. 3. Proliferation and survival ability of OE progenitor cells. (A) EdU labeling for the measurement of proliferation potency. Scale bar is 20 μ m. (B) Ratio of EdU⁺/DAPI. (C) Cell apoptosis was analyzed by flow cytometry. (D) Bar graph showing cell apoptosis. (E) Cell viability detected by CCK8 assay. n = 5 per group. * p < 0.05, ** p < 0.01, *** p < 0.001 compared to the control group. EdU, 5-Ethynyl-2'-Deoxyuridine; CCK8, Cell Counting Kit-8.

3.4 Cultured OE Progenitor Cells from Rats Exposed to CUMS Show Decreased Potency for Neuronal Differentiation

To study neuronal differentiation, purified OE progenitor cells were cultured for 3 days in DMEM/F12 sup-

plemented with fetal bovine serum and B27. OE progenitor cells obtained from depressive-like rats showed reduced mRNA expression of β -3 tubulin using reverse transcription polymerase chain reaction (RT-PCR) (p = 0.03; Fig. 4A), as well as reduced expression of β -3 tubulin pro-

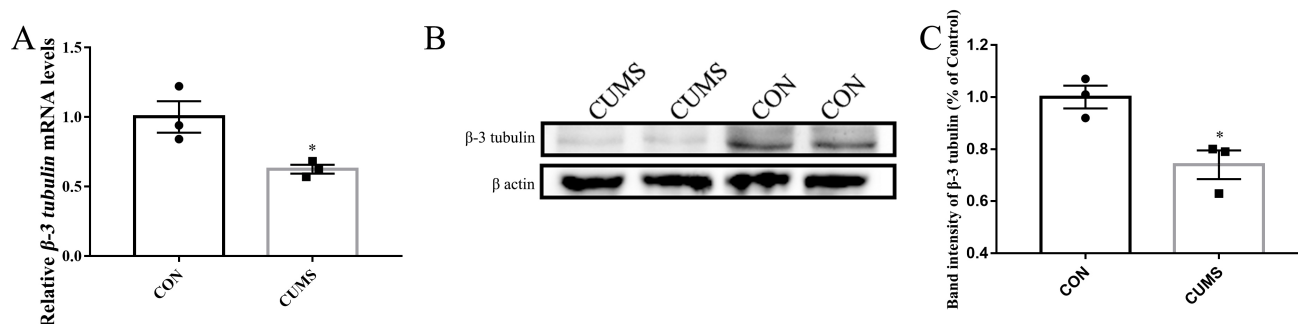


Fig. 4. OE progenitor cells derived from depressive-like rats showed reduced potential for neuronal differentiation. (A) Relative mRNA expression of β -3 tubulin. (B) Representative immunoblotting of β -3 tubulin. (C) Relative band intensity showing protein expression of β -3 tubulin. $n = 3$ per group. * $p < 0.05$ compared to the control group.

tein using Western blot ($p = 0.03$; Fig. 4B,C). These results indicate that exposure of rats to CUMS reduces the neuronal differentiation ability of OE progenitor cells.

3.5 Cultured OE Progenitor Cells Derived from Rats Exposed to CUMS Showed Decreased Expression of mRNA Associated with Neural Stemness

To further evaluate the expression of mRNA related to neural stemness, we performed RT-PCR of cultured OE progenitor cells. Compared to the control group, OE progenitor cells obtained from rats exposed to CUMS showed reduced expression of mRNA related to neural stemness (*NES*: $p = 0.002$, *SOX2*: $p = 0.04$, *OLIG2*: $p = 0.42$; Fig. 5).

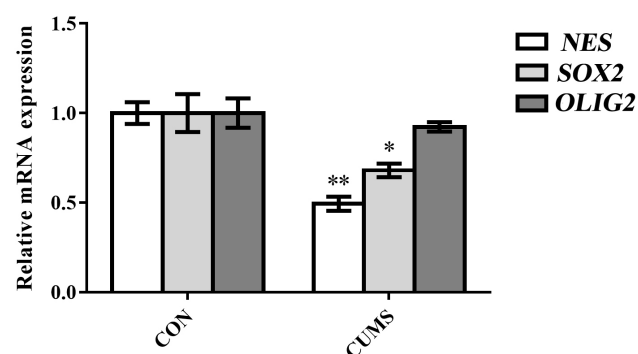


Fig. 5. Relative mRNA expression associated with neural stemness in OE progenitor cells. $n = 3$ per group. * $p < 0.05$, ** $p < 0.01$ compared to the control group.

4. Discussion

In the present study the state of depression in CUMS-induced depressive-like rats was found to correlate with olfactory dysfunction. We subsequently investigated the expansion and neuronal differentiation potency of primary cultured OE progenitor cells. As expected, OE progenitor cells derived from rats exposed to CUMS exhibited decreased potency for proliferation, survival, and neuronal differentiation. In addition, reduced mRNA expression as-

sociated with neural stemness was also observed in OE progenitor cells derived from depressive-like rats.

Despite anhedonia, we observed reduced olfactory sensing function in depressive-like rats. This could be due to the fact that emotional and olfactory processing are both regulated by the shared brain area, such as the amygdala [15]. An earlier study suggested that rats exposed to CUMS showed a reduced number of OSN in the OE [16]. As this peripheral neural tissue has the potential to regenerate throughout life, we speculated that the reduced number of OSN may contribute to the dysfunction of OE progenitor cells. To further test this idea, we evaluated the quality of OE progenitor cells in primary culture. The neural progenitor cell type is capable of multi-lineage differentiation and self-renewal. In the current study, OE progenitor cells had the ability to differentiate into neuron-like cells, as detected by RT-PCR and immunoblotting. Moreover, OE progenitor cells derived from depressive-like rats showed reduced ability for proliferation and neuronal differentiation. Other studies have also reported cellular dysfunction of OE progenitor cells obtained from patients with schizophrenia, Parkinson's disease, or bipolar disorder [17,18]. Previous studies in this active research area have suggested that decreased neurogenesis in the hippocampus might be responsible for anhedonia in depression. We have provided further evidence that decreased neurogenesis in the OE may be partly responsible for the olfactory dysfunction during depression. The genes associated with neural stemness are crucial for the maintenance of neural progenitor cells [19]. The present study also found that mRNA expression associated with neural stemness was reduced in OE progenitor cells obtained from depressive-like rats. Together, these results indicate that a poor quality of OE progenitor cells may contribute to the impairment of olfactory sensing function during depression.

OE progenitor cells are emerging as a robust tool for the pathobiological analysis of neuropsychiatric diseases. These cells have many advantages over other stem cell models. First, OE can be obtained dynamically with minimal trauma. Therefore, various pathological alterations in

OE progenitor cells may correspond better to the different disease conditions. Second, unlike induced pluripotent stem cells or induced neuronal cells, OE progenitor cells are obtained directly from neural tissue and do not need genetic reprogramming, thus more closely resembling the natural state. Third, as the unique peripheral tissue connected to the olfactory pathway, OE progenitor cells may serve as a window to explore pathobiological changes in the neurocircuitry of the OE, olfactory bulb, and olfactory cortex. Lastly, the OE progenitor cell model should be more convincing for the exploration of disease-specific changes since it has the same genetic background as the subject. Experimental studies examining protein localization/abundance, epigenetic markings, and gene expression have already reported disease-specific changes in several neuropsychiatric diseases using this *in vitro* cell model [20]. Of note, long non-coding RNA (lncRNA)-microRNA (miRNA)-messenger RNA (mRNA) network is emerging as potential diagnostic biomarkers for depression [21], we speculate that olfactory progenitor cells may be a robust tool for this field.

Olfactory signals are regulated by both internal and external stimuli [22]. Internal stimuli such as neurotransmitters, paracrine signals and hormones can affect the survival of olfactory sensory neurons and progenitor cells. Further research is needed to identify the initial factors in the brain that reduce the expansion and neuronal differentiation potency of OE progenitor cells. External stimuli such as environmental pathogens and toxicants that irritate nasal inflammation may also impact the OE microenvironment, thereby affecting olfactory sensing function. An epidemiological study found that patients who suffer chronic nasosinusitis have a higher prevalence of depression [23]. The relationship between environmental factors and depression will be interesting to explore in future work.

There are several limitations to this study. First, additional phenotypic and histochemical studies of OE and OE-derived progenitor cells from depressive patients may allow more convincing conclusions to be drawn. Second, comparison of the differentially expressed genes in OE progenitor cells between depressed subjects and normal controls is needed to further elucidate the mechanism of olfactory impairment. Finally, the extent to which rodent models of depression can accurately reflect the pathophysiological changes occurring in depressed patients requires further investigation [24].

5. Conclusions

This study suggests that olfactory dysfunction and anhedonia occur simultaneously in a rat model of depression. Importantly, these results indicate that olfactory dysfunction during depression may be due to the reduced expansion and neuronal differentiation potential of OE progenitor cells. OE progenitor cells may be a robust and feasible tool for the investigation of depression.

Availability of Data and Materials

Data available upon reasonable request.

Author Contributions

LC, YX, JC and YL designed the research study. LC and YX performed the research. LC and YX conducted experiments. JC analyzed the data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The animal study protocol was approved by the Institutional Review Board of The Affiliated Nanhua Hospital (protocol code: 2023-1LY-01).

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Conflict of Interest

The authors declare no conflict of interest.

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