

Characterization and Turnover of CD73/IP₃R3-positive Microvillar Cells in the Adult Mouse Olfactory Epithelium

Sandra Pfister^{1,2,*}, Maren G. Dietrich^{1,3,*}, Corinne Sidler¹, Jean-Marc Fritschy¹, Irene Knuesel¹ and Rebecca Elsaesser^{1,4}

¹Institute of Pharmacology and Toxicology, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland and ²Neuroscience Center Zurich, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland ³Present address: Division of Endocrinology, Diabetes and Clinical Nutrition, University Hospital Zurich, Raemistrasse 100, CH-8091 Zurich, Switzerland ⁴Present address: Carl Zeiss Microscopy GmbH, Carl Zeiss Gruppe, Carl-Zeiss-Promenade 10, 07745 Jena, Germany

Correspondence to be sent to: Irene Knuesel, Institute of Pharmacology and Toxicology, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland. e-mail: knuesel@pharma.uzh.ch

*These authors contributed equally to the work.

Accepted June 22, 2012

Abstract

The main olfactory epithelium consists of 4 major cell types: sensory neurons, supporting cells, microvillar cells, and basal progenitor cells. Several populations of microvillar olfactory cells have been described, whose properties are not yet fully understood. In this study, we aimed to clarify the classification of microvillar cells by introducing a specific marker, CD73. Furthermore, we investigated the turnover of CD73-microvillar cells during adult life. Using direct and indirect immunofluorescence in adult main olfactory epithelium, we first demonstrate that ecto-5'-nucleotidase (CD73) is a reliable marker for microvillar cells reported previously to express phospholipase C β 2 (PLC β 2) along with type 3 IP₃ receptors (IP₃R3) and transient receptor potential channels 6 (TRPC6), as well as for cells labeled by transgenic expression of tauGFP driven by the IP₃R3 promoter. The ubiquitous CD73 immunoreactivity in the microvilli of these 2 cell populations indicates that they correspond to the same cell type (CD73-microvillar cell), endowed with a signal transduction cascade mobilizing Ca²⁺ from intracellular stores. These microvillar cells respond to odors, possess a basal process, and do not degenerate after bulbectomy, suggesting that they contribute to cellular homeostasis in the olfactory epithelium. Next, we examined whether CD73-microvillar cells undergo turnover in the adult olfactory epithelium. By combining CD73 immunofluorescence and BrdU pulse labeling, we show delayed BrdU incorporation in a small fraction of CD73-positive microvillar cells, which persists for several weeks after BrdU administration. These findings indicate that CD73-microvillar cells likely differentiate from proliferating progenitor cells and have a slow turnover despite their apical position in the olfactory epithelium. These combined properties are unique among olfactory cells, in line with the possibility that they might regulate cellular homeostasis driven by extracellular ATP and adenosine.

Key words: BrdU, CD73, neurogenesis, neuronal homeostasis, olfaction

Introduction

The postnatal olfactory epithelium (OE) is a pseudostratified epithelium containing several distinct cell types, notably olfactory sensory neurons, supporting cells, microvillar cells (MVCs), as well as 2 types of progenitor cells (horizontal and globose basal cells) and Bowman's gland and duct cells. The nasal cavity is directly exposed to air-borne contaminants;

as a consequence, cells in the nasal epithelia are prone to damage. Therefore, olfactory cells typically have a short lifespan and get replaced from progenitor cells throughout adult life (Graziadei et al. 1978; Schwob 2002; Gulbransen and Finger 2005). In the OE, the basal germinal zone, from which regeneration occurs, contains 2 cell populations with stem cell characteristics, the globose (GBCs) and horizontal

basal cells (HBCs) (Mackay-Sim and Kittel 1991; Huard et al. 1998). GBCs are multipotent and have the highest proliferation rate in the OE (Caggiano et al. 1994; Goldstein et al. 1998; Jang et al. 2003; Chen et al. 2004). HBCs are postulated to represent relatively quiescent, multipotent progenitors; extensive injury of the neuroepithelium can induce proliferation of HBCs to replenish the pools of neuronal and nonneuronal cells (Carter et al. 2004; Leung et al. 2007; Iwai et al. 2008; Packard et al. 2011). Interestingly, the supporting cells arranged in a single layer at the apical surface possess the capability to self-renew in the unperturbed OE. Nevertheless, after massive damage to the OE they get replaced from progenitor cells, as well (Schwob et al. 1995; Weiler and Farbman 1998). The continuous turnover of olfactory neurons and their replenishment after damage is critical to maintain the functional integrity of the OE.

In this process, the role and the fate of MVCs are not well understood. Part of the difficulty arises from their incomplete morphological and functional characterization. Although MVCs are clearly distinct from supporting cells, which also possess microvilli, the nomenclature of the various subpopulations described in rodent and human OE (Moran et al. 1982a, 1982b; Rowley et al. 1989; Carr et al. 1991; Menco and Jackson 1997a, 1997b; Braun and Zimmermann 1998; Asan and Drenckhahn 2005; Elsaesser et al. 2005; Hansen and Finger, 2008; Lin et al. 2008; Hegg et al. 2010) is controversial. In previous work, we have described 1 subpopulation of MVCs, endowed with an inositol-triphosphate (IP_3)-mediated signal transduction cascade, including phospholipase C beta 2 (PLC β_2), type-3 IP_3 receptors (IP_3R3), and transient receptor potential channels 6 (TRPC6) (Elsaesser et al. 2005; Montani et al. 2006). These flask-shaped cells, representing about 5% of all olfactory cells, are evenly distributed throughout the OE and are situated at the most superficial layer intermingled with supporting cells. They most likely correspond Jourdan's "type B cells" (Jourdan 1975), as well as MVCs described by Moran et al. (1982a) and microvillous cells type 2/4 (Menco and Jackson 1997a, 1997b; Menco and Morrison 2003).

PLC β_2 -MVCs are excitable and convert extracellular signals in a Ca^{++} response (Elsaesser et al. 2005; Montani et al. 2006). Interestingly, they selectively express the neuroproliferative factor neuropeptide Y (NPY), suggesting a role in the control of neural proliferation in the postnatal OE by stimulus-induced release of NPY (Hansel et al. 2001; Montani et al. 2006; Jia and Hegg, 2012). In particular, adenosine-5'-triphosphate (ATP) has been shown to be such a stimulus, as the release of NPY is triggered by ATP (Kanekar et al. 2009; Jia et al. 2011) and ATP increases the expression of NPY in MVCs in vivo (Jia and Hegg, 2010).

However, Hegg et al. (2010) recently reported that MVCs expressing tauGFP⁺ under transgenic control of the IP_3R3 gene promoter were immunonegative for PLC β_2 , despite many common features with the MVCs described in our studies. Therefore, 1 goal of this study was to clarify the

relationship between PLC β_2 -MVCs and IP_3R3 -MVCs. To this end, we investigated ecto-5'-nucleotidase (CD73) as a possible marker of both cell populations in the mouse OE. CD73 is a glycosyl phosphatidyl inositol (GPI-) linked, membrane bound glycoprotein that catalyzes the extracellular hydrolysis of 5'-AMP to adenosine, which may subsequently activate adenosine receptors (Zimmermann 1992) or be recycled after intracellular transport. Thereby, CD73 is involved in various physiological processes mediated by adenosine including hypoxia, inflammation, antinociception, epithelial ion transport, and modulation of blood-brain barrier functions (Koszalka et al. 2004; Thompson et al. 2004; Colgan et al. 2006; Mills et al. 2008; Niemelä et al. 2008; Sowa et al. 2010a, 2010b). CD73 has been reported in the rat OE to label dark/horizontal cells at the basal side, MVCs at the luminal side, and Bowman's duct cells (Braun and Zimmermann, 1998). Here, we used direct and indirect immunofluorescence for CD73 to demonstrate unambiguously that this marker is expressed in PLC- β_2 / IP_3R3 -MVCs of the adult mouse OE, thereby establishing CD73 as a useful tool to study this major population of MVCs.

As a first step toward their functional characterization, we addressed important open issues in the field of OE regeneration, namely whether CD73-MVCs get replaced in the adult animal, at which rate, and from which progenitor cells. To answer these questions, we monitored the fate of CD73-MVCs pulse-labeled with the thymidine analog 5-bromo-2'-deoxyuridine (BrdU) combined with immunofluorescence staining for CD73.

Material and methods

Animals

$IP_3R3^{tm(tauGFP)}$ transgenic mice in which the first exon of the *Itpr3* gene is replaced by the coding region of the fusion protein tau-eGFP (Hegg et al. 2010) were kindly provided by Dr Diego Restrepo (University of Colorado Denver, CO). Adult male IP_3R3^+/IP_3R3^- tauGFP⁺ mice were used for immunohistochemistry. Additionally, immunohistochemistry was performed in tissue from adult (6–8 week old) male C57BL/6J wild-type mice bred in our Institute. All experimental procedures were approved by the Cantonal Veterinary Office in Zurich. The mice were housed 3–6 animals per cage in a 12-h light/dark cycle with food and water provided ad libitum.

Mouse olfactory epithelium tissue preparation

Mice were given a single intraperitoneal injection of 180 mg/kg BrdU (Sigma-Aldrich, Switzerland, #B5002) dissolved in 0.9% NaCl with an injection volume of 6.6 mL/kg body weight. At various time spans after BrdU injection (1, 3, 5, 7, 10, 14, 21, 28, and 42 days), mice were deeply anesthetized with Nembutal (50 mg/kg, i.p.) and perfused transcardially

with phosphate buffered saline (PBS) followed by aldehyde fixation (4% paraformaldehyde, 15% saturated picric acid, 150mM sodium phosphate buffer, pH 7.4). Thereafter, the noses were rapidly dissected and stored for 2h in fixative at 4 °C. After washing, they were decalcified in 5% ethylenediamine tetraacetic acid (EDTA, pH 7.4) for 7 days at 4 °C. Next, the specimens were cryoprotected overnight in 30% sucrose in PBS and subsequently frozen and stored at -80 °C. The specimens were embedded with Neg-50 Frozen Section Medium (Richard-Allan Scientific, MI) and coronal sections of 20 µm thickness were cut and mounted on gelatine-coated slides.

Whole-mount preparation: Animals were decapitated and the OE was dissected out and transferred into fixative. The tissue was fixed for 1 h at 4 °C. After washing, the tissue was processed for immunohistochemistry.

DNA denaturation for BrdU staining

The sections were air-dried before being washed in PBS and incubated in 0.2 N HCl at room temperature (RT) for 5 min. Thereafter, they were transferred into 4 N HCl and incubated at 37 °C for 30 min. After denaturation, the sections were washed 4 times in PBS and processed for immunohistochemistry.

Immunofluorescence staining

The primary antibodies used are listed in [Table 1](#); they were diluted in PBS containing 5% normal serum and 0.2% Triton X-100. Thereafter, sections were incubated overnight at 4 °C, washed 3 times in PBS for 10 min and incubated for 45 min at RT with secondary antibodies raised in goat or donkey and conjugated to Alexa488 (Molecular Probes) or Cy3 (Jackson ImmunoResearch, West Grove, PA). They were diluted in PBS containing 5% normal serum (Alexa488 1:1000; Cy3 1:500; Cy5 1:500). Direct immunofluorescence staining of CD73 was performed with Cy3-conjugated anti-CD73 antibodies (1:1500). The sections were air-dried and coverslipped using DAKO fluorescence mounting medium (Dako North America, CA).

In order to detect CD73 and BrdU simultaneously, the immunofluorescence staining protocol was slightly adjusted. To this end, sections were incubated overnight at RT with rat-anti-CD73 antibody (1:1000) containing 5% normal serum and 0.2% Triton X-100. Following three 10-min washing steps, sections were incubated for 30 min at RT with biotinylated secondary antibody (1:500) diluted in PBS containing 5% normal serum and 0.02% Triton X-100. After washing, the slides were transferred into fixative for 12 min at 4 °C. This was followed by washing in PBS three times for 10 min each. DNA denaturation was applied by incubating the sections firstly for 5 min in 0.2 N HCl at RT and subsequently for 30 min in 4 N HCl at 37 °C. Next, sections were washed 3 times in PBS before incubating for 20 h at RT with FITC-conjugated anti-BrdU antibody (1:100) in PBS containing 5% NGS and 0.02% Triton X-100. Slides were rinsed in PBS and transferred into Cy3-conjugated streptavidin solution and incubated for 30 min at RT. Thereafter, they were washed, air-dried, and coverslipped with DAKO mounting medium.

Image processing and analysis

Sections processed for immunofluorescence were analyzed by confocal microscopy (LSM-710, Zeiss, Jena, Germany) using 40× (NA 1.3) or 63× (NA 1.4) and sequential acquisition of separate channels. Z-stacks of consecutive sections (5–8; 1024×1024 pixel; spaced 1 µm in z) were acquired with the pinhole set at 1 Airy unit. For visual display, image stacks were projected in the z-dimension and merged using the image analysis software Imaris (Bitplane, Zurich, Switzerland).

Three animals per time point post-BrdU injection were used to quantify the number of CD73-MVCs and BrdU-positive CD73-MVCs, respectively. Random sampling fields containing CD73-immunoreactive cells were selected at 2 anteroposterior levels of the main sensory OE. The first level contained all ethmoid turbinates and the second level composed the ethmoid turbinates located below the rostral tip of the olfactory bulb. Four sampling fields in each area were acquired using a 40× objective (N.A., 1.2). Stacks of

Table 1 List of antibodies

Antibody	Specificity	Manufacturer	Description/Nr	Dilution
ACIII	Wei et al. 1998; Wong et al. 2000	Santa Cruz Biotechnology, Inc.	Rabbit polyclonal; #sc-588	1:1000
BrdU: FITC	Vanderlaan and Thomas, 1985; Kondo et al. 2010	AbD Serotec, Oxford	Rat monoclonal, IgG2a conjugated to FITC-liquid; #OBT0030F	1:100
CD73	Yamashita et al. 1998; Eliopoulos et al. 2005; Kobie et al. 2006	eBioscience, Inc.; San Diego	Rat monoclonal, IgG1; #16-0731	1:1000/1:1500
IP ₃ R3	Blondel et al. 1993; Leite et al. 2003	BD Bioscience	Mouse monoclonal, IgG2a, #610312	1:500
OMP	Baker et al. 1989; Cummings et al. 2000	Wako, Chemicals, Richmond	Goat polyclonal, #544-10001	1:500
PLC β2	Ali et al. 1997; Perez et al. 2002	Santa Cruz Biotechnology, Inc.	Rabbit polyclonal, Q-15, #sc-206	1:500

11–12 confocal layers were projected in 2D and used for cell counts. Cells were only considered as double-labeled if the BrdU⁺ nucleus was clearly visible.

Statistical analysis was performed by Kruskal–Wallis test using GraphPad Prism (GraphPad Software, Inc. Version 4.01). Statistical significance was set at $P < 0.05$.

Results

CD73 is a selective marker for microvillar cells in the adult mouse olfactory epithelium

In previous studies, we investigated PLC β 2-MVCs as a major microvilli-bearing cell type in the OE characterized by expression of a phosphatidyl inositol-mediated signal transduction pathway and postulated their involvement in the control of neuronal proliferation in the postnatal OE (Elsaesser et al. 2005). Here, we detected the membrane-bound CD73 (ecto-5'-nucleotidase) as an unequivocal marker for PLC β 2-MVCs. Using anti-CD73 antibodies in whole-mount specimen of adult mice revealed immunoreactive cells that were evenly scattered throughout the sensory OE (Figure 1A). In double-labeling experiments with CD73 and PLC β 2, CD73-immunoreactivity was colocalized with microvilli of PLC β 2-positive cells (Figure 1A). Staining of transverse sections of the OE demonstrated that anti-CD73 antibodies labeled cells that strongly resembled PLC β 2-MVCs (Elsaesser et al. 2005) based on their density, shape, and localization (Figure 1B–D). The selectivity of CD73 as a marker for PLC β 2-MVCs was confirmed by double immunofluorescence staining. These results show that these 2 proteins were extensively co-associated within the same cells throughout the OE (Figure 1A,1B), with 304 out of 330 cells (92%) from 5 animals being double-labeled for CD73 and PLC β 2. Similar results were obtained for IP₃R3 immunoreactivity. In wild-type mice, IP₃R3 and CD73 were systematically codetected in the same cells (Figure 1C). To confirm that CD73 is not expressed in olfactory neurons, parallel triple-labeling experiments were performed with olfactory marker protein (OMP) and adenylyl cyclase III (ACIII). As seen as high magnification, CD73 was never colocalized with either OMP or ACIII at the apical pole (Figure 1D), but was located underneath the layer of ACIII-positive cilia (Figure 1D'). In addition, as reported by Braun and Zimmermann (1998) in the rat OE, CD73 may also be expressed by Bowman's duct/gland cells and dark/horizontal basal cells. However, we could not confirm these observations in adult mouse OE, due to nonspecific binding of anti-rat IgGs (secondary antibodies) in the basal lamina and in the lamina propria. Moreover, using direct immunofluorescence for CD73, no staining was detectable in these structures. In conclusion, our data indicates that monoclonal anti-CD73 antibodies can be used as a reliable marker for PLC β 2-MVCs.

IP₃R3-eGFP microvillar cells are immunoreactive for CD73

Recently, Hegg et al. (2010) described MVCs expressing IP₃R3 using a transgenic mouse strain. This cell type has several features in common with PLC β 2-MVCs. Both of them bear microvilli at their apical protrusions, they possess axon-like processes that do not penetrate the basal lamina, and they do not degenerate after bulbectomy. Furthermore, they respond to odors with an increase of intracellular Ca²⁺ and do not express neuronal markers (Elsaesser et al. 2005; Hegg et al. 2010). In order to clarify whether they represent the same cell type, we examined CD73 immunoreactivity in IP₃R3⁺/IP₃R3⁻ tauGFP mice. Whole-mount preparations revealed that every IP₃R3-tauGFP-positive cell in adult OE was capped by CD73 immunostaining (Figure 2A). Likewise, in transversally cut sections we detected that CD73-immunofluorescence was selectively co-associated with IP₃R3-tauGFP-positive cells (Figure 2B). In a sample of 400 randomly selected CD73-immunoreactive cells in whole mount and tissue sections from 4 adult mice, 98% were GFP-positive, suggesting systematic association of these 2 markers. Furthermore, we used anti-PLC β 2 antibodies to test the distribution of a marker of PLC β 2-MVCs in IP₃R3-tauGFP-positive cells. As expected, PLC β 2 immunoreactivity was present in virtually all tauGFP-positive cells in whole-mount preparation and in tissue sections of 3 animals (97% out of 300 GFP-positive cells; Figure 2C,2D). Therefore, these results indicate that these 2 populations of MVCs (PLC β 2-MVCs and IP₃R3-MVCs) correspond to the same cell type, and can be termed CD73-MVCs.

Microvillar cell turnover

There are many open questions about the role and regulation of MVCs in the postnatal OE. In particular, it is not known whether differentiated MVCs undergo cell division and thereby self-renew or whether they originate from a population of olfactory progenitor cells in adult life. Here, we addressed these issues for CD73-MVCs, using an immunohistochemical protocol allowing the simultaneous detection of CD73 and BrdU in the same tissue section. Mice were injected once with BrdU and the proportion of BrdU-labeled CD73-MVCs in the olfactory sensory epithelium was assessed at 1–21 days postinjection (dpi). We analyzed 3 mice per time point and counted CD73-MVCs cells bilaterally in 4 sampling areas on 2 different sections resulting in 16 fields of view. As expected, many cells were immunoreactive for either CD73 or BrdU and only a small percentage was double-labeled. At 1 dpi, BrdU immunoreactivity was mainly visible in the basal germinative zone. The majority of BrdU-positive cells in this region likely constitute the progenitor cell population (Figure 3A). Nevertheless, there were also a few BrdU-immunoreactive cells detectable at the most superficial layer of the epithelium (Figure 3B). However, there were no BrdU/CD73 double-labeled cells detectable at 1 dpi (Figure 3B). The first BrdU-positive CD73-MVCs were

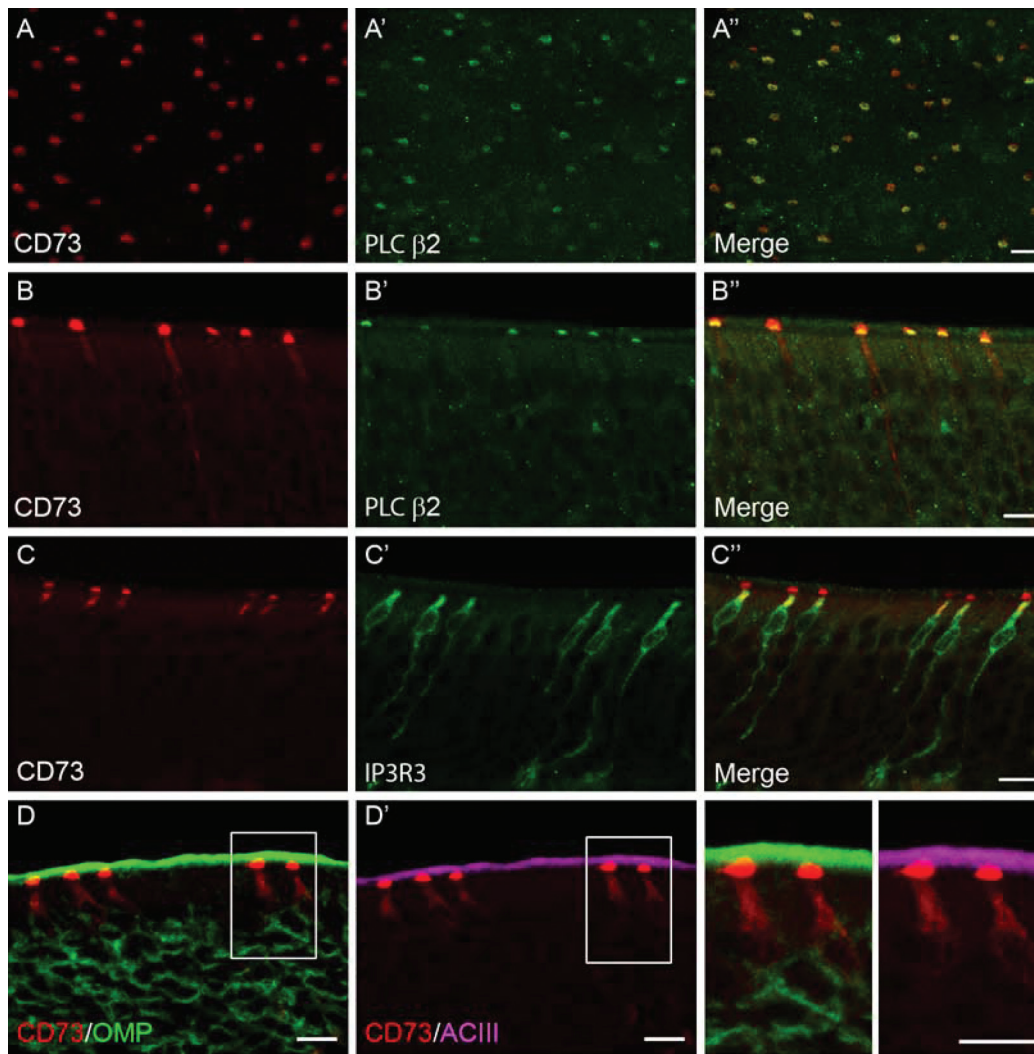


Figure 1 CD73 as a marker of a major MVCs population in the olfactory epithelium. (A) Double immunofluorescence staining on whole-mount tissue preparations using anti-CD73 antibody (red, left panel) and anti-PLC β 2 antibody (green, middle panel, A') revealed co-association of these 2 proteins in microvilli (merged, A''). (B) Double immunofluorescence labeling against CD73 (red, left) and PLC β 2 (green, B') on coronal sections stained the same cells (merged, B''). (C) Using an anti-IP₃R3 antibody (green, middle panel, C') stained cells that were co-associated with CD73 (red, left). The merged image is shown on the right (C''). (D) Triple-immunofluorescence staining on coronal sections using anti-CD73 antibody (red, left and middle panel), anti-OMP antibody (green, left), and anti-ACIII antibody (magenta, middle). The OMP-immunoreactive receptor neurons were not co-associated with CD73-positive cells (D) nor did ACIII overlap with CD73 on cilia (D'). The boxed areas are enlarged on the right. Scale bars: 10 μ m.

observed at 3 dpi. Thus, these cells seem to require 3 days between S-phase and the earliest detectable expression of CD73. Double-labeled cells were observed more frequently at 5, 7, and 10 dpi (Figure 3C,3D), clearly indicating that CD73-MVCs get replaced in the adult animal. At 10 dpi, the fraction of BrdU-positive CD73-MVCs was maximal with a mean value of 2.9% CD73-MVCs being double-labeled at this time point. Later on, the number of BrdU-labeled CD73-MVCs decreased to 0.5% at 21 dpi. These values were obtained from a total number of 583 CD73-positive cells at 10 dpi and 675 at 21 dpi. In line with this time-course, Kruskal–Wallis analysis yielded a significant overall time effect on the proportion of BrdU-positive CD73-MVCs ($N = 3$ per time point; $H = 17.126$; $P = 0.008$; Figure 3E).

To extend our observations to later time points (21, 28, and 42 dpi), we treated a separate cohort of mice with 6 BrdU injections (twice daily for 3 days). Under these conditions, BrdU-positive CD73-MVCs remained detectable even at 42 dpi, but without a significant overall time effect (Kruskal–Wallis; $N = 2$ –3 per time point, $H = 2.889$, $P = 0.2359$; Figure 3E). This finding, together with the observation that a relatively low percentage of CD73-MVCs was BrdU-positive at any time point, indicated that MVCs represent a stable cell population with a long lifespan. In line with this conclusion, quantification of the total number of CD73-MVCs revealed no significant differences in the 2 cohorts of mice analyzed (1 BrdU injection: $H = 10.939$; $P = 0.090$; 6 BrdU injections, $N = 2$ –3 per time point, $H = 5.556$, $P = 0.0662$).

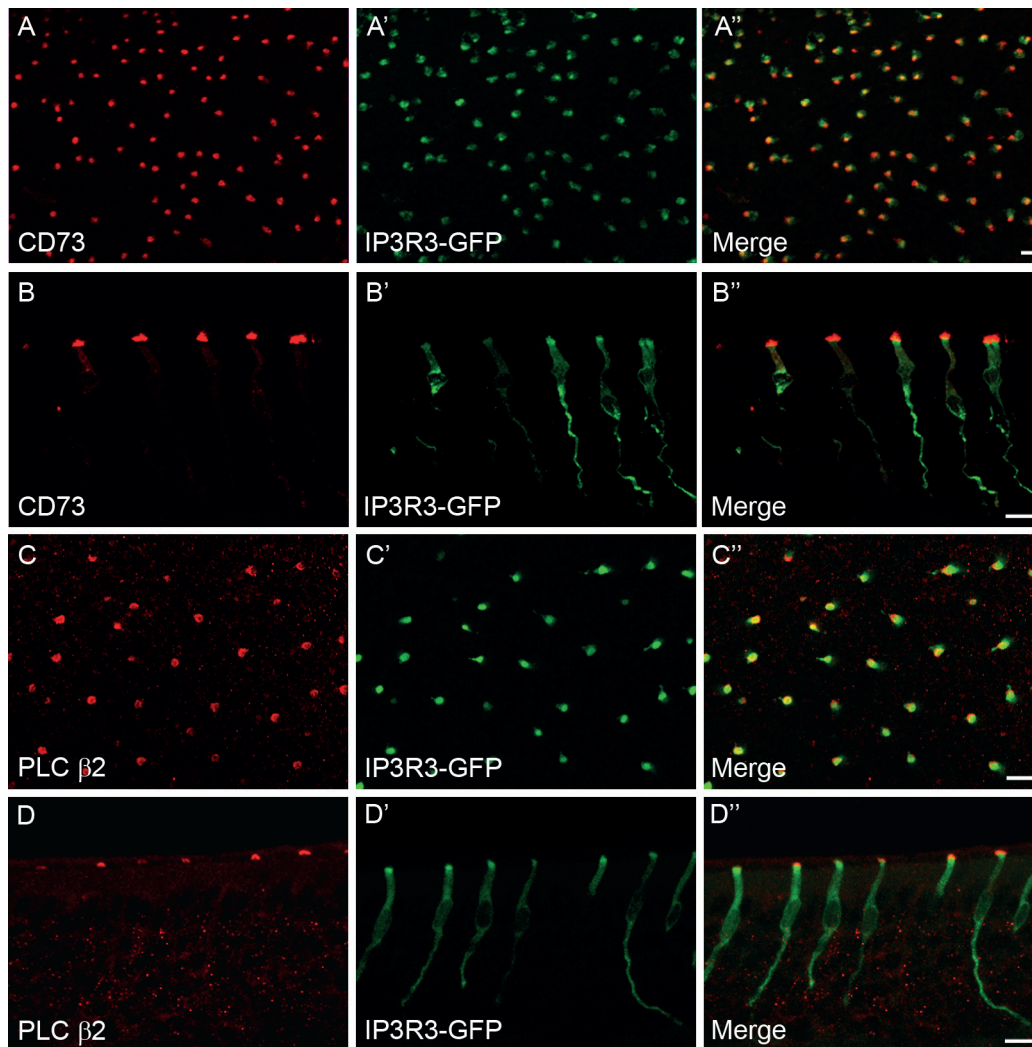


Figure 2 Identification of IP₃R3-GFP-positive CD73-MVCs. Immunofluorescence staining was performed on tissue sections from IP₃R3⁺/IP₃R3⁻ tauGFP⁺ mice. **(A,B)** CD73-immunoreactivity (red, left) is detected in IP₃R3GFP-positive cells (green, A',B') as shown in whole-mount tissue preparations (A) and coronal sections of the olfactory epithelium (B). **(C,D)** Immunofluorescence staining using anti-PLC β2 (red, left) antibody revealed its co-association with IP₃R3-GFP MVCs (green, C', D') in whole-mount tissue (C) and coronal sections (D). Scale bars: A = 20 μm, B–D = 10 μm.

After 1 BrdU injection, we counted on average 240 ± 64 CD73-MVCs per mouse (mean \pm SEM; $N = 3$), representing 18 ± 2.8 CD73-MVCs per sampling field. These values are similar to those counted in mice examined after 6 BrdU injections (236 ± 38 ; 17.3 ± 1.8 , respectively).

Discussion

The present results clarify the nomenclature of MVCs in the adult mouse OE by demonstrating that PLC β2-MVCs (Elsaesser et al. 2005; Montani et al. 2006) and IP₃R3-MVCs (Hegg et al. 2010) represent the same cell type, characterized in addition by prominent expression of CD73 at their apical pole. Accordingly, the failure to detect PLC-β2 in these MVCs in previous studies was likely due to technical reasons, such as high sensitivity of antibodies to tissue

fixation. This cell population, which we propose to name CD73-MVCs, is endowed with multiple elements of the IP₃-mediated signal transduction cascade, including IP₃R3 and PLC β2. Furthermore, CD73-MVCs most likely also express Gq/11 and TRPC6, because Gq/11 has been reported in IP₃R3-MVCs and TRPC6 in PLC β2-MVCs (Elsaesser et al. 2005; Hegg et al. 2010). Conversely, it is highly unlikely that CD73-MVCs correspond to TrpM5-positive MVCs described by Hansen and Finger (2008) and Lin et al. (2008), because the latter cells have a clearly different morphology and do not express PLC β2, TRPC6, or IP₃R3. Therefore, in adult mouse OE, there are at least 2 main, distinct types of MVCs. Further studies will help elucidating their specific role and determine possible species differences. In particular, the relevance of CD73 for the function of MVCs will be an important topic of future investigations. There is an

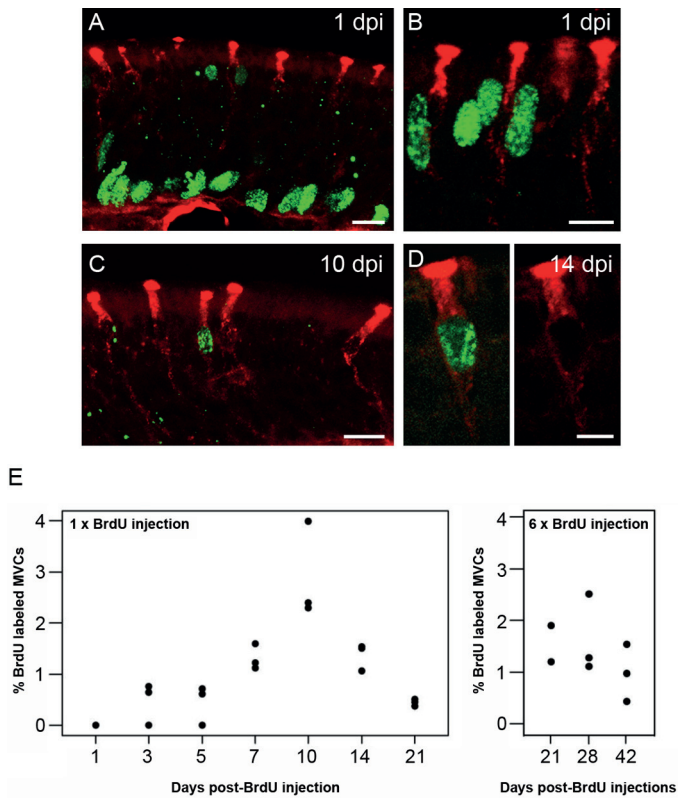


Figure 3 BrdU pulse-chase labeling combined with immunofluorescence staining for CD73 provides evidence for generation of adult-born CD73-MVCs. (A–D) Double immunofluorescence staining on coronal sections using anti-CD73 antibody (red) and anti-BrdU antibody (green). (A) At 1 dpi, most of the BrdU-positive nuclei are present at the basal part of the olfactory epithelium. (B) BrdU-positive nuclei (green) were detected at the apical side of the epithelium at 1 dpi, but were not present in CD73-MVCs (red, apical part). (C,D) BrdU-labeled CD73-MVCs are detectable at 10 dpi (C) and 14 dpi (D). (E) Fraction of MVCs double-labeled for BrdU and CD73 at different time points after BrdU injection. Each dot represents the median value from an individual mouse. The left graph represents the data after a single BrdU injection, and the right graph after multiple BrdU injections (see main text). In both experiments, there was no difference in the total number of CD73-MVCs (1× BrdU: median, 25th–75th percentile: 258, 198–297; 6× BrdU: 217, 164–273) counted at the various time points. The first double-labeled cells appeared at 3 dpi. The percentage of BrdU-labeled CD73-MVCs gradually increased, peaked at 10 dpi and thereafter declined. Kruskal–Wallis test confirmed a statistical effect of time ($N = 3$ for time point; $H = 17.126$; $P = 0.008$). After 1 BrdU injection, 240 ± 64 CD73-MVCs were counted on average per mouse and after 6 BrdU injections 236 ± 38 , respectively. Scale bars: A–C = 10 μm , D = 5 μm .

increasing evidence for purinergic signaling having a prominent role in contributing to the control of progenitor cell proliferation in the adult OE (Hassenklöver et al. 2009; Jia et al. 2009; Jia and Hegg, 2010; Jia et al. 2011; Jia and Hegg 2012). CD73 catalyzes the hydrolysis of AMP to adenosine and, therefore, might contribute to rapid termination of purinergic signaling, notably on release of ATP from degenerating cells.

Adult stem cells are present in many tissues and have the ability to replace the majority of somatic cells. Several

types of somatic cells that are exposed to noxious stimuli undergo a lifelong cycle of cell death and replacement. In the OE, the germinative zone lies adjacent to the basal lamina and contains multipotent cells. Olfactory cells, including sensory neurons, are continuously restored throughout life. This study showed that also CD73-MVCs undergo a turnover. We detected the earliest BrdU-labeled CD73-MVCs at 3 dpi and observed a peak of newly generated CD73-MVCs at 10 dpi. This temporal profile is very similar to the maturation of other sensory cells. For example, olfactory sensory neurons need approximately 1 week to mature. BrdU-OMP double-labeled cells were reported to appear 7 days after BrdU injection (Miragall and Monti Graziadei 1982; Schwob et al. 1992; Kondo et al. 2010). Solitary chemosensory cells lying in the nonolfactory nasal epithelium express elements of their transduction cascade, α -gustducin, within 3 days after completing mitosis (Gulbransen and Finger 2005). In Type II taste vallate bud cells, α -gustducin and BrdU were coexpressed starting from 2.5 dpi and reaching a peak at 6.5 dpi (Farbman 1980; Cho et al. 1998). The signaling molecule for taste transduction, PLC $\beta 2$, has been shown to be expressed in taste bud cells from day 5 and reached maximum at day 12 (Hamamichi et al. 2006). The timing of CD73 expression in CD73-MVCs after 3 days exiting S-phase indicates that MVCs might mature at a rate similar to other sensory cells.

The majority of epithelial cells is generated from a population of undifferentiated progenitor cells (Chen et al. 2004). These progenitor cells have the capacity to replace cells and maintain the epithelial homeostasis. Olfactory neurons are among the best-studied example of how regeneration from stem cells occurs. The cell lineage relationship has been extensively described; starting from undifferentiated precursor cell types to mature neurons. Evidence indicates that olfactory neurons are generated by GBCs, at least in undamaged tissue (Caggiano et al. 1994; Huard et al. 1998). As 1 exception, supporting cells have the capacity to proliferate and can replace themselves (Graziadei et al. 1979; Weiler and Farbman 1998). Although self-renewal of CD73-MVCs cannot be excluded at this stage, it would imply that both daughter cells do not express detectable CD73 during the first days after exiting the S-phase, which appears unlikely.

Therefore, we suggest that CD73-MVCs do not divide after differentiation, but rather derive from a population of olfactory progenitor cells. The identity of this progenitor cell remains elusive. CD73-MVCs might originate from GBCs, like olfactory neurons. Other possible candidates are the HBCs, in keeping with the relatively rare occurrence of BrdU-labeled CD73-MVCs. As a third possibility, Bowman's gland/duct cells need to be considered, because Bowman's gland/duct cells have the capacity to replenish supporting cells (Huard et al. 1998) and have been reported to express CD73 in the OE of young rats (Braun and Zimmermann 1998). However, working with mouse tissue, we could not confirm the presence of CD73 in cells of the Bowman's gland/duct and we obtained no clear evidence for

BrdU-labeling in this organ, precluding a definitive conclusion about this possibility.

The proportion of BrdU-labeled CD73-MVCs reaches a maximum at 10 dpi and declines thereafter. This time course indicates either that some newly generated CD73-MVCs fail to complete differentiation and degenerate, or that some of their progenitors undergo several cycles of division prior to becoming postmitotic and differentiate. The first possibility would be in line with previous reports on the lifespan of newborn cells. For instance, roughly half of newly generated taste bud cells are eliminated within the first few days after birth (Hamamichi et al. 2006). The number of newly generated olfactory sensory neurons that express OMP increases gradually from 7 to 14 days and thereafter starts to decrease. Moreover, a large fraction of BrdU-positive cells underneath the OMP-positive cell layer dies within 14 days after birth (Kondo et al. 2010). In analogy to sensory neurons, which depend on signals from the olfactory bulb and from locally released factors within the OE (Mackay-Sim and Kittel 1991; Schwob et al. 1992; Carr and Farbman 1993; Kondo et al. 2010), newly generated CD73-MVCs might need to receive trophic support for their survival or be vulnerable to exposure to noxious stimuli.

It is the current understanding that the degree of exposure of a cell type to noxious stimuli is loosely correlated with its turnover; for example, airway epithelial cells lining the tracheobronchial tree have a significant longer turnover than cells in the nasal cavity (Basbaum and Jany 1990). Consequently, solitary chemosensory cells, located in a remarkably vulnerable location at the anterior end of the nasal cavity, have an estimated turnover of only 20 days (Gulbransen and Finger 2005). Likewise, olfactory sensory neurons in a slightly more protected position at the posterior part of the nasal cavity have a lifespan of 1–2 months (Moulton 1974; Graziadei and Graziadei 1979; Graziadei et al. 1979; Miragall and Monti Graziadei 1982; Mackay-Sim and Kittel 1991; Schwob et al. 1992; Schwob 2002). Exceptions are the olfactory supporting cells, involved in forming the apical layer of the OE, which are considered to have a long lifespan and be replaced at a low rate (Naguro and Iwashita 1992; Weiler and Farbman 1998). Because CD73-MVCs are intermingled with the supporting cells at the most superficial layer in close contact with the incoming airstream, they are prone to cellular damage, pointing to a short turnover. However, we detected only few double-labeled cells indicating that CD73-MVCs, like their neighboring supporting cells, have a slow turnover and a long lifespan. Indeed, even at 42 dpi, we still were able to detect BrdU-labeled CD73-MVCs in the OE.

In conclusion, in this study, we classified a major population of MVCs by demonstrating CD73 expression in PLC β 2-MVCs and IP₃R3-MVCs, which suggests that these 2 MVC types correspond to 1 population, termed CD73-MVCs. In addition, our results showed that CD73-MVCs undergo turnover and are, therefore, similar to

the majority of somatic cells that can be replaced throughout life, most likely to protect the organism against damage. Because of the postulated involvement of MVCs in olfactory neurogenesis, it will be essential to further investigate their function and their own regulation for a better understanding of the molecular and cellular pathways underlying progenitor cell proliferation and differentiation.

Funding

The work was supported by the Swiss National Science Foundation [National Research Program NRP63, 406340-128112 to R.E. and I.K.] and by the 'Forschungskredit' of the University of Zurich to R.E.

Acknowledgements

The authors thank all collaborators of the Animal Services of the Institute of Pharmacology and Toxicology for animal husbandry and care, and Dr Wolfgang Härtig (University of Leipzig) for CD73 antibody conjugation to Cy3.

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