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*Research Paper*

# Culture and purification of human fetal olfactory ensheathing cells using different attachment rates combined with intermittent NT3 nutrition ☆

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## Abstract

**Objective:**To explore a simple and pragmatic method to obtain sufficient olfactory ensheathing cells from human fetus by selective attachment of harvested cells combined with intermittent NT3 nutrition. **Methods:**DMEM/F12 culture solution including 10% fetal bovine serum or NT3 was used to culture olfactory ensheathing cells intermittently every 48 h. The cell state and growth rates of OECs were observed, and P75 staining was used to estimate the purity of the cells. **Results:**Human fetal OECs were positive with P75 immunocytochemical staining. OECs in dipolar or tripolar shape formed networks by their processes *in vitro*. The purity of OECs in "good state" was about 95% at 9 d and 83% on 12 d, respectively. **Conclusion:**The method of using different attachment rates combined with intermittent NT3 addition is a simple and effective way to culture and purify OECs.

*Keywords*: olfactory ensheathing cell; cell culture; purification; NT3

## INTRODUCTION

Olfactory ensheathing cell(OEC) is a special kind of cell that resides in both peripheral and central nerves. It is different from astrocyte and schwann cells, but possesses characteristics of the two. OEC can secrete neurotrophic factors[1-6], inhibit inflammatory reaction, promote neurite regeneration and transmigration to the injured region. So to recover the function of the injured spinal cord, OEC transplantation is considered one of the most promising methods for spinal injury therapy[7-10]. Until now, many methods including immuno-affinity adsorption, immunomagnetic beads, chemicals, selective attachment and starvation without serum have been tried for culture and purification of OECs[11-15], but none of them is completely effective. In the present study, we combined selective attachment with intermittent neurotrophin-3 addition, to explore a simple and useful method for OEC culture and purification, which will be beneficial for the further research of OECs.

## MATERIALS AND METHODS

### Materials

12 artificial abortion fetus(4~6 months) were obtained from the maternity department of the Second Hospital Affiliated to Xi' an Jiaotong University. DMEM-F12 medium and fetal bovine serum(FBS) were purchased from Gibco company. NT3, polylysine and rabbit anti-human $p75^{NTR}$ mAb were from Sigma company (USA). SABC kit was from Huamei biological company(China). Dissecting microscope, phase-contrast microscopy and stereomicroscope S6D were from Leica company (Germany).

### Culture and purification of human OECs

The olfactory bulbs were detached from abortion embryos and placed in chilled calcium and magnesium free

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Hank's balanced salt solution(HBSS) supplemented with 1% penicillin/streptomycin. After 3 times washing, the menings and blood vessels of the olfactory bulbs were totally peeled off, then the olfactory nerve and glomerular layer were carefully dissected and washed 3 times again with ice-cold HBSS. The remaining olfactory nerves and glomerular layers were cut into 1 $mm^3$ pieces, and incubated with 0.25% trypsin at 37℃ for 15 min. The trypsinization was stopped by a media consisting of Dulbecco's minimum essential medium (DMEM) and Ham's F-12 medium(DF12;1:1 mixture) with 10% heat-inactivated fetal bovine serum (DF12-10S). The cell suspension was centrifuged at 1000 r/min for 5 min and resuspended in DF12-10S. The tissue was triturated with a flamed polished Pasteur pipette(approximately 15~20 times) and filtered through a 75 μm cell strainer to obtain a single cell suspension. The cell density was adjusted to $1 \times 10^9$/L. The single cell suspension was seeded into 25 $cm^2$ flask and incubated for 3~12 hours at 37℃, then the supernate with floating cells was transferred into another flask for continuous 12~36 hour incubating, (the actual incubation time was based on the observation under microscopy). The last supernate with floating cells was seeded into poly-L-lysine-coated flasks(or plates) and incubated at 37℃. Two days later, the culture medium was changed to non-serum DF12 medium with 50 μg/L NT3. After 2 days incubating with NT3 medium, the medium was changed to DF12 medium with 100 ml/L FBS again; in other words, the cells were incubated in NT3 medium without serum and DF12 medium with serum was applied every 48 hours. Cells were observed under microscopy and photos were taken.

### Immunocytochemistry and purity studies

Cells contaminating OEC culture included fibroblasts, astrocytes and sparing neurons, but astrocytes and neurons were mostly unable to survive *in vitro*. OEC and fibroblasts show different appearances and OECs exprsss $P75^{NTR}$ while astrocytes and fibroblasts don't express it. So the purity of OECs was identified by morphology and immunocytochemistry staining with monoclonal rabbit anti-human p75 low-affinity nerve growth factor receptor(p75-LNGFR). Coverslips were taken from plates which had OECs cultured at 3, 6, 9, 12, 15 days. Coverslips were rinsed with 0.01mol/L PBS for 3 times. Cells were fixed with 4% paraformaldehyde 30 mins at room temperature. Endogenous peroxidase activity was quenched by immersion of the slips for 30 min in a 3% hydrogen peroxide solution. After three rinses in PBS, slips were incubated for 30 min with 0.25% Triton X-100. Slips were incubated for 1h with a blocking solution(after another three washes with PBS) including 1% bovine serum albumin. Primary antibodies were diluted(1:200) and applied to slips for 2 h at room temperature. The slips were incubated for 1 h with biotinylated secondary goat anti-rabbit IgG antibodies (1:200), then treated with components of the avidin-biotin complex, according to manufacturer's recommendations. Negative control immunostaining was performed by omitting the primary antibody. All images were visualized with a LEICA microscope. Ten fields of view were taken for each coverslip randomly, counting for P75-positive cells.

## RESULTS

### OECs morphology

By selective attachment, most OECs attached on the 2nd day. A few cells protruded short and small neurite and had good refraction. At the same time, spindle-shaped fibroblasts could also (although rarely) be found in diffused distribution. On the 4th day(NT3 incubating for 48h), OECs became bi- or tripolar, with slender and straight neurite, and showed better refraction than that of the scattered fibroblasts(***Fig 1A***). On the 6th day (serum-medium incubating for another 48 h), most OECs with larger body were appulsive, forming networks as processes elongated. A few fibroblasts with less refraction could be found on the bottom of the flask.

On the 8th day(NT3 incubating for the second 48 h), OECs kept growing. The cells' processes got longer and the small cell network fused together, while no change was found in fibroblasts. On the 10th day (serum-medium incubating for the second 48 h), the quantity of OECs increased, cells' bodies enlarged, neurite elongated and fused cell networks extended on the bottom of the flask. At this time, the fibroblasts also grew(***Fig 1B***). On the 12th day(NT3 incubating for the third 48 h), the widespread OEC network got compacted, and clumps of fibroblasts were observed. Some OECs that situated over or around fibroblasts lost cell refraction and strong neurite, and even died. On the 14th day (serum-medium incubating for the fourth 48h), fibroblasts showed obvious proliferation and extensive spreading. Most OECs over the fibroblasts died and detached, while only a few OECs survived, with slender and curved processes. OECs networks away from fibroblast clumps still existed, but got less compacted.

### Immunocytochemical staining of OEC

Immuncytochemistry of OECs showed $P75^{NTR}$ positive with yellow color, intense stain in cells bodies and slight in processes. While fibroblasts showed $P75^{NTR}$ expression was negative by immuncytochemistry(***Fig 2***).

### Measurement of OECs purification

Puritiy of OECs was identified according to mor-

A:OEC exserting process on 4d; B:OEC processes forming network on 10d.

***Fig 1*** Morphological observation of OEC(× 200)

OECs immunostained positive for $P75^{NTR}$ showed as yellow, while fibroblasts showed negative. A1:OEC; A2: Fibroblast; B:OEC processes forming network.

***Fig 2*** Observation of OEC with $P75^{NTR}$(Immunocytochemical staining,× 400)

phological observation and immunocytochemical staining. At 3~9 days, the purity was 95%. After this time, purity of OECs decreased owing to fibroblast proliferation, so by the 12th day, the purity was 83%.

## DISCUSSION

Neurotrophins are neurotrophic proteins or neuropeptides that can nourish both central and peripheral nerve cells. Neurotrophins, such as nerve growth factor, brain-derived neurotrophic factor, NT3 and glia cell line-derived neurotrophic factor, are very important for survival, growth, differentiation and normal function of the nerve cells[16-18]. John et al[19] found that NGF, BDNF and NT3 can promote proliferation and purification of the primary cultured adult nasal OECs. Because NT3 is the most effective, it was selected to assist the culture and purification of OECs in this study. At first, DF12 medium containing both NT3 and FBS was tried and compared with DF12 medium containing only NT3. The results showed that cells in DF12 medium with only NT3 grew quickly and well in the early stages, and cells had large bodies, slender and straight prominence and good refraction. But as time passed, proliferation of OECs became progressively slower, the cell bodies became thin and small, the neurite attenuated and became curved, and cells lost refraction and even died. The cells different appearance in different stages indicated that NT3 could stimulate proliferation of OECs, but it was not sufficient to take the place of the nutritional support of serum for continual cell growth. Cells in DF12 medium containing both NT3 and FBS grew very quickly, but the much quicker proliferation of fibroblast leaded to failure of OECs purification. It was postulated that NT3 might synergia the serum to accelerate the growth of fibroblast, so single NT3 addition might be better for OECs purification.

It was found that OECs in DF12 medium containing only NT3 kept growing well, while fibroblasts proliferated weakly, and often died owing to lack of serum. When cells were changed into the serum-medium, the fibroblasts couldn't recover to quick proliferation because of the confluence of most OECs. Meanwhile, OECs recovered to 'good state' gradually. The cells were changed into medium(containing NT3) again when the fibroblast showed quick proliferation in serum-medium, so as to make fibroblasts in a continual weak growth and keep proliferation and activity of OECs. During this course, NT3 started gene expression by connecting with tropomyosin receptor kinase(Trk) and P75 receptor on OEC surface, to promote cell proliferation. P75 receptor might play a role in enhancing the affinity of NT3 to Trk receptor and in regulating the phosphokinase activity. Further studies are still needed to understand the mechanism that allows NT3 to promote OEC proliferation.

In the study, many factors were also found to affect state of OECs. Younger fetal age and shorter time to draw the olfactory bulb would result in cells with 'good state' and fast attachment. On the contrary, cells from fetus with older age or longer drawing time would be in bad state and attached slowly, and the result was consistent with that from a study by Lü RB et al[20]. So the interval time of different cell attachments should be adjusted according to precise observation under microscope to obtain a higher purity of OECs. This will prevent either losing a lot of cells due to fast attaching or too much mixture being caused by slow attaching. Therefore, culture of OECs should be based on the principle of individualization and good results can only be possibly obtained under an individualized culture.

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