Chapter 12

Olfactory Receptors in Human Airway Epithelia

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Abstract

Olfactory receptors (OR) represent one of the largest gene families in the human genome. In spite of a significant progress in deciphering the physiological functions of olfactory receptors, how the majority of these G-protein-coupled receptors are activated is still mostly a mystery. Consequently, for the majority of OR genes there are currently no assigned physiological or behavioral functions. Deciphering ligand specificities and physiological significance of human ORs is important for understanding how the human olfactory genome encodes odors, and how such odors drive human behavior in health and disease. Although OR genes were originally thought to be restricted to the olfactory epithelium, several recent studies indicated that some members of the OR family might be acting outside the canonical chemosensory system. In a recent study, we have shown that the human airway epithelial cells can also act as chemosensory cells by directly sensing the inhalation of noxious bitter compounds, which can lead to increased mucociliary clearance, and hence may serve as a protective mechanism against inhaled toxins and microorganisms. Whether the airway epithelium can detect chemicals via other sensory pathways has not been reported to date. As a step in this direction, we describe methods for studying the cellular and subcellular localization of olfactory receptor proteins and mRNAs in human airways in both primary in vitro cultures and tissue sections.

Key words Olfactory receptors, Pulmonary epithelium, Confocal immunofluorescence, Homo sapiens, Primate, Lung

1 Introduction

The rat olfactory receptor (OR) family was first described in 1991 (1). Since then, many additional publications established members of the family as the principle molecular olfactory receptors in the vertebrate main olfactory epithelium where they detect inhaled volatile chemicals (e.g., (2–5)). The human genome contains at least 339 genes that encode for functional OR proteins and 297 OR pseudogenes (3). Although OR genes are clearly playing an olfactory role, the vast majority of the members of this superfamily are orphan receptors, i.e., the specific ligand(s) that activate them are unknown. Nevertheless, decoding the ligand specificity of

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human ORs is important for understanding how the human olfactory genome encodes odors, and how such odors drive human behavior in health and disease. Furthermore, natural genetic variations in the ability of individuals to respond physiologically and behaviorally to specific odors can teach us much about the evolution of our own species (6).

Although the main olfactory epithelium is thought to be the sole organ for the detection of volatile chemicals, some OR genes seem to be acting outside the canonical chemosensory system. Examples include the role of OR1D2 in the chemotactic response of human sperm to Bourgeonal (7), and the presence of OR genes in the gut epithelium (8).

We have recently demonstrated that the human airway epithelium can act as a chemosensory organ. Ciliated airway cells express members of bitter taste receptor family and are responsive to bitter compounds (9). During our investigation of human airways taste functions we also discovered that several OR genes are present in these tissues. Since primary airway cultures are readily available, this finding suggests that the human airway epithelium could serve as an excellent in vitro model for studying human olfactory functions. Here we focus on methods for describing the cellular and subcellular localization of olfactory receptor proteins and mRNAs in human airways in both primary in vitro cultures and tissue sections (Fig. 1). These methods can be easily modified for studies of olfactory receptors in other mammalian species.

2 Materials

2.1 Immunofluorescence

1. Xylene.
2. 100 % Ethanol.
3. 95 % Ethanol.
4. Phosphate-buffered saline (PBS): Dissolve the following components in 800 ml distilled H_2O. (8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, 0.24 g of KH_2PO_4), and adjust pH to 7.4 with HCl, add distilled H_2O to final volume 1 l. Autoclave and store the solution at room temperature.
5. Paraformaldehyde (PFA, Sigma): 4 % PFA was freshly prepared with PBS, aliquoted and stored at −20 °C.
6. SuperBlock blocking buffer (Thermo Scientific).
7. VECTASHIELD Hard set mounting medium with DAPI (Vector Laboratories).
8. Antigen Unmasking solution (Vector Laboratories).
9. PBS with Triton (PBST): Add 0.3 ml Triton X-100 to 1 l PBS.

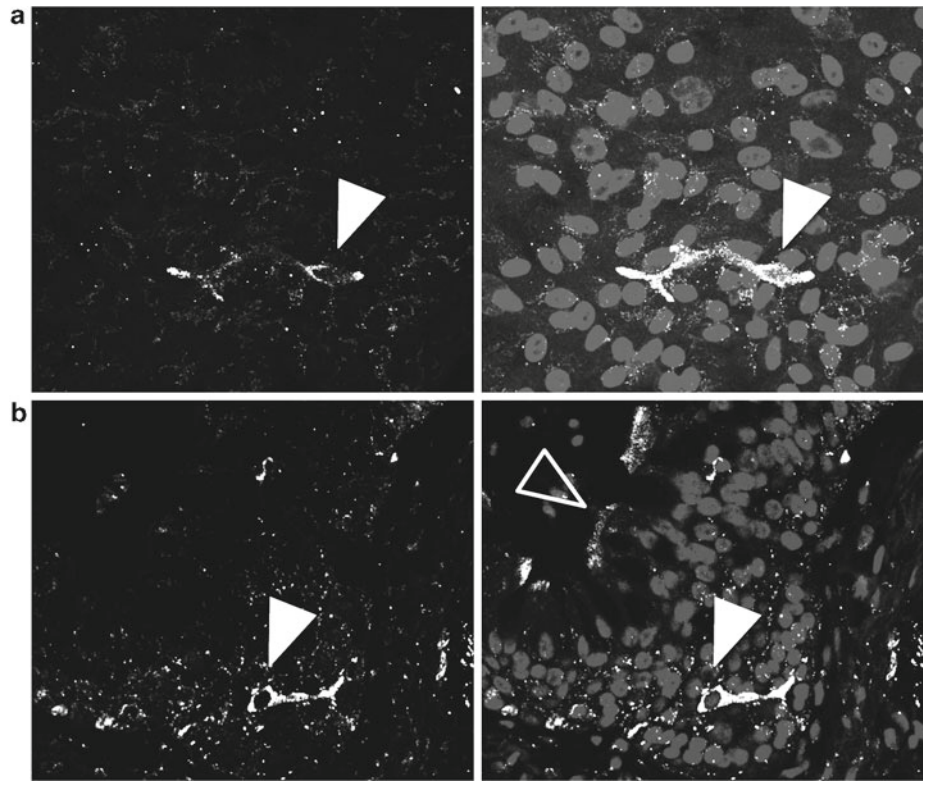

Fig. 1 Human olfactory receptor 2H3 (OR2H3) expression in airway tissues. (**a**) Pulmonary olfactory cell in human primary airway culture. (**b**) Pulmonary olfactory cell in human airway paraffin section. *Left panel*, anti-OR2H3 (Abcam); *right panel*, anti-OR2H3 channel merged with DAPI (nuclear stain). *White arrowheads*, cell bodies; *empty arrowhead*, ciliated lumen of a major airway. Note that the olfactory cell body is at the basal layer of the stratified epithelium

2.2 Double Immunofluorescence Staining

1. Conjugated Fab: Alexa Fluor 488-conjugated AffiniPure Fab Fragment Donkey anti-rabbit IgG (H + L) (Jackson ImmunoResearch).
2. Unconjugated Fab: AffiniPure Fab Fragment Donkey anti-rabbit IgG (H + L) (Jackson ImmunoResearch).

2.3 In Situ Hybridization

1. In vitro RNA transcription kit (e.g., MAXIscript, Ambion).
2. Hybridization solution: 50 % Formamide, 10 % Dextran Sulfate, 1× Denhardt's solution (Invitrogen), 10 mM Tris–HCl pH 7.5, 60 mM NaCl, 1 mM EDTA, 0.25 % SDS, 1 mg/ml yeast tRNA (Sigma).
3. Proteinase K (Roche): 1 mg/ml Proteinase K stock solution was prepared in 0.1 M Tris–HCl pH 7.5 and 50 mM EDTA.
4. 10× SSC (Thermo Scientific).

5. 5× MABT stock: 500 ml maleic acid pH 7.5, 750 mM NaCl, 0.5 % v/v Tween 20.
6. Formamide (Sigma).
7. DIG-labeled RNA probe.
8. Fluorescein-labeled RNA probe.
9. Anti-fluorescein-POD antibody (Roche), anti-DIG-POD antibody (Roche).
10. TSA™ plus Fluorescein System (PerkinElmer).
11. TSA™ plus Cyanine 3 System (PerkinElmer).

3 Methods

3.1 Preparation of Primary Cultures and Tissue Sections for Immunofluorescence Confocal Microscopy

3.1.1 Primary Cultures

This section assumes that primary human airway cultures are available. A detailed protocol for how to obtain such cultures from human donors is available (10). The protocol below requires the use of fully differentiated human primary airway cultures (high density of ciliated epithelial cells) in Millicell Cell Culture Inserts (Millipore). We find that this protocol works well regardless of the Millicell membrane type.

1. Wash inserts briefly with PBS (both sides).
2. Fix with 4 % paraformaldehyde in PBS for 15 min at room temperature.
3. Wash inserts twice with PBS.
4. Cover inserts with ice-cold methanol for 10 min on ice.
5. Wash twice with PBS.

3.1.2 Paraffin Sections

1. Deparaffinize slides in xylene, 10 min.
2. Wash slides in 100 % ethanol, 2× 10 min.
3. Wash slides in 90 % ethanol, 10 min.
4. Wash slides in 70 % ethanol, 10 min.
5. Rinse slides in distilled water.
6. If antigen unmasking is required, place slides in antigen unmasking solution at room temperature. Then bring slides to boil in antigen unmasking solution using a microwave for 10–20 min. Cool slides back to room temperature.
7. Wash slides twice with distilled water.
8. Wash slides with PBS.

3.2 Antibody Staining

This protocol can be used for a single or multiple protein targets, as long as the primary antibodies have been produced in different hosts. For a double staining of receptors with antibodies that were produced in a single host, please refer to Subheading 3.3.

1. Incubate slides with superblock blocking buffer for 30 min at room temperature.
2. Incubate slides with primary antibody at the appropriate dilution in superblock blocking buffer for 1–2 h at room temperature or overnight at 4 °C. For very rare targets, longer incubation time might increase specific signal.
3. Rinse slides with PBS/0.3 % Triton X-100.
4. Incubate slides with fluorochrome-conjugated secondary antibody at superblock blocking buffer for 30 min at room temperature.
5. Rinse in PBS/0.3 % Triton X-100.
6. Cover tissue with Vectashield Mounting medium and place a coverslip of appropriate size and thickness.
7. Seal coverslip by covering its edges with nail polish.
8. Examine slides immediately or store at 4 °C in dark.

3.3 Double Staining with Primary Antibodies from a Single Host Species

1. Incubate sections with superblock blocking buffer for 30 min at room temperature.
2. Incubate sections with primary antibody at appropriate dilution in superblock blocking buffer for 1–2 h at room temperature or overnight at 4 °C.
3. Wash sections with PBS/0.3 % Triton X-100.
4. Incubate with excess Alexa Fluor 488-conjugated AffiniPure Fab (2). If this results in high background, try a lower concentration of the probe-conjugated Fab followed by further blocking with excess *unconjugated* AffiniPure Fab. Since the first antigen will generate a lower signal, typically the more abundant protein should be labeled first (see Notes 6 and 7).
5. Rinse in PBS/0.3 % Triton X-100.
6. Incubate with the second primary antibody at appropriate dilution in superblock blocking buffer, followed by Probe-conjugated secondary antibody.
7. Rinse in PBS/0.3 % Triton X-100.
8. Rinse coverslip slides with Vectashield Mounting medium.
9. Seal slides by painting around edges of coverslips with nail polish. Examine slides immediately or store at 4 °C in dark.

3.4 In Situ Hybridization on Paraffin Sections Using TSA-Based Double Immunofluorescent Probes

This protocol is intended for the detection of two genes simultaneously by using confocal microscopy. It can be easily modified for using chromogenic detection reagents.

3.4.1 Generating Riboprobes for In Situ Hybridization

We use a commercial kit for generating in vitro RNA probes (riboprobes). Kits are available from multiple commercial sources. This protocol will focus on generating the template for the probe. We generate a DNA template by amplifying the probe from a cDNA template or genomic DNA. We also typically use the T7 RNA polymerase. Using T3 or SP6 polymerases can yield similar results.

1. We find that riboprobes with a length of 300–600 bp work well (1). To design primers we use the freely available PCR oligomer design tool available on the NCBI webpage (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (See Note 1).
2. RNA polymerases require a specific promoter sequences. We typically add the sequence for the T7 polymerase (TAATACGACTCACTATAGGG) to the 5′ end of each designed primer.
3. Two version of each primer should be synthesized with and without the T7 promoter sequence at the 5′ end.
4. For each template two PCR reaction should be set:
   (a) For the antisense probe use a reverse primer with a T7 promoter sequence and forward primer without the T7 promoter.
   (b) For a control sense probe use the reverse primer without the T7 promoter and the forward primer with the T7 promoter sequence.
5. Run the PCR reaction on a 1 % agarose gel and purify the expected PCR band. Sequence the PCR fragment to ensure it has amplified the correct template. This is especially important of genomic DNA was used as a template for the PCR.
6. To generate the riboprobe, mix the following component on ice:
   (a) PCR DNA template (100–200 ng).
   (b) 2 μl of 10× RNA labeling mix (DIG or fluorecein; Roche) (see Note 5).
   (c) 2 μl of 10× transcription buffer (each polymerase requires its own buffer) (see Note 2).
   (d) 2 μl of RNA polymerase (SP6, T7, or T3).
7. DEPC-treated water to bring the final volume to 20 μl. Mix briefly and incubate at 37 °C for 2 h. Add 2 μl of 0.2 M EDTA (pH 8) to stop the reaction (see Notes 3 and 4).

3.4.2 In Situ Hybridization

1. Deparaffinize slides in xylene, 10 min.
2. Wash slides in 100 % ethanol, 2× 10 min.
3. Wash slides in 90 % ethanol, 10 min.
4. Wash slides in 70 % ethanol, 10 min.
5. Wash slides in 50 % ethanol, 10 min.
6. Rinse slides in distilled water. From this point onwards do not allow the slides to dry, as this might result in high background signal.
7. Cover sections with 20 µg/ml proteinase K in 0.1 M Tris–HCl pH 7.5 and 0.05 M EDTA for 10–20 min at 37 °C. This step might require optimization in terms of time of incubation and enzyme concentration. Under-digestion might lead to a lower specific signal, while over-digestion might lead to high background and poor tissue morphology.
8. Rinse slide 5× in distilled water.
9. Incubate slides in ice-cold 20 % (v/v) acetic acid for 20 s to permeabilize cells.
10. Dehydrate the sections for approximately 1 min each in 70 % EtOH, 95 % EtOH, and 100 % EtOH then air dry.
11. Mark around the tissue section with a PAP Pen to create a hydrophobic barrier. Add 100 µl hybridization solution to each section. Volume might vary pending the original area of the tissue section. Pre-hybridize the slides for 1 h in a humidified hybridization chamber (plastic dish with wet paper towels would suffice) at the desired hybridization temperature. While pre-hybridizing, dilute one or two probes (DIG-labeled RNA probe and Fluorescein-labeled RNA probe) in hybridization solution, and heat the probes for 95 °C for 2 min on a heat block. Chill on ice immediately to prevent rehybridization. Drain off the hybridization solution. Add 50–100 µl of diluted probe per section (ensure the entire sample is covered). Incubate in the hybridization chamber at 65 °C overnight.
12. Wash 3× 5 min at 37–45 °C with 50 % formamide/2× SSC.
13. Wash 15 min at 37–45 °C with 2× SSC.
14. Wash 30 min at 37–45 °C with 0.2× SSC.
15. Wash twice in MABT for 30 min at room temperature.
16. Incubate with superblock blocking buffer for 30 min at room temperature.
17. Add the anti-fluorescein-POD antibody (3) at the appropriate dilution in superblock blocking buffer. Incubate for 1–2 h at room temperature or 4 °C overnight.

18. Wash slides with MABT, 5× 10 min at room temperature. Wash twice for 5 min each in PBS.
19. Add TSA Plus Fluorescein Solution and incubate at room temperature for 5–30 min. Reaction time must be determined empirically for each probe (4).
20. Wash with 30 %, 50 %, 75 %, and 100 % methanol in PBS for 10 min each.
21. Incubate with 3 % H_2O_2 in PBS for 10–30 min to inactivate the first peroxidase (5).
22. Wash three times with PBS for 10 min.
23. Incubate with superblock blocking buffer for 30 min at room temperature.
24. Add the anti-DIG-POD antibody at the appropriate dilution in superblock blocking buffer. Incubate for 1–2 h at room temperature or 4 °C overnight.
25. Wash slides with MABT, 5× 10 min at room temperature. Wash 2× 5 min each in PBS.
26. Add TSA Plus Cy3 Solution and incubate at room temperature for 5–30 min. Reaction time must be determined empirically for each probe.
27. Wash twice with PBS for 10 min.
28. Cover tissue with Vectashield Mounting medium and place a coverslip of appropriate size and thickness.
29. Seal coverslip by covering its edges with nail polish.
30. Examine slides immediately or store at 4 °C in the dark.

4 Notes

1. The choice of size and sequence of the RNA probe is very important for a successful *in situ* hybridization. We introduce T7 promoter sequences to the 5¢ terminal of primers, which allows us to transcribe sense and antisense probes from a PCR fragment. Cloning a cDNA or a PCR product into a vector with a T7 promoter can substitute a PCR template for in vitro transcription.
2. In vitro transcription can fail for various reasons. One step we found to be important is to make sure that the enzyme buffer is fully reconstituted after thawing. For best results, role the tube fast between your gloved hands for about 10–15 s.
3. Although some protocols recommend removal of the DNA template with a DNase treatment prior to hybridization, we find that it is unnecessary since the amount of ssRNA molecules is significantly higher than the template dsDNA. Furthermore, DNase treatment and subsequent RNA purification often result in a significantly lower probe yields.

4. A fast way to know that your transcription was successful is to run 1 μl of your transcription reaction on a 1 % agarose gel. You should see a somewhat fuzzy but very dominant band at roughly the size of your DNA PCR template. If the band looks smeared, it is better to repeat rather than continue with the protocol.
5. We find that riboprobes labeled with DIG give a stronger signal than probes labeled with fluorescein. Consequently, we typically use the DIG-labeled probe for transcripts that are expressed at lower levels and fluorescein-labeled probes for more abundant transcripts.
6. For double immunostaining with primary antibodies from a single host, we use a relatively high concentration of conjugated Fab. We use Alexa Fluor 488-conjugated AffiniPure Fab antibody to saturate the first primary antigen. Since Fab is monovalent antibody, saturation of the first primary antibody blocks any subsequent binding of any additional antibodies.
7. Our protocol often results in high background of the first antigen tested, which often requires careful optimization. To increase signal-to-noise ratio, we typically lower the concentration of conjugated Fab, which is then followed by a saturation step of the sample by using excess of unconjugated Fab.

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