EXPERIMENTAL CELL RESEARCH 317 (2011) 2823–2834

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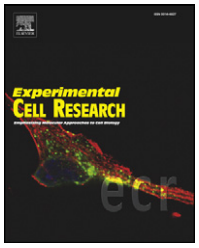

Research Article

# RhoA–ROCK–Myosin pathway regulates morphological plasticity of cultured olfactory ensheathing cells

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ARTICLE INFORMATION

*Article Chronology:*
Received 2 April 2011
Revised version received 9 August 2011
Accepted 9 September 2011
Available online 17 September 2011

*Keywords:*
Olfactory ensheathing cells
RhoA
Morphological plasticity
Regeneration
Myosin

ABSTRACT

Olfactory ensheathing cells (OECs) are glial cells in the olfactory system with morphological and functional plasticity. Cultured OECs have the flattened and process-bearing shape. Reversible changes have been found between these two morphological phenotypes. However, the molecular mechanism underlying the regulation of their morphological plasticity remains elusive. Using RhoA FRET biosensor, we found that the active RhoA signal mainly distributed in the lamellipodia and/or filopodia of OECs. Local disruption of these active RhoA distributions led to the morphological change from the flattened into process-bearing shape and promoted process outgrowth. Furthermore, RhoA pathway inhibitors, Toxin-B, C3, Y-27632 or over-expression of DN-RhoA blocked serum-induced morphological change of OECs from the process-bearing into flattened shape, whereas the activation of RhoA pathway by lysophosphatidic acid (LPA) promoted the morphological change from the process-bearing into flattened shape. Finally, ROCK–Myosin–F-actin as a downstream of RhoA pathway was involved in morphological plasticity of OECs. Taken together, these results suggest that RhoA–ROCK–Myosin pathway mediates the morphological plasticity of cultured OECs in response to extracellular cues.



## Introduction

Olfactory ensheathing cells (OECs) are a type of glial cells in the olfactory system with axonal growth-promoting properties. They have been discovered to promote the growth of olfactory sensory axons and the regeneration of injured axons after being transplanted into nerve injury sites [1,2]. Current studies have shown that OECs have enormous potential for therapeutic use in treatment of axonal injuries and demyelinating diseases [3–7].

Initially, cultured OECs are described as antigenically and morphologically heterogeneous and comprised of both astrocyte-like cells and Schwann cell-like cells [4,8–11]. However, recent studies have shown that cultured OECs are a single cell type with morphological plasticity [12–15]. Cultured OECs have flattened shape with a flat sheet-like

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*Abbreviations:* OECs, Olfactory ensheathing cells; FBS, fetal bovine serum; PLL, poly-L-lysine; LPA, lysophosphatidic acid; FRET, fluorescence resonance energy transfer; ROCK, Rho associated kinase; MLC, myosin light chain.

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doi:10.1016/j.yexcr.2011.09.004

morphology and process-bearing shape with a long fusiform bipolar or multi-polar morphology. The morphology of OECs is affected by some extracellular factors such as cultured media [10,14,16–18], endothelin-1 [14] and extracellular matrix [19,20], and intracellular factors such as cAMP [12,14,16,18]. Interestingly, several studies have shown that reversible changes have been found between these two shapes by using time-lapse imaging or addition of cAMP or endothelin-1 [12,14,15]. However, the molecular mechanism underlying the regulation of these reversible changes is still unknown.

The Rho subfamily of small GTPases (including RhoA, Rac1 and Cdc42) are the central players in cell morphology and motility in many cell types through regulating the assembly of actin cytoskeleton, adhesion formation and membrane protrusion. RhoA signals in the formation and maturation of focal adhesion complex associated with actin stress fiber bundles, whereas Rac1 and Cdc42 stimulate the formation of cell protrusions in association with filopodia and lamellipodia [21,22]. RhoA signaling pathways have been reported in mediating the guidance of OEC migration [23–25]. However, whether RhoA pathway regulates the morphological plasticity of cultured OECs remains unclear. In the present study, we demonstrate the role of RhoA signaling pathway in the morphological plasticity of OECs.

## Materials and methods

### *Primary culture of olfactory ensheathing cells*

Primary culture of OECs were prepared from olfactory bulb of adult male Sprague–Dawley rats and purified by differential cell adhesiveness as described previously [12,26–29]. Briefly, the meninges were carefully removed from the olfactory bulb under the dissecting microscope and the olfactory nerve layer was peeled away from the glomerular and deeper layers of the olfactory bulb, then dissociated with 0.125% trypsin (Sigma, St Louis, MO) and incubated at 37 °C for 15 min. Trypsinization was stopped by DMEM/F12 (1:1, vol/vol, Gibco, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (FBS, Hyclone, Logan, UT). The tissue was centrifuged for 10 min at 500 *g*, and the pellet was triturated using a flame-polished Pasteur pipette and plated on uncoated 25 $cm^2$ culture flask (Corning, LY) two times, each for 36 h at 37 °C in 5% $CO_2$. The non-adhesive cell suspension was collected and then seeded onto 12-well plates (Corning) pre-coated with poly-L-lysine (PLL, 0.1 mg/ml, Sigma), and incubated with DMEM/F-12 containing 10% FBS, 2 μM forskolin (Sigma) and 10 ng/ml bFGF-2 (Sigma) as mitogen. The media were changed every 3 days. For experiments, the purified OEC cells were replanted onto square coverslips (8 mm) coated with laminin (10 μg/ml) at a density of $2\times10^5$ cells. OECs were identified by double immunostaining for p-75 and GFAP, or p-75 and S-100, or S-100 and GFAP (Fig. S1). The overall purity of OECs was around 95%. We adopted the previous criteria and method to define and count morphological phenotypes of OECs [12,14]. Process-bearing shape of OECs had very little cytoplasm and two or more fine processes that were longer than the width of the cell body, and flattened shape of OECs had a large area of cytoplasm surrounding the nucleus and either fewer than two processes or processes that were shorter than the width of the cell body. According to these criterions, we counted the total number of each morphological phenotype under bright filed microscopy, and counted the number of positive cells expressed OECs markers at same field under fluorescence microscopy. The purity of each morphological phenotype of OECs was analyzed by determining the percentage of cells expressing OEC marker (p-75, S-100, GFAP) in total counted cells. Quantitative data were from three different cell cultures. Cells were counted in at least 20 randomly selected fields from one coverslip and 100 cells for each shape per coverslip ($n=4$) were counted.

### *FRET-based imaging of active RhoA with three-channel microscopy*

The fluorescence resonance energy transfer (FRET) probe pRaichu-RhoA (YFP–RBD–RhoA–CFP) for monitoring the subcellular RhoA activity was kindly provided by Dr. M. Matsuda (Osaka University) [30]. Cells transfected with the FRET probe were imaged on a Nikon Ti microscope with a 40 oil lens (N.A. 1.30) using the Perfect Focus System and were illuminated by a polychrome IV monochromator (TILL Photonics). Filter sets for FRET imaging are CFP (excitation 436 nm, emission, 480/40 HQ, DM 455), FRET (excitation 436 nm, emission, 535/30 HQ, DM 515) and YFP (excitation 510 nm, emission, 535/30 HQ, DM 515). Images of the three channels were recorded simultaneously by using the Cascade 512B CCD (Roper Scientific). Background images were subtracted from the raw images before carrying out FRET calculation. Corrected FRET ($FRET^C$) was calculated on a pixel-by-pixel basis for the image using the following equation: $FRET^C = FRET\text{-}a\times YFP\text{-}b\times CFP$, where FRET, CFP and YFP corresponded to background-subtracted images, acquired through the FRET, CFP and YFP channels, respectively. "a" and "b" were the fraction of bleed-through of YFP and CFP fluorescence through the FRET channel, respectively, and the two values were determined by using cells transfected with YFP or CFP alone. We used the following equation: $E = FRET^C/(CFP + FRET^C)\times 100\%$ to quantify the FRET signal by using MetaFluo and Image J software (PixFRET Plug-in) [31–33].

### *Immunocytochemistry and cytoskeleton staining*

Briefly, OECs were fixed with fresh 4% paraformaldehyde in 0.1 M PBS (pH 7.4) for 20 min. After washed by PBS, cells were permeabilized with 0.2% Triton X-100 in 0.1 M PBS for 5 min, followed by incubation in blocking buffer (5% normal goat serum and 0.2% Triton X-100 in 0.1 M PBS, pH 7.4) for 1 h, and incubated overnight at 4 °C with polyclonal antibodies against p-75 (1:500, Promega, Madison, WI), or GFAP (1:500, Sigma) and with a monoclonal anti-p-MLC antibody (1:200, Cell Signaling Technology) or anti-S-100 antibody (1:500, Sigma) or anti-p-75 antibody (1:200, Chemicon), diluted in the blocking buffer. Cells were washed three times with PBS and incubated for 1 h at room temperature with an appropriate fluorescence-conjugated secondary antibody (1:1000, Molecular probe, Eugene, OR), and then visualized by confocal fluorescence microscopy (FV1, 000, Olympus). No positive signal was observed in control incubations with no primary antibody. For visualization of F-actin, cells were incubated with rhodamine-conjugated phalloidin (1:60, Molecular probe) at room temperature for 1 h.

### *Plasmids and cell transfection*

EGFP-tagged WT-RhoA and DN-RhoA were used previously [34]. For cultured OEC transfection, we used rat Astrocyte Nucleofector Kit (Amaxa) according to the manufacturer's instructions. Briefly,